WHAT'S NEW IN ENDOCRINOLOGY?

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PUBLISHED IN: Frontiers in Endocrinology

frontiers Research Topics

ISSN 1664-8714
ISBN 978-2-88963-392-0
DOI 10.3389/978-2-88963-392-0

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WHAT'S NEW IN ENDOCRINOLOGY?

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Citation: Holly, J. M. P., LeRoith, D., eds. (2020). What's New in Endocrinology?. Lausanne: Frontiers Media SA. doi: 10.3389/978-2-88963-392-0

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frontiers in Endocrinology

EDITORIAL
published: 03 December 2019
doi: 10.3389/fendo.2019.00838

Editorial: What's New in Endocrinology?

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Keywords: obesity, reproduction, stress, cancer, aging

Editorial on the Research Topic

What's New in Endocrinology?

As it approaches a decade since Frontiers in Endocrinology was launched, the Chief Editors have commissioned a series of articles to reflect the continuing dynamic evolution of the science at the Frontiers of Endocrinology. These articles highlight recent breakthroughs or advances, new technologies, or challenges in the field of endocrinology. As with any dynamic field the frontiers are ever changing and these articles exemplify some of the recent developments together with some of the new questions and challenges for the future. The articles cover many different areas of endocrinology including issues involved in some of the biggest health challenges facing today's society such as stress, obesity, reproduction, cancer, and aging.

The greatest challenge to health provision in virtually every country across the globe is obesity; the scale of the epidemic threatens to outstrip resources in even the richest of societies (1). The burden of morbidity arising from obesity and its sequelle of type 2 diabetes, cardiovascular disease, and cancer present many challenges that are not restricted to those providing healthcare but reaching across society. Big questions of how we feed ourselves and how we live our lives need to be addressed (2); but some of the most fundamental questions center on how the body regulates energy balance: essentially an endocrine question. Obesity is the direct result of a chronic imbalance between energy intake and energy expenditure with the excess energy stored in adipose tissue. Adipose tissue was traditionally considered a relatively inert tissue comprising cells that just functioned as stores of excess energy in the form of lipids. This changed during the 1990's when it was discovered that adipocytes were an important source of key hormones such as leptin and adiponectin with important roles in regulating both energy intake and expenditure (3). Interest in adipose tissue was enhanced further around a decade ago when imaging techniques using labeled glucose revealed that humans possess brown adipose tissue (BAT) (4). Whereas, white adipocytes contain large stores of fats, in contrast brown adipocytes contain large numbers of mitochondia in which uncoupling protein 1 (UCP1) enables energy to be dissipated as heat rather than being stored. Until then it was considered that brown adipocytes were an important site of thermogenesis and hence energy expenditure in rodents but were considered absent from humans. This raised the potential of a new strategy to address human obesity by targeting BAT to enhance energy expenditure. In a comprehensive article Carpentier et al. review the challenges that have been encountered in the subsequent decade of research into BAT in humans. They address key questions such as the true extent of BAT in humans; whether the original imaging techniques underestimated the total mass of BAT and to what extent "beiging" of white adipocytes, with the induction of UCP1, can occur in humans. These are critical questions that could establish whether beige and brown adipocytes in humans could be an effective target to bring about changes in energy expenditure that are of therapeutic benefit.

OPEN ACCESS

Edited and reviewed by:
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Specialty section:
This article was submitted to Clinical Diabetes, a section of the journal Frontiers in Endocrinology

Received: *08 July 2019*
Accepted: *18 November 2019*
Published: *03 December 2019*

Citation:
Holly JMP and LeRoith D (2019) Editorial: What's New in Endocrinology? Front. Endocrinol. 10:838. doi: 10.3389/fendo.2019.00838

In addition to the advances in our understanding of energy expenditure there have been major progress in our knowledge of the endocrine controls of energy intake via hormones that control hunger and satiety and hence determine food intake (5). Life within modern societies is increasingly stressful and whether this impacts on the controls of appetite and satiety resulting in over-eating is an interesting question. The effects of stress on the gut-brain signaling pathways and the neuropeptides involved are reviewed by Stengel and Taché.

The management of obesity remains a huge health challenge. Numerous dietary and lifestyle changes have been proposed and all have achieved modest weight loss that is invariably soon regained. The pharmaceutical industry has invested considerably in developing medications to treat the huge potential market with a long trail of failures with many concerns regarding adverse effects and long-term safety (2). While nutritionists, physicians, and pharmacologists have floundered in the quest to control the obesity epidemic; surgeons have developed a number of bariatric procedures that result in effective and sustained weight loss. One of the surprising observed effects of bariatric surgery has been the remarkable correction of the metabolic disturbances often resulting in complete remission of type 2 diabetes. That this effect is often apparent within days of surgery and before any appreciable weight loss has challenged many of our dogmas of the links between obesity and its associated metabolic disturbances. Both Ma and Vella and Laferrère and Pattou have reviewed the potential new insights into metabolic endocrinology that these observations may provide. The clues from the anatomical differences between the different surgical procedures are reviewed by Ma and Vella. They describe how such anatomical distinctions can provide insights into the various hormonal pathways and in particular the interactions between regions of the gut and the pancreas. They also touch on other interesting, less well-appreciated effects, such as how the surgery and endocrine changes can alter the perception of sweet-taste and hence alter calorie intake. The most studied surgical intervention is Roux-enY gastric bypass and Laferrère and Pattou review these studies with an emphasis on what these studies have revealed regarding the gut endocrine system. They highlight the many new questions raised in relation to the role of satiety hormones, incretins, and bile acids. Our concepts of bile acids have been transformed from being regarded as just soaps that aid in the uptake of dietary fats to being a previously unappreciated complex endocrine system.

While the obesity epidemic has added to the focus on type 2 diabetes it has also become clear that there has been a 3% annual increase in the incidence of type 1 diabetes and Jacobsen et al. provide an overview of the development of strategies to prevent this and avoid the need for lifelong treatment. In order to prevent type I diabetes it is important to understand the natural history of the development of the autoimmunity that results in pancreatic beta cell destruction and the onset of type 1 diabetes. The challenges of studying populations prior to the disease onset and how this is being addressed around the world are described. These studies have informed the various trials for primary prevention in subjects at risk and secondary prevention in those already exhibiting evidence of autoimmunity. To date these studies have had limited success and new and future strategies are discussed.

In a fine exposition of how studying one component can help inform on how inter-connected the endocrine system has become; Eiden and Jiang review how new observations of adrenal chromaffin cells have contributed to our understanding of how we coordinate response to stress. The adrenal medulla has conventionally been considered the source of epinephrine to coordinate the cardiovascular, neuronal, and metabolic responses to stress. In this overview they describe recent observations of sympathetic nervous system regulation of chromaffin cell function and its secretion of not just epinephrine but also a rich cocktail of novel bioactive peptides. This new evidence is synthesized into a broader understanding of how metabolic, cardiovascular, and inflammatory responses are integrated. They also highlight interesting new questions that have arisen from this work; such as whether the sensory nervous system and immune/inflammatory systems are looped-in together via the adrenal medullary stress response and what are the broader endocrine functions of the many bioactive peptides secreted from the chromaffin cells.

Population control and reproductive health remain major health issues globally. The important role of androgens both in ovarian follicle selection to ensure mono-follicular ovulation in women and in the normal cyclical secretion of estrodiol is reviewed by Franks and Hardy. They focus on recent advances regarding the role of androgens in the development of polycystic ovary syndrome (PCOS), which remains the most common endocrine disorder in women of reproductive age.

One of the recent advances in techniques for maintaining fertility in women has been ovarian tissue cryopreservation (OTC) and transplantation. Originally developed to assist prepubertal girls and young women faced with reductions in the ovarian reserve due to pathologies, such as malignancies, or due to aggressive therapies that damage the ovary, Kristensen and Andersen discuss the many issues and challenges with extending this technique to more broader applications. Using this technique to restore fertility to women with anovulatory PCOS is discussed. The many issues surrounding the more controversial application of the technique to enable healthy women to postpone childbearing into their more advanced years is also addressed.

During pregnancy a woman's endocrine system kicks into overdrive with most hormones adapting to enable the mother to meet the additional metabolic demands and to provide an optimal environment in which the fetus can develop. Among all of these hormonal changes vitamin D plays an under-appreciated role both in ensuring adequate calcium availability for fetal bone development and in enhancing maternal tolerance to the presence of paternal and fetal alloantigens. Recent advances in our understanding of the part played by vitamin D-binding protein (VDBP) in facilitating these roles is reviewed by Karras et al.. The ongoing questions regarding the role of VDBP in important clinical issues such as preeclampsia, preterm birth, and gestational diabetes are discussed.

Neuropeptide G protein-coupled receptors (GPCRs) are over-expressed in many different cancers; not just the relatively rare

neuroendocrine tumors but also in some common cancers such as small cell lung cancers. The potential targeting of specific GPCRs for the development of novel cancer therapies is reviewed by Moody et al.. The development of agents that target receptors for bombesin, neurotensin, vasoactive intestinal peptide, and somatostatin are described in relation to the detection and treatment of both endocrine and non-endocrine cancers.

Recent advances in the genetics, biochemical characterization, and imaging of pheochromocytomas (PCCs) and paragangliomas (PGLs) are reviewed by Alrezk et al.. These are challenging cancers to treat and although most can be cured by surgery on rare occasions they metastasize and for these there are currently no approved treatments. The potential of systemic therapies, that have largely been developed to treat other cancers, is reviewed by Jimenez. Different strategies that have been designed to target each of the accepted "hallmarks" are discussed in relation to their application to treating PCCs and PGLs. These include different strategies to inhibit angiogenesis, cell proliferation, invasion, and metastasis, to enhance the induction of cell death and the recently developed immunotherapies.

As more and more people survive into advanced ages the problems of the elderly become an increasing burden on clinical services. The prevalence of thyroid nodules in people over the age of 60 years is extremely high (50–70%) and although most of these are benign (85–95%) it is important to distinguish the few that can become malignant and require surgery. The current status of molecular markers for the differential diagnosis of malignant, vs. benign, thyroid nodules is reviewed by Sahli et al.. The limitations of current genetic markers, their cost-effectiveness and the next generation of tests currently being evaluated are discussed.

Another common ailment associated with aging is osteopenia, which leads to the high prevalence of fractures seen in the elderly population. The challenges of identifying individuals at risk of fractures and the uses and limitations of current therapies for osteopenia are discussed by Ramchand and Seeman. The relative merits of antiresorptive and anabolic therapies are discussed as are the alternative strategies of combining these therapies or using them sequentially.

The increasing speed of technological advances provides endocrinologists with ever more powerful tools for investigation, diagnosis and treatment. As the pressures of modern lifestyles involve major changes in how we live and eat and demographics markedly increase the elderly population the challenges endocrinologists face in the clinic are constantly evolving. This collection of articles illustrates the variety of these challenges across the different specialties within endocrinology and the dynamic nature of modern endocrinology.

AUTHOR CONTRIBUTIONS

Both authors have made a substantial, direct and intellectual contribution to the work and approved it for publication.

ACKNOWLEDGMENTS

We want to thank all the authors of this Research Topic for their excellent contributions, and the dedicated reviewers for their insightful comments that helped maintain the articles at the highest standards. We also gratefully acknowledge the continuous support of the Frontiers staff.

REFERENCES

1. Tremmel M, Gerdtham UG, Nilsson PM, Saha S. Economic burden of obesity: a systematic literature review. *Int J Environ Res Public Health*. (2017) 14:E435. doi: 10.3390/ijerph14040435
2. Samaras K, Tevaearai H, Goldman M, le Coutre J, Holly JMP. With obesity becoming the new normal, what should we do? *Front Endocrinol*. (2019) 10:250. doi: 10.3389/fendo.2019.00250
3. Kershaw EE, Flier JS. Adipose tissue as an endocrine organ. *J Clin Endocrinol Metab*. (2004) 89:2548–56. doi: 10.1210/jc.2004-0395
4. Virtanen KA, Lidell ME, Orava J, Heglind M, Westergren R, Niemi T, et al. Functional brown adipose tissue in healthy adults. *N Engl J Med*. (2009) 360:1518–25. doi: 10.1056/NEJMoa0808949
5. Ahima RS, Antwi DA. Brain regulation of appetite and satiety. *Endocrinol Metab Clin North Am*. (2008) 37:811–23. doi: 10.1016/j.ecl.2008.08.005

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Endocrinology

REVIEW
published: 06 March 2018
doi: 10.3389/fendo.2018.00070

Understanding Pre-Type 1 Diabetes: The Key to Prevention

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While the incidence of type 1 diabetes continues to rise by 3% each year, the ability to prevent this disease remains elusive. Hybrid closed loop devices, artificial pancreas systems, and continuous glucose monitoring technology have helped to ease the daily burden for many people living with type 1 diabetes. However, the artificial pancreas is not a cure; more research is needed to achieve our ultimate goal of preventing type 1 diabetes. The preceding decades have generated a wealth of information regarding the natural history of pre-type 1 diabetes. Islet autoimmunity in the form of multiple autoantibodies is known to be highly predictive of progression to disease. Staging systems have been devised to better characterize pre-type 1, direct mechanistic understanding of disease, and guide the design of prevention studies. However, there are no evidence-based recommendations for practitioners caring for autoantibody patients other than to encourage enrollment in research studies. Close monitoring of high-risk patients in natural history studies markedly reduces diabetic ketoacidosis rates at diagnosis and research participation is critical to finding a means of preventing type 1 diabetes. The discovery of an effective preventative strategy for type 1 diabetes will justify universal risk screening for all children.

Keywords: prevention, type 1 diabetes, staging, mechanisms, autoimmune diseases

OPEN ACCESS

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Specialty section:
This article was submitted to Diabetes, a section of the journal Frontiers in Endocrinology

Received: *12 December 2017*
Accepted: *16 February 2018*
Published: *06 March 2018*

Citation:
Jacobsen LM, Haller MJ and Schatz DA (2018) Understanding Pre-Type 1 Diabetes: The Key to Prevention. Front. Endocrinol. 9:70. doi: 10.3389/fendo.2018.00070

INTRODUCTION

As is the case for type 2 diabetes, the incidence and prevalence of type 1 diabetes is increasing annually. It is estimated that more than 542,000 children worldwide have type 1 diabetes. With the diagnosis of type 1 diabetes rising by 2–3% per year, 86,000 children are expected to developed type 1 diabetes each year (1, 2). Recent data from Thomas et al. suggests that this is an underestimate when both children and adults diagnosed with type 1 diabetes are included. They estimate that over 40% of all new cases of type 1 diabetes occur over the age of 30 years (3). While advancements and innovation are occurring for those affected with type 1 diabetes in the artificial pancreas arena and therapeutic interventions for new-onset diabetes clinical trials, a focus on the prevention of type 1 diabetes is crucial. Prevention revolves around the identification and interdiction of this immune-mediated process.

Intensive insulin therapy in the Diabetes Control and Complications Trial has led to decreases in, but not the absence of, microvascular and macrovascular complications of diabetes (4). Insulin pump therapy and continuous glucose monitoring are setting the course for widespread use of a closed loop

Abbreviations: HLA, human leukocyte antigen; HbA1c, hemoglobin A1c; GLP-1, glucagon-like peptide-1; NOD, non-obese diabetic.

system, but insulin itself is not a cure. Despite advances, recent data from the T1D Exchange show no improvement in metabolic control over the past 5 years (5).

The etiology and precise mechanisms leading to type 1 diabetes remain elusive. Nevertheless, considerable progress has been made in our understanding of the natural history of "pre-type 1 diabetes." Such natural history study advances have led to the earlier diagnosis of type 1 diabetes and less diabetic ketoacidosis (DKA) at onset in those followed prospectively (6–8). These studies are a platform for studying mechanisms and staging of the disease to enable use of preventative therapies. No formal pre-type 1 diabetes evidence-based guidelines exist, but as endocrine and diabetes providers, we are responsible for (1) understanding the risk of progression to type 1 diabetes, (2) preventing DKA, and (3) advocating for prevention trials, mechanistic and natural history studies, and their continued funding and support.

AT-RISK POPULATION

To prevent type 1 diabetes, our understanding of the natural history of pre-type 1 diabetes and the mechanisms culminating in the autoimmune destruction of beta cells must continue to advance. Large international cohorts have been studied from birth in both relatives of patients with type 1 diabetes and more recently in the general population who are at high genetic risk. These studies have selected infants based on high-risk human leukocyte antigen (HLA) alleles, most commonly HLA-DR3/4, DQB1*0201/DQB1*0302. Monozygotic twins have a lifetime 50–70% risk of developing type 1 diabetes (9). In the US, the risk of developing type 1 diabetes is 1 in 20 in first-degree relatives and in the general population is 1 in 300 (9). By studying these individuals over time, risk predictors of progression to type 1 diabetes were determined, and none were more pronounced than the presence of islet autoantibodies. Islet autoantibodies develop in 90–95% of those destined to develop type 1 diabetes (10). These include islet cell autoantibodies (ICA) detected by indirect immunofluorescence (11) and insulin autoantibodies (IAA), glutamic acid decarboxylase autoantibodies (GADA), and insulinoma associated-2 autoantibodies (IA-2A) measured by radiobinding assays, and more recently zinc transporter 8 autoantibodies (10). Newer, more sensitive assay methods including electrochemiluminescence have been developed (12).

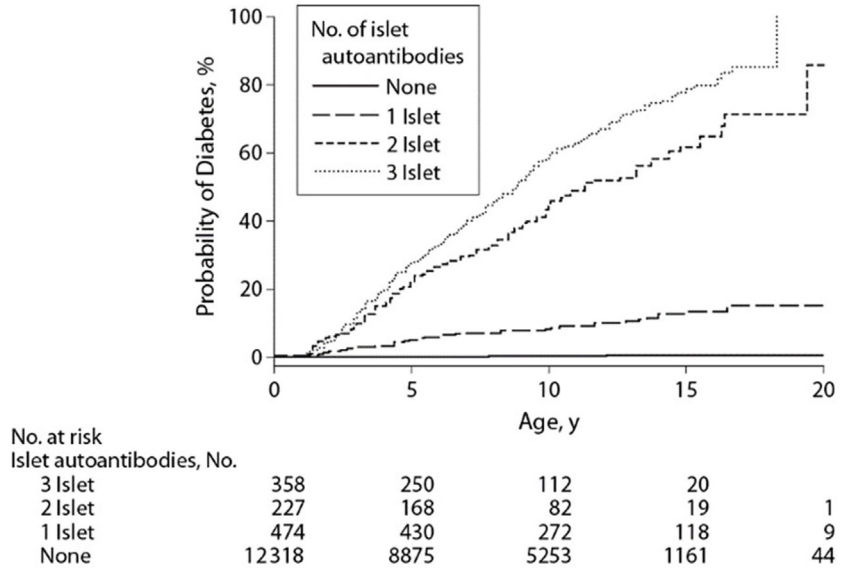
The forethought to initiate long-term natural history studies greatly increased our knowledge of islet autoimmunity, the precursor of clinical disease. Large birth cohorts including Germany's BABYDIAB (started in 1989), Finland's Diabetes Prediction and Prevention (DIPP; started in 1994), and Colorado's Diabetes Autoimmunity Study in the Young (DAISY; started in 1993) demonstrated a peak in islet autoimmunity development within the first 2–5 years of life and more rapid disease progression in those who developed autoantibodies in these early years compared to later childhood and adulthood (13, 14). IAA development occurs first in these young children with IgG1 subclass predominance and is more likely to be associated with the DR4 allele (13, 15). While islet autoantibodies detected in cord blood are most likely maternal in origin, Germany's BABYDIAB demonstrated lower risk in offspring of mothers with type 1 diabetes who had GADA or IA-2A in cord blood than those who were autoantibody negative offspring of type 1 diabetes mothers (16). In the most definitive study to date, The Environmental Determinants of Diabetes in the Young (TEDDY) study (started in 2004) is examining gene/environment interactions and subsequent development of islet autoimmunity and clinical disease (17). To date, this multicountry study (Germany, Finland, Sweden, US), has confirmed what appears to be two waves of separate islet autoantibody appearance—IAA within the first 18–24 months and GADA around age 3 years (15), as well as confirmed the correlation with genetic risk and importantly age (18, 19). Recent publications identify possible risk augmenters seen within the TEDDY cohort including non-HLA genes, single nucleotide polymorphisms, and other autoimmune diseases (20).

Combining data derived from the aforementioned DIPP, DAISY, and, BABYDIAB birth cohort studies, Ziegler et al. demonstrated the risk of progression to type 1 diabetes based on the age of appearance of autoimmunity and number of autoantibodies. This young cohort of genetically high-risk children with one autoantibody had a 10-year risk of progression to diabetes of 14.5%. Children with two or more autoantibodies were at markedly increased risk of progression to type 1 diabetes at 5-year (43.5%), 10-year (69.7%), and 15-year (84.2%) follow-up. Sixty percent of children with multiple autoantibodies progressed to diabetes (median age of 6.1 years) compared to 10% of children with a single autoantibody (median age 5.2 years) (**Figure 1**) (21).

STAGING OF TYPE 1 DIABETES

These birth cohort studies, in addition to non-birth cohorts such as the large Diabetes Prevention Trial-Type 1 (DPT-1, 1994–2003) and the Type 1 Diabetes TrialNet Pathway to Prevention (PTP; 2004–present) studies have allowed us to further characterize the time period before diagnosis. Although the actual diagnosis of diabetes has traditionally been based on American Diabetes Association (ADA) criteria (22), it is clear that the onset of the disease *per se*, often occurs years before the onset of symptoms. Thus, pre-type 1 diabetes is a unique physiologic state where autoimmunity is present and progression to metabolic derangement and clinical onset can be predicted especially in younger children and adolescents. As such, the ADA, JDRF, and Endocrine Society released a joint position statement for the staging of pre-type 1 diabetes (**Figure 2**) (23). Stage 1 is defined by the presence of 2 or more islet autoantibodies with normoglycemia (normal glucose tolerance on 2-h OGTT). Stage 2 shows progression to dysglycemia (impaired glucose tolerance) in the setting of 2 or more islet autoantibodies, and stage 3 occurs when a patient meets ADA criteria for the diagnosis of diabetes.

Why is this distinction important? The overall goal of all those who care for children and adults with type 1 diabetes is to cure the disease (obviously preventing its recurrence) as well as prevention of the disease in those at risk and destined to subsequently develop clinical onset. Participants screened and followed as part of natural history studies whether enrolled in prevention trials or not have a decreased incidence of DKA at diagnosis compared to those screened but not followed and those not screened (6–8). While DKA rates at onset of type 1 diabetes vary widely between



No. at risk Islet autoantibodies, No.					
3 Islet	358	250	112	20	
2 Islet	227	168	82	19	1
1 Islet	474	430	272	118	9
None	12318	8875	5253	1161	44

FIGURE 1 | Development of diabetes in children stratified for islet autoantibody outcome. The numbers at risk represent the children receiving follow-up at age 0, 5, 10, 15, and 20 years. Reproduced with permission from Ziegler et al. (21).

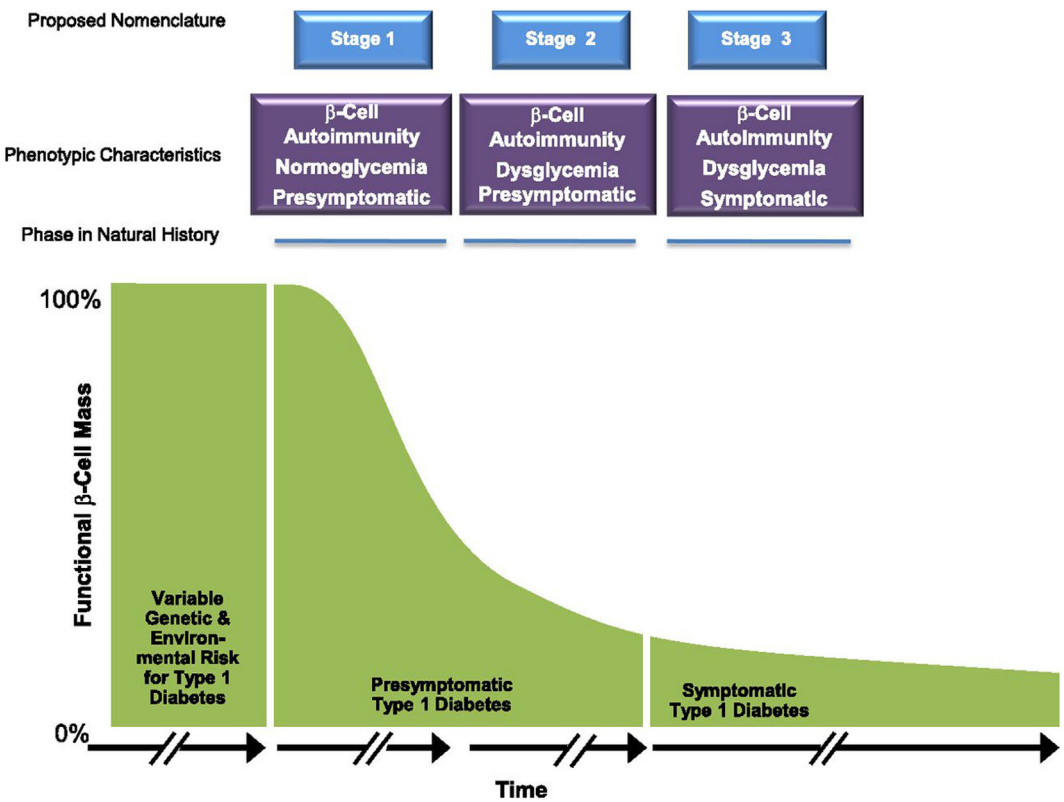


FIGURE 2 | Early stages of type 1 diabetes. Reproduced with permission from Insel et al. (23).

countries (16–67%) (24), there has clearly been a decrease shown within the TEDDY study; rates of DKA in those under 5 years of age were significantly less (11.3%) compared to national population-based registries (US SEARCH 36.4%, Swediabkids 16.9%, Finnish Pediatric Diabetes Register 18.7%, and German DPV Register 32.2%) (6). This lower rate of DKA was even more

significant in the youngest age group (<2 years old) where DKA is more common and carries a higher mortality (DKA rates of 15% in TEDDY versus 39.5–54% in population-based registries) (6). Thus, as a consequence of close monitoring in natural history studies, there is decreased morbidity and mortality. Several recent studies have also demonstrated long-term glycemic benefits seen for those patients who did not experience the severe metabolic derangement of DKA at diagnosis (25, 26).

Understanding the likelihood of progression to disease in different groups is crucial. In prevention studies, a clinical endpoint, such as type 1 diabetes, can take years to reach and result in lengthy, expensive clinical trials. Limiting enrollees to those already in stage 2 may see endpoints reached more rapidly. That said, curtailing studies to those with advanced autoimmunity limit those available to enroll and can slow recruitment. Additionally, non-interventional, mechanistic-focused studies in stage 1 and 2 subjects can be designed to further our understanding of the etiopathogenesis of disease. The classification of pre-type 1 diabetes provides a uniform way in which researchers and clinicians can converse while promoting more individualized medical management (23). Clinical trial design, subject selection, and risk versus benefit analysis can all be improved by the use of these risk-stratified groups.

Biomarkers, if validated, would be useful in understanding the natural history of the disease, heterogeneity, and as well as in clinical trials to shorten studies. C-peptide preservation is well studied as a metabolic endpoint in intervention trials. Samples collected during OGTT can be used as absolute values (fasting and peak C-peptide) or as part of a multivariable equation such as Index60 derived from a proportional hazards regression model as predictors of progression to type 1 diabetes (27–29). Markers of beta cell-related stress, damage, and death are currently under investigation.

Our knowledge of rates of progression, risk factors and the heterogeneity of the disease has been greatly advanced by these studies. Recently, it has been proposed that stage 1 and 2 could also be referred to by the terminology autoimmune beta cell disorder (ABCD), though this is new and may be controversial nomenclature (30–32). Semantically, ABCD may promote understanding among primary care providers, pharmaceutical companies, and funding organizations as to the importance of this unique physiologic state and increase awareness and urgency to prevent this disease. Additionally, as the population of islet autoantibody-positive patients grows, what will be the appropriate counseling, monitoring, and management of these patients?

The cost of population screening and the parental anxiety associated with early monitoring for a disease must also be kept in mind (33). Parental anxiety studied within TEDDY has shown increased anxiety with genetic screening that decreases if no further risk is incurred (islet autoimmunity); however, with increasing positive islet autoantibodies increases in parental anxiety may occur (and slowly lessens with time) (34). Early population-based genetic and/or autoantibody screening programs are currently in progress (35). Public awareness campaigns for the earlier diagnosis of type 1 diabetes have shown mixed results but with some decreasing DKA at onset of type 1 diabetes (36).

PRACTICAL APPROACH TO PRE-TYPE 1 DIABETES

A practical approach to the management of islet autoantibody positivity starts with how islet autoantibody positivity is determined. This may occur through (1) testing of islet autoantibodies in patients found to have a mildly elevated blood glucose found incidentally (not meeting criteria for diabetes), (2) hyperglycemia detected during an acute illness that resolves but in whom autoantibody testing was done, (3) screening in the setting of multiple other autoimmune conditions, (4) screening of family members of probands diagnosed with clinical type 1 diabetes as part of a research study, or (5) population screening (only in the context of research). After confirmation of either one or more islet autoantibodies (specific autoantibody assays mentioned previously), the patient should be counseled if possible (based on age, number/type of autoantibodies and glycemia status) and referred to centers participating in available research studies (in the US, the NIH-funded TrialNet umbrella). If the patient does not qualify for any trials or does not wish to participate in a trial or research follow-up, the primary care provider, endocrinologist, or diabetologist should consider performing a hemoglobin A1c (HbA1c) and/or random/fasting/post-prandial blood glucose self-monitoring. An OGTT may be done to detect early clinical diabetes (Stage 3).

Initiation of insulin is not recommended in the pre-type 1 phase. Initiation of insulin early in disease was previously thought to provide for beta cell rest and recovery even without severe metabolic derangement or markedly elevated HbA1c. More recent studies, such as the DPT-1, have shown that this is not the case, and others looking at intensive insulin therapy initiated shortly after diabetes diagnosis fail to show increased preservation of C-peptide compared to conventional treatment (37, 38). Management questions that will require more study include the optimal time to start insulin in a patient diagnosed with stage 3 disease without symptoms ("silent diabetes"). Other important areas of pre-type 1 diabetes management to consider include the potential benefit of intense diet and exercise or GRAS (Generally Regarded as Safe) therapies. Will there be a role for adjunctive therapies other than insulin such as glucagon-like peptide-1 agonists or metformin? These and other preventative therapies are also being studied.

PREVENTION TRIALS IN TYPE 1 DIABETES

Recently Completed Studies

Prevention trials may have multiple endpoints and the populations treated may differ. Those found to be genetically at risk for developing islet autoimmunity are targeted with primary prevention strategies that are typically of low risk. Secondary prevention studies aim to slow down or halt the destruction of beta cells in those who have islet autoantibodies. Multiple approaches have been used with limited success to date (**Table 1**). These include dietary changes, antigen-based therapy, immunomodulatory, and immunosuppression therapies. Primary dietary prevention

TABLE 1 | Overview of recently completed, current and planned clinical trials aimed at prevention of type 1 diabetes.

Recently completed	Trial to Reduce IDDM in the Genetically at Risk (TRIGR)
	BABYDIET study in Germany
	Finnish Dietary Intervention Trial for the Prevention of Type 1 Diabetes (FINDIA)
	TrialNet Nutritional Intervention to Prevent (NIP) Type 1 diabetes study
	Type 1 Diabetes Prediction and Prevention (DIPP) study in Finland
	Pre-POINT (Primary Oral/Intranasal INsulin Trial) and Pre-POINT-early in Germany
	Diabetes Prevention Trial-Type 1 (DPT-1)
	TrialNet Oral Insulin study
	Australian Intranasal Insulin Trial-I (INIT I)
	Diabetes Prevention—Immune Tolerance (DIAPREV-IT) study
	European Nicotinamide Diabetes Intervention Trial
Current	TrialNet Teplizumab (anti-CD3) trial
	TrialNet Abatacept (CTLA4-Ig) trial
	The CoRD Study with autologous cord blood in Australia
	DIAPREV-IT2 study
	Australian Intranasal Insulin Trial-II (INIT II)
Future	TrialNet Aldomet (methyldopa) study
	TrialNet Hydroxychloriquine
	TrialNet Rituximab and Abatacept
	Fr1da Insulin Intervention in Germany
	Adjunctive therapies such as glucagon-like peptide-1 receptor agonists

strategies beginning in the mid-1990s included the Trial to Reduce IDDM in the Genetically at Risk evaluating the role of a hydrolyzed casein-based formula (free of intact bovine insulin) compared to cow's milk-based formula, BABYDIET looking at a gluten-free diet in the first year of life, the Finnish Dietary Intervention Trial for the Prevention of Type 1 Diabetes (FINDIA) with insulin-free bovine formula, and the TrialNet Nutritional Intervention to Prevent Type 1 diabetes study with docosahexaenoid acid. All studies failed to show efficacy other than FINDIA which demonstrated some delay in the development of autoantibodies (39–41).

Antigen therapy was established with the hope of inducing peripheral tolerance by exposure of the naïve immune system to an antigen found in the target organ (beta cell) or through induction of anergy of already present autoreactive T cells (42, 43). The Type 1 Diabetes Prediction and Prevention (DIPP) study in Finland screened cord blood samples for high-risk HLA genotypes and followed children for the subsequent development of autoantibodies. Children were treated with intranasal insulin or placebo and the outcome was no different between groups. In Germany, the Pre-POINT (Primary Oral/Intranasal INsulin Trial) study administered different doses of oral insulin (and intranasal insulin) to high-risk HLA individuals prior to the development of autoantibodies. This small study demonstrated mechanistic/immunological effects including elevated serum IgG and salivary IgA binding to insulin and an increase in regulatory T cells (39). This led to the ongoing Pre-POINT-early study including children 6-months to 2-years old looking for induction of CD4+ T cell and antibody responses against insulin with dose escalation of oral insulin.

Insulin was first targeted as an autoantigen in the late 1980s starting in the non-obese diabetic (NOD) mouse model. In the early 1990s, two insulin-based therapies were conducted in the DPT-1 network. In separate studies, oral insulin and intravenous/subcutaneous insulin were administered to those of intermediate and high-risk, respectively. No difference was seen except in an *ad hoc* analysis of a subgroup—those with positive ICA, elevated IAA titers (≥80 nU/mL), and normal glucose tolerance—a projected delay of 4.8 years in onset was observed. An even greater delay was observed in those with higher IAA levels (>300 nU/mL). The protective effect continued even after the end of the study (39). The large TrialNet Oral Insulin study (2007–2016) recently completed and demonstrated no effect in the overall cohort although a delay was noted in a stratum of high-risk subjects (with loss of first phase insulin response) treated with oral insulin (44). Further analyses through a small mechanistic trial of participants receiving oral insulin has completed and is being analyzed. Other modes of insulin delivery aiming to achieve immune tolerance have also been employed including intranasal insulin administration in the Australian Intranasal Insulin Trial-I and II (INIT I and II) (45).

Glutamic acid decarboxylase (GAD), another islet autoantigen, as a vaccination in the new onset and prevention time period has failed to provide preservation of beta cell function and effective delay in type 1 diabetes onset, respectively (39). The use of GAD together with an aluminum adjuvant (Diamyd®), in the Diabetes Prevention—Immune Tolerance (DIAPREV-IT) study, has shown increases in GADA titers but no delayed onset of disease (46). The addition of high dose vitamin D to Diamyd for the DIAPREV-IT2 study is ongoing. A non-antigen-based therapy, the European Nicotinamide Diabetes Intervention Trial study, in which relatives who had developed islet cell antibodies were randomized to 5 years of nicotinamide versus placebo, showed no difference in the rate of diabetes development (39).

Current Studies

In addition to the oral insulin and GAD-alum studies mentioned above there are other ongoing antigen-based prevention (and intervention) trials including those using multiple peptide mixtures from known islet autoantigens with the aim of inducing immunological tolerance to beta cells (47). More recently, there has been a focus on immunologic modulation in prevention studies after promising efficacy results in new-onset studies (38). Attempts to restore self-tolerance, promote Tregs, and reduce Teff have been evaluated with several different classes of drugs including anti-CD3. Based on data from well-designed new-onset studies (Protégé and AbATE trials) (48), TrialNet has just completed enrollment in a high-risk population of relatives with two or more autoantibodies and dysglycemia (stage 2 disease) using anti-CD3 (tepiluzimab). Abatacept, CTLA4, co-stimulation blockade was chosen for transition from intervention to prevention trials after it demonstrated a slowed rate of beta cell decline that was maintained 1 year after therapy cessation (39). The TrialNet Abatacept prevention study is underway and is still recruiting individuals

with stage 1 disease (multiple autoantibodies and normal glucose tolerance).

A cellular therapy approach seeking the promotion of tolerizing Treg cells using autologous cord blood is underway in Australia (The CoRD Study). This open label pilot study is recruiting multiple islet autoantibody-positive first-degree relatives. Simultaneously, the DIAPREVI-IT2 and the INIT II studies mentioned above have built off their predecessors and are looking to add new therapies or enlarge the group studied.

Future Studies

Once efficacy, safety, and feasibility (and hopefully mechanism) is demonstrated in new-onset type 1 diabetes patients receiving immune and other therapies they should be moved into the prevention arena. Due to the number of potential therapeutic targets—both immune and non-immune—multi-agent (cocktail) therapy targeting multiple aspects of this disease is likely to be needed. The timing of initiation and duration of treatment are also important areas of study.

Type 1 Diabetes TrialNet has recently expanded to other therapeutics that have been approved and tested safe in other conditions and populations. One is the use of methyldopa to inhibit the communication between antigen presenting cells through MHC Class II signaling in susceptible HLA-DQ8 haplotypes. This focused, small mechanistic study will enroll participants with HLA-DQ8, 1 or more autoantibodies and stage 1 or stage 2 disease. Second, hydroxychloroquine, after its success in rheumatoid arthritis, will be tested in stage 1 individuals. The rationale for this therapeutic, historically used to treat malaria, includes modulation of T cells and interleukins, specifically reductions in Th17 cells in the NOD mouse model of type 1 diabetes. Hydroxychloroquine also has been shown to improve glucose metabolism and insulin sensitivity in type 2 diabetes (49).

In Germany, the Fr1da study, performing general population screening for islet autoantibodies, will also be conducting the Fr1da Insulin Intervention looking at oral insulin in multiple autoantibody-positive subjects enrolled in the natural history study and progression to dysglycemia. This study serves many important purposes, mainly, the feasibility of population screening and seamless enrollment into a prevention study.

Many exciting trials will finish enrollment and follow-up in the next couple of years. As is the challenge with prevention trials, waiting for a clinical endpoint is costly and time-consuming. Large numbers of patients need to be screened and well-powered studies require large numbers of participants, which limit the number of studies able to be performed. Other clinically relevant endpoints are being explored and small, brief studies are being designed to test mechanistic outcomes.

CONCLUSION

We have gained incredible knowledge and understanding of the natural history of type 1 diabetes thanks to the international natural history and birth cohort studies described in this review. The presence of increased genetic risk (HLA) and multiple autoantibodies currently provides the most reliable means of predicting type 1 diabetes. However, additional clinical, metabolic, and genetic factors can be assessed to fine-tune that risk. While whole population screening for HLA risk or islet autoimmunity is not yet justified, several groups continue to create networks that will be poised to provide this screening as soon as meaningful prevention is identified. We await the results of several prevention clinical trials and other innovations as we labor to develop a sustainable method of preventing the complex autoimmune process that leads to type 1 diabetes.

As we identify more patients with islet autoimmunity, we must contemplate how to best care for them. All individuals found to have 1 or more islet autoantibodies should, ideally, be referred to contact a type 1 diabetes clinical research center which can be reached through type 1 diabetes supporting agencies, including the JDRF, ADA, NIDDK, and the Type 1 Diabetes TrialNet. Specifically, TrialNet has more than 200 clinical and affiliate centers across the US and worldwide (www.trialnet.org). Through this connection, individuals can decide if enrollment in a prevention clinical trial and/or natural history study is feasible as this is the best way to advance the field of prevention research.

Because of these efforts, a growing number of children are diagnosed with type 1 diabetes prior to the onset of clinical symptoms. There remain no firm guidelines for the follow-up and monitoring of individuals with 1 or more autoantibodies in the general community. Anecdotal evidence is provided and case studies emanating from birth cohorts like TEDDY will help to clarify management options that will undoubtedly vary based on institutional and country-specific preferences. The DPT-1, the Type 1 Diabetes TrialNet international clinical trial collaboration, the SEARCH for Diabetes in Youth Study, Fr1DA, and others will continue to provide valuable information with regards to the potential for population-based screening and the management of patients diagnosed with early type 1 diabetes.

In closing, whether it be through targeted screening of relatives for autoantibodies or population-based screening for high-risk HLA, we must continue to study the natural history of type 1 diabetes and identify patients with beta cell autoimmunity. We feel that primary care providers and subspecialists alike must continue to work together to identify these patients and encourage them to participate in research. Only through these concerted efforts will we move closer to our ultimate goal of preventing and reversing type 1 diabetes.

AUTHOR CONTRIBUTIONS

DAS conceptualized this proposal and reviewed/edited the manuscript, LMJ reviewed the current literature and wrote the manuscript, MJH reviewed/edited the manuscript. All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

FUNDING

Supported by grants U01 DK 60987-01, UO1DK60987-03, 1UC4DK104216-01 from NIH/NIDDK and by UL1 TR000064 from the NIH/NCATS Clinical and Translational Science Award to the University of Florida.

REFERENCES

1. International Diabetes Federation. *Diabetes Atlas*. 5th ed. Brussels: IDF (2011).
2. Centers for Disease Control and Prevention. *National Diabetes Statistics Report, 2017*. Atlanta, GA: Centers for Disease Control and Prevention, US Department of Health and Human Services (2017).
3. Thomas NJ, Jones SE, Weedon MN, Shields BM, Oram RA, Hattersley AT. Frequency and phenotype of type 1 diabetes in the first six decades of life: a cross-sectional, genetically stratified survival analysis from UK Biobank. *Lancet Diabetes Endocrinol* (2017) 6(2):122–9. doi:10.1016/S2213-8587(17)30362-5
4. The Diabetes Control and Complications Trial Research Group, Nathan DM, Genuth S, Lachin J, Cleary P, Crofford O, et al. The effect of intensive treatment of diabetes on the development and progression of long-term complications in insulin-dependent diabetes mellitus. *N Engl J Med* (1993) 329:977–86. doi:10.1056/NEJM199309303291401
5. Miller KM, Foster NC, Beck RW, Bergenstal RM, DuBose SN, DiMeglio LA, et al. Current state of type 1 diabetes treatment in the U.S.: updated data from the T1D exchange clinic registry. *Diabetes Care* (2015) 38(6):971–8. doi:10.2337/dc15-0078
6. Elding Larsson H, Vehik K, Bell R, Dabelea D, Dolan L, Pihoker C, et al. Reduced prevalence of diabetic ketoacidosis at diagnosis of type 1 diabetes in young children participating in longitudinal follow-up. *Diabetes Care* (2011) 34(11):2347–52. doi:10.2337/dc11-1026
7. Lundgren M, Sahlin Å, Svensson C, Carlsson A, Cedervall E, Jönsson B, et al. Reduced morbidity at diagnosis and improved glycemic control in children previously enrolled in DiPiS follow-up. *Pediatr Diabetes* (2014) 15(7):494–501. doi:10.1111/pedi.12151
8. Hekkala AM, Ilonen J, Toppari J, Knip M, Veijola R. Ketoacidosis at diagnosis of type 1 diabetes: effect of prospective studies with newborn genetic screening and follow up of risk children. *Pediatr Diabetes* (2018)19(2):314–9. doi:10.1111/pedi.12541
9. Maahs DM, West NA, Lawrence JM, Mayer-Davis EJ. Chapter 1: epidemiology of type 1 diabetes. *Endocrinol Metab Clin North Am* (2010) 39(3):481–97. doi:10.1016/j.ecl.2010.05.011
10. Wenzlau JM, Hutton JC. Novel diabetes autoantibodies and prediction of type 1 diabetes. *Curr Diab Rep* (2013) 13(5):608–15. doi:10.1007/s11892-013-0405-9
11. Decochez K, Keymeulen B, Somers G, Dorchy H, De Leeuw IH, Mathieu C, et al. Use of an Islet cell antibody assay to identify type 1 diabetic patients with rapid decrease in C-peptide levels after clinical onset. *Diabetes Care* (2000) 23:1072–8. doi:10.2337/diacare.23.8.1072
12. Miao D, Steck AK, Zhang L, Guyer KM, Jiang L, Armstrong T, et al. Electrochemiluminescence assays for insulin and glutamic acid decarboxylase autoantibodies improve prediction of type 1 diabetes risk. *Diabetes Technol Ther* (2015) 17(2):119–27. doi:10.1089/dia.2014.0186
13. Achenbach P, Bonifacio E, Koczwara K, Ziegler AG. Natural history of type 1 diabetes. *Diabetes* (2005) 54(Suppl 2):S25–31. doi:10.2337/diabetes.54.suppl_2.S25
14. Nokoff N, Rewers M. Pathogenesis of type 1 diabetes: lessons from natural history studies of high-risk individuals. *Ann N Y Acad Sci* (2013) 1281:1–15. doi:10.1111/nyas.12021
15. Krischer JP, Lynch KF, Schatz DA, Ilonen J, Lernmark Å, Hagopian WA, et al. The 6 year incidence of diabetes-associated autoantibodies in genetically at-risk children: the TEDDY study. *Diabetologia* (2015) 58(5):980–7. doi:10.1007/s00125-015-3514-y
16. Koczwara K, Bonifacio E, Ziegler AG. Transmission of maternal islet antibodies and risk of autoimmune diabetes in offspring of mothers with type 1 diabetes. *Diabetes* (2004) 53:1–4. doi:10.2337/diabetes.53.1.1
17. TEDDY Study Group. The environmental determinants of diabetes in the young (TEDDY) study: study design. *Pediatr Diabetes* (2007) 8(5):286–98. doi:10.1111/j.1399-5448.2007.00269.x
18. Krischer JP, Liu X, Lernmark Å, Hagopian WA, Rewers MJ, She JX, et al. The influence of type 1 diabetes genetic susceptibility regions, age, sex, and family history to the progression from multiple autoantibodies to type 1 diabetes: a TEDDY study report. *Diabetes* (2017) 66(12):3122–9. doi:10.2337/db17-0261
19. Steck AK, Vehik K, Bonifacio E, Lernmark Å, Ziegler AG, Hagopian WA, et al. Predictors of progression from the appearance of islet autoantibodies to early childhood diabetes: the environmental determinants of diabetes in the young (TEDDY). *Diabetes Care* (2015) 38:808–13. doi:10.2337/dc14-2426
20. Krischer JP, Lynch KF, Lernmark Å, Hagopian WA, Rewers MJ, She JX, et al. Genetic and environmental interactions modify the risk of diabetes-related autoimmunity by 6 years of age: the TEDDY study. *Diabetes Care* (2017) 40:1194–202. doi:10.2337/dc17-0238
21. Ziegler AG, Rewers M, Simell O, Simell T, Lempainen J, Steck A, et al. Seroconversion to multiple islet autoantibodies and risk of progression to diabetes in children. *JAMA* (2013) 309(23):2473–9. doi:10.1001/jama.2013.6285
22. American Diabetes Association. 2. Classification and diagnosis of diabetes. *Diabetes Care* (2017) 40(Suppl 1):S11–24. doi:10.2337/dc17-S005
23. Insel RA, Dunne JL, Atkinson MA, Chiang JL, Dabelea D, Gottlieb PA, et al. Staging presymptomatic type 1 diabetes: a scientific statement of JDRF, the endocrine society, and the American Diabetes Association. *Diabetes Care* (2015) 38(10):1964–74. doi:10.2337/dc15-1419
24. Usher-Smith JA, Thompson M, Ercole A, Walter FM. Variation between countries in the frequency of diabetic ketoacidosis at first presentation of type 1 diabetes in children: a systematic review. *Diabetologia* (2012) 55:2878–94. doi:10.1007/s00125-012-2690-2
25. Duca LM, Wang B, Rewers M, Rewers A. Diabetic ketoacidosis at diagnosis of type 1 diabetes predicts poor long-term glycemic control. *Diabetes Care* (2017) 40(9):1249–55. doi:10.2337/dc17-0558
26. Shalitin S, Fisher S, Yackbovitch-Gavan M, de Vries L, Lazar L, Lebenthal Y, et al. Ketoacidosis at onset of type 1 diabetes is a predictor of long-term glycemic control. *Pediatr Diabetes* (2017) 19(2):320–8. doi:10.1111/pedi.12546
27. Sosenko JM, Skyler JS, DiMeglio LA, Beam CA, Krischer JP, Greenbaum CJ, et al. A new approach for diagnosing type 1 diabetes in autoantibody-positive individuals based on prediction and natural history. *Diabetes Care* (2015) 38(2):271–6. doi:10.2337/dc14-1813
28. Veijola R, Koskinen M, Helminen O, Hekkala A. Dysregulation of glucose metabolism in preclinical type 1 diabetes. *Pediatr Diabetes* (2016) 17(Suppl 22):25–30. doi:10.1111/pedi.12392
29. Xu P, Krischer JP; Behalf of the Type 1 Diabetes TrialNet Study Group. Prognostic classification factors associated with development of multiple autoantibodies, dysglycemia, and type 1 diabetes – a recursive partitioning analysis. *Diabetes Care* (2016) 39(6):1036–44. doi:10.2337/dc15-2292
30. Bonifacio E, Mathieu C, Nepom GT, Ziegler AG, Anhalt H, Haller MJ, et al. Rebranding asymptomatic type 1 diabetes: the case for autoimmune beta cell disorder as a pathological and diagnostic entity. *Diabetologia* (2017) 60:35–8. doi:10.1007/s00125-016-4144-8
31. Jacobsen LM, Anhalt H, Haller MJ. Presymptomatic screening for autoimmune β-cell disorder (ABCD): baby steps towards prevention? *Pediatr Diabetes* (2018) 19(1):11–3. doi:10.1111/pedi.12620
32. Becker D, Insel R. Screening, staging, and naming of presymptomatic type 1 diabetes. *Pediatr Diabetes* (2018) 19:7–10. doi:10.1111/pedi.12613
33. Meehan C, Fout B, Ashcraft J, Schatz DA, Haller MJ. Screening for type 1 diabetes risk to reduce DKA is not economically viable. *Pediatr Diabetes* (2015) 16:565–72. doi:10.1111/pedi.12313
34. Johnson SB, Lynch KF, Roth R, Schatz D; TEDDY Study Group. My child is islet autoantibody positive: impact on parental anxiety. *Diabetes Care* (2017) 40(9):1167–72. doi:10.2337/dc17-0166
35. Fr1da. *Fr1da Study in Bavaria: Type 1 Diabetes – Early Detection – Early Treatment*. (2018). Available from: https://www.typ1diabetes-frueherkennung.de/
36. Deylami R, Townson J, Mann M, Gregory JW. Systematic review of publicity interventions to increase awareness amongst healthcare professionals and the public to promote earlier diagnosis of type 1 diabetes in children and young people. *Pediatr Diabetes* (2017):1–8. doi:10.1111/pedi.12565
37. Buckingham B, Beck RW, Ruedy KJ. Effectiveness of early intensive therapy on β-cell preservation in type 1 diabetes-randomized controlled trial. *Diabetes Care* (2013) 36(12):4030–5. doi:10.2337/dc13-1074
38. Diabetes Prevention Trial–Type 1 Diabetes Study Group. Effects of insulin in relatives of patients with type 1 diabetes mellitus. *N Engl J Med* (2002) 346:1685–91. doi:10.1056/NEJMoa012350
39. Jacobsen L, Schatz D. Current and future efforts toward the prevention of type 1 diabetes. *Pediatr Diabetes* (2016) 17(Suppl 22):78–86. doi:10.1111/pedi.12333
40. Writing Group for the TRIGR Study Group. Effect of hydrolyzed infant formula vs conventional formula on risk of type 1 diabetes the TRIGR randomized clinical trial. *JAMA* (2018) 319(1):38–48. doi:10.1001/jama.2017.19826

41. Ziegler AG, Danne T, Dunger DB, Berner R, Puff R, Kiess W, et al. Primary prevention of beta-cell autoimmunity and type 1 diabetes – the global platform for the prevention of autoimmune diabetes (GPPAD) perspectives. *Mol Metab* (2016) 5:255–62. doi:10.1016/j.molmet.2016.02.003
42. Peakman M, von Herrath M. Antigen-specific immunotherapy for type 1 diabetes: maximizing the potential. *Diabetes* (2010) 59(9):2087–93. doi:10.2337/db10-0630
43. Tian J, Kaufman DL. Antigen-based therapy for the treatment of type 1 diabetes. *Diabetes* (2009) 58(9):1939–46. doi:10.2337/db09-0451
44. Writing Committee for the Type 1 Diabetes Trial Net Oral Insulin Study Group, Krischer JP, Schatz DA, Bundy B, Skyler JS, Greenbaum CJ. Effect of oral insulin on prevention of diabetes in relatives of patients with type 1 diabetes: a randomized clinical trial. *JAMA* (2017) 318(19):1891–902. doi:10.1001/jama.2017.17070
45. Simmons KM, Michels AW. Type 1 diabetes: a predictable disease. *World J Diabetes* (2015) 6(3):380–90. doi:10.4239/wjd.v6.i3.380
46. Larsson HE, Lundgren M, Jonsdottir B, Cuthbertson D, Krischer J. DiAPREV-IT Study Group. Safety and efficacy of autoantigen-specific therapy with 2 doses of alum-formulated glutamate decarboxylase in children with multiple islet autoantibodies and risk for type 1 diabetes: A randomized clinical trial. *Pediatr Diabetes* (2017). doi:10.1111/pedi.12611
47. King's College London. *New Immunotherapy Trial for Type 1 Diabetes*. (2018).Availablefrom:https://www.kcl.ac.uk/newsevents/news/newsrecords/2016/03%20March/New-immunotherapy-trial-for-Type-1-diabetes.aspx
48. Sherry N, Hagopian W, Ludvigsson J, Jain SM, Wahlen J, Ferry RJ Jr, et al. Teplizumab for treatment of type 1 diabetes (Protégé study): 1-year results from a randomised, placebo-controlled trial. *Lancet* (2011) 378(9790):487–97. doi:10.1016/S0140-6736(11)60931-8
49. Hage MP, Al-Badri MR, Azar ST. A favorable effect of hydroxychloroquine on glucose and lipid metabolism beyond its anti-inflammatory role. *Ther Adv Endocrinol Metab* (2014) 5(4):77–85. doi:10.1177/2042018814547204

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Endocrinology

REVIEW
published: 18 April 2018
doi: 10.3389/fendo.2018.00179

Preoperative Molecular Markers in Thyroid Nodules

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The need for distinguishing benign from malignant thyroid nodules has led to the pursuit of differentiating molecular markers. The most common molecular tests in clinical use are Afirma® Gene Expression Classifier (GEC) and Thyroseq® V2. Despite the rapidly developing field of molecular markers, several limitations exist. These challenges include the recent introduction of the histopathological diagnosis "Non-Invasive Follicular Thyroid neoplasm with Papillary-like nuclear features", the correlation of genetic mutations within both benign and malignant pathologic diagnoses, the lack of follow-up of molecular marker negative nodules, and the cost-effectiveness of molecular markers. In this manuscript, we review the current published literature surrounding the diagnostic value of Afirma® GEC and Thyroseq® V2. Among Afirma® GEC studies, sensitivity (Se), specificity (Sp), positive predictive value (PPV), and negative predictive value (NPV) ranged from 75 to 100%, 5 to 53%, 13 to 100%, and 20 to 100%, respectively. Among Thyroseq® V2 studies, Se, Sp, PPV, and NPV ranged from 40 to 100%, 56 to 93%, 13 to 90%, and 48 to 97%, respectively. We also discuss current challenges to Afirma® GEC and Thyroseq® V2 utility and clinical application, and preview the future directions of these rapidly developing technologies.

Keywords: thyroid cancer, non-invasive follicular thyroid neoplasm with papillary-like nuclear features, molecular test, Afirma, Thyroseq

OPEN ACCESS

Edited by:
Derek LeRoith, Icahn School of Medicine at Mount Sinai, United States

Reviewed by:
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Specialty section:
This article was submitted to Thyroid Endocrinology, a section of the journal Frontiers in Endocrinology

Received: *14 February 2018*
Accepted: *03 April 2018*
Published: *18 April 2018*

Citation:
Sahli ZT, Smith PW, Umbricht CB and Zeiger MA (2018) Preoperative Molecular Markers in Thyroid Nodules. Front. Endocrinol. 9:179. doi: 10.3389/fendo.2018.00179

INTRODUCTION

Thyroid nodules are common among adults over the age of 60 years, with a prevalence of 50–70% (1, 2). Moreover, the incidence of thyroid cancer in the United States has increased by 211% between 1975 and 2013 (3), due to both an improved detection of small (<2 cm) thyroid nodules by thyroid ultrasonography and a true increase in thyroid cancer incidence (4). Nevertheless, the vast majority (85–95%) of thyroid nodules are benign (5). For this reason, the ability to distinguish between benign and malignant nodules is important in order to spare patients unnecessary diagnostic surgery.

Fine needle aspiration (FNA) to facilitate this distinction first became widely practiced in the early 1980s (6), and is widely recognized as the gold standard initial diagnostic procedure in the differential diagnosis of thyroid nodules, being accurate, safe, and cost effective (7, 8). The sensitivity (Se), specificity (Sp), positive predictive value (PPV), negative predictive value (NPV), false-negative (FN) rate, and false-positive (FP) ranges of an FNA are 88.2–97.0%, 47.0–98.2%, 52.0–98.0%, 89.0–96.3%, 0.5–10.0%, and 1.0–7.0%, respectively (9).

In 2009, the Bethesda classification system for FNA reporting was introduced by the National Cancer Institute, and recently, revised, included six categories based upon cytopathological features, with an associated malignancy rate and standardized management recommendation for each

category (**Table 1**) (10). FNA reliably establishes the diagnosis of a benign or malignant nodule in 70–80% of all cases (11) and has decreased the proportion of benign nodules unnecessarily resected from 86 to 50% (12). However, 20–30% of FNA cases have indeterminate or suspicious cytological results that include Bethesda III, IV, and V categories (12) and, of these, 6–75% are malignant on final surgical pathology (13, 14). Due to the uncertainty of malignancy in these patients, their management has been challenging, usually including a repeat FNA or a diagnostic lobectomy. For this reason, the need for distinguishing benign from malignant lesions in this subset of thyroid nodules has led to the pursuit of differentiating molecular markers.

Interest in achieving this distinction increased in 2002 with the recognition of the oncogenic role of the *BRAF V600E* mutation in approximately 58–69% of papillary thyroid cancers (PTC) (15, 16). However, genetic testing for *BRAF V600E* alone for the detection of PTCs is inadequate for clinical decision making due to its low sensitivity of 60% for PTC (17). Indeed, our group first published its use in indeterminate and suspicious thyroid lesions and found it to add minimal clinical value (18). In addition to studying the diagnostic utility of *BRAF V600E*, numerous studies have investigated the association of *BRAF V600E* and patient prognosis. However, the correlation between *BRAF V600E* and clinical features of PTCs has yielded inconsistent results. Some studies report that *BRAF V600E* is associated with a more advanced phenotype including an increased risk of lymph node metastasis, cancer recurrence, and patient mortality (19–21), while others report no such associations (22). Moreover, thyroid cancer with *BRAF V600E* and *TERT* promoter mutations has been associated with worse clinico-pathological outcomes (23, 24). *BRAF* testing can also be useful in deciding treatment in the setting of known metastatic thyroid cancer. Direct tyrosine kinase inhibitors, such as vemurafenib (25), dabrafenib (26), and sorafenib (27), have been shown to be effective in *BRAF V600E* metastatic thyroid cancers.

Since mutational analysis of single genes has not proven adequate in guiding management decisions in indeterminate or suspicious thyroid nodules, attention turned to using panels of molecular markers. Currently, the most common molecular tests in clinical use are Afirma® Gene Expression Classifier (GEC) and Thyroseq® V2 (28). Introduced in 2011 by Veracyte, the Afirma® GEC has been considered a "rule-out" malignancy test. It includes a 142-gene expression molecular assay and uses microarray technology to measure the mRNA expression profiles to determine whether a thyroid nodule is "suspicious" or "benign." The test's primary aim is to spare patients with cytologically indeterminate FNA samples unnecessary diagnostic surgery (29). Among indeterminate/suspicious nodules (Bethesda Type III–V), the test has both a high Se (92%) and high NPV (93%) (30) (**Table 2**). In contrast, it has a low Sp (52%) and PPV (47%), and cannot accurately identify malignant lesions alone.

ThyroSeq® v2, introduced in 2014 by CBL Path, is designed to identify malignant thyroid nodules by next generation sequencing (NGS), detecting 14 thyroid cancer-related genetic mutations, including *RAS* and *BRAF* mutations, 42 types of gene fusions associated with thyroid cancer, including *PAX*8/*PPARγ* and *RET*/*PTC* rearrangements, and mRNA expression levels for 16 genes; it is therefore considered a "rule-in" malignancy test (29). Among Bethesda Type III – IV nodules, the test is marketed as having a high Se, Sp, PPV, and NPV of 90–91%, 92–93%, 77–83%, and 96–97%, respectively, as well as having the ability to stratify risk based on the mutation detected (52, 53) (**Table 3**).

In this manuscript, we review the current published literature surrounding Afirma® GEC and Thyroseq® V2, discuss current challenges to their utility; and clinical application, and preview the future directions of these rapidly developing technologies.

CLINICAL MANAGEMENT OF INDETERMINATE THYROID NODULES

The 2015 American Thyroid Association (ATA) (60) and the 2016 American Association of Clinical Endocrinologists (AACE) (9) clinical guidelines recommend "considering," molecular testing for indeterminate nodules. If molecular testing is being considered, ATA recommends that patients "should be counseled regarding the potential benefits and limitations of testing and about the possible uncertainties in the therapeutic and long-term clinical implications of results" (strong recommendation, low-quality evidence) (60). However, long-term outcome data on the use of molecular markers for therapeutic decision-making is currently unavailable. A recent report estimated that standard application of the GEC for all indeterminate thyroid nodules would result in only a 7.2% decrease in thyroidectomy volume (61). Similarly, two studies by our group showed that molecular markers did not significantly affect the surgical decision-making process, where only 7.9–8.4% of patients had altered clinical management as a result of molecular testing (39, 62).

TABLE 1 | The Bethesda system for reporting thyroid cytopathology (10).

Bethesda group	Diagnostic category	Abbreviation	Malignancy rate	
			Before NIFTP reclassification (NIFTP malignant)	After NIFTP reclassification (NIFTP benign)
I	Non-diagnostic/unsatisfactory	–	5–10%	No change
II	Benign	B	0–3%	No change
III	Atypia of undetermined significance/follicular lesion of undetermined significance	AUS/FLUS	10–30%	6–18%
IV	Follicular neoplasm/suspicious for follicular neoplasm	FN/SFN	25–40%	10–40%
V	Suspicious for malignancy	SM	50–75%	45–60%
VI	Malignant	M	97–99%	94–96%

TABLE 2 | Afirma Gene Expression Classifier (GEC) literature review.

Author	Year	Study type	Follow-up period	Bethesda category	Sample size	GEC suspicious	Total surgery	Malignant nodules	NIFTP	Diagnostic value			
										Se (%)	Sp (%)	PPV (%)	NPV (%)
Afirma® GEC (30)	2012	Prospective, blinded multicenter	301 days (Median)	Total	265	165	265	85	–	92	52	47	93
				III	129	74	129	31		90	53	38	95
				IV	81	49	81	20		90	49	37	94
				V	55	42	55	34		94	52	76	85
Hang et al. (31)	2017	Retrospective, single institution	–	III	133	112	133	27	21	100	24	42	100
				IV	42	39	42	6	4	90	6	23	67
Harrison et al. (32)	2017	Retrospective, single institution	–	III	100	–	37	13	–	–	–	35	–
				IV	10	–	4	1		–	–	25	–
Kay-Rivest et al. (33)	2017	Retrospective, multicenter	–	III, IV	172	83	77	38	–	–	–	49	–
Samulski et al. (34)	2016	Retrospective, single institution		Total	294	133	128	33	11	93	17	39	81
			–	III	166	60	60	15	5	95	19	40	88
				IV	122	73	68	18	6	92	15	39	75
Wu et al. (35)	2016	Prospective, single institution	–	Total	245	132	128	63	–	94	31	57	83
				III	217	115	107	55		93	29	58	79
				IV	28	17	21	8		100	38	50	100
Yang et al. (36)	2016	Retrospective, single institution	–	Total	189	94	67	32	–	100	15	51	100
				III	165	81	55	26		100	7	49	100
				IV	24	13	12	6		100	50	67	100
Chaudhary et al. (37)	2016	Retrospective, single institution	–	Total	158	85	73	28	–	100	15	100	20
				III	89	41	45	8		100	18	20	100
				IV	69	44	41	21		100	11	55	100
Abeykoon et al. (38)	2016	Retrospective, single institution	–	III, IV	34	17	16	12	–	–	–	49	–
Noureldine et al. (39)	2016	Prospective, single institution	–	III, IV	99	89	89	37	–	97	9	42	83
Al-Qurayshi et al. (40)	2016	Retrospective, single institution	–	Total	154	96	112	50	–	78	40	51	69
				III	114	66	84	36		78	52	55	76
				IV	40	30	28	14		79	0	44	0
Witt (41)	2016	Retrospective, single institution		III, IV	47	15	15	6	–	–	–	40	–
Wong et al. (42)	2016	Retrospective, single institution	–	III, IV	63	63	63	8	14	–	–	35	–
Zhu et al. (43)	2015	Retrospective, single institution	–	III, IV	45	21	10	6	–	–	–	60	–
Celik et al. (44)	2015	Retrospective, single institution		Total	40	23	20	10	–	100	20	56	100
			–	III	11	6	6	4		100	0	67	Null
				IV	29	17	14	6		100	25	50	100
Marti et al. (45)	2015	Retrospective, multicenter	–	III: 103 IV: 62	165	104	70	27	–	100	16	43	100
Brauner et al. (46)	2015	Retrospective, multicenter	–	III, IV, and Hurthle Cell	72	45	47	6	–	100	8	14	100

(*Continued*)

TABLE 2 | Continued

Author	Year	Study type	Follow-up period	Bethesda category	Sample size	GEC suspicious	Total surgery	Malignant nodules	NIFTP	Diagnostic value			
										Se (%)	Sp (%)	PPV (%)	NPV (%)
McIver et al. (47)	2014	Prospective, single institution	9.5 months	Total	72	44	36	6	–	83	10	16	75
				III	9	–	5	1		100	0	20	Null
				IV	63	–	31	5		80	12	15	75
Lastra et al. (48)	2014	Retrospective, single institution	–	Total	132	62	50	22	–	100	7	54	100
				III	68	23	18	11		100	0	61	Null
				IV	64	39	32	11		100	10	37	100
Aragon Han et al. (49)	2014	Retrospective, single institution	–	III, IV	37	36	37	16	–	100	5	44	100
Alexander et al. (50)	2013	Retrospective, multicenter	8.5 months	Total	339	148	132	54*	–	98	13	44	91
				III	165	66	48	23		–	–	–	–
				IV	79	73	65	24		–	–	–	–
				V	13	9	8	6		–	–	–	–
Harrell et al. (51)	2013	Retrospective, single institution		Total	58	36	35	18	–	94	24	57	80
			–	III	–	22	–	–		100	33	64	100
				IV	–	8	–	–		75	0	38	0

TABLE 3 | Thyroseq V2 literature review.

Author	Year	Study type	Follow-up period	Bethesda category	Sample size	Thyroseq suspicious	Total surgery	Malignant nodules	NIFT-P	Diagnostic value			
										Se (%)	Sp (%)	PPV (%)	NPV (%)
ThyroSeq® v2 (53)	2015	Retrospective, Single Institution	–	III	465	31	95	22	–	91	92	77	97
ThyroSeq® v2 (52)	2014	Single Institution	–	IV	143	42	143	39	–	90	93	83	96
Taye et al. (54)	2017	Prospective, Multicenter	–	III, IV	156	51	63	10	3	91	45	27	96
Valderrabano et al. (55)	2017	Retrospective, Single Institution	–	Total	190	45	102	15	5	70	77	42	91
				III	104	22	52	5	2	43	71	19	89
				IV	86	23	50	10	3	85	84	65	94
Shrestha et al. (56)	2016	Retrospective, Single Institution	–	Total	39	23	39	14	–	93	60	57	94
				III	27	15	27	9		89	61	53	92
				IV	12	8	12	5		100	57	63	100
Khatami et al. (57)	2016	Retrospective, Single Institution	3–6 months	III, IV, V	42	7	7	4	–	–	–	57	–
Toraldo et al. (58)	2016	Prospective, Single Institution	–	III, IV	148	51	45	20	–	95	60	66	94
Shrestha et al. (59)	2016	Retrospective, Single Institution	–	III	41	–	41	–	–	83	62	90	48
				IV	14	–	14	–	–	80	56	50	90

For these reasons, patient benefit from molecular marker use in routine clinical practice is likely marginal. Moreover, the AACE 2016 guidelines recommend molecular testing to complement cytologic evaluation in indeterminate nodules (Grade A recommendation), but only when the "results are expected to influence clinical management" (Grade A recommendation) (9). Testing for detection of *BRAF*, *RET/PTC*, *PAX8/PPRG*, and *RAS* mutations can be considered (Grade B recommendation). Furthermore, with the exception of *BRAF V600E*, there is insufficient evidence "to recommend in favor of or against the use of mutation testing as a guide to determine the extent of surgery" (Grade A recommendation) (9).

Importantly, molecular testing is not recommended in patients with an indeterminate thyroid nodule if other indications for surgery are present such as a nodule greater than 4 cm, compressive symptoms, or personal preference (63). The utility of molecular testing in Bethesda Type V nodules at institutions with a high prevalence of malignancy is low, and provides little additional benefit from a "positive" test result due to the similar PPV as that of a Bethesda Type V FNA result. Moreover, a diagnostic lobectomy would still be recommended in the case of a "negative" result. Finally, a limitation of the current molecular markers is their insufficient data to recommend use among pediatric patients (≤18 years) (64). Until these tests can be validated using this patient population, they cannot be routinely used to complement the indeterminate FNA cytology results.

CURRENT PUBLISHED LITERATURE

Current published literature regarding Afirma® and Thyroseq® V2 validation studies are summarized in **Tables 2** and **3**. The data were summarized from the results of a PubMed search for English language studies that reported diagnostic accuracy in observational clinical settings published for GEC and Thyroseq® V2 up to November 30, 2017. References from the retrieved articles were also searched for additional studies. Inclusion criteria included reporting molecular marker diagnostic accuracy or enough information to calculate sensitivity, specificity, NPV, and PPV among Bethesda Type III or IV lesions. All calculations were made using the available published information. To adhere to current clinical guidelines, non-invasive follicular thyroid neoplasm with papillary-like nuclear features (NIFTP) and malignant pathologies were categorized as malignant or "requiring resection." Of the published literature, only two studies included pediatric patients in their cohort (31, 32).

Among Afirma® GEC studies, Se, Sp, PPV, and NPV ranged from 75 to 100%, 5 to 53%, 13 to 100%, and 20 to 100%, respectively. Among Thyroseq® V2 studies, Se, Sp, PPV, and NPV ranged from 40 to 100%, 56 to 93%, 13 to 90%, and 48 to 97%, respectively. Valderrabano et al. report that the wide variation among reported diagnostic values can be explained by different defining characteristics of the study populations such as institutional prevalence of malignancy sample size, Bethesda Type included or combination thereof used, the proportions of each Bethesda Type, the definition of "benign" used in the study, and Hürthle cell (HC) predominance (65). Furthermore, among post-validation studies, the molecular test outcome itself influenced the clinical management.

Supporting this, numerous studies have reported a lower specificity or higher false positive rate in GEC tests among indeterminate nodules with HC predominance. Brauner et al. reported 86% (37/43) patients with a GEC suspicious result had unnecessary surgery (46). The authors include a grouped cohort analysis of 122 HC predominant nodules between 2012 and 2014, showing 85 of 95 (89.5%) benign pathologies identified as GEC suspicious (46). Another study by Parajuli et al. reported on GEC's increase in false positive rate among HC predominant nodules, but did not observe the same increase in Thyroseq® V2 (66). Additional studies assessing Thyroseq®'s performance in HC predominant nodules are lacking.

One component of GEC, the Medullary Thyroid Cancer (MTC) Classifier, has been far less studied (**Table 4**). Among the few existing studies, Se, Sp, PPV, and NPV ranged from 91 to 100%, 100%, 98 to 100%, and 99 to 100%, respectively. Further evaluation of the MTC Classifier is needed to establish its clinical efficacy.

CURRENT CHALLENGES OF MOLECULAR MARKER ASSAYS

Current challenges to the application of molecular markers are fourfold: (A) the recent introduction of the histopathological diagnosis NIFTP, (B) the correlation of genetic mutations within both benign and malignant pathologic diagnoses, (C) the lack of follow-up of molecular marker negative nodules, and (D) the cost-effectiveness of molecular markers.

Non-Invasive Follicular Thyroid Neoplasm With Papillary-Like Nuclear Features

In March 2015, Nikiforov et al. introduced the new histopathological term NIFTP, previously known as encapsulated follicular

TABLE 4 | Medullary thyroid cancer (MTC) classifier and diagnostic value.

Author	Year	Study type	Follow-up period	Bethesda category	Sample size	MTC suspicious	Surgery	MTC prev.	Diagnostic value			
									Se (%)	Sp (%)	PPV (%)	NPV (%)
Pankratz (67)	2016	Retrospective, single institution	–	–	27	26	27	27	96	–	–	–
Kloos (68)	2016	Prospective, blinded multicenter	–	III–VI	10,488	43	43	42	–	–	98	–
Alexander et al. (30)	2012	Prospective, blinded multicenter	301 days (median)	III–VI	441	4	441	4	100	100	100	100
Training set (69)	2010	Prospective, blinded multicenter	–	–	220	22	220	20	91	100	100	99
Chudova et al. (69)	2010	Prospective, blinded multicenter	–	I–VI	48	0	48	0	–	100	–	99

variant of papillary thyroid cancer, representing an indolent entity with very low risk of recurrence (70). Major diagnostic characteristics of NIFTP include features of FVPTC, such as a follicular growth pattern and nuclear features of PTC (enlargement, crowding, elongation, irregular contours, grooves, pseudoinclusions, and chromatin clearing), but a lack of vascular or capsular invasion, key features differentiating NIFTP from FVPTC.

This new diagnosis represents a dramatic shift in thyroid pathology where an estimated 61% of lesions previously classified as FVPTCs will now be classified as NIFTP, thus decreasing the percentage of "malignancies" on final pathology compared with FNA. On pre-operative cytology, NIFTP is associated with FNA Bethesda Category III, IV, V, or VI in 15, 56, 27, and 2% of tumor samples, respectively (71). As a consequence, this has created a shift in the malignancy rate associated with each Bethesda category. Strickland et al. evaluated a cohort of 655 FNAs with subsequent resection specimens (72). When taking into account the new NIFTP diagnosis, indeterminate, and suspicious FNA samples of Bethesda III, IV, and V had an absolute decrease in rate of malignancy by 17.6, 8.0, and 41.5%, respectively (72). Similarly, Faquin et al. reported an absolute decrease in rate of malignancy in Bethesda III, IV, and V by 13.6, 15.1, and 23.4%, respectively (73).

Despite NIFTP's extremely low-recurrence rate of 0.6% (two cases), there remains disagreement regarding NIFTP's true malignant potential (74–80). Despite its likely benign and, at worst, indolent nature, current ATA guidelines recommend lobectomy as definitive therapy for NIFTP. More importantly, however, and, apropos of this review, Afirma® GEC and Thyroseq® V2 validation studies occurred before the establishment of NIFTP as a distinct entity. Because of this one needs to be circumspect about the real utility of these marker panels. And, as a consequence, these molecular diagnostic panels require recalibration to appropriately account for the newly introduced entity, NIFTP; a lesion that should likely not be considered malignant (70, 81, 82).

Correlating Mutations to Pathology

The correlation between presence of mutations and malignancy is imprecise. Among 967 Bethesda Type III, IV, and V nodules, the detection of any mutation conferred the risk of histologic malignancy of 88, 87, and 95%, respectively (83). However, even in nodules with no detected mutations, the malignancy rates were 6, 14, and 28%, respectively. A systematic review by our group included 8,162 patients, of whom 42.5% had benign lesions (84). Among the benign lesions, *RAS* mutations, *RET/PTC* rearrangements, and *PAX8/PPAR-gamma* rearrangements were present up to 48, 68, 55% of the time, respectively. Thus, benign nodules frequently harbor mutations, while some malignant lesions harbor no detected mutations. The combination of the variable and potentially high level of mutations among benign nodules may explain the low specificity and PPV seen in Afirma. Furthermore, their prominence in benign lesions may also challenge the reported PPV of Thyroseq V2.

This issue is further complicated when an indolent tumor, such as NIFTP, should be resected according to current ATA guidelines. NIFTP is commonly associated with *RAS* mutations (8/27, 29.6%) and its diagnosis is incompatible with the presence of *BRAF V600E* mutations (70, 79). Moreover, Nikiforov et al. describe that 22% (6/27) of NIFTP samples harbor no detectable mutations. To conform to the recommendation that this indolent lesion be resected, new validation studies must show the reliable identification of NIFTP by molecular markers, an unlikely occurrence given the fact that benign lesions also harbor them.

Molecular Marker Negative Nodule Follow-Up

Despite a number of studies exploring the diagnostic value of GEC and Thyroseq® V2, the current published literature includes discrepancies in the follow-up of molecular marker negative nodules and their consideration as a benign pathology (85, 86). Consequently, this may lead to inaccuracies in diagnostic value calculation. A systematic review by Duh et al. (85) highlighted these issues. They included 12 studies and discussed the exclusion of cytologically indeterminate, GEC benign nodules from diagnostic performance calculations (malignant versus benign), leading to an erroneous decrease in Sp and NPV. This is due to the lack of surgical pathology specimens to establish a definitive diagnosis, as well as a lack of follow-up of GEC benign nodules to establish a reference diagnosis. To establish a diagnostic "reference standard" in these nodules that have not undergone surgery and include them in calculations, the authors argue that they should be considered as "true negative" only if no suspicious changes are noted on scheduled interval ultrasound examinations. However, even the natural history of benign thyroid nodules has been described to involve size changes. Indeed, a 5-year prospective study involving 1,567 sonographically or cytologically benign thyroid nodules showed nodule growth in 11.1% (87). However, thyroid cancer was diagnosed in five original nodules (0.3%), of which, only two had an increase in size. Furthermore, a retrospective study ranging from 1 month to 5 years, reported that 39% of the 268 benign thyroid nodules showed at least a 15% change in nodule volume (88). Only one of the 74 repeat-FNAs was malignant. The authors conclude that an increase in nodule volume alone is not a reliable predictor of malignancy.

Two studies have described their experience with follow-up of GEC benign nodules on ultrasound. A study by Angel et al. including 56 patients with cytologically indeterminate, GEC benign nodules followed for a median of 13 months exhibited similar growth (≥20% in two dimensions or ≥50% in volume) to cytologically benign nodules (86). Furthermore, in a grouped cohort analysis by Kloos et al. of 443 GEC benign patients in six studies with a reported follow-up time of 7–26 months, 380 patients (85.8%) were spared unnecessary surgery (89). Clearly, the currently available follow-up periods are inadequate for a definitive assessment, and larger, prospective studies are needed to further evaluate the behavior of cytologically indeterminate,

TABLE 5 | Molecular marker cost.

	Afirma®	ThyroSeq V2
Cost	$4,875 (Afirma® GEC & MTC) $975 (Afirma® MTC) $475 (Afirma® BRAF)	$3,200
Patient insurance coverage	$300 (Afirma® GEC & MTC) $80 (Afirma® MTC) $50 (Afirma® BRAF)	$300
Estimated cost-effectiveness	Standard of care $12,172 versus GEC $10,719 (91) Standard of care $11,505 versus GEC $13,027 (92)	Standard of care $11,505 versus Thyroseq® cost $7,683 (92)

molecular marker "negative" thyroid nodules to help guide recommendations for management.

Cost-Effectiveness

The cost-effectiveness of GEC and Thyroseq® has also been an intense area of research. The cost for GEC and MTC is $4,875 while the cost for Afirma® MTC alone is $975 and that of Afirma® BRAF is $475 (**Table 5**). The cost of Thyroseq® V2 is $3200 (29). Despite these high costs, insured patient costs are capped at $300 for either GEC or ThyroSeq® V2. Numerous studies have reported on the cost-effectiveness of both GEC (90, 91) and Thyroseq® V2 (92).

A 5-year cost effectiveness study of routine use of GEC reported 74% fewer operations for benign nodules with no increase in untreated cancers. Compared with standard clinical management based only on indeterminate FNA results, GEC may lower overall costs (standard cost $12,172 versus GEC cost $10,719) and improve quality of life for patients (91). Another study reported that to be cost effective, GEC's specificity would have to be greater than 68% and decrease the number of unnecessary surgeries performed on benign nodules by more 50% (90). However, a study by Yip et al. compared the average cost per patient with Bethesda IV nodules larger than 1 cm extending 10 years from follicular neoplasm diagnosis in three groups: standard of care, GEC, and Thyroseq®. The authors reported a 13% increase in average cost per patient when using GEC at $13,027 (range $12,373–$13,666) when compared with the standard of care $11,505 (range $10,676–$12,347), but a 30% reduction in those using Thyroseq® cost $7,683 (range $7,174–$8,333) (92).

Despite the conflicting results of GEC cost-effectiveness studies and the paucity of analyses on Thyroseq®, these studies highlight the need to closely examine cost-effectiveness with the use of genetic studies. However, the true impact of thyroid molecular tests on a population's health care costs can only be determined taking cancer prevalence into account. Furthermore, cost-effectiveness also depends on proper education to ensure that these tests are only used in indicated clinical settings.

CONCLUSION AND FUTURE DIRECTION

Molecular markers are a rapidly developing field despite the current limitations. Veracyte and CBL Path have begun to address the challenges discussed here. In 2017, Veracyte launched the Afirma® Genomic Sequencing Classifier (GSC), its newest product which tests a total of 10,196 genes. The Afirma® GSC test system is composed of a series of classifiers including parathyroid (mRNA expression), MTC (mRNA expression), *BRAF* (mRNA expression + variants), and *RET/PTC* fusion (fusion transcripts). GSC also includes a follicular content index (mRNA expression), HC index (mRNA expression and mitochondrial transcripts), and Hürthle neoplasm index (mRNA expression and chromosomal level loss of heterozygosity). The reliance on mRNA could prevent detection of mutations undetectable in transcriptome-based assays, such as telomerase reverse transcriptase (hTERT) promoter mutations. Veracyte states that GSC addresses the weaknesses of GEC by significantly increasing molecular test specificity, using a validation cohort with 15 NIFTP samples, and improving the diagnostic performance among HC lesions. Among Bethesda Type III and IV nodules, Veracyte quotes a Se, Sp, NPV, and PPV of 91, 68, 96, and 50%, respectively, when compared with the GEC parameters of 89 50, 93, and 46%, respectively. Among HC lesions, Sp has increased from 11.8 to 58.8%.

Similarly, in 2017 CBL Path sought to address similar issues with the release of Thyroseq® V3 (93). It has an expanded its assay from 56 to 112 genes, detecting mutations, gene fusions, gene expression alterations, and copy number variations. In a prospective double-blind multicenter study using 257 nodules with Bethesda Types III–V (including 11 NIFTP samples, 10 HC carcinomas, 34 HC adenomas, and 5 hyperplastic nodules with HC predominance), Thyroseq® V3 is reported to have an increased diagnostic value, including a Se of 98% (from 96.9%) and specificity of 81.8% (from 74.0%). Moreover, HC samples had an Se and Sp of 100.0 and 66.7%, respectively.

As experience accumulates with these next generation tests, we will gain a better understanding of how well they mitigate the limitations and challenges addressed herein. As our understanding of genetic drivers of malignant cancers and our understanding of NIFTP's malignant potential becomes clearer, molecular markers will continue to more accurately identify malignant nodules as well as to spare patients from unnecessary surgery.

AUTHOR CONTRIBUTIONS

ZS conducted the literature review. ZS, PS, and CU drafted and revised the manuscript. MZ drafted and revised the manuscript and, as the senior and corresponding author, takes full responsibility.

REFERENCES

1. Gharib H. Changing trends in thyroid practice: understanding nodular thyroid disease. *Endocr Pract* (2004) 10(1):31–9. doi:10.4158/EP.10.1.31

2. Guth S, Theune U, Aberle J, Galach A, Bamberger C. Very high prevalence of thyroid nodules detected by high frequency (13 MHz) ultrasound examination. *Eur J Clin Invest* (2009) 39(8):699–706. doi:10.1111/j.1365-2362.2009.02162.x

3. Howlader N, Noone A, Krapcho M, Miller D, Bishop K, Altekruse S, et al. *SEER Cancer Statistics Review, 1975–2013*. Bethesda, MD: National Cancer Institute (2016).
4. Hahn SY, Shin JH, Lim HK, Jung SL, Oh YL, Choi IH, et al. Preoperative differentiation between noninvasive follicular thyroid neoplasm with papillary-like nuclear features (NIFTP) and non-NIFTP. *Clin Endocrinol* (2017) 86(3):444–50. doi:10.1111/cen.13263
5. Brito JP, Yarur AJ, Prokop LJ, McIver B, Murad MH, Montori VM. Prevalence of thyroid cancer in multinodular goiter versus single nodule: a systematic review and meta-analysis. *Thyroid* (2013) 23(4):449–55. doi:10.1089/thy.2012.0156
6. Nguyen G-K, Lee MW, Ginsberg J, Wragg T, Bilodeau D. Fine-needle aspiration of the thyroid: an overview. *Cytojournal* (2005) 2(1):12. doi:10.1186/1742-6413-2-12
7. Werga P, Wallin G, Skoog L, Hamberger B. Expanding role of fine-needle aspiration cytology in thyroid diagnosis and management. *World J Surg* (2000) 24(8):907–12. doi:10.1007/s002680010163
8. Gharib H, Goellner J, Johnson D. Fine-needle aspiration cytology of the thyroid. A 12-year experience with 11,000 biopsies. *Clin Lab Med* (1993) 13(3):699–709.
9. Gharib H, Papini E, Garber J, Duick D, Harrell R, Hegedüs L, et al. American Association of Clinical Endocrinologists, American College of Endocrinology, and Associazione Medici Endocrinologi Medical guidelines for clinical practice for the diagnosis and management of thyroid nodules—2016 update. *Endocr Pract* (2016) 22(5):622–39. doi:10.4158/EP161208.GL
10. Cibas ES, Ali SZ. The 2017 Bethesda system for reporting thyroid cytopathology. *J Am Soc Cytopathol* (2017) 6(6):217–22. doi:10.1016/j.jasc.2017.09.002
11. Bongiovanni M, Spitale A, Faquin WC, Mazzucchelli L, Baloch ZW. The Bethesda system for reporting thyroid cytopathology: a meta-analysis. *Acta Cytol* (2012) 56(4):333–9. doi:10.1159/000339959
12. Cibas ES, Ali SZ. The Bethesda system for reporting thyroid cytopathology. *Am J Clin Pathol* (2009) 132(5):658–65. doi:10.1309/AJCPPHLWMI3JV4LA
13. Baloch ZW, LiVolsi VA, Asa SL, Rosai J, Merino MJ, Randolph G, et al. Diagnostic terminology and morphologic criteria for cytologic diagnosis of thyroid lesions: a synopsis of the National Cancer Institute thyroid fine-needle aspiration state of the science conference. *Diagn Cytopathol* (2008) 36(6):425–37. doi:10.1002/dc.20830
14. Mazzaferri EL. Management of a solitary thyroid nodule. *N Engl J Med* (1993) 328(8):553–9. doi:10.1056/NEJM199302253280807
15. Cancer Genome Atlas Research Network. Integrated genomic characterization of papillary thyroid carcinoma. *Cell* (2014) 159(3):676–90. doi:10.1016/j.cell.2014.09.050
16. Cohen Y, Xing M, Mambo E, Guo Z, Wu G, Trink B, et al. BRAF mutation in papillary thyroid carcinoma. *J Natl Cancer Inst* (2003) 95(8):625–7. doi:10.1093/jnci/95.8.625
17. Pusztaszeri MP, Krane JF, Faquin WC. BRAF testing and thyroid FNA. *Cancer Cytopathol* (2015) 123(12):689–95. doi:10.1002/cncy.21614
18. Cohen Y, Rosenbaum E, Clark DP, Zeiger MA, Umbricht CB, Tufano RP, et al. Mutational analysis of BRAF in fine needle aspiration biopsies of the thyroid: a potential application for the preoperative assessment of thyroid nodules. *Clin Cancer Res* (2004) 10(8):2761–5. doi:10.1158/1078-0432.CCR-03-0273
19. Xing M, Alzahrani AS, Carson KA, Viola D, Elisei R, Bendlova B, et al. Association between BRAF V600E mutation and mortality in patients with papillary thyroid cancer. *JAMA* (2013) 309(14):1493–501. doi:10.1001/jama.2013.3190
20. Kebebew E, Weng J, Bauer J, Ranvier G, Clark OH, Duh Q-Y, et al. The prevalence and prognostic value of BRAF mutation in thyroid cancer. *Ann Surg* (2007) 246(3):466. doi:10.1097/SLA.0b013e318148563d
21. Xing M, Westra WH, Tufano RP, Cohen Y, Rosenbaum E, Rhoden KJ, et al. BRAF mutation predicts a poorer clinical prognosis for papillary thyroid cancer. *J Clin Endocrinol Metab* (2005) 90(12):6373–9. doi:10.1210/jc.2005-0987
22. Fugazzola L, Puxeddu E, Avenia N, Romei C, Cirello V, Cavaliere A, et al. Correlation between B-RAFV600E mutation and clinico-pathologic parameters in papillary thyroid carcinoma: data from a multicentric Italian study and review of the literature. *Endocr Relat Cancer* (2006) 13(2):455–64. doi:10.1677/erc.1.01086
23. Liu R, Bishop J, Zhu G, Zhang T, Ladenson PW, Xing M. Mortality risk stratification by combining BRAF V600E and TERT promoter mutations in papillary thyroid cancer: genetic duet of BRAF and TERT promoter mutations in thyroid cancer mortality. *JAMA Oncol* (2017) 3(2):202–8. doi:10.1001/jamaoncol.2016.3288
24. Liu X, Qu S, Liu R, Sheng C, Shi X, Zhu G, et al. TERT promoter mutations and their association with BRAF V600E mutation and aggressive clinicopathological characteristics of thyroid cancer. *J Clin Endocrinol Metab* (2014) 99(6):E1130–6. doi:10.1210/jc.2013-4048
25. Kim KB, Cabanillas ME, Lazar AJ, Williams MD, Sanders DL, Ilagan JL, et al. Clinical responses to vemurafenib in patients with metastatic papillary thyroid cancer harboring BRAFV600E mutation. *Thyroid* (2013) 23(10):1277–83. doi:10.1089/thy.2013.0057
26. Falchook GS, Millward M, Hong D, Naing A, Piha-Paul S, Waguespack SG, et al. BRAF inhibitor dabrafenib in patients with metastatic BRAF-mutant thyroid cancer. *Thyroid* (2015) 25(1):71–7. doi:10.1089/thy.2014.0123
27. Brose MS, Nutting CM, Jarzab B, Elisei R, Siena S, Bastholt L, et al. Sorafenib in radioactive iodine-refractory, locally advanced or metastatic differentiated thyroid cancer: a randomised, double-blind, phase 3 trial. *Lancet* (2014) 384(9940):319–28. doi:10.1016/S0140-6736(14)60421-9
28. Zhang M, Lin O. Molecular testing of thyroid nodules: a review of current available tests for fine-needle aspiration specimens. *Arch Pathol Lab Med* (2016) 140(12):1338–44. doi:10.5858/arpa.2016-0100-RA
29. Nishino M. Molecular cytopathology for thyroid nodules: a review of methodology and test performance. *Cancer Cytopathol* (2016) 124(1):14–27. doi:10.1002/cncy.21612
30. Alexander EK, Kennedy GC, Baloch ZW, Cibas ES, Chudova D, Diggans J, et al. Preoperative diagnosis of benign thyroid nodules with indeterminate cytology. *N Engl J Med* (2012) 367(8):705–15. doi:10.1056/NEJMoa1203208
31. Hang J-F, Westra WH, Cooper DS, Ali SZ. The impact of noninvasive follicular 1 thyroid neoplasm with papillary-like nuclear features on the performance of Afirma gene expression classifier. *Cancer Cytopathol* (2017) 125(9):683–91. doi:10.1002/cncy.21879
32. Harrison G, Sosa JA, Jiang X. Evaluation of the Afirma gene expression classifier in repeat indeterminate thyroid nodules. *Arch Pathol Lab Med* (2017) 141(7):985–9. doi:10.5858/arpa.2016-0328-OA
33. Kay-Rivest E, Tibbo J, Bouhabel S, Tamilia M, Leboeuf R, Forest V-I, et al. The first Canadian experience with the Afirma® gene expression classifier test. *J Otolaryngol Head Neck Surg* (2017) 46(1):25. doi:10.1186/s40463-017-0201-7
34. Samulski TD, LiVolsi VA, Wong LQ, Baloch Z. Usage trends and performance characteristics of a "gene expression classifier" in the management of thyroid nodules: an institutional experience. *Diagn Cytopathol* (2016) 44(11):867–73. doi:10.1002/dc.23559
35. Wu JX, Young S, Hung ML, Li N, Yang SE, Cheung DS, et al. Clinical factors influencing the performance of gene expression classifier testing in indeterminate thyroid nodules. *Thyroid* (2016) 26(7):916–22. doi:10.1089/thy.2015.0505
36. Yang SE, Sullivan PS, Zhang J, Govind R, Levin MR, Rao JY, et al. Has Afirma gene expression classifier testing refined the indeterminate thyroid category in cytology? *Cancer Cytopathol* (2016) 124(2):100–9. doi:10.1002/cncy.21624
37. Chaudhary S, Hou Y, Shen R, Hooda S, Li Z. Impact of the Afirma gene expression classifier result on the surgical management of thyroid nodules with category III/IV cytology and its correlation with surgical outcome. *Acta Cytol* (2016) 60(3):205–10. doi:10.1159/000446797
38. Abeykoon JP, Mueller L, Dong F, Chintakuntlawar AV, Paludo J, Mortada R. The effect of implementing gene expression classifier on outcomes of thyroid nodules with indeterminate cytology. *Horm Cancer* (2016) 7(4):272–8. doi:10.1007/s12672-016-0263-4
39. Noureldine SI, Najafian A, Han PA, Olson MT, Genther DJ, Schneider EB, et al. Evaluation of the effect of diagnostic molecular testing on the surgical decision-making process for patients with thyroid nodules. *JAMA Otolaryngol Head Neck Surg* (2016) 142(7):676–82. doi:10.1001/jamaoto.2016.0850
40. Al-Qurayshi Z, Deniwar A, Thethi T, Mallik T, Srivastav S, Murad F, et al. Association of malignancy prevalence with test properties and performance of the gene expression classifier in indeterminate thyroid nodules. *JAMA Otolaryngol Head Neck Surg* (2016) 143(4):403–8. doi:10.1001/jamaoto.2016.3526
41. Witt RL. Outcome of thyroid gene expression classifier testing in clinical practice. *Laryngoscope* (2016) 126(2):524–7. doi:10.1002/lary.25607

42. Wong KS, Angell TE, Strickland KC, Alexander EK, Cibas ES, Krane JF, et al. Noninvasive follicular variant of papillary thyroid carcinoma and the Afirma gene-expression classifier. *Thyroid* (2016) 26(7):911–5. doi:10.1089/thy.2015.0644
43. Zhu Q-L, Faquin WC, Samir AE. Relationship between sonographic characteristics and Afirma gene expression classifier results in thyroid nodules with indeterminate fine-needle aspiration cytopathology. *AJR Am J Roentgenol* (2015) 205(4):861–5. doi:10.2214/AJR.14.13984
44. Celik B, Whetsell CR, Nassar A. Afirma GEC and thyroid lesions: an institutional experience. *Diagn Cytopathol* (2015) 43(12):966–70. doi:10.1002/dc.23378
45. Marti JL, Avadhani V, Donatelli LA, Niyogi S, Wang B, Wong RJ, et al. Wide inter-institutional variation in performance of a molecular classifier for indeterminate thyroid nodules. *Ann Surg Oncol* (2015) 22(12):3996–4001. doi:10.1245/s10434-015-4486-3
46. Brauner E, Holmes BJ, Krane JF, Nishino M, Zurakowski D, Hennessey JV, et al. Performance of the Afirma gene expression classifier in Hürthle cell thyroid nodules differs from other indeterminate thyroid nodules. *Thyroid* (2015) 25(7):789–96. doi:10.1089/thy.2015.0049
47. McIver B, Castro MR, Morris JC, Bernet V, Smallridge R, Henry M, et al. An independent study of a gene expression classifier (Afirma) in the evaluation of cytologically indeterminate thyroid nodules. *J Clin Endocrinol Metab* (2014) 99(11):4069–77. doi:10.1210/jc.2013-3584
48. Lastra RR, Pramick MR, Crammer CJ, LiVolsi VA, Baloch ZW. Implications of a suspicious Afirma test result in thyroid fine-needle aspiration cytology: an institutional experience. *Cancer Cytopathol* (2014) 122(10):737–44. doi:10.1002/cncy.21455
49. Han PA, Olson MT, Fazeli R, Prescott JD, Pai SI, Schneider EB, et al. The impact of molecular testing on the surgical management of patients with thyroid nodules. *Ann Surg Oncol* (2014) 21(6):1862–9. doi:10.1245/s10434-014-3508-x
50. Alexander EK, Schorr M, Klopper J, Kim C, Sipos J, Nabhan F, et al. Multicenter clinical experience with the Afirma gene expression classifier. *J Clin Endocrinol Metab* (2013) 99(1):119–25. doi:10.1210/jc.2013-2482
51. Harrell R, Bimston D. Surgical utility of Afirma: effects of high cancer prevalence and oncocytic cell types in patients with indeterminate thyroid cytology. *Endocr Pract* (2013) 20(4):364–9. doi:10.4158/EP13330.OR
52. Nikiforov YE, Carty SE, Chiosea SI, Coyne C, Duvvuri U, Ferris RL, et al. Highly accurate diagnosis of cancer in thyroid nodules with follicular neoplasm/suspicious for a follicular neoplasm cytology by ThyroSeq v2 next-generation sequencing assay. *Cancer* (2014) 120(23):3627–34. doi:10.1002/cncr.29038
53. Nikiforov YE, Carty SE, Chiosea SI, Coyne C, Duvvuri U, Ferris RL, et al. Impact of the multi-gene ThyroSeq next-generation sequencing assay on cancer diagnosis in thyroid nodules with atypia of undetermined significance/follicular lesion of undetermined significance cytology. *Thyroid* (2015) 25(11):1217–23. doi:10.1089/thy.2015.0305
54. Taye A, Gurciullo D, Miles BA, Gupta A, Owen RP, Inabnet WB, et al. Clinical performance of a next-generation sequencing assay (ThyroSeq v2) in the evaluation of indeterminate thyroid nodules. *Surgery* (2018) 163(1):97–103. doi:10.1016/j.surg.2017.07.032
55. Valderrabano P, Khazai L, Leon ME, Thompson ZJ, Ma Z, Chung CH, et al. Evaluation of ThyroSeq v2 performance in thyroid nodules with indeterminate cytology. *Endocr Relat Cancer* (2017) 24(3):127–36. doi:10.1530/ERC-16-0512
56. Shrestha RT, Evasovich MR, Amin K, Radulescu A, Sanghvi TS, Nelson AC, et al. Correlation between histological diagnosis and mutational panel testing of thyroid nodules: a two-year institutional experience. *Thyroid* (2016) 26(8):1068–76. doi:10.1089/thy.2016.0048
57. Khatami R, Heimann A, Hwang S, Shroyer K, Wu M. ThyroSeq V2 application study for indeterminate thyroid FNAs with surgical follow-up; experience at a university hospital. *Am J Clin Pathol* (2016) 5(5):S80–1. doi:10.1093/ajcp/aqw165.018
58. Tolerado G, Godley FA, Cerda SR, Mcaneny D, Doherty GM, Braverman L, et al. *Large Independent Prospective Study to Evaluate the Performance of ThyroSeq2 Multigene Next Generation Sequencing Panel Analysis on Cancer Diagnosis in Thyroid Nodules with Indeterminate Cytopathology*. ThyroSeq Press Release: ThyroSeq (2016). Report No.: 1050–7256.
59. Shrestha RT, Evasovich MR, Shahi M, Radulescu AI, Amin K, Burmeister L. Surgical outcomes of Thyroseq® markers at the university of Minnesota. *Endocrine Society*. Boston (2016).
60. Haugen BR, Alexander EK, Bible KC, Doherty GM, Mandel SJ, Nikiforov YE, et al. 2015 American Thyroid Association management guidelines for adult patients with thyroid nodules and differentiated thyroid cancer: the American Thyroid Association guidelines task force on thyroid nodules and differentiated thyroid cancer. *Thyroid* (2016) 26(1):1–133. doi:10.1089/thy.2015.0020
61. Dedhia PH, Rubio GA, Cohen MS, Miller BS, Gauger PG, Hughes DT. Potential effects of molecular testing of indeterminate thyroid nodule fine needle aspiration biopsy on thyroidectomy volume. *World J Surg* (2014) 38(3):634–8. doi:10.1007/s00268-013-2430-x
62. Noureldine SI, Olson MT, Agrawal N, Prescott JD, Zeiger MA, Tufano RP. Effect of gene expression classifier molecular testing on the surgical decision-making process for patients with thyroid nodules. *JAMA Otolaryngol Head Neck Surg* (2015) 141(12):1082–8. doi:10.1001/jamaoto.2015.2708
63. Ferris RL, Baloch Z, Bernet V, Chen A, Fahey TJ III, Ganly I, et al. American thyroid association statement on surgical application of molecular profiling for thyroid nodules: current impact on perioperative decision making. *Thyroid* (2015) 25(7):760–8. doi:10.1089/thy.2014.0502
64. Francis GL, Waguespack SG, Bauer AJ, Angelos P, Benvenga S, Cerutti JM, et al. Management guidelines for children with thyroid nodules and differentiated thyroid cancer: the American Thyroid Association guidelines task force on pediatric thyroid cancer. *Thyroid* (2015) 25(7):716–59. doi:10.1089/thy.2014.0460
65. Valderrabano P, Leon ME, Centeno BA, Otto KJ, Khazai L, McCaffrey JC, et al. Institutional prevalence of malignancy of indeterminate thyroid cytology is necessary but insufficient to accurately interpret molecular marker tests. *Eur J Endocrinol* (2016) 174(5):621–9. doi:10.1530/EJE-15-1163
66. Parajuli S, Jug R, Ahmadi S, Jiang X. Hurthle cell predominance impacts results of GEC and molecular panel performance in indeterminate thyroid nodules. *J Am Soc Cytopathol* (2017) 6(5):S70–1. doi:10.1016/j.jasc.2017.06.175
67. Pankratz DG, Hu Z, Kim SY, Monroe RJ, Wong MG, Traweek ST, et al. Analytical performance of a gene expression classifier for medullary thyroid carcinoma. *Thyroid* (2016) 26(11):1573–80. doi:10.1089/thy.2016.0262
68. Kloos RT, Monroe RJ, Traweek ST, Lanman RB, Kennedy GC. A genomic alternative to identify medullary thyroid cancer preoperatively in thyroid nodules with indeterminate cytology. *Thyroid* (2016) 26(6):785–93. doi:10.1089/thy.2016.0001
69. Chudova D, Wilde JI, Wang ET, Wang H, Rabbee N, Egidio CM, et al. Molecular classification of thyroid nodules using high-dimensionality genomic data. *J Clin Endocrinol Metab* (2010) 95(12):5296–304. doi:10.1210/jc.2010-1087
70. Nikiforov YE, Seethala RR, Tallini G, Baloch ZW, Basolo F, Thompson LD, et al. Nomenclature revision for encapsulated follicular variant of papillary thyroid carcinoma: a paradigm shift to reduce overtreatment of indolent tumors. *JAMA Oncol* (2016) 2(8):1023–9. doi:10.1001/jamaoncol.2016.0386
71. Maletta F, Massa F, Torregrossa L, Duregon E, Casadei GP, Basolo F, et al. Cytological features of "noninvasive follicular thyroid neoplasm with papillary-like nuclear features" and their correlation with tumor histology. *Hum Pathol* (2016) 54:134–42. doi:10.1016/j.humpath.2016.03.014
72. Strickland KC, Howitt BE, Marqusee E, Alexander EK, Cibas ES, Krane JF, et al. The impact of noninvasive follicular variant of papillary thyroid carcinoma on rates of malignancy for fine-needle aspiration diagnostic categories. *Thyroid* (2015) 25(9):987–92. doi:10.1089/thy.2014.0612
73. Faquin WC, Wong LQ, Afrogheh AH, Ali SZ, Bishop JA, Bongiovanni M, et al. Impact of reclassifying noninvasive follicular variant of papillary thyroid carcinoma on the risk of malignancy in the Bethesda System for Reporting Thyroid Cytopathology. *Cancer Cytopathol* (2015) 124(3):181–7. doi:10.1002/cncy.21631
74. Piana S, Frasoldati A, Di Felice E, Gardini G, Tallini G, Rosai J. Encapsulated well-differentiated follicular-patterned thyroid carcinomas do not play a significant role in the fatality rates from thyroid carcinoma. *Am J Surg Pathol* (2010) 34(6):868–72. doi:10.1097/PAS.0b013e3181dbee07

75. Vivero M, Kraft S, Barletta JA. Risk stratification of follicular variant of papillary thyroid carcinoma. *Thyroid* (2013) 23(3):273–9. doi:10.1089/thy.2012.0369
76. Ganly I, Wang L, Tuttle RM, Katabi N, Ceballos GA, Harach HR, et al. Invasion rather than nuclear features correlates with outcome in encapsulated follicular tumors: further evidence for the reclassification of the encapsulated papillary thyroid carcinoma follicular variant. *Hum Pathol* (2015) 46(5):657–64. doi:10.1016/j.humpath.2015.01.010
77. Rosario PW, Penna GC, Calsolari MR. Noninvasive encapsulated follicular variant of papillary thyroid carcinoma: is lobectomy sufficient for tumours ≥1 cm? *Clin Endocrinol* (2014) 81(4):630–2. doi:10.1111/cen.12387
78. Baloch ZW, LiVolsi VA. Encapsulated follicular variant of papillary thyroid carcinoma with bone metastases. *Mod Pathol* (2000) 13(8):861–5. doi:10.1038/modpathol.3880153
79. Howitt BE, Paulson VA, Barletta JA. Absence of BRAF V600E in non-infiltrative, non-invasive follicular variant of papillary thyroid carcinoma. *Histopathology* (2015) 67(4):579–82. doi:10.1111/his.12680
80. Liu Z, Zhou G, Nakamura M, Koike E, Li Y, Ozaki T, et al. Encapsulated follicular thyroid tumor with equivocal nuclear changes, so-called well-differentiated tumor of uncertain malignant potential: a morphological, immunohistochemical, and molecular appraisal. *Cancer Sci* (2011) 102(1):288–94. doi:10.1111/j.1349-7006.2010.01769.x
81. Sahli ZT, Umbricht CB, Schneider EB, Zeiger MA. Thyroid nodule diagnostic markers in the face of the new diagnosis, NIFT-P: time for a reset? *Thyroid* (2017) 27(11):1393–9. doi:10.1089/thy.2017.0238
82. Jiang X, Harrison GP, Datto MB. Young investigator challenge: molecular testing in noninvasive follicular thyroid neoplasm with papillary-like nuclear features. *Cancer Cytopathol* (2016) 124(12):893–900. doi:10.1002/cncy.21802
83. Nikiforov YE, Ohori NP, Hodak SP, Carty SE, LeBeau SO, Ferris RL, et al. Impact of mutational testing on the diagnosis and management of patients with cytologically indeterminate thyroid nodules: a prospective analysis of 1056 FNA samples. *J Clin Endocrinol Metab* (2011) 96(11):3390–7. doi:10.1210/jc.2011-1469
84. Najafian A, Noureldine S, Azar F, Atallah C, Trinh G, Schneider EB, et al. RAS mutations, and RET/PTC and PAX8/PPAR-gamma chromosomal rearrangements are also prevalent in benign thyroid lesions: implications thereof and a systematic review. *Thyroid* (2017) 27(1):39–48. doi:10.1089/thy.2016.0348
85. Duh Q-Y, Busaidy NL, Rahilly-Tierney C, Gharib H, Randolph G. A systematic review of the methods of diagnostic accuracy studies of the Afirma gene expression classifier. *Thyroid* (2017) 27(10):1215–22. doi:10.1089/thy.2016.0656
86. Angell TE, Frates MC, Medici M, Liu X, Kwong N, Cibas ES, et al. Afirma benign thyroid nodules show similar growth to cytologically benign nodules during follow-up. *J Clin Endocrinol Metab* (2015) 100(11):E1477–83. doi:10.1210/jc.2015-2658
87. Durante C, Costante G, Lucisano G, Bruno R, Meringolo D, Paciaroni A, et al. The natural history of benign thyroid nodules. *JAMA* (2015) 313(9):926–35. doi:10.1001/jama.2015.0956
88. Alexander EK, Hurwitz S, Heering JP, Benson CB, Frates MC, Doubilet PM, et al. Natural history of benign solid and cystic thyroid nodules. *Ann Intern Med* (2003) 138(4):315–8. doi:10.7326/0003-4819-138-4-200302180-00010
89. Kloos RT, Hanna JW, Haroldson J, Barth N. Systematic review of clinical utility and longer term follow up of Afirma GEC testing. *Endocr Soc* (2016). doi:10.1210/endo-meetings.2016.THPTA.14.SAT-284
90. Labourier E. Utility and cost-effectiveness of molecular testing in thyroid nodules with indeterminate cytology. *Clin Endocrinol* (2016) 85(4):624–31. doi:10.1111/cen.13096
91. Li H, Robinson KA, Anton B, Saldanha IJ, Ladenson PW. Cost-effectiveness of a novel molecular test for cytologically indeterminate thyroid nodules. *J Clin Endocrinol Metab* (2011) 96(11):E1719–26. doi:10.1210/jc.2011-0459
92. Yip L, Apfel A, Singh A, Yoo J, McCoy K, Stang MT, et al. Comprehensive cost analysis of available molecular tests for thyroid nodules with follicular neoplasm cytology. *International Thyroid Congress and American Thyroid Association*. Lake Buena Vista, FL (2015).
93. Steward D, Carty S, Sippel R, Yang P, Sosa J, Sipos J, et al. Clinical validation of ThyroSeq V3® performance in thyroid nodules with indeterminate cytology: a prospective blinded multi-institutional validation study. *American Thyroid Association*. Victoria, Canada (2017).

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Endocrinology

MINI REVIEW
published: 23 May 2018
doi: 10.3389/fendo.2018.00259

Deconvoluting the Biological Roles of Vitamin D-Binding Protein During Pregnancy: A Both Clinical and Theoretical Challenge

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The teleological purpose of an ongoing pregnancy is to fulfill its fundamental role of a successful, uncomplicated delivery, in conjunction with an optimal intrauterine environment for the developing fetus. Vitamin D metabolism is adapted to meet both these demands during pregnancy; first by stimulation of calcium absorption for adequate intrauterine bone mineral accrual of the fetus, and second, by enhancing systemic and local maternal tolerance to paternal and fetal alloantigens. Vitamin D-binding protein (VDBP) is one of the key biomolecules that optimize vitamin D homeostasis and also contributes as an immune regulator for a healthy, ongoing pregnancy. In this regard, recent results indicate that dysregulation of VDBP equilibrium could be a risk factor for adverse fetal, maternal, and neonatal outcomes, including preeclampsia, preterm birth, and gestational diabetes. Moreover, it has been hypothesized to be also implicated in the interpretation of vitamin D status in the pregnant state. The aim of this review is to assess available literature regarding the association of VDBP with clinical outcomes during pregnancy, as a potential biomarker for future clinical practice, with a discourse on current knowledge gaps and future research agenda.

Keywords: vitamin D-binding protein, 25-hydroxyvitamin D, Gc-globulin, pregnancy, clinical outcomes, polymorphisms

OPEN ACCESS

Edited by:
Derek LeRoith, Icahn School of Medicine at Mount Sinai, United States

Reviewed by:
Ludwig Wildt, Innsbruck Medical University, Austria
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Specialty section:
This article was submitted to Reproduction, a section of the journal Frontiers in Endocrinology

Received: *11 March 2018*
Accepted: *07 May 2018*
Published: *23 May 2018*

Citation:
Karras SN, Koufakis T, Fakhoury H and Kotsa K (2018) Deconvoluting the Biological Roles of Vitamin D-Binding Protein During Pregnancy: A Both Clinical and Theoretical Challenge. Front. Endocrinol. 9:259. doi: 10.3389/fendo.2018.00259

INTRODUCTION

The vitamin D-binding protein (VDBP), also known as group-specific component of serum (Gc-globulin), is a member of the albumin, α-fetoprotein, and α-albumin/afamin gene family and the major plasma carrier protein of vitamin D and its metabolites (1, 2). Vitamin D sterols are important for preserving normal serum calcium levels and electrolyte homeostasis. In addition to its specific sterol-binding capacity, VDBP has been shown to be involved in a plethora of other essential biological functions, including actin scavenging to fatty acid transport and macrophage activation and chemotaxis (2).

The teleological purpose of an ongoing pregnancy is to fulfill its fundamental role of a successful, uncomplicated delivery, in conjunction with an optimal intrauterine environment for the developing fetus. Vitamin D is adapted to meet both these demands during pregnancy; first by stimulation of calcium absorption for adequate intrauterine bone mineral accrual of the fetus, and second, by enhancing systemic and local maternal tolerance to paternal and fetal alloantigens (1–3). In this context, it is believed that VDBP is one of the key biomolecules that optimize vitamin D homeostasis and

also contributes as an immune regulator for a healthy, ongoing pregnancy. VDBP concentrations are increased in the pregnant state; however, the functional significance of this fact has not, so far, fully been clarified (3). There are emerging theories, that in clinical terms and under certain conditions, biodynamics of VDBP compound could reflect the health status of an ongoing pregnancy, as well as being predictors of neonatal birth parameters or adverse outcomes (2). From an analytical aspect, VDBP could interfere with available assays and confound interpretation of maternal and neonatal vitamin D status.

The aim of this review is to assess available literature regarding the association of VDBP with clinical outcomes during pregnancy, as a potential biomarker for future clinical practice, with a discourse on current knowledge gaps and future research agenda.

OVERVIEW OF VDBP BIODYNAMICS

Non-Pregnant State

In humans, vitamin D_3 (cholecalciferol) is naturally obtained through sunlight in the UVB range of 290–315 nm, through a membrane enhanced thermal-dependent isomerization reaction, which results in 7-dehydrocholesterol conversion into vitamin D_3 (4). Alternatively, vitamin D, either as D_2 or D_3, can enter the body from its absorption in the intestine. In either case, D_2 or D_3 then diffuse into the circulation through the capillary bed and reversibly bound to the vitamin D-binding (globulin) protein (VDBP) (5). VDBP is a 58 kDa glycosylated α-globulin that carries the lipophilic vitamin D in the plasma until it reaches target tissues (6). It is composed of 458 amino acid residues in length and folds into a disulfide-bonded, triple-domain structure. The latter is further divided into two repeated, homologous domains of 186 acids (domains I and II) and a shorter domain of 86 residues at the C-terminus (domain III) (7). It is considered as the principal transporter of vitamin D molecules. Liver is the main organ where VDBP is synthesized, whereas it is also expressed in kidney, gonads and fat tissue (8).

Vitamin D_3 undergoes its first step of activation, namely 25-hydroxylation in the liver, by the mitochondrial form of 25-hydroxylase (CYP27A1), which appears to be a bifunctional cytochrome P450 enzyme (9, 10). The product of the 25-hydroxylation step, 25-hydroxyvitamin D_3 [25(OH)D_3] (calcidiol), is the major circulating form of vitamin D_3. In humans, it is present in plasma at concentrations that range between 10 and 40 ng/ml (25–125 nM) (11–13). The second step of activation occurs mainly in the kidney (14) by the cytochrome P450 enzyme, 25(OH)D-1^{α}-hydroxylase (CYP27B1). This process leads to the formation of the active metabolite of vitamin D_3, 1, 25-dihydroyxvitamin D_3 [1, 25 (OH) $2D_3$] (calcitriol) (15, 16).

The majority of circulating 25(OH)D and 1,25-dihydroxyvitamin D is tightly bound to VDBP, with a smaller amount (10–15%) bound to albumin. Less than 1% of circulating vitamin D metabolites exists in a free, unbound form (4–6).

Apart from its function as a carrier protein, affinity for VDBP is the major parameter regulating the half-life of a vitamin D metabolite in the systemic circulation (17–19). The "free hormone hypothesis" suggests that only free steroid hormones are physiologically active, because their lipophilic ability allows them to passively diffuse across cell membranes. According to the "free hormone hypothesis," it is only the free 25(OH)vitamin D_3 that is taken up into the tubular epithelium, to be converted by CYP27B1 to calcitriol. Similarly, in the case of calcitriol, biological actions are mediated through passive diffusion of the free calcitriol to its cognate nuclear vitamin D receptor (VDR), which is a high-affinity ligand-activated transcription factor (13, 14).

Vitamin D-binding protein was believed to solely regulate the amount of free 25(OH)D available in the circulation (20). However, a landmark study by Nykjaer et al. (21) revealed an important transport system that affects vitamin D metabolism, being the megalin–cubilin endocytotic system. In this case, 25(OH)D/VDBP complex in the circulation is endocytosed into the proximal tubular cell *via* the apical-membrane receptor, megalin, the largest member of the LDL receptor super family (22). Megalin-mediated endocytosis of 25(OH)D/VDBP, also requires the receptor-associated protein and cubilin, a protein required for sequestering VDBP on the cell surface prior to its internalization by megalin (21). This system is a key player in the delivery of 25(OH)D to the 25-hydroxyvitamin D-1-α-hydroxylase in the kidney (21), since 25(OH)D molecules bound to VDBP taken up *via* this receptor pathway, are converted to calcitriol. The megalin–cubilin system has been also recognized in the placenta and several other tissues (22, 23). These results underline that VDBP, in addition to its carrier protein functions and regulation of free vitamin D fractions available in the circulation, presents pleiotropic actions: contributes significantly to renal and extra-renal production of calcitriol and ensures vitamin D molecule reabsorption in the kidney, by preventing urinary loss of vitamin D.

Pregnancy

Systemic Circulation

A limited number of studies have determined longitudinal increase of VDBP concentrations during pregnancy (24, 25). The magnitude of the increase varies: highest concentrations reach a 40–50% increase compared to non-pregnant women, with a maximum at the beginning of the third trimester before starting to decrease at term. VDBP increase was accompanied by an increase in calcitriol concentrations in most available studies (24, 25). As expected, a negative association between free 25(OH)D and VDBP concentrations was evident, resulting in a consistent decrease of free 25(OH)D from 15 to 36 weeks of gestational age (25). On the other hand, as the affinity of calcitriol is much lower for VDBP compared to 25(OH)D, a significant increase in both total 1,25(OH)2D and VDBP levels was observed during pregnancy, while the free 1,25(OH)2D concentrations remained nearly constant (26, 27). However, whereas the increase in total 1,25(OH)2D and VDBP concentrations in the pregnant state has been repeatedly reported in different studies (24, 26), reports on the free 1,25(OH)2D are conflicting. In general, an increase in 1,25(OH)2D—being of both renal and placental origin—throughout pregnancy seems to be replicated by the majority of data (28–30). Nevertheless, observed discrepancies between different trials may actually be the result of complex interactions between calcitriol concentrations and a plethora of factors, including VDBP levels, and stimulation by prolactin, insulin-like growth factor 1, and parathyroid hormone (PTH)-related protein, while

they are probably unaffected by PTH, which has been shown to decrease during pregnancy (30). Moreover, these studies used different laboratory methods of assessment of calcitriol concentrations; thus, interpretation of their results may be problematic.

Interestingly, Chun and colleagues have recently proposed a viable hypothesis considering a role for VDBP in tissue discrimination of $25(OH)D_2$ and $25(OH)D_3$ (31). Given that $25(OH)_2$ binds to VDBP with lower affinity than $25(OH)D_3$, the kidney would preferentially use the latter metabolite. Differently, cells in the immune system might profit of a greater pool of $25(OH)D_2$ for antimicrobial peptide induction (31), which is of outmost importance for enhancing systemic and local maternal tolerance to paternal and fetal alloantigens immune tolerance induction (32).

Placenta

Placenta has its own mechanisms regulating vitamin D metabolism. The decidua facilitates nutritional fetal–maternal exchange and serves as an endocrine tissue by secreting a plethora of biomolecules. In addition, it provides "immunological stability and tolerance" to accommodate the developing fetus. The 1^{α}-hydroxylase, the 24-hydroxylase, the VDBP, and VDR have all been detected either in trophoblast cultures or in freshly obtained, placental tissue (33–37). Undoubtedly, the placenta is able to metabolize vitamin D, providing active $1,25(OH)_2$ D *in vitro*. VDBP is expressed on the cell-surface of human placental trophoblasts during normal human pregnancy (37). This observation has led to the suggestion that the rise in VDBP concentrations during pregnancy could be the result of high turnover rate of trophoblasts, which are in direct contact with maternal blood (31). VDBP has been also demonstrated to affect the expression of specific placental aminotransporters, which may be involved in the regulation of amino acid transfer to the offspring during *in utero* development (38).

Vitamin D-binding protein could be possibly connected with the management of large amounts of progesterone produced by the placental trophoblast during the second and third trimesters of pregnancy, which could theoretically displace vitamin D from VDBP (25, 26). Under these conditions, VDBP may additionally play the role of a major plasma progesterone transport protein at least during late gestation; however, relevant data are still scarce and this hypothesis warrants further clarification. Although these mechanisms are still under research investigation, the above observations support the multifunctional role of VDBP both as a regulator of vitamin D homeostasis and as an immunomodulator at a systemic and placental level, during pregnancy. In accordance with this hypothesis, VDBP dysregulation has been implicated in the pathogenesis of several adverse outcomes during pregnancy, which will be further discussed below. **Figure 1** provides a schematic overview of the physiological functions of VDBP during pregnancy.

VDBP AS A MARKER OF A HEALTHY ONGOING PREGNANCY: CLINICAL IMPLICATIONS

During the past few years, there has been increasing research effort to discover novel biomarkers that could effectively predict adverse pregnancy and fetal outcomes. In this setting, the dysfunction of the immunoregulatory biological properties of circulating VDBP during pregnancy, as well as specific VDBP polymorphisms, have been the objective of several clinical studies.

Association With Type 1 Diabetes (T1D), Gestational Diabetes, and Adipokines

In a recent nested case-control study, concentrations of VDBP and 25(OH)D throughout pregnancy between 113 women whose offspring later developed T1D and 220 controls, were evaluated (39). VDBP and 25(OH)D significantly increased by gestational week and were lower in cases than in controls. Lower third trimester VDBP concentrations tended to be associated with higher risk of T1D in the offspring (39). Moreover, in a study among Chinese women, the risk allele-A of rs3733359 of VDBP gene was correlated with an increased risk of gestational diabetes mellitus, in the obese subgroup (40). Similar to other autoimmune disorders, the involvement of VDBP in the pathogenesis of T1D, may lay on a positive correlation between its levels and macrophage activation (41). Higher levels and frequencies of serum anti-VDBP autoantibodies were identified in patients with T1D than in healthy controls, suggesting VDBP as a possible autoantigen in T1D (42). Given that VDBP exerts immunomodulatory characteristics and contributes to the transport of vitamin D metabolites, reduced serum concentrations may be related, in a direct or indirect way, to the autoimmune functional deterioration of pancreatic β-cells in the disease.

Recent results from our group, in maternal–neonatal pairs at birth, demonstrated an independent positive correlation of VDBP with adipokines, adiponectin, and irisin, which remained significant after adjustment for multiple parameters, including weeks of gestation, maternal age, and Body Mass Index, in both mothers and neonates (not for irisin in the case of neonates) (43). Further mechanistic studies are required to elucidate whether VDBP plays a carrier or regulatory role for adiponectin and/or irisin during pregnancy and its potential effects on offspring anthropometry in late childhood and adolescence.

