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Improved Nutrition in Early Life and Cardiometabolic Outcomes in Guatemala

By

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Doctor of Philosophy

Nutrition and Health Sciences Program

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Abstract

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There is a global epidemic of cardiometabolic diseases. Metabolic flexibility, which can be assessed through meal challenges, is integral to cardiometabolic health. Previous research drew inconsistent linkages between early-life nutrition and cardiometabolic conditions in adulthood. *The overarching goal* of this dissertation is to investigate the pathways through which early nutrition affects long-term cardiometabolic health.

This research is nested within the Institute of Nutrition of Central America and Panama (INCAP) Longitudinal Study in Guatemala, a cluster randomized controlled trial conducted in 1969-77 with subsequent follow-up studies. In 2015-17, plasma samples were obtained both at the fasted state and two-hour after a meal challenge. We performed assays for lipids, glycemic markers, and inflammation markers.

First, through difference-in-difference modeling and mediation analysis, we examined the role of leptin. We observed that leptin partially mediates the pathway between early-life nutrition and glycemic status (only in women). The mediation was associated with improved pancreatic β-cell function, and not with reduced insulin resistance. Second, to describe the metabolic flexibility in this population, we assessed the postprandial biomarker responses, and compared the responses across strata of cardiometabolic phenotypes. We observed that the underlying pathways, particularly glycemic pathway, differed across cardiometabolic phenotypes. Subsequently, we compared metabolic flexibility between those who were exposed to improved nutrition in early life versus others. At the multi-marker level, overall postprandial biomarker responses did not differ by early-life nutritional exposure. However, response in the glycemic domain differed between exposure groups. At the single-marker level, glucose response was attenuated in the improved nutrition group. These findings strengthened our previous observation of reduced fasting glycemia among those exposed to improved nutrition. We postulated that nutrition improvements in early life contribute to euglycemia by enhancing metabolic flexibility.

By integrating assessments of metabolic flexibility into a long-term cohort study, we extended previous research in this population. We also provided context for this work through a systematic review and meta-analysis to summarize global evidence on the association of nutrition interventions in early life and cardiometabolic outcomes. Altogether, the findings support evidence-based maternal and child nutrition interventions to promote long-term cardiometabolic health and to avoid unintended consequences.

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CHAPTER 1: INTRODUCTION

1.1 Background

1.1.a Global burden of cardiometabolic diseases

Over the past few decades, a global epidemiology transition in disease patterns has taken place: there has been a steady decrease in communicable diseases, accompanied by a gradual increase in non-communicable diseases (NCD) worldwide (1). Despite the success in reducing mortality rates in NCDs such as cardiovascular diseases as a result of medical advancement and public health efforts, these conditions place increasingly heavy burdens on the health systems, especially in aging populations (2). From 1990 to 2013, the global average life expectancy increased by more than five years, largely attributable to a decline in neonatal and child mortality (1). Regional and country-level differences are large, as are variations in age and sex; But the general trend points to even more aging populations and heavier NCD burdens across the board (3). The direct and indirect healthcare cost associated with managing and treating chronic conditions is astronomic; the loss of disability-adjusted life years is even more difficult to conceptualize and quantify (1).

Cardiometabolic diseases (CMDs) are a subset of NCDs. A cluster of chronic conditions are referred to as CMDs due to their overlapping biological pathways and risk factors, such as insulin resistance, hyperlipidemia, and inflammation. CMDs include cardiovascular diseases, diabetes, obesity, non-alcoholic fatty acid disease, and associated complications (4). These conditions may result from a spectrum of factors, such as diet, environmental exposure, and genetics (4). CMDs are an emerging contributor to global health burden. Cardiovascular mortality accounts for approximately 50% of NCD deaths worldwide, and 70% of

cardiovascular-related deaths took place in low- and middle-income countries in 2015 (5). Equally disconcerting is the high prevalence of diabetes. On average, diabetes mellitus claims 21 lives per 100,000 (and approximately 870 disability-adjusted life year, or DALY, per 100,000) in 2015, contributing to 2.7% of global deaths (6). There is an upward trend of diabetes mortality rates and DALYs worldwide (6). Some low- and middle-income countries (LMICs) are heavily affected. For example, in recent data from Guatemala, cardiovascular disease and type 2 diabetes were ranked the first and the third leading causes of deaths, respectively (6).

Occurring alongside the epidemiology transition is a "nutrition transition", featuring globalization in agricultural systems and food supply networks and associated changes in food accessibility, dietary patterns, body composition, and other health outcomes in populations over long periods of time (7). While changes in food processing have provided greater accessibility to more diverse food options with longer shelf-life worldwide, many of these food items, however, are nutrient-poor and calorie rich (7). Nutrition transition, combined with a trend towards sedentary lifestyles and other drawbacks of unplanned urbanization, contributes to the epidemic of global obesity and associated complications (7, 8). Not only are the epidemiology and nutrition transitions worldwide phenomena, they are becoming more pronounced in low- and middle-income countries (LMICs) than in high-income countries (6). Approximately 80% of non-communicable disease mortality in the world took place in low- and middle-income settings, disproportionately affecting countries undergoing rapid social changes, but with sub-optimal health infrastructure to cope with these changes (2, 9).

1.1.b The long-term cardiometabolic impact of nutrition in early life

The developmental origins of health and diseases (DOHaD) paradigm links fetal and early life factors to long-term health consequences (10, 11). DOHaD centers around the concept

of developmental plasticity and the benefits of adapting to fetal environments. It also emphasizes a consequential mismatch between early-life environment (genetic predispositions, epigenetic modifications, and other environmental factors) and later environments, paving the path to reduced metabolic flexibility and increased susceptibility to cardiometabolic perturbations later in life (2). For instance, if the mother is exposed to caloric restriction during pregnancy, ontogenic alternations and metabolic adaptation may occur in preparation for a low-energy food environment (12). When this early predisposition is mismatched with a later obesogenic environment, the "thrifty" metabolic system, along with other metabolic alterations, will eventually lead to the onset and progression of CMD (13).

Life course epidemiology is a useful tool in studying the long-term impact of early nutrition (14). Building upon the concept of DOHaD, life course epidemiology provides a more holistical analysis of the health and disease patterns throughout the life course of populations, beyond the focus on the in utero developmental phase (14, 15). From the perspective of life course epidemiology, components that could affect long-term health outcomes include genetic predisposition, critical windows for growth and development (such as during the first trimester of pregnancy), sensitive periods (such as adolescence), as well as cumulative effects of lifelong exposure to nutritional and environmental factors (14). Incorporating a life course perspective into epidemiological studies will allow us to understand the determinants of current disease patterns, and to evaluate nutrition investments in early life that may provide long-lasting benefits (5, 14, 16).

The period from conception to two years of age, which corresponds to a period of rapid growth and programming, is believed to be a vulnerable phase of human development (17). This period, commonly referred to as "the first 1000 days" is in the focus in nutrition research and

public health work. First, there is now definitive evidence supporting the same growth potential of children across the globe (18). The variances in the actual growth patterns are attributable to dietary, environmental, and social factors, which are identifiable and largely modifiable (17). Second, the first 1000 days is a period when fetal and early-life programming occurs, including altered organ development and metabolic programming in response to external signals such as maternal diet (17, 19). With the globalization of food system and changes in nutrition environment, populations that were chronically malnourished in early life may be exposed to a different external environment later in life. This mismatch between the "hypothesized early-life environment" that controlled fetal programming and the actual external environment has been found to contribute to higher risks for CMDs in adulthood at the population level (20, 21).

1.1.c Significance of this work

The classic design of nutritional research usually involved an assessment of nutritional status and associated health effects within a relatively short period of time. This type of cross-sectional studies, albeit effective in establishing important associations between nutrition and health, cannot supply much needed evidence on *how, over time,* nutrition influences our health. Nutritional needs change according to different life stages (14). Even within the same chronological stage, the focus on nutritional demand varies. Maternal nutritional status, for instance, have profound effects on fetal development: in the earlier phases of pregnancy, development or vital organs is rapid and protein- and energy-intensive. Based on observational studies, nutritional status and exposure at different fetal develop stages have varied long-term impact on later health (22-24). This dissertation work is situated within a prospective study, the Institute of Nutrition of Central America and Panama (INCAP) Longitudinal Cohort Study (which is described in more details in **Chapter 4**) that was initiated in 1969-77 as a cluster

randomized controlled trial, which provides valuable longitudinal data on the topic of early nutrition and long-term health association (25).

This dissertation work serves to further explore the association of early-life nutrition and CMDs, using information from biomarkers. The INCAP researchers and staff has worked closely with the communities for over five decades. During the most recent follow-up study in 2015-17 (when biological samples used in this dissertation were collected), we observed high prevalence of cardiometabolic conditions, and a large proportion of the study population also had subclinical risk factors that may lead to higher prevalence of cardiometabolic diseases as they age (16). We need to elucidate the underlying pathways between the early-life nutritional exposure statuses and cardiometabolic outcomes observed in this study population.

Biomarkers are valuable in assessing cardiometabolic diseases. Measuring biomarkers in the fasted state can help fill the gaps in many population studies through elucidating biochemical, physiological, and pathophysiological mechanisms (26, 27). It is important to include relevant biomarkers in longitudinal studies, especially from the perspective of life course epidemiology. In this dissertation work, we selected a panel of conventional and emerging biomarkers to characterize cardiometabolic profiles in the study population and compared the profiles across strata of cardiometabolic conditions and early-life nutritional exposure status. We analyzed these novel and established biomarkers individually and combined, based on both *a priori* and data-driven methodologies. A more in-depth review of relevant CMD biomarkers is in included as **Chapter 3** of this dissertation.

While biomarker levels in the fasted state can provide a snapshot at the current metabolic status, they do not provide any information how these biomarkers would be affected under stress. One unique aspect of this work is the availability of data on two-hour response to a meal

challenge in a population with a relatively large sample size in a low- and middle-income setting. Relatively little is known, even under controlled clinical conditions in high-income countries, about the dynamic responses of various biomarkers and their indication for human health (28, 29). The mixed-component meal challenge, described in more details in **Chapter 4**, mimics the stress induced by a typical meal containing fat and sugar. Responses to this physiologic stress was assessed through two-hour changes in biomarker levels. It is considered a more robust and accurate approach to examine the changes in biomarker levels in response to meal challenges (30). Through investigating the dynamic postprandial responses of biomarkers, we identified key differences in metabolic flexibility between those with and without cardiometabolic risks.

1.2 Specific Aims

The overarching goal of this dissertation is to examine the pathways through which nutrition supplementation in early life can improve long-term cardiometabolic health outcomes in a chronically malnourished population that is undergoing nutrition transition.

Specific Aim 1: Investigate the role of leptin in the diverging association of early nutrition with cardiometabolic conditions observed in this study population, including decreased risk for type 2 diabetes and increase risk for obesity.

H2.1: Early-life exposure to improved nutrition promotes organ development, which determines future risk for obesity and the concentration of leptin.

H2.2: Leptin has glucose-lowering effects among participants who were exposed to improved nutrition in early life in this population.

Approach: Through literature review, I built the basis for the effects of early nutritional exposure on ontogeny for metabolically active tissues. Using fasting biomarkers collected from

the Institute of Nutrition of Central America and Panama (INCAP) Longitudinal Cohort Study, I examined the proportional association between leptin and adiposity. Through mediation analysis and difference-in-difference modeling strategy, I tested the hypothesis that leptin has glucose-lowering effects and can mediate the pathway between early-life nutritional exposure and long-term glycemic status.

Specific Aim 2: Describe adulthood cardiometabolic health status of the study population through assessment of metabolic flexibility, as characterized through meal-induced biomarker responses in this population.

H2.1: Metabolic flexibility can be characterized by meal-induced responses in selected biomarkers to represent cardiometabolic pathways.

H2.2: Meal-induced responses in biomarkers differ across strata of cardiometabolic conditions.

Approach: A mixed-component meal challenge was administered in the study population. I assayed fasting and postprandial plasma samples to obtain biomarkers that represent four cardiometabolic pathways, including lipids, glycemic markers, and pro- and anti-inflammation markers. I used least squares regression to assess postprandial biomarker responses. I compared the responses across strata of cardiometabolic phenotypes to examine metabolic flexibility. I analyzed the structural association among biomarkers using structural equation modeling, to further characterize the cardiometabolic profile of the study population.

Specific Aim 3: Investigate the association of early-life nutrition with adulthood metabolic flexibility in cardiometabolic pathways, using a single- and multi-biomarker approach.

H3.1: Early-life exposure to improved nutrition, compared with other exposure status, is associated with improved metabolic flexibility, as characterized by biomarker responses at the single-biomarker level.

H3.2: Early-life exposure to improved nutrition, compared with other exposure status, is associated with improved metabolic flexibility, as characterized by biomarker responses at the multi-biomarker level.

Approach: At the single-biomarker level, I combined difference-in-difference modeling strategy and least squares regression to compare the postprandial biomarker responses between those who were exposed to improved nutrition in early life in the INCAP study versus other. At the multi-biomarker level, I used two data reduction techniques to test the hypothesis from two opposite directions: I used multivariate analysis of variance (MANOVA) to compare global (all combined) and domain specific (by cardiometabolic pathway) biomarker responses between the exposure groups. I then used linear discriminant analysis (LDA), a data-driven technique, to examine whether we can predict exposure group membership using available biomarker information.

1.3 Overview of the Chapters

The rest of the dissertation is structured as follows: Chapter 2 is a systematic review and meta-analysis that centered around global evidence regarding the type and the timing of maternal and child nutrition interventions and their associations with long-term cardiometabolic health. Chapter 3 present the rationale for the selected cardiometabolic biomarkers, their significance in nutrition research, and the relevance to this work. Chapter 4 described in details the methodologies used in this work, including the analytical assays and the statistical tools. The

next 3 chapters (Chapters 5 to 7) explore different aspects of the early nutrition-CMD paradigm, as described in the Specific Aims. They represent three individual manuscripts submitted for publication in peer-reviewed journals. Chapter 8 summarizes the key findings and public health implications of this work, and to provide recommendations for future research.

CHAPTER 2: LITERATURE REVIEW PART I

Early-life nutrition interventions and associated long-term cardiometabolic outcomes: a systematic review and meta-analysis

2.1 Abstract

Background: Early-life nutrition interventions may have lifelong cardiometabolic benefits. Most evidence on this topic is derived from observational studies. We evaluated the association of early-life nutrition interventions (randomized controlled trials) and long-term cardiometabolic outcomes.

Methods: Through electronic literature search of PubMed, CABI Global Health, EMBASE, and Cochrane, with manual reference check and weekly new publication alert from PubMed, we identified 8312 records, and eventually included 55 records in this review. We conducted qualitative and quantitative synthesis and evaluated risk of bias.

Results: We included 55 reports from 38 cohorts in 20 countries. Interventions were initiated as early as conception, and the longest till seven years of age (except for one ongoing study till young adulthood). The cohorts were followed up between three and 73 years. We identified seven types of interventions (protein-energy supplements, long-chain polyunsaturated fatty acids, single micronutrient, multiple micronutrients, infant and young child feeding, dietary counselling, and other interventions) and four categories of cardiometabolic outcomes (biomarkers, cardiovascular physiology, body size and composition, and sub-clinical and clinical outcomes). Most findings were null. The primary exception was an overall effect on fasting glucose concentration (standardized mean difference, SMD = -0.06; 95% confidence interval: -0.09, -0.02; heterogeneity $p = 0.70$). Albeit statistically insignificant, there was an inverse effect on total cholesterol (-0.08; -0.17, 0.02; heterogeneity $p < 0.01$) and a positive effect on body

mass index (0.04; -0.04, 0.11; heterogeneity $p = 0.01$). No effect was found for blood pressure (heterogeneity $p < 0.01$ for both systolic and diastolic blood pressure). Ongoing and personalized dietary counselling was associated with predominantly favorable outcomes. Breastfeeding was associated with better cardiometabolic outcomes. The timing of intervention mattered, with earlier initiation conferring greater benefit.

Conclusion: Glucose homeostasis benefited from early-life nutrition interventions, but there is a risk of unintended consequences, including higher adiposity. Nutrition interventions in early life must be evidence-based and tailored to the target populations to promote long-term cardiometabolic health.

2.2 Introduction

There is much interest in the role of nutrition in early life, often conceptualized as the 'first 1000 days' from conception to the second birthday, on child growth and development (1). The potential for nutrition in early life to impact also on adult outcomes has been explored. Early studies by Ravelli *et al* and a large body of work by Barker and colleagues led to the formulation of the Developmental Origins of Health and Disease paradigm, positing that insults in fetal and early post-natal periods alter the child's growth and development and affect the risk of later cardiometabolic disease (2-4).

Epidemiological evidence from observational studies is abundant, suggesting associations between various early-life nutritional exposures and long-term health consequences (5-7). For instance, six or more months of breastfeeding, compared with shorter duration, is associated with lower odds for diabetes and obesity (8). A recent systematic review reported that low vitamin D status during pregnancy was associated with greater weight in the offspring at 9 months of age (9). Famine studies are generally considered pseudo-experimental, which drew lessons from the unfortunate "natural experiments" to explore the consequences of severe nutritional deprivation in early life (5). The famine studies identified early gestation as a critical window of development, as well as numerous morbidity and mortality consequences of severe nutritional deprivation in early life (10-13).

Populations that were malnourished in childhood and subsequently exposed to an obesogenic environment are particularly susceptible to cardiometabolic disturbances, which inevitably contributes to a vicious cycle of early-life malnutrition and increased risk in adulthood, and may even predispose the future generations to higher risks (7). In a world of ageing population and increasingly heavy burden of non-communicable diseases, particularly

cardiometabolic diseases, we consider it urgent and critical to investigate the potentials of early-life nutrition investments in preventing long-term illnesses (14).

There is limited evidence from experimental studies, despite considering this type of study to be more indicative of causal associations (15). Nutrition interventions in early life were usually designed to provide short-term benefits, such as promoting infant growth and preventing childhood diseases (16, 17). Randomized trials conducted among pregnant women and young children were designed to address these shorter-term outcomes. We therefore aimed at summarizing the evidence on the association between early-life nutrition interventions in the form of randomized controlled trials and their long-term influences on cardiometabolic diseases and associated risk factors.

2.3 Methods

Electronic literature search

We developed search terms based on three main domains, including “early life”, “nutrition interventions”, and “cardiometabolic outcomes”. We included additional qualifiers to specify the concept of “early life” and the duration of follow-up. In the first screening phase, we did not restrict the type of trial, the category of cardiometabolic outcomes, the language, nor the publication date. We searched the following data bases: PubMed, CABI Global Health, EMBASE, and Cochrane. We also set up PubMed email alert to screen new studies published after the initial search (completed in Feb. 2019). We received ongoing, weekly alerts of new publications till September 27, 2019, and added new records from the weekly list if they met the inclusion criteria.

Screening process

We obtained 9230 records through the electronic literature search process, and an additional 220 records were added through other sources, including searches within known longitudinal studies, manual reference checks, and a search of the gray literature. After removing duplicates, we retained 8312 records for first-pass, title screening. After removing 7413 records through this initial screening process, we conducted a second round of title and abstract screening of the remaining 899 records, and eventually identified 136 records for full-text review and data extraction.

The inclusion criteria for the articles to be included in the final analysis were: nutrition interventions, randomized controlled trial (acceptable if randomization by cluster or block), intervention conducted in early life (prenatal, maternal, gestational, antenatal, the first 1000 days, or in early childhood), with three or more years of follow up, and outcome(s) relevant to cardiometabolic diseases and risk factors.

Exclusion criteria included: not original research articles (e.g., review, trial protocol, summary of outcomes in a single cohort across decades) (n = 19); observational studies (e.g., cross-sectional analysis) nested within cohorts that had an original randomized controlled trial (n = 6); interventions related to early life development and childcare, but that were not nutritional in nature (n = 1); did not have strictly randomized assignment for the exposure variable (n = 8); did not follow up with the cohort for three or more years, or the children were not yet born after maternal intervention (n = 13); no primary outcome of interest (e.g., reported neurodevelopment and dietary pattern) (n = 5); reported maternal outcome but not outcome in the children (n = 1); previously un-identified duplicates (n = 3); reported the same outcomes from the same population as another included study, but from an earlier time point (n = 1 insufficient number of unit in cluster randomization trials (less than 20 clusters randomized) and without appropriate

statistical methods to adjust for this insufficiency (e.g., small-sample corrections or variance-weighted cluster-level analyses) (n = 2) (18); or the analysis not based on original assignment, but used difference-in-difference modeling strategy (n = 2).). Famine studies were excluded due to the quasi-experimental exposure assignment and the inability to isolate the nutritional aspects of the famine from other stressors (n = 20); **Figure 1** provides the Preferred Reporting Items for Systematic Review and Meta-Analysis (PRISMA) flow chart.

Data collection

After the screening process, a total of 55 records were retained for data extraction and synthesis of results. We identified seven categories of interventions, and four categories of cardiometabolic outcomes.

The interventions were classified into seven categories. 1) Protein-energy supplementation: in this type of intervention, the study participants (or their mothers) were provided supplements that contain mainly protein and energy, some of these supplements may include micronutrients, but the focus was protein and calories. 2) Long-chain polyunsaturated fatty acids (LCPUFA) supplementation. Some studies also included micronutrients along with the LCPUFA. 3) Single micronutrient supplementation: only one micronutrient provided in this type of trial. 4) micronutrient supplementation (MMS): two or more micronutrients were provided as supplements. 5) Infant and young child feeding (IYCF), and milk supplementation: all trials related to promoting or practicing IYCF were included in this category. A few studies that could have been included in the previous categories were included as IYCF trials as long as IYCF was the main purpose (e.g., LCPUFA-supplemented infant formula). 6) Dietary counselling: either the participants or their caretakers were provided dietary counselling. 7)

Other interventions: in this category we included all other types of trials that do not fit in any of the previous categories, including a food-based intervention and probiotics trials.

The four major categories of cardiometabolic outcomes were: 1) Biomarkers – mainly glucose homeostasis, lipid profile, and inflammation; 2) Cardiovascular physiology; 3) Body size and body composition; and 4) Sub-clinical and clinical cardiometabolic outcomes.

We extracted information on basic description of the study, funding and conflict of interest, details of methods, participant profile, and both qualitative and quantitative results. We used the most recent World Bank classification to determine the income levels of the countries where these studies were situated (19).

Qualitative and quantitative analysis

We first described the publication year, country, cohort, sample size, intervention age, duration of follow up, summary of the intervention, and outcome category. We evaluated risk for bias in six domains based on the Quality in Prognostic Studies (QUIPS) tool: study participants, study attrition, prognostic factor measurement, outcome measurement, study confounding, and statistical reporting (20). The research group that developed this tool advised against assigning an overall bias score across all domains, therefore we reported bias assessment by each domain for the studies in a qualitative manner. We also provided summary of key findings and outcome measurements. To facilitate interpretation of the results, we used bolded and capitalized words to indicate the key message, for instance, “NULL”, “HIGHER”, or “LOWER” value. In this review, the comparison was always the intervention group minus the control group, regardless of how the original paper presented the results.

For quantitative synthesis, we conducted meta-analysis using the packages “meta”, “metafor”, and “esc” in R version 3.6.1 (R Core Team, Foundation for Statistical Computing,

Vienna, Austria) (21-23). We included four major outcomes in meta-analysis: fasting glucose concentration, total cholesterol concentration, blood pressure (systolic and diastolic), and body mass index. These outcomes were included based on an additional set of criteria: three or more studies reported this outcome; the reporting format was consistent (e.g., no transformation of the raw data such as z-score or logarithm in some studies but not others); qualitative synthesis showed that not all studies reported null results for this outcome. We pre-calculated effect size for each outcome by converting either unstandardized regression coefficient or mean and standard deviation to effect size Hedge's *g*, which is bias-corrected standardized mean difference (SMD). We used a random effects model to generate overall effect size for each outcome with between-study heterogeneity test, including Higgin's & Thompson's I^2 (percentage of variability) and Tau-square (τ^2, between-study variance). We then plotted the meta-analysis results as Forest plots. For each of the selected outcomes, we also provided the corresponding funnel plot to assess publication bias.

2.4 Results

Summary of study characteristics

The 55 publications were from 38 cohort studies in 20 countries, including 12 high-income and eight low- and middle-income countries (Table 1, Figure 2). Publication dates ranged between 1997 and 2019 (Table 1). Interventions were conducted as early as conception, and the longest continued till seven years of age, with the exception of one study – the Special Turku Coronary Risk Factor Intervention Project for Children (STRIP), which was an ongoing dietary counselling intervention that lasted till early adulthood. The cohorts were followed up for between three and 73 years (Table 1). Most of the included studies were in the low to moderate

bias category across domains of bias assessment, except for Forsyth et al. 2003, which has high risk for bias in four domains (Table 2) (24). We did not observe publication bias for any of the selected outcomes (Supplemental Figure 1).

Outcome category 1: biomarkers

Meta-analysis showed that, fasting glucose concentration was significantly lower in intervention groups versus control groups across seven types of interventions (SMD = -0.06, 95% confidence interval, CI: -0.09, -0.02), with very low heterogeneity (I^2 = 0%, p = 0.70) (Figure 3-A). There was also a decrease in total cholesterol in the intervention groups, but it was not statistically significant (SMD = -0.08, 95% CI: -0.17, 0.02). There was moderate heterogeneity in the group (I^2 = 55%, p < 0.01) (Figure 3-B).

Qualitative evidence showed predominantly favorable outcomes in biomarkers of glycemic function, lipids and apolipoproteins, as well as inflammation, with a few exceptions (Supplemental Table 1). Two protein-energy trials that presented biomarker outcomes showed favorable effects, including marginally lower fasting glucose, and lower insulin and HOMA score (25, 26). One LCPUFA trial reported higher insulin resistance, whereas the other reported lower insulin concentration and insulin resistance (27). Two multiple micronutrients interventions reported favorable biomarker outcomes, including lower total cholesterol and lower inflammation marker (28, 29). Most IYCF studies reported null findings, and studies with significant findings were inconsistent in terms of the direction of impact. Based on the same study in preterm infants, Singhal et al. reported lower C-reactive protein and LDLc-to-HDLc ratio in banked breastmilk versus preterm formula group, but also higher insulin resistance (30, 31). They further reported lower leptin concentration (relative to fat mass) in the intervention group (32). In addition, Toftlund et al. 2018 reported lower cholesterol concentration in

breastmilk than in preterm formula group (33). Dietary counselling (with evidence coming primarily from the STRIP study) reported several favorable biomarker outcomes in the intervention groups, including lower insulin resistance, lower circulation fatty acids, higher serum poly-unsaturated fatty acids, and lower serum cholesterol (34-37). All other results were null (Supplemental Table 1).

Outcome category 2: cardiovascular physiology

Meta-analysis showed that there was no statistically significantly difference between intervention and control groups in blood pressure (systolic: SMD = -0.00, 95% CI: -0.07, 0.06; diastolic: SMD = -0.01, 95% CI: -0.09, 0.07), and there were substantial heterogeneity in the two sets of comparisons (I^2 = 77% and 55%, respectively, $p < 0.01$ both). (Figure 3-C)

Approximately half (nine out of 20) of studies reported significant results in this category (Supplemental Table 1). The favorable findings were as follows: one protein-energy trial reported lower augmentation index (26). One single micronutrient trial reported marginally lower diastolic blood pressure, but only in overweight children (38). Two IYCF trials reported lower blood pressure, and one reported marginally lower heart rate (24, 39, 40). Two dietary counseling studies (both from the STRIP study) reported lower blood pressure and better endothelial functions (mainly in boys for the latter) (41, 42). The unfavorable findings included the following: one LCPUFA study reported higher blood pressure in boys; one IYCF trial reported higher blood pressure in the intervention group, but only in girls (43, 44). All other results were null (Supplemental Table 1).

Outcome category 3: body size and body composition

Based on meta-analysis, the intervention groups had higher body mass index than the control groups, but the estimate was not statistically significant (SMD = 0.04, 95% CI: -0.04, 0.11). There was moderate heterogeneity in this group (I^2 = 45%, p = 0.01). (Figure 3-D)

Several studies reported higher risk for obesity based on anthropometry measurements, including one maternal LCPUFA trial and six IYCF trials (high- versus low-protein formula, milk supplementation versus no supplementation, baby-friendly hospital versus control, enriched vs. un-enriched formula) (44-51) (Supplemental Table 1). Some potentially positive outcomes were reported as well, including larger head circumference and lower waist circumference reported two LCPUFA trials. One multiple-micronutrient trial reported lower body mass index-for-age z score, whereas one IYCF trial (breastfeeding versus formula feeding) reported lower level of early rapid growth (27, 29, 33, 43). All other results were null (Supplemental Table 1).

Outcome *category* 4: subclinical and clinical outcomes

because of the xx studies in this category, only eight studies reported subclinical or clinical cardiometabolic outcomes, and the findings were inconsistent (Supplemental Table 1). One IYCF trial (high- versus low-protein formula) reported higher risk for obesity (51). On the contrary, findings from the STRIP study (dietary counselling) reported lower overweight prevalence among girls (52). Both Nupponen et al. 2015 (dietary counselling) and Stewart et al. 2009 (folic acid plus vitamin A supplementation versus vitamin A alone) reported lower risk for metabolic syndrome (53, 54). In addition, Pahkala et al. 2013 (dietary counselling) reported lower risk for poor cardiovascular health. (55) The remaining studies reported null findings (Supplemental Table 1). Available data for this outcome category is not suitable for meta-analysis.

Timing of intervention

Two studies directly examined the difference in timing of the interventions. Ekström et al. 2016 observed that, when the same supplementary food item was administered immediately after detection of pregnancy, instead of at 20 weeks of gestion, there was improved lipid profile (56) (Table 1, Supplemental Table 1). Hawkesworth et al. 2011 reported that delivering the same maternal protein-energy biscuit pre- versus post-delivery was associated with marginally lower fasting glucose concentration in the offspring (25) (Table 1, Supplemental Table 1).

Across different LCPUFA supplementation studies, we observed that, providing LCPUFA supplementation to mothers appeared to have minimal impact on with cardiometabolic indicators in offsprings (39, 57-61) (Table 1, Supplemental Table 1). Vinding et al. 2018 reported that offspring in the maternal fish oil supplementation group, compared with placebo group, had higher body mass index from birth to 6 years of age (50). In contrast, when LCPUFA was provided directly to the infants, there was an association with lower insulin resistance and lower waist circumference later in life (27) (Table 1, Supplemental Table 1).

2.5 Discussion

We conducted this systematic review and meta-analysis as an effort to synthesize up-to-date information regarding the long-term cardiometabolic impact of nutrition interventions (randomized controlled trials) in early life. The primary findings of this review include: 1) Across different types of interventions, there was an overall favorable effect on glucose homeostasis. There was also a marginally favorable effect on total cholesterol and a borderline unfavorable effect on obesity risk. 2) Ongoing and personalized dietary counselling was the only intervention that reported predominantly beneficial cardiometabolic outcomes. 3) Among IYCF studies, breastfeeding was more beneficial than formula feeding, although breastfeeding-

promotion alone did not yield observable long-term benefits. 4) Timing of intervention have direct effect on outcome, and earlier exposure to improved nutrition was more beneficial than later.

In this review, we observed mostly null to modest findings in terms of long-term cardiometabolic impacts, unlike the relatively clear associations drawn from famine studies (12, 62, 63). It is possible that the human body is rather resilient: unless undergoing severe energy and nutrient deprivation early in life, metabolic programming either would not occur, or the programming effects could be offset or compensated for in the long term. It is plausible to assume that most cardiometabolic disturbances in early life do not persist into adulthood, although some evidence suggested the lifelong tracking of blood pressure level (64). The underlying mechanisms of early-life nutrition and long-term impact is not fully elucidated (5). A few possible mechanisms include in-utero growth restriction, ontogenic alterations, metabolic adaptation, and epigenetic modifications (5, 65). Factors in the broader social, economic, and environmental context should be taken into consideration as well (5, 66).

Glucose homeostasis appeared to have benefited from various types of early-life nutrition interventions, the most prominent being protein-energy supplementation in relatively malnourished populations (25, 26). The availability of sufficient amount of protein in early life likely supports the development of essential metabolic organs, especially the pancreas, where insulin, glucagon, and other key glycemic regulators are produced (67). Research showed that protein supplementation promotes brain development, which is a major organ that relies solely on glucose as fuel (68). A few other types of interventions also improved glucose homeostasis. Dietary counselling reduced fasting glucose concentration and insulin resistance (69, 70). Gestational supplementation with multiple micronutrients versus only iron and folic acid, as well

as LCPUFA supplementation during infancy also had beneficial effects on glucose concentration, indicating the importance of a balanced micronutrient profile and improved lipid intake (27, 56).

The increased risk for obesity was mainly observed in interventions that provided "enriched" supplements, which provided either higher energy or key macronutrients beyond average needs (49, 51). In well-nourished populations, higher protein intake in infancy may increase the availability of branched-chain amino acids that can enhance the release of insulin-like growth factor 1 (IGF-1), which is known to stimulate weight gain and body fat deposition (51). One study, however, provided body composition data with short intervals over the follow-up period (birth to 6y). They reported that despite the increase in body mass index, there was a proportional increase in lean, bone, and fat mass in the intervention group (LCPUFA) (50). Since BMI does not directly measure lean mass and fat mass (48), results based solely on BMI should be interpreted with caution. It is possible that these interventions stimulated growth proportionally in different types of tissues, without compromising metabolic functions. More research is needed to ascertain this finding and investigate the underlying mechanisms.

The STRIP study of dietary counselling in Finland was the only cohort that reported consistently positive results across all categories of cardiometabolic outcomes, including lower insulin resistance, favorable lipid profile, lower blood pressure, better endothelial function, lower risk for metabolic syndrome, and better indicators for cardiovascular health (35, 37, 41, 52, 53, 55, 69-71). It is possible that only intensive interventions such as STRIP can ensure long-term benefits, especially given its behavioral-change nature and personalized design. All other interventions involved changes in one or several nutritional components directly provided through the study, without requiring additional lifestyle modifications. Chronic diseases are

usually the result of cumulative exposures, which may not be sufficiently prevented through interventions that start and end early in life (72). However, it is important to note that the STRIP cohort received continuing intervention from infancy till young adulthood, making the "longitudinal" nature of the outcome less compelling (73).

Protein-energy supplementation trials are a common type of nutrition intervention, especially in low- and middle-income countries, where the need for maternal and child nutrition investments is high (74). For instance, the Institute of Nutrition of Central America and Panama (INCAP) Longitudinal Study reported numerous positive health and human capital outcomes associated with protein-energy supplementation in early life (74, 75). In a recent follow-up study, however, INCAP researchers observed diverging effect of the supplementation on cardiometabolic outcomes, including protective effect against diabetes but increased risk for obesity (76). In this review, we have observed similar conflicting impacts of protein-energy supplementations on cardiometabolic outcomes in various studies (25, 26). Similar to the quasi-experimental nature of famine studies (which focus on deprivation), the INCAP study could be viewed as a quasi-experimental trial with a focus on remediating chronic undernutrition. Its various findings are, by nature of the context, different from those obtained in high-income countries. It is important to further investigate the effect of relatively higher protein and energy intake in early life, and to compare the results between malnourished and well-nourished populations.

IYCF was a major category in this review, because we combined behavior change trials and supplementation trials. The one IYCF behavior change trial (PROBIT in Belarus), reported mainly null results after implementing policies based on the Baby-Friendly Hospital Initiative (47, 77-79). Across supplementation trials with IYCF focus, infants who were breastfed,

compared with those fed with formula, had lower C-reactive protein, cholesterol, heart rate, and blood pressure later in life, regardless of the baseline characteristics of the cohorts, formula composition, or duration of follow-up (33, 39, 40, 80). Among formula-fed groups, participants receiving high-protein or nutrient-enriched (versus low-protein or standardized) formula had higher level of early rapid growth and higher fat mass during follow-up (46, 48, 49, 51). This is possibly due to the relatively higher percentage of protein and lower percentage of fat in the formula, which is rather different from breastmilk that typically contains 3-5% fat and 0.8-0.9% protein (81). High protein intake in early life may alter fat distribution in healthy children during developmental processes, including potentially higher subcutaneous fat layer (46). We therefore urge public health researchers and practitioners to strictly follow evidence-based programming.

Most studies with a single micronutrient supplementation showed no clear long-term cardiometabolic benefits, except for reduced risk of metabolic syndrome in the group receiving folic acid supplementation (54). Multiple micronutrient supplementations seemed to be associated with better lipid profile and glycemic status, lower concentration of inflammation markers, and lower body mass index (29, 56). Micronutrients are essential in early-life development, and it has been reported that antenatal micronutrient supplementation increases birth weight, which in itself has long-term implications (54). It is possible that, despite short-term benefits of single micronutrient trials, the effects are overshadowed by life-long exposure in other aspects. In contrast, multiple micronutrient supplementations may promote growth and early development in a holistic way for the effects to be long-lasting. Given the relative ease and convenience of micronutrient-centered trials, we should continue investigating their long-term benefits.

Regarding the timing of interventions, we have observed that earlier than later enrollment in the trials had more beneficial effects on lipid profile and glucose homeostasis (25, 56, 82, 83). However, when comparing different studies with LCPUFA supplementation, interventions targeting infants, compared with maternal interventions, had more cardiometabolic benefits (24, 39, 43, 44, 50, 59-61). We did not observe a clear pattern regarding timing in this review due to the limited number of studies with relevant information. There is some evidence in the literature to support the differential impact of nutrition at various time points during pregnancy, in infancy and early childhood. The most well studied famine is the Dutch Famine, which helped distinguish famine exposure at different trimesters of pregnancy and in infancy for relatively accurate analysis. Earlier exposure to famine (e.g., in the 1st trimester) than later exposure (e.g., after birth) had associations with more severe long-term outcomes including insulin resistance and increased adiposity (10-13). Similarly, researchers reported that prenatal exposure (compared with later exposure) to the Chinese Famine was associated with significantly higher risk for hyperglycemia in two consecutive generations (84-86). Famine research from Bangladesh reported underweight as an outcome following in utero famine exposure, but overweight following postnatal famine exposure (87). Observations based on the Ukraine Famine also identified early gestation as a critical window of development (63).

There are a few limitations in this review. We combined different types of interventions as an effort to summarize the impact early-life nutrition interventions as conducted by researchers around the world. This may lead to over generalization of the results. The trials in high-income settings may differ from those in low-resource settings, because the populations did not have the same potential to benefit from nutrition interventions. We did, however, provide details in the results and discussion sections to help interpret the results. The studies were also

conducted at different time points in early life, with varying lengths of follow-up periods. We encourage the readers to refer to the tables and supplemental materials, whenever necessary, for details in study design and outcome. We also caution against unfounded extrapolation of the results.

This review is the first to summarize information related to early-life nutrition interventions and long-term cardiometabolic impacts with a focus on randomized controlled trials. We emphasized both the type and the timing of interventions in association with different categories of outcomes. In addition, we did not restrict publication year or the language of the articles, hence providing wide coverage of relevant results. In sum, this systematic review and meta-analysis serves both as a reference manual to refine and improve nutrition interventions to yield more long-term gains, and as a preventative measure to identify any intervention that may have unintended negative effects.

From a public health programming point of view, it may not be feasible to implement long-term, intensive, and individualized dietary counselling in most settings. However, it is possible to incorporate dietary counseling into other types of study designs, including various types of macro- and micro-nutrient supplementations. It is also advisable to incorporate individualized dietary and lifestyle counselling into primary healthcare to ensure sustainability. We suggest that researchers and public health practitioners shift their point of view regarding nutrition interventions from disease-centered prevention to long-term, sustainable, health promotion strategies.

2.6 Tables, figures, and supplemental materials

Table 1. Description of studies included in this systematic review

Type	Study (n = 55) [1]	Year	Country (n = 20)	Cohort [2] (n = 38)	Sample size (% Female)[3]	Intervention age range	Duration of follow up	Description of the intervention [Individual or cluster randomization] [4]	Cardio-metabolic outcomes
Protein-energy supplementation									
1	Hawkesworth et al. (Trial 1) [5] (25)	2011	Gambia	[U] Gambia (2 trials)	Trial 1: 1317 (47.9%) (Trial 2 see single micronutrient)	20-week gestation to delivery (Mothers)	17 y	[Cluster: N_{INT} = 16, N_{CON} = 16] Intervention group: Pre-delivery (20-week gestation till delivery) provision of protein-energy biscuit (2 biscuits/day, max 1015 kcal energy, 22g protein, 56g fat, 47mg calcium, and 1.8mg iron) Control group: Post-delivery (delivery till 20 weeks postpartum) provision of the same protein-energy biscuit	Biomarkers Cardiovascular physiology Body size & composition
2	Hawkesworth et al. (82)	2009	Gambia	[U] Gambia (1 trial)	1317 (47.9%)	20-week gestation to delivery [Mothers]	17 y	Same as Hawkesworth et al. 2011	Cardiovascular physiology
3	Hawkesworth et al. (83)	2008	Gambia	[U] Gambia (1 trial)	1317 (47.9%)	20-week gestation to delivery [Mothers]	17 y	Same as Hawkesworth et al. 2011	Body size & composition
4	Kinra et al. (26)	2008	India	[U] ICDS	1131 (46.1%)	In utero to 6 y [Mothers, infants, and	18 y	[Cluster: N_{INT} = 15, N_{CON} = 14] Intervention group: Protein-calorie supplement “upma", a	Biomarkers

						young children]		local preparation providing 2.51 MJ and 20 g protein to the women and half this amount to the children. No other nutrients. Plus integrated child development services (ICDS) Control group: "Control group has similar ICDS services equivalent to intervention group (uptake was lower)	Cardiovascular physiology Body size & composition
5	Macleod et al. (88)	2013	UK	Sorrento Study	65 (53.5%)	In utero [Mothers]	23 y	[Individual: N_{INT} = 44, N_{CON} = 21] Intervention group 1: Protein, energy, and vitamins: vitamins plus 1810 KJ daily, 90% of energy as carbohydrate (glucose syrup) and 10% as protein Intervention group 2: Energy and vitamins: vitamins plus 1810 KJ daily of carbohydrate Control group: Vitamins only (vitamin A 0.75mg, thiamine 1.4mg, riboflavin 1,7mg, pyridoxine 2.0mg, nicotinamide 18mg, ascorbic acid 60mg, calciferol 2.5 µg daily delivered in sachets for dissolving in water)	Biomarkers Cardiovascular physiology Body size & composition
Long-chain polyunsaturated fatty acids supplementation									

1	Asserhøj et al. (43)	2009	Denmark	[U] Maternal lactation fish oil	98 (44.9%)	0 to 4 months postpartum (during lactation) [Mothers]	7 y	[Individual: N_{INT} = 64, N_{CON} = 34] Intervention group: Fish-oil supplement (0.6 g/d eicosapentaenoic acid and 0.8 g/d DHA) Control group: Olive oil supplement	Cardiovascular physiology Body size & composition
2	Brei et al. (57)	2016	Germany	INFAT	114 (NA)	In utero [Mothers]	5 y	[Individual: N_{INT} = 58, N_{CON} = 56] Intervention group: Daily, 1200 mg long-chain polyunsaturated fatty acids, LCPUFAs (1020mg DHA + 180mg EPA + 9mg vitamin E) as fish oil capsules. Plus individualized dietary counselling aimed at reducing n-6:n-3 ratio Control group: General recommendations regarding healthy nutrition during pregnancy	Body size & composition
3	Foster et al. (89)	2017	United States	[U] Obese pregnancy	63 (41.3%)	26.6-week gestation to delivery [Mothers]	4 y	[Individual: N_{INT} = 34, N_{CON} = 29] Intervention group: DHA (800 mg/day) supplementation, from 25–29 weeks gestation (mean 26.6 weeks) till end of pregnancy Control group: placebo (corn/soy oil), same timing	Body size & composition

4	Gutierrez-Gomez et al. (58)	2017	Mexico	POSGRAD	524 (45.8%)	18- to 22-week gestation to delivery [Mothers]	4 y	[Individual: N_{INT} = 276, N_{CON} = 248] Intervention group: 400 mg/day DHA (LCPUFA) in capsules Control group: placebo (a mixture of corn and soy oils)	Biomarkers
5	Muhlhausler et al. (90)	2016	Australia	DOMInO	1531 (50.0%)	In utero [Mothers]	5 y	[Individual: N_{INT} = 770, N_{CON} = 761] Intervention group: Three 500-mg capsules DHA-rich fish oil/d (w800 mg DHA/d and 100 mg EPA/d) Control group: Three 500-mg vegetable-oil capsules (without DHA)/d	Biomarkers Body size & composition
6	Rytter et al. (60)	2012	Denmark	[U] Maternal pregnancy fish Oil	180 (54.4%)	30-week gestation to delivery [Mothers]	19 y	[Individual: N_{INT} = 108, N_{CON} = 72] Intervention group 1: Fish-oil capsules (2.7g n-3 LCPUFA/d) "Intervention" group 2: No capsule Control group: Olive oil capsules	Cardiovascular physiology
7	Rytter et al. (59)	2011 (1)	Denmark	[U] Maternal pregnancy fish Oil	135 (54.8%)	30-week gestation to delivery [Mothers]	19 y	Same as Rytter et al. 2012	Biomarkers Body size & composition
8	Rytter et al. (61)	2011 (2)	Denmark	[U] Maternal pregnancy fish Oil	180 (53.9%)	30-week gestation to delivery [Mothers]	19 y	Same as Rytter et al. 2012	Biomarkers

9	See et al. (27)	2018	Australia	[U] Infant fish oil	322 (51.6%)	0 to 6 months postpartum [Infants]	5 y	[Individual: N_{INT} = 165, N_{CON} = 157] Intervention group: Daily 650 mg encapsulated n-3 LCPUFA in the form of ethyl esters (280 mg DHA and 110 mg EPA) Control group: Olive oil (66.6% omega-9 oleic acid)	Biomarkers Cardiovascular physiology Body size & composition
10	Vinding et al. (50)	2018	Denmark	$COPSAC_{2010}$	523 (49.0%)	24-week gestation to 1-week postpartum [Mothers]	6 y	[Individual: N_{INT} = 263, N_{CON} = 260] Intervention group: Fish oil: 2.4 g n-3 LCPUFA Control group: Placebo were lookalike control supplementation capsules of olive oil (72% n-9 oleic acid and 12% n-6 linoleic acid	Body size & composition
Single micronutrient supplementation									
1	Belizan et al. (38)	1997	Argentina	[U] Maternal calcium	518 (46.2%)	20-week gestation to delivery	7 y	[Individual: N_{INT} = 257, N_{CON} = 261] Intervention group: 2g/day of elemental calcium Control group: Placebo	Cardiovascular physiology Body size & composition
2	Hawkesworth et al. (Trial 2) [5] (25)	2011	Gambia	[U] Gambia (2 trials)	Trial 2: 389 (NA) (Trial 1 see protein-energy)	20-week gestation to delivery [Mothers]	17 y	[Individual: N_{INT} = 193, N_{CON} = 196] Intervention group: Calcium supplementation (1500 mg/day elemental calcium as 3750 mg calcium carbonate) Control group: Placebo	Cardiovascular physiology Body size & composition

