The Lipidomic Features of Amniotic Fluid Associated with Fetal Macrosomia

by

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Abstract

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Background: The *in utero* metabolic environment promoting macrosomia is yet to be fully characterized. Strong evidence suggests that lipid dysregulation may underlie its development. Therefore, characterizing the lipidomic profile of amniotic fluid (AF) in pregnancies complicated with macrosomia can provide important insights to the pathological mechanisms involved. Given the wide array of the associated maternal characteristics, our investigation addressed macrosomia in two maternal groups: healthy normal-weight (NW) and over-weight/obese (Ov/Ob) otherwise healthy mothers. Our objectives were: 1) to compare the AF lipidome of large-for-gestational age (LGA) with appropriate-for-gestational age (AGA) infants in NW and Ov/Ob mothers; 2) to investigate the associations of LGA lipidomic features with AF concentrations of glucose and insulin; 3) to contrast the LGA AF lipidome between NW and Ov/Ob mothers. **Methods**: In this nested case-control study, bio-banked AF samples collected during routine amniocentesis performed at early 2nd trimester were analyzed. Untargeted lipidomics analysis was conducted using LC-QToF-MS platform. Univariate and multivariate statistical methods including PCA and PLS-DA were used to extract features with significant differences and high variable importance in projection scores. **Results**: One sphingomyelin specie (SM 14:0;O2/20:1) was markedly elevated in the AF of LGA infants and discriminated LGAs from AGAs in both NW and Ov/Ob mothers. SM 14:0;O2/20:1 also discriminated LGAs between NW and Ov/Ob mothers. NW mothers of LGAs showed enrichment of AF phosphatidic acid (PA) species when compared to AGAs, while the opposite trend (i.e. depletion of PA was evident in Ov/Ob mothers of LGAs. The comparison between Ov/Ob and NW mothers of LGAs revealed a depletion of several PA, phosphatidylcholine, and SM species along with elevated ceramide levels in Ov/Ob mothers. Insulin and glucose were not different between LGAs and AGAs and did not correlate to any of the differentially abundant lipidomic features. **Conclusion**: Macrosomia is associated with unique lipidomic features evident in early pregnancy AF of normoglycemic NW and Ov/Ob mothers. Maternal obesity clearly complicates the lipidomic signature of LGA pregnancies, which may result from differential placental metabolism in Ov/Ob mothers. SM 14:0;O2/20:1 consistently characterizes LGA complicated pregnancies which warrants further investigation of its role in macrosomia.

Résumé

Les caractéristiques lipidomiques du liquide amniotique associées à la macrosomie fœtale

Introduction: L'environnement métabolique in utero favorisant la macrosomie n'est pas encore entièrement caractérisé. De solides preuves suggèrent que la dysrégulation lipidique pourrait être à l'origine de son développement. Par conséquent, caractériser le profil lipidomique du liquide amniotique (LA) dans les grossesses compliquées par la macrosomie peut fournir des informations importantes sur les mécanismes pathologiques impliqués. Étant donné la large gamme de caractéristiques maternelles associées, notre recherche a porté sur la macrosomie dans deux groupes de mères : les mères en bonne santé de poids normal (PN) et les mères en surpoids/obèses (SP/Ob) autrement en bonne santé. Nos objectifs étaient : 1) comparer le lipidome du LA des nouveau-nés grands pour l'âge gestationnel (GAG) avec celui des nouveau-nés appropriés pour l'âge gestationnel (AAG) chez les mères PN et SP/Ob ; 2) étudier les associations des caractéristiques lipidomiques des nouveau-nés GAG avec les concentrations de glucose et d'insuline dans le LA ; 3) comparer le lipidome du LA des nouveau-nés GAG entre les mères PN et SP/Ob. **Méthodes**: Dans cette étude cas-témoins imbriquée, des échantillons de LA bio-banqués, recueillis lors d'amniocentèses de routine effectuées au début du deuxième trimestre, ont été analysés. Une analyse lipidomique non ciblée a été réalisée à l'aide de la plateforme LC-QToF-MS. Des méthodes statistiques univariées et multivariées, comme PCA et PLS-DA, ont été utilisées pour extraire des caractéristiques présentant des différences significatives et une importance variable élevée dans les scores de projection. **Résultats**: Une espèce de sphingomyéline (SM 14:0;O2/20:1) était nettement élevée dans le LA des nouveau-nés GAG et les a distingués des nouveau-nés AAG chez les mères PN et SP/Ob. La SM 14:0;O2/20:1 a distingué également les nouveau-nés GAG entre les mères PN et SP/Ob. Les mères PN des nouveau-nés GAG ont présenté un enrichissement des espèces d'acide phosphatidique (AP) dans le LA par rapport aux nouveau-nés AAG, tandis que une diminution de l'AP était évidente chez les mères SP/Ob des nouveau-nés GAG. La comparaison entre les mères SP/Ob et PN des nouveau-nés GAG a révélé une diminution de plusieurs espèces d'AP, de phosphatidylcholine et de SM, ainsi que des taux élevés de céramide chez les mères SP/Ob. Les taux d'insuline et de glucose n'étaient ni différents entre les mères des nouveau-nés GAG et AAG, ni corrélés à aucune des caractéristiques lipidomiques différemment abondantes. **Conclusion**: La macrosomie est

associée à des caractéristiques lipidomiques uniques qui sont visibles au début de la grossesse dans le LA des mères normoglycémiques PN et SP/Ob. L'obésité maternelle complique clairement la signature lipidomique des grossesses GAG, qui pourrait résulter du métabolisme placentaire différentiel chez les mères SP/Ob. La SM 14:0;O2/20:1 caractérise systématiquement les grossesses GAG, ce qui justifie une recherche plus approfondie de son rôle dans la macrosomie.

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Distinct Contribution to Knowledge

This research work is the first to profile the human amniotic fluid lipidome at early pregnancy in relation to birth weight outcomes, specifically macrosomia. The following summarizes the main findings of this thesis:

1. We provide evidence for a distinct lipidomic signature of the intrauterine compartment in early pregnant mothers who deliver large-for-gestational age infants in comparison to appropriate-for-gestational age infants. We demonstrated that this signature is influenced by maternal weight status, with sphingomyelins and phosphatidic acid species being key features of the associated lipid dysregulation.
2. Other important features were detected in amniotic fluid and related strongly to birth weight, such as acyl coenzyme A, phosphatidylcholines, and triglycerides.
3. We are the first to reveal that the impact of maternal obesity is also evident in the amniotic fluid lipidome. Despite similar pregnancy outcomes, overweight and obese mothers exhibited a unique lipidomic profile reflecting specific metabolic changes that are different from normal-weight women. Discriminating features included ceramides, hexosylceramides, phosphatidylcholines, triglycerides, in addition to sphingomyelins and phosphatidic acid species.
4. We showed that amniotic fluid concentrations of glucose and insulin were not related to the lipidomic features distinguishing large-for-gestational age from appropriate-for-gestational age infants nor normal-weight from overweight and obese mothers.

Contribution of Authors

This thesis was written in manuscript format and all chapters are authored by Isra'a Haj Hussein and supervised by Dr. Kristine Koski. It includes the following three manuscripts which are either published or in preparation for publication:

- Haj-Husein, I.; Kubow, S.; Koski, K.G. Untargeted Lipidomic Profiling of Amniotic Fluid Reveals Dysregulated Lipid Metabolism in Healthy Normal-Weight Mothers with Fetal Macrosomia. Nutrients 2024, 16, 3804. https://doi.org/10.3390/nu16223804
- Haj-Husein, I.; Kubow, S.; Koski, K.G. The lipidomic Features of Amniotic Fluid Associated with Fetal Macrosomia in Overweight and Obese Mothers.
- Haj-Husein, I.; Kubow, S.; Koski, K.G. Distinct Lipidomic Features of Large-for-Gestational Age Infants between Normal-Weight and Overweight/Obese Mothers.

For all manuscripts, Isra'a Haj Hussein, was responsible for conceptualization of the research work, study design, sample collection and sorting of the amniotic fluid biobank, coordination of lipidomics analysis, statistical and bioinformatics analysis, data interpretation and manuscripts writing.

Dr. Kristine Koski supervised the whole research project, revised and approved the final manuscripts. Dr. Kristine Koski served as the PhD candidate supervisor.

Dr. Stan Kubow participated in supervising the research work, revised and approved the final manuscripts.

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General Discussion

List of Abbreviations

AC	Acylcarnitine
Acer	Acylceramide
AF	Amniotic fluid
AGA	Appropriate-for-gestational age
AGPAT	Acylglycerol-3-phosphate acyltransferase
ANCOVA	Analysis of covariance
BW	Birth weight
BMI	Body mass index
Car	Acyl carnitine
Cer	Ceramide
DDA	Data-Dependent Analysis
DA	Differentially abundant
DG	Diglyceride
DHA	Docosahexaenoic acid
DNFS	De novo fatty acid synthesis
FDR	False Discovery Rate
GDM	Gestational diabetes mellitus
GPAT	Glycerol-3-phosphate acyltransferase
HexCer	Hexosyl ceramide
hPGH	Human placental growth hormone

hPL	Human placental lactogen
IRS-1	Insulin receptor substrate-1
LGA	Large-for-gestational age
LPC	Lysophosphatidylcholine
LPE	Lysophosphatidylethanolamine
LPI	Lysophosphatidylinositol
mTOR	Mammalian target of rapamycin
MUFAs	Monounsaturated fatty acids
NAT	N-acyl amine
NEFAs	Non esterified fatty acids
NW	Normal weight
Ov/Ob	Overweight/Obese
PCA	Principal Component Analysis
PC	Phosphatidylcholine
PG	Phosphatidylglycerol
PHTs	Primary human trophoblast cells
PLS-DA	Partial least squares discriminant analysis
PUFAs	Polyunsaturated fatty acids
QC	Quality control
QToF	Quadrupole Time-of-Flight
RSD	Relative standard deviation
SFA	Saturated fatty acids
SM	Sphingomyelin

SPTLC3	Serine palmitoyl transferase subunit 3
ST	Sterol
TGs	Triglycerides
TMIC	The Metabolomics Innovation Center
UHPLC	Ultra-high performance liquid chromatography
VIP	Variable importance in projection
WE	Wax ester

Chapter 1: Introduction

Macrosomia is a term used to describe fetal overgrowth, and although there is no consensus on a clear-cut definition criterion, it is commonly defined as a birthweight (BW) above 4000 g. Large-for-gestational age (LGA) is an alternative term for fetal overgrowth, and it is defined as a BW above the 90th percentile for a given gestational age. Worldwide, the prevalence of macrosomia ranges from 0.5% in India to 14.9% in Algeria (1). In Canada, the rate of LGA was 9.6% in 2022 (2). Macrosomia is associated with an increased risk of several maternal and neonatal complications, most notably cesarean section and fetal shoulder dystocia (3, 4, 5). The metabolic, endocrine, immune and epigenetic alterations associated with fetal macrosomia are likely to exert an in-utero programming effect that further perpetuates the risk of several health conditions later in life, including obesity, type 2 diabetes mellitus (T2DM), and cardiovascular diseases (6, 7, 8, 9). Such increased risk has been frequently reported by epidemiological studies (10, 11, 12). Gestational diabetes mellitus (GDM) and maternal pre-pregnancy obesity are consistently reported among the strongest contributors to fetal macrosomia (3, 13-18), and given their increasing prevalence worldwide (19, 20, 21), they provide the most common context to investigate macrosomia and its etiopathology.

The underlying mechanisms of macrosomia are not yet determined. Macrosomia has often been viewed as a physiological response to maternal hyperglycemia, and research has focused on targeting glycemic control to normalize the odds of macrosomia, however, with limited success (9, 22, 23, 24). In the meanwhile, the role of maternal lipid metabolism became well recognized, with evidence indicating that its markers predict BW and fetal adiposity better than glucose (9, 23, 24). In support of such role, several metabolomics and lipidomics studies revealed significant alterations in maternal lipid metabolism in direct relation to fetal overgrowth (25, 26, 27).

However, these studies mainly focused on maternal blood and primarily during mid-to-late pregnancy, while the studies that investigated the intrauterine compartment are limited to cord blood or placental tissue collected after parturition. To our knowledge, there are no studies investigating the lipidomic profile of the intrauterine compartment early in pregnancy, which has the potential to provide deep insights on the *in-utero* environment promoting augmented fetal adiposity.

Amniotic fluid (AF) occupies the amniotic cavity. It surrounds the developing fetus, provides thermal and mechanical protection, and supports fetal growth and health (2, 28). AF is thought to arise as a transudate of maternal and/or fetal plasma (29), as fluids originating from maternal plasma can easily diffuse through the simple epithelium of the embryonic skin and across surfaces of the amnion, placenta, and umbilical cord. Furthermore, the bidirectional flow of fluids across the non-keratinized fetal skin contributes to AF composition specifically at early pregnancy, and uniquely reflects on the interaction between maternal and fetal metabolism (29). Fetal skin keratinization begins at 19-20 weeks and it is fully attained at 25 weeks of gestation (30), hence the importance of the time at which amniocentesis is performed to collect AF in relation to its composition. Studies on human and animal fetuses were able to demonstrate that AF swallowing impacts fetal growth and that conditions of esophageal ligation or atresia of the gastrointestinal tract are associated with lower birth weights (31, 32).

Total lipid content of AF increases with advancing gestation, reaching a maximum concentration near term, and this is mainly related to fetal surfactant production and pulmonary secretions into AF (33), which significantly increase during the second half of pregnancy (34). Hence, the dominance of phospholipids among AF lipids is well justified, as it reaches about 25% of AF lipids at term. The main circulating phospholipids of AF throughout pregnancy are

phosphatidylcholines, sphingomyelins, phosphatidylserines, phosphatidylethanolamines, and phosphatidylinositols (35, 36). Other major classes include free fatty acids, cholesterol, and glycerides (35, 36).

Although it is considered as a dynamic complex mixture that represents both maternal and fetal metabolism especially at early pregnancy, AF is an underutilized resource for studying and identifying perturbed metabolic pathways and biomarkers that are indicative of fetal health (30, 37). This is likely because of the invasive nature of amniocentesis (38). Within our research context, there are no studies profiling the AF lipidome in relation to macrosomia, which is what we aimed to provide.

1.1 Design Rationale

Epidemiological studies investigating LGA deliveries emphasize a wide spectrum of maternal characteristics with reference to pre-pregnancy body mass index (BMI) and glycemic status, despite having obesity and GDM as well-established risk factors. Jolly *et al*. (2003) studied about 350,000 completed singleton pregnancies in the UK and reported that from about 35,000 LGA infants born, 45% were from mothers with normal BMI, 50% from over-weight and obese mothers, while only 6% were from mothers with GDM or pre-existing diabetes (16). Similarly, Hua et al. (2019) studied 16,896 singleton pregnancies not complicated with diabetes in China and reported that 77.8% of LGAs were delivered to mothers with normal BMI (39). Therefore, it is compelling to address features of lipid alteration in LGA pregnancies in these two major maternal populations: Healthy normoglycemic mothers with normal body weight/BMI and normoglycemic overweight/obese mothers how are otherwise healthy. In specific, we are asking what are the lipidomic features characterizing LGA from appropriate-for-gestational age (AGA) pregnancies within normal-weight and overweight/obese maternal populations? And how similar are the

lipidomic features of LGA pregnancies between normal-weight and overweight/obese mothers? We aimed to address these questions using early pregnancy AF, to reflect more on the intrauterine environment. Up to our knowledge, this is the first research project to utilize AF in this research context.

1.2 Objectives

Using second trimester AF samples, we aimed to:

1) Contrast the lipidomic profiles between LGA and AGA infants in each of the following maternal groups: a) Healthy mothers with normal-weight (1st study), b) Overweight/obese otherwise-healthy mothers (2nd study).
2) Contrast the lipidomic profiles of LGA infants between mothers with normal-weight mothers and mothers with overweight/obesity.
3) Investigate the associations of LGA lipidomic features with AF concentrations of glucose and insulin

1.3 Hypotheses

For our first two studies, we hypothesize that the AF lipidome of LGA mothers will be distinguishable from that of AGA mothers in both maternal populations: healthy normal-weight mothers and overweight/obese mothers. We also hypothesize that the differential features will correlate with AF concentration of glucose and insulin. Undetected lipid dysregulation and glucose intolerance may underly LGA pregnancies. For our third study, we hypothesize that LGA mothers, regardless of whether they are normal-weight or overweight/obese, will exhibit similar lipidomic profiles and similar glucose and insulin concentrations, given their similar pregnancy outcomes.

Chapter 2: Literature Review

2.1 Maternal Metabolic Adaptations during Pregnancy

Complex physiological and metabolic adaptations occur during pregnancy which ultimately ensures an optimal supply of nutrients to the *in-utero* compartment. Maternal nutrition and metabolic health status greatly affect the transfer of nutrients to the fetus, thereby affecting fetal nutrition and growth (23). Understanding maternal metabolism of glucose and lipids, as relevant to our research context, in addition to their placental transport mechanisms is essential to understand the underlying pathophysiology of macrosomia. The following sections are dedicated to briefly describe the current knowledge on these topics.

2.1.1 Maternal Glucose Metabolism

The pregnancy period can be divided into two major phases based on the prevailing metabolic features. The first half is considered an anabolic phase driven by enhanced insulin secretion, which, as an anabolic hormone, enhances maternal accumulation of nutrient stores (40, 41). While fasting insulin remains normal (42), first phase insulin secretion doubles by the end of the first trimester (12 to 14 weeks) in comparison to pre-pregnancy insulin response, which is estimated by measuring area under the glucose curve during 0 to 5 minutes after glucose infusion in an intravenous glucose tolerance test (43). Such anabolic metabolism and the consequent increase in maternal energy stores will support the accelerated fetal growth during the second half of gestation, which is regarded as a catabolic phase (40, 41).

Alterations in insulin secretion and action are more prominent during the second half of gestation, which is considered a catabolic phase. Insulin sensitivity is greatly reduced (42, 43) and this triggers a maternal catabolic state, in which more fat is utilized for maternal energy supply while glucose is essentially spared for fetal growth (40). Estimates of insulin sensitivity using the

hyperinsulinemia-euglycemic clamp in late gestation show a significant decrease of about 56% when compared to pre-pregnancy levels (43). The increase in several hormones' levels, i.e. pregnancy orchestrating hormones, such as progesterone, estrogen, human placental lactogen (hPL), and human placental growth hormone (hPGH), is thought to mediate insulin resistance during pregnancy (44).

Concomitant to the state of insulin resistance, insulin secretion is greatly augmented with advancing gestation. By 34 to 36 weeks, fasting insulin was found to increase by 65%, while first and second phase insulin response showed a 3.5- and 3-fold increase respectively, compared to pre-pregnancy levels (45). Furthermore, fasting maternal levels of glucose decrease with advancing gestation. Although the exact underlying mechanism for maternal hypoglycemia is not fully understood, a number of factors may contribute to this drop in glucose levels, including the dilutional effect of increased plasma volume, which occurs progressively throughout normal gestation, and the increase in feto-placental glucose uptake and utilization during late gestation, a period of accelerated fetal growth (41).

2.1.2 Placental Glucose Transport

Glucose transport is mediated through facilitated diffusion, which is dependent on glucose concentration gradient. A number of glucose transporters (GLUTs) are present in both membranes of the syncytiotrophoblast (46), a single cellular layer covering the chorionic villi and separating maternal and fetal circulation (47). Maternal blood in the intervillous space contacts the microvillus membrane of the syncytiotrophoblast, while its basal membrane is adjacent to fetal capillaries containing fetal blood (47). Insulin-independent GLUT1, is considered as the primary and most abundant placental glucose transporting protein (48). Insulin-responsive GLUT4 is

expressed in placental stromal cells and is thought to play a role in the transport and the conversion of glucose to glycogen in response to fetal insulin (49).

Insulin membrane receptors are also found in the placenta throughout the whole gestational period. The role insulin plays on placental glucose uptake and transfer is still not fully elucidated. Earlier research suggested that the placenta was insulin resistant (50). However, newer reports indicated otherwise (51, 52). Basak *et al*. (2019), for example, reported a significant increase in GLUT1 expression and consequently glucose uptake in early gestation derived trophoblast cells upon stimulation with insulin, and that silencing of GLUT1 diminished insulin-stimulated glucose uptake (51). In this regard, it is important to note that glucose transporters, other than GLUT1, play an insignificant role in the first trimester placenta, and that GLUT1 is essential for embryonic development and implantation (51). Moreover, GLUT1 expression in syncytiotrophoblast basal membrane was found to correlate positively with birth weight (53), while its expression and activity on the apical microvillous membrane were down regulated in pregnancies complicated with preeclampsia, which had significantly lower birth weights compared to normal pregnancies (54).

Insulin receptors are thought to be expressed in a spatio-temporal manner. Their expression changes from being primarily on the maternal side of the syncytiotrophoblast in early pregnancy to the placental endothelium of fetal capillaries towards the end of pregnancy. Therefore, these receptors are more responsive to maternal insulin in early pregnancy, then fetal insulin plays the predominant role by late pregnancy (55, 56). Insulin anabolic effects include glycogen synthesis in placental endothelial cells and lipid droplets formation in term trophoblasts (55). In fact, about 40-50% of placental glucose uptake is directed to the fetus, while the remainder is utilized by the

placenta itself (40). Maternal insulin secretory response at early pregnancy was found to positively associate with placental volume at early pregnancy and placental weight at birth (57).

2.1.3 Maternal Lipid Metabolism

Maternal lipid metabolism undergoes major changes in response to changes in glucose metabolism (40). The first two thirds of gestation are characterized by an increase in maternal fat depot accumulation, which is a result of the anabolic metabolism featured with an increased insulin secretion, enhanced lipogenesis, hyperphagia, and perhaps an enhanced insulin responsiveness of the adipose tissue (40, 58). The activity of adipose tissue lipoprotein lipase (LPL) is either unchanged or increases during early gestation. LPL facilitates the accumulation of maternal fat depots as it hydrolyzes the triglyceride content of circulating triglyceride-rich lipoproteins, i.e. chylomicrons and very low density lipoproteins (VLDL) (58).

Late gestation or the last trimester is characterized by a maternal catabolic state, which is driven by insulin resistance. The impact on adipose tissue metabolism includes decreased fatty acid synthesis consequent to decreased LPL activity. The latter results in reduced tissue uptake of triglycerides and hence contributes to the development of maternal hypertriglyceridemia, which is one of the most striking changes in maternal lipid metabolism. Concomitant to insulin resistance is also the increase in adipose tissue lipolytic activity. Pregnancy hormones contribute to insulin resistance, such as hPL, hPGH, progesterone and estrogen, as mentioned previously (44). Several cytokines secreted by the adipose tissue, known as adipocytokines, also mediate insulin resistance in pregnancy, including leptin, tumor necrosis factor-α (TNF-α), interleukin-6, visfatin, and resistin. Simultaneously, cytokines associated with enhanced insulin sensitivity, adiponectin and apelin, were found to decrease. This effect, i.e. insulin resistance and the associated lipolytic activity, is not only mediated by the adipose tissue, but also by the placenta, as these

adipocytokines are mostly derived from the placenta during pregnancy (58, 74). The increase in non esterified fatty acids (NEFAs) resulting from active adipose tissue lipolysis further induces insulin resistance, as does the reduction of transcription factor peroxisome proliferator-activated receptor gamma (PPAR-γ). The latter is known to be highly expressed in adipose tissue and it helps regulate adipogenesis and lipolysis through binding to several genes and inducing the synthesis of proteins like adiponectin and LPL (58).

The ultimate results of changes in maternal lipid metabolism are clearly manifested in maternal lipid profile. Maternal hyperlipidemia mainly corresponds to an increase in triglyceride level (40, 58). Cholesterol and phospholipids also rise but to a lower extent than that of triglycerides (40, 58). Unlike a modest non-significant increase during the first trimester, blood levels of total triglycerides increase markedly during the second and third trimesters, more than 2.5 times higher than the levels of non-pregnant women (59, 60, 61). Triglycerides increase in all lipoprotein fractions, but the greatest increase corresponds to VLDLs triglycerides (58). Interestingly, disruption of maternal hyperlipoproteinemia in animal studies, by lipid lowering drugs, showed a negative impact on fetal development. In contrast, maternal plasma triglyceride levels significantly correlate with birth weight (40), which indicates a role of maternal lipids in predicting fetal adiposity. A concept we will expand on in relation to the pathophysiology of macrosomia.

2.1.4 Placental Transport of Lipids

Placental transport of lipids has not been fully elucidated, but its concept has been developed based on the available data and extrapolation from other tissues. The vast majority of circulating maternal fatty acids are found as complex lipids in lipoproteins, and only about 1-3% are found as NEFAs in maternal blood (58). Specific receptors for lipoproteins mediate endocytosis of

lipoproteins into the syncytiotrophoblast and its content of triglycerides is hydrolyzed by intracellular lipases. Lipoproteins can also be hydrolyzed extracellularly by placental lipase as they bind to surface receptors on the syncytiotrophoblast (58, 62). The extracellularly released NEFAs and those derived directly from maternal circulation are up-taken by fatty acid binding proteins (FABPs), which facilitate the intracellular channeling of fatty acids, and these include a placenta-specific protein found exclusively on the microvillous membrane (p-FABPpm), fatty acid transport proteins (FATP)-1, FATP-4, and fatty acid translocase (FAT/CD36) (58, 59, 63). In addition, fatty acids can cross the phospholipid bilayer membrane of syncytiotrophoblast by simple diffusion (59).

In the syncytiotrophoblast, there are cytoplasmic FABPs. The major types are isomers that were first identified in the heart (H-FABP) and liver (L-FABP) (59). Cytoplasmic FABPs bind to fatty acids inside the syncytiotrophoblast and facilitate their channeling into various metabolic routes, such as β-oxidation, conversion into eicosanoids, and re-esterification to form phospholipids and triglycerides which can be stored as intracellular lipid droplets (58). NEFAs can cross the basal membrane of the syncytiotrophoblast through simple diffusion or by the FABPs located on the basal membrane. Once released into fetal circulation, carrier proteins such as albumin, the principal carrier, and α-fetoprotein transport NEFAs to fetal liver (59), where they are metabolized or esterified to form lipoproteins (58). Furthermore, fatty acids may also be esterified into glycerides and phospholipids and then released into fetal circulation (40).

It is important to note that placental transport of fatty acids seems to be dependent on the transplacental concentration gradient of NEFAs relative to the available binding proteins, i.e. NEFAs to albumin. Due to a rapid increase in fetal utilization of fatty acids, fetal concentration of circulating NEFAs drops markedly during the second half of pregnancy. Maternal circulating

levels of NEFAs, on the other hand, increase with advancing gestation, reaching about three times the concentration of fetal plasma at term. The albumin levels are also higher on the fetal side than on the maternal side, unlike fetal lipoproteins which decrease with advancing gestation. Consequently, the ratio of NEFAs to albumin on the fetal side drops to about one quarter the ratio on the maternal side at term. Such difference creates a pull on intracellular NEFAs within the syncytiotrophoblast towards fetal plasma (59).

