PERSONALIZED NUTRITION

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PUBLISHED IN: Frontiers in Nutrition

frontiers Research Topics

ISSN 1664-8714
ISBN 978-2-88966-811-3
DOI 10.3389/978-2-88966-811-3

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PERSONALIZED NUTRITION

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Citation: Blaak, E. E., Roche, H. M., Afman, L., eds. (2021). Personalized Nutrition. Lausanne: Frontiers Media SA. doi: 10.3389/978-2-88966-811-3

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frontiers in Nutrition

EDITORIAL
published: 25 March 2021
doi: 10.3389/fnut.2021.669307

Editorial: Personalized Nutrition

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Keywords: personalized nutrition, precision-based nutrition, microbiome, web-based intervention, childhood, plant-based diets

Editorial on the Research Topic

Personalized Nutrition

Worldwide, the prevalence of obesity has grown dramatically since the 1980s (http://www.who.int/news-room/fact-sheets/detail/obesity-and-overweight). Driven by easy access to energy-dense foods and a sedentary lifestyle, obesity has become a major global health and socio-economic problem in the twenty-first century (1), leading to insulin resistance (IR), a pro-inflammatory and hypoxic milieu, and type 2 diabetes (T2D) (2). More recently there has also been concern that the prevalence of obesity-related cancers will rise. While dietary guidelines are evidence-based and valuable for the general population to maintain health, it has become increasingly clear that it may be important to personalize dietary advice to obtain more effective prevention and treatment of chronic metabolic diseases. Indeed, lifestyle intervention, focused on personalization of general guidelines for a healthy diet and physical activity at an individual level, may reduce diabetes and metabolic syndrome risk by more than 50% in different settings worldwide (2–4). Nevertheless, within these tailored interventions, 30% of the participants do not respond and/or adhere to the intervention. More insight is required on factors determining adherence to a healthy diet, as well as the biological mechanisms underpinning divergent responses to dietary interventions, with respect to metabolic outcomes and subsequent potential pathologies, in order to optimize the effectiveness of intervention.

In the Research Topic *Personalized Nutrition*, important dietary strategies are addressed that focus on the delivery of personalized advice, web-based vs. face-to-face (Al-Alwadhi et al.), precision-based strategies, methodological considerations based on diet-microbiome interactions (Johnson et al.; Nestel et al.), insight on mechanisms underlying impaired glucose metabolism in childhood and puberty (Cominetti et al.), as well as a study design of a Randomized Controlled Trial focused on the impact of plant-based vs. animal-based diets (Dawczynski).

In the review of Al-Awadhi et al., evidence for the effectiveness of web-based and face-to-face dietary interventions on dietary change is assessed to give more insight into which method may be most effective at delivering personalized nutrition. In total, 19 peer-reviewed randomized controlled trials were identified for inclusion in the review. Findings from personalized web-based nutrition interventions showed that they may be successful at inducing short-term dietary change. Nevertheless, there appears to be insufficient evidence that personalized web-based interventions are as effective as personalized face-to-face interventions, indicating the need for further controlled comparative studies and cost-benefit analyses.

In addition to focusing on the delivery of personalized advice, more information is required on whether precision-based strategies focused on a metabolic or microbial phenotype may be effective at improving the success of nutritional intervention with respect to metabolic health (5).

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Specialty section:
This article was submitted to Nutrition and Metabolism, a section of the journal Frontiers in Nutrition

Received: *18 February 2021*
Accepted: *02 March 2021*
Published: *25 March 2021*

Citation:
Blaak EE, Roche HM and Afman LA (2021) Editorial: Personalized Nutrition. Front. Nutr. 8:669307. doi: 10.3389/fnut.2021.669307

Microbial responses to foods are very personalized. A landmark study within this field by Zeevi et al. (6) showed that, despite high interpersonal variability in post-meal glucose profiles, personalized diets created with the help of a machine learning algorithm, including data reflecting dietary habits, physical activity, and gut microbiota, may be used to successfully resolve elevated postprandial glucose responses. Additionally, a 2020 study [PREDICT, (7)] again showed that there is large variation in how individuals respond to different meals in a large, well-characterized cohort. Subsequently, the influence of person-specific factors, such as gut microbiome and genetics, on their response was estimated, as was the influence of meal macronutrient composition. Nestel et al. studied whether postprandial glucose response 60 min after a standardized breakfast (PPGR) was associated with gut microbial α-diversity (primary outcome) and explored whether postprandial responses of glucose and insulin were associated with specific microbiome taxa, colonic fermentation markers, and abiotic factors in 31 healthy individuals. They observed that the gut microbiome, measures of colonic fermentation, and abiotic factors (fecal characteristics and intestinal transit time) were not shown to be significantly associated with variability in postprandial responses. This may be related to the fact that contributions may be subtle. Indeed, the PREDICT study showed that the gut microbiome explained just 6% of the variability in glycemic responses (7). However, the sample size is relatively small in the Nestel study. Nevertheless, interesting associations were observed between intestinal transit time and fasting glucose, as well as between fasting breath hydrogen and fasting insulin, which may require further study.

Studying diet-microbiome-host metabolism is complex due to high interindividual variation in both dietary intake and microbial composition, and the difficulty of controlling diet and microbiome covariation in observational and interventional studies. In the excellent review by Johnson et al., variables that should be considered and controlled for in study designs are discussed. The review explores several other factors that need to be considered in future studies, including the requirement of multiple consecutive microbiome samples per study time point or phase and multiple days of dietary history prior to each microbiome sample whenever feasible. Additionally, the difficulty in quantifying dietary intake and analyzing microbial composition is discussed. Although the effects of diet on the microbiome may be observed within a day, subsequent impacts on host metabolism may take much longer to occur. The authors also recognize and review that diet-microbiome interactions may be personalized with consequences for initial stratification and/or sample size.

Personalized or precision-based strategies may also be relevant for different life stages since consumer needs, as well as health profiles, may vary across the lifespan. The obesity pandemic is also of great concern in children, with 124 million children worldwide currently obese (https://www.who.int/end-childhood-obesity/en/). Importantly, Cominetti et al. addressed how temporal glycemic variations during childhood relate to physiological changes in central energy metabolism and substrate oxidation in the EarlyBird study. The EarlyBird study is a non-interventional, prospective cohort study that recruited 307 healthy UK children at age 5, and followed them annually throughout childhood for 12 years with longitudinal data on blood metabolite profile, anthropometry, and respiratory exchange ratio. They showed that fasting glycemia increases steadily during childhood, which is accompanied by increased insulin concentrations, and was positively associated with LDL and VLDL blood lipid signature, as well as greater fasting respiratory exchange ratio, reflecting a shift toward carbohydrate oxidation. Metabolome data show increased flux through glycolytic pathways, and complex changes in amino acids. These findings raise an important question: at what point do physiological changes, such as increasing fasting glycemia, begin to have pathophysiological consequences on cardiometabolic health? Greater understanding of the relation to the underlying mechanisms and pathogenic consequences may provide insights in to the relation with optimal intervention "windows" or time points.

Currently, consumers are driving demand for more plant-based diets and lifestyles in Europe. However, not much is known on the long-term effects on cardiometabolic health of specific lifestyles like flexitarians, vegetarians, and vegans. The biological impact of plant-based dietary interventions is very difficult to implement, wherein isocaloric dietary control groups allow true definition of relative efficacy, independent of energy and macronutrient composition. In the article by Dawczynski, a study design is presented of the NuEva study. This study aims to reveal the impact of chosen nutritional habits (Western diet, flexitarians, vegetarians, vegans) on health status and disease risk with respect to physiological benefits or possible pathophysiological consequences, resulting from long-term implementation of the studied diets. It is a 12-month parallel-designed trial (with 12 months follow-up) with at least 55 participants for each diet (vegetarian, vegan, flexitarian [rare meat/sausage consumption, once or twice per week]), and participants who consume a traditional Western diet as the control group. Major questions that will be answered are: Is it possible to ensure an adequate intake of all essential nutrients; are land-based n-3 PUFA from linseed oil a suitable source to ensure an adequate status of n-3 LC-PUFA; and what impact has each diet had on health and disease and in particular, cardiovascular risk? This study may provide important information on more personalized guidance of groups with specific dietary habits.

In conclusion, it is increasingly evident that one size does not fit all. Thus, personalized or precision-based dietary intervention strategies provide the opportunity to increase the effectiveness of dietary prevention and treatment of chronic metabolic diseases. The present Research Topic addresses different interesting aspects related to personalized delivery of dietary guidance, metabolic profiling of specific age groups, as well as precision-based strategies and methodological considerations based on diet-microbe-host interactions. Overall, we still need more research on the most optimal implementation of dietary advice as well as to relating (epi)genetic, microbial, and metabolic phenotypes to intervention outcomes to define more optimal diets for individuals with or predisposed to chronic metabolic diseases. In this, the different etiologies toward T2D,

cardiometabolic diseases, and possibly obesity-related cancers have to be considered. More prospective trials are required to provide the evidence for the implementation of these approaches.

AUTHOR CONTRIBUTIONS

EB wrote the editorial. All authors revised the manuscript.

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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.

frontiers in Nutrition

REVIEW
published: 12 June 2020
doi: 10.3389/fnut.2020.00079

A Guide to Diet-Microbiome Study Design

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Intense recent interest in understanding how the human gut microbiome influences health has kindled a concomitant interest in linking dietary choices to microbiome variation. Diet is known to be a driver of microbiome variation, and yet the precise mechanisms by which certain dietary components modulate the microbiome, and by which the microbiome produces byproducts and secondary metabolites from dietary components, are not well-understood. Interestingly, despite the influence of diet on the gut microbiome, the majority of microbiome studies published to date contain little or no analysis of dietary intake. Although an increasing number of microbiome studies are now collecting some form of dietary data or even performing diet interventions, there are no clear standards in the microbiome field for how to collect diet data or how to design a diet-microbiome study. In this article, we review the current practices in diet-microbiome analysis and study design and make several recommendations for best practices to provoke broader discussion in the field. We recommend that microbiome studies include multiple consecutive microbiome samples per study timepoint or phase and multiple days of dietary history prior to each microbiome sample whenever feasible. We find evidence that direct effects of diet on the microbiome are likely to be observable within days, while the length of an intervention required for observing microbiome-mediated effects on the host phenotype or host biomarkers, depending on the outcome, may be much longer, on the order of weeks or months. Finally, recent studies demonstrating that diet-microbiome interactions are personalized suggest that diet-microbiome studies should either include longitudinal sampling within individuals to identify personalized responses, or should include an adequate number of participants spanning a range of microbiome types to identify generalized responses.

Keywords: microbiome, diet, dietary intake, study design, methodology, personalized nutrition

OPEN ACCESS

Edited by:
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Specialty section:
This article was submitted to Nutrition and Metabolism, a section of the journal Frontiers in Nutrition

Received: *06 February 2020*
Accepted: *04 May 2020*
Published: *12 June 2020*

Citation:
Johnson AJ, Zheng JJ, Kang JW, Saboe A, Knights D and Zivkovic AM (2020) A Guide to Diet-Microbiome Study Design. Front. Nutr. 7:79. doi: 10.3389/fnut.2020.00079

INTRODUCTION

Microbiome features and metabolites have been increasingly linked to states of health and disease (1, 2), and diet is appreciated as one of the key drivers of this relationship. The microbes residing in the human gastrointestinal tract depend on their hosts for sources of fermentable substrate. Microbes metabolize the end-products of human digestion and indigestible dietary substrates to produce a wide variety of diet-derived and secondary metabolites. The microbes themselves and their metabolites signal the immune and nervous systems through known and unknown

mechanisms, ultimately affecting human physiology, and disease development or progression. Unlike the known relationships between the essential nutrients and disease, which focus on single-nutrient relationships such as that between vitamin C and scurvy, these new diet-microbe-disease, and diet-metabolite-disease relationships are more complex. Microbe-disease relationships likely depend on the production of diet-derived metabolites such as short chain fatty acids (SCFAs) or on the production of secondary metabolites created by microbes prior to entering host circulation (**Figure 1**). While mechanisms have been identified for certain unique diet-microbe-metabolite relationships (3, 4) and a growing body of evidence suggests that the microbiome is playing an important role in drug metabolism (5), the complexity of the changing landscape of each individual's gastrointestinal tract has proven difficult to study. With each individual person's microbiome acting as an ecosystem, the majority of mechanisms explaining exactly how diet alters the microbiome and conversely how the microbiome alters dietary inputs to impact health remain uncharacterized.

High interindividual variation in both dietary intake and microbiome composition contribute to our poor understanding of the relationships between the foods we eat and how they impact the bacterial communities that reside within our bodies (6). It is difficult to control for diet and microbiome covariation in observational and interventional studies. In this article we discuss the limitations and complexity involved in planning dietary intervention studies with microbiome outcomes. We identify variables that should be considered, controlled for, and recorded in study designs. Finally, we attempt to make practical suggestions for study designs moving forward that appropriately incorporate and control for nutritional factors such as food intake and dietary patterns.

A BRIEF REVIEW OF DIET-MICROBIOME INTERACTIONS

Past efforts to understand diet-microbiome relationships have linked dietary features to microbial composition (7, 8), but few diet-microbe-metabolite pathways have been decoded. Most of the exploration of diet-microbe-metabolites has been focused on SCFA production from fiber. However, other metabolites are gaining increased interest. Products of bacterial proteolytic fermentation from dietary proteins have been linked to negative health conditions such as diabetic kidney disease (9) and insulin resistance in individuals without diabetes (10). A large focus on the research in this space has centered on the microbial production of trimethylamine (TMA) from host dietary choline, which after absorption and conversion to trimethylamine N-oxide (TMAO), becomes a circulating microbe-host co-metabolite that is associated with increased risk for cardiovascular disease (3). Blocking microbial TMA production in a mouse model has been demonstrated to reduce atherosclerotic lesion development (11). The production of secondary bile acids by microbes have also been linked to health outcomes. For example, the relationship between cream-derived saturated fat, host production of taurocholic acid, and *Bilophila wadsworthia* has been identified in animal models as potentially important for the development of inflammatory bowel diseases in genetically susceptible individuals (12). Microbial production of secondary bile acids may also impact host metabolism by producing ligands that bind to the farnesoid X receptor (FXR) (13) and the G protein-coupled bile acid receptor-1 (also known as the Takeda G-protein-coupled receptor-5[TGR5]) (14). A large amount of work remains to be done in this space for these relationships to be exploited for their therapeutic potential because the majority of metabolites measured by untargeted mass spectroscopy remain uncharacterized (15).

A growing body of literature suggests that microbial responses to foods are personalized. For example, efforts to improve blood glucose control based on microbiome features suggest that food-microbe pathways could improve health in a personalized manner (16). The abundance of *Prevotella*, in particular, has been described as a way to differentiate between responders and non-responders with improved glucose metabolism following consumption of bread high in barley fiber (17, 18). Similar findings of responder and non-responder microbiome features have been reported after consumption of whole grain sourdough or white bread (19). Microbial responses to fiber-specific dietary interventions have also revealed responder and non-responder phenotypes related to the production of SCFAs (20, 21). Beyond treating dysbiotic states and predicting biochemical responses to food, changing diet or providing prebiotics in a personalized way could also theoretically change the microbial community to be more accepting of new members from a fecal transplant or after administration of probiotics. The future of personalized nutrition will likely need to harness all of these mechanisms, but the knowledge base needed to design microbiome-informed, personalized nutrition interventions is currently limited.

Diet is attractive as a modifier of gut microbiome composition because it itself is modifiable. If we can modify diet to modify the microbiome or the metabolites produced by the microbiome, then we can potentially prevent or modulate disease outcomes. There are two ways that dietary intake can interact with the microbiome to impact health. First, specific diets, dietary patterns, food components, and foods may change gut microbiome composition in a predictable way. For example, many studies have found an enrichment in protein and fat metabolizing microbial species or genes in Western diets, which are enriched in animal products and deficient in fiber, and conversely an enrichment in saccharolytic microbes in diets high in fermentable fiber (22–24). Second, diet-derived metabolites may themselves be modified by sets of microbes or individual microbial strains and affect host physiology (25). For example, the soy isoflavone daidzein is a phytoestrogen that can be metabolized to equol by gut microbes, and this gut microbe-derived metabolite has significantly stronger estrogenic activity than its precursor (26). Although equol production is common in animals, only ∼30% of humans harbor microbes that convert daidzein to equol, with important implications on the estrogenic potential of soy-derived isoflavones on human health (26). However, while diet may predictably change some

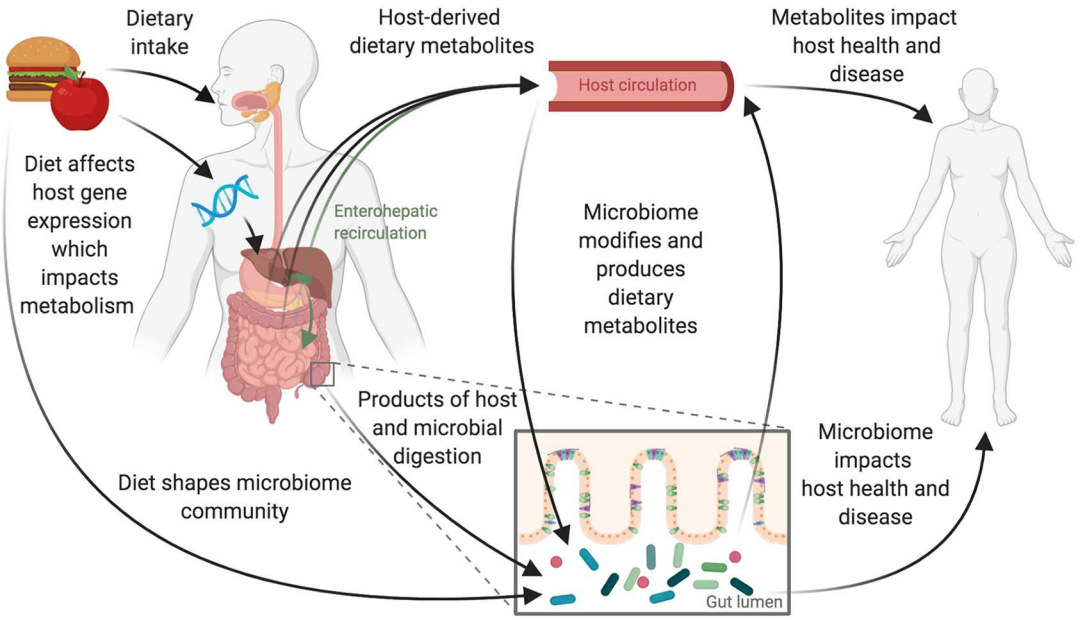

FIGURE 1 | Diet and the gut microbiome interact to influence host health. Dietary intake and the gastrointestinal microbiome interact to affect host physiology and disease. These interactions are complex and likely depend on interplay between the host's genetics and immune system and the composition and function of the gut microbiome. Consumption of foods begins the digestive process, leading to the production of host-derived metabolites primarily absorbed through the stomach and small intestine. Host genetics impacts this metabolism and can also be influenced by diet with some diet-gene interactions altering host gene expression. Food and dietary products that reach the large intestine can shape the microbiome directly. These products are also acted upon by microbes to produce microbially derived metabolites. Host-derived metabolites that have previously entered host-circulation may also pass into the large intestine via biliary enterohepatic recirculation or by transport across the gut epithelium resulting in additional opportunities for the host and microbes to jointly act upon dietary derived metabolites.

aspects of the microbiome and while certain dietary components may be predictably modified by certain microbes harbored by different groups of individuals, many food-microbe interactions are variable and dependent on the individual (6, 16).

Altering the microbiome through dietary intake is not a new concept. One hundred years ago John C. Torrey stated that, "It is now well-known that diet exercises a profound influence on the determination of the types of bacteria developing in the intestinal tract (27)." Scientists of the early 1900s accepted that the intestinal flora, as they referred to what we now call the microbiome, changed quickly in response to changes in diet. These findings were built on a foundation of animal studies (28), but they incorporated minimal evidence from humans (29). Likewise, modern diet-microbiome understanding has grown out of strong foundations in animal models—primarily mice (12, 30)—with other non-human primates also contributing to our understanding (31). Increasingly, there is a need to translate findings from animal models to free-living humans and recent work has made strides toward this effort. The body of diet-microbiome literature has recently been reviewed elsewhere (15, 25, 32–34).

CURRENT DIET ASSESSMENT PRACTICES AND THEIR LIMITATIONS IN DIET-MICROBIOME STUDIES

The advent and increasing availability and affordability of sequencing technology has resulted in an explosion of diet-microbiome literature. This is easily illustrated with a PubMed search of "diet" and "microbiome." In 2009 there were 100 papers published and in 2019 there were 2,204 papers published that were identified using these search terms. As of January 2020, there are 9,544 papers that are returned on PubMed using these terms, and of those over half were published in the last 3 years. This increase in publications has been accompanied by a growing awareness of the limitations we face while attempting to measure and analyze the highly complex interactions between microbes, dietary exposures, and host phenotypes.

Measurement of dietary intake remains particularly challenging. While the methods for collection and analysis of microbiome data have improved over the past decade, there has been little change in the analysis and collection of dietary data with many human studies relying on food frequency questionnaires (FFQs) or self-administered single day food records or 24-h dietary recalls. Each of these methods is prone to reporting errors and is associated with advantages and disadvantages, as has been reviewed extensively elsewhere (35). Importantly, while certain dietary assessment approaches may be adequate for estimating total caloric intake, dietary diversity, or the intake of certain foods and food categories, they may not capture the level of detail needed to discover relationships between diet and the gut microbiome. Although improved methods for the collection and assessment of diet in microbiome studies are desperately needed to address these issues, the development and adoption of new dietary assessment techniques

will take time. In the meantime, careful consideration of key variables when planning studies and integrating dietary data with microbiome outcomes is recommended.

Accurately measuring and assessing dietary intake using self-report and nutritional biomarkers is challenging (36, 37). When choosing a dietary assessment technique for a microbiome study the method selected will ultimately impact the research questions that can be answered using the data. Like most research decisions, to assess diet it is necessary to weigh competing options in terms of time, quality, and cost. In this case, time includes both investigator time for collection and review, and participant response time and burden due to effort expended to record diet. Participant time is influenced by the timeframe of dietary record keeping. Low-burden dietary assessments include the administration of a single 24-h recall or record or a single FFQ to capture dietary history over the preceding weeks, months, or years. More time-intensive longitudinal dietary assessments, using detailed daily records over an extended period of weeks, months, or even years have a high participant burden. Quality relates to how closely the data from the dietary assessment accurately captures and reflects actual dietary intake, and how well the data capture the level of detail necessary for microbiome-related outcomes such as the inclusion of microbially-important diet-derived chemicals in nutrient databases (38). Cost is also multifactorial, including the cost of any nutritional software or physical measurement collection devices like scales and measuring cups, to the cost of trained personnel to collect or enter daily 24-h recalls, and the cost needed to pay participants to encourage participation and complete record keeping. Typically, researchers can only pick two of these three competing interests—time, quality, or cost. Therefore, researchers usually choose to minimize participant time burden and overall cost at the expense of quality. What this means in practice is that research teams frequently use FFQs or dietary screeners instead of more time- and cost-intensive methods like multi-day diet records or recalls administered by trained personnel.

Dietary measurement by FFQ has both advantages and disadvantages (39, 40). The primary advantage is that FFQs are convenient. They are very easy to administer and take less participant time than other methods. A study participant simply indicates the frequency at which they consume specific foods and how much they consume, and these answers are used to estimate total caloric intake, as well as the intake of the major macronutrients and micronutrients. However, because FFQs were developed to quantify broad dietary patterns or indices of healthy eating, they are limited in a number of ways and cannot capture diet as accurately as other methods (41). While far from optimal, dietary patterns estimated by FFQ have provided some insight into the way habitual diet contributes to microbiome composition; long-term FFQ-determined nutrient intake has been associated with microbial composition, and relationships found between FFQ-determined dietary patterns and the abundance of microbial genera (7, 42–44). These findings have been supported by research showing that changing dietary patterns, either experimentally or through natural experiments that take advantage of seasonal eating habits or immigration, affect microbiome composition (45–47). However, most FFQs are not designed to provide data that can link specific foods to specific changes in microbial species composition or functional pathways.

Despite the identification of some signals from microbiome studies that use FFQs for dietary analysis, the technique is simply not specific enough to untangle the complex relationships between foods and the microbiome. We have shown previously that microbiome composition more closely covaries with food intake, not nutrient intake (6), indicating that reliance on existing nutrient composition variables is insufficient and that foods themselves are important when exploring diet-microbiome covariation. However, even well-conducted 24-h recalls and food records fail to sufficiently capture the complexity of foods in ways that are meaningful to microbes. For example, one brand of bread may include 7 different whole grains as well as nuts and seeds, while another brand may be made with sprouted wheat. During diet entry both of these breads may be coded as whole-wheat bread and therefore downstream analysis will treat them as identical when they are different. This variation could contribute to some of the personalized diet-microbiome results that have been reported with respect to blood glucose response (16, 19).

In instances where researchers recognize the need to collect more finely resolved dietary information, it is common in the microbiome literature to read about techniques that use researcher-developed food questionnaires, independently created app-based collection methods (16, 48), and consumer-facing tools for nutrient analysis rather than using validated techniques for dietary collection or nutrient analysis tools developed with a research focus (23). When collecting 24-h recalls or asking participants for 24-h food records, inclusion of properly trained staff can improve data quality. At a minimum, participants recording their intake should be trained using detailed examples showing the level of detail necessary to complete an accurate record. This should include a discussion of serving sizes, ingredient specificity, preparation methods, and the inclusion of commonly forgotten foods and additives. All records should be reviewed with the participant with a focus on identifying misreported or commonly forgotten foods. Methods for dietary intake assessment are improving and technology using computerized recalls and records can greatly reduce researcher burden when collecting dietary data. Regardless of collection method, when records or recalls are coded for analysis using dietary research software, consideration for consistency with data entry, coding, and cleaning of dietary data is important to allow for robust analysis of nutrient composition and food intake downstream.

In the current dietary record collection environment, different dietary collection tools and FFQs rely on different underlying databases, which makes comparison across studies and cohorts difficult, if not almost impossible. Even within the English speaking regions of the Americas, United Kingdom, and Australasia, food records are ultimately mapped back to different databases depending on the software tools used for analysis (49). Each database provides nutrient composition data, but the source of that data varies. The analysis methods for specific

nutrients can also vary by database which leads to different nutrient level outcomes in databases from different countries. Beyond nutrient composition, the naming conventions used to identify foods are not identical across databases leading to issues when comparing data collected using different tools. Additionally, food-grouping structures vary, with no universally accepted method adopted across studies, or databases. Efforts to establish a shared food ontology that can harmonize dietary data collected in different global regions and by different tools are in progress, incorporating other food features such as preparation method (50). Food preparation and cooking methods alter the chemical properties of foods, such as the changes that occur to sweet potatoes during cooking, that then impact the effects of that food on the microbiome (51), adding another layer of complexity to measurement of diet. The food matrix (52) is likely to play an important role in the relationship between diet and the microbiome and should also be considered. Specific information such as the ripeness of fruits is not currently captured in any food databases. However, this level of detail may be highly relevant for certain food-microbe interactions. For example, it is known that when bananas are unripe or green the starch contained within them is resistant starch (53), which is fermentable by microbes, whereas in ripe bananas that resistant starch has broken down into simpler starch and glucose molecules, which are absorbable by the host, and no longer provide any fermentable substrate for the gut microbes. Beyond food preparation and ripeness, recent research addressing eating behaviors around ultra-processed foods shows that controlling for energy and macronutrient content alone may be insufficient for dietary interventions (54). Food sourcing, processing or cooking methods (51), additives or emulsifiers (55, 56), artificial sweeteners (57), and conventional or organic farming methods (58) likely also need to be taken into consideration.

In addition to the microbiome changes induced by the biochemical components of foods, foods themselves contain bacteria that affect the gut microbiome. From a health perspective, fermented dairy such as yogurt and cheeses, are the most commonly recognized foods that contain "beneficial" microbes (59). These foods are sources of microbes that can transiently populate the human gut (23). Fresh, non-fermented foods have long been recognized as a source of food-borne pathogens and are the target of public health interventions to prevent the spread of food-borne disease. Despite this recognition, we know surprisingly little about the microbial composition in other non-fermented foods. Recently, crops that are not usually considered to transfer bacteria, such as apples, have been shown to harbor a microbiome that depends on growth and farming practices (58). Indeed, dietary patterns that include more food types, particularly fermented foods like yogurt, contain a higher abundance of microbes relative to less diverse diets (1×10^9 colony forming units [CFU] vs. 1×10^6 CFU in a day's worth of meals) (60). Consideration for the microbial load of a dietary pattern is important because the engraftment of non-pathogenic food-borne bacteria depends, in part, on other dietary components. For example, higher abundances of parmesan-cheese-associated bacteria are present after consumption of milk products (61). Ideally, we need to consider these microbial features of diet when planning and analyzing microbiome-diet studies.

CONSIDERING DAY-TO-DAY VARIATION IN BOTH MICROBIOME AND DIET

As the number of longitudinal microbiome studies steadily increases in the literature, controlling for within-individual variation is an increasingly important consideration during study design. Here we present reasoning that supports including multiple consecutive microbiome samples per study timepoint or phase, and multiple days of dietary history prior to each microbiome sample whenever feasible; thus capturing longitudinal change within an individual by measuring changes over time or before and after a dietary intervention, while also using repeated sampling around set timepoints to control for within-person variation. To support these recommendations, we provide suggestions for microbiome sample collection, transport, and storage for diet-microbiome studies and explore questions that researchers designing studies in this space should consider.