3	Palmer et al. (91)	2019	Nepal	NNIPS-2	290 (47.9%)	In utero till lactation [Mothers]	13 y	[Cluster: N_{INT} = 180, N_{CON} = 90] Intervention group 1: Vitamin A (700ug retinol equivalent) Intervention group 2: Beta-carotene (42mg) Control group: Placebo	Biomarkers
4	Taylor et al. (92)	2015	UK	Aberdeen Folic Acid Supplementation Trial	2928 (NA)	In utero [Mothers]	73 y	[Individual: N_{INT} = 951, N_{CON} = 1977] Intervention group 1: 0.2mg folic acid per day Intervention group 2: 5 mg folic acid per day Control group: Placebo	Clinical and sub-clinical outcomes
Multiple micronutrient supplementation									
1	Ekström et al. (Trial 1) [6] (56)	2016	Bangladesh	MINIMat	Trial 1: 1335 (47.0%) (Trial 2 see other intervention)	In utero [Mothers]	4.5 y	[Individual: N_{INT} = 435, N_{CON} = 900] Intervention group: Multiple micronutrient supplementation (MMS) Control group: Iron (60mg) and folic acid (400 ug) (IFA)	Biomarkers
2	Hiller et al. (93)	2007	Australia	ACT	179 (NA)	Less than 24-week gestation to delivery [Mothers]	8 y	[Individual: N_{INT} = 91, N_{CON} = 88] Intervention group: 1.8g/day of calcium Control group: Placebo	Cardiovascular physiology
3	Mannan et al. (29)	2016	Bangladesh	MINIMat	540 (42.0%)	In utero [Mothers]	9 y	[Individual: N_{INT} = 167, N_{CON} = 373]	Biomarkers Body size & composition

								Intervention group: Multiple micronutrient supplementation (MMS) – 60mg iron, 400 ug folic acid, plus 15 micronutrients Control group: Iron (60mg) and folic acid (400 ug) (IFA)	
4	Stewart et al. (94)	2011	Nepal	[U] Antenatal MMS	545 (NA)	In utero [Mothers]	8 y	[Cluster: 426 total units for 5 arms] Intervention group 1: Folic acid (400 ug) Intervention group 2: Folic acid + iron (60mg ferrous fumarate) Intervention group 3: Folic acid + iron + zinc (30mg zinc sulfate); Intervention group 4: Folic acid + iron + zinc + 11 vitamins and minerals (10 mg vitamin D as cholecalciferol, 10 mg vitamin E as d-a tocopherol, 1.6 mg thiamine, 1.8 mg riboflavin, 20 mg niacin, 2.2 mg vitamin B-6, 2.6 mg vitamin B-12, 100 mg vitamin C, 65 mg vitamin K as phylloquinone, 2.0 mg Cu, 100 mg Mg Control group: vitamin A (retinyl palmitate) 1000 ug RE of preformed vitA	Biomarkers

5	Stewart et al. (54)	2009	Nepal	[U] Antenatal MMS	3524 (NA)	In utero [Mothers]	8 y	Same as Stewart et al. 2011	Clinical and sub-clinical outcomes
Infant and young child feeding, and milk supplementation									
1	De Jong et al. (39)	2011	Netherlands	The Groningen LCPUFA study	341 (48.1%)	0 to 8 months postpartum [Infants]	9 y	[Individual: N_{INT} = 91, N_{CON} = 250] Intervention group: Breastfeeding (BF) Intervention/control group: LCPUFA-supplemented formula group (LF): The LCPUFAs were provided as mix of phospholipids (15%) and triglycerides (85%) to mimic the composition of breast milk. Control group: Standard formula group (CF): Standard formula consisted of Nutrilon Premium	Cardiovascular physiology Body size & composition
2	Forsyth et al. (24)	2003	4 European countries: UK, Italy, Belgium, & Germany	[U] LCPUFA infant formula	147 (46.9%)	0 to 4 months postpartum [Infants]	5 y	[Individual: N_{INT} = 71, N_{CON} = 76] Intervention group: LCPUFA-supplemented infant formula Control group: Un-supplemented infant formula	Cardiovascular physiology
3	Gruszfeld et al. (46)	2016	5 European countries:	CHOP	183 (48.6%)	0 to 12 months postpartum	5 y	[Individual: N_{INT} = 86, N_{CON} = 97]	Body size & composition

			Belgium, Germany, Italy, Poland, & Spain			[Infants]		Intervention group: high-protein formula (2.05 g/dl for infants, and 3.2 g/dl as follow-up formula) (HP) Control group: low-protein formula (1.25 g/dl for infants, and 1.6 g/dl as follow-up formula) (LF), equal energy as HP group Also has breastfeeding (BF) as observational group	Clinical and sub-clinical outcomes
4	Gruszfeld et al. (95)	2015	5 European countries: Belgium, Germany, Italy, Poland, & Spain	CHOP	183 (48.6%)	0 to 12 months postpartum [Infants]	5 y	[Individual: N_{INT} = 92, N_{CON} = 91] Same as Gruszfeld et al. 2016	Biomarkers Cardiovascular physiology Body size & composition
5	Kennedy et al. (44)	2010	UK	[U] UK preterm LCPUFA	107 (47.7%)	0 to 9 months postpartum [Preterm infants]	10 y	[Individual: N_{INT} = 50, N_{CON} = 57] Intervention group: LCPUFA-supplemented infant formula Control group: Un-supplemented infant formula	Cardiovascular physiology Body size & composition
6	Kramer et al. (77)	2007	Belarus	PROBIT	13889 (48.3%)	During postpartum stay in hospitals [Mothers]	6.5 y	[Cluster: N_{INT} = 16, N_{CON} = 16] Intervention group: Based on the Baby-Friendly Hospital Initiative to promote and support breastfeeding,	Cardiovascular physiology Body size & composition

								particularly among mothers who have chosen to initiate breastfeeding Control group: The control maternity hospitals and polyclinics continued the practices and policies in effect at the time of randomization	
7	Martin et al. (47)	2017	Belarus	PROBIT	13557 (48.5%)	During postpartum stay in hospitals [Mothers]	16 y	Same as Kramer et al. 2007	Cardiovascular physiology Body size & composition
8	Martin et al. (79)	2014	Belarus	PROBIT	13616 (48.5%)	During postpartum stay in hospitals [Mothers]	11.5 y	Same as Kramer et al. 2007	Biomarkers Cardiovascular physiology Clinical and sub-clinical outcomes
9	Martin et al. (78)	2013	Belarus	PROBIT	13879 (48.5%)	During postpartum stay in hospitals [Mothers]	11.5 y	Same as Kramer et al. 2007	Body size & composition
10	Singhal et al. (48)	2010	UK	[U] SGA cohort	243 (55.2%)	Trial 1 – 0 to 9 months postpartum;	8 y	Trial 1: [Individual: N_{INT} = 70, N_{CON} = 83] Intervention group 1: Nutrient-enriched formula, 28% more protein and 6% more energy	Body size & composition

						Trial 2 – 0 to 6 months postpartum [Small for gestational age infants]		than control formula, plus more micronutrients Control group 1: Standard term formula Trial 2: [Individual: N_{INT} = 41, N_{CON} = 49] Intervention group 2: Nutrient-enriched formula, 43% more protein and 12% more energy than control formula, plus more micronutrients Control group 2: Standard term formula Also has breastfeeding group as an observational reference	
11	Singhal et al. (80)	2004	UK	[U] Preterm cohort	216 (50.5%)	Postpartum till weight reached 2000 g or was discharged home (median 4 weeks) [Preterm infants]	16 y	Trial 1: [Individual: N_{INT} = 66, N_{CON} = 64] Intervention group 1: Banked breastmilk from donation Control group 1: Nutrient-enriched preterm formula, enriched in protein (20g) and fat (45g) but not carbohydrate (70g/L) Trial 1: [Individual: N_{INT} = 44, N_{CON} = 42] Intervention group 2: Standard term formula (15g protein, 38 g fat, 70g/L carb)	Biomarkers

								Control group 1: Nutrient-enriched preterm formula, enriched in protein (20g) and fat (45g) but not carbohydrate (70g/L)	
12	Singhal et al. (96)	2003	UK	[U] Preterm cohort	216 (50.5%)	Postpartum till weight reached 2000 g or was discharged home (median 4 weeks) [Preterm infants]	16 y	Same as Singhal et al. 2004	Biomarkers
13	Singhal et al. (32)	2002	UK	[U] Preterm cohort	216 (50.5%)	Postpartum till weight reached 2000 g or was discharged home (median 4 weeks) [Preterm infants]	16 y	Same as Singhal et al. 2004	Biomarkers
14	Singhal et al. (40)	2001	UK	[U] Preterm cohort	216 (50.5%)	Postpartum till weight reached 2000 g or was discharged home (median 4 weeks)	16 y	Same as Singhal et al. 2004	Cardiovascular physiology

15	Toftlund et al. (33)	2018	Denmark	[U] Preterm cohort	235 (48.5%)	0 to 4 moths postpartum [Preterm infants]	6 y	[Individual: N_{INT} = 71, N_{CON} = 164] Intervention group: Breastfeeding Control group: preterm formula feeding Also have comparison within breastfeeding group: fortified vs. unfortified human milk	Biomarkers Cardiovascular physiology Body size & composition
16	Totzauer et al. (49)	2018	5 European countries (Belgium, Germany, Italy, Poland, & Spain)	CHOP	440 (47.6%)	0 to 12 months postpartum [Infants]	6 y	Same as Gruszfeld et al. 2016	Body size & composition
17	Weber et al. (51)	2014	5 European countries: Belgium, Germany, Italy, Poland, & Spain	CHOP	518 (NA)	0 to 12 months postpartum [Infants]	6 y	Same as Gruszfeld et al. 2016	Body size & composition Clinical and sub-clinical outcomes
18	Williams et al. (97)	2012	UK	BCG	569 (45.4%)	0 to 5 y [Mothers and infants]	27 y	[Individual: N_{INT} = 531, N_{CON} = 38] Intervention group: Free (cow) milk supplements [through the provision of tokens equating to a half-pint (284 ml) of milk per	Biomarkers

								day] for pregnant women and their infants up to 5 years Control group: Breastfeeding only	
Dietary counselling									
1	Costa et al. (98)	2017	Brazil	[U] São Leopoldo dietary counselin g	305 (43.6%)	0 to 1 y [Mothers]	8 y	[Individual: N_{INT} = 126, N_{CON} = 179] Intervention group: During each home visit, mothers received dietary advice in accordance with the baby's age. Control group: standard care	Biomarkers
2	Hakanen et al. (52)	2006	Finland	STRIP	585 (NA)	7 months to 10 y [Mother and children]	10 y	[Individual: original N_{INT} = 540, N_{CON} = 522] Intervention group: received individualized dietary counseling at 1- to 3-month intervals until the child was 2y and biannually thereafter. The main focus was on replacing intake of saturated fat with unsaturated fat Control group: Basic health education routinely given at Finnish well-baby clinics and by school health care. Biannually until 7y and annually thereafter	Clinical and sub-clinical outcomes

3	Kaitosaari et al. (69)	2006	Finland	STRIP	167 (50.9%)	7 months to 9 y [Mother and children]	9 y	Same as Hakanen et al. 2006	Biomarkers
4	Lehtovirta et al. (35)	2018	Finland	STRIP	450 (48.4%)	7 months to 20 y [Mother and children]	20 y	Same as Hakanen et al. 2006	Biomarkers
5	Niinikoski et al. (71)	2012	Finland	STRIP	446 (46.8%)	7 months to 19 y [Mother and children]	19 y	Same as Hakanen et al. 2006	Biomarkers
6	Niinikoski et al. (41)	2009	Finland	STRIP	524 (48.1%)	7 months to 15 y [Mother and children]	15 y	Same as Hakanen et al. 2006	Cardiovascular physiology
7	Nupponen et al. (53)	2015	Finland	STRIP	514 (49.0%)	7 months to 20 y [Mother and children]	20 y	Same as Hakanen et al. 2006	Clinical and sub-clinical outcomes
8	Oranta et al. (70)	2013	Finland	STRIP	518 (48.1%)	7 months to 20 y [Mother and children]	20 y	Same as Hakanen et al. 2006	Biomarkers Body size & composition
9	Pahkala et al. (55)	2013	Finland	STRIP	394 (45.2%)	7 months to 19 y [Mother and children]	19 y	Same as Hakanen et al. 2006	Clinical and sub-clinical outcomes
10	Raitakari et al. (42)	2005	Finland	STRIP	369 (50.7%)	7 months to 19 y	19 y	Same as Hakanen et al. 2006	Biomarkers

						[Mother and children]			Cardiovascular physiology Body size & composition
11	Simell et al. (37)	1999	Finland	STRIP	748 (48.1%)	7 to 36 months [Mother and children]	3 y	Same as Hakanen et al. 2006	Biomarkers Body size & composition
Other interventions									
1	Ekström et al. (Trial 2) [6] (56)	2016	Bangladesh	MINIMat	Trial 2: 1335 (47.0%) (Trial 1 see multiple micronutrients)	In utero [Mothers]	4.5 y	[Individual: N_{INT} = 672, N_{CON} = 663] Intervention group: Early timing of invitation (immediately after detection of pregnancy) to food supplement, 608 kcal/day, 6 days a week Control group: Usual timing of invitation (around 20-week gestation) to food supplementation	Biomarkers
2	Luoto et al. (99)	2010	Finland	[U] Probiotics Study	113 (39.8%)	4 weeks before delivery till 6 months postpartum [Mothers and infants]	10 y	[Individual: N_{INT} = 54, N_{CON} = 59] Intervention group: Probiotic supplementation: 1 1010 colony-forming units of Lactobacillus rhamnosus in capsules once a day for 4 weeks before expected	Body size & composition Clinical and sub-clinical outcomes

								delivery. After delivery, given either to the mothers (if breastfeeding), or to the children mixed in water for 6 months Control group: microcrystalline cellulose	
3	Videhult et al. (100)	2015 (1)	Sweden	[U] Probiotics Study	120 (56.6%)	4 to 13 months postpartum [Infants]	8.8 y	[Individual: N_{INT} = 58, N_{CON} = 62] Intervention group: daily intake of cereals with probiotic LF19 (*Lactobacillus paracasei ssp. paracasei* strain F19) Control group: daily intake of cereals without LF19	Biomarkers Body size & composition
4	Videhult et al. (101)	2015 (2)	Sweden	[U] Probiotics Study	120 (56.6%)	4 to 13 months postpartum [Infants]	8.8 y	Same as Videhult et al. 2015 (1)	Biomarkers

[1] The studies were ordered by type of intervention, then listed in alphabetical order (A to Z), followed by chronological order (newer to older). Some studies were in more than one intervention categories, but they were only included in one category based on the most dominant feature of the study (e.g., infant feeding with LCPUFA-enriched infant formula could be in categories infant formula and macronutrient supplementation, but was only presented as IYCF study)

[2] Cohort abbreviations: when marked "[U]", it means unofficial study name for the purpose of this review only. The rest are official cohort study names, including: ACT, Australian Calcium Trial; BCG, the Barry-Caerphilly Growth Study; CHOP, the European Childhood Obesity Project; $COPSAC_{2010}$, Mother-child cohort Copenhagen Prospective Studies on Asthma in Childhood 2010; DOMInO: DHA to Optimize Mother Infant Outcome Trial; ICDS, Integrated Childhood Development Services; INFAT, Impact of Nutritional Fatty Acids during Pregnancy and Lactation on Early Human Adipose Tissue Development; MINIMat, Maternal and Infant Nutrition Interventions in Matlab Trial; NNIPS-2, Nepal Nutrition Intervention Project – Sarlahi;

POSGRAD, the Prenatal Omega-3 Fatty Acid Supplementation, Growth, and Development Trial; PROBIT, the Promotion of Breastfeeding Intervention Trial; STRIP, Special Turku Coronary Risk Factor Intervention Project for Children.

[3] Sample size refers to the main cohort whose cardiometabolic outcomes were assessed (e.g., if maternal intervention, it refers to the offspring). Sample size may differ for different sets of analysis within the same cohort population.

[4] N_{INT}, sample size in intervention group (or number of intervention clusters in cluster randomization; N_{CON}, sample size in control group (or number of control clusters in cluster randomization).

[5] This study reported results of two trials within the same population: 1) protein energy biscuits and 2) calcium supplementation

[6] This study has two sets of interventions: 1) multiple micronutrient supplement, 2) a food-based intervention

Table 2. Risk of bias assessment for included studies

Study	Domains of bias evaluation (low, moderate, or high risk of bias) *					
	Study participants	**Study attrition**	**Prognostic factor measurement**	**Outcome measurement**	**Study confounding**	**Statistical analysis and reporting**
Asserhøj 2009	Moderate	Moderate	Low	Low	Low	Low
Belizan 1997	Low	Moderate	Low	Low	Moderate	Low
Brei 2016	Low	Moderate	Low	Low	Moderate	Low
Costa 2017	Low	Moderate	Low	Low	Low	Low
de Jong 2011	Low	Low	Low	Low	Moderate	Moderate
Ekström 2016	Low	Low	Low	Low	Moderate	Moderate
Forsyth 2003	Low	Moderate	High	High	High	High
Foster 2017	Moderate	Low	Low	Low	Moderate	Moderate
Gruszfeld 2015	Low	Moderate	Low	Low	High	Moderate
Gruszfeld 2016	Low	High	Low	Low	Low	Low
Gutierrez-Gomez 2017	Low	Moderate	Low	Low	Moderate	Low
Hakanen 2006	Low	Moderate	Low	Low	Moderate	Low
Hawkesworth 2008	Low	Moderate	Low	Low	Low	Low
Hawkesworth 2009	Low	Moderate	Low	Low	Low	Low
Hawkesworth 2011	Low	Moderate	Low	Low	Low	Low
Hiller 2007	Moderate	High	Low	Low	Moderate	Moderate
Kaitosaari 2006	Low	Low	Low	Low	Low	Moderate
Kennedy 2010	Low	Moderate	Low	Low	Moderate	Low
Kinra 2008	Low	Low	Low	Low	Low	Low
Kramer 2007	Low	Low	Low	Low	Low	Moderate
Lehtovirta 2018	Low	Moderate	Low	Low	Low	Moderate

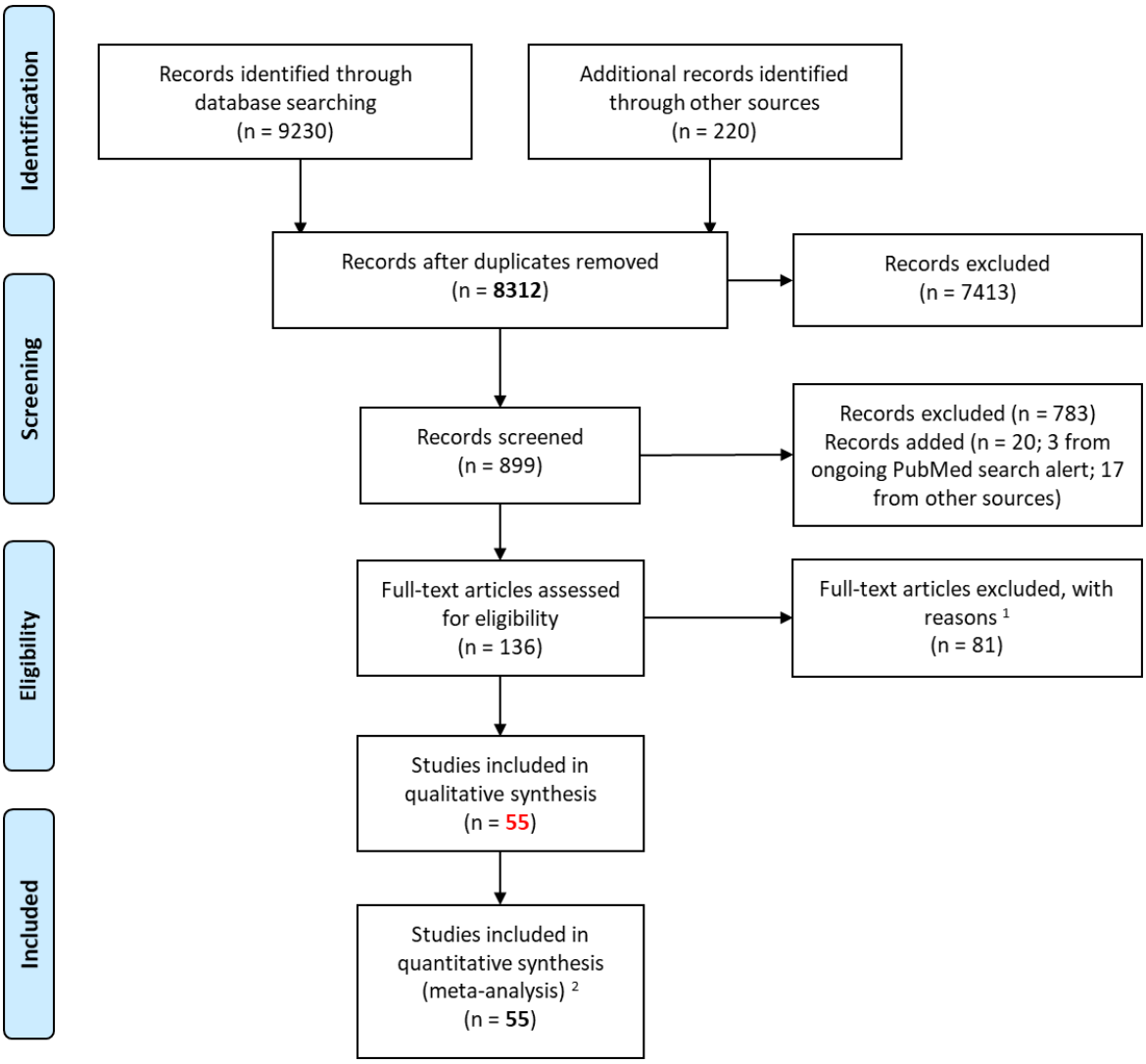
Luoto 2010	Moderate	Low	Low	Low	Moderate	Low
Macleod 2013	High	Moderate	Low	Low	Low	Moderate
Mannan 2016	Low	High	Low	Low	Low	Moderate
Martin 2013	Low	Low	Low	Low	Low	Low
Martin 2014	Low	Low	Low	Low	Low	Low
Martin 2017	Low	Low	Low	Low	Low	Low
Muhlhausler 2016	Low	Low	Low	Low	Low	Low
Niinikoski 2009	Low	Moderate	Low	Low	Moderate	Moderate
Niinikoski 2012	Low	Moderate	Low	Low	Moderate	Moderate
Nupponen 2015	Low	Moderate	Low	Low	Moderate	Low
Oranta 2013	Low	Moderate	Low	Low	Moderate	Moderate
Pahkala 2013	Low	Moderate	Low	Low	Moderate	Low
Palmer 2019	Low	High	Low	Low	Low	Low
Raitakari 2005	Low	Moderate	Low	Low	Moderate	Moderate
Rytter 2011	Low	Moderate	Low	Low	Low	Low
Rytter 2011	Low	Moderate	Low	Low	Low	Low
Rytter 2012	Low	Moderate	Low	Low	Low	Low
See 2018	Moderate	Moderate	Low	Low	Moderate	Moderate
Simell 1999	Low	Low	Low	Low	High	Moderate
Singhal 2001	Moderate	High	Low	Low	Low	Low
Singhal 2002	Moderate	High	Low	Low	Low	Moderate
Singhal 2003	Moderate	High	Low	Low	Low	Low
Singhal 2004	Moderate	High	Low	Low	Low	Low
Singhal 2010	Low	Moderate	Low	Low	Low	Low
Stewart 2009	Low	Low	Low	Low	Low	Low
Stewart 2011	Low	Moderate	Low	Low	Low	Low
Taylor 2015	Low	Low	Low	Low	Low	Low

Toftlund 2018	Moderate	Moderate	Low	Low	Moderate	Moderate
Totzauer 2018	Low	Moderate	Low	Low	Low	Low
Videhult 2015 (1)	Low	Low	Low	Low	Moderate	Moderate
Videhult 2015 (2)	Low	Low	Low	Low	Moderate	Low
Vinding 2018	Low	Low	Low	Low	Low	Low
Weber 2014	Low	Moderate	Low	Low	Low	Low
Williams 2012	Low	Moderate	Low	Low	Low	Low

* Bias assessment followed specific criteria by each domain:

1. Study participants: high bias – the relationship between predictor and outcome is very likely to be different for participants and eligible nonparticipants. Moderate bias – the relationship may be different. Low bias – the relationship is unlikely to be different.
2. Study attrition: high bias – the relationship between predictor and outcome is very likely to be different for completing and non-completing participants. Moderate bias – the relationship may be different. Low bias – the relationship is unlikely to be different.
3. Prognostic factor measurement: high bias – the measurement of the predictor is very likely to be different for different levels of the outcome of interest. Moderate bias – the measurement may be different. Low bias – the measurement is unlikely to be different.
4. Outcome measurement: high bias – the measurement of the outcome is very likely to be different related to the baseline level of the predictor. Moderate bias – the measurement may be different. Low bias – the measurement is unlikely to be different.
5. Study confounding: high bias – the observed effect of the predictor on the outcome is very likely to be distorted by another factor related to the predictor and outcome. Moderate bias – the effect may be distorted. Low bias – the effect is unlikely to be distorted.
6. Statistical analysis and reporting: high bias – the reported results are very likely to be spurious or biased related to analysis or reporting. Moderate bias may be spurious or biased. Low bias – unlikely to be spurious or biased.

Figure 1. PRISMA flow chart study inclusion/exclusion



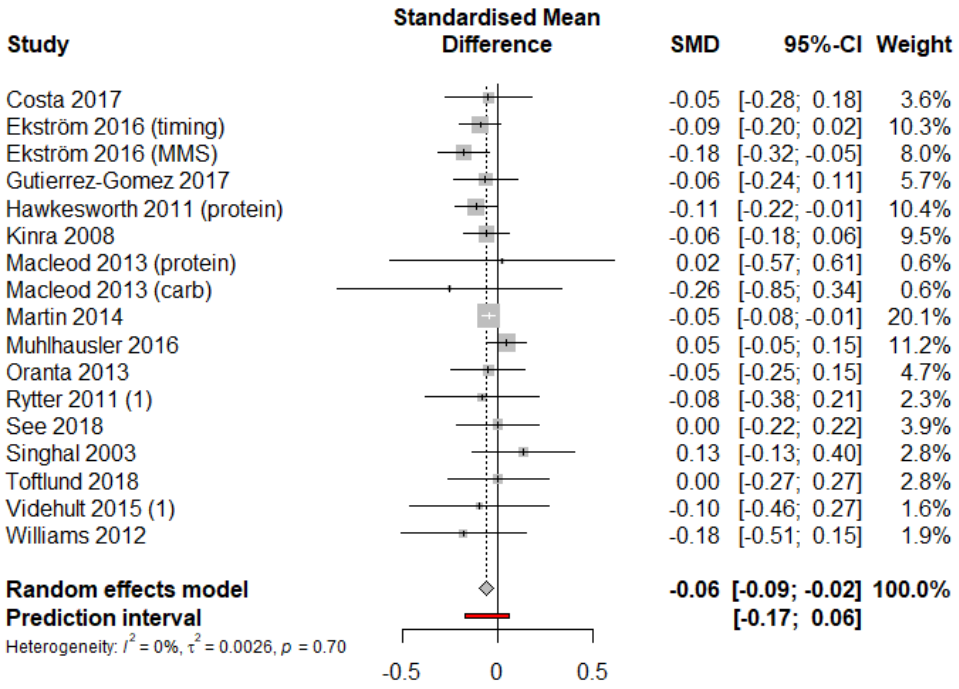
Legend:

1. Reasons for exclusion: not original research article (n = 19); observational studies nested within original RCT cohort (n = 6); exposure was not nutritional intervention (n = 1); exposure not randomized (n = 8); follow-up too short (< 3y) (n = 13); no primary outcome of interest (n = 5); reported maternal outcome but not children's outcome (n = 1); previously un-identified duplicates (n = 3); similar outcomes in a previously included study, but from an earlier time point (n = 1); famine studies or follow-up study of several acute malnutrition in childhood (n = 20); insufficient number of unit in cluster randomization and without appropriate statistical methods (n = 2); the analysis was not based on original assignment (n = 2).
2. For each category of outcome, the number of included studies varied in meta-analysis.
3. PRISMA flow diagram template from: Moher D, Liberati A, Tetzlaff J, Altman DG, The PRISMA Group (2009). Preferred Reporting Items for Systematic Reviews and Meta-Analyses: The PRISMA Statement. PLoS Med 6(7): e1000097.

Figure 2. Map of the world indicating the countries and cohorts included

Figure 3. Meta-analysis forest plots by cardiometabolic outcomes

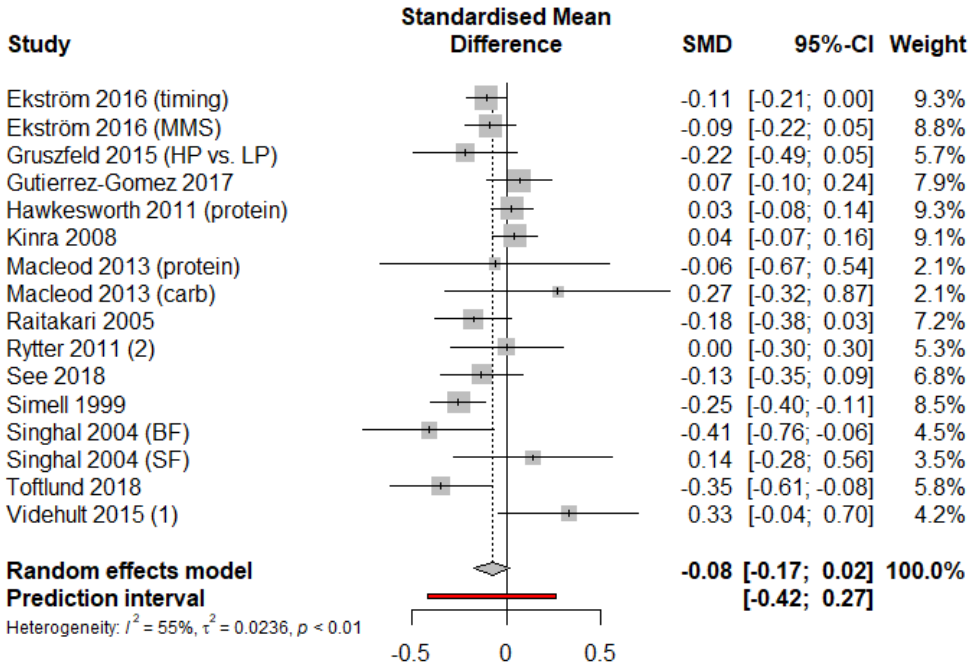
A) Fasting glucose concentration



Legend:

- Ekström: timing referred to timing of invitation to food trial (early vs. late); MMS referred to multiple micronutrient vs. iron and folic acid supplementation
- Hawkesworth: only the first trial (protein-energy biscuits) reported fasting glucose, and not the second trial (calcium supplementation) in this paper
- Macleod: protein referred to protein, carbohydrate, and vitamin vs. vitamin only; carb referred to carbohydrate and vitamin vs. vitamin only
- (1) means the same study identifier, but not a duplicate. See Table 1 for studies with the same IDs.

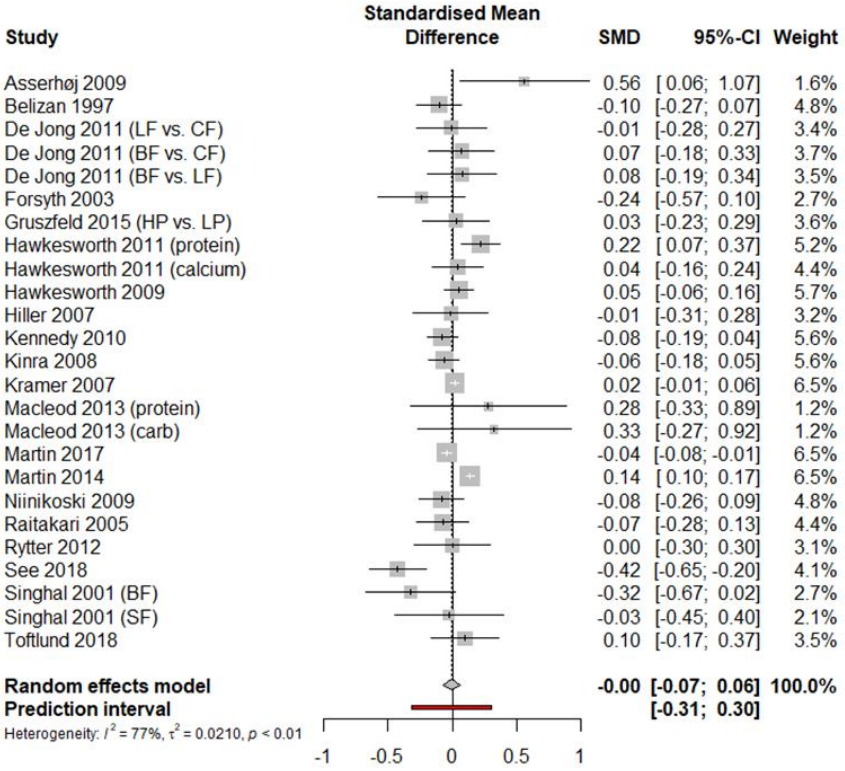
B) Total cholesterol concentration

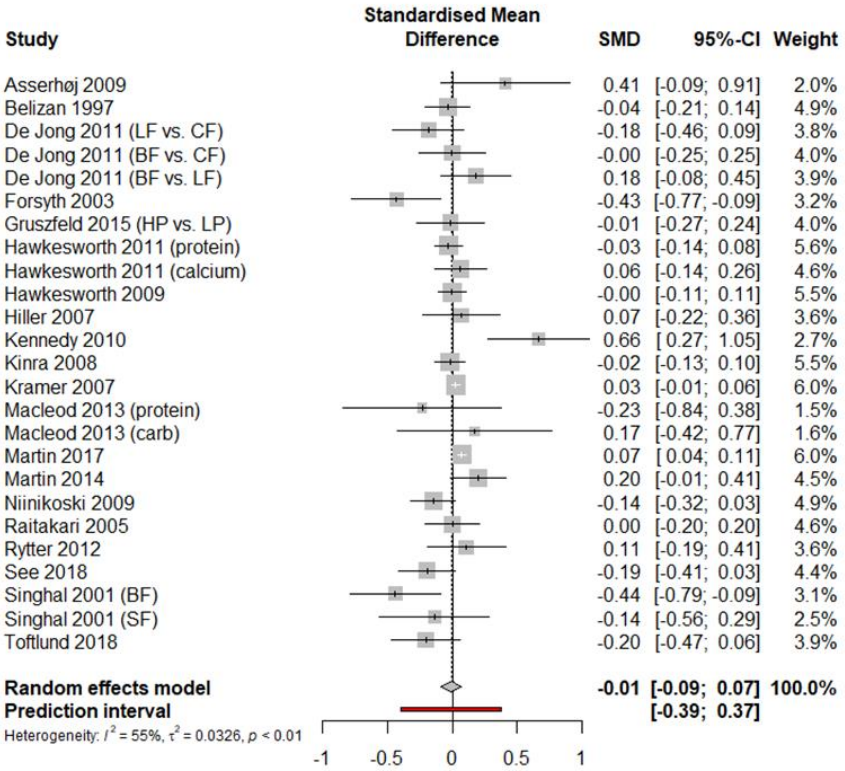


Legend:

- Ekström: timing referred to timing of invitation to food trial (early vs. late); MMS referred to multiple micronutrient vs. iron and folic acid supplementation
- Gruszfeld: high protein vs. low protein infant formula
- Hawkesworth: only the first trial (protein-energy biscuits) reported fasting glucose, and not the second trial (calcium supplementation) in this paper
- Macleod: protein referred to protein, carbohydrate, and vitamin vs. vitamin only; carb referred to carbohydrate and vitamin vs. vitamin only
- (1) or (2) means the same study identifier, but not a duplicate. See Table 1 for studies with the same IDs.
- Singhal: BF referred to banked breastmilk vs. preterm formula; SF referred to standardized term formula vs. preterm formula

C) Systolic (left) and diastolic (right) blood pressure

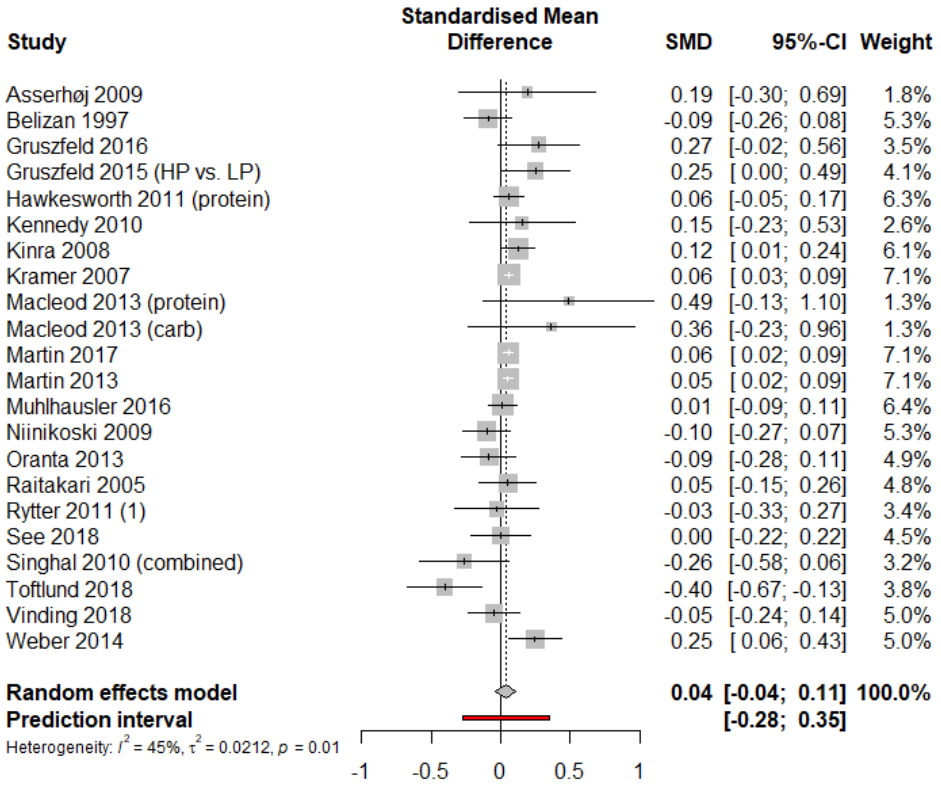




Legend:

- De Jong: LF referred to long-chain PUFA supplemented formula; CF referred to standardized control formula; BF referred to breastfeeding
- Gruszfeld: high protein vs. low protein infant formula
- Hawkesworth 2011: protein referred to the first trial, which was protein-energy biscuits (early vs. late supplementation); calcium referred to the second trial, which was calcium supplementation vs. placebo
- Macleod: protein referred to protein, carbohydrate, and vitamin vs. vitamin only; carb referred to carbohydrate and vitamin vs. vitamin only
- Singhal: BF referred to banked breastmilk vs. preterm formula; SF referred to standardized term formula vs. preterm formula

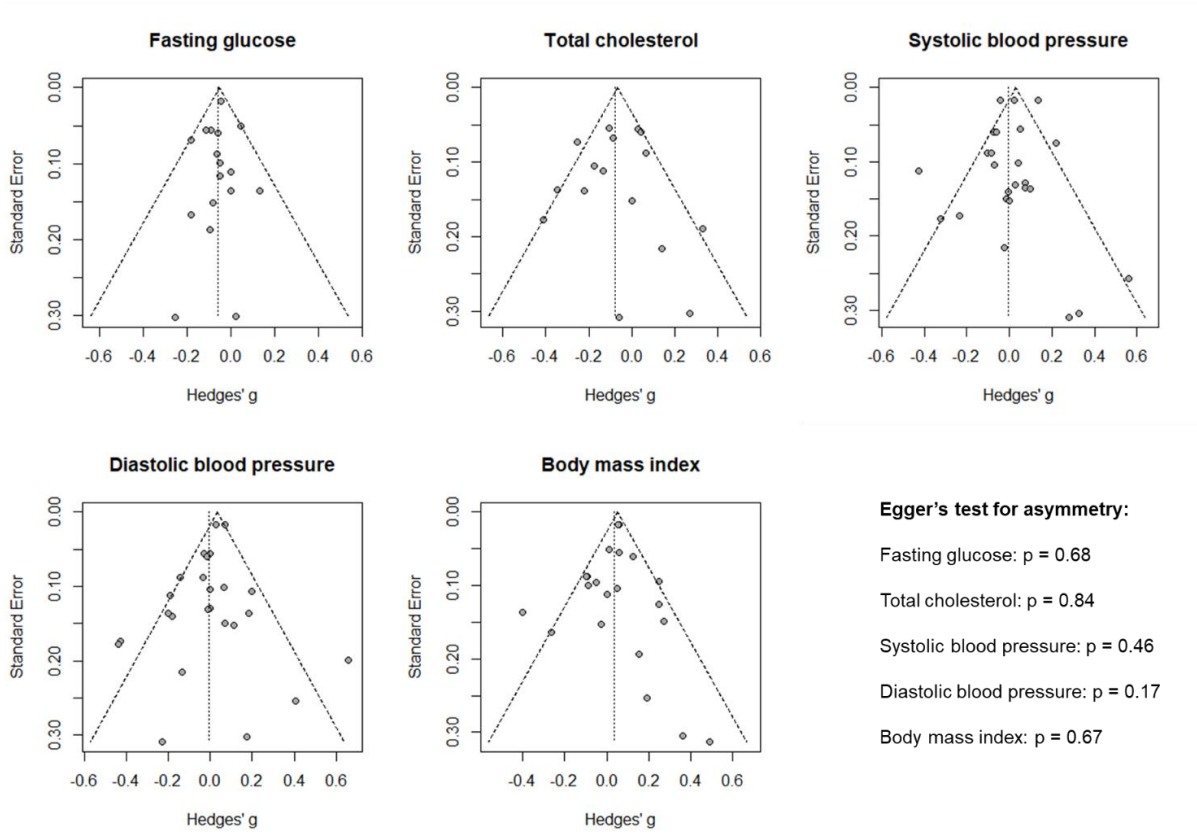
D) Body mass index



Legend:

- Gruszfeld: high protein vs. low protein infant formula
- Hawkesworth: only the first trial (protein-energy biscuits) reported BMI, and not the second trial (calcium supplementation) in this paper
- Macleod: protein referred to protein, carbohydrate, and vitamin vs. vitamin only; carb referred to carbohydrate and vitamin vs. vitamin only
- (1) means the same study identifier, but not a duplicate. See Table 1 for studies with the same IDs.
- Singhal: combined two trials, both compared standardized formula (with different protein and energy content) vs. enriched preterm formula

Supplemental Figure 1: Funnel plots to assess publication bias



Supplemental Table 1 (See Appendix II of the dissertation)

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CHAPTER 3: LITERATURE REVIEW PART II

Cardiometabolic biomarkers

3.1 Introduction

A biological marker, or biomarker, is defined as "*a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to an intervention*" (31, 32). Biomarkers could be biochemical, functional, or clinical indices of status. In nutrition and health sciences research, biomarkers serve to assist in the assessment of dietary exposure, nutritional status, interactions among nutrients, metabolic processes, and nutrition-related health outcomes (26, 33). In this field of research, biomarkers fall into two broad domains: those used to assess the "input" – nutrient intake and nutritional status, and those used to assess the associated "outcomes" – health and disease status. Biomarkers in the latter domain are useful in investigating health status, supporting clinical diagnosis, or predicting future disease risks (26).

The objective nature of biomarkers gives it advantage over self-reported or questionnaire-based data collection (34). To assess nutritional status, for instance, it is much more accurate to measure biomarker levels than to rely on food consumption data (34). Serum hemoglobin, ferritin, or transferrin saturation are much better indicators of iron status than dietary data, even the ones collected though repeated 24-hour recall (35). Similarly, it is more reliable to confirm clinical diagnosis for cardiometabolic diseases based on biomarker cut-off points than to rely solely on clinical symptoms (26). For example, abnormally high glucose concentration (specifically, fasting glucose of 126 mg/dL or higher, or two hours following an oral glucose tolerance test, 200 mg/dL or higher) is regarded as the key diagnostic criteria for diabetes, instead of symptoms alone, such as excessive thirst and frequent urination (36).

In this dissertation work, I mainly focused on biomarkers for health and diseases (the "outcomes" domain). With the global burden of cardiometabolic diseases steadily on the rise, it is necessary to characterize cardiometabolic profiles, detect sub-clinical cardiometabolic perturbations, and to distinguish "healthy" and "unhealthy" cardiometabolic phenotypes through various biomarkers (4). Measuring biomarkers can help fill the gaps in conventional population studies through elucidating underlying biological mechanisms (26, 27). Research hitherto has drawn associations among inflammation, oxidative stress, and an array of cardiometabolic conditions (37-39). For instance, with respect to cardiovascular diseases, not only have we identified key biomarkers (such as oxidized low-density lipoprotein-cholesterol), but also relevant inflammation markers (such as C-reactive protein) (26, 27). My goal is to build upon the existing knowledge to further investigate a panel of cardiometabolic biomarkers.

Many gaps remain unfilled in using biomarkers to assess and predict cardiometabolic diseases. There is a need to further explore the association between early nutrition and long-term health from a perspective of life course epidemiology, with the assistance of existing and emerging biomarkers. We need to elucidate the pathways through which early nutritional exposure affect cardiometabolic health later in life. Biomarkers are well suited in this type of analysis, especially when they have known biological functions. The predictive power of condition-specific biomarkers needs to be evaluated as well, both from fasting steady-state condition and after acute stress condition such as physical activity or meal consumption, and can be used to assess systemic responses (30). Before an individual develop a cardiometabolic condition, their body may go through 'latent' phases when the pathophysiological changes are subclinical. These subclinical changes could be captured in the form of a dynamic response to external stress signals, and may not be detected through a single measurement in the fasted state

(30). Therefore, many researchers proposed that measuring biomarker responses to external metabolic stress is more accurate in predicting cardiometabolic conditions than is measuring the biomarkers from fasting biospecimens (29, 40). This aspect of biomarker needs to be assessed at the population level as well.

