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Special 2021 frontiers in endocrinology collection for the 100th anniversary of insulin discovery

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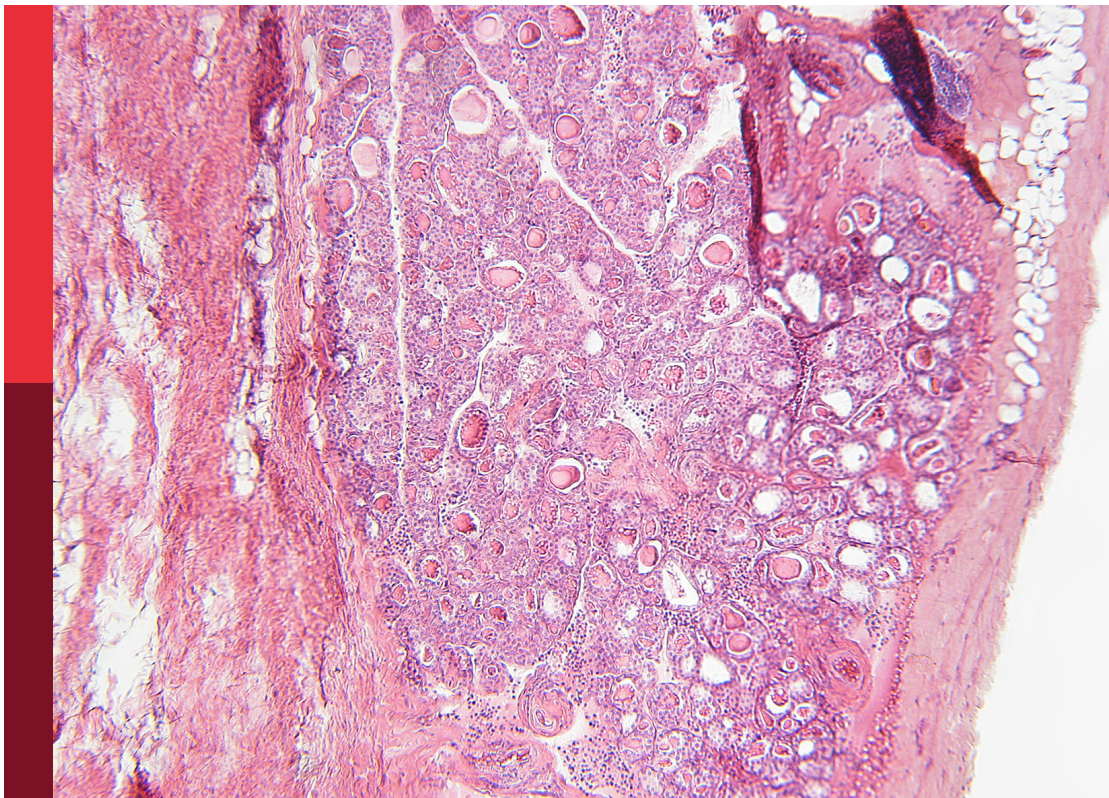

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Special 2021 frontiers in endocrinology collection for the 100th anniversary of insulin discovery

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Editorial: Special 2021 Frontiers in Endocrinology collection for the 100th anniversary of insulin discovery

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Editorial on the Research Topic

Special 2021 Frontiers in Endocrinology collection for the 100th anniversary of insulin discovery

To commemorate the 100th anniversary of the discovery and successful purification of insulin (1) at the University of Toronto by Banting, Best, Collip and McLeod in 1921 (earning the Nobel Prize to Banting and McLeod in 1923), Frontiers in Endocrinology organized a special collection related to insulin and insulin-like peptides, with Jeff M.P. Holly and Pierre De Meyts as supervisory editors. The aim was to bring together a collection of articles to celebrate the anniversary and the continued breadth of studies of this remarkable hormone. The articles could be submitted to the various sections of the journal.

The review by Irwin discusses the evolution of the insulin gene in the superfamily of insulin-like genes in multicellular invertebrates and vertebrates, as well as the recent discovery of insulin-like peptides in some fish viruses (2). Initial efforts to study the evolutionary aspects of insulin-like sequences relied on the sequencing of isolated peptides, but in the past 20 years, an increasing number of protein sequences have been predicted from the complete genome sequences of organisms. In most vertebrates a single insulin gene has been found, however multiple copies, some with differing gene structures, have been found in some species (e.g. rats, mice, *Xenopus laevis*, as well as teleost fishes that have undergone an ancestral whole genome duplication). In the vertebrate suborder hystricomorphs (guinea pigs and relatives), highly divergent insulin sequences are due to an accelerated rate of evolution. This is also the case in some species of New World monkeys. Several species of fish and mammals have accumulated increased amounts of sequence changes that affected sites of proteolytic processing of the prohormone precursor, resulting in either a single protein chain or two-chain proteins with an A- or B- chain that is extended to include the complete C-peptide sequence but remain biologically functional. The author concludes: "As we complete more genomes and microbiomes, it is certain that we will discover more insulin-like sequences with novel aspects to their sequences, structures, and functions. However, sequence will not tell us function. Experimental

work is still needed to identify the functions of these novel insulin-like sequences, which may uncover new roles for insulin in biology".

In a comprehensive (238 references) and well-illustrated review, Dhayalan et al. describe how the analysis of dominant diabetes-associated mutations in the human insulin gene (*INS)* provided critical insight into the folding mechanisms of proinsulin. Those mutations impair pancreatic β-cell function due to toxic misfolding of proinsulin, endoplasmic reticulum (ER) stress, intracellular proteotoxicity and impaired insulin secretion. Most mutations introduce or remove a cysteine (Cys), leading to an impaired thiol group, but non-Cys mutations affect and identify key determinants of folding efficiency. These studies suggest that the susceptibility of proinsulin to impaired foldability ("biosynthesis at the edge of non-foldability") is one factor constraining insulin's evolution. A similar process may occur in the natural history of Type 2 DM due to *INS* overexpression in response to insulin resistance.

In his Hypothesis and Theory article, Tatar addresses the regulation of aging in *Drosophila* and *C. elegans* by the single insulin/IGF-like receptor signaling. It is known that mutations of this receptor tyrosine kinase (InR in *Drosophila* and DAF-2 in *C. elegans)* slow aging and extend lifespan in these model invertebrates. The receptor appears to act in two modes. The first extends lifespan while slowing reproduction and reducing growth. The second strongly extends lifespan without impairing growth and reproduction; conferring "longevity assurance". The author hypothesizes that a recent model ("threshold model" or "stability model") for the function of receptor tyrosine kinases developed by Zinkle and Mohammadi (3) may explain how insulin receptor structure can modulate aging. Strong ligands (with fast on-rate and slow off-rate) – like DILP5 – stabilize the receptor kinase dimer and permits phosphorylation of substrates with both high and low stability thresholds of activation, resulting in robust induction of reproduction, impairing survival as a consequence of trade-offs. Weaker ligands (with slow on-rate and fast off-rate) – like DILP2 – induce only moderate kinase dimer stability and only phosphorylates sites with low stability thresholds of activation, reducing reproduction and extending lifespan by avoiding reproductive costs. Space is lacking here to go into further details of the author's interesting speculation regarding the specific effects of the seven DILPs; this paper deserves careful reading.

Two contributions address the mode of insulin administration to diabetic patients. Masierek et al. review thoroughly the past, present and future of insulin pens, today the leading mode of insulin delivery, replacing the syringe used earlier in the last 100 years. This includes the original NovoPen in 1985, the reusable, durable pens, the disposable, prefilled pens and the modern smart insulin pens. The authors provide comprehensive tables where they review in painstaking detail all the clinical trials and all types of pens from all companies including type of insulin, participants, study design and results. This represents a considerable amount of work and may be the ultimate reference source on this topic. An original research article by Pan et al. describes a randomized, open-label, cross-over trial conducted over eight healthy adult male volunteers evaluating the absorption of needle-free insulin aspart using the QS-M needle-free jet injector from Beijing QS Medical Technology Co injected in different body parts (abdomen, upper arm and thighs). They conclude that injection sites did not affect the absorption of insulin in needle-free injections.

Two contributions addressed the pathogenesis of type 1 diabetes, a condition resulting from an absolute insulin deficiency, typically secondary to autoimmunity. In the first one, March et al. discuss the impact of nutrition and obesity on the pathogenesis of youth-onset type 1 diabetes and its complications. Over the past several decades, there has been a dramatic rise in the prevalence of overweight and obesity in pediatric populations, including among children and adolescents with type 1 diabetes (T1D). With this changing prevalence, there are youths who are now presenting with overlapping characteristics of the different types of diabetes, including features of both autoimmunity and insulin resistance, described as "double diabetes". The authors have postulated that the increased insulin demands of obesity may accelerate the presentation of autoimmune T1D (the "accelerator hypothesis"). Various clinical studies investigating this link are described. Different pathophysiological mechanisms may explain how obesity contributes to insulitis and autoimmunity, blurring the boundary with type 2 diabetes (T2D). Insulin resistance, insulin demand and inflammation are plausible underlying mechanisms. Hormonal differences in sex steroids likely play a role in potentiating the risk for autoimmune progression. As therapeutic guidelines to manage this condition are limited, various therapeutic approaches are discussed including targeted nutritional therapies, metformin, GLP-1 agonists, DPP4-inhibitors, SGLT 1/2 inhibitors. No therapeutic options are currently approved to treat the obese youth with T1D, and future research is needed to understand which therapies might be the safest and most effective. Another review by Frommer and Kahaly examines the genetic link between T1D and autoimmune thyroid disease (AITD). T1D and AITD often cluster in individuals and families, seen as autoimmune polyendocrinopathy (AP), due to a common genetic background between TID and AITD. The major common genetic predisposition is the HLA antigens DQ2 and DQ8, tightly linked with DR3 and DR4. In addition, functional SNPs (single nucleotide polymorphisms) or rare variants of various genes that are involved in immune regulation confer susceptibility to both T1D and AITD: including *CTLA4*, *PTPN22*, *IL2Ra*, *VDR* and *TNF*. Other genes are also suspected to increase susceptibility to T1D and AITD: *CD40*, *FOXP3*, *MICA*, *INS-VNTR*, *CLEC16A*, *ERBB3*, *IFIH1*, and various cytokine genes. Furthermore, the following genes have been found in various independent GWAS studies to be associated with T1D and AITD: *BACH2*, *CCR5*, *SH2B3* and *RAC2*, indicating a strong genetic link for T1D and AITD. Consequently, all patients with T1D should be screened for AITD, and vice versa.

Two mini-reviews examine the role of two enzymes that play an important role in insulin secretion or action and metabolism, and their disorders. Possik et al. examine the role of a mammalian glycerol-3-phosphate phosphatase (recently discovered by them) in β-cell, liver and adipocyte metabolism, and cardiometabolic diseases like T2D, obesity and non-alcoholic liver steatosis. The metabolism of carbohydrates, amino acids and fats converges in generating a three-carbon molecule, glycerol, either in the free form or as glycerol-3-phosphate (Gro3P), which constitutes the backbone of glycerolipids. Free glycerol is believed to be produced and

released from cells by the hydrolysis of glycerolipids in higher animals including humans. An enzyme that would generate glycerol directly from Gro3P, a Gro3P phosphatase (G3PP) was thought to be present only in plants and lower organisms. However, Prentki's group identified in 2016 a previously described phosphoglycolate phosphatase (PGP) as the mammalian G3PP. In this review, they discuss this discovery and the most plausible physiological function of G3PP in controlling glucose, lipid and energy metabolism. They discuss the importance of G3PP in preventing glucotoxicity/nutri-stress and the control of glucose-stimulated insulin secretion (GSIS) in β-cells, in the regulation of lipogenesis in adipose tissue, and in slowing down hepatic glucose production. Finally, they address the regulation of G3PP and its role in cardiometabolic diseases. In the second mini-review, Kuefner examines the role of the secretory phospholipases A_2 ($sPLA_2$) superfamily, commonly found in mammalian tissue and snake venoms, in insulin resistance and metabolism. The $sPLA_2$s are low molecular weight enzymes that hydrolyze glycerophospholipids to generate a non-esterified free fatty acid and a lysophospholipid. Twelve isoforms have been identified so far. Most studies of $sPLA_2$s have addressed their roles in cardiovascular disease, inflammation, antimicrobial actions, and membrane remodeling. A discussion on how $sPLA_2$s may regulate or impact glucose metabolism, insulin signaling and metabolism has been lacking. Recent research has identified 7 of the $sPLA_2$s as modulators of glucose metabolism through mechanisms involved in insulin signaling and obesity. This review goes a long way in filling our gap of knowledge on these issues.

Finally, two reviews address the crosstalk between two endocrine systems, the pituitary hormones and the thyroid, and the insulin/IGF-1 systems. Schernthaner – Reiter et al. provide a brief review on the interaction of the pituitary hormone axes with glucose metabolism through direct or indirect effects on insulin secretion and function. The emphasis is on the actions on glucose metabolism of pituitary adenomas causing Cushing's disease or acromegaly, but the authors also discuss prolactinomas, GH-deficiency, hypogonadism and hypothyroidism. Smith's comprehensive and nicely illustrated review (134 references) discusses the two-way interplay between thyroid hormones and TSH on one hand, and growth hormone and IGF-1, on the other hand. There is a focus on Grave's disease (GD) and thyroid-associated ophthalmopathy (TAO), and the role of IGF-1 receptors. The clinical trials of the IGF-1R blocking monoclonal antibody teprotumumab in TAO are reviewed in detail, showing an effective and well-tolerated treatment. Other potential indications are discussed.

All in all, this collection of articles represents a nice sampling of studies going from very basic to clinical applications, a suitable tribute to the 100th anniversary of a pioneering hormone that remains the subject of a very fertile research field.

Author contributions

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

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REVIEW
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Type 1 Diabetes and Autoimmune Thyroid Disease—The Genetic Link

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Type 1 diabetes (T1D) and autoimmune thyroid disease (AITD) are the most frequent chronic autoimmune diseases worldwide. Several autoimmune endocrine and non-endocrine disorders tend to occur together. T1D and AITD often cluster in individuals and families, seen in the formation of autoimmune polyendocrinopathy (AP). The close relationship between these two diseases is largely explained by sharing a common genetic background. The HLA antigens DQ2 (*DQA1*0501-DQB1*0201*) and DQ8 (*DQA1*0301-DQB1*0302*), tightly linked with DR3 and DR4, are the major common genetic predisposition. Moreover, functional single nucleotide polymorphisms (or rare variants) of various genes, such as the *cytotoxic T-lymphocyte- associated antigen (CTLA4)*, the *protein tyrosine phosphatase non-receptor type 22 (PTPN22)*, the *interleukin-2 Receptor (IL2Ra)*, the *Vitamin D receptor (VDR)*, and the *tumor-necrosis-factor-α (TNF)* that are involved in immune regulation have been identified to confer susceptibility to both T1D and AITD. Other genes including cluster of differentiation *40 (CD40)*, the *forkhead box P3 (FOXP3)*, the *MHC* Class I *Polypeptide-Related Sequence A (MICA)*, *insulin variable number of tandem repeats (INS-VNTR)*, the *C-Type Lectin Domain Containing 16A (CLEC16A)*, the *Erb-B2 Receptor Tyrosine Kinase 3 (ERBB3)* gene, the *interferon-induced helicase C domain-containing protein 1 (IFIH1)*, and various cytokine genes are also under suspicion to increase susceptibility to T1D and AITD. Further, *BTB domain and CNC homolog 2* (*BACH2)*, *C-C motif chemokine receptor 5* (*CCR5)*, *SH2B adaptor protein 3* (*SH2B3)*, and *Rac family small GTPase 2* (*RAC2)* are found to be associated with T1D and AITD by various independent genome wide association studies and overlap in our list, indicating a strong common genetic link for T1D and AITD. As several susceptibility genes and environmental factors contribute to the disease aetiology of both T1D and AITD and/or AP subtype III variant (T1D+AITD) simultaneously, all patients with T1D should be screened for AITD, and vice versa.

Keywords: type 1 diabetes, autoimmune thyroid disease, genetic link, susceptibility genes, HLA antigens, single nucleotide polymorphisms, autoimmune polyendocrinopathy

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EPIDEMIOLOGY AND SEROLOGY

Type 1 diabetes (T1D) and autoimmune thyroid disease (AITD) are two frequent autoimmune endocrine disorders. The prevalence of T1D is increasing worldwide and has nearly doubled in the past 40 years, with a prevalence of 9.5 per 10,000 people worldwide. The incidence worldwide is 15 per 100,000, ranging from 0.1 per 100,000 in China and Venezuela to 40.9 per 100,000 in Finland and in Arab countries between 2.4 in Oman and 29.0 in Saudia Arabia (1, 2). The highest incidence rates are at ages 5 to 9 years in girls and 10 to 14 in boys. The gender ratio is almost balanced with a slight preponderance of males (3–7). AITD, encompassing Graves' disease (GD) and Hashimoto's thyroiditis (HT), is defined as the presence of positive thyroid-related Ab. It is most commonly associated with other autoimmune glandular and non-glandular disorders. AITD peaks in the fourth decade for GD or fifth and sixth decade for HT. AITD is frequently combined with T1D in populations of various ancestries, occurring together more often than expected by the population prevalence of each disease GD is less prevalent than HT, only affecting approximately 1–1.5% of the general population, but is the underlying cause of 50–80% of cases of autoimmune hyperthyroidism. While subclinical hyperthyroidism can be diagnosed in only 0.12% of the non-diabetic population, it is prevalent in 6–10% of T1D patients (8–10).

The association of at least two autoimmune-induced glandular disorders is defined as autoimmune polyendocrinopathy (AP). Several non-endocrine autoimmune diseases can also be present (11, 12). AP is divided into a very rare, monogenic juvenile type I and one polygenic adult type II with three variants or subtypes, which are distinguished according to age of presentation, disease combinations, and modes of inheritance (13–17). The most prevalent AP type is the disease association of T1D and AITD in the same individual defined as subtype III variant. While the AP juvenile type has an annual incidence of 1–2:100,000, prevalence numbers are 1:600–1:900, 1:4,000, and 1:25,000 in Iranian Jews, Italians, and Finns, respectively. The adult AP form is far more common, with a worldwide incidence and prevalence of 1.4–4.5:100,000 and 14–45:1,000,000, respectively. Due to the high number of cases remaining unreported, the prevalence may approximate 1:20,000 (11, 18, 19). No accumulation in individual ethnic groups that deviate from the worldwide prevalence is known. The manifestation peak of adult AP is in the fourth-to-fifth decade depending on the combination of endocrine components with a female predominance of 75% (16, 20). T1D is the main cost driver in AP (21). The inheritance pattern seems to be autosomal dominant with incomplete penetrance, while several genetic loci may interact with environmental factors, such as deficiency of vitamin D and selenium, high iodine intake, and exposure to irradiation. The exact underlying pathogenic mechanisms are however not yet completely characterized. Also, chemical contaminants such as polybrominated diethyl ethers, polychlorinated biphenyls and their metabolites, binding to thyroid transport proteins and disrupting the thyroid function by displacing thyroxine, may be involved (22).

T1D is a T-cell mediated chronic disorder characterized by the loss of insulin-producing pancreatic β-cells and the appearance of insulitis. Biomarkers for T1D are autoantibodies (Ab) to islet cell antigens (ICA), tyrosine phosphatase (IA2), glutamic acid decarboxylase-65 (GAD), insulin (IAA), and zinc transporter ZnT8Solute carrier family 30 member 8 (SLC30A8). GD is defined by the presence of autoimmune-induced hyperthyroidism together with the presence of thyrotropin receptor autoantibodies (TSH-R-Ab) in general and stimulatory TSH-R-Ab (TSAb) in particular (23–29). HT is defined as primary hypothyroidism with an atrophic thyroid gland, an increased serum level of serum thyroid peroxidase (TPO) Ab and/or blocking TSH-R-Ab (TBAb) (24, 26, 30–32). As many as 25% of adolescents with T1D have thyroid-related Ab (15, 33–36). Long-term follow-up suggests that 30% of patients with T1D will develop AITD (10, 11, 15). TPO-Ab are present in 15–30% of adults and in 5–22% of children with T1D, while only 2–10 and 1–4%, respectively, are present in healthy controls (10, 19, 37). Up to 50% of TPO-Ab positive T1D patients develop an AITD. The diagnosis of AP III variant involves serological measurement of organ-specific Ab and subsequent functional testing of baseline thyrotropin (TSH), follicle-stimulating hormone (FSH), luteinizing hormone (LH), free triiodothyronine (fT_3) and thyroxine (fT_4), estradiol, cortisol, testosterone and fasting morning glucose as well as serum sodium (Na^+), potassium (K^+), and calcium (38).

Numerous reports on the individual susceptibility genes for T1D and/or AITD have been published, however scarce data are available pertaining to joint susceptibility. Therefore, in the present review we focus on the genetic link between these two clinically relevant endocrine autoimmune diseases and outline the underlying pathogenetic mechanisms causing AP.

PATHOGENESIS

In glandular autoimmunity, auto-aggression is considered multifactorial (39, 40). Antigen presenting cells (APC), such as ubiquitous dendritic cells, initiate the principal antigen-specific immune response (41). In non-lymphoid organs, immature dendritic cells pick up and fracture antigen molecules to subsequently migrate to the secondary lymphoid organs presenting their HLA class I or II associated antigen fragments. Together with chemokines/cytokines, antigen-specific T helper (Th) cells are activated *via* expansion of Th1 cells. They start to proliferate and exert tissue destructive activities. They further activate cytotoxic T lymphocytes, which stimulate the humoral immune response *via* expansion of Th2 cells and B-lymphocytes (42, 43). Activation of mononuclear phagocytes, another class of APC that also produce pro-inflammatory mediators accompanies the Th1 response. Auto-aggression occurs when immune tolerance, such as T suppressor cells that usually down regulate the overactive immune responses, is lost (41).

The regulatory T cell population (Treg) that inhibits the activation of $CD4^+$ and CD25 T effector cells and regulates auto-aggressive T and B cell impacts the potential development of

human autoimmune diseases (44). Naive $CD4^+$ T cells, upon encountering their cognate, differentiate into effector cells, such as Th1, Th2, Th17, or Treg. These are further characterized by their cytokine production profiles and immune regulatory functions (44):

- Th1 cells regulate antigen presentation and immunity against intracellular pathogens and produce tumor necrosis factor alpha (TNF) and interferon gamma (IFN-γ).
- Th2 cells mediate humoral responses and immunity against parasites. They are important mediators of allergic diseases and produce interleukins (IL)-4, IL-5, and IL-13.
- Th17 cells, which develop *via* an independent lineage from Th1 or Th2 cells, participate in inflammation and autoimmunity processes and express IL-17, IL-17F, IL-21, IL-22, and IL-26.
- Treg mediate immune suppression by secretion of transforming growth factor-β (TGF-β) and IL-10 and express Forkhead box P3 (FOXP3) transcription factor (45).

The innate immune system steers the differentiation of Th cells by providing T cell receptor (TCR), and co-stimulatory signals as well as an appropriate cytokine microenvironment, which ultimately leads to the preferential induction of one specific cell lineage over the other. Of upmost importance for Th1, Th2, and Th17 cell differentiation are IFN-γ, IL-12 and IL-4, while IL-6 and TGF-β potently initiate Th17 differentiation (45).

Further possible mechanisms of autoimmunity include the hygiene hypotheses, which could play an important role in the worldwide increase of autoimmune diseases. It states that pathogens, parasites and commensal microorganisms protect against a variety of autoimmune conditions. Hence, the reduction in infection rates is likely to be one of several factors that has led to an increase in the frequency of certain autoimmune diseases (46). This is paired with changes in diet, especially the increase in food consumption of saponins, lectins, gliadin, and capsaicin, which can increase intestinal permeability, thus leading to increased uptake of endotoxin and inflammation. The increase in autoimmunity may enhance intake of epitopes cross-reactive with self. However, as the increase in autoimmunity did not occur directly after the switch from hunter-gatherer diet to the early agricultural diet, an underlying immunoregulatory deficit was necessary. The hygiene hypotheses does therefore not fully explain the rise in autoimmunity. It seems that genetic, molecular mimicry, and viral hypotheses are incoherent without a major simultaneous environmental change to weaken background immunoregulation, so that certain genotypes, in the presence of certain triggers, can develop these autoimmune diseases (47, 48).

Further mechanisms of co-occurrence other than the genetic-link could be shared, i.e., molecular amino acid signatures in the HLA-DR peptide-binding pocket predisposing to both T1D and AITD. A positively charged (Lys-71, Arg-74) HLA-DR pocket 4 was found to be critical for the development of both T1D and AITD, by accommodating autoantigenic peptides that may initiate both diseases or may facilitate the anchoring of the T-cell receptor to the peptide- MHC II complex (49). While differences exist in the pathogenesis of both diseases, epidemiological data revealed clustering of T1D and AITD in the same individual as well as in families, suggesting a common genetic basis for both diseases. Studies performing family-based association analyses in multiplex families demonstrated a joint genetic susceptibility that involves complex gene-gene and genetic-epigenetic interactions. As the molecular basis for the interactions between susceptibility genes in complex diseases remains unknown, the cumulative effect of increased statistical risk, as well as molecular interactions between susceptibility genes or their products, determine disease phenotype and severity (50–52). Even in countries with low disease incidence familial clustering has been observed (1).

THE GENETIC LINK

Both T1D and AITD are multifactorial autoimmune endocrine diseases, with several susceptibility genes and environmental factors contributing to disease aetiology (15). Confirmed by genetic studies, a few genes confer risk for developing both AITD and T1D. These genes are denominated as joint susceptibility genes for AP III variant, suggesting that the gene product is implicated in the pathogenesis of both diseases (53, 54). Both whole-genome linkage screening and candidate gene studies have identified these genes (51, 55–57). Comprehensive analysis of gene-gene interactions in T1D showed that the more susceptibility risk alleles an individual carries, the higher the relative risk of developing disease, with a resulting odds ratio of 61. HLA remains the most important contributor to the overall risk, while additional gene interactions are likely to confer either protection or susceptibility (1, 58).

HLA Genes

Several genetic studies of patients with autoimmune diseases have shown specific contribution of HLA alleles, mostly HLA class II, to the genetic predisposition to autoimmune diseases. This is crucial for understanding their pathogenesis. There is a correlation between carriage of certain HLA class II alleles and an increased probability of developing the most common autoimmune diseases, including T1D and AITD. Several HLA class I and II alleles have been identified to influence both T1D and AITD, as well as AP (59–62). The HLA gene complex is located on chromosome 6p21 (**Figure 1**) and encodes the major histocompatibility complex (MHC) proteins in humans. MHC class I proteins are heterodimers that consist of a long α-chain containing a transmembrane domain and a short universal β2-microglobulin chain while MHC class II proteins consist of long α- and β- chains carrying extracellular, transmembrane and short cytoplasmic domains. While HLA corresponding to MHC class I present peptides from inside the cell, HLA corresponding to MHC class II present antigens from outside the cell to T-lymphocytes. Class I genes encode for HLA antigens A, B, and C, class II genes encode for α- and β-chains of the heterodimeric HLA class II antigens DR, DP and DQ. Class III genes encode for complement components (63).

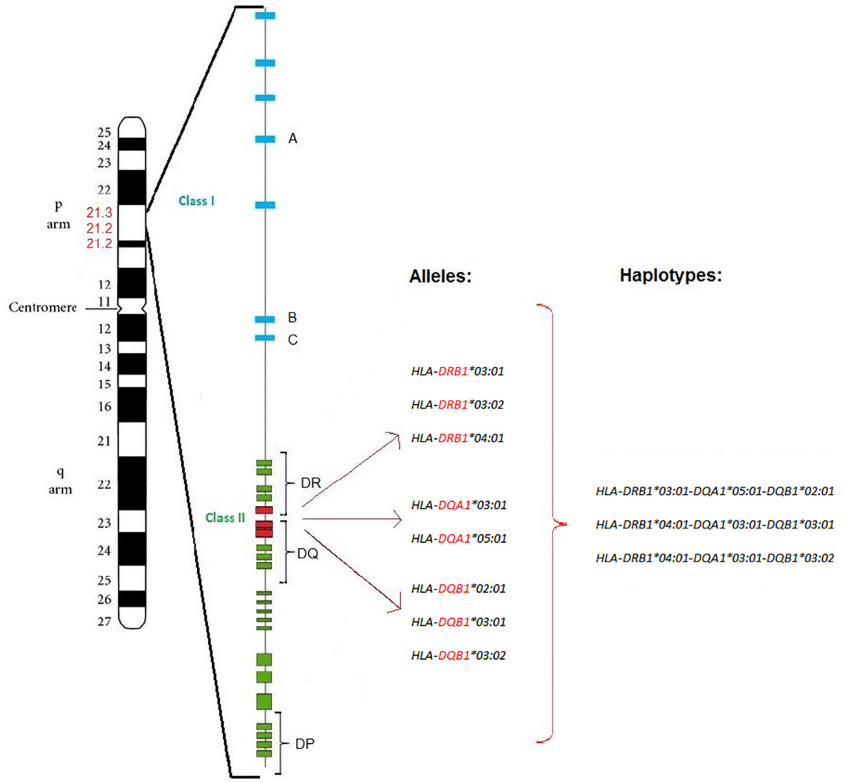

FIGURE 1 | T1D and AITD associated HLA alleles and haplotypes. Position of the HLA gene complex on the p arm of chromosome 6p21 (6p21.1-6p21.3) with around 3,500 kilo bases. The HLA class I region (blue) is located at the telomere side, while the HLA class II region (green) is located at the centromere side. *DRB1*, *DQA1*, and *DQB1* alleles and haplotypes of the HLA class II (red) are associated with both T1D and AITD (AP III variant) in Caucasian subjects. Major susceptibility alleles in Caucasians are *HLA-DRB1-03:01*, *HLA-DRB1-03:02*, *HLA-DRB1-04:01*, *HLA-DQA1-03:01*, *HLA-DQA1-05:01*, *HLA-DQB1-02:01*, *HLA-DQB1-03:01*, and *HLA-DQB1-03:02*. The resulting haplotypes are *HLA-DRB1-03:01-DQA1-05:01-DQB1-02:01*, *HLA-DRB1-04:01-DQA1-03:01-DQB1-03:01*, and *HLA-DRB1-04:01-DQA1-03:01-DQB1-03:02*.

HLA class II molecules are synthesized in the endoplasmic reticulum. The immune response develops after the 13–18 amino-acid antigenic peptide is presented by APC, i.e., dendritic cells, B cells or macrophages, using the HLA class II molecule, and is recognized by the respective T-cell receptor on the $CD4^+$ T-cell surface (64). HLA-DM, a nonconventional HLA class II molecule, is not polymorphic and cannot interact with antigenic peptides. However, it catalyzes the binding of antigenic peptide to HLA-DR. The HLA class II–peptide complexes are then delivered to the plasma membrane to present peptides to $CD4^+$ T cells (65–67).

If an immune response is initiated, $CD4^+$ T cells activate B cells for a subsequent production of specific autoantibodies and contribute to the recruitment of macrophages to the immune response. HLA molecules with point substitutions within the antigen-binding groove vary in their efficiency of binding and presentation of self-peptides followed by the initiation of an autoimmune response (68–70). Since HLA class II can present both exogenous and endogenous peptides to $CD4^+$ T cells, many biomedical studies have focused on its role in the initiation of autoimmune responses. In patients with autoimmune diseases, such as T1D and AITD, autoantibodies are synthesized and lymphocytes often infiltrate into the target organ, leading to inflammation and even partial destruction. Because antigen presentation and further T cell activation are considered key components of the immune response, studying the peculiarities of antigen presentation as well as the structure and features of HLA proteins in particular is of utmost importance. Interestingly, the autoimmune diseases accompanied by Ab production are typically associated with HLA class II, while the diseases not accompanied by Ab production are more commonly associated with certain HLA class I alleles (71, 72). Although certain HLA Class II molecules may initiate the $CD4^+$ T-cell mediated autoimmune response, HLA Class I molecules induce autoantigen-specific $CD8^+$ T cell-mediated cytotoxicity, explaining with a plausible immunological rationale, and the genetic association of both HLA Class I and Class II molecules with glandular autoimmunity. This combination has synergistic and complementary effects on various stages of the autoimmune response (73).

HLA-DR3 is the major HLA class II allele contributing to the joint susceptibility for AITD and T1D. HLA haplotypes DR3-

*DQB1*02:01* and DR4-*DQB1*03:02* contribute to AP III variant. The haplotypes *HLA-DRB1*03:01-DQA1*05:01-DQB1*02:01* and *HLA-DRB1*04:01-DQA1*03:01-DQB1*03:02* are significantly overrepresented in AP III compared to controls (72). The haplotypes for the AITD HT (*DRB1*04:01-DQA1*03:01-DQB1*03:02/03:01)* and GD (*DRB1*03:01-DQA1*05:01-DQB1*02:01*), as well as the two haplotypes identified for T1D (*HLA-DQA1*03:01-DQB1*03:02* and *HLA-DQA1*05:01-DQB1*02:01*) are nearly identical. Hence, these HLA alleles and haplotypes confer susceptibility to both T1D and AITD (74–76). Interestingly, in Arabic populations, the haplotype *DRB1*03:01-DQB1*02:01* was associated with T1D in individuals from Bahrain, Lebanon, and Tunisia, while the Japanese high-risk haplotypes *DRB1*04:05-DQB1*04:01* and *DRB1*09:01- DQB1*03:03* as well as the Caucasian haplotypes *DRB1*03:01-DQB1*02:01* and *DRB1*04:01-DQB1*03:02* were associated with T1D in individuals from Egypt, Morocco, Kuwait, Saudi Arabia, and Algeria (2).

The amino acid at position 57 of the DQ-β chain confers resistance or susceptibility to disease. An aspartic acid on position 57 in both alleles has a protective effect on T1D, while its absence provides susceptibility. In individuals in whom both alleles are non-Asp, the relative risk of T1D has been estimated to be 30 to 107 (1). The specific class II HLA-DR variant containing arginine at position 74 (*DR-1-Arg74*) increases the risk for AITD by blocking thyroglobulin peptides, especially Tg.2098. While bound to *DR-1-Arg74*, it could block continuous T cell activation and the autoimmune response in AITD. Of note, the position 74 in the β chain is exceptionally important, since this amino acid residue is located where the peptide-binding motif of HLA overlaps with the T-cell receptor docking site (77). The *HLA-DR* binding pocket has a unique amino acid signature. Four islet and thyroid peptides (Tg.1571, GAD.492, TPO.758, and TPO.338) were identified with the ability to bind to the *HLA-DR* binding pocket, being presented by antigen-presenting cells and elicited a T cell response. Both thyroid and islet peptides can bind to this flexible binding pocket and induce thyroid and islet specific T cell responses, thus triggering T1D and AITD in the same individual (78). Potential mechanisms how HLA genes activate endocrine autoimmunity and predispose to both T1D and AITD, even if the autoantigenic peptides are distinct, are shown in **Figure 2**. Either one APC expresses both pancreas (islet) as well as thyroid autoantigens (peptides) which are embedded in pockets with HLA class II molecules and presented to the T cell within the immunological synapse, or two APC express either a pancreas or thyroid antigen, however both APC share a common amino acid which facilitates the anchoring of T cells (1, 79, 80).

Another risk allele for both T1D and AITD is *HLA-DQB1* with Alanine at position 57 (Ala57), whereas Aspartic acid (Asp57) is protective. *HLA-DQB1* confers strong susceptibility by Ala57 homozygosity in monoglandular and polyglandular autoimmunity (81). *HLA-DQB1* Ala57 heterozygous women have an increased risk for AITD, whereas males have an increased risk for T1D, revealing sex-dependent increased susceptibility (81). The amino acid residue 57 is responsible for the formation of the DQA1– DQB1 heterodimer. If the aspartic acid is substituted by a neutral amino acid at this position, the HLA molecule will be able to present insulin fragments (82). HLA haplotypes DR3-DQ2 and DR4-DQ8 are associated with the presence of GAD and insulin Ab in patients with recent onset T1D (83). The specific *HLA-DR9/DQ9* subtype might be associated to both T1D and AITD, as it was found in Japanese patients exhibiting both diseases (84). However, the most universal haplotypes positively associated with these diseases are DR3-DQ2 and DR4-DQ8 (85). In the Japanese population, the DRB1-DQB1 haplotypes *DR4, DR8* and *DR9* confer susceptibility to T1D and AITD (86). Moreover, the major susceptible HLA class II haplotype in Japanese associated with AP III was *DRB1*04:05-DQB1*04:01* (87), and the haplotype *DRB1*09:01* was associated with the co-occurrence of T1D and AITD in Japan (88).

Sex alters the *class I HLA-A* association with T1D and AITD (60). In addition, patients with T1D and AITD share a common genetic background of HLA class II antigens DQ2 *(DQA1*05:01-DQB1*02:01)* and DQ8 *(DQA1*03:01-DQB1*03:02)* and overlapping functional single-nucleotide polymorphisms of various susceptibility genes involved in the immune regulation (10).

Population based differences are found in the association of HLA with T1D and AITD. Linkage analysis showed that the HLA class II alleles DQ and DR are strongly associated with both diseases. While DR3 is a major risk allele in German, Belgian, Filipino and Chinese Han populations, it does not confer susceptibility in the Japanese population. DR4 is major risk allele in Western Europe, America, South Asia and Africa but is not found in the Chinese Han population. DQA1*03:01, *05:01, and BQB1*02:01 confer risk in Caucasians, while DQB1*03:02 additionally confers risk in USA and DQB1*03:03 in Japan. DQB1*04:01 occurs in lower frequency in Caucasians but is a major risk allele in the Chinese Han population, probably due to its linkage disequilibrium with DRB1*04:05, which with DQB1*03:02 are further major risk alleles in the Chinese Han population (89). In the Northern Indian population, HLA-DRB1*03, DQA1*05, and DQB1*02 were associated with T1D, while worldwide in Caucasians HLA-DR4 (DRB1*04:01/04/05) and DQ8 (DQA1*03:01-DQB1*03:02) are associated with T1D. While in Northern India HLA-DRB1*04 and DRB1*03 were increased in patients with T1D, only the DRB1*03 allele was strongly associated with T1D and DRB1*04 was only increased in combination with DRB1*03. In Southern India however, both DRB1*03 and DRB1*04 contributed equally towards disease predisposition (73).

Single Nucleotide Polymorphisms in Non-HLA Genes

Additionally to *HLA* genes, several other genes, including the *cytotoxic T-lymphocyte associated protein 4 (CTLA4*, c.+6230G>A, rs3087243) and (*CTLA4*, c.49A>G, rs231775), the *protein tyrosine phosphatase non-receptor type 22 (PTPN22*, c.+1858 C>T, rs2476601), the *interleukin 2 receptor subunit alpha (IL2Ra, c.*A>G rs10795791), the *vitamin D receptor*

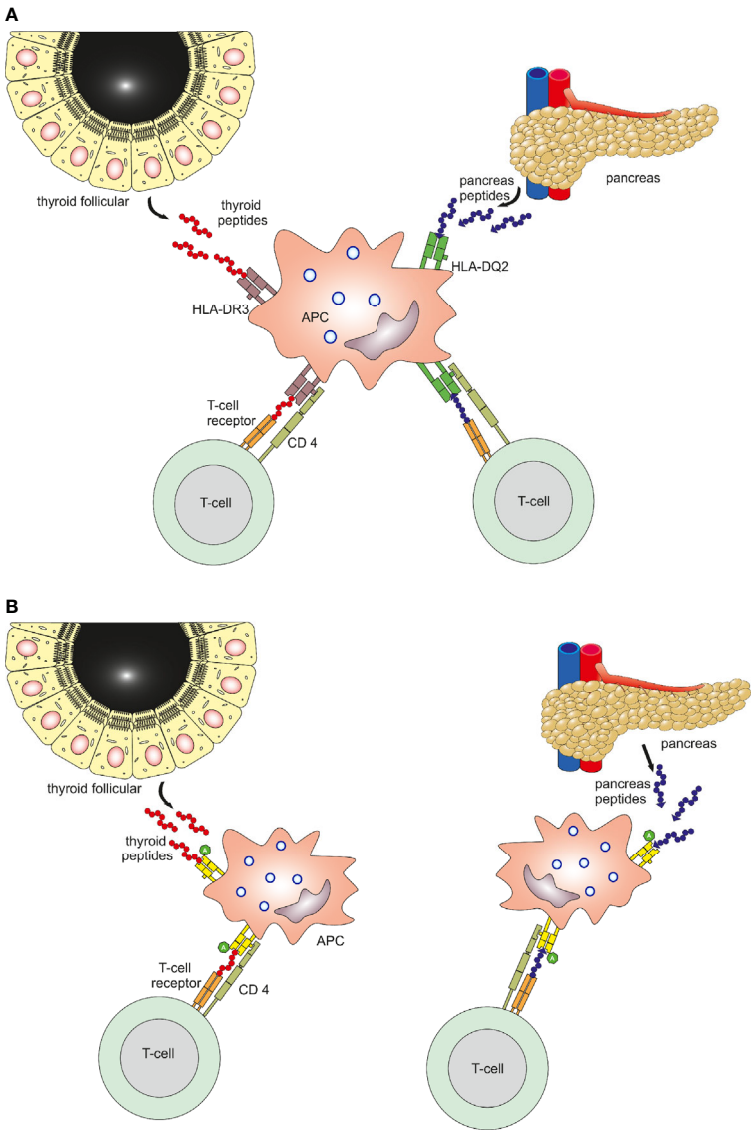

FIGURE 2 | Binding mechanisms of islet and thyroid peptides. Two potential mechanisms for immunologically targeting several glands in patients with polyglandular autoimmunity. Panel **(A)** One APC expresses both pancreas as well as thyroid autoantigens (peptides) which are embedded in pockets with HLA class II molecules and presented to the T cell within the immunological synapse. Panel **(B)** Two APC express either a pancreas or thyroid antigen, however both APC share a common amino acid, which facilitates the anchoring of T cells.

(VDR, Bsm I rs1544410; Apa I rs7975232; Taq I rs731236), as well as the *tumor necrosis factor (TNF, c.*-863G>A, rs1800630) have been reported as susceptibility genes for T1D and AITD (**Table 1**). Many other genes, such as cluster of differentiation *40* (*CD40), forkhead box P3 (FOXP3), MHC class I polypeptide-related sequence A (MICA), insulin variable number of tandem repeats (INS-VNTR), C-type lectin domain containing 16 (CLEC16A), erb-B2 receptor tyrosine kinase 3 (ERBB3)* gene, the *interferon induced with helicase C domain 1 (IFIH1)* gene and various cytokine genes, are assumed to be involved (90, 91).

A major and confirmed susceptibility gene across different ethnic groups, for both T1D and AITD, is the *CTLA4* gene. Being located on chromosome 2q33, it encodes a receptor expressed on T cells and serves as a negative regulator of T-cell activation. *CTLA4* is involved in the interaction between T lymphocytes and APC. APC activate T lymphocytes by presenting an antigenic peptide bound to a HLA class II protein on the cell surface to the T lymphocyte receptor (92). On the surface of $CD4^+$ T lymphocytes, co-stimulatory signals on the APC surface interact with receptors, such as CTLA4 during antigen presentation. *CTLA4* downregulates T lymphocyte activation (93).

TABLE 1 | Mutual non-HLA susceptibility genes in Type 1 diabetes and autoimmune thyroid disease.

Gene	Location	Function	Mutations/ Polymorphisms	Mutation/Polymorphism Phenotype
CONFIRMED				
CTLA4	2q33	Encodes a receptor that is expressed on T cells and is a negative regulator of T-cell activation.	+6230G>A (rs3087243) 49A>G (rs231775)	CTLA4 function or expression is affected by the threonine-to alanine substitution in the signal peptide of the CTLA4 protein, which leads to less efficient glycosylation in endoplasmic reticulum and reduced surface expression of CTLA4 protein. Increases susceptibility to T1D, AITD, and AP.
PTPN22	1p13	Encodes lymphoid tyrosine phosphatase, a strong inhibitor of T-cell activation, expressed in B and T lymphocytes. Inhibits T lymphocyte antigen receptor signaling pathway	+1858C>T (rs2476601)	Polymorphisms lead to increased T-cell activation and enhanced susceptibility to T1D, AITD, and AP.
IL2Ra	10p15	Differentiation factor actively suppresses auto-reactive T cells *via* CD25 and regulates function of natural killer cells, B cells and Treg.	CD25 (rs10795791)	Polymorphisms in the CD25 gene region might affect function of Treg. Increased susceptibility to T1D and AITD, especially GD
VDR	12q13.11	Expressed on immune cells and directly inhibits activated T cells. Reduces production of pro-inflammatory cytokines (IFNγ).	Bsm I (rs1544410) Aps I (rs7975232) Taq I (rs731236)	Polymorphisms lead to increased T-cell activation and enhanced susceptibility to T1D.
TNF	6p21, within HLA class III region of MHC.	Located on chromosome 6p21, within HLA class III region of MHC.	-308 (rs1800629)	Increased transcription and production of TNF protein through polymorphisms in promoter region, higher levels of TNFα transcription facilitate inflammatory response in autoimmunity. Increases susceptibility to T1D and AITD.
SUSPECTED				
CD40 Gene	20q13.12	Influences both humoral and cell-mediated immune responses.	–1T>C (rs1883832)	Polymorphisms in the Kozak sequence are associated with autoimmunity, especially AITD.
FOXP3	p arm of X chromosome (Xp11.23)	FOXP3 transcription factor occupies the promoters for genes involved in regulatory T-cell function, controls regulatory T-cell differentiation, and is considered the master regulator of Treg development and function.	-2383C>T (rs3761549)	Polymorphisms might affect function of Treg hence increasing susceptibility to T1D and AITD.
MICA	p arm of chromosome 6 (6p21.33)	Encodes highly polymorphic cell surface glycoprotein. Protein expressed in two isoforms (MICA1 and MICA2), by alternative splicing. MICA protein is a ligand for receptors on natural killer cells and the stress-induced antigen is broadly recognized by intestinal epithelial T cells.	MICA*A5 variant	Polymorphisms increase susceptibility for organ-specific autoimmune diseases.
INS-VNTR	11p15	Translation of pre-proinsulin, precursor of mature insulin. VNTR region of 14 to 15 bp consensus sequence upstream of insulin gene in insulin promoter.	Short class I: (26–63 repeats), Intermediate class II: (64–139 repeats) Larger class III: (140–210 repeats)	Polymorphisms might affect translation of pre-proinsulin, and production mature insulin. Increased susceptibility for T1D in AP type III.
CLEC16A	16p13	Encodes a member of the C-type lectin domain containing family.	rs12708716	Polymorphisms increase susceptibility for organ-specific autoimmune diseases, T1D and AITD
ERBB3	12q13	Encodes a member of the epidermal growth factor receptor (EGFR) family of receptor tyrosine kinases.	rs2292399	Polymorphisms increase risk for organ-specific autoimmunity, especially T1D and AITD.
IFIH1	2q24.3	Belongs to the pattern recognition receptor (PPR) family and is a sensor of double-stranded RNA, initiating antiviral activity through IFN production.	A946T (rs1990760)	Polymorphisms are associated with both T1D and AITD.
IL1R1	2q14.2	Encodes a competitive inhibitor of interleukin-1–induced proinflammatory activity	VNTR	Polymorphisms are associated with autoimmunity, especially AITD.
IL4 Genes	5q31.1	Is involved in the regulation of immune and inflammatory responses.	-590C>T	Polymorphisms are associated with autoimmunity, especially AITD.

CTLA4, cytotoxic T-lymphocyte associated protein 4; PTPN22, protein tyrosine phosphatase non-receptor type 22; IL2Ra, interleukin 2 receptor subunit alpha; VDR, Vitamin D receptor; TNF, Tumor necrosis factor; CD40, cluster of differentiation 40; FOXP3, forkhead box P3; MICA, MHC Class I Polypeptide-Related Sequence A; INS-VNTR, insulin variable number of tandem repeats; CLEC16A, C-Type Lectin Domain Containing 16; ERBB3, Erb-B2 Receptor Tyrosine Kinase 3 gene; IFIH1, interferon induced with helicase C domain 1; IL1R1, interleukin-1 receptor antagonist; IL4, interleukin 4; GD, Graves' disease.

Also related to autoimmunity is a 3' untranslated region (3' UTR), $(AT)_n$ microsatellite polymorphism with longer and shorter repeats of AT. Longer repeats are associated with decreased inhibitory function of *CTLA4* and a reduced control of T cell proliferation by correlating with a shorter half-life of the CTLA4 mRNA than shorter repeats (94). Both single-nucleotide polymorphisms (SNP) or rare variants +6230G>A (rs3087243) and 49A>G (rs231775) promote development of

autoimmunity, e.g., T1D and AITD by decreasing CTLA4 function. In patients with T1D and AITD (AP III), the *CTLA4* G/G genotype of the +6230 G>A SNP is increased significantly (38, 95). In families with both T1D and AITD, a preferential transmission of the G allele of the *CTLA4* 49A>G SNP in exon 1 can be seen. The 49A>G SNP in the signal peptide of the CTLA4 protein results in a threonine-to-alanine substitution, leading to a less efficient glycosylation in the endoplasmic reticulum and reduced surface expression of the CTLA4 protein. This results in increased T-cell activation by affecting *CTLA4* function and/or expression (37, 38).

Located on chromosome 1p13, the *PTPN22* gene encodes the lymphoid tyrosine phosphatase (LYP), one of the strongest inhibitors of T cell activation. It inhibits the T lymphocyte antigen receptor-signaling pathway by binding to protein kinase Csk and limiting the response to antigens. It is expressed in immature and mature B and T lymphocytes. LYP, associated with the molecular adaptor protein CBL, regulates CBL function in the T cell antigen receptor signaling pathway, as it also binds to Csk, limiting the response to antigens (10, 37, 38). The minor T allele of the +1858 (rs2476601) C>T transition was observed to be associated with T1D, AITD, and AP III (96). The SNP causes a tryptophan for arginine substitution in the LYP protein at codon 620. Distinct isoforms of the protein are encoded through two transcript variants by alternative splicing of this gene (97–99). In addition, patients carrying the minor T allele of the *PTPN22* +1858C>T SNP, had a twofold increased frequency of the *HLA-DRB1*03* allele (57). While the minor T allele is known to be involved in altered T lymphocyte activation, a novel SNP in the promoter region of the *PTPN22 gene G1123C*, has been found to be associated with both T1D and AITD in Asian patients (98). Additional polymorphisms may also be causative (54).

The *IL2Ra* gene encoding CD25 is located on chromosome 10p15 and impacts production and function of regulatory T cells, actively suppressing autoreactive T cells in the periphery as a differentiation factor and *via* CD25. It regulates the function of natural killer cells and B cells and plays an important role in the development and function of Treg. A polymorphism in the CD25 gene region (rs10795791) thereby influences the development of the autoimmune diseases and is known to be associated with both T1D and AITD, especially GD (55, 100).

The *VDR* gene is expressed on immune cells and reduces the production of pro-inflammatory cytokines by directly inhibiting activated T cells. Vitamin D3 exerts its immune modulatory function through suppression of activated T cells, resulting in improvement of phagocytosis and suppression of gamma-interferon (IFNγ) production. It therefore may reduce the occurrence of T1D in humans. Three SNPs, BsmI (rs1544410), ApaI (rs7975232), and TaqI (rs731236), have been associated with T1D (101–103), while the BsmI and TaqI polymorphisms are also significantly associated with an increased AITD risk (104). Also, the VDR polymorphism FokI has been associated with both T1D and AITD in the Brazilian population (105, 106).

The *TNF* gene is located within the HLA class III region of the MHC between HLA-B loci of class I and HLA-D loci of class II on chromosome 6p21. It encodes the pro-inflammatory cytokine TNF. Both the uncommon A allele of the G>A genotype of the -308 (rs1800629) SNP and the C/C genotype in the promoter region of the gene are associated with increased transcription and production of the TNF protein, which has been both implicated in the pathogenesis of autoimmune diseases as well as confers susceptibility to both T1D and AITD. The endogenous production of TNF is influenced by TNF promoter polymorphisms, thereby affecting messenger RNA (mRNA) and protein expression levels. Higher levels of TNF transcription may facilitate the inflammatory response in autoimmunity (91, 107). These SNP show a strong association with the occurrence of T1D and AITD in Asian and Caucasian populations (54, 108). Finally, *HLA-DRB1*03* and *TNF* -308*A alleles were strongly associated in patients with AP III. These findings indicate similar immunogenetics of T1D and AITD (109–111).

Further genes are still a matter of discussion regarding their contribution to T1D and AITD. The *CD40* gene-encoded protein also belongs to the TNF receptor superfamily and is mainly expressed in B-lymphocytes, monocytes, thyrocytes, orbital connective tissue, macrophages and dendritic cells, while the CD40 cell surface receptor is expressed on the surface of mature B cells, but not on plasma cells. CD40 influences both humoral and cell-mediated immune responses by interacting with the CD40 ligand on T cells (112). The CD40 ligand (CD40L) binds to the CD40 receptor and is predominantly expressed by activated $CD4^+$ T cells, thus activating B-cells and other APC (113). The interaction of CD40-CD40L is vital for the activation of humoral immunity through triggering B cells and production of Ab. CD40 was associated with uncontrolled HLA class II expression and intercellular adhesion molecule 1 (ICAM-1) overexpression in the thyroid follicular cells of patients with AITD (114). Thyroid follicular cells might be able to function as APC under special circumstances (115). The CD40 −1 T>C SNP (rs1883832) in the Kozak sequence is associated with AITD, especially GD (116–118).

CD247 codes for CD3-zeta, a component of the TCR-CD3 signaling complex on T cells. CD3-zeta functions as an amplifier of TCR signaling and the CD3-zeta tyrosine phosphorylation is one of the first events occurring after TCR engagement (119).

The *FOXP3* gene, located on the p arm of the X chromosome (Xp11.23), modulates the differentiation of regulatory T cells. It contains 11 coding exons and belongs to the forkhead/winged-helix family of transcriptional regulators. The FOXP3 transcription factor controls Treg differentiation and is considered the master regulator of Treg development by occupying the promoters for genes involved in Treg function. Genetic variants through mutations of the FOXP3 regulatory pathway reduces function and affects thymocytes developing within the thymus that during thymopoiesis are transformed into mature Tregs, promoting the development of autoimmunity (54, 90, 120). Both a haplotype consisting of 25 repeats of a microsatellite on allele 10 and the T allele of a C>T SNP at position -283 (rs1883832) were related with AP III. Because the microsatellite is located past the zinc finger domain of the *FOXP3*

gene, it affects downstream splicing, thereby impeding the function of the gene (121).

MICA is located on the p arm of chromosome 6 (6p21.33) and an associated locus within the MHC region. It encodes a highly polymorphic cell surface glycoprotein expressed in two isoforms formed by alternative splicing, MICA1 and MICA2. MICA2 is lacking exon 3. The MICA protein is expressed in epithelial and intestinal cells and is a ligand for receptors on the surface of natural killer cells. It acts as a possibly stress-induced antigen that is broadly recognized by intestinal epithelial T cells, binding to CD8 T cells carrying the integral membrane protein receptor natural killer group 2, member D (NKG2D) as well as natural killer cells. When engaged, the NKG2D–MICA complex results in the activation of T cell responses and natural killer cells against epithelial stressor cells expressing MICA on their surface. It is therefore also connected to organ-specific autoimmune diseases such as T1D and AITD (54, 90, 122, 123).

The *INS gene* region, encoding Insulin, is located on chromosome 11p5. Based on the number of the VNTRs, three classes are differentiated. An association of the *insulin VNTR class I alleles* with AP III has been found (51). A short class I *VNTR* penta-allelic 86-bp tandem repeat in the regulatory 5′-UTR and a longer class III *VNTR* 600 bp tandem repeat polymorphism are related to lower and higher INS expression in the thymus, respectively (54, 90, 124). As insulin binds to insulin- like growth factor 1 receptors and exert its functions and the insulin-like, growth factor 1 receptor overlaps with TSH-R signaling in AITD, DNA alternations affecting the insulin expression influence the TSH-R signaling in AITD.

The *CLEC16A* gene, located on chromosome 16p13, contains a C-type lectin domain. The encoded protein is detected in immune cells, is implicated in pathogen recognition, and might predispose for immune mediated diseases e.g. T1D and AITD (125). The SNP rs2903692 shows a G>A transition significantly associated with both T1D and AITD, with the G allele increasing the risk for AP III (56).

The *ERBB3* gene is located on chromosome 12q13. The SNP rs2292399 in intron 7 of ERBB3 has been shown to be associated with AITD and T1D with the *A allele* increasing the risk for AP III (56).

The *IFIH1* gene, located at 2q24.3 chromosome, belongs to the pattern recognition receptor (PPR) family and is a cytosolic RNA sensor. The gene product, as a sensor of double-stranded RNA, initiates antiviral activity through the induction of IFN regulatory factors 3 and 7, leading to IFN production and apoptosis of virally infected cells, also promoting innate and adaptive immune responses. The SNP *rs1990760* is a non-synonymous polymorphism, an alanine to threonine amino acid change at codon 946A>T, within the *IFIH1* coding region located in the HNF-3b binding site. The polymorphism is associated with both T1D and AITD, especially GD. The correlation between viral infections and development of autoimmune diseases such as T1D and AITD makes *IFIH1* a good susceptibility gene candidate (126–129).

Cytokine genes are also potential candidate genes for development of autoimmunity, especially AITD, since they are involved in the regulation of immune and inflammatory responses (130). The *interleukin-1 receptor antagonist* (*IL-1RA)* gene, located on chromosome 2q14.2, encodes a competitive inhibitor of interleukin-1–induced pro-inflammatory activity. *IL1R1* modulates several interleukin-1–related immune and inflammatory responses and inhibits the activities of interleukin 1, interleukin 1-α and interleukin 1-β. A *VNTR in intron 2* within the *IL1R1* gene has been associated with AITD (131, 132). Genes encoding *interleukin 4* (*IL4*), located on chromosome 5q31.1, are also involved in the regulation of immune and inflammatory responses. IL4 is produced by activated T cells and acts as a pleiotropic cytokine with immunomodulatory functions and is a ligand for the IL-4 receptor. A polymorphism at position −590C>T in the *IL4* gene is associated with AITD (54, 133).

A list of susceptibility genes from several international publications obtained by a NCBI PubMed (https://pubmed.ncbi.nlm.nih.gov/) search for T1D, AITD, and the co-occurrence of both diseases from various populations worldwide is shown in **Table 2**. In detail, in Japan, CTLA4 is strongly associated with T1D or AITD but not with AP III (87, 136). Furthermore, ERBB3, CLEC16A, and CTLA4 are associated with the co-occurrence of thyroid autoimmunity and T1D in the Japanese population (56, 87, 88). CTLA4, PTPN22, IFIH1, INS, CD247, and NAA25, are associated with both T1D and AITD in Sweden (119). The VDR polymorphism FokI is associated with both T1D and AITD in the Brazilian Hispanic population (105). The chemokine receptor type (CCR) 5 - 32 bp deletion (Δ32) is associated with both diseases and with celiac disease in Poland (141). Finally, the signal transducer and activator of transcription (STAT)-4 is associated with both T1D and AITD in Korea (140), SESN3 with AITD in the Chinese Han population (139) and SLC26A4 is associated with AITD in the Tunisian population (138). Non-HLA genes like CTLA4 and PTPN22, which have been associated with both T1D and AITD in many Caucasian populations could only be associated with either T1D or AITD in certain Asian and Arab populations (2, 135, 137). Differences of association could also be found in IFIH1 and INS. Interestingly, population differences of IFIH1 susceptibility were also found within Caucasians populations in Europe (134, 135). This raises the question of how strong the population-based differences actually are and if they only occur between populations of different races or even within populations of the race.

Further, CTLA4, PTPN22, IL2RA, BACH2, CCR5, SH2B3, and RAC2 are found to be associated with T1D and AITD by various independent genome wide association studies and overlap in our list, indicating a strong common genetic link for T1D and AITD.

CONCLUSION

The coexistence of different organ-specific and non- organ-specific autoimmune diseases in the same individual or family

TABLE 2 | List of population based susceptibility genes for T1D, AITD, and AP subtype III variant.