Maternal cholesterol is also effectively transported across the placental syncytiotrophoblast. Placental lipoprotein receptors mediate the uptake of cholesterol-carrying lipoproteins. Their content of cholesteryl esters is hydrolyzed, and cholesterol is secreted out of the basal side via protein-mediated processes or by aqueous diffusion (64). Other lipids crossing the placenta include glycerol, which is transported through simple diffusion, however, the rate of transfer is lower than other metabolites with similar molecular characteristics, such as glucose and alanine (40). Ketone bodies cross the placenta by simple diffusion or through carrier mediated processes. Their placental transfer is considered very efficient and concentration dependent until equal concentration is attained between fetal plasma and maternal plasma (65).

2.2 Amniotic Fluid

2.2.1 Origin and Development

As it occupies the amniotic cavity and surrounds the developing fetus, amniotic fluid (AF) provides a significant protective environment against mechanical and thermal adverse events. It also presents a reservoir of fluid, nutrients, and antimicrobial peptides which serve to support fetal growth and health (28, 66). During early gestation, i.e. from fertilization to 8 weeks, fluids originating from maternal plasma can easily diffuse through the simple epithelium of the

embryonic skin and across the surfaces of the amnion, placenta, and umbilical cord. Therefore, the AF is thought to arise as a transudate of maternal or fetal plasma (67).

By 8 weeks of gestation, fetal urinary system starts to produce urine into the amniotic sac. Skin keratinization begins at 19 to 20 weeks and is fully attained at 25 weeks, which prevents the bidirectional diffusion of fluids across the fetal skin (28, 68). Thereafter, fetal urine production becomes the predominant contributor to AF (300 ml/kg fetal weight/day). AF also contains secretions from fetal pulmonary and gastrointestinal systems. In fact, fetal lung surfactant production contributes significantly to AF content, particularly during the second half of gestation. It is estimated that the lung fluid production is about one third of urine production, and half of these fluids are swallowed by the fetus (67).

Fetal swallowing is considered the predominant route of AF elimination during the second half of gestation (67). It was observed as early as 16 weeks, and the swallowed fluid is absorbed through the fetal gastrointestinal system to be either recycled through kidneys or transferred to the maternal circulation through the placenta (69). The second route is the intramembranous pathway which allows the transfer of fluids across the amniotic membranes back to fetal circulation, via hydrostatic and oncotic forces. The human amnion consists of a single layer of epithelial cells, and intercellular channels between the tight junctions of amniocytes allow the backflow of AF fluids into fetal vessels (68). It is estimated that the amount of AF eliminated by swallowing and intramembranous flow approximately equals the amount produced by urine production and lung secretions, therefore maintaining the regulation of AF volume during the second half of gestation (67). In addition to bulk flow of fluid, bidirectional flow of water, through diffusion, between the amniotic and maternal compartments continue throughout pregnancy, however, with no net change in fluid volume (69).

2.2.2 Composition and Volume

The AF is about 98-99% water (70), and its composition reflects fetal developmental stages throughout gestation. During early gestation, the AF is a dialysate that is identical to fetal and maternal plasma but contains minimal protein (69). It also contains an increased concentration of sugar alcohols, which are the products of anaerobic metabolism (67). As urine production starts to contribute to AF, AF composition starts to diverge from that of plasma. Since fetal urine is hypotonic, AF osmolality and sodium concentration decreases, and it progressively becomes a hypotonic fluid (250–260 mOsm/liter near term), with increasing concentrations of urea, uric acid, and creatinine (69). It is estimated that AF osmolality decreases to approximately 85-90% of maternal plasma during the second half of pregnancy, while urinary byproducts increase two to three times higher than fetal plasma (67).

A linear relationship between AF volume and fetal size is observed during the first half of gestation, which may be explained by the predominant contribution of fetal skin dialysis to AF volume. AF volume more than doubles from 12 weeks to 16 weeks of gestation, which is the time at which amniocentesis is often performed. As fetal skin keratinization is fully accomplished, the relationship is no longer linear, and a greater variance in volume is observed from 20 weeks on. An average volume of 800 ml is reached by 40 weeks of gestation, beyond which volume starts to decrease sharply (67, 69).

2.2.3 Characteristics of AF Lipid Profile

Early studies carried out during the past century helped establish the major characteristics of the AF lipid profile (71-74), which mainly includes the following lipid classes: Phospholipids, sterols, hydrocarbons, glycerides, and free fatty acids (72, 73). Total lipids increase with advancing gestation (72, 73), and phospholipids are considered the largest lipid class (about 25% of AF

lipids), followed by free fatty acids, cholesterol esters and free cholesterol, hydrocarbons, and glycerides (72, 73). The main circulating phospholipid in AF is lecithin (i.e. phosphatidylcholine). Sphingomyelin, phosphatidylethanolamine, phosphatidylserine, and phosphatidylinositol are also major circulating phospholipids (72-74). Furthermore, squalene is the major hydrocarbon, and glycerides are mostly enriched with saturated and monounsaturated fatty acids in term AF. Palmitic and palmitoleic (16:1) acids are the most prominent fatty acids in glycerides (72).

A more comprehensive analysis of AF total fatty acids at weeks 12 to 20 revealed the presence of 32 fatty acids, the majority of which were saturated fatty acids (SFAs) (50.9%), whereas monounsaturated fatty acids (MUFAs) and polyunsaturated fatty acids (PUFAs) constituted 24.7% and 23.7% respectively. Trans fatty acids were also detected in AF, but in much lower quantities (1.1%). In line with previous results, the major fatty acids found, which had a relative proportion of > 5%, were palmitic, stearic, oleic, linoleic, and arachidonic acids. Minor fatty acids detected (1 to 5%) included myristic, arachidic acid (C20:0), behenic acid (C22:0), lignoceric acid (C24:0), palmitoleic acid (C16:1n-7), vaccenic acid (C18:1n-7), nervonic acid (C24:1n-9), and docosahexaenoic acid (C22:6n-3, DHA) (75).

AF concentration of cholesterol was found to increase after the first trimester (75-77). Cholesterol precursors, including lanosterol, dihydrolanosterol, and lathosterol, were detected in AF in very low levels at 15 weeks of gestation, but their levels significantly increase after 19 weeks, indicating an increase in fetal synthesis of cholesterol (76). Similar findings were also reported for other cholesterol precursors, namely 7-dehydrocholesterol and desmosterol, which were also found to significantly increase during the second half of gestation (77). It was suggested that during the first half of gestation, the fetus relies on maternal supply of cholesterol, as

evidenced by the presence of β-sitosterol in AF, which is a phytosterol not synthesized in humans and only supplied by maternal diet (76, 77).

2.3 Macrosomia: Overview

Although there is no clear-cut definition for fetal macrosomia, it is generally defined by a birth weight > 4000 g. The use of a clinical value that is independent of gestational age, such as LGA, is usually preferred. LGA is defined as having a birth weight above the 90th percentile for gestational age. Macrosomia is associated with increased risks of several maternal and neonatal complications (3). A meta-analysis study concluded that deliveries of macrosomic neonates were associated with about 2-fold increased risk of emergency cesarean section, perineal injuries, and postpartum hemorrhage (5), which is excessive blood loss after delivery and a leading cause of maternal mortality worldwide (4). In addition, a more pronounced risk of neonatal complications in macrosomic neonates was reported, including shoulder dystocia and birth fractures, with increased risks of 9.45 and 6.43 folds, respectively (5). Furthermore, children born with macrosomia appear to face an increased risk of several health issues later in life, including obesity (11), type 2 diabetes mellitus (T2DM), cardiovascular diseases (10) and certain types of cancers, such as lung cancer and colon cancer (78), despite some inconsistent evidence. In case of T2DM for example, U-shaped and J-shaped curves were reported for its risk in association with birth weight (12, 10). In contrast, two meta-analyses reported an inverse relation between birth weight and T2DM risk (79, 80). Until the dispute is resolved, the well-established metabolic factors associated with an increased risk of macrosomia are likely to exert an early in-utero programming effect, and, hence, impact adult health one way or the other.

A number of risk factors were found to significantly contribute to fetal macrosomia. GDM is a well-established risk factor (3). In a meta-analysis study, He *et al*. (2015) reported that GDM

was independently associated with macrosomia with an adjusted odds ratio (OR_{adj}) of 1.7 (13). Maternal pre-pregnancy overweight/obesity is also consistently reported as a strong contributor (81-85). Furthermore, Usta *et al*. (2017) investigated macrosomia risk factors in a group of more than 4000 non-GDM women and reported the following: in addition to pre-pregnancy maternal overweight and obesity, which had an OR_{adj} of 3.17 and 5.64 respectively, gestational weight gain ≥ 12kg (OR_{adj}=5.45), parity > 1 (OR_{adj}= 1.76), male fetal sex (OR_{adj}= 1.89), and maternal age ≥ 30 years (OR_{adj}=1.49) significantly increased the odds of macrosomic infants (15). Similar results were reported by Nkwabong and Tangho (2015). In addition, they found that paternal BMI ≥ 30 and post-term delivery (OR 1.9) were also significant risk factors (86).

2.3.1 Pathophysiology

Pederson hypothesis is one of the very early theories formulated to justify fetal overgrowth frequently encountered with type-1 diabetic mothers. Pederson J. stated that: "maternal hyperglycemia results in fetal hyperglycemia and, hence, in hypertrophy of fetal islet tissue with insulin-hypersecretion. This again means a greater fetal utilization of glucose. This phenomenon will explain several abnormal structures and changes found in the newborn". In other words, Pederson suggested that the fetal hyperinsulinemia, as a consequence of excess glucose flux from mother to fetus, induces overgrowth of fetal tissues. This concept is well-supported by scientific evidence (22). Hyperglycemia is among the major metabolic factors investigated for its association with macrosomia. Maternal fasting glucose concentrations were found positively associated with birth weight at mid-to-late gestation (25-32 weeks) in non-GDM mothers (87-89). However, this association was not significant at gestational weeks 14-16 and 18, as reported by Voldner *et al.* (2010) and Kulkarni *et al*. (2013) respectively (87, 89). In addition, cord blood levels of insulin are found to significantly correlate with fetal birth weight (90-92). A similar relation with AF

insulin levels was also found (93), although it is not consistently reported (91, 94). Moreover, in animal models, insulin injection or infusion into rat or monkey fetuses significantly increases their body weight (95, 96).

However, Pederson hypothesis still fails to justify the following two major aspects in relation to macrosomia. First, GDM mothers with optimal glucose control and obese mothers with normal glucose tolerance are consistently reported to have a higher risk of delivering macrosomic infants (77, 97-99). Feig *et al*. (2017) reported an incidence of 53% LGA infants and 36% extremely-LGA infants in T1DM mothers despite optimal levels of glycosylated hemoglobin with the use of continuous glucose monitoring, which improved birth weight outcomes (100). Voormolen *et al*. (2018) reported an incidence of 30% LGA infants for mothers with type T1DM, T2DM, and GDM, despite the use of continuous glucose monitoring to achieve optimal glucose levels (101). These rates are much higher than the reported figures of LGA for the general population. In Canada, for example, the rate of LGA infants in 2022 was 9.6% (2). Therefore, although improvement in glucose control attenuates the odds of macrosomia, other factors are likely to play a role in pathophysiology, given the high rates of macrosomia not resolved with glucose control.

Secondly, while Pederson hypothesis suggests that excess glucose, along with hyperinsulinemia, derives the augmented fetal adiposity, the relative contribution of de novo lipogenesis to neonatal birth weight and adiposity is not yet well-established (23, 89, 102). De novo fatty acid synthesis (DNFS), which converts carbohydrates to fatty acids that can be oxidized for energy or esterified to form storage triglycerides, has been demonstrated in vitro in fetal liver, subcutaneous adipose tissue, and other fetal tissues as well. Insulin was indeed found to stimulate DNFS in fetal subcutaneous adipose tissue (85). The capacity for DNFS seems to be very limited before 28 weeks of gestation and increases markedly after (103). DNFS in fetal liver was also

found diminished in comparison to the adult liver, based on in vitro results from hepatocytes (aged 16 weeks of gestation) (102). Interestingly, measurement of lipid content in different fetal tissues at 8, 16, 23, 24, 26 and 28 weeks of gestation showed 2.5 and 3 times increase in liver and brain lipids, respectively, from 8 to 16 weeks of gestation while remained constant thereafter. The increase in the lipid content of these tissues at early gestation is not a function of DNFS and maternal fatty acids must be the precursors of fetal lipids, at least before 28 weeks (103).

Furthermore, evidence from adult human and animal studies also indicate that high fat diet (104, 105), and long chain NEFAs, PUFAs in particular, suppress DNFS (106, 107). Long chain PUFAs are biomagnified by the placenta in fetal circulation (7), which may also exert a suppressive effect on fetal DNFS. PUFAs can also induce adipocyte differentiation, a process that occurs as early as 14 weeks of gestation and is further subjected to hormonal regulation. Insulin, as expected, potentiates the adipogenic effects of PUFAs (108), and increases the activity of placental lipoprotein lipase, which hydrolyses the lipid content of maternal lipoproteins to be transported to the fetus (9). Moreover, the largest development in fetal adipose tissue occurs during the third trimester and it coincides with the physiological increase in maternal levels of triglycerides (TGs) and NEFAs (102). Such increase is augmented and initiated much earlier in pregnancies complicated with GDM and obesity (23, 109-111). Therefore, it may be reasonable to assume that fetal esterification of maternally supplied lipids, rather than DNFS, plays a major role in fetal adiposity.

In further support of this concept, hypertriglyceridemia is frequently reported as a significant marker for macrosomia in healthy mothers during late gestation (88, 89, 112). Early in pregnancy, i.e. at 12-14 weeks, maternal triglycerides were also significantly higher in healthy mothers who gave birth to macrosomic infants than those with normal birth weight (113). Interestingly, Wang

et al. (2018) found that each 1 mmol/L increase in triglycerides, at late gestation, resulted in 27% increase in macrosomia risk, while each 1 mmol/L HDL-cholesterol increase resulted in 37% decrease in macrosomia risk among healthy mothers (112).

In GDM women, similar results, i.e. higher maternal triglycerides in association with macrosomia, were reported in mid-to-late gestation (114, 115, 116). Maternal triglycerides and NEFAs, but not glucose, were reported to positively correlate with neonatal adiposity at late gestation (116). In fact, maternal glucose levels were found to explain no more than 5% of BW variability in diabetic mothers (81). This corresponded with gene expression changes in women with GDM and type 1 diabetes, of which 67% were related to lipid transport pathways and 9% to glucose transport pathways, compared to healthy women (82).

In obese mothers, maternal fasting and postprandial triglycerides had a stronger association with newborn fat percentage than glucose had at early (14-16 weeks) and late pregnancy (26-28 weeks) (111). Correlations with fasting and postprandial glucose levels were not even reported at early pregnancy in some studies (89, 111). In conclusion, there seems to be strong evidence for the involvement of maternal lipids in the pathophysiology of macrosomia. Such association is further confirmed by lipidomics profiling of maternal blood. The following sections will further describe placentas reviewed in the following sections.

2.3.2 Lipidomic Alterations in Relation to Birth Weight, GDM, and Maternal Obesity

Several studies investigated the metabolomic and lipidomic profile of human maternal and cord blood in relation to birth weight (BW). Disparate and inconsistent results are evident, but a comprehensive review based on the major classes of lipids and the timing at which screening was performed during pregnancy can help draw some general lines or conclusions. Given that maternal obesity and GDM are major risk factors contributing to macrosomia, it is also important to review

their characterizing lipidomic features and their alignment with features of macrosomia. Of note, in this review, only lipid-related species are extracted from metabolomic results. Reviewing the analysis results of maternal blood obtained during pregnancy was prioritized over cord blood which is obtained at the time of delivery. Labor-induced changes in the lipidomic profile are likely to occur and, hence, cord blood may not reflect the actual lipidomic characteristics of maternal or *in utero* environment during pregnancy. Furthermore, the below mentioned lipidomic features associated with increased BWs mainly represented profiles of healthy mothers who were not diagnosed with diabetes or GDM, and the majority of such reported studies adjusted for maternal age and pre-pregnancy body mass index (BMI).

2.3.2.1 Phospholipids

Alterations in this lipid class are frequently reported. Early in pregnancy, at 11 to 14 gestation weeks, blood levels of different species of phospholipids were reported to decrease in relation to BW in healthy mothers, including phosphatidylcholine (PC), lysophosphatidylcholine (LPC), lysophosphatidylethanolamine (LPE), and lysophosphatidylinositol (LPI) (25, 26, 27), however, during second and third trimesters, associations were not consistently reported (25, 26, 83, 84, 117, 118). Similarly, early in pregnancies complicated with GDM, lower blood levels of several phospholipids including PCs, particularly with polyunsaturated fatty acyl chains, were reported in comparison to controls (119-121). In addition, PCs with fatty acyl chains of higher saturation may be elevated in GDM women; Lu *et al*. (2016) identified four lipid biomarkers as predictive of GDM risk, among which was PC (C32:1, positively correlated) (120). Furthermore, the majority of studies on GDM mothers, during second and third trimesters, indicate reductions in phospholipids, including LPCs, LPI, and lysophosphatidylserine (LPS), with various lengths and saturation degrees of fatty acyl chains (85, 119, 121-123). In relation to maternal pre-pregnancy

obesity, Hellmuth *et al.* (2017) reported lower levels of PCs with acyl chains of higher unsaturation degrees, such as C42:6, while higher levels of PCs with species of lower unsaturation, such as C30:3 and C32:3 (124). No significant differences in lysoglycerophospholipids were reported in relation to maternal BMI (84, 124, 125).

A lower ratio of PCs to PEs, which are major constituents of cellular membranes, was found to perturb membrane fluidity and integrity (126). Reduced cellular PC levels were also found to promote lipid droplet formation and contribute to metabolic disorders (127). Plasma lipidomic studies on obese, prediabetic, and diabetic patients reveal reduced PC levels (126), which is line with the reduction in PCs with PUFAs associated with increased BWs, GDM and maternal obesity, indicating similar pathological mechanisms. However, the increase in PCs with more saturated acyl chains noted in GDM and maternal obesity further complicates the interpretation of lipidomic changes. At any rate, although the influence of PC fatty acid composition remains largely unknown (126), it has been suggested to significantly modulate insulin sensitivity, with positive relation to the percentage of PUFAs and a negative one to saturated fatty acids such as C16:0 (128, 129).

The reduction in LPCs reported in association with higher BWs and GDM pregnancies was also found to be tightly associated with insulin resistance in type 2 diabetic and prediabetic patients (130). Elevated levels are associated with insulin sensitivity and anti-inflammatory effects (131). However, contradicting results do exist (132). Furthermore, reduced levels of LPI and LPS are also indicative of alterations in glucose homeostasis. LPI has a stimulatory effect on insulin secretion and counteracts diabetic symptoms (124). LPS stimulates glucose uptake in both skeletal myocytes and adipocytes (133). It is also noteworthy to mention that not only lysoglycerophospholipids were found lower in GDM women, Dudzik *et al*. (2014) also reported a higher ratio of saturated to unsaturated acyl chains of lysoglycerophospholipids (122). Acyl

moieties are also thought to influence the metabolic effects of lysoglycerophospholipids, with saturated species promoting inflammation and those with long chain PUFAs protecting against induced inflammation (134). This aligns with a state of low-grade chronic inflammation well established in pregnancies complicated with GDM (135).

Furthermore, alterations in several species of sphingomyelins (SMs) were reported. In GDM mothers, blood levels of SMs, with mostly saturated and monounsaturated fatty acids are reduced in all three trimesters (119, 122, 123). In healthy mothers at early second trimester, similar SM species were also reported to decrease in association with increased BWs (27), while SMs with polyunsaturated fatty acids were found to increase (26). In contrast, SMs with mostly SFAs and MUFAs were found to positively correlate with maternal pre-pregnancy BMI during early second trimester (136), which is in line with results on adult obesity and insulin resistance (137). Furthermore, genetic ablation of enzymes synthesizing SMs in mice models was found to decrease SMs in cellular membranes, increase insulin sensitivity, and enhance insulin signaling in liver, white adipose tissue, and skeletal muscles (138, 139), in addition to reducing obesity induced by a high fat diet (138). Therefore, the reduction of SMs with SFAs and MUFAs in GDM pregnancies and association with increased BWs is unexpected and difficult to explain given, the role of insulin resistance in both conditions.

Plasmalogens, which are membrane ether-linked phospholipids, are thought to have an important antioxidant role in protecting membranes and lipoproteins, given their vinyl ether linkage which is particularly susceptible to oxidation (126, 140). Reduced levels of plasmalogens are reported in association with obesity and diabetes (141, 142). Both conditions are known to share a state of heightened oxidative stress (143). However, changes in plasmalogens during pregnancy are less evident in our research context. The reduction in alkyl-acyl-PCs, which are also

ether-linked phospholipids, was reported in GDM pregnancies (118, 144) and in association with maternal obesity (124). Only Odinokova *et al*. (2019) reported a significant increase in PC plasmalogen (PC-O 30:5) in association with macrosomia at early pregnancy (26), which contradicts with the anticipated changes based on obesity and diabetic studies (141, 142).

In reference to cord blood, significant associations, both positive and negative, of different species of phospholipids in relation to BW were reported, including LPCs, PCs, ether-PCs, and SMs (145-152). Cord LPCs are consistently reported to positively correlate with BW (145-149), which contradicts with the reduced maternal levels during pregnancy in relation to increased BWs and GDM. A possible explanation to elevated LPC cord levels is labor-induced hypoxia, which is known to activate phospholipases and lead to enhanced LPC production (149, 153). Larger birth weights are likely to be associated with a longer duration of giving birth and hence longer acute periods of hypoxia, which translates to a stronger activation of phospholipases and higher levels of LPCs (149). Therefore, results from cord blood profiling of phospholipids may not be reliable to represent alterations in lipidomic features if labor-induced hypoxia is not controlled for.

2.3.2.2 NEFAs and Ketone Bodies

The majority of metabolomics studies do not conclude on a relation between BW and the overall levels of maternal NEFAs (25-27, 84, 117, 118, 136), however, Sandler *et al*. (2017) reported a positive association at early third trimester (83). Among ketone bodies, 3-hydroxybutyric acid and its acyl carnitine were found to positively correlate with BW in maternal blood at third trimester and in cord blood as well (83, 117, 154). In relation to maternal obesity, the majority of NEFAs were positively associated with maternal BMI during all the three trimesters (136, 155), and higher levels of 3-hydroxybutyric acid and its acyl carnitine were also evident (83, 136, 155). Such results may indicate enhanced lipolysis and fatty acid oxidation,

which is likely a result of insulin resistance encountered with higher BWs and obesity. In GDM mothers, maternal blood alterations in several species of NEFAs and fatty acid derivatives, both positive and negative, were reported during all three trimesters (119, 122, 123, 156, 157). Similar to obesity and higher BWs, elevated levels of ketone bodies were also reported in GDM women during second and early third trimesters (122, 156), indicating enhanced lipolysis and fatty acid oxidation, which is typically encountered in pregnancies with uncontrolled diabetes (158).

In contrast, cord levels of NEFAs, in total or as specific species, were negatively associated with BW (145-148, 150, 159). Cord omega-3 fatty acids, such as linolenic acid and docosahexaenoic acid (DHA), are also frequently reported to negatively correlate with BW (145, 147, 150, 159), which is paradoxical to the reported effects of omega-3 supplementation, i.e. prolonged pregnancy and increased BW. It was suggested that reduced levels may indicate higher fetal accretion or higher utilization in metabolic pathways such as enhanced eicosanoid metabolism (147). Similarly, lower levels of arachidonic acid and DHA were observed in the placentas of obese mothers at birth, however, this result was justified as a possible disruption of the physiological long chain-PUFA biomagnification, leading to decreased availability of these fatty acids to the fetus (160). The underlying etiology for this reduction remains to be elucidated.

2.3.2.3 Triglycerides, Cholesterol and Related Species

A positive association of maternal TGs and BW was reported during all three trimesters (25, 83, 117, 118), which is a relation consistently indicated in non-metabolomic studies as discussed earlier. TGs were also positively associated with maternal pre-pregnancy BMI (83, 144, 155). In relation to GDM, the majority of studies reported no significant alterations in maternal TGs, however, Furse *et al*. (2019) found an increase in several diglyceride (DG) and TG species with low unsaturation degrees while reporting a decrease in TG species with higher unsaturation, such

as TG (C48:6, C46:5) (121). Similarly, Lu *et al.* (2016) found two TG species with low unsaturation (C51:1, C48:1) to be positive predictors of GDM (120). With regards to cholesterol and BW, a positive correlation was reported at third trimester (83). In GDM and maternal obesity, no significant results were reported for maternal cholesterol species (119, 120, 122, 123, 156, 157), except for an increase in cholesteryl ester (C18:3) at early second trimester of GDM pregnancies (121).

In cord blood, total TGs and diglycerides were negatively associated with BW in healthy mothers (146, 147, 154), which may indicate increased accretion by fetal tissues. Untargeted metabolomic studies screening for cholesterol did not indicate any significant associations of cholesterol or its esters to BW (146, 147, 151, 152, 159).

2.3.2.4 Acylcarnitines

Short chain acylcarnitines (ACs), such as acetylcarnitine, were reported to positively correlate with BW in maternal and cord blood (83, 117, 152, 154, 161). Elevated levels were also reported in obese (154, 189, 162) and GDM mothers (122, 163). Medium and long chain ACs were found to negatively correlate with maternal fasting glucose levels during an oral glucose tolerance test at about 28 gestation weeks (155). However, contradicting results were reported in GDM women, with either an increase (163) or a decrease in long chain ACs (122). It was suggested that increased muscular fatty acid oxidation resulting from insulin resistance depletes long and medium chain ACs while accumulates acetyl-CoA. The latter is metabolized to acetylcarnitine to permit its mitochondrial efflux, hence, its observed increase (122). In relation to BW, the association is less clear, as different species of long and medium chain ACs were reported to either increase or decrease (117, 147), while only short chain ACs being consistently higher with higher BWs.

2.3.2.5 Lipidomic Alterations of Amniotic Fluid

In this research context, and to our knowledge, only three studies investigated the metabolomic profile of AF which was only in relation to GDM. No major changes in second trimester AF of GDM mothers compared to normal mothers were reported by Graca et al. (2010) and Graca et al. (2012) (164, 165). A more recent work by O'Neill et al. (2018) showed significant alterations pertaining to lipid metabolic pathways in GDM mothers, which included increased essential fatty acids and their longer chain derivatives, glycerol, and ketone bodies. These features typically suggest aggravated insulin resistance. Furthermore, pronounced elevations in several species of saturated and unsaturated SMs were found in GDM AF (99).

