Does the metabolomic profile differ by physical activity level in pregnancy?

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Contents

PREFACE TO THIS THESIS

The original aim of this thesis was to characterize the metabolomic profile of mid- and late-gestation serum, cord blood, and term placenta from pregnant individuals differing by physical activity status. This comprehensive analysis was intended to provide a detailed understanding of how physical activity influences gestational parent, fetal, and placental metabolome.

Initially, we planned to run our samples through the Department of Biology's core facility in collaboration with Dr. Harris. However, after developing the protocol, we encountered technical issues with their machine that could not be resolved. Due to logistical reasons and budget constraints, using other vendors was not feasible as they were not well-versed in the use of human samples, were prohibitively expensive and the lab lacked sufficient funding at the time.

Fortunately, we identified the core facility at Carleton University as a potential alternative. This facility primarily focused on lipidomics and approached metabolomic experiments with caution. Consequently, we had to design a new protocol that met their standards, which took considerable time and effort. By the time the new protocol was finalized and the lab secured the necessary funding, the remaining time was insufficient for my thesis timeline.

As a result, this thesis presents data from an initial analysis conducted in collaboration with my supervisor's collaborator (Dr. Jane Shearer) at the University of Calgary, focusing on mid-pregnancy serum and cord blood samples. While I was not involved in the metabolite extraction and LC-MS analysis of these samples, I was responsible for all subsequent data analysis and interpretation.

Despite these challenges and changes to the original plan, the findings presented here offer valuable insights into the metabolomic association with physical activity during pregnancy and lay the groundwork for future research in this area. My supervisor, Dr. Kristi Adamo, and my committee members Dr. Cory Harris, Dr. Eric Doucet, and Dr. Pascal Imbeault supported the modifications necessary to my thesis due to these unforeseen circumstances.

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I am deeply grateful to all members of the Adamo lab for their support, dedication, and friendship. Your collective efforts have made this research endeavor both productive and enjoyable. I would like to give special thanks to our postdoctoral fellow, Dr. Ana Carolina Rosa Da Silva, whose mentorship and training in the field of metabolomics have been invaluable.

I would also like to thank all my family, especially my parents. Without their encouragement, sacrifices, and endless support, I would not have had the opportunity to embark on this incredible journey.

Lastly, I want to express my sincere appreciation to all the pregnant individuals who participated in the PLACENTA study. Your generous contribution of time and enthusiasm during such a significant period in your life to support future parents and children everywhere is truly remarkable. I extend my sincerest gratitude to you. Thank you for your invaluable participation.

ABSTRACT

Introduction: Gestational physical activity (PA) is known as an effective strategy to reduce the risk of several pregnancy and birth complications, including gestational diabetes, gestational hypertension, and macrosomia. Despite the well-established health-promoting impacts of gestational PA, the underlying mechanisms behind these benefits remain poorly understood. Metabolites, which can provide a readout of the cellular, systemic metabolism, and environmental exposures, can be valuable in understanding these mechanisms. In non-pregnant individuals, there is a growing body of evidence indicating that PA leaves a signature fingerprint on the metabolome. Despite these findings, there is limited data on the metabolomic profile of the gestational parent (gesP) and no data regarding cord blood (CB) or placental metabolome associations with PA.

Method: An untargeted metabolomics approach was utilized to (1) analyze the serum metabolome associations with the PA status of the pregnant individuals, (2) investigate associations between umbilical CB metabolome and gesP PA status, and (3) develop a protocol for metabolite extraction from placenta samples to enable future comprehensive analyses of gesP, CB, and placenta metabolomes responses to gestational PA. Metabolomic analysis was performed using Ultra-Performance Liquid Chromatography-Quadrupole Time-of-Flight Mass Spectrometry (Agilent Technologies Inc., Canada).

Results: A total of 75 metabolites were annotated in the serum samples. Contrary to our hypothesis, the differences in measured metabolomic profiles of gesPs based on PA levels were minimal, with only pipecolic acid significantly lower in the inactive gesPs. Higher pipecolic acid and PC(18:1e/8-HEPE) emerged as potential biomarkers of lower PA status. The CB metabolome, however, displayed more pronounced differences aligned with our hypothesis. Several key metabolites, including phenylalanine, valine, threonine, lysophosphatidylcholines (18:1, 18:2, and 16:0), O-acetylcarnitine, and 5-hydroxyindole-3-acetic acid, were significantly lower in the CB of babies born to active pregnancies. Additionally, a protocol for placenta sample metabolite extraction was successfully developed, addressing challenges with solvent compatibility, extraction dilution, and the need for filtration steps.

