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The role of maternal-fetal metabolism in the DOHaD hypothesis: Insights from a metabolomics perspective

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Contents

Abbreviations

BMI	Body mass index
FA	Fatty acid
DI	Desaturation index
DOHaD	Developmental Origins of Health and Disease
FDR	False discovery rate
GDM	Gestational diabetes mellitus
GI	Glycaemic index
GWG	Gestational weight gain
HDL	High density lipoproteins
IR	Insulin resistance
LC	Liquid chromatography
LDL	Low density lipoproteins
MS	Mass spectrometry
NCD	Non-communicable diseases
NEFA	Non-esterified fatty acid
SCD-1	Stearoyl-CoA desaturase-1
T2D	Type 2 diabetes
VLDL	Very low-density lipoproteins
WHO	World Health Organization

Publications List

This dissertation is based on the following publications:

Publication I: E. Shokry, L. Marchioro, O. Uhl, M. G. Bermudez, J. A. Garcia-Santos, M. T. Segura, et al., and B. Koletzko, "Impact of maternal BMI and gestational diabetes mellitus on maternal and cord blood metabolome: results from the PREOBE cohort study," *Acta Diabetol*, vol. 56, no. 4, pp. 421–430, 2019

Publication II: L. Marchioro, A. A. Geraghty, O. Uhl, E. Shokry, E. C. O'Brien, B. Koletzko, and F. M. McAuliffe, "Effect of a low glycaemic index diet during pregnancy on maternal and cord blood metabolomic profiles: results from the ROLO randomized controlled trial," *Nutr Metab (Lond)*, vol. 16, p. 59, 2019

Publication III: L. Marchioro, C. Hellmuth, O. Uhl, A. A. Geraghty, E. C. O'Brien, M. K. Horan, B. Koletzko, and F. M. McAuliffe, "Associations of maternal and fetal SCD-1 markers with infant anthropometry and maternal diet: Findings from the ROLO study," *Clin Nutr*, 2019

Summary

The Developmental Origins of Health and Disease (DOHaD) hypothesis proposes that, besides genetics and lifestyle, also prenatal and early postnatal factors can shape the risk for non-communicable metabolic diseases, such as cardiovascular diseases and type 2 diabetes. On the path to non-communicable metabolic disorders, childhood obesity is a major risk factor. At the same time, childhood obesity is a feasible and thus widely used endpoint for clinical and epidemiological studies starting at birth or during pregnancy. Gestation is indeed a critical period, as it has the potential to modulate long-term health outcomes in both mother and offspring. The aim of this doctoral project was to investigate metabolic pathways and mechanisms affected by maternal obesity, gestational diabetes mellitus (GDM), and nutrition during pregnancy by using targeted metabolomics data.

This work contains three analyses conducted using data from two European pregnancy studies, the PREOBE and ROLO trials. The combined inspection of amino acids, fatty acids, acylcarnitines, intermediates of the TCA cycle and phospholipids gives insights on amino acid and lipid catabolism for energy production and in lipid mobilization.

In the first publication, I showed, using data from the PREOBE study, that elevated maternal body mass index (BMI) and GDM are associated with systemic disturbances in branched chain amino acids and lipid metabolism and in their placental transport.

In the second publication, I showed that the consumption of a low glycaemic index diet during pregnancy, as promoted by the interventional ROLO study, did not affect fetal metabolism, but hinted towards an increased lipid mobilization and fatty acid catabolism on the maternal side.

In the third publication, I made use of the extensive dietary and anthropometry data collected within the ROLO study to investigate the potentially obesogenic effect of the enzyme Stearoyl-CoA Desaturase 1 (SCD-1) and its nutritional regulation. I showed that only one of the typical pathways by which SCD-1 acts (via stearic acid) was associated with infant adiposity up to two years of age, but it could not be regulated by dietary intakes during pregnancy. Conversely, the alternative pathway (via palmitic acid) did show potential for modulation by absolute and relative carbohydrate and fat dietary intakes, but was not associated with anthropometry during childhood.

In conclusion, this work contributed to our understanding of the impact of BMI, GDM and nutrition during pregnancy on systemic and specific metabolic pathways. Based on these results, more attention should be dedicated to promoting a favourable metabolic status and positive dietary habits in the pre-conceptional period, for the long-term benefits of the mothers and their offspring.

Zusammenfassung

Laut der DOHaD Hypothese (Developmental Origins of Health and Disease - Entwicklungbiologische Ursachen von Gesundheit und Krankheit) können nicht nur genetische und Lifestylefaktoren, sondern auch vorgeburtliche und frühkindliche Stimuli das spätere Risiko für nicht-übertragbare Erkrankungen wie kardiovaskuläre Krankheiten und Typ 2 Diabetes modifizieren. Übergewicht und Adipositas im Kindesalter stellen einen bestätigten Risikofaktor für spätere metabolische Krankheiten dar. Gleichzeitig ist kindliche Adipositas ein guter und oft benutzter Endpunkt von klinischen und epidemiologischen Schwangerschafts- und Geburtsstudien. Die Schwangerschaft ist ein besonders kritischer Zeitraum, da die veränderte metabolische Homöostase die langfristige metabolische Gesundheit von Mutter und Kind prägen kann. Das Ziel dieser Doktorarbeit war es, anhand von Targeted Metabolomics Daten zu untersuchen, wie sich mütterliches Übergewicht und Adipositas, Gestationsdiabetes Mellitus (GDM) und Ernährung in der Schwangerschaft auf Stoffwechselwege und -mechanismen auswirken.

Diese Arbeit umfasst drei Teilanalysen, die anhand von Daten aus zwei europäischen Schwangerschaftsstudien (PREOBE und ROLO) durchgeführt wurden. Die gleichzeitige Betrachtung von Aminosäuren, Fettsäuren, Acylcarnitinen, Zwischenprodukten vom Zitratzyklus (TCA) und Phospholipiden ermöglicht einen Überblick über die Transport- und Abbauprozesse von Aminosäuren und Lipiden zum Zweck der Energiegewinnung.

In der ersten Publikation zeigte ich anhand von Daten aus der PREOBE Studie, dass erhöhter mütterlicher Body-Mass-Index (BMI) und GDM mit Störungen im Stoffwechsel und im Transport durch die Plazenta von verzweigtkettigen Aminosäuren und Lipiden assoziiert sind.

In der zweiten Publikation zeigte ich, dass die Umstellung auf eine Ernährung mit niedrigem glykämischen Index während der Schwangerschaft, wie in der ROLO Studie untersucht, keine Änderung im fötalen Metabolismus bewirken konnte, jedoch mit einer erhöhten Mobilisierung von mütterlichen Lipiden und erhöhtem Fettsäurenkatabolismus assoziiert war.

In der dritten Publikation nutzte ich die umfassenden Ernährungs- und Anthropometriedaten aus der ROLO Studie, um den potenziellen adipogenischen Effekt des Enzyms Stearoyl-CoA Desaturase-1 (SCD-1) sowie ihre ernährungsbedingte Regulierung zu untersuchen. Ich zeigte, dass nur einer der typischen Stoffwechselwege, über die SCD-1 agiert (und zwar über Stearinsäure), mit Kinderadipositas bis zum zweiten Lebensjahr assoziiert war. Allerdings konnte dieser Weg nicht durch Ernährung in der Schwangerschaft moduliert werden. Im Gegensatz dazu zeigte der alternative Weg (über Palmitinsäure) Potential für Regulierung durch die absolute und relative Aufnahme von Kohlenhydraten und Fett, es wurde aber keine Assoziation mit anthropometrischen Messungen gefunden.

Diese Arbeit trägt dazu bei, den Einfluss von BMI, GDM und Ernährung in der Schwangerschaft auf systemische und spezifische Stoffwechselwege besser zu verstehen. Die Ergebnisse legen dabei nahe, dass dem metabolischen Status sowie den Essensgewohnheiten bereits vor der Schwangerschaft mehr Aufmerksamkeit geschenkt werden sollte, um die langfristige Gesundheit von Müttern und Kindern zu verbessern.

Chapter 1

Introduction

In this work, I used targeted metabolomics data to investigate how selected pre-conceptional and pregnancy exposures interact with maternal and fetal metabolism, and to explore their potential within the Developmental Origins of Health and Disease (DOHaD) hypothesis framework [1–3].

In this first chapter, I will clarify and give insights into the three keywords of this thesis's title – maternal-fetal metabolism, the DOHaD hypothesis, and metabolomics – and give some context regarding the relevance of this research question.

1.1 Non-Communicable Diseases and Childhood Obesity

As for 2019, one of the ten threats to global health proposed by the World Health Organization (WHO) are non-communicable diseases (NCD) [4]. The umbrella term NCD refers to those chronic diseases, such as cardiovascular diseases, cancer, and type 2 diabetes (T2D), which result as consequence of genetic, environmental and lifestyle factors [5]. NCD have not only been the leading cause of death worldwide for the last two decades, but they are also associated with disability and diminished quality of life [6–8].

One relevant intermediate outcome and risk factor for the onset of NCD is childhood overweight and obesity, defined as having an age-standardized body mass index (BMI, measured in kg/m^2) above the 85^{th} and 95^{th} percentile of the WHO growth reference (corresponding to 1 and 2 standard deviations above the mean), respectively [9, 10]. There is substantial evidence that children who are overweight or obese have up to five times higher chances to remain so and to develop metabolic syndrome (the co-occurrence of three or more of the following conditions: elevated waist circumference, elevated triglycerides, elevated blood pressure, elevated blood glucose and reduced HDL cholesterol [11]) in adult age than children with normal weight [12–14]. Regional and worldwide prevalences of childhood overweight and obesity are alarmingly on the rise. In 2016, about 50 million girls and 74 million boys between 5 and 19 years of age were estimated to be obese, corresponding to 5.6% and 7.8% worldwide, respectively; in 1975, the same figures did not exceed 1.3% [9]. In 2016, additional 216 million children and adolescents were classified as overweight but not obese [9].

These data remark the importance and the urgency to tackle childhood overweight and obesity, and, subsequently, the onset of NCD, in a sustainable fashion. According to the DOHaD hypothesis, it is never too early to engage in prevention [15].

1.2 The DOHaD Hypothesis and Early Metabolic Programming

The DOHaD hypothesis is the result of a body of research produced over the last century linking perinatal and early life factors with adult health and disease.

Between 1930s and 1980s, several researchers reported sparse correlations between fetal and early life factors and later mortality and morbidity [16,17]. Among these, the endocrinologist Günter Dörner noted that the concentration of hormones, metabolites and neurotransmitters in critical phases of early development had a "programming" effect on brain structure and endocrinological diseases in later life [18, 19]. The foundations for the systematic investigation of perinatal factors and adult health were provided by a series of research conducted in late 1980s – early 1990s in Southampton by the epidemiologist David Barker and colleagues. In geographical studies from England and Wales, they observed that adult metabolic and coronary heart diseases were strongly associated with fetal and infant weight, growth, and nutrition [20–24]. Given the evidence of these studies, they formed the hypothesis that poor nutrition during fetal and early postnatal life are detrimental in the development of several tissues and organs (such as cardiac muscle and pancreatic beta cells), thus increasing the risk for diseases onset in later life. This hypothesis became quickly popular as the "thrifty phenotype hypothesis" or "fetal origins hypothesis"– even though the former designation was proposed with respect to T2D [25,26], while the latter referred to cardiovascular diseases [23,27]. Shortly afterwards, investigations on cohorts from all over the world began to flourish [17]. Some notable examples were provided by analyses by Ravelli, Roseboom and others, who used the natural experiment provided by the Dutch Famine of 1944-1945 (a hunger wave triggered by the combination of war events and an extremely severe winter) to corroborate the findings by Barker et al. [28,29]. Several studies on animal models conducted in the 1990s confirmed the biological foundation of the fetal origins hypothesis [16, 17]. The hypothesis was renamed "Fetal Origins of Health and Disease" (FOAD), despite the criticisms of many scientists who recognized that also the early postnatal period, and not only the fetal life, had the potential to modulate adult health [17, 19]. The term "Developmental Origins of Health and Disease" (DOHaD) was finally introduced in 2003 to indicate both the theory and the related international society. Since its establishment in the scientific panorama, the DOHaD debate has increasingly moved out of laboratories and medical journals to reach policy makers, economists, and social scientists, not without harsh criticisms [30–33]. Currently, pre- and periconceptional health of women in reproductive age is one of the top priorities of the DOHaD society [30, 34].

The role of childhood overweight and obesity in the DOHaD research is pivotal. On the one hand, as seen in the previous section, childhood obesity is a worrying precursor of adult obesity and related comorbidities. On the other hand, it is a practical endpoint for clinical investigations, since it occurs in a relatively short time frame and can be followed more closely than adult events [35]. As formulated by the Early Nutrition Programming Project and the Early Nutrition Academy, the metabolic roots of childhood obesity can be ascribed to three main pathways [35–37]:

- the *fuel-mediated in utero hypothesis*: when the intrauterine milieu favours fetal overnutrition;
- the *mismatch hypothesis*: when inadequate fetal or early postnatal growth is compensated by an obesogenic environment, leading to overnutrition;
- the *accelerated postnatal growth hypothesis*: when excessive early postnatal feeding induces a too rapid growth, e.g. by a too short breastfeeding period or by an increased protein intake in early life.

This categorisation shows the indisputable importance of the maternal-fetal environment in the development of childhood obesity.

1.3 Pregnancy: A Mother-Fetus Symbiosis

In the 37 to 40 weeks from conception until (term) birth, mother and fetus build a single system in terms of endocrine regulation [38], immune response [39], and energy homeostasis [40].

Metabolically, pregnancy is characterized by a first anabolic period (corresponding to first and second trimester) followed by a catabolic phase during the third trimester [40–42]. During the anabolic period, the body prepares to meet the enhanced metabolic demand of late pregnancy and lactation by increasing maternal appetite and calories intake (hyperphagia), and thus fat storages, while maintaining normal glucose utilization and increasing insulin sensitivity [41, 42]. During the catabolic phase, maternal fat is mobilized, which results higher concentrations of circulating triglycerides (hyperlipidemia) [43, 44]. An increase in total and (V)LDL-cholesterol, albeit not as large as for triglycerides, also takes place [43, 44]. These changes in lipids are accompanied, if not favoured, by an increase in peripheric insulin resistance (IR) [40–42].

A critical time frame for the increased IR is between the 20^{th} and the 28^{th} gestational week (around the fifth month of gestation), where underlying glucose disturbances can be exacerbated and manifest themselves as gestational diabetes mellitus (GDM) [40, 42]. GDM is broadly defined as a degree of IR that begins or is first identified during pregnancy and disappears shortly after birth, and is a well known risk factor for adverse pregnancy outcomes and development of T2D in the mother [45, 46]. Due to the heterogeneity of the diagnostics criteria, a more precise definition of GDM is virtually non-existent [45, 46]; moreover, the debate about diagnostics of GDM has heated up again in the past decade, after the Hyperglycaemia and Adverse Pregnancy Outcomes (HAPO) study showed that clinically adverse outcomes are also present in case of high IR below the current thresholds for GDM [47].

These major adaptations in maternal metabolism are prompted by the pregnancy-specific hormonal stimulation and ensure an appropriate and sustained nutrient supply for fetal development and growth. Throughout gestation, glucose is the main source for fetal growth. The amount of glucose produced by the fetus is minimal until birth, and up to 95% of fetal glucose is provided by maternal circulation via facilitated diffusion through the placenta [40, 48, 49]. This facilitated diffusion is regulated by the gradient in maternal-fetal glucose concentrations, since circulating glucose in the fetus is approximately 70-80% as much as in the mother [40]. Maternal insulin, on the contrary, does not cross the placenta [50], and the fetus is able to synthesize its own insulin from the 10^{th} - 12^{th} gestational week onwards – although insulin is mostly used as growth-stimulating hormone than as metabolic regulator [40]. Other nutrients relevant for fetal growth, such as amino acids, cholesterol, and (essential) fatty acids, are transported through the placenta by simple diffusion or via active mechanisms [49–51].

The metabolic adaptations described in the previous paragraphs, together with the increase in mass in fetus and placenta, cause the well known increase in weight during pregnancy, formally known as gestational weight gain (GWG). In 1990, the Institute of Medicine (IOM) published a set of guidelines to evaluate the appropriate GWG in women with different pre-pregnancy BMI; these guidelines have been updated in 2010 and are presented in table 1.1. Weight gains below and above the given frame are defined as inadequate and excessive, respectively.

Given the tight entanglement of maternal and fetal metabolism, it should not surprise that adverse exposures for the mother are also inconvenient for the baby. Regarding the offspring's risk for overweight and obesity during childhood, epidemiological studies have identified strong evidence for maternal smoking, elevated BMI prior to and during pregnancy, and excessive GWG as the most relevant risk factors [53–62]. Other maternal exposures related to infant overweight and obesity, but for which the evidence is less striking, include maternal diabetes (pre-existing or gestational),

Table 1.1: 2010 Institute of Medicine guidelines for gestational weight gain (GWG). Adapted from [52].

Pre-pregnancy BMI (kg/m^2)	Total GWG (kg)	Rates of GWG in 2^{nd} and 3^{rd} trimester (kg)
Underweight (BMI $<$ 18.5)	12.5 - 18	0.51 (0.44 - 0.58)
Normal weight (18.5 $\leq$ BMI $<$ 25)	11.5 - 16	0.42 (0.35 - 0.50)
Overweight (25 $\leq$ BMI $<$ 30)	7 - 11.5	0.28 (0.23 - 0.33)
Obese (BMI $\geq$ 30)	5 - 9	0.22 (0.17 - 0.27)

psychosocial stress and depression, and inadequate (excessive or deficient) nutrition [53, 54, 63–69].

However, the investigation of the mechanisms by which these exposures can modulate the risk for childhood obesity is all but straightforward. Both these exposures and their effects are systemic, i.e., not limited to one tissue or organ, but rather affecting the whole body; therefore, the classic laboratory approaches can only partially explain the underlying pathways. Comprehensive literature providing information about the underlying mechanisms is necessarily fragmentary and limited [50, 51, 58, 59]. In other words, to study how these exposures play a role in the DOHaD framework, a systems biology approach is needed.

1.4 Metabolomics: 'The Apogee of the Omics Trilogy'

The advances in analytical techniques, computational power and statistical methods of the past decades have laid the ground for the development of high throughput methods, i.e., techniques able to simultaneously identify and quantify pools of genes or biological compounds in a sample (tissue or biofluid). These techniques have proved to be very effective in the investigation of systemic disorders, where the information needs to be broad enough to cover the physiology of the whole organism, but detailed enough to provide sufficient accuracy at molecular level. The suffix '-omics' has come in use to define the measurement and analysis of biological molecules in a comprehensive fashion [70–73].

'Metabolomics' is the omics science that deals with the metabolites, i.e. small molecules (typically $<$ 1500 Dalton) which are substrates, intermediates or products of metabolic reactions [74, 75]. The complete set of metabolites in an organism is also referred to as the 'metabolome' [76]. The number of metabolites (both endogenous and exogenous) in the human body is itself a debated matter, depending on both the definition of metabolites (some consider 1000 Dalton to be the upper limit for a metabolite) and the advances on analytical techniques. The Human Metabolome Database (http://www.hmdb.ca/), the best-established online data bank for human metabolites, first published in 2007, has expanded from 2180 to 40153 entries in just eleven years [77, 78].

The advantage of metabolomics over the other omics techniques lies in its proximity to the phenotype. By measuring molecules and compounds which are rapidly exchanged and transformed by chemical reactions between cells and tissues, it is often said that metabolomics provides a 'snapshot of the physiological status of the cells' [79], and it has been shown that the metabolome can be used to investigate the prompt response to instantaneous challenges [80]. For these reasons, Patti, Yanes and Siuzdak have given metabolomics, in their famous 2012 Nature article, the epithet of 'apogee of the omics trilogy' [74].