Considerations for Sample Size and Consecutive Microbiome Samples

Diet explains around 5–20% of the variation in microbiome (6, 46, 62, 63). In every cohort there is a large amount of variation between individuals that is not driven by diet and likely depends on environmental differences, early life exposures, or immune and other host differences (64, 65). Without establishing a baseline microbiome for each study participant, it is difficult to assess the effects of dietary interventions on the microbiome. Moving toward designs that can account for inter-individual variation is necessary to improve microbiome studies (66). Studies can minimize the impact of inter-individual variation by increasing the number of participants enrolled in a study. The number of participants needed to assess the effects of a particular dietary intervention on the gut microbiome likely varies depending on the research question and anticipated taxon difference in a case-control setting. Study populations of 400–500 individuals are necessary to power case-control microbiome studies to detect differences in dominant taxon proportions of between 5 and 9% (62), suggesting that these sizes should be sufficient to detect differences of dietary origin. This also suggests that a substantial amount of cross-sectional research in the diet-microbiome space may be underpowered to detect dietary impacts on microbiome composition and raises questions regarding when cross-sectional study designs should be used, if at all, to assess diet-microbiome interactions. We expect that in the majority of cases longitudinal studies in which subjects serve as their own control will be more fruitful than cross-sectional studies, and we expect that cross-sectional studies will require large sample sizes.

Increasing sample size will boost power to identify diet-induced microbiome differences but does little to account for the dynamic nature of the microbiome. Increasingly, data support the need for repeated sampling of the microbiome to account for intra-individual variation with 3–5 daily sequential fecal

samples in aggregate providing better results over single samples in inflammatory bowel disease cohorts (67) and in healthy individuals (6, 68). Averaging repeated samples and looking at changes within an individual over time allows for greater power to detect microbiome differences by removing within-person noise; thereby reducing the need to dramatically increase sample sizes. In both healthy and disease cohorts, little improvement is seen with the collection of more than 7–9 serial samples (67, 68). The caveat for the collection of 3–5 serial samples per person is that the strongest data for this recommendation comes from cohorts of healthy individuals. While some data from inflammatory bowel disease cohorts suggests this is sufficient to capture variability (67), there are insufficient data for most other microbiome-related conditions where the microbiome may have large compositional swings that are not captured with this density of sampling.

In addition to considerations regarding sample size and sampling frequency, researchers should take into consideration participant selection as a means to improve diet-microbiome research (**Figure 2**). When enrolling participants for dietary intervention trials it can be useful to consider the baseline microbiome of participants. In situations where a 1-2 week turn-around from collection to sequence is possible, there are opportunities to consider participant stratification or exclusion by baseline microbiome composition during recruitment. Current sequencing timeframes likely prevent the incorporation of this type of baseline assessment. However, with advances in fast, affordable sequencing this may be possible in the future. If we imagine a future where baseline microbiome-typing is feasible, then it should be possible to select or stratify participants with the goal of including a range of microbiome compositions at baseline to capture the breadth of possible responses to dietary interventions, or the converse goal of selecting only participants with a certain type of microbiome, for example, *Prevotella*-dominant, to increase power. In study designs for dietary interventions where the length of the intervention required makes a cross-over design difficult, stratified randomization or an interspersed treatment design that incorporates baseline microbiome could make parallel arm studies more feasible. As an alternative, selection for a specific baseline microbiome phenotype should improve the ability to assess how interventions depend on the presence of particular microbes. As a thought experiment, we consider the situation of studying a specific prebiotic. A researcher in this situation may want to choose only people who have the targeted microbe that is expected to be enriched by that prebiotic, or to choose people who have a low abundance of fiber-degrading microbes to see how the intervention affects that subgroup. Importantly, microbial phenotyping at baseline would allow for randomization into study arms using a stratification design that incorporates baseline microbiome composition, thereby avoiding the situation where after randomization clusters of individuals with similar microbial compositions are assigned to the same treatment arm (6).

Strategies for Fecal Sample Collection and Sequencing

Repeated and longitudinal microbiome sampling strategies require careful consideration of fecal sample collection, transportation, and storage to prevent unintentional bias due to the potential for the composition of these living communities to change after collection. Comparisons of fecal microbiome collection, transport, and storage methods have been detailed in several recent publications (69–72). When considering longitudinal studies and repeated microbiome sampling the collection process selected for the study has a large impact on participant compliance and will also dictate the parameters that should be considered in terms of temperature and transport.

Several commercial collection methods are readily available for researchers and others can be easily assembled from standard laboratory supplies. Prior to choosing a collection kit researchers should consider the volume of sample necessary for sequencing and other planned analyses, as well as the conditions and available resources associated with the study design. Swab fecal collection requires very little fecal material and allows participants to easily obtain specimens from used toilet paper or collected stool samples. Swab collection has been used successfully in large scale citizen science projects like the American Gut Project (73). The American Gut Project sampling methodology which uses a swab to take a small amount of fecal material from a stool or soiled toilet paper did not significantly alter microbial diversity and composition as measured by 16S amplicon sequencing compared to other methods (74). However, swabs might not be ideal for studies where larger amounts of fecal material are necessary, for example where metabolomics is required in addition to sequencing (75).

For studies that require a large volume of fecal material, collection of an entire bowel movement can be completed using a stool collection hat or container. The collection of the entire bowel movement may be important for some studies considering that the distribution of microbial species in a single bowel movement is not homogenous (76, 77). Fecal sampling location within a single stool sample may affect the abundance of specific microbes, reflecting the diverse microbial communities residing in small niches throughout the gastrointestinal tract (78). Collection of the entire bowel movement presents significant logistical challenges including the need for study participants to collect and transport large sample volumes. When collecting whole stool samples researchers should work closely with participants to agree upon collection times. Fresh samples should be transported to the research facility quickly where study personnel can process, aliquot, and store the sample. Consideration should be given to the timing of bowel movements and participant or researcher schedules when incorporating whole stool collections into protocols.

For most studies, participant self-collection of a few grams of fecal material into a collection container using a sterile spatula or scoop provides ample material for deep shotgun sequencing or even metabolomics without undue participant or researcher burden. Subsampling of a stool sample can over- or under-represent the relative abundance of certain taxa and under-report low abundance taxa (79). However, the differences in composition relative to whole stool samples are minor compared to inter-individual variability (80); making this a viable collection methodology for clinical studies. Participants can be trained to collect a fecal sample using a stool collection hat and then to transfer a portion of the whole stool sample into one or

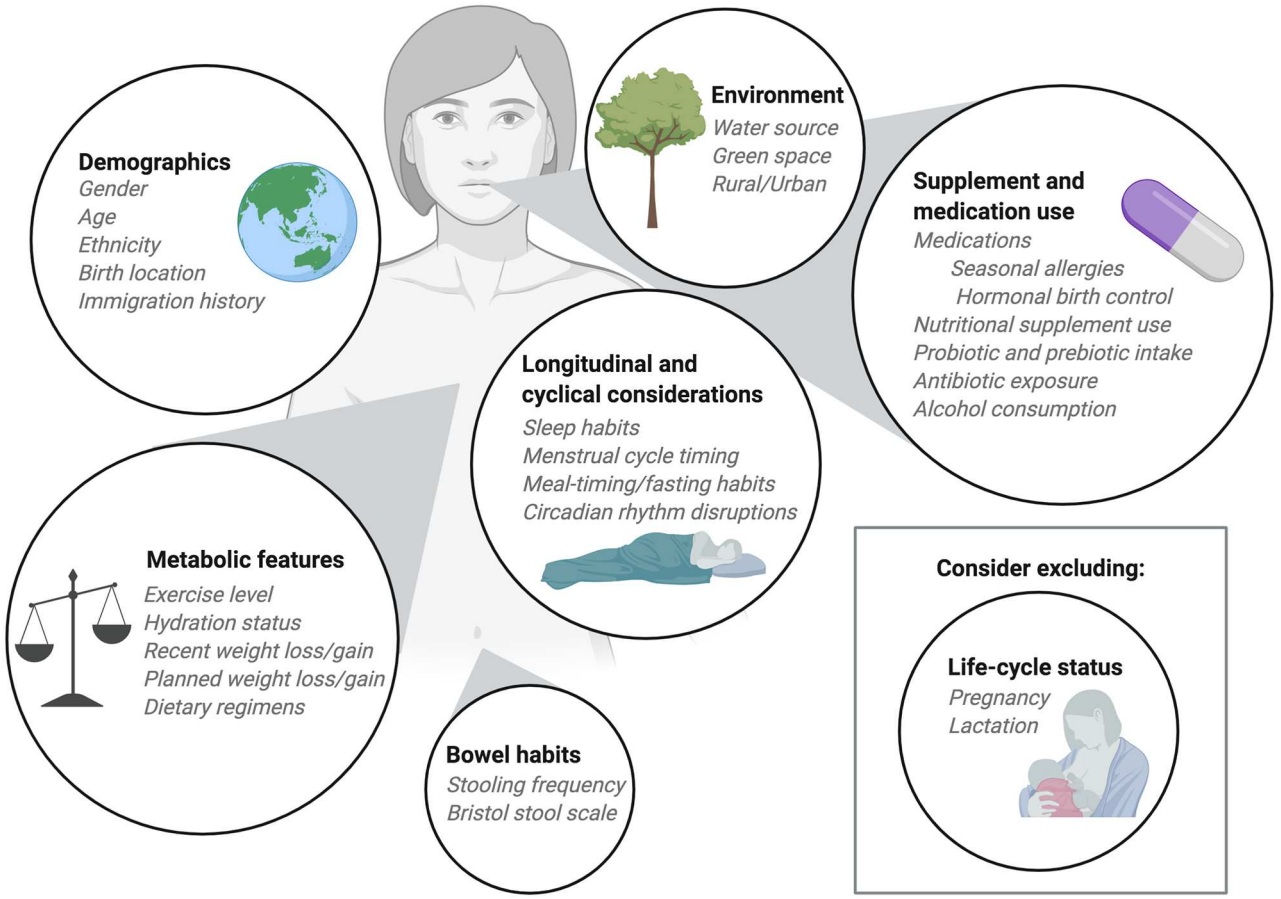

FIGURE 2 | Considerations for participant enrollment and data collection. When planning diet-microbiome studies researchers should take into consideration numerous participant features when determining inclusion and exclusion criteria. Demographic information should be collected with a focus on features and exposures known to impact the microbiome. Information about medication use, including commonly consumed over-the-counter medications should be recorded. Depending on the research question, researchers may choose to exclude participants who consume supplements, prebiotics, or probiotics. Alternatively, researchers may ask participants to maintain consistent use of nutritional products throughout the study period. Recent infection and antibiotic exposures should be considered as part of the inclusion and exclusion criteria as should alcohol consumption. In longitudinal studies, normal cycles including sleep cycles, menstrual cycles, and meal timing or fed/fasted cycles should be collected as covariates. The metabolic status of participants can be an important covariate and, when possible, exercise level and hydration status should be recorded and held constant. For research with metabolic outcomes, exclusion of participants who have recently experienced weight loss or weight gain may be important. Typically, we exclude pregnant and lactating participants from our dietary intervention studies, but in some instances it may be appropriate to include these individuals. Finally, bowel habits should be considered at enrollment and during study participation. When collecting multiple consecutive stool samples from participants the frequency of stooling can impact study retention and complicate study timelines so it can be helpful to interview participants about their usual bowel habits before enrollment.

more tubes. One benefit of this collection strategy is that the participant can transfer fresh samples into one or more small cryovials or microcentrifuge tubes which can be immediately stored in regular freezer boxes; minimizing sample handling and diminishing sample degradation from freeze-thaw cycles (81).

Regardless of which sampling and collection technique is selected, the collection container can be prefilled with preservative, which allows for the sample to be shipped and stored at ambient temperature. If samples are collected without preservatives then they need to be temperature controlled from the point of collection (e.g., placed in coolers with ice packs or dry ice, temporary storage in home freezers) until they can be stored appropriately at −80°C in a research facility. Immediate sample storage at −80°C is considered the gold standard regardless of the presence of a preservative. In most human studies, practical considerations around the timing of bowel movements, location relative to the research facility, or availability of refrigeration requires the use of a preservative to prevent microbial growth in these samples. Preservatives can allow samples to be stored at ambient temperatures for as long as 60 days without impacting technical reproducibility (82); although the exact number of days varies by preservative (74, 83). Commonly used preservatives like 95% ethanol and RNA*later* result in high reproducibility and stability, as do some commercially available kits like the OMNIgene gut kit (71). Many preservatives can interfere with fecal metabolites (84), however when multiple analyses (DNA, RNA, and metabolites) are required from the same sample 95% ethanol may have advantages over other methods (82). Overall,

as long as the preservation and collection method is standardized within the study, many methods provide acceptable stability and reproducibility.

Choosing a DNA sequencing technique for the analysis of collected fecal samples is another important consideration. When selecting a method, the desired taxonomic and functional resolution required to address study hypotheses should be carefully considered. The primary sequencing methods available for microbiome classification are 16S amplicon sequencing and metagenomic sequencing. The 16S ribosomal RNA gene is conserved across bacteria and contains nine hypervariable regions (V1-V9) that have amassed changes throughout evolutionary history. Amplification and sequencing of the variable regions within this gene (i.e., V3-V4) (85) allows for a low-cost and rapid way to detect the taxonomic composition of a sample (86). However, this method is relatively low-resolution and does not allow for accurate elucidation of bacterial taxonomy beyond the genus level. Since this method involves the use of primers to amplify the target bacterial gene, bias may also be introduced that affects the outcome of sequencing (87, 88). Therefore, this method would not be desirable when more detailed assessment of taxonomy is desired.

An alternative to 16S sequencing is deep whole metagenomic shotgun sequencing, which involves the sequencing of all microbial genomes in a sample. This method provides resolution to species level of taxonomy, and can also detect eukaryotic species and viruses to provide information on other microbes in a sample (86). Amplification bias is less likely to occur with this primer-free approach, but one downfall is that it is significantly more expensive, which may affect the feasibility of use for larger-scale studies. Shallow shotgun sequencing is an alternative to deep shotgun metagenomic sequencing. This method is significantly cheaper and has been shown to provide accurate resolution of species despite having a lower sequencing depth in comparison to deep sequencing (89). Shallow shotgun sequencing has proven effective in assessing longitudinal covariation of the microbiome with dietary intake demonstrating that this is a viable approach for cost-effective longitudinal diet-microbiome studies (6). Additional approaches for and analysis of microbiome data with a clinical focus have recently been thoroughly reviewed including the considerations for when to include metatranscriptomics and metabolomics approaches in analysis pipelines (90).

Collecting Multiple Days of Dietary History Prior to Each Microbiome Sample

For observational studies where dietary intake is considered as a confounder for the microbiome outcome of interest, the decision to collect dietary data is driven by different factors than when dietary intake is the exposure of interest. We do not think that all microbiome studies need to collect dietary data. In many well-designed microbiome studies it may not be necessary to collect dietary data and the decision to collect dietary data should be weighed carefully with the researchers' hypotheses and planned analyses. In observational studies where diet will be a clear confounder that cannot be controlled for through other study design parameters, investigators should collect detailed information about diet.

There are additional factors causing personalization of diet-microbiome interactions. Of particular importance is the effect of transit time on microbiome-diet study planning and analysis. Transit time has been shown to affect the microbiome directly. Fukuyama et al. conducted a longitudinal study on human participants receiving an intervention to induce iso-osmotic diarrhea, a less extreme method to mimic a physiological disturbance of gut microbiome than antibiotic-induced cleanse, and revealed that rapid transit time affects microbiome composition. This study pointed out that although rapid transit, usually resulting from diarrhea, can cause dysbiosis, the gut microbiome communities revert to their pre-cleanout states within ∼5 days after the cleanout, depending on the specific strains of bacteria (91). Because of the differences in transit time between people when sampling a single microbiome time point in a study, different durations of dietary history may be represented in a single stool sample for different people. In people who have a bowel movement 3 times per day one may observe diet-microbiome interactions at shorter time intervals, whereas individuals who have bowel movements less frequently, on the order of once a day or every other day, may have longer dietary time intervals represented in a single stool sample. More research is needed into how to best manage this temporal variation when analyzing diet-microbiome interactions. We have demonstrated that using several days of recent dietary history in models can improve the ability to pair diet and microbiome covariation (6). Other approaches to manage variation in transit time include the provision of foods labeled with dyes to measure transit time at the start of a study, or the ingestion of radio-opaque labels coupled with x-ray to empirically measure transit time. The latter has the distinct benefit of avoiding the ingestion of potentially microbiome-active food dyes, however it also comes with increased investigator and participant risk and burden.

We recommend the collection of dietary recalls or records from 2-3 days prior to the sample collection period (**Figure 3**). In a cohort where transit time is measured and known it may be possible to optimize the dietary collection window relative to each stool sample to capture the dietary intake that best corresponds to each microbiome sample. When transit time is unknown or cannot be measured, then 3 days should be sufficient to capture most of the dietary intake from the days prior to a stool sample (6). Diet should be collected in a way that ultimately allows for the analysis of food choices, and not simply nutrient totals, to provide opportunity to assess data for food sources of nutrients and capture some of the dietary complexity and diversity that is not apparent from macronutrient totals. The timing of meals and other eating behaviors also impact gut microbiome composition (92). The microbiome responds to circadian rhythms, potentially driven by meal timing and fed-fast cycles (93). As this is not generally controllable in free-living human studies, fecal collection time should be recorded and used as a covariate in analysis to determine relationships relative to meal timing and fasting intervals.

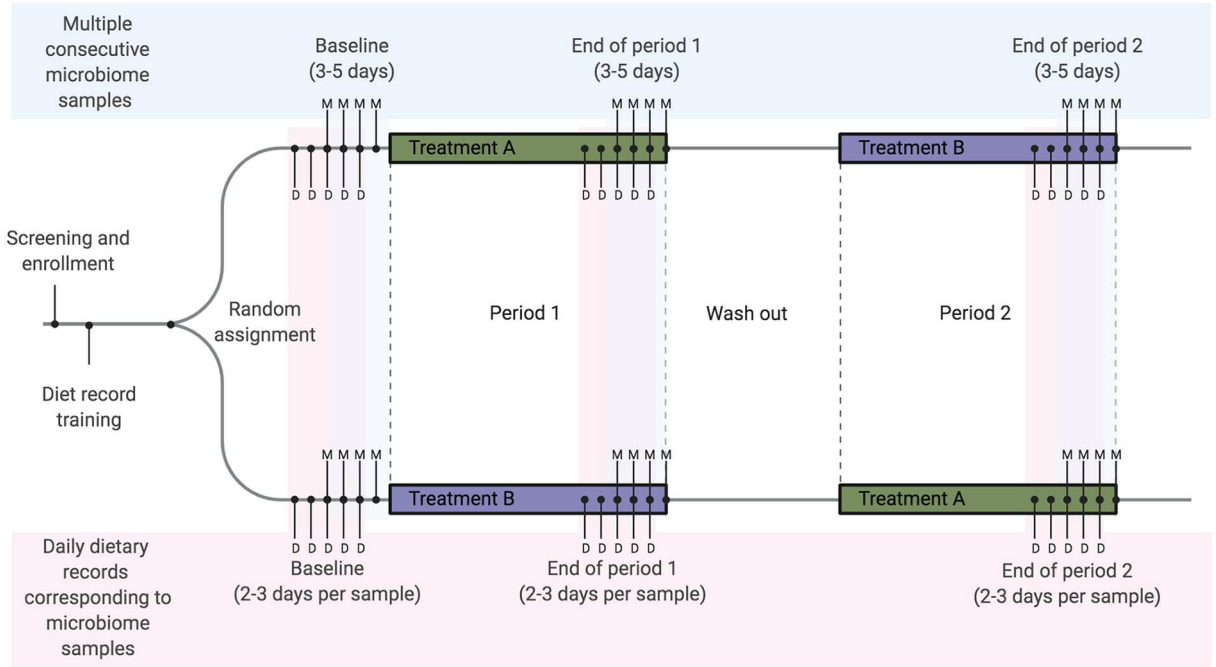

FIGURE 3 | Example diet and microbiome sampling timeline for a longitudinal cross-over dietary intervention study. We recommend that diet-microbiome studies longitudinally follow individual participants and include multiple consecutive microbiome samples per study timepoint or period. As an example, a cross-over study design using repeated longitudinal sampling would include a total of 9–15 microbiome samples per person over three sampling time points. Here we show the mid-range of 4 samples per time point (labeled with M). Optimally, for each microbiome sample collected, 2–3 days of dietary records will be collected from each time point for a total of 15–18 dietary records over the study. In this example 2 days of records are collected prior to each sample (labeled with D). These records overlap, resulting in a total of 5 days of dietary records at each time point.

RECOMMENDATIONS WHEN CONTROLLING FOR HABITUAL DIET

Dietary intake is more variable than microbiome composition both within and between people (6), and studies of diet in the microbiome space must take this dietary variation into account whenever feasible. Measurement of dietary variation may be particularly important when studying diverse populations, because while dietary intake in the US is highly variable, dietary intake in other parts of the world may be homogenous from day-to-day and vary only over longer time periods (46, 47). Considerations for dietary measurement can vary depending on study design and outcomes measured. Considerations for different types of studies are included below.

Even if we are able to account for baseline microbiome differences with study design and pre-study microbiome-typing, we may also want to account for differences in habitual dietary intake at baseline. One way to control for variation in dietary intake in microbiome studies would be to switch participants onto an identical, standardized baseline diet prior to the intervention period. In practice, this is not trivial because the shift to the standardized diet can have its own effects on the microbiome, and thus may obscure the effects of the intervention. For example, Gurry et al. used a meal replacement beverage (Ensure) in an attempt to standardize the diet of their cohort prior to testing the effects of specific foods and supplements, but changing participants to this standardized beverage induced changes to their gut microbiomes including some diversity loss, and the changes were not consistent across all participants (94). From this study, it is difficult to determine whether the microbiome diversity loss was due to the colon cleanse which the study participants also underwent, or from the change to the standardized Ensure diet. This example raises several additional questions that are outside the scope of this article: Should a control diet be representative of habitual intake? What should a standardized baseline diet consist of in terms of macronutrient and micronutrient composition and food diversity? Are there ways to more accurately represent typical diets made of real food by considering more than just macronutrient and micronutrient composition? If so, what features should be considered? Should control diets be liquid meal replacements or standardized whole-food diets?

Based on currently available information, we recommend controlling variation in dietary intake by *stabilizing* diet, rather than by *standardizing* diet. This ensures consistency of individual diet during microbiome-focused intervention studies but does not attempt to modify habitual diet. The simplest approach is to ask participants to maintain their normal diet. More complex approaches would assign each subject to a specific constrained diet based on their own recent dietary intake data. Ultimately the best dietary approach will be study-dependent, and should be considered carefully when planning interventions to improve the ability to draw strong conclusions.

RECOMMENDATIONS FOR DIETARY INTERVENTIONS

Dietary intervention studies are likely the best way to understand the impact of dietary components on the microbiome, but themselves come with some caveats. Cross-over intervention studies, where each participant undergoes both the target intervention and the control arm, with a washout period between treatments, is the most optimal study design for studying the effects of a dietary intervention because each participant acts as their own control. Cross-over designs have been successfully implemented in diet-microbiome studies (19, 24, 95), and allow the researcher to conduct a within-person comparison instead of a between-person comparison, reducing the sample size needed to detect differences by at least half, and reducing the confounding effects of inter-individual variation (96). Cross-over studies are more difficult to conduct than parallel studies for several reasons including longer study duration and higher participant burden, and it is imperative that the washout period be long enough to prevent carryover effects (97). Proper control of factors such as randomization of order, verifying a return to baseline diet during the washout period, and general compliance with study protocols over a longer study duration are critical and typically require a higher level of study personnel training and time. A lead-in period both prior to the start of the study and during the latter half of the washout period are also recommended to acclimate participants and to stabilize any relevant diet and lifestyle factors (98). Despite these obstacles, cross-over study designs hold promise for understanding the effects of diets on the gut microbiome due to their unique ability to not just account for or diminish the noise of inter-individual variability, but to allow the study of personal responses, which can lead the way to personalized diets, and personalized microbiome-oriented recommendations.

Many studies have assessed the intake of grains and grain products (99, 100), and whole foods including walnuts (101), almonds (102), broccoli (103) and other cruciferous vegetables (104), and pistachios (105) for their impact on the microbiome. The literature on whole-food interventions has been recently reviewed (106). These studies tend to reveal modest changes in microbiome structure. However, it is also interesting to consider how intervening with food products alters habitual intake and how changes to habitual diet can affect an intervention in ways that may not be fully appreciated.

The short-term impact of diet on the microbiome beta-diversity is well-known, while the finer details and specific impacts of individual foods are still poorly understood. Results from mouse models revealed that large changes in diet can cause rapid gut microbiome compositional changes within a small number of days (107, 108). In human studies, gut microbiome composition responds at a similar rate. For example, David et al. showed that dietary interventions change microbiome composition significantly starting only 1 day after the intervention reached the distal gut (23). Largely based on these data, dietary intervention studies use a minimum of 3 to 5 days for maintenance diet before intervention or between crossing-over diets (109). Other intervention designs, in contrast, apply longer maintenance diets between different interventions (19, 95, 99, 100, 110). The rationale used to determine dietary intervention length is not always justified, likely due to the lack of strong evidence for best practices in the field. It is still unclear how long dietary interventions would need to be to shift community membership such that even after removal of the intervention the microbes persist (111). In fact, some researchers have suggested that the inability of gut microbiome changes to persist could be an evolutionary advantage resulting from the constant switching of diets in the hunter-gatherer era (23, 112). Determining the length of dietary intervention when the outcome is not a microbiome change but rather a biomarker change in the host, will be more challenging. The length of an intervention required to see differences in host phenotype is likely on the order of weeks or months, depending on the markers of interest (111). This is particularly salient for cross-over intervention studies, since the duration of the washout period is critical to ensure that there are no carryover effects, which can confound the results.

An additional consideration for planning dietary intervention studies where study foods will be provided to the study participants is preference. It is imperative to know whether a study participant will be willing to eat all of the study foods and meals provided. Ideally, the study meal plans, with all the intended ingredients and preparation methods, should be clearly explained in advance, and potential participants should be asked during screening whether they are willing to follow all study protocols, to prevent the recruitment of non-compliant participants. If substitutions need to be made after the participant has already been recruited into the study, these substitutions may impact the effects of the intervention on the microbiome. For example, in a recent study we tested the effects of a Mediterranean style diet compared to a fast food diet of burgers and fries (24). Even in this small group of only 10 healthy, young subjects who reported to be omnivores and willing to consume foods provided to them, 3 of the participants did not like certain foods and ingredients and their preferences were accommodated to ensure that they were at least receiving the intended calorie levels and macronutrient breakdowns. One participant did not like chickpeas so his lunch salad contained black beans instead. Another participant did not like balsamic vinegar so his salad only contained olive oil instead of vinaigrette dressing. Yet another participant did not like walnuts so these were replaced with almonds in the mixed nut snack. While these substitutions seem reasonable in terms of maintaining consistent macronutrient and micronutrient levels, the poly- and oligosaccharide composition of the fermentable component of chickpeas and black beans, for example, may be different enough to elicit differential microbiome responses.

Dietary interventions with microbiome outcomes should include placebos and blinding where possible; these considerations are not unique to microbiome studies (113). When dietary interventions are implemented thought should be given to how adherence and compliance will be assessed. The best practice in feeding trials is to provide all meals to participants and to collect plate waste to determine true intake. In studies

where that level of control is not possible, or when dietary interventions are implemented using instruction, researchers should include multiple dietary records throughout the study period to see how well-participants comply with recommended intake instruction. Furthermore, when designing feeding trials and interventions there are numerous axes of dietary intake beyond diet make-up in terms of foods and food quantity that ideally should be controlled. These include meal timing, length or duration, and location—all features that affect the food environment. Researchers should also consider capturing qualitative metrics surrounding hunger and satiety when they are relevant for specific dietary intervention questions.

Ultimately, the results of even extremely well-controlled dietary intervention studies are likely to be affected by personalized diet-microbiome interactions. The individual variation across people's microbiomes arises from a combination of factors beginning in infancy, likely including birth mode and antibiotic exposure (64, 65). Individual microbiome responses also likely stem from variation in biogeography along the gastrointestinal tract, stochastic or random events, and complex community interactions. For example, a recent well-controlled study measuring microbiome response to either fast food or the Mediterranean diet demonstrated individual variation. Most participants showed consistent and comparable changes in response to dietary intervention. However, there was a small number of participants whose microbiome shifts were in opposite directions when compared to the majority (24). Individual responses to interventions and dietary intake are documented across multiple studies (6, 16, 19). These kinds of observations create difficulties for analysis, particularly when using group means for comparisons, but there is strong evidence now that individual-specific responses should be expected and modeled.

IMPROVED DIETARY ASSESSMENT METHODOLOGIES COULD IMPROVE DIET-MICROBIOME UNDERSTANDING

Participant compliance and the cost of participant time remain an obstacle for researchers to attain authentic dietary information in a practical way. Improvement in dietary assessment by shortening the time of data collection and reducing participant workload would improve the overall quality of dietary intervention trials. Recent work has applied the technique of metabarcoding to evaluate the plant component of diet from stool samples using the *trn*L-P6 marker gene (114). Similar techniques have previously been used to assess diet in non-human primate populations (115, 116); demonstrating that DNA from plant species persists through digestion and can be identified in stool. While these methods may not be able to quantify protein or kilocalorie content, they should provide a method to identify plant diversity and could provide a non-invasive measure of diet quality. Importantly, plant diversity has previously been linked to microbiome features (73) and ways to quantify this may improve future studies of diet and the microbiome without increasing participant burden. Moreover, detection of dietary intake within stool samples will allow for perfect matching between dietary and microbiome features without concern for transit time or self-report.