2.3.3 Concluding Remarks

Overall, the lipidomic features characterizing increased BWs overlap to a great extent with those of GDM, which may indicate the central role of insulin resistance in influencing maternal lipidome in both conditions. The overlap in the lipidomic profile between maternal overweight/obesity and increased BWs is less obvious, specifically with regards to glycerophospholipids and sphingolipids, which indicates a distinct, yet unknown, lipidomic profile associated with LGA pregnancies within the overweight/obese maternal population. Furthermore, there seems to be a divergence of some lipidomic features between maternal blood and in utero fluids, such as AF and cord blood. Such disparity may stem from placental lipid transport and metabolism. For example, sphingomyelins with saturated and monounsaturated acyl species are elevated in GDM AF (99) while depleted in GDM maternal blood (119, 122, 123), in comparison to healthy mothers. The majority of cord blood lipidomic changes associated with increased BWs contradict with those of maternal blood, including TGs, DGs, NEFAs, and LPCs, despite the fact that labor induced changes may influence such lipidome features. Hence, studying biological fluids

which can represent the fetal compartment, such as the AF, may provide further insights to the pathological changes of the lipidome influencing and/or being influenced by the in utero metabolic environment of macrosomic fetuses.

Chapter 3:

Untargeted Lipidomic Profiling of Amniotic Fluid Reveals Dysregulated Lipid Metabolism in Healthy Normal-Weight Mothers with Fetal Macrosomia

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3.1 Abstract

Background: Alterations in maternal lipid metabolism have been elucidated by several studies in relation to macrosomia. However, the lipidome of the intrauterine compartment associated with macrosomia, particularly in early pregnancy, remains largely unknown. Objectives: (1) To compare the lipidomic profile of early 2nd trimester amniotic fluid (AF) of healthy mothers with normal body mass index who gave birth to large-for-gestational age (LGA) versus appropriate for- gestational age (AGA) infants; and (2) to examine if insulin and glucose concentrations in AF were associated with the AF lipidomic profile. Methods: In this nested case–control study, bio-banked AF samples were collected from pregnant women undergoing routine amniocentesis at 12–22 weeks of gestation. A subsample of 15 LGA infants (cases) were contrasted with 15 AGA infants (controls). An untargeted lipidomics analysis using liquid chromatography quadrupole time of- flight mass spectrometry was conducted. Univariate and multivariate statistical analyses (principal component analysis and partial least-squares discriminant analysis) were used to extract differentially abundant (DA) features with high variable importance in projection (VIP) scores. Results: LGA AF was characterized by elevations of 30 phosphatidic acid species. Among other DA features, sphingomyelin (SM 14:0;O2/20:1) had the highest VIP score and was markedly elevated in LGA AF. Neither insulin nor glucose was associated with 2nd trimester AF lipidomic profiles in these healthy, normal-weight mothers. Conclusion: These findings provide evidence of early dysregulated lipid metabolism in healthy, normal-weight mothers with LGA infants.

Keywords: lipidomics; lipids; fetal macrosomia; birth weight; amniotic fluid

3.2 Introduction

Macrosomia and large-for-gestational age (LGA) are alternative terms used to describe fetal overgrowth, with higher risk for cesarean section, fetal shoulder dystocia [1], obesity, type 2 diabetes mellitus, and cardiovascular diseases later in life [2,3]. While maternal obesity and diabetes are strong factors, epidemiological studies emphasize a wide spectrum of maternal characteristics in association with macrosomia. In fact, a large proportion of LGA infants are born to mothers with normal glucose tolerance and body mass index (BMI) [4,5]. Macrosomia has often been viewed as a physiological response to maternal hyperglycemia, although it does not fully explain it even in diabetic pregnancies [6]. The role of maternal lipid metabolism, on the other hand, has become well recognized, with evidence indicating that its markers predict birth weight and fetal adiposity better than glucose [7,8]. Mothers of macrosomic infants were also found to have higher placental gene and protein expression levels of fatty acid binding and transport proteins, which indicate upregulated placental lipid transport and metabolism [9].

Several lipidomics studies have revealed significant alterations in maternal blood lipids in direct relation to fetal overgrowth [10,11]. For instance, reductions in different species of phosphatidylcholines, lysophosphatidylcholines, and lysophosphatidylinositols have been observed in early pregnant mothers in association with high birth weights [12–14]. These changes align with the lipidomic features associated with insulin resistance [15–17]. Alterations during the second half of pregnancy are complex and not consistent, but overall, they also indicate insulin resistance. Maternal short-chain acylcarnitines, such as acetylcarnitine, were reported to positively correlate with birth weight [18,19]. It was suggested that increased muscular fatty acid oxidation resulting from insulin resistance accumulates acetyl-CoA, which may justify the observed increase in acetylcarnitine [20]. Nevertheless, researchers investigated either the maternal side or the

intrauterine compartment (i.e., cord blood/placenta) specifically at birth. The lipidome of the intrauterine compartment characterizing macrosomia at early pregnancy is still unknown and has the potential to provide further insights into its pathophysiology.

Amniotic fluid (AF) is thought to arise as a transudate of maternal and/or fetal plasma [21]. It uniquely reflects the interaction between maternal, placental, and fetal metabolism [22], especially at early pregnancy before fetal skin keratinization is fully attained at 25 weeks of gestation, which ends the bidirectional flow of fluids across the skin [23]. Moreover, fetal swallowing of AF, which begins as early as 16 weeks [23], was found to impact fetal growth, and conditions of esophageal ligation or atresia of the gastrointestinal tract are associated with lower birth weights [24,25]. Such dynamically changing fluid offers the potential to elucidate the metabolic perturbations affecting the intrauterine compartment in adverse maternal and fetal health conditions. Although it is considered an underutilized resource, given the invasive nature of amniocentesis as a procedure [26], several studies have provided important metabolic clues about gestational diabetes, prematurity, and other conditions using AF [27,28]. In the context of gestational diabetes, for example, profiling the AF metabolome provided evidence for the divergence between maternal and intrauterine lipid metabolism, with elevations in AF saturated and monounsaturated sphingomyelins (SMs) [27], whereas similar SM species were reported as lower in maternal circulation [20,29]. Such findings highlight the complexity of the associated metabolic dysregulation.

To date, there are no studies profiling the human AF lipidome in relation to macrosomia. Therefore, our objective was to compare the lipidomic profile of AF obtained at early second trimester between healthy normal-weight mothers who gave birth to LGA and AGA infants. At

the same time, we aimed to examine if AF insulin and glucose concentrations were associated with these AF lipidomic profiles.

3.3 Methods

Study Design

In this nested case–control study, AF samples were selected from a biobank of samples previously collected from pregnant women who had undergone routine amniocentesis at early second trimester. Our inclusion criteria included healthy mothers with normal BMI (18.5–24.9 kg/m^2) [30] who gave birth to either LGA or AGA infants. Women who had pre-existing health conditions, such as pre-pregnancy diabetes or other endocrine disorders, and those who developed gestational diabetes or hypertensive disorders of pregnancy were excluded. This exclusion criterion aimed to eliminate the confounding effect of these disorders and to better isolate the pathological mechanisms mediating LGA development. All relevant information was extracted from the participants' medical records. A sample of 15 LGA infants (cases, birth weight >90th percentile for gestational age and sec) was randomly selected and matched with 15 AGA infants (controls, birth weight between 10th and 90th percentile for gestational age and sex). This sample size was deemed an acceptable starting point for our study given the exploratory nature of our work [31]. Individual 1:1 matching was conducted based on sex, amniocenteses week or the gestational age at the time of amniocenteses (±1 week), pre-pregnancy maternal BMI (±2 kg/m^2), and maternal age (±2 years). A flowchart illustrating sample selection from the AF biobank is provided in Supplementary data (Figure S1). Samples were shipped in dry ice for lipidomic profiling at The Metabolomics Innovation Center (TMIC, Edmonton, AB, Canada).

Study Population and Characteristics of the AF Biobank

Participants' recruitment was carried out in 2000–2004 at St. Mary's Hospital Centre (Montreal, QC, Canada). Ethics approval was obtained from the McGill University and St. Mary's Hospital Centre Institutional Review Boards. Pregnant women were recruited while undergoing routine amniocentesis for age-related genetic testing (15.1 ± 0.1 weeks, range 12–22 weeks). Women with multiple pregnancies and fetuses with genetic anomalies were exc1uded. Consents were obtained from participating women to allow the investigators to analyze their AF for various constituents on the remaining fluid once genetic testing was completed. The consent form also provided permission to obtain information from medical charts on maternal health, age, height, and pre-pregnancy weight, and infant sex, birth weight, and gestational age at delivery. As birth weight can be influenced by gestational length and fetal sex, Kramer birth weight percentile charts were used to control for these factors [32]. Aliquots of AF samples were stored at −80°C since collection. Samples with meconium staining or blood were excluded. AF samples were analyzed for glucose and insulin as previously described [33]. Briefly, glucose was analyzed using an adapted hexokinase assay kit (No. 6082; Abbott Laboratories, North Chicago, IL, USA) for use with a microplate reader. Insulin was analyzed using the Beckman Access Ultrasensitive assay system (Beckman Coulter, Brea, CA, USA), which measures insulin to within 0.03–300 μIU/L.

Global Lipidomics Analysis

For lipid extraction, a modified Folch liquid–liquid extraction protocol was applied [34]. Each aliquot of 200 μL of sample was evaporated to dryness before its weight was determined. A corresponding amount of water was added to samples to resuspend before mixing with dichloromethane, methanol, and an internal standard mixture, which consisted of 15 deuterated lipids from different lipid subclasses: lysophosphatidylcholine (LPC d5-18:1),

lysophosphatidylethanolamine (LPE d5-18:1), fatty acid (FA d3-16:0), cholesterold3, phosphatidylglycerol (PG d5-16:0/18:1), phosphatidylserine (PS d5-16:0/18:1), phosphatidic acid (PA d5-16:0/18:1), phosphatidylinositol (PI d5-16:0/18:1), ceramide (Cer d3-16:0/18:1), phosphatidylcholine (PC d5-16:0/18:1), phosphatidylethanolamine (PE d5-16:0/18:1), monoglyceride (MG d5-18:1), diglyceride (DG d5-18:1/16:0), triglyceride (TG d5-16:0/18:1/16:0), steryl ester (CE d3-18:1). A clean-up step was performed with water. Samples were equilibrated at room temperature for 10 min and centrifuged at 16,000× g for 10 min at 4°C. An aliquot of the organic layer was evaporated to dryness with a nitrogen blowdown evaporator. The residue was immediately re-suspended in mobile phase B (10 mM ammonium formate in 95:5 (v/v) isopropanol:water), vortexed for 1 min, and diluted with mobile phase A (10 mM ammonium formate in 5:4:1 (v/v) methanol:acetonitrile:water).

Reversed-phase chromatography was conducted using ultra-high-performance liquid chromatography (UHPLC) with a Vanquish Flex Duo Tandem Binary UHPLC system (Thermo Fisher Scientific, Waltham, MA, USA) and a Waters Acquity CSH C18 column (5 cm × 2.1 mm, 1.7 μm particle size). Microflow-based lipidomic profiling of extracts was performed using UHPLC linked to a Bruker Impact II Quadrupole time-of-flight (QToF) mass spectrometer (Bruker Daltonics, Billerica, MA, USA), with separate injections for each ionization polarity and the following conditions: injection volume of 4.0 μL for positive ionization and 12.0 μL for negative ionization; flow rate of 210 to 300 μL/min; NovaMT 20-min-gradient; column temperature of 45°C; and a mass range of 150–1500 m/z. MS/MS analysis was performed in auto-MS/MS mode with collision energies of 10–60 eV. Chromatograms were processed with an intensity threshold of 3000 cts; signal-to-noise ratio threshold of 3; minimum peak length of 6 spectra; retention time tolerance of 6 s for correction and 4 s for alignment; m/z tolerance of 20.0 ppm for peak picking

and 20.0 ppm and 5.0 mDa for alignment. MS/MS spectra were acquired for all samples for alignment and identification using NovaMT LipidScreener V1 (Nova Medical Testing Inc., Edmonton, AB, Canada) [35].

For quality control (QC), a pooled mixture composed of one aliquot from each sample was prepared. The pooled mixture was split into multiple aliquots of equal volume, evaporated to dryness with a nitrogen blowdown evaporator, purged with nitrogen, and stored at −80°C. One QC aliquot was extracted with each randomized batch of samples; 13 samples per batch, as this analysis was part of a larger project with a larger cohort of samples. Samples within each batch were injected in-between two injection replicates of the corresponding QC aliquot. Multiple QC aliquots were also injected before and after all samples to ensure technical stability.

Data Processing

The detected features from all samples were merged into one feature-intensity table. A three-tier annotation approach based on MS/MS spectral similarity match, retention time, and accurate mass match was employed for lipid identification. A filtering and scoring approach embedded in NovaMT LipidScreener was employed to calculate MS/MS match scores (when available), restrict the number of matches, and select the best identification. The approach uses parameters like the expected retention time range, expected adducts and isoforms, fatty acyl chain length, ionization efficiency and sensitivity of each lipid subclass, and m/z error to make the best identification choice for each feature among all isomeric and isobaric possibilities [35].

Features identified in Tier 1 and 2 were based on MS/MS identification. Specifically, Tier 1 included features that had an MS/MS match score of ≥500 and a precursor m/z error ≤ 20.0 ppm and 5.0 mDa. Features with an MS/MS match score of <500 and a precursor m/z error ≤ 20.0 ppm and 5.0 mDa were included in Tier 2. Features not identified by MS/MS (Tier 3) were imported

into the Lipid Maps database for identification based on accurate mass match and a m/z error of ≤ 20.0 ppm and 5.0 mDa. All features identified in Tiers 1, 2, and 3 were combined for normalization and statistical analysis. The classification and shorthand notation of lipids followed the guidance of LipidMaps, MSDial and the Update on LIPID MAPS classification, nomenclature, and shorthand notation for MS-derived lipid structures [36, 37].

Data Analysis

Missing values were substituted by (1) the median intensity of the sample group (LGA/AGA) for features detected in at least 75% of injections within the group; (2) the minimum intensity within the group for features detected in at least 50% of injections; or (3) the global minimum for all samples and QCs for features detected in less than 50% of injections within the group. Features not detected in ≥80% of injections within at least one sample group or QCs were filtered out.

To correct for ion suppression effects and any sources of technical variations, the identified features/lipids were first normalized by internal standards. The positively identified lipids were matched to one of 15 deuterated internal standards of different lipid classes that were spiked into samples before extraction, according to lipid class similarity and the expected retention time range for each class. Intensity ratios, i.e., the intensity of each lipid/feature divided by the intensity of the matched internal standard, were calculated for data normalization. Second, the identified lipids were median-normalized, i.e., the intensity ratio of each feature was divided by the median intensity ratio of all identified lipids within each sample experiment. Non-informative features (e.g., internal standards and common contaminants) and near-constant features were filtered out during data processing.

The following steps of data analysis were performed using R (version 4.3.2). Features filtered out included those with a relative standard deviation (RSD) greater than 25% for QC injections and features with near-constant values between groups, i.e., the top 30% of features with the lowest RSD among all samples. EigenMS normalization [38] was applied after several attempts of using other data transformation/normalization methods, such as log transformation, autoscaling, probabilistic quotient normalization, and variance stabilization normalization. However, only EigenMS normalization was robust enough to allow the detection of significantly different features between the comparison groups (LGA versus AGA) upon statistical analysis of the data. Each feature was tested for normality (from each comparison group) and for homogeneity of variance, using the Shapiro–Wilk test and Bartlett's test, respectively. A p value > 0.01 was used to conclude a normal distribution and equal variances. Based on the results of these tests for each feature, univariate analysis was applied; with parametric (t-test) or non-parametric (Wilcoxon Rank Sum) tests and with either equal or non-equal variances. This was followed by False Discovery Rate (FDR) correction, using Benjamini–Hochberg adjustment. An adjusted p value < 0.05 was considered statistically significant. Insulin and glucose were also assessed similarly using this univariate analysis approach.

For differentially abundant (DA) features, i.e., significantly different between LGA and AGA, analysis of covariance (ANCOVA) was used to adjust for the effects of covariates: maternal BMI and age, fetal sex, and amniocentesis week. Linear regression models were fitted for each DA feature as the dependent variable and birth weight group (LGA/AGA) as the classification variable along with the covariates. To examine linear correlations of glucose and insulin with birth weight and DA features, Pearson and Spearman's rank correlation testing were used for normally and non-normally distributed features, respectively. DA lipid features with identical identification

were averaged for correlation analysis. Correction for multiple comparisons was also performed using Benjamini–Hochberg adjustment for ANCOVA and correlation testing. Log_2 fold change was calculated for DA features as log2(mean feature intensity LGA/AGA). Principal component analysis (PCA) was applied to visualize the clustering of samples. Partial least squares discriminant analysis (PLS-DA) and variable importance in projection (VIP) were applied to extract features with high contribution to group separation. Only for PLS-DA analysis, the dataset was pareto-scaled, i.e., each feature was mean-centered and divided by the square root of its standard deviation. MixOmics (v6.25.1) and RVAideMemoire (v0.9-83-7) R packages, which offer sets of functions designed for multivariate analysis of biological data, were used to optimize the PLS-DA model and test its significance.

3.4 Results

Characteristics of Sample Population

Maternal and infant characteristics are shown in Table 3.1. Except for birth weight, no significant differences in maternal and infant characteristics were observed between LGA and AGA groups, mostly because of the matching criteria we applied.

Global Assessment of Lipidomic Features

Mass accuracy for data acquisition was assessed using internal standards. Out of 15 deuterated lipids/internal standards spiked in each sample and QC, 14 were detected in positive ionization and 12 were detected in negative ionization. The maximum mass error of internal standards detected was 1.44 ppm or 0.78 mDa for positive ionization and 0.81 ppm or 0.72 mDa for negative ionization, showing good mass accuracy. The average RSD (%) values for internal standards' peak intensities as detected in QC samples were 13% in positive mode and 15% in

negative mode. The average RSD (%) values of all detected features in QC samples before and after internal standard and median intensity normalization were 21.8% and 12%, respectively.

The analytical approach detected 4985 unique peaks in all samples, of which 2584 peaks were identified. In Tier 1 and Tier 2, 966 and 122 features were identified, respectively, at the species or molecular species level, with either full composition of fatty acyl/alkyl residues or summed composition if individual residues were not specified in the source database. The remaining 1496 features were putatively identified in Tier 3 based on mass match and at the species level, i.e., only summed composition of fatty acyl/alkyl residues is provided. Multiple peaks were often annotated as the same lipid at the molecular species level (most identifications for Tiers 1 and 2) or at the species level (Tier 3), corresponding to similar lipids with minor differences in their structures, such as the position of double bonds, position of functional groups, and stereochemistry. Such differences between lipid isomers cannot be distinguished by the employed untargeted LC-MS/MS approach and require sophisticated targeted methods. Hence, several peaks had identical identifications.

The identified lipids included 49 distinct lipid subclasses, of which triglycerides (TGs), hexosyl-ceramides, and phosphatidylcholines had the highest relative abundances, with percentages of 15%, 11.9%, and 11.5%, respectively (Figure 3.1). Sphingolipids and glycerophospholipids constituted more than 60% of all lipids identified. The distribution and total count of identified lipids within lipid categories and subclasses, along with their identification tiers, are provided in Supplementary data (Table S1, Figure S2). After applying the RSD filters, i.e., removing features with near-constant values and those with RSD > 25% in QC injections, 1780 features remained.

Univariate Analyses

Univariate analysis detected 42 features as significantly different between LGA and AGA samples. These features corresponded to 38 identified lipids, given that some features had similar identification (Table 3.2). All DA features were Tier 3-identified except for two features, which were Tier 1-identified as sphingomyelins (SMs). The majority of DA features (34 out of 42) were phosphatidic acid (PA) species, and they were all enriched in LGA AF samples. In addition, a few other species, including SM 14:0;O2/20:1, SM 39:1;O2, acyl-CoA (CoA 14:1), and wax ester (WE 22:1;O4), were elevated, while two TGs (TG 51:1 and TG 54:9;O), phosphatidylcholine (PC O-44:3), and SM 14:1;O2/21:1 were depleted in LGA samples compared to AGA. All DA features were still significantly different between LGA and AGA groups after adjusting for covariates using ANCOVA. A volcano plot was generated to visualize features' intensity fold changes and statistical significance (Figure 3.2). Among DA features, SM 14:0;O2/20:1, followed by PA species and CoA 14:1 had the highest intensity fold changes ($\log_2$ fold change > 1). Insulin and glucose levels were not significantly different between LGA and AGA AF.

Multivariate Analyses

Unsupervised clustering of samples using PCA showed a clear separation between LGA and AGA groups as shown in PCA scores plot (Figure 3.3a), indicating different lipidomic trends between the two groups. A PLS-DA model was fit with 8 components (Figure 3.3b). The first two components explained 12% and 10% of the data variation. Three-fold cross validation with 100 repeats yielded a mean classification error rate of 0.4 ± 0.12%, and the model was significant upon permutation testing (p value = 0.001). VIP analysis showed that SM 14:0;O2/20:1 had the highest VIP score, followed by all DA PA species (Figure 3.4). All DA features had a VIP score > 2. In other words, DA features had the highest contribution to group separation based on PLS-DA

classification model. Results from correlation analysis showed that insulin and glucose did not correlate significantly with any of the DA features nor with birth weight (adjusted p value > 0.05).

3.5 Discussion

To our knowledge, this is the first study to profile the AF lipidome in relation to LGA as a pregnancy outcome. Untargeted lipidomics of AF revealed a unique signature of lipid metabolism characterizing fetal overgrowth during early pregnancy in healthy normal-weight mothers. This signature manifested mainly as a marked elevation of SM 14:0;O2/20:1, and many PA species with ether/ester bonds, different chain lengths, and degrees of saturation. This result is clearly different from the lipidomic features of maternal blood reported in association with higher birth weights. The general observation from previous studies indicates a reduction of maternal blood phosphatidylcholines and lysophosphatidylcholines in association with LGA birth weights [12–14,39]. Different prevailing features of LGA lipidome between maternal blood and AF are expected, given that AF uniquely reflects the interaction between maternal, placental, and fetal lipid metabolism, especially at early pregnancy.

Marked Elevation in Phosphatidic Acid Species

PA is a key intermediate in lipid metabolism and is implicated in insulin signaling pathways [40]. Cellular accumulation of PA was found to inhibit insulin signaling [41,42], an effect that seems to depend on PA composition of fatty acids and the synthetic pathways producing PA [43]. However, our sample of pregnant women were all healthy, with normal BMI, and none had any complications during pregnancy, like gestational diabetes. Therefore, it is not reasonable to assume that the observed increase in PA directly indicates maternal insulin resistance, although we cannot exclude the possibility of some degree of insulin resistance in these women. Also, elevated PA levels did not resemble a major feature of maternal blood or AF lipidome in association with

gestational diabetes, which serves as a clear model for insulin resistance [20,29], although a few species have been reported to be elevated in patients with gestational diabetes [44].

A potential explanation of the observed elevation of PA in LGA AF could be related to enhanced placental stimulation of PA synthesis, secondary to increased maternal flux of TGs, non-esterified fatty acids (NEFAs), glucose, and growth factors such as insulin, which are positively associated with birth weight [45–47]. In that regard, both oleic acid [48] and insulin [49], acting as a growth factor, induce PA synthesis in primary human trophoblast cells (PHTs), which activates mammalian target of rapamycin (mTOR) [50]. Interestingly, activation of mTOR signaling was evident in the placentas of LGA infants [51]. Placental mTOR acts as a nutrient sensor, regulating nutrient delivery and fetal growth [48]. While amino acid uptake and transport are induced by mTOR activation in PHTs, the effect on placental lipid transport and metabolism is unknown [52]. It is noteworthy, however, that in cultured preadipocytes, activation of mTOR complex 1 promotes lipogenesis and inhibits lipolysis [53].

Alterations in Sphingomyelin Species

Among the DA features, two SMs were Tier 1-identified, i.e., with high confidence in structural identification. SM 14:0;O2/20:1 was markedly higher in LGA AF, with the highest VIP score and intensity fold change, while SM 14:1;O2/21:1 was lower. Not much is known about these two specific SM molecular species, and 14-carbon sphingoid bases are not common in human tissues [54]. However, the presence of SMs with 14-carbon sphingoid bases in AF can be well justified, given the high expression levels of serine palmitoyl transferase subunit 3 (SPTLC3) in placenta and trophoblast cells [55]. SPTLC3 has a high activity towards lauryl-CoA and myristoyl-CoA and primarily generates sphingoid bases with 14- or 16-carbon backbones [56]. It seems that monounsaturated SM species, at the sum composition, were particularly higher in LGA AF, as SM

14:0;O2/20:1 and SM 39:1;O2 were significantly elevated. In maternal blood, the opposite trend can be observed, as lower levels of monounsaturated SMs were reported in relation to birth weight among early pregnant mothers [13,57]. These opposite trends of alterations may indicate the divergence between the intrauterine and maternal lipid metabolism, which is likely the result of placental metabolism.

Alterations in Triglyceride and Fatty Acid Species

Another example of the unique intrauterine lipid profile that differs from the maternal side is the reduction of AF TGs. While maternal hypertriglyceridemia is frequently reported as a significant marker for macrosomia [45,58,59], our results showed that LGA infants had lower AF levels of two TG species, TG 54:9;O and TG 51:1. In line with this reduction is the negative association reported for cord blood TGs and birth weight [11,60]. Enhanced placental and fetal uptake and storage of fatty acids could explain the reduction of TGs observed in AF and cord blood. Furthermore, circulating myristic acid (C14:0), as a NEFA, was previously reported to correlate positively with maternal levels of TGs [61], which, as mentioned above, strongly predicts the risk of LGA. Myristic acid is known to activate several enzymes, such as desaturases, through protein myristoylation [62], which may explain the increase in its activated and monounsaturated form, acyl-CoA 14:1, we noted in LGA AF.

No differences in glucose and insulin

Our results did not indicate any difference in AF concentration of glucose or insulin between LGA and AGA pregnancies, which is in line with previous studies [63–65]. However, one study reported that AF insulin is significantly associated with the risk of macrosomia among women with a positive glucose challenge test [66]. Furthermore, our analysis did not reveal any correlations between the DA lipids and AF concentration of glucose or insulin. Therefore, no

evidence for glucose or insulin alterations in association with LGA pregnancies can be found based on the analysis of our AF samples.

Limitations

Among the limitations of this study is the small sample size, which aligns with the exploratory nature of the work. The lack of information on the dietary intake of participants and their glycemic and lipid profiles has limited our ability to draw stronger conclusions of LGA AF lipidome in relation to maternal metabolic health. The advanced maternal age of participants may limit the generalizability of our findings. Long storage duration of our AF samples is another limitation. However, given that all samples were stored for similar durations, it can be assumed that the effects of storage on the lipidome were consistent across all samples, hence, maintaining the relationships with birth weight. Aliquoting AF samples prior to storage at −80 °C also helped minimize the freeze–thaw cycles. Furthermore, although the employed identification method used a nine-tier approach to filter and select the best identification choice for each detected feature, most features identified as DA, such as PA species, were Tier-3 identified. This identification tier was based on mass match rather than MS/MS identification, which implies lower confidence in the identification. However, the fact that 34 DA features were consistently identified as PA reduces the possibility of misidentifying PA as an important DA lipid.