In this chapter, I provided an overview of selected cardiometabolic biomarkers. The second section of this chapter presented a brief summary of conventional and emerging biomarkers that, at the fasted state, are indicative of cardiometabolic health and disease status. In the third section, I discussed the importance and added benefit of testing changes in biomarker concentrations in response to external stress signals, such as a meal challenge. In the last section of this chapter, I presented and explained three major conceptual frameworks that guided my dissertation research, at both the population level and the molecular level.

3.2 Cardiometabolic Biomarkers at the Fasted State

Cardiometabolic diseases are interwoven conditions with overlapping biochemical mechanisms, including but were not limited to abdominal adiposity, insulin resistance, dyslipidemia, inflammation, and oxidative stress (4). In my dissertation work, I focused on three major categories of biomarkers are of interest: lipids and apolipoproteins, glycemic markers, and (pro- and anti-) inflammation markers. I would also like to highlight a specific category of markers under the umbrella of inflammation markers: adipose tissue-derived cytokines, or adipokines. The first two columns of Table 3.1 briefly summarized the significance of these cardiometabolic biomarkers.

3.2.a Lipids and Apolipoproteins

Lipids are an important category of cardiometabolic biomarkers due to their predictive strength in cardiovascular disease risks (29, 41). Lipoproteins are transporters of lipids that have an external hydrophilic layer and an inner hydrophobic space that contains varying proportions of triglycerides (TG) and cholesterol esters. As the primary system for the transport and delivery of dietary and endogenously synthesized triglycerides and cholesterol to peripheral tissues, lipoprotein metabolism is highly dynamic in fasted as well as fed states (42).

Lipoproteins are a heterogeneous group of particles. With meal consumption, all newly absorbed fats, including triglycerides (TG), cholesterol, and phospholipids, are packaged into chylomicrons for the delivery of exogenous TG to peripheral tissue. With the unloading of TG, the resulting chylomicron remnants have been suggested to have downstream atherogenic implications (43, 44). In the fasted state, the liver continuously synthesized TG and cholesterol for secretion in the form of very low-density lipoproteins (VLDL), low-density lipoproteins (LDL), and high-density lipoproteins (HDL). As VLDL delivers endogenous TG to peripheral tissues, it is converted to cholesterol-rich LDL. Clinical conditions that are characterized by elevated intrahepatic fatty acids, for instance obesity, type 2 diabetes, and non-alcoholic fatty liver diseases, are associated with increased production of VLDL with subsequent elevations in plasma levels of VLDL and LDL (45). While VLDL and LDL contribute to the delivery of lipids to peripheral tissues, HDL is responsible for the transport of cholesterol from the periphery to the liver, the so-called reverse cholesterol transport. It should be noted that the lipid contents of the various lipoprotein classes are in a dynamic equilibration under the action of several plasma enzymes, including lipoprotein lipase, hepatic triglyceride lipase, cholesteryl ester transfer protein, lecithin-cholesterol acyl transferase and lipoprotein-associated phospholipase A2.

Apolipoproteins (apo) are specific proteins that are part of the hydrophylic coat of lipoproteins and are responsible for mediating the interactions of lipoproteins with various enzymes and surface receptors. ApoA-I is needed to form HDL, which in turn allows for the binding of other antioxidant enzymes. It is notable that apoA-I itself does not have antioxidant properties. ApoB is the major structural apolipoprotein of the class of atherogenic lipoproteins that include VLDL and LDL in the fasted state and chylomicrons in the fed state. There are two forms of apoB, with apoB-100 being synthesized in the liver as part of VLDL/LDL and apoB-48 being synthesized primarily in the intestine as part of chylomicrons. Since apoB-48 is a truncated form of apoB-100 with only 48% of its amino acids, most immunoassays for apoB cannot distinguish between these two forms. Research in apolipoproteins as biomarkers gained popularity due to their disease-specific predictive strengths, and the high population-attributable risks of cardiovascular diseases associated with them (46). Researchers have found that, not only are apolipoprotein better indicators of coronary heart disease risks than any cholesterol index, but the apoB-to-apoA-I ratio is also superior than total cholesterol-to-HDLc ratio in predicting vascular risks (47). This is due to the high variability of the cholesterol contents in lipoproteins, apoB and apoA-I levels therefore reflect the true number of particles available for transport.

Non-esterified fatty acids (NEFA) are free fatty acids that usually have dose-response relationship with insulin signaling. TG must be hydrolyzed into NEFA and monoglycerides in order to go across the endothelium for storage. In the context of high TG concentration (e.g., following a high-fat meal), not all NEFA can move through the endothelium. This can lead to high plasma NEFA, which are bound to albumin and transported back to the liver and become substrate for more hepatic TG and VLDL synthesis. In *fasted* state, low insulin in plasma means that hormone-sensitive lipase (HSL) promotes the utilization of stored TG. This leads to the

release of NEFA and monoglycerides into the circulation, which is then transported back to the liver. In *fed* state, high plasma insulin inhibits HSL, which contributes to net movement for NEFA from chylomicrons to enter the cells for storage. Therefore, NEFA concentration typically reduces following a meal. However, when there is increased insulin resistance, HSL is not sufficiently inhibited in fed state, and the plasma would contain both new NEFA and stored NEFA. Therefore, among individuals with insulin resistance, postprandial NEFA reduction is attenuated, and there may even be an increase in NEFA. This feeds back to the negative cycle: more NEFA flux back to the liver, providing more substrate for endogenous VLDL and TG production. Elevated or insufficient reduction in postprandial NEFA is usually a result of chronic exposure to hypercaloric diet. High NEFA can activate pro-inflammatory genes and serve a potent stimulator of systemic oxidative stress and inflammation, which is core elements to the onset and development of metabolic syndrome and other cardiometabolic conditions (28).

It is important to note that, TG and cholesterol are essential agents to support normal biological functions. It is under abnormal conditions that subsequent cardiometabolic consequences may emerge, such as when the transportation is disturbed, the proportion of "good" (e.g., HDLc) versus "bad" cholesterol (e.g., LDLc) is reduced, the remnants are not efficiently cleared from the circulation (e.g., fasting hyperlipidemia), or when the lipoproteins are chemically altered (e.g., oxidized LDL). For example, LDL remnants, when encounter free radicals in the body, can be altered into oxidized LDL (oxLDL) (48, 49). OxLDL can initiate the atherogenic processes by first causing endothelial injury, which will then recruit monocytes as protective inflammatory responses to the injured sites (49). Afterwards, monocytes morph into macrophages to release inflammatory cytokines and to accumulate cholesterol esters to form

foam cells (48). The long-term downstream consequences include an array of cardiometabolic conditions, such as atherosclerosis, heart attack, and stroke (49).

In sum, assessing the concentrations of lipids and apolipoproteins can provide us with important diagnostic and prognostic information about cardiometabolic status and future risks.

3.2.b Glycemic Measurements

Glucose, the most abundant form of monosaccharide, is a subcategory of carbohydrates, which is one of three major sources of energy for the human body. While many tissues can utilize fat, protein, and carbohydrate as energy sources, a few vital organs such as the brain and red blood cells rely solely on glucose (42).

Glucose metabolism involves multiple metabolically active tissues, including (but is not limited to) the endocrine pancreas, liver, muscle, and the brain. Pancreatic α-, β- and δ-cells secrete glucagon, insulin, and somatostatin, respectively, to participate in intricate glucose regulation. Liver is where the most glucose metabolic processes take place, including gluconeogenesis, glucose storage in the form of glycogen, and pentose phosphate pathway. Uptake of glucose by muscle is important in adjusting glucose concentration in the circulation, and glucose can also be stored as glycogen in the skeletal muscle tissues. Brain consumes approximately 20% of glucose-derived energy to support its vital functions – both neuronal and non-neuronal. Peripheral tissues also use glucose to generate adinosine triphosphate (ATP) to support their respective functions. The update of glucose by peripheral tissue is another essential element in systematic glucose regulation.

Due to its indispensable role as the main source of energy in the human body, blood glucose concentrations are tightly regulated by a complex system. At the fasted state, the concentration is usually 70 to 100 mg/dL (36). Glucose concentration fluctuates after food

consumption, and the fluctuation can be predicated based on empirical data, thus forming the basis for oral glucose tolerance tests (OGTT). Our study design also selected 0h and 2h in relation to a meal challenge as time points for observation based on the OGTT rationale (More details in Chapter 4). While hypoglycemia (low blood sugar) can have a wide range of symptoms and require prevention or treatment, it is beyond the scope of the current work. I mainly focused on the harmful impacts of hyperglycemia and diabetes in this dissertation, as they are among the most common cardiometabolic conditions and are of growing global health concern (50). In population studies, researches can refer to the normal distribution of glucose concentration to identify cut-off points for high blood glucose. According to the American Diabetes Association, hyperglycemia (or pre-diabetes) is defined as fasting plasma glucose ≥ 100 mg/dL and ≤ 125 mg/dL, or two-hour post-challenge plasma glucose level ≥ 140 mg/dL and < 200 mg/dL among participants who were not using diabetic medication. Type 2 diabetes mellitus is defined as a fasting plasma glucose of 126 mg/dL or more, two-hour postprandial glucose of 200 mg/dL or more, or use of diabetes medication (36).

Why is excess glucose detrimental to cardiometabolic health? Apart from the overt symptoms of diabetes (including polyphagia, polyuria, fatigue, and weight loss) that interfere with the normal lives of the patients, the long-term consequences are serious or even fatal (51). Diabetes-related complications range from damage to large and small blood vessels (which can lead to cardiovascular diseases), to kidney failure, ophthalmological symptoms (and even blindness), amputation of limbs due to tissue infection and cell death, and even damage to the nerves (51, 52). At the molecular and biochemical level, excess glucose harms health through many pathways through glucose metabolites: fructose 1,6-bisphosphate can activate the protein kinase C and hexosamine metabolism pathways to accumulate reactive oxygen species (ROS);

glyceraldehyde 3-phosphate can trigger downstream oxidative phosphorylation and glycation; excess glucose itself can also be utilized in the sorbitol metabolism pathway and lead to ROS generation. These pathways converge in apoptosis, oxidative stress, and inflammation, all of which lead to cardiometabolic perturbations and diseases (42).

Insulin is an anabolic hormone that is essential in glucose homeostasis. It is a peptide protein that is synthesized and secreted by the pancreatic β-cells (53). Unlike the tightly controlled range of glucose concentration, the concentration of insulin varies considerably. Its secretion is acutely responsive to elevated blood concentrations of glucose. In healthy individuals, insulin promotes cellular glucose uptake, suppresses hepatic gluconeogenesis, and regulates carbohydrate, lipid, and protein metabolism. Insulin resistance refers to attenuated or diminished biological responses to a normal or even increased insulin level (53). Insulin resistance at the cellular level usually manifests as deficient glucose uptake by muscle and adipose tissue, and increase free fatty acid flux in the liver, which can then be linked to perturbation in lipoprotein production and regulation (see section 3.2.a for more details). Because of the dynamic relationship between insulin and glucose, a classic mathematical model was developed to quantify this association, namely the homeostasis model assessment (HOMA) (54). Insulin resistance (IR) could be reflected by HOMA-IR, and pancreatic β-cell function by HOMA-B. Both indices require fasting glucose and insulin measurements and are useful tools in population studies.

3.2.c Biomarkers for Inflammation

Inflammation biomarkers are of interest in nutrition research because they are a barometer of general inflammatory status. They can both be influenced by dietary components,

as well as in turn affect nutritional biomarker measurements (such as elevating serum ferritin concentration) (55).

Acute inflammation is part of the normal immune response of human body to combat infection, wounds, and any tissue damage (26). The typical signs of acute inflammation include redness, swelling, heat, pain, and loss of function. These symptoms are the results of a common set of cellular pathways that involve the release of inflammatory cytokines. Under normal circumstances, these cytokines perform intended function to assist in tissue repair. They also should be effectively cleared from local tissue and from the circulation in order to prevent further damage to local tissue from the destructive side effects of inflammation. Nevertheless, when the trigger of inflammation is not eliminated or properly controlled, the ongoing inflammation may become pathological.

Chronic inflammation can leave varying degrees of health consequences and is recognized as a key player in the development of cardiometabolic diseases (28, 39). When chronic inflammation is marked by significantly activated inflammatory cells at the site of tissue damage and in the circulation, the resulting diseases have severe clinical manifestations, including inflammatory bowel disease and rheumatoid arthritis. However, when chronic inflammation is of a “lower grade”, with a moderate (but persistent) elevation in systemic inflammatory responses, the resulting cardiometabolic perturbations could take years or decades to manifest in clinical outcomes such as insulin resistance and atherosclerosis (56). This progressive development, difficult to detect in the earlier stages, is usually irreversible in the later stages when clinically diagnosable (26).

Inflammation state is a balance between pro- and anti-inflammatory cytokines, some of which could be altered acutely by environmental stress signals. Pro-inflammatory cytokines

initiate and sustain inflammation. C-reactive protein, a protein of hepatic origin, is essential in host defense and activating acute-phase responses. It is a predictor of cardiovascular diseases, independent of other factors (57). Persistent elevation in CRP can induce oxidation of LDL, activate pro-inflammatory genes, and lead to atherogenic developments. Interleukin-6 (IL-6), an interleukin promptly produced at the site of infection or tissue injury, has potent inflammatory function, including the induction of acute-phase proteins like CRP in the liver (26). Circulating concentration of IL-6 is usually elevated in obesity. Hepatic elevation of IL-6 and free fatty acids influx collectively contribute to systemic inflammation and hepatic insulin resistance. Tumor necrosis factor-α (TNF-α), a cell signaling protein that makes up part of the "acute phase reaction", strongly promotes systemic inflammation. It is expressed on a wide variety of cells and organs to activate acute-phase protein synthesis. It is central to activating the NF-κB pathway (26). Monocyte chemoattractant protein-1 (MCP-1) is a key chemokine that regulates migration of monocytes and macrophages in the early stage of endothelial infiltration. It is highly expressed at the site atherosclerotic plaques and is responsive to various pro-inflammatory cytokine. It is thus predictive of atherogenic risk and plays an important role in obesity and type 2 diabetes (58). Resistin, a peptide produced by adipose tissue, immune cells, and epithelial cells, serves as an important link between obesity and insulin resistance. Its name originated from 'resistance to insulin', and it has since emerged as a biomarker for assessing a range of cardiometabolic risks including insulin resistance, inflammation, and metabolic disturbances (59).

IL-10 is an important anti-inflammation cytokine that is also known as "human cytokine synthesis inhibitory factor". As the name suggests, IL-10 can inhibit cytokine synthesis via inhibition of the NF-κB pathway. It helps decrease obesity, insulin resistance, and leptin

resistance. Interestingly, IL-6 can up-regulate IL-10 secretion, which serves as feedback inhibition to control systemic inflammation (26). The TNF receptors are also protective against (TNF-inducted) inflammation processes. At relatively high concentrations, soluble TNF receptors (e.g., TNFsR II) inhibits TNF functions by competing with membrane-bound TNF receptors. Adiponectin (more details in the following paragraph), a versatile cytokine produced by the adipose tissue, also exert anti-inflammatory effects.

Adipokines are adipose tissue-derived cytokines. Leptin and adiponectin are the most widely studied adipokines (60). A few cytokines mentioned previously are also derived from adipose tissue, including TNF-α, MCP-1, and resistin (61). When leptin was first discovered in animal models, the scientific community hoped that it would be the ultimate "cure" for obesity, resembling how the discovery of insulin transformed the treatment for diabetes (62, 63). Nevertheless, leptin is a versatile hormone that bridges the immune system and neuroendocrine system. Apart from its anorexigenic function in energy homeostasis that decreases energy intake and increases energy consumption, it is also now recognized as inflammatory mediator (64). Circulating level of leptin increase in proportion to overall adiposity, but it is also affected by the type and location of adipose tissue. In normal conditions, leptin participates in glucose homeostasis, improves insulin function, and decreases dyslipidemia. When leptin resistance is developed (usually in obesity), however, there is a lack of anticipated salutary metabolic outcomes due to the inability of leptin to send appropriate signals to the brain (65).

Adiponectin circulates in much higher concentrations than leptin. Contrary to leptin, adiponectin has negative correlation with adiposity. It is observed that the concentration of adiponectin also decreased before the development of type 2 diabetes. Adiponectin enhances insulin sensitivity and has anti-inflammatory and anti-atherogenic effects (66). Measuring the

concentrations of leptin and adiponectin together can help established a balanced view of the adipokines in the system. Leptin-to-adiponectin ratio is also regarded as a marker for atherogenic risks (67).

Under pathological conditions such as obesity and insulin resistance (the former can also lead to the latter), there is increased fatty acid flux that can trigger inflammatory pathways and increase the circulating concentrations of pro-inflammatory cytokines. When accompanied by decrease in adipokines (adiponectin and leptin), we may observe decreased insulin sensitivity. Additionally, the accumulation of pro-inflammatory macrophages around adipose tissue also activates the NF-κB pathway and cause atherogenic effects. Taken together, the long-term consequences of such prolonged inflammation include type 2 diabetes, cardiovascular diseases, non-alcholic steatohepatitis, and other cardiometabolic conditions. Measuring circulating levels of inflammation biomarkers can be informative in determining systemic inflammation status and in predicting future cardiometabolic risks (26).

3.3 Stress-Induced Responses in Biomarkers

A nutritionally balanced diet supports growth, promotes physical strength, enhances mental health, and positively influences the psycho-neuro-endocrinological processes (30). On the flip side of this coin is the pathophysiological damage an imbalanced diet can cause. Researchers have postulated that, repeated exposure to fat- and sugar-containing diets (e.g., the dietary patterns characterized as “Western”) may accumulate systemic insults. Oxidative stress and inflammation may be triggered by remnants of macronutrients in excess, or the inability to perform intended functions due to deficiency in essential micronutrients (28).

How does nutrition determine health or diseases? Four major mechanisms have been identified that link nutrition and cardiometabolic disturbances: metabolism, inflammation, oxidative stress, and psychological stress (30). International scientific organizations have set forth standards to help diagnose diseases and identifying future disease risks using many biomarkers (36, 68). The cut-off points for disease classification, however, is often debated, especially given the associated implications. For instance, hyperglycemia, when referred to as 'pre-diabetes', may be interpreted by the patients as an irreversible first step in developing type 2 diabetes (69). This may obstruct the lifestyle changes and medical attention needed to attenuate the condition (69). Apart from the convenience for clinical diagnosis, it is unadvisable to characterize cardiometabolic profiles using solely biomarkers at the fasted state. Fasting biomarkers tend to reflect a snapshot of the "resting" status without accounting for the regulatory and compensatory mechanisms that maintain system homeostasis (70).

Fixed cut-off points do not take into consideration the phenotypic flexibility of the human body, which refers to the metabolic adaptation that allows changes within a reasonable range in response to external stress signals (71). These adaptive responses have considerable clinical implications. For example, our blood pressure is considered normal so long as it is more than 120 over 80 mmHg and less than 140 over 90 mmHg. Similar to the long-standing recognition of homeostasis in medical research, phenotypical flexibility is more appropriate in nutrition research because it can help identify an acceptable range of certain biomarkers. It is also useful in characterizing healthy and resilient cardiometabolic phenotypes(70, 71). The responses of biomarkers to external stress is in line with the concept of phenotypic flexibility. It is not the resting status that matters the most, it is whether, and how fast, can the body return to homeostasis. The changes, both in amount and in timing, can provide rich information on the

cardiometabolic health and readiness of the body to respond to external stress while maintaining homeostasis (30).

Relatively little is known, even under controlled clinical conditions, about the dynamic responses of various biomarkers and their indication of health (28, 29). In controlled laboratory settings, stress-based methods have been developed to test the range of phenotypic flexibility, such as metabolic stress models (for instance a meal challenge), infectious stress models (such as an early-response vaccination test), and tissue damage models (such as an physical activity and ultraviolet exposure test) (71, 72). A meal challenge model that mimics external dietary stress is particularly ideal in studying metabolic responses (71). A meal challenge commonly involves administering a meal of known nutritional composition and assessing biomarker changes from pre- to post-meal challenge. In this type of dynamic models, information at several levels are of value – the fasting status, the peak of reaction, and the time course trajectory (if data from multiple time points are available) (71).

In conventional metabolic stress models, researchers usually focus on one nutrient and collect biomarkers known to be directly affected by intake of this nutrient, such as the glucose tolerance test (73). Mounting evidence suggests that researchers need to sufficiently characterize responses in the whole metabolic system to mixed-component (and not just single-nutrient) meal challenges (30). For one, humans rarely consume one nutrient at a time, but rather a meal of composite macro- and micro-nutrient profile. For another, even the observed response in a single biomarker may be a reflection of interaction among a few nutrients (74). It is necessary to focus on interlinked biomarkers representing major pathways to characterize the overall metabolic responses, such as lipid and glycemic pathways (75-78). More research is needed to investigate the shift in these biomarkers under disease conditions. We also lack definitive evidence when it

comes to markers of inflammation, a process that leads to various cardiometabolic conditions under pathological circumstances.

In response to a high-fat and high-sugar diet, postprandial hyperglycemia and hyperlipidemia are the core components contributing to unfavorable cardiometabolic changes, involving mechanisms such as inflammation and oxidative stress (28, 40). Postprandial hyperglycemia may be more predictive of future cardiometabolic events than fasting plasma glucose or fasting HbA1c value alone (40). Postprandial lipemia was also proposed as a better alternative to fasting triglyceride concentrations as predictor for cardiovascular disease risks (28). Table 3.1 is a brief summary of the cardiometabolic biomarkers introduced in this chapter. I also summarized potential mechanisms of their expected postprandial responses.

Table 3.1 Summary of cardiometabolic biomarkers and expected two-hour postprandial responses

Biomarker	**Significance in Cardiometabolic health**	**Expected Postprandial Response** *	**Potential Mechanisms**
Lipids			
Total cholesterol	• Predictive of cardiovascular disease (CVD) events	=	• The fat content in our meal challenge is not large enough (compared to dietary average of approx. 500 grams) to acutely change total cholesterol concentration.
Triglycerides	• Predictive of CVD events	↑	• Postprandial triglycerides usually show pronounced elevation within an hour

			• Can remain elevated for 5-8 hours following a fat-containing meal.
HDL-cholesterol	• Protective against CVD events • Limits oxidant and inflammatory processes	Slight ↓	• In the short term (e.g., 1-2h) as chylomicrons enter the circulation, they tend to pull apoC-II and C-III away from HDL, leading to HDL clearance.
LDL-cholesterol	• Predictive of CVD events	Slight ↑ or =	• Depending on the fat content, metabolism efficiency, and cholesterol transportation, LDL may have slight two-hour elevation, but more subtle than TG. • At the two-hour time point, we may not capture peak response of LDLc
Non-HDLc	• Predictive of CVD events • Higher postprandial response may indicate increased atherogenic risks	Slight ↓ or =	• If TC and HDLc did not change dramatically, non-HDLc would remain the same. • Higher postprandial response may indicate increased atherogenic risks

Apolipoprotein A-I	• Protective against CVD events	Slight ↑ or =	• As apolipoprotein specific to HDLc, apoA-I should display similar pattern in postprandial change as that of HDLc
Apolipoprotein B	• Predictive of CVD events	Slight ↑	• It may increase with the secretion of chylomicrons, and delayed clearance of VLDL
Non-esterified fatty acids	• Predictive of CVD events • Activates pro-inflammatory genes	↓ or ↑	• In healthy individuals, Postprandial release of free fatty acids from adipocyte is usually suppressed by hormone sensitive lipase (acutely responsive to insulin). • In individuals with insulin resistance, the reduction may be attenuated, and there may even be postprandial increase in NEFA
Glycemic Measurements			
Insulin	• Diabetes/pre-diabetes assessment • Anabolic functions	↑↑	• As a major metabolism regulating hormone, insulin should respond significantly to the meal challenge to achieve glucose lowering effects. • Different patterns were observed by other researchers. The two-hour postprandial time point may not capture the highest level of insulin response. Responses may differ by disease phenotypes
Glucose	• Diabetes/pre-diabetes assessment	↑	• Two-hour postprandial glucose level should gradually return to fasting state. • It may still be elevated but not significantly, so at the two-hour time point we may not capture peak response of glucose

Pro-Inflammation Markers			
Note: these markers would likely respond depending on the state of the individuals (e.g., those with poor metabolic flexibility would expect an increase in pro-inflammation cytokines); It may also depend on the quantity and quality of the experimental meal, compared with their usual diet. The trigger we provided may or may not be sufficient to induce observable changes.			
High-sensitivity C-Reactive Protein	• Acute phase protein • Inflammation marker • Predictive of CVD events	↑	• Rapid reaction to stress signal: it may react to the meal challenge in our study rapidly, and the two-hour time point may not capture peak response of hsCRP • The response also differs by the type of diet (e.g., high fat versus high fiber)
Interleukin-6	• Inflammation marker • Associated with CRP level	↑	• Released by neutrophils & macrophages; partly induced by TNF-a • It triggers liver to release acute phase proteins, such as CRP • Timing for the assessment of IL-6 response is critical, and it varied in previous studies. The two-hour time point may not capture peak response
Leptin	• Catabolic hormone • Satiety regulation (central nervous system)	↑ or =	• As a satiety hormone with catabolic function and pro-inflammatory effects, its level may increase in response to the meal. But exact time frame is unclear

	• Proportional to adiposity mass • Glucose homeostasis		
Resistin	• Important link between obesity, insulin resistance, and type 2 diabetes	↑	• Resistin is an adipocyte-specific hormone, and is an important link between obesity, IR, and diabetes • The two-hour time point may not have captured peak response
Monocyte Chemoattractant Protein-1	• Several diseases are characterized by monocytic infiltration • Important role in obesity and diabetes	↑	• As an important chemoattractant, MCP-1 regulates migration and infiltration of monocytes and macrophages, thus is predictive of atherogenic risk • The two-hour time point may not have captured peak response
Anti-Inflammation Markers			
Interleukin-10	• Anti-inflammation • Inhibits the NFkB pathway	↑	• IL-10 is a potent anti-inflammatory cytokine that can inhibit the NFkB pathway o It helps decrease obesity, insulin resistance, and leptin resistance • May be up regulated to counter postprandial inflammation
Adiponectin	• Anti-inflammation	↓	• As another important adipose tissue-derived cytokine, works together with leptin in

	• Anti-obesity hormone • Sensitive to insulin		metabolism control, and can counter insulin resistance • It may also act against leptin's inflammatory effects, thus is anti-inflammatory • The two-hour time point may not have captured the peak response of adiponectin
Soluble TNF receptor II	• Anti-inflammation • TNF-α is central to activating NF-κB pathway, and its receptor is protective against inflammation	↓	• Soluble TNF receptors act as TNF antagonists, inhibiting TNFa-mediated proinflammatory effects • It may have reduced due to meal-induced inflammation • Maybe related to immunological functions too

* Symbols: "=" no significant change; "↑" postprandial increase (double-arrow denoted significant increase); "↓" postprandial decrease (double arrow denoted significant decrease)

Abbreviations: TC, total cholesterol; TG, triglycerides; HDLc, high density lipoprotein cholesterol; LDLc, low density lipoprotein cholesterol; apo, apolipoprotein; NEFA, non-esterified fatty acid; hsCRP, high sensitivity C-reactive protein; IL, interleukin; MCP-1, monocyte chemoattractant protein 1; TNFsR, soluble TNF receptor II.

3.4 Biomarker-Centered Conceptual Frameworks

I developed three central conceptual frameworks to guide this work. Framework 1 is an overview of the impact of early-life nutritional exposure on current cardiometabolic profile, which was characterized by fasting and postprandial biomarkers that are summarized in this chapter. Framework 2 has a specific focus on the role of leptin in the pathway between early-life

nutritional exposure and long-term risks for obesity and diabetes, using biomarkers assessed at the fasted state. Framework 3 focuses on postprandial hyperglycemia and hyperlipidemia and the associated pathways that eventually lead to cardiometabolic disturbances. I have provided detailed narrative for each of the framework in this section.

3.4.a Conceptual Framework 1

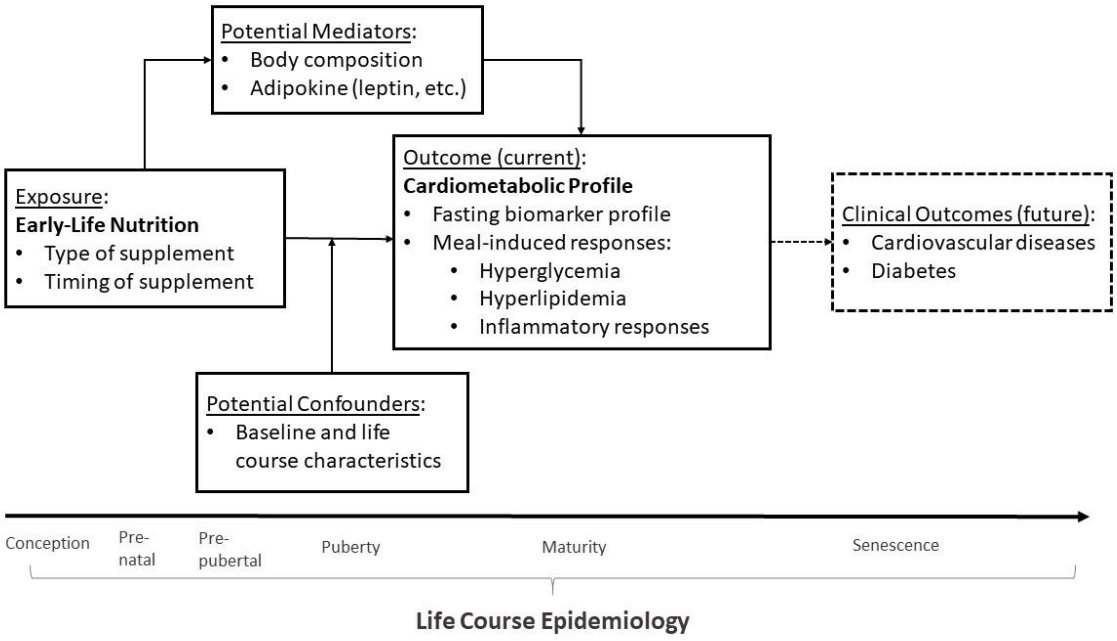


Figure 3.1 Conceptual framework of the cardiometabolic impact of early-life exposure to improved nutrition

This is the core conceptual framework for my dissertation work. At the bottom of the framework is a timeline incorporating the concept of life course epidemiology: early-life exposure to improved nutrition can have life-long impact, and other life course factors also play a role in affecting cardiometabolic outcomes in adulthood. It is a gradual and cumulative process, with early life being a critical period for nutritional investment to achieve long-term health improvements. On the left side, the “exposure” box indicated the nutritional exposure in this

research, which is the timing and the type of early-life nutritional supplementation. More details about the exposure can be found in Chapter 4. Our Main focus for current outcome is cardiometabolic profile, which is both a direct gauge of the cardiometabolic profile and a surrogate indicator for future clinical outcomes. To quantify current cardiometabolic profile, we used information based on both fasting biomarker data and postprandial responses of the biomarkers, individually and collectively. In this framework, we also paid attention to potential mediators and confounders, and the details of statistical methodology used to account for these factors can be found in Chapter 4.

3.4.b Conceptual Framework 2

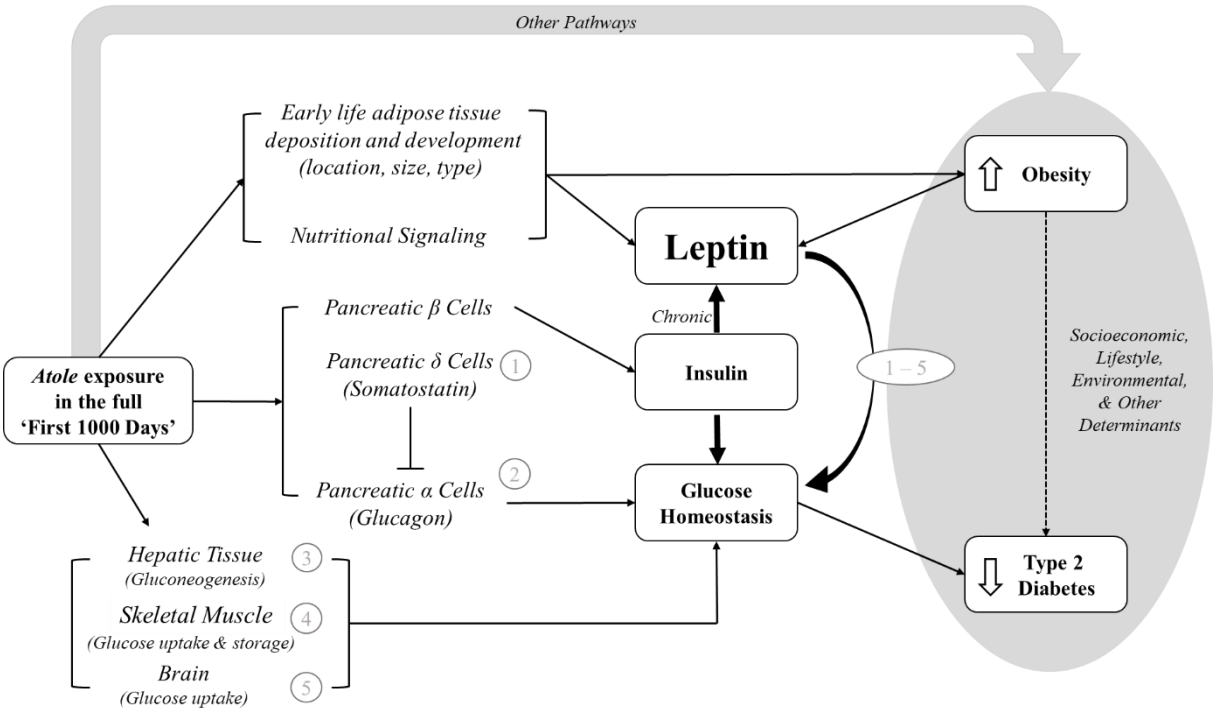


Figure 3.2 Conceptual framework ontogenic impact of early-life nutritional exposure, and the role of leptin in mediating long-term differential impacts on cardiometabolic diseases

The second conceptual framework was designed with a focus on the role of fasting biomarkers. I used this framework to elucidate the differential impact of early-life exposure to improved nutrition on two long-term cardiometabolic diseases: obesity (increased risk associated with the exposure) and type 2 diabetes (reduced risk associated with the exposure), which was observed in a previous study in the same population (16). From left to right, this framework connected four main factors including early-life nutritional exposure, ontogeny (organ development) and its impact on three fasting biomarkers (leptin, insulin, and glucose), the mechanisms through which leptin performs glucose regulation, and cardiometabolic outcomes (obesity and type 2 diabetes).

The "exposure" box referred to early-life (the first 1000 days) exposure to *atole* supplementation (improved nutrition) (17). Exposure to improved nutrition can affect the development of metabolically active tissues: adipose tissue (inside the first set of square brackets component in the graph), the endocrine pancreas (inside the second set of square brackets, with three cell types), hepatic tissue, skeletal muscle, and the brain (the bottom left square brackets) (19, 79-84).

Early life adipose tissue development can be affected by improved nutrition, and changes could be made in the depot (location), size of fat cells, and type of adipose tissue (white versus brown). Adiposity, in combination with nutritional signaling, can affect leptin level, as well as pre-determine future obesity risk. Therefore, leptin could be an indicator for overall adiposity in adulthood. Pancreatic β-cells, important for insulin secretion and indispensable in glucose control, are sensitive to maternal diet. Exposure to improved nutrition in early life may improve insulin function, and insulin can chronically up-regulate leptin levels in both production and secretion. Improved early nutrition can facilitate the development of pancreatic α- and δ-cells as

well, which exerted glycemic regulation through glucagon and somatostatin, respectively. Glucagon raises blood glucose level, whereas somatostatin is a pituitary-secreted hormone that can locally inhibit glucagon release from the pancreas. Skeletal muscle actively participates in glucose storage and utilization. It follows a different development trajectory from that of adipose tissue, and is actually 'in competition' against adipose tissue in early stages because of shared origins from stem cells. It is plausible that exposure to improved nutrition in early life helped guide more stem cells to prioritize the path of myogenesis over adipogenesis, which predetermined adulthood muscle mass and intramuscular fat content. Improved nutrition in early life can affect hepatic tissue as well, which is central to gluconeogenesis and glucose storage. Brain and other peripheral tissues that actively utilize (or depend solely on) glucose may have also benefited from early life exposure to improved nutrition (19, 79-84).

Leptin actively participates in glucose homeostasis and counters insulin resistance through a few mechanisms that are independent of food intake (indicated by the numbers 1 to 5 in the framework). Leptin can reduce lipolysis in the entire body, which leads to lower rates of hepatic gluconeogenesis by reducing substrates to liver. Leptin also has impact on skeleton muscle and other peripheral tissues to increase glucose uptake. Further, based on results from animal studies, leptin may have effects on pancreatic α-cells as an antagonist to glucagon, thus lowering blood glucose concentration. It has also been reported that, through hypothalamic pathways, leptin regulates the release of somatostatin from pancreatic δ-cells, which may indirectly affect glucose concentration. Leptin can also affect glucose metabolism through its direct effects on the central nervous system (19, 79-84).

The other components in the framework were introduced in grey shaded areas. They were not the focus of our biomarker analyses but should be taken into consideration to form a holistic

view. The grey arrow at the top of the graph indicated "other pathways" that are related to social, economic, and lifestyle factors. The grey oval area surrounding the cardiometabolic outcomes indicated that there are many other contributors to obesity and type 2 diabetes. More details about the methods and results related to this conceptual framework can be found in Chapter 5.

3.4.c Conceptual Framework 3

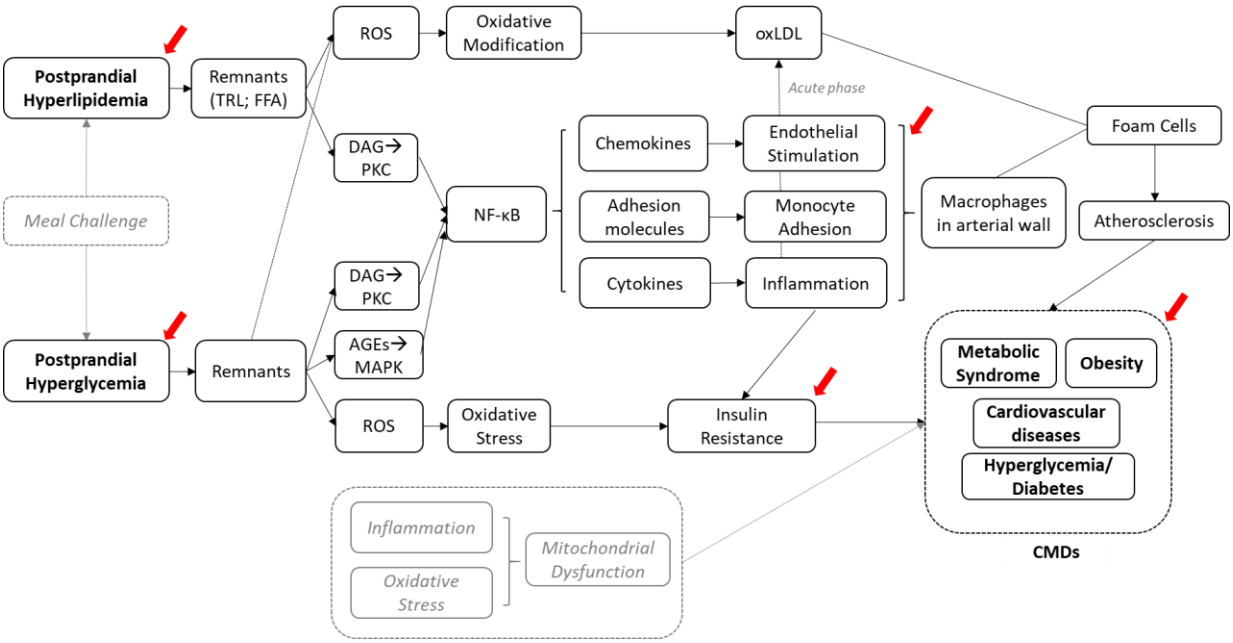


Figure 3.3 From postprandial hyperlipidemia and hyperglycemia to cardiometabolic diseases

Abbreviations: TRL – triglyceride-rich lipoprotein; FFA – free fatty acids; ROS – reactive oxygen species; oxLDL – oxidized low-density lipoprotein; DAG – diacylglyceride; PKC – protein kinase C; NF-κB – nuclear factor kappa-light-chain-enhancer of activated B cells; AGEs – advanced glycation end-products; MAPK – mitogen-activated protein kinase; CMDs – cardiometabolic diseases.

The third conceptual framework focuses on the role of postprandial hyperglycemia and hyperlipidemia in the development of cardiometabolic diseases, through potential mechanisms such as inflammation, oxidative stress, insulin resistance, and endothelial processes. Postprandial

hyperlipidemia and hyperglycemia is normal metabolic responses to a meal challenge. However, if the levels are too high, of if the remnants are not efficiently and effectively cleared from the circulation within a regular time frame, the remnants can trigger downstream pathways (28, 85).

Hyperlipidemia can produce remnants including triglyceride-rich lipoprotein (TRL, the hypertriglyceridemia observed postprandially reflects a collective elevation in chylomicrons, very low-density lipoproteins, and remnants of these particles) and free fatty acids (FFA). They can induce the production of reactive oxygen species (ROS), which when come in contact with low-density lipoprotein, can chemically alter it into oxidized LDL (oxLDL). TRL and FFA can also initiate the protein kinase C (PKC) pathway, which can trigger the nuclear factor kappa light chain enhancer of activated B cells (NFκB) pathway – a potent pro-inflammatory pathway. Similarly, the remnants from hyperglycemia can also produce ROS (which may induce insulin resistance), and can initiate the NFκB processes through both PKC and MAP Kinase signaling pathways. Another message in this framework is that hyperlipidemia and hyperglycemia have additive effects on endothelial and oxidative processes.

The NFκB pathways is multifaceted and complex, and in this framework I only focused on the downstream effects relevant to cardiometabolic perturbations: it can recruit chemokines, adhesion molecules, and cytokines. These agents then lead to endothelial stimulation, monocyte adhesion, and inflammation, all of which contribute to subsequent formation of foam cells in the endothelial wall. Long-term consequences include atherscleros is and associated cardiovascular diseases. Inflammation, which is increasingly recognized an important component of cardiometabolic perturbations, can also contribute to insulin resistance, oxidative stress, mitochondrial dysfunction. The red arrows in the graph indicated the biochemical pathways represented by cardiometabolic-sensitive biomarkers in this research.

CHAPTER 4: METHODOLOGY

4.1 Overview of Methodology

This dissertation work was nested within the Institute of Nutrition of Central America and Panama (INCAP) Longitudinal Study in Guatemala (86). There were four main components: 1) Laboratory assays: fasting and postprandial plasma samples were collected from consented participants (1,112 fasting, 1,027 postprandial samples were included in the final analyses). I was responsible for the analysis of all biomarkers under the auspices of the Biomarker Core Laboratory at Atlanta Veterans Affairs Health Care System. 2) Statistical analyses using fasting biomarkers: We used the difference-in-difference modeling strategy to investigate the impact of early life exposure to improved nutrition on fasting biomarker concentrations in adulthood. We explored pathways that could potentially mediated the early nutrition and adulthood cardiometabolic disease association. We also provided some explanations regarding the differential effect of early nutrition reported by previous studies, with a focus on the diverging impact on obesity (early life exposure to improved nutrition increased the odds for adulthood obesity) and type 2 diabetes (decreased risk). Through this set of analysis, we identified leptin – an adipose tissue-derived hormone – as a key player in glucose homeostasis. 3) Statistical analyses using postprandial responses of biomarkers: we are among the first study in low- and middle-income settings to have collected data on meal challenge-induced changes in biomarkers. We described the dynamic and varied responses of each biomarker to this external stress in controlled lab setting, and further investigated the differences in biomarker responses across strata of cardiometabolic phenotypes. 4) Data-driven exploratory analysis of biomarkers: we used data-driven approaches to reduce the dimensionality of biomarker data, and to identify and ascertain biochemical processes (e.g., glucose metabolism and inflammation). We characterized

the patterns and structural relationships of postprandial biomarker changes. We compared these observations of meal-induced responses across strata of cardiometabolic phenotypes.

4.2 Study Population

This dissertation work is a follow-up study of the Institute of Nutrition of Central America and Panama (INCAP) Longitudinal Study initiated in 1969-77 in Guatemala. The study cohort included residents from four villages (San Juan, Conacaste, Espiritu Santo, and Santo Domingo) in the eastern region of Guatemala (87). These four villages were selected based on population size, relative population density (to allow for adequate sample size and access to centrally located feeding stations), homogeneity in population, and language (due to the language constraints of initial psychometric testing instruments) (86). Guatemala is a lower-middle income country in Central America, bordering Mexico, Belize, Honduras, and El Salvador. Chronic undernutrition in the study area was prevalent in the 1960s to 70s, resulting in high prevalence (45%) of stunting in the participating villages (88). Over the past four to five decades, there has been considerable economic development in the villages where our participants reside, but a nutrition transition was also underway, parallel to the economic advancements in the same areas (89).

The original INCAP study was a randomized controlled trial (1969-77) conducted in the era when protein deficiency was the focus of research on malnutrition in resource-poor settings (86). In the initial trial, a total of 2,392 children were recruited as the core cohort members (our current work has also focused on this core cohort). They were either under the age of seven years at study launch or were born into the villages to mothers who have been enrolled during their pregnancy (87). The randomization was at the village level, and one large village and one small

village were paired per each randomization arm, for a total of four villages. In each village, a nutritional supplement was provided throughout the study duration. The two 'intervention villages' received *atole,* which was a protein- and energy-containing nutritional made from dry skimmed milk, sugar, and *Incaparina* (a vegetable protein mixture developed by INCAP). The two 'control villages' received *fresco*, which was a refreshing beverage that did not contain protein and had low calorie content, with matching micronutrient profile to that of *atole* (86). The supplements were provided twice a day (mid-morning and mid-afternoon to facilitate access and to avoid replacing main meals of the participants), seven days a week for a total of seven years in the selected villages. Study participants could have been exposed to either *atole* or *fresco* through maternal intake (prenatally), breastmilk (younger infants who were breastfed), or through the child's own consumption (no longer exclusively breastfed). Relevant information was fastidiously collected and recorded in this study (86).

Through seven successive follow-up studies (conducted within five study periods), a rich dataset is available from the INCAP Longitudinal Study (16, 25). The most recent study period (2015-17) was initiated by core INCAP researchers upon observing the high prevalence of obesity and diabetes (as well as 'pre-diabetes'). This study period involved the collection of biological samples to assess cardiometabolic disease risks. The hypothesis is that early nutrition investment is associated with long-term cardiometabolic profiles, as assessed by biomarkers.