The aim of metabolomics in basic and clinical research is 'to understand pathophysiological processes [...] as well as to search for new diagnostic or prognostic biomarkers' [81]. Metabolomics is increasingly being used in the DOHaD framework [82, 83]. Recently, there have been attempts to integrate metabolomics and multi-omics tests in epidemiological and prognostic studies, but routines and protocols have to be established yet [71–73, 84].

The analytical techniques typically used in metabolomics are mass spectrometry (MS), possibly combined to gas or liquid chromatography (LC) for the species separation, and nuclear magnetic resonance [85]. Regarding the choice of the metabolites to measure, two different approaches can be distinguished. In untargeted metabolomics, all compounds identified in the sample are quantified, independently whether or not their name or chemical properties are known. In targeted metabolomics, the measurement is limited to a predefined set of well characterized metabolites [74, 85]. Despite requiring a prior knowledge of the underlying pathways and a more intensive samples preparation, targeted metabolomics data are characterized by a high specificity and an absolute quantification of the metabolites, which in turn facilitates the post-analytical treatment and the interpretation of the results [85].

In the next chapter, I will present the data and methods I used to investigate the entanglement of maternal BMI, GDM, and nutrition during pregnancy (especially carbohydrates and fats) with maternal and fetal metabolism, as well as the main results of the analyses.

Chapter 2

Summary of Analyses and Results

In this chapter, I will present the clinical studies that provided the data analysed in this project. Then, I will give an overview about the metabolomics measurements. Finally, I will give a summary of the specific research questions, methods and results from each of the publication included in this thesis.

2.1 The PREOBE and ROLO Studies

The data used in this thesis stem from two European pregnancy studies: the PREOBE and ROLO studies.

PREOBE

The PREOBE study was a prospective observational study conducted in Granada, Spain, between 2008 and 2012 [86] (ClinicalTrials.gov identifier: NCT01634464). A total of 331 women were followed throughout pregnancy, categorized into four groups: normal weight and normal glycaemic control (n = 132), overweight and normal glycaemic control (n = 56), obesity and normal glycaemic control (n = 64), and GDM (all weight categories, n = 79, diagnosed via the criteria established in the Third International Workshop-Conference on Gestational Diabetes Mellitus [86, 87]). The aim of the study was to monitor short- and long-term anthropometrics and metabolic parameters in mothers and children depending on maternal pre-pregnancy BMI or GDM. The authors of the study reported differences in maternal iron status, placental weights, and in maternal and cord blood vitamin concentrations [86]. The study aimed for a longitudinal follow up of the mother/child dyads until six years after birth; currently published analyses have mainly focused on cognitive and neurodevelopmental outcomes in the children until 18 months of age [88, 89].

ROLO

The ROLO study was a randomized controlled trial about diet in pregnancy conducted in Dublin, Ireland, between 2007 and 2011 [90] (ISRCTN identifier: ISRCTN54392969). The study tested the hypothesis that, in a population at risk for recurrent fetal macrosomia (birth weight $>$ 4000 g), a low glycaemic index (GI) diet during pregnancy might be beneficial in reducing birth weight. The GI of a carbohydrate-containing food is a value between 0 and 100 corresponding to the spike in blood glucose after its ingestion [91]. A diet low in GI has proven beneficial in the glycaemic control in pregnant and non-pregnant populations [92]. Already in 1988, Fraser had suggested that the increased IR associated with late pregnancy might be not physiological but diet-induced, and

reported that, in a small randomized trial, women consuming a high-fibre diet (low in GI) did not experience the expected rise in IR [93]. As mentioned in section 1.3, glucose can cross the placenta following the maternal-fetal concentration gradient, and the stimulated fetal insulin serves then as a growth hormone. Thus, the rationale of the ROLO study was that favouring a low GI diet might reduce the maternal glucose concentrations, and, in turn, the maternal-fetal glucose gradient, which ultimately would limit fetal growth [92, 94]. In the trial, 800 secundigravid women with a previous macrosomic child were recruited, and the half of it was randomly assigned to receive an educational session and counselling about low GI diet during pregnancy. The expected reduction in birth weight (102 g) was not achieved, and neither was the occurrence of macrosomia [90]; however, mothers in the intervention group had a lower GWG and trends for better glycaemic control and self-reported dietary fibre intake [90, 95]. The follow up of mothers and children who took part in the ROLO study is currently ongoing, and data about the 6 months, 2 years and 5 years visits have already been published [96, 97].

Maternal and cord blood samples of the PREOBE and ROLO studies were sent to the laboratory of the Division of Metabolic and Nutritional Medicine of the von Hauner Children's Hospital, University Hospital, Munich (Germany), for the metabolomic analysis. Table 2.1 shows the number of samples according to study, blood source, and time of collection.

Table 2.1: Overview of metabolomics samples analysed.
The superscript ^{f} indicates that the samples were collected after overnight fasting.

Blood source	PREOBE	ROLO
Maternal^{f}, approx. 13th week	-	75
Maternal^{f}, approx. 28th week	-	110
Maternal, delivery	200	-
Cord	124	136

2.2 Targeted Metabolomics Measurements

Blood is a particularly suited specimen for the investigation of metabolomics throughout pregnancy, since it can be easily collected at routine visits with a minimally invasive procedure, and it has proven to deliver good information about the metabolic progression of pregnancy [82, 98]. Cord blood has been similarly investigated in the metabolomics community, as it is especially suited to investigate the exchange of material in the fetoplacental unit under abnormal conditions (such as low birth weight or asphyxia [99]).

The samples were quantified using a targeted approach via liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS). In both studies and blood sources, we measured the following five metabolites classes according to published methods:

- amino acids (AA), related to protein metabolism [100];
- non esterified fatty acids (NEFA), which mirror the status of the adipose tissue [101, 102];
- acylcarnitines (AC), which are intermediates of amino acid and fatty acid catabolism [103];
- TCA cycle intermediates and ketoacids, involved in energy metabolism [104, 105];

- phospholipids (PL), including phosphatidylcholines (PC), lysophosphatidylcholines (LPC) and sphingomyelins (SM), which are important membrane constituents and play a role in transport and signalling mechanisms [103].

The simultaneous overview of these five metabolite classes enables insights in transport of lipids and in the catabolism of lipids and AA for energy production.

2.3 Specific Research Questions, Methods, and Results

2.3.1 Publication I: Maternal BMI and GDM in the PREOBE Cohort

In line with the aim and design of the PREOBE study, in this analysis we investigated the association of BMI and GDM with maternal and cord blood metabolites [1].

Data and Methods

Two hundred maternal and 124 cord blood samples, including data from 119 mother/child dyads, were available for the analysis. As mentioned in table 2.1, the maternal blood samples were collected at delivery. Less than 30% of the mothers gave birth via scheduled caesarean section, and were thus fasted for at least eight hours, but no information about the fasting status was available for the remaining ones. Fasting status is an important confounder in metabolomics analysis [106]. Therefore, we flagged the non-GDM mothers with glucose values $\geq$ 126 mg/dl and removed them from further analysis. The value of 126 mg/dl (7 mmol/l) was proposed in a recommendation paper by the International Association of Diabetes and Pregnancy Study Groups Consensus Panel to diagnose overt diabetes during pregnancy [87]. Thus, non-GDM mothers with a glucose value above this threshold must have been fed at time of blood samples collection. Following this rationale, we excluded 27 non-GDM mothers from the analysis. Obviously, we could not employ this method to identify fed GDM mothers, which were all considered in the analysis.

After exclusion of the subjects with ascertained fed status or high missingness in the data, the analysis of maternal blood included 67 normal weight, 50 overweight/obese, and 45 GDM study participants, whereas the analysis of cord blood was conducted on 49, 40 and 27 participants, respectively. For each of the 202 valid metabolites, the concentration values were regressed on log2(BMI) and GDM status, with covariates adjustment. In particular, we included BMI as a continuous variable, since we observed statistical artefacts in the models residuals when using BMI classes. We could not investigate the co-occurrence of overweight/obesity and GDM, as originally planned, due to the low number of high BMI mothers in the GDM groups.

When a high number of tests is performed, like in this analysis, the chance of false positives increases. This is known in statistics as the *multiple testing problem*, and a number of corrections have been developed to keep the global significance at the nominal alpha level (usually 0.05) [107]. In this analysis, we applied false discovery rate (FDR) adjustment within each blood source and exposure of interest. In this as well as in the following publications, we referred to associations with adjusted p-value < 0.05 as *significant*, but we also inspected *trends*, e.g. associations with uncorrected p-value < 0.05 but adjusted p-value ≥ 0.05.

Results and Interpretation

In both maternal and cord blood, GDM showed more strong associations with the metabolome than BMI.

Regarding BMI, we found no significant associations in maternal or cord blood. In both blood sources there were trends for positive associations of BMI and branched chain amino acids (BCAA), and, in cord blood only, for a negative associations with antiinflammatory markers. We hypothesized that, due to the larger availability of lipid depots in mothers with elevated BMI, fatty acids might be the preferred oxidative substrates for glucose production, thus sparing the BCAA which eventually accumulate in maternal blood. The increased concentrations and reduced catabolism on the maternal side favour an accumulation on the fetal side as well, which eventually might contribute to fetal growth. In GDM, the sum of hexoses (mainly glucose) was, as expected, significantly elevated both in maternal and cord blood. Further findings in the GDM group included lower PC and LPC in maternal blood and reduced free and esterified carnitines in cord blood. Based on observations regarding methyl group transferase, we proposed that the low concentrations of PC are due to a decreased synthesis. GDM is a state of oxidative stress, and glutathione can counterbalance reactive oxygen species; however, for the synthesis of glutathione, S-adenosylmethionine (SAM) is needed, which is also a precursor for the synthesis of PC from phosphatidylethanolamines [108]. Thus, we proposed that the lower concentrations of PC are concomitant to the need to fight oxidative stress. The lower levels of carnitines presented a challenge to the current paradigm that fetal carnitine relies on placental transport from the mother, since the maternal carnitines did not present such a reduction. We hypothesized that the increased fetal glucose might reduce the need of placental fatty acid oxidation, thus less carnitines might be transported in the placenta and, eventually, to the fetal circulation. Of course, we could not exclude a confouding effect of the fasting status, which might have blunted or enhanced the associations with GDM.

The full publication can be found in chapter 3, page 12.

2.3.2 Publication II: Low GI Diet in the ROLO Study

In the ROLO study, beneficial effects of a low GI diet were observed in mothers, but not in their babies (see also section 2.1). This analysis aimed to investigate (1) how the intervention was able to modulate maternal metabolism, and (2) if the low GI diet, if not by lowering birth weight, had affected fetal metabolism at all [2].

Data and Methods

Data from 51 and 136 study participants were used for the maternal and cord blood analysis, respectively. Each metabolite was analysed separately in maternal and cord blood (in a univariate fashion); the models included the metabolite concentration as dependent variable, the RCT group as independent variable, and covariates appropriate for the respective blood source. In particular, for maternal blood, the metabolite at 28 weeks was used as outcome, and the metabolite at 13 weeks as confounder; this longitudinal adjustment is the reason behind the small sample size. More than 200 metabolites, sums and ratios were included in each analysis, thus significance was defined in each blood source as Bonferroni-adjusted 0.05 alpha level. We performed sensitivity analyses via adjustment for additional confounders, trimming of highest and lowest 2.5% of the data, and multivariate methods (random forest, LASSO and elastic net, for maternal blood only).

Results and Interpretation

None of the metabolites in maternal or cord blood was significantly associated with the RCT group after Bonferroni correction. However, when considering the unadjusted p-values, many maternal PL and AC with 16 or 18 carbon atom chains and low saturation levels were higher in the intervention

than in the control group.A similar picture was observed also in the sensitivity analyses, especially after the adjustment for LDL cholesterol. We interpreted these results as indicative in an increased mobilization of fat due to the low GI diet, possibly accompanied by a shift to fat as source of maternal fuel (due to the increased levels of AC and fatty acid oxidation markers).

In the cord blood, only three metabolites had uncorrected p-values slightly below the 0.05 alpha level, so these associations were not further investigated. Regarding cord blood, our results confirmed that no short-term changes in the fetal/neonatal metabolism were induced by a low GI diet during pregnancy.

The manuscript is available in chapter 4, page 23.

2.3.3 Publication III: SCD-1 Activity in the ROLO Study

In this third analysis, we made use of the extensive dietary and anthropometry data collected within the ROLO study to investigate the modulation and effects of the enzyme Stearoyl-CoA desaturase-1 (SCD-1) during pregnancy [3].

SCD-1, also known as Delta-9-Desaturase, is responsible for inserting a double bond in even chain fatty acids with 14 to 18 carbon atoms, thus transforming them into their cis-monounsaturated counterpart [109]. Palmitic (16:0) and stearic acid (18:0) are the preferred substrates [109]. The ratios 16:1/16:0 and 18:1/18:0, also known as desaturation indices (DI), are used to estimate the activity of SCD-1 [109]. High levels of SCD-1 activity have often been reported in association with elevated BMI, and studies on animal models have proposed that SCD-1 plays an aetiological role in this association [110]. From the same animal models, it is known that SCD-1 expression and activity are upregulated by insulin, carbohydrate and saturated fat intake, and are downregulated by leptin and polyunsaturated fat [109, 111].

We aimed to investigate two questions: (RQ 1) whether maternal and cord SCD-1 markers are associated with infant adiposity up to 2 years of age, and (RQ 2) if carbohydrate and fat intake during pregnancy were able to modulate SCD-1 activity.

Data and Methods

For the first research question, we used the infant anthropometry measures collected at birth, 6 months and 2 years follow up visits. These measures included, but were not limited to, weight, height, BMI, and measures of skinfolds. Whenever possible, we calculated the corresponding WHO growth standard z-scores [112]. For the second question, we drew on the three-days food diaries collected during each pregnancy trimester. We analysed absolute and relative intakes of carbohydrates, sugars, fat (total, saturated, monounsaturated and polyunsaturated), as well as their mutual relative proportions. We estimated SCD-1 activity in 75 early and 110 late pregnancy and in 136 cord blood samples via the desaturation indices NEFA 18:1/18:0 (DI_{18}) and 16:1/16:0 (DI_{16}), and calculated their Spearman correlations to leptin and insulin. Given the form *dependent variable* $\sim$ *independent variable* + *{confounder}*, we constructed the main models for each research question as follows:

RQ 1 Anthropometry measure $\sim$ DI + {maternal BMI} + {child sex and age *(if the outcome was not a z-score)*} + {measurement at birth *(if the outcome was at 6 months or 2 years)*};

RQ 2 DI $\sim$ Dietary intake + {maternal BMI}

for each DI, anthropometry measure and dietary intake. The sensitivity analyses included adjustment for leptin and insulin as well as other confounders, and the use of Box-Cox transformation for models whose residuals showed issues of non-normality. P-values adjustment was performed via FDR correction within each time point of anthropometry/DI and DI/diet association, i.e., separately for

anthropometry at birth/DI in early pregnancy, anthropometry at birth/DI in late pregnancy, and so on.

Results and Interpretation

We found that DI_{18}, but not DI_{16}, both in maternal and cord blood was strongly associated with weight and BMI, as well as their z-scores, and weight-for-length at birth; late pregnancy maternal DI_{18} even showed two associations with 2 years z-scores at uncorrected 0.05 alpha level. Thus, it seems that SCD-1 with stearic acid might act upon central adiposity.

Conversely, dietary intakes did not show significant associations after FDR correction, but its associations at uncorrected 0.05 alpha level were almost exclusively with DI_{16}. Absolute intakes of both carbohydrates and all types of fat were more often positively associated with DIs, while the relative intakes of carbohydrates and sugars to fat associated negatively. These results suggest that not only the quantity, but also the composition, of the consumed macronutrients should be considered in studies examining SCD-1. Moreover, we explained the discrepancies between our results and published literature by considering the confounding effects of long-term diet and the hormonal regulation during pregnancy, as well as by observing that the diet compositions used in animal models are not compatible with real life intakes (e.g. high-fat diet consisting of 60 or 70% of fat versus real life intakes which seldom exceed 45%).

In both research questions, leptin and insulin did not mediate any of the associations, and their own Spearman correlations with the DI were also weak.

For the complete analysis, see chapter 5, page 35.

2.4 Conclusive Remarks

Our analysis on the PREOBE study remarked that elevated BMI and GDM represent a state of inflammation, in which the availability and placental transport of critical nutrients such as BCAA and glucose are dysregulated.

From the intervention in the ROLO trial, we have gained insights that switching to a low GI diet during pregnancy could not modify the offspring's metabolism. However, in the mothers, it was associated with a modest but consistent increase in fat mobilization and in its usage as fuel source.

By using metabolites ratios as surrogate biomarkers, we have learned that SCD-1 activity acting on stearic acid seems to be involved in the genesis of infant adiposity and might thus play an obesogenic role, but that this association could not be modulated by diet. On the contrary, when biomarkers based on palmitic acid were used, both the absolute and relative intakes of fat and carbohydrates were associated with SCD-1 activity, but this pathway would not affect the child's risk for overweight and obesity. Implicitly, our results hint at substrate-dependent regulatory mechanisms and action of SCD-1 in-utero, which should be considered in further studies.

This thesis contributed to clarifying putative mechanistic pathways linking maternal BMI, GDM and diet during pregnancy with childhood overweight and obesity. Elevated BMI and GDM are systemic metabolic risks with a major impact on the fetoplacental unit. The attainment of a healthy metabolic status and the development of positive nutritional habits should be promoted not only during pregnancy, but also before. The results of these analyses strengthen the current recommendation of the DOHaD society to engage already pre-conceptionally in improving long-term health in pregnant women and their children.

Chapter 3

Publication I: Maternal BMI and GDM in the PREOBE Cohort

The following pages present the publication E. Shokry, L. Marchioro, O. Uhl, M. G. Bermudez, J. A. Garcia-Santos, M. T. Segura, et al., and B. Koletzko, "Impact of maternal BMI and gestational diabetes mellitus on maternal and cord blood metabolome: results from the PREOBE cohort study," *Acta Diabetol*, vol. 56, no. 4, pp. 421–430, 2019.

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I contributed to this publication by conducting the statistical analysis, reviewing, editing and approving the manuscript.

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ORIGINAL ARTICLE

Impact of maternal BMI and gestational diabetes mellitus on maternal and cord blood metabolome: results from the PREOBE cohort study

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Abstract

Aims Maternal obesity and gestational diabetes mellitus (GDM) were frequently reported to be risk factors for obesity and diabetes in offspring. Our goal was to study the impact of maternal prepregnancy BMI (pBMI) and GDM on both maternal and cord blood metabolic profiles.

Methods We used LC–MS/MS to measure 201 metabolites comprising phospholipids (PL), amino acids, non-esterified fatty acids (NEFA), organic acids, acyl carnitines (AC), and Krebs cycle metabolites in maternal plasma at delivery and cord plasma obtained from 325 PREOBE study participants.

Results Several metabolites were associated with pBMI/GDM in both maternal and cord blood ($p < 0.05$), while others were specific to either blood sources. BMI was positively associated with leucine, isoleucine, and inflammation markers in both mother and offspring, while β-hydroxybutyric acid was positively associated only in cord blood. GDM showed elevated levels of sum of hexoses, a characteristic finding in both maternal and cord blood. Uniquely in cord blood of offspring born to GDM mothers, free carnitine was significantly lower with the same tendency observed for AC, long-chain NEFA, PL, specific Krebs cycle metabolites, and β-oxidation markers.

Conclusions Maternal BMI and GDM are associated with maternal and cord blood metabolites supporting the hypothesis of transgenerational cycle of obesity and diabetes.

Keywords Gestational diabetes · Intrauterine environment · Maternal obesity · Maternal phenotypes · Metabolomics

Introduction

Obesity and overweight have become a global epidemic [1]. In women of reproductive age, obesity poses a risk to maternal health, with consequences from gestational diabetes

Managed by Antonio Secchi.

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(GDM) [2] and adverse pregnancy outcomes to later type-2 diabetes (T2D) and cardiovascular diseases [3]. It is also associated with adverse outcomes for child at birth and during neonatal, infant and later life periods [4].