VDBP and the Risk of Adverse Pregnancy Outcomes

Vitamin D-binding protein has been also implicated recently in the pathogenesis of preeclampsia. A small pilot study showed that VDBP in the first trimester of pregnancy was upregulated in women who developed early-onset preeclampsia (EOPE) compared to controls, suggesting a hypothetical VDBP utility, as a biomarker for the diagnosis of EOPE (44). These results were in accordance with a previous cohort study, which included 239 pregnant women, 107 with preeclampsia, and 132 controls, where phenotype frequency distribution of serum group-specific component (Gc) and haptoglobin (Hp) was determined (45). The results indicated a significant statistical difference in phenotype frequency distribution of the Gc-system. Gc 2-1 phenotype was expressed significantly in women with preeclampsia compared to controls, suggesting a potential utility of Gc 2-1 phenotype as a genetic marker for

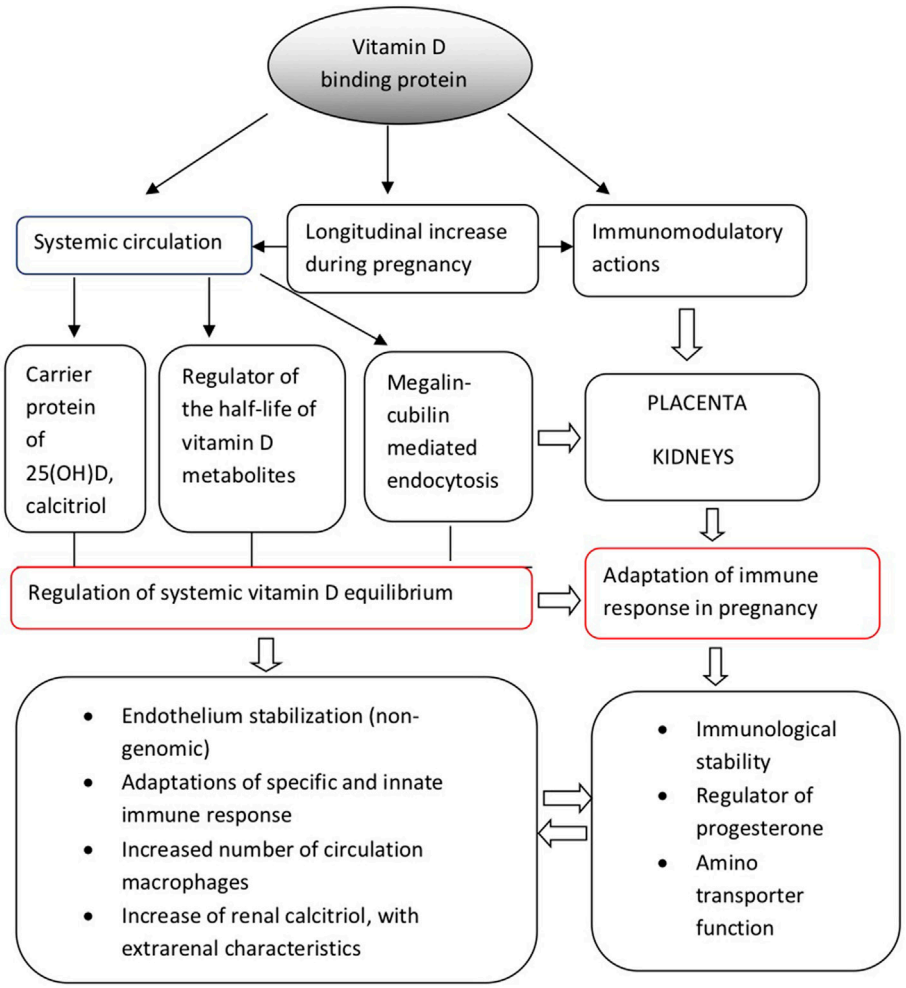

FIGURE 1 | Physiological functions of vitamin D-binding protein during pregnancy.

early preeclampsia detection (45). However, a recent study by Powe et al. showed no significance variances between first trimester VDBP concentrations between cases with preeclampsia and controls, neither association with first trimester blood pressure (46). In contrast to previous studies, Tannetta et al. showed that actin-free VDBP plasma levels tended to be lower in early onset preeclampsia compared to normal pregnancies, still not statistically significantly (47). It becomes evident that the heterogeneity of baseline 25(OH)D concentrations across trimesters of pregnancy of the populations included in these studies could contribute to this discordance. Of major interest, Behrouz et al. demonstrated that VDBP of placental origin is a target for auto-antibodies detected in sera of preeclamptic women, indicating a strong autoimmune component in the pathogenesis of the disorder (48).

Vitamin D-binding protein has been also suggested to contribute to the development of an optimal intrauterine environment for the developing fetus as well as to a successful, uncomplicated delivery. Results from the Southampton Women's Survey (33) indicate that maternal both 25(OH)D and VDBP concentrations were positively linked to placental expression of certain genes related to placental amino acid transport. On that basis, in a recent study by Wookey et al., a significant reduction of placental VDBP concentrations in women with idiopathic fetal growth restriction, as compared to normal pregnancy controls, was demonstrated (49).

On the other hand, albumin/VDBP ratio was proven to be more efficacious than fetal fibronectin in predicting spontaneous preterm delivery in symptomatic women within 7 days (50). VDBP concentrations in cervicovaginal fluid (CVF) of pregnant women successfully predicted spontaneous labor onset within 3 days, with positive and negative predictive values of 82.8 and 95.3%, respectively (51). VDBP was estimated to be 3.9-fold higher in the CVF of asymptomatic women that subsequently presented preterm premature rupture of the fetal membranes (PROM), as compared to gestation-matched controls (52).

Potential explanations for the VDBP rise in CVF in pregnancies with high risk for preterm birth could be the increased cell death and inflammation of the fetal membranes leading to increased permeability of blood vessels and augmented VDBP

deglycosylation, as an effect of the immune response (52). In addition, VDBP synthesis is known to be enhanced by pro-inflammatory cytokines, such as IL-6 (51). Given that the results of different studies regarding the VDBP concentrations in EOPE are conflicting, it has been also suggested that a potential reduction of VDBP plasma levels in EOPE, may reflect the dysfunction of the actin scavenging system, which is known to cleave extracellular actin and hinder repolymerization, inhibiting its thrombotic effects (47).

VDBP and 25(OH)D Status During Pregnancy and Lactation

GC Single Nucleotide Polymorphism (SNPs) rs12512631 and rs7041 were determined in the peripheral blood of 356 pregnant individuals and were found to significantly interplay with the maternal and cord-blood concentrations of 25(OH)D and birth weight (49). Low 25(OH)D concentrations in the maternal and cord blood were significantly associated with decreased birth weight among infants of mothers carrying the rs12512631 "C" allele, but not in those born to mothers homozygous for the "T" allele. In addition, low 25(OH)D concentrations in cord blood were significantly linked with reduced birth weight only among infants born to mothers being carriers of the rs7041 "G" allele (53).

Vitamin D-binding protein polymorphisms have been also reported to affect vitamin D status and attained 25(OH)D concentrations after supplementation. In this regard, GC rs2282679 polymorphism was found to positively correlate with achieved 25(OH)D status, following gestational cholecalciferol supplementation (54).

There is also evidence that polymorphisms in VDBP gene may be related to 25(OH)D status during pregnancy. The minor allele for rs7041 was related to increased 25(OH)D and rs4588 was associated with decreased 25(OH)D, among pregnant women (55). Chinese pregnant women with VDBP Gc-1f and Gc-1s genotypes had higher plasma 25(OH)D concentrations compared to women with Gc-2 (56). VDBP is known to increase during pregnancy; however, this phenomenon was observed only in women with rs7041 GG or GT genotypes, while pregnant TT carriers did not manifest greater VDBP concentrations compared to TT non-pregnant controls (57).

The impact of genotype on VDBP changes during pregnancy may reflect placental vitamin D transport and thus regulate the availability of vitamin D to the mother and fetus. A different study demonstrated higher VDBP concentrations in healthy pregnant women compared to non-pregnant controls, presenting comparable vitamin D intake, suggesting that metabolic alterations, possibly involving the placenta, may occur during pregnancy that aim to increase vitamin D supply (58). In addition, genetic and ethnic variations in VDBP polymorphisms could also explain the different responses after vitamin D supplementation during pregnancy (32).

On the other hand, a recent study that explored the association between 25(OH)D and VDBP concentrations in lactating mother–neonate pairs, concluded that the high maternal and the neonatal serum VDBP concentrations may be related to falsely low vitamin D concentrations, as suggested by the normal serum calcium (Ca), phosphorus (P), magnesium (Mg), Alkaline Phosphatase (ALP), and PTH levels (59). Even when maternal and neonatal serum vitamin D concentrations were consistent with each other in terms of profound hypovitaminosis D (<10 ng/ml), this definition was not enough to establish vitamin D deficiency, without taking into account other regulatory factors of the vitamin D biological network, including Ca, P, and PTH concentrations.

VDBP and Infertility

Vitamin D-binding protein has been also considered to be involved in the pathogenesis of idiopathic infertility. A recent pilot case-control study, including 39 infertile premenopausal women and 29 fertile controls, identified that VDBP concentrations were lower in the infertile group, compared to controls (60). In the same study, total 25(OH)D concentrations did not significantly differ between the two groups; however, free and bioavailable vitamin D concentrations were higher among the infertile women. The genotype distribution of GC rs1155563 and rs2298849 SNPs was compared between 154 women with endometriosis-associated infertility and 347 controls; still, no statistically significant differences were detected (61).

In a cohort of 165 healthy women, aged between 26 and 75 years, it was found that postmenopausal women had higher 25(OH)D, VDBP, and estradiol concentrations than premenopausal subjects, and that estradiol was independently correlated to VDBP (62). The work by Pirani et al. demonstrated that estradiol treatment increased the uptake of labeled VDBP by hepatocytes isolated from female animals, but not from male animal cells, indicating that the estradiol effect may lay on the presence of estrogen receptors (63). Interestingly, in infertile women undergoing *in vitro* fertilization, VDBP concentrations were not found to fluctuate as estradiol changes throughout the follicular phase of the menstrual cycle (64). These findings are suggestive of the regulatory role that other factors—besides the already well known, such as age, gender and race—may play in determining VDBP concentrations. **Table 1** summarizes key characteristics and findings of studies examined the relationship between VDBP and pregnancy-related clinical outcomes. **Figure 2** provides a schematic overview of the main pathophysiologic aspects of the VDBP network, during pregnancy.

CRITICAL APPRAISAL OF AVAILABLE EVIDENCE AND REASONS FOR DISCREPANCIES BETWEEN STUDY RESULTS

Available evidence in the field manifest several limitations, with the most prominent being the wide heterogeneity between study design, included populations, explored outcomes, and analytical methods. Therefore, any interpretation of studies results should be made with caution. Regarding genetic studies in particular, they often present specific methodological issues, including inadequate power to reveal potential gene-disease associations, population stratification as a result of genetic and environmental

TABLE 1 | Main characteristics and findings of key studies examined the relation between VDBP and pregnancy-related clinical outcomes.

Study ID	First author, year (reference)	Included subjects	Investigated outcome(s)	Key findings
1	Sørensen, 2016 (39)	113 pregnant women whose offspring later developed T1D/220 pregnant controls	VDBP concentrations during pregnancy and subsequent risk for T1D development in the offspring	Lower third trimester VDBP concentrations associated with higher risk of T1D in the offspring
2	Wang, 2015 (40)	692 women with GDM/802 pregnant controls	Whether Vitamin D related SNPs predispose to GDM development	rs3733359 allele-A was correlated with an increased GDM risk, in the obese subgroup
3	Kolialexi, 2017 (44)	5 pregnant women with EOPE/5 pregnant controls	Identification of potential biomarkers for EOPE	VDBP in the first trimester was upregulated to 3.38-fold in the EOPE group
4	Mekbeb, 1990 (45)	107 pregnant women with PE/132 pregnant controls	Relation between GC phenotype and PE risk	Gc 2-1 phenotype was expressed significantly in PE group compared to controls
5	Powe, 2010 (46)	39 pregnant women with PE/131 pregnant controls	Relation between first trimester VDBP levels and subsequent PE risk	No association between VDBP concentrations, subsequent PE development and first trimester BP
6	Cleal, 2015 (33)	85 pregnant women	Relation between maternal VDBP levels and placental expression of genes related to placental amino acid transport	VDBP levels were positively associated with placental expression of specific genes involved in amino acid transport
7	Wookey, 2017 (49)	Placentae from 18 pregnant women with FGR/17 gestation-matched healthy control subjects	Whether VDBP expression is altered in FGR-associated placental dysfunction	Significant reduction of placental VDBP concentrations in women with idiopathic FGR compared to controls
8	Liong, 2015 (50)	12 pregnant women with preterm delivery/129 women as validation cohort	Identification of CVF biomarkers predictive of spontaneous preterm birth in women with symptoms of preterm labor	Albumin/VDBP ratio is more efficacious than fetal fibronectin in predicting spontaneous preterm delivery in symptomatic women within 7 days
9	Liong, 2013 (52)	5 pregnant women with PROM/10 gestation-age matched controls	Identification of differentially expressed proteins in the CVF of asymptomatic women before the clinical manifestation of preterm PROM	VDBP was significantly increased (3.9-fold) in the PROM group
10	Tannetta, 2014 (47)	10 non-pregnant women/10 women with normal pregnancy/10 women with EOPE/10 women with LOPE	Investigation of the actin scavenging system in PE	Actin-free VDBP plasma levels were lower in EOPE compared to normal pregnancies, still not statistically significantly
11	Szczepańska, 2015 (61)	154 women with endometriosis-associated infertility/347 controls	Identification of genetic risk factors for endometriosis-associated infertility	Genotype distribution of GC rs1155563 and rs2298849 SNPs did not differ between patients and controls
12	Behrouz, 2013 (48)	5 human placentas from normotensive pregnant women/sera from 20 normal and 20 women with severe PE	Investigation of placental proteins as targets for auto-antibodies in PE patients	VDBP of placental origin is a target for auto-antibodies detected in sera of women with PE
13	Chun, 2017 (53)	Maternal and umbilical cord blood from 356 pregnant women and their infants	Relation between maternal GC SNPs, 25(OH)D concentrations and infant birth weight	Low 25(OH)D concentrations in the maternal and cord blood were significantly associated with decreased birth weight among infants of mothers carrying the rs12512631 'C' allele
14	Moon, 2017 (54)	682 pregnant women (351 placebo, 331 cholecalciferol)	Relation between GC SNPs and the response to gestational cholecalciferol supplementation	GC rs2282679 positively correlated with achieved 25(OH)D status
15	Baca, 2018 (55)	882 Black and 1796 White pregnant women	Relationship between maternal vitamin D receptor, GC, and CYP27B1 SNPs and 25(OH)D concentrations	The minor allele for rs7041 was related to increased 25(OH)D and rs4588 was associated with decreased 25(OH)D levels
16	Shao, 2017 (56)	759 pregnant women	Relationship between vitamin D pathway genes, gene–environment interactions, and vitamin D levels	Gc-1f and Gc-1s genotypes had higher plasma 25(OH)D levels compared to women with Gc-2 genotype
17	Ganz, 2018 (57)	26 third-trimester pregnant/28 lactating/21 non-pregnant and non-lactating women consuming a single amount of vitamin D	Metabolic effects of GC rs7041 SNP on vitamin D biomarkers	Increased VDBP concentrations were observed only in pregnant women with GG or GT genotypes
18	Park, 2016 (58)	26 healthy pregnant/28 lactating/21 non-pregnant and non-lactating women consuming a single amount of vitamin D	The impact of the reproductive state on vitamin D biomarkers	Higher VDBP concentrations were observed in healthy pregnant women compared to non-pregnant controls

(*Continued*)

TABLE 1 | Continued

Study ID	First author, year (reference)	Included subjects	Investigated outcome(s)	Key findings
19	Doneray, 2018 (59)	30 mother-neonate pairs with serum 25(OH)D < 10 ng/ml/30 mother–neonate pairs with serum 25(OH) D > 20 ng/ml	Relationship between serum 25(OH)D and VDBP levels in mother-neonate pairs	The maternal and neonatal vitamin D concentrations were negatively correlated with their VDBP concentrations
20	Franasiak, 2017 (60)	39 infertile premenopausal women/29 regularly cycling fertile controls	Differences in VDBP concentrations between fertile and infertile women	VDBP concentrations were lower in the infertile group, compared to controls
21	Karras, 2018 (43)	70 pairs of newly delivered neonates and their mothers	Relationship between vitamin D, VDBP, and the adipokines, adiponectin, and irisin in mothers and neonates at birth and their effects on neonate anthropometric outcomes	Independent positive correlation of maternal VDBP levels with adiponectin and irisin concentrations. Strong association of VDBP and adiponectin but not irisin was found in neonates

VDBP, Vitamin D-binding protein; T1D, type 1 diabetes; GDM, gestational diabetes mellitus; SNP, single nucleotide polymorphism; PE, preeclamsia; EOPE, early onset preeclampsia; LOPE, late onset preeclampsia; Gc, group-specific component; BP, blood pressure; FGR, fetal growth restriction; CVF, cervicovaginal fluid; PROM, preterm premature rupture of the fetal membranes; 25(OH)D, 25-hydroxyvitaminn D.

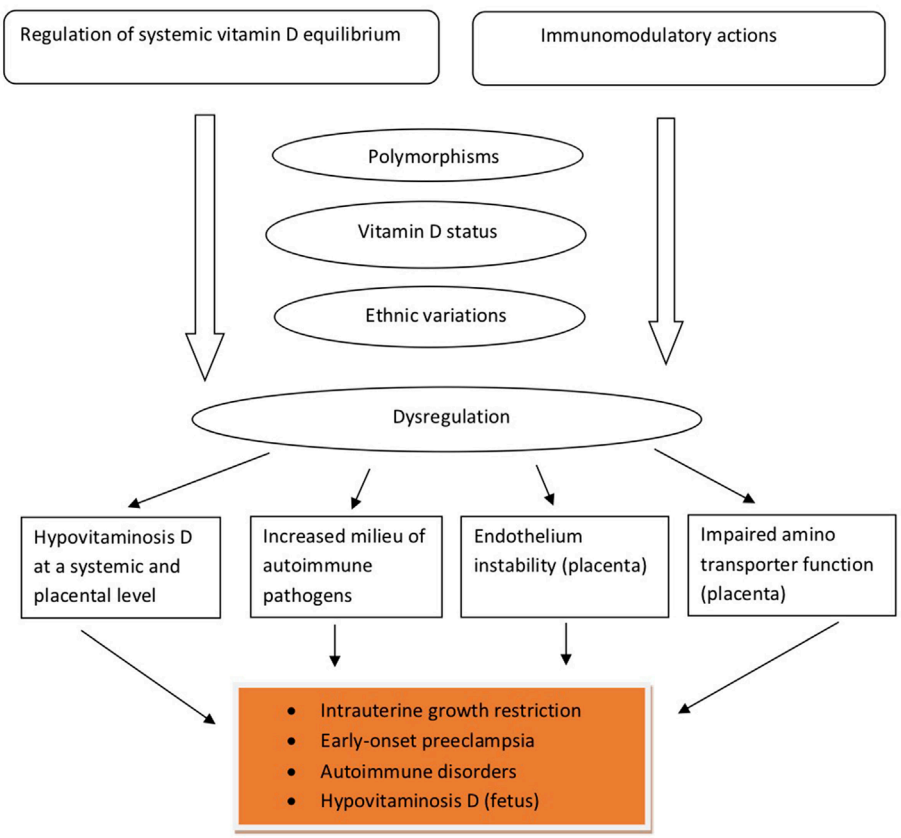

FIGURE 2 | The main pathophysiologic aspects of the vitamin D-binding protein network in pregnancy.

heterogeneity between studied populations, departure from Hardy–Weinberg equilibrium, hence, they tend to produce inconclusive and conflicting results (65).

Ethnic differences in VDBP polymorphisms could also result in differences in 25(OH)D status in pregnant cohorts across the same geographical region (66, 67), as well as the gap between observational and supplementation studies (68, 69). We have previously described in detail (69) the main reasons behind the aforementioned gap between observational and interventional studies, with regard to the role of Vitamin D in pregnancy. These reasons can be summarized as follows: 1. various study designs (lack of a precise outcome in conjunction with timing of supplementation, enrollment of participants with varied vitamin D status); 2. difficulties in the interpretation of vitamin

D equilibrium (lack of determination of plasma half-life); 3. administration of a wide range of regimens, in terms of dose, bolus, and form; 4. geographical dissimilarities (vitamin D needs could vary significantly within a country, particularly in areas with a wide range of latitude gradient); 5. alterations of vitamin D metabolism during pregnancy and 6. supplementation of individuals with low baseline 25(OH)D concentrations would be more likely to have beneficial effects compared to subjects with higher baseline status. It is highly likely that the above handicaps also affect the reproducibility of study results related to VDBP status during pregnancy, since Vitamin D and VDBP are parts of a common biological network with complex interactions between its various components.

In addition, laboratory assessment of VDBP concentrations during pregnancy may be challenging. Different analytical methods have been developed and used in the conducted studies, so far. The monoclonal immunoassay technique recognizes an epitope near the polymorphic region of VDBP and thus has different affinities for the different VDBP haplotypes: this issue probably affects the results of the assay. As a consequence, it presented uncoupling results compared to a polyclonal immunoassay method (70). Hoofnagle et al. developed a liquid chromatography–tandem mass spectrometric (LC-MS/MS) assay, where plasma proteins can be cleaved into peptides making their specific detection and quantification possible (71). LC-MS/MS method gave results similar to the polyclonal immunoassay, but different from those of the monoclonal immunoassay (70, 71). In addition, although the existence of various vitamin D forms (such as epimers) has been established, their clinical significance remains obscure. Furthermore, recent data show that at least one epimer form has activity *in vitro* (72, 73). With the development of more advanced assays, a thorough understanding of the interplay among the various vitamin D forms could be achieved.

GAPS IN EXISTING KNOWLEDGE AND FUTURE RESEARCH AGENDA

It becomes evident from the above that VDBP plays some role in the progression of normal pregnancy and that it is also implicated in the pathogenesis of some of the commonest pregnancy complications, in a way—however—that is not yet completely understood. The fact that VDBP seems to be involved in the pathogenesis of numerous and heterogeneous clinical entities, underlines its pluralistic role in vitamin D homeostasis.

Despite the intensive research work having been conducted during the past few years in terms of the role of Vitamin D in pregnancy, it is clear that existing data regarding VDBP is still very limited. The understanding of the physiology of the VDBP network is extremely useful; however, focus of future research on the association between VDBP and adverse pregnancy outcomes may have multiple benefits. First, the establishment of a novel biomarker for the early detection of endangered pregnancies, which can be translated into daily clinical benefit and second, the further decryption of the complex pathophysiological aspects of pregnancy's abnormalities.

For this purpose, additional clinical trials are required, characterized by interventional and randomized design in order to reduce potential bias, adequate power, and targeted on populations with high-risk for adverse outcomes. Future mechanistic studies from different ethnic groups are needed to investigate the regulatory and immune functions of VDBP during pregnancy and other reproduction outcomes.

AUTHOR CONTRIBUTIONS

SK and TK drafted the first edition of the paper. All authors drafted the final version and contributed to the final revisions. Last revision was made by SK.

REFERENCES

1. Couchman L, Moniz CF. Analytical considerations for the biochemical assessment of vitamin D status. *Ther Adv Musculoskelet Dis* (2017) 9:97–104. doi:10.1177/1759720X17692500
2. Davey RX. Vitamin D-binding protein as it is understood in 2016: is it a critical key with which *to* help *to* solve the calcitriol conundrum? *Ann Clin Biochem* (2017) 54:199–208. doi:10.1177/0004563216677100
3. Brannon PM, Picciano MF. Vitamin D in pregnancy and lactation in humans. *Annu Rev Nutr* (2011) 31:89–115. doi:10.1146/annurev.nutr.012809.104807
4. Holick MF. Vitamin D: photobiology, metabolism and clinical applications. In: Degroot L, editor. *Endocrinology*. Philadelphia: Saunders (1995). p. 990–1014.
5. Speeckaert MM, Speeckaert R, van Geel N, Delanghe JR. Vitamin D binding protein: a multifunctional protein of clinical importance. *Adv Clin Chem* (2014) 63:1–57. doi:10.1016/B978-0-12-800094-6.00001-7
6. Haddad JG. Plasma vitamin D-binding protein (Gc-globulin): multiple tasks. *J Steroid Biochem Mol Biol* (1995) 53:579–82. doi:10.1016/0960-0760(95)00104-8
7. Swamy N, Head JF, Weitz D, Ray R. Biochemical and preliminary crystallographic characterization of the vitamin D sterol-and actin-binding by human vitamin D-binding protein. *Arch Biochem Biophys* (2002) 402:14–23. doi:10.1016/S0003-9861(02)00033-4
8. McLeod J, Cooke N. The vitamin D-binding protein, alpha-fetoprotein, albumin multigene family: detection of transcripts in multiple tissues. *J Biol Chem* (1989) 264:21760–9.
9. Bhattacharyya MH, DeLuca HF. The regulation of the rat liver calciferol-25-hydroxylase. *J Biol Chem* (1973) 248:2969–73.
10. Andersson S, Davis DL, Dahlback H, Jornvall H, Russell DW. Cloning, structure and expression of the mitochondrial cytochrome P450 sterol 26-hydroxylase, a bile acid biosynthetic enzyme. *J Biol Chem* (1989) 246:8222–9.
11. Okuda KI, Usui E, Ohyama Y. Recent progress in enzymology and molecular biology of enzymes involved in vitamin D metabolism. *J Lipid Res* (1995) 36:1641–52.
12. Guo YD, Strugnell S, Back DW, Jones G. Transfected human liver cytochrome P-450 hydroxylates vitamin D analogs at different side-chain positions. *Proc Natl Acad Sci U S A* (1993) 90:8668–72. doi:10.1073/pnas.90.18.8668
13. Fukushima M, Susuki Y, Tohira Y, Nishi Y, Susuki M, Sasaki S, et al. 25-Hydroxylation of 1-alpha-hydroxyvitamin D3 in vivo and in perfused rat liver. *FEBS Lett* (1976) 65:211–4. doi:10.1016/0014-5793(76)80482-6
14. Cheng JB, Motola DL, Mangelsdorf DJ, Russell DW. De-orphanization of cytochrome P450 2R1: a microsomal vitamin D 25-hydroxylase. *J Biol Chem* (2003) 278:38084–93. doi:10.1074/jbc.M307028200
15. Cheng JB, Levine MA, Bell NH, Mangelsdorf DJ, Russell DW. Genetic evidence that the human CYP2R1 enzyme is a key vitamin D 25-hydroxylase. *Proc Natl Acad Sci U S A* (2004) 101:7711–5. doi:10.1073/pnas.0402490101
16. Hollis BW, Wagner CL. Clinical review: the role of the parent compound vitamin D with respect to metabolism and function: why clinical dose intervals can affect clinical outcomes. *J Clin Endocrinol Metab* (2013) 98:4619–28. doi:10.1210/jc.2013-2653

17. Smith JE, Goodman DS. The turnover and transport of vitamin D and of a polar metabolite with the properties of 25-hydroxycholecalciferol in human plasma. *J Clin Invest* (1971) 50:2159–67. doi:10.1172/JCI106710
18. Kissmeyer A, Mathiasen IS, Latini S, Binderup L. Pharmacokinetic studies of vitamin D analogues: relationship to vitamin D binding protein (DBP). *Endocrine* (1995) 3:263–6. doi:10.1007/BF03021403
19. Vieth R, Kessler MJ, Pritzker KP. Species differences in the binding kinetics of 25-hydroxyvitamin D3 to vitamin D binding protein. *Can J Physiol Pharmacol* (1990) 68:1368–71. doi:10.1139/y90-207
20. Haddad JG, Hillman L, Rojanasathit S. Human serum binding capacity and affinity for 25-hydroxyergocalciferol and 25- hydroxycholecalciferol. *J Clin Endocrinol Metab* (1976) 43:86–91. doi:10.1210/jcem-43-1-86
21. Nykjaer A, Dragun D, Walther D, Vorum H, Jacobsen C, Herz J, et al. An endocytic pathway essential for renal uptake and activation of the steroid 25-(OH) vitamin D3. *Cell* (1999) 96:507–15. doi:10.1016/S0092-8674(00)80655-8
22. Marzolo MP, Farfan P. New insights into the roles of megalin/LRP2 and the regulation of its functional expression. *Biol Res* (2011) 44:89–105. doi:10.4067/S0716-97602011000100012
23. Jassil NK, Sharma A, Bikle D, Wang X. Vitamin D binding protein and 25-hydroxyvitamin D levels: emerging clinical applications. *Endocr Pract* (2017) 23:605–13. doi:10.4158/EP161604.RA
24. Bouillon R, Van Assche FA, Van Baelen H, Heyns W, De Moor P. Influence of the vitamin D-binding protein on the serum concentration of 1,25-dihydroxyvitamin D3: significance of the free 1,25-dihydroxyvitamin D3 concentration. *J Clin Invest* (1981) 67:589–96. doi:10.1172/JCI110072
25. Zhang J, Lucey A, Horgan R, Kenny L, Kiely M. Impact of pregnancy on vitamin D status: a longitudinal study. *Br J Nutr* (2014) 112:1081–7. doi:10.1017/S0007114514001883
26. Bikle DD, Gee E, Halloran B, Haddad JG. Free 1,25-dihydroxyvitamin D levels in serum from normal subjects, pregnant subjects, and subjects with liver disease. *J Clin Invest* (1984) 74:1966–71. doi:10.1172/JCI111617
27. van Hoof HJ, de Sevaux RG, van Baelen H, Swinkels LM, Klipping C, Ross HA, et al. Relationship between free and total 1,25-dihydroxyvitamin D in conditions of modified binding. *Eur J Endocrinol* (2001) 144:391–6. doi:10.1530/eje.0.1440391
28. Kumar R, Cohen WR, Silva P, Epstein FH. Elevated 1,25-dihydroxyvitamin D plasma levels in normal human pregnancy and lactation. *J Clin Invest* (1979) 63:342–4. doi:10.1172/JCI109308
29. Reddy GS, Norman AW, Willis DM, Goltzman D, Guyda H, Solomon S, et al. Regulation of vitamin D metabolism in normal human pregnancy. *J Clin Endocrinol Metab* (1983) 56:363–70. doi:10.1210/jcem-56-2-363
30. Souberbielle JC, Cavalier E, Delanaye P, Massart C, Brailly-Tabard S, Cormier S, et al. Serum calcitriol concentrations measured with a new direct automated assay in a large population of adult healthy subjects and in various clinical situations. *Clin Chim Acta* (2015) 451:149–53. doi:10.1016/j.cca.2015.09.021
31. Chun RF, Peercy BE, Orwoll ES, Nielson CM, Adams JS, Hewison M. Vitamin D and DBP: the free hormone hypothesis revisited. *J Steroid Biochem Mol Biol* (2014) 144:132–7. doi:10.1016/j.jsbmb.2013.09.012
32. Karras SN, Wagner CL, Castracane VD. Understanding vitamin D metabolism in pregnancy: from physiology to pathophysiology and clinical outcomes. *Metabolism* (2017). doi:10.1016/j.metabol.2017.10.001
33. Cleal JK, Day PE, Simner CL, Barton SJ, Mahon PA, Inskip HM, et al. Placental amino acid transport may be regulated by maternal vitamin D and vitamin D-binding protein: results from the Southampton Women's Survey. *Br J Nutr* (2015) 113:1903–10. doi:10.1017/S0007114515001178
34. Bikle DD. Vitamin D metabolism, mechanism of action, and clinical applications. *Chem Biol* (2014) 21:319–29. doi:10.1016/j.chembiol.2013.12.016
35. Wagner CL, Hollis BW, Kotsa K, Fakhoury H, Karras SN. Vitamin D administration during pregnancy as prevention for pregnancy, neonatal and postnatal complications. *Rev Endocr Metab Disord* (2017) 18:307–22. doi:10.1007/s11154-017-9414-3
36. Karras SN, Fakhoury H, Muscogiuri G, Grant WB, van den Ouweland JM, Colao AM, et al. Maternal vitamin D levels during pregnancy and neonatal health: evidence to date and clinical implications. *Ther Adv Musculoskelet Dis* (2016) 8:124–35. doi:10.1177/1759720X16656810
37. Kovacs CS. The role of vitamin D in pregnancy and lactation: insights from animal models and clinical studies. *Annu Rev Nutr* (2012) 32:97–123. doi:10.1146/annurev-nutr-071811-150742
38. Ma R, Gu Y, Zhao S, Sun J, Groome LJ, Wang Y. Expressions of vitamin D metabolic components VDBP, CYP2R1, CYP27B1, CYP24A1, and VDR in placentas from normal and preeclamptic pregnancies. *Am J Physiol Endocrinol Metab* (2012) 303:E928–35. doi:10.1152/ajpendo.00279.2012
39. Sørensen IM, Joner G, Jenum PA, Eskild A, Brunborg C, Torjesen PA, et al. Vitamin D-binding protein and 25-hydroxyvitamin D during pregnancy in mothers whose children later developed type 1 diabetes. *Diabetes Metab Res Rev* (2016) 32:883–90. doi:10.1002/dmrr.2812
40. Wang Y, Wang O, Li W, Ma L, Ping F, Chen L, et al. Variants in vitamin D binding protein gene are associated with gestational diabetes mellitus. *Medicine (Baltimore)* (2015) 94:e1693. doi:10.1097/MD.0000000000001693
41. Blanton D, Han Z, Bierschenk L, Linga-Reddy MV, Wang H, Clare- Salzler M, et al. Reduced serum vitamin D-binding protein levels are associated with type 1 diabetes. *Diabetes* (2011) 60:2566–70. doi:10.2337/db11-0576
42. Kodama K, Zhao Z, Toda K, Yip L, Fuhlbrigge R, Miao D, et al. Expression-based genome-wide association study links vitamin D-binding protein with autoantigenicity in type 1 diabetes. *Diabetes* (2016) 65:1341–9. doi:10.2337/db15-1308
43. Karras SN, Polyzos SA, Newton DA, Wagner CL, Hollis BW, Ouweland JVD, et al. Adiponectin and vitamin D-binding protein are independently associated at birth in both mothers and neonates. *Endocrine* (2018) 59:164–74. doi:10.1007/s12020-017-1475-2
44. Kolialexi A, Tsangaris GT, Sifakis S, Gourgiotis D, Katsafadou A, Lykoudi A, et al. Plasma biomarkers for the identification of women at risk for early-onset preeclampsia. *Expert Rev Proteomics* (2017) 14:269–76. doi:10.1080/14789450.2017.1291345
45. Mekbeb T. The association of serum proteins with preeclampsia. *Ethiop Med J* (1990) 28:9–14.
46. Powe CE, Seely EW, Rana S, Bhan I, Ecker J, Karumanchi SA, et al. First trimester vitamin D, vitamin D binding protein, and subsequent preeclampsia. *Hypertension* (2010) 56:758–63. doi:10.1161/HYPERTENSIONAHA.110.158238
47. Tannetta DS, Redman CW, Sargent IL. Investigation of the actin scavenging system in pre-eclampsia. *Eur J Obstet Gynecol Reprod Biol* (2014) 172:32–5. doi:10.1016/j.ejogrb.2013.10.022
48. Behrouz GF, Farzaneh GS, Leila J, Jaleh Z, Eskandar KS. Presence of autoantibody against two placental proteins, annexin A1 and vitamin D binding protein, in sera of women with pre-eclampsia. *J Reprod Immunol* (2013) 99:10–6. doi:10.1016/j.jri.2013.04.007
49. Wookey AF, Chollangi T, Yong HE, Kalionis B, Brennecke SP, Murthi P, et al. Placental vitamin D-binding protein expression in human idiopathic fetal growth restriction. *J Pregnancy* (2017) 2017:5120267. doi:10.1155/2017/5120267
50. Liong S, Di Quinzio MK, Fleming G, Permezel M, Rice GE, Georgiou HM. New biomarkers for the prediction of spontaneous preterm labour in symptomatic pregnant women: a comparison with fetal fibronectin. *BJOG* (2015) 122:370–9. doi:10.1111/1471-0528.12993
51. Liong S, Di Quinzio MK, Fleming G, Permezel M, Georgiou HM. Is vitamin D binding protein a novel predictor of labour? *PLoS One* (2013) 8:e76490. doi:10.1371/journal.pone.0076490
52. Liong S, Di Quinzio MK, Heng YJ, Fleming G, Permezel M, Rice GE, et al. Proteomic analysis of human cervicovaginal fluid collected before preterm premature rupture of the fetal membranes. *Reproduction* (2013) 145:137–47. doi:10.1530/REP-12-0264
53. Chun SK, Shin S, Kim MY, Joung H, Chung J. Effects of maternal genetic polymorphisms in vitamin D-binding protein and serum 25-hydroxyvitamin D concentration on infant birth weight. *Nutrition* (2017) 35:36–42. doi:10.1016/j.nut.2016.10.006
54. Moon RJ, Harvey NC, Cooper C, D'Angelo S, Curtis EM, Crozier SR, et al. Response to antenatal cholecalciferol supplementation is with common vitamin D-related genetic variants. *J Clin Endocrinol Metab* (2017) 102:2941–9. doi:10.1210/jc.2017-00682
55. Baca KM, Govil M, Zmuda JM, Simhan HN, Marazita ML, Bodnar LM. Vitamin D metabolic loci and vitamin D status in Black and White pregnant women. *Eur J Obstet Gynecol Reprod Biol* (2018) 220:61–8. doi:10.1016/j.ejogrb.2017.11.013
56. Shao B, Jiang S, Muyiduli X, Wang S, Mo M, Li M. Vitamin D pathway gene polymorphisms influenced vitamin D level among pregnant women. *Clin Nutr* (2017). doi:10.1016/j.clnu.2017.10.024

57. Ganz AB, Park H, Malysheva OV, Caudill MA. Vitamin D binding protein rs7041 genotype alters vitamin D metabolism in pregnant women. *FASEB J* (2018) 32(4):2012–20. doi:10.1096/fj.201700992R
58. Park H, Brannon PM, West AA, Yan J, Jiang X, Perry CA, et al. Vitamin D metabolism varies among women in different reproductive states consuming the same intakes of vitamin D and related nutrients. *J Nutr* (2016) 146:1537–45. doi:10.3945/jn.116.229971
59. Doneray H, Yesilcibik RS, Laloglu E, Ingec M, Orbak Z. Serum vitamin D and vitamin D-binding protein levels in mother-neonate pairs during the lactation period. *Ital J Pediatr* (2018) 44:15. doi:10.1186/s13052-018-0448-2
60. Franasiak J, Shapses S, Sun W, Scott R, Wang X. Vitamin D binding protein is lower in infertile patients compared to fertile controls: a case control study. *Fertil Res Pract* (2017) 3:14. doi:10.1186/s40738-017-0042-0
61. Szczepańska M, Mostowska A, Wirstlein P, Skrzypczak J, Misztal M, Jagodziński PP. Polymorphic variants in vitamin D signaling pathway genes and the risk of endometriosis-associated infertility. *Mol Med Rep* (2015) 12:7109–15. doi:10.3892/mmr.2015.4309
62. Pop LC, Shapses SA, Chang B, Sun W, Wang X. Vitamin D-binding protein in healthy pre- and postmenopausal women: relationship with estradiol concentrations. *Endocr Pract* (2015) 21:936–42. doi:10.4158/EP15623.OR
63. Pirani T, Chen J, Vieira A. Effects of estradiol on the endocytic transport of vitamin D carrier protein in hepatocytes. *Biochim Biophys Acta* (2013) 1830:3421–6. doi:10.1016/j.bbagen.2013.01.025
64. Franasiak JM, Wang X, Molinaro TA, Green K, Sun W, Werner MD, et al. Free vitamin D does not vary through the follicular phase of the menstrual cycle. *Endocrine* (2016) 53:322–6. doi:10.1007/s12020-016-0946-1
65. Zintzaras E, Koufakis T, Ziakas PD, Rodopoulou P, Giannouli S, Voulgarelis M. A meta-analysis of genotypes and haplotypes of methylenetetrahydrofolate reductase gene polymorphisms in acute lymphoblastic leukemia. *Eur J Epidemiol* (2006) 21:501–10. doi:10.1007/s10654-006-9027-8
66. Karras S, Paschou SA, Kandaraki E, Anagnostis P, Annweiler C, Tarlatzis BC, et al. Hypovitaminosis D in pregnancy in the Mediterranean region: a systematic review. *Eur J Clin Nutr* (2016) 70:979–86. doi:10.1038/ejcn.2016.12
67. Karras SN, Anagnostis P, Annweiler C, Naughton DP, Petroczi A, Bili E, et al. Maternal vitamin D status during pregnancy: the Mediterranean reality. *Eur J Clin Nutr* (2014) 68:864–9. doi:10.1038/ejcn.2014.80
68. Karras SN, Anagnostis P, Bili E, Naughton D, Petroczi A, Papadopoulou F, et al. Maternal vitamin D status in pregnancy and offspring bone development: the unmet needs of vitamin D era. *Osteoporos Int* (2014) 25:795–805. doi:10.1007/s00198-013-2468-5
69. Karras SN, Anagnostis P, Naughton D, Annweiler C, Petroczi A, Goulis DG. Vitamin D during pregnancy: why observational studies suggest deficiency and interventional studies show no improvement in clinical outcomes? A narrative review. *J Endocrinol Invest* (2015) 38:1265–75. doi:10.1007/s40618-015-0363-y
70. Henderson CM, Lutsey PL, Misialek JR, Laha TJ, Selvin E, Eckfeldt JH, et al. Measurement by a novel LC-MS/MS methodology reveals similar serum concentrations of vitamin D-binding protein in Blacks and Whites. *Clin Chem* (2016) 62:179–87. doi:10.1373/clinchem.2015.244541
71. Hoofnagle AN, Eckfeldt JH, Lutsey PL. Vitamin D-binding protein concentrations quantified by mass spectrometry. *N Engl J Med* (2015) 373:1480–2. doi:10.1056/NEJMc1502602
72. Karras SN, Kotsa K, Angeloudi E, Zebekakis P, Naughton DP. The road not so travelled: should measurement of vitamin D epimers during pregnancy affect our clinical decisions? *Nutrients* (2017) 9:90. doi:10.3390/nu9020090
73. Karras S, Petroczi A, Anagnostis P, Naughton DP. Vitamin D epimer: a significant bioregulator or an inactive compound? *Osteoporos Int* (2015) 26:2345–6. doi:10.1007/s00198-015-3198-7

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Endocrinology

REVIEW
published: 28 May 2018
doi: 10.3389/fendo.2018.00277

Treatment for Patients With Malignant Pheochromocytomas and Paragangliomas: A Perspective From the Hallmarks of Cancer

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Malignant pheochromocytomas and paragangliomas affect a very small percentage of the general population. A substantial number of these patients have a hereditary predisposition for the disease and consequently, bear the risk of developing these tumors throughout their entire lives. It is, however, unclear why some patients with no hereditary predisposition develop these tumors, which frequently share a similar molecular phenotype with their hereditary counterparts. Both hereditary and sporadic tumors usually appear at an early age, and affected people often die before reaching their expected lifespans. Unfortunately, there is currently no systemic therapy approved for patients with this orphan disease. Therefore, pheochromocytomas and paragangliomas are very challenging malignancies. The recognition of genetic and molecular abnormalities responsible for the development of these tumors as well as the identification of effective therapies for other malignancies that share a similar pathogenesis is leading to the development of exciting clinical trials. Tyrosine kinase inhibitors, radiopharmaceutical agents, and immunotherapy are currently under evaluation in prospective clinical trials. A phase 2 clinical trial of the highly specific metaiodobenzylguanidine, iobenguane ^{131}I, has provided impressive results; this radiopharmaceutical agent may become the first approved systemic therapy for patients with malignant pheochromocytoma and paraganglioma by the United States Food and Drug Administration. Nevertheless, systemic therapies are still not able to cure the disease. This review will discuss the development of systemic therapeutic approaches using the hallmarks of cancer as a framework. This approach will help the reader to understand where research efforts currently stand and what the future for this difficult field may be.

Keywords: pheochromocytoma, paraganglioma, tyrosine kinase inhibitors, radionuclides, immunotherapy

OPEN ACCESS

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Specialty section:
This article was submitted to Cancer Endocrinology, a section of the journal Frontiers in Endocrinology

Received: *11 April 2018*
Accepted: *11 May 2018*
Published: *28 May 2018*

Citation:
Jimenez C (2018) Treatment for Patients With Malignant Pheochromocytomas and Paragangliomas: A Perspective From the Hallmarks of Cancer. Front. Endocrinol. 9:277. doi: 10.3389/fendo.2018.00277

INTRODUCTION

Pheochromocytomas and paragangliomas are neuroendocrine tumors derived from the paraganglia. Most pheochromocytomas and sympathetic paragangliomas secrete excessive amounts of catecholamines that predispose to elevated blood pressure, palpitations, sweats, anxiety, and gastrointestinal disease (1, 2). Patients are prone to develop a catecholamine crisis characterized by a hypertensive emergency and cardiovascular events. The excessive secretion of catecholamines is confirmed by measuring the plasma concentrations of metanephrines (3). Most patients have localized tumors and subsequently, they are cured with surgery (4, 5).

Malignant pheochromocytomas and paragangliomas are rare endocrine cancers. Approximately 100–200 new cases are diagnosed every year in the United States (6). The definition of these malignancies rests on the presence of metastases because there is currently no histological, biochemical, molecular, or genetic marker that can clearly differentiate benign from malignant tumors (7, 8). Therefore, the World Health Organization has recommended classifying pheochromocytomas and paragangliomas as metastatic or nonmetastatic, as a substantial number of patients with clinical predictors of metastases can be diagnosed and treated before the malignant cells spread to distant sites (7, 9). Metastatic pheochromocytomas and paragangliomas (MPPGs) frequently spread to regional and distant lymph nodes, bones, liver, and lungs (10, 11). Metastases are rarely found in the pancreas, breast, central nervous system, or skin (12). As expected, patients with MPPG have shorter overall survival (OS) durations than do patients with non-MPPGs (10).

Patients with MPPG are mainly treated with systemic chemotherapy and radiopharmaceutical agents such as conventional ^{131}Iodine-metaiodobenzylguanidine (13). The understanding on these treatments is difficult as it mainly derives from small, retrospective studies (14). Subsequently, there are no guidelines for the treatment of patients with MPPG. Progress toward systemic therapies for patients with MPPG has been slow (13) owing to the rarity of the disease and the lack of a reliable animal model that can mimic a human MPPG phenotype (15, 16). However, the recognition of the fundamental genetic and metabolic characteristics of MPPG and clinical experience with other cancers that share similar pathogenetic processes have led to the identification of new therapeutic horizons (16–18). Approximately 30% of patients with MPPG harbor a germline mutation of the succinate dehydrogenase subunit B of the mitochondrial enzymatic complex 2 gene (*SDHB*) (19). Tumors with *SDHB* mutations are characterized by abnormal angiogenesis and a hypervascular phenotype (20). *SDHB* tumors also display intense DNA hypermethylation and upregulation of the epithelial-to-mesenchymal transition, which fosters distant spread (21–23). In addition, these tumors express cell membrane glucose transporters and activate glucose phosphorylation to support their energetic demands (24). Because *SDHB*-associated MPPGs are very avid for glucose, positron emission tomography with fludeoxyglucose (FDG-PET) is a sensitive test to identify the disease (25). Interestingly, many patients with MPPG do not harbor germline mutations; their tumors are considered apparently sporadic. However, many apparently sporadic tumors exhibit a very similar molecular phenotype to the one observed in *SDHB*-associated tumors (20). In addition, gastrointestinal stromal tumors and renal cell clear cell, medullary thyroid, and pancreatic neuroendocrine carcinomas share some crucial pathogenetic characteristics with MPPG (26–29). Therapeutic progress on these tumors has helped in identifying potential therapies for patients with MPPG (30).

Scientific efforts have identified several biological capabilities, called the "hallmarks of cancer," that are essential for the formation of cancer in humans (31). These hallmarks are distinctive and complementary abilities acquired by cancer cells that enable tumor growth and metastatic dissemination. Cancer cells have the ability to sustain proliferative signaling, evade growth-inhibiting signals, evade apoptosis, enable replicative immortality, sustain angiogenesis, and invade and metastasize (31). In addition, they can reprogram energy metabolism and evade immune destruction (31). They provide a solid conceptual foundation for understanding the biology of cancer (31). As in other cancers, the survival of MPPG cells likely depends on a combination of these hallmarks. This review will discuss the development of systemic therapeutic approaches for patients with MPPG using the hallmarks of cancer as a framework. We will also assess the value of surgical resection and traditional therapies such as chemotherapy for patients with MPPG.

SURGERY

The early resection of a pheochromocytoma or a sympathetic paraganglioma may cure the disease. In fact, more than 90% of patients with nonmetastatic disease treated with surgery are alive 5 years after initial diagnosis (10). The surgical approach (i.e., open laparotomy or laparoscopy) must be carefully selected on the basis of the presence of clinical predictors of aggressiveness, such as the size and location of the primary tumor and the presence of *SDHB* mutations (32). In patients with subdiaphragmatic primary tumors larger than 5 cm, an open laparotomy allows better visualization of the lymph nodes and is associated with a lower risk of tumor rupture than are laparoscopic procedures (32).

Over the last 20 years, clinical experience has suggested that it may be best to observe most patients with head and neck paragangliomas (33). Because of their parasympathetic origin, it is exceedingly rare to find a head and neck paraganglioma that secretes noradrenaline; consequently, these patients are not prone to hormonal syndromes. In addition, these tumors are rarely metastatic (34) and subsequently, no TNM staging has been proposed yet for head and neck paragangliomas (30). Most importantly, their intimate contact with neurovascular structures increases the risk of intraoperative vascular accidents and postoperative low cranial nerve neuropathy (35).

Patients with MPPG will most likely not be cured by surgery unless they present with only regional lymph node metastases or small, localized, and resectable distant metastases. Nevertheless, patients with noncurable MPPG may still benefit from surgical resection of the primary tumor (32). Resection of the primary tumor may decrease the catecholamine surge associated with these tumors and improve hormonal symptoms (32); patients may consequently have a lower risk for cardiovascular and gastrointestinal morbidity. Furthermore, resection of the primary tumor is associated with an improvement in OS regardless of performance status, tumor burden, genetic profile, or hormonal status (32), likely because of a lower rate of metastatic spread, as patients exhibit similar OS rates irrespective of their hormonal status (32).

CHEMOTHERAPY

Understanding the role of chemotherapy in patients with MPPG is challenging. Chemotherapy decreases the tumor's ability to sustain proliferative signaling, which underlies its abnormal cell growth and division. However, chemotherapy does not induce complete responses; in fact, retrospective studies have shown variable responses. The difficulties faced by clinicians are

highlighted by a recent systematic review and meta-analysis of all published studies on the topic of chemotherapy for MPPG (14). Of 459 potential studies, only 4 (<1%) were of high enough quality for inclusion in the meta-analysis (36–39). These four studies included consecutive patients, had an adequate description of diagnostic and therapeutic interventions, employed a clear definition of and evaluation criteria for tumor response, and had few or no lost patients during follow-up. The results of this meta-analysis suggested that approximately 37% of patients with MPPG respond to systemic chemotherapy with a combination of cyclophosphamide, vincristine, and dacarbazine (14). Patients generally did not have complete responses. However, some had improved blood pressure control and apparent improvement in the symptoms of catecholamine excess attributable to a reduction in tumor size or stabilization of disease (14). Only one study—the largest one—suggested that MPPG patients whose tumors responded to chemotherapy had longer OS than did patients without a tumor response (36). This study was also the only one that clearly indicated chemotherapy for patients with progressive disease (36). Therefore, the results of this meta-analysis may have overestimated the rate and scale of MPPG response to chemotherapy (14). Toxicity related to chemotherapy varies and duration of therapy has not been determined yet. A maintenance regimen with dacarbazine or temozolomide may improve chemotherapy long-term efficacy (16, 40).

THE HALLMARKS OF CANCER AND MPPG

The tumor growth observed in patients with MPPG clearly demonstrates that sustained proliferative signaling allows the excessive activation of the cell division cycle in these tumors. As in other malignancies, this process is in part mediated by tyrosine kinase receptors. The interaction of growth factors with these receptors activates signaling pathways that modulate the cell cycle and cell growth; these signals also control cell survival and energy metabolism (20). MPPGs are frequently characterized by a tumor environment of pseudohypoxia, which leads to deregulation of cellular energetics, abnormal activation of proliferative pathways, tumor inflammation and necrosis, and activation and recruitment of cells that prevent immune system recognition of the tumor. MPPGs associated with *SDHB* mutations and other MPPGs associated with an environment of pseudohypoxia (i.e., those with germline mutations in regulatory genes of the other subunits of the mitochondrial enzymatic complex 2, fumarase, or the protein von Hippel-Lindau disease) exhibit a phenotype characterized by large intratumor concentrations of vascular endothelial growth factors (VEGFs), platelet-derived growth factor beta (PDGF-β), epidermal growth factors, fibroblast growth factors, and others; their cognate receptors are also overexpressed by these tumors (20, 41). The stabilization of the hypoxia-inducible factor (HIF) under conditions of pseudohypoxia is responsible for the overexpression of genes responsible for the synthesis of these growth factors and their receptors (20). Hereditary mutations also confer advantages to specific clones that benefit from, for instance, the activation of epigenetic mechanisms such as DNA hypermethylation (22, 42). Mutations of the *EPAS1* gene, which codes for the HIF-2α, have been described in 6% of patients with pheochromocytoma and paraganglioma and strongly suggest a pathogenic and, therapeutically speaking, targetable role for hypoxia (43, 44). This gene controls several proteins involved in cell division, angiogenesis, and red blood cells production (43). In addition, somatic activating mutations of certain receptors may result in structural modifications that lead to independent signaling activation. Recently, activating mutations of the c-Met receptor have been described in MPPG (45).

Along with the sustained proliferative signaling supported by pseudohypoxia, MPPG cells exhibit deregulation of cellular energetics (46). These tumors compensate for pseudohypoxia with increased expression and activity of glucose transporters and glycolytic regulatory enzymes such as hexoquinases and pyruvate and lactate dehydrogenases (17). The resulting negative energetic balance may lead to necrosis, which in turn leads to activation of inflammatory cells that contribute to tumor cell growth and angiogenesis. Neoangiogenesis, necrosis, and inflammation, DNA hypermethylation, and other mechanisms activate the epithelial-to-mesenchymal and mesenchymal-to-epithelial transition pathways that lead to the development of metastases (31). Activating c-Met mutations may also facilitate the distant spread observed in some MPPGs (45).

Somatic mutations in other genes may also activate downstream pathways. For example, *RAS* mutations have been described in pheochromocytomas and paragangliomas (47, 48). These mutations predict a constitutive activation of the mitogen-activated protein kinase and phosphoinositide 3-kinase (PI3K) pathways, leading to abnormal proliferation of pheochromocytoma and paraganglioma cells. *RAS* mutations have not, however, yet been associated with a clear MPPG phenotype (47, 48).

Metastatic pheochromocytomas and paraganglioma cells require unlimited replicative capacity in order to create a macroscopic tumor. In fact, MPPGs are frequently characterized by large primary tumors and massive metastases (32). This implies that MPPGs are able to overcome the two mechanisms that prevent cell immortality: senescence and death/crisis. The telomeres that protect the ends of the chromosomes are strongly involved in the regulation of this hallmark (31). The maintenance of telomeric DNA is linked with tumor cell immortalization. The polymerase telomerase extends telomeric DNA by adding telomere repeat segments, preventing senescence; the loss or erosion of telomeres may trigger the senescence process (49). Recently, somatic mutations of telomerase have been identified in MPPG (50). In addition, noncanonical roles of telomerase and its subunit telomerase reverse transcriptase may also contribute to the development of MPPG (50). Mutations in *ATRX* which is involved in chromatin remodeling have been described in some MPPG tumors (51, 52).

In order to survive, MPPG cells also require mechanisms that allow them to evade apoptosis and immune system recognition. TP53-inactivating mutations have been described in some MPPGs, and abnormal activation of the PI3K and mechanistic target of rapamycin pathways has also been observed in MPPGs (18, 53). Somatic *NF1* mutations may inhibit autophagy in MPPG cells (54). Recently, a great deal of interest in oncology has been

focused on the identification of therapies that may enhance the immune system recognition of tumor cells. Several mechanisms that prevent immune system recognition have been described in cancers characterized by hypoxia and pseudohypoxia (55).

It is important to emphasize that like in other malignancies, the microenvironment determines the joint success of the hallmarks of cancer (56). Preclinical studies have shown that the production of lactate by cancer activated fibroblasts stimulates the migration of *SDHB* silent pheochromocytoma cells (57). Furthermore, clinical evidence reveals that MPPG cells—irrespective of their genotype—are very much attracted by the bone microenvironment (58). As it will described later, this finding supports exploring medications such as cabozantinib for patients with bone metastases. **Figure 1** summarizes the hallmarks of cancer in the context of MPPG.

NOVEL THERAPIES FOR MPPG ACCORDING TO THEIR EFFECTS ON ONE OR MORE HALLMARK CAPABILITIES

Inhibition of Angiogenesis and Proliferative Signaling: Pazopanib and Sunitinib

Pazopanib and sunitinib block the VEGF-1, -2, and -3, PDGF-α and -β, c-Kit, fms-related tyrosine kinase 3, and ret proto-oncogene (RET) receptors. As such, these medications prevent neoangiogenesis, cell growth, and cell migration and may induce apoptosis (59, 60).

Sunitinib was the first tyrosine kinase inhibitor recognized as a potential treatment for patients with MPPG (61). A retrospective study of 17 patients treated with sunitinib provided useful information that has helped in designing prospective trials (62). These patients did not have a response to chemotherapy or had contraindications to chemotherapy. Thirteen patients had measurable disease, and four patients had predominant bone metastases. Three patients discontinued sunitinib therapy early because of adverse events and were not evaluable for objective response; 10 patients with measurable disease were evaluable, and the objective response rate (ORR) was 30%. In addition, one patient had stable disease with some degree of regression. Patients with partial responses and stable disease also saw improvement in their symptoms of catecholamine excess; their blood pressure normalized, and two patients discontinued all antihypertensive therapies for some time. Six patients had no response to sunitinib. The four patients with nonmeasurable disease (bone metastases) exhibited a substantial reduction of glucose uptake as assessed *via* FDG-PET imaging and had improved blood pressure control (62).

Sunitinib treatment had clinical benefits in both carriers of *SDHB* mutations and patients with apparently sporadic tumors (62). The progression-free survival (PFS) (the length of response

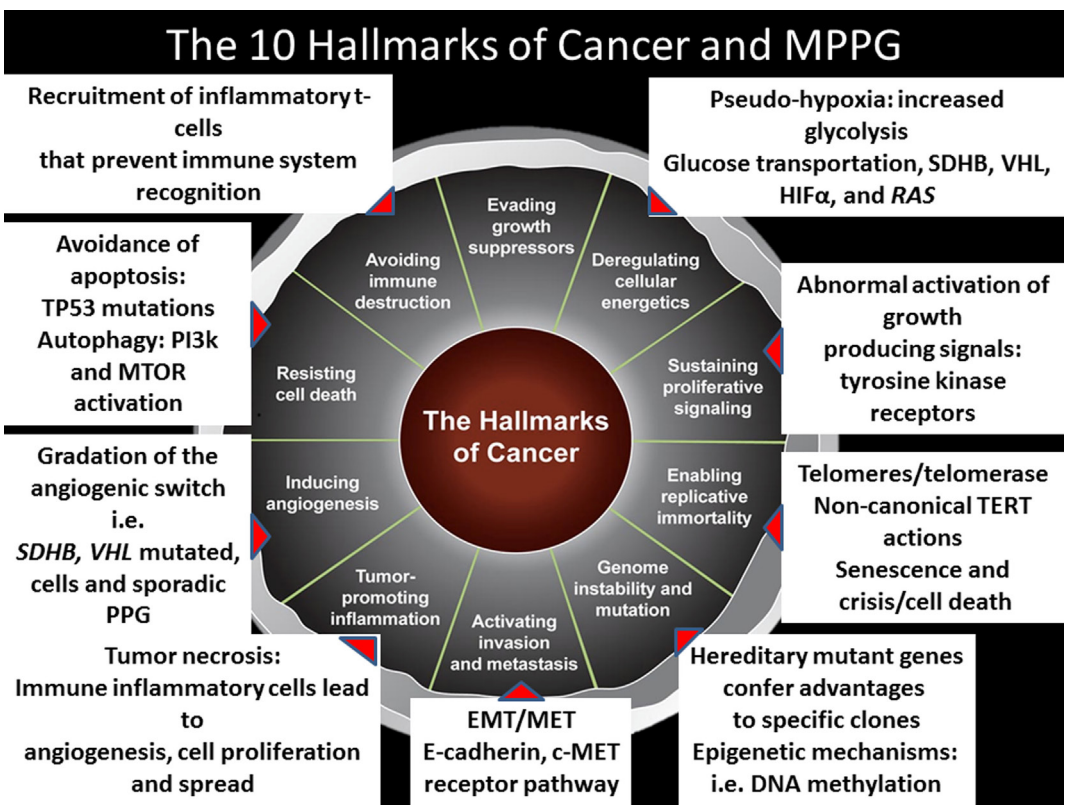

FIGURE 1 | The 10 hallmarks of cancer and metastatic pheochromocytomas and paragangliomas (MPPGs). This figure describes the 10 hallmarks of cancer and several mechanisms identified to date in patients with MPPG that contribute to their tumor development.

duration) was, however, not very impressive (4.1 months). The study was in fact an intention-to-treat analysis that included the three nonevaluable patients in the final evaluation of PFS. Some patients, nonetheless, exhibited a durable response to sunitinib; the longest response lasted 3 years. The study concluded that some patients with MPPG may benefit from antiangiogenic therapies such as sunitinib (62). However, the dose of sunitinib must be carefully chosen, and adverse events should be prevented or treated aggressively with dose adjustment and/or interventions to prevent exacerbation of hypertension or symptoms such as pain. The FIRSTMAPPP trial (NCT01371201) is a multinational phase 2 study evaluating sunitinib in patients with MPPG. The intervention group receives 37.5 mg sunitinib daily. This dose of sunitinib is lower than the dose currently approved for the treatment of patients with kidney cancer (50 mg daily, 4 weeks on, 2 weeks off). The lower dose may be associated with a better safety profile.

Pazopanib was tested in a phase 2 clinical trial involving patients with MPPG (63). The intervention group received 400 mg pazopanib daily for 2 weeks of the first cycle, then 800 mg for 2 weeks of the second cycle, followed by 800 mg daily for the whole of the subsequent cycles. Patients needed to have measurable disease, as the primary endpoint was ORR. Of the seven recruited patients, only one patient exhibited a confirmed partial response of −57%. This patient's response to pazopanib lasted for approximately 2 years. Four patients had disease progression. Almost every patient exhibited hypertension; severe hypertension was noted in 50% of patients, including one patient who developed Takotsubo cardiomyopathy. The serious cardiovascular adverse events happened once the dose of pazopanib was titrated to 800 mg daily. The trial was terminated because of poor accrual (63).

This trial aimed to evaluate pazopanib as a potential therapeutic option for MPPG patients because previous comparative studies in patients with kidney cancer suggested that pazopanib was better tolerated than sunitinib (64, 65). Later, however, it was recognized that these studies had several pitfalls related to patient selection and quality-of-life assessment (66); more importantly, clinical practice indicated that patients treated with sunitinib and pazopanib had, in reality, similar compliance patterns. Thus, clinical considerations, including the physician's experience, should determine treatment choices. Pazopanib could prove to be an effective medication to treat MPPG. Nevertheless, it is important to remember that MPPGs are very challenging tumors; in addition to a large tumor burden, MPPGs frequently secrete excessive amounts of catecholamines that predispose patients to cardiovascular disease. A pazopanib dose of 800 mg daily was likely too high to tolerate.

Inhibition of Angiogenesis: Axitinib

Pseudohypoxia activates angiogenesis through stimulating the synthesis and secretion of VEGFs by MPPG cells. Axitinib is a potent antiangiogenic medication that has been approved by regulatory agencies for the treatment of patients with kidney cancer. Axitinib inhibits VEGFR-2 but does not inhibit other receptors involved in angiogenesis such as PDGFR-β. For this reason, axitinib is expected to cause fewer adverse events than multi-tyrosine kinase inhibitors that also target angiogenesis (67).

A phase 2 clinical trial of axitinib (NCT01967576) enrolled 11 patients (68). The primary endpoint was PFS, and secondary endpoints included ORR and safety. The intervention was 5 mg axitinib given twice daily. The dose of axitinib was increased to 7–10 mg twice daily in patients who did not experience side effects more severe than grade 2 hypertension. Approximately 36% of patients achieved a partial response, and 54% had stable disease. Of those with stable disease, half had some degree of regression. Only one patient exhibited disease progression. The ORR was 36% (68). No patients tolerated the starting dose of 5 mg twice daily for a long period of time because they developed hypertension. Hypertension was common and frequently serious. About 82% of patients had grade 3–4 hypertension and required dose reduction or discontinuation of therapy (68). The trial is currently closed for recruitment.

Inhibition of Angiogenesis, Proliferative Signaling, and Invasion and Metastasis: Cabozantinib

Cabozantinib is a multi-tyrosine kinase inhibitor approved for the treatment of patients with medullary thyroid and clear cell renal cell carcinomas. Cabozantinib is perhaps, the most potent antiangiogenic medication available in clinical practice. Cabozantinib inhibits VEGFR-2 as well as the RET and c-Met receptor pathways (69, 70). Although the inhibition of the RET receptor pathway may not be of interest for the treatment of the great majority of patients with MPPG (malignant pheochromocytomas are an exceptional phenotype of patients with multiple endocrine neoplasia type 2, which is associated with RET mutations) (71), the inhibition of the c-Met pathway may indeed be of interest. MET activation is a universal mechanism that drives cell survival, invasion, and metastasis in many cancers and cooperates with the VEGFR pathways to promote tumor angiogenesis (72). Upregulation of the c-MET pathway occurs as a consequence of the VEGFR inhibition, leading to tumor resistance to antiangiogenic medications and escape from VEGFR inhibition (72). Inhibition of the c-Met pathway may delay the development of tumor resistance and improve clinical outcomes (73). Patients with kidney cancer treated with cabozantinib exhibit significantly longer PFS than do patients treated with sunitinib (74). Cabozantinib is also an interesting medication to study in MPPG patients because of its potential impact on the bone microenvironment. Cabozantinib has been associated with palliation of bone pain, improvement of anemia, modulation of bone turnover, and bone scan resolution in patients with malignancies frequently associated with bone metastases (75). MPPG frequently spreads to the bones, predisposing patients to overwhelming skeletal-related events (58, 76).

A phase 2 clinical trial of cabozantinib is currently ongoing (NCT02302833). The primary endpoint of this study is ORR. The trial includes an exploratory branch of patients with MPPG with predominant bone metastases. The intervention is 60 mg cabozantinib daily with dose titration to 40 or 20 mg depending on patients' toleration of adverse events. Patients require objective evidence of disease progression to be included in the trial. Preliminary results in 10 patients with measurable disease showed an ORR of 40% (77). Half of patients had stable disease, and only

one patient did not have a response to therapy. All patients with stable disease had tumor regression; the clinical benefit rate was 90% (77). In addition, all patients with bone metastases exhibited a reduction of glucose uptake as demonstrated by FDG-PET. Patients with hormonally active tumors associated with partial responses or stable disease exhibited improvement of symptoms of catecholamine excess, including diabetes mellitus and hypertension (77). No patients experienced severe hypertension. However, most patients required dose reduction because of grade 2 fatigue or hand-foot syndrome. Two patients required dose reduction because of asymptomatic grade 3 elevation of pancreatic enzymes and formation of a rectal fistula, respectively (77). This clinical trial is actively recruiting participants.

Induction of Cell Death and Prevention of Replicative Immortality: Iobenguane ^{131}I and ^{177}Lu-DOTATATE

The radiopharmaceutical metaiodobenzylguanidine (MIBG) was created in 1979 (78). MIBG is labeled with ^{131}I at the meta-position and is taken up by the noradrenaline transporter. Once inside the tumor cell, MIBG releases lethal radiation that causes severe DNA damage, inhibiting cell proliferation and causing cell death. Up to 80% of MPPG patients have tumors that express the noradrenaline transporter in the cell membrane (79). Responses to MIBG are, however, limited, with only 30% of MPPG patients seeing a clinical benefit (80). The limited benefits associated with MIBG are likely attributable in part to its manufacturing process (81). MIBG is produced by a simple isotope exchange that leaves a large amount of unlabeled MIBG, called cold MIBG, in each dose. Cold MIBG may compete with labeled MIBG for the noradrenaline transporter, preventing the uptake of labeled MIBG and decreasing the tumor's exposure to radiation. MIBG delivers low levels of radioactivity per dose (~1.59 MBq/μg) (82). In addition, cold MIBG may compete with noradrenaline for the noradrenaline transporter, increasing the concentrations of circulating noradrenaline and predisposing to cardiovascular events during or shortly after the drug's administration (83). Iobenguane ^{131}I is also MIBG labeled with ^{131}I at the meta-position. Unlike conventional MIBG, iobenguane ^{131}I is produced from a solid-phase ultratrace precursor that eliminates the presence of cold MIBG. Iobenguane ^{131}I is, then, a highly specific radiopharmaceutical agent that delivers very high levels of radioactivity per dose (~92.5 MBq/μg). Furthermore, iobenguane ^{131}I may be associated with a lower rate of cardiovascular events than conventional MIBG (84).

A phase 1 dose-escalation study of iobenguane ^{131}I in patients with MPPG determined the maximum tolerated dose to be 296 MBq/kg (8 mCi/kg) (85). A pivotal phase 2b clinical trial of iobenguane ^{131}I was then developed, with an intervention of 2–500 mCi doses of iobenguane ^{131}I separated by a period of at least 3 months depending on bone marrow toxicity. This trial recruited 81 patients, 68 of whom had MIBG-avid tumors and received at least one therapeutic dose of iobenguane ^{131}I. Fifty patients received two doses of iobenguane ^{131}I (86). The trial's primary endpoint was clinical: the number of patients who had at least a 50% reduction in the dose and number of antihypertensives for at least 6 months. Secondary endpoints included ORR, OS, and safety (86). One-quarter of patients achieved the primary endpoint, and many of the patients who did not achieve the primary endpoint nonetheless had improvement of hypertension with a reduction of less than 50% in the dose and number of antihypertensives (86). Almost all patients had a tumor response. Partial responses and stable disease were noted in 30 and 68%, respectively, of patients treated with two doses (86). The proportion of patients who experienced a partial response increased over time, suggesting that iobenguane ^{131}I has persistent antitumor effects (86). Overall, 90% of patients treated with two doses continued to have a partial response or stable disease 12 months after receiving the initial dose (86). The most common treatment-emergent adverse events were consistent with expected radiation-related risks: bone marrow suppression, nausea and vomiting, fatigue, and dizziness. Hematological toxicities resolved within 4–8 weeks and without the need for stem cell transplantation (86). On the basis of these findings, the United States Food and Drug Administration granted breakthrough therapy and fast-track designation to iobenguane ^{131}I for the treatment of MPPG.

Peptide receptor radionuclide therapy (PRRT) is a molecular therapy used to treat neuroendocrine tumors. Examples of PRRT agents include ^{177}Lu-DOTATATE and ^{90}Y-DOTATE. ^{177}Lu-DOTATATE is approved for the treatment of patients with somatostatin receptor-positive gastroenteropancreatic neuroendocrine tumors. This radiopharmaceutical binds to the somatostatin receptors present at the tumor cell membrane, delivering lethal radiation. MPPGs usually express somatostatin receptors. In fact, the sensitivity of ^{68}Ga-DOTATATE positron emission tomography/computed tomography imaging in patients with MPPG seems to be higher than that of MIBG scans (87, 88). This observation makes ^{177}Lu-DOTATATE an interesting medication to evaluate in clinical trials. Initial prospective studies of ^{177}Lu-DOTATATE and ^{90}Y-DOTATE included occasional patients with MPPG. Response rates were disappointing, with less than 10% of these patients exhibiting a clinical benefit (89, 90). This observation contrasted with the higher sensitivity of octreotide scintigraphy compared with MIBG scans. Investigators hypothesized that MPPGs may have inappropriate expression of somatostatin receptor subtypes and/or processing errors that caused the lack of response to octreotide and its analogs (91). In fact, molecular studies in a few MPPG specimens have found minimal or no expression of the somatostatin receptor 2 (92). DOTATATE mainly targets this receptor (93). Recently, however, the interest in ^{177}Lu-DOTATATE for the treatment of patients with MPPG has been reactivated. A retrospective study of patients with MPPG treated with MIBG ($n = 16$), ^{90}Y-DOTATE ($n = 12$), or ^{177}Lu-DOTATATE ($n = 2$) suggested that PRRT offered better OS and PFS than did conventional MIBG (94). Nevertheless, this study had several limitations. The sample size was very small, the treatment groups for comparison were quite heterogeneous, and the authors did not conduct a multivariate or propensity score analysis to reduce bias. Nevertheless, individual clinical observations suggested that some patients benefited from PRRT (94). In a more recent retrospective study of 20 patients with MPPG treated with ^{177}Lu-DOTATATE, 29% had partial responses and 62% had stable disease 3 months after therapy (95). Fourteen patients had

hypertension, and only six patients had disease progression before treatment was provided. Nine patients received radiosensitizing chemotherapy. Some reduction in the dosage of antihypertensive medications was observed in 62% of the patients with hypertension (95). However, the small and heterogeneous sample of this study, the fact that most patients had stable disease before treatment, and the simultaneous use of chemotherapy hinder the interpretation of this study's results. Therefore, a prospective clinical trial is required to prove the efficacy of PRRT in patients with MPPG.

Regulation of Cellular Energetics: HIF Inhibitors

Several medications that inhibit the HIF-2α pathway have been tested in patients with cancer. Most of these medications have been shown not to be very effective and have not moved from phase 1 to phase 2 clinical trials (96). Crystallography identified a large protein cavity within the HIF-2α PAS-B domain. This cavity is the target of potent 130 HIF-2α inhibitors (PT2385 and PT2977) (97). Recently, PT2385 was evaluated in a phase 2 clinical trial for heavily pretreated patients with progressive renal cancer (98). Several patients had partial responses, and one patient had a complete response. Interestingly, side effects were minimal (98). These drugs have not been tested in patients with MPPG. An investigator-initiated proposal for a phase 2 clinical trial of PT2977 for MPPGs is under evaluation by regulatory agencies.

Enhancement of Immune System Tumor Recognition: Interferon and Pembrolizumab

Pseudohypoxia causes inactivation of cytotoxic T-cell lymphocytes, activation of immune-suppressive monocytes, increased adenosine production, and increased expression of the immune checkpoint protein programmed death-ligand 1 (PD-L1) and its receptor, among many other immune system disarrangements (55, 99, 100).

One of the first immunotherapies introduced to clinical practice was interferon alpha-2b. Interferon alpha-2b activates natural killer cells that can recognize and destroy cancer cells and has been used for the treatment of patients with gastroenteropancreatic neuroendocrine tumors, melanoma, and kidney cancer. Some of these patients had clinical benefits, with disease stabilization and occasional partial responses (101). These findings correlated with histological evidence of tumor necrosis. For many years, interferon alpha-2b and octreotide analogs were considered the pillars of treatment for patients with gastroenteropancreatic neuroendocrine tumors (102). As MPPGs are also neuroendocrine tumors, occasionally patients were also treated with interferon alpha-2b. A recent retrospective study of 14 patients with progressive MPPG who were treated with interferon alpha-2b showed that 12 patients had disease stabilization and 2 had partial responses (103). Baseline PFS was 9.4 months, while the PFS of patients treated with interferon alpha-2b was 17.2 months. This study suggested that immunotherapy could have a positive impact on patients with MPPG (103). Prospective studies of interferon alpha-2b in other malignancies had several methodological problems (101); furthermore, the side effects of this drug, including fatigue, depression, flu-like syndrome, renal failure, and liver toxicity, substantially alter patients' quality of life and frequently lead to therapy discontinuation (104). Consequently, interest in interferon alpha-2b has declined.

Over the last decade, several novel immune therapies have been developed. These medications target immune-related molecular pathways such as the cytotoxic T-lymphocyte-associated protein 4 and PD-L1/PD-1 pathways. These pathways, among others, play an important role in the recognition of the cancer cell by the immune system. These immune checkpoint inhibitors are approved for the treatment of several malignancies, including melanoma, kidney cancer, and non-small cell lung cancer. Prospective studies have demonstrated disease stabilization, partial responses, and sometimes disease resolution (105–107). Although serious adverse events—mainly autoimmune events—have been described, for the most part patients tolerate these medications well (105–107). A phase 2 clinical trial of the PD-1 inhibitor pembrolizumab for patients with MPPG is currently underway. This study hypothesizes that the administration of single-agent pembrolizumab to patients with PD-L1-positive MPPG will result in a nonprogression rate of greater than 20% (based on RECIST 1.1 criteria) at 27 weeks (nine cycles). The study is actively recruiting patients (NCT02721732).

WHERE ARE WE GOING?

Clinical and basic research studies have revealed that replicative immortality, upregulated and sustained proliferative signaling, genome instability (single mutations), inflammation, deregulation of cellular energetics, angiogenesis, and activation of mechanisms responsible for invasion and metastasis are hallmarks of the development of MPPG that could be effectively targeted with available treatments. MPPG treatments, then, can be categorized on the basis of their actions on one or more of these hallmark capabilities. As detailed above, each potential therapy for patients with MPPG typically targets only one or two hallmark capabilities. Preliminary results from prospective clinical trials and observations derived from retrospective studies strongly suggest that angiogenesis is a predominant druggable hallmark of MPPG. Patients treated with angiogenesis inhibitors have indeed exhibited clinical benefits such as tumor size reduction and improvement of hormonal symptoms. However, although individual clinical responses may sometimes last for several years, these responses are, in general, transitory (108). The development of resistance is not exclusive to therapies that target angiogenesis; it is also expected with other therapies because they target a limited number of MPPG hallmark capabilities (109). In fact, cancer cells may, over time, acquire other capabilities that lead to treatment resistance, tumor recurrence, and disease progression.

In addition, each of the hallmark capabilities is regulated by partially redundant molecular pathways, and individual targeted therapies may not fully downregulate all of the molecular pathways responsible for a specific hallmark capability. Therefore, some MPPG cells may survive and adapt over time to the biological impositions of targeted therapies. These adaptive mechanisms may include the development of new mutations, the

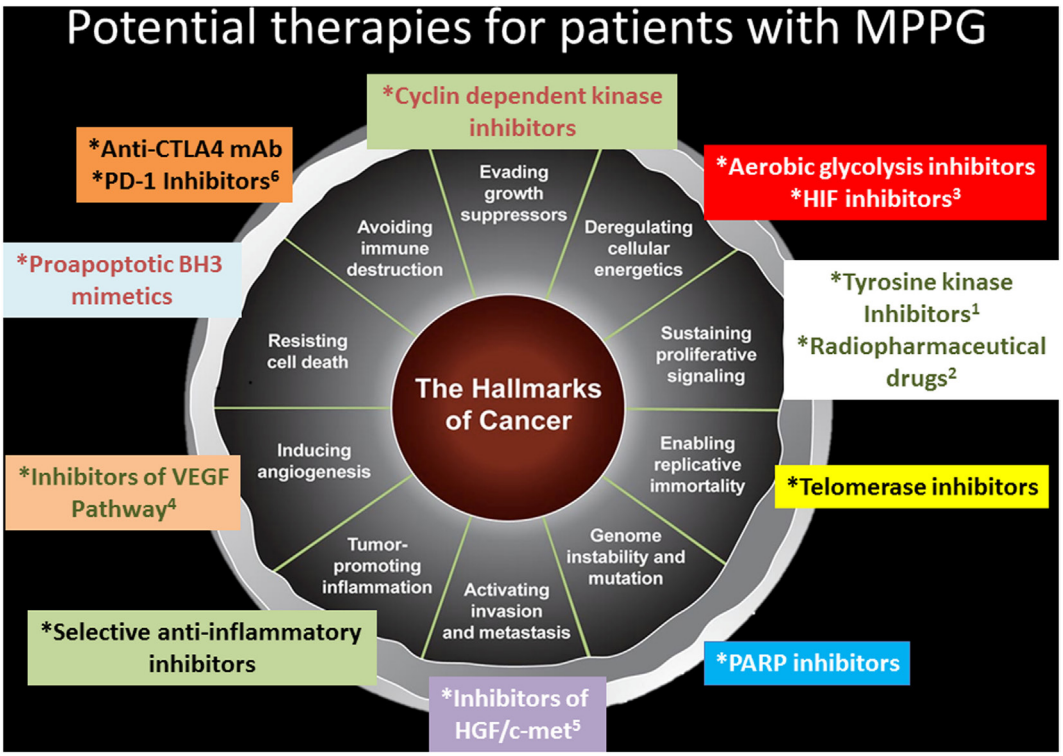

FIGURE 2 | Potential therapies for patients with metastatic pheochromocytomas and paraganglioma (MPPG). This figure describes potential therapies for patients with MPPG. Some of these therapies are currently evaluated in clinical trials: [1]axitinib, cabozantinib, lenvatinib, pazopanib, and sunitinib; [2]iobenguane ^{131}I, ^{177}Lu-DOTATATE; [3]PT2977; [4]axitinib, cabozantinib, lenvatinib, pazopanib, and sunitinib; [5]cabozantinib; and [6]pembrolizumab.

remodeling of cells' epigenetic characteristics, and modifications of their microenvironment. Single medications or combined therapies that target several hallmark capabilities at the same time may produce a higher rate of response and more durable benefits. However, patients receiving such treatments might be more prone to develop severe adverse events than are patients treated with medications that target only one or two capabilities. Therefore, the dose and administration of these drugs must be carefully calibrated in order to achieve the best possible therapeutic response while minimizing the severity of side effects. The phase 2 clinical trial of cabozantinib discussed above, for instance, is exploring this concept. It is also important to develop clinical trials that combine therapies with different and complementary mechanisms of action. There are currently no such trials for patients with MPPG, and any such trial will likely need to be preceded by a phase 1 trial to evaluate dosage and safety.

Our current knowledge about the hallmarks of cancer suggests that exploring other therapeutic options for patients with MPPG may be helpful. Telomerase inhibitors that can modulate replicative immortality, poly (ADP-ribose) inhibitors that can stabilize the genome, selective anti-inflammatory medications, inhibitors of the hepatocyte growth factor and c-Met pathways that can stop invasion and metastasis, proapoptotic Bcl-2 homology domain 3 inhibitors that can prevent resistance to cell death, cyclin-dependent kinase inhibitors that can enhance the activity of growth suppressors, aerobic glycolysis inhibitors, and epidermal growth factor receptor inhibitors that can prevent sustained proliferative signaling all are potential medications to evaluate in clinical trials (**Figure 2**).

Metastatic pheochromocytomas and paraganglioma is an orphan disease with no Food and Drug Administration-approved therapies. The final results of the phase 2 pivotal clinical trial with iobenguane ^{131}I are sound and impressive (86). Given the rarity of MPPG, it would be very difficult to develop a phase 3 clinical trial, so regulatory agencies should consider approving iobenguane ^{131}I for the treatment of patients with MIBG-avid MPPG. If such approval is granted, iobenguane ^{131}I may become the first-line treatment for many patients with MPPG. Clinical trials with multi-tyrosine kinase inhibitors, HIF inhibitors, ^{177}Lu-DOTATATE, and pembrolizumab would become therapeutic options to explore in patients with non-MIBG-avid MPPG, patients with MIBG-avid tumors that do not respond to iobenguane ^{131}I, and patients who have contraindications for iobenguane ^{131}I therapy.

CONCLUSION

The available treatments for MPPG are, so far, not curative; research is needed to evaluate therapies with novel mechanisms of action. The use of tyrosine kinase inhibitors, radionuclide agents, and immune therapy may improve the outcomes of patients with MPPG and should be studied in clinical trials. It is always important to treat and prevent hormonal complications and symptoms that derive from direct drug toxicity, so drug doses

must be carefully selected. Clinical trials combining therapies that target several hallmarks of MPPG in a simultaneous or sequential manner are an essential goal of MPPG research.

AUTHOR CONTRIBUTIONS

CJ has written this invited review manuscript. CJ created the manuscript structure, abstract, sections, figures, and chose the references. CJ is the only author of this manuscript.

ACKNOWLEDGMENTS

The author acknowledges Dr. Amy Ninetto from the Department of Scientific Publications at The University of Texas MD Anderson Cancer Center for the thorough editing provided to this manuscript. The author would like to thank very much Ms. Natalie Papadam, Mr. Peter Liu, the Team Nat Foundation, Ms. Margaret Cazalot, Ms. Marle Granek, and Dr. Catherine Cotton for their immense support to develop clinical trials for patients with malignant pheochromocytomas and paragangliomas. Thanks to their inspiration and support, patients have new hope.

FUNDING

This publication was supported by the Team Nat Foundation and the Cazalot Fund for research on pheochromocytoma and paraganglioma.

REFERENCES

1. Falhammar H, Kjellman M, Calissendorff J. Initial clinical presentation and spectrum of pheochromocytoma: a study of 94 cases from a single center. *Endocr Connect* (2018) 7:186–92. doi:10.1530/EC-17-0321
2. Thosani S, Ayala-Ramirez M, Roman-Gonzalez A, Zhou S, Thosani N, Bisanz A, et al. Constipation: an overlooked, unmanaged symptom of patients with pheochromocytoma and sympathetic paraganglioma. *Eur J Endocrinol* (2015) 173:377–87. doi:10.1530/EJE-15-0456
3. Lenders JW, Duh QY, Eisenhofer G, Gimenez-Roqueplo AP, Grebe SK, Murad MH, et al. Pheochromocytoma and paraganglioma: an endocrine society clinical practice guideline. *J Clin Endocrinol Metab* (2014) 99:1915–42. doi:10.1210/jc.2014-1498
4. Abadin SS, Ayala-Ramirez M, Jimenez C, Dickson PV, Liang Y, Lazar AJ, et al. Impact of surgical resection for subdiaphragmatic paragangliomas. *World J Surg* (2014) 38:733–41. doi:10.1007/s00268-013-2443-5
5. Grubbs EG, Rich TA, Ng C, Bhosale PR, Jimenez C, Evans DB, et al. Long-term outcomes of surgical treatment for hereditary pheochromocytoma. *J Am Coll Surg* (2013) 216:280–9. doi:10.1016/j.jamcollsurg.2012.10.012
6. Grogan RH, Mitmaker EJ, Duh QY. Changing paradigms in the treatment of malignant pheochromocytoma. *Cancer Control* (2011) 18:104–12. doi:10.1177/107327481101800205
7. Lam AK. Update on adrenal tumours in 2017 World Health Organization (WHO) of endocrine tumours. *Endocr Pathol* (2017) 28:213–27. doi:10.1007/s12022-017-9484-5
8. Wu D, Tischler AS, Lloyd RV, DeLellis RA, de Krijger R, van Nederveen F, et al. Observer variation in the application of the pheochromocytoma of the adrenal gland scaled score. *Am J Surg Pathol* (2009) 33:599–608. doi:10.1097/PAS.0b013e318190d12e
9. Ayala-Ramirez M, Feng L, Johnson MM, Ejaz S, Habra MA, Rich T, et al. Clinical risk factors for malignancy and overall survival in patients with pheochromocytomas and sympathetic paragangliomas: primary tumor size and primary tumor location as prognostic indicators. *J Clin Endocrinol Metab* (2011) 96:717–25. doi:10.1210/jc.2010-1946
10. Jimenez C, Rohren E, Habra MA, Rich T, Jimenez P, Ayala-Ramirez M, et al. Current and future treatments for malignant pheochromocytoma and sympathetic paraganglioma. *Curr Oncol Rep* (2013) 15:356–71. doi:10.1007/s11912-013-0320-x
11. Baudin E, Habra MA, Deschamps F, Cote G, Dumont F, Cabanillas M, et al. Therapy of endocrine disease: treatment of malignant pheochromocytoma and paraganglioma. *Eur J Endocrinol* (2014) 171:R111–22. doi:10.1530/EJE-14-0113
12. Srinivasan R, Kini U, Babu MK, Jayaseelan E, Pradeep R. Malignant pheochromocytoma with cutaneous metastases presenting with hemolytic anemia and pyrexia of unknown origin. *J Assoc Physicians India* (2002) 50:731–3.
13. Plouin PF, Fitzgerald P, Rich T, Ayala-Ramirez M, Perrier ND, Baudin E, et al. Metastatic pheochromocytoma and paraganglioma: focus on therapeutics. *Horm Metab Res* (2012) 44:390–9. doi:10.1055/s-0031-1299707
14. Niemeijer ND, Alblas G, van Hulsteijn LT, Dekkers OM, Corssmit EP. Chemotherapy with cyclophosphamide, vincristine and dacarbazine for malignant paraganglioma and pheochromocytoma: systematic review and meta-analysis. *Clin Endocrinol* (2014) 81:642–51. doi:10.1111/cen.12542
15. Lepoutre-Lussey C, Thibault C, Buffet A, Morin A, Badoual C, Benit P, et al. From Nf1 to Sdhb knockout: successes and failures in the quest for animal models of pheochromocytoma. *Mol Cell Endocrinol* (2016) 421:40–8. doi:10.1016/j.mce.2015.06.027
16. Jimenez P, Tatsui C, Jessop A, Thosani S, Jimenez C. Treatment for malignant pheochromocytomas and paragangliomas: 5 years of progress. *Curr Oncol Rep* (2017) 19:83. doi:10.1007/s11912-017-0643-0
17. Dahia PL. Pheochromocytoma and paraganglioma pathogenesis: learning from genetic heterogeneity. *Nat Rev Cancer* (2014) 14:108–19. doi:10.1038/nrc3648
18. Favier J, Amar L, Gimenez-Roqueplo AP. Paraganglioma and phaeochromocytoma: from genetics to personalized medicine. *Nat Rev Endocrinol* (2015) 11:101–11. doi:10.1038/nrendo.2014.188
19. Gimenez-Roqueplo AP, Favier J, Rustin P, Rieubland C, Crespin M, Nau V, et al. Mutations in the SDHB gene are associated with extra-adrenal and/or malignant phaeochromocytomas. *Cancer Res* (2003) 63:5615–21.
20. Dahia PL, Ross KN, Wright ME, Hayashida CY, Santagata S, Barontini M, et al. A HIF1alpha regulatory loop links hypoxia and mitochondrial signals in pheochromocytomas. *PLoS Genet* (2005) 1:72–80. doi:10.1371/journal.pgen.0010008
21. Loriot C, Burnichon N, Gadessaud N, Vescovo L, Amar L, Libe R, et al. Epithelial to mesenchymal transition is activated in metastatic pheochromocytomas and paragangliomas caused by SDHB gene mutations. *J Clin Endocrinol Metab* (2012) 97:E954–62. doi:10.1210/jc.2011-3437
22. Letouze E, Martinelli C, Loriot C, Burnichon N, Abermil N, Ottolenghi C, et al. SDH mutations establish a hypermethylator phenotype in paraganglioma. *Cancer Cell* (2013) 23:739–52. doi:10.1016/j.ccr.2013.04.018
23. Loriot C, Domingues M, Berger A, Menara M, Ruel M, Morin A, et al. Deciphering the molecular basis of invasiveness in Sdhb-deficient cells. *Oncotarget* (2015) 6:32955–65. doi:10.18632/oncotarget.5106
24. van Berkel A, Rao JU, Kusters B, Demir T, Visser E, Mensenkamp AR, et al. Correlation between in vivo 18F-FDG PET and immunohistochemical markers of glucose uptake and metabolism in pheochromocytoma and paraganglioma. *J Nucl Med* (2014) 55:1253–9. doi:10.2967/jnumed.114.137034
25. Timmers HJ, Kozupa A, Chen CC, Carrasquillo JA, Ling A, Eisenhofer G, et al. Superiority of fluorodeoxyglucose positron emission tomography to other functional imaging techniques in the evaluation of metastatic SDHB-associated pheochromocytoma and paraganglioma. *J Clin Oncol* (2007) 25:2262–9. doi:10.1200/JCO.2006.09.6297
26. Henegan JC Jr, Gomez CR. Heritable cancer syndromes related to the hypoxia pathway. *Front Oncol* (2016) 6:68. doi:10.3389/fonc.2016.00068
27. Casey RT, Warren AY, Martin JE, Challis BG, Rattenberry E, Whitworth J, et al. Clinical and molecular features of renal and pheochromocytoma/paraganglioma tumor association syndrome (RAPTAS): case series and

literature review. *J Clin Endocrinol Metab* (2017) 102:4013–22. doi:10.1210/jc.2017-00562
28. Scarpa A, Chang DK, Nones K, Corbo V, Patch AM, Bailey P, et al. Whole-genome landscape of pancreatic neuroendocrine tumours. *Nature* (2017) 543:65–71. doi:10.1038/nature21063
29. Roy S, Narang BK, Rastogi SK, Rawal RK. A novel multiple tyrosine-kinase targeted agent to explore the future perspectives of anti-angiogenic therapy for the treatment of multiple solid tumors: cabozantinib. *Anticancer Agents Med Chem* (2015) 15:37–47. doi:10.2174/1871520614666140902153840
30. Roman-Gonzalez A, Jimenez C. Malignant pheochromocytoma-paraganglioma: pathogenesis, TNM staging, and current clinical trials. *Curr Opin Endocrinol Diabetes Obes* (2017) 24:174–83. doi:10.1097/MED.0000000000000330
31. Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. *Cell* (2011) 144:646–74. doi:10.1016/j.cell.2011.02.013
32. Roman-Gonzalez A, Zhou S, Ayala-Ramirez M, Shen C, Waguespack SG, Habra MA, et al. Impact of surgical resection of the primary tumor on overall survival in patients with metastatic pheochromocytoma or sympathetic paraganglioma. *Ann Surg* (2017). doi:10.1097/SLA.0000000000002195
33. Jansen TTG, Timmers H, Marres HAM, Kunst HPM. Feasibility of a wait-and-scan period as initial management strategy for head and neck paraganglioma. *Head Neck* (2017) 39:2088–94. doi:10.1002/hed.24871
34. Smith JD, Harvey RN, Darr OA, Prince ME, Bradford CR, Wolf GT, et al. Head and neck paragangliomas: a two-decade institutional experience and algorithm for management. *Laryngoscope Investig Otolaryngol* (2017) 2:380–9. doi:10.1002/lio2.122
35. Bacciu A, Medina M, Ait Mimoune H, D'Orazio F, Pasanisi E, Peretti G, et al. Lower cranial nerves function after surgical treatment of Fisch class C and D tympanojugular paragangliomas. *Eur Arch Otorhinolaryngol* (2015) 272:311–9. doi:10.1007/s00405-013-2862-9
36. Ayala-Ramirez M, Feng L, Habra MA, Rich T, Dickson PV, Perrier N, et al. Clinical benefits of systemic chemotherapy for patients with metastatic pheochromocytomas or sympathetic extra-adrenal paragangliomas: insights from the largest single-institutional experience. *Cancer* (2012) 118:2804–12. doi:10.1002/cncr.26577
37. Tanabe A, Naruse M, Nomura K, Tsuiki M, Tsumagari A, Ichihara A. Combination chemotherapy with cyclophosphamide, vincristine, and dacarbazine in patients with malignant pheochromocytoma and paraganglioma. *Horm Cancer* (2013) 4:103–10. doi:10.1007/s12672-013-0133-2
38. Szalat A, Fraenkel M, Doviner V, Salmon A, Gross DJ. Malignant pheochromocytoma: predictive factors of malignancy and clinical course in 16 patients at a single tertiary medical center. *Endocrine* (2011) 39:160–6. doi:10.1007/s12020-010-9422-5
39. Huang H, Abraham J, Hung E, Averbuch S, Merino M, Steinberg SM, et al. Treatment of malignant pheochromocytoma/paraganglioma with cyclophosphamide, vincristine, and dacarbazine: recommendation from a 22-year follow-up of 18 patients. *Cancer* (2008) 113:2020–8. doi:10.1002/cncr.23812
40. Hadoux J, Favier J, Scoazec JY, Leboulleux S, Al Ghuzlan A, Caramella C, et al. SDHB mutations are associated with response to temozolomide in patients with metastatic pheochromocytoma or paraganglioma. *Int J Cancer* (2014) 135:2711–20. doi:10.1002/ijc.28913
41. Castro-Vega LJ, Buffet A, De Cubas AA, Cascon A, Menara M, Khalifa E, et al. Germline mutations in FH confer predisposition to malignant pheochromocytomas and paragangliomas. *Hum Mol Genet* (2014) 23:2440–6. doi:10.1093/hmg/ddt639
42. Killian JK, Kim SY, Miettinen M, Smith C, Merino M, Tsokos M, et al. Succinate dehydrogenase mutation underlies global epigenomic divergence in gastrointestinal stromal tumor. *Cancer Discov* (2013) 3:648–57. doi:10.1158/2159-8290.CD-13-0092
43. Toledo RA, Qin Y, Srikantan S, Morales NP, Li Q, Deng Y, et al. In vivo and in vitro oncogenic effects of HIF2A mutations in pheochromocytomas and paragangliomas. *Endocr Relat Cancer* (2013) 20:349–59. doi:10.1530/ERC-13-0101
44. Toledo RA. New HIF2alpha inhibitors: potential implications as therapeutics for advanced pheochromocytomas and paragangliomas. *Endocr Relat Cancer* (2017) 24:C9–19. doi:10.1530/ERC-16-0479
45. Toledo RA, Qin Y, Cheng ZM, Gao Q, Iwata S, Silva GM, et al. Recurrent mutations of chromatin-remodeling genes and kinase receptors in pheochromocytomas and paragangliomas. *Clin Cancer Res* (2016) 22:2301–10. doi:10.1158/1078-0432.CCR-15-1841
46. Kluckova K, Tennant DA. Metabolic implications of hypoxia and pseudohypoxia in pheochromocytoma and paraganglioma. *Cell Tissue Res* (2018) 372(2):367–78. doi:10.1007/s00441-018-2801-6
47. Stenman A, Welander J, Gustavsson I, Brunaud L, Backdahl M, Soderkvist P, et al. HRAS mutation prevalence and associated expression patterns in pheochromocytoma. *Genes Chromosomes Cancer* (2016) 55:452–9. doi:10.1002/gcc.22347
48. Oudijk L, de Krijger RR, Rapa I, Beuschlein F, de Cubas AA, Dei Tos AP, et al. H-RAS mutations are restricted to sporadic pheochromocytomas lacking specific clinical or pathological features: data from a multi-institutional series. *J Clin Endocrinol Metab* (2014) 99:E1376–80. doi:10.1210/jc.2013-3879
49. Blasco MA. Telomeres and human disease: ageing, cancer and beyond. *Nat Rev Genet* (2005) 6:611–22. doi:10.1038/nrg1656
50. Dwight T, Flynn A, Amarasinghe K, Benn DE, Lupat R, Li J, et al. TERT structural rearrangements in metastatic pheochromocytomas. *Endocr Relat Cancer* (2018) 25:1–9. doi:10.1530/ERC-17-0306
51. Fishbein L, Khare S, Wubbenhorst B, DeSloover D, D'Andrea K, Merrill S, et al. Whole-exome sequencing identifies somatic ATRX mutations in pheochromocytomas and paragangliomas. *Nat Commun* (2015) 6:6140. doi:10.1038/ncomms7140
52. Comino-Mendez I, Tejera AM, Curras-Freixes M, Remacha L, Gonzalvo P, Tonda R, et al. ATRX driver mutation in a composite malignant pheochromocytoma. *Cancer Genet* (2016) 209:272–7. doi:10.1016/j.cancergen.2016.04.058
53. Burnichon N, Buffet A, Parfait B, Letouze E, Laurendeau I, Loriot C, et al. Somatic NF1 inactivation is a frequent event in sporadic pheochromocytoma. *Hum Mol Genet* (2012) 21:5397–405. doi:10.1093/hmg/dds374
54. Petrilli AM, Fuse MA, Donnan MS, Bott M, Sparrow NA, Tondera D, et al. A chemical biology approach identified PI3K as a potential therapeutic target for neurofibromatosis type 2. *Am J Transl Res* (2014) 6:471–93.
55. Chouaib S, Noman MZ, Kosmatopoulos K, Curran MA. Hypoxic stress: obstacles and opportunities for innovative immunotherapy of cancer. *Oncogene* (2017) 36:439–45. doi:10.1038/onc.2016.225
56. Singh A, Settleman J. EMT, cancer stem cells and drug resistance: an emerging axis of evil in the war on cancer. *Oncogene* (2010) 29:4741–51. doi:10.1038/onc.2010.215
57. D'Antongiovanni V, Martinelli S, Richter S, Canu L, Guasti D, Mello T, et al. The microenvironment induces collective migration in SDHB-silenced mouse pheochromocytoma spheroids. *Endocr Relat Cancer* (2017) 24:555–64. doi:10.1530/ERC-17-0212
58. Ayala-Ramirez M, Palmer JL, Hofmann MC, de la Cruz M, Moon BS, Waguespack SG, et al. Bone metastases and skeletal-related events in patients with malignant pheochromocytoma and sympathetic paraganglioma. *J Clin Endocrinol Metab* (2013) 98:1492–7. doi:10.1210/jc.2012-4231
59. Hao Z, Sadek I. Sunitinib: the antiangiogenic effects and beyond. *Onco Targets Ther* (2016) 9:5495–505. doi:10.2147/OTT.S112242
60. van Geel RM, Beijnen JH, Schellens JH. Concise drug review: pazopanib and axitinib. *Oncologist* (2012) 17:1081–9. doi:10.1634/theoncologist.2012-0055
61. Santarpia L, Habra MA, Jimenez C. Malignant pheochromocytomas and paragangliomas: molecular signaling pathways and emerging therapies. *Horm Metab Res* (2009) 41:680–6. doi:10.1055/s-0029-1214381
62. Ayala-Ramirez M, Chougnet CN, Habra MA, Palmer JL, Leboulleux S, Cabanillas ME, et al. Treatment with sunitinib for patients with progressive metastatic pheochromocytomas and sympathetic paragangliomas. *J Clin Endocrinol Metab* (2012) 97:4040–50. doi:10.1210/jc.2012-2356
63. Jasim S, Suman VJ, Jimenez C, Harris P, Sideras K, Burton JK, et al. Phase II trial of pazopanib in advanced/progressive malignant pheochromocytoma and paraganglioma. *Endocrine* (2017) 57:220–5. doi:10.1007/s12020-017-1359-5
64. Motzer RJ, Hutson TE, Cella D, Reeves J, Hawkins R, Guo J, et al. Pazopanib versus sunitinib in metastatic renal-cell carcinoma. *N Engl J Med* (2013) 369:722–31. doi:10.1056/NEJMoa1303989
65. Escudier B, Porta C, Bono P, Powles T, Eisen T, Sternberg CN, et al. Randomized, controlled, double-blind, cross-over trial assessing treatment preference for pazopanib versus sunitinib in patients with metastatic renal cell carcinoma: PISCES Study. *J Clin Oncol* (2014) 32:1412–8. doi:10.1200/JCO.2013.50.8267

66. Ruiz-Morales JM, Swierkowski M, Wells JC, Fraccon AP, Pasini F, Donskov F, et al. First-line sunitinib versus pazopanib in metastatic renal cell carcinoma: results from the international metastatic renal cell carcinoma database consortium. *Eur J Cancer* (2016) 65:102–8. doi:10.1016/j.ejca.2016.06.016
67. Keating GM. Axitinib: a review in advanced renal cell carcinoma. *Drugs* (2015) 75:1903–13. doi:10.1007/s40265-015-0483-x
68. Emmanuel M, Velarde M, Elaine-Bates S, Daerr R, Adams K, Fojo T. Phase II clinical trial of axitinib in metastatic pheochromocytomas and paraganlgiomas (P/PG): preliminary results. *Presented at ASCO Annual Meeting*. Chicago, IL, USA (2015).
69. Al-Salama ZT, Keating GM. Cabozantinib: a review in advanced renal cell carcinoma. *Drugs* (2016) 76:1771–8. doi:10.1007/s40265-016-0661-5
70. Hoy SM. Cabozantinib: a review of its use in patients with medullary thyroid cancer. *Drugs* (2014) 74:1435–44. doi:10.1007/s40265-014-0265-x
71. Thosani S, Ayala-Ramirez M, Palmer L, Hu MI, Rich T, Gagel RF, et al. The characterization of pheochromocytoma and its impact on overall survival in multiple endocrine neoplasia type 2. *J Clin Endocrinol Metab* (2013) 98:E1813–9. doi:10.1210/jc.2013-1653
72. Trusolino L, Bertotti A, Comoglio PM. MET signalling: principles and functions in development, organ regeneration and cancer. *Nat Rev Mol Cell Biol* (2010) 11:834–48. doi:10.1038/nrm3012
73. Yakes FM, Chen J, Tan J, Yamaguchi K, Shi Y, Yu P, et al. Cabozantinib (XL184), a novel MET and VEGFR2 inhibitor, simultaneously suppresses metastasis, angiogenesis, and tumor growth. *Mol Cancer Ther* (2011) 10:2298–308. doi:10.1158/1535-7163.MCT-11-0264
74. Choueiri TK, Halabi S, Sanford BL, Hahn O, Michaelson MD, Walsh MK, et al. Cabozantinib versus sunitinib as initial targeted therapy for patients with metastatic renal cell carcinoma of poor or intermediate risk: the alliance A031203 CABOSUN trial. *J Clin Oncol* (2017) 35:591–7. doi:10.1200/JCO.2016.70.7398
75. Smith M, De Bono J, Sternberg C, Le Moulec S, Oudard S, De Giorgi U, et al. Phase III study of cabozantinib in previously treated metastatic castration-resistant prostate cancer: COMET-1. *J Clin Oncol* (2016) 34:3005–13. doi:10.1200/JCO.2015.65.5597
76. Gravel G, Leboulleux S, Tselikas L, Fassio F, Berraf M, Berdelou A, et al. Prevention of serious skeletal-related events by interventional radiology techniques in patients with malignant paraganglioma and pheochromocytoma. *Endocrine* (2018) 59:547–54. doi:10.1007/s12020-017-1515-y
77. Jimenez C, Pool M, Busaidy N, Habra MA, Waguespack S, Jessop A. A phase 2 study to evaluate the effects of cabozantinib in patients with unresectable metastatic pheochromocytomas and paragangliomas. *International Symposium on Pheochromocytoma and Paraganglioma*. Sydney, Australia (2017).
78. Bruchelt G, Klingebiel T, Treuner J, Beck J, Lode H, Seitz G, et al. Radiolabeled metaiodobenzylguanidine (mibg) in diagnosis and therapy of neuroblastoma – results from basic research (review). *Int J Oncol* (1995) 6:705–12.
79. Bhatia KS, Ismail MM, Sahdev A, Rockall AG, Hogarth K, Canizales A, et al. 123I-metaiodobenzylguanidine (MIBG) scintigraphy for the detection of adrenal and extra-adrenal phaeochromocytomas: CT and MRI correlation. *Clin Endocrinol* (2008) 69:181–8. doi:10.1111/j.1365-2265.2008.03256.x
80. van Hulsteijn LT, Niemeijer ND, Dekkers OM, Corssmit EP. (131)I-MIBG therapy for malignant paraganglioma and phaeochromocytoma: systematic review and meta-analysis. *Clin Endocrinol* (2014) 80:487–501. doi:10.1111/cen.12341
81. Vallabhajosula S, Nikolopoulou A. Radioiodinated metaiodobenzylguanidine (MIBG): radiochemistry, biology, and pharmacology. *Semin Nucl Med* (2011) 41:324–33. doi:10.1053/j.semnuclmed.2011.05.003
82. Vaidyanathan G, Zalutsky MR. No-carrier-added synthesis of meta-[131I] iodobenzylguanidine. *Appl Radiat Isot* (1993) 44:621–8. doi:10.1016/0969-8043(93)90179-E
83. Gonias S, Goldsby R, Matthay KK, Hawkins R, Price D, Huberty J, et al. Phase II study of high-dose [131I]metaiodobenzylguanidine therapy for patients with metastatic pheochromocytoma and paraganglioma. *J Clin Oncol* (2009) 27:4162–8. doi:10.1200/JCO.2008.21.3496
84. Barrett JA, Joyal JL, Hillier SM, Maresca KP, Femia FJ, Kronauge JF, et al. Comparison of high-specific-activity ultratrace 123/131I-MIBG and carrier-added 123/131I-MIBG on efficacy, pharmacokinetics, and tissue distribution. *Cancer Biother Radiopharm* (2010) 25:299–308. doi:10.1089/cbr.2009.0695
85. Noto RB, Pryma DA, Jensen J, Lin T, Stambler N, Strack T, et al. Phase 1 study of high-specific-activity I-131 MIBG for metastatic and/or recurrent pheochromocytoma or paraganglioma. *J Clin Endocrinol Metab* (2018) 103:213–20. doi:10.1210/jc.2017-02030
86. Jimenez C, Pryma DA, Chin BB, Noto RB, Dillon JS, Solnes L, et al. *AZEDRA® (iobenguane I 131) in Patients with Metastatic and/or Recurrent and/or Unresectable Pheochromocytoma or Paraganglioma: Results of a Multicenter, Open-Label, Pivotal Phase 2b Study*. NANETS: Philadelphia, PA (2017).
87. Maurice JB, Troke R, Win Z, Ramachandran R, Al-Nahhas A, Naji M, et al. A comparison of the performance of (6)(8)Ga-DOTATATE PET/CT and (1)(2)(3)I-MIBG SPECT in the diagnosis and follow-up of phaeochromocytoma and paraganglioma. *Eur J Nucl Med Mol Imaging* (2012) 39:1266–70. doi:10.1007/s00259-012-2119-7
88. Tan TH, Hussein Z, Saad FF, Shuaib IL. Diagnostic performance of (68) Ga-DOTATATE PET/CT, (18)F-FDG PET/CT and (131)I-MIBG scintigraphy in mapping metastatic pheochromocytoma and paraganglioma. *Nucl Med Mol Imaging* (2015) 49:143–51. doi:10.1007/s13139-015-0331-7
89. Forrer F, Riedweg I, Maecke HR, Mueller-Brand J. Radiolabeled DOTATOC in patients with advanced paraganglioma and pheochromocytoma. *Q J Nucl Med Mol Imaging* (2008) 52:334–40.
90. van Essen M, Krenning EP, Kooij PP, Bakker WH, Feelders RA, de Herder WW, et al. Effects of therapy with [177Lu-DOTA0, Tyr3]octreotate in patients with paraganglioma, meningioma, small cell lung carcinoma, and melanoma. *J Nucl Med* (2006) 47:1599–606.
91. Mundschenk J, Unger N, Schulz S, Hollt V, Schulz S, Steinke R, et al. Somatostatin receptor subtypes in human pheochromocytoma: subcellular expression pattern and functional relevance for octreotide scintigraphy. *J Clin Endocrinol Metab* (2003) 88:5150–7. doi:10.1210/jc.2003-030262
92. Kolby L, Bernhardt P, Johanson V, Wangberg B, Muth A, Jansson S, et al. Can quantification of VMAT and SSTR expression be helpful for planning radionuclide therapy of malignant pheochromocytomas? *Ann N Y Acad Sci* (2006) 1073:491–7. doi:10.1196/annals.1353.051
93. Reubi JC, Schar JC, Waser B, Wenger S, Heppeler A, Schmitt JS, et al. Affinity profiles for human somatostatin receptor subtypes SST1-SST5 of somatostatin radiotracers selected for scintigraphic and radiotherapeutic use. *Eur J Nucl Med* (2000) 27:273–82. doi:10.1007/s002590050034
94. Nastos K, Cheung VTF, Toumpanakis C, Navalkissoor S, Quigley AM, Caplin M, et al. Peptide receptor radionuclide treatment and (131)I-MIBG in the management of patients with metastatic/progressive phaeochromocytomas and paragangliomas. *J Surg Oncol* (2017) 115:425–34. doi:10.1002/jso.24553
95. Kong G, Grozinsky-Glasberg S, Hofman MS, Callahan J, Meirovitz A, Maimon O, et al. Efficacy of peptide receptor radionuclide therapy for functional metastatic paraganglioma and pheochromocytoma. *J Clin Endocrinol Metab* (2017) 102:3278–87. doi:10.1210/jc.2017-00816
96. Ban HS, Naik R, Kim HM, Kim BK, Lee H, Kim I, et al. Identification of targets of the HIF-1 inhibitor IDF-11774 using alkyne-conjugated photoaffinity probes. *Bioconjug Chem* (2016) 27:1911–20. doi:10.1021/acs.bioconjchem.6b00305
97. Chen W, Hill H, Christie A, Kim MS, Holloman E, Pavia-Jimenez A, et al. Targeting renal cell carcinoma with a HIF-2 antagonist. *Nature* (2016) 539:112–7. doi:10.1038/nature19796
98. Courtney KD, Infante JR, Lam ET, Figlin RA, Rini BI, Brugarolas J, et al. Phase I dose-escalation trial of PT2385, a first-in-class hypoxia-inducible factor-2alpha antagonist in patients with previously treated advanced clear cell renal cell carcinoma. *J Clin Oncol* (2018) 36(9):867–74. doi:10.1200/JCO.2017.74.2627
99. Labiano S, Palazon A, Bolanos E, Azpilikueta A, Sanchez-Paulete AR, Morales-Kastresana A, et al. Hypoxia-induced soluble CD137 in malignant cells blocks CD137L-costimulation as an immune escape mechanism. *Oncoimmunology* (2016) 5:e1062967. doi:10.1080/2162402X.2015.1062967
100. Hatfield SM, Sitkovsky M. A2A adenosine receptor antagonists to weaken the hypoxia-HIF-1alpha driven immunosuppression and improve immunotherapies of cancer. *Curr Opin Pharmacol* (2016) 29:90–6. doi:10.1016/j.coph.2016.06.009
101. Dillman RO. Cancer immunotherapy. *Cancer Biother Radiopharm* (2011) 26:1–64. doi:10.1089/cbr.2010.0902
102. Kaltsas GA, Besser GM, Grossman AB. The diagnosis and medical management of advanced neuroendocrine tumors. *Endocr Rev* (2004) 25:458–511. doi:10.1210/er.2003-0014

103. Hadoux J, Terroir M, Leboulleux S, Deschamps F, Al Ghuzlan A, Hescot S, et al. Interferon-alpha treatment for disease control in metastatic pheochromocytoma/paraganglioma patients. *Horm Cancer* (2017) 8:330–7. doi:10.1007/s12672-017-0303-8
104. Kirkwood JM, Bender C, Agarwala S, Tarhini A, Shipe-Spotloe J, Smelko B, et al. Mechanisms and management of toxicities associated with high-dose interferon alfa-2b therapy. *J Clin Oncol* (2002) 20:3703–18. doi:10.1200/JCO.2002.03.052
105. Robert C, Schachter J, Long GV, Arance A, Grob JJ, Mortier L, et al. Pembrolizumab versus ipilimumab in advanced melanoma. *N Engl J Med* (2015) 372:2521–32. doi:10.1056/NEJMoa1503093
106. Motzer RJ, Escudier B, McDermott DF, George S, Hammers HJ, Srinivas S, et al. Nivolumab versus everolimus in advanced renal-cell carcinoma. *N Engl J Med* (2015) 373:1803–13. doi:10.1056/NEJMoa1510665
107. Reck M, Rodriguez-Abreu D, Robinson AG, Hui R, Csoszi T, Fulop A, et al. Pembrolizumab versus chemotherapy for PD-L1-positive non-small-cell lung cancer. *N Engl J Med* (2016) 375:1823–33. doi:10.1056/NEJMoa1606774
108. Azam F, Mehta S, Harris AL. Mechanisms of resistance to antiangiogenesis therapy. *Eur J Cancer* (2010) 46:1323–32. doi:10.1016/j.ejca.2010.02.020
109. Kleczko EK, Heasley LE. Mechanisms of rapid cancer cell reprogramming initiated by targeted receptor tyrosine kinase inhibitors and inherent therapeutic vulnerabilities. *Mol Cancer* (2018) 17:60. doi:10.1186/s12943-018-0816-y

Conflict of Interest Statement: The author declares that the submitted work was not carried out in the presence of any personal, professional, or financial relationships that could potentially be construed as a conflict of interest.

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REVIEW
published: 26 June 2018
doi: 10.3389/fendo.2018.00324

What Has Bariatric Surgery Taught Us About the Role of the Upper Gastrointestinal Tract in the Regulation of Postprandial Glucose Metabolism?

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The interaction between the upper gastrointestinal tract and the endocrine system is important in the regulation of metabolism and of weight. The gastrointestinal tract has a heterogeneous cellular content and comprises a variety of cells that elaborate paracrine and endocrine mediators that collectively form the entero-endocrine system. The advent of therapy that utilizes these pathways as well as the association of bariatric surgery with diabetes remission has (re-)kindled interest in the role of the gastrointestinal tract in glucose homeostasis. In this review, we will use the changes wrought by bariatric surgery to provide insights into the various gut–pancreas interactions that maintain weight, regulate satiety, and limit glucose excursions after meal ingestion.

Keywords: incretin hormones, bariatric surgery, gastric emptying, gastric accommodation, insulin secretion, insulin action, vagus nerve

OPEN ACCESS

Edited by:
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Reviewed by:
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Specialty section:
This article was submitted to Diabetes, a section of the journal Frontiers in Endocrinology

Received: *19 April 2018*
Accepted: *31 May 2018*
Published: *26 June 2018*

Citation:
Ma J and Vella A (2018) What Has Bariatric Surgery Taught Us About the Role of the Upper Gastrointestinal Tract in the Regulation of Postprandial Glucose Metabolism? Front. Endocrinol. 9:324. doi: 10.3389/fendo.2018.00324

BACKGROUND

In the United States, the prevalence of obesity is rapidly increasing with 65% of adults and 17% of adolescents and children classified as being overweight or obese (1). Obesity is associated with multiple diseases, such as type 2 diabetes, non-alcoholic steatohepatitis, and osteoarthritis, as well as being associated with an increased frequency of the risk factors for cardiovascular disease (2). Approximately 9% of national health-care costs have been attributed to excess weight (3). Because of the evidence that weight reduction ameliorates or corrects the comorbidities of obesity, the US Preventive Services Task Force has recommended that body mass index (BMI) is routinely assessed and weight management recommended for obese patients (4).

Behavioral intervention with lifestyle and dietary modification usually achieves modest weight loss (4). While generally safe, most regain the weight lost within 5 years. Pharmacotherapy for obesity is considered for patients who have failed efforts at lifestyle modification and who have a BMI $\geq$ 30 kg/m^2 or a BMI $\geq$ 27kg/m^2 in the presence of comorbidities such as diabetes (5). However, there have been significant concerns about the long-term safety of such medications and many of the currently available medications have limited efficacy (6).

Bariatric surgery, sometimes referred to as metabolic surgery, is usually considered for patients who have a BMI $\geq$ 40 kg/m^2 or a BMI $\geq$ 35 kg/m^2 associated with comorbidities such as type 2 diabetes (5). Restrictive surgeries such as adjustable gastric banding (AGB) and sleeve

gastrectomy (SG) limit the capacitance of the stomach. Roux-en-Y gastric bypass (RYGB) is the most commonly performed bypass procedure and produces gastric restriction together with selective malabsorption. RYGB involves creation of a gastric pouch by separating the stomach across the fundus. Drainage of this 10–30 ml pouch is achieved by a gastrojejunostomy. The distal end of the jejunum is anastomosed ~150 cm below the gastrojejunostomy effectively bypassing the distal stomach, duodenum, and proximal jejunum. Duodenal switch (DS) is a variation of biliopancreatic diversion and involves a SG with division of the duodenum below the pylorus. The distal ileum is anastomosed to the short stump of the duodenum producing a ~100 cm channel for nutrient absorption. The other end of the duodenum is closed and the remaining small bowel connected onto the enteral limb 75–100 cm from the ileocecal valve (2).

Observational and prospective studies have suggested that bariatric surgery is the most effective intervention for weight loss producing an average weight loss of 30–35% that is maintained in ~60% of patients at 5 years (7). This has led to a dramatic increase in the number of procedures performed annually from 13,365 in 1998 (8) to 216,000 in 2016 according to the data released by American Society for Metabolic and Bariatric Surgery (9). In a meta-analysis of 136 studies of bariatric surgery, which included a total of 22,094 patients, Buchwald et al. reported that within studies examining type 2 diabetes after bariatric surgery, 1,417 of 1,846 (76%) patients experienced complete resolution. When categorized by operative procedure, there were clear differences in efficacy. Diabetes resolved in 98.9% of patients undergoing biliopancreatic diversion or DS. In contrast, the rate was 83.7% for RYGB and 47.9% for AGB (10). A retrospective review of 257 patients who underwent the long-limb modification of RYGB (400–500 cm Roux limb length) at our institution reported resolution of type 2 diabetes in 94% of patients (11). Recent prospective, randomized controlled trials have, however, reported lower remission rates for diabetes with RYGB, although it remains superior to medical therapy (12–14). Setting aside the superiority of one procedure over the other in terms of inducing diabetes remission [which is likely related to residual β-cell function at the time of the procedure (15, 16) as well as the magnitude of weight loss (17)], obvious differences between procedures can be used to explore the role of the gastrointestinal tract in metabolism. RYGB is sometimes complicated by the occurrence of hyperinsulinemic hypoglycemia (18). Its incidence is uncertain although it has been suggested that excessive glucagon-like peptide-1 (GLP-1) secretion after RYGB (19) may be the cause of this phenomenon, but this is unlikely (20). The condition has been the subject of an extensive review recently (21) (**Figure 1**).

The anatomic differences among bariatric procedures result in differences in enteroendocrine secretion (**Table 1**): postprandial GLP-1 concentrations are lower after SG compared to RYGB in the comparative studies undertaken in humans (22–26). On the other hand, a liquid meal, especially after gastric restriction, may

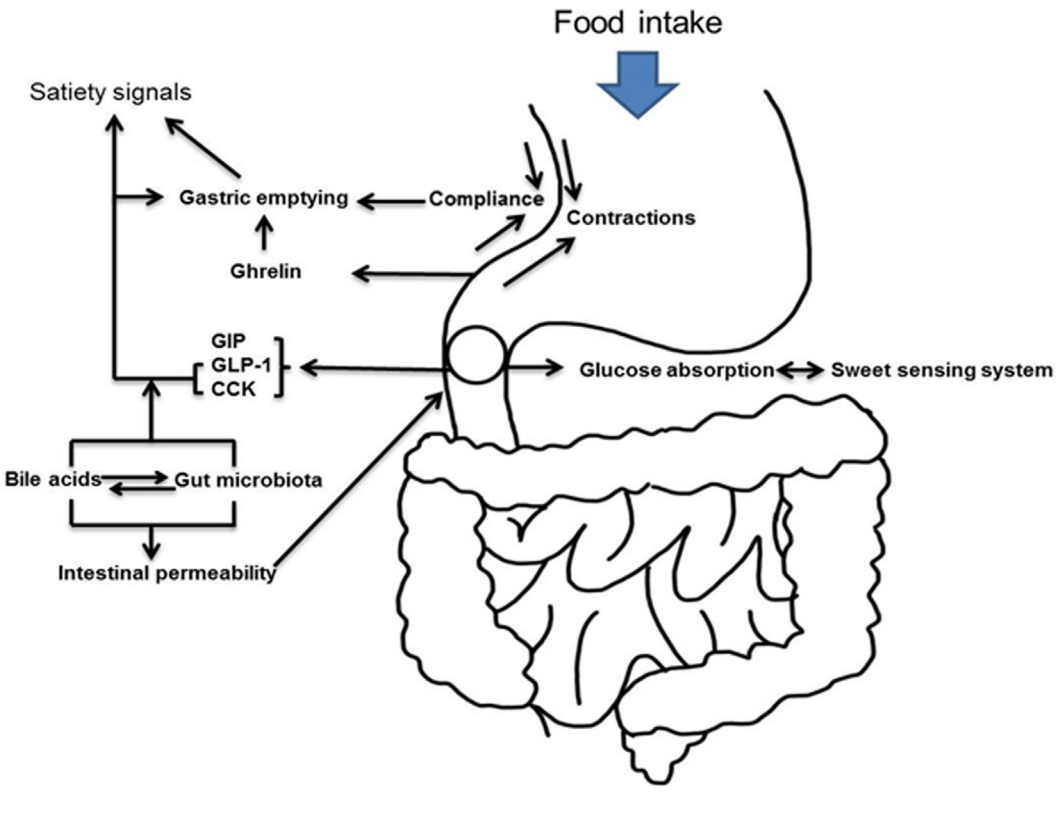

FIGURE 1 | Schematic representation of upper gastrointestinal tract and hyperglycemia.

TABLE 1 | Efficacy of different bariatric surgeries.

Mechanism	SG	RYGB	AGB	DS
Weight loss	↓	↓	↓	↓
Amelioration of diabetes	71.6%	83.7%	47.9%	98.9%
Adverse effects	Band slippage, stoma obstruction, intractable postoperative vomiting	Dumping syndrome, dyspepsia, abdominal pain	Band erosion, leakage from the balloon	Gastrointestinal leaks and constipation
Plasma ghrelin	↓	↓	↓	↓
Plasma GLP-1	↑	↑	↑	↑
Plasma GIP	↑	↑	↔	N/A
Plasma CCK	↑	↑	↔	↑

Increase, ↑; decrease, ↓; no change, ↔; NA, no available evidence; SG, sleeve gastrectomy; RYGB, Roux-en-Y gastric bypass; AGB, adjustable gastric banding; DS, duodenal switch; GLP-1, glucagon-like peptide-1; GIP, gastric inhibitory polypeptide; CCK, cholecystokinin.

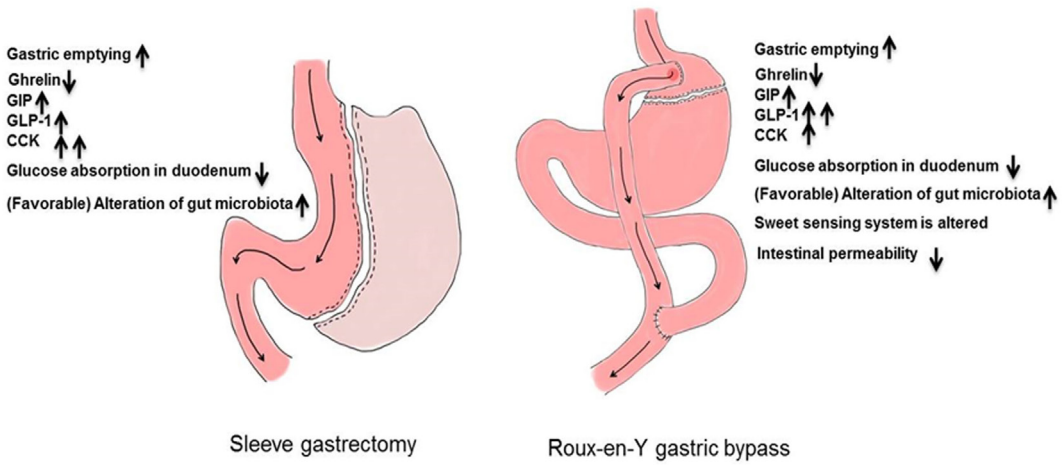

FIGURE 2 | Upper intestinal adaptions after bariatric surgery.

not recreate conditions present after a solid meal (27). Indeed, liquid emptying especially after restrictive gastric surgery is dependent on fasting gastric volume (28, 29). SG removes the capacitance function of the stomach and decreases ghrelin concentrations to a greater extent than does RYGB (24, 25, 30). This is more apparent when acyl-ghrelin is measured (22, 31, 32). Whether these differences can explain metabolic outcomes will be explored in detail below (**Figure 2**).