Ideally, researchers could follow a meal through the gut and collect data about that meal and the microbial community changes that are induced by it. Technology that allows easier sampling throughout the gastrointestinal tract using capsules to capture information directly from within the small intestine and colon is a promising advance (117). The ideal approach, though costly and often impractical due to high participant burden, is to couple this type of measurement with the direct analysis of food composition and content from duplicate plates, which can provide precise estimates of exposure to specific components (118), and even dietary microbe information (60).

Most of the technology-based approaches for dietary assessment still depend on self-reported dietary intake (49). The computerization of the automated multiple pass method (AMPM) is commonly used for dietary recalls (119). In the US, this method is widely available to researchers using the Automated Self-Administered 24-h (ASA24) dietary assessment tool. ASA24 can be used to apply the AMPM method at scale in large studies as it has acceptable validity relative to interviewer administered recall (120). Numerous other web-based and computerized approaches for dietary assessment are available in other countries (49). Using a computer-administered approach such as ASA24 is not without limitations and ASA24 in particular may be most appropriate when studying computer-savvy adults (121, 122). Other exploratory technology such as image-based diet capture could improve dietary assessment and reduce participant burden. Feature recognition from images is difficult (123). Even informed humans struggle with food image recognition projects, with dietetics students accurately identifying foods from images 79.5% of the time (124). Image-assisted methods for dietary assessment with mobile and wearable technologies (125) to detect eating occasions, mastication, and hand-to-mouth gestures have great potential and will likely harness machine learning (126); we expect these approaches to continue to improve in accuracy in the coming decade. Efforts to capture and quantify dietary intake from photos taken with a smartphone or from a combination of cameras and wearable devices have not been applied extensively to microbiome datasets. Available dietary assessment technology options using these types of approaches and created for research purposes in the US include eButton (127), Remote Food Photography Method (128), and Technology Assisted Dietary Intake Assessment (125).

Given the still-emerging technologies in dietary assessment, exploration of alternative methods of collecting dietary data is an important area of future work. In the short-term, we may need to shift the paradigm in dietary assessment from a nutrition-centric perspective to a microbiologist-centric perspective and to design and validate a microbiome-focused food intake questionnaire. A tool like this should divide foods into groups that have particular relevance for the microbiome based on fiber type or phytocompound composition known or suspected to affect the microbiome. There may be other proxies that we can capture as latent variables for diet that discard previous dietary

TABLE 1 | Summary of current and future recommendations for dietary factors in diet-microbiome studies.

	Feasible now	Ideal now	Future needs
Study design and sampling protocols	Include large sample sizes for cross-sectional studies (400–500 participants).	Dense longitudinal sampling during and after interventions.	Stratify longitudinal studies by baseline microbiome composition.
	Collect multiple fecal samples (e.g., for 3 consecutive days) per study time point. Design longitudinal studies and favor cross-over intervention studies over parallel study designs.	Collect more fecal samples (e.g., for up to 7 consecutive days) per study time point, or daily sampling throughout an entire study.	Sequence microbiome during recruitment to enroll predicted responders.
Dietary assessment	In addition to or in lieu of using food frequency questionnaires (FFQ), participants report food intake using multiple 24-h dietary recalls or 3-day diet records.	Participants report food intake using multiple 3-day dietary records paired with each microbiome sample collected and receive instruction or training preferably by a dietetic professional.	Measure biochemical markers for intake of specific foods or dietary components (e.g., plant DNA in stool, metabolomic markers in urine or blood) and use technology to accurately capture dietary intake.
Dietary intervention	Participants stabilize their diet by consuming a consistent diet (e.g., the same breakfast, lunch and dinner for 3 days) individualized and based on their habitual dietary intake, prior to and/or during each study time point or set of sample collections.	Intervention meals are consumed at the same time, location, and within the same length of time and compliance is assessed by weighing plate waste.	Participants consume all of their study foods at the research center, consume only foods provided by the study but take the food with them and receive specific instructions, or consume their own foods but generate duplicate plates from which food composition can be measured directly.
Dietary data analysis	Conduct analysis of nutrient intake and food intake in terms of food groups and healthy eating indices.	Include detailed longitudinal analysis of food intake using methods that account for the multivariate nature of dietary data and relationships between foods.	Connect dietary intake data to food databases that contain extensive information about foods and food components and use machine learning approaches to compare with microbiome data.

Table 1 highlights suggestions for improving studies investigating relationships between diet and the gut microbiome. We include suggestions that can be implemented currently with minimal additional resources, those that would be ideally implemented but require additional resources, and those that could be implemented in the future after further research and development.

norms and assumptions such as macronutrients and kilocalories while still collecting key information relevant to microbiome analysis. There is likely also a role for nutritional biomarkers or blood metabolites to characterize individual diet-microbiome interactions, particularly when personalization is considered relative to microbiome changes (129). Combined, advances in these areas as well as in microbiome sequencing and analysis techniques have the potential to greatly improve the quality of diet-microbiome studies to move beyond correlational analysis and develop understanding of causative relationships.

FUTURE DIRECTIONS AND CONCLUSIONS

In this article, we have presented a variety of considerations including both practical recommendations that can be immediately incorporated into studies and aspirational recommendations that will require greater effort for implementation (**Table 1**). Ultimately, we believe that microbiome research will benefit from more rigor and more informed design in dietary assessment and intervention. We have also raised several questions that do not yet have good answers. Moving forward, the field is in need of more well-controlled longitudinal studies and controlled feeding trials that can isolate the impact of specific changes in dietary intake on microbial communities in human hosts. With well over 9,000 unique foods represented in most nutritional databases and an estimated 26,000 unique food chemicals in the larger food supply (38) it is difficult to determine which foods are the most promising candidates for intervention trials. Studies with strong dietary data collection methods will therefore play an important role in identifying potential diet-derived bioactive compounds and their food sources that can be investigated more closely with well-designed interventional studies.

AUTHOR CONTRIBUTIONS

AJ wrote the manuscript and created the figures. JZ, JK, and AS wrote sections of the manuscript and assisted with figure planning. DK and AZ planned the review and contributed to editing and revising the manuscript. All authors read and approved the submitted version. All figures were created with BioRender.com.

FUNDING

This work was supported by USDA National Institute of Food and Agriculture, Hatch project CA-D-NTR-2575-H (to AZ), NIH Grant No. R01AI121383 (to DK), and Minnesota Partnership for Biotechnology and Genomics Grant No. #18.08 (to DK).

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Conflict of Interest: AJ and DK have received research support from General Mills. AJ has received speaking fees from Abbott Nutrition. DK serves as CEO of CoreBiome, a company involved in the commercialization of microbiome analysis. CoreBiome is now a wholly owned subsidiary of OraSure. These interests have been reviewed and managed by the University of Minnesota in accordance with its Conflict of Interest policies.

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

frontiers in Nutrition

ORIGINAL RESEARCH
published: 28 August 2020
doi: 10.3389/fnut.2020.00139

Contributions of Fat and Carbohydrate Metabolism to Glucose Homeostasis in Childhood Change With Age and Puberty: A 12-Years Cohort Study (EARLYBIRD 77)

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OPEN ACCESS

Edited by:
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Reviewed by:
*Stefan Camps, Singapore Institute for Clinical Sciences (A*STAR), Singapore*
Stephen F. Burns, Nanyang Technological University, Singapore

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Specialty section:
This article was submitted to Nutrition and Metabolism, a section of the journal Frontiers in Nutrition

Received: *30 April 2020*
Accepted: *17 July 2020*
Published: *28 August 2020*

Citation:
Cominetti O, Hosking J, Jeffery A, Pinkney J and Martin F-P (2020) Contributions of Fat and Carbohydrate Metabolism to Glucose Homeostasis in Childhood Change With Age and Puberty: A 12-Years Cohort Study (EARLYBIRD 77). Front. Nutr. 7:139. doi: 10.3389/fnut.2020.00139

Puberty—a period when susceptibility to the onset of Type 2 diabetes (T2D) increases—is marked with profound physiological and metabolic changes. In the EarlyBird cohort, children who developed impaired fasting glycemia in adolescence already exhibited higher fasting blood glucose at 5 years of age, independent of their body mass index (BMI), suggesting that pubertal factors may modify existing predisposition. Understanding how the physiological changes during childhood influence glucose homeostasis and how the central energy metabolism may help deciphering the mechanisms that underlie the risk of developing T2D in children and adults. We investigated these associations by analyzing glycemic variations with molecular markers of central energy metabolism, substrate oxidation status and pubertal stages in the EarlyBird cohort. The EarlyBird study is a non-interventional, prospective cohort study, that recruited 307 healthy UK children at age 5, and followed them annually throughout childhood for 12 years. Longitudinal data on blood biochemistry, respiratory exchange ratio, and anthropometry, available from 150 children were integrated with fasting glycemia. The gradual rise in blood glucose during childhood associates with age-dependent changes in molecular processes and substrate oxidation status, namely (i) greater pre-pubertal fat utilization, ketogenesis, and fatty acid oxidation, and (ii) greater pubertal carbohydrate oxidation and glycolytic metabolism (Cori and Cahill Cycles) associated with different amino acid exchanges between muscle and other tissues (proline, glutamine, alanine). Since children's metabolic and nutritional requirements evolve during childhood, this study has potential clinical implications for the development of nutritional strategies for disease prevention in children.

Keywords: amino acids, biochemistry, glucose, longitudinal study, phenotyping, substrate oxidation, puberty, type 2 diabetes

INTRODUCTION

Diabetes is now one of the most common non-communicable diseases in the world, affecting over 422 million people according to the World Health Organization (WHO) (1). It has been forecasted that one in every three individuals born in the US in the year 2000 will develop diabetes during their lifetime (2). The principal form of diabetes accounting for these projections is Type 2 diabetes (T2D). As a result of the rising prevalence of obesity, T2D is a growing concern in children (3), with puberty being a period of increasing susceptibility to the onset of diabetes (4, 5).

Normal pubertal growth, along with its underlying physiological endocrine changes, affects body composition, muscle mass and strength, and processes including bone development, erythropoiesis, and substrate utilization (6). These key physiological processes are accompanied by changes in biochemical processes, and in turn may influence aerobic and anaerobic fitness (6). Aerobic fitness increases with the development of the cardiovascular and respiratory systems, and skeletal muscle. In addition, anaerobic fitness is influenced by muscle mass, as well as by size-independent factors (e.g., glycolytic metabolism, muscle architecture, neural control), and tends to increase throughout puberty (7). Such complex and rapid changes in biochemical and physiological parameters influence numerous metabolic functions, including total and resting energy expenditure and physical activity. These changes may be important in determining susceptibility to the development of T2D.

Basal metabolism is the energy required for cellular and tissue maintenance. It increases rapidly up to the age of ∼2 years, and reaches a plateau in late adolescence when growth velocity and the growth of muscle mass decreases (8). Growth has additional energy requirements to those of basal metabolism, and is unique to this early stage of life. Energy cost for growth has two components; the energy needed for biosynthetic processes in growing tissues and the energy stores deposited in those tissues (e.g., fat and protein) (8). The role of resting energy expenditure and weight gain in children is subject to controversy, with some evidence that lower energy expenditure may be a factor predisposing to childhood obesity (9, 10). During adolescence, the pubertal growth spurt may be associated with a substantial fall in resting energy expenditure, independent of adiposity (11), which may influence long-term body composition and metabolic health outcomes. Food provides macronutrients (carbohydrates, protein, and fats) that are utilized by the body as sources of energy.

The EarlyBird study is a landmark prospective cohort study investigating the origins of T2D in children. This cohort of healthy children has been followed from age 5 to 16 years with annual clinical, anthropometric, and physiological measurements (12). A total of 17% of the initially healthy children in the EarlyBird cohort showed impaired fasting glycemia (IFG) by the age of 15, a risk factor for future diabetes. The children who developed IFG already exhibited higher fasting blood glucose levels at 5 years of age, compared with those who did not subsequently develop IFG, and this effect was independent of BMI (12). Recently, we reported that the occurrence of an early defect in β-cell function among children who go on to develop prediabetes (12), was genetically determined and independent of BMI (13). These studies also revealed how genetic markers are associated with normal glycemic trajectories during childhood. However, prediabetes did not appear until puberty, when insulin resistance was at its highest (12). We successfully applied a longitudinal analysis to explore the metabolite signatures that precede or follow the development of greater levels of insulin resistance (IR) during childhood (14, 15). These analyses provide key insights into metabolite pathways (ketogenesis, fatty acid oxidation, branched-amino acids), with distinct patterns according to chronological age, and different contribution to glucose metabolism (14, 15). In addition, we reported that interpretation of HbA1c for the diagnosis of impaired fasting glycemia was limited due to factors other than glycaemia systematically influencing the variance of HbA1c in youth (16). Understanding how changes in these molecular pathways associate with changes in glucose homeostasis and dietary substrate oxidation in healthy children is necessary to inform the design of preventive measures, such as individualized nutritional recommendations.

The aim of the present study was to determine how temporal glycemic variations during childhood relate to physiological changes in central energy metabolism and substrate oxidation. Therefore, we investigated the associations between individual metabolites, respiratory exchange ratio and fasting glucose concentrations in the EarlyBird cohort, taking into account critical covariates of age, growth, puberty, adiposity, and physical activity.

METHODS

Study Population

The study was conducted in accordance with the principles of the Declaration of Helsinki II. Ethical approval was granted by the Plymouth Local Research Ethics Committee (1999), and parents gave written consent and children verbal assent. The EarlyBird Diabetes Study incorporates a 1995/1996 birth cohort recruited in 2000/2001 when the children were 5 years old (307 children, 170 boys) (17). Most of the children were white Caucasian and five children out of 307 were of mixed race, reflecting the ethnic mix of the city of Plymouth. According to the pediatric thresholds for overweight and obesity proposed by the International Obesity Task Force (IOTF), 13% of the EarlyBird boys and 26% of girls were overweight at baseline, which included 4 and 5%, respectively who were obese. The thresholds approximate to the 91st and 98th centiles of the 1990 BMI reference curves for the UK, and are deemed to correspond to equivalent thresholds in adulthood. The collection of data from the EarlyBird cohort is composed of clinical and anthropometric variables measured on an annual basis from the age of 5 to 16.

Impaired fasting glycemia was selected as an objective criterion to identify children with an additional risk for future diabetes. For blood metabolic phenotyping, subjects were purposively selected to include children who had shown impaired fasting glucose at one or more time-points during the course

of childhood as reported previously (12), and gender-matched normoglycemic children. A total of 150 participants [63 children (44 boys and 19 girls) who had shown IFG by age 16 years and 87 children (61 boys and 26 girls) who had not] were selected. Metabonomics data were available for 130 of these children. Out of the 55 children who had shown impaired fasting glucose in this subset, seven had a first degree relative with T2D or T1D.

Anthropometrics

Height and weight were measured every 6 months from age 5 years. BMI was derived from direct measurement of height (Leicester Height Measure; Child Growth Foundation, London, U.K.) and weight (Tanita Solar 1632 electronic scales), performed in duplicate and averaged. BMI SD scores were calculated from the British 1990 standards (18). Moderate to vigorous physical activity (MVPA) was measured annually from 5 years by accelerometry (Acti-Graph) (19). Children were asked to wear the accelerometers for 7 consecutive days at each annual follow-up visit, and only recordings that captured at least 4 days (each day incorporating at least 9 h wear time) were used. Pubertal timing was evaluated by means of age at peak height velocity (APHV), determined as the tangential velocity at the middle time point of three consecutive height measurements taken 6 months apart. Chronologic age does not necessarily correlate with physiologic or somatic (i.e., related to the body) pubertal changes (20). The most common way to describe the sequence of changes in secondary sexual characteristics is that published by Marshall and Tanner, commonly referred as Tanner stages (21, 22). The distribution of age at which each tanner stage was reached for boys and girls is provided in **Supplementary Tables 1, 2**, respectively. There are five Tanner stages. The first stage (Pre-pubertal stage) of puberty begins around 6–8 years of age, long before any physical changes are noticeable. The second stage of puberty usually begins around 9–11 years for girls and 11–13 years for boys. However, it is normal for this to vary by up to 5 years. The second stage marks the beginning of sexual development and physical changes, during which boys and girls experience a large growth spurt. The stages 3 and 4 mark particularly development of secondary sexual characteristics, whilst the stage 5 marks the end of puberty and the staging into the body of an adult. Peripheral blood was collected annually after an overnight fast, blood serum samples were stored at −80°C.

Laboratory Assessment

The children were fasted overnight for 10 h before venesection. The Homeostatic Model Assessment of Insulin Resistance (HOMA2IR) and the Homeostatic Model Assessment of Beta Cell Function (HOMA2B) were determined each year from fasting glucose (Cobas Integra 700 analyzer; Roche Diagnostics) and insulin (DPC IMMULITE) (cross-reactivity with proinsulin, 1%) using the homeostasis model assessment program, which has been validated in children (23).

Respiratory Exchange Ratio

Resting energy expenditure was measured by indirect calorimetry using a ventilated flow through hood technique (Gas Exchange Measurement, Nutren Technology Ltd, Manchester, UK). Performance tests reportedly showed a mean error of 0.3 ± 2.0% in the measurement of oxygen consumption and 1.8 ± 1% in that of carbon dioxide production. Measurements were performed in a quiet thermoneutral room (20°C) after an overnight fasting period of at least 6 h, to minimize any effect attributable to the thermic effect of food. Data were collected for a minimum of 10 min and the respiratory exchange ratio was calculated as an indicator for substrate oxidation.

Blood Metabolic Profiling

Serum samples collected from each child at every age between 5 and 16 years old were subjected to metabonomic analysis. For technical feasibility and to ensure optimal data reproducibility for cohort analysis, a threshold of 1,800 blood serum samples (e.g., 150 different subjects) was determined. Metabolic profiling was carried out by means of proton nuclear magnetic resonance spectroscopy (^{1}H NMR) spectroscopy, as reported previously (14, 15). Briefly, 400 μL of blood serum were mixed with 200 μL of deuterated phosphate buffer solution 0.6 M KH_2PO_4. ^{1}H NMR metabolic profiles of serum samples were acquired with a Bruker Avance III 600 MHz spectrometer equipped with a 5 mm cryoprobe at 310 K (Bruker Biospin, Rheinstetten, Germany) and processed using TOPSPIN (version 2.1, Bruker Biospin, Rheinstetten, Germany) software package. Based on an internal database of reference compounds, representative signals of metabolites were integrated. The signals are expressed in an arbitrary unit corresponding to a peak area normalized to total metabolic profiles. ^{1}H NMR spectroscopy being a quantitative method, metabolite peak areas are proportional to metabolite concentrations, and thus their changes are representative of absolute change in metabolite concentrations in the serum. This metabonomics approach targeted the major metabolic pathways, including central energy metabolism, amino acids, carboxylic acids, and lipoproteins and in a highly reproducible manner across more than 1,700 serum samples. In particular, ^{1}H-NMR spectroscopy of human blood serum enables the monitoring of signals related to lipoprotein-bound fatty acyl groups found in triglycerides, phospholipids and cholesteryl esters, together with peaks from the glyceryl moiety of triglycerides and the choline head group of phosphatidylcholine.

Statistical Analysis

Mixed effects modeling was used to assess the association between individual metabolites and fasting glucose, taking into account age, BMI z-scores, and physical activity. Controlling for maturational and growth status is crucial in life course studies, and age at peak height velocity (APHV) is a key measure of maturity that was also taken into account. Random intercepts were included as well as age (categorized to allow for non-linear change in glucose over time), gender, BMI z-score, APHV, MVPA (number of minutes spent in moderate-vigorous physical activity), and individual metabolites (in separate models) as fixed effects. Each metabolite was transformed to a z-score (i.e., standardized with mean of 0 and standard deviation of 1) for analysis. Modeling was

carried out in R software (www.R-project.org) using the lmer function in the package lme4 (24) and *p*-values calculated using the Satterthwaite approximation implemented in the lmerTest package (25). Both unadjusted and Bonferroni-adjusted *p*-values are presented. Additional Spearman Correlation analysis was conducted between fasting glucose and serum metabolites, HOMA indexes, HbA1c, and respiratory exchange ratio.

RESULTS

Influence of Chronological Age and Pubertal Stage on Population Demographics

Clinical and anthropometric characteristics of the children for the 12-year period are summarized in **Table 1** and **Supplementary Figure 1**. For both genders, there was an increase in fasting glucose throughout childhood, concomitant with increasing BMI-z-score and respiratory exchange ratio, and decreasing physical activity (MVPA). As previously reported, fasting insulin and HOMA-IR decreased until around 8 years, and then increased during puberty until the age of 14 years, before decreasing until the age of 16 years. This pattern was dependent on the time of APHV and BMI z-scores (15).

Mean fasting glucose concentrations increased from 4.3 and 4.4 mmol at age 5, to 5.2 and 5.0 mmol at age 16, for boys and girls, respectively (**Table 1**). Interestingly, these increases were marked by two plateaus, first between 8 and 11 years of age, then between 13 and 16 years of age (**Supplementary Figure 1**). Mirroring the changes in blood glucose concentrations, an evolving pattern was observed in the RER and age. RQ values reflect metabolic substrate utilization for energy production. From age 5 to 7, RER values were around 0.9, and increased toward their maximum values of 1 between the age of 11 and 13, followed by a slight decrease toward 0.95 from 14 years of age (**Supplementary Figure 1**). **Figure 1** describes the age-dependent changes in clinical and glycemic parameters in relation to male and female pubertal development. Parameters were plotted according to Tanner stage (21, 22). Tanner stage was self-reported at each time-point, and the same Tanner stage may be reported at more than one time-point. Therefore, for each child, the parameter for each Tanner stage is represented once by selecting only the value at the first occurrence (e.g., for Tanner Stage 1, values at age 5 were selected).

Longitudinal Association of Fasting Glucose and Serum Metabolites

Using data at all ages simultaneously, mixed effects modeling was applied to assess the association between fasting glucose concentrations and individual metabolites. Several blood metabolites including amino acids, organic acids, and lipids showed statistically significant associations with fasting glucose concentrations in longitudinal models, independently of BMI z-score, physical activity, and APHV. Data are reported to statistical significance and in alphabetic order for different metabolic pathways and metabolites (**Table 2**).

TABLE 1 | Characteristics of the studied Earlybird cohort.

	Age (years)	Boys	Girls
Fasting glucose (mmol/L^{-1})	5	4.3 ± 0.4	4.4 ± 0.4
	6	4.5 ± 0.4	4.4 ± 0.3
	7	4.6 ± 0.5	4.6 ± 0.4
	8	4.8 ± 0.3	4.7 ± 0.4
	9	4.8 ± 0.5	4.9 ± 0.3
	10	4.9 ± 0.3	4.8 ± 0.3
	11	4.8 ± 0.4	4.8 ± 0.3
	12	4.9 ± 0.4	5.1 ± 0.4
	13	5.2 ± 0.3	5.1 ± 0.4
	14	5.2 ± 0.3	5.2 ± 0.5
	15	5.2 ± 0.3	5.2 ± 0.4
	16	5.2 ± 0.3	5.0 ± 0.4
HOMA-IR	5	0.6 ± 0.49	0.81 ± 0.37
	6	0.48 ± 0.44	0.63 ± 0.41
	7	0.37 ± 0.23	0.46 ± 0.20
	8	0.44 ± 0.26	0.60 ± 0.44
	9	0.59 ± 0.39	0.89 ± 0.49
	10	0.88 ± 0.41	1.15 ± 0.71
	11	0.85 ± 0.54	1.19 ± 0.70
	12	0.96 ± 0.56	1.89 ± 1.30
	13	1.10 ± 0.57	1.61 ± 1.00
	14	1.15 ± 0.68	1.57 ± 0.97
	15	0.93 ± 0.56	1.45 ± 1.54
	16	0.83 ± 0.64	0.96 ± 0.84
Fasting insulin (mU/L^{-1})	5	4.3 ± 3.5	5.7 ± 2.6
	6	3.4 ± 3.2	4.4 ± 2.9
	7	2.6 ± 1.5	3.2 ± 1.3
	8	3.0 ± 1.7	4.1 ± 3.0
	9	4.1 ± 2.7	6.1 ± 3.4
	10	6.0 ± 2.9	8.0 ± 5.1
	11	5.8 ± 3.8	8.3 ± 4.9
	12	6.6 ± 3.9	13.1 ± 9.2
	13	7.4 ± 4.0	11 ± 6.9
	14	7.7 ± 4.7	10.7 ± 6.6
	15	6.3 ± 3.7	9.9 ± 10.9
	16	5.6 ± 4.4	6.6 ± 5.9
BMI Z-scores	5	0.22 ± 1.1	0.3 ± 1.23
	6	0.19 ± 0.99	0.58 ± 0.96
	7	0.23 ± 1.06	0.64 ± 0.93
	8	0.2 ± 0.92	0.46 ± 1.04
	9	0.33 ± 1.05	0.57 ± 0.98
	10	0.33 ± 1.04	0.72 ± 1.11
	11	0.33 ± 1.11	0.66 ± 1.16
	12	0.37 ± 1.16	0.75 ± 1.16
	13	0.49 ± 1.21	0.86 ± 1.21
	14	0.33 ± 1.16	0.86 ± 1.17
	15	0.37 ± 1.12	0.89 ± 1.25
	16	0.51 ± 1.13	0.87 ± 1.14
Moderate-vigorous physical activity (min/day)	5	55.1 ± 19.9	46.2 ± 26.6

(Continued)

TABLE 1 | Continued

	Age (years)	Boys	Girls
	6	61.9 ± 23.6	54.8 ± 17.5
	7	63 ± 24.1	52.6 ± 17.2
	8	61.3 ± 23.1	46.7 ± 15
	9	60.9 ± 23.9	40.8 ± 17.3
	10	59.1 ± 26.5	37.2 ± 14.7
	11	57.3 ± 27.4	34.3 ± 20.2
	12	59.6 ± 27.5	35.2 ± 19.6
	13	55.1 ± 23.9	36.4 ± 22.2
	14	49.5 ± 26.6	40.6 ± 30.4
	15	48.3 ± 23.6	31.6 ± 18.1
	16	44.5 ± 23.7	32.1 ± 22.9
Respiratory exchange ratio	5	0.89 ± 0.09	0.92 ± 0.06
	6	0.88 ± 0.08	0.88 ± 0.09
	7	0.90 ± 0.06	0.92 ± 0.08
	8	0.97 ± 0.09	0.99 ± 0.09
	9	0.95 ± 0.1	0.96 ± 0.14
	10	0.95 ± 0.1	0.93 ± 0.1
	11	1.01 ± 0.07	1.01 ± 0.07
	12	0.99 ± 0.06	1.00 ± 0.07
	13	1.00 ± 0.07	1.02 ± 0.09
	14	0.96 ± 0.08	0.96 ± 0.09
	15	0.95 ± 0.3	0.93 ± 0.09
	16	0.95 ± 0.15	0.95 ± 0.09
Age at peak height velocity (years)		13.0 (12.8–13.4)	11.6 (10.8–12.3)

Age at peak height is reported as median with interquartile range, and other data are reported as mean +/- standard deviation.

Of note, the analysis described positive associations of alanine and lactate with fasting glucose. In addition, the LDL and VLDL-related blood lipid signature was positively associated with fasting glucose concentrations throughout childhood. Most other amino acid metabolites, HDL and phosphocholine-related lipids were negatively associated with fasting glucose throughout childhood. The analysis also described how blood ketone bodies (3-D-hydroxybutyrate, acetoacetate), Krebs cycle intermediates (citrate, formate), glycine-related metabolites (dimethylglycine, creatine, creatinine) were negatively associated with the fasting glucose trajectories.

Age Dependent Correlation of Blood Metabolites With Fasting Glucose

Additional cross-sectional correlation analysis of metabolites, insulin traits, HbA1c, respiratory exchange ratio, and BMI-z-scores with fasting glucose for each year was conducted using Spearman rank correlation. Data for the 12-year period were reported using heatmaps in **Figure 2,** for which the variables are ordered according to the temporal profile of their correlation with fasting glucose.

The heatmap plot highlights the negative correlations of α-keto-isovalerate, 3-methyl-2-oxovalerate, 2-ketobutyrate, 3-D-hydroxybutyrate, acetoacetate, citrate, and leucine with fasting glucose at each age, throughout childhood. In addition, positive associations of glucose with alanine, lactate, LDL, and VLDL related blood lipids were observed in the early years, between 5 and 9 years of age.

The same correlation analysis using HbA1c as endpoint variable was also performed and reported in **Supplementary Figure 2**. Fasting glucose and HbA1c showed positive correlations during childhood, and similar correlation patterns are observed between metabolites and fasting glucose and HbA1c. Yet, fasting glucose shows stronger statistically significant correlation with metabolites, and with insulin and insulin resistance, than HbA1c during childhood.

Metabolite Changes According to Pubertal Stages and Metabolic Pathways

Major changes in levels of blood serum metabolites suggested changes in protein and amino acid levels, as well as lipid metabolic pathways. Therefore, blood biochemical patterns involved in central carbon metabolism, branched chain amino acids (BCAA), fatty acid oxidation, and ketogenesis were displayed according to their respective metabolic pathways (**Figure 3**, **Supplementary Figures 3–5**). Data are reported as a function of the pubertal stages for boys and girls.