GDM is defined as glucose intolerance that is first recognized during pregnancy. Risk factors have been identified (including obesity, age and family history); however, the underlying mechanisms remain an enigma. GDM might be due to a pancreatic β-cell defect similar to that in type-1 diabetes (T1D), or to a dormant, pre-existing insulin resistance (IR) clinically manifesting as GDM during the diabetogenic state of late pregnancy [5]. Women developing GDM show impaired ability to stimulate glucose disposal and to suppress both glucose production and fatty acid (FA) levels [5]. Maternal obesity and GDM, each and combined, predispose to adverse short- and long-term infant outcomes [4, 6, 7].

To provide an understanding of the connection between early metabolic programming and the increased incidence of metabolic diseases resulting from the disruption of the intrauterine environment, associations between maternal prepregnancy Body Mass Index/GDM and maternal–cord blood metabolic profiles were studied.

Experimental section

Participants

The study was performed on data from the PREOBE study, a prospective observational cohort study (NCT01634464). Study procedures, group classification and subject inclusion/exclusion criteria have been described by Berglund et al. [8].

The study was approved by the Bioethical Committees for Clinical Research of the University Hospital San Cecilio, the Mother-Infant University Hospital of Granada, Spain.

Sample and data collection

Maternal venous blood samples were drawn into EDTA-containing tubes at delivery. Vein umbilical cord blood samples were obtained after clamping the cord. Blood samples were processed and plasma aliquots were stored at − 80 °C until analysis. Samples for metabolomics analyses were available for 200 mothers and 124 newborns, with an overlap for 119 mother/children pairs. At recruitment, information was collected on maternal anthropometry and used to calculate pBMI [8]. Gestational weight gain (GWG) at 34 weeks and data regarding clinical outcome at delivery were collected from medical records. Biochemical analysis of serum samples was performed as previously reported [8].

Since the maternal fasting status at time of blood collection was unknown, we used a fasting blood glucose threshold (126 mg/dl) previously validated for diabetes to identify non-diabetic, non-fasting mothers. Namely, if a non-diabetic mother presents a plasma glucose value above the threshold, this indicates non-fasted status. 27 non-GDM mothers were defined as non-fasted and excluded from the analysis. Such a distinction was obviously not possible for GDM mothers [9].

Targeted metabolomics assays

Targeted metabolomics involved analysis of 400 metabolites comprising: polar lipids [acylcarnitines (AC), diacyl-phosphatidylcholines (PCaa), acyl-alkyl-phosphatidylcholines (PCae), sphingomyelines (SM), acyl-lysophosphatidylcholines (LPCa), alkyl-lysophosphatidylcholines (LPCe)], sum of hexoses (H1), amino acids (AA), keto acids, non-esterified FA (NEFA), and Krebs cycle metabolites. NEFA analysis was performed by liquid chromatography with tandem mass spectrometry (LC–MS/MS) as previously reported [10]. Polar lipids were analysed with flow-injection analysis tandem mass spectrometry (FIA–MS/MS) [11]. A formula CX:Y was assigned for polar lipids and NEFA where X: length of carbon chain, Y: number of double bonds, OH: indicates presence of hydroxyl group. Letters ‘a’ and ‘e’ indicate that the acyl chain is bound via an ester or ether bond to the backbone, respectively. AA analysis was done by derivatization and separation by ion-pair LC–MS/MS [12]. AA were coded according to IUPAC abbreviations.

Six plasma quality control (QC) samples per batch were consistently measured with the samples. Concentrations were calculated in µmol/l; the analytical process was controlled and post-processed by Analyst 1.6.1 and R software (R version 3.4.3).

Metabolomics quality control (QC) and preprocessing

First, the QC for metabolomics measurements was done using a threshold of 20% and 30% for the intra- and inter-batch coefficient of variation, respectively (with allowance of max. 1 outlier measurement > 2 IQR from the next measurement). In total, 202 metabolites passed the QC. Measurements lying away than 1.5 standard deviations (SD) from the next closest measurement were removed. We corrected for batch effects by dividing metabolite concentrations by the ratio intra-batch median/inter-batch median. Then, the measurements were split into two datasets corresponding to maternal and cord blood.

Within each dataset, analytes with > 40% missing values were excluded. Sums were computed: Σ branched chain AA (BCAA), ΣLPCa, ΣPCaa, ΣPCae. Also, ratios were computed: ΣPCaa/ΣPCae, reflecting oxidative stress [13]; ΣLPCa/ΣPCaa, as a lipid biomarker of inflammation; (LPCa16:0 + LPCa18:0)/ΣPCaa as a proinflammatory biomarker [14]; (LPCa18:1 + LPCa 18:2)/ΣPCaa as

an anti-inflammatory biomarker [15]; AC ratios (AC16:0/free carnitine (Carn) and AC2:0/AC16:0) as markers of carnitine palmitoyl transferase-1 activity (CPT1) and FA β-oxidation, respectively [16]. Moreover, FA ratios were used to estimate activities of stearoyl-CoA desaturase-1 (SCD-1; 16:1/16:0, SCD-16 and 18:1/18:0, SCD-18) [17] and AA ratios Asn/Asp and Gln/Glu as indicators for anaplerosis or replenishing of Krebs cycle metabolites. Analytes, sums and ratios were log2-transformed after inspection of boxplots and quantile–quantile plots. Outliers were defined as points lying further away than 1 SD from the next measurement and were excluded.

Statistical analysis

Baseline characteristics

The 'overweight' and 'obese' groups were merged into one category (pBMI was considered a continuous variable). Differences between the covariates in the three groups were evaluated via Kruskal–Wallis and Fisher tests for continuous and categorical covariates, respectively.

Associations of maternal and cord blood metabolome with BMI and GDM

The associations between metabolite concentrations in maternal/cord blood and the exposures of interest were determined using multiple linear regression models with covariates adjustment. The final model used log2 of the metabolite concentration as outcome and log2(pBMI) and GDM as independent variables, GWG at 34 weeks, maternal age, mode of delivery, and infant sex as covariates. Parity was not included in the model due to high missingness (> 50%). A sensitivity analysis including gestational age showed no differences in the effect size and was not included in the final models. For these models, median values of 157 and 111 observations were used for maternal and cord blood, respectively. Results were depicted in Manhattan plots with|log10 (P)|-values on y-axis and metabolites on x-axis, with the direction on y-axis and the magnitude indicating the sign and strength of the association, respectively. We used false discovery rate (FDR) to minimize the occurrence of false positives (type I errors), a common issue in multiple testing. FDR controls for the expected proportion of false predictions relative to the total number of predictions at the level of significance. Nevertheless, because of the exploratory nature of the analysis, we also inspected associations with uncorrected $p < 0.05$ ('trends').

Results

Baseline characteristics of the two populations used in the analyses are summarized in Table 1A, B.

In a nutshell, our analyses of maternal and cord blood metabolites showed highly significant associations of GDM with several maternal and, to a lesser extent, cord blood metabolites, yet weaker associations with pBMI (Figs. 1, 2, 3, 4).

Maternal prepregnancy BMI

For pBMI, none of the maternal metabolites were significant after correction for multiple testing. All BCAA, SM32:2, 34:2, PCaa38:4 and alpha-aminoadipic acid (AAA) showed positive trends with pBMI, while PCae36:1 showed a negative trend. Also, scatterplot inspection revealed positive trends for AC3:0, 4:0, 5:0, and 9:0 (Fig. 1).

In cord blood, BCAA behaved similarly except Val, while Cys showed a negative trend. Similarly, β-hydroxybutyric acid (BHBA), was positively associated with pBMI concomitant with a negative tendency for α-ketoglutaric acid. There were positive tendencies with NEFA22:4 and ΣPCaa/ΣPCae ratio with pBMI, the latter due to reduced PCae levels, while Σ(LPCa18:1 + LPCa18:2)/ΣPCaa, an anti-inflammatory biomarker, showed a negative tendency (Fig. 2).

Maternal gestational diabetes mellitus

H1 (about 90–95% glucose, 5% other hexoses) was significantly higher in GDM in both maternal and, especially, cord blood (Figs. 3, 4). In both maternal and cord blood, Asn/Asp and Gln/Glu ratios were elevated in GDM subjects, with decrease in Asp and Glu. Another common finding is the overall decrease in the majority of phospholipids (PL). Most maternal LPCs and PCaa were decreased in GDM mothers, with strongly significant associations for LPC16:0, PCaa38:3,38:5, and some SM, especially SM32.2. PCae were not affected by GDM, thus resulting in a negative association for ΣPCaa/ΣPCae ratio.

In cord blood, LPC concentrations showed no difference with maternal GDM status, while there was a tendency for PCae and some PCaa to be decreased in GDM babies (PCae38:0 being significant after FDR correction). Interestingly, Carn was significantly lower in GDM babies with the same tendency for all AC, especially short-chain AC and acetyl carnitine. Markers for CPT1 activity and β-oxidation were higher and lower, respectively, in GDM babies. Also, an overall decrease in cord blood long-chain NEFAs and Krebs metabolites was observed in association with GDM,

Table 1 Demographics characteristics of study participants

	Healthy, normal weight ($n=67$)	Healthy, overweight/obese ($n=50$)	Gestational diabetes (GDM) ($n=45$)	p value
(A) Maternal samples				
Maternal age (years)	31.00 ± 6.00	31.00 ± 4.75	34.00 ± 6.00	0.003***
Prepregnancy BMI (kg/m^2)	21.87 ± 2.66	28.83 ± 4.31	26.29 ± 8.60	< 0.001****
GWG (34 weeks) (kg)	12.50 ± 4.47 [1]	9.20 ± 7.90 [1]	6.20 ± 9.60	< 0.001****
Gestational age (weeks)	39.00 ± 1.00	40.00 ± 2.00 [1]	39.00 ± 2.00 [4]	0.067*
Smoking—no	51 (85%) [7]	41 (91%) [5]	31 (91%) [11]	0.623
Mode of delivery—elective C-section	10 (15%)	17 (34%)	20 (44%)	0.002***
Fetal sex—female	33 (50%)	26 (52%)	20 (44%)	0.764
Parity—nulliparous	12 (46%) [41]	13 (54%) [26]	11 (50%) [23]	0.955
Maternal glucose(mg/dl)	76.00 ± 21.50	84.00 ± 30.75	90.00 ± 38.50 [3]	0.001****
Maternal triglycerides (mg/dl)	213.00 ± 93.00	232.00 ± 94.00	205.00 ± 76.25 [3]	0.257
Maternal LDL cholesterol (mg/dl)	139.00 ± 44.50	141.50 ± 51.50	115.00 ± 63.00 [4]	0.031**
Maternal HDL cholesterol (mg/dl)	69.00 ± 24.00	67.50 ± 20.00	65.00 ± 19.00 [4]	0.032**
Fetal (cord) glucose (mg/dl)	72.00 ± 23.00 [38]	67.00 ± 28.00 [19]	73.00 ± 23.00 [22]	0.12
Fetal (cord) triglycerides (mg/dl)	43.00 ± 14.25 [37]	43.00 ± 27.00 [19]	48.50 ± 17.50 [21]	0.867
Fetal (cord) LDL cholesterol (mg/dl)	30.50 ± 16.75 [37]	27.00 ± 9.50 [19]	25.00 ± 10.00 [22]	0.212
Fetal (cord) HDL cholesterol (mg/dl)	29.00 ± 18.25 [37]	26 ± 9.00 [19]	27.00 ± 8.50 [22]	0.364
	Healthy, normal weight ($n=49$)	Healthy, overweight/obese ($n=40$)	GDM ($n=27$)	p value
(B) Cord blood samples				
Maternal age (years)	31.00 ± 6.00	31.50 ± 4.50	35.00 ± 6.50	0.002***
Prepregnancy BMI (kg/m^2)	21.87 ± 2.19	30.11 ± 5.34	26.64 ± 8.40	< 0.001****
GWG (34 weeks) (kg)	12.00 ± 3.90	8.10 ± 7.65 [1]	4.70 ± 10.55	< 0.001****
Gestational age (weeks)	39.00 ± 2.00	40.00 ± 2.00 [1]	39.00 ± 2.00 [1]	0.005***
Smoking—no	38 (84%) [4]	32 (86%) [3]	18 (90%) [7]	0.932
Mode of delivery—elective C-section	6 (12%)	16 (40%)	11 (41%)	0.003***
Fetal sex—female	27 (55%)	20 (50%)	11 (41%)	0.497
Parity—nulliparous	11 (50%) [27]	11 (50%) [18]	7 (50%) [13]	1
Maternal glucose (mg/dl)	80.00 ± 25.50 [2]	87.00 ± 32.50 [5]	105.00 ± 44.00 [2]	0.026**
Maternal triglycerides (mg/dl)	217.00 ± 108.50 [2]	231.00 ± 84.00 [5]	208.00 ± 68.00 [2]	0.47
Maternal LDL cholesterol (mg/dl)	139.00 ± 52.50 [2]	144.00 ± 49.50 [5]	111.50 ± 30.00 [3]	0.009***
Maternal HDL cholesterol (mg/dl)	68.00 ± 27.50 [2]	64.00 ± 20.00 [5]	59.50 ± 23.25 [3]	0.146
Fetal (cord) glucose (mg/dl)	73.00 ± 30.00 [20]	63.00 ± 29.00 [9]	77.00 ± 31.00 [10]	0.069*
Fetal (cord) triglycerides (mg/dl)	42.00 ± 16.50 [19]	45.00 ± 28.50 [9]	47.00 ± 22.00 [9]	0.957
Fetal (cord) LDL cholesterol (mg/dl)	28.00 ± 16.50 [19]	29.00 ± 8.75 [10]	27.00 ± 9.00 [10]	0.553
Fetal (cord) HDL cholesterol (mg/dl)	29.00 ± 20.25 [19]	25.50 ± 10.00 [10]	25.00 ± 14.00 [10]	0.265

Values are expressed in 'median ± interquartile range' or 'absolute number (percentage)'. Numbers in square brackets indicate the numbers of missing observations. p values refer to Kruskal–Wallis test (for continuous covariates) or Chi square test (for categorical covariates)

****$p < 0.001$, ***$p < 0.01$, **$p < 0.05$, *$p < 0.1$

most notably NEFA26:1, malic, and succinic acids with an elevation of 3-methyl-2-oxobutanoic acid.

Similar and contrasting associations in GDM and obesity

In cord blood, Asn/Asp ratio was positively associated with pBMI and elevated (though not statistically significant) in GDM subjects. Ile was positively associated with pBMI and negatively with GDM.

Some maternal PCaa and SM were slightly elevated in overweight/obese and significantly reduced in GDM mothers.

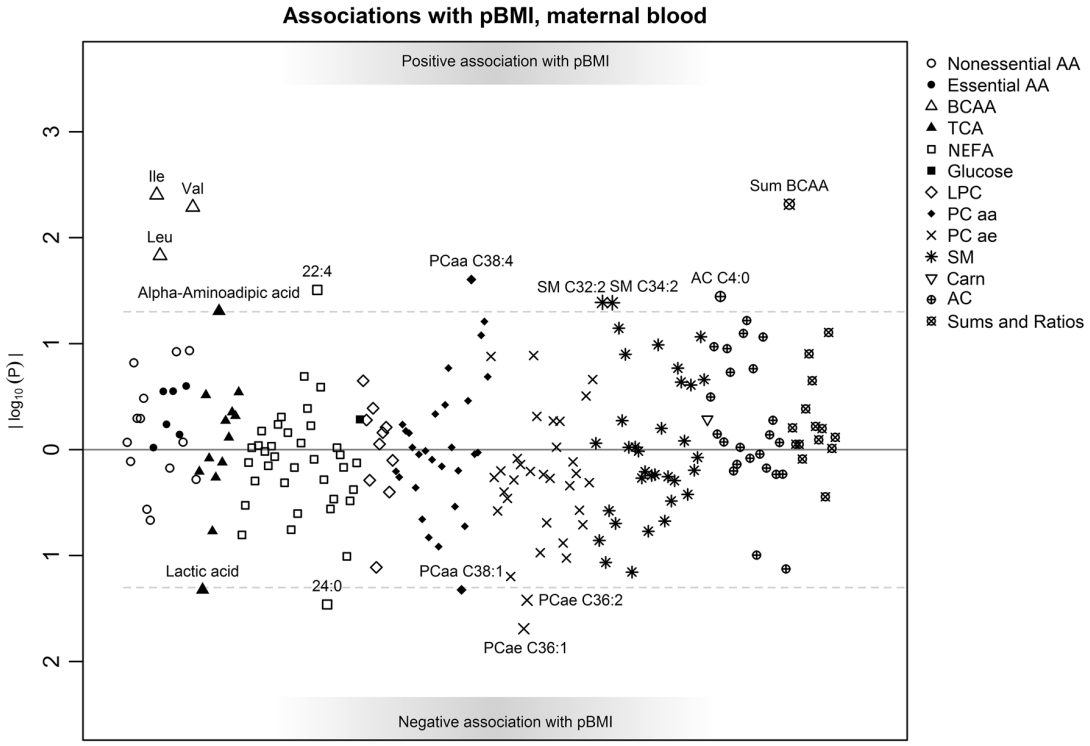

Fig. 1 Manhattan plot showing association of metabolites in maternal plasma with maternal prepregnancy BMI (pBMI). The *y*-axis represents the log10 *p* value of pBMI; the direction (positive or negative) corresponds to the sign of the estimate for pBMI. *p* value and estimate were calculated in the linear model with the log2 metabolite concentration as dependent variable and log2 pBMI, GDM, GWG at 34 weeks, fetal sex and mode of delivery as independent variables. The dashed lines represent the uncorrected 0.05 significance level. *AA* amino acid, *AC* acylcarnitines, *BCAA* branched chain amino acid, *H1* sum of hexoses, *LPCa* acyl-lysophosphatidylcholines, *LPCe* alkyl–lysophosphatidylcholines, *NEFA* non-esterified fatty acid, *PCaa* diacyl-phosphatidylcholines, *PCae* acyl-alkyl-phosphatidylcholines, *TCA* tricarboxylic acid, *SM* sphingomyelines

Discussion

Influence of obesity on maternal and cord blood metabolome

In high pBMI, the most characteristic common features between maternal and cord blood were the elevated BCAA levels, which are essential AA promoting protein synthesis and turnover and glucose metabolism. In cord blood, BCAA are transplacentally transported from maternal to fetal side to be utilized for fetal growth and protein metabolism, and their transport is highly enhanced in late pregnancy to provide for the increased fetal nutrient demands [18]. This reflects other observations linking elevated BCAA and their metabolic by-products to obesity and metabolic syndrome [19]. Explanations were given showing BCAA as a consequence of obesity, others as a cause. Some authors attributed elevated BCAA levels to increased dietary protein intake, or excessive protein breakdown in skeletal muscle due to obesity-related IR [20]. Another hypothesis involves downregulation of BCAA oxidation enzymes, especially those involved in first pass BCAA metabolism, and was supported by animal and human studies [20]. There is also a speculation about role of gut microbiome in BCAA de-novo synthesis, thus contributing to their circulating levels [20].

We hypothesize that increased lipid availability associated with obesity favours FAO for satisfying energy needs. This reduces catabolism of other fuels as AA and glucose, sparing them for transfer to the fetus for fetal growth, and consequently leads to BCAA accumulation in maternal blood [21]. However, such an interpretation fails to explain the encountered elevated AC3:0 and AC5:0 levels, end products of BCAA catabolism, as well as the elevated AC4:0 and AC9:0, which were in agreement with previous reports from multiple cohorts of obese and IR subjects [19, 20].