CALORIC RESTRICTION—CHANGES IN GASTRIC VOLUME, COMPLIANCE, AND ACCOMMODATION

Restrictive procedures reduce gastric volume—indeed, the postoperative period of any form of bariatric surgery is characterized by a significant degree of caloric restriction (33). Fasting blood glucose and insulin resistance improves within 6 days of gastric bypass and occurs before any weight loss (34). This had been observed previously with very low-calorie diets outside of bariatric surgery (35), suggesting that caloric restriction at least partially, explains the acute improvement in glucose control after bariatric surgery (14, 36, 37). Six weeks of caloric restriction (700–900 kcal/day) decreases fasting and postprandial glycemia by lowering fasting endogenous glucose production and improving β-cell function (38). Jackness et al. showed that very low caloric restriction (500 kcal/day) produced a similar improvement in β-cell function as those who underwent post-RYGB (39). Indeed, caloric restriction after RYGB outweighs the effect of GLP-1 on glucose metabolism [as studied by the use of a competitive antagonist of GLP-1 at its receptor (40)]. Of course outside of the mechanical restrictions induced by bariatric surgery, there are difficulties with long-term compliance with regimens of caloric restriction. However, in an open-label, cluster-randomized trial 24% patients achieved weight losses of 15 kg or more in 12 months, and 86% experienced diabetes remission (41).

Multiple factors influence satiation including stomach capacitance and emptying. The stomach increases in volume in anticipation of food ingestion (42). In the normal stomach, this volume expansion is not associated with an increase in gastric wall stiffness (or increased intraluminal pressure) suggesting a change in the mechanical properties of the stomach wall (increased compliance) to accommodate the capacitive function of the stomach (43). This function is primarily fulfilled by the proximal stomach, which serves as a food reservoir, while the distal stomach triturates food to a size that can pass the pylorus. The physical nature, particle size, fat, and caloric content of food

alter emptying rate (27). Although nutrient and non-caloric liquids empty rapidly, solids are initially retained in the stomach while antral contractions propel particles toward the closed pylorus. Food particles are emptied once they are ~2 mm in diameter (27). Restrictive procedures eliminate the function of the proximal stomach displacing food to the distal stomach and accelerating emptying. It is uncertain if restriction of accommodation alone alters gastric emptying (44). In SG, a functioning pylorus is retained and gastric volume is usually larger than the pouch created after RYGB. Post-RYGB it has been assumed that there is little neuromuscular control on gastric emptying since the pylorus is bypassed [although this may not be correct (45)]. Surgical vagotomy (which occurs in RYGB when the gastric pouch is created) alters gastric accommodation but may not change emptying (46) and typically does not have durable effects on weight (47).

Gastric emptying plays an important role in determining the magnitude of change in glucose concentrations after nutrient ingestion (48). Indeed, variation in the rate of gastric emptying alters peak insulin response after 75 g oral glucose, in both healthy subjects and patients with type 2 diabetes (49, 50). Fasting gastric volume affects the rate of emptying of a liquid challenge (51). Interventions that delay gastric emptying have the potential to regulate glycemia in patients with diabetes. Accelerating nutrient flow to the small intestine with erythromycin increases the postprandial glycemic response (52), whereas slowing gastric emptying with Xenin-25, a 25-amino acid neurotensin-related peptide, reduces postprandial blood glucose (53). Although it is important to match the rate of gastric emptying and the onset and offset of insulin action, significantly lower insulin requirements are observed in patients with type 1 diabetes with gastroparesis than those without, during the first hour of the postprandial period (54). Delaying gastric emptying is also a mechanism of action of some antidiabetic medications, such as GLP-1 analogs and pramlintide (55, 56).

Gut hormones can modulate food intake over and above that caused by mechanical restriction after bariatric surgery (22). Ghrelin increases food intake after esophagectomy or gastrectomy (57, 58). Neuronal GLP-1R mediates the anorectic effects of GLP-1 (59). Inhibition of GLP-1 action with Exendin-9,39 after RYGB accelerates gastric emptying (45). Taken together, these observations suggest that factors other than anatomy contribute to the upper gastrointestinal response to food ingestion. The attraction of certain foods decreases after RYGB (60) and appetite may be altered by enteroendocrine secretion (61, 62). A potential mechanism is *via* GLP-1, which alters gastrointestinal transit, gastric accommodation (45, 46, 63), and has direct effects on hypothalamic nuclei outside of the blood–brain barrier (64). GLP-1 and GLP-1 receptor agonists decrease food intake and cause weight loss (65, 66). GLP-1 also modulates taste sensitivity in rodents (67–70). The peripheral concentrations of GLP-1 observed in the early postprandial period in subjects post-RYGB, exceed concentrations observed after infusion at 0.75 pmol/kg/min, and are similar to those observed after infusion at 1.5 pmol/kg/min—both infusion rates that alter gastrointestinal function (71). It is, therefore, reasonable to consider that the postprandial rise in GLP-1 might affect feeding behavior after RYGB, and to a lesser extent SG, where the increase in GLP-1 is less marked (22–26). The elevated postprandial concentrations of GLP-1 observed after RYGB are unlikely to be the cause of diabetes remission after bariatric surgery. We (45) and others (72) have shown that inhibition of GLP-1 actions in the postprandial period has limited effects on glucose concentrations in people after RYGB. This is in agreement with data from mice deficient in the GLP-1 receptor that lost the same amount of weight as wild-type mice (73). This is also the case after SG in humans (74) and in mice deficient in the GLP-1 receptor (75). On the other hand, SG decreases acyl-ghrelin concentrations, presumably due to excision of a large part of the ghrelin-secreting stomach, which should decrease appetite (22, 31, 32). Fasting after SG is not associated with a rise in (low) ghrelin concentrations, in contrast to RYGB (22, 24, 25, 30–32).

In an effort to circumvent the costs and complications associated with bariatric surgery, various attempts have been made to develop endoscopically placed devices that might cause weight loss. One such device is a synthetic sleeve placed post-pylorus under endoscopic control. The rationale underlying such a device is to ensure that nutrients are prevented from coming in contact with the absorptive surfaces of the proximal small bowel (76). Unfortunately, such a device is prone to migration, bleeding, and bolus obstruction. A placebo-controlled study utilizing the device as treatment for type 2 diabetes was terminated prematurely because of a ~3% incidence of hepatic abscess in subjects using the device (77). Other devices such as intra-gastric balloons to induce early satiety are under study.

GHRELIN

Ghrelin is a 28-amino acid peptide and is the only orexigenic hormone recognized in humans. It is secreted from the gastric mucosa and hypothalamus in both rodents and humans. There are two forms of circulating ghrelin, unacylated and acylated ghrelin (AG) (78). In the fasting state, AG is elevated (~110 pM) and decreases (~70 pM) in response to food ingestion. Patients with Prader–Willi syndrome—a syndrome characterized by excessive feeding behavior—have high concentrations of circulating ghrelin (79). Fasting and postprandial acyl-ghrelin levels are decreased following SG, compared to Roux-en-Y gastric bypass (RYGB), which may play a role in weight loss (26). SG involves removal of the gastric fundus—the primary source of ghrelin synthesis and secretion. Exogenous ghrelin administration increases energy intake in both rodents (80) and humans (81). Infusion of ghrelin in patients after esophagectomy (58) or gastrectomy (57) increases caloric intake and appetite. Although the contribution of ghrelin to normal physiology is unclear, it has been demonstrated that ghrelin can directly inhibit insulin secretion (82). Pharmacologic concentrations of ghrelin or ghrelin receptor agonists accelerate gastric emptying, suppress insulin secretion, and increase glucagon secretion (83). In a randomized controlled phase Ib clinical trial, ghrelin accelerated gastric emptying and improved gastrointestinal symptoms in patients with type 2 diabetes (84). Ghrelin receptor agonists are being developed as potential therapies for gastroparesis (85).

INCRETIN HORMONES

The incretin effect is a phenomenon first observed several decades ago when intravenous glucose produced lower insulin concentrations, despite higher glucose concentrations than observed after ingestion of an equivalent amount of glucose (86). This observation has subsequently been confirmed with isoglycemic infusion studies (87). The subsequent discovery of glucagon-like immunoreactivity in the gut led to the realization that proglucagon is synthesized in enteroendocrine cells intercalated between enterocytes and distributed throughout the intestine. GLP-1 and gastric inhibitory polypeptide (GIP) are two incretin hormones, which stimulate postprandial insulin secretion (88). GLP-1 is released from L-cells, most densely located in the distal small intestine and colon, although they are also located more proximally in the duodenum and jejunum (89). There is some evidence in rodent models of paracrine GLP-1 secretion within pancreatic islets (90). GIP is secreted from K cells (which reside mainly in the duodenum and upper jejunum) in response to nutrient ingestion. The early secretion of GLP-1 might involve an indirect neural or hormonal mechanism (91). The later secretion of GLP-1 is dependent on direct contact of nutrients in the small intestine with L-cells (92). Targeted delivery of lauric acid in enteric-coated pellets to the ileum and colon can stimulate substantial endogenous GLP-1 release and attenuate postprandial glycemia (93). To stimulate its receptor, GLP-1 requires the presence of 2 N-terminal amino acids, which are cleaved by the enzyme, dipeptidyl peptidase-4 (DPP-4), rendering the truncated form (GLP-1-9,36) inactive. Because of the widespread distribution of DPP-4, the active form of GLP-1 has a short half-life in the circulation (94). GLP-1 receptor agonists that are not substrates of DPP-4 and DPP-4 inhibitors are approved for the treatment of type 2 diabetes. They lower fasting and postprandial glucose concentrations (66). In addition to stimulating insulin secretion, pharmacologic concentrations of GLP-1 (and GLP-1 receptor agonists) inhibit gastric emptying, and suppress glucagon secretion. Moreover, GLP-1 and GLP-1 receptor agonists increase satiety, leading to a reduction in weight (95).

Although GIP secretion is preserved, the insulinotropic effect of GIP is diminished in type 2 diabetes. Unlike GLP-1, GIP stimulates glucagon secretion during hypoglycemia (96, 97) and has no effect on gastric emptying. Circulating concentrations of GIP are related to BMI (98), which suggests a role of GIP in energy metabolism. In mice, high GIP concentrations promote obesity and insulin resistance (99). However, recent study shows that there is a synergistic effect of GIP and GLP-1 co-agonists in weight lowering (100) and glycemic improvement in patients with type 2 diabetes than mono-agonist (101). Addition of a dual GIP/GLP-1 receptor agonist (NNC0090-2746) to metformin improved glycemic control with accompanying reductions in body weight and circulating cholesterol (102). The molecular mechanism underneath the metabolic improvements is not known. The effects of GIP on glucose metabolism are an area of ongoing investigation, which will hopefully be accelerated by the development of a specific GIP receptor antagonist (103).

CHOLECYSTOKININ (CCK)

Cholecystokinin is secreted from the I-cells by exposure to nutrients in the duodenum and upper jejunum. Fat is a strong stimulus for CCK secretion, followed by protein, whereas carbohydrate is a weaker stimulus of CCK secretion. CCK concentrations increase from fivefold to tenfold after ingestion of a mixed meal and inhibit gastric emptying through activation of CCK-1 receptors (104). Physiological concentrations of CCK delay entry of glucose into the duodenum, reducing postprandial glucose excursions (105). In rats, CCK decreased hepatic glucose production to maintain glucose homeostasis by inhibiting CCK-A receptors and triggering a gut–brain–liver neuronal axis (106). In humans, CCK dose-dependently presents early satiety and reduces the energy intake at a buffet style meal, which was attenuated by the CCK-1 antagonist, loxiglumide (107). However, the long-term effects of CCK administration in humans and its role in obesity therapy are not clear.

ROLE OF THE VAGUS—VAGAL BLOCKADE/VAGOTOMY

The gastrointestinal tract is innervated by the parasympathetic and sympathetic divisions of the autonomic nervous system. The parasympathetic innervation originates from the dorsal motor nucleus of the vagus (DMV) in the medulla (108), while the sympathetic supply derives from the prevertebral ganglia (109). Gastric motility is partially controlled by the vagus nerve, a mixed motor, and sensory nerve. The sensory axons of the vagus receive afferent inputs from gastrointestinal receptors and then project to the nucleus of the solitary tract (110). Nucleus of the solitary tract (NTS) neurons activate vagal motor neurons in the nucleus ambiguus and the dorsomedial nucleus to regulate the smooth muscle contractions in the stomach and duodenum, with these neural loops being known as vagovagal reflexes (111). Bilateral truncal vagotomy (112), aiming for treating of peptic ulcer surgery, and electrical vagal blockade (113) results in delayed gastric emptying, and weight loss—at least in the short term. The gastric vagal branches are often damaged during bariatric surgery (114). It remains controversial whether vagal innervation of the portal hepatis contributes to the beneficial effects of RYGB on food intake, energy expenditure, and body weight (115). Electrical vagal blockade does not seem to have significant effects on glucose metabolism (116).

Obese subjects exhibit decreased heart rate variability likely due to an imbalance of sympathetic and parasympathetic activity (117). Overactivity of the sympathetic nervous system is more significant in obese subjects with type 2 diabetes than in those subjects without diabetes (118). Weight reduction following RYGB and AGB in severely obese patients is associated with an increase in heart rate variability (119). The underlying mechanism(s) remain unknown but the improvement in autonomic function does not appear to be related to improved insulin action (120). It has been posited that these changes in autonomic function could arise from crosstalk between the gastrointestinal tract and the central nervous system (121) generated by a neuro-inflammatory reflex (122) arising from the gut microflora.

THE TASTE SIGNALING SYSTEM

It is increasingly recognized that bariatric surgery may alter food preference and taste, in particular, the perception of sweet taste. This likely contributes to the reduction in energy intake after surgery (123). Both SG and RYGB result in a reduction of the frequency of food craving and the hedonic component of taste perception (124). Subjects experience a decreased desire to consume sweet and fatty flavors after RYGB (60) and SG (125).

The sweet taste signaling system includes heterodimeric G protein-coupled receptors, composed of the taste receptors (TRs), T1R2 + T1R3 heterodimers, which are activated by the binding of sweet compounds such as monosaccharides and disaccharides (126). These receptors are G-protein coupled (gustducin), and activation increases phospholipase C-β_2 activity, which ultimately results in the release of Ca^{2+} from intracellular stores and the opening of a transient receptor potential ion channel TRPM5. The resulting membrane depolarization activates gustatory afferents (127). Sweet TRs are found in the tongue, gastrointestinal tract, pancreas, adipose tissue, brain, and bone (128). Expression of T1R2 + T1R3 also occurs in the entero-endocrine L cells (129), suggesting that the sweet sensing system in the gut is involved in incretin secretion. T1R3 knockout mice exhibit impaired GLP-1 secretion and glucose intolerance (130). Intragastric infusion of nutrients with lactisole, a T1R2/T1R3 blocker, attenuates GLP-1 and peptide YY secretion in humans (131, 132). The expression of sweet taste receptors and downstream molecule transcripts are disordered in models of type 2 diabetes (133). T1R2 expression is reciprocally regulated by luminal glucose in health, but not in patients with type 2 diabetes; during acute hyperglycemia, T1R2 transcript levels decrease in response to duodenal glucose infusion in healthy subjects, but increase in subjects with type 2 diabetes (134).

In addition to changes in oral taste sensitivity, the expression of T1R2 and T1R3 is decreased in the small intestine of rats after bariatric surgery; this occurs in parallel with elevation of GLP-1 (135). Functional magnetic resonance imaging or positron emission tomography demonstrates a decrease in neural activity in the brain reward areas in response to high-calorie foods (136).

PERMEABILITY AND GLUCOSE TRANSPORT

The proximal small intestine initiates carbohydrate absorption after digestion. Glucose absorption is mediated by the sodium glucose co-transporter-1 (SGLT1) across the apical cell membrane and partially by the glucose transporter 2 (GLUT-2) at high glucose concentrations (137). The small intestine has a maximal capacity of glucose absorption of about 0.5 g/min (or 2 kcal/min) per 30 cm (138). The absorptive rate depends on the exposure rate of glucose, region, and length of the small intestine, and the expression of glucose transporters (139). The inhibition of motility and blood flow in the small intestine also attenuates glucose absorption (140). Plasma concentration of 3-O-methyl-glucose, a glucose analog that is not metabolized, is normally used to measure the absorption rate of glucose. Physiologically, enhanced glucose absorption in the proximal gut would increase blood glucose concentrations; acute hyperglycemia itself appears to enhance glucose absorption (141). Rodent models of diabetes exhibit small intestinal hyperplasia and increased absorption of glucose from intestinal mucosa (142). It is unclear to what extent inhibition of SGLT-1 can alter glucose absorption in a way that is relevant to postprandial glycemic control in diabetes.

Active glucose transport and intestinal permeability are increased in obesity and diabetes. For a given caloric intake, this could alter the nutrient load entering the portal circulation (143–146). Changes in intestinal thickness and transcription of SGLT-1 and GLUT-2 occur after RYGB (147, 148). Foregut exclusion decreases glucose absorption in rodents (149). However, it is currently not known, and if so, the extent to which RYGB and SG alter the rate of active intestinal glucose absorption or the rate of passive intestinal permeability.

Intestinal integrity provides a physical barrier to luminal bacteria, toxins, and antigens from the external environment. In health, it allows the passage of water and nutrients. Increased paracellular permeability, following disruption of the intestinal tight junctions enables bacteria to leak out of the intestinal lumen into the blood stream (150). Factors that influence permeability include the gut microbiome and fatty acids (whether ingested directly or as products of bacterial fermentation) (151). Bile acids could alter gut permeability through the G-protein-coupled bile acid receptor (TGR5), a cell surface receptor, which occurs at a high level expression in the human placenta and spleen and is also found in multiple tissues such as the lung, liver, adipocytes, and the gastrointestinal tract (152). A systematic review of 14 studies suggests that that fasting and postprandial lipopolysaccharide (LPS) are increased in patients with diabetes (153). LPS is the core component of the outer membrane of Gram-negative bacteria. Metabolic endotoxemia is defined by a twofold to threefold increase in plasma LPS concentration (154). Rosiglitazone is the most effective in the lowering the LPS in patients with type 2 diabetes, but the extent to which this contributes to the glucose-lowering effects of this compound are unknown (155).

BILE ACID METABOLISM

Bile acids are synthesized in hepatocytes *via* cytochrome P450-mediated oxidation of cholesterol and then secreted into the intestinal lumen through the biliary system. 95% of intestinal bile acids are reabsorbed in the distal gut and transported back to the liver by the enterohepatic circulation (156, 157). CCK induces production of bile, contraction of the gall bladder, and relaxation of the sphincter of Oddi, to deliver bile into the duodenum (158). Bile acids promote digestion and absorption of lipids in the gastrointestinal tract as well as participate in the regulation of glucose and energy homeostasis (159), acting through two specific receptors, the farnesoid X receptor (FXR) and TGR5.

FXR is expressed in the liver and the intestine in humans and is a member of the nuclear receptor super-family. It can be activated by both primary and secondary conjugated bile acids (160, 161). Similar to other nuclear receptors, FXR translocates to the cell nucleus and subsequently induces expression of

the small heterodimer partner (SHP). SHP is involved in bile acids synthesis by downregulating the gene transcription of cholesterol 7 alpha-hydroxylase (CYP7A1), a rate-limiting enzyme in bile acid synthesis. The activation of TGR5 triggers the production of intracellular cAMP and secondary active the mitogen activated protein kinase signaling pathway to perform different functions in various organs. For instance, TGR5 is expressed in rodent and human pancreatic islets and regulates insulin secretion (162). TGR5 in enteroendocrine L cells stimulates secretion of GLP-1 (163). In addition, TGR5 may regulate energy homeostasis through activating deiodinases to convert the prohormone thyroxine (T4) into the active hormone triiodothyronine (T3) (164, 165).

Circulating bile acids' concentrations after meal ingestion are decreased in obese subjects compared to lean controls (166). This difference is no longer significant after bariatric surgery (167, 168). The effects of SG on body weight and glucose tolerance are attenuated in the absence of FXR (169) and in TGR5 knock-out mice (170). In a diet-induced obesity mouse model, diversion of bile flow to the ileum produces similar metabolic benefits to RYGB (171), while the ability of RYGB to decrease body weight and improve glucose tolerance is substantially reduced in the absence of FXR. Bile acids may stimulate insulin secretion *via* activation of FXR and inhibition of ATP-dependent K + channels (172). It has been suggested that the changes in bile acid composition and concentrations induced by bariatric surgery can contribute to metabolic changes *via* FXR and TGR5-signaling pathways.

However, in humans, the contribution of bile acid changes to metabolic improvements after bariatric procedures is less clear. One study reported that total plasma bile acid concentrations increased twofold after RYGB but decreased after AGB, despite similar weight loss (173). Longitudinal study suggests that there are two phasic increases in plasma bile acid concentrations in a cohort of RYGB patients at 1 month and up to 24 months after surgery (168). This time course differs from the time course of metabolic resolution suggesting that they are unrelated phenomena.

THE GUT MICROBIOME

The human gut microbiome consists of 10–100 trillion of microorganisms, primarily bacteria, in the digestive tract (174). The composition of the gut microbiome influences digestion, absorption, inflammation, and intestinal motility. Over the past decade, several studies have demonstrated that gut microbial populations are closely associated with metabolic disorders such as dyslipidemia, obesity, and diabetes (175). The gut microbiome is established early in life (176). Exposure to antibiotics alters the normal distribution of intestinal flora and is associated with changes in metabolism in some (177) but not all studies (178). Diet and lifestyle and geography are the primary influencers of the distribution of intestinal flora (179).

In humans, gut microbiota produce glycoside hydrolases and polysaccharide lyases, which facilitate digestion of sucrose, lactose, and starch (180). Undigested polysaccharides are subject to fermentation by intestinal bacterial leading to the production of short-chain fatty acids, which can provide 5–10% energy consumption (181). Gut microbiota is also involved in signaling of FXR and TGR5 by modifying the bile acid pool (182). In fact, bile acids interact with gut microbiota by direct effects on the mucosal defense, membrane integrity, oxidative and pH stress to increase the growth of bile acid-metabolizing bacteria (183).

D-lactate acidosis is a rare complication of jejuno-ileal bypass surgery or patients with short bowel syndrome (SBS) (184). D-lactate production is mainly dependent on the colonic microbiome (184). Notably, in patients with SBS or after jejuno-ileal bypass surgery, delivery of an increased amount of undigested carbohydrates to the colon can result in excess D-lactate accumulation (185, 186). *Bacteroides thetaiotaomicron* abundance is decreased in obese subjects compared to lean individuals (187). Patients with type 2 diabetes may exhibit decreased abundance of butyrate-producing bacteria and an increase in various opportunistic pathogens (188). Dietary fiber intake in patients with type 2 diabetes increases acetate and butyrate-producing bacteria improves glycemic control (189). Use of metformin is accompanied by increased abundance of *Escherichia* and a decrease of *Intestinibacter* (190). Impaired glucose tolerance is reversed after the transfer of metformin-altered microbiota to germ-free mice (191). Acarbose alters bile acid metabolism through changes in gut microbial populations (192). This results in great interest in microbiota alteration on improvement of metabolic parameters. Recently, transplantation of fecal microbiota or "bacteriotherapy" seems a promising therapeutic method for metabolic syndrome (193).

Individuals with obesity exhibit markedly decreased abundance of *B. thetaiotaomicron*. However, the abundance of this microbe increased after SG despite similar metabolic outcomes suggesting that this is incidental to the improvements in glucose metabolism after bariatric surgery (187). Randomized trials are warranted in the future to further assess the gut mechanism after bariatric surgeries in humans.

CONCLUSION

The upper gastrointestinal tract plays a primary role in the regulation of glucose excursions in response to meal ingestion by determining the rate of gastric emptying and indirectly by regulating appetite and satiation, barrier integrity, and nutrient absorption. Bariatric surgery has helped improve our knowledge of the mechanisms underlying gut–pancreas interactions and may enable development of effective dietary or pharmacological strategies in the management of diabetes.

AUTHOR CONTRIBUTIONS

JM wrote the paper with input from AV regarding content and layout. AV edited the draft for clarity and content.

ACKNOWLEDGMENTS

AV's research is supported by the Mayo Clinic General Clinical Research Center (UL1 TR000135) and by the National Institutes of Health (DK78646, DK116231).

REFERENCES

1. Ogden CL, Carroll MD, Curtin LR, McDowell MA, Tabak CJ, Flegal KM. Prevalence of overweight and obesity in the United States, 1999–2004. *JAMA* (2006) 295(13):1549–55. doi:10.1001/jama.295.13.1549
2. Kushner RF. Obesity management. *Gastroenterol Clin North Am* (2007) 36(1):191–210, viii. doi:10.1016/j.gtc.2007.01.004
3. Elmer PJ, Brown JB, Nichols GA, Oster G. Effects of weight gain on medical care costs. *Int J Obes Relat Metab Disord* (2004) 28(11):1365–73. doi:10.1038/sj.ijo.0802774
4. U.S. Preventive Services Task Force. Screening for obesity in adults: recommendations and rationale. *Ann Intern Med* (2003) 139(11):930–2. doi:10.7326/0003-4819-139-11-200312020-00012
5. National Institutes of Health. Clinical guidelines on the identification, evaluation, and treatment of overweight and obesity in adults – the evidence report. *Obes Res* (1998) 6(Suppl 2):51S–209S.
6. Bachman KH. Obesity, weight management, and health care costs: a primer. *Dis Manag* (2007) 10(3):129–37. doi:10.1089/dis.2007.103643
7. Maggard MA, Shugarman LR, Suttorp M, Maglione M, Sugerman HJ, Livingston EH, et al. Meta-analysis: surgical treatment of obesity. *Ann Intern Med* (2005) 142(7):547–59. doi:10.7326/0003-4819-142-7-200504050-00013
8. Santry HP, Gillen DL, Lauderdale DS. Trends in bariatric surgical procedures. *JAMA* (2005) 294(15):1909–17. doi:10.1001/jama.294.15.1909
9. English WJ, DeMaria EJ, Brethauer SA, Mattar SG, Rosenthal RJ, Morton JM. American Society for Metabolic and Bariatric Surgery estimation of metabolic and bariatric procedures performed in the United States in 2016. *Surg Obes Relat Dis* (2018) 14(3):259–63. doi:10.1016/j.soard.2017.12.013
10. Buchwald H, Avidor Y, Braunwald E, Jensen MD, Pories W, Fahrbach K, et al. Bariatric surgery: a systematic review and meta-analysis. *JAMA* (2004) 292(14):1724–37. doi:10.1001/jama.292.14.1724
11. Nelson WK, Fatima J, Houghton SG, Thompson GB, Kendrick ML, Mai JL, et al. The malabsorptive very, very long limb Roux-en-Y gastric bypass for super obesity: results in 257 patients. *Surgery* (2006) 140(4):517–22, discussion 22–3. doi:10.1016/j.surg.2006.06.020
12. Mingrone G, Panunzi S, De Gaetano A, Guidone C, Iaconelli A, Leccesi L, et al. Bariatric surgery versus conventional medical therapy for type 2 diabetes. *N Engl J Med* (2012) 366(17):1577–85. doi:10.1056/NEJMoa1200111
13. Schauer PR, Kashyap SR, Wolski K, Brethauer SA, Kirwan JP, Pothier CE, et al. Bariatric surgery versus intensive medical therapy in obese patients with diabetes. *N Engl J Med* (2012) 366(17):1567–76. doi:10.1056/NEJMoa1200225
14. Ikramuddin S, Korner J, Lee WJ, Connett JE, Inabnet WB, Billington CJ, et al. Roux-en-Y gastric bypass vs intensive medical management for the control of type 2 diabetes, hypertension, and hyperlipidemia: the Diabetes Surgery Study randomized clinical trial. *JAMA* (2013) 309(21):2240–9. doi:10.1001/jama.2013.5835
15. Still CD, Wood GC, Benotti P, Petrick AT, Gabrielsen J, Strodel WE, et al. Preoperative prediction of type 2 diabetes remission after Roux-en-Y gastric bypass surgery: a retrospective cohort study. *Lancet Diabetes Endocrinol* (2014) 2(1):38–45. doi:10.1016/S2213-8587(13)70070-6
16. Nguyen KT, Billington CJ, Vella A, Wang Q, Ahmed L, Bantle JP, et al. Preserved insulin secretory capacity and weight loss are the predominant predictors of glycemic control in patients with type 2 diabetes randomized to Roux-en-Y gastric bypass. *Diabetes* (2015) 64(9):3104–10. doi:10.2337/db14-1870
17. Schauer PR, Bhatt DL, Kirwan JP, Wolski K, Brethauer SA, Navaneethan SD, et al. Bariatric surgery versus intensive medical therapy for diabetes – 3-year outcomes. *N Engl J Med* (2014) 370(21):2002–13. doi:10.1056/NEJMoa1401329
18. Service GJ, Thompson GB, Service FJ, Andrews JC, Collazo-Clavell ML, Lloyd RV. Hyperinsulinemic hypoglycemia with nesidioblastosis after gastric-bypass surgery. *N Engl J Med* (2005) 353(3):249–54. doi:10.1056/NEJMoa043690
19. Goldfine AB, Mun EC, Devine E, Bernier R, Baz-Hecht M, Jones DB, et al. Patients with neuroglycopenia after gastric bypass surgery have exaggerated incretin and insulin secretory responses to a mixed meal. *J Clin Endocrinol Metab* (2007) 92(12):4678–85. doi:10.1210/jc.2007-0918
20. Vella A, Service FJ. Incretin hypersecretion in post-gastric bypass hypoglycemia – primary problem or red herring? *J Clin Endocrinol Metab* (2007) 92(12):4563–5. doi:10.1210/jc.2007-2260
21. Salehi M, Vella A, McLaughlin T, Patti ME. Hypoglycemia after gastric bypass surgery: current concepts and controversies. *J Clin Endocrinol Metab* (2018). doi:10.1210/jc.2018-00528
22. Yousseif A, Emmanuel J, Karra E, Millet Q, Elkalaawy M, Jenkinson AD, et al. Differential effects of laparoscopic sleeve gastrectomy and laparoscopic gastric bypass on appetite, circulating acyl-ghrelin, peptide YY3-36 and active GLP-1 levels in non-diabetic humans. *Obes Surg* (2014) 24(2):241–52. doi:10.1007/s11695-013-1066-0
23. Kashyap SR, Bhatt DL, Wolski K, Watanabe RM, Abdul-Ghani M, Abood B, et al. Metabolic effects of bariatric surgery in patients with moderate obesity and type 2 diabetes: analysis of a randomized control trial comparing surgery with intensive medical treatment. *Diabetes Care* (2013) 36(8):2175–82. doi:10.2337/dc12-1596
24. Peterli R, Steinert RE, Woelnerhanssen B, Peters T, Christoffel-Courtin C, Gass M, et al. Metabolic and hormonal changes after laparoscopic Roux-en-Y gastric bypass and sleeve gastrectomy: a randomized, prospective trial. *Obes Surg* (2012) 22(5):740–8. doi:10.1007/s11695-012-0622-3
25. Peterli R, Wolnerhanssen B, Peters T, Devaux N, Kern B, Christoffel-Courtin C, et al. Improvement in glucose metabolism after bariatric surgery: comparison of laparoscopic Roux-en-Y gastric bypass and laparoscopic sleeve gastrectomy: a prospective randomized trial. *Ann Surg* (2009) 250(2):234–41. doi:10.1097/SLA.0b013e3181ae32e3
26. Lee WJ, Chong K, Ser KH, Lee YC, Chen SC, Chen JC, et al. Gastric bypass vs sleeve gastrectomy for type 2 diabetes mellitus: a randomized controlled trial. *Arch Surg* (2011) 146(2):143–8. doi:10.1001/archsurg.2010.326
27. Camilleri M. Clinical practice. Diabetic gastroparesis. *N Engl J Med* (2007) 356(8):820–9. doi:10.1056/NEJMcp062614
28. Maddern GJ, Jamieson GG. Fundoplication enhances gastric emptying. *Ann Surg* (1985) 201(3):296–9. doi:10.1097/00000658-198503000-00006
29. Jamieson GG, Maddern GJ, Myers JC. Gastric emptying after fundoplication with and without proximal gastric vagotomy. *Arch Surg* (1991) 126(11):1414–7. doi:10.1001/archsurg.1991.01410350108017
30. Cummings DE, Overduin J. Gastrointestinal regulation of food intake. *J Clin Invest* (2007) 117(1):13–23. doi:10.1172/JCI30227
31. Lee WJ, Chen CY, Chong K, Lee YC, Chen SC, Lee SD. Changes in postprandial gut hormones after metabolic surgery: a comparison of gastric bypass and sleeve gastrectomy. *Surg Obes Relat Dis* (2011) 7(6):683–90. doi:10.1016/j.soard.2011.07.009
32. Malin SK, Samat A, Wolski K, Abood B, Pothier CE, Bhatt DL, et al. Improved acylated ghrelin suppression at 2 years in obese patients with type 2 diabetes: effects of bariatric surgery vs standard medical therapy. *Int J Obes (Lond)* (2014) 38(3):364–70. doi:10.1038/ijo.2013.196
33. Riccioppo D, Santo MA, Rocha M, Buchpiguel CA, Diniz MA, Pajecki D, et al. Small-volume, fast-emptying gastric pouch leads to better long-term weight loss and food tolerance after Roux-en-Y gastric bypass. *Obes Surg* (2018) 28(3):693–701. doi:10.1007/s11695-017-2922-0
34. Wickremesekera K, Miller G, Naotunne TD, Knowles G, Stubbs RS. Loss of insulin resistance after Roux-en-Y gastric bypass surgery: a time course study. *Obes Surg* (2005) 15(4):474–81. doi:10.1381/0960892053723402
35. Kelley DE, Wing R, Buonocore C, Sturis J, Polonsky K, Fitzsimmons M. Relative effects of calorie restriction and weight loss in noninsulin-dependent diabetes mellitus. *J Clin Endocrinol Metab* (1993) 77(5):1287–93. doi:10.1210/jc.77.5.1287
36. Steven S, Hollingsworth KG, Small PK, Woodcock SA, Pucci A, Aribasala B, et al. Calorie restriction and not glucagon-like peptide-1 explains the acute improvement in glucose control after gastric bypass in type 2 diabetes. *Diabet Med* (2016) 33(12):1723–31. doi:10.1111/dme.13257
37. Perry RJ, Peng L, Cline GW, Wang Y, Rabin-Court A, Song JD, et al. Mechanisms by which a very-low-calorie diet reverses hyperglycemia in a rat model of type 2 diabetes. *Cell Metab* (2017) 27(1):210–7.e3. doi:10.1016/j.cmet.2017.10.004
38. Sathananthan M, Shah M, Edens KL, Grothe KB, Piccinini F, Farrugia LP, et al. Six and 12 weeks of caloric restriction increases beta cell function and lowers fasting and postprandial glucose concentrations in people with type 2 diabetes. *J Nutr* (2015) 145(9):2046–51. doi:10.3945/jn.115.210617
39. Jackness C, Karmally W, Febres G, Conwell IM, Ahmed L, Bessler M, et al. Very low-calorie diet mimics the early beneficial effect of Roux-en-Y gastric bypass on insulin sensitivity and beta-cell function in type 2 diabetic patients. *Diabetes* (2013) 62(9):3027–32. doi:10.2337/db12-1762

40. Lips MA, de Groot GH, van Klinken JB, Aarts E, Berends FJ, Janssen IM, et al. Calorie restriction is a major determinant of the short-term metabolic effects of gastric bypass surgery in obese type 2 diabetic patients. *Clin Endocrinol (Oxf)* (2014) 80(6):834–42. doi:10.1111/cen.12254
41. Lean ME, Leslie WS, Barnes AC, Brosnahan N, Thom G, McCombie L, et al. Primary care-led weight management for remission of type 2 diabetes (DiRECT): an open-label, cluster-randomised trial. *Lancet* (2017) 391: 541–51. doi:10.1016/S0140-6736(17)33102-1
42. Bredenoord AJ, Chial HJ, Camilleri M, Mullan BP, Murray JA. Gastric accommodation and emptying in evaluation of patients with upper gastrointestinal symptoms. *Clin Gastroenterol Hepatol* (2003) 1(4):264–72. doi:10.1053/S1542-3565(03)00130-7
43. Delgado-Aros S, Kim DY, Burton DD, Thomforde GM, Stephens D, Brinkmann BH, et al. Effect of GLP-1 on gastric volume, emptying, maximum volume ingested, and postprandial symptoms in humans. *Am J Physiol Gastrointest Liver Physiol* (2002) 282(3):G424–31. doi:10.1152/ajpgi.2002.282.3.G424
44. Camilleri M, Breen M, Ryks M, Burton D. Proximal and overall gastric emptying of solids in patients with reduced gastric volume accommodation compared to matched controls. *Dig Dis Sci* (2011) 56(6):1729–34. doi:10.1007/s10620-011-1615-0
45. Shah M, Law JH, Micheletto F, Sathananthan M, Dalla Man C, Cobelli C, et al. Contribution of endogenous glucagon-like peptide 1 to glucose metabolism after Roux-en-Y gastric bypass. *Diabetes* (2014) 63(2):483–93. doi:10.2337/db13-0954
46. Delgado-Aros S, Vella A, Camilleri M, Low PA, Burton DD, Thomforde GM, et al. Effects of glucagon-like peptide-1 and feeding on gastric volumes in diabetes mellitus with cardio-vagal dysfunction. *Neurogastroenterol Motil* (2003) 15(4):435–43. doi:10.1046/j.1365-2982.2003.00422.x
47. Kral JG. Effects of truncal vagotomy on body weight and hyperinsulinemia in morbid obesity. *Am J Clin Nutr* (1980) 33(2 Suppl):416–9. doi:10.1093/ajcn/33.2.416
48. Ma J, Pilichiewicz AN, Feinle-Bisset C, Wishart JM, Jones KL, Horowitz M, et al. Effects of variations in duodenal glucose load on glycaemic, insulin, and incretin responses in type 2 diabetes. *Diabet Med* (2012) 29(5):604–8. doi:10.1111/j.1464-5491.2011.03496.x
49. Marathe CS, Rayner CK, Lange K, Bound M, Wishart J, Jones KL, et al. Relationships of the early insulin secretory response and oral disposition index with gastric emptying in subjects with normal glucose tolerance. *Physiol Rep* (2017) 5(4):e13122. doi:10.14814/phy2.13122
50. Marathe CS, Horowitz M, Trahair LG, Wishart JM, Bound M, Lange K, et al. Relationships of early and late glycemic responses with gastric emptying during an oral glucose tolerance test. *J Clin Endocrinol Metab* (2015) 100(9):3565–71. doi:10.1210/JC.2015-2482
51. Vazquez-Roque MI, Camilleri M, Vella A, Carlson P, Laugen J, Zinsmeister AR. Association of TCF7L2 allelic variations with gastric function, satiation, and GLP-1 levels. *Clin Transl Sci* (2011) 4(3):183–7. doi:10.1111/j.1752-8062.2011.00284.x
52. Parthasarathy G, Kudva YC, Low PA, Camilleri M, Basu A, Bharucha AE. Relationship between gastric emptying and diurnal glycemic control in type 1 diabetes mellitus: a randomized trial. *J Clin Endocrinol Metab* (2017) 102(2):398–406. doi:10.1210/jc.2016-2809
53. Chowdhury S, Reeds DN, Crimmins DL, Patterson BW, Laciny E, Wang S, et al. Xenin-25 delays gastric emptying and reduces postprandial glucose levels in humans with and without type 2 diabetes. *Am J Physiol Gastrointest Liver Physiol* (2014) 306(4):G301–9. doi:10.1152/ajpgi.00383.2013
54. Ishii M, Nakamura T, Kasai F, Onuma T, Baba T, Takebe K. Altered postprandial insulin requirement in IDDM patients with gastroparesis. *Diabetes Care* (1994) 17(8):901–3. doi:10.2337/diacare.17.8.901
55. Meier JJ, Rosenstock J, Hincelin-Mery A, Roy-Duval C, Delfolie A, Coester HV, et al. Contrasting effects of lixisenatide and liraglutide on postprandial glycemic control, gastric emptying, and safety parameters in patients with type 2 diabetes on optimized insulin glargine with or without metformin: a randomized, open-label trial. *Diabetes Care* (2015) 38(7):1263–73. doi:10.2337/dc14-1984
56. Hinshaw L, Schiavon M, Dadlani V, Mallad A, Dalla Man C, Bharucha A, et al. Effect of pramlintide on postprandial glucose fluxes in type 1 diabetes. *J Clin Endocrinol Metab* (2016) 101(5):1954–62. doi:10.1210/jc.2015-3952
57. Adachi S, Takiguchi S, Okada K, Yamamoto K, Yamasaki M, Miyata H, et al. Effects of ghrelin administration after total gastrectomy: a prospective, randomized, placebo-controlled phase II study. *Gastroenterology* (2010) 138(4):1312–20. doi:10.1053/j.gastro.2009.12.058
58. Yamamoto K, Takiguchi S, Miyata H, Adachi S, Hiura Y, Yamasaki M, et al. Randomized phase II study of clinical effects of ghrelin after esophagectomy with gastric tube reconstruction. *Surgery* (2010) 148(1):31–8. doi:10.1016/j.surg.2009.11.026
59. Sisley S, Gutierrez-Aguilar R, Scott M, D'Alessio DA, Sandoval DA, Seeley RJ. Neuronal GLP1R mediates liraglutide's anorectic but not glucose-lowering effect. *J Clin Invest* (2014) 124(6):2456–63. doi:10.1172/JCI72434
60. Miras AD, Jackson RN, Jackson SN, Goldstone AP, Olbers T, Hackenberg T, et al. Gastric bypass surgery for obesity decreases the reward value of a sweet-fat stimulus as assessed in a progressive ratio task. *Am J Clin Nutr* (2012) 96(3):467–73. doi:10.3945/ajcn.112.036921
61. Stefater MA, Wilson-Perez HE, Chambers AP, Sandoval DA, Seeley RJ. All bariatric surgeries are not created equal: insights from mechanistic comparisons. *Endocr Rev* (2012) 33(4):595–622. doi:10.1210/er.2011-1044
62. Shah M, Vella A. Effects of GLP-1 on appetite and weight. *Rev Endocr Metab Disord* (2014) 15(3):181–7. doi:10.1007/s11154-014-9289-5
63. Vella A, Camilleri M, Rizza RA. The gastrointestinal tract and glucose tolerance. *Curr Opin Clin Nutr Metab Care* (2004) 7(4):479–84. doi:10.1097/01.mco.0000134375.01310.97
64. Williams DL, Grill HJ, Cummings DE, Kaplan JM. Vagotomy dissociates short- and long-term controls of circulating ghrelin. *Endocrinology* (2003) 144(12):5184–7. doi:10.1210/en.2003-1059
65. Zander M, Madsbad S, Madsen JL, Holst JJ. Effect of 6-week course of glucagon-like peptide 1 on glycaemic control, insulin sensitivity, and beta-cell function in type 2 diabetes: a parallel-group study. *Lancet* (2002) 359(9309):824–30. doi:10.1016/S0140-6736(02)07952-7
66. Drucker DJ, Nauck MA. The incretin system: glucagon-like peptide-1 receptor agonists and dipeptidyl peptidase-4 inhibitors in type 2 diabetes. *Lancet* (2006) 368(9548):1696–705. doi:10.1016/S0140-6736(06)69705-5
67. Dotson CD, Geraedts MC, Munger SD. Peptide regulators of peripheral taste function. *Semin Cell Dev Biol* (2013) 24(3):232–9. doi:10.1016/j.semcdb.2013.01.004
68. Elson AE, Dotson CD, Egan JM, Munger SD. Glucagon signaling modulates sweet taste responsiveness. *FASEB J* (2010) 24(10):3960–9. doi:10.1096/fj.10-158105
69. Martin B, Dotson CD, Shin YK, Ji S, Drucker DJ, Maudsley S, et al. Modulation of taste sensitivity by GLP-1 signaling in taste buds. *Ann N Y Acad Sci* (2009) 1170:98–101. doi:10.1111/j.1749-6632.2009.03920.x
70. Shin YK, Martin B, Golden E, Dotson CD, Maudsley S, Kim W, et al. Modulation of taste sensitivity by GLP-1 signaling. *J Neurochem* (2008) 106(1):455–63. doi:10.1111/j.1471-4159.2008.05397.x
71. Sathananthan A, Man CD, Micheletto F, Zinsmeister AR, Camilleri M, Giesler PD, et al. Common genetic variation in GLP1R and insulin secretion in response to exogenous GLP-1 in nondiabetic subjects: a pilot study. *Diabetes Care* (2010) 33(9):2074–6. doi:10.2337/dc10-0200
72. Vetter ML, Wadden TA, Teff KL, Khan ZF, Carvajal R, Ritter S, et al. GLP-1 plays a limited role in improved glycemia shortly after Roux-en-Y gastric bypass: a comparison with intensive lifestyle modification. *Diabetes* (2015) 64(2):434–46. doi:10.2337/db14-0558
73. Ye J, Hao Z, Mumphrey MB, Townsend RL, Patterson LM, Stylopoulos N, et al. GLP-1 receptor signaling is not required for reduced body weight after RYGB in rodents. *Am J Physiol Regul Integr Comp Physiol* (2014) 306(5):R352–62. doi:10.1152/ajpregu.00491.2013
74. Jimenez A, Mari A, Casamitjana R, Lacy A, Ferrannini E, Vidal J. GLP-1 and glucose tolerance after sleeve gastrectomy in morbidly obese subjects with type 2 diabetes. *Diabetes* (2014) 63(10):3372–7. doi:10.2337/db14-0357
75. Douros JD, Lewis AG, Smith EP, Niu J, Capozzi M, Wittmann A, et al. Enhanced glucose control following vertical sleeve gastrectomy does not require a beta-cell glucagon-like peptide 1 receptor. *Diabetes* (2018). doi:10.2337/db18-0081
76. de Jonge C, Rensen SS, Verdam FJ, Vincent RP, Bloom SR, Buurman WA, et al. Endoscopic duodenal-jejunal bypass liner rapidly improves type 2 diabetes. *Obes Surg* (2013) 23(9):1354–60. doi:10.1007/s11695-013-0921-3
77. Cohen R, Galvao Neto MP, Roux CL. Another look at the (endoscopic duodenal liner) ENDO trial, or how to avoid the burial of a valuable

antidiabetic tool. *Surg Obes Relat Dis* (2016) 12(3):702–4. doi:10.1016/j.soard.2015.08.503
78. Spiegel K, Tasali E, Leproult R, Scherberg N, Van Cauter E. Twenty-four-hour profiles of acylated and total ghrelin: relationship with glucose levels and impact of time of day and sleep. *J Clin Endocrinol Metab* (2011) 96(2):486–93. doi:10.1210/jc.2010-1978
79. Cummings DE, Clement K, Purnell JQ, Vaisse C, Foster KE, Frayo RS, et al. Elevated plasma ghrelin levels in Prader Willi syndrome. *Nat Med* (2002) 8(7):643–4. doi:10.1038/nm0702-643
80. Tschop M, Smiley DL, Heiman ML. Ghrelin induces adiposity in rodents. *Nature* (2000) 407(6806):908–13. doi:10.1038/35038090
81. Wren AM, Seal LJ, Cohen MA, Brynes AE, Frost GS, Murphy KG, et al. Ghrelin enhances appetite and increases food intake in humans. *J Clin Endocrinol Metab* (2001) 86(12):5992. doi:10.1210/jcem.86.12.8111
82. Dezaki K, Damdindorj B, Sone H, Dyachok O, Tengholm A, Gylfe E, et al. Ghrelin attenuates cAMP-PKA signaling to evoke insulinostatic cascade in islet beta-cells. *Diabetes* (2011) 60(9):2315–24. doi:10.2337/db11-0368
83. Yanagi S, Sato T, Kangawa K, Nakazato M. The homeostatic force of ghrelin. *Cell Metab* (2018) 27(4):786–804. doi:10.1016/j.cmet.2018.02.008
84. Shin A, Camilleri M, Busciglio I, Burton D, Stoner E, Noonan P, et al. Randomized controlled phase Ib study of ghrelin agonist, RM-131, in type 2 diabetic women with delayed gastric emptying: pharmacokinetics and pharmacodynamics. *Diabetes Care* (2013) 36(1):41–8. doi:10.2337/dc12-1128
85. Shin A, Camilleri M, Busciglio I, Burton D, Smith SA, Vella A, et al. The ghrelin agonist RM-131 accelerates gastric emptying of solids and reduces symptoms in patients with type 1 diabetes mellitus. *Clin Gastroenterol Hepatol* (2013) 11(11):1453–9.e4. doi:10.1016/j.cgh.2013.04.019
86. Elrick H, Stimmler L, Hlad CJ Jr, Arai Y. Plasma insulin response to oral and intravenous glucose administration. *J Clin Endocrinol Metab* (1964) 24:1076–82. doi:10.1210/jcem-24-10-1076
87. Perley MJ, Kipnis DM. Plasma insulin responses to oral and intravenous glucose: studies in normal and diabetic subjects. *J Clin Invest* (1967) 46(12):1954–62. doi:10.1172/JCI105685
88. Vilsboll T, Krarup T, Madsbad S, Holst JJ. Both GLP-1 and GIP are insulinotropic at basal and postprandial glucose levels and contribute nearly equally to the incretin effect of a meal in healthy subjects. *Regul Pept* (2003) 114(2–3):115–21. doi:10.1016/S0167-0115(03)00111-3
89. Eissele R, Goke R, Willemer S, Harthus HP, Vermeer H, Arnold R, et al. Glucagon-like peptide-1 cells in the gastrointestinal tract and pancreas of rat, pig and man. *Eur J Clin Invest* (1992) 22(4):283–91. doi:10.1111/j.1365-2362.1992.tb01464.x
90. Chambers AP, Sorrell JE, Haller A, Roelofs K, Hutch CR, Kim KS, et al. The role of pancreatic preproglucagon in glucose homeostasis in mice. *Cell Metab* (2017) 25(4):927–34.e3. doi:10.1016/j.cmet.2017.02.008
91. Brubaker PL, Anini Y. Direct and indirect mechanisms regulating secretion of glucagon-like peptide-1 and glucagon-like peptide-2. *Can J Physiol Pharmacol* (2003) 81(11):1005–12. doi:10.1139/y03-107
92. Little TJ, Doran S, Meyer JH, Smout AJ, O'Donovan DG, Wu KL, et al. The release of GLP-1 and ghrelin, but not GIP and CCK, by glucose is dependent upon the length of small intestine exposed. *Am J Physiol Endocrinol Metab* (2006) 291(3):E647–55. doi:10.1152/ajpendo.00099.2006
93. Ma J, Checklin HL, Wishart JM, Stevens JE, Jones KL, Horowitz M, et al. A randomised trial of enteric-coated nutrient pellets to stimulate gastrointestinal peptide release and lower glycaemia in type 2 diabetes. *Diabetologia* (2013) 56(6):1236–42. doi:10.1007/s00125-013-2876-2
94. Kieffer TJ, McIntosh CH, Pederson RA. Degradation of glucose-dependent insulinotropic polypeptide and truncated glucagon-like peptide 1 in vitro and in vivo by dipeptidyl peptidase IV. *Endocrinology* (1995) 136(8):3585–96. doi:10.1210/endo.136.8.7628397
95. Burcelin R, Gourdy P. Harnessing glucagon-like peptide-1 receptor agonists for the pharmacological treatment of overweight and obesity. *Obes Rev* (2017) 18(1):86–98. doi:10.1111/obr.12465
96. Christensen M, Vedtofte L, Holst JJ, Vilsboll T, Knop FK. Glucose-dependent insulinotropic polypeptide: a bifunctional glucose-dependent regulator of glucagon and insulin secretion in humans. *Diabetes* (2011) 60(12):3103–9. doi:10.2337/db11-0979
97. Christensen M, Calanna S, Sparre-Ulrich AH, Kristensen PL, Rosenkilde MM, Faber J, et al. Glucose-dependent insulinotropic polypeptide augments glucagon responses to hypoglycemia in type 1 diabetes. *Diabetes* (2015) 64(1):72–8. doi:10.2337/db14-0440
98. Calanna S, Christensen M, Holst JJ, Laferrere B, Gluud LL, Vilsboll T, et al. Secretion of glucose-dependent insulinotropic polypeptide in patients with type 2 diabetes: systematic review and meta-analysis of clinical studies. *Diabetes Care* (2013) 36(10):3346–52. doi:10.2337/dc13-0465
99. Nasteska D, Harada N, Suzuki K, Yamane S, Hamasaki A, Joo E, et al. Chronic reduction of GIP secretion alleviates obesity and insulin resistance under high-fat diet conditions. *Diabetes* (2014) 63(7):2332–43. doi:10.2337/db13-1563
100. Finan B, Ma T, Ottaway N, Muller TD, Habegger KM, Heppner KM, et al. Unimolecular dual incretins maximize metabolic benefits in rodents, monkeys, and humans. *Sci Transl Med* (2013) 5(209):209ra151. doi:10.1126/scitranslmed.3007218
101. Schmitt C, Portron A, Jadidi S, Sarkar N, DiMarchi R. Pharmacodynamics, pharmacokinetics and safety of multiple ascending doses of the novel dual glucose-dependent insulinotropic polypeptide/glucagon-like peptide-1 agonist RG7697 in people with type 2 diabetes mellitus. *Diabetes Obes Metab* (2017) 19(10):1436–45. doi:10.1111/dom.13024
102. Frias JP, Bastyr EJ III, Vignati L, Tschop MH, Schmitt C, Owen K, et al. The sustained effects of a dual GIP/GLP-1 receptor agonist, NNC0090-2746, in patients with type 2 diabetes. *Cell Metab* (2017) 26(2):343–52.e2. doi:10.1016/j.cmet.2017.07.011
103. Gasbjerg LS, Gabe MBN, Hartmann B, Christensen MB, Knop FK, Holst JJ, et al. Glucose-dependent insulinotropic polypeptide (GIP) receptor antagonists as anti-diabetic agents. *Peptides* (2018) 100:173–81. doi:10.1016/j.peptides.2017.11.021
104. Chandra R, Liddle RA. Cholecystokinin. *Curr Opin Endocrinol Diabetes Obes* (2007) 14(1):63–7. doi:10.1097/MED.0b013e3280122850
105. Liddle RA, Rushakoff RJ, Morita ET, Beccaria L, Carter JD, Goldfine ID. Physiological role for cholecystokinin in reducing postprandial hyperglycemia in humans. *J Clin Invest* (1988) 81(6):1675–81. doi:10.1172/JCI113505
106. Cheung GW, Kokorovic A, Lam CK, Chari M, Lam TK. Intestinal cholecystokinin controls glucose production through a neuronal network. *Cell Metab* (2009) 10(2):99–109. doi:10.1016/j.cmet.2009.07.005
107. Beglinger C, Degen L, Matzinger D, D'Amato M, Drewe J. Loxiglumide, a CCK-A receptor antagonist, stimulates calorie intake and hunger feelings in humans. *Am J Physiol Regul Integr Comp Physiol* (2001) 280(4):R1149–54. doi:10.1152/ajpregu.2001.280.4.R1149
108. Travagli RA, Hermann GE, Browning KN, Rogers RC. Brainstem circuits regulating gastric function. *Annu Rev Physiol* (2006) 68:279–305. doi:10.1146/annurev.physiol.68.040504.094635
109. Furness JB. Novel gut afferents: intrinsic afferent neurons and intestinofugal neurons. *Auton Neurosci* (2006) 125(1–2):81–5. doi:10.1016/j.autneu.2006.01.007
110. Chang HY, Mashimo H, Goyal RK. Musings on the wanderer: what's new in our understanding of vago-vagal reflex? IV. Current concepts of vagal efferent projections to the gut. *Am J Physiol Gastrointest Liver Physiol* (2003) 284(3):G357–66. doi:10.1152/ajpgi.00478.2002
111. Broussard DL, Altschuler SM. Brainstem viscerotopic organization of afferents and efferents involved in the control of swallowing. *Am J Med* (2000) 108(Suppl 4a):79S–86S. doi:10.1016/S0002-9343(99)00343-5
112. Smith DK, Sarfeh J, Howard L. Truncal vagotomy in hypothalamic obesity. *Lancet* (1983) 1(8337):1330–1. doi:10.1016/S0140-6736(83)92437-6
113. Sarr MG, Billington CJ, Brancatisano R, Brancatisano A, Toouli J, Kow L, et al. The EMPOWER study: randomized, prospective, double-blind, multicenter trial of vagal blockade to induce weight loss in morbid obesity. *Obes Surg* (2012) 22(11):1771–82. doi:10.1007/s11695-012-0751-8
114. Browning KN, Fortna SR, Hajnal A. Roux-en-Y gastric bypass reverses the effects of diet-induced obesity to inhibit the responsiveness of central vagal motoneurones. *J Physiol* (2013) 591(9):2357–72. doi:10.1113/jphysiol.2012.249268
115. Shin AC, Zheng H, Berthoud HR. Vagal innervation of the hepatic portal vein and liver is not necessary for Roux-en-Y gastric bypass surgery-induced hypophagia, weight loss, and hypermetabolism. *Ann Surg* (2012) 255(2):294–301. doi:10.1097/SLA.0b013e31823e71b7
116. Sathananthan M, Ikramuddin S, Swain JM, Shah M, Piccinini F, Dalla Man C, et al. The effect of vagal nerve blockade using electrical impulses on glucose

metabolism in nondiabetic subjects. *Diabetes Metab Syndr Obes* (2014) 7:305–12. doi:10.2147/DMSO.S65733
117. Karason K, Molgaard H, Wikstrand J, Sjostrom L. Heart rate variability in obesity and the effect of weight loss. *Am J Cardiol* (1999) 83(8):1242–7. doi:10.1016/S0002-9149(99)00066-1
118. Lips MA, de Groot GH, De Kam M, Berends FJ, Wiezer R, Van Wagensveld BA, et al. Autonomic nervous system activity in diabetic and healthy obese female subjects and the effect of distinct weight loss strategies. *Eur J Endocrinol* (2013) 169(4):383–90. doi:10.1530/EJE-13-0506
119. Maser RE, Lenhard MJ, Irgau I, Wynn GM. Impact of surgically induced weight loss on cardiovascular autonomic function: one-year follow-up. *Obesity (Silver Spring)* (2007) 15(2):364–9. doi:10.1038/oby.2007.554
120. Maser RE, Lenhard MJ, Peters MB, Irgau I, Wynn GM. Effects of surgically induced weight loss by Roux-en-Y gastric bypass on cardiovascular autonomic nerve function. *Surg Obes Relat Dis* (2013) 9(2):221–6. doi:10.1016/j.soard.2011.11.014
121. Casellini CM, Parson HK, Hodges K, Edwards JF, Lieb DC, Wohlgemuth SD, et al. Bariatric surgery restores cardiac and sudomotor autonomic C-fiber dysfunction towards normal in obese subjects with type 2 diabetes. *PLoS One* (2016) 11(5):e0154211. doi:10.1371/journal.pone.0154211
122. Parekh PJ, Nayi VR, Johnson DA, Vinik AI. The role of gut microflora and the cholinergic anti-inflammatory neuroendocrine system in diabetes mellitus. *Front Endocrinol* (2016) 7:55. doi:10.3389/fendo.2016.00055
123. Kenler HA, Brolin RE, Cody RP. Changes in eating behavior after horizontal gastroplasty and Roux-en-Y gastric bypass. *Am J Clin Nutr* (1990) 52(1):87–92. doi:10.1093/ajcn/52.1.87
124. Nance K, Eagon JC, Klein S, Pepino MY. Effects of sleeve gastrectomy vs. Roux-en-Y gastric bypass on eating behavior and sweet taste perception in subjects with obesity. *Nutrients* (2017) 10(1):E18. doi:10.3390/nu10010018
125. Van Vuuren MAJ, Strodl E, White KM, Lockie PD. Taste, enjoyment, and desire of flavors change after sleeve gastrectomy-short term results. *Obes Surg* (2017) 27(6):1466–73. doi:10.1007/s11695-016-2497-1
126. Nelson G, Hoon MA, Chandrashekar J, Zhang Y, Ryba NJ, Zuker CS. Mammalian sweet taste receptors. *Cell* (2001) 106(3):381–90. doi:10.1016/S0092-8674(01)00451-2
127. Liu D, Liman ER. Intracellular Ca^{2+} and the phospholipid PIP2 regulate the taste transduction ion channel TRPM5. *Proc Natl Acad Sci U S A* (2003) 100(25):15160–5. doi:10.1073/pnas.2334159100
128. Laffitte A, Neiers F, Briand L. Functional roles of the sweet taste receptor in oral and extraoral tissues. *Curr Opin Clin Nutr Metab Care* (2014) 17(4):379–85. doi:10.1097/MCO.0000000000000058
129. Young RL, Sutherland K, Pezos N, Brierley SM, Horowitz M, Rayner CK, et al. Expression of taste molecules in the upper gastrointestinal tract in humans with and without type 2 diabetes. *Gut* (2009) 58(3):337–46. doi:10.1136/gut.2008.148932
130. Kokrashvili Z, Mosinger B, Margolskee RF. T1r3 and alpha-gustducin in gut regulate secretion of glucagon-like peptide-1. *Ann N Y Acad Sci* (2009) 1170:91–4. doi:10.1111/j.1749-6632.2009.04485.x
131. Steinert RE, Gerspach AC, Gutmann H, Asarian L, Drewe J, Beglinger C. The functional involvement of gut-expressed sweet taste receptors in glucose-stimulated secretion of glucagon-like peptide-1 (GLP-1) and peptide YY (PYY). *Clin Nutr* (2011) 30(4):524–32. doi:10.1016/j.clnu.2011.01.007
132. Gerspach AC, Steinert RE, Schonenberger L, Graber-Maier A, Beglinger C. The role of the gut sweet taste receptor in regulating GLP-1, PYY, and CCK release in humans. *Am J Physiol Endocrinol Metab* (2011) 301(2):E317–25. doi:10.1152/ajpendo.00077.2011
133. Feng R, Qian C, Liu Q, Jin Y, Liu L, Li S, et al. Expression of sweet taste receptor and gut hormone secretion in modelled type 2 diabetes. *Gen Comp Endocrinol* (2017) 252:142–9. doi:10.1016/j.ygcen.2017.08.008
134. Young RL, Chia B, Isaacs NJ, Ma J, Khoo J, Wu T, et al. Disordered control of intestinal sweet taste receptor expression and glucose absorption in type 2 diabetes. *Diabetes* (2013) 62(10):3532–41. doi:10.2337/db13-0581
135. Bueter M, Miras AD, Chichger H, Fenske W, Ghatei MA, Bloom SR, et al. Alterations of sucrose preference after Roux-en-Y gastric bypass. *Physiol Behav* (2011) 104(5):709–21. doi:10.1016/j.physbeh.2011.07.025
136. Ochner CN, Laferrere B, Afifi L, Atalayer D, Geliebter A, Teixeira J. Neural responsivity to food cues in fasted and fed states pre and post gastric bypass surgery. *Neurosci Res* (2012) 74(2):138–43. doi:10.1016/j.neures.2012.08.002
137. Kellett GL. The facilitated component of intestinal glucose absorption. *J Physiol* (2001) 531(Pt 3):585–95. doi:10.1111/j.1469-7793.2001.0585h.x
138. Duchman SM, Ryan AJ, Schedl HP, Summers RW, Bleiler TL, Gisolfi CV. Upper limit for intestinal absorption of a dilute glucose solution in men at rest. *Med Sci Sports Exerc* (1997) 29(4):482–8. doi:10.1097/00005768-199704000-00009
139. Holst JJ, Gribble F, Horowitz M, Rayner CK. Roles of the gut in glucose homeostasis. *Diabetes Care* (2016) 39(6):884–92. doi:10.2337/dc16-0351
140. Thazhath SS, Marathe CS, Wu T, Chang J, Khoo J, Kuo P, et al. The glucagon-like peptide 1 receptor agonist exenatide inhibits small intestinal motility, flow, transit, and absorption of glucose in healthy subjects and patients with type 2 diabetes: a randomized controlled trial. *Diabetes* (2016) 65(1):269–75. doi:10.2337/db15-0893
141. Fischer E, Lauterbach F. Effect of hyperglycaemia on sugar transport in the isolated mucosa of guinea-pig small intestine. *J Physiol* (1984) 355:567–86. doi:10.1113/jphysiol.1984.sp015439
142. Adachi T, Mori C, Sakurai K, Shihara N, Tsuda K, Yasuda K. Morphological changes and increased sucrase and isomaltase activity in small intestines of insulin-deficient and type 2 diabetic rats. *Endocr J* (2003) 50(3):271–9. doi:10.1507/endocrj.50.271
143. Nguyen NQ, Debreceni TL, Bambrick JE, Chia B, Wishart J, Deane AM, et al. Accelerated intestinal glucose absorption in morbidly obese humans: relationship to glucose transporters, incretin hormones, and glycemia. *J Clin Endocrinol Metab* (2015) 100(3):968–76. doi:10.1210/jc.2014-3144
144. Seimon RV, Brennan IM, Russo A, Little TJ, Jones KL, Standfield S, et al. Gastric emptying, mouth-to-cecum transit, and glycemic, insulin, incretin, and energy intake responses to a mixed-nutrient liquid in lean, overweight, and obese males. *Am J Physiol Endocrinol Metab* (2013) 304(3):E294–300. doi:10.1152/ajpendo.00533.2012
145. Damci T, Nuhoglu I, Devranoglu G, Osar Z, Demir M, Ilkova H. Increased intestinal permeability as a cause of fluctuating postprandial blood glucose levels in type 1 diabetic patients. *Eur J Clin Invest* (2003) 33(5):397–401. doi:10.1046/j.1365-2362.2003.01161.x
146. Horton F, Wright J, Smith L, Hinton PJ, Robertson MD. Increased intestinal permeability to oral chromium (51 Cr)-EDTA in human type 2 diabetes. *Diabet Med* (2014) 31(5):559–63. doi:10.1111/dme.12360
147. Nguyen NQ, Debreceni TL, Bambrick JE, Chia B, Deane AM, Wittert G, et al. Upregulation of intestinal glucose transporters after Roux-en-Y gastric bypass to prevent carbohydrate malabsorption. *Obesity (Silver Spring)* (2014) 22(10):2164–71. doi:10.1002/oby.20829
148. Camilleri M, Madsen K, Spiller R, Greenwood-Van Meerveld B, Verne GN. Intestinal barrier function in health and gastrointestinal disease. *Neurogastroenterol Motil* (2012) 24(6):503–12. doi:10.1111/j.1365-2982.2012.01921.x
149. Pal A, Rhoads DB, Tavakkoli A. Foregut exclusion disrupts intestinal glucose sensing and alters portal nutrient and hormonal milieu. *Diabetes* (2015) 64(6):1941–50. doi:10.2337/db14-1578
150. Suzuki T. Regulation of intestinal epithelial permeability by tight junctions. *Cell Mol Life Sci* (2013) 70(4):631–59. doi:10.1007/s00018-012-1070-x
151. Camilleri M, Lasch K, Zhou W. Irritable bowel syndrome: methods, mechanisms, and pathophysiology. The confluence of increased permeability, inflammation, and pain in irritable bowel syndrome. *Am J Physiol Gastrointest Liver Physiol* (2012) 303(7):G775–85. doi:10.1152/ajpgi.00155.2012
152. Kawamata Y, Fujii R, Hosoya M, Harada M, Yoshida H, Miwa M, et al. A G protein-coupled receptor responsive to bile acids. *J Biol Chem* (2003) 278(11):9435–40. doi:10.1074/jbc.M209706200
153. Gomes JMG, Costa JA, Alfenas RCG. Metabolic endotoxemia and diabetes mellitus: a systematic review. *Metabolism* (2017) 68:133–44. doi:10.1016/j.metabol.2016.12.009
154. Cani PD, Amar J, Iglesias MA, Poggi M, Knauf C, Bastelica D, et al. Metabolic endotoxemia initiates obesity and insulin resistance. *Diabetes* (2007) 56(7):1761–72. doi:10.2337/db06-1491
155. Al-Attas OS, Al-Daghri NM, Al-Rubeaan K, da Silva NF, Sabico SL, Kumar S, et al. Changes in endotoxin levels in T2DM subjects on anti-diabetic therapies. *Cardiovasc Diabetol* (2009) 8:20. doi:10.1186/1475-2840-8-20
156. Kuipers F, Bloks VW, Groen AK. Beyond intestinal soap – bile acids in metabolic control. *Nat Rev* (2014) 10(8):488–98. doi:10.1038/nrendo.2014.60
157. Staels B, Fonseca VA. Bile acids and metabolic regulation: mechanisms and clinical responses to bile acid sequestration. *Diabetes Care* (2009) 32 (Suppl 2):S237–45. doi:10.2337/dc09-S355

158. Reeve JR Jr, Eysselein VE, Rosenquist G, Zeeh J, Regner U, Ho FJ, et al. Evidence that CCK-58 has structure that influences its biological activity. *Am J Physiol* (1996) 270(5 Pt 1):G860–8.
159. Vella A, Camilleri M. The gastrointestinal tract as an integrator of mechanical and hormonal response to nutrient ingestion. *Diabetes* (2017) 66(11): 2729–37. doi:10.2337/dbi17-0021
160. Makishima M, Okamoto AY, Repa JJ, Tu H, Learned RM, Luk A, et al. Identification of a nuclear receptor for bile acids. *Science* (1999) 284(5418):1362–5. doi:10.1126/science.284.5418.1362
161. Teodoro JS, Rolo AP, Palmeira CM. Hepatic FXR: key regulator of whole-body energy metabolism. *Trends Endocrinol Metab* (2011) 22(11):458–66. doi:10.1016/j.tem.2011.07.002
162. Kumar DP, Rajagopal S, Mahavadi S, Mirshahi F, Grider JR, Murthy KS, et al. Activation of transmembrane bile acid receptor TGR5 stimulates insulin secretion in pancreatic beta cells. *Biochem Biophys Res Commun* (2012) 427(3):600–5. doi:10.1016/j.bbrc.2012.09.104
163. Camilleri M, Vazquez-Roque MI, Carlson P, Burton D, Wong BS, Zinsmeister AR. Association of bile acid receptor TGR5 variation and transit in health and lower functional gastrointestinal disorders. *Neurogastroenterol Motil* (2011) 23(11):995–9, e458. doi:10.1111/j.1365-2982.2011.01772.x
164. Watanabe M, Houten SM, Mataki C, Christoffolete MA, Kim BW, Sato H, et al. Bile acids induce energy expenditure by promoting intracellular thyroid hormone activation. *Nature* (2006) 439(7075):484–9. doi:10.1038/nature04330
165. Baxter JD, Webb P. Metabolism: bile acids heat things up. *Nature* (2006) 439(7075):402–3. doi:10.1038/439402a
166. Haeusler RA, Camastra S, Nannipieri M, Astiarraga B, Castro-Perez J, Xie D, et al. Increased bile acid synthesis and impaired bile acid transport in human obesity. *J Clin Endocrinol Metab* (2016) 101(5):1935–44. doi:10.1210/jc.2015-2583
167. Jansen PL, van Werven J, Aarts E, Berends F, Janssen I, Stoker J, et al. Alterations of hormonally active fibroblast growth factors after Roux-en-Y gastric bypass surgery. *Dig Dis* (2011) 29(1):48–51. doi:10.1159/000324128
168. Albaugh VL, Flynn CR, Cai S, Xiao Y, Tamboli RA, Abumrad NN. Early increases in bile acids post Roux-en-Y gastric bypass are driven by insulin-sensitizing, secondary bile acids. *J Clin Endocrinol Metab* (2015) 100(9):E1225–33. doi:10.1210/jc.2015-2467
169. Ryan KK, Tremaroli V, Clemmensen C, Kovatcheva-Datchary P, Myronovych A, Karns R, et al. FXR is a molecular target for the effects of vertical sleeve gastrectomy. *Nature* (2014) 509(7499):183–8. doi:10.1038/nature13135
170. McGavigan AK, Garibay D, Henseler ZM, Chen J, Bettaieb A, Haj FG, et al. TGR5 contributes to glucoregulatory improvements after vertical sleeve gastrectomy in mice. *Gut* (2017) 66(2):226–34. doi:10.1136/gutjnl-2015-309871
171. Flynn CR, Albaugh VL, Cai S, Cheung-Flynn J, Williams PE, Brucker RM, et al. Bile diversion to the distal small intestine has comparable metabolic benefits to bariatric surgery. *Nat Commun* (2015) 6:7715. doi:10.1038/ncomms8715
172. Dufer M, Horth K, Wagner R, Schittenhelm B, Prowald S, Wagner TF, et al. Bile acids acutely stimulate insulin secretion of mouse beta-cells via farnesoid X receptor activation and K(ATP) channel inhibition. *Diabetes* (2012) 61(6):1479–89. doi:10.2337/db11-0815
173. Kohli R, Bradley D, Setchell KD, Eagon JC, Abumrad N, Klein S. Weight loss induced by Roux-en-Y gastric bypass but not laparoscopic adjustable gastric banding increases circulating bile acids. *J Clin Endocrinol Metab* (2013) 98(4):E708–12. doi:10.1210/jc.2012-3736
174. Ursell LK, Metcalf JL, Parfrey LW, Knight R. Defining the human microbiome. *Nutr Rev* (2012) 70(Suppl 1):S38–44. doi:10.1111/j.1753-4887.2012.00493.x
175. Hartstra AV, Bouter KE, Backhed F, Nieuwdorp M. Insights into the role of the microbiome in obesity and type 2 diabetes. *Diabetes Care* (2015) 38(1):159–65. doi:10.2337/dc14-0769
176. Yatsunenko T, Rey FE, Manary MJ, Trehan I, Dominguez-Bello MG, Contreras M, et al. Human gut microbiome viewed across age and geography. *Nature* (2012) 486(7402):222–7. doi:10.1038/nature11053
177. Schulfer A, Blaser MJ. Risks of antibiotic exposures early in life on the developing microbiome. *PLoS Pathog* (2015) 11(7):e1004903. doi:10.1371/journal.ppat.1004903
178. Reijnders D, Goossens GH, Hermes GD, Neis EP, van der Beek CM, Most J, et al. Effects of gut microbiota manipulation by antibiotics on host metabolism in obese humans: a randomized double-blind placebo-controlled trial. *Cell Metab* (2016) 24(1):63–74. doi:10.1016/j.cmet.2016.06.016
179. Gomez A, Petrzelkova KJ, Burns MB, Yeoman CJ, Amato KR, Vlckova K, et al. Gut microbiome of coexisting BaAka pygmies and Bantu reflects gradients of traditional subsistence patterns. *Cell Rep* (2016) 14(9):2142–53. doi:10.1016/j.celrep.2016.02.013
180. El Kaoutari A, Armougom F, Gordon JI, Raoult D, Henrissat B. The abundance and variety of carbohydrate-active enzymes in the human gut microbiota. *Nat Rev Microbiol* (2013) 11(7):497–504. doi:10.1038/nrmicro3050
181. McNeil NI. The contribution of the large intestine to energy supplies in man. *Am J Clin Nutr* (1984) 39(2):338–42. doi:10.1093/ajcn/39.2.338
182. Fiorucci S, Distrutti E. Bile acid-activated receptors, intestinal microbiota, and the treatment of metabolic disorders. *Trends Mol Med* (2015) 21(11):702–14. doi:10.1016/j.molmed.2015.09.001
183. Wahlstrom A, Sayin SI, Marschall HU, Backhed F. Intestinal crosstalk between bile acids and microbiota and its impact on host metabolism. *Cell Metab* (2016) 24(1):41–50. doi:10.1016/j.cmet.2016.05.005
184. Kowlgi NG, Chhabra L. D-lactic acidosis: an underrecognized complication of short bowel syndrome. *Gastroenterol Res Pract* (2015) 2015:476215. doi:10.1155/2015/476215
185. Vella A, Farrugia G. D-lactic acidosis: pathologic consequence of saprophytism. *Mayo Clin Proc* (1998) 73(5):451–6. doi:10.4065/73.5.451
186. Boccia S, Torre I, Santarpia L, Iervolino C, Del Piano C, Puggina A, et al. Intestinal microbiota in adult patients with short bowel syndrome: preliminary results from a pilot study. *Clin Nutr* (2017) 36(6):1707–9. doi:10.1016/j.clnu.2016.09.028
187. Liu R, Hong J, Xu X, Feng Q, Zhang D, Gu Y, et al. Gut microbiome and serum metabolome alterations in obesity and after weight-loss intervention. *Nat Med* (2017) 23(7):859–68. doi:10.1038/nm.4358
188. Qin J, Li Y, Cai Z, Li S, Zhu J, Zhang F, et al. A metagenome-wide association study of gut microbiota in type 2 diabetes. *Nature* (2012) 490(7418):55–60. doi:10.1038/nature11450
189. Zhao L, Zhang F, Ding X, Wu G, Lam YY, Wang X, et al. Gut bacteria selectively promoted by dietary fibers alleviate type 2 diabetes. *Science* (2018) 359(6380):1151–6. doi:10.1126/science.aao5774
190. Forslund K, Hildebrand F, Nielsen T, Falony G, Le Chatelier E, Sunagawa S, et al. Disentangling type 2 diabetes and metformin treatment signatures in the human gut microbiota. *Nature* (2015) 528(7581):262–6. doi:10.1038/nature15766
191. Wu H, Esteve E, Tremaroli V, Khan MT, Caesar R, Manneras-Holm L, et al. Metformin alters the gut microbiome of individuals with treatment-naive type 2 diabetes, contributing to the therapeutic effects of the drug. *Nat Med* (2017) 23(7):850–8. doi:10.1038/nm.4345
192. Gu Y, Wang X, Li J, Zhang Y, Zhong H, Liu R, et al. Analyses of gut microbiota and plasma bile acids enable stratification of patients for antidiabetic treatment. *Nat Commun* (2017) 8(1):1785. doi:10.1038/s41467-017-01682-2
193. de Groot PF, Frissen MN, de Clercq NC, Nieuwdorp M. Fecal microbiota transplantation in metabolic syndrome: history, present and future. *Gut Microbes* (2017) 8(3):253–67. doi:10.1080/19490976.2017.1293224

Conflict of Interest Statement: AV is an investigator in an investigator-initiated study sponsored by Novo Nordisk. He has consulted for vTv Therapeutics, XOMA, Sanofi-Aventis, Novartis, and Bayer in the past 5 years. JM has no relevant disclosures.

frontiers in Endocrinology

REVIEW
published: 28 June 2018
doi: 10.3389/fendo.2018.00347

Cryopreservation of Ovarian Tissue: Opportunities Beyond Fertility Preservation and a Positive View Into the Future

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In current years, ovarian tissue cryopreservation (OTC) and transplantation is gaining ground as a successful method of preserving fertility in young women with primarily cancer diseases, hereby giving them a chance of becoming biological mothers later on. However, OTC preserves more than just the reproductive potential; it restores the ovarian endocrine function and thus the entire female reproductive cycle with natural levels of essential hormones. In a female population with an increased prevalence in the loss of ovarian function due to induced primary ovarian insufficiency (POI) and aging, there is now, a need to develop new treatments and provide new opportunities to utilize the enormous surplus of follicles that most females are born with and overcome major health issues associated with the lack of ovarian hormones. Cell/tissue-based hormone replacement therapy (cHRT) by the use of stored ovarian tissue could be one such option comprising both induction of puberty in prepubertal POI girls, treatment of POI and premature menopause, and as primary prevention at the onset of menopause. In the current review, we explore known and entirely new applications for the potential utilization of OTC including cHRT, social freezing, culture of immature oocytes, and a modern ovarian resection for women with polycystic ovaries, and discuss the indications hereof.

Keywords: ovarian tissue cryopreservation, fertility preservation, cell/tissue-based hormone replacement therapy, induction of puberty, ovarian resection, social freezing, *in vitro* maturation

OPEN ACCESS

Edited by:
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Specialty section:
This article was submitted to Reproduction, a section of the journal Frontiers in Endocrinology

Received: *13 April 2018*
Accepted: *11 June 2018*
Published: *28 June 2018*

Citation:
Kristensen SG and Andersen CY (2018) Cryopreservation of Ovarian Tissue: Opportunities Beyond Fertility Preservation and a Positive View Into the Future. Front. Endocrinol. 9:347. doi: 10.3389/fendo.2018.00347

INTRODUCTION

The follicle constitutes the functional unit of the ovary and produces steroids and peptide hormones to regulate the female reproductive cycle. During the follicular phase more than 90% of the available oestradiol is produced by a single selected preovulatory follicle, which in the luteal phase is transformed to the corpus luteum that secretes progesterone in high concentrations. The unique physical distribution of the follicular reserve within the ovary, with the vast majority of small resting follicles located in the outer cortical region and the growing stages of follicles located in the inner medullary region, represents a perfect opportunity to preserve an organ function without freezing the entire organ. By isolating the cortical region, containing 90% of the follicular reserve, human ovarian tissue has been successfully cryopreserved for fertility preservation in young women with cancer diseases for over two

decades. Subsequent transplantation of thawed ovarian tissue has restored ovarian endocrine function in 95% of the patients and resulted in the birth of over 130 children worldwide (1, 2). Half of all the children born have been conceived by natural conception, which highlights the exceptional ability of this procedure to actually restore an entire organ function in contrast to conventional fertility preserving strategies like oocyte and embryo freezing, in which the fertility potential is fixed by the number of oocytes retrieved and IVF is the only option to conceive. Moreover, ovarian tissue cryopreservation (OTC) can be performed from day to day and is therefore the only fertility preserving strategy for girls and young women who needs to undertake acute gonadotoxic treatment, and for prepubertal girls who do not yet produce mature oocytes for freezing.

Primary ovarian insufficiency (POI) is defined as the cessation of the ovarian function before the age of 40 years, and is a common cause of infertility in women. Its incidence is estimated to be as high as 1 in 100 by the age of 40, and 1 in 1,000 by the age of 30 years (3, 4). Spontaneous POI include genetic, autoimmunological, inflammatory, enzyme deficiency, metabolic, or very often idiopathic causes, whereas induced POI occurs mainly due to oncological treatment such as surgery (bilateral oophorectomy), chemotherapy and radiotherapy (5). POI not only interferes with a woman's reproductive potential, but the cessation of sex steroid production is also associated with an increased risk of osteoporosis, cardiovascular disease, earlier mortality, obesity, sexual dysfunction, dementia, and cognitive decline (6–8).

Up until now fertility preservation has been the primary goal of OTC in young girls and women diagnosed with a malignancy or genetic disease threatening to destroy their ovarian reserve. However, the restoration of an organ function, the steroidogenic capacity of the transplanted ovarian tissue and the unique access to immature oocytes from small antral follicles have now evoked new perspectives and ideas to expand the utilization of OTC beyond its traditional purpose of fertility preservation for medical reasons. These novel ideas approach a broader population of women and include utilization of OTC for cell/tissue-based hormone replacement therapy (cHRT), non-medical reasons, optimizing culture systems for immature oocytes, and performing a modern ovarian resection for women with polycystic ovaries (**Figure 1**). This kind of ideas which are technical possible, but not yet proven, will undoubtedly raise controversy in the field and a plethora of questions concerning the ethics, safety, cost-effectiveness, superiority, and implications of the proposed procedures. In this paper, we aim to explore known and entirely new applications for the utilization of OTC and discuss the indications for such procedures.

CELL/TISSUE-BASED HORMONE REPLACEMENT THERAPY (cHRT)

More efficient cancer treatments combined with a general increase in life expectancy have led to a higher frequency of POI in a growing population of cancer survivors and aging women. It is widely accepted that the pillar of treatment of POI is pharmacologic hormone replacement therapy (pHRT), at least until the average age of natural menopause. Pharmacologic HRT with estrogen alone or estrogen and progestogens in combination is known to effectively compensate for the loss of ovarian hormone production, but only when it is delivered at an optimal dosage, frequency, and at an appropriate time (8, 9). Unfortunately, pharmacological delivery methods of HRT are not integrated into the hypothalamic-pituitary (HP) axis which would facilitate feedback and regulation of dosage and timing of circulating hormone levels. As such, pharmacological hormone delivery results in consistently higher serum concentrations as compared to those associated with normal functional ovaries.

New approaches using cHRT offer a potential physiological solution to timely control of hormone delivery and the ability to restore the functionality of the HPO axis. OTC can be used as cHRT as thawed ovarian tissue grafted into the pelvis cavity restores the natural hormone milieu and endocrine function of the ovary which have been consistently documented by a rise in estradiol and a decrease in FSH and LH levels returning to premenopausal levels 4–5 months after transplantation leading to cessation of menopausal symptoms and renewed menstrual cycles in the vast majority of transplanted patients (1, 10, 11). Restored hormone production may even be the desired effect rather than fertility restoration in some patients (10), and a more appealing alternative to pHRT for some women. In Denmark around a dozen young women who entered menopause due to cancer treatment have had frozen/thawed tissue transplanted only to become a "normal woman" again and avoid menopause (10), The average duration of graft function is approximately 5 years, but the function can persist for over 9 years (10), depending on the follicular density at the time of OTC (2). The transplantation procedure may be repeated multiple times in order to stretch the longevity of the tissue, if sufficient amounts of ovarian tissue has been stored, hereby extending the lifespan of ovarian endocrine activity up to 11 years or more (12, 13). If patients are merely in the need of sex steroid production, a heterotopic graft site like the abdominal wall or a subcutaneous site may be preferred over an orthotopic site as it requires less invasive surgery and would obviate the need for general anesthesia and a laparoscopic procedure. A cHRT approach combining a heterotopic graft site with multiple transplantations extending the longevity of banked ovarian tissue can therefore facilitate prolonged ovarian endocrine function and have implications way beyond fertility preservation.

Inducing Puberty in Pre-pubertal POI Girls

Survivors of childhood cancer represent a rapidly growing population of patients in which temporary or permanent POI is a common side effect of the cytotoxic treatments. In addition, POI often has a genetic background with more than 50 genes in which mutations can be causative and many other genes that may be implicated (14). These genes can affect various processes such as gonadal development, DNA replication/ meiosis and DNA repair, hormonal signaling, immune function, and metabolism, and include conditions like Turner syndrome, sickle cell anemia, thalassemia, and

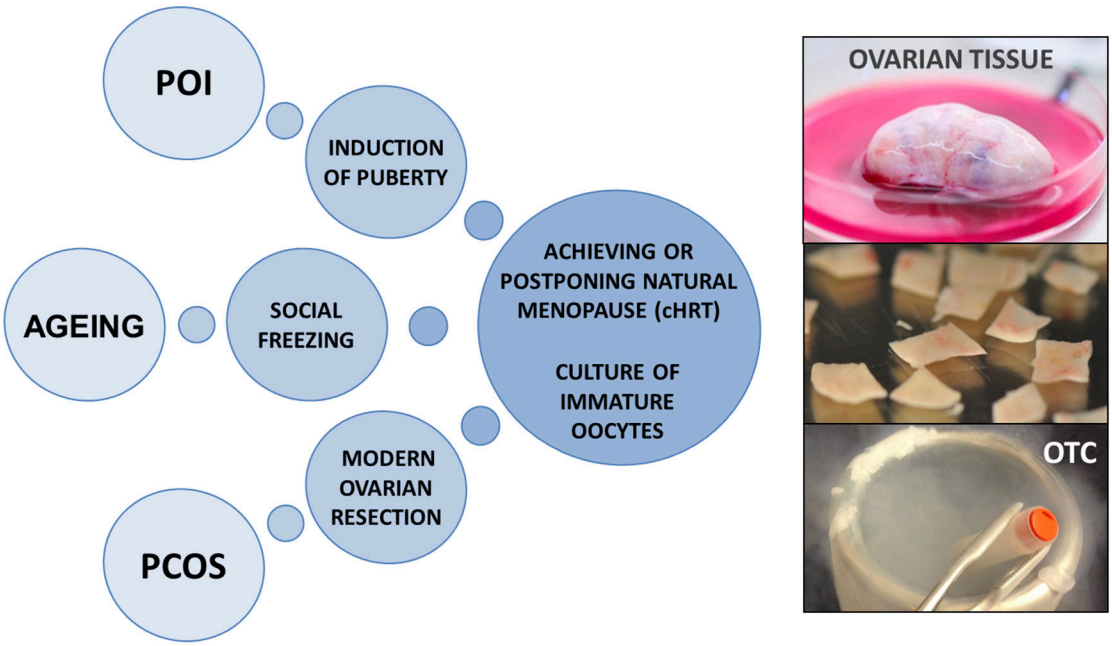

FIGURE 1 | Potential applications for ovarian tissue cryopreservation beyond fertility preservation in young women with cancer diseases. OTC, ovarian tissue cryopreservation; cHRT, cell/tissue-based hormone replacement therapy; POI, premature ovarian insufficiency; PCOS, polycystic ovary syndrome.

galactosemia. These young girls often experience delayed puberty or need pHRT to induce puberty to enable the pubertal growth spurt as well as development of secondary sexual characteristics. However, pharmacological delivery of increasing doses of estrogen followed with progesterone only address some aspects of puberty and does not completely match the physiological complexity of the hormonal milieu during puberty. Moreover, there is only limited data on the long-term safety of exogenous hormones in childhood cancer survivors and pHRT can cause potentially significant side effects (15, 16).

Cell/tissue-based HRT can therefore be an attractive approach to provide a more physiological hormonal milieu for induction of puberty in pre-pubertal POI girls. In 2012, Poirot and co-workers published the first case report showing that stored ovarian tissue from pre-pubertal girls can provide adequate endogenous sex steroid hormone levels to induce puberty (17). In this case, a 13-year old girl developed POI after being treated for sickle-cell disease at the age of 10, and after having 3 out of 23 stored ovarian pieces transplanted, she entered puberty. A second case report was published shortly after with another 13-year old girl who had developed POI following treatment for Ewing sarcoma at the age of 9 (18). In both cases, a unilateral oophorectomy was performed before treatment and a small proportion of the frozen ovarian cortical fragments (i.e., 10–20% of the stored tissue) were transplanted to a heterotopic location in the former case and to the remaining ovary in the latter. Puberty was successfully induced in both girls although the graft function failed after nearly 2 years.

It can be discussed whether or not young girls should choose to save their ovarian tissue for later use to achieve fertility instead (19), but using only a small percentage (i.e., 10–20%) of the stored tissue might be worth it for some young girls to feel "normal" during a critical period of their emotional and social life as the physical maturation process directly affects body and brain to alter children's needs, interests, and moods. Several studies have suggested that young childhood cancer survivors with POI face significant psychosexual dysfunction and that the symptoms of POI could severely stunt social growth and exacerbate anxiety and feelings of isolation (20, 21). Therefore, cHRT could potentially be beneficial in the long run at both the physiological and psychological level, and the preferred choice for some pre-pubertal POI girls in order for them to experience the life of every other teenager and becoming a woman with physiological levels of sex hormones. However, the preferred treatment for young girls with POI is still pHRT, as it is cheap, simple, and ready available and does not include any surgical intervention. Moreover, the theoretical superiority of physiological hormone levels over the pharmacological levels for induction of puberty has not been proven in any human clinical trials and awaits further research.

Achieving or Postponing Natural Menopause

One century ago the average life expectancy corresponded to the natural age of menopause around the age of 50 years, however, nowadays the majority of women live beyond 80 years in many developed countries, and the demographic structure

is changing toward an increasingly aging population in which women will spend 30–40% of their lives being postmenopausal. In combination with the increasing population of cancer survivors experiencing POI these developments in society calls for preventive strategies to alleviate and decrease short- and long-term consequences and health risks associated with the lack of ovarian endocrine function.

The use of pHRT has been vigorously debated for several decades. In the 1980's and 90's pHRT was administered to millions of women to relieve menopausal symptoms and by the mid-1990s estrogen became the biggest-selling prescription drug in the US. However, it all changed with the publication of the results from the Women's Health Initiative (WHI) randomized clinical trials in 2002 (22), suggesting pHRT to cause an increased risk of breast cancer, after which many women discontinued pHRT or avoided starting pHRT at all ages, including before age 50 years (23, 24). One of the big misunderstandings was that the results from the WHI studies were inappropriately extrapolated from women in the late postmenopausal stage (aged >60 years) to younger women in the early stage of postmenopause (50–59 years), and even further to women experiencing premature or early natural menopause. The WHI study was built upon numerous observational studies and clinical trials consistently demonstrating benefits in the prevention of chronic diseases, which include reduced coronary heart disease (CHD) and mortality, when pHRT was initiated near the onset of menopause (25–30). The WHI study was therefore designed to test the effects of pHRT in women initiating treatment a decade or more after menopause. This is where the "timing hypothesis" comes into play and suggest that different clinical effects occurs if hormones are initiated close to the onset of menopause compared with several years later (31). Basic and animal studies together with clinical studies have now shown that the timing of pHRT can be crucial with respect to especially CHD, cognitive decline, and dementia (8, 31–34). In 2005, the Multi-Institutional Research on Alzheimer Genetic Epidemiology study showed that the risk of dementia was reduced in women who initiated hormonal therapy at age 50–63 years, but was not reduced in women who started hormonal treatment at ages 64–71 or 72–99 years (35).

Reassessment of clinical trials in women initiating pHRT close to the onset of menopause together with more recent studies and meta-analyses now show a long list of benefits with estrogen alone therapy, and risks are considered rare. Beyond symptomatic relief, improvements in quality of life and a reduction in osteoporosis, estrogen-based therapy has now been shown to be cost-effective and have a very favorable risk–benefit profile in healthy women under the age of 60. Specifically, estrogen alone therapy have been shown to consistently decrease CHD in women under 60 years of age by up to 40% and to decrease all-cause mortality by 20–40% (36–38). Moreover, a long-term follow up on the WHI study showed that estrogen alone therapy resulted in a significant decrease in the total risk of cancer by 20% in women aged 50–59 years (39).

The majority of studies show benefits with estrogen alone rather than with estrogen plus progestogen, however, no particular HRT regimen can currently be advocated (31). Moreover, the use of age-appropriate estrogen doses has been reported to be crucial to maximize cardiovascular benefits while minimizing risk of adverse effects such as venous thromboembolism and stroke (32). This is where cHRT comes into the picture and we hypothesize that transplantation of stored ovarian tissue could be used to restore endocrine ovarian function at the onset of menopause or for women entering menopause prematurely. In 2015, we proposed that ovarian tissue frozen in the young years could be transplanted back at the time of menopause to provide continued endogenous, physiological levels of steroid production to postpone menopause (40). A small portion of stored ovarian tissue may be transplanted subcutaneously during local anesthesia multiple times providing menstrual cycles for a prolonged period, using the woman's own tissue and follicles to sustain menstrual cycles with natural variations in the whole armamentarium of hormones. In this way, natural fertility will also be avoided and prevent senior women to conceive spontaneously. Recent animal studies have demonstrated that supraphysiological plasma levels of estrogen were required for pHRT to achieve benefits in bone health that were comparable to those achieved by cHRT (using ovarian cell constructs) at much lower plasma hormone concentrations (41). Such studies suggest a potential benefit of physiological secreted sex steroid hormones under the control of the HPO axis at which effects can be obtained at lower and safer plasma levels.

In theory, cHRT using OTC at a young age could be used as a physiological solution to prevent the massive medical legacy of osteoporosis and menopause-related conditions in the large population of aging women, however, currently the majority of women would probably not subject themselves to an elective surgery purely for delaying menopause and it can be argued whether or not any women would prefer to have regular menstrual cycles up to 60 years of age, when readily available pHRT compounds can deliver the hormones without the bleeding. Nevertheless, OTC could potentially be justified in some women already undergoing pelvic surgery for other reasons for example, a Caesarean section or an appendectomy where ovarian tissue could be collected and frozen as an adjunct. This is, however, also an approach which is highly debatable and may not be ethically and medically appropriate as no studies have been conducted that directly or indirectly compare the benefit and disadvantage of ovarian tissue removal for future cHRT.

The group of women for whom the use of cHRT are most likely to become a clinical option initially is the thousands of women worldwide who have already had ovarian tissue frozen for fertility preservation due to different malignancies and genetic conditions. For various reasons, a large number of these women will not have used their stored tissue by the time they reach menopause, either prematurely or at the natural age of menopause. Some of these women might wish to use their store tissue for postponing menopause or to achieve natural menopause which is nowadays recommended by many scientific societies (8, 31). Studies have shown that women who become postmenopausal before the age of 50 years and do not receive any treatment have an increased risk for cardiovascular disease-related mortality compared to women receiving pHRT (42–46). Other studies have shown an almost doubled long-term risk of

cognitive impairment or dementia in women who underwent oophorectomy before menopause (47). A risk that was eliminated if estrogen therapy was initiated after the surgery and continued up to age 50 years or longer (47), which is advertising a need for sustained ovarian function up to at least the age of natural menopause.

However, many concerns and unanswered questions exist; Is it safe? To whom is the risk-benefit profile actually favorable? Are physiological hormones better than pharmaceutical? Careful consideration of risks and benefits, individually structured counseling and close monitoring are needed for each woman who may want to use their ovarian tissue for primary prevention or postponing menopause. To summarize, cHRT in the form of stored ovarian tissue or ovarian cell constructs (41, 48, 49) could potentially be used at the onset of natural or induced menopause as an ideal time to institute preventative strategies that could potentially increase the quality and length of women's lives. However, this is again technical possible but not proven in a clinical setting with women in this age group, and it can be argued that pHRT should be preferred in all cases as it is the cheapest and simplest way to provide hormones, and no real evidence for the superiority of physiological hormone levels exists. Therefore, more research in the area of physiological and pharmacological hormones is needed in order for this potential application to be adopted clinically in the future.

OTC FOR NON-MEDICAL REASONS

In many countries women also have the opportunity to preserve their fertility due to personal reasons and hereby postpone childbearing. The increasing age of childbearing observed in most high-income countries is often depicted as a result of women selectively choosing to pursue a career and other life goals before having children, however, evidence now suggests that the primary reason is the lack of a partner who is willing to commit to parenthood (50, 51). The traditional method of fertility preservation for these social indications is vitrification of mature oocytes. In recent studies, it has become evident that most of the women who cryopreserve oocytes for non-medical reasons are in their mid to late 30 s, well educated, socio-economically advantaged and single (52–54). Data concerning the reproductive experiences and outcomes of this growing group of women is still very limited, but two severe issues with today's policy for non-medical oocyte freezing have emerged. First of all, women are too old when they decide to store oocytes as the mean age at the time of freezing is around 37 years (53, 55), which may be deemed suboptimal (56). Additionally, one in five patients in this group is a low responder and combined with the advanced maternal age this results in one quarter of the women having fewer than 8–10 mature oocytes frozen which is considered necessary for a reasonable chance of success (57). The second issue is that the majority of the women with frozen oocytes do not come back to collect them and the utilization rate of the stored oocytes is currently below 10% (53, 55). Collectively, it appears that especially reproductive aged women may only to a limited extend benefit from this approach.

A proposition would be to use OTC as an alternative to oocyte vitrification in connection with fertility preservation for non-medical reasons. In contrast to mature oocytes, transplanted ovarian tissue will give the woman the opportunity to conceive spontaneously without IVF, which many women and their potential future partners would probably appreciate. If the woman ends up not needing the stored ovarian tissue for fertility, then the tissue is not wasted, but could instead be used as cHRT to alleviate postmenopausal symptoms later on or avoiding POI. Thus, OTC may serve multiple purposes depending on the need for either fertility or endocrine function and provide a better justification of such intervention. In addition, by storing ovarian tissue, and not just a fixed number of mature oocytes, a range of potential new treatments, which are currently being developed, might become available for these patients in the future. These treatments include *in vitro* follicle activation (58), culture of human preantral follicles (48, 59, 60), autologous transfer of mitochondria to oocytes (61), and *in vitro* maturation of immature oocytes (62).

OTC requires at least two surgical procedures to collect and graft ovarian tissue and it might be overwhelming and excessive for some patients. Therefore, OTC may only be a preferable option over oocyte vitrification for some groups of patients in particular women of advanced maternal age with a low ovarian reserve or poor responders which require multiple cycles of controlled ovarian stimulation. For these groups of patients, their future use of stored ovarian tissue could potentially be enhanced by *in vitro* follicle activation (IVA) in which the resting pool of follicles is activated mechanically and/or biochemically prior to transplantation. In 2013, Kawamura et al. were the first to test IVA in a clinical setting and enabled POI patients without menstrual cycles for several years to conceive with their own oocytes by activating a residual pool of resting follicles (58). This technique has so far resulted in three live births in Japan and two pregnancies in other clinics (58, 63–65).

Taken together, oocyte cryopreservation is the clinical available option for non-medical freezing, but it can be argued that women seeking fertility preservation for non-medical reasons should be presented with both fertility preserving options and the potential beneficial applications for cHRT and future use, and then the woman can help decide which method is most suitable for her. It should be highlighted that age is the most limiting and critical factor for both methods, and the success rate always depends hereof, which means that the issue with low ovarian reserve will apply for both methods, and low return rates are probably an inevitable factor in this group of women.

A MODERN OVARIAN RESECTION FOR ANOVULATORY PCOS WOMEN

A completely new way of utilizing OTC is in the context of surgical treatment for anovulatory women with polycystic ovaries. Polycystic ovary syndrome (PCOS) is one of the most common endocrine disorders among women in their reproductive age and is a predominant cause of anovulatory infertility. In 1930s, Stein and Leventhal developed the ovarian

wedge resection (OWR) in which the ovarian volume was surgically reduced during laparotomy (66). For decades OWR was the only treatment for PCOS as it effectively restored regular menstrual cyclicity for a period of time in 80–90% of women with PCOS and allowed conception in 45–65% of those who underwent surgery (66). However, the procedure later became less popular due to significant side effects; primarily a significant incidence of postoperative pelvic adhesion formation adding to the infertility-issue (67–69). Furthermore, follow-up studies revealed that OWR was not always curative and a significant number of women, 30–35%, reverted into a state of anovulation or oligo-ovulation (69, 70). With the introduction of clomiphene citrate (CC) and human menopausal gonadotropins in the 1960s the surgical treatment of PCOS was less used, and today CC is used as the first-line therapy for ovulation induction in women with anovulatory PCOS (71, 72). However, 10–15% of women are CC-resistant and will not ovulate in response to CC, and in those who do respond, not all will conceive. Laparoscopic ovarian drilling (LOD) using a unipolar electrode is currently recommended as a successful second-line treatment for ovulation induction in women with CC-resistant PCOS, as it has been shown to be just as effective as gonadotropin treatment, but less expensive and not associated with an increased risk of multiple pregnancy, ovarian hyperstimulation syndrome (OHSS) or pregnancy loss (71, 73–75). In the 1980's the first studies on LOD showed that ovulation was restored in 92% of patients, with a pregnancy rate of up to 80% (76). Furthermore LOD allows multiple attempts of conception, but without intensive monitoring, and is preferred by the majority of patients (75). The main shortcomings of LOD are postoperative adhesion formations and the potential risk of affecting the ovarian reserve in case of excessive damage (75).

We therefore hypothesize that the development of a modern version of an ovarian resection in which ovarian biopsies are excised surgically during laparoscopy and subsequently frozen could potentially provide an alternative to traditional LOD and OWR in CC-resistant women with PCOS. In this case, the surgical intervention should be performed in a way in which whole pieces of cortical and medullary tissue is removed in amounts similar to what is destroyed during LOD, and the ovarian cortex could then be isolated and frozen to preserve the follicles for potential later use instead of wasting them when performing LOD or OWR. Thus, in cases were too much tissue is removed during the surgical procedure the stored ovarian tissue can be grafted as back-up, or potentially be used for cHRT if the patient later on enters menopause prematurely or for fertility at an advanced reproductive age. In 1970's it was incidentally noted that ovarian biopsy alone (taken for diagnostic purposes) could induce regular menstrual cyclicity and conception in some women with PCOS (77), and in 1980's OWR was also performed through multiple ovarian biopsies and reported successful using a laparoscopic approach with less adhesion formations (78). Like LOD the proposed modern version of an ovarian resection through excision of ovarian biopsies would restore regular ovulation and allow spontaneous pregnancy, and in patients who remain anovulatory following the procedure most of them would have an increased responsiveness of the ovary to CC-treatment or respond less aggressively to exogenous hormone stimulation. Taken together, the proposed procedure using OTC to advance a surgical treatment for PCOS patients is completely theoretical and has not been tested in a clinical setting, however, this approach could potentially utilize the excessive—and in this case harmful—follicle pool in a beneficial way by relieving PCOS symptoms immediately and at the same time securing ovarian endocrine function later on in life. The efficacy and advantages of the proposed procedure now needs to be proven and compared to LOD in a clinical setting to justify any potential use in the future.

OPTIMIZING CULTURE SYSTEMS FOR IMMATURE OOCYTES

By freezing ovarian cortical tissue for fertility preservation, it is possible to gain access to a wide range of immature oocytes, including early preantral and small antral stage follicles, which can be isolated and collected from surplus or donated ovarian tissues (62, 79). This provides a rare opportunity to characterize the basic molecular mechanisms controlling and regulating human follicular growth and maturation in unstimulated ovaries of fertile women (80, 81), and to develop and optimize culture conditions for immature oocytes (59, 62), which could provide additional fertility for young women with a wide range of indications. Thus, utilization of immature oocytes could in this way augment the overall fertility from the OTC procedure. Moreover, young cancer survivors with a risk of ovarian involvement from the underlying cancer cannot have their stored ovarian tissue transplanted safely, and current advances in culture systems for the earliest stages of follicles could potentially provide fertility for these women in the future. In a recent paper, Telfer et al. succeeded with *in vitro* growth of unilaminar follicles (IVG) in a multi-step culture model which supported the development and maturation to the Metaphase II stage (60).

Several studies have now shown that a considerable number of immature oocytes can be collected from small antral follicles visible on the surface of the ovary or released to the dissection medium during the preparation for OTC (62, 82–84). These oocytes can be matured, vitrified and used to augment fertility to the patient (62, 84–86). The maturation rate has been shown to vary between ∼30 and 60% (87), and the first three live births resulting from a cryopreserved embryo obtained from *in vitro* matured (IVM) oocytes has been reported (82, 88, 89). However, there is plenty of room for improvement and currently IVM is mainly used as an additional fertility preserving option in combination with OTC or to avoid OHSS in PCOS patient. A more widely use of IVM is currently not accepted due to the fact that implantation and developmental potential of embryos derived from IVM oocytes has consistently been reported to be significantly lower in comparison to *in vivo* matured oocytes (90, 91).

One explanation for the inefficiency of IVM is that IVM protocols have changed little, if any, since the first reported birth obtained by IVM oocytes in 1994 using a non-specific

medium (MEM) with addition of FSH, LH and estradiol as hormones and maternal serum as supplements (92). In recent reports, commercial maturation media have been used for IVM in both patients with PCOS and women undergoing fertility preservation, but there is no real evidence that the formula is appropriate for oocyte maturation *in vitro* (91). Thus, IVM in connection with OTC provides a unique platform to compare multiple culture media compositions and improve basal maturation conditions for IVM. Another explanation for the decreased implantation and pregnancy rate with the use of IVM derived embryos is insufficient endometrial receptivity. The limited time between oocyte recovery and embryo transfer is potentially insufficient to allow full completion of the endometrial proliferative phase, which could compromise the formation of a secretory endometrium and the chances of embryonic implantation (91). A study by De Vos et al. has shown that implantation and pregnancy rates were much higher in replacement cycles using frozen embryos obtained after IVM compared to cycles in which embryos were transferred fresh after IVM (93). Thus, the relatively lower clinical efficiency that currently characterizes IVM might derive not exclusively from reduced oocyte quality caused by inadequate IVM conditions, but also from inadequate endometrial receptivity. Therefore, the notion that IVM oocytes are intrinsically less developmentally competent will need to be reconsidered.

Despite the current limitations, IVM continue to attract growing interest in consideration of potential novel applications in human ART. However, to advance more sophisticated IVM systems able to reproduce more physiologically and efficiently the process of oocyte maturation, we need more research material and settings such as provided by OTC, in which we can gain a better understanding of the biological mechanisms transforming a fully-grown oocyte into a mature fertilizable gamete.

CONCLUSION

OTC in prepubertal girls and young women is becoming an increasingly well-established method of fertility preservation in many clinics worldwide. However, by storing ovarian tissue more than just the reproductive potential is preserved. The steroidogenic capacity and endocrine function of the tissue could potentially expand the utilization of OTC and the ovarian reserve to other beneficial applications throughout the female lifespan and several indications beyond cancer. With an increasing prevalence in loss of ovarian function due to POI and aging we now need to explore these opportunities and outweigh the cost-benefit and risk-benefit ratios associated with potential new treatments. Nonetheless, it is important to recognize that fertility and endocrine function restored with ovarian tissue go hand in hand and that both are the result of follicular activity.

In conclusion, the described and suggested applications for OTC in this review are technically possible, however they have not been validated clinically (with the exception of puberty induction), and therefore the suggested applications should be regarded as an optimistic view on the potential future use of OTC. Moreover, the suggested applications for OTC are thought provoking to many people and the controversial opinions hereof should of course be recognized. Finally, the burden of repeated surgical interventions in connection with OTC must in all cases be weighed against the simple, cheap, and readily available pHRT medications which already exist and are administered to millions of women worldwide.

AUTHOR CONTRIBUTIONS

SK and CA both contributed to the conception and writing of this paper.

ACKNOWLEDGMENTS

The Research Pools of Rigshospitalet, The ReproUnion network, and the Novo Nordisk foundation is thanked for financial support.

REFERENCES

1. Gellert SE, Pors SE, Kristensen SG, Bay-Bjørn AM, Ernst E, Yding Andersen C. Transplantation of frozen-thawed ovarian tissue: an update on worldwide activity published in peer-reviewed papers and on the Danish cohort. *J Assist Reprod Genet.* (2018) 35:561–70. doi: 10.1007/s10815-018-1144-2
2. Donnez J, Dolmans MM. Fertility preservation in women. *N Engl J Med.* (2017) 377:1657–65. doi: 10.1056/NEJMra1614676
3. Emre SY, Balik KI, Bauthan O. Ovarian aging and premature ovarian failure. *J Turk Ger Gynecol Assoc.* (2014) 15:190–6. doi: 10.5152/jtgga.2014.0022
4. Fenton AJ. Premature ovarian insufficiency: pathogenesis and management. *J Midlife Health* (2015) 6:147–53. doi: 10.4103/0976-7800.172292
5. Nelson LM. Clinical practice. primary ovarian insufficiency. *N Engl J Med.* (2009) 360:606–14. doi: 10.1056/NEJMcp0808697
6. Tao XY, Zuo AZ, Wang JQ, Tao FB. Effect of primary ovarian insufficiency and early natural menopause on mortality: a meta-analysis. *Climacteric* (2016) 19:27–36. doi: 10.3109/13697137.2015.1094784
7. Eastell R. Management of osteoporosis due to ovarian failure. *Med Pediatr Oncol.* (2003) 41:222–7. doi: 10.1002/mpo.10341
8. Rocca WA, Grossardt BR, Shuster LT. Oophorectomy, estrogen, and dementia: a 2014 update. *Mol Cell Endocrinol.* (2014) 389:7–12. doi: 10.1016/j.mce.2014.01.020
9. Clarkson TB, Melendez GC, Appt SE. Timing hypothesis for postmenopausal hormone therapy: its origin, current status, and future. *Menopause* (2013) 20:342–353. doi: 10.1097/GME.0b013e3182843aad
10. Jensen AK, Kristensen SG, Macklon KT, Jeppesen JV, Fedder J, Ernst E, et al. Outcomes of transplantations of cryopreserved ovarian tissue to 41 women in Denmark. *Hum Reprod.* (2015) 30:2838–45. doi: 10.1093/humrep/dev230
11. Donnez J, Dolmans MM, Pellicer A, Diaz-Garcia C, Sanchez Serrano M, Schmidt KT, et al. Restoration of ovarian activity and pregnancy after transplantation of cryopreserved ovarian tissue: a review of 60 cases of reimplantation. *Fertil Steril.* (2013) 99:1503–13. doi: 10.1016/j.fertnstert.2013.03.030

12. Andersen CY, Silber SJ, Bergholdt SH, Jorgensen JS, Ernst E. Long duration of function of ovarian tissue transplants: case reports. *Reprod. Biomed. Online* (2012) 25:128–132. doi: 10.1016/j.rbmo.2012.03.014
13. Donnez J, Dolmans MM (2015) Ovarian tissue freezing: current status. *Curr Opin Obstet Gynecol.* 27:222–30. doi: 10.1097/GCO.0000000000000171
14. Qin Y, Jiao X, Simpson JL, Chen ZJ. Genetics of primary ovarian insufficiency: new developments and opportunities. *Hum Reprod Update* (2015) 21:787–808. doi: 10.1093/humupd/dmv036
15. Fish JD. Part 1: hormone replacement for survivors of childhood cancer with ovarian failure–when is it worth the risk? *J Pediatr Adolesc Gynecol.* (2011) 24:98–101. doi: 10.1016/j.jpag.2010.11.001
16. Gore L, DeGregori J, Porter CC. Targeting developmental pathways in children with cancer: what price success? *Lancet Oncol.* (2013) 14:e70–8. doi: 10.1016/S1470-2045(12)70530-2
17. Poirot C, Abirached F, Prades M, Coussieu C, Bernaudin F, Piver P. Induction of puberty by autograft of cryopreserved ovarian tissue. *Lancet* (2012) 379:588. doi: 10.1016/S0140-6736(11)61781-9
18. Ernst E, Kjærsgaard M, Birkebæk NH, Clausen N, Andersen CY. Stimulation of puberty in a girl with chemo- and radiation therapy induced ovarian failure by transplantation of a small part of her frozen/thawed ovarian tissue. *Eur J Cancer* (2013) 49:911–4. doi: 10.1016/j.ejca.2012.09.028
19. Anderson RA, Hindmarsh PC, Wallace WH. Letter to the editor: induction of puberty by autograft of cryopreserved ovarian tissue in a patient previously treated for Ewing sarcoma. *Eur J Cancer* (2013) 49:2960–1. doi: 10.1016/j.ejca.2013.04.031
20. van Dijk EM, van Dulmen-den Broeder E, Kaspers GJ, van Dam EW, Braam KI, Huisman J. Psychosexual functioning of childhood cancer survivors. *Psychooncology* (2008) 17:506–11. doi: 10.1002/pon.1274
21. Zebrack BJ, Foley S, Wittmann D, Leonard M. Sexual functioning in young adult survivors of childhood cancer. *Psychooncology* 2010; 19:814–22. doi: 10.1002/pon.1641
22. Rossouw JE, Anderson GL, Prentice RL, LaCroix AZ, Kooperberg C, Stefanick ML, et al. Risks and benefits of estrogen plus progestin in healthy postmenopausal women: principal results from the women's health initiative randomized controlled trial. *JAMA* (2002) 288:321–33. doi: 10.1001/jama.288.3.321
23. Buist DS, Newton KM, Miglioretti DL, Beverly K, Connelly MT, Andrade S, et al. Hormone therapy prescribing patterns in the United States. *Obstet Gynecol.* (2004) 104(5 Pt 1):1042–50. doi: 10.1097/01.AOG.0000143826.38439.af
24. Hersh AL, Stefanick ML, Stafford RS. National use of postmenopausal hormone therapy: annual trends and response to recent evidence. *JAMA* (2004) 291:47–53. doi: 10.1001/jama.291.1.47
25. Nachtigall LE, Nachtigall RH, Nachtigall RD, Beckman EM. Estrogen replacement therapy. II. A prospective study in the relationship between carcinoma and cardiovascular and metabolic problems. *Obstet Gynecol.* (1979) 54:74–9. doi: 10.1097/00006250-197907000-00017
26. Stampfer MJ, Willett WC, Colditz GA, Rosner B, Speizer FE, Hennekens CH. A prospective study of postmenopausal estrogen therapy and coronary heart disease. *N Engl J Med.* (1985) 313:1044–49. doi: 10.1056/NEJM198510243131703
27. Petitti DB, Perlman JA, Sidney S. Noncontraceptive estrogens and mortality: long-term follow-up of women in the Walnut Creek Study. *Obstet Gynecol.* (1987) 70:289–93.
28. Paganini-Hill A, Ross RK, Henderson BE. Postmenopausal oestrogen treatment and stroke: a prospective study. *BMJ* (1988) 297:519–22. doi: 10.1136/bmj.297.6647.519
29. Falkeborn M, Persson I, Adami HO, Bergström R, Eaker E, Lithell H, et al. The risk of acute myocardial infarction after oestrogen and oestrogen-progestin replacement. *Br J Obstet Gynaecol.* (1992) 99:821–28. doi: 10.1111/j.1471-0528.1992.tb14414.x
30. Grodstein F, Manson JE, Colditz GA, Willett WC, Speizer FE, Stampfer MJ. A prospective, observational study of postmenopausal hormone therapy and primary prevention of cardiovascular disease. *Ann Intern Med.* (2000) 133:933–41. doi: 10.7326/0003-4819-133-12-200012190-00008
31. Lobo RA. Hormone-replacement therapy: current thinking. *Nat Rev Endocrinol.* (2017) 13:220–31. doi: 10.1038/nrendo.2016.164
32. Maclaran K, Stevenson JC. Primary prevention of cardiovascular disease with HRT. *Womens Health* (2012) 8:63–74. doi: 10.2217/WHE.11.87
33. Clarkson TB. The new conundrum: do estrogens have any cardiovascular benefits? *Int. J. Fertil. Womens Med.* (2002) 47, 61–68.
34. Rossouw JE, Prentice RL, Manson JE, Wu L, Barad D, Barnabei VM, et al. Postmenopausal hormone therapy and cardiovascular disease by age and years since menopause. *JAMA* (2007) 297:1465–77. doi: 10.1001/jama.297.13.1465
35. Henderson VW, Benke KS, Green RC, Cupples LA, Farrer LA. Postmenopausal hormone therapy and Alzheimer's disease risk: interaction with age. *J Neurol Neurosurg Psychiatry* (2005) 76:103–5. doi: 10.1136/jnnp.2003.024927
36. Henderson BE, Paganini-Hill A, Ross RK. Decreased mortality in users of estrogen replacement therapy. *Arch Intern Med.* (1991) 151:75–8. doi: 10.1001/archinte.1991.00400010095012
37. Grady D, Rubin SM, Petitti DB, Fox CS, Black D, Ettinger B, et al. Hormone therapy to prevent disease and prolong life in postmenopausal women. *Ann Intern Med.* (1992) 117:1016–37. doi: 10.7326/0003-4819-117-12-1016
38. Manson JE, Aragaki AK, Rossouw JE, Anderson GL, Prentice RL, LaCroix AZ et al. Menopausal hormone therapy and long-term all-cause and cause-specific mortality: the women's health initiative randomized trials. *JAMA* (2017) 318:927–8. doi: 10.1001/jama.2017.11217
39. Manson JE, Chlebowski RT, Stefanick ML, Aragaki AK, Rossouw JE, Prentice RL, et al. Menopausal hormone therapy and health outcomes during the intervention and extended poststopping phases of the women's health initiative randomized trials. *JAMA* (2013) 310:1353–68. doi: 10.1001/jama.2013.278040
40. Andersen CY, Kristensen SG. Novel use of ovarian follicular pool to postpone menopause and delay osteoporosis. *Reprod Biomed Online* (2015) 31:128–31. doi: 10.1016/j.rbmo.2015.05.002
41. Sittadjody S, Saul JM, McQuilling JP, Joo S, Register TC, Yoo JJ, et al. *In vivo* transplantation of 3D encapsulated ovarian constructs in rats corrects abnormalities of ovarian failure. *Nat Commun.* (2017) 8:1858. doi: 10.1038/s41467-017-01851-3
42. Rivera CM, Grosshardt BR, Rhodes DY, Brown RD Jr, Roger VL, Melton LJ 3rd, et al. Increased cardiovascular mortality after early bilateral oophorectomy. *Menopause* (2009) 16:15–23. doi: 10.1097/gme.0b013e31818888f7
43. Parker WH, Feskanich D, Broder MS, Chang E, Shoupe D, Farquhar CM, et al. Long-term mortality associated with oophorectomy compared with ovarian conservation in the Nurses Health Study. *Obstet Gynecol.* (2013) 121:709–16. doi: 10.1097/AOG.0b013e3182864350
44. Salpeter SR, Cheng J, Thabane L, Buckley NS, Salpeter EE. Bayesian meta-analysis of hormone therapy and mortality in younger postmenopausal women. *Am J Med.* (2009) 122:1016–22. doi: 10.1016/j.amjmed.2009.05.021
45. Schierbeck LL, Rejnmark L, Tofteng CL, Stilgren L, Eiken P, Mosekilde L, et al. Effect of hormone replacement therapy on cardiovascular events in recently postmenopausal women: randomised trial. *BMJ* (2012) 345:e6409. doi: 10.1136/bmj.e6409
46. Savolainen-Peltonen H, Tuomikoski P, Korhonen P, Hoti F, Vattulainen P, Gissler M, et al. Cardiac death risk in relation to the age at initiation or the progestin component of hormone therapies. *J Clin Endocrinol Med.* (2016) 101:2794–801. doi: 10.1210/jc.2015-4149
47. Rocca WA, Bower JH, Maraganore DM, Ahlskog JE, Grossardt BR, de Andrade M, et al. Increased risk of cognitive impairment or dementia in women who underwent oophorectomy before menopause. *Neurology* (2007) 69:1074–83. doi: 10.1212/01.wnl.0000276984.19542.e6
48. Laronda MM, Rutz AL, Xiao S, Whelan KA, Duncan FE, Roth EW, et al. A bioprosthetic ovary created using 3D printed microporous scaffolds restores ovarian function in sterilized mice. *Nat Commun.* (2017) 8:15261. doi: 10.1038/ncomms15261
49. Day JR, David A, Cichon AL, Kulkarni T, Cascalho M, Shikanov A. Immunoisolating poly(ethylene glycol) based capsules support ovarian tissue survival to restore endocrine function. *J Biomed Mater Res A.* (2018) 106:1381–89. doi: 10.1002/jbm.a.36338
50. Hammarberg K, Clarke VE. Reasons for delaying childbearing—a survey of women aged over 35 years seeking assisted reproductive technology. *Aust Fam Phys.* (2005) 34:187–9.
51. Holton S, Fisher J, Rowe H. To have or not to have? Australian women's childbearing desires, expectations and outcomes. *J Popul Res.* (2011) 28:353–79. doi: 10.1007/s12546-011-9072-3

52. Hodes-Wertz B, Druckenmiller S, Smith M, Noyes N. What do reproductive-age women who undergo oocyte cryopreservation think about the process as a means to preserve fertility? *Fertil Steril* (2013) 100:1343–49. doi: 10.1016/j.fertnstert.2013.07.201
53. Baldwin K, Culley L, Hudson N, Mitchell H, Lavery S. Oocyte cryopreservation for social reasons: demographic profile and disposal intentions of UK users. *RBMO* (2015) 31:239–45. doi: 10.1016/j.rbmo.2015.04.010
54. Stoop D, Maes E, Polyzos NP, Verheyen G, Tournaye H, Nekkebroeck J. Does oocyte banking for anticipated gamete exhaustion influence future relational and reproductive choices? A follow-up of bankers and nonbankers. *Hum Reprod.* (2015) 30:338–44. doi: 10.1093/humrep/deu317
55. Hammarberg K, Kirkman M, Pritchard N, Hickey M, Peate M, McBain J, et al. Reproductive experiences of women who cryopreserved oocytes for non-medical reasons. *Hum Reprod.* (2017) 32:575–81. doi: 10.1093/humrep/dew342
56. Mesen TB, Mersereau JE, Kane JB, Steiner AZ. Optimal timing for elective egg freezing. *Fertil Steril.* (2015) 103:1551–56. doi: 10.1016/j.fertnstert.2015.03.002
57. Cobo A, Garcia-Velasco JA. Why all women should freeze their eggs. *Curr Opin Obstet Gynecol.* (2016) 28:206–10. doi: 10.1097/GCO.0000000000000269
58. Kawamura K, Cheng Y, Suzuki N, Deguchi M, Sato Y, Takae S, et al. Hippo signaling disruption and AKT stimulation of ovarian follicles for infertility treatment. *Proc Natl Acad Sci USA.* (2013) 110:17474–79. doi: 10.1073/pnas.1312830110
59. Yin H, Kristensen SG, Jiang H, Rasmussen A, Andersen CY. Survival and growth of isolated pre-antral follicles from human ovarian medulla tissue during long-term 3D culture. *Hum Reprod.* (2016) 31:1531–9. doi: 10.1093/humrep/dew049
60. McLaughlin M, Albertini DF, Wallace WHB, Anderson RA, Telfer EE. Metaphase II oocytes from human unilaminar follicles grown in a multi-step culture system. *Mol Hum Reprod.* (2018) 24:135–42. doi: 10.1093/molehr/gay002
61. Kristensen SG, Pors SE, Andersen CY. Improving oocyte quality by transfer of autologous mitochondria from fully grown oocytes. *Hum Reprod.* (2017) 32:725–32. doi: 10.1093/humrep/dex043
62. Yin H, Jiang H, Kristensen SG, Andersen CY. Vitrification of *in vitro* matured oocytes collected from surplus ovarian medulla tissue resulting from fertility preservation of ovarian cortex tissue. *J Assist Reprod Genet.* (2016) 33:741–6. doi: 10.1007/s10815-016-0691-7
63. Suzuki N, Yoshioka N, Takae S, Sugishita Y, Tamura M, Hashimoto S, et al. Successful fertility preservation following ovarian tissue vitrification in patients with primary ovarian insufficiency. *Hum Reprod.* (2015) 30:608–15. doi: 10.1093/humrep/deu353
64. Kawamura K, Cheng Y, Sun YP, Zhai J, Diaz-Garcia C, Simon C, et al. Ovary transplantation: to activate or not to activate. *Hum Reprod.* (2015) 30:2457–60. doi: 10.1093/humrep/dev211
65. Zhai J, Yao G, Dong F, Bu Z, Cheng Y, Sato Y, et al. *In vitro* activation of follicles and fresh tissue autotransplantation in primary ovarian insufficiency patients. *J Clin Endocrinol Metab.* (2016) 101:4405–12. doi: 10.1210/jc.2016-1589
66. Stein IF, Leventhal ML. Amenorrhea associated with bilateral polycystic ovaries. *Am J Obstet Gynecol.* (1935) 29:181–91. doi: 10.1016/S0002-9378(15)30642-6
67. Kistner RW. Peri-tubal and peri-ovarian adhesions subsequent to wedge resection of the ovaries. *Fertil Steril.* (1969) 20:35. doi: 10.1016/S0015-0282(16)36902-3
68. Buttram VC, Vaquero C. Post ovarian wedge resection adhesive disease. *Fertility Sterility* (1975) 26:874. doi: 10.1016/S0015-0282(16)41351-8
69. Toaff R, Toaff ME, Peyser MR. Infertility following wedge resection of the ovaries. *Am J Obst Gynecol.* (1976) 124:92. doi: 10.1016/0002-9378(76)90018-1
70. Adashi EY, Rock JE, Guzick D, Wentz AC, Jones GS, Jones HW Jr. Fertility following bilaterial ovarian wedge resection: a critical analysis of 90 consecutive cases of polycystic ovary syndrome. *Fertil Steril* (1981) 36:320. doi: 10.1016/S0015-0282(16)45732-8
71. Thessaloniki ESHRE/ASRM-Sponsored PCOS Consensus Workshop Group. Consensus on infertility treatment related to polycystic ovary syndrome. *Fertil Steril.* (2008) 89:505–22. doi: 10.1016/j.fertnstert.2007.09.041
72. Brown J, Farquhar C, Beck J, Boothroyd C, Hughes E. Clomiphene and anti-oestrogens for ovulation induction in PCOS. *Cochrane Database Syst Rev.* (2009) 4:CD002249. doi: 10.1002/14651858.CD002249.pub4
73. Amer SAK. Laparoscopic ovarian surgery for polycystic ovarian syndrome. In: Dunlop W, Ledger WL, editors. *Recent Advances in Obstetrics and Gynaecology, 24th Edn*. London: Royal Society of Medicine Press Ltd. (2008). p. 227–43.
74. Flyckt RL, Goldberg JM. Laparoscopic ovarian drilling for clomiphene-resistant polycystic ovary syndrome. *Semin Reprod Med.* (2011) 29:138–46. doi: 10.1055/s-0031-1272476
75. Abu Hashim H, Al-Inany H, De Vos M, Tournaye H. Three decades after Gjönnaess's laparoscopic ovarian drilling for treatment of PCOS; what do we know? An evidence-based approach. *Arch Gynecol Obstet.* (2013) 288:409–22. doi: 10.1007/s00404-013-2808-x
76. Gjönnaess H (1984) Polycystic ovarian syndrome treated by ovarian electrocautery through the laparoscope. *Fertil Steril.* 41:20–5. doi: 10.1016/S0015-0282(16)47534-5
77. Yuzpe AA, Rioux J-E (1975) The value of laparoscopic ovarian biopsy. *J Repr Med.* 15:57.
78. Campo S, Garcea N, Caruso A, Siccardi P. Effect of celioscopic ovarian resection in patients with polycystic ovaries. *Gynecol Obstet Invest.* (1983) 15:213–22. doi: 10.1159/000299413
79. Kristensen SG, Rasmussen A, Byskov AG, Andersen CY. Isolation of pre-antral follicles from human ovarian medulla tissue. *Hum Reprod.* (2011) 26:157–66. doi: 10.1093/humrep/deq318
80. Kristensen SG, Andersen K, Clement CA, Franks S, Hardy K, Andersen CY. Expression of TGF-beta superfamily growth factors, their receptors, the associated SMADs and antagonists in five isolated size-matched populations of pre-antral follicles from normal human ovaries. *Mol Hum Reprod.* (2014) 20:293–308. doi: 10.1093/molehr/gat089
81. Kristensen SG, Ebbesen P, Andersen CY. Transcriptional profiling of five isolated size-matched stages of human preantral follicles. *Mol Cell Endocrinol.* (2015) 401:189–201. doi: 10.1016/j.mce.2014.12.012
82. Segers I, Mateizel I, Van Moer E, Smitz J, Tournaye H, Verheyen G, et al. *In vitro* maturation (IVM) of oocytes recovered from ovariectomy specimens in the laboratory: a promising "*ex vivo*" method of oocyte cryopreservation resulting in the first report of an ongoing pregnancy in Europe. *J Assist Reprod Genet.* (2015) 32:1221–31. doi: 10.1007/s10815-015-0528-9
83. Escriba MJ, Grau N, Escrich L, Novella-Maestre E, Sánchez-Serrano M. Spontaneous *in vitro* maturation of oocyte prior to ovarian tissue cryopreservation in natural cycles of oncologic patients. *J Assist Reprod Genet.* (2012) 29:1261–5. doi: 10.1007/s10815-012-9860-5
84. Imesch P, Scheiner D, Xie M, Fink D, Macas E, Dubey R, et al. Developmental potential of human oocytes matured *in vitro* followed by vitrification and activation. *J Ovarian Res.* (2013) 6:30. doi: 10.1186/1757-2215-6-30
85. Fasano G, Moffa F, Dechene J, Englert Y, Demeestere I. Vitrification of *in vitro* matured oocytes collected from antral follicles at the time of ovarian tissue cryopreservation. *Reprod Biol Endocrinol.* (2011) 9:150. doi: 10.1186/1477-7827-9-150
86. Kedem A, Yerushalmi GM, Brengauz M, Raanani H, Orvieto R, Hourvitz A, et al. Outcome of immature oocytes collection of 119 cancer patients during ovarian tissue harvesting for fertility preservation. *J Assist Reprod Genet.* (2018) 35:851–56. doi: 10.1007/s10815-018-1153-1
87. Shirasawa H, Terada Y. *In vitro* maturation of human immature oocytes for fertility preservation and research material. *Reprod Med Biol.* (2017) 16:258–67. doi: 10.1002/rmb2.12042
88. Prasath EB, Chan ML, Wong WH, Lim CJ, Tharmalingam MD, Hendricks M, et al. First pregnancy and live birth resulting from cryopreserved embryos obtained from *in vitro* matured oocytes after oophorectomy in an ovarian cancer patient. *Hum Reprod.* (2014) 29:276–78. doi: 10.1093/humrep/det420
89. Uzelac PS, Delaney AA, Christensen GL, Bohler HC, Nakajima ST. Live birth following *in vitro* maturation of oocytes retrieved from extracorporeal ovarian tissue aspiration and embryo cryopreservation for 5 years. *Fertil Steril.* (2015) 104:1258–60. doi: 10.1016/j.fertnstert.2015.07.1148
90. Gremeau AS, Andreadis N, Fatum M, Craig J, Turner K, McVeigh E, et al. *In vitro* maturation or *in vitro* fertilization for women with polycystic ovaries? A case-control study of 194 treatment cycles. *Fertil Steril.* (2012) 98:355–60. doi: 10.1016/j.fertnstert.2012.04.046

91. Coticchio G, Dal-Canto M, Guglielmo MC, Mignini-Renzini M, Fadini R. Human oocyte maturation *in vitro*. *Int J Dev Biol.* (2012) 56:909–18. doi: 10.1387/ijdb.120135gv
92. Trounson A, Wood C, Kausche A. *In vitro* maturation and the fertilization and developmental competence of oocytes recovered from untreated polycystic ovarian patients. *Fertil Steril.* (1994) 62:353–62. doi: 10.1016/S0015-0282(16)56891-5
93. De Vos M, Ortega-hrepich C, Albuz FK, Guzman L, Polyzos NP, Smitz J, et al. Clinical outcome of non-hCG-primed oocyte *in vitro* maturation treatment in patients with polycystic ovaries and polycystic ovary syndrome. *Fertil Steril.* (2011) 96:860–64. doi: 10.1016/j.fertnstert.2011.07.1108

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Endocrinology

REVIEW
published: 29 June 2018
doi: 10.3389/fendo.2018.00345

Neuropeptide G Protein-Coupled Receptors as Oncotargets

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Neuropeptide G protein-coupled receptors (GPCRs) are overexpressed on numerous cancer cells. In a number of tumors, such as small cell lung cancer (SCLC), bombesin (BB) like peptides and neurotensin (NTS) function as autocrine growth factors whereby they are secreted from tumor cells, bind to cell surface receptors and stimulate growth. BB-drug conjugates and BB receptor antagonists inhibit the growth of a number of cancers. Vasoactive intestinal peptide (VIP) increases the secretion rate of BB-like peptide and NTS from SCLC leading to increased proliferation. In contrast, somatostatin (SST) inhibits the secretion of autocrine growth factors from neuroendocrine tumors (NETs) and decreases proliferation. SST analogs such as radiolabeled octreotide can be used to localize tumors, is therapeutic for certain cancer patients and has been approved for four different indications in the diagnosis/treatment of NETs. The review will focus on how BB, NTS, VIP, and SST receptors can facilitate the early detection and treatment of cancer.