Gene	Diseases	Country	Ethnecity	Reference
CTLA4	T1D	Middle East & North Africa	Arab	Hatem Zayed, 2016 (2)
	T1D	Poland	Caucasoid	Hanna Borysewicz-Sanczyk, 2020 (134)
	T1D	Great Britain	Caucasoid	Suna Onengut-Gumuscu 2015 (135)
	AITD	Great Britain, China	Caucasoid, Asian	Yul Hwangbo, 2018 (136)
	T1D AITD	Japan	Asian	Takuya Awata, 2008 (56)
	T1D AITD	Japan	Asian	Hisakuni Yamashita, 2011 (88)
	T1D AITD	Japan	Asian	Ichiro Horie, 2012 (87)
	T1D AITD	Sweden	Caucasoid	Dan Holmberg, 2016 (119)
PTPN22	T1D	Tunisia	Arab	Hatem Zayed, 2016 (2)
	T1D	Great Britain	Caucasoid	Suna Onengut-Gumuscu 2015 (135)
	AITD	Jordan	Arab	Asem Alkhateeb, 2013 (137)
	AITD	Great Britain	Caucasoid	Yul Hwangbo, 2018 (136)
	T1D AITD	Sweden	Caucasoid	Dan Holmberg, 2016 (119)
IL2RA	T1D	Japan	Asian	Hisakuni Yamashita, 2011 (88)
	T1D	Great Britain	Caucasoid	Suna Onengut-Gumuscu 2015 (135)
	T1D	Poland	Caucasoid	Hanna Borysewicz-Sanczyk, 2020 (134)
	AITD	Great Britain	Caucasoid	Yul Hwangbo, 2018 (136)
IFIH1	T1D	Japan	Asian	Hisakuni Yamashita, 2011 (88)
	T1D	Poland	Caucasoid	Hanna Borysewicz-Sanczyk, 2020 (134)
	T1D	Great Britain	Caucasoid	Suna Onengut-Gumuscu 2015 (135)
	T1D AITD	Sweden	Caucasoid	Dan Holmberg, 2016 (119)
INS	T1D	Japan	Asian	Hisakuni Yamashita, 2011 (88)
	T1D	Great Britain	Caucasoid	Suna Onengut-Gumuscu 2015 (135)
	T1D AITD	Sweden	Caucasoid	Dan Holmberg, 2016 (119)
CLEC16A	T1D AITD	Japan	Asian	Takuya Awata, 2008 (56)
	T1D AITD	Japan	Asian	Hisakuni Yamashita, 2011 (88)
ERBB3	T1D AITD	Japan	Asian	Takuya Awata, 2008 (56)
	T1D AITD	Japan	Asian	Hisakuni Yamashita, 2011 (88)
CD28	T1D	Tunisia	Arab	Hatem Zayed, 2016 (2)
CD40	GD	Japan	Asian	Naoya Inoue, 2012 (118)
CD69	T1D	Great Britain	Caucasoid	Suna Onengut-Gumuscu 2015 (135)
CD226	T1D	Great Britain	Caucasoid	Suna Onengut-Gumuscu 2015 (135)
CD247	T1D	Tunisia	Arab	Hatem Zayed, 2016 (2)
	T1D AITD	Sweden	Caucasoid	Dan Holmberg, 2016 (119)
TNF	T1D	Great Britain	Caucasoid	Suna Onengut-Gumuscu 2015 (135)
IL2	T1D	Great Britain	Caucasoid	Suna Onengut-Gumuscu 2015 (135)
IL10	T1D	Great Britain	Caucasoid	Suna Onengut-Gumuscu 2015 (135)
IL21	T1D	Great Britain	Caucasoid	Suna Onengut-Gumuscu 2015 (135)
IL27	T1D	Great Britain	Caucasoid	Suna Onengut-Gumuscu 2015 (135)
IL7R	T1D	Japan	Asian	Hisakuni Yamashita, 2011 (88)
	T1D	Great Britain	Caucasoid	Suna Onengut-Gumuscu 2015 (135)
IL15	T1D	Tunisia	Arab	Hatem Zayed, 2016 (2)
VDR	T1D AITD	Brazil	Hispanic	Denise Barreto Mory et al., 2016 (105)
NAA25	T1D AITD	Sweden	Caucasoid	Dan Holmberg, 2016 (119)
TRB	T1D	Tunisia	Arab	Hatem Zayed, 2016 (2)
SLC26A4	AITD	Tunisia	Arab	Rihab Kallel-Bouattour, 2017 (138)
SESN3	AITD	Chinese Han	Asian	Wei Liu, 2018 (139)
FCRL3	GD	Japan	Asian	Naoya Inoue, 2012 (118)
	GD	China	Asian	Yul Hwangbo, 2018 (136)
ZFAT	HT	Japan	Asian	Naoya Inoue, 2012 (118)
STAT4	T1D AITD	Korea	Asian	Yongsoo Park, 2011 (140)
BANK1	T1D	Tunisia	Arab	Hatem Zayed, 2016 (2)
ZAP70	T1D	Tunisia	Arab	Hatem Zayed, 2016 (2)
SMOC2	AITD	Jordan	Arab	Asem Alkhateeb, 2013 (137)
BACH2	T1D	Great Britain	Caucasoid	Suna Onengut-Gumuscu 2015 (135)
	AITD	Great Britain	Caucasoid	Yul Hwangbo, 2018 (136)
IKZF1	T1D	Great Britain	Caucasoid	Suna Onengut-Gumuscu 2015 (135)
IKZF3	T1D	Great Britain	Caucasoid	Suna Onengut-Gumuscu 2015 (135)
IKZF4	T1D	Great Britain	Caucasoid	Suna Onengut-Gumuscu 2015 (135)
AFF3	T1D	Great Britain	Caucasoid	Suna Onengut-Gumuscu 2015 (135)
CCR5	T1D	Great Britain	Caucasoid	Suna Onengut-Gumuscu 2015 (135)
	T1D AITD CD	Poland	Caucasoid	Bartosz Słomiński, 2017 (141)
CCR7	T1D	Great Britain	Caucasoid	Suna Onengut-Gumuscu 2015 (135)

(Continued)

TABLE 2 | Continued

Gene	Diseases	Country	Ethnecity	Reference
GLIS3	T1D	Great Britain	Caucasoid	Suna Onengut-Gumuscu 2015 (135)
SH2B3	T1D	Great Britain	Caucasoid	Suna Onengut-Gumuscu 2015 (135)
	HT	United States	Caucasoid	Yul Hwangbo, 2018 (136)
GRP183	T1D	Great Britain	Caucasoid	Suna Onengut-Gumuscu 2015 (135)
RASGRP1	T1D	Great Britain	Caucasoid	Suna Onengut-Gumuscu 2015 (135)
CTSH	T1D	Great Britain	Caucasoid	Suna Onengut-Gumuscu 2015 (135)
DEXI	T1D	Great Britain	Caucasoid	Suna Onengut-Gumuscu 2015 (135)
BCAR1	T1D	Great Britain	Caucasoid	Suna Onengut-Gumuscu 2015 (135)
FUT2	T1D	Great Britain	Caucasoid	Suna Onengut-Gumuscu 2015 (135)
UBASH3A	T1D	Great Britain	Caucasoid	Suna Onengut-Gumuscu 2015 (135)
ICOSLG	T1D	Great Britain	Caucasoid	Suna Onengut-Gumuscu 2015 (135)
RAC2	T1D	Great Britain	Caucasoid	Suna Onengut-Gumuscu 2015 (135)
	GD	China	Asian	Yul Hwangbo, 2018 (136)
TSHR	GD	Great Britain, China	Caucasoid, Asian	Yul Hwangbo, 2018 (136)
RNASET2	AITD	Great Britain, China	Caucasoid, Asian	Yul Hwangbo, 2018 (136)
CHRNA9	GD	China	Asian	Yul Hwangbo, 2018 (136)
MMEL1	GD	Great Britain	Caucasoid	Yul Hwangbo, 2018 (136)
PRICKLE1	GD	Great Britain	Caucasoid	Yul Hwangbo, 2018 (136)
ITGAM	GD	Great Britain	Caucasoid	Yul Hwangbo, 2018 (136)
GPR174	GD	China	Asian	Yul Hwangbo, 2018 (136)
SLAMF6	GD	China	Asian	Yul Hwangbo, 2018 (136)
ABO	GD	China	Asian	Yul Hwangbo, 2018 (136)
LINC01550	GD	China	Asian	Yul Hwangbo, 2018 (136)
TG	GD	China	Asian	Yul Hwangbo, 2018 (136)
FOXE1	HT	United States	Caucasoid	Yul Hwangbo, 2018 (136)
VAV3	HT	United States, Japan	Caucasoid, Asian	Yul Hwangbo, 2018 (136)
CAPZB	HT	United States	Caucasoid	Yul Hwangbo, 2018 (136)
PDE8B	HT	United States	Caucasoid	Yul Hwangbo, 2018 (136)
TRIB2	AITD	Great Britain	Caucasoid	Yul Hwangbo, 2018 (136)
LPP	AITD	Great Britain	Caucasoid	Yul Hwangbo, 2018 (136)
FAM76B	AITD	Great Britain	Caucasoid	Yul Hwangbo, 2018 (136)

T1D, Type 1 diabetes; AITD, Autoimmune thyroid disease; GD, Graves' disease; HT, Hashimoto's thyroiditis; CD, Celiac disease; CTLA4, cytotoxic T-lymphocyte associated protein 4; PTPN22, protein tyrosine phosphatase non-receptor type 22; IL2RA, interleukin 2 receptor subunit alpha; IFIH1, interferon-induced with helicase C domain 1; INS, insulin gene; CLEC16A, C-Type Lectin Domain Containing 16; ERBB3, Erb-B2 Receptor Tyrosine Kinase 3; CD28, cluster of differentiation 28; CD40, cluster of differentiation 40; CD3z, cluster of differentiation 3z; CD247, cluster of differentiation 247; IL7R, interleukin-7 receptor; IL15, interleukin-15; VDR, Vitamin D receptor; NAA25, N-Alpha-Acetyltransferase 25; TCRβ, T-cell receptor beta; SLC26A4, solute carrier family 26 member 4; CCR)5-(Δ32), Chemokine receptor type (CCR)5 - 32-bp deletion (Δ32); SESN3, Sestrin3; FCRL3, Fc receptor-like protein 3; ZFAT, Zinc Finger And AT-Hook Domain Containing; STAT4, Signal transducer and activator of transcription 4; BANK1, B Cell Scaffold Protein With Ankyrin Repeats 1; ZAP70, Zeta-chain-associated protein kinase 70; SMOC2, SPARC-related modular calcium-binding protein 2; BACH2, BTB domain and CNC homolog 2; IKZF1, IKAROS family zinc finger 1; IKZF3, IKAROS family zinc finger 3; IKZF4, IKAROS family zinc finger 4; AFF3, AF4/FMR2 family member 3; CCR5, C-C motif chemokine receptor 5; CCR7, C-C motif chemokine receptor 7; GLIS3, GLIS family zinc finger 3; SH2B3, SH2B adaptor protein 3; GRP183, G protein-coupled receptor 183; RASGRP1, RAS guanyl releasing protein 1; CTSH, cathepsin H; DEXI, Dexi homolog; BCAR1, BCAR1 scaffold protein, Cas family member; FUT2, fucosyltransferase 2; UBASH3A, ubiquitin associated and SH3 domain containing A; ICOSLG, inducible T cell costimulator ligand; RAC2, Rac family small GTPase 2; TSHR, thyroid stimulating hormone receptor; RNASET2, ribonuclease T2; CHRNA9, cholinergic receptor nicotinic alpha 9 subunit; MMEL1, membrane metalloendopeptidase like 1; PRICKLE1, prickle planar cell polarity protein 1; ITGAM, integrin subunit alpha M; GPR174, G protein-coupled receptor 174; SLAMF6, SLAM family member 6; ABO: ABO, alpha 1-3-N-acetylgalactosaminyltransferase and alpha 1-3-galactosyltransferase; LINC01550, long intergenic non-protein coding RNA 1550; TG, thyroglobulin; FOXE1, forkhead box E1; VAV3, vav guanine nucleotide exchange factor 3; CAPZB, capping actin protein of muscle Z-line subunit beta; PDE8B, phosphodiesterase 8B; TRIB2, tribbles pseudokinase 2; LPP, LIM domain containing preferred translocation partner in lipoma; FAM76B, family with sequence similarity 76 member B.

could be explained by sharing a common genetic background as well as a defective immune regulation. HLA regions, including DR3, DR4, in association with DQ2 and DQ8 are strongly associated with T1D, AITD, and AP III in Caucasians. HLA haplotypes in patients with AITD (HT and GD), as well as T1D were found to be nearly identical. However, in Arab and Asian populations, HLA susceptibility alleles and haplotypes differ from the ones found in European and American Caucasian populations. Also, population based differences are detected in the association between glandular autoimmunity with both HLA and non-HLA genes. Certain non-HLA susceptibility genes, such as *CTLA4, PTPN22, IL2Ra, VDR*, and *TNF* are involved in the activation of Treg that can react or cross-react with autoantigens. Therefore, polymorphisms in these genes confer further susceptibility to T1D, AITD, and AP III in Caucasians. The contribution of further genes e.g., *CD40, FOXP3, MICA, INS-VNTR, CLEC16A, ERBB3, IFIH1*, and various cytokine genes has not been definitely and/or fully clarified. In conclusion, the combined influence of genetic, epigenetic and environmental factors may lead to the onset of autoimmune disorders in different organs of the same subject or within families. Therefore, genetic screening is useful in patients with monoglandular autoimmunity e.g., T1D and Addison's disease, as well as their first-degree relatives. In view of the possible long interval between the first manifestation of AP and the subsequent development of further autoimmune endocrinopathies, regular and long-term observation of patients is warranted. Furthermore, screening for autoimmune endocrine diseases is

recommended regularly, especially for the offspring of patients with T1D and AITD, and AP III variant.

AUTHOR CONTRIBUTIONS

LF conceptualized and designed the study, acquired and analyzed the data as well as drafted the article. GK Project initiation, conception and design, analysis and interpretation of data, drafting and critical revision of the article, as well as approval of the final version to be published. All authors contributed to the article and approved the submitted version.

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Aging Regulated Through a Stability Model of Insulin/Insulin Growth Factor Receptor Function

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Mutations of the insulin-like receptor in *Drosophila* extend lifespan. New research suggests this receptor operates in two modes. The first extends lifespan while slowing reproduction and reducing growth. The second strongly extends lifespan without impairing growth or reproduction; it confers longevity assurance. The mutation that confers longevity assurance resides in the kinase insert domain, which contains a potential SH2 binding site for substrate proteins. We apply a recent model for the function of receptor tyrosine kinases to propose how insulin receptor structure can modulate aging. This concept hypothesizes that strong insulin-like ligands promote phosphorylation of high threshold substrate binding sites to robustly induce reproduction, which impairs survival as a consequence of trade-offs. Lower levels of receptor stimulation provide less kinase dimer stability, which reduces reproduction and extends lifespan by avoiding reproductive costs. Environmental conditions that favor diapause alter the expression of insulin ligands to further repress the stability of the interacting kinase domains, block phosphorylation of low threshold substrates and thus induce a unique molecular program that confers longevity assurance. Mutations of the insulin receptor that block low-phosphorylation site interactions, such as within the kinase insert domain, can extend lifespan while maintaining overall dimer stability. These flies are long-lived while maintaining reproduction and growth. The kinase insert domain of *Drosophila* provides a novel avenue from which to seek signaling of the insulin/insulin-like growth factor system of humans that modulate aging without impacting reproduction and growth, or incurring insulin resistance pathology.

Keywords: aging, *Drosophila*, insulin receptor, insulin, IGF, reproduction, longevity, insulin resistance

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INTRODUCTION

Mutations of the insulin/IGF tyrosine kinase receptor slow aging in *Drosophila* and *C. elegans*, and perhaps as well in humans (1–3). These invertebrates have single insulin/IGF-like receptors, InR in *Drosophila* and DAF-2 in *C. elegans*. Besides aging, these receptors regulate traits including development, growth, metabolism, reproduction, sleep, behavior, and Dauer/diapause (4–10). In mammals, a family of insulin, IGF, relaxin, and insulin-like peptides modulate many functions including metabolism, cell cycle, development, reproduction, cognition, and vascular physiology (11–13), where adult insulin and IGF1 signals *via* three dimeric receptors [IR, IGF1-R, IR/IGF1R

hybrid (14)]. In contrast, the single invertebrate insulin-like receptors respond to a number of unique insulin-like ligands, seven in *Drosophila* and as many as 40 in *C. elegans* (15, 16). Despite their centrality, little is understood about how these invertebrate insulin-like ligands control such an array of distinct phenotypes. Here we explore a potential solution. We integrate new observations derived from single amino acid substitutions of *Drosophila* InR (17) with the receptor tyrosine kinase (RTK) threshold model of Zinkle and Mohammadi (18). We will propose that the level of insulin-stimulated dimer stability determines which substrate binding sites are activated to impact specific traits. Mutations of *InR* may slow aging because they reduce overall receptor dimer stability or because they directly modify binding sites. This model suggests how insulin-like receptors might slow aging without insulin-resistance and how diverse *Drosophila* insulin-like ligands control unique sets of traits. The model provides a framework to understand where and how modified insulin/IGF signaling can affect human aging.

THE THRESHOLD MODEL OF RECEPTOR TYROSINE KINASE SIGNALING

Receptor tyrosine kinases (RTK) are single-pass transmembrane proteins that transduce extracellular ligand binding into kinase activity. Strongly bound ligands are thought to induce sustained kinase activity to promote outputs distinct from those of weak ligands, which produce transient or low kinase activity; the *intensity and duration* of intracellular signaling pathways determines the cellular response (19). As reviewed in Zinkle and Mohammadi (18), this process was first proposed for rat PC12 cells where the duration of MAPK activation differentially promotes neurite outgrowth *versus* cell proliferation, independent of ligand or receptor identity (19). In a second example, isoforms of fetal growth factor (FGF) ligand FGF8a and FGF8b differentially induce the midbrain to differentiate or expand. This specificity, however, is based on the relative abundance of each isoform and the associated magnitude of Ras/MAPK induction, not upon the ligand identity (20).

RTK also phosphorylate binding sites within their juxtamembrane (JM), C-terminal tail, and kinase domains. These sites recruit adapter proteins including those with Src homology 2 (SH2), phosphotyrosine-binding (PTB), and SH3 domain-binding sites. The identity of recruited substrate specifies which transduction pathways the receptor activates (21–23). Thus, mutation of one docking site can alter one particular outcome without affecting others, for instance when mutation of the Grb2-recruitment site on the canine kidney cell MET receptor blocks tubulogenesis without disrupting cell dissociation (24). In this view, the *quality* of the receptor-protein interaction determines the cellular response.

Zinkle and Mohammadi (18) integrate how the *intensity of activation* and the *quality of interactions* determine RTK function. Ligand binding causes receptor tyrosine kinase protomers to dimerize or in the case of IR preformed dimers cause the intracellular domains to structurally reorient (25). Repositioning of IR intracellular domains is induced when insulin binds multiple ectodomain sites upon both protomers to affect hinge motions that bring each internal kinase domains into proximity, permitting them to asymmetrically transphosphorylate A-loop tyrosine residues (26–28). This transactivation stimulates subsequent kinase activity to phosphorylate endodomain tyrosine residues and substrate binding proteins. Central to the model (18), the level of stability between the repositioned intracellular domains determines which endodomain tyrosine residues are phosphorylated, where adaptor binding sites have unique phosphorylation thresholds. High affinity insulin ligands will have fast on-rates and slow off-rates at receptor binding sites and thus continuously stabilize the dimer to phosphorylate both low- and high-threshold sites (**Figure 1A**). Relatively weak or transient ligands will have slower on-rates and faster off-rates and consequently induce weak dimer stability that only activates binding sites with low phosphorylation thresholds. As a general point for the model relevant for any RTK, although thresholds are ordered, cellular responses need not be nested because signals from a high threshold site can inhibit the output from lower threshold sites (**Figure 1B**).

Overall, Zinkle and Mohammadi synthesize both perspectives of RTK operation: the intensity and duration of dimer stability regulates which binding proteins are activated, and these substrates specify the cellular outcome of the stimulated receptor. Here we develop how this threshold model helps explain control of aging by insulin-like receptors. First we describe longevity-extending mutations of *Drosophila InR* and *C. elegans daf-2*, and introduce known adaptor proteins of InR.

THE *DROSOPHILA* AND *C. ELEGANS* INSULIN-LIKE RECEPTORS

Gems, Patel, and colleagues classified multiple mutations of the *C. elegans* insulin-like receptor *daf-2* (29, 30). "Class 1" mutants include substitutions in the extracellular CR, L2, and FnIII domains. These induce dauer, an alternative quiescent developmental stage, and promote adult longevity. "Class 2" substitutions reside in the L1 ligand pocket, the CR ectodomain, and the intracellular tyrosine kinase domain. These alleles induce dauer and extend lifespan, but also variously affect feeding, reproduction, movement, and growth (29). Class 1 and Class 2 alleles stimulate unique transcriptional profiles (30). To explain these differences, Patel (30) suggested Class 1 mutants reduce DAF-2 abundance and thus activate the transcription factor DAF-16/FOXO. Class 2 alleles were thought to increase receptor perdurance and thereby reduce interaction with Ras-associated substrates while retaining signal induction of PI3K/Akt. From extensive phenotypic analyses, these authors suggest the DAF-2 receptor has two distinct functional outputs.

We recently studied how mutations in *Drosophila InR* affect aging (17). InR is generated from three alternative 5'UTRs (31, 32) to produce isoforms differing by a 368-amino acid C-terminal tail

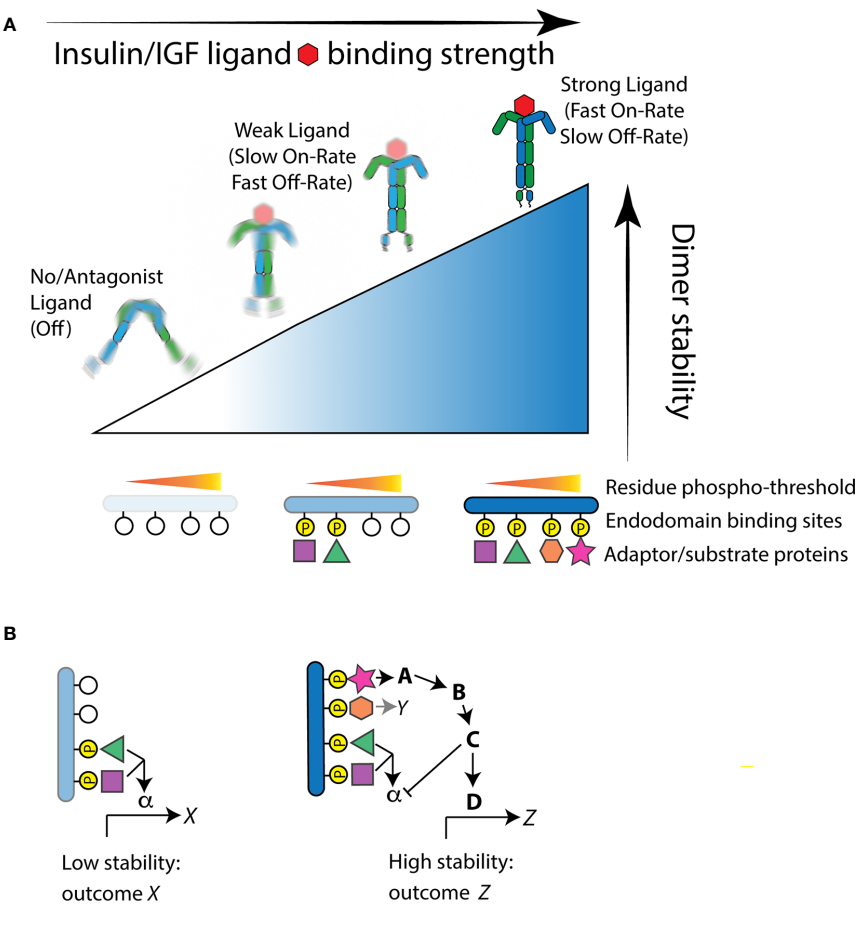

FIGURE 1 | The Receptor Tyrosine Kinase threshold model of Zinkle and Mohammadi applied to the insulin-like receptor. **(A)** Structural reorientation of insulin-like protomers is induced by ligand binding. Strong binding ligands have fast-on/slow-off rates and produce highly stable interactions of the internal kinase domains. This permits phosphorylation of endodomain substrate binding sites that have high as well as low thresholds, thus recruiting a full complement of available substrate proteins. Weaker ligands produce moderate kinase domain interaction stability and thus only induce residue phosphorylation at sites with a relatively low stability threshold. In the absence of ligand, or when the receptor is bound by an antagonist ligand, protomers fail to reorient or are highly unstable. In this state, the kinase domains do not phosphorylate substrate residue sites and few if any substrate binding protein are engaged. **(B)** Signal feedback among hierarchical thresholds can produce unnested signaling outcomes. As an example: In weak stability activation of the receptor, low threshold binding protein interactions activate a signal pathway through the substrate protein α to induce a transcriptional program *X*. This program is not necessarily activated, however, when the receptor gains greater stability, even though the substrate protein α is recruited. A high threshold substrate interaction that activates binding protein A may simultaneously propagate signaling to induce the transcriptional program *Z* and repress signaling otherwise propagated by α.

(33–35). Based on our analysis of codon substitutions, InR appears to modulate aging through distinct modes (**Table 1**). As transheterozygotes, Mode 1 alleles increase survival, decrease egg production, reduce body size, and repress insulin-stimulated Akt phosphorylation (17). Among genotypes from these alleles, lifespan negatively correlates with egg production (**Figure 2A**), consistent with theory for how aging arises when selection optimizes fitness (47). These pro-longevity mutations produce amino acid substitutions in the extracellular FnIII domain (extracellular V*810*D), and in conserved residues of the kinase A-loop and the kinase C-lobe (**Figure 2B**). As a group, these substitutions are likely to destabilize protomer endodomain interaction or directly inhibit kinase catalytic function (27, 28).

Mode 2 is represented by the dominant allele InR^{353} (17). Adult heterozygotes (wildtype/InR^{353}) have robustly increased lifespan but remarkably so without decreasing reproduction or growth (**Table 1**). Unlike Mode 1 flies, tissue from the InR^{353} heterozygotes strongly induces pAkt in response to insulin—they are not insulin resistant. When InR^{353} is combined with Mode 1 alleles, adults lay fewer eggs and lifespan is increased by the combined effects of reduced survival costs of reproduction added to the longevity assured by InR^{353} (**Figure 2A**).

The InR^{353} substitution Arg*1466*Cys lies within the kinase insert domain (KID) (**Figure 2C**), an unstructured peptide segment that interrupts the kinase domain of many RTKs (48). Arg*1466* of Drosophila is homologous to Arg*1092* of the human

TABLE 1 | Phenotypes of *Drosophila* insulin/IGF receptor and substrate protein mutations.

Genotype	Lifespan increaseDays (proportion)	Net fecundity, proportion	Adult size, proportion	Ref
Mode 2: Increase longevity without reduced fecundity or growth				
WT/*InR*^{353}	10–16 d (1.2–1.4)	1.6	1.0	(17)
WT/*chico*^{1}	14–18 d (1.3–1.4)	2.0	1.0	(36)
	10 d (1.4)	0.80	1.0	(37)
	3–16 d (1.1–1.4)			(38)
	12–22 d (1.2–1.5)			(39)
	8 d (1.1)			(40)
	10 d (1.2)			(41)
Mode 1: Increase longevity with reduced fecundity or growth				
***InR*^{74}, *InR*^{E19}, *InR*^{211}**	6–14 d (1.2–1.4)	0.05–0.75	0.81–0.88	(17)
***chico*^{1}/*chico*^{1}**	16 d (1.3)	sterile	0.40–0.50	(36)
	16 d (1.6)		0.35	(37)
	12–22 d (1.2–1.5)			(38)
	18 d (1.4)			(42)
				(41)
Lnk/Lnk (SH2B1)	5–8d (1.0–1.1)	<0.2	0.60–0.65	(43)
				(44)
				(45)
InR-DN	9–13 d (1.2–1.4)	0.19–0.86	0.55	(46)
UAS-p110	5 d (1.1)	0.73		(46)

Compiled from sources that together describe lifespan and reproduction (female); and adult size when available. Values for lifespan are the average gain in median survival relative to wildtype controls, in days and as a proportion relative to control. When shown, range is among replicate trials within the publication. Fecundity: net egg production per female across the measured duration of each genotype relative to wildtype. Adult size based on mass or wing area, as a proportion relative to wildtype. Empty cells: data not available. Upper table compiles ***Mode 2*** *genotypes: longevity is extended without reduced fecundity or impaired growth; representing longevity assurance. Lower table compiles* ***Mode 1*** *genotypes: longevity is extended while reproduction and growth are impaired; representing life history trade-offs.*

insulin receptor (49). In humans, the insulin receptor mutation Arg*1092*Glu produces Donohue syndrome where heterozygotes are largely normal while homozygotes are strongly insulin resistant, small, and inviable (50). Overall, the function of kinase insert domains is poorly understood but whereas the human IR and IGFR domains are short, the longer *Drosophila* KID contains a potential SH2 binding motif (Tyr*1477*-Leu-Asn; **Figure 2C**). This site may recruit an adaptor protein, potentially Grb2 as seen in the KID of mammalian PDGFR, CSF1R, and Kit (48). We hypothesize the *InR*^{353} substitution disrupts this receptor-protein interaction to induce longevity assurance—a homeostatic program that increases somatic survival independent of reproductive trade-offs (51).

ADAPTOR AND SUBSTRATE PROTEINS OF *DROSOPHILA* INR

A number of receptor-adaptor protein interactions are documented for the *Drosophila* insulin receptor. The C-terminal tail of InR recruits Chico (homolog of IRS1-4), although apparently without phosphorylating this substrate (52, 53). The tail likewise contains Y*XX*M motifs to recruit the p85/p60 subunit of PI3-kinase (34), and P*XX*P sequences for the SH2/SH3 adapter Dock (homolog of mammalian Nck) (54). Dock modulates photoreceptor axon guidance but does not affect growth. No data address if protein interactions with the C-terminal tail affect aging.

The juxtamembrane domain (JM) of InR also recruits Chico, using NP*X*Y residues conserved in the human insulin receptor (42, 53). Interaction between InR and Chico is mediated by the SH2B1 adaptor protein Lnk (44, 45, 55). In mammals, SH2B1 is recruited to insulin receptor A-loop phosphotyrosines (56, 57). In *Drosophila*, Lnk colocalizes InR and Chico to promote phosphorylation of Akt (55). Genetic loss of Lnk extends longevity, reduces body size, and represses fecundity (**Table 1**).

Mutation of *chico* itself slows aging (**Table 1**). Appropriate for the centennial of insulin discovery, *chico* is debated to harken back to 1919, potentially as an allele of the mutation *flipper* identified by Bridges and Mohr (see https://flybase.org/reports/FBgn0000675). Modern *chico* mutant alleles are transposon insertions initially characterized to elevate lipids, and impair cell size and number (42, 58). Homozygotes of the mutant *chico*^{1} are small, long-lived, and sterile; wildtype/*chico*^{1} heterozygotes are also long-lived and similar to wildtype/*InR*^{353} these adults have normal growth and fertility (36–38) (**Table 1**).

Chico is a substrate adapter protein. It recruits SH2/SH3 domain-containing proteins including the p85/p60 subunit of PI3K and the Grb2 homolog Drk (Downstream of receptor kinase) (42, 53, 59). Oldham expressed *chico*-transgenes in *chico*^{1} homozygotes (59). Wildtype *chico*-transgenes rescued body size and fertility. Transgenes that only restored Grb2/Drk binding did not rescue these traits while those that restored p60/PI3K restored growth and reproduction. Slack (40) used this design to study aging. The exceptional longevity of *chico*^{1} heterozygotes reverted to normal by addition of a wildtype *chico* transgene but not when the *chico* transgene contained only functional p60/PI3K sites or only functional Grb2/Drk sites. Overall, Chico controls p60/PI3K/Akt to modulate growth, metabolism, and longevity, but its effects through Grb2/Drk appear to be limited to aging.

As in mammals, activated InR phosphorylates Akt to repress *Drosophila* Foxo, the homolog of mammalian FOXO1-4 and *C. elegans* DAF-16. As seen for *daf-16*, *foxo* is required for insulin receptor mutations to extend *Drosophila* lifespan (39, 46). Gene targets of these transcription factors in both invertebrates reveal many distal mechanisms to slow aging (60–62). Parallel to Akt-Foxo, Drosophila Grb2/Drk regulates Ras to control signaling through Erk (63). Slack (40) demonstrated Chico acts through Ras-Erk to regulate the E-twenty-six transcription factor Anterior Open (Aop). Aop is required for *chico* mutations to extend lifespan, however no data yet shows if this interaction is downstream of InR rather than other potential IRS-regulatory receptors (64).

These observations provide three touchpoints. First, mutations of *InR* may affect aging through altered kinase activity while another may act by altering adapter protein interaction. Second, *InR*^{353} and *chico*^{1} are dominant alleles that produce long-lived adults that are unexpectedly large and

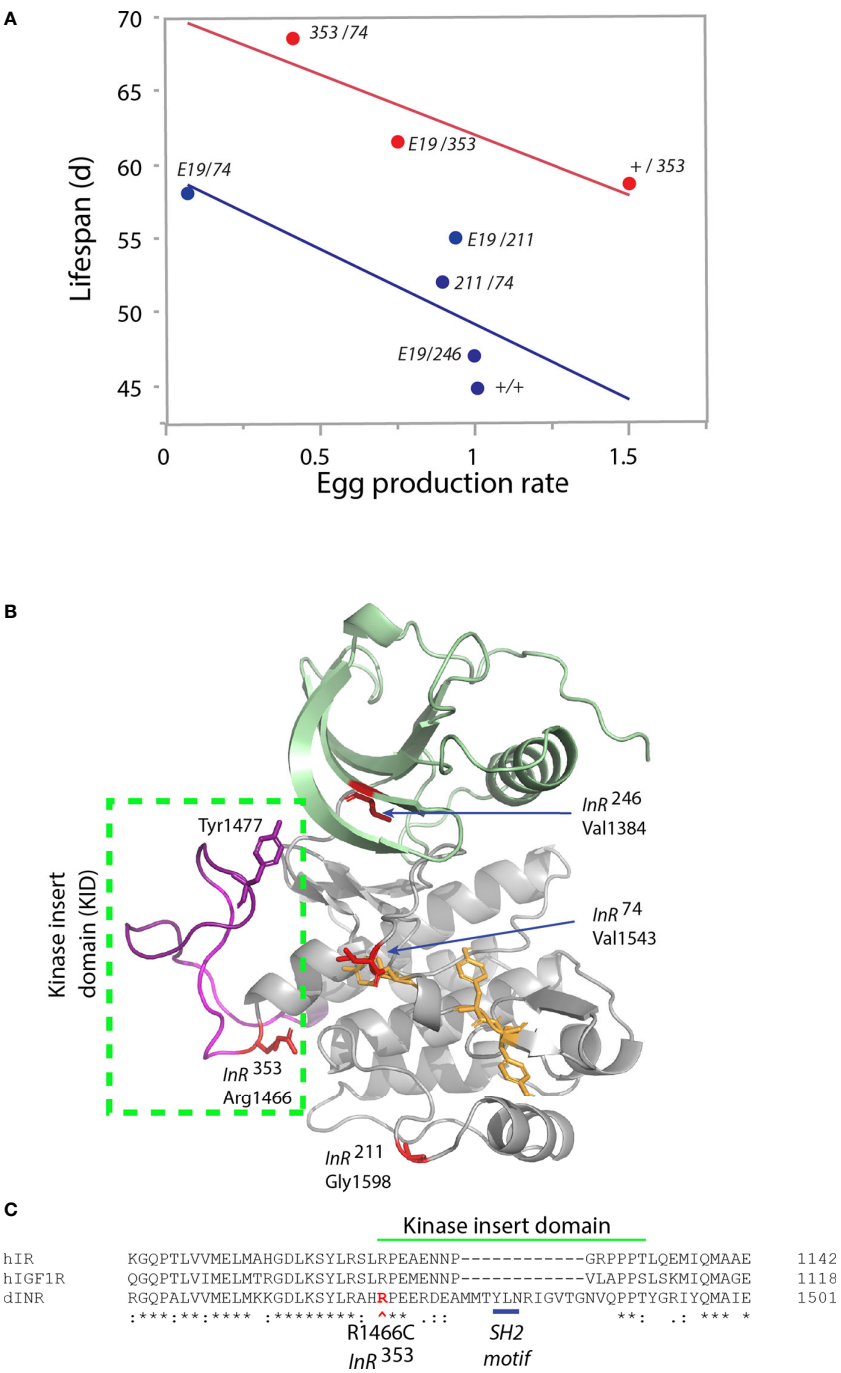

FIGURE 2 | Characteristics of single amino acid substitution of the *Drosophila* insulin-like receptor. **(A)** Relationship of genotype average median lifespan relative to rate of egg production, from Yamamoto (17). Egg production rate is number of eggs produced daily scaled by the ovary size (number of ovarioles: ovary subunits). Lifespan is the average of median survival among replicate trials and independent genetic accessions of each genotype. Blue regression: genotypes lacking the InR^{353} allele ("353" in figure). Red regression: genotypes that include one InR^{353} allele. "E19" is the allele InR^{E19}, a V*810*D substitution in the extracellular FnIII domain (17). Other alleles (InR^{74}, InR^{211}, InR^{246}; in figure "74," "211," "246") are substitutions within the kinase domain. The +/+ genotype is wildtype coisogenic will all mutant alleles, co-derived by homologous recombination; details in Yamamoto (17). **(B)** Model of kinase domain structure for the *Drosophila* insulin-like receptor. Subdomains: N-lobe (green), C-lobe (gray), A-loop tyrosines (yellow), Kinase Insert Domain (magenta) within box. Substitution residues for kinase domain mutations in red; Tyrosine*1477* of the proposed SH2 binding site within the KID. **(C)** Partial amino acid alignment of the C-lobe to include region of the KID in human IR, human IGF1R and *Drosophila* InR [alignments and nomenclature from (17)]. Site of the InR^{353} substitution in red (R*1466*C), site of the proposed SH2 motif underlined in blue.

fecund. Third, Chico appears to signal through SH2-Grb2/Drk-Ras to modulate aging without affecting growth or reproduction, while we suggest the InR kinase insert domain contains an unrecognized SH2 binding motif. The Arg*1466*Cys substitution of InR^{353} within the KID may destabilize Grb2/Drk direct signaling to slow aging. These observations can be integrated with the RTK threshold model to hypothesize how InR regulates aging.

HYPOTHESIS: STABILITY THRESHOLDS TO REGULATE AGING

Zinkle and Mohammadi (18) propose stimulated RTK have varied levels of dimer stability that progressively phosphorylate adaptor binding sites, each with a characteristic threshold. Activated binding sites interact with specific adaptor proteins to stimulate unique cellular outcomes. We envision this model operates within insulin-like receptors (**Figure 3A**). In *Drosophila* InR, sites with high thresholds may include those that recruit Lnk and Chico while sites with a relatively Low phosphorylation threshold might recruit Grb2/Drk. In conditions favoring full reproduction, abundant, strong insulin ligands interact with InR to stabilize protomer kinase domain interaction. Strong transphosphorylation and extensive kinase activity phosphorylate both low (Grb2/Drk) and high threshold receptor binding sites (Chico, Lnk), and efficiently phosphorylate substrate proteins. The activated substrates transduce signals through Akt, TOR, Ras, AMPK, and GSK to promote growth and reproduction. These conditions are permissive for aging because lifetime reproductive success is optimized through the balance of egg production with associated survival costs.

In restricted conditions such as limited diet, adults secrete fewer or different DILPs (65). We propose this moderately reduces dimer stability to a level that dampens kinase activity while Akt is still phosphorylated. The receptor propagates less intense signaling, which reduces reproduction and correspondingly increases survival. In extreme conditions, such a season that induces diapause, we propose the endocrine state minimizes InR dimer stability so that low threshold residues become dephosphorylated. The SH2 motif of the kinase insert domain may represent such a site. It may be activated in normal conditions by insulin ligands to induce Grb2/Drk-Ras/Erk signaling. However, in diapause conditions key insulin ligands are repressed (67). We hypothesize this will destabilize InR dimers to dephosphorylate the SH2/Grb/Drk site of the KID, and thereby blunt Erk signaling to release somatic maintenance programs that retards somatic aging. Because of the hierarchy within the threshold model, high-phosphorylation threshold sites of InR will not be activated in this state of low dimer stability; Akt will not transduce pro-reproductive signaling. This mechanism models InR regulation of reproductive diapause; it simultaneously stalls reproduction and assures somatic survival until favorable environmental conditions return (68, 69).

This model may explain how some insulin receptor mutations slow aging without affecting reproduction or insulin sensitivity (Mode 2). We hypothesize the Arg*1466*Cyr substitution disrupts how Grb/Drk is recruited to the SH2 binding motif of the KID. This mutation, however, does not destabilize the dimer and heterozygous receptors therefore phosphorylate Akt and retain kinase activity that propagate reproduction and growth. Although, balancing this hypothesis, the drug Trametinib, a selective MEK1 and MEK2 inhibitor, extends fly lifespan while reducing fecundity (70). In contrast, Mode 1 mutations have reduced kinase activity and are therefore insulin resistant (17). We propose these mutations somewhat increase dimer instability, but not to an extent that dephosphorylates Try*1477*. Fecundity and growth are reduced with moderate loss of dimer stability, and longevity is increased by mitigating survival costs of reproduction.

DROSOPHILA INSULIN-LIKE LIGANDS

In this threshold model, receptor dimer stability will be modulated by the quantity, quality, and bioavailability of insulin-like ligands. *Drosophila* has seven insulin-like loci, *dilp1-7* (49, 66, 71). Based on *dilp* sequence from 12 *Drosophila* species, Gronke (66) concluded these ligand peptides contain conserved cysteine disulfide bridges, bioactive A and B chains, and functional signal peptides (**Figure 3B**). DILP1, DILP6, and DILP7 are notable for their extended B-chain N termini. DILP6 has a short C-peptide sequence and may thus more resemble mammalian IGF. An alternative insulin-like peptide was subsequently identified, *dilp8*, which encodes a relaxin-like ligand that stimulates G protein-coupled signaling (72, 73). The insulin-like peptide genes are expressed in varied tissues from embryo to adult, and early work showed mutants of these loci affect growth and metabolism (49, 74, 75). In normal adults, *dilp2-3*, *and 5* are primarily produced in median neurosecretory cells (MNC) where they are released into the brain, into secondary endocrine organs, and into circulation (74). In contrast, adult *dilp1* is only expressed in MNC during reproductive diapause (76). The MNCs derive from anterior neuroectoderm of the fly embryo, orthologous to vertebrate adenohypophyseal placoid that is the developmental source of mammalian islet-like endocrine cells (77). As well, *dilp6* is expressed in the fat body, a tissue with liver- and adipose-related function (78, 79).

Synthetic and recombinant peptides have been used to reveal the function of individual DILPs. Dimeric recombinant DILP5 binds human insulin receptors in a manner consistent with negative cooperativity (80), and when injected into rats and Drosophila the recombinant hormone transiently lowers circulating sugar. DILP5 also interacts with the insect-binding protein Imp-L2 (80, 81), likely to antagonize circulating insulin (82, 83). Notably, elevated Imp-L2 is associated with extended lifespan, even in conditions where *dilp2*, *dilp3*, and *dilp5* mRNA are elevated (84–86). It is not known which insulins aside from DILP5 bind to Imp-L2.

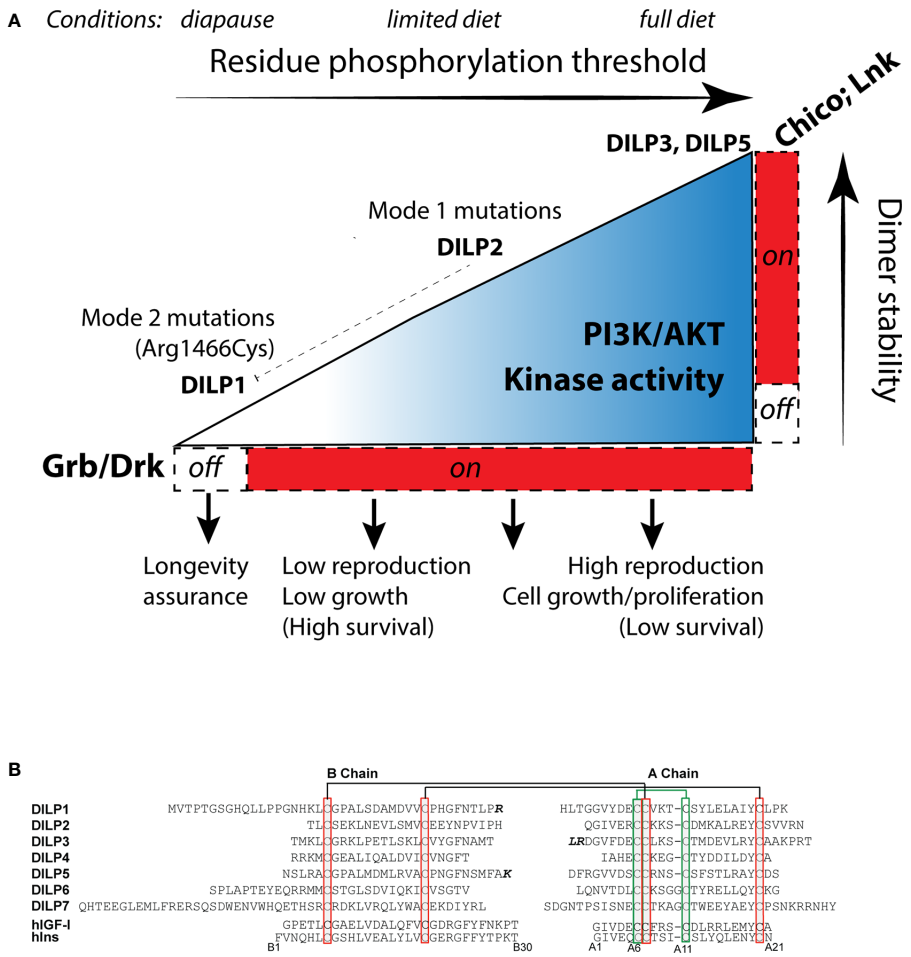

FIGURE 3 | How insulin-like receptors and ligands may modulate aging relative to reproduction, growth and metabolism through the Receptor Tyrosine Kinase threshold model. **(A)** The environment determines the level of insulin-like receptor dimer stability through control of *Drosophila* insulin like peptides (DILP). Peptides with high binding activity (DILP5, perhaps DILP3) promote stable protomer kinase interaction, leading to strong kinase catalytic activity and phosphorylation of high threshold substrate binding sites, activation of the adaptor proteins LNK, Chico and Grb, and signal transduction through AKT and Ras. This stimulates reproduction and growth. Limited diet (or moderating environments) reduce DILP5 and DILP3 but retain DILP2 expression (65). DILP2 has reduced kinase dimer stability, potentially sufficient to activate AKT and RAS through PI3K and Grb/Drk recruitment but with less kinase catalytic activity. This state sustains less reproduction. DILP2 represses expression of *dilp1*. In extreme environments, flies enter diapause and express *dilp1*. We propose DILP1 is a competitive receptor antagonist. Kinase dimer stability is minimized. High/moderate threshold sites required to activate Akt are not phosphorylated, ceasing reproduction. Low threshold sites required to activate Grb/Drk are not activated. This impairs Ras signaling, which induces systems to support somatic survival (longevity assurance). This state produces reproductive diapause. The InR^{353} mutation (Arg*1466*Cys), we propose, inhibits phosphorylation of Grb/Drk by the KID but does not (as observed) affect the ability of the receptor to induce phosphorylation of Akt. The mutation unleashes the longevity assurance program of diapause while bypassing the loss of dimer stability that would otherwise inhibit reproduction. **(B)** Sequences of the *Drosophila* insulin-like peptides DILP1-7, B- and A-chains, using cleavage sites predicted by Gronke (66), with potential alternatives where the additional residues are noted in bold. Chains are aligned across bridge cysteines. Human insulin and insulin growth factor 1 for comparison, numbered from insulin.

A synthetic DILP2 was compared to DILP5 when these peptides stimulated Drosophila S2 cells in culture (87). These peptides induced broadly similar signaling elements (Akt, Erk, S6K) and transcriptional profiles, but they also revealed unique outputs. DILP5 produced high, continuous phosphorylation of Akt whereas DILP2 only induced a transient response. In a phosphoproteomic scan, DILP2 equally increased and decreased the number of total phosphorylation sites while DILP5 overwhelmingly increased total phosphorylation. Several specific proteins were differentially phosphorylated by these peptides. Notably, glycogen phosphorylase did not respond to DILP5 but the enzyme was dephosphorylated and inactivated in cells stimulated by DILP2, a response typical for human insulin. Conversely, elevated glycogen phosphorylase activity was found in *dilp2* mutant flies, which are long lived, while transgenic expression of *GlyP* was sufficient to extend lifespan. These data demonstrate measurable differences among specific DILPs acting through a common receptor. And they remind us that the action

of insulin-like peptides in aging can involve non-genomic, cellular metabolic regulation independent of canonical FOXO transcription factors.

Understanding DILP function *in vivo* is complicated because mutation of one *dilp* changes the expression of others (66). Nonetheless, abundant data shows longevity is extended when *dilp2* is reduced alone or with other insulins (66, 79, 88). *dilp2* expression in adults is greatest on diet of low protein and high sugar (65). In contrast, adult *dilp1* is absent under normal conditions soon after eclosion, but is elevated 14-fold in *dilp2* mutants and 4-fold during diapause (76, 89). Post (89) demonstrated that dilp1 is required for loss of dilp2 to extend lifespan, but dilp1 is not required for the loss of dilp2 to induce dilp3 and dilp5 or stimulate phosphorylation of Akt. In contrast, loss of *dilp2* represses pErk in a *dilp1* dependent manner. DILP1 and DILP2 appear to have countervailing functions associated with diapause, longevity, and Erk signaling.

These observations suggest how Drosophila insulin-like peptides might regulate the outcomes of InR. We tentatively propose DILP5 (and perhaps DILP3) strongly stabilizes InR dimers; DILP2 transiently stabilizes the dimer; DILP1 inhibits InR stability and competitively blocks other insulin-like ligands. In good environments, DILP2, DILP3, and DILP5 promote dimer stability and kinase activity. This activates pAKT and pERK signal transduction to promote growth and reproduction. In this state DILP2 simultaneously represses *dilp1*. Conditions of limited diet repress *dilp3* and *dilp5* but not *dilp2* (65); dimer stability is moderately reduced. This state still phosphorylates Akt but diminishes kinase signaling, which down-regulates reproduction and improves survival. At the extreme, in diapause, *dilp1* is transcribed. Abundant DILP1 inhibits the binding of other insulin ligands to the receptor, minimizes dimer stability, prevents Akt phosphorylation to retard reproduction, and extinguishes Grb2/Drk-Erk signaling to induce systems of longevity assurance.

This sketch is speculative and incomplete. No work yet reveals how DILP1 or DILP2 interact with InR, or how any DILP affects dimer stability or substrate protein interaction. We have not considered DILP6, perhaps the most IGF-like fly ligand, which non-autonomous affects aging through its action in the fat body (78, 79). Little functional data are available for DILP3 despite its abundance in adults. There is much work ahead.

THE PARADOX OF INSULIN RESISTANCE AND LONGEVITY

How could altered insulin-like signaling support healthy human aging as found in *C. elegans* and *Drosophila*? One solution argues the domain-defined functions of the invertebrate insulin-like receptor are distributed across the mammalian IR and IGFR receptors. The Arg*1466*Cys substitution of the *Drosophila* kinase insert domain promotes longevity without impairing growth and reproduction, or incurring loss of kinase activity (stimulated pAkt). Similar outcomes arise in *chico* heterozygotes and when the SH2/Grb site of Chico is blocked. None of these genotypes are particularly hyperglycemic or insulin resistant (17, 40). Instead, insulin resistance occurs in InR genotypes where we predict the mutations reduce stability of activated protomers. These outcomes suggest we identify where the longevity assurance function of the *Drosophila* KID translates to human IR or IGFR. While the human kinase insert domains share the KID sequence Arg-Pro-Glu where Arg*1466*Cys is substituted in Drosophila InR^{353}, the human KID are small and lack the SH2 motif proposed for *Drosophila*. It is possible in the evolution of insulin-like receptors that some ancestral KID functions were integrated into the four insulin receptor substrates of mammals, as may also be the case of the *Drosophila* IRS-like C-terminal tail. In particular, IRS2 contains SH2 binding motifs that recruit Grb2, and mice mutant for IRS2 are long-lived (90). This property of IRS2 could involve interactions with IGF1R (91, 92). Notably, human polymorphisms in *IGF1R* are associated with survival to extreme age (3), and mice heterozygous for *IGF1R* are long-lived in some genetic backgrounds (93). It would be interesting to explore how these *IGF1R* genotypes affect specific phospho-sites of IRS2, and whether they alter Grb/Ras/Erk signaling.

If human aging can be modulated by IGFR-IRS2, insulin resistance is not required to slow aging (94), which is otherwise a paradox attributable to invertebrate models. Insulin resistance and slow aging indeed covary in *Drosophila* but the traits are decoupled in a mutation of the KID that potentially avoids loss of high dimer-stability signaling. Parallel benefits in humans might occur through elements of IGFR-mediated signaling rather than through reduced insulin sensitivity.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the author, without undue reservation.

AUTHOR CONTRIBUTIONS

The author confirms being the sole contributor of this work and has approved it for publication.

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REVIEW
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Nutrition and Obesity in the Pathogenesis of Youth-Onset Type 1 Diabetes and Its Complications

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Since the 1980s, there has been a dramatic rise in the prevalence of overweight and obesity in pediatric populations, in large part driven by sedentary lifestyles and changing dietary patterns with more processed foods. In parallel with the rise in pediatric obesity in the general population, the prevalence of overweight and obesity has increased among children and adolescents with type 1 diabetes. Adiposity has been implicated in a variety of mechanisms both potentiating the risk for type 1 diabetes as well as exacerbating long-term complications, particularly cardiovascular disease. Treatment options targeting the unique needs of obese pediatric patients, both before and after diagnosis of type 1 diabetes, are limited. In this review, we discuss the history of the epidemiology of the obesity epidemic in the context of pediatric type 1 diabetes, highlight the possible role of obesity in type 1 diabetes pathogenesis and review the concept of "double diabetes". The impact of obesity at and after diagnosis will be discussed, including noted differences in clinical and biochemical markers, lipid abnormalities, and long-term cardiovascular complications. Finally, we will review the existing literature on pharmacologic and nutritional interventions as potential treatment strategies for youth with coexisting type 1 diabetes and obesity.

Keywords: type 1 diabetes, obesity, nutrition, double diabetes, cardiovascular complications

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INTRODUCTION

Autoimmune type 1 diabetes is one of the most common chronic conditions of childhood, with the incidence increasing worldwide (1–3). Our understanding of the diverse forms of diabetes has evolved since the 1950s, with several different classification systems proposed until the most recent distinction of type 1 and type 2 diabetes in the 1990s (4). This classification focused on pathogenesis, separating diabetes resulting from absolute insulin deficiency, typically secondary to autoimmunity,

Abbreviations: BMI, body mass index; DPV, Diabetes Patienten Verlaufsdokumentation registry; ADA, American Diabetes Association; ISPAD, International Society for Pediatric and Adolescent Diabetes; DCCT, Diabetes Control and Complications Trial; EDIC, Epidemiology of Diabetes Interventions and Complications study; PTP, Pathway to Prevention; ceBMI, cumulative excess BMI; HOMA-IR, homeostatic model assessment of insulin resistance; ER, endoplasmic reticulum; SNPs, single nucleotide polymorphisms; CVD, cardiovascular disease; eGDR, estimated glucose disposal rate; EDC, Epidemiology of Diabetes Complications study; CACTI, Coronary Artery Calcification in Type 1 Diabetes; GLP 1, glucagon-like peptide 1; DPP4, dipeptidyl peptidase 4; SGLT, sodium glucose cotransporter.

or from progressive loss of insulin secretion in the setting of insulin resistance. However, continued advances have unraveled the variable genetic, immunologic, and metabolic factors that contribute to diabetes, and our current concept of type 1 diabetes in youth acknowledges significant heterogeneity of this disease. One noteworthy factor affecting this disease is the rise in childhood overweight and obesity (5). A known risk factor for insulin resistance, obesity is now a frequently recognized comorbidity in type 1 diabetes and may compound both the risk for and subsequent complications of type 1 diabetes in youth.

In this review, we highlight the epidemiological trends in obesity and new onset type 1 diabetes, including possible etiological explanations. With the rising prevalence of overweight and obesity at diagnosis of type 1 diabetes and persisting over time, we will address three important questions. First, does obesity contribute to the increased incidence of type 1 diabetes in youth and how? Second, what are the short- and long-term impacts of obesity on type 1 diabetes and its complications? And finally, can targeted treatment strategies optimize outcomes in youth with coexisting obesity and type 1 diabetes?

CHANGING TRENDS IN DIABETES EPIDEMIOLOGY

Parallel Epidemics: Obesity and Type 1 Diabetes

The obesity epidemic is one of the defining global public health issues of our time. Over the latter half of the 20th century, the prevalence of overweight and obesity in pediatric populations increased dramatically in the United States. Approximately one-third of American children are overweight or obese (6). Recent estimates among children age 2–19 years point to 17% of children with obesity [body mass index (BMI) ≥ 95th percentile] and 5.8% with extreme obesity (BMI at or above 120% of the sex-specific 95th percentile), with some leveling-off in the prevalence of obesity in younger age groups in recent years (5). Similar trends in pediatric overweight and obesity have been observed worldwide (7, 8), though the rates of obesity in the developing world continue to accelerate (9). At the heart of the obesity epidemic are sedentary lifestyles and the "westernized diet", consisting of increased processed foods, refined sugars, and saturated fat. Metabolic derangements resulting from this diet promote weight gain and contribute to cardiovascular disease, diabetes, and cancer (10). Ultra-processed foods, both nutrient poor and calorically dense, are increasingly linked to higher risk for all-cause mortality in the United States and other countries (11, 12). Added sugars in processed foods are a significant portion of the diet of American children beginning at a young age (13), a concerning finding given the evidence for long-term health problems. Longitudinal data links obesity in childhood to adverse health outcomes in adulthood, including hypertension, fatty liver disease, dyslipidemia, and type 2 diabetes (14–16). In addition to the significant morbidity and mortality associated with adult obesity, there are enormous economic costs that are projected to continue to increase over the next decades (17).

In parallel with the obesity epidemic, the incidence of type 1 diabetes in youth has also been increasing worldwide (1–3). The multi-center SEARCH for Diabetes in Youth Study reported a 1.8% per year increase in the incidence of type 1 diabetes from 2002–2012 adjusted for age, sex, and race/ethnicity; the incidence was higher for racial/ethnic minorities at 4.2% increase per year in Hispanic youth and 2.2% increase per year in African American youth (18). In Europe, the EURODIAB study has examined trends in incidence from 1989 to 2013. Initial findings demonstrated an incidence of 3.2–4.1% per year from 1989–2003 with a subsequent leveling off; however, a follow-up investigation found a persistent and steady rise in the incidence of type 1 diabetes in more recent years of approximately 3% per year, suggesting possible cyclicity in a changing incidence rate (19, 20). No clear etiologic factor has been identified to explain this pattern. Various environmental triggers have been explored, including pathogens, nutritional changes, and obesity (21), though the increasing incidence of type 1 diabetes has continued despite some slowing in the pediatric obesity epidemic in developed countries (9).

Obesity in Children and Adolescents With Type 1 Diabetes

With the increasing incidences of both type 1 diabetes and obesity, the prevalence of overweight and obesity in youth with type 1 diabetes at diagnosis has also increased. Unlike type 2 diabetes, where obesity is a known risk factor, children with new-onset type 1 diabetes in the past were not overweight at diagnosis and traditionally thought to be thin. Libman et al. was one of the first to identify the changing presentation of youth with diabetes in a study examining new onset insulin dependent diabetes in cohorts of children from 1979–1998 (22). Over a 20-year period, the prevalence of overweight and obesity at diagnosis increased threefold, from 12.6% in the first decade to 36.8% in the second decade. The prevalence of overweight and obesity at onset increased nearly five times among those with confirmed autoimmunity from the 1980s to the 1990s, suggesting that the observation was not driven by increasing cases of type 2 diabetes. Subsequent studies have similarly identified a rise in BMI at diagnosis of type 1 diabetes over time (23–25).

Following diagnosis, several groups have examined the prevalence of overweight and obesity cross-sectionally in children and adolescents with type 1 diabetes at least one year into diagnosis. In single-center reports, the estimated prevalence ranged from 25–40% of youth, with most approaching 35%, in children with a mean duration of diabetes of 5.6–8.7 years (26–29). In recent years, larger, registry-based assessments have confirmed these findings, as illustrated in **Figure 1**. The US-based SEARCH for Diabetes in Youth Study found that 34.7% of participants with type 1 diabetes were overweight or obese (30); another United States-based multicenter collaborative, the Type 1 Diabetes Exchange, estimated this prevalence to be 36% in their population with a mean duration of 6.8 ± 4.1 years (31). In contrast, the European-based, prospective Diabetes Patienten Verlaufsdokumentation (DPV) Registry estimated a lower prevalence of 15.3% of children being overweight/obese in their population with a mean duration of 4.7 ± 3.0 years (33).

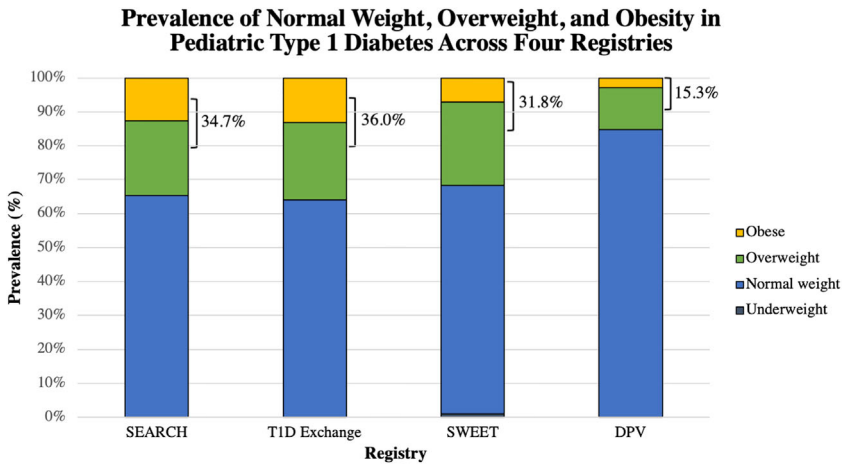

FIGURE 1 | Graphical representation of the proportion of reported normal weight, overweight, and obese youth with type 1 diabetes in each of these four registries: SEARCH (30), T1D Exchange (31), SWEET (32), and the DPV (33). The combined percentage of overweight and obese is shown. The proportion of youth who were underweight was available only for the SWEET registry. SWEET data was reported separately for males and females; a weighted average was obtained and is reported here.

Most recently, the SWEET registry, an international consortium consisting of 26 countries, found the prevalence to be 29.6% of male patients and 34% of female patients who had a mean duration of diabetes approximately 5.5 years (32). The higher preponderance of overweight/obesity among females has been noted in other studies (26, 29–31, 33, 34), but is not a consistent finding. In the United States, racial/ethnic minorities with type 1 diabetes are more likely to be overweight/obese (30, 31) consistent with national trends in BMI by race/ethnicity among youth without diabetes (35). Importantly, the prevalence of obesity appears to be similar to youth without diabetes in most cases (23, 26, 29), though some have suggested that it may be higher among youth with type 1 diabetes (27, 34).

Several plausible explanations have been reported to explain the rise in obesity in youth with type 1 diabetes. Certainly, this trend mirrors the worldwide epidemic of obesity, and youth with diabetes are likely not immune to the westernized diet despite frequent nutrition counseling. Indeed, children and adolescents with type 1 diabetes generally do not meet the American Diabetes Association (ADA) and International Society for Pediatric and Adolescent Diabetes (ISPAD) dietary recommendations. More than a decade ago, our group found that adolescents with type 1 diabetes consume a higher percentage of their total calories from fat compared to peers without diabetes (36). A subsequent systematic review confirmed that children with type 1 diabetes tend to eat diets higher in saturated fat and also lower in fruits, vegetables, and whole grains, than recommended (37). These findings were echoed in more recent studies examining dietary habits in younger children (38) and adolescents (39) with type 1 diabetes. Diets with a higher percentage of total energy intake coming from fat rather than carbohydrates contribute to both weight gain and, ultimately, adverse lipid profiles (40). Carbohydrate counting, a system by which the insulin dose is calculated based upon the planned number of carbohydrates to be consumed, became widely popularized in the 2010s to increase dietary choice and flexibility for individuals with type 1 diabetes. Whether carbohydrate counting itself has contributed to weight gain is met with some controversy, with studies finding conflicting results (41). A recent study by Donzeau et al. examined carbohydrate counting versus fixed dose insulin for meals and found no change in BMI z-score in youth over one year of follow-up, though the fairly low starting mean BMI z-score (0.7 ± 0.8) of this population and possible selection bias may limit the generalizability of the findings (42).

In addition to diet, inadequate physical activity may also impact glycemia and weight in youth with type 1 diabetes. The guidelines recommend 60 min of moderate to vigorous physical activity per day (43); however, a large portion of youth with diabetes do not meet these guidelines (44). Similar to children without diabetes, various home, peer, and environmental factors may affect physical activity in youth with type 1 diabetes (45), though fear of hypoglycemia and the need for adequate supervision to monitor blood glucoses may be additional considerations (46). Regular activity has been shown to lower hemoglobin A1c, with additional benefits on weight, cardiovascular risk, and well-being (46). In the T1D Exchange cohort, youth who rated themselves as active (defined as 5–7 days/week) rather than inactive (defined as 0–1 days/week) were less likely to be overweight (OR 0.72, 95% CI 0.54–0.96) or obese (OR 0.70, 95% CI 0.49–0.99) in univariate analyses. Despite the evidence that children and adolescents with type 1 diabetes may not follow a healthy diet or get sufficient physical activity, we lack sophisticated studies examining temporal trends in relation to weight gain in this population, specifically.

An additional factor is our revised approach to treatment of type 1 diabetes, specifically with insulin therapy. The Diabetes Control and Complications Trial (DCCT) definitively identified

intensive insulin therapy as the gold standard of treatment to prevent the development and progression of long-term micro- and macrovascular complications (47–49). As a result, both increased doses and frequency of dosing of insulin have become standard of care. In the 18 years following the DCCT as part of the Epidemiology of Diabetes Interventions and Complications (EDIC) trial, the prevalence of overweight increased by 47% and obesity increased seven times to 22.7% among participants (50). Prior randomization to the intensive insulin therapy group was predictive of elevated BMI. More recent studies have similarly correlated higher insulin dose and continuous subcutaneous insulin infusion with increased weight gain (28, 31, 33); in one circumstance, each unit increase in hemoglobin A1c decreased the odds of overweight by 8% (OR 0.92, 95% CI 0.87 0.97) (31), again suggesting that intensive treatment to improve glycemic control can result in weight gain. There are likely two explanations. With more insulin, the body is better able to utilize the calories from the food consumed, and in the setting of poor dietary choices and sedentary behavior, this can contribute to weight gain. Another potential adverse effect of intensive insulin therapy, hypoglycemia, may also result in excessive carbohydrate consumption to either treat or prevent low blood sugars, further adding to weight gain (51). This has improved with long-acting insulin analogues and insulin pumps, and may improve further with newer hybrid closed loop technologies, which can minimize hypoglycemia through automated basal rate adjustment (52, 53).

OBESITY AND THE PATHOGENESIS OF TYPE 1 DIABETES

The Accelerator Hypothesis and Double Diabetes

With the changing prevalence of obesity at onset of type 1 diabetes in youth, it has become apparent that there are youth presenting with overlapping characteristics of the different types of diabetes, including features of both autoimmunity and insulin resistance, described as "double diabetes". Data from our group suggested a higher than expected frequency of a family history of type 2 diabetes in youth with type 1 diabetes from our Children's Hospital of Pittsburgh Diabetes Registry, leading us to postulate that the increased insulin demands of obesity may accelerate the presentation of autoimmune type 1 diabetes (54, 55). In further support of this theory, we later observed features of insulin resistance, including obesity and acanthosis nigricans in islet autoantibody positive youth with new onset insulin-dependent diabetes (56). A similar conclusion was drawn by T. Wilkin who developed the "accelerator hypothesis", which proposes that obesity-induced insulin resistance places increased burden on islets in genetically at-risk individuals, inducing autoimmune destruction and/or accelerating its course (57). These hypotheses have called into question the traditional classification structure of type 1 and type 2 diabetes, considering them instead to be two extremes on a spectrum of disease. Wilkin argued that the primary factor differentiating diabetes type is the *tempo* of progression to overt clinical disease, driven by the interplay between β-cell reserve and insulin sensitivity. This phenomenon of double diabetes, has also been described as developing after onset of diabetes. A series of case reports have described combined features of autoimmunity and insulin resistance in children, including a notable case from our group describing a 5-year-old child diagnosed with type 1 diabetes who ultimately developed obesity, acanthosis nigricans, and severe insulin resistance (58). Indeed, youth-onset type 1 diabetes is characterized by significant disease heterogeneity which may in part relate to weight. The complex relationship between environmental factors and genetic risk for type 1 or 2 diabetes likely plays a role in autoimmunity pathogenesis and presentation of clinical disease.