In overweight/obese women, elevated levels of maternal hormones such as leptin, insulin, and IL-6 were discovered to play a key role in activating the mammalian target of rapamycin complex-1 (mTORC1) signalling and AA transporter activity [22], causing enhanced fetal growth. Moreover, evidence was found that placental BCAA uptake is further

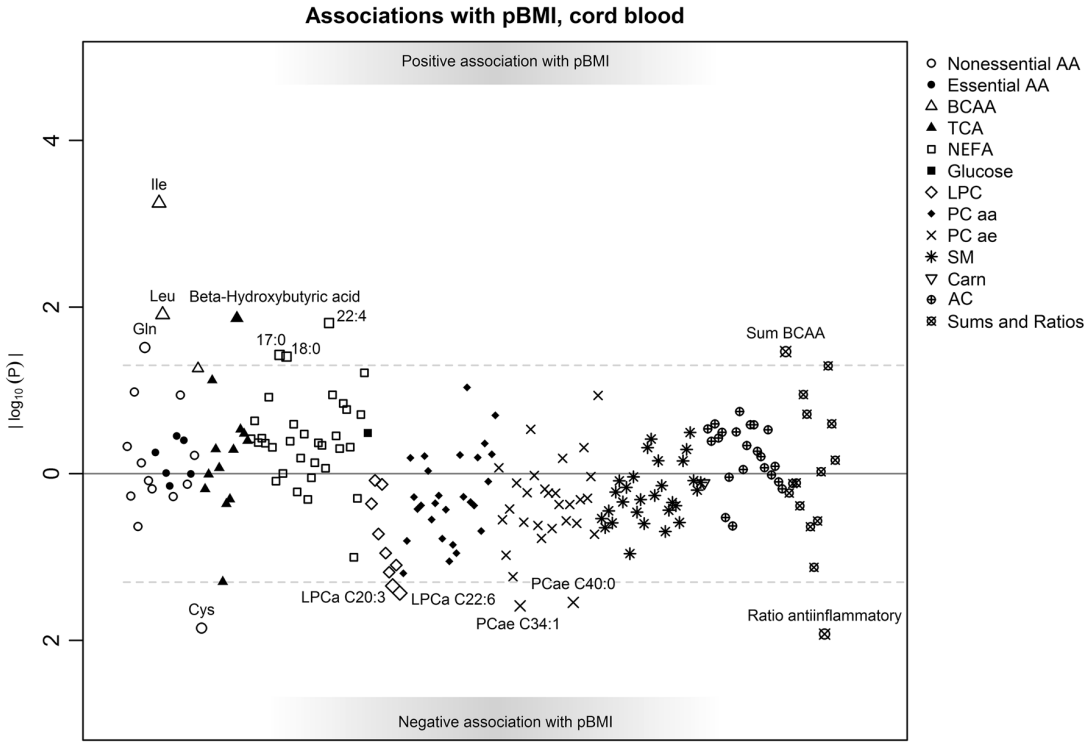

Fig. 2 Manhattan plot showing association of cord blood metabolites with maternal pBMI. For abbreviations and explanation of the plot, see Fig. 1 legend

increased in pregnancies with enhanced maternal ketogenesis and β-oxidation rate [23].

In addition, we confirmed associations between pBMI and metabolites commonly related to inflammation, such as alpha-aminoadipic acid (AAA), LPC, and Cys. Low-grade inflammation, a common finding in obesity and diabetes, happens when excessive fat accumulation induces the release of adipokines which, in turn, lead to reactive oxygen species (ROS) production [24]. In maternal blood, mothers with high pBMI showed elevated AAA levels, whose excessive production was suggested to result from increased breakdown of Lys through oxidative stress and ROS [25]. AAA has also been identified as a potential modulator of glucose homeostasis in humans and an important factor mediating central obesity and diabetes [26]. In line with this picture, cord blood Σ(LPCa18:1 + LPCa18:2)/ΣPCaa levels, an anti-inflammatory marker, negatively associated with maternal pBMI [15]. Decreased LPCa18:1 and LPCa18:2 levels were previously associated with obesity and obesity-related factors [11, 27]. It is believed that not only the absolute concentration, but also the LPC acyl composition could be linked to inflammation, obesity, and atherogenesis, with a higher saturated-to-unsaturated LPC ratio being observed in inflammation [28]. We focused on four LPC metabolites (LPC containing 16:0, 18:0, 18:1, and 18:2 FA) and their ratios to PC because they were demonstrated to be among the most abundant serum metabolites [28]. The anti-inflammatory effect of LPC18:1 may be linked to decreased superoxide production and platelet aggregation [29] and was previously reported to be decreased among other unsaturated LPC in studies involving obese versus normal subjects [27]. Cord blood Cys was reduced suggesting that the existing oxidative stress drives Cys towards glutathione biosynthesis to fight ROS, thus enhancing its consumption and decreasing its cord blood levels [28].

Interestingly, maternal SM32:2 showed a weak positive trend with pBMI. Our group found the same tendency in another cohort of pregnant women [30] as well as a strong positive association with BMI and waist circumference in young adults [11] and children (unpublished results).

BHBA, a ketone body produced from the oxidation of fatty acids and utilized as an energy source particularly in the neonatal period and under starving conditions, was elevated only in cord blood in association with pBMI. Ketone bodies produced in maternal circulation are readily transported to the fetus as a source of energy. There are reports supporting free flow of ketone bodies from maternal to fetal circulation [31].

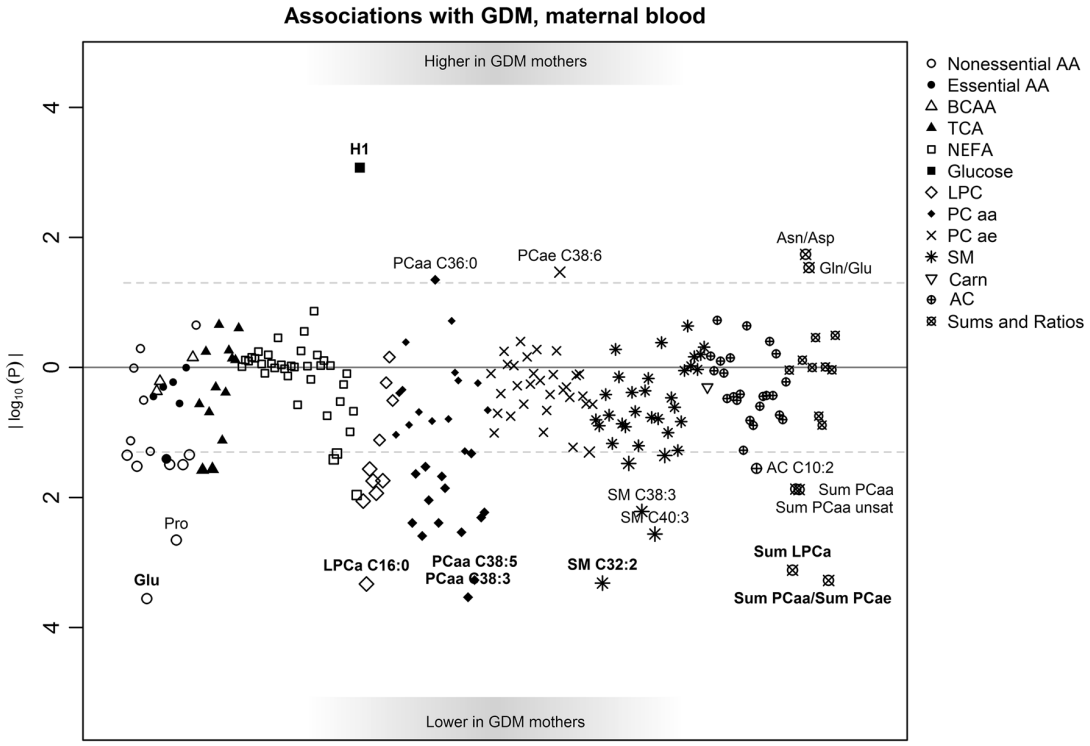

Fig. 3 Manhattan plot showing association of metabolites in maternal plasma with gestational diabetes mellitus (GDM). The *y*-axis represents the log10 *p* value of GDM; the direction (positive or negative) corresponds to the sign of the estimate for GDM. For abbreviations and explanation of the plot, see Fig. 1 legend. Associations significant after FDR correction are marked with bold names for the metabolite

Influence of GDM on maternal and cord blood metabolome

High pBMI and GDM share some common associations with some metabolites in maternal/cord blood which can be explained on the same basis, since obesity also presents increased IR and altered glucose tolerance, typical of GDM [8]. Trends for higher Asn/Asp levels and lower levels of some Krebs cycle metabolites were encountered in cord blood, which agrees with previous reports on these metabolites in association with IR and hyperglycaemia during pregnancy and reduced use of these metabolites for Krebs cycle replenishment [20, 32].

In GDM, we found common metabolite associations in both maternal and cord blood. Expectedly, H1 was increased reflecting maternal hyperglycaemia and enhanced transplacental transfer of glucose. Glucose is transported down its concentration gradient from maternal to fetal circulation through "GLUT1-mediated transport" or facilitated glucose transporters in basal membrane (BM) of the syncytiotrophoblast (STB) epithelium of the human term placenta [33]. Increased GLUT1 expression and activity in the BM of STB in GDM women either treated with diet alone or with insulin therapy were reported [34]. This may lead to fetal hyperglycemia and hyperinsulinemia and was proposed as a mechanism causing fetal overgrowth in GDM [34]. Another important common finding is overall trend of reduced PL levels, especially maternal LPC and PCaa. Reduced synthesis of PC and, in turn, LPC, might explain this finding being a result of reduced hepatic de-novo synthesis of PC from phosphoethanolamines (PE) and *S*-adenosylmethionine (SAM), which is catalysed by phosphatidyl–ethanolamine methyl transferase (PEMT). To our understanding, the existing oxidative stress associated with GDM drives Cys metabolism towards glutathione biosynthesis as a defensive mechanism leading to a decrease in SAM and thus decreased PC and LPC [28, 35]. Decreased synthesis of PC, a major PL in the very low-density lipoprotein (VLDL) causes fat and cholesterol accumulation in the liver (hepatic steatosis) [35, 36]. These hypotheses, however, apply only to PCaa, since PCae did not behave similarly, therefore ΣPCaa/ΣPCae ratio was decreased in GDM mothers. This could be explained by considering that ether lipids represent only a small portion of the total PL (in our study, the total PCae concentration is < 10% of PCaa), and their intracellular levels are quite low in the liver [37], so their decreased synthesis might be too small to be detected.

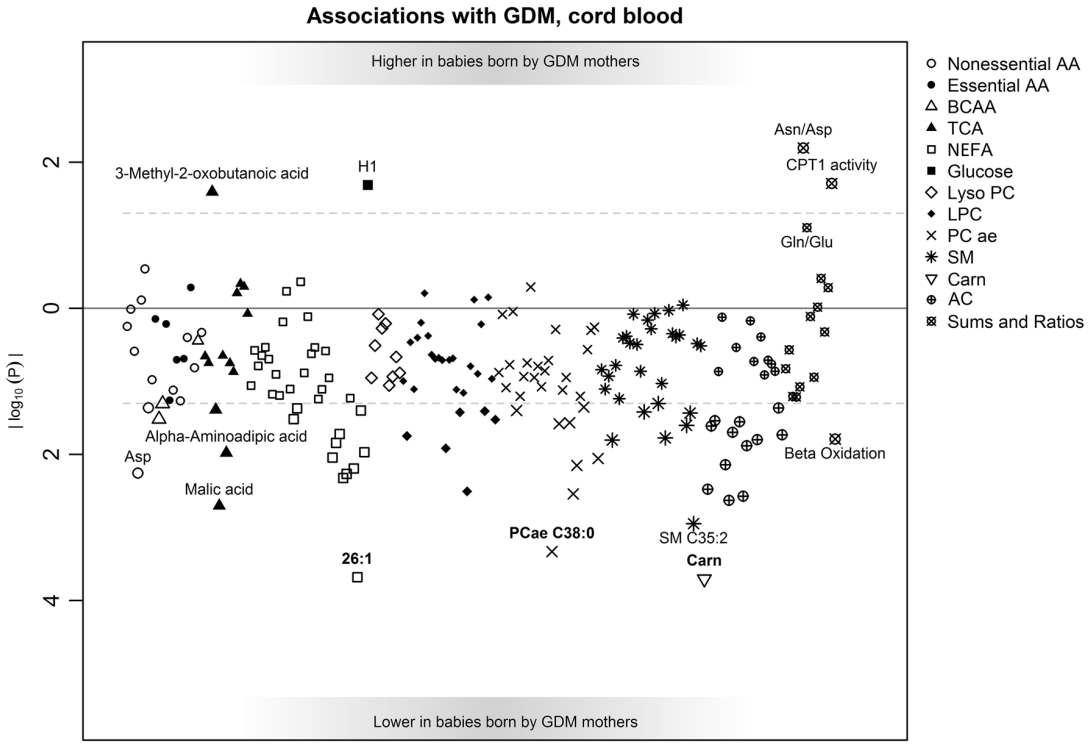

Fig. 4 Manhattan plot showing association of metabolites in cord blood with GDM. For abbreviations and explanation of the plot, see Fig. 1 legend. Associations significant after FDR correction are marked with bold names for the metabolite

Trends for negative association with SM might be explained by either reduced synthesis [20] or increased breakdown [38]. The reduced de-novo synthesis was explained by redirection of phosphatidic acid to triglycerides (TG) rather than PL synthesis [28]. As for the increased breakdown, it was reported that GDM is associated with increased breakdown of SM resulting in ceramides, which induce inflammation and β-cell apoptosis and are negatively correlated with insulin sensitivity [28, 38].

Only in cord blood, we observed a significant decrease in Carn levels and an overall decrease in AC levels. There were different reports on Carn levels. One study reported low Carn in children with T1D [39]. In GDM pregnancies, data on Carn are scarce, but increased Carn levels in GDM mothers [40] and their offspring have been reported without clear explanation [41].This correlation of fetal and maternal Carn levels was explained due to the fetus's inability to synthetize Carn and its reliance on placental transfer [41]. Thus, one would expect that low fetal Carn would be secondary to low maternal levels. However, we found decreased fetal Carn with no differences for maternal Carn in the GDM group, along with a decrease in long-chain NEFA and fatty acid oxidation (FAO), as reflected in increased AC16:0/Carn and decreased AC2:0/AC16:0 ratios. A plausible explanation would be the reduced transport of Carn, and thus reduced initiation of FAO, as a consequence of fetal hyperglycemia. This matches the previously reported reduction in FAO, concomitant with elevated TG formation, in placentae of GDM mothers [42]. Thus, the whole picture suggests that GDM is associated with reduced placental transport of NEFAs and Carn along with incomplete or reduced FAO, which was reported in IR and diabetes [43].

Maternal blood lactic acid tended to be lower in GDM, although one might expect an elevation with increased IR, typical for GDM [44]. A plausible explanation would be the liver's ability to utilize circulating lactate for reconversion to glucose through the Cori cycle [45].

The proposed mechanisms of metabolic alterations associated with obesity and GDM are depicted in Suppl. Fig. S1. Results from linear models for all investigated metabolites are listed in Suppl. Table S2.

Conclusion

Our study investigated the association of > 200 intermediates of energy metabolic pathways in maternal and cord blood with maternal BMI and GDM status. We observed consistent associations of BMI with BCAA as well as pronounced

association of several markers related to inflammation and β-oxidation with GDM.

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Compliance with ethical standards

Conflict of interest None of the authors reports conflicts of interest.

Ethical approval All procedures followed were in accordance with the ethical standards of the bioethical Committees for clinical research of the Clinical University Hospital San Cecilio, the Mother-Infant University Hospital of Granada, and with the Helsinki Declaration of 1975, as revised in 2008.

Informed consent Written informed consent was obtained from all participants at the study entry.

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Chapter 4

Publication II: Low GI Diet in the ROLO Study

The following pages present the publication L. Marchioro, A. A. Geraghty, O. Uhl, E. Shokry, E. C. O'Brien, B. Koletzko, and F. M. McAuliffe, "Effect of a low glycaemic index diet during pregnancy on maternal and cord blood metabolomic profiles: results from the ROLO randomized controlled trial," *Nutr Metab (Lond)*, vol. 16, p. 59, 2019.

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I contributed to this publication by analysing and interpreting the data, writing, reviewing, editing and approving the manuscript.

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RESEARCH Open Access

Effect of a low glycaemic index diet during pregnancy on maternal and cord blood metabolomic profiles: results from the ROLO randomized controlled trial

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Abstract

Background: Elevated post-prandial blood glucose during pregnancy has been associated with adverse pregnancy and offspring outcomes, such as maternal gestational diabetes and excessive foetal growth. The ROLO Study is a randomized controlled trial (RCT) investigating the effect of a low glycaemic index (GI) diet in pregnancy to prevent foetal macrosomia (birth weight > 4000 g). We described the impact of a low-GI diet on the maternal and feto-placental unit metabolism by studying how the ROLO intervention affected maternal and cord blood metabolomes.

Methods: Fasting maternal plasma samples pre- and post-intervention of 51 pregnant women and 132 cord blood samples were measured with a targeted metabolomics approach using liquid-chromatography coupled to tandem mass spectrometry. The differences between RCT groups were explored via multivariate models with covariates correction. Significance was set at Bonferroni-corrected level of 0.05.

Results: A total of 262 metabolites species, sums and ratios were investigated. While no metabolite reached statistical significance after Bonferroni correction, many maternal phospholipids and acylcarnitines were elevated in the intervention group at uncorrected 0.05 alpha level. Most species contained saturated and monounsaturated fatty acid chains with 16 or 18 carbon atoms. In cord blood, no differences were identified between RCT groups.

Conclusions: A low-GI diet in pregnancy was associated with a trend to modest but consistent changes in maternal lipid and fatty acid metabolism. The intervention seemed not to affect foetal metabolism. Our exploratory findings may be used to direct further investigations about low GI diets before and during pregnancy, to improve patient care for pre-conceptional and pregnant women with lipid dysregulations and potentially modulate the offspring's risk for future metabolic diseases.

Trial registration: Current Controlled Trials ISRCTN54392969.

Keywords: Glycaemic index, Macrosomia, Pregnancy, Dietary intervention, Metabolomics, Maternal blood, Cord blood, Lipid metabolism, Fatty acid metabolism

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Background

Pregnancy is a period of major endocrine and metabolic changes which modulate both maternal and child's health [10]. Pregnancy exposures such as gestational diabetes mellitus (GDM), elevated maternal pre-pregnancy body-mass-index (BMI) and gestational weight gain (GWG) are risk factors for type 2 diabetes, overweight, and metabolic syndrome not only in the mother [3, 10, 27] but also in the offspring, as suggested by the numerous indications for the Developmental Origins of Health and Disease (DOHaD) hypothesis [12, 24]. Therefore, it is important to understand the mechanisms driving these changes and to build the foundation for acting timely to prevent the onset of disease in mothers and children.

One possible intervention strategy involves targeting maternal blood glucose levels. Elevated fasting and postprandial blood glucose levels, even in absence of overt pre-existing diabetes or GDM, have been associated with adverse outcomes for mother and child [33]. Nutritional and dietary measures to ameliorate glycaemic control are standard in pregnant women with diabetes and GDM [21]. One dietary parameter of interest is the glycaemic index (GI). The GI of a carbohydrate-containing food, expressed on a scale from 0 to 100, quantifies the peak in the blood sugar concentrations after ingestion of the food [25]; therefore, the consumption of low GI foods is considered desirable to achieve good glycaemic control.

Several randomized control trials to investigate the effect of low-GI diet on maternal and new-born outcomes have been conducted, reporting favourable effects on maternal glycaemic control but heterogeneous results regarding offspring outcomes [48]. In particular, there is a lack of knowledge regarding how a low-GI diet may impact maternal and feto-placental metabolism at a molecular level in a real-environment clinical setting. In this study, we aim to provide insights into this question using a metabolomics approach. Metabolomics is the omics branch investigating small (< 1.5 kDa) intermediates and products of metabolic reactions and is an established tool in metabolism research, with potential applications in precision medicine and personalized patient care [2, 23, 37]. Ultimately, this exploratory study could inform clinical practise on treatments for pregnant women, aimed at increasing maternal wellbeing and decreasing the offspring's risk for future metabolic conditions.