Keywords: cancer GPCR, cancer RTK, bombesin, neurotensin, vasoactive intestinal peptide, pituitary adenylate cyclase activating polypeptide (PACAP), somatostatin, cancer signal transduction

OPEN ACCESS

Edited by:
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Reviewed by:
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Carsten Grötzinger, Charité Universitätsmedizin Berlin, Germany

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Specialty section:
This article was submitted to Systems and Translational Endocrinology, a section of the journal Frontiers in Endocrinology

Received: *15 May 2018*
Accepted: *11 June 2018*
Published: *29 June 2018*

Citation:
Moody TW, Ramos-Alvarez I and Jensen RT (2018) Neuropeptide G Protein-Coupled Receptors as Oncotargets. Front. Endocrinol. 9:345. doi: 10.3389/fendo.2018.00345

INTRODUCTION

G protein-coupled receptors (GPCRs) have 7 transmembrane (T M) domains and they interact with G proteins comprised of α, β, and γ subunits (1). The activated GPCRs undergoes a conformation change dissociating the G-protein into a GTP-bound α subunit and β, γ dimer. GPCRs for bombesin (BB) and neurotensin (NTS) interact with Gq/11, whereas receptors for vasoactive intestinal peptide (VIP) interact with Gs and somatostatin (SST) receptors interact with Gi/0 (2). BB and NT receptors cause phosphatidylinositol (PI) turnover resulting in the elevation of cytosolic Ca^{2+} and activation of protein kinase (PK) C. VIP receptors activate adenylyl cyclase resulting in elevated cAMP whereas SST receptors reduce the elevation of cAMP stimulation caused by VIP.

Neuropeptides modulate neural activity in the brain in a paracrine manner, however, they function as autocrine growth factors in cancer (3). BB, NTS, and VIP stimulate the growth of small cell lung cancer (SCLC) cells whereas SST inhibits growth (3). BB and the structurally related gastrin-releasing peptide (GRP) bind with high affinity to the GRP receptor or BB_2R; NTS binds high affinity to NTSR1; VIP binds with high affinity to VPAC1/VPAC2 and SST as well as octreotide/lanreotide bind with high affinity to SSTR2/SSTR5 but reduced affinity to SSTR1, SSTR3, and SSTR4. The agonist-GPCR complex is internalized and the GPCR recycle to the membrane but the peptide is metabolized in lysosomes.

Because cancers frequently over-express GPCRs, the cancer GPCRs can be used to deliver neuropeptide-drug conjugates into the cancer cell (4). In contrast, GPCR antagonists bind to the GPCR at the cell surface but are not internalized. PD176252 (GRPR antagonist) and SR48692 (NTSR1 antagonist) inhibit cancer growth (5–7). This review will focus on how neuropeptide GPCRs are oncotargets for the early detection and treatment of cancer.

GRPR, NEUROMEDIN B RECEPTOR AND BBR SUBTYPE 3

SCLC has high levels of the BB-like peptide GRP (8, 9). GRP is derived from a 148 amino acid prepropeptide (10). After removal of the N-terminal 23 amino acid signal sequence, pro-GRP$^{1-125}$ is metabolized by a prohormone convertase to GRP, which contains 27 amino acids and has an amidated C-terminal. GRP (proGRP$^{1-27}$) is readily metabolized in the blood but SCLC patients have elevated pro-GRP (11). Antibodies to proGRP$^{31-98}$ have been used to detect high concentrations of proGRP (>100 pg/ml) in the serum of patients with SCLC. Because proGRP is elevated in the serum of 71% of the SCLC patients it may be a biomarker for SCLC (12). BB or GRP, but not proGRP bind with high affinity to the GRPR. The C-terminal octapeptide of BB or GRP can be neutralized by mAb 2A11. mAb2A11 inhibits the growth of SCLC *in vitro* and in mouse models *in vivo* (13). In a clinical trial, 2A11 was well tolerated and one patient had SCLC remission whereas four patients had stable disease out of 13 patients treated (14). The results indicate that the GRP precursor may be a biomarker for SCLC.

Table 1 shows that the GRPR, which is localized to chromosome xp22, contains 384 amino acids and is a member of the class A/Rhodopsin-like GPCR (15, 16). The neuromedin B (NMB) R or BB_1R, which is localized to chromosome 6q24, contains 390 amino acids whereas BB receptor subtype-3 (BRS-3), which is localized to chromosome xq26, contains 399 amino acids. The NMBR and BRS-3 have about 50% sequence homology with the GRPR (17, 18). The GRPR binds GRP and NMB with high and low affinity, respectively. The NMBR binds GRP and NMB with low and high affinity, respectively. The orphan receptor BRS-3 binds both GRP and NMB with low affinity but MK5046 binds with high affinity (7). The universal agonist BA1, (D-Tyr^6, β-Ala^{11}, Phe ^{13}, Nle^{14}) BB$^{6-14}$, binds with high affinity to the GRPR, NMBR, and BRS-3. Numerous amino acids in TM domains 6 and 7 as well in extracellular loops (EL) 1, 2, and 3 of the GRPR are essential for high affinity binding of GRP (4). While the GPCRs of each family have a similar sequence, the pharmacological profile is different.

BB-drug conjugates were synthesized which are cytotoxic for lung cancer cells. The topoisomerase-1 inhibitor camptothecin (CPT) was coupled with a linker to the N-terminal of BA1. Surprisingly, the resulting CPT-L2-BA1 bound with higher affinity to the GRPR, NMBR, and BRS-3 than did BA1 (19). CPT-L2-BA1 was an agonist which increased PI turnover and was internalized. The CPT-L2-BA1 was metabolized in the lysosome leading to the release of CPT (20). Also, BB agonists have been coupled to paclitaxel (21), doxorubicin (22), marine toxins (23), magainin II (24), and siRNA to the GRPR (25) resulting in decreased cancer cellular proliferation. Doxorubicin was coupled to a GRPR antagonist and the resulting AN-215 was cytotoxic for gastric, colon, lung, ovarian, endometrial, breast, and pancreatic cancer (26). RC-3095, a GRPR antagonist, was tested in 25 patients with solid tumors. RC-3095 had minimal toxicity but a partial response was only seen in 1 patient (27). Unfortunately, these BB-drug conjugates will not only kill cancer cells, but normal cells with excessive BBR.

BBR antagonists were developed which inhibit the growth of cancer cells. Peptide antagonists such as RC-3095 or (Psi^{13,14}, Leu^{14})BB blocked the GRPR, and they inhibited the growth of cancer cells (27, 28). Small molecule antagonists such as PD168368 were synthesized which inhibit the growth of cancer cells which have NMBR (6). Also, bantag-1 is a peptide antagonist for BRS-3 (29). The BB receptor antagonists inhibited the growth of lung cancer cells *in vitro* and *in vivo* using nude mice bearing lung cancer xenografts. GRPR, NMBR, and BRS-3 mRNA was detected in 11/13 lung cancer cell lines (7). All lung cancer cell lines tested had at least 1 type of BBR and many cell lines had all 3 receptors.

In contrast, a high density of GRPR but not NMBR or BRS-3 were detected in most prostate and breast cancer cells (30). GRPR agonists were labeled with ^{111}In, ^{64}Cu, ^{99m}Tc, ^{68}Ga, ^{18}F for imaging studies. Using a ^{99m}Tc-BB$^{2-14}$ analog 14 prostatic lesions were visualized in patients (31). Using a ^{99m}Tc-RGD-BB analog, tumors were visualized in 6/6 breast cancer patients (32). Using a ^{64}Cu-BB$^{6-14}$ analog, tumors were visualized in 3 of 4 prostate cancer patients (33). It remains to be determined if the imaging of GRPR will be useful in the early detection of breast and/or prostate cancer.

Many of the growth effects of BB-like peptides on non-SCLC (NSCLC) cells may result from transactivation of receptor tyrosine kinases (RTK) such as the epidermal growth factor receptor (EGFR). Activation of the NMBR in NSCLC cells causes PI turnover leading to increased phosphorylation of the EGFR (**Figure 1**). Addition of NMB to NSCLC cells increases the tyrosine phosphorylation of the EGFR after 1 min leading to the tyrosine phosphorylation of ERK after 2 min (34). The transactivation of the EGFR that is regulated by the NMBR is inhibited by the tyrosine kinase inhibitor (TKI) gefitinib or the NMBR antagonist PD168368. The transactivation process in NSCLC cells is mediated by the EGFR ligand transforming growth factor (TGF)α (**Figure 1**). The inactive precursor pro-TGFα is metabolized by matrix metalloprotease (MMP) enzymes in the membrane to biologically active TGFα which is secreted and binds to the EGFR. The transactivation of the EGFR caused by addition of NMB to NSCLC cells is inhibited by GM6001 (MMP inhibitor) or anti-TGFα Ab. The transactivation process requires reactive oxygen species (ROS). Addition of N-acetyl cysteine (antioxidant) or tiron (superoxide scavenger) impaired the ability of NMB to increase EGFR tyrosine phosphorylation. The ROS may oxidize Cys^{773} of the EGFR increasing its tyrosine kinase activity and/ or oxidize protein tyrosine phosphatases (PTP) impairing their ability to metabolize phosphotyrosine (35, 36). The results indicate that GPCRs

TABLE 1 | Peptide GPCRs (human).

Receptor	GRPR	NMBR	BRS-3	NTSR1	NTSR2	VPAC1	VPAC2	PAC1
Chromosome	xp22	6q24	xq26	20q13	2p25	3p22	7q36	7p14
Amino acids	384	390	399	418	410	457	438	468
G-protein	Gq	Gq	Gq	Gq	Gq	Gs	Gs	Gs, Gq
Agonist	BB, GRP	NMB	MK5046	NTS	NTS	VIP	VIP	Maxidillin
	BA1	BA1	BA1	JMV449	Levocabastine	PACAP	PACAP	PACAP
						(Lys^{15}, Arg^{16}, Leu^{17})VIP$^{1-7}$ GRF$^{8-27}$	R025-1553	
Antagonist	RC3095 (Psi^{13,14}, Leu^{14})BB	PD168368	Bantag1	SR142948A	SR142948A	VIPhyb	VIPhyb	PACAP(6–38)
	PD176252			SR48692				

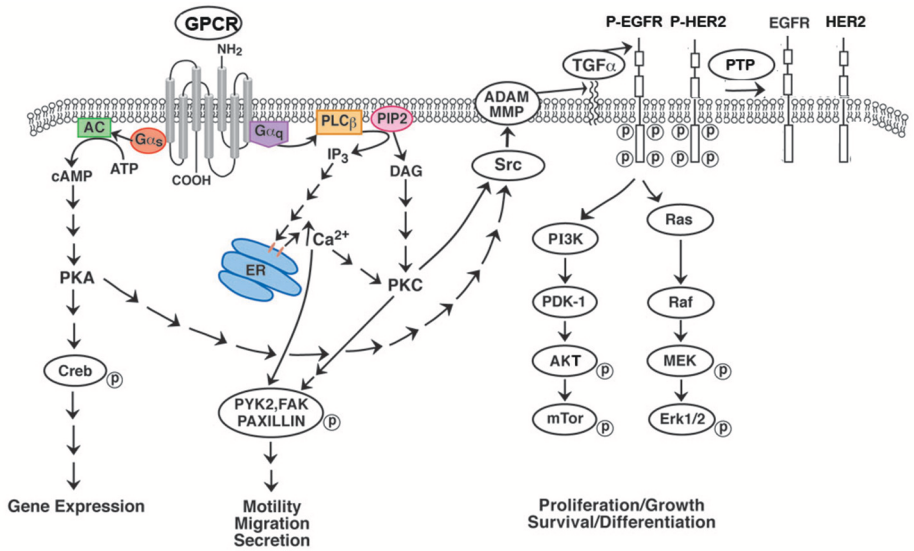

FIGURE 1 | Effect of GPCR's on RTK transactivation. GPCRs for BB and NTS couple to Gq and causes metabolism of PIP_2 to DAG (activates PKC) and IP_3 (elevates cytosolic Ca^{2+}). Addition of NTS or BB to NSCLC cells increases phosphorylation of PYK2, FAK or paxillin leading to increased cellular migration. GPCR for VIP interact with Gs activating adenylyl cyclase and increasing cAMP. The cAMP activates PKA leading to CREB phosphorylation and altered gene expression. GPCR for PACAP interact with both Gq and Gs. GPCR activate Src and MMP resulting in the production of EGFR ligands such as TGFα. When TGFα binds to the EGFR, tyrosine kinase activity is increased leading to phosphorylated EGFR homodimers or EGFR-HER2 heterodimers. The RTK activates the Ras-Raf-MEK-ERK pathway leading to increased cellular proliferation. The RTK activates the PI3K-PKD-AKT-mTOR pathway leading to increased cellular survival. The phosphorylated EGFR/HER2 is dephosphorylated by protein tyrosine phosphatase (PTP).

regulate the transactivation of receptor tyrosin kinases (RTKs) in NSCLC cells.

The EGFR contains 1,186 amino acids with a 621 and extracellular domains I and III bind EGF or TGFα with high affinity (37). The EGFR has a 23 amino acid TM domain and a 542 intracellular domain with tyrosine kinase activity. Lys^{721} is essential for binding ATP and the phosphorylation of protein substrates. Upon binding of ligand, the EGFR can undergo homodimerization resulting in the phosphorylation of Tyr^{1068}, Tyr^{1086}, Tyr^{1148}, and Tyr^{1174}. Alternatively, the EGFR can form heterodimers with HER2. The MAPK and PI3K/Akt pathways are downstream of the EGFR and are important for EGFR mediated proliferation and cancer cellular survival, respectively. Currently, we are investigating if GPCRs transactivate additional RTK such as HER2, HER3 or HER4 in cancer cells.

NMB increases the proliferation of NSCLC cells. In contrast, PD168368 and gefitinb inhibit the growth of NSCLC cells (34). Surprisingly, combinations of the NMBR antagonist with the EGFR TKI reduced the proliferation of NSCLC cells in a synergistic manner. The results indicate that GPCR antagonists potentiate the action of TKI in NSCLC. Traditionally NSCLC which kills 130,000 U.S. citizens annually is treated with combination chemotherapy, however, the 5 year survival rate is only 15%. The EGFR is mutated is approximately 13% of

the NSCLC patients and those with the L858R mutation have increased tyrosine kinase activity and sensitivity to TKI such as gefitinib or erlotinib (38, 39). Traditionally NSCLC patients are treated with combination chemotherapy, however, the 5 year survival rate is only 15% (40).

NEUROTENSIN RECEPTORS

NTS is present in numerous SCLC cell lines (9, 41). NTS is derived from a 170 amino acid precursor and metabolized to a biologically active peptide which contains 13 amino acids (42). NTS and its C-terminal fragment NTS^{8-13} bind with high affinity to the NTSR1, which is localized to chromosome 20q13, contains 418 amino acids, and is a class A/Rhodopsin-like GPCR. **Table 1** shows that the NTSR2 which is on chromosome 2p25 contains 410 amino acids and binds NTS and levocabastine with high affinity. The NTS^{-}-NTSR1 complex has been crystallized and NTS^{8-13} sits on top of the NTS binding pocket and interacts with TM domains 6 as well as 7 and EL 2 as well as 3 (43). Both NT and BB receptors have short N-terminals which have little effect on ligand binding (44). The nonpeptide NTSR1 antagonist SR48692 binds deep into the NTSR1 binding pocket and blocks the effects of NTS agonists. Also, SR142948A blocks both the NTSR1 and NTSR2. The NTSR3 is not a GPCR but is sortilin.

NTS binds with high affinity to SCLC cells (45). Addition of NTS to cancer cells causes PI turnover leading to increases PKC activity and elevation of cytosolic Ca^{2+} (46–48). In contrast, the NTSR2 agonist levocabastin has little effect on lung cancer cells. The effects of NTS on second messenger production and proliferation was antagonized by SR48692 (5). NTS addition to cancer cells causes phosphorylation of various proteins such as focal adhesion kinase (FAK) or ERK (49, 50). The phosphorylated ERK increases the expression of c-fos and c-jun leading to cellular proliferation (51). NTS stimulates proliferation whereas SR48692 inhibits the proliferation of lung cancer cells (5). NT addition to NSCLC cells increased EGFR tyrosine phosphorylation 5-fold (52). NT (5 nM) half-maximally increased EGFR transactivation after 2 min. NTS or NTS^{8-13} but not NT^{1-8} or levocabastine increase EGFR tyrosine phosphorylation. The NTSR1 regulation of EGFR transactivation is inhibited by SR48692, gefitinib, PP1, GM6001, TGFα antibodies and antioxidants. SR48692 and gefitinib inhibit the proliferation of NSCLC cells in a synergistic manner. Previously, JMV449, a NT^{8-13} analog, was found to increase expression of the EGFR, HER2, and HER3 after 24 h (53). JMV449 addition to cells increase MMP activity resulting in HB-EGF and neuregulin 1 release, which activates the EGFR and HER3, respectively.

NTSR1 regulates the EGFR transactivation in numerous cancers including colon, foregut neuroendocrine, lung, and prostate cancer (47, 52, 54, 55). Lung cancer and gastric cancer patients whose tumors had high densities of NTSR1 had decreased survival (53, 56). Addition of NTS to NSCLC cells caused tyrosine phosphorylation of the EGFR in a PLC-dependent manner (52). Phosphorylated β-catenin dissociates from E-cadherin and increases the expression of NTSR1. Wnt/β-catenin signaling increases the expression of E-cadherin leading to epithelial to mesenchymal transitions and cancer metastasis (57). Recently, 3BP-227, a SR142948A analog, was radiolabeled and used to image tumors containing NTSR1. In nude mice containing HT29 colon cancer tumors ^{177}Lu-3BP-227 localized to the tumors with high tumor-to-kidney or tumor-to-liver ratios using whole-body SPECT/CT techniques (58). In 5 out of 6 patients with ductal pancreatic adenocarcinoma tumor uptake of ^{177}Lu-3BP-227 was observed (59). It remains to be determined if ^{177}Lu-3BP-227 will improve survival of patients whose tumors are enriched in NTSR1.

VIPRs AND PITUITARY ADENYLATE CYCLASE ACTIVATING POLYPEPTIDE RECEPTOR

The biological activities of the VIP and PACAP family of peptides are mediated by 3 GPCR (VPAC1, VPAC2, and PAC1), which are members of the classB/secretin-like receptors (60). **Table 1** shows that VPAC1, which is localized to chromosome 3p22, contains 457 amino acids with a 112 amino acid N-terminal. VPAC2, which is localized to chromosome 7q36, contains 438 amino acids with a 103 amino acid N-terminal. PAC1, which is localized to chromosome 7p14, contains 468 amino acids with a 125 amino acid N-terminal. PAC1 has about 50% sequence homology with VPAC1 or VPAC2 (60). The large N-terminal extracellular domain of PAC1 has antiparallel β-sheets and binds to the C-terminal of PACAP (61, 62). The PAC1 receptor has 3 closed transitional states (G1-G3) and one open state named G4 (63). The N-terminal of PACAP, which activates PAC1 binds to EL and TM domains (64). VPAC1, VPAC2, and PAC1 interact with Gs resulting in elevated cAMP, however, PAC1 interacts with Gq as well resulting in PI turnover (65). VIP, which contains 28 amino acids, is derived from a 170 amino acid precursor protein. PACAP-27 as well as PACAP-38 is derived from a 176 amino acid precursor protein and 67% of the amino acids in PACAP-27 and VIP are identical (60). VPAC1 and VPAC2 binds VIP and PACAP-27 or PACAP-38 with high affinity, whereas PAC1 binds PACAP-27 or PACAP-38 with high affinity but VIP with low affinity. Maxidillin, a 61 amino acid peptide isolated from sand fly, binds with high affinity to PAC1 but not VPAC1 or VPAC2 (66). Recently, a number of PACAP-38 analogs were synthesized which prefer PAC1 relative to VPAC1 or VPAC2 by over an order of magnitude (67). VIPhybrid is a peptide antagonist which binds with moderate affinity to VPAC1 or VPAC2, whereas, PACAP(6–38) is a peptide antagonist for PAC1 (68). Selective non-peptide antagonists for VPAC1, VPAC2 or PAC1 remain unknown.

VPAC1 is present in numerous cancers including breast, colon, liver, lung, neuroblastoma, pancreatic, and prostate cancers in high densities (69). VPAC2 is present in moderate densities in gastric pancreatic adenocarcinomas, gastric leiomyomas, thyroid cancer, and sarcomas (70). PAC1 is present in brain, breast, colon lung, neuroendocrine, pancreatic, pituitary, and prostate cancer as well as neuroblastoma/pheochromocytoma (71). In SH1SY5Y neuroblastoma cells, numerous PAC1 splice variants (SV) were detected in the N-terminal and intracellular loop (IL)

3 (72). PAC1 has 18 exons and deletion of exons 5,6 or 4-6 reduce the N-terminal by 7, 21 (short) or 57 amino acids (very short) (73). The short PAC1 but not the very short PAC1 bind PACAP-38 with high affinity and elevate cAMP (74). Alternative splice variants (SV) of IL3 result in the addition of an additional 28 amino acid segment (hip) to PAC1null (75). Addition of a different set of 28 amino acids to IL3 of the PAC1 results in the hop SV. Finally, both SVs can be added resulting in PAC1hiphop. The order of potency to increase PI turnover was PAC1hop > PAC1null = PAC1hiphop > PAC1hip (76). Thus binding of PACAP and second messenger production can be altered by PAC1 deletions and SVs.

VPAC1 can be utilized to image cancer tumors. ^{18}F(Arg^{15,21})VIP localized to T47D breast cancer cells in nude mice (77) and ^{64}Cu-TP3982 localized to mammary tumors in MMTVneu transgenic mice (78). ^{99m}Tc-TP3982 was used to image breast tumors in 5 patients (79). The VPAC1-agonist complex internalizes in cancer cells and the ligand is metabolized in lysosomes. VPAC1 has been used to deliver VIP analogs containing cytotoxic CPT, paclitaxel, ellipticin or geldanomycin to cancer cells (80–83). The actions of VIP are antagonized by peptides such as VIP$^{10-28}$ or VIPhybrid (84). Addition of VIP to cancer cells results in elevated cAMP which activates PKA. Activation of PKA results in CREB phosphorylation which increases nuclear oncogene expression of c-Myc leading to increased proliferation (**Figure 1**). VIP increases the proliferation of lung cancer cells whereas VIPhybrid inhibits proliferation (84). Addition of PACAP-27 or PACAP-38 to lung cancer cells containing VPAC1, VPAC2 or PAC1 increases cAMP, however, it causes PI turnover in cells containing PAC1. When PI is metabolized, ERK becomes phosphorylated. Phosphorylated ERK increased the expression of the nuclear oncogenes c-fos and c-jun leading to increased cancer cellular proliferation. PACAP(6–38) inhibits the proliferation of lung cancer cells *in vitro* and *in vivo* (85).

VIP may be a promoter of carcinogenesis. VPAC1 density is higher in mammary cancer than adjacent normal tissue using rat and mouse models (86). Specific binding of ^{125}I-VIP to mouse mammary tumors was inhibited with high affinity by (Lys^{15}, Arg^{16}, Leu^{17}) VIP$^{1-7}$GRF$^{8-27}$ (VPAC1 peptide agonist) but not Ro25-1553 (VPAC2 peptide agonist). Retinoic acid, a chemopreventive agent, down-regulates VPAC1 expression in breast and lung cancer cells (87, 88). Finally VIPhybrid inhibits mammary carcinogenesis in C3(1)SV40T antigen mice (89).

Addition of PACAP-27 or PACAP-38 but not VIP causes transactivation of the EGFR in NSCLC cells (90). The PAC1 regulation of EGFR tyrosine phosphorylation is inhibited by PACAP(6–38), gefitinib, PP2, GM6001, and ROS inhibitors. Diphenyleneiodonium (DPI), a NADPH oxidase (NOX) inhibitor impaired the ability of PACAP to increase EGFR tyrosine phosphorylation. NOX-4, which produces ROS, is present in NSCLC cells (91). PACAP-27 addition to NSCLC cells increased ROS which was inhibited by DPI. VIP addition to breast cancer cells increased EGFR and HER2 phosphorylation (92). The EGFR which dimerizes, may form homodimers with itself or heterodimers with HER2. VPAC1 regulation of EGFR transactivation was blocked by JV-1-53 (VPAC1 antagonist), PP2 or H89 (PKA inhibitor). In contrast, the PAC1 regulation of EGFR transactivation in NSCLC cells was inhibited by U73122 (phospholipase C inhibitor) but not H89. The results indicate that the EGFR can be transactivated by GPCR which interact with Gq or Gs.

SST RECEPTORS

SST occurs endogenously in two principal forms (SST-14, SST-28) and their action is mediated by 5 related subtypes of GPCRs (SSTR1-5) (93, 94). SST receptors are not only widely expressed on normal tissues, but also are frequently overexpressed by many neoplasms, particularly NETs [i.e., carcinoids/pancreatic neuroendocrine tumors (panNETs)] (93–95). SST has a wide range of physiological actions and they are primarily inhibitory (93, 94).

SST and its receptors represent the prototype for a clinically successful peptide/peptide receptor oncotarget. It is the only peptide-GPCR system which has multiple approved indications (four different indications) for the treatment of a class of human neoplasms, NETs. Furthermore, its results in NETs have potential applicability for its clinical utility in a number of other neoplasms.

The initial approved indication for SST analogs was its use in hormone-excess states. Depot long-acting formulations of synthetic SST analogs (octreotide-LAR, lanreotide autogel) (**Figure 2**) are the drugs of choice to control various functional NET syndromes due to the ectopic release of a biologically active peptides by the NETs (93, 96–99). This includes the control of such widely different functional NET syndromes as the carcinoid syndrome (diarrhea, flushing) due to metastatic carcinoid tumor; severe diarrhea due to VIPomas; rash due to glucagonomas; acromegaly due to excessive growth hormone release primarily by pituitary adenomas, and a number of others (93, 96–100). Almost all (>90%) of the well-differentiated forms of these NETs (>95%) overexpress somatostatin receptor subtype 2 which has high affinity for octreotide and therefore it is generally effective in controlling the hormone-excess state and in many, their growth. Some NETs such as pituitary adenomas do not overexpress SSTR2 with the result that octreotide/lanreotide are only effective in 20–70% of patients (101). One solution to this has been the development of next generation somatostatin analogs such as pasireotide, which has high affinity for multiple subtypes (SSTR5 > SSTR2 > SSTR3 > SSTR1), which has been shown to be effective in these patients and is approved for use in the treatment of a acromegaly.

The second approved indication for SST analogs is for its anti-proliferative activity on NETs. In numerous pre-clinical studies and animal studies, it was shown that SST analogs have anti-proliferative effects on NETs, as well as number of other human tumors (102–107) Two double-blind Phase 3 studies (108, 109) in patients with advanced NETs treated with lanreotide/octreotide increased the patient's progressive free survival (PFS), which lead to FDA approval. Recent meta-analyses (106, 110) of SST analogs anti-proliferative effects in all publications (106) or the above two studies (110), in patients with advanced NETs, demonstrate

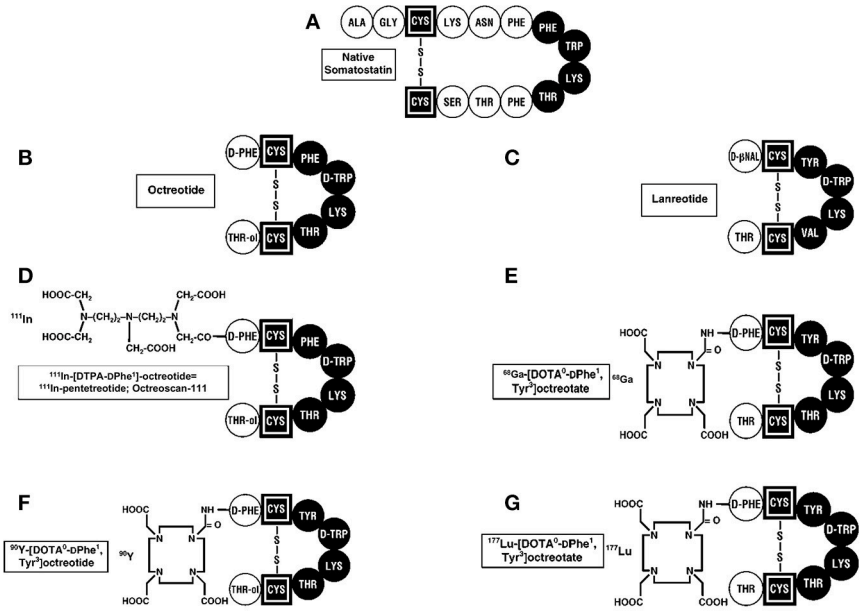

FIGURE 2 | Structures of SST and synthetic analogs used clinically. **(A)** The 14 amino acid SST is shown and essential amino acids are in black. The SST synthetic analogs, octreotide **(B),** and lanreotide **(C)**, which have 8 amino acids, are approved to treat patients with neuroendocrine tumors (NETs) producing hormone-excess states (VIPoma, carcinoid syndrome) and for their anti-proliferative activity in patients with advanced, aggressive NETs. ^{111}InDTPA pentetreotide **(D)** as well as ^{68}GaDOTATATE **(E)** are used for SSTR imaging in patients with NETs. ^{90}Y-DOTA **(F)** and ^{177}Lu-DOTA **(G)** -labeled SST analogs are used for their antitumor activity during PRRT by targeting the cytotoxic radiolabel to the tumor.

good anti-proliferative activity, significant benefit from their use resulting in disease control (HR 0.51, $p < 0.01$), with the response rates vs. placebo being 58 vs. 32% and a good safety profile. In general, these studies demonstrate that SST analog treatment in patients with advanced NETs result in a tumoristatic effect primarily, rather than a decrease in the tumor size. SST analogs are now recommended in recent guidelines as well as expert reviews as one of the initial treatments for controlling tumor growth in patients with advanced NETs, especially those with well-differentiated NETs, and slower growth rates (99, 111–115). Numerous *in vitro* and animal studies report that SST receptors are expressed on a number of other non-endocrine tumors, and that SST analogs have anti-proliferative activity in these tumors (93, 94, 97, 103, 104, 107, 116, 117). No controlled trials have established the use of SST analogs for anti-proliferative effects in patients with non-endocrine tumors.

The third approved indication for SST analogs in patients with NETs is for imaging of the tumor. SST receptor imaging (SRI) was originally approved for the use of ^{111}In-labeled pentetreotide with SPECT/CT scanning (Octreoscan), which is now replaced by the use of ^{68}Ga-DOTATATE PET/CT scanning, which has greater resolution, sensitivity and high specificity (118–122). Almost all well differentiated NETs overexpress the somatostatin receptor subtypes (SSTR2 > SSTR5 > SSTR3) that bind this radiolabeled agonist with high affinity (118, 119, 123). A systematic review (122) demonstrated that the use of ^{68}Ga-DOTATATE PET/CT scanning changed the management of the patient in a mean of 44% (range, 16–71%). SRI is now essential for the staging and management of NET patients and is the most sensitive method to allow whole body scanning rapidly to present a complete assessment of the extent of the tumor (112, 118, 121).

The fourth approved indication for SST analogs in patients with NETs is their use to target cytotoxic radiolabeled SST analogs to the tumor in patients with advanced NETs, as an anti-tumor therapy (called PRRT for peptide receptor radionuclide therapy) (124–127). Numerous animal studies as well as uncontrolled studies on patients with advanced NETs, demonstrated this approach resulted in tumor stabilization in progressive tumors as well tumor shrinkage in a significant number of patients with acceptable side-effects (124–127). Various SST analogs were coupled to linkers (DOTATATE, DOTATOC, DOTANOC) (**Figure 2**) and to different radiolabels including ^{111}Indium,^{90} Yttrium, and ^{177}Lutetium (127, 128). A recent double-blind controlled trial (129) demonstrated that ^{177}Lutetium-DOTATATE treatment in patients with advanced midgut carcinoids, resulted in marked increase in progression-free survival and a preliminary result demonstrating increased overall survival, with acceptable safely profile. This has led to FDA approval for this treatment in patients with advanced NETs. Almost all of the early studies performed with SRI and PRRT used SST analogs that were agonists because of the belief the peptide should be internalized to provide the best imaging and radionuclide delivery to the tumor. Recent studies demonstrate that SST receptor antagonists recognize more binding sites on the tumor, provide superior tumor targeting to agonists (118, 130, 131) and also demonstrate

greater membrane binding suggesting it will be superior for PRRT.

Unfortunately, the over-expression of SST receptors is limited to a subset of tumors and is not seen in many of the more frequent adenocarcinomas, such as breast, colon, lung or prostate and therefore the specific ligands developed for NETs will not be useful in these tumors. However, many of these other more common tumors over-express a number of other GPCRs including receptors for the BB, NTS, VIP/PACAP family (68, 132–134). Furthermore, in many cases selective ligands that are radiolabeled have been developed and studies in animal models and some cases in small numbers of humans with different diseases, support this approach (68, 132–134). Whether in the future they will become established for the imaging of these tumors or for the delivery of cytotoxic substances is unknown.

CONCLUSIONS

Neuropeptide GPCR may play an important role in cancer proliferation, angiogenesis, and metastasis. Most of the research conducted on NTS, BB, and VIP has been at the preclinical level. NTSR1 and BBR are class A/Rhodopsin-like receptors which interact with Gq and cause PI turnover. Nonpeptide antagonists are available for the BBR and NTSR which inhibit the proliferation of cancer cells. NTS, BB, and VIP conjugates, which kill cancer cells, have been developed. BB, NTS or PACAP stimulate the proliferation of NSCLC cells in an EGFR-dependent manner. The transactivation of the EGFR is blocked by SR48692 (NTSR1 antagonist), PD176252 (GRPR antagonist) or PACAP(6–38) (PAC1 antagonist) as well as gefitinib (EGFR TKI). The GPCR antagonists potentiate the ability of TKI to reduce NSCLC growth *in vitro*. It remains to be determined if GPCR antagonists will potentiate the action of TKI *in vivo*. GPCR antagonists potentiate the effects of chemotherapeutic drugs (135, 136). VIPhybrid, a VPAC1 antagonist, potentiates the effects cytotoxicity of taxol in breast cancer *in vitro* and *in vivo*. Also, VIPhybrid potentiated the cytotoxicity of cisplatin, doxorubicin, gemcitabine, irinotecan or vinorelbine on colon cancer *in vitro*.

VIP and PACAP are class B/Secretin-like receptors which interact with Gs and stimulate adenylyl cyclase. PAC1 has numerous splice variants, which alter second messenger production. VIP has been coupled to radioisotopes, e.g., ^{18}F, ^{65}Cu, and ^{99m}Tc to image tumors in animal models. High affinity non-peptide antagonists need to be developed for PAC1 VPAC1 and VPAC2. The use of peptide coated nanoparticles which contain chemotherapeutics is being investigated (137). The GPCR can be used to direct neuropeptide coated nanoparticle to the tumor. Recently, cholecystokinin antagonists were found to potentiate the effects of immune checkpoint inhibitors at impairing the growth of pancreatic tumors *in vivo* (138). Thus GPCR antagonists can potentiate the effects of various drugs in cancer treatment.

The use of SST analogs and their receptors are now an established part of clinical practice and provide the basis for 4 different FDA approved indications in patients with NETs. Long acting formulations of octreotide or lanreotide are the predominant therapeutic agents used to control excess secretion of peptides or growth hormone causing clinical syndromes in NET patients. Large clinical trials have recently led to the FDA approval of lanreotide/octreotide analogs to reduce NET growth in patients with advanced disease resulting in increased progression-free survival. SST receptor imaging using initially ^{111}In-pentetreotide, and more recently, ^{68}Ga-DOTATATE, which takes advance of the over-expression of SSTRs by NETs is the most sensitive method to image tumor location/extent in these patients. Lastly, numerous animal studies and non- prospective clinical studies, demonstrated that patients with advanced NETs expressing SSTRs could be treated with radiolabeled SST analogs with good antitumor effects. Recently, a large double -blind study in patients with advanced ileal carcinoid NETs confirmed this result leading to FDA approval for this approach using ^{177}Lu labeled octreotate. The SST research shows that neuropeptide GPCRs can be used as oncotargets to detect and treat a human cancer.

A goal is to advance the cancer research on BB, NTS, and VIP. SST is inhibitory in nature and was initially used to inhibit secretions from NETs. Few effective therapies were available and octreotide effectively controlled the symptoms of patients with VIPomas, glucagonomas, GRFomas, insulinomas or gastrinomas. In contrast, BB, NTS, and VIP are stimulatory in nature and a substantial effort has been made to improve the half-life of peptide agonists in the blood and develop specific high affinity antagonists which are stable. SSTRs are present in over 90% of the NETs. BB, NTS, and VIPRs are not so universal in epithelial cancers. By precision medicine, the overexpression of GRPCRs for BB, NTS or VIP in the biopsy specimen will dictate which GPCR should be targeted. To illustrate if the tumor overexpresses NTSR1, the patient may be treated with SR48692 alone or in combination with another drug. Additional clinical trials are needed in BB, NT, and VIP research so that these peptide GPCR's can be used as oncotargets to treat epithelial cancers of the breast, colon, lung, and prostate.

AUTHOR CONTRIBUTIONS

TM, IR-A and RJ are responsible for the writing of the manuscript and the figure preparation.

FUNDING

This research is supported by the intramural program of the NCI and NIDDK of the NIH.

ACKNOWLEDGMENTS

The authors thank Drs. D. Wink and T. Leto for helpful discussions.

REFERENCES

1. Almendro V, Garcia-Recio S, Gascon P. Tyrosine kinase receptor transactivation associated to Gprotein- coupled receptors. *Curr Drug Targets* (2010) 11:1160–80. doi: 10.2174/138945010792006807
2. Lappano R, Maggiolinin M. Pharmacotherpeutic targeting of G protein-coupled receptors in oncology: examples of approved therapies and emerging concepts. *Drugs* (2017) 77:951–65. doi: 10.1007/s40265-017-0738-9
3. Moody TW, Moreno P, Jensen RT. Neuropeptides as lung cancer growth factors. *Peptides* (2015) 72:106–11. doi: 10.1016/j.peptides.2015.03.018
4. Jensen RT, Battey JF, Spindel ER, Benya RV. Mammalian bombesin receptors: nomenclature, distribution, pharmacology, signaling and functions in normal and disease states. *Pharmacol Rev.* (2008) 60:1–42. doi: 10.1124/pr.107.07108
5. Moody TW, Chiles J, Casibang M, Moody E, Chan D, Davis TP. SR48692 is a neurotensin receptor antagonist which inhibits the growth of small cell lung cancer cells. *Peptides* (2001) 22:109–15. doi: 10.1016/S0196-9781(00)00362-4
6. Moody TW, Leyton J, Garcia L, Jensen RT. Nonpeptide gastrin releasing peptide receptor antagonists inhibit the proliferation of lung cancer cells. *Eur J Pharmacol.* (2003) 474:21–9. doi: 10.1016/S0014-2999(03)01996-4
7. Moreno P, Mantey SA, Lee SH, Ramos-Alvarez I, Moody TW, Jensen RT. A possible new target in lung cancer cells:The orphan receptor, bombesin receptor subtype-3. *Peptides* (2018) 101:213–26. doi: 10.1016/j.peptides.2018.01.016
8. Moody TW, Pert CB, Gazdar AF, Carney DN, Minna JD. High levels of intracellular bombesin characterize human small cell lung carcinoma. *Science* (1981) 214:1246–48. doi: 10.1126/science.6272398
9. Wood SM, Wood JR, Ghatei MA, Lee YC, O'Shaughnessy D, Bloom SR. Bombesin somatostatin and neurotensin-like immunoreactivity in bronchial carcinoma. *J Clin Endocrinol Metab.* (1981) 53:1310–14.
10. Yang HJ, Gu Y, Chen C, Xu C, Bao YX. Diagnostic value of pro-gastrin-releasing peptide for small cell lung cancer:A meta-analysis. *Clin Chem Lab Med.* (2011) 49:1039–48. doi: 10.1515/CCLM.2011.161
11. Korse CM, Taal BG, Bonfrer JM, Vincent A, val Velthuysen M, Baas P. An elevated progastrin releasing peptide level in patients with well-differentiated neuroendocrine tumours indicates a primary tumour in the lung and predicts a shorter survival. *Ann Oncol.* (2011) 22:2625–30. doi: 10.1093/annonc/mdr007
12. Wojcik E, Rychik U, Skotnicki P, Jakubowicz J, Kulpa JK. Utility of proGRP determinations in cancer patients. *Clin Lab.* (2010) 56:527–34.
13. Cuttitta F, Carney DN, Mulshine J, Moody TW, Fedorko J, Fischler A, et al. Bombesin-like peptides can function as autocrine growth factors in human small cell lung cancer. *Nature* (1985) 316:823–26. doi: 10.1038/316823a0
14. Kelley MJ, Linnoila RI, Avis IL, Georgiakis MS, Cuttitta, F, Mulshine JL et al. Antitumor activity of a monoclonal antibody directed against GRP in patients with small cell lung cancer. *Chest* (1997) 112:255–61. doi: 10.1378/chest.112.1.256
15. Battey JF, Way J, Corjay MH, Shipira H, Kasano K, Hawkins R, et al. Molecular cloning of the BB/GRP receptor from Swiss 3T3 cells. *Proc Natl Acad Sci USA.* (1991) 88:395–9. doi: 10.1073/pnas.88.2.395
16. Spindel ER, Giladi E, Brehm P, Goodman RA, Segerson TP. Cloning and functional characterization of a complementary DNA encoding the murine fibroblast BB/GRP receptor. *Mol Endocrin.* (1990) 4:1956–63. doi: 10.1210/mend-4-12-1956
17. Wada E, Way J, Shapira H, Kusano K, Lebacq-Verheyden AM, Coy D, et al. cDNA cloning, characterization and brain region-specific expression of a neuromedin-B preferring bombesin receptor. *Neuron* (1991) 6:421–30. doi: 10.1016/0896-6273(91)90250-4
18. Fathi Z, Corjay MH, Shapira H, Wada E, Benya R, Jensen R et al. BRS-3:a novel bombesin receptor subtype selectively expressed in testis and lung carcinoma cells. *J Biol Chem.* (1993) 268:5979–84.
19. Moody TW, Mantey SA, Pradhan TK, Schumann M, Nakagawa T, Martinez A, et al. Development of high affinity camptothecin-bombesin conjugates that have targeted cytotoxicity for bombesin receptor-containing tumor cells. *J Biol Chem.* (2004) 279:23580–89. doi: 10.1074/jbc.M401938200
20. Moody TW, Sun LC, Mantey SA, Pradhan T, Mackey LV, Gonzalez N, et al. *In vitro* and *in vivo* antitumor effects of cytotoxic camptothecin bombesin conjugates are mediated by specific interaction with cellular bombesin receptors. *J Pharmacol Exp Ther.* (2006) 318:1265–72. doi: 10.1124/jpet.106.104141
21. Safavy A, Raisch KP, Khazaeli MB, Buchsbaum DJ, Bonner JA. Paclitaxel derivatives for targeted therapy of cancer:Toward the development of smart taxanes. *J Med Chem.* (1999) 42:419–24. doi: 10.1021/jm990355x
22. Wang K, Sun X, Wang Y, Yang F, Wang H. Breast cancer targeted chemotherapy based on doxorubicin-loaded bombesin peptide modified nanocarriers. *Drug Deliv.* (2015) 23:2697–702. doi: 10.3109/10717544.2015.1049721
23. Moody TW, Pradhan T, Manety SA, Jensen RT, Dyba M, Moody D, et al. Bombesin marine toxin conjugates inhibit the growth of lung cancer cells. *Life Sci.* (2008) 82:855–865. doi: 10.1016/j.lfs.2008.01.019
24. Liu S, Yang H, Wan L, Cai HW, Li SF, Li YP, et al. Enhancement of cytotoxicity of anti-microbial peptide magainin II in tumor cells by bombesin-targeted delivery. *Acta Pharmacol Sin.* (2011) 32:79–88. doi: 10.1038/aps.2010.162
25. Sioud M, Mobergslien A. Efficient siRNA targeted delivery into cancer cells by gastrin-releasing peptides. *Bioconjug Chem.* (2012) 23:1040–49. doi: 10.1021/bc300050j
26. Hohla, F, Schally AV. Targeting gastrin releasing peptide receptors: new options for therapy and diagnosis of cancer. *Cell Cycle* (2010) 9:1738–41. doi: 10.4161/cc.9.9.11347
27. Schwartzmann G, DiLeone LP, Horowitz M, Schunemann D, Cancella A, Pereira AS et al. A phase I trial of the bombesin/gastrin-releasing peptide (BN/GRP) antagonist RC3095 in patients with advanced solid malignancies. *Invest New Drugs* (2006) 24:403–12. doi: 10.1007/s10637-006-6886-5
28. Mahmoud S, Staley J, Taylor J, Bodgen A, Moreau JP, Coy D, et al. (Psi^{13,14}) Bombesin analogues inhibit the growth of small cell lung cancer *in vitro* and *in vivo*. *Cancer Res.* (1991) 51:1798–802.
29. Nakamura T, Ramos-Alvarez I, Iordanskaia T, Moreno P, Mantey SA, Jensen RT. Molecular basis for high affinity and selectivity of the peptide antagonist Bantag-1 for the orphan BB3 receptor. *Biochem Pharmacol.* (2016) 115:64–7. doi: 10.1016/j.bcp.2016.06.013
30. Reubi JC, Wenger S, Schumuckli-Mauer J, Schaer JC, Gugger M. Bombesin receptor subtypes in human cancers: detection with the universal radioligand (125)I-[D-TYR(6), beta-ALA(11), PHE (13), NLE (14)] bombesin (6-14). *Clin Cancer Res.* (2002) 8:1139–46.
31. DeVincentis G, Remediani S, Varvarigou AD, DiSanto G, Lori F, Laurenti C, et al. Role of 99mTc-bombesin scan in diagnosis and staging of prostate cancer. *Cancer Biother Radiopharm.* (2004) 19:81–4. doi: 10.1089/108497804773391711
32. Chen Q, Ma Q, Chen M, Chen B Wen Q, Jia B, et al. An exploratory study of 99mTc-RGD-BBN peptide scintimamography in the assessment of breast malignant lesions compared to 99mTc-3P4-RGD2. *PLoS ONE* (2015) 10:e0123401. doi: 10.1371/journal.pone.0123401
33. Kahkonen E, Jambor I, Kemppainen J, Lehtio K, Gronroos TJ, Kulsma A, et al. *In vivo* imaging of prostate cancer using [68Ga]-labeled bombesin analog BAY86-7548. *Clin Cancer Res.* (2013) 19:5434–43. doi: 10.1158/1078-0432.CCR-12-3490
34. Moody TW, Berna MJ, Mantey S, Sancho V, Ridnour L, Wink DA, et al. Neuromedin B receptors regulate EGF receptor tyrosine phosphorylation in lung cancer cells. *Eur J Pharmacol.* (2010) 637:38–45. doi: 10.1016/j.ejphar.2010.03.057
35. Truong TH, Carroll KS. Redox regulation of the EGFR signaling through cysteine oxidation. *Biochemistry* (2012) 51:9954–65. doi: 10.1021/bi301441e
36. Heppner DE, van der Vliet A. Redox-dependent regulation of the epidermal growth factor receptor signaling. *Redox Biol.* (2016) 8:24–7. doi: 10.1016/j.redox.2015.12.002
37. Lemmon MA, Schlessinger J. Cell signaling by receptor tyrosine kinases. *Cell* (2010) 141:1117–34. doi: 10.1016/j.cell.2010.06.011
38. Lynch TJ, Bell DW, Sordella R, Gurubhagavatula S, Okimoto RA, Brannigan BW, et al. Activating mutations in the epidermal growth factor receptor underlying responsiveness of non-small-cell lung cancer to gefitinib. *N Eng J Med.* (2004) 350:2129–39. doi: 10.1056/NEJMoa040938
39. Paez JB, Janne PA, Lee JC, Tracy S, Greuich H, Gabriel S et al. EGFR mutations in lung cancer: Correlation with clinical response to gefitinib. *Science* (2004) 304:1497–1500. doi: 10.1126/science.1099314

40. Schrump DS, Carter D, Kelsey CR, Marks LB, Giaccone G. Non-small cell lung cancer. In: DeVita Jr V, Lawrence TS, Rosenberg SA, editors. *Cancer: Principles and Practice of Oncology*. Philadelphia, PA: Lippincott, Williams and Wilkins (2011) p. 799–847.
41. Moody TW, Carney DN, Korman LY, Gazdar A, Minna JD. Neurotensin is produced by and secreted from classic small cell lung cancer cells. *Life Sci.* (1985) 36:1727–32. doi: 10.1016/0024-3205(85)90555-7
42. Kitabgi P. Neurotensin and neuromedin N are differentially processed from a common precursor by prohormone convertases in tissues and cell lines. *Results Probl Cell Differ.* (2010) 50:85–96. doi: 10.1007/400_2009_27
43. White JE, Noinaj N, Shibata Y, Love J, Kloss B, Xu F, et al. Structure of the agonist-bound neurotensin receptor. *Nature* (2012) 490:508–13. doi: 10.1038/nature11558
44. Kitabgi P. Functional domains of the subtype 1 neurotensin receptor (NTS1). *Peptides* (2006) 27:2461–68. doi: 10.1016/j.peptides.2006.02.013
45. Allen AE, Carney DN, Moody TW. Neurotensin binds with high affinity to small cell lung cancer cells. *Peptides* (1988) 9:57–61. doi: 10.1016/0196-9781(88)90224-0
46. Staley J, Fiskum G, Davis TP, Moody TW. Neurotensin elevates cyutosolic calcium in small cell lung cancer cells. *Peptides* (1989) 10:121–7. doi: 10.1016/0196-9781(89)90015-6
47. Muller KM, Tveteraas IH, Aasrum M, Odegard J, Dawood M, Dajani O, et al. Role of protein kinase C and epidermal growth factor receptor signaling in growth stimulation by neurotensin in colon carcinoma cells. *BMC Cancer* (2011) 11:421. doi: 10.1186/1471-2407-11-421
48. Guha S, Rey O, Rozengurt E. Neurotensin induces protein kinase C-dependent protein kinase D activation and DNA synthesis in human pancreatic carcinoma cell lune PANC-1. *Cancer Res.* (2002) 62:1632–40.
49. Leyton J, Garcia-Marin L, Jensen RT, Moody TW. Neurotensin causes tyrosine phosphorylation of focal adhesion kinase in lung cancer cells. *Eur J Pharmacol.* (2002) 442:179–86. doi: 10.1016/S0014-2999(02)01539-X
50. Kisfalvi K, Guha S, Rozengurt E. Neurotensin and EGF induce synergistic stimulation of DNA synthesis by increasing the duration of ERK signaling to ductal pancreatic cancer cells. *J Cell Physiol.* (2012) 202:880–90. doi: 10.1002/jcp.20187
51. Harrison RJ, McNeil GP, Dobner PR. Synergistic activation of neurotensin/neuromedin N gene expression by c-Jun and glucocorticoids: Novel effects of Fos family proteins. *Mol Endocrinol.* (1995) 9:981–93.
52. Moody TW, Chan DC, Mantey SA, Moreno P, Jensen RT. SR48692 inhibits non-small cell lung cancer proliferation in an EGF receptor-dependent manner. *Life Sci.* (2014) 100:25–34. doi: 10.1016/j.lfs.2014.01.072
53. Younes M, Wu Z, Dupouy S, Lupo AM, Mourra N, Takahashi T, et al. Neurotensin (NTS) and its receptor (NTSR1) causes EGFR, HER2, and HER3 over-expression and their autocrine/paracrine activation in lung tumors, confirming responsiveness to erlotinib. *Oncotarget* (2014) 5:8252–59. doi: 10.18632/oncotarget.1633
54. Hassan S, Dobner PR, Carraway RE. Involvement of MAP kinase, PI-3 kinase and EGF-receptor in the stimulatory effect of neurotensin on DNA synthesis in PC3 cells. *Regul Pept.* (2004) 120:155–66. doi: 10.1016/j.regpep.2004.03.004
55. DiFlorio A, Sancho V, Moreno P, Della Fave G, Jensen RT. Gastrointestinal hormones stimulate growth of foregut neuroendocrine tumors by transactivating the EGF receptor. *Biochem Biophys Acta* (2013) 1833:573–82. doi: 10.1016/j.bbamcr.2012.11.021
56. Zhou Z, Xie J, Cai Y, Yang S, Chen Y, Wu H. The significance of NTR1 expression and its correlation with β-catenin and EGFR in gastric cancer. *Diagn Pathol.* (2015) 100:128. doi: 10.1186/s13000-015-0356-3
57. Ye Y, Long X, Zhang L, Chen J, Liu P, Li H, et al. NTS/NTR1 co-expression enhances epithelial-to-mesenchymal transition and promotes tumor metastasis by activating the Wnt/β-catenin signaling pathway in hepatocellular carcinoma. *Oncotarget* (2016) 7:70303–22. doi: 10.18632/oncotarget.11854
58. Schulz J, Rohracker M, Stiebler M, Goldschmidt J, Stober F, Noriega M, et al. Proof of therapeutic efficacy of a ^{177}Lu-labeled neurotensin receptor 1 antagonists in a colon carcinoma xenograft model. *J Nucl Med.* (2017) 58:936–41. doi: 10.2967/jnumed.116.185140
59. Baum RP, Singh A, Schuchardt C, Kulkarni HR, Klette I, Wiessalla S, et al. ^{177}Lu-3BP-227 for neurotensin receptor 1-targeted therapy of metastatic pancreatic adenocarcinoma: first clinical results. *J Nucl Med.* (2018) 59:809–14. doi: 10.2967/jnumed.117.193847
60. Harmar AJ, Fahrenkrug J, Gozes I, Laburthe M, May V, Pisegna JR, et al. Pharmacology and functions of receptors for vasoactive intestinal peptide and pituitary adenylate cyclase-activating polypeptide:IUPHAR review 1. *Br J Pharmacol.* (2012) 166:4–17. doi: 10.1111/j.1476-5381.2012.01871.x
61. Sun C, Song D, Davis-Taber R, Barrett LW, Scott V, Richardson PL, et al. Solution structure and mutational analysis of pituitary adenylate cyclase-activating polypeptide binding to the extracellular domain of PAC1-Rs. *Proc Natl Acad Sci USA.* (2007) 104:7875–7880. doi: 10.1073/pnas.0611397104
62. Kumar S, Ploszak A, Zhang C, Swaminathan K, Xu HE. Crystal structure of the PAC1R extracellular domain unifies a consensus fold for hormone recognition by class B G-protein coupled receptors. *PLoS ONE* (2011) 6:e19682. doi: 10.1371/journal.pone.0019682
63. Liao C, Zhao X, Brewer M, May V, Li J. Conformational transitions of the pituitary adenylate cyclase-activating polypeptide receptor, a human class B GPCR. *Sci Rep.* (2017) 7:5427. doi: 10.1038/s41598-017-05815-x
64. Furness SGB, Wootten D, Christopoulos A, Sexton PM. Consequences of splice variation on secretin family G protein-coupled receptor function. *Br J Pharmacol.* (2012) 166:98–109. doi: 10.1111/j.1476-5381.2011.01571.x
65. Vaudry D, Falluel-Morel A, Vourgault S, Basille M, Burel D, Wurtz O, et al. Pituitary adenylate cyclase-activating polypeptide and its receptor:20 years after discovery. *Pharmacol Rev.* (2009) 61:283–357. doi: 10.1124/pr.109.001370
66. Lerner EA, Ribeiro JM, Nelson RJ, Lerner MR. Isolation of maxadilan, a potent vasodilatory peptide from the salivary glands of the sand fly Lutzomyia longipalpis. *J Biol Chem.* (1991) 266:11234–236.
67. Ramos-Alvarez I, Mantey SA, Nakamura T, Nuche-Berenguer B, Moreno P, Moody TW, et al. A structure-function study of PACAP using conformationally restricted analogs: identification of PAC1 receptor-selective PACAP agonists. *Peptides* (2015) 66:26–42. doi: 10.1016/j.peptides.2015.01.009
68. Moody TW, Nuche-Berenguer B, Jensen RT. VIP/PACAP, and their receptors in cancer. *Curr Opin Endocrinol Diabetes* (2016) 23:38–47. doi: 10.1097/MED.0000000000000218
69. Reubi JC, Laderach U, Waser B, Gebbers JO, Robberecht P, Laissue JA. Vasoactive intestinal peptide/pituitary adenylate cyclase activating peptide receptor subtypes in human tumors and their tissues of origin. *Cancer Res.* (2000) 60:3105–12.
70. Tang B, Yong X, Xie R, Li QW, Yang SM. Vasoactive intestinal peptide receptor-based imaging and treatment of tumors. *Int J Oncol.* (2014) 44:1023–31. doi: 10.3892/ijo.2014.2276
71. Moody TW, Jensen RT. VIP/PACAP receptors. In: Kastin AJ, editor. *Handbook of Biologically Active Peptides*, *Second Edition*. San Diego, CA: Academic Press. (2013), p. 556–561.
72. Lutz EM Ronaldson E, Shaw P, Johnson MS, Holland PJ, Mitchell R. Characterization of novel splice variants of the PAC1 receptor in human neuroblastoma cells: consequences for signaling by VIP and PACAP. *Mol Cell Neurosci.* (2006) 31:193–209. doi: 10.1016/j.mcn.2005.09.008
73. Blechman J, Levkowitz G. Alternative splicing of the pituitary adenylate cyclase activating polypeptide receptor PAC1: mechanisms of fine tuning of brain activity. *Front Endocrinol.* (2013) 4:55. doi: 10.3389/fendo.2013.00055
74. Pisegna J, Wank S. Cloning and characterization of the signal transduction of four splice variants of the human pituitary adenylate cyclase activating polypeptide receptor: evidence for dual coupling to adenylate cyclase and phospholipase C. *J Biol Chem.* (1996) 271:17267–74. doi: 10.1074/jbc.271.29.17267
75. Usiyama M, IkeldaR, Sugawara H, Yoshida M, Mori K, Kangawa K, et al. Differential intracellular signaling through PAC1 isoforms as a result of alternative splicing in the first extracellular domain and the third intracellular loop. *Mol Pharmacol.* (2007) 72:103–11. doi: 10.1124/mol.107.035477
76. Moody TW, Jensen RT. PACAP and Cancer. In: Reglodi D, Tamas A, Editors. *Pituitary Adenylate Cyclase Activating Polypeptide-PACAP*. Basel: Springer International Publishing AG. (2016) p. 795–814.
77. Moody TW, Leyton J, Unsworth E, John C, Lang L, Eckelman WC. (Arg^{15}, Arg^{21}) VIP: Evaluation of biological activity and localization to breast cancer tumors. *Peptides* (1998) 19:585–92. doi: 10.1016/S0196-9781(97)00459-2

78. Thakur ML, Devadhas D, Zhang K, Pestell RG, Wang C, McCue P, et al. Imaging spontaneous MMTV neu transgencic mammary tumors: targeting metabolic activity versus genetic products. *J Nucl Med.* (2010) 51:106–11. doi: 10.2967/jnumed.109.069542
79. Thakur ML, Marcus CS, Saeed S, Pallela V, Minami C, Diggles L, et al. Imaging tumors in humans with Tc-99m-VIP. *Ann NY Acad Sci.* (2000) 921:37–44. doi: 10.1111/j.1749-6632.2000.tb06949.x
80. Moody TW, Mantey SA, Fuselier JA, Coy D, Jensen RT. Vasoactive intestinal peptide-camptothecin conjugates inhibit the proliferation of breast cancer cells. *Peptides* (2007) 28:1883–90. doi: 10.1016/j.peptides.2007.04.017
81. Moody TW, Czerwindki G, Tarasova NI, Moody DL, Michejda CJ. The development of VIP ellipticin conjugates. *Regul Pept.* (2004) 123:187–92. doi: 10.1016/j.regpep.2004.03.021
82. Oryuksel H, Mohanty PS, Rubinstein J. VIP-grafted sterically stabilized phospholipid nanomicellar 17-allylamino-17-dimethoxy geldanomycin: a novel targeted nanomedicine for breast cancer. *Int J Pharm.* (2009) 365:157–61. doi: 10.1016/j.ijpharm.2008.08.024
83. Onyuksel H, Jeon E, Rubinstein I. Nanomicellar paclitaxel increases cytotoxicity of multidrug resistant breast cancer cells. *Cancer Lett.* (2009) 274:327–30. doi: 10.1016/j.canlet.2008.09.041
84. Moody TW, Zia F, Draoui M, Brenneman DE, Fridkin M, Davidson A, et al. A vasoactive intestinal peptide antagonist inhibits non-small cell lung cancer growth. *Proc Natl Acad Sci USA.* (1993) 90:4345–49. doi: 10.1073/pnas.90.10.4345
85. Zia F, Fagarasan M, Bitar K, Coy DH, Pisegna JR, Wank SA, et al. Pituitary adenylate cyclase activating peptide receptors regulate the growth of non-small cell lung cancer cells. *Cancer Res.* (1995) 55:4886–91.
86. Dagar S, Sekosan M, Rubinstein I, Onyeksel H. Detection of VIP receptors in MNU-induced breast cancer in rats: implications for breast cancer targeting. *Breast Cancer Res Treat.* (2001) 65:49–54. doi: 10.1023/A:1006406617497
87. Madsen B, Georg B, Vissing H, Fahrenkrug J. Retinoic acid down-regulates the expression of vasoactive intestinal polypeptide receptor type-1 in human breast carcinoma cell lines. *Cancer Res.* (1998) 58:4845–50.
88. Jakowlew SB, Zakowicz H, Moody TW. Retinoic acid down-regulates VPAC(1) receptors and TGF-beta-3 but up-regulates TGF-beta 2 in lung cancer cells. *Peptides* (2000) 2:1831–7. doi: 10.1016/S0196-9781(00)00344-2
89. Moody TW, Dudek J, Zakowicz H, Walters J, Jensen RT, Petricoin E, et al. VIP receptor antagonists inhibit mammary carcinogenesis in C3(1)SV40T antigen mice. *Life Sci.* (2004) 74:345–57. doi: 10.1016/j.lfs.2003.07.043
90. Moody TW, Osefo N, Nuche-Berenguer B, Ridnour L, Wink D, Jensen RT. Pituitary adenylate cyclase-activating polypeptide causes tyrosine phosphorylation of the epidermal growth factor receptor in lung cancer cells. *J Pharm Exp Ther.* (2012) 341:873–81. doi: 10.1124/jpet.111.190033
91. Beaudreau HE, Casterline BW, Burke DJ, Leto TL. Wild-type and mutant p53 differentially regulate NADPH oxidase-4 in TGF-β-mediated migration of human lung cancer epithelial cells. *Br J Cancer* (2014) 110:2569–82. doi: 10.1038/bjc.2014.165
92. Valdehita A, Bajo AM, Schally AV, Varga JL, Carmena MJ, Prieto JC. VIP induces transactivation of the EGFR and HER2 in human breast cancer cells. *Mol Cell Endocrinol.* (2009) 302:41–8. doi: 10.1016/j.mce.2008.11.024
93. Rai U, Thrimawithana TR, Valery C, Young SA. Therapeutic uses of somatostatin and its analogues: current view and potential applications. *Pharmacol Ther.* (2015) 152:98–110. doi: 10.1016/j.pharmthera.2015.05.007
94. Sun L, Coy DH. Somatostatin and its analogs. *Curr Drug Targets* (2016) 17:529–37. doi: 10.2174/1389450116666141205163548
95. Reubi JC, Krenning E, Lamberts SW, Kvols L. *In vitro* detection of somatostatin receptors in human tumors. *Metabolism* (1992) 41:104–10. doi: 10.1016/0026-0495(92)90042-9
96. Ito T, Lee L, Jensen RT. Carcinoid-syndrome:recent advances, current status and controversies. Curr Opin Endocrinol Diabetes Obes. (2018) 25:22–35. doi: 10.1097/MED.0000000000000376.
97. Pokuri VK, Fong MK, Iyer R. Octreotide and lanreotide in gastroenteropancreatic neuroendocrine tumors. *Curr Oncol Rep.* (2016) 18:7. doi: 10.1007/s11912-015-0492-7
98. Narayanan S, Kunz PL. Role of somatostatin analogues in the treatment of neuroendocrine tumors. *Hematol Oncol Clin North Am.* (2016) 30:163–77. doi: 10.1016/j.hoc.2015.09.008
99. Ito T, Lee L, Jensen RT. Treatment of symptomatic neuroendocrine tumor syndromes: recent advances and controversies. *Expert Opin Pharmacother.* (2016) 17:2191–205. doi: 10.1080/14656566.2016.1236916
100. Oberg K, Lamberts SW. Somatostatin analogues in acromegaly and gastroenteropancreatic neuroendocrine tumors: past, present and future. *Endocr Relat Cancer* (2016) 23:R551–66. doi: 10.1530/ERC-16-0151
101. Gadelha MR, Wildemberg LE, Bronstein MD, Gatto F, Ferone D. Somatostatin receptor ligands in the treatment of acromegaly. *Pituitary* (2017) 20:100–8. doi: 10.1007/s11102-017-0791-0
102. Susini C, Buscail L. Rationale for the use of somatostatin analogs as antitumor agents. *Ann. Oncol.* (2006) 17:1733–42. doi: 10.1093/annonc/mdl105
103. Schally AV, Szepeshazi K, Nagy A, Comaru-Schally AM, Halmos G. New approaches to therapy of cancers of the stomach, colon and pancreas based on peptide analogs. *Cell Mol Life Sci.* (2004) 61:1042–68. doi: 10.1007/s00018-004-3434-3
104. Msaouel P, Galanis E, Koutsilieris M. Somatostatin and somatostatin receptors:implications for neoplastic growth and cancer biology. *Expert Opin Investig Drugs* (2009) 18:1297–316. doi: 10.1517/13543780903176399
105. Verslype C, Carton S, Borbath I, Delaunoit T, Demetter P, Demolin G, et al. The anti-proliferative effect of somatostatin analogs:clinical relevance in patients with neuroendocrine gastro-entero-pancreatic tumours. *Acta Gastroenterologica Belgica* (2009) 72:54–8.
106. Michael M, Garcia-Carbonero R, Weber MM, Lombard-Bohas C, Toumpanakis C, Hicks RJ. The anti-proliferative role of lanreotide in controlling growth of neuroendocrine tumors: a systematic review. *Oncologist* (2017) 22:272–85. doi: 10.1634/theoncologist.2016-0305
107. Keskin O, Yalcin S. A review of the use of somatostatin analogs in oncology. *Onco Targets Ther.* (2013) 6:471–83. doi: 10.2147/OTT.S39987
108. Caplin ME, Pavel M, Cwikla JB, Phan AT, Raderer M, Sedlackova E, et al. Lanreotide in metastatic enteropancreatic neuroendocrine tumors. *N Engl J Med.* (2014) 371:224–33. doi: 10.1056/NEJMoa1316158
109. Rinke A, Muller HH, Schade-Brittinger C, Klose KJ, Barth P, Wied M, et al. Placebo-controlled, double-blind, prospective, randomized study on the effect of octreotide LAR in the control of tumor growth in patients with metastatic neuroendocrine midgut tumors:a report from the PROMID study group. *J Clin Oncol.* (2009) 27:4656–63. doi: 10.1200/JCO.2009.22.8510
110. Merola E, Panzuto F, Delle Fave G. Antiproliferative effect of somatostatin analogs in advanced gastro-entero-pancreatic neuroendocrine tumors:a systematic review and meta-analysis. *Oncotarget* (2017) 8:46624–34. doi: 10.18632/oncotarget.16686
111. Ito T, Igarashi H, Jensen RT. Therapy of metastatic pancreatic neuroendocrine tumors (pNETs): recent insights and advances. *J Gastroenterol.* (2012) 47:941–60. doi: 10.1007/s00535-012-0642-8
112. Pavel M, O'Toole D, Costa F, Capdevila J, Gross D, Kianmanesh R, et al. ENETS consensus guidelines update for the management of distant metastatic disease of intestinal, pancreatic, bronchial neuroendocrine neoplasms (NEN) and NEN of unknown primary site. *Neuroendocrinology* (2016) 103:172–85. doi: 10.1159/000443167
113. Kunz PL, Reidy-Lagunes D, Anthony LB, Bertino EM, Brendtro K, Chan JA, et al. Consensus guidelines for the management and treatment of neuroendocrine tumors. *Pancreas* (2013) 42:557–77. doi: 10.1097/MPA.0b013e31828e34a4
114. Massironi S, Conte D, Rossi RE. Somatostatin analogues in functioning gastroenteropancreatic neuroendocrine tumors:literature review, clinical recommendations and schedules. *Scand J Gastroenterol.* (2016) 51:513–23. doi: 10.3109/00365521.2015.1115117
115. Enzler T, Fojo T. Long-acting somatostatin analogues in the treatment of unresectable/metastatic neuroendocrine tumors. *Seminars in Oncol.* (2017) 44:141–56. doi: 10.1053/j.seminoncol.2017.07.001
116. Hasskarl J, Kaufmann M, Schmid HA. Somatostatin receptors in non-neuroendocrine malignancies:the potential role of somatostatin analogs in solid tumors. *Future Oncol.* (2011) 7:895–913. doi: 10.2217/fon.11.66
117. Smith M C, Maggi M, Orlando C. Somatostatin receptors in non-endocrine tumors. *Dig Liver Dis.* (2004) 36:S78–85. doi: 10.1016/j.dld.2003.11.019
118. Ito T, Jensen RT. Molecular imaging in neuroendocrine tumors:recent advances, controversies, unresolved issues, and roles in

management. *Curr Opin Endocrinol Diabetes Obes.* (2017) 24:15–24. doi: 10.1097/MED.0000000000000300
119. Dromain C, Deandreis D, Scoazec JY, Goere D, Ducreux M, Baudin E, et al. Imaging of neuroendocrine tumors of the pancreas. *Diagn Interv Imaging* (2016) 97:1241–57. doi: 10.1016/j.diii.2016.07.012
120. Deppen SA, Blume J, Bobbey AJ, Shah C, Graham MM, Lee P, et al. 68Ga-DOTATATE compared with 111In-DTPA-Octreotide and conventional imaging for pulmonary and gastroenteropancreatic neuroendocrine tumors: a systematic review and meta-analysis. *J Nucl Med.* (2016) 57:872–78. doi: 10.2967/jnumed.115.165803
121. Ambrosini V, Nanni C, Fanti S. The use of gallium-68 labeled somatostatin receptors in PET/CT imaging. *PET Clin.* (2014) 9:323–29. doi: 10.1016/j.cpet.2014.03.008
122. Barrio M, Czernin J, Fanti S, Ambrosini V, Binse I, Du L, et al. The impact of somatostatin receptor-directed PET/CT on the management of patients with neuroendocrine tumor: a systematic review and meta-analysis. *J Nucl Med.* (2017) 58:756–761. doi: 10.2967/jnumed.116.185587
123. Mizutani G, Nakanishi Y, Watanabe N, Honma T, Obana Y, Seki T, et al. Expression of somatostatin receptor (SSTR) subtypes (SSTR-1, 2A, 3, 4 and 5) in neuroendocrine tumors using real-time RT-PCR method and immunohistochemistry. *Acta Histochem Cytochem.* (2012) 45:167–76. doi: 10.1267/ahc.12006
124. Bodei L, Kwekkeboom DJ, Kidd M, Modlin IM, Krenning EP. Radiolabeled somatostatin analogue therapy of gastroenteropancreatic cancer. *Semin Nucl Med.* (2016) 46:225–38. doi: 10.1053/j.semnuclmed.2015.12.003
125. Brabander T, Teunissen JJ, van Eijck CH, Franssen GJ, Feelders RA, de Herder WW, et al. Peptide receptor radionuclide therapy of neuroendocrine tumors. *Best Pract Res Clin Endocrinol Metab.* (2016) 30:103–14. doi: 10.1016/j.beem.2015.10.005
126. Bodei L, Cwikla JB, Kidd M, Modlin IM. The role of peptide receptor radionuclide therapy in advanced/metastatic thoracic neuroendocrine tumors. *J Thorac Dis.* (2017) 9S1511–23. doi: 10.21037/jtd.2017.09.82
127. Kwekkeboom DJ, Krenning EP. Peptide receptor radionuclide therapy in the treatment of neuroendocrine tumors. *Hematol Oncol Clin North Am.* (2016) 30:179–91. doi: 10.1016/j.hoc.2015.09.009
128. Kwekkeboom DJ, de Herder WW, Kam BL, van Eijck CH, Van Essen M, Kooij PP, et al. Treatment with the radiolabeled somatostatin analog [177 Lu-DOTA 0, Tyr3]octreotate:toxicity, efficacy, and survival. *J Clin Oncol* (2008) 26:2124–30. doi: 10.1200/JCO.2007.15.2553
129. Strosberg JR, Wolin EM, Chasen B, Kulke M, Bushnell DL, Caplin ME, et al. NETTER-1 phase III: progression-free survival, radiographic response, and preliminary overall survival results in patients with midgut neuroendocrine tumors treated with 177-Lu-Dotatate. *J Clin Oncol.* (2016) 34:194. doi: 10.1200/jco.2016.34.4_suppl.194
130. Dalm SU, Nonnekens J, Doeswijk GN, de Blois E, van Gent DC, Konijnenberg M W, et al. Comparison of the therapeutic response to treatment with a 177-lutetium labeled somatostatin receptor agonist and antagonist in preclinical models. *J Nucl Med.* (2016) 57:260–5. doi: 10.2967/jnumed.115.167007
131. Fani M, Nicolas GP, Wild D. Somatostatin receptor antagonists for imaging and therapy. *J Nucl Med.* (2017) 58:61S−66S. doi: 10.2967/jnumed.116.186783
132. Reubi JC. Peptide receptors as molecular targets for cancer diagnosis and therapy. *Endocr Rev.* (2003) 24:389–427. doi: 10.1210/er.2002-0007
133. Reubi JC, Macke HR, Krenning EP. Candidates for peptide receptor radiotherapy today and in the future. *J Nucl Med.* (2005) 46:67S−75S.
134. Sancho V, Di Florio A, Moody TW, Jensen RT. Bombesin receptor-mediated imaging and cytotoxicity: review and current status. *Curr Drug Deliv.* (2011) 8:79–134. doi: 10.2174/156720111793663624
135. Moody TW, Leyton J, Chan D, Brenneman DC, Fridkin M, Gelber E, et al. VIP receptor antagonists and chemotherapeutic drugs inhibit the growth of breast cancer cells. *Breast Cancer Res Treat.* (2001) 68:55–64. doi: 10.1023/A:1017994722130
136. Gelber E, Granoth R, Fridkin M, Dreznik Z, Brenneman DE, Moody TW, et al. A lipophilic vasoactive intestinal peptide analog enhances the anti-proliferative effect of chemotherapeutic agents on cancer cell lines. *Cancer* (2001) 92:2172–2180. doi: 10.1002/1097-0142(20011015)92:8<2172::AID-CNCR1560>3.0.CO;2-4
137. Khan M, Huang T, Lin CY, Wu J, Fan BM, Blan ZX. Exploiting cancer's phenotypic guise against itself: targeting ectopically expressed peptide G-protein coupled receptors for lung cancer therapy. *Oncotarget* (2017) 8:104615–637. doi: 10.18632/oncotarget18403
138. Smith JP, Wang S, Nadella S, Jablonski SA, Weiner LM. Cholecystokinin receptor antagonist alters pancreatic cancer microenvironment and increases efficacy of immune checkpoint antibody therapy in mice. *Cancer Immunol Immunother.* (2018) 67:195–207. doi: 10.1007/s00262-017-2077-9

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Endocrinology

REVIEW
published: 07 August 2018
doi: 10.3389/fendo.2018.00447

Brown Adipose Tissue Energy Metabolism in Humans

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The demonstration of metabolically active brown adipose tissue (BAT) in humans primarily using positron emission tomography coupled to computed tomography (PET/CT) with the glucose tracer 18-fluorodeoxyglucose (^{18}FDG) has renewed the interest of the scientific and medical community in the possible role of BAT as a target for the prevention and treatment of obesity and type 2 diabetes (T2D). Here, we offer a comprehensive review of BAT energy metabolism in humans. Considerable advances in methods to measure BAT energy metabolism, including nonesterified fatty acids (NEFA), chylomicron-triglycerides (TG), oxygen, Krebs cycle rate, and intracellular TG have led to very good quantification of energy substrate metabolism *per* volume of active BAT *in vivo*. These studies have also shown that intracellular TG are likely the primary energy source of BAT upon activation by cold. Current estimates of BAT's contribution to energy expenditure range at the lower end of what would be potentially clinically relevant if chronically sustained. Yet, ^{18}FDG PET/CT remains the gold-standard defining method to quantify total BAT volume of activity, used to calculate BAT's total energy expenditure. Unfortunately, BAT glucose metabolism better reflects BAT's insulin sensitivity and blood flow. It is now clear that most glucose taken up by BAT does not fuel mitochondrial oxidative metabolism and that BAT glucose uptake can therefore be disconnected from thermogenesis. Furthermore, BAT thermogenesis is efficiently recruited upon repeated cold exposure, doubling to tripling its total oxidative capacity, with reciprocal reduction of muscle thermogenesis. Recent data suggest that total BAT volume may be much larger than the typically observed 50–150 ml with ^{18}FDG PET/CT. Therefore, the current estimates of total BAT thermogenesis, largely relying on total BAT volume using ^{18}FDG PET/CT, may underestimate the true contribution of BAT to total energy expenditure. Quantification of the contribution of BAT to energy expenditure begs for the development of more integrated whole body *in vivo* methods.

Keywords: brown adipose tissue, energy metabolism, obesity, type 2 diabetes, molecular imaging, positron emission tomography, tracer methods

OPEN ACCESS

Edited by:
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Specialty section:
This article was submitted to Diabetes, a section of the journal Frontiers in Endocrinology

Received: *31 May 2018*
Accepted: *20 July 2018*
Published: *07 August 2018*

Citation:
Carpentier AC, Blondin DP, Virtanen KA, Richard D, Haman F and Turcotte ÉE (2018) Brown Adipose Tissue Energy Metabolism in Humans. Front. Endocrinol. 9:447. doi: 10.3389/fendo.2018.00447

INTRODUCTION

Since 1980, the global prevalence of obesity has doubled (1). In 2015, overweight and obesity accounted for 4 million deaths worldwide, including 3.3 million from cardiovascular diseases and type 2 diabetes (T2D) (1). Restricting energy intake by reducing food consumption, increasing satiety and/or fat malabsorption, is the chief weight-loss mechanism of most medical and surgical treatments of obesity and has profound anti-diabetic effects (2–5). Increasing exercise- and non-exercise activity-related thermogenesis is the other cornerstone of obesity and T2D management. Simultaneously targeting multiple mechanisms of energy homeostasis is advantageous for the treatment of obesity (6). However, targeting energy expenditure unrelated to physical activity remains largely underexplored. Consequently, a number of unexploited mechanism may help fill a gap as an adjunct to current treatments for obesity and T2D.

One emerging, highly modifiable homeostatic mechanism for energy expenditure in humans is BAT thermogenesis. BAT may contribute as much as 60% of "non-shivering" thermogenesis in small mammals (7, 8), enabling their survival in the cold without reliance on shivering to produce heat (9, 10). BAT is currently considered a prime target for the treatment of obesity and T2D (11–15). Although the relative role of BAT on energy expenditure, thermogenesis and substrate utilization is dominant in rodents, the contribution of BAT to energy homeostasis in humans is more controversial. A detailed discussion on the different factors implicated in BAT and WAT "browning" such as immune cell-mediated modulation of adipose tissue sympathetic innervation (16) [please see (17) and (18) for review] is beyond the scope of the present review. The aim of the present article is to review the evidence for a role of BAT in energy substrate metabolism and thermogenesis in humans.

THE DEFINITION OF BAT

BAT is a heat-producing adipose tissue located in interscapular, subscapular, axillary, perirenal, and periaortic regions in rodents (19). In infants, the predominant interscapular distribution found in small mammals also occurs (20–22), but regresses with age and is lost at adulthood. The typical supraclavicular and paravertebral BAT distribution seen in adults appears to develop with puberty in boys and girls (23, 24). BAT cells differ from white adipose tissue (WAT) cells (25, 26). The former cells contain numerous small lipid vacuoles and a large number of well-developed mitochondria, whereas the latter are characterized by a single large lipid vacuole and a few mitochondria. BAT cells in WAT depots, called "beige" or "brite" adipocytes, have also been shown in rodents and humans (26). Histologically, "beige" cells demonstrate an intermediate phenotype between classical BAT adipocytes and classical white adipocytes (26).

The hallmark of BAT cells at the molecular level in animals and humans alike is the high level of expression of uncoupling protein-1 (UCP1). UCP1 is found in the inner membrane of BAT cells' mitochondria (19, 27). UCP1 uncouples mitochondrial respiration from adenosine-5′-triphosphate (ATP) synthesis (28). When activated, it causes a leak that dissipates the electrochemical proton gradient that builds up across the inner mitochondrial membrane during BAT fatty acid oxidation. This electrochemical proton gradient drives the conversion of adenosine-5′-diphosphate (ADP) to ATP by ATP synthase. As a consequence, the presence of active UCP1 abolishes the negative feedback inhibition exerted by high ATP and/or low ADP levels on mitochondrial Krebs' cycle and respiration, leading to very high rate of fatty acid oxidation that directly produces heat. Because of its large amount of active UCP1 proteins, BAT is thus the only organ that literally can "burn" fat.

UCP1 is activated by long chain fatty acids (19, 28, 29), but the mechanism by which it uncouples mitochondrial respiration has long been debated (30–32). UCP1 is an anion/H^+ symporter that binds avidly long chain fatty acids, making it in effect a proton translocator (33). BAT is richly innervated by sympathetic nervous system efferent fibers and sympathetic activation is the physiological activator of BAT thermogenesis (19, 34–37). The release by these fibers of noradrenaline stimulates BAT intracellular triglyceride (TG) lipolysis, releasing long chain fatty acids that in turn activate UCP1 and BAT thermogenesis (**Figure 1**). We provided *in vivo* experimental evidence for this model by showing that nicotinic acid administration, an inhibitor of intracellular TG lipolysis, blocks acute cold-stimulated BAT thermogenesis in rats (38) and in humans (39). Recent investigations using genetic deletion of genes essential for intracellular TG lipolysis in mice models have, however, casted doubt about the essential role of intracellular TG lipolysis-derived fatty acids to activate BAT thermogenesis (40, 41). However, direct *in vivo* assessment of BAT thermogenesis was not measured and BAT of these genetic mouse models displayed a large increase in utilization of circulating fatty acids and glucose. It is therefore likely that intracellular TG and, if the later are unavailable circulating fatty acids, play an important role for the activation of BAT thermogenesis.

It is clear that BAT cells can stem from different cell lineages and display different molecular signatures depending on whether they are harvested from classical BAT or classical WAT depots (42–45). This molecular signature of supraclavicular BAT depots in humans may also be much more similar to that of "beige" adipocytes than that of "classical BAT" of rodents (46). Despite these differences, UCP1 content and function appear similar between human and mouse BAT (47). The distinct molecular signature of BAT could potentially be exploited for the *in vivo* identification and quantification of BAT. For example, targeting of a relatively BAT specific molecule, programed death ligand-1, was recently proposed for PET imaging and to quantify BAT in mice (48). However, from an integrative physiology and clinical perspective, it is the unique thermogenic potential of BAT, not its molecular signature, that matters. The presence of BAT in human adults has been noticed earlier from pathological investigations (49–51). Despite this early pathological description, the presence of functional BAT in adult humans was widely acknowledged only with the use of positron emission tomography coupled with computed tomography (PET/CT) with the glucose analogue 18-fluoro-deoxyglucose (^{18}FDG) (52–57). It is the very intense metabolic activity of otherwise metabolically quiescent fat tissue, at least with regards to glucose metabolism, that led the scientific

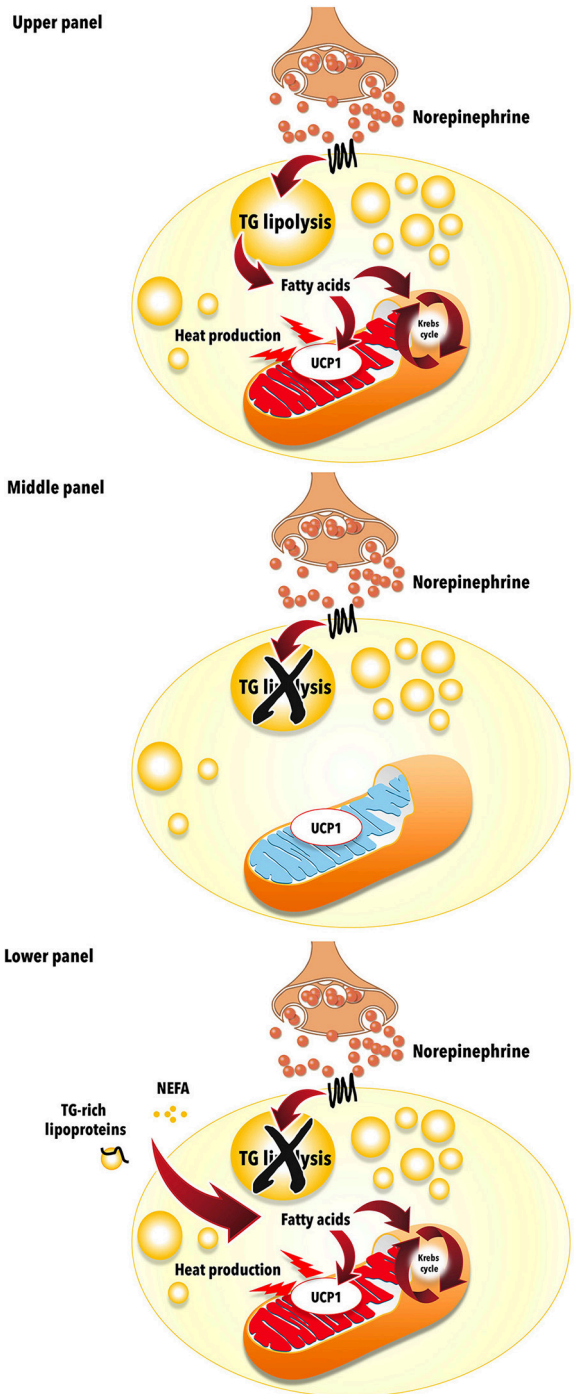

FIGURE 1 | Uncoupling protein 1 (UCP1)-mediated brown adipose tissue thermogenesis. **Upper panel:** Brown adipose tissue UCP1-mediated thermogenesis is activated by fatty acids produced via norepinephrine-induced intracellular triglyceride (TG) lipolysis during cold exposure. **Middle panel:** Acute pharmacological inhibition of intracellular TG lipolysis blunts brown adipose tissue thermogenesis via reduction of intracellular fatty acids availability. **Lower panel:** Genetic deletion-mediated inhibition of intracellular TG lipolysis in brown adipose tissues leads to increased reliance on circulating nonesterified fatty acids (NEFA) and triglyceride (TG)-rich lipoproteins to sustain UCP1-mediated thermogenesis. The mitochondrion illustration was obtained free of copyright from Pixabay (www.pixabay.com, 2018).

community to finally acknowledge BAT as an organ of interest for energy balance and as a potential therapeutic target for obesity and T2D.

Currently, ^{18}FDG PET/CT is considered the "gold-standard" method to identify BAT in humans (58), although BAT glucose metabolism does not accurately reflect BAT thermogenic activity (see section on glucose metabolism below) (59). The presence of BAT is defined according to the combination of two tissue characteristics on static (whole body) ^{18}FDG PET/CT acquisition (**Figure 2**): (1) unusually high ^{18}FDG (glucose) uptake for an adipose tissue, i.e., ^{18}FDG PET standard uptake value normalized for lean mass higher than that of the upper range normally seen in classical WAT; and (2) a tissue radio-density on CT that is compatible with the presence of adipose tissue. Using ^{18}FDG PET/CT, most of the glucose-utilizing BAT volume ("^{18}FDG positive fat") is constituted by multiple small adipose depots scattered in the supraclavicular, paravertebral, pericardial, and suprarenal regions (54, 56, 57, 60). Using ^{18}FDG PET/CT, measured BAT volume in humans varies over two orders of magnitude, from a few to hundreds of milliliters (59). Three-dimensional mapping of adipose tissue depots with ^{18}FDG PET/CT showed that up to 4.3% of total body adipose tissue mass accounts for depots that may display significant glucose uptake upon cold exposure (61). However, the proportion of this adipose tissue mass that was demonstrated as BAT mass using ^{18}FDG PET/CT is very small, especially in obese individuals. It is important to note that accurate quantification of total BAT volume of metabolic activity by the addition of numerous small regions, typically less than 1 cm^3 each, is very challenging using PET for a number of technical reasons that were discussed in more details elsewhere (59, 62). ^{18}FDG positive fat sites are also determined by a series of environmental and biological factors including outdoor temperature preceding PET/CT scanning procedures, age, sex, body fat content, central adiposity, the presence of diabetes, circadian rhythm, and the use of some drugs such as β-adrenergic blockers (54, 55, 60, 63–70). The prevalence of spontaneously detectable ^{18}FDG positive fat sites range from 2 to 7% in large cohorts of patients evaluated for cancer, but reaches 70–100% during experimental cold exposure (58, 59). ^{18}FDG positive BAT volume and/or activity also significantly increases within weeks of cold acclimation (71–74). Glucose uptake in BAT is profoundly influenced by insulin sensitivity (see section on glucose metabolism below). Because of these technical and biological reasons, ^{18}FDG PET/CT therefore likely underestimates true BAT volume in humans, especially in people with obesity and T2D.

Despite emerging methods using other PET tracers (48, 75–79), single-photon emission computed tomography (67, 80), magnetic resonance imaging (MRI), and spectroscopy (MRS) (81–89), near infrared spectroscopy (90, 91), contrast ultrasound (92), microwave radiometry (93), and optoacoustic imaging (94), ^{18}FDG PET/CT currently remains the best method to define the presence and to measure BAT volume in humans (95–98) (99). The lack of a method that directly measure total BAT volume and BAT-specific thermogenesis, however, constitutes an important gap to fill in order to accurately define the true contribution of BAT to energy homeostasis in humans.

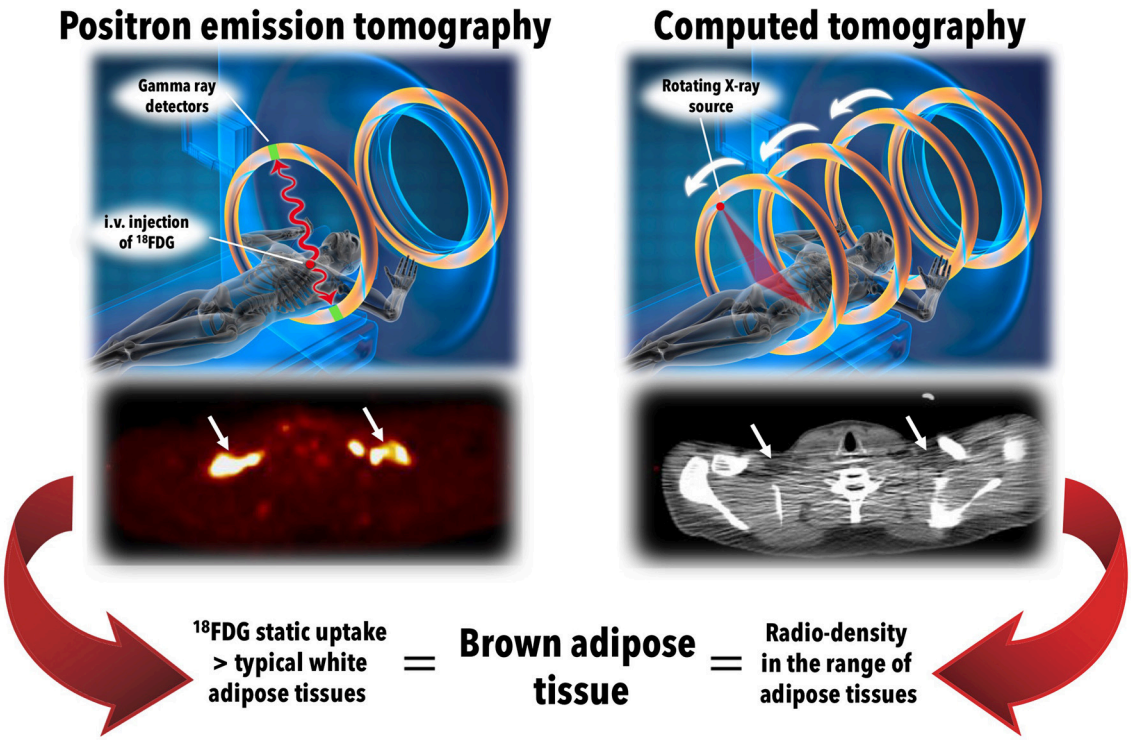

FIGURE 2 | The standard definition of brown adipose tissue *in vivo* in humans. Brown adipose tissue is currently defined *in vivo* in humans by the combination of two radiological features: (1) 18-fluorodeoxyglucose (^{18}FDG) uptake above a set threshold higher than that usually observed in white adipose tissues using positron emission tomography (**left panels**); and (2) a radio-density that is compatible with the presence of adipose tissue using computed tomography (**right panels**). After intravenous (i.v.) injection of ^{18}FDG, whole body (static) positron emission tomography scanning is performed, giving quantitative tissue bio-distribution of the tracer into brown adipose tissues. This tissue tracer uptake is co-registered with tissue radio-density measured using computed tomography. The middle left and right panels show positron emission tomography and computed tomography transverse views, respectively, of supraclavicular brown adipose tissue in a healthy individual during a standardized cooling protocol. Source of illustration: Shutterstock (www.shuterstock.com, 2018, no. 100687138).

ENERGY SUBSTRATES UTILIZATION BY BAT

Glucose

The demonstration of large increase in BAT glucose uptake with the activation of BAT oxidative metabolism led to the suggestion that BAT metabolic activation could be exploited to increase glucose clearance and utilization and treat diabetes (100, 101). This possibility was furthermore supported by recent epidemiological observations showing an association between increased glycosylated hemoglobin and increased incidence of diabetes with higher outdoor temperature (68, 102). Additionally, it was shown that the incidence of gestational diabetes rises by 6% for every 10°C increase in mean 30-day outdoor air temperature (103). Cold-induced whole body glucose disposal was shown to increase only in ^{18}FDG BAT positive individuals (104) and BAT activation with cold exposure is furthermore associated with improved glucose homeostasis and insulin sensitivity in patients with T2D (105, 106).

There are, however, obvious problems with this hypothesis. First, cold exposure increases muscle *glut4* cell membrane expression and stimulates shivering and deep muscle glucose uptake, even when care is applied to limit muscle shivering (105, 107). Therefore, this muscle metabolic activity likely contributes to some cold-induced increase in whole body glucose disposal. Second, although cold-induced BAT glucose uptake *per* volume of tissue is indeed usually higher than that of other tissues in healthy subjects (107, 108), total volume of ^{18}FDG-positive BAT amounts to <150 ml in most healthy individuals (59). ^{18}FDG-positive BAT volume is also much smaller in individuals with obesity and T2D (109). This imposes an important limitation to the capacity of BAT metabolism to significantly impact systemic glucose clearance. For example, using whole body ^{18}FDG PET acquisitions during standardized cold exposure in healthy subjects, we showed that BAT accounted for ∼1% of total body glucose utilization as compared to ∼50% for skeletal muscles (107) (**Figure 3**). Based on calculations that we previously described (110), glucose partitioning was 4, 8, 6, and 10% in the heart, liver, visceral WAT, and sub-cutaneous WAT, respectively (**Figure 4**).

Unfortunately, dynamic ^{18}FDG PET acquisition allowing precise quantification of BAT glucose uptake rate has been used by only a few investigators. The group of University of Turku in Finland has reported BAT glucose uptake rates during acute cold exposure in the order of 90–120 nmol.g^{-1}.min^{-1} in healthy individuals and of 35 nmol.g^{-1}.min^{-1} in obese

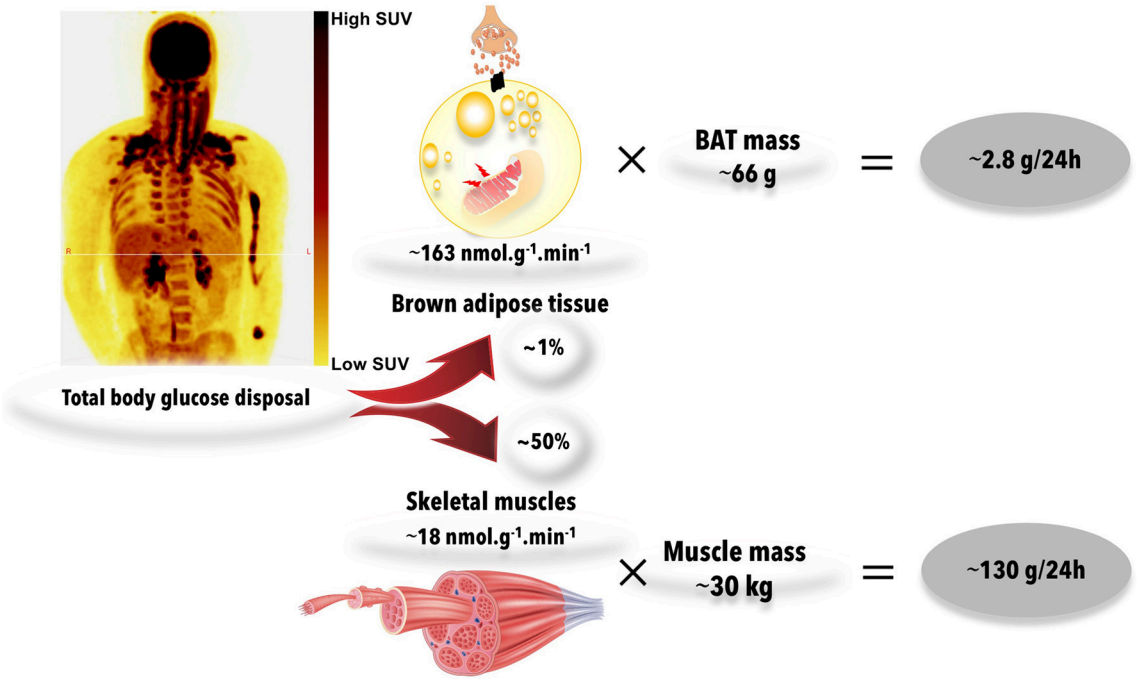

FIGURE 3 | Whole body glucose uptake into brown adipose tissues and muscles during acute cold exposure. During mild cold exposure, glucose uptake is stimulated in brown adipose tissue, but also in several centrally-located skeletal muscles. Brown adipose tissue glucose uptake is ~8-fold higher than that of skeletal muscles, on average, per gram of tissue during mild cold exposure. However, total mass of brown adipose tissue is about 0.2% of that of skeletal muscles. Therefore, brown adipose tissue and skeletal muscle glucose uptake account for ~1 and 50%, respectively, of systemic glucose disposal. The figures presented were calculated from previously published data in young healthy individuals, before cold acclimation (39). BAT, brown adipose tissue; SUV, standard uptake value. Source of muscle illustration: Shutterstock (www.shuterstock.com, 2018, no. 404668558).

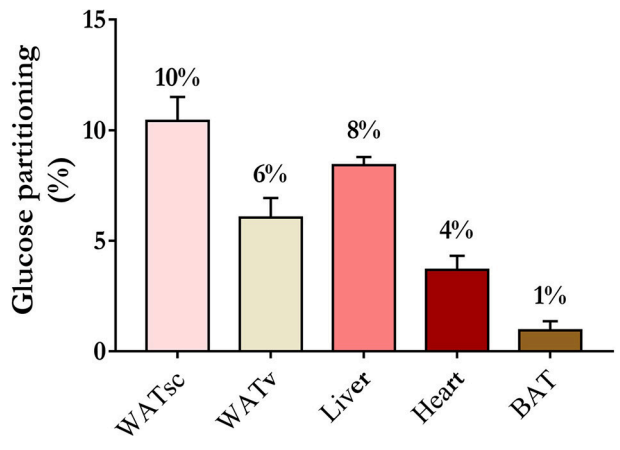

FIGURE 4 | Organ-specific glucose partitioning during acute cold exposure. The figures presented were calculated from a previously published study in young healthy individuals, before cold acclimation (39), based on calculations that we detailed previously (110). BAT, brown adipose tissue; WATsc, sub-cutaneous white adipose tissues; WATv, visceral white adipose tissue.

individuals (57, 111, 112). Our group at *Université de Sherbrooke* reported BAT glucose uptake rates during acute cold exposure at fasting ranging from 80 ± 14 nmol.g^{-1}.min^{-1} in non-cold-acclimated healthy individuals to 209 ± 50 nmol.g^{-1}.min^{-1} in cold-acclimated healthy individuals (39, 73, 108) (**Figure 5** and **Table 1**). We found BAT glucose uptake during cold exposure in the postprandial period in the range of 50 nmol.g^{-1}.min^{-1}, i.e., not very different from those measured in the fasting state (115). Although these rates of glucose uptake are two to three-fold higher *per* volume of tissue than that measured in skeletal muscles, the much larger muscle vs. BAT mass translates into organ-specific uptake that is two orders of magnitude higher in the former (39). Furthermore, we found BAT glucose uptake rates to be much lower in older, overweight subjects without or with T2D, in the range of ~10 nmol.g^{-1}.min^{-1} (109). In absolute terms, we found rates of BAT glucose uptake ranging from ~0.1 μmol/min in overweight individuals without and with T2D to ~3 μmol/min in healthy individuals during acute cold exposure. Using simultaneous quantification of BAT glucose uptake with dynamic ^{18}FDG PET acquisition and systemic glucose utilization with conventional glucose tracer method, we found that acutely cold-activated BAT glucose uptake accounted for <1% of systemic glucose turnover in healthy men (39, 108, 109). It is therefore unlikely that BAT activation may significantly contribute to improve systemic glucose metabolism, especially in subjects with impaired glucose metabolism.

BAT glucose uptake has been extensively used as a surrogate marker of BAT thermogenesis in humans on the basis of correlative observations between BAT thermogenic activity and glucose uptake. Indeed, the presence and metabolic activity

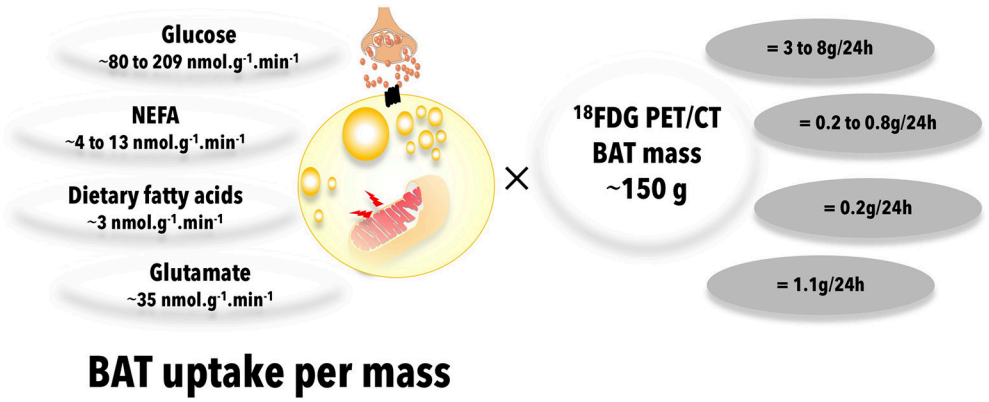

FIGURE 5 | Brown adipose tissue uptake of energy substrates. Total brown adipose tissue uptake of energy substrates is calculated from published quantitative, dynamic positron emission tomography or microdialysis experiments in humans, multiplied by a typical total brown adipose tissue mass reported in the literature. Data from (73, 108, 113), (108, 113, 114), (115), and (116) were used to calculate glucose, NEFA, dietary fatty acid, and glutamate BAT uptake, respectively. ^{18}FDG, 18-fluorodeoxyglucose; BAT, brown adipose tissue; NEFA, nonesterified fatty acids; PET/CT, positron emission tomography coupled with computed tomography.

TABLE 1 | Upper and lower estimates of brown adipose tissue plasma glucose, nonesterified fatty acid, dietary fat, and glutamate uptake rates in humans.

Substrate	Mean uptake (nmol.g^{-1}.min^{-1})	Molar mass (g.mol^{-1})	Absolute uptake assuming BAT mass of 150g (g.day^{-1})	Notes	References
Glucose	80	180.156	3.11	Healthy men, non-cold acclimated, acute cold exposure	(39, 73, 108)
	209		8.13	Healthy men, post-cold acclimation, acute cold exposure	(73)
NEFA	4	275.446	0.24	Obese subjects, room temperature	(114)
	13		0.77	Healthy men, acute cold exposure	(108, 109)
Dietary fat	3	275.446	0.18	Healthy men, postprandial and acute cold exposure	(115)
Glutamate	35	147.13	1.11	Healthy men, acute cold exposure	(116)

NEFA, nonesterified fatty acids.

of ^{18}FDG positive BAT are associated with increased plasma catecholamines and inversely related to central obesity in patients with pheochromocytoma (117). Cold-induced BAT glucose uptake correlates with BAT sympathetic activity *in vivo* (118) and unilateral sympathetic denervation has been shown to reduce supraclavicular BAT glucose uptake in a patient (119). In mice however, β3-adrenergic-stimulated BAT glucose uptake does not need the presence of UCP1 and activation of BAT thermogenesis (120, 121). Extrapolated over a 24 h period, BAT glucose uptake in healthy individuals in our hands sums up only to a maximum utilization of 5 g of glucose, or ∼23 kcal. Obviously, this energy expenditure rate assumes that BAT fully oxidizes the glucose it takes up. The classical studies by Ma and Foster (122), however, demonstrated more than three decades ago that a large fraction of glucose taken up by BAT is metabolized and released as lactate or serves for glyceroneogenesis (123) or perhaps *de novo* lipogenesis and does not contribute to increased BAT oxidative metabolism (**Figure 6**). Activated BAT glucose uptake exceeds increase in blood flow, suggesting non-thermogenic utilization of glucose by BAT in humans (124). A recent study using the adipose tissue microdialysis technique applied to supra-clavicular BAT also demonstrated that a large fraction of glucose taken up by BAT upon acute cold exposure is released as lactate *in vivo* in healthy subjects (116). The later study also independently confirmed the magnitude of glucose uptake in BAT measured by the ^{18}FDG PET dynamic acquisition method. Thus, glucose uptake is not a good method to quantify BAT oxidative metabolism and thermogenesis, even in healthy subjects.

^{18}FDG BAT positive individuals are more insulin sensitive and cold-induced BAT glucose uptake and stimulation of blood flow are blunted in obese individuals (112). BAT glucose uptake is reduced with genetic variants associated with insulin resistance (125), glucocorticoid treatment (126), fasting-induced insulin resistance (127). Chronic ephedrine administration which may induce insulin resistance leads to reduced BAT glucose uptake despite increased weight loss (128). BAT glucose uptake tends to be higher after bariatric surgery-induced weight loss in obese individuals (129, 130). Exercise, which increases muscle glucose uptake and improves whole body insulin sensitivity, does not however necessarily lead to increase in insulin-mediated BAT glucose uptake (131). Insulin stimulates BAT glucose uptake without stimulating blood flow, suggesting that insulin signaling increases BAT glucose uptake independent of BAT thermogenic activation (111). We found that older, overweight individuals without and with T2D display a ∼10-fold reduction in BAT glucose uptake rate vs. young healthy

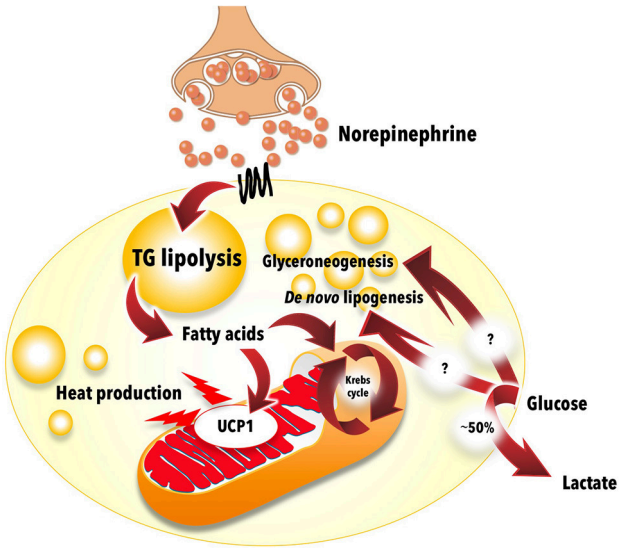

FIGURE 6 | Glucose metabolism in brown adipose tissue. Most of the glucose taken up by brown adipose tissue during cold exposure does not contribute to thermogenesis. Experimental data show that approximately half of the glucose molecules are excreted from brown adipose tissue as lactate. Most of the remaining glucose likely contributes to glycerol production (glyceroneogenesis) and/or fatty acid synthesis (*de novo* lipogenesis) for intracellular triglyceride synthesis. The mitochondrion illustration was obtained free of copyright from Pixabay (www.pixabay.com, 2018).

subjects despite no reduction in BAT NEFA uptake and thermogenic activity upon acute cold exposure (109). Reduced BAT glucose uptake is furthermore associated with increased BAT fat content (88, 109, 132). Thus, as in lean tissues and WAT (133), excess lipid deposition appears to be a marker of BAT insulin resistance. Total BAT volume of ^{18}FDG uptake has been associated with plasma NEFA appearance rate and oxidation and with WAT insulin sensitivity during cold exposure (109, 134). Thus, BAT glucose uptake may be a marker of WAT metabolic flexibility. In the presence of obesity, T2D or other insulin resistance states, BAT glucose uptake is an especially poor surrogate marker for BAT thermogenic activity.

Typical BAT cells expressing UCP1, large mitochondrial content and numerous small lipid vacuoles are present in supraclavicular adipose depots, independent of the presence of spontaneously active BAT based on ^{18}FDG PET (135). Because ^{18}FDG PET/CT is currently the only method capable of measuring BAT volume of metabolic activity, the above considerations clearly point to the absence of a reliable method to quantify BAT volume and, therefore, total thermogenic activity in humans. Unfortunately, all of the figures thus far reported with regards to the contribution of BAT to fatty acid utilization and whole body thermogenesis (see below) have been calculated using total BAT volume from ^{18}FDG PET/CT. Therefore, these figures are likely underestimated, especially in subjects with any degree of insulin resistance.

Circulating Fatty Acids

Utilization of circulating fatty acids by BAT may occur through two different pools in circulation: (1) NEFA; and (2) triglyceride-rich lipoproteins (TRL). Plasma NEFA are produced mostly by WAT, either via intracellular TG lipolysis or via LPL-mediated lipolysis of circulating TRL (i.e., NEFA spillover of TRL into the systemic circulation) (136). The circulating NEFA pool is tightly regulated by the sympathetic system and circulating insulin level via β-adrenergic stimulation and insulin signaling-mediated inhibition, respectively, of intracellular WAT lipolysis. Although plasma membrane fatty acid transporters (137) and local blood flow (138) are known to modify local tissue NEFA uptake, tissue NEFA transport rate is mostly regulated by the plasma NEFA concentration and by the tissue's rate of fatty acid oxidation. TRL include: (1) chylomicrons, produced by the intestine and transporting dietary fatty acids into the circulation; and (2) VLDL, produced by the liver and transporting TG from NEFA and lipoprotein-derived fatty acids recycled in the liver and fatty acids produced *de novo* from carbohydrates in the liver (110, 139). These two TRL circulating pools are mostly regulated through clearance mainly mediated by the activity of LPL, although increase in liver's VLDL-TG secretion rate also contributes to the increase of TG in circulation with obesity and T2D. Local tissue uptake of fatty acids from circulating TRL is mostly under the control of local tissue LPL-mediated lipolysis (140).

As can be expected from stimulation of the sympathetic system activity, acute cold exposure leads to robust increase in plasma NEFA levels and appearance rate (39, 108, 109, 141). Upregulation of genes of lipid utilization was shown in BAT with cold exposure in humans (134). Only a few studies however reported BAT-specific uptake rates of plasma NEFA. In all instances, this has been performed using the PET tracer ^{18}F-fluoro-6-thiaheptadecanoic acid [^{18}FTHA), a long-chain fatty acid analog that is taken up at similar rate than palmitate and that is trapped into the mitochondrial matrix and non-oxidative fatty acid metabolic pathways (142). These characteristics make this tracer, when administered intravenously, an excellent method to measure tissue-specific plasma NEFA uptake rate, but not tissue oxidative or non-oxidative metabolism. Using ^{18}FTHA PET, BAT NEFA uptake was reported similar in healthy (~5.7 nmol.g^{-1}.min^{-1}) and obese subjects (~3.9 nmol.g^{-1}.min^{-1}, non-significant vs. healthy) at room temperature (**Figure 5** and **Table 1**), only slightly higher than the NEFA uptake rate observed in subcutaneous neck WAT (~4.7 and ~3.4 nmol.g^{-1}.min^{-1}, respectively) (114). In the later study, slight but significant increase in BAT NEFA uptake rate was shown 6 months after bariatric surgery in obese individuals (~5.0 nmol.g^{-1}.min^{-1}) (114). Interestingly, BAT NEFA uptake was inversely correlated with age, waist circumference and percent body fat and directly correlated with HDL cholesterol level (114). Using ^{18}FTHA PET, we reported BAT NEFA uptake rates ~13 nmol.g^{-1}.min^{-1} in healthy young men acutely exposed to cold (108, 109) (**Figure 5** and **Table 1**). We found BAT NEFA uptake in the same range as that observed in skeletal muscles and two to three-fold higher than that of subcutaneous WAT of the neck. In contrast to

glucose uptake, BAT NEFA uptake *per* volume of tissue was the same in older, overweight participants without or with T2D compared to healthy young men (109). Because of the overlap in NEFA uptake between BAT and WAT and the limited experience with ^{18}FTHA PET for BAT imaging, it has been thus far impossible to use this method to measure BAT volume, as performed using ^{18}FDG PET/CT. Thus, current estimates of BAT total contribution to NEFA uptake is limited by the use of ^{18}FDG PET/CT to measure BAT volume. Using the latter, we calculated that BAT may metabolize ∼7 μmol.min^{-1} of plasma NEFA in healthy men exposed to cold, but only 0.1 μmol.min^{-1} in older overweight subjects without or with T2D (109). Extrapolated over a 24-h period, this amounts to up to 0.6 g of fat, or <3 kcal. Using simultaneous intravenous stable isotopic palmitate tracer, we calculated that BAT contribution to whole body NEFA metabolism is <1% (108, 109). Given the likely underestimation of BAT volume using ^{18}FDG PET/CT, however, it is possible that BAT contribution to plasma NEFA metabolism could be higher. Cold-induced BAT NEFA uptake was shown to be associated with BAT thermogenesis (143). Therefore, the use of BAT NEFA uptake as a surrogate of BAT thermogenesis remains a viable alternative to glucose uptake. However, the use of PET NEFA tracers that can measure tissue oxidative and non-oxidative metabolic rates, as for example ^{11}C-palmitate, will be needed to ensure that BAT NEFA uptake is quantitatively linked to BAT oxidative metabolism and not fatty acid esterification into BAT TG droplets.

Animal studies showing that activated BAT utilizes a large fraction of circulating TRL led to the hypothesis that active BAT may reduce circulating lipoprotein-TG and cholesterol in humans (144, 145). Angiopoietin-like 4 (ANGPTL4) is down-regulated in BAT during cold exposure in mice, leading to LPL-stimulated TG lipolysis and fatty acid uptake in BAT (146). Activated BAT in mice stimulates the formation of lipoprotein remnants from more buoyant TRL (147). Thus, in rodents, metabolically active BAT exerts significant impact on circulating TRL metabolism. Lower plasma TG and increased HDL-c has been observed in subjects with metabolically active BAT determined by ^{18}FDG PET/CT (148). Experimental acute cold exposure in humans does not however lead to significant reduction in plasma TG levels (108, 109) and may even lead to small increase in TG and cholesterol levels in some instances (106, 141). To our knowledge, we published the only study that measured directly BAT uptake of fatty acids transported by TRL in humans (115). To achieve this, we used the oral ^{18}FTHA PET method that we validated to measure organ-specific dietary fatty acid uptake (149). This method measures relative tissue uptake (partitioning) of dietary fatty acids from direct transport through chylomicron-TG and recycling from WAT metabolism as NEFA [see our recent review for a detailed discussion on the method (110)]. We demonstrated significant, albeit small, BAT dietary fatty acid uptake after administration of a standard meal during acute cold exposure in healthy young men (115). Rate of BAT dietary fatty acid uptake was calculated at ∼3 nmol.g^{-1}.min^{-1} (**Figure 5** and **Table 1**), two to three-fold higher than in the neck subcutaneous WAT and skeletal muscles, respectively. Because of the small BAT volume, again determined using ^{18}FDG PET/CT, BAT only contributed to 0.3% of whole body dietary fatty acid partitioning. In contrast to what has been observed in mice (144), BAT contribution's to whole body dietary fatty acid metabolism was much lower than that of the liver, the heart, skeletal muscles, and even WAT (115). Furthermore, we found that 4-week cold acclimation that significantly increased BAT oxidative metabolism in the participants did not increase BAT dietary fatty acid uptake (115). There was no relation between BAT oxidative metabolism and BAT dietary fatty acid uptake, suggesting that the latter is not a main energy substrate for BAT thermogenesis in humans, at least during acute cold exposure.

Cold-induced changes in plasma concentrations of some non-prominent fatty acids has been reported (106), but there was no demonstration that these changes were indeed due to increase in BAT metabolism. Cold exposure induces 12,13-dihydroxy-9Z-octadecenoic acid production in BAT and in circulation, which in turn may contribute to stimulate BAT fatty acid uptake in mice (150). In a cross-sectional study in healthy men, lysophosphatidylcholine-acyl C16:1 was shown to correlate with BAT volume and metabolic activity assessed by ^{18}FDG PET/CT (151). The physiological and clinical relevance of these observations are unclear at the moment.

Other Substrates in Circulation

BAT expresses glycerol kinase at higher levels than WAT and thus has the potential to utilize glycerol (152). Furthermore, this enzyme's expression is increased in BAT by cold exposure and β-adrenergic stimulation (153, 154). Recent experiments in mice showed that glycerol kinase is a downstream target of PPARγ and that its inhibition leads to reduced UCP1 expression, isoproterenol-stimulated cellular respiration and intracellular TG synthesis (155). A very recent study using adipose tissue microdialysis technique applied to supra-clavicular BAT in humans demonstrated reduced glycerol release by BAT vs. WAT at room temperature, suggesting that glycerol can be recycled to a greater extent in BAT compared to WAT (116).