Conclusions

While insulin and glucose were not different between LGA and AGA AF, several lipid species were DA, of which PA species were dominantly and markedly elevated in LGA AF. We suspect that elevated levels of maternal lipids and growth factors, such as insulin, induce higher placental synthesis of PA, leading to the observed elevation of PA in LGA AF. Furthermore, elevated SM 14:0;O2/20:1 strongly distinguished LGA from AGA AF; however, the role and the

significance of this SM need further investigation. Overall, our results may provide a mechanistic clue about the positive association between birth weight and maternal circulating lipids and growth factors. Furthermore, this study signifies the role of altered lipid metabolism in promoting augmented fetal adiposity. Future research with both comprehensive and targeted lipidomic profiling is needed to establish our results and expand upon them. More focus on the early stages of pregnancy, tissues from the intrauterine compartment, and the metabolic pathways involved in the synthesis of PA and SMs with 14-carbon sphingoid bases may provide further insights into the underlying mechanisms promoting macrosomia.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/nu16223804/s1, Figure S1:Flowchart of sample selection from AF biobank; Figure S2: Distribution of identified lipids among lipid subclasses and identification tiers; Table S1: Total count of identified lipids within lipid categories, subclasses, and identification tiers.

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Institutional Review Board Statement: The study was conducted in accordance with the Declaration of Helsinki and approved by the Institutional Review Board of McGill University (Approval No. A03-M03-98) and St. Mary's Hospital Centre.

Informed Consent Statement: Informed consent was obtained from all participants included in the study.

Data Availability Statement: The raw data supporting the conclusions of this article will be made available by the corresponding author on reasonable request.

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3.7 Tables and Figures

Table 3. 1: Maternal and infant characteristics

	LGA (n=15)	AGA (n=15)
Maternal		
Age (years)	37.2 ± 1.5	37.3 ± 1.7
Pre-pregnancy weight (kg)	61.6 ± 7.4	58.5 ± 6.6
Height (m^2)	1.68 ± 0.08	1.64 ± 0.09
BMI (kg/m^2)	21.7 ± 1.7	21.7 ± 1.6
Amniocenteses week (weeks)	15.2 ± 0.8	15.2 ± 0.7
Infant		
Sex (n = female/male)	5/10	5/10
Birth weight (grams) *	4314 ± 333	3532 ± 346
Gestational age at birth (weeks)	40.2 ± 1.1	39.74 ± 0.7

Data are presented as mean ± SD. * Indicates a significant difference between LGA and AGA (p value < 0.05).

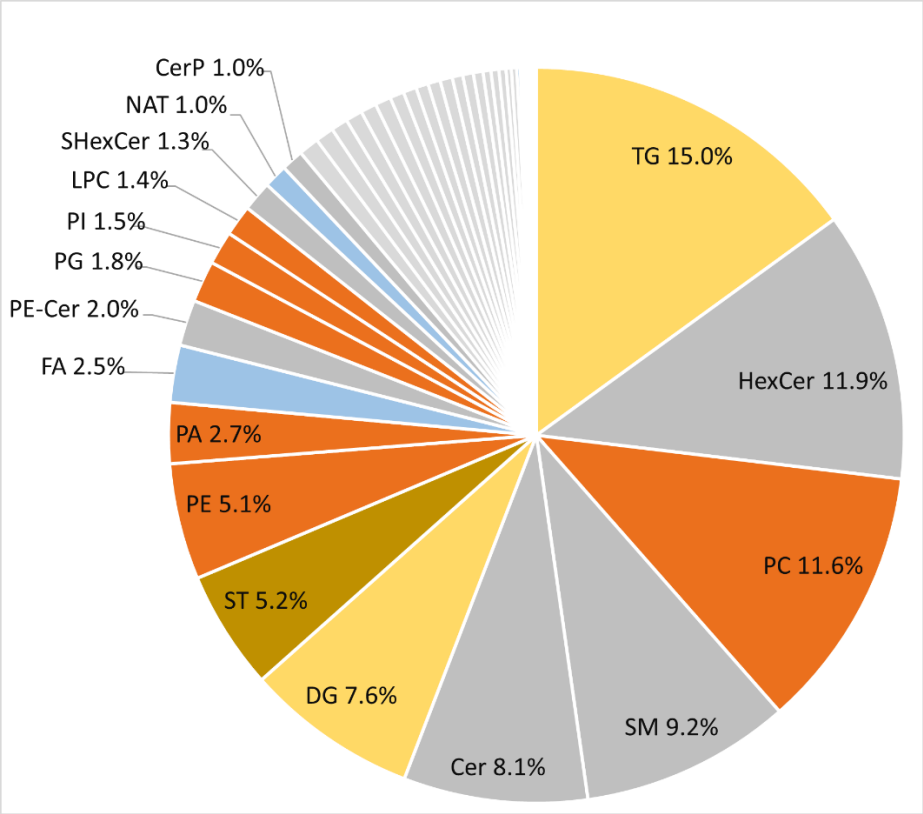
Table 3. 2: Differentially abundant features between LGA and AGA infants.

Feat. No	Identified Feature/Lipid	Log_2 Fold Change	Adjusted *p*-value
Features enriched in LGA AF			
1	PA O-39:2 [R]	1.63	< 0.001
2	PA O-40:2 [R]	1.64	< 0.001
3	PA O-40:3 [R]	1.65	< 0.001
4	PA O-40:2 [R]	1.81	< 0.001
5	PA O-34:2	1.59	< 0.001
6	PA O-61:7	2.11	< 0.001
7	PA O-39:1	1.32	< 0.001
8	PA O-45:3	1.37	< 0.001
9	PA O-41:1	1.96	< 0.001
10	PA O-37:1	1.37	< 0.001
11	CoA 14:1	1.08	< 0.001
12	PA O-57:0	1.51	< 0.001
13	PA O-35:0	1.45	< 0.001
14	PA O-30:2	1.61	< 0.001
15	PA 37:0	1.37	< 0.001
16	PA 34:1	1.35	< 0.001
17	SM 14:0;O2/20:1	2.51	< 0.001
18	PA 33:3	2.08	< 0.001
19	PA O-45:2	1.38	< 0.001
20	PA 39:2	1.83	< 0.001
21	PA O-38:2	1.61	< 0.001
22	PA O-39:2 [R]	1.83	< 0.001
23	PA O-41:0	1.93	< 0.001
24	PA O-43:1	1.79	< 0.001
25	PA O-45:1	1.55	< 0.001
26	PA O-61:9	1.56	< 0.001
27	PA O-45:0;O	1.51	< 0.001
28	PA O-38:1	1.95	< 0.001

29	PA O-39:0	1.66	0.002
30	PA O-60:7	1.60	0.002
31	PA O-36:0	1.52	0.003
32	PA O-35:3	1.84	0.003
33	PA O-40:3 [R]	1.33	0.003
34	PA O-40:3 [R]	1.35	0.003
35	WE 22:1;O4	0.54	0.017
36	PA O-62:12	0.47	0.028
37	SM 39:1;O2	0.51	0.045
38	PA 27:2	0.45	0.047
Features depleted in LGA AF			
39	TG 51:1	–0.68	< 0.001
40	TG 54:9;O	–0.78	0.002
41	SM 14:1;O2/21:1	–0.88	0.006
42	PC O-44:3	–0.89	0.018

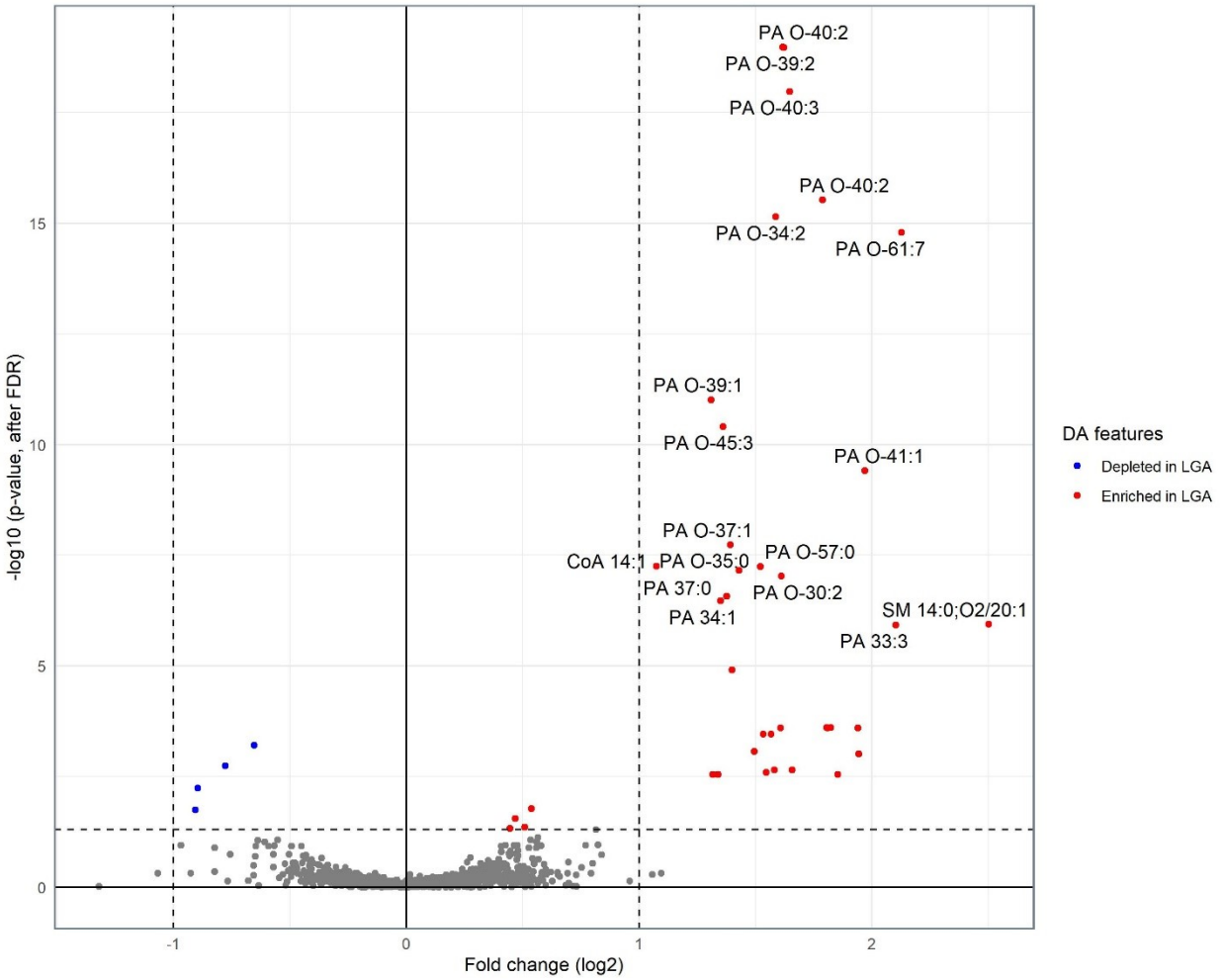
All features listed were Tier 3 identified, except SM 14:0;O2/20:1 and SM 14:1;O2/21:1 which were Tier 1 identified, i.e., based on MS/MS identification. R Indicates features with replicate identification. Abbreviations: PA: phosphatidic acid, CoA: acyl coenzyme A, SM: sphingomyelin, WE: wax ester, TG: triglycerides, PC: phosphatidylcholine. Shorthand notation of lipid species; PA 37:0 denotes a PA with a total of 37 carbons and 0 double bonds in its two ester-linked fatty acyl chains; PA O-39:2 denotes a PA with an ester-linked fatty acid group and an ether/vinyl ether-linked fatty alcohol group containing a total of 39 carbons and 2 double bonds; SM 14:0;O2/20:1 denotes a SM in which the sphingoid base contains 14 carbons, 0 double bonds, and 2 hydroxyl groups, and the fatty acid contains 20 carbons and 1 double bond; SM 39:1;O2 denotes a SM in which the sphingoid base and fatty acid contain a total of 39 carbons, 1 double bond, and 2 hydroxyl groups.

Figure 3. 1: Relative abundance of the identified lipids' subclasses.



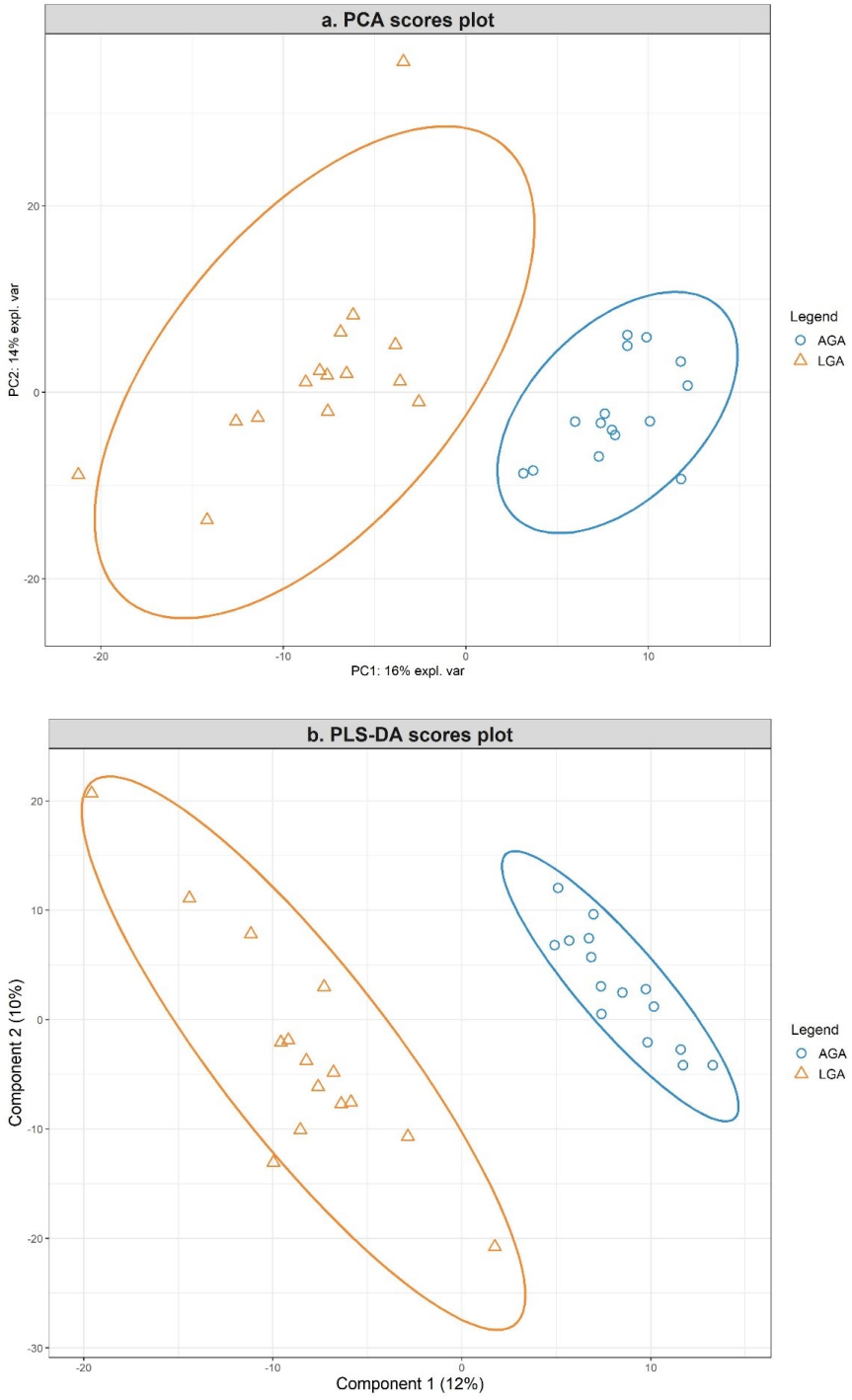
The abundance of each lipid class, among detected and identified peaks, is illustrated. Percentages were calculated by dividing the total count of identified lipids within each subclass by the total count of detected and identified peaks, i.e., 2584 peaks. Only subclasses with a relative abundance of ≥ 1% are shown. Abbreviations: Cer: ceramides, CerP: ceramide 1-phosphates, DG: diacylglycerols, FA: fatty acids, HexCer: hexosyl ceramides, LPC: lysophosphatidylcholines, NAT: N-acyl amines, PA: phosphatidic acids, PC: phosphatidylcholines, PE: phosphatidylethanolamines, PE-Cer: ceramide phosphoethanolamines, PG: phosphatidylglycerols, PI: phosphatidylinositols, SHexCer: sulfoglycosphingolipids, SM: sphingomyelins, ST: sterols, TG: triglycerides.

Figure 3. 2: Volcano plot analysis of identified lipids.



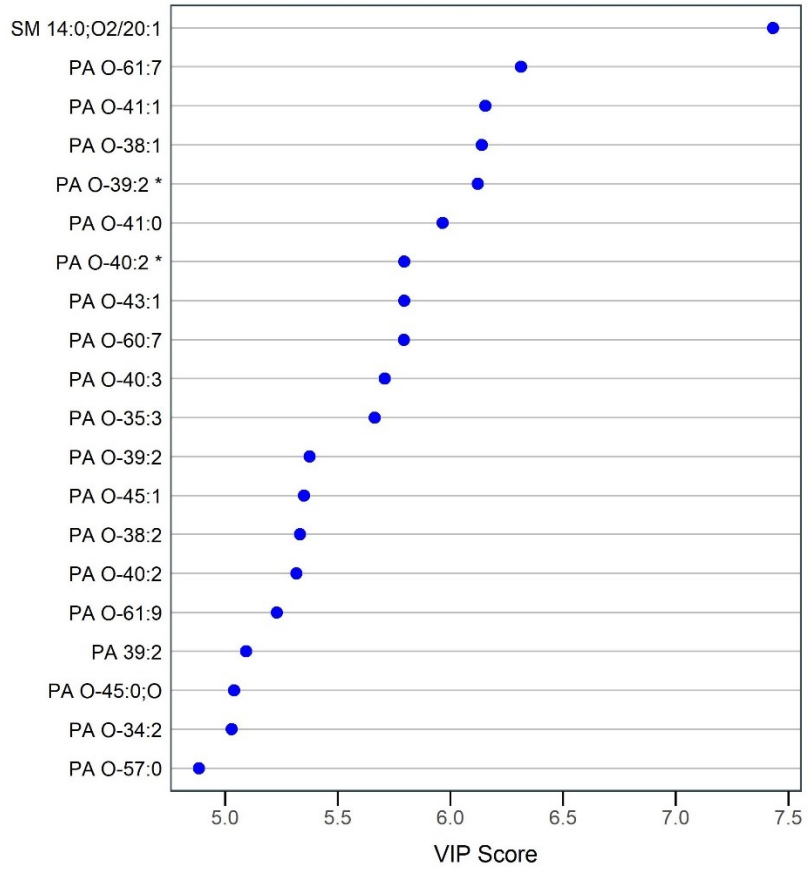
Features with an adjusted *p* value < 0.05 are colored in red if their intensities were higher in LGA compared to AGA AF and in blue if they were lower. Only features with an adjusted *p* value < 0.00001 are annotated. Abbreviations: CoA: acyl coenzyme A, PA: phosphatidic acid, SM: sphingomyelin.

Figure 3. 3: PCA and PLS-DA scores plots.



(a) PCA 2D scores plot. (b) PLS-DA scores plot (mean classification error rate: 0.4 ± 0.12% based on an 8-component model, p value < 0.001 from 999 permutations based on 3-fold cross validation with 100 repeats).

Figure 3. 4: Variable importance in projection (VIP) scores.



The top 20 VIP scores of the first component from PLS-DA analysis are presented. All lipids displayed were significantly enriched in LGA compared to AGA AF. * Indicates features with replicate identification.

3.8 Supplementary Data

Untargeted lipidomic profiling of amniotic fluid reveals dysregulated lipid metabolism in healthy normal-weight mothers with fetal macrosomia

Haj-Husein I, Kubow S, and Koski K.G.

- Methods: Sample selection

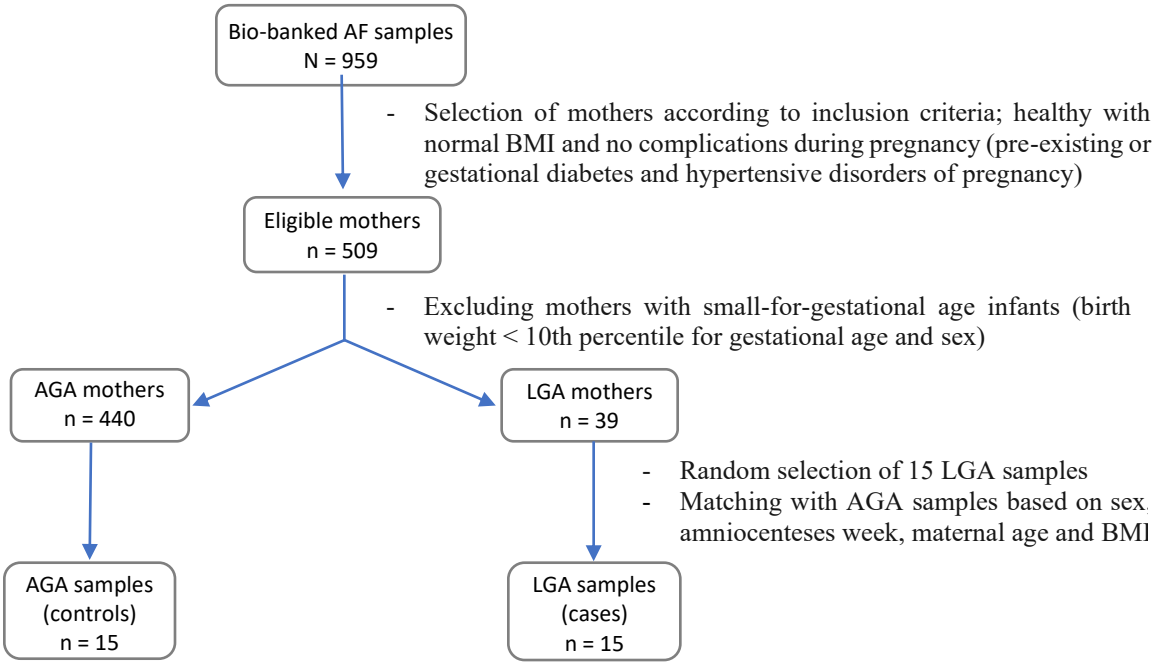


Figure S1: Flowchart of sample selection from AF biobank

- Results: Identified lipidomic features of AF

Table S1: Total count of identified lipids within lipid categories, subclasses, and identification tiers

Lipid Category	Subclass	Tier 1	Tier 2	Tier 3	Total count (%)
Sterols	ST	4	10	121	135
Sterols	CE			1	1
Sterols-Total					136 (5.3%)
Sphingolipids	ACer			11	11
Sphingolipids	Cer	79	2	128	209
Sphingolipids	CerP			25	25
Sphingolipids	GlcCer			4	4
Sphingolipids	HexCer	128	7	172	307
Sphingolipids	PE-Cer	7	4	41	52
Sphingolipids	PI-Cer			14	14
Sphingolipids	SHexCer	11	2	21	34
Sphingolipids	SPBP			1	1
Sphingolipids	SM	130	31	78	239
Sphingolipids	M(IP)2C			1	1
Sphingolipids	MIPC			2	2
Sphingolipids-Total					899 (34.8%)
Glycerophospholipids	LPA			3	3
Glycerophospholipids	LPC	22	7	6	35
Glycerophospholipids	LPE	7	10	2	19
Glycerophospholipids	LPG		1	5	6
Glycerophospholipids	LPI			6	6
Glycerophospholipids	PA	3	2	64	69
Glycerophospholipids	PC	237	1	62	300
Glycerophospholipids	PE	95	2	35	132
Glycerophospholipids	PG	3		44	47
Glycerophospholipids	PI	18	3	17	38
Glycerophospholipids	PIP			16	16
Glycerophospholipids	PS	4		10	14
Glycerophospholipids	BMP	1		1	2
Glycerophospholipids	GP		3		3
Glycerophospholipids-Total					690 (26.7%)
Glycerolipids	TG	133	1	254	388
Glycerolipids	DG	25	3	168	196
Glycerolipids	MG	1	1	7	9
Glycerolipids	GlcADG	12			12
Glycerolipids	DGCC		1		1
Glycerolipids	DGDG		1		1
Glycerolipids	MGDG	13	7	1	21

Glycerolipids	SQDG	1			1
Glycerolipids-Total					629 (24.3%)
Fatty Acyls	CAR	1	1	16	18
Fatty Acyls	CoA			1	1
Fatty Acyls	FA	18	6	41	65
Fatty Acyls	FAHFA	9	1	4	14
Fatty Acyls	HC			8	8
Fatty Acyls	NA	1	9	8	18
Fatty Acyls	NAE			12	12
Fatty Acyls	NAT			27	27
Fatty Acyls	FAG			1	1
Fatty Acyls	WE			15	15
Fatty Acyls-Total					179 (6.9%)
Other	PK			19	19
Other	PR		1	22	23
Other	SL	3	5	1	9
Other-Total					51 (2%)
Grand Total		966	122	1496	2584

Percentage of relative abundance for each lipid class was calculated based on the total number of identified features (n = 2584). Abbreviations: ACer: Acylceramides, BMP: Monoacylglycero-phosphomonoradylglycerols, CAR: Fatty acyl carnitines, CE: Steryl esters, Cer: Ceramides, CerP: Ceramide 1-phosphates, CoA: Fatty acyl CoAs, DG: Diglycerides, DGCC: Hydroxymethyl-choline, DGDG: Digalactosyldiacylglycerols, FA: Fatty acids, FAG: Fatty acyl glycosides of mono- and disaccharides, FAHFA: Fatty acid estolides, GlcADG: Glycosyldiacylglycerols, GlcCer: Glucuronosphingolipids, GP: Other Glycerophospholipids, HC: Hydrocarbons, HexCer: Hexosyl ceramides, LPA: Lysophosphatidic acids, LPC: Lysophosphatidylcholines, LPE: Lysophosphatidylethanolamines, LPG: Lysophosphatidylglycerols, LPI: Lysophosphatidylinositols, MG: Monoglycerides, MGDG: Monogalactosyldiacylglycerols, MIPC: Ceramide phosphoinositols, M(IP)2C: Ceramide phosphoinositols, NA: Nitrogenated fatty acids, NAE: N-acyl ethanolamines, NAT: N-acyl amines, PA: Phosphatidic acids, PC: Phosphatidylcholines, PE: Phosphatidylethanolamines, PE-Cer: Ceramide phosphoethanolamines, PG: Phosphatidylglycerols, PI: Phosphatidylinositols, PI-Cer: Ceramide phosphoinositols, PIP: Glycerophosphoinositol monophosphates, PK: Polyketides, PR: Prenol Lipids, PS: Phosphatidylserines, SHexCer: Sulfoglycosphingolipids, SL: Saccharolipids, SM: Sphingomyelins, SPBP: Sphingoid base-1 phosphates, SQDG: Sulfoquinovosyldiacylglycerols, ST: Sterol, TG: Triglycerides, WE: Wax esters and diesters.