Clinical Studies Implicating Obesity in Autoimmunity and Type 1 Diabetes

Various studies have investigated the role of birth weight, weight gain, and obesity in the development of islet autoimmunity, progression from single to multiple antibodies, and development of type 1 diabetes. The earliest analyses included population-based cohort and epidemiological case-control studies which correlated a diagnosis of type 1 diabetes with elevated birth weight and early childhood weight gain in infancy as compared to a referent population (59–62). However, these findings were not universal across all populations studied, as others found no supportive evidence linking weight and presentation of type 1 diabetes (63, 64). Subsequently, several prospective cohort studies from birth identified a higher rate of early weight gain and absolute BMI z-score as predictors for the development of islet autoimmunity (65–69). Notably, The Environmental Determinants of Diabetes in the Young (TEDDY) study, a multi-country cohort following children at-risk for type 1 diabetes based upon their HLA-DR-DQ genotype, has examined the associations between weight in the first few years of life with progression from single to multiple antibodies and subsequent development of type 1 diabetes. Weight z-scores at 12 and 24 months were associated with increased risk for progression to multiple antibodies (67), and a higher rate of weight gain in early childhood was associated with progression from autoimmunity to type 1 diabetes in those with the initial presenting autoantibody being directed against glutamic acid decarboxylase (69). Additionally, there may be some relationship between weight gain and earlier age at presentation of type 1 diabetes (23, 61). These studies provide some support for the accelerator hypothesis by suggesting that birth weight and excessive weight gain in early years may hasten diabetes onset, though with little evidence for an association with the onset of islet autoimmunity.

Additional investigations have examined the possible connection between BMI with the progression of type 1 diabetes after autoimmunity onset in older children and adults. Initial comparisons of autoantibody status by BMI at diagnosis were inconclusive. A cross-sectional assessment of 263 children under age 19 years at onset found no significant association between the number of antibodies and measures of adiposity, including BMI and waist circumference (70). A second study

examining the TrialNet Pathway to Prevention (PTP) cohort, which follows individuals at risk for type 1 diabetes based upon family history and islet autoantibody status, also explored the relationship between BMI, BMI percentile, and insulin resistance (measured by HOMA-IR) with the progression from autoimmunity to diagnosis. Similarly, they found no association between any of these variables with the risk for developing type 1 diabetes (71). However, these studies lacked the ability to assess temporal trends. Recent, sophisticated analyses have examined the "cumulative excess BMI" (ceBMI) in the TrialNet PTP subjects, a calculated value describing the persistent elevation of BMI over time beyond the overweight threshold (BMI ≥ 25 kg/m^2 in adults or ≥ 85th percentile for age and sex in children). In children, ceBMI ≥ 0 kg/m^2, indicating persistent overweight/obesity over time, was associated with a higher rate of progression from a single to multiple β-cell autoantibodies in children 9 years of age or older and without high-risk HLA genotypes (72). Furthermore, ceBMI ≥ 0 kg/m^2 was also associated with a 63% greater risk to develop type 1 diabetes following islet autoimmunity in children, adjusted for age, sex, and autoantibody number (p=0.0009) (73). In adults, ceBMI also increased the risk for progression to type 1 diabetes, though only in certain age and gender cohorts, namely men over 35 years of age and women younger than 35 years age, suggesting some influence from sex hormones (74).

In further support of the accelerator hypothesis, insulin resistance likely adversely affects β-cell function in youth with type 1 diabetes and may promote immune activation in at risk individuals. Among pooled European cohorts of youth with type 1 diabetes, higher BMI at diagnosis was associated with greater decline in fasting C-peptide levels among teens 10–18 years at 1 year follow-up even when adjusting for glycemia, suggesting that BMI-related insulin resistance is contributing to β -cell dysfunction in this population (75). Resulting immune activation was suggested by a study examining T-cell autoreactivities to neuronal diabetes-associated autoantigens, which are typically observed early in type 1 diabetes pathology before antibodies emerge (76). Youth with the highest quintile of BMI or elevated waist circumference at onset of insulin dependent diabetes had higher islet associated T-cell autoreactivities, suggesting that T-cell autoimmunity may be promoted by visceral adiposity-associated insulin resistance. Furthermore, all but one youth without autoantibodies had evidence of T-cell autoimmunity, suggesting some immune activation among youth who might otherwise be classified as having type 2 diabetes.

Obese youth with new onset type 1 diabetes also have altered pro-inflammatory profiles which may contribute to the acceleration of their presentation. Adipocytes secrete a variety of pro- or anti-inflammatory adipokines which correlate with insulin resistance (77). Obese children with new onset type 1 diabetes are more likely to have higher pro-inflammatory markers (leptin, visfatin, chemerin, TNF-α, CRP) and lower anti-inflammatory markers (adiponectin, omentin) compared to non-obese peers with type 1 diabetes (78). While two or more autoantibodies at diagnosis, suggesting a higher degree of autoimmunity, are associated with higher adiponectin and lower leptin levels, these relationships were negated when adjusting for BMI, suggesting that adiposity is the primary driver of abnormal adipokines (79). Together, these studies examining ceBMI, autoimmune profiles, and inflammatory markers provide reasonable evidence to hypothesize that excessive weight gain may contribute to progression of islet autoimmunity to type 1 diabetes, perhaps through insulin resistance, altered adipokines and cytokines, and β-cell stress.

Proposed Underlying Pathophysiology

Though no confirmed pathway exists linking obesity and the rising incidence of type 1 diabetes, different pathophysiological mechanisms may explain how obesity contributes to insulitis and autoimmunity, blurring the boundary with type 2 diabetes. Obesity-induced insulin resistance, insulin demand and inflammation are possible underlying mechanisms. Expanding adipose tissue through adipocyte hypertrophy leads to hypoxia and stress of the cellular endoplasmic reticulum (ER) (80). Subsequent adipocyte necrosis both activates and recruits tissue-specific macrophages, which expand in number from only 5–10% of stromal cells to 40–50%. These macrophages secrete a variety of pro-inflammatory cytokines (TNF-α, IL-6, and IL-1 β) and chemokines (CCL1, CXCL1, and CXCL2), which further activate the immune system (81). Locally, these pro-inflammatory signals impair insulin action and promote insulin resistance, ultimately leading to reduced glucose disposal and a failure to suppress both lipolysis and hepatic glucose production. In turn, insulin resistance, hyperglycemia, elevated free fatty acids, and cytokines promote additional ER and mitochondrial stress, perpetuating this cycle.

This milieu creates low-grade, chronic inflammation which may have downstream effects for β -cells. Insulin resistance and resulting inflammation are hypothesized to increase the β-cell secretory demand, placing added stress on the β-cell and resulting in local cytokine release (IFN-γ), neo-antigen formation, and β-cell apoptosis, thus triggering an immune response and insulitis (82, 83). In response to inflammatory signals, β-cells under stress may secrete chemokines, facilitating leukocyte recruitment and contributing to islet inflammation and destruction (84). Cytokines, specifically IL-1β and IFN-γ, may also trigger β-cell apoptosis directly *via* upregulation of the NF-κβ transcription pathway in islets (81). Interestingly, the pattern of immune cell activation in islets may differ between type 1 and 2 diabetes; adaptive immune cells predominate in type 1 compared to innate immune cells in type 2 diabetes (83). Additional studies are needed to further clarify the immune dysregulation that may occur in type 2 diabetes to underscore any underlying similarities in pathophysiological mechanisms. In sum, insulitis is likely driven by secretion of chemokines from activated immune cells and β-cells themselves in response to pro-inflammatory stimuli (cytokines, fatty acids), furthering leukocyte inflammation and contributing to β-cell death and, ultimately, type 1 diabetes (84). At present, it is unclear if obesity may be one environmental mechanism that perpetuates insulitis caused by an underlying unknown trigger, or if obesity itself

could be a primary driver of insulitis; however, the data described above currently suggests the former.

Type 2 Diabetes Genetic Risk

Genetic risk factors typically associated with type 2 diabetes may modulate the risk of autoimmune progression and development of clinical type 1 diabetes. The genetic risk factor most strongly associated with type 2 diabetes is TCF7L2 single nucleotide polymorphisms (SNPs). TCF7L2 SNPs are strongly associated with various aspects of type 2 diabetes, including insulin resistance, impaired insulin secretion, altered insulin processing, diminished suppression of glucagon by glucose, and increased fasting hepatic glucose release (85). These SNPs have been identified in children and adults with a single autoantibody at diagnosis of type 1 diabetes (86, 87) and seem to be inversely correlated with high-risk HLA genotypes (88). Those with these SNPs may present with a distinct phenotype characterized by less apparent islet cell damage. Oral glucose tolerance test stimulated glucose area under the curve was lower (p-0.0127) and area under the curve C-peptide was higher (p=0.008) in subjects with TCF7L2 SNPs identified at diagnosis (86). Furthermore, subjects with TCF7L2 SNPs may have altered progression from single to multiple autoantibodies, affected by both the initial antibody and the presence of overweight or obesity (89).

Summary of Evidence

Taken together, obesity may augment the risk for autoimmune progression and development of type 1 diabetes when considering longitudinal assessments of excess weight. Hormonal differences in sex steroids likely play a role in potentiating this risk, given some findings in teens undergoing physiological insulin resistance of puberty (75) and differences observed in adults based upon sex and age (74). Proposed mechanisms include obesity-induced insulin resistance and inflammation increasing β-cell secretory demand, stress, and apoptosis, with autoimmune activation. Genetic factors traditionally associated with type 2 diabetes may also augment this risk. Perhaps most telling, the Diabetes Prevention Trial Type 1 Risk Score incorporates body mass index into its modern prediction model, highlighting the important, albeit complex, role of obesity in type 1 diabetes (90).

ADDED RISK FOR MICRO- AND MACROVASCULAR COMPLICATIONS

Insulin Resistance and Cardiometabolic Markers

Premature CVD is a major cause of morbidity and mortality in adults with type 1 diabetes (91). Traditional risk factors for cardiovascular disease in the general population, including hypertension, dyslipidemia, and smoking, do not fully account for the increased CVD risk among those with type 1 diabetes. Insulin resistance emerged as the proposed link to explain the heightened risk for both microvascular and macrovascular complications (92). Applying a calculated measure of insulin resistance, the estimated Glucose Disposal Rate (eGDR) (93), to subjects in the longitudinal Pittsburgh Epidemiology of Diabetes Complications (EDC) study, increasing insulin resistance was associated with a higher risk of nephropathy (94), peripheral vascular disease (women only) (95), and coronary artery disease (96). This relationship in adult patients was further elucidated using data from the DCCT, where eGDR at baseline was significantly inversely associated with higher risk for nephropathy, retinopathy, cardiovascular events, and macrovascular disease (97). More recently, the Coronary Artery Calcification in Type 1 Diabetes (CACTI) study confirmed these findings using hyperinsulinemic euglycemic clamp data comparing adult subjects with type 1 diabetes to age, BMI, and physical activity-matched controls without diabetes (98). Insulin resistance correlated with the presence and progression of coronary artery calcifications, a surrogate marker for coronary artery disease and strong predictor of adverse outcomes. The subjects with type 1 diabetes had impaired insulin-mediated free fatty acid suppression, suggesting that lipotoxicity over time may be a potential mechanistic explanation.

Youth with type 1 diabetes may also have adipose, hepatic, and peripheral insulin resistance regardless of obesity and other features of metabolic syndrome (99). Early CVD factors are present in youth with type 1 diabetes despite their shorter duration of diabetes, also believed to be related to their decreased insulin sensitivity. In hyperinsulinemic euglycemic clamps, youth with type 1 diabetes are more insulin resistant compared to matched healthy peers and have lower functional exercise capacity, a marker of cardiovascular function and predictor of mortality (100). These findings were observed in normal weight youth with no features of Metabolic Syndrome, suggesting that some degree of insulin resistance is present regardless of adiposity. Using a clamp-derived measure, insulin sensitivity is inversely correlated with more atherogenic cardiovascular risk factors, including BMI z-score, total cholesterol, low density lipoprotein-cholesterol, blood pressure, and C-reactive protein (101), as well as arterial stiffness, measured by pulse wave velocity, in a longitudinal assessment (102).

Obesity Compounds and Increases Risk for Complications

Though insulin resistance may account for much of the baseline cardiovascular risk profile in type 1 diabetes, the increased prevalence of obesity in this population likely augments this risk. Obese adults with type 1 diabetes have higher risk for micro- and macro-vascular comorbidities independent of glucose control (103). In the adult population from DCCT, the quartile with the highest weight gain in the intensively treated group also had higher BMI, blood pressure, triglycerides, total cholesterol, LDL-cholesterol, and apolipoprotein B compared to all other quartiles, indicative of a more atherogenic profile (104). These findings persisted in the EDIC follow-up of the DCCT and were associated with greater intima media thickness (105). Though the impact of obesity on cardiovascular complications is thought to be mediated by Metabolic Syndrome (e.g. hypertension, dyslipidemia), the CACTI study identified obesity as a risk factor for both the presence and progression of coronary artery calcifications independent of pre-existing metabolic abnormalities (106). How this translates to long-term cardiovascular morbidity

and mortality is less clear (107). The Pittsburgh EDC study demonstrated that waist circumference, a surrogate of visceral adiposity, increases the risk for long-term mortality in type 1 diabetes (108). In the DCCT/EDIC studies, the rate of adverse cardiac events was initially similar between those who did or did not have excessive weight gain in the intensively treated group, with some divergence after 14 years, when the event rate in excessive weight gainers approached that of conventionally treated patients (109). The lack of a difference in events within 14 years of follow-up was attributed to both better management of cardiovascular risk factors (e.g. anti-hypertensive and lipid lowering medications) as well as the improved nutrition counseling this group received irrespective of overweight/obesity.

The potential compounding effect of obesity on insulin resistance and complications may appear as early as adolescence. Indeed, overweight and obese youth with type 1 diabetes are more likely to have coexisting hypertension, abnormal lipids, and elevated alanine aminotransferase compared to healthy weight peers (110, 111). In the SEARCH registry, youth with obesity and persistently elevated hemoglobin A1c over time had the highest risk for adverse cardiovascular markers and microvascular complications compared to those with either elevated hemoglobin A1c and normal weight or obesity with fairly-well controlled A1c (112). In clamp studies, our group found that obese youth with type 1 diabetes were more insulin resistant compared to non-obese peers with type 1 diabetes. Furthermore, insulin sensitivity correlated with cardiovascular risk factors, including pulse wave velocity, even when adjusting for the degree of obesity, suggesting that insulin resistance, augmented by excess weight, is the underlying factor contributing to cardiovascular complications (113). Overall, children do not meet ADA and ISPAD-determined clinical targets for glycemic control (114, 115), placing them at risk for long-term micro and macrovascular disease. Children with coexisting features of type 1 and 2 diabetes, or double diabetes, may be at heightened risk. Given the substantial changes in insulin therapy over time as a result of the DCCT with new analogs and continuous subcutaneous insulin infusion being used in children, the long-term impact on weight gain, insulin resistance, and cardiovascular health merits further study of intervention strategies.

THERAPEUTIC APPROACHES

Despite the more pronounced long-term risks of coexisting obesity and type 1 diabetes, there are limited therapeutics or guidelines to manage this condition beyond what has been traditionally available. One particular challenge for providers is the lack of a clinically meaningful definition for double diabetes which can guide treatment (116). Though glycemia is the most important predictor of outcomes, the degree of insulin resistance associated with obesity and type 1 diabetes is also important in predicting future complications, and this is not addressed by insulin titration alone. Escalating insulin doses to achieve glycemic goals may also further compound weight gain, exacerbating obesity-related complications. Adequate counseling on a healthy diet and adequate physical activity should be paramount in the care of these patients, following recommended guidelines from leading diabetes organizations (43, 46). In addition, the use of targeted nutritional therapies or pharmaceuticals for the treatment of type 2 diabetes have been proposed as adjuvant treatments for children and adults with obesity complicating type 1 diabetes; the evidence for these therapies is summarized in **Table 1**.

Targeted Nutritional Therapies

Diet is an essential component to type 1 and 2 diabetes management and is necessary to achieve optimal glycemic control (129). Both the ADA and ISPAD promote nutrition counseling and education that is tailored to the unique psychosocial and cultural needs of that family (43, 130). As a guide, children should receive approximately 45–50% of their energy from carbohydrates, 15–20% from protein, and <35% from fat (with saturated fat <10%) (130). The dietary recommendations must be commensurate with the insulin regimen, as certain approaches (e.g. sliding scales/algorithms) require fixed- macronutrient intake for each meal time. The diet for children and adolescents with type 1 diabetes is not only important for glycemic control, but also for appropriate growth and development while minimizing unnecessary weight gain. In adults with type 1 diabetes, both the Mediterranean and Dietary Approaches to Stop Hypertension (DASH) diets have been shown to promote weight loss as well as optimize glucose levels (131).

The low carbohydrate or very low carbohydrate (ketogenic) diets have gained recent popularity as part of the treatment for type 1 diabetes. They require significant carbohydrate restriction, at times to no more than 20–70 g/day. The studies examining the effectiveness of this diet are heterogeneous and often small, limiting their interpretation. Some evidence suggests there may be a beneficial reduction in hemoglobin A1c, daily insulin dose, and body weight mostly in adults, though this may be at the expense of insufficient insulin, dyslipidemia or increased hypoglycemic events (132). Use of this diet in children, especially if normal weight, is highly controversial, as carbohydrate intake and appropriate insulin dosing are critical for growth. A case series documented poor growth, significant fatigue, and adverse lipid profiles in six children with type 1 diabetes on a low carbohydrate diet (133). At present, the low carbohydrate diets are not recommended for youth with type 1 diabetes, including as treatment for obesity. The relationship between any of these diets and insulin resistance has not been studied. Similarly, studies have not examined whether weight control in children at risk for type 1 diabetes can help prevent the onset of the disease.

Metformin

Metformin is considered first line therapy in both adolescents and adults with type 2 diabetes. Its mechanism is believed to be improved insulin sensitivity by inhibiting hepatic gluconeogenesis, resulting in reduced hepatic glucose output, and increasing peripheral uptake of glucose in the muscle (134). Metformin has been proposed as an adjunct therapy in both youth and adults with type 1 diabetes to address peripheral insulin resistance regardless of

TABLE 1 | Summary of Evidence for Adjunctive Pharmaceuticals in Type 1 Diabetes.

Drug Category	Mechanism	Summary of Evidence
Biguanides (Metformin)	Improves insulin sensitivity by blocking hepatic gluco-neogenesis	• Adequately powered studies in youth have found no improvement in hemoglobin A1c (117–119) • May result in a modest reduction in daily insulin dose and BMI (117, 119–121) • Possible cardioprotective effects, though evidence is limited (119, 122, 123)
GLP-1 agonists	Stimulates insulin release and inhibits glucagon secretion in a glucose-dependent manner; induces satiety	• Across multiple trials in adults, small improvement in hemoglobin A1c (-0.21%) (124) • Mean weight loss of approximately 3.5 kg (124) • May lower daily bolus insulin (124) • No available studies in youth with type 1 diabetes
DPP4-inhibitors	Blocks degradation of endogenous GLP-1	• Across multiple trials in adults, no improvement in hemoglobin A1c, BMI, or insulin dose (125, 126) • No available studies in youth with type 1 diabetes
SGLT 1/2 inhibitors	Blocks sodium-glucose transporter in the proximal tubule of the kidney resulting in glycosuria	• Across multiple trials in adults, small reduction in hemoglobin A1c (-3.9%) (127) • Daily insulin dose reduced by ~10% (127) • Body weight reduced by ~4% (127) • No available studies in youth with type 1 diabetes examining change in weight (128)

weight; relevant pediatric studies are summarized in **Table 2**. Numerous randomized controlled trials have attempted to explore the efficacy of metformin in this context. Initial studies examined adolescents with poor glycemic control, only some of whom were overweight and obese, with few finding a modest improvement in hemoglobin A1c (120, 121, 135, 136) and others finding no difference (137). These studies were small, of variable duration, and frequently used non-standard doses of metformin, limiting their interpretation. An adequately powered trial using low dose metformin (1,000 mg daily) in adolescents with sub-optimal glycemic control (hemoglobin A1c >8.5%), only approximately one-third of whom were overweight or obese, found no difference in hemoglobin A1c levels from baseline to six months (117). Two additional studies specifically examined use of metformin in overweight or obese adolescents (118, 119). A large randomized trial found that though 2,000 mg of daily metformin led to a small decline in hemoglobin A1c compared to placebo at the mid-way point, at 6 months there was no difference in hemoglobin A1c compared to placebo (119).

Despite the lack of clinical change in hemoglobin A1c over the course of these trials, many studies demonstrated a reduction in body mass index (117, 119, 121), waist circumference (117), and insulin dose per unit of body weight (117, 119–121). Indeed, the aforementioned large randomized trial of 2,000 mg metformin daily in obese adolescents demonstrated that approximately one quarter of the intervention group had at least a 25% reduction in their total daily insulin dose per unit body weight (compared to 1% in placebo group p=0.003) and at least a 10% reduction in their BMI z-score (compared to 7% in placebo group p=0.01) (119). In addition, there was a small reduction in total body fat by approximately 2 kg as determined by dual-energy x-ray absorptiometry in the metformin group compared to placebo (p<0.001).

Taken together, these findings have mirrored those in adults, which similarly have found no improvement in hemoglobin A1c, though reductions in insulin dose and body weight may occur (122, 138). The noted reductions in insulin dose were thought to imply improved insulin resistance. Two studies examined this

TABLE 2 | Summary of Clinical Trials of the Use of Metformin in Youth with Type 1 Diabetes.

Citation	N	Intervention	Comparison?	Duration	Effect on HbA1c	Effect on dailyInsulin Dose	Effect on Weight
Gomez et al. J Pediatr Endocrinol Metab. 2002. (135)	10	Variable dose metformin	No	6 months	Decrease by 11% of baseline	No change	No change
Hamilton et al. Diabetes Care. 2003. (120)	27	Weight-based dose metformin (up to 2000 mg/day)	Placebo	3 months	-0.6% (p=0.03)	-0.16 units/kg (p=0.01)	No change
Urakami et al. Pediat Int. 2005. (121)	9	500-750 mg twice daily metformin	No	12 months	-1.1% (p<0.01)	-6.7 units (p<0.01)	-0.7 kg/m^2 (p<0.05)
Nadeau et al. Pediatr Diabetes. 2015. (117)	74	1000 mg daily metformin	Placebo	6 months	No change	No change	No change
Nwosu et al. PLoS One. 2015. (118)	28	1000 mg daily metformin	Placebo	9 months	No change	No change	No change
Libman et al. JAMA. 2015. (119)	140	2000 mg daily metformin	Placebo	6 months	No change	-0.1 units/kg (p<0.001)	-0.1 BMI z-score (p<0.001)

directly using hyperinsulinemic euglycemic clamps, finding improvement in both whole-body insulin sensitivity and peripheral insulin resistance in those adolescents taking metformin (136, 139). How this actually relates to long-term cardiovascular complications is less clear, as studies are inconclusive on whether the addition of metformin impacts intermediate measures of hypertension, lipids, inflammatory markers, or carotid intima media thickness (119, 122). Through the use of advanced magnetic resonance imaging techniques, metformin may lead to some benefit in measures of aortic pulse wave velocity and wall sheer stress among youth, suggesting cardioprotective effects (123). The potential downstream benefits, particularly as pertains to the risk for micro- and macro-vascular complications, warrant further investigation.

At present, use of metformin is not recommended by the ADA for youth with type 1 diabetes (43), and though it is discussed as a consideration in the guidelines for adults with type 1 diabetes, it is not explicitly recommended (140). Real-world studies evaluating how often metformin is used within a type 1 diabetes population and which patients receive this adjunct therapy are limited. Among both the T1D Exchange and DPV registries, including close to 50,000 patients (both youth and adults), metformin was used in about 3.5% of individuals in the T1D Exchange and only 1.3% in the DPV (141). Adjuvant therapy was rare in youth under 13 years of age. An additional analysis of the DPV registry examined 525 youth with type 1 diabetes treated with metformin in addition to insulin compared to over 57,000 youth on insulin alone (142). In this observational study, the children treated with metformin tended to be slightly older and were more commonly female. Median BMI z-score of the metformin-treated youth was +1.86 (+1.33 to +2.58) compared to a median BMI z-score of +0.51 (−0.12 to +1.15) in youth on insulin alone ($p<0.001$). Furthermore, hemoglobin A1c was significantly higher among those on metformin, as was the presence of comorbidities, including polycystic ovary syndrome, hypertension, elevated liver enzymes, and dyslipidemia. Those youth treated with metformin required a significantly higher insulin dose per unit body weight than those on insulin alone. Over time, the metformin-treated group had modest improvements in BMI and insulin dose. The study concluded that metformin is used rarely in youth with type 1 diabetes outside of the research setting, most often in obese female adolescents, perhaps to treat concomitant polycystic ovary syndrome, and the benefits of this therapy are not fully realized.

GLP-1 Receptor Agonists and DPP-4 Inhibitors

Glucagon-like-peptide-1 (GLP-1) is an incretin hormone secreted by the gut which stimulates insulin release and inhibits glucagon secretion in a glucose-dependent manner, resulting in control of meal-induced glycemic excursions (143). GLP-1 also delays gastric emptying and acts centrally to decrease appetite (144), which can help to promote weight loss. GLP-1 receptor signaling may also support islet health by inhibiting β-cell apoptosis, promoting proliferation of β-cells, and reducing ER stress, with the net result of preserving or enhancing residual function; however, restoration of β-cell function has not been proven in human studies of islet transplantation or type 1 diabetes (145). GLP-1 is rapidly degraded by the dipeptidyl peptidase-4 enzyme (DDP-4), limiting the longevity of its effect. Both GLP-1 receptor agonists and DPP-4 inhibitors are recent advances for the treatment of type 2 diabetes, and in the case of GLP-1 agonists, for obesity in the absence of diabetes. In addition to myriad studies in adults, the GLP-1 agonist, Liraglutide, has been shown to be effective in reducing hemoglobin A1c in youth with type 2 diabetes (146) and promoting weight loss in obese adolescents with no evidence of dysglycemia (147).

While this treatment has not yet been studied in obese children with type 1 diabetes, there is some evidence supporting the use of GLP-1 agonists in adults with type 1 diabetes. A meta-analysis examined the net impact of GLP-1 agonists on glycemic control, weight, and insulin dose in seven randomized controlled trials in 206 adult subjects with type 1 diabetes also treated with insulin over study durations of 3–15 months (124). Overall, there was a net, albeit modest, improvement in hemoglobin A1c by −0.21% (95% CI −0.40, −0.02, $p=0.03$), without increased risk of hypoglycemia, and mean weight loss of 3.53 kg (95% CI −4.86, −2.19, $p<0.05$) in adults taking GLP-1 agonists compared to placebo. In the few studies that examined total daily insulin dose, specifically differentiating bolus and basal insulin, use of a GLP-1 agonist was associated with a 6% reduction in daily bolus insulin dose (95%CI, −10%, −2%, $p=0.001$) with no change in absolute daily insulin dose. Taken together, the authors suggest a potential benefit to adding GLP-1 to insulin therapy in adults with type 1 diabetes and offer different mechanisms by which GLP-1 may mediate these improvements. Two explanations may be improved insulin sensitivity (148) and/or reduced appetite and carbohydrate intake (149), resulting in decline in bolus insulin requirements and weight loss (124). In contrast, DPP-4 inhibitors are less promising. Two meta-analyses examined the efficacy and safety of DPP-4 inhibitors in adults with type 1 diabetes; neither found any significant improvement in hemoglobin A1c, BMI, or insulin dosage with treatment, though significant heterogeneity among studies limited the analyses (125, 126).

Altogether, adult studies have identified a slight improvement in glycemic control, weight, and bolus insulin dose among patients with type 1 diabetes on combined therapy of insulin and GLP-1 agonists, but not DPP-4 inhibitors. The therapeutic mechanisms underpinning these improvements are poorly understood based upon the available studies, though the theoretical advantage to these medications may predominantly be weight loss in the setting of overweight/obesity, which may improve insulin sensitivity. To date, no studies have examined the use of either of these therapies in youth with type 1 diabetes, and they are not currently recommended for this population.

SGLT-1 and 2 Inhibitors

Sodium-glucose cotransporter (SGLT) inhibitors are another fairly recent therapeutic advancement approved for the treatment of type 2 diabetes which have been proposed as an adjunct treatment for type 1 diabetes. These medications block SGLT-2 in the proximal tubule of the kidney, resulting in glycosuria and natriuresis; some medications also block SGLT-1, which can delay glucose absorption from the intestinal tract (150). In adults, SGLT inhibitors are believed to have added cardioprotective effects (151). A meta-

analysis examined 10 randomized controlled trials of both SGLT-2 and combined SGLT-1/2 inhibitors, including over 5961 patients with type 1 diabetes with follow-up of 12–52 weeks (127). Across pooled studies, these medications resulted in a reduction in body weight of approximately 4% and associated reductions in hemoglobin A1c by −0.39% (95% CI −0.43 to −0.36) and fasting glucose by −1.13 mmol/L (95% CI −1.36 to −0.90). In addition, total daily insulin dose, basal insulin dose, and short acting insulin dose were all reduced by approximately 10%. Thus far in youth with type 1 diabetes, one study has examined safety and effect of SGLT-2 inhibitors, though did not comment on any effect on weight (128).

These medications essentially compensate for some food indiscretions by lowering the renal threshold, promoting the excretion of excess glucose following a meal. By reducing hyperglycemia, these drugs likely facilitate weight loss; both are proposed mechanisms for improved insulin sensitivity in adults with type 2 diabetes (152, 153). No investigations have evaluated whether the same metabolic effects are present in individuals with type 1 diabetes regardless of adiposity, and use of these medications in this population remains fairly cautionary due to the increased risk for euglycemic ketoacidosis (127, 154). Similar to the therapies described before, SLGT inhibitors are not currently recommended for adjuvant therapy in pediatric type 1 diabetes with obesity.

In all, the options to treat obese youth with type 1 diabetes remain limited. The prevention and management of obesity continues to rely upon early, consistent, and individualized dietary counseling both at diagnosis and at least annually at follow-up appointments. No therapeutic options are currently approved for this purpose, and future research is needed to understand which therapies might be safe and effective in weight reduction, optimizing glycemic control, and in preventing long-term complications.

DISCUSSION AND FUTURE DIRECTIONS

Overweight and obesity are increasingly common in youth and adults with type 1 diabetes, perhaps a consequence of changing cultural practices surrounding diet, nutrition and exercise over the past several decades. Genetic risk and environmental factors may lead to coexisting features of type 1 and 2 diabetes, with added implications for both pathogenesis and long-term health. Data supporting these outcomes led to the concept of double diabetes. In the setting of widespread use of intensive insulin therapy, poor dietary choices and over-treatment of hypoglycemia may both contribute to weight gain. Further study is needed to understand whether use of new technologies, particularly closed loop hybrid insulin pumps and automated insulin delivery systems, may help reduce the risk of hypoglycemia and thus result in decreased weight gain by lessening the need for treatments with added food intake.

It is possible that obesity has a role in potentiating the risk for type 1 diabetes, similarly to type 2 diabetes. Obesity causes augmentation of insulin resistance, which may increase both insulin demand, stressing the islets. Though longitudinal studies have examined the role of excessive weight gain in autoimmune development and progression, future research is needed to better elucidate the mechanisms by which obesity contributes to increased stress on the insulin secretory functions and inflammation, possibly leading to or exacerbating β-cell pathology.

There is limited evidence for any beneficial effect of currently available adjuvant therapies apart from lifestyle adjustments, and these all need further investigation for both the delay of type 1 diabetes and the prevention of complications in those with double diabetes. Better measurement systems are needed to identify those with significant insulin resistance who may be at higher risk for complications that are easily adaptable for busy clinical settings. Additional investigations are needed to examine whether the use of targeted therapies for weight loss can also modulate inflammation and insulin resistance and reduce the long-term risk for cardiovascular complications. Given our current fund of knowledge, it is critical to consider how we counsel patients and families about excess weight gain for children diagnosed with type 1 diabetes to prevent double diabetes in adolescence and adulthood. Perhaps most important are practical, evidenced-based strategies to promote lay awareness of the dangers of weight gain in the type 1 diabetes population and focus efforts on obesity prevention.

AUTHOR CONTRIBUTIONS

All authors contributed to the conceptualization of this manuscript and identified source articles. CM drafted the manuscript. All authors contributed to the article and approved the submitted version.

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Evolution of the Insulin Gene: Changes in Gene Number, Sequence, and Processing

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Insulin has not only made major contributions to the field of clinical medicine but has also played central roles in the advancement of fundamental molecular biology, including evolution. Insulin is essential for the health of vertebrate species, yet its function has been modified in species-specific manners. With the advent of genome sequencing, large numbers of insulin coding sequences have been identified in genomes of diverse vertebrates and have revealed unexpected changes in the numbers of genes within genomes and in their sequence that likely impact biological function. The presence of multiple insulin genes within a genome potentially allows specialization of an insulin gene. Discovery of changes in proteolytic processing suggests that the typical two-chain hormone structure is not necessary for all of inulin's biological activities.

Keywords: insulin, gene duplication, evolution, adaptive evolution, vertebrates, proteolytic processing, virus

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INTRODUCTION

Insulin is well-characterized as a key regulator of blood glucose levels in vertebrates (1). Insulin related peptides have also been identified in a number of other metazoan species and have been shown to contribute to various aspects of physiology in these species (2–4). The discovery of insulin 100 years ago, led to a revolution in clinical medicine, as it allowed an effective treatment for diabetes (5). Since its discovery, the treatment of diabetes using insulin, derivatives of insulin, and other peptides has and continues to evolve (6). There still is no cure. In addition to its critical role in the history of clinical medicine, insulin has played key roles in the development of many revolutionary technologies that are now commonplace in molecular biology, including protein sequencing (7) and the deduction of the three-dimensional structures of proteins (8). A key discovery made with insulin, but with important implications for many other bioactive peptides, is the role of proteolytic processing in regulating its biological action (9, 10). Since the sequencing of human insulin more than 60 years ago (7), a large number of insulin protein sequences have been determined due to its importance in medicine, as well as its small size and relative ease at isolation (11, 12). Over the past 20 years, as we entered the genomic era, an increasing number of insulin sequences have been predicted from the complete genome sequences of organisms. Genomic sequences have led to improvements of our understanding of not only human genetics and disease (13, 14), but also nearly all other areas of biology (15). The new insulin sequences identified from genome sequences have revealed an increased diversity in the number of insulin genes within

species and has revealed that changes in the proteolysis processing of the proinsulin precursor likely contributes to the diversity of the biological actions of insulin.

SUPERFAMILY OF INSULIN-LIKE GENES

While insulin was first identified in mammals, it soon became evident that peptides with sequences similar to insulin can be found in diverse multicellular animals, including many non-vertebrate species such as insects and worms (2–4). In many of these species, the insulin-like peptides were found through directed efforts to identify peptides with similarity to insulin, but increasingly, they are now being reported from searches of genome sequences. Multiple insulin-like genes have been characterized in the genomes of many non-vertebrate species that are due to lineage-specific gene duplication events (3, 4). A parallel set of duplications of insulin-like genes has also occurred within vertebrates. In addition to the insulin gene (*INS*), nine other genes encoding peptides with similarity to insulin both in their primary sequences and secondary structures have been identified in the human genome, including 2 insulin-like growth factors (*IGF1* and *IGF2*), 4 insulin-like factors (*INSL3*, *INSL4*, *INSL5*, and *INSL6*), and three relaxins (*RLN1*, *RLN2*, and *RLN3*) (16, 17). While the relationships among these genes cannot be fully resolved by phylogenetic analysis, due to their short protein lengths, information derived from their locations within the genome has helped to largely resolve the order and timing of the gene duplication events that generated this gene family (18–21). As summarized in **Figure 1A**, these studies suggest that the initial gene duplication event that originated this gene family separated an ancestor for the insulin and insulin-like growth factor (*IGF1* and *IGF2*) genes from an ancestor of the insulin-like (*INSL*) and relaxin (*RXN*) genes. This duplication was then followed by a duplication that separated the insulin gene from an ancestor of the insulin-like growth factor (*IGF1* and *IGF2*) genes. Both of these gene duplication events occurred before the two genome duplications that are associated with the origin of vertebrates (18).

The human insulin gene (*INS*) is a small gene of 1,425 base pairs located on chromosome 11 and is composed of 3 exons separated by two introns (29). The first exon of this gene is composed entirely of 5' untranslated sequence, with all of the coding region, which encodes the 110 amino acid long proinsulin precursor protein, distributed across exons 2 and 3 (29). The N-terminal portion of the proinsulin precursor protein sequence is a signal peptide that allows secretion from pancreatic beta cells and is removed by signal peptidase to yield proinsulin (10, 30).

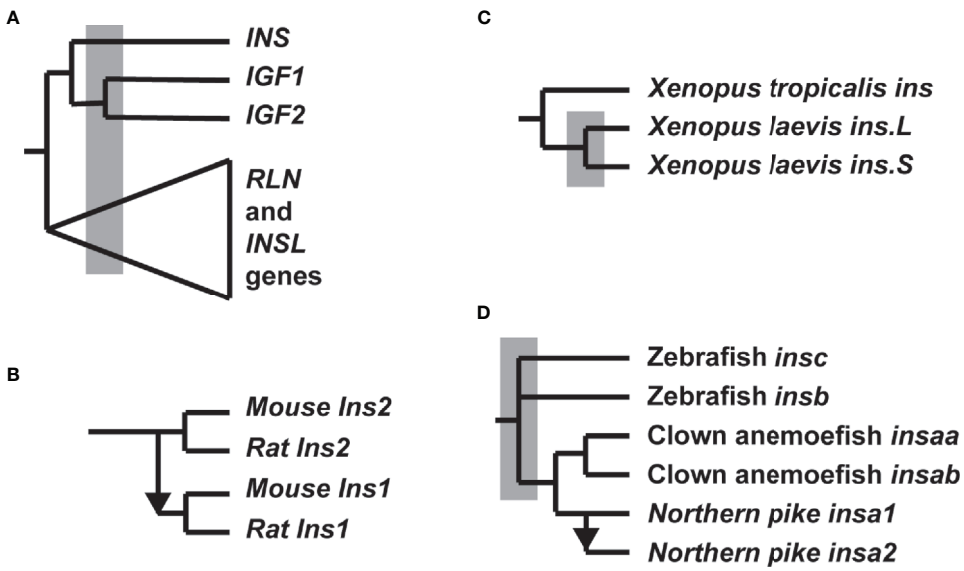

FIGURE 1 | Duplication of insulin-like genes in vertebrates. **(A)** Phylogeny of the insulin supergene family members inferred from their sequences and their genomic locations (18–21). The divergence of the multiple human relaxin (*RLN1*, *RLN2*, and *RLN3*) and insulin-like peptide (*INSL3*, *INSL4*, *INSL5*, and *INSL6*) are indicated by the triangle. Grey box indicates the two genome duplications (2R) that occurred near the origin of vertebrates. **(B)** Origin of the duplicated rodent insulin genes. The *Ins1* gene originated by a retroposition event, shown by the arrow, in the common ancestor of the mouse (*Mus musculus*) and rat (*Rattus norvegicus*), while *Ins2* is located at the locus-of origin (22–26). **(C)** Duplication of the insulin gene in the frog *Xenopus laevis* (27). A pair of insulin genes, *ins.L* and *ins.S*, are found in the *Xenopus laevis* genome due to a genome duplication that has occurred since its divergence from the diploid frog *Xenopus tropicalis*. The grey box indicates the genome duplication. **(D)** Summary of the duplications of insulin genes in bony fish (28). A triplication of the insulin gene, yielding *insa*, *insb*, and *insc* genes, occurred in an early ancestor of teleost fish (the order of the duplications events yielding the *insa*, *insb*, and *insc* is unresolved). These duplications occurred at about the same time as the fish-specific genome duplication (the genome duplication is shown as a grey box) – it is unclear whether if any of the insulin gene duplications were due to the genome duplication. A latter duplication of the *insa* gene in an ancestor of a subset of teleost fish resulted in an *insaa* and an *insab* gene in many fish (e.g., clownfish; *Amphiprion ocellaris*). Insulin genes within some fish genomes also originated *via* retroposition, as indicated by the arrow, with the Northern pike (*Esox lucius*) *insa2* gene being an example.

Further processing by prohormone convertase enzymes releases the internal C-peptide to produce the two-chain insulin hormone, composed of A- and B-chains linked by disulphide bridges (10, 30). The proteolytic processing of proinsulin to yield a two-chain insulin hormone contrasts with that of the insulin-like growth factors, where both are single chain peptides that retain the C-peptide-like sequences (31, 32). Insulin genes from diverse species representing multiple classes of vertebrates, including fish, frogs, and birds, have been characterized that share with the human gene a similar three exon (two coding) gene structure, and encode homologous protein sequences (30, 33). In most vertebrates only a single insulin gene has been found, however, multiple copies of this gene, including some with differing gene structures, have been found in some species.

DUPLICATED INSULIN GENES

Rats (*Rattus norvegicus*) and mice (*Mus musculus*) were the first vertebrates found to each have two insulin proteins, which were subsequently found to be encoded by a pair of genes (22). While the insulin 2 genes (*Ins2*) have a gene structure similar to that of the human gene (three exons and two introns), the insulin 1 gene (*Ins1*) was found to be composed of only 2 exons, with all of the coding sequence contained in the second exon (23). These two genes have a relatively recent origin (see **Figure 1B**), in the common ancestor of mice and rats (22–26). Further study of these genes indicated that the *Ins1* gene originated from an aberrant mRNA transcript that was initiated about 500 bases upstream of the normal mRNA start site and partially processed to remove only intron 2 sequences before being reverse transcribed and inserted into the genome (23, 24, 26). Despite the differences in the structures of these two genes, both are equally expressed (22, 24). Since the mouse and rat *Ins1* genes only have ~500 bases of 5' flanking sequence homologous to the *Ins* genes of other mammals, this indicates that only a limited amount of sequence is needed for efficient beta-cell-specific expression of the insulin gene. But why do mice and rats have two insulin genes that have identical expression patterns, while other species can survive with a single gene? This might suggest that the two genes differ in function, perhaps specializing function at different sites or times, thus generating a need to retain both genes. While some evidence for differences in the selective constraints acting on the two insulin genes has been detected (25), no evidence for different functions have been found, thus a convincing explanation has not been reached. Intriguingly, duplicated insulin genes that originated by retroposition, like the rodent *Ins1* gene, have also been described in several species of bony fish through three independent origins (28). While most of the retroposed insulin-like sequences in fish appeared to be pseudogenes, the gene sequence of a potentially retroprocessed insulin gene in the Northern pike (*Esox lucius*) has an intact coding sequence (**Figure 1C**) that is potentially functional (28). Further study is needed to determine whether this retroposed Northern pike insulin gene is expressed and if its encoded protein has a physiological function. These observations also indicate that the vertebrate insulin gene is expressed in the germ cells of a number of species, thus allowing it to be retroprocessed and integrated into the genome allowing to be passed on to the next generation, raising questions about the possible function of insulin in germ line cells.

Duplicated copies of the insulin gene have also been found in several other vertebrate species (**Figures 1C, D**), where these genes retain the three exon and two intron gene structure and potentially have large amounts of flanking sequences that would allow their continued expression. The frog *Xenopus laevis*, which experienced a recent genome duplication (**Figure 1D**), was the first published example (34). Unlike the duplicated rodent insulin genes, the two *Xenopus laevis* insulin genes were found to display differing developmental gene expression patterns that might suggest diverging functions (34), and a reason why both are retained, however they also had overlapping gene expression in the adult pancreas (35). A genome duplication, the fish-specific whole genome duplication (3R), was experienced by an ancestor of teleost fish (36), thus the discovery of a second insulin gene in several fish genome sequences (37) was not a surprise. Characterization of the two zebrafish (*Danio rerio*) insulin genes (*insa* and *insb*) provided evidence that the two genes had distinct expression patterns and potentially differing functions (38), supporting a hypothesis that fish genes have diverged in function, potentially subfunctionalizing so that each now is responsible for a subset of the ancestral functions of insulin. More recently, a third insulin gene (*insc*) has been found in some, but not all, fish (**Figure 1C**), with all three of these genes originating in an early teleost, thus it is unclear which, if any, originated through the fish-specific whole genome duplication (28). The role of this third insulin gene in fish physiology is unknown. Does it also possess a subset of ancestral insulin functions, or has it gained new function? In addition to these three types of insulin genes found in fish, additional lineage-specific duplications of insulin genes were found, including some species [e.g., carp (*Cyprinus carpio*)] that are associated with additional genome duplications on these lineages (28). In addition to these lineage-specific duplications due to genome duplications, a duplication of the *insa* insulin gene, resulting in the *insaa* and *insab* genes, occurred early in the diversification of teleost fish (**Figure 1C**) yielding a large number of species with these gene duplicate. Intriguingly, most of the proteins encoded by the *insab* genes have amino acid substitutions that are predicted to impair proteolytically processing that generates the typical two-chain insulin hormone (28). While these sequences retain signal peptides, which would allow secretion, and cysteine residues that allow disulphide bridge formation, this raises the possibility that they yield an unprocessed inulin-like protein that retains an insulin-like protein structure and has an unknown function (28).

EVOLUTION OF INSULIN SEQUENCES

In addition to changes in the numbers and structure of insulin genes, sequences of insulin genes have also changed. Typically, genes evolve at a near steady rate, but occasionally they display

episodes of more rapid change, which are hypothesized to signal a change in gene function. Studies of mammalian insulins have provided support for this hypothesis. Insulin sequences from the guinea pig (*Cavia porcellus*) and relatives (rodents of the suborder Hystricomorpha) are well known for having insulin sequences with highly divergent sequences (39, 40). The biological activities of these insulins also differ, acting more as a growth factor than as a metabolic hormone (41, 42). These changes, in sequence and function, have been accompanied with an acceleration of the rate of evolution of the insulin protein sequence in the guinea pig and relatives (40, 43, 44). Similar, but less dramatic, episodes of accelerated evolution of insulin sequences have been observed in some species of New World monkeys (44, 45), species that have insulin hormones with lower potency (46).

CHANGES IN PROTEOLYTIC PROCESSING

Recent surveys of fish and mammalian insulin coding sequences have identified several species that have accumulated increased amounts of sequence change (28, 44). However, in contrast to the sequences from the rodent suborder of Hystricomorpha and New World monkeys, the striking changes in these sequences were at sites involved in proteolytic processing. Insulin is composed of two peptide chains linked by disulphide bonds, with both peptide chains generated from a single precursor protein (9, 10). Studies on insulin emphasized the importance of proteolytic processing in the generation of bioactive peptides (2, 9, 10, 47). The insulin precursor undergoes two types of proteolytic processing to generate a functional hormone: 1) removal of its signal peptide, which is necessary for secretion, and 2) removal of the C-peptide to generate the two-chain molecule linked by disulphide bonds (10, 48). Evolutionary changes have occurred at all of these proteolytic sites. Changes in the sites of proteolytic processing of the insulin precursor had previously been observed, especially at the signal peptidase cleavage site and at the B-chain/C-peptide processing site (12). While some earlier studies have suggested altered proteolytic processing of proinsulin in some species of the most divergent classes of vertebrates, jawless and cartilaginous fish (49, 50), recent studies of insulin gene sequences obtained from the genomic sequences of some species of bony fish and mammals predict amino acid replacements that abolish proteolytic processing at both the B-chain/C-peptide and the C-peptide/A-chain processing sites of insulin (28, 44). Almost all of the proinsulin sequences predicted by teleost fish *ins* genes encode sequences that can be processed into two-chain insulin hormones, except for those encoded by *insab* genes (28). Most proinsulin sequences encoded by the *insab* genes contain amino acid substitutions at their B-chain/C-peptide processing site that likely prevent proteolytic cleavage, with many of them also having substitutions that should impair processing at the C-peptide/A-chain site (**Figure 2**), thus leading to improperly processed insulin molecules (28). Similarly, insulin gene sequences from two bats (*Myotis brandtii* and *M. lucifugus*) predict substitutions at both the A-chain/C-peptide and C-peptide/B-chain processing sites that should prevent processing (**Figure 2**), while the insulin sequences from several species of Afrotheria [e.g., aardvark (*Orycteropus afer*)] likely have altered C-peptide/A-chain processing (**Figure 2**) (44). Thus, it is likely that the insulin genes of many vertebrate species generate a final protein product that is composed of either a single protein chain or are two-chain protein molecules that have an A- or a B-chains that is extended to include the complete C-peptide sequence (28, 44). Intriguingly, all of these insulin sequences with altered protein processing retain conserved cysteine residues that are

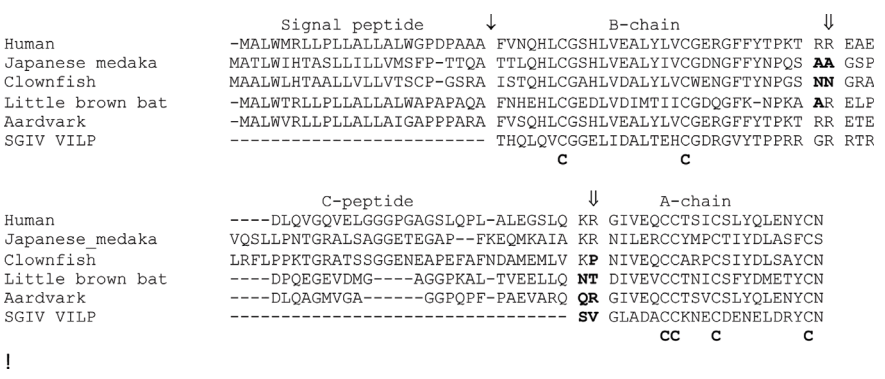

FIGURE 2 | Changes in in the processing of proinsulin-like sequences found in vertebrates. An alignment of the human (*Homo sapiens*) proinsulin protein sequence with selected examples showing potentially altered proteolytic processing. The examples include the insulin proteins encoded by the *insab* genes from two fish (28) [Japanese medaka (*Oryzias latipes*) and Clownfish (*Amphiprion ocellaris*)], two mammals (44) [little brown bat (*Myotis lucifugus*) and aardvark (*Orycteropus afer*)], and the Singapore grouper iridovirus viral insulin-like peptide (SGIV-VILP) (51). SGIV-VILP would be produced by vertebrate cells infected by the Singapore grouper iridovirus. Protein sequences are shown in single letter code, with ↓ indicating the signal peptidase cleavage site and ⇓ the indicating the prohormone convertase processing sites for the human insulin sequence. Domains of the human proinsulin sequence are shown above the alignment. Amino acid replacements in the sequences, relative to the human sequence, which are predicted to impair proteolytic processing are shown in bold. Conserved cysteine residues involved in disulphide bridging are shown below the alignment.

important for disulphide bridging, thus, these proteins potentially retain three-dimensional structures that are similar to insulin and are also biologically functional.

VIRAL INSULIN-LIKE PEPTIDES

Most studies on the function of insulin assume that this peptide is of endogenous origin, or from relatively closely related species. Indeed, humans have been treated with insulin from several other mammalian sources (5). Recent studies analyzing the sequences of viral genomes have revealed that some viruses that infect vertebrates could be another source of insulin-like peptides, with these peptides having the potential to affect physiology and pathophysiology (51). Altindis et al. (51) identified four viruses, which infect fish, whose genomes predict peptides with similarity with insulin, which they called viral insulin-like peptides (VILPs). While these new VILPs sequences share similarity with insulin, differences exist at the regions corresponding to the B-chain/C-peptide and C-peptide/A-chain processing sites (**Figure 2**), thus, and might not generate two-chain molecules (51). However, like the incompletely processed vertebrate insulin sequences described above, the VILPs share the conserved cysteine residues involved in disulphide bridging and are requisite to the maintain the 3D structure (51). Some VILPs have been shown to bind to insulin receptors and regulate glucose metabolism in mice (51, 52), indicating that it may function in its proinsulin form, and that they might have a pathophysiological role beneficial to the viruses. Insulin-like molecules are used as toxins by cone snails (53), thus the use of an insulin-like peptide by a virus in pathophysiology should not be a surprise. It has long been known that full length proinsulin can bind and activate the insulin receptor (54), as do the single-chain IGF1 and IGF2 hormones with their specific receptors, thus, the incompletely processed insulin molecules encoded by genes in fish, mammals, or viruses could impact physiology or pathophysiology.

PERSPECTIVES AND FUTURE DIRECTIONS

The biology of insulin as well as the evolution of insulin have been studied for many years (2, 9, 12) yet new discoveries and insights have been gained from the analysis of the rapidly increasing amount of genomic data. Studies into the evolution of the genes for the insulin receptor, enzymes involved in producing the mature hormone and downstream signaling partners should also improve our understanding of the biology of insulin. Genomic sequences, together with improved bioinformatic search algorithms, allow unbiased searches for sequences with similarity to insulin (or your favorite protein) in genomes, revealing sequences that might not have been found in more directed searches for bioactive peptides. As we complete more genomes and microbiomes, it is certain that we will discover more insulin-like sequences with novel aspects to their sequences, structures, and functions. However, sequence will not tell us function. Experimental work is still needed to identify the functions of these novel insulin-like sequences, which may uncover new roles for insulin in biology.

AUTHOR CONTRIBUTIONS

The author confirms being the sole contributor of this work and has approved it for publication.

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REVIEW
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The Interaction of Insulin and Pituitary Hormone Syndromes

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Pituitary hormone axes modulate glucose metabolism and exert direct or indirect effects on insulin secretion and function. Cortisol and growth hormone are potent insulin-antagonistic hormones. Therefore impaired glucose tolerance, elevated fasting glucose concentrations and diabetes mellitus are frequent in Cushing's disease and acromegaly. Also prolactinomas, growth hormone (GH) deficiency, hypogonadism and hypothyroidism might be associated with impaired glucose homeostasis but usually to a lesser extent. Therefore glucose metabolism needs to be closely monitored and treated in patients with pituitary adenomas. Correction of the pituitary dysfunction is frequently followed by improvement of glucose homeostasis.

Keywords: insulin, cortisol, growth hormone, prolactin, thyroid hormones, sex hormones

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INTRODUCTION

Pituitary hormone excess and deficiency syndromes impair the action of insulin to a varying extent by direct and indirect effects. This is most prevalent and most pronounced in Cushing's disease and acromegaly but also observed in prolactinomas, GH-deficiency, hypogonadism and hypothyroidism (1). The impairment of glucose metabolism is related to the extent and duration of hormone excess and contributes to the co-morbidities, increased cardiovascular risk and to the increased mortality in patients with pituitary adenomas (2, 3). Occasionally impaired glucose tolerance or diabetes mellitus is the presenting symptom of Cushing's syndrome or acromegaly (4, 5). In many instances, cure or amelioration of the hormonal imbalance by pituitary surgery, medical or radio-therapy induces improvement of glucose homeostasis (6, 7). On the contrary, some drugs used for therapy of pituitary adenomas or hormone deficiencies negatively impact glucose metabolism (8). In any case parameters of glucose metabolism should be closely monitored at presentation and throughout active disease but also after cure of the underlying pituitary disease. Consequent treatment of diabetes mellitus, impaired glucose tolerance and elevated fasting glucose concentrations is mandatory and in the absence of strong evidence for these special conditions should in general follow established guidelines for the general population. This article summarizes the effects of functioning and non-functioning pituitary adenomas on glucose metabolism.

CUSHING'S DISEASE

Interactions between insulin and cortisol are manifold (2). Cortisol is a potent insulin-antagonistic hormone inhibiting insulin secretion, stimulating glucagon secretion and disrupting insulin signaling. Cortisol inhibits insulin release and reduces GLP-1 production and thereby also

insulin secretion (**Figure 1**). Cortisol induces the expression of key gluconeogenic enzymes and increases hepatic glucose production and gycogenolysis (**Table 1**). In the muscle cortisol reduces the translocation of the insulin-dependent glucose transporter 4 (GLUT4) to the plasma membrane thereby impairing glucose uptake and by activating glycogen synthase kinase-3 suppresses glycogen synthesis and promotes protein degradation (9) (**Table 1**). In addition cortisol's lipolytic activity with increase in circulating free fatty acids contributes to insulin resistance (**Table 1**). Thus it is not surprising that cortisol excess is associated with disturbed glucose homeostasis in patients with Cushing's disease. Impaired glucose tolerance has been reported in 21-64% at diagnosis and diabetes mellitus in 20-47% (10–15). No data are available in the literature on the percentage of patients requiring insulin therapy at diagnosis. But there are several reports on diabetic ketoacidosis as a presenting symptom of Cushing's disease where diabetes was cured or remitted with the control of cortisol excess (4, 16, 17). Possible explanations are cortisol's stimulation of lipolysis and increase in free fatty acids, suppression of insulin secretion and ketogenesis in the liver.

Vice versa, in a series of 200 overweight patients with type 2 diabetes and a HbA1c >8% devoid of specific clinical symptoms of Cushing's syndrome, 17 patients were identified with 2 abnormal screening tests for hypercortisolism but only 3 patients received a definitive diagnosis of Cushing's disease while 1 patient had a cortisol producing adrenal adenoma (18).

TABLE 1 | Metabolic effects of insulin and alterations in Cushing's disease and acromegaly.

Organ	Insulin	Cushing's disease	Acromegaly
Liver			
Gluconeogenesis	↓	↑	↑
Glycogenolysis	↓	↑	↑
Muscle			
Glucose uptake	↑	↓	↓
Proteolysis	↓	↑	↓
Glycogen synthesis	↑	↓	↑
Adipose Tissue			
Lipolysis	↓	↑	↑
Glucose uptake	↑	↓	↓

In addition, a recent study in 384 newly diagnosed patients with type 2 diabetes without a persuasive cushingoid phenotype reported a 5% prevalence of hypercortisolism including 1 patient with a pituitary macroadenoma and 9 patients with an adrenal adenoma (19). The low prevalence of Cushing's disease in patients with type 2 diabetes and the high prevalence of type 2 diabetes in the general population, does not justify the recommendation that all type 2 diabetes patients should be screened for hypercortisolism, but it should be borne in mind even in the absence of characteristic signs, symptoms and comorbidities. An increased activity of the hypothalamic-pituitary adrenal axis has been observed in patients with type 2 diabetes mellitus with asymptomatic autonomic imbalance with

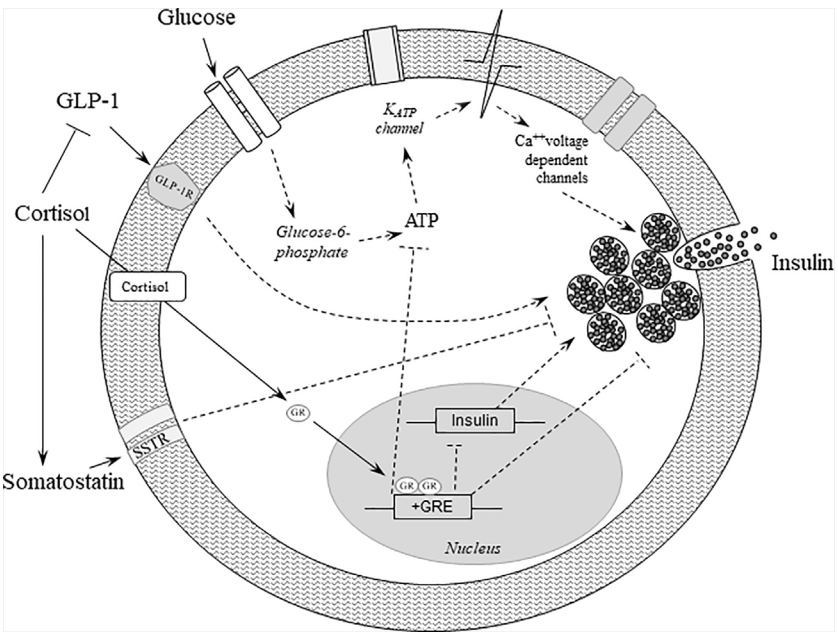

FIGURE 1 | Effects of glucocorticoids on beta-cell function. Binding of glucose to the GLUT2 receptor increases oxidative metabolism and ATP synthesis, thereby leading to the closure of the K_{ATP} channels, which in turn stimulates membrane depolarization and insulin secretion. Cortisol binds to and activates the glucocorticoid receptor, which translocates to the nucleus and initiates several cascades culminating in repression of insulin transcription and inhibition of insulin release. In addition, cortisol further deteriorates beta-cell function by reducing GLP-1 production and its positive effects on insulin secretion, and by increasing the secretion of somatostatin, which in turn negatively impacts insulin gene transcription and insulin secretion. Dashed lines represent indirect effects. ATP, adenosine triphosphate; Ca, Calcium; GLP-1, glucagon-like peptide 1; GLP-1R, glucagon-like peptide 1 receptor; GLUT2, glucose transporter 2; GR, glucocorticoid receptor; GRE, glucocorticoid response element.

prevalent parasympathetic failure (20). Hypercortisolism may also be caused by alcoholism, severe depression, anxiety disorders and severe obesity (21).

Remission or reduction of hypercortisolism by transsphenoidal surgery, medical - or radio-therapy frequently improves glucose homeostasis but induces remission of diabetes or prediabetes in only a minority of patients (6, 7, 22, 23). This might be related to the delay of diagnosis and thus prolonged exposure to cortisol excess especially in patients predisposed to diabetes mellitus. In contrast, the second generation somatostatin analog pasireotide has substantial negative effects on glucose homeostasis despite a reduction or normalization of hypercortisolism (8, 24). Pasireotide's higher affinity to somatostatin receptor subtype 5 (SSTR5) and lower affinity to SSTR2 compared to first generation somatostatin analogs is presumed to result in a greater suppression of insulin secretion and lesser decrease in glucagon secretion (25). In addition pasireotide suppresses also the secretion of GLP-1 and GIP (26). Close monitoring of glucose concentrations after initiation and throughout therapy with pasireotide treatment is mandatory (8, 24). Mifepristone, a glucocorticoid and progesterone receptor antagonist, has been shown to improve insulin sensitivity and decrease fasting plasma glucose concentrations and HbA1c concentrations in patients with Cushing's disease and impaired glucose tolerance or diabetes mellitus (27).

No strong evidence on how to treat disturbed glucose metabolism in Cushing's disease exists. Considering pathophysiological mechanisms insulin sensitizing drugs counteracting insulin resistance should be considered as first choice. This has also been suggested by a guideline of the Italian Society for the Study of Diabetes and the Italian Endocrinological Society (28). Due to the negative effects of thiazolidinediones on bones (29) and fluid retention which would add to the ones of hypercortisolism metformin either as monotherapy or, if glycemic targets cannot be reached, in combination with other oral antidiabetic drugs, GLP-1 receptor agonists or insulin should be considered as first choice. SGLT-2 inhibitors might induce the so called "euglycemic" ketoacidosis and therefore appear not to be optimally suited. They add to cortisol's metabolic actions by increasing glucagon secretion and ketogenesis in the liver as well as reabsorption of ketone bodies in the kidney (30) and their side effect of genitourinary infections may be especially disadvantageous in patients with Cushing's syndrome who are already at an increased risk of infection. In contrast, GLP-1 receptors agonists which besides their insulin stimulating effect also suppress glucagon secretion and appetite appear to be an attractive alternative. GLP-1 infusion in a patient with Cushing's disease induced diabetes has indeed demonstrated similar insulinotropic, glucagonostatic and glucose-lowering actions as in patients with type 2 diabetes (31).

ACROMEGALY

Growth hormone (GH) induces lipolysis with increased circulating free fatty acids leading to insulin resistance in the liver and peripheral muscle (32–34) (**Table 1**). Thereby hepatic glucose production, gluconeogenesis and glycogenolysis in the liver are stimulated, whereas glucose uptake in the muscle is reduced (**Table 1**). The contribution of GH-induced elevated IGF-1 concentrations to these effects is not completely elucidated (34). While IGF-1 has insulin-agonistic actions and has been used for treatment of severe insulin resistance, IGF-1 reduction with the GH antagonist pegvisomant improves glycemic control in acromegalic patients with diabetes or impaired glucose tolerance despite elevated GH (35, 36).

GH excess leads to impaired glucose homeostasis in a considerable part of patients with acromegaly. In more than half of the patients, diabetes is diagnosed before acromegaly (37) highlighting the need to increase the awareness of this association. At diagnosis, 22 – 40.5% of patients with acromegaly exhibit elevated fasting glucose concentrations and/or impaired glucose tolerance and 12 – 34.9% diabetes mellitus (37–42). In two studies that reported details on diabetes treatment the percentage of patients requiring insulin therapy ranged from 17.6 to 24% (37, 41). Also several cases of diabetic ketoacidosis have been reported as presenting symptom/condition in patients with acromegaly (5, 43–46). This association must be attributed to extremely severe insulin resistance as well as GH's lipolytic action with an increase in free fatty acids and the suppression of insulin secretion. In all reported cases of acromegaly-associated ketoacidosis, diabetes resolved after remission of acromegaly.

Glucose homeostasis frequently improves following remission of GH excess by transsphenoidal surgery or pharmacological disease control but glucose metabolism remains impaired in many patients (47, 48).

Pharmacological treatment of acromegaly has varying effects on glucose homeostasis. Treatment of mild acromegaly with bromocriptine has been reported to be associated with favorable effects that are more pronounced with the more potent dopamine agonist cabergoline (49, 50). Most studies evaluating the first generation somatostatin analogs have demonstrated neutral to mild adverse effects (51). The second generation somatostatin analog pasireotide has been shown to induce impaired glucose tolerance, overt diabetes or worsen glycemic control especially in patients with pre-existing disturbed glucose metabolism (52). The mechanisms by which pasireotide exerts these effect have been described in the section on Cushing's disease. In contrast, the GH-antagonist pegvisomant has been shown to improve glucose metabolism whether applied as monotherapy or added to treatment with somatostatin analogs (36).