Weir et al., using the microdialysis technique in supraclavicular BAT, reported significantly higher uptake of glutamate (i.e., ∼35 nmol.g^{-1}.min^{-1}) by this tissue (**Figure 5** and **Table 1**) vs. WAT (i.e., ∼12 nmol.g^{-1}.min^{-1}) upon acute cold exposure (116). Uptake of glutamate in BAT, but not in WAT, was also significantly increased by acute cold exposure in the later study (by ∼10 nmol.g^{-1}.min^{-1}), suggesting the use of this substrate for energy production or, alternatively, for anaplerosis. However, cold-induced increase in glucose uptake was about 10-fold (by ∼120 nmol.g^{-1}.min^{-1}), showing that glutamate is a minor BAT substrate compared to glucose (116). Weir et al. also demonstrated net release of lactate (∼150–200 nmol.g^{-1}.min^{-1}) and pyruvate (∼5 nmol.g^{-1}.min^{-1}) by BAT that increased non significantly with acute cold exposure (by ∼50 and ∼1 nmol.g^{-1}.min^{-1}, respectively) (116). This release of lactate and pyruvate accounted for approximately half of the glucose that was taken up by BAT in response to cold.

To our knowledge, there has been thus far no attempt to quantify BAT utilization rate of ketones or amino acids *in vivo* in humans. Based on cardiac utilization rate of these substrates (156), it is however unlikely that they amount to a significant

proportion of energy substrate utilization compared to fatty acids and glucose under most physiological circumstances.

Intracellular Triglycerides

Intracellular TG content of BAT can be quantified using CT or magnetic resonance imaging and spectroscopy (MRI/MRS). The former technique, as applied currently by most groups in the field of research on brown adipose tissue, is semi-quantitative and can only provide relative content of lipids in a tissue by comparing its radio-density (quantified in Hounsfield units). MRS is the gold-standard method for non-invasive quantification of tissue triglycerides (as opposed to total lipid content) and directly reports TG vs. water content of a tissue (62). BAT CT radio-density is strongly correlated with %TG by MRS (157). MRI can also provide quantitative fat-to-water ratios in BAT, that is lower than that observed in WAT (87). However, because of the large overlap observed in fat-to-water ratios, it is not possible to systematically distinguish metabolically active from non-active BAT or even WAT depots using quantification of adipose tissue fat fraction (158). These methods are sensitive enough to demonstrate association of fat fraction in BAT with biologically and clinically relevant end-points. For example, BAT fat fraction has been associated with systemic insulin resistance, central obesity or T2D (109, 114, 159) and with BAT NEFA uptake (157). BAT fat fraction is also reduced in obese individuals 6 months after bariatric surgery and this reduction is associated with reduction in BMI and insulin resistance (114).

Early observation demonstrated that BAT radio-density increases rapidly during acute cold exposure in rats and humans (160). Numerous studies have now demonstrated that BAT TG content is hydrolyzed within 1–3 h through sympathetically-stimulated intracellular lipolysis, as observed using CT or MRI/MRS to monitor shifts of BAT water-to-fat ratio (73, 87, 108, 109, 115, 132, 158, 159, 161). This reduction in BAT TG content during acute cold exposure is specific to BAT and does not occur in WAT or in shivering muscles (**Figure 7**). It has also been related to whole body insulin sensitivity (132) and with plasma NEFA appearance rate (109). However, in contrast to cold-stimulated BAT glucose uptake, the rapid cold-induced reduction of BAT intracellular TG content is independent of age and T2D status, at equivalent cold exposure (109).

Assuming a total body BAT mass of 168 g [our ^{18}FDG PET/CT data, (108)] and cold-induced reduction of BAT TG fraction from 81 to 76% (i.e., from 136 to 128 g of TG) (132), ∼8 g of TG (∼72 kcal) is mobilized from BAT over 2 hours of very mild cold exposure (**Figure 7**). *In vitro* experiments have suggested that up to 50% of fatty acids hydrolyzed by BAT could be released into the extracellular media (162) and subsequently oxidized or re-esterified elsewhere. It is therefore not possible to determine the precise contribution of fatty acids released by intracellular TG lipolysis to BAT thermogenesis currently, because the intracellular metabolic fate of fatty acids utilized by BAT has not yet been determined *in vivo* in humans. From BAT microdialysis data, glycerol release of BAT during cold exposure amounts to ∼22 nmol.g^{-1}.min^{-1} vs. ∼10 nmol.g^{-1}.min^{-1} at room temperature (116). It is not possible to directly measure tissue fatty acid release using microdialysis, but assuming that 3 fatty acid molecules are produced from intracellular TG *per* molecule of glycerol released, this cold-induced glycerol release (∼12 nmol.g^{-1}.min^{-1}) could represent up to ∼36 nmol.g^{-1}.min^{-1} of fatty acids released by intracellular TG lipolysis in BAT. Assuming a fatty acid composition of ∼30 palmitate, 30 linoleate, and 40% oleate (i.e., an average molar mass of 274.04 g.mol^{-1}) (163), and an average BAT mass of 168 g (108), this would sum up to 2.4 g of fat over 24 h, or ∼21 kcal. These figures, however, are likely underestimated given significant intracellular recycling of glycerol by BAT (116). Therefore, current estimates of fatty acid metabolism from BAT intracellular TG mobilization range between ∼3 to up to 96 g over 24 h with sustained activation. Again, these figures depend on total BAT volume measured using ^{18}FDG PET/CT and, therefore, are likely underestimated. It is also not known what proportion of these fatty acids are oxidized directly by BAT vs. released in circulation *in vivo* in humans.

We have shown in animals (38) and in humans (39) that BAT TG content is the primary source of energy that fuels BAT thermogenesis. We used nicotinic acid to inhibit intracellular BAT TG lipolysis *in vivo*, and to fully arrest BAT water-to-fat ratio shift and oxidative metabolism upon acute cold exposure. We also showed that blocking BAT thermogenesis with nicotinic acid reduced BAT glucose uptake by 26% (i.e., equivalent to 62 mg of glucose over the course of the study), with no change in systemic glucose turnover (39). This reduction in glucose uptake was most likely due to the abolished cold-induced increase in BAT oxidative metabolism with nicotinic acid, since fatty acids from intracellular TG activate UCP1-mediated mitochondrial energy uncoupling (19, 28, 29). Likewise, although not measured in our study, BAT NEFA uptake was likely also driven down by nicotinic acid-mediated inhibition of plasma NEFA appearance from WAT (39). Cold-stimulated BAT blood flow was unaffected by nicotinic acid, demonstrating that cold-induced water-to-fat ratio shift is indeed due to BAT TG disappearance, as opposed to increased blood flow. Importantly, muscle shivering rose reciprocally, compensating for the reduction in BAT thermogenesis. Given the small magnitude of the nicotinic acid-induced reduction of BAT glucose uptake and the currently estimated small contribution of plasma NEFA utilization by BAT in humans (see section Circulating fatty acids above), it is unlikely that these off-target effects of nicotinic acid confounded nicotinic acid effect through inhibition of intracellular TG lipolysis on BAT thermogenesis. In mice, gene deletion of key enzymes of BAT intracellular TG lipolysis induces a major increase in BAT utilization of fatty acids in circulation, thus substituting for BAT TG in order to sustain cold-induced thermogenesis (40, 41). In summary, these *in vivo* evidences suggest a major role for intracellular TG as fuel for BAT thermogenesis.

CONTRIBUTION OF BAT TO THERMOGENESIS AND ENERGY EXPENDITURE

Some indirect evidences suggest a significant role for BAT in cold-induced thermogenesis in humans. Cold-induced increase in whole body energy expenditure is related to the presence

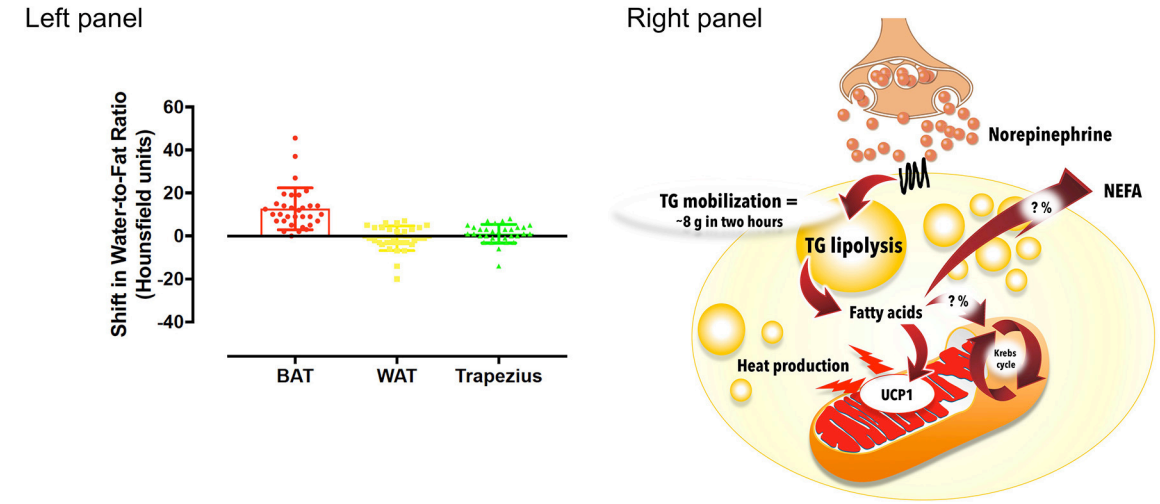

FIGURE 7 | Intracellular triglyceride (TG) mobilization in brown adipose tissue during cold exposure. **Left panel:** Brown adipose tissue (BAT), white adipose tissue (WAT) and trapezius muscle change in radio-density during standardized acute cold exposure from previously published studies of our group (39, 73, 108, 109). **Right panel:** During cold-induced brown adipose tissue metabolic activation, up to 8 g of intracellular triglycerides can be mobilized within 2 h. The metabolic fate of the fatty acids that are mobilized is currently unknown. Although these fatty acids likely constitute most of the energy substrates driving brown adipose tissue thermogenesis, a fraction of them may also be released in circulation to be utilized by other tissues. NEFA, nonesterified fatty acids; UCP1, uncoupling protein 1. The mitochondrion illustration was obtained free of copyright from Pixabay (www.pixabay.com, 2018).

of ^{18}FDG positive BAT (164, 165). Cold-stimulated BAT blood flow is also associated with cold-induced whole body energy expenditure (111) and is blunted in obesity (112). Seasonal variation of cold-induced whole body energy expenditure is larger in ^{18}FDG BAT positive subjects (166). Total ^{18}FDG BAT volume is correlated with higher core body temperature during experimental cooling procedures in one study (167). Living in a mildly cold environment increases energy expenditure and, using ^{18}FDG PET, BAT activation was shown to be a significant determinant of this response (168). We found that inhibition of BAT thermogenesis using nicotinic acid administration leads to reciprocal increase in muscle shivering to combat cold, suggesting a physiologically significant role for BAT in cold-induced thermogenesis (39). We also reported increased skeletal muscle energy coupling with cold acclimation—which is expected to reduce heat production at the same shivering intensity—suggesting an important role for BAT thermogenesis during cold acclimation (113).

Other indirect evidences suggest a role for BAT in energy expenditure and caloric balance in humans. The presence of metabolically active BAT assessed using ^{18}FDG PET/CT is associated with reduced adiposity, especially with aging (56, 164, 169), with higher resting energy expenditure (56, 170), and with less ectopic fat deposition in the liver (171). Athletes, however, tend to have lower BAT volume and activity based on ^{18}FDG PET/CT despite higher whole body energy expenditure (172, 173). UCP1 and beta-3 adrenergic receptor polymorphisms have been associated with lower BAT glucose metabolic activity and increased visceral fat with aging (174). Upstream stimulatory factor 1 deficiency that was shown to activate BAT metabolism in mice, is associated with improved insulin sensitivity, lipid profile and cardiometabolic risk in humans (175). Cold acclimation that increases BAT metabolic activity has been shown to reduce weight in some (72), but not all studies (73, 74, 104, 105, 176). The presence of ^{18}FDG PET BAT predicts capsinoids, catechin-, and caffeine-stimulated whole body energy expenditure (177, 178). Treatment with capsinoids leads to increased BAT glucose uptake and supraclavicular temperature determined by near-infrared spectroscopy (90). Vagus nerve stimulation therapy associated with weight loss increases energy expenditure, which is associated with increased BAT glucose uptake (179). Ephedrine-stimulated BAT metabolic activity is blunted in obesity (180). However, other studies have found that Isoprenaline and ephedrine did not activate BAT metabolic activity despite increasing whole body energy expenditure in lean men (181, 182). Significant BAT contribution to energy expenditure is nevertheless supported by the β3-adrenergic agonist mirabegron-mediated increase in energy expenditure (+203 ± 40 kcal/day), associated with an increase in ^{18}FDG BAT activity (183). However, this treatment is also associated with increased pulse rate and blood pressure, suggesting increased energy expenditure from the cardiovascular system.

Hypothyroidism and hyperthyroidism are conditions that reduce and increase, respectively, whole body energy expenditure. Although one study reported increased BAT glucose uptake with hyperthyroidism (184), others have reported no change in spontaneously occurring (185) or cold-induced BAT metabolic activity (186). Likewise, BAT activation has been observed with cancer cachexia (187, 188). Higher ^{18}FDG BAT volume predicts less adipose tissue accumulation during cancer treatment in children (189). Association was also observed

between reduction of BAT glucose uptake and chemotherapy-induced weight gain in women treated for breast cancer (190).

Some role for BAT in diet-induced thermogenesis has been proposed on the basis of preclinical studies (191). Postprandial increase in energy expenditure was reported to be higher in ^{18}FDG BAT positive vs. BAT negative individuals, with lower respiratory quotient, but without significant change in total 24 h energy expenditure (192). BAT glucose uptake increases after meal intake, but is not related to diet-induced thermogenesis (193). Overfeeding, which increases energy expenditure (194), does not activate BAT glucose uptake (195). We showed no change in postprandial BAT dietary fatty acid and glucose uptake during cold exposure prior to vs. after cold acclimation for 4 weeks that activated BAT thermogenic activity 2 to 3-fold (115). This suggests that cold-induced BAT activation does not change organ-specific postprandial glucose or dietary fatty acid partitioning between organs. Interestingly, our study showed a non-statistically significant trend toward greater cold-induced increase in BAT radio-density postprandially after cold acclimation (115). A very recent study from the Turku group (196) demonstrated meal-induced BAT oxygen consumption equivalent to that observed with mild cold stimulation, together with significant reduction of BAT NEFA uptake and a trend toward higher BAT radio-density. Again, this suggests a more important role for intracellular TG vs. circulating substrates to fuel BAT thermogenesis in humans. The study of U Din et al. (196) estimated at ∼13 kcal *per* day this meal-induced BAT contribution to energy expenditure. However, this calculation extrapolated BAT thermogenesis measured within the first postprandial hour to the entire day, which likely overestimates the contribution of this postprandial BAT thermogenesis to energy expenditure. Animal studies have consistently shown a decrease in classical BAT thermogenesis associated with a decrease in norepinephrine turnover with prolonged fasting (197). To our knowledge, there is no data available on the effect of prolonged or intermittent fasting on BAT activity or thermogenesis in humans.

The fact that BAT significantly takes up glucose, fatty acids, or other energy substrates from the circulation, and that it rapidly mobilizes its own TG content upon cold exposure does not prove that BAT contributes to thermogenesis and, therefore, to energy expenditure. Although direct BAT heat production was suggested by infrared spectroscopy [see (99) for review], this method cannot ascertain that heat difference measured on the surface of the skin overlying supraclavicular BAT is indeed produced by this organ. Likewise, measurement of BAT blood flow is an indirect measure of BAT thermogenesis and was shown in some instances to be dissociated from BAT thermogenesis (39). Supraclavicular BAT biopsies have been used to show higher *ex vivo* thermogenic activity in BAT vs. subcutaneous fat (134, 198). These biopsy methods are however incapable of measuring the true *in vivo* contribution of BAT to thermogenesis.

We used ^{11}C-acetate, a tracer that allows quantification of Krebs' cycle rate through measure of $^{11}CO_2$ BAT production with dynamic PET acquisition to demonstrate more direct evidence of BAT's contribution to cold-induced thermogenesis in humans (108). In the latter study, we found that cold-induced increase in tissue thermogenesis was observed in BAT, but not in neck WAT or skeletal muscles. Using this tracer, we found that BAT thermogenesis can be increased by 2 to 3-fold by acclimation to cold (73, 115), that it is not reduced in T2D vs. healthy individuals despite major reduction in BAT glucose uptake (109), and that it is blunted by inhibition of intracellular TG lipolysis with nicotinic acid (39). However, this method does not directly quantify BAT thermogenesis as $^{11}CO_2$ tissue production is only a surrogate of tissue oxygen consumption.

Direct measurement of BAT oxygen consumption has been performed by the groups of Otto Muzik (199, 200) and that of the Turku PET Centre (143) using $^{15}O_2$ dynamic PET acquisition. Using this method during very mild, short-term [60 min), but poorly controlled cold exposure, Muzik et al. estimated BAT thermogenesis to range between 15 to 25 kcal/day (200). The Turku group reported BAT thermogenesis figures in the range of ∼7 kcal/day at room temperature to ∼10 kcal/day during mild cold exposure in healthy subjects (143). Although BAT oxygen consumption *per* gram of tissue is 2 to 10-fold higher than that observed in WAT and skeletal muscles at room temperature or during mild cold exposure (143), the small BAT total tissue mass makes its relative contribution to basal and cold-induced thermogenesis very small. However, the small BAT total mass was again determined using ^{18}FDG PET, which may lead to underestimation of the contribution of BAT to thermogenesis. Unfortunately, the current PET scanners with a limited field of view ranging from 16 to 24 cm in most instances do not allow total body dynamic acquisition during $^{15}O_2$ or ^{11}C-acetate administration. It is therefore not possible to simultaneously measure oxidative metabolism in all organs and all adipose tissue depots of the body. Furthermore, the very rapid tissue metabolism of these tracers makes impossible sequential dynamic acquisitions in different regions from the same tracer administration and safety considerations limit the number of sequential PET tracer administrations that can be made as part of experimental studies in humans. Therefore, the currently available methods cannot accurately determine total BAT contribution to thermogenesis.

Recently, radiological 3D mapping of possibly metabolically active adipose tissues has suggested a much greater metabolic potential for BAT (61). Using measures of total BAT volume from the later study (ranging from 510 to 2358 ml) (61) with the data on BAT oxidative metabolism *per* gram of tissue measured by U Din et al. (143) [0.007 ml.g^{-1}.min^{-1} at room temperature and 0.012 ml.g^{-1}.min^{-1} during cold exposure), and assuming energy expenditure of 4.801 kcal *per* liter of oxygen consumed (201) and an adipose tissue density of 0.925 g.ml^{-1} (202), BAT contribution to thermogenesis could range from 27–123 kcal *per* day at room temperature to 46–211 kcal *per* day during mild cold exposure (**Figure 8**). Accurate determination of oxidative metabolism over total body BAT volume will be critical to quantify the true potential of BAT in energy expenditure in humans.

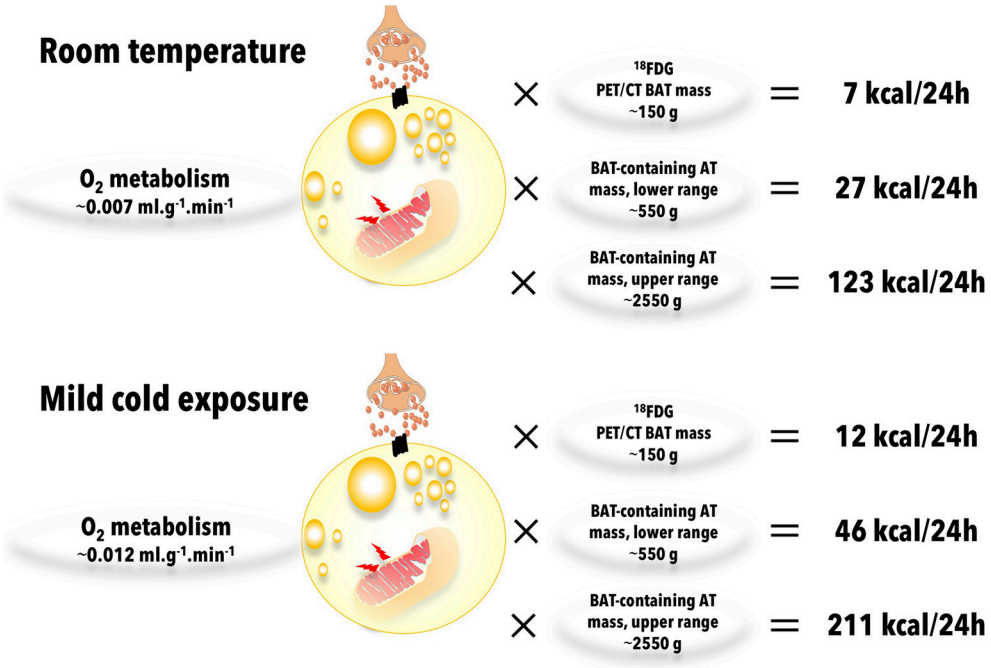

FIGURE 8 | Brown adipose tissue (BAT) oxidative metabolism and contribution to total body energy expenditure. Brown adipose tissue oxygen consumptions are from U Din et al. (143) and brown adipose tissue-containing adipose tissue (AT) mass range is from Leitner et al. (61). Calculations were made assuming energy expenditure of 4.801 kcal *per* liter of oxygen consumed (201) and an adipose tissue density of 0.925 g.ml^{-1} (202). AT, adipose tissue.

IS THERE A CONTRIBUTION OF WAT BROWNING TO THERMOGENESIS AND ENERGY EXPENDITURE?

In addition to the "classical" BAT depots, WAT "browning" (or "*beiging*") may also contribute to thermogenesis, although this is still hotly debated (11, 203–206). A detailed discussion on the mechanisms and cellular adaptations of WAT "browning" is beyond the scope of this review and has been the subject of excellent recent papers (17, 207, 208). "Beige" cells express functional UCP1 and their development appears to be *Prdm16*-dependent, as classical BAT adipocytes (26, 44, 209). In rodents, chronic cold exposure, treatment with β_3-adrenergic or PPARγ agonists, and exercise (38, 209–213) were shown to induce "browning" of WAT preferentially in subcutaneous depots, while reduced "browning" is seen with aging (214).

Although more controversial, there is also some evidence for physiologically significant WAT "browning" in humans. Ageing is associated with reduced white adipose tissue "browning" (169, 215, 216). Perirenal fat in women expresses UCP1 after exposure to cold environment, but difference in UCP1 expression does not translate into change in adipocyte respiration rate (217). Visceral fat "browning" *per* ^{18}FDG PET/CT and histopathological examination has been shown in patients with pheochromocytoma or paraganglioma, associated with increased energy expenditure and diabetes (61, 218). Visceral fat glucose uptake was reduced by alpha blockade and removal of the tumor, associated with weight gain and reversal of diabetes. Cold acclimation however does not lead to "browning" of abdominal subcutaneous WAT in humans (71).

Seeing WAT "browning" using molecular markers, histological examination or even with ^{18}FDG PET does not necessarily imply significant contribution to thermogenesis and energy expenditure. For example, PPARγ agonist treatment in rodents, while inducing WAT "browning" and BAT volume expansion, induces a reduction in sympathetic tone and thermogenesis (219–221). Recently, treatment with thiazolidinedione was shown to increase "browning" of subcutaneous WAT while reducing classical BAT glucose uptake and promoting weight gain *in vivo* in humans (222). We have shown in rats that chronic cold exposure or beta-3 adrenergic agonist treatment, while leading to robust "browning" of WAT as assessed by histological examination, UCP1 gene and protein expression, and mitochondrial DNA content, do not lead to significant increase in WAT thermogenic activity as assessed by ^{11}C-acetate PET (213). This lack of WAT thermogenic activation contrasted with a very robust thermogenic activation of classical BAT simultaneously assessed by ^{11}C-acetate PET in the same animals. It is also important to note that despite significant WAT "browning," WAT UCP1 expression and mitochondrial DNA content remain one to two orders of magnitude below that observed in classical BAT (213, 223, 224). The total volume of WAT "browning" as well as its thermogenic activity have yet to be measured in humans. Based on the data available, it cannot be concluded at the moment that WAT "browning" plays

a significant role in energy expenditure through mitochondrial uncoupling to similar extent as in "classical BAT" depots.

WAT "browning" can nevertheless potentially contribute to energy expenditure through activation of futile metabolic cycles. These cycles include TG lipolysis/esterification, activation of Na^+-K^+-ATPase, creatine/phosphocreatine cycling, and ATP-dependent Ca^{2+} cycling (206, 225, 226). For example, in the absence of UCP1, energy-consuming and heat-producing WAT metabolic adaptations nevertheless occur, including smaller multilocular lipid droplets, larger mitochondrial content, and increased sarcoendoplasmic reticulum Ca^{2+}-ATPase expression (227). These adaptations are also associated with enhanced capacity of WAT to oxidize fatty acids (227), an outcome that can also be obtained using energy restriction or omega-3 supplementation (228). The group of Shingo Kajimura recently demonstrated UCP1-independent increased beige fat *ex vivo* glucose uptake, oxidation and thermogenesis mediated through enhanced ATP-dependent Ca^{2+} cycling from sarco/endoplasmic reticulum Ca^{2+}-ATPase 2b and ryanodine receptor 2 (226). UCP1-independent BAT and beige fat thermogenesis from creatine/phosphocreatine cycling has also been demonstrated *in vitro* in human and murine cells and *in vivo* in mice (225).

During cold exposure, there is substantial activation of intracellular TG lipolysis in BAT and in WAT (see sections on Circulating fatty acids and Intracellular triglycerides above). Major stimulation of TG/fatty acid cycling is therefore expected to occur in both tissues, especially in BAT where glycerol can be utilized directly to a greater extent (116), and where glyceroneogenesis (123) and glycolysis (see section Glucose) occur at much faster rates. Of course, intracellular reesterification of fatty acids is fueled by Krebs' cycle and is accounted for when BAT oxidative metabolism is measured by the $^{15}O_2$ or ^{11}C-acetate PET methods. Nevertheless, part of the fatty acids produced by intracellular lipolysis in BAT could potentially be reesterified in other organs, leading to some additional energy expenditure. The extent of adipose tissue metabolic adaptation to prolonged cold exposure or pharmacological activation of BAT thermogenesis has not been determined thus far. However, adaptation of WAT TG/fatty acid cycling can reach impressive levels in humans. For example, using stable isotope tracer methodology in morbidly obese subjects before and 3 days, 3 months and 1 year after they underwent bariatric surgery, we showed that WAT NEFA production rate and storage capacity can be sustained at the same level despite a more than 3-fold reduction in adipose tissue mass (229), likely resulting in much enhanced energy expenditure *per* adipose tissue mass. Whether such WAT adaptations may contribute to chronic shift of total body energy balance remains to be tested.

CLINICAL IMPLICATIONS OF BAT THERMOGENESIS AND ENERGY SUBSTRATE UTILIZATION

The obesity epidemic is mainly driven by a chronic positive energy balance—a difference of $< 0.1\%$ between daily intake and expenditure—that is sustained over years. The average weight gain which when sustained over young adulthood leads to obesity by middle age is an incremental ∼0.5–0.7 kg per year (230, 231). On a daily basis, this is an energy surplus of a mere ∼12–17 kcal i.e., less than a 5 g cube of sugar, for an average energy density of 8840 kcal/kg of body (232). It is common among people who lose weight, to experience a drop in total energy expenditure—on average, ∼25 kcal/day per kg of weight loss (232). This phenomenon, however, frustrates attempts by most people to maintain healthy weight. The inter-individual variability of this drop in energy expenditure can also significantly influence the rate of diet-induced weight loss (232, 233). When greater weight loss is maintained over time, the health advantages of lifestyle- and/or bariatric surgery-induced weight loss tend to be more significant (234–236). However, when as little as a ∼2-kg weight loss is maintained over a span of 10 years—i.e., an average negative balance of ∼48 kcal per day—the incidence of T2D is curbed by as much as 18–34% (237). Therefore, small shifts in energy balance that are sustained over time can exert major effects on health outcomes.

As discussed above, current estimates of BAT contribution to energy expenditure in humans are in the range of ∼7–25 kcal *per* day based on $^{15}O_2$ PET data recorded at room temperature or during mild acute cold exposure. Although small, these figures may be underestimated because of the current limitations of ^{18}FDG PET to measure total BAT volume, especially in obese and T2D individuals. Whether enhanced TG/fatty acid recycling could add to BAT (or "beige" adipose tissue) thermogenesis has not been quantified in humans. It is also clear that BAT thermogenesis can be recruited to a significant extent with cold acclimation (73) and that its absence or stimulation lead to changes in cold-induced muscle shivering and non-shivering thermogenesis (39, 113). Activation of BAT thermogenesis may therefore be useful for people with occupational cold exposure. Attempts to activate BAT using cold over a prolonged period of time in humans did not result in weight loss in most (73, 74, 104, 105, 176) but not all studies (72), likely because of compensatory increase in energy intake. Circulating endocannabinoids increase with cold exposure (238), suggesting a possible mechanism for cold-induced increase in energy intake. It is also important to note that obese individuals need to reach lower skin temperature to induce cold-induced thermogenesis, due to increased body heat generation (i.e., increased resting energy expenditure driven by larger lean mass), not the generally falsely assumed increased fat layer insulation (239). The well-documented beneficial effect of chronic cold exposure on total body insulin sensitivity (74, 104, 105, 176) is driven by muscle, not BAT thermogenic activity (107). Therefore, cold-induced activation of BAT thermogenesis cannot by itself be proposed to shift caloric balance in humans. However, the possibility that it may serve as an adjunct preventive or therapeutic avenue to lifestyle interventions and/or appetite-suppressant drugs for obesity begs further investigations.

Rodent studies have suggested that BAT and/or WAT "browning" may be implicated in some of the beneficial metabolic effects of physical exercise [see (208, 240–242) for recent reviews on this topic]. At least four mechanisms have been evoked to drive such effects. First, physical exercise is associated with increased adrenergic stimulation, which may lead to direct

activation of BAT thermogenesis and WAT "browning." Second, exercise induces secretion of myokines such as irisin or meteorin like, cardiac natriuretic peptides, and fibroblast growth factor 21, that have all been implicated in BAT metabolic activity (243–245). Third, BAT-derived circulating factor such as IL-6 (246) or the recently discovered 12,13 diHOME (247) have been implicated in systemic metabolic improvement in mice. Fourth, exercise may lead to improved leptin and insulin signaling in the brain, with enhanced pro-opio-melanocortin neurons activation leading to WAT browning (241). However, exercise has long been shown to reduce cold-induced thermogenesis and cold acclimation in rats (248, 249), likely because heat produced by exercise down-regulates the sympathetic stimulatory output signal to BAT. As mentioned above, athletes have lower BAT volume and activity based on ^{18}FDG PET/CT (172, 173) and BAT glucose uptake is reduced following short-term exercise training in healthy men (130). Different exercise duration, intensity and type can result in different adrenergic and systemic stress responses. Furthermore, as discussed above, BAT glucose uptake cannot be taken as a reliable index of BAT thermogenesis. To our knowledge, there is no study published in humans that has measured BAT thermogenesis *per se* in response to exercise. It is therefore difficult to draw definitive conclusions on the effect of exercise on BAT thermogenic activity in humans given the data currently available.

From epidemiological (retrospective) studies that have been published, there seems to be a graded South-to-North incremental prevalence of spontaneously active BAT. For example, the reported prevalence in Boston (latitude: 42°21′30″ North) is 4% (54) whereas it is 6.8% in Sherbrooke (latitude: 45°24′30″ North) (60). As it has been discussed above, cold acclimation leads to clear increase in BAT thermogenic activity and capacity in humans (73) and, therefore, cold exposure is an important driver of this South-to-North incremental prevalence of metabolically active BAT. Whether ethnic background differences may explain changes in BAT metabolic activity is more controversial. For example, direct comparison between subjects of South Asian vs. Europid descent showed either no change in BAT ^{18}FDG uptake (250), or reduced BAT ^{18}FDG volume (but not activity) (170). In the latter study, all participants were Dutch (therefore living at the same latitude) and participants from South Asian descent also displayed lower resting energy expenditure and lower cold-induced non-shivering thermogenesis. The same research group later demonstrated that this blunted thermogenic response in subjects from South Asian descent may be due to increased endocannabinoid tone (238). From the limited data available, we can conclude that cold exposure is clearly linked to differences of BAT activity between different populations, and that genetic/ethnic background differences may also play a role in modulating BAT metabolic responses. More data are needed to address this issue.

Despite encouraging results of BAT metabolic activation with a single-dose administration of mirabegron, a beta-3 adrenergic agonist (183), this class of agents has proven ineffective for the treatment of obesity or T2D in early clinical trials (251, 252). Furthermore, cardiovascular safety is of concern given the increase in heart rate and blood pressure observed with this class of medication, likely mediated through beta-1 and/or beta-2 spillover effects. Capsinoids, catechins and caffeine activate BAT glucose uptake and whole body energy expenditure (177, 178), but with variable results on clinical outcomes (253–256). The effect of beta-3 adrenergic agonists, capsinoids, and catechins on BAT thermogenesis however remains to be determined in humans. The intriguing possibility that BAT thermogenesis could be used as a *personalized medicine* approach to guide the use of these agents for the prevention or treatment of obesity also remains unexplored.

CONCLUSION

BAT is a fascinating organ that possesses a very large thermogenic potential *per* mass of tissue. This tissue has an astounding capacity to rapidly mobilize its own TG content upon cold exposure. Fatty acids from this intense intracellular lipolysis are likely the main substrates for BAT thermogenesis, although the metabolic fate of fatty acids in BAT *in vivo* in humans has not yet been reported. The contribution of BAT and "beige" adipose tissue to thermogenesis through accelerated TG/fatty acid cycling also need to be quantified. BAT is of clear physiological relevance for cold-induced thermogenesis and is integral to a multisystem adaptive response to cold. The current estimated contribution of BAT to energy expenditure is however low due to its small volume measured using ^{18}FDG PET. The latter method has major limitations for accurate measurement of BAT volume, likely leading to underestimation of the true contribution of this tissue to thermogenesis, especially in individuals with obesity and T2D. The current estimates of BAT thermogenesis are at the lower end of energy expenditure shifts that could lead to clinical benefits if sustained without off-target side-effects over the long term. Currently estimated plasma glucose, NEFA or lipoprotein utilization by BAT is also too low to be deemed of clinical relevance to treat T2D or lipid disorders. The development of novel imaging methods for accurate quantification of BAT volume is however required to delineate the true potential of targeting BAT thermogenesis to prevent and/or treat cardiometabolic disorders. With the demonstration of a slightly higher contribution to thermogenesis, it is still possible that metabolic activation of BAT could serve as an effective adjunct therapeutic target to existing treatments for obesity and T2D. Whether monitoring of the effect of clinical interventions on BAT thermogenesis may help personalize treatment selection for obesity and/or T2D also needs to be addressed in future studies.

AUTHOR CONTRIBUTIONS

AC wrote the first draft of the manuscript and drafted all figures, except **Figure 4**. DB critically reviewed the manuscript, drafted **Figure 4**, and contributed to draft **Figures 3**, **7**. KV, DR, FH, and ÉT critically reviewed the manuscript and contributed to draft **Figure 2**.

ACKNOWLEDGMENTS

AC holds the GSK Chair in Diabetes of Université de Sherbrooke. Original work from our group in the field of human brown adipose tissue metabolism has been funded by grants from the Canadian Diabetes Association, the Canadian Institutes of Health Research (Grant no. 299962) and the Natural Sciences and Engineering Research Council of Canada. KV received financial support from Academy of Finland (259926). We thank Ms. Anick Turgeon for drafting the illustrations of **Figures 1–8**.

REFERENCES

1. GBD 2015 Obesity Collaborators, Afshin A, Forouzanfar MH, Reitsma MB, Sur P, Estep K, et al. Health effects of overweight and obesity in 195 Countries over 25 years. *N Engl J Med* (2017) 377:13–27. doi: 10.1056/NEJMoa1614362
2. Plourde CE, Grenier-Larouche T, Caron-Dorval D, Biron S, Marceau S, Lebel S, et al. Biliopancreatic diversion with duodenal switch improves insulin sensitivity and secretion through caloric restriction. *Obesity* (2014) 22:1838–46. doi: 10.1002/oby.20771
3. Grenier-Larouche T, Carreau AM, Carpentier AC. Early metabolic improvement after bariatric surgery: the first steps toward remission of type 2 diabetes. *Can J Diabetes* (2017) 41:418–25. doi: 10.1016/j.jcjd.2016.10.013
4. Lean ME, Leslie WS, Barnes AC, Brosnahan N, Thom G, Mccombie L, et al. Primary care-led weight management for remission of type 2 diabetes (DiRECT): an open-label, cluster-randomised trial. *Lancet* (2017) 391:513–630. doi: 10.1016/S0140-6736(17)33102-1
5. Narayanaswami V, Dwoskin LP. Obesity: current and potential pharmacotherapeutics and targets. *Pharmacol Ther.* (2017) 170:116–47. doi: 10.1016/j.pharmthera.2016.10.015
6. Khera R, Murad MH, Chandar AK, Dulai PS, Wang Z, Prokop LJ, et al. Association of pharmacological treatments for obesity with weight loss and adverse events: a systematic review and meta-analysis. *JAMA* (2016) 315:2424–34. doi: 10.1001/jama.2016.7602
7. Foster DO, Frydman ML. Tissue distribution of cold-induced thermogenesis in conscious warm- or cold-acclimated rats reevaluated from changes in tissue blood flow: the dominant role of brown adipose tissue in the replacement of shivering by nonshivering thermogenesis. *Can J Physiol Pharmacol.* (1979) 57:257–70. doi: 10.1139/y79-039
8. Heldmaier G, Buchberger A. Sources of heat during nonshivering thermogenesis in Djungarian hamsters: a dominant role of brown adipose tissue during cold adaptation. *J Comp Physiol B* (1985) 156:237–45. doi: 10.1007/BF00695778
9. Himms-Hagen J. Brown adipose tissue thermogenesis: interdisciplinary studies. *FASEB J.* (1990) 4:2890–8. doi: 10.1096/fasebj.4.11.2199286
10. Klingenspor M. Cold-induced recruitment of brown adipose tissue thermogenesis. *Exp Physiol.* (2003) 88:141–8. doi: 10.1113/eph8802508
11. Blondin DP, Carpentier AC. The role of BAT in cardiometabolic disorders and aging. *Best Pract Res Clin Endocrinol Metab.* (2016) 30:497–513. doi: 10.1016/j.beem.2016.09.002
12. Schlein C, Heeren J. Implications of thermogenic adipose tissues for metabolic health. *Best Pract Res Clin Endocrinol Metab.* (2016) 30:487–96. doi: 10.1016/j.beem.2016.08.002
13. Schrauwen P, Van Marken Lichtenbelt WD. Combatting type 2 diabetes by turning up the heat. *Diabetologia* (2016) 59:2269–79. doi: 10.1007/s00125-016-4068-3
14. Virtanen KA. The rediscovery of BAT in adult humans using imaging. *Best Pract Res Clin Endocrinol Metab.* (2016) 30:471–7. doi: 10.1016/j.beem.2016.09.001
15. Scheele C, Nielsen S. Metabolic regulation and the anti-obesity perspectives of human brown fat. *Redox Biol.* (2017) 12:770–5. doi: 10.1016/j.redox.2017.04.011
16. Wolf Y, Boura-Halfon S, Cortese N, Haimon Z, Sar Shalom H, Kuperman Y, et al. Brown-adipose-tissue macrophages control tissue innervation and homeostatic energy expenditure. *Nat Immunol.* (2017) 18:665–74. doi: 10.1038/ni.3746
17. Villarroya F, Cereijo R, Villarroya J, Gavalda-Navarro A, Giralt M. Toward an understanding of how immune cells control brown and beige adipobiology. *Cell Metab.* (2018) 27:954–61. doi: 10.1016/j.cmet.2018.04.006
18. Bolus WR, Hasty AH. Contributions of innate type 2 inflammation to adipose function. *J Lipid Res.* (2018). doi: 10.1194/jlr.R085993
19. Cannon B, Nedergaard J. Brown adipose tissue: function and physiological significance. *Physiol Rev.* (2004) 84:277–359. doi: 10.1152/physrev.00015.2003
20. Aherne W, Hull D. Brown adipose tissue and heat production in the newborn infant. *J PatholBacteriol.* (1966) 91:223–34. doi: 10.1002/path.1700910126
21. Hu HH, Tovar JP, Pavlova Z, Smith ML, Gilsanz V. Unequivocal identification of brown adipose tissue in a human infant. *J Magn Reson Imaging* (2012) 35:938–42. doi: 10.1002/jmri.23531
22. Lidell ME, Betz MJ, Dahlqvist Leinhard O, Heglind M, Elander L, Slawik M, et al. Evidence for two types of brown adipose tissue in humans. *Nat Med.* (2013) 19:631–4. doi: 10.1038/nm.3017
23. Drubach LA, Palmer EL, Connolly LP, Baker A, Zurakowski D, et al. Pediatric brown adipose tissue: detection, epidemiology, and differences from adults. *J Pediatr.* (2011) 159:939–44. doi: 10.1016/j.jpeds.2011.06.028
24. Gilsanz V, Smith ML, Goodarzian F, Kim M, Wren TA, Hu HH. Changes in brown adipose tissue in boys and girls during childhood and puberty. *J Pediatr.* (2012) 160:604–9.e601. doi: 10.1016/j.jpeds.2011.09.035
25. Cinti S. The adipose organ: morphological perspectives of adipose tissues. *Proc Nutr Soc.* (2001) 60:319–28. doi: 10.1079/PNS200192
26. Smorlesi A, Frontini A, Giordano A, Cinti S. The adipose organ: white-brown adipocyte plasticity and metabolic inflammation. *Obes Rev.* (2012) 13(Suppl. 2):83–96. doi: 10.1111/j.1467-789X.2012.01039.x
27. Ricquier D. Respiration uncoupling and metabolism in the control of energy expenditure. *Proc Nutr Soc.* (2005) 64:47–52. doi: 10.1079/PNS2004408
28. Nicholls DG, Locke RM. Thermogenic mechanisms in brown fat. *Physiol Rev.* (1984) 64:1–64. doi: 10.1152/physrev.1984.64.1.1
29. Mozo J, Emre Y, Bouillaud F, Ricquier D, Criscuolo F. Thermoregulation: what role for UCPs in mammals and birds? *Bio sci Rep.* (2005) 25:227–49. doi: 10.1007/s10540-005-2887-4
30. Garlid KD, Jaburek M, Jezek P. The mechanism of proton transport mediated by mitochondrial uncoupling proteins. *FEBS Lett.* (1998) 438:10–4. doi: 10.1016/S0014-5793(98)01246-0
31. Klingenberg M, Echtay KS. Uncoupling proteins: the issues from a biochemist point of view. *Biochim Biophys Acta* (2001) 1504:128–43. doi: 10.1016/S0005-2728(00)00242-5
32. Gonzalez-Barosso MMRE. The role of fatty acids in the activity of the uncoupling protein. *Curr Chem Biol* (2009) 3:180–8. doi: 10.1079/PNS2003336
33. Fedorenko A, Lishko PV, Kirichok Y. Mechanism of fatty-acid-dependent UCP1 uncoupling in brown fat mitochondria. *Cell* (2012) 151:400–13. doi: 10.1016/j.cell.2012.09.010
34. Bachman ES, Dhillon H, Zhang CY, Cinti S, Bianco AC, Kobilka BK, et al. betaAR signaling required for diet-induced thermogenesis and obesity resistance. *Science* (2002) 297:843–5. doi: 10.1126/science.1073160
35. Jimenez M, Leger B, Canola K, Lehr L, Arboit P, Seydoux J, et al. Beta(1)/beta(2)/beta(3)-adrenoceptor knockout mice are obese and cold-sensitive but have normal lipolytic responses to fasting. *FEBS Lett.* (2002) 530:37–40. doi: 10.1016/S0014-5793(02)03387-2
36. Lowell BB, Bachman ES. Beta-Adrenergic receptors, diet-induced thermogenesis, and obesity. *J Biol Chem.* (2003) 278:29385–8. doi: 10.1074/jbc.R300011200
37. Collins S, Cao W, Robidoux J. Learning new tricks from old dogs: beta-adrenergic receptors teach new lessons on firing up adipose tissue metabolism. *Mol Endocrinol.* (2004) 18:2123–31. doi: 10.1210/me.2004-0193

38. Labbe SM, Caron A, Bakan I, Laplante M, Carpentier AC, Lecomte R, et al. *in vivo* measurement of energy substrate contribution to cold-induced brown adipose tissue thermogenesis. *FASEB J* (2015) 29:2046–58. doi: 10.1096/fj.14-266247
39. Blondin DP, Frisch F, Phoenix S, Guerin B, Turcotte EE, Haman F, et al. Inhibition of intracellular triglyceride lipolysis suppresses cold-induced brown adipose tissue metabolism and increases shivering in humans. *Cell Metab.* (2017) 25:438–47. doi: 10.1016/j.cmet.2016.12.005
40. Schreiber R, Diwoky C, Schoiswohl G, Feiler U, Wongsiriroj N, Abdellatif M, et al. Cold-induced thermogenesis depends on ATGL-mediated lipolysis in cardiac muscle, but not brown adipose tissue. *Cell Metab.* (2017) 26:753–63.e757. doi: 10.1016/j.cmet.2017.09.004
41. Shin H, Ma Y, Chanturiya T, Cao Q, Wang Y, Kadegowda AKG, et al. Lipolysis in brown adipocytes is not essential for cold-induced thermogenesis in mice. *Cell Metab* (2017) 26:764–77.e765. doi: 10.1016/j.cmet.2017.09.002
42. Seale P, Bjork B, Yang W, Kajimura S, Chin S, Kuang S, et al. PRDM16 controls a brown fat/skeletal muscle switch. *Nature* (2008) 454:961–7. doi: 10.1038/nature07182
43. Seale P, Kajimura S, Spiegelman BM. Transcriptional control of brown adipocyte development and physiological function–of mice and men. *Genes Dev.* (2009) 23:788–97. doi: 10.1101/gad.1779209
44. Kajimura S, Seale P, Spiegelman BM. Transcriptional control of brown fat development. *Cell Metab.* (2010) 11:257–62. doi: 10.1016/j.cmet.2010.03.005
45. Walden TB, Hansen IR, Timmons JA, Cannon B, Nedergaard J. Recruited vs. nonrecruited molecular signatures of brown, "brite," and white adipose tissues. *Am J Physiol Endocrinol Metab.* (2012) 302:E19–31. doi: 10.1152/ajpendo.00249.2011
46. Wu J, Bostrom P, Sparks LM, Ye L, Choi JH, Giang AH, et al. Beige adipocytes are a distinct type of thermogenic fat cell in mouse and human. *Cell* (2012) 150:366–76. doi: 10.1016/j.cell.2012.05.016
47. Porter C, Herndon DN, Chondronikola M, Chao T, Annamalai P, Bhattarai N, et al. Human and mouse brown adipose tissue mitochondria have comparable UCP1 function. *Cell Metab.* (2016) 24:246–55. doi: 10.1016/j.cmet.2016.07.004
48. Ingram JR, Dougan M, Rashidian M, Knoll M, Keliher EJ, Garrett S, et al. PD-L1 is an activation-independent marker of brown adipocytes. *Nat Commun.* (2017) 8:647. doi: 10.1038/s41467-017-00799-8
49. Hull D. The structure and function of brown adipose tissue. *Br Med Bull.* (1966) 22:92–6. doi: 10.1093/oxfordjournals.bmb.a070447
50. Heaton JM. The distribution of brown adipose tissue in the human. *J Anat.* (1972) 112:35–9.
51. Huttunen P, Hirvonen J, Kinnula V. The occurrence of brown adipose tissue in outdoor workers. *Eur J Appl Physiol Occup Physiol.* (1981) 46:339–45. doi: 10.1007/BF00422121
52. Hany TF, Gharehpapagh E, Kamel EM, Buck A, Himms-Hagen J, Von Schulthess GK. Brown adipose tissue: a factor to consider in symmetrical tracer uptake in the neck and upper chest region. *EurJ Nucl Med Mol Imaging* (2002) 29:1393–8. doi: 10.1007/s00259-002-0902-6
53. Nedergaard J, Bengtsson T, Cannon B. Unexpected evidence for active brown adipose tissue in adult humans. *AmJ Physiol Endocrinol Metab.* (2007) 293:E444–52. doi: 10.1152/ajpendo.00691.2006
54. Cypess AM, Lehman S, Williams G, Tal I, Rodman D, Goldfine AB, et al. Identification and importance of brown adipose tissue in adult humans. *N Engl J Med.* (2009) 360:1509–17. doi: 10.1056/NEJMoa0810780
55. Saito M, Okamatsu-Ogura Y, Matsushita M, Watanabe K, Yoneshiro T, Nio-Kobayashi J, et al. High incidence of metabolically active brown adipose tissue in healthy adult humans: effects of cold exposure and adiposity. *Diabetes* (2009) 58:1526–31. doi: 10.2337/db09-0530
56. Van Marken Lichtenbelt WD, Vanhommerig JW, Smulders NM, Drossaerts JM, Kemerink GJ, Bouvy ND, et al. Cold-activated brown adipose tissue in healthy men. *N Engl J Med.* (2009) 360:1500–8. doi: 10.1056/NEJMoa0808718
57. Virtanen KA, Lidell ME, Orava J, Heglind M, Westergren R, Niemi T, et al. Functional brown adipose tissue in healthy adults. *N Engl J Med.* (2009) 360:1518–25. doi: 10.1056/NEJMoa0808949
58. Chen KY, Cypess AM, Laughlin MR, Haft CR, Hu HH, Bredella MA, et al. Brown adipose reporting criteria in imaging studies (BARCIST 1.0): recommendations for standardized FDG-PET/CT experiments in humans. *Cell Metab.* (2016) 24:210–22. doi: 10.1016/j.cmet.2016.07.014
59. Blondin DP, Labbe SM, Turcotte EE, Haman F, Richard D, Carpentier AC. A critical appraisal of brown adipose tissue metabolism in humans. *Clin Lipidol.* (2015) 10:259–80. doi: 10.2217/clp.15.14
60. Ouellet V, Routhier-Labadie A, Bellemare W, Lakhal-Chaieb L, Turcotte E, Carpentier AC, et al. Outdoor temperature, age, sex, body mass index, and diabetic status determine the prevalence, mass, and glucose-uptake activity of 18F-FDG-detected BAT in humans. *J Clin Endocrinol Metab.* (2011) 96:192–9. doi: 10.1210/jc.2010-0989
61. Leitner BP, Huang S, Brychta RJ, Duckworth CJ, Baskin AS, Mcgehee S, et al. Mapping of human brown adipose tissue in lean and obese young men. *Proc Natl Acad Sci USA.* (2017) 114:8649–54. doi: 10.1073/pnas.1705287114
62. Schrauwen-Hinderling VB, Carpentier AC. Molecular imaging of postprandial metabolism. *J Appl Physiol.* (2018) 124:504–11. doi: 10.1152/japplphysiol.00212.2017
63. Parysow O, Mollerach AM, Jager V, Racioppi S, San Roman J, Gerbaudo VH. Low-dose oral propranolol could reduce brown adipose tissue F-18 FDG uptake in patients undergoing PET scans. *Clin Nucl Med.* (2007) 32:351–7. doi: 10.1097/01.rlu.0000259570.69163.04
64. Soderlund V, Larsson SA, Jacobsson H. Reduction of FDG uptake in brown adipose tissue in clinical patients by a single dose of propranolol. *Eur J Nucl Med Mol Imaging* (2007) 34:1018–22. doi: 10.1007/s00259-006-0318-9
65. Lee P, Greenfield JR, Ho KK, Fulham MJ. A critical appraisal of the prevalence and metabolic significance of brown adipose tissue in adult humans. *Am J Physiol Endocrinol Metab.* (2010) 299:E601–6. doi: 10.1152/ajpendo.00298.2010
66. Persichetti A, Sciuto R, Rea S, Basciani S, Lubrano C, Mariani S, et al. Prevalence, mass, and glucose-uptake activity of (1)(8)F-FDG-detected brown adipose tissue in humans living in a temperate zone of Italy. *PLoS ONE* (2013) 8:e63391. doi: 10.1371/journal.pone.0063391
67. Bahler L, Deelen JW, Hoekstra JB, Holleman F, Verberne HJ. Seasonal influence on stimulated BAT activity in prospective trials: a retrospective analysis of BAT visualized on 18F-FDG PET-CTs and 123I-mIBG SPECT-CTs. *J Appl Physiol.* (2016) 120:1418–23. doi: 10.1152/japplphysiol.00008.2016
68. Lee P, Bova R, Schofield L, Bryant W, Dieckmann W, Slattery A, et al. Brown adipose tissue exhibits a glucose-responsive thermogenic biorhythm in humans. *Cell Metab.* (2016) 23:602–9. doi: 10.1016/j.cmet.2016.02.007
69. Green AL, Bagci U, Hussein S, Kelly PV, Muzaffar R, Neuschwander-Tetri BA, et al. Brown adipose tissue detected by PET/CT imaging is associated with less central obesity. *Nucl Med Commun.* (2017) 38:629–35. doi: 10.1097/MNM.0000000000000691
70. Steinberg JD, Vogel W, Vegt E. Factors influencing brown fat activation in FDG PET/CT: a retrospective analysis of 15,000+ cases. *Br J Radiol.* (2017) 90:20170093. doi: 10.1259/bjr.20170093
71. Van Der Lans AA, Hoeks J, Brans B, Vijgen GH, Visser MG, Vosselman MJ, et al. Cold acclimation recruits human brown fat and increases nonshivering thermogenesis. *J Clin Invest.* (2013) 123:3395–403. doi: 10.1172/JCI68993
72. Yoneshiro T, Aita S, Matsushita M, Kayahara T, Kameya T, Kawai Y, et al. Recruited brown adipose tissue as an antiobesity agent in humans. *J Clin Invest.* (2013) 123:3404–8. doi: 10.1172/JCI67803
73. Blondin DP, Labbe SM, Tingelstad HC, Noll C, Kunach M, Phoenix S, et al. Increased brown adipose tissue oxidative capacity in cold-acclimated humans. *J Clin Endocrinol Metab.* (2014) 99:E438–46. doi: 10.1210/jc.2013-3901
74. Hanssen MJ, Van Der Lans AA, Brans B, Hoeks J, Jardon KM, Schaart G, et al. Short-term cold acclimation recruits brown adipose tissue in obese humans. *Diabetes* (2016) 65:1179–89. doi: 10.2337/db15-1372
75. Eriksson O, Mikkola K, Espes D, Tuominen L, Virtanen K, Forsback S, et al. The cannabinoid receptor-1 is an imaging biomarker of brown adipose tissue. *J Nucl Med.* (2015) 56:1937–41. doi: 10.2967/jnumed.115.156422
76. Hwang JJ, Yeckel CW, Gallezot JD, Aguiar RB, Ersahin D, Gao H, et al. Imaging human brown adipose tissue under room temperature conditions with (11)C-MRB, a selective norepinephrine transporter PET ligand. *Metabolism* (2015) 64:747–55. doi: 10.1016/j.metabol.2015.03.001

77. Madar I, Naor E, Holt D, Ravert H, Dannals R, Wahl R. Brown adipose tissue response dynamics: *in vivo* insights with the voltage sensor 18F-fluorobenzyl triphenyl phosphonium. *PLoS ONE* (2015) 10:e0129627. doi: 10.1371/journal.pone.0129627
78. Muzik O, Mangner TJ, Leonard WR, Kumar A, Granneman JG. Sympathetic innervation of cold-activated brown and white fat in lean young adults. *J Nucl Med.* (2017) 58:799–806. doi: 10.2967/jnumed.116.180992
79. Ran C, Albrecht DS, Bredella MA, Yang J, Yang J, Liang SH, et al. PET Imaging of Human Brown Adipose Tissue with the TSPO Tracer [(11)C]PBR28. *Mol Imaging Biol.* (2017) 20:188–93. doi: 10.1007/s11307-017-1129-z
80. Admiraal WM, Holleman F, Bahler L, Soeters MR, Hoekstra JB, Verberne HJ. Combining 123I-metaiodobenzylguanidine SPECT/CT and 18F-FDG PET/CT for the assessment of brown adipose tissue activity in humans during cold exposure. *J Nucl Med.* (2013) 54:208–12. doi: 10.2967/jnumed.112.111849
81. Holstila M, Virtanen KA, Gronroos TJ, Laine J, Lepomaki V, Saunavaara J, et al. Measurement of brown adipose tissue mass using a novel dual-echo magnetic resonance imaging approach: a validation study. *Metabolism* (2013) 62:1189–98. doi: 10.1016/j.metabol.2013.03.002
82. Hu HH, Perkins TG, Chia JM, Gilsanz V. Characterization of human brown adipose tissue by chemical-shift water-fat MRI. *AJR Am J Roentgenol.* (2013) 200:177–83. doi: 10.2214/AJR.12.8996
83. Van Rooijen BD, Van Der Lans AA, Brans B, Wildberger JE, Mottaghy FM, Schrauwen P, et al. Imaging cold-activated brown adipose tissue using dynamic T2*-weighted magnetic resonance imaging and 2-deoxy-2-[18F]fluoro-D-glucose positron emission tomography. *Invest Radiol.* (2013) 48:708–14. doi: 10.1097/RLI.0b013e31829363b8
84. Crane JD, Mottillo EP, Farncombe TH, Morrison KM, Steinberg GR. A standardized infrared imaging technique that specifically detects UCP1-mediated thermogenesis *in vivo*. *Mol Metab.* (2014) 3:490–4. doi: 10.1016/j.molmet.2014.04.007
85. Reddy NL, Jones TA, Wayte SC, Adesanya O, Sankar S, Yeo YC, et al. Identification of brown adipose tissue using MR imaging in a human adult with histological and immunohistochemical confirmation. *J Clin Endocrinol Metab.* (2014) 99:E117–121. doi: 10.1210/jc.2013-2036
86. Franz D, Karampinos DC, Rummeny EJ, Souvatzoglou M, Beer AJ, Nekolla SG, et al. Discrimination between brown and white adipose tissue using a 2-point dixon water-fat separation method in simultaneous PET/MRI. *J Nucl Med.* (2015) 56:1742–7. doi: 10.2967/jnumed.115.160770
87. Gifford A, Towse TF, Walker RC, Avison MJ, Welch EB. Characterizing active and inactive brown adipose tissue in adult humans using PET-CT and MR imaging. *Am J Physiol Endocrinol Metab.* (2016) 311:E95–104. doi: 10.1152/ajpendo.00482.2015
88. Holstila M, Pesola M, Saari T, Koskensalo K, Raiko J, Borra RJ, et al. MR signal-fat-fraction analysis and T2* weighted imaging measure BAT reliably on humans without cold exposure. *Metabolism* (2017) 70:23–30. doi: 10.1016/j.metabol.2017.02.001
89. Deng J, Neff LM, Rubert NC, Zhang B, Shore RM, Samet JD, et al. MRI characterization of brown adipose tissue under thermal challenges in normal weight, overweight, and obese young men. *J Magn Reson Imaging* (2018) 47:936–47. doi: 10.1002/jmri.25836
90. Nirengi S, Homma T, Inoue N, Sato H, Yoneshiro T, Matsushita M, et al. Assessment of human brown adipose tissue density during daily ingestion of thermogenic capsinoids using near-infrared time-resolved spectroscopy. *J Biomed Opt.* (2016) 21:091305. doi: 10.1117/1.JBO.21.9.091305
91. Nirengi S, Yoneshiro T, Saiki T, Aita S, Matsushita M, Sugie H, et al. Evaluation of brown adipose tissue using near-infrared time-resolved spectroscopy. *Adv Exp Med Biol.* (2016) 876:371–6. doi: 10.1007/978-1-4939-3023-4_46
92. Flynn A, Li Q, Panagia M, Abdelbaky A, Macnabb M, Samir A, et al. Contrast-enhanced ultrasound: a novel noninvasive, nonionizing method for the detection of brown adipose tissue in humans. *J Am Soc Echocardiogr.* (2015) 28:1247–54. doi: 10.1016/j.echo.2015.06.014
93. Crandall J, O JH, Gajwani P, Leal JP, Mawhinney DD, Sterzer F, et al. Measurement of brown adipose tissue activity using microwave radiometry (MRAD) and FDG PET/CT. *J Nucl Med.* (2018) doi: 10.2967/jnumed.117.204339 . [Epub ahead of print].
94. Reber J, Willershauser M, Karlas A, Paul-Yuan K, Diot G, Franz D, et al. Non-invasive measurement of brown fat metabolism based on optoacoustic imaging of hemoglobin gradients. *Cell Metab* (2018) 27:689–701.e684. doi: 10.1016/j.cmet.2018.02.002
95. Bahler L, Holleman F, Booij J, Hoekstra JB, Verberne HJ. Interobserver and intraobserver variability for the assessment of brown adipose tissue activity on 18F-FDG PET-CT. *Nucl Med Commun.* (2016) 37:363–71. doi: 10.1097/MNM.0000000000000450
96. Sampath SC, Sampath SC, Bredella MA, Cypess AM, Torriani M. Imaging of brown adipose tissue: state of the art. *Radiology* (2016) 280:4–19. doi: 10.1148/radiol.2016150390
97. Becker AS, Zellweger C, Schawkat K, Bogdanovic S, Phi Van VD, Nagel HW, et al. In-depth analysis of interreader agreement and accuracy in categorical assessment of brown adipose tissue in (18)FDG-PET/CT. *Eur J Radiol.* (2017) 91:41–6. doi: 10.1016/j.ejrad.2017.03.012
98. Chondronikola M, Beeman SC, Wahl RL. Non-invasive methods for the assessment of brown adipose tissue in humans. *J Physiol.* (2018) 596:363–78. doi: 10.1113/JP274255
99. Ong FJ, Ahmed BA, Oreskovich SM, Blondin DP, Haq T, Konyer NB, et al. Recent advances in the detection of brown adipose tissue in adult humans: a review. *Clin Sci.* (2018) 132:1039–54. doi: 10.1042/CS20170276
100. Hankir MK, Cowley MA, Fenske WK. A BAT-centric approach to the treatment of diabetes: turn on the brain. *Cell Metab.* (2016) 24:31–40. doi: 10.1016/j.cmet.2016.05.003
101. Giralt M, Villarroya F. Mitochondrial uncoupling and the regulation of glucose homeostasis. *Curr Diabetes Rev.* (2017) 13:386–94. doi: 10.2174/1573399812666160217122707
102. Blauw LL, Aziz NA, Tannemaat MR, Blauw CA, De Craen AJ, Pijl H, et al. Diabetes incidence and glucose intolerance prevalence increase with higher outdoor temperature. *BMJ Open Diabetes Res Care* (2017) 5:e000317. doi: 10.1136/bmjdrc-2016-000317
103. Booth GL, Luo J, Park AL, Feig DS, Moineddin R, Ray JG. Influence of environmental temperature on risk of gestational diabetes. *CMAJ* (2017) 189:E682–9. doi: 10.1503/cmaj.160839
104. Chondronikola M, Volpi E, Borsheim E, Porter C, Annamalai P, Enerback S, et al. Brown adipose tissue improves whole-body glucose homeostasis and insulin sensitivity in humans. *Diabetes* (2014) 63:4089–99. doi: 10.2337/db14-0746
105. Hanssen MJ, Hoeks J, Brans B, Van Der Lans AA, Schaart G, Van Den Driessche JJ, et al. Short-term cold acclimation improves insulin sensitivity in patients with type 2 diabetes mellitus. *Nat Med.* (2015b) 21:863–5. doi: 10.1038/nm.3891
106. Iwen KA, Backhaus J, Cassens M, Waltl M, Hedesan OC, Merkel M, et al. Cold-induced brown adipose tissue activity alters plasma fatty acids and improves glucose metabolism in men. *J Clin Endocrinol Metab.* (2017) 102:4226–34. doi: 10.1210/jc.2017-01250
107. Blondin DP, Labbe SM, Phoenix S, Guerin B, Turcotte EE, Richard D, et al. Contributions of white and brown adipose tissues and skeletal muscles to acute cold-induced metabolic responses in healthy men. *J Physiol.* (2015) 593:701–14. doi: 10.1113/jphysiol.2014.283598
108. Ouellet V, Labbe SM, Blondin DP, Phoenix S, Guerin B, Haman F, et al. Brown adipose tissue oxidative metabolism contributes to energy expenditure during acute cold exposure in humans. *J Clin Invest.* (2012) 122:545–52. doi: 10.1172/JCI60433
109. Blondin DP, Labbe SM, Noll C, Kunach M, Phoenix S, Guerin B, et al. Selective impairment of glucose but not fatty acid or oxidative metabolism in brown adipose tissue of subjects with type 2 *Diabetes* (2015) 64:2388–97. doi: 10.2337/db14-1651
110. Noll C, Carpentier AC. Dietary fatty acid metabolism in prediabetes. *Curr Opin Lipidol.* (2017) 28:1–10. doi: 10.1097/MOL.0000000000000369
111. Orava J, Nuutila P, Lidell ME, Oikonen V, Noponen T, Viljanen T, et al. Different metabolic responses of human brown adipose tissue to activation by cold and insulin. *Cell Metab.* (2011) 14:272–9. doi: 10.1016/j.cmet.2011.06.012
112. Orava J, Nuutila P, Noponen T, Parkkola R, Viljanen T, Enerback S, et al. Blunted metabolic responses to cold and insulin stimulation in brown adipose tissue of obese humans. *Obesity* (2013) 21:2279–87. doi: 10.1002/oby.20456

113. Blondin DP, Daoud A, Taylor T, Tingelstad HC, Bezaire V, Richard D, et al. Four-week cold acclimation in adult humans shifts uncoupling thermogenesis from skeletal muscles to brown adipose tissue. *J Physiol.* (2017) 595:2099–113. doi: 10.1113/JP273395
114. Dadson P, Hannukainen JC, Din MU, Lahesmaa M, Kalliokoski KK, Iozzo P, et al. Brown adipose tissue lipid metabolism in morbid obesity: effect of bariatric surgery-induced weight loss. *Diabetes Obes Metab.* (2018) 20:1280–8. doi: 10.1111/dom.13233
115. Blondin DP, Tingelstad HC, Noll C, Frisch F, Phoenix S, Guerin B, et al. Dietary fatty acid metabolism of brown adipose tissue in cold-acclimated men. *Nat Commun.* (2017) 8:14146. doi: 10.1038/ncomms14146
116. Weir G, Ramage LE, Akyol M, Rhodes JK, Kyle CJ, Fletcher AM, et al. Substantial metabolic activity of human brown adipose tissue during warm conditions and cold-induced lipolysis of local triglycerides. *Cell Metab.* (2018) 27:1348–55.e4. doi: 10.1016/j.cmet.2018.04.020
117. Wang Q, Zhang M, Ning G, Gu W, Su T, Xu M, et al. Brown adipose tissue in humans is activated by elevated plasma catecholamines levels and is inversely related to central obesity. *PLoS ONE* (2011) 6:e21006. doi: 10.1371/journal.pone.0021006
118. Bahler L, Verberne HJ, Admiraal WM, Stok WJ, Soeters MR, Hoekstra JB, et al. Differences in sympathetic nervous stimulation of brown adipose tissue between the young and old, and the lean and obese. *J Nucl Med.* (2016) 57:372–7. doi: 10.2967/jnumed.115.165829
119. Ulaner GA, Samstein R, Cahlon O, Weber WA, Rimner A. Unilateral suppression of brown fat on FDG PET/CT in horner syndrome. *Clin Nucl Med.* (2016) 41:797–8. doi: 10.1097/RLU.0000000000001319
120. Hankir MK, Kranz M, Keipert S, Weiner J, Andreasen SG, Kern M, et al. Dissociation between brown adipose tissue (18)F-FDG uptake and thermogenesis in uncoupling protein 1-deficient mice. *J Nucl Med.* (2017) 58:1100–3. doi: 10.2967/jnumed.116.186460
121. Olsen JM, Csikasz RI, Dehvari N, Lu L, Sandstrom A, Oberg AI, et al. beta3-Adrenergically induced glucose uptake in brown adipose tissue is independent of UCP1 presence or activity: Mediation through the mTOR pathway. *Mol Metab.* (2017) 6:611–9. doi: 10.1016/j.molmet.2017.02.006
122. Ma SW, Foster DO. Uptake of glucose and release of fatty acids and glycerol by rat brown adipose tissue in vivo. *Can J Physiol Pharmacol.* (1986) 64:609–14. doi: 10.1139/y86-101
123. Reshef L, Olswang Y, Cassuto H, Blum B, Croniger CM, Kalhan SC, et al. Glyceroneogenesis and the triglyceride/fatty acid cycle. *J BiolChem.* (2003) 278:30413–6. doi: 10.1074/jbc.R300017200
124. Cypess AM, Doyle AN, Sass CA, Huang TL, Mowschenson PM, Rosen HN, et al. Quantification of human and rodent brown adipose tissue function using 99mTc-methoxyisobutylisonitrile SPECT/CT and 18F-FDG PET/CT. *J Nucl Med.* (2013) 54:1896–901. doi: 10.2967/jnumed.113.121012
125. Latva-Rasku A, Honka MJ, Stancakova A, Koistinen HA, Kuusisto J, Guan L, et al. A partial loss-of-function variant in AKT2 is associated with reduced insulin-mediated glucose uptake in multiple insulin-sensitive tissues: a genotype-based callback positron emission tomography study. *Diabetes* (2018) 67:334–42. doi: 10.2337/db17-1142
126. Thuzar M, Law WP, Ratnasingam J, Jang C, Dimeski G, Ho KKY. Glucocorticoids suppress brown adipose tissue function in humans: a double-blind placebo-controlled study. *Diabetes Obes Metab.* (2018) 20:840–8. doi: 10.1111/dom.13157
127. Hanssen MJ, Wierts R, Hoeks J, Gemmink A, Brans B, Mottaghy FM, et al. Glucose uptake in human brown adipose tissue is impaired upon fasting-induced insulin resistance. *Diabetologia* (2015) 58:586–95. doi: 10.1007/s00125-014-3465-8
128. Carey AL, Pajtak R, Formosa MF, Van Every B, Bertovic DA, Anderson MJ, et al. Chronic ephedrine administration decreases brown adipose tissue activity in a randomised controlled human trial: implications for obesity. *Diabetologia* (2015) 58:1045–54. doi: 10.1007/s00125-015-3543-6
129. Vijgen GH, Bouvy ND, Teule GJ, Brans B, Hoeks J, Schrauwen P, et al. Increase in brown adipose tissue activity after weight loss in morbidly obese subjects. *J Clin Endocrinol Metab.* (2012) 97:E1229–33. doi: 10.1210/jc.2012-1289
130. Rachid B, Van De Sande-Lee S, Rodovalho S, Folli F, Beltramini GC, Morari J, et al. Distinct regulation of hypothalamic and brown/beige adipose tissue activities in human obesity. *Int J Obes.* (2015) 39:1515–22. doi: 10.1038/ijo.2015.94
131. Motiani P, Virtanen KA, Motiani KK, Eskelinen JJ, Middelbeek RJ, Goodyear LJ, et al. Decreased insulin-stimulated brown adipose tissue glucose uptake after short-term exercise training in healthy middle aged men. *Diabetes Obes Metab.* (2017) 19:1379–88. doi: 10.1111/dom.12947
132. Koskensalo K, Raiko J, Saari T, Saunavaara V, Eskola O, Nuutila P, et al. Human brown adipose tissue temperature and fat fraction are related to its metabolic activity. *J Clin Endocrinol Metab.* (2017) 102:1200–7. doi: 10.1210/jc.2016-3086
133. Grenier-Larouche TL, Noll C, Richard D, Carpentier AC. Metabolic inflexibility of white and brown adipose tissues in abnormal fatty acid partitioning of type 2 diabetes. *Int J Obesity* (2012) 2:S37–42. doi: 10.1038/ijosup.2012.21
134. Chondronikola M, Volpi E, Borsheim E, Porter C, Saraf MK, Annamalai P, et al. Brown adipose tissue activation is linked to distinct systemic effects on lipid metabolism in humans. *Cell Metab.* (2016) 23:1200–6. doi: 10.1016/j.cmet.2016.04.029
135. Lee P, Zhao JT, Swarbrick MM, Gracie G, Bova R, Greenfield JR, et al. High prevalence of brown adipose tissue in adult humans. *J Clin Endocrinol Metab.* (2011) 96:2450–5. doi: 10.1210/jc.2011-0487
136. Carpentier AC, Labbe SM, Grenier-Larouche T, Noll C. Abnormal dietary fatty acid metabolic partitioning in insulin resistance and type 2 diabetes. *Clin Lipidol.* (2011) 6:703–16. doi: 10.2217/clp.11.60
137. Hames KC, Vella A, Kemp BJ, Jensen MD. Free fatty acid uptake in humans with CD36 deficiency. *Diabetes* (2014) 63:3606–14. doi: 10.2337/db14-0369
138. Labbe SM, Croteau E, Grenier-Larouche T, Frisch F, Ouellet R, Langlois R, et al. Normal postprandial nonesterified fatty acid uptake in muscles despite increased circulating fatty acids in type 2 diabetes. *Diabetes* (2011) 60:408–15. doi: 10.2337/db10-0997
139. Bjornson E, Adiels M, Taskinen MR, Boren J. Kinetics of plasma triglycerides in abdominal obesity. *Curr Opin Lipidol.* (2017) 28:11–8. doi: 10.1097/MOL.0000000000000375
140. Goldberg IJ, Eckel RH, Abumrad NA. Regulation of fatty acid uptake into tissues: lipoprotein lipase- and CD36-mediated pathways. *J.Lipid Res.* (2009) 50(Suppl.):S86–90. doi: 10.1194/jlr.R800085-JLR200
141. Hoeke G, Nahon KJ, Bakker LEH, Norkauer SSC, Dinnes DLM, Kockx M, et al. Short-term cooling increases serum triglycerides and small high-density lipoprotein levels in humans. *J Clin Lipidol.* (2017) 11:920–928.e922. doi: 10.1016/j.jacl.2017.04.117
142. Ci X, Frisch F, Lavoie F, Germain P, Lecomte R, Van Lier JE, et al. The effect of insulin on the intracellular distribution of 14(R,S)-[(18)F]Fluoro-6-thia-heptadecanoic acid in rats. *Mol Imaging Biol.* (2006) 8:237–44. doi: 10.1007/s11307-006-0042-7
143. U. Din M, Raiko J, Saari T, Kudomi N, Tolvanen T, Oikonen V, et al. Human brown adipose tissue [(15)O]O2 PET imaging in the presence and absence of cold stimulus. *Eur J Nucl Med Mol Imaging* (2016) 43:1878–86. doi: 10.1007/s00259-016-3364-y
144. Bartelt A, Bruns OT, Reimer R, Hohenberg H, Ittrich H, Peldschus K, et al. Brown adipose tissue activity controls triglyceride clearance. *Nat Med.* (2011) 17:200–5. doi: 10.1038/nm.2297
145. Hoeke G, Kooijman S, Boon MR, Rensen PC, Berbee JF. Role of brown fat in lipoprotein metabolism and atherosclerosis. *Circ Res.* (2016) 118:173–82. doi: 10.1161/CIRCRESAHA.115.306647
146. Dijk W, Heine M, Vergnes L, Boon MR, Schaart G, Hesselink MK, et al. ANGPTL4 mediates shuttling of lipid fuel to brown adipose tissue during sustained cold exposure. *Elife* (2015) 4:e08428. doi: 10.7554/eLife.08428
147. Hoeke G, Wang Y, Van Dam AD, Mol IM, Gart E, Klop HG, et al. Atorvastatin accelerates clearance of lipoprotein remnants generated by activated brown fat to further reduce hypercholesterolemia and atherosclerosis. *Atherosclerosis* (2017) 267:116–26. doi: 10.1016/j.atherosclerosis.2017.10.030
148. Shao X, Yang W, Shao X, Qiu C, Wang X, Wang Y. The role of active brown adipose tissue (aBAT) in lipid metabolism in healthy Chinese adults. *Lipids Health Dis.* (2016) 15:138. doi: 10.1186/s12944-016-0310-8
149. Labbe SM, Grenier-Larouche T, Croteau E, Normand-Lauziere F, Frisch F, Ouellet R, et al. Organ-specific dietary fatty acid uptake in humans using positron emission tomography coupled to computed

tomography. *Am J Physiol Endocrinol Metab* (2011) 300:E445–53. doi: 10.1152/ajpendo.00579.2010
150. Lynes MD, Leiria LO, Lundh M, Bartelt A, Shamsi F, Huang TL, et al. The cold-induced lipokine 12,13-diHOME promotes fatty acid transport into brown adipose tissue. *Nat Med.* (2017) 23:631–7. doi: 10.1038/nm.4297
151. Boon MR, Bakker LEH, Prehn C, Adamski J, Vosselman MJ, Jazet IM, et al. LysoPC-acyl C16:0 is associated with brown adipose tissue activity in men. *Metabolomics* (2017) 13:48. doi: 10.1007/s11306-017-1185-z
152. Chakrabarty K, Chaudhuri B, Jeffay H. Glycerokinase activity in human brown adipose tissue. *J Lipid Res.* (1983) 24:381–90.
153. Kawashita NH, Festuccia WT, Brito MN, Moura MA, Brito SR, Garofalo MA, et al. Glycerokinase activity in brown adipose tissue: a sympathetic regulation? *Am J Physiol Regul Integr Comp Physiol.* (2002) 282:R1185–90. doi: 10.1152/ajpregu.00419.2001
154. Festuccia WT, Kawashita NH, Garofalo MA, Moura MA, Brito SR, Kettelhut IC, et al. Control of glyceroneogenic activity in rat brown adipose tissue. *AmJ Physiol Regul Integr Comp Physiol.* (2003) 285:R177–82. doi: 10.1152/ajpregu.00713.2002
155. Lasar D, Rosenwald M, Kiehlmann E, Balaz M, Tall B, Opitz L, et al. Peroxisome proliferator activated receptor gamma controls mature brown adipocyte inducibility through glycerol kinase. *Cell Rep.* (2018) 22:760–73. doi: 10.1016/j.celrep.2017.12.067
156. Lopaschuk GD. Metabolic modulators in heart disease: past, present, and future. *Can J Cardiol.* (2017) 33:838–49. doi: 10.1016/j.cjca.2016.12.013
157. U. Din M, Raiko J, Saari T, Saunavaara V, Kudomi N, Solin O, et al. Human brown fat radiodensity indicates underlying tissue composition and systemic metabolic health. *J Clin Endocrinol Metab* (2017) 102:2258–67. doi: 10.1210/jc.2016-2698
158. Mccallister A, Zhang L, Burant A, Katz L, Branca RT. A pilot study on the correlation between fat fraction values and glucose uptake values in supraclavicular fat by simultaneous PET/MRI. *Magn Reson Med.* (2017) 78:1922–32. doi: 10.1002/mrm.26589
159. Raiko J, Holstila M, Virtanen KA, Orava J, Saunavaara V, Niemi T, et al. Brown adipose tissue triglyceride content is associated with decreased insulin sensitivity, independently of age and obesity. *Diabetes Obes Metab.* (2015) 17:516–9. doi: 10.1111/dom.12433
160. Baba S, Jacene HA, Engles JM, Honda H, Wahl RL. CT hounsfield units of brown adipose tissue increase with activation: preclinical and clinical studies. *J Nucl Med.* (2010) 51:246–50. doi: 10.2967/jnumed.109.068775
161. Lundstrom E, Strand R, Johansson L, Bergsten P, Ahlstrom H, Kullberg J. Magnetic resonance imaging cooling-reheating protocol indicates decreased fat fraction via lipid consumption in suspected brown adipose tissue. *PLoS ONE* (2015) 10:e0126705. doi: 10.1371/journal.pone.0126705
162. Mottillo EP, Desjardins EM, Crane JD, Smith BK, Green AE, Ducommun S, et al. Lack of adipocyte AMPK exacerbates insulin resistance and hepatic steatosis through brown and beige adipose tissue function. *Cell Metab.* (2016) 24:118–29. doi: 10.1016/j.cmet.2016.06.006
163. Carpentier A, Frisch F, Cyr D, Genereux P, Patterson BW, Giguere R, et al. On the suppression of plasma non-esterified fatty acids by insulin during enhanced intravascular lipolysis in humans. *Am J Physiol Endocrinol Metab.* (2005) 289:E849–56. doi: 10.1152/ajpendo.00073.2005
164. Vijgen GH, Bouvy ND, Teule GJ, Brans B, Schrauwen P, Van Marken Lichtenbelt WD. Brown adipose tissue in morbidly obese subjects. *PLoS ONE* (2011) 6:e17247. doi: 10.1371/journal.pone.0017247
165. Yoneshiro T, Aita S, Matsushita M, Kameya T, Nakada K, Kawai Y, et al. Brown adipose tissue, whole-body energy expenditure, and thermogenesis in healthy adult men. *Obesity* (2011) 19:13–6. doi: 10.1038/oby.2010.105
166. Yoneshiro T, Matsushita M, Nakae S, Kameya T, Sugie H, Tanaka S, et al. Brown adipose tissue is involved in the seasonal variation of cold-induced thermogenesis in humans. *Am J Physiol Regul Integr Comp Physiol.* (2016) 310:R999–1009. doi: 10.1152/ajpregu.00057.2015
167. Chondronikola M, Volpi E, Borsheim E, Chao T, Porter C, Annamalai P, et al. Brown adipose tissue is linked to a distinct thermoregulatory response to mild cold in people. *Front Physiol* (2016) 7:129. doi: 10.3389/fphys.2016.00129
168. Chen KY, Brychta RJ, Linderman JD, Smith S, Courville A, Dieckmann W, et al. Brown fat activation mediates cold-induced thermogenesis in adult humans in response to a mild decrease in ambient temperature. *J Clin Endocrinol Metab.* (2013) 98:E1218–23. doi: 10.1210/jc.2012-4213
169. Yoneshiro T, Aita S, Matsushita M, Okamatsu-Ogura Y, Kameya T, Kawai Y, et al. Age-related decrease in cold-activated brown adipose tissue and accumulation of body fat in healthy humans. *Obesity* (2011) 19:1755–60. doi: 10.1038/oby.2011.125
170. Bakker LE, Boon MR, Van Der Linden RA, Arias-Bouda LP, Van Klinken JB, Smit F, et al. Brown adipose tissue volume in healthy lean south Asian adults compared with white Caucasians: a prospective, case-controlled observational study. *Lancet Diabetes Endocrinol.* (2014) 2:210–7. doi: 10.1016/S2213-8587(13)70156-6
171. Yilmaz Y, Ones T, Purnak T, Ozguven S, Kurt R, Atug O, et al. Association between the presence of brown adipose tissue and non-alcoholic fatty liver disease in adult humans. *Aliment Pharmacol Ther.* (2011) 34:318–23. doi: 10.1111/j.1365-2036.2011.04723.x
172. Vosselman MJ, Hoeks J, Brans B, Pallubinsky H, Nascimento EB, Van Der Lans AA, et al. Low brown adipose tissue activity in endurance-trained compared with lean sedentary men. *Int J Obes.* (2015) 39:1696–702. doi: 10.1038/ijo.2015.130
173. Singhal V, Maffazioli GD, Ackerman KE, Lee H, Elia EF, Woolley R, et al. Effect of chronic athletic activity on brown fat in young women. *PLoS ONE* (2016) 11:e0156353. doi: 10.1371/journal.pone.0156353
174. Yoneshiro T, Ogawa T, Okamoto N, Matsushita M, Aita S, Kameya T, et al. Impact of UCP1 and beta3AR gene polymorphisms on age-related changes in brown adipose tissue and adiposity in humans. *Int J Obes.* (2013) 37:993–8. doi: 10.1038/ijo.2012.161
175. Laurila PP, Soronen J, Kooijman S, Forsstrom S, Boon MR, Surakka I, et al. USF1 deficiency activates brown adipose tissue and improves cardiometabolic health. *Sci Transl Med.* (2016) 8:323ra313. doi: 10.1126/scitranslmed.aad0015
176. Lee P, Smith S, Linderman J, Courville AB, Brychta RJ, Dieckmann W, et al. Temperature-acclimated brown adipose tissue modulates insulin sensitivity in humans. *Diabetes* (2014) 63:3686–98. doi: 10.2337/db14-0513
177. Yoneshiro T, Aita S, Kawai Y, Iwanaga T, Saito M. Nonpungent capsaicin analogs (capsinoids) increase energy expenditure through the activation of brown adipose tissue in humans. *Am J Clin Nutr.* (2012) 95:845–50. doi: 10.3945/ajcn.111.018606
178. Yoneshiro T, Matsushita M, Hibi M, Tone H, Takeshita M, Yasunaga K, et al. Tea catechin and caffeine activate brown adipose tissue and increase cold-induced thermogenic capacity in humans. *Am J Clin Nutr.* (2017) 105:873–81. doi: 10.3945/ajcn.116.144972
179. Vijgen GH, Bouvy ND, Leenen L, Rijkers K, Cornips E, Majoie M, et al. Vagus nerve stimulation increases energy expenditure: relation to brown adipose tissue activity. *PLoS ONE* (2013) 8:e77221. doi: 10.1371/journal.pone.0077221
180. Carey AL, Formosa MF, Van Every B, Bertovic D, Eikelis N, Lambert GW, et al. Ephedrine activates brown adipose tissue in lean but not obese humans. *Diabetologia* (2013) 56:147–55. doi: 10.1007/s00125-012-2748-1
181. Cypess AM, Chen YC, Sze C, Wang K, English J, Chan O, et al. Cold but not sympathomimetics activates human brown adipose tissue in vivo. *Proc Natl Acad Sci USA.* (2012) 109:10001–5. doi: 10.1073/pnas.1207911109
182. Vosselman MJ, Van Der Lans AA, Brans B, Wierts R, Van Baak MA, Schrauwen P, et al. Systemic beta-adrenergic stimulation of thermogenesis is not accompanied by brown adipose tissue activity in humans. *Diabetes* (2012) 61:3106–13. doi: 10.2337/db12-0288
183. Cypess AM, Weiner LS, Roberts-Toler C, Franquet Elia E, Kessler SH, Kahn PA, et al. Activation of human brown adipose tissue by a beta3-adrenergic receptor agonist. *Cell Metab.* (2015) 21:33–8. doi: 10.1016/j.cmet.2014.12.009
184. Lahesmaa M, Orava J, Schalin-Jantti C, Soinio M, Hannukainen JC, Noponen T, et al. Hyperthyroidism increases brown fat metabolism in humans. *J Clin Endocrinol Metab.* (2014) 99:E28–35. doi: 10.1210/jc.2013-2312
185. Zhang Q, Miao Q, Ye H, Zhang Z, Zuo C, Hua F, et al. The effects of thyroid hormones on brown adipose tissue in humans: a PET-CT study. *Diabetes Metab Res Rev.* (2014) 30:513–20. doi: 10.1002/dmrr.2556
186. Gavrila A, Hasselgren PO, Glasgow A, Doyle AN, Lee AJ, Fox P, et al. Variable cold-induced brown adipose tissue response to *Thyroid* hormone status. *Thyroid* (2017) 27:1–10. doi: 10.1089/thy.2015.0646

187. Beijer E, Schoenmakers J, Vijgen G, Kessels F, Dingemans AM, Schrauwen P, et al. A role of active brown adipose tissue in cancer cachexia? *Oncol Rev.* (2012) 6:e11. doi: 10.4081/oncol.2012.e11
188. De Vos-Geelen J, Fearon KC, Schols AM. The energy balance in cancer cachexia revisited. *Curr Opin Clin Nutr Metab Care* (2014) 17:509–14. doi: 10.1097/MCO.0000000000000106
189. Chalfant JS, Smith ML, Hu HH, Dorey FJ, Goodarzian F, Fu CH, et al. Inverse association between brown adipose tissue activation and white adipose tissue accumulation in successfully treated pediatric malignancy. *Am J Clin Nutr.* (2012) 95:1144–9. doi: 10.3945/ajcn.111.030650
190. Gadea E, Thivat E, Merlin C, Paulon R, Kwiatkowski F, Chadeyras JB, et al. Brown adipose tissue activity in relation to weight gain during chemotherapy in breast cancer patients: a pilot study. *Nutr Cancer* (2014) 66:1092–6. doi: 10.1080/01635581.2014.948212
191. Carpentier AC. Acute adaptation of energy expenditure predicts diet-induced weight loss: revisiting the thrifty phenotype. *Diabetes* (2015) 64:2714–6. doi: 10.2337/db15-0553
192. Hibi M, Oishi S, Matsushita M, Yoneshiro T, Yamaguchi T, Usui C, et al. Brown adipose tissue is involved in diet-induced thermogenesis and whole-body fat utilization in healthy humans. *Int J Obes.* (2016) 40:1655–61. doi: 10.1038/ijo.2016.124
193. Vosselman MJ, Brans B, Van Der Lans AA, Wierts R, Van Baak MA, Mottaghy FM, et al. Brown adipose tissue activity after a high-calorie meal in humans. *Am J Clin Nutr.* (2013) 98:57–64. doi: 10.3945/ajcn.113.059022
194. Thearle MS, Pannacciulli N, Bonfiglio S, Pacak K, Krakoff J. Extent and determinants of thermogenic responses to 24 hours of fasting, energy balance, and five different overfeeding diets in humans. *J Clin Endocrinol Metab.* (2013) 98:2791–9. doi: 10.1210/jc.2013-1289
195. Schlogl M, Piaggi P, Thiyyagura P, Reiman EM, Chen K, Lutrin C, et al. Overfeeding over 24 hours does not activate brown adipose tissue in humans. *J Clin Endocrinol Metab.* (2013) 98:E1956–1960. doi: 10.1210/jc.2013-2387
196. U. Din M, Saari T, Raiko J, Kudomi N, Maurer SF, Lahesmaa M, et al. Postprandial oxidative metabolism of human brown fat indicates thermogenesis. *Cell Metab*. (2018). doi: 10.1016/j.cmet.2018.05.020. [Epub ahead of print].
197. Young JB, Landsberg L. Suppression of sympathetic nervous system during fasting. *Obes Res.* (1997) 5:646–9. doi: 10.1002/j.1550-8528.1997.tb00590.x
198. Vijgen GH, Sparks LM, Bouvy ND, Schaart G, Hoeks J, Van Marken Lichtenbelt WD, et al. Increased oxygen consumption in human adipose tissue from the "brown adipose tissue" region. *J Clin Endocrinol Metab.* (2013) 98:E1230–4. doi: 10.1210/jc.2013-1348
199. Muzik O, Mangner TJ, Granneman JG. Assessment of oxidative metabolism in brown fat using PET imaging. *Front Endocrinol.* (2012) 3:15. doi: 10.3389/fendo.2012.00015
200. Muzik O, Mangner TJ, Leonard WR, Kumar A, Janisse J, Granneman JG. 15O PET measurement of blood flow and oxygen consumption in cold-activated human brown fat. *J Nucl Med.* (2013) 54:523–31. doi: 10.2967/jnumed.112.111336
201. Leonard WR. Measuring human energy expenditure and metabolic function: basic principles and methods. *J Anthropol Sci.* (2010) 88:221–30.
202. Martin AD, Daniel MZ, Drinkwater DT, Clarys JP. Adipose tissue density, estimated adipose lipid fraction and whole body adiposity in male cadavers. *Int J Obes Relat Metab Disord.* (1994) 18:79–83.
203. Keipert S, Jastroch M. Brite/beige fat and UCP1 - is it thermogenesis? *Biochim Biophys Acta* (2014) 1837:1075–82.
204. Kiefer FW. Browning and thermogenic programing of adipose tissue. *Best Pract Res Clin Endocrinol Metab.* (2016) 30:479–85. doi: 10.1016/j.beem.2016.09.003
205. Castro E, Silva TEO, Festuccia WT. Critical review of beige adipocyte thermogenic activation and contribution to whole-body energy expenditure. *Horm Mol Biol Clin Investig.* (2017) 31. doi: 10.1515/hmbci-2017-0042
206. Sepa-Kishi DM, Ceddia RB. White and beige adipocytes: are they metabolically distinct? *Horm Mol Biol Clin Investig* (2018) 33. doi: 10.1515/hmbci-2018-0003
207. Hu J, Christian M. Hormonal factors in the control of the browning of white adipose tissue. *Horm Mol Biol Clin Investig* (2017) 31. doi: 10.1515/hmbci-2017-0017
208. Aldiss P, Betts J, Sale C, Pope M, Budge H, Symonds ME. Exercise-induced 'browning' of adipose tissues. *Metabolism* (2018) 81:63–70. doi: 10.1016/j.metabol.2017.11.009
209. Seale P, Conroe HM, Estall J, Kajimura S, Frontini A, Ishibashi J, et al. Prdm16 determines the thermogenic program of subcutaneous white adipose tissue in mice. *J Clin Invest.* (2011) 121:96–105. doi: 10.1172/JCI44271
210. Morroni M, Barbatelli G, Zingaretti MC, Cinti S. Immunohistochemical, ultrastructural and morphometric evidence for brown adipose tissue recruitment due to cold acclimation in old rats. *Int J Obes Relat Metab Disord.* (1995) 19:126–31.
211. Bostrom P, Wu J, Jedrychowski MP, Korde A, Ye L, Lo JC, et al. A PGC1-alpha-dependent myokine that drives brown-fat-like development of white fat and thermogenesis. *Nature* (2012) 481:463–8. doi: 10.1038/nature 10777
212. Ohno H, Shinoda K, Spiegelman BM, Kajimura S. PPARgamma agonists induce a white-to-brown fat conversion through stabilization of PRDM16 protein. *Cell Metab.* (2012) 15:395–404. doi: 10.1016/j.cmet.2012.01.019
213. Labbe SM, Caron A, Chechi K, Laplante M, Lecomte R, Richard D. Metabolic activity of brown, "beige," and white adipose tissues in response to chronic adrenergic stimulation in male mice. *Am J Physiol Endocrinol Metab.* (2016) 311:E260–8. doi: 10.1152/ajpendo.00545.2015
214. Sbarbati A, Morroni M, Zancanaro C, Cinti S. Rat interscapular brown adipose tissue at different ages: a morphometric study. *Int J Obes.* (1991) 15:581–7.
215. Zingaretti MC, Crosta F, Vitali A, Guerrieri M, Frontini A, Cannon B, et al. The presence of UCP1 demonstrates that metabolically active adipose tissue in the neck of adult humans truly represents brown adipose tissue. *FASEB J.* (2009) 23:3113–20. doi: 10.1096/fj.09-133546
216. Nedergaard J, Bengtsson T, Cannon B. Three years with adult human brown adipose tissue. *AnnNYAcadSci.* (2010) 1212:E20–36. doi: 10.1111/j.1749-6632.2010.05905.x
217. Van Den Beukel JC, Grefhorst A, Hoogduijn MJ, Steenbergen J, Mastroberardino PG, Dor FJ, et al. Women have more potential to induce browning of perirenal adipose tissue than men. *Obesity* (2015) 23:1671–9. doi: 10.1002/oby.21166
218. Sondergaard E, Gormsen LC, Christensen MH, Pedersen SB, Christiansen P, Nielsen S, et al. Chronic adrenergic stimulation induces brown adipose tissue differentiation in visceral adipose tissue. *Diabet Med.* (2015) 32:e4–8. doi: 10.1111/dme.12595
219. Berthiaume M, Sell H, Lalonde J, Gelinas Y, Tchernof A, Richard D, et al. Actions of PPARgamma agonism on adipose tissue remodeling, insulin sensitivity, and lipemia in absence of glucocorticoids. *Am J Physiol Regul Integr Comp Physiol.* (2004) 287:R1116–23. doi: 10.1152/ajpregu.00339.2004
220. Festuccia WT, Oztezcan S, Laplante M, Berthiaume M, Michel C, Dohgu S, et al. Peroxisome proliferator-activated receptor-gamma-mediated positive energy balance in the rat is associated with reduced sympathetic drive to adipose tissues and thyroid status. *Endocrinology* (2008) 149:2121–30. doi: 10.1210/en.2007-1553
221. Festuccia WT, Blanchard PG, Oliveira TB, Magdalon J, Paschoal VA, Richard D, et al. PPARgamma activation attenuates cold-induced upregulation of thyroid status and brown adipose tissue PGC-1alpha and D2. *Am J Physiol Regul Integr Comp Physiol.* (2012) 303:R1277–85. doi: 10.1152/ajpregu.00299.2012
222. Loh RKC, Formosa MF, Eikelis N, Bertovic DA, Anderson MJ, Barwood SA, et al. Pioglitazone reduces cold-induced brown fat glucose uptake despite induction of browning in cultured human adipocytes: a randomised, controlled trial in humans. *Diabetologia* (2018) 61:220–30. doi: 10.1007/s00125-017-4479-9
223. Shabalina IG, Petrovic N, De Jong JM, Kalinovich AV, Cannon B, Nedergaard J. UCP1 in brite/beige adipose tissue mitochondria is functionally thermogenic. *Cell Rep.* (2013) 5:1196–203. doi: 10.1016/j.celrep.2013.10.044
224. Kalinovich AV, De Jong JM, Cannon B, Nedergaard J. UCP1 in adipose tissues: two steps to full browning. *Biochimie* (2017) 134:127–37. doi: 10.1016/j.biochi.2017.01.007
225. Kazak L, Chouchani ET, Jedrychowski MP, Erickson BK, Shinoda K, Cohen P, et al. A creatine-driven substrate cycle enhances energy expenditure and thermogenesis in beige fat. *Cell* (2015) 163:643–55. doi: 10.1016/j.cell.2015.09.035

226. Ikeda K, Kang Q, Yoneshiro T, Camporez JP, Maki H, Homma M, et al. UCP1-independent signaling involving SERCA2b-mediated calcium cycling regulates beige fat thermogenesis and systemic glucose homeostasis. *Nat Med.* (2017) 23:1454–65. doi: 10.1038/nm.4429
227. Ukropec J, Anunciado RP, Ravussin Y, Hulver MW, Kozak LP. UCP1-independent thermogenesis in white adipose tissue of cold-acclimated Ucp1-/- mice. *JBiolChem.* (2006) 281:31894–908. doi: 10.1074/jbc.M606114200
228. Flachs P, Rossmeisl M, Kuda O, Kopecky J. Stimulation of mitochondrial oxidative capacity in white fat independent of UCP1: a key to lean phenotype. *Biochim Biophys Acta* (2013) 1831:986–1003. doi: 10.1016/j.bbalip.2013.02.003
229. Grenier-Larouche T, Carreau AM, Geloen A, Frisch F, Biertho L, Marceau S, et al. Fatty Acid metabolic remodeling during type 2 *diabetes* remission after bariatric surgery. *Diabetes* (2017) 66:2743–55. doi: 10.2337/db17-0414
230. Hivert MF, Langlois MF, Berard P, Cuerrier JP, Carpentier AC. Prevention of weight gain in young adults through a seminar-based intervention program. *Int J Obes.* (2007) 31:1262–9. doi: 10.1038/sj.ijo.0803572
231. Dutton GR, Kim Y, Jacobs DR Jr, Li X, Loria CM, Reis JP, et al. 25-year weight gain in a racially balanced sample of U.S. adults: The CARDIA study. *Obesity* (2016) 24:1962–8. doi: 10.1002/oby.21573
232. Sanghvi A, Redman LM, Martin CK, Ravussin E, Hall KD. Validation of an inexpensive and accurate mathematical method to measure long-term changes in free-living energy intake. *Am J Clin Nutr.* (2015) 102:353–8. doi: 10.3945/ajcn.115.111070
233. Reinhardt M, Thearle MS, Ibrahim M, Hohenadel MG, Bogardus C, Krakoff J, et al. A human thrifty phenotype associated with less weight loss during caloric restriction. *Diabetes* (2015) 64:2859–67. doi: 10.2337/db14-1881
234. Nguyen KT, Billington CJ, Vella A, Wang Q, Ahmed L, Bantle JP, et al. Preserved insulin secretory capacity and weight loss are the predominant predictors of glycemic control in patients with type 2 diabetes randomized to Roux-en-Y gastric bypass. *Diabetes* (2015) 64:3104–10. doi: 10.2337/db14-1870
235. Sjoholm K, Pajunen P, Jacobson P, Karason K, Sjostrom CD, Torgerson J, et al. Incidence and remission of type 2 diabetes in relation to degree of obesity at baseline and 2 year weight change: the Swedish Obese Subjects (SOS) study. *Diabetologia* (2015) 58:1448–53. doi: 10.1007/s00125-015-3591-y
236. Dandanell S, Skovborg C, Praest CB, Kristensen KB, Nielsen MG, Lionett S, et al. Maintaining a clinical weight loss after intensive lifestyle intervention is the key to cardiometabolic health. *Obes Res Clin Pract.* (2017) 11:489–98. doi: 10.1016/j.orcp.2016.09.009
237. Knowler WC, Fowler SE, Hamman RF, Christophi CA, Hoffman HJ, Brenneman AT, et al. 10-year follow-up of diabetes incidence and weight loss in the diabetes prevention program outcomes study. *Lancet* (2009) 374:1677–86. doi: 10.1016/S0140-6736(09)61457-4
238. Kantae V, Nahon KJ, Straat ME, Bakker LEH, Harms AC, Van Der Stelt M, et al. Endocannabinoid tone is higher in healthy lean South Asian than white Caucasian men. *Sci Rep.* (2017) 7:7558. doi: 10.1038/s41598-017-07980-5
239. Nahon KJ, Boon MR, Doornink F, Jazet IM, Rensen PCN, Abreu-Vieira G. Lower critical temperature and cold-induced thermogenesis of lean and overweight humans are inversely related to body mass and basal metabolic rate. *J Therm Biol.* (2017) 69:238–48. doi: 10.1016/j.jtherbio.2017.08.006
240. Dewal RS, Stanford KI. Effects of exercise on brown and beige adipocytes. *Biochim Biophys Acta* (2018). doi: 10.1016/j.bbalip.2018.04.013
241. Rodrigues K, Pereira RM, De Campos TDP, De Moura RF, Da Silva ASR, Cintra DE, et al. The role of physical exercise to improve the browning of white adipose tissue via POMC neurons. *Front Cell Neurosci.* (2018) 12:88. doi: 10.3389/fncel.2018.00088
242. Townsend LK, Wright DC. Looking on the "brite" side exercise-induced browning of white adipose tissue. *Pflugers Arch.* (2018). doi: 10.1007/s00424-018-2177-1. [Epub ahead of print].
243. Bordicchia M, Liu D, Amri EZ, Ailhaud G, Dessi-Fulgheri P, Zhang C, et al. Cardiac natriuretic peptides act via p38 MAPK to induce the brown fat thermogenic program in mouse and human adipocytes. *J Clin Invest.* (2012) 122:1022–36. doi: 10.1172/JCI59701
244. Rao RR, Long JZ, White JP, Svensson KJ, Lou J, Lokurkar I, et al. Meteorin-like is a hormone that regulates immune-adipose interactions to increase beige fat thermogenesis. *Cell* (2014) 157:1279–91. doi: 10.1016/j.cell.2014.03.065
245. Hanssen MJ, Broeders E, Samms RJ, Vosselman MJ, Van Der Lans AA, Cheng CC, et al. Serum FGF21 levels are associated with brown adipose tissue activity in humans. *Sci Rep.* (2015) 5:10275. doi: 10.1038/srep10275
246. Stanford KI, Middelbeek RJ, Townsend KL, An D, Nygaard EB, Hitchcox KM, et al. Brown adipose tissue regulates glucose homeostasis and insulin sensitivity. *J Clin Invest.* (2013) 123:215–23. doi: 10.1172/JCI62308
247. Stanford KI, Lynes MD, Takahashi H, Baer LA, Arts PJ, May FJ, et al. 12,13-diHOME: An Exercise-Induced Lipokine that Increases Skeletal Muscle Fatty Acid Uptake. *Cell Metab* (2018) 27:1111–20.e1113. doi: 10.1016/j.cmet.2018.03.020
248. Arnold J, Leblanc J, Cote J, Lalonde J, Richard D. Exercise suppression of thermoregulatory thermogenesis in warm- and cold-acclimated rats. *Can J Physiol Pharmacol.* (1986) 64:922–6. doi: 10.1139/y86-158
249. Arnold J, Richard D. Exercise during intermittent cold exposure prevents acclimation to cold rats. *J Physiol.* (1987) 390:45–54. doi: 10.1113/jphysiol.1987.sp016685
250. Admiraal WM, Verberne HJ, Karamat FA, Soeters MR, Hoekstra JB, Holleman F. Cold-induced activity of brown adipose tissue in young lean men of South-Asian and European origin. *Diabetologia* (2013) 56:2231–7. doi: 10.1007/s00125-013-2938-5
251. Redman LM, De Jonge L, Fang X, Gamlin B, Recker D, Greenway FL, et al. Lack of an effect of a novel beta3-adrenoceptor agonist, TAK-677, on energy metabolism in obese individuals: a double-blind, placebo-controlled randomized study. *J Clin Endocrinol Metab.* (2007) 92:527–31. doi: 10.1210/jc.2006-1740
252. Arch JR. Challenges in beta(3)-adrenoceptor agonist drug development. *Ther Adv Endocrinol Metab.* (2011) 2:59–64. doi: 10.1177/2042018811398517
253. Snitker S, Fujishima Y, Shen H, Ott S, Pi-Sunyer X, Furuhata Y, et al. Effects of novel capsinoid treatment on fatness and energy metabolism in humans: possible pharmacogenetic implications. *Am J Clin Nutr.* (2009) 89:45–50. doi: 10.3945/ajcn.2008.26561
254. Chen IJ, Liu CY, Chiu JP, Hsu CH. Therapeutic effect of high-dose green tea extract on weight reduction: a randomized, double-blind, placebo-controlled clinical trial. *Clin Nutr.* (2016) 35:592–9. doi: 10.1016/j.clnu.2015.05.003
255. Dostal AM, Arikawa A, Espejo L, Kurzer MS. Long-term supplementation of green tea extract does not modify adiposity or bone mineral density in a randomized trial of overweight and obese postmenopausal women. *J Nutr.* (2016) 146:256–64. doi: 10.3945/jn.115.219238
256. Dostal AM, Samavat H, Espejo L, Arikawa AY, Stendell-Hollis NR, Kurzer MS. Green tea extract and catechol-o-methyltransferase genotype modify fasting serum insulin and plasma adiponectin concentrations in a randomized controlled trial of overweight and obese postmenopausal women. *J Nutr.* (2016) 146:38–45. doi: 10.3945/jn.115.222414

Conflict of Interest Statement: The GSK Chair in Diabetes of the *Université de Sherbrooke* held by AC has been created in part through a donation of $1 million by GSK to the *Université de Sherbrooke*. AC has been recently funded by Janssen Canada to perform an investigator-initiated trial on the effect of treatment with canagliflozin on cardiac energy metabolism and function. AC has no other disclosures related to the content of this manuscript. AC holds research funding from the Canadian Institutes of Health Research, Canadian Diabetes Association, *Fonds de recherche Québec—Santé*, GSK, Janssen, Merck, Pfizer, AstraZeneca, Aventis, NovoNordisk, Eli Lilly, UniQure, Caprion Biosciences. AC has participated in advisory boards for the companies UniQure, Merck, Janssen and AstraZeneca and made one conference sponsored by AstraZeneca. The research and work at the basis of the present manuscript were not supported by any of these sources, aside from the GSK Chair in Diabetes of the *Université de Sherbrooke*.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Endocrinology

MINI REVIEW
published: 10 August 2018
doi: 10.3389/fendo.2018.00452

Androgen Action in the Ovary

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Androgen production by the ovary is an essential requirement for normal cyclical secretion of estradiol but its physiological role extends to important actions on both preantral and antral follicle development, including promotion of granulosa cell proliferation. It is likely only in mature antral follicles that androgens encourage apoptosis and consequent follicle atresia, and this may be an important mechanism to ensure mono-follicular ovulation in primates, including humans. Recent studies have provided new insight into the mechanism of androgen signaling in the ovary which involves both genomic and non-genomic effects that are complementary in effecting a cellular response. In polycystic ovary syndrome, a condition characterized by intra-ovarian androgen excess, aberrant development of both preantral and antral follicles is a salient feature. We present evidence that local action of androgens plays a part in such abnormalities. Finally, we review the role of androgens in follicle atresia and conclude that the effects are part of the normal physiology of follicle maturation.

Keywords: androgen receptor, follicle development, apoptosis, polycystic ovary syndrome, genomic and non-genomic actions, follicle atresia

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Edited by:
Jeff M. P. Holly,
University of Bristol, United Kingdom

Reviewed by:
Katja Teerds,
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Francisco Prat,
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Specialty section:
This article was submitted to Experimental Endocrinology, a section of the journal Frontiers in Endocrinology

Received: *01 June 2018*
Accepted: *24 July 2018*
Published: *10 August 2018*

Citation:
Franks S and Hardy K (2018) Androgen Action in the Ovary. Front. Endocrinol. 9:452. doi: 10.3389/fendo.2018.00452

INTRODUCTION

Whilst it is well recognized that androgens are an essential substrate for estradiol production by the ovary, the perception persists that androgens have an adverse effect on ovarian follicular development, even under physiological conditions but especially in an environment of androgen excess. This review will focus on the variety of androgen action on normal ovarian function and on the role of androgen excess in the etiology and ovarian manifestations of polycystic ovary syndrome (PCOS), the commonest endocrine disorder in women of reproductive age.

PHYSIOLOGY OF ANDROGEN ACTION IN THE OVARY

Androgens and Antral Follicle Function

The cyclical production of estradiol depends upon the availability of androgen, as a steroid precursor and, of course, cyclical changes in gonadotrophins. Under the influence of tonic levels of LH, androgens are produced by the theca cells of antral follicles. In the human ovary, LH receptors are present in theca cells but normally only appear in granulosa cells in mature follicles greater than 10 mm in diameter (i.e., the antral follicle that is most likely to go on to ovulate) (1). FSH receptors are present exclusively in the granulosa cells. Androgens (predominantly androstenedione and testosterone) diffuse across the basal lamina of the follicle to the granulosa layer where, under the control of FSH, they are converted to estrogen by the action of CYP19 (aromatase) (2). This co-ordinated interaction of gonadotrophins within the follicle is often referred to as the 2-cell, 2-gonadotophin process (2). Androgens may also have a role in the demise of antral follicles that form part of the cohort that undergo further growth in response to the early follicular phase rise in FSH

but regress in the late follicular phase as FSH levels fall (1, 3). This is a physiological mechanism that ensures that in humans (and non-human primates), mono-follicular ovulation is the rule. The ability of androgens to induce atresia in antral follicles has often been cast as a deleterious effect, particularly under conditions of androgen excess (notably PCOS) but the role of androgens may be rather more nuanced than has been described, as suggested below (see "Androgens and follicle atresia revisited").

Androgen Receptor Expression and Androgen Action Throughout Follicle Development

Although androgen receptor (AR) is found in all three components of the ovarian follicle, granulosa, theca and oocyte (4, 5), AR RNA and protein are most abundant in granulosa cells. In the primate ovary, there is little expression of AR in oocyte and theca of antral follicles (6) and in the human fetal ovary, AR expression is confined to somatic cells (7). Gene expression in the human ovary is high in granulosa cells of small antral follicles but reduces in pre-ovulatory follicles (8). AR expression is present in preantral follicles in rodent (9), ovine (10) as well as primate ovary, suggesting a physiological role in follicle development and function over and above the provision of substrate for estrogen production. In the human ovary, AR gene expression can be detected in human preantral follicles from the primary stage onwards (11), whilst AR protein can be observed from the primordial stage, gradually increasing during follicle development so that 100% of multi-layered, preantral follicles express AR protein (12) (**Figure 1**). AR gene expression is prominent in human antral follicles but it is noteworthy that peak expression is in small antral follicles (of around 6 mm in diameter) but is much reduced in larger (about 15 mm) antral follicles and lower still in the mature, preovulatory follicle (8). These changes in the level of AR expression during the later stages of follicle development may be important in terms of androgen action on survival or loss of follicles during a normal ovulatory cycle (see section on "Androgens and follicle atresia revisited" below).

There is plentiful evidence to show that androgens stimulate the growth of both preantral and antral follicles in various species (13–20). Androgen action appears be important for normal follicle development and function. Mice lacking AR in the ovary have impaired follicle maturation and reduced litter size (21–23). Recently, Walters and colleagues have shown that in a neurone-specific AR knockout mouse, there is significant disruption of the hypothalamic regulation of gonadotrophins, associated with abnormalities of ovarian follicular development (24). The potent androgen, dihydrotestosterone (DHT) stimulates protein expression of Ki67 (a marker of cell proliferation) in granulosa cells of mouse preantral follicles without effect on apoptosis (20). Androgen also increases responsiveness of granulosa cells to FSH in terms of both growth and expression of key genes involved in steroidogenesis (15, 20, 25, 26). These results reflect those of a seminal series of studies in primate ovary in which Bondy and colleagues demonstrated that *in vivo* exposure to androgen leads to growth of both preantral and antral follicles and was associated with increased expression of FSH receptor (FSHR) in granulosa cells (18, 27, 28). AR expression was found to be positively correlated with that of Ki67 and inversely related to apoptotic cell count (27).

In isolated, mouse preantral follicles, incubation with DHT greatly enhances expression of the steroid acute regulatory protein (StAR, a key regulator of steroidogenesis) in response to FSH (20). In the same model, both testosterone and DHT interact with members of the TGFβ superfamily, most noticeably reducing gene expression of anti-Mullerian hormone (AMH)

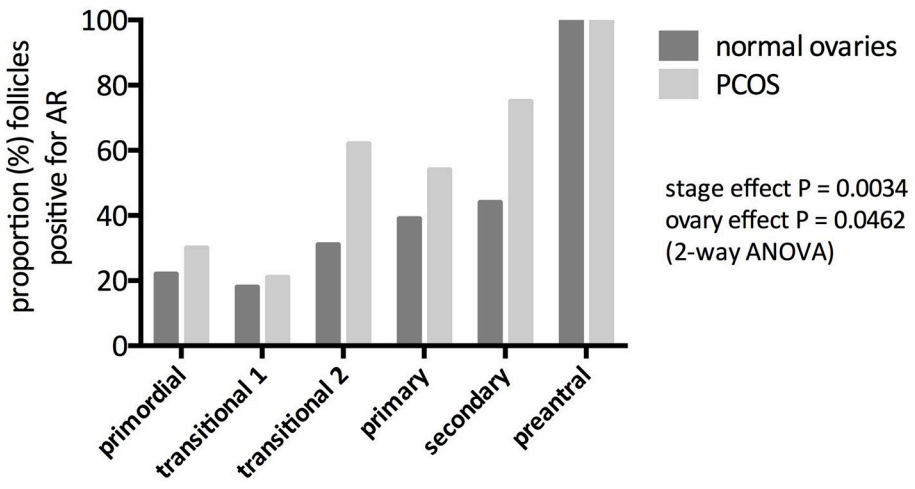

FIGURE 1 | Immunohistochemical identification of androgen receptor (AR) protein in preantral follicles of women with and without PCOS. Bars represent the proportion of follicles that stain positive for AR protein. AR expression increased significantly with increasing stage of follicle development in both normal and PCOS (stage effect) but here was a significantly greater abundance of AR in PCO follicles (ovary effect). From Webber (12).

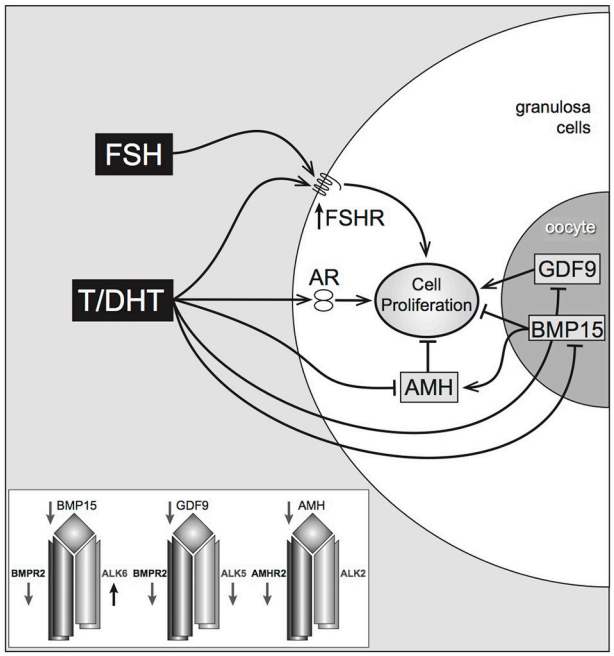

FIGURE 2 | Proposed pathways of androgen action on preantral follicle growth. Testosterone or DHT act via the androgen receptor, increasing granulosa cell proliferation. This may be mediated directly, or indirectly by increased FSHR (stimulating GC proliferation) or decreased AMH (reducing AMH inhibition). AMH can be further reduced by androgen-induced reduction of oocyte-specific BMP, which normally stimulates AMH levels. Inset box summarizes androgen-induced inhibition of TGFβ ligands, and type I and II TGFβ receptors, with the exception of *Alk6* Laird et al. (20).

(produced by granulosa cells) and bone morphogenetic protein-15 (BMP-15) (produced by the oocyte) both of which may have inhibitory effects on follicle growth (20) (although BMP-15, particularly in the presence of GDF-9 signaling, may also have a stimulatory action on follicle growth) (29). The positive effect of DHT on FSHR expression and the negative effect on the growth-inhibitory AMH and BMP-15, suggest that DHT-stimulated growth in preantral follicles is a complex phenomenon that relies upon a balance of endocrine and local growth factor actions (**Figure 2**). Conversely, there is evidence that BMPs 4, 6, and 7 have inhibitory actions on androgen production by bovine theca (30).

Androgen Signaling in the Ovary

The classic mode of androgen action, as for most steroids, involves binding of androgen to AR in the cell cytoplasm and translocation of the hormone-receptor complex to the nucleus where it binds to a specific sequence in the promotor of the relevant target gene and promotes gene transcription (31, 32). Whilst there is clear evidence that this pathway is operational in both physiological and pathological actions of androgens, recent work suggests that the pathway(s) of androgen signaling are more complex and involve rapid effects that do not involve classic nuclear receptor action on transcription ie non-genomic actions (22, 33, 34). These non-genomic actions have been highlighted in the work of Sen and colleagues who describe transactivation of the epidermal growth factor receptor (EGFR) by androgen. They show that androgens can activate the MAPK kinase pathway (by phosphorylation of ERK), which, classically, transduces rapid growth factor signaling (33, 35–37). In this sense, androgens appear to have growth factor properties. The action of androgens on ERK activation appear to be mediated by matrix metalloproteases (MMPs) and by paxillin (PXN), an adaptor protein which is also implicated in translocation of AR to the nucleus (22, 36). In this way, it is proposed that genomic and non-genomic actions of androgens can be co-ordinated and may work in concert (37). PXN is able to induce expression of the microRNA miR-125b which has an anti-apoptotic effect, hence promoting androgen induced follicle survival (33).

The important finding of an interaction of androgen with the EGFR is supported by data from our own laboratory. Exposure of mouse preantral follicles to a combination of DHT and EGF in culture results in stimulation of growth that is greater than either treatment alone. Furthermore, incubation of follicles with DHT in the presence of an EGF receptor inhibitor results in attenuation of the growth-promoting effect of DHT, strongly suggesting that the effect of androgen on proliferation of granulosa cells is, in part, mediated by activation of EGFR (38).

The EGF signaling pathway is one of several growth factor-signaling pathways with which androgens may interact. As previously mentioned, DHT influences expression of oocyte- and granulosa cell-derived growth factors of the TGFβ superfamily (20). Androgen can enhance both synthesis and action of insulin-like growth factors (IGFs) (18, 28). IGFs signal primarily though the PI3Kinase (PI3K) pathway and recently, Sen and colleagues have provided evidence that androgens also directly activate the PI3K signaling pathway, leading to a complex cascade of events that involve, in an initial and rapid effect, phosphorylation, and thereby inhibition, of the polycomb group protein enhancer of zeste homolog 2 (Ezh2) (34). Ezh2 appears to be a factor in regulation of LH action in the preovulatory follicle. In the longer term (24–48 h) the micro RNA miR-101 is induced which, in turn, greatly reduces the expression of Ezh2 protein (34). These findings give further support to their hypothesis that androgen action is likely to involve both genomic and non-genomic events that are closely co-ordinated.

ANDROGENS AND POLYCYSTIC OVARY SYNDROME

Increased Androgen Production in PCOS

Polycystic ovary syndrome (PCOS) is the commonest endocrine disorder in women of reproductive age (39, 40). Although there is a wide spectrum of clinical presentation, it is typically characterized by infrequent or absent ovulation together with clinical and/or biochemical evidence of androgen excess. The biochemical hall mark of PCOS is excess androgen production, predominantly of ovarian origin (39, 41).

Androgen Action in Polycystic Ovaries

The systemic effects of androgen excess include cutaneous manifestations (hirsutism, acne, androgenetic alopecia) and predisposition to metabolic derangement (including increased risk of type 2 diabetes mellitus) (42–49). But there are also local actions within the ovary that are characteristic of PCOS. Anovulation in PCOS is distinguished by arrest of growth of antral follicles at 5–8 mm (50). The mechanism of follicle arrest is complex but is likely to be due to the abnormal endocrine environment that includes excessive secretion of LH, insulin and androgens, all of which may contribute to premature arrest of follicles (1, 50, 51).

There is, in addition, clear evidence for aberrant development of preantral follicles in the ovaries of women with PCOS. The density of preantral follicles is increased compared with that in normal ovaries and there is a higher proportion of primordial follicles that have been activated and have started to grow (52) with evidence for accumulation ("stockpiling" at the primary stage (53). Small preantral follicles in PCOS show higher expression of the proliferation marker, mini-chromosome maintenance protein-2 (MCM-2) than that in size matched follicles of normal ovaries (54) and demonstrate prolonged survival in culture (55). These changes in early follicle development can be attributed, at least in part, to the effects of androgen. As described above, androgens stimulate preantral follicle growth in mice, rats, sheep, cows and primates (16–18, 20, 56–58). In the prenatally androgenised sheep, histological examination of ovarian cortical tissue reveals an increase in the proportion of growing preantral follicles and a reciprocal reduction in the proportion of primordial follicles, a pattern that mimics that seen in human cortical tissue in women with PCOS (52, 58).

An interesting question is whether the androgen-growth factor interactions, referred to above, play a part in aberrant early follicle development in PCOS. In that context, it has been shown that both gene and protein expression of the type 1 IGF receptor is increased in preantral follicles of women with PCOS (59). Furthermore, there are differences between normal and polycystic ovaries in growth responsiveness to IGF1 of follicles during culture of cortical tissue which suggest that PCOS follicles have been exposed to enhanced action of IGFs *in vivo* (59).

It remains unclear whether these aberrations in early follicle development contribute to the characteristic arrest of antral follicles in PCOS but the premature appearance of LH receptors in small antral follicles may provide a clue. Androgens induce FSHR expression and the acquisition of LH receptors in the dominant follicle that is destined to ovulate is an FSH-dependent event. As yet, we know little about FSHR expression in follicles of women with PCOS but it is noteworthy that cultured granulosa cells from small antral follicles in polycystic ovaries are hyper-responsive to FSH in terms of estradiol production (60, 61).

Androgen and the Developmental Origins of PCOS

The impact of excess androgen extends beyond the systemic and local effects described above Data from animal models of PCOS suggest that exposure to excess androgen during fetal life may play as significant part in the development of PCOS (62–67). In rodents, exposure to androgen in postnatal life can also reproduce some of reproductive and metabolic sequelae of PCOS (56, 57). Studies using large animal models (sheep, rhesus monkey) provide information that is perhaps more relevant to human PCOS, particularly as ovarian function is similar in terms of follicle development and mono-ovulatory cycles (62, 64, 66, 67). At critical stages during pregnancy, these animals are given very large doses of testosterone which are sufficient to overload the "buffering" of androgen action that occurs during normal pregnancy [elevated maternal plasma levels of sex hormone-binding globulin (SHBG) and activation of placental aromatase] that prevent the fetus being exposed to excess maternal androgen. The fetus is therefore androgenised and, in postnatal life, shows features which replicate many of the characteristics of PCOS including ovarian hyperandrogenism, infrequent or absent ovulation and metabolic dysfunction (65, 66). These findings raise the possibility that PCOS is a developmental disorder in which "programming" by excess androgen plays a key role—probably by epigenetic modification (64, 68). In human development, the source of excess androgen is unlikely to be maternal androgen (thanks to the protective effect of high, maternal, circulating levels of SHBG and placental aromatase). It is more plausible that, in human PCOS, the source of excess androgen is the ovary itself. We have postulated that the polycystic ovary is genetically predisposed to secrete excess androgen and that androgen excess is manifest, perhaps in the fetal ovary but more likely during the "mini-puberty" of infancy and/or at the onset of puberty itself (64, 68, 69). Certainly, there is strong evidence for a genetic basis of PCOS. Recent genome-wide association studies have indeed identified loci (such as DENND1A) that can be implicated in androgen production by theca cells (70–73).

Further evidence to support the notion that developmental programming by excess androgens plays a part in the origins of PCOS comes from data in women with congenital adrenal hyperplasia (CAH) due to 21-hydroxylase deficiency. Here, of course, the adrenal, rather than ovary is the source of excess androgen in fetal life and beyond. Women with a well-established diagnosis of CAH commonly (80% or more) have polycystic ovaries on ultrasound (74, 75) and, indeed, may have associated endocrine abnormalities including elevated serum levels of LH (76).

ANDROGENS AND FOLLICLE ATRESIA REVISITED

There is, as has been illustrated in this review, ample evidence to support the contention that androgens have a positive and, indeed obligatory, role in normal follicle growth and function. These phenomena call into question the widely perceived view that androgens are predominantly detrimental to normal ovarian function. Nevertheless, there is clear evidence that androgens have the ability to inhibit proliferation and promote apoptosis in mature antral follicles as, for example, shown in the rat ovary (77). These apparently paradoxical phenomena can perhaps be best explained by taking into account the stage of follicle

development. In the menstrual cycle of humans and non-human primates, mono-follicular ovulation is the rule. In such cycles, a single, "dominant" follicle is selected from the cohort of perhaps 5–10 small antral follicles that are recruited by the early follicular phase rise in FSH. Thereafter, it is the follicle that is most responsive to FSH that continues on the path to ovulation whereas the subsidiary follicles are unable to progress because of the negative feedback on effect on FSH of rising circulating levels of estradiol (and inhibin) (1, 50). FSH deficiency clearly plays a role in the atresia of the smaller follicles but, in this context, intra-follicular androgen concentrations appear also to play an important role.