4.3 Sources of Data

In this dissertation work, we defined improved nutrition in early life as those who received *atole* during the full first 1000 days (from conception to two years of age) (16). Data on timing and type of exposure to the nutritional supplements were available at baseline (1969-77

data), which allows for assessing the effects of different early nutrition exposures on long-term health outcomes, as well as investigating associations of other non-randomized exposures with the outcomes. Determination of the timing of exposure (full exposure during the first 1000 days, partial or no exposure – either too old or too young for full first 1000 day exposure) was presented in Table 4.1. With the open-cohort nature of the initial cluster-randomized controlled trial, cohort members entered the study at different ages, and were exposed for different length of time. To account for this complexity of exposure variable, INCAP researchers have developed a modeling strategy, based on the concept of difference-in-difference (DD) estimation strategy that is commonly used in the field of economics. More details about DD modeling is described in this statistical analysis section) (90).

Table 4.1: Timing of exposure to nutritional supplementations

Start date of trial based on village size	**Too old for full exposure in the first 1000 days**	**Full exposure in the first 1000 days**	**Too young for full exposure in the first 1000 days**
Large villages: Jan 1, 1969	DOB earlier than Sep 24, 1969	DOB between Sep 24, 1969 and Feb 28, 1975	DOB later than Feb 28, 1975
Small villages: May 1, 1969	DOB earlier than Jan 22, 1970	DOB between Jan 22, 1970 and Feb 28, 1975	DOB later than Feb 28, 1975

Note: The trial ended in all four villages (large and small) on Feb 28, 1977. First 1000 days was calculated as from conception to two years of age; and conception was assumed to be 9 months before DOB in exact number of days

We investigated the following cardiometabolic outcomes, including metabolic syndrome, obesity, hyperglycemia (pre-diabetes), and type 2 diabetes mellitus. metabolic syndrome (MetS) was defined as having ≥ 3 of the following five components: 1) abdominal obesity (waist circumference ≥ 88 cm for women; ≥ 102 cm for men); 2) fasting glucose ≥ 100 mg/dL or

diabetic medication use; 3) triglycerides ≥ 150 mg/dL or statin use; 4) HDL-cholesterol < 40 mg/dL in men or < 50 mg/dL in women, and; 5) systolic blood pressure (SBP) > 130 mmHg, diastolic blood pressure (DBP) > 85 mmHg, and/or hypertension medication use (91). Normal weight was defined as body mass index (BMI) between 18.5 and 25 kg/m^2; overweight was BMI between 25 and 30 kg/m^2; and obesity status was defined as BMI ≥ 30 kg/m^2 (92). Hyperglycemia (prediabetes) was defined as fasting plasma glucose ≥ 100 mg/dL and ≤ 125 mg/dL, or two-hour post-challenge plasma glucose level ≥ 140 mg/dL and < 200 mg/dL among participants who were not using diabetic medication (36). Type 2 diabetes was defined as a fasting plasma glucose of 126 mg/dL or more, post-challenge glucose of 200 mg/dL or more, or use of diabetes medication (36).

Relevant sociodemographic data and health information, including age, sex, lifestyle factors, and current health and disease status, are also available from the baseline and subsequent 5 follow-up visits over the past 45 years. Anthropometry and body composition data were collected during the most recent study period and, include weight and height, tricipital, abdominal and subscapular skinfolds, and hip, thigh, and abdominal circumferences. Body mass index (BMI) was calculated as Quetelet Index (weight in kg/ height in m^2). In addition, a more accurate, independentmeasurement of body composition was also available based on total body water measurement as assessed by deuterium oxide dilution. After an overnight fast, the participants were given 30 g dose (safe for all types of patients) of deuterium oxide, which are stable tracers to be tested. The basic assumption of this methodology is that neutral fat does not bind water, nitrogen, or electrolytes, hence allowing for the separated assessment of lean body mass and fat mass (93).

The main domains of data were collected for three generations of cohort members and included: G1 – pregnant and lactating women enrolled in the initial study, whose children were considered the main cohort members; G2 – the main study participants were enrolled as children 0-7 years of age; and G3 – the offsprings of G2. The initial study (1969-77) was designed as a RCT focusing on the influence of intrauterine and preschool malnutrition on behavior, and mental and physical development. The second data collection period (1989-99) focused on adolescents and young adult health. The third data collection period (1991-99) included three sub-studies to investigate birthweight, generational effects, and cardiovascular risk factors. The fourth period (2002-2004) studied human capital and economic productivity, The fifth period (2005-2007) investigated the flow of resources across three generations (87).

The current study (2015-17) includes a comprehensive physical examination with plasma and urine collection to assess cardiometabolic conditions and risk factorsin all available participants of the originalcohort . All living members of the cohort were contacted and invited to join in the current study period, and there were no *a priori* exclusion criteria (except for pregnant women, who were rescheduled to 6 months after delivery). Biological samples are available from 1,115 participants (67% of a traceable sample of 1,661 at the beginning of this study period). A unique feature of the current study period is the ability to assess the acute response to a physiologic meal challenge of mixed composition. Analysis of plasma samples collected at 2hr after meal consumption allows for concurrent assessment of oral glucose tolerance and meal-induced oxidative stress. The freshly prepared challenge was in the form of a liquid shake and consisted of 25 g safflower oil, 52 g sugar, 12 g *Incaprina* powder (a plant-based protein mixture developed by INCAP), and 170 ml lactose-free skim milk. The shake was selected to minimize variability in absorption rate among participants. As quality controls,

several batches of individual shakes prepared on different days were sent to a commercial reference laboratory for detailed composition analysis (Covance Inc., https://www.covance.com). On average each 100 g of the shake had 164.7 cal (31% from fat), containing 3.4 g protein, 25.2 g carbohydrate, and 5.7 g fatty acids, including 1.8 g saturated fatty acids, 3.0 g monounsaturated fatty acids and 0.9 g polyunsaturated fatty acids. Exactly two hours after the meal challenge, the phlebotomist drew venous blood a second time. According to study protocol, those with glucose > 180 mg/dL at fasting state (n =85) were not given the meal challenge for safety consideration.

4.4 Laboratory Methods

The fasting and postprandial plasma samples were aliquoted into cryovials, stored at 4°C and transported on ice to the central laboratory at -80°C within eight hours. All samples were shipped on dry ice to Atlanta, GA, US and stored at -80°C until analysis. One aliquot for 1,115 fasting plasma samples and one aliquot for 1,030 postprandial plasma samples were available for the current analysis. Three women were excluded due to lactation or pregnancy status (which tend to influence body adiposity measurements and biomarker concentrations). The final sample size was 1,112 for fasting plasma and 1,027 for postprandial plasma.

Prior to laboratory assays, we sorted plasma samples into 28 batches in a randomized manner, balanced by residence, location of data collection, and timing of exposure to *atole* or *fresco* (Table 4.2 presented the sorting results). This sorting step prevented overlaying potential systematic bias in the location of data collection (with different personnel) with bias in laboratory batches. It should be noted that while most participants were enrolled at the village of their birth, several were seen at the clinic in Guatemala City or in other villages.

Table 4.2: Sample size based on the laboratory sorting step

Fasting Plasma							
Location of data collection (Hechoen)		**Timing of exposure**					
		Too old for full exposure		**Full exposure in first 1000 days**		**Too young for full exposure**	
		n	%	n	%	n	%
Village 1		54	4.84	54	4.84	31	2.78
Village 2		110	9.87	113	10.13	37	3.32
Village 3		88	7.89	75	6.73	37	3.32
Village 4		68	6.1	55	4.93	28	2.51
5 (Capital)	Birth village = 6 or 14	45	4.04	45	4.04	13	1.17
	Birth village = 3 or 8	14	1.26	21	1.88	7	0.63
6 (Sanarate)	Birth village = 6 or 14	54	4.84	43	3.86	14	1.26
	Birth village = 3 or 8	48	4.3	52	4.66	9	0.81
Postprandial Plasma							
Location of data collection (Hechoen)		**Timing of exposure**					
		Too old for full exposure		**Full exposure in first 1000 days**		**Too young for full exposure**	
		n	%	n	%	n	%

Village 1		52	5.02	54	5.22	30	2.9
Village 2		97	9.37	102	9.86	34	3.29
Village 3		77	7.44	69	6.67	34	3.29
Village 4		62	5.99	53	5.12	28	2.71
5 (Capital)	Birth village = 6 or 14	43	4.15	44	4.25	13	1.26
	Birth village = 3 or 8	13	1.26	19	1.84	6	0.58
6 (Sanarate)	Birth village = 6 or 14	46	4.44	42	4.06	13	1.26
	Birth village = 3 or 8	48	4.64	48	4.64	8	0.77

Note: the sorting process included: 1) calculate the sample size in each category as presented in this table; 2) balancing the characteristics by ensuring in each new batch (total batch=28), we included individuals from almost all cells above; 3) we did the sorting step for fasting sample first, then paired the postprandial samples for the same individual. Therefore, in each batch, sample size may vary (max=80), depending on the availability of postprandial samples.

We characterized cardiometabolic profile using selected biomarkers, including lipid profiles, glycemic markers, and inflammation markers deemed pertinent to the research questions. We assayed lipid profile, glycemic status, and inflammatory status in all available plasma samples. Lipid profile included the following biomarkers: total cholesterol (TC, mg/dL), triglycerides (TG, mg/dL), high-density lipoprotein cholesterol (HDLc, mg/dL), low-density

lipoprotein cholesterol (LDLc, mg/dL), non-HDLc (subtracting HDLc from TC concentration, mg/dL), non-esterified fatty acids (NEFA, Eq/L), apolipoprotein A-I (apoA-I, mg/dL), and apolipoprotein B (apoB, mg/dL). All lipids were assessed using the AU480 Chemical Analyzer (Beckman Coulter Diagnostics, Fullerton CA, US), but the specific methods varied: TC and TG – enzymatic methods (Sekisui Diagnostics P.E.I. Inc., Canada); HDLc and LDLc – homogeneous methods (Sekisui Diagnostics P.E.I. Inc. Canada); ApoA-I and ApoB – immunoturbidimetric assays (Kamiya Biomedical Company, WA, US); NEFA – colorimetric methods (Wako Chemicals Corporation, Richmond VA, US). Glycemic status was assessed by glucose (mg/dL) (enzymatic methods, Sekisui Diagnostics, PA, US), insulin (mIU/L) (immunoturbidimetric assay, Kamiya Biomedical Company, WA, US). Inflammatory status was assessed by fasting plasma levels of several biomarkers, including pro-inflammatory markers such as high-sensitivity C-reactive protein (hsCRP, mg/dL), interleukin-6 (IL-6, pg/mL), leptin (ng/mL), resistin (ng/mL), and monocyte chemoattractant protein-1 (MCP-1, pg/mL); as well as anti-inflammatory biomarkers including interleukin-10 (IL-10, pg/mL), adiponectin (μg/mL), and soluble tumor necrosis factor receptor II (TNFsR, ng/mL). HsCRP was assayed using immunoturbidimetric assay (Kamiya Biomedical Company, WA, USA), and all other cytokines (IL-6, leptin, resistin, MCP-1, IL-10, adiponectin, and TNFsR) were assayed with ELISA kits (Boster Biological Technology, CA, USA). Among these inflammatory markers, leptin, adiponectin, and resistin were adipose tissue-derived cytokines. Table 4.3 summarized the biomarkers assayed in our study.

Table 4.3: List of biomarkers assayed in our study

Biomarker	Unit	Lab Method	Final sample size, n (%)
Lipids			
Total cholesterol (TC)	mg/dL	Enzymatic methods	Fasting: 1112 (99.7 %) Postprandial: 1030 (99.5%)
Triglycerides (TG)	mg/dL	Enzymatic methods	Fasting: 1113 (99.8 %) Postprandial: 1030 (99.5%)
High-density lipoprotein cholesterol (HDLc)	mg/dL	Homogeneous method	Fasting: 1113 (99.8 %) Postprandial: 1030 (99.5%)
Low-density lipoprotein cholesterol (LDLc)	mg/dL	Homogeneous method	Fasting: 1113 (99.8 %) Postprandial: 1030 (99.5%)
Non-HDLc	mg/dL	Calculated as TC minus HDLc	Fasting: 1112 (99.7 %) Postprandial: 1030 (99.5%)
Apolipoprotein A-I (ApoA-I)	mg/dL	Immunoturbidimetric method	Fasting: 1113 (99.8 %) Postprandial: 1030 (99.5%)
Apolipoprotein B (ApoB)	mg/dL	Immunoturbidimetric method	Fasting: 1112 (99.7 %) Postprandial: 1030 (99.5%)
Non-esterified fatty acids (NEFA)	Eq/L	Calorimetric method	Fasting: 1113 (99.8 %) Postprandial: 1030 (99.5%)
Glycemic markers			
Insulin	mIU/L	Immunoturbidimetric assay	Fasting: 1113 (99.8 %) Postprandial: 1030 (99.5%)
Glucose	mg/dL	Enzymatic method	Fasting: 1112 (99.7 %)

			Postprandial: 1030 (99.5%)
Pro-inflammation markers			
High sensitivity C-reactive protein (hsCRP)	mg/dL	Immunoturbidimetric assay	Fasting: 1113 (99.8 %) Postprandial: 1030 (99.5%)
Interleukin-6 (IL-6)	pg/mL	Enzyme-linked immunosorbent assay (ELISA)	Fasting: 347 (31.1 %) Postprandial: 315 (30.4 %)
Leptin	ng/mL	ELISA	Fasting: 1115 (100.0 %) Postprandial: 746 (72.1%)
Resistin	ng/mL	ELISA	Fasting: 1033 (92.6 %) Postprandial: 615 (59.4 %)
Monocyte chemoattractant protein-1 (MCP-1)	pg/mL	ELISA	Fasting: 974 (87.4 %) Postprandial: 900 (87.0 %)
Anti-inflammation markers			
IL-10	pg/mL	ELISA	Fasting: 845 (75.8 %) Postprandial: 741 (71.6 %)
Adiponectin	μg/mL	ELISA	Fasting: 1034 (92.7 %) Postprandial: 616 (59.5 %)
Soluble TNF receptor II (TNFsR)	ng/mL	ELISA	Fasting: 718 (64.4 %) Postprandial: 616 (59.5 %)

4.5 Safety and Confidentiality

Since the work involved only laboratory assays and data analysis, and did not involve direct contact with human subjects, there was no direct potential harm to study participants. Confidentiality was ensured by the following means: all data were de-identified for analytical

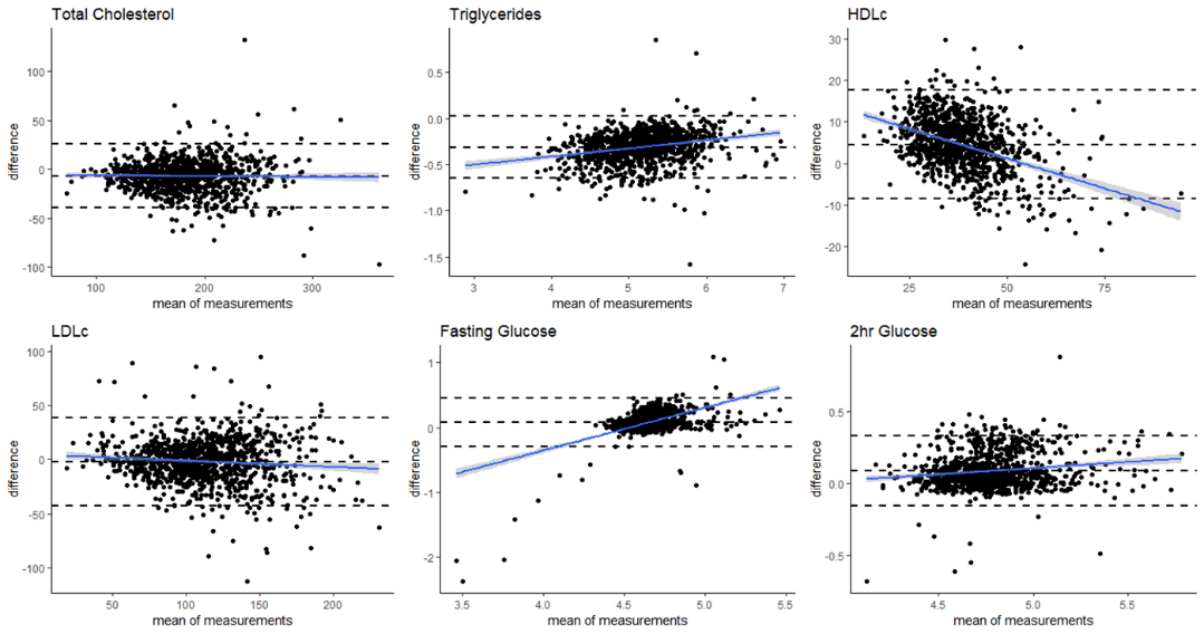
purposes; data were electronically shared through secured online portals (mainly through Emory Box or on a flash drive between IRB-approved researchers); data analysis were all conducted by me (IRB approved doctoral candidate), and were be stored on a password-secured computer that only I could access.

In addition, I received CITI training in laboratory safety, and completed additional certification in various safety protocols required by the Veterans Affairs Medical Center - VAMC Research Safety/Biosafety course and a one-year refresher, including training modules in biological hazards, chemical hazards, physical hazards, emergency response for research, and security in research areas. I also completed additional training through the Talent Management System (TMS), including Intellectual Property Agreement, Occupational Health and Safety training, VA Privacy and Information Security Awareness and Rules of Behavior Course, and the Information 201 course. Because my work involved human studies and contact with human samples, I received safety training in Bloodborne Pathogens. I used laboratory protection when processing human plasma samples.

4.6 Ascertainment of Data Quality

Laboratory Data Quality Control: To ensure strict quality assurance and control of the plasma samples, the following standard quality control (QC) procedures were performed in the laboratory (after samples are received and properly stored): 1) we used a quality control product (pooled plasma sample that was aliquoted and stored at -80oC, and were run together with each batch of study samples) to track potential drift in the analysis over different reagent lots. A quality control log was maintained daily to document routine steps, outliers, abnormal observation, or any mistakes and subsequent corrective measures; 2) QC statistics were calculated daily, by batch, and across every eight batches. Outliers were identified using two sets

of scatterplots: pre-post scatterplot; and %-delta change plot based on fasting concentrations. All outliers were either confirmed (if biologically plausible and reproduceable) or discarded (if implausible or unable to reproduce) by repeating lab procedures for the specific samples. 3) Reliability was examined for the repeated measures of TC, TG, HDLc, LDLc, fasting and postprandial glucose for a random subset of samples in each batch. See Figure 4.1 for a set of Bland-Altman plots to ensure reliability (< 5% of outliers in each plot) between measurements taken in the field sites in Guatemala versus at the VA laboratory in the US.



Note: TG, FBG, and 2hPG data were log-transformed to correct for skewness.

Figure 4.1 Bland-Altman plot for reliability assessment: field measurements compared with VA measurements.

Other Data Quality Control: Electronic data was shared by the Guatemala INCAP team with Emory. I was granted access to the secure shared drive that contained all waves of historical INCAP data. Data quality assurance included the following methods: 1) For data based on field

questionnaires, I first flagged implausible values, and then communicate with the Guatemala data team to compare the electronic data values with those recorded on the paper forms. 2) I performed exploratory data analysis to check ranges and distributions of variables in the data. 3) For all data (including questionnaire and lab-based data), I examined them for plausibility. I was assisted by the field team and Emory colleagues (including the previous doctoral student working with the datasets) to ensure the consistency in our selected variables.

4.7 Statistical Analysis

4.7.a Missing Data

Merged laboratory and field data were checked for missingness for each variable. An Excel file will be maintained to document data missingness. For outcome variables (e.g., all biomarkers), the missingness were used as is in subsequent analyses in order to not bias or neutralize the results. For fasting biomarkers, the ones with over 5% missing included 69.5% for IL-6, 35.6% for TNFsR, 25.7% for IL-10, 13.6% for MCP-1, and 7.4% for resistin. For postprandial biomarkers, missingness (> 5.0%) in women included 71.0% for IL-6, 38.0% for TNFsR, 26.4% for IL-10, 12.7% for MCP-1, 8.8% for resistin, and 8.6% for adiponectin; In men: 69.1% for IL-6, 33.3% for TNFsR, 25.2% for IL-10, 12.7% for MCP-1, 5.0% for resistin, and 5.0% of adiponectin.

For control variables, I used multiple imputation method (“Hmisc” package in R) to account for missingness for the following variables: maternal height (missing 20.5%), maternal age at child birth (missing 1.6%), and maternal education (missing 3.5%). It is notable that in the INCAP study, attrition has been reported in the most recent study wave. Albeit missing not

completely at random, attrition was not shown to bias the association between early-life nutritional exposure and a range of long-term outcomes (16).

4.7.b Power calculation

Power calculation based on expected sample size and modeling assumptions: Since the work was nested within an existing cohort with fixed sample size, we conducted *post hoc* power calculation as follows: we assumed a type I error of 0.05 and specify conservative, two-tailed tests for all calculations. We based the estimation of effect sizes on Cohen's suggestions: 0.02, 0.15, and 0.35 represent small, medium, and large effect sizes (94). We used the G*Power 3 software for the power calculations (95). Based on an expected sample size of 1,112 in with 10 independent variables and an effect size of 0.02, we can expect to achieve 92% power with an F test. Overall conclusion related to power calculation: The focus was to perform *post hoc* power calculation of the interaction term: atole/fresco * timing of exposure. All markers except IL-6 had >80% power for the interaction term to detect at least medium effect size (0.5), for most of the markers, small effect size (0.2). For IL-6, even with full sample (1,112), we are not powered to detect the small effect size.

4.7.c Data Inspection, Transformation, and Univariate Data Analysis

All data were inspected for distributions and ranges. Appropriate presentation methods for distribution were used (mean ± standard deviation for normally distributed variables, and median, inter-quartile range for skewed variables). Transformation (log transformation and z-score calculation) were used when appropriate to: 1) improve distribution; 2) fulfill linear model assumption ("ggfortify" package in R); and 3) convert postprandial biomarker responses (%-delta, calculated as the difference between pre- and post-prandial concentration of each

biomarker divided by their pre-prandial level, presented as a percentage) into z-score (values minus mean, divided by standard deviation) to make the changes comparable across all biomarkers. We refrained from performing multiple transformations on the same variables to ensure interpretability of the outcomes.

In addition, we describe the population in terms of their sociodemographic characteristics and CMD profiles. First, descriptive analyses to summarize key characteristics of the study participants were performed. Comparisons among groups (by sex or by early-life nutritional exposure status) were conducted using the Student's *t* test, $\chi 2$ test, or non-parametric tests (Mann-Whitney *U* test), depending on the type of data. Both fasting concentrations of biomarkers and two-hour changes following the meal challenge were also described.

4.7.d Modeling Strategies

To investigate the association of early-life nutritional exposure and cardiometabolic profile, the selected biomarkers were treated as outcome variables in the following analyses. Crude and adjusted regression analyses were constructed to explore the association. Linear least squared regression was used for continuous outcomes including body adiposity measurements (body mass index, waist circumference, and percent body fat, fasting biomarker concentrations, and postprandial biomarker responses). Logistic regression was used for cardiometabolic diseases and associated phenotypes (obesity, glycemic gradient including normal glycemia, hyperglycemia, and type 2 diabetes, and metabolic syndrome). The modeling strategy is as follows: 1) variable specification; 2) check regression assumptions and assess collinearity among variables; 3) interaction assessment; 4) confounding assessment (following '10%' rule, comparing coefficients with and without confounding terms in the models); and 5) precision assessment (compare confidence intervals). In addition, pooled and stratified (by sex) analysis

were conducted to account for sex-specific differences. In the pooled models, we also tested stratum heterogeneity by adding an interaction term between sex and the key independent variable: the independent (exposure) variable is described in detail in the following paragraph.

Because the initial randomized control trial in the four Guatemalan villages was an open cohort for seven years, the cohort members have entered the study at different time points in their childhood. They were exposed to the two supplements for varying durations, and previous studies found time-dependent differences in the timing (and relatively length) of exposure, emphasizing the window from conception to two years of age. Therefore, a difference-in-difference estimation strategy was used to model the exposure variable based on intent-to-treat analytical principles. For each cohort member, three variables were used for exposure status: 1) exposure to *atole* or *fresco* (because the randomization was at village level, to simultaneously capture the randomization status and village-level effects, we used village identifier in place of the binary variable); 2) timing of exposure: full exposure during the first 1000 days, or partial to no exposure in this period of time; and 3) the interaction term between variables #1 and #2, which estimates the difference-in-difference effect of *atole* vs. *fresco* for a given duration and timing of exposure. Figure 4.2 showed the DD method in the INCAP study.

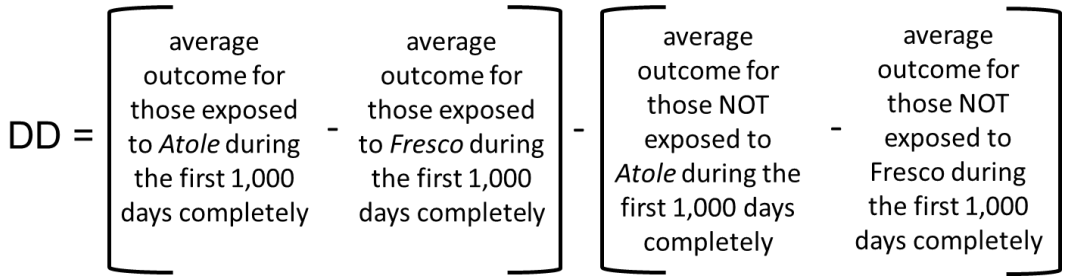


Figure 4.2 Difference-in-difference (DD) modeling strategy in the INCAP study

The model could be represented by the following formula:

$$Y_{i,j} = \alpha + \beta_1 X_i + \beta_2 Z_j + (\gamma_1 V_1 + \ldots + \gamma_m V_m) + \boldsymbol{\delta X_i \times Z_j} + \varepsilon \quad (1)$$

$$P(X) = \alpha + \beta_1 X_i + \beta_2 Z_j + (\gamma_1 V_1 + \ldots + \gamma_m V_m) + \boldsymbol{\delta X_i \times Z_j} + \varepsilon \quad (2)$$

$$\text{Logit P(X)} = ln\left(\frac{P(X)}{1-P(X)}\right)$$

Formula (1) represents linear models, and formula (2) represents logistic models in log odds, or logit form, where $Logit\ P(X) = ln\left(\frac{P(X)}{1-P(X)}\right)$. $Y_{i,j}$ is continuous outcome variables to be modeled (e.g., biomarker levels and postprandial changes) (Eq. 1). $P(X)$ is categorical outcome variables to be modeled (e.g., disease phenotypes) (Eq 2). X_i is variable 1 (*atole* vs. *fresco* treatment), Z_j is variable 2 (timing of exposure), ε is the error term that captures residual variances. $X_i \times Z_j$ is variable 3, which is the exposure variable of interest. Our focus in this model is to test the significance of coefficient δ for variable 3. The V_1-V_m terms are potential covariates to be included in the model (described further below). For logistic models, Logit P(X) was used (Eq. 3).

It is worth noting that in previous studies conducted by the INCAP researchers, sensitivity analyses were conducted to test whether it is reasonable to combine those who were partially exposed. Some of these individuals were conceived prior to the beginning of the RCT in 1969 (thus were 'too old' to have exposure for the whole first 1,000 days), whereas some were born after 1975 (thus were 'too young' to have full exposure during the first 1,000 days) (See Table 4.1 for exact dates used in our calculation). It is reasonable to assume that these two groups of individuals may have considerable differences in sociodemographic characteristics, and the treatment itself may impact their growth differently. Through sensitivity analyses,

however, INCAP researchers have not found significant difference in previous study outcomes when treating the exposure group differently (90). In addition, the sample sizes are small in these two partially exposed groups, hence making combining them more statistically reasonable when comparing to the full exposure group.

Both formulas above included potential covariates in parenthesis (the *V* terms). The DD modeling strategy already accounts for the following effects: secular trends (by including variable 2), and fixed inter-group differences (by including variable 1). The following characteristics may affect the early nutrition-CMD association, thus will be controlled for: childhood characteristics (socioeconomic status, or SES, tertiles in childhood, maternal age at childbirth, maternal height, and maternal grades); adulthood characteristics (SES in 2015, grades of schooling completed by the participant, and residence in Guatemala City); and adiposity measurements (BMI for total adiposity and waist-to-height ratio for central distribution of adiposity). Anthropometry was not controlled for when the outcome assessed were adiposity. The selection of covariates to be included in the final models will depend on both statistical testing and empirical evidences from previous INCAP study and other relevant studies.

Building upon the difference-in-difference modeling strategies, mediation analysis was used to ascertain potential mediators, featuring the adipokine leptin. Mediation analyses were conducted separately for men and women, because leptin level is known to differ by sex, and the outcomes of interest (in this case, obesity and glycemic measurements including glucose concentration and

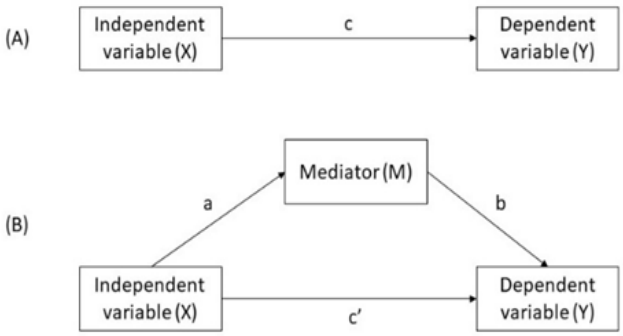


Figure 4.3 Mediation

homeostasis assessment models, or HOMA) are sex-dependent. The conventional mediation method was used (see Figure 4.3 for graphic illustration) (96). X is our exposure variable (early-life exposure to improved nutrition), Y is outcome variables (glycemic measurements), and M is mediator (leptin concentration). Graph (A) shows the relationship between X and Y without mediation, whereas graph (B) illustrates a mediated effect between X and Y. The formula of mediation analysis could be written as follows:

$$Y = i_1 + cX + \varepsilon_1 \quad (3)$$

$$Y = i_1 + c'X + bM + \varepsilon_2$$

$$M = i_3 + aX + \varepsilon_3$$

All terms in formula (3) are the same as shown in Figure 4.3 (The error terms, ε, denotes residuals). The testing of mediation effect will follow these steps first proposed by Baron and Kenny in 1986 (97). First, we will test whether the relationship between *X* and *Y* is significant (test coefficient *c*). This step is controversial because when mediation effect is strong, *c* may not be significant (96). Therefore, regardless of the significance of *c*, the analysis continues. Second, we will test whether *X* significantly predicts the hypothesized *M* (test coefficient *c'*). Subsequently, we will test whether *M* is significantly related to *Y*, when both *X* and *M* are in the model (test coefficient *b*). Finally, we will check whether *c* is larger in absolute value than *c'*. If the regressions are linear, then mediation effect equals to either $\hat{a}\hat{b}$ or $\hat{c}$- $\hat{c}'$ (the hat denotes observed values), and they are algebraically equivalent.

4.7.e Data Reduction Technique

Data dimensionality reduction techniques were used, including principal component analysis (PCA), exploratory and confirmatory factor analysis (EFA and CFA, respectively).

Confirmatory factor analysis was also assessed using structural equation modeling, SEM), multi-variate analysis of variance (MANOVA), linear discriminatory analysis (LDA) to analyze the postprandial biomarker changes. This set of analysis is data-driven and exploratory in nature.

For biomarkers using fasting specimen, we used principal component analysis (PCA) will be used to first inspect whether there are a few principal components that can explain most variances in the dataset. PCA is an ideal exploratory data analysis methodology in this context, which helps reduce the dimensionality of data through identifying linear combinations of existing variables (in our case, multiple biomarkers that potentially assess similar underlying biochemical processes) (98).

We then used Exploratory factor analysis (EFA) to first derive hypothesis regarding biomarker clusters (factors), because factor analysis is a measurement model that can identify latent variables that may simultaneously explain multiple variables (99). Confirmatory factor analysis (CFA, in the form of structural equation modeling, SEM) in this dissertation for instance maximum likelihood testing, will then be used to validate that the number of factors selected fits the data well. We split the data into two random halves, using the first half to 'train' the model and the remaining half to validate the trained model. The factor analysis model can be presented as follows (99).

$$x_1 = \mu_1 + \beta_{11}f_1 + \beta_{12}f_2 + \ldots + \beta_{1m}f_m + \varepsilon_1 \quad (4)$$

$$x_2 = \mu_2 + \beta_{21}f_1 + \beta_{22}f_2 + \ldots + \beta_{2m}f_m + \varepsilon_2$$

…

$$x_p = \mu_p + \beta_{p1}f_1 + \beta_{p2}f_2 + \ldots + \beta_{pm}f_m + \varepsilon_p$$

In the formula above, x_1 to x_p are the first through the pth observed variables (biomarker levels), whereas f_1 to f_m are the first through mth latent factors that were not directly measured. The β values are factor loadings, which could be interpreted as correlations that associate each latent factor with the observed variables (100). The ε values represent the residual variance of each observed variable that are not completely explained by latent factors after loading. Through this process, we aim at identifying m factors ($m << p$) to characterize metabolic flexibility and cardiometabolic profile in the population, as assessed by meal-induced biomarker responses.

Multivariate analysis of variance (MANOVA) was used to test mean differences in postprandial biomarker responses between exposure groups, by cardiometabolic processes (lipid, glycemic, or inflammatory domain) and by global (all biomarkers) comparison (101). We used linear discriminant analysis (LDA) to predict group membership (e.g., nutrition exposure group) based on collective biomarker responses (102, 103). For LDA, we partitioned the data into two random parts, 80% of the data were used to train the LDA models, and the remaining 20% were used to test the established models. We obtained one linear discriminant (LD) that is a linear combination of the multivariate data to maximize the between-group differences. Prior to conducting the multivariate analyses, we examined the correlation matrix across all biomarker responses.

4.8 Methodologies in the Core Chapters

The subsequent three chapters each expands on the methodologies used in the specific research context: **Chapter 5** involved DD modeling strategy and mediation analysis to investigate selected fasting biomarkers in the early-life nutrition-cardiometabolic profile associations; **Chapter 6** presented cross-sectional analysis of the dynamic responses of all

biomarkers, as well as the structural relationship among all biomarkers. We compared the individual biomarker responses across strata of cardiometabolic phenotypes to identify “healthy” and “unhealthy” stress responses; **Chapter 7** combined DD, MANOVA, and LDA to compare individual, domain-specific, and global biomarker responses between those who were exposed to *atole* in the full first 1000 days versus other exposure status.

CHAPTER 5: MANUSCRIPT FOR SPECIFIC AIM 1

Leptin partially mediates the association between early-life nutritional supplementation and long-term glycemic status among women in a Guatemalan longitudinal cohort

5.1 Abstract

Background: Early-life exposure to improved nutrition was associated with decreased risk for diabetes but increased risk for obesity. Leptin positively correlates with adiposity and has glucose lowering effects, thus may mediate the association of early-life nutrition and long-term glycemic status.

Objective: To investigate the role of leptin in the differential association between early-life nutrition and the risks for obesity and diabetes.

Design: We analyzed data from a Guatemalan cohort who were randomized at the village level to receive nutritional supplements as children. We conducted mediation analysis to examine the role of leptin in the associations of early-life nutrition and adult cardiometabolic outcomes.

Results: Among 1,112 study participants aged (mean ± standard deviation) 44.1 ± 4.2 years, 60.6% were women. Cardiometabolic conditions were common: 40.2% of women and 19.4% of men were obese, and 53.1% of women and 41.0% of men were hyperglycemic or diabetic. Leptin concentration (median and interquartile range) was 15.2 ng/mL (10.2-17.3 ng/mL) in women and 2.7 ng/mL (1.3-5.3 ng/mL) in men. Leptin was positively correlated with body mass index (Spearman's ρ was 0.6 in women, 0.7 in men). Women exposed to improved nutrition in early life had 2.8 ng/mL (95% confidence interval: 0.3, 5.3 ng/mL) higher leptin and tended to

have lower fasting glucose (-0.8 mmol/L; -1.8, 0.2 mmol/L, NS) than unexposed women. There were no significant differences in leptin (-0.7 ng/mL; -2.1, 0.8 ng/mL) or fasting glucose (0.2 mmol/L; -0.5, 0.9 mmol/L) in men exposed to improved nutrition in early life compared with unexposed men. Leptin mediated 34.9% of the pathway between early-life nutrition and fasting glucose in women. The mediation in women was driven by improved pancreatic β-cell function. We did not observe the mediation effect in men.

Conclusions: Leptin mediated the glucose-lowering effect of early-life nutrition in women but not in men.

A Note on Copyright:

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5.2 Introduction

The period from conception to two years of age (the first 1000 days) is a critical window of early-life development (1). Nutritional status during this window has been reported to affect health status in later years. Sub-optimal nutrition in the first 1000 days is associated with increased risk for obesity, type 2 diabetes, and cardiovascular diseases (1-4). This cluster of interwoven cardiometabolic diseases is an emerging contributor to the global disease burden and has becoming increasingly prevalent in low- and middle-income countries (5). For instance, recent data from Guatemala ranked cardiovascular diseases and diabetes as the first and third leading causes of mortality, respectively, and together they account for one third of total deaths (5).

Early-life nutritional exposure affects long-term cardiometabolic health through epigenetic, pathophysiological, and other mechanisms (1). Many of these mechanisms are not well understood. From a developmental perspective, early-life nutrition affects the ontogeny of metabolically active tissues (6). Animal models have provided relevant evidence: malnutrition caused structural and functional changes in the placenta and metabolic organs, and these changes were associated with long-term cardiometabolic disturbances (7). In human studies, it is challenging to distinguish the impact of early-life nutrition from other determinants of cardiometabolic perturbations. Longitudinal cohort studies are valuable resources in meeting this challenge (8). More than 40 years following a nutrition trial, Ford *et al* reported that, although early-life exposure to improved nutrition was associated with reduced risk of diabetes, odds of obesity were increased (9). Previous research explored the linkages between early-life nutrition and long-term risks for increased adiposity (10). However, the same factors could not explain the observed reduction in diabetes risk (9).

Cardiometabolic diseases including obesity and diabetes share underlying biochemical pathways (11). Leptin, an adipose-tissue derived hormone, is proportional to body fat mass. Leptin also participates in the programming of obesity through a leptin-dependent feedback loop (12). Leptin concentration in early life is important in the development of metabolic profile (13). Leptin is a key signaling molecule in glucose homeostasis (14-16). As a catabolic agent in metabolism, leptin reduces hepatic gluconeogenesis by limiting substrates to liver (14, 15). It has impact on skeletal muscle and other peripheral tissues to increase glucose uptake (15). Leptin can also regulate glucose homeostasis through pancreatic-secreted hormones (17). Because of the versatile functions of leptin, it is important to investigate the specific mechanisms – whether leptin mainly reduces insulin resistance (assessed by the homeostasis assessment model, HOMA-IR) or improves pancreatic β-cell function (HOMA-B) (18).

We therefore assessed the contribution of leptin to glucose homeostasis in a group of adults who participated in a randomized nutritional supplementation trial in early life. We postulated that leptin might help explain the differential effects of early-life nutrition on long-term risks for diabetes and obesity.

5.3 Subjects and methods

Study population

From Jan 1, 1969 to Feb 28, 1977, investigators at the Institute of Nutrition of Central America and Panama (INCAP) carried out a randomized controlled trial in four villages in southeastern Guatemala. Details of the initial trial and successive follow-up studies have been reported elsewhere (9, 19). Briefly, participants in four villages were randomized to receive either *atole* (the treatment group) or *fresco* (the control group) twice daily for the duration of the

study. *Atole* is a protein- and energy-containing supplementation, whereas *fresco* is a low-energy drink with no protein. A total of 2,392 children were included either because they were under 7 y at study launch or were born during the original study period. In the follow-up conducted from 2015 to 2017, 1,661 cohort members (69.4 % of the original cohort) were eligible for participation. The remaining cohort members had died (15.4 %), emigrated (10.4 %), or were lost to follow up (4.7 %). Of the 1,661 eligible cohort members, 500 (30.1%) could not be contacted or declined to participate in this wave. An additional 49 (2.9%) individuals were excluded during the current wave because they either did not attend scheduled clinical exams, did not have plasma samples required for this set of analysis, or were pregnant or lactating at the time of data collection. The final sample size was 1,112. (**Supplementary Figure 1**) As previously reported, the loss-to-follow up at this examination was not differential in terms of the randomization (9).

Data Collection

Cohort members were invited to attend centralized clinics (one in each study site) after an overnight fast. After obtaining informed consent in Spanish, trained phlebotomists collected venous blood in ethylenediaminetetraacetic acid (EDTA) from each participant. Blood samples were kept on ice and centrifuged within two hours of collection. On the day of sample collection, we aliquoted plasma samples and stored them at -20 ^{o}C. Once a month, these samples were transported on dry ice to INCAP headquarters in Guatemala City, where we assayed fasting and postprandial glucose concentrations (mg/dL, converted to mmol/L for analysis) using enzymatic colorimetric methods (Cobas C111 analyzer, ROCHE, Indianapolis, IN, USA). The remaining plasma samples were immediately stored at -80°C, and were shipped on dry ice in three

installments to Atlanta, GA, US and stored at -80^{o}C until analysis. For laboratory assays conducted in the Biomarker Core Laboratory (Foundation for Atlanta Veterans Education and Research, Atlanta Veterans Affairs Medical Center), samples were thawed at 4^{o}C over a weekend in batches of 40 participants. The plasma samples were randomized into 28 batches, balanced by location of data collection, village of birth at the beginning of the INCAP Longitudinal Study, and timing of exposure to the nutritional supplements to prevent overlaying potential systematic bias in study design with bias in laboratory batches. We assayed insulin (mIU/L) using immunoturbidimetric methods (Kamiya Biomedical Company, WA, US). We assayed fasting leptin (ng/mL) in duplicates by ELISA (Boster Biological Technology, Pleasanton, CA, USA). For quality assurance, we repeated the assays for samples with implausible values, usually between one to eight samples per batch (2.5% to 20%), and the frequency was once every other week. Overall, across all batches, approximately 5% of all samples were re-analyzed. We plotted the concentrations to identify outliers within each batch and examined batch effects. In addition, we performed quality check collectively for the first half and the second half of the data by examining their comparability, and by identifying outliers and re-running the selected samples.

Anthropometry: Trained research staff measured body weight (kg), height (cm), and waist circumference (cm) of all study participants in duplicates using standardized methods. Body mass index (BMI) was calculated as weight (kg) divided by height (m) squared. Waist-to-height ratio (WHtR) was calculated as waist (cm) divided by height (cm). Body composition was assessed using the deuterium oxide (D_2O) dilution technique (20). (Fourier Infrared analysis, FTIR, Shimadzu 8400S). Total body water was determined based on mathematical models from the D_2O dilution, and fat free mass was calculated using a hydration constant of 0.732 (21). Fat

mass, calculated as the difference between body mass and fat free mass, is presented as body fat percentage.

Cardiometabolic outcomes: We focused on obesity, central obesity, hyperglycemia, and type 2 diabetes mellitus (T2DM) to characterize cardiometabolic status in the study population. Obesity was defined as BMI $\geq 30 kg/m^2$. Central obesity was defined as waist circumference $\geq$ 88 cm for women and $\geq$ 102 cm for men (22). Hyperglycemia was defined as fasting plasma glucose $\geq$ 100 mg/dL and $\leq$ 125 mg/dL, or two-hour post-challenge plasma glucose level $\geq$ 140 mg/dL and < 200 mg/dL among participants who were not using diabetic medication (23). (The two-hour postprandial plasma glucose was obtained after a mix-component meal challenge designed to mimic an oral glucose tolerance test.) T2DM was defined as a fasting plasma glucose of 126 mg/dL or more, post-challenge glucose of 200 mg/dL or more, or use of diabetes medication.(23) We calculated HOMA-IR as the product of fasting glucose (mmol/L) and fasting insulin (mIU/L) divided by 22.5, and HOMA-B as the product of fasting insulin and 20, divided by the value of fasting glucose minus 3.5 (24).

Statistical analysis

We had over 80% statistical power to detect medium effect size (Cohen's $d = 0.5$) for the difference-in-difference exposure variable for all biological markers. We described the sociodemographic characteristics of the population, pooled and separately by sex. We used Student's *t*-test or Mann-Whitney *U* test, when appropriate, for comparisons of characteristics (sociodemographic information, cardiometabolic risk factors, and biomarker concentrations) between male and female participants. We treated missingness for the following variables using

bootstrapped multiple imputation: maternal height (missing 20.6%), maternal age at childbirth (missing 1.6%), and maternal schooling (missing 3.5%).

Following previously described modeling strategies, we constructed difference-in-difference models to investigate the intention-to-treat impact of exposure to *atole* in the full first 1000 days versus partial or no exposure on cardiometabolic disease risk factors (9). The primary outcome variables were fasting glucose, HOMA-IR, HOMA-B, and leptin concentration, and the secondary variables were body mass index, waist circumference, percent body fat, and fasting insulin concentration. As there are multiple sibling sets in our data, we controlled for clustering at the household level by generating cluster-robust estimate of the variance matrix. We built a series of models. Our base model (Model 1) included three independent variables: (a) the treatment variable: receiving either *atole* or *fresco* during the nutritional supplementation trial. Because the randomization was at the village level, we used birth village in place of the binary '*atole* versus *fresco*' variable to control for village-level random effects (controlling for differences between the villages at baseline); (b) timing of exposure: exposed to the either *atole* or *fresco* during the full first 1000 days versus otherwise; and (c) the interaction term between the treatment and timing of exposure, which is our target difference-in-difference exposure variable. This variable represented participants who were exposed to *atole* during the full first 1000 days versus those with partial or no exposure in this timeframe. We controlled for birth year and sex in the base models. When biomarkers and T2DM were the dependent variables in the base models, we also adjusted for body mass index and waist-to-height ratio.

The difference-in-difference approach controls for within-village fixed effects that might otherwise differ between individuals. However, there were still potential between-group

differences using this approach. We therefore built adjusted models that sequentially added childhood characteristics (socioeconomic status, or SES, tertiles in early life, maternal age at childbirth, maternal height, and maternal schooling – Model 2), adulthood characteristics (SES in 2015, grades of schooling completed by the participant, and residence in Guatemala City – Model 3), and adiposity measurements (BMI for total adiposity and waist-to-height ratio for central distribution of adiposity – Model 4). We presented Model 3 (for anthropometric measurements as dependent variables) and Model 4 (for biomarkers as dependent variables) as the adjusted models. For pooled models, we assessed stratum heterogeneity by sex through testing an interaction term between sex and the difference-in-difference exposure variable. Even when stratum heterogeneity was not detected, we also conducted sex-specific analysis due to the biological differences between the two sexes, especially due to the significantly higher leptin concentration in women than men.