Such a data visualization illustrates a rapid decrease in the level of ketogenesis from the early pubertal stages. These changes were associated with decreased levels of acetate, formate and the major Krebs cycle intermediate citrate, and are indicative of a profound remodeling of fatty acid oxidation in children's metabolism during the transition from early childhood to adolescence. In contrast to the changes in lipid metabolism, glucose and alanine concentrations increased steadily during puberty, whilst lactate concentration increased primarily in the early period of pubertal development. Such variations in blood biochemical profiles probably reflect changes in energy and carbohydrate metabolism during puberty, with alanine and lactate concentrations reflecting changing activity in the Cori and Cahill cycles.

Throughout puberty, changes in amino acid metabolism are more complex. Overall, children show a decreased blood concentration of several compounds, including glutamate, arginine, and glycine. In addition, complex patterns in the metabolism of branched amino acids (BCAA) are described. Whilst circulating levels of BCAA catabolic products decreased during puberty, circulating levels of BCAA evolved differently, and seemed to exhibit sexual dimorphism (e.g., Valine) (**Supplementary Figure 3**). Of note, several other blood amino acid profiles displayed distinct differences between boys and girls in late puberty. For instance, boys showed a distinct increase in glutamine and proline in late puberty, whilst girls showed decreases in histidine, asparagine, and citrulline (**Supplementary Figure 4**). Finally, creatinine metabolism shows a consistent pattern throughout puberty, with creatinine concentrations increasing steadily, and more markedly in boys from mid-puberty (**Supplementary Figure 5**).

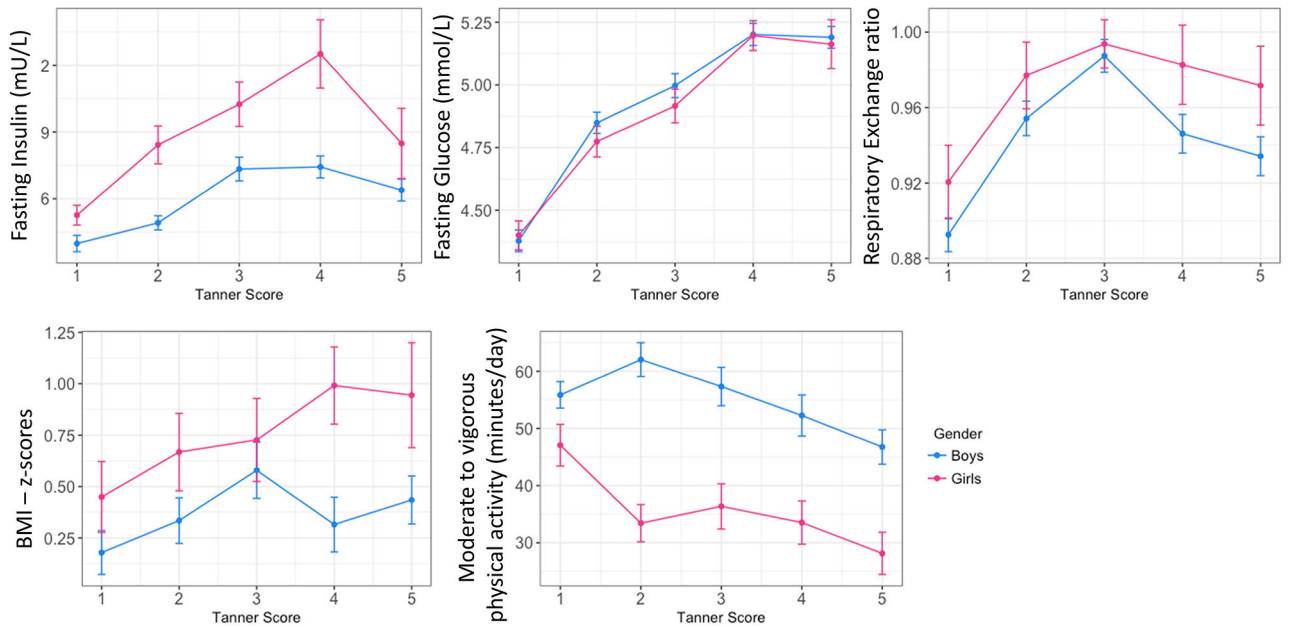

FIGURE 1 | Overview of main glycemic and physiological trajectories in childhood: longitudinal status according to pubertal stage in boys and girls. Data are plotted as mean ± standard error.

DISCUSSION

As children grow and develop, changes in metabolism are directly related to total energy requirements (e.g., basal metabolism, physical activity, and growth) (26). Growth and development are associated with complex endocrine changes. In particular, the growth hormone (GH)/insulin-like growth factor 1 (IGF-1) axis has a fundamental impact on glucose homeostasis and metabolism throughout childhood, by influencing glycogenolysis, gluconeogenesis, and lipolysis (27). This description of puberty-related changes in molecular processes and substrate utilization for energy production significantly extends the existing literature. Although HbA1c retains a positive association with glucose throughout childhood in our cohort, it is weak, and their trends diverge from 10 years (16). These findings therefore limit the interpretation of HbA1c for the diagnosis of impaired fasting glycemia during childhood and suggest that factors other than glycaemia systematically influence the variance of HbA1c in youth (16). Our additional study reveals stronger associations of fasting glycemia with changes in insulin resistance as well as metabolites when compared to HbA1c, which suggests that analysis of temporal glycemic variations may encapsulate more comprehensively the changes in physiological and metabolite pathways during childhood. In this uniquely well-characterized cohort of healthy children, the transition from childhood to adolescence was associated with increasing fasting glucose concentrations and a complex remodeling of central energy metabolism, including amino acid and fatty acid molecular pathways.

In the EarlyBird cohort, the gradual rise in the fasting respiratory exchange ratio describes an increased carbohydrate oxidation throughout childhood. Yet, these fasting respiratory exchange ratio values are high in comparison to adults, where fasting respiratory exchange ratio would remain between 0.8 and 0.90 (28). Higher fasting respiratory exchange ratio values in adults (29) and in adolescents (30) may be linked to reduced metabolic flexibility (i.e., reduced ability to switch from fat to carbohydrate oxidation). Whilst there is limited published literature on healthy children, in the Earlybird cohort, we did not see statistically significant differences in fasting respiratory exchange ratio between normoglycemic children and those with impaired fasting glycemia. Since the maximum values are observed around 11–13 years of age, a period of height growth spurt and important growth in lean mass tissues, our observations may suggest a period of reduced metabolic flexibility during puberty. Finally, a potential limitation in the interpretation of the respiratory exchange ratio is that the measurements were conducted in the fasted state, and conclusions should not necessarily be extrapolated to the post-prandial state.

Prior to puberty, we identified that pre-pubertal children oxidize more fat relative to total energy expenditure than adults and pubertal children, an observation consistent with previous reports (31). In addition, pre-pubertal children are known to oxidize fats preferentially over carbohydrates during low to moderate intensity exercise as well, when compared with post-pubertal children and adults (32–35). Boisseau et al. reported that higher fat oxidation in pre-pubertal children was associated with a distinctive metabolic phenotype, namely

TABLE 2 | Estimates and *p*-values from mixed effects models examining the association between metabolites and fasting glucose.

Metabolic pathway	Metabolite	^{1}H NMR chemical shift (ppm)	Coef	SE	*p*-value (unadjusted)	*p*-value (adjusted)
Alcohol	Methanol	3.31	−0.004	0.010	0.737	1.000
Amino acid derivatives	2-ketobutyrate	1.07	−0.111	0.012	<0.0001	<0.0001
Amino acid derivatives	3-Methyl-2oxovalericacid	1.08	−0.117	0.012	<0.0001	<0.0001
Amino acid derivatives	Alpha-ketoisovalerate	1.14	−0.041	0.012	0.001	0.044
Amino acid derivatives	Dimethylglycine	2.93	−0.057	0.013	<0.0001	<0.0001
Amino acid derivatives	N-acetyl proteins	2.03	−0.055	0.014	<0.0001	0.003
Amino acid derivatives	Taurine	3.29	−0.042	0.012	<0.0001	0.027
Amino acid derivatives	Trimethylamine	2.87	−0.066	0.013	<0.0001	<0.0001
Amino acid derivatives	Trimethylamine-N-Oxide	3.25	0.027	0.013	0.042	1.000
Amino acids	Alanine	1.48	0.050	0.012	<0.0001	0.003
Amino acids	Arginine	1.71	−0.096	0.013	<0.0001	<0.0001
Amino acids	Asparagine	2.85	−0.087	0.014	<0.0001	<0.0001
Amino acids	Citrulline	3.14	−0.045	0.014	<0.0001	0.039
Amino acids	Glutamate	2.35	−0.038	0.013	0.004	0.171
Amino acids	Glutamine	2.45	−0.027	0.014	0.053	1.000
Amino acids	Glycine	3.57	−0.052	0.016	0.001	0.063
Amino acids	Histidine	7.06	−0.064	0.013	<0.0001	<0.0001
Amino acids	Lysine	1.76	−0.061	0.012	<0.0001	<0.0001
Amino acids	Phenylalanine	7.42	−0.038	0.012	0.002	0.093
Amino acids	Proline	3.34	0.017	0.014	0.199	1.000
Amino acids	Serine	3.96	−0.062	0.014	<0.0001	<0.0001
Amino acids	Threonine	4.29	−0.022	0.010	0.036	1.000
Amino acids	Tyrosine	7.2	0.008	0.013	0.503	1.000
Branched chain amino acids	Isoleucine	1.01	−0.099	0.013	<0.0001	<0.0001
Branched chain amino acids	Leucine	0.96	−0.144	0.011	<0.0001	<0.0001
Branched chain amino acids	Valine	1.05	−0.112	0.012	<0.0001	<0.0001
Glycolysis related	Citrate	2.66	−0.107	0.013	<0.0001	<0.0001
Glycolysis related	Glucose	3.25	0.109	0.013	<0.0001	<0.0001
Glycolysis related	Lactate	1.33	0.023	0.012	0.049	1.000
Ketone bodies	3-D-Hydroxybutyrate	1.18	−0.158	0.011	<0.0001	<0.0001
Ketone bodies	Acetate	1.91	−0.042	0.017	0.015	0.642
Ketone bodies	Acetoacetate	2.29	−0.112	0.009	<0.0001	<0.0001
Lipids	Lipid (mainly HDL, fatty acid CH_3 moieties)	0.83	−0.053	0.015	<0.0001	0.016
Lipids	Lipid (mainly HDL, fatty acid $(CH_2)_n$ moieties)	1.23	−0.004	0.013	0.74	1.000
Lipids	Lipid (mainly LDL, fatty acid CH_3 moieties)	0.87	0.063	0.014	<0.0001	0.001
Lipids	Lipid (mainly LDL, fatty acid $(CH_2)_n$ moieties)	1.27	0.060	0.014	<0.0001	0.001
Lipids	Lipid (mainly VLDL, fatty acid (CH_2) moieties)	1.5	0.062	0.014	<0.0001	0.001
Lipids	Lipid ^{1}H signal	5.18–5.21	0.031	0.015	0.037	1.000
Lipids	Phosphocholine containing lipids	3.21	−0.067	0.016	<0.0001	0.001
Organic acid	Creatine	3.93	−0.088	0.016	<0.0001	<0.0001
Organic acid	Creatinine	4.05	−0.060	0.014	<0.0001	<0.0001
Organic acid	Formate	8.45	−0.064	0.013	<0.0001	<0.0001

Coef, coefficient indicating the directions of the associations between the metabolite and fasting glycemia overtime; SE, standard error for the coefficient.

increased blood free fatty acid and glycerol, which are indicators of fat mobilization from peripheral stores and increased lipolysis (35). Our study has also shown that pre-pubertal children have higher levels of ketogenesis, as noted by higher serum levels of ketones. Two ketone bodies, namely 3-D-hydroxybutyrate and acetoacetate, decreased linearly during the first two pubertal stages for both sexes, to reach minima that remained constant throughout the rest of childhood. Ketogenesis is generally stimulated when fatty acid β-oxidation and production of acetyl-CoA exceeds the processing capacity of the Krebs cycle. The

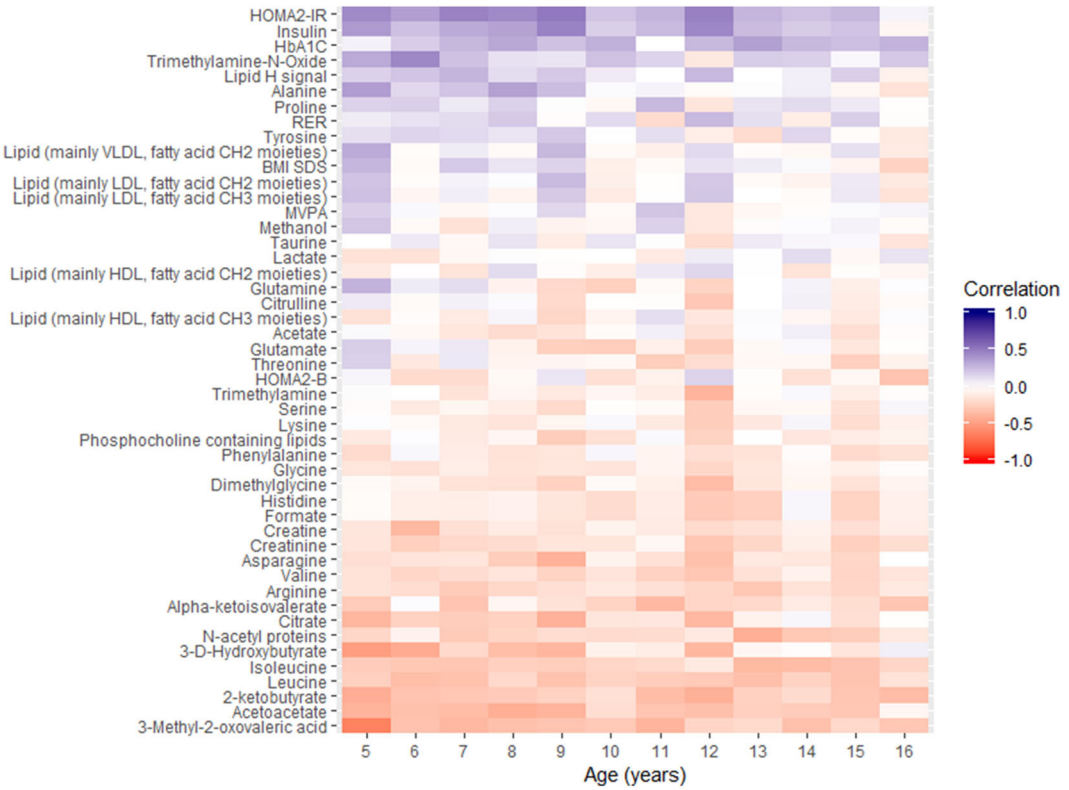

FIGURE 2 | Overview of spearman correlations between fasting glucose and other parameters as a function of chronological age. MVPA, moderate to vigorous physical activity; RER, respiratory exchange ratio.

decreased concentration of serum citrate and formate with puberty illustrates the decreased contribution of fatty acids to the pool of acetyl-coA entering the Krebs for energy production. These patterns describe an overall decreasing fatty acid oxidation, via β-oxidation and ketogenesis, from pre-pubertal to pubertal stage. Whereas 3-D-hydroxybutyrate showed the largest decrease in concentration, levels of acetoacetate remained more stable (constant levels), which suggests that there may be different contributions to ketogenesis from protein and lipid metabolism during puberty.

In addition, serum lipoprotein levels in childhood are known to vary with age, as a result of the hormonal changes of puberty, with reports of complex pattern and interactions according to age, gender and insulin resistance (36–38). Some studies in normal weight children reported that levels of triglycerides (mainly in VLDL) increased whereas total cholesterol and LDL-cholesterol decreased during puberty in both sexes (36, 37). Other reports describe distinct and gender-specific patterns from mid-puberty, namely increased triglycerides and decreased HDL cholesterol in boys, and the opposite pattern in girls (38). Our observations suggest that changes in the serum LDL and VLDL fatty acid signature are positively associated with fasting glycemia throughout childhood. We previously reported how IR development in the Earlybird cohort was marked by decreased phospholipids (mainly in HDL particles) and increased LDL fatty acid signature in both males and females in the EarlyBird cohort (15). Such an observation further illustrates the remodeling of lipid mobilization and metabolism that underpins structural growth and changing energy storage (36, 37).

As puberty commences and progresses, there are major changes in many physiological processes, which in turn modify fuel mobilization and utilization (39, 40). Jones and Kostyak reported higher fat oxidation in children (5–10 years) compared with adults—an adaptative process that might support normal growth requirements, such as higher rates of protein synthesis, lipid storage, and bone growth. Such higher requirements are captured in dietary recommendations for fat consumption, which suggest reduction in fat intake from childhood to adulthood (40, 41). For children 1–3 years of age, and 4–18 years of age, the Acceptable Macronutrient Distribution Range (AMDR) for total fat is 30–40% of energy, and 25–35% of energy, respectively (40, 41). In adults, the AMDR for fat has been set at 20–35% of energy (40, 41). The novel molecular insights into lipid metabolism before and during puberty, revealed in the present study, may help to further refine the dietary recommendations in terms of quantity and quality of lipids required for optimal growth and development of children before and during puberty.

Girls and boys are indistinguishable in muscle strength until puberty, at which time strength and aerobic performance increases more rapidly in boys (7, 20). Our analysis also revealed

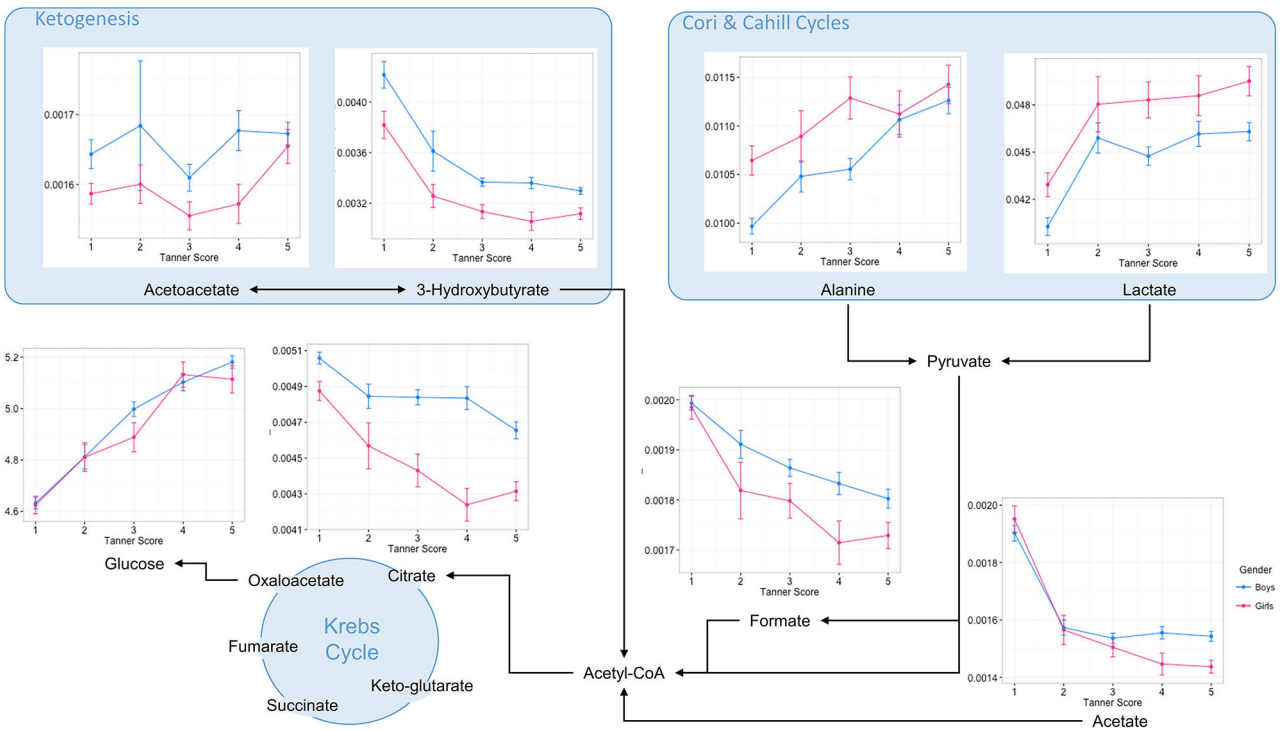

FIGURE 3 | Overview of central energy metabolic pathways with selected blood serum metabolite patterns according to pubertal stages. Data are plotted as mean ± standard error of metabolite integrals (arbitrary units) and glucose concentration (Mmol/L) measured at the first occurrence of pubertal stages for boys and girls, respectively.

that serum creatinine increased from mid puberty more rapidly in boys than in girls, whilst being negatively correlated with fasting glucose. It is likely that the gender difference in muscle mass and function is driven primarily by the large difference in free testosterone concentrations that emerges with the onset of puberty (42). However, boys are more insulin sensitive than girls, especially during puberty, and it is possible that differences in the action of insulin may also contribute to gender difference in muscle mass and function. The gender-specific pattern of creatinine was associated with greater increases in serum leucine, valine, glutamine and proline in boys. Our observations agree with a recent report on whole blood amino acid patterns in puberty from the LIFE Child Cohort by Hirschel et al. (43). Serum creatinine is known to be affected by age, gender, ethnicity, dietary protein intake, and lean mass (44). During puberty, the bodies of boys exhibit a different tempo of bone, muscle, and cartilage/tendon growth, which is reflected in the gender-specific patterns of creatinine and amino acids. Amino acids play a major role as building blocks for protein synthesis and as regulators of key metabolic pathways for cell maintenance and growth (45). Previous studies reported that during puberty, growth is driven by maintaining a greater rate of protein synthesis than that of breakdown (46, 47). Arslanian et al. described lower protein oxidation and proteolysis during puberty when compared to pre-puberty, whereas protein synthesis was unchanged (46). In addition, they showed that during puberty whole body proteolysis is resistant to suppression by insulin (46). Blood amino acid concentrations reflect both the availability of amino acids and changes in amino acid influx or efflux between muscle and other tissues as a result of their utilization (e.g., by protein synthesis) or catabolism (protein turnover) (48, 49). In particular, proline, alanine, and glutamine are used as a source of energy metabolism through the anaplerotic pathway of the Krebs cycle in skeletal muscle (50). Since the efficiency of carbohydrate oxidation increases during puberty, we may hypothesize that increasing glycolytic metabolism reduces the mobilization of these amino acids into the anaplerotic pathway, and further contributes to higher circulating concentrations. The observed elevation of blood lactate and alanine concentrations with age reflects changes in the Cori and Cahill cycles. Since Cori and Cahill cycle shuttle lactate and alanine from the muscles to the liver, where the nitrogen enters the urea cycle for gluconeogenesis, this phenotype further illustrates the pubertal changes in glycolytic metabolism.

Last, several metabolites of one-carbon metabolism—glycine, dimethylglycine and creatine—showed a negative association with fasting glucose trajectories. This transmethylation pathway closely interconnects choline, betaine and homocysteine metabolism, and is of major importance for numerous cellular functions, such as DNA methylation, phosphatidylcholine, and protein synthesis (51, 52). Previous reports described how glycine and dimethylglycine metabolism is linked to glucose

homeostasis and diabetes and may be genetically determined (53). In particular, lower circulating levels were associated with lower insulin sensitivity and higher fasting glucose (53), which is in agreement with our novel observations. With a potential role of the one-carbon cycle in the developmental origins of T2D (54), the biological implication of such a signature in the course of childhood would benefit from further clinical investigations.

It is recognized that there are several potential limitations with the present study. Importantly, the sample size was limited, and being an exploratory study, it was not possible to undertake an a priori power calculation. Furthermore, while less-invasive methods for measuring IR, such as the HOMA are well-suited for repeat measurements in cohort studies of children, it is recognized that a potential limitation is that IR measured by HOMA correlates only modestly with clamp-derived measures of IR, and also that HOMA IR already correlates highly with fasting insulin in normoglycaemic subjects (55, 56). However, if fasting insulin secretion is impaired, the direction of error is that HOMA underestimates IR. Despite these acknowledged limitations, HOMA is considered as a valid method for measuring IR in pediatric research (57).

CONCLUSION

This study demonstrates that normal pubertal growth and development is accompanied by complex and extensive remodeling of metabolism and fuel oxidation, reflecting the changing energy requirements of puberty. The full complexity of this process is revealed by blood metabolic profiling. Fasting glycemia increases steadily throughout childhood and is accompanied by increasing concentration of insulin and rising respiratory exchange ratio. These metabolic changes are influenced by the endocrine changes of puberty, including the GH/IGF axis. As a result, the fuel economy shifts away from fatty acid oxidation and toward carbohydrate oxidation. The metabolic signatures indicate reduced fatty acid oxidation and ketogenesis, increased flux through Cori and Cahill cycles, and complex changes in amino acids with gender differences reflecting the emerging contrasts in body composition. There are gradual rises in LDL and VLDL particles and remodeling of one carbon metabolism. All of these changes represent normal physiological development.

These findings raise the important question at what point do physiological changes, such as increasing fasting glycemia begin to have pathophysiological consequences and raise concern for future cardiometabolic health? It is possible to speculate that the metabolic changes we have observed, especially the shift away from fat oxidation, and reduced ketogenesis, is maladaptive in the context of obesity, and may also be liable to perpetuate the obese state. Therefore, the reduced metabolic flexibility of puberty makes this a vulnerable period for excessive weight gain. Weight gain and obesity further exacerbate the physiological insulin resistance of puberty and fasting glycemia, and will favor atherogenic changes in the lipid profile and pathways, such as one carbon metabolism. This is in line with our other findings which suggested that weight gain and increasing insulin resistance will exacerbate hyperglycaemia (15) in adolescence, especially in those who also have genetic impairment of pancreatic beta cell function (13, 15).

Finally, these findings will have implications for guidance on child nutrition. Since fat, protein, and carbohydrate requirements change during pubertal development, this study suggests that macronutrient requirements for optimum healthy growth and development and reduction in risk of cardiometabolic disease may need to take into account metabolic changes at puberty and gender differences. We speculate that increasing respiratory exchange ratio and reduced ketogenesis may justify reduction in dietary fat relative to carbohydrate at adolescence, in order to reduce the risks of weight gain and insulin resistance. This nutritional change might be necessary earlier in girls, reflecting their earlier onset of puberty and growth spurt. The avoidance of adolescent weight gain is also emphasized, in view of the maladaptive metabolic effects of insulin resistance, and in order to reduce long term cardiometabolic risks. Since growth and energy metabolism are dependent also on the presence of small quantities of several micronutrients, further analyses should explore the potential influence of key enzyme cofactors on metabonomic profiles and implications for cardiometabolic risk. This knowledge has the potential to open-up the development of new and age-specific strategies for the prevention of cardiometabolic disease in children, through more evidence-based guidance on lifestyle and personalized dietary interventions.

DATA AVAILABILITY STATEMENT

The datasets presented in this article are not readily available because subject in particular, to ethical and privacy considerations. Requests to access the datasets should be directed to jonathan.pinkney@plymouth.ac.uk and francois-pierre.martin@rd.nestle.com.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Plymouth Local Research Ethics Committee. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

F-PM designed the study. AJ and F-PM were involved in the acquisition of the data. OC, F-PM, JH, and JP contributed to the analysis, data interpretation, and drafted the manuscript. JP was guarantor of the work. All authors approved the final version.

ACKNOWLEDGMENTS

We acknowledge the life and work of our former colleague Terence Wilkin (1945–2017), Professor of Endocrinology and Metabolism, whose vision and original thinking led to the creation of the EarlyBird Study and the establishment of the

collaboration that made possible the studies reported here. We thank the EarlyBird children, their parents and all EarlyBird team members for their contribution to the study. We thank Ondine Walter for biobanking, sample handling and preparation at Nestlé, and for support for compliance with the Human Research Act. We thank Christian Darimont and Jörg Hager for scientific discussion during the preparation of the manuscript. The EarlyBird study was supported by Bright Future Trust, The Kirby Laing Foundation, Peninsula Medical Foundation, Diabetes UK, and the EarlyBird Diabetes Trust. JH and AJ have received funding from the Nestlé Group. The metabonomic analysis reported in this paper was funded by Nestlé Research.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnut.2020.00139/full#supplementary-material

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Conflict of Interest: The authors declare that this study received funding from Bright Future Trust, The Kirby Laing Foundation, Peninsula Medical Foundation, Diabetes UK, the EarlyBird Diabetes Trust, and Nestlé Research. Nestlé Research had the following involvement with the study: metabonomics data generation and analysis, interpretation of data, writing of this article and decision to submit it for publication. The other funders were not involved in the study design, collection, analysis, interpretation of data, the writing of this article or the decision to submit it for publication. JH, JP, and AJ are employees of Plymouth University Peninsula School of Medicine and Dentistry. F-PM and OC are employees of Nestlé Research. JH and AJ have received funding from Nestlé Research. The authors have no other dualities of interest to declare.

frontiers in Nutrition

CLINICAL TRIAL
published: 14 January 2021
doi: 10.3389/fnut.2020.594850

The Gut Microbiome and Abiotic Factors as Potential Determinants of Postprandial Glucose Responses: A Single-Arm Meal Study

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OPEN ACCESS

Edited by:
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Specialty section:
This article was submitted to Nutrition and Metabolism, a section of the journal Frontiers in Nutrition

Received: *14 August 2020*
Accepted: *07 December 2020*
Published: *14 January 2021*

Citation:
Nestel N, Hvass JD, Bahl MI, Hansen LH, Krych L, Nielsen DS, Dragsted LO and Roager HM (2021) The Gut Microbiome and Abiotic Factors as Potential Determinants of Postprandial Glucose Responses: A Single-Arm Meal Study. Front. Nutr. 7:594850. doi: 10.3389/fnut.2020.594850

The gut microbiome has combined with other person-specific information, such as blood parameters, dietary habits, anthropometrics, and physical activity been found to predict personalized postprandial glucose responses (PPGRs) to various foods. Yet, the contributions of specific microbiome taxa, measures of fermentation, and abiotic factors in the colon to glycemic control remain elusive. We tested whether PPGRs 60 min after a standardized breakfast was associated with gut microbial α-diversity (primary outcome) and explored whether postprandial responses of glucose and insulin were associated with specific microbiome taxa, colonic fermentation as reflected by fecal short-chain fatty acids (SCFAs), and breath hydrogen and methane exhalation, as well as abiotic factors including fecal pH, fecal water content, fecal energy density, intestinal transit time (ITT), and stool consistency. A single-arm meal trial was conducted. A total of 31 healthy (24 female and seven male) subjects consumed a standardized evening meal and a subsequent standardized breakfast (1,499 kJ) where blood was collected for analysis of postprandial glucose and insulin responses. PPGRs to the same breakfast varied across the healthy subjects. The largest inter-individual variability in PPGRs was observed 60 min after the meal but was not associated with gut microbial α-diversity. In addition, no significant associations were observed between postprandial responses and specific taxa of the gut microbiome, measures of colonic fermentation, ITT, or other abiotic factors. However, fasting glucose concentrations were negatively associated with ITT, and fasting insulin was positively associated with fasting breath hydrogen. In conclusion, the gut microbiome, measures of colonic fermentation, and abiotic factors were not shown to be significantly associated with variability in postprandial responses, suggesting that contributions of the gut microbiome, colonic fermentation, and abiotic factors to PPGRs may be subtle in healthy adults.