Hillier and colleagues demonstrated a biphasic action of androgens in the ovaries of a non-human primate, the marmoset. In small antral follicles, androgens augmented FSH action on aromatase activity whereas, in larger follicles, androgen had a clear inhibitory effect (26). In a classic study, McNatty and colleagues measured estradiol and androstenedione concentrations in a large number of individual, healthy and atretic human ovarian follicles. Androgen concentrations were similar in healthy and atretic follicles but atretic follicles were characterized by much lower levels of estradiol (3). This has been interpreted as an indication that a high androstenedione to estradiol ratio (i.e., an excess of androgen over estrogen) contributes to (if not causes) follicle atresia. However, it can also be viewed as an effect of FSH deficiency, which itself is the major reason for demise of subsidiary follicles. Nevertheless, the striking finding that AR expression, which is high in small antral follicles, is drastically reduced in the healthy, preovulatory follicle (8) points to the removal, or reduction, of a potentially deleterious effect of androgen on granulosa cell survival and function in the mature follicle.

SUMMARY

In this review, we have provided evidence that androgens have a clear and important physiological role in follicle development, at all stages, and in estrogen production by antral follicles. In PCOS, androgen excess may contribute to aberrant preantral and antral follicle function in PCOS although other endocrine (and paracrine) factors play a part. The role of androgens in causing follicle atresia, in a normal cycle, has probably been exaggerated. FSH deficiency is likely to be the major cause of atresia in subsidiary follicles in mono-ovulatory species but in these estrogen deficient, androgen dominated follicles, androgen action may contribute to follicle loss.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

REFERENCES

1. Hillier SG. Current concepts of the roles of follicle stimulating hormone and luteinizing hormone in folliculogenesis. *Hum Reprod.* (1994) 9:188–91. doi: 10.1093/oxfordjournals.humrep.a138480
2. Hillier SG, Whitelaw PF, Smyth CD. Follicular oestrogen synthesis: the 'two-cell, two-gonadotrophin' model revisited. *Mol Cell Endocrinol.* (1994) 100:51–4. doi: 10.1016/0303-7207(94)90278-X
3. Westergaard L, Christensen IJ, McNatty KP. Steroid levels in ovarian follicular fluid related to follicle size and health status during the normal menstrual cycle in women. *Hum Reprod.* (1986) 1:227–32. doi: 10.1093/oxfordjournals.humrep.a136390
4. Lenie S, Smitz J. Functional AR signaling is evident in an *in vitro* mouse follicle culture bioassay that encompasses most stages of folliculogenesis. *Biol Reprod.* (2009) 80:685–95. doi: 10.1095/biolreprod.107.067280
5. Walters KA, Handelsman DJ. Role of androgens in the ovary. *Mol Cell Endocrinol.* (2018) 465:36–47. doi: 10.1016/j.mce.2017.06.026
6. Hillier SG, Tetsuka M, Fraser HM. Location and developmental regulation of androgen receptor in primate ovary. *Hum Reprod.* (1997) 12:107–11. doi: 10.1093/humrep/12.1.107
7. Fowler PA, Anderson RA, Saunders PT, Kinnell H, Mason JI, Evans DB, et al. Development of steroid signaling pathways during primordial follicle formati on in the human fetal ovary. *J Clin Endocrinol Metab.* (2011) 96:1754–62. doi: 10.1210/jc.2010-2618
8. Jeppesen JV, Kristensen SG, Nielsen ME, Humaidan P, Dal Canto M, Fadini R, et al. LH-receptor gene expression in human granulosa and cumulus cells from antral and preovulatory follicles. *J Clin Endocrinol Metab.* (2012) 97: E1524–31. doi: 10.1210/jc.2012-1427
9. Tetsuka M, Whitelaw PF, Bremner WJ, Millar MR, Smyth CD, Hillier SG. Developmental regulation of androgen receptor in rat ovary. *J Endocrinol.* (1995) 145:535–43. doi: 10.1677/joe.0.1450535
10. Juengel JL, Heath DA, Quirke LD, McNatty KP. Oestrogen receptor alpha and beta, androgen receptor and progesterone receptor mRNA and protein localisation within the developing ovary and in small growing follicles of sheep. *Reproduction* (2006) 131:81–92. doi: 10.1530/rep.1.00704
11. Rice S, Ojha K, Whitehead S, Mason H. Stage-specific expression of androgen receptor, follicle-stimulating hormone receptor, and anti-Mullerian hormone type II receptor in single, isolated, human preantral follicles: relevance to polycystic ovaries. *J Clin Endocrinol Metab.* (2007) 92:1034–40. doi: 10.1210/jc.2006-1697
12. Webber LJ. *Prentral Follicle Development in Normal and Polycystic Ovaries*. PhD Thesis, Imperial College London, Chapter 5 (2005).
13. Nimrod A, Lindner HR. A synergistic effect of androgen on the stimulation of progesterone secretion by FSH in cultured rat granulosa cells. *Mol Cell Endocrinol.* (1976) 5:315–20.
14. Hillier SG, Knazek RA, Ross GT. Androgenic stimulation of progesterone production by granulosa cells from preantral ovarian follicles: further *in vitro* studies using replicate cell cultures. *Endocrinology* (1977) 100:1539–49.
15. Wang XN, Greenwald GS. Synergistic effects of steroids with FSH on folliculogenesis, steroidogenesis and FSH- and hCG-receptors in hypophysectomized mice. *J Reprod Fertil.* (1993) 99:403–13. doi: 10.1530/jrf.0.0990403
16. Murray AA, Gosden RG, Allison V, Spears N. Effect of androgens on the development of mouse follicles growing in vitro. *J Reprod Fertil.* (1998) 113:27–33. doi: 10.1530/jrf.0.1130027
17. Yang MY, Fortune JE. Testosterone stimulates the primary to secondary follicle transition in bovine follicles *in vitro*. *Biol Reprod.* (2006) 75:924–32. doi: 10.1095/biolreprod.106.051813
18. Vendola KA, Zhou J, Adesanya OO, Weil SJ, Bondy CA. Androgens stimulate early stages of follicular growth in the primate ovary. *J Clin Invest.* (1998) 101:2622–9. doi: 10.1172/JCI2081
19. Qureshi AI, Nussey SS, Bano G, Musonda P, Whitehead SA, Mason HD. Testosterone selectively increases primary follicles in ovarian cortex grafted onto embryonic chick membranes: relevance to polycystic ovaries. *Reproduction* (2008) 136:187–94. doi: 10.1530/REP-07-0172

20. Laird M, Thomson K, Fenwick M, Mora J, Franks S, Hardy K. Androgen stimulates growth of mouse preantral follicles *in vitro*: interaction with follicle-stimulating hormone and with growth factors of the TGFbeta superfamily. *Endocrinology* (2017) 158:920–35. doi: 10.1210/en.2016-1538
21. Shiina H, Matsumoto T, Sato T, Igarashi K, Miyamoto J, Takemasa S, et al. Premature ovarian failure in androgen receptor-deficient mice. *Proc Natl Acad Sci USA*. (2006) 103:224–9. doi: 10.1073/pnas.0506736102
22. Sen A, Hammes SR. Granulosa cell-specific androgen receptors are critical regulators of ovarian development and function. *Mol Endocrinol.* (2010) 24:1393–403. doi: 10.1210/me.2010-0006
23. Walters KA, Middleton LJ, Joseph SR, Hazra R, Jimenez M, Simanainen U, et al. Targeted loss of androgen receptor signaling in murine granulosa cells of preantral and antral follicles causes female subfertility. *Biol Reprod.* (2012) 87:151. doi: 10.1095/biolreprod.112.102012
24. Walters KA, Edwards MC, Tesic D, Caldwell ASL, Jimenez M, Smith JT, et al. The Role of Central Androgen Receptor Actions in Regulating the Hypothalamic-Pituitary-Ovarian Axis. *Neuroendocrinology* (2018) 106:389–400. doi: 10.1159/000487762
25. Dorrington JH, McKeracher HL, Chan AK, Gore-Langton RE. Hormonal interactions in the control of granulosa cell differentiation. *J Steroid Biochem.* (1983) 19:17–32.
26. Harlow CR, Shaw HJ, Hillier SG, Hodges JK. Factors influencing follicle-stimulating hormone-responsive steroidogenesis in marmoset granulosa cells: effects of androgens and the stage of follicular maturity. *Endocrinology* (1988) 122:2780–7.
27. Weil SJ, Vendola K, Zhou J, Adesanya OO, Wang J, Okafor J, et al. Androgen receptor gene expression in the primate ovary: cellular localization, regulation, and functional correlations. *J Clin Endocrinol Metab.* (1998) 83:2479–85.
28. Vendola K, Zhou J, Wang J, Bondy CA. Androgens promote insulin-like growth factor-I and insulin-like growth factor-I receptor gene expression in the primate ovary. *Hum Reprod.* (1999) 14:2328–32.
29. Fenwick MA, Mora JM, Mansour YT, Baithun C, Franks S, Hardy K. Investigations of TGF-beta signaling in preantral follicles of female mice reveal differential roles for bone morphogenetic protein 15. *Endocrinology* (2013) 154:3423–36. doi: 10.1210/en.2012-2251
30. Glister C, Richards SL, Knight PG. Bone morphogenetic proteins (BMP)−4,−6, and−7 potently suppress basal and luteinizing hormone-induced androgen production by bovine theca interna cells in primary culture: could ovarian hyperandrogenic dysfunction be caused by a defect in thecal BMP signaling? *Endocrinology* (2005) 146:1883–92. doi: 10.1210/en.2004-1303
31. Simental JA, Sar M, Lane MV, French FS, Wilson EM. Transcriptional activation and nuclear targeting signals of the human androgen receptor. *J Biol Chem.* (1991) 266:510–8.
32. Askew EB, Minges JT, Hnat AT, Wilson EM. Structural features discriminate androgen receptor N/C terminal and coactivator interactions. *Mol Cell Endocrinol.* (2012) 348:403–10. doi: 10.1016/j.mce.2011.03.026
33. Sen A, Prizant H, Light A, Biswas A, Hayes E, Lee HJ, et al. Androgens regulate ovarian follicular development by increasing follicle stimulating hormone receptor and microRNA-125b expression. *Proc Natl Acad Sci USA*. (2014) 111:3008–13. doi: 10.1073/pnas.1318978111
34. Ma X, Hayes E, Biswas A, Seger C, Prizant H, Hammes SR et al. Androgens Regulate Ovarian Gene Expression Through Modulation of Ezh2 Expression and Activity. *Endocrinology* (2017) 158:2944–54. doi: 10.1210/en.2017-00145
35. Sen A, O'Malley K, Wang Z, Raj GV, Defranco DB, Hammes SR. Paxillin regulates androgen- and epidermal growth factor-induced MAPK signaling and cell proliferation in prostate cancer cells. *J Biol Chem.* (2010) 285:28787–95. doi: 10.1074/jbc.M110.134064
36. Sen A, De Castro I, Defranco DB, Deng FM, Melamed J, Kapur P, et al. Paxillin mediates extranuclear and intranuclear signaling in prostate cancer proliferation. *J Clin Invest.* (2012) 122:2469–81. doi: 10.1172/JCI62044
37. Prizant H, Gleicher N, Sen A. Androgen actions in the ovary: balance is key. *J Endocrinol.* (2014) 222: R141–51. doi: 10.1530/JOE-14-0296
38. Thomson K, Mora, J,M.,Laird, M., Hardy, K. Investigating a role for the epidermal growth factor receptor in androgen signalling within mouse preantral follicle development. *Reprod Abstracts.* (2015) 2:O016. doi: 10.1530/repabs.2.O016
39. Franks S. Polycystic ovary syndrome. *N Engl J Med.* (1995) 333:853–61. doi: 10.1056/NEJM199509283331307
40. Ehrmann DA. Polycystic ovary syndrome. *N Engl J Med.* (2005) 352:1223–36. doi: 10.1056/NEJMra041536
41. Gilling-Smith C, Story H, Rogers V, Franks S. Evidence for a primary abnormality of thecal cell steroidogenesis in the polycystic ovary syndrome. *Clin Endocrinol (Oxf)* (1997) 47:93–9.
42. Dunaif A, Graf M, Mandeli J, Laumas V, Dobrjansky A. Characterization of groups of hyperandrogenic women with acanthosis nigricans, impaired glucose tolerance, and/or hyperinsulinemia. *J Clin Endocrinol Metab.* (1987) 65:499–507.
43. Robinson S, Chan SP, Spacey S, Anyaoku V, Johnston DG, Franks S. Postprandial thermogenesis is reduced in polycystic ovary syndrome and is associated with increased insulin resistance. *Clin Endocrinol (Oxf)* (1992) 36:537–43. doi: 10.1111/j.1365-2265.1992.tb02262.x
44. Robinson S, Henderson AD, Gelding SV, Kiddy D, Niththyananthan R, Bush A, et al. Dyslipidaemia is associated with insulin resistance in women with polycystic ovaries. *Clin Endocrinol (Oxf)* (1996) 44:277–84.
45. Dunaif A. Insulin resistance and the polycystic ovary syndrome: mechanism and implications for pathogenesis. *Endocr Rev.* (1997) 18:774–800.
46. Dunaif A. Perspectives in polycystic ovary syndrome: from hair to eternity. *J Clin Endocrinol Metab.* (2016) 101:759–68. doi: 10.1210/jc.2015-3780
47. Moran LJ, Misso ML, Wild RA, Norman RJ. Impaired glucose tolerance, type 2 diabetes and metabolic syndrome in polycystic ovary syndrome: a systematic review and meta-analysis. *Hum Reprod Update.* (2010) 16:347–63. doi: 10.1093/humupd/dmq001
48. Joham AE, Ranasinha S, Zoungas S, Moran L, Teede HJ. Gestational diabetes and type 2 diabetes in reproductive-aged women with polycystic ovary syndrome. *J Clin Endocrinol Metab.* (2014) 99:E447–52. doi: 10.1210/jc.2013-2007
49. Couto Alves A, Valcarcel B, Makinen VP, Morin-Papunen L, Sebert S, Kangas AJ, et al. Metabolic profiling of polycystic ovary syndrome reveals interactions with abdominal obesity. *Int J Obes.* (2017) 41:1331–40. doi: 10.1038/ijo.2017.126
50. Franks S, Stark J, Hardy K. Follicle dynamics and anovulation in polycystic ovary syndrome. *Hum Reprod Update.* (2008) 14:367–78. doi: 10.1093/humupd/dmn015
51. Willis DS, Watson H, Mason HD, Galea R, Brincat M, Franks S. Premature response to luteinizing hormone of granulosa cells from anovulatory women with polycystic ovary syndrome: relevance to mechanism of anovulation. *J Clin Endocrinol Metab.* (1998) 83:3984–91.
52. Webber LJ, Stubbs S, Stark J, Trew GH, Margara R, Hardy K, et al. Formation and early development of follicles in the polycystic ovary. *Lancet* (2003) 362:1017–21. doi: 10.1016/S0140-6736(03)14410-8
53. Maciel GA, Baracat EC, Benda JA, Markham SM, Hensinger K, Chang RJ, et al. Stockpiling of transitional and classic primary follicles in ovaries of women with polycystic ovary syndrome. *J Clin Endocrinol Metab.* (2004) 89:5321–7. doi: 10.1210/jc.2004-0643
54. Stubbs SA, Stark J, Dilworth SM, Franks S, Hardy K. Abnormal preantral folliculogenesis in polycystic ovaries is associated with increased granulosa cell division. *J Clin Endocrinol Metab.* (2007) 92:4418–26. . doi: 10.1210/jc.2007-0729
55. Webber LJ, Stubbs SA, Stark J, Margara RA, Trew GH, Lavery SA, et al. Prolonged survival in culture of preantral follicles from polycystic ovaries. *J Clin Endocrinol Metab.* (2007) 92:1975–8. doi: 10.1210/jc.2006-1422
56. Manneras L, Cajander S, Holmang A, Seleskovic Z, Lystig T, Lönn M, et al. A new rat model exhibiting both ovarian and metabolic characteristics of polycystic ovary syndrome. *Endocrinology* (2007) 148:3781–91. doi: 10.1210/en.2007-0168
57. van Houten EL, Kramer P, McLuskey A, Karels B, Themmen AP, Visser JA. Reproductive and metabolic phenotype of a mouse model of PCOS. *Endocrinology* (2012) 153:2861–9. doi: 10.1210/en.2011-1754
58. Forsdike RA, Hardy K, Bull L, Stark J, Webber LJ, Stubbs S, et al. Disordered follicle development in ovaries of prenatally androgenized ewes. *J Endocrinol.* (2007) 192:421–8. doi: 10.1677/joe.1.07097
59. Stubbs SA, Webber LJ, Stark J, Rice S, Margara R, Lavery S, et al. Role of Insulin-like growth factors in initiation of follicle growth in normal and

polycystic human ovaries. *J Clin Endocrinol Metab.* (2013) 98:3298–305. doi: 10.1210/jc.2013-1378
60. Erickson GF, Garzo VG, Magoffin DA. Insulin-like growth factor-I regulates aromatase activity in human granulosa and granulosa luteal cells. *J Clin Endocrinol Metab.* (1989) 69:716–24. doi: 10.1210/jcem-69-4-716
61. Mason HD, Willis DS, Beard RW, Winston RM, Margara R, Franks S. Estradiol production by granulosa cells of normal and polycystic ovaries: relationship to menstrual cycle history and concentrations of gonadotropins and sex steroids in follicular fluid. *J Clin Endocrinol Metab.* (1994) 79:1355–60.
62. Franks S. Animal models and the developmental origins of polycystic ovary syndrome: increasing evidence for the role of androgens in programming reproductive and metabolic dysfunction. *Endocrinology* (2012) 153:2536–8. doi: 10.1210/en.2012-1366
63. Roland AV, Moenter SM. Reproductive neuroendocrine dysfunction in polycystic ovary syndrome: insight from animal models. *Front Neuroendocrinol.* (2014) 35:494–511. doi: 10.1016/j.yfrne.2014.04.002
64. Abbott DH, Dumesic DA, Franks S. Developmental origin of polycystic ovary syndrome - a hypothesis. *J Endocrinol.* (2002) 174:1–5. doi: 10.1677/joe.0.1740001
65. Hogg K, Young JM, Oliver EM, Souza CJ, McNeilly AS, Duncan WC. Enhanced thecal androgen production is prenatally programmed in an ovine model of polycystic ovary syndrome. *Endocrinology* (2012) 153:450–61. doi: 10.1210/en.2011-1607
66. Abbott DH, Nicol LE, Levine JE, Xu N, Goodarzi MO, Dumesic DA. Nonhuman primate models of polycystic ovary syndrome. *Mol Cell Endocrinol.* (2013) 373:21–8. doi: 10.1016/j.mce.2013.01.013
67. Padmanabhan V, Veiga-Lopez A. Sheep models of polycystic ovary syndrome phenotype. *Mol Cell Endocrinol.* (2013) 373:8–20. doi: 10.1016/j.mce.2012.10.005
68. Gorry A, White DM, Franks S. Infertility in polycystic ovary syndrome: focus on low-dose gonadotropin treatment. *Endocrine* (2006) 30:27–33. doi: 10.1385/ENDO:30:1:27
69. Jayasena CN, Franks S. The management of patients with polycystic ovary syndrome. *Nat Rev Endocrinol.* (2014) 10:624–36. doi: 10.1038/nrendo.2014.102
70. Chen ZJ, Zhao H, He L, Shi Y, Qin Y, Shi Y, et al. Genome-wide association study identifies susceptibility loci for polycystic ovary syndrome on chromosome 2p16.3, 2p21 and 9q33.3. *Nat Genet.* (2011) 43:55–9. doi: 10.1038/ng.732
71. McAllister JM, Modi B, Miller BA, Shi Y, Qin Y, Shi Y, et al. Overexpression of a DENND1A isoform produces a polycystic ovary syndrome theca phenotype. *Proc Natl Acad Sci USA.* (2014) 111: E1519–27. doi: 10.1073/pnas.14005 74111
72. Hayes MG, Urbanek M, Ehrmann DA, Armstrong LL, Lee JY, Sisk R, et al. Genome-wide association of polycystic ovary syndrome implicates alterations in gonadotropin secretion in European ancestry populations. *Nat Commun.* (2015) 6:7502. doi: 10.1038/ncomms8502
73. Day FR, Hinds DA, Tung JY, Stolk L, Styrkarsdottir U, Saxena R, et al. Causal mechanisms and balancing selection inferred from genetic associations with polycystic ovary syndrome. *Nat Commun.* (2015) 6:8464. doi: 10.1038/ncomms9464
74. Hague WM, Adams J, Rodda C, , Brook CG, de Bruyn R, Grant DB, et al. The prevalence of polycystic ovaries in patients with congenital adrenal hyperplasia and their close relatives. *Clin Endocrinol (Oxf)* (1990) 33:501–10.
75. Carmina E, Dewailly D, Escobar-Morreale HF, Kelestimur F, Moran C, Oberfield S, et al. Non-classic congenital adrenal hyperplasia due to 21-hydroxylase deficiency revisited: an update with a special focus on adolescent and adult women. *Hum Reprod Update* (2017) 23:580–99. doi: 10.1093/humupd/dmx014
76. Barnes RB, Rosenfield RL, Ehrmann DA, Cara JF, Cuttler L, Levitsky LL, et al. Ovarian hyperandrogynism as a result of congenital adrenal virilizing disorders: evidence for perinatal masculinization of neuroendocrine function in women. *J Clin Endocrinol Metab.* (1994) 79:1328–33.
77. Billig H, Furuta I, Hsueh AJ. Estrogens inhibit and androgens enhance ovarian granulosa cell apoptosis. *Endocrinology* (1993) 133:2204–12. doi: 10.1210/endo.133.5.8404672

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Endocrinology

REVIEW
published: 28 August 2018
doi: 10.3389/fendo.2018.00498

Gut-Brain Neuroendocrine Signaling Under Conditions of Stress—Focus on Food Intake-Regulatory Mediators

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The gut-brain axis represents a bidirectional communication route between the gut and the central nervous system comprised of neuronal as well as humoral signaling. This system plays an important role in the regulation of gastrointestinal as well as homeostatic functions such as hunger and satiety. Recent years also witnessed an increased knowledge on the modulation of this axis under conditions of exogenous or endogenous stressors. The present review will discuss the alterations of neuroendocrine gut-brain signaling under conditions of stress and the respective implications for the regulation of food intake.

Keywords: food intake, gastrointestinal functions, gut-brain axis, hypothalamus, peptides

OPEN ACCESS

Edited by:
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Specialty section:
This article was submitted to Neuroendocrine Science, a section of the journal Frontiers in Endocrinology

Received: *31 May 2018*
Accepted: *08 August 2018*
Published: *28 August 2018*

Citation:
Stengel A and Taché Y (2018) Gut-Brain Neuroendocrine Signaling Under Conditions of Stress—Focus on Food Intake-Regulatory Mediators. Front. Endocrinol. 9:498. doi: 10.3389/fendo.2018.00498

INTRODUCTION

Peripheral signals reach the brain *via* neuronal and humoral pathways. The neuronal connection from the gut to the brain through vagal afferents originating from pseudo-unipolar cell bodies located in the nodose ganglia is the most extensively investigated (1, 2). The vagus nerve is composed of over 80% of afferent fibers which convey chemical and mechanosensory signals involved in the regulation of food intake and body weight (3). Peptide hormones predominantly produced in the gut interact with cognate G protein seven transmembrane domain receptors localized on nodose ganglia neurons (4). The expression of these receptors is modulated by feeding and fasting (2, 5) underlining the importance of vagal pathways in the control of energy homeostasis.

Stress influences the expression or circulating levels of several gastrointestinal peptides involved in the regulation of metabolic status under conditions of hunger or satiety (6). The impact of these alterations on the stress response has subsequently been investigated. The present review will highlight the impact of stress on peptidergic gut-brain hormones primarily involved in the regulation of food intake along with the functional implications.

MODULATION OF GUT-BRAIN SIGNALING UNDER CONDITIONS OF STRESS

Ghrelin

Ghrelin has been identified in the rat stomach (7) which is by far the major site of synthesis as indicated by the pronounced decrease of circulating ghrelin levels following gastrectomy (8).

Ghrelin is produced in gastric endocrine X/A-like cells (human nomenclature: P/D_1 cells) (9) and bears a unique fatty acid residue on its third amino acid essential to bind to its receptor, the growth hormone secretagogue receptor 1a (GHSR1a) (7) now also designated as the ghrelin receptor (GRLN) (10). The enzyme catalyzing this acylation was identified later on and named ghrelin-*O*-acyltransferase (GOAT) (11, 12). Double labeling showed that GOAT immunoreactive cells co-labeled with ghrelin expression in rodents (13) and humans (14). In addition, the finding that GOAT was detected in the pancreas (15) and circulation of rodents (13) and humans (16) supports additional extragastric acylation of the peptide.

Early on, ghrelin has been reported to stimulate food intake after peripheral and central injection in animals (17) and peripheral infusion in humans (18) leading to an increased body weight after repeated injections (19). Expression of the GHSR1a on vagal afferents and the blunting of the peptide's orexigenic action by vagotomy or selective reduction of the GHSR1a in nodose ganglia support a major role of the vagus in mediating ghrelin's action in rats (20, 21). Nonetheless, ghrelin was also shown to cross the blood-brain barrier in both directions (22) indicative of an additional humoral mode of signaling. In the hypothalamus, the GHSR1a is expressed on neuropeptide Y (NPY)/agouti-related peptide (AgRP) neurons of the arcuate nucleus (23, 24). Neuroanatomical and functional studies using optogenetics indicate that ghrelin expressed in axon terminals innervating hypothalamic nuclei increases NPY/AgRP activity (25, 26). In addition, the ghrelin-induced stimulation of food intake is abolished in NPY/AgRP knockout mice (27) demonstrating an essential role of these signaling pathways in mediating ghrelin's orexigenic action in the hypothalamus.

The predominant form of circulating ghrelin is, however, the non-acylated form, des-acyl ghrelin (28, 29). Des-acyl ghrelin initially received little attention due to its lack of affinity to the GHSR1a (7). Nonetheless, des-acyl ghrelin exhibits several biological actions such as decreasing anxiety after intraperitoneral injection in receptor knockout mice (30). Peripheral or intracerebroventricular pretreatment with des-acyl ghrelin blunts the orexigenic action of ghrelin in rats (31) and mice (32). Des-acyl ghrelin's action takes place in a subset of arcuate nucleus neurons distinct from those activated by ghrelin (32). Studies using fluorescein des-acyl ghrelin injected intracerebroventricularly in mice demonstrate that the peptide binds selectively and mainly on arcuate neurons in a GHSR1a independent manner (32). However, to date the receptor mediating des-acyl ghrelin's effects remains to be identified.

Exposure to various acute or chronic stressors influences ghrelin expression and circulating levels although the response varies with the modality of stressors and experimental conditions as detailed in a previous review (6). Tail pinch or starvation increases gastric ghrelin mRNA expression in mice (33). Several other acute stressors including psychological (water avoidance stress, trier social stress test), physical (cold ambient temperature, restraint at 18° C, cholecystectomy, colectomy, cold pressure test) or metabolic (fasting) increase circulating ghrelin levels (34–43). It is to note that in the clinical setting the acute social stress test-induced rise in circulating ghrelin and cortisol levels was not associated with binge eating (35). Likewise, chronic stressors such as repeated restraint in rats, social defeat in mice or trauma in humans also induce a sustained elevation of circulating ghrelin levels lasting for months after the cessation of the stress making ghrelin a persistent biomarker of chronic stress (44–50). The rise in ghrelin may represent a compensatory action to counteract chronic stress-induced anxiety and depression-like behavior (49, 51, 52). Indeed, ghrelin increased the rewarding aspect of food (46) and body weight observed under these conditions, effects no longer observed in GHSR1a knockout mice (47).

By contrast, conditions of stress associated with inflammation decrease ghrelin expression or circulating levels (6). In detail, immune stress triggered by intraperitoneal injection of lipopolysaccharide results in a rapid decline in ghrelin levels associated with a decrease in circulating GOAT protein concentration likely contributing to the reduced acylation in rats (53). Abdominal surgery associated with gastric inflammation (54) decreases acyl and des-acyl ghrelin levels (55, 56) and food intake (57) in rats, an effect blunted by rikkunshito, a herbal medicine stimulating the release of ghrelin (58). Central vagal stimulation, which normalizes the gastric inflammatory response (54), prevents the reduction of plasma ghrelin (55). Chronic inflammatory stress elicited by adjuvant-induced arthritis in rats or rheumatoid arthritis in humans reduces circulating ghrelin levels (59). There are also reports that a psychological stressor such as novelty stress in mice decreases plasma levels of ghrelin and food intake, alterations prevented by rikkunshito (60, 61). Chronic restraint stress or exposure to foot-shock downregulates ghrelin mRNA expression in the mouse hypothalamus (62) and reduces plasma levels of ghrelin in rats (63). These alterations were associated with decreased food intake and body weight gain in mice (62).

Whether the differential alterations of ghrelin by stressors reflect differences in species, metabolic status and/or stressor-related specific recruitment of central and/or peripheral signaling pathways regulating ghrelin release (56, 64) warrant further investigations. Moreover, it cannot be ruled out that difference in modalities to determine ghrelin (total vs. acyl, radioimmunoassay vs. enzyme-linked immunosorbent assay, commercial vs. custom-made kits) might also affect the levels reported.

However, while stressors modulate circulating ghrelin levels, there is also evidence that ghrelin stimulates the hypothalamic-pituitary adrenal axis (33). The peptide injected peripherally upregulates hypothalamic corticotropin-releasing factor (CRF) (33), a key peptide involved in the stress response (65). Recent studies indicate that ghrelin acts *via* the inhibition of hypothalamic GABAergic signaling on CRF neurons in the paraventricular nucleus of the hypothalamus (PVN) (66). In hypothalamic 4B cells *in vitro,* ghrelin stimulates CRF promoter activity through activation of protein kinase A and phospholipase C pathways resulting in increased CRF mRNA levels (67). These data suggest a bidirectional interaction between CRF and the ghrelin signaling system.

Nesfatin-1

Nesfatin-1 has been first detected in the rat hypothalamus as an 82-amino acid peptide derived from nucleobindin2 (NUCB2)

(68). Subsequent research showed a more widespread brain distribution (69) as well as a 10-fold higher expression of NUCB2 mRNA in the stomach indicating that the upper gut is a major site of production (70). Interestingly, immunohistochemical double labeling showed that NUCB2/nesfatin-1 (the antibody recognizes both nesfatin-1 and the full length NUCB2) co-localizes with ghrelin indicating the production in the same gastric endocrine cell type, namely X/A-like cells in rats (70). This finding was later confirmed in humans where these cells are named P/D_1 cells (14). Nesfatin-1 is able to reach the brain humorally but likely also acts *via* the vagus nerve as intraperitoneal injection induces Fos expression in neurons of the nucleus of the solitary tract that receives input from vagal afferents (71). However, the putative receptor mediating nesfatin-1's effects is still unknown. Converging evidence points toward a G protein-coupled receptor (72, 73). A recent autoradiographic study indicates widespread binding of ^{125}I-labeled nesfatin-1 in the brain with signals in the cortex, PVN, area postrema, dorsal motor nucleus of the vagus nerve and cerebellum (74) supporting its centrally mediated pleiotropic effects (75).

The anorexigenic effect of nesfatin-1 has been early on described in several species including rats (68, 76), mice (77), chicks (78), and goldfish (79) following intracerebroventricular injection. In contrast to the convergent findings on the robust food intake-reducing effects of centrally injected nesfatin-1, only one study in mice reported an anorexigenic effect after acute intraperitoneal injection of nesfatin-1 at high doses (71), while other studies in rats (76) and mice (77) showed no effect. Similarly, data following chronic peripheral administration did not produce consistent results: while a reduction of food intake was observed in rats (80), no effect was detected in mice (81). Taken together, the effect of peripheral nesfatin-1 on food intake seems less robust and may not be the primary function of peripherally produced nesfatin-1. By contrast, consistent reports showed that the peptide may play an important role in glucose-stimulated pancreatic insulin release in rats (82) and humans (83).

Convergent findings support an involvement of nesfatin-1 in the stress response. First, several stressors activate nesfatin-1 immunoreactive neurons in the brain, namely psychological (restraint, water avoidance stress) (84–87), physical (abdominal surgery) (88), immunological (injection of lipopolysaccharide) (89) as well as a combination of stressors (chronic variable mild stress) (90). Second, water avoidance stress (91) and injection of lipopolysaccharide (92) elevate circulating levels of nesfatin-1 likely due to the release of the peptide associated with the upregulation of NUCB2 mRNA expression assessed by RT-qPCR and NUCB2/nesfatin-1 protein concentration measured by Western blot in the stomach (92). Third, intracerebroventricular injection of nesfatin-1 increases plasma adrenocorticotropic hormone (ACTH) and corticosterone in rats, an effect likely occurring in the hypothalamus as *in vitro* nesfatin-1 stimulates cytosolic Ca^{2+} in CRF-containing cells of the PVN (86). Therefore, nesfatin-1 exerts its stress-mediating effect likely *via* downstream CRF signaling. Lastly, circulating NUCB2/nesfatin-1 levels are positively correlated with perceived stress in a human female obese population (93) suggesting a potential role in the mediation of stress in humans as well. Interestingly, suicide victims showed altered NUCB2 mRNA expression in a midbrain nucleus implicated in stress-related mood alterations, the Edinger-Westphal nucleus, with an 1.8-fold increase in males and a 2.7-fold decrease in females compared to control subjects who died without any diagnosed neurodegenerative or psychiatric disorder (94).

Urocortins

Belonging to the CRF family, urocortins (Ucns) have been identified, namely Ucn1, a 40-amino acid (aa) peptide sharing 45% sequence identity with rat/human (r/h) CRF (95), Ucn2, a 39-aa peptide sharing 34% identity with r/h CRF and 42% with r/h Ucn1 (96, 97) and Ucn3, a 38-aa peptide sharing 26% homology with r/h CRF and 21% with r/h Ucn1 (98). Ucn1 binds to both CRF receptors, CRF_1 and CRF_2, with equal affinity, whereas Ucn2 and Ucn3 bind to the CRF_2 receptor with high selectivity (99).

Besides their widespread brain distribution extensively reviewed elsewhere (100), Ucns are also expressed in the periphery, namely the heart, skeletal muscle, spleen, kidney, adipose tissue, ovary, skin (101) as well as the gastrointestinal tract including liver, pancreas, stomach, small, and large intestine (101–108).

Peripheral injection of Ucn1 inhibits food intake in different species including mice (109–111) and sheep (112). In rodents, Ucn1 reduces meal frequency and size and can induce conditioned taste aversion (113) and reduces body weight upon repeated peripheral administration (109). Reports showed that the food intake-reducing effect of Ucn1 is more potent compared to that of CRF, Ucn2, Ucn3, cholecystokinin (CCK) and leptin (109, 110, 113); moreover, a synergistic interaction between Ucn1 and CCK on satiety has been demonstrated (114). The anorexia induced by peripheral Ucn1 is mediated *via* the CRF_2 receptor based on the observation that the selective CRF_2 antagonists, antisauvagine-30 and astressin$_2$-B, unlike selective CRF_1 antagonists, suppress the Ucn1-induced reduction of food intake (110, 112, 115). The finding that CRF_2 knockout mice do not display a reduction of food intake after intraperitoneal injection of Ucn1 further corroborates the implication of this receptor subtype (115). The mechanism through which peripherally injected Ucn1 influences food intake is still to be elucidated. It is unlikely to be mediated by vagal afferent signaling as capsaicin treatment did not alter the anorexigenic response of the peptide in mice (110). Moreover, Ucn1 barely enters the brain through the blood-brain barrier (116). However, CRF_2 receptors are densely expressed in brain areas outside of the blood-brain barrier, namely the area postrema (117, 118), and neurons at this site are activated by peripheral Ucn1 (119) suggesting a possible pathway.

Ucn2 and Ucn3 injected peripherally also reduce *ad libitum* food intake during the dark phase as well as the refeeding response to a fast with Ucn2 being more potent compared to Ucn3 in mice (110, 111, 114, 115, 120), rats (113), and fish (121). The automated dark phase food intake monitoring showed that Ucn2 reduces meal size (increased satiation), while meal frequency (indicative of satiety) is not altered in mice (115).

Interestingly, under re-feeding conditions after a fast, meal size is also reduced, however, meal frequency is increased (decreased satiety) (115). It is important to note that Ucn2, unlike Unc1, does not induce signs of taste aversion (113) pointing toward a specific food intake-reducing effect. Moreover, Ucn2 acts synergistically with CCK to reduce food intake, an effect also observed *in vitro* when recording gastric vagal afferent activity (114). This supports a vagal mode of transmission corroborated by the expression of the CRF_2 receptor in the nodose ganglia (122, 123).

Various stressors upregulate the peripheral expression of Ucns. Injection of lipopolysaccharide, an immunological stressor, increases the expression of Ucn1, Ucn2 and Ucn3 mRNA in gastric mucosa and submucosa plus muscle layers (107) which is associated with the reduction of food intake under these conditions (53). Ucn1 and Ucn3 immunoreactivity in blood vessels and submucous neurons of the ileum is also increased following *Schistosoma mansoni*-induced inflammation (124). Likewise, blood monocyte-derived dendritic cells display largely increased Ucn1 mRNA and protein expression following stimulation with *Bacteroides vulgatus* or *Fusobacterium varium* (125). There is also evidence that psychological stressors (chronic social stress) upregulates Ucn2 mRNA expression in the pig colon (126), and maternal deprivation increases duodenal Ucn2 and CRF_2 receptor mRNA, whereas CRF_1 mRNA is decreased in rats (127).

Cholecystokinin

CCK is mainly produced in I cells scattered within the upper small intestine with more prominent distribution in the duodenum (128). These cells harbor the feature to be in direct contact with other cells *via* pseudopods (129). Several forms of CCK have been detected including CCK-5,−7,−8,−18,−22,−25,−33,−39, and−58 (representing the number of amino acids) with CCK-8 being the most commonly studied form (128). The demonstration that CCK-58 is the only form detected in the circulation when using a new method for blood processing suggests that the shorter forms are products of degradation (29).

The first described biological action of CCK was the stimulation of gallbladder contraction along with the stimulation of the production and release of pancreatic enzymes and secretion [for review see Sayegh (128)]. The food intake-suppressing effect of CCK was initially reported in rats, and later extended to rabbits, monkeys, pigs, sheep and humans [for review see Sayegh (128)]. Both forms of CCK, CCK-8 and CCK-58 were shown to decrease dark phase food intake following intraperitoneal injection in *ad libitum* fed rats by reducing meal size (130). However, CCK-58 does not shorten the subsequent inter-meal interval as observed following injection of CCK-8 providing evidence for a more sustained effect of CCK-58 (130). Moreover, CCK also suppresses gastric emptying in rats (131) and humans (132) contributing to its anorexigenic effect.

CCK interacts with two receptor subtypes, CCK_A (alimentary), expressed in the gastrointestinal tract and on vagal afferents and CCK_B (brain), predominantly expressed in the brain (133). CCK is postprandially released from duodenal I cells with lipids and proteins being the most potent stimulators (134–136). Released CCK binds to vagal CCK_A expressing afferents and activates neurons in the nucleus of the solitary tract to inhibit food intake, with vagotomy abolishing both the CCK-induced neuronal activation in the brain (137) as well as the anorexigenic effect (138).

A combination of immunological stress using infection with *Giardia lamblia* and psychological stress using the water avoidance model increases CCK levels in the colonic mucosa of mice (139). The stress-induced visceral hypersensitivity could be blocked using the CCK_A antagonist, L-364718, and the CCK_B antagonist, L-365260 following psychological but not immunological stress (139) giving rise to a role of CCK in visceral sensitivity under selective stress conditions. By contrast, acute or chronic intraperitoneal injection of CCK exerts a protective effect on the impairment of memory functions under conditions of chronic restraint stress (140, 141). Moreover, OLETF rat pups lacking the CCK_A display a higher separation-induced ultrasonic vocalization (142) as a surrogate for increased experience of stress. The link between the stress response and CCK signaling was further corroborated by the observation that a well-established immunological stressor, lipopolysaccharide, increases CCK mRNA expression in PVN CRF-containing neurons (143). Intraperitoneal injection of CCK stimulates neuronal activation in noradrenergic A2 neurons (144) as well as increases corticosterone levels to comparable magnitudes observed after injection of CRF (145). Also repetitive intracerebroventricular injections of cortagine, a CRF_1 agonist, increases CCK mRNA as well as CCK_B protein expression in the mouse amygdala and hippocampus resulting in heightened anxiety behavior as assessed using the elevated plus maze and open field test, an effect reversed by intracerebroventricular injection of the CCK_B antagonist, LY225910 (146). This anxiety-inducing effect of CCK has also been observed in pharmacological provocation studies following intracerebroventricular injection of CCK-8 in rats that reduced exploratory behavior in the light/dark paradigm (147). In humans, intravenous injection of CCK-4 was shown to induce anxiety and panic symptoms (148, 149). Lastly, tail pinch stress-induced eating in rats (150) is reduced by intracerebroventricular injection of CCK-8 (151). Collectively these observations are indicative of a modulation of the stress response by CCK signaling.

Glucagon-Like Peptide 1

Glucagon-like peptide (GLP-1) is produced by endocrine L cells of the small intestine and processed into two biologically active forms, GLP-1_{7-36} amide and GLP-1_{7-37} (152) with GLP-1_{7-36} amide being the predominant form in the human circulation (153). GLP-1 is released postprandially with a biphasic pattern: an early peak of GLP-1 secretion occurs ∼15 min after meal intake that involves humoral (154, 155) and vagal (156) stimulation, while a later and larger peak is related to the direct contact of L cells with food components (157).

Peripheral but also central administration of GLP-1, in addition to the well-described incretin effect, results in a decrease of food intake in animals (157–159) and humans (160). In addition, the slowing of gastric and intestinal transit (161, 162) is likely to contribute to the food intake-reducing effect.

GLP-1 signals to the brain *via* the vagus nerve expressing the GLP-1 receptor (163) as shown by the suppression of the anorexigenic effect of peripherally injected GLP-1 by vagotomy (164, 165). It is to note that GLP-1 is also expressed in the brainstem nucleus of the solitary tract that projects to the PVN (166), and local knockdown of the pro-glucagon gene in the nucleus of the solitary tract increases food intake and also body weight gain (167). Since lesioning of these connections blunts the anorexigenic effect of peripherally injected GLP-1 (164) the gut-vagal-brainstem-hypothalamus connection is essential for the mediation of GLP-1's food intake-suppressing effect. Nonetheless, GLP-1 is able to cross the blood-brain barrier by simple diffusion (168). However, the rapid degradation of the peptide by circulating DPPIV (169) points toward the importance of neural and/or paracrine signaling.

GLP-1 can modulate a number of stress responses. Under basal conditions, GLP-1_{7-36} amide injected peripherally stimulates circulating corticosterone levels in mice and rats as well as cortisol levels in healthy human subjects (170). Other studies showed that targeted knockdown of the GLP-1 receptor in single-minded 1-expressing neurons of the PVN reduces hypothalamic-pituitary-adrenal axis responses to acute and chronic stress and this was associated with reduced anxiety-like behavior and a prevention of body weight reduction under conditions of chronic stress (171). Similarly, injection of the GLP-1 receptor inverse agonist, exendin-$_{(9-39)}$ into the dorsal subregion of the lateral septum blocks the acute restraint stress-induced anorexigenic effect in rats (172). While these studies support GLP-1's permissive role in the activation of stress signaling pathways, other reports showed that mice lacking the GLP-1 receptor display an increased corticosterone response to novel environment stress (173).

Several protective effects of GLP-1 have been reported under conditions of stress. GLP-1 injected intracerebroventricularly prevents gastric mucosal lesions induced by a combination of cold and restraint stress, an effect blocked by exendin-$_{(9-39)}$ (174). Subcutaneous injection of liraglutide, a GLP-1 analog, inhibits visceral allodynia induced by injection of lipopolysaccharide or repeated water avoidance stress (175). In humans with alcohol dependence, treatment with the GABA-B receptor agonist, baclofen at a dose of 30 mg/day increases circulating levels of GLP-1 (176), possibly associated with a reduced craving for alcohol. Moreover, GLP-1 receptor activation reverses the restraint stress-induced activation of bone marrow sca-1^{high}c-$Kit^{high}CD48^{low}CD150^{high}$ proliferation of hematopoietic stem cells in mice, thereby reducing the inflammatory response (177). In a study using geniposide as GLP-1 agonist these anti-inflammatory effects were associated with an amelioration of depression-like behaviors following repeated restraint stress (178). Also *in vitro* GLP-1_{7-36} prevents various stressors (e.g., H_2O_2 and amyloid β_{1-42})-induced death of murine hippocampal HT22 cells, an effect likely mediated *via* increased phosphorylation of Akt and ERK1/2 (179).

Peptide YY

Peptide YY (PYY) is derived from L cells located in the distal small intestine and colon (180). The peptide circulates in two forms, PYY_{1-36} and PYY_{3-36}, which is the predominant form in the blood (181) resulting from the processing by dipeptidyl peptidase IV (182). PYY is well established to reduce food intake in animals and humans following peripheral injection *via* binding to the Y_2 receptor (183). This was demonstrated by the blunting of the peptide's anorexigenic effect by the Y_2 antagonist, BIIE0246 (184) and knockout of the Y_2 receptor (183) in rodents. The anorexigenic gut-brain mode of action may involve the vagus nerve and humoral pathways. The Y_2 receptor is expressed on vagal afferents (185) and vagotomy blocks the anorexigenic effect of PYY (186) In addition, PYY can also cross the blood-brain barrier in a non-saturable manner (187). There is evidence that peripherally injected PYY or PYY_{3-36} activates brain nuclei

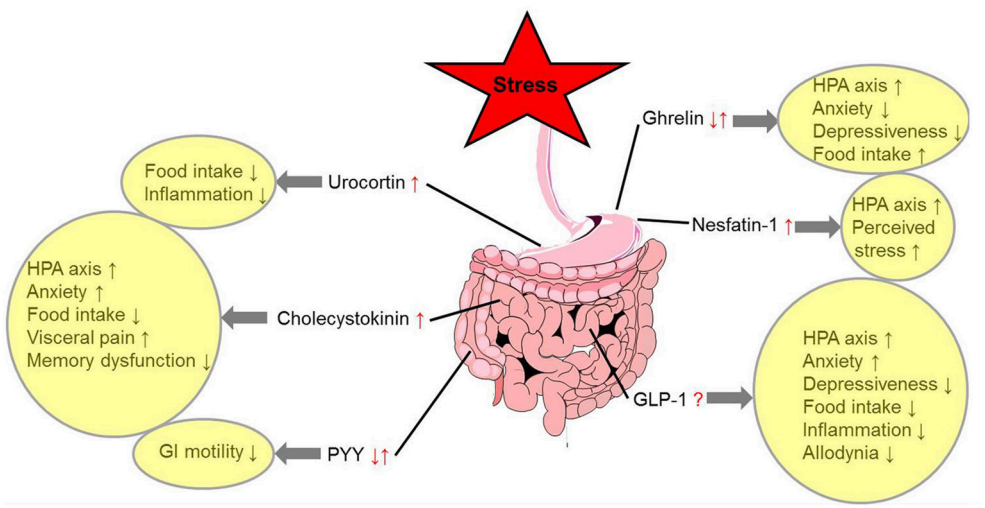

FIGURE 1 | Alterations of gut-brain peptides under conditions of stress and functional implications. ↓, decrease; ↑, increase; ?, unknown effect; GLP-1, glucagon-like peptide 1; GI, gastrointestinal; HPA axis, hypothalamus-pituitary-adrenal axis; PYY, peptide YY.

such as the nucleus of the solitary tract (188) and hypothalamic nuclei (189) which are known to regulate food intake. In the brain, PYY microinjected directly into the arcuate nucleus, a nucleus involved in the regulation of food intake and expressing the Y_2 receptor (190), reduces food intake. This is achieved by decreasing the activity of neuropeptide Y-containing neurons and activating proopiomelanocortin-containing cells (183).

Repetitive water avoidance stress decreases circulating PYY levels compared to non-stressed rats (191). Likewise, in humans a well-established psychological stressor, the Trier social stress test, reduces circulating PYY levels in normal weight and obese women (192). However, in mice, water immersion stress results in increased plasma PYY levels (193). Other studies showed that mice lacking PYY have an enhanced restraint stress-induced fecal pellet output and upper gastrointestinal transit (194); therefore, the peptide might play a modulatory role in the stress response. Whether the contrasting effects of stress on PYY release are related to species or stress modality differences remains to be further investigated.

SUMMARY

Various stressors alter the expression or circulating levels of several gut-brain peptidergic hormones involved in the regulation of hunger and satiety. While most anorexigenic peptides are upregulated under conditions of stress (nesfatin-1, Ucns, and CCK), others were shown to be differentially regulated dependent on the type of stressors (ghrelin and PYY), and for GLP-1 conclusive data are lacking so far. In addition, there is a further activation of the hypothalamus-pituitary-adrenal axis induced by specific gut peptides (ghrelin, nesfatin-1, CCK, and GLP-1) acting *via* neuronal and/or humoral gut-brain signaling highlighting the PVN as key responsive area orchestrating the stress response. This results in an increased perception of stress (nesfatin-1) and an alteration of anxiety and depressiveness (ghrelin, CCK, and GLP-1) with the PVN and Edinger-Westphal nucleus playing an important role in the behavioral responses. While most peptides contribute to stress-induced anorexia (Ucns, CCK, and GLP-1), ghrelin can stimulate food intake under these conditions (**Figure 1**). Despite the fact that our knowledge on these regulatory pathways greatly increased during the past years, the interactions between these peptides (114, 195) under stress conditions should be further investigated along with the possible translation of these findings—derived mainly from animal models—to humans.

AUTHOR CONTRIBUTIONS

AS drafted the manuscript. AS and YT reviewed and finalized the manuscript.

ACKNOWLEDGMENTS

This work was supported by funding of the German Research Foundation STE 1765/3-2, Charité University Funding UFF 89/441-176 (AS) and National Institute of Health grant DK-41301 (Animal Model Core) and VA Senior Career Scientist Award (YT).

REFERENCES

1. Cummings DE, Overduin J. Gastrointestinal regulation of food intake. *J Clin Invest.* (2007) 117:13–23. doi: 10.1172/JCI30227
2. Dockray GJ. Gastrointestinal hormones and the dialogue between gut and brain. *J Physiol.* (2014) 592:2927–41. doi: 10.1113/jphysiol.2014.270850
3. de Lartigue G, Diepenbroek C. Novel developments in vagal afferent nutrient sensing and its role in energy homeostasis. *Curr Opin Pharmacol.* (2016) 31:38–43. doi: 10.1016/j.coph.2016.08.007
4. de Lartigue G. Putative roles of neuropeptides in vagal afferent signaling. *Physiol Behav.* (2014) 136:155–69. doi: 10.1016/j.physbeh.2014.03.011
5. Prinz P, Stengel A. Control of food intake by gastrointestinal peptides: mechanisms of action and possible modulation in the treatment of obesity. *J Neurogastroenterol Motil.* (2017) 23:180–96. doi: 10.5056/jnm16194
6. Stengel A, Wang L, Taché Y. Stress-related alterations of acyl and desacyl ghrelin circulating levels: mechanisms and functional implications. *Peptides* (2011) 32:2208–17. doi: 10.1016/j.peptides.2011.07.002
7. Kojima M, Hosoda H, Date Y, Nakazato M, Matsuo H, Kangawa K. Ghrelin is a growth-hormone-releasing acylated peptide from stomach. *Nature* (1999) 402:656–60. doi: 10.1038/45230
8. Ariyasu H, Takaya K, Tagami T, Ogawa Y, Hosoda K, Akamizu T, et al. Stomach is a major source of circulating ghrelin, and feeding state determines plasma ghrelin-like immunoreactivity levels in humans. *J Clin Endocrinol Metab.* (2001) 86:4753–8. doi: 10.1210/jcem.86.10.7885
9. Date Y, Kojima M, Hosoda H, Sawaguchi A, Mondal MS, Suganuma T, et al. Ghrelin, a novel growth hormone-releasing acylated peptide, is synthesized in a distinct endocrine cell type in the gastrointestinal tracts of rats and humans. *Endocrinology* (2000) 141:4255–61. doi: 10.1210/endo.141.11.7757
10. Davenport AP, Bonner TI, Foord SM, Harmar AJ, Neubig RR, Pin JP, et al. International union of pharmacology. LVI Ghrelin receptor nomenclature, distribution, and function *Pharmacol Rev.* (2005) 57:541–6. doi: 10.1124/pr.57.4.1
11. Gutierrez JA, Solenberg PJ, Perkins DR, Willency JA, Knierman MD, Jin Z, et al. Ghrelin octanoylation mediated by an orphan lipid transferase. *Proc Natl Acad Sci USA.* (2008) 105:6320–5. doi: 10.1073/pnas.0800708105
12. Yang J, Brown MS, Liang G, Grishin NV, Goldstein JL. Identification of the acyltransferase that octanoylates ghrelin, an appetite-stimulating peptide hormone. *Cell* (2008) 132:387–96. doi: 10.1016/j.cell.2008.01.017
13. Stengel A, Goebel M, Wang L, Taché Y, Sachs G, Lambrecht NW. Differential distribution of ghrelin-O-acyltransferase (GOAT) immunoreactive cells in the mouse and rat gastric oxyntic mucosa. *Biochem Biophys Res Commun.* (2010) 392:67–71. doi: 10.1016/j.bbrc.2009.12.169
14. Stengel A, Hofmann T, Goebel-Stengel M, Lembke V, Ahnis A, Elbelt U, et al. Ghrelin and NUCB2/nesfatin-1 are expressed in the same gastric cell and differentially correlated with body mass index in obese subjects. *Histochem Cell Biol.* (2013) 139:909–18. doi: 10.1007/s00418-013-1087-8
15. An W, Li Y, Xu G, Zhao J, Xiang X, Ding L, et al. Modulation of ghrelin O-acyltransferase expression in pancreatic islets. *Cell Physiol Biochem.* (2010) 26:707–16. doi: 10.1159/000322338
16. Goebel-Stengel M, Hofmann T, Elbelt U, Teuffel P, Ahnis A, Kobelt P, et al. The ghrelin activating enzyme ghrelin-O-acyltransferase (GOAT) is present in human plasma and expressed dependent on body mass index. *Peptides* (2013) 43:13–9. doi: 10.1016/j.peptides.2013.02.011
17. Wren AM, Small CJ, Ward HL, Murphy KG, Dakin CL, Taheri S, et al. The novel hypothalamic peptide ghrelin stimulates food intake and growth hormone secretion. *Endocrinology* (2000) 141:4325–8. doi: 10.1210/endo.141.11.7873
18. Wren AM, Seal LJ, Cohen MA, Brynes AE, Frost GS, Murphy KG, et al. Ghrelin enhances appetite and increases food intake in humans. *J Clin Endocrinol Metab.* (2001) 86:5992. doi: 10.1210/jcem.86.12.8111

19. Tschöp M, Smiley DL, Heiman ML. Ghrelin induces adiposity in rodents. *Nature* (2000) 407:908–13. doi:10.1038/35038090
20. Date Y, Murakami N, Toshinai K, Matsukura S, Niijima A, Matsuo H, et al. The role of the gastric afferent vagal nerve in ghrelin-induced feeding and growth hormone secretion in rats. *Gastroenterology* (2002) 123:1120–8. doi: 10.1053/gast.2002.35954
21. Okada T, Waise TMZ, Toshinai K, Mita Y, Sakoda H, Nakazato M. Analysis of peripheral ghrelin signaling via the vagus nerve in ghrelin receptor-restored GHSR-null mice. *Neurosci Lett*. (2018) 681:50–5. doi: 10.1016/j.neulet.2018.05.035
22. Banks WA, Tschöp M, Robinson SM, Heiman ML. Extent and direction of ghrelin transport across the blood-brain barrier is determined by its unique primary structure. *J Pharmacol Exp Ther*. (2002) 302:822–7. doi: 10.1124/jpet.102.034827
23. Guan XM, Yu H, Palyha OC, Mckee KK, Feighner SD, Sirinathsinghji DJ, et al. Distribution of mRNA encoding the growth hormone secretagogue receptor in brain and peripheral tissues. *Brain Res Mol Brain Res*. (1997) 48:23–9.
24. Willesen MG, Kristensen P, Romer J. Co-localization of growth hormone secretagogue receptor and NPY mRNA in the arcuate nucleus of the rat. *Neuroendocrinology* (1999) 70:306–16. doi: 10.1159/000054491
25. Cowley MA, Smith RG, Diano S, Tschöp M, Pronchuk N, Grove KL, et al. The distribution and mechanism of action of ghrelin in the CNS demonstrates a novel hypothalamic circuit regulating energy homeostasis. *Neuron* (2003) 37:649–61. doi: 10.1016/S0896-6273(03)00063-1
26. Zhang X, van den Pol AN. Hypothalamic arcuate nucleus tyrosine hydroxylase neurons play orexigenic role in energy homeostasis. *Nat Neurosci*. (2016) 19:1341–7. doi: 10.1038/nn.4372
27. Chen HY, Trumbauer ME, Chen AS, Weingarth DT, Adams JR, Frazier EG, et al. Orexigenic action of peripheral ghrelin is mediated by neuropeptide Y and agouti-related protein. *Endocrinology* (2004) 145:2607–12. doi: 10.1210/en.2003-1596
28. Patterson M, Murphy KG, Le Roux CW, Ghatei MA, Bloom SR. Characterization of ghrelin-like immunoreactivity in human plasma. *J Clin Endocrinol Metab*. (2005) 90:2205–11. doi: 10.1210/jc.2004-1641
29. Stengel A, Keire D, Goebel M, Evilevitch L, Wiggins B, Taché Y, et al. The RAPID method for blood processing yields new insight in plasma concentrations and molecular forms of circulating gut peptides. *Endocrinology* (2009) 150:5113–8. doi: 10.1210/en.2009-0697
30. Mahbod P, Smith EP, Fitzgerald ME, Morano RL, Packard BA, Ghosal S, et al. Desacyl ghrelin decreases anxiety-like behavior in male mice. *Endocrinology* (2018) 159:388–99. doi: 10.1210/en.2017-00540
31. Inhoff T, Mönnikes H, Noetzel S, Stengel A, Goebel M, Dinh QT, et al. Desacyl ghrelin inhibits the orexigenic effect of peripherally injected ghrelin in rats. *Peptides* (2008) 29:2159–68. doi: 10.1016/j.peptides.2008.09.014
32. Fernandez G, Cabral A, Cornejo MP, De Francesco PN, Garcia-Romero G, Reynaldo M, et al. Des-Acyl ghrelin directly targets the arcuate nucleus in a ghrelin-receptor independent manner and impairs the orexigenic effect of ghrelin. *J Neuroendocrinol*. (2016) 28:12349. doi: 10.1111/jne.12349
33. Asakawa A, Inui A, Kaga T, Yuzuriha H, Nagata T, Fujimiya M, et al. A role of ghrelin in neuroendocrine and behavioral responses to stress in mice. *Neuroendocrinology* (2001) 74:143–7. doi: 10.1159/000054680
34. Kristenssson E, Sundqvist M, Astin M, Kjerling M, Mattsson H, Dornonville De La Cour C, et al. Acute psychological stress raises plasma ghrelin in the rat. *Regul Pept*. (2006) 134:114–7. doi: 10.1016/j.regpep.2006.02.003
35. Rouach V, Bloch M, Rosenberg N, Gilad S, Limor R, Stern N, et al. The acute ghrelin response to a psychological stress challenge does not predict the post-stress urge to eat. *Psychoneuroendocrinology* (2007) 32:693–702. doi: 10.1016/j.psyneuen.2007.04.010
36. Maruna P, Gurlich R, Rosicka M. Ghrelin as an acute-phase reactant during postoperative stress response. *Horm Metab Res*. (2008) 40:404–9. doi: 10.1055/s-2008-1065329
37. Raspopow K, Abizaid A, Matheson K, Anisman H. Psychosocial stressor effects on cortisol and ghrelin in emotional and non-emotional eaters: influence of anger and shame. *Horm Behav*. (2010) 58:677–84. doi: 10.1016/j.yhbeh.2010.06.003
38. Kontoravdis N, Vassilikostas G, Lagoudianakis E, Pappas A, Seretis C, Panagiotopoulos N, et al. Effect of acute surgical stress on serum ghrelin levels. *Gastroenterology Res*. (2012) 5:97–102. doi: 10.4021/gr455e
39. Geliebter A, Carnell S, Gluck ME. Cortisol and ghrelin concentrations following a cold pressor stress test in overweight individuals with and without night eating. *Int J Obes*. (2013) 37:1104–8. doi: 10.1038/ijo.2012.166
40. Jung YS, Kim MY, Lee HS, Park SL, Lee KJ. Effect of DA-9701, a novel prokinetic agent, on stress-induced delayed gastric emptying and hormonal changes in rats. *Neurogastroenterol Motil*. (2013) 25:254–259:e166. doi: 10.1111/nmo.12053
41. Gluck ME, Yahav E, Hashim SA, Geliebter A. Ghrelin levels after a cold pressor stress test in obese women with binge eating disorder. *Psychosom Med*. (2014) 76:74–9. doi: 10.1097/PSY.0000000000000018
42. Wang L, Goebel-Stengel M, Yuan PQ, Stengel A, Taché Y. Corticotropin-releasing factor overexpression in mice abrogates sex differences in body weight, visceral fat, and food intake response to a fast and alters levels of feeding regulatory hormones. *Biol Sex Differ* (2017) 8:2. doi: 10.1186/s13293-016-0122-6
43. Carnell S, Grillot C, Ungredda T, Ellis S, Mehta N, Holst J, et al. Morning and afternoon appetite and gut hormone responses to meal and stress challenges in obese individuals with and without binge eating disorder. *Int J Obes*. (2018) 42:841–9. doi: 10.1038/ijo.2017.307
44. Lutter M, Sakata I, Osborne-Lawrence S, Rovinsky SA, Anderson JG, Jung S, et al. The orexigenic hormone ghrelin defends against depressive symptoms of chronic stress. *Nat Neurosci*. (2008) 11:752–3. doi: 10.1038/nn.2139
45. Zheng J, Dobner A, Babygirija R, Ludwig K, Takahashi T. Effects of repeated restraint stress on gastric motility in rats. *Am J Physiol Regul Integr Comp Physiol*. (2009) 296:R1358–1365. doi: 10.1152/ajpregu.90928.2008
46. Chuang JC, Perello M, Sakata I, Osborne-Lawrence S, Savitt JM, Lutter M, et al. Ghrelin mediates stress-induced food-reward behavior in mice. *J Clin Invest*. (2011) 121:2684–92. doi: 10.1172/JCI57660
47. Patterson ZR, Khazall R, Mackay H, Anisman H, Abizaid A. Central ghrelin signaling mediates the metabolic response of C57BL/6 male mice to chronic social defeat stress. *Endocrinology* (2013) 154:1080–91. doi: 10.1210/en.2012-1834
48. Elbassuoni EA. Gender differences in ghrelin response to chronic immobilization stress in rats: possible role of estrogen. *Gen Physiol Biophys*. (2014) 33:111–20. doi: 10.4149/gpb_2013061
49. Huang HJ, Zhu XC, Han QQ, Wang YL, Yue N, Wang J, et al. Ghrelin alleviates anxiety- and depression-like behaviors induced by chronic unpredictable mild stress in rodents. *Behav Brain Res*. (2017) 326:33–43. doi: 10.1016/j.bbr.2017.02.040
50. Yousufzai M, Harmatz ES, Shah M, Malik MO, Goosens KA. Ghrelin is a persistent biomarker for chronic stress exposure in adolescent rats and humans. *Transl Psychiatry* (2018) 8:74. doi: 10.1038/s41398-018-0135-5
51. Labarthe A, Fiquet O, Hassouna R, Zizzari P, Lanfumey L, Ramoz N, et al. Ghrelin-derived peptides: a link between appetite/reward, GH axis, and psychiatric disorders? *Front Endocrinol*. (2014) 5:163. doi: 10.3389/fendo.2014.00163
52. Morin V, Hozer F, Costemale-Lacoste JF. The effects of ghrelin on sleep, appetite, and memory, and its possible role in depression: a review of the literature. *Encephale* (2018) 44:256–63. doi: 10.1016/j.encep.2017.10.012
53. Stengel A, Goebel M, Wang L, Reeve JR Jr, Taché Y, Lambrecht NW. Lipopolysaccharide differentially decreases plasma acyl and desacyl ghrelin levels in rats: potential role of the circulating ghrelin-acylating enzyme GOAT. *Peptides* (2010) 31:1689–96. doi: 10.1016/j.peptides.2010.06.015
54. Yuan PQ, Taché Y. Abdominal surgery induced gastric ileus and activation of M1-like macrophages in the gastric myenteric plexus: prevention by central vagal activation in rats. *Am J Physiol Gastrointest Liver Physiol*. (2017) 313:G320–29. doi: 10.1152/ajpgi.00121.2017
55. Stengel A, Goebel M, Luckey A, Yuan PQ, Wang L, Taché Y. Cold ambient temperature reverses abdominal surgery-induced delayed gastric emptying and decreased plasma ghrelin levels in rats. *Peptides* (2010) 31:2229–35. doi: 10.1016/j.peptides.2010.08.026
56. Stengel A, Goebel-Stengel M, Wang L, Shaikh A, Lambrecht NW, Rivier J, et al. Abdominal surgery inhibits circulating acyl ghrelin and ghrelin-O-acyltransferase levels in rats: role of the somatostatin receptor

subtype 2. *Am J Physiol Gastrointest Liver Physiol*. (2011) 301:G239–48. doi: 10.1152/ajpgi.00018.2011
57. Stengel A, Goebel-Stengel M, Wang L, Luckey A, Hu E, Rivier J, et al. Central administration of pan-somatostatin agonist ODT8-SST prevents abdominal surgery-induced inhibition of circulating ghrelin, food intake and gastric emptying in rats. *Neurogastroenterol Motil*. (2011) 23:e294–308. doi: 10.1111/j.1365-2982.2011.01721.x
58. Wang L, Mogami S, Yakabi S, Karasawa H, Yamada C, Yakabi K, et al. Patterns of brain activation and meal reduction induced by abdominal surgery in mice and modulation by rikkunshito. *PLoS ONE* (2015) 10:e0139325. doi: 10.1371/journal.pone.0139325
59. Otero M, Nogueiras R, Lago F, Dieguez C, Gomez-Reino JJ, Gualillo O. Chronic inflammation modulates ghrelin levels in humans and rats. *Rheumatology* (2004) 43:306–10. doi: 10.1093/rheumatology/keh055
60. Saegusa Y, Takeda H, Muto S, Nakagawa K, Ohnishi S, Sadakane C, et al. Decreased plasma ghrelin contributes to anorexia following novelty stress. *Am J Physiol Endocrinol Metab*. (2011) 301:E685–696. doi: 10.1152/ajpendo.00121.2011
61. Yamada C, Saegusa Y, Nakagawa K, Ohnishi S, Muto S, Nahata M, et al. Rikkunshito, a Japanese kampo medicine, ameliorates decreased feeding behavior via ghrelin and serotonin 2B receptor signaling in a novelty stress murine model. *Biomed Res Int*. (2013) 2013:792940. doi: 10.1155/2013/792940
62. Jeong JY, Lee DH, Kang SS. Effects of chronic restraint stress on body weight, food intake, and hypothalamic gene expressions in mice. *Endocrinol Metab*. (2013) 28:288–96. doi: 10.3803/EnM.2013.28.4.288
63. Rostamkhani F, Zardooz H, Goshadrou F, Baveisi M, Hedayati M. Stress increased ghrelin secretion from pancreatic isolated islets in male rats. *Gen Physiol Biophys*. (2016) 35:109–17. doi: 10.4149/gpb_2015037
64. Ibrahim Abdalla MM. Ghrelin - physiological functions and regulation. *Eur Endocrinol*. (2015) 11:90–5. doi: 10.17925/EE.2015.11.02.90
65. Bale TL, Vale WW. CRF and CRF receptors: role in stress responsivity and other behaviors. *Annu Rev Pharmacol Toxicol*. (2004) 44:525–57. doi: 10.1146/annurev.pharmtox.44.101802.121410
66. Cabral A, Portiansky E, Sanchez-Jaramillo E, Zigman JM, Perello M. Ghrelin activates hypophysiotropic corticotropin-releasing factor neurons independently of the arcuate nucleus. *Psychoneuroendocrinology* (2016) 67:27–39. doi: 10.1016/j.psyneuen.2016.01.027
67. Kageyama K, Kumata Y, Akimoto K, Takayasu S, Tamasawa N, Suda T. Ghrelin stimulates corticotropin-releasing factor and vasopressin gene expression in rat hypothalamic 4B cells. *Stress* (2011) 14:520–9. doi: 10.3109/10253890.2011.558605
68. Oh IS, Shimizu H, Satoh T, Okada S, Adachi S, Inoue K, et al. Identification of nesfatin-1 as a satiety molecule in the hypothalamus. *Nature* (2006) 443:709–12. doi: 10.1038/nature05162
69. Goebel M, Stengel A, Wang L, Lambrecht NW, Taché Y. Nesfatin-1 immunoreactivity in rat brain and spinal cord autonomic nuclei. *Neurosci Lett*. (2009) 452:241–46. doi: 10.1016/j.neulet.2009.01.064.
70. Stengel A, Goebel M, Yakubov I, Wang L, Witcher D, Coskun T, et al. Identification and characterization of nesfatin-1 immunoreactivity in endocrine cell types of the rat gastric oxyntic mucosa. *Endocrinology* (2009) 150:232–8. doi: 10.1210/en.2008-0747
71. Shimizu H, Oh IS, Hashimoto K, Nakata M, Yamamoto S, Yoshida N, et al. Peripheral administration of nesfatin-1 reduces food intake in mice: the leptin-independent mechanism. *Endocrinology* (2009) 150:662–71. doi: 10.1210/en.2008-0598
72. Brailoiu GC, Dun SL, Brailoiu E, Inan S, Yang J, Chang JK, et al. Nesfatin-1: distribution and interaction with a G protein-coupled receptor in the rat brain. *Endocrinology* (2007) 148:5088–94. doi: 10.1210/en.2007-0701
73. Ozcan M, Gok ZB, Kacar E, Serhatlioglu I, Kelestimur H. Nesfatin-1 increases intracellular calcium concentration by protein kinase C activation in cultured rat dorsal root ganglion neurons. *Neurosci Lett*. (2016) 619:177–81. doi: 10.1016/j.neulet.2016.03.018
74. Prinz P, Goebel-Stengel M, Teuffel P, Rose M, Klapp BF, Stengel A. Peripheral and central localization of the nesfatin-1 receptor using autoradiography in rats. *Biochem Biophys Res Commun*. (2016) 470:521–7. doi: 10.1016/j.bbrc.2016.01.113
75. Dore R, Levata L, Lehnert H, Schulz C. Nesfatin-1: functions and physiology of a novel regulatory peptide. *J Endocrinol*. (2017) 232:R45–65. doi: 10.1530/JOE-16-0361
76. Stengel A, Goebel M, Wang L, Rivier J, Kobelt P, Mönnikes H, et al. Central nesfatin-1 reduces dark-phase food intake and gastric emptying in rats: differential role of corticotropin-releasing factor2 receptor. *Endocrinology* (2009) 150:4911–9. doi: 10.1210/en.2009-0578
77. Goebel M, Stengel A, Wang L, Taché Y. Central nesfatin-1 reduces the nocturnal food intake in mice by reducing meal size and increasing inter-meal intervals. *Peptides* (2011) 32, 36–43. doi: 10.1016/j.peptides.2010.09.027
78. Heidarzadeh H, Zendehdel M, Babapour V, Gilanpour H. The effect of Nesfatin-1 on food intake in neonatal chicks: role of CRF1 /CRF2 and H1/ H3 receptors. *Vet Res Commun*. (2018) 42:39–47. doi: 10.1007/s11259-017-9706-9
79. Kerbel B, Unniappan S. Nesfatin-1 suppresses energy intake, co-localises ghrelin in the brain and gut, and alters ghrelin, cholecystokinin and orexin mRNA expression in goldfish. *J Neuroendocrinol*. (2012) 24:366–77. doi: 10.1111/j.1365-2826.2011.02246.x
80. Mortazavi S, Gonzalez R, Ceddia R, Unniappan S. Long-term infusion of nesfatin-1 causes a sustained regulation of whole-body energy homeostasis of male Fischer 344 rats. *Front Cell Dev Biol*. (2015) 3:22. doi: 10.3389/fcell.2015.00022
81. Yin Y, Li Z, Gao L, Li Y, Zhao J, Zhang W. AMPK-dependent modulation of hepatic lipid metabolism by nesfatin-1. *Mol Cell Endocrinol*. (2015) 417:20–6. doi: 10.1016/j.mce.2015.09.006
82. Gonzalez R, Reingold BK, Gao X, Gaidhu MP, Tsushima RG, Unniappan S. Nesfatin-1 exerts a direct, glucose-dependent insulinotropic action on mouse islet beta- and MIN6 cells. *J Endocrinol*. (2011) 208:R9–16. doi: 10.1530/JOE-10-0492
83. Riva M, Nitert MD, Voss U, Sathanoori R, Lindqvist A, Ling C, et al. Nesfatin-1 stimulates glucagon and insulin secretion and beta cell NUCB2 is reduced in human type 2 diabetic subjects. *Cell Tissue Res*. (2011) 346:393–405. doi: 10.1007/s00441-011-1268-5
84. Goebel M, Stengel A, Wang L, Taché Y. Restraint stress activates nesfatin-1-immunoreactive brain nuclei in rats. *Brain Res*. (2009) 1300:114–124. doi: 10.1016/j.brainres.2009.08.082
85. Okere B, Xu L, Roubos EW, Sonetti D, Kozicz T. Restraint stress alters the secretory activity of neurons co-expressing urocortin-1, cocaine- and amphetamine-regulated transcript peptide and nesfatin-1 in the mouse Edinger-Westphal nucleus. *Brain Res*. (2010) 1317:92–9. doi: 10.1016/j.brainres.2009.12.053
86. Yoshida N, Maejima Y, Sedbazar U, Ando A, Kurita H, Damdindorj B, et al. Stressor-responsive central nesfatin-1 activates corticotropin-releasing hormone, noradrenaline and serotonin neurons and evokes hypothalamic-pituitary-adrenal axis. *Aging (Albany NY)* (2010) 2:775–84. doi: 10.18632/aging.100207
87. Goebel-Stengel M, Wang L, Stengel, A, Taché Y. Localization of nesfatin-1 neurons in the mouse brain and functional implication. *Brain Res*. (2011) 1396:20–34. doi: 10.1016/j.brainres.2011.04.031
88. Stengel A, Goebel M, Wang L, Taché Y. Abdominal surgery activates nesfatin-1 immunoreactive brain nuclei in rats. *Peptides* (2010) 31:263–270. doi: 10.1016/j.peptides.2009.11.015
89. Bonnet MS, Pecchi E, Trouslard J, Jean A, Dallaporta M, Troadec JD. Central nesfatin-1-expressing neurons are sensitive to peripheral inflammatory stimulus. *J Neuroinflammation* (2009) 6:27. doi: 10.1186/1742-2094-6-27
90. Xu L, Bloem B, Gaszner B, Roubos EW, Kozicz T. Stress-related changes in the activity of cocaine- and amphetamine-regulated transcript and nesfatin neurons in the midbrain non-preganglionic Edinger-Westphal nucleus in the rat. *Neuroscience* (2010) 170:478–88. doi: 10.1016/j.neuroscience.2010.07.001
91. Xu YY, Ge JF, Qin G, Peng YN, Zhang CF, Liu XR, et al. Acute, but not chronic, stress increased the plasma concentration and hypothalamic mRNA expression of NUCB2/nesfatin-1 in rats. *Neuropeptides* (2015) 54:47–53. doi: 10.1016/j.npep.2015.08.003
92. Stengel A, Goebel-Stengel M, Jawien J, Kobelt P, Taché Y, Lambrecht NW. Lipopolysaccharide increases gastric and circulating NUCB2/nesfatin-1 concentrations in rats. *Peptides* (2011) 32:1942–7. doi: 10.1016/j.peptides.2011.07.006

93. Hofmann T, Stengel A, Ahnis A, Busse P, Elbelt U, Klapp BF. NUCB2/nesfatin-1 is associated with elevated scores of anxiety in female obese patients. *Psychoneuroendocrinology* (2013) 38:2502–10. doi: 10.1016/j.psyneuen.2013.05.013
94. Bloem B, Xu L, Morava E, Faludi G, Palkovits M, Roubos EW, et al. Sex-specific differences in the dynamics of cocaine- and amphetamine-regulated transcript and nesfatin-1 expressions in the midbrain of depressed suicide victims vs. controls. *Neuropharmacology* (2012) 62:297–303. doi: 10.1016/j.neuropharm.2011.07.023
95. Vaughan J, Donaldson C, Bittencourt J, Perrin MH, Lewis K, Sutton S, et al. Urocortin, a mammalian neuropeptide related to fish urotensin I and to corticotropin-releasing factor. *Nature* (1995) 378:287–92. doi: 10.1038/378287a0
96. Reyes TM, Lewis K, Perrin MH, Kunitake KS, Vaughan J, Arias CA, et al. Urocortin II: a member of the corticotropin-releasing factor (CRF) neuropeptide family that is selectively bound by type 2 CRF receptors. *Proc Natl Acad Sci USA*. (2001) 98:2843–8. doi: 10.1073/pnas.051626398
97. Vaughan JM, Donaldson CJ, Fischer WH, Perrin MH, Rivier JE, Sawchenko PE, et al. Posttranslational processing of human and mouse urocortin 2: characterization and bioactivity of gene products. *Endocrinology* (2013) 154:1553–64. doi: 10.1210/en.2012-2011
98. Lewis K, Li C, Perrin MH, Blount A, Kunitake K, Donaldson C, et al. Identification of urocortin III, an additional member of the corticotropin-releasing factor (CRF) family with high affinity for the CRF2 receptor. *Proc Natl Acad Sci USA*. (2001) 98:7570–5. doi: 10.1073/pnas.121165198
99. Grace CR, Perrin MH, Cantle JP, Vale WW, Rivier JE, Riek R. Common and divergent structural features of a series of corticotropin releasing factor-related peptides. *J Am Chem Soc*. (2007) 129:16102–14. doi: 10.1021/ja0760933
100. Stengel A, Taché Y. CRF and urocortin peptides as modulators of energy balance and feeding behavior during stress. *Front Neurosci*. (2014) 8:52. doi: 10.3389/fnins.2014.00052
101. Boorse GC, Denver RJ. Widespread tissue distribution and diverse functions of corticotropin-releasing factor and related peptides. *Gen Comp Endocrinol*. (2006) 146:9–18. doi: 10.1016/j.ygcen.2005.11.014
102. Kozicz T, Arimura A. Distribution of urocortin in the rat's gastrointestinal tract and its colocalization with tyrosine hydroxylase. *Peptides* (2002) 23:515–21. doi: 10.1016/S0196-9781(01)00639-8
103. Li C, Chen P, Vaughan J, Blount A, Chen A, Jamieson PM, et al. Urocortin III is expressed in pancreatic beta-cells and stimulates insulin and glucagon secretion. *Endocrinology* (2003) 144:3216–24. doi: 10.1210/en.2002-0087
104. Porcher C, Peinnequin A, Pellissier S, Meregnani J, Sinniger V, Canini F, et al. Endogenous expression and *in vitro* study of CRF-related peptides and CRF receptors in the rat gastric antrum. *Peptides* (2006) 27:1464–75. doi: 10.1016/j.peptides.2005.10.023
105. Kimura T, Amano T, Uehara H, Ariga H, Ishida T, Torii A, et al. Urocortin I is present in the enteric nervous system and exerts an excitatory effect via cholinergic and serotonergic pathways in the rat colon. *Am J Physiol Gastrointest Liver Physiol*. (2007) 293:G903–10. doi: 10.1152/ajpgi.00066.2007
106. van der Meulen T, Xie R, Kelly OG, Vale WW, Sander M, Huising MO. Urocortin 3 marks mature human primary and embryonic stem cell-derived pancreatic alpha and beta cells. *PLoS ONE* (2012) 7:e52181. doi: 10.1371/journal.pone.0052181
107. Yuan PQ, Wu SV, Taché Y. Urocortins and CRF type 2 receptor isoforms expression in the rat stomach are regulated by endotoxin: role in the modulation of delayed gastric emptying. *Am J Physiol Gastrointest Liver Physiol*. (2012) 303:G20–31. doi: 10.1152/ajpgi.00547.2011
108. Yuan PQ, Wu SV, Pothoulakis C, TachéNY. Urocortins and CRF receptor type 2 variants in the male rat colon: gene expression and regulation by endotoxin and anti-inflammatory effect. *Am J Physiol Gastrointest Liver Physiol*. (2016) 310:G387–398. doi: 10.1152/ajpgi.00337.2015
109. Asakawa A, Inui A, Ueno N, Makino S, Fujino MA, Kasuga M. Urocortin reduces food intake and gastric emptying in lean and ob/ob obese mice. *Gastroenterology* (1999) 116:1287–92.
110. Wang L, Martinez V, Rivier JE, Taché Y. Peripheral urocortin inhibits gastric emptying and food intake in mice: differential role of CRF receptor 2. *Am J Physiol Regul Integr Comp Physiol*. (2001) 281:R1401–10. doi: 10.1152/ajpregu.2001.281.5.R1401
111. Tanaka C, Asakawa A, Ushikai M, Sakoguchi T, Amitani H, Terashi M, et al. Comparison of the anorexigenic activity of CRF family peptides. *Biochem Biophys Res Commun*. (2009) 390:887–91. doi: 10.1016/j.bbrc.2009.10.069
112. Weisinger RS, Blair-West JR, Burns P, Denton DA, Mckinley MJ, Purcell B, et al. The inhibitory effect of hormones associated with stress on Na appetite of sheep. *Proc Natl Acad Sci USA*. (2000) 97:2922–7. doi: 10.1073/pnas.040577997
113. Fekete EM, Zhao Y, Szucs A, Sabino V, Cottone P, Rivier J, et al. Systemic urocortin 2, but not urocortin 1 or stressin 1-A, suppresses feeding via CRF2 receptors without malaise and stress. *Br J Pharmacol*. (2011) 164:1959–75. doi: 10.1111/j.1476-5381.2011.01512.x
114. Gourcerol G, Wang L, Wang YH, Million M, Taché Y. Urocortins and cholecystokinin-8 act synergistically to increase satiation in lean but not obese mice: involvement of corticotropin-releasing factor receptor-2 pathway. *Endocrinology* (2007) 148:6115–23. doi: 10.1210/en.2007-0678
115. Wang L, Stengel A, Goebel M, Martinez V, Gourcerol G, Rivier J, et al. Peripheral activation of corticotropin-releasing factor receptor 2 inhibits food intake and alters meal structures in mice. *Peptides* (2011) 32:51–9. doi: 10.1016/j.peptides.2010.10.017
116. Kastin AJ, Akerstrom V. Differential interactions of urocortin/corticotropin-releasing hormone peptides with the blood-brain barrier. *Neuroendocrinology* (2002) 75:367–74. doi: 10.1159/000059433
117. Sakai N, Yamamoto T. Conditioned taste aversion and c-fos expression in the rat brainstem after administration of various USs. *Neuroreport* (1997) 8:2215–20. doi: 10.1097/00001756-199707070-00025
118. Bittencourt JC, Sawchenko PE. Do centrally administered neuropeptides access cognate receptors?: an analysis in the central corticotropin-releasing factor system. *J Neurosci*. (2000) 20:1142–56. doi: 10.1523/JNEUROSCI.20-03-01142.2000
119. Wang L, Martinez V, Vale W, Taché Y. Fos induction in selective hypothalamic neuroendocrine and medullary nuclei by intravenous injection of urocortin and corticotropin-releasing factor in rats. *Brain Res*. (2000) 855:47–57. doi: 10.1016/S0006-8993(99)02200-3
120. Terashi M, Asakawa A, Cheng KC, Koyama KI, Chaolu H, Ushikai M, et al. Effects of peripherally administered urocortin 3 on feeding behavior and gastric emptying in mice. *Exp Ther Med*. (2011) 2:333–5. doi: 10.3892/etm.2011.200
121. Zhang X, Wu Y, Hao J, Zhu J, Tang N, Qi J, et al. Intraperitoneal injection urocortin-3 reduces the food intake of Siberian sturgeon (*Acipenser baerii*). *Peptides* (2016) 85:80–8. doi: 10.1016/j.peptides.2016.09.007
122. Mercer JG, Lawrence CB, Copeland PA. Corticotropin-releasing factor binding sites undergo axonal transport in the rat vagus nerve. *J Neuroendocrinol*. (1992) 4:281–6. doi: 10.1111/j.1365-2826.1992.tb00169.x
123. Lawrence AJ, Krstew EV, Dautzenberg FM, Ruhmann A. The highly selective CRF(2) receptor antagonist K41498 binds to presynaptic CRF(2) receptors in rat brain. *Br J Pharmacol*. (2002) 136:896–904. doi: 10.1038/sj.bjp.0704783
124. Buckinx R, Bagyanszki M, Avula LR, Adriaensen D, Van Nassauw L, Timmermans JP. Expression of corticotropin-releasing factor and urocortins in the normal and Schistosoma mansoni-infected mouse ileum. *Cell Tissue Res*. (2015) 359:453–63. doi: 10.1007/s00441-014-2012-8
125. Koido S, Ohkusa T, Kan S, Takakura K, Saito K, Komita H, et al. Production of corticotropin-releasing factor and urocortin from human monocyte-derived dendritic cells is stimulated by commensal bacteria in intestine. *World J Gastroenterol*. (2014) 20:14420–9. doi: 10.3748/wjg.v20.i39.14420
126. Li Y, Song Z, Kerr KA, Moeser AJ. Chronic social stress in pigs impairs intestinal barrier and nutrient transporter function, and alters neuro-immune mediator and receptor expression. *PLoS ONE* (2017) 12:e0171617. doi: 10.1371/journal.pone.0171617.
127. Estienne M, Claustre J, Clain-Gardechaux G, Paquet A, Taché Y, Fioramonti J, et al. Maternal deprivation alters epithelial secretory cell lineages in rat duodenum: role of CRF-related peptides. *Gut* (2010) 59:744–51. doi: 10.1136/gut.2009.190728
128. Sayegh AI. The role of cholecystokinin receptors in the short-term control of food intake. *Prog Mol Biol Transl Sci*. (2013) 114:277–316. doi: 10.1016/B978-0-12-386933-3.00008-X

129. Chandra R, Samsa LA, Vigna SR, Liddle RA. Pseudopod-like basal cell processes in intestinal cholecystokinin cells. *Cell Tissue Res*. (2010) 341:289–97. doi: 10.1007/s00441-010-0997-1
130. Goebel-Stengel M, Stengel A, Wang L, Ohning G, Taché Y, Reeve JRJr. CCK-8 and CCK-58 differ in their effects on nocturnal solid meal pattern in undisturbed rats. *Am J Physiol Regul Integr Comp Physiol*. (2012) 303:R850–60. doi: 10.1152/ajpregu.00365.2011
131. Moos AB, Mclaughlin CL, Baile CA. Effects of CCK on gastrointestinal function in lean and obese Zucker rats. *Peptides* (1982) 3:619–22.
132. Chey WY, Hitanant S, Hendricks J, Lorber SH. Effect of secretin and cholecystokinin on gastric emptying and gastric secretion in man. *Gastroenterology* (1970) 58:820–7.
133. Dufresne M, Seva C, Fourmy D. Cholecystokinin and gastrin receptors. *Physiol Rev*. (2006) 86:805–47. doi: 10.1152/physrev.00014.2005
134. Liddle RA, Green GM, Conrad CK, Williams JA. Proteins but not amino acids, carbohydrates, or fats stimulate cholecystokinin secretion in the rat. *Am J Physiol*. (1986) 251:G243–8. doi: 10.1152/ajpgi.1986.251.2.G243
135. Greenberg D, Smith GP, Gibbs J. Intraduodenal infusions of fats elicit satiety in sham-feeding rats. *Am J Physiol*. (1990) 259:R110–18. doi: 10.1152/ajpregu.1990.259.1.R110
136. Brennan IM, Luscombe-Marsh ND, Seimon RV, Otto B, Horowitz M, Wishart JM, et al. Effects of fat, protein, and carbohydrate and protein load on appetite, plasma cholecystokinin, peptide YY, and ghrelin, and energy intake in lean and obese men. *Am J Physiol Gastrointest Liver Physiol*. (2012) 303:G129–40. doi: 10.1152/ajpgi.00478.2011
137. Bonaz B, De Giorgio R, Taché Y. Peripheral bombesin induces c-fos protein in the rat brain. *Brain Res*. (1993) 600:353–7.
138. Smith GP, Jerome C, Cushin BJ, Eterno R, Simansky KJ. Abdominal vagotomy blocks the satiety effect of cholecystokinin in the rat. *Science* (1981) 213:1036–7. doi: 10.1152/ajpregu.1993.264.5.R912
139. Hsu LT, Hung KY, Wu HW, Liu WW, She MP, Lee TC, et al. Gut-derived cholecystokinin contributes to visceral hypersensitivity via nerve growth factor-dependent neurite outgrowth. *J Gastroenterol Hepatol*. (2016) 31:1594–603. doi: 10.1111/jgh.13296
140. Sadeghi M, Radahmadi M, Reisi P. Effects of repeated treatment with cholecystokinin sulfated octapeptide on passive avoidance memory under chronic restraint stress in male rats. *Adv Biomed Res*. (2015) 4:150. doi: 10.4103/2277-9175.161577
141. Sadeghi M, Reisi P, Radahmadi M. The effects of CCK-8S on spatial memory and long-term potentiation at CA1 during induction of stress in rats. *Iran J Basic Med Sci*. (2017) 20:1368–76. doi: 10.22038/IJBMS.2017.9619
142. Hurwitz I, Malkesman O, Stern Y, Schroeder M, Lavi-Avnon Y, Shayit M, et al. Stress and pain responses in rats lacking CCK1 receptors. *Peptides* (2006) 27:1483–9. doi: 10.1016/j.peptides.2005.10.009
143. Juaneda C, Lafon-Dubourg P, Ciofi P, Sarrieau A, Wenger T, Tramu G, et al. CCK mRNA expression in neuroendocrine CRH neurons is increased in rats subjected to an immune challenge. *Brain Res*. (2001) 901:277–80. doi: 10.1016/S0006-8993(01)02365-4
144. Maniscalco JW, Rinaman L. Overnight food deprivation markedly attenuates hindbrain noradrenergic, glucagon-like peptide-1, and hypothalamic neural responses to exogenous cholecystokinin in male rats. *Physiol Behav*. (2013) 121:35–42. doi: 10.1016/j.physbeh.2013.01.012
145. Sander LD, Porter JR. Influence of bombesin, CCK, secretin and CRF on corticosterone concentration in the rat. *Peptides* (1988) 9:113–7.
146. Sherrin T, Todorovic C, Zeyda T, Tan CH, Wong PT, Zhu YZ, et al. Chronic stimulation of corticotropin-releasing factor receptor 1 enhances the anxiogenic response of the cholecystokinin system. *Mol Psychiatry* (2009) 14:291–307. doi: 10.1038/sj.mp.4002121
147. Macneil G, Sela Y, Mcintosh J, Zacharko RM. Anxiogenic behavior in the light-dark paradigm follwoing intraventricular administration of cholecystokinin-8S, restraint stress, or uncontrollable footshock in the CD-1 mouse. *Pharmacol Biochem Behav*. (1997) 58:737–46.
148. Kellner M, Yassouridis A, Jahn H, Wiedemann K. Influence of clonidine on psychopathological, endocrine and respiratory effects of cholecystokinin tetrapeptide in patients with panic disorder. *Psychopharmacology* (1997) 133:55–61.
149. Kellner M, Wiedemann K, Yassouridis A, Levengood R, Guo LS, Holsboer F, et al. Behavioral and endocrine response to cholecystokinin tetrapeptide in patients with posttraumatic stress disorder. *Biol Psychiatry* (2000) 47:107–11. doi: 10.1016/S0006-3223(99)00118-3
150. Levine AS, Morley JE. Cholecystokinin-octapeptide suppresses stress-induced eating by inducing hyperglycemia. *Regul Pept*. (1981) 2:353–7.
151. Goebel-Stengel M, Stengel A, Wang L, Taché Y. Orexigenic response to tail pinch: role of brain NPY(1) and corticotropin releasing factor receptors. *Am J Physiol Regul Integr Comp Physiol*. (2014) 306:R164–74. doi: 10.1152/ajpregu.00335.2013
152. Mojsov S, Heinrich G, Wilson IB, Ravazzola M, Orci L, Habener JF. Preproglucagon gene expression in pancreas and intestine diversifies at the level of post-translational processing. *J Biol Chem*. (1986) 261:11880–9.
153. Orskov C, Rabenhoj L, Wettergren A, Kofod H, Holst JJ. Tissue and plasma concentrations of amidated and glycine-extended glucagon-like peptide I in humans. *Diabetes* (1994) 43:535–9.
154. Roberge JN, Brubaker PL. Regulation of intestinal proglucagon-derived peptide secretion by glucose-dependent insulinotropic peptide in a novel enteroendocrine loop. *Endocrinology* (1993) 133:233–40. doi: 10.1210/endo.133.1.8319572
155. Reimer RA, Darimont C, Gremlich S, Nicolas-Metral V, Ruegg UT, Mace K. A human cellular model for studying the regulation of glucagon-like peptide-1 secretion. *Endocrinology* (2001) 142:4522–8. doi: 10.1210/endo.142.10.8415
156. Rocca AS, Brubaker PL. Role of the vagus nerve in mediating proximal nutrient-induced glucagon-like peptide-1 secretion. *Endocrinology* (1999) 140:1687–94. doi: 10.1210/endo.140.4.6643
157. Dailey MJ, Moran TH. Glucagon-like peptide 1 and appetite. *Trends Endocrinol Metab*. (2013) 24:85–91. doi: 10.1016/j.tem.2012.11.008
158. Tang-Christensen M, Larsen PJ, Goke R, Fink-Jensen A, Jessop DS, Moller M, et al. Central administration of GLP-1-(7-36) amide inhibits food and water intake in rats. *Am J Physiol*. (1996) 271:R848–56. doi: 10.1152/ajpregu.1996.271.4.R848
159. Chelikani PK, Haver AC, Reidelberger RD. Intravenous infusion of glucagon-like peptide-1 potently inhibits food intake, sham feeding, and gastric emptying in rats. *Am J Physiol Regul Integr Comp Physiol*. (2005) 288:R1695–706. doi: 10.1152/ajpregu.00870.2004
160. Gutzwiller JP, Goke B, Drewe J, Hildebrand P, Ketterer S, Handschin D, et al. Glucagon-like peptide-1: a potent regulator of food intake in humans. *Gut* (1999) 44:81–6.
161. Tolessa T, Gutniak M, Holst JJ, Efendic S, Hellstrom PM. Glucagon-like peptide-1 retards gastric emptying and small bowel transit in the rat: effect mediated through central or enteric nervous mechanisms. *Dig Dis Sci*. (1998) 43:2284–90.
162. Schirra J, Nicolaus M, Roggel R, Katschinski M, Storr M, Woerle HJ, et al. Endogenous glucagon-like peptide 1 controls endocrine pancreatic secretion and antro-pyloro-duodenal motility in humans. *Gut* (2006) 55:243–51. doi: 10.1136/gut.2004.059741
163. Nakagawa A, Satake H, Nakabayashi H, Nishizawa M, Furuya K, Nakano S, et al. Receptor gene expression of glucagon-like peptide-1, but not glucose-dependent insulinotropic polypeptide, in rat nodose ganglion cells. *Auton Neurosci*. (2004) 110:36–43. doi: 10.1016/j.autneu.2003.11.001
164. Abbott CR, Monteiro M, Small CJ, Sajedi A, Smith KL, Parkinson JR, et al. The inhibitory effects of peripheral administration of peptide YY(3-36) and glucagon-like peptide-1 on food intake are attenuated by ablation of the vagal-brainstem-hypothalamic pathway. *Brain Res*. (2005) 1044:127–31. doi: 10.1016/j.brainres.2005.03.011
165. Krieger JP, Arnold M, Pettersen KG, Lossel P, Langhans W, Lee SJ. Knockdown of GLP-1 receptors in vagal afferents affects normal food intake and glycemia. *Diabetes* (2016) 65:34–43. doi: 10.2337/db15-0973
166. Larsen PJ, Tang-Christensen M, Holst JJ, Orskov C. Distribution of glucagon-like peptide-1 and other preproglucagon-derived peptides in the rat hypothalamus and brainstem. *Neuroscience* (1997) 77:257–70.
167. Barrera JG, Jones KR, Herman JP, D'alessio DA, Woods SC, Seeley RJ. Hyperphagia and increased fat accumulation in two models of chronic CNS glucagon-like peptide-1 loss of function. *J Neurosci*. (2011) 31:3904–13. doi: 10.1523/JNEUROSCI.2212-10.2011

168. Orskov C, Poulsen SS, Moller M, Holst JJ. Glucagon-like peptide I receptors in the subfornical organ and the area postrema are accessible to circulating glucagon-like peptide I. *Diabetes* (1996) 45:832–5.
169. Hansen L, Deacon CF, Orskov C, Holst JJ. Glucagon-like peptide-1-(7-36)amide is transformed to glucagon-like peptide-1-(9-36)amide by dipeptidyl peptidase IV in the capillaries supplying the L cells of the porcine intestine. *Endocrinology* (1999) 140:5356–63. doi: 10.1210/endo.140.11.7143
170. Gil-Lozano M, Perez-Tilve D, Alvarez-Crespo M, Martis A, Fernandez AM, Catalina PA, et al. GLP-1(7-36)-amide and Exendin-4 stimulate the HPA axis in rodents and humans. *Endocrinology* (2010) 151:2629–40. doi: 10.1210/en.2009-0915
171. Ghosal S, Packard AEB, Mahbod P, Mcklveen JM, Seeley RJ, Myers B, et al. Disruption of glucagon-like peptide 1 signaling in Sim1 neurons reduces physiological and behavioral reactivity to acute and chronic stress. *J Neurosci*. (2017) 37:184–93. doi: 10.1523/JNEUROSCI.1104-16.2016
172. Terrill SJ, Maske CB, Williams DL. Endogenous GLP-1 in lateral septum contributes to stress-induced hypophagia. *Physiol Behav*. (2018) 192:17–22. doi: 10.1016/j.physbeh.2018.03.001
173. Maclusky NJ, Cook S, Scrocchi L, Shin J, Kim J, Vaccarino F, et al. Neuroendocrine function and response to stress in mice with complete disruption of glucagon-like peptide-1 receptor signaling. *Endocrinology* (2000) 141:752–62. doi: 10.1210/endo.141.2.7326
174. Isbil Buyukcoskun N, Gulec G, Cam Etoz B, Ozluk K. Central effects of glucagon-like peptide-1 on cold-restraint stress-induced gastric mucosal lesions. *Turk J Gastroenterol*. (2007) 18:150–6.
175. Nozu T, Miyagishi S, Kumei S, Nozu R, Takakusaki K, Okumura T. Glucagon-like peptide-1 analog, liraglutide, improves visceral sensation and gut permeability in rats. *J Gastroenterol Hepatol*. (2018) 33:232–9. doi: 10.1111/jgh.13808
176. Farokhnia M, Sheskier MB, Lee MR, Le AN, Singley E, Bouhlal S, et al. Neuroendocrine response to GABA-B receptor agonism in alcohol-dependent individuals: results from a combined outpatient and human laboratory experiment. *Neuropharmacology* (2018) 137:230–9. doi: 10.1016/j.neuropharm.2018.04.011
177. Zhu E, Hu L, Wu H, Piao L, Zhao G, Inoue A, et al. Dipeptidyl peptidase-4 regulates hematopoietic stem cell activation in response to chronic stress. *J Am Heart Assoc*. (2017) 6:e006394. doi: 10.1161/JAHA.117.006394
178. Zhao Y, Li H, Fang F, Qin T, Xiao W, Wang Z, et al. Geniposide improves repeated restraint stress-induced depression-like behavior in mice by ameliorating neuronal apoptosis via regulating GLP-1R/AKT signaling pathway. *Neurosci Lett*. (2018) 676:19–26. doi: 10.1016/j.neulet.2018.04.010
179. Yoshino Y, Ishisaka M, Tsujii S, Shimazawa M, Hara H. Glucagon-like peptide-1 protects the murine hippocampus against stressors via Akt and ERK1/2 signaling. *Biochem Biophys Res Commun*. (2015) 458:274–9. doi: 10.1016/j.bbrc.2015.01.098
180. Suzuki K, Iwasaki K, Murata Y, Harada N, Yamane S, Hamasaki A, et al. Distribution and hormonal characterization of primary murine L cells throughout the gastrointestinal tract. *J Diabetes Investig*. (2018) 9:25–32. doi: 10.1111/jdi.12681
181. Grandt D, Schimiczek M, Beglinger C, Layer P, Goebell H, Eysselein VE, et al. Two molecular forms of peptide YY (PYY) are abundant in human blood: characterization of a radioimmunoassay recognizing PYY 1-36 and PYY 3-36. *Regul Pept*. (1994) 51:151–9.
182. Mentlein R, Dahms P, Grandt D, Kruger R. Proteolytic processing of neuropeptide Y and peptide YY by dipeptidyl peptidase IV. *Regul Pept*. (1993) 49:133–44.
183. Batterham RL, Cowley MA, Small CJ, Herzog H, Cohen MA, Dakin CL, et al. Gut hormone PYY(3-36) physiologically inhibits food intake. *Nature* (2002) 418:650–4. doi: 10.1038/nature00887
184. Abbott CR, Small CJ, Kennedy AR, Neary NM, Sajedi A, Ghatei MA, et al. Blockade of the neuropeptide Y Y2 receptor with the specific antagonist BIIE0246 attenuates the effect of endogenous and exogenous peptide YY(3-36) on food intake. *Brain Res*. (2005)1043:139–44. doi: 10.1016/j.brainres.2005.02.065
185. Burdyga G, De Lartigue G, Raybould HE, Morris R, Dimaline R, Varro A, et al. Cholecystokinin regulates expression of Y2 receptors in vagal afferent neurons serving the stomach. *J Neurosci*. (2008) 28:11583–92. doi: 10.1523/JNEUROSCI.2493-08.2008
186. Koda S, Date Y, Murakami N, Shimbara T, Hanada T, Toshinai K, et al. The role of the vagal nerve in peripheral PYY3-36-induced feeding reduction in rats. *Endocrinology* (2005) 146:2369–75. doi: 10.1210/en.2004-1266
187. Nonaka N, Shioda S, Niehoff ML, Banks WA. Characterization of blood-brain barrier permeability to PYY3-36 in the mouse. *J Pharmacol Exp Ther*. (2003) 306:948–53. doi: 10.1124/jpet.103.051821
188. Bonaz B, Taylor I, Taché Y. Peripheral peptide YY induces c-fos-like immunoreactivity in the rat brain. *Neurosci Lett*. (1993) 163:77–80.
189. Stadlbauer U, Arnold M, Weber E, Langhans W. Possible mechanisms of circulating PYY-induced satiation in male rats. *Endocrinology* (2013) 154:193–204. doi: 10.1210/en.2012-1956
190. Broberger C, Landry M, Wong H, Walsh JN, Hokfelt T. Subtypes Y1 and Y2 of the neuropeptide Y receptor are respectively expressed in pro-opiomelanocortin- and neuropeptide-Y-containing neurons of the rat hypothalamic arcuate nucleus. *Neuroendocrinology* (1997) 66:393–408. doi: 10.1159/000127265
191. Liang C, Luo H, Liu Y, Cao J, Xia H. Plasma hormones facilitated the hypermotility of the colon in a chronic stress rat model. *PLoS ONE* (2012) 7:e31774. doi: 10.1371/journal.pone.0031774
192. Kiessl GR, Laessle RG. Stress inhibits PYY secretion in obese and normal weight women. *Eat Weight Disord*. (2016) 21:245–9. doi: 10.1007/s40519-015-0231-y
193. Yamada C, Mogami S, Kanno H, Hattori T. Peptide YY causes apathy-like behavior via the dopamine D2 receptor in repeated water-immersed mice. *Mol Neurobiol*. (2018) 55:7555–66. doi: 10.1007/s12035-018-0931-1
194. Forbes SC, Cox HM. Peptide YY, neuropeptide Y and corticotrophin-releasing factor modulate gastrointestinal motility and food intake during acute stress. *Neurogastroenterol Motil*. (2014) 26:1605–14. doi: 10.1111/nmo.12428
195. Barrachina MD, Martinez V, Wang L, Wei JY, Taché Y. Synergistic interaction between leptin and cholecystokinin to reduce short-term food intake in lean mice. *Proc Natl Acad Sci USA*. (1997) 94:10455–60.

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Endocrinology

REVIEW
published: 06 September 2018
doi: 10.3389/fendo.2018.00505

Advances and Unmet Needs in the Therapeutics of Bone Fragility

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The prevalence of fragility fractures increases as longevity increases the proportion of the elderly in the community. Until recently, the majority of studies have targeted women with osteoporosis defined as a bone mineral density (BMD) T score of < -2.5 SD, despite evidence that the population burden of fractures arises from women with osteopenia. Antiresorptive agents reduce vertebral and hip fracture risk by ∼50 percent during 3 years but efficacy against non-vertebral fractures, 80% of all fractures in the community, is reported in few studies, and of those, the risk reduction is only 20–30%. Recent advances in the use of antiresorptives and anabolic agents has addressed some of these unmet needs. Zoledronic acid is now reported to reduce vertebral and non-vertebral fractures rates in women with osteopenia. Studies using teriparatide demonstrate better vertebral and clinical (symptomatic vertebral and non-vertebral) antifracture efficacy than risedronate. Abaloparatide, a peptide sharing amino acid sequences with teriparatide, reduces vertebral and non-vertebral fractures. Romosozumab, a monoclonal antibody suppressing sclerostin, reduces vertebral and non-vertebral fractures within a year of starting treatment, and does so more greatly than alendronate. Some recent studies signal undesirable effects of therapy but provide essential cautionary insights into long term management. Cessation of denosumab is associated with a rapid increase in bone remodeling and the uncommon but clinically important observation of increased multiple vertebral fractures suggesting the need to start alternative anti-resorptive therapy around the time of stopping denosumab. Antiresorptives like bisphosphonates and denosumab suppress remodeling but not completely. Antifracture efficacy may be limited, in part, as a consequence of continued unsuppressed remodeling, particularly in cortical bone. Bisphosphonates may not distribute in deeper cortical bone, so unbalanced intracortical remodeling continues to cause microstructural deterioration. In addition, suppressed remodeling may compromise the material composition by increasing matrix mineral density and glycosylation of collagen. As antiresorptive agents do not restore microstructural deterioration existing at the time of starting treatment, under some circumstances, anabolic therapy may be more appropriate first line treatment. Combining antiresorptive and anabolic therapy is an alternative but whether anti-fracture efficacy is greater than that achieved by either treatment alone is not known.

Keywords: anabolic agents, antiresorptive agents, bone fragility, fractures, microstructure, osteoporosis, osteopenia

OPEN ACCESS

Edited by:
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Specialty section:
This article was submitted to Bone Research, a section of the journal Frontiers in Endocrinology

Received: *10 July 2018*
Accepted: *10 August 2018*
Published: *06 September 2018*

Citation:
Ramchand SK and Seeman E (2018) Advances and Unmet Needs in the Therapeutics of Bone Fragility. Front. Endocrinol. 9:505. doi: 10.3389/fendo.2018.00505

INTRODUCTION

Bone remodeling, a sequential process of bone resorption and formation, occurs throughout life renewing the composition of the mineralized matrix volume (1). During young adulthood, bone remodeling is balanced—an equal volume of bone is resorbed and subsequently replaced so no net loss or gain occurs (2). Around midlife, bone formation by the osteoblasts of the basic multicellular units (BMUs) decreases, producing remodeling imbalance (3). In addition, as a consequence of the estrogen deficiency accompanying menopause, remodeling imbalance worsens and the rate of remodeling increases—less bone is deposited than was resorbed by each of the many BMUs initiated upon the three (intracortical, endocortical, trabecular) components of the endosteal (inner) bone surface (4). Estrogen therapy, by influencing the lifespan of osteoblasts and osteoclasts, may reverse the estradiol dependent component of the remodeling imbalance (5–7).

There is a reduction in total mineralized bone matrix volume and the decreasing total mineralized bone matrix volume becomes deteriorated in its microstructure. Unbalanced remodeling upon trabeculae cause them to thin, perforate and disappear. Unbalanced intracortical remodeling initiated upon the intracortical canal surfaces enlarge them, they coalesce and fragment the cortex. With advancing age bone loss from the trabecular compartment lessens because trabeculae with their surfaces disappear (remodeling requires a surface to be initiated upon). Bone loss becomes predominantly cortical as intracortical surface area increases facilitating initiation of unbalanced intracortical remodeling (8, 9). The microstructural deterioration produces bone fragility out of proportion to the bone loss producing it (10).

The burden of fragility fractures is increasing in absolute terms because longevity is increasing the proportion of the population over 65 years of age (11). Reducing the burden of fractures is an unmet need because there are unresolved issues in detection of individuals at high risk for fracture that need to be addressed (12). For example, identifying methods able to detect individuals at imminent risk for fracture is a challenge. Commonly used tools such as bone densitometry lack sensitivity. A BMD T score threshold of − 2.5 SD designated as "osteoporosis" identifies only 30–40% of women having fragility fractures (13–15).

The word "osteoporosis" is often used synonymously with bone fragility but women with osteopenia are not free of the risk of fracture (13, 15). Indeed, most women and men sustaining fragility fractures have osteopenia and many have so-called "normal" BMD (14). Women with osteopenia at risk for fracture can be identified by measuring microstructural deterioration (16, 17) but high-resolution imaging methods are not yet widely available. The use of clinical risk factor assessment tools such as FRAX have met with variable success (18, 19). Challenges also arise in the uptake and adherence to therapy, in part, because of concerns regarding the serious but uncommon long term adverse effects of therapy (20, 21).

Antiresorptive agents are the first line and most commonly used treatments for prevention and treatment of bone fragility (22). Apart from denosumab, which virtually abolishes remodeling, most antiresorptives slow unbalanced remodeling so microstructural deterioration continues to occur albeit more slowly (23). This lower rate of remodeling reduces fracture risk compared to untreated women in whom rapid remodeling continues to deteriorate the skeleton. This is a relative risk reduction. In absolute terms, fracture risk does not decrease during antiresorptive therapy because microstructural deterioration present is not reversed and the slow continued unsuppressed and unbalanced remodeling continues to deteriorate bone. This, in part, may explain why fracture risk reduction with antiresorptives is modest. Teriparatide increases bone matrix volume predominantly through remodeling based bone formation (24). It is likely that the anabolic effect of abaloparatide, which acts via the same receptor as teriparatide, is also remodeling based like teriparatide, although rigorous assessment of its mechanism of action has not been undertaken (25). Both reduce the risk of vertebral and non-vertebral fractures (26, 27) but no adequately designed trials have been done to determine whether hip fracture risk is reduced.

Several comprehensive literature reviews are available (28, 29). We confine this manuscript to defining advances that have taken place in existing therapies and new therapies that are available or may become available soon, particularly the development of anabolic agents, therapies that partly reconstruct the skeleton but are also not without their limitations.

ANTI-RESORPTIVE THERAPY

Bisphosphonates

Bisphosphonates are currently first line treatment and the most common antiresorptive therapy used. The antiresorptive efficacy of bisphosphonates depend on inhibition of farnesyl pyrophosphate (FPP) synthase, required for osteoclast resorptive function, as well as their affinity for mineral which influences uptake, distribution, and retention in the bone (30–33). High affinity binding agents, like alendronate, have a reduced ability to penetrate and distribute widely in deeper cortical matrix so that when osteoclasts remodel cortical bone they encounter matrix free of bisphosphonates and continue to resorb bone.

This may partly account for the finding of less reduction in porosity with alendronate than denosumab (34). The reduction in porosity is the net result of fewer cavities being excavated plus the incomplete filling of the resorption cavities excavated shortly before treatment (35). Likewise, ibandronate is avidly bound to matrix mineral and is present in lower concentrations in cortical than trabecular bone. Studies of ibandronate in ovariectomized cynomolgus monkeys demonstrate reduced remodeling and improved trabecular bone strength but not intracortical remodeling suppression or improved cortical bone strength (36). Lower binding affinity of risedronate than alendronate results in wider distribution of risedronate through the bone and may contribute to its earlier suppression of bone remodeling as reported in animal models and claims of possible earlier fracture risk reduction (37, 38).

Several advances have been made in the study of zoledronic acid. This drug is usually given as an annual 5 mg infusion for 3 years. Fracture risk is reduced in postmenopausal women

with osteoporosis, by 77% for clinical (symptomatic) vertebral fractures, 25% for non-vertebral, and 41% for hip fractures (39). Zoledronic acid was also associated with a 28% reduction in mortality after hip fracture, independent of its effects on fracture risk reduction (40, 41).

Discontinuation of zoledronic acid at 3 years, followed by no treatment for 3 years, was associated with minimal reduction in BMD but 30 new morphometric vertebral fractures occurred compared to 14 in those treated for 6 years (odds ratio = 0.51; $P = 0.035$) (42) In further follow-up, women treated for 6 years and 3 years off treatment did not have more fractures than women treated for 9 years. However, the loss of the inception cohort and small numbers of events make interpretation problematic (43).

Several very insightful studies have been published regarding zoledronic acid treatment. In a *post-hoc* analysis of pooled data from the HORIZON studies, there were comparable ∼30% reductions in clinical fractures in those who received a single infusion or three or more annual infusions (44). Whether differing baseline characteristics influenced this outcome is not clear, but given the protracted remodeling suppression with this drug, it is of interest to determine the appropriate regimen needed for efficacy and safety.

This has been evaluated by Reid et al. in postmenopausal women with osteopenia randomized to a single dose of zoledronic acid 1, 2.5, or 5 mg. These doses resulted in a similar increase in spine and hip BMD in the 2.5 and 5 mg groups at 2 years but not at 5 years (45), where increases were greater in the 5 mg than 2.5 mg group (46).

More recently, Reid et al. addressed two important unmet needs. There is a lack of information regarding the antifracture efficacy of drugs given to women with osteopenia, the source of over 60% of all fragility fractures (13). There is also little information regarding the prevention of non-vertebral fractures, 80% of all fractures in the community (11). The investigators evaluated 2,000 women with osteopenia, mean age 71 years, treated with zoledronic acid 5 mg or placebo every 18-months for 4 doses. After 6 years of follow up, treated women had a 34% reduction in non-vertebral fractures (HR 0.66, 95% CI 0.51–0.85) (47).

Denosumab

Denosumab is a fully humanized monoclonal antibody directed against RANK-ligand, a major regulator of osteoclast development which inhibits osteoclast recruitment, activity and survival. Denosumab (60 mg subcutaneously administered every 6 months) produces almost complete suppression of bone remodeling. Treatment for 3 years resulted in a 68% reduction in vertebral fractures, 40% reduction in hip fracture and 20% reduction in non-vertebral fractures (48).

Remodeling suppression with denosumab is greater than that achieved with any other antiresorptive agent (49). This greater suppression of remodeling accounts for greater increases in BMD achieved in postmenopausal women treated with denosumab compared to bisphosphonates (34, 50–52). Whether these BMD differences translate to differences in fracture risk reduction between the two groups is not known.

No trials have been conducted with a placebo control group beyond 3 years. Because of this, whatever the fracture rate, it is not possible to infer with confidence that fracture rates reported are attributable to the drug rather than healthy user bias. In the open label extension of the FREEDOM study (53), all participants were treated with denosumab and followed up for 7 years. Ten years of denosumab treatment was associated with an acceptable safety profile, sustained suppression of bone remodeling, continued increase in BMD without plateau and "low" fracture rates. The lack of controls and the large number of participants withdrawn from the trial, suggest that the finding of low and further reductions in fracture rates should be cautiously interpreted.

In the long-term group, BMD increased by 21.7% in the lumbar spine and 9.0% in the femoral neck at 10 years compared to FREEDOM baseline. The mechanism of increase in BMD may be due to progressive secondary mineralization. It is plausible that in the face of suppressed remodeling, continued slow age-related modeling based bone formation becomes detectable as reported in studies of histomorphometry conducted in cynomolgus monkeys (54, 55). Dempster et al. also report some evidence of modest trabecular modeling based bone formation after 3 months of denosumab therapy (56). Further studies are needed to determine whether antiresorptive therapy permits expression of any existing modeling based bone formation when remodeling is suppressed.

An important insight into treatment with denosumab is the report that cessation of denosumab caused a rapid rise in bone remodeling markers with a transitory overshoot above baseline, a decline in BMD in 12 months and, uncommonly, an increased risk in multiple vertebral fractures (57, 58). Occurrence of multiple vertebral fractures were initially reported in several case series (59–64). In a subsequent *post hoc* analysis of the FREEDOM and FREEDOM Extension trials (65), vertebral fracture rate after discontinuation of denosumab increased to 7.1 per 100 participant years vs. 8.5 per 100 participant years in the placebo group. In those who developed one or more vertebral fractures, the proportion of multiple vertebral fractures (>2) was higher in those discontinuing denosumab (60.7%) than in those discontinuing placebo (38.7%, $P = 0.049$). The risk of multiple vertebral fractures after stopping denosumab was greatest in those with a prior vertebral fracture, either before or during treatment (odds 3.9, 95% CI 2.1–7.2) (65). Investigators recommend commencement of an alternative antiresorptive agent soon after cessation of denosumab, although the agent to use and when to start needs further evaluation.

At present, there is no evidence that the increased risk of vertebral fractures is due to the rapid increase in remodeling. The term "rebound" is used but cessation of remodeling suppression produces expansion of the reversible remodeling space with any antiresorptive (66). When an antiresorptive is stopped, the effect has similarities to the onset of menopause. There is a rapid increase in the number of remodeling units starting to excavate bone and hence a rapid rise in remodeling markers. With denosumab, given the drug is not retained in the skeleton like bisphosphonates, the increased number of resorptive cavities is not offset as it is with bisphosphonate cessation. With

bisphosphonate cessation, increased resorption may lead to release of bisphosphonate and readsorption into matrix slowing the decrease in BMD. In the absence of denosumab, the rapid increase in number of resorption cavities is not inhibited and may create stress concentrators which pre-dispose to microcrack propagation and increased vertebral fracture risk (67, 68). Whether this increase in resorption is amplified by rapid differentiation of existing osteoclast precursors remaining undifferentiated until denosumab is stopped is not known.

While this "rebound" or "overshoot" in remodeling markers is reported, remodeling returns to its pretreatment level and the reduction in BMD returns to baseline leaving a residual higher BMD than untreated controls (58, 69). If there was accelerated loss of bone, beyond that found early after stopping treatment due to many more excavated cavities than those incompletely refilling after starting denosumab, then BMD should decline to levels no different to untreated controls (70). Bone fragility is likely to occur after stopping treatment because the accelerated remodeling occurs in the setting of an already deteriorated skeleton (as antiresorptive agents do not reverse microstructural deterioration present at the time of starting treatment). This is not the same after menopause where there is little, if any, deterioration before menopause.

Selective Estrogen Receptor Modulators

Raloxifene, a selective estrogen receptor modulator (SERM), reduces the rate of bone remodeling by about 20–30% as determined using circulating bone remodeling markers (71). It produces a modest transitory increase in BMD during early treatment, as fewer new resorption cavities are excavated while the many more cavities excavated before treatment refill, albeit incompletely. With more protracted treatment, unbalanced remodeling continues at 20–30% slower rate than before treatment so microstructural deterioration continues (70). The decline in BMD during prolonged therapy is well documented (71, 72) and probably accounts, in part, for the modest vertebral fracture risk reduction and lack of evidence for non-vertebral fracture risk reduction (71).

Raloxifene appears to reduce vertebral fractures with small and perhaps transient effects on BMD. It is of interest that pre-clinical studies demonstrate an increase in the material strength produced by increases in skeletal-bound water with minimal effect on tissue mineral composition or microdamage accumulation (73–75). These findings provide a novel target for future pharmacological interventions to improve bone strength and lower fracture risk. This is of particular interest given the concerns of protracted remodeling suppression by bisphosphonates and denosumab which are likely to pre-dispose to loss of toughness and atypical femoral fractures (AFFs).

Treatment of osteoporosis in patients with AFFs is challenging; withholding an antiresorptive will result in ongoing structural decay predisposing to fragility fracture, conversely, if an antiresorptive is continued, structural decay will slow but material composition will be compromised predisposing to AFFs (76). One approach may be to use a weaker antiresorptive such as raloxifene, which improves bone toughness with minimal effects on tissue mineral composition or microdamage accumulation. The efficacy of raloxifene in this context has not been established.

ANABOLIC AGENTS

Teriparatide (PTH 1-34) and abaloparatide are available for clinic use, romosozumab is a modeling based anabolic agent that is still under investigation.

Teriparatide and Abaloparatide

The anabolic effects of PTH 1-34 are ∼70% remodeling based. Abaloparatide, which shares amino acid sequences with both parathyroid hormone-related protein (PTHrP) and PTH, acts via the same receptor as teriparatide (PTHR1). Both teriparatide and abaloparatide increase trabecular thickness and improve trabecular microstructure (77–79). There is a transitory phase of increased cortical porosity produced with PTH 1-34 (80, 81). Whether the anabolic effect of abaloparatide is accompanied by less resorptive activity and less cortical porosity needs to be confirmed (27, 82, 83).

Parathyroid hormone analogs have consistently been reported to reduce vertebral fractures but evidence for non-vertebral fracture risk reduction reported by Neer et al. has not been replicated (84). No randomized controlled studies have been done to evaluate anti-hip fracture efficacy, an omission that needs to be addressed.

Abaloparatide also reduces vertebral and non-vertebral fractures (27). In a phase 3 clinical trial, 2463 ambulatory postmenopausal women, of which 1901 completed the study, were randomized to 18 months of abaloparatide (80 μg daily), placebo or open-label teriparatide (20 μg daily). New morphometric vertebral fractures occurred in 0.58% ($n = 4$) of the abaloparatide group, 4.22% ($n = 30$) of the placebo group (relative risk 0.14, 95% CI 0.05–0.39), and 0.84% ($n = 6$) of the teriparatide group. The Kaplan-Meier estimated event rate for non-vertebral fracture was 2.7% for abaloparatide, 4.7% for placebo (HR 0.57, 95% CI 0.32–1.00), and 3.3% for teriparatide ($P = 0.22$ compared to placebo and $P = 0.44$ compared to abaloparatide) (27). Major osteoporotic fractures were reduced with abaloparatide compared to placebo or teriparatide, Kaplan-Meier estimated event rate for placebo was 6.2% (HR 0.30, 95% CI 0.15–0.61) and for teriparatide was 3.1% (HR 0.45, 95% CI 0.21–0.95, $P = 0.03$) (27).

While these findings are encouraging, the claim that abaloparatide produced an earlier and more efficacious fracture risk reduction than teriparatide is problematic because of an increase in number of subjects who fractured in the first few weeks of treatment in the placebo and teriparatide group that is unlikely to be associated with therapy (27). Differences in fracture rates in the two treatment arms in the second and third 6 months of the 18-month trial were minimal (83). In addition, evidence that the anabolic effect is accompanied by less bone resorption with abaloparatide than teriparatide is also not well founded (83).

Increases in BMD with abaloparatide were greater (∼1%) than those with teriparatide at the total hip and femoral neck at all time points and ∼2% at the lumbar spine at 6 and 12 months (both $P <$ 0.001). At 18-months, BMD in the lumbar spine was no different

between the two groups (27). These difference in BMD at the femoral neck and total hip were attributed to the net difference in resorption and formation markers claimed to be surrogates of more net bone deposition with abaloparatide than teriparatide, a problematic interpretation for a range of reasons (83, 85).

Romosozumab

Romosozumab is a humanized monoclonal antibody against sclerostin, an endogenous inhibitor of bone formation. Treatment results in an increase in modeling based bone formation and evidence of decreases in bone resorption. Two studies support the antifracture efficacy of this anabolic agent (86, 87). Cosman et al. enrolled 7,180 postmenopausal women with osteoporosis to monthly romosozumab (210 mg) or placebo for 12 months followed by denosumab (60 mg 6 monthly) for 12 months (86). At 12 months, risk reductions were reported for vertebral fractures by 73% ($P < 0.001$), for clinical fractures by 36% ($P = 0.008$) and non-vertebral fractures by 24% ($P = 0.10$). At 24 months, vertebral fracture risk was reduced by 75% ($P < 0.001$) (86).

Saag et al. (87). assigned 4,093 postmenopausal women with osteoporosis and a fragility fracture to romosozumab (210 mg) or weekly alendronate (70 mg) for 12 months then open label alendronate in both groups. Over 24 months, romosozumab/alendronate reduced vertebral fracture risk by 48% ($P < 0.001$), clinical fractures by 27% ($P < 0.001$), non-vertebral fracture by 19% ($P = 0.04$), and hip fracture by 38% ($P = 0.02$). At 12 months, romosozumab reduced new vertebral (risk ratio 0.63, 95% CI 0.47–0.85) and clinical (HR 0.72, 95% CI 0.54–0.96) fractures compared to alendronate. Non-vertebral fracture risk was also reduced by 26% with romosozumab, but this difference was not statistically significant ($P = 0.06$) (87). The use of romosozumab is under FDA review after results of the trial demonstrated a higher incidence of adjudicated serious cardiovascular events with romosozumab (50/2040) compared to alendronate (38/2014) at the end of 12 months, which did not persist in the 24-month open label extension (87). These findings were not replicated in the much larger placebo-controlled FRAME study (88).

Recent work by McClung et al. (89). report loss of benefit of romosozumab soon after cessation of therapy. Three hundred and sixty-four postmenopausal women with low bone mass were treated with romosozumab for 24 months and then randomized to either denosumab or placebo for a further 12 months. Treatment with romosozumab led to a continued increase in BMD over 2 years with further accrual in those that transitioned to denosumab, whereas BMD returned toward pre-treatment levels in those that transitioned to placebo (89).

Given the inability of antiresorptives to reverse existing microstructural deterioration, and the evidence that anabolic therapy may partly restore bone microstructure, is there evidence supporting better antifracture efficacy using anabolic therapy than antiresorptive therapy. Kendler et al. studied 1360 postmenopausal women with severe osteoporosis randomized to teriparatide (20 μg daily) or risedronate (35 mg daily) over 2 years (26). Overall, 72% of participants received at least one bone targeted treatment prior to study entry, most commonly a bisphosphonate (59% in the teriparatide group and 57% in the risedronate group) and median duration of bisphosphonate treatment was 3.5 years (IQR 1.1–7.0) in the teriparatide group and 3.6 years (IQR 1.3–6.1) in the risedronate group. At 2 years, treatment with teriparatide resulted in a 56% (risk ratio 0.44, 95% CI 0.29–0.68) reduction in incident vertebral fractures with a reduction in non-vertebral fractures that did not achieve statistical significance; 25 (4.0%) in the teriparatide group vs. 38 (6.1%) in the risedronate group (hazard ratio (HR) 0.66, 95% CI 0.39–1.10, $P = 0.10$) (26). In a subgroup analyses, these changes were consistent across a range of characteristics of the participants (90).