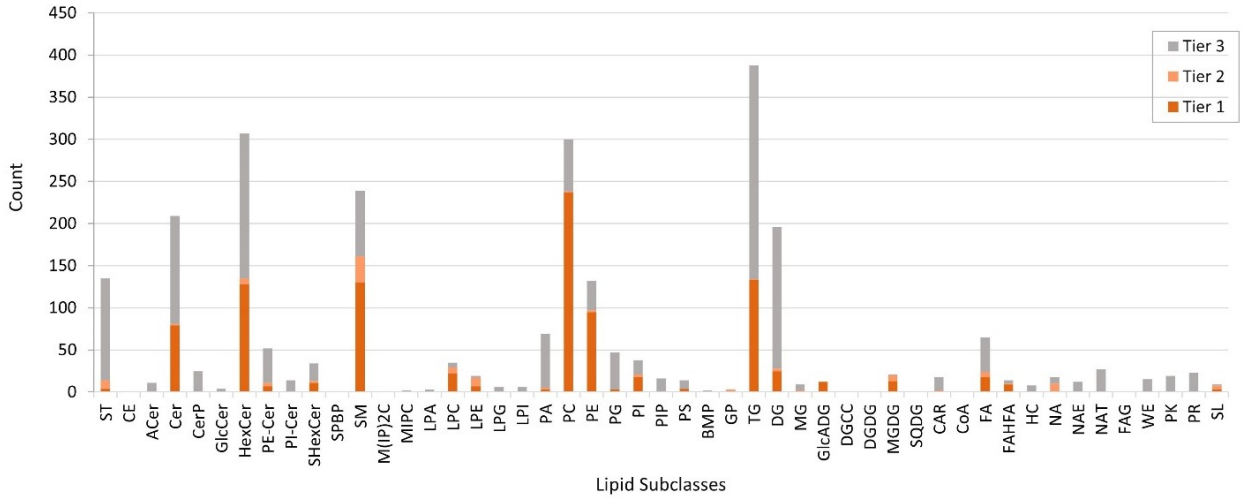


Figure S2: Distribution of identified lipids among lipid subclasses and identification tiers

Connecting Statement I

Our first study was designed to address the lack of research investigating early pregnancy lipidome of the intrauterine compartment in relation to LGA birth weights. Therefore, we conducted global lipidomics analysis of AF from healthy normal-weight mothers who gave birth to LGA infants in comparison to AGA infants. We started with this maternal group since epidemiological studies showed that a large proportion of LGA infants are indeed born to healthy mothers with normal body weight and no diabetic complications during pregnancy (16, 39). This maternal group represents our reference to identify the lipidomic features associated with LGA pregnancies without any interaction from other pathological conditions. Given that maternal obesity is a well-established risk factor for macrosomia and overweight/obese mothers represent a significant proportion of LGA mothers (16, 39), our next study aimed to query the AF lipidome in relation to LGA/AGA birthweights among overweight/obese otherwise-healthy mothers. The results of this study should shadow those of the first study unless there is impact of maternal obesity on LGA AF lipidome. These two studies should also allow for a comparison of LGA lipidomic features between maternal blood and amniotic fluid, since the available studies profiling early pregnancy maternal blood in relation to birth weight included mainly healthy normal-weight and overweight/obese mothers as study participants (5-7, 136).

Chapter 4:

The lipidomic Features of Amniotic Fluid Associated with Fetal Macrosomia in Overweight and Obese Mothers

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4.1 Abstract

Introduction: Maternal obesity is strongly associated with an increased risk of macrosomia. While the precise mechanisms underlying macrosomia remain unclear, investigating the lipidomic profile of the intrauterine compartment can provide further insights to the associated lipid dysregulation which seems to play a significant role. Our objectives were: 1) To examine differences in the lipidomic profile of amniotic fluid (AF) between overweight/obese (Ov/Ob) otherwise healthy mothers who gave birth to large-for-gestational age (LGA) in comparison to appropriate-for-gestational age (AGA) infants; 2) To examine the association between AF concentration of insulin and glucose with the lipidomic features characterizing LGA infants. Methods: Untargeted lipidomics analysis using LC-QToF-MS was conducted to profile AF samples of LGA (n=14, cases) and AGA infants (n=14, controls) selected and matched from a biobank of AF samples, which were collected during routine amniocentesis at about 15 weeks of gestation. Univariate and multivariate statistical analyses (PCA and PLS-DA) were applied. Results: LGA AF of Ov/Ob mothers had significantly lower levels of 15 phosphatidic acid species as compared to AGAs. Only one sphingomyelin molecular specie (SM 14:0;O2/20:1) was markedly elevated and had the highest variable importance in projection score. Insulin and glucose were not different between LGA and AGA AF and did not correlate to any of the differentially abundant features. Conclusion: Distinct lipidomic signature of LGA AF in Ov/Ob mothers was evident and it indicates that lipid dysregulation manifests at early pregnancy.

4.2 Introduction

Maternal obesity is thought to be associated with *in utero* epigenetic, metabolic, endocrine, and immune alterations, which can create a long-lasting signature promoting obesity and metabolic dysfunction later in life (1, 2). It is not surprising that the risk of macrosomia, or fetal overgrowth,

is three-fold higher in obese mothers compared to those with normal weight (3). While the underlying cause of macrosomia remains unclear, maternal lipid alterations seem to play a significant role in fetal adiposity. In fact, maternal hyperlipidemia may have a greater impact than hyperglycemia, which has long been considered the primary factor promoting macrosomia according to Pederson hypothesis (4, 5). Several studies demonstrated an independent relationship of maternal triglycerides (TGs) and none esterified fatty acids (NEFAs) to birth weight (6-8). Furthermore, the physiological increase in maternal TGs and NEFAs, which occur during the third trimester and coincides with the largest development in fetal adipose tissue, was found to be augmented and initiated much earlier in pregnancies complicated with obesity (4, 6). Such hyperlipidemic profile at early pregnancy may justify the high prevalence of macrosomia among obese mothers.

Maternal lipid alterations were evident in several lipidomics and metabolomics studies in association with higher birth weights (9, 10). Maternal obesity, itself, has its own lipidomic features of dysregulation, some of which aligns with the features associated with macrosomia, such as elevated maternal levels of short chain acyl carnitines (11, 12, 13), which are thought to indicate glucose intolerance and higher muscular fatty acid oxidation (14). However, not all obese mothers give birth to macrosomic or large-for-gestational age (LGA) infants. This implies that specific metabolic and lipidomic characteristics may distinguish obese mothers who do deliver LGA infants. These features are yet to be fully described. While previous studies have profiled maternal blood during pregnancy or placenta and cord blood at parturition in relation to birth weight, the lipidomic environment of the intrauterine compartment promoting macrosomia at early pregnancy remains under-investigated. Profiling amniotic fluid (AF) offers the opportunity to provide insights on the output of lipid metabolism and its interaction between maternal, placental, and fetal

compartments, especially at early pregnancy (15, 16). Therefore, our objective was to contrast the lipidomic profile of AF, obtained at early second trimester, between overweight/obese (Ov/Ob) mothers giving birth to LGA versus appropriate for gestational age (AGA) infants. We also aimed at investigating the association of AF glucose and insulin with the characteristic lipidomic features of LGA infants.

4.3 Methods

AF Biobank and Sample Selection

From a biobank of pre-collected AF samples, samples of Ov/Ob mothers, i.e., those with pre-pregnancy body mass index (BMI) ≥ 25 kg/m^2, who gave birth to LGA infants (cases; n =14) were randomly selected and matched with the available pool of Ov/Ob mothers of AGA infants (controls; n = 14). Matching criteria included infant's sex, gestational age at amniocenteses (± 1 week), pre-pregnancy BMI (± 3kg/m^2), and maternal age (± 3 years). Infants born with birth weights above the 90th percentile for gestational age and sex were considered LGA and those between 10th and 90th percentile were considered AGA, using birth weight for gestational age and sex percentiles (17). Our inclusion criteria for this nested case-control study included only mothers who gave birth to full term infants and had no adverse complications during pregnancy such as gestational diabetes (GDM) or preeclampsia. AF samples were collected from participants during second trimester amniocentesis performed for prenatal genetic testing at St. Mary's Hospital Centre (Montreal, Canada). The biobank collection of AF samples took place during the years 2000-2004. Only women with singleton pregnancies and no genetic anomalies were originally recruited. All participants provided written informed consents and the ethics approvals was obtained from McGill University and St. Mary's Hospital Centre Institutional Review Boards (IRBs). AF samples free of blood and meconium were aliquoted and stored at -80°C. Lipidomic profiling of selected

samples was performed at the Metabolomics Innovation Center (TMIC, Edmonton, Canada). Glucose and insulin were previously analyzed using a hexokinase assay (Abbott Laboratories, North Chicago, IL) and the Beckman Access ultrasensitive immunoenzymatic assay (Beckman Coulter, Brea, CA), respectively.

Lipidomics Analysis

AF samples were extracted using a modified Folch extraction method with dichloromethane, methanol, and water. Before extraction, an internal standard mixture composed of 15 deuterated lipids was added to samples. Sample extracts were analyzed by reversed-phase liquid chromatography using a Thermo Vanquish UHPLC equipped with a Waters Acquity CSH C18 column, linked to a Bruker Impact II Quadrupole Time-of-Flight (QToF) mass spectrometer. Separate injections for each ionization polarity were applied, and NovaMT LipidScreener 1.0.0 (Nova Medical Testing Inc.) was used for spectral processing and identification. For quality control (QC), a pooled mixture was prepared from all AF samples and then split into multiple aliquots. During analysis, each randomized batch of 13 samples was injected between two replicate injections of one QC aliquot, as this analysis was part of a project with a larger number of samples. More details on sample preparation and analysis can be found in our previous work (18).

A feature-intensity table was compiled for all detected features from all samples. Only features detected in ≥80% of injections within QC samples or at least one sample group (LGA or AGA) were retained. For feature identification, a three-tier annotation approach was used. Tier 1 included lipids identified with an MS/MS match score of ≥500 and a precursor m/z error ≤20.0 ppm and 5.0 mDa. Tier 2 included lipids identified with an MS/MS match score of <500 and a precursor m/z error ≤20.0 ppm and 5.0 mDa. Features which could not be identified using tandem-MS (tier 3) were annotated based on mass match only with a m/z error of ≤20.0 ppm and 5.0 mDa,

using the Lipid Maps database. NovaMT LipidScreener, which employs an internal filtering and scoring approach, was used to calculate MS/MS match scores, restrict the number of possible matches, and select the best identification. Matches with m/z errors exceeding 5.0 mDa or 20.0 ppm or having unexpected retention times or adducts were excluded.

Different isomeric lipids have identical masses, chemical formulas, and MS/MS fragmentation patterns with only minor differences, such as the position of double bonds, functional groups, or stereochemistry. These lipids cannot be distinguished using our employed untargeted LC-MS/MS approach. Hence, multiple peaks were annotated as the same lipid. The classification and shorthand notation of lipids followed the guidance of LipidMaps, MS-Dial and the Update on LIPID MAPS classification, nomenclature, and shorthand notation for MS-derived lipid structures (19-21).

Data Analysis

To control for any technical sources of variations, all identified features were first normalized using internal standards. Based on lipid class similarity and the expected retention time range for each class, the identified lipids were matched to one of 15 deuterated internal standards added to all samples before extraction. The intensity of each identified lipid was divided by the intensity of the matched internal standard. Second, the intensity ratio of each identified lipid was median-normalized, i.e., divided by the median intensity ratio of all identified lipids within each sample experiment. Near constant and non-informative features, such as internal standards and common contaminants were filtered out.

Data analysis was performed using R (version 4.3.2). Filters applied included a relative standard deviation (RSD) filter to remove features with RSD > 25% for QC samples, i.e., features with low consistency of signal intensity detection in QC samples. The top 30% of features with

the lowest RSD among all samples were also filtered out as near-constant features. To eliminate any bias trends from our data, EigenMS normalization was applied to filtered features (22), and it was robust enough to allow the detection of differentially abundant (DA) features between LGA and AGA groups with statistical analysis.

Univariate Analysis

A tailored approach with parametric (t-test) or non-parametric (Wilcoxon Rank Sum) tests were applied with equal or non-equal variances. Each feature from each comparison group was tested for normality using the Shapiro-Wilk test and for homogeneity of variance using Bartlett's test. A p value > 0.01 was used to conclude normal distribution and equal variance, respectively. Insulin and glucose were also assessed using this univariate analysis approach. To adjust for the effect of covariates (amniocentesis week, maternal BMI and age, and fetal sex) on differentially abundant (DA) features, analysis of covariance (ANCOVA) was used. Ethnicity was not adjusted for in the analysis due to limited variation in the data. The majority of participants were Caucasian (North American or European; 21 out of 28), while the remaining represented a mixture of ethnic groups with sample sizes too small for meaningful stratification: Asian (n = 2), Black (Caribbean or African; n = 1), and Hispanic or Middle Eastern (n = 4). Given P values were corrected for false Discovery Rate (FDR) using Benjamini-Hochberg adjustment for univariate analysis and ANCOVA testing. Adjusted p value < 0.05 was considered significant.

Multivariate Analysis

Principal component analysis (PCA) was applied to visualize the clustering of samples. For supervised modeling, partial least squares discriminant analysis (PLS-DA) and variable importance in projection (VIP) were applied to build a classification model and select features with high contribution to sample classification. Only for PLS-DA analysis, pareto-scaling was applied

to reduce the effect of different features' variance on VIP analysis. Pearson and Spearman correlation analysis were used for normally and non-normally distributed features respectively, with the objective of assessing correlations between DA features, insulin, glucose, and birth weight. The intensities of features with identical identification were averaged for correlation analysis, and Benjamini-Hochberg adjustment was applied.

Extended analysis

Given that univariate analysis yielded similar DA species, mainly phosphatidic acid (PA) species, to what was obtained in our previous study (18), in which we compared LGA to AGA AF of normal-weight (NW) mothers, we extended the analysis to include four groups: LGAs of NW mothers, LGAs of Ov/Ob mothers, AGAs of NW mothers, and AGAs of Ov/Ob mothers. We aimed to compare all PA species detected, in addition to one other DA specie mainly against AGA AF of NW mothers. Data was combined for all groups after RSD filtration, followed by EigenMS normalization with four comparison groups. A subset of data including only PA features (53 features) and SM 14:0;O2/20:1 was retained. Linear regression models were fitted for each feature, as the dependent variable, birth weight group (LGA/AGA) and maternal weight group (NW and Ov/Ob) as classification variables, and their interaction component along with covariates; amniocentesis week, maternal age, and fetal sex. Benjamini-Hochberg adjustment was used to correct the models' p values based on 54 p values. For features with significant interaction component, pairwise comparisons were conducted among the four groups with Bonferroni's adjustment for multiple comparisons. Adjusted p value < 0.05 was considered significant.

4.4 Results

Our LGA and AGA groups were essentially similar in all maternal and infant characteristics, except for birth weight (Table 4.1). LC-MS analysis enabled the detection of 4985 unique peaks

in all samples, of which 2584 peaks were identified. Tandem MS spectra was identifiable for 966 (Tier 1) and 122 (Tier 2) features. The remaining 1496 features were identified at the species level, i.e., sum composition is provided, based on mass match (Tier 3). Lipids identified comprised 49 distinct lipid subclasses. Major subclasses included glycerolipids with mainly triglycerides (15% of total identified lipids) and diglycerides (7.6%), sphingolipids with mainly hexosyl-ceramides (11.9%), sphingomyelins (9.3%), and ceramides (8.1%), and glycerophospholipids with phosphatidylcholines (PCs, 11.6%) (Fig. 4.1).

Univariate Analysis

Univariate analysis was applied over 1780 features, which remained after applying the RSD filters, and 22 features were significantly different between LGA and AGA samples (Table 4.2). Of the 22 DA features, 20 lipids were uniquely identified, i.e., some features had replicate identifications. Fig. 4.2 shows a volcano plot of these features/identified lipids. Among the 22 DA features, 17 were Tier 3 identified, i.e., based on mass match, as PA species which were all depleted in LGA samples. A sphingomyelin molecular specie (SM 14:0;O2/20:1, Tier 1 identified based on MS/MS) was the only lipid enriched in LGA samples and had a high fold change of 9.5. Other depleted lipids included a hexosyl ceramide (HexCer), acylceramide (ACer), PC and acyl-CoA. All DA features were still significantly different between groups after adjusting for covariates. Insulin and glucose were not different between LGA and AGA AF samples.

Multivariate Analysis

3D-PCA and PLS-DA scores plots are shown in Fig. 4.3. A third principal component in PCA was needed to visualize the separation between LGA and AGA samples. A PLS-DA classification model with 8 components was fit. Each of the first two PLD-DA components captured 9% of the variance. The classification error rate, obtained through a three-fold cross

validation approach with 100 repeats, was 7.5 ± 0.46%, and the model was significant upon permutation testing (999 permutations, *p* value = 0.002). Features with the highest VIP scores, i.e. the highest contribution to group separation, were SM 14:0;O2/20:1 and acyl CoA 14:1, followed by several PA species, as shown in Fig. 4.4. All DA features had a VIP score > 2. Correlation analysis (Fig. 4.5) showed that insulin and glucose did not correlate significantly with any of the DA lipids nor with birth weight. SM 14:0;O2/20:1 correlated negatively and significantly with all DA features (average r value = -0.6, adjusted *p* value < 0.05).

Extended analysis

Multiple linear regression showed significant interaction component between maternal weight groups and birth weight groups for 34 PA species and SM 14:0;O2/20:1 (adjusted *p* value < 0.05). The majority of PA species were significantly higher in LGA AF of NW mothers, as well as in LGA and AGA of Ov/Ob mothers when compared to AGA of NW mothers as a reference group. SM 14:0;O2/20:1 was significantly higher in LGA AF of NW and Ov/Ob mothers in comparison to AGA of NW mothers (adjusted *p* value < 0.05). No significant difference in AF SM 14:0;O2/20:1 was found between AGA of NW and AGA of Ov/Ob mothers (adjusted *p* value > 0.05). A heatmap showing differential intensities of these lipidomic features with significant interactions across groups is provided in Fig. 4.6. Among features with no significant interaction components, 6 PA species; PA O-62:12, PA O-37:1, PA O-58:10, PA O-45:3, PA O-60:10, and PA O-60:11, had significant main effects for the birth weight group, with higher levels in LGA compared to AGA AF.

4.5 Discussion

Untargeted lipidomics analysis of AF revealed some interesting characteristics of the intrauterine environment promoting macrosomia specifically in Ov/Ob mothers. LGA lipidomic

profile of AF was distinct from that of AGA mothers, with mainly reductions of many PA species and a few other species including acyl CoA 14:1. SM 14:0;O2/20:1 was also profoundly elevated in LGA AF with high discriminating power, i.e. VIP score, against AGA infants. These results were unexpected given the results of our previous work with LGA AF of healthy NW mothers (18). In contrast to Ov/Ob mothers, LGA compared to AGA AF of NW mothers had marked elevations of many PA species, along with acyl CoA 14:1. The only similarity seems to be the elevation of SM 14:0;O2/20:1. Therefore, clearly maternal obesity and its associated metabolic derangements further complicate the lipidomic profile of LGA pregnancies.

In our previous work on LGA AF of NW mothers, we hypothesized that the increase in PA species observed was induced by higher maternal circulating levels of TGs, NEFAs, and growth factors, such as insulin (18), i.e., factors which are known to correlate positively with birth weight (6, 23, 24). Our interpretation was based on studies reporting elevated PA levels in primary human trophoblast cells as a results of fatty acid treatment or insulin treatment (25, 26). Hence, placenta may be the source of elevated PA in AF. Higher maternal levels of TG and NEFAs are also associated with maternal obesity (6, 27), therefore, higher PA should be expected in AF of Ov/Ob mothers based on our hypothesis, which is indeed the case. The majority of PA species identified in AF were significantly higher in Ov/Ob mothers of both AGAs and LGAs in comparison to NW mothers of AGAs as a reference group, as shown in Fig. 4.6. In line with our results is the activation of placental mTOR signaling, of which PA is a key regulator (28), which was reported to positively associate with maternal BMI and with birth weight (29).

The underlying mechanism for the reduction of PA species in LGA compared to AGA AF of Ov/Ob mothers is unknown, however, it may be a result of higher metabolic utilization of PA species. Maternal obesity is known to be associated with heavier placentas (30), increased

placental lipid content of TGs, PCs, and cholesterol esters, higher rates of lipid esterification, and upregulation of proteins involved in lipid esterification pathways (31). While both the LGA and AGA groups compared are born to Ov/Ob mothers, it is possible that in placentas of Ov/Ob mothers of LGAs, PA is being converted to TG and other phospholipids at higher rates and therefore lower levels of PA are evident in LGA AF of Ov/Ob mothers. This may also explain the reduction observed in CoA 14:1 in LGA of Ov/Ob compared to AGA of Ov/Ob mothers.

SM 14:0;O2/20:1 was markedly elevated in LGA compared to AGA AF of Ov/OB mothers, and it had the highest VIP score in discriminating between the two groups. SMs with sphingoid bases other than sphingosine 18:0 or 18:1 are not commonly reported in human tissues (32). Mammalian serine palmitoyl transferase (SPT), which catalyzes the first step in *de novo* synthesis of sphingolipids, exhibits high preference for saturated fatty acyl-CoAs containing 16 carbon atoms (±1), leading to the predominant formation of 18-carbon sphingoid bases upon condensation with serine (32). However, SPT subunit 3, which is highly expressed in the placenta and trophoblast cells (40), preferentially synthesizes 14-carbon sphingoid bases. This explains the presence of SMs with 14-carbon sphingoid bases in AF (33). SMs are known to influence cellular fatty acid uptake and promote the formation of large lipid droplets (34). Yet, it is hard to conclude on the metabolic significance of SM 14:0;O2/20:1 elevation in LGA AF due to the lack of information about it.

Ceramides are known to be associated with insulin resistance and obesity (4). However, despite the high VIP scores for a few ceramide species (VIP > 2), these ceramides were not differentially abundant between LGA and AGA AF of Ov/Ob mothers. Other modified ceramides, HexCer 8:0;O2/34:8 and ACer 47:6;O2 were, on the other hand, significantly depleted in LGA AF of Ov/Ob mothers. The role of HexCers, which are formed by the enzymatic addition of hexose

molecules (glucose or galactose) to ceramides, is not fully elucidated, it but seems to be tissue dependent. Overexpression of glucosylceramide synthase, for example, increases glucosylceramides and enhances insulin signalling in myocytes but impairs it in adipocytes (35). In the context of gestational metabolic health, specifically in relation to insulin resistance, the available literature shows that GDM mothers have lower circulating levels of a few HexCer species (36), but higher placental levels of them (37, 38). Therefore, it is hard to interpret the reduction observed in LGA AF of HexCer 8:0;O2/34:8, as it is for ACer 47:6;O2. It is noteworthy that the metabolic conversion of ceramides to ACers and their storage in hepatic lipid droplets, as observed in mice fed a high-fat diet, has been proposed as a protective mechanism to manage excess toxic ceramides within cells (39).

Conclusion

In this study, we provide evidence for a distinct lipidomic profile of AF in early pregnant Ov/Ob mothers giving birth to LGA compared to AGA infants. The driver of such lipidomic differences is unclear, but placental metabolism is likely involved in such alterations. Higher metabolic utilization of intermediate lipid species may explain the observed reduction of PA and CoA 14:1 in LGA compared to AGA AF of Ov/Ob mothers. SMs with 14-carbon sphingoid bases possibly play a role in LGA complicated pregnancies, given the consistent elevation of SM 14:0;O2/20:1 in LGA compared to AGA AF. Moreover, AF glucose and insulin were not different and did not correlate with any of the DA lipidomic features, nor with birth weight. Nonetheless, this exploratory study had a number of limitations, including the small sample size and prolonged storage duration of AF samples. Furthermore, not all lipidomic features were identified by tandem mass spectrometry, and hence their identification was based on mass match only and they were not fully characterized. There is also the inherent limitation of lipidomics in providing only a static

image of lipid differences, rather than the dynamic changes in metabolic pathways, which can be overcome by use of flux based lipidomics. Overall, further investigation using lipidomic profiling, preferably of maternal blood and AF, is needed to gain deeper insights into the lipidomic alterations associated with macrosomia.

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4.7 Tables and Figures

Table 4. 1: Maternal and infant characteristics.

	LGA (n=14)	AGA (n=14)
Maternal		
Age (years)	38.3 ± 2.2	38.2 ± 2.2
Pre-pregnancy weight (kg)	79.7 ± 14.6	78.7 ± 12.5
Height (m^2)	1.62 ± 0.06	1.62 ± 0.06
BMI (kg/m^2)	30.1 ± 4.4	29.9 ± 4.0
Amniocenteses week (weeks)	15.4 ± 0.9	15.4 ± 1.0
Infant		
Sex (n = female/male)	5/9	5/9
Birth weight (grams) *	4295.4 ± 260.3	3599.5 ± 363.6
Gestational age at birth (weeks)	40.0 ± 0.9	39.9 ± 1.1

- Mean ± SD.