Materials and methods

Study participants and data collection

This was a secondary analysis conducted on data from the ROLO study. The ROLO study (Randomised cOntrol trial of LOw glycaemic index diet versus no dietary intervention to prevent recurrence of foetal macrosomia, 2007–2011, Dublin, Ireland) tested the hypothesis of a low-GI diet in pregnant women to reduce birth weight in secundigravida with a previous macrosomic child (birth weight > 4000 g); the intervention group (n = 394) received an educational session about low-GI diet at the beginning of the second trimester, while the standard group (n = 406) received standard care only (trial registration: Current Controlled Trials ISRCTN54392969) [46].

Recruitment and the first study visit took place at the end of the first pregnancy trimester (median: 13th gestation week) and rapidly followed by the educational session (median: 15th week); additional visits were held at 28th and 34th weeks of gestation.

Maternal age at delivery, early pregnancy weight and BMI, weight at 34th week, gestational age at delivery, newborn's sex, weight and length were documented. Gestational weight gain (GWG) was defined as weight at last measured visit (38th or 40th gestational week) after subtraction of early pregnancy weight; for cases with missing weight at 38th or 40th week, GWG was imputed by adding the overall ROLO median GWG between 34th and 38th week to the weight measured at 34th week. Newborn's ponderal index at birth was calculated as 100 · birth weight (g) / birth length^3 (cm^3).

Maternal fasting blood samples were collected at recruitment and again at the 28th week. Cord blood was collected at delivery. Total, HDL and LDL cholesterol were measured via Roche cholesterol oxidase method and direct HDL Roche 3rd generation method, respectively, on the cobas C702 module of the Roche Cobas 8000 analyser (Roche Diagnostics GmbH, Penzberg, Germany); the Friedewald equation was used to estimate LDL-cholesterol concentrations [7].

Three-days food diaries were collected in each pregnancy trimester and evaluated by a research dietitian via WISP software version 3.0 (Tinuviel Software, Llanfechell, UK) [32]. From these data, the absolute GI intake and the proportion of energy derived from saturated, monounsaturated and polyunsaturated fat intake, expressed as percentage of total energy intake (% kcal), were derived.

Metabolomics measurements

For subgroups of evaluable mother/child pairs, aliquots of the collected samples were provided for metabolomics analysis. Plasma samples were measured in a targeted approach using liquid chromatography coupled to tandem mass spectrometry (LC/MS-MS) in the laboratory of the Division of Metabolic and Nutritional Medicine, Dr. von Hauner Children's Hospital (LMU Munich). Five classes of metabolites were analysed: amino acids (AA), non-esterified fatty acids (NEFA), acylcarnitines (AC), branched chain keto acids (BCKA) and intermediates of TCA cycle (TCA), and phospholipids (PL) (including sphingomyelins (SM), diacyl-phosphatidylcholines (PCaa), acyl-alkyl-phosphatidylcholines (PCae) and lysophosphatidylcholines (LPC)). After preparation, samples were

randomly distributed in 4 96-wells batches with maternal blood (1–4) and 3 batches with cord blood (5–7). In each batch, up to 80 test samples were measured together with 6 quality control (QC) samples (prepared as pooled mixture of the samples from batch 1, for maternal blood, or from batch 5, for cord blood) and 10 standards used for quantification. The injection of the samples was randomized in each run, with QC and standards being injected regularly every 6–7 test samples. Measurements and QC were performed separately for each blood source.

Samples preparation

Proteins of 50 μL plasma were precipitated on a plate with PTFE filter elements by adding 450 μL methanol including internal standards (ISD). After centrifugation the filtrate was split into aliquots for the analyses of individual methods.

Amino acids

Fifty μL of the filtrate was used for the derivatization to AA butyl ester with hydrochloric acid in 1-butanol according to the method described by Harder et al. [15]. A set of labeled amino acid standards (set A, Cambridge Isotope Laboratories) mixed with L-Asparagine (15 N2, 98%, Cambridge Isotope Laboratories) and L-Tryptophan (Indole-D5, 98%, Cambridge Isotope Laboratories) was used as internal standard (ISD). After evaporation, the residues were dissolved in water/methanol (80:20) with 0.1% formic acid and determined by LC-MS/MS equipped with 150 × 2.1 mm, 3.5 μm particle size C18 HPLC column (X-Bridge, Waters, Milford, USA) and 0.1% heptafluorobutyric acid as ion pair reagent in mobile phase A (water) and B (methanol). MS detection was performed with a triple quadrupole mass spectrometer (API2000, Sciex, Darmstadt, Germany) with atmospheric pressure chemical ionization source (APCI) operating in positive ion ionization mode.

NEFA

Fifty μL of the filtrate was diluted with100 μL methanol and injected to a LC-MS/MS operating in negative electrospray ionization (ESI) mode for identification of NEFA as described by Hellmuth et al. [16]. Uniformly 13C-labeled palmitic acid was used as ISD. Samples were injected to an HPLC system (1200, Agilent, Waldbronn, Germany) with a UPLC diphenyl column (Pursuit UPS Diphenyl, Agilent, Waldbronn, Germany). Five mM ammonium acetate and 2.1 mM acetic acid in water were used as mobile phase A and acetonitrile/isopropanol (80/20) as mobile phase B. A hybrid triple quadrupole mass spectrometer (4000 QTRAP, Sciex, Darmstadt, Germany) operating in negative ESI multiple reaction monitoring mode (MRM) mode was used for MS detection. This method allows for the separation of NEFA species differing in chain length and number of double bonds, but not in the position of double bonds. The analytical process was post-processed using Analyst software version 1.6.2.

BCKA and TCA

Organic and keto-acids were measured by a modified method based on previously published procedures [4, 30]. D3-methylmalonic acid (Cambridge Isotope Laboratories, Teweeksbury, MA, USA) was used as ISD. One hundred μL of the supernatant were evaporated to dryness and resuspended in 50 μL water. Five μL of the extracted samples were injected by HPLC system (1200, Agilent, Waldbronn, Germany) on a Kinetex F5 core-shell HPLC column, 150 × 2.1 mm, 2.6 μm particle size (Kinetex F5, Phenomenex, Aschaffenburg, Germany) for chromatographic separation of molecular species. The mobile phase A was water with 1% formic acid and mobile phase B was composed of methanol/isopropanol (50/50) with 1% formic acid. A gradient elution at a flow rate of 250 μL/min was held constant for 1 min with 1% B, raised to 65% B within 6 min, and turned back to initial conditions of 1%B within 0.5 min. The triple quadrupole mass spectrometer (4000QTRAP, Sciex, Darmstadt, Germany) was operated in negative scheduled MRM mode using ESI.

Phospholipids

Phospholipids were analyzed as described by Uhl et al. [45] using LPC (13:0) and PC (14:0/14:0) (Avanti Polar Lipids, Alabaster, Alabama, USA) as ISD. Thirty μL of the centrifuged supernatant were mixed for 20 min at 600 rpm with 500 μl methanol containing 1.2 mM ammonium acetate. Phospholipids were analyzed by flow-injection analysis (FIA) in a triple quadrupole mass spectrometer (QTRAP4000, Sciex, Darmstadt, Germany) coupled to a LC system (1200 Agilent, Waldbronn, Germany). ESI was used in positive ionization mode. MS/MS analysis was run in positive MRM mode with 184 Da (choline head group) as product ion for the PL. Analyst 1.6.2 software, followed by in-house processing with the statistical software R [44], was used for post-processing. The number of carbon atoms (XX) and double bonds (Y) is expressed in the form C XX:Y.

Acylcarnitines

D3-carnitine-C2, D3-carnitine-C8 and D3-carnitine-C16 (all Cambridge Isotope Laboratories, Teweeksbury, MA, USA) were used as ISDs. FIA with isocratic elution with 76% isopropanol, 19% methanol and 5% water was used to measure acylcarnitines. The mass spectrometer (4000 QTRAP, Sciex, Darmstadt, Germany) was equipped with ESI and operated in positive ionization mode.

Quality control

To ensure precision of the measured samples, 6 QC samples, pooled from the test samples, were measured in each batch. Batches with a coefficient of variation (CV) > 25% were excluded. If at least 75% of the batches for a metabolite passed the intra-batch quality control, the inter-batch CV was calculated, and the metabolite was kept if CV < 30%. In each batch, at most one QC sample was allowed to be an outlier (defined as measurement further away than 1.5 interquartile range (IQR) from the next measurement) and removed.

After quality control, 6 sums and ratios were additionally calculated: sums of PCaa, PCae, total PC, total SM, ratio of total SM to total PC, ratios of NEFA 18:1/18:0 and 16:1/16:0 depicting SCD-1 activity [6], and five ratios of AC 2:0 to mid-chain AC (AC 14:0, 16:0, 16:1, 18:0, 18:1) depicting fatty acid oxidation (FAO) [29].

Statistical treatment

Data preparation

QC and statistical treatment of the data were performed using the statistical software R version 3.4.3 [44].

To ensure interpretability of the results, only subjects with covariates information, mothers with longitudinal metabolomics data (full set analysis) and babies born after the 37th gestational weeks were included. The final sample sizes for maternal and cord analyses were thus 51 and 132 subjects, respectively. Metabolomics outliers identification and removal was performed before models calculation within each blood source and visit time point; outliers were defined as concentration values further away than 3 standard deviations from the next measurements.

Covariables are presented descriptively as median (IQR) or as absolute number (percentage), stratified by blood source and RCT arms. Variables were compared in the two RCT arms using Mann Whitney U-tests.

Main models

For each metabolite, a generalized additive model (GAM) was calculated using the function gam() from the R package mgcv [47]. In the following notations, s(·) indicates a non-linear effect and 1|· the random intercept.

The models for maternal metabolites were calculated as follows: metabolite at 28 weeks ~ RCT group + maternal BMI + metabolite at 13 weeks + s (sample storage time) + 1|batch number. Full results are presented in Additional file 1. Maternal age was included in a first step, but since preliminary results showed weak to no associations with maternal age, the variable was removed to preserve statistical power. For some metabolites of interest, a sensitivity analysis was conducted by re-calculating the models after trimming the highest and lowest 5 concentration values. Additional univariate and multivariate sensitivity analyses (including the association of selected metabolites with dietary fat intakes) and their results are presented in Additional file 2.

The models for cord metabolites were calculated as follows: metabolite ~ RCT group + maternal BMI + gestational age + foetal sex + s (sample storage time) + 1|batch number. As sensitivity analysis, the following covariates were included one at a time in the model: ponderal index (PI) of the new-born, maternal GWG, cord HDL, LDL and total cholesterol. Since the results did not substantially change, these are not presented. Additionally, the calculation of the main model was repeated by including only those maternal/child dyads for which also maternal blood was analysed.

Significance and reported values

From these models, the standardized beta estimates, uncorrected and Bonferroni-corrected *p*-values and 95% confidence interval of the beta estimate for the RCT variable are reported. Associations with Bonferroni-corrected p-values < 0.05 were defined as 'significant', associations with uncorrected p-values < 0.05 were defined as 'trends'. False discovery rate (FDR) p-values correction was also applied, but, since the significant metabolites did not differ between the two approaches, we used only Bonferroni due to its easier interpretation. Metabolites with uncorrected RCT p-value < 0.05 were visually inspected via grouped boxplots. Results of these models are presented in graphical form via Manhattan plots.

Results

Covariates

The covariates, stratified for the two subpopulations (maternal blood samples/cord blood samples), are presented in Table 1. Maternal samples from 51 mothers (control/intervention: 26/25) and 132 cord blood samples (68/64) were included in the analysis; both maternal and cord blood were analysed for 48 mother/child dyads. Only GI in trimester 2 (i.e. after the intervention) and gestational age were significantly different between the RCT arms.

Metabolites

Two hundred twenty-nine analytes were used in the analyses for maternal blood, 197 in cord blood. A total of 257 analytes passed the quality control in at least one of the blood sources, 170 of which in both. These were: sum of hexoses (H1), 22 AA, 33 NEFA, 26 AC (including free carnitine), 8 TCA, 2 BCKA, 7 LPC, 24 PCaa, 26 PCae, and 21 SM. In both blood sources it was additionally possible to investigate sums and ratios.

RCT and maternal blood

After Bonferroni correction, no significant differences were found (see Fig. 1 and Additional file 1). However,

Table 1 Demographic and clinical variables of the subjects included in the analyses

	Maternal blood			Cord blood		
	Control group ($n = 26$)	Intervention group ($n = 25$)	*p*-value	Control group ($n = 68$)	Intervention group ($n = 64$)	*p*-value
Maternal anthropometry and blood parameters						
Maternal BMI (kg/m^2)	24.34 ± 4.40	26.36 ± 5.33 [1]	NS	25.38 ± 4.27	26.24 ± 4.36 [1]	NS
Maternal age (years)	33.37 ± 5.73	32.30 ± 6.89	NS	33.00 ± 5.04	32.45 ± 6.31	NS
Gestational weight gain (kg)	13.20 ± 4.06 [7]	12.18 ± 4.74 [7]	NS	13.20 ± 4.59 [13]	12.40 ± 4.69 [16]	NS
Fasting glucose, 13th week (mmol/l)	4.50 ± 0.40 [1]	4.40 ± 0.50	NS	4.50 ± 0.40 [4]	4.50 ± 0.40 [2]	NS
Fasting glucose, 28th week (mmol/l)	4.60 ± 0.42 [2]	4.50 ± 0.30	NS	4.50 ± 0.60 [3]	4.50 ± 0.50 [2]	NS
Total cholesterol, 13th week (mmol/l)	4.77 ± 0.92 [9]	4.81 ± 1.01 [8]	NS	4.76 ± 1.06 [28]	5.25 ± 1.32 [25]	0.072
Total cholesterol, 28th week (mmol/l)	6.31 ± 1.30 [8]	6.39 ± 1.06 [9]	NS	6.16 ± 1.13 [26]	6.46 ± 1.30 [24]	NS
HDL cholesterol, 13th week (mmol/l)	0.80 ± 0.26 [9]	0.93 ± 0.38 [8]	NS	0.78 ± 0.28 [28]	0.87 ± 0.40 [25]	NS
HDL cholesterol, 28th week (mmol/l)	0.97 ± 0.32 [8]	1.04 ± 0.34 [9]	NS	0.93 ± 0.39 [26]	1.02 ± 0.40 [24]	NS
LDL cholesterol, 13th week (mmol/l)	3.35 ± 0.64 [9]	3.45 ± 1.03 [8]	NS	3.34 ± 0.89 [28]	3.64 ± 0.90 [25]	NS
LDL cholesterol, 28th week (mmol/l)	4.71 ± 1.40 [8]	4.62 ± 1.22 [9]	NS	4.26 ± 1.33 [26]	4.75 ± 1.23 [24]	NS
LDL cholesterol, difference 28th - 13th week (mmol/l)	0.83 ± 1.47 [9]	1.06 ± 0.60 [9]	NS	0.98 ± 1.17 [29]	1.01 ± 0.83 [27]	NS
Maternal diet						
Daily energy intake T1 (kcal)	1803.28 ± 357.75 [4]	1873.09 ± 420.48 [5]	NS	1876.03 ± 506.89 [10]	1793.15 ± 467.24 [13]	NS
Daily energy intake T2 (kcal)	1839.30 ± 279.74 [4]	1775.72 ± 532.19 [4]	NS	1932.25 ± 434.53 [10]	1759.49 ± 534.65 [11]	0.085
Daily energy intake T3 (kcal)	1915.07 ± 334.97 [4]	1800.89 ± 450.74 [3]	NS	2022.50 ± 494.67 [10]	1768.26 ± 473.59 [10]	0.027
Daily GI T1	57.10 ± 6.76 [4]	57.09 ± 3.57 [6]	NS	57.13 ± 5.45 [11]	56.98 ± 4.38 [14]	NS
Daily GI T2	58.52 ± 2.99 [4]	55.25 ± 2.95 [6]	0.004	57.66 ± 3.93 [11]	55.65 ± 3.42 [14]	0.001
Daily GI T3	58.60 ± 3.97 [4]	56.61 ± 3.72 [6]	NS	57.76 ± 4.83 [11]	56.15 ± 4.21 [14]	NS
Total fat intake T1 (g)	71.35 ± 22.41 [4]	70.32 ± 26.29 [5]	NS	76.10 ± 22.08 [10]	72.13 ± 25.26 [13]	NS
Saturated fat intake T1 (g)	30.26 ± 11.38 [4]	27.74 ± 11.65 [5]	NS	31.12 ± 10.23 [10]	27.35 ± 14.41 [13]	0.057
Monounsaturated fat intake T1 (g)	22.59 ± 7.08 [4]	23.26 ± 6.53 [5]	NS	23.81 ± 6.42 [10]	23.91 ± 7.22 [13]	NS
Polyunsaturated fat intake T1 (g)	10.87 ± 6.05 [4]	10.73 ± 5.37 [5]	NS	10.87 ± 5.65 [10]	10.80 ± 5.11 [13]	NS
Total fat intake T2 (g)	71.80 ± 13.20 [4]	70.49 ± 26.74 [4]	NS	75.01 ± 24.54 [10]	70.25 ± 25.53 [11]	0.077
Saturated fat intake T2 (g)	26.97 ± 10.95 [4]	24.51 ± 8.20 [4]	NS	29.56 ± 10.96 [10]	24.51 ± 11.56 [11]	0.019
Monounsaturated fat intake T2 (g)	21.91 ± 4.80 [4]	22.75 ± 7.53 [4]	NS	23.34 ± 8.38 [10]	22.63 ± 8.73 [11]	NS
Polyunsaturated fat intake T2 (g)	11.39 ± 5.60 [4]	10.79 ± 6.03 [4]	NS	12.23 ± 5.41 [10]	10.55 ± 4.76 [11]	NS
Total fat intake T3 (g)	75.28 ± 22.81 [4]	73.48 ± 30.10 [3]	NS	79.31 ± 22.91 [10]	70.82 ± 29.12 [10]	NS
Saturated fat intake T3 (g)	28.49 ± 10.77 [4]	29.32 ± 18.93 [3]	NS	29.92 ± 12.11 [10]	26.37 ± 13.87 [10]	NS
Monounsaturated fat intake T3 (g)	23.33 ± 6.30 [4]	24.43 ± 8.99 [3]	NS	25.56 ± 7.54 [10]	21.56 ± 10.81 [10]	NS
Polyunsaturated fat intake T3 (g)	10.17 ± 5.23 [4]	13.01 ± 5.69 [3]	NS	12.04 ± 5.32 [10]	11.34 ± 5.36 [10]	NS
Total fat intake T1 (%kcal)	0.37 ± 0.09 [4]	0.34 ± 0.06 [5]	NS	0.38 ± 0.07 [10]	0.35 ± 0.08 [13]	NS
Saturated fat intake T1 (%kcal)	14.76 ± 5.36 [4]	13.49 ± 4.31 [5]	NS	15.16 ± 4.63 [10]	13.55 ± 4.09 [13]	NS
Monounsaturated fat intake T1 (%kcal)	11.68 ± 3.59 [4]	11.20 ± 2.93 [5]	NS	11.76 ± 3.14 [10]	11.34 ± 3.75 [13]	NS
Polyunsaturated fat intake T1 (%kcal)	5.71 ± 2.18 [4]	5.31 ± 1.74 [5]	NS	5.64 ± 1.92 [10]	5.09 ± 2.33 [13]	NS
Total fat intake T2 (%kcal)	0.36 ± 0.06 [4]	0.36 ± 0.06 [4]	NS	0.36 ± 0.07 [10]	0.35 ± 0.06 [11]	NS
Saturated fat intake T2 (%kcal)	13.39 ± 3.91 [4]	12.93 ± 4.72 [4]	NS	13.60 ± 3.87 [10]	12.56 ± 4.36 [11]	NS
Monounsaturated fat intake T2 (%kcal)	11.21 ± 2.81 [4]	10.76 ± 2.78 [4]	NS	11.57 ± 2.56 [10]	10.90 ± 3.14 [11]	NS
Polyunsaturated fat intake T2 (%kcal)	5.63 ± 1.86 [4]	5.93 ± 1.86 [4]	NS	5.57 ± 1.91 [10]	5.48 ± 1.97 [11]	NS
Total fat intake T3 (%kcal)	0.36 ± 0.06 [4]	0.36 ± 0.04 [3]	NS	0.36 ± 0.06 [10]	0.36 ± 0.06 [10]	NS
Saturated fat intake T3 (%kcal)	13.50 ± 4.34 [4]	14.10 ± 4.27 [3]	NS	13.73 ± 4.00 [10]	13.24 ± 3.76 [10]	NS

Table 1 Demographic and clinical variables of the subjects included in the analyses *(Continued)*

	Maternal blood			Cord blood		
	Control group ($n = 26$)	Intervention group ($n = 25$)	*p*-value	Control group ($n = 68$)	Intervention group ($n = 64$)	*p*-value
Monounsaturated fat intake T3 (%kcal)	11.36 ± 2.62 [4]	11.60 ± 2.67 [3]	NS	11.39 ± 2.07 [10]	11.33 ± 2.72 [10]	NS
Polyunsaturated fat intake T3 (%kcal)	5.52 ± 1.91 [4]	5.93 ± 1.61 [3]	NS	5.43 ± 2.07 [10]	5.59 ± 1.63 [10]	NS
New-born anthropometry						
Gestational age (days)	280 ± 10.00 [1]	284 ± 8.00	0.022	282 ± 12.25	284 ± 10.00	0.048
Child sex - female	17 (65%)	11 (44%)	NS	39 (57%)	30 (47%)	NS
Birth weight (g)	3860 ± 460.00	4090 ± 480.00	NS	3995 ± 532.50	4105 ± 550.00	NS
Birth length (cm)	52.00 ± 3.00 [6]	52.75 ± 1.88 [7]	NS	52.50 ± 2.75 [13]	53.00 ± 3.00 [15]	NS
Birth ponderal index (100 g/cm^3)	2.60 ± 0.28 [6]	2.81 ± 0.50 [7]	NS	2.73 ± 0.44 [13]	2.70 ± 0.41 [15]	NS

Values are reported in absolute number (%) or median ± interquartile range. Numbers in square brackets indicate the amount of missing values. *P*-values were calculated via Mann-Whitney U-tests and chi-square tests. *Abbreviations: GI* glycaemic index, *T1/T2/T3* (Pregnancy) Trimester 1/2/3, *NS* non significant

40 metabolites were higher in intervention than control arm at uncorrected 0.05 level (see Table 2): two NEFA (16:1 and 18:1), eight mid-chain AC (with chain length from 8 to 18 carbon atoms), three LPC (with chain lengths of 16 and 18 carbon atoms), 15 PCaa and PCae (13 of which containing a 16- or 18-carbon atom saturated or monounsaturated fatty acid (FA) chain), and 12 SM (with saturated or monounsaturated FA chains). The sum of SM and two FAO markers were also significantly higher (uncorrected 0.05 level) in the intervention group; no other sum or ratio was different between the groups.