As with Cushing's disease, no clear evidence exists as to the optimal treatment of impaired glucose tolerance and diabetes associated with acromegaly. From a pathophysiological viewpoint substances counteracting insulin resistance appear to be the first choice also in acromegaly (53). Metformin should be preferred to glitazones due to the adverse effects on bone and fluid retention of the latter. As mentioned in the section on Cushing's disease this has also been supported by guideline of the Italian Society for the Study of Diabetes and the Italian

Endocrinological Society (28). SGLT2 inhibitors appear not to be the first choice due to their mechanism of action adding to GH's effects on glucose metabolism by facilitating the so-called euglycemic ketoacidosis (see Cushing section). In fact, a case of rapid onset diabetic ketoacidosis has been reported after addition of the SGLT2 inhibitor empaglflozin to metformin, sitagliptin and gliclazide in a patient with unrecognized acromegaly and type 2 diabetes (54). DPP4-inhibitors and GLP-1 receptor agonists might be used along with metformin but insulin remains the therapy of choice in cases with severe hyperglycemia.

EFFECTS OF EXCESS/DEFICIENCY OF OTHER PITUITARY HORMONES ON GLUCOSE HOMEOSTASIS

In contrast to the frequent and sometimes severe effects of hypercortisolism and GH excess, the deficiency or excess of other pituitary hormones have also been shown to affect glucose homeostasis but mostly to a less severe extent. In these hormonal imbalances factors regulating insulin sensitivity and secretion are often acting in opposite directions and affected by concomitant deficiencies of other pituitary hormones. In addition to the extent of hormone deficiency or excess, changes in appetite regulation, energy expenditure, body weight and body composition might contribute to the adverse effect on glucose homeostasis. It is therefore not possible to reduce observed clinical changes to single pathomechanisms that are often derived from animal or *in vitro* studies and might not apply to humans. In the following part some of the postulated pathways that might contribute to the impaired glucose homeostasis will be briefly discussed.

Whereas GH deficiency according to the negative effects of elevated GH levels on glucose homeostasis would be expected to result in beneficial effects the findings of available studies on this topic are inconsistent. Normal, reduced as well as increased insulin sensitivity have been reported in GH deficient patients (55, 56). GH deficiency in adults has been reported to induce insulin resistance by inhibiting glucose storage rate and glycogen synthase activity in peripheral tissues (57). In addition, increased fat mass and decreased muscle mass contribute to insulin resistance in these patients. In a retrospective analysis of 6050 patients with adult-onset GH deficiency, a significantly increased prevalence of diabetes has been reported (58).

Hyperprolactinemia is associated with insulin resistance which improves with dopamine agonist therapy which might be related to the therapy associated weight loss and an activation of insulin signaling (59–62). The pathomechanisms by which prolactin impairs glucose homeostasis are unclear, some effects would rather enhance insulin sensitivity. Central prolactin infusion in rats increases food intake, but prolactin receptor knockout mice show reduced adiposity and prolactin deficiency had only negligible effects on body weight, body composition, serum lipids and adiponectin concentrations (63, 64). It also has been demonstrated that prolactin induces increased expression of *Pparg*, *Xbp1s*, and *GLUT4* in visceral and subcutaneous adipose tissue and elevated circulating adiponectin levels thereby increasing insulin sensitivity in mice (65). Prolactin induced hypogonadism might contribute to the metabolic alterations in hyperprolactinemia.

Gonadotrophin deficiency due to mass effects of pituitary adenomas or gonadotrophin suppression due to cortisol or prolactin excess might induce insulin resistance in men and women that can be improved by hormone replacement therapy. This can be deduced from studies in men and women with hypogonadism unrelated to pituitary diseases (66–69). Pathophysiological mechanisms by which testosterone deficiency might impair glucose metabolism in men include reduced insulin receptor expression and intracellular insulin signaling, reduced GLUT4 expression and membrane translocation in skeletal muscle and liver cells as well as in adipose tissue (70, 71). Also key enzymes of the glycolysis pathway and mitochondrial oxidative phosphorylation are increased by testosterone. In addition, promotion of differentiation of pluripotent stem cells into adipocytes rather than myocytes in testosterone deficiency and the resulting increase in fat mass and decrease in muscle mass might contribute to development of insulin resistance (71). Estrogen deficiency might impair glucose homeostasis through several pathomechanisms. 17β estradiol has been shown to suppress hepatic glucose production and gluconeogenesis through inhibition of the transcription factor Foxo1via activation of estrogen receptorα-phosphoinositide 3-kinase-Akt signaling (72). Estradiol improves also insulin-induced glucose uptake and GLUT4 translocation to the plasma membrane in adipocytes (73). In addition estrogens are also involved in the regulation of energy expenditure and food intake and have been reported to protect pancreatic β cells from various injuries as well as enhancing insulin biosynthesis through activation of the estrogen receptorα (74).

Hypothyroidism has also been associated with insulin resistance and risk of type 2 diabetes. Insulin resistance could be restored by thyroid hormone replacement (75, 76). The interplay between thyroid hormones and appetite regulation, energy expenditure, sympathetic activity and glucose and lipid metabolism is complex (77). Among other effects T3 exerts an acute hypoglycemic effect by activating the phosphatidylinositol 3-kinase signaling cascade which is also involved in lowering serum and hepatic triglycerides. Thyroid hormones cause an increase in ATP utilization accelerating lipolysis/fatty acid oxidation and increased protein turnover. T3 increases also the translocation of GLUT4 to the plasma membrane in skeletal muscle and adipose tissue and this might contribute to the hypothyroidism associated impaired glucose tolerance (77).

In conclusion, disturbances of glucose metabolism are frequently found in patients with pituitary adenomas, and can be especially severe in Cushing's disease and acromegaly. Hyperglycemia and diabetes have serious consequences increasing cardiovascular morbidity and mortality and therefore deserve special attention and consequent treatment at any stage of the disease. Cardiovascular endpoint studies have

revolutionized the therapy of patients with diabetes, but they are lacking in the context of diabetes due to pituitary diseases. Therapy of the underlying pituitary disease frequently ameliorates glucose homeostasis.

AUTHOR CONTRIBUTIONS

All authors contributed to the article and approved the submitted version.

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REVIEW
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Insulin-Like Growth Factor Pathway and the Thyroid

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The insulin-like growth factor (IGF) pathway comprises two activating ligands (IGF-I and IGF-II), two cell-surface receptors (IGF-IR and IGF-IIR), six IGF binding proteins (IGFBP) and nine IGFBP related proteins. IGF-I and the IGF-IR share substantial structural and functional similarities to those of insulin and its receptor. IGF-I plays important regulatory roles in the development, growth, and function of many human tissues. Its pathway intersects with those mediating the actions of many cytokines, growth factors and hormones. Among these, IGFs impact the thyroid and the hormones that it generates. Further, thyroid hormones and thyrotropin (TSH) can influence the biological effects of growth hormone and IGF-I on target tissues. The consequences of this two-way interplay can be far-reaching on many metabolic and immunologic processes. Specifically, IGF-I supports normal function, volume and hormone synthesis of the thyroid gland. Some of these effects are mediated through enhancement of sensitivity to the actions of TSH while others may be independent of pituitary function. IGF-I also participates in pathological conditions of the thyroid, including benign enlargement and tumorigenesis, such as those occurring in acromegaly. With regard to Graves' disease (GD) and the periocular process frequently associated with it, namely thyroid-associated ophthalmopathy (TAO), IGF-IR has been found overexpressed in orbital connective tissues, T and B cells in GD and TAO. Autoantibodies of the IgG class are generated in patients with GD that bind to IGF-IR and initiate the signaling from the TSHR/IGF-IR physical and functional protein complex. Further, inhibition of IGF-IR with monoclonal antibody inhibitors can attenuate signaling from either TSHR or IGF-IR. Based on those findings, the development of teprotumumab, a β-arrestin biased agonist as a therapeutic has resulted in the first medication approved by the US FDA for the treatment of TAO. Teprotumumab is now in wide clinical use in North America.

Keywords: growth factor, hormone, goiter, autoimmune, Graves' disease, ophthalmopathy, thyroid

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INTRODUCTION

Among the most ubiquitous regulatory factors governing functions in the mammalian body are those belonging to the insulin and insulin-like growth factor-I (IGF-I) family, including their respective receptors, binding and related proteins, and extensive signaling pathways that mediate/modulate their actions. Discovery of insulin has been attributed to several individuals, but most

prominent among them are Paulescu [cited in reference (1)] in France and Banting, Best and colleagues (2) in Toronto, Canada, working independently. Therapeutic insulin was first administered to patients with diabetes mellitus in 1922, representing a seismic event ranking among the most impactful in modern medicine. In aggregate, this body of work concerning the discovery and characterization of insulin opened the door to our current understanding of normal and pathological carbohydrate metabolism with implications extending far beyond. Its "first cousin", sulfation factor, was first described nearly four decades later by Daughaday and colleagues (3). Sulfation factor underwent a name change to somatomedin C in 1972 (4) and finally to its current designation, IGF-I, in the early 1980s (5). Subsequent to their discovery, the actions of both insulin and IGF-I have been characterized extensively and found to be overlapping in many regards. Their physiological roles and impact on target tissues and cells have been slowly disentangled but their functional promiscuity and that of their respective receptors continue to intrigue students of the field.

In this article, I focused on the growth hormone (GH) and IGF-I pathways and have attempted to briefly review their numerous intersections in the hypothalamic-pituitary-thyroid axis. The relevance of IGF-IR in the pathogenesis of thyroid-associated ophthalmopathy (TAO), the most serious extra-thyroidal autoimmune manifestation of Graves' disease (GD), has only come into prominence over the past 2 decades (6–8). As I will describe, IGF-IR plays not only a critical role in the pathogenesis of TAO, but can be effectively targeted as a therapeutic strategy for managing the disease. The concept of IGF-IR playing an important role in the development of TAO or targeting the protein as a strategy for treating the disease had been met with overwhelming skepticism when it was first proposed (9, 10). Despite these dismissive views, teprotumumab, an inhibitory monoclonal antibody directed at IGF-IR, was recently approved specifically for the treatment of TAO. It is now in clinical use in North America; however, considerably more information will be required if we are to fully understand its mechanism of action in ameliorating the signs and symptoms of TAO and the entirety of its off-target consequences on the human body.

IGF-I and Its Associated Pathway

The molecular structure of IGF-I is closely related to insulin (11) (**Figure 1**). IGF-I comprises 70 amino acids and mediates growth during childhood and adolescence (12). IGF-II contains 67 amino acids. Both IGF-I and IGF-II have three intramolecular disulfide bridges. While IGF-I synthesis was initially considered to be driven by GH as a systemically active mediator, recent decades have witnessed increasing insight into its roles as a paracrine and autocrine factor. Mediating the actions of insulin and IGF-I are their respective, closely related receptors (IGF-IR and IR, respectively), both belonging to the tyrosine kinase family (**Figure 2**). The cell surface receptors of both ligands also exhibit substantial structural and functional relatedness and may have evolved from gene duplication of a common precursor (13).

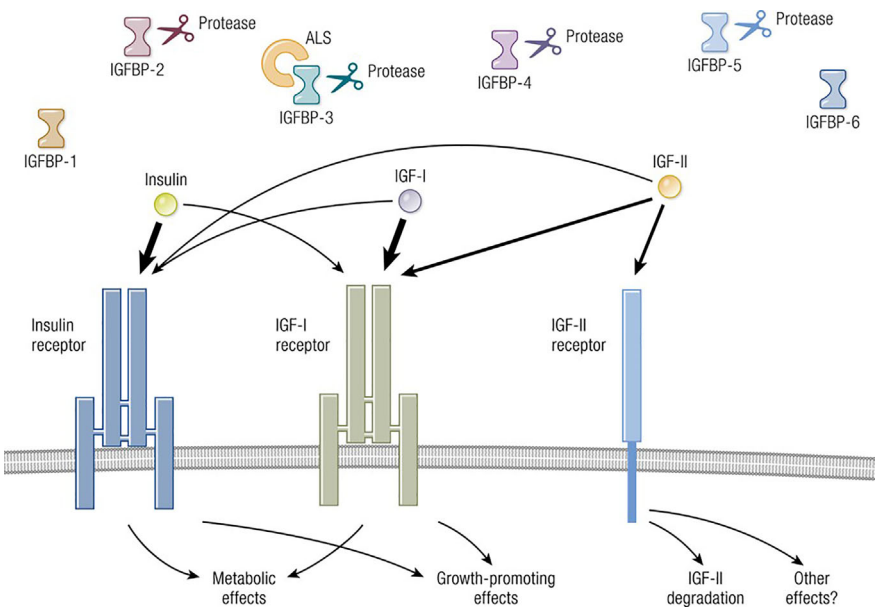


FIGURE 1 | Overview of the IGF-I pathway, including its multiple ligands, modulatory binding proteins and receptors. Ligands including IGF-I, IGF-II, and insulin share important structural and functional relationships including the promiscuous utilization of multiple receptors, such as IGF-IR, IGF-IIR, and IR. The pathway also contains IGF-binding proteins (IGFBPs) which can function either independently or may require ligation. IGF-IR plays a central role in mediating growth regulation. In contrast, the insulin receptor is the primary regulator of metabolism and carbohydrate handling. IGF-IIR (mannose-6-phosphate receptor) influences IGF-II degradation and participates in the biology of the Golgi apparatus. Biological impact of both IGF-I and IGF-II action is modulated by six thus-far identified IGFBPs. Adapted from Lowe WL. Insulin-like growth factors. Science & Medicine 1996; 3; 65.

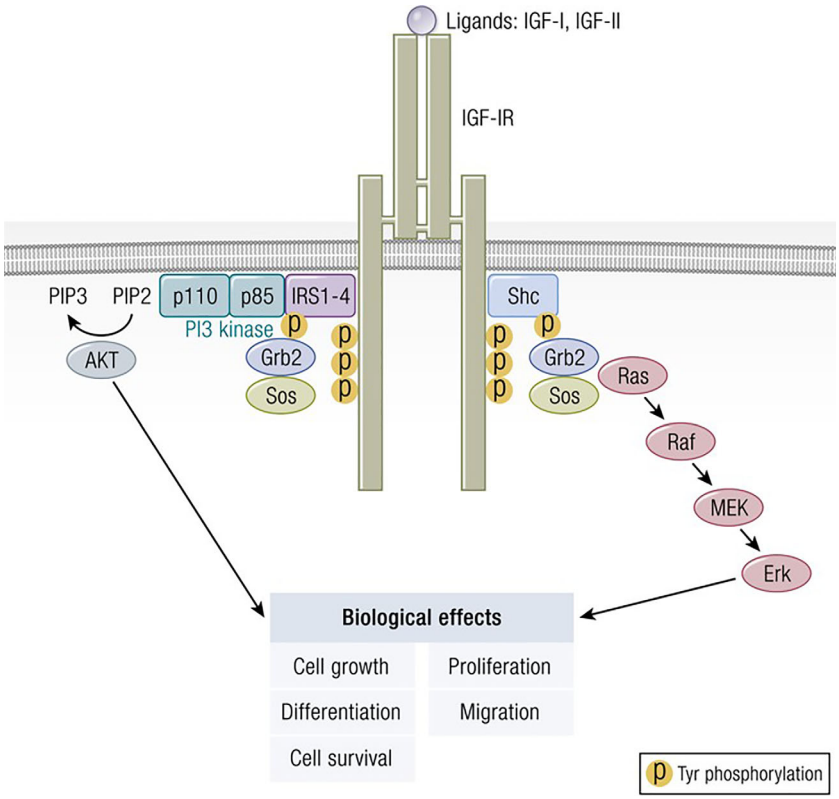


FIGURE 2 | Classical model of signaling through the IGF-IR. IGF-I ligation of IGF-IR most commonly results in canonical mitogen activated protein kinase (MAPK)/Ras-Raf-Erk and phosphatidylinositol-3-kinase/AKT/mTOR (PI3K/AKT) phosphorylation, critical to downstream signaling and the activation of targeted genes associated with IGF-I responses. Adapted from Worrall et al. Novel Mechanisms of Regulation of IGF-1R Action: Functional and Therapeutic Implications. Pediatr Endocrinol Rev. 2013 10:473-484.

They form IR/IR and IGF-IR/IGF-IR as well as IR/IGF-IR hybrids, the particular protein pairings of which might be determined stochastically. Both possess heterotetrameric protein structures comprising extracellular domains containing ligand binding sites located in two α subunits. Two β subunits consist of extracellular, transmembrane and intracellular domains containing the kinase domains, the ATP binding site, and multiple potential tyrosine phosphorylation sites (14). The α and β subunits are linked by two disulfide bonds. Both insulin and IGF-I engage in promiscuous interactions with each other's receptor, causing substantial overlap in the physiological and pathological consequences of activating the IGF-IR and IR pathways. While IGF-IR primarily functions as an integral membrane receptor the activation of which provokes tyrosine kinase autophosphorylation, recent studies have revealed the nuclear translocation of IGF-IR as well as other components of the IGF-I pathway (15). Similar to many other cell-surface receptors, the density of IGF-IR determines in part the pattern of signaling downstream from that receptor (16). It has been proposed that unligated IGF-IR can initiate signaling mediated through microRNAs and imprinted genes (17). Several factors can upregulate IGF-IR gene expression while others reduce target gene transcriptional activity (8). In addition to IGF-IR, a second receptor, IGF-IIR, aka the mannose-6-phosphate receptor, can bind IGF-II (18) and plays roles in trafficking molecules to the Golgi and the endosomal-lysosomal system (19). IGF-IIR does not mediate tyrosine phosphorylation-dependent signaling analogous to that attributed to IGF-IR and IR. Besides the two receptors, the IGF-I pathway includes six IGF-I binding proteins and nine IGFBP related proteins (20, 21). Their potential relevance to the thyroid has been reviewed recently in detail (8).

IMPACT OF GH AND ITS PRIMARY EFFECTOR, IGF-I, ON THE THYROID GLAND

The relationship between GH, IGF-I and the thyroid gland is complex, frequently reciprocal and temporally spans the interval during fetal development throughout adult life (22, 23). Much of what we understand about the impact of the IGF-I pathway on the thyroid derives from observations made in states of hormone excess such as acromegaly (22, 24). Porcine thyroid cells express both IGF-IR and IR (25) as do human thyroid cancer cells and

normal thyroid (26). Thyrospheres derived from healthy tissue express both isoforms of IR, namely IR-A and IR-B, as well as IGF-IR, IGF-I and IGF-II, levels which decline following cell differentiation. Interestingly, between the two insulin receptor isoforms, IR-A expression dominates thyrospheres while IR-B is more abundant in differentiated thyrocytes. IGF-I binding is saturable and Scatchard analysis discloses a single binding class with a Ka 4.3 X 10^{-10} and 49 X 10^{3} sites per cell. IGF-I stimulates DNA synthesis and thyroid cell proliferation, actions synergistic with those of epidermal growth factor. IGF-I and insulin have important roles in regulating not only proliferation but also thyroid cell differentiation. In studies of FRTL-5 rat thyroid cells, IGF-II but not IGF-I was found to be synthesized and released (27). IGF-II promotes DNA synthesis in FRTL-5. On the other hand, medullary thyroid carcinoma cells were found to express both IGF-I and IGF-II (28, 29). A p21 Ras mutation can induce IGF-I expression and release in immortalized human thyroid epithelial cells (30). In porcine thyroid follicles, TSH and iodine were found to regulate IGF-I mRNA levels (31). In those studies, IGF-I transcript was undetectable in the absence of TSH, effects that were mimicked by forskolin. In contrast, Iodine downregulated the levels of this mRNA. Similar TSH-dependent effects on IGF-I were demonstrated in another report (32). Interestingly, differentiation of mouse embryonic stem cells to thyroid epithelial cells requires insulin/IGF-I, suggesting the complexities of these factors in thyroid development (33). The incidence of thyroid nodules was found to be increased substantially in children with extreme insulin resistance (34) bringing clinical relevance to the basic observations made in the laboratory.

Signaling through both IGF-IR and IR is considered essentially indispensable in the normal function of the thyroid gland, both through their independent support of thyroid epithelial survival and vitality and also by virtue of the interactions of their downstream pathways with those of the thyrotropin receptor (TSHR) (35). The patterns of effects in FRTL-5 cells suggest that IGF-I and insulin have distinct actions on specific gene expression, both independent of and in concert with TSH (36). Among the IGF-IR/IR-activated pathways in thyroid cells are Erk 1/2, and Akt, while TSHR is G protein coupled and results in the generation of cAMP. Examination of IGF-IR and insulin receptor docking proteins has revealed a role for insulin receptor substrate 2 (IRS-2) in mediating the proliferative actions of IGF-I, both *in vitro* and *in vivo* (37). Iodide uptake and organification are among the specific aspects of thyroid metabolism under dual TSH and IGF-I/insulin control. These processes in turn are mediated through the activities of the sodium iodide symporter and thyroperoxidase, respectively. In a study of mice with conditional double-thyroid knockout of IGF-IR and IR, neonatal thyroid glands were smaller, exhibited repressed FOXE1 expression, and manifested defective folliculogenesis (38). At postnatal day 14, mTOR-dependent epithelial cell proliferation and serum TSH were detectable. By week 50, lesions resembling papillary carcinoma had developed, coincident with ErbB activation. The authors concluded that both IGF-IR and IR are critical to follicle formation in the developing thyroid. IGF-IR engagement in cultured thyroid epithelial cells has been shown to activate the expression of chemokines and cytokines such as IL-16 and RANTES (39). Both insulin and IGF-I can regulate the rate of major histocompatibility complex class 1 (MHC class 1) gene transcription (40). These effects are similar to those of TSH in FRTL-5 cells. IGF-I and insulin in combination with TSH can suppress the transcription of the Mac-2BP gene in these cells by decreasing the binding of an upstream specific factor to a gene promoter site (41). IGF-I and cAMP appear to differentially activate the PI3 kinase pathway in FRTL-5 cells, leading to G1 cyclin-cyclin dependent kinase activation and DNA synthesis (42). IGF-I induces several anti-apoptotic proteins in thyroid cells, including Fas-associated death domain-like interleukin-1-converting enzyme-inhibitory protein, actions mediated through activation of the NF-κB pathway (43). Quercetin inhibition of FRTL-5 proliferation is mediated by alterations in insulin and IGF-I signaling involving the Akt pathway (41). It has been suggested that lithium might influence IGF-IR coupled G_i-proteins during the G1 phase of FRTL-5 cell cycling (44). With regard to thyroid cancer, the IGF-I pathway has been directly implicated in the transformation of thyroid epithelial cells (45). It remains uncertain whether the relationship between IGF-I action, IGF-IR signaling, or any of the other components of the IGF-I pathway differ in their relationship to thyroid cancers when compared to other forms of neoplastic disease. Elevated levels of the IGF-IEc isoform have been reported in differentiated papillary thyroid carcinoma and are associated with advanced disease (46).

THE THYROID IN ACROMEGALY

The hypothalamic-pituitary-thyroid axis can be affected at several levels in acromegaly, including those imposed directly and indirectly on the gland itself. For instance, interpretation of laboratory thyroid testing in this condition can be complicated. Both basal and pulsatile TSH secretion are often reduced while thyroxine levels can remain unaffected (47). This phenomenon may result from enhanced thyroid sensitivity to the actions of TSH caused by either growth hormone, IGF-I, or both. In their very recent report, Natchev and colleagues studied a cross-section of 146 acromegalic patients and found that secondary hypothyroidism and hyperthyroidism are commonly encountered (48). Thyroid gland volume is frequently impacted (49) and frank thyroid enlargement in acromegaly is not unusual. Thyromegaly was first recognized in acromegaly by Rolleston in 1936 (24). Gland volume in acromegaly correlates with serum GH and IGF-I levels. Distinguishing which of these hormones might be impacting thyroid enlargement in a particular case is frequently not possible (50). The incidence of both euthyroid and hyperthyroid goiters is increased in acromegaly, a consequence potentially independent of TSH (51). In the absence of TSH, it appears that GH has little or no effect on thyroid size (52). Surgical cure of acromegaly can result in reduced thyroid volume (50). Sera from patients manifesting

untreated acromegaly can induce [^{3}H] thymidine incorporation into the DNA of FRTL-5 cells more than sera from healthy control donors (53). The mitogenic activities of both normal and acromegalic sera can be attenuated by pretreating them with neutralizing monoclonal anti-IGF-I antibodies. Thyroid enlargement in acromegaly can be nodular, multinodular or diffuse (48, 54). The nodules in this condition are described as "stiff", a quality thought to result from localized fibrosis (55). Besides benign goiter, the incidence of thyroid carcinoma is said to be increased in acromegaly (56, 57). In fact, both benign and malignant thyroid nodules are commonplace in these patients (54). Patients with acromegaly are at increased risk for a variety of cancers besides those of the thyroid. These include those of the colon, prostate, and breast, but thyroid cancers may be the most common malignancy associated with the disease (58). IGF-I can promote tumor progression and perhaps facilitate neoplastic initiation (59). McCune-Albright syndrome occurring in adults can be associated with toxic multinodular goiter and an increased risk of thyroid cancer (60). Of the thyroid cancers associated with acromegaly, papillary tumors can occur, especially in those patients manifesting other endocrine and non-endocrine neoplasms. These include pheochromocytoma, growth hormone-producing pituitary adenoma, and duodenal adenocarcinoma (61, 62).

INTERPLAY BETWEEN IGF-I AND THYROID HORMONE METABOLISM AND PATHWAYS

As suggested above, entanglement between the actions of GH, IGF-I and thyroid hormones is complex and can become unmasked with excesses or deficiencies in one or both pathways. Childhood deficiency of GH can occur as an isolated defect, whereas those occurring in adults are typically associated with a constellation of pituitary-hypothalamic insufficiencies and are mostly associated with anatomic lesions. Exogenous GH administration in GH-deficient children has been shown to reduce circulating levels of T_4, although the mechanism(s) involved has yet to be established. Some studies have revealed a GH-dependent increase in T_4 to T_3 conversion (63) and that GH may support physiological T_4 to T_3 conversion. Further, GH deficiency may impair T_3 generation (64). GH may also work centrally, either through direct actions or those mediated through IGF-I to reduce the synthesis and release of TSH from the pituitary (65). T_3 and IGF-I influence divergently GH synthesis and release from cultured pituitary cells in monolayer (66). The enhancement of GH synthesis by T_3 is mediated through enhanced gene transcription while the inhibition by IGF-I of GH induction appears to reflect changes in posttranscriptional events. Insulin and thyroid hormones can act synergistically by mutual enhancement. For instance, T_3 can upregulate the expression of glutamic acid decarboxylase in pure cortical neuronal cultures, actions requiring the presence of insulin (67). T_3 (and thyroxine to a lesser extent) enhances the increased sulfation of embryonic cartilage by sera from either intact or hypophysectomized rats, effects mediated at least in part by IGF-I (68). IGF-I and thyroid hormones interact on growth plate chondrocytes where IGF-I enhances Wnt-4 expression and β-catenin activation (69). In that study, T_3 was shown to enhance IGF-1R signaling and its upregulation of PI3K/Akt/GSK-3β signaling. IGF-I increases levels of thyroid transcription factor-2, actions similar to those of insulin (70). T_3 and IGF-I have synergistic, enhancing effects on the expression of fast-type sarcoplasmic-reticulum Ca^{++}-ATPase in L6 myocytes (71). T_3 acts on the rate of gene transcription while IGF-I enhances mRNA and protein stabilities. These interactions can translate into clinically relevant events. For instance, exogenous GH attenuates thyroid hormone action in patients with Turner syndrome (72).

IMPACT OF THYROIDAL STATUS ON THE GH/IGF-I AXIS

Normal growth requires intactness of both GH/IGF-I and thyroid hormone pathways. Thyroid hormones influence IGF-I levels in the pituitary and in peripheral tissue compartments (73), including expression and release of GH from the pituitary (74, 75) and the circulating levels of both GH and IGF-I (76). Animals harboring the mutant transcription factor, Pit-1, (Snell dwarf mice), exhibit multiple deficiencies in anterior pituitary hormones resulting in defective B cell development (77). This defect can be partially ameliorated with exogenous GH or IGF-I but a more complete remediation can be accomplished with administration of exogenous thyroxine. Thus, the regulatory actions of GH/IGF-I and TSH/thyroid hormones appear to overlap in bone marrow cells. Evidence suggests that not all thyroid hormone effects on the IGF-I pathway are mediated through GH expression and release but that other, GH-independent mechanisms may also be involved (78). Some contradictory findings have been reported in human dysthyroidemia. Plasma levels of IGF-I were found to be reduced in hypothyroid individuals; these were increased substantially with adequate thyroid hormone replacement. Basal IGF-I levels are lower in hypothyroid patients compared to those who are euthyroid (79). In another report, serum IGF-I levels were lower in hyperthyroid patients while levels trended toward elevation in hypothyroid individuals (80). Other components of the IGF-I pathway are influenced by thyroid hormone. Thyroxine replacement therapy appears to increase serum levels of IGFBP1 (81). In 18 day old rats, thyroid hormones can regulate the expression of hepatic IGFBP2 mRNA and serum protein levels through a mechanism independent of GH (78). Thus thyroid hormone effects on IGFBP2 in these animals diverge from those of IGF-I where its effects are mediated through GH. Serum IGFBP3 and IGFBP4 levels are reduced in hypothyroid animals (82). Thyroidectomized patients in whom thyroid hormone replacement has been withdrawn exhibit a reduction in circulating IGFBP1 levels (83) while thyroxine treatment increases them (81). It appears that the impact of thyroid

hormone status on the GH/IGF pathway may change with maturation; further some of these developmental-stage sensitive effects are mediated through GH (78). Propylthiouracil treatment in rats results in substantially increased IGF-II binding site density on thyroid epithelial cells (84). In contrast, thyroid hormones induce IGF-IR mRNA expression in rat epiphyseal chondrocytes (85) and enhance IGF-I binding in rat pituitary cells (86). They regulate IGF-IR expression in rat heart and lung, both during animal development and in adults (87). In aggregate, these findings suggest the complex interactions shared by the two pathways. They support the important roles played by each in the maintenance of both endocrine functions at multiple levels.

IGF-I AND INSULIN ENHANCE THE ACTIONS OF TSH AND THYROID-STIMULATING IMMUNOGLOBULINS: EVIDENCE FOR INTERPLAYING PATHWAYS

Several factors are necessary for thyroid epithelial cells to function normally. Among these are IGF-I and TSH, both of which are critical to thyroid hormone synthesis (88). Of these two molecules, TSH and its cognate receptor, TSHR, represent the primary regulatory pathway for thyroid development, growth and function. Several 'well-travelled" IGF-IR signaling pathways cross-talk with those downstream from TSHR in thyroid epithelial cells. These interactions can either enhance or modulate TSHR-mediated biosynthetic events, depending on their context. Among the intersecting signaling cascades, the p42/44 mitogen-activated protein kinase (MAPK) pathway is pivotal (89). TSHR signaling through p42/44 MAPK is independent of cAMP but dependent on the receptor's coupling to G13 protein (89). So too, is this pathway of central importance to the signaling initiated through IGF-I. The interplay between the actions of IGF-I, insulin and TSH in the thyroid involves interactions between tyrosine kinase receptors (RTKs) and G protein coupled receptors (GPCRs) (**Figure 3**). These receptor classes share scaffolding proteins, namely β-arrestin 1 and 2. The apparently critical role played by β-arrestin 1 in downregulating IGF-IR expression through ubiquination is mediated by an MDM2 E3 Ligase dependent mechanism (90). β-arrestin also mediates IGF-IR signaling through the Erk pathway (91). Studies in Ewing's sarcoma cells treated with figitumumab, an IGF-IR antagonist, revealed that this antibody functioned as an IGF-1R-biased agonist through β-arrestin1 recruitment to the receptor, enhancing IGF-IR ubiquination and provoking Erk phosphorylation in the absence of Akt activation (92). The scope of potential RTK/GPCR hybrid formation is not limited to that involving the IGF-I/insulin family. These hybrids have been observed with other RTKs, including the epidermal growth factor receptor (93). Initial observations that IGF-I and insulin can amplify effects of TSH on thyroid epithelial cells were conducted in FRTL-5 cells (94). Ingbar and colleagues found that both IGF-IR- and IR-activating ligands could provoke proliferation and DNA synthesis in these cultured cells. Their initial report was soon followed by more complete characterizations from that group as well as studies emanating from other laboratories (95). Those studies disclosed that IGF-I could consistently enhance TSH actions in thyroid epithelial cells and demonstrated synergism with regard to cell proliferation and tyrosine kinase activation. As an example, TSH and IGF-I synergistically enhance levels of 1,2-diacylglycerol in FRTL-5 cells, resulting in increased DNA synthesis (96). On the other hand, TSH and IGF-I have distinctly different effects on immediate early gene expression in rat thyroid epithelial cells (97). Thus, despite similarities, the two pathways should be viewed as non-equivalent but frequently intersecting. Tsui et al. subsequently reported that signaling initiated by both rhTSH and GD-IgG could be attenuated *in vitro* by 1H7, a monoclonal anti-IGF-IR-inhibitory antibody (98). Those investigators also reported that TSHR and IGF-IR co-localize in orbital fibroblasts, *in situ* in orbital fat and in primary human thyroid epithelial cells. Further, the two receptor proteins co-precipitate in monoclonal antibody-utilizing pull-down studies (98). Those studies were followed by others demonstrating that the conditional knock-out of IGF-IR in thyroid diminishes its responsiveness to TSH (99). Conversely, selective, combined over-expression of IGF-I and IGF-IR in thyroid of transgenic mice exhibits enhanced sensitivity to endogenous TSH (100). Extensive crosstalk of the multiple downstream signal transduction pathways utilized by the two receptors has been identified (101–104). Those pathways include Erk 1/2 (98). Further, TSH can enhance IGF-I signaling in thyroid, actions mediated through the generation of cAMP (105). Thus, there exists substantial molecular rationale for considering the importance of a functional interplay between IGF-IR and TSHR and for targeting this protein complex therapeutically. Congruent with that possibility, Chen et al. (106) found that the fully human IGF-IR inhibiting antibody, teprotumumab, examined earlier in clinical trials for multiple cancers (107–111), could also attenuate the actions of both IGF-I and TSH in cultured $CD34^+$ fibrocytes. IGF-I was recently found to enhance the expression of TSHR in orbital fibroblasts (112).

EMERGING RELEVANCE OF IGF-IR IN THE PATHOGENESIS OF GD AND TAO

The development of GD remains an only partially understood process. These deficits have resulted historically in suboptimal medical management of both the thyroid dysfunction and TAO components of this vexing condition (113) (**Figure 4**). Initial insights that the IGF-I pathway might be involved in the pathogenesis of TAO emerged from the study of Weightman et al. (114). These investigators reported detecting IgGs collected from patients with GD (GD-IgG) that were capable of displacing radiolabeled IGF-I from binding sites on the surface of orbital fibroblasts coming from these individuals (GD-OF). Pritchard et al. demonstrated subsequently that these bindings sites were

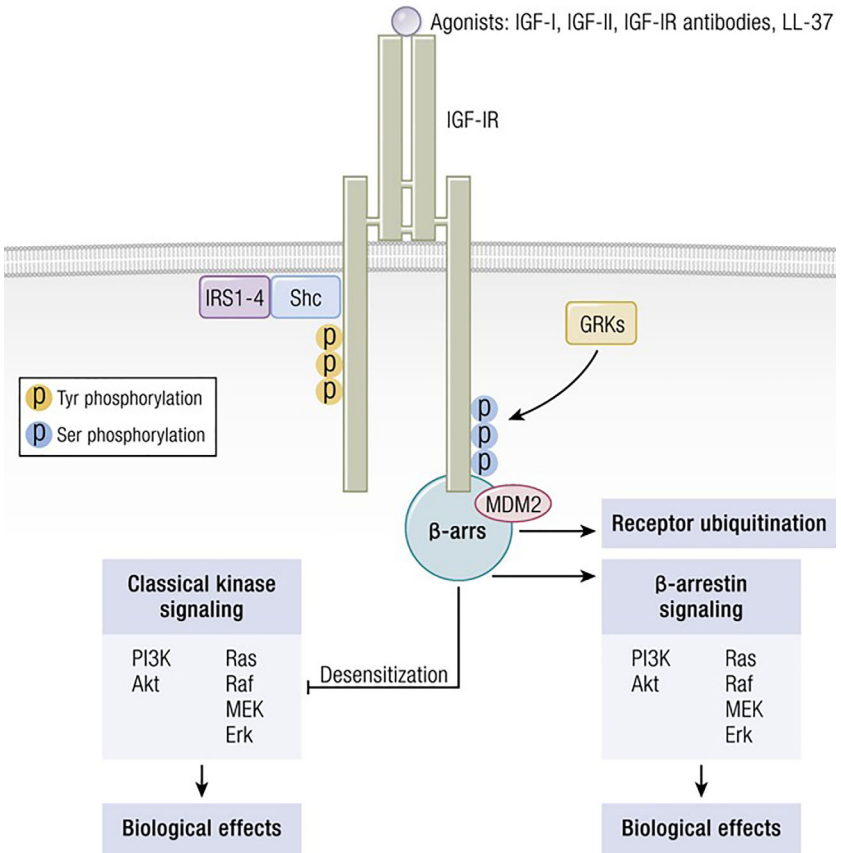


FIGURE 3 | IGF-IR can form functional RTK/GPCR hybrids, thus aggregating tyrosine kinase and GPCR signaling. Ligand-dependent activation of classical kinase-dependent signaling with β-arrestin recruitment provokes GRK-dependent serine phosphorylation located in the IGF-IR C-domain. β-arrestin activates kinase desensitization and ubiquitination and initiates kinase-independent signaling through MAPK. Adapted from Worrall et al. Novel Mechanisms of Regulation of IGF-1R Action: Functional and Therapeutic Implications. Pediatr Endocrinol Rev. 2013 10:473-484.

IGF-IR rather than one or more of the IGFBPs (9). Further, Pritchard et al. reported that GD-IgGs can initiate signaling in GD-OF, resulting in the activation of the PI_3 kinase/FRAP/mTOR/$p70^{s6\ kinase}$ pathway and the induction of target genes encoding IL-16 and "Regulated Upon Activation, Normal T Cell Expressed and Presumably Secreted" (RANTES), two T cell chemoattractants (115). A major issue remaining to be clarified concerns whether the agonistic autoantibodies generated in TAO that induce responses in GD-OF and fibrocytes act directly through TSHR, IGF-IR or both. Whether the anti-IGF-IR antibodies represent those that stimulate or block the receptor's activation or do neither (neutral) remains another open question.

IGF-IR is over-expressed by GD-OF (9) as well as T cells (116) and B cells (117). This increased IGF-IR expression in patients with GD is undetectable in the unaffected monozygotic twin of a sibling with GD, strongly suggesting that non-genetic factors are responsible, at least in part, for the increased receptor levels associated with the disease (118). IGF-I and GD-IgG purified from patients with GD were found to enhance hyaluronan accumulation in cultured GD-OF but had no effect in orbital fibroblasts from healthy donors (119). In contrast, rhTSH failed to influence glycosaminoglycan synthesis. Further, IGF-I appears to skew the accumulating HA molecules toward higher molecular weight species and to promote the proliferation of perimysial GD-OF (120). The effects of hyaluronan on proliferation of these fibroblasts was found to involve differential effects on membrane polarization, where high molecular weight hyaluronan results in depolarization and low molecular weight hyaluronan causes membrane hyperpolarization and inhibits proliferation.

As mentioned above, subsequent studies have disclosed the physical and functional interactions between IGF-IR and TSHR occurring in thyroid epithelial cells, GD-OF and *in situ* in TAO orbital fat (98). Those studies of Tsui et al. also demonstrated that inhibiting IGF-IR could attenuate Erk 1/2 p42/44 activation, regardless of whether the signaling was initiated by either TSHR or IGF-IR. They revealed that the actions of rhTSH, rhIGF-I and GD-IgG could be inhibited, strongly suggesting that the two receptors are functionally linked. Based on those findings, we proffered that IGF-IR might be targeted therapeutically with either monoclonal antibody or small molecule inhibitors of IGF-IR for TAO (10). The capacity for IGF-IR to crosstalk with

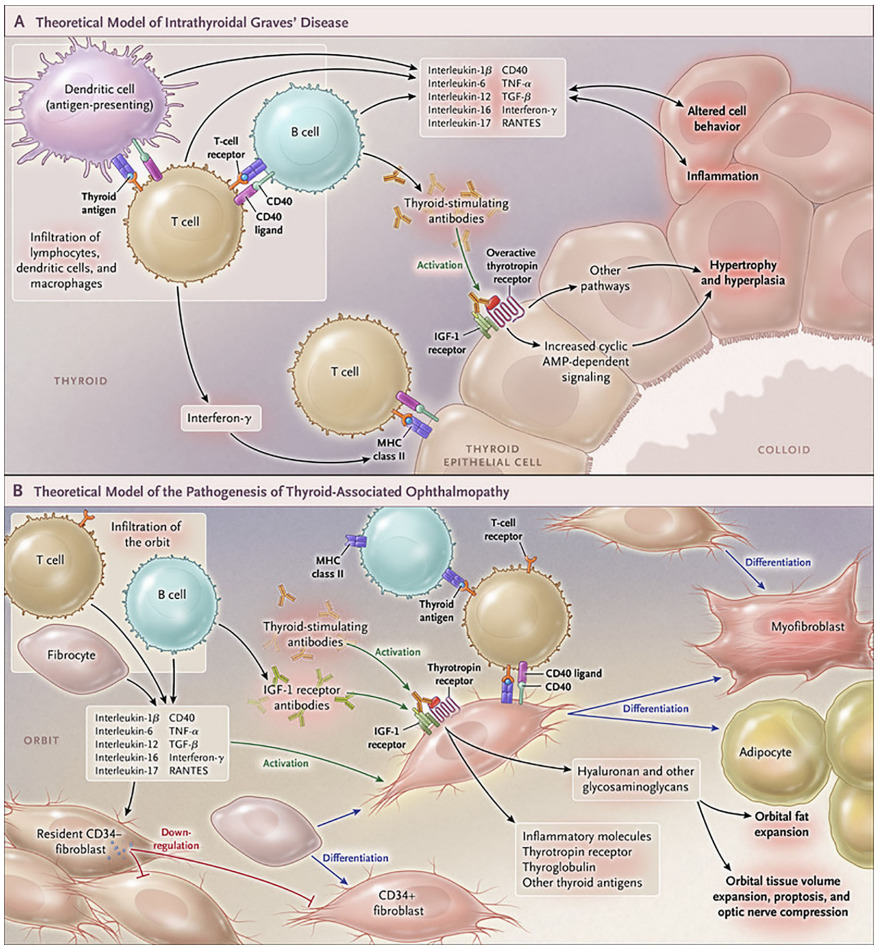


FIGURE 4 | Cartoon proposed model of Graves' disease and thyroid-associated ophthalmopathy (TAO) pathogenesis. **(A)** Thyroid-stimulating immunoglobulins (TSI) drive the over-production of thyroid hormones by activating the thyrotropin receptor (TSHR), thus overriding the normal regulatory role of thyrotropin on the process. B and T cells and antigen-presenting cells which infiltrate the gland produce interleukins 1β, 6, 12, 13, interferon γ, tumor necrosis factor α, and CD40 ligand. These cytokines then activate thyroid epithelial cells, promote inflammation, and induce genes ordinarily unexpressed by these cells such as major histocompatibility complex II (MHC II). Anti-thyroid drugs are useful therapeutically by reducing excess thyroid hormone production as well as modulating the expression of pathogenic intrathyroidal cytokines. **(B)** The pathogenesis of TAO also involves the infiltration of professional immune cells. Orbital fibroblasts exhibit particularly robust responses to inflammatory mediators. Among these cells are $CD34^+$ fibroblasts which we propose derive from fibrocytes, monocyte-derived progenitor cells that traffic from bone marrow. Fibrocytes circulate in Graves' disease at higher frequency than that found in healthy individuals. When cultivated from the peripheral circulation, fibrocytes express several thyroid-specific proteins, including thyrotropin receptor (TSHR), thyroglobulin, thyroperoxidase and sodium-iodide symporter. They also express MHC constitutively and can present antigens. When exposed to the appropriate culture conditions, they undergo differentiation into myofibroblasts (through Smad pathway activation by TGF-β) and adipocytes (through the activation of PPAR-γ). Many of the genes expressed by fibrocytes are detected at considerably lower levels in $CD34^+$ orbital fibroblasts. We have found recently that these lower levels of expression result from the actions of Slit2 which acts through its cognate receptor, Roundabout 1 (ROBO1). When activated, $CD34^+$ fibrocytes and $CD34^+$ fibroblasts generate several pro-inflammatory or anti-inflammatory cytokines, including interleukins 1β, 6, 8, 10, 12, 16, tumor necrosis factor α, and regulated on activation, normal T expressed and secreted (RANTES), CXCL-12 and CD40-CD154. Both $CD34^+$ and $CD34^-$ orbital fibroblasts cell-surface display insulin-like growth factor-I receptor (IGF-IR). Orbital fibroblasts express three mammalian hyaluronan synthase (HAS) isoenzymes and UDP glucose dehydrogenase and synthesize hyaluronan, the glycosaminoglycan associated with expanding orbital tissue in TAO. The vast majority of hyaluronan synthesis occurs in $CD34^-$ orbital fibroblasts. From N. Engl. J. Med, Smith T.J. and Hegedus L., Graves' Disease, 375; 1552-1565. Copyright © (2016) Massachusetts Medical Society. Reprinted with permission.

additional proteins is becoming increasingly recognized (121). Several years after the initial observation of Tsui et al., β-arrestin was found to function as a scaffold for both TSHR and IGF-IR (122), This association potentially accounts for protein:protein crosstalk as underlying the apparent importance of IGF-IR activity in TSHR signaling. Similar associations have been identified in other receptor complexes.

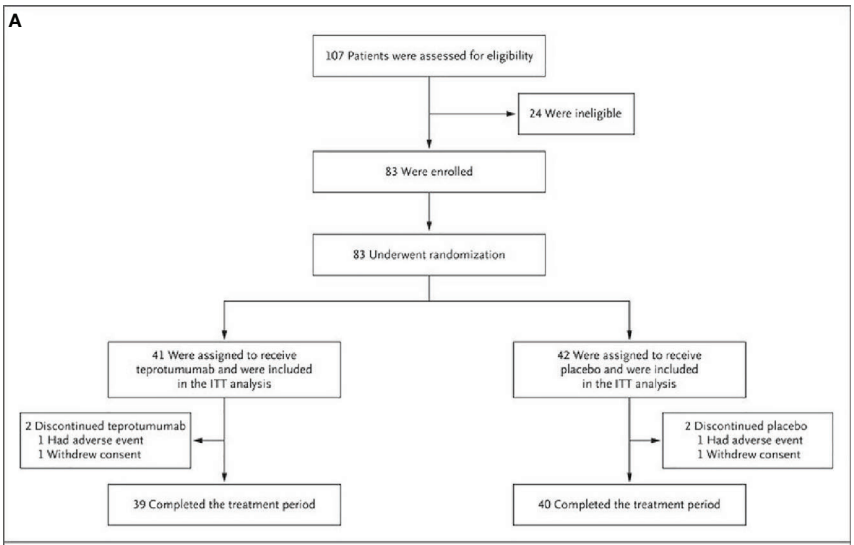
CULMINATION OF EVIDENCE THAT IGF-IR REPRESENTS A CLINICALLY IMPORTANT THERAPEUTIC TARGET IN TAO

To test the central hypothesis that IGF-IR represents not only a critical component in the pathogenesis of TAO, but can also be therapeutically targeted, two placebo-controlled, multicenter

clinical trials of teprotumumab have been conducted (123, 124). The two studies were designed similarly. The drug was developed as an antineoplastic agent and had already been administered to hundreds of patients with a broad range of cancers (107–111). In general, teprotumumumab (AKA R1507) was well-tolerated in those earlier studies, frequently involving fragile patients; however, the drug was not devoid of adverse events which were more severe in younger patients (125). Like other biologicals targeting IGF-IR simultaneously under development at several other pharmaceutical companies, the efficacy of teprotumumab was found inadequate for sustaining its development program by Roche (107, 109–111, 126). The failure of teprotumumab in the cancer space had made it available for potential repurposing in TAO.

The initial trial of teprotumumab in TAO was organized by River Vision Development starting in 2010. This study, a multicenter phase 2 trial, involved the recruitment of 88 patients within 9 months of developing ocular manifestations of GD (123). The trial enrolled patients with moderate to severe, active TAO between 18 and 75 years of age between July 2, 2013 and September 23, 2015. Patients were clinically euthyroid (within 50% above or below the normative range for serum thyroxine and triiodothyronine levels) at baseline. None had undergone orbital radiotherapy or remedial surgery for TAO. Further, none had received > 1 gram of prednisolone or equivalent for the treatment of TAO. For those with systemic exposure, a uniform steroid washout period of 6 weeks was required prior to study enrolment. Each patient was randomized to receive either placebo or teprotumumab in a 1:1 ratio. Doses were administered as 8 infusions, each at 3 week intervals over a 24-week treatment phase. The initial (partial) dose (10 mg/Kg B.W.) was followed by doses of 20 mg/Kg B.W. The primary response endpoint was the aggregate of 1) ≥ 2-point improvement in clinical activity score (CAS) using a 7-point scale AND 2) ≥ 2 mm proptosis reduction. Both responses must have occurred in the more severely affected (study) eye assessed at 24 weeks. This response must have occurred in the absence of a similar worsening in the contralateral (fellow) eye. Secondary responses included reduction from baseline in proptosis ≥ 2 mm, improvement from baseline in CAS ≥ 2 points, (both measured as continuous independent variables), improved subjective diplopia, and improved quality of life using a validated instrument (GO-QOL) (127). The results of the study were as follows: Twenty-nine of 42 patients in the intention to treat cohort receiving teprotumumab achieved the primary response at 24 weeks compared to 9/45 individuals in the placebo group ($p<0.001$). The differences between the two treatment groups emerged at week 6 of treatment ($p<0.001$). These highly significant differences continued over the duration of the treatment phase ($p<0.001$ at all clinical assessments). The time to first response was significantly shorter in those patients receiving teprotumumab. Further, more subjects receiving the active drug achieved a "high" primary response (≥3 mm proptosis reduction AND CAS reduction ≥ 3 points, $p<0.001$). With regard to the secondary endpoints, reduction in CAS and proptosis from baseline was significantly different in the two treatment groups as was improvement in the visual function scale of GO-QOL. Subjective diplopia response rates were significantly higher in those receiving teprotumumab compared with the placebo group. The drug safety profile from that trial was encouraging (123). The most common adverse events were muscle cramping and hyperglycemia, most commonly seen in patients with baseline abnormalities in glycemic control or frank diabetes mellitus. The worsening of glycemic control was easily managed with adjustment of diabetes medication. Further, these changes reverted to baseline following completion of the treatment phase of the trial. At analysis it was discovered that an imbalance of smokers occurred with more representation of tobacco users in the placebo group.

A second, phase 3 trial, was initiated by Horizon Pharmaceuticals (now Horizon Therapeutics) after a licensing agreement was established with River Vision in 2017. Eighty-three patients with moderate to severe TAO who had disease and demographic characteristics very similar to those included in the phase 2 trial were enrolled at performance sites in North America and Europe. This occurred from October 24, 2017 until August 31, 2018 (124) (**Figure 5**). The participating investigators represented a subset of those enrolling patients in the phase 2 trial. Subjects aged 18-80 years, were randomized to receive either teprotumumab (n=41) or placebo (n=42). Like the initial study, this trial was also placebo-controlled, double-masked, and all patients were clinically euthyroid, and were judged to manifest active, moderate to severe TAO. All had eye disease ≤ 9 months in duration. Individuals who had previously undergone orbital surgery, had received tocilizumab or rituximab or who had been treated with high-dose glucocorticoids for TAO (excepting those receiving < 1 gm prednisone equivalent following a 6-week washout period) were excluded. The primary outcome was changed from that in the initial study to a reduction in proptosis ≥ 2 mm in the study eye at week 24. The aggregate of ≥ 2 mm reduction in proptosis and improved CAS ≥ 2 points (the overall response and the primary response in the phase 2 trial) was among the secondary end points. Others included improved CAS > 2 points, reduction in proptosis, both measured as independent variables from baseline, reduced diplopia ≥ 1 Gorman scale grade and mean change in the GO-QOL questionnaire score. Trial results of the phase 3 study were congruent with those observed in phase 2. In addition, the skewed distribution of smokers in the two treatment groups in the earlier trial was successfully corrected. More patients receiving active drug experienced a ≥ 2mm proptosis reduction at week 24 when compared to those receiving placebo (teprotumumab 83% versus placebo controls 10%, $p<0.001$) (**Figure 6**). The necessary number to treat was 1.36. Further, all secondary endpoints were more frequently achieved in patients receiving teprotumumab than those in the placebo group. A few patients underwent orbital imaging at a single performance site at baseline and again at week 24. Those studies were conducted off protocol and revealed that both orbital fat and extraocular muscle volumes were reduced in 6 patients undergoing imaging (**Figure 7**). The phase 3 trial included an extension where all non-responders were offered



B

Efficacy Outcomes	Teprotumumab	Placebo	Difference (95% CI)	P Value
Primary outcome of proptosis response at wk 24				
In the ITT population — % of patients (no./total no.)	83 (34/41)	10 (4/42)	73 (59 to 88)	<0.001
In the per-protocol population — % of patients (no./total no.)	88 (29/33)	12 (4/34)	76 (61 to 92)	<0.001
Secondary outcomes in the ITT population				
Overall response at wk 24 — % of patients (no./total no.)	78 (32/41)	7 (3/42)	71 (56 to 86)	<0.001
Clinical Activity Score of 0 or 1 at wk 24 — % of patients (no./total no.)	59 (24/41)	21 (9/42)	36 (17 to 55)	<0.001
Mean change in proptosis from baseline through wk 24 — mm	−2.82±0.19	−0.54±0.19	−2.28 (−2.77 to −1.80)	<0.001
Diplopia response at wk 24 — % of patients (no./total no. with diplopia at baseline)	68 (19/28)	29 (8/28)	39 (16 to 63)	0.001
Mean change in GO-QOL score from baseline through wk 24	13.79±2.07	4.43±2.10	9.36 (4.08 to 14.64)	<0.001

FIGURE 5 | **(A)** Trial profile describing enrollment, randomization, and follow-up. Random assignment to subgroups receiving intravenous infusions of either teprotumumab (10 mg/Kg B.W. for test infusion and 20 mg/Kg for subsequent infusions) or placebo. Infusions administered every 3 weeks for 21 weeks eight infusions in total. **(B)** Efficacy Endpoints describing the phase 3 trial of teprotumumab in patients with moderate to severe active TAO. CMH weighting was used to estimate the common risk difference and the 95% Confidence Interval (95% CI) of the common risk difference for the primary and secondary endpoints of overall responder, percent with CAS 0 or 1, and diplopia responder; least squares mean difference was calculated for secondary endpoints of change in proptosis from baseline and change in Graves' Orbitopathy quality of life (GO-QOL) questionnaire from baseline using the Mixed-Model Repeated-Measures (MMRM) analysis of covariance (ANCOVA). From N. Engl. J. Med, Douglas R.S, Kahaly G.J., Patel A., Sile E.H.Z., Thompson R. et al. Teprotumumab for the treatment of active thyroid eye disease. 382; 341-352. Copyright © (2020) Massachusetts Medical Society.

teprotumumab as an open label, regardless of whether or not they had received the active drug or placebo during the 24-week treatment phase. A similar fraction of patients responded to the drug as did those in the initial intervention phase. Follow-up data, including those from the extension study of this phase 3 trial, reveal that a majority of both proptosis and diplopia responders at Week 24 maintained their responses (56% and 58%, respectively). The aggregate results from the two trials indicate that clinical improvement of moderate to severe, active TAO is very similar to the best outcomes of the ophthalmic remedial surgeries thus far reported in the literature.

Aggregate safety data from the two trials suggest that teprotumumab was well-tolerated. Several adverse events, most mild to moderate in severity, were identified. Among the most common was hyperglycemia, especially in individuals who were diabetic or glucose intolerant at baseline. Grade 2-3 hyperglycemia developed in some patients with pre-study diabetes mellitus who were receiving teprotumumab. These were managed by increasing diabetes medications. No ketoacidosis occurred in this group of patients. Baseline diabetes medication requirements returned to pre-study levels following the completion of the 24-week treatment phase. A few patients in both treatment arms not having histories of carbohydrate intolerance developed grade 1 hyperglycemia. Other adverse events include hearing abnormalities, muscle cramps, hair loss, dysgeusia, and diarrhea. These uniformly resolved or improved substantially after the treatment phase of the trials had been completed.

TEPROTUMUMAB BECOMES FIRST EVER FDA-APPROVED MEDICAL THERAPY FOR TAO

Based on the results of the two clinical trials conducted for teprotumumab in TAO, the FDA approved its use in that disease in January, 2020 (128). Several important exclusions in the profiles of eligible patients who were enrolled in those studies have resulted in additional questions needing answers during the post-approval period. For instance, all trial subjects must have

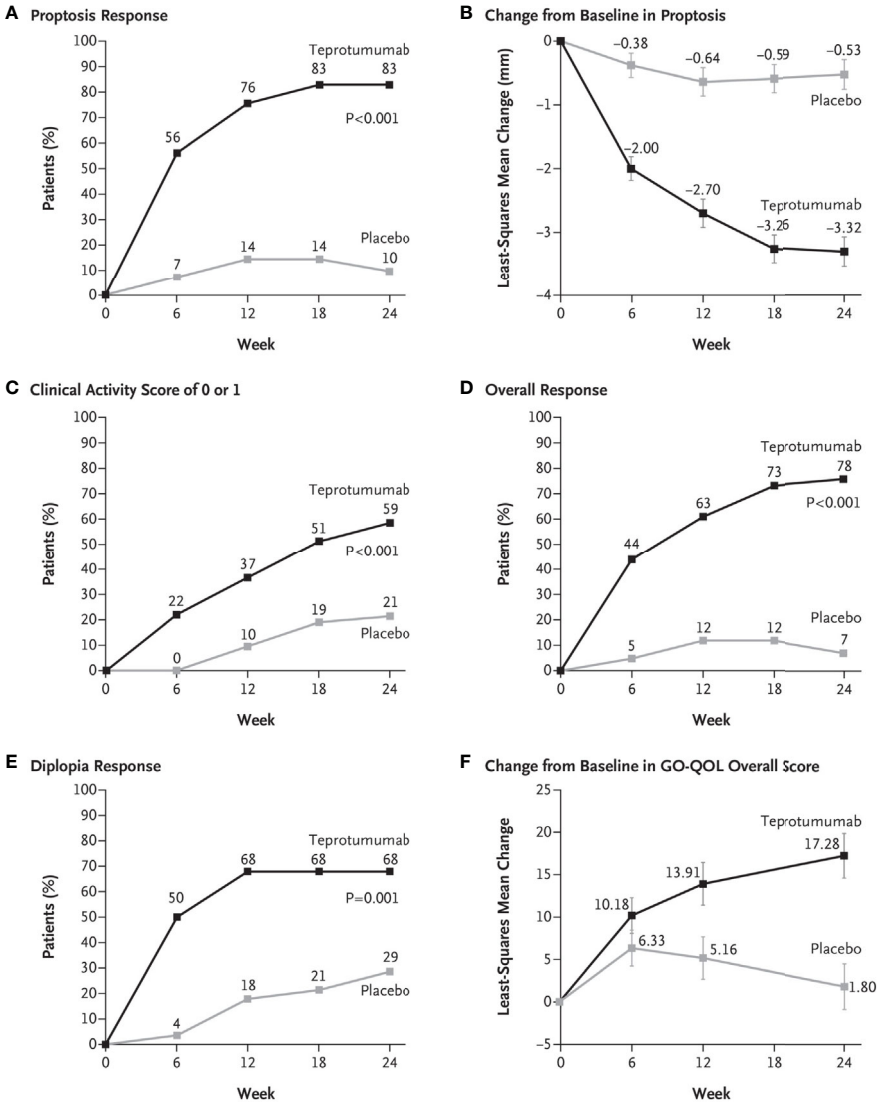


FIGURE 6 | **(A)** Proptosis responder analysis (percent of patients with ≥2 mm reduction in proptosis from baseline in study eye). **(B)** Change from baseline in proptosis (least squares mean ± standard error). **(C)** Percent of patients with clinical activity score (CAS) of 0 or 1 in study eye. **(D)** Overall responder rate (percent of patients with ≥2-point reduction in CAS and ≥2 mm reduction in proptosis from baseline in study eye). **(E)** Diplopia response (percent of patients with improvement of at least 1 grade from baseline). **(F)** Change from baseline in transformed GO-QOL score (least squares mean ± standard error). From N. Engl. J. Med, Douglas R.S, Kahaly G.J., Patel A., Sile E.H.Z., Thompson R. et al. Teprotumumab for the treatment of active thyroid eye disease. 382; 341-352. Copyright © (2020) Massachusetts Medical Society. Reprinted with permission.

manifested TAO for ≤ 9 months prior to their study enrolment, leaving uncertain whether more chronic, less active disease might respond to teprotumumab. Enrollment criteria were stringently skewed toward early disease since *a priori* reasoning suggested that the most active patients were more likely to respond. The relatively short term follow-up of the studies has left the question of long-term therapeutic durability of the drug. The efficacy of the drug in stable disease is being addressed, not only in the formal study currently under development but also by monitoring real-world experience. Potential effectiveness of teprotumumab in vision threatening TAO resulting from compressive optic neuropathy is unknown since patients with signs of impending vision loss were excluded from both trials. Those open questions are also under study. Single case reports are now appearing suggesting that the drug may be beneficial in apparently stable, longer term disease (129) and in optic neuropathy (130, 131). Clearly more extensive clinical experience with the drug will be necessary before teprotumumab can be considered a reliable treatment option in long-standing or vision-threatening TAO. An important goal of this medical

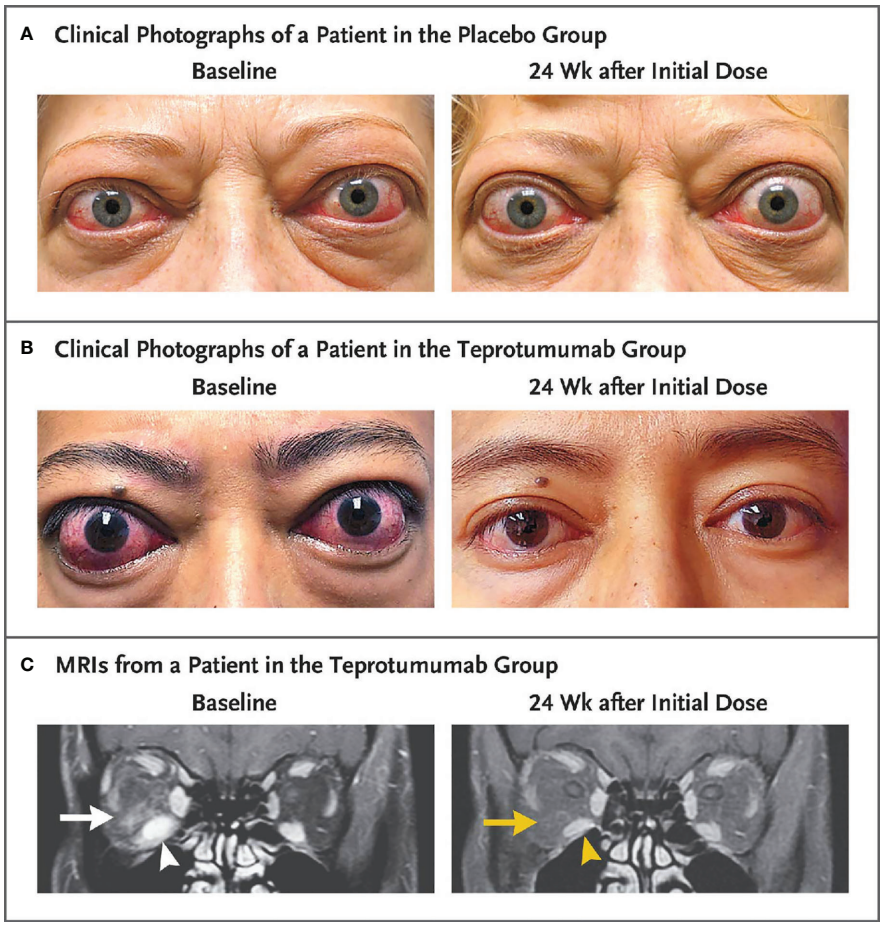


FIGURE 7 | Facial photographic images and MRIs at Baseline and 24 Weeks following treatment with either placebo or teprotumumab in patients enrolled in the Phase 3 trial. Panel **(A)** Clinical photographs of a patient receiving placebo. At baseline, the patient exhibits substantial proptosis (left eye, 29 mm and right eye, 27 mm) as well as multiple inflammatory signs (left eye Clinical Activity Score of 7 and right eye 5). At week 24, considerable proptosis and inflammatory signs remain. **(B)** Images of a teprotumumab-treated patient. Baseline proptosis (both eyes 24 mm), edema, upper and lower eyelid retraction, and multiple inflammatory signs (CAS 5 bilaterally). At week 24, considerable bilateral reductions in proptosis (–5 mm) and CAS (–4 points). **(C)** Coronal, contrast-enhanced, fat-saturated, T1-weighted MRIs in a single patient receiving teprotumumab at baseline and at week 24. Note marked enhancement of the inferior rectus muscle (white arrowhead) and orbital fat (white arrow) as well as inferior rectus muscle enlargement. At week 24, resolved inferior rectus muscle (yellow arrowhead) enhancement and orbital fat (yellow arrow). The muscle volume was reduced by 49% (yellow arrowhead). Proptosis reduction decreased from 23 mm at baseline to 18 mm at week 24. From N. Engl. J. Med, Douglas R.S, Kahaly G.J., Patel A., Sile E.H.Z., Thompson R. et al. Teprotumumab for the treatment of active thyroid eye disease. 382; 341-352. Copyright © (2020) Massachusetts Medical Society. Reprinted with permission.

therapy is to lessen reliance on either routine surgical rehabilitation for chronic disease or urgent surgical intervention in sight-threatening TAO.