Combined Antiresorptive and Anabolic Therapy

Combining antiresorptive and anabolic therapy is a missed opportunity for two reasons (70). First, no studies have been done demonstrating greater antifracture efficacy than achieved by either treatment alone. This is a valid reason for a cautionary approach to the uptake of this regimen. The second reason is the widely held belief that antiresorptive therapy suppresses, "blunts," remodeling based bone formation by PTH (91–93). This is largely based on two influential papers and the accompanying editorial in the New England Journal of Medicine (91, 93, 94). The notion of blunting was based on the assumption that a higher BMD or higher P1NP mean more bone formation and a lack of response means less bone formation.

Comparator studies that use changes in BMD and bone remodeling markers as the outcome variable are problematic endpoints. Remodeling based anabolic therapy increases bone matrix volume by replacing more fully mineralized bone with young less fully mineralized bone. Modeling based anabolic therapy adds young less fully mineralized bone to existing older bone. Imaging using radiation transmission often results in a net reduction in BMD because young less mineralized bone transmits rather than attenuates photons leading to the inference that bone "loss" and fragility have occurred. Antiresorptives slow remodeling. Matrix no longer "turned over" undergoes more complete mineralization increasing BMD leading to the inference that bone "volume" or "mass" has increased, and that bone strength has increased even though the matrix becomes less ductile.

As an example, even if an increase or lack of an increase in BMD is accepted on face value, examination of Figures 1 to 3 of the study by Black et al. does not support the notion of blunting (91). Relative to PTH alone, combined therapy (i) did *not* produce a smaller increment in spine or femoral neck BMD, (ii) *did* produce a greater increase in total hip BMD, (iii) *did* reduce the decline in distal radius BMD, (iv) *did* prevent the reduction in total hip and femoral neck vBMD produced by PTH alone. Curiously, the increase in total hip and femoral neck cortical volume by PTH, a *modeling* effect, was prevented by combined therapy. Moreover, combined therapy increased

trabecular vBMD less than PTH alone but this may be a benefit, not blunting. The antiresorptive might prevent PTH mediated increase in intracortical remodeling, cortical porosity and the increase in cortical fragments that look like "trabeculae" (35). Blunting of the rise in P1NP and CTX is likely to be the result of suppressed remodeling, not a reduction in the net volumes of bone deposited or resorbed respectively (85). If blunting of the BMD response was due to fewer BMUs then blunting should be *more* severe with co-administration of PTH with zolendronate, denosumab, or osteoprotegerin (OPG, an endogenous inhibitor of RANKL) than with alendronate. The opposite is reported, and many studies report additive effects (95–98).

The difficulties in using BMD are also present using high-resolution peripheral computed tomography. Tsai et al. (80). report that combined PTH 1-34 and denosumab increased cortical vBMD yet PTH 1-34 reduced it and denosumab had no effect. Combined therapy increased cortical matrix mineral density yet PTH 1-34 decreased it and denosumab had no effect. Combined therapy had no effect on porosity yet PTH 1-34 increased it while denosumab had no effect. These findings do not add up, probably because there are methodological challenges in segmenting (separating) the cortical and trabecular compartments and quantifying porosity and trabecular density because low image resolution and changes in matrix mineral density influence the quantification of microstructure (8, 99).

Sequential Therapy

Anabolic to Anti-resorptive

Cessation of anabolic treatment results in loss of the benefits. Antiresorptives maintain or increase BMD, particularly denosumab because it is the most efficacious in suppressing remodeling. In the DATA-Switch study, 2 years of PTH 1-34 followed by 2 years of denosumab resulted in further increases in BMD (100). At 48 months, women treated with combined PTH 1-34 and denosumab for 2 years followed by denosumab alone had greater gains in BMD than those treated with PTH 1-34 followed by denosumab. Whether this results in fewer fractures is not known but it is likely that stopping any of the treatments will result in the loss of benefits and eventual increase in fracture risk.

In another publication of the abaloparatide trial by Miller et al. (27), Bone et al. (101) administered alendronate after abaloparatide which maintained the fracture risk reduction relative to placebo also given alendronate after 18 months. This design does not address the question of whether stopping abaloparatide produces loss of benefits as found with PTH, which requires an arm with abaloparatide given placebo. Any comparisons of abaloparatide/alendronate and placebo/alendronate is flawed as the placebo group is likely to have undergone bone loss and microstructural deterioration during 18 months. The likelihood is however, that stopping abaloparatide will result in loss of benefits. This has recently been reported with romosozumab; where stopping treatment was accompanied by loss of the benefits achieved by the modeling dependent anabolic effect (89).

Anti-resorptive to Anabolic

Two recent trials evaluating antiresorptive therapy followed by an anabolic agent have been conducted (100, 102). In the DATA-switch study, women receiving denosumab had a reduction in hip BMD during 12 months of PTH 1-34 followed by a gradual increase in BMD, but remained lower than women in the PTH 1-34 to denosumab group and the PTH 1-34/denosumab to denosumab group (100). Spine BMD decreased in the first 6 months but then increased to a value no different to the above two groups at 48 months. Bone remodeling markers increased in the denosumab to PTH 1-34 group by over 200% relative to baseline values. Whether bone fragility increases is difficult to determine given the decline in BMD may be due to the replacement of more mineralized with less mineralized new bone by remodeling or addition of under mineralized bone by modeling which then becomes mineralized. High remodeling is found using anabolic agents but fracture rates do not increase, they decrease. Nevertheless, cessation of denosumab is associated with rapid increases in remodeling and multiple vertebral fractures; whether this might be prevented or worsened by PTH 1-34 is not known.

In an unblinded study comparing romosozumab (120 mg monthly) vs. PTH 1-34 (20 μg daily) in postmenopausal women previously treated with bisphosphonates, total hip BMD increased with romosozumab by 2.6% compared to a decrease of 0.6% with PTH 1-34 (102). Both drugs increased spine BMD (romosozumab 9.8% vs. PTH 1-34 5.4%). At the hip, romosozumab increased cortical vBMD whilst PTH 1-34 decreased it. Trabecular vBMD was similarly increased with both drugs (102). The comparison of BMD changes is problematic given BMD may decrease when a large volume of bone that is still unmineralized is deposited and the effects on microstructure which contribute disproportionately to bone strength are not taken into account.

CONCLUSION

Fragility fractures are a public health burden. Advances are occurring, but several challenges remain unmet. Most fractures occur in women with osteopenia yet methods of identifying the women forming the population burden of fractures remain to be identified. Even when women at risk are identified, the uptake and adherence to therapy is poor for reasons that are not well defined. Antiresorptive agents are first line approaches to therapy even though these agents do not restore bone volume or the microstructural deterioration present at the time of treatment. Most, if not all, controlled trials are 3 years duration and long-term efficacy is unknown. Anabolic therapies have not been as comprehensively studied. Although newer agents are emerging and vertebral fracture risk reduction is confirmed, less evidence is available for non-vertebral fracture risk reduction, and no anabolic agent has been evaluated for hip fracture risk reduction in randomized controlled studies. Bone densitometry was a good beginning but most fractures in the community arise in persons with a BMD T-score less reduced than −2.5 SD and so they are not offered treatment. Whether assessment of bone

microstructure might help identify and target therapy more effectively remains an unmet challenge, but it is an opportunity in need of exploration because bone fragility is caused by microstructural deterioration.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

REFERENCES

1. Hattner R, Epker BN, Frost HM. Suggested sequential mode of control of changes in cell behaviour in adult bone remodelling. *Nature* (1965) 206:489–90. doi: 10.1038/206489a0
2. Parfitt AM. Skeletal heterogeneity and the purposes of bone remodelling: implications for the understanding of osteoporosis. In: Marcus RFD, Kelsey J, editors. *Osteoporosis*. San Diego, CA: Academic (1996). p. 315–39.
3. Lips P, Courpron P, Meunier PJ. Mean wall thickness of trabecular bone packets in the human iliac crest: changes with age. *Calcif Tissue Res.* (1978) 26:13–7. doi: 10.1007/BF02013227
4. Vedi S, Compston JE, Webb A, Tighe JR. Histomorphometric analysis of dynamic parameters of trabecular bone formation in the iliac crest of normal British subjects. *Metab Bone Dis Relat Res.* (1983) 5:69–74. doi: 10.1016/0221-8747(83)90004-8
5. Vedi S, Compston JE. The effects of long-term hormone replacement therapy on bone remodeling in postmenopausal women. *Bone* (1996) 19:535–9. doi: 10.1016/S8756-3282(96)00227-X
6. Vedi S, Purdie DW, Ballard P, Bord S, Cooper AC, Compston JE. Bone remodeling and structure in postmenopausal women treated with long-term, high-dose estrogen therapy. *Osteoporos Int.* (1999) 10:52–8. doi: 10.1007/s001980050194
7. Eriksen EF, Langdahl B, Vesterby A, Rungby J, Kassem M. Hormone replacement therapy prevents osteoclastic hyperactivity: a histomorphometric study in early postmenopausal women. *J Bone Miner Res.* (1999) 14:1217–21. doi: 10.1359/jbmr.1999.14.7.1217
8. Zebaze RM, Ghasem-Zadeh A, Bohte A, Iuliano-Burns S, Mirams M, Price RI, et al. Intracortical remodelling and porosity in the distal radius and post-mortem femurs of women: a cross-sectional study. *Lancet* (2010) 375:1729–36. doi: 10.1016/S0140-6736(10)60320-0
9. Parfitt AM. Misconceptions (2): turnover is always higher in cancellous than in cortical bone. *Bone* (2002) 30:807–9. doi: 10.1016/S8756-3282(02)00735-4
10. Schaffler MB, Burr DB. Stiffness of compact bone: effects of porosity and density. *J Biomech.* (1988) 21:13–6. doi: 10.1016/0021-9290(88)90186-8
11. Johnell O, Kanis JA. An estimate of the worldwide prevalence and disability associated with osteoporotic fractures. *Osteoporos Int.* (2006) 17:1726–33. doi: 10.1007/s00198-006-0172-4
12. Khosla S, Cauley JA, Compston J, Kiel DP, Rosen C, Saag KG, et al. Addressing the crisis in the treatment of osteoporosis: a path forward. *J Bone Miner Res.* (2017) 32:424–30. doi: 10.1002/jbmr.2888
13. Siris ES, Chen YT, Abbott TA, Barrett-Connor E, Miller PD, Wehren LE, et al. Bone mineral density thresholds for pharmacological intervention to prevent fractures. *Arch Intern Med.* (2004) 164:1108–12. doi: 10.1001/archinte.164.10.1108
14. Sanders KM, Nicholson GC, Watts JJ, Pasco JA, Henry MJ, Kotowicz MA, et al. Half the burden of fragility fractures in the community occur in women without osteoporosis. When is fracture prevention cost-effective? *Bone* (2006) 38:694–700. doi: 10.1016/j.bone.2005.06.004
15. Pasco JA, Seeman E, Henry MJ, Merriman EN, Nicholson GC, Kotowicz MA. The population burden of fractures originates in women with osteopenia, not osteoporosis. *Osteoporos Int.* (2006) 17:1404–9. doi: 10.1007/s00198-006-0135-9
16. Bala Y, Zebaze R, Ghasem-Zadeh A, Atkinson EJ, Iuliano S, Peterson JM, et al. Cortical porosity identifies women with osteopenia at increased risk for forearm fractures. *J Bone Miner Res.* (2014) 29:1356–62. doi: 10.1002/jbmr.2167
17. Boutroy S, Van Rietbergen B, Sornay-Rendu E, Munoz F, Bouxsein ML, Delmas PD. Finite element analysis based on *in vivo* HR-pQCT images of the distal radius is associated with wrist fracture in postmenopausal women. *J Bone Miner Res.* (2008) 23:392–9. doi: 10.1359/jbmr.071108
18. Shepstone L, Lenaghan E, Cooper C, Clarke S, Fong-Soe-Khioe R, Fordham R, et al. Screening in the community to reduce fractures in older women (SCOOP): a randomised controlled trial. *Lancet* (2018) 391:741–7. doi: 10.1016/S0140-6736(17)32640-5
19. Rubin KH, Rothmann MJ, Holmberg T, Hoiberg M, Moller S, Barkmann R, et al. Effectiveness of a two-step population-based osteoporosis screening program using FRAX: the randomized Risk-stratified Osteoporosis Strategy Evaluation (ROSE) study. *Osteoporos Int.* (2018) 29:567–78. doi: 10.1007/s00198-017-4326-3
20. Shane E, Burr D, Abrahamsen B, Adler RA, Brown TD, Cheung AM, et al. Atypical subtrochanteric and diaphyseal femoral fractures: second report of a task force of the American Society for Bone and Mineral Research. *J Bone Miner Res.* (2014) 29:1–23. doi: 10.1002/jbmr.1998
21. Khosla S, Burr D, Cauley J, Dempster DW, Ebeling PR, Felsenberg D, et al. Bisphosphonate-associated osteonecrosis of the jaw: report of a task force of the American Society for Bone and Mineral Research. *J Bone Miner Res.* (2007) 22:1479–91. doi: 10.1359/jbmr.0707onj
22. Russell RG, Watts NB, Ebetino FH, Rogers MJ. Mechanisms of action of bisphosphonates: similarities and differences and their potential influence on clinical efficacy. *Osteoporos Int.* (2008) 19:733–59. doi: 10.1007/s00198-007-0540-8
23. Seeman E, Delmas PD, Hanley DA, Sellmeyer D, Cheung AM, Shane E, et al. Microarchitectural deterioration of cortical and trabecular bone: differing effects of denosumab and alendronate. *J Bone Miner Res.* (2010) 25:1886–94. doi: 10.1002/jbmr.81
24. Lindsay R, Cosman F, Zhou H, Bostrom MP, Shen VW, Cruz JD, et al. A novel tetracycline labeling schedule for longitudinal evaluation of the short-term effects of anabolic therapy with a single iliac crest bone biopsy: early actions of teriparatide. *J Bone Miner Res.* (2006) 21:366–73. doi: 10.1359/JBMR.051109
25. Moreira CA, Fitzpatrick LA, Wang Y, Recker RR. Effects of abaloparatide-SC (BA058) on bone histology and histomorphometry: the ACTIVE phase 3 trial. *Bone* (2017) 97:314–9. doi: 10.1016/j.bone.2016.11.004
26. Kendler DL, Marin F, Zerbini CAF, Russo LA, Greenspan SL, Zikan V, et al. Effects of teriparatide and risedronate on new fractures in post-menopausal women with severe osteoporosis (VERO): a multicentre, double-blind, double-dummy, randomised controlled trial. *Lancet* (2018) 391:230–40. doi: 10.1016/S0140-6736(17)32137-2
27. Miller PD, Hattersley G, Riis BJ, Williams GC, Lau E, Russo LA, et al. Effect of abaloparatide vs placebo on new vertebral fractures in postmenopausal women with osteoporosis: a randomized clinical trial. *JAMA* (2016) 316:722–33. doi: 10.1001/jama.2016.11136
28. Khosla S, Hofbauer LC. Osteoporosis treatment: recent developments and ongoing challenges. *Lancet Diabetes Endocrinol.* (2017) 5:898–907. doi: 10.1016/S2213-8587(17)30188-2
29. Reid IR. Short-term and long-term effects of osteoporosis therapies. *Nat Rev Endocrinol.* (2015) 11:418–28. doi: 10.1038/nrendo.2015.71
30. van Beek E, Pieterman E, Cohen L, Lowik C, Papapoulos S. Farnesyl pyrophosphate synthase is the molecular target of nitrogen-containing bisphosphonates. *Biochem Biophys Res Commun.* (1999) 264:108–11. doi: 10.1006/bbrc.1999.1499
31. Kavanagh KL, Guo K, Dunford JE, Wu X, Knapp S, Ebetino FH, et al. The molecular mechanism of nitrogen-containing bisphosphonates as antiosteoporosis drugs. *Proc Natl Acad Sci USA.* (2006) 103:7829–34. doi: 10.1073/pnas.0601643103
32. Fisher JE, Rogers MJ, Halasy JM, Luckman SP, Hughes DE, Masarachia PJ, et al. Alendronate mechanism of action: geranylgeraniol, an intermediate in

the mevalonate pathway, prevents inhibition of osteoclast formation, bone resorption, and kinase activation *in vitro*. *Proc Natl Acad Sci USA.* (1999) 96:133–8. doi: 10.1073/pnas.96.1.133
33. Dunford JE, Thompson K, Coxon FP, Luckman SP, Hahn FM, Poulter CD, et al. Structure-activity relationships for inhibition of farnesyl diphosphate synthase *in vitro* and inhibition of bone resorption *in vivo* by nitrogen-containing bisphosphonates. *J Pharmacol Exp Ther.* (2001) 296:235–42.
34. Zebaze RM, Libanati C, Austin M, Ghasem-Zadeh A, Hanley DA, Zanchetta JR, et al. Differing effects of denosumab and alendronate on cortical and trabecular bone. *Bone* (2014) 59:173–9. doi: 10.1016/j.bone.2013.11.016
35. Zebaze R, Seeman E. Cortical bone: a challenging geography. *J Bone Miner Res.* (2015) 30:24–9. doi: 10.1002/jbmr.2419
36. Smith SY, Recker RR, Hannan M, Muller R, Bauss F. Intermittent intravenous administration of the bisphosphonate ibandronate prevents bone loss and maintains bone strength and quality in ovariectomized cynomolgus monkeys. *Bone* (2003) 32:45–55. doi: 10.1016/S8756-3282(02)00923-7
37. Allen MR, Turek JJ, Phipps RJ, Burr DB. Greater magnitude of turnover suppression occurs earlier after treatment initiation with risedronate than alendronate. *Bone* (2011) 49:128–32. doi: 10.1016/j.bone.2010.07.011
38. Silverman SL, Watts NB, Delmas PD, Lange JL, Lindsay R. Effectiveness of bisphosphonates on nonvertebral and hip fractures in the first year of therapy: the risedronate and alendronate (REAL) cohort study. *Osteoporos Int*. (2007) 18:25–34. doi: 10.1007/s00198-006-0274-z
39. Black DM, Delmas PD, Eastell R, Reid IR, Boonen S, Cauley JA, et al. Once-yearly zoledronic acid for treatment of postmenopausal osteoporosis. *N Engl J Med.* (2007) 356:1809–22. doi: 10.1056/NEJMoa067312
40. Lyles KW, Colon-Emeric CS, Magaziner JS, Adachi JD, Pieper CF, Mautalen C, et al. Zoledronic acid and clinical fractures and mortality after hip fracture. *N Engl J Med*. (2007) 357:1799–809. doi: 10.1056/NEJMoa074941
41. Colon-Emeric CS, Mesenbrink P, Lyles KW, Pieper CF, Boonen S, Delmas P, et al. Potential mediators of the mortality reduction with zoledronic acid after hip fracture. *J Bone Miner Res.* (2010) 25:91–7. doi: 10.1359/jbmr.090704
42. Black DM, Reid IR, Boonen S, Bucci-Rechtweg C, Cauley JA, Cosman F, et al. The effect of 3 versus 6 years of zoledronic acid treatment of osteoporosis: a randomized extension to the HORIZON-Pivotal Fracture Trial (PFT). *J Bone Miner Res*. (2012) 27:243–54. doi: 10.1002/jbmr.1494
43. Black DM, Reid IR, Cauley JA, Cosman F, Leung PC, Lakatos P, et al. The effect of 6 versus 9 years of zoledronic acid treatment in osteoporosis: a randomized second extension to the HORIZON-Pivotal Fracture Trial (PFT). *J Bone Miner Res.* (2015) 30:934–44. doi: 10.1002/jbmr.2442
44. Reid IR, Black DM, Eastell R, Bucci-Rechtweg C, Su G, Hue TF, et al. Reduction in the risk of clinical fractures after a single dose of zoledronic Acid 5 milligrams. *J Clin Endocrinol Metab.* (2013) 98:557–63. doi: 10.1210/jc.2012-2868
45. Grey A, Bolland MJ, Horne A, Mihov B, Gamble G, Reid IR. Duration of antiresorptive activity of zoledronate in postmenopausal women with osteopenia: a randomized, controlled multidose trial. *CMAJ* (2017) 189:E1130–6. doi: 10.1503/cmaj.161207
46. Grey A, Bolland M, Mihov B, Wong S, Horne A, Gamble G, et al. Duration of antiresorptive effects of low-dose zoledronate in osteopenic postmenopausal women: a randomized, placebo-controlled trial. *J Bone Miner Res*. (2014) 29:166–72. doi: 10.1002/jbmr.2009
47. Reid I, Horne A, Mihov B, Stewart A, Garratt L, Bolland M, et al. Abstracts of the ECTS Congress 2018 - P068 Zoledronate every 18 months for 6 years in osteopenic postmenopausal women reduces non-vertebral fractures and height loss. *Calcif Tissue Int.* (2018) 102:S1–159. doi: 10.1007/s00223-018-0418-0
48. Cummings SR, San Martin J, McClung MR, Siris ES, Eastell R, Reid IR, et al. Denosumab for prevention of fractures in postmenopausal women with osteoporosis. *N Engl J Med.* (2009) 361:756–65. doi: 10.1056/NEJMoa0809493
49. Reid IR, Miller PD, Brown JP, Kendler DL, Fahrleitner-Pammer A, Valter I, et al. Effects of denosumab on bone histomorphometry: the FREEDOM and STAND studies. *J Bone Miner Res.* (2010) 25:2256–65. doi: 10.1002/jbmr.149
50. Miller PD, Pannacciulli N, Brown JP, Czerwinski E, Nedergaard BS, Bolognese MA, et al. Denosumab or zoledronic acid in postmenopausal women with osteoporosis previously treated with oral bisphosphonates. *J Clin Endocrinol Metab.* (2016) 101:3163–70. doi: 10.1210/jc.2016-1801
51. Brown JP, Roux C, Ho PR, Bolognese MA, Hall J, Bone HG, et al. Denosumab significantly increases bone mineral density and reduces bone turnover compared with monthly oral ibandronate and risedronate in postmenopausal women who remained at higher risk for fracture despite previous suboptimal treatment with an oral bisphosphonate. *Osteoporos Int.* (2014) 25:1953–61. doi: 10.1007/s00198-014-2692-7
52. Roux C, Hofbauer LC, Ho PR, Wark JD, Zillikens MC, Fahrleitner-Pammer A, et al. Denosumab compared with risedronate in postmenopausal women suboptimally adherent to alendronate therapy: efficacy and safety results from a randomized open-label study. *Bone* (2014) 58:48–54. doi: 10.1016/j.bone.2013.10.006
53. Bone HG, Wagman RB, Brandi ML, Brown JP, Chapurlat R, Cummings SR, et al. 10 years of denosumab treatment in postmenopausal women with osteoporosis: results from the phase 3 randomised FREEDOM trial and open-label extension. *Lancet Diabetes Endocrinol.* (2017) 5:513–23. doi: 10.1016/S2213-8587(17)30138-9
54. Kostenuik PJ, Smith SY, Jolette J, Schroeder J, Pyrah I, Ominsky MS. Decreased bone remodeling and porosity are associated with improved bone strength in ovariectomized cynomolgus monkeys treated with denosumab, a fully human RANKL antibody. *Bone* (2011) 49:151–61. doi: 10.1016/j.bone.2011.03.769
55. Ominsky MS, Libanati C, Niu QT, Boyce RW, Kostenuik PJ, Wagman RB, et al. Sustained modeling-based bone formation during adulthood in cynomolgus monkeys may contribute to continuous BMD gains with denosumab. *J Bone Miner Res.* (2015) 30:1280–9. doi: 10.1002/jbmr.2480
56. Dempster DW, Zhou H, Recker RR, Brown JP, Recknor CP, Lewiecki EM, et al. Remodeling- and modeling-based bone formation with teriparatide versus denosumab: a longitudinal analysis from baseline to 3 months in the AVA study. *J Bone Miner Res.* (2018) 33:298–306. doi: 10.1002/jbmr.3309
57. Miller PD, Wagman RB, Peacock M, Lewiecki EM, Bolognese MA, Weinstein RL, et al. Effect of denosumab on bone mineral density and biochemical markers of bone turnover: six-year results of a phase 2 clinical trial. *J Clin Endocrinol Metab.* (2011) 96:394–402. doi: 10.1210/jc.2010-1805
58. Bone HG, Bolognese MA, Yuen CK, Kendler DL, Miller PD, Yang YC, et al. Effects of denosumab treatment and discontinuation on bone mineral density and bone turnover markers in postmenopausal women with low bone mass. *J Clin Endocrinol Metab.* (2011) 96:972–80. doi: 10.1210/jc.2010-1502
59. Anastasilakis AD, Makras P. Multiple clinical vertebral fractures following denosumab discontinuation. *Osteoporos Int.* (2016) 27:1929–30. doi: 10.1007/s00198-015-3459-5
60. Aubry-Rozier B, Gonzalez-Rodriguez E, Stoll D, Lamy O. Severe spontaneous vertebral fractures after denosumab discontinuation: three case reports. *Osteoporos Int.* (2016) 27:1923–5. doi: 10.1007/s00198-015-3380-y
61. Popp AW, Zysset PK, Lippuner K. Rebound-associated vertebral fractures after discontinuation of denosumab-from clinic and biomechanics. *Osteoporos Int.* (2016) 27:1917–21. doi: 10.1007/s00198-015-3458-6
62. Lamy O, Gonzalez-Rodriguez E, Stoll D, Hans D, Aubry-Rozier B. Severe rebound-associated vertebral fractures after denosumab discontinuation: 9 clinical cases report. *J Clin Endocrinol Metab.* (2017) 102:354–8. doi: 10.1210/jc.2017-00096
63. Polyzos SA, Terpos E. Clinical vertebral fractures following denosumab discontinuation. *Endocrine* (2016) 54:271–2. doi: 10.1007/s12020-016-1030-6
64. Anastasilakis AD, Polyzos SA, Makras P, Aubry-Rozier B, Kaouri S, Lamy O. Clinical features of 24 patients with rebound-associated vertebral fractures after denosumab discontinuation: systematic review and additional cases. *J Bone Miner Res.* (2017) 32:1291–6. doi: 10.1002/jbmr.3110
65. Cummings SR, Ferrari S, Eastell R, Gilchrist N, Jensen JB, McClung M, et al. Vertebral fractures after discontinuation of denosumab: a *post hoc* analysis of the randomized placebo-controlled freedom trial and its extension. *J Bone Miner Res.* (2018) 33:190–8. doi: 10.1002/jbmr.3337
66. Parfitt AM. Morphological basis of bone mineral measurements: transient and steady state effects of treatment in osteoporosis. *Miner Electrolyte Metab.* (1980) 4:273–87.

67. Dempster DW. Exploiting and bypassing the bone remodeling cycle to optimize the treatment of osteoporosis. *J Bone Miner Res.* (1997) 12:1152–4. doi: 10.1359/jbmr.1997.12.8.1152
68. Einhorn TA. Bone strength: the bottom line. *Calcif Tissue Int.* (1992) 51:333–9. doi: 10.1007/BF00316875
69. Miller PD, Bolognese MA, Lewiecki EM, McClung MR, Ding B, Austin M, et al. Effect of denosumab on bone density and turnover in postmenopausal women with low bone mass after long-term continued, discontinued, and restarting of therapy: a randomized blinded phase 2 clinical trial. *Bone* (2008) 43:222–9. doi: 10.1016/j.bone.2008.04.007
70. Seeman E, Martin TJ. Co-administration of antiresorptive and anabolic agents: a missed opportunity. *J Bone Miner Res.* (2015) 30:753–64. doi: 10.1002/jbmr.2496
71. Ettinger B, Black DM, Mitlak BH, Knickerbocker RK, Nickelsen T, Genant HK, et al. Reduction of vertebral fracture risk in postmenopausal women with osteoporosis treated with raloxifene: results from a 3-year randomized clinical trial. Multiple Outcomes of Raloxifene Evaluation (MORE) Investigators. *JAMA* (1999) 282:637–45. doi: 10.1001/jama.282.7.637
72. Kaufman JM, Palacios S, Silverman S, Sutradhar S, Chines A. An evaluation of the Fracture Risk Assessment Tool (FRAX(R)) as an indicator of treatment efficacy: the effects of bazedoxifene and raloxifene on vertebral, nonvertebral, and all clinical fractures as a function of baseline fracture risk assessed by FRAX(R). *Osteoporos Int.* (2013) 24:2561–9. doi: 10.1007/s00198-013-2341-6
73. Allen MR, Iwata K, Sato M, Burr DB. Raloxifene enhances vertebral mechanical properties independent of bone density. *Bone* (2006) 39:1130–5. doi: 10.1016/j.bone.2006.05.007
74. Allen MR, Hogan HA, Hobbs WA, Koivuniemi AS, Koivuniemi MC, Burr DB. Raloxifene enhances material-level mechanical properties of femoral cortical and trabecular bone. *Endocrinology* (2007) 148:3908–13. doi: 10.1210/en.2007-0275
75. Gallant MA, Brown DM, Hammond M, Wallace JM, Du J, Deymier-Black AC, et al. Bone cell-independent benefits of raloxifene on the skeleton: a novel mechanism for improving bone material properties. *Bone* (2014) 61:191–200. doi: 10.1016/j.bone.2014.01.009
76. Ramchand SK, Chiang CY, Zebaze RM, Seeman E. Recurrence of bilateral atypical femoral fractures associated with the sequential use of teriparatide and denosumab: a case report. *Osteoporos Int*. (2016) 27:821–5. doi: 10.1007/s00198-015-3354-0
77. Chen P, Miller PD, Recker R, Resch H, Rana A, Pavo I, et al. Increases in BMD correlate with improvements in bone microarchitecture with teriparatide treatment in postmenopausal women with osteoporosis. *J Bone Miner Res.* (2007) 22:1173–80. doi: 10.1359/jbmr.070413
78. Bahar H, Gallacher K, Downall J, Nelson CA, Shomali M, Hattersley G. Six weeks of daily abaloparatide treatment increased vertebral and femoral bone mineral density, microarchitecture and strength in ovariectomized osteopenic rats. *Calcif Tissue Int.* (2016) 99:489–99. doi: 10.1007/s00223-016-0171-1
79. Bilezikian JP, Hattersley G, Fitzpatrick LA, Harris AG, Shevroja E, Banks K, et al. Abaloparatide-SC improves trabecular microarchitecture as assessed by trabecular bone score (TBS): a 24-week randomized clinical trial. *Osteoporos Int.* (2018) 29:323–8. doi: 10.1007/s00198-017-4304-9
80. Tsai JN, Uihlein AV, Burnett-Bowie SM, Neer RM, Derrico NP, Lee H, et al. Effects of two years of teriparatide, denosumab, or both on bone microarchitecture and strength (DATA-HRpQCT study). *J Clin Endocrinol Metab.* (2016) 101:2023–30. doi: 10.1210/jc.2016-1160
81. Macdonald HM, Nishiyama KK, Hanley DA, Boyd SK. Changes in trabecular and cortical bone microarchitecture at peripheral sites associated with 18 months of teriparatide therapy in postmenopausal women with osteoporosis. *Osteoporos Int.* (2011) 22:357–62. doi: 10.1007/s00198-010-1226-1
82. Leder BZ, O'Dea LS, Zanchetta JR, Kumar P, Banks K, McKay K, et al. Effects of abaloparatide, a human parathyroid hormone-related peptide analog, on bone mineral density in postmenopausal women with osteoporosis. *J Clin Endocrinol Metab.* (2015) 100:697–706. doi: 10.1210/jc.2014-3718
83. Martin TJ, Seeman E. Abaloparatide is an anabolic, but does it spare resorption? *J Bone Miner Res.* (2017) 32:11–6. doi: 10.1002/jbmr.3042
84. Neer RM, Arnaud CD, Zanchetta JR, Prince R, Gaich GA, Reginster JY, et al. Effect of parathyroid hormone (1-34) on fractures and bone mineral density in postmenopausal women with osteoporosis. *N Engl J Med.* (2001) 344:1434–41. doi: 10.1056/NEJM200105103441904
85. Seeman E, Nguyen TV. Bone remodeling markers: so easy to measure, so difficult to interpret. *Osteoporos Int.* (2016) 27:33–5. doi: 10.1007/s00198-015-3374-9
86. Cosman F, Crittenden DB, Adachi JD, Binkley N, Czerwinski E, Ferrari S, et al. Romosozumab treatment in postmenopausal women with osteoporosis. *N Engl J Med.* (2016) 375:1532–43. doi: 10.1056/NEJMoa1607948
87. Saag KG, Petersen J, Brandi ML, Karaplis AC, Lorentzon M, Thomas T, et al. Romosozumab or alendronate for fracture prevention in women with osteoporosis. *N Engl J Med.* (2017) 377:1417–27. doi: 10.1056/NEJMoa1708322
88. Cosman F, Crittenden DB, Ferrari S, Khan A, Lane NE, Lippuner K, et al. FRAME Study: the foundation effect of building bone with 1 year of romosozumab leads to continued lower fracture risk after transition to denosumab. *J Bone Miner Res.* (2018) 33:1219–26. doi: 10.1002/jbmr.3427
89. McClung MR, Brown JP, Diez-Perez A, Resch H, Caminis J, Meisner P, et al. Effects of 24 months of treatment with romosozumab followed by 12 months of denosumab or placebo in postmenopausal women with low bone mineral density: a randomized, double-blind, phase 2, parallel group study. *J Bone Miner Res.* (2018) 33:1397–406. doi: 10.1002/jbmr.3452
90. Geusens P, Marin F, Kendler DL, Russo LA, Zerbini CA, Minisola S, et al. Effects of teriparatide compared with risedronate on the risk of fractures in subgroups of postmenopausal women with severe osteoporosis: the VERO trial. *J Bone Miner Res.* (2018) 33:783–94. doi: 10.1002/jbmr.3384
91. Black DM, Greenspan SL, Ensrud KE, Palermo L, McGowan JA, Lang TF, et al. The effects of parathyroid hormone and alendronate alone or in combination in postmenopausal osteoporosis. *N Engl J Med.* (2003) 349:1207–15. doi: 10.1056/NEJMoa031975
92. Finkelstein JS, Leder BZ, Burnett SM, Wyland JJ, Lee H, de la Paz AV, et al. Effects of teriparatide, alendronate, or both on bone turnover in osteoporotic men. *J Clin Endocrinol Metab.* (2006) 91:2882–7. doi: 10.1210/jc.2006-0190
93. Finkelstein JS, Hayes A, Hunzelman JL, Wyland JJ, Lee H, Neer RM. The effects of parathyroid hormone, alendronate, or both in men with osteoporosis. *N Engl J Med.* (2003) 349:1216–26. doi: 10.1056/NEJMoa035725
94. Khosla S. Parathyroid hormone plus alendronate–a combination that does not add up. *N Engl J Med*. (2003) 349:1277–9. doi: 10.1056/NEJMe038143
95. Cosman F, Eriksen EF, Recknor C, Miller PD, Guanabens N, Kasperk C, et al. Effects of intravenous zoledronic acid plus subcutaneous teriparatide [rhPTH(1-34)] in postmenopausal osteoporosis. *J Bone Miner Res.* (2011) 26:503–11. doi: 10.1002/jbmr.238
96. Tsai JN, Uihlein AV, Lee H, Kumbhani R, Siwila-Sackman E, McKay EA, et al. Teriparatide and denosumab, alone or combined, in women with postmenopausal osteoporosis: the DATA study randomised trial. *Lancet* (2013) 382:50–6. doi: 10.1016/S0140-6736(13)60856-9
97. Kostenuik PJ, Capparelli C, Morony S, Adamu S, Shimamoto G, Shen V, et al. OPG and PTH-(1-34) have additive effects on bone density and mechanical strength in osteopenic ovariectomized rats. *Endocrinology* (2001) 142:4295–304. doi: 10.1210/endo.142.10.8437
98. Samadfam R, Xia Q, Goltzman D. Co-treatment of PTH with osteoprotegerin or alendronate increases its anabolic effect on the skeleton of oophorectomized mice. *J Bone Miner Res.* (2007) 22:55–63. doi: 10.1359/jbmr.060915
99. Zebaze R, Ghasem-Zadeh A, Mbala A, Seeman E. A new method of segmentation of compact-appearing, transitional and trabecular compartments and quantification of cortical porosity from high resolution peripheral quantitative computed tomographic images. *Bone* (2013) 54:8–20. doi: 10.1016/j.bone.2013.01.007

100. Leder BZ, Tsai JN, Uihlein AV, Wallace PM, Lee H, Neer RM, et al. Denosumab and teriparatide transitions in postmenopausal osteoporosis (the DATA-Switch study): extension of a randomised controlled trial. *Lancet* (2015) 386:1147–55. doi: 10.1016/S0140-6736(15)61120-5
101. Bone HG, Cosman F, Miller PD, Williams GC, Hattersley G, Hu MY, et al. ACTIVExtend: 24 months of alendronate after 18 months of abaloparatide or placebo for postmenopausal osteoporosis. *J Clin Endocrinol Metab.* (2018) 103:2949–57. doi: 10.1210/jc.2018-00163
102. Langdahl BL, Libanati C, Crittenden DB, Bolognese MA, Brown JP, Daizadeh NS, et al. Romosozumab (sclerostin monoclonal antibody) versus teriparatide in postmenopausal women with osteoporosis transitioning from oral bisphosphonate therapy: a randomised, open-label, phase 3 trial. *Lancet* (2017) 390:1585–94. doi: 10.1016/S0140-6736(17)31613-6

Conflict of Interest Statement: ES has received research support and lecture fees from Amgen, Allergan, and Eli Lilly; he is a director of the board and shareholder of StraxCorp.

The remaining author declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Endocrinology

REVIEW
published: 10 September 2018
doi: 10.3389/fendo.2018.00530

Weight-Independent Mechanisms of Glucose Control After Roux-en-Y Gastric Bypass

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Roux-en-Y gastric bypass results in large and sustained weight loss and resolution of type 2 diabetes in 60% of cases at 1–2 years. In addition to calorie restriction and weight loss, various gastro-intestinal mediated mechanisms, independent of weight loss, also contribute to glucose control. The anatomical re-arrangement of the small intestine after gastric bypass results in accelerated nutrient transit, enhances the release of post-prandial gut hormones incretins and of insulin, alters the metabolism and the entero-hepatic cycle of bile acids, modifies intestinal glucose uptake and metabolism, and alters the composition and function of the microbiome. The amelioration of beta cell function after gastric bypass in individuals with type 2 diabetes requires enteric stimulation. However, beta cell function in response to intravenous glucose stimulus remains severely impaired, even in individuals in full clinical diabetes remission. The permanent impairment of the beta cell may explain diabetes relapse years after surgery.

Keywords: gastric bypass surgery, beta cell function, glucagon-like peptide 1 (GLP-1), bile acids, microbiome, sodium glucose transporter 1 (SGLt1), type 2 diabetes

OPEN ACCESS

Edited by:
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Specialty section:
This article was submitted to Diabetes, a section of the journal Frontiers in Endocrinology

Received: *21 June 2018*
Accepted: *22 August 2018*
Published: *10 September 2018*

Citation:
Laferrère B and Pattou F (2018) Weight-Independent Mechanisms of Glucose Control After Roux-en-Y Gastric Bypass. Front. Endocrinol. 9:530. doi: 10.3389/fendo.2018.00530

The prevalence of severe obesity, defines as body mass index (BMI) above 40 kg/m^2, is increasing. It is affecting women more than men, and African American women (16.9%) more than Caucasian (9.3%), or Hispanic (8.9%) women (1). The number of bariatric surgeries performed yearly in the US has increased only minimally in the last few years and was estimated at 216,000 in 2016. Hence, only a small percentage of people meeting criteria for bariatric surgery, the most efficient and durable form of weight loss, actually benefit from it. Roux-en-Y gastric bypass (RYGB) was the dominant type of surgery performed in the US up to 2011. Vertical sleeve gastrectomy (VSG) is now the most performed surgery and represented 58% of all bariatric procedures in 2016 (2, 3). However, RYGB is the surgical model that has been studied the most to investigate gut mechanisms, independent of weight loss, that may contribute to post-operative glucose control. In addition, there are more long-term data on clinical remission of type 2 diabetes (T2D) after RYGB. Hence, this review will be more RYGB-centric.

The remarkable effect of bariatric surgery on T2D has generated considerable attention from the surgical, as well as the research community, in the last 12 years. Non-randomized observational studies have shown that bariatric surgery results not only in diabetes remission, but also decreases micro- and macro-vascular complications, cardiovascular disease risk and events, non-alcoholic steato-hepatitis (NASH) (4) and cancers (5–12). Cohort studies have shown increased longevity after bariatric surgery (10, 13). The effect of bariatric surgery on T2D remission is of particular interest. Both observational studies (14) and randomized controlled trials (RCTs) (15) show rates

of remission varying from 15 to 100%, depending, in part, of the definition of diabetes remission (16, 17). Determinants of diabetes remission have been reviewed in meta-analysis (18) and the IDF-ADA Translational symposium (19). Pre-intervention β-cell function, use of insulin, known duration of T2D, HbA1C, age, surgery type, weight loss amount, genomics biomarkers, and the duration of follow-up after surgery remission, are all predictors of remission (20–25). The duration of follow up is certainly one of the key variables. In the Swedish Obesity Study (SOS), the rate of T2D remission decreases from 72%, at 2 years, to 36% at 10 years (5). Adams et al. show a decrease in the rate of remission from 75% at 2 years to 51% at 21 years in 84 patients with little attrition (90% follow up) (12). Arterburn et al. using electronic medical records, studied a large cohort of 4, 434 individuals with uncontrolled diabetes prior to surgery; of the 68.2% patients who initially remit their diabetes at 5 years, one third experience diabetes relapse 5–8 years after RYGB surgery (11). Overall, clinical parameters pre-intervention, surgery type, and post-surgery weight loss amount predict about 70% of remission rate. Predictive scores such as DiaRem (26) and ABCD (27) have been developed.

Pooling data from observational studies (14) and RTCs (15, 28–30), the rate of T2D remission is about 60% 2 years after RYGB. The mechanism by which RYGB results in this remarkable high rate of diabetes remission is not fully elucidated. The key question is whether diabetes remission is entirely weight loss dependent or not. If it is weight loss driven, then research should focus on the mechanisms, likely centrally mediated, by which patients eat less, lose about 30% of their total body weight and are able to keep the weight off, all goals unmatched with diet and exercise alone (31), or with pharmacotherapy (32). If some weight loss independent effects are at play in diabetes remission, they are likely gut-mediated. However, although RYGB results in many alterations of gut-mediated endocrine mechanisms, some of which play a role in post-prandial glucose control, their role in diabetes remission has not been fully demonstrated. The understanding of these mechanisms is crucial as it may help identify novel targets for the treatment of T2DM.

Calorie restriction with large (25–30%) and sustained (33–35) weight loss, are clearly important factors in the remission of diabetes after RYGB. They remove the chronic insult on the β-cell resulting from nutrient excess, i.e., glucose and lipid toxicity (36, 37), decrease inflammation (38–41), decrease fat mass and ectopic fat depots (42–44), and improve insulin sensitivity (29), all important modulators of metabolism. However, the benefit of the surgery on glucose control is apparent very rapidly, within days after RYGB surgery, prior to large amount of weight loss (45). In addition, the clinical observations that surgeries that alters the gastro intestinal track, such as RYGB, VSG, or biliopancreatic diversion (BPD), result in greater and more rapid diabetes remission than purely restrictive surgeries such as adjusted gastric banding (AGB), have prompted investigations of gastro-intestinal mediated mechanisms of glucose improvement. The regulation of blood glucose is complex and necessitates cross talk between the central nervous system, the endocrine pancreas, the liver, and the intestine (46). The intestine is the first line of contact with the environment, i.e., nutrient calorie load and composition, and plays a central role in post-prandial glucose control. The small intestine signals other organs via nutrient sensing, glucose transport, satiety and incretin hormones, bile acids metabolism, and the microbiome. Many of these intestinal pathways, reviewed below, contribute to glucose control, independent of weight loss, after RYGB (47).

The gut endocrine system regulates satiety and insulin secretion, both key factors in body weight and glucose control (48). Bariatric surgery alters the gut endocrine system in a favorable way to decrease appetite and improved glucose metabolism (49). After RYGB, ingested food empties rapidly from the small gastric pouch into the alimentary limb, and mix with the biliary and pancreatic exocrine secretion in the common limb (**Figure 1**). The rapid emptying of the reduced gastric pouch results in accelerated nutrient transit (50, 51) and alters the post-prandial gastro-intestinal hormonal chain of event. It enhances the release of satiety hormone such as cholecystokinin (CCK) (52, 53), peptide yy (PYY) (54), glucagon like peptide 1 (GLP-1) (55–57) and oxyntomodulin (58). A few clinical studies, using octreotide, demonstrated the role of gut peptides in increased satiety and decreased food reward after RYGB surgery (59, 60) (**Figure 2**). The release of the incretins GLP-1 (51, 61, 62), and of glucose dependent insulin peptide (GIP), in some (63–65) but not all (66) studies, is also enhanced by the accelerated transit; this improves the incretin effect on insulin secretion (55–57, 63, 67, 68) and lowers post-prandial glycemia (20). This exaggerated post-prandial release of GLP-1 occurs rapidly after the surgery (69), is independent of weight loss (51, 70) and can be entirely abolished by administration of the meal and/or glucose in the gastric remnant via a gastrostomy (67, 68). Although mean glucose levels improved after RYGB, the pattern of glucose levels during meals shows greater variability with earlier and higher glucose peaks, and lower post-prandial glycemia, at times in the hypoglycemic range, even if often asymptomatic. A small percentage of individuals experience debilitating neuroglycopenia after RYGB (71, 72), in relation to altered counter regulatory hormone response (73), increased insulin sensitivity (29) and decreased insulin clearance (74). The infusion of the GLP-1 receptor blocker exendin 9–39 prevents the large post-prandial insulin secretion and corrects the post-prandial neuroglycopenia; this illustrates the effect of endogenous GLP-1 on post-prandial glycemic control (75). The effect of exendin 9–39 on post-prandial glucose in individuals with normoglycemia, however, is more modest. Although exendin 9–39 can blunt post-prandial insulin secretion (76, 77), it results only in modest worsening of the glycemia (77–79).

The role of enhanced endogenous GLP-1 on the control of insulin secretion in response to oral glucose after RYGB is well demonstrated; its long-term implication on diabetes remission however remains elusive (79). Beta-cell function, assessed in response to intravenous glucose stimulus, improves only minimally and remains impaired in individuals in clinical diabetes remission and sustained weight loss, up to 3 years after RYGB (76). The reversal of post-prandial hyperinsulinemic hypoglycemia by the administration of food directly via gastrostomy in the remnant stomach, rather than

per os, highlights the absence of permanent amelioration of the pancreatic endocrine function years after RYGB (80, 81). Therefore the increased meal-related insulin secretion after RYGB depends on enteric stimulation rather than on improved beta cells responsiveness to glucose (82) or to incretin stimuli (83). The persistent defect of beta cell function, overcome during meals, may explain, in part, the potential for diabetes relapse years after RYGB, in older patients who eat a less restrictive diet and regain some weight.

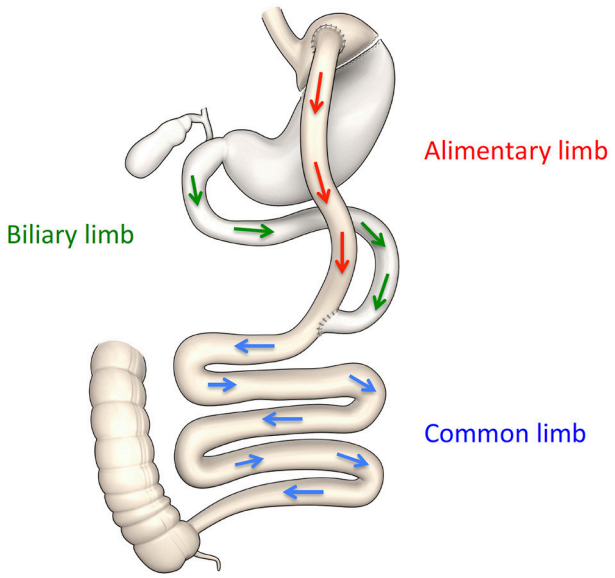

FIGURE 1 | Schematic representation of anatomical changes after RYGB.

In addition to the intestinal endocrine function, other aspects of the gastro intestinal track play an important role in glucose control (84). The remodeling and reprogramming of the gastrointestinal track modifies intestinal glucose metabolism and glucose absorption and contributes to whole body metabolism after RYGB (85, 86). Interestingly this may not be the case after VSG (87) (**Table 1**). Troy et al. show increased intestinal gluconeogenesis after RYGB in mice, and effect abolished in GLUT-2 knockout mice (88). Others have shown an increased expression of genes involved in intestinal glucose transport and gluconeogenesis, in a rat bypass model (89–91), in association with decreased insulin resistance (92). Saeidi et al. demonstrated in rats that the active remodeling of the gastrointestinal tract increased intestinal cholesterol and glucose utilization, and contributed significantly to the improvement of whole body glucose metabolism after RYGB (93). Intestinal glucose transport is one of the key determinants of post-prandial glucose. The sodium-glucose transporter 1 (SGLT1) is responsible for the sodium-dependent, active uptake of glucose across the apical membrane of the small intestine (94). The expression of SGLT-1 increases after duodenal jenunal bypass (DJB) in rats (90) and after RYGB in humans (95). Baud et al. demonstrated, in a well characterized RYGB model in mini pigs (96), that the intestinal uptake of ingested glucose is blunted in the bile-deprived alimentary limb (**Figure 1**). Glucose absorption can be restored

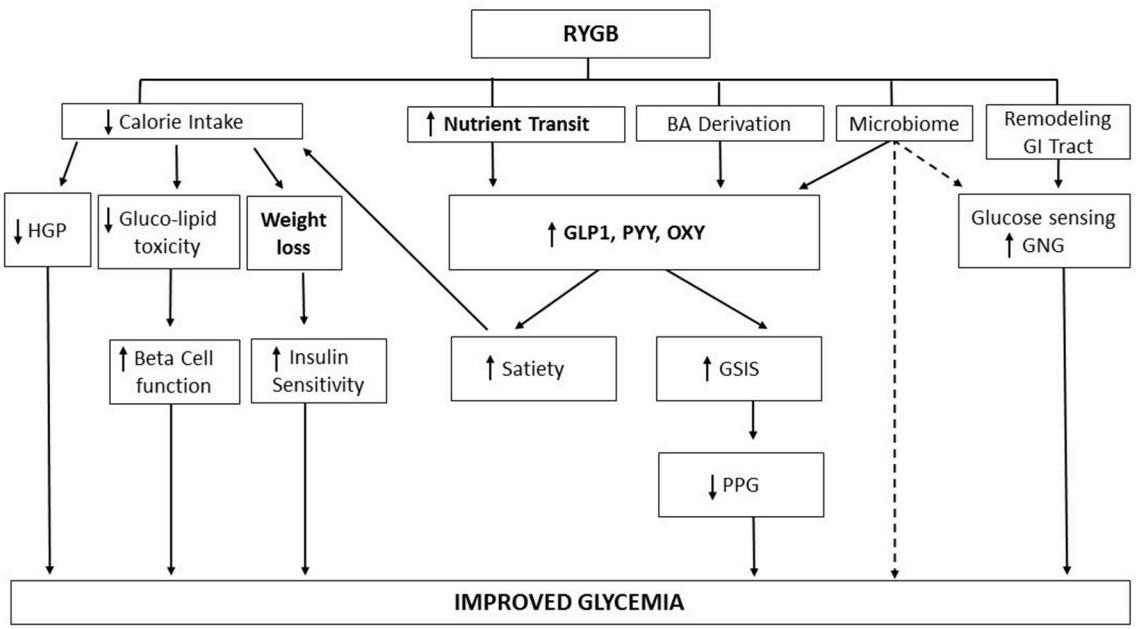

FIGURE 2 | Mechanistic model of improved glycemia after RYGB. RYGB improves glucose metabolism via weight loss, and via weight-independent mechanisms, including stimulation of gut peptides, alteration of bile acids enterohepatic cycle, remodeling of the gastrointestinal track, and alteration of the microbiome. Solid lines: evidence based mechanisms; dashed lines: possible mechanisms. RYGB, Roux-en-Y gastric bypass surgery; HGP, hepatic glucose production; BA derivation, bile acids derivation; GI, gastro intestinal; GNG, gluconeogenesis; GLP-1, glucagon-like-peptide 1; PYY, peptide YY; OXY, oxyntomodulin; GSIS, glucose-stimulated insulin secretion; PPG, post-prandial glucose; ↑: increase; ↓: decrease.

TABLE 1 | Mechanisms of glycemic control after RYGB, VSG, and AGB.

	RYGB	VSG	AGB
Weight loss	+++	++	+
Accelerated nutrient transit	+	+	↔
↑ GLP-1, PYY, OXY	++	+/–	↔
Bile acid derivation	+	–	–
Circulating bile acid pool	↑	↑/↔	↓/↔
Remodeling GI tract	+	–	–
Microbiome	+	+	+

by the addition of either bile or sodium to the glucose meal, and is blocked with phlorizin. These studies provide direct evidence of a novel mechanism, via the reduction of active glucose-sodium transport, of decreased post-prandial glycemia after RYGB (96). More research is needed to understand the role of altered glucose transport, intestinal neoglucogenesis, and re-programming of the intestine on short and long-term glucose control after RYGB in humans, and its contribution to diabetes remission.

The change of bile acids metabolism after RYGB has been studied as potential mechanism of improved glucose control after bariatric surgery (97). Bile acids are synthesized by the liver, stored in the gallbladder and released in the duodenum in response to ingestion of nutrient. In non-operated individuals, after food intake, the chime, bile acids, and pancreatic exocrine secretions mix to enhance intestinal lipid digestion and absorption. After RYGB, in the absence of gastric fundus and pylorus, the ingested food empties rapidly from the gastric pouch; it then mix with bile acids and pancreatic secretions only in the common limb (**Figure 1**), precluding any duodenal absorption of nutrient (96). In addition to their role on lipid absorption, bile acids act as signaling molecules to regulate metabolism and inflammation (98). Bile acids are ligands of the nuclear receptor farnesoid X receptor (FXR) and the Takeda-G-protein- membrane receptor-5 TGR5 (99, 100), both receptors present in several organs that regulate metabolism. The role of the intestinal bile acids receptors as key regulators of glucose homeostasis was reviewed recently (101). The circulating bile acids concentrations (total molar sum) increase after RYGB in the fasted (102–106) and postprandial (77, 107–113) states in humans, as well as in rats and mini-pigs (114). The composition of the bile acids pool is also altered, and could contribute to the improvement of metabolism (106, 113). The rise of circulating bile acids after the surgery is delayed, occurs only a few months after the surgery and seems to be sustained overtime (106, 111, 113, 115). The underlying mechanisms of the elevated concentrations of circulating bile acids are unknown. Contrary to RYGB, calorie restriction and weight loss, either with (109) or without (112) AGB, decrease circulating bile acids concentrations (**Table 1**). Therefore, the rise in circulating bile acids after RYGB is not weight loss dependent. Experimental bile diversion, similarly to ileal transposition (116), are associated with increased circulating bile acids, increased postprandial GLP-1, weight loss and improved glucose tolerance (117–119). Possible explanations for the increased systemic pool of bile acids after RYGB are: increased hepatic synthesis and/or intestinal reabsorption, decreased fecal excretion and/or hepatic uptake or change in the microbiota. The increase in the peripheral but not in the portal circulation indicate that increase in bile acids systemic concentration after RYGB can be explained, in part, by a decrease of hepatic recapture, as shown after RYGB in mini pig (120). Whether the elevated systemic concentration of bile acids after RYGB in humans (113) is accompanied by increased concentration of luminal bile acids is unknown. One study in rats, show no change in luminal bile acids metabolism after RYGB and VSG (98, 121). Intestinal FXR is an important modulator of whole body metabolism. Pharmacological intestinal-specific activation of FXR reduces insulin resistance and stimulates adipose tissue browning, reduces lipids, inflammation, and atherosclerosis, while intestinal FXR inhibition favors non-alcoholic hepatic steatosis (NASH) (122). The effect of VSG on the improvement of glucose tolerance is reduced in FXR knock out (KO) mice (123). Bile acids, via activation of TGR5 signaling on the L cells, stimulate GLP-1 and participate in the control of glucose homeostasis (124–126). TGR5 seems to be required for the anti-hyperglycemic effect of VSG, as shown by two independent reports of VSG in TGR-5 KO mice (127, 128).

In all, results from clinical and animal studies suggest an important role of altered bile acids pool, composition, re-routing and signaling that may contribute to the metabolic effects of RYGB or VSG (**Table 1**). The elegant experiments of bile acids derivation and FXR and TGR5 KO propose a role for luminal bile acids in the improvement of metabolism after bariatric surgery. The clinical translation of these data, however, is still elusive. The temporal dissociation between the immediate rise of GLP-1 and the delayed increase in circulating bile acids, makes it less likely that the two processes are linked, at least in the early months after RYGB. Important information on intraluminal bile acids concentration after RYGB (or VSG) in humans is lacking. The composition, and therefore the function of the bile acids differs amongst species and add to the difficulty of translational research in this field. Finally, the large variability of the circulating concentrations of bile acids in humans studies (115) point out to other mechanisms, perhaps diet and/or microbiome dependent, that may modulate their composition and function.

Specific composition of the gut microbiome associates with pathological conditions such as cardiovascular disease, and with certain phenotypes like obesity and insulin resistance (129, 130). The link between gut microbiota composition and metabolic status is established through transplantation studies in humans and animals (131). However, the mechanism by which the gut microbiome maintains health or contributes to diseases is unknown. The change in microbiota composition, diversity and function is proposed as mechanism of some of the metabolic alterations after bariatric surgery (132–138). Transplantation of gut microbiota from RYGB mice (139) or patients (140) to germ-free mice reduces weight, fat mass, and induces metabolic improvements. Together, these studies indicate a possible link between gut flora modifications and metabolic changes after RYGB. Proposed mechanisms involve changes in glucose transport and sensing, GLP-1, short-chain

fatty acids, lipogenesis, food intake, energy expenditure, adipose tissue metabolism, bile acids metabolism (141–146). One study however showed no link between alteration of the microbiome signature after RYGB and VSG and luminal metabolism of bile acids (121). The translational applicability of germ free mice experiments to humans is questionable. Human bariatric microbiota studies are often short term, lack controlled condition (diet, antibiotics and other drugs, metabolic status), favor description of composition rather than function of microbiome, and are based on feces, rather than luminal flora analysis. Future research will help identify whether the pre-surgery microbiome signature can be used to predict the metabolic response to the surgical intervention, and /or whether the change of microbiome composition and function can identify novel pathways of improved metabolism after various types of surgeries.

An important variable often overlooked in cross sectionals studies, is the change over time of many of the mechanisms described above. The accelerated nutrient transit time and stimulated GLP-1 release both occur immediately after RYGB and are sustained over time. However, the variance of the GLP-1 response increases between 1 month and 3 years post-surgery (147). We (113) and others (111) have demonstrated a temporal change of the pool of circulating bile acids after RYGB. Gut adaptation (hypertrophy, density of endocrine cells, glucose sensing, GNG) and the microbiome, are likely to undergo temporal transformation, in part, diet dependent. These data show the complexity of the gut physiology and adaptability, the difficulty of clinical studies, and the importance of longitudinal long-term studies for a better understanding of the contribution of the gut on post-prandial glycemia as well as diabetes remission.

In summary, RYGB results in T2DM remission as a result of large and sustained weight loss. RYGB also triggers weight independent gastro-intestinal mechanisms, including the stimulation of the incretins, the modulation of intestinal glucose transport and metabolism, the alteration of the entero-hepatic bile acids cycle, and change in the microbiome. These gut-related systems are inter-related as bile diversion impairs upper intestinal glucose uptake, nutrient malabsorption and bile acids can stimulate GLP-1, and the microbiome modulates many of these gastrointestinal targets. The mechanisms described above are likely to act in concert to contribute, with weight loss and calorie restriction, to glucose control after bariatric surgery (**Figure 2**). However, more clinical research needs to be done to understand the molecular mechanisms by which these different systems interact to improve glucose metabolism and to result in diabetes remission. The lack of normalization of beta cell function in response to IV glucose stimulus may be an important determinant of the future risk of diabetes relapse after RYGB surgery.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

ACKNOWLEDGMENTS

We thank Kapila Patel for her help with the formatting of references and Figures. BL and some of the work that she refers to in this review, were supported, in part, by grant by the National Institutes of Health (NIH) R01DK067561, P30DK26687-30, P30DK063608, and in part by the National Center for Advancing Translational Sciences, NIH, through Grant Number UL1TR001873. The content is solely the responsibility of the authors and does not necessarily represent the official views of the NIH. FP was supported by the Fédération Francophone de Recherche sur le Diabète (FFRD).

REFERENCES

1. Flegal KM, Kruszon-Moran D, Carroll MD, Fryar CD, Ogden CL. Trends in obesity among adults in the united states, 2005 to 2014. *JAMA* (2016) 315:2284–91. doi: 10.1001/jama.2016.6458
2. *Estimate of Bariatric Surgery Numbers, 2011-2016*. American Society for Metabolic and Bariatric Surgery (2016).
3. Abraham A, Ikramuddin S, Jahansouz C, Arafat F, Hevelone N, Leslie D. Trends in bariatric surgery: procedure selection, revisional surgeries, and readmissions. *Obes Surg.* (2016) 26:1371–7. doi: 10.1007/s11695-015-1974-2
4. Lassailly G, Caiazzo R, Buob D, Pigeyre M, Verkindt H, Labreuche J, et al. Bariatric surgery reduces features of nonalcoholic steatohepatitis in morbidly obese patients. *Gastroenterology* (2015) 149:379–88. doi: 10.1053/j.gastro.2015.04.014
5. Sjöström L, Lindroos AK, Peltonen M, Torgerson J, Bouchard C, Carlsson B, et al. Lifestyle, diabetes, and cardiovascular risk factors 10 years after bariatric surgery. *N Eng J Med.* (2004) 351:2683–93. doi: 10.1056/NEJMoa035622
6. Adams TD, Davidson LE, Litwin SE, Kolotkin RL, LaMonte MJ, Pendleton RC, et al. Health benefits of gastric bypass surgery after 6 years. *JAMA* (2012) 308:1122–31. doi: 10.1001/2012.jama.11164
7. Sjöström L, Narbro K, Sjöström CD, Karason K, Larsson B, Wedel H, et al. Effects of bariatric surgery on mortality in swedish obese subjects. *N Eng J Med.* (2007) 357:741–52. doi: 10.1056/NEJMoa066254
8. Sjöström L, Gummesson A, Sjöström CD, Narbro K, Peltonen M, Wedel H, et al. Effects of bariatric surgery on cancer incidence in obese patients in Sweden (Swedish Obese Subjects Study): a prospective, controlled intervention trial. *Lancet Oncol.* (2009) 10:653–62. doi: 10.1016/S1470-2045(09)70159-7
9. Sjöström L, Peltonen M, Jacobson P, Sjöström CD, Karason K, Wedel H, et al. Bariatric surgery and long-term cardiovascular events. *JAMA* (2012) 307:56–65. doi: 10.1001/jama.2011.1914
10. Adams TD, Gress RE, Smith SC, Halverson RC, Simper SC, Rosamond WD, et al. Long-term mortality after gastric bypass surgery. *N Eng J Med.* (2007) 357:753–61. doi: 10.1056/NEJMoa066603
11. Arterburn DE, Bogart A, Sherwood NE, Sidney S, Coleman KJ, Haneuse S, et al. A multisite study of long-term remission and relapse of type 2 diabetes mellitus following gastric bypass. *Obes Surg.* (2013) 23:93–102. doi: 10.1007/s11695-012-0802-1
12. Adams TD, Davidson LE, Litwin SE, Kim J, Kolotkin RL, Nanjee MN, et al. Weight and metabolic outcomes 12 years after gastric bypass. *N Eng J Med.* (2018) 378:93–6. doi: 10.1056/NEJMoa1700459
13. Sjostrom L, Peltonen M, Jacobson P, Ahlin S, Andersson-Assarsson J, Anveden A, et al. Association of bariatric surgery with long-term remission of type 2 diabetes and with microvascular and macrovascular complications. *JAMA* (2014) 311:2297–304. doi: 10.1001/jama.2014.5988

14. Singh AK, Singh R, Kota SK. Bariatric surgery and diabetes remission: Who would have thought it? *Indian J Endocrinol Metab.* (2015) 19:563–76. doi: 10.4103/2230-8210.163113
15. Schauer PR, Mingrone G, Ikramuddin S, Wolfe B. Clinical outcomes of metabolic surgery: efficacy of glycemic control, weight loss, and remission of diabetes. *Diabetes Care* (2016) 39:902–11. doi: 10.2337/dc16-0382
16. Pournaras DJ, Aasheim ET, Søvik TT, Andrews R, Mahon D, Welbourn R, et al. Effect of the definition of type II diabetes remission in the evaluation of bariatric surgery for metabolic disorders. *Br J Surg.* (2011) 99:100–3. doi: 10.1002/bjs.7704
17. Mas-Lorenzo A, Benaiges D, Flores-Le-Roux JA, Pedro-Botet J, Ramon JM, Parri A, et al. Impact of different criteria on type 2 diabetes remission rate after bariatric surgery. *Obes Surg.* (2014) 24:1881–7. doi: 10.1007/s11695-014-1282-2
18. Wang GF, Yan YX, Xu N, Yin D, Hui Y, Zhang J-P, et al. Predictive factors of type 2 diabetes mellitus remission following bariatric surgery: a meta-analysis. *Obes Surg.* (2015) 25:199–208. doi: 10.1007/s11695-014-1391-y
19. Panunzi S, Carlsson L, De Gaetano A, Peltonen M, Rice T, Sjöström L, et al. Determinants of diabetes remission and glycemic control after bariatric surgery. *Diabetes Care* (2016) 39:166–74. doi: 10.2337/dc15-0575
20. Shah A, Laferrère B. Diabetes after bariatric surgery. *Can J Diabetes* (2017) 41:401–6. doi: 10.1016/j.jcjd.2016.12.009
21. Vu L, Switzer NJ, De Gara C, Karmali S. Surgical interventions for obesity and metabolic disease. *Best Pract Res Clin Endocrinol Metab.* (2013) 27:239–46. doi: 10.1016/j.beem.2012.12.001
22. Pedersen HK, Gudmundsdottir V, Pedersen MK, Brorsson C, Brunak S, Gupta R. Ranking factors involved in diabetes remission after bariatric surgery using machine-learning integrating clinical and genomic biomarkers. *NPJ Genom Med.* (2016) 1:16035. doi: 10.1038/npjgenmed.2016.35
23. Nannipieri M, Baldi S, Mari A, Colligiani D, Guarino D, Camastra S, et al. Roux-en-Y gastric bypass and sleeve gastrectomy: mechanisms of diabetes remission and role of gut hormones. *J Clin Endocrinol Metab.* (2013) 98:4391–9. doi: 10.1210/jc.2013-2538
24. Blanco J, Jiménez A, Casamitjana R, Flores L, Lacy A, Conget I, et al. Relevance of beta-cell function for improved glycemic control after gastric bypass surgery. *Surg Obes Relat Dis.* (2014) 10:9–13. doi: 10.1016/j.soard.2013.07.020
25. Mathus-Vliegen EM. Long-term weight loss after bariatric surgery in patients visited at home outside the study environment. *Obes Surg.* (2006) 16:1508–19. doi: 10.1381/096089206778870003
26. Still CD, Wood GC, Benotti P, Petrick AT, Gabrielsen J, Strodel WE, et al. A probability score for preoperative prediction of type 2 diabetes remission following RYGB surgery. *Lancet Diabetes Endocrinol.* (2014) 2:38–45. doi: 10.1016/S2213-8587(13)70070-6
27. Lee WJ, Hur KY, Lakadawala M, Kasama K, Wong SK, Chen SC, et al. Predicting success of metabolic surgery: age, body mass index, C-peptide, and duration score. *Surg Obes Relat Dis.* (2013) 9:379–84. doi: 10.1016/j.soard.2012.07.015
28. Schauer PR, Bhatt DL, Kirwan JP, Wolski K, Aminian A, Brethauer SA, et al. Bariatric surgery versus intensive medical therapy for diabetes — 5-year outcomes. *N Eng J Med.* (2017) 376:641–51. doi: 10.1056/NEJMoa1600869
29. Mingrone G, Cummings DE. Changes of insulin sensitivity and secretion after bariatric/metabolic surgery. *Surg Obes Relat Dis.* (2016) 12:1199–205. doi: 10.1016/j.soard.2016.05.013
30. Mingrone G, Panunzi S, De Gaetano A, Guidone C, Iaconelli A, Nanni G, et al. Bariatric–metabolic surgery versus conventional medical treatment in obese patients with type 2 diabetes: 5 year follow-up of an open-label, single-centre, randomised controlled trial. *Lancet* (2015) 386:964–73. doi: 10.1016/S0140-6736(15)00075-6
31. Fothergill E, Guo J, Howard L, Kerns Jennifer C, Knuth Nicolas D, Brychta R, et al. Persistent metabolic adaptation 6 years after "The Biggest Loser" competition. *Obesity* (2016) 24:1612–9. doi: 10.1002/oby.21538
32. Kushner RF. Weight loss strategies for treatment of obesity: lifestyle management and pharmacotherapy. *Prog Cardiovasc Dis.* (2018). doi: 10.1016/j.pcad.2018.06.001. [Epub ahead of print].
33. Sjöström L. Review of the key results from the Swedish Obese Subjects (SOS) trial – a prospective controlled intervention study of bariatric surgery. *J Intern Med.* (2012) 273:219–34. doi: 10.1111/joim.12012
34. Courcoulas A, Christian N, Belle S, Berk P, Flum D, Garcia L. Longitudinal Assessment of Bariatric Surgery (LABS) consortium. Weight change and health outcomes at 3 years after bariatric surgery among individuals with severe obesity. *JAMA* (2013) 310:2416–25. doi: 10.1001/jama.2013.280928
35. Purnell JQ, Selzer F, Wahed AS, Pender J, Pories W, Pomp A, et al. Type 2 diabetes remission rates after laparoscopic gastric bypass and gastric banding: results of the longitudinal assessment of bariatric surgery study. *Diabetes Care* (2016) 39:1101–7. doi: 10.2337/dc15-2138
36. Kim JW, Yoon KH. Glucolipotoxicity in pancreatic β-Cells. *Diabetes Metab J.* (2011) 35:444–50. doi: 10.4093/dmj.2011.35.5.444
37. Poitout V, Robertson RP. Minireview: secondary β-cell failure in type 2 diabetes—a convergence of glucotoxicity and lipotoxicity. *Endocrinology* (2002) 143:339–42. doi: 10.1210/endo.143.2.8623
38. Shoelson SE, Herrero L, Naaz A. Obesity, inflammation, and insulin resistance. *Gastroenterology* (2007) 132:2169–80. doi: 10.1053/j.gastro.2007.03.059
39. Tilg H, Moschen AR. Inflammatory mechanisms in the regulation of insulin resistance. *Mol Med.* (2008) 14:222–31. doi: 10.2119/2007-00119
40. Kratz M, Hagman Derek K, Kuzma Jessica N, Foster-Schubert Karen E, Chan Chun P, Stewart S, et al. Improvements in glycemic control after gastric bypass occur despite persistent adipose tissue inflammation. *Obesity* (2016) 24:1438–45. doi: 10.1002/oby.21524
41. Hagman DK, Larson I, Kuzma JN, Cromer G, Makar K, Rubinow KB, et al. The short-term and long-term effects of bariatric/metabolic surgery on subcutaneous adipose tissue inflammation in humans. *Metabolism* (2017) 70:12–22. doi: 10.1016/j.metabol.2017.01.030
42. Luo RB, Suzuki T, Hooker JC, Covarrubias Y, Schlein A, Liu S, et al. How bariatric surgery affects liver volume and fat density in NAFLD patients. *Surg Endosc.* (2018) 32:1675–82. doi: 10.1007/s00464-017-5846-9
43. Immonen H, Hannukainen JC, Kudomi N, Pihlajamäki J, Saunavaara V, Laine J, et al. Increased liver fatty acid uptake is partly reversed and liver fat content normalized after bariatric surgery. *Diabetes Care* (2018) 41:368–71. doi: 10.2337/dc17-0738
44. Toro-Ramos T, Goodpaster Bret H, Janumala I, Lin S, Strain Gladys W, Thornton John C, et al. Continued loss in visceral and intermuscular adipose tissue in weight-stable women following bariatric surgery. *Obesity* (2014) 23:62–9. doi: 10.1002/oby.20932
45. Pories WJ, Swanson MS, MacDonald KG, Long SB, Morris PG, Brown BM, et al. Who would have thought it? An operation proves to be the most effective therapy for adult-onset diabetes mellitus. *Ann Surg.* (1995) 222:339–52
46. Schwartz MW, Seeley RJ, Tschöp MH, Woods SC, Morton GJ, Myers MG, et al. Cooperation between brain and islet in glucose homeostasis and diabetes. *Nature* (2013) 503:59–66. doi: 10.1038/nature12709
47. Jain A, Le Roux CW, Puri P, Tavakkoli A, Gletsu-Miller N, Laferrère B, et al. Proceedings of the 2017 ASPEN Research Workshop-Gastric Bypass: role of the Gut. *JPEN J Parenter Enteral Nutr.* (2018) 42:279–95. doi: 10.1002/jpen.1121
48. Drucker DJ. The role of gut hormones in glucose homeostasis. *J Clin Invest.* (2007) 117:24–32. doi: 10.1172/JCI30076
49. Quercia I, Dutia R, Kotler DP, Belsley S, Laferrère B. Gastrointestinal changes after bariatric surgery. *Diabetes Metab.* (2014) 40:87–94. doi: 10.1016/j.diabet.2013.11.003
50. Wang G, Agenor K, Pizot J, Kotler DP, Harel Y, Van Der Schueren BJ, et al. Accelerated gastric emptying but no carbohydrate malabsorption 1 year after gastric bypass surgery (GBP). *Obes Surg.* (2012) 22:1263–7. doi: 10.1007/s11695-012-0656-6
51. Stano S, Alam F, Wu L, Dutia R, Ng SN, Sala M, et al. Effect of meal size and texture on gastric pouch emptying and glucagon-like peptide 1 after gastric bypass surgery. *Surg Obes Relat Dis.* (2017) 13:1975–83. doi: 10.1016/j.soard.2017.09.004
52. Steinert RE, Feinle-Bisset C, Asarian L, Horowitz M, Beglinger C, Geary N. Ghrelin, CCK, GLP-1, and PYY(3–36): secretory controls and physiological roles in eating and glycemia in health, obesity, and after RYGB. *Physiol Rev.* (2016) 97:411–63. doi: 10.1152/physrev.00031.2014

53. Ockander L, Hedenbro JL, Rehfeld JF, Sjölund K. Jejunoileal bypass changes the duodenal cholecystokinin and somatostatin cell density. *Obes Surg.* (2003) 13:584–90. doi: 10.1381/096089203322190781
54. Olivan B, Teixeira J, Bose M, Bawa B, Chang T, Summe H, et al. Effect of weight loss by diet or gastric bypass surgery on peptide YY3-36 levels. *Ann Surg.* (2009) 249:948–53. doi: 10.1097/SLA.0b013e3181a6cdb0
55. Vidal J, Nicolau J, Romero F, Casamitjana R, Momblan D, Conget I, et al. Long-term effects of Roux-en-Y gastric bypass surgery on plasma glucagon-like peptide-1 and islet function in morbidly obese subjects. *J Clin Endocrinol Metab.* (2009) 94:884–91. doi: 10.1210/jc.2008-1620
56. le Roux CW, Welbourn R, Werling M, Osborne A, Kokkinos A, Laurenius A, et al. Gut hormones as mediators of appetite and weight loss after Roux-en-Y gastric bypass. *Ann Surg.* (2007) 246:780–5. doi: 10.1097/SLA.0b013e3180caa3e3
57. Morinigo R, Moize V, Musri M, Lacy AM, Navarro S, Marin JL, et al. Glucagon-like peptide-1, peptide YY, hunger, and satiety after gastric bypass surgery in morbidly obese subjects. *J Clin Endocrinol Metab.* (2006) 91:1735–40. doi: 10.1210/jc.2005-0904
58. Laferrère B, Swerdlow N, Bawa B, Arias S, Bose M, Olivan B, et al. Rise of oxyntomodulin in response to oral glucose after gastric bypass surgery in patients with type 2 diabetes. *J Clin Endocrinol Metab.* (2010) 95:4072–6. doi: 10.1210/jc.2009-2767
59. de Hollanda A, Casals G, Delgado S, Jiménez A, Viaplana J, Lacy AM, et al. Gastrointestinal hormones and weight loss maintenance following Roux-en-Y gastric bypass. *J Clin Endocrinol Metab.* (2015) 100:4677–84. doi: 10.1210/jc.2015-3065
60. Goldstone AP, Miras AD, Scholtz S, Jackson S, Neff KJ, Pénicaud L, et al. Link between increased satiety gut hormones and reduced food reward after gastric bypass surgery for obesity. *J Clin Endocrinol Metab.* (2016) 101:599–609. doi: 10.1210/jc.2015-2665
61. Laferrère B, Teixeira J, McGinty J, Tran H, Egger JR, Colarusso A, et al. Effect of weight loss by gastric bypass surgery versus hypocaloric diet on glucose and incretin levels in patients with type 2 diabetes. *J Clin Endocrinol Metab.* (2008) 93:2479–85. doi: 10.1210/jc.2007-2851
62. Morinigo R, Lacy AM, Casamitjana R, Delgado S, Gomis R, Vidal J. GLP-1 and changes in glucose tolerance following gastric bypass surgery in morbidly obese subjects. *Obes Surg.* (2006) 16:1594–601. doi: 10.1381/096089206779319338
63. Laferrère B, Heshka S, Wang K, Khan Y, McGinty J, Teixeira J, et al. Incretin levels and effect are markedly enhanced 1 month after Roux-en-Y gastric bypass surgery in obese patients with type 2 diabetes. *Diabetes Care* (2007) 30:1709–16. doi: 10.2337/dc06-1549
64. Purnell JQ, Johnson GS, Wahed AS, Dalla Man C, Piccinini F, Cobelli C, et al. Prospective evaluation of insulin and incretin dynamics in obese adults with and without diabetes for 2 years after Roux-en-Y gastric bypass. *Diabetologia* (2018) 61:1142–54. doi: 10.1007/s00125-018-4553-y
65. Lee CJ, Brown TT, Cheskin LJ, Choi P, Moran TH, Peterson L, et al. Effects of meal composition on postprandial incretin, glucose and insulin responses after surgical and medical weight loss. *Obes Sci Pract.* (2015) 1:104–9. doi: 10.1002/osp4.17
66. Xiong SW, Cao J, Liu XM, Deng XM, Liu Z, Zhang FT. Effect of modified Roux-en-Y gastric bypass surgery on GLP-1, GIP in patients with type 2 diabetes mellitus. *Gastroenterol Res Pract.* (2015) 2015:625196. doi: 10.1155/2015/625196
67. Lindqvist A, Spégel P, Ekelund M, Mulder H, Groop L, Hedenbro J, et al. Effects of ingestion routes on hormonal and metabolic profiles in gastric-bypassed humans. *J Clin Endocrinol Metab.* (2013) 98:E856–61. doi: 10.1210/jc.2012-3996
68. Pournaras DJ, Aasheim ET, Bueter M, Ahmed AR, Welbourn R, Olbers T, et al. Effect of bypassing the proximal gut on gut hormones involved with glycemic control and weight loss. *Surg Obes Relat Dis.* (2012) 8:371–4. doi: 10.1016/j.soard.2012.01.021
69. Falken Y, Hellstrom PM, Holst JJ, Naslund E. Changes in glucose homeostasis after Roux-en-Y gastric bypass surgery for obesity at day three, two months, and one year after surgery: role of gut peptides. *J Clin Endocrinol Metab.* (2011) 96:2227–35. doi: 10.1210/jc.2010-2876
70. Bose M, Teixeira J, Olivan B, Bawa B, Arias S, Machineni S, et al. Weight loss and incretin responsiveness improve glucose control independently after gastric bypass surgery. *J Diabetes* (2010) 2:47–55. doi: 10.1111/j.1753-0407.2009.00064.x
71. Raverdy V, Baud G, Pigeyre M, Verkindt H, Torres F, Preda C, et al. Incidence and predictive factors of postprandial hyperinsulinemic hypoglycemia after Roux-en-Y gastric bypass: a five year longitudinal study. *Ann Surg.* (2016) 264:878–85. doi: 10.1097/sla.0000000000001915
72. Patti ME, Goldfine AB. Hypoglycemia after gastric bypass: the dark side of GLP-1. *Gastroenterology* (2014) 146:605–8. doi: 10.1053/j.gastro.2014.01.038
73. Abrahamsson N, Börjesson JL, Sundbom M, Wiklund U, Karlsson FA, Eriksson JW. Gastric bypass reduces symptoms and hormonal responses in hypoglycemia. *Diabetes* (2016) 65:2667–75. doi: 10.2337/db16-0341
74. Salehi M, Gastaldelli A, D'Alessio DA. Altered islet function and insulin clearance cause hyperinsulinemia in gastric bypass patients with symptoms of postprandial hypoglycemia. *J Clin Endocrinol Metab.* (2014) 99:2008–17. doi: 10.1210/jc.2013-2686
75. Salehi M, Gastaldelli A, D'Alessio DA. Blockade of glucagon-like peptide 1 receptor corrects postprandial hypoglycemia after gastric bypass. *Gastroenterology* (2014) 146:669–80 e2. doi: 10.1053/j.gastro.2013.11.044
76. Dutia R, Brakoniecki K, Bunker P, Paultre F, Homel P, Carpentier AC, et al. Limited recovery of beta-cell function after gastric bypass despite clinical diabetes remission. *Diabetes* (2014) 63:1214–23. doi: 10.2337/db13-1176
77. Jorgensen NB, Dirksen C, Bojsen-Moller KN, Jacobsen SH, Worm D, Hansen DL, et al. Exaggerated glucagon-like peptide 1 response is important for improved beta-cell function and glucose tolerance after Roux-en-Y gastric bypass in patients with type 2 diabetes. *Diabetes* (2013) 62:3044–52. doi: 10.2337/db13-0022
78. Shah M, Law JH, Micheletto F, Sathananthan M, Dalla Man C, Cobelli C, et al. Contribution of endogenous glucagon-like peptide 1 to glucose metabolism after Roux-en-Y gastric bypass. *Diabetes* (2014) 63:483–93. doi: 10.2337/db13-0954
79. Vidal J, de Hollanda A, Jiménez A. GLP-1 is not the key mediator of the health benefits of metabolic surgery. *Surg Obes Relat Dis.* (2016) 12:1225–9. doi: 10.1016/j.soard.2016.02.029
80. McLaughlin T, Peck M, Holst J, Deacon C. Reversible hyperinsulinemic hypoglycemia after gastric bypass: a consequence of altered nutrient delivery. *J Clin Endocrinol Metab.* (2010) 95:1851–5. doi: 10.1210/jc.2009-1628
81. Davis DB, Khoraki J, Ziemelis M, Sirinvaravong S, Han JY, Campos GM. Roux en Y gastric bypass hypoglycemia resolves with gastric feeding or reversal: Confirming a non-pancreatic etiology. *Mol Metab.* (2018) 9:15–27. doi: 10.1016/j.molmet.2017.12.011
82. Salehi M, Gastaldelli A, D'Alessio David A. Beta-cell sensitivity to glucose is impaired after gastric bypass surgery. *Diabetes Obes Metab.* (2017) 20:872–8. doi: 10.1111/dom.13165
83. Dirksen C, Bojsen-Moller KN, Jorgensen NB, Jacobsen SH, Kristiansen VB, Naver LS, et al. Exaggerated release and preserved insulinotropic action of glucagon-like peptide-1 underlie insulin hypersecretion in glucose-tolerant individuals after Roux-en-Y gastric bypass. *Diabetologia* (2013) 56:2679–87. doi: 10.1007/s00125-013-3055-1
84. Fujita Y, Kojima H, Hidaka H, Fujimiya M, Kashiwagi A, Kikkawa R. Increased intestinal glucose absorption and postprandial hyperglycaemia at the early step of glucose intolerance in Otsuka Long-Evans Tokushima Fatty Rats. *Diabetologia* (1998) 41:1459–66. doi: 10.1007/s0012500 51092
85. le Roux CW, Borg C, Wallis K, Vincent RP, Bueter M, Goodlad R, et al. Gut hypertrophy after gastric bypass is associated with increased glucagon-like peptide 2 and intestinal crypt cell proliferation. *Ann Surg.* (2010) 252:50–6. doi: 10.1097/SLA.0b013e3181d3d21f
86. Hansen CF, Bueter M, Theis N, Lutz T, Paulsen S, Dalbøge LS, et al. Hypertrophy dependent doubling of L-cells in Roux-en-Y gastric bypass operated rats. *PLOS ONE* (2013) 8:e65696. doi: 10.1371/journal.pone.0065696
87. Mumphrey MB, Hao Z, Townsend RL, Patterson LM, Berthoud H-R. Sleeve gastrectomy does not cause hypertrophy and reprogramming of intestinal glucose metabolism in rats. *Obes Surg.* (2015) 25:1468–73. doi: 10.1007/s11695-014-1547-9
88. Troy S, Soty M, Ribeiro L, Laval L, Migrenne S, Fioramonti X, et al. Intestinal gluconeogenesis is a key factor for early metabolic changes after gastric

bypass but not after gastric lap-band in mice. *Cell Metab.* (2008) 8:201–11. doi: 10.1016/j.cmet.2008.08.008
89. Yan Y, Zhou Z, Kong F, Feng S, Li X, Sha Y, et al. Roux-en-Y gastric bypass surgery suppresses hepatic gluconeogenesis and increases intestinal gluconeogenesis in a T2DM rat model. *Obes Surg.* (2016) 26:2683–90. doi: 10.1007/s11695-016-2157-5
90. Kim M, Son YG, Kang YN, Ha TK, Ha E. Changes in glucose transporters, gluconeogenesis, and circadian clock after duodenal–jejunal bypass surgery. *Obes Surg.* (2015) 25:635–41. doi: 10.1007/s11695-014-1434-4
91. Sun D, Wang K, Yan Z, Zhang G, Liu S, Liu F, et al. Duodenal–jejunal bypass surgery up-regulates the expression of the hepatic insulin signaling proteins and the key regulatory enzymes of intestinal gluconeogenesis in diabetic goto–kakizaki rats. *Obes Surg.* (2013) 23:1734–42. doi: 10.1007/s11695-013-0985-0
92. Gutierrez-Repiso C, Garcia-Serrano S, Moreno-Ruiz FJ, Alcain-Martinez G, Rodriguez-Pacheco F, Garcia-Fuentes E. Jejunal gluconeogenesis associated with insulin resistance level and its evolution after Roux-en-Y gastric bypass. *Surg Obes Relat Dis.* (2017) 13:623–30. doi: 10.1016/j.soard.2016.11.021
93. Saeidi N, Meoli L, Nestoridi E, Gupta NK, Kvas S, Kucharczyk J, et al. Reprogramming of intestinal glucose metabolism and glycemic control in rats after gastric bypass. *Science* (2013) 341:406–10. doi: 10.1126/science.1235103
94. Wright EM, Hirayama BA, Loo DF. Active sugar transport in health and disease. *J Intern Med* (2007) 261:32–43. doi: 10.1111/j.1365-2796.2006.01746.x
95. Nguyen NQ, Debreceni TL, Bambrick JE, Chia B, Deane AM, Wittert G, et al. Upregulation of intestinal glucose transporters after Roux-en-Y gastric bypass to prevent carbohydrate malabsorption. *Obesity* (2014) 22:2164–71. doi: 10.1002/oby.20829
96. Baud G, Daoudi M, Hubert T, Raverdy V, Pigeyre M, Hervieux E, et al. Bile Diversion in Roux-en-Y gastric bypass modulates sodium-dependent glucose intestinal uptake. *Cell Metab.* (2016) 23:547–53. doi: 10.1016/j.cmet.2016.01.018
97. Albaugh VL, Banan B, Ajouz H, Abumrad NN, Flynn CR. Bile acids and bariatric surgery. *Mol Aspects Med.* (2017) 56:75–89. doi: 10.1016/j.mam.2017.04.001
98. Chávez-Talavera O, Tailleux A, Lefebvre P, Staels B. Bile acid control of metabolism and inflammation in obesity, type 2 diabetes, dyslipidemia, and nonalcoholic fatty liver disease. *Gastroenterology* (2017) 152:1679–94.e3. doi: 10.1053/j.gastro.2017.01.055
99. Pols TWH, Noriega LG, Nomura M, Auwerx J, Schoonjans K. The bile acid membrane receptor TGR5 as an emerging target in metabolism and inflammation. *J Hepatol.* (2011) 54:1263–72. doi: 10.1016/j.jhep.2010.12.004
100. Lefebvre P, Cariou B, Lien F, Kuipers F, Staels B. Role of bile acids and bile acid receptors in metabolic regulation. *Physiol Rev.* (2009) 89:147–91. doi: 10.1152/physrev.00010.2008
101. Trabelsi MS, Lestavel S, Staels B, Collet X. Intestinal bile acid receptors are key regulators of glucose homeostasis. *Proc Nutr Soc.* (2017) 76:192–202. doi: 10.1017/S0029665116002834
102. Nakatani H, Kasama K, Oshiro T, Watanabe M, Hirose H, Itoh H. Serum bile acid along with plasma incretins and serum high-molecular weight adiponectin levels are increased after bariatric surgery. *Metabolism* (2009) 58:1400–7. doi: 10.1016/j.metabol.2009.05.006
103. Pournaras DJ, Glicksman C, Vincent RP, Kuganolipava S, Alaghband-Zadeh J, Mahon D, et al. The role of bile after Roux-en-Y gastric bypass in promoting weight loss and improving glycaemic control. *Endocrinology* (2012) 153:3613–9. doi: 10.1210/en.2011-2145
104. Simonen M, Dali-Youcef N, Kaminska D, Venesmaa S, Kakela P, Paakkonen M, et al. Conjugated bile acids associate with altered rates of glucose and lipid oxidation after Roux-en-Y gastric bypass. *Obes Surg.* (2012) 22:1473–80. doi: 10.1007/s11695-012-0673-5
105. Gerhard GS, Styer AM, Wood GC, Roesch SL, Petrick AT, Gabrielsen J, et al. A role for fibroblast growth factor 19 and bile acids in diabetes remission after Roux-en-Y gastric bypass. *Diabetes Care* (2013) 36:1859–64. doi: 10.2337/dc12-2255
106. Ferrannini E, Camastra S, Astiarraga B, Nannipieri M, Castro-Perez J, Xie D, et al. Increased bile acid synthesis and deconjugation after biliopancreatic diversion. *Diabetes* (2015) 64:3377–85. doi: 10.2337/db15-0214
107. Patti ME, Houten SM, Bianco AC, Bernier R, Larsen PR, Holst JJ, et al. Serum bile acids are higher in humans with prior gastric bypass: potential contribution to improved glucose and lipid metabolism. *Obesity* (2009) 17:1671–7. doi: 10.1038/oby.2009.102
108. Werling M, Vincent RP, Cross GF, Marschall HU, Fandriks L, Lonroth H, et al. Enhanced fasting and post-prandial plasma bile acid responses after Roux-en-Y gastric bypass surgery. *Scand J Gastroenterol.* (2013) 48:1257–64. doi: 10.3109/00365521.2013.833647
109. Kohli R, Bradley D, Setchell KD, Eagon JC, Abumrad N, Klein S. Weight loss induced by Roux-en-Y gastric bypass but not laparoscopic adjustable gastric banding increases circulating bile acids. *J Clin Endocrinol Metab.* (2013) 98:E708–12. doi: 10.1210/jc.2012-3736
110. Ahmad NN, Pfalzer A, Kaplan LM. Roux-en-Y gastric bypass normalizes the blunted postprandial bile acid excursion associated with obesity. *Int J Obes.* (2013) 37:1553–9. doi: 10.1038/ijo.2013.38
111. Steinert RE, Peterli R, Keller S, Meyer-Gerspach AC, Drewe J, Peters T, et al. Bile acids and gut peptide secretion after bariatric surgery: a 1-year prospective randomized pilot trial. *Obesity* (2013) 21:E660–8. doi: 10.1002/oby.20522
112. Sachdev S, Wang Q, Billington C, Connett J, Ahmed L, Inabnet W, et al. FGF 19 and bile acids increase following Roux-en-Y gastric bypass but not after medical management in patients with type 2 diabetes. *Obes Surg.* (2016) 26:957–65. doi: 10.1007/s11695-015-1834-0
113. Dutia R, Embrey M, O'Brien S, Haeusler RA, Agenor KK, Homel P, et al. Temporal changes in bile acid levels and 12alpha-hydroxylation after Roux-en-Y gastric bypass surgery in type 2 diabetes. *Int J Obes.* (2016) 40:554. doi: 10.1038/ijo.2015.250
114. Spinelli V, Lalloyer F, Baud G, Osto E, Kouach M, Daoudi M, et al. Influence of Roux-en-Y gastric bypass on plasma bile acid profiles: a comparative study between rats, pigs and humans. *Int J Obes* (2016) 40:1260–7. doi: 10.1038/ijo.2016.46
115. Jørgensen NB, Dirksen C, Bojsen-Møller KN, Kristiansen VB, Wulff BS, Rainteau D, et al. Improvements in glucose metabolism early after gastric bypass surgery are not explained by increases in total bile acids and fibroblast growth factor 19 concentrations. *J Clin Endocrinol Metab.* (2015) 100:E396–406. doi: 10.1210/jc.2014-1658
116. Tsuchiya T, Kalogeris TJ, Tso P. Ileal transposition into the upper jejunum affects lipid and bile salt absorption in rats. *Am J Physiol Gastrointest Liver Physiol.* (1996) 271:G681–91. doi: 10.1152/ajpgi.1996.271.4.G681
117. Han H, Wang L, Du H, Jiang J, Hu C, Zhang G, et al. Expedited biliopancreatic juice flow to the distal gut benefits the diabetes control after duodenal-jejunal bypass. *Obes Surg.* (2015) 25:1802–9. doi: 10.1007/s11695-015-1633-7
118. Weng SG, Zhang B, Wang X, Chen H. Effects of duodenal-jejunal exclusion and new bilio-pancreatic diversion on blood glucose in rats with type 2 diabetes mellitus. *Obes Surg.* (2017) 27:2067–72. doi: 10.1007/s11695-017-2599-4
119. Kohli R, Setchell KD, Kirby M, Myronovych A, Ryan KK, Ibrahim SH, et al. A surgical model in male obese rats uncovers protective effects of bile acids post-bariatric surgery. *Endocrinology* (2013) 154:2341–51. doi: 10.1210/en.2012-2069
120. Chávez-Talavera O, Baud G, Spinelli V, Daoudi M, Kouach M, Goossens JF, et al. Roux-en-Y gastric bypass increases systemic but not portal bile acid concentrations by decreasing hepatic bile acid uptake in minipigs. *Int J Obes.* (2017) 41:664–8. doi: 10.1038/ijo.2017.7
121. Duboc H, Nguyen CC, Cavin J-B, Ribeiro-Parenti L, Jarry A-C, Rainteau D, et al. Roux-en-Y gastric-bypass and sleeve gastrectomy induces specific shifts of the gut microbiota without altering the metabolism of bile acids in the intestinal lumen. *Int J Obes.* (2018). doi: 10.1038/s41366-018-0015-3. [Epub ahead of print].
122. Mazuy C, Helleboid A, Staels B, Lefebvre P. Nuclear bile acid signaling through the farnesoid X receptor. *Cell Mol Life Sci.* (2015) 72:1631–50. doi: 10.1007/s00018-014-1805-y
123. Ryan KK, Tremaroli V, Clemmensen C, Kovatcheva-Datchary P, Myronovych A, Karns R, et al. FXR is a molecular target for the effects of vertical sleeve gastrectomy. *Nature* (2014) 509:183–8. doi: 10.1038/nature13135

124. Kuhre RE, Wewer Albrechtsen NJ, Larsen O, Jepsen SL, Balk-Møller E, Andersen DB, et al. Bile acids are important direct and indirect regulators of the secretion of appetite- and metabolism-regulating hormones from the gut and pancreas. *Mol Metab.* (2018) 11:84–95. doi: 10.1016/j.molmet.2018.03.007
125. Brighton CA, Rievaj J, Kuhre RE, Glass LL, Schoonjans K, Holst JJ, et al. Bile Acids Trigger GLP-1 release predominantly by accessing basolaterally located G protein–coupled bile acid receptors. *Endocrinology* (2015) 156:3961–70. doi: 10.1210/en.2015-1321
126. Katsuma S, Hirasawa A, Tsujimoto G. Bile acids promote glucagon-like peptide-1 secretion through TGR5 in a murine enteroendocrine cell line STC-1. *Biochem Biophys Res Commun.* (2005) 329:386–90. doi: 10.1016/j.bbrc.2005.01.139
127. Ding L, Sousa Kyle M, Jin L, Dong B, Kim BW, Ramirez R, et al. Vertical sleeve gastrectomy activates GPBAR-1/TGR5 to sustain weight loss, improve fatty liver, and remit insulin resistance in mice. *Hepatology* (2016) 64:760–73. doi: 10.1002/hep.28689
128. McGavigan AK, Garibay D, Henseler ZM, Chen J, Bettaieb A, Haj FG, et al. TGR5 contributes to glucoregulatory improvements after vertical sleeve gastrectomy in mice. *Gut* (2017) 66:226–34. doi: 10.1136/gutjnl-2015-309871
129. Clemente Jose C, Ursell Luke K, Parfrey Laura W, Knight R. The impact of the gut microbiota on human health: an integrative view. *Cell* (2012) 148:1258–70. doi: 10.1016/j.cell.2012.01.035
130. Cox LM, Blaser MJ. Pathways in microbe-induced obesity. *Cell Metab.* (2013) 17:883–94. doi: 10.1016/j.cmet.2013.05.004
131. Udayappan SD, Hartstra AV, Dallinga-Thie GM, Nieuwdorp M. Intestinal microbiota and faecal transplantation as treatment modality for insulin resistance and type 2 diabetes mellitus. *Clin Exp Immunol.* (2014) 177:24–9. doi: 10.1111/cei.12293
132. Zhang H, DiBaise JK, Zuccolo A, Kudrna D, Braidotti M, Yu Y, et al. Human gut microbiota in obesity and after gastric bypass. *Proc Natl Acad Sci U S A.* (2009) 106:2365–70. doi: 10.1073/pnas.0812600106
133. Furet JP, Kong LC, Tap J, Poitou C, Basdevant A, Bouillot JL, et al. Differential adaptation of human gut microbiota to bariatric surgery–induced weight loss: links with metabolic and low-grade inflammation markers. *Diabetes* (2010) 59:3049–57. doi: 10.2337/db10-0253
134. Kong LC, Tap J, Aron-Wisnewsky J, Pelloux V, Basdevant A, Bouillot JL, et al. Gut microbiota after gastric bypass in human obesity: increased richness and associations of bacterial genera with adipose tissue genes. *Am J Clin Nutr.* (2013) 98:16–24. doi: 10.3945/ajcn.113.058743
135. Graessler J, Qin Y, Zhong H, Zhang J, Licinio J, Wong ML, et al. Metagenomic sequencing of the human gut microbiome before and after bariatric surgery in obese patients with type 2 diabetes: correlation with inflammatory and metabolic parameters. *Pharmacogenomics J.* (2012) 13:514–22. doi: 10.1038/tpj.2012.43
136. Murphy R, Tsai P, Jüllig M, Liu A, Plank L, Booth M. Differential changes in gut microbiota after gastric bypass and sleeve gastrectomy bariatric surgery vary according to diabetes remission. *Obes Surg.* (2017) 27:917–25. doi: 10.1007/s11695-016-2399-2
137. Palleja A, Kashani A, Allin KH, Nielsen T, Zhang C, Li Y, et al. Roux-en-Y gastric bypass surgery of morbidly obese patients induces swift and persistent changes of the individual gut microbiota. *Genome Med.* (2016) 8:67. doi: 10.1186/s13073-016-0312-1
138. Anhê FF, Varin TV, Schertzer JD, Marette A. The gut microbiota as a mediator of metabolic benefits after bariatric surgery. *Can J Diabetes* (2017) 41:439–47. doi: 10.1016/j.jcjd.2017.02.002
139. Liou AP, Paziuk M, Luevano JM, Machineni S, Turnbaugh PJ, Kaplan LM. Conserved shifts in the gut microbiota due to gastric bypass reduce host weight and adiposity. *Sci Transl Med.* (2013) 5:178ra41. doi: 10.1126/scitranslmed.3005687
140. Tremaroli V, Karlsson F, Werling M, Ståhlman M, Kovatcheva-Datchary P, Olbers T, et al. Roux-en-Y gastric bypass and vertical banded gastroplasty induce long-term changes on the human gut microbiome contributing to fat mass regulation. *Cell Metab.* (2015) 22:228–38. doi: 10.1016/j.cmet.2015.07.009
141. Begley M, Gahan CG, Hill C. The interaction between bacteria and bile. *FEMS Microbiol Rev.* (2005) 29:625–51. doi: 10.1016/j.femsre.2004.09.003
142. Ridlon JM, Kang DJ, Hylemon PB. Bile salt biotransformations by human intestinal bacteria. *J Lipid Res.* (2006) 47:241–59. doi: 10.1194/jlr.R500013-JLR200
143. Sayin SI, Wahlstrom A, Felin J, Jantti S, Marschall HU, Bamberg K, et al. Gut microbiota regulates bile acid metabolism by reducing the levels of tauro-beta-muricholic acid, a naturally occurring FXR antagonist. *Cell Metab.* (2013) 17:225–35. doi: 10.1016/j.cmet.2013.01.003
144. Swann JR, Want EJ, Geier FM, Spagou K, Wilson ID, Sidaway JE, et al. Systemic gut microbial modulation of bile acid metabolism in host tissue compartments. *Proc Natl Acad Sci.* (2011) 108(Suppl. 1):4523–30. doi: 10.1073/pnas.1006734107
145. Duca FA, Lam TK. Gut microbiota, nutrient sensing and energy balance. *Diabetes Obes Metab.* (2014) 16:68–76. doi: 10.1111/dom.12340
146. Bauer PV, Duca FA, Waise TM, Rasmussen BA, Abraham MA, Dranse HJ, et al. Metformin alters upper small intestinal microbiota that impact a glucose-SGLT1-sensing glucoregulatory pathway. *Cell Metab.* (2018) 27:101–17.e5. doi: 10.1016/j.cmet.2017.09.019
147. Van der Schueren BJ, Homel P, Alam M, Agenor K, Wang G, Reilly D, et al. Magnitude and variability of the glucagon-like peptide-1 response in patients with type 2 diabetes up to 2 years following gastric bypass surgery. *Diabetes Care* (2012) 35:42–6. doi: 10.2337/dc11-1472

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Endocrinology

REVIEW
published: 27 November 2018
doi: 10.3389/fendo.2018.00515

Update of Pheochromocytoma Syndromes: Genetics, Biochemical Evaluation, and Imaging

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Pheochromocytomas and paragangliomas (PCCs/PGLs) are rare commonly benign neuroendocrine tumors that share pathology features and clinical behavior in many cases. While PCCs are chromaffin-derived tumors that arise within the adrenal medulla, PGLs are neural-crest-derived tumors that originate at the extraadrenal paraganglia. Pheochromocytoma-paraganglioma (PPGL) syndromes are rapidly evolving entities in endocrinology and oncology. Discoveries over the last decade have significantly improved our understanding of the disease. These include the finding of new hereditary forms of PPGL and their associated susceptibility genes. Additionally, the availability of new functional imaging tools and advances in targeted radionuclide therapy have improved diagnostic accuracy and provided us with new therapeutic options. In this review article, we present the most recent advances in this field and provide an update of the biochemical classification that further reflects our understanding of the disease.

Keywords: pheochromocytoma, paraganglioma, genetics, biochemical classification, DOTATATE, PRRT

OPEN ACCESS

Edited by:
Derek LeRoith, Icahn School of Medicine at Mount Sinai, United States

Reviewed by:
Luiz Eduardo Armondi Wildemberg, Instituto Estadual do Cérebro Paulo Niemeyer, Brazil
Agnese Barnabei, Istituto Nazionale del Cancro Regina Elena, Italy

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Specialty section:
This article was submitted to Cancer Endocrinology, a section of the journal Frontiers in Endocrinology

Received: *24 April 2018*
Accepted: *16 August 2018*
Published: *27 November 2018*

Citation:
Alrezk R, Suarez A, Tena I and Pacak K (2018) Update of Pheochromocytoma Syndromes: Genetics, Biochemical Evaluation, and Imaging. Front. Endocrinol. 9:515. doi: 10.3389/fendo.2018.00515

INTRODUCTION

According to the 2017 World Health Organization (WHO) classification of endocrine tumors, pheochromocytomas (PCCs) are tumors of the chromaffin cells that arise within the adrenal medulla (1), whereas paragangliomas (PGLs) are neural crest-derived neuroendocrine tumors (NETs) that can originate at any level of extra-adrenal paraganglia (from the skull base to the pelvic floor) (2). PCCs and PGLs arising from sympathetic paraganglia are characterized by catecholamine production whereas PGLs distributed along the parasympathetic chains of the head and neck (NHPGL) tend to be silent or pseudo-silent tumors (3–5).

The field of pheochromocytoma-paraganglioma (PPGL) is rapidly evolving. Many discoveries over the last decade have significantly improved our understanding of the disease. The identification of new hereditary forms of PPGL has led to the highest rate of germline susceptibility in cancer genetics at almost 40% (6, 7). In addition, other PPGL-related genes have been discovered and there are currently over 22 susceptibility genes identified. The genotype-phenotype correlation shown in many studies often dictates the clinical presentation of syndromic forms of the disease. These include associated biochemical profile, tumor location, malignant potential, aggressive clinical behavior, and overall prognosis. Furthermore, genetic identification provides valuable information for establishing a treatment plan and procures the rational for an appropriate guidance for follow-up surveillance.

The involvement of the Krebs cycle and the respiratory chain, mainly represented by the involvement of succinate dehydrogenase (SDH) in the etiology of PPGL, is perhaps the most important discovery in this area. Mutations of genes that encode any of the subunits A, B, C, or D or the complex assembly factor 2 (AF2) account for a group of overlapping yet distinct hereditary syndromes termed SDHx. These forms are considered the most common of all hereditary PPGL syndromes accounting for ∼30% of them (6, 7). Over the past 6 years, ten new genes have been discovered. These new genes include hypoxia-inducible factor 2 alpha (*HIF2A)*—also known as endothelial PAS domain-containing protein 1 (*EPAS1*) (8, 9), fumarate hydratase (*FH)* (10), HRAS proto-oncogene (*H-RAS)* (11), prolyl hydroxylase 1 (*PHD1)*—also known as egl nine homolog 2 (*EGLN2*) (12), malate dehydrogenase 2 (*MDH2)* (13), chromatin remodeler ATRX (*ATRX)* (14, 15), H3 histone family member 3A (*H3F3A*) (15), cold-shock domain containing E1 (*CSDE1)* (15), coactivator 3 mastermind-like (*MAML3*) fusion genes [upstream binding transcription factor, RNA polymerase I] *(UBTF)–MAML3* (15), and iron regulatory protein 1 (*IRP1)* (16). Multiplicity regarding mosaicism underlines also in some syndromes (8, 9, 17, 18). Each of these genes mutations affects a specific metabolic pathway. As a result, The Cancer Genomic Atlas (TCGA) group proposed a comprehensive system to classify PPGL-susceptibility genes into a molecular level. Based on genomic analysis, the system divides PPGL-related genes into four major clusters: a pseudohypoxia subtype (subdivided into tricarboxylic acid (TCA) cycle-dependent and *VHL/EPAS1*-dependent), a kinase-signaling subtype, a Wnt signaling subtype, and a cortical admixture subtype (**Figure 1**). These integrative efforts show that PPGL can be driven either by germline, somatic, or fusion genes mutations in 27, 39, and 7% of the cases, respectively (15).

Abbreviations: 3-MT; 3-methoxytyramine; ^{18}FDA, ^{18}F-fluorodopamine; ^{18}F-DOPA, ^{18}F-fluorodopa; ^{18}F-FDG, 18F-fluorodeoxyglucose; ^{68}Ga, ^{68}Galium; ^{90}Y, ^{90}Ytrium; ^{131}I-MIBG, ^{131}I-MIBG, metaiodobenzylguanidine; ^{111}In, ^{111}Indium; ^{177}Lu, ^{177}Lutethium; AFAP1, apoptosis protease activator protein 1; AKT, serine/threonine kinase; ATRX, chromatin remodeler ATRX; CT, computerized tomography; CRG, growth regulatory factors; CSDE1, cold-shock domain containing E1; CVD, cyclophosphamide, vincristine and dacarbazine; DA, dopamine; DIPG, diffuse intrinsic pontine glioma; DOTA, tetraazacyclododecanetetraacetic acid; DOTATATE, DOTA-Tyr3-octreotate; DS, direct sequencing; E, epinephrine; EGLN1/2, egl nine homolog 1 and 2; EPAS1, PAS domain-containing protein 1; EPO erythropoietin; EpoR, erythropoietin receptor; ERK, extracellular mitogen-activated protein kinase 1; FDA, food and drug administration; FH, fumarate hydratase; HNPGL, head and neck paraganglioma; GTC, giant cell tumor of the bone; GTP, guanosine-5'-triphosphate; H3F3A, H3 histone family member 3A; NHPGL head and neck PGL; HIF2α hypoxia-inducible factor 2 alpha; HIF2A, hypoxia-inducible factor 2 alpha; HLPC, high-performance liquid chromatography; HLRCC, leiomyomatosis and renal cell cancer; H-RAS, HRAS proto-oncogene; IDH1/2, isocitrate dehydrogenase 1 and 2; IHC, immunohistochemistry; IRP1 iron regulatory protein; MCUL, multiple cutaneous leiomyomatosis; MDH1/2, malate dehydrogenase type 1 and 2, MAML3; coactivator 3 mastermind-like; MAPK, mitogen-activated protein kinase; MAX, myc-associated factor X gene; Men1, multiple endocrine neoplasia 1; LC-MS, liquid chromatography tandem-mass spectrometry; MEK, mitogen-activated protein kinase; MLPA, multiplex ligation-dependent probe amplification; MN, metanephrine; MRI, magnetic resonance imaging; mRNA, messenger ribonucleic acid; mTOR, mammalian target of rapamycin; NE norepinephrine (NE); NETs neuroendocrine tumors; NF1, neurofibromin 1; NIH, National Institutes of Health; NMN, normetanephrine; NET, neuroendocrine tumor; PCC, pheochromocytoma; PET, positron emission tomography; PGLs, paraganglioma; PHD1/2, prolyl hydroxylase 1 and 2; PI3K, phosphatidyl-inositol-3-kinase; PPGL, pheochromocytoma-paraganglioma; PRRT, peptide receptor radionuclide therapy; RF, radiofrequency; SDH, succinate dehydrogenase subunits A/B/C/D; SDHAF2, succinate dehydrogenase complex assembly factor 2; SPECT, single photon emission computed tomography; SSA, somatostatin analogs; SSTR, somatostatin receptor; TCA, tricarboxylic acid, TA, thermoablation; TCGA, The Cancer Genomic Atlas, TFG, transcription factors genes; TMEM127, transmembrane protein 127; TMZ, temozolomide; TSG, tumor suppressor gene; UBTF, upstream binding transcription factor; VHL, von Hippel Lindau; WES, whole exome sequencing; WHO, World Health Organization.

GENETICS

Advances in genetics over the last decade have allowed the implementation of whole exome sequencing (WES) in developed countries as the new standard screening tool for genetic testing in patients with a suspected hereditary form of PPGL. However, the application of this technology is limited to specialized centers in developing countries. When available, the cost of testing is often a barrier for wide implementations of WES. Immunohistochemistry (IHC) is an alternative method in these cases, given its affordability and feasibility. It is used as screening tool where negative IHC for a gene can serve as an indirect indicator of the presence of a mutation in the gene of interest. This technique is especially useful in the context of suspected *SDHx* mutations (19–22). False-positive or false-negative results (19–22) are not uncommon, therefore, IHC should be interpreted with caution.

Integrative patient evaluation is essential as the clinical presentation, along with radiological and biochemical profile will guide clinicians toward the correct genetic diagnosis. This holds true when WES is not readily available. In these cases screening for specific gene or gene panels for a subgroup of susceptibility genes can be an alternative option. Therefore, we emphasize the importance of phenotype profile recognition (**Table 1**, **Figure 2**).

CLUSTER 1/PSEUDOHYPOXIA SUBTYPE

1A. TCA Cycle-Related

FH:

FH is a tumor suppressor gene in the TCA cycle that encodes FH enzyme, which converts fumarate into malate (31). Deficiency in FH results in accumulation of the precursor metabolite fumarate, which shares structural similarities with succinate, leading to prolyl-hydroxylase (PHD) inactivation and HIF stabilization (32, 33). *FH* has an autosomal dominant inheritance with variable expressivity. Patients typically present with multiple cutaneous leiomyomatosis (MCUL). Leiomyomas are smooth muscle tumors that arise from the skin and uterus in these patients. When associated with renal cell cancer, this syndrome is referred to as hereditary leiomyomatosis and renal cell cancer (HLRCC) also known as Reed's syndrome (23). PPGL is a rare second manifestation of the syndrome and tends to be malignant and/or multiple and present with a predominant noradrenergic profile. To date, only few cases have been reported and both pathogenic germline and somatic mutations have been described (10, 34). Age of presentation varies widely from as

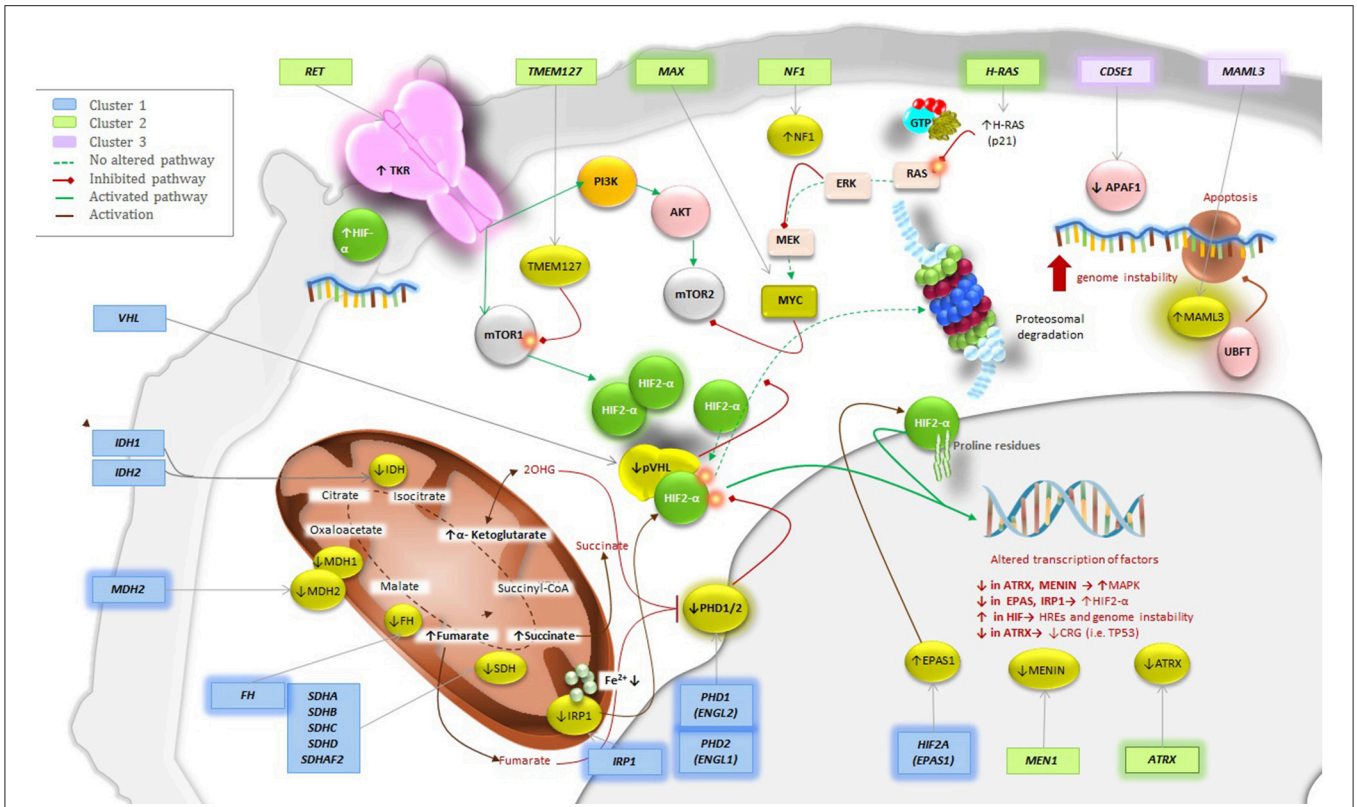

FIGURE 1 | Genetics and molecular pathways for PPGLs Pathways for the New Genes; Placing the New into Perspective. Mutations of the highlighted genes have been discovered to play a role in the pathogenesis of PPGL. These genes can be classified in cluster 1, 2, or 3. Cluster 1 would be implemented with *FH*, *MDH2, PHD1 (EGLN2), EPAS1/HIF2A* and the most recently discovered *IRP1* that controls cellular iron metabolism and negatively regulates HIF2α mRNA translation. Cluster 2 would include *H-RAS* and *ATRX,* which belongs to the SWI/SNF family of chromatin remodeling proteins, as their upregulation will activate the RAS/RAF/ERK signaling pathway resulting in tumor formation. Finally, cluster 3 would be implemented with both *CSDE1* and *UBFT* fusion at *MAML3.* Alterations of any of this genes will result in increase of target genes involved in Wnt receptor and Hedgehog signaling pathways.

young as 6- to 70-years-old. More recently, a study of a cohort reported by the National Institutes of Health (NIH) revealed co-secreting dopamine (DA) tumors in some *FH* mutated patients (24).

1B. *VHL/EPAS1*-Related

EPAS1/HIF2A:

EPAS1 (*HIF2A*) is an oncogene that encodes the EPAS1; a transcription factor related to oxygen level responses (35). In 2012, Zhuang et al. were the first to identify a gain-of-function somatic mutation in exon 12 of *EPAS1* (G1588A and C1589T) in patients with PPGLs. These mutations caused defects on the proline residues at the hydroxylation site of HIF-2α leading to its reduced degradation and stabilization (8, 9). Since then, other cases of PPGL with polycythemia have been reported. Among them, somatic mutations of *HIF2A* at exon 12 are responsible in most cases of the disease, and only one case was caused by germline mutation of *HIF2A* at exon 9 (36). Furthermore, both germline mosaicism/somatic mutations have been described in additional studies (18, 36). Somatic *HIF2A*-related PPGL affects predominantly females and patients typically presents with PPGL, somatostatinoma and polycythemia (Pacak-Zhuang syndrome) (8). However, penetrance can vary from patient to patient and incomplete forms of the disease have been reported. In particular, the presence of somatostatinoma has not been described in males yet. PPGLs in these patients are often multiple or recurrent with elevated norepinephrine (NE), normetanephrine (NMN), DA, and erythropoietin (EPO) and once third of the patients present with metastatic disease (37). Also, ∼70% of the patients have been found to have ocular abnormalities with bilateral dilated capillaries and fibrosis overlying the optic disc being the most common findings. Therefore, an early referral to an ophthalmologist acquainted with the retinal findings of this syndrome is strongly recommended (38). Surgery of the PPGL, often results in an improvement of *HIF2A*-induced polycythemia but treatment of the disease still requires intermittent phlebotomies, and control blood pressure to prevent complications resulting from polycythemia.

Cluster 2/Kinase Signaling Group

H-RAS:

Located on chromosome 11p.15.5, *H-RAS* is a proto-oncogene that, encodes H-RAS factor (also known as transforming

TABLE 1 | Genetics and clinical profile for the newly discovered forms of PPGLs.

Gene	Syndrome	Biochemical profile	Date of discovery	Gene role	Clinical presentation	Gene type	Cluster	Inheritance	References
FH	HLRCC	Noradrenergic	2012	TSG; encodes FH that catalyzes the reversible hydration of fumarate to l-malate in the TCA cycle. Increase in fumarate leads to stabilization of HIF	Multifocal, metastatic, associated with RCC and leiomyomatosis	G	1	AD	(10, 23–25)
HIF2A or EPAS1	Pacak-Zhuang	Noradrenergic	2012	Oncogene; encodes EPAS1; transcription factor related to oxygen level responses and activated in hypoxic conditions	Triad of PPGLs, polycythemia, and somatostatinoma. Ocular abnormalities occur in 70%	S/M	1	N/A	(8, 9, 24, 26)
H-RAS		Adrenergic	2013	Proto-oncogene; encodes H-RAS (P21), that once bound to GTP, activates the RAS/RAF/ERK signaling pathway leading to cell proliferation	Unilateral PCC, sporadic, benign	S	2	N/A	(11, 27)
H3F3A		Unknown	2013	Encodes the histone H3.3 protein. responsible for chromatin regulation	Giant cell tumors of the bones (GCT), PCCs, bladder and periaortic PPGL	S	*	N/A	(28)
EGLN2 (PHD1)		Noradrenergic	2015	TSG; encodes PHD1, enzyme which in normal oxygen conditions, hydroxylates specific proline residues of the HIF-α subunits for posterior degradation in the proteasome	Polycythemia associated with recurrent PPGLs, and normal or mild elevated EPO	G	1	**	(12)
MDH2		Noradrenergic	2015	TSG; encodes MDH2 that catalyzes the reversible oxidation of malate to oxaloacetate in the TCA cycle. Increase in malate, fumarate and succinate leads to stabilization of HIF	Multiple PGLs	G	1	AD	(13)
ATRX	ATRX	Noradrenergic	2015	Encodes the transcriptional regulator ATRX	Clinically more aggressive and metastatic PGL	S	*	N/A	(29)
CSDE1		Adrenergic	2016	Tumor suppressor gene. Involved in normal development through messenger RNA stability internal initiation of translation, and cell-type-specific apoptosis.	Sporadic, metastatic, recurrent PPGL	S	*	N/A	(15)
UBTF-MAML3 fusion		Adrenergic	2016	Oncogene. In PPGLs, unique hypomethylation profile mRNA overexpression of target gene involved in Wnt receptor and Hedgehog signaling pathways	Sporadic, recurrent PGL. New prognostic factor of poor outcome	F	3	N/A	(15)
IRP1	IRP1	Noradrenergic	2017	TSG; encodes IRP1, that controls cellular iron metabolism and negatively regulates HIF2α mRNA translation under iron-deficient conditions. Deficiency of IRP1 protein increases HIF2α	Sporadic, adrenal PCC	S	1	N/A	(30)

S, somatic; G, germline; M, mosaicism; F, fusion; RCC, renal cell carcinoma; HLRCC, hereditary leiomyomatosis and renal cell cancer; PCC, pheochromocytoma; PPGL, pheochromocytoma-paraganglioma; TSG, tumor suppressor gene; GCT, giant cell tumor.

N/A, Not Applicable in the setting of somatic mutations.

AD, Autosomal Dominant.

**Not classified by clusters, **Unknown.*

protein p21). H-RAS, once bound to guanosine-5'-triphosphate (GTP), activates the RAS/RAF/ERK signaling pathway that will ultimately result in cell proliferation (27). Pathogenic mutations in *H-RAS* were firstly identified in 2013. WES of tumor DNA in four cases with phenotype suggesting an underlying pathogenic genetic variant revealed the presence of 2 hotspot mutations in *H-RAS* (G13R and Q61K) in two of them. In all four samples, known susceptibility genes had been previously excluded (11). All four cases were male patients with unilateral, sporadic/benign tumors (three PCCs and one abdominal PGL), along with elevation in plasma catecholamines. Further validation in a cohort of 58 additional samples obtained showed an accumulated frequency of 6.9% (4/58) of missense somatic mutations in *H-RAS*: G13R ($n = 1$), Q61K ($n = 1$), and Q61R ($n = 2$) (11).

Cluster 3/*WNT* Signaling Group

CSDE1:

Formerly known as upstream of N-ras (*UNR*), *CSDE1* is a tumor suppressor gene located at chromosome 1p13.2 that encodes CSDE1 factor, which is mainly involved in development and has several functions including messenger RNA stability, internal initiation of translation, cell-type-specific apoptosis and neuronal differentiation (39, 40). The association of *CSDE1* to PPGL was recently described by TCGA group in a cohort study of 176 PPGL patients, in which four were found to have a somatic mutation— two frameshift, and two splice-site—, in *CSDE1* (15). Underexpression of CDSE1 has been reported in several tumors (41). Mutations in this gene result in downregulation of the apoptosis protease activator protein 1 (APAF1), which controls apoptosis in PCC cells under normal conditions (42, 43). Clinically, patients presented with sporadic cases, and some of them with recurrence or metastatic disease proposing a more aggressive form of PPGLs (15).

UBTF-MAML3 Fusion:

MAML3 (4q.31.1) is an oncogene that had been previously associated with other tumor types (44, 45). On the other hand, *UBTF* (17q21.31), encodes UBTF required for the expression of rRNA subunits (46). The association of the fusion with PPGL was identified by TCGA group in 2017 when analysis from RNA sequencing of the same cohort mentioned above revealed that ten tumors (eight primary, and two primary-metastatic) were positive for a *MAML3* fusion gene. Patients presented with a unique and expansive hypomethylation profile that was correlated with mRNA overexpression of target genes involved in developmental pathways, such as Wnt receptor signaling and Hedgehog signaling, that were significantly overexpressed including miR-375, β-catenin, DVL3, and GSK3 (15). MAML3-related PPGLs had the highest Ki-67 index expression, and some patients presented with aggressive disease (15). Finding *UBTF-MAML3* fusion predicts a poor prognosis, as compared with other syndromic forms of PPGL (15).