We conducted sex-specific mediation analysis to investigate the role of leptin in the difference-in-difference models. We used the Baron & Kenny method (25). (Refer to **Figure 2** in the results section for annotations) The direct model included the glycemic measurements (fasting glucose, HOMA-IR, and HOMA-B, respectively) as the outcome and the difference-in-difference exposure variable as the predictor [Glycemic measurement = $\hat{c}$ *(Exposure to *atole* in the full first 1000 days) + (control variables) + ε_1]. The standardized regression coefficient of the exposure variable was the total effect $\hat{c}$. The mediation model has the same outcome and predictor with leptin being added as a mediator [Glycemic measurement = $\hat{c}'$ * (Exposure to *atole* in the full first 1000 days) + $\hat{b}$ * (Fasting leptin) + (control variables) + ε_2]. Then, treating leptin as the outcome, the exposure variable has a coefficient $\hat{a}$ [Leptin = $\hat{a}$ * (Exposure to *atole* in the full first 1000 days) + (control variables) + ε_3]. The mediation effect is the product of $\hat{a}$

and $\hat{b}$, which represents the indirect pathway between exposure and outcome. The mediation percentage is indirect effect $\hat{a}\hat{b}$ divided by the total effect $\hat{c}$. When the indirect pathway $\hat{a}\hat{b}$ suppresses the total direct effect $\hat{c}$ (e.g., the sign flipped), we did not report the mediation percentage. Control variables were included as in the unmediated difference-in-difference models. We ruled out moderated mediation by testing potential moderating effect by leptin through adding an interaction term between leptin and the exposure variable. We confirmed the results through simulation exercises using the statistical package "mediation" in R, bootstrapped 1,000 times (25, 26). We used the "RMediation" package to obtain the 95% confidence interval of the mediation effect (27).

We also conducted a sensitivity analysis to account for potential hormonal impact on biomarker concentrations: we compared the mediation results between post-menopausal women and other women. We categorized women who did not have menstruation for ≥ 12 consecutive months at the time of data collection as postmenopausal (28).

We conducted all analyses in R version 3.6.0 (R Core Team 2018, Foundation for Statistical Computing, Vienna, Austria). Statistical significance was set a priori at p value < 0.05. All p-values were two-sided.

Research ethics: The study was approved by the Institutional Review Board at Emory University and the Ethics Review Committee of INCAP. All study participants provided written informed consent in Spanish.

5.4 Results

The sample included 1,112 Guatemalan adults (60.6% women) with a mean (± standard deviation) age of 44.2 y (± 4.3 y) for women and 43.9 y (± 4.1 y) for men (**Table 1**). Approximately one in five participants were exposed to *atole* during the full first 1000 days. Men and women were similar in most sociodemographic factors investigated. Women had higher BMI, waist circumference, and percent body fat than men (Table 1). Based on BMI, approximately 40% of women and 20% of men were obese. Based on sex-specific waist circumference standards, almost 90% of women and 20% of men were centrally obese. Over 30% of all participants were hyperglycemic. More women than men (16.5% and 9.2%, respectively) had T2DM, and more women than men (less than 10% for both) were taking medications to manage their diabetic condition (Table 1). Compared with men, women had higher fasting concentrations of insulin, glucose, HOMA-IR, HOMA-B, and leptin. Leptin concentration was positively correlated with both BMI (Spearman's ρ was 0.6 for women and 0.7 for men) and waist circumference (Spearman's ρ was 0.6 for women and 0.8 for men) (**Figure 1**).

Based on results from adjusted models, the mean concentration of glucose was 0.8 mmol/L lower (95% CI: -1.8, 0.2 mmol/L) in women who were exposed to *atole* in early life than unexposed women. Among women, leptin concentration was 2.8 ng/mL higher (95% CI: 0.3, 5.3 ng/mL) and HOMA-IR was 0.5 lower (95% CI: -1.5, 0.6) in the exposed group than in the unexposed group (**Table 2**). Among men, being in the exposure group was associated with lower leptin concentration (-0.7 ng/mL; 95% CI: -2.1, 0.8 ng/mL) and higher fasting glucose concentration (0.2 mmol/L; 95% CI: -0.5, 0.9 mmol/L). Exposure to *atole* during the first 1000 days was positively associated with a few measurements of fatness (Table 2). In pooled analysis,

exposure group was associated with 1.3 kg/m^2 higher BMI (95% CI: 0.2, 2.3 kg/m^2) and 2.7 cm (95% CI: 0.1, 5.2 cm) larger waist circumference. Among men, exposure group was associated with 1.7 kg/m^2 higher BMI (95% CI: 0.3, 3.1 kg/m^2), 5.2 cm (95% CI: 2.0, 8.5 cm) larger waist circumference, and 2.8% increased body fat percentage (95% CI: 0.4, 5.1 %). We did not observe significant stratum heterogeneity by sex in pooled models.

Among women, leptin mediated the pathway between early-life *atole* exposure and fasting glucose concentration (**Figure 2**). In adjusted models for women, leptin mediated 34.9 % of the nutrition exposure-glucose association (mediation effect = -0.3 mmol/L; 95% CI: -0.5, -0.1 mmol/L) (Figure 2A). Leptin did not mediate the pathway between early-life nutritional exposure and HOMA-IR in women (Figure 2B), but it did mediate the pathway to HOMA-B: indirect effect through leptin was 8.1 mmol/L (95% CI: 1.8, 14.9 mmol/L) and direct effect in the mediation model was -7.6 mmol/L (95% CI: -36.6, 21.4 mmol/L) (Figure 2C). We did not observe any mediation effect of leptin on fasting glucose, HOMA-IR, or HOMA-B in men (Figure 2A to 2C). We confirmed the results of mediation analysis by bootstrapped simulation: as shown in **Supplementary Figure 2**, average causal mediation effect (ACME) was consistent in bootstrapped simulation results for all three glycemic measurements by sex.

In sensitivity analyses, mediation analysis showed significant mediating effect of leptin between early-life *atole* exposure and fasting glucose concentration in all other women (38.7% mediated), but not in postmenopausal women. Nevertheless, the coefficients (*a*, *b*, *c*, and *c'*) in the two sets of mediation models were similar between postmenopausal women and other women. (**Supplementary Figure 3**).

5.5 Discussion

To our knowledge, this study is among the first to use biomarker data at the population level to investigate potential biochemical mechanisms through which early-life nutritional exposure can have long-term cardiometabolic impacts. Previous papers from the INCAP study have documented associations between early-life exposure to *atole* and positive health and human capital outcomes (1). In the current paper, we showed that leptin partially mediated the association of early-life nutritional exposure and glycemic measurements in women. We did not observe the same mediation effect in men. The mediation in women was mainly driven by improved pancreatic β-cell function (leptin was associated with increase in HOMA-B via the indirect pathway in mediation analysis), and not by reduction in insulin resistance (no mediation for HOMA-IR). We confirmed that the protein- and energy-containing nutritional supplement, *atole*, had mixed effects on long-term cardiometabolic risk factors: early-life exposure to *atole* was associated with lower fasting glucose concentration (in women). The same exposure, however, was also associated with increased odds for overall and central adiposity (mainly in pooled models). As hypothesized, we observed a positive correlation between leptin and adiposity measurement in both sexes, both overall adiposity and central adiposity. We also observed significantly higher leptin concentration in women than in men.

This set of analysis was guided by a conceptual framework to draw linkages between early-life nutrition, human ontogeny, and relevant cardiometabolic pathways, emphasizing the role of leptin (**Figure 3**). Previous research suggested that early-life exposure to improved nutrition affects the development of metabolically active tissues, including adipose tissue, skeletal muscle, pancreas, liver, and the brain (6, 7, 29). Nutritional exposure influences leptin concentration through adipose tissue and other pathways, including nutrition signaling, hormonal regulation, and psycho-neurological regulations (30). Measurements of overall and central

adiposity in adulthood reflected two different sources of adiposity: adipose tissue influenced by early-life nutritional exposure (cell size, depot, and the type of adipose tissue), and increase in adiposity due to an obesogenic environment. All of these factors predicted obesity risk and determined circulating leptin concentrations (30, 31). Conversely, increased leptin level may also increase later adiposity through the leptin-dependent feedback loop, especially when there is physiologic leptin insensitivity (12). Indeed, we observed a positive association between leptin concentration and measurements of overall and central adiposity for both men and women.

As a catabolic hormone, leptin plays a central role in glucose regulation: It promotes glucose uptake by skeletal muscle and other peripheral tissues, reduces hepatic gluconeogenesis, and has direct effects on the central nervous system (14-16). Researchers have found that early-life exposure to improved nutrition can help guide more stem cells to prioritize myogenesis over adipogenesis, which predetermines adulthood muscle mass and intramuscular fat content (32). Improved nutritional exposure in early life also supports the development of hepatic tissue, which is central to gluconeogenesis and glucose storage. Brain and other peripheral tissues that actively utilize glucose also benefit from early-life exposure to improved nutrition. This is consistent with earlier reports of strong association between *atole* supplementation and increased lean mass and larger head circumference in the INCAP population (33, 34). Our current data suggest that exposure to *atole* in early life may have positively affected tissue development in this chronically malnourished population by providing the basis for leptin to exert euglycemic regulation.

Leptin also participates in glucose homeostasis through its effects – both acute and chronic – on the pancreas (17, 35, 36). Leptin can lower glucose concentration through inhibiting glucagon release from pancreatic α-cells, countering its glucose-raising effect (17). Pancreatic β-

cells are sensitive to maternal diet and in utero nutritional environment and can play an important role in insulin secretion (37). Insulin can, in turn, chronically up-regulate both the production and secretion of leptin (38). However, in obese individuals with chronic leptin resistance, leptin can impair pancreatic β-cell function and disrupt insulin secretion (36). In this study we observed a positive association between leptin and HOMA-B, suggesting that this population may not be leptin resistant despite the high prevalence of obesity.

Consistent with a recent meta-analysis that reported difference in the association of leptin and diabetes between men and women, we also noted that leptin had sex-specific mediation effects in our study (39). Previous research indicated that, due to differences in metabolic programming, women are predisposed to obesity and metabolic syndrome and men to diabetes – although this difference was not observed in our study (40). Sensitivity analysis between postmenopausal women and all other women indicated that the observed sex-specific differences may not be explained merely by hormonal differences. Although the leptin pathway is partially explanatory in the sex-specific differences, other factors may play a role as well, including potential impact of early-life nutrition on myogenesis and adipogenesis, which contribute to differences in body composition between men and women. Adiposity is not only affected by early-life nutritional status, but also by external factors such as occupational, environmental, and lifestyle differences between the two sexes (40, 41). Men are engaged in more manual work and physical activity than women in this study population. Long-term improvements in work capacity and wages were also documented among men who received improved nutrition in early childhood in this population (1). These factors can affect adiposity, circulating leptin concentration, and glycemic status.

Our observation that leptin statistically mediated the pathways between early nutrition and long-term glycemic status supports the biological postulations. Nevertheless, population-level data have not shown a consistent association between leptin and diabetes or fasting glucose concentration: leptin was used in animal models to reverse diabetes, but epidemiological data in human populations mainly reported a null to positive correlation between leptin and diabetes, with only a few exceptions (39, 42-45). It is possible that the participants who were exposed to *atole* in early life did not develop resistance to leptin even when their risk for obesity increased, thus allowing leptin to perform its expected catabolic functions. This postulation warrants further investigation.

There are a few limitations to our current analysis. First, there was missingness in several confounding factors. We used multiple imputation methods to attenuate any potential bias. Second, the biomarker data reflect a cumulative effect of early-life nutritional exposure and ensuing lifestyle and environmental factors over a span of close to 50 years. We do not have ontogeny information and all relevant exposure data throughout the life course to confirm several assumptions made in the conceptual framework, but we have reviewed literature in animal models to help discuss the biological plausibility. In addition, although the 'first 1000 days' is an important concept, the actual developmental processes do not follow this exact timeframe. Lastly, our study population included only Guatemalan adults within a relatively narrow age range (born during 1962-77). When considering the generalizability of our study, findings from other similar studies should be taken into consideration to properly interpret the results.

At a mean age of 44 years, both men and women in this Guatemalan cohort had high prevalence of cardiometabolic conditions. We identified a positive association between leptin and body adiposity in both sexes, as well as the mediation effect of leptin on long-term glucose

regulation among female participants. The underlying reasons for the observed sex-specific differences in leptin mediation effect should be further investigated.

5.6 Tables and figures

Table 1: Selected characteristics of the study population by sex

Characteristics [1-4]	Women (*n* = 674) Mean (SD) or %	Men (*n* = 438) Mean (SD) or %
Sociodemographic Characteristics		
Age, y	44.2 (4.3)	43.9 (4.1)
Exposure to *atole* in the first 1000 days, %	22.4	21.9
Maternal height, cm	148.9 (5.0)	149.0 (4.9)
Maternal age at child birth, y	26.8 (7.0)	27.1 (7.4)
Maternal education level, y	1.2 (1.6)	1.4 (1.7)
Socioeconomic status tertiles in childhood, %		
Poorest	34.3	31.7
Middle	33.7	33.3
Wealthiest	32.1	34.9
Socioeconomic status tertiles in 2015, %		
Poorest	32.5	33.3
Middle	34.7	30.4
Wealthiest	32.8	36.3
Total grades completed, y	3.3 (2.2)	3.6 (2.1)
Residing in Guatemala City, %	18.3	19.2
Anthropometry		
Height, cm	151.5 (5.3)	163.9 (6.1) ***

BMI, kg/m^2	29.3 (5.3)	26.6 (4.2) ***
Obese, %	40.2	19.4 ***
Waist Circumference, cm	101.8 (12.4)	94.2 (10.2) ***
Central Obesity, %	89.7	21.0 ***
Waist-to-height ratio	0.7 (0.1)	0.6 (0.1) ***
Body fat, percentage	42.2 (5.9)	28.8 (6.7) ***
Glycemic Conditions		
Hyperglycemia, %	36.6	31.8
Type 2 Diabetes, %	16.5	9.2 ***
Diabetes medication, %	9.5	4.3 **
Biomarkers, Median (IQR)		
Fasting Insulin, mIU/L	14.7 (9.1 – 22.2)	9.6 (6.2 – 16.4)***
Fasting Glucose, mmol/L	5.6 (5.2 – 6.1)	5.4 (5.2 – 5.8) **
HOMA-IR	3.9 (2.3 – 6.3)	2.4 (1.5 – 4.4) ***
HOMA-B	135.0 (82.6 – 199.4)	93.1 (61.2 – 152.0)***
Fasting Leptin, ng/mL	15.2 (10.2 – 17.3)	2.7 (1.3 – 5.3) ***

[1] Medians and interquartile range are provided for continuous variables with skewed distributions, including insulin, glucose, leptin, HOMA-IR, and HOMA-B

[2] Total sample size is 1,112, except the following variables: maternal height (n=883, missing 20.6%), maternal age at child birth (n=1094, missing 1.6%), maternal education (n=1073, missing 3.5%).

[3] Definitions: Obesity is defined as body mass index of 30 kg/m^2 or higher. Hyperglycemia is defined according to the American Diabetes Association (ADA) diagnostic criteria as a fasting

plasma glucose of 100–125 mg/dL or 2-h post-challenge glucose of 140–199 mg/dL among participants not reporting use of diabetes medication. HOMA-IR = fasting insulin (μIU/L) × fasting glucose (mmol/L)/22.5. HOMA-B = 20 × fasting insulin (μIU/L)/fasting glucose (mmol/L) − 3.5].

[4] P-values were based on Student's *t*-test between men and women, and Independent 2-group Mann-Whitney U test were used to compare biomarkers between men and women. $^{*} < 0.05$, $^{**} < 0.01$, $^{***} < 0.001$.

Abbreviations: BMI, body mass index (kg/m^2); HOMA, the homeostasis model assessment; HOMA-B, HOMA for β-cell function; HOMA-IR, HOMA for insulin resistance; IQR, interquartile range; SD, standard deviation.

Table 2: Difference-in-difference estimates for exposure to *atole* during the full first 1000 days versus partial or no exposure in predicting cardiometabolic risk factors

		Women	Men	Pooled [1-3]
		β (95% CI)	β (95% CI)	β (95% CI)
Anthropometry				
BMI, kg/m^2	Base	1.0 (-0.4, 2.5)	1.4 (-0.1, 2.9)	1.2 (0.1, 2.2)
	Adjusted	0.9 (-0.6, 2.4)	1.7 (0.3, 3.1) *	1.3 (0.2, 2.3) *
Waist circumference, cm	Base	1.1 (-2.5, 4.6)	4.6 (1.1, 8.0) *	2.3 (-0.2, 4.9)
	Adjusted	1.0 (-2.6, 4.6)	5.2 (2.0, 8.5) **	2.7 (0.1, 5.2)
Body fat %	Base	0.8 (-0.9, 2.5)	2.3 (-0.2, 4.7)	1.3 (-0.2, 2.8)
	Adjusted	0.7 (-1.0, 2.4)	2.8 (0.4, 5.1) *	1.4 (-0.03, 2.9)
Biomarkers				
Fasting Insulin, mIU/L	Base	0.8 (-2.8, 4.4)	-1.5 (-4.4, 1.3)	-0.2 (-2.8, 2.4)
	Adjusted	0.7 (-3.1, 4.5)	-0.8 (-3.7, 2.2)	-0.02 (-2.8. 2.7)
Fasting Glucose, mmol/L	Base	-0.8 (-1.7, 0.1)	0.2 (-0.6, 0.9)	-0.4 (-1.1, 0.2)
	Adjusted	-0.8 (-1.8, 0.2)	0.2 (-0.5, 0.9)	-0.4 (-1.0, 0.3)
HOMA-IR	Base	-0.2 (-1.3, 0.8)	0.2 (-0.9, 1.3)	-0.1 (-0.9, 0.7)
	Adjusted	-0.5 (-1.5, 0.6)	-0.1 (-1.0, 0.8)	-0.3 (-1.1, 0.4)
HOMA-B	Base	4.5 (-26.6, 35.6)	0.3 (-34.4, 35.1)	1.5 (-22.5, 25.5)
	Adjusted	-7.6 (-36.6, 21.4)	-10.2 (-39.5, 19.2)	-9.0 (-30.8, 12.7)
Fasting Leptin, ng/mL	Base	2.6 (0.2, 5.1) *	-0.8 (-2.3, 0.6)	1.2 (-0.4, 2.8)

Adjusted	2.8 (0.3, 5.3) *	-0.7 (-2.1, 0.8)	1.2 (-0.4, 2.8)

[1] For anthropometric measurements each as a dependent variable, the base models were: Anthropometry = birth villages + timing of exposure + (*atole* versus *fresco*) * (timing of exposure) + birth year + sex. The adjusted models controlled for childhood characteristics (childhood socioeconomic status tertiles dummy variables, maternal age at childbirth, maternal height, and maternal schooling) and adult characteristics (2015 socioeconomic status tertiles dummy variables, grades of schooling completed, and Guatemala city residence). The coefficients presented were for the interaction term (*atole* versus *fresco*) * (timing of exposure).

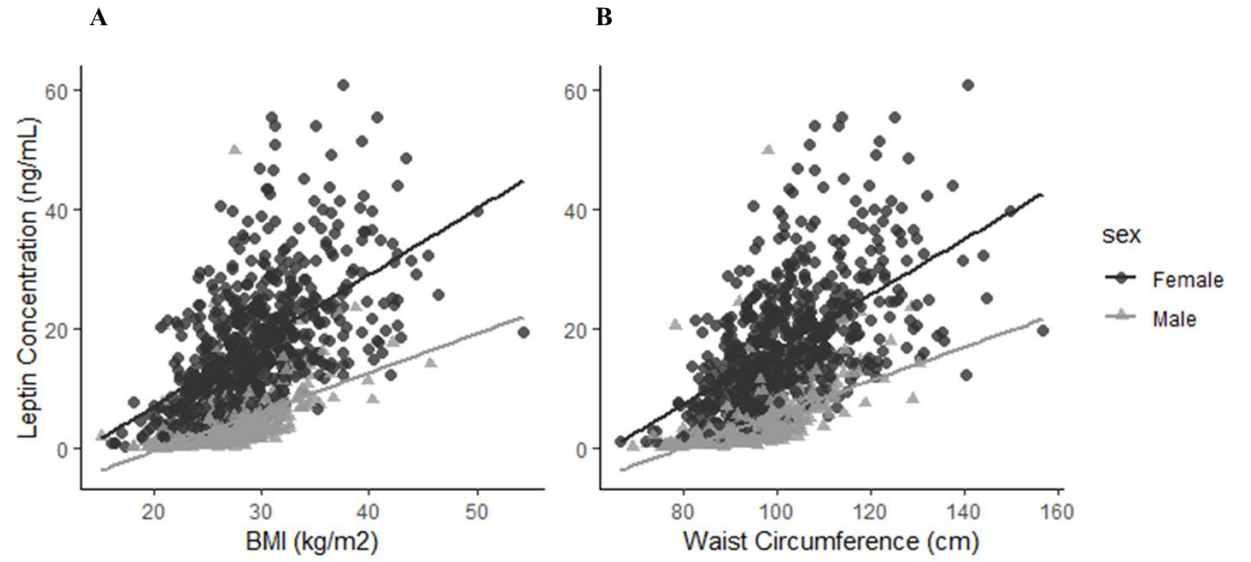
[2] For biomarkers each as a dependent variable, the base models were: Biomarker = birth villages + timing of exposure + (*atole* versus *fresco*) * (timing of exposure) + birth year + sex. The adjusted models controlled for childhood characteristics (childhood socioeconomic status tertiles dummy variables, maternal age at childbirth, maternal height, and maternal schooling), adult characteristics (2015 socioeconomic status tertiles dummy variables, grades of schooling completed, and Guatemala city residence), and measurements of overall and central adiposity (body mass index and waist-to-height ratio. The coefficients presented were for the interaction term (*atole* versus *fresco*) * (timing of exposure).

[3] For pooled models, we tested stratum heterogeneity by sex through constructing the interaction term between sex and the difference-in-difference exposure variable. None of these tests had a p-value <0.05. We did not adjust for sex in sex-specific models.

P-values: $^{*} < 0.05$, $^{**} < 0.01$, $^{***} < 0.001$

Abbreviations: BMI, body mass index; CI, confidence internal; HOMA, the homeostasis model assessment; HOMA-B, HOMA for β-cell function; HOMA-IR, HOMA for insulin resistance.

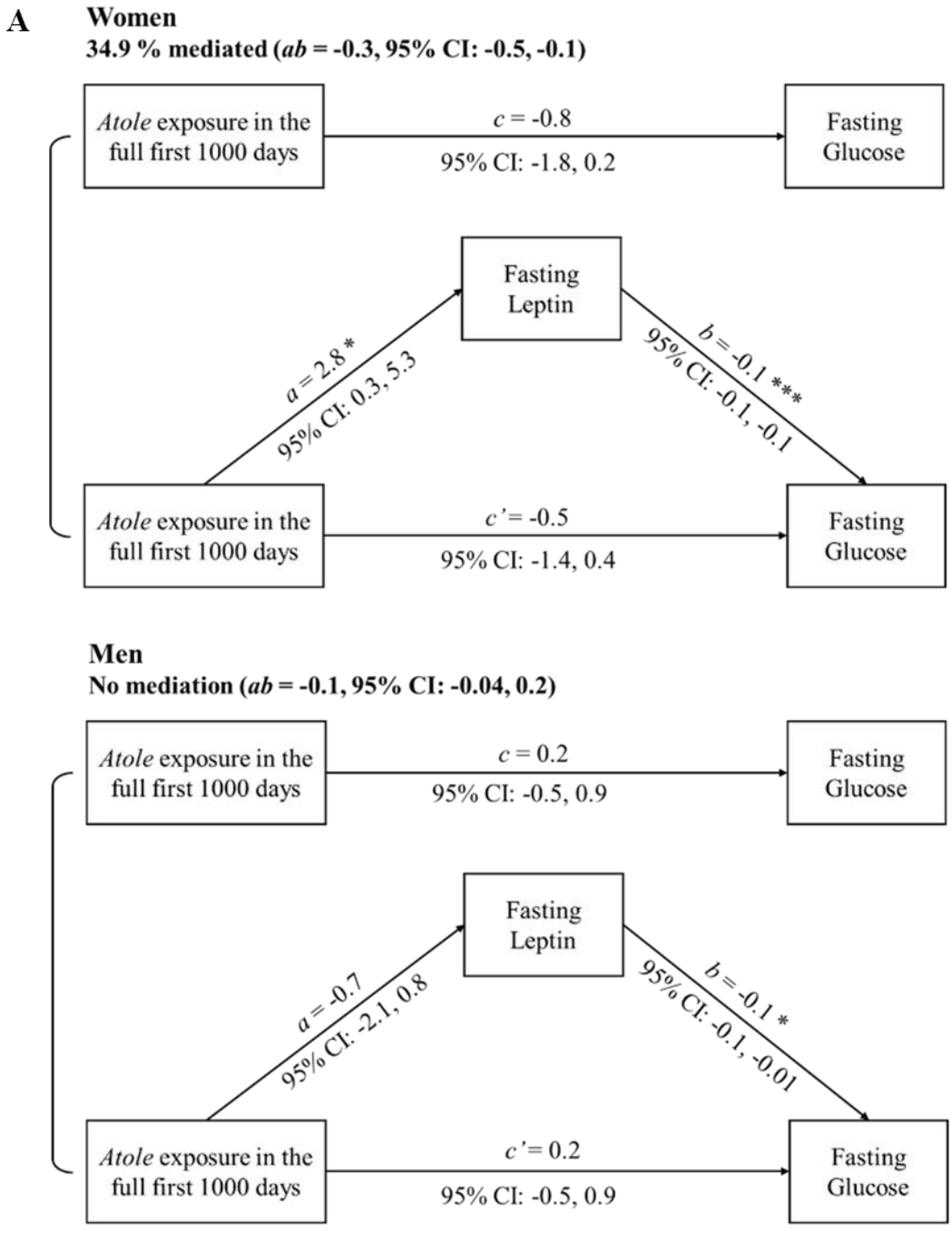
Figure 1. Correlation between fasting leptin concentration measurements of overall and central adiposity



Legend:

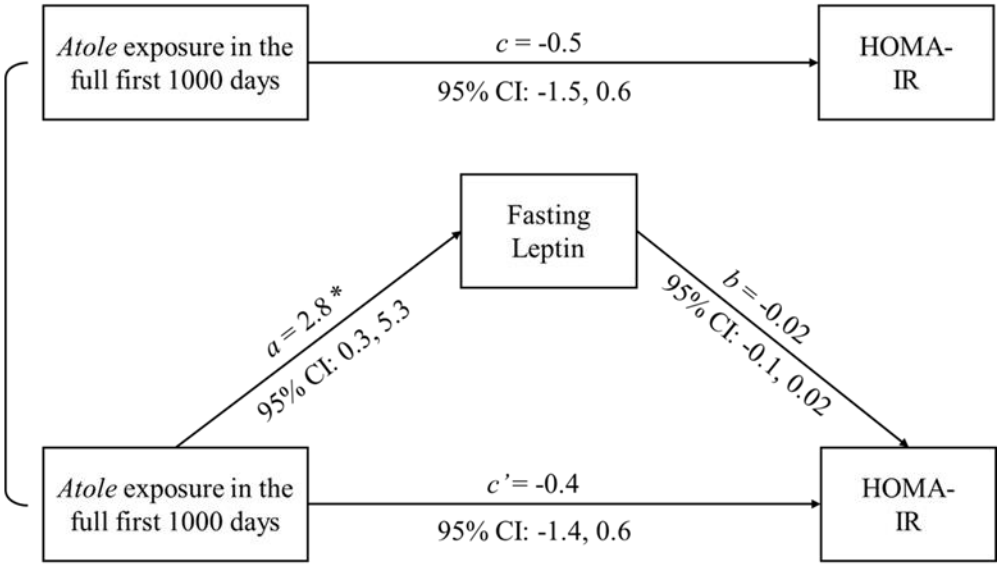
1. Spearman's ρ for leptin and BMI (n = 1,112): women = 0.6, men = 0.7
2. Spearman's ρ for leptin and waist circumference (n = 1,112): women = 0.6, men = 0.8
3. Fitted lines were based on simple linear regression models
4. Abbreviation: BMI, body mass index

Figure 2. Mediation analysis of leptin in the pathway between exposure to *atole* in the full first 1000 days and three glycemic measurements



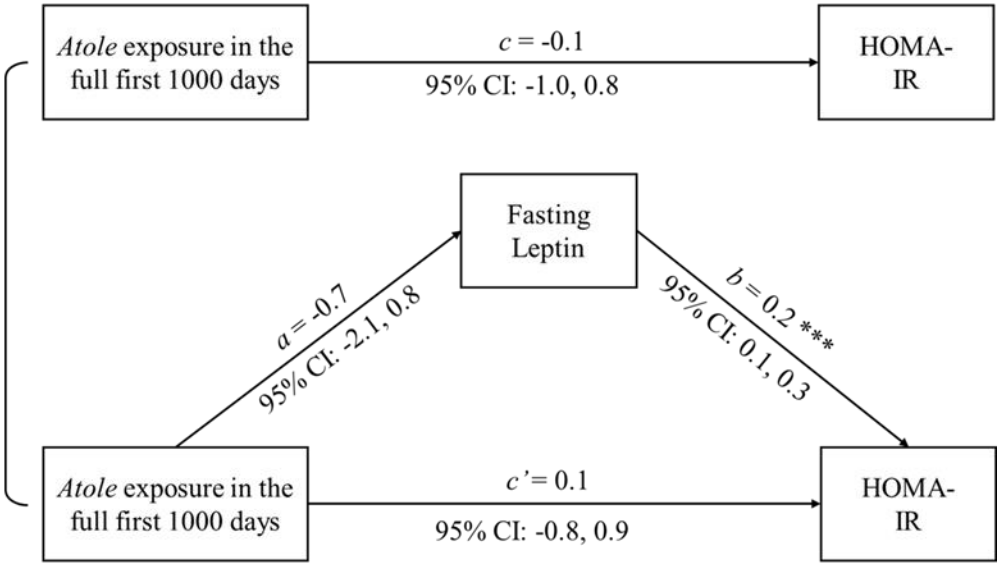
B **Women**

No mediation (*ab* = -0.1, 95% CI: -0.2, 0.02)



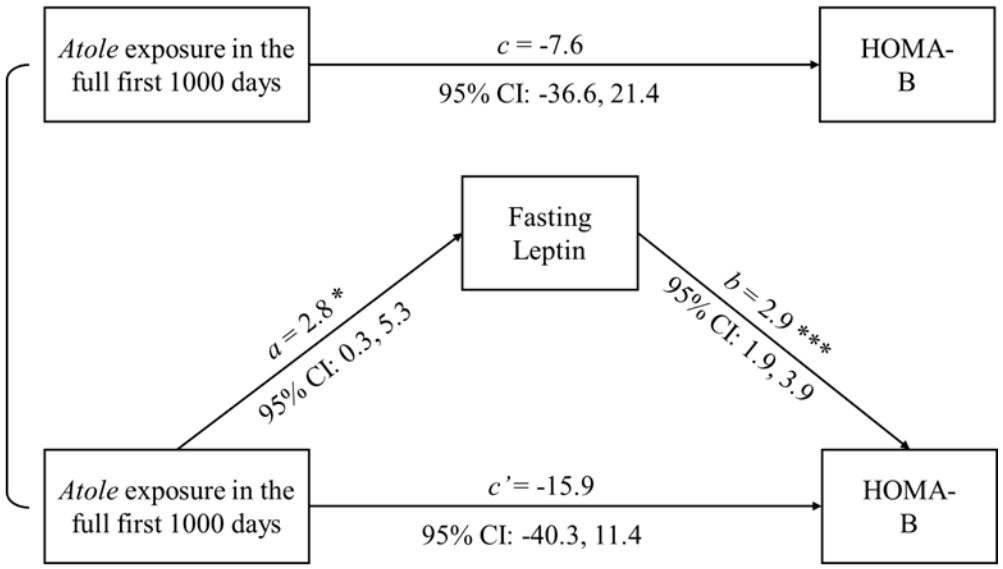
Men

No mediation (*ab* = -0.1, 95% CI: -0.4, 0.1)



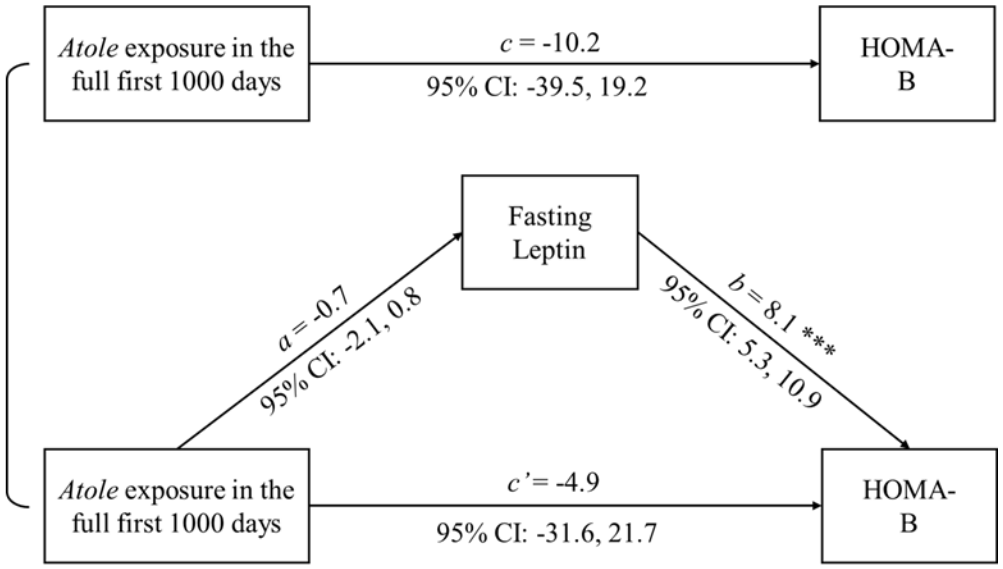
C **Women**

Suppression effect (*ab* = 8.1, 95% CI: 1.8, 14.9)



Men

No mediation (*ab* = -5.3, 95% CI: -15.5, 4.6)

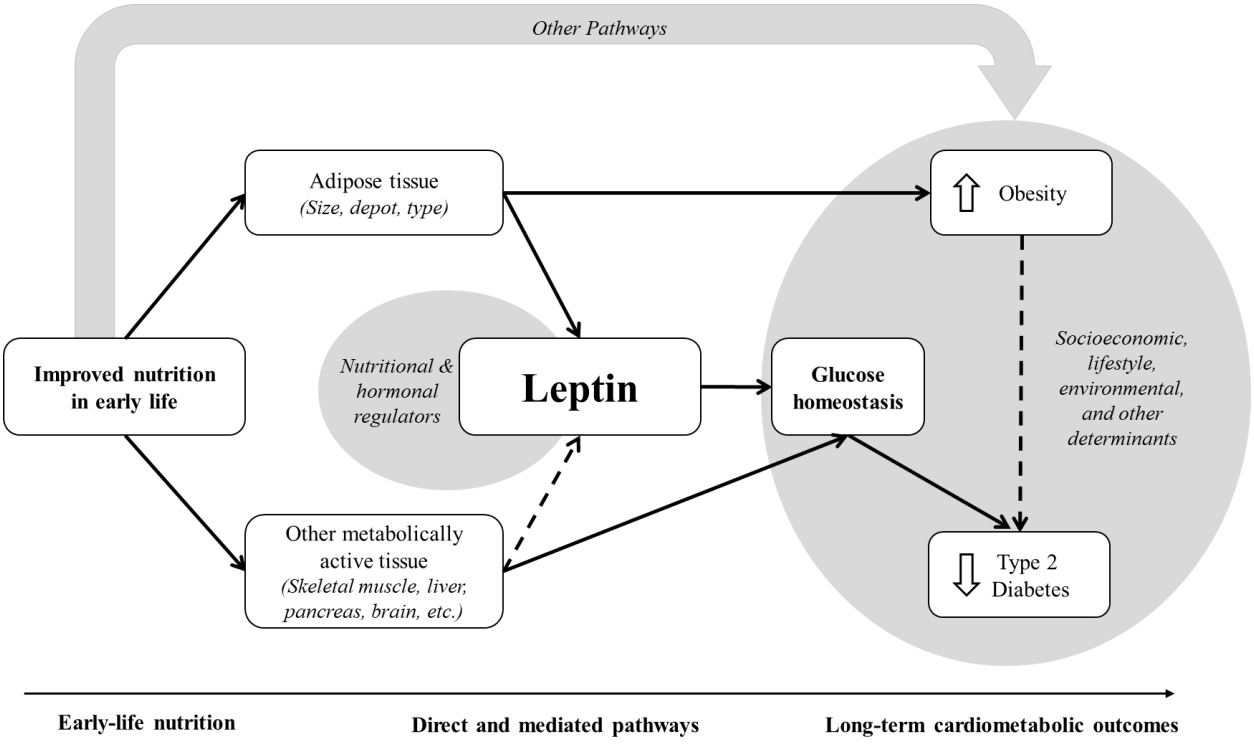


Legend:

1. Total effect $c = c' + ab$; Mediation effect = ab; Percentage mediated = $ab/c * 100\%$
2. Direct model: Glycemic measurements = c * (Exposure to *atole* in the full first 1000 days) + (control variables) + ε

3. Mediation model: (i) Glycemic measurements = *c'* * (Exposure to *atole* in the full first 1000 days) + *b* * (Fasting leptin) + (control variables) + *ε'*; (ii) Leptin = *a* * (Exposure to *atole* in the full first 1000 days) + (control variables) + *ε''*
4. Control variables included birth villages, timing of exposure, birth year, childhood characteristics (socioeconomic status, or SES, in childhood, maternal age at childbirth, maternal height, and maternal schooling), adult characteristics (SES in 2015, grades of schooling completed, Guatemala city residence), and anthropometry (BMI and waist-to-height ratio)
5. P-values: * < 0.05, ** < 0.01, *** < 0.001
6. Abbreviations: HOMA, the homeostasis model assessment; HOMA-B, HOMA for β-cell function; HOMA-IR, HOMA for insulin resistance

Figure 3. Conceptual framework centering leptin in pathways between early nutrition and long-term cardiometabolic outcomes



Legend: The conceptual framework mapped out simplified pathways between exposure to improved nutrition in early life and the ontogenic effects on metabolically active tissues, including adipose tissue, hepatic tissue, pancreas, skeletal muscle, and brain tissue. Leptin is proportional to adipose tissue mass. Leptin is a key glucose lowering agent in this conceptual framework, which helped elucidate the differential associations between early nutrition and two cardiometabolic outcomes, obesity and type 2 diabetes. Other non-biological determinants were summarized in the shaded ovals, following the grey shaded arrow, but were not the focus of our analysis.

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CHAPTER 6: MANUSCRIPT FOR SPECIFIC AIM 2

Meal challenge-induced biomarker responses differed by cardiometabolic phenotypes in a Guatemalan adult population

6.1 Abstract

Objective: To describe the responses of biomarkers induced by a mixed-component meal challenge, and to compare them across cardiometabolic phenotypes.

Methods: In this study of 1,027 participants (mean ± SD age 44.0 ± 4.2 y, 59.4% women), fasting and two-hour postprandial plasma were assayed for lipids, glycemic, and inflammatory biomarkers. We compared individual biomarker responses (postprandial relative change, %Δ) across strata of glycemic and adiposity phenotypes using linear regression models. We also investigated the relationships among all %Δ using structural equation modeling.

Results: Meal-induced increase in glucose was higher in participants who had nondiabetic hyperglycemia (3.9%; 95% confidence interval: 1.4, 6.4%) or diabetes (21.3%; 16.6, 26.0%), compared with normoglycemia (%Δ = 11.1). Overweight and obese participants also exhibited greater postprandial increase in glucose (4.5%; 1.7, 7.3% and 5.1%; 2.1, 8.1% more, respectively), compared with normal weight group (%Δ = 9.2). Postprandial insulin response was blunted in diabetic participants (-49.6%; -91.5, 7.8%), but not in hyperglycemic participants, compared with normoglycemic participants (%Δ = 238.0). Insulin responses were also attenuated in the obese group (-54.6%; -83.1, -26.2%) compared with normal weight participants (%Δ = 247.6). Leptin concentration decreased after meal (%Δ = -17.7 and -18.5 in normoglycemic and normal weight participants, respectively), and the reduction was attenuated in hyperglycemia

(4.3%; 0.04, 8.4%) and in obesity (6.2%; 0.9, 11.5%). We identified three latent variables for postprandial %Δ (F1 to F3). F1 and F3 were lipid-dominant, whereas F2 was glycemic response-dominant.

Conclusions: Meal-induced biomarker responses, especially glycemic responses, differed among participants, as stratified by cardiometabolic phenotypes. Structurally, lipids and glycemic markers clustered separately. These findings may help elucidate the mechanisms for the development of cardiometabolic conditions.

6.2 Introduction

Major cardiometabolic phenotypes are associated with the most common non-communicable diseases, such as obesity, cardiovascular disease, and diabetes (1). Globally, the prevalence of adulthood obesity has doubled since 1980, reaching 603.7 million in 2015 (2). Cardiometabolic mortality is high: cardiovascular diseases account for almost a half of global non-communicable disease mortality (17.5 out of 38 million), and diabetes contributed to 1.5 million deaths (3, 4). Key molecular mechanisms that contribute to the onset and progression of cardiometabolic conditions include inflammation and oxidative stress (1). Among the many different stress signals that can initiate these biochemical processes, a recurring daily activity that is directly linked to inflammation and oxidative stress is meal consumption (5).

Following the consumption a meal containing fat and sugar, acute and transient hyperlipidemia and hyperglycemia are key metabolic perturbations that determine the extent of cardiometabolic insults (5, 6). The impact of postprandial hyperlipidemia and hyperglycemia on inflammation and oxidative stress has been reported to be independent and cumulative (7). Acute increases in lipids during the post-absorptive period may induce a state of inflammation (8). Non-esterified fatty acids released by hydrolysis of dietary triglycerides can also trigger endothelial cells to express adhesion molecules and produce inflammatory cytokines (9). Similarly, meal-induced elevations in glucose also play a role in inducing inflammation and oxidative stress (10, 11). In vivo studies have demonstrated that consumption of glucose resulted in increased production of tumor necrosis factor alpha (TNF-α) and interleukin-6 by peripheral blood mononuclear cells (12, 13). In addition, exposure to high glucose can result in modification of proteins, leading to the formation of pro-inflammatory advanced glycation end products (10). However, under ideal conditions, homeostasis would be readily restored with the stimulation of anti-inflammatory and antioxidant processes (14, 15).

The ability to maintain homeostasis, in the context of intermittent and recurrent exposure to a high-fat and high-sugar environment, is a measure of overall cardiometabolic health (16) This ability is referred to as phenotypic flexibility (17). Failure to maintain homeostasis may eventually lead to chronic metabolic perturbation. In controlled laboratory settings, stress-based methods have been developed to test the range of phenotypic flexibility (17-19). A meal challenge model that mimics dietary stress is particularly ideal in studying metabolic responses (17, 18). As an effort to maximize the acute responses, many in vivo acute studies have chosen meal challenges that provide large doses of fat, glucose, and calories (7, 20, 21). It is our hypothesis that responses to meals that contain physiologic amounts of fat and glucose may provide a better understanding of the cumulative metabolic disturbances leading to abnormal cardiometabolic conditions. We also hypothesized that these responses may differ by various cardiometabolic phenotypes.

In this sample of 1,027 adults, we assessed the effect of a mixed-component meal challenge on responses in biomarkers that represent four cardiometabolic processes, including lipids, glycemic responses, as well as pro- and anti-inflammatory responses. We compared the differences in biomarker responses across strata of cardiometabolic phenotypes. We then described the structural relationships amongst these responses from a data-driven perspective to confirm empirically recognized cardiometabolic pathways.

6.3 Material and methods

Study population

This is an assessment of meal-induced changes in biomarkers in a cohort of 1,027 (59.4% women) free-living adults in Guatemala. The study participants were a subset of individuals who

were enrolled in the Institute of Nutrition of Central America and Panama (INCAP) Longitudinal study initiated in 1969 (22). Excluded from the current study (2015-17) are individuals who were not fasted at the time of the clinic visit (n = 27), or who had known diabetes diagnosis or had fasting glucose of 180 mg/dL or higher (n = 85).

Meal challenge procedure

After obtaining informed consent, a trained phlebotomist drew venous blood from each participant upon verifying fasting state (eight hours or more). Participants were given a freshly prepared milkshake, consisting of 25 g safflower oil, 52 g sugar, 12 g *Incaparina* powder (a soy and maize-based protein mixture developed by INCAP), in 170 ml lactose-free skim milk. Each 100 g of the shake provided 164.7 cal (31% from fat), containing 3.4 g protein, 25.2 g carbohydrate, and 5.7 g fatty acids, including 3.0 g monounsaturated fatty acids and 0.9 g polyunsaturated fatty acids (fatty acids were calculated as triglycerides). Exactly two hours after the consumption of the shake, the phlebotomist drew a second venous blood sample.

Laboratory methods

Pre- and post-prandial glucose concentrations were assayed using enzymatic colorimetric methods (Cobas C111 analyzer, ROCHE, Indianapolis, IN, USA), before the samples were frozen for storage at -80 ^{o}C. Plasma samples were shipped in dry ice to the US and fasting and post-challenge plasma samples were assayed for lipids, glycemic markers, and inflammation markers. The samples were thawed at 4 ^{o}C in batches, each containing approximately 40 pairs of samples.

All other measurements were performed on the AU480 automatic chemistry analyzer (Beckman Coulter Diagnostics, Fullerton CA, US). We assayed total cholesterol (TC) and

triglycerides (TG) using enzymatic methods (Sekisui Diagnostics, PA, US). We assayed high-density lipoprotein cholesterol (HDLc) and low-density lipoprotein cholesterol (LDLc) using homogeneous method (Sekisui Diagnostics, PA, US). Non-HDLc concentration was calculated as subtracting HDLc from TC concentration. Apolipoproteins (apoA-I and apoB) were assayed using immunoturbidimetric assay (Kamiya Biomedical Company, WA, US). We assayed non-esterified fatty acids (NEFA) using calorimetric methods (Wako Chemicals Corporation, Richmond VA, US). Insulin and hsCRP were assayed using immunoturbidimetric method (Kamiya Biomedical Company, WA, US). Cytokines, including leptin, resistin, monocyte chemoattractant protein-1 (MCP-1), interleukin-10 (IL-10), and adiponectin, were determined in duplicates by ELISA (Boster Biologicals Technology, CA, USA).