ARE DISEASE INDICATIONS BEYOND TAO IN STORE FOR TEPROTUMUMAB?

The IGF-I pathway regulates a vast array of physiological and pathological processes in most mammalian tissues. The effective and well-tolerated treatment with teprotumumab of TAO suggests that this pathway might be beneficially targeted in many other diseases. With regard to GD, pretibial myxedema, a potentially debilitating and disfiguring condition seen in a subset of those with TAO, might also improve with the drug. In fact, a recent case report from Varma et al. suggests that long-standing pretibial myxedema might also respond to teprotumumab (132). Shortly after the original observations concerning anti-IGF-IR antibody involvement in TAO (9, 115), similar findings were reported in rheumatoid arthritis (RA) (133). That study demonstrated that synovial fibroblasts from patients with RA responded to their own IgGs as well as to GD-IgG in inducing IL-16 and RANTES expression. Thus, it remains possible that teprotumumab and other IGF-IR inhibitors might prove effective in in the treatment of RA and allied autoimmune diseases. Investigators have speculated that teprotumumab might prove effective in arresting the deleterious lung tissue remodeling associated with coronavirus-19 (134).

It remains possible that additional indications for the therapeutic targeting of IGF-I and its pathway will become identified as the experience with teprotumumab broadens.

AUTHOR CONTRIBUTIONS

The author confirms being the sole contributor of this work and has approved it for publication.

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Conflict of Interest: The author declares US Patents covering the use of IGF-1 receptor inhibitors in autoimmune disease and consultancy for Horizon Therapeutics Consultant Immunovant.

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MINI REVIEW
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New Mammalian Glycerol-3-Phosphate Phosphatase: Role in β-Cell, Liver and Adipocyte Metabolism

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Cardiometabolic diseases, including type 2 diabetes, obesity and non-alcoholic fatty liver disease, have enormous impact on modern societies worldwide. Excess nutritional burden and nutri-stress together with sedentary lifestyles lead to these diseases. Deranged glucose, fat, and energy metabolism is at the center of nutri-stress, and glycolysis-derived glycerol-3-phosphate (Gro3P) is at the crossroads of these metabolic pathways. Cellular levels of Gro3P can be controlled by its synthesis, utilization or hydrolysis. The belief that mammalian cells do not possess an enzyme that hydrolyzes Gro3P, as in lower organisms and plants, is challenged by our recent work showing the presence of a Gro3P phosphatase (G3PP) in mammalian cells. A previously described phosphoglycolate phosphatase (PGP) in mammalian cells, with no established physiological function, has been shown to actually function as G3PP, under physiological conditions, particularly at elevated glucose levels. In the present review, we summarize evidence that supports the view that G3PP plays an important role in the regulation of gluconeogenesis and fat storage in hepatocytes, glucose stimulated insulin secretion and nutri-stress in β-cells, and lipogenesis in adipocytes. We provide a balanced perspective on the pathophysiological significance of G3PP in mammals with specific reference to cardiometabolic diseases.

Keywords: glycerol-3-phosphate phosphatase, phosphoglycolate phosphatase, glycerolipid/free fatty acid cycle, insulin secretion, nutri-stress, cardiometabolic diseases, type 2 diabetes, obesity

INTRODUCTION

Metabolism of macronutrients including carbohydrates, amino acids and fats converges on the generation of a three-carbon moiety, glycerol, either in the free form or as glycerol-3-phosphate (Gro3P), which forms the backbone of glycerolipids in almost all the species. Glycerolipids, including triglycerides and phospholipids make up a large part of the fat in our body, either as depot fat or as membrane components (1). The glycerol moiety of these glycerolipids is derived from glucose metabolism, dietary fat and *via* glyceroneogenesis, particularly under fasting and high sucrose diet conditions (2–5). It is generally believed that free glycerol is produced and released from cells mainly during the hydrolysis of glycerolipids (6) in higher animals, including humans (4, 5).

Although some early studies indicated the likely presence of a specific enzyme in animal cells that can generate glycerol directly from the hydrolysis of Gro3P, such enzyme was known to be present only in plants and lower organisms (7–9).

The present review focuses on the identification of a specific Gro3P phosphatase (G3PP) in mammalian cells and its most plausible physiological function, specifically addressing its role in controlling glucose, lipid and energy metabolism. We also present a balanced view on the physiological relevance of the various suggested substrates of this enzyme, with a discussion on the importance of G3PP in preventing glucotoxicity/nutri-stress and the control of glucose stimulated insulin secretion (GSIS) in β-cells, in the regulation of lipogenesis in liver and adipose tissue, and in slowing down hepatic glucose production. Finally, we address the regulation of G3PP and its role in cardiometabolic diseases.

EARLY EVIDENCE FOR THE PRESENCE OF Gro3P PHOSPHATASE IN ANIMAL CELLS

Even though lipolysis is considered to be the main source of free glycerol in mammalian cells, few earlier studies suggested that glycerol may be formed *via* non-lipolytic pathways during glycolysis. Thus, as much as 15 to 20% of plasma glycerol was thought to be derived from non-adipose tissue sources including perirenal fat or skeletal muscle lipolysis or possibly by the hydrolysis of Gro3P in long-term fasting human subjects, on the basis of stable isotope labeling (10) and in rats and monkeys (11), but no specific enzyme was described for this process. Similarly, it was noticed that significant levels of glycerol are derived directly from glucose in ischemic rat brain (12) and ischemic cardiac tissue (13). In addition, in the fish *Osmerus mordax* (Rainbow smelt) high concentrations of glycerol are generated as a cryoprotective mechanism directly from glucose, glycogen and amino acids and not from lipolysis, probably involving a glycerol-3-phosphatase, even though no specific enzyme was identified (14, 15).

Lipolysis, measured as glycerol release, was implicated in the regulation of GSIS by pancreatic β-cells (16, 17), and it was assumed that glycerolipid hydrolysis is the only source of free glycerol in mammalian cells (4, 5). However, despite the loss of adipose triglyceride lipase (ATGL), which catalyzes the first step of triglyceride hydrolysis (18, 19), glycerol is still produced in significant quantities in the pancreatic islets from whole-body ATGL-KO (20) and β-cell specifc ATGL-KO (21) mice, at high concentrations of glucose, suggesting a non-lipolytic origin of glycerol. We reported that orlistat, a powerful pan-lipase inhibitor, totally inhibits GSIS as well as lipolysis, measured as free fatty acid (FFA) release, in pancreatic β-cells (22), but not glycerol release at elevated glucose concentrations (>10 mM), suggesting that pancreatic β-cells can produce glycerol from glucose, *via* non-lipolytic pathways (22). This observation led us to conduct a thorough search using BLAST analysis for mammalian proteins that are homologous to the known microbial (yeast and bacteria) glycerol-3-phosphate phosphatase enzymes (8, 23), and to the identification of previously described phosphoglycolate phosphatase (PGP), as the potential mammalian G3PP (22).

PGP is an evolutionarily conserved enzyme that can hydrolyze various phospho-metabolites, under normal physiological as well as stress conditions (24, 25). In lower organisms and plants, PGP and G3PP are products of separate genes and hydrolyze 2-phosphoglycolate and Gro3P, respectively. However, in mammalian cells, a single gene (*PGP*) product, i.e., G3PP, appears to catalyze the hydrolysis of Gro3P under normal physiological conditions, and also 2-phosphoglycolate and other phospho-substrates produced in stress conditions, as reviewed here.

MAMMALIAN PGP: NAMES, SUBSTRATE SPECIFICITY AND ENZYME KINETICS

In mammals, *PGP* gene product has been described with different names and ascribed different functions. This protein was first described in human red blood cells (RBC) as phosphoglycolate phosphatase, based on the sequence similarity to the plant and bacterial PGP enzymes, with similar substrate specificity, i.e., high activity towards 2-phosphoglycolate (26, 27). Later, this protein was called as aspartate-based, ubiquitous, Mg^{2+}-dependent phosphatase (AUM), showing hydrolytic activity with phosphotyrosine containing peptides, with implications in the modulation of epidermal growth factor receptor (EGFR) signaling (25, 28–30). However, considering the extremely low cellular levels of 2-phosphoglycolate under physiological conditions and as the catalytic efficiency of purified PGP towards the phosphotyrosine peptides was ∼1,000-fold less than that of classical tyrosine phosphatases like PTP1B, TCPTP, or SHP1, the proposed physiological role of mammalian PGP in the hydrolysis of either 2-phosphoglycolate or phosphotyrosine residues is questionable.

The more likely function of RBC PGP was suggested to be the hydrolysis of 2-phospholactate, formed as a by-product of pyruvate kinase (31). More recently, PGP was shown to dephosphorylate 4-phosphoerythronate and 2-phospholactate, toxic by-products of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and pyruvate kinase, respectively. The kinetic parameters and the available intracellular substrate concentrations likely provide a clue about the more appropriate physiological substrate of G3PP in mammalian cells. The reported Km of the purified mouse PGP/G3PP enzyme for 2-phosphoglycolate is 766μM; for 4-phosphoerythronate it is 247μM and for 2-phospholactate it is 174 μM (32). However, it was reported by Collard et al. (32), that in wildtype HCT116 cells 4-phosphoerythronate and also 2-phosphoglycolate are at below detection limits (<2-3 μM). Even if one assumes that normally expressed PGP/G3PP is maintaining the concentration of these metabolites at low level, the enzyme should be able to act on them. This is kinetically not favourable for PGP/G3PP expressed at normal level as these metabolites are

present at nearly 100-300 fold lower concentration in wildtype cells than their respective Km values for PGP/G3PP. In addition, the catalytic rate of GAPDH to produce 4-phosphoerythronate is ~3500 fold lower than its normal function and similarly the formation of 2-phospholactate by pyruvate kinase is several orders of magnitude lower (32). Therefore, the possibility that 2-phosphoglycolate, 4-phosphoerythronate and 2-phospholactate serve as 'physiological' substrates for PGP/G3PP is remote, unlike glycerol-3-phosphate, which is present at above its Km concentration in cells normally, and is readily available as substrate for G3PP. However, as 4-phosphoerythronate and 2-phospholactate accumulate when PGP expression is suppressed (32–34), the possibility that PGP/G3PP may act on these substrates with very low efficiency due to their extremely low intracellular concentrations, cannot be discounted. Even though purified recombinant mouse PGP was found to show high catalytic efficiency with 2-phosphoglycolate, 4-phosphoerythronate, and 2-phospholactate, and relatively lower activity with Gro3P (22, 32), it also shows very high activity with the non-physiological substrate p-nitrophenol phosphate (30). Studies from our laboratory demonstrated that PGP actually functions as a G3PPin many cell types, hydrolysing Gro3P, normally produced in all the cells and available in sufficient concentrations (1 to 5 mM; Km, ~1 mM) to serve as a substrate for this enzyme (22, 24). Hence, the name G3PP is more appropriate for the *PGP* gene product, and is now accepted by most protein databases (Uniprot, NCBI Protein, PDB, etc.). Thus, the observed catalytic efficiencies of purified G3PP/PGP *in vitro* may not have much relevance physiologically, as it is the availability of substrate that dictates the activity of a given enzyme. Therefore, on the basis of available evidence, we suggest that the protein encoded by *PGP* in mammalian cells is poised to act on Gro3P, as its normal physiological function, but may assume a detoxification role to hydrolyze toxic phospho-metabolites, such as 2-phosphoglycolate, 4-phosphoerythronate or 2-phospholactate, which may buildup in the cells under stress conditions (22, 24, 25, 32). Additional studies, particularly *in vivo*, are needed to ascertain this possibility.

CONTROL OF GLUCOSE, LIPID AND ENERGY METABOLISM BY G3PP/PGP

Glycolytically derived Gro3P is at the crossroads of glucose, lipid and energy metabolism in all cells, as it is the starting substrate for glycerolipid synthesis and also participates in the electron shuttle to transfer cytosolic reducing equivalents to mitochondrial electron transport chain for ATP synthesis (22, 24). Hydrolytic control of Gro3P in the cells by G3PP adds another level of metabolic regulation in animal cells that was not recognized previously, as the existence of G3PP is only recently established in mammalian cells. Significant evidence accumulated in the last five years suggests an important role for G3PP/PGP in the regulation of glucose and lipid metabolism in pancreatic islets, hepatocytes and adipocytes (**Figure 1**), which is summarized below.

Pancreatic Islets

Cellular levels of Gro3P in INS-1(832/13) β-cells (35, 36) under physiological but elevated glucose concentrations (~10-16 mM) are sufficient to serve as the substrate for G3PP catalysis. In most cells, dihydroxyacetone-3-phosphate (DHAP) formed during glycolysis is partly converted to Gro3P. Pancreatic β-cells are not equipped to phosphorylate glycerol to Gro3P, as these cells express low levels of glycerol kinase (18, 37, 38). Modulation of cellular Gro3P levels by altering G3PP expression can impact the associated metabolic pathways. Thus, suppression of G3PP expression in rat pancreatic islets and INS-1(832/13) β-cells was found to lower glycerol production from glucose, in association with marked elevation of Gro3P, glycerolipid synthesis, glycolysis and glucose driven respiration, whereas overexpression of human G3PP in these cells produced opposite changes (22). In agreement with its anticipated role in the Gro3P shuttle to transfer electrons from the cytosol to mitochondria, the expression level of G3PP/PGP in rat pancreatic islets is inversely related to glucose-driven ATP synthesis. Interestingly, all the listed effects were apparent at elevated glucose (10-16 mM) with minimal changes at low (2-4 mM glucose). Thus, under elevated glucose concentration conditions, G3PP is able to control both glucose and lipid metabolism in β-cells (22).

Hepatocytes

As in the case of pancreatic β-cells, Gro3P level in isolated rat hepatocytes (22) incubated at 5 or 25 mM glucose concentration was found to be sufficient to serve as substrate for G3PP. In hepatocytes, Gro3P can be formed from DHAP during glycolysis or by the phosphorylation of glycerol by glycerol kinase expressed in these cells (4). Similar to what was noticed in β-cells, suppression of G3PP expression in rat hepatocytes decreased glycerol production from glucose, increased intracellular Gro3P, glycerolipids, glycolysis, glucose driven respiration, and gluconeogenesis, whereas opposite changes were seen with overexpression of human G3PP in these cells (22). Interestingly, increased G3PP activity in hepatocytes also led to elevated fatty acid β-oxidation even at high glucose concentrations, which normally lower fatty acid oxidation (22). Importantly and as noted in ß-cells, these effects following changes in the expression levels of G3PP were prominent at high (25 mM) but less marked at low (5 mM) physiological basal glucose.

Adipocytes

Adipocytes, from both white and brown adipose depots, synthesize and store large amounts of triglycerides and hydrolyze the same to release glycerol and FFA in response to hormonal and other cues. It is generally accepted that most of the glycerol released from adipocytes is of lipolytic origin. White adipocytes are known to conduct anaerobic glycolysis and produce lactate in large amounts from glucose (39, 40). It has recently been shown that in the presence of either glucose or fructose, mature differentiated 3T3-L1 adipocytes release copious amounts of lactate and glycerol, that cannot be accounted for

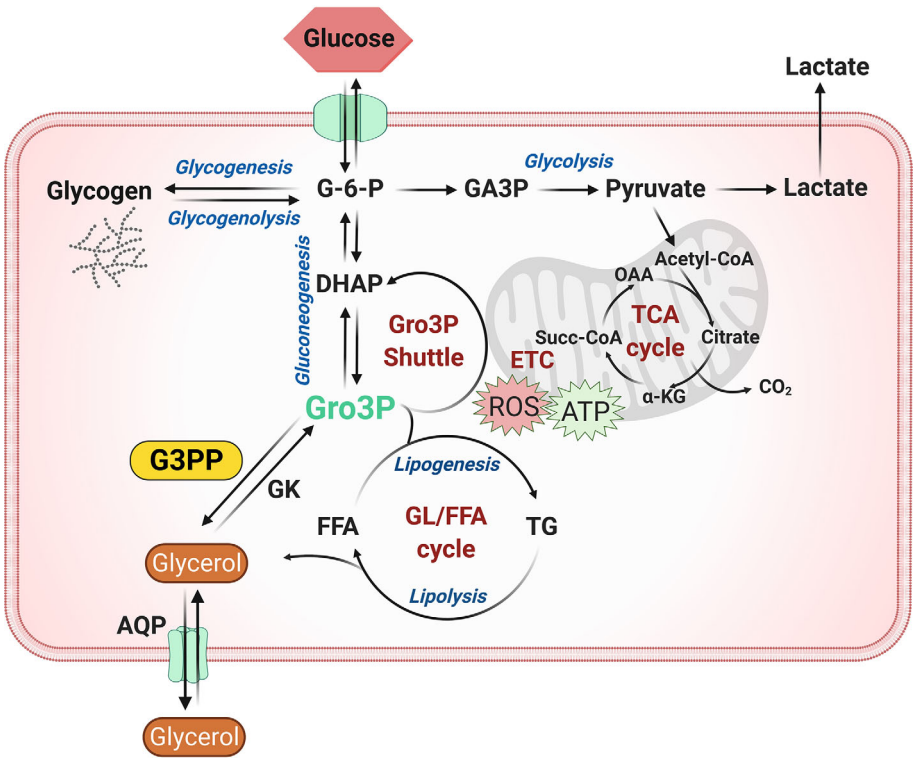

FIGURE 1 | Role of G3PP in intermediary metabolism. Glycerol-3-phosphate (Gro3P) is a central metabolite at the intersection of four important pathways in most cells: 1) glycolysis; 2) glycerolipid synthesis and the glycerolipid/free fatty acid (GL/FFA) cycle; 3) gluconeogenesis (liver and kidney) and 4) energy metabolism *via* electron transfer shuttle to mitochondria. Gro3P can be produced from glucose *via* glycolysis or from lipolysis-derived glycerol by glycerol kinase. The cellular levels and availability of Gro3P are regulated by Gro3P phosphatase (G3PP), which dephosphorylates Gro3P to form glycerol. Under conditions of excess glucose supply, a buildup of Gro3P in the cell may cause an overflow of glucose carbons into glycolysis, lipid synthesis and electron transfer, leading to accumulation of fat and also elevated production of reactive oxygen species (ROS) in mitochondria. G3PP may act as a detoxification enzyme to protect the cells from glucotoxicity, excess fat synthesis and storage and oxidative damage by hydrolyzing Gro3P to glycerol, a less harmful molecule, that exits the cell through aquaglyceroporins. α-KG, α-ketoglutarate; AQP, aquaglyceroporin; DHAP, dihydroxyacetone phosphate; ETC, electron transport chain; FFA, free fatty acid; G-6-P, glucose-6-phosphate; G3PP, glycerol-3-phosphate phosphatase; GA3P, glyceraldehyde-3-phosphate; GK, glycerol kinase; GL/FFA cycle, glycerolipid/free fatty acid cycle; Gro3P, glycerol-3-phosphate; OAA, oxaloacetate; ROS, reactive oxygen species; Succ-CoA, succinyl-CoA; TCA cycle, tricarboxylic acid cycle; TG, triglycerides.

their triglyceride stores and lipolysis, suggesting the presence of a mechanism for glycerol production from Gro3P, even though an enzyme for such reaction was not identified in adipocytes (41). In the later studies, these authors showed that G3PP/PGP is expressed in the primary rat adipocytes and contributes to glycerol production and that its expression is much higher than that of glycerol kinase, thereby ensuring removal of glycerol produced from Gro3P *via* aquaporin-7 in these cells (42). The same group studied glycerol metabolism at various glucose concentrations (3, 5, 7, or 14 mM) with and without insulin. When medium glucose was at basal level, most glycerol came from lipolysis, but when glucose was high, the release of glycerol *via* breakup of Gro3P was predominant (43). Under conditions of excess glucose supply, adipocytes take-up glucose and metabolize *via* glycolysis to Gro3P. But if not enough FFA is simultaneously available, the produced Gro3P is hydrolyzed to export glycerol *via* aquaporin-7. Adipocytes do not have a significant level of *de novo* lipogenesis machinery (44) unlike hepatocytes, so they cannot generate acyl-CoA (*de novo*) to esterify Gro3P to triglyceride. It is necessary to hydrolyze Gro3P, as it can be toxic when present in excess and generate toxic reactive oxygen species or even inhibit glycolysis, unless removed as glycerol. Thus, G3PP appears to play an important role in regulating glycolysis and glycerolipid metabolism in adipocytes at elevated glucose levels primarily (42, 45).

Evidence From Embryonic Cells With Inactive Mutant G3PP

Regulation of glycerolipid metabolism by G3PP/PGP has recently been shown to be essential during development. Thus, in E8.5 mouse embryos expressing catalytically inactive G3PP/PGP ($PGP^{D34N/D34N}$) elevated diacylglycerols and triglycerides were noticed, indicating increased lipogenesis from accumulating Gro3P in these knock-in embryos (46). As the $PGP^{D34N/D34N}$ knock-in mouse embryos do not survive beyond E11.5, it appears that G3PP/PGP is essential during development, possibly due to its role in glucose and glycerolipid metabolism (46).

Overall the data indicate that G3PP is a glucose concentration-dependent enzyme at the nexus of glucose and

lipid metabolism that plays a role in glucose, energy and lipid metabolism primarily at elevated concentrations of glucose and cellular Gro3P.

PHYSIOLOGICAL ROLES OF G3PP/PGP

As G3PP/PGP plays a role in glucose, lipid, and energy metabolism (**Figure 1**), this enzyme is likely implicated in various physiological and pathological processes related to nutrient excess. It was earlier suggested that G3PP/PGP plays a role in the removal of 2-phosphoglycolate generated during DNA repair processes (46), but this is questionable as in mammalian cells, 2-phosphoglycolate levels are either very low under normal conditions (22) or below detectable levels, even under stress conditions (46, 47). Similarly, the 2-phosphoglycolate hydrolytic ability of G3PP/PGP has been implicated (48) in the control of the bifunctional glycolytic enzyme 1,3-diphosphoglycerate mutase/2,3-diphosphoglycerate (DPG) phosphatase, which regulates hemoglobin binding to oxygen in the RBC. DPG phosphatase hydrolyzes 2,3-diphosphoglycerate, which is known to lower the affinity of hemoglobin for oxygen and thus promote the release of oxygen from oxyhemoglobin (49). The proposed role for PGP in RBC in this process is to control the levels of 2-phosphoglycolate, which potently activates the DPG phosphatase (48). However, the concentration of 2-phosphoglycolate in RBC (~4 μM) is nearly 200 fold less than its Km for G3PP/PGP (26), and at such low concentration, 2-phosphoglycolate may not be available as a substrate for G3PP/PGP in RBC (50), questioning this role of G3PP/PGP in RBC.

Detoxification of Metabolic By-Products of Enzymatic Reactions

Toxic metabolic side products are generated in cells due to mutations in enzymes, changes in metabolic flux or due to the enzyme reaction with alternative substrates at low rates as the specificity of many enzymes is not absolute. Thus, it was shown that GAPDH can also catalyze the conversion of the pentose phosphate pathway metabolite erythrose-4-phosphate to 4-phosphoerythronate (51), though at a much lower rate (32). Similarly, pyruvate kinase was shown to phosphorylate lactate to 2-phospholactate (52). 4-Phosphoerythronate and 2-phospholactate were shown to inhibit 6-phosphogluconate dehydrogenase and phosphofructokinase-2, respectively (32). As 4-phosphoerythronate and 2-phospholactate accumulate only in *PGP* deleted HCT116 cells (32) and malarial parasite Plasmodium (33, 53), it was suggested that G3PP/PGP can act like a 'metabolic repair enzyme' and hydrolyze these two toxic metabolites. However, as the concentrations of these metabolites in wild type cells with normal expression of G3PP/PGP are much lower than their corresponding Km for purified mouse G3PP/PGP, it is plausible that only under stress conditions when these metabolites accumulate significantly, G3PP/PGP may hydrolyze them and act as a detoxification enzyme of various phospho-metabolites, besides its action on Gro3P.

Adipose Tissue and Liver Metabolism

Increased adiposity leads to obesity and is an important risk factor for type 2 diabetes (T2D) and other cardiometabolic diseases. Synthesis and storage of triglycerides is critical in the expansion of adipose tissue, and recent studies demonstrated a role for G3PP/PGP in the control of lipogenesis by the hydrolysis of Gro3P in primary white adipocytes (42). We reported that the expression level of G3PP in mouse visceral, subcutaneous and brown fat is nutritionally regulated (fed *vs.* fasting state and low *vs.* high-fat diet (20). The precise role of G3PP in various adipose depots and whole-body energy homeostasis remains to be defined.

Liver plays key role in the synthesis and secretion of lipoproteins and gluconeogenesis and these processes are dependent on the availability of Gro3P in hepatocytes. We reported that in isolated rat primary hepatocytes incubated at elevated glucose (25 mM), the levels of Gro3P and glycolysis as measured by lactate release and glycerolipid synthesis as indicated by diacylglycerol and triglyceride levels, were all higher when G3PP/PGP expression was suppressed using RNAi. Opposite effects were noted in hepatocytes with human G3PP overexpression (22). All these effects due to variations in the expression level of G3PP were glucose concentration dependent as they were much less at low 5 mM glucose. Interestingly, the toxic metabolite 2-phosphoglycolate was found at very low levels in hepatocytes but was measurable in these cells at 5 and 25 mM glucose, but its concentration did not change upon RNAi-suppression of G3PP, indicating that G3PP has little or no role in modulating 2-phosphoglycolate levels under normal conditions. In addition, we noticed that adenoviral vector mediated overexpression of G3PP in rat liver, led to decreased gluconeogenesis from glycerol and also enhanced secretion of high density lipoprotein, *in vivo* (22). Thus, G3PP/PGP in adipocytes and hepatocytes likely has a significant role in the regulation of their physiological functions.

Influence of Nutrients, Hormones and Dietary State

Considering that G3PP/PGP is an important metabolic enzyme that regulates glucose and lipid metabolism and nutri-stress, expression of this enzyme is likely regulated by multiple factors including nutritional status. Thus, we observed that overnight fasting in mice lowered G3PP expression at mRNA and protein levels in brown adipose tissue while its expression increased in visceral white adipose tissue (22). It was suggested that such depot specific inverse change in G3PP expression ensures supply of lipolysis-derived glycerol upon fasting, from white adipose to liver and kidney for gluconeogenesis, rather than being used for re-esterification. However, feeding of 60% high fat diet to mice led to elevated G3PP expression in brown adipose but decreased in white adipose depots, so that Gro3P is made available for the esterification of excess FFA for storage as triglycerides. High fat diet increased G3PP mRNA expression in heart and also testis also, probably as a defense mechanism to prevent build-up of fat in these organs, which otherwise may have pathological consequences (22). Expression of G3PP was found to be

unaltered by increasing glucose concentration (7 to 14 mM) in differentiated 3T3-L1 adipocytes (42) and also in primary visceral white adipocytes from rats (45) and exogenous insulin was also without any effect on G3PP expression at various glucose concentrations (43). Conversely, the expression of three G3PP/PGP homologues (K09H11.7; C53A3.2; F44E7.2) in *C. elegans* was found to be upregulated upon exposure to high glucose concentration, accompanied by elevated glycerol production (54). Interestingly, it was described that G3PP mRNA expression in female rat perigonadal white adipose tissue is much higher than in males, but if this corresponds with elevated G3PP activity in female adipose tissue is not known (45). It is yet to be determined if sex hormones influence the expression of G3PP in any tissue, even though testis as a whole organ is found to have the most expression of G3PP among various body tissues (22). Thus, the expression of G3PP appears to be controlled by nutritional status in a tissue and organism dependent manner, which adds another level of regulation of energy metabolism, and further work is needed to fully understand the hormonal regulation of this enzyme's expression.

Insulin Secretion

Insulin secretion in pancreatic β-cells is driven by the intracellular metabolism of glucose and other fuels, in particular by the so-called metabolic coupling factors derived from glycolysis, mitochondrial and lipid metabolism, such as the ATP/ADP ratio and monoacylglycerol (38). Our earlier studies showed that changes in the expression level of G3PP/PGP in β-cells alters glucose and lipid metabolism and the glycerolipid/FFA cycle in ß-cells, which are known to produce coupling factors for glucose induced insulin secretion. Thus, downregulation of G3PP in INS1(832/13) β-cells and rat islets was found to enhance GSIS in association with elevated production of ATP and glycerolipids, such as diacylglycerols, which are known to act as signals to promote insulin secretion, while G3PP over-expression led to slowed down GSIS response accompanied by reduced ATP and glycerolipid production (22). Thus, G3PP/PGP is a new player in ß-cell metabolic signaling and insulin secretion.

G3PP IN HUMAN DISEASES

Considering that Gro3P occupies a central position in glucose, lipid and energy metabolism, enzymes that generate and use this metabolite are likely to have important regulatory roles and any defects in these enzymes can have pathological consequences. Thus, glycerol kinase deficiency in humans is an X-chromosome linked disease and is reported to be associated with abnormalities in lipid metabolism, susceptibility to diabetes, metabolic acidosis, etc. (55). Glycerol kinase knockout mice were found to have severely disturbed fat metabolism, hyperglycerolemia and postnatal growth retardation and death by 3-4 days of age (56). Mitochondrial glycerol-3-phosphate dehydrogenase deletion in livers was found to lead to hepatic steatosis due to enhanced lipogenesis in mice (57) and a rare case of genetic deficiency of this enzyme was found to be associated with mental retardation (58). In addition, cytosolic glycerol-3-phosphate dehydrogenase KO mice were found to have elevated body weight, increased compensatory gluconeogenesis from alanine, increased fatty acid oxidation in skeletal muscle, but reduced gluconeogenesis from glycerol (59). Mutations in cytosolic glycerol-3-phosphate dehydrogenase were shown to be associated with transient hypertriglyceridemia in children (60). Similarly, Gro3P acyltransferases, which esterify Gro3P to lysophosphatidic acid, were implicated in obesity and hepatic steatosis and insulin resistance (61). Collectively, it appears that all the enzymes that are directly involved in the synthesis and utilization of Gro3P play critical roles in the whole body metabolism and their compromised activity can have pathological consequences.

Despite the fact that *PGP* gene is essential for mammalian embryogenesis and development (46), there are no studies showing a direct "cause and effect" relationship between G3PP/PGP and the pathogenesis of human diseases. However, considering the role of G3PP/PGP in the regulation of glucose, lipid and energy metabolism, it may have a protective function against cardiometabolic diseases due to excess nutrient fuel supply.

Nutri-Stress and Glucolipotoxicity

Excess supply of nutrient fuels is the primary cause of cellular dysfunction in various organs, including pancreatic islets, heart muscle and liver, that eventually causes cardiometabolic diseases (62, 63), and we have recently termed this as 'nutri-stress' (64). Indeed, toxicity manifested as apoptosis in tissues due to the combined presence of excess glucose and fatty acids is called glucolipotoxicity (65–67). We have proposed that the diversion of glucose carbons to glycerol is deployed as a defense mechanism by pancreatic β-cells to evade toxic effects of excess glucose, as β-cells cannot re-use glycerol, which leaves the cell (35). Thus, the expression level of G3PP, which is responsible for the direct conversion of glucose carbons to glycerol, was found to be inversely related to glucotoxicity and glucolipotoxicity in β-cells (22). Elevated G3PP/PGP expression in β-cells has also been shown to slow-down the β-cell response to secrete increased levels of insulin in the presence of high concentrations of glucose (22), an effect that is anticipated to prevent hyperinsulinemia as well as β-cell exhaustion and dysfunction. Thus, the emerging view is that G3PP/PGP plays an important role in the β-cells not only to regulate glucose and lipid metabolism but also as a glucose excess security valve to alleviate metabolic stress in these cells and to prevent hyperinsulinemia, which leads to insulin resistance, obesity and T2D in the face of excess nutrient supply. In addition to β-cells, G3PP/PGP expression level is also relevant in preventing excess synthesis and storage of fat in the liver and thus hepatic steatosis, and also in slowing down hepatic glucose production, a significant problem in T2D (22).

Cardiometabolic Disorders

Chronic nutri-stress is the root cause of cardiometabolic disorders such as metabolic syndrome, T2D, obesity, atherosclerosis, and non-alcoholic fatty liver disease (64, 68). Nutri-stress promotes lipogenesis with associated accumulation of fat in adipose tissue and other tissues (18, 35, 37), oxidative

damage due to excessive ROS production *via* mitochondrial metabolism and leads to aggravated local and systemic inflammation (69). The increased cellular level of Gro3P derived from glucose metabolism is likely at the center of many of these pathogenic pathways. Therefore, curtailing the excess buildup of Gro3P by G3PP likely alleviates the metabolic stress and fuel surfeit toxicity to the cells. Thus, *in vitro* and *in vivo* studies in rats with G3PP overexpression suggested several beneficial effects of elevated G3PP activity in the liver and pancreatic islets against metabolic complications due to excess nutrients (22).

CONCLUSIONS AND PERSPECTIVE

Despite the earlier belief that mammals do not possess a G3PP like enzyme, there is an overwhelming evidence now, as summarized in this review, that indeed such an enzyme exists in mammalian cells and plays a major role in the regulation of metabolic and physiological processes, disturbances of which could lead to cardiometabolic diseases. Activity of G3PP in pancreatic β-cells appears to be an important player in the regulation of GSIS and also in preventing nutri-stress. In other cell types, including hepatocytes and adipocytes, G3PP seems to control glucose and lipid metabolism and excessive fat buildup. There are still several knowledge gaps concerning the role of G3PP in other organs, including muscle and heart, whose functions are affected in cardiometabolic diseases. In addition, the proposed role of this enzyme as a metabolic repair enzyme needs to be further studied for its relevance in mammalian cells under normal physiological conditions. Several of these knowledge gaps can be addressed using appropriate animal models, including tissue-specific knockout and graded overexpression models. More detailed genetic studies are also needed focusing on the association of the *PGP* gene and its variations with cardiometabolic diseases. Considering that elevated activity of G3PP protects against nutri-stress, excess fat buildup, hyperinsulinemia, hepatic glucose production and fatty liver disease, this enzyme can be a potential therapeutic target for cardiometabolic diseases.

AUTHOR CONTRIBUTIONS

EP and AA-M contributed equally to this review. MP and SM conceptualized the topic and the outline for the review. EP, AA-M, and M-LP collected literature information and compiled it. EP and MP prepared the Figure. SM and MP wrote the review and edited it. RA and FA-M contributed by analytical reading of the review and valuable suggestions to improve. All authors contributed to the article and approved the submitted version.

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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.

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Secretory Phospholipase A2s in Insulin Resistance and Metabolism

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The phospholipases A_2 (PLA_2) superfamily encompasses enzymes commonly found in mammalian tissues and snake venom. Many of these enzymes have unique tissue distribution, function, and substrate specificity suggesting distinct biological roles. In the past, much of the research on secretory PLA_2s has analyzed their roles in inflammation, anti-bacterial actions, and atherosclerosis. In recent studies utilizing a variety of mouse models, pancreatic islets, and clinical trials, a role for many of these enzymes in the control of metabolism and insulin action has been revealed. In this review, this research, and the unique contributions of the PLA_2 enzymes in insulin resistance and metabolism.

Keywords: phospholipase A2, metabolism and obesity, Type 2 diabetes, insulin resistance, glucose homeostasis, lipid metabolism

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INTRODUCTION

Metabolic syndrome constitutes an array of pathophysiologies, including obesity, glucose intolerance, and dyslipidemia. The most common feature between these pathophysiologies is insulin resistance, a condition in which cells fail to respond normally to insulin. One factor associated with insulin resistance is triacylglycerol accumulation in muscle, liver, and fat (1, 2). While triacylglycerols are a marker for insulin resistance (3), they may not be causal. Instead, cellular lipids with signaling roles (diacylglycerols, fatty acids, phospholipids, etc.) may fill this role, as accumulation of a variety of lipid species can cause insulin resistance (4–6).

One group of enzymes that produce these lipids are secretory phospholipases A_2 ($sPLA_2$s), a protein family found in mammalian tissues and snake venom which hydrolyze glycerophospholipid *sn-2* ester bonds, generating a non-esterified free fatty acid and a lysophospholipid. Secretory phospholipases A_2s are low molecular weight enzymes (~14 kDa), most of which require millimolar amounts of Ca^{2+} to function. Twelve $sPLA_2$ isoforms have been identified thus far, of which ten are catalytically active (IB, IIA, IIC, IID, IIE, IIF, III, V, X, XIIA), and two are dormant (XIIB and otoconin-90) (7, 8). These enzymes have varied expression patterns and substrate preferences, signifying diversity in their biological roles. Over the past couple decades of research, the majority of studies concerning $sPLA_2$s dealt with their roles in cardiovascular disease, inflammation, antimicrobial actions, and membrane remodeling. However, a discussion on how sPLA2s may regulate or impact glucose metabolism, insulin signaling, and metabolism is lacking. Recent research has identified that at least 7 of these $sPLA_2$s modulate glucose metabolism, presumably by generation of fatty acids or lipoproteins that influence lipid metabolism and mobilization, alterations in fatty acid oxidation, or other mechanisms involved in insulin signaling and obesity (**Table 1**).

TABLE 1 | Metabolic roles of sPLA2 isoforms.

$sPLA_2$ Isoforms	Primary Localization	Metabolic Implications	Reference
PLA2G1B	Pancreas, lung	Promotes weight gain; increases TG and cholesterol levels through elevated LPC intestinal absorption	(9–16)
PLA2G2A	Platelets, liver, leukocytes, paneth cells, adipose tissue	Controversial; promotes weight gain, insulin resistance in rats. Improves metabolic parameters in mice.	(17–21)
PLA2G2D	Lymph tissue dendritic cells	Undocumented; May be metabolically beneficial due to release of anti-inflammatory FAs/ lipid mediators	(22–25)
PLA2G2E	Adipose	Controversial; *Pla2g2e*$^{-/-}$ male mice display blunted lipolysis and elevated TG storage. Other experiments using *Pla2g2e*$^{-/-}$ female mice found reduced lipid accumulation.	(26–29)
PLA2G5	Adipose, bronchial epithelial cells, hepatocytes, islets, macrophages, cardiomyocytes	Protective of diet-induced obesity and insulin resistance; pushes adipose tissue macrophages from M1➔M2 state.	(26)
PLA2G10	Lung, adrenal gland, brain, heart, adipose	Protective of diet-induced obesity. Improves TG clearance in adipose and suppresses glucocorticoid production in adrenal cells	(27, 30–34)
PLA2G12B	Liver, small intestine, kidneys	Strong regulation over hepatic lipoprotein packaging and VLDL secretion; expression protects from hepatosteatosis	(8, 35–45)

Tissue specific expression patterns and metabolic roles for the secretory phospholipases A2.

PLA2G1B

The metabolic role of PLA2G1B has been clarified with *Pla2g1b* knockout (*Pla2g1b*$^{-/-}$) mice or pancreatic acinar cell-specific PLA2G1B overexpression. PLA2G1B is mainly expressed in pancreatic acinar cells and the lungs, and only displays its enzymatic activity after feeding, as this causes the enzyme's release into the pancreatic fluid and subsequent secretion into the intestinal lumen where it is proteolytically cleaved from an inactive state to its active form (46). Activated PLA2G1B contributes to lipid metabolism and absorption of lysophospholipids, particularly lysophosphatidylcholine (LPC). *Pla2g1b*$^{-/-}$ mice on a C57BL/6 background fed a hypercaloric diet (58.5% fat, 25% sucrose, 16.5% protein) for either 3 or 10 weeks are resistant to diet-induced obesity (9). *Pla2g1b*$^{-/-}$ mice showed a 37% reduction in plasma triglyceride (TG) levels primarily due to a decrease in hepatic VLDL production and an increase in TG-rich lipoprotein clearance. *Pla2g1b*$^{-/-}$ mice also displayed a 61% reduction in plasma cholesterol following 10 weeks on the hypercaloric diet compared to age-matched wild-type controls. Notably, *Pla2g1b*$^{-/-}$ *Ldlr*$^{-/-}$ mice fed the same hypercaloric diet for 10 weeks displayed a similar phenotype, including reductions in fasting glucose, insulin, and plasma lipids (10). Similarly, wild-type mice consuming a high-fat, high-carbohydrate diet supplemented with the general $sPLA_2$ inhibitor, methyl indoxam, showed a reduction in body weight after 10 weeks (11). This decrease in body weight was accompanied by enhanced glucose tolerance and suppression of post-prandial plasma lysophospholipid levels. Transgenic mice over-expressing the human *PLA2G1B* in pancreatic acinar cells gained more weight when given the hypercaloric high-fat/high-carb diet, and these mice also had reduced glucose tolerance and insulin resistance (12).

Given the strong evidence supporting PLA2G1B inhibition as an avenue for improving metabolic health, a discussion on the mode of action is warranted. Absorption of LPC into the portal blood, plasma, and livers was reduced in *Pla2g1b*$^{-/-}$ mice fasted for 12 hours followed by a glucose-lipid mixed meal (13). These data suggest that phospholipid digestion in the intestinal lumen and absorption of the digested lysophospholipid product through the portal blood is caused directly by PLA2G1B enzymatic activity following a meal (13). While PLA2G1B is the major enzyme for phospholipid hydrolysis within the intestinal lumen, other lipolytic enzymes may compensate in its absence to preserve lipid and cholesterol absorption (14). In regards to the enhanced glucose tolerance in *Pla2g1b*$^{-/-}$ mice, there is evidence that LPC alone has an adverse effect on hyperglycemia as shown with a glucose tolerance test (GTT) (13). Furthermore, *Pla2g1b*$^{-/-}$ mice have elevated fatty acid oxidation which can be directly suppressed by LPC, suggesting PLA2G1B enzymatic products reduce fatty acid oxidation (15).

Taken together, studies on the metabolic impact of PLA2G1B inhibition indicate it has metabolic effects in response to feeding. Recent experiments provide evidence that the benefits of PLA2G1B inhibition mimic those seen in response to bariatric surgery in mice, including prevention of dyslipidemia, and protection and remission from diet-induced Type 2 diabetes (16).

PLA2G2A

PLA2G2A is induced by several cytokines and second messengers including interleukins 1 and 6 (IL-1 and IL-6), tumor necrosis factor (TNFα), lipopolysaccharides (LPS), and cyclic AMP, suggesting a pro-inflammatory role (47–49). The Pla2g2a knockout (*Pla2g2a*$^{-/-}$) BALB/c mice have less joint inflammation than their wild-type counterparts under inflammatory conditions (50). With the focus of PLA2G2A studies being on its role in inflammation and atherosclerosis, studies on the actions of PLA2G2A in metabolism are quite limited. One consistent observation of the metabolic studies involving PLA2G2A is that its expression is up-regulated in response to a high-fat diet (17–19). In male Wistar rats, *Pla2g2a* expression was elevated 6-fold after 16 weeks on a

high-carbohydrate high-fat (HCHF) diet (17). At 8 weeks of age, these rats were orally administered the PLA2G2A inhibitor KH064, which drastically reduced weight gain, fat mass, and prevented an increase of adipocyte crown formation and macrophage infiltration seen in the wild-type rats (17). Inhibition of Pla2g2a by KH064 was also accompanied by an increase in lipolytic gene expression, attributed to an increase in hormone-sensitive lipase (HSL) phosphorylation. Lastly, treatment with KH064 improved glucose tolerance and insulin sensitivity as assessed by GTTs and ITTs (17).

The metabolic phenotype of male C57BL/6 mice genetically engineered to overexpress the human *PLA2G2A* gene has also been assessed (51). PLA2G2A overexpression improved glucose clearance and insulin sensitivity in GTTs and ITTs, thereby alleviating obesogenic symptoms in response to HFD (18, 20). C57BL/6 mice normally do not express the murine *Pla2g2a* due to a frameshift mutation in exon 3 (52). Overexpression of human *PLA2G2A* protected mice from weight gain on a high-fat diet compared to wild-type C57BL/6 mice after 10 weeks. *PLA2G2A* expression also enhanced oxygen consumption (VO_2) and energy expenditure. The expression of thermogenic genes in brown adipose tissue (BAT), including uncoupling protein 1 (UCP1), peroxisome proliferator-activated receptor γ coactivator 1α (PGC-1α), and Sirtuin-1 (SIRT1) was elevated (18, 20). *PLA2G2A*-expressing primary adipocytes from epididymal and inguinal white adipose tissue (WAT), and interscapular BAT showed elevated abundance of several proteins involved in adaptive thermogenesis compared to C57BL/6 wild-type adipocytes (20). To accompany this phenotype, mice expressing *PLA2G2A* also had reduced 6-hour fasting blood glucose levels and an increase in glucose transporter type 4 (GLUT4) in BAT suggesting that *PLA2G2A* enhances BAT glucose utilization (20).

PLA2G2A contributes to the inflammatory response, but the enzyme's role in obesity and metabolism is still unclear. Contributing to this, current investigations of PLA2G2As metabolic role used different designs. The human PLA2G2A gene was expressed in mice, whereas the rat Pla2g2a enzyme was inhibited pharmacologically (17, 51). Overexpression of *PLA2G2A* in mice may alter the expression of other secretory phospholipases in a variety of tissues, which could influence metabolism. Similarly, the activity of various $sPLA_2$ isozymes has not been examined in response to PLA2G2A inhibition by KH064. Moreover, food intake following KH064 administration was not reported in this study, and the impact of KH064 on intestinal lipid absorption was not assessed.

PLA2G2E

The expression of PLA2G2E is elevated in the white adipose tissue (WAT) and BAT of female C57BL/6 mice fed a HFD (26). Conversely, female *Pla2g2e*$^{-/-}$ mice gain less weight on a HFD over 18 weeks, with marked reductions in fat mass, hepatic lipid deposition, plasma aspartate aminotransferase (AST), and alanine aminotransferase (ALT) levels. Thus, the level of PLA2G2E may affect adiposity and liver metabolism. PLA2G2E preferentially hydrolyzes PE and PS on very low-density lipoprotein (VLDL), low-density lipoprotein (LDL), and high-density lipoprotein (HDL), although with weak enzymatic activity compared to other $sPLA_2$s (27, 28). Mass spectrometry of the lipoprotein particles from *Pla2g2e*$^{-/-}$ mice revealed a reduction in phospholipids (PL), triglyceride (TG), and cholesterol accumulation in VLDL, LDL, and HDL (26). These data suggest that Pla2g2e may promote obesity through elevated hepatic lipogenesis and VLDL assembly in the liver. Anionic phospholipids decrease the affinity for ApoE to bind the LDL receptor (LDL-r), which could impact lipoprotein particle clearance (53). Finally, whether lysophosphatidylserine (LPS) or lysophosphatidylethanolamine (LPE) impacts metabolism has yet to be determined.

In contrast, another study using male *Pla2g2e*$^{-/-}$ mice discovered the enzyme regulates lipolysis in adipocytes, likely through enhanced ERK1/2 signaling (29). Pla2g2e$^{-/-}$ mice had increased epididymal fat compared to C57BL/6 wild-type mice and accumulated more TG in the SVF isolated from adipose tissue. Over-expression of Pla2g2e in OP9 stem cells or treatment of 3T3-L1 cells with Pla2g2e protein reduced lipid accumulation and increased release of free glycerol, indicative of elevated lipolysis (29). These *Pla2g2e*$^{-/-}$ animals had reduced ERK1/2 signaling and HSL, the intracellular lipase responsible for hydrolyzing TG to FFAs. Finally, treatment of adipocytes with mouse Pla2g2e protein induced ERK1/2 signaling, demonstrating that Pla2g2e regulates adipocyte lipolysis through ERK/HSL signaling (29).

Currently, the understanding of the role of PLA2G2E in obesity and metabolism is limited to the two studies described above which report contrasting phenotypes with *Pla2g2e*$^{-/-}$ mice. A notable difference between these studies is the use of females *versus* males, and the contrasting results suggest sex-specific differences in Pla2g2e activity and its impact on obesity may be involved.

PLA2G5

PLA2G5 is mainly expressed in WAT and protects from diet-induced obesity. PLA2G5 expression is elevated in response to HFD feeding in female C57BL/6 mice (26). When placed on a HFD, *Pla2g5*$^{-/-}$ mice gained a large amount of weight, mainly from increased visceral fat mass (26). In GTTs and ITTs, the *Pla2g5*$^{-/-}$ mice had impaired glucose tolerance and insulin resistance. Furthermore, there was a striking induction of plasma ALT levels and hepatic fat deposition, indicating exacerbated hepatosteatosis (26). PLA2G5 preferentially hydrolyzes phosphatidylcholine (PC) in low-density lipoprotein (LDL) (54). In the *Pla2g5*$^{-/-}$ mice phospholipid, cholesterol, and TG levels were considerably higher in LDL (26). When transgenic mice overexpressing PLA2G5 in adipocytes were put on HFD, they showed better insulin sensitivity and a decreased expression of pro-inflammatory genes in WAT (26). These data suggest PLA2G5-mediated hydrolysis of PC and other phospholipids may reduce local

adipose tissue inflammation which appears to have a beneficial impact on whole-body insulin sensitivity.

PLA2G5 also modulates bone marrow-derived macrophage (BMDM) polarization. BMDM treated with palmitic acid (PA) or lipopolysaccharides induces the inflammatory response. Addition of recombinant PLA2G5 enzyme augments the expression of M2 markers *Arg1* and *Cd206* in the BMDM, suggesting that PLA2G5 has anti-inflammatory effects (26). The capacity for PLA2G5 to push macrophage polarization from an M1 to M2 state broadens its impact as the metabolic benefits of M2 macrophages are well documented (55–57). Genetic deletion of Th2 or M2 inducers increases the risk for metabolic disorders (56), and M2 macrophage infusion into obese mice has proven to be effective in treating obesity and improving insulin sensitivity (57). In humans, M2 macrophages are more prevalent in adipose tissue from lean individuals (55). While additional work needs to be done to elucidate the effects of PLA2G5 on metabolism, these actions appear to be partly mediated by the fatty acid and/or PC-released induction of M2 macrophage polarization in adipose tissue.

The role of PLA2G5 in glucose-stimulated insulin secretion (GSIS) is complex. GSIS is decreased in isolated pancreatic islets from PLA2G5 knockout mice and in pancreatic MIN6 cells following siRNA-mediated PLA2G5 knockdown (58). Additionally, PLA2G5 overexpression in MIN6 cells enhanced GSIS and increased AA release into the media with no change in prostaglandin E2 (PGE2) abundance (58). In contrast to the studies with isolated islets, the GSIS of $Pla2g5^{-/-}$ mice was increased compared to WT mice (58). The elevated GSIS was attributed to increased pancreatic islet size and number of proliferating cells in the pancreatic β-islets of the Pla2g5 KO mice. The *in vivo* data from this study suggests a reduction in the release of AA, a fatty acid contained in over 30% of glycerolipids in rodent islets (59), is beneficial for insulin secretion and β-cell proliferation. Amino acids generally induce GSIS, while inhibition of the release of AA inhibits GSIS (60). Paradoxically, a major metabolite of AA is PGE2, a well-known inhibitor of GSIS (61–63). The role of AA metabolites in GSIS will be discussed further in the next section regarding Pla2g10. The data suggest that PLA2G5 regulates insulin secretion and β-cell proliferation, which may be dependent on the amount of AA released *versus* the amount of AA used for PGE2 production.

PLA2G10

Studies with transgenic mice provided evidence that PLA2G10 mediates adipogenesis and has thereby led to the hypothesis that it protects from diet-induced obesity (30, 31). PLA2G10 binds to zwitterionic phospholipids such as PC with high affinity, releasing AA and LPC. PLA2G10 is expressed in a variety of tissues including the lungs, adrenal glands, pancreas, brain, heart, and adipose tissue (27, 32–34). $Pla2g10^{-/-}$ mice on a C57BL/6 background gain more weight and adipose mass over 40 weeks compared to chow-fed wild-type mice (30). The effects of Pla2g10 deletion are directly on WAT, as there were no changes in food intake or respiration. Pre-adipocytes prepared from WAT of $Pla2g10^{-/-}$ mice accumulated more TG when induced to differentiate *ex vivo*, suggesting that PLA2G10 has a direct effect in adipose tissue to reduce lipid accumulation. Similarly, when the OP9 cell line was modified to overexpress PLA2G10, TG accumulation was reduced following differentiation (30). In addition, the cells had reduced expression of multiple lipogenic genes including sterol regulatory element-binding protein 1c (SREBP-1c), stearoyl-CoA desaturase-1 (SCD-1), and fatty acid synthase (FAS) (31, 64–66). Interestingly, the reduction in lipogenic gene expression arose from the ability of PLA2G10 to generate lipid products that suppress liver X receptor (LXR) activity.

PLA2G10 is expressed in adrenal cells and has a regulatory role in adrenal corticosteroid production. Overexpression of PLA2G10 in C57BL/6 mice resulted in a 30-40% reduction in corticosteroid production, and this effect was reversed by methyl indoxam administration (31). The elevated glucocorticoid level did not arise from elevated ACTH or ACTH responsiveness. PLA2G10 overexpression dramatically reduced expression of the LXR-target gene steroidogenic acute regulatory protein (StAR), a nuclear-encoded mitochondrial protein that mediates the rate-limiting step of steroid synthesis (67). As in the adipocytes, Pla2g10 generated a ligand that reduced LXR activity.

Pla2G10 is also expressed in the pancreatic beta cells and suppresses GSIS (68). Like Pla2g5, Pla2g10 generates AA. However, this pool of AA is converted to prostaglandin E2, which binds to the EP3 receptor. EP3 elevates cAMP leading to decreased insulin secretion. Why Pla2g5 and Pla2g10 have opposite effects on GSIS is unclear. However, Pla2g10 has to be proteolytically activated, and this may give it access to a different pool of phospholipids. Finally, older Pla2g10 KO mice appear to be protected from age-related glucose intolerance.

The current data on PLA2G10 indicate it impacts multiple aspects of metabolism and hormonal action. PLA2G10 expression reduces weight gain and overall adiposity in mice. Concerning hormone actions, Pla2g10 decreases corticosteroid production in adrenal cells (31). Excessive use or production of glucocorticoids will induce insulin resistance, weight gain, and adiposity while also exacerbating Type 2 diabetes (69–72). However, Pla2g10 reduces GSIS, giving this phospholipase a complex contribution to the overall metabolic state.

PLA2G12B

PLA2G12B is the only phospholipase implicated in metabolism showing no catalytic activity due to a point mutation in its active site, and thus it is hypothesized to act as a ligand for receptors that are currently unidentified (8). Using $Pla2g12b^{-/-}$ mice fed an *ad libitum* chow diet, knockout of the *Pla2g12b* gene increased TG, cholesterol, and free fatty acids in the liver, resulting in severe hepatosteatosis (35). Hepatocyte nuclear factor-4alpha (HNF-4α) and its co-activator PGC-1α induce *Pla2g12b* expression (35, 36), resulting in the induction of genes involved in lipoprotein packaging (microsomal triglyceride transfer protein, MTP) and

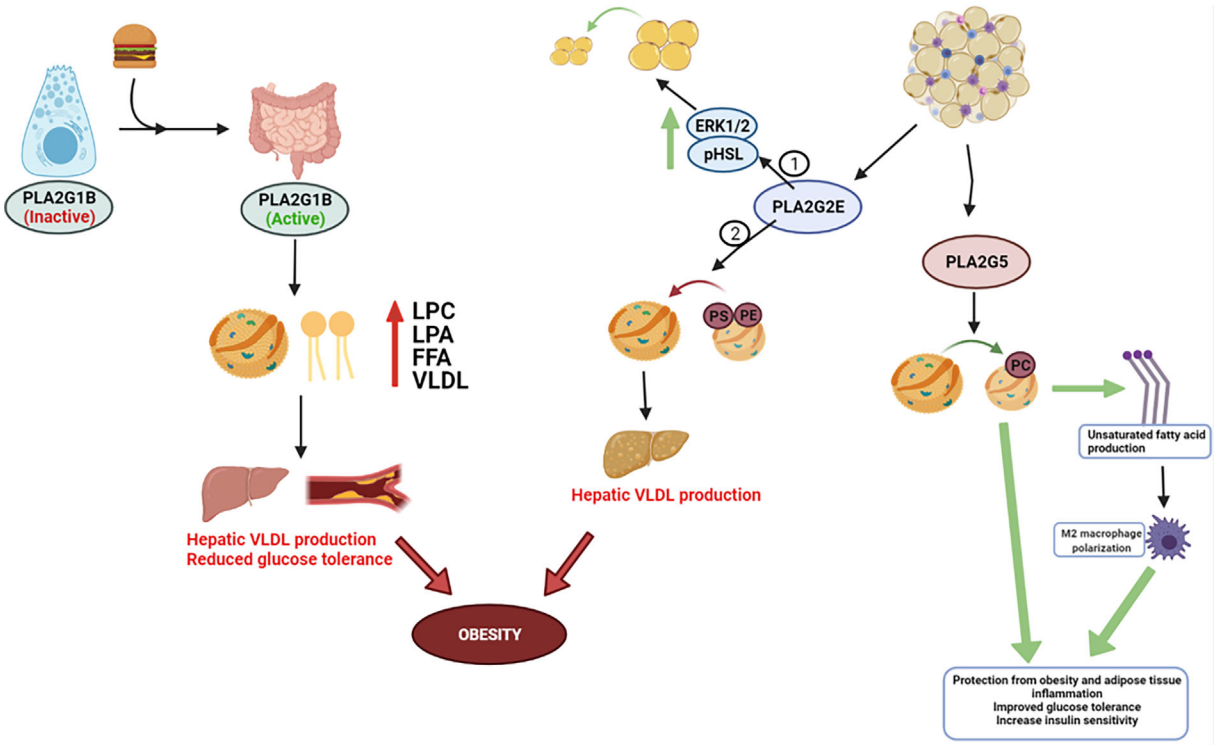

FIGURE 1 | Metabolic role for PLA2s. PLA2G1B is released by pancreatic acinar cells into the pancreatic juice following a meal and then secreted into the intestinal lumen, where it eventually becomes activated. Phospholipid digestion by PLA2G1B results in elevated lysophosphatidylcholine (LPC), lysophosphatidic acid (LPA), and free fatty acids absorption in the portal blood, plasma, and liver, and increases hepatic very-low density lipoprotein (VLDL) production. Elevated enzymatic activity in the intestinal lumen can progress obesity, reduce glucose tolerance, and exacerbate insulin resistance – most notably due to elevated release/absorption of LPC and overarching dyslipidemia. PLA2G2E and PLA2G5 are expressed in WAT. 2 studies have contradicting results in PLA2G2Es metabolic role – 1 study discovered PLA2G2E increases ERK1/2 signaling and HSL phosphorylation to increase lipolysis, whereas Study 2 found PLA2G2E may promote obesity through hepatic lipogenesis and VLDL production. PLA2G5 preferentially hydrolyzes PC-rich phospholipids and promotes M2 macrophage polarization, which appears to have a beneficial impact on LDL lipid normalization and whole-body insulin sensitivity.

VLDL secretion (37, 38). Moreover, liver-specific *HNF-4α*$^{-/-}$ mice are phenotypically similar to *Pla2g12b*$^{-/-}$ mice, as both lines have reduced serum TG and cholesterol levels and display severe hepatosteatosis (35, 39). These observations suggest that PLA2G12B is one gene involved in the control of lipid metabolism downstream of HNF-4α. Infection of mice with an adenovirus encoding *Pla2g12b* into *Pla2g12b*$^{-/-}$ mice improves hepatic VLDL secretion and restores the decline in serum TGs (35). These data indicate that PLA2G12B is under the regulation of HNF-4α and plays a metabolic role by regulating lipoprotein packaging and VLDL secretion in the liver. More recent data has shown that estrogen-related receptor γ (ERRγ) transcriptionally regulates PLA2G12B through binding to its promoter region to regulate hepatic VLDL-TG secretion (73). In *Pla2g12b*$^{-/-}$ mice, ERRγ fails to regulate VLDL-TG secretion and large hepatic lipid droplets result. Importantly, ERRγ is implicated in a wide range of physiologic roles including metabolic homeostasis, especially hepatic glucose metabolism (40). The findings associating PLA2G12B with ERRγ may be one avenue by which ERRγ mediates hepatic glucose production.

CONCLUSION

The recent studies discussed in this review show that sPLA$_2$s can influence metabolic diseases such as Type 2 diabetes and obesity, at least partially through alterations in lipid production and mobilization (**Figure 1**); and while controversy exists regarding whether elevated lipids directly cause insulin resistance, pre-clinical and clinical data indicate an association between elevated lipids and lipoproteins with insulin resistance (74–77). Furthermore, one issue in analyzing the metabolic impact of sPLA2s is many of the isoforms effect intertwined pathologies such as atherosclerosis, heart disease, and cancer (41, 55, 57, 64–67, 69–72). For this reason, future studies on sPLA$_2$s should consider their roles based on tissue localization, as their distinct functions may be altered based on the tissue being analyzed. Furthermore, the expression and compensation of other sPLA$_2$ isoforms in transgenic animal models is another factor that might result in large phenotypic changes, and thus should also be observed to advance what we know about sPLA$_2$s in metabolic diseases.

AUTHOR CONTRIBUTIONS

The author confirms being the sole contributor of this work and has approved it for publication.

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Structural Lessons From the Mutant Proinsulin Syndrome

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Insight into folding mechanisms of proinsulin has been provided by analysis of dominant diabetes-associated mutations in the human insulin gene (*INS*). Such mutations cause pancreatic β-cell dysfunction due to toxic misfolding of a mutant proinsulin and impairment in *trans* of wild-type insulin secretion. Anticipated by the "Akita" mouse (a classical model of monogenic diabetes mellitus; DM), this syndrome illustrates the paradigm endoreticulum (ER) stress leading to intracellular proteotoxicity. Diverse clinical mutations directly or indirectly perturb native disulfide pairing leading to protein misfolding and aberrant aggregation. Although most introduce or remove a cysteine (Cys; leading in either case to an unpaired thiol group), non-Cys-related mutations identify key determinants of folding efficiency. Studies of such mutations suggest that the hormone's evolution has been constrained not only by structure-function relationships, but also by the susceptibility of its single-chain precursor to impaired foldability. An intriguing hypothesis posits that *INS* overexpression in response to peripheral insulin resistance likewise leads to chronic ER stress and β-cell dysfunction in the natural history of non-syndromic Type 2 DM. Cryptic contributions of conserved residues to folding efficiency, as uncovered by rare genetic variants, define molecular links between biophysical principles and the emerging paradigm of *Darwinian medicine*: Biosynthesis of proinsulin at the edge of non-foldability provides a key determinant of "diabesity" as a pandemic disease of civilization.

Keywords: protein folding, protein structure, folding efficiency, hormone, metabolism

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INTRODUCTION

The Centennial of insulin's discovery (1) coincides with renewed interest in cellular mechanisms of biosynthesis. The mature hormone is the post-translational product of a single-chain precursor, proinsulin (2, 3). Diverse dominant mutations in the human insulin gene (*INS*) have been identified associated with diabetes mellitus (DM) (4–10). Such mutations impair oxidative folding of nascent proinsulin in the endoplasmic reticulum (ER) of pancreatic β-cells (11, 12). Originally identified as a monogenic cause of permanent neonatal-onset DM (7, 13–15), this syndrome (designated mutant

Abbreviations: DM, diabetes mellitus; ER, endoplasmic reticulum; GA, Golgi apparatus; MIDY, mutant *INS*-gene-induced diabetes of youth; MODY, maturity-onset diabetes of the young, and NMR, nuclear magnetic resonance. Amino acids are designated by standard one- and three-letter codes.

INS-gene-induced diabetes of youth; MIDY) can also present in childhood (16) or adolescence (17) (maturity-onset diabetes of the young; MODY). Such variation in onset is ascribed to mutation-specific differences in extent of perturbed folding (12, 18). The spectrum of phenotypes may also reflect polygenic differences in how the β-cell responds to chronic ER stress (19, 20).