RCT and cord blood

No significant difference was found (see Fig. 2), and weak trends for elevated values in the intervention group were identified for only 3 metabolites: NEFA 14:1 and 15:1 and AC 20:0 (see Additional file 1). The subanalysis including only overlapping mother/child dyads delivered a similar picture, with PCaa 30:0, AC 4:0 and the branched-chain AA (BCAA) Valine (Val) showing a trend for lower values in the intervention group. Val, in particular, was more strongly different than the other two analytes (uncorrected $p = 0.005$). This subpopulation did not significantly differ in the baseline characteristics from the total population.

Discussion

In this study, we explored the impact of a dietary intervention promoting a low-GI diet during pregnancy on the metabolome of pregnant women and cord blood of their offspring. We found that the low-GI diet was associated with consistently higher concentrations of phospholipids (PL) and acylcarnitines (AC) in maternal blood

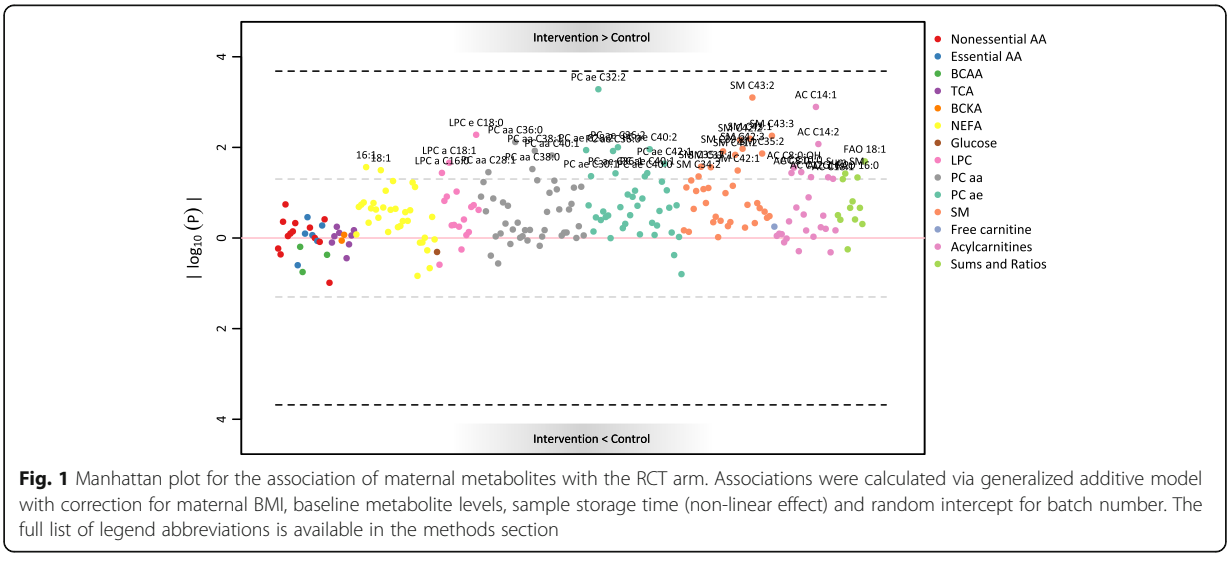

Fig. 1 Manhattan plot for the association of maternal metabolites with the RCT arm. Associations were calculated via generalized additive model with correction for maternal BMI, baseline metabolite levels, sample storage time (non-linear effect) and random intercept for batch number. The full list of legend abbreviations is available in the methods section

Table 2 Results from maternal blood analysis. Since no metabolite was significant after Bonferroni correction, only results with uncorrected $p < 0.05$ are presented here (for complete results, including Bonferroni correction: see Additional file 2). Beta > 0 indicates higher values of the analyte in the intervention group

Analyte	Analyte group	Beta (Std.)	95% CI (Std.)	*p*-value
16:1	NEFA	0.61	(0.09, 1.12)	0.027
18:1	NEFA	0.62	(0.07, 1.17)	0.032
AC C8:0	Acylcarnitines	0.58	(0.05, 1.11)	0.037
AC C8:0:OH	Acylcarnitines	0.59	(0.09, 1.1)	0.028
AC C10:0	Acylcarnitines	0.6	(0.06, 1.14)	0.035
AC C12:1	Acylcarnitines	0.57	(0.03, 1.11)	0.045
AC C14:1	Acylcarnitines	0.87	(0.38, 1.37)	0.001
AC C14:2	Acylcarnitines	0.73	(0.21, 1.25)	0.008
AC C16:2	Acylcarnitines	0.51	(0.03, 1)	0.046
AC C18:1	Acylcarnitines	0.57	(0.02, 1.12)	0.049
LPC a C16:0	LPC	0.52	(0.05, 0.98)	0.037
LPC a C18:1	LPC	0.62	(0.11, 1.13)	0.022
LPC e C18:0	LPC	0.65	(0.22, 1.07)	0.005
PC aa C28:1	PC aa	0.48	(0.05, 0.92)	0.035
PC aa C36:0	PC aa	0.64	(0.19, 1.09)	0.008
PC aa C38:0	PC aa	0.54	(0.07, 1.02)	0.03
PC aa C38:1	PC aa	0.65	(0.17, 1.13)	0.012
PC aa C40:1	PC aa	0.55	(0.13, 0.97)	0.015
PC ae C28:2	PC ae	0.58	(0.15, 1.01)	0.011
PC ae C30:1	PC ae	0.53	(0.03, 1.02)	0.043
PC ae C32:2	PC ae	0.77	(0.37, 1.16)	5.21e-04
PC ae C36:0	PC ae	0.63	(0.16, 1.1)	0.012
PC ae C36:1	PC ae	0.54	(0.05, 1.04)	0.037
PC ae C36:2	PC ae	0.73	(0.2, 1.26)	0.01
PC ae C40:0	PC ae	0.53	(0.03, 1.03)	0.044
PC ae C40:1	PC ae	0.55	(0.05, 1.05)	0.037
PC ae C40:2	PC ae	0.51	(0.13, 0.88)	0.011
PC ae C42:1	PC ae	0.47	(0.08, 0.86)	0.023
SM C34:2	SM	0.53	(0.03, 1.03)	0.044
SM C35:1	SM	0.58	(0.08, 1.07)	0.027
SM C35:2	SM	0.61	(0.15, 1.06)	0.014
SM C37:1	SM	0.61	(0.09, 1.13)	0.027
SM C39:2	SM	0.59	(0.15, 1.02)	0.012
SM C41:2	SM	0.58	(0.14, 1.03)	0.015
SM C42:1	SM	0.52	(0.06, 0.98)	0.032
SM C42:2	SM	0.71	(0.22, 1.19)	0.007
SM C42:3	SM	0.62	(0.16, 1.07)	0.011
SM C43:1	SM	0.74	(0.23, 1.24)	0.006
SM C43:2	SM	0.84	(0.38, 1.29)	7.94e-04
SM C43:3	SM	0.68	(0.23, 1.14)	0.006
Sum SM	Sums and Ratios	0.55	(0.05, 1.06)	0.038
FAO 16:0	Sums and Ratios	0.52	(0.02, 1.02)	0.046
FAO 18:1	Sums and Ratios	0.62	(0.11, 1.12)	0.02

Abbreviations: *CI* Confidence interval, *Std.* Standardized estimate, *NEFA* Non-esterified fatty acid, *AC* Acylcarnitine, *LPC a* Lysophosphatidylcholine with acyl bond, *PC aa* diacyl-phosphatidylcholine, *PC ae* acyl-alkyl-phosphatidylcholine, *SM* Sphingomyelin, *FAO* Fatty acid oxidation

(though non-significant after correction for multiple testing), while cord blood metabolome was not substantially affected by the intervention.

Maternal blood

All changes identified in maternal blood between control and intervention groups were related to fatty acids (FA), either non-esterified or in the form of acyl esters or PL chains. PLs are membranes lipids whose abundance is associated with both endogenous metabolism and dietary intake [14, 22]. The amount and composition of dietary fat consumed by the mothers did not change over pregnancy or due to the intervention, thus an exogenous change in fat intake can be excluded. It is possible that the low-GI diet modifies metabolism towards the release of fat from adipose tissue, hence the usage of fat as source of energy (as seen in the higher FAO markers and AC) and their transport (via PL).

A recent study by Hernandez-Alonso et al. found that a low-GI diet over 6 months was associated with changes in amino acid concentrations (both positively and negatively) and with a marked decrease in phospholipids, particularly SM and LPC, when compared both intra-subject to the patient's own baseline levels or inter-subjects against patients following high GI or low fat diets [17]. In our results, subjects in the intervention group showed no differences regarding AA, but higher levels of phospholipids, especially those containing FA with 16 and 18 carbon atoms chains, than subjects in the control group. To evaluate these discrepancies, it should be noted that the populations and study design largely differed: Hernandez-Alonso et al. investigated overweight and obese men and women in a calories-restricted setting for over 6 months, while our population was composed of pregnant women in the second half of gestation who embraced an isocaloric, low-GI diet for 12 weeks. In particular, a 6 months calories-restricted diet can be seen as a prolonged catabolic state, while in pregnancy a first anabolic state spanning until the end of the first trimesters is followed by an accelerated catabolic state in the third trimester [19, 20, 29];

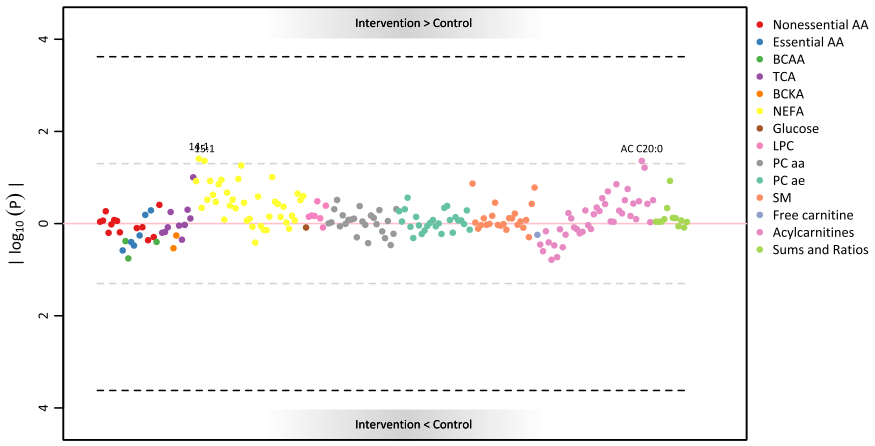

Fig. 2 Manhattan plot for the association of cord metabolites with the RCT arm. Associations were calculated via generalized additive model with correction for maternal BMI, gestational age, foetal sex, sample storage time (non-linear effect) and random intercept for batch number. The full list of legend abbreviations is available in the methods section

therefore, our data, collected at the end of the second trimester, might represent the peak of the anabolic phase or the begin of the catabolic phase.

All AA modulated by low-GI diet in Hernandez-Alonso's study have been observed to decrease during gestation [29]; this progression has been linked to the increased placental uptake for foetal protein synthesis [29] and it is possible that the foetal needs might dominate over their regulation due to low-GI diet. Maternal phospholipids, especially PL and SM, have been observed to rise during pregnancy [29, 41]; this rise has been attributed to oestrogens [42] and similar differences in phospholipids have also been observed in young adult women taking hormonal contraceptives [38]. Moreover, a systematic review conducted by Goff et al. in 2013 found that low-GI diet reduces total and LDL cholesterol but does not affect HDL cholesterol [13] (meta-analysis estimate for reduction in LDL for a low-GI diet of 9–20 weeks from a total population of 1281 study subjects: − 0.16 mmol/l, 95% CI: − 0.24 to − 0.08). These results refer to intra-subject differences pre- and post-intervention. In our data, neither the absolute LDL concentrations at 28th week nor their difference to the baseline levels were different between the groups. During pregnancy, a marked increase in circulating lipoproteins occurs (e.g., LDL cholesterol is expected to increase from < 2.59 mmol/l in non-pregnant population to up to 5.8 mmol/l at the end of gestation) [1], as the high foetal demand for cholesterol is matched solely by maternal supply during the first two trimesters [18]. It is plausible that, also in the case of phospholipids, the cholesterol- and phospholipids-lowering effect of a low-GI diet might be inhibited by the major endocrinologic changes enacted to provide for the increased needs of mother and foetus.

Nevertheless, some of the species higher in the intervention group (total SM, SM C42:2 and C42:3) were found to be associated with LDL lipoproteins in pregnant women in a recent publication by Rauschert et al. [36]. Moreover, the absolute differences in GI in the population under investigation were very modest (see Table 2), so it is possible that a more intensive dietary intervention beginning pre-conceptually might in fact be beneficial for prospective mothers with potential lipid dysregulations.

As for how such changes are enacted, an interesting study in mice by Stavrovskaya et al. [40] found that GI and fat composition work synergistically in affecting the FA composition of cardiolipins, a subclass of mitochondrial PLs; that is, the changes in the FA composition of cardiolipids were more pronounced if the diet was high GI and high in trans or saturated fat than if either component was present alone. In our data, we could not test this hypothesis due to lack of variability in the dietary fat intake. Nevertheless, our results, combined with the findings from Stavrovskaya et al., urge further investigation of the mechanisms linking fat intake and GI to lipids composition and fat metabolism.

Cord blood

Cord blood metabolome was largely unimpacted by the intervention. We did find a small difference in the concentration of Val between the RCT groups in the analysis of the overlapping subjects which might be indeed be ultimately linked to the lower maternal GI intake (BCAA levels correlate with and might cause insulin resistance [31], while a low GI diet should prevent it); however, since the finding was not replicated in the larger cohort and no other BCAA was different between the study groups, this result has to be interpreted carefully.

In general, data about the impact of lifestyle interventions on cord blood metabolome are scarce; however, our results from the ROLO cohort are in line with previous findings from the UPBEAT study [35], where a lifestyle intervention in pregnant women with obesity, while beneficial to the mothers, did not affect cord blood metabolome [34]. In particular, the enhanced availability of maternal lipids was not mirrored in higher transport to the foetus. This is not surprising, since placental FA transport is subject to complex regulatory mechanisms [5, 11, 39]. Nevertheless, there is the need of a deeper understanding of how maternal diet may influence placental transport, as the data to this regard are scarce [43].

Despite the ROLO trial found no effect of the intervention on birth and early infancy anthropometry measures [46], epidemiological studies show that the effects of in-utero exposures on metabolic health might become evident later in life [24, 26], e.g. because modulated via epigenetic changes [8, 28]. "Subtle but widespread" changes in the DNA methylation were found in the cord blood of babies from the ROLO study, as reported by Geraghty et al. [9]. In other words, the intervention might have not impacted foetal metabolism and early infancy anthropometry, but the in-utero exposure to a low-GI diet might still show its beneficial effects in later stages in life.

Strengths and limitations

The strengths of our analysis were the large panel of metabolites (268 analytes, sums and ratios) studied with the same LC-MS/MS targeted approach, the availability of data from both maternal and cord samples, and the extensive dietary data. A major limitation of our study was the small sample sizes, which nevertheless did not prevent from inspecting the trends in maternal and cord blood. The small magnitude of the difference in GI between the study group and the lack of hormonal measurements as confounding factors might also have obscured additional differences and associations from being identified.

Conclusions

Our analysis showed that a low-GI dietary intervention in pregnancy was associated with modest but consistent increases in maternal plasma phospholipids and utilization of fat as source of fuel, while cord blood was not affected by this intervention. Our study was the first to investigate the effect of a low-GI diet in a pregnant population. Our results were partially in agreement with studies conducted on non-pregnant subjects, and we ascribe discrepancies in the findings to the pregnancy-specific metabolic adaptations enacted to ensure sufficient nutrients to the developing foetus, for which more research is needed. Our exploratory findings may be used to direct further investigations about low-GI diets before and during pregnancy, to improve patient care for pre-conceptional and pregnant women with lipid dysregulations and potentially modulate the offspring's risk for future onset of metabolic diseases.

Additional files

Additional file 1: Results of the generalized additive models. (XLSX 83 kb)
Additional file 2: Sensitivity analyses for maternal blood. (DOCX 25 kb)

Abbreviations

AA: Amino acids; AC: Acylcarnitine; BMI: Body-mass index; CV: Coefficient of variation; FA: Fatty acid; FAO: Fatty acid oxidation; GDM: Gestational diabetes mellitus; GI: Glycaemic index; GWG: Gestational weight gain; ISD: Internal standard; LC: Liquid chromatography; LPC: Lysophosphatidylcholine; MS: Mass spectrometry; NEFA: Non-esterified fatty acids; PCaa: Diacyl-phosphatidylcholine; PCae: Acyl-alkyl-phosphatidylcholine; PL: Phospholipids; QC: Quality control; RCT: Randomized controlled trial; SM: Sphingomyelin

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The data presented are part of the PhD thesis accomplished by Linda Marchioro at the Faculty of Medicine of the LMU Munich.

Authors' contributions

Conceived and designed the experiments: FMMA, BK. Collected the data: AAG, ECOB. Conducted experiments: OU. Analysed and interpreted the data: LM, ES. Contributed reagents/materials/analysis tools: FMMA, BK, OU. Wrote the paper: LM. Reviewed, edited, and approved the paper: all authors. Provided funding: FMMA, BK.