Stratification by cardiometabolic phenotypes

Hyperglycemia was defined as fasting plasma glucose ≥ 100 mg/dL and ≤ 125 mg/dL, or two-hour post-challenge plasma glucose level ≥ 140 mg/dL and < 200 mg/dL among participants who were not using diabetic medication (23). Type 2 diabetes was defined as a fasting plasma glucose of 126 mg/dL or more, post-challenge glucose of 200 mg/dL or more, or use of diabetes medication. (23)

Normal weight was defined as body mass index (BMI) between 18.5 and 24.9 kg/m^2. Overweight was BMI between 25 and 29.9 kg/m^2. Obesity status was defined as BMI ≥ 30 kg/m^2 (24). Abdominal obesity was defined as waist circumference larger than 88 cm in women and more than 102 cm in men (25).

Statistical analysis

We examined the reliability between biomarkers assayed on the day of blood draw at each site in Guatemala (all assayed at the same time as glucose with the Cobas C111 analyzer, ROCHE, Indianapolis, IN, USA) and those assayed in the US (described in this article) for TC, TG, HDLc, and LDLc using Bland-Altman plots (26). We observed less than 5% of outliers in each plot, indicating high reliability. Missingness of biomarkers included: 25.8% of IL-10, 12.8% of MCP-1, 7.3% of resistin, and 7.2% of adiponectin.

We used Student's *t*-test for comparisons between men and women. For each biomarker, we described fasting concentrations and two-hour postprandial changes in concentration by sex. Postprandial relative change (%Δ) was expressed as:

$$\frac{Postprandial\ concentration - Fasting\ concentration}{Fasting\ concentration} x\ 100\%$$

We compared the postprandial biomarker changes across two sets of cardiometabolic phenotypes, including glycemic phenotypes and overall adiposity phenotypes. There were three glycemic phenotypes, including normoglycemia (reference group), hyperglycemia, and type 2 diabetes. For adiposity phenotypes, we included three groups based on BMI categories, including those who were normal weight (reference group), those who were overweight, and those who had obesity. %Δ of each biomarker was modelled as the dependent variable in linear regression models. We controlled for age and sex. We added BMI to the glycemic phenotype models, and added fasting glucose concentration to the adiposity phenotype models (except for glucose response outcome). To illustrate the response patterns in figures, we replaced the dependent variables (%Δ) with the standardized Z-scores to improve visual comparability across markers. For %Δ of each biomarker, the Z-scores was calculated as: $\frac{Observed\ (\%\Delta) - Mean\ (\%\Delta)}{Standard\ Deviation\ (\%\Delta)}$

To describe the relationships among postprandial changes, we first calculated the correlation matrix among the %Δ. Subsequently, we divided the dataset into two random halves.

Using the first half, we conducted exploratory factor analysis (EFA) to identify the number of factors (latent variables) and to select manifest variables (%Δ) that loaded onto each factor (**Supplemental Table 1**). Using the second half of the data, we tested the EFA-identified latent variables and corresponding manifest variables (the cut point for inclusion in the confirmatory analysis was loading > |0.3|) by constructing a structural equation model ("lavaan" and "semPlot" packages in R) (27, 28). Both latent and manifest variables were standardized. We presented the completely standardized solution but omitted the residuals for visual clarity.

We carried out all analyses in R version 3.6.0 (R Core Team, Foundation for Statistical Computing, Vienna, Austria). Statistical significance was set at p value < 0.05. All p-values were two-sided.

Research ethics

The study was approved by the Institutional Review Board at Emory University and the Ethics Review Committee of INCAP. All study participants provided written informed consent in Spanish.

6.4 Results

Characteristics and fasting cardiometabolic profile of the study population, by sex

Among the 1,027 participants in our study, 610 (59.4%) were women (**Table 1**). The mean (standard deviation) age was 44.1 (4.3) years for women and 43.9 (4.1) years for men. Abnormal glucose was more prevalent in women than in men, 40% of women versus 30% of men had hyperglycemia (p = 0.03), and 8% of women versus 5% of men had type 2 diabetes (p = 0.03). The prevalence of overweight was similar in both sexes (38.5% of women and 44.4% of men, NS). However, more than 40% of women were obese as compared to only 20% men (p <

0.001). In addition, 90.0% of women and 20.1% of men had abdominal obesity ($p < 0.001$) (Table 1).

Fasting concentrations of most lipids differed between men and women ($p < 0.001$ for TC, HDLc, LDLc, non-HDLc, apoA-I, apoB, and NEFA; $p = 0.05$ for TG) (**Table 2**). Fasting insulin and glucose concentrations were higher in women than in men ($p < 0.001$ and $p = 0.01$, respectively). Among pro-inflammation biomarkers, hsCRP and leptin differed by sex ($p < 0.001$) and both were higher in women than in men. The anti-inflammatory cytokine adiponectin was higher in women than in men ($p < 0.001$) (Table 2).

Postprandial changes in biomarker concentrations

TG, insulin, glucose, and IL-10 had the highest relative increase in both men and women (Table 2). Two biomarkers (NEFA and leptin) had relatively large magnitude of reductions in their levels for both men and women. Postprandial biomarker responses differed between men and women for NEFA (larger reduction in women than in men), insulin and glucose (larger increased in women than in men), and leptin (larger reduction in men than in women) ($p < 0.001$ for all) (Table 2). The overall correlations among biomarker responses are shown in **Figure 1**: lipids (all except TG and NEFA) had positive correlations in postprandial responses, and the response for hsCRP was positively correlated with those of the lipids. Insulin and glucose responses were positively correlated, whereas glucose responses had negative correlations with that of all lipids. There were weak ($< |0.10|$) correlations among most cytokines.

Postprandial changes in biomarkers across strata of cardiometabolic phenotypes

The interpretation of the linear regression coefficients depends on the direction of the postprandial change in the reference groups (**Tables 3 and 4**). For biomarkers that had postprandial increase in the reference group (for instance TG), a positive coefficient (β) in $\%\Delta$

showed that the comparison group had a larger magnitude of increase than the reference group did, whereas a negative β meant a smaller magnitude of increase than the reference group. For biomarkers that had postprandial reduction in the reference group (for instance NEFA), a positive β should be interpreted as a smaller magnitude of reduction in the comparison group than in the reference group, whereas a negative β showed a larger magnitude of reduction in the comparison group than in the reference group.

Insulin responded positively to the meal challenge in all three glycemic phenotypes, but the response was 49.6% less in magnitude (95%CI: -91.5, -7.8%) in diabetic participants than in the normoglycemic reference group (Table 3, **Figure 2**). Glucose responses increased in a gradient manner in hyperglycemia (3.9%, 95%CI: 1.4, 6.4%) and in diabetes (21.3%, 95%CI: 16.6, 26.0%) (Figure 2). Leptin decreased across all three groups, but the reduction was attenuated in participants with hyperglycemia (4.3%, 95%CI: 0.04, 8.4%) and diabetes (2.1%, 95%CI: -6.0, 10.2%, NS). MCP-1 reduction was larger in magnitude in hyperglycemic participants (-5.6%, 95%CI: -10.9, -0.2%) and in diabetic individuals (-7.6%, 95%CI: -17.4, 2.2%, NS) (Table 3, Figure 2).

Across strata of adiposity, TC had higher response (1.5%, 95%CI: 0.4, 2.7%) in obese participants, compared with the normal weight reference group (Table 4, **Figure 3**). Insulin response was lower in both overweight (-9.4%, 95% CI: -35.5, 16.7%) and obese group (-54.6%, 95% CI: -83.1, -26.2%), compared with the reference group. Glucose was significantly higher in both overweight (4.5%, 95% CI: 1.7, 7.3%) and obese groups (5.1%, 95% CI: 2.1, 8.1%), comparing with the reference group (Figure 3). Leptin responses decreased in all three strata, but it decreased less in magnitude in obese participants (6.2%, 95%CI: 0.9, 11.5%) (Table 4, Figure

3). Obese participants had an increased response tendency in all lipids and pro-inflammatory markers, compared with the reference group. (Figure 3)

Structural relationships among biomarker responses

The structural equation model (guided by EFA results) showed three main latent variables. (**Figure 4**) %Δ of several lipids (TC, HDLc, LDLc, apoA-I, and apoB, standardized parameter values all > 0.50) and hsCRP loaded on latent variable 1 (F1). TC and hsCRP responses had negative correlation of -0.41. The %Δ of insulin and glucose (standardized parameter values > 0.50), as well as NEFA (-0.23) loaded on latent variable 2 (F2). TG (0.10) and leptin (0.01) responses loaded on latent variable 3 (F3). There was positive correlation between F1 and F3 (1.98) and between F1 and F2 (0.11). We observed a negative correlation between F2 and F3 (-2.33). Fit indices showed that, although this structure fitted the data well in EFA (Supplemental Table 1), it is less so in the confirmatory analysis (Figure 4), with comparative fit index (CFI) of 0.889, Tucker Lewis Index (TLI) of 0.849, root mean square error of approximation (RMSEA) of 0.100, and standardized root mean square residual (SRMR) of 0.071.

6.5 Discussion

Through a standardized, mixed-component meal challenge, we assessed the relationship among meal-induced lipid, glycemic, and inflammatory responses, as well as their differences based on cardiometabolic phenotypes in the present study. When comparing the individual biomarker responses across two sets of cardiometabolic phenotypes, we observed larger magnitude in postprandial insulin increase, lower glucose response, and greater reduction in leptin concentration in the metabolically healthy reference groups. Glycemic responses,

especially insulin response, were the most pronounced differences across the phenotypes. Using data-driven technique, we also confirmed empirically recognized cardiometabolic pathways in response to the meal challenge. These results are important in characterizing the impact of a meal containing physiologic amount of macronutrients on cardiometabolic disturbances.

In our study, postprandial insulin relative elevation was significantly higher in reference groups, compared with their unhealthy counterparts. Interestingly, we observed a gradient effect in insulin responses across strata of cardiometabolic phenotypes: although the glucose response was already greater in hyperglycemic participants, their insulin response was actually greater than the reference group, indicating reasonable insulin sensitivity. It was among diabetic participants that we observed significantly lower insulin response, accompanied by further elevated glucose response. Postprandial insulin response was also attenuated in a gradient manner from overweight to obesity status, compared with normal weight participants. These two sets of gradient patterns support the concept of phenotypic flexibility: at pre-clinical stage, despite some signs of cardiometabolic disturbances, the body maintained homeostasis through other pathways. Only when the disturbances proceeded to clinical stage did we observe significant differences in biomarker responses (30).

We examined the postprandial changes as stratified by glycemic phenotypes. After adjusting for age, sex, and BMI, we observed that responses in lipids were comparable for individuals who had normoglycemia, hyperglycemia, and type 2 diabetes. As expected, meal-induced increases in glucose were higher in individuals with hyperglycemia and highest in individuals with type 2 diabetes. At two-hour after meal consumption, insulin increases were comparable for participants with either normoglycemia or hyperglycemia but was significantly blunted in individuals with newly identified type 2 diabetes, consistent with impaired insulin

metabolism. All three groups stratified by glycemic status had reduction in leptin levels, which was attenuated in hyperglycemia (significant) and diabetes (not significant). Similarly, while both groups of participants who had hyperglycemia and diabetes exhibit further reduction in MCP-1 levels after meal consumption, statistical significance was not demonstrated in type 2 diabetes. The variability in response in this smaller group of diabetic individuals may account for the lack of statistical significance. A previous study reported that postprandial glycemia triggered acute increase in the biomarkers associated with vascular remodeling (and hence future cardiovascular diseases) in diabetic patients who were overweight but healthy (31). We suggest that future studies investigate the associations among these markers in both metabolically healthy and unhealthy adults.

We then examined the postprandial responses as stratified by adiposity phenotypes. After adjusting for age and sex, there was a modest increase in TC in individuals with increased BMI as compared to individuals with normal BMI. However, statistical significance was observed only in obese and not in overweight participants. This may reflect the delayed clearance of intestinal chylomicrons transporting newly absorbed triglycerides and cholesterol (32). Postprandial increases in plasma TG were also higher in overweight and obese individuals as compared to normal weight participants. Among obese participants, there was significantly greater meal-induced increase in glucose and reduction in insulin, although the difference in insulin response was significant only in obese individuals. While postprandial reduction in leptin was comparable for normal and overweight individuals, it was blunted in obese individuals. With respect to anti-inflammatory response, meal-induced increase in IL-10 was reduced with higher BMI (significant in overweight but not in obese participants).

Despite a lack of statistical significance, we also observed higher overall lipid responses and pro-inflammatory responses among participants with obesity. The pronounced decrease in insulin response and higher glucose response among obese participants supports a close linkage between obesity and glucose dysregulation (33). However, inflammatory responses in each biomarker varied considerably. MCP-1 is a potent atherogenic chemoattractant that regulates migration and infiltration of monocytes and macrophages, and it is an important agent in the obesity-diabetes association (34). The observed reduction in MCP-1 in hyperglycemia and diabetes, as well as the increase in the same cytokine in overweight and obesity was not readily interpretable. Further investigation is needed to explain the lack of pattern in the inflammatory responses.

As expected, the difference in cardiometabolic characteristics between men and women in our cohort translated into significant differences in fasting levels for all of the metabolites with the exception of TG, resistin, MCP-1, and IL-10. However, meal-induced responses (%Δ) were different for only a select subset of biomarkers, including NEFA, insulin, glucose, and leptin. Meal-induced increases in both insulin and glucose were greater in women as compared to men. Postprandial reduction in NEFA was more pronounced in women while the reduction in leptin was greater for men. Meal consumption was associated with a reduction in MCP-1 among men but an increase among women. Previous research have reported that women had higher postprandial glucose uptake rate, better insulin secretion, and lower oxidative stress than men (35, 36). The different outcomes observed in our study may be due to the significantly higher prevalence of central obesity in women than men in our study, the use of different doses of fat and glucose in the meal challenge, or the time point for data collection.

With respect to the relationship among postprandial changes in biomarkers, we identified two lipid-dominant latent variables (F1 and F3) and one glucose-dominant latent variable (F2). Except for hsCRP and leptin, none of the pro- and anti-inflammatory markers contributed to the model. We noted a positive correlation between F1 and F3, which may be due to the close association of postprandial changes in TG and all other lipid responses. We observed that lipid responses correlated among themselves (except TG and NEFA), and were associated with inflammatory response as represented by hsCRP. A possible molecular process behind lipid-induced inflammation is the activation of NF-κB, which induces the transcription of genes encoding several pro-inflammatory cytokines and chemokines (10). We also observed a negative correlation between F2 and F3, which may reflect the difference in time to maximum response between lipids and glycemic markers. Previous studies have reported that TG elevation can sustain for 5-8 hours following a fat-containing meal (37, 38). Insulin and glucose responses may occur earlier than lipids, with the maximum changes occurring around the two-hour mark, based on a study in a diabetic population (39). In addition, previous research found that adding glucose to a fat-abundant meal delayed chylomicron response, which may account for the modest changes in TG in our study as well (40).

A few more observations from the structural models were noteworthy: F2 underscored close association between glycemic markers and NEFA. The release of NEFA from adipose tissue is usually suppressed by hormone sensitive lipase that is acutely inhibited to insulin level.(5) Therefore in a physiological state, NEFA is expected to fall rapidly after a mix-component meal due to the suppression of fat mobilization by insulin.(41) We indeed observed this reduction in our study. The unexpected negative correlation between TG and hsCRP responses, might be accounted for by the differences in time course of TG and hsCRP changes

after meal consumption. Based on a previous study, maximal changes in hsCRP occurred earlier than the peak change in chylomicron-TG, which was in line with our observation (39).

Adipokines were previously found to be important in predicting future cardiometabolic risks due to their effects on insulin sensitivity and inflammation (42). Leptin, a versatile adipokine, decreased significantly two hours after the meal challenge, which was plausible based on its catabolic functions, but was unexpected in terms of its pro-inflammatory effect (43). In our study, postprandial leptin was higher in obese participants, compared with normal weight ones. This may be attributable to the positive correlation between leptin and adiposity, and to the common presence of leptin resistance in the obesity status (44). The smaller magnitude of postprandial leptin reduction among hyperglycemic participants was worth noting, especially that it was not observed among diabetic participants. A few possible mechanisms may explain this phenomenon. First, higher glucose concentration in the "unhealthy" groups required more leptin to perform its catabolic effects (45). Second, leptin response differed by body fatness, and by disease status (46). It is possible that the reaction time and the extent to which leptin exerts its impact differ between hyperglycemic and diabetic conditions.

There were a few limitations to this study. We only obtained two time points of biomarker responses, which may not characterize the exact response trajectory of the biomarkers. The lack of response in some biomarkers may be due to a true resilience to stress, or perhaps the peak functioning time was not captured at the two-hour time point. In addition, the reference groups within each cardiometabolic disease category may contain other cardiometabolic phenotypes. For instance, normoglycemic participants may be categorized as overweight. We recognized this possibility for overlapping categorization, and controlled for relevant variables (e.g., BMI in glycemic models) in the regression models to minimize the effect. Similarly, we

did not carry out the same analysis for metabolic syndrome, despite it being a widely studied cardiometabolic condition. This is because metabolic syndrome is a complex, non-clinical definition that includes both measurements of adiposity and glucose intolerance.

To our knowledge, this is the first study in low- and middle-income country setting to test meal-induced stress response in a cohort of adults. Very few studies in similar settings have comprehensive panel of cardiometabolic biomarkers as ours, let alone testing a metabolic stress model to assess phenotypic flexibility. Our study is also relatively large-scale with sufficient statistical power to detect significant changes (over 80% power for most biomarkers to detect a medium effect size, except IL-10 and MCP-1). Another strength of our study is that the meal challenge provided macronutrients within a physiological (and not pathologically high) range, which enabled us to observe biologically plausible responses. This may also explain the modesty in the response levels in a few biomarkers, since we did not artificially magnify the external stress to trigger responses that may lack clinical implication. In addition, we conducted the analyses and interpreted the data based on both individual biomarker responses and overall patterns.

Daily exposure to fat- and sugar-containing diets may gradually contribute to cardiometabolic disturbances. We tested an overall response in the metabolic system following a mix-component meal challenge that provided physiologic amount of macronutrients. We observed that the postprandial responses, particularly glycemic responses, differed across strata of cardiometabolic phenotypes. We also documented the structural relationships amongst the biomarker responses. We emphasized the concept of phenotypic flexibility, especially through the observed gradient effect in glycemic responses from pre-clinical to clinical conditions. Building upon these results, researchers should adopt a holistic view to understand how the

pathways overlap and compensate for each other in response to external stress signals. Future studies with larger sample sizes should aim to compare the structural equation models across cardiometabolic phenotypes to further elucidate the nutritional mechanisms for the onset and progression of cardiometabolic conditions. We also consider it valuable to identify an “ideal” range of responses across multiple cardiometabolic pathways, which provides a more dynamic assessment model for cardiometabolic health.

6.6 Tables, figures, and supplemental materials

Table 1: Selected sociodemographic and cardiometabolic characteristics [1] of the study population, by sex

	Women (n = 610) **Mean (SD) or %**	**Men (n = 417)** **Mean (SD) or %**	**p value [2]**
Age, years	44.1 (4.3)	43.9 (4.1)	0.26
Glycemic status, %			
Hyperglycemia	40.2	33.4	0.03
Type 2 diabetes	8.4	4.8	0.03
Body mass index, kg/m^2	29.3 (5.8)	26.6 (4.7)	<0.001
Weight status, %			
Overweight	38.5	44.4	0.07
Obesity	41.3	19.4	<0.001
Waist Circumference, cm	101.7 (13.6)	93.9 (11.3)	<0.001
Abdominal obesity, %	90.0	20.1	<0.001

[1] Definitions: Hyperglycemia: fasting plasma glucose of 100–125 mg/dL or two-hour post-challenge glucose of 140–199 mg/dL among participants not reporting use of diabetes medication. Type 2 diabetes: fasting plasma glucose > 125 mg/dL, or two-hour post-challenge glucose ≥ 200 mg/dL, or reporting use of diabetes medication. Overweight: BMI ≥ 25 kg/m^2 &

BMI < 30 kg/m^2; Obesity: BMI ≥ 30 kg/m^2. Abdominal obesity: waist circumference > 88 cm for women; > 102 cm for men.

[2] P-values based on Student's *t*-test (continuous variables) or chi-squared test (categorical variables)

Abbreviation: SD, standard deviation.

Table 2. Fasting and two-hour postprandial changes in concentrations of biomarkers, by sex

Biomarkers	Fasting concentration [1]			Postprandial change (%Δ) [2]		
Mean (standard deviation)	**Women (n = 610)**	**Men (n = 417)**	**p-value [3]**	**Women (n = 610)**	**Men (n = 417)**	**p-value [4]**
Lipids						
TC (mmol/L)	4.7 (1.0)	4.4 (1.0)	<0.001	0.3 (6.3)	0.1 (7.6)	0.68
TG (mmol/L)	4.3 (2.3)	4.6 (3.0)	0.05	15.2 (18.9)	15.9 (18.2)	0.56
HDLc (mmol/L)	1.1 (0.2)	1.0 (0.2)	<0.001	1.3 (6.3)	0.8 (7.0)	0.27
LDLc (mmol/L)	3.0 (0.8)	2.7 (0.9)	<0.001	0.1 (7.7)	0.5 (11.4)	0.54
Non-HDLc (mmol/L)	3.7 (0.9)	3.4 (0.9)	<0.001	-0.04 (7.4)	0.6 (10.6)	0.26
ApoA-I (g/L)	1.1 (0.3)	1.0 (0.3)	<0.001	0.5 (6.3)	0.9 (8.5)	0.37
ApoB (g/L)	0.9 (0.3)	0.8 (0.3)	<0.001	-0.5 (8.4)	0.2 (9.9)	0.23
NEFA (mEq/L)	1.0 (0.4)	0.8 (0.4)	<0.001	-52.5 (28.0)	-42.2 (31.6)	< 0.001
Glycemic measurements						
Insulin (pmol/L)	122.0 (73.9)	90.4 (72.5)	<0.001	264.2 (170.8)	196.0 (167.6)	< 0.001
Glucose (mmol/L)	5.7 (0.9)	5.5 (0.7)	0.01	19.7 (18.5)	7.3 (20.8)	< 0.001
Pro-inflammation markers						
hsCRP (mg/L)	3.8 (3.7)	2.1 (2.9)	<0.001	0.6 (12.1)	1.5 (17.2)	0.35
Leptin (ng/mL)	17.9 (10.1)	4.1 (4.7)	<0.001	-10.4 (25.0)	-22.0 (29.7)	< 0.001
Resistin (ng/mL)	2.2 (2.5)	2.3 (3.8)	0.56	-4.0 (18.7)	-2.2 (23.5)	0.30
MCP-1 (pg/mL)	93.7 (98.6)	99.1 (70.2)	0.34	15.8 (111.2)	-6.3 (35.4)	0.01
Anti-inflammation markers						
IL-10 (pg/mL)	70.0 (190.0)	93.8 (235.8)	0.14	15.8 (111.2)	15.3 (110.1)	0.95
Adiponectin (µg/mL)	12.5 (8.7)	9.1 (7.4)	<0.001	-1.3 (24.8)	4.4 (103.4)	0.38

[1] Missingness (> 5.0%): 25.8% of IL-10, 12.8% of MCP-1, 7.3% of resistin, and 7.2% of adiponectin.

[2] Postprandial change (%Δ) equals the difference between postprandial and fasting biomarker concentrations, divided by fasting concentrations, presented as percentages.

[3] Student's *t*-test, comparing the fasting biomarker concentrations between men and women.

[4] Student's *t*-test, comparing the postprandial changes (%Δ) between men and women.

Abbreviations: TC, total cholesterol; TG, triglycerides; HDLc, high density lipoprotein cholesterol; LDLc, low density lipoprotein cholesterol; apo, apolipoprotein; NEFA, non-esterified fatty acid; hsCRP, high sensitivity C-reactive protein; MCP-1, monocyte chemoattractant protein 1; IL-10, interleukin 10.

Table 3. Comparison of postprandial changes in biomarkers among people differing by glycemic phenotypes

Postprandial Changes in Biomarkers	**Reference: Normoglycemia n = 570**	**Hyperglycemia n = 384**		**Type 2 Diabetes n = 71**	
%Δ [1]	**Mean (SD)**	**Mean (SD)**	**In relation to reference β (95% CI) [2]**	**Mean (SD)**	**In relation to reference β (95% CI) [2]**
Lipid responses					
TC	0.0 (6.6)	0.6 (7.5)	0.4 (-0.5, 1.3)	-0.1 (4.9)	-0.3 (-2.0, 1.4)
TG	15.0 (18.2)	15.9 (19.8)	0.6 (-1.9, 3.1)	17.0 (15.6)	1.7 (-2.9, 6.4)
HDLc	0.9 (6.4)	1.4 (7)	0.4 (-0.5, 1.2)	0.7 (5.4)	-0.3 (-2.0, 1.3)
LDLc	0.2 (9.5)	0.6 (9.6)	0.5 (-0.7, 1.8)	-0.6 (5.8)	-0.5 (-2.9, 1.8)
Non-HDLc	0.2 (8.7)	0.4 (9.7)	0.1 (-1.0, 1.3)	-0.2 (5.3)	-0.3 (-2.5, 1.9)
ApoA-I	0.6 (7.7)	0.8 (7.1)	0.1 (-0.9, 1.0)	0.2 (5.1)	-0.4 (-2.3, 1.4)
ApoB	0.0 (9.1)	-0.3 (9.3)	-0.4 (-1.6, 0.8)	-1.4 (6.3)	-1.3 (-3.6, 0.9)
NEFA	-47.9 (29.9)	-49.4 (30.2)	-1.2 (-5.1, 2.7)	-46.8 (29)	2.1 (-5.5, 9.3)
Glycemic responses					
Insulin	238 (179.8)	243 (168.8)	8.9 (-13.4, 31.2)	188.7 (123.8)	-49.6 (-91.5, -7.8) *
Glucose	11.1 (16.1)	16.3 (19.8)	3.9 (1.4, 6.4) **	34.8 (36)	21.3 (16.6, 26.0) ***
Pro-inflammatory responses					
hsCRP	1.4 (15.3)	0.3 (12.7)	-1.1 (-3.0, 0.8)	1.7 (15.7)	0.5 (-3.1, 4.1)
Leptin	-17.7 (27.5)	-11.1 (26.7)	4.3 (0.04, 8.4) *	-12.8 (30)	2.1 (-6.0, 10.2)
Resistin	-3.4 (21.8)	-3.1 (19.8)	0.5 (-3.1, 4.2)	-3.1 (19)	0.8 (-6.1, 7.8)
MCP-1	0.3 (40.0)	-5.0 (34.2)	-5.6 (-10.9, -0.2) *	-6.9 (31.6)	-7.6 (-17.4, 2.2)
Anti-inflammatory responses					
IL-10	15.6 (100.7)	14.8 (116.2)	2.0 (-16.0, 20.0)	20 (148.3)	7.8 (-24.7, 40.4)
Adiponectin	2.6 (91.4)	-0.8 (19.6)	-3.6 (-15.7, 8.4)	-0.9 (19.3)	-3.2 (-26.5, 20.1)

[1] Postprandial change (%Δ) equals the difference between postprandial and fasting biomarker concentrations, divided by fasting concentrations, presented as percentages.

[2] Regression models adjusted for sex, age, and body mass index

* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

Abbreviations: β, regression coefficient; CI, confidence interval; TC, total cholesterol; TG, triglycerides; HDLc, high density lipoprotein cholesterol; LDLc, low density lipoprotein cholesterol; apo, apolipoprotein; NEFA, non-esterified fatty acid; hsCRP, high sensitivity C-reactive protein; MCP-1, monocyte chemoattractant protein 1; IL-10, interleukin 10.

Table 4. Comparison of postprandial changes in biomarkers among people differing by adiposity phenotypes

Postprandial Changes in Biomarkers	**Reference: Normal weight (n = 262)**	**Overweight (n = 420)**		**Obese (n = 333)**	
%Δ [1]	**Mean (SD)**	**Mean (SD)**	**In relation to reference β (95% CI) [2]**	**Mean (SD)**	**In relation to reference β (95% CI) [2]**
Lipid response					
TC	-0.6 (7.3)	0.3 (6.3)	0.8 (-0.2, 1.9)	1.0 (7.2)	1.5 (0.4, 2.7) **
TG	13.6 (19.4)	16.4 (20)	2.9 (-0.01, 5.8)	16.1 (16)	2.8 (-0.3, 6.0)
HDLc	0.6 (6.7)	1.3 (6.8)	0.6 (-0.4, 1.7)	1.3 (6.3)	0.5 (-0.6, 1.6)
LDLc	0.2 (10.9)	0.4 (9.3)	0.2 (-1.2, 1.7)	0.2 (8)	0.1 (-1.5, 1.7)
Non-HDLc	-0.2 (10.5)	0.3 (7.9)	0.5 (-0.8, 1.9)	0.7 (8.7)	1.1 (-0.4, 2.6)
ApoA-I	-0.1 (9.4)	1.0 (6.6)	1.1 (0.01, 2.3) *	0.9 (6.2)	1.1 (-0.2, 2.3)
ApoB	-0.6 (9.7)	0.1 (8.6)	0.8 (-0.6, 2.2)	-0.3 (9)	0.6 (-0.9, 2.2)
NEFA	-49.7 (32)	-46.3 (30.2)	5.0 (0.4, 9.6) *	-49.9 (27.7)	3.3 (-1.6, 8.4)
Glycemic responses					
Insulin	247.6 (203.8)	247.2 (171.6)	-9.4 (-35.5, 16.7)	212.8 (141.2)	-54.6 (-83.1, -26.2) ***
Glucose	9.2 (21.6)	14.9 (18.6)	4.5 (1.7, 7.3) **	17.9 (16.9)	5.1 (2.1, 8.1) **
Pro-inflammatory responses					
hsCRP	0.8 (17.1)	1.1 (15)	0.4 (-1.9, 2.6)	1.2 (10.6)	0.9 (-1.6, 3.3)
Leptin	-18.5 (34.9)	-17.7 (26.5)	0.3 (-4.7, 5.3)	-9.7 (20.9)	6.2 (0.9, 11.5) *
Resistin	-5.8 (21.8)	-1.7 (21.5)	4.3 (-0.01, 8.6)	-3.6 (19.4)	3.0 (-1.7, 7.6)
MCP-1	-6.7 (37.5)	-0.7 (36.8)	5.0 (-1.2, 11.2)	-1.1 (38.3)	4.1 (-2.7, 10.9)
Anti-inflammatory responses					
IL-10	24.4 (121.5)	4.8 (85.4)	-21.4 (-41.8, -1.0) *	17.3 (116.9)	-9.2 (-31.0, 12.6)

Adiponectin	-4.2 (20.2)	5.3 (106.2)	10.1 (-4.3, 24.5)	-0.4 (22.3)	5.7 (-9.8, 21.2)

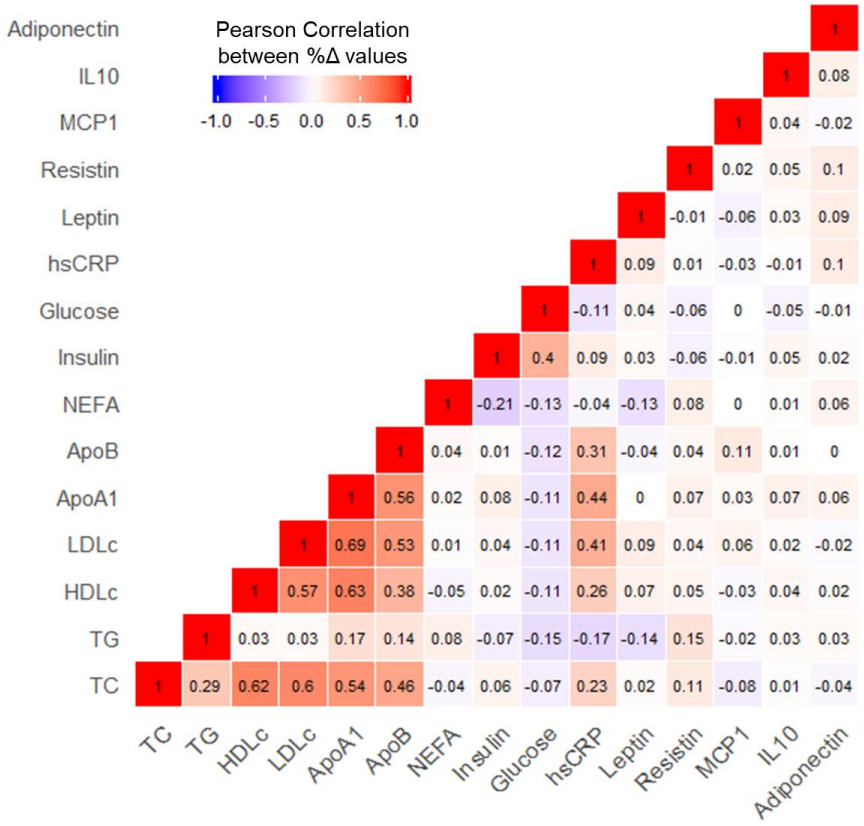
[1] Postprandial change (%Δ) equals the difference between postprandial and fasting biomarker concentrations, divided by fasting concentrations, presented as percentages

[2] Regression models adjusted for sex and age

* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

Abbreviations: β, regression coefficient; CI, confidence interval; TC, total cholesterol; TG, triglycerides; HDLc, high density lipoprotein cholesterol; LDLc, low density lipoprotein cholesterol; apo, apolipoprotein; NEFA, non-esterified fatty acid; hsCRP, high sensitivity C-reactive protein; MCP-1, monocyte chemoattractant protein 1; IL-10, interleukin 10.

Figure 1. Correlation matrix of postprandial biomarker changes

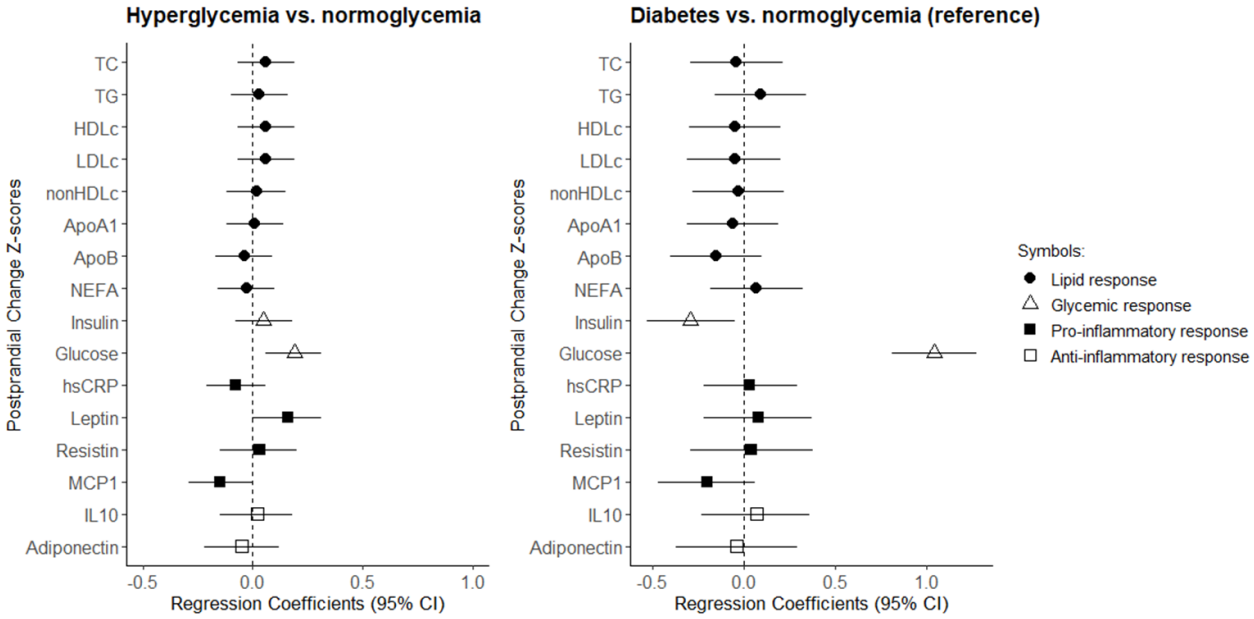


Legend:

1. The values in each cell represented Pearson's correlation coefficient.
2. Postprandial change (%Δ) of each biomarker equals the difference between postprandial and fasting biomarker concentrations, divided by fasting concentrations, presented as percentages.

Abbreviations: TC, total cholesterol; TG, triglycerides; HDLc, high density lipoprotein cholesterol; LDLc, low density lipoprotein cholesterol; apo, apolipoprotein; NEFA, non-esterified fatty acid; hsCRP, high sensitivity C-reactive protein; MCP-1, monocyte chemoattractant protein 1; IL-10, interleukin 10.

Figure 2. Standardized comparison of postprandial biomarker changes among participants differing by glycemic phenotypes

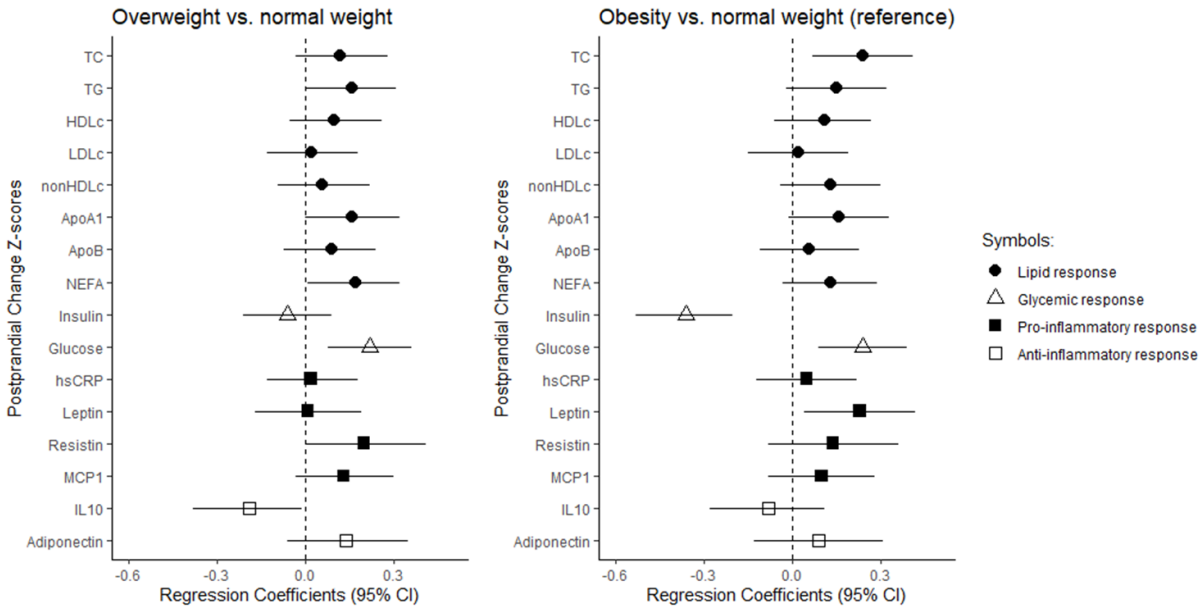


Legend:

1. Sample sizes: 570 normoglycemia, 384 hyperglycemia, and 71 diabetes.
2. This figure presented linear regression results to compare postprandial biomarker responses across strata of glycemic phenotypes (those with normal glycemia served as reference group) Each regression model had one biomarker as the dependent variable, and the three-level glycemic phenotype variable (dummy variables) as the independent variable. We controlled for age, sex, and body mass index in each model.
3. Postprandial change (%Δ) equals the difference between postprandial and fasting biomarker concentrations, divided by fasting concentration. Standardized Z-scores were calculated as postprandial response (%Δ) minus the mean and divided by the standard deviation for each biomarker.

Abbreviations: TC, total cholesterol; TG, triglycerides; HDLc, high density lipoprotein cholesterol; LDLc, low density lipoprotein cholesterol; apo, apolipoprotein; NEFA, non-esterified fatty acid; hsCRP, high sensitivity C-reactive protein; MCP-1, monocyte chemoattractant protein 1; IL-10, interleukin 10.

Figure 3. Standardized comparison of postprandial biomarker changes among participants differing by adiposity phenotypes

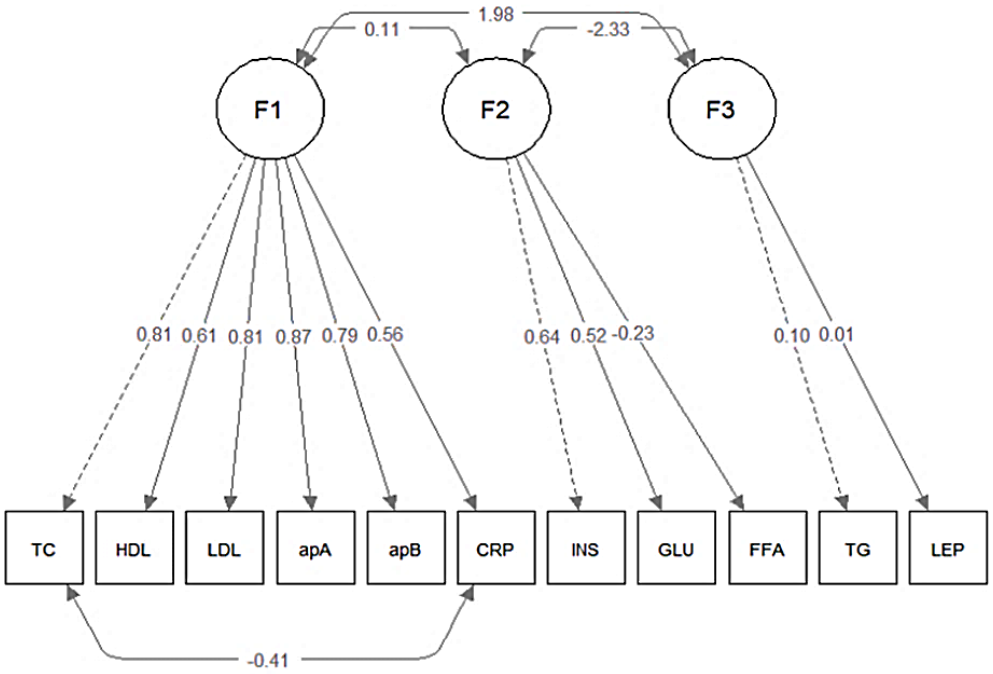


Legend:

1. Sample sizes: 262 normal weight, 420 overweight, and 333 obese.
2. This figure presented linear regression results to compare postprandial biomarker responses across strata of adiposity phenotypes (those with normal weight served as reference group) Each regression model had one biomarker as the dependent variable, and the three-level adiposity phenotype variable (dummy variables) as the independent variable. We controlled for age and sex in each model.
3. Postprandial change (%Δ) equals the difference between postprandial and fasting biomarker concentrations, divided by fasting concentration. Standardized Z-scores were calculated as postprandial response (%Δ) minus the mean and divided by the standard deviation for each biomarker.

Abbreviations: TC, total cholesterol; TG, triglycerides; HDLc, high density lipoprotein cholesterol; LDLc, low density lipoprotein cholesterol; apo, apolipoprotein; NEFA, non-esterified fatty acid; hsCRP, high sensitivity C-reactive protein; MCP-1, monocyte chemoattractant protein 1; IL-10, interleukin 10.

Figure 4. Structural equation modeling for postprandial biomarker changes in the study



Legend:

1. Model fit statistics: CFI = 0.889, TLI = 0.847; RMSEA = 0.100 (90%CI: 0.079, 0.122); SRMR = 0.071
2. All manifest variables (boxes) were postprandial biomarker changes, or %Δ values. All latent variables (circles) were derived from loadings in exploratory factor analysis (EFA) on the other random half of the data (refer to Supplemental Table 1).
3. Dashed lines indicated fixed parameters, and continous lines indicated free parameters.
4. The residuals were omitted in the graph for visual clarity. Values were standardized parameter values.

Abbreviations: F, factor (latent variable), TC, total cholesterol; TG, triglycerides; HDLc, high density lipoprotein cholesterol; LDLc, low density lipoprotein cholesterol; apo,

apolipoprotein; FFA, free fatty acids, same as NEFA or non-esterified fatty acid; hsCRP, high sensitivity C-reactive protein; MCP-1, monocyte chemoattractant protein 1; IL-10, interleukin 10.

Supplemental Table 1. Exploratory factor analysis loadings

Manifest variables	**Factor 1**	**Factor 2**	**Factor 3**
(%Δ)	**(0.213)**	**(0.075)**	**(0.063)**
(proportional variance)			
TC	0.696	--	0.192
TG	--	--	0.730
HDLc	0.890	--	-0.115
LDLc	0.862	--	--
ApoA-I	0.832	--	0.111
ApoB	0.500	--	0.242
NEFA	--	-0.305	--
Insulin	--	0.770	--
Glucose	--	0.644	--
hsCRP	0.369	--	--
Leptin	0.174	--	-0.380
Resistin	--	--	0.209
MCP-1	--	--	0.254
IL-10	0.172	--	0.146
Adiponectin	--	--	--

1. Cut-off for including the items in subsequent confirmatory factor analysis/structural equation modeling: loading $\geq |0.3|$

2. EFA model fit: TLI = 0.93, RMSEA = 0.05 (90%CI: 0.02, 0.07)

Abbreviations: TC, total cholesterol; TG, triglycerides; HDLc, high density lipoprotein cholesterol; LDLc, low density lipoprotein cholesterol; apo, apolipoprotein; NEFA, non-esterified fatty acid; hsCRP, high sensitivity C-reactive protein; MCP-1, monocyte chemoattractant protein 1; IL-10, interleukin 10.

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CHAPTER 7: MANUSCRIPT FOR SPECIFIC AIM 3

Postprandial glycemic response differed by early-life nutritional exposure in a longitudinal cohort: a single- and multi-biomarker approach

7.1 Abstract

Purpose: Populations malnourished in early life are at increased risk for cardiometabolic diseases. We assessed if improved nutrition predicts cardiometabolic function, as assessed by postprandial biomarker responses.

Methods: Participants had been randomized at the village level to receive one of two nutritional supplements as children. At mean age 44 y (range 37 – 53 y), we obtained plasma samples before and 2h after a mixed-component meal challenge. We assayed biomarkers including lipids, glycemic measurements, and inflammatory cytokines. We compared postprandial biomarker responses among those who received the improved nutrition intervention from conception through to their second birthday (the first 1000 days) to those with other exposure status, including those who received the improved nutrition intervention at other ages, and those who received the less nutritious supplement.

Results: Among 1,027 participants (59.4% female), 22.9% were exposed to improved nutrition in the first 1000 days. Insulin increased the most in response to the meal challenge (over two folds), and non-esterified fatty acids decreased the most (by half). Glucose increased postprandial by 11.4% in the exposed group, compared with 15.7% in the unexposed group ($p < 0.05$), which remained significant after adjusting for confounders (-4.7%; 95% confidence

interval: -9.3%, -0.01%). Responses to the prandial challenges for the other biomarkers did not differ by intervention group (all $p > 0.05$).