Keywords: gut microbiome, personalized nutrition, individuality, intestinal transit time, abiotic factors, colonic fermentation

INTRODUCTION

Elevated postprandial glucose response (PPGR) is associated with type 2 diabetes mellitus (T2DM), which is a worldwide growing concern (1). Previous research has uncovered high variability in postprandial glucose and insulin responses of different people to the same food (1–5), supporting the need for personalized nutrition in contrast to the prevalent "one size fits all" approach to dietary guidance. Recent studies (3–5) have found that the gut microbiome composition is associated with variations in PPGR to different foods. However, the contribution of specific bacterial taxa of the gut microbiome to the variability in PPGR and the underlying mechanisms remain elusive. High gut microbial α-diversity has been suggested as a marker of a healthy gut (6, 7), which is associated with intake of a high diversity of vegetables and fruits (8). However, high gut microbial α-diversity can be confounded by a long intestinal transit time (ITT) (9, 10), which favors growth of slow-growing species and thereby increase microbial richness (11). Abiotic factors such as pH, ITT, and stool consistency are determinants of gut microbial composition, diversity, and metabolism (9–12). Furthermore, ITT is a determinant of stool pH and short-chain fatty acid (SCFA) concentrations (13), as well as microbial hydrogen and methane production in the colon (14). Together, this emphasizes that abiotic factors are fundamental determinants of the gut microbial composition and metabolism, suggesting that individual differences in abiotic factors could explain differences in the gut microbiome and be associated with variations in PPGR. Therefore, we hypothesized as the primary outcome that the individual PPGR 60 min after a standardized meal test is inversely associated with gut microbial diversity in healthy subjects. In addition, we explored whether PPGR after a standardized meal is associated with baseline gut microbial composition and metabolism as reflected by fecal SCFAs and breath hydrogen and methane exhalation, as well as abiotic factors including fecal pH, fecal water content, fecal energy density, ITT, and stool consistency assessed by the Bristol Stool Scale (BSS).

MATERIALS AND METHODS

Protocol

The study (MIGLUCOSE) was conducted as a single-arm meal study at the Department of Nutrition, Exercise and Sports, University of Copenhagen, Denmark, from October to December 2018. The study was approved by The Ethical Committee of the Capital Region of Denmark (H-18032846) and conducted according to the Declaration of Helsinki, and the handling of personal data was endorsed by the Faculty of Science, University of Copenhagen (514-0052/18-5000). All subjects signed an informed consent form before participating in the study. The study was registered at ClinicalTrials.gov (ID: NCT03686293).

Participants

A total of 31 healthy Danish subjects (24 women and seven men) were recruited and completed the test day. The inclusion criteria were as follows: male and female; aged 18–40 years; body mass index (BMI) < 27 kg/m^2; no history of chronic or infectious diseases; no medical conditions and not taking medication known to influence any of the outcome measures; no blood donations or participation in another scientific study within the three previous months; no oral antibiotics, diarrhea inhibitors, or laxatives taken within the previous six months; and no pregnant or lactating women. The inclusion criteria regarding age and BMI were chosen to target a rather homogenous and healthy group of adults. The participants were recruited from October until December 2018 through poster boards at educational institutions in Copenhagen and on the websites www.forsøgsperson.dk, https://nexs.ku.dk/om_nexs/forsogspersoner/, and www.sundhed.dk and via social media (Twitter, Facebook, Instagram, and LinkedIn). One participant turned 41 years between the period of giving written informed consent and the study day.

Prior to the Meal Test

Ten days prior to the test day, subjects were asked not to consume any sweet corn (maize). Five days prior to the test day, subjects were asked to consume 100 g of provided sweet corn with no other foods, 2 h before dinner. In the subsequent five consecutive days, the subjects filled out a defecation diary where they noted down the time and date of the sweet corn consumption and the time and date of when they observed sweet corn excreted in their feces. Based on this, the subjects' ITT was estimated.

The subjects were asked to abstain from alcohol intake, strenuous exercise, and painkillers containing paracetamol 24 h before the test day. The participants were asked to consume a standardized meal on the evening before the test day, which they prepared according to a provided recipe (wheat spaghetti with lentils and tomato sauce), followed by at least 12 h of fasting where they were only allowed to consume 500 ml of water. Finally, participants were instructed to collect a fecal sample between 4 p.m. the day before the test day and 8 a.m. on the morning of the test day. However, one participant handed in a fecal sample collected in the morning the day before the test day, and one subject delivered the fecal sample the morning after the test day. Subjects assessed the fecal consistency by the BSS, which is a validated surrogate measure for gastrointestinal transit time (15).

Meal Test

On the test day, the participants were asked to refrain from brushing their teeth and smoking. Avoidance of smoking was to avoid the contamination of exogenous hydrogen from cigarette smoke when conducting the breath exhalation measurements. The avoidance of teeth brushing was done to preserve saliva samples, which were collected for microbiome profiling but not included in the present study. Additionally, the participants were instructed to arrive at the study site using the least strenuous form of transportation. On arrival at the study site, subjects delivered the collected fecal sample, which had been kept cold in a cooling bag with freeze elements upon collection at home and during transportation to the laboratory. In the fasting condition, anthropometric measurements (height and body weight) were obtained, and a peripheral venous catheter was placed inside the elbow joint of the subjects from which blood could be drawn.

Each participant consumed 500 mg of paracetamol (1 × 500 mg tablet, Panodil, GlaxoSmithKline Dungarvan Ltd.), allowing for measurement of gastric emptying (16) and 150 ml water followed by a carbohydrate rich standardized breakfast (1,499 kJ, **Supplementary Table 1**) consisting of two slices of white toast bread (60 g) with butter (8 g) and jam (20 g) and 250 ml of orange juice (macronutrient breakdown: 60.9 g of carbohydrate, 9.1 g of fat, and 6.6 g of protein). The participants were asked to consume the test meal within 15 min. Blood samples were drawn into EDTA plasma (for glucose determination) and serum (for insulin determination and mass spectrometry) tubes before the meal (fasting) and at 15, 30, 60, 90, and 120 min after starting the test meal. Finally, breath exhalation samples were collected before the meal (fasting) by exhaling air into a provided sampling bag.

Compliance

Compliance to the standardization procedures was evaluated by self-assessment questionnaires on the test day. In addition, to assess whether the participants had in fact consumed the standardized meal including lentils the evening before the test day, we performed ultra-performance liquid chromatography–mass spectrometry (UPLC-MS) on serum samples as previously published (17) and checked for the presence of tryptophan betaine (247.1448 [M + H], retention time 2.85 min), a biomarker of chickpeas and lentils peaking in blood 4–6 h after consumption before slowly being excreted (18). Tryptophan betaine was detected in considerable amounts in all serum samples except from one male subject (**Supplementary Figure 1**), suggesting that all participants except one had been compliant and consumed the standardized evening meal containing lentils. The non-compliant subject was excluded from further analyses.

Biochemical Analysis of Blood

EDTA plasma samples were upon collection immediately put on ice, until they were centrifuged for precipitation of blood cells and stored at −80°C. The serum samples were left to clot at room temperature for 30 min, and the supernatants were collected into cryotubes and stored at −80°C. Glucose was measured in plasma samples by using Pentra ABX 400 (HORIBA ABX, Montpellier, France). The detection limit was 0.11 mmol/L, and the reference interval for fasting glucose was 4.2–6.3 mmol/L. Serum insulin levels were measured by using Immulite 2000 XPi (Siemens Healthcare Diagnostics Ltd., Llaneris Gwynedd LL554EL, United Kingdom). The detection limit was 14.4 pmol/L. Measurements below detection limit were set to half the detection limit (7.2 pmol/L). Prior to analysis of insulin and glucose, both the instrument's performances were validated using external and internal insulin and glucose controls. For the external glucose controls, the coefficient of variation (CV) of glucose was 1.2% [low-level control (5.3–5.5 mmol/L)] and 0.7% [high-level control (14.5–14.8 mmol/L)]. For the internal glucose controls (range 4.5–4.7 mmol/L), the CV was 1.2%. For the external insulin controls, the CV of insulin was 2.0% [low-level control (70.1–74.3 pmol/L)] and 3.3% [high-level control (358.0–397.0 pmol/L)]. For the internal insulin controls (range 48.9–56.7 pmol/L), the CV was 4.1%.

Breath Exhalation Measurements

Concentrations of hydrogen and methane were measured in all breath samples using the M.E.C. Lactotest 202 Xtend (M.E.C. R&D sprl, Brussels, Belgium), as a proxy of colonic fermentation (19).

Fecal Measurements

Fecal samples were homogenized in sterile water 1:1, and pH was determined using a digital pH meter (Lutron PH-208, Taiwan). The homogenized samples were aliquoted to cryotubes and stored at −80°C. SCFAs were quantified in feces by UPLC-MS as previously described (17). Fecal water content was determined by drying fecal samples for 48 h at 50°C or until complete dryness. Gross energy density of fecal samples was determined by combusting ∼150 mg of dry feces in a bomb calorimeter C6000 (IKA, Staufen, Germany) using benzoic acid as a calibrator (IKA® C 723). The energy density in the dried feces was converted into energy density per gram wet feces.

Microbiome Profiling

DNA was extracted from ∼100 mg of homogenized feces by Bead-Beat Micro AX Gravity method (A&A Biotechnology, Gdynia, Poland) with several adjustments mentioned below. A NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies Inc., Wilmington, USA) was used to assess the purity of the extracted DNA. The DNA concentration was measured using Qubit dsDNA BR Assay Kit (Thermo Fisher Scientific Inc., Waltham, USA). The gut microbiota composition of 27 subjects was successfully analyzed by 16S ribosomal RNA (rRNA) gene amplicon sequencing of the V3 region on the Illumina NextSeq platform (Illumina Inc.) with the Mid Output Kit v2 (300 cycles) as previously described (20). The raw dataset containing pair-ended reads with matching quality scores were fused and clipped in the USEARCH pipeline using fastq_mergepairs and fastq_filter scripts. The UNOISE was used to purge the dataset from chimeric reads and to build zero radius Operational Taxonomic Units (zOTUs). As a reference database, the Greengenes (13.8) 16S rRNA gene collection was employed. The acquired 16S rRNA gene amplicon data were pre-processed using the web-based tool MicrobiomeAnalyst (21). The average count per sample was 65,142 reads (ranging from 16,647 to 151,790). After zOTUs that either were low abundant (<10 counts) or had low prevalence (<10%) across samples were filtered out, 1,215 zOTUs remained. The data were then rarefied to the minimum library size (16,647 reads) to make sure that all samples were comparable. The subjects' fecal α-diversity was assessed by both the Shannon index and zOTU richness. For correlation analyses, core zOTUs (17/480) and bacterial genera (19) present in at least 30% of the samples were used.

Statistical Analysis

Based on a pilot experiment, we expected to obtain a correlation coefficient of 0.5 between gut microbial diversity and postprandial glucose levels at 60 min. With alpha set to 0.05 and beta set to 0.20, we calculated that 29 participants would be needed. Statistical analyses were conducted in R (version 3.4.2) and GraphPad Prism (version 8.2.0). The area

TABLE 1 | Clinical characteristics of the 31 healthy subjects.

Characteristic	Total group[a]
Sex (f/m)	24/7
Age (years)	28.4 (±5.6)
Weight (kg)	64.6 (±10.6)
Body mass index (kg/m^2)	22.0 (±2.2)
Fasting plasma glucose (mmol/L)	5.4 (±0.3)
Fasting serum insulin (pmol/L)	35.1 (±23.9)
Intestinal transit time[b] (h)	28.4 (±15.3)
Stool consistency[c]	3.4 (±1.3)
Fasting breath hydrogen (ppm)	31.1 (±27.6)
Fasting breath methane (ppm)	7.3 (±20.1)

[a] *Values are means (±SD). Missing values are reported in* ***Supplementary Figure 2****.*
[b] *Intestinal transit time was estimated by the time it took sweet corn to travel through the subjects' gastrointestinal system.*
[c] *Stool consistency was assessed by the Bristol Stool Scale, which ranges from 1 to 7, with 1 indicating hard stools and 7 indicating watery diarrhea.*

under the curves (AUCs) for glucose and insulin concentrations during the test period were calculated by using the trapezoidal method, where the total AUCs were determined using the entire area above zero, using the following formula: $AUC = x\text{t}\ ((y1 + y2)/2)$. The *ggplot2* package (version 3.4.4) was used to calculate the 95% confidence interval (CI) for the insulin and glucose concentrations from 0 to 120 min. Correlations between baseline and postprandial glucose and insulin measures (30, 60, 90, and 120 min), individual microbiome features, and other colonic factors, such as fecal SCFAs, breath hydrogen and methane exhalation, ITT, stool consistency, fecal pH, fecal water content, and fecal energy density, were calculated using the standard Spearman's rank correlation, as implemented in the *ppcor* R package (version 3.4.4) (22). Random forest predictions of fasting and postprandial glucose and insulin responses by microbiota and abiotic variables composed of 1,001 trees and were computed using the default settings of the randomForest function implemented in the randomForest R package (23). For explorative correlation analyses, *p*-values were adjusted for multiple testing by the Benjamini–Hochberg false discovery rate (24). A $p < 0.05$ was considered statistically significant. Heatmaps were generated in GraphPad Prism. The flow of the participants from enrolment until analysis and missing data is shown in **Supplementary Figure 2**.

RESULTS

All 31 subjects (**Table 1**) completed the procedures prior to the test day and ingested the test meal without any problems. However, one male subject was excluded from analysis, as tryptophan betaine, a biomarker of lentils (**Supplementary Figure 1**), was not detected in his blood, suggesting that this subject had not consumed the standardized evening meal prior to the test day. The 30 remaining subjects were apparently healthy. We did however notice that seven subjects had a fasting glucose in the prediabetic range (5.6–6.9 mmol/L).

Gut Microbiome and Postprandial Glucose and Insulin Responses

Large inter-individual variations in glucose and insulin responses to the standardized breakfast were as expected observed in the 30 subjects (**Figure 1**). The individual glucose and insulin responses were weakly correlated across all individuals (**Supplementary Figure 3**), emphasizing the personal nature of glucose regulation. The inter-individual variability [as measured by the population CV (SD/mean, %)] in PPGRs was evident at all time points: 15 min (7%), 30 min (13%), 60 min (18%), 90 min (14%), and 120 min (11%). The largest inter-individual variability in PPGRs was, as hypothesized prior to the study, observed 60 min after the meal (18%), which is why we focused on this time point for the subsequent correlation analyses.

We hypothesized prior to the study that an inverse association would exist between postprandial plasma glucose at 60 min and gut microbial α-diversity as reflected by zOTU richness. However, no correlations were observed between the PPGR after 60 min and gut microbial α-diversity as reflected by observed zOTU richness ($p = 0.52$, rho = 0.13) and Shannon index ($p = 0.12$, rho = 0.31) (**Figure 2A**; **Supplementary Figure 4**). We next investigated whether relative abundances of core bacterial genera or zOTUs (prevalent in at least 30% of subjects) were associated with fasting and postprandial glucose and insulin responses. However, no core bacterial genera or zOTUs were significantly associated with fasting or postprandial glucose and insulin responses after adjusting for multiple testing (adjusted $p > 0.05$) (**Figures 2B,C**).

Associations Between Abiotic Factors, Measures of Colonic Fermentation, and Fasting and Postprandial Responses

We furthermore explored whether abiotic factors known to influence gut microbial composition, diversity, and metabolism were related to the fasting or postprandial state. ITT, determined by the time it took sweet corn to travel through the subjects' gastrointestinal system (median 21.5 h; range 10–55 h), was as expected negatively associated with stool consistency assessed by BSS (Spearman $r = -0.37$, $p = 0.049$; **Supplementary Figure 5A**) and fecal water content (Spearman $r = -0.40$, $p = 0.029$; **Supplementary Figure 5B**). In addition, fecal water content was positively associated with BSS (Spearman $r = 0.56$, $p = 0.0017$; **Supplementary Figure 5C**). Both ITT and stool consistency were consistently associated with fecal branched SCFAs (isobutyrate, methylbutyrate, and isovalerate) and fecal energy density (**Figure 3**). However, none of the abiotic factors (ITT, BSS, fecal water content, fecal pH, and fecal energy density) and measures of colonic fermentation (fecal SCFAs, breath hydrogen, and methane concentrations) were associated with postprandial glucose and insulin responses, respectively (**Figure 3**). Only ITT was negatively associated with fasting glucose (Spearman $r = -0.49$, adjusted $p = 0.04$), whereas the positive association between stool consistency and fasting glucose did not remain significant after adjustment for multiple testing (unadjusted $p = 0.049$, adjusted $p = 0.23$) (**Figure 3**). In addition, a positive association between fasting breath hydrogen

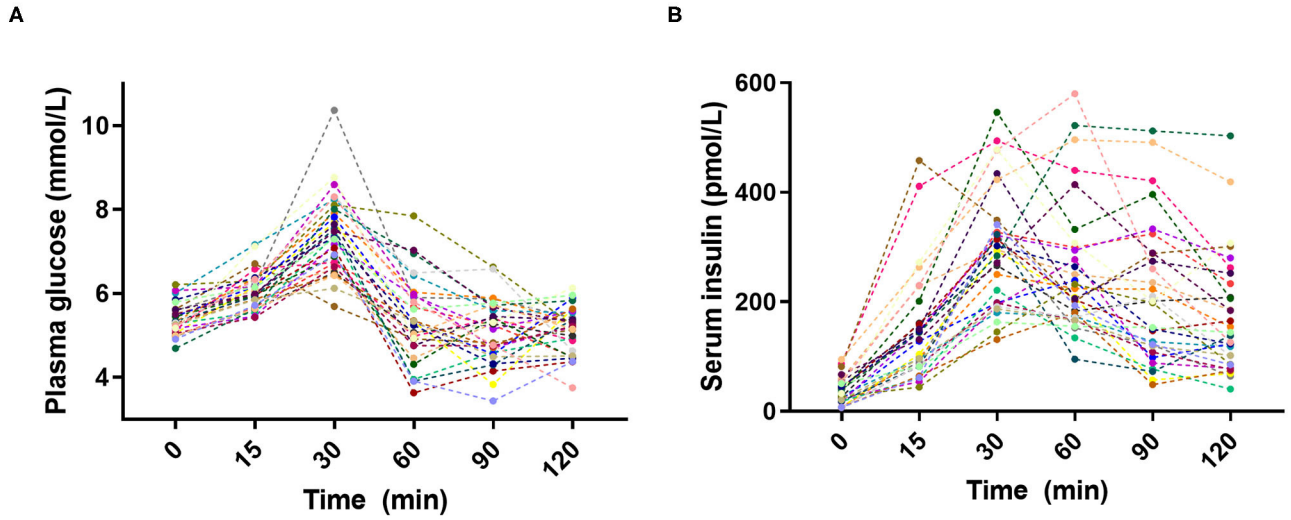

FIGURE 1 | Inter-individual variation in **(A)** plasma glucose and **(B)** serum insulin postprandial responses to the standardized breakfast ($n = 30$).

concentrations and fasting insulin was observed (adjusted $p = 0.016$) (**Figure 3**). Finally, we used random forest to rank the importance of the gut microbiota and abiotic variables in predicting fasting and postprandial glucose and insulin responses. Overall, the random forest corroborated the observed correlations (**Supplementary Figure 6**).

Associations Between Bacterial Genera, Short-Chain Fatty Acids, and Intestinal Transit Time

Since ITT was found to associate with both fasting glucose and several branched SCFAs, we correlated ITT, stool consistency, and fecal SCFA concentrations against the relative abundance of bacterial genera (**Figure 4**). A longer ITT and a firmer stool were associated with higher abundance of *Coprococcus* and *Blautia* but lower abundance of *Lachnospira*. Additionally, *Coprococcus* was positively associated with the branched SCFAs isobutyrate, methylbutyrate, isovalerate, and caproate (**Figures 3**, **4**). In contrast, the relative abundance of *Faecalibacterium* was negatively correlated with fecal isovalerate concentrations. Finally, the abundance of *Dialister* was positively associated with fecal valerate and caproate.

DISCUSSION

We set out to explore whether PPGRs were associated with specific taxa of the gut microbiome, measures of colonic fermentation, and abiotic factors in healthy adults. In agreement with other studies (3–5), we did observe that PPGRs are highly variable across individuals despite the recruitment of a rather homogenous group of young, healthy adults and our standardization procedures prior to the meal test. We hypothesized that the PPGR 60 min after the standardized breakfast would be inversely associated with the subjects' gut microbial diversity, which has been suggested as marker of a healthy gut (6, 7). However, we did not observe any associations between PPGRs and the gut microbial diversity nor with any other specific taxa of the gut microbiome or abiotic factors in healthy adults. This might be due to the rather small sample size of 30 individuals of which runs the risk of false negative, type 2 error. Furthermore, the fact that only six participants were males might decrease the generalizability. Given the limited sample size, the very homogenous group of adults, and the imbalance in sex ratio, the effects of both sex, age, and BMI were not included in our data analysis. Therefore, our findings are explorative and should be validated in larger cohorts.

Previous studies have reported that the gut microbiome contributes to explaining PPGR to identical foods (1, 3–5). The recent PREDICT study, including more than 1,000 individuals, found that the gut microbiome composition, derived from 16S rRNA high-throughput sequencing of baseline stool samples, explained 6% of postprandial glycemia and that meal composition, genetics, meal context (i.e., meal timing, exercise, sleep, and circadian rhythm), and serum glycemic markers are more important determinants of postprandial glycemia (5). In light of our limited sample size, this explains why we were not able to identify any specific taxa of the gut microbiome being significantly associated with variability in PPGR in healthy subjects. Given that glucose mainly is absorbed in the small intestine, the influence of the colonic microbiome on glucose homeostasis is most likely to occur indirectly through production of SCFA and bile acid metabolism (25). However, even elimination of the gut microbiota with antibiotics has little or no effect on glucose metabolism in humans (26, 27), suggesting that the influence of the colonic microbiome on PPGR may be subtle. Altogether, this suggests that the gut microbiome mainly contributes to predictive models of PPGR because the gut microbiome reflects the individual's long-term dietary practices

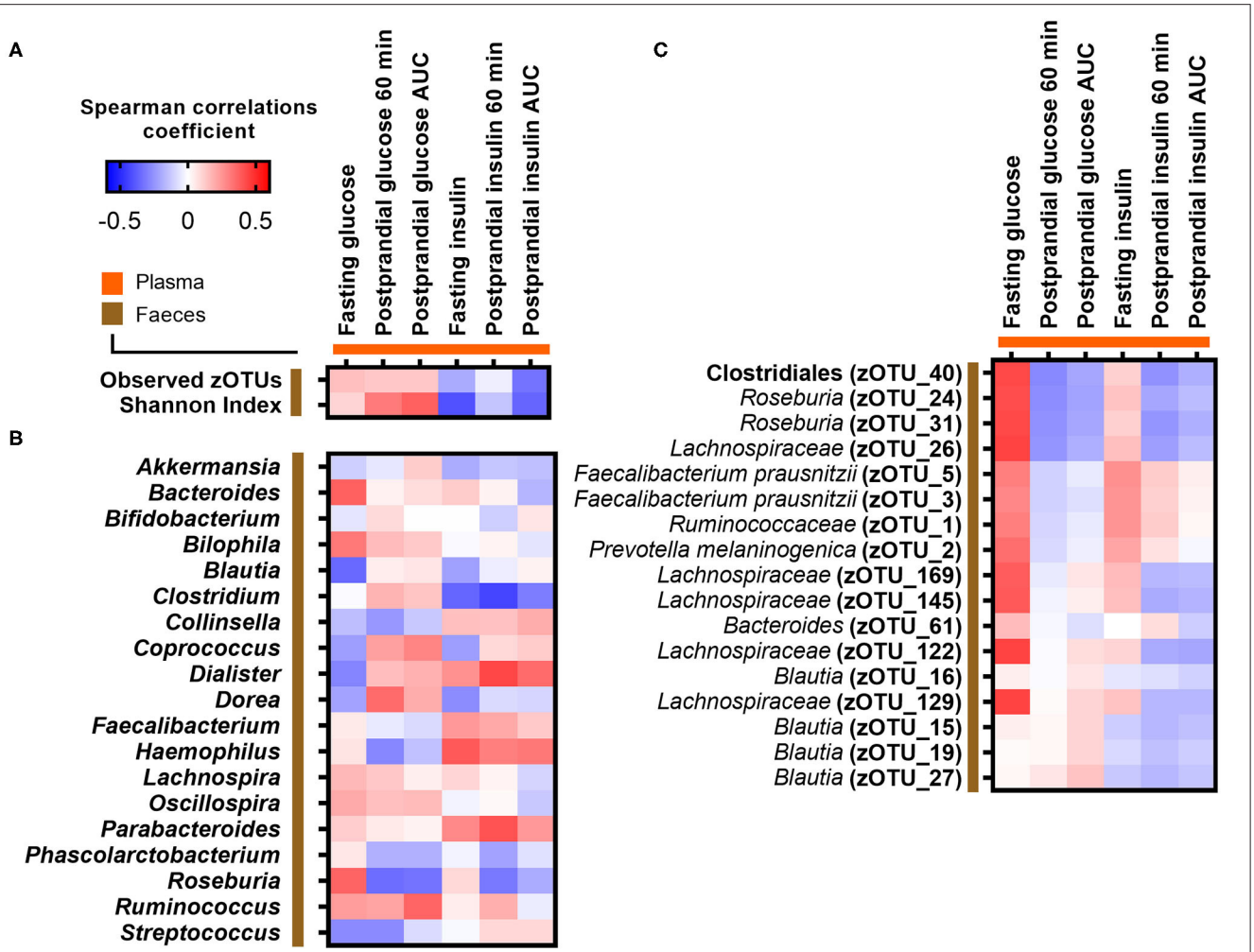

FIGURE 2 | Correlations between fasting and postprandial glucose and insulin measures and **(A)** gut microbial α-diversity, **(B)** abundance of core bacterial genera, and **(C)** core zOTUs. Correlations were calculated based on Spearman's rank correlation ($n = 27$). No associations remained significant after adjustment for multiple testing.

(28, 29), physical activity (30), intake of medicine (31), and differences in ITT (9).

Another modifiable variable, which could possibly influence glucose homeostasis, is ITT (32). Today, there is little consensus on the gold standard for measuring ITT. Scintigraphy (33), wireless SmartPills (34), and radio-opaque marker (9) methods are commonly used to determine ITT. However, other less expensive alternatives include a tasteless, non-absorbable, blue dye (35) and stool consistency assessed by BSS, a validated surrogate measure of ITT (15). Here, we found that ITT, estimated by sweet corn transit time, is commensurate with literature values obtained from wireless motility capsules (36) and correlated well with both stool consistency and fecal water content, confirming that this is a cheap and suitable alternative for estimating ITT. Also, fecal water content was as expected strongly correlated with BSS, suggesting that determination of fecal water may be used as a continuous and more objective measure of ITT rather than BSS, which is a subjective measure of stool consistency.

We observed a negative association between ITT and fasting glucose. While small intestinal motility and flow of luminal content are determinants of glucose absorption (37), little is known about the relation between ITT and glucose homeostasis (32). Numerous appetite hormones secreted from enteroendocrine cells in the intestine such as cholecystokinin (CCK), glucagon-like peptide 1 (GLP-1), and peptide YY (PYY), can regulate intestinal motility, satiety, and gastric emptying and thereby regulate glucose homeostasis (25). Therefore, such hormones could be considered in future mechanistic studies aimed at establishing the role of ITT in determining glucose homeostasis.