MIDY patients are heterozygous. Although one wild-type (WT) insulin allele would ordinarily be sufficient to maintain metabolic homeostasis, studies of the Akita mouse [a corresponding mouse model (21–23)] first demonstrated biochemical dominance: misfolding of the variant proinsulin impairs wild-type (WT) biosynthesis (24, 25). Analogous biochemical interference occurs in β-cell lines (11, 12, 18). ER stress leads to distorted organelle architecture, impaired glucose-stimulated β-cell secretion and eventual cell death (26, 27). Discovery of the mutant proinsulin syndrome has stimulated renewed interest in structural mechanisms of disulfide pairing (28–33) as a critical step in the biosynthesis of insulin (2, 3, 34). The central importance of such mechanisms—both in β-cells and as a general model for oxidative protein folding—have motivated extensive cell-based and animal studies (35–39). Together, these efforts have deepened the biophysical understanding of classical structure-functional relationships in the insulin molecule (9, 10, 19, 40) in relation to cellular mechanisms of biosynthesis (4, 10, 41, 42).

The goal of this review is to provide a structural perspective on *INS* mutations in human proinsulin [for clinical background and history of discovery, see (43)]. A starting point is provided by a general biophysical paradigm: that key interactions in intermediate stages of protein folding often foreshadow spatial relationships in the native state (44–46). Accordingly and in the reverse direction, we will regard the classical crystal structure of insulin (47) as a framework for interpreting folding mechanisms. Given this context, we will restrict our attention to mutations in (or adjoining) the well-organized insulin moiety of proinsulin (48) (**Figure 1A** and **Table 1**). Whereas traditional structure-activity relationships (SAR) pertain to receptor binding (9), contributions of the same residues to folding efficiency may be inapparent once the native structure is reached. The growing MIDY/MODY database of *INS* mutations (**Figure 1B** and **Table S1**) may be exploited to decipher this hidden layer of meaning. As a seeming paradox in Darwinian medicine (49, 50), the biophysical non-robustness of proinsulin biosynthesis suggests that the hormone has evolved to the precarious edge of foldability (40, 51, 52). We envisage that foundational principles of protein folding, structure and stability will be

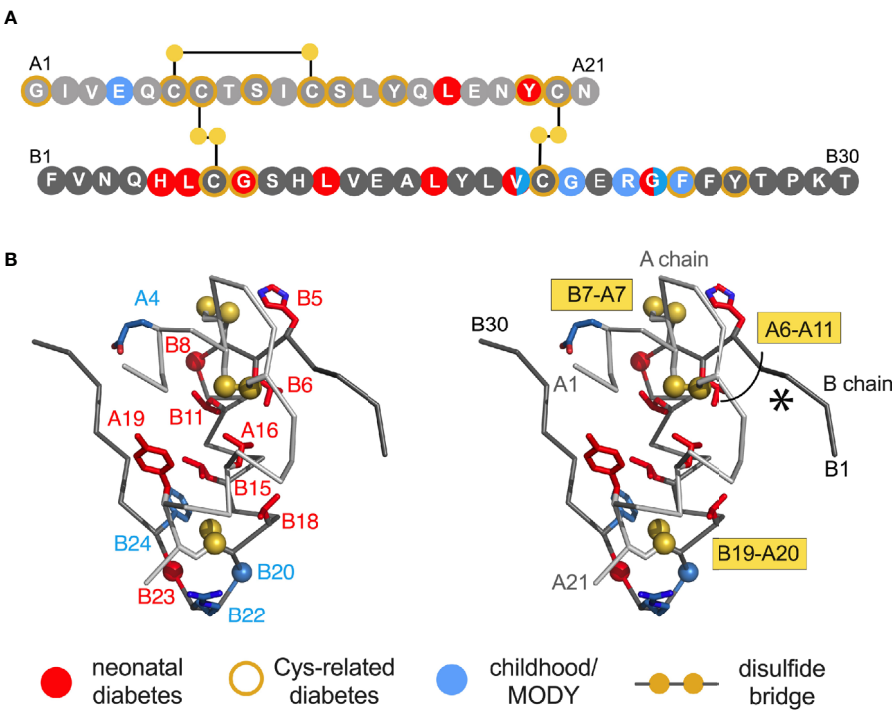

FIGURE 1 | Clinical mutations in *INS* gene. **(A)** Sequence of insulin showing positions of clinical mutations. Residues are labelled by standard single letter code (bold white). The A chain is shown as light gray circles (upper sequence), and B chain as dark gray circles (lower sequence). Color code: neonatal- or delayed onset is indicated by filled red or blue circles, respectively. Sites of Cys-related mutations are highlighted by gold borders (**Tables 1A, B**). Disulfide bridges are indicated by filled gold circles connected by black lines. **(B)** Stereo view of insulin monomer (C_α-trace ribbon model; PDB entry 4INS) (47). Non-Cys-related mutations are highlighted as in **(A)**; side chains are shown in red as labeled (**Tables 1C, D**). The C_α atoms of Gly^{B8}, Gly^{B20} and Gly^{B23} are respectively shown as red, blue and red spheres (one-third Van der Waals radii), and sulfur atoms likewise as gold spheres. The A- and B chain ribbons are shown in light and dark gray, respectively. For clarity, symbols are also defined at bottom.

TABLE 1 | Sites of clinical mutations in proinsulin[a,b].

A) removal of a Cys	
Cys31 [B7]	Tyr
Cys43 [B19]	Gly, Ser, Tyr, Ala
Cys95 [A6]	Tyr, Ser
Cys96 [A7]	Arg, Ser, Tyr
Cys100 [A11]	Tyr
Cys109 [A20]	Tyr, Phe, Arg
B) addition of a Cys[c]	
Gly32 [B8]	Cys
Phe48 [B24]	Cys
Tyr50 [B26]	Cys
Arg89 [Cpep+2]	Cys
Gly90 [A1]	Cys
Ser98 [A9]	Cys
Ser101 [A12]	Cys
Tyr103 [A14]	Cys
Tyr108 [A19]	Cys
C) neonatal non-Cys-related mutations	
His29 [B5]	Asp, Gln
Leu30 [B6]	Pro, Gln, Val, Arg
Gly32 [B8]	Ser, Arg, Val
Leu35 [B11]	Pro, Gln
Leu39 [B15]	Pro, Val
[B15-B16]del	His
Val42 [B18]	Gly
Gly47 [B23]	Val
Leu105 [A16]	Pro
Tyr108 [A19]	Asp or Stop
D) childhood or MODY mutations	
His29 [B5]	Tyr
Leu30 [B6]	Met
Val42 [B18]	Ala
Gly44 [B20]	Arg
Arg46 [B22]	Gln
Gly47 [B23]	Asp
Phe48 [B24]	Ser
Glu93 [A4]	Lys

[a]*Residue numbers refer to preproinsulin; positions in the mature A- and B chains are given in brackets.*

[b]*References are given in* ***Table S1****.*

[c]*Cys insertions have also been observed in the signal sequence and C domain (see* ***Table S1****).*

found to rationalize the distribution of MIDY/MODY mutations and broad spectrum of clinical presentations.

Studies of Insulin Biosynthesis

Essential background is provided by the molecular biology of the insulin gene (53–57). In brief, *INS* encodes a single-chain precursor polypeptide, designated *preproinsulin*. Its signal peptide is cleaved on ER translocation. The translocated polypeptide (reduced proinsulin) contains a C domain between B- and A domains (thus connecting Thr^{B30} to Gly^{A1}) (58). Folding in the ER requires specific pairing of three disulfide bridges (cystines B7-A7, B19-A20 and A6-A11). These bridges (gold spheres in **Figure 1B**) stabilize the protein and its receptor-binding surface (31, 59–67). Heteronuclear NMR studies of proinsulin (as an engineered monomer) have defined a folded insulin core with flexible C domain (48) as suggested by earlier studies (68–73). The contribution of each disulfide bridge to structure, stability, and activity have been extensively investigated (31, 59–61, 63–67). Together, these bridges provide both interior struts (cystines B19-A20 and A6-A11) and an external staple between chains (A7-B7). Mispairing of insulin's cysteines (to form disulfide isomers) markedly impairs stability and activity (74–76).

Processing of proinsulin by prohormone convertases (PC1/3 and PC2) liberates the mature hormone (3, 77). Such conversion, occurring in the Golgi apparatus (GA) and immature secretory granules (78), ensures hormonal activity as proinsulin binds more weakly than insulin to the insulin receptor (IR) (79). Insulin and C-peptide are stored within glucose-regulated secretory granules (80) with microcrystalline assembly of Zn^{2+}-stabilized hexamers (81–83). Evolution of such assembly foreshadowed its pharmacologic exploitation in clinical formulations (84). The marked susceptibility of the Zn^{2+}-free insulin monomer (the active form of the hormone) to fibrillation complicated its manufacture and clinical use in the immediate decades after its discovery in 1921, thus recapitulating evolutionary constraints faced by β-cells due to the implicit threat of toxic misfolding (34, 85). This perspective has been reinforced by studies of a mouse model lacking the β-cell zinc transporter (86). Although key to the stable pharmaceutical formulation of "first-generation" rapid-acting insulin analogs [*lispro* and *aspart* (87), otherwise exhibiting heightened susceptibility to fibrillation (88)], in β-cells such assembly occurs only after exit from the ER and so cannot mitigate toxic misfolding of proinsulin variants.

Unlike native biosynthesis, chemical synthesis of insulin has traditionally employed isolated A- and B-chain peptides (89). The success of insulin chain combination implies that chemical information required for folding is contained within A- and B-chain sequences (90, 91). Hundreds of analogues have been prepared by this protocol, facilitating pharmaceutical innovation (87, 92). Despite the general robustness of insulin chain combination, synthesis of certain analogues has been confounded by low yields (30, 93–99). In selected cases such limitations have been overcome through the use of proinsulin or foreshortened single-chain synthetic intermediates ["mini-proinsulins" (100–103)]. Chemical protein synthesis *via* native ligation of peptide segments (104, 105) has also enabled synthetic access to the proinsulin molecule (106). In addition to their practical utility, such synthetic advances promise to provide insight into structural mechanisms of disulfide pairing (31, 95, 107–109). Sites of mutation among MIDY patients in large measure coincide with past difficulties in synthetic efforts.

Oxidative Folding Mechanisms

An historic foundation for studies of MIDY mutations in proinsulin has been provided by basic studies of protein folding over the past sixty years. Whereas studies of isolated peptides motifs and model globular domains were often designed to circumvent the complexity of disulfide pairing (28, 29, 110), oxidative protein folding has provided an attractive opportunity to define intermediates investigated by chemical trapping of partial folds (111). An extensive literature pertains to such disulfide-rich globular proteins as bovine pancreatic trypsin inhibitor (44, 112–114), hen egg white lysozyme (115–118) and α-lactalbumin (119–122). Insights from these model proteins

and their application to proinsulin and homologous polypeptides underlie efforts to interpret INS mutations associated with toxic misfolding.

Chemical-trapping studies of proinsulin and homologous proteins have provided evidence for preferential accumulation of one- and two-disulfide intermediates (28, 29, 123, 124). These intermediates define a series of partial folds and corresponding trajectories on successive free-energy landscapes ["landscape maturation"; **Figure 2A**). The landscapes (maturing from shallow to steep; left to right in **Figure 2A**)] are each associated with (a) stepwise stabilization on successive disulfide pairing and (b) a corresponding ensemble of dynamic trajectories constrained by the bridges. Chemical-trapping studies are thus consistent with both multiple folding trajectories on funnel-shaped landscapes and a preferred sequence of specific disulfide intermediates (125) in general accordance with biophysical principles (126–128).

Physiological interpretation of proinsulin refolding studies has been limited by its aggregation near neutral pH [thereby imposing a technical requirement for pH > 9) (29, 129)]. This limitation has been circumvented through the use of mini-proinsulin and IGF-I as more tractable models (28, 29, 59, 62, 108, 110, 123, 130). A structural pathway was proposed based on spectroscopic studies of equilibrium models (31, 59–61, 63–67, 131); this scheme highlights initial formation of cystine A20-B19 within a hydrophobic cluster of conserved side chains between the C-terminal A-chain α-helix and central B-chain α-helix (75, 76, 95). Because in the refolding of mini-proinsulin and IGF-I the A20-B19 disulfide bridge is the first to form (as the only one-disulfide intermediate to accumulate) (28, 29, 110), its pairing defines a biophysical milestone, formation of a specific folding nucleus (31, 131, 132). The predominance of cystine A20-B19 among populated intermediates motivated design of equilibrium models based on pairwise Ala- or Ser substitution of the other cystines (31, 59–67). Such analogues exhibit reduced α-helix content with native-like structure near cystine B19-A20 (31). Mutations in the putative B19-A20-related folding nucleus impair insulin chain combination, biosynthetic expression, and secretion of single-chain precursors in yeast (63, 97, 132–134).

^{1}H-NMR spectra of one- and two-disulfide analogues exhibit progressive chemical-shift dispersion with successive disulfide pairing. These data are in accordance with stepwise structural stabilization in the landscape paradigm illustrated above (59, 131). Despite the predominance of A20-B19 pairing as an initial step, folding subsequently proceeds in parallel *via* multiple channels. Mini-proinsulin, for example, can rapidly form cystine A7-B7 or slowly undergo pairing of A6-A11. Although it is not apparent that pairing of cysteines distant in the sequence (such as A7 and B7) should be favored relative to pairing of nearby cysteines (A6 and A11), pairwise substitution of cystine A7-B7 (by Ser) destabilizes insulin more markedly than does pairwise substitution of A6-A11 (132). These findings suggest that nascent structure in the one-disulfide [B19-A21] intermediate either more effectively aligns Cys^{A7} and Cys^{B7} or more significantly impairs pairing of Cys^{A6} and Cys^{A11}. These on-pathway two-disulfide intermediate may interconvert with non-native disulfide isomers as off-pathway kinetic traps. The danger posed by such traps has been highlighted in studies of IGF-I and its non-native disulfide isomers (28, 135). Relative isomer stabilities (as probed in a mini-IGF model) are

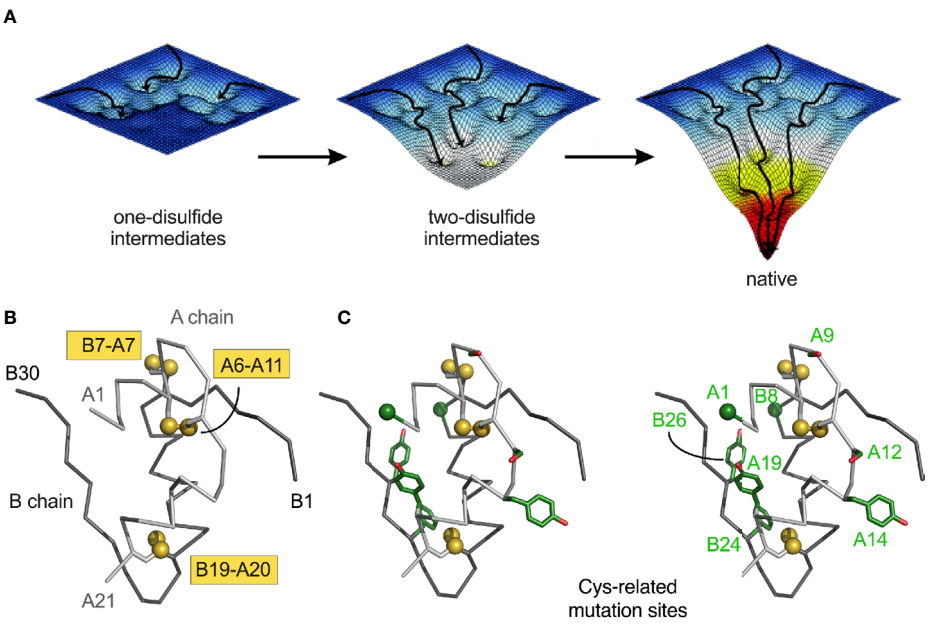

FIGURE 2 | Energy landscape paradigm. **(A)** Landscape maturation: successive disulfide pairing enables a sequence of folding trajectories on ever-steeper funnel-shaped free-energy landscapes. **(B)** Ribbon model of insulin showing the three native disulfide bonds (yellow boxes). Coordinates were obtained from PDB entry 4INS (47). The A- and B chains are shown in light- and dark gray, respectively. **(C)** Stereo view of insulin with Cys substitutions highlighted in green (**Table 1B**). Side chains are shown as sticks; Cys-related sulfur atom and alpha-carbons of Gly^{A1} and Gly^{B8} represented as spheres (one-third Van der Waals radii).

specified by N-terminal residues in the B domain (136, 137). Although the refolding of proinsulin is more stringent, related non-native disulfide isomers (76) may readily be generated by disulfide exchange on addition of a chemical denaturant (75). Corresponding insulin isomers are molten globules whose stability and cooperativity are marginal (76).

Non-native disulfide isomers of proinsulin and related polypeptides have also been observed in transfection studies of mammalian cells (27, 30, 138–140). These studies have exploited electrophoretic mobility differences between native and non-native disulfide isomers in non-denaturing gels [as demonstrated by Arvan, Liu and colleagues (138)]. Less compact structures of non-native states are presumably associated with slower mobilities. Formation of non-native proinsulin isomers has thus been observed on transfection of expression constructs in a variety of mammalian cell lines. Although non-native proinsulin isomers are generally not secreted, mutations can enhance mispairing in the ER (139, 140). Extent of cellular misfolding does not correlate with *in vitro* thermodynamic stability, suggesting that the ER machinery does not evaluate free energies of unfolding (ΔG_u) as a criterion of quality-control.

Studies of proinsulin variants containing N-terminal substitutions or deletions suggest that contributions of specific side chains to foldability may not be apparent in the native state (141). The substituted side chains may perturb the relative stabilities or kinetic accessibility of disulfide intermediates, for example, disproportionately to effects on the native state, once achieved. Such residues may also contribute to interactions of the nascent polypeptide with ER chaperones and its oxidative machinery (142). Indeed, the ER of β-cells may contain a lineage-specific set of chaperones and foldases required for proinsulin biosynthesis. Defining such a β-cell-specific "ER proteome" defines a key frontier of current research. Cell-type-specific differences in ER proteomes are likely to underlie the inefficient folding and secretion of proinsulin in the majority of human cell lines (143).

Foreshortened "mini-proinsulins" (144) can misfold in yeast to form a metastable disulfide isomer as the predominant secretion product. Such quantitative misfolding indicates that the ER folding machinery of a eukaryotic cell can selectively direct folding into a non-ground-state conformation. Characterizing this alternative pairing scheme and assessing its structural resemblance to the native fold would be of broad interest. Because the aberrant protein is not degraded prior to ER trafficking (*i.e.*, it passes ER quality-control checkpoints), such analogues provide models of "stealth" misfolding, in turn leading to secretion of a protein caught in a kinetic trap. As described in the following two sections, clinical mutations in proinsulin conversely exemplify "non-stealth" misfolding leading to activation of the unfolded protein response (UPR) (145–150).

MONOGENIC DIABETES AND THE *INS* GENE

The majority of *INS* mutations cause permanent neonatal-onset DM (**Figure 1** and **Table S1**) (14). Because impaired β-cell function develops prior to maturation of the immune system, the patients present with auto-antigen-negative DM. Similar phenotypes may be caused by mutations in other genes (151), most frequently a heterozygous activating mutation in the β-cell voltage-gated potassium channel (either *KCNJ11* or *ABCC8*, respectively encoding its Kir6.2 and Sur1 subunits) (152, 153). The resulting diabetic phenotype in this genetic background may be transient or permanent. It is important to recognize this subset of neonates or toddlers as in favorable cases they can successfully be treated with oral agents that inhibit the channel (sulfonylureas) rather than by insulin injections (151).

Dominant *INS* mutations are the second most common genetic cause of permanent neonatal DM (7, 13, 14, 16). Such mutations occur in each region of preproinsulin: its signal peptide, B-, C- and A domains (**Table S1**) (9, 10). The majority result in the addition or removal of a cysteine, leading in either case to an odd number of potential pairing sites (**Figure 1A**). Mutations have been found at each of insulin's six canonical cysteines, generally associated with neonatal onset (**Figure 2B**, **Table 1A**). An additional cysteine may be introduced at various positions in the insulin moiety (**Figure 2C** and **Table 1B**). The resulting odd number of thiol groups leads in general to misfolding and aggregation (11, 12, 18). Even in this context structure may matter, as it is possible that some sites of Cys introduction lead more readily to aberrant intra- or intermolecular disulfide pairing than others, depending on the conformational properties of oxidative folding intermediates and their interactions surfaces. Such biophysical variability would be expected to be associated with differences in ER stress and hence age of DM onset.

Among human MIDY mutations is the same "Akita" substitution (Cys^{A7}→Tyr) as in the *Ins2* gene of the *Mody4* mouse (21–23); this dominant murine substitution has thus been characterized as a model of the human syndrome (25–27). The variant murine proinsulin *in vitro* undergoes partial unfolding with increased aggregation (154). Analogous perturbations were found in human insulin- and proinsulin analogues lacking cystine A7-B7 (66, 132). Heterozygous expression of related *Ins2* allele Cys^{A6}→Ser in the mouse also causes DM (155).

Identification of identical human and murine mutations at position A7 suggests that the mechanisms of neonatal DM have shared pathogenetic features independent of species (21–23, 25–27). Although β-cell degeneration in the Akita mouse remains incompletely understood, early defects have been observed in the folding and trafficking of both wild-type and variant proinsulins. These defects are associated with elevated markers of ER stress, electron-dense deposits in abnormal ER and GA, and mitochondria swelling—together leading to a progressive decline in β-cell mass (25–27). Evidence for the clinical relevance of these findings has been obtained by the construction of innovative fluorescent proinsulin fusion proteins and their use in cell lines and transgenic mice to detect subcellular localization and aggregation (35–38).

Deciphering Determinants of Foldability

The Akita variant is representative of a mutant proinsulin with an odd number of cysteines. However, a distinct subset of MIDY- or MODY-associated mutations does not involve cysteine (**Table 1C**). Although widely scattered in the sequence, these mutations occur more often in the B domain than in the A

domain—and not at all in the C domain. Because the variant proinsulins retain the six canonical cysteines and yet pair inefficiently, such mutations are of special biophysical interest. A structural overview is provided in **Figures 3–5** as described in turn.

Structural relationships in insulin were examined using the monomer derived from a representative wild-type T_6 zinc insulin hexamer [PDB entry 4INS (47)]. NMR studies have shown that the conformation of an engineered insulin monomer in solution closely resembles the T-state protomer in a zinc insulin hexamer as characterized by X-ray crystallography (60, 157–159). Short- and medium-range NOEs are consistent with spatial relationships in the T state (159). Although positions of C-terminal B-chain residues (B25-B30) are generally less well defined than in crystallographic dimers and hexamers, classical attachment of B24-B28 β-strand to the α-helical core is maintained in solution. The free monomer thus

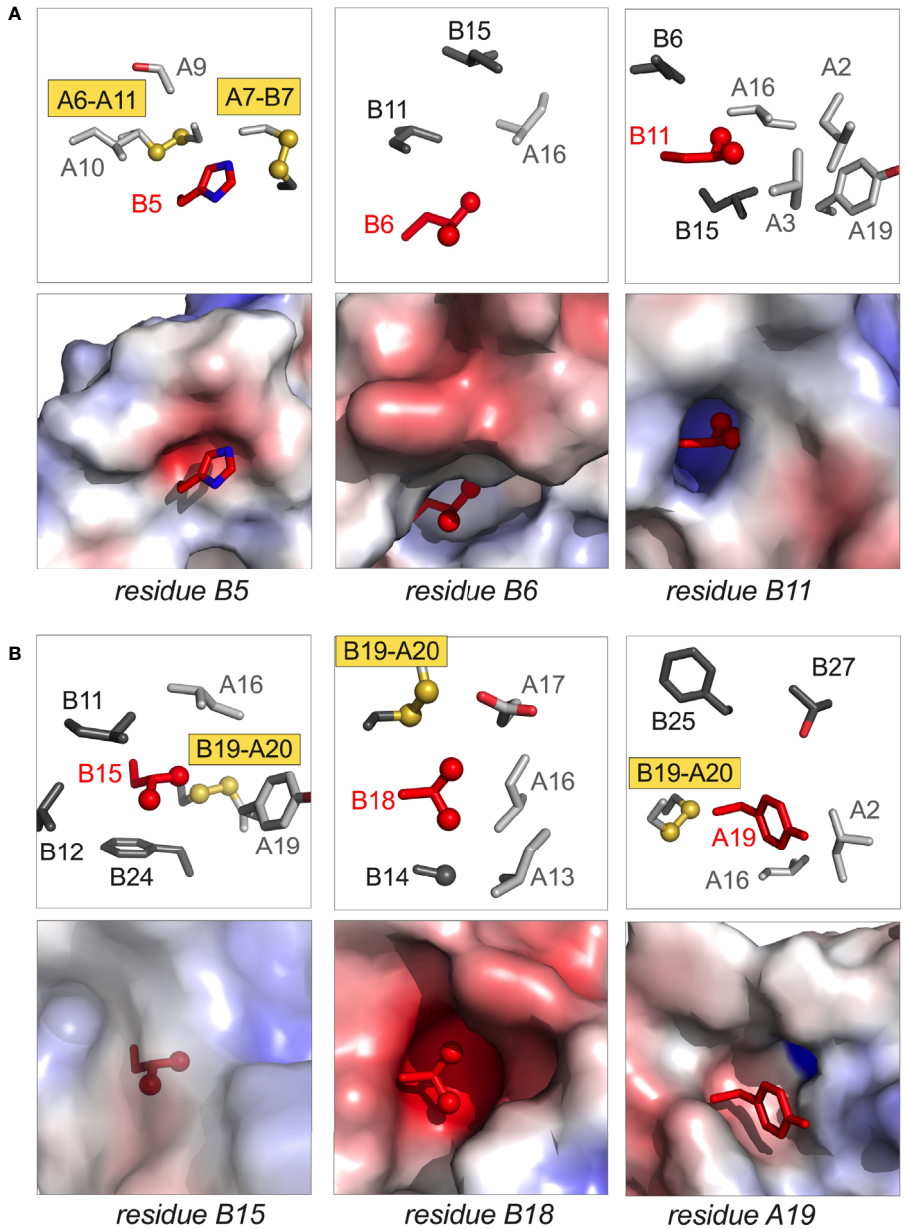

FIGURE 3 | Structural sites of neonatal-onset mutations. **(A)** Spatial environments of residues B5, B6, and B11; **(B)** spatial environments of residues B15, B18 and A19. In each panel the highlighted side chain is shown in red; in each pair of images, stick models are shown in upper panels whereas electrostatic surfaces (calculated in absence of indicated side chain) are shown in the lower panels. In stick models, side chains belonging to the A- and B chains are respectively shown in light and dark gray; Cys-related sulfur atoms (gold) and aliphatic methyl groups (red) are represented as spheres (one-third Van der Waals radii). Coordinates were obtained from PDB entry 4INS (47).

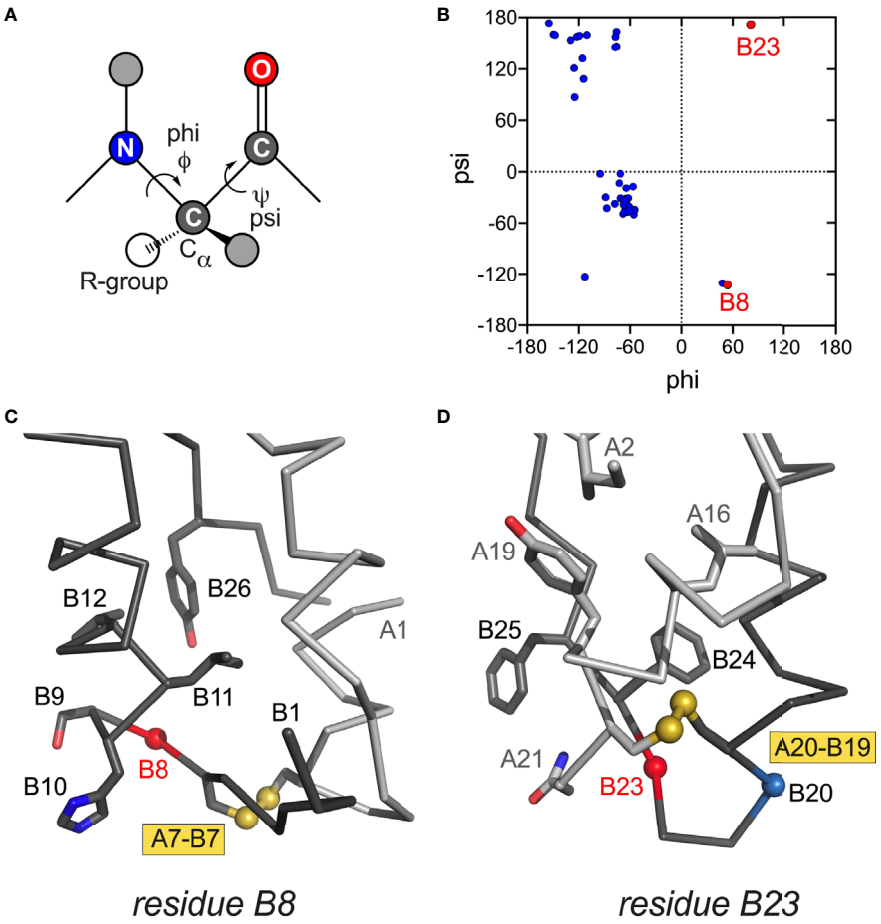

FIGURE 4 | Conformations and structural environments of conserved glycines at positions B8 and B23. **(A)** Main-chain dihedral angles phi (φ) and psi (ψ) in a peptide. **(B)** Ramachandran plot of crystallographic insulin T state (PDB entry 4INS; φ/ψ angles generated using PyMOL and plotted using GraphPad Prism software). Residues B8 and B23 are as labeled (red). In canonical T state Gly^{B8} and Gly^{B23} lie within β-turns with positive φ–angles, thereby residing on the *right* side of Ramachandran plane in regions unfavorable or "forbidden" for L-amino acids. In R state Gly^{B8} residues in α-helix and so on the *left* side of Ramachandran plot (not shown). **(C)** Canonical environment of Gly^{B8} in the T-state β-turn; key nearby side chains are shown. **(D)** Canonical environment of Gly^{B23} in B20-B23 β-turn, shared by T- and R states. Residue B23 is near the side chain of Asn^{A21}, and the positive B23 φ angle enables formation of an inter-chain hydrogen bond (A21 side-chain carboxamide NH … O=C B23). Nearby side chains are shown. α-Carbon traces of the A- and B chains are shown in light- and dark gray, respectively. Coordinates were obtained from PDB entry 4INS (47).

does not exhibit a major change in B-chain conformation [hinge opening of the B20-B23 β-turn (160)]. The present analysis has thus focused on spatial relationships in T-state monomers (as extracted from crystal structures) because of their higher resolution (relative to NMR ensembles) and likely pertinence to proinsulin (48).

N-terminal segment. In NMR-derived structures of insulin as a Zn^{2+}-free monomer (60, 157, 158), residues B1-B6 are extended (asterisk in **Figure 1B**); B7-B10 comprise a β-turn adjoining the central α-helix. Similar features occur in the crystallographic T-state protomers within Zn^{2+} hexamers (47, 161). The N-terminal five residues favor A7-B7 disulfide pairing *in vitro* (136, 137) and to overall efficiency of proinsulin folding in cell lines (141). These residues are dispensable for receptor binding (162). Although Phe^{B1} has not been identified to date as a site of clinical mutation, studies of *des*-B1 analogues nonetheless suggest that its loose T-state-specific packing against a nonpolar A-chain surface (principally the otherwise exposed side chain of Leu^{A13}) contributes to disulfide specification (12). Sites of clinical mutation (His^{B5}, Leu^{B6} and Gly^{B8}; broadly conserved among vertebrate insulins) have been well characterized (30, 96, 98, 99).

i. *Position B5.* In the native state His^{B5} packs within an inter-chain crevice, making one or more hydrogen bonds to carbonyl oxygens in the A chain (**Figure 3A**, left). Clinical mutations are Asp, Gln and Tyr (**Table 1C**); in mammalian cell culture substitution of His^{B5} by Asp blocks the folding and secretion of human proinsulin (30). Although some substitutions impair chain combination (30), Arg^{B5} (found in non-mammalian insulins) is well tolerated. We imagine that His^{B5} and Arg^{B5} form analogous inter-chain hydrogen bonds in the course of disulfide pairing; this hypothesis is in

accordance with the respective crystal structures of WT and Arg^{B5}-insulin (99). Ala^{B5}-insulin (as an engineered monomer) exhibits decreased stability (30), presumably due to the absence of these hydrogen bonds and to a cavity penalty (163, 164). Its solution structure is nonetheless similar to the parent His^{B5} monomer (30), suggesting that critical perturbations in an oxidative folding intermediate can be inapparent in the native state, once reached.

ii. *Position B6*. Leu^{B6} inserts into a deep inter-chain cavity bounded by the invariant side chains of Leu^{B11}, Leu^{B15} and Leu^{A16} (**Figure 3A**, middle). Neonatal-onset mutations are Arg, Gln, Pro and Val (**Table 1C**). Each variant would be expected to be destabilizing in this environment: Arg and Gln *via* insertion of charged or polar functions into a nonpolar cavity, Pro and Val *via* introduction of packing defects. Substitution of the branched and nonpolar side chain of Leu^{B6} by the linear non-polar side chain Met by contrast leads to MODY (**Table 1D**). Delay in clinical onset presumably follows the structural biology: we envision that Met^{B6} can be accommodated within the B6-related cavity but with less optimal packing interactions.

iii. *Position B8*. Special structural principles pertain to position B8. Neonatal-onset mutations are Arg, Ser and Val (**Table 1C**; also Cys in **Table 1B**). In an insulin or proinsulin monomer in solution (48, 60) Gly^{B8} exhibits a positive ϕ dihedral angle [as in the crystallographic T state (47)] and so occupies a position in the Ramachandran plane ordinarily forbidden to L-amino acids (**Figures 4A, B**). In a protein-folding intermediate an L-amino-acid side chain at B8 would presumably change the orientation of Cys^{B7} and so impair its pairing with Cys^{A7} (**Figure 4C**) (98). The side chain itself would be expected to project into solvent.

Kent, Weiss and colleagues described synthetic studies of human proinsulin variants containing L-Ala or D-Ala at B8 (109). Such protein diastereomers exhibited L-specific impairment of specific disulfide pairing; D-Ala^{B8} was well tolerated, presumably due to its enforcement of a positive ϕ angle favorable to [B7-A7] pairing. These findings corroborated prior studies of mini-proinsulin analogues (134, 165) and insulin chain combination (96). In the latter stereospecific B-chain libraries were exploited to demonstrate that L-substitution at B8 generally impair chain combination whereas yield was generally enhanced by D-substitutions (96). Together, these studies rationalize the invariance of Gly^{B8} among vertebrate insulins and insulin-related polypeptides and the diversity of clinical mutations at this site. Interestingly, Ser^{B8}-insulin (but not Ala^{B8}-insulin) exhibits substantial biological activity despite its reduced foldability (109). Indeed, its solution structure retain native-like features. Decreased thermodynamic stability was nonetheless observed, presumably due to an unfavorable local main-chain conformation on the right side of the Ramachandran plot (166).

Central α-helix. Nascent α-helical structure in the B chain has been observed in one- and two-disulfide analogues containing the key [A20-B19] disulfide bridge (31, 59, 63, 66, 67, 132). Neonatal-onset mutations have been identified at positions B11, B15 and B18 (**Table 1C**) as described in turn.

(i, ii) *Helicogenic residues B11 and B15*. Leu^{B11} and Leu^{B15} each contribute to segmental α-helical propensity (167, 168) and to the nascent clustering of nonpolar residues (31, 131). We imagine that mutations at these sites (Pro or Gln at B11, Pro or Val at B15; **Table 1C**) would impede nascent α-helix formation and in turn initial [B19-A20] disulfide pairing. In the mature structure the B11 side chain is buried within a cavity abutting the nonpolar inner surface of the A chain (**Figure 3A**, right) whereas the B15 side chain packs within a shallower neighboring inter-chain crevice delimited by Cys^{B19} and Phe^{B24} (**Figure 3B**, left). Should native disulfide pairing be achieved, we would expect that that mutations Pro^{B11} and Pro^{B15} would profoundly perturb native structure, stability and self-assembly. Gln^{B11} and Val^{B15} would also be destabilizing, but likely less so than Pro. Gln^{B11} would fit within the B11-related cavity, but its carboxamide group would impose an electrostatic penalty; the smaller, β-branched side chain of Val^{B15} would be predicted to attenuate segmental α-helical propensity (167, 168) and impose a cavity penalty (163).

The importance of Leu^{B11} and Leu^{B15} to folding efficiency was first demonstrated in a model organism. Ala substitutions at these positions (although compatible with α-helix) were found to hinder secretion of mini-proinsulin in *S. cerevisiae* (134). Insulin chain combination was likewise impaired by interchange of Leu^{B11} and Val^{B12}, presumably due to perturbed long-range packing (93). Native spacing between Cys^{B7} and Cys^{B19}—and hence length of the central B-chain α-helix—are also likely to influence the efficiency of disulfide pairing as a complex MIDY mutation combines a point mutation with deletion with an adjacent residue: Leu^{B15}-Tyr^{B16} are replaced by His (43), leaving an even number of residues between the B-chain cysteines.

(iii) *Non-helicogenic residue B18*. Val^{B18} packs near cystine [B19-A20] in a solvent-exposed inter-chain crevice. This environment is polar on one side (due to Glu^{A17}) and non-polar on other sides (due to the cystine, Ala^{B14}, Leu^{A13} and Leu^{A16}). Although the β-branched side chain of Val is not in principle favorable within an α-helix (167, 168), its mutation to Gly (also of low helical propensity; **Table 1C**) would enhance main-chain flexibility and introduce an inter-chain packing defect; each perturbation could reduce efficiency of [B19-A20] disulfide pairing. In the native state ^1H-^2H exchange studies in D_2O have established that the main-chain amide proton of Val^{B18} is the most highly protected site in insulin (159). Extending this to variant on-pathway folding intermediates, we propose that enhanced segmental conformational fluctuations and decreased thermodynamic stability could each contribute to impaired biosynthesis.

B20-B23 β-Turn. The B chain contains a U-turn between its central α-helix and C-terminal β-strand (B24-B28). This super-secondary motif requires a solvent-exposed β-turn (Gly-Glu-Arg-Gly tetrapeptide motif). Like Gly^{B8} (above), the flanking glycines each exhibit positive ϕ angles associated with a specific pattern of hydrogen bonds within the turn (47). Discussed more fully in the following section (MODY), mutation of Gly^{B23} to Val

is associated with neonatal-onset DM (**Table 1C**). Cell-based and biophysical studies of this mutation have demonstrated profound perturbations (97). Qualitative NMR studies suggest that the β-branched side chain leads to transmitted perturbations in the position or conformation of the following B24-B27 segment (12).

A-chain mutations. Studies of peptide models have suggested that initial pairing of cystine [B19-A20] is coupled to nascent α-helical conformations of the A16-A19 segment, coincident with nonlocal hydrophobic collapse of Leu^{A16} and Tyr^{A19} within a folding nucleus (31, 131). Indeed, substitutions at these sites were found to impair the yield of insulin chain combination (94, 95, 156). In accordance with the above mechanism and such synthetic experience, recent clinical studies have uncovered neonatal-onset MIDY mutations Pro^{A16} and Asp^{A19} (**Table 1C**).

The structural environments of α-helical residues A16 and A19 are distinctive. Whereas Tyr^{A19} projects from a non-polar crevice (lined in part by cystine [B19-A20]) to expose its *para*-hydroxyl group (**Figure 3B**, right), the side chain of Leu^{A16} is inaccessible to solvent (**Figure 5**). Asp^{A19} would place a negative charge within the non-polar confines of the core. Pro^{A16} would perturb segmental main-chain conformation and (when modelled in a native-like framework) introduce both side-chain steric clash and a destabilizing cavity. The essential contribution of Leu^{A16} to protein-folding intermediates has been demonstrated through studies of Val^{A16}-proinsulin and Val^{A16}-insulin (156). Although this substitution is compatible with a native-like crystal structure (essentially identical to WT insulin), Val^{A16} markedly impairs both insulin chain combination and cellular folding of the variant proinsulin (156). Because Val^{A16}-insulin also exhibits high biological activity (156), the evolutionary invariance of Leu at this position presumably reflects its cryptic yet key contribution to folding efficiency.

MIDY mutations have not been identified in the N-terminal A-domain α-helix (residues A1-A8). Their absence may simply reflect incomplete sampling of patients to date; however, it is also possible that non-cysteine residues in this segment are tangential in the mechanism of disulfide pairing. Indeed, successful combination of variant A chains containing Gly at positions A1-A2, A1-A4 or A1-A4 (in each case with WT B chain S-sulfonate) provided evidence that an N-terminal A-chain α-helical conformation is not required for native disulfide pairing (95). Such dispensability is in accord with a putative structural pathway in which segmental folding of this α-helix is a late event.

FROM MIDY TO MODY

INS mutations may also be associated with onset of DM in childhood or adolescence (**Table 1D**) (169–171); diagnoses may be carried as auto-antibody-negative presumed Type 1 DM or Type 2 DM. Substitution of Val^{B18} (**Figure 3B**, center) by Ala (172) was identified as a MODY allele (DM onset <25 years of age, autoantigen negative) in a three-generation Italian pedigree (three siblings, the parent and presumed grandfather) (172). Unlike MIDY patients with neonatal onset, birth weights were normal. The Ala mutation at position B18 would be expected to enhance segmental α-helical propensity (167, 168), but introduce a destabilizing cavity (163, 164) adjacent to the critical [B19-A20] disulfide bridge. Unlike the perturbations introduced by Gly^{B18} (above), these effects would offset to yield, rationalizing a mild net impairment of initial disulfide pairing.

Four additional MODY mutations occur within the B20-B23 β-turn and its aromatic anchor at B24: Gly^{B20}→Arg, Arg^{B22}→Gln, Gly^{B23}→Asp and Phe^{B24}→Ser (**Figure 6**). Although the mechanism by which Gln^{B22} causes MODY is not apparent, L-amino-acid substitutions of Gly^{B20} or Gly^{B23} would be expected to alter their respective ϕ dihedral angles. It has previously been reported that Ala substitutions impair the expression of mini-proinsulin in *S. cerevisiae* and impede chain combination, whereas efficient disulfide pairing *in vitro* can be rescued by D-Ala substitutions (97). That B23 mutations may cause either neonatal onset (Val^{B23}) or delayed onset (Asp^{B23}) suggests that details of side-chain chemistry influence folding efficiency.

Ser^{B24} (originally designated insulin *Los Angeles*) is associated with variable genetic penetrance with hyperinsulinemia. The latter finding indicates that Ser^{B24}-proinsulin can in fact fold in the β-cell ER, undergo proper trafficking and processing to mature Ser^{B24}-insulin (173). In cell culture the variant proinsulin nonetheless induces ER stress, albeit at a level below MIDY variants (12). In vivo mutational induction of mild or moderate ER stress can presumably cause (depending on other genetic risk alleles and environmental factors) slow but progressive loss of β-cell mass (174, 175) as in the Akita mouse (24, 176).

The final MODY-associated mutation occurs on the surface of the A domain: Glu^{A4}→Lys (**Table 1D** and **Figure 7A**). That this substitution should perturb the folding of proinsulin seems surprising given (a) the absence of structural constraints at this position in insulin and (b) the broad tolerance of insulin chain combination to substitutions within the N-terminal A-chain α-helix (95). We speculate that Lys^{A4} introduces a subtle perturbation in proinsulin through electrostatic repulsion of the dibasic element at the CA junction (red box in **Figure 7B**). In particular, nascent α-helical structure in the A1-A8 segment may be stabilized by a salt bridge between "Arg^{A0}" (*i.e.*, the final residue of the C domain [position 89 of preproinsulin]; **Figure 7A**) and WT Glu^{A4} (**Figure 7C**). Such an interaction, together with Gly^{A1}, could in essence provide a favorable N-Cap (177), which could overcome the adverse helical propensities of the three β-branched residues in this segment (Ile^{A2}, Val^{A3} and Thr^{A8}). This contribution would not pertain to insulin chain combination due to the absence of Arg^{A0} (an analogous C-capping salt bridge from Glu^{A4} to the A1 α-amino group would be blocked by its deprotonation at the reaction pH of 10.5).

DIVERSITY OF INS-RELATED DISEASE MECHANISMS

For completeness, we note that mutations in the insulin gene that are not associated with impaired folding can nonetheless

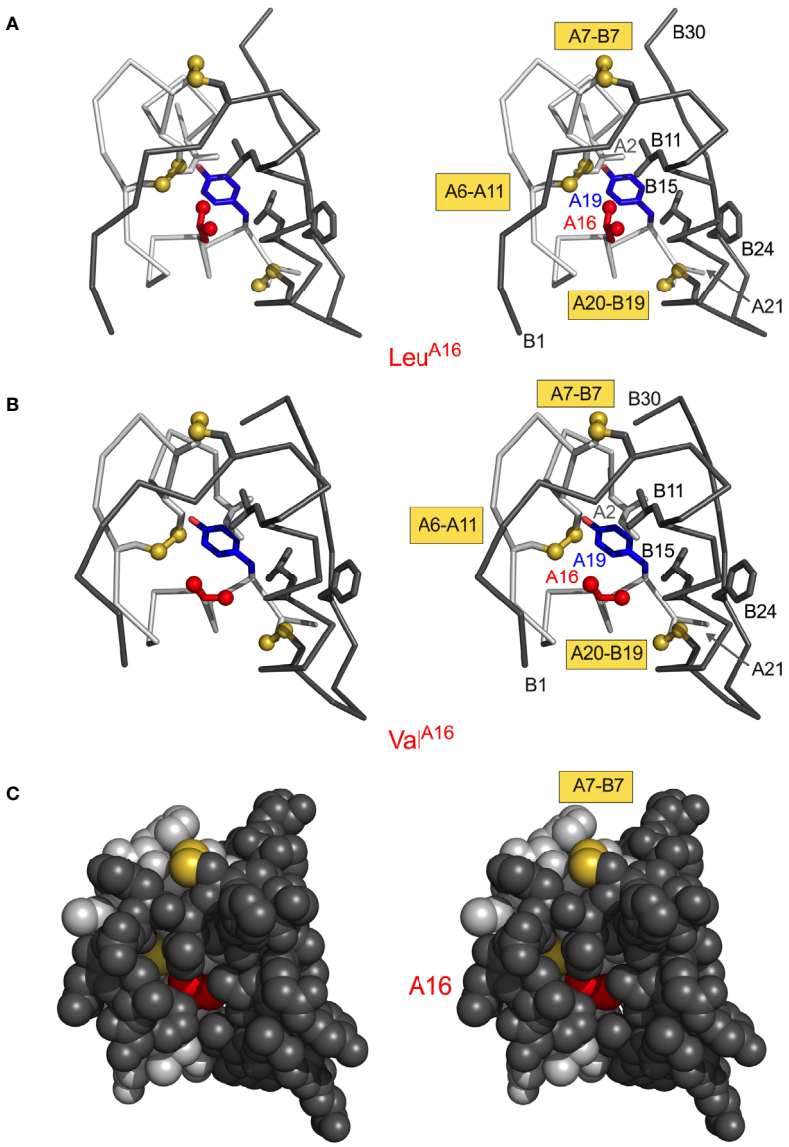

FIGURE 5 | Structural environment of conserved position A16. **(A)** Leu^{A16} packs in core of insulin monomer: ribbon model (stereo pair) showing Leu^{A16} (red) in relation to Tyr^{A19} (blue) and internal side chains Ile^{A2} (light gray), Leu^{B11} (dark gray) and Leu^{B15} (dark gray). A- and B-chain ribbons are shown in light- and dark gray, respectively; disulfide bridges are shown as gold spheres. Molecular coordinates were obtained from PDB entry 4INS (47). **(B)** Corresponding ribbon model (same orientation) of "non-foldable" analogue Val^{A16} (PDB entry 3GKY) (156). Structural similarities highlight cryptic folding defect. **(C)** Stereo space-filling model showing limited exposure of internal Val^{A16} side chain (red) between B-chain surface (dark gray, overlying surface) and A-chain surface (light gray). The solvent-exposed A7–B7 disulfide bridge is shown in gold (top); internal cystine side chains A6–A11 and A20–B19 are not visible.

be associated with adult-onset DM phenotypes of variable penetrance (57) (**Table S1**). Such heterogeneity is in accord with "Murphy's Law of genetics": in a complex pathway or set of mechanisms, what can go wrong will go wrong. For example, insulin variants *Wakayama* and *Chicago* (*i.e.*, classical insulinopathies Val^{A3}→Leu and Phe^{B25}→Leu respectively) markedly impede receptor binding (173) in association with mutant hyperinsulinemia (178). These mutations directly perturb the hormone-receptor interface (160). A complementary example is provided by diabetes-associated mutation His^{B10}→Asp, which enhances receptor binding (179). Although Asp^{B10} would introduce a favorable electrostatic interaction at the hormone-receptor interface, in β-cells Asp^{B10}-proinsulin exhibits inappropriate sorting to a constitutive granule (180, 181). Unlike glucose-regulated secretory granules, constitutive granules lack prohormone convertases, and so the patients exhibit mutant hyperproinsulinemia. Yet another syndrome is characterized by impaired prohormone processing leading to circulation of a split proinsulin with reduced activity (182).

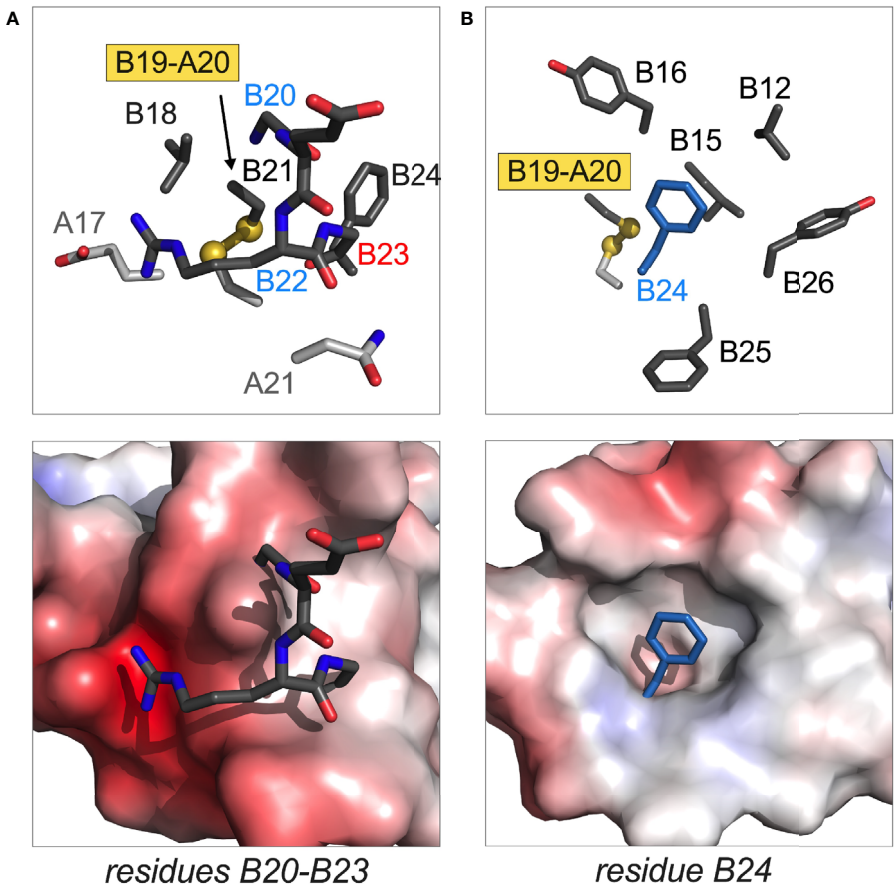

FIGURE 6 | Structural sites of MODY mutations. **(A)** Residues B20 and B22 in B-chain B20-B23 β-turn; **(B)** residue B24 anchoring this β-turn and adjoining B24-B28 β-strand. In each pair of images, stick models are in upper panel and electrostatic surfaces in lower panel. The latter highlights the groove or cavity occupied by the designated structural element; blue and red surfaces are coded by positive or negative electrostatic potential. In stick models main-chain atoms in A- or B main chains are shown in light or dark gray, respectively. Disulfide bridges are shown as balls and sticks with sulfur atoms in gold (one-third Van der Waals radii). The side chain of Phe^{B24} in **(B)** is shown as dark blue stick (not related to electrostatic potential). Coordinates were obtained from PDB entry 4INS (47).

EVOLUTION AT THE EDGE OF FOLDABILITY

Protein evolution is generally enjoined by overlapping biological constraints, including biosynthesis, structure, and function (**Figure 8A**). Particular residues in insulin may thus contribute to one or more critical mechanisms, including nascent foldability in the ER, protection from intra- or extracellular toxic misfolding, trafficking from the ER through the GA to glucose-regulated secretory granules, self-assembly within these granules, disassembly of Zn^{2+}-insulin hexamer in the portal circulation and in turn receptor binding. The stringency of these overlapping constraints rationalizes the limited sequence variation among vertebrate insulins (47).

Evolutionary constraints may be coincident or opposing at a given position. An example of a coincident constraint is the concurrent contributions of invariant Phe^{B24} to core packing, dimerization and receptor binding. Opposing constraints call for compromise. An example is provided by Gly^{B8}, invariant as an achiral amino acid free to roam in the Ramachandran plane. Systematic studies of L- or D substitutions have suggested that at B8 kinetic determinants of foldability are at odds with conformational requirements of receptor binding (96, 98). Whereas a positive ϕ dihedral angle (enforced by a D-substitution) facilitates disulfide pairing, a D side chain impedes receptor binding. Conversely, negative dihedral angle (like that of an L-amino acid) impair folding efficiency but may be compatible with receptor binding (96, 98). These opposing requirements presumably underlie the invariance of glycine – the only achiral amino acid – at a site of conformational change. A switch in conformation of Gly^{B8} between the right side of the Ramachandran plot and the left (respectively corresponding to positive or negative ϕ angles) was anticipated by the classical TR transition among zinc insulin hexamers (187). Although such allostery may pertain only to hexamers (99), the TR transition exemplified the long-range transmission of conformational change (188) —a theme central to the transmembrane propagation of an insulin signal *via* receptor reorganization

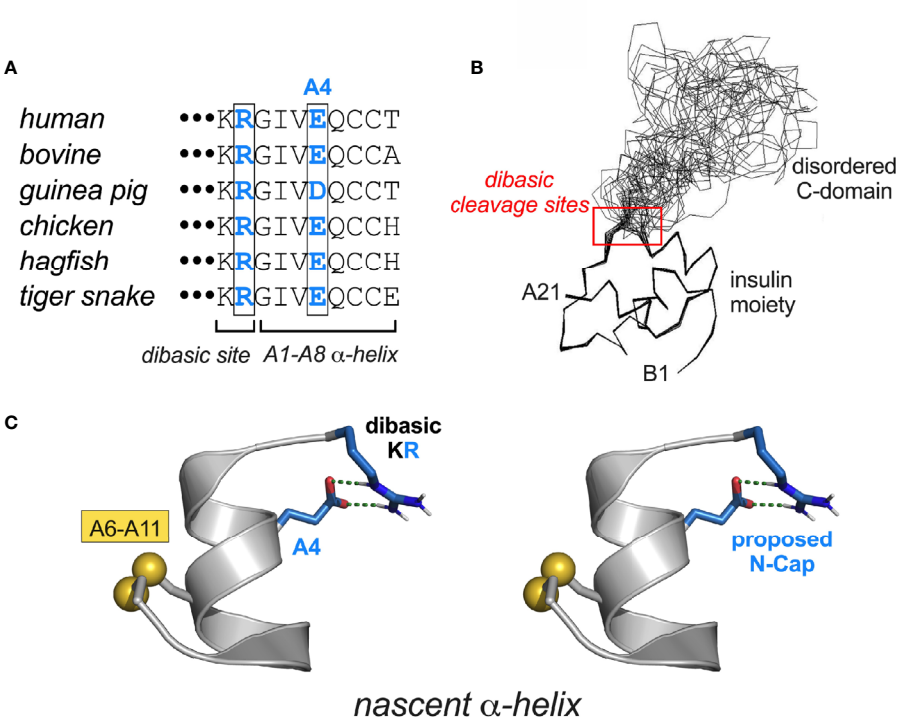

FIGURE 7 | MIDY-related mutations at CA junction. **(A)** Vertebrate sequence alignment showing conserved KR dibasic site (CA junction) and acidic side chain at residue A4. **(B)** Solution structure of proinsulin (line drawing) showing the folded insulin moiety and disordered C-domain (48). The dibasic RR (BC junction) and KR sites (CA junction) are within red box. **(C)** Proposed stabilization of a nascent α-helix in proinsulin folding intermediate by junctional (*i*, *i*+4) salt bridge between residues Arg and Glu^{A4} (blue in panels **A**, **C**). The putative salt bridge was modeled as an α-helical N-Cap element (177) using PyMOL.

(189–191). The examples posed by clinical mutations at B24 and B8 (**Table 1C**) suggest that premature adoption of the hormone's receptor-engaged conformation within β-cells (either by proinsulin in the ER or GA or by insulin in secretory granules) may trigger toxic misfolding.

Recent co-crystal and cryo-EM-derived structures of insulin bound to receptor fragments have demonstrated the function of a protective hinge in B chain (160, 189, 190, 192). Mechanisms of hormone-receptor recognition [for review, see (191)], extend to IGF-I as visualized in a landmark series of homologous cryo-EM-derived structures of respective receptor ectodomain complexes (189, 190, 193–195). As predicted based on studies of "anomalous" insulin analogues (157, 196, 197), detachment of the C-terminal β-strand (residues B24-B28) enables both its own binding in a groove between receptor elements L1 and αCT (respectively at the N- and C-terminal ends of the IR α-subunit); the latter element also packs against the N-terminal A-chain α-helix.

Insulin's B-chain hinge—opened on receptor binding—may represent an evolutionary response to the danger of proteotoxicity. This danger, aggravated by exposure of non-polar surfaces, is intrinsic to the coupled folding/misfolding landscapes wherein the true ground state is defined by β-sheet-rich amyloid (**Figure 8B**). Models of insulin amyloid as superhelices of protofilaments have been derived at low resolution by cryo-EM (**Figure 8C**). Studies of insulin fibrils by infrared and Raman spectroscopy have demonstrated a predominance of β-sheet (198–200) in accordance with fibril X-ray diffraction (201–203). Despite the striking biophysical features of fibrils as a universal thermodynamic ground state of polypeptides (**Figure 8B**) (85), oligomeric intermediates in the pathway of fibrillation pose the greater cytopathic danger (**Figure 8D**) (204).

Recent evolutionary studies of insulin have highlighted the importance of Phe^{B24}, whose conserved aromatic ring plays multiple roles: anchoring the native B-chain β-strand, stabilizing the α-helical core, and contributing to both self-assembly (47) and hinge opening on receptor binding (197). In the open state the aromatic ring binds within a classical nonpolar pocket at the hormone-receptor interface (189, 190, 195). On substitution of Phe^{B24} by Gly, native function is paradoxically retained (157). Comparative studies of "register shift" analogues indicate that an alternative mode of receptor binding supervenes in which Phe^{B25} takes the place of the missing Phe^{B24} (52); residues B20-B24 form a flexible pentaloop rather than an aromatic-anchored β-turn (205). This alternative binding mode is apparently disallowed in evolution due to toxic misfolding of Gly^{B24}-proinsulin (as evidenced impaired folding efficiency, induction of ER stress and impaired secretability in transfected cell models) and possibly by the heightened

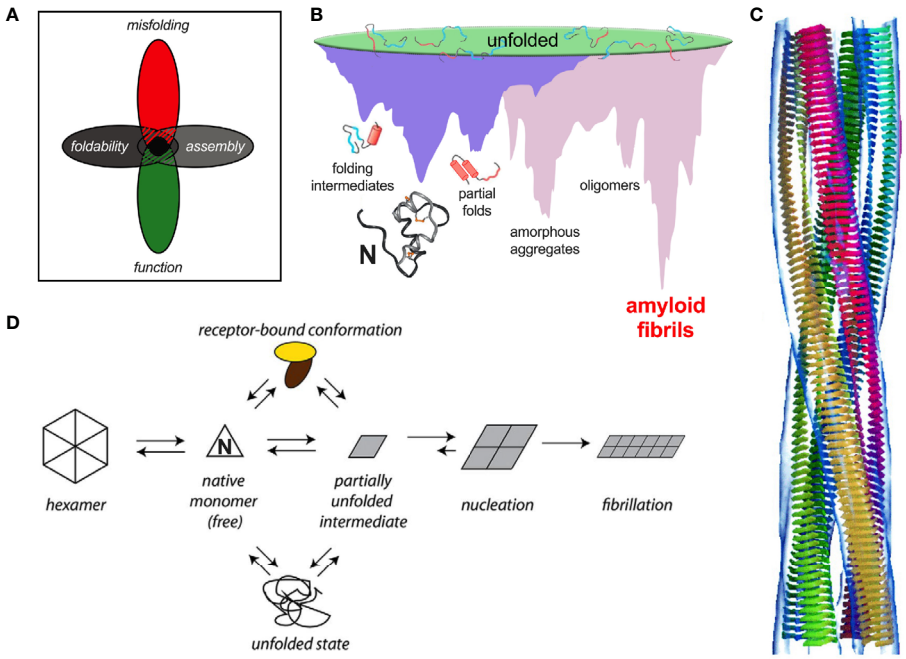

FIGURE 8 | Evolutionary constraints and insulin fibrillation. **(A)** Venn diagram showing intersection of multiple constraints: function, foldability, misfolding, and assembly (183). **(B)** Energy landscape of protein folding (purple) and coupled landscape of aggregation (pink) (184). Cytotoxic oligomers may form as off-pathway intermediates en route to amyloid. **(C)** Models of protofilament packing based on low-resolution cryo-EM images. The image is reproduced from the reference (185), Copyright (2002) National Academy of Sciences. **(D)** General scheme of insulin fibrillation *via* a partially unfolded monomeric intermediate (parallelogram at center) (186). The native state (triangle) is protected by classic self-assembly (far left). Disassembly leads to an equilibrium between native and partially folded monomers. The receptor-bound conformation of insulin (top) may also participate in this equilibrium. This partial fold may unfold completely (bottom) as an off-pathway event or aggregate to form an amyloidogenic nucleus en route to a proto-filament (right).

susceptibility of Gly^{B24}-insulin analogues to fibrillation (52). Evidence for the paradoxical evolution of vertebrate insulins to the edge of foldability has been provided by biophysical studies of a native-like variant, Tyr^{B24}-insulin (40). Although providing the C-terminal B-chain β-turn and β-strand with an homologous "aromatic anchor," Tyr^{B24} is also disallowed due its perturbation of biosynthesis and induction of ER stress. Indeed, of the 20 natural amino acids, only Phe at position B24 enables the efficient biosynthesis of proinsulin (40). We speculate that such marked sensitivity to mutation—signifying the paradoxical non-robustness of an adaptive landscape (40)—will be found at many or most sites associated with neonatal-onset DM (**Table 1C**).

Because, to our knowledge, clinical mutations that selectively perturb insulin's hexameric structure and storage in secretory granules have not been described, this review has not focused on these processes. Any such perturbations would be downstream from the major sites of perturbation in the MIDY syndrome: misfolding in the ER and impaired trafficking through the GA. It is possible, however, that processes in the secretory granule are affected by Ser^{B24} and Asp^{B10} in concert with other perturbations. (i) *Phe^{B24}→Ser*. The invariant aromatic ring of Phe^{B24} packs at the dimer interface. Its substitution by Ser^{B24} impairs self-assembly (as monitored by gel-filtration) and leads to accelerated disassembly of the R_6 hexamer once formed (40). Receptor binding and biological activity are low. (ii) *His^{B10}→Asp*. The conserved imidazole ring of His^{B10} coordinates the axial zinc ions at the trimer interface of insulin hexamers (47). Genetic variant Asp^{B10} causes a diabetes syndrome characterized by baseline mutant proinsulinemia due to constitutive secretion (180) as the mutation perturbs specific trafficking to glucose-regulated secretory granules (34, 57). The corresponding substitution in insulin blocks both zinc binding and trimer formation *in vitro* (206, 207). Asp^{B10}-insulin exhibits increased affinity for both IR and IGF-1R with prolonged residence times in association with augmented mitogenic signaling (179, 208–211).

AN EVOLUTIONARY HYPOTHESIS

Given the ancestral history of metazoan insulin-like proteins over the past 540 million years and its broad radiation among diverse body plans (212–214), why might vertebrate proinsulins be susceptible to misfolding and lacking in mutational robustness? A possible answer is given by the history of the *INS* gene as traced by the late D.F. Steiner and coworkers (215–218). This seminal study characterized an insulin-like gene encoding an insulin-like protein (ILP) in an extant protochordate (amphioxus; *Branchiostoma californiensis*) (**Figure 9A**). The predicted polypeptide precursor pro-ILP contains a C-terminal peptide resembling the D and E domains of vertebrate IGFs, suggesting an intermediate form

linking the ancestral proto-insulin gene with modern IGF genes. In accordance with this perspective, *ILP* is the only *INS*-like gene in amphioxus; its genome also contains a single gene encoding a putative insulin-IGF receptor (216) and a single gene encoding a putative IGF-binding protein (IGFBP) (220, 221).