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Availability of data and materials

The datasets during and/or analysed during the current study available from the corresponding author on reasonable request.

Ethics approval and consent to participate

The ROLO Study had received approval from the ethics committee of the National Maternity Hospital (June 2006) and all study participants were included in the study after giving written informed consent.

Consent for publication

Not applicable.

Competing interests

The authors declare no conflicts of interest i.e. circumstances that involve the risk that the professional judgment or acts of primary interest may be unduly influenced by a secondary interest.

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Publisher's Note

Chapter 5

Publication III: SCD-1 Activity in the ROLO Study

The following pages present the publication L. Marchioro, C. Hellmuth, O. Uhl, A. A. Geraghty, E. C. O'Brien, M. K. Horan, B. Koletzko, and F. M. McAuliffe, "Associations of maternal and fetal SCD-1 markers with infant anthropometry and maternal diet: Findings from the ROLO study," *Clin Nutr*, 2019.

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I contributed to this publication by designing the research, analysing the data, interpreting the results, writing, reading and approving the manuscript. I had primary responsibility for the final content.

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Original article

Associations of maternal and fetal SCD-1 markers with infant anthropometry and maternal diet: Findings from the ROLO study

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SUMMARY

Background: Elevated stearoyl-CoA desaturase 1 (SCD-1) activity showed associations with obesity in cross-sectional studies. In non-pregnant populations, nutrition regulates SCD-1 transcription and activity.
Objective: To investigate the longitudinal associations of maternal and fetal SCD-1 activity markers with infant anthropometry up to 2 years of age, and to explore how selected dietary intakes modulate SCD-1 activity in pregnancy.
Methods: As a secondary analysis from the ROLO intervention study, which was conducted in a population at risk for macrosomia, non-esterified fatty acids (NEFA) from maternal plasma at 13 and 28 weeks' gestation and in cord blood were measured via liquid-chromatography-mass-spectrometry. Fatty acid ratios 18:1/18:0 and 16:1/16:0 were used as markers for SCD-1 activity ('desaturation indices', DIs). Relationships of DIs with infant anthropometry up to 2 years of age and maternal dietary parameters during pregnancy were investigated using adjusted linear regression models and *p*-values correction for multiple testing.
Results: 18:1/18:0, but not 16:1/16:0, was associated with measures of infant anthropometry at birth (maternal and fetal markers) and up to 2 years of age (maternal markers only). Dietary intakes did not show strong associations with 18:1/18:0, but 16:1/16:0 was associated with absolute and relative dietary intakes.
Conclusions: In a population at risk for macrosomia, maternal SCD-1 activity measured via 18:1/18:0 was involved in the fetal programming of infant obesity, but could not be substantially modulated by short-term diet in pregnancy.
Clinical trial registration: ISRCTN Registration number: ISRCTN54392969 (http://www.isrctn.com/ISRCTN54392969).

1. Introduction

The rapidly increasing prevalence of obesity and related non-communicable diseases in adults and children makes it necessary to engage in precautionary interventions to prevent its onset in early childhood [1–3]. According to the 'early programming' hypothesis, the in-utero and early life environments are critical for the development of long-term metabolism and risk for metabolic conditions [4,5]. Metabolic mechanisms that influence fetal metabolism in-utero have yet to be fully elucidated. Dietary interventions during pregnancy are a possible intervention strategy

Abbreviations: SCD-1, stearoyl-CoA desaturase-1; AT, adipose tissue; BMI, body-mass index; CB, cord blood; DI, desaturation index; EP, early pregnancy; FA, fatty acid; FDR, false discovery rate; GDM, gestational diabetes mellitus; LP, late pregnancy; MUFA, monounsaturated fatty acid; NEFA, non-esterified fatty acid; PUFA, polyunsaturated fatty acid; SFA, saturated fatty acid.

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to modify maternal metabolism and act on programming mechanisms [6].

One enzyme that is likely to be involved in such programming mechanisms is stearoyl-CoA desaturase-1 (SCD-1, or Delta-9-desaturase). SCD-1 plays a key role in the fatty acid (FA) metabolism, since it catalyzes the formation of monounsaturated fatty acids (MUFA) from saturated fatty acids (SFA), preferentially with chain length of 18 or 16 carbon atoms [7,8]. Elevated SCD-1 activity levels have been repeatedly associated with obesity and increased insulin resistance [9–15]. Nevertheless, a causal link between SCD-1 and obesity has never been proven, and it has been proposed that "SCD-1 serves as a molecular switch in the promotion or prevention of lipid-induced disorders" [16]. The mechanisms by which SCD-1 activity may impact lipogenesis and insulin resistance are not clear, but mediating effects of oleate itself (18:1, n-9) or protein UBXD8 have been proposed [17,18].The ratios 18:1/18:0 and 16:1/16:0, named 'desaturation indices' (DIs), are a widely used surrogate measure of SCD-1 activity in-vivo [8].

Because of its relevance in metabolic homeostasis, SCD-1 regulation is finely tuned. Leptin, insulin, fat and sugar intake are known regulators of SCD-1 transcription and the effect of hormones is under investigation [11,19]. Some authors have argued that SCD-1 regulation and effects are dependent on the substrate (16:0 vs. 18:0) and location of SCD-1 activity (liver or adipose tissue) [20,21].

Few studies have investigated SCD-1 during pregnancy. In mice models, diets high in fat and/or sugars in pregnancy increased SCD-1 activity in the offspring [22,23]. In humans, DIs in cord blood were increased in children born to mothers with gestational diabetes mellitus (GDM) [24] and they have been found to positively correlate with birth weight in a German birth cohort [25].

In this explorative analysis, we investigated the involvement of maternal and fetal SCD-1 activity with infant adiposity up to two years of age. Secondarily, we investigated if and how the maternal intake of sugars, carbohydrate, fats and their ratios (all known regulators of SCD-1), as well as the closely related glycemic index and load, can modulate SCD-1 during pregnancy.

2. Material and methods

2.1. Data and samples collection

The present study is a secondary analysis conducted on a subpopulation of the ROLO Study [26]. The ROLO Study (Randomized cOntrolled trial of LOw glycemic index diet versus no dietary intervention to prevent recurrence of fetal macrosomia) was a randomized controlled trial of diet in pregnancy conducted at the National Maternity Hospital, Dublin, Ireland, between 2007 and 2011. The study was approved by the ethical commission of the National Maternity Hospital, Dublin, Ireland in June 2006 [26]. Briefly, 800 secundigravida mothers with a previous macrosomic child (birth weight > 4000 g) and without underlying medical disorders (including previous GDM) were recruited in the first trimester of pregnancy and randomly assigned to receive either a dietary education session about a low glycemic index (GI) diet in addition to routine antenatal care, or routine antenatal care only [27].

Fasting blood samples were acquired at study entry in early pregnancy (EP, approximately 13 weeks) and in late pregnancy (LP, 28 weeks); a sample of cord blood (CB) was collected at birth after the delivery of the baby and before the delivery of the placenta, and glucose was measured in each blood sample. Leptin, maternal insulin and cord blood C-peptide (marker of insulin secretion) as well as triglycerides were measured as previously published [28]. Maternal anthropometry was measured at study entry and used to calculate early pregnancy body-mass index (BMI). Weight was additionally measured at 34 weeks' gestation and again at 40 weeks, if delivery had not yet occurred. Dietary data were collected via 3-days food diaries in trimester 1, 2 and 3 of gestation [27] and processed using NetWISP version 3.0 (Tinuviel Software, Llanfechell, Anglesey, UK). Carbohydrate (including sugars) and fat (total/saturated/monounsaturated/polyunsaturated) intakes were expressed in grams (g), energy from carbohydrates and fat was calculated as percentage of total kilocalories (%kcal), ratios of carbohydrate/sugars-to-fat intakes were calculated and expressed as g/g. The use of dietary supplements was recorded as a binary variable from food frequency questionnaires administered at the first antenatal visit [29]. Child anthropometry was measured at birth, 6 months (6 m) and 2 years (2 y) follow up visits. The following parameters were measured at all three time points: weight; length, abdominal circumference; biceps, triceps, subscapular and thigh (leg) skinfolds [28,30]. From these measures, BMI as well as weight-for-length, total sum of skinfolds, subscapular-to-triceps skinfold ratio and waist-to-length ratio were computed; the corresponding WHO z-scores were calculated for weight-for-age, length-for-age, BMI-for-age and weight-for-length. At 6 m, information about breastfeeding of the child was collected.

Non-esterified fatty acids were measured in 75 maternal blood samples from early pregnancy (EP), 110 from late pregnancy (LP) and 136 cord blood (CB) samples with a targeted metabolomics approach in the laboratory of the Division of Metabolic and Nutritional Medicine of the Dr. von Hauner Children's Hospital, Munich, Germany.

2.2. Analytical measurement of non-esterified fatty acids (NEFA)

50 μl of plasma were thawed and diluted with 450 μl methanol containing internal standards corresponding to different metabolites groups. After centrifugation (4000 rpm, 10 min, room temperature), 100 μl of the supernatant were analyzed as previously reported [31]. 10 μl of the supernatant were injected to an HPLC system (1200 Agilent, Waldbronn, Germany) coupled to a hybrid triple quadrupole mass spectrometer (4000 QTRAP, Sciex, Darmstadt, Germany) operating in negative ESI MRM mode. Chromatographic separation was performed using an UPLC diphenyl column (Pursuit UPS Diphenyl, Agilent, Waldbronn, Germany) with 5 mM ammonium acetate and 2.1 mM acetic acid in water as mobile phase A and acetonitrile/isopropanol (80:20) as mobile phase B and with an eluent flow rate of 700 mL/min. The analytical process was post-processed using Analyst software version 1.6.2.

Other four metabolites classes were measured (amino acids, organic acids, phospholipids and acylcarnitines); details about the measurements are available as online supplemental material (Supplement 1).

2.3. Data processing

The data processing and the statistical analyses were performed in R version 3.1.3.

The quality of the metabolomics measurements was assessed via a 20% intra-batch, 30% inter-batch coefficient of variation (CV) criterion. Batches or metabolites which did not meet these conditions were discarded from the analysis. Metabolites measurements were corrected for storage time and batch number via linear mixed models.

Final maternal weight was defined as last measured weight measured before delivery. When this information was not available, final weight at 40 weeks' gestation (corresponding to the median gestational age of 280 days) was estimated by adding 2.67 kg

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(median weight gain between 34 and 40 weeks' gestation in the complete ROLO population) to the last weight measured at the 34 weeks' visit. With this method, 63 values were imputed. Gestational weight gain (GWG) was subsequently defined by subtracting early pregnancy maternal weight from the final weight.

Only data from term babies (>36th gestation week) were included in the analysis. For continuous variables, normality was visually assessed via histograms and quantile–quantile plots. Dietary data were log-transformed and observations further away than 2.5 standard deviations (SD) from the next one were excluded.

2.4. Statistical analysis

2.4.1. Preliminary analyses

2.4.1.1. Baseline dietary/fatty acids associations. To consider the dietary origins of the fatty acids, we first inspected the association between the FA and the dietary fat intakes used in the analysis. This explorative analysis was carried out via Spearman correlations (without *p*-values correction for multiple testing) and inspection of scatterplots with LOESS curves. Results with $p < 0.05$ are reported as significant.

2.4.1.2. Desaturation indices. The desaturation indices (DIs), markers for SCD-1 activity, were calculated as the ratios 18:1/18:0 (DI_{18}) and 16:1/16:0 (DI_{16}). DIs were plotted for inspection; one data point lying further away than 2.5 SD away from the closest measurement was removed. The longitudinal progression of the DIs across the three blood sources was qualitatively inspected via boxplots.

2.4.1.3. RCT and baseline characteristics. We investigated preliminarily whether the following measures were associated with SCD-1 markers: maternal age, early pregnancy BMI and arm circumference, gestation days, GWG (total and per week), maternal fasting blood glucose, insulin, leptin and triglycerides in early and late pregnancy, cord blood glucose, c-peptide, leptin and triglycerides, as well as RCT group and child sex. The association with continuous covariates was investigated via Spearman correlations and scatterplots with LOESS curve, with dichotomous and categorical covariates via ANOVA and boxplots (no correction for multiple testing was performed in this part of the analysis). Significance was defined at 0.05 alpha level.

2.4.2. Main analysis

2.4.2.1. Infant anthropometry. In the first part of the main analysis, associations between child anthropometry and DIs were investigated via linear regressions with adjustment for maternal BMI, plus, if the outcome was not a z-score, child sex and age (at 6 m and 2 y). Models on 6 m and 2 y child anthropometry outcomes were adjusted for anthropometry at birth to remove the confounding effect of birth parameters. According to the results from the preliminary analysis for baseline characteristics, the following covariates were added one at a time to the model to avoid confounding effects in the results interpretations: RCT group, maternal age, leptin and insulin/c-peptide from the same blood time point as the DI under examination (since leptin and insulin are themselves regulator of SCD-1), maternal fasting glucose from late pregnancy (to consider maternal glycemic control even in non-GDM levels), and any breastfeeding (only at 6 m and 2 y) (data not shown).

2.4.2.2. Diet. In the second part of the main analysis, we investigated how the selected dietary parameters (carbohydrates, sugars, fats and their ratios, glycemic index/load) were associated with SCD-1 activity markers at a later time point. We used multiple linear regressions with SCD-1 markers as outcome, the dietary parameter as independent variables and maternal early pregnancy BMI as confounder. Additional models were calculated including maternal age, maternal leptin and insulin from the same trimester at the same time (late pregnancy maternal leptin and insulin for cord blood samples), late pregnancy maternal fasting glucose and the respective total kilocalories intake, and dietary supplements intake as covariates (data not shown). Since the diagnostics plots showed statistical issues, the same analysis was additionally performed after 1-parameter Box–Cox transformation of the dietary variables to confirm the results (data not shown).

2.4.2.3. Presentation of the results. We used false detection rate (FDR) within each pair of SCD-1/anthropometry or Diet/SCD-1 time points to adjust for multiple testing. For associations with an uncorrected *p*-value < 0.05, positive adjusted R^2, at least 10 observations per model covariate, and that were stable for at least three additional covariates ($p < 0.1$), we inspected partial residual plots [32] and model diagnostics plots. For brevity, we report here only results that were also confirmed by the additional analyses and visual inspection of the plots. The complete results are available as online Supplemental Tables 2 and 3 [33].

3. Results

3.1. Population characteristics

Table 1 presents the baseline characteristics of the population, according to the availability of early pregnancy maternal blood (EP MB, approximately 13 weeks gestation) late pregnancy maternal blood (LP MB, 28 weeks gestation) and cord blood (CB). Maternal anthropometry, blood values, as well as selected dietary and anthropometry data are presented (i.e. only variables which had a significant association with SCD-1 markers, see results below); the complete overview is available online (Supplemental Table 1).

3.1.1. Plasma fatty acids and dietary fat intakes

Within this exploratory analysis, only four associations showed statistical significance (uncorrected $p < 0.05$), all of which were negative and related to intakes from trimester 1: EP 16:1 and CB 16:0 with polyunsaturated fat intake, CB 18:0 with the saturated fat intake, and LP 18:0 with the proportion of saturated-to-total fat. Only the latter association was confirmed by the scatterplot inspection (Spearman: −0.26, $p = 0.03$).

3.2. SCD-1 and baseline characteristics

Figure 1 presents the progression of the DIs in the three blood sources.

Despite several baseline characteristics were significantly associated with SCD-1 markers (uncorrected $p < 0.05$), the inspection of the scatterplots revealed statistical artefacts and these associations were not interpreted. The only significant associations identified by the tests and confirmed by visual inspection of the plots were LP DI_{16} and maternal pre-pregnancy BMI (LP: Spearman: 0.21, $p = 0.03$) as well as between CB DI_{18} and cord blood triglycerides (Spearman: 0.40, $p = 0.01$). In particular, we found no evidence for associations of leptin and insulin with SCD-1 activity markers (correlation coefficients and *p*-values are available online in Supplement 2).

3.3. SCD-1 and child anthropometry

Table 2 reports the significant associations found for this analysis and supported by the scatterplots and model diagnostics. Even though both DI were inspected, only DI_{18}, and not DI_{16}, showed

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Table 1
Characteristics of the mothers/infants in the analysis. Baseline data are reported according to metabolomic data availability at each time point. Data are expressed in median ± interquartile range or as number (percentage). The numbers in brackets indicate the amount of missing observations. For anthropometry and dietary data, only variables which presented significant associations with SCD-1 markers are presented (see also Tables 2 and 3). Complete data are available in Supplemental Table 1.