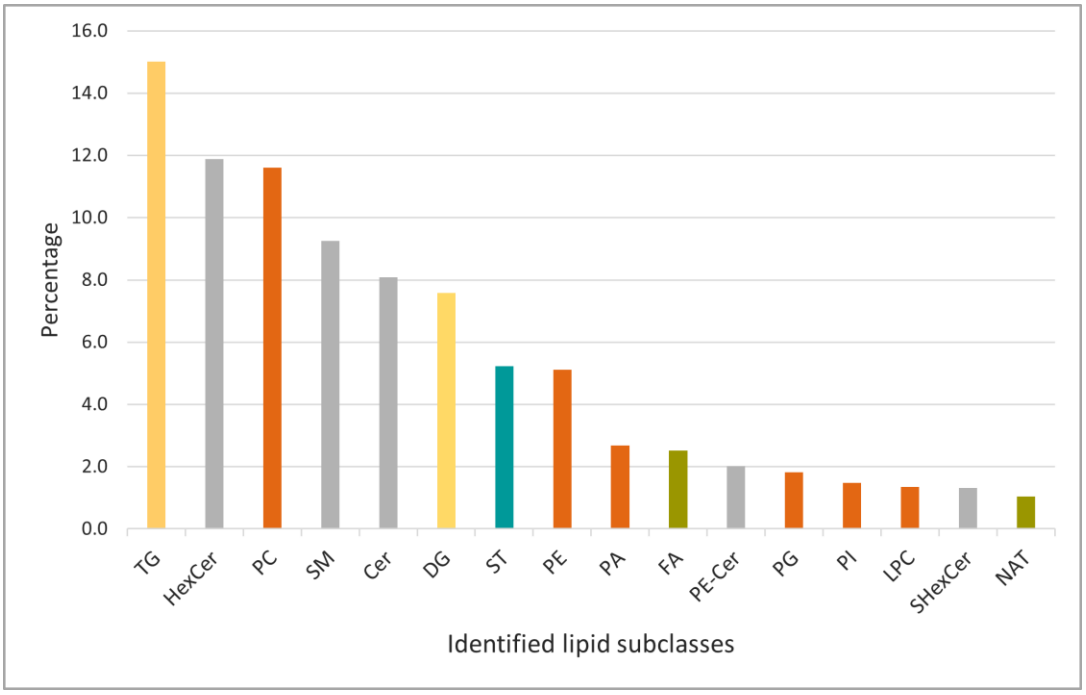
* Significantly different between LGA and AGA (p value < 0.05).

Table 4. 2: Differentially abundant features between LGA and AGA infants.

Feat. No	Identified Feature/Lipid	Log2 Fold Change	Adjusted *p* value
Features enriched in LGA AF			
1	SM 14:0;O2/20:1 *	3.25	< 0.01
Features depleted in LGA AF			
2	PA O-40:2 [R]	-0.42	< 0.000
3	PA O-39:0	-0.53	< 0.01
4	PA O-38:2	-0.64	< 0.01
5	PA O-39:2 [R]	-0.93	< 0.01
6	PA O-40:2 [R]	-0.70	< 0.01
7	PA O-30:2	-0.93	< 0.01
8	PA O-61:7	-0.92	< 0.01
9	PA O-45:0;O	-0.82	< 0.01
10	PA O-45:1	-0.74	< 0.01
11	PA 33:3	-1.04	< 0.01
12	PA O-39:2 [R]	-0.61	< 0.01
13	PA O-34:2	-0.66	< 0.01
14	PA O-45:2	-1.38	< 0.05
15	CoA 14:1	-2.30	< 0.05
16	PA 39:2	-0.41	< 0.05
17	PA O-39:1	-0.80	< 0.05
18	PA O-35:3	-0.67	< 0.05
19	PA O-57:0	-0.78	< 0.05
20	HexCer 8:0;O2/34:8 **	-0.58	< 0.05
21	PC 16:1_18:1 *	-0.73	< 0.05
22	ACer 47:6;O2	-0.72	< 0.05

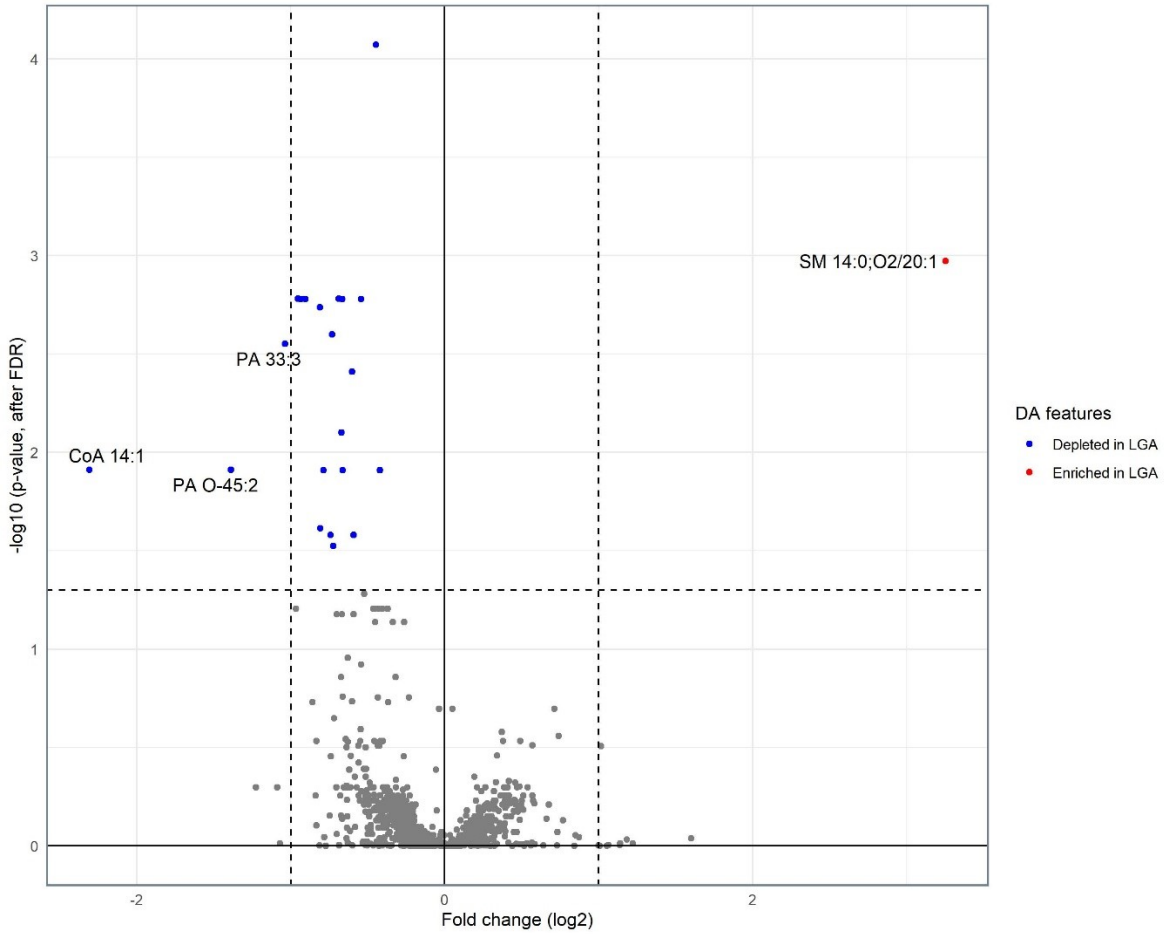
- Log2 fold change was calculated as log2(mean feature intensity LGA/AGA).
- All features listed were Tier 3 identified, i.e., based on mass match, except for those with an asterisk which were based on MS/MS identification; * for Tier 1 identified and ** for Tier 2 identified features.
- [R] Indicates features with replicate identification.
- ACer: Acylceramide, CoA: Fatty acyl CoA, HexCer: Hexosyl ceramides, PA: Phosphatidic acid, PC: Phosphatidylcholine, SM: Sphingomyelin.

Figure 4. 1: Relative abundance of identified lipid subclasses.



- Only subclasses with relative abundance > 1% of total identified lipids are presented.
- Cer: Ceramides, DG: Diglycerides, FA: Fatty acid, HexCer: Hexosyl ceramides, LPC: Lysophosphatidylcholines, NAT: N-acyl amines, PA: Phosphatidic acids, PC: Phosphatidylcholines, PE: Phosphatidylethanolamines, PE-Cer: Ceramide phosphoethanolamines, PG: Phosphatidylglycerols, PI: Phosphatidylinositols, SHexCer: Sulfoglycosphingolipids, SM: Sphingomyelins, ST: Sterol, TG: Triacylglycerols.

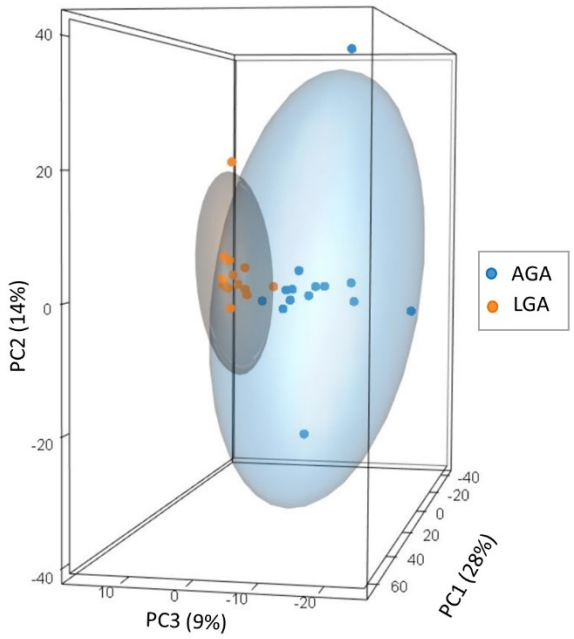
Figure 4. 2: Volcano plot analysis of identified lipids.



- Features with an adjusted *p* value < 0.05 are colored. Red indicates higher intensity in LGA compared to AGA and blue indicates lower intensity.
- Annotation was only added to features with an adjusted *p* value < 0.05 and log fold change > 1 or < -1.
- CoA: Coenzyme A, PA: Phosphatidic acid, SM: Sphingomyelin.

Figure 4. 3: PCA and PLS-DA scores plots.

a) PCA 3D scores plot



b) PLS-DA scores plot

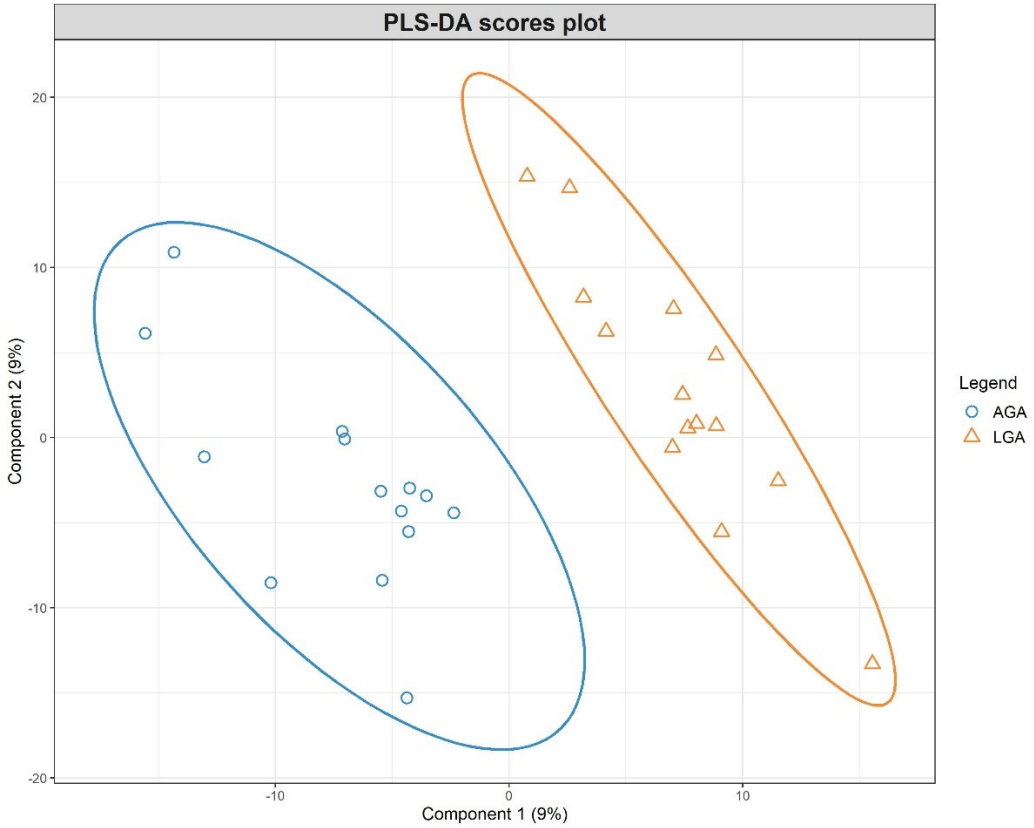
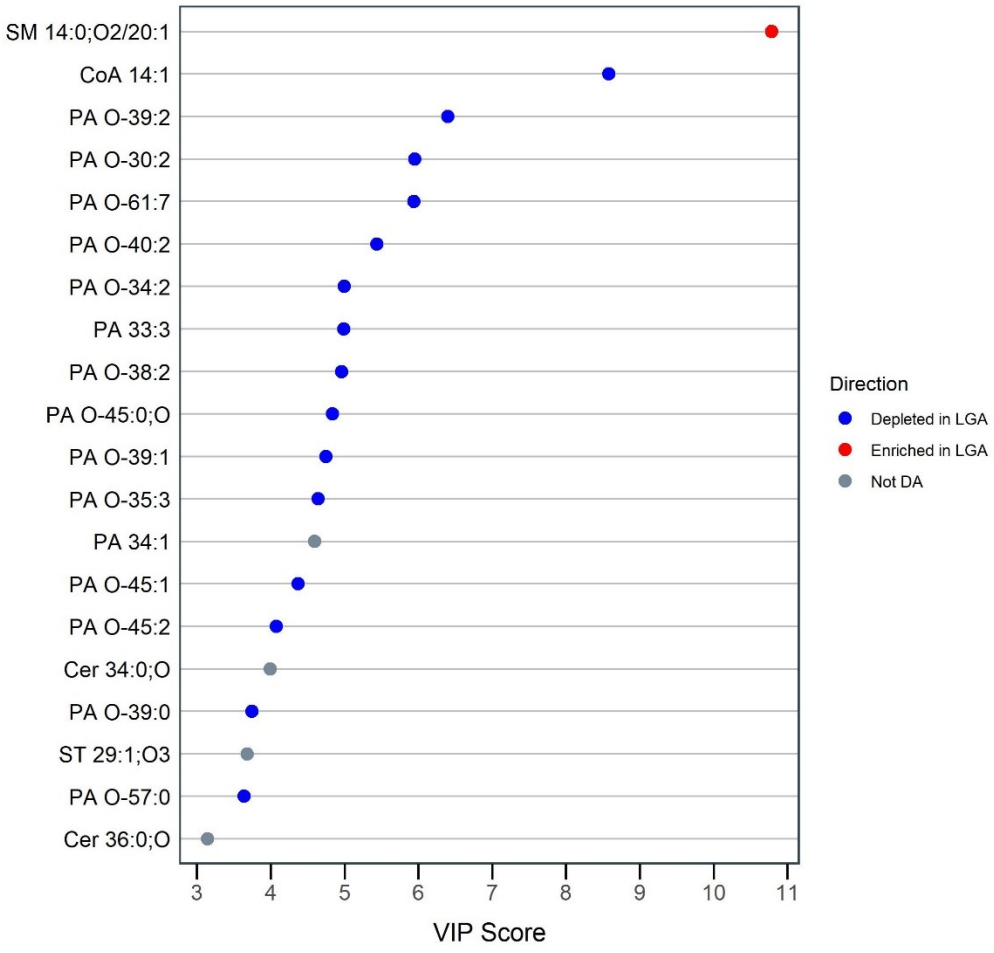
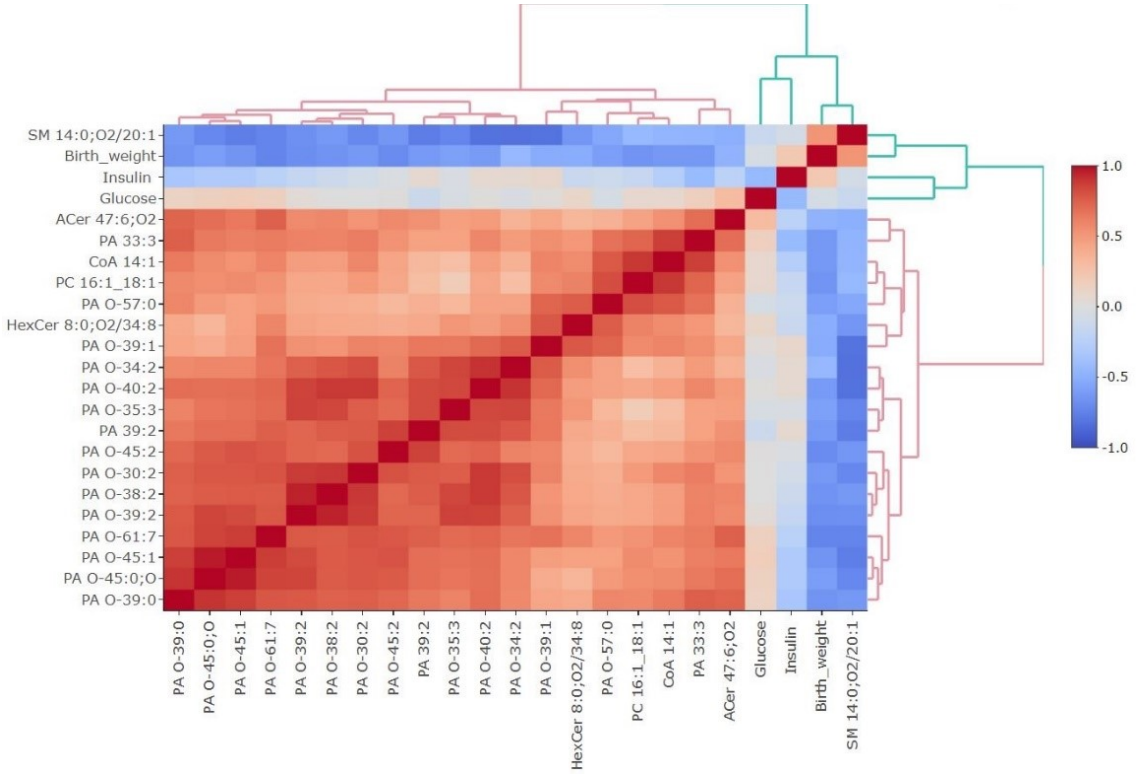


Figure 4. 4: Variable importance in projection (VIP) scores.



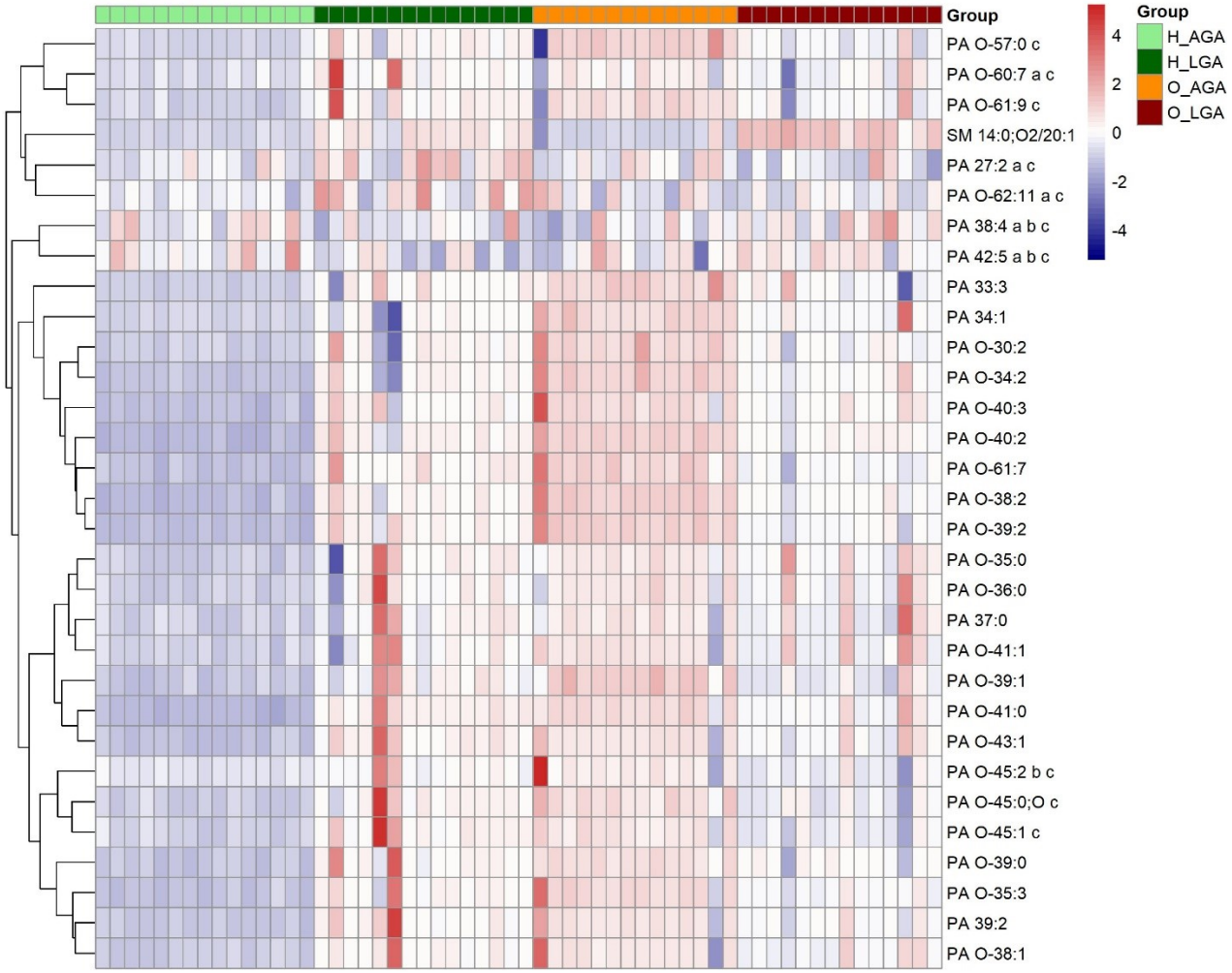
- The top 20 VIP scores of the first component from PLS-DA analysis are presented.
- All lipid species reported in this figure were DA except for ceramides (Cer 34:0;O and Cer 36:0;O), sterol (ST 29:1;O3), and phosphatidic acid (PA 34:1).

Figure 4. 5: Correlation heatmap.



- The correlation heatmaps displays the correlation coefficients of Pearson or Spearman's correlation analysis, based on the normality of features tested.
- No significant correlations were found for insulin and glucose with any of the DA features nor with birth weight (adjusted p value > 0.05). The majority of correlations between DA features were significant (adjusted p value < 0.05).

Figure 4. 6: Intensity heatmap of PA species across birth weight and maternal weight groups.



- All features showed significantly higher intensities in all groups compared to AGA of NW mothers, as a reference group, with adjusted *p* values < 0.05, except for features marked with letters: (a) no significant difference with AGA of Ov/Ob; (b) no significant difference with LGA of NW; (c) no significant difference with LGA of Ov/Ob mothers
- Intensities of features with similar identifications were averaged, given their similar significance pattern.

Connecting Statement II

In the previous two studies, we profiled the AF lipidome in relation to LGA/AGA pregnancies in healthy normal-weight and overweight/obese mothers. Statistical and bioinformatics analyses revealed a number of significant lipidomic features characterizing LGA from AGA AF, some of which were similar between normal-weight and overweight/obese mothers, while others were different or had a different direction of change. In addition, insulin and glucose were not correlated to birth weight nor to DA lipidomic features of LGAs in any of these maternal groups. Clearly, maternal obesity complicates LGA lipidomic profile and influences lipid metabolism of the intrauterine compartment, which requires further investigation. In the following study, we aimed to address this issue by contrasting AF lipidome between normal-weight and overweight/obese mothers who gave birth to LGA infants. This comparison should further clarify the characteristics of LGA AF lipidome as a function of maternal body weight. Blood lipidomic features of maternal obesity overlap to some extent with those of macrosomia as discussed in the literature review. Therefore, and given the similar pregnancy outcomes, we expect that LGA mothers regardless of their pre-pregnancy weight status will exhibit similar AF lipidomic profiles and similar glucose and insulin levels.

Chapter 5:

Distinct Lipidomic Features of Large-for-Gestational Age Infants between Normal-Weight and Overweight/Obese Mothers

5.1 Abstract

Introduction: Alterations in lipid metabolism are reported in association with maternal obesity and fetal macrosomia, i.e. large-for-gestational age (LGA) infants with some overlapping features. In this study we aimed to investigate the impact of maternal obesity on the lipidomic profile of amniotic fluid (AF) in LGA complicated pregnancies. Methods: From bio-banked AF samples, healthy normal-weight mothers (NW) of LGA infants (n=15) were randomly selected, matched and compared to overweight/obese mothers (Ov/Ob) of LGAs (n=14). Untargeted lipidomics analysis was conducted followed by univariate (t-test and Wilcoxon Rank Sum test) and multivariate (PCA and PLS-DA) methods to extract differentially abundant (DA) features. Results: Ov/Ob mothers had elevated levels of 24 identified lipids, which included mainly ceramides and triglycerides. On the negative side, 37 lipids were depleted including mainly phosphatidic acid, phosphatidylcholine, and sphingomyelin species. Conclusion: Maternal obesity complicates the AF lipidome of LGA mothers, with evident alterations that are likely indicative of insulin resistance and distinct placental lipid metabolism associated with obesity.

5.2 Introduction

The pathophysiology of fetal macrosomia has been under investigation for decades. Epidemiological studies revealed a strong association between maternal obesity and macrosomia, or large for gestational age (LGA) infants. Overweight and obese mothers have a 1.5- and 2-times higher risk of delivering an LGA infants, respectively, compared to normal-weight mothers (1). Yet, studies on maternal characteristics of LGA infants report a high percentage of these infants being born to healthy normal-weight mothers. For example, Jolly *et al*. studied about 350,000 completed singleton pregnancies in the UK and reported that from about 35,000 LGA infants born, 45% were born to mothers with normal body mass index (BMI), 50% to overweight and obese, and 6% to mothers with GDM or pre-existing diabetes (2). Similarly, Hua *et al*. studied 16,896 singleton pregnancies not complicated with diabetes in China and reported that 77.8% of LGAs were born to mothers with normal BMI (3). These reports raise the question of how similar the metabolic profile is between normal-wight mothers and overweight/obese mothers, both giving birth to LGA infants.

Advancements in the field of omics has provided important insights to the metabolomic and lipidomic alterations associated with maternal obesity and macrosomia. In fact, an overlap of maternal blood lipidomic features can be observed between the two conditions, such as the reduction of phosphatidylcholines and lysophosphatidylcholines in association with maternal obesity (4) and higher birth weights (5-7). Higher levels of short chain acylcarnitines are also observed in both conditions (8-10), which may indicate increased muscular fatty acid oxidation as a result of insulin resistance (11). In our previous work, we profiled the amniotic fluid (AF) lipidome of LGA in contrast to appropriate for gestational age (AGA) pregnancies early in the second trimester. Our analysis revealed an elevation of sphingomyelin (SM 14:0,O2/20:1) in LGA

AF compared to AGA AF in two maternal populations, normal-weight and overweight/obese mothers (12, 13). Other features, however, were the exact opposite between the two maternal populations, such as higher phosphatidic acid (PA) species in LGA AF of normal-weight mothers whereas lower PA in LGA AF of overweight/obese mothers when compared to their AGA counterparts (12, 13). In this study, we aimed to contrast LGA pregnancies between normal-weight and overweight/obese mothers.