ILP was thus proposed to combine the functions of insulin, IGF-I and IGF-II prior to the duplication the proto-insulin gene and specialization of distinct factors (215). Evolutionary changes in intron-exon structures are shown in greater detail in **Supplemental Figure S1A**. In this scheme conversion of a metazoan proto-insulin gene to ILP would have been effected by a nonsense-to-sense mutation at the end of the A-domain-encoding sequence in the putative proto-insulin gene; conversion of ILP to proto-lGF would have been effected by an upstream shift in the intron donor site into the B-domain-encoding exon. IGF-I and -II genes subsequently evolved from the posited proto-IGF by insertion of an intron into the E-encoding domain followed by gene duplication.

Thus, predating the divergence of insulin and IGFs as distinct gene products, ILP retains framework residues conserved among vertebrate insulins and IGFs, including the six canonical cysteines, Leu^{B6}, Leu^{B11}, Leu^{B15}, Val^{B18}, Leu^{A16} and Tyr^{A19}—hotspots for MIDY mutations (**Table 1C**). Whereas mammalian insulins contain Leu^{B17}, however, residue B17 in ILP is Phe as in IGFs. Similarly, ILP residue A8 is Tyr, resembling the homologous Phe in IGF-I and IGF-II but unlike Ala^{A8} or Thr^{A8} in mammalian insulins (215, 218). ILP would not be expected to undergo insulin-like self-assembly: (a) it lacks a His at position B10 and so would not be expected to coordinate zinc ions; and (b) dimerization would be predicted to be impaired by ILP residues Ala^{B12} and Ser^{B26} (in place of Val^{B12} and Tyr^{B26}) (183, 222). Representative vertebrate insulin B-chain sequences and IGF-I B-domain sequences are shown in **Supplemental Figure S1B**.

Given the evolutionary framework established by Steiner and coworkers (215–217), we hypothesize that the primordial insulin/IGF precursor protein folded as a heterodimer in partnership with a proto-IGFBP. Such heterodimeric folding occurs in vertebrate IGF-IGFBP systems (221, 223–226) and appears to compensate for the ambiguous refolding properties of

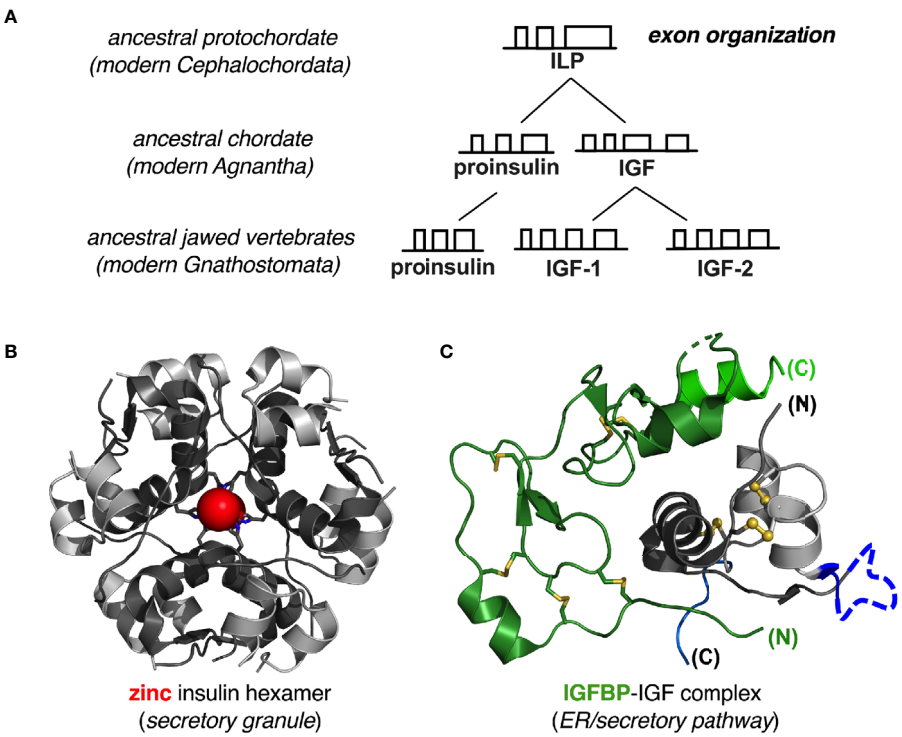

FIGURE 9 | Evolution of insulin-like genes in vertebrates. **(A)** Steiner and colleagues proposed that a primordial insulin-like gene in protochordates was the common ancestor of vertebrate insulin/IGF factors (215, 217). Amphioxus ILP represents this ancestral gene (as a "living fossil") prior to gene duplication in early agnathan vertebrates. Further gene duplication results in distinct IGF-I and IGF-II genes found in all gnathosomes (jawed vertebrates). Independent gene duplication events occurred during the evolution of invertebrate insulin-like peptides (not shown). See **Supplemental Figure S1A** for further details of exonic structure. **(B)** Proinsulin folds autonomously, and the mature hormone is stabilized by native self-assembly: ribbon model of classical zinc-insulin hexamer. The A- and B chains are shown in light- and dark gray, respectively; the two overlying axial zinc ions are shown as red spheres (enlarged for emphasis at twice Van der Waals radii), each coordinated by three His^{B10} side chains (sticks). **(C)** IGF-I and IGF-II fold together with specific IGF binding proteins: ribbon model of illustrative IGFBP-IGF complex. We hypothesize that heterodimeric folding "rescues" the foldability of the IGFs, whose folding properties would otherwise be ambiguous. Color codes as follows: IGF domain A (light gray), domain B (dark gray), domain C (dark blue) and domain D (light blue); IGFBP N-terminal domain (dark green) and C- terminal domain (light green). Molecular coordinates were respectively obtained from PDB entries 1MSO (zinc insulin hexamer) (186) and 2DSP (IGF-1/IGFBP-4 complex) (219).

IGFs *in vitro* (28, 29, 123, 130). We envisage that in heterodimeric folding requirements of foldability are relaxed in each partner (when considered in isolation). Crystal structures of human IGF-I/IGFBP complexes [illustrated in a representative case in **Figure 9C** (219)] exhibit extensive engagement of IGF surfaces adjoining disulfide bridges and sites of MIDY mutations. This model predicts that the foldability of pro-IGF variants in mammalian cells would be more robust to MIDY-like mutations than is proinsulin—*but only in cells co-expressing one or more IGFBPs*.

Proinsulin by contrast folds in the ER as an autonomous monomer, aided by chaperonins and oxidoreductases but not, to our knowledge, by specific proinsulin-binding proteins. Native zinc-mediated self-assembly of insulin (**Figure 9B**) can include proinsulin [which can form corresponding hexamers (69)], but such self-assembly occurs in secretory granules and not in the zinc-poor environment of the ER. Although IGFBPs do not bind to insulin or proinsulin, this model predicts that engineered proinsulin-binding proteins may be designed to enhance the foldability of WT and variant proinsulins. Although such artificial proteins are unlikely to find therapeutic application, they may be of interest as reagents to probe the mechanism of proinsulin folding *in vivo*, including steps susceptible to misfolding.

STRUCTURAL DETERMINANTS OF ER QUALITY CONTROL

Arvan and colleagues have studied the ER folding of proinsulin in β-cell lines using a systematic set of variants that can form only one or two disulfide bonds; to this end, specific disulfide bridges were removed by pairwise mutagenesis (227). These constructs differed in biosynthetic properties and so provided probes of quality-control determinants. Their results demonstrated that cystines A20-B19 and A7-B7 (but not cystine A6-A11) are critical to enable native folding and ER exit. Prior biophysical studies of an insulin analogue lacking cystine A7-B7 (due to pairwise Ser substitution) demonstrated a more marked decrease in stability and chain-combination efficiency relative to analogous analogues lacking A6-A11 (60, 132). Further studies on single-chain insulin analogues (67) and IGF-1-related peptides and peptide fragments (29, 31, 60) provided evidence for a kinetic pathway in which pairing of cystine A20-B19 provides a required first step to stabilize a native-like molten mini-core (31). Native-like NOEs were observed in such a one-disulfide peptide model even in the absence of stable secondary structure (31). Together, these cell-based and *in vitro* studies suggest possible structural features that might be sensed by ER quality control: as a general principle, the more destabilizing the disulfide intermediate or isomer destabilizing, the greater the degree of exposed non-polar surfaces and in turn the intervention of detection and degradation by the quality-control machinery.

Whereas variant proinsulin polypeptides without interdomain disulfide bridges cannot be secreted (227), non-native disulfide isomers can accumulate and evade ER quality control (138, 140). Early work from the Arvan laboratory demonstrated secretion of mispaired disulfide isomers in cells using various single-chain insulin constructs (138, 140). Indeed, prior studies of IGF-I revealed that its oxidative refolding *in vitro* yielded two isoenergetic products (28, 123). Although these had similar α-helical propensities and thermodynamic stabilities, 2D ^{1}H-NMR spectra were remarkable for distinct well-dispersed patterns of chemical shifts, indicative of different three-dimensional structures (28). Unlike IGF-I and its disulfide "swapped" isomer, insulin disulfide isomers are less stable and less well-ordered than is native insulin (75, 76). The respective N-terminal segments of proinsulin and IGF-I contribute to such salient differences in the fidelity of disulfide specification and relative stability (228, 229). The chain asymmetry of non-Cys-related MIDY mutations—more in the B domain than in the A domain (**Figure 1**)—is consistent with a hierarchical disulfide pathway in which nascent structure in the B domain provides a structural template for folding of the A domain (95).

Mutations that impair the foldability of proinsulin (or efficiency of insulin chain combination (95) can nonetheless be compatible with high activity (108). This lack of correlation suggests that determinants of quality control in the ER differ from determinants of receptor binding. A prominent example is provided by substitution of invariant Leu^{A16} by Val (156). This cavity-associated mutation (not [yet] seen among MIDY patients) markedly impairs both cellular folding of Val^{A16}-proinsulin and chain combination, and yet substantial biological activity is retained once the native state is reached (94, 156). Similarly, folding of Ser^{B8}-proinsulin is significantly reduced *in vitro*, yet IR affinity is similar to WT insulin (109). A recent study reported that substitution of Phe^{B24} by Tyr (also not [yet] seen among MIDY patients) blocks cellular folding (40) whereas the corresponding two-chain insulin analogue retains substantial activity in a rat model of DM.

CONCLUDING REMARKS

We imagine that insulin's conserved side chains, as exemplified by Phe^{B24}, play different roles in the course of a complex conformational "life cycle." If so, this would represent a marked compression of structural information within a short protein sequence. The present cryo-EM revolution promises to provide snapshots of structures through this life cycle, likely to be extended by solid-state NMR-based models of non-native insulin aggregates and fibrils. As we celebrate the Centennial of insulin's discovery in Toronto in 1921 (1, 230)—and coincidentally the gold anniversary of its high-resolution crystal structure at Oxford in 1971 (161)—it is remarkable to appreciate how much remains to be discovered in relation to biosynthesis, folding, function and evolution. Further, in a related review article, J. S. Flier and C. R. Kahn have discussed how the discovery of insulin has defined a milestone in the history of molecular medicine that extends beyond the insulin molecule itself (231).

This review has focused on the structural lessons of the mutant proinsulin syndrome (4–6). Patient-derived experiments of nature are providing an opportunity to investigate biophysical principles at

the intersection of cell biology and human genetics. As envisioned by classical diffusion-collision and framework models (232), folding of globular proteins (such as proinsulin) represent the coalescence of discrete subdomains (233, 234). Even as funnel-like energy landscapes make possible parallel events in folding (126), the existence of preferred trajectories (235) is implied by disulfide trapping studies of insulin-related polypeptides. Biophysical studies of these trajectories and equilibrium models promise to deepen a structural understanding of MIDY/MODY mutations. Sites of mutation reflect mechanisms of folding or misfolding that may not be apparent in the native state (9, 10, 19, 51). Many of the principles discussed here were foreshadowed in pioneering efforts toward the total chemical synthesis of insulin wherein specific disulfide pairing posed a key challenge to chain combination (89).

Foldability is an evolved property (236), highlighting the general threat of toxic misfolding as a hidden constraint in protein evolution. In the genetics of proteotoxic diseases these principles connect bench to bedside. Critical questions for continuing investigation include: can over-expression of the WT *INS* gene in response to peripheral insulin resistance likewise tax the folding capacity of the β-cell and induce ER stress analogous to that of the mutant proinsulin syndrome? Might structural mechanisms of misfolding due to MIDY mutations broadly inform a hidden landscape of toxic aggregation awaiting WT biosynthesis? A key frontier in molecular metabolism is thus defined by the role of the UPR and chronic ER stress in the progression of non-canonical Type 2 DM (9, 24, 25). Molecular dissection of how β-cells respond to the challenge of proinsulin overexpression (237) is of compelling translational interest as a strategy to arrest the progression of prediabetes to frank diabetes (9, 38, 238). Structural lessons of the mutant proinsulin syndrome may thus inform UPR-based approaches to mitigate the growing pandemic of diabesity.

AUTHOR CONTRIBUTIONS

MW oversaw preparation of the manuscript and wrote the first draft. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2021.754693/full#supplementary-material

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REVIEW
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The Review of Insulin Pens—Past, Present, and Look to the Future

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Currently, there are about 150–200 million diabetic patients treated with insulin globally. The year 2021 is special because the 100th anniversary of the insulin discovery is being celebrated. It is a good occasion to sum up the insulin pen technology invention and improvement which are nowadays the leading mode of an insulin delivery. Even though so many years have passed, insulin is still administered subcutaneously, that is why devices to deliver it are of great importance. Insulin pens have evolved only through the last decades (the reusable, durable pens, and the disposable, prefilled pens) and modern smart insulin pens have been developed in the last few years, and both types of the devices compared to traditional syringes and vials are more convenient, discrete in use, have better dosing accuracy, and improve adherence. In this review, we will focus on the history of insulin pens and their improvement over the previous decades.

Keywords: insulin, pen, diabetes mellitus, prefilled pen, smart pen

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INTRODUCTION

The International Diabetes Federation (IDF) estimates that over 537 million people all over the world are currently struggling with diabetes mellitus (DM) (1) and there are about 150–200 million of them treated with insulin (2). The history of insulin dates back to the last century, when in 1921 Frederick Banting and Charles Best with the support of John Macleod and James Collip discovered insulin and thereby revolutionized the treatment of DM (3–5). The first injection of insulin on January 11, 1922, to a 14-year-old boy with the use of reusable glass-bodied syringes (6) started an entirely new era of diabetes management (4, 5) and led to the improvement of insulin delivery methods (3, 5). Even though insulin has been used for 100 years already, its administration remains subcutaneous where insulin pens which evolved only through the last four decades are the leading method of its delivery (about 60% of patients treated with insulin use insulin pens all over the world) (7–9). Insulin pen utility is not the same in different regions of the world. According to a report from the year 2008, insulin pens were used by only 15% of patients in the US, compared with 80%–90% in Europe, and it was suspected that it could be due to limited education regarding the benefits of insulin pens but also their higher price (10). The situation has changed in the next years where data from the year 2011 indicate that the number of patients initiating vial/syringe in the US decreased from 2005 to 2011 to approximately 30% while patients initiating pens increased to approximately 60% (11). According to a IQVIA® report for the period from June 2020 till June 2021 prepared for the purpose of this manuscript [data not published (12)], the usage

of pens in US rose to 59% where in Europe it is comparably high and assessed to be 93.6%. Insulin pens have numerous advantages over traditional vial and syringe injections, among others easy use especially for patients with vision problems or manual dexterity, accuracy of delivering small doses of insulin, and discretion of use (13). It is worth noting that aspects of insulin administration may also contribute to the treatment outcomes even though the type of insulin and its efficacy and safety are the primary factors to consider. It is important to underline that each insulin-producing company has its own insulin pen dedicated to use with the produced insulin. It was proved in some studies that patients who use insulin pens are more adherent to the treatment regimen and have less hypoglycemic events compared to insulin vial users (14–18). Also, numerous studies report that patients' preference for insulin pens exceeds that for vials or syringes (19–21) and portability of insulin pens improves patients' convenience (22). However, it is important to note that the superiority of insulin pens in achieving and maintaining glycemic control has been questioned, and this question has not been resolved up to day (23). American and European guidelines underline the necessity of undertaking patient preference when selecting diabetes treatment especially when treatment is accoutered with pain due to injection (24). That is why recently a study assessing the patient perspective of injectable treatment among patients with type 2 diabetes (T2DM) has been performed and showed that there are some features of the injection device that patients choose more often which may help in future improvement of insulin pens (25). Development of insulin pens is parallel to the development of newer insulin formulation where insulin pen must adapt to changes to dosing and timing requirements like it is in case of modern ultra-long-acting insulin analogue icodec, administered once weekly, which is under development (26). This year, the discovery of insulin turns 100 years, and this provides an opportunity to reflect on its administration methods over the past years.

In this review, we will focus on the history of insulin pens and their improvement over the previous decades, starting from the first-generation insulin pens throughout modern smart insulin pens (**Figure 1**). It must be noticed that clinical trials in relation to the newest smart insulin pens and insulin pen caps are very limited to date, that is why information related to this new technology comes also from manufacturer websites and commercial data resources.

FIRST-GENERATION INSULIN PENS

The first insulin injections were made with large and heavy glass or metal syringes and reusable needles (4, 7, 24–26). Syringe was the only possible way of delivering insulin in clinical practice for the next several decades (4, 7, 27). This method of administration had several and serious disadvantages including poor dose accuracy, lack of social acceptance, and fear of injections (7, 27, 28). These inconveniences of the vial and syringe led to the manufacture of insulin pens. Majority of insulin pens are proprietary devices and are developed to work with specific insulin from the same manufacturer (29). Insulin pens are classified into two categories: being reusable (durable) or prefilled (disposable). The reusable insulin pen is loaded by the patient with replaceable insulin cartridges, and the prefilled insulin pen has the insulin reservoir cartridge already installed and the pen is discarded when the cartridge is empty. Both types of insulin pens can contain a maximum of 3 ml of insulin (30) and can deliver insulin in 0.5-, 1-, or 2-unit (U) increments up to 160 U with the use of a needle which has to be attached to the insulin pen.

REUSABLE INSULIN PENS

In 1985, Novo Nordisk has launched the first reusable insulin pen injector called NovoPen® to overcome barriers of the vial and syringe (31) and started a series of NovoPen® insulin injectors. The new device was a combination of the syringe and insulin vial in one mechanism, resembling a fountain pen (31). NovoPen® contained a disposable, replaceable 1.5-ml insulin cartridge connected with a single-use needle and one-unit incremental dosing (29, 30) which was ready to use whenever needed. This allowed patients to administer multiple, preprandial injections discreetly, and their daily schedule became more flexible (32–34). First studies related to insulin pen comprised only several patients in 1995 (31), but as the development of the devices has grown up, also the number of patients studied increased to several hundreds per study in 2002 (35) and up to several thousands in 2020 (27, 36). Initially, insulin cartridges dedicated to insulin pen contained short-acting insulin for numerous injections before meals and basal insulin was injected with conventional syringes (37). Soon after, in 1988 a new insulin pen NovoPen® 2 was presented to administer NPH and premixed insulins (38–40). Analogically as with short-acting insulins, majority of patients using the device to administer basal or mixed insulin preferred to continue the therapy with pens (38–40). In 1992, NovoPen® 3 was launched which had a maximum dose that could be administered at one time which increased to 70 U (from 36 U with NovoPen® 2) and the dialed doses could be reset without insulin waste. Soon after, in 1996 NovoPen® 1.5 was released which had a smaller insulin cartridge and was shorter in length, followed by NovoPen® 3 Demi to administer 0.5 U dose increments in 1999 and NovoPen® Junior in 2003 which was designed with vibrant colors and developed specifically for children with diabetes. In 2005, NovoPen® 4 was introduced which required reduced force to perform an injection, which had dose increments of 1.0 U and a maximum dose of 60 U (41). Moreover, NovoPen® 4 was reported as simpler to learn and easier to use for both insulin-naïve and currently using NovoPen® 3 patients (42). Following the release of NovoPen®s, other manufacturers have also introduced reusable insulin pens, including the HumaPen® range (Eli Lilly and Company, Indianapolis, IN, USA) and the OptiPen® Pro, OptiClik®, and ClikSTAR® pens (Sanofi, Bridgewater, NJ, USA) The inconvenience of the first insulin pens was no possibility of dialing backward without wasting insulin, but the thing changed with the introduction of NovoPen® 3 and HumaPen Ergo® (35, 41). This option translated to device acceptability in comparison with previous generations of insulin injectors and syringes (43). With time, the option of insulin-free dialing forward and backward became a prevailing way of setting the insulin doses. All mentioned insulin pens had the trigger placed at on the opposite

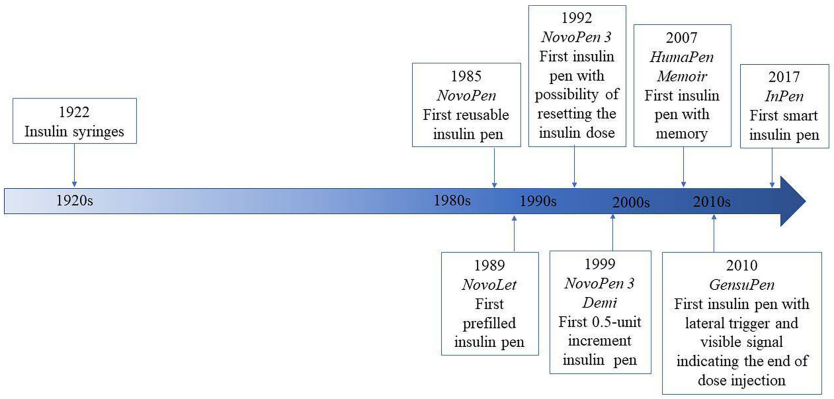

FIGURE 1 | Timeline of insulin pen history.

site of the needle attach end, but there are also insulin pens with a side-mounted release button used for half-automatic insulin delivery, first developed in AutoPen (44), and this mechanism was also present later on in 2010 in GensuPen® and in 2017 in GensuPen® 2 insulin pens (27, 45). Such a mechanism ensured patients about proper insulin administration, simplified the way of injection, and was convenient for elderly patients (27). Moreover, it was proven that the GensuPen® 2 injector in comparison to NovoPen® 4 (Novo Nordisk, Bagsværd, Denmark) and HumaPen Ergo® (Eli Lilly, Indianapolis, IN) requires reduced force for insulin administration, especially at high doses of the drug (46).

In recent years, further improvement in insulin pen function has been made and there are several ones which possess the memory function of the last dose taken. In 2007, Eli Lilly released the world's first digital insulin pen with memory function, namely, HumaPen Memoir (47). Soon after, in 2010, Novo Nordisk launched NovoPen® Echo (48), the first insulin pen with memory function and half-unit dosing feature. Most of the insulin pens available in the market have the feature to deliver insulin in 1-unit increments, and only a few deliver in half-units. 0.5-increment insulin pens are designed for patients who need small insulin doses, and the available ones are HumaPen Luxura HD, Humalog® Junior KwikPen®, NovoPen® Demi, Junior, Echo, JuniorSTAR®, and InPen™. Based on the trials' outcomes, children, adolescents, and their parents appreciated both the memory function and simplicity of junior devices (49, 50).

Cited studies related to reusable insulin pens are summarized in **Table 1**, and the technical characteristics of reusable insulin pens are presented in **Table 2**.

TABLE 1 | Reusable insulin pens.

Study, year	Device studied/ device compared	Type of insulin	Participants	Study design	Results
Berger et al., 1985 (31)	NovoPen®	Short-acting human insulin (Actrapid HM)	16 adults (10 females, 6 males) aged 21–45 years with T1DM	6-week randomized, controlled, crossover study. During the first treatment period (3 weeks), the patients were instructed to take short-acting insulin with the new device and during the next 3 weeks to take the insulin with their conventional syringes. Intermediate/long-lasting insulin was taken with usual syringes in both study periods.	No significant differences ($p > 0.05$) in blood glucose profile, HbA1c, and hypoglycemia frequency were found between syringes and new device use. 14 patients found that the new device made their life easier.
Saurbrey et al., 1985 (51)	NovoPen®	Short-acting insulin (Actrapid HM)	16 adults (10 females, 6 males) aged 21–45 years with T1DM	10-month follow-up study of the study by Berger et al.	15 patients were still using the NovoPen®. There were no differences in mean blood glucose, HbA1c, and number of hypoglycemia ($p > 0.05$). No significant difference between HbA1c values was found between the outcomes after 6 weeks and 10 months of NovoPen® use.
Jefferson et al., 1985 (32)	NovoPen®	Short-acting, human insulin	11 adolescents (7 boys, 4 girls) aged 12–16	3-month observational study. During 4 weeks of the run-in period, the patients were prepared to the study by optimizing the blood glucose levels, and in the end of the fourth week, the therapy was	10 patients completed the study. There was a non-significant reduction of HbA1c. Moreover, mean blood values were lowered but only in pre-lunch measurements were

(Continued)

TABLE 1 | Continued

Study, year	Device studied/ device compared	Type of insulin	Participants	Study design	Results
		(Actrapid HM)	years with T1DM	changed from a conventional to multiple-injection regimen (MIR). Next, the patients started a 3-month observation of the MIR treatment with NovoPen® and a single injection of Human Monotard insulin using a conventional syringe.	significantly reduced ($p < 0.02$). Greater flexibility of timing and size of meals was an overriding advantage of NovoPen® use in the final interview.
Walters et al., 1985 (52)	NovoPen®	Short-acting, human insulin (Actrapid HM)	31 patients (20 males, 11 females) aged 16–57 years with T1DM	48-week observational study. After 4 weeks of run-in period, the participants started therapy with MIR using NovoPen® with one injection of Human Monotard from the usual syringe.	27 patients completed the study. Reduction of mean HbA1c values was observed (11.5% in week 0 vs. 10.3% in week 48, $p < 0.01$). In the final interview, the device was well accepted and 27 patients would like to continue the treatment with NovoPen®.
Dahl-Jorgensen et al., 1986 (33)	NovoPen®	Short-acting, human insulin (Actrapid HM)	10 adults (5 males, 5 females) aged 21–34 years with IDDM.	6- to 9-month observational study. Patients who had used MIR therapy from conventional syringe for a minimum of 1 year previously started using NovoPen® for short-acting insulin injections (Actrapid HM). A single injection of NPH insulin (Insulatard) was maintained.	HbA1c increased during the pen injector treatment (from 8.8% to 9.3%; $p < 0.01$). All but one patient had technical problems with NovoPen®. All participants desired to continue using the pen injector because of the simplicity of the device and greater flexibility of meal time.
Jensen et al., 1986 (53)	NovoPen®	Short-acting, human insulin (Actrapid HM)	20 adults (11 males, 9 females) aged 19–53 years with IDDM.	24-week observational study. Study started with 8 weeks of run-in period. Next, the patients started multiple injections insulin therapy with NovoPen® and a single injection of intermediate-acting insulin (Protaphane) from the conventional syringe.	HbA1c improved during the study (from mean 8.7% to mean 7.9%; $p < 0.05$). The frequency of hypoglycemia was significantly reduced during the training period (from 1.2 attack/patient/week to 0.3 attack/patient/week; $p < 0.01$).
Jorgensen et al., 1988 (38)	Insuject-X (NovoPen® 2)	Intermediate-acting NPH-insulin (Insulatard Human)	50 adults (28 males, 22 females) aged 18–56 years with IDDM	6-month randomized, control, crossover trial. All participants were using MIR of soluble insulin (Velosulin Human) from pen injector (Insuject) with a single injection of NPH insulin (Insulatard Human) from the conventional syringe before the study. The first group was continuing multiple injections with the pen injector and NPH insulin from the conventional syringe in the first 3 months of the trial. In the next study period, the group started to administer NPH insulin (Ultratard Human) in the pen injector Insuject-X. The second study group started the trial in the reverse order.	No differences in the metabolic control were found between both study groups. In the final questionnaires, 86% of the patients found the NPH pen injector less complicated to use than usual syringes. All but 2 patients wished to continue using Insuject-X in the future.
Murray et al., 1988 (34)	NovoPen®	Short-acting, human insulin (Actrapid HM)	78 adults (44 females, 34 males) aged 18–60 years with T1DM	20-week randomized, controlled trial. After a 6-week run-in period of twice-daily injections with fast and intermediate-acting insulin, patients were randomized into 2 groups. One of them (37 patients) was continuing the two-step insulin regimen with usual syringes. The second group (41 patients) started another regimen with 3 times daily injections of Actrapid made with NovoPen® and a single injection of ultralente insulin (Ultratard).	No significant differences ($p > 0.05$) in blood glucose profile, HbA1c, and frequency of hypoglycemia were found between the study groups. Patients presented a high level of satisfaction with NovoPen® for the effect on lifestyle (78%) and increased flexibility (81%). 95% of patients preferred using NovoPen® than conventional syringes. In a questionnaire before the study, 47% of the participants revealed that a rigorous daily schedule for meals and activity was the most important disadvantage. At the end of the study, only 21% and 10% respectively still considered these problems as inconvenient. Moreover, patients expressed greater flexibility of meal times and all but one wanted to continue MIR with NovoPen®.
Saurbrey et al., 1988 (54)	NovoPen®	Short-acting, human insulin (Actrapid HM)	21 adult patients (9 females, 12 males) with T1DM	20-week randomized, controlled, crossover trial. Comparison of intensified conventional treatment (ICT) with continuous subcutaneous insulin injection (CSII). In the first study period (10 weeks), the patients were treated with MIR using NovoPen® with Actrapid insulin plus a single injection of intermediate-acting insulin	19 patients completed the study. HbA1c declined significantly in both groups with no differences between the responses (ICT 7.6%; CSII 8.7%). Mean blood glucose was slightly lower in CSII ($p < 0.05$). There were no differences in frequency of hypoglycemia

(Continued)

TABLE 1 | Continued

Study, year	Device studied/ device compared	Type of insulin	Participants	Study design	Results
				(Monotard HM). In the next 10-week period, the participants were treated by CSII with a Medix or Auto-Syringe pump.	between ICT and CSII. In the questionnaire, all patients found NovoPen® is better than conventional therapy. Moreover, 12 patients would choose ICT with NovoPen® and 6 ones CSII for the future treatment.
Houtzagers et al., 1989 (55)	NovoPen®	Short-acting, human insulin (Actrapid HM)	16 adults (11 males, 5 females) aged 18–63 years with T1DM.	48-week randomized, controlled, crossover trial. Study started with an 8-week run-in period after which patients were included to 2 study periods lasting 24 weeks each. Participants were allocated randomly in one of the study groups: twice-daily syringe injections with human short-acting (Actrapid HM) and intermediate-acting isophane insulin (NPH; Protaphane HM) or 3 times daily preprandial injections of human short-acting insulin (Actrapid HM) with a single injection of human ultralente insulin (Ultratard HM).	The mean daily home blood glucose concentration was significantly lower in the pen-injector group (7.1 ± 0.4 vs. 8.2 ± 0.5 mmol l^{-1}, $p < 0.05$). Neither HbA1c nor fructosamine outcomes did not differ between the syringe and pen injector groups. At the end of the study, 13 patients decided to continue the MIR with NovoPen®.
Houtzagers et al., 1989 (56)	NovoPen®	Short-acting, human insulin (Actrapid HM)	16 adults (11 males, 5 females) aged 18–65 years with T1DM	12-month randomized, controlled, crossover trial. Following an 8-week run-in period, participants were randomly allocated to twice daily injections of combined human short-acting (Actrapid HM) and intermediate-acting isophane (NPH) insulin (Protaphane HM) with a conventional syringe or administration of human short-acting insulin (Actrapid HM) in 3 preprandial injections from NovoPen® with a single-syringe injection of human ultralente insulin (Ultratard HM).	HbA1c was not significantly different in both study groups (8.2 ± 0.4 vs. 7.6 ± 0.4%). In the questionnaires completed at the end of the study periods, the patients using the pen injector presented significantly less state anxiety ($p < 0.05$) and tended to experience a better self-concept as having diabetes ($p < 0.06$).
Tallroth et al., 1989 (57)	NovoPen®	Short-acting insulin (Actrapid HM)	18 adults (16 males, 2 females) aged 31.0 ± 7.4 years with T1DM	6-month randomized, controlled, crossover trial. Patients were randomly allocated into group A or B. Group A started a 3-month study period with premeal injections of short-acting insulin with NovoPen® and intermediate-acting insulin with ordinary syringes. In the following 3 months, the therapy was continued with three daily insulin injections of intermediate- and short-acting insulin from conventional syringe. Group B participated in the study in the reverse order.	Both groups expressed improved mood and well-being in general during multiple insulin injections. Moreover, increased experience of freedom and less content meal times during pen injector treatment were noted. Metabolic control outcomes differ significantly neither in group A nor B after 6 in the end of the study.
Tubiana-Rufi et al., 1989 (58)	NovoPen®	Short-acting, human insulin (Actrapid HM)	15 adolescents (8 boys, 7 girls) aged 5–19.5 years with IDDM	6- to 24-month observational study. Patients, previously treated with 2 daily injections of mixed insulin, started the therapy with multiple injections of short-acting human insulin (Actrapid HM) using NovoPen® before each meal. A single dose of long-lasting insulin (Ultratard HM) was injected separately with the conventional syringe.	Significant improvement in metabolic control was observed in the insufficiently controlled group of patients (n = 8) where HbA1c decreased from 8.4 ± 1.8% to 7.3 ± 1.2% ($p < 0.05$) in the first 6 months of NovoPen® therapy. No more metabolic improvement was observed. The long-term acceptability of multiple injections with NovoPen® was excellent; 100% patients experienced the pen injector as a progress, and 80% would like to continue the treatment in the future.
Engstrom, 1990 (39)	NovoPen®	Intermediate-acting insulin NPH (Protaphane HM)	40 patients with IDDM	24-week randomized, controlled, crossover trial. Before the study, all participants were treated with multiple injections of short-acting insulin with the pen injector and single injection of basal NPH insulin from the conventional syringe. In the first 12 weeks, one group started using NovoPen® to inject NPH insulin and the second one continued using usual syringes to administer isophane insulin. The second period was followed in the reverse order.	Outcomes of metabolic control were similar in both study groups. Total soluble insulin doses were significantly higher (31.3 vs. 29.9 U/day, $p = 0.02$), similarly the ones before breakfast (11.1 vs. 10.6 U/day, $p = 0.04$) when NovoPen® with NPH insulin were used. All but one patient found it easy to resuspend the isophane insulin in the penfill and was confident in the dose accuracy. 38 (of 40 patients) decided to continue using NovoPen® for basal insulin injections.
Henderson et Tindall, 1990 (40)	NovoPen® 2	Premixed insulin (Actraphane)	32 patients with IDDM	3-month observational study. Two groups took part in the trial: volunteers testing NovoPen® II [12 patients (9 males, 3 females)] and the ones continuing twice daily injections with conventional	67% of patients found the NovoPen® II easy to use, but only half found it more convenient than usual syringes. No significant differences were found in the questionnaire outcomes

(Continued)

TABLE 1 | Continued

Study, year	Device studied/ device compared	Type of insulin	Participants	Study design	Results
				syringe [20 patients (12 males, 8 females)]. The NovoPen® II group completed the quality-of-life (QoL) questionnaire at the beginning of the trial and 3 months later while the control group filled in the one 3 times (to test the reliability of the survey): during the first visit to clinic, 2 weeks later, and at the end of the study.	between the study groups—NovoPen® II did not markedly alter patients' QoL.
Kadiri et al., 1998 (59)	NovoPen® 3	Intermediate-acting insulin NPH (Insulatard HM) or premixed one (Mixtard HM)	96 adults with NIDDM	24-week, open, randomized, crossover trial. Patients with NIDDM and secondary failure (fasting blood glucose > 7.8 mmol/l and HbA1c >25% above the upper limit). All patients were treated with OHAs and diet for at least 1 year before entering the study. The trial consisted of two 12-week periods of insulin administration. Group A started with NovoPen® 3 in Period 1 and crossed over to syringe/vial use in Period 2. Group B followed the study in the inverse order.	78 patients completed the study. Pain during injections was significantly reduced in the NovoPen® 3 periods ($p = 0.0018$), including patients in group B who reported lower injection pain using NovoPen® 3 after syringes/vials ($p = 0.0003$). Acceptance of the injections was significantly higher in the NovoPen® group ($p = 0.0059$). 89.5% of patients preferred NovoPen® 3 to syringes and vials.
Stocks et al., 2001 (43)	HumaPen® Ergo vs. NovoPen® 3 and vial/ syringes	Intermediate-acting insulin NPH or premixed 30/70 one	70 insulin-requiring patients (aged 13–65 years, mean 44.6) with T1DM and T2DM	5-7 week, multicenter, observational study. Patients administering insulin at least 3 months prior to study entry were asked to answer the questionnaire to assess the level of satisfaction with their current delivery device. Next, participants were instructed how to use HumaPen® Ergo and started injecting insulin in their previous regimen with the new injector. After 5–7 weeks, in the end of the study, patients were asked to answer the questionnaire regarding the acceptability of HumaPen® Ergo, compared with their previous devices.	>70% of both syringe and NovoPen® 3 users rated HumaPen® Ergo as easy to use in all aspects. The main advantages of the new device were ease of holding during injection, possibility of correcting the doses and the procedure of cartridge changing. At the end of the study, 74% of syringe users and 72% of previous injector users decided to continue administering insulin with HumaPen® Ergo.
Ristic et al., 2002 (35)	HumaPen® Ergo	Intermediate-acting insulin NPH or premixed 30/70 one	230 patients with T1DM (23%) or T2DM (73%) and 24 HCPs	5- to 7-week multicenter, observational study (consisted of two open-label studies with identical design). Participants who were using another injector before the study started the insulin administration with HumaPen® Ergo. The visits took place in the beginning of the study, after the next 3 weeks, and again in the 7th week of the study. The acceptability of the HumaPen® Ergo was evaluated with a questionnaire in the end of the trial. The HCPs assessed the pen injector with the same criteria as the patients.	Participants considered HumaPen® Ergo as easy/very easy in learning to use (97%), reading the dose (95%), correcting the dose (97%), and holding during injection (62%). Most of patients (Study 1/2: 89%/93%) found the pen easier/much easier to correct the dose than the previously used injector. 60%/ 69% of the study group would continue using HumaPen® Ergo and recommend the model to the others HCPs and would recommend the injector because of the ease in dialing back with no insulin waste (80%) and reading the dose (74%).
Summers et al., 2004 (21)	Insulin injection pen device (IIPD) vs. vial and syringe	N/A	242 respondents with T1DM and T2DM (99 insulin users and 143 insulin nonusers) aged 18–83 years (mean 53.4 ± 13.2 years)	US residents completed an email survey with a 19-item self-administered questionnaire. Items were designed to evaluate patients' experience with IIPD and vial and syringe. The results were analyzed on a 5-point Likert-type scale. Higher scores mean greater agreement. The survey examined ease of use, activity interference, and social acceptability of IIPD and vial and syringe.	Overall preference for the IIPD was higher than that for vial and syringes among both groups (insulin users and nonusers), mainly because of social acceptability. However, current insulin users claimed that social acceptability and ease of use were the most significant predictors of preference vial and syringes. For insulin non-users, these preference predictors were activity interference and also ease of use.
Larbig et al., 2005 (44)	AutoPen® 24	N/A	40 adults (20 men, 20 women mean aged 49.3 ± 15.1 years), 20 patients with T1DM and 20 ones with T2DM	6-month multicenter, open, randomized, crossover study. Before the study, the patients were trained to handle the insulin pens properly. Group A started the study with AutoPen® 24 and after 3 months switched to OptiPen® Pro. Group B followed the study in the reverse order. All the patients participated in all three visits every 12 ± 2 weeks each. After every study period, the patients completed a standardized patient experience and preference questionnaires.	Both groups presented similar metabolic control and number of hypoglycemic episodes. AutoPen® 24 presented a high level of acceptance in patients (in comparison with OptiPen® Pro) and was preferred by older patients with T2DM.

(Continued)

TABLE 1 | Continued

Study, year	Device studied/ device compared	Type of insulin	Participants	Study design	Results
Goksen et al., 2006 (60)	OptiPen® Pro-1	NPH insulin	32 patients (mean age 17.0 ± 4.4 years) with T1DM	6-month observational study. Patients were treated with NPH insulin for at least 6 months before the study. In the beginning of the trial, they were transferred to glargine insulin administered with OptiPen® Pro-1. After 6 months of observations, the patients were asked to complete an inquiry form and rate the OptiPen® Pro-1 on a scale (0 = worst, 5 = best).	Patients rated the pen as 5 (9% of patients), 4 (38.4%), 3 (26.4%), 2 (11.7%), 1 (8.8%), and 0 (2.9%). Leakage from the injector was noted in 58.8% of subjects, and 38.2% of the ones reported a problem with a dosage button (it was not locking when it was fully depressed after the injection). 61.7% of patients exchanged the pen for an insulin syringe or insulin detemir.
Venekamp et al., 2006 (61)	HumaPen® Memoir	Lispro insulin (Humalog®) and human NPH one	300 participants (aged 18–75 years) with T1DM (38%) or T2DM (62%).	6- to 10-week multicenter, open-label, single-arm study. The study involved 3 office visits in 6–10 weeks. Patients (who were regularly using pen injectors prior the study) started injections of basal/prandial doses with HumaPen® Memoir. Moreover, patients were recording any complaints that they had during the trial. The complaints were categorized as functional or non-functional. Participants had a possibility to call the investigators if any help with the injector was needed during the study.	287 patients completed all 3 visits. There were 33 (10.5%) non-functional and 24 (7.6%) functional complaints reported (15 user-related and 8 electronic failures), but none of them resulted in a serious adverse event. No pen-related hypoglycemia and 2 pen-related hyperglycemias were reported. 81.4% of participants preferred the HumaPen® Memoir than their recent injectors.
Olsen et al., 2010 (49)	NovoPen® Echo	N/A	205 participants (79 children aged 7–18 years with T1DM, 78 parents and 48 HCPs).	Observational study. Participants were asked to assess the usability of the device they were using before and the NovoPen® Echo. Firstly, they completed specially designed tasks (setting up the pen, adjusting and injecting a dose, operating the memory function and subjective assessment). Afterward, participants filled up rating scales (1 = most favorable; 6 = least favorable) to rank each pen.	NovoPen® Echo was highly rated for the design and overall appearance (1.71 ± 0.79) in comparison with NovoPen® Junior (2.02 ± 0.93) and HumaPen® Luxura HD (2.36 ± 1.01). Moreover, 94% parents and 89% children/adolescents found the memory function very easy/easy to use. 80% participants preferred NovoPen® Echo to the other pens ($p < 0.0001$).
Israel-Bultman et al., 2011 (62)	NovoPen® 4	Human insulin or analogues	1854 adults with T1DM or T2DM	12-week, open-label, observational study. The study investigated the preference of NovoPen® 4 usage among patients who previously administered insulin with other pen injectors (NovoPen® 3, HumaPen® Ergo, OptiPen Pro). During the first visit, participants completed the Investigator's Questionnaire and received a NovoPen® 4 with a complete instruction on how to use it. Moreover, patients' satisfaction with the previous treatment was analyzed with validated DTSQ. In the final visit (after 12 weeks), the new treatment was evaluated and patients completed the Investigator's Questionnaire again.	Patients' satisfaction improved from 26.5 to 30.5 in DTSQ score ($p < 0.0001$). 83.3% of patients found NovoPen® 4 easier to use overall ($p < 0.0001$), and over 70% of them declared that the new device was less complicated to set, read, correct, inject, and change the cartridge than in the previous injectors. 97.2% of healthcare professionals would recommend the NovoPen® 4 to the other patients.
Sommavilla et al., 2011 (42)	NovoPen® 4 vs. NovoPen® 3	N/A	117 participants: 82 current NovoPen® 3 users (mean age 48.5 ± 1.6 years) and 34 insulin-naïve patients (mean age 61.8 years ± 1.9) with T1DM or T2DM	Multicenter, open-label, crossover study. In the first step of the study, the group of patients currently using NovoPen® 3 were asked to handle NovoPen® 4 and complete a sequence of tasks within 5 min. The second, crossover part of the trial concerned both groups of patients (NovoPen® 3 users and insulin-naïve patients). The first half of every group received a time-recorded training about using NovoPen® 3 before completing a series of tasks. In the end of the tasks, the patients were asked to evaluate handling the device in a questionnaire. In the second step, the participants completed the same sequence of tasks with another device—NovoPen® 4. The other half of the study groups assessed the injectors in the reverse order.	Current NovoPen® 3 users completed the tasks with NovoPen® 4 in an average time of 1.94 min (range, 0.57–4.98 min). Survey responses presented less difficulty and more confidence in handling NovoPen® 4 than NovoPen® 3 in both groups. 96.3% NovoPen® 3 users and 100% insulin-naïve patients preferred to use NovoPen® 4 ($p < 0.0001$).
Klonoff et al., 2013 (50)	JuniorSTAR®	N/A	167 participants (nurses working with children with T1DM, children/ adolescents	Observational study. In the study, the following participated: 109 nurses working with children with T1DM; 16 parents of children aged < 5 years; 8 children aged 6–12 years; 12 parents of children aged 6–12 years and 22 adolescents aged 13–18 years.	98% of the study population found that the insulin injector helped patients achieve a high level of dose dialing accuracy (93% of children/parents and 100% of nurses). The key advantages of the JuniorSTAR® (found in at least 84% of all participants) are

(Continued)

TABLE 1 | Continued

Study, year	Device studied/ device compared	Type of insulin	Participants	Study design	Results
			with T1DM and their parents)	Participants were asked to assess the JuniorSTAR® pen injector on 3 five-point scales: - when rating the product: 1 = very poor; 5 = very good or 1 = very difficult; 5 = very easy, - when asked to agree/disagree: 1 = completely disagree; 5 = completely agree. Positive response means a percentage of either a 4 or a 5 score.	practicality, ease of carrying (84%), ease of reading the dose (96%), ease of dialing back (87%), and a suitable injection force (87%). When the respondents were asked to describe the pen in one word, the most common replies were as follows: practical, easy, and simple.
Grabner et al., 2013 (18)	Pen vs. vial	Glargine insulin	2,531 insulin-naive patients with T2DM (1384 pen and 1147 vial users)	Retrospective, observational cohort study. Patients were included into the study using data from HealthCore Integrated Research Database. Patients were treated with at least 1 oral antidiabetic or glucagon-like peptide-1 receptor agonist (GLP-1) at baseline. The observations were provided 6 months before first insulin use (first insulin prescription) and 12 months later (follow-up period). The analysis covered 1-year outcomes including treatment persistence and adherence, HbA1c, hypoglycemia rates and healthcare costs.	Patients initiating insulin therapy with pens (glargine) were more persistent (60.6% vs. 50.1%, $p < 0.001$), adherent (medication possession ratio, 0.73 vs. 0.57, $p < 0.001$) and with lower HbA1c levels in follow-up (mean adjusted change, -1.05 vs. 0.73, $p < 0.001$) in comparison to vial patients. In both cohorts, hypoglycemia occurred at similar rates (3.8% vs. 5.2% respectively, $p = 0.21$). Study drug costs were higher among pen users ($1164 vs. $762, $p < 0.001$).
Asche et al., 2013 (15)	Pen vs. vial	Aspart insulin	11,588 adults patients from the MarketScan database (6,065 pen users and 5,523 vial ones) and 8,294 adults from the LifeLink database (4,512 pen users and 3,782 vial ones) with T2DM and T1DM	Longitudinal retrospective analysis based on the MarketScan and IMS LifeLink databases. Study groups contained patients initiating treatment with insulin aspart administered by pen or vial and syringe. The data were collected based on outpatient pharmacy claims data. During the 12-month post-index period, patients had at least 2 claims for the index treatment.	Vial and syringe use was characterized by 35% greater odds of at least one hypoglycemic episode than pen use ($p < 0.001$) in the MarketScan database and 44% greater odds in the LifeLink database ($p < 0.001$). Use of vial and syringes was associated with 89% and 62.7% (respectively, both $p < 0.001$)) greater healthcare costs because of hypoglycemic events than use of pens.
Ahmann et al., 2014 (20)	Pen vs. vial	Glargine insulin	405 insulin-naïve adults with T2DM (aged 18–85 years)	Randomized, open-label, crossover study. Patients received basal insulin (glargine) in one of two treatment sequences (2 weeks of using pen followed by 2 weeks of using vial and syringe or vice versa). Patient device preference was evaluated by the Insulin Injection Preference Questionnaire in the first end point (at week 4—the end of the crossover period). Then, patient preference and HCP recommendation were assessed with one global item and 3 others (blood glucose control, reluctance to use insulin, long-term insulin use) using a 5-point scale (1 = not preferred, 5 = preferred/recommended). Next, patients were re-randomized to pen or vial and syringe group for further observation (6, 10, and 30 weeks) to evaluate clinical end-points (HbA1c, fasting blood glucose levels) and safety outcomes (hypoglycemia, adverse events).	Pens were preferred by patients and strongly recommended by HCPs over vials and syringes ($p < 0.001$). Corresponding responses were observed by both groups (patients and HCPS) in the three subscale items. Fasting glucose levels, HbA1c levels, and hypoglycemia rates were comparable in both pen and vial/syringe users.
Lasalvia et al, 2016 (23)	Pen vs. vial	Glargine, detemir, NPH, aspart, premixed human 30/ 70, lispro	Study groups generally composed of adults with T2DM.	Meta-analysis. 10,348 articles from 8 different databases, of which 17 studies were selected: 7 experimental and 10 analytical. Studies concerned a comparison of insulin administration by pen devices with vial and syringes. HbA1c, hypoglycemia, adherence, persistence, patient preference, and QoL were analyzed.	Pen devices presented better results in mean HbA1c change, frequency of hypoglycemia, adherence, and persistence in comparison with vial and syringes. Among patients with good metabolic control (HbA1c < 7%) no difference was observed. Tendency to prefer pen devices was observed, however unvalidated tools were used in the analysis.

(Continued)

TABLE 1 | Continued

Study, year	Device studied/ device compared	Type of insulin	Participants	Study design	Results
Gorska-Ciebiada et al., 2020 (36)	GensuPen®	Short- and long-acting insulins, premixed human 30/70, 40/60 and 50/50 ones	4,513 adults (mean age 65.3 ± 10.2 years) with T2DM	12-week, multicenter, observational trial EGIDA II (Education and GensuPen® In Diabetology II) Participants were divided into 2 groups: A—treated with GensuPen®; B—treated with other pens. Before the study, all the subjects were educated by trained HCPs. Patients were asked to complete the questionnaires regarding injection parameters, pain scale, and satisfaction of the treatment before (visit 1) and after the study (visit 2).	Patients' utility, comfort, and satisfaction with the treatment increased, wherein group A presented a greater increase. In both study groups, mean glucose levels (from self-control diaries) were significantly lower after 3 months of the trial, but group A presented a greater difference between visits 1 and 2. In both groups, a significant decrease in sensation of pain was observed, with a greater decrease in group A. Moreover, education of the patients could help to improve the metabolic control and technique of insulin injections, reduce BMI and pain sensation.
Masierek M et al., 2020 (27)	GensuPen®	Gensulin® R, Gensulin® N, and premixed insulins M30, M40, and M50	10,309 adults (mean 63.3 ± 12.0 years) with T2DM	4-week multicenter, prospective, observational, open-label study. The trial consisted of one visit in the office (during study enrolment) and two telephone contacts (performed 7 days after enrolment and 4 weeks ± 7 days later). All patients were educated about the proper use of GensuPen® and maintained on Gensulin® (Gensulin® R, N or premixed M30, M40, M50). Moreover, participants had an opportunity to contact dedicated helpline in case of any technical problems with the injector. During the first telephone contact, patients were asked about any problems and needed information regarding GensuPen® use. The next call (after the study) was aimed at assessing patients' safety and comfort concerning GensuPen®. The interview was based on two questionnaires concerning evaluation of the GensuPen® and comparing the new injector with previously used ones (if applicable).	GensuPen® was rated as very good in confirmation of successful administration (92.0%), setting a dose (87.8%), trigger location (80.9%), and injection force (75.0%). Adverse events occurred in 0.6% of participants and none was serious. Moreover, the overall safety of the device was rated as high (severe hypoglycemia affected only 0.2% of the study group).
Boye et al., 2021 (25)		N/A	504 adults (251 UK, 253 US) treated with injections of insulin (49.6%) or GLP-1 receptor agonist (50.4%)	Observational, online survey study. Patients treated with insulin or GLP-1 receptor agonist were presented with a list of 17 characteristics of injectable medication and ask to indicate which were most important for them.	The most frequently selected characteristics were confidence in administering the correct dose (n = 300, 59.5%); ease of selecting the correct dose (n = 268, 53.2%); overall ease of using the injection device (n = 239, 47.4%); frequency of injections (n = 223, 44.2%); ease of carrying the device when necessary to inject away from home (n = 190, 37.7%). Respondents least often chose dose escalation (n = 79, 15.7%); handling the needle (n = 74, 14.7%); connectivity to an electronic device (n = 70, 13.9%); and the time required to prepare and inject each dose (n = 62, 12.3%).

DM, diabetes mellitus; T2DM, type 2 diabetes mellitus; GLP-1, glucagon-like peptide 1; IIPD, insulin injection pen device; DTSQ, Diabetes Treatment Satisfaction Questionnaire. N/A, not applicable.

PREFILLED (DISPOSABLE) INSULIN PENS

Prefilled (disposable) insulin pens, like reusable ones, are loaded with 3 ml (300 U) of insulin, and some of the patients find it easier to operate than the reusable insulin pens because there is no need to replace the cartridge (83). In 1989, Novo Nordisk launched the world's first disposable, prefilled insulin pen namely NovoLet® (84) followed by FlexPen® introduced in 2001 (41) and Next Generation FlexPen (NGFP) in 2008 (85) and FlexTouch®, a reengineered version of the FlexPen® with a novel injection mechanism, in 2011 (86).

Other prefilled insulin pens include SoloSTAR® (Sanofi) launched in 2008, KwikPen® (Eli Lilly) launched in 2007 (87), and Junior KwikPen® launched in 2017, a half-unit insulin pen (88). Similarly to reusable insulin pens, prefilled ones when compared to vials and syringes were rated as much easier to handle, discreet in public use, confident in proper dose delivery, and preferred by majority of patients (with T1DM and T2DM), healthcare professionals (89–91), and patients' caregivers (parents, relatives) (92). Moreover, both non-experienced healthcare practitioners and needle-naïve patients found the prefilled insulin pens much easier to teach and learn (93, 94).

TABLE 2 | Characteristics of reusable insulin pens.

Pen device	Type of insulin/company	Dose range (dose increment)	Memoir	Dialing forward and backward without wasting insulin	Special characteristics
NovoPen® 3 (63)	Novo Nordisk 3-ml cartridges	2–70 units (1 unit)	No	No	N/A
NovoPen® 1.5 (41)	Novo Nordisk 1.5-ml cartridges	1–40 units (1 unit)	No	No	Shorter device.
NovoPen® 3 Demi (64)	Novo Nordisk 3-ml cartridges	1–35 units (0.5 unit)	No	No	First 0.5-unit increment pen.
NovoPen® Junior (65)	Novo Nordisk 3-ml cartridges	1–35 units (0.5 unit)	No	No	Vibrant colors.
NovoPen® 4 (66)	Novo Nordisk 3-ml cartridges	1–60 units (1 unit)	No	Yes	Audible confirmatory dosing click. Safety feature preventing selection of a dose greater than the amount of insulin left in the cartridge.
NovoPen® Echo (67)	Novo Nordisk 3-ml cartridges	0.5–30 units (0.5 unit)	Yes	Yes	Two color variants and choice of skins available. Electronic display showing the last dose of insulin administrated.
NovoPen® 5 (68)	Novo Nordisk 3-ml cartridges	1–60 units (1 unit)	Yes	Yes	2 color variants, electronic display showing the last dose of insulin administrated.
AutoPen 24® (69)	Sanofi Aventis 3-ml cartridges	1–21 units (1 unit) or 2–42 units (2 units)	No	No	Side-mounted release button.
AutoPen® Classic (70)	Eli Lilly or Wockhardt 3-ml cartridges	1–21 units (1 unit) or 2–42 units (2 units)	No	No	Side-mounted release button.
AutoPen 2® (71)	N/A	1–72 units (1 unit)	No	Yes	Side-mounted release button. Dose correction button. Identity rings for different types of insulin.
OptiPen® Pro 1 (72)	Sanofi-Aventis 3-ml cartridges	1–60 units (1 unit)	No	Yes	Digital display to set the insulin dose.
OptiPen® Pro 2 (72)	Sanofi-Aventis 3-ml cartridges	2–60 units (2 units)	No	Yes	Digital display to set the insulin dose.
HumaPen® Ergo (2002) (73)	Eli Lilly 3-ml cartridges	1–60 units (1 unit)	Yes	Yes	N/A
HumaPen® Ergo II (74)	Eli Lilly 3-ml cartridges	1–60 units (1 unit)	Yes	Yes	N/A
HumaPen® Luxura (75)	Eli Lilly 3-ml cartridges	1–60 units (1 unit)	Yes	Yes	2 color variants.
HumaPen® Luxura HD (76)	Eli Lilly 3-ml cartridges	1–30 units (0.5 unit)	Yes	Yes	N/A
HumaPen® Memoir (77)	Eli Lilly 3-ml cartridges	1–60 units (1 unit)	Yes	Yes	Digital display with time, date and dose of insulin.
OptiClik® (78)	Lantus 3 ml (Sanofi-Aventis) Cartridge System	1–80 units (1 unit)	No	No	Digital display.
BerliPen® 301 (79)	Berlinsulin H or Liprolog 3-ml cartridges	1–21 units (1 unit)	No	No	Side-mounted release button.
BerliPen® 302 (79)	Berlinsulin® H or Liprolog 3-ml cartridges	1–42 units (2 units)	No	No	Side-mounted release button.
BerliPen® Areo 3 (80)	Berlinsulin® H or Liprolog 3-ml cartridges	1–60 units (1 unit)	No	Yes	5 color variants.
GensuPen® (27)	Gensulin® 3-ml cartridges	1–40 units (2 units)	No	Yes	Side-mounted release button. End-of-dose indicator.
GensuPen® 2 (45)	Gensulin® 3-ml cartridges	1–60 units (1 unit)	No	Yes	Side-mounted release button. End-of-dose indicator. 3 color variants
JuniorSTAR® (81)	Lantus®, Apidra® or Insuman® (Sanofi-Aventis) 3-ml cartridges	1–30 units (0.5 unit)	No	Yes	3 color variants.
TactiPen® (Itango) (82)	Sanofi-Aventis 3-ml cartridges	1–60 units (1 unit)	No	Yes	4 color variants.

N/A, not applicable.

For years, insulin pens were used with insulin 100 U/ml, but since the development of higher-concentration insulins, also new insulin pens for 200 and 300 U/ml have been manufactured and used since 2017, namely, Humalog® 200 U/ml KwikPen® (Eli Lilly) (95), Tresiba® 200 U/ml prefilled FlexTouch® (Novo Nordisk) (96), and Glargine U300 SoloSTAR® insulin pen (Sanofi-Aventis) (97). However, we must consider that disposable pens are less environment friendly and this is a globally growing importance nowadays (98). One can just imagine that if a patient is using approximately 40 units of insulin a day there is about 50 prefilled plastic pens thrown away every year and accounting for thousands of patients using insulin pens the number of insulin pens being thrown away per year is accounted in millions. Based just on a small study form Bosnia and Herzegovina published in 2020, it was predicted that only in this small country there were 3.2 million pens used and dispensed annually (99).

Cited studies related to prefilled insulin pens are summarized in **Table 3**, and the technical characteristics of prefilled pens are presented in **Table 4**.

TABLE 3 | Prefilled insulin injectors.