	Subpopulation with EP MB metabolomics measurements ($n = 72$)	Subpopulation with LP MB metabolomics measurements ($n = 108$)	Subpopulation with CB metabolomics measurements ($n = 133$)
Maternal and pregnancy data			
Maternal age at delivery (years)	32.79 ± 5.95	32.97 ± 5.94	32.84 ± 5.90
Early pregnancy BMI (kg/m^2)	25.72 ± 4.78 [1]	25.92 ± 4.60 [1]	25.86 ± 4.37 [1]
Early pregnancy arm circumference (cm)	28.80 ± 3.05 [5]	29.00 ± 3.80 [6]	28.95 ± 3.73 [7]
RCT group – intervention	32 (44%)	58 (54%)	64 (48%)
GWG (40 weeks) (kg)	12.55 ± 4.70 [16]	12.57 ± 4.70 [23]	12.57 ± 4.54 [30]
Gestation days (days)	282 ± 12.00	284 ± 10.50	283 ± 11.00
Child sex – female	36 (50%)	59 (55%)	70 (53%)
Blood parameters			
Insulin EP MB (μIU/ml)	14.14 ± 14.46 [14]	14.01 ± 13.47 [24]	13.22 ± 13.83 [30]
Insulin LP MB (μIU/ml)	15.86 ± 14.09 [10]	18.95 ± 14.10 [20]	16.90 ± 14.92 [23]
C-peptide CB (ng/ml)	0.48 ± 0.80 [5]	0.42 ± 0.78 [8]	0.41 ± 0.78 [7]
Leptin EP MB (ng/ml)	16.01 ± 13.61 [11]	16.61 ± 13.76 [19]	16.23 ± 14.14 [24]
Leptin LP MB (ng/ml)	19.59 ± 15.38 [13]	19.59 ± 18.03 [19]	19.31 ± 17.72 [25]
Leptin CB (ng/ml)	22.93 ± 29.37 [8]	23.84 ± 28.01 [20]	24.02 ± 28.63 [20]
Child anthropometry			
Birth BMI (kg/m^2)	14.50 ± 1.81 [15]	14.31 ± 1.90 [23]	14.375 ± 1.87 [29]
Birth waist-to-length (cm/cm)	0.64 ± 0.05 [23]	0.64 ± 0.05 [46]	0.637 ± 0.05 [55]
Birth weight (g)	4019 ± 502.50	4100 ± 572.50	4060 ± 540.00
Birth weight-for-length (kg/cm)	0.07 ± 0.01 [15]	0.08 ± 0.01 [23]	0.076 ± 0.01 [29]
Birth z-BMI-for-age	0.84 ± 1.27 [15]	0.76 ± 1.46 [23]	0.75 ± 1.43 [29]
Birth z-weight-for-age	1.51 ± 0.87	1.56 ± 1.00	1.57 ± 0.97
Birth z-weight-for-length	0.15 ± 1.86 [15]	0.04 ± 1.92 [23]	0.10 ± 1.91 [29]
2 y z-BMI-for-age	0.09 ± 1.15 [27]	−0.05 ± 1.45 [45]	0.07 ± 1.44 [56]
2 y z-weight-for-age	0.48 ± 1.06 [27]	0.46 ± 1.23 [45]	0.48 ± 1.24 [56]
2 y z-weight-for-length	0.11 ± 1.02 [24]	0.04 ± 1.32 [38]	0.11 ± 1.35 [49]
Diet			
Tri1 carbohydrate (g)	224.30 ± 69.41 [12]	225.16 ± 64.00 [19]	225.83 ± 75.55 [23]
Tri1 monounsaturated fat (g)	22.91 ± 7.42 [12]	23.62 ± 7.10 [19]	23.78 ± 6.91 [23]
Tri1 polyunsaturated fat (g)	10.67 ± 5.26 [13]	11.23 ± 4.90 [20]	10.80 ± 5.14 [24]
Tri1 sugars (g)	92.09 ± 53.21 [12]	92.22 ± 43.02 [20]	92.99 ± 45.13 [24]
Tri2 saturated fat (g)	27.27 ± 11.99 [11]	26.60 ± 10.77 [16]	26.97 ± 11.81 [21]
Tri2 monounsaturated fat (g)	22.63 ± 8.60 [11]	22.69 ± 7.98 [16]	22.83 ± 8.15 [21]
Tri2 saturated fat energy (%kcal)	13.18 ± 4.39 [11]	13.42 ± 4.26 [16]	13.50 ± 4.06 [21]
Tri2 monounsaturated fat energy (%kcal)	10.86 ± 3.03 [11]	11.23 ± 2.93 [16]	11.26 ± 2.96 [21]
Tri2 polyunsaturated: total fat ratio (g/g)	0.15 ± 0.06 [12]	0.16 ± 0.06 [17]	0.16 ± 0.06 [22]
Tri2 polyunsaturated: saturated fat ratio (g/g)	0.40 ± 0.19 [12]	0.44 ± 0.20 [17]	0.42 ± 0.20 [22]
Tri2 carbohydrate: saturated fat ratio (g/g)	8.29 ± 3.47 [13]	8.27 ± 3.58 [18]	8.34 ± 3.58 [23]
Tri2 carbohydrate: monounsaturated fat ratio (g/g)	10.17 ± 4.49 [11]	9.85 ± 3.82 [17]	9.90 ± 4.07 [22]
Tri2 carbohydrate: polyunsaturated fat ratio (g/g)	20.29 ± 8.08 [11]	19.87 ± 7.21 [17]	20.11 ± 7.06 [22]
Tri3 carbohydrate energy (%kcal)	50.40 ± 6.51 [10]	49.08 ± 7.72 [15]	49.88 ± 6.71 [20]
Tri3 total fat energy (%kcal)	35.72 ± 5.97 [10]	36.12 ± 6.18 [15]	36.08 ± 6.25 [20]
Tri3 saturated fat energy (%kcal)	13.27 ± 3.93 [11]	13.86 ± 4.05 [16]	13.50 ± 3.82 [21]
Tri3 monounsaturated fat energy (%kcal)	11.12 ± 2.69 [12]	11.47 ± 2.66 [17]	11.24 ± 2.54 [22]
Tri3 carbohydrate: total fat ratio (g/g)	3.17 ± 1.12 [10]	3.08 ± 1.05 [15]	3.09 ± 0.98 [20]
Tri3 sugars: total fat ratio (g/g)	1.32 ± 0.63 [10]	1.22 ± 0.55 [15]	1.27 ± 0.49 [20]
Tri3 carbohydrate: saturated fat ratio (g/g)	8.49 ± 3.67 [10]	7.71 ± 3.36 [15]	8.07 ± 3.18 [20]
Tri3 sugars: saturated fat ratio (g/g)	3.29 ± 1.86 [11]	3.12 ± 1.52 [15]	3.23 ± 1.51 [21]
Tri3 carbohydrate: monounsaturated fat ratio (g/g)	10.05 ± 4.25 [10]	9.83 ± 3.80 [15]	9.95 ± 3.86 [20]
Tri3 sugars: monounsaturated fat ratio (g/g)	4.19 ± 2.46 [11]	3.98 ± 1.94 [15]	4.03 ± 2.04 [21]
Tri3 sugars: polyunsaturated fat ratio (g/g)	8.49 ± 4.23 [11]	7.89 ± 4.04 [16]	8.22 ± 4.08 [21]
Tri3 glycemic index	57.39 ± 5.25 [15]	56.87 ± 5.15 [20]	56.92 ± 4.85 [26]

Abbreviations: EP MB: early pregnancy maternal blood; LP MB: late pregnancy maternal blood; CB: cord blood; GWG (40 weeks): gestational weight gain (measured at 40 weeks gestation).

trends associations with infant anthropometry (uncorrected $p < 0.05$). All trends were positive. 7 of them remained significant after FDR correction: maternal LP DI_{18} with birth weight-for-length, BMI, weight, as well as BMI-for-age and weight-for-length z-scores, and fetal DI_{18} with birth waist-to-length. Two additional associations were identified but not confirmed by the plots inspection (for completeness, see online Supplemental Table 2).

No trends with 6 m anthropometry measures were identified. Maternal LP DI_{18} correlated positively with 2 y weight-for-length and BMI-for-age z-scores, though the association was not significant after correction for multiple testing.

3.4. Diet and SCD-1

No association of DI and dietary intake was significant at 0.05 alpha level after FDR correction, but 8 associations of CB DIs with trimester 3 intakes had p (FDR) = 0.087.

An additional 13 trends (uncorrected p-value < 0.05) were confirmed graphically and by the sensitivity analyses (see Table 3).

Four trends involving DI_{18} were found: in EP, a negative association with trimester 1 carbohydrate intake; in LP, a positive association with trimester 2 ratios of polyunsaturated-to-total/saturated fat; in CB, a negative association with trimester 3 GI.

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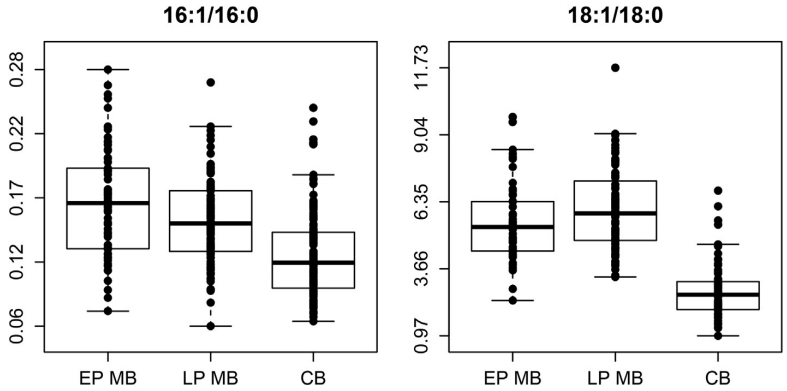

Fig. 1. Longitudinal progression of SCD-1 markers. Abbreviations: EP MB: early pregnancy maternal blood; LP MB: late pregnancy maternal blood; CB: cord blood. Ratios are expressed in [μmol/l]/[μmol/l].

Table 2
Significant associations of DI_{18} between infant anthropometry and desaturation indices. The results were calculated via multiple linear regression with adjustments for maternal BMI at study entry and, where applicable, child sex and age, as well as previous measurement (for 6 m and 2 y models). In bold: FDR-corrected *p*-values < 0.05.

Blood source	Measure	*n*	Beta	SE	*p* Value (raw)	*p* Value (FDR)	Adjusted R^2 (%)
EP MB	Birth BMI (kg/m^2)	46	0.374	0.158	0.023	0.254	5.6
EP MB	Birth weight-for-length (kg/m)	46	0.002	0.001	0.032	0.254	6.0
LP MB	Birth BMI (kg/m^2)	69	0.420	0.120	8.75E−04	**0.007**	14.4
LP MB	Birth weight (g)	87	99.200	32.774	0.003	**0.019**	8.9
LP MB	Birth weight-for-length (kg/m)	69	0.002	0.001	2.24E−04	**0.005**	19.1
LP MB	Birth z-BMI-for-age	69	0.298	0.083	5.91E−04	**0.007**	14.9
LP MB	Birth z-weight-for-age	87	0.177	0.060	0.004	**0.019**	7.8
LP MB	Birth z-weight-for-length	69	0.282	0.111	0.013	0.054	6.5
LP MB	2 y z-weight-for-age	50	0.152	0.070	0.036	0.173	35.9
LP MB	2 y z-weight-for-length	45	0.278	0.117	0.022	0.173	19.7
CB	Birth BMI (kg/m^2)	61	0.416	0.184	0.028	0.137	7.9
CB	Birth waist-to-length (cm/cm)	44	0.017	0.005	0.003	**0.036**	16.7
CB	Birth weight-for-length (kg/m)	61	0.002	0.001	0.028	0.137	11.5
CB	Birth z-BMI-for-age	61	0.290	0.129	0.028	0.137	5.0

Abbreviations: DI: desaturation index; *n*: number of observations in the model; FDR: false discovery rate; EP MB: early pregnancy maternal blood; LP MB: late pregnancy maternal blood; CB: cord blood; SE: standard error of the beta estimate.

Regarding the remaining 17 trends involving DI_{16}, it could be observed that maternal markers were more often associated with absolute intakes of fats and carbohydrates, while fetal markers showed more associations with ratios thereof, and particularly with carbohydrates/sugars-to-fat ratios.

In relation to the timing, first trimester intakes showed trends with maternal SCD-1 activity only, while they did not affect cord blood markers.

The complete results for the dietary analysis are available as online supplemental material (Supplemental Table 3).

4. Discussion

4.1. Compartmentalization of desaturation pathways

We observed an involvement of maternal DI_{18} with fetal anthropometry up to 2 years of age, while fetal DI_{18} was not associated with later anthropometry measures. Neither maternal nor fetal DI_{16} was associated with infant anthropometry. Maternal and fetal DI_{18} showed limited potential for dietary regulation during pregnancy, but trends for associations of DI_{16} with dietary intakes and composition were identified.

Differences in the two indices had already been reported. For example, Yee et al. observed that levels of DI_{18}, but not DI_{16}, were significantly different in the cord blood of babies born by GDM vs. non GDM mothers [24]. The same authors had previously observed, using in-vitro models from human hepatic cells, that SCD-1 inhibition differed according to the source of saturated fatty acids, thus suggesting a compartmentalized regulation of the enzyme activity [20]. Our results support the hypothesis of compartmentalized regulation and effects of SCD-1 activity according to the substrate; more in detail, they seem to suggest that the dietary modulation during pregnancy of SCD-1 activity does not affect fetal programming, since different pathways are involved.

4.2. Child anthropometry and maternal adipose status

Elevated maternal and fetal levels of DI_{18} were consistently associated with higher measures of body mass and central adiposity in the offspring. Interestingly, the sum of skinfolds did not show any association with SCD-1 markers. This seems to point towards a programming effect of maternal SCD-1 (DI_{18}) in the total mass but not in the body composition of the offspring, at least not in early infancy.

We did not identify associations of early pregnancy BMI with maternal DI_{18} (observed by Hellmuth et al. [34]) or with fetal SCD-1 markers (partially observed by Yee et al. in the offspring of GDM mothers [24]). With respect to these discrepancies, one has to recall

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Table 3
Significant associations between desaturation indices and trimester-specific maternal nutrient intakes (log) (results from multiple linear regression with adjustments for maternal BMI at study entry and, for trimesters 2 and 3, RCT group).

Blood source	DI	Trimester of dietary intake	Dietary intake (log)	*n*	Beta	SE	*p* Value (raw)	*p* Value (FDR)	Adjusted R^2 (%)
EP MB	DI 18	1	Carbohydrate	46	−1.507	0.628	0.021	0.322	7.8
EP MB	DI 16	1	Monounsaturated fat	59	−0.02	0.01	0.047	0.322	18.7
EP MB	DI 16	1	Polyunsaturated fat	58	−0.025	0.011	0.032	0.322	19.7
EP MB	DI 18	1	Sugars	46	−0.855	0.333	0.014	0.322	9.4
LP MB	DI 16	1	Sugars	85	−0.014	0.007	0.035	0.874	9.5
LP MB	DI 16	2	Saturated fat	89	−0.016	0.007	0.023	0.331	9.4
LP MB	DI 16	2	Monounsaturated fat	89	−0.013	0.006	0.041	0.331	8.4
LP MB	DI 16	2	Saturated fat energy (%kcal)	89	−0.024	0.011	0.028	0.331	9.1
LP MB	DI 18	2	Polyunsaturated: total fat ratio (g/g)	74	1.287	0.436	0.004	0.174	10.6
LP MB	DI 18	2	Polyunsaturated: saturated fat ratio (g/g)	74	0.834	0.298	0.007	0.174	9.6
LP MB	DI 16	2	Carbohydrate: saturated fat ratio (g/g)	87	0.018	0.009	0.04	0.331	8.5
CB	DI 16	2	Carbohydrate: monounsaturated fat ratio (g/g)	108	0.017	0.008	0.036	0.653	4.0
CB	DI 16	2	Carbohydrate: polyunsaturated fat ratio (g/g)	108	0.017	0.008	0.039	0.653	3.9
CB	DI 16	2	Monounsaturated fat energy (%kcal)	109	−0.021	0.011	0.049	0.653	3.4
CB	DI 16	3	Carbohydrate energy (%kcal)	110	0.052	0.021	0.015	0.087	5.1
CB	DI 16	3	Total fat energy (%kcal)	110	−0.045	0.017	0.011	0.087	5.7
CB	DI 16	3	Saturated fat energy (%kcal)	109	−0.029	0.011	0.013	0.087	5.6
CB	DI 16	3	Monounsaturated fat energy (%kcal)	108	−0.026	0.013	0.044	0.189	4.3
CB	DI 16	3	Carbohydrate: total fat ratio (g/g)	110	0.026	0.01	0.009	0.087	5.9
CB	DI 16	3	Sugars: total fat ratio (g/g)	110	0.017	0.007	0.012	0.087	5.5
CB	DI 16	3	Carbohydrate: saturated fat ratio (g/g)	110	0.022	0.008	0.006	0.087	6.6
CB	DI 16	3	Sugars: saturated fat ratio (g/g)	109	0.016	0.006	0.012	0.087	5.9
CB	DI 16	3	Carbohydrate: monounsaturated fat ratio (g/g)	110	0.022	0.008	0.009	0.087	5.9
CB	DI 16	3	Sugars: monounsaturated fat ratio (g/g)	109	0.014	0.006	0.025	0.131	4.7
CB	DI 16	3	Sugars: polyunsaturated fat ratio (g/g)	109	0.013	0.006	0.036	0.169	4.2
CB	DI 18	3	Glycemic index	59	−4.5	1.793	0.015	0.087	11.8

Abbreviations: DI: desaturation index; *n*: number of observations in the model; FDR: false discovery rate; EP MB: early pregnancy maternal blood; LP MB: late pregnancy maternal blood; CB: cord blood; SE: standard error of the beta estimate.

that the intrinsic bias in the baby size (the study only recruited mothers at high risk for a macrosomic child) might have reduced the variability necessary to reproduce these associations.

4.3. Dietary regulation and hormonal involvement

We found evidence for a modest but widespread regulation of DI_{16}, while DI_{18}, the SCD-1 marker involved in infant anthropometry, showed limited associations with dietary intakes during pregnancy.

Potential for dietary regulation of SCD-1 transcription and activity has been reported in non-pregnant populations and most proposed mechanisms involve sterol regulatory element-binding protein 1 (SREBP-1) [11,19]. In pregnant population, one study in mice observed a regulatory effect of maternal diet on offspring SCD-1 transcription and activity [22], but this was not replicated in humans [24]. In general, polyunsaturated fat was reported to downregulated SCD-1 transcription and activity, while carbohydrate, sugars and saturated fat were observed to upregulate it. These findings were only partially replicated in our study; we did find that higher SCD-1 activity markers were associated with elevated ratios of carbohydrates/sugars-to-fat, but we also found negative correlations with absolute carbohydrates and fat intakes.

Several factors might contribute to the discrepancy of our results with findings from animal models. First, in controlled animal studies the relative amount of fat or carbohydrate intake can be as elevated as 60 or 70%, respectively [21], while in our population (and, more generally, in real life) the intakes of total fat and carbohydrates are around 32–40% and 45–55% of total kcal intake, respectively. Thus the regulatory effect of diet on SCD-1 under real-life conditions in humans might be smaller than that reported in controlled animal experiments.

Second, the effects might additionally be confounded by long-term diet, which could also explain why we observed a strong association between maternal DI_{16} and maternal BMI, but not between DI_{16} and infant anthropometry. Seet and colleagues observed dysregulated DI_{16}, but not DI_{18}, in dams born to rats exposed to a high-fat diet prior to and during pregnancy [22], therefore a confounding effect of long-term dietary intake in their results cannot be excluded.

A third possibility is that a shift in SCD-1 regulation occurs during pregnancy, as it evolves from an initial anabolic state (during the first half) into a catabolic state (during the second half). Most studies have observed SCD-1 at one single point during pregnancy [22,24,25], while we have observed it both in early and in late pregnancy. The hypothesis of a regulatory shift might explain surprising findings such as negative associations with carbohydrates and sugars intakes (in the first half of pregnancy) and positive associations with polyunsaturated fat (in late pregnancy). Also the involvement of sex hormones should be further investigated to this regard. Estrogen has been shown to regulate SCD-1 transcription, mainly via mechanisms involving estrogen receptor alpha (ERα) and peroxisome proliferator-activated receptor gamma (PPAR-γ), mostly in the negative direction, but the experiments so far have been conducted in very inhomogeneous settings regarding species and hormonal status of the population. Gene expression of SCD-1 was reduced in ERα-knockout rats [35] and in post-menopausal women with estradiol supplementation [36]; no difference in gene expression, but elevated SCD-1 protein content, was observed in ovariectomized rats with supplementation of 17β-estradiol, progesterone, or both [37]. Nevertheless, we did not observe strong associations or confounding effects of leptin and insulin on SCD-1.

In summary, our results show that, in our population, neither dietary intake nor leptin or insulin are involved in the regulation of markers of SCD-1 activity associated with infant anthropometry, and we conjecture that long-term diet and hormones would be stronger regulator of SCD-1 during pregnancy.

4.4. Strengths and limitations

The ROLO dataset provided the unique opportunity to observe the associations between markers of SCD-1 activity during

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pregnancy and early infancy child anthropometry. We furthermore linked SCD-1 activity to maternal exposures such as diet in gestation and early pregnancy BMI. The exhaustive data about dietary intake made it possible to investigate the effect of carbohydrates, sugars and fat; however, a healthy diet index was not available, and we could not distinguish between sources and types of sugars (mainly fructose and glucose) and fat intakes (e.g. from nuts and seeds, oily fish, or processed oils).

Our LC-MS method does not distinguish the position of the double bond, but it is known from literature that oleic acid (18:1 n-9) is the most abundant 18:1 isoform in fasting plasma [38].

Our results concerning anthropometry were only in partial agreement with published studies [24,25]. With respect to this, it should not be neglected that the ROLO population has an intrinsic bias, since the babies were at higher risk for macrosomia. It is possible that this population shares common epigenetic traits related to the previous macrosomia. Another possible explanation for these discrepancies is of statistical nature, that is, the elevated population birth weight might have reduced the variability in the birth anthropometry measures.

Finally, we could not test our hypothesis that sex hormones regulate SCD-1, since the remaining biological material from the analyzed samples did not suffice for the measurement of hormones.

5. Conclusion

In this explorative analysis we found that maternal and fetal SCD-1 activity markers are associated with measures of infant adiposity up to 2 years of age, thus representing an involvement of SCD-1 in the long-term metabolic programming of the offspring. Nevertheless, we did not find any strong regulatory effect of short-term diet during gestation on SCD-1, and conjectured that this regulation might be preferentially regulated by long-term diet or pregnancy hormones. Moreover, our results support the hypothesis of a differential regulation of SCD-1 when using stearic or palmitic acid as substrates.

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Authors' contributions

Designed research: LM, CH, AGG, ECOB, BK, FMM; conducted research: AGG, ECOB, MKH, JMD; provided essential reagents or provided essential materials: OU, BK; analyzed data or performed statistical analysis: LM, CH, FK; interpreted results: LM, CH, AGG, ECOB; wrote paper: LM, CH; read and approved the paper: all authors. LM had primary responsibility for final content.

Conflict of interest

The authors have no conflict of interest.

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Appendix A. Supplementary data

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