5.3 Methods

Study Design, AF Biobank, and Lipidomics Profiling

In a nested case control design, AF samples of LGA and AGA infants were selected and stratified based on maternal body mass index (BMI), from our biobank of AF samples. LGA infants were defined as having birth weights above the 90th for gestational age and sex (14). BMI criteria for normal, overweight, and obese were used to classify maternal groups into normal-weight (NW) and overweight/obese (Ov/Ob) mothers. LGA AF samples were matched and compared between NW mothers (n=15) and Ov/Ob mothers (n=15). Matching was based on sex, amniocenteses week or the gestational age at the time of amniocenteses (± 1 week), and maternal age (± 4 years). Our inclusion criteria included only mothers who gave birth to full term infants and had no adverse complications during pregnancy such as gestational diabetes or preeclampsia. The biobank of AF was collected from consenting pregnant women undergoing routine amniocentesis at early second trimester at St. Mary's Hospital Centre (Montreal, Canada). Only women with no genetic anomalies and singleton pregnancies were originally recruited. The ethics approval was granted by the Institutional Review Boards (IRBs) at McGill University and St. Mary’s Hospital Centre.

AF samples were stored at -80°C since their collection. Untargeted lipidomics profiling of samples was performed using LC-QToF-MS platform at the Metabolomics Innovation Center (TMIC, Edmonton, Canada), as detailed in our previous study (12). AF glucose and insulin were previously assayed using a hexokinase assay (Abbott Laboratories, North Chicago, IL) and the Beckman Access ultrasensitive immunoenzymatic assay (Beckman Coulter, Brea, CA), respectively.

Data Analysis

Lipid identification followed a three-tier annotation approach. Detected features were either annotated based on MS/MS identification (Tier 1 and 2) or mass match (Tier 3). NovaMT LipidScreener employs a filtering and scoring approach and it was used to generate MS/MS match scores for detected features. Tier 1 identified features had an MS/MS match score of ≥500 and a precursor m/z error ≤20.0 ppm and 5.0 mDa. Tier 2 identified features had an MS/MS match score of <500 and a precursor m/z error ≤20.0 ppm and 5.0 mDa. Tier 3 included features which could not be identified using tandem-MS, and hence were annotated using mass match only based on Lipid Maps database, with a m/z error of ≤20.0 ppm and 5.0 mDa.

Raw intensities of identified lipids were normalized by internal standards; 15 deuterated lipids of different classes which were spiked into samples before lipid extraction. The intensity of each identified lipid was divided by the intensity of the class-matching internal standard. The intensity ratio of each identified lipid was then median-normalized, by dividing it by the median intensity ratio of all identified lipids within each sample experiment as indicated by TMIC. The following data analysis steps were performed using R (version 4.3.2). Features with low reproducibility, i.e., features with a relative standard deviation (RSD) > 25% as detected in quality control samples, were filtered out. Near-constant features were also filtered out by removing the

top 30% of features with the lowest RSD among all samples. Finally, EigenMS normalization was applied to the data set of filtered features with NW mothers and Ov/Ob mothers of LGAs as comparison groups (15).

Univariate analysis was applied to find differentially abundant features (DA), using either parametric (t-test) or non-parametric (Wilcoxon Rank Sum) tests and with equal or non-equal variances. Shapiro-Wilk test and Bartlett's test with a p value > 0.01 were used to conclude a normal distribution and equal variances, respectively. To adjust for covariates, linear regression models were fitted with each DA feature as the dependent variable, maternal groups (NW and Ov/Ob mothers) as the classification variable, along with the following covariates: Amniocentesis week, maternal age, and fetal sex. Ethnicity was not adjusted for given that the majority of mothers were Caucasian (North American or European; 24 out of 29). Benjamini-Hochberg adjustment was used to correct for multiple testing in both univariate and regression analyses. An adjusted p value < 0.05 was used to conclude significance.

Multivariate analysis included supervised and unsupervised clustering methods, i.e., principal component analysis (PCA) and partial least squares-discriminant analysis (PLS-DA) for descriptive modeling. Pareto-scaling was applied to features before PLS-DA analysis, and variable importance in projection (VIP) was used to estimate the relative importance of each feature in the PLS-DA classification model. Optimization and permutation testing of the PLS-DA model was performed using MixOmics and RVAideMemoire packages. Furthermore, linear correlations of glucose and insulin were examined in relation to birth weight and DA features. Pearson and Spearman's rank correlation testing were used for normally and non-normally distributed features, respectively. For DA features with replicate identification, features' intensities were averaged for correlation analysis. Benjamini-Hochberg adjustment was also applied for correlation analysis.

5.4 Results

General maternal and infant characteristics of LGA infants included in this study are listed in Table 5.1. These characteristics were not different (p value > 0.05) between NW and Ov/Ob mothers of LGAs, except for maternal weight, height, and BMI. The applied lipidomics approach detected 4985 unique peaks in all samples, of which 2584 peaks were identified, encompassing 49 distinct lipid subclasses. Triglycerides (TGs), hexosyl-ceramides, and phosphatidylcholines (PC) resembled the largest detected and identified lipid subclasses. Further details on quality control data and lipid subclasses identified in AF samples can be found in our previous study (12). Following the application of RSD filters, 1780 features remained for univariate and multivariate analysis.

Univariate analysis detected 61 features/lipids as DA (adjusted p value < 0.05) between Ov/Ob and NW mothers of LGA infants, corresponding to 60 identified lipids when replicate identification is excluded. Twenty-four features were enriched in Ov/Ob mothers compared to NW mothers of LGAs and mainly included ceramides and TGs. Thirty-seven features were depleted in including PA species, PCs, SMs, and hexosylceramides (Table 5.2). Fig. 5.1 shows volcano plot analysis of all identified lipids illustrating significance versus log2 fold changes between Ov/Ob and NW mothers of LGAs. Several ceramide species had the highest positive log2 fold changes (>1), i.e., they were highly enriched in Ov/Ob compared to NW mothers, while triglyceride (TG 58:7; O3) and PA O-45:2 had the lowest negative log2 fold changes (< -1), i.e., highly depleted in Ov/Ob compared to NW mothers. AF glucose and insulin were essentially similar between Ov/Ob and NW mothers of LGAs (adjusted p value > 0.05).

Group separation could not be visualized with PCA scores plot (Fig. 5.2a). Supervised clustering using PLS-DA provided clear separation (Fig. 5.2b). A PLS-DA model was fit with 4

components, the first two explained 19.5% of the total variance. The model was significant (p value = 0.001) upon permutation testing and provided 5.66 ± 0.47% classification error rate. VIP analysis obtained from the PLS-DA model showed that SM 14:0;O2/20:1 had the highest VIP score, i.e., contributed the most to the separation between Ov/Ob and NW mothers, followed by several ceramides and PA species, as shown in Fig. 5.3. Among DA features, 51 feature had a VIP score > 2.0. Finally, no significant correlations were found for glucose or insulin with birth weight nor with any of the DA features (adjusted p value > 0.05).

5.5 Discussion

The impact of maternal obesity on the intrauterine lipidomic signature at an early stage of pregnancy is an area with scarcity of information. To our knowledge, this is the first study to profile the AF lipidome as a function of maternal body weight in LGA complicated pregnancies. AF lipidome of Ov/Ob mothers was distinct from that of NW mothers in several major features, which included elevated levels of ceramides, TGs and one specific sphingomyelin (SM 14:0,O2/20:1). On the other hand, different species of PA, PC, and SMs were depleted in Ov/Ob mothers as compared to NW mothers of LGAs.

Alterations in Ceramide Species

Ov/Ob mothers had elevated levels of several ceramide species, i.e., identified at sum composition, and molecular species with different sphingoid bases, ranging from 14 to 24 carbon atoms, and different acyl chains. These ceramides were also highly discriminating between NW and Ov/Ob mothers of LGA infants. Elevation of ceramides is a common feature of obesity (16-18) and ceramide levels correlate positively with measures of insulin resistance (16, 18). In fact, higher levels of Cer (d18:1/16:0) were found in the adipose tissue of diabetic obese compared to non-diabetic obese patients (19), which signifies the role of ceramides as an indicator for insulin

resistance. In the context of maternal obesity and diabetes, results are less consistent. Obese mothers had lower circulating ceramides (20), and similar placental ceramides as compared to non-obese normoglycemic mothers (21). In contrast, several ceramides were found higher in early pregnant women developing GDM (22, 23). Placental ceramides were also found elevated with poor glycemic control (21) and insulin treatment in diabetic mothers (24). Therefore, researchers suggested that elevated ceramides reflect glycemic control and insulin resistance rather than the state of obesity per se (20), despite some contradicting results (25).

Our results of elevated ceramides in Ov/Ob mothers of LGAs may therefore indicate higher insulin levels and insulin resistance in these women. However, the fact that some ceramides had 14-carbon sphingoid bases suggest that these ceramides originate from the placenta. These sphingoid bases are not common in human tissues (26), and they are preferentially synthesized by serine palmitoyl transferase subunit 3 (SPTLC3), which is highly expressed in the placenta and trophoblast cells (27, 40). Furthermore, three hexosylceramide species were depleted in AF of Ov/Ob mothers while one was enriched. Remodeling and different utilization of different species is likely, but the general trend seems to be the reduction of hexosylceramide species, which are ceramides with added hexoses, either glucose or galactose. In line with our results is the negative association reported for pre-pregnancy BMI and plasma hexosylceramides (4). However, the interpretation and implications of such reduction are challenging to conclude, given their complex and tissue-dependent effects; hexosylceramides enhance insulin signaling in myocytes while impair it in adipocytes (28).

Alterations in SM Species

In our previous work, we found that AF SM 14:0;O2/20:1 was consistently elevated in LGA compared to AGA pregnancies in both NW and Ov/Ob mothers (12, 13). Furthermore, SM

14:0;O2/20:1 was not significantly different between AGA AF of NW and Ov/Ob mothers (13). The significance of such elevation cannot be interpreted due to a lack of information on this specific SM. However, given its 14-carbon sphingoid base, it is likely to be a product of placental lipid metabolism similar to the elevated ceramide species observed. On the other hand, ten SM species were depleted in AF of Ov/Ob compared to NW mothers of LGAs, five of which were identified with 14-carbon sphingoid bases and long acyl chains. Maternal obesity was inconsistently associated with higher and lower circulating SM species (4, 29). The lower abundance of SMs, circulating and placental, seems to better characterize GDM versus non-GDM women (25, 30-32), despite some contradicting results; elevated SM species were reported in the AF of GDM compared to non-GDM mothers, specifically in female fetuses (33). Therefore, our results of depleted SMs in AF of Ov/Ob mothers might indicate insulin resistance in these women. It is also possible that the reduction in SMs results from increased hydrolysis to ceramides, which we found at higher levels in Ov/Ob mothers of LGAs, similar to what has been suggested for GDM (30).

Elevation of TGs and Reduction of PA and PCs

Analysis of placental tissues obtained from obese women revealed higher content of TGs, PCs, and cholesterol esters along with higher lipid esterification rates in comparison to lean women (34). Also, the expression of genes involved in TG synthesis such as Lipin 1, which dephosphorylates phosphatidic acid to diacylglycerol, was also found higher in placentas of obesity prone rats (35). Based on such findings, higher metabolic utilization of PA species towards TG synthesis may provide explanation to the reduction of PA and the elevation of TGs we observed in the AF of Ov/Ob mothers. Another possible mechanism contributing to the lower PA levels in LGA AF is the inhibition of phospholipase D by ceramides, which hydrolyzes PCs to produce PA

(36, 37). Therefore, assuming a placental origin of AF PA, the observed elevation of ceramides, likely also of placental origin, may be responsible for the reduction of PA in LGA AF. However, the observed reduction of AF PCs is in obvious contradiction with their elevation in obese placental tissues collected throughout pregnancy (34). Yet, few depleted PC species were still observed in obese and GDM placentas compared to healthy mothers (38, 39), which may align with our results.

Conclusion

The lipidomic signature of maternal obesity is evident in LGA complicated pregnancies. Despite similar birth weight outcomes, obese mothers showed an unfavourable elevation of ceramides which may indicate some degree of insulin resistance, although these women were not diagnosed with GDM during their pregnancy. Unfortunately, no further details are available regarding the results of oral glucose tolerance test, which is routinely performed to rule out GDM during pregnancy. In any case, AF glucose and insulin were not different between the two groups of NW and Ov/Ob mothers, and their concentrations did not correlate with any of the DA lipids characterising maternal obesity. Furthermore, as placental lipid metabolism associated with maternal obesity favours the synthesis and accumulation of triglycerides, this may explain some of the lipidomic alterations we observed in the AF of Ov/Ob mothers. The small sample size and long storage duration of AF samples may limit the strength of our results. However, our work had an exploratory nature, and more research is needed to further investigate and establish our observations.

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5.7 Tables and Figures

Table 5. 1: Maternal and infant characteristics.

LGA infants	NW mothers (n=15)	Ov/Ob mothers (n=14)
Maternal		
Age (years)	37.2 ± 1.5	38.3 ± 2.2
Pre-pregnancy weight (kg)	61.6 ± 7.4*	79.7 ± 14.6
Height (m^2)	1.68 ± 0.08*	1.62 ± 0.06
BMI (kg/m^2)	21.7 ± 1.7*	30.1 ± 4.4
Amniocenteses week (weeks)	15.2 ± 0.8	15.4 ± 0.9
Infant		
Sex (n = female/male)	5/10	5/9
Birth weight (grams)	4314 ± 333	4295 ± 260
Gestational age at birth (weeks)	40.2 ± 1.1	40.0 ± 0.9

* Significantly different between NW and Ov/Ob mothers (p value < 0.05).

Table 5. 2: Differentially abundant features between Ov/Ob and NW mothers of LGA infants.

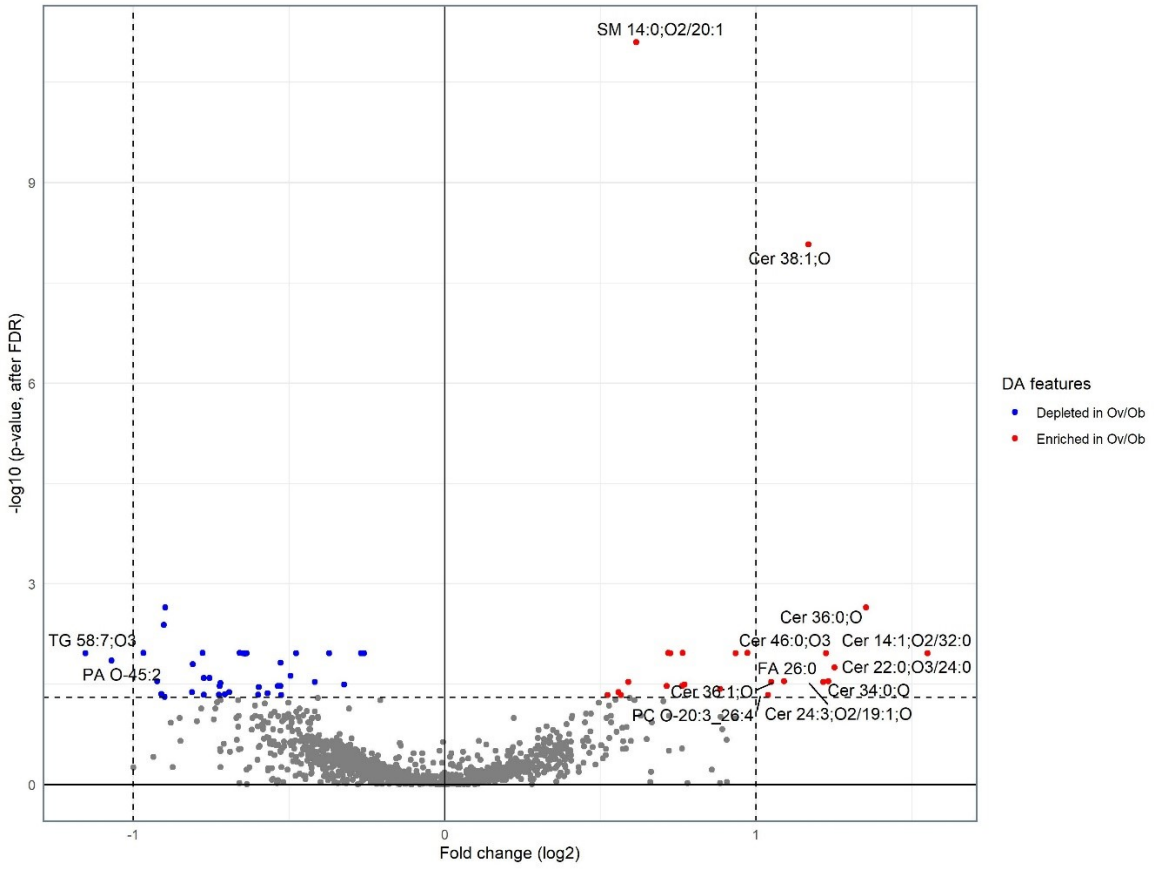
Feat. No	Identified Feature/Lipid	Log2 Fold Change	Adjusted *p*-value
Features enriched in AF of Ov/Ob mothers			
1	Car 34:1;O4	0.69	< 0.05
2	Cer 14:0;O3/30:0;O *	0.91	< 0.05
3	Cer 14:1;O2/29:0 *	0.61	< 0.05
4	Cer 14:1;O2/32:0 *	1.57	< 0.05
5	Cer 18:0;O3/25:0 *	0.81	< 0.05
6	Cer 22:0;O3/24:0 *	1.27	< 0.05
7	Cer 24:3;O2/19:1;O *	1.17	< 0.05
8	Cer 32:0;O	0.89	< 0.05
9	Cer 34:0;O	1.21	< 0.05
10	Cer 36:0;O	1.37	< 0.01
11	Cer 36:1;O	1.06	< 0.05
12	Cer 38:1;O	1.16	< 0.000
13	Cer 46:0;O3	1.18	< 0.05
14	FA 26:0 *	1.10	< 0.05
15	HexCer 15:3;O2/22:2 *	0.76	< 0.05
16	NAT 27:4;O4	0.49	< 0.05
17	PC O-20:3_26:4 *	1.01	< 0.05
18	SM 14:0;O2/20:1 *	0.64	< 0.000
19	ST 21:0;O3;Hex	0.55	< 0.05
20	TG 51:1	0.71	< 0.05
21	TG 54:9;O	0.93	< 0.05
22	TG 57:9;O3	0.57	< 0.05
23	TG O-78:0;O2	0.79	< 0.05
24	WE 22:0;O4	0.72	< 0.05
Features depleted in AF of Ov/Ob mothers			
25	DG 61:3;O2	-0.73	< 0.05

26	FA 22:6 **	-0.56	< 0.05
27	HexCer 16:0;O2/22:0;O *	-0.91	< 0.05
28	HexCer 22:1;O2/18:0;O *	-0.81	< 0.05
29	HexCer 37:1;O4	-0.67	< 0.05
30	PA O-35:3	-0.31	< 0.05
31	PA O-38:1	-0.32	< 0.05
32	PA O-39:1	-0.37	< 0.05
33	PA O-39:2	-0.50	< 0.05
34	PA O-41:0	-0.26	< 0.05
35	PA O-43:1	-0.30	< 0.05
36	PA O-45:0;O	-0.67	< 0.05
37	PA O-45:1	-0.88	< 0.01
38	PA O-45:2	-1.09	< 0.05
39	PA O-61:7	-0.50	< 0.05
40	PC 16:1_22:6 *	-0.73	< 0.05
41	PC 38:6;O	-0.49	< 0.05
42	PC 39:6	-0.56	< 0.05
43	PC 51:6	-0.54	< 0.05
44	PC 55:4;O	-0.73	< 0.05
45	PC 56:4;O	-0.75	< 0.05
46	PC 9:0_27:0 *	-0.77	< 0.05
47	PC O-14:0_6:0 *	-0.64	< 0.05
48	PC O-18:3_23:0 *	-0.65	< 0.05
49	PG dO-40:0	-0.65	< 0.05
50	PR 52:2;O4	-0.54	< 0.05
51	SM 14:0;O2/24:0 *	-0.95	< 0.05
52	SM 14:0;O2/25:0 *	-0.81	< 0.05
53	SM 14:0;O2/25:1 *	-0.86	< 0.01
54	SM 14:0;O2/28:2 *	-0.77	< 0.05
55	SM 14:0;O2/28:2	-0.57	< 0.05
56	SM 39:1;O2	-0.62	< 0.05
57	SM 40:3;O2	-0.61	< 0.05
58	SM 46:5;O2	-0.72	< 0.05
59	SM 54:6;O6	-0.85	< 0.05
60	SM 58:11;O2 *	-0.90	< 0.05
61	TG 58:7;O3	-1.11	< 0.05

- Car: Acyl carnitine, Cer: Ceramide, DG: Diglyceride, FA: Fatty acid, HexCer: Hexosyl ceramide, NAT: N-acyl amine, PA: Phosphatidic acid, PC: Phosphatidylcholine, PG: Phosphatidylglycerol, PR: Prenol Lipid, SM: Sphingomyelin, ST: Sterol, TG: Triglyceride, WE: Wax ester.

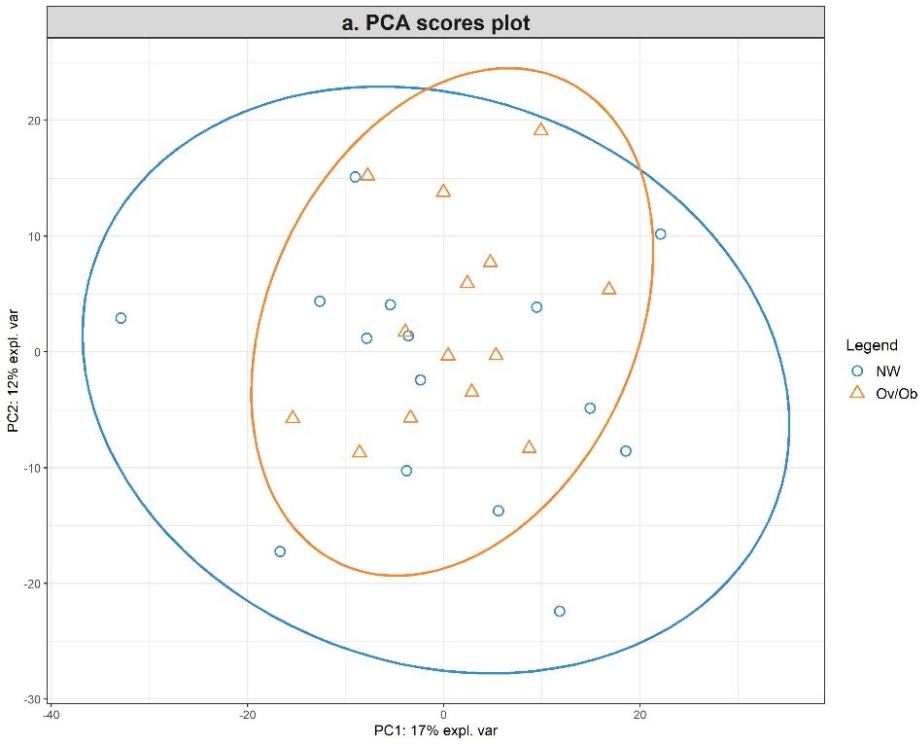
- All features listed were Tier 3 identified, i.e., based on mass match, except for those with an asterisk which were based on MS/MS identification; * for Tier 1 identified and ** for Tier 2 identified features.

Figure 5. 1: Volcano plot analysis of identified lipids.



- Features with an adjusted *p* value < 0.05 are colored; red to denote higher intensity in Ov/Ob compared to NW mothers and blue to denote lower intensities.
- Annotation was only added to highly significant features (adjusted *p* value < $0.1*10^{-7}$) and features with log2 fold change > 1 or < -1 (adjusted *p* value < 0.05).
- Cer: Ceramide, PA: Phosphatidic acid, PC: Phosphatidylcholine, SM: Sphingomyelin, TG: Triglyceride.

Figure 5. 2: Multivariate analysis of identified lipids between Ov/Ob and NW mothers of LGA infants.



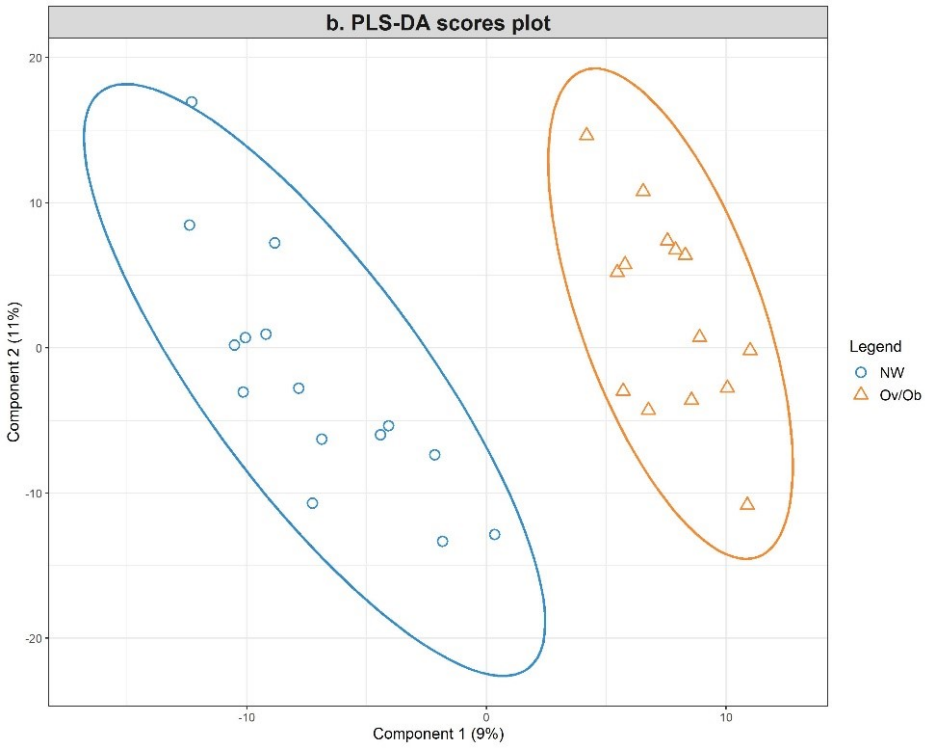
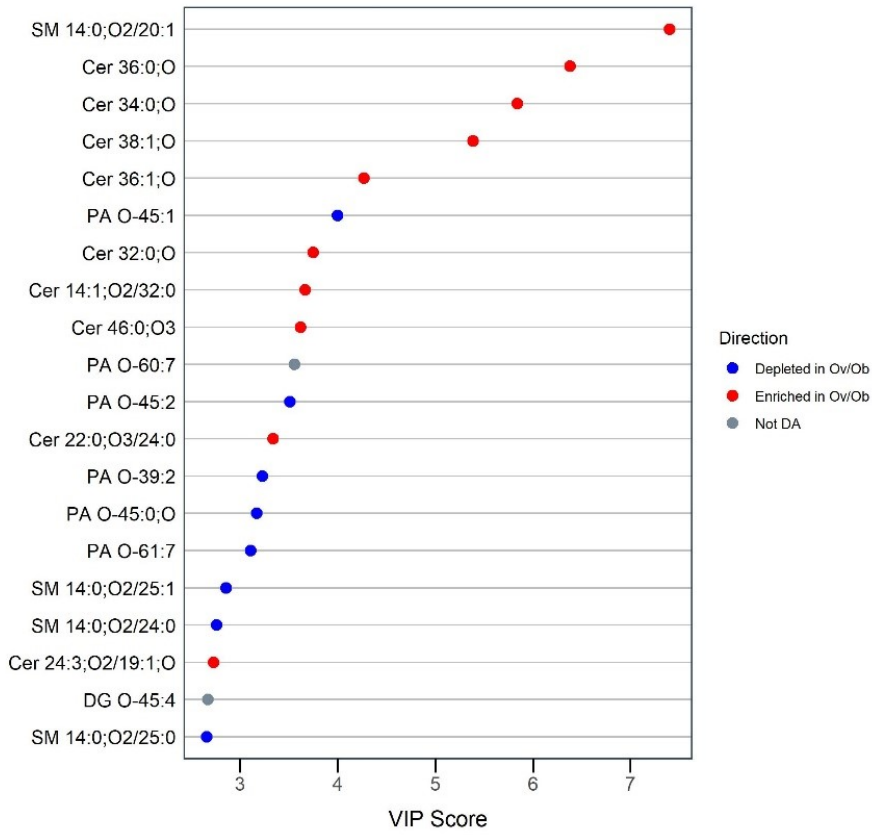

b. PLS-DA scores plot
Component 2 (11%)
Component 1 (9%)
20
10
0
-10
-20
-10
0
10
Legend
NW
Ov/Ob

Figure 5. 3: Variable importance in projection (VIP) scores.