Study, year	Device studied/ device compared	Participants	Study design	Outcomes
Korytkowski et al., 2003 (89)	FlexPen® vs. vial and syringe	121 adults aged 28–81 years with T1DM and T2DM	8-week multicenter, randomized, open-label, comparative, two-period crossover trial. During the 4-week run-in period, the patients continued the therapy with the previous devices (i.e., their own pens or syringes), to administer a mixture of 70% aspart protamine suspension and 30% aspart insulin. Insulin doses were optimized. Then patients were randomly allocated to one of the study groups. Half of the participants started the trial using prefilled, disposable pens for 4 weeks, and next they were crossed over to a vial/syringe group for another 4 weeks. The second group followed the study in the reverse order. Patients' preference was assessed based on the Patient Preference Questionnaire in the final visit of the second treatment period.	103 patients completed the study. Most of the patients (78%) preferred the pen over vial and syringe methods, and 85% found the FlexPen® more discreet in public. Ease of pen use was greater for 74% of respondents, and 85% of them considered the insulin dose scale much easier to read in the pen injector. However, metabolic control was comparable in both FlexPen® and vial and syringe group and patients' HbA1c improved during the study ($p < 0.05$).
Niskanen et al., 2004 (100)	FlexPen® vs. Humalog® Pen	137 patients (mean aged 62.3 ± 9.2 years) with T2DM	24-week randomized, multinational, multicenter, open-label, 2-period crossover trial. After a 2-week run-in period, patients were randomly involved into a 12-week treatment period with BIAsp 30 (30% of soluble insulin aspart and 70% protaminated insulin aspart) or Mix25 (25% soluble insulin lispro and 75% neutral protamine lispro) using FlexPen® or Humalog® Pen. Next, participants were crossed over to the second treatment period with another type of insulin and pen device. In the final questionnaire, patients' preference for the pen injectors was assessed.	FlexPen® received the highest rates for all device features assessed in the final questionnaires (all $p < 0.005$). 32.4% of patients experienced problems with Humalog® Pen when only 9.0% with FlexPen® ($p < 0.001$). 74.6% of respondents preferred to continue using FlexPen® (in comparison with 14.3% preferred Humalog® Pen, $p < 0.001$).
Haak et al., 2007 (101)	SoloSTAR®, Humalog®/ Humulin pen, FlexPen®, and prototype Pen X	510 patients aged 11–82 years (232 adults with T2DM receiving only OHAs and 278 insulin users with T1DM or T2DM).	Multicenter, observational study The trial consisted of 1-hour face-to-face interviews aimed at evaluating the usability of the devices and patients' preferences. Firstly, participants were asked to prepare the device and deliver a 40-unit dose relying on their intuition and/or relevant manuals. Any training and maintenance was not provided. Next, respondents evaluated abovementioned procedures for each pen in a five-point scale (1 = poor, 5 = excellent).	Significant majority of patients prepared the SoloSTAR® properly and performed a correct injection with the device in comparison with the other pens ($p < 0.05$). Moreover, most of the patients (53%) preferred to use SoloSTAR® than Flex Pen® (31%) and Humalog®/Humulin pen (15%).
Ignaut et al., 2008 (87)	FlexPen® (NovoLog® Mix 70/30) vs. KwikPen® (Humalog® Mix75/25)	50 insulin pen device (25 FlexPen®s and 25 KwikPen®s)	In this study, ergonomic features, injection force (as glide force (GF), and glide force variability (GFV)) were measured and compared in FlexPen® and KwikPen® injectors.	FlexPen® was lighter than KwikPen® and had a smaller diameter at the cartridge holder and dose window while KwikPen® presented a shorter overall pen length with a shorter thumb reach at both 30- and 60-unit dose settings. For both the 30-unit and 60-unit doses, maximum GF was lower in KwikPen® than in FlexPen® (3.42 vs. 5.36 lb and 3.61 vs. 5.62, respectively, both $p < 0.0001$).
Asakura et al., 2009 (93)	FlexPen® vs. vial and syringe	60 HCPs (30 insulin experienced	Multicenter, observational study The first part of the study consisted of insulin delivery training among insulin-naïve participants. Next, respondents were	Insulin therapy-naïve HCPs preferred FlexPen® and found it much easier to handle than vial and syringe ($p < 0.001$). Moreover, the pen was more accurate

(Continued)

TABLE 3 | Continued

Study, year	Device studied/ device compared	Participants	Study design	Outcomes
		and 30 insulin-naïve ones)	randomized into 2 study groups, one of group performed an injection of 10 U with FlexPen® (Day 1) and then with vial/syringe (Day 2). The second group followed the tasks in the reverse order. Subsequently, insulin-naïve HCPs assessed the devices and made an overall comparison in the evaluating questionnaires (rate range: 1 = very poor, 5 = excellent). The second part of the trial depended on the randomized, accuracy testing of the two devices (FlexPen® and vial/syringe) by 30 insulin-experienced and 20 insulin-naïve HCPs. After injecting 10 U of insulin, devices were weighed and the outcomes were converted into insulin units (0.1 g = 10 U).	than syringe when used by both insulin experienced and non-experienced HCPs ($p < 0.001$).
Asakura et Jensen, 2009 (102)	FlexPen® vs. OptiClik®	61 adults (mean aged 61.9 ± 12.3 years) with T2DM	Randomized, open-label, crossover study. All study groups were insulin-device-naïve. Participants were randomized into intuitiveness and instruction time group and then randomized again to the subgroups starting injections with FlexPen® or OptiClik®. The intuitiveness group had to make an injection into a cushion without any training or manual. At the end the study, the group completed a intuitiveness and device understanding questionnaire. The second group received an instruction before injecting a dose. Both groups completed the important features of the device questionnaire. Afterward, everyone received the injectors again and became instructed how to use each pen. In the end, patients fulfilled questionnaires regarding ease of use and overall preference.	FlexPen® required less instruction time and was more intuitive for most of patients ($p < 0.001$). None in the instruction time group considered FlexPen® difficult to learn, but 45% of the group found OptiClik® difficult/very difficult to learn. Moreover, respondents rated FlexPen® (in comparison to OptiClik®) as simpler to use (77% vs. 12%, $p < 0.001$), easier to inject (67% vs. 13%, $p < 0.001$), and more convenient 71% vs. 12%, $p < 0.001$). Analogically, most of the respondents preferred using FlexPen® than OptiClik® (82% vs. 13%, $p < 0.001$).
Ignaut et al., 2009 (90)	KwikPen® vs. vials and syringes and KwikPen® vs. FlexPen®	232 adults (aged 40–75 years) with T1DM or T2DM	1-day, open-label, randomized, crossover study. The study assessed the preference of using KwikPen® vs. vial/syringe and next, KwikPen® vs. FlexPen® among insulin users. Dose accuracy, ease of use (*via* insulin device assessment battery), and respondents' preference (*via* insulin device preference battery) for each pen were examined, and both pens were evaluated with the final preference questionnaire.	KwikPen® was the most preferable device (over both vial and syringe and FlexPen®) because of its appearance, quality, discretion, convenience, public use, ease of learn and use, reliability, dose confidence, and following insulin regimen. KwikPen® was considered as overall the most satisfying device, willingly recommended to others.
Yakushiji et al., 2010 (103)	OptiClik®, SoloSTAR®, MirioPen, and FlexPen®	22 (50% male, 50% female) respondents (11 experienced and 11 non-experienced with insulin injectors) aged 25–57 years.	Observational study Non-experienced participants were educated how to use the injectors. All the respondents made 2 injections with 5 examined devices. The first one was a self-injection in the prosthetic skin attached in the respondents' flank. The second injection was made to the prosthetic skin placed in the upper arm of the mock patient (other injection). Every injection contained 10 units of saline. In the end, both self- and other injections with every device were evaluated in the questionnaire and rated from 1 to 5.	FlexPen® was rated as the best device for self-injections. However, FlexPen® was also selected the worst one for the other-injections because it was too long, was less stable, and had inadequate visibility of the dial. OptiClik® was evaluated as the best device for other injection but the second worst one to self-injection.
Bailey et al., 2011 (104)	FlexTouch® vs. KwikPen®	160 participants: 79 patients with T1DM or T2DM and 81 HCPs (40 physicians, 41 nurses)	1-day, randomized, crossover study. Respondents were randomly assigned to one of the groups (starting the study with FlexTouch® or KwikPen®) and then crossover to test the second pen. Participants were trained how to use the devices before the test injections. Next, both patients and HCPs made multiple injections (with randomly altered doses including 20, 40, and 60 U) into a foam cushion and answered questions concerning ease of use, confidence, and preferences.	FlexTouch® (compared to KwikPen®) was rated as most preferred device (86% vs. 7%; $p < 0.001$), easier to use (85% vs. 4%; $p < 0.001$), and recommended to others (88% vs. 6%; $p < 0.001$). Additionally, FlexTouch® was characterized as the better device in the injections for ease of depressing the push button and ease of injecting the doses ($p < 0.001$ for all). FlexTouch® was found as the most confident in correcting and completing insulin delivery (73% vs. 6%; $p < 0.001$).
Hancu et al., 2011 (105)	SoloSTAR®	6481 adults (mean aged 54 years) with T1DM or T2DM	6- to 8-week, multinational, multicenter, open, prospective, observational product/device registry study. At the first, registry visit participants were included to the insulin therapy with the new pens (LANTUS SoloSTAR® and/or Apidra SoloSTAR®) and completed a questionnaire regarding their previous experience with insulin injectors (if applicable). Last visit (after 6–8 weeks of SoloSTAR® use) purposed to assess the acceptance of the new disposable pen and compare patients'	6,364 participants were included to the analysis of patient satisfaction. 77.1% patients had used insulin before inclusion in the study. In the trial, SoloSTAR® was used to administer glargine (97.3%) and/or glulisine (36%) insulin. Most of patients found the new disposable injector as "excellent/good" in learning to use (98.3%), ease

(Continued)

TABLE 3 | Continued

Study, year	Device studied/ device compared	Participants	Study design	Outcomes
			experience with the ones used prior the trial. Moreover, series of questions have been asked to evaluate the study period.	of use (97.9%), selecting the dose (97.6%), and reading the dose (95.1%). SoloSTAR® was "much easier/easier" for over 80% of the study group (in comparison with previously used pens) because of ease of use (88.4%) and injecting a dose (84.5%). Furthermore, 98% patients desired to continue using SoloSTAR® in the future.
Oyer et al., 2011 (106)	FlexTouch® vs. SoloSTAR®	120 participants: - 59 patients with T1DM or T2DM -61 HCPs (30 physicians, 31 nurses)	1-day multicenter, open-label, randomized, crossover study. Respondents were randomly assigned into the study groups (starting test with FlexTouch® or SoloSTAR®). Participants were instructed how to use the pen and performed test injections into a foam cushion, dosing 20, 40, and 80 U. In the following step, both study groups were crossed over to test another pen device. Each pen device was assessed separately (in a form evaluating handling and operation of the pen). Moreover, in the final questionnaire respondents completed regarding their preferences.	A significant majority of participants (88%) preferred FlexTouch® over SoloSTAR® (10%). They considered FlexTouch® (vs. SoloSTAR®) easier to use (83% vs. 9%), willingly recommended to others (83% vs. 8%; $p < 0.001$), very/fairly easy to reach the push-button and inject the doses ($p < 0.001$ for all), more confident in correct insulin delivery (76% vs. 6%; $p < 0.001$), and managing daily injections (88% vs. 58%).
Buysman et al., 2011 (17)	FlexPen® (Levemir) vs. vials (NPH)	1,876 patients with T2DM (1082 Levemir FlexPen® users and 794 NPH vial ones)	Retrospective analysis from a large geographically diverse US health insurance plan. Patients were divided into 2 study groups—initiating basal insulin therapy with Levemir FlexPen® or NPH in vials. Patients were defined as adherent to therapy if their medication possession ratio (MPR) was at least 80% in the 12-month follow-up period. Patients' persistence was defined as the lack of gaps in insulin therapy during the follow-up period.	Patients beginning therapy with Levemir FlexPen® had 39% higher adjusted odds of achieving an MPR ≥80% in comparison to patients with NPH vials (OR 1.39, 95% CI: 0.55–0.70). Moreover, analysis of persistence presented that patients initiating Levemir FlexPen® had a 38% lower hazard of discontinuation compared to NPH vial users (HR 0.62, 95% CI: 0.55–0.70)
Campos et al., 2012 (91)	FlexTouch® vs. vial and syringe	120 participants: - 60 patients with T1DM or T2DM, - 60 HCPs (30 physicians, 30 nurses)	1-day randomized, multicenter, open-label, crossover study. Participants were trained how to use the devices. Next, test injections into foam cushion (dosing 20, 55, and 80 U) were made with both vial and syringe and FlexTouch®. Then, respondents separately rated the devices in respect of ease and confidence of use.	FlexTouch® (compared to vial and syringe) was found a preferred device (88% vs. 5%; $p < 0.001$), easier to use (91% vs. 6%; $p < 0.001$), and willingly recommended (91% vs. 3%; $p < 0.001$). Moreover, participants considered FlexTouch® easier to use, more stable during injection, and better in depressing the push-button and reading the dose scale (all $p < 0.001$). Patients and HCPs using FlexTouch® were also more confident in properly insulin delivery and metabolic control than the ones using vial and syringe ($p < 0.001$).
Lajara et al., 2012 (94)	FlexTouch® vs. vial and syringe	120 participants: - 30 needle-naïve patients, - 30 vial and syringe-experienced patients, - 30 physicians, - 30 nurses.	1-day randomized, multicenter, open-label, crossover study. All participants received an instruction on how to use the injection device. Then they were asked to make a test injection into a foam cushion (dosing 20, 55, and 80 U) with FlexTouch® or vial and syringe (in a random order) and answer questions on confidence and ease of use (1 = very difficult/not at all confident; 5 = very easy/very confident). In the next step, respondents followed the abovementioned procedures with another device. Finally, all participants completed a preference questionnaire to evaluate both methods.	Both HCPs (nurses: 100% vs. 0%; physicians 87% vs. 7%), needle-naïve (83% vs. 7%), and vial- and syringe-experienced (73% vs. 7%) patients preferred FlexTouch® over vial and syringe for ease of teaching. Moreover, the insulin pen was rated as very/fairly easy for depressing the push-button (physicians: 93% vs. 80%; nurses: 97% vs. 80%; vial and syringe-experienced patients: 93% vs. 90% and needle-naïve ones: 100% vs. 77%).
Nadeau et al., 2012 (107)	FlexTouch® vs. KwikPen® (FT vs. KP) and FlexTouch® vs. SoloSTAR® (FT vs. SS)	FT vs. KP: 160 participants (79 patients with T1DM or T2DM and 81 HCPs) FT vs. SS: 120 participants (59 patients with T1DM or T2DM and 61 HCPs)	1-day, randomized, crossover study. The study consisted of 2 comparison groups: FT to KP and FT to SS. Participants were randomized to start the trial with FT or another pen device and then were crossed over to test the second injector. All respondents were educated about using the devices. Both patients and HCPs were asked to make multiple injections of different doses with each pen (FT vs. KP study: 20, 40, 60 U; FT vs. SS study: 20, 40, 80 U). In the end, participants answered the questions regarding ease of use, learning and teaching, confidence in use, and preference.	FlexTouch® was rated as very/fairly easy to inject, particularly in the maximum dose (compared to KP or SS: ≥80% vs. ≤38% and ≤23%) and very/rather confident in the ability to manage daily injections. FT was also considered as easier to teach and learn to use than KP and SS (all $p < 0.001$) and preferred for learning and teaching (≥39% vs. ≤4% for KP and ≤6% for SS). Most of the patients and HCPs would recommend FT (≥95%) than KP (≤72%) and SS (≤71%).

(Continued)

TABLE 3 | Continued

Study, year	Device studied/ device compared	Participants	Study design	Outcomes
Pfutzner et al., 2012 (108)	InnoLet® vs. FlexTouch®	90 patients (mean aged 62 ± 8 years) with T1DM or T2DM, with or without impaired dexterity and visual impairment	Patients became stratified into 4 study groups: A—visually impaired with T1DM and impaired dexterity; B—visually impaired with T2DM and impaired dexterity; C—visually impaired with T1DM or T2DM; D—patients without any impairment with T1DM or T2DM. Participants were asked to perform some test injections (dosing 10, 30, and 50 U) and complete a standardized questionnaire assessing the handling of the pen device. The procedure was repeated with a second insulin injector. In the end, patients evaluated the study by completing a comparative questionnaire.	FlexTouch® was preferred in all study groups including 100% of group D (unimpaired patients). Only a few patients with visual/dexterity impairment preferred InnoLet® (group A—13%, group B—3%, group C—14%).
Schipper et al., 2012 (109)	FlexTouch® vs. InnoLet®	90 patients (mean aged 62 ± 8 years) with T1DM or T2DM	Patients were assigned to the study groups in random order. Participants (educated how to use the devices) were asked to perform a mock injections (with 10-, 30-, and 50-U doses) and complete a final 41 item standardized questionnaire to assess the device. Patients rated each pen in a five-point scale (1 = very easy, 5 = very difficult) regarding injection confidence and performance, dose setting, general handling, and others.	FlexTouch® (FT) was found better than InnoLet® (IL) for the injection procedure (FT: 1.2 ± 0.1 vs. IL: 2.1 ± 0.4; $p < 0.001$), general handling (1.3 ± 0.2 vs. 2.3 ± 0.7; $p < 0.001$), confidence of dosing (1.4 ± 0.2 vs. 2.1 ± 0.9; non-significant). Dose setting was ranked equally (FT: 1.6 ± 0.3, IL: 1.7 ± 0.4, non-significant). 92.2% of patients would recommend FT (IL only 30.0%).
Pfutzner et al., 2013 (92)	FlexTouch® vs. vial and syringe	120 participants: - 40 patients with T1DM or T2DM, - 20 caregivers (i.e. parents, relatives) - 20 physicians, - 40 nurses/ certified diabetes educators	1-day single-center, randomized, crossover study. Participants (in random order) were asked to perform testing injections into laboratory tubes (doses of 5, 25, 43, and 79 U) with the devices. Dosing accuracy was measured, and patients completed final questionnaires (device assessment questionnaire, patient perception questionnaire). Next, respondents were crossed over to test another device. At the end of the trial, all participants answered the questions in the device preference questionnaire.	FlexTouch® presented significantly better dosing accuracy when used by all cohorts and at all doses ($p < 0.005$ for all doses). The pen injector was rated significantly higher than vial and syringe in both device preference questionnaire (93% vs. 2% for vial and syringe; $p < 0.001$)) and patient perception questionnaire (in all aspects).
Pfutzner et al., 2014 (110)	FlexTouch® (U100 and U200) vs. SoloSTAR®	64 adults with T1DM or T2DM and 64 HCPs (32 physicians, 32 nurses)	Multicenter, randomized, open-label, crossover study. The study consisted of one visit. Participants were asked to make 4–6 injections into a foam cushion (dosing 2, 20, 40, 80, 120, and 160 units). Next, they were asked to complete a questionnaire to evaluate the device. These procedures were repeated in each of the three analyzed injectors. After the tests, participants answered the final, overall questions.	Significant majority of participants preferred to use FlexTouch® U100 (93.0%) and U200 (91.4%), even dexterity-impaired and pen-naïve patients in comparison with SoloSTAR® ($p < 0.001$), respectively.
Cheen et al., 2014 (14)	FlexPen® (NovoMix 30) vs. vial and syringe (Mixtard 30)	955 patients	Retrospective, single-center, longitudinal study. Data were collected from the outpatient clinics database of the largest acute care hospital in Singapore. During 24 months of the observation adherence, compliance (as medication possession ratio - MPR) and persistence were measured, based on electronic medical and pharmacy refill records.	Mean MPR was comparable in vial/syringe and pen users (83.8% ± 26.9% vs. 86.0% ± 23.2% respectively, $p = 0.266$). Persistent with therapy was higher among pen users (odds ratio = 1.36; 95% CI, 1.01–1.86) after adjusting for sociodemographic and clinical covariates.
Friedrichs et al., 2015 (111)	SoloSTAR® (SS), FlexPen® (FP), KwikPen® (KP), and FlexTouch® (FT 1 and 2)	20 pen-experienced patients (mean aged 55 ± 14 years) with T1DM or T2DM	Patients were asked to dial up from zero to maximum and next, dial down from maximum to zero with each pen. Dialing up and down was recorded with a video, and the torque of the devices was analyzed. Next, 16 pen-experienced people with T2DM rated the subjective comfort for each insulin injector after dialing up and down again.	SS was rated as most comfortable in dialing up by 8 and dialing down by 6 of the 16 respondents; analogically, FP was ranked by 5 and 8, respectively; FT1: 2 and 1; KP: 1 and 1. FT2 was evaluated as least comfortable by 12 and 10 patients. Comfort of up- and down-dialing was considered "very comfortable" for SS by 15 patients each and next, FP (12 and 14), KP (10 each), and FT1 (9 and 7). FT2 was ranked "less/not comfortable" by 10 and 11 respondents, respectively.
Slabaugh et al., 2015 (16)	Pen vs. vial	3,172 insulin-naïve patients with T2DM (aged 18–89	Retrospective, observational study. The study analyzed data from Medicare Advantage with Prescription Drug insurance database. Patients initiating basal insulin administration with pens vial/syringes were observed.	Adjusted mean PDC was significantly higher in the pen cohort than the vial one (0.67 vs. 0.50 respectively, $p < 0.001$), the same as mean MPR (0.75 vs. 0.57 respectively, $p < 0.0001$). Adjusted

(Continued)

TABLE 3 | Continued

Study, year	Device studied/ device compared	Participants	Study design	Outcomes
		years), 1,231 vial users and 1941 pen ones	Persistence and adherence (as proportion of days covered—PDC and medication possession ratio—MPR) were measured during the 12-month follow-up period.	odds for adherence (PDC at least 80%) presented a positive association with insulin pen use (odds ratio = 2.19, 95% CI: 1.86–2.59). The adjusted risk of non-persistence was lower among pen users (hazard ratio = 0.42, 95% CI: 0.38–0.45).
Warren et al., 2019 (112)	FlexTouch® (200 U/ml) vs. SoloSTAR® (100 U/ml)	145 patients with T2DM using ≥ 81 units of insulin a day	32-week randomized, multicenter, open-label, crossover study. Patients became randomly assigned to one of the study groups and started a treatment with insulin degludec (200 U/ml, 3 ml FlexTouch®) or glargine (100 U/ml, 3 ml SoloSTAR®). After 16 weeks, participants were crossed over to another insulin therapy. Patients' preference and treatment impact were assessed in the final PRO questionnaires.	Most of the patients found FlexTouch® "extremely easy" for learning (62.5% vs. 43.0%, $p < 0.01$), maintaining (63.2% vs. 42.2%), and adjusting a dose (63.2 vs. 44.4%). Moreover, respondents considered FlexTouch® (compared to SoloSTAR®) as very/extremely confident in using the injector (60.3 vs. 36.3%) and its accuracy (50.7% vs. 30.4%). A significant majority of patients preferred therapy with FlexTouch® (59% vs. 22%), would like to continue (67% vs. 15%), and willingly recommend the injector (67% vs. 14%) in comparison with SoloSTAR®.

T1DM, type 1 diabetes mellitus; T2DM, type 2 diabetes mellitus; HCPs, healthcare professionals; MPR, medical possession ratio; U, units of insulin; SS, SoloSTAR®; FP, FlexPen®; KP, KwikPen®; FT1, FlexTouch® 1; FT2, FlexTouch® 2; PDC, proportion of days covered.

TABLE 4 | Technical characteristics of prefilled insulin injectors.

Pen device	Type of insulin	Company	Dose range (dose increment)	Memoir	Dialing forward and backward without wasting insulin	Special characteristics
FlexPen®	NovoRapid® NovoLog® Protaphane® Levemir® NovoMix 30® NovoMix 50®	Novo Nordisk	0–60 units (1 unit)	No	Yes	Compatibility with insulin smart caps
FlexTouch® 1	Tresiba® Ryzodeg®		0–80 units (1 unit)			
FlexTouch® 2	Tresiba®		0–160 units (2 units)			
KwikPen® Junior	Liprolog® Humalog®®	Eli Lilly	0–30 units (0.5 unit)			
KwikPen® U-100	Liprolog® Humalog®® Humulin R® Abasaglar® Humalog® Mix25 Humalog® Mix50		0–60 units (1 unit)			
KwikPen® U-200	Humalog®		0–60 units (1 unit)			
KwikPen® U-500	Humulin R®		0–300 units (5 units)			
SoloSTAR®	Lispro® Lantus® Insuman Basal® Insuman Rapid® Apidra® Toujeo®	Sanofi-Aventis	0–80 units (1 unit)			

NEXT-GENERATION INSULIN PENS (SMART INSULIN PENS) AND INSULIN PEN CAPS

Nowadays, the way of health delivery is becoming more digital than ever before where face-to-face visits are often replaced by telephone or video contacts and continuous glucose monitoring or glucometer data can be revived through cloud-based data sharing technology which was very pronounced in the COVID-19 era. One of the key problems for patients with T1DM and T2DM treated with multiple daily insulin (MDI) is omitting or late insulin doses which has been found in the study which analyzed data from a continuous glucose monitoring system (CGM) (113). It was also described lately in the study with a Bluetooth®-enabled insulin pen cap that all of the patients taking part in the study missed the insulin doses and it could be intentionally missed because of inconvenience or eating pattern or just forgotten (113). It is important to note that it was also calculated already a years ago that omitting only two meal-related insulin doses per week is associated with a 0.4% increase in HbA1c value (114). Another problem with MDI is that patients rely on numeracy skills while deciding about the meal insulin dose, and it has been proven that these skills are many times not good enough which leads to errors in insulin dosing and to poor glycemic control (115–117). Because patients treated with MDI have to make their insulin dosing decisions without access to the amount and timing of previous insulin doses or residual active insulin, this can, on the other hand, cause overlapping of insulin boluses and put a patient at risk of hypoglycemia (118). That is why smart insulin pens and pen caps were and are being developed to overcome these barriers. Information coming from business research indicates that the smart insulin pen market value will significantly increase by the year 2027 in Latin America, the Middle East, and Africa (119) with the greatest market growth in Europe with a trend toward increased use of smart insulin pen market seen also in North America (120).

SMART INSULIN PENS

Smart insulin pens are digital, connected insulin pens which go beyond memory function and automatically transmit information about time and amount of insulin administered to the user's mobile device and can remind about the insulin dose and help to calculate the bolus (7). The clinical data from the smart insulin pen are transferred wirelessly *via* Bluetooth® technology to an application (app) available for smartphones (7, 121, 122). Therefore, smart insulin pens require the use of an app to collect the data sent from the pen but eliminate the need for manual self-report logbooks (121). Thus, smart insulin pens can help to overcome the challenges that users of pen injectors have to deal with on a daily basis. Smart insulin pens are a relatively new invention, so it should come as no surprise that a few studies have been conducted in this field to date (121). In 2017, the world's first US Food and Drug Administration (FDA)-approved insulin smart pen which uses Bluetooth® technology, namely, InPen™ (Companion Medical, San Diego, Ca, USA), was launched, and in November 2020 its new version was launched by Medtronic (123). This pen combines the insulin pen with a smartphone app which has the ability to record and store data of insulin injections and recommend doses, as well as display glycemia and related data on the paired smartphone app (124–126). InPen™ is designed for use with rapid-acting insulin U-100 Lilly Humalog® and Novo Nordisk NovoLog® (127). InPen™ is the first of its kind of smart insulin pen that allows to prepare reports for healthcare professionals, reminds about missed doses, and tracks insulin on board, but also alerts the user about an exposure of the device to abnormal (very high or very low) temperatures that may inactivate insulin (124–126, 128). What is likewise important, in InPen™ the dose can be increased or decreased in half-unit steps, and therefore the dose administered is very precise (128, 129). Later on, several new smart insulin pens emerged on the market, namely, ESYSTA® pens (Emperra), Pendiq 2.0 pens (Pendiq), and NovoPen® 6 (Novo Nordisk). It cannot escape the attention that insulin pen injectors may help not only patients but also diabetes care teams. They provide accurate information about missed doses as well as injection times in relation to meals and dose sizes, which is useful in making correct therapeutic decisions and giving personalized treatment plans (121, 130–132). The first study of clinical outcomes using a smart insulin pen was reported in 2020 (133). This investigation was conducted in Sweden and indicated that among patients with T1DM using smart insulin pens, clinical outcomes improved at lower costs compared to standard care. What is even more important, this research suggested that smart insulin pens have the potential to improve glycemic control and decrease glucose variability (133, 134).

INSULIN PEN CAPS

Insulin pen caps are another device which does not have a clear definition but displays the quantity of insulin in the pen and integrate the insulin-related information with a mobile app. Insulin pen caps are usually attached to the side or fit in the end of the pen.

A first-of-its-kind smart pen cap for insulin pens (Bigfoot Unity™ Diabetes Management System) launched by Bigfoot Biomedical received FDA clearance in May 2021. This insulin pen cap is integrated with Abbott's FreeStyle Libre 2 system and translated continuous monitored glucose data into on-demand insulin dose recommendations displayed on the pen cap screen. It is the first and only device which integrates a continuous glucose monitoring system (CGMS) to insulin dose recommendation (135).

Another smart cap integrated with a dedicated mobile app is GoCap (Common Sensing company) (136). The integration with the application helps calculate the meal or correct boluses, preventing overdosing by active insulin display (125, 136). Moreover, individual reminders allow to keep the schedule of basal insulin (136). Similarly, Insulclock® is an electronic device attached into the insulin pen and connected with a smartphone app and has an insulin reminder system to reduce insulin omissions (137); this device helps to improve glycemic control and reduce glycemic variability with improved adherence in a

recent pilot, randomized study among T1DM (138) and among T2DM patients (139). Another two devices do not connect with any mobile app but present an interactive display (Timesulin®) or flash diode (Dukada® Trio), which define the time of last insulin injection (140, 141). The GoCap device received FDA approval (125). Clinical trials which compare different insulin pen caps are not available yet.

Cited studies related to smart insulin pens and their technical characteristics are summarized in **Tables 5** and **6**. As for the studies related to insulin pen caps and thier technical details the summery is provided in **Tables 7** and **8**, accordingly.

CONCLUSIONS

Insulin remains the primary medication in the treatment of T1DM and is often used therapy in T2DM. The methods and

TABLE 5 | Smart insulin pens.

Study, year	Device studied/ device compared	Type of insulin/ company (number of users)	Participants	Study design	Results
Adolfsson et al., 2020 (133)	NovoPen® 6	Basal and/or bolus insulin: deguldec (n = 21), detemir (n = 1), aspart (n = 79), human insulin (n = 1), faster-acting insulin (n = 1)	94 participants (48 men and 46 women; aged 18–83 years, mean 40.1 years) with T1DM	Multicenter, prospective, observational, proof-of-concept study, Participants were using continuous glucose monitoring (CGM) and administered bolus and/or basal insulin with NovoPen® 6. During each healthcare professional (HCP) visit, pen and CGM data were downloaded. The analysis included time in range (TIR; sensor glucose 3.9–10.0 mmol/l), time in hyperglycemia (>10 mmol/l), and hypoglycemia (L1: 3.0–<3.9 mmol/l; L2: <3.0 mmol/l). Missed bolus done (MBD) injections were meals without bolus injection within -15 and +60 min from the start of a meal. These outcomes were compared between the baseline (until visit 1) and follow-up periods (at least 5 HCP visits).	TIR increased (+1.9, 95% CI: 0.8–3.0 h/day, p < 0.001) from baseline to follow-up period with a reduction in time in hyperglycemia (-1.8; 95% CI:- 3.0–(-0.6) h/day, p = 0.003) and L2 hypoglycemia (-0.3; 95% CI: -0.6–(-0.1) h/day; p = 0.005) but with no change in time in L1 hypoglycemia. MBD injections decreased by 43% over the study (p = 0.002).
Jendle et al., 2021 (134)	NovoPen® 6	Basal and/or bolus insulin: deguldec (n = 21), detemir (n = 1), aspart (n = 79), human insulin (n = 1), faster-acting insulin (n = 1)	94 participants (48 men and 46 women; aged 18–83 years, mean 40.1 years) with T1DM	Multicenter prospective, observational, proof-of-concept study, continuation of Swedish study (Adolfsson et al., 2020 (80)) Clinical outcomes and healthcare costs (in 2018 Swedish krona, SEK) were projected to estimate cost-effectiveness of smart insulin pen use over patients' lifetime.	Smart insulin pen use was associated with improvement of mean discounted life expectancy (+0.90 years) and quality-adjusted life expectancy (+1.15 quality-adjusted life-years). Moreover, using smart injectors was a source of cost savings (direct SEK 124,270; indirect SEK 373,725) in comparison to standard care. The abovementioned profits were a result of projected lower frequency and delayed onset of diabetes complications versus standard care.
Vigersky et al., 2021 (142)	InPen™	Bolus insulin	529 individuals with non-optimal glycemic control (423 ones with glucose management indicator (GMI) >8.0% and 106 ones with GMI >9.5%)	Observational study CGM data were collected and compared before and up to 90 days after initiating InPen™ use. The outcomes were evaluated including means sensor glucose (SG), GMI, TIR, time above range (TAR), and time below range (TBR).	Patients with suboptimal metabolic control (GMI >8.0%) presented increased TIR (+2.3%, 0.6 h/day), reduced GMI (0.1%), SG (-4.3 mg/dl), and TAR (-2.4%) with no change in TBR, in comparison to pre-InPen™ use. Participants with poorest glycemic control at baseline (GMI >9.5) had TIR improvement by +5.0% (1.2 h/day), GMI by -0.4%, SG by -14.9 mg/dl, and TAR by 5.1% (1.2 h/day) with no change in TBR. From the first month to 90-days, post-InPen™ use bolus frequency decreased (from 3.7 to 3.6/ day and 3.3 to 3.2/day, respectively) and total rapid-acting daily dose of insulin increased (from 26.29 to 27.19 U/day and 27.57 to 29.24 U/day, respectively). All mentioned results were significant (p < 0.05).

T1DM, type 1 diabetes mellitus; CGM, continuous glucose monitoring; HCP, healthcare professional; TIR, time in range (70–180 mg/dl; 3.9–10.0 mmol/l); GMI, glucose management indicator; SG, sensor glucose; TAR, time above range (>180 mg/dl; >10.0 mmol/l); TBR, time below range (<70 mg/dl; <3.9 mmol/l); U, units of insulin.

TABLE 6 | Technical characteristics of smart insulin pens.

Pen device (year of introduction)	Company	Insulin producing company/insulin compatibilities		Cartridge volume and insulin concentration	Dose range (dose increment)	Monitors active insulin on board/ bolus dose calculator	Reports to download/ connects with company app on smartphones	Special characteristics	Battery lifetime/ application service
InPen™ (2017) (123, 143)	Companion Medical	Lilly Novo Nordisk	Humalog® NovoLog® Fiasp®	3 ml (100 IU/ml)	0.5–30 units (0.5 unit)	Yes	Yes	Integrates with CGM, insulin injection reminder, temperature sensor	1 year/ Android, Apple
ESYSTA® BT pen (144)	Emperra	Novo Nordisk	NovoRapid® NovoMix® 30 Levemir® Actrapid® Actraphane® 30/-50 Protaphane®	3 ml (100 IU/ml)	1–60 units (1 unit)	Yes	Yes	Stores 1,000 records, displays of the last insulin dose	6 months, replaceable/ Android, Apple
		Sanofi-Aventis	Lantus® Apidra® Insuman® Rapid Insuman® Comb 15/-25/-50 Insuman® Basal						
		Lilly	Huminsulin® Normal Huminsulin® Profil III Huminsulin® Basal (NPH) Humalog® Humalog® Mix 25/-50 Abasaglar®						
		Berlin-Chemie	Berlinsulin® H Normal Berlinsulin® H 30/70 Berlinsulin® H Basal Liprolog® Mix 25/-50 Pen Liprolog®						
		B. Braun	Insulin B. Braun Rapid® Insulin B. Braun Comb® Insulin B. Braun Basal®						
Pendiq 2.0 (145)	Pendiq	Novo Nordisk	NovoRapid® Fiasp® NovoLog®	3 ml (100 IU/ml)	0.5–60 units (0.1 unit)	No	Yes	Low battery/insulin level alarms, data transmit to a computer with USB cable, stores 1,000 records	Rechargeable with USB charger/ Android, Apple
		Lilly Sanofi-Aventis Berlin-Chemie	Humalog® Apidra® Lispro® Liprolog®						
NovoPen® 6 (2019) (146)	Novo Nordisk	Novo Nordisk	NovoRapid® NovoLog® Actrapid® Fiasp® Levemir® Tresiba®	3 ml (100 IU/ml)	1–60 units (1 unit)	Yes	Yes	Dose memory, uses NFC to transfer data	4 to 5 years/ Android, Apple

NFC, near-field communication.

TABLE 7 | Insulin pen caps.

Study, year	Device studied/ device compared	Type of insulin/company	Participants	Study design	Results
Gomez -Peralta F. et al. (2019) (137)	Insulclock®/ none	Humulin NPH, Abasaglar, Humalog®, Humalog® Junior, Humalog® Mix25, Humalog® Mix50, and Humalog® 200/Eli Lilly	9 volunteers with T1DM	Performance and functionalities tests	Insulclock® detected seven types of insulin pens with a 97% correct classification rate. Most of the doses were accurately detected (deviation = 0), with relative errors ranging from 3% to 7% across different dosages among 556 injections.
Gomez-Peralta F. et al. (2020) (138)	Insulclock®/ standard pen (masked device)	Humalog® KwikPen®/Eli Lilly	16	Randomized, single-center, prospective, open-label, pilot study	Insulclock® led to the decrease in mean glucose (-27.0 mg/dl [1.5 mmol/l]; p = 0.013), glucose standard deviation (SD) (-14.4 mg/dl [0.8 mmol/l]; p = 0.003), and time above range (TAR) (-12.5%, p = 0.0026), and an increase in time in range (TIR) (+7%; p = 0.038) in the overall population.
Galindo et al. (2021) (139)	Insulclock®/ standard pen (masked device)	Lantus®/Sanofi-Aventis	80 patients with uncontrolled T2DM on basal insulin	Randomized, 26-week, prospective, crossover, pilot study	Patients in the active phase were characterized by lower mean daily blood glucose (147.0 ± 34 vs. 157.6 ± 42 mg/dl, $p < .01$) and greater reduction of HbA1c (-0.98% vs. -0.72%, $p = .006$) but with no significant changes in treatment adherence, insulin omission, and insulin mistiming.

TABLE 8 | Technical characteristics of insulin pen caps.

Smart insulin pen cap (year of introduction)	Company	Insulin producing company/insulin compatibilities		Mobile app/ company	Bluetooth/ USB	Special characteristics	Battery lifetime
Timesulin® (2010) (141)	Bigfoot Biomedical	Eli Lilly Novo Nordisk Sanofi-Aventis	KwikPen® FlexPen® FlexTouch® SoloSTAR®	No	Yes/no	Records the time since the last injection	1 year
Dukada® Trio (2012) (140)	Dukada®	Novo Nordisk Sanofi-Aventis	FlexPen® SoloSTAR®	No	No/no	Flexible grip features, a light above the needle	6–8 months, replaceable
GoCap (2013) (136)	Common Sensing	Sanofi-Aventis Novo Nordisk Eli Lilly	SoloSTAR® FlexPen® KwikPen®	Yes/Apple, Android	Yes/yes	Shows the quantity of insulin in the pen, time and type of insulin injection displays in app	10 -days, rechargeable with micro-USB cable
Insulclock® (2019) (137)	Insulcloud	Sanofi-Aventis	SoloSTAR®	Yes/Apple, Android	Yes/No	Indicates the time, type, and amount of insulin administrated. App remained about food/glucose input, temperature fluctuations	Rechargeable with micro-USB cable

tools for insulin administration are various and have been constantly evolving for over the last 100 years. Insulin pens have changed the lives of millions of people who suffer from diabetes and now are the most widespread way of administering insulin. They are safe, simple to use, convenient, efficient, and less painful than conventional vials and syringes. An increasing number of modern, yet useful features may help to improve patients' quality of life. Technology evolves to improve adherence and glycemic outcomes, optimize delivery, and reduce dosing errors. Studies performed up to date, summarized in this review, indicate that insulin pens came a long way from a very simple device produced in the year 1985 up till the newest insulin smart pens, and the further improvement is on the way.

AUTHOR CONTRIBUTIONS

Conceptualization: MMas, KN, and JG. Writing—original draft preparation: MMas, KN, OJ, HK, MMac, and JG. Review and editing: MMas, KN, OJ, HK, and JG. Visualization: MMas, OJ, and KN. All authors contributed to the article and approved the submitted version.

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The Absorption of Needle-Free Insulin Aspart Through Jet Injector in Different Body Parts of Healthy Individuals

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The absorption of needle-free fast-acting insulin injected into different body parts of healthy male subjects was studied in an attempt to provide clinical guidance for diabetic patients who take needle-free insulin injections in terms of providing reference in the clinical guidance regarding the correct use of needle-free insulin injections among diabetic patients. This randomized, open-label, cross-over trial was conducted on eight healthy adult male volunteers, in which the skin thickness at three injection sites (abdomen, upper arm, and thigh), the time to peak, peak rate, and area under the glucose infusion rate (GIR) curve of plasma insulin were measured through the hyperinsulin-normal glucose clamp test after the injection of insulin aspart with a needle-free syringe at three different sites to analyze the correlation between insulin absorption index at different injection sites and skin thickness. The values of the skin thickness of the abdomen, upper arm, and thigh measured by ultrasonic wave were 2.45 ± 0.34 mm, 2.18 ± 0.50 mm, and 1.93 ± 0.55 mm, respectively. There was a significant difference in the skin thickness of the abdomen and thigh (P = 0.014). The hyperinsulin-normal glucose clamp model was successfully established for each subject. Approximately 0–2 h after injection of insulin aspart with needle-free syringes, the area under the GIR-time curve of the abdomen, upper arm, and thigh was 29,400.75 ± 2,645.00 ml, 30,230.50 ± 4,937.87 ml, and 30,179.63 ± 6,188.57 ml, respectively. There was no significant difference in the area under the GIR curve between any two injection sites (P >0.05). The time to peak of GIR at different injection sites was 38.68 ± 13.57 min in the abdomen, 40.86 ± 12.70 min in the upper arm, and 37.03 ± 13.29 min in the thigh, respectively, in which no significant difference was found between each of them (P >0.05). The GIR curve after injection at the three different sites was consistent with each other. There was no significant difference in insulin absorption after the injection of insulin aspartate into the abdomen, upper arm, and thigh with a needleless syringe in healthy male adult volunteers, and there was no correlation between skin thickness at the injection site and insulin absorption. Injection sites did not affect the absorption of insulin in needle-free injections.

Keywords: needleless syringe, hyperinsulin-normal glucose clamp, injection site, insulin absorption, healthy adult male

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1 INTRODUCTION

Diabetes mellitus (DM) is a clinical syndrome characterized by chronic hyperglycemia due to inadequate insulin secretion and/or deficient insulin action. Chronic complications of diabetes can occur because of prolonged poor glycemic control (1). Good glycemic control can improve the progression of diabetes-related complications.

Diabetes is a progressive disease. In type 1 diabetes, insulin must be used to maintain life, while in many patients with type 2 diabetes, it is necessary to increase the drug dose or choose combination drug therapy for better glycemic control (2). When the combined use of oral hypoglycemic agents is not effective or conjunctive, insulin or non-insulin injections should be used to control hyperglycemia and reduce the risk of diabetic complications. For patients with a long course of disease or multiple complications, insulin therapy has become the most important and even necessary measure to control blood glucose.

Insulin administration technology has undergone a long process of continuous innovation (3). The advent of insulin pens has improved insulin delivery. Compared with syringes, it is simpler, safe, effective, operable, and easy to carry and improves the treatment compliance of a patient to a certain extent. With the development of injection technology, needleless syringes have been accepted by a large number of patients due to their advantages, such as good injection experience, less subcutaneous injury, and faster peak time of efficacy, thus becoming a new choice for diabetic patients with insulin injection.

Needle-free injectors, called jet injectors, are usually used for insulin administration among patients who have injection-related anxiety or phobia. Additionally, the use of these jet injectors has been reported to markedly improve and accelerate the absorption of rapid-acting insulin from subcutaneous tissues and into the blood (4). These injectors often deliver insulin at a very high velocity, reaching more than 100 m/s across the subcutaneous tissue while distributing insulin over a larger area compared to syringes (5). Recent evidence shows that the use of the euglycemic clamp technique is effective among healthy individuals in terms of shortening the time-to-peak plasma insulin levels by as much as 50% compared to the normal insulin injection pen (6).

When patients start to use insulin injection therapy, it is necessary to choose the injection site for each injection. Current studies have shown that when insulin is injected into patients through a syringe, injection sites may impact on the insulin absorption and blood glucose control of patients (7). However, there are still no relevant studies on needle-free insulin injections. In this study, the normal glucose clamp technique was used to observe the effects of the injection at different injection sites in the body with needle-free syringes on the absorption of insulin aspart.

2 MATERIALS AND METHODS

2.1 Research Subjects

In this randomized, open-label, cross-over trial, we included healthy male subjects (18–75 years of age) at the Beijing Hospital, Beijing, China, during March, 2021. The inclusion criteria included the following: (1) healthy male subjects with no diabetes or any other endocrine abnormalities, (2) clinical examination and laboratory assessment revealing no abnormalities, (3) body mass index (BMI) ranging from 18 to 32 kg/m^2 with no change in body weight of more than 10 kg in the 3 months preceding screening, and (4) hemoglobin ≥12 g/L during screening.

On the other hand, patients were excluded if they had any of the following criteria: (1) participation in other clinical trials within the 3 months preceding the conduct of this trial, (2) severe recurrent hypoglycemia, (3) history of complications related to poor glycemic control such as diabetic ketoacidosis or hyperosmolar coma in the previous 6 months, (4) serious cardiovascular events within the previous 6 months, (5) immunocompromised state, (6) end-stage renal disease requiring dialysis, (7) history of cancer in the previous 5 years, (8) mental disorders, (9) long-term alcohol and drug abuse, (10) chronic skin lesions at the injection sites, (11) allergy to insulin or its preparations, and (12) hemoglobin disorders such as thalassemia or sickle cell anemia or anemia of any cause.

2.2 Trial Ethics

This clinical trial was carried out in Beijing Hospital and was approved by the Drug Clinical Trial Ethics Committee of Beijing Hospital (Approval Number: 2020BJYYEc-026-01) (ChiCTR2100049569). This study was conducted in accordance with the Helsinki Declaration, Quality Management Practice for Pharmaceutical Clinical Trials (GCP) and relevant regulations. The subjects were informed of the nature, purpose, and possible adverse reactions before being enrolled in the trial, and all participants gave informed consent before conducting the study protocol.

2.3 Research Methods

The establishment of the hyperinsulin-normal glucose clamp model was carried out in four main steps. The first step is the establishment of venous blood collection and infusion channels. Subjects were placed in the supine position, and then a superficial venipentesis was performed on one side of the forearm in the opposite direction of the heart, and an indwelling catheter was placed to maintain the channel with heparin saline to facilitate blood collection. Arterial blood was arterialized by heating the arm of the subject with a constant temperature electric blanket (50–60°C). A venous cannula was placed from the midcubital vein on the other side for insulin and glucose infusion. The second step involved injecting of short-acting human insulin at the beginning of the experiment into an injection pump through the mid-cubitus vein. The infusion rate for the first 10 min was 2.0 mU/kg/min, which rapidly increased the blood insulin level; subsequently, continuous infusion was carried out at a rate of 1.0 mU/kg/min for 290 min. During the period, 0.1 ml of venous blood was taken from the superficial venous channel of the forearm of the subjects to measure the blood glucose value as follows: Blood samples were collected every 5 min during the period of 120–0 min before drug administration and every 5 min during the period from 0 to 60 min (inclusive) after drug administration. Blood was sampled every 10 min during the

period from 61 to 180 min (inclusive) after drug administration. The third step was the determination of the target blood glucose value: venous blood was collected every 10 min to measure blood glucose with the glucose oxidase method with the rapid blood glucose detector of Johnson & Johnson (China) Medical Equipment Co., Ltd.). The mean value of three points was taken as the baseline blood glucose value, and the difference between the mean value of baseline blood glucose and 0.28 mmol/L (5 mg/dl) was taken as the target blood glucose value of the subjects in this clamp test. The fourth and final step involved the determination of glucose infusion rate (GIR), where, in the test, the input rate of 20% glucose solution in an infusion pump was adjusted according to the results of blood glucose testing to maintain the blood glucose level at the target value. The GIR and corresponding times were recorded.

2.3.1 The Overall Research Steps

The participation time of each subject was defined as the period from the time when the participants agreed to enter the group to participate in the study to the end of the final follow-up, including screening period of 1 week, the first phase of 1 week, the second phase of 1 week, the third stage of 1 week and the follow-up period of 1 week, so each participant is expected to participate in the study for 5 weeks (the overall flow chart is shown in (**Figure 1**). The skin thickness of the abdomen, upper arm, and lateral thigh in all subjects was measured by ultrasound and recorded. All the subjects fasted for 12 h before the study and then started the phase I trial (start time was recorded): Under the condition of maintaining the same temperature, humidity, environment, and other external factors, all subjects began the study at the same time. After the model of normal glucose clamp was established, all the subjects were injected with insulin aspart (NovoRapid. Penfill, Novo Nordisk. AS) through the QS-M needle-free jet injector (Beijing QS Medical Technology Co., Ltd., China), which was approved by China Food and Drug Administration (FDA) in 2012. The dose was calculated at 0.2 IU/kg. Meanwhile, blood glucose was continuously monitored for 3 h, exogenous glucose infusion rate was adjusted, and GIR changes were recorded. Blood glucose was measured every 5 min 1 h before drug administration and every 10 min 2 h after drug administration. The washout period started at the end of the first phase of the experiment. It was ensured that the subjects kept a normal light diet for a week (weight change within ±5%). The second stage test was started after blood glucose went normally (the start time was recorded): the normal glucose clamp model was reestablished according to the method in the first cycle. The injection experiment was conducted after the successful establishment of the model. The experimental method and the blood sampling time were the same as before, except the injection site was changed into the upper arm. Furthermore, the results were recorded. The washout period started at the end of the first phase of the experiment. It was ensured that the subjects kept a normal light diet for a week. The third stage test was started after blood glucose had gone normally (the start time was recorded): the normal glucose clamp model was reestablished according to the method in the first cycle, and the injection experiment was conducted after the successful establishment of the model. The experimental method and the blood sampling time were the same as before, except the injection site was moved to the lateral thigh. Furthermore, the results were recorded. At the end of each experimental period, the healthy subjects were required to remain under observation for 4 h. Before they left the test center, their vital signs were detected, blood glucose levels were monitored, and adverse events were closely observed and recorded. They can leave with no abnormalities. Follow-up was conducted 7 ± 3 days after the end of the third cycle experiment.

2.4 Statistical Analysis

Statistical analysis was performed by SAS 9.4 software (SAS Software Institute in North Carolina, USA). Unless otherwise specified here, all the statistical analysis tests adopted two-sided hypothesis tests, and the level of hypothesis test was set at $\alpha = 0.05$. That is, P-values less than or equal to 0.05 were considered statistically significant. In normally distributed data, the means and standard deviations (SD) were reported for continuous variables, and the numbers and percentages were reported for categorical variables. On the other hand, for skewed data, the median and interquartile ranges (IQR) were reported for continuous variables. For grade indicators, such as various scoring values, frequency tables were listed, the median and quartile spacing were used for the statistical description, and the rank sum test was performed. For the counting index, frequency tables and percentages were used for description. Chi-square or exact probability methods were used for the test.

The difference in skin thickness at the injection site, the difference in time to peak glucose infusion rate at each site, the difference in peak value of glucose infusion rate at each site, and the difference in area under the GIR curve at each injection site are all compared pairwise by paired T-test. $P < 0.05/2 = 0.017$ (the test level of 0.05 needs to be corrected to reduce class I errors)) means that the difference between the two positions is statistically significant. $P > 0.05/2 = 0.017$ (the test level of 0.05 needs to be corrected to reduce class I errors) means that the difference between the two positions is not statistically significant.

A correlation test was conducted between the thickness of the injection site and the time to peak and the peak rate of plasma insulin. The area under the GIR curve, $P < 0.05$ indicates that the correlation is statistically significant, and $P > 0.05$ means that there is no statistical significance in the correlation. The maximum value of the absolute value of the correlation coefficient R is 1, and the minimum value is 0. The closer to 1, the greater the correlation is. The positive number of the correlation coefficient indicates that there is a positive correlation, and the negative number indicates that there is a negative correlation.

3 RESULTS

3.1 Demographic Characteristics of the Subjects

Eight subjects were finally included in the analysis, with an average age ranging from 18 to 33 (average = 27.9; mean = 27.47; SD = 13.51) years, a median height of 174 cm (174.00 ± 0.44),

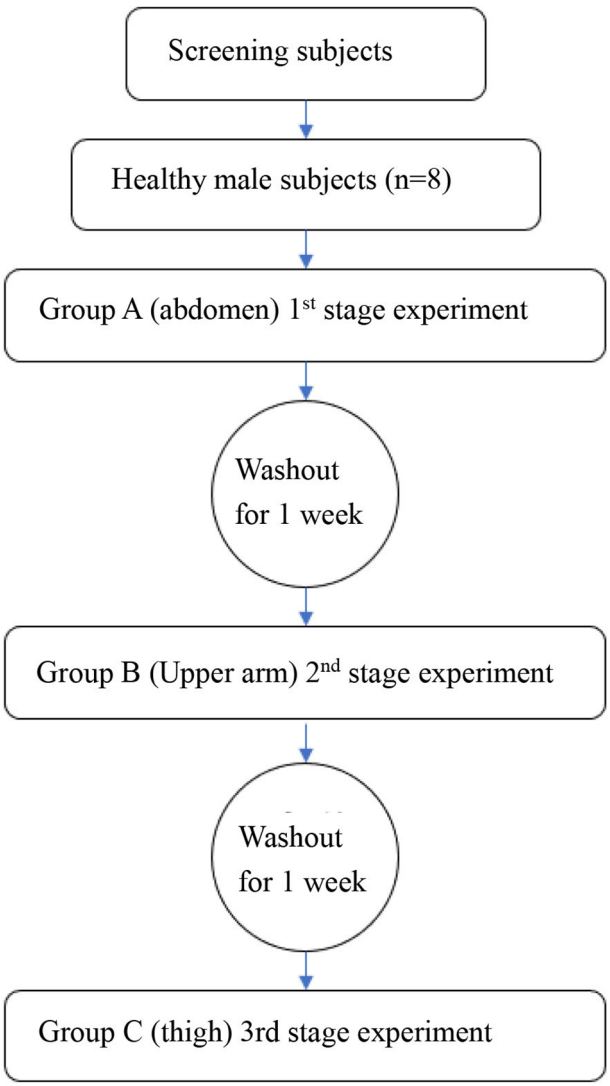

FIGURE 1 | The overall research step. The machines and methods for measuring skin were supplemented. A Samsung RS-80A ultrasonic diagnostic instrument and a L3-12AMHz linear array probe were applied for exploration. All subjects took the supine position, and the skin thickness was measured on the upper abdomen, lateral upper arm, and lateral thigh.

and a mean BMI of 22.42 ± 5.15 kg/m^2. All subjects had no previous family history of diabetes, liver or kidney diseases. All of the participants were not sensitive to the injected insulin aspart, and all of them had a normal hemoglobin level of ≥12 g/L. The baseline demographic characteristics of the included subjects are shown in **Table 1**.

3.2 Difference in Skin Thickness of the Injection Site of the Subjects

All subjects fasted for 12 h before the study. The skin thickness of the abdomen (about 1 cm above the pubic symphysis, higher than the part about 1 cm below the costal margin and on the right of the area surpassing 2.5 cm around the periumbilical), the upper arm (the central 1/3 in the lateral part of the right upper arm) and the lateral thighs (the upper 1/3 part on the anterolateral right thigh) were measured by ultrasound and recorded, as shown in **Table 2**. The skin thickness of the abdomen, upper arm, and thigh was 2.45 ± 0.34 mm, 2.18 ± 0.50 mm, and 1.93 ± 0.55 mm, respectively. Statistical analysis by paired t-test showed that there was a significant difference in the skin thickness of the abdomen and thigh (P = 0.014).

3.3 Insulin Absorption at Different Injection Sites

The hyperinsulin-normal glucose clamp technique was used in this study. During the test, the infusion rate of 20% glucose solution was adjusted according to the blood glucose test results to maintain the target blood glucose level. **Figure 2** shows that the serum glucose

TABLE 1 | Demographic characteristics of the subjects.

	Age (Years old)	Body Height (cm)	Body Weight (kg)	BMI (kg/m^2)
N (Missing)	8 (0)	8 (0)	8 (0)	8 (0)
Mean ± SD	27.47 ± 13.51	174.00 ± 0.44	67.99 ± 8.74	22.42 ± 5.15
Median	27.90	174.00	67.50	22.01
Min, Max	19.97, 32.74	161.00, 183.00	53.00, 82.00	19.07, 26.17

SD, Standard deviation; N, Number.

concentrations of the three different injection sites were basically the same as the target blood glucose, and the blood glucose level within 3 h was basically maintained at the target blood glucose level, indicating that the normal glucose clamp model was successfully established. Under these conditions, the glucose infusion rate reflected the sensitivity of the tissue to exogenous insulin. As can be seen from **Figure 3**, the glucose infusion rate curves of the abdomen, upper arm, and thigh were basically the same after injection.

The time to peak of glucose infusion rate at different injection sites was 38.68 ± 13.57 min for abdominal injection, 40.86 ± 12.70 min for upper arm injection, and 37.03 ± 13.29 min for thigh injection, as shown in **Table 3**. Paired t-tests showed that P >0.05, indicating that there was no statistically significant difference in the time to peak glucose infusion rate between any two injection sites in the abdomen, upper arm or thigh.

The peak values of the glucose infusion rate at different injection sites were 297.75 ± 18.15 for abdominal injection, 310.38 ± 57.39 for upper arm injection, and 301.38 ± 57.78 for thigh injection, respectively, as shown in **Table 3**. The paired t-test showed that P >0.05, indicating that there was no statistically significant difference in the peak value of glucose infusion rate between any two injection sites in the abdomen, upper arm, or thigh.

The changes in plasma insulin content at different injection sites are compared in **Table 3**. The results of the paired t-test showed that P >0.05, which meant that there was no statistically significant difference in the area under the glucose infusion rate-time curve between any two injection sites in the abdomen, upper arm, or thigh 0–2 h after insulin injection, indicating that the absorption of insulin at different injection sites was similar.

The correlations between skin thickness at different injection sites, time to peak, and peak rate of plasma insulin, and area under the GIR curve are shown in **Table 4**. The correlation coefficients were all small (R was −0.084, 0.006, and −0.055, respectively), and P-values were all greater than 0.05, indicating that there was no statistically significant correlation between skin thickness at the injection sites and time to peak, peak rate of plasma insulin, and area under the GIR curve.

4 DISCUSSION

For diabetic patients who cannot control the disease with oral hypoglycemic drugs, the supplementation of exogenous insulin is one of the main therapeutic methods for better glycemic control. In the past decades, great progress has been made in insulin therapy, new insulin preparations, and administration methods have been developed, but there are still many obstacles, challenges, and uncertainties (8). The main reasons for patient refusal were inconvenient of insulin treatment (51.6%) and fear of injection (48.2%) (9). Due to the long course of disease, patients receiving insulin injections generally ignore the injection site rotation strategy and often reuse needles, which leads to subcutaneous fat hypertrophy (10–15). This, in turn, affects glycemic control, resulting in unexplained hypoglycemia and a significant increase in blood glucose change levels (11, 16, 17), which leads to an increase in insulin costs (18).

Needleless injection is a new type of insulin administration technology that is simple, safe, effective, operable, and easy to carry. It can reduce the fear of syringes and significantly improve the quality of life of patients (18). Insulin injected by injection shows a specific tapered diffusion pattern in subcutaneous tissues with a relatively large surface area. This diffusion pattern enhances the absorption of insulin in the blood circulation, thus achieving a more direct hypoglycemic effect (10). The injection of insulin aspart by jet can enhance insulin absorption and shorten the duration of hypoglycemic action. This curve is more similar to the pattern of endogenous insulin secretion, which can achieve better postprandial insulin coverage and correct postprandial glucose fluctuations (6).

TABLE 2 | Difference in skin thickness of the injection site.

Site	Skin thickness (mm)	Paired T	P-value
Abdomen	2.45 ± 0.34	1.676	0.138
Upper arm	2.18 ± 0.50		
Abdomen	2.45 ± 0.34	3.241	0.014
Thigh	1.93 ± 0.55		
Upper arm	2.18 ± 0.50	0.977	0.361
Thigh	1.93 ± 0.55		

The results of paired t-test above showed that P <0.05/2 = 0.017 (the test level of 0.05 should be corrected here to reduce class I error) indicated that the difference between the two parts was statistically significant, P >0.05/2 = 0.017 (the test level of 0.05 needs to be corrected here to reduce class I error) indicated that the difference between the two parts is not statistically significant.

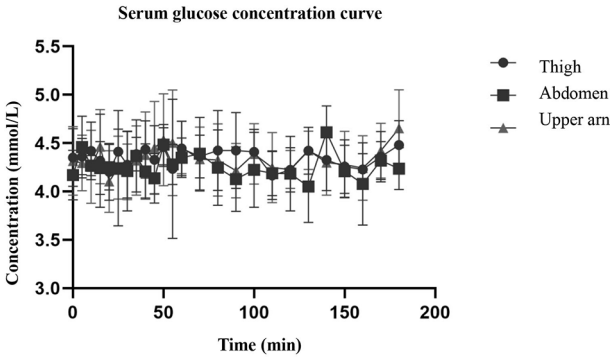

FIGURE 2 | The figure shows that the serum glucose concentrations of the three different injection sites were basically the same as the target blood glucose, and the blood glucose level within 3 h was basically maintained at the target blood glucose level.

Previous studies have shown (19) that in patients with type 1 and type 2 diabetes, insulin absorption is significantly faster and postprandial hyperglycemia is significantly reduced after the administration of a needleless jet syringe. The improvement of early postprandial glucose control may be particularly beneficial for patients with difficulty controlling postprandial glucose fluctuations (20). Compared with traditional needle-injected insulin therapy, needle-free insulin therapy showed a better hypoglycemic effect and significantly improved the satisfaction of patients with insulin therapy. Additionally, needle-free syringes also have better safety (21). However, there are few studies on whether needle-free injection is affected by skin thickness at different injection sites.

The hyperinsulin-normal glucose clamp technique was used in this study. The observational record indices under study included: (1) changes in the plasma insulin levels of the subjects; (2) changes in the GIR curve of the subjects; and (3) skin conditions of the subjects at the injection sites (injection reaction, skin thickness). Through the comparison of these indicators, the differences in drug absorption and safety of insulin injected with needle-free syringes in different parts of the body were studied.

Although the skin thickness at the three injection sites was different, especially that of the abdomen and thigh, there was a significant difference (**Table 2**), but the serum glucose concentration curve of the three parts after insulin injection (**Figure 2**) was basically consistent with the glucose infusion rate curve (**Figure 3**). A significant difference was not seen between time and peak (**Table 3**) and peak value (**Table 3**) of glucose infusion rate and the area under the GIR curve of plasma insulin in each part (**Table 4**). During the test, the infusion rate of 20% glucose solution was adjusted according to the blood glucose test results to maintain the target blood glucose level. **Figure 2** shows that the serum glucose concentrations at the three different injection sites were basically the same as the target blood glucose level and the blood glucose level within 3 hours was basically maintained at the target blood glucose level. The glucose infusion rate reflects the use of glucose by the tissue and its sensitivity to insulin. With the hyperinsulin-normal blood glucose clamp model in this study, GIR reflects the sensitivity of

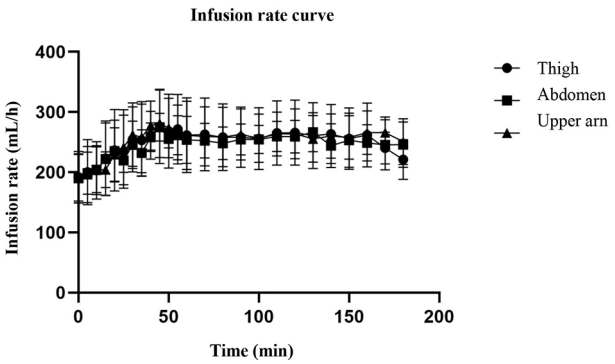

FIGURE 3 | The curve of glucose infusion rate. The figure shows that the serum glucose concentrations of the three different injection sites were basically the same as the target blood glucose, and the blood glucose level within 3 h was basically maintained at the target blood glucose level. As can be seen from the figure, the glucose infusion rate curves of the abdomen, upper arm, and thigh were basically the same after injection.

TABLE 3 | The differences in time-to-peak, peak values, and area under the glucose infusion rate curve at different injection sites.

Variable	Thigh	Upper Arm	Thigh	Abdomen	Upper Arm	Abdomen
Time to peak						
Time	37.03 ± 13.29	40.86 ± 12.70	37.03 ± 13.29	38.68 ± 13.57	40.86 ± 12.70	38.68 ± 13.57
Paired T	-0.584		-0.277		0.344	
P	0.578		0.79		0.741	
Peak values of GIR						
Rate (ml/h)	301.38 ± 57.78	310.38 ± 57.39	301.38 ± 57.78	297.75 ± 18.15	310.38 ± 57.39	297.75 ± 18.15
Paired T	–0.568		0.204		0.739	
P	0.588		0.844		0.484	
Area under the GIR curve (0–2 h)						
Mean (SD)	30,179.63 ± 6,188.57	30,230.50 ± 4,937.87	30,179.63 ± 6,188.57	29,400.75 ± 2,645.00	30,230.50 ± 4,937.87	29,400.75 ± 2,645.00
Paired T	0.043		–0.539		–0.726	
P	0.967		0.607		0.491	

P, P-value; GIR, Glucose infusion rate. The results of paired t-test above showed that P <0.05/3 = 0.017 (the test level of 0.05 should be corrected here to reduce class I error) indicated that the difference between the two parts was statistically significant, P >0.05/3 = 0.017 (the test level of 0.05 needs to be corrected here to reduce class I error) indicated that the difference between the two parts was not statistically significant.

the tissue to exogenous insulin. It can be seen from **Figure 3** that glucose infusion rate curves after injections at three different positions in the abdomen, upper arm, and thigh were basically consistent with each other, which showed that exogenous insulin sensitivity was not affected by different injection sites. We also analyzed the correlation between the injection site thickness and the time to peak and rate of plasma insulin, and the area under the GIR curve, and did not find a statistically significant correlation, indicating that insulin absorption was similar at different injection sites, which was independent of the skin thickness at the injection sites. Studies on routine insulin injections have found that the thickness of injection sites and local blood flow both affect insulin absorption (22). For the first time, we conducted a study to compare the absorption of needle-free insulin injected in different parts of the body. Furthermore, we found that the absorption of insulin was not related to the skin thickness at the injection site, indicating that the absorption of the drug by jet injection (needleless) was different from that by conventional needle injection. The exact mechanism needs further study.

That being said, our study has several major limitations, and therefore, our findings should be carefully interpreted. First, the small sample size of our population made it difficult to reach statistically significant differences among the different injection sites in terms of insulin absorption. Second, subjects were followed-up for a short period of 1 week, and we are not confident whether there would be a significant variation in the absorption rate over a longer period. Third, our population was made up of normal, healthy individuals, who were administered a single dose of insulin. Therefore, future studies of larger sample sizes, longer follow-up periods, and conducted among diabetic patients who are injected with insulin regularly are still warranted to validate our findings and estimate the difference in the absorption of rapid-acting insulin through jet injectors in different body parts of diabetic patients. Finally, another point that should be carefully taken into consideration is the BMI of recruited subjects because it is evident that the absorption of insulin, regardless of body part, is variable based on BMI (23). In our study, recruited subjects had low BMI, so we are not confident whether or not our findings would be consistent among subjects with high BMI. Therefore, we recommend putting this point into consideration when conducting future studies.

The results in this study showed that the injection of insulin aspart with a needleless syringe into the abdomen, upper arm and thigh of healthy male adult volunteers had no effect on insulin absorption, and the skin thickness at the injection site had no correlation with insulin absorption. This study provided some evidence that diabetic patients took turns in needle-free insulin injections at different parts of the body, and therefore, it can be used as hypothesis-generating article for directing future research.

TABLE 4 | Correlation between skin thickness at injection sites, time to peak and peak rate of plasma insulin and the area under GIR curve.

Indices	Skin thickness	
	(r)	P-value
Time to peak	–0.084	0.695
Peak rate	0.006	0.976
Area under GIR curve	–0.055	0.800

P <0.05 meant the correlation was statistically significant, P <0.05 indicated no statistical significance. The maximum value of the absolute value of the correlation coefficient R is 1, and the minimum value is 0. The closer to 1, the greater the correlation is. The positive number of correlation coefficient indicates that there is a positive correlation, and the negative number indicates that there is a negative correlation.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material. Further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Beijing Hospital. The patients/participants

provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

QP and LG designed the manuscript. LZ, AG, and DY collected the data. XW, YZ, and LG analyzed the data. All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

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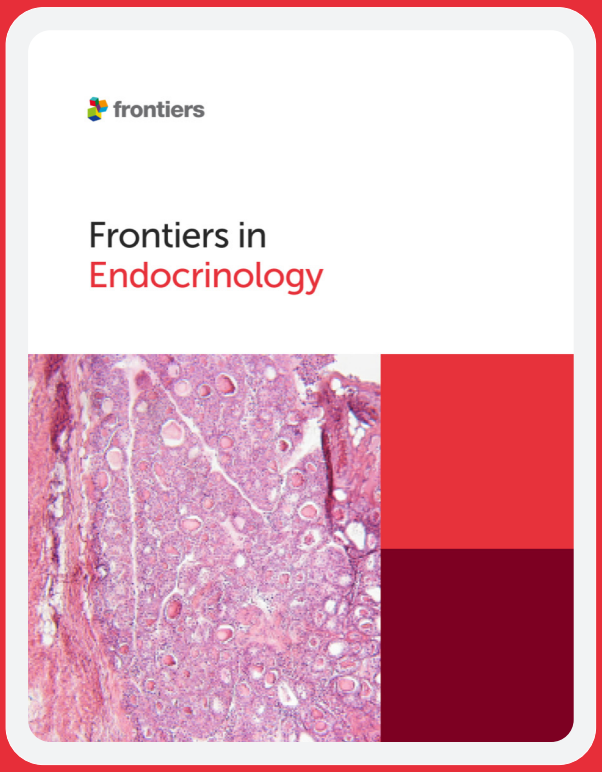

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