ITT and stool consistency have previously been associated with gut microbial composition, diversity, and metabolism (9, 11, 38). Here, we found ITT and a firm stool to be positively associated with the relative abundance of *Coprococcus* and

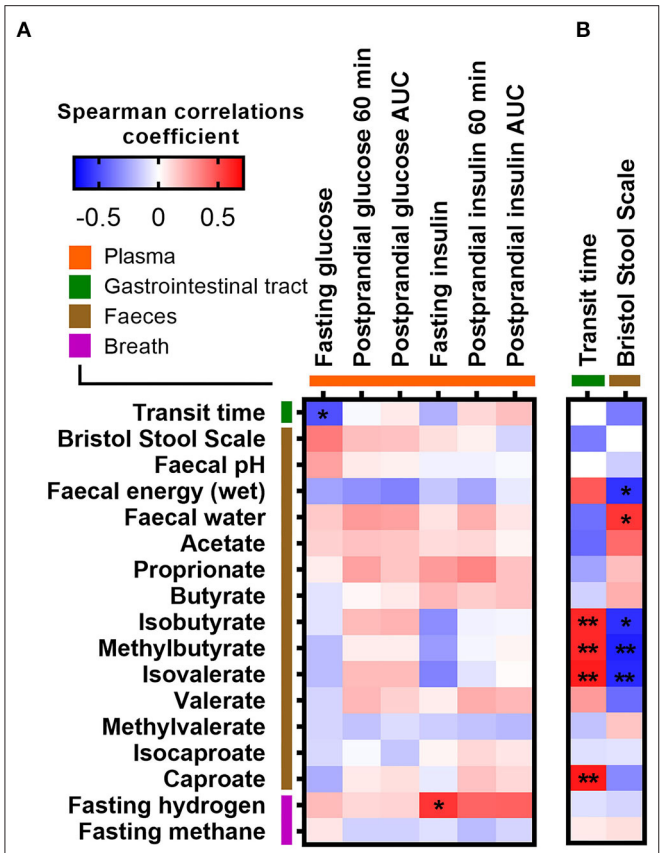

FIGURE 3 | Correlations between measures of colonic fermentation, abiotic factors, and **(A)** fasting and postprandial glucose and insulin measures, as well as with **(B)** measurements of intestinal transit time, respectively. Correlations were calculated based on Spearman's rank correlation ($n = 27$). Significant associations are represented by asterisks (*adjusted $p < 0.05$, **adjusted $p < 0.01$).

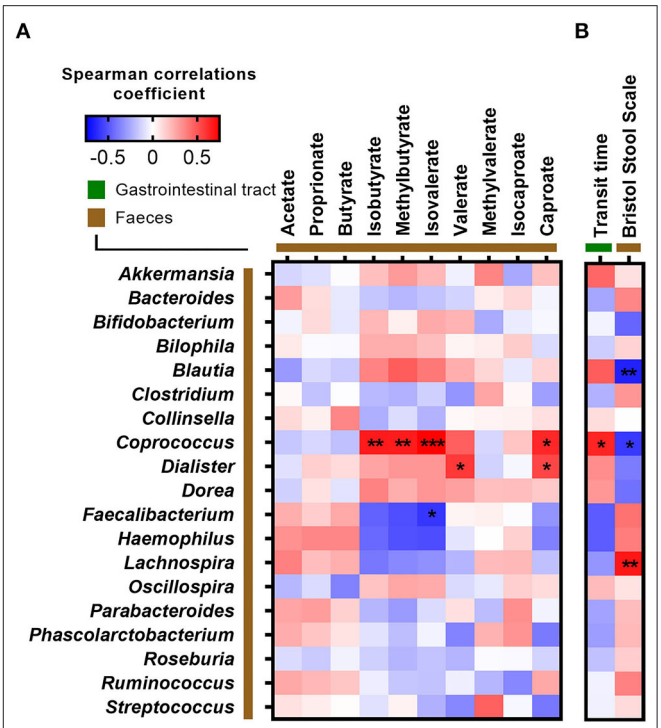

FIGURE 4 | Correlations between fecal bacteria at genus level and **(A)** short-chain fatty acids and **(B)** measurements of intestinal transit time. Correlations were calculated based on Spearman's rank correlation ($n = 27$). Significant associations are represented by asterisks (*adjusted $p < 0.05$, **adjusted $p < 0.01$, ***adjusted $p < 0.001$).

fecal concentrations of branched SCFAs and furthermore a firm stool to be positively associated with fecal wet energy density and the relative abundance of *Blautia* and negatively associated with *Lachnospira*. This is in agreement with studies showing that amino acid fermentation increases in the distal colon when carbohydrate is depleted following a prolonged transit time (9, 39). Of notice, strong correlations were observed between *Coprococcus*, *Dialister*, and branched SCFAs. Previous studies have reported that these species produce SCFAs (40, 41). However, it remains unknown whether these species also produce branched SCFAs.

Finally, we observed that increased fasting insulin levels were associated with increased fasting breath hydrogen levels. Breath hydrogen exhalation reflects colonic fermentation (42) and studies have shown that ingestion of complex carbohydrates increases breath hydrogen exhalation (43, 44). In the current study, participants consumed a standardized evening meal containing lentils before fasting, prior to the test day, which was confirmed by the presence of tryptophan betaine in the blood. As lentils are high in resistant starch and fermented by the colonic microbiota (45), breath hydrogen measured on the test day likely, at least partly, reflected colonic fermentation of the lentils. Therefore, we speculate that individuals with increased colonic fermentation, as reflected by breath hydrogen, could have an increased release and absorption of glucose into the blood, resulting in more insulin being released into the bloodstream in the fasting state. In contrast, concentrations of fecal SCFAs were most likely not related to the evening meal before, since the stool sample was collected prior or shortly after the evening meal or in the early morning on the test day. This may explain why fecal SCFAs were not associated with fasting insulin. The inconsistent correlations between fasting insulin and these two different measures of colonic fermentation suggest that fecal SCFAs and breath hydrogen are not necessarily reflecting colonic fermentation at the same point in time.

In conclusion, we did not observe any associations between PPGRs and the gut microbial diversity nor with any specific taxa of the gut microbiome, measures of colonic fermentation, or abiotic factors in healthy adults, suggesting that the contributions of the gut microbiome and abiotic factors to PPGR may be subtle in healthy adults. We did however observe associations between ITT and fasting glucose, as well as between fasting breath hydrogen and fasting insulin, which could be used to generate new hypotheses for mechanistic research on the complex interactions between ITT, gut microbiome, and glucose homeostasis.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: NCBI, PRJNA665627 (https://www.ncbi.nlm.nih.gov/search/all/?term=PRJNA665627).

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Ethical Committee of the Capital Region of Denmark. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

LD and HR: conception and design of the study. NN and JH: recruitment, collection of data, and biological samples. NN, LH, LK, and DN: fecal microbiota. JH and MB: fecal energy, fecal pH, fecal water determination. HR: liquid chromatography mass spectrometry. JH: fecal SCFAs. NN, JH, and HR: data analysis and interpretation and manuscript drafting. All authors read, revised and approved the final manuscript.

FUNDING

This study was supported by the Novo Nordisk Foundation (NNF19OC0056246; PRIMA—toward Personalized dietary Recommendations based on the Interaction between diet, Microbiome and Abiotic conditions in the gut). The funder had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

ACKNOWLEDGMENTS

We want to thank Cecilie Fryland Appeldorff and Hanne Lysdal Petersen for their help with the meal study and laboratory assistance at the Department of Nutrition, Exercise and Sports, University of Copenhagen, as well as Le Lene Susanne Simon Stevner for assistance with preparing the protocol for the ethical committee.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnut.2020.594850/full#supplementary-material

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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.

frontiers in Nutrition

REVIEW
published: 27 January 2021
doi: 10.3389/fnut.2020.570531

Insights Into the Delivery of Personalized Nutrition: Evidence From Face-To-Face and Web-Based Dietary Interventions

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Prevention strategies for non-communicable diseases (NCDs) are a global priority as it has been estimated that NCDs will account for around 73% of worldwide mortality by the year 2020. The adoption of diets that are low in saturated fat, free sugars, and red and processed meats and higher in unsaturated fats, wholegrains, fruit, and vegetables have been shown to reduce the risk of NCDs. With increasing internet use, several nutrition interventions are now being conducted online as well as face-to-face, however it is unclear which delivery method is most effective. Although a consumer preference toward face-to-face dietary advice delivery has been identified previously, interest in delivering web-based dietary advice, and in particular personalized nutrition (PN), has been rising, as internet delivery may be less costly and more scalable. This review compares published face-to-face and web-based dietary interventions to give insight into which dietary method might be more effective for PN. In total, 19 peer-reviewed randomized controlled trials were identified for inclusion in the review. The majority of face-to-face nutrition interventions were successful at facilitating dietary change. Results from web-based nutrition interventions suggested that personalized web-based nutrition interventions may be successful at inducing short-term dietary change compared to standardized dietary interventions, however, minimal evidence of long-term impact has been found across both delivery methods. Results of a trial that compared face-to-face with web-based diet intervention found significantly greater dietary changes in the face-to-face group compared to web-based and control groups. Further controlled comparative studies and cost-benefit analysis are needed to assess whether web-based methods can be used in place of face-to-face interventions for achieving dietary change.

Keywords: face-to-face nutrition, personalized, personalized nutrition, web-based, dietary change

OPEN ACCESS

Edited by:
Lydia Afman, Wageningen University and Research, Netherlands

Reviewed by:
Sonia de Pascual-Teresa, Consejo Superior de Investigaciones Científicas (CSIC), Spain
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Specialty section:
This article was submitted to Nutrition and Metabolism, a section of the journal Frontiers in Nutrition

Received: *08 June 2020*
Accepted: *08 December 2020*
Published: *27 January 2021*

Citation:
Al-Awadhi B, Fallaize R, Zenun Franco R, Hwang F and Lovegrove JA (2021) Insights Into the Delivery of Personalized Nutrition: Evidence From Face-To-Face and Web-Based Dietary Interventions. Front. Nutr. 7:570531. doi: 10.3389/fnut.2020.570531

INTRODUCTION

According to the World Health Organization, minimal physical activity (PA), obesity and poor dietary habits are major risk factors for non-communicable diseases (NCDs), which include cardiovascular diseases (CVD), type 2 diabetes and several cancers (1). In 2018, NCDs were responsible for around 89% of annual deaths in the UK and are the main cause of more than 2

million deaths annually in the European Union (1). Given that obesity is a major risk factor for NCDs, the adoption of a healthy lifestyle that includes a balanced diet and increased PA is essential to reduce the risk of NCDs (2).

Several studies have shown that the adoption of a diet that is relatively high in polyunsaturated fatty acids (PUFA), monounsaturated fatty acids (MUFA), potassium, fruit, vegetables; or moderately low in fat, saturated fatty acids (SFA), sodium, and dietary cholesterol may reduce the development of certain cancers and CVD (3–6). Despite public health campaigns, a significant proportion of the public is still not adopting this type of eating pattern (7), therefore additional counsel and intervention methods are necessary. Dietary advice can be delivered in several ways—via group or individual settings, over the phone, by text message, face-to-face with a dietitian/nutritionist (in person or via video call) or online and can therefore be given verbally and/or in written form. Face-to-face advice is typically provided by registered dietitians or nutritionists and involves tailoring or personalizing nutrition information to the individuals' requirements and lifestyle with the aim of facilitating behavior change.

Following technological advances, written methods of assessing dietary intakes and delivering dietary advice are being replaced or supplemented with computerized, web-based and mobile methods (8, 9). Based on findings from two systematic reviews, around 30 dietary trials have implemented remote methods to deliver the dietary information which included web-based, e-mails, videos, printed materials, and text messages (10, 11). Currently, most strategies used to either prevent or reduce obesity and CVD are based on standard public health recommendations and are therefore targeted at a population rather than individual level. For example, based on public overconsumption of salt and salt-rich products, public health messages aim to decrease consumption of salt as a protective method against stroke and other CVD (12). Nevertheless, more effective prevention strategies are necessary as NCDs continue to increase in number world-wide (2, 13).

Personalized nutrition (PN), that is, nutrition that is tailored toward an individual's or group's specific dietary requirements, has been identified as an important component of effective dietary intervention (14). PN may be more effective than general nutrition information as the advice is perceived as more personally relevant (15). One of the largest PN dietary intervention trial to date, Food4Me, used a web-based model to evaluate the efficacy of different levels of PN compared with standard population-based dietary advice, and found that PN improved dietary intake significantly more than non-personalized advice (16). Whilst Food4Me was delivered online, the research team identified a consumer preference toward face-to-face PN (17). However, face-to-face nutrition can be expensive, time consuming and may not be accessible to everyone (18). The use of web-responsive applications, websites, or emails provide an alternative method to face-to-face nutrition counseling that can reach a larger population. Web-based PN also allows individuals to access dietary interventions at home and therefore away from the usual clinical setting (19). Thus, interest in web-based health education messages has increased in recent years.

Given the differences in cost and reach between face-to-face and web-based nutrition, it will be useful to evaluate which method is more effective. Few trials have directly assessed the effectiveness of web-based nutrition intervention compared with face-to-face nutrition intervention. The purpose of this review is to assess evidence for the effectiveness of web-based and face-to-face dietary interventions on dietary change and to give insight into which method may be more effective at delivering PN.

METHODS

This review focuses on dietary change trials delivered in person/face-to-face or via the web in adult populations. A literature search was undertaken in PUBMED, Google Scholar, and MEDLINE to identify the effect of communicating dietary advice (to change dietary habits) in face-to-face and web-based settings. Terms used in the searches were face-to-face nutrition, nutrition interviews, weight-loss, dietary advice, web-based nutrition interventions, online, one-to-one nutrition counseling, online face-to-face nutrition, online one-to-one nutrition, Internet nutrition advice, obesity, dietary changes, and personalized nutrition. All terms were paired for outcome measures (dietary change). Only articles that were written or translated into English were included in the search.

Study Selection

A total of 417 peer-reviewed and accepted manuscripts (from 1990 to 2020) reporting on RCT were identified; 19 were included in the review after screening (see **Figure 1**). Only randomized control trials (RCT) that reported original data on the effect of communicating dietary advice in a face-to-face setting or web-based nutrition interventions were included. Studies were excluded if the design trial was not a RCT or if the main focus of the trial was not dietary change. Face-to-face nutrition interventions included studies that utilized either individualized (one-to-one) settings or nutrition advice delivered in group settings. The focus of this paper was on dietary change for the healthy and overweight/obese population, therefore, studies were excluded if they were conducted with people with eating disorders, pregnant women and if the goal of the intervention was for treatment of a specific medical condition, with the exception of disorders with asymptomatic risk factors such as hypertension, hypercholesterolemia or impaired glucose tolerance.

RESULTS

Of the 19 RCT identified, 6 focused on face-to-face dietary interventions and 12 on web-based; 1 paper compared a face-to-face with a web-based dietary intervention. Furthermore, 16 out of the 19 articles incorporated PN as opposed to a standard dietary intervention.

Face-To-Face Dietary Interventions

The face-to-face intervention studies identified ($n = 6$) were long-term trials (>6 months) that assessed the impact of dietary

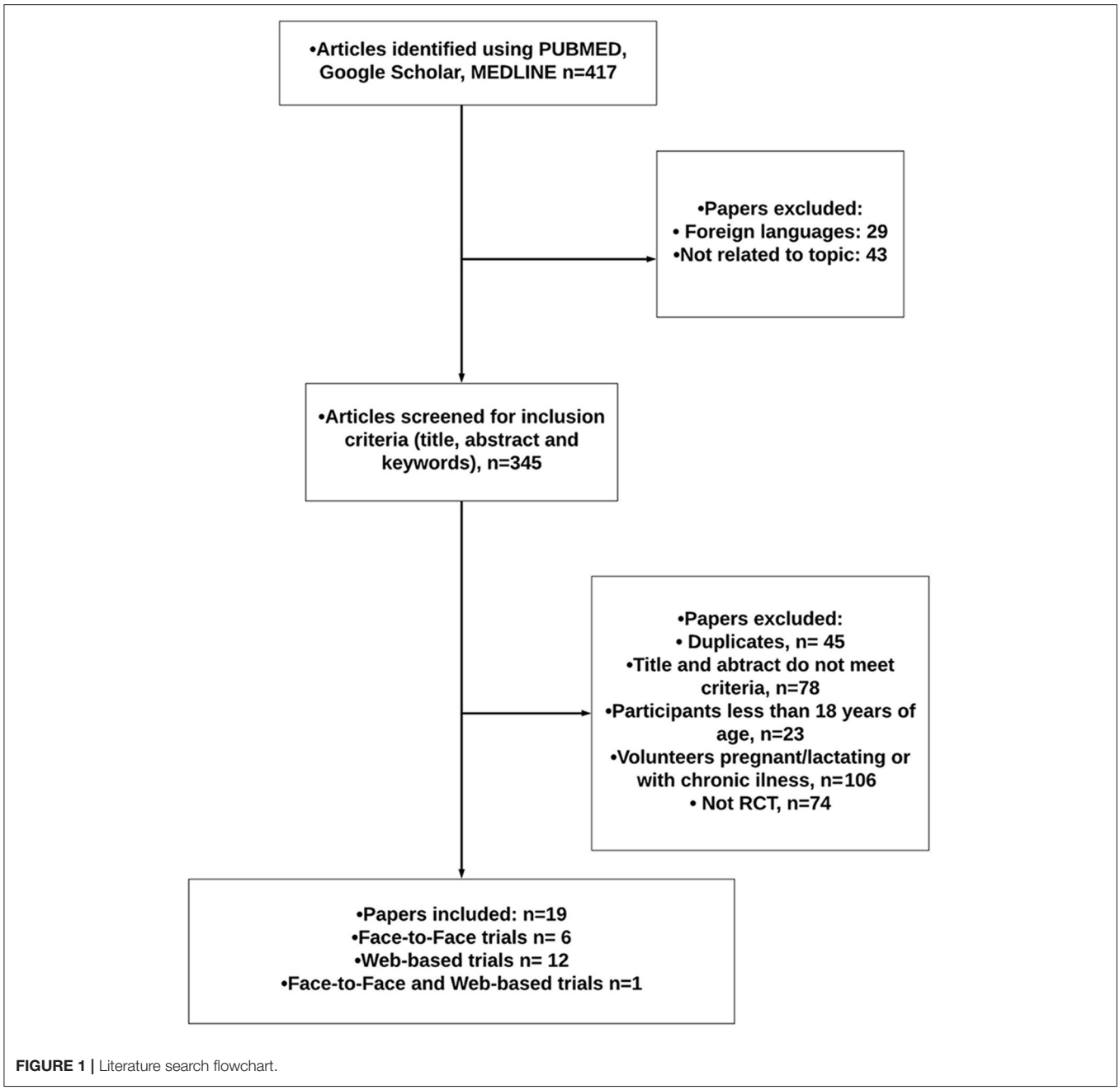

FIGURE 1 | Literature search flowchart.

counseling on a variety of diet-related outcomes (e.g., specific nutrient intake, food groups/items). Four out of six of the face-to-face trials compared PN to generalized nutrition advice. Outcomes were either measured or self-reported (see **Table 1**).

Aldana et al. Baron et al. and Maskarinec et al. all observed beneficial dietary changes following face-to-face intervention, when compared with no treatment controls. In the 6-month trial by Aldana et al. ($n = 348$) participants who received face-to-face lifestyle-modification intervention (40 h of diet and lifestyle group sessions with a registered dietitian) significantly improved F&V servings by 2.3 servings/d and decreased % daily intake of dietary fat by 8.2% with the exception of servings from whole grains and protein (% energy) when compared to baseline levels, and to control group participants (21). Baron et al. examined the effectiveness of a 12-month PN face-to-face vs. group nutrition intervention that aimed at reducing blood lipid levels vs. no treatment control. A total of 368 subjects were randomly allocated to either one of the two dietary intervention groups or a no treatment control group. The intervention groups were given dietary advice by a registered nurse either in a one-to-one or group setting. Following the trial, both intervention groups self-reported increased intakes of fiber by 47%, PUFA by 26%,

TABLE 1 | Face-to-face dietary interventions.

Author	Number (*n*) and study characteristics	Baseline dietary data	Dietary assessment	Outcome(s)	Study findings
Coates et al. (20)	$n = 2,207$ (I, $n = 1324$; C, $n = 883$) 18-month trial comparing the effectiveness of low-fat diets among post-menopausal women from several ethnic origins.	Fat (%): I group 39.74%, C group 39.08% F/V: 3.2 servings/d, C group 3.2 servings/d	FFQ	Fat (%): I group decreased by 13.3 vs. 2.3% in C group at 6 mo. and by −14.17 vs. −2.54% at 18 mo.; F/V: consumption increased by 0.5 serving/d in I group vs. 0.05 serving/d in C group at 6 mo. and by 0.8 serving/d and 0.1 serving/d in C group at 18 mo. Weight change: N/A	I group decreased percentage daily dietary fat intake and increased F&V intakes compared to baseline levels, but this change was non-significant. No change was seen for wholegrain foods.
Aldana et al. (21)	$n = 348$ (Diet, $n = 174$; C, $n = 174$) 6-month trial to determine the impact of a lifestyle-modification intervention receiving counseling compared to controls with no intervention.	Fat %: Diet group 36.7%, C group 34.6% F/V: 4.6 servings/d, C group 5.0 servings/d	FFQ	Fat (%): diet group lowered by 8.2% vs. increase of 1% in C group. F/V: diet group F increased by 0.9 serving/d vs. no change in C group and V increased by 1.4 serving/d vs. 0.1 in C group. Wholegrains: diet increased by 0.7 serving/d vs. decrease of 0.5 serving/d in C group. PA (steps/week): diet increased by 12,372 steps/week vs. 5,661 steps/week in C group. Weight change: Diet group −4.5 kg, C group −0.6 kg	At 6 months, diet group participants experienced significant improvements in all nutrition and PA variables except calories from protein and whole-grain servings ($p < 0.001$).
Maskarinec et al. (22)	$n = 29$ (I, $n = 13$; C, n = 16) 6-month trial examining the effectiveness of increasing fruit and vegetable intakes among healthy women via personalized dietary sessions and group activities.	F/V: I group 3.2 servings/d, C group 3.3 servings/d	FFQ + 3 day DR	F/V: mean consumption increased in the I group by 5.1 serving/d at 3 mo. vs. 0.9 serving/d mean consumption in C group at 6 mo., F/V consumption in I group increased by 4.7 serving/d and C group increased by 0.8 servings/day compared to baseline. Weight change: N/A	Increased average F&V consumption in the I group at 3 and 6 months whereas minimal differences in intakes were found in the C group ($p < 0.001$).
Steptoe et al. (23)	$n = 271$ (behavioral counseling; $n =$ 13; basic counseling, $n = 135$) 12-month trial comparing brief nutrition counseling to behavioral dietary counseling	F/V: Behavioral counseling 3.7 servings/d, basic counseling 3.6 servings/d	FFQ	F/V: increased by 1.5 in behavioral counseling vs. 0.9 in basic counseling (5-a-day % increase) increased by 42% in behavioral counseling vs. 27% in basic counseling group. Weight change: N/A	Increased F&V intake in the behavioral counseling group compared to the basic counseling group at 12-months ($p < 0.021$). % 5-a-day was also significantly higher in the behavioral group compared to the basic group ($p < 0.019$).
Roderick et al. (24)	$n = 956$ (I, $n = 473$; C, $n = 483$) 12-month trial assessing the effectiveness of face-to-face dietary advice to generalized health information on serum cholesterol levels, diet, and weight	Serum Cholesterol: I group 6.0 mmol/, C group 6.2 mmol/l F/V: N/A Fat (%): I group 34.3%, C group 34.2%	FFQ	Serum cholesterol: I group decreased serum cholesterol by 0.20 mmol/l compared to C group 0.04 mmol/l. F/V: I group increased consumption of F by 0.76 serving/week and V by 0.33 serving/week vs. change of 0.28 F serving/week and −0.25 serving/week in C group. Fat (%): I group decreased by −2.4% vs. C group by −0.9%. Weight change: I group −0.1 vs. 0.44 in C group.	I group had lower mean serum cholesterol compared to C group. I group participants reduced their weight and intakes of dietary fat and saturated fat; this difference was not statistically significant.
Carpenter et al. (25)	$n = 98$ (Face-to-face group, $n = 30$; web-based, $n = 33$; C, $n = 35$) 14-week trial to assess the efficacy of group behavioral counseling via	Modified Healthy Eating Index: face-to-face group 61.2, C group 59.0 Fat score:	3 day DR	Modified Healthy Eating Index: face-to-face group increased fruit score by 2.2 vs. a reduction of 0.18 in web-based and 0.54 in C groups. Face-to-face group	Face-to-face group significantly improved scores compared to web-based ($p = 0.04$) and C group ($p = 0.002$).

(Continued)

TABLE 1 | Continued

Author	Number (*n*) and study characteristics	Baseline dietary data	Dietary assessment	Outcome(s)	Study findings
	weekly meetings or correspondence to improve diet quality	Face-to-face group 4.9, C group 4.9		increased fat score by 2 vs. 0.81 in web-based group and 0.39 in C group. Weight change: N/A	
Baron et al. (26)	$n = 368$ (I, $n = 187$; C, $n = 181$) 12-month trial. examining the effectiveness of a dietary intervention that aimed at reducing blood lipid levels	Fiber: I group males 20.4 g/d & females 18.9 g/d, C group males 19.3 g/d & females 16.4 g/d SFA(%): I group males 67% used SFA & females 51%, C group males 47% used SFA & females 55% PUFA(%): I group males 20 and 19% females used PUFA, C group males 26 and 21% of females used PUFA	FFQ	Fiber (%): at 12 mo., I group reported to have increased daily % fiber by 52% male participants and 42% in female participants vs. 1% increase in males and 3% in reported fiber intakes in C group. SFA (%): I group males decreased SFA use % by 55% and females by 38 vs. 5% decrease in C group male participants and 0% fat change in females. PUFA (%): 22% increase in I group male participants and 30% in female vs. 1% in C group participants. Weight change: I group N/A or non-significant, C group –significant, C group nts.ts	I group reported increased intakes of fiber, PUFA and decreased use of saturated fat, minimal changes were reported in the C group. Differences between groups were statistically significant ($p < 0.001$).

N, total number of participants; I, intervention; C, control; F/V, fruits and vegetables; F, fruit; V, vegetables; PUFA, polyunsaturated fatty acids; FFQ, food frequency questionnaire; DR, diet recall.

and decreased intake of SFA by 40%; whereas minimal changes were reported in the no treatment control group and differences between groups were statistically significant (26). Results of the trial by Maskarinec et al. ($n = 29$ women) found that face-to-face personalized dietary counseling targeted towards F&V significantly increased dietary F&V intake (4.7 servings/d), when compared with general written dietary recommendations provided to controls (0.8 servings/d) ($P < 0.001$) (22).

Beneficial impacts of face-to-face interventions targeting dietary fat intake were reported by both Roderick et al. and Coates et al., however the results did not significantly differ compared with the control groups and to baseline values. Roderick et al. compared the impact of face-to-face PN intervention and generalized health information (usual care) on dietary intake. The 12-month study was targeted toward fat-loss (%), cholesterol reduction and changes in weight and a total of 956 participants were randomly assigned to either the intervention or a usual care control group. Following the trial, the intervention group had lower intakes of total dietary fat (2.4%) and lower mean serum cholesterol (0.20 mmol/l) compared to minimal changes in the control group, however these were not statistically significant between the groups and to baseline values (24). The 18-month trial by Coates et al. compared the effectiveness of low-fat diets in post-menopausal women ($n = 208$) that were randomly assigned to a low-fat intervention group or a control group that received paper-form Dietary Guidelines for Americans (27). In this study, participants in the intervention group were required to attend face-to-face group sessions with a nutritionist. At 6 months, the intervention group participants reduced their percentage of daily dietary fat intake (13.3%), but this change was non-significant from baseline. A non-significant increase in F&V intake was also observed in the intervention group (0.5 servings/d). Similar results were seen at 12 and 18 months of the trial (20).

Results of a 12-month trial that compared the impact of PN behavioral counseling to controls, that received brief nutrition counseling on improving F&V intakes, in 271 low-income adults found significantly increased F&V (1.5 servings/d) intakes in the individualized PN group compared to the control group. However, all F&V intake was based on self-report and participants received only two dietary consultations during the whole trial period (at baseline and week 2) that were restricted to 15 min (23).

Outcomes of the face-to-face dietary interventions suggest that in-person dietary advice, provided either individually or in group sessions, improved dietary intakes compared to controls. The majority of the trials have demonstrated significant improvements in number of F/V servings and dietary fat (%) intake in the face-to-face nutrition counseling groups compared to the control groups.

Web-Based Dietary Interventions

Twelve PN web-based trials were identified (6 long term >6 months, 6 short term, 3–16 weeks) that assessed the effectiveness of web-based interventions at improving dietary change (**Table 2**).

Two of the trials examined the effectiveness of the Nutrition for a Lifetime System (NLS) web-based program, with comparable results (29, 38). The NLS is an automated computerized intervention based in supermarkets and provided

TABLE 2 | Web-based dietary interventions.