Conclusion: Early-life exposure to improved nutrition was associated with more favorable postprandial glucose response in this population. We did not observe a difference in overall cardiometabolic responses between the exposure groups.

Key words: early life, nutritional intervention, cardiometabolic diseases, diabetes, obesity, inflammation

7.2 Introduction

The first 1000 days from conception to the second birthday is a critical window for growth and development [1,2]. During this period of time, balanced maternal diet and optimal infant and young child feeding is essential in supporting growth and developing the psycho-neuro-endocrinological systems [3]. The developmental origins of health and disease (DOHaD) paradigm centers around the lifelong consequences of exposures in early life [4]. An important aspect of DOHaD is identifying the mismatch between early-life factors (genetic predisposition, epigenetic modification, and early-life environment) and the environment later in life [5]. For instance, undernutrition in the first 1000 days signals the need for nutrient and energy preservation, thus shaping the metabolic system accordingly. When later encountering an obesogenic environment, the metabolic system is ill-equipped to respond to cardiometabolic disturbances and properly restore homeostasis [5].

The phenomenon of developmental mismatch is observed in populations around the world that are undergoing nutrition transition [6,7]. Nutrition transition is a concept that describes the shift in dietary trends, along with changes in activities and body composition, at the population level over long periods of time [7]. In low- and middle-income countries, nutrition transition is usually observed in the dietary changes from traditional, local, majority plant-based diets to processed, energy-dense but nutrient-poor diets [8]. Parallel to the global nutrition transition is a shift in disease patterns: from the early 1990s till recent years, there has been a 41% decrease in communicable diseases and neonatal disorders worldwide [9]. However, a simultaneous 40% increase in non-communicable diseases was observed, with cardiometabolic diseases being the most important subset therein [9].

The association of early-life nutrition and adulthood cardiometabolic diseases is difficult to ascertain if we focus solely on the clinical diagnosis. Pre-clinical cardiometabolic perturbations, such as the cardiometabolic syndrome, may occur well before clinical diagnosis could be made [10]. These perturbations can manifest as altered or impaired phenotypic flexibility, which is a more sensitive measure of overall cardiometabolic status than assessments at the fasted state [11]. The global burden of pre-clinical cardiometabolic conditions is rapidly increasing and should be a target for prevention of future cardiometabolic diseases and associated complications. For example, intermediate hyperglycemia affected one in five men and one third of women in Guatemala, a country that is undergoing nutrition transition [12]. Hyperglycemia and other forms of glucose dysregulation, if left uncontrolled, are known to progress to type 2 diabetes (8).

We have previously reported that exposure to improved nutrition in the first 1000 days was associated with long-term cardiometabolic status at the fasted state, including reduced glucose concentration [13,14]. We do not, however, have any information on how early-life nutritional improvements may impact phenotypic flexibility, which could be assessed via metabolic challenge models [15]. A mixed-component meal challenge simultaneously tests several cardiometabolic pathways, and may provide valuable insights into stress response capacity of the cardiometabolic system [15,16]. Hence, we tested the hypothesis that early-life nutritional exposure modifies meal-induced biomarker responses in a population that is undergoing nutrition transition.

7.3 Methods

Study population

We conducted a follow-up study of the Institute of Nutrition of Central America and Panama (INCAP) Longitudinal Study (17). Between 1969 and 1977, a total of 2,392 children were randomized at the village level to receive either *atole* (the "improved nutrition" supplement) or *fresco* (the "control" supplement). *Atole* is a dietary supplement in the form of porridge that provides 6.4 g protein, 0.4 g fat, and 90 kcal per 100 mL. Fresco is a low-calorie drink (all calories from carbohydrates, 33 kcal per 100 mL) that does not contain protein. The consumption of the supplements was meticulously documented for each participant, and more details were reported elsewhere (17).

In the current analysis, we dichotomized the participants into "exposed group" and "unexposed group", considering both the type and the timing of early-life nutritional exposure. We used the first 1000 days as proxy for early life, calculated as 266 days before birth (the average length of pregnancy) till two years after birth. The exposed group included participants who received *atole* during the full first 1000 days. The unexposed group included the rest of the participants, who either received *atole* but not during the entire period of first 1000 days or received *fresco*.

The participants were followed up in several subsequent study waves to track the growth and development of the children, as well as human capital-related information in their adolescence and adulthood (18). Attrition was as follows: at the time of data collection, 369 (15.4%) cohort members have died, 249 (10.4%) emigrated, and 113 (4.7%) were been lost to follow up. Out of the 1,661 eligible members, 134 could not be reached, and 366 declined to participate. Of the remaining participants who provided consent, 16 did not attend the scheduled

clinical exam, 6 women were pregnant or lactating at the scheduled time for data collection, 27 did not have fasting plasma samples required for this set of analysis, and an additional 85 did not have postprandial samples. Our final sample size for this analysis is 1,027 (42.9% of the original cohort) (Supplemental Figure 1).

Meal challenge procedure

During the 2015-17 data collection, we administered a standardized, mixed-component meal challenge to test the metabolic stress response among the participants. For each study participant who provided informed consent, a trained phlebotomist drew venous blood after confirming fasting status (an overnight fast of eight hours or longer). Following a safety protocol, we did not give participants the meal challenge if their fasting glucose concentration was >180 mg/dL. For all other participants, we administered the meal challenge in the form of a milk shake, which has macronutrient composition within the physiological range. The shake was a mixture of 25 g safflower oil, 52 g sugar, 12 g *Incaparina* powder (a plant-based protein mixture developed by INCAP), and 170 ml lactose-free skim milk. Each 100 g of the meal challenge contained 164.7 calories (31% from fat), 3.4 g protein, 25.2 g carbohydrate, and 5.7 g fatty acids (fatty acids were calculated as triglycerides). At the two-hour mark after the meal challenge, the phlebotomist drew venous blood a second time.

We collected additional relevant data during this study wave, including anthropometry measurements and cardiometabolic status. Body mass index (BMI) was calculated as weight (kg) divided by height-squared (m^2) (19). All cardiometabolic conditions and risk factors were defined according to established standards, including overweight, obesity, central obesity, metabolic syndrome (MetS), hyperglycemia, and type 2 diabetes (19-21).

Cardiometabolic biomarkers

We assayed 17 biomarkers that represented four cardiometabolic pathways. Lipids included total cholesterol (TC), triglycerides (TG), high-density lipoprotein cholesterol (HDLc), low-density lipoprotein cholesterol (LDLc), apolipoprotein A-I and B (apoA-I, apoB), and non-esterified fatty acids (NEFA). Glycemic markers included insulin and glucose. Pro-inflammation markers included high-sensitivity C-reactive protein (hsCRP), interleukin-6 (IL-6), leptin, resistin, and monocyte chemoattractant protein 1 (MCP-1). Anti-inflammation markers included IL-10, adiponectin, and soluble TNF receptor II (TNFsR).

Fasting and postprandial glucose concentrations were assayed in Guatemala, before the plasma samples were frozen for storage at -80 °C. The samples were shipped in dry ice to a biomarker core laboratory in the US, where a trained lab personnel thawed the samples at 4 °C in 28 batches for all other assays to be performed (one batch at a time). Samples in each batch included pre- and post-challenge samples from the same individuals, balanced on the location of data collection, village of birth during the INCAP trial, and timing of exposure to the nutritional supplements. We presented the details of laboratory methods for each biomarker in Supplemental Table 1.

Statistical methods

We describe selected sociodemographic information, cardiometabolic conditions, and cardiometabolic risk factors in the study population. We compared these characteristics between the two early-life nutritional exposure groups, using Student's *t*-test, chi-squared test, or Mann-Whitney *U* test, where appropriate.

The main outcome variables are meal-induced biomarker responses. For each biomarker, we designated meal-induced response as %Δ, calculated as:

$\frac{Postprandial\ concentration - Fasting\ concentration}{Fasting\ concentration} x\ 100\%$. To ensure visual comparability of the responses, we then calculated standardized Z-scores for each %Δ: $\frac{\text{Observed (\%}\Delta\text{)} - Mean\ (\%\Delta)}{Standard\ Deviation\ (\%\Delta)}$.

We first analyzed the postprandial responses using single-biomarker approach. We constructed difference-in-difference (DD) models for each %Δ (comparison of raw values) and corresponding Z-scores (visual comparison). In each DD model, the exposure variable is an interaction term between the type of exposure (*atole* versus *fresco*) and the timing of exposure (in the full first 1000 days versus other). We controlled for birth village (since it was village-level randomization, this variable can account for the type of supplementation and control for undocumented characteristics at the village level), timing of exposure, age, sex, and BMI. In models with lipid responses (TC, TG, HDLc, LDLc, apoA-I, apoB, and NEFA) as outcomes, we also controlled for lipid-lowering medication use. In models with glycemic responses, we also controlled for diabetes medication use. We tested sex-specific stratum heterogeneity by adding an interaction term between the DD exposure variable and sex. We reported significant interactions by sex, but the final models did not include this variable.

We then used multi-biomarker approaches to analyzed overall (global) and domain-specific postprandial responses. Prior to conducting the multivariate analyses, we examined the correlation matrix across all biomarker responses. To extract multivariate information and reduce the data dimensionality in postprandial biomarker responses, we conducted two sets of analysis. Multivariate analysis of variance (MANOVA) was used to test mean differences in postprandial biomarker responses between exposure groups, and linear discriminant analysis (LDA) was used to predict group membership based on collective biomarker responses.

We used MANOVA (base R), in combinations with DD modeling (previously described) to compare differences in %Δ between exposure groups. We conducted both domain-specific

(separately for lipid, glycemic, pro-inflammatory, and anti-inflammatory responses) and global (all biomarker responses combined) comparisons. We then conducted LDA (package "MASS") to test whether the global biomarker responses adequately predict group separation by early-life nutritional exposure status [22]. We partitioned the data into two random parts, 80% of the data were used to train the LDA models, and the remaining 20% were used to test the established models. For the first set of LDA model, we obtained one linear discriminant that is a combination of the multivariate data of all postprandial %Δ to maximize between-group differences. We removed IL-6 in this model to improve sample size. For the second set of LDA model, we only retained the glycemic domain (insulin and glucose responses) to calculate the linear discriminant to distinguish the two exposure groups.

We used R version 3.6.1 (R Core Team, Foundation for Statistical Computing, Vienna, Austria) for all our analyses. Statistical significance was set *a priori* at p value < 0.05. All p-values were two-sided.

Research ethics: The study was approved by the Institutional Review Board at Emory University and the Ethics Review Committee of INCAP. All study participants provided written informed consent in Spanish.

7.4 Results

Description of the study population

One fifth of the population were exposed to *atole* during the full first 1000 days (235, 22.9%), and 60.4% and 59.1% were women in the exposed and unexposed group, respectively (not statistically significant, or NS). Due to the nature of the study design, those in the exposed group were younger than in the unexposed group (mean ± standard deviation 42.2 ± 1.6 y and 44.6 ± 4.6 y, respectively, p < 0.001) (Table 1). Cardiometabolic conditions and risk factors were

similar between the two groups, with slightly over one third of the participants categorized as obese in both groups (NS). Type 2 diabetes affected 5.1% of the exposed and 7.5% of the unexposed participants (NS), whereas over half in each group (53.4% of exposed and 56.2% of unexposed) had metabolic syndrome (NS) (Table 1). The fasting concentrations of the biomarkers were similar between the two early-life nutritional exposure groups (Table 1). Glucose, resistin, adiponectin, and TNFsR concentrations were significantly lower in the exposed group than in the unexposed group ($p = 0.03$, < 0.001, 0.02, and < 0.001, respectively) (Table 1).

Comparison between exposure groups at the single-biomarker level

Early-life exposure to *atole* was associated with attenuated postprandial glucose response. The exposed group had 4.7% (95%CI: -9.3% to -0.01%) smaller magnitude of postprandial glucose increase than the unexposed group (the %Δ was 11.4 ± 17.3 in exposed group, compared with 15.7 ± 21.2 in the unexposed group, $p < 0.05$). The remaining biomarker responses were non-differential between the two exposure groups (Table 2, Figure 1). The most pronounced differences between the two groups was in glycemic responses and pro- and anti-inflammatory responses, but no clear pattern was observed in terms of inflammatory responses (Figure 1).

Biomarkers that had postprandial increase in both exposure groups: Insulin increased the most after the meal challenge in both exposure groups, and the %Δ was 232.4 ± 163.5 in the exposed group, compared with 237.7 ± 175.5 in the unexposed group (NS). TG and IL-10 increased by approximately 15% in both exposure groups. The %Δ of TG was slightly higher in the exposed group (16.0 ± 19.2) than in the unexposed group (15.3 ± 18.5, NS), whereas the %Δ of IL-10 was slightly lower in the exposed group (14.8 ± 84.3) than in the unexposed group (15.8

± 116.9). hsCRP increased more in the exposed group (1.6 ± 14.9) than in the unexposed group (0.8 ± 14.3, NS) (Table 2, Figure 1).

Biomarkers that had postprandial decrease in both exposure groups: NEFA decreased the most in both exposure groups, with %Δ of -45.1 ± 31.9 in exposed group and -49.3 ± 29.3 in unexposed group (NS). Postprandial leptin reduction was milder in the exposed group (-13.5 ± 31.7) than in the unexposed group (-15.3 ± 26.2, NS). TNFsR also decreased less in the exposed (-8.1 ± 16.9) than in the unexposed group (-9.6 ± 14.2, NS) (Table 2, Figure 1).

Biomarkers that had mixed postprandial changes in two exposure groups: In addition, IL-6 decreased in the exposed group (-1.4 ± 68.8) but increased in the unexposed group (9.7 ± 81.3, NS). MCP-1 increased in the exposed group (1.1 ± 37.1) but decreased in the unexposed group (-3.3 ± 37.5, NS). Adiponectin increased by 11.4% in the exposed group but decreased by 1.4% in the unexposed group (NS) (Table 2, Figure 1).

We did not observe any stratum heterogeneity by sex except for MCP-1 ($p = 0.04$ for the interaction between exposure variable and sex) (Table 2).

Comparison between exposure groups at the multi-biomarker level

MANOVA results indicated that the multivariate mean of glycemic biomarker responses differed by early-life nutritional exposure ($p = 0.03$). Apart from a borderline significant difference observed in the lipid response domain ($p = 0.06$), we did not observe any other domain-specific or global difference between the two groups ($p > 0.05$ for all) (Table 3). LDA results confirmed that neither the global multivariate biomarker responses nor responses in the glycemic domain alone predicted exposure group. Figure 2 indicated that the center and spread of the two groups significantly overlapped in both sets of LDA models.

7.5 Discussion

Our study is the first of its kind in linking early-life nutrition to long-term cardiometabolic status through a combination of single- and multi-biomarker approaches. Testing biomarker responses to meal challenge can help gauge the phenotypic flexibility of the cardiometabolic system. Cumulative disturbances throughout the life course, similar to the meal challenge in this study, eventually lead to cardiometabolic diseases such as type 2 diabetes and cardiovascular diseases. Therefore, it is important to assess whether and how early-life exposure to improved nutrition can attenuate such disturbances later in life. Regarding our main hypothesis, we did not observe an overall difference in postprandial biomarker responses between those who were exposed to improved nutrition in the first 1000 days and those who were unexposed. We did, however, observe a modest reduction in the glucose response in the exposed group, compared with the unexposed. This finding was consistent at both the single-biomarker and the multi-biomarker levels.

The one favorable difference that we observed is worth noting – the exposed group differed in the domain of glycemic responses from the unexposed group. At the individual biomarker level, the exposed group had a smaller magnitude of postprandial glucose response. In previous studies within the same population, researchers reported that early-life exposure to improved nutrition was associated with lower fasting glucose concentration [13,23]. We consider it equally, if not more, important that postprandial glucose response was also attenuated among participants with improved nutrition in the first 1000 days. The smaller magnitude in postprandial glucose response indicated improved capacity of the metabolic system to regulate glucose within a tight range. This finding further substantiated the euglycemic effect of having improved early-life nutrition in our study population. Mechanisms that link early-life nutritional

exposure with long-term glycemic regulation is not yet fully elucidated. We postulate that potential mechanisms include positive effects of improved nutrition on ontogeny, especially on the development of metabolically active tissues [2,24-26]. Based on results from animal studies, pancreas may be the key metabolic organ in this linkage [27,28].

Overall, participants who were exposed to improved nutrition in the first 1000 days did not differ significantly from the unexposed group in phenotypic flexibility, as assessed by collective biomarker responses in our study. Chronic malnutrition was prevalent in our study population when they were enrolled in the initial INCAP trial [29]. The improved protein and calorie content provided in the form of *atole* was hypothesized to attenuate the mismatch between undernutrition signals in early life and the increasingly obesogenic environment that they later encountered. The fact that we did not observe significant improvements in terms of adulthood cardiometabolic health among the exposed group may be a result of multiple factors. First, we know that *atole* improved early growth and development, and the benefits extended to human capital gains and intellectual capacities in early adulthood [2]. The lack of benefit in cardiometabolic health may be due to different pathways that additional protein and energy affect growth and development. Gruszfeld *et al.* reported that high-protein (versus low-protein) infant formula was associated with increased body mass in childhood, perhaps due to increasing availability of long-chain amino acids, which stimulates IGF-1 and thus promotes fat distribution [30]. Higher protein and energy may improve early linear growth, cognitive development, but may not directly benefit the metabolic tissues. In addition, cardiometabolic perturbations are a result of both early-life nutrition and cumulative exposures in subsequent years, with the latter being more indicative of current status. Therefore, it is possible that early protective effects of

improved nutrition in the first 1000 days is insufficient to fend off long-term negative impact due to lifestyle factors and obesogenic environment.

Insulin increased two hours after the meal challenge by more than two folds, but the increase did not differ significantly between the two exposure groups. Nevertheless, glucose response was attenuated in the exposed group. This is interesting because insulin is the major glycemic regulating hormone that rapidly respond to postprandial glucose signals [31]. It is possible that in our study population, glycemic regulation was improved by early nutrition through other mechanisms, such as the leptin-mediated glucose-lowering pathways [32,33]. Since glucose concentration is strictly regulated under physiological conditions, it is possible that the cardiometabolic system mobilized other compensatory pathways to improve glycemic regulation [34]. Despite the potential of nutritional improvements in early life to promote the growth and development of endocrine pancreas, its effect on insulin production and secretion may be limited in the long term. On the other hand, our observation may have been limited by the availability of only two data points surrounding the meal challenge. Postprandial insulin response varies greatly and can be influenced by numerous factors [31]. It is possible that insulin reached peak reaction sooner than the two-hour time point, hence helped reduce glucose concentration. But our assessment did not capture the highest level of such response. This postulation warrants further investigation, preferably through trajectory analysis with multiple data points following the meal challenge.

Most lipid responses were modest or null, except a 15% increase in TG and a 50% in NEFA. There were no statistically significant differences the lipid domain between two exposure groups. After a fat-containing meal, such as the meal challenge in this study, postprandial TG usually elevate within an hour, and can remain elevated for 5 to 8 hours [35,36]. Postprandial

increase in TG is a predictor of future cardiovascular diseases [37]. In physiological state, NEFA is excepted to fall rapidly after a mix-component meal due to the suppression of fat mobilization by insulin [38]. We indeed observed this sharp decrease of NEFA. In both of the exposure groups, NEFA reduced to approximately half of the fasting concentration two hours after the meal challenge, which adequately reflected the insulin-driven suppression of postprandial release of free fatty acids from adipocyte [38].

Leptin and adiponectin showed interesting patterns in this study. At the fasted state, leptin was lower in exposed than unexposed group (NS), whereas adiponectin was higher in exposed than unexposed group (NS). Two hours after the meal challenge, we observed that adiponectin increased more in the exposed than unexposed group, but the decrease of leptin was abated in the exposed group. Evidence is mounting that, apart from their main functions of regulating feeding behaviors, these two adipokines have important role in inflammation and insulin resistance [39]. Leptin and adiponectin have opposing effects, with leptin being pro-inflammatory and adiponectin being anti-inflammatory [40]. Leptin-to-adiponectin ratio is positively associated with metabolic syndrome and other cardiometabolic disturbances. Therefore, we postulate that early-life exposure to improved nutrition may help reduce leptin-to-adiponectin ratio, both at the fasted state and through dynamic assessment. In addition, leptin has complex biological functions beyond appetite control and pro-inflammation, therefore the attenuation in postprandial leptin response in the exposed group is not necessarily an unfavorable observation. More research is needed to elucidate the mechanisms behind this observation.

We tested the sex-specific stratum heterogeneity in postprandial responses and concluded that the association of early nutrition and adulthood cardiometabolic responses did not differ by sex, except for MCP-1. This is notable because at the fasted state, concentrations of many

cardiometabolic biomarkers differ between the two sexes at physiological state [41]. Leptin, for example, is significantly higher in women than in men due to hormonal regulation of the production and secretion of leptin [42]. These findings, if replicable in other populations, may have clinical implications: despite the sex-specific differences of cardiometabolic mechanisms and outcomes, early nutrition-associated long term phenotypic flexibility may be non-differential between the two sexes [43].

There were a few limitations in our study. Despite the innovative challenge model, we only had two time points available. We did not observe any pattern in the postprandial responses in pro- and anti-inflammation markers. It is possible that we did not observe the peak action of these biomarkers at exactly two hours after the meal challenge. There is limited information in the literature to indicate whether there is great inter- and intra-person variability throughout the day regarding postprandial biomarker responses. In our study, we tested the responses in all participants following an overnight fast as an attempt to attenuate inter-person variability. Another limitation is the varying degrees of missingness across the selected biomarkers. Because of the limited funds, certain assays were discontinued when interim analysis failed to show significant changes between fasting and postprandial samples within the same individuals. Nevertheless, the samples were processed in randomly grouped batches, and the missingness was batch-based. Therefore, the overall results should be representative of the study population despite the missingness. In addition, glucose response may be slower after a mixed-component meal than a standardized oral glucose tolerance test [44]. This may have unmeasured effect on glucose response in this study. Lastly, because of our focus on the metabolic processes and mechanistic investigation, we did not examine the role of economic and sociocultural factors in the association between early-life nutrition and long-term health outcomes. Previous INCAP

studies have reported favorable outcomes in human capital and economic productivity in those who were exposed to *atole* in early life [2,45]. Socioeconomic changes inevitably alter lifestyle choices that have health implications, which may have influenced the outcomes we observed in this study [46,47].

This is a longitudinal study with a randomized controlled trial as the starting point, which enables us to provide strong measurements of association between the exposure and the outcomes. We also further distinguished four cardiometabolic pathways, including lipid response, glycemic response, as well as pro- and anti-inflammatory responses [15]. Previously, researchers have suggested that multiple biomarkers should be used in concordance for each chronic disease, and even for different stages of the same disease [11,15]. We therefore tested the stress responses of the whole cardiometabolic system using key biomarkers, instead of focusing on clinically diagnosed disease status. At the multivariate level, we summarized the information of biomarker responses from two mutually compensatory aspects: we first compared between-group differences in the overall responses (MANOVA), then used the multivariate information to predict group membership (LDA).

Our study population were malnourished in childhood and have been undergoing a nutrition transition. Despite the cumulative benefits that were observed in this population in terms of physical growth, cognitive development, and human capital gains, a recent study from this population reported mixed association between early-life nutritional exposure and cardiometabolic risk factors [2,23]. We considered it urgent and necessary to test the DOHaD

7.6 Tables, figures, and supplemental materials

Table 1: Selected characteristics of the study population, between participants exposed to *atole* in the full first 1000 days versus unexposed

Characteristics [1]	**Exposed (n = 235)**	**Unexposed (n = 792)**	***p* value** [2]
Age (years), Mean (SD)	42.2 (1.6)	44.6 (4.6)	< 0.001
Female, %	60.4	59.1	0.77
Body mass index (kg/m^2), Mean (SD)	28.3 (4.6)	28.2 (5.2)	0.76
Obesity, %	31.9	32.6	0.92
Type 2 diabetes, %	5.1	7.5	0.27
Metabolic syndrome, %	53.4	56.2	0.50
Fasting lipids, Median (IQR)			
TC (mmol/L)	4.6 (3.9, 5.3)	4.6 (3.9, 5.2)	0.43
TG (mmol/L)	1.7 (1.2, 2.4)	1.7 (1.2, 2.4)	0.72
HDLc (mmol/L)	1.0 (0.9, 1.2)	1.0 (0.9, 1.2)	0.88
LDLc (mmol/L)	2.8 (2.2, 3.5)	2.8 (2.3, 3.4)	0.84
ApoA-I (g/L)	1.1 (0.9, 1.3)	1.7 (0.9, 1.2)	0.14
ApoB (g/L)	0.8 (0.7, 1.0)	0.8 (0.6, 0.84)	0.17
NEFA (mEq/L)	0.8 (0.6, 1.1)	0.8 (0.6, 1.1)	0.44
Fasting glycemic markers, Median (IQR)			
Insulin (pmol/L)	78.5 (49.3, 133.7)	88.2 (54.2, 139.6)	0.09
Glucose (mmol/L)	5.4 (5.0, 5.7)	5.5 (5.2, 5.8)	0.03

Fasting pro-inflammation markers, Median (IQR)			
hsCRP (mg/L)	1.8 (0.9, 3.7)	1.9 (0.9, 4.0)	0.34
IL-6 (pg/mL)	5.5 (2.1, 12.2)	5.1 (2.4, 13.0)	0.99
Leptin (ng/mL)	11.6 (3.2, 19.8)	9.8 (3.3, 18.5)	0.62
Resistin (ng/mL)	1.3 (0.8, 2.1)	1.7 (1.0, 2.7)	< 0.001
MCP-1 (pg/mL)	82.0 (54.8, 126.3)	80.2 (51.1, 120.6)	0.22
Fasting anti-inflammation markers, Median (IQR)			
IL-10 (pg/mL)	14.2 (4.6, 56.3)	22.6 (6.7, 67.6)	0.10
Adiponectin (μg/mL)	7.9 (4.7, 12.0)	9.0 (4.9, 15.9)	0.02
TNFsR (ng/mL)	2.2 (1.6, 3.0)	2.6 (2.0, 3.3)	<0.001

[1] Definitions: Obesity: BMI ≥ 30 kg/m^2; Metabolic syndrome: having three or more of the following five components: 1) abdominal obesity (waist circumference ≥ 88 cm for women; ≥ 102 cm for men); 2) fasting glucose ≥ 110 mg/dL or diabetic medication use; 3) triglycerides ≥ 150 mg/dL or statin use; 4) HDL-cholesterol < 40 mg/dL in men or < 50 mg/dL in women, and; 5) systolic blood pressure (SBP) > 130 mmHg, diastolic blood pressure (DBP) > 85 mmHg, and/or hypertension medication use. Hyperglycemia: fasting plasma glucose of 100–125 mg/dL or two-hour post-challenge glucose of 140–199 mg/dL among participants not reporting use of diabetes medication. Type 2 diabetes: fasting plasma glucose > 125 mg/dL, or two-hour post-challenge glucose ≥ 200 mg/dL, or reporting use of diabetes medication

[2] P-values based on Student's *t*-test (continuous variables with normal distribution), Mann-Whitney *U* test (continuous variables with skewed distribution), or chi-squared test (categorical variables)

Abbreviations: Apo, apolipoprotein; HDLc, high-density lipoprotein cholesterol; hsCRP, high sensitivity C-reactive protein; IL, interleukin; IQR, interquartile range; LDLc, low density lipoprotein cholesterol; MCP-1, monocyte chemoattractant protein 1; NEFA, non-esterified fatty acid; SD, standard deviation; TC, total cholesterol; TG, triglycerides; TNFsR, soluble TNF receptor II.

Table 2. Comparison of postprandial changes in biomarkers between participants exposed to *atole* in the full first 1000 days versus unexposed

Postprandial biomarker responses (%Δ) [1]	**Unexposed Mean (SD)**	**Exposed Mean (SD)**	**β (95% CI)** [2,3]
Lipid responses			
TC	0.2 (6.2)	0.6 (8.7)	1.2 (-0.6, 2.9)
TG	15.3 (18.5)	16.0 (19.2)	1.7 (-2.9, 6.4)
HDLc	0.9 (6.5)	1.7 (6.9)	0.4 (-1.2, 2.1)
LDLc	0.4 (9.2)	-0.1 (9.9)	-0.2 (-2.6, 2.1)
ApoA-I	0.6 (7.2)	1.0 (7.6)	0.5 (-1.4, 2.3)
ApoB	-0.1 (8.7)	-0.8 (10.1)	0.6 (-1.7, 2.8)
NEFA	-49.3 (29.3)	-45.1 (31.9)	-2.9 (-10.2, 4.4)
Glycemic responses			
Insulin	237.7 (175.5)	232.4 (163.5)	3.6 (-38.4, 45.5)
Glucose	15.7 (21.2)	11.4 (17.3)	-4.7 (-9.3, -0.01) *
Pro-inflammatory responses			
hsCRP	0.8 (14.3)	1.6 (14.9)	3.1 (-0.5, 6.7)
IL-6	9.7 (81.3)	-1.4 (68.8)	-28.7 (-67.7, 10.4)
Leptin	-15.3 (26.2)	-13.5 (31.7)	1.4 (-6.5, 9.2)
Resistin	-3.1 (20.9)	-4.0 (21.1)	-1.1 (-7.9, 5.8)
MCP-1	-3.3 (37.5)	1.1 (37.1)	4.4 (-5.6, 14.4)
Anti-inflammatory responses			
IL-10	15.8 (116.9)	14.8 (84.3)	5.3 (-28.9, 39.5)

Adiponectin	-1.4 (23.5)	11.4 (149.3)	3.6 (-4.1, 11.4)
TNFsR	-9.6 (14.2)	-8.1 (16.9)	3.5 (-1.4, 8.3)

[1] Postprandial change (%Δ) equals the difference between postprandial and fasting biomarker concentrations, divided by fasting concentrations, presented as percentages.

[2] Exposed vs. unexposed (reference group). Each model had the %Δ of one biomarker as dependent variable, and the independent variable is an interaction term between the type (*atole* or *fresco*) and the timing (full first 1000 days versus other) of exposure, and adjusted for age, sex, body mass index, type of exposure (used birth village instead of the binary variable), and timing of exposure. We also adjusted for usage of lipid-lowering medication in the lipid response models, and adjusted for usage of anti-diabetic medication in the glycemic response models.

[3] To test stratum heterogeneity by sex, we added the interaction term between sex and the exposure variable (sex * type of exposure * timing of exposure) to the model. Only significant for MCP-1 ($p = 0.04$).

* $p < 0.05$

Abbreviations: TC, total cholesterol; TG, triglycerides; HDLc, high density lipoprotein cholesterol; LDLc, low density lipoprotein cholesterol; apo, apolipoprotein; NEFA, non-esterified fatty acid; hsCRP, high sensitivity C-reactive protein; IL, interleukin; MCP-1, monocyte chemoattractant protein 1; TNFsR, soluble TNF receptor II.

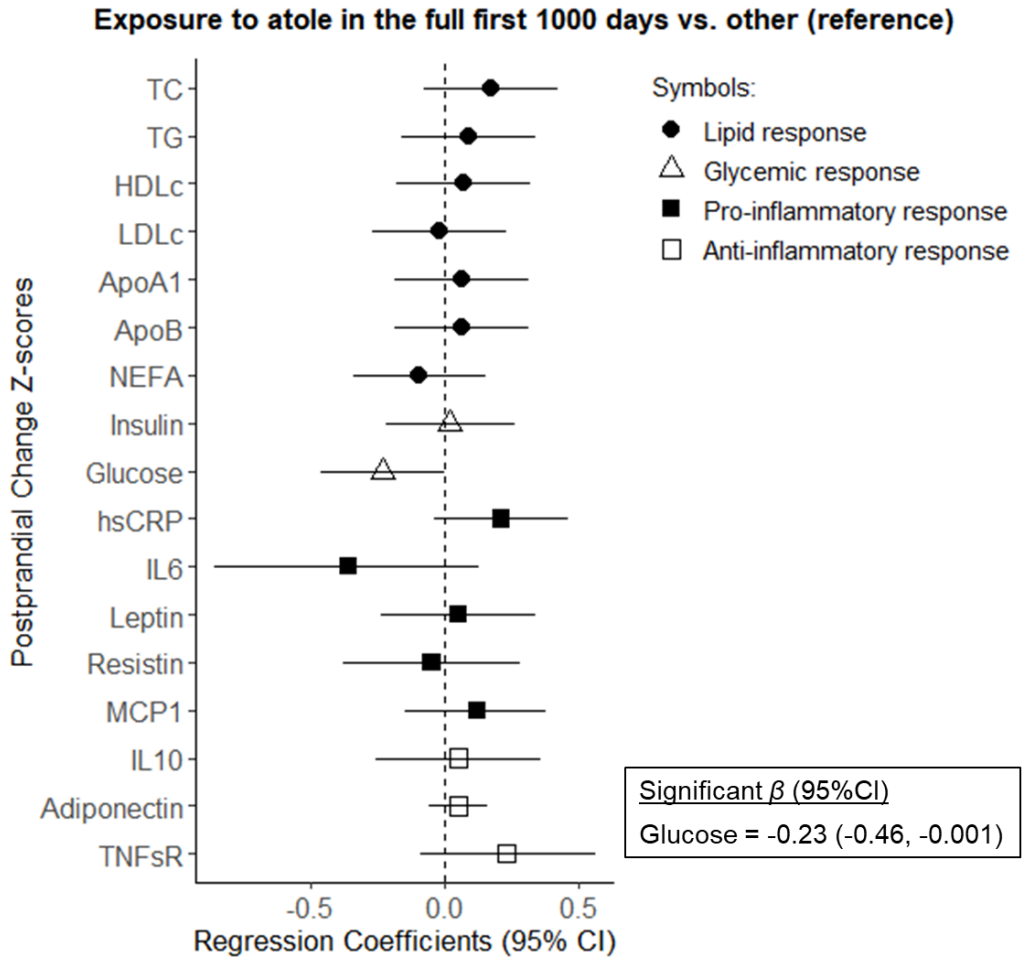
Table 3. Multivariate analysis of variance (MANOVA) of postprandial biomarker responses, between participants exposed to *atole* in the full first 1000 days versus unexposed

Type of comparison [1]	**Hotelling-Lawley Trace**	***F* statistic**	***df***	***df* error**	***p* value**
Domain-specific comparisons					
Lipid responses	0.02	1.93	7	612	0.06
Glycemic responses	0.01	3.49	2	614	0.03
Pro-inflammatory responses	0.05	1.34	5	121	0.25
Anti-inflammatory responses	0.01	1.81	3	458	0.14
Global comparison [2]	0.22	1.38	17	108	0.16

[1] Each MANOVA model had early-life nutritional exposure variable (type of exposure * timing of exposure), age, sex, type of exposure, timing of exposure, body mass index, and smoking status.

[2] Global comparisons referred to an overall comparison across all four domains (lipid, glycemic, and pro- and anti-inflammatory biomarker responses)

Figure 1. Standardized regression results of postprandial biomarker responses, between participants exposed to *atole* in the full first 1000 days versus unexposed



Legend:

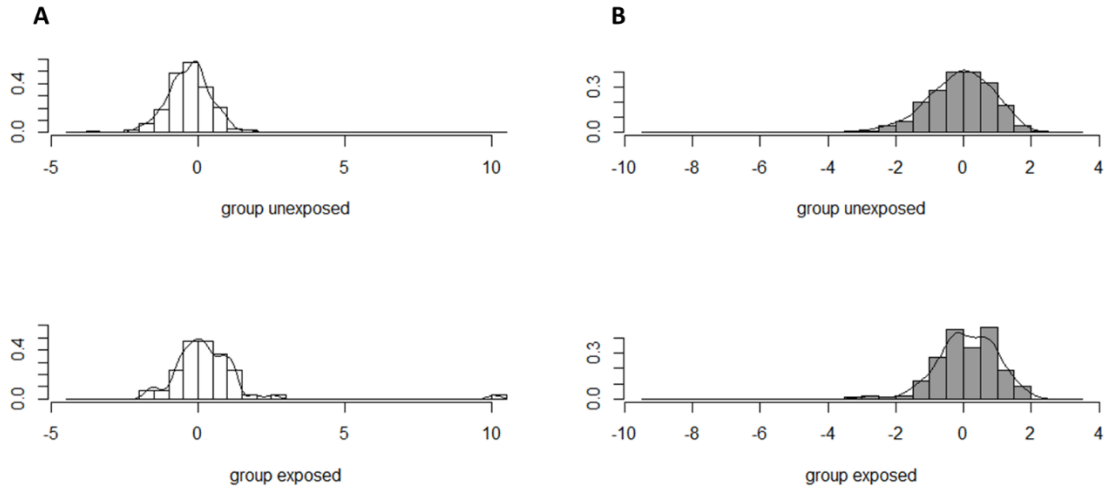
1. This figure presents linear regression results to compare postprandial biomarker responses between the participants who were exposed to *atole* in the full first 1000 days (n=235) versus other (n=792). Each regression model had one biomarker response z-score as the dependent variable. The independent (exposure) variable is an interaction term between the type (*atole* or *fresco*) and the timing (full first 1000 days versus other) of exposure, and adjusted for age, body mass index, village of birth (in place of type of

exposure), and timing of exposure. We also adjusted for lipid-lowering medication in the lipid models, and adjusted for anti-diabetic medication in the glycemic models.

2. Postprandial change (%Δ) equals the difference between postprandial and fasting biomarker concentrations, divided by fasting concentration. Standardized Z-scores were calculated as %Δ minus the mean and divided by the standard deviation for each biomarker.

Abbreviations: TC, total cholesterol; TG, triglycerides; HDLc, high density lipoprotein cholesterol; LDLc, low density lipoprotein cholesterol; apo, apolipoprotein; NEFA, non-esterified fatty acid; hsCRP, high sensitivity C-reactive protein; IL, interleukin; MCP-1, monocyte chemoattractant protein 1; TNFsR, soluble TNF receptor II.

Figure 2. Linear discriminant analysis based on postprandial biomarker responses to predict group membership



Legend:

1. The graphs were based on linear discriminant analysis to predict group membership by early-life nutrtional exposure status (exposed to *atole* in the full first 1000 days versus unexposed)
2. The histograms represent linear combination of the multivariate biomarker responses that allow for the greatest separation between groups. The x-axis shows distribution of the linear combination by each group, and the y-axis represents density.
3. A, postprandial responses in all markers: coefficients of linear discriminant (LD) for the prediction of early-life nutritional exposure = 0.49*TC – 0.38*TG – 0.11*HDLc + 0.47*LDLc – 0.85*ApoA1 – 0.26*ApoB + 0.23*NEFA + 0.16*Insulin – 0.33*Glucose + 0.10*hsCRP – 0.29*Leptin – 0.08*Resistin + 0.73*MCP1 – 0.18*IL10 + 0.14*Adiponectin + 0.02*TNFsR
4. B, glycemic domain (glucose and insulin responses): LD = -0.21*Insulin – 0.90*Glucose

Supplemental Figure 1: Participant flow chart

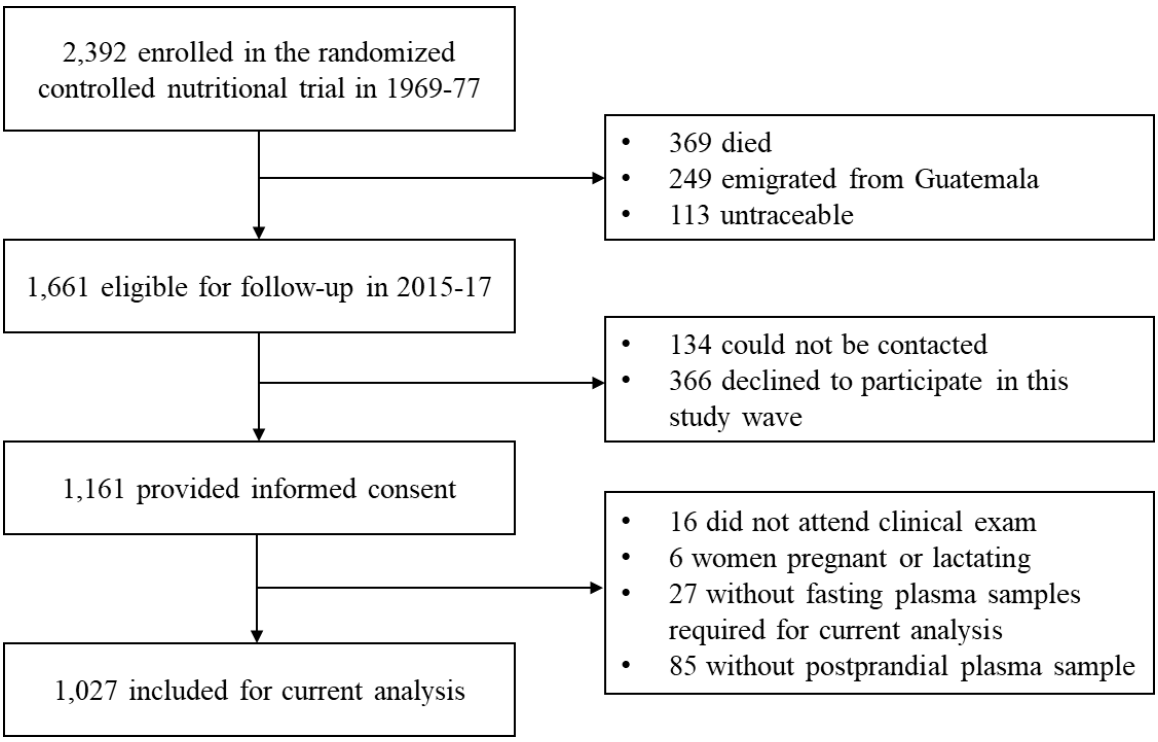


Figure legend:

This figure is an updated version of the participant flow chart, entitled 'Figure: Trial Profile' in the published paper: Ford ND, Behrman JR, Hoddinott JF, Maluccio JA, Martorell R, Ramirez-Zea M, Stein AD. Exposure to improved nutrition from conception to age 2 years and adult cardiometabolic disease risk: a modelling study. Lancet Glob Health 2018;6(8):e875-e84.

Supplemental Table 1: Methods used in the determination of biomarker concentrations from plasma samples

Biomarker	Metric unit	SI unit (conversion factor)	Laboratory method
Lipids			
Total cholesterol (TC) [1]	mg/dL	mmol/L (0.02586)	Enzymatic method (Sekisui Diagnostics, PA, US)
Triglycerides (TG) [1]	mg/dL	mmol/L (0.01129)	Enzymatic method (Sekisui Diagnostics, PA, US)
High-density lipoprotein cholesterol (HDLc) [1]	mg/dL	mmol/L (0.02586)	Homogeneous method (Sekisui Diagnostics, PA, US)
Low-density lipoprotein cholesterol (LDLc) [1]	mg/dL	mmol/L (0.02586)	Homogeneous method (Sekisui Diagnostics, PA, US)
Apolipoprotein A-I (ApoA-I) [1]	mg/dL	g/L (0.01)	Immunoturbidimetric assay (Kamiya Biomedical Company, WA, US)
Apolipoprotein B (ApoB) [1]	mg/dL	g/L (0.01)	Immunoturbidimetric assay (Kamiya Biomedical Company, WA, US)

Non-esterified fatty acids (NEFA)[1]	mEq/L	mmol/L (1.0)	Calorimetric methods (Wako Chemicals Corporation, Richmond VA, US).
Glycemic markers			
Insulin [1]	mIU/L	pmol/L (6.9444)	Immunoturbidimetric method (Kamiya Biomedical Company, WA, US).
Glucose [2]	mg/dL	mmol/L (0.0555)	Enzymatic colorimetric methods (Cobas C111 analyzer, ROCHE, Indianapolis, IN, USA)
Pro-inflammation markers			
High-sensitivity C-reactive protein (hsCRP) [1]	mg/L	Same as metric unit	Immunoturbidimetric method (Kamiya Biomedical Company, WA, US).
Interleukin-6 (IL-6)	pg/mL	N/A	Enzyme-linked immunosorbent assay, ELISA (Boster Biologicals Technology, CA, USA)
Leptin	ng/mL	N/A	ELISA (Boster Biologicals Technology, CA, USA)
Resistin	ng/mL	N/A	ELISA (Boster Biologicals Technology, CA, USA)

Monocyte chemoattractant protein (MCP-1)	pg/mL	N/A	ELISA (Boster Biologicals Technology, CA, USA)
Anti-inflammation markers			
Interleukin-10 (IL-10)	pg/mL	N/A	ELISA (Boster Biologicals Technology, CA, USA)
Adiponectin	μg/mL	N/A	ELISA (Boster Biologicals Technology, CA, USA)
Soluable TNF receptor II (TNFsR)	ng/mL	N/A	ELISA (Boster Biologicals Technology, CA, USA)

[1] These markers were assayed simultaneously for each batch through the AU480 automatic chemical analyzer (Beckman Coulter Diagnostics, Fullerton CA, US)

[2] Glucose was assayed in field laboratory in Guatemala, the rest in the US biomarker core laboratory

7.7 References

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CHAPTER 8: SUMMARY AND CONCLUSIONS

8.1 Summary of Main Findings

8.1.a Overview

In order to critically evaluate the findings presented in the previous chapters, let us re-visit the aims of the research that I presented in the first chapter. *The overarching goal* of this work is to investigate whether early-life exposure to improved nutrition is associated with cardiometabolic health in adulthood, as characterized by biomarkers, in the Institute of Nutrition of Central America and Panama (INCAP) population. Cohort members in the INCAP study were chronically malnourished in early life, as reflected by high stunting prevalence (17, 88). They also underwent nutrition transition that gradually exposed them to a more obesogenic environment (16). The combination of early-life malnutrition and cumulative exposure to a transitional food environment makes them highly susceptible to cardiometabolic disturbances.

Through a systematic review and meta-analysis (Chapter 2), we summarized the most up-to-date global evidence on the long-term cardiometabolic impact of nutritional interventions in early life. This systematic review and meta-analysis highlighted that, despite tremendous efforts in research on early life nutrition, the long-term gain was difficult to measure, except for an ongoing intervention in the form of personalized dietary counselling in a high-income setting (104). There was, however, an overall favorable effect on glucose homeostasis and an overall unfavorable effect on obesity risk. Among studies that focused on infant and young child feeding, breastfeeding was more beneficial than formula feeding in terms of long-term cardiometabolic benefits. However, breastfeeding-promotion alone – if not coupled with actual behavioral change strategies – did not yield observable benefits later in life. Lastly, although

rarely investigated or reported in the selected trials, timing of the nutritional interventions may have an impact on outcome with earlier exposure being more beneficial than later in life.