- The top 20 VIP scores of the first component from PLS-DA analysis are shown.
- All the appearing lipids were DA (adjusted *p* value < 0.05) except for DG O-45:4 and PA O-60:7.

Chapter 6: General Discussion

6.1 Lipidomic Profiling of AF in LGA Complicated Pregnancies: Main Results

Untargeted profiling of AF revealed distinct lipidomic features of mothers giving birth to LGA compared to AGA infants at early pregnancy. This suggests that alterations in lipid metabolism are observable not only in maternal blood as indicated by other studies (25-27), but also in the intrauterine compartment of LGA complicated pregnancies. Furthermore, while we examined LGA lipidomic features in apparently healthy mothers with no diabetic complications or any other metabolic disorders, maternal pre-pregnancy weight alone showed a significant influence on the lipidomic signature of LGA AF. The main two features of LGA AF lipidome as observed by our three studies are discussed in the following sections:

6.1.1 Alterations in PA Species

In our first study, we found elevations of many PA species in LGA AF of NW mothers compared to AGA AF. Our proposed hypothesis to explain this finding was based on the current knowledge we have about macrosomia pathophysiology; the strong association between maternal TGs, NEFAs, and insulin levels with birth weight as reported in clinical studies (89, 111, 166), and the results of fatty acids and growth factors' treatment of cultured PHT cells (167, 168). We hypothesized that the elevation in PA levels in LGA AF is a result of enhanced placental synthesis of PA secondary to enhanced maternal flux of TG, NEFAs and growth factors such as insulin. Enhanced PA synthesis may be derived from enhanced *de novo* synthesis of PA or enhanced hydrolysis of phosphatidylcholines by phospholipase D, as depicted in Fig 6.1, both of which have been reported in PHT cells upon treatment with fatty acids and insulin, respectively (167, 168).

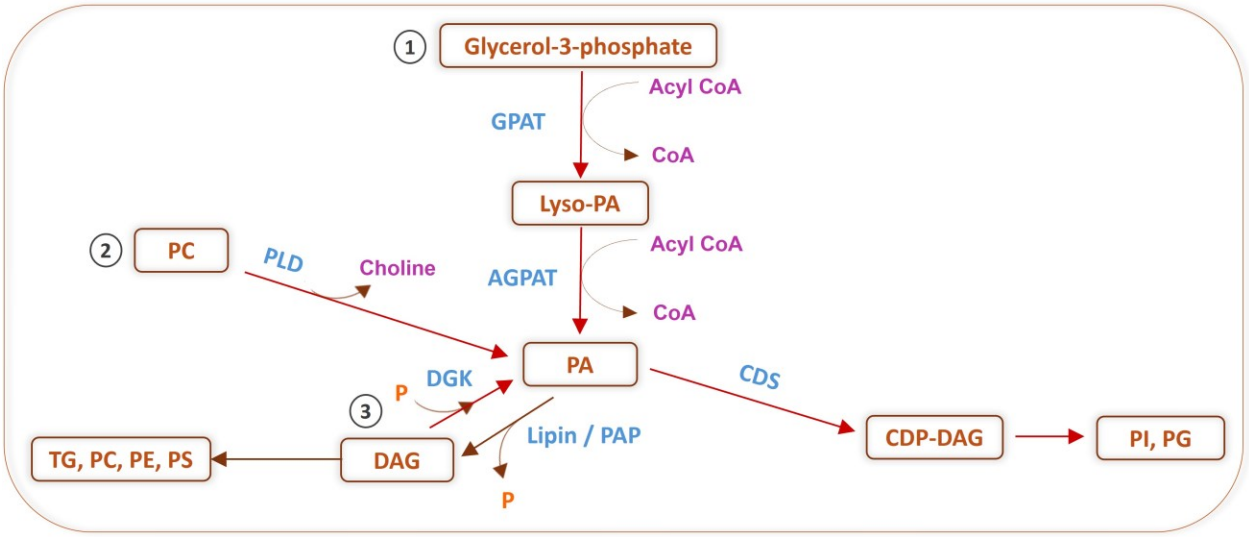


Figure 6. 1: Metabolic pathways of PA synthesis and utilization.

Different metabolic pathways can generate PA. (1) De novo synthesis of PA is the result of two sequential acylation steps; Glycerol-3-phosphate acyltransferase (GPAT) first acylates glycerol 3-phosphate or dihydroxyacetone phosphate forming lysophosphatidic acid (Lyso-PA). The latter undergoes a second acylation mediated by acylglycerophosphate acyltransferase (AGPAT) to produce PA. (2) Hydrolysis of membrane phospholipids, such as phosphatidylcholine (PC), through the action of phospholipase D generates PA by removing the choline group. PA can be dephosphorylated by PA phosphohydrolase (PAP) to produce diacylglycerol (DAG). DAG serves as a precursor for the synthesis of triglycerides and various glycerophospholipids, including PC, phosphatidylethanolamine, and phosphatidylserine. (3) DAG can also be phosphorylated by DAG kinase (DGK) to regenerate PA. Cytidyldiphosphate-DAG synthase (CDS) converts PA into cytidyldiphosphate-DAG (CDP-DAG), which can be metabolized into glycerophospholipids such as phosphatidylglycerol and phosphatidylinositol (212, 213).

In support of our hypothesis, birth weight was found to correlate positively with the activation of mTOR complexes in term placentas collected after birth (169). Activation of mTOR complexes is dependent on and regulated by PA (170). Upon treatment of PHT cells with oleic acid, increased *de novo* synthesis of PA was associated with increased amino acid uptake through System A amino acid transporter (167). Activation of System A amino acid transporter was also found to correlate positively with birth weight (169). Interestingly, amino acids' concentration in

AF was found to correlate positively with maternal nutrient intake of fats (171). Such observations may further support our hypothesis/interpretation of higher PA in LGA AF.

The impact of placental activation of mTOR signalling through de novo synthesis of PA on placental fatty acid uptake and metabolism is currently lacking. However, in cultured preadipocytes, mTOR complex 1 activation is thought to promote lipogenesis and inhibit lipolysis (197). Also, enhanced de novo synthesis of PA involves the activation of acylglycerol-3-phosphate acyltransferase (AGPAT), which acylates lysophosphatidic acid to produce PA. AGPAT has an established role in lipid storage, as it mediates the synthesis of TGs and PCs from PA (198). PA, itself, facilitates the coalescence of cellular lipid droplets and promotes the formation of supersized lipid droplets (199). Hence, it is likely that the effects of induced PA synthesis play a role in augmented fetal adiposity, apart from only reflecting higher maternal flux of lipids to the intrauterine compartment.

It is also unknown whether higher PA levels in AF can have a direct effect on fetal organs and tissues through its non-keratinized epithelial skin layer or through AF swallowing. The latter was observed as early as 16 weeks of gestation and complete skin keratinization is attained at 25 weeks (30). Of interest are the effects of PA on insulin signalling; accumulation of PA in mouse hepatocytes was found to inhibit insulin signalling, whether achieved through overexpression of phospholipase D, diacylglycerol kinase, or glycerol-3-phosphate acyltransferase (GPAT) (200). Increased cellular levels of PA in L6 myotubes, which was achieved upon treatment with linoleic acid, was found to inhibit insulin signaling, through reducing tyrosine phosphorylation of insulin receptor substrate-1 (IRS-1) (201). Therefore, higher PA may induce insulin resistance in fetal tissues very early during prenatal development, potentially affecting metabolic programming and

increasing the risk of metabolic disorders later in life. Interestingly, LGAs are shown to have significantly lower insulin sensitivity soon after birth in comparison to AGA infants (202, 203).

Contrary to the previously mentioned results of our first study, we found lower PA levels in LGA compared to AGA AF of Ov/Ob mothers (second study) and lower PA in LGA of Ov/Ob mothers compared to LGA AF of NW mothers (third study), which was very unexpected. These findings would essentially refute our proposed hypothesis/interpretation for PA elevation in LGA compared to AGA AF of NW mothers, as reported in the first study. This is simply because elevated circulating levels of maternal TGs, NEFAs and growth factors are well-reported not only in association with birth weight (89, 111, 166) but also with maternal obesity (111, 172). Therefore, elevated PA should be observed in LGA AF of Ov/Ob mothers based on the same logic we interpreted our first study results with. To further clarify this conundrum, we examined all detected PA species among the four groups of mothers: NW mothers of LGAs and AGA, and Ov/Ob mothers of LGAs and AGAs. The results consistently showed higher levels of about 36 PA species in all three groups: LGA of NW, AGA of Ov/Ob, and LGA of Ov/Ob, compared to AGA AF of NW mothers. Therefore, our hypothesis maintains its validity based on our findings.

Nevertheless, the mechanisms underlying the lower PA we observed in LGA AF of Ov/Ob mothers in comparison to AGA of Ov/Ob and LGA of NW mothers remains unknown. We speculate that it could be a result of distinct placental metabolism associated with maternal obesity. Placentas of obese mothers are known to have higher lipid content of TGs, PCs, and cholesterol esters, along with higher esterification rates, and increased expression of key enzymes and proteins involved in fatty acid uptake, transport, and metabolism, such as fatty acid translocase (FAT/CD36), lipoprotein lipase (173, 174), acetyl-CoA carboxylase, steroyl-CoA desaturase 1, and diacylglycerol O-acyltransferase-1 (175). Therefore, it is possible that the lower PA in LGA

AF of Ov/Ob mothers, compared to AGA of Ov/Ob and LGA of NW mothers, is a result of higher metabolic utilization towards synthesizing glycerides and other phospholipids, given that PA is a key intermediate in lipid biosynthesis pathways (176). It was even suggested that the lipid accumulative phenotype of placentas in obese mothers serve to protect the fetus against hyperlipidemia (173). This may be specifically more accurate in LGA pregnancies of Ov/Ob mothers.

Furthermore, higher ceramides observed in LGA AF of Ov/Ob mothers compared to LGA of NW mothers may provide another possible explanation for the reduced PA in LGA AF of Ov/Ob mothers. Ceramides inhibit phospholipase D, lowering PA production from PC, and stimulate phosphatidate phosphohydrolase-2, which converts PA into TGs and PCs (177, 178). While we only observed the elevation of ceramide species in LGA AF of Ov/Ob mothers compared to LGA of NW mothers, several ceramide species had high VIP scores and were also elevated but did not reach statistical significance in LGA compared to AGA AF of Ov/Ob mothers. In other words, our results may also support a role for ceramides in the reduction of PA observed in LGA compared to AGA AF of Ov/Ob mothers.

6.1.2 Consistent Elevation of SM 14:0;O2/20:1

Across our three comparisons, SM 14:0;O2/20:1 was consistently elevated when LGA AF was compared to AGA AF in both Ov/Ob and NW mothers, as well as when LGA AF was compared between NW and Ov/Ob mothers. It also had the highest VIP score in all three comparisons. It was only similar between AGA AF of NW and Ov/Ob mothers. This may indicate an important role of SM 14:0;O2/20:1 in mediating fetal overgrowth or at least characterizing the intrauterine lipidome of LGA pregnancies. In maternal blood lipidome, a general observation is that higher birth weights are associated with lower levels of SMs with saturated and

monounsaturated acyl chains (27) and with higher levels of SMs with polyunsaturated acyl chains (26). Although these SMs were reported at the sum composition level, i.e., the specific composition of sphingoid bases and acyl chains are unknown, they most likely contain the common 18-carbon sphingoid bases (211). Therefore, these SM species can not be compared to the molecular species with 14-carbon sphingoid bases which we found of significance in LGA AF. Interestingly, placentas of GDM mothers showed elevation of SM d14:0 /22:0 in comparison to non-GDM mothers (179), which is similar in composition to the SM molecular specie (SM 14:0;O2/20:1) we report in association with LGA AF. This similarity may indicate similar pathological mechanisms, but this needs to be further investigated.

6.1.3 Lack of Association to AF Glucose and Insulin Concentration

Another consistent result for our three studies was the essentially similar concentration of AF glucose and insulin between LGAs and AGAs of both NW and Ov/Ob mothers. Similar concentrations were also observed for Ov/Ob and NW mothers of LGA infants. In addition, none of the DA features in any of the three studies correlated significantly to AF glucose and insulin concentrations before and after correction for multiple testing. These results are in line with previous studies (193-195). However, there are contradicting results, as one study found a significant association between AF insulin and risk of macrosomia among women with a positive glucose challenge test, i.e., having blood glucose levels above 7.2 mmol/l one hour after consuming a 50-gram glucose solution (196). The latter study may be more relevant to the impact of pronounced glucose intolerance on AF insulin, and the strong association between gestational diabetes and macrosomia. However, this is not the case of mothers included in our studies, since none of them had gestational diabetes. Nevertheless, our small sample size may have obscured minor true differences.

It is of importance to note that while research, such as ours, have focused on the association between altered lipid metabolism and macrosomia or birth weight in general, this does not refute or disapprove the role of glucose intolerance/insulin resistance in promoting fetal overgrowth. The argument is whether maternal glucose or fat levels resemble the underlying adipogenic factor for the fetus (23). In other words, even in diabetic mothers, 67% of placental gene expression changes were related to lipid transport pathways while only 9% corresponded to glucose transport pathways, in comparison to healthy women (82). Our results further stress that alterations in lipid metabolism are evident in the intrauterine compartment unlike glucose or insulin, and it is likely to reflect higher maternal flux of lipids to the fetus.

6.2 Implications on the Metabolic Profile of LGA Mothers

The ultimate objective of profiling the lipidome associated with LGA pregnancies is to characterize or identify mothers with a high risk of developing an LGA infant. This is to control the underlying metabolic factors leading to fetal overgrowth early enough to prevent it. Our project aimed to investigate LGA AF lipidome in two maternal groups, NW and Ov/Ob, that represent significant proportions of LGA mothers (16). We unfortunately do not have further information on their metabolic health, such as the detailed results of their oral glucose tolerance test, which could have explained some of the lipidomic features observed. Nevertheless, clinically, all mothers included are considered healthy. They were not diabetic or hyperlipidemic, and had no adverse complications during their pregnancy, such as gestational diabetes, to justify any of the lipidomic alterations observed in our studies. Therefore, it is important to reflect on their metabolic health in light of our results.

Healthy individuals vary in their postprandial TG responses, with factors like meal composition, physical activity and age playing an important role in the regulation of postprandial

TG levels (180). Among overweight and obese individuals, central adiposity and insulin resistance were reported as predictors of postprandial lipemic responses (181). Moreover, plasma lipidomic profiling of healthy young and non-obese women in response to a high-fat meal revealed differential lipidomic responses 5 hours postprandial. These responses divided participants into two groups, designated as slow and fast TG metabolizers. The group of slow metabolizers exhibited a higher area under the curve (AUC) for total triglyceride species and insulin, despite having similar baseline characteristics and fasting triglycerides and glucose as the fast metabolizers group (182). Based on such results, we may assume that LGA mothers exhibit postprandial lipidomic responses similar to those of slow TG metabolizers. This assumption is supported by the strong association between maternal TG levels and birth weight even in healthy NW mothers (88). It is also supported by the elevation of PA we report in LGA compared to AGA AF of NW mothers, which is likely a product of higher placental synthesis second to enhanced maternal flux of lipids as we assumed.

The picture is less clear in case of Ov/Ob mothers, given the lower AF levels of PA in LGAs compared to AGAs which is probably a result of distinct placental lipid metabolism. Still, Ov/Ob mothers of LGAs have higher PA compared to NW mothers of AGAs, which again points to elevated TG levels. In addition, they had higher AF ceramides in comparison to NW mothers of LGAs, which probably indicates some degree of insulin resistance (183). Although these mothers were not diagnosed with GDM during their course of pregnancy, they may have had a subtype of gestational glucose intolerance that did not meet the diagnostic criteria of GDM (184). In such case, these mothers are also likely to be slow TG metabolizers, given that measures of insulin resistance positively relate to fasting TGs (185), as well as postprandial TG levels as reported by plasma lipidomics (182). While these are only speculations, the objective here is to highlight the

association between maternal lipid profile and AF lipidome as mediating factors of macrosomia. Unfortunately, clinical testing of maternal lipid profile, let alone postprandial lipid screening, is currently not part of the routine medical practice (186), despite well established associations of maternal lipids to pregnancy outcomes. Bridging this gap, we believe, can help prevent adverse maternal and infant outcomes such as macrosomia.

6.3 Lipidomics: Methodological Aspects and Limitations

Untargeted lipidomics analysis is an advanced complex method used to comprehensively characterize all lipids in a biological sample. This analysis can offer insights into the metabolic mechanisms underlying a disease state and aid in identifying biomarkers that accurately predict specific health outcomes (187). However, despite advancements in the field, several aspects still represent major challenges for lipidomics research work, including lipid extraction, identification, and pathway analysis (188, 189). Standardization of procedures is still lacking, therefore, the approach adopted in our research is justified and discussed in relation to these aspects.

6.3.1 Lipid Extraction and Identification

The choice of a lipid extraction method is very important because of its impact on the type of lipids detected, hence the findings of the work. There is no perfect method to cover the entire lipidome, given the diverse structural and physicochemical properties of thousands of lipids (188). Among the well established and commonly used methods is Folch extraction method (187, 190), which utilizes a mixture of chloroform/methanol as a two-phase organic/aqueous system that will partition lipids from water soluble molecules and precipitate the protein found in biological fluids (187). Folch extraction method offers wide coverage and effective recovery for a broad range of lipid classes (190). Our analytical approach applied a modified Folch liquid-liquid extraction

protocol, in which dichloromethane was used instead of chloroform, and this is to avoid the high carcinogenic risk of chloroform and the regulatory restrictions associated with its use (191).

LC-MS analysis, as employed in our work, is widely regarded as the mainstream method for lipidomics research, due to its superior separation efficiency and high sensitivity (192). Chromatographic separation of different lipid classes and species prior to MS analysis has several advantages, as it reduces sample complexity, lowers ion suppression and enhancement effects, and improves signal to noise ratio in MS analysis. This improves sensitivity and efficiency of the analysis and allows for more accurate identification of lipid species (187, 192). For mass spectra acquisition, TMIC employed Data-Dependent Analysis (DDA), which typically includes a full MS scan followed by automatic selection of the most intense ions within a defined mass-to-charge (m/z) range. Selected ions are then fragmented to produce product ions and detailed MS/MS spectra (204). While our employed method produces high quality spectra, we acknowledge that it is biased towards high intensity ions, as lower intensity ions will not be selected for fragmentation and their identification will rely only on matching their m/z ratios with reference libraries (205).

Lipid identification, i.e., annotation of LC-MS detected peaks/features, is one of the main bottlenecks in lipidomics research. For identification, each feature is queried against various libraries of all known lipid species. The query is based on several parameters including retention time, mass match, and MS/MS spectral match if available (187). The three-tier identification approach employed by TMIC aimed to classify the identified features in terms of identification reliability or confidence. Tiers 1 and 2 identification was based on accurate mass match and MS/MS spectral similarity match, while Tier 3 identification was based only on accurate mass match. The structural information provided by MS/MS spectral data provides the highest confidence levels. Tier 1 was defined by an MS/MS match score ≥ 500, referring to the highest

confidence level as adopted by TMIC, followed by Tier 2 with an MS/MS match score of < 500. Of note, MS/MS match score refers to the similarity between the observed and theoretical fragmentation pattern. Tier 3 has the lowest confidence level of identification (205, 206).

Nevertheless, it is important to note that TMIC also employed a 9-tier scoring and filtering approach to select the best possibility for identification. This approach uses parameters like m/z error, expected retention time range for each lipid subclass, expected adducts and isoforms, fatty acyl chain length (number of carbons), and ionization efficiency and sensitivity of each lipid subclass. Therefore, the best identification choice for each feature among all isomeric and isobaric possibilities was made (205, 206). While the results of our threes studies included several features that were tier 1 and 2 identified, among the important DA features were PA species which were all tier 3 identified. The fact that many of the detected DA features in each of the three studies were consistently identified as PA reduces the risk of misidentifying PA as an important DA lipid. However, we do acknowledge that tier 3 identifications have a low confidence level.

6.3.2 Pathway Analysis

Enrichment analysis and pathway analysis are necessary to provide a more informative biological context of the observed alterations in lipidomic features. However, a critical initial step is to map lipidomic features based on their unique identifiers (IDs) to lipid pathway libraries such as KEGG (Kyoto Encyclopedia of Genes and Genomes) and HMDB (Human Metabolome Database) pathway databases. However, given that full structural elucidation of lipid molecular species is not often attained by the analytical platforms, it is quite challenging to annotate the identified lipidomic features with unique IDs from major reference libraries, such as LipidMaps, HMDB, or PubChem (207).

In case of our results, about half of the identified features, which remained after applying RSD filters, were tier 3 identified, i.e., only the sum composition is provided. Therefore, only a minority of the identified features had reference IDs for mapping. Despite the attempts to address this limitation using the available tools, such as BridgeDB (208), Goslin (209), and MetabolomicsWorkBench (210) R packages, which can be used to extract the sum formulas and exact masses and search for corresponding lipid IDs across different libraries, the quality of the data obtained was not deemed sufficient for pathway analysis. Still, although pathway analysis could not be conducted, the types of subclasses emerging as DA lipids were homogenous enough to suggest some potentially impacted metabolic pathways, which is fairly adequate for the preliminary nature of our work.

6.4 Summary and conclusion

Our studies are the first to investigate the association of AF lipidomic profile with fetal overgrowth at early second trimester of pregnancy. Studies were designed to stratify the maternal population based on pre-pregnancy maternal BMI and exclude any clinically overt metabolic disorders with onset before or during the whole pregnancy period. This design aimed to replicate the metabolic phenotype of two maternal subgroups representing the largest proportions of LGA mothers according to Jolly *et al*. (16). Further to this, the AF lipidome of these two maternal subgroups with LGA infants, i.e., NW and Ov/Ob mothers, were contrasted to capture any differences that arise from the state of maternal obesity.

Our first two hypotheses stated that differences in AF lipidome between LGA and AGA mothers of both NW and Ov/Obesity were highly expected to arise and that lipidomic alterations are likely to correlate with AF concentration of glucose and insulin. Indeed, a distinct lipidomic signature was evident in LGA AF of NW and Ov/Ob mothers, however, with some differences between the two maternal groups. A unique SM, SM 14:0;O2/20:1, was consistently enriched in LGA AF of both maternal groups. On other hand, a significant number of PA species were enriched in LGA, compared to AGA AF of NW mothers, while depleted in LGA AF of Ov/Ob mothers. Both LGA groups had higher PA in comparison to AGAs of NW mothers, which may reflect enhanced placental synthesis second to increased maternal levels of lipids and growth factors. The contradiction between NW and Ov/Ob mothers in terms of PA trend of change is likely a result of unique placental metabolism associated with maternal obesity. However, the second part of our hypotheses, concerning lipidomic associations with AF glucose and insulin, was not valid based on our results.

Our third hypothesis assumed a similar lipidomic profile for LGA AF between NW and Ov/Ob mothers. However, lipidomic profiling showed significant differences between the two groups with several elevated species of ceramides in Ov/Ob mothers, which may indicate reduced insulin sensitivity in these mothers. Consistent elevation of SM 14:0;O2/20:1 was also evident in Ov/Ob mothers of LGAs compared to NW mothers, while other several SM and PA species were depleted.

Despite the preliminary nature of our studies, our findings are novel and clearly indicate alterations in lipid metabolism of the intrauterine compartment, specifically AF of LGA pregnancies. These alterations may reflect and relate to altered maternal and placental lipid metabolism, as we suspect. In addition, we clearly demonstrated that despite similar pregnancy outcome of LGA infants, AF lipidome is still distinct between NW and Ov/Ob mothers. The driver of such alterations requires further investigation, but it strongly implies the significance of lipid metabolism in promoting macrosomia at an early stage of pregnancy.

It is of value to acknowledge the limitations of our research work for other researchers to refine methodologies, address potential biases, and build upon our findings with improved accuracy and reliability. Among the main limitations are: 1) Our sample size was rather small to provide enough power and statistically-robust conclusions; 2) Lack of details on maternal metabolic indicators, such as glycemic and lipid profiles in addition to dietary intake, have limited our ability to interpret the results of LGA AF lipidome in light of maternal metabolic health; 3) Despite the use of advanced analytical platform with high sensitivity and mass resolution, structural elucidation and sufficiently reliable identification was not attained for many of the detected features, which limits our ability to draw strong conclusion from our findings.

Nevertheless, our research work is exploratory in nature and has provided valuable preliminary insights to the underlying metabolic environment of macrosomia using AF, a biological fluid that is not routinely accessed in standard clinical practice. Future research with both comprehensive and targeted lipidomic profiling is needed to establish our results and expand upon them. More focus on the early stages of pregnancy, tissues from the intrauterine compartment, and in relation to fasting and postprandial maternal lipid and glycemic profiles will help further delineate the pathophysiology of macrosomia.

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(For Introduction, Literature Review, Connecting Statements and Discussion)

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