Author	Number (*n*) and study characteristics	Baseline dietary data	Dietary assessment	Outcome(s)	Study findings
Delichatsios et al. (28)	$n = 298$ (I, $n = 148$; C, $n = 150$) 6-month trial comparing the effectiveness a web-based dietary program that aimed at improving the overall health of individuals.	F intake (servings/d): I group 2.9, C group 2.7 V intake (servings/d): I group 4.1, C group 3.8 Fiber (g/d): I group 22, C group 21	FFQ	F/V: consumption of fruit increased in the I group by 1.1 serving/day compared to C group. No difference for vegetables. Fiber: increased by 4.0 g/d in I group compared to C group. Weight change: N/A	I group significantly increased their fruit from baseline levels compared to C group ($p < 0.05$).
Anderson et al. (29)	$n = 277$ (I, $n = 129$; C, $n = 148$) 6-month trial comparing the impact of a web-based intervention on the food choices made by supermarket shoppers.	Fat (%): I group 33.2%, C group 32.7% F/V(servings/1,000 Kcal): I group 2.8, C group 2.8 Fiber (servings/1,000 Kcal): I group 8.9, C group 8.9	FFQ + Food shopping receipts	Fat (%): decreased by 9% in I group vs. increased by 2% in C group. F/V (%): consumption increased by 20% in I group vs. 2.8% decrease in C group. Fiber (%) 19% in I group vs. 4% decrease in C group. Weight change: N/A	I group decreased their fat intake by 9% ($p < 0.05$) and increased their serving sizes from F&V by 20% and ($p < 0.01$) increased their total fiber intake by 19% ($p < 0.001$). C group increased their total fat intake and had slightly lower fiber intake.
Stevens et al. (30) Celis-Morales et al. (16)	$n = 616$ (I, $n = 308$; C, $n = 308$) 4-month trial to assess the efficacy of a web-based counselling intervention to minimise risk of diet-related cancers. $n = 1,269$ (PN diet, $n = 312$; PN diet + phenotype, $n = 324$; PN diet + phenotype + genotype, $n = 321$; C, $n = 312$) 6-month trial to examine the effectiveness of PN advice on dietary change in comparison to "one size fits all" advice.	Fat (%): I group 33.1, C group 32.2 F/V (Servings/d): I group 5.1, C group 5.0 Red and processed meat (g/d): PN I groups 79.2, C group 74.4 Salt (g/d): PN I groups 7.4, C group 7.3 HEI: PN I groups 49.1, C group 49.5	24 h DR FFQ	Fat (%): I group lowered fat by 2.84 vs. C group increased by 0.48. F/V: I group increased by 0.54 serving/d vs. lowered by 0.51 serving/d in C group. Weight change: N/A Red and processed meat intake decreased by 8.5%, salt intake decreased by 6.3%, energy intake decreased by 4.4% in all three PN intervention group compared to C group. HEI increased by 2.7% in PN intervention groups compared to C group. Weight change: PN I groups −2.8 kg, C group −0.5 kg	At 4-months, I group had significantly increased F/V servings/day ($p < 0.001$) and decreased daily fat % intake significantly compared to C group ($p < 0.009$). At 6-months, PN intervention groups improved intakes of red and processed meats, salt, had lower energy intakes and increased HEI significantly compared to C group ($p < 0.05$).
Brug et al. (31)	$n = 507$ (I, n =178; C, $n = 169$) 6-week trial examining the effect of online personalised nutrition information on fat intake and fruit and vegetable intakes.	F/V (servings/d): I group 2.5, C group 2.6 Fat (fat points/d): I group 29.0, C group 28.0	FFQ	F/V: minimal increase in I group in F intake by 0.008 serving/d and V by 0.04 serving/d and C group decreased F intake by 0.04 serving/d and increased V by 0.06 serving/d. Fat: fat points/d decreased by 2.1 in I group compared to 0.8 in C group. Weight change: N/A	Minimal increase in F&V consumption was found in the I group from baseline levels. Fat intake decreased significantly in the I group ($p < 0.001$) and C group ($p < 0.05$) compared to baseline.
Block et al. (32)	$n = 481$ (I with phone calls, $n = 162$; I without phone calls, $n = 160$; C group, $n = 159$) 8-week trial to assess whether an interactive CD-ROM can enhance the diet of low-income women.	F/V (servings/d): N/A	Diet survey + 24 h DR	F/V: I with phone calls increased by 1.32 serving/d vs. I without phone calls by 1.20 serving/d vs. 0.71 serving/d in C group. Weight change: N/A	After 2 months, both intervention groups significantly increased F/V consumption compared to C group ($p < 0.016$ I with phone calls group, $p < 0.052$ I without Phone calls group).
Alexander et al. (33)	$n = 2,540$ (I1, $n = 848$; I2, $n = 845$; C, $n = 847$) 12-months trial to assess F&V intake by comparing online tailored to non-tailored dietary interventions.	F/V (servings/d): I1 3.3, I2 3.2, C group 3.4	FFQ	F/V: I1 increased by 2 servings/d vs. I2 increased by 2.8 servings/d vs. C increased by 2 servings/d. Weight change: N/A	Average F&V servings increased by more than 2 servings across all study arms ($p < 0.001$). Greatest increase in I2 compared to C group at 12 months ($P = 0.05$).
Irvine et al. (34)	$n = 517$ (I, $n = 260$; C, $n = 257$) 2-month trial comparing the effectiveness of an interactive computer -based program on the dietary intake of individuals.	Fat (DHQ): I group 2.5, C group 3.0 F/V (DHQ): 3.0, C group 3.1	FFQ	Fat, F/V: I group decreased fat DHQ score by 0.5 SD and increased F&V intake by 0.93 SD compared to baseline levels vs. a 0.41 SD decrease in fat score	After 1 month, the I group reduced their fat intake compared to the C group ($p < 0.001$). I group significantly increased F&V consumption

(Continued)

TABLE 2 | Continued

Author	Number (*n*) and study characteristics	Baseline dietary data	Dietary assessment	Outcome(s)	Study findings
				and 0.88 increase in F&V intake in C group. Weight change: N/A	compared to controls ($p < 0.001$). I group maintained these dietary changes after a 60 day-follow up.
Oenema et al. (35) Collins et al. (36)	$n = 782$ (I, $n = 261$; G, $n = 260$; C, $n = 261$) 3-week trial examining effectiveness of a short-term computer tailored nutrition intervention that aimed at decreasing saturated fat intakes and increasing fruit and vegetable intakes and to raise personal dietary awareness. $n = 65$ (I, $n = 34$; C, $n = 31$) 3-month trial examining the effectiveness of the SHED IT web-based intervention on weight loss and dietary change in overweight/obese men.	Fat (points): I group 19.8, G group 20.0, C group 20.3 F/V (servings/d): I group 4.0, G group 3.9, C group 4.0 PSF: I group 1.5, C group 1.5 Fat (%): I group 35%, C group 35% SFA (%):I group 15%, C group 15%	FFQ FFQ	Fat (points): I group decreased by 0.6 vs. 0.8 in G vs. 0.4 in C group. F/V: V intake Increased by 0.1 serving/d in I group vs. lowered by 0.1/serving/d in GI vs. lowered by 0.1 serving/d in C group. Weight change: N/A PSF: decreased in both I and C group to 1.3. Fat (%) I and C group reduced to 32%. SFA I and C group reduced to 13%. Weight change: I group −5.3 kg, C group −3.5 kg	I group significantly increased their awareness of the benefits of consuming a diet high in fruits and vegetables ($p < 0.05$) and low in fat compared to G and C group. Both I group and C group significantly reduced daily energy intakes ($p > 0.001$), percentage energy from fat ($p < 0.05$) and SFA ($p < 0.001$).
Vandelanotte et al. (37)	$n = 771$ (simultaneous group 1, $n = 189$; sequential group 2, $n = 180$; sequential group 3, $n = 204$; C, $n = 194$) 6-month trial comparing the effectiveness of a computer-individualised intervention on dietary fat intake and physical activity	Fat (%): I1 40.8, I2 + I3 38.0, C group 35.3 PA (min/week): I1 532, I2+I3 514, C group 720	FFQ	Fat (%): I1 decreased by 11.5%, I2 + I3 groups by 8.6% compared to 2.1% in C group. PA: increased by 61 min/week in I1 and by 93 min/week in I2 + I3 and by 45 min/week in C group. Weight change: N/A	I1, I2, and I3 groups significantly increased their PA scores ($p < 0.001$), and reduced fat intakes ($p < 0.001$) when compared to C group participants.
Winett et al. (38)	$n = 141$ (I, $n = 54$; C, $n = 51$) 10-week trial examining the effectiveness of a computer-based program "The Nutrition for a Lifetime System" (NLS) that aimed at helping shoppers at supermarkets to decrease intakes of fat and increase intakes of fruits, vegetables and fiber	F/V (servings/d): I group 1.4 C group 1.4 Fat (%): I group 38.4, C group 38.7 Fiber (g/1,000 Kcal): I group 6.2, C group 6.9	Food shopping receipts	F/V, Fat, Fiber: F/V I group increased F/V intake by 0.29 serving/1,000 Kcal compared to −0.12 serving/1,000 Kcal in C group. Fat (%): I group decrease by 3.2% compared to 0.7% increase in C group. Fiber: I group increased by 1.24 g/1,000 Kcal compared to decrease of 0.61 g/1,000 Kcal in C group Weight change: N/A	I group significantly reduced their fat intake and increased their intakes of fiber and F&V compared to C group participants ($p < 0.001$).

N, total number of participants; I, intervention; C, control; I1, intervention 1; I2, intervention 2; I3, intervention 3; G, general nutrition information; F/V, fruits or vegetables; F, fruits; V, vegetables; SD, standard deviation; PA, physical activity; DHQ, diet habits questionnaire; FFQ, food frequency questionnaire; DR, diet recall; HEI, healthy eating index; N/A, not available; PSF, portion size factor.

PN feedback on personal behavior change goals. The first 6-month trial by Anderson et al. compared the impact of the computer-based intervention on the food choices made by supermarket shoppers (29). Participants ($n = 277$) were randomly assigned to either a PN computer-based intervention group that received the NLS web-based program or a no-treatment control group. At 6-months, participants in the intervention group significantly decreased their fat (9%) intake from baseline and significantly increased intakes of F&V (20%) and fiber (19%) (29). Similar findings were achieved in a trial by Winett et al. that examined the effectiveness of the NLS. Participants ($n = 127$) were randomly assigned to either the PN NLS or a control group for 10-weeks and were asked to return supermarket weekly purchase receipts. Participants in the PN NLS intervention completed the NLS computer program weekly and control group participants did not receive dietary advice. Participants in the PN NLS group significantly reduced their fat (3.2%) intake and increased their intakes of fiber (1.2 g/1,000 kcal) and F&V (0.29 g/1,000 kcal) compared to control group participants ($P < 0.001$) (38).

Results of the EU Food4Me 6-month web-based dietary intervention study found that web-based PN, regardless of the level of personalization, was more effective at improving healthy eating, when compared to controls that received standardized web-based dietary advice (16). Participants ($n = 1,269$) were randomized to PN dietary advice, PN dietary advice + phenotype, PN dietary advice + phenotype + genotype or to a generalized dietary advice control group. At 6 months,

participants in the PN groups had significantly lower intakes of red and processed meat (8.5%), salt (6.3%), daily energy intakes (4.4%), and significantly improved their overall Healthy Eating Index (HEI) (2.6%) scores compared to participants in the control group $P < 0.05$ (16).

Results of the 3 other long-term web-based dietary interventions also found that web-based PN interventions can result in significant dietary improvements when compared to a control group (28, 33, 37). Results of a 12-month trial on 2,540 volunteers that compared two PN web-based interventions (PN intervention, PN + motivational e-mail counseling intervention) to a non-personalized intervention control group, indicated that the PN intervention with motivational counseling resulted in significantly greater F&V intakes (2.8 servings/day) when compared to controls (2 servings/day) ($P = 0.05$). All groups increased F&V intakes significantly at the end of the trial compared to baseline values (33). A 6-month dietary intervention study by Delichatsios et al. examined the effectiveness of a web-based PN program that aimed at improving several aspects of diet quality. Adults ($n = 298$) were randomized to either the PN intervention group who received weekly sessions with a computer automated voice program or the control group that received web-based PA information. At 6 months, intervention group participants increased intakes of fruit (1.1 servings/d) and fiber (4.0 g/d) significantly compared to control group ($P < 0.05$) (28). A 6-month trial by Vandelanotte et al. that compared a PN web-based dietary change intervention to a wait-list control, produced similar results. Participants ($n = 771$) were randomly assigned to four groups; the first group received PA and fat intake information at baseline, second group received PA information at baseline and fat intake information at 3 months, group 3 received at baseline the fat intake information and PA information at 3 months or a group 4 wait-list control group. All PN intervention groups significantly increased their PA scores (77 min/week), and reduced fat intakes (10%) when compared to control group participants (37).

Results of the 6 short-term web-based dietary change trials found significant differences between intervention and control groups (30–32, 34–36). A 12-week trial by Brug et al. examined the effectiveness of a PN web-based intervention on total fat and F&V intakes. Participants ($n = 347$) were randomized to the PN intervention group, that received online feedback based on their dietary intakes, or a non-personalized control group that received generalized nutrition related information. Participants in the PN group significantly decreased their fat score by 9% compared to baseline levels and to the control group ($P < 0.01$). However, fruit consumption in the PN group remained similar to baseline (31). Findings from a 12-week SHED-IT intervention on 65 overweight/obese men that assessed dietary, PA and weight loss changes using PN web-based feedback reports were successful at reducing fat and saturated fatty acid intakes. Participants were randomized to a web-based group that received personalized feedback reports on specific dietary areas (sodium, fiber, saturated fatty acids, and calorie intake) or to a control group that were provided with a dietary and PA handbook. Trial results have shown significant improvements in both groups in portion size factor (PSF) (1.3), fat (32%), and SFA (13%) compared to baseline values, however, non-significant differences were found between the groups ($P < 0.05$) (36). A 12-week trial by Irvine et al. evaluated the effectiveness of a PN interactive behavioral change computer-based program on the dietary intake of individuals. Participants ($n = 517$) were randomized to either an intervention or control group. After 1 month, the intervention group significantly reduced their fat intake diet habit questionnaire (DHQ) score (2.27) and increased their DHQ score for F&V intakes (3.36) compared to controls ($P < 0.001$). Furthermore, the intervention group maintained these dietary changes after a 60-day follow up (34). An 8-week-long trial assessed the efficacy of web-based dietary change programs to improve the dietary intake of 481 low-income women. Subjects were allocated to a PN web-based group, PN web-based + phone-calls with researcher group or a non-diet related control group. Results of the trial indicated F&V intakes increased in both groups (1.3 servings/d) compared to controls, which reached borderline significance ($P = 0.05$) (32). Research by Oenema et al. studied the effectiveness of a short-term web-based PN intervention that aimed at improving dietary awareness. A total of 782 subjects were randomly assigned to a PN intervention group or a general nutrition control group or a control group that did not receive any information for a 3-week period. The intervention group significantly increased their awareness of the benefits of consuming a diet high in F&V (0.6 points) and low in fat (0.1 servings/d) compared to the control groups (35). Comparable results were found in a 4-month trial by Stevens et al. that examined the effectiveness of a web-based PN intervention to improve dietary intake compared to controls. A total of 616 women were randomized to either the intervention group that received access to a web-based PN program in addition to two counseling sessions or a control that received non-diet related information. At the end of the trial, the PN intervention group significantly increased F&V intake (0.54 servings/d) and decreased fat intake (2.8%) compared to controls ($P < 0.001$) (30).

Results of these trials suggested that web-based PN dietary advice was effective at enhancing dietary change compared to standardized controls. Outcomes of 10 trials indicated significant improvements in either F/V or % daily fat intake in the groups that received web-based PN dietary advice in comparison to control group participants.

Face-To-Face Compared With Web-Based Dietary Intervention

A 6-month cognitive and behavioral pilot study by Carpenter et al. compared the effectiveness of face-to-face and web-based interventions on dietary change. A total of 98 volunteers were randomized to a face-to-face group, a web-based with personalized email feedback group or a control group. The face-to-face group met with a counselor once/week for the first 16 weeks and biweekly for the remaining 8 weeks. The web-based group received weekly PN emails and had access to a general website about dietary change. The control group also received access to the general website. At the end of the trial, the face-to-face group had significantly increased their modified

healthy index score (2.2%) compared to PN web-based (−0.18%) ($P = 0.04$) and control groups (−0.54%) ($P = 0.02$) (25).

DISCUSSION

The interventions reviewed were difficult to compare as they varied considerably in sample size, duration, study design, and contact with participants. Results of the face-to-face nutrition intervention trials indicated that frequent face-to-face nutrition counseling was effective at achieving and maintaining dietary change in both PN and group face-to-face sessions. These results are in line with a recent systematic review of 26 RCT by Mitchell et al. that assessed the effectiveness of PN dietary consultations in primary health care, out of which 18 trials demonstrated significant improvements in either anthropometric outcomes (including weight change) or dietary change including increased fiber, calcium, improvements in salt, and reduced fat intakes compared to comparator groups (39). However, a number of limitations were found in the reviewed trials which included small sample size for long-term trials (25, 36), low numbers of participants in intervention groups which may have underpowered study outcomes (36), control groups receiving no intervention during trial period (21, 26), low adherence rates and high attrition rates (28).

When it came to the delivery of web-based dietary advice, results of the reviewed trials have indicated that PN web-based dietary advice is more effective at improving dietary change, especially consumption of F&V compared to generalized controls. This finding was supported further in a systematic review and meta-analysis of 13 RCT by Celis-Morales et al. that assessed the effectiveness of web-based dietary interventions at enhancing F&V intakes. Results of the systematic review have suggested that personalized web-based nutrition interventions were more effective at improving F&V intakes compared to non-personalized interventions (40).

A number of limitations of the present review should be acknowledged. Firstly, there were differences in the trial designs and a few studies lacked a description of what the control group received during the intervention; this highlights the importance of detailed trial reporting. Moreover, during the review process, it was difficult to determine whether some of the trials were conducted over the web (using a computer) or delivered on a computer-based application as there were no clear definitions used. The lack of detailed descriptions of the type of face-to-face dietary counseling provided e.g., consultation with a health practitioner was a limitation as well as minimal information about the type of usual care provided to control group participants in several trials. It was also difficult to compare the overall effect between the trials as they differed in design. In addition, the majority of the trials have used FFQs to collect dietary data which are subject to recall report bias as they require participants to report dietary intakes over previous weeks or months. A further limitation is the lack of weight-loss data in most of the trials which would have added further data on the effects of dietary change on health outcomes. Moreover, the male:female ratio was not equal as most studies were carried out in women, calling for future trials to target men. As all of the included trials were diet related and based on self-report, this may question the validity of the dietary intake information provided and outcome measures.

Moreover, limited work has focused on comparing the delivery of PN face-to-face and PN web-based dietary interventions, and more comparative trials are needed to demonstrate the strengths and weaknesses of each strategy. Face-to-face trials that targeted dietary change in specific population groups were successful at achieving dietary change; however, face-to-face consultation is costly and is not generally available to the public (20, 41, 42).

CONCLUSION

Findings from web-based nutrition interventions and their impact on dietary change suggest that personalized/enhanced web-based nutrition interventions may be successful at inducing short-term dietary change compared to non-personalized dietary interventions. Although face-to-face nutrition interventions were generally successful at enhancing dietary change, those targeting dietary fat yielded inconsistent results which may indicate the need for further long-term research. There still remains insufficient evidence to suggest that web-based PN interventions are as effective as PN face-to-face interventions, therefore, further controlled comparative studies and cost-benefit analysis are needed.

AUTHOR CONTRIBUTIONS

BA-A conducted the review of the literature and wrote the manuscript. RF and JL contributed to the drafting of the manuscript. All authors made a critical review of the draft.

FUNDING

BA-A received funding from the Public Authority of Applied Education and Training, Kuwait.

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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.

frontiers in Nutrition

METHODS
published: 02 February 2021
doi: 10.3389/fnut.2020.608854

A Study Protocol for a Parallel-Designed Trial Evaluating the Impact of Plant-Based Diets in Comparison to Animal-Based Diets on Health Status and Prevention of Non-communicable Diseases—The Nutritional Evaluation (NuEva) Study

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OPEN ACCESS

Edited by:
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Reviewed by:
Costas A. Anastasiou, Harokopio University, Greece
Emilia Vassilopoulou, International Hellenic University, Greece

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Specialty section:
This article was submitted to Nutrition and Metabolism, a section of the journal Frontiers in Nutrition

Received: *21 September 2020*
Accepted: *29 December 2020*
Published: *02 February 2021*

Citation:
Dawczynski C (2021) A Study Protocol for a Parallel-Designed Trial Evaluating the Impact of Plant-Based Diets in Comparison to Animal-Based Diets on Health Status and Prevention of Non-communicable Diseases—The Nutritional Evaluation (NuEva) Study. Front. Nutr. 7:608854. doi: 10.3389/fnut.2020.608854

Background and Aims: Currently, there is a continuing upward trend for plant-based lifestyles in Germany and Europe. The implementation of vegetarian and vegan lifestyles is characterized by omitting defined food groups such as fish, meat, sausage (vegetarians), or dairy products and honey (vegans). This carries the risk of an undersupply of valuable nutrients. The NuEva study is designed to examine this hypothesis and to evaluate the impact of plant-based diets on health status and disease risk.

Methods: The NuEva study is a parallel-designed trial with at least 55 participants for each diet (vegetarian, vegan, flexitarian [rare meat/sausage consumption, once or twice per week]), and participants who consume a traditional Western diet as the control group. In the screening period critical nutrients are identified for the studied diets by analysis of a broad spectrum of nutrients in the human samples (fatty acids, vitamins, minerals, trace elements, nutrient metabolites).

Results: Based on the data from the screening period, defined menu plans, ensuring an adequate nutrient intake in accordance with the nutritional guidelines are prepared for each group. The plans are adapted and personalized to individual energy requirements based on the basal metabolic rate and physical activity level. The compliance with the NuEva concept and their impact on nutrient status and cardiovascular risk factors are validated during the intervention period of the NuEva study over 1 year. To investigate the impact of the studied diets on the microbiome, feces samples are collected at the beginning and after the 12 months intervention period (follow up: 12 months).

Conclusion: The NuEva study is designed to investigate the impact of common diets on health and disease status, with focus on prevention of cardiovascular diseases.

In addition, the effectiveness of the prepared nutritional coaching strategy, ensuring optimal nutrient intake in accordance with the guidelines, is validated during the intervention period of the NuEva study.

Clinical Trial Registration: Registered under ClinicalTrials.gov Identifier no. NCT03582020.

Keywords: nutrients, vegetarians, vegans, western diet, nutritional concepts

IMPORTANCE

Currently, there is a continuing upward trend for vegetarian and vegan lifestyles. The Vegetarierbund Deutschland e.V.[1] postulates that in 2015, 7.8 million Germans were vegetarians and 900,000 were vegan, whereby about 2,000 vegetarians and 200 vegans join the community in Germany every day. This is also a global trend. It has been estimated, that worldwide, about 1 billion people have adopted a vegetarian-vegan lifestyle.

The implementation of the vegetarian-vegan lifestyle is characterized by omitting defined food groups such as fish, meat, sausage (vegetarians), or additionally dairy products, and honey (vegans). This bears the risk of undersupply of valuable nutrients. Critical aspects of the vegetarian-vegan lifestyle are low intakes of vitamin B12, vitamin D, long-chain omega-3 fatty acids (n-3 LC-PUFA), calcium, iron, zinc, as well as a high phytate intake (1). On the other hand, a vegetarian/vegan diet, usually rich in fruits, vegetables, whole-grains, legumes, nuts, and various soy products, is characterized by a low intake of saturated fat and cholesterol and a high intake of dietary fiber, carotenoids, vitamins and health-promoting phytochemicals (2, 3). Vegan and vegetarian diets are associated with cardiovascular and other health benefits, but little is known about the associations of critical nutrients in plant-based diets and the resulting effects on brain function and mental health.

The Western diet, which is the major nutritional style in Germany, is characterized by high intake of energy, saturated fat, salt, and simple/added sugar and, on the other side, a comparably low intake of vegetables and fruits, resulting in low intake of dietary fibers, PUFA and secondary plant compounds[2] (4). Obviously, these dietary habits are associated with an increase of cardiovascular risk factors (5, 6).

RATIONALE OF THE NuEva STUDY

The NuEva study aims to reveal the impact of chosen nutritional habits (Western diet, flexitarians, vegetarians, vegans) on health status and disease risk with respect to physiological benefits or possible pathophysiological consequences, resulting from long-term implementation of the studied diets.

In this context the central issues of the NuEva study are:

i) Is it possible to ensure an adequate intake of all essential nutrients?
ii) Are land-based n-3 PUFA from linseed oil a suitable source to ensure an adequate status of n-3 LC-PUFA?
iii) What impact has each of the studied diets on state of health and disease and, in particular, on cardiovascular risk?

As the Western diet is the main nutritional style in Germany, it is used as an appropriate comparator for evaluating the physiological impact, resulting from alternative diets, e.g., the vegetarian, vegan and flexitarian diet. There are inherent risks, in both the rising popularity of veganism and vegetarianism and the prevalence of the Western diet because of the likelihood of over- and undersupply of valuable nutrients. This highlights the need of an extensive data collection to serve recommendations, that are based on scientific evidence.

METHODS AND ANALYSIS

Study Design

For the NuEva study, at least 55 participants for each diet (vegans, vegetarians, flexitarians, and participants who consume a traditional Western diet) are recruited in summer/autumn 2018 according to the following inclusion and exclusion criteria (**Table 1**). The 2-year trial is carried out in central Germany (recruiting area: central East Germany: Jena-Halle-Leipzig). The allocation ratio is 1:1:1:1. The long-term trial in parallel design consists of four study periods (**Figure 1**). The Follow-up period ends in December 2020 (optional: winter 2021).

Run-in Period—Assessment of Dietary Habits and Lifestyle

To record and document the variety in dietary practices within and among each group, the run-in phase of the NuEva study includes full self-reports of the individual dietary habits using a food frequency protocol (FFP) which had to be maintained over 5 days (**Figure 1**). The FFP consists of a list of foods that are normally consumed in the Western diet and the corresponding portion information. The participants have to mark the consumption of the listed foods with a line. Food and beverages that are not listed can be added. The FFP based on the template "Freiburger Ernährungsprotokoll" which was provided by the software package PRODI®. The template was adapted on the NuEva study by adding plant-based foods, e.g., tofu, seitan, tempeh, legumes. The daily nutrient intake was calculated by the software package PRODI®. The protocol to record physical activity was adapted to a template which is provided by the German Institute of Nutrition Research (DIfE) (7).

[1] Available online at: https://vebu.de/veggie-fakten/entwicklung-in-zahlen/vegan-trend-fakten-zum-veggie-+/

[2] Available online at: https://www.bmel.de/SharedDocs/Downloads/Ernaehrung/NVS_ErgebnisberichtTeil2.pdf?__blob=publicationFile

TABLE 1 | Inclusion and exclusion criteria in the NuEva study.

INCLUSION CRITERIA

- Healthy volunteers
- Female (pre-menopause) and male gender
- Age: 18 to < 65 years
- BMI < 30 kg/m^2
- Adherence to one of the four diets (Western diet, flexitarians, vegetarians, vegans) confirmed by lifestyle and food questionnaires (FFPs over 5d, run-in)
- Precondition: stable eating habits for at least 1 year before enrolment

EXCLUSION CRITERIA

- Patients with any acute or chronic disease (tumor, infection, other), gastrointestinal disease, diabetes mellitus (type I, II), chronic renal disease, diseases of the parathyroid, diseases necessitating regular phlebotomies, or other chronic diseases, which could affect the results of the present study
- Use of prescription medicine, which could affect results of the study, including systemic glucocorticoids, and lipid-lowering agents
- Dependency on alcohol or drugs
- Simultaneous participation in other clinical studies
- Inability (physically or psychologically) to comply with the procedures required by the protocol
- Changes in eating habits

Treatments precluding participation (at least 3 months prior to study start) and/or during the trial:

- Weight loss or weight gain (> 3 kg)
- Fundamental changes in dietary habits (precondition: stable eating habits for at least 1 year before enrolment)
- Hormone replacement therapy
- Elite athletes (>15 h of strenuous physical activity per week)
- Pregnancy or lactation
- Transfusion of blood in the last 3 months before blood sample taking

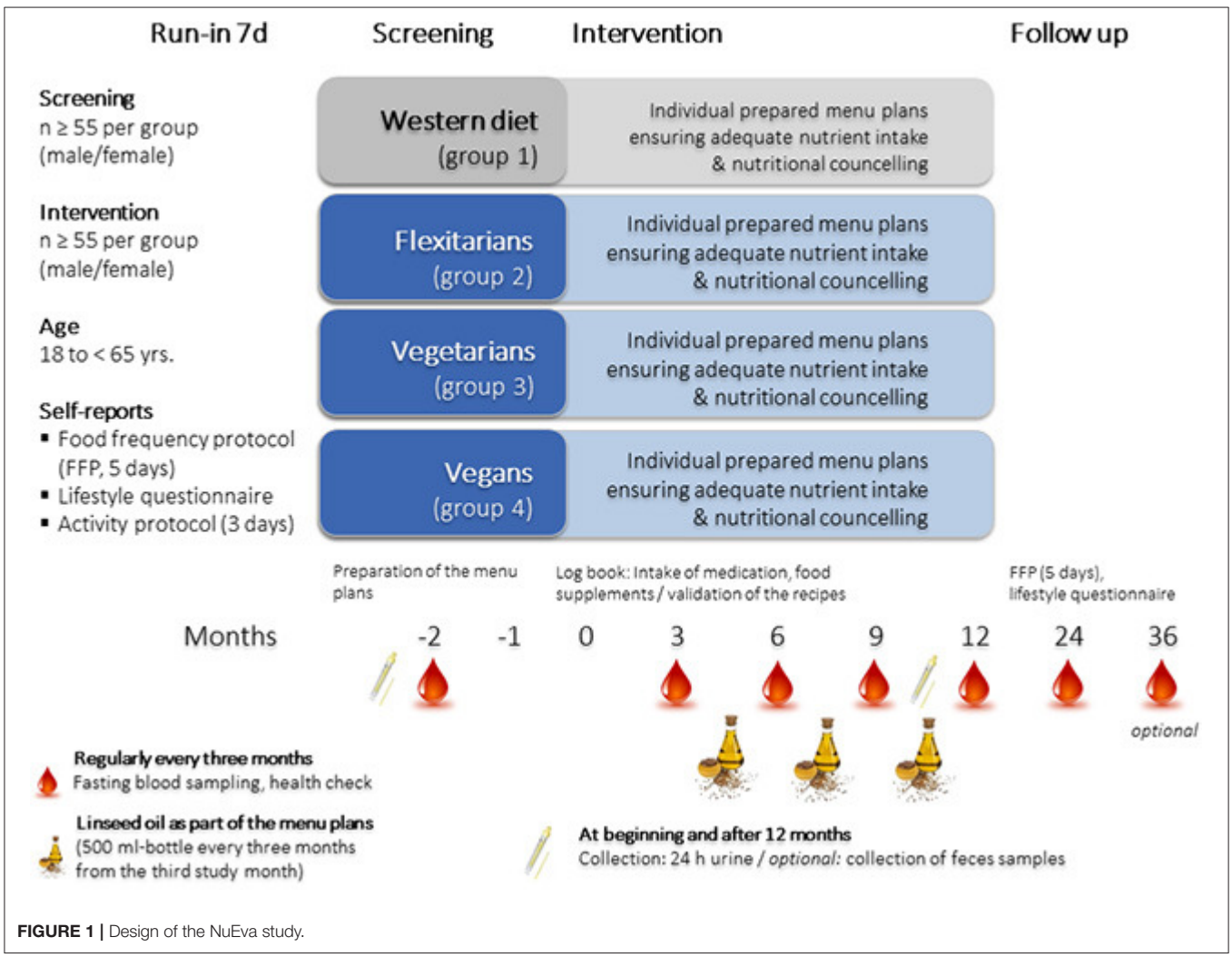

FIGURE 1 | Design of the NuEva study.