8.1.b Specific Aim 1 Summary and Discussion

Through Specific Aim 1, we investigated the role of leptin in the diverging association of early nutrition with cardiometabolic conditions observed in this study population, including decreased risk for type 2 diabetes and increase risk for obesity. In Chapter 5, we first confirmed the proportional association between leptin and adiposity. We then established that leptin has glucose-lowering effects and partially mediates the pathway between early-life nutritional exposure and long-term glycemic status (only observed in women). The mechanism may be through improving pancreatic β cell function, and not through countering insulin resistance. Admittedly, although we did find evidence in mediation effect by leptin between early nutrition and adulthood glycemic status, it is insufficient to fully elucidate the diverging impact of early-life nutrition.

The diverging association between early nutrition and adulthood cardiometabolic conditions featured a decreased risk for Type 2 diabetes and an increase risk for obesity in our study population. This observation was first made by Ford and colleagues, using data from the same study population (16). The beneficial effects in glucose regulation and detrimental effects on body size and composition have also been reported by other studies based on protein-energy supplementation in early life (105, 106). We could view this diverging association from two perspectives.

First, we could assume that increased body mass index and body fat percentage indeed represent unfavorable changes in body composition. From this perspective, it is peculiar that the average glycemic status was better in the “improved nutrition” group, since obesity is a known

risk factor for hyperglycemia and that adipose tissue plays a key role in this association (56, 107).

Alternately, the different sources of adiposity may explain the inconsistency of early life nutrition intervention on obesity and diabetes. By “different sources of adiposity”, I am referring to adipose tissue gained through early-life exposure to improved nutrition versus adiposity accumulated through a later obesogenic environment. I postulated that, early-life exposure to improved nutrition may have beneficial effects on fat depot (more subcutaneous and less visceral), the types of fat (more brown than white adipose tissue), and fat cell mass (smaller, less inflammatory-prone adipocytes). In this chronically malnourished population, it is possible that early exposure to supplements with better energy and protein content affected the developmental processes of adipose tissue and other metabolically active tissues. Therefore, despite having higher amount of overall fat mass (higher BMI), the exposed individuals may have lower grades of chronic inflammation and may be less leptin resistant, which can also contribute to euglycemia. Early nutrition-related gain in adiposity may be metabolically healthier than obesity attributable to a later obesogenic environment, especially given the mismatch between the in utero and later nutritional environment in this population. This hypothesis warrants further investigation, as we do not have all relevant data to investigate it.

8.1.c Specific Aim 2 Summary and Discussion

Through Specific Aim 2, we described adulthood cardiometabolic health status of the study population through assessment of metabolic flexibility, as characterized through meal-induced biomarker responses in this population. Prior to the dynamic assessment, we first observed that this population had high prevalence of cardiometabolic conditions such as diabetes

and obesity. They were also at increased risk for future cardiometabolic perturbations, as indicated by the prevalence of pre-clinical conditions such as of metabolic syndrome.

In Chapter 6, we described the postprandial relative changes in selected biomarkers in terms of their respective meal-induced responses, and the structural relationships among the responses. Interestingly, despite the differences across most fasting biomarkers between men and women, no significant sex-specific differences were observed in the postprandial responses other than in non-esterified fatty acids, glucose, insulin, and leptin. We then compared the individual biomarker responses across strata of cardiometabolic conditions to identify "healthy" versus "unhealthy" characteristics. We found that, in individuals with relatively "healthy" cardiometabolic phenotypes, their responses to the prandial challenge involved larger magnitude in postprandial insulin increase, lower glucose response, and greater reduction in leptin concentration. There was also a gradient effect of gradually increased disturbance in glycemic responses from "healthy" to "pre-clinical", and to "clinical" phenotypes.

Through structural equation modeling, we found that lipids and glycemic markers clustered separately. Inflammatory responses (represented by CRP) was associated with lipid responses, whereas the substantial reduction in non-esterified fatty acids was strongly correlated with glycemic responses. Two-hour postprandial responses in triglycerides and leptin were inversely correlated, and the latent variable formed by triglycerides and leptin was negatively correlated with the glycemic responses. These structural associations may be valuable in characterizing "ideal" cardiometabolic responses through systematic biomarker assessment, instead of merely relying on a single marker.

8.1.d Specific Aim 3 Summary and Discussion

Through Specific Aim 3, we investigated the association of early-life nutrition with adulthood metabolic flexibility in cardiometabolic pathways, using a single- and multi-biomarker approach. In Chapter 7, we compared the biomarker responses between those who were exposed to improved nutrition in early life versus the unexposed. We did not observe a statistically significant difference between the exposed and unexposed participants other than a modest and favorable change in glycemic response, both at the individual biomarker level (glucose response) and at the multi-variate domain level (glucose and insulin response, collectively): those who were exposed to improved nutrition in early life showed attenuation in two-hour glucose elevation. This is meaningful because it provides more evidence supporting the euglycemic regulation among the participants who were exposed to improved nutrition in early childhood. We have already found a favorable association between this exposure and glucose concentration at the fasted state (16). The current results strengthened the previous findings by showing beneficial effects of early nutrition in dynamic glucose control, not just at the fasted state, which should be further explored in future studies.

8.2 Limitations

The research presented in this dissertation has several limitations. First, this is a set of follow-up study nested within the INCAP Longitudinal Study cohort (86). The original cohort had 2,392 individuals, whereas our current sample size is 1,112 (46.5% of original cohort) for fasting biomarker-based analysis, and 1,027 (42.9%) for postprandial analyses. For a 50-year longitudinal study, attrition of more than half is common (please refer to Chapter 2, systematic review and meta-analysis for attribution in other studies), but this level of attrition significantly

reduced our statistical power and affected the overall interpretation of the findings. According to a recent study by INCAP researchers, the attrition appeared to be non-differential regarding the initial randomization (16). Therefore, it is possible that the findings in this dissertation work were unbiased regarding early-life nutritional exposure.

There were varying degrees of missingness in cytokines, including 70.1% missing for IL-6, 36.1% for TNFsR, 25.8% for IL-10, 12.8% for MCP-1, 7.3% for resistin, and 7.2% for adiponectin (See Chapter 4, Table 4.3). The differences in missingness was due to the laboratory method used (ELISA), which was carried out on a need-based schedule. Because of the limited funds, certain assays were discontinued when the interim analysis failed to show significant changes between fasting and postprandial samples within the same individuals. We stopped assaying IL-6 after 12.5 (out of 28) batches, because we did not obtain biologically plausible data for IL-6. We stopped assaying the postprandial samples for these TNFsR, resistin, and adiponectin after Batch 17, because we did not observe significant meal-induced changes in these markers. In order to generate preliminary data for future studies, we added MCP-1, a biomarker not considered in the initial research proposal. Since the samples were processed in randomly grouped batches, the overall results should be representative of the study population, despite the missingness. While this did not interfere with neither the descriptive analysis nor the linear and logistic models, the lack of complete case did not allow for multivariate analysis using data reduction techniques. We meticulously documented and reported sample sizes in all analyses for clear interpretation of the results.

In view of the large number of participants in this field study, we were limited by the availability of only one timed sample after the meal challenge. The study observed significant meal-induced differences in only TG, INS, NEFA and leptin. The lack of significant postprandial

changes in other biomarkers could be due to the time points selected, as each marker may have followed different time-course trajectory in response to the meal challenge. Future studies should aim at collecting repeated samples at additional time points to allow for trajectory analysis of the biomarker responses to capture peak action, slope of response, and time to return to homeostasis (108).

We did not distinguish the depot and type of adipose tissue in the current work. It has been previously reported that all abdominal adiposity are not created equal – hepatic adiposity and subcutaneous adiposity have significantly different metabolic implications. The former is associated with insulin resistance and dyslipidemia including heightened endogenous fatty acids synthesis. Future research should ideally provide information about the different sources of adipose tissues to facilitate the interpretation of the results.

Another source of bias may come from the nutrient content (particularly energy and micronutrients) in the two supplements, *atole* and *fresco*. In this dissertation, our emphasize was largely on protein content. The micronutrient profile actually changed in *fresco* supplements on from none to the same amount as in *atole* on Oct. 1, 1977 (Martorell R. Food Nutr Bull, article under review)(109). Second, the pattern of consumption of the two supplements by mothers and children was not homogeneous due to differences in community perception, consistency, serving temperature, and other factors. In addition, younger children consumed more *atole* than *fresco* (approx. 3y and younger) versus older children (3-7y), and the pattern reversed in older children. Pregnant women, on the other hand, consumed more *fresco* and had higher micronutrient consumption associated with it, although *atole* still contributed more energy for pregnant women (Martorell R. Food Nutr Bull, article under review). Micronutrient deficiencies and disease implications are increasingly recognized in nutrition research and policy (110). Future analyses

using this dataset should take these variances into account for more accurate interpretation of the outcomes.

Due to the longitudinal nature of this work that spanned five decades since the initiation of the original trial, it is possible that many confounding factors affected the key association we investigated. These factors may be difficult or impossible to measure, including pathogen load, obesogenic environment assessment, and various lifestyle factors across five decades of their lives. Because we analyzed the impact of early-life nutrition through difference-in-difference modeling strategy, this could introduce new confounders that were not balanced by the initial randomization of *atole* versus *fresco*. We have, however, attempted to control for potential confounders in all our models to minimize these observable biases. In addition, data in genetics, epigenetics, and ontogeny were not collected in this cohort. These data, although beyond the scope of this dissertation work, may help reveal key information in the mechanistic association between early-life nutritional exposure and subsequent developmental impacts.

In this dissertation work, we focused on the biochemical and metabolic aspects of the associations between early life and cardiometabolic functions, and did not investigate the impact of socioeconomic and lifestyle factors in the association. INCAP researchers have previously reported the impact of *atole* exposure on socioeconomic status (higher wages among men in the exposure than unexposed group), human capital and economic productivity (particularly improved among those exposed to *atole* before 3y) (17, 111). Lifestyle factors and the increased likelihood of exposure to calorie-dense diet in this LMIC setting among those with better socioeconomic status may contribute to the progression of cardiometabolic diseases (112, 113). It will be necessary in the next phase of this study to incorporate the biochemical findings with economic and sociocultural aspects to investigate the cumulative impact of these factors.

The results in this dissertation are also limited by external validity, or generalizability, because it was conducted within a relatively homogeneous group of individuals with similar cultural background and environmental exposure. These Guatemalan individuals also lacked ethnical diversity due to a challenge at the beginning of the study to translate the instruments from Spanish to Mayan, resulting in the omission of any non-Ladino communities (86). The results in this dissertation should be interpreted with caution, especially when there is need to extrapolate the outcomes to other populations.

8.3 Strengths and Innovations

Our major strengths lie in the fact that we not only have cardiometabolic markers at the fasted state, but also gathered meal challenge-induced biomarker responses for dynamic assessment of sub-clinical disturbances and metabolic flexibility (71). We have selected markers that represent four cardiometabolic pathways, including lipids, glycemic markers, and pro- and anti-inflammatory markers. Even within controlled laboratory setting in high-income countries, this level of large-scale pre-post biomarker data is rare. We consider it increasingly important in elucidating what is inside the “black box” of biological pathways when designing, implementing, and evaluation public health programs, especially given the ubiquitous biochemical functions of nutritional supplements.

All analyses in this dissertation were guided by conceptual frameworks central to our hypotheses (refer to Chapter 3). We analyzed fasting biomarkers directly in association with early-life nutritional exposure, because most biomarkers at the fasted state are well studied. Subsequently, we investigated the postprandial changes in biomarkers for the individual markers, and collectively by cardiometabolic pathways. We then used multivariate and data reduction

techniques (MANOVA and structural equation modeling) to analyze the postprandial biomarker responses. We also conducted principal component analysis and cluster analysis in support of the core strategies (data not presented in this dissertation). We believe that this comprehensive set of analytical strategies strengthened the hypothesis testing.

This study is relatively large-scale, including over a thousand community dwelling individuals in the setting of a low- and middle-income country (at the initiation of the original INCAP study in the 1960s, Guatemala was a low-income country, and it has risen to be a higher-middle income economy as of 2019). Building upon strong community rapport, we were able to not only collect and meticulously document early-life nutritional exposure data, but also continuously follow up with this population to glean insights into the changes across five decades of their lives. In the 2015-17 data collection wave, we obtained biological samples, which made this dissertation work possible. These data are unique and valuable, especially considering the resource-restricted setting in the earlier phases of this study.

The population is ideal for our study from a "developmental origins of health and diseases (DOHaD)" perspective, especially regarding the mismatch between early and later nutritional contexts in this population. This chronically malnourished population had one of the highest stunting prevalence in the world in early childhood and the protein-energy supplementation was important in supporting their growth and development (114). Despite the supplementation, it is possible that the cohort members underwent in utero (and during early infancy) metabolic programming to prepare them for a nutrition-limited environment. Unfortunately, this population has also experienced an ongoing nutrition transition, rendering them more vulnerable to the detrimental effects of an obesogenic environment. Based on our assessment, 40% of women and 20% of men in our cohort were obese, and approximately 40%

of all were pre-diabetic, with other cardiometabolic risk factors predicting future diseases. This work is therefore very timely and critical. We consider it essential at the current stage to study the determinants of cardiometabolic perturbations to both understand the developmental mechanisms and explore future preventive options.

8.4 Public Health Implications

Nutrition matters. Public health nutritionists are (naturally) in agreement with this statement. However, I often found it difficult to explain *why* and *how* nutrition matters to the lay audiences, beyond the body of common knowledge people already harbor. For instance, most people seem to be familiar with concepts such as "loading half of your plate with vegetables is good for you", but it does not stop a completely healthy person from purchasing, unnecessarily, bottles of multi-vitamin supplements instead of improving his/her overall diet quality. The acute effects of nutrition are well known, and one may argue that these direct associations (e.g., treating scurvy with ascorbic acid) made the early phase of nutrition research possible (115). However, I wish to drive home this message that, yes, nutrition matters, and its impacts are so profound that early-life nutritional exposure can shape health in adulthood, and even the health of future generations (116). Through this work, I would like to emphasize *the importance of nutrition as a life-long determinant of health and diseases, rather than merely a matter of short-term or within-day dietary choices.*

Prior to this work, there were a few major gaps in the literature in terms of maternal and child health and nutrition, as well as in life course epidemiology. First, we did not have a consensus regarding the global evidence of long-term impact of early-life nutritional interventions. Through a systematic review and meta-analysis, we filled this gap and provided

insights into the long-term cardiometabolic impact of various types of maternal and child nutritional interventions (Chapter 2). Second, researchers reported inconsistent cardiometabolic outcomes associated with nutritional interventions in early life, and the mechanisms have yet been thoroughly explored. Through this dissertation work, we offered several potential mechanistic explanations, for instance through leptin-mediated pathways (Chapter 5). Third, to our knowledge, no large-scale study in resource-limited settings have collected dynamic data related to a meal challenge. We filled this gap and provided both cross-sectional and longitudinal analysis of the postprandial response data, individually and combined, for the selected cardiometabolic biomarkers.

Our work emphasized the importance of *evidence-based design and implementation of public health programs*. For instance, our work showed the long-term euglycemic effect of early-life supplementation of a protein- and energy-containing supplementation, and offered mechanistic explanation of how this benefit may be achieved (Chapter 5). There is a wealth of knowledge for us to learn and explore. As an example, prior to providing large dosage of vitamin A supplementation to mothers and neonates in a low-resource setting, it is advisable to consider vitamin A as a bio-activator that may upregulate other biological functions and lead to sub-clinical toxicity, even in malnourished populations (117). It is extremely important to consult the literature about the global evidence regarding the positive and negative impact of vitamin A supplementation, and in which populations, under what circumstances were the interventions implemented. Different populations have vastly different baseline nutritional status and may contribute to the heterogeneity of intervention outcomes (118). As public health professionals, we must always prioritize evidence-based interventions to avoid unintended consequences on the growth, development, and long-term health outcomes of the target population.

We should pay more attention to the *nutritional composition* in maternal and child health interventions. In the INCAP study, the nutritional exposure of interest was *atole*, a protein- and energy-containing supplement (86). In our systematic review (Chapter 2), we discussed in details about the importance of protein in early life, as well as recent controversies surrounding the unintended effects that excessive protein (and energy) may have on growth and development. The cohort members in the INCAP study were malnourished in childhood, therefore a nutritional supplement that did not replace their main meals served to promote growth and development (111). I presume that we could reach a consensus about the importance of protein in early life, particularly in chronically malnourished populations. However, recent evidence from well-nourished populations suggested that high protein intake in early infancy may affect later abdominal fat distribution, and increase the risk for cardiovascular diseases in adulthood (119). High-protein and low-fat diet, which is often assumed to be "healthier" than the opposite composition, may lead to higher level of early rapid growth and higher fat mass in childhood. This may be a result of metabolic programming that contributes to a more energy-preserving, or "thrifty" body type (120). In addition, it is important to shifted from a single-nutrient view to the promotion of appropriate food consumption and improved diet quality (121).

Aging populations around the world is increasingly facing challenges of non-communicable diseases (6). Many of these diseases are preventable, and it is essential to first recognize the *disease phenotypes and their underlying linkages*. For example, obesity and Type 2 diabetes are two different cardiometabolic conditions based on their clinical diagnostic criteria. Nevertheless, scientific evidence pointed to the intricate associations between these two conditions, with the former also serving as a risk factor for the latter (56). They share many underlying pathways, and it is important to investigate these shared pathways and their

implications on assessing systematic phenotypic flexibility and cardiometabolic capacities (see Chapter 6). In public health (and preventive medicine) work, we should pay more attention to homeostasis when we consider the onset and development of cardiometabolic conditions. By understanding homeostasis, it is more likely that we modify and improve public health nutrition programs to support and improve systemic phenotypic flexibility. We should focus on what our body can maintain and achieve, and not on what needs to be fixed after it is already "broken".

Our work also displayed how public health programs could benefit from the rich information in *biomarkers*. Chapter 3 of this dissertation provided a brief review of cardiometabolic biomarkers (and their expected postprandial responses) that can serve to enrich studies at the population level. Despite the value that biomarkers add to nutrition and health sciences research, it is necessary to recognize the difficulty in collecting biomarker data rather than using other non-invasive data collection methods (such as self-reported health status). This difficulty is further magnified in large-scale population studies in low-resource settings (122). For one, the collection process of many biological samples is either invasive (e.g., plasma) or inconvenient (e.g., breast milk) for the participants. It not only adds more barriers for participation, but also carries higher risk in terms of research ethics. For another, in resource-poor settings, it is difficult to conform to the stringent protocol required to preserve biological samples, including sterilization practice to avoid point-of-contact contamination, proper laboratory tools and equipment for sample collection, storage, transportation, and performing lab assays (122). In the long term, there are also barriers in sample storage and proper disposition following biohazard protocols. Nonetheless, we can overcome these barriers through meticulous planning and good collaboration. Numerous researchers in resource-limited settings successfully

implemented projects and programs involving biological samples – including the INCAP study, on which this dissertation work is based (25).

I would like to emphasize a caveat regarding maternal and child nutrition. Based on our findings (and findings from many other studies with a maternal and child health component), it certainly appears that mothers and associated factors (maternal nutritional status, maternal behavior, and their sociodemographic characteristics) are among the most important determinants of the health of their children. However, it is important to avoid criticizing, shaming, or stigmatizing women who cannot breastfed, those who were obese, those who had gestational diabetes, or those who do not have the knowledge or resource to provide optimal nutrition to their children and themselves. Public health professionals should strive to view these circumstances as opportunities for improvement, and not as sources of failure.

8.5 Future Directions

In line with the Lancet series on "double burden of malnutrition", results presented in this dissertation work emphasized a holistic view on the spectrum of under- to over-nutrition (123). I recommend future studies to adopt a life course perspective and understand that, double burden of malnutrition does not just co-exist within the same population across different individuals; it could also be viewed as a continuum of conditions with interlinked underlying mechanisms within the same individuals. By investing in early-life nutrition and to support a life course, repeated, and individualized intervention regime, it is more likely that we can simultaneously tackle under- and over-nutrition (124).

Big data analytics should be applied to this body of work. If companies like Target can (very accurately) predict consumer behavior through their purchase history, we should strive to

adapt similar analytical strategies to predict long-term health outcomes through early-life factors. Scientific evidence is sound in terms the "critical window" (e.g., the first 1000 days) for nutritional investment to yield long-term benefits (17, 125). More research is now needed to understand the interaction between genetic predisposition and environmental factors, including the epigenetic mechanisms associating early-life nutrition and long-term cardiometabolic status. It is empirical to fully grasp *when* and *what* the "critical windows" are for nutritional investment. For one, different nutrients affect the growth and development at various stages of fetal and early childhood stages. For another, different genetic disposition determines that individuals react to nutritional investments in early life to a different extent (125).

Another area where big data analytics are increasingly applied include metabolomics (126). Metabolomics can help us understanding the metabolic systems to further elucidating the nutrition-health longitudinal associations. By investigating the networks of metabolites, we are more likely to understand the intricate pathways associated with the early nutrition-chronic condition paradigm, including the psycho-neuro-endocrine network (71).

There is growing interest in the research community in Mendelian randomization, which is a methodology that controls for genetic factors related to study outcomes but not related to other behavioral and environmental factors (127). Should we incorporate this method into our future research, it will help remove confounders that are conventionally difficult to account for (e.g., apoB level associated with genetic variants), reduce the risk of reverse causality, as well as strengthen the association between exposure and outcome (should there be one). In addition, the concept of "intergenerational Mendelian randomization" is highly relevant to our work, as it takes into account the mother's genotype as a determinant for the health of her offspring (127).

As for myself, I would like to pursue advanced epidemiology training, especially in longitudinal data analysis. Given the broad, long-term goal of my research (to clarify whether improved nutrition in early life can improve short- and long-term health outcomes), I believe that advanced training in longitudinal data analysis is conducive to continuing and supplementing my work in this area. In addition, parallel to my dissertation work, I have started learning and practicing cluster analysis, principal component analysis, factor analysis, and structural equation modeling. I would like to systematically improve my skills in the realm of big data analytics, including machine learning. I believe that these analytical skills can help predict long-term health outcomes based on exposures in early life.

8.6 Conclusions

In summary, this dissertation contributed to the literature by highlighting the following: first, we provided the most up-to-date global evidence on the potential of early-life nutritional investments to reduce adulthood disease burden and to promote cardiometabolic health later in life. Second, we confirmed the significant associations between early-life nutritional factors and cardiometabolic health in middle age. We should continue investigating the underlying mechanisms and apply the knowledge to public health work. Last but not least, biomarker assessment – both at the fasted state and as a dynamic assessment tool in response to metabolic challenges – offers great potential in future public health work, especially in this era of non-communicable disease “epidemic”. We believe that our work holds value in multiple disciplines, including nutrition research, public health, and laboratory sciences.

APPENDIX I: Lay Summary of the Dissertation

Cardiometabolic diseases, such as diabetes and heart diseases, are on the rise globally. There may be a link between nutrition in early life (for instance, during pregnancy or early infancy) and cardiometabolic diseases in adulthood. During the early phases of one's life, good nutrition plays an important role in supporting the physical and functional developments; poor nutrition, on the other hand, can hinder growth and may lead to future cardiometabolic diseases. In addition, there has been a shift in diets around the world, and populations are increasingly exposed to high-fat and high-sugar meals, which may exacerbate the process of developing cardiometabolic diseases. It is critical to understand possible mechanisms that link nutrition in early life and long-term cardiometabolic diseases.

In our work, we studied cohort members of the Institute of Nutrition of Central America and Panama (INCAP) Longitudinal Study, who were enrolled in the study in their childhood (1969-77) and were followed up for five decades since then. In the initial study, the members were given one of two nutrition supplements – an "improved nutrition" gruel that contains protein and energy, or a drink with no protein and lower energy. In the current analyses using data from 2015-17, we focused on analyzing markers in the blood to understand cardiometabolic mechanisms. We collected two sets of blood samples: 1) fasting samples (from 1,112 participants), or blood collected after they ceased eating overnight (eight or more hours), and 2) postprandial samples (from 1,027 participants), or blood collected two hours after they were given a fluid meal, which mimics the nutrient content of a regularly available diet (moderate amount of protein, fat, and sugar). The changes of markers in blood from fasting to postprandial samples within the same individuals are used to assess how well their systems maintain cardiometabolic balance when facing external challenges (i.e., the meal).

We found that: 1) If the participants received the “improved nutrition” gruel very early in life (from the mother’s pregnancy to their two-year birthday), they had better blood sugar levels but also higher body mass index in adulthood, compared with the other participants. Leptin, a hormone that is produced by fat tissue, may play a role in the association between early nutrition and lower blood sugar in adulthood. 2) When assessing the capacity of their systems to maintain balance, we found that the extent of postprandial changes in many markers were different across participants who had various health conditions (e.g., obese versus normal weight participants). The differences were the most pronounced in markers related to blood sugar control. 3) The participants who were exposed to improved nutrition in early life had better blood sugar control. We did not observe impact on other markers, such as in cholesterol responses or in inflammatory processes.

Overall, our study suggested that, nutrition in early life indeed has long-term impact on cardiometabolic health. The impacts are nuanced due to the human body’s ability to maintain balance despite external changes. More studies should be conducted to elucidate the mechanisms behind why or why not maternal and child nutrition interventions are effective in promoting long-term cardiometabolic health.

APPENDIX II: Supplemental Table for Chapter 2

Supplemental Table 1 for Chapter 2: Summary of main findings of included studies, by type of cardiometabolic outcomes

Study	**Categories of Cardiometabolic Outcome**			
Intervention type	**Biomarkers (n = 28)**	**Cardiovascular physiology (n = 20)**	**Body size & composition (n = 29)**	**Clinical and sub-clinical outcomes (n = 8)**
Protein-energy supplementation				
Hawkesworth et al. 2011 (Trial 1) *Mean age at follow up: 14y*	• Main treatment effects: **marginally LOWER** fasting glucose in pre-delivery group, **NULL** for the rest • Measurements: Total cholesterol, HDLc, LDLc, triglycerides, glucose, insulin, low HDLc	• Main treatment effects: **NULL** • Measurements: systolic blood pressure and diastolic blood pressure	• Main treatment effects: **NULL** • Measurements: BMI, fat mass index, and lean mass index	N/A
Hawkesworth et al. 2009	N/A	• Main treatment effects: **NULL**	N/A	N/A

Mean age at follow up: 14y		• Measurements: systolic blood pressure, diastolic blood pressure, pulse pressure, and mean arterial pressure		
Hawkesworth et al. 2008 *Mean age at follow up: 14y*	N/A	N/A	• Main treatment effects: **NULL** • Measurements: height, weight, BMI, percent body fat, trunk fat, fat mass index, fat-free mass index, by sex	N/A
Kinra et al. 2008 *Mean age at follow up: 16y*	• Main treatment effects: **LOWER** insulin and HOMA score • Measurements: total cholesterol,	• Main treatment effects: **LOWER** augmentation index, **NULL**	• Main treatment effects: **HIGHER** height, **marginally**	N/A

	LDLc, HDLc, triglycerides, glucose, insulin, HOMA score	for blood pressure • Measurements: systolic blood pressure, diastolic blood pressure, and augmentation index	**LOWER** fat-free mass index • Measurements: height, BMI, fat mass index, fat-free mass index, central-peripheral skinfold ratio	
Macleod et al. 2013 *Mean age at follow up: 23y*	• <u>Main treatment effects</u>: all **NULL** • Measurements: Fasting glucose, 30min post load glucose, 120min post load glucose, fasting insulin, 30 min post load insulin, 120 min post load insulin, HbA1c, fasting C-peptide, beta cell function, insulin	• <u>Main treatment effects</u>: all **NULL** • Measurements: systolic blood pressure and diastolic blood pressure	• <u>Main treatment effects</u>: all **NULL** • Measurements: BMI, total fat (EDXA)	N/A

	sensitivity, insulin resistance, total cholesterol, HDLc, triglycerides, IGF-1, IGFBP3			
Long-chain polyunsaturated fatty acids supplementation				
Asserhøj et al. 2009 *Mean age at follow up: 5y*	N/A	• <u>Main treatment effects</u>: **HIGHER** blood pressure (boys) • Measurements: systolic blood pressure, diastolic blood pressure, and mean arterial pressure	• <u>Main treatment effects</u>: **HIGHER** head circumference, otherwise **NULL** • Measurements: head circumference, height, weight, BMI, body fat percentage, waist-to-height ratio	N/A
Brei et al. 2016	N/A	N/A	• <u>Main treatment effects</u>: **NULL**	N/A

Mean age at follow up: 5y			• Measurements: sum of 4 skinfold thickness, fat mass, weight, height, BMI percentile, head circumference, arm circumference, and waist circumference	
Foster et al. 2017 *Mean age at follow up: 4y*	N/A	N/A	• Main treatment effects: **NULL** • Measurements: BMI z-score, arm circumference, arm skinfold z-score	N/A
Gutierrez-Gomez et al. 2017 *Mean age at follow up: 4y*	• Main treatment effects: **NULL** • Measurements: insulin, glucose, triglycerides, total cholesterol,	N/A	N/A	N/A

	HDLc, LDLc, non-HDLc, apoB, total cholesterol-to-HDLc			
Muhlhausler et al. 2016 *Mean age at follow up: 3y (body size & composition), 5y (biomarkers, body size & composition)*	• <u>Main treatment effects</u>: **HIGER** insulin resistance, insulin (boys and girls), fasting glucose (boys) • Measurements: Fasting glucose, insulin, HOMA-IR, combined and by sex	N/A	• <u>Main treatment effects</u>: **NULL** • Measurements: BMI, BMI z score, BMI percentile, body fat percentage, body weight, weight z score, total fat mass, fat-free mass, total body water, impedance index, height and z score, height increase, head circumference, waist circumference,	N/A

			hip circumference, wait-to-hip ratio	
Rytter et al. 2012 *Mean age at follow up: 19y*	N/A	• <u>Main treatment effects</u>: **NULL** • Measurements: systolic blood pressure, diastolic blood pressure, heart rate, heart rate variability (RR, SDNN, SDNNindex, RMSSD, PNN50, and HR_{EA})	N/A	N/A
Rytter et al. 2011 (1) *Mean age at follow up: 19y*	• <u>Main treatment effects</u>: all **NULL**	N/A	• <u>Main treatment effects</u>: all **NULL**	N/A

	• Measurements: insulin, glucose, HbA1c, HOMA-IR, leptin, adiponectin, hsCRP, IGF-1		• Measurements: BMI, waist circumference	
Rytter et al. 2011 (2) *Mean age at follow up: 19y*	• <u>Main treatment effects</u>: all **NULL** • Measurements: Total cholesterol, HDLc, LDLc, triglycerides, apoA-1, apoB, sdLDL	N/A	N/A	N/A
See et al. 2018 *Mean age at follow up: 5y*	• <u>Main treatment effects</u>: **LOWER** insulin concentration and insulin resistance • Measurements: glucose, insulin, HOMA-IR, cholesterol, HDLc, LDLc, triglycerides,	• <u>Main treatment effects</u>: **NULL** • Measurements: heart rate, systolic blood pressure, and diastolic blood pressure	• <u>Main treatment effects</u>: **LOWER** waist circumference • Measurements: Waist circumference, arm circumference, weight, height, BMI, head circumference,	N/A

	hsCRP, adiponectin, leptin,		abdominal skinfold, suprailiac skinfold, subscapular skinfold, triceps skinfold	
Vinding et al. 2018 *Mean age at follow up: 6y*	N/A	N/A	• <u>Main treatment effects</u>: **HIGHER** BMI from 0 to 6 years, **NULL** for obesity risk at 6y (authors: proportional increase in lean, bone, and fat mass at 6y) • Measurements: BMI z score, total body fat, percent body fat, percent trunk fat, lean mass, lean mass percentage, trunk lean	N/A

			mass, total bone mineral content, total bone mineral density	
Single micronutrient supplementation				
Belizan et al. 1997 *Mean age at follow up: 7y*	N/A	• <u>Main treatment effects</u>: **NULL**, with **marginally LOWER** diastolic blood pressure in overweight children only (BMI > 17.5) • Outcomes: systolic blood pressure, diastolic blood pressure, blood pressure by	• <u>Main treatment effects</u>: all **NULL** • Outcomes: Weight, height, BMI	N/A

		different BMI group		
Hawkesworth et al. 2011 (Trial 2) *Mean age at follow up: 14y*	N/A	• <u>Main treatment effects</u>: **NULL** • Measurements: systolic blood pressure and diastolic blood pressure	• <u>Main treatment effects</u>: **NULL** • Measurements: BMI, fat mass index, and lean mass index	N/A
Palmer et al. 2019 *Mean age at follow up: 24y*	• <u>Main treatment effects</u>: **NULL** • Measurements: thymulin	N/A	N/A	N/A
Taylor et al. 2015 *Mean age at follow up: 73y*	N/A	N/A	N/A	• <u>Main treatment effects</u>: all **NULL** • Outcomes: All-cause mortality, cardiovascular mortality, all

				cancer mortality, breast cancer mortality, all cancer morbidity, breast cancer morbidity
Multiple micronutrient supplementation				
Ekström et al. 2016 (Trial 1) *Mean age at follow up: 4.5y*	• <u>Main treatment effects</u>: **LOWER** total cholesterol, LDLc, and ApoB in early invitation group • Measurements: ApoA-1, apoB, total cholesterol, HDLc, LDLc, LDL-to-HDL ratio, triglycerides, glucose, insulin, HOMA-IR, IGF-1, IGFBP-1, IGF-1/IGFBP-1, CRP, Oxidative stress	N/A	N/A	N/A

Hiller et al. 2007 *Age range at follow up: 4-8y*	N/A	• Main treatment effects: **NULL** • Outcomes: systolic blood pressure and diastolic blood pressure	N/A	N/A
Mannan et al. 2016 *Mean age at follow up: 9y*	• Main treatment effects: **LOWER** inflammation marker (sTfR, hepcidin), **LOWER** vitamin A concentration (mainly boys) • Measurements: sTfR, ferritin, folate, vitamin B12, hepcidin, zinc, vitamin A,	N/A	• Main treatment effects: **LOWER** body mass-for-age z score • Measurements: height-for-age z-score, BMI-for-age z score	N/A
Stewart et al. 2011	• Main treatment effects: overall **NULL**	N/A	N/A	N/A

Mean age at follow up: 7y	• Measurements: HOMA-IR			
Stewart et al. 2009 *Mean age at follow up: 7y*	N/A	N/A	N/A	• <u>Main treatment effects</u>: **LOWER** metabolic syndrome (MetS) in the folic acid group, and **LOWER** risk for microalbuminuria in folic acid group and folic acid + iron + zinc group, vs. control • Outcomes: MetS and MetS components, including high glucose, low HDLc, high

				triglycerides, high blood pressure, and high waist circumference; Microalbuminuria
Infant and young child feeding, and milk supplementation				
De Jong et al. 2011 *Mean age at follow up: 9y*	N/A	• <u>Main treatment effects</u>: **marginally LOWER** heart rate in breastfed than formula-fed children, overall considered **NULL** • Measurements: heart rate (all null: blood pressure)	• <u>Main treatment effects</u>: **NULL** • Measurements: weight, body length, BMI, and head circumference	N/A

Forsyth et al. 2003 *Mean age at follow up: 6y*	N/A	• <u>Main treatment effects</u>: **LOWER** blood pressure [conflict of interest alert] • Measurements: systolic blood pressure, diastolic blood pressure, and mean blood pressure	N/A	N/A
Gruszfeld et al. 2016 *Mean age at follow up: 5y*	N/A	N/A	• <u>Main treatment effects</u>: **HIGHER** pre-peritoneal fat layer (tissue accumulation), **NULL** for the rest • Measurements: subcutaneous	• <u>Main treatment effects</u>: **NULL** • Measurements: overweight, obesity

			fat layer, peritoneal fat layer, subcutaneous fat area, peritoneal fat area, ratio of subcutaneous/peritoneal fat area, waist circumference, and BMI	
Gruszfeld et al. 2015 *Mean age at follow up: 5y*	• Main treatment effects: overall **NULL** • Measurements: IGF-1, insulin, total cholesterol, LDLc, HDLc, triglyceride, apoA-1, apoB, apoB to A1 ratio	• Main treatment effects: overall **NULL** • Measurements: systolic blood pressure and diastolic blood pressure	• Main treatment effects: overall **NULL** • Measurements: BMI, weight, height	N/A
Kennedy et al. 2010	N/A	• Main treatment effects:	• Main treatment effects: **HIGHER**	N/A

Mean age at follow up: 10.8y		**HIGHER** blood pressure (girls only) • Measurements: systolic blood pressure, diastolic blood pressure, and mean arterial blood pressure	weight and adiposity (girls only) • Measurements: Weight SD scores, height, head circumference, biceps skinfold, Ln sum of skinfolds	
Kramer et al. 2007 *Mean age at follow up: 6.5y*	N/A	• <u>Main treatment effects</u>: **NULL** • Measurements: systolic blood pressure and diastolic blood pressure	• <u>Main treatment effects</u>: **NULL** • Measurements: BMI, BMI percentile, height, leg length, head circumference, waist circumference, hip circumference, waist-to-hip ratio, mid-thigh	N/A

			circumference, mid-upper arm circumference, triceps skinfold thickness	
Martin et al. 2017 *Mean age at follow up: 16y*	N/A	• <u>Main treatment effects</u>: **NULL** • Measurements: systolic blood pressure and diastolic blood pressure	• <u>Main treatment effects</u>: **HIGHER** risk for overweight and obesity, including more rapid growth in height followed by more rapid weight gain • Measurements: BMI, fat mass index, fat-free mass index, percent body fat, waist circumference, waist-to-height ratio, standing height, BMI percentile, birth weight,	N/A

			birth length, birth BMI, weight gain, statue gain, & BMI gain	
Martin et al. 2014 *Mean age at follow up: 11.5y*	• Main treatment effects: **NULL** • Measurements: fasting glucose, insulin, HOMA-IR, HOMA-B, adiponectin, apoA-1	• Main treatment effects: **NULL** • Measurements: systolic blood pressure and diastolic blood pressure	N/A	• Main treatment effects: **NULL** • Measurements: metabolic syndrome
Martin et al. 2013 *Mean age at follow up: 11.5y*	N/A	N/A	• Main treatment effects: **NULL** • Measurements: BMI, BMI percentile fat mass index, fat-free mass index, percent body fat, waist circumference, hip	N/A

			circumference, waist-to-hip ratio, leg length, head circumference, mid-upper arm circumference, triceps skinfold, subscapular skinfold, insulin-like growth factor (IGF-1)	
Singhal et al. 2010 *Mean age at follow up: 6.5y*	N/A	N/A	• <u>Main treatment effects</u>: **HIGHER** fat mass in both nutrient-enriched formula groups that promoted faster weight gain in infancy • Measurements: BMI, adjusted BMI, fat mass, fat-free mass,	N/A

			sum of skinfold thickness, measured with different methods	
Singhal et al. 2004 *Mean age at follow up: 14.8y*	• Main treatment effects: **LOWER** C-reactive protein and LDLc-to-HDLc ratio in banked breastmilk group vs. preterm formula • Measurements: total cholesterol, LDLc, HDLc, LDLc-to-HDLc ratio, total/HDLc ratio, apoA-1, apoB, apoB to A1 ratio, C-reactive protein	N/A	N/A	N/A
Singhal et al. 2003 *Mean age at follow up: 14.8y*	• Main treatment effects: **HIGHER** insulin resistance (32-23	N/A	N/A	N/A

	split insulin) in combined intervention group (banked breastmilk and term formula, vs. preterm formula) • Measurements: Insulin, proinsulin, 32-23 split proinsulin, and glucose			
Singhal et al. 2002 *Mean age at follow up: 14.8y*	• <u>Main treatment effects</u>: **LOWER** leptin concentration relative to fat mass in combined intervention group (banked breastmilk and term formula, vs. preterm formula) • Measurements: Leptin concentration, leptin/fat mass,	N/A	N/A	N/A

	leptin/percentage of fat mass			
Singhal et al. 2001 *Mean age at follow up: 14.8y*	N/A	• Main treatment effects: **LOWER** blood pressure in banked breastmilk group vs. preterm formula • Measurements: systolic blood pressure, diastolic blood pressure, and mean arterial blood pressure	N/A	N/A
Toftlund et al. 2018 *Mean age at follow up: 6y*	• Main treatment effects: **LOWER** cholesterol in breastmilk than	• Main treatment effects: **NULL**	• Main treatment effects: **LOWER** level of early rapid	N/A

	in preterm formula group; **NULL** otherwise • Measurements: Fasting glucose, fasting insulin, HbA1c, total cholesterol, triglycerides, HDLc, LDLc, creatinine	• Measurements: systolic blood pressure, diastolic blood pressure, and mean arterial blood pressure	growth in breastfeeding than formula group • Measurements: BMI, fat mass, fat-mass index, fat free mass, fat free mass index, abdominal fat mass	
Totzauer et al. 2018 *Mean age at follow up: 6y*	N/A	N/A	• <u>Main treatment effects</u>: **HIGHER** fat mass (also higher values at 1y follow-up, with a continuous increase over time) • Measurements: Sum of 2 skinfold thickness, percent body fat, fat mass	N/A

			index, fat-free mass index, and BMI	
Weber et al. 2014 *Mean age at follow up: 6y*	N/A	N/A	• Main treatment effects: **HIGHER** BMI • Measurements: weight, height, and BMI (raw and imputed values)	• Main treatment effects: **HIGHER** risk for obesity • Outcomes: obesity (raw and imputed values)
Williams et al. 2012 *Mean age at follow up: 25y*	• Main treatment effects: **NULL** • Measurements: Fasting glucose, fasting insulin, insulin sensitivity index, corrected insulin responses (insulin secretion)	N/A	N/A	N/A
Dietary Counselling				
Costa et al. 2017	• Main treatment effects: **NULL**	N/A	N/A	N/A

Mean age at follow up: 8y	• Measurements: fasting glucose, fasting insulin, and HOMA-IR			
Hakanen et al. 2006 *Mean age at follow up: 10y*	N/A	N/A	N/A	• <u>Main treatment effects</u>: **LOWER** overweight prevalence (girls only) • Measurements: Overweight and obesity (and growth trend over the years)
Kaitosaari et al. 2006 *Mean age at follow up: 9y*	• <u>Main treatment effects</u>: **LOWER** insulin resistance • Measurements: HOMA-IR	N/A	N/A	N/A
Lehtovirta et al. 2018 *Mean age at follow up: 20y*	• <u>Main treatment effects</u>: **LOWER** circulation fatty acids and lipoprotein	N/A	N/A	N/A

	subclass lipids (especially in boys). **HIGHER** serum PUFA • Measurements: lipid concentration in medium-sized VLDL particles and in small-sized VLDL particles, serum PUFA-to-SFA ratio			
Niinikoski et al. 2012 *Mean age at follow up: 19y*	• <u>Main treatment effects</u>: **LOWER** LDLc (boys and girls), total cholesterol (boys), triglycerides (boys), VLDL-TG (boys) • Measurements: triglycerides, total cholesterol, LDLc, HDLc, IDLc, VLDL-	N/A	N/A	N/A

	TG, apoA-1, apoB, apoB to apoA-1 ratio			
Niinikoski et al. 2009 *Mean age at follow up: 15y*	N/A	• <u>Main treatment effects</u>: **LOWER** blood pressure (with a meaningful population-attributable amount) • Measurements: systolic blood pressure and diastolic blood pressure	N/A	N/A
Nupponen et al. 2015 *Mean age at follow up: 20y*	N/A	N/A	N/A	• <u>Main treatment effects</u>: **LOWER** relative risk of metabolic syndrome

				(MetS, boys and girls), blood pressure (boys and girls), and triglycerides (boys) • Measurements: MetS and MetS components, including high waist circumference (with different cut-off points), high fasting glucose, high blood pressure, high triglycerides, and low HDLc, by sex
Oranta et al. 2013	• <u>Main treatment effects</u>: **LOWER** insulin resistance	N/A	• <u>Main treatment effects</u>: **NULL**	N/A

Age range at follow up: 15-20y	• Measurements: glucose, insulin, HOMA-IR		• Measurements: BMI, waist circumference	
Pahkala et al. 2013 *Mean age at follow up: 19y*	N/A	N/A	N/A	• <u>Main treatment effects</u>: **LOWER** risk of poor cardiovascular health, including **LOWER** blood pressure • Measurements: American Heart Association definition of "Ideal Cardiovascular Health" Matrices, including no smoking, good BMI, physically active,

				healthy diet, low total cholesterol, blood pressure, and glucose
Raitakari et al. 2005 *Mean age at follow up: 11y*	• Main treatment effects: **NULL** • Measurements: triglycerides, total cholesterol, LDLc, HDLc	• Main treatment effects: **BETTER** endothelial function (mainly in boys) • Measurements: systolic blood pressure, diastolic blood pressure, brachial artery diameter, increase in blood flow after cuff release,	• Main treatment effects: **NULL** • Measurements: weight, height, BMI	N/A

		nitrate-mediated dilation, area under dilation vs time curve, maximum flow-mediated dilation		
Simell et al. 1999 *Mean age at follow up: 19y*	• <u>Main treatment effects</u>: **LOWER** serum cholesterol (total, HDL, non-HDL, mainly in girls) • Measurements: total cholesterol, HDLc, LDLc, non-HDLc	N/A	• <u>Main treatment effects</u>: **NULL** • Measurements: weight, height	N/A
Other *interventions*				
Ekström et al. 2016 (Trial 2) *Mean age at follow up: 4.5y*	• <u>Main treatment effects</u>: **LOWER** HDLc, glucose, and IGF-1 in MMS than IFA group	N/A	N/A	N/A

	• Measurements: ApoA-1, apoB, total cholesterol, HDLc, LDLc, LDL-to-HDL ratio, triglycerides, glucose, insulin, HOMA-IR, IGF-1, IGFBP-1, IGF-1/IGFBP-1, CRP, Oxidative stress			
Luoto et al. 2010 *Mean age at follow up: 10y*	N/A	N/A	• <u>Main treatment effects</u>: all **NULL** (authors: early gut microbiota modulation with probiotics may restrain excessive weight gain during the first years of life) • Measurements: BMI (ages 2y, 4y, 7y, 10y, and overall	• <u>Main treatment effects</u>: **NULL** • Outcomes: overweight and obesity

			ANCOVA results for growth pattern)	
Videhult et al. 2015 (1) *Mean age at follow up: 10y*	• Main treatment effects: all **NULL** • Measurements: total cholesterol, triglycerides, HDLc, LDLc, apoA-1, apoB, apoB to A1 ratio, AST, ALT, insulin, glucose, HOMA-IR	N/A	• Main treatment effects: all **NULL** • Measurements: weight, height, sagittal abdominal diameter, BMI z score, trucal fat percentage, android fat percentage, gynoid fat percentage, fat-free mass	N/A
Videhult et al. 2015 (2) *Mean age at follow up: 10y*	• Main treatment effects: all **NULL** • Measurements: C-peptide, ghrelin, GIP, GLP-1, glucagon, insulin, leptin,	N/A	N/A	N/A

	HMW adiponectin, PAI-1, resistin, visfatin, hsCRP			

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