PROPOSED CLASSIFICATION FOR NEW GENES ACCORDING TO TCGA

Three additional genes were not included in the molecular classification done by TCGA (15). However, based on their signaling pathways, we believe that all three should be included as part of the cluster 1 or pseudohypoxia signaling group for future updates. While *MDH2* is a part of the TCA cycle, both *PHD1* (*EGLN2*) and *IRP1* belong to the *VHL/EPAS1*-related subtype (**Figure 1**) and have been very recently described.

MDH2:

MDH2 encodes the MDH2 enzyme, which catalyzes the reversible oxidation of malate to oxaloacetate in the TCA cycle. Deficiency of MDH2 has shown to lead to the accumulation of malate, fumarate, and succinate on Drosophila models (47). Thus, *MDH2* should be classified as a cluster 1-TCA cycle-related gene. In 2015, Cascon et al. were the first to report a case with a germline mutation in *MDH2*: a male patient diagnosed with multiple PGLs (13). Later, five asymptomatic relatives were tested, and two were found to be positive. Subsequent updates showed elevation of NE and the presence of a hypermethylator phenotype similar to *SDHx*-related PPGLs (48). Interestingly, no malate accumulation was found on tumor cells, but a high fumarate/succinate ratio was observed (13).

PHD1 (EGLN2):

PHD1 (*EGLN2*) encodes PHD1 enzyme, which in normal oxygen conditions hydroxylates specific proline residues of the HIF-α subunits for their subsequent degradation by proteasome. Deficiency of this enzyme prevents degradation of HIF-α and resulting in HIF stabilization leading to a global activation of signaling pathways that lead to tumorigenesis (49). *PHD1* should therefore be classified as cluster 1-*VHL/EPAS1*-related gene. In 2008, Ladroue et al. were the first to describe the association of mutations in *PHD2* with polycythemia and PPGLs (50). Seven years later, Yang et al. reported the first mutation in *PHD1* (*EGLN2*), found to be associated with PPGL, along with an additional case with *PHD2* (*EGLN1*) germline mutation. These two patients presented with multiple recurrent PPGL, polycythemia with normal or mildly elevated EPO, and were negative for *HIF2A* mutation. Both patients presented with catecholamine related symptoms including: headaches, episodic chest pain, anxiety, and hypertension. Both cases revealed a noradrenergic profile. In the patient with the mutation in *PHD1 (EGLN2)*, sensitivity of erythroid progenitors to EPO and erythropoietin receptor (EpoR) activity were inappropriately increased and resulted in polycythemia with no or mild increase in EPO levels with increased EpoR expression (12).

IRP1:

IRP1 is a tumor suppression gene that encodes IRP1, a bi-functional protein that controls cellular iron metabolism and negatively regulates HIF2α mRNA translation under iron-deficient conditions (51, 52). Thus, deficiency of IRP1 increases

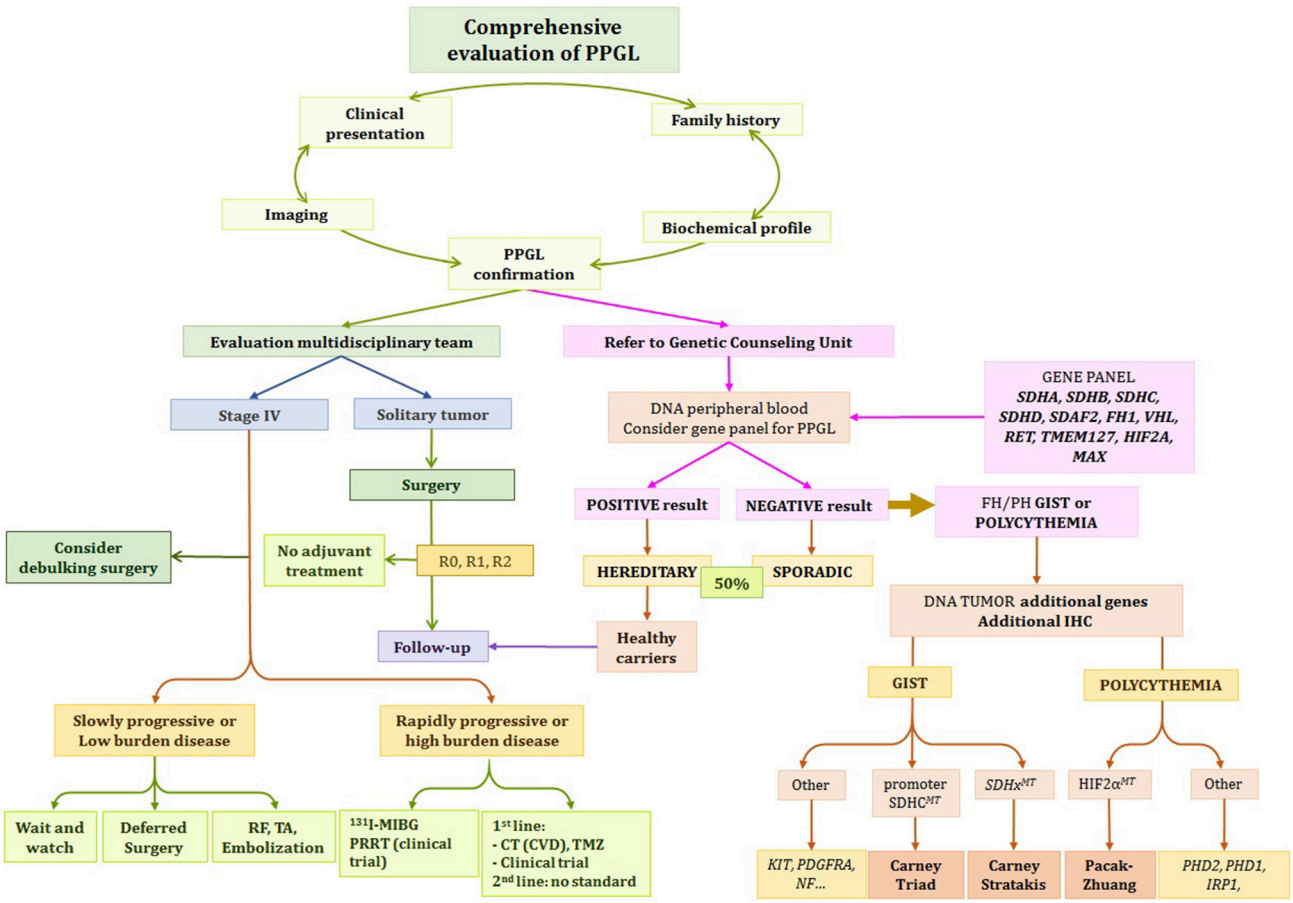

FIGURE 2 | Proposed Algorithm for Evaluation and Management of PPGL Patient. *Depending on clinical status, growth acceleration, symptoms, and genetic status, the options include: observation, systemic therapy, chemotherapy, PRRT, ^{131}I-MIBG, or referral to clinical trials. R0, microscopically margin-negative resection; R1, microscopically margin-positive resection; R2, macroscopically margin-positive resection; stage IV, metastatic disease (1).

HIF2α by dissociating sequences of HIF2α mRNA from iron-responsive element and suppressing protein translation. Activation of HIF-2α leads to an increase of transcription of EPO and EpoR. Recently, *IRP1* association with PCC was reported by Pang et al. in a patient with a medical history of polycythemia vera with a proven *JAK2* mutation, hypertension, diaphoresis, and abdominal pain that led to the diagnosis of PCC years later. An investigational gene panel consisting of 54 tumor-associated genes was negative for patient's peripheral blood DNA. Subsequent tumor DNA sequencing revealed a somatic, loss of function mutation in *IRP1* located on exon 3 splicing site (16).

Driver Mutation Gene With Unknown Classification

H3F3A

Located on chromosome 1, *H3F3A* encodes the histone H3.3 protein (53). Histones are responsible for nucleosome formation, and as a chromatin regulator, mutations of this gene will affect DNA methylation, chromatin remodeling, or nucleosome positioning (54). Defects in *H3F3A* have been linked with diffuse intrinsic pontine glioma (DIPG) (55, 56), chondroblastoma, and giant cell tumor of the bone (GCT) (57). Association with PPGL was initially reported in 2013 as a case report (58). Three years later, in 2016, Toledo et al. analyzed 43 samples of 41 patients using exome or transcriptome sequencing and detected a postzygotic *H3F3A* mutation in three tumors from one patient with a history of recurrent GCT. This patient presented with bilateral PCCs and developed bladder and several periaortic PGLs later. Family history of PPGL was absent. Further analysis showed that this mutation was identical to an oncogenic driver of sporadic GCT (c.103 G > T, p.G34W) (57, 58). Furthermore, additional variants in other chromatin remodeling genes (*KMT2B, EZH2, SETD2, ATRX, JMJD1C, KDM2B*) were reported in this study (28). Additionally, two kinase receptor-encoding genes (*MERTK, MET*) were found (28). Also, the investigators detected a somatic mutation in the main hotspot residue of the fibroblast growth factor receptor 1 gene (28), which is known to play a role in other cancers, such as glioblastomas (59). Further studies are needed to clarify the role of these genes in the pathogenesis of PPGL.

DISEASE MODIFYING GENE

ATRX

Located on the X chromosome, *ATRX* encodes the transcriptional regulator ATRX, which belongs to the SWI/SNF family of chromatin remodeling proteins. ATRX plays a role in the histone deposition in telomeres, chromosome segregation in the cell cycle and transcription regulation (60–62). Germline mutations in *ATRX* have been reported as a cause of X-linked alpha thalassemia mental retardation syndrome (ATRX syndrome) (63). In 2015, using WES, Fishbein et al. reported somatic *ATRX* mutations in two *SDHB*-related frozen tumors. They found somatic *ATRX* mutations in 12.6% of the samples, along with an alternative lengthening of telomeres seen on fluorescence *in situ* hybridization (FISH) and presenting with more aggressive disease (29). Later, the first case of an *ATRX* driver mutation was reported in a patient with a metastatic composite PCC-PGL, clinically with anemia, weight loss, and hepatic metastases. WES showed a somatic loss of function on the *ATRX* gene, along with downregulation of genes involved in the neuronal development and homeostasis (*NLGN4, CD99,* and *CSF2RA*) and upregulation of *Drosha* gene related with RNA processing and alternative lengthening of telomeres (14). Somatic mutations have been reported in association with co-existing mutations in the isocitrate dehydrogenase 1 and 2 (*IDH 1/2*) genes in both adult and pediatric patients with astrocytic tumors (64). In addition, ATRX may play a driver mutation role for sporadic PPGL (14) and truncated ATRX could potentially play a synergistic role with *SDHx* in tumor initiation and might be a predisposition for a more aggressive disease (15).

Biochemical Evaluation

Biochemical evaluation has made tremendous strides forward since the 1950's when colorimetric assays were first implemented, and later replaced with the more accurate high-performance liquid chromatography (HPLC) in the 1980s. Currently, liquid chromatography tandem-mass spectrometry (LC-MS) has become the new gold standard due to its accuracy and reproducibility. A value three times the upper range of normal is a positive result. However, some patients present with pseudo-silent PPGL and high tumor burden resulting in a late diagnosis. Therefore, any values above the normal range should be carefully considered as positive in the setting of prospective screening in hereditary forms of the disease, given early proactive surveillance has made pre-detection of tumors very possible.

Besides screening and diagnoses, biochemical phenotype of the tumor is a useful tool for PPGL syndromic assessment. PPGLs can be classified according to their biochemical profile. This classification allows the establishment of an algorithm and addresses specific causative genes. Here, we present an update of the different PPGL-related biochemical phenotypes.

A- Truly biochemically silent phenotype: Often associated with *SDHx* syndromes, truly silent PPGLs are mostly located in the head and neck area (HNPGL). Among HNPGL, carotid body tumors are the most frequent (65), followed by glomus vagale, jugulotympanic, and laryngeal PGLs.

B- Biochemically pseudo-silent phenotype: In this category, PPGLs present with levels of catecholamines and metanephrines that can be "normal" or "near-normal" in a misleading way. This category should be distinguished from group A since PPGLs in this category are indeed catecholamine-producing tumors. However, detection of elevated metanephrine and normetanephrine falls below the limit of detection due to either low tumor burden or catecholamine production fluctuations. This usually happen in patients with very small (less than 5–7 mm) PPGLs.

C- Noradrenergic phenotype: Characterized by increased levels of NE/NMN (66, 67), noradrenergic PPGLs are commonly located outside the adrenals (66, 67), NE acts on both α (1 and 2) and β (1, 2, and 3) adrenoreceptors with predominant effect on α. These patients present less frequently with paroxysmal symptomatology. Sustained hypertension and tachycardia are the most common symptoms. However, hypertensive crisis, myocardial infarction, lethal tachyarrhythmia, and acute intramural hemorrhage have been reported (68, 69). This phenotype is commonly seen in the cluster 1/ pseudohypoxia group (15), including both *VHL* and *SDHx* mutations (70).

D- Adrenergic phenotype: These PPGLs are characterized by either purely elevated epinephrine (E)/metanephrine (MN) (66), or in both E/MN and NE/NMN. This phenotype can be accurately identified when the plasma level of free MN is greater than 10% of the sum of NMN and MN (66). Adrenergic PPGLs are often located in the adrenal gland (67). Epinephrine represents a higher level of cellular differentiation of adrenal PPGL when compared with tumors derived from paraganglia (71, 72). Epinephrine activates α-1 and α-2 adrenoceptors with a higher affinity compared to NE (73) but also affects β-2 adrenoceptors. Paroxysmal symptomatology due to a rapid metabolism (74) has been related to the concomitant use of medications like histamine, tricyclic antidepressants (TCA), anesthetics, and tyramine-rich food (75). In many cases these patients are found to have hyperglycemia and hyperlipidemia secondary to the stimulus of lipolysis, glycogenolysis, and gluconeogenesis (68). This phenotype is commonly seen in the cluster 2/kinase signaling group (15).

E- Dopaminergic phenotype: These PPGLs are characterized by high levels of DA/3-methoxytyramine (3-MT) with normal or near-normal levels of E/MN and NE/NMN (75, 76). Tumors are commonly extra-adrenal and primarily located in the head and neck region (77, 78). Patients can be either asymptomatic or have atypical symptoms like abdominal pain, diarrhea, nausea/vomiting, hypotension, and weight loss. These symptoms are most likely related to dopamine receptor stimulation on the smooth muscle (79), gastrointestinal tract (80), and central nervous system (81). This subset of patients has been traditionally classified as biochemically "silent". Elevated levels of DA/3MT together with NE have been reported in approximately 65% of patients with *SDHx* mutations, especially in *SDHB* (76, 82).

Experts Recommendations and Meta-Analysis

The Endocrine Society 2014 guidelines recommend initial screening with either plasma or urine fractionated MN/NMN, with the consensus that values three to four times higher than the upper reference limit are almost always diagnostic for PPGLs (83). A recent meta-analysis done by Därr et al., compared the accuracy of plasma and urine metanephrines in 1,039 patients with PPGL. The study also compared immunoassay methods—HPLC and LC-MS—and differences between supine vs. seated position during sampling. Results in terms of sensitivity/specificity/accuracy showed that supine sampling was more sensitive in tumor detection (95 vs. 89% [$p < 0.02$]). Furthermore, the supine position has a higher specificity than 24-h urine samples (95 vs. 90% [$p < 0.03$]) and the highest accuracy (95%), especially when measured with HPLC and LC-MS over immunoassay (84) as LC-MS eliminates drug interference providing more accurate results.

When evaluating pediatric patients with PPGL, considerations for age-adjusted reference values are crucial to determine the tests positivity. Higher limits for E/MN along with lower limits for NE/NMN were seen in children when compared with adults (85). Finally, we wish to note that LC-MS is not widely available in all countries. Cost of technology itself as well as a lack of trained staff stand as barriers to full implementations of LC-MS (86).

Imaging

The latest Endocrine Society Guidelines (83) emphasize that consideration for any imaging modality in PPGLs requires prior positive biochemical evidence of disease except for the presence of personal or family history of HNPGL related or not to a hereditary form of the disease. For a general work-up, computed tomography (CT) is recommended as the anatomic imaging modality of choice due to its excellent spatial resolution. Magnetic resonance imaging (MRI) is recommended for children, pregnant women, or patients with HNPGL or metastatic disease. Regarding functional imaging the panel of experts suggests the use of ^{123}I-metaiodobenzylguanidine (MIBG) scintigraphy in patients with metastatic disease when treatment with radiotherapy using ^{131}I-MIBG is considered or when the risk of metastasis or recurrence of the disease is high based on tumor size. However, if metastatic disease is confirmed, the use of ^{18}F-fluorodeoxyglucose (^{18}F-FDG) positron emission tomography hybridized with CT (PET/CT) is preferred (83).

We believe PPGL is a disease "born to be filmed" as the availability of high-value modalities for imaging provides a versatile algorithm to evaluate the different scenarios of the disease: in the initial evaluation setting, in treatment planification, and for tumor response assessment. This is due to the expression of various transporters and receptors in the surface of PPGL cells. Receptors that can be exploited for imaging include the NE transporter, glucose transporter (GLUT), amino acid transporter, and somatostatin receptor (SSTR) (87, 88). Currently, there are three types of PET/CT radiopharmaceuticals that exert their actions through these receptors: ^{18}F-FDG, ^{18}F-fluorodopa (^{18}F-FDOPA), and ^{68}Galium (^{68}Ga)- tetraazacyclododecanetetraacetic acid (DOTA) analogs (89–91). Functional imaging using PET/CT has proven to be superior to ^{123}I-MIBG SPECT not only for the higher detection, sensitivity, localization, and resolution, but also for reducing indeterminate or equivocal findings by about 20 to 40% (92). Among these modalities, ^{68}Ga- DOTA-Tyr3-octreotate (DOTATATE) PET/CT has emerged as the preferred modality in NETs in general. This is due to the high affinity of the radiolabeled compound for the SSTR type 2 ($SSTR_2$) (93) that can more accurately predict a tumor response to treatment to radiolabeled somatostatin analogs in patients with avidity for the tracer. In terms of tumor detection, ^{68}Ga-DOTATATE PET/CT has proven to be exceptional with a 98.6% overall detection rate in patients with *SDHB* metastatic PPGL, superior to anatomic imaging (CT/MRI), and other functional scans (^{18}F-FDG, ^{18}F-FDOPA, and ^{18}F-FDA PET/CT) (94). Similar results were also observed in sporadic cases with a sensitivity of 97.6% (89, 95) and in patients with HNPGL (96). Regarding specific mutations, ^{68}Ga-DOTATATE PET/CT resulted inferior in the evaluation of patients with polycythemia/PPGL— including both *HIF2A* and *PHD1*-related tumors—(97), *FH* or *MAX* mutations. In these patients— polycythemia associated to PPGL—, the combination of ^{18}F-FDOPA PET/CT and ^{18}F-FDA PET/CT resulted superior with a lesion-base detection rate of ∼98%, vs. 35.3% for the ^{68}Ga-DOTATATE PET/CT group (95% CI, 25.0–47.2%) (97). With respect to *FH*-related tumors, in a patient reported ^{68}Ga-DOTATATE PET/CT showed an overall detection of 66% when compared to ^{18}F-FDOPA PET/CT (98). In reference to *MAX*-related PCCs, in a recent study evaluating six patients, ^{68}Ga-DOTATATE accuracy was lower than ^{18}F-DOPA PET/CT (99). Based on these studies authors suggest allocating both ^{18}F-FDOPA and ^{18}F-FDA PET/CT in the diagnostic algorithm of polycythemia/PPGL patients and designate ^{18}F-FDOPA PET/CT as first functional imaging modality of choice in the diagnoses and follow-up of both *MAX* and *FH*-related patients. If the availability of ^{18}F-FDA and ^{18}F-FDOPA PET/CT is limited, use of other imaging modalities like ^{123}I-MIBG single-photon emission CT (SPECT) is recommended.

Regarding the pediatric population, ^{68}Ga-DOTATATE PET/CT has shown to be superior in a cohort of nine children with *SDHx*-related PPGL with a detection rate of 98.4% when compared to ^{18}F-FDG PET/CT (100). However, these results are intriguing as another study reported that the sensitivity of ^{68}Ga-DOTATATE PET/CT is lower for abdominal lesions in children, with a detection rate of 66.7% (100, 101), warranting additional larger studies in this population. Thus, the use of more than one functional imaging modality is recommended in the pediatric group and the use of both ^{68}Ga-DOTATATE and ^{18}F-FDG PET/CT is highly recommended in children with small lesions, when there is a high likelihood of metastatic disease and in those patients with *SDHx* mutations.

The utility of functional imaging using somatostatin analogs, was recently extended and exploited to open new doors for targeted radiotherapy using the both ^{177}Lutetium (^{177}Lu) or ^{90}Yttrium (^{90}Y). The 'so-called' peptide receptor radionuclide therapy (PRRT) has shown to a very effective therapeutic option in patients with advanced midgut NETs in a phase 3 clinical

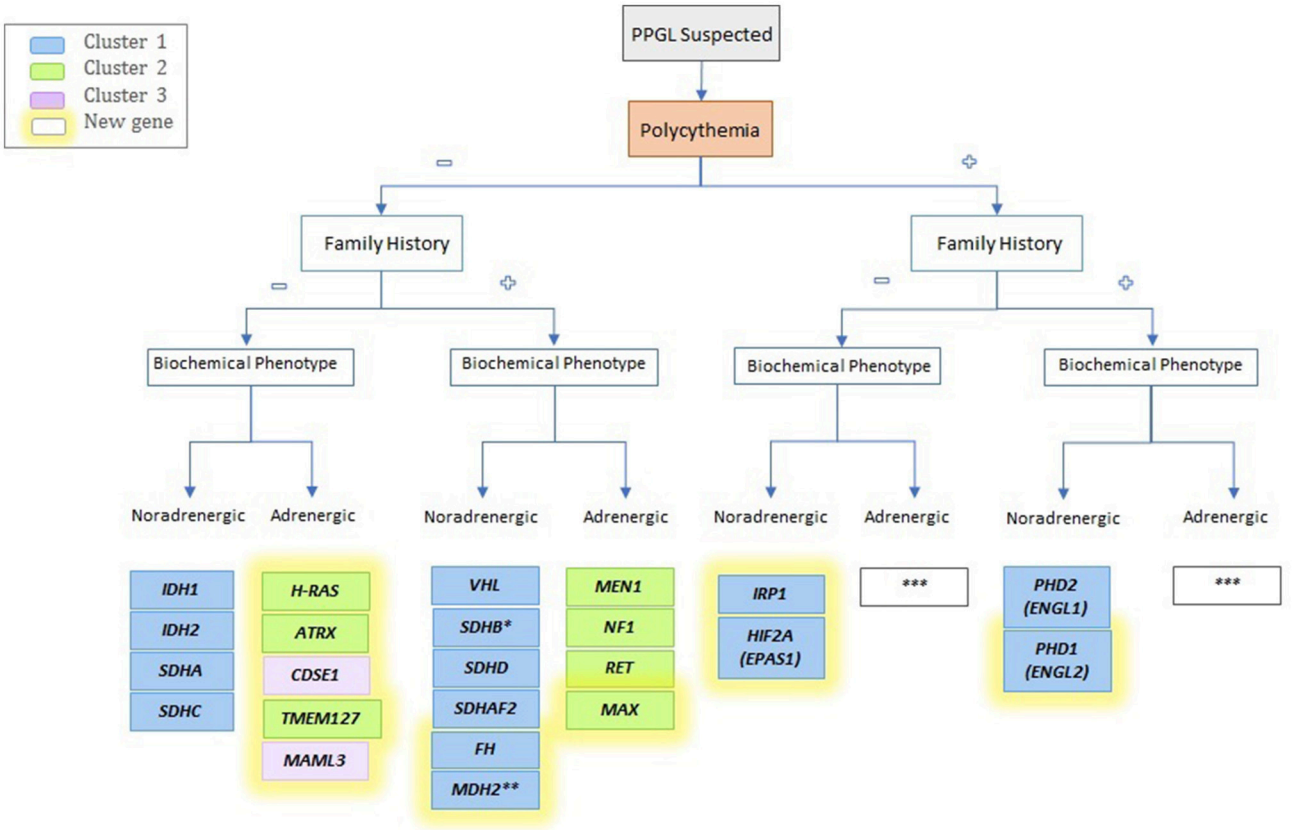

FIGURE 3 | Schematic algorithm for identifying a possible driver mutation of the newly identified genes mutation. *Often family history is absent; **Unknown; ***Not described yet.

trial called NETTER-1 published in 2017 (102). As a result, ^{177}Lu-DOTATATE (Lutathera®) has been recently approved by the food and drug administration (FDA) for the treatment of advanced midgut NETs. Applications of PRRT in PPGL have been assessed also in two small cohorts of patients with mediastinal or HNPGL (103) and with inoperable HNPGL (104), with promising outcomes in terms of tumor response and control of symptoms. A higher number of patients ($N = 20$) received four cycles of Lutathera® with encouraging results in terms of control of symptoms (decreased medication requirements), circulating chromogranin A, tumor response and control of disease with a median progression-free survival (PFS) of 29 months (105). When comparing PRRT with ^{131}I-MIBG in 22 patients with metastatic/progressive PPGL, PRRT showed increased PFS and tumor response rate, as well as increased event-free and overall survival (OS) (106). An ongoing prospective clinical trial at the NIH evaluating PRRT for progressive PPGL will provide definite answers regarding the utility and safety of PRRT in PPGL (NCT03206060).

The role of SSTR antagonists appears to be promising in the field of NETs. SSTR antagonists recognie more binding sites on SSTRs allowing better tumor visualization (107). Their clinical utility in functional imaging was first demonstrated in 2011 (108). A second-generation of $SSTR_2$ antagonists that include DOTA-JR11 showed higher tumor uptake when combined with ^{68}Ga-DOTA (1.3 times), or ^{68}Ga-NODAGA (1.7 times) as compared with DOTA analogs (109). In the preclinical setting, DOTA-JR11 was superior to ^{177}Lu in H69 cell lines, and *in vivo* therapy experiments achieved a higher uptake, median survival rate, and a longer delay in tumor growth (110). Regarding pharmacokinetics and dosimetry, DOTA-JR11 showed very promising results when tested in human embryonic kidney cells with a higher uptake and longer tumor residence time. With an escalating dose DOTA-JR11 demonstrated an improved safety profile and the potential decrease toxicity (111). In *in vitro* studies including both NETs and non-NETs, DOTA-JR11 showed a higher affinity for SSTTRs when compared to the $SSTR_2$ agonist ^{125}I-Tyr3-octreotide in NETs (2.5 to 40 times). Interestingly, the group of other non-NETs tumors— such as breast cancer, renal cell carcinoma, medullary thyroid cancer, and non-Hodgkin lymphoma—, was also targeted with high affinity, potentially opening a new door for exploring this modality in tumors where typically SSTR have played little role, if any (112). Currently, an ongoing clinical trial for metastatic and unresectable progressive, well-differentiated carcinoid is trying to elucidate the role of SSTR antagonist DOTA-JR11 both in diagnostic and therapeutic settings (NCT02609737).

CONCLUSION

In this article, we presented some of the latest advances in the rapidly evolving field of PPGL and we focused on genetic, biochemical and imaging discoveries over the last 6 years. In addition, we proposed an updated biochemical classification and provided a novel algorithm for identifying newly diagnosed PPGL (**Figure 3**). Ten new susceptibility genes related to PPGL have been described in the last 10 years, including new germline as well as somatic mutations. The last can be presented as mosaicisms and result in syndromic forms of the disease. Also, functional imaging modalities continue to improve, and lesion-base detection studies are more reliable and accurate. The wide availability of ^{68}Ga-DOTATATE PET/CT following the approval of the radiopharmaceutical compound by the FDA, is revolutionizing the way we diagnose not only PPGLs but also NETs in general. The expression of SSTR^2 on PPGL cells has allowed the exploitation of this modality as a therapeutic option in these patients using Lutathera®, which was recently approved for use in midgut NETs. Current ongoing clinical trials in PPGL will determine safety, tolerability profile, and also efficacy in terms of clinical benefit and control of disease. Hopefully the availability of these diagnostic modalities will be more implemented in developing countries in the future. The expense associated with these technologies stands as a barrier; therefore, referral to centers of excellence that specialize in PPGL is warranted and highly advisable as implementation of theranostics into an algorithm is required for establishing different therapeutic options.

We are now entering a new and exciting era for PPGL that will allow us to advance one step further toward personalized medicine, making precision medicine for PPGL a step closer (**Figure 2**).

AUTHOR CONTRIBUTIONS

RA: writing and editing MS, creating **Table 1** and **Figure 3**, proposing the idea of an update in the biochemical classification; AS: writing MS; IT: writing, editing and reviewing MS, creating **Figures 1, 2**; KP: creating outline and reviewing MS.

FUNDING

The author(s) disclosed receipt of the following financial support for the research, authorship, and/or publication of this article: This study was funded by National Institutes of Health (grant number: Z1AHD008735), by the Spanish Society of Medical Oncology Foundation (F SEOM 2015) and the PHEiPAS patient Alliance (2016).

ACKNOWLEDGMENTS

This research was supported, in part, by the Intramural Research Program of the NIH, NICHD. We would like to thank the NIH for the enormous support in patient care and research of this disease Library Writing Center for manuscript editing assistance; In addition, we wish to thank Ying Pang for reviewing the *IRP1* section.

REFERENCES

1. Tischler ASKRR, Gill A, Kawashima A, Kimura N, Komminoth P, Papathomas TG, et al. Tumours of the adrenal medulla and extra-adrenal paraganglia. In: Lloyd RV, Osamura RY, Klöppel G, Rosai J, editors. *WHO Classification of Tumours of Endocrine Organs 4th Edition*. Lyon: International Agency for Research on Cancer (IARC) (2017). pp. 179–207.
2. Whalen RK, Althausen AF, Daniels GH. Extra-adrenal pheochromocytoma. *J Urol*. (1992) 147:1–10. doi: 10.1016/S0022-5347(17)37119-7
3. Kimura N, Miura Y, Nagatsu I, Nagura H. Catecholamine synthesizing enzymes in 70 cases of functioning and non-functioning phaeochromocytoma and extra-adrenal paraganglioma. *Virchows Arch A Pathol Anat Histopathol*. (1992) 421:25–32. doi: 10.1007/BF01607135
4. Mena J, Bowen JC, Hollier LH. Metachronous bilateral nonfunctional intercarotid paraganglioma (carotid body tumor) and functional retroperitoneal paraganglioma: report of a case and review of the literature. *Surgery* (1993) 114:107–11.
5. van Duinen N, Steenvoorden D, Kema IP, Jansen JC, Vriends AH, Bayley JP, et al. Increased urinary excretion of 3-methoxytyramine in patients with head and neck paragangliomas. *J Clin Endocrinol Metab*. (2010) 95:209–14. doi: 10.1210/jc.2009-1632
6. Castro-Vega LJ, Letouzé E, Burnichon N, Buffet A, Disderot PH, Khalifa E, et al. Multi-omics analysis defines core genomic alterations in pheochromocytomas and paragangliomas. *Nat Commun*. (2015) 6:6044. doi: 10.1038/ncomms7044
7. Jochmanova I, Pacak K. Genomic landscape of pheochromocytoma and paraganglioma. *Trends Cancer* (2018) 4:6–9. doi: 10.1016/j.trecan.2017.11.001
8. Pacak K, Jochmanova I, Prodanov T, Yang C, Merino MJ, Fojo T, et al. New syndrome of paraganglioma and somatostatinoma associated with polycythemia. *J Clin Oncol*. (2013) 31:1690–8. doi: 10.1200/JCO.2012.47.1912
9. Zhuang Z, Yang C, Lorenzo F, Merino M, Fojo T, Kebebew E, et al. Somatic HIF2A gain-of-function mutations in paraganglioma with polycythemia. *N Engl J Med*. (2012) 367:922–30. doi: 10.1056/NEJMoa1205119
10. Castro-Vega LJ, Buffet A, De Cubas AA, Cascón A, Menara M, Khalifa E, et al. Germline mutations in FH confer predisposition to malignant pheochromocytomas and paragangliomas. *Hum Mol Genet*. (2014) 23:2440–6. doi: 10.1093/hmg/ddt639
11. Crona J, Delgado Verdugo A, Maharjan R, Stålberg P, Granberg D, Hellman P, et al. Somatic mutations in H-RAS in sporadic pheochromocytoma and paraganglioma identified by exome sequencing. *J Clin Endocrinol Metab*. (2013) 98:E1266–71. doi: 10.1210/jc.2012-4257
12. Yang C, Zhuang Z, Fliedner SM, Shankavaram U, Sun MG, Bullova P., et al. Germ-line PHD1 and PHD2 mutations detected in patients with pheochromocytoma/paraganglioma-polycythemia. *J Mol Med*. (2015) 93:93–104. doi: 10.1007/s00109-014-1205-7
13. Cascón A, Comino-Méndez I, Currás-Freixes M, de Cubas AA, Contreras L, Richter S, et al. Whole-exome sequencing identifies MDH2 as a new familial paraganglioma gene. *J Natl Cancer Inst*. (2015) 107:djv053. doi: 10.1093/jnci/djv053
14. Comino-Méndez I, Tejera ÁM, Currás-Freixes M, Remacha L, Gonzalvo P, Tonda R., et al. ATRX driver mutation in a composite malignant pheochromocytoma. *Cancer Genet*. (2016) 209:272–7. doi: 10.1016/j.cancergen.2016.04.058
15. Fishbein L, Leshchiner I, Walter V, Danilova L, Robertson AG, Johnson AR, et al. Comprehensive molecular characterization of

pheochromocytoma and paraganglioma. *Cancer Cell* (2017) 31:181–93. doi: 10.1016/j.ccell.2017.01.001
16. Pang Y, Lu Y, Caisova V, Liu Y, Bullova P, Huynh TT, et al. Targeting NAD(+)/PARP DNA repair pathway as a novel therapeutic approach to SDHB-mutated cluster i pheochromocytoma and paraganglioma. *Clin Cancer Res*. (2018) 24:3423–32. doi: 10.1158/1078-0432.CCR-17-3406
17. Carney JA. Carney triad: a syndrome featuring paraganglionic, adrenocortical, and possibly other endocrine tumors. *J Clin Endocrinol Metab*. (2009) 94:3656–62. doi: 10.1210/jc.2009-1156
18. Buffet A, Smati S, Mansuy L, Ménara M, Lebras M, Heymann MF., et al. Mosaicism in HIF2A-related polycythemia-paraganglioma syndrome. *J Clin Endocrinol Metab*. (2014) 99:E369–73. doi: 10.1210/jc.2013-2600
19. Castelblanco E, Santacana M, Valls J, de Cubas A, Cascón A, Robledo M, et al. Usefulness of negative and weak-diffuse pattern of SDHB immunostaining in assessment of SDH mutations in paragangliomas and pheochromocytomas. *Endocr Pathol*. (2013) 24:199–205. doi: 10.1007/s12022-013-9269-4
20. Menara M, Oudijk L, Badoual C, Bertherat J, Lepoutre-Lussey C, Amar L, et al. SDHD immunohistochemistry: a new tool to validate SDHx mutations in pheochromocytoma/paraganglioma. *J Clin Endocrinol Metab*. (2015) 100:E287–91. doi: 10.1210/jc.2014-1870
21. Pai R, Manipadam MT, Singh P, Ebenazer A, Samuel P, Rajaratnam S. Usefulness of Succinate dehydrogenase B (SDHB) immunohistochemistry in guiding mutational screening among patients with pheochromocytoma-paraganglioma syndromes. *APMIS* (2014) 122:1130–5. doi: 10.1111/apm.12269
22. Santi R, Rapizzi E, Canu L, Ercolino T, Baroni G, Fucci R, et al. Potential pitfalls of SDH immunohistochemical detection in paragangliomas and phaeochromocytomas harbouring germline SDHx gene mutation. *Anticancer Res*. (2017) 37:805–12. doi: 10.21873/anticanres.11381
23. Tomlinson IP, Alam NA, Rowan AJ, Barclay E, Jaeger EE, Kelsell D., et al. Germline mutations in FH predispose to dominantly inherited uterine fibroids, skin leiomyomata and papillary renal cell cancer. *Nat Genet*. (2002) 30:406–10. doi: 10.1038/ng849
24. Gupta G, Pacak K, Committee AAS. Precision medicine: an update on genotype/biochemical phenotype relationships in pheochromocytoma/paraganglioma patients. *Endocr Pract*. (2017) 23:690–704. doi: 10.4158/EP161718.RA
25. Clark GR, Sciacovelli M, Gaude E, Walsh DM, Kirby G, Simpson MA., et al. Germline FH mutations presenting with pheochromocytoma. *J Clin Endocrinol Metab*. (2014) 99:E2046–50. doi: 10.1210/jc.2014-1659.
26. Yang C, Sun MG, Matro J, Huynh TT, Rahimpour S, Prchal JT., et al. Novel HIF2A mutations disrupt oxygen sensing, leading to polycythemia, paragangliomas, and somatostatinomas. *Blood* (2013) 121:2563–6. doi: 10.1182/blood-2012-10-460972
27. Adari H, Lowy DR, Willumsen BM, Der CJ, McCormick F. Guanosine triphosphatase activating protein (GAP) interacts with the p21 ras effector binding domain. *Science* (1988) 240:518–21. doi: 10.1126/science.2833817
28. Toledo RA, Qin Y, Cheng ZM, Gao Q, Iwata S, Silva GM, et al. Recurrent mutations of chromatin-remodeling genes and kinase receptors in pheochromocytomas and paragangliomas. *Clin Cancer Res*. (2016) 22:2301–10. doi: 10.1158/1078-0432.CCR-15-1841
29. Fishbein L, Khare S, Wubbenhorst B, DeSloover D, D'Andrea K, Merrill S., et al. Whole-exome sequencing identifies somatic ATRX mutations in pheochromocytomas and paragangliomas. *Nat Commun*. (2015) 6:6140. doi: 10.1038/ncomms7140
30. Pang Y, Gupta G, Yang C, Wang H, Huynh TT, Abdullaev Z, et al. A novel splicing site IRP1 somatic mutation in a patient with pheochromocytoma and JAK2(V617F) positive polycythemia vera: a case report. *BMC Cancer* (2018) 18:286. doi: 10.1186/s12885-018-4127-x
31. Pollard PJ, Brière JJ, Alam NA, Barwell J, Barclay E, Wortham NC, et al. Accumulation of Krebs cycle intermediates and over-expression of HIF1alpha in tumours which result from germline FH and SDH mutations. *Hum Mol Genet*. (2005) 14:2231–9. doi: 10.1093/hmg/ddi227
32. Dahia PL. Pheochromocytoma and paraganglioma pathogenesis: learning from genetic heterogeneity. *Nat Rev Cancer*. (2014) 14:108–19. doi: 10.1038/nrc3648
33. Isaacs JS, Jung YJ, Mole DR, Lee S, Torres-Cabala C, Chung YL., et al. HIF overexpression correlates with biallelic loss of fumarate hydratase in renal cancer: novel role of fumarate in regulation of HIF stability. *Cancer Cell* (2005) 8:143–53. doi: 10.1016/j.ccr.2005.06.017
34. Clark GR, Sciacovelli M, Gaude E, Walsh DM, Kirby G, Simpson MA., et al. Germline FH mutations presenting with pheochromocytoma. *J Clin Endocrinol Metab*. (2014) 99:E2046–50. doi: 10.1210/jc.2014-1659
35. Tian H, McKnight SL, Russell DW. Endothelial PAS domain protein 1 (EPAS1), a transcription factor selectively expressed in endothelial cells. *Genes Dev*. (1997) 11:72–82. doi: 10.1101/gad.11.1.72
36. Lorenzo FR, Yang C, Ng Tang Fui M, Vankayalapati H, Zhuang Z, Huynh T, et al. A novel EPAS1/HIF2A germline mutation in a congenital polycythemia with paraganglioma. *J Mol Med.* (2013) 91:507–12. doi: 10.1007/s00109-012-0967-z
37. Därr R, Nambuba J, Del Rivero J, Janssen I, Merino M, Todorovic M, et al. Novel insights into the polycythemia-paraganglioma-somatostatinoma syndrome. *Endocr Relat Cancer* (2016) 23:899–908. doi: 10.1530/ERC-16-0231
38. Pacak K, Chew EY, Pappo AS, Yang C, Lorenzo FR, Wilson MW., et al. Ocular manifestations of hypoxia-inducible factor-2alpha paraganglioma-somatostatinoma-polycythemia syndrome. *Ophthalmology* (2014) 121:2291–3. doi: 10.1016/j.ophtha.2014.06.019
39. Mihailovich M, Militti C, Gabaldón T, Gebauer F. Eukaryotic cold shock domain proteins: highly versatile regulators of gene expression. *BioEssays* (2010) 32:109–18. doi: 10.1002/bies.200900122
40. Kobayashi H, Kawauchi D, Hashimoto Y, Ogata T, Murakami F. The control of precerebellar neuron migration by RNA-binding protein Csde1. *Neuroscience* (2013) 253:292–303. doi: 10.1016/j.neuroscience.2013.08.055
41. Cerami E, Gao J, Dogrusoz U, Gross BE, Sumer SO, Aksoy BA, et al. The cBio cancer genomics portal: an open platform for exploring multidimensional cancer genomics data. *Cancer Discovery* (2012) 2:401–4. doi: 10.1158/2159-8290.CD-12-0095
42. Dormoy-Raclet V, Markovits J, Malato Y, Huet S, Lagarde P, Montaudon D, et al. Unr, a cytoplasmic RNA-binding protein with cold-shock domains, is involved in control of apoptosis in ES and HuH7 cells. *Oncogene* (2007) 26:2595–605. doi: 10.1038/sj.onc.1210068
43. Elatmani H, Dormoy-Raclet V, Dubus P, Dautry F, Chazaud C, Jacquemin-Sablon H. The RNA-binding protein Unr prevents mouse embryonic stem cells differentiation toward the primitive endoderm lineage. *Stem Cells* (2011) 29:1504–16. doi: 10.1002/stem.712
44. Enlund F, Behboudi A, Andrén Y, Oberg C, Lendahl U, Mark J, et al. Altered notch signaling resulting from expression of a WAMTP1-MAML2 gene fusion in mucoepidermoid carcinomas and benign Warthin's tumors. *Exp Cell Res.* (2004) 292:21–8. doi: 10.1016/j.yexcr.2003.09.007
45. Amelio AL, Fallahi M, Schaub FX, Zhang M, Lawani MB, Alperstein AS, et al. CRTC1/MAML2 gain-of-function interactions with MYC create a gene signature predictive of cancers with CREB-MYC involvement. *Proc Natl Acad Sci USA.* (2014) 111:E3260–8. doi: 10.1073/pnas.1319176111
46. Wang K, Singh D, Zeng Z, Coleman SJ, Huang Y, Savich GL, et al. MapSplice: accurate mapping of RNA-seq reads for splice junction discovery. *Nucleic Acids Res*. (2010) 38:e178. doi: 10.1093/nar/gkq622
47. Wang L, Lam G, Thummel CS. Med24 and Mdh2 are required for Drosophila larval salivary gland cell death. *Dev Dyn*. (2010) 239:954–64. doi: 10.1002/dvdy.22213
48. Letouzé E, Martinelli C, Loriot C, Burnichon N, Abermil N, Ottolenghi C, et al. SDH mutations establish a hypermethylator phenotype in paraganglioma. *Cancer Cell*. (2013) 23:739–52. doi: 10.1016/j.ccr.2013.04.018
49. Kaelin WG Jr, Ratcliffe PJ. Oxygen sensing by metazoans: the central role of the HIF hydroxylase pathway. *Mol Cell*. (2008) 30:393–402. doi: 10.1016/j.molcel.2008.04.009
50. Ladroue C, Carcenac R, Leporrier M, Gad S, Le Hello C, Galateau-Salle F, et al. PHD2 mutation and congenital erythrocytosis with paraganglioma. *N Engl J Med*. (2008) 359:2685–92. doi: 10.1056/NEJMoa0806277
51. Meyron-Holtz EG, Ghosh MC, Rouault TA. Mammalian tissue oxygen levels modulate iron-regulatory protein activities *in vivo*. *Science* (2004) 306:2087–90. doi: 10.1126/science.1103786
52. Wilkinson N, Pantopoulos K. IRP1 regulates erythropoiesis and systemic iron homeostasis by controlling HIF2alpha mRNA translation. *Blood* (2013) 122:1658–68. doi: 10.1182/blood-2013-03-492454

53. Wells D, Hoffman D, Kedes L. Unusual structure, evolutionary conservation of non-coding sequences and numerous pseudogenes characterize the human H3.3 histone multigene family. *Nucleic Acids Res.* (1987) 15:2871–89. doi: 10.1093/nar/15.7.2871
54. Lehninger BPJ. Principles of biochemistry. In: Nelson DL, editors. MCC, 4th edition. New York, NY: W. H. Freemand and Co (2004), p. 1119.
55. Khuong-Quang DA, Buczkowicz P, Rakopoulos P, Liu XY, Fontebasso AM, Bouffet E, et al. K27M mutation in histone H3.3 defines clinically and biologically distinct subgroups of pediatric diffuse intrinsic pontine gliomas. *Acta Neuropathol.* (2012) 124:439–47. doi: 10.1007/s00401-012-0998-0
56. Wu G, Broniscer A, McEachron TA, Lu C, Paugh BS, Becksfort J., et al. Somatic histone H3 alterations in pediatric diffuse intrinsic pontine gliomas and non-brainstem glioblastomas. *Nat Genet.* (2012) 44:251–3. doi: 10.1038/ng.1102
57. Behjati S, Tarpey PS, Presneau N, Scheipl S, Pillay N, Van Loo P, et al. Distinct H3F3A and H3F3B driver mutations define chondroblastoma and giant cell tumor of bone. *Nat Genet.* (2013) 45:1479–82. doi: 10.1038/ng.2814
58. Iwata S, Yonemoto T, Ishii T, Araki A, Hagiwara Y, Tatezaki SI. Multicentric giant cell tumor of bone and paraganglioma: a case report. *JBJS Case Connect.* (2013) 3:e23. doi: 10.2106/JBJS.CC.L.00155
59. Rand V, Huang J, Stockwell T, Ferriera S, Buzko O, Levy S., et al. Sequence survey of receptor tyrosine kinases reveals mutations in glioblastomas. *Proc Natl Acad Sci USA.* (2005) 102:14344–9. doi: 10.1073/pnas.0507200102
60. Clynes D, Gibbons RJ. ATRX and the replication of structured DNA. *Curr Opinion Genet Dev.* (2013) 23:289–94. doi: 10.1016/j.gde.2013.01.005
61. Kernohan KD, Vernimmen D, Gloor GB, Berube NG. Analysis of neonatal brain lacking ATRX or MeCP2 reveals changes in nucleosome density, CTCF binding and chromatin looping. *Nucleic Acids Res.* (2014) 42:8356–68. doi: 10.1093/nar/gku564
62. Ratnakumar K, Bernstein E. ATRX: the case of a peculiar chromatin remodeler. *Epigenetics* (2013) 8:3–9. doi: 10.4161/epi.23271
63. Gibbons RJ, Picketts DJ, Villard L, Higgs DR. Mutations in a putative global transcriptional regulator cause X-linked mental retardation with alpha-thalassemia (ATR-X syndrome). *Cell* (1995) 80:837–45. doi: 10.1016/0092-8674(95)90287-2
64. Liu XY, Gerges N, Korshunov A, Sabha N, Khuong-Quang DA, Fontebasso AM, et al. Frequent ATRX mutations and loss of expression in adult diffuse astrocytic tumors carrying IDH1/IDH2 and TP53 mutations. *Acta Neuropathol.* (2012) 124:615–25. doi: 10.1007/s00401-012-1031-3
65. Persky MS, Setton A, Niimi Y, Hartman J, Frank D, Berenstein A. Combined endovascular and surgical treatment of head and neck paragangliomas–a team approach. *Head Neck.* (2002) 24:423–31. doi: 10.1002/hed.10068
66. Eisenhofer G, Lenders JW, Goldstein DS, Mannelli M, Csako G, Walther MM, et al. Pheochromocytoma catecholamine phenotypes and prediction of tumor size and location by use of plasma free metanephrines. *Clin Chem.* (2005) 51:735–44. doi: 10.1373/clinchem.2004.045484
67. van der Harst E, de Herder WW, de Krijger RR, Bruining HA, Bonjer HJ, Lamberts SW, et al. The value of plasma markers for the clinical behaviour of phaeochromocytomas. *Eur J Endocrinol.* (2002) 147:85–94. doi: 10.1530/eje.0.1470085
68. Pacak K. Phaeochromocytoma: a catecholamine and oxidative stress disorder. *Endocr Regul.* (2011) 45:65–90. doi: 10.4149/endo_2011_02_65
69. Zuber SM, Kantorovich V, Pacak K. Hypertension in pheochromocytoma: characteristics and treatment. *Endocrinol Metab Clin North Am.* (2011) 40:295–311, vii. doi: 10.1016/j.ecl.2011.02.002
70. Eisenhofer G, Pacak K, Huynh TT, Qin N, Bratslavsky G, Linehan WM., et al. Catecholamine metabolomic and secretory phenotypes in phaeochromocytoma. *Endocr Relat Cancer.* (2011) 18:97–111. doi: 10.1677/ERC-10-0211
71. Huynh TT, Pacak K, Wong DL, Linehan WM, Goldstein DS, Elkahloun AG, et al. Transcriptional regulation of phenylethanolamine N-methyltransferase in pheochromocytomas from patients with von Hippel-Lindau syndrome and multiple endocrine neoplasia type 2. *Ann N Y Acad Sci.* (2006) 1073:241–52. doi: 10.1196/annals.1353.026
72. Nilsson H, Jögi A, Beckman S, Harris AL, Poellinger L, Påhlman S. HIF-2alpha expression in human fetal paraganglia and neuroblastoma: relation to sympathetic differentiation, glucose deficiency, and hypoxia. *Exp Cell Res.* (2005) 303:447–56. doi: 10.1016/j.yexcr.2004.10.003
73. Pacak K, Eisenhofer G. An assessment of biochemical tests for the diagnosis of pheochromocytoma. *Nat Clin Pract Endocrinol Metab.* (2007) 3:744–5. doi: 10.1038/ncpendmet0615
74. Ito Y, Fujimoto Y, Obara T. The role of epinephrine, norepinephrine, and dopamine in blood pressure disturbances in patients with pheochromocytoma. *World J Surg.* (1992) 16:759–63; discussion. 763–754.
75. Pacak K, Eisenhofer G, Ilias I. Diagnosis of pheochromocytoma with special emphasis on MEN2 syndrome. *Hormones* (2009) 8:111–6. doi: 10.14310/horm.2002.1227
76. Van Der Horst-Schrivers AN, Osinga TE, Kema IP, Van Der Laan BF, Dullaart RP. Dopamine excess in patients with head and neck paragangliomas. *Anticancer Res.* (2010) 30:5153–58.
77. Righini Ch, Pecher M, Halimi S, Magne JL, Reyt E. [Malignant carotid paraganglioma. A case report]. *Ann Otolaryngol Chir Cervicofac.* (2003) 120:103–8.
78. van Duinen N, Corssmit EP, de Jong WH, Brookman D, Kema IP, Romijn JA. Plasma levels of free metanephrines and 3-methoxytyramine indicate a higher number of biochemically active HNPGL than 24-h urinary excretion rates of catecholamines and metabolites. *Eur J Endocrinol.* (2013) 169:377–82. doi: 10.1530/EJE-13-0529
79. Goldberg LI. Cardiovascular and renal actions of dopamine: potential clinical applications. *Pharmacol Rev.* (1972) 24:1–29.
80. Marmon LM, Albrecht F, Canessa LM, Hoy GR, Jose PA. Identification of dopamine1A receptors in the rat small intestine. *J Surg Res.* (1993) 54:616–20. doi: 10.1006/jsre.1993.1094
81. Shen WW. Vomiting, chemoreceptor trigger zone, and dopamine. *Psychosomatics* (1989) 30:118–9. doi: 10.1016/S0033-3182(89)72333-1
82. Eisenhofer G, Lenders JW, Timmers H, Mannelli M, Grebe SK, Hofbauer LC, et al. Measurements of plasma methoxytyramine, normetanephrine, and metanephrine as discriminators of different hereditary forms of pheochromocytoma. *Clin Chem.* (2011) 57:411–20. doi: 10.1373/clinchem.2010.153320
83. Lenders JW, Duh QY, Eisenhofer G, Gimenez-Roqueplo AP, Grebe SK, Murad MH, et al. Pheochromocytoma and paraganglioma: an endocrine society clinical practice guideline. *J Clin Endocrinol Metab.* (2014) 99:1915–42. doi: 10.1210/jc.2014-1498
84. Därr R, Kuhn M, Bode C, Bornstein SR, Pacak K, Lenders JWM., et al. Accuracy of recommended sampling and assay methods for the determination of plasma-free and urinary fractionated metanephrines in the diagnosis of pheochromocytoma and paraganglioma: a systematic review. *Endocrine* (2017) 56:495–503. doi: 10.1007/s12020-017-1300-y
85. Weise M, Merke DP, Pacak K, Walther MM, Eisenhofer G. Utility of plasma free metanephrines for detecting childhood pheochromocytoma. *J Clin Endocrinol Metab.* (2002) 87:1955–60. doi: 10.1210/jcem.87.5.8446
86. Eisenhofer G, Peitzsch M, McWhinney BC. Impact of LC-MS/MS on the laboratory diagnosis of catecholamine-producing tumors. *TrAC Trends in Anal Chem.* (2016) 84:106–16. doi: 10.1016/j.trac.2016.01.027
87. Ilias I, Pacak K. Current approaches and recommended algorithm for the diagnostic localization of pheochromocytoma. *J Clin Endocrinol Metab.* (2004) 89:479–91. doi: 10.1210/jc.2003-031091
88. Pacak K, Ilias I, Adams KT, Eisenhofer G. Biochemical diagnosis, localization and management of pheochromocytoma: focus on multiple endocrine neoplasia type 2 in relation to other hereditary syndromes and sporadic forms of the tumour. *J Intern Med.* (2005) 257:60–8. doi: 10.1111/j.1365-2796.2004.01425.x
89. Archier A, Varoquaux A, Garrigue P, Montava M, Guerin C, Gabriel S., et al. Prospective comparison of (68)Ga-DOTATATE and (18)F-FDOPA PET/CT in patients with various pheochromocytomas and paragangliomas with emphasis on sporadic cases. *Eur J Nucl Med Mol Imaging* (2016) 43:1248–57. doi: 10.1007/s00259-015-3268-2
90. Garrigue P, Bodin-Hullin A, Balasse L, et al. The Evolving Role of Succinate in Tumor Metabolism: An (18)F-FDG-Based Study. *J Nucl Med.* (2017) 58:1749–55. doi: 10.2967/jnumed.117.192674
91. Taieb D, Hicks RJ, Pacak K. PET Imaging for endocrine malignancies: from woe to go. *J Nucl Med.* (2017) 58:878–80. doi: 10.2967/jnumed.117.189688
92. Schiesser M, Veit-Haibach P, Muller MK, Weber M, Bauerfeind P, Hany T, et al. Value of combined 6-[18F]fluorodihydroxyphenylalanine PET/CT

for imaging of neuroendocrine tumours. *Br J Surg*. (2010) 97:691–97. doi: 10.1002/bjs.6937

93. Sagman U, Mullen JB, Kovacs K, Kerbel R, Ginsberg R, Reubi JC. Identification of somatostatin receptors in human small cell lung carcinoma. *Cancer* (1990) 66:2129–33. doi: 10.1002/1097-0142(19901115)66:10<2129::AID-CNCR2820661015>3.0.CO;2-T
94. Janssen I, Blanchet EM, Adams K, Chen CC, Millo CM, Herscovitch P, et al. Superiority of [68Ga]-DOTATATE PET/CT to other functional imaging modalities in the localization of SDHB-associated metastatic pheochromocytoma and paraganglioma. *Clin Cancer Res*. (2015) 21:3888–95. doi: 10.1158/1078-0432.CCR-14-2751
95. Janssen I, Chen CC, Millo CM, Ling A, Taieb D, Lin FI., et al. PET/CT comparing (68)Ga-DOTATATE and other radiopharmaceuticals and in comparison with CT/MRI for the localization of sporadic metastatic pheochromocytoma and paraganglioma. *Eur J Nucl Med Mol Imaging* (2016) 43:1784–91. doi: 10.1007/s00259-016-3357-x
96. Janssen I, Chen CC, Taieb D, Patronas NJ, Millo CM, Adams KT, et al. 68Ga-DOTATATE PET/CT in the localization of head and neck paragangliomas compared with other functional imaging modalities and CT/MRI. *J Nucl Med*. (2016) 57:186–91. doi: 10.2967/jnumed.115.161018
97. Janssen I, Chen CC, Zhuang Z, Millo CM, Wolf KI, Ling A, et al. Functional imaging signature of patients presenting with polycythemia/paraganglioma syndromes. *J Nucl Med*. (2017) 58:1236–42. doi: 10.2967/jnumed.116.187690
98. Nambuba J, Därr R, Janssen I, Bullova P, Adams KT, Millo C, et al. Functional imaging experience in a germline fumarate hydratase mutation-positive a patient with pheochromocytoma and paraganglioma. *AACE Clin Case Rep Summer* (2016) 2:e176–81. doi: 10.4158/EP15759.CR
99. Taïeb D, Jha A, Guerin C, Pang Y, Adams KT, Chen CC, et al. 18F-FDOPA PET/CT imaging of MAX-related pheochromocytoma. *J Clin Endocrinol Metab*. (2018) 103:1574–82. doi: 10.1210/jc.2017-02324
100. Jha A, Ling A, Millo C, Gupta G, Viana B, Lin FI, et al. Superiority of (68)Ga-DOTATATE over (18)F-FDG and anatomic imaging in the detection of succinate dehydrogenase mutation (SDHx)-related pheochromocytoma and paraganglioma in the pediatric population. *Eur J Nucl Med Mol Imaging* (2018) 45:787–97. doi: 10.1007/s00259-017-3896-9
101. Babic B, Patel D, Aufforth R, Assadipour Y, Sadowski SM, Quezado M, et al. Pediatric patients with pheochromocytoma and paraganglioma should have routine preoperative genetic testing for common susceptibility genes in addition to imaging to detect extra-adrenal and metastatic tumors. *Surgery* (2017) 161:220–7. doi: 10.1016/j.surg.2016.05.059
102. Strosberg J, Krenning E. 177Lu-dotatate for midgut neuroendocrine tumors. *N Engl J Med*. (2017) 376:1391–2. doi: 10.1056/NEJMoa1607427
103. Zovato S, Kumanova A, Demattè S, Sansovini M, Bodei L, Di Sarra D, et al. Peptide receptor radionuclide therapy (PRRT) with 177Lu-DOTATATE in individuals with neck or mediastinal paraganglioma (PGL). *Horm Metab Res*. (2012) 44:411–4. doi: 10.1055/s-0032-1311637
104. Puranik AD, Kulkarni HR, Singh A, Baum RP. Peptide receptor radionuclide therapy with (90)Y/ (177)Lu-labelled peptides for inoperable head and neck paragangliomas (glomus tumours). *Eur J Nucl Med Mol Imaging* (2015) 42:1223–30. doi: 10.1007/s00259-015-3029-2
105. Kong G, Grozinsky-Glasberg S, Hofman MS, et al. Efficacy of Peptide Receptor Radionuclide Therapy (PRRT) for Functional Metastatic Paraganglioma and Phaeochromocytoma. *J Clin Endocrinol Metab*. (2017) 102:3278–87. doi: 10.1210/jc.2017-00816
106. Nastos K, Cheung VTF, Toumpanakis C, Navalkissoor S, Quigley AM, Caplin M, et al. Peptide receptor radionuclide treatment and (131)I-MIBG in the management of patients with metastatic/progressive phaeochromocytomas and paragangliomas. *J Surg Oncol*. (2017) 115:425–34. doi: 10.1002/jso.24553
107. Ginj M, Zhang H, Waser B, Cescato R, Wild D, Wang X., et al. Radiolabeled somatostatin receptor antagonists are preferable to agonists for in vivo peptide receptor targeting of tumors. *Proc Natl Acad Sci USA*. (2006) 103:16436–41. doi: 10.1073/pnas.0607761103
108. Wild D, Mäcke HR, Waser B, Reubi JC, Ginj M, Rasch H, et al. 68Ga-DOTANOC: a first compound for PET imaging with high affinity for somatostatin receptor subtypes 2 and 5. *Eur J Nucl Med Mol Imaging* (2005) 32:724. doi: 10.1007/s00259-004-1697-4
109. Fani M, Braun F, Waser B, Beetschen K, Cescato R, Erchegyi J, et al. Unexpected sensitivity of sst2 antagonists to N-terminal radiometal modifications. *J Nucl Med*. (2012) 53:1481–9. doi: 10.2967/jnumed.112.102764
110. Dalm SU, Nonnekens J, Doeswijk GN, de Blois E, van Gent DC, Konijnenberg MW, et al. Comparison of the therapeutic response to treatment with a 177Lu-labeled somatostatin receptor agonist and antagonist in preclinical models. *J Nucl Med*. (2016) 57:260–5. doi: 10.2967/jnumed.115.167007
111. Nicolas GP, Mansi R, McDougall L, Kaufmann J, Bouterfa H, Wild D, et al. Biodistribution, pharmacokinetics, and dosimetry of (177)Lu-, (90)Y-, and (111)In-labeled somatostatin receptor antagonist OPS201 in comparison to the agonist (177)Lu-dotatate: the mass effect. *J Nucl Med*. (2017) 58:1435–41. doi: 10.2967/jnumed.117.191684
112. Reubi JC, Waser B, Macke H, Rivier J. Highly increased 125I-JR11 antagonist binding *in vitro* reveals novel indications for sst2 targeting in human cancers. *J Nucl Med*. (2017) 58:300–6. doi: 10.2967/jnumed.116.177733

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Endocrinology

REVIEW
published: 04 December 2018
doi: 10.3389/fendo.2018.00711

What's New in Endocrinology: The Chromaffin Cell

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Recent advances in understanding the intracellular and intercellular features of adrenal chromatin cells as stress transducers are reviewed here, along with their implications for endocrine function in other tissues and organs participating in endocrine regulation in the mammalian organism.

Keywords: chromaffin cell, catestatin, PACAP, interleukin 6, gap junction, NCS-Rapgef2

OPEN ACCESS

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Specialty section:
This article was submitted to Neuroendocrine Science, a section of the journal Frontiers in Endocrinology

Received: *17 August 2018*
Accepted: *12 November 2018*
Published: *04 December 2018*

Citation:
Eiden LE and Jiang SZ (2018) What's New in Endocrinology: The Chromaffin Cell. Front. Endocrinol. 9:711. doi: 10.3389/fendo.2018.00711

INTRODUCTION

It is sometimes useful to view the sprawling field of endocrinology through a narrow window to view advances that might otherwise be lost in the larger and more chaotic picture. Here, we take the opportunity to review briefly recent advances in our understanding of the regulation of the mammalian adrenal medulla, the storehouse for secretion of epinephrine that is critical for cardiovascular, neuronal, and metabolic homeostatic control, especially during stress. We compare the accepted view of the adrenal medulla of the twentieth century, still promulgated in most textbooks, and what we know about its function given recent information over the past decade or so. In each of five areas, we present "foundational" data, and then review emerging information sets that have significantly changed how the adrenal medulla is viewed as a stress-transducing endocrine tissue, and in addition shed significant light on the function of other mammalian endocrine organs and systems.

The first area in which significantly new developments in our understanding of chromaffin cell function have occurred is the emergence of the neuropeptide PACAP, rather than acetylcholine, as the physiologically dominant splanchnicoadrenomedullary synaptic neurotransmitter during stress. A second is the discovery in adrenal medulla of a neuroendocrine-specific cyclic AMP effector that propagates signaling for gene regulation in response to stress into the chromaffin cell. Third, peptides found in adrenal medulla and released from it may have important paracrine and autocrine roles, while their roles as both biomarkers and physiological actors is still unfolding. A fourth emerging concept is that chromaffin cells of the adrenal medulla work together via altered gap junction coupling and cellular adhesion during certain physiological states, predominantly in stress. Finally, we are becoming aware, with acquisition of new *in vivo* and cell culture data, that the adrenal medulla is not only the original "stress transducer" of the body, but also a regulatory nexus for the secretion of peptides important in stress-inflammation interactions and in integration of stress responding with the sensory nervous system. We will take each of these points in order, and end by summarizing how an updated view of adrenomedullary function has illuminated endocrine cell and organ function in general (**Figure 1**).

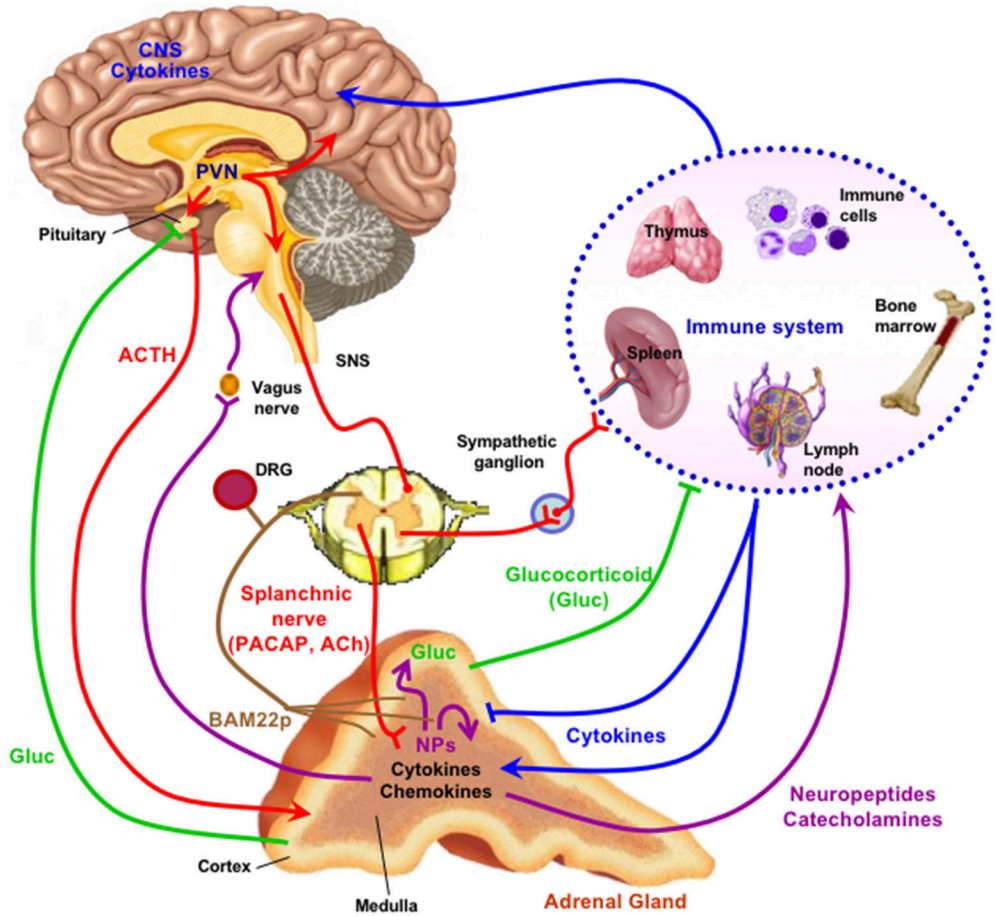

FIGURE 1 | The adrenal medulla as a stress transducer and neuroimmunoinflammatory and cardiovascular regulator. Cytokines are delivered to receptors on chromaffin cells as blood-borne messengers or via mobile secreting cells such as monocytes/macrophages; neurotransmitters PACAP and ACh are delivered to receptors on chromaffin cells via the splanchnic nerve. Activation of chromaffin cell signaling pathways (see **Figure 2**) increase expression of genes encoding neuropeptides (NPs), other mediators, catecholamine biosynthetic enzymes, and adhesion factors and connexins that increase cell-cell communication among chromaffin cells, and amplify catecholamine, neuropeptide, and chromogranin output in response to stress. Secreted neuropeptides such as galanin may in turn act upon the adrenal cortex to modulate glucocorticoid output; catecholamines act as hormones at cardiovascular and other targets; chromogranins and their derived peptides have both autocrine and paracrine actions in the adrenal medulla and also as hormones; BAM-22P may act on receptors on sensory neurons innervating the adrenal gland. For further explication of the figure, see the text. Note that there is some degree of species specificity to expression of cytokines and neuropeptides in adrenal medulla, so that the schematic offered here may differ in some particulars depending on the species, including *H. sapiens*, under consideration (1–8). Figure adapted from (9).

PACAP AS A STRESS TRANSMITTER AT THE SPLANCHNICOADRENOMEDULLARY SYNAPSE

Almost 70 years ago, Rex Coupland properly defined the first peripheral synapse, through electron microscopical observation, detailing the innervation of chromaffin cells of the golden hamster adrenal medulla by the cholinergic sympathetic pre-ganglionic fibers of the splanchnic nerve (10). Somewhat earlier, the storage of epinephrine (and norepinephrine) within secretory granules was characterized (11). The morphological, cell biological, and biochemical aspects of chromaffin cell function were integrated by several additional observations. These included the ultrastructural observation of the epinephrine-containing secretory granules presenting an omega-shaped profile to the extracellular space of the chromaffin cell by Coupland; the determination that a large protein (chromogranin A) was contained in and secreted from the adrenal medulla upon splanchnic nerve stimulation [see for original pertinent literature (12, 13)][1]; and finally the observation that cholinergic and nerve stimulation was calcium-dependent (14). A rich cascade of concepts followed, and culminated in Douglas's idea of stimulus-secretion coupling

[1]As often as possible, in the interests of brevity, review articles in which the historical documentation of discovery can be perused, will be cited in lieu of the primary literature.

(15), in analogy to muscle stimulus-contraction coupling. In this case, a transmitter released from the splanchnic nerve excited the chromaffin cell through an ionotropic receptor, calcium influx occurred, and exocytosis followed, with the release of granular proteins and catecholamines into the circulation. The transmitter released from the splanchnic nerve (and at all other preganglionic sympathetic/sympathoadrenal synapses) of course was acetylcholine. It caused cellular depolarization through the nicotinic cholinergic receptor, and exocytotic secretion ensued (16). Subsequently, the splanchnicoadrenomedullary synapse became the paradigm for peripheral neurotransmission at sympathetic as well as parasympathetic ganglia.

The ability of acetylcholine to generate an action potential by acting upon sodium channels on chromaffin cells (and post-ganglionic sympathetic and parasympathetic neurons) explained its secretagogue activity for epinephrine release from the adrenal medulla (16). Similar nicotinic receptor activation on post-ganglionic sympathetic and parasympathetic neurons was responsible for norepinephrine and acetylcholine release (respectively) at autonomic neuroeffector junctions in heart, spleen, lymphatic tissue, and elsewhere throughout the body. The possibility for additional complexity, however, in the neurotransmitter coding of the autonomic nervous system began to arise in the late 70s with several laboratories exploring the long-term changes in chromaffin cells driven by stress-induced catecholamine secretion. Wakade and colleagues discovered that the adrenal medulla may release its entire complement of catecholamines during a bout of secretion lasting several hours, yet remain competent for secretion afterwards due to heroic repletion of the gland: at the end of a secretory bout that releases all of the epinephrine present in the adrenal medulla at the start of secretion, there remains as much epinephrine as before (17). The gland re-synthesizes its entire store of accumulated epinephrine in a few hours. How does this occur? Costa and colleagues explored the induction of the rate-limiting enzyme for catecholamine biosynthesis, tyrosine hydroxylase (TH), and determined that through both transcriptional and post-translation activation mechanisms, long-term splanchnicoadrenomedullary stimulation would induce TH and this would in turn increase the rate of catecholamine biosynthesis in a compensatory way (18). Possible mechanisms for this (since nicotinic stimulation was limited in its ability to induce TH as well as peptide synthesis in the gland) included the activation of muscarinic cholinergic receptors (19). Thoenen et al., however, noted that reflex stimulation of chromaffin cells *in situ* by whole-animal treatment with reserpine caused an induction of TH that could not be blocked with nicotinic or muscarinic blockers, and opined that either a novel type of cholinergic receptor must exist or, presciently, that a non-cholinergic substance might be released along with acetylcholine to allow TH induction (20). Ip and Zigmond posited something similar at sympathetic ganglion, where stimulation of the sympathetic trunk at 10 Hz (frequency typical of the stress response) caused in the superior cervical ganglion the induction of TH by a mechanism that could be pharmacologically explained only partially through an action of acetylcholine release from preganglionic terminals (21). The situation languished until the discovery of the neuropeptide pituitary adenylate cyclase-activating polypeptide (PACAP) (22) by Miyata et al. Despite its name, and its discovery as a hypothalamic peptide able to stimulate cAMP production in perfused anterior pituitary hemi-organs (23), three remarkable properties of PACAP were soon discovered. First, it was ubiquitously expressed in brain, and not only hypothalamus; second, it was present in peripheral tissues; third, application of PACAP in various contexts potently stimulated epinephrine secretion from the adrenal gland *in vivo*, from perfused adrenal gland *ex vivo*, and from chromaffin cells in culture [(24–27) and references therein].

The development of PACAP-deficient mice allowed characterization of antibodies that were unambiguous in detecting PACAP (compared to its structurally similar congener VIP) in the cholinergic nerve terminals of the splanchnic nerve within the adrenal gland (28). The PACAP knock-out mouse also allowed examination of the role of PACAP on adrenomedullary performance *in vivo*. Reflex stimulation of the splanchnic nerve after hypoglycemia induced by insulin shock increased plasma epinephrine levels, and adrenomedullary TH activity: both effects were blunted or abolished in PACAP-deficient mice, and correction of hypoglycemia did not occur, a fatal outcome unless prevented by intraperitoneal injection of PACAP or glucose itself (28). Later experiments conducted in adrenal slices *ex vivo* showed that epinephrine secretion upon splanchnic nerve stimulation at high frequency, but not at low stimulation rates, does not occur in slices from PACAP-deficient mice or from wild-type mice when perfused with the PACAP antagonist PACAP(6-38) (29, 30).

What have we learned? First, PACAP is present at each of the splanchnic nerve terminals that contain cholinergic secretory vesicles and innervate the mouse adrenal chromaffin cells. Second, stress-induced catecholamine secretion and biosynthetic enzyme regulation is PACAP-dependent, regardless of whether the stressor is metabolic/systemic (e.g., insulin-induced hypoglycemia) or exteroceptive/psychogenic (e.g., restraint stress). Second, this dependence can be shown to exist at the level of the adrenal gland itself, since adrenal slices taken from PACAP-deficient mice, in which the morphology and neurochemistry of the splanchnicoadrenomedullary synapse is normal, fail to release epinephrine in response to high-frequency stimulation of the splanchnic nerve stump, while basal (low-frequency) catecholamine secretion is unaffected. These experiments appear to satisfy the criterion for PACAP as the neurotransmitter responsible for stress transduction at the adrenomedullary synapse (see **Figure 2A**) (31, 32).

How generalizable is the concept that PACAP, and not acetylcholine, is the major autonomic stress neurotransmitter? This involves answering two questions. First, in how many other mammalian species is PACAP the splanchnicoadrenomedullary transmitter? Second, is PACAP the stress transducing transmitter at other autonomic synapses of the sympathetic and parasympathetic nervous systems? With respect to the

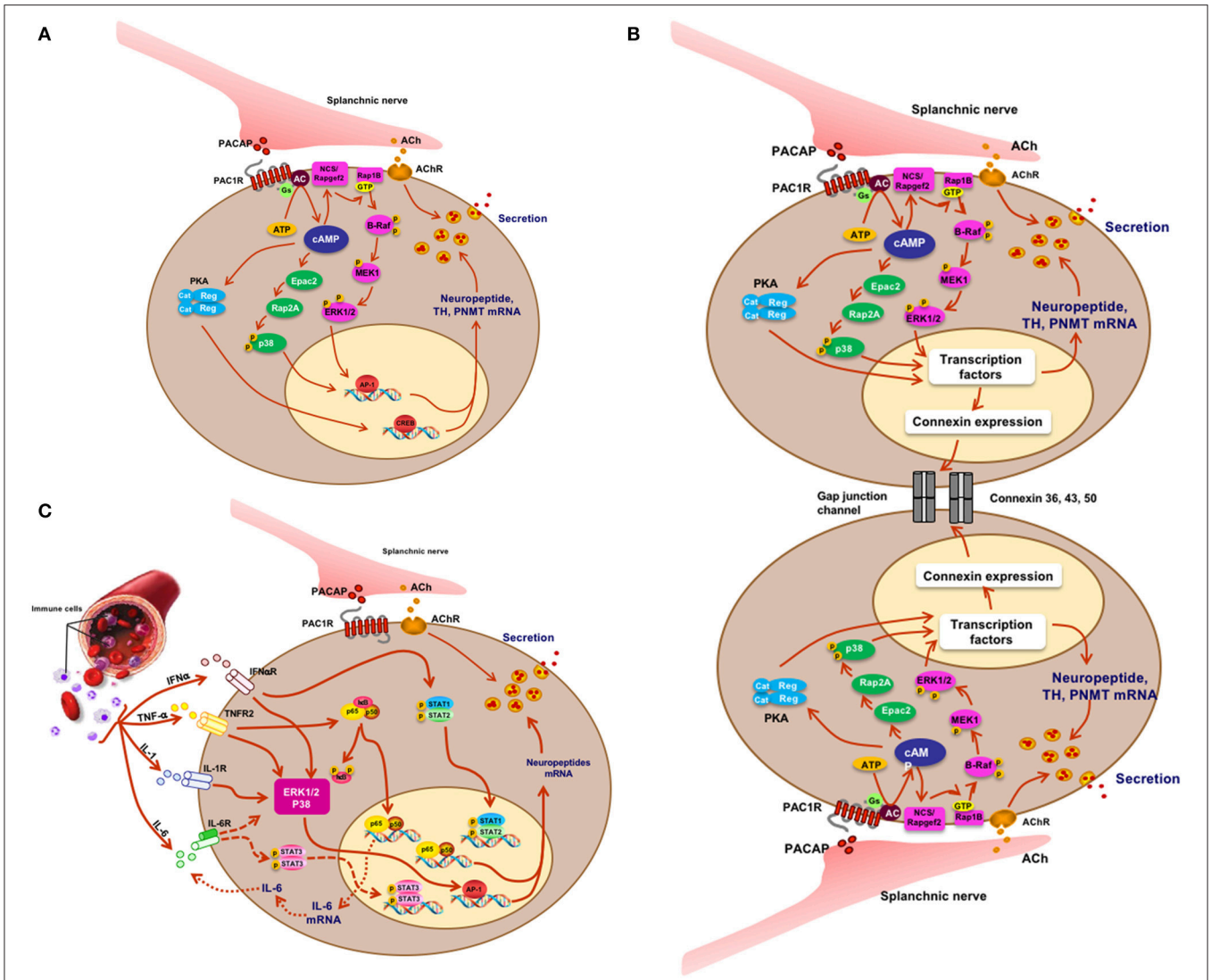

FIGURE 2 | Signaling and physiology of the chromaffin cell. **(A)** Acetylcholine and PACAP (sympathetic pre-ganglionic) release from splanchnic nerve and stimulus-secretion-synthesis coupling in the chromaffin cell. **(B)** Mechanistic depiction of enhanced cell-cell interaction and hormone secretion under conditions of stress in the chromaffin cell. There is as yet no direct proof for PACAP or acetylcholine as the principal mediator of what might be termed the "gap junction response." **(C)** IFN, TNF, IL-1, and IL-6 effects on cognate receptors on chromaffin cells and cellular sequelae in the chromaffin cell. Splanchnic nerve input affords synergistic as well as antagonistic interactions between cytokines and PACAP under physiological conditions in which both stress and inflammation may play a role. Figure adapted from (9).

first question, catecholamine secretion is provoked by PACAP, either *in vivo* or in chromaffin cells in culture, in all mammals examined so far, including mice, rats, cows, dogs, and humans [reviewed in (27)]. Regarding the second, there is a significant body of evidence for receptivity of sympathetic post-ganglionic neurons, in culture, to PACAP, as evidenced by enhanced secretion as well as signaling for gene expression (33, 34). Post-ganglionic neurons of the system, notably in the heart, are also PACAP responsive (35). Thus, findings in the findings in the adrenal medulla likely herald a new perspective in adding the neuropeptide PACAP as the third major neurotransmitter of the autonomic nervous system, in addition to acetylcholine and norepinephrine.

Gs-GPCR SIGNALING FOR SECRETION AND BIOSYNTHESIS IN THE CHROMAFFIN CELL

Hormone-secreting endocrine tissues must maintain their stores of secreted material, catecholamines from chromaffin cells, insulin from beta cells of the pancreas, hypophyseal hormones from their depots in anterior pituitary cells, incretins from endocrine cells of the gut, and so forth, in order to maintain patency for future endocrine response. This can occur via parallel but uncoupled processes if hormone secretion and biosynthesis both occur at about the same constitutive rate, but this is rarely the case, as hormone secretion tends to be episodic

in response to metabolic and other homeostatic organismic demands, when the endocrine cell is called upon by first messenger secretagogues to release its characteristic hormone into the bloodstream to act at distant sites. Timely repletion of hormone content in endocrine tissue following a bout of secretion in these cases can occur in one of two ways. The first is a chronic overproduction of prohormone protein by the endocrine cell relative to storage capacity. In this case, excess hormone is degraded at times of low secretory demand, and diverted to the secretory pathway at times of high secretory demand. This type of regulation appears to be the case for chromogranin A in adrenal medulla: because chromogranin A is a constituent of secretory granule biogenesis itself, the chromaffin cell makes no more chromogranin than it does secretory granules. When the secretory granule complement is complete, excess chromogranin is apparently degraded in the trans-Golgi network and when the granule complement is depleted by a bout of secretion, this excess is re-directed toward granule production, so that chromogranin levels are maintained during secretion even when there is no compensatory up-regulation of chromogranin mRNA abundance compared to that of other co-secreted neuropeptides (36, 37). For (pro)hormones that are only a fraction of the total secretory granule protein content, however, a non-default regulatory mechanism for compensating hormone loss through secretion with enhanced biosynthesis must exist (this actually includes chromogranins themselves, in cells in which they are not the dominant secreted protein).

The concept of stimulus-secretion-synthesis coupling was introduced into the neuroendocrine lexicon with the realization that first messengers that act as secretagogues, facilitating the release of stored hormones into the bloodstream, may simultaneously signal to the endocrine cell to engage the genetic machinery that allows repletion of hormone (38). For hormones that are produced by prohormone processing within the secretory granule, this is reflected in enhanced transcription of the prohormone gene and subsequently enhanced translation of its mRNA into prohormone protein. In the adrenal medulla, in which secreted material is a mixture of protein (chromogranins and neuropeptides) and small molecules (catecholamines and ATP), stimulus-secretion synthesis coupling must accommodate both types of secreted material. Thus, secretagogue (first-messenger) signaling leads immediately to calcium influx, then to activation of the secretory apparatus and release from the cell of the contents of the secretory vesicle/granule. Simultaneously, downstream signaling from the first messenger must engage pathways leading to (i) stimulation of transcription of prohormone protein-encoding genes and (ii) stimulation of biosynthetic enzymes responsible for catecholamine and other small-molecule biosynthesis. In the case of catecholamine production, this means activation of both the enzymatic activity, via phosphorylation, of the rate-limiting enzyme for catecholamine biosynthesis, tyrosine hydroxylase (TH) and increased production of the TH protein itself, via increased transcription from the TH gene (39).

The detailed cellular mechanisms of signaling for hormone repletion in the adrenal medulla was much-studied throughout the Twentieth century and in many ways was paradigmatic for understanding this process in other endocrine cells and tissues. The second messenger most often evoked as mediating stimulus-synthesis coupling is calcium, the same second messenger that triggers secretin in most endocrine cells. However, in the adrenal medulla, cyclic AMP was implicated early on in both transcriptional and post-translation effects on TH activation. Later, examination of stimulus-secretion-synthesis coupling related to co-stored peptides such as substance P, NPY, enkephalin, and galanin revealed that while cyclic AMP appears (as in anterior pituitary cells such as corticotropes) to be the main second messenger for activation of gene transcription of the latter, the classical pathway to cAMP-dependent gene activation by the third messenger protein kinase A (PKA) was insufficient to explain how this signaling could occur.

The concept that cAMP's actions are mediated solely through activation of the serine/threonine protein kinase protein kinase A (PKA) was a durable one for several decades after its promulgation by Kuo and Greengard (40). However, toward the end of the Twentieth century it became increasingly apparent that not only could cAMP gate calcium channels on the surface of specialized cells, such as those of the olfactory mucosa (41), but that there were a plethora of cAMP-dependent and PKA-independent actions of first messengers that strongly suggested the existence of other cAMP effectors within mammalian, especially neuroendocrine cells. In the late 90s, two groups discovered two proteins among a family of Rapgef proteins (for guanine nucleotide exchange factors activating the signaling kinase Rap), variously called Epac1 and 2 (the currently most common name), or Rapgef3 and 4, or cAMP-GEF1 and −2 (42–44). These proteins clearly expanded the range of cAMP effectors within mammalian cells from protein kinases to guanine nucleotide exchange factors, with their own sets of downstream effectors (mainly MAP kinases) distinct from cytoplasmic substrates such as glycogen synthase, and nuclear transcription factors such as CREB, activated by PKA.

Our own laboratory noted in 2012 that galanin biosynthesis in the chromaffin cell was regulated by the stress-associated secretagogue PACAP, via a cyclic AMP signaling pathway independent of PKA, and requiring activation of the MAP kinase ERK (45). The cAMP effector linking activation of adenylate cyclase and ERK by PACAP was identified by both loss- and gain-of-function experiments, in cellula, as the gene product of the guanine nucleotide exchange factor Rapgef2 (46). This enzyme had been deemed to be insensitive to regulation by cAMP by both *in vitro* biochemical criteria and by sequence comparison to known cAMP binding proteins (47, 48). However, others had identified Rapgef2 as a cyclic nucleotide-regulated protein in neuroendocrine (melanoma) cells (49, 50). We have subsequently identified two mRNA variants transcribed from the Rapgef2 gene, which we have termed NN (for non-neuronal)-Rapgef2 and NCS (for neuritogenic cAMP sensor)-Rapgef2. The latter transcript appears to be generated exclusively in neuronal and endocrine cell types/tissues in adult rodents, and is responsible for linking cAMP elevation and ERK activation not only in chromaffin cells, but in neurons of the central nervous system (51). Re-assessment of cAMP signaling with respect to its parcellation between protein kinase A, and the guanine

nucleotide exchange factors Epac and NCS-Rapgef2, is likely to occur throughout the field of endocrinology in the coming years. The characterization of the cellular assignments of these three cAMP effectors in signaling not only within the adrenal medulla, but in anterior pituitary, pancreatic islets, enterochromaffin cells, and other endocrine and neuronal cells is presently underway in several laboratories (51–56).

THE ROLE OF PEPTIDES SECRETED FROM THE ADRENAL MEDULLA

As mentioned previously, the finding that the adrenal medulla secretes bioactive neuropeptides in addition to catecholamines was a major development for neurochemistry and neuroendocrinology in the early 1980s, and in fact paved the way for the discovery of the mRNAs encoding the opiate peptides (57–60). Its historical roots exist in the discovery of the chromogranins as secreted proteins of the adrenal medulla (vida infra); the discovery that enkephalin peptides are a major constituent of the secretory granules of the adrenal medulla (57, 58); and the realization that chromogranin A is itself a prohormone for bioactive peptides including pancreastatin, vasostatin, catestatin, and others (61–64). The adrenal medulla-specific proenkephalin-derived BAM-22P was identified via systematic peptidomics analysis of the adrenal medullary "peptide storehouse" (65). Adrenomedullin was discovered via a specific proteomics-based search for novel cAMP-elevating neuropeptides in pheochromocytoma peptide extracts (66). The presence of a rich secretory cocktail, comprising the chromogranin-derived, enkephalin-derived, and other neuropeptides, as well as the neuropeptides substance P, galanin, and NPY raised the important question of what the function of such released peptides might be. The answer(s) to these questions was initially frustrated by a lack of knowledge about where the corresponding receptors for peptides exist, and thus whether they were most likely to play an autocrine, paracrine, or hormonal function *in vivo* (63). The molecular nature of the receptor for catestatin, one of the CgA-derived peptides of the adrenal medulla, is still uncertain. However, O'Connor and colleagues were able to show convincingly that catestatin is able to modulate acetylcholine-induced catecholamine secretion from chromaffin cells, without affecting secretion caused by potassium or barium depolarization or ionophore-stimulated release, suggesting that its receptor either is, or acts immediately downstream of, the cholinergic nicotinic receptor of the chromaffin cell (67). More recently, it has been demonstrated in PC12 cells that catestatin affects not only acetylcholine-induced but also PACAP-induced catecholamine secretion, making its role in stress-related as well as basal adrenomedullary function of likely physiological importance (68). Likewise, substance P has been proposed as an autocrine regulator of catecholamine secretion and biosynthesis, based on its negative modulation of nicotinic receptor activation in chromaffin cells (69–71).

Catestatin, however, is truly protean in its autocrine and hormonal roles throughout the body. Perhaps the most striking finding about catestatin is its ability to rescue a chromogranin A-deficient phenotype in mice (72). Catestatin/chromogranin-mediated effects permeate all functions of the adrenal chromaffin cell (72). This includes the formation of secretory granules themselves (73), although this function appears to be shared with other granin proteins in chromaffin cells (74), and modulation of catecholamine release (75). Physiologically, catestatin extends the range of the adrenal medulla as an endocrine organ beyond the scope of catecholamine effects on cardiovascular function and metabolism, to endocrine (catestatin) modulation of the secretion, metabolism, and morphology of endocrine cells which are its targets (including the chromaffin cell) as well as its effects on modulation of beta cell function, immune regulation, and muscle and neuronal cellular metabolism (76, 77). As well, several other chromogranin A-derived peptides, including vasostatin, have potent cardiovascular effects that make the adrenal medulla a multi-pronged regulator of cardiovascular as well as metabolic function, and therefore link stress transduction even more tightly to cardiovascular and metabolic physiology (78).

The protean role of chromogranin-derived peptides, at every level of adrenomedullary function, has opened up an important chapter of endocrinology by sharpening appreciation for the role of peptide hormones, and their receptors, as highly adaptive components of the evolution of the endocrine network through constant adaptation of peptides to new regulatory roles, especially through tissue-specific expression of various peptides due to cell-specific prohormone processing. Such is the case of BAM22P. This 22-amino acid proenkephalin-derived peptide (YGGFMRRVGRPEWWMDYQKRYG) is found almost exclusively in the adrenal medulla, most likely because the only partial processing of proenkephalin in the adrenal gland, unlike in neuronal cells, results in a plethora of "incompletely" processed peptides. These however, are not only intermediates. Only distantly related to the mu opiate receptor, which is mainly liganded by leu- and met-enkephalin *in vivo*, the BAM22P receptor shows a more than 50-fold higher affinity for BAM22P than for met-enkephalin, which is contained within the primary sequence of the BAM22P peptide (79). The exclusive presence of the BAM22P receptor in sensory neurons, and the high levels of BAM22P in adrenal medulla, suggests that this peptide may be a specific first messenger for adrenomedullary communication with sensory nerves that abundantly innervate the adrenal gland (**Figure 1**). A link between the major stress-transducing endocrine organ, the adrenal medulla, and the sensory nervous system at this anatomical locus is intriguing to consider.

THE CONCEPT OF ORGAN PLASTICITY IN STRESS RESPONDING OF THE ADRENAL MEDULLA

It is by now well-known that endocrine tissues develop as "colonies" of cells devoted to a single secretory mission, but it was initially thought that each cell within this colony acts more-or-less independently of its neighbors in responding to first-messenger secretagogues with hormone release: a massively parallel rather than a highly integrated response. In the secretion of vasopressin, however, several investigators promulgated the

concept of "concerted" actions of the entire secretory cell cohort (80). This seemed to account for the dynamics of secretion in this system more comprehensively, and mechanisms of this type are now being explored and discovered in other cell types. "Concerted secretion" could occur by one of two (or both) mechanisms. One of these is paracrine/autocrine regulation, in which substances secreted from a single secretory cell interact with receptors on the same cell (autocrine regulation) or neighboring cells of the same (also autocrine) or another (paracrine) type, to modulate their secretion. In any event, the concept of autocrine/paracrine regulation was easily applied to the adrenal medulla, in part to explain the actions of hormones besides catecholamines (see previous section) which appeared to be released from the adrenal medulla in too low levels to be bona fide hormones, thus begging for an additional function to explain their production in and secretion from chromaffin cells. A second mode of modulating the secretory response of endocrine cells as an organ collective consists in stimulus-dependent changes in cellular adhesion mediating cell-cell interactions called gap junctions that can affect secretory performance (**Figure 2B**). Gap junctions are electrical connections that lower the resistance between cells that are connected by them. Connexins are the proteins that make up the hexameric hemichannels that form gap junctions: when two hemichannels on adjacent cells are apposed, a gap junction is formed. In 2001 Martin et al. (81) made the seminar observation that connexins are expressed by rat adrenal chromaffin cells. The history of the gap junction in adrenal medulla and other endocrine tissues is capably summarized by Colomer et al. (82): suffice it to say here that the initial demonstration of the chromaffin cell gap channel was facilitated initially by the realization that the disparate cellular resistances of isolated chromaffin cells in culture are considerably greater than those of chromaffin cells in intact adrenomedullary cells, with these differences in input resistance logically inferred to arise from the existence of gap junctions between the latter and not the former (81, 83). A series of *in vivo* experiments have been performed by the Guerineau laboratory to assess the role(s) of gap junctions in adrenomedullary function *in vivo* [summarized in (84)]. Rat adrenomedullary slices were examined *ex vivo* both before and after 5 days of cold stress (85). Morphological remodeling of both splanchnicoadrenomedullary synapses and chromaffin cells at their borders of adjacency was observed, along with increased dye permeation between cells at gap junctions, and increased electrical coupling following exogenous depolarization of individual chromaffin cells. Further experiments have indicated that stress *in vivo* causes increased catecholamine secretion in response to electrical stimulation that is blocked by pharmacological inhibition of gap junction formation. Detail of biochemical constitution of gap junctions, and gap-junction dependence of stress-induced enhancement of catecholamine secretion from chromaffin cells *in vivo* have followed (82, 86). Clearly there is, in addition to a shift from predominantly cholinergic to PACAPergic neurotransmission at the adrenomedullary synapse from rest to stress upon initial exposure to stressors, a profound reorganization of the gland itself that prepares it to anticipate further challenge by stress, and/or to protect the integrity of the gland from deleterious effects of increased functional load. At this time, it is unclear whether acetylcholine, PACAP or a combination of the two transmitters mediates this organotypic adaptive response.

The role of gap junctions in adrenomedullary function is likely to be a generalizable phenomenon, not only to other endocrine organs (87) but also to neuronal communication: the stress stimulus selected by Guerineau for analysis of the adrenal medulla is one that is relatively slight for that organ (88) but plays a major role in regulation of norepinephrine output from post-ganglionic sympathetic nerves. It remains to be seen if principal ganglion cells of the sympathetic nervous system respond to stress as the adrenal medulla does, and if PACAP, or acetylcholine, or both are the principal regulator(s) of this important phenomenon. Of utmost importance, this work in aggregate reminds investigators of the limitations of studying endocrine phenomena in isolated cells, in which there is much insight to be gained, but in which much about how endocrine tissues actually function *in vivo* can be overlooked.

ADRENOMEDULLARY FUNCTION DURING INFLAMMATORY RESPONSES

The inflammatory response is complex and involves virtually all organs, as cytokines are both secreted into the bloodstream and affect tissues hormonally, and because cytokine-secreting cells of the immune system are mobile and ubiquitous, migrating to various locations in response to local and systemic inflammatory challenge. The discovery of LPS and cytokine receptors on chromaffin cells was a key event in focusing on the role of the adrenal medulla in coordinating some aspects of the immune response (**Figure 1**). Working out the pathways for cytokine signaling and its ramifications for chromaffin cell parachymal function, and adrenomedullary participation in systemic inflammation, has been an intriguing enterprise, and worth noting within the purview of "what's new in endocrinology."

It was noted a number of years ago that neuropeptide biosynthesis is complexly modulated by the cytokines IL-1 and TNF-alpha in bovine chromaffin cells in culture, with positive regulation of VIP biosynthesis and down-regulation of met-enkephalin, and amplification of the effects of cyclic AMP elevation on VIP and substance P production by both cytokines (89). More recently, Anouar and colleagues demonstrated the existence of TNF-alpha receptor expression in the bovine adrenal medulla *in vivo* (90, 91), and the regulation of gene transcription and peptide production of galanin and chromogranin in response to this cytokine (91). Subsequently, Bunn and colleagues postulated a role for TNF in inflammation-induced VIP biosynthesis and TH induction in rodent adrenal medulla *in vivo* (9, 92). A number of other studies converge to create a picture of cytokine regulation of adrenomedullary peptide production for a counter-regulatory role in inflammation: exposure of chromaffin cells to cytokines or administration of LPS *in vivo* (S. Bunn, personal communication) results in enhanced production of galanin, which is reported in turn to be a positive regulator of

glucocorticoid production in the adrenal cortex (see **Figure 2C**). The inflammatory cytokines IL-1 and TNF (9) also increase the production of IL-6 mRNA in chromaffin cells, revealing an occult source of this anti-inflammatory peptide that implicates a second mechanism of down-regulation of the "cytokine storm" associated with inflammation. This anti-inflammatory mechanism is also linked to the stress-transducing function of the adrenal gland, emphasizing further the highly integrative role of the adrenal medulla as stress transducer across multiple other (metabolic, neuronal, immune) endocrine regulatory domains (**Figures 1**, **2**).

SUMMARY AND FUTURE PROSPECTS

The simple and classical view that the adrenal medulla releases catecholamines under stress in response to acetylcholine release from the splanchnic nerve provided a framework for much fruitful investigation of the detailed cellular physiology underlying the function of the chromaffin cell. This view has been enriched over the past 20 years, as summarized in **Figures 1**, **2**. The adrenal medulla is now viewed as a more complex, and more integrative, stress transducer. Basal secretion, under the influence solely of acetylcholine, is seen as important to cardiovascular function as well. Additional first messengers besides acetylcholine, including PACAP and cytokines, bring organismic information to the chromaffin cell. The secretory products of the chromaffin cells also have important, yet-to-be-discovered paracrine, autocrine and hormonal roles. These allow integration of immune, inflammatory, sensory, and cardiovascular surveillance and regulation by the adrenal medulla. Chromaffin cells themselves not only operate as individual cellular elements that amplify signal transduction by being organized into an endocrine organ, but also modulate the performance of this cellular cohort by chromaffin cell-chromaffin cell coordination through gap junctions. Each of these themes is likely to be generalizable to other endocrine organs to some degree: PACAP at sympathetic ganglia as well as the sympathoadrenal junction; cytokine regulation of other peripheral endocrine depots such as the pancreas; gap junction function in pituitary and likely elsewhere; parcellated cAMP signaling in pancreas and brain as well as the adrenal; catestatin function throughout the cardiovascular system. Leveraging the sense that 'everything is connected to everything else' (93) does not mean, however, that the mammalian endocrine network is chaotic, or therapeutically opaque. Rather, apprehending directionality in endocrine regulation probably requires a better sense of what is unique about each endocrine organ, including the adrenal medulla, in addition to the predominant hormone that is released from it.

Some rather large questions remain, both about the adrenal medulla in particular, and its characteristics as an endocrine organ in general. What are the ways in which acetylcholine and PACAP collaborate in adrenomedullary function in homeostasis and stress responding? Are these two messengers specialized for "basal" or "rest and digest" vs. "stress" or "fight or flight" responding, or is their interaction more intricate than that? Does acetylcholine/PACAP co-transmission contribute importantly to transmission elsewhere in the autonomic nervous system, i.e., at both sympathetic and parasympathetic synapses? Is the adrenomedullary stress response "looped in" to both the immune-inflammatory and sensory nervous systems? Might this be a potential key to immune and sensory regulation in stress and the balance between pro- and anti-inflammatory regulation that allows glucocorticoids and cytokines to counter-balance without either being overcome in sepsis or autoimmune dysregulation? Might signaling via catestatin, BAM-22P, substance P, galanin and other secretory products of the adrenal medulla lead to regulatory sequelae that can be exploited in disease treatment? Are the signaling mechanisms newly discovered in the adrenal medulla generalizable to other endocrine organs and even the nervous system? Is metabologenomics of the chromaffin cell a paradigm that will create diagnostic/prognostic opportunities for endocrinopathies beyond pheochromocytoma (94, 95)? Finally, we would be remiss not to note again that the adrenal medulla is an organ with pronounced mammalian species differences: how much of what we have learned about its function in rodents and other mammals is directly applicable to *H. sapiens*? It is our hope that this bouquet of questions will stimulate further research leading to the discovery of new aspects of chromaffin cell function. These in turn will beget additional questions, keeping the adrenal medulla at the forefront of endocrinology, despite our recurrent conviction that we know all we need to know about this deceptively simple endocrine organ.

AUTHOR'S NOTE

Much of the content of this review concerns work performed in our own and colleagues' laboratories and its precedents that are only now emerging in the literature. We apologize in advance for any myopia about progress in chromaffin cell endocrinology emerging from other laboratories that this review may have overlooked.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

REFERENCES

1. Andreis PG, Tortorella C, Ziolkowska A, Spinazzi R, Malendowicz LK, Neri G, et al. Evidence for a paracrine role of endogenous adrenomedullary galanin in the regulation of glucocorticoid secretion in the rat adrenal gland. *Int J Mol Med*. (2007) 19:511–5. doi: 10.3892/ijmm.19.3.511
2. Mazzocchi G, Malendowicz LK, Rebuffat P, Gottardo L, Nussdorfer GG. Expression and function of vasoactive intestinal peptide, pituitary adenylate cyclase-activating polypeptide, and their receptors in the human adrenal gland. *J Clin Endocrinol Metab*. (2002) 87:2575–80. doi: 10.1210/jcem.87.6.8571
3. Spinazzi R, Andreis PG, Nussdorfer GG. Neuropeptide-Y and Y-receptors in the autocrine-paracrine regulation of adrenal gland under physiological

and pathophysiological conditions (Review). *Int J Mol Med.* (2005) 15:3–13. doi: 10.3892/ijmm.15.1.3
4. Nussdorfer GG. Paracrine control of adrenal cortical function by medullary chromaffin cells. *Pharmacol Rev*. (1996) 48:49 5–530.
5. Bornstein SR, Haidan A, Ehrhart-Bornstein M. Cellular communication in the neuroadrenocortical axis: Role of vasoactive intestinal polypeptide (VIP). *Endocrine Res*. (1996) 22:819–29.
6. Bornstein SR, Rutkowski H, Vrezas I. Cytokines and steroidogenesis. *Mol. Cell. Endocrinol.* (2004) 215:135–41. doi: 10.1016/j.mce.2003.11.022
7. Path G, Bornstein SR, Ehrhart-Bornstein M, Scherbaum WA. Interleukin-6 and the interleukin-6 receptor in the human adrenal gland: expression and effects on steroidogenesis. *J Clin Endocrinol Metab*. (1997) 82:2343–9. doi: 10.1210/jcem.82.7.4072
8. Ehrhart-Bornstein M, Hinson JP, Bornstein SR, Scherbaum WA, Vinson GP. Intraadrenal interactions in the regulation of adrenocortical steroidogenesis. *Endocr Rev*, (1998) 19:101–43. doi: 10.1210/edrv.19.2.0326
9. Bunn SJ, Ait-Ali D, Eiden LE. Immune-neuroendocrine integration at the adrenal gland: cytokine control of the adrenomedullary transcriptome. *J Mol Neurosci.* (2012) 48:413–9. doi: 10.1007/s12031-012-9745-1
10. Coupland RE. Electron microscopic observations on the structure of the rat adrenal medulla. The ultrastructure, I. and organization of chromaffin cells in the normal adrenal medulla. *J Anat.* (1965) 99: 231–54.
11. Hillarp NA. Isolation and some biochemical properties of the catechol amine granules in the cow adrenal medulla. *Acta Physiol Scand.* (1958) 43:82–96. doi: 10.1111/j.1748-1716.1958.tb01579.x
12. Winkler H, Fischer-Colbrie R. The chromogranins A and B: the first 25 years and future perspectives. *Neuroscience* (1992) 49:497–528. doi: 10.1016/0306-4522(92)90222-N
13. Helle KB. The granin family of uniquely acidic proteins of the diffuse neuroendocrine system: comparative and functional aspects. *Biol Rev Camb Philos Soc.* (2004) 79:769–94. doi: 10.1017/S146479310400644X
14. Douglas WW, Rubin RP. The role of calcium in the secretory response of the adrenal medulla to acetylcholine. *J Physiol*. (1961) 159:40–57. doi: 10.1113/jphysiol.1961.sp006791
15. Douglas WW. Stimulus-secretion coupling: The concept and clues from chromaffin and other cells. *Br J Pharmacol.* (1968) 34:451–74. doi: 10.1111/j.1476-5381.1968.tb08474.x
16. Marley PD. Desensitization of the nicotinic secretory response of adrenal chromaffin cells. *Trends Pharmacol Sci.* (1988) 9:102–7. doi: 10.1016/0165-6147(88)90177-0
17. Wakade AR, Wakade TD, Malhotra RK. Restoration of catecholamine content of previously depleted adrenal medulla *in vitro*: importance of synthesis in maintaining the catecholamine stores. *J Neurochem*. (1988) 51:820–29. doi: 10.1111/j.1471-4159.1988.tb01817.x
18. Chuang DM, Costa E. Biosynthesis of tyrosine hydroxylase in rat adrenal medulla after exposure to cold. *Proc Natl Acad Sci USA*. (1974) 71: 4570–4.
19. Evinger MJ, Ernsberger P, Regunathan S, Joh TH, Reis DJ. A single transmitter regulates gene expression through two separate mechanisms: cholinergic regulation of phenylethanolamine N-methyltransferase mRNA via nicotinic and muscarinic pathways. *J Neurosci.* (1994) 14:2106–16. doi: 10.1523/JNEUROSCI.14-04-02106.1994
20. Thoenen H, Mueller RA, Axelrod J. Trans-synaptic induction of adrenal tyrosine hydroxylase. *J Pharm Exp Ther.* (1969) 169:249–54.
21. Ip NY, Perlman RL, Zigmond RE. Acute transsynaptic regulation of tyrosine 3-monooxygenase activity in the rat superior cervical ganglion: evidence for both cholinergic and noncholinergic mechanisms. *Proc Natl Acad Sci USA*. (1983) 80:2081–85. doi: 10.1073/pnas.80.7.2081
22. Miyata A, Arimura A, Dahl RR, Minamino N, Uehara A, Jiang L, et al. Isolation of a novel 38 residue-hypothalamic polypeptide which stimulates adenylate cyclase in pituitary cells. *Biochem Biophys Res Commun.* (1989) 164:567–74. doi: 10.1016/0006-291X(89)91757-9
23. Arimura A, Pituitary Adenylate Cyclase-Activating Polypeptide (PACAP): discovery and current status of research. *Regul. Pept.* (1992) 37:287–303. doi: 10.1016/0167-0115(92)90621-Z
24. Edwards AV, Jones CT. Adrenal cortical and medullary responses to acetylcholine and vasoactive intestinal peptide in conscious calves. *J Physiol.* (1993) 468:515–27. doi: 10.1113/jphysiol.1993.sp019785
25. Geng G, Gaspo R, Trabelsi F, Yamaguchi N. Role of L-type Ca2+ channel in PACAP-induced adrenal catecholamine release *in vivo*. *Am J Physiol.* (1997) 42:R1339–45. doi: 10.1152/ajpregu.1997.273.4.R1339
26. Lamouche S, Martineau D, Yamaguchi N. Modulation of adrenal catecholamine release by PACAP *in vivo*. *Regul Integr Comp Physiol.* (1999) 45:R162–70. doi: 10.1152/ajpregu.1999.276.1.R162
27. Hamelink C, Weihe E, Eiden LE. PACAP: an "emergency response" co-transmitter in the adrenal medulla. In: Vaudry H. Arimura A, editors. *Pituitary Adenylate Cyclase-Activating Polypeptide*. Norwell, MA: Kluwer-Academic Press (2003), p. 227–50.
28. Hamelink C, Tjurmina O, Damadzic R, Young WS, Weihe E, Lee HW, et al. Pituitary adenylate cyclase activating polypeptide is a sympathoadrenal neurotransmitter involved in catecholamine regulation and glucohomeostasis. *Proc Natl Acad Sci USA*. (2002) 99:461–66. doi: 10.1073/pnas.012608999
29. Stroth N, Kuri BA, Mustafa T, Chan SA, Smith CB, Eiden LE et al. PACAP controls adrenomedullary catecholamine secretion and expression of catecholamine biosynthetic enzymes at high splanchnic nerve firing rates characteristic of stress transduction in male mice. *Endocrinology* (2013) 154:330–9. doi: 10.1210/en.2012-1829
30. Kuri BA, Chan SA, Smith CB. PACAP regulates immediate catecholamine release from adrenal chromaffin cells in an activity-dependent manner through a protein kinase C-dependent pathway. *J Neurochem* (2009) 110:1214–25. doi: 10.1111/j.1471-4159.2009.06206.x
31. Smith CB, Eiden LE. Is PACAP the major neurotransmitter for stress transduction at the adrenomedullary synapse? *J Mol Neurosci.* (2012) 48:403–12. doi: 10.1007/s12031-012-9749-x
32. Eiden LE, Emery AC, Zhang L, Smith CB. PACAP signaling in stress: insights from the chromaffin cell. *Pflugers Arch*. (2018) 470:79–88. doi: 10.1007/s00424-017-2062-3
33. Beaudet MM, Parsons RL, Braas KM, May V. Mechanisms mediating pituitary adenylate cyclase-activating polypeptide depolarization of rat sympathetic neurons. *J Neurosci.* (2000) 20:7353–61. doi: 10.1523/JNEUROSCI.20-19-07353.2000
34. May V, Beaudet MM, Parsons RL, Hardwick JC, Gauthier EA, Durda JP, et al. Mechanisms of pituitary adenylate cyclase activating polypeptide (PACAP)-induced depolarization of sympathetic superior cervical ganglion (SCG) neurons. *Ann N Y Acad Sci.* (1998) 865:164–75. doi: 10.1111/j.1749-6632.1998.tb11175.x
35. Tompkins JD, Ardell JL, Hoover DB, Parsons RL. Neurally released pituitary adenylate cyclase-activating polypeptide enhances guinea pig intrinsic cardiac neurone excitability. *J Physiol.* (2007) 582:87–93. doi: 10.1113/jphysiol.2007.134965
36. Eiden LE, Iacangelo A, Hsu CM, Hotchkiss AJ, Bader MF, Aunis D. Chromogranin A synthesis and secretion in chromaffin cells. *J. Neurochem.* (1987) 49:65–74.
37. Fischer-Colbrie R, Iacangelo A, Eiden LE. Neural and humoral factors separately regulate neuropeptide Y, enkephalin, and chromogranin A and B mRNA levels in rat adrenal medulla. *Proc Natl Acad Sci USA*. (1988) 85:3240–4.
38. Eiden LE, Giraud P, Dave JR, Hotchkiss AJ, Affolter HU. Nicotinic receptor stimulation activates both enkephalin release and biosynthesis in adrenal chromaffin cells. *Nature* (1984) 312:661–3. doi: 10.1038/312661a0
39. Haycock JW, Wakade AR. Activation and multiple-site phosphorylation of tyrosine hydroxylase in perfused rat adrenal glands. *J Neurochem.* (1992) 58:57–64. doi: 10.1111/j.1471-4159.1992.tb09276.x
40. Kuo JF, Greengard P. Cyclic nucleotide-dependent protein kinases. IV. Widespread occurrence of adenosine 3′,5′-monophosphate-dependent protein kinase in various tissues and phyla of the animal kingdom. *Proc Natl Acad Sci USA*. (1969) 64:1349–55. doi: 10.1073/pnas.64.4.1349
41. Brown RL, Strassmaier T, Brady JD, Karpen JW. The pharmacology of cyclic nucleotide-gated channels: emerging from the darkness. *Curr Pharm Des.* (2006) 12:3597–613. doi: 10.2174/138161206778522100

42. de Rooij J, Zwartkruis FJ, Verheijen MH, Cool RH, Nijman SM, Wittinghofer A. Epac is a Rap1 guanine-nucleotide-exchange factor directly activated by cyclic AMP. *Nature* (1998) 396:474–7.
43. Bos JL. Epac: a new cAMP target and new avenues in cAMP research. *Nat Rev Mol Cell Biol.* (2003) 4:733–8. doi: 10.1038/nrm1197
44. Kawasaki H, Springett GM, Mochizuki N, Toki V, Nakaya V, Matsuda M, et al. A family of cAMP-binding proteins that directly activate Rap1. *Science* (1998) 282:2275–9. doi: 10.1126/science.282.5397.2275
45. Emery AC, Eiden LE. Signaling through the neuropeptide GPCR PAC1 induces neuritogenesis via a single linear cAMP- and ERK-dependent pathway using a novel cAMP sensor. *FASEB J.* (2012) 26:3199–211. doi: 10.1096/fj.11-203042
46. Emery AC, Eiden MV, Mustafa T, Eiden LE. Rapgef2 connects GPCR-Mediated cAMP signals to ERK activation in neuronal and endocrine cells. *Sci Signal.* (2013). 6:ra51. doi: 10.1126/scisignal.2003993
47. Kuiperij HB, de Rooij J, Rehmann H, van Triest M, Wittinghofer A, Bos JL, et al. Characterisation of PDZ-GEFs, a family of guanine nucleotide exchange factors specific for Rap1 and Rap2. *Biochim Biophys Acta*. (2003) 1593:141–9. doi: 10.1016/S0167-4889(02)00365
48. Kannan N, Wu J, Anand GS, Yooseph S, Neuwald AF, Venter JC, et al. Evolution of allostery in the cyclic nucleotide binding module. *Genome Biol.* (2007) 8:R264. doi: 10.1186/gb-2007-8-12-r264
49. Pham N, Cheglakov I, Koch CA, de Hoog CL, Moran MF, Rotin D, et al. The guanine nucleotide exchange factor CNrasGEF activates ras in response to cAMP and cGM. *Curr Biol.* (2000) 10:555–8. doi: 10.1016/S0960-9822(00)00473-5
50. Amsen EM, Pham N, Pak Y, Rotin D. The guanine nucleotide exchange factor CNrasGEF regulates melanogenesis and cell survival in melanoma cells. *J Biol Chem.* (2006) 281:121–8. doi: 10.1074/jbc.M507595200
51. Jiang SZ, Xu W, Emery AC, Gerfen CR, Eiden MV, Eiden LE. NCS-Rapgef2, the protein product of the neuronal Rapgef2 Gene, is a specific activator of D1 dopamine receptor-dependent ERK phosphorylation in mouse brain. *eNeuro* (2017) 4:1–17. doi: 10.1523/ENEURO.0248-17.2017
52. Holz GG, Kang G, Harbeck M, Roe MW, Chepurny OG. Cell physiology of cAMP sensor Epac. *J Physiol.* (2006) 577:5–15. doi: 10.1113/jphysiol.2006.119644
53. Lopez De Jesus M, Stope MB, Oude Weernink PA, Mahlke Y, Börgermann C, Ananaba VN, et al. Cyclic AMP-dependent and Epac-mediated activation of R-Ras by G protein-coupled receptors leads to phospholipase D stimulation. *J Biol Chem.* (2006) 281:21837–47. doi: 10.1074/jbc.M604156200
54. Keiper M, Stope MB, Szatkowski D, Bohm A, Tysack K, Vom Dorp F, et al. Epac- and Ca2+ -controlled activation of Ras and extracellular signal-regulated kinases by Gs-coupled receptors. *J Biol Chem.* (2004) 279:46497–508. doi: 10.1074/jbc.M403604200
55. Roscioni SS, Elzinga CR, Schmidt M. Epac: effectors and biological functions. *Naunyn Schmiedebergs Arch Pharmacol.* (2008) 377:345–57. doi: 10.1007/s00210-007-0246-7
56. Emery AC, Liu XH, Xu W, Eiden MV, Eiden LE. Cyclic adenosine 3',5'-monophosphate elevation and biological signaling through a secretin family Gs-coupled G protein-coupled receptor are restricted to a single adenylate cyclase isoform. *Mol Pharmacol.* (2015) 87:928–35. doi: 10.1124/mol.115.098087
57. Livett BG, Dean DM, Whelan LG, Udenfriend S, Rossier J. Co-release of enkephalin and catecholamines from cultured chromaffin cells. *Nature* (1981) 289:317–9.
58. Lewis RV, Stern AS, Kilpatrick DL, Gerber LD, Rossier J, Stein S et al. Marked increases in large enkephalin-containing polypeptides in the rat adrenal gland following denervation. *J Neurosci.* (1981) 1:80–82.
59. Comb M, Seeburg PH, Adelman J, Eiden L, Herbert E. Primary structure of the human Met- and Leu-enkephalin precursor and its mRNA. *Nature* (1982) 295:663–6.
60. Lewis RV, Stern AS, Kimura S, Rossier J, Stein S, Udenfriend S. An about 50,000-dalton protein in adrenal medulla: a common precursor of [Met]- and [Leu]enkephalin. *Science* (1980) 208:1459–61.
61. Eiden LE. Is chromogranin a prohormone? *Nature* (1987) 325:301. doi: 10.1038/325301a0
62. Taylor CV, Taupenot L, Mahata SK, Mahata M, Wu H, Yasothornsrikul S, et al. Formation of the catecholamine release-inhibitory peptide catestatin from chromogranin A. Determination of proteolytic cleavage sites in hormone storage granules. *J Biol Chem.* (2000) 275:22905–15. doi: 10.1074/jbc.M001232200
63. Helle KB, Angeletti RH. Chromogranin A: a multipurpose prohormone? *Acta Physiol Scand.* (1994) 152:1–10.
64. Iacangelo AL, Eiden LE. Chromogranin A: current status as a precursor for bioactive peptides and a granulogenic/sorting factor in the regulated secretory pathway. *Regul Pept.* (1995) 58:65–88.
65. Mizuno K, Minamino N, Kangawa K, Matsuo H. A new family of endogenous "big" Met-enkephalins from bovine adrenal medulla: purification and structure of docosa- (BAM-22P) and eicosapeptide (BAM-20P) with very potent opiate activity. *Biochem Biophys Res Commun.* (1980) 97:1283–90.
66. Kitamura K, Kangawa K, Kawamoto M, Ichiki Y, Nakamura S, Matsuo H, et al. Adrenomedullin: a novel hypotensive peptide isolated from human pheochromocytoma. *Biochem Biophys Res Commun.* (1993) 192:553–60. doi: 10.1006/bbrc.1993.1451
67. Mahata S.K, Mahata M, Parmer RJ, O'Connor DT. Desensitizaton of catecholamine release. The novel catecholammine release-inhibitory peptide catestatin (chromogranin A344–364) acts at the receptor to prevent nicotinic cholinergic tolerance. *J Biol Chem.* (1999) 274:2920–8.
68. Sahu BS, Mahata S, Bandyopadhyay K, Mahata M, Avolio E, Pasqua T, et al. Catestatin regulates vesicular quanta through modulation of cholinergic and peptidergic (PACAPergic) stimulation in PC12 cells. *Cell Tissue Res.* 1–20. doi: 10.1007/s00441-018-2956-1
69. Houchi H, Teraoka K, Oka M, Murakumo Y, Morita K. Substance P inhibits catecholamine biosynthesis stimulated by carbamylcholine in cultured adrenal chromaffin cells. *Biochem Pharmacol.* (1993) 45:1165–7.
70. Bunn SJ, Marley PD, Livett BG. Receptor stimulated formation of inositol phosphates in cultures of bovine adrenal medullary cells: the effects of bradykinin, bombesin and neurotensin. *Neuropeptides* (1990) 15:187–94.
71. Livett BG, Kozousek V, Mizobe F, Dean DM. Substance P inhibits nicotinic activation of chromaffin cells. *Nature* (1979) 278:256–7.
72. Mahapatra NR, O'Connor DT, Vaingankar SM, Hikim AP, Mahata M, Ray S, et al. Hypertension from targeted ablation of chromogranin A can be rescued by the human ortholog. *J Clin Invest.* (2005) 115:1942–52. doi: 10.1172/JCI24354
73. Kim T, Tao-Cheng JH, Eiden LE, Loh YP. Chromogranin A, an "on/off" switch controlling dense-core secretory granule biogenesis. *Cell* (2001) 106:499–509. doi: 10.1016/S0092-8674(01)00459-7
74. Montesinos MS, Machado JD, Camacho M, Diaz J, Morales YG, Alvarez de la Rosa D, et al. The crucial role of chromogranins in storage and exocytosis revealed using chromaffin cells from chromogranin A null mouse. *J Neurosci.* (2008) 28:3350–8. doi: 10.1523/JNEUROSCI.5292-07.2008
75. Tsigelny IF, Kouznetsova VL, Biswas N, Mahata SK, O'Connor DT. Development of a pharmacophore model for the catecholamine release-inhibitory peptide catestatin: virtual screening and functional testing identify novel small molecule therapeutics of hypertension. *Bioorg Med Chem.* (2013) 21:5855–69. doi: 10.1016/j.bmc.2013.07.008
76. Stadinski BD, Delong T, Reisdorph N, Reisdorph R, Powell RL, Armstrong M, et al. Chromogranin A is an autoantigen in type 1 diabetes. *Nat Immunol.* (2010) 11:225–31. doi: 10.1038/ni.1844
77. Kennedy BP, Mahata SK, O'Connor DT, Ziegler MG. Mechanism of cardiovascular actions of the chromogranin A fragment catestatin *in vivo*. *Peptides* (1998) 19:1241–8.
78. Helle KB. The chromogranin A-derived peptides vasostatin-I and catestatin as regulatory peptides for cardiovascular functions. *Cardiovasc Res.* (2009) 85:9–16. doi: 10.1093/cvr/cvp266
79. Lembo PM, Grazzini E, Groblewski T, O'Donnell D, Roy MO, Zhang J, et al. Proenkephalin A gene products activate a new family of sensory neuron–specific GPCRs. *Nat Neurosci.* (2002) 5:201–9. doi: 10.1038/nn815
80. Brown CH, Scott V, Ludwig M, Leng G, Bourque CW. Somatodendritic dynorphin release: orchestrating activity patterns of vasopressin neurons. *Biochem Soc Trans.* (2007) 35:1236–42. doi: 10.1042/BST0351236
81. Martin AO, Mathieu MN, Chevillard C, Guérineau NC. Gap junctions mediate electrical signaling and ensuing cytosolic Ca2+ increases

between chromaffin cells in adrenal slices: a role in catecholamine release. *J Neurosci.* (2001) 21:5397–405. doi: 10.1523/JNEUROSCI.21-15-05397.2001

82. Colomer C, Martin AO, Desarménien MG, Guérineau NC. Gap junction-mediated intercellular communication in the adrenal medulla: an additional ingredient of stimulus-secretion coupling regulation. *Biochim Biophys Acta* (2011) 1818:1937–51. doi: 10.1016/j.bbamem.2011.07.034
83. Cena V, Nicolas GP, Sanchez-Garcia P, Kirpekar SM, Garcia AG. Pharmacological dissection of receptor-associated and voltage-sensitive ionic channels involved in catecholamine release. *Neuroscience* (1983) 10:1455–62.
84. Guerineau NC. Gap junction communication between chromaffin cells: the hidden face of adrenal stimulus-secretion coupling. *Pflugers Arch.* (2018) 470:89–96. doi: 10.1007/s00424-017-2032-9
85. Colomer C, Olivos Ore LA, Coutry N, Mathieu MN, Arthaud S, Fontanaud P, et al. Functional remodeling of gap junction-mediated electrical communication between adrenal chromaffin cells in stressed rats. *J Neurosci.* (2008) 28:6616–26. doi: 10.1523/JNEUROSCI.5597-07.2008
86. Desarmenien MG, Jourdan C, Toutain B, Vessières E, Hormuzdi SG, Guérineau NC. Gap junction signalling is a stress-regulated component of adrenal neuroendocrine stimulus-secretion coupling *in vivo. Nat Commun.* (2013) 4:2938. doi: 10.1038/ncomms3938
87. Farnsworth NL, Benninger RK. New insights into the role of connexins in pancreatic islet function and diabetes. *FEBS Lett.* (2014) 5881278–87. doi: 10.1016/j.febslet.2014.02.035
88. Goldstein DS, Kopin IJ. Adrenomedullary, adrenocortical, and sympathoneural responses to stressors: a meta-analysis. *Endocr Regul.* (2008) 42:111–9.
89. Eskay RL, Eiden LE. Interleukin-1 alpha and tumor necrosis factor alpha differentially regulate enkephalin, vasoactive intestinal polypeptide, neurotensin, and substance P biosynthesis in chromaffin cells. *Endocrinology* (1992) 130:2252–8.
90. Ait-Ali D, Turquier V, Grumolato L, Yon L, Jourdain M, Alexandre D, et al. The proinflammatory cytokines tumor necrosis factor-alpha and interleukin-1 stimulate neuropeptide gene transcription and secretion in adrenochromaffin cells via activation of extracellularly regulated kinase 1/2 and p38 protein kinases, and activator protein-1 transcription factors. *Mol. Endocrinol.* (2004) 18:1721–39. doi: 10.1210/me.2003-0129
91. Ait-Ali D, Turquier V, Tanguy Y, Thouënnon E, Ghzili H, Mounien L, et al. Tumor necrosis factor (TNF)-alpha persistently activates nuclear factor-kappaB signaling through the type 2 TNF receptor in chromaffin cells: implications for long-term regulation of neuropeptide gene expression in inflammation. *Endocrinology* (2008) 149:2840–52. doi: 10.1210/en.2007-1192
92. Douglas SA, Sreenivasan D, Carman FH, Bunn SJ. Cytokine interactions with adrenal medullary chromaffin cells. *Cell Mol Neurobiol.* (2010) 30:1467–75. doi: 10.1007/s10571-010-9593-x
93. Commoner B. *The Closing Circle: Nature, Man And Technology*. New York, NY: Penguin Random House (1971).
94. Curras-Freixes M, Piñeiro-Yañez E, Montero-Conde C, Apellániz-Ruiz M, Calsina B, Mancikova V, et al. PheoSeq: a targeted next-generation sequencing assay for pheochromocytoma and paraganglioma diagnostics. *J Mol Diagn.* (2017) 19:575–88. doi: 10.1016/j.jmoldx.2017.04.009.
95. Lenders JWM, Eisenhofer G. Update on modern management of pheochromocytoma and paraganglioma. *Endocrinol Metab.* (2017) 32:152–61. doi: 10.3803/EnM.2017.32.2.152

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