NuEva participants kept the activity log for 3 days (pre-condition: 1 day on the weekend). To document medical history, an appropriate questionnaire was developed for the Nueva study. This questionnaire based on a comparable questionnaire available from the LURIC study (8). Available questionnaires from the German National Consumption Survey II (NVS II) and the German health interview and examination survey for adults (DEGS1) are used to consider also the socio-economic status as a confounding factor (9). In detail, the questionnaire include a set of questions about marital status, household size, educational achievement, income, and occupation as well as employment status. To evaluate differences on the occurrence of depressive symptoms between the studied dietary habits, the Patient Health Questionnaire-9 and the Patient Health Questionnaire-15 are used (10, 11).

Screening Period—Assessment of Nutrient Status and Health Status

Blood samples, 24 h urine and a fecal sample are collected to examine nutrient status and chosen risk factors for CVD and type-2 diabetes mellitus, parameters reflecting thyroid function and bone health, and inflammatory markers (**Figure 1**; **Table 2**). In addition, a health check is conducted, which includes measurement of weight, height, waist circumference, blood pressure, basal metabolic rate and bioelectrical impedance analysis (BIA) according to standard procedures (**Table 2**).

Intervention Period—Validation of the NuEva Concept

Based on the screening data and published scientific data, critical nutrients, whose intakes are too low or too high in comparison to the recommendations, are identified. These weak points are addressed by the personalized nutritional concepts which are prepared for each diet. The compliance with the nutritional concepts and its physiological impact on health and disease status are studied by regular analyses of the study parameters (**Table 2**). Therefore, blood samples are taken every 3 months (five times), and 24 h urine is collected at the beginning and at the end of the intervention period (**Figure 1**). Fecal samples are also collected at the beginning and after 12 months of the NuEva study to evaluate the impact of the studied diets on the composition of the gut microbiome (**Figure 1**).

Follow-Up

After the implementation of the NuEva concept (intervention period), a 12-months period (or optional 24-months period) without nutritional coaching follows (**Figure 1**).

The Nutritional Coaching Concept

The NuEva coaching concept is developed to improve nutritional behavior based on three columns: (1). personalized menu plans that are adapted to individual needs and requirements, (2). regular nutritional counseling units including feedback, and tracking, and (3). various incentive strategies (**Figure 2**).

Menu Plans

Participants of the NuEva study receive daily menu plans with optimized nutrient profiles over a period of 1 year. In total, participants receive 20 menu plans for each season (autumn, winter, spring, summer) as part of the nutritional counseling units every 12 weeks. The menu plans are validated by regular fasting blood sampling and health checks every 3 months (**Figure 1**). The regularly analysis of the study parameters, e.g., fatty acid distribution in plasma and erythrocyte lipids allows an evaluation of the compliance with the menu plans as the characteristic fatty acid profiles of the menu plans will be reflected by changes on fatty acid distribution in plasma and erythrocyte lipids (12).

Based on the analysis of the nutrient status in the screening period, critical nutrients are identified for each participant and summarized for each group. Based on this and published scientific data, menu plans and recommendations are prepared for each group and personalized for each participant, ensuring adequate nutrient intake. The plans are adapted to individual energy requirements based on the basal metabolic rate and the physical activity level (PAL).

The menu plans are in accordance with the following hallmarks of the studied diet forms:

Western diet: daily consumption of meat, sausage or fish
Flexitarians: rare consumption of meat, sausage or fish (predominantly high-quality products), $\leq$ 2 times/week
Ovo-lacto vegetarians: no consumption of meat, sausage or fish
Vegans: no consumption of foods from animal origin

The NuEva menu plans of all four groups are characterized by:

1. Adequate amounts of energy, carbohydrates, protein and fat in accordance with the guidelines of the German Society of Nutrition (13)
2. Defined intake of saturated fatty acids (SFA, $<$ 10% of daily energy), monounsaturated fatty acids (MUFA, $>$ 10% of daily energy), PUFA ($\sim$10% of daily energy) and at least two grams of alpha-linolenic acid per day, e.g., through linseed oil (5–10 ml per day between study month 3 and 12; **Figure 1**).
3. Encouraged consumption of vegetables, fruits, cereals
4. Intake of $>$ 40 g dietary fiber per day based on the MoKaRi concept (14)
5. Salt (maximum of 6 g per day) and sugar reduction (maximum of 50 g per day)
6. Reduced intake of highly processed, calorie-rich, low-nutrient foods (fast foods, convenience products)
7. Optimized intake of vitamins, minerals, and trace elements by commercially available foods, considering seasonal availability of vegetables and fruits.

The daily recipes and menu plans are adapted to the individual energy requirement depending on age, gender, basal metabolic rate, and physical activity. Therefore, the menu plans are prepared in eight energy levels, including detailed information about the type and amount of food for breakfast, morning snack, lunch, afternoon snack and dinner. For each meal, a well-designed step-by-step description for preparation is provided (**Figure 3**).

The software package PRODI® (Nutri-Science, Stuttgart, Germany, version 6.4) for professional dietary counseling and therapy was used to calculate the nutrient content of the menu

TABLE 2 | Primary and secondary outcome measures in the NuEva study.

Primary outcome measure **(plasma, five times plus follow-up)**

- Low density lipoprotein (LDL)/high density lipoprotein (HDL) ratio

Secondary measures **(plasma/serum, five times plus follow-up)**

- Total cholesterol, triacylglycerides, lipoprotein (a), apolipoprotein AI, B
- Anthropometric and physiological data (height, weight, waist circumference, blood pressure, bioelectric impedance measurement)
- Vitamins (A, D, E, B1, B2, B6, folic acid, B12) and vitamin B12 status marker (holotranscobalamin, methylmalonic acid, homocysteine)
- Minerals [calcium, sodium, potassium, iron, and iron status markers (ferritin, transferrin)]
- Trace elements (copper, zinc, manganese, total selenium and selenoprotein P, glutathione peroxidase (GPx) activity)
- Fatty acid distribution in plasma lipids and erythrocyte lipids (> 90 fatty acids)
- Additional cardiovascular risk factors (high sensitive c-reactive protein, uric acid)
- Diabetes risk factors [fasting glucose, fasting insulin, hemoglobin A1c (HbA1c)]
- Clotting markers (alpha prothrombin time, fibrinogen, international normalized ratio, quick value) and blood count
- Cholesterol synthesis markers/cholesterol resorption markers
- Bone turnover (bone-specific alkaline phosphatase, osteocalcin, β-crosslaps (CTX), N-terminal propeptide of type I procollagen (PINP), parathyroid hormone)
- Thyroid function [thyroid-stimulating hormone, free thyroxine (T4), free triiodothyronine (T3)]
- Optional: targeted metabolic profiling (AbsoluteIDQ p180 Kit from Biocrates), inclusive unbound free fatty acid profiles in plasma

Secondary measures **(24 h urine, at the beginning and after 12 months)**

- Albumin, creatinine
- Sodium, magnesium, zinc, selenium, iodine
- Heavy metals (arsenic, lead, cadmium, mercury)

Secondary measures **(feces collection, at the beginning and after 12 months)**

- Distribution of the gut microbiome

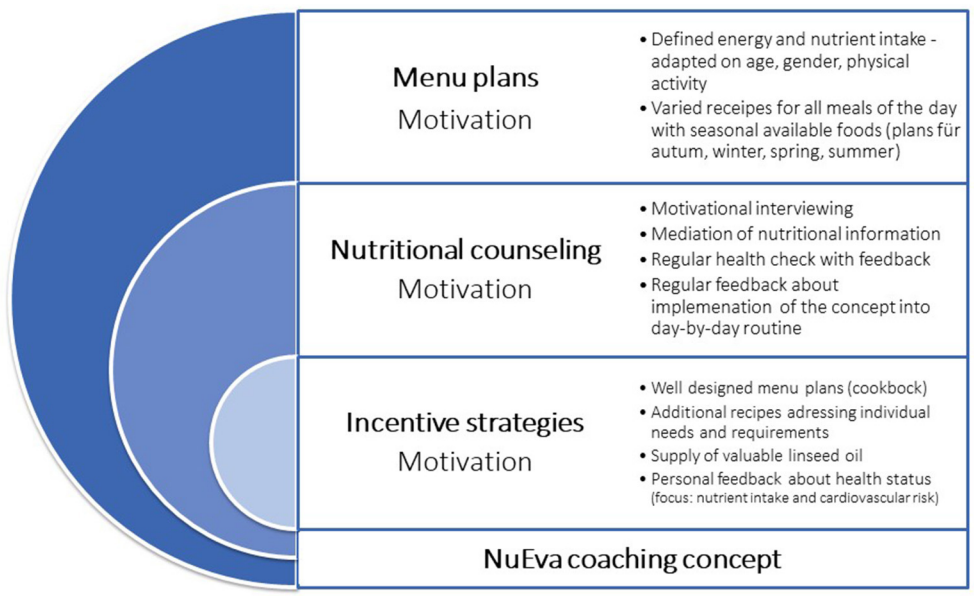

FIGURE 2 | The NuEva coaching concept.

plans. The recipes for each meal originated from cookbooks. The uniqueness of the menu plans comes from their balanced nutrient profile which considers the individual requirement of each participant. Thus, selected nutrient-rich foods were added to conventional recipes in order to meet the NuEva criteria for daily nutrient intake. For example, fatty cold water fishes are selected to ensure adequate intake of n-3 LC-PUFA, vitamin B12, vitamin D, iodine, and protein for participants who follow the flexitarian diet or the Western diet. Dairy products with moderate fat content are included to help flexitarians, vegetarians and adherents of the Western diet to reach the recommended intake of B vitamins, calcium and other minerals but also high-value protein. Linseed, rapeseed and olive oils, mixed nuts and seeds are added to meet the criteria for fat quality in all diets. Vegetables, chosen fruits, nuts and seeds, cereals, cacao and pulses, such as peas, beans, or lentils are added to ensure optimal intake of vitamins, minerals, trace elements, dietary fibers, protein and secondary plant compounds, e.g., carotenoids and polyphenols (all groups). For vegans special foods, enriched with vitamin B12 and calcium but also the recommendation to supplement vitamin B12 are included to reach the recommended intake of these valuable

nutrients. The daily menu plans are provided as well-designed cookbooks with one page for each day on wipeable paper (**Figure 3**).

Lifestyle Coaching—Focus: Nutritional Habits

The regular nutritional counseling units are implemented by using the Motivational Interview technique, based on the transtheoretical model (15–18). In detail, the screening period of the NuEva study starts with participants in the contemplation ("getting ready") stage. The participants are interested in nutritional facts and wanted to be informed about the pros and cons of their previous nutritional patterns. If they decide to participate in the intervention period, they continue to the next level, called preparation ("ready") stage. From this point, participants are intending to take action in the immediate future and may begin taking small steps toward changing their nutritional behavior. During the first 6 months of the nutritional coaching program (intervention period), the participants move up to the action level ("current action"). This stage is characterized by changes in their nutritional behavior by implementing the personalized menu plans. In the further course of the intervention period, the participants maintain the learned nutritional behavior by continuous usage of the menu plans (maintenance stage "monitoring"). After the intervention period, participants hopefully get into the termination stage. On this level, subjects will continue the learned healthy eating habits to ensure an adequate nutrient intake, depending on the characteristics of their individual diet.

The nutritional counseling concept includes the following hallmarks (**Figure 2**):

1. Supply of 80 personalized menu plans, including practical hints to facilitate implementation into the day-to-day routine
2. Providing nutritional facts on the following topics: energy requirement, physiological impact of chosen nutrients (e.g., fats, dietary fibers, sugar, salt) and nutrient-dense foods such as vegetables and fruits, dairy products or plant-based alternatives, and beverages
3. Individual feedback about the course of the study parameters which reflect state of health (**Table 2**)
4. Defining individual goals depending on the course of the study parameters
5. Exchange about the feasibility of the NuEva concept in the day-by-day routine.

Besides the supply of the personalized menu plans, regular talks and feedback loops are important hallmarks of the NuEva concept (**Figure 2**). Through them, personal information about life circumstances, individual needs and possible barriers of implementing the NuEva concept are discussed. Based on this, individual solutions to improve compliance are sought. The talks are conducted in a user-friendly environment to improve the well-being of the participants.

Outcome Measures

The NuEva study aims to investigate the impact of the four studied diets on health status and established risk factors for non-communicable diseases (**Table 2**). The analyses are conducted every 3 months over 1 year (plus follow-up after further 12 [optional 24] months; **Figure 1**).

STATISTICAL METHODS

Data management will be conducted using the statistical software system R version 3.5.0. The design allows the comparison of the study outcomes between the studied diets (screening) and the course of the study parameters over the intervention and follow-up periods, respectively. Descriptive statistics will be used to describe each stage of the study (run-in, screening, intervention, and follow-up). Datasets from the screening period will be analyzed using (generalized) linear models, e.g., analysis of covariance (ANCOVA). The endpoints will be adjusted for potential confounding factors, including, but not limited to, gender, age, and BMI. The datasets from the intervention and the follow-up periods will be tested using a repeated measurement analysis with a significance level of 5%. Previously, data will be tested for normal distribution and homogeneity of variances, and adjustments for multiple testing will also be considered.

The power calculation for the NuEva study based on a cross-sectional study which was designed to compare physiological markers and cardiovascular risk markers, such as blood lipids between habitual meat-eaters and habitual vegetarians (19). One hundred and thirty-nine healthy male subjects (vegans: $n = 18$, ovo-lacto vegetarians: $n = 43$, moderate meat eaters: $n = 60$, and high = meat eaters: $n = 18$) aged 20–55 years were recruited in Melbourne. Based on this study, meat eaters had a significantly higher cluster of cardiovascular risk factors compared with vegetarians, including increased body mass index, waist to hip ratio, plasma total cholesterol, LDL cholesterol, and triacylglycerol levels, ratio of total cholesterol/HDL and LDL/HDL, and plasma factor VII activity.

Li et al. (19) revealed a LDL/HDL ratio of 2.89 ± 0.91 in the group "high meat" (corresponding to the NuEva group: Western diet) and a significant lower LDL/HDL ratio of 2.25 ± 0.71 in the group "vegan" (corresponding to the NuEva group: vegan). Based on this data, a sample size of 44 per group had 80% power to achieve a difference of 0.6 (difference between $\mu 1 = 2.89$, $\mu 2 = 2.25$), assuming that the standard deviation is 0.9 (using a 2-side *t*-test with 0.05 as significance level; Power calculation by G*Power 3.1.9.2). Because of the complex study design and the long-term interval of the NuEva study, a dropout rate of 25% per group was assumed. Thus, at least 55 participants per group (n total = 220) were enrolled in the NuEva study (**Figure 1**).

DISCUSSION

Cardiovascular diseases are the leading cause of death worldwide, and the rising upward trend from 12.3 million in the year 1990 to more than 17.6 million in 2016 is alarming (20). Several risk factors contribute to an earlier onset and accelerated progression of CVD. While gender, age, and genetic predisposition are beyond control, most risk factors result from lifestyle. The Global Burden of Disease Study stated that more than 9.1 million premature deaths from CVD worldwide are attributable to dietary risks, which equals 52% of all CVD-related deaths

Autumn day 1

Ingredients for one person (one portion)

			Breakfast
3	slices	150 gram	rye bread
2	teaspoon	10 gram	jam
5	teaspoon	25 gram	peanut butter
0.5		65 gram	banana

			Snack
		250 gram	yoghurt (plant-based, Alpro®)
3	teaspoon	15 gram	cacao powder (unsweetened)
1	teaspoon	5 gram	linseed oil
6	teaspoon	30 gram	amaranth (puffed)

			Lunch
			roasted autumn salad

			Snack
1	piece	110 gram	banana cake
		250 milliliter	Drink, plant-based (Alpro®)

			Dinner
			chick pea curry

Preparation - Breakfast

Spread a slice of bread with peanut cream and place sliced banana on the bread.

Roasted autumn salad

Ingredients for one person (one portion)

125	gram		beetroot (raw)	2	teaspoon		olive oil
125	gram		pumpkin (Hokkaido)			*Dressing*	
125	gram		Brussels sprouts	1	tablespoon		balsamic vinegar
0.5			onion	50	milliliter		vegetable stock
			iodized salt, pepper	1	teaspoon		maple syrup
			rosemary	1	tablespoon		parsley

Preparation - Lunch

Pre heat the oven to 180 degrees Celsius. Peel and quarter the beetroot. Brush and quarter Brussels sprouts. Peel and dice the onion. Wash the pumpkin, remove the seeds and dice the pumpkin. Put beetroot, Brussels sprouts, onion and pumpkin on a baking sheet and mix with salt, pepper, rosemary

Figure 3 | Continued.

and oil. Cook the vegetables in the oven on a medium rack for 20-25 minutes and allow cooling for about 30 minutes. Make a dressing from vinegar, broth, salt, pepper, and sweetener.

Chickpea curry

Ingredients for one person (one portion)

1		carrot
0.5		onion
1	teaspoon	ginger
0.5		clove of garlic
2	teaspoon	rapeseed oil
		curry powder
		iodized salt, pepper
200	gram	canned tomato
5	teaspoon	water
250	gram	chickpeas

Preparation - Dinner

Peel carrot and cut diagonally into 1 cm thick slices. Finely chop the onion, thinly peel ginger. Finely chop ginger and garlic.
Heat the oil in a saucepan; fry the onions in a medium heat for 2 minutes. Add carrots and fry with occasional stirring until the onions are glassy. Fry ginger and garlic for a short time. Dust the turkey powder and fry briefly, season with salt and pepper.
Add pizza tomato and water; bring to the boil and cook over medium heat for 12-15 minutes. Rinse chickpeas cold in a colander and drain. Add to the curry and heat.

Banana cake

Ingredients for eight portions

Crush the peeled bananas with a fork. Add margarine, sugar, salt and mix.
Mix the flour with baking powder and add to the moist ingredients. Mix everything well and then put in a box shape. Bake at 180 ° C for 45 minutes to 1 hour.

4		bananas
80	gram	margarine
1	teaspoon	linseeds
1	teaspoon	baking powder
		iodized salt
120	gram	sugar
150	gram	spelled flour type 630

Figure 3 | Example for a menu plan (vegan). Menu plan for a woman (25 ≤ 51 years), with a physical activity level (PAL) of 1.6 which corresponds to a daily energy intake of 2,100 kcal (13).

in the year 2016 (20). The traditional Western Diet is marked by high intakes of calories, salt, saturated fat, and simple sugar, while consumption of MUFA and PUFA, whole grain fibers, fish, vegetables, fruits, vitamin D, or potassium is inadequate (21). In addition, unfavorable developments such as (i) a lack of the natural sense of appetite, hunger or satiety in combination with (ii) an oversupply of food, and (iii) the lack of knowledge about relations between food intake and health risks are marking the Western lifestyle and may contribute to the increased cardiovascular risk.

On the other hand, a reduction of animal products in the daily diet correlates with a reduction of cardiovascular risk factors. The *Dietary Approaches to Stop Hypertension (DASH)* emphasizes the consumption of fruits, vegetables, fat-free/low-fat dairy, whole grains, nuts, and legumes and limits saturated fat, cholesterol, red and processed meats, sweets, and added sugars, salt and sugar-sweetened beverages (22). This dietary pattern is associated with decreased incidence of CVD [RR, 0.80 (0.76–0.85)] and diabetes [RR, 0.82 (0.74–0.92)] in prospective cohort studies (n = 942,140). Besides, the implementation of the *DASH* diet improves blood pressure, total cholesterol, LDL cholesterol, HbA1c, fasting blood insulin, and body weight in controlled trials (n = 4,414). Thus, the *DASH diet* is widely recommended by international diabetes and heart association guidelines. Comparable results are found for the *Portfolio diet*, a plant-based dietary pattern rich in nuts, plant protein, viscous fiber and plant sterols (23).

In accordance, vegetarians have lower BMI, blood pressure, serum total cholesterol and LDL cholesterol concentrations than non-vegetarians (3). In addition, reduced rates of death from ischemic heart disease, and lower incidence of hypertension, stroke, type-2 diabetes mellitus, and certain cancers are described. Viguiliouk et al. (24) also reported that vegetarian dietary patterns improve glycemic control, LDL cholesterol, non-HDL cholesterol, and body weight (nine trials, n = 664). Glenn et al. (25) confirmed the favorable effects of vegetarian dietary patterns on cardio-metabolic risk factors. They included seven prospective cohort studies (197,737 participants, 8,430 events). This meta-analysis indicates, that vegetarian dietary patterns are associated with reductions in CHD mortality (∼22%) and incidence (∼28%) but not with CVD and stroke mortality in individuals with or without diabetes.

In summary, current data indicate an inverse association between consumption of plant-based diets and cardiovascular risk factors and CHD risk, respectively. The reduction in animal products in the daily diet is associated with a decrease in energy, fat, and particularly SFA. On the other hand, long-term implementation of plant-based dietary patterns bears the risk for inadequate intake of essential nutrients such as vitamin B12, n-3 LC-PUFA, minerals (focus: calcium) and trace elements (focus: iron, iodine, zinc, selenium). This could impair bone health and may favor the development of chronic-inflammatory symptoms or depressive symptoms, respectively (26–29).

The NuEva study is designed to identify critical nutrients for the studied diets, varying in their content of animal products and to validate the derived nutritional concepts to counteract nutrient deficiencies.

In this context, the supply of n-3 LC-PUFA and in particular, the conversion of alpha linolenic acid (C18:3, n-3, ALA) from linseed oil is also an important aspect of the NuEva study. In most countries and, particularly, in subgroups such as vegetarians or vegans, ALA-rich sources, such as walnuts, flaxseed, soybeans, and canola oils, provide most of the dietary n-3 PUFA. In humans, the enzymatic conversion of ALA into n-3 LC-PUFA is inefficient. In particular, endogenous conversion into docosahexaenoic acid (C22:6, n-3, DHA) is limited (30, 31). Plant-based diets are very low in n-3 LC-PUFA such as eicosapentaenoic acid (C20:5, n-3, EPA, <5 mg/d). The intake of DHA varies depending on consumption of DHA-enriched eggs on average <33 mg/d (32). The dominating PUFA in vegetarian/vegan diets is the n-6 PUFA linoleic acid (C18:2, n-6, LA) from seeds and oils from sunflower, soy, safflower or pumpkin. Its intake ranges from ∼15 g/d (5.7% of daily energy) in omnivores to ∼30 g/d (10–12% of daily energy) in vegetarian/vegan diets (33). The relatively high LA/ALA ratio in the vegetarian/vegan diet could impair the conversion efficiency from ALA to EPA and DHA (24, 25). High intake of n-3 LC-PUFA is considered to improve cardiovascular risk factors and to reduce the incidence of cardiovascular outcomes (34–39). However, the evidence for similar health-promoting effects of land-based n-3 PUFA is weak (37, 38). Thus, an adequate supply of n-3 LC-PUFA is eligible to maintain or improve health.

Adequate nutrient intake also modulates the complex process of bone mineralization and resorption. Thus, plant-based dietary regimens are traditionally considered healthy, but their real impact on bone health still needs to be examined. Plant-based diets are alkaline and tending to contain more protective nutrients, including magnesium, potassium, vitamin K, and antioxidant and anti-inflammatory phytonutrients that favor bone mineral density (BMD). The intake of dairy products as sources of calcium, high-value protein, and vitamin D may also improve bone health. On the other hand, vegetarian and vegan diets contain lower amounts of protein, vitamin D, vitamin B12, n-3 LC-PUFA, and calcium (40, 41). Moreover, the intake of oxalic acid and phytic acid, and the lower bioavailability of zinc in plant-based diets may impair the absorption and retention of calcium. Knurick et al. (42) compared BMD from young, non-obese adults consuming meat-based (n = 27), lacto-ovo vegetarian (n = 27), or vegan (n = 28) diets for ≥1 year. Based on 24 h diet recalls, the plant-based diets are associated with reduced protein intake by ∼30% compared to omnivores. An association between dietary protein and BMD was found for those following a vegan diet. Veronese and Reginster (29) confirm that vegetarian diets (particularly vegan ones) are associated with significantly lower BMD values in comparison with meat eaters. The NuEva study aims to evaluate the impact of the studied diets on markers reflecting bone health as well as to validate the developed nutritional concepts concerning their impact on bone mineralization and resorption.

Strengths and limitations of this study:

- The NuEva study is designed to identify critical nutrients relating to the implementation of the studied diets (vegetarians, vegans, flexitarians, Western diet)

- Based on the screening data, personalized nutritional concepts are prepared to counteract under- or oversupply of essential nutrients.
- The personalized nutritional concepts are validated in the intervention period of the NuEva study.
- The design of the NuEva study provides the opportunity to identify differences in the conversion of ALA into n-3 LC-PUFA depending on the studied dietary patterns, e.g., varying in the concurrent intake of n-6 PUFA such as linoleic acid.
- The study allows assessing physiological benefits or possible physiological consequences resulting from the implementation of the studied nutritional habits with focus on cardiovascular risk, but also diabetes risk, inflammatory markers, bone health, thyroid function and microbiome.
- The lack of a control group without menu plans is a limitation of the study design.
- Despite the multifarious approach of the NuEva concept the individual compliance with the proposed dietary strategies stills an uncertainty factor that cannot be captured in total.
- The menu plans were prepared with the software PRODI® version 6.4 (Nutri-Science, Stuttgart, Germany) for professional dietary counseling and therapy. The calculations on nutrients composition based on the "Bundeslebensmittelschlüssel" and further nutrition tables[3]. In this context, limitation occur due to differences between nutrient profiles calculated with the software and the nutrient composition of the foods that are really consumed. Variations can depend on sorts, preparation conditions, feeding conditions.

ETHICS AND DISSEMINATION

- All human investigations are conducted according to the principles expressed in the Declaration of Helsinki. Written informed consent is a precondition for enrollment of each participant. The study protocol, including the protocols for subject recruitment, assessment, analysis of study parameters, participant information, was reviewed and approved by the Ethical Committee of the Friedrich Schiller University of Jena (number: 5504-03/18).
- The NuEva study was registered before launching (Trial Registration: ClinicalTrials.gov Identifier: NCT03582020).
- At first, a manuscript with the results of the screening period will be published in a peer-reviewed journal. Secondly, separate manuscripts will be written on the results of the intervention period, addressing each focus of the NuEva study such as cardiovascular risk, diabetes risk, inflammatory markers, bone health, thyroid function and microbiome, but also the conversion of land-based n-3 PUFA into their long-chain metabolites. These manuscripts will also be submitted for publication in peer-reviewed journals.

[3] Available online at: https://www.nutri-science.de/software/prodi.php

DATA AVAILABILITY STATEMENT

The article includes the description of a human trial (protocol). No datasets are presented in this article. Requests to access results or datasets should be directed to the corresponding author.

ETHICS STATEMENT

All human investigations are conducted according to the principles expressed in the Declaration of Helsinki. Written informed consent was a precondition for enrollment of each participant. The study protocol, including the protocols for subject recruitment, assessment, analysis of study parameters, participant information, was reviewed and approved by the Ethical Committee of the Friedrich Schiller University of Jena (number: 5504-03/18).

AUTHOR CONTRIBUTIONS

CD planned and performed the NuEva study (inclusive recruiting and conduction of the nutritional counseling units as part of the NuEva concept). She was responsible for preparation of the used information materials and personalized menu plans, sample collection, management of the study data, analysis of the study parameters, and their statistical evaluation. CD wrote the paper and approved the final version of the manuscript.

FUNDING

This work was supported by the German Federal Ministry of Research and Education (grant number 01EA1708).

ACKNOWLEDGMENTS

We thanks all participants of the NuEva study for participating on the trial. Many thanks go to M.Sc. Maren Biernath and further students of Nutritional Science for the supervised preparation of the personalized menu plans. We thank PD Dr. Kristin Dawczynski for supervision as medical doctor, Muriel Steiner for taking the blood samples and Ute Helms, Susann Schröpfer, and M.Sc. Benjamin Schenz for carrying out the pre-analytic of the human samples according to standard procedures and Prof. Dr. Peter Schlattmann for supervision of the statistical procedures.

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