Conclusion: Higher gestational PA is associated with differences in fetal (CB) metabolome, while the effects on gesP metabolome are minimal. Gestational PA likely mitigates the risk of birth and pregnancy complications by modulating metabolic pathways that the identified metabolites are involved in. Many of these metabolites are linked to oxidative stress and inflammation, suggesting that increased PA enhances antioxidant capacity and reduces inflammation, leading to better cardiometabolic health, mainly in the fetus. Furthermore, a placenta tissue extraction protocol was designed to address the unique challenges associated with this multifunctional organ, laying the groundwork for future comprehensive investigations of gesP, CB, and placenta response to PA.

ABBREVIATIONS

AA: Amino Acid
A-gesP: Active gestational parents
AUC: Area Under the Curve
BCAA: Branched-Chain Amino Acid
BMI: Body Mass Index
CB: Cord Blood
CPM: Count per Minute
DOHaD: Developmental Origins of Health and Disease
FA: Fatty Acid
FC: Fold Change
FDR: False Discovery Rate
GC-MS: Gas Chromatography-Mass Spectrometry
GDM: Gestational Diabetes Mellitus
GesP: Gestational Parent
HDL: High-Density Lipoprotein
HIIT: High-Intensity Interval Training
IB: Inactive pregnancies
I-gesP: Inactive gestational parents
IR: Insulin Resistance
LC-MS: Liquid Chromatography-Mass Spectrometry
LC-PUFA: Long-Chain Polyunsaturated Fatty Acid
LDL: Low-Density Lipoprotein
LPA: Light Physical Activity
LPC: Lysophosphatidylcholine
MeOH: methanol
MS: Mass Spectrometry
m/z: Mass-to-Charge Ratio
MVPA: Moderate to Vigorous Physical Activity
MPA: Moderate Physical Activity
NMR: Nuclear Magnetic Resonance
PA: Physical Activity
PC: Phosphatidylcholine
PLS-DA: Partial Least Squares Discriminant Analysis
PPARγ: Peroxisome Proliferator-Activated Receptor Gamma
PUFA: Polyunsaturated Fatty Acid
QC: Quality Control
RT: Retention Time
ROC: Receiver Operating Characteristic
ROS: Reactive Oxygen Species
SM: Sphingomyelin
T2D: Type 2 Diabetes
TCA: Tricarboxylic Acid (Cycle)
TG: Triglyceride
VIP: Variable Importance in Projection
VLDL: Very Low-Density Lipoprotein
VPA: Vigorous Physical Activity

Chapter 1: Thesis Overview

1.1. Introduction and Review of the Literature

1.1.1. Developmental Origins of Health and Disease Theory

For years, the prevailing belief was that one's health during a particular life stage resulted from gene-lifestyle interactions across that phase of the lifecourse (1). In the last decades, this notion has evolved into an approach that considers cumulative exposures throughout the lifecourse to determine the disease risk. The Developmental Origins of Health and Disease (DOHaD) theory posits that exposures related to behavioural or environmental factors before conception, *in-utero*, and in the early postnatal period can impact fetal development and the newborn phenotype (1). The prenatal period is a sensitive phase of fetal tissue and organ development (2). Environmental exposures during this period can potentially lead to permanent alterations in the structure and functions of fetal organs and metabolism and therefore have long-lasting health impacts on the offspring (3). Examples of such exposures include smoking, infant feeding methods, infection, stress, nutritional, and as our team posits; physical activity (PA) status of the birthing parent (1, 4). Therefore, studying these behavioural exposures in early developmental phases can offer a window into understanding the origins of health and disease in the birthing parent and offspring.

1.1.2. Gestational Physical Activity Guidelines

The term ‘physical activity’ refers to any bodily movement that engages the skeletal muscles and requires more energy than the resting levels (5). In nearly all medical fields, PA is recognized as an effective independent strategy to promote health and reduce disease risks, the obstetrics field is no exception (6). PA during gestation can reduce the risk of several pregnancy and fetal complications including excessive gestational weight gain (7), gestational diabetes mellitus (GDM), pre-eclampsia, gestational hypertension (8), urinary incontinence (9), macrosomia (10), and the need for cesarean section (11), along with better psychological well-being (12, 13) (Figure 1). To achieve a clinically significant reduction in the risk of these disorders, the *2019 Canadian Guideline for PA Throughout Pregnancy* recommends at least 150 minutes of

moderate-intensity PA (MPA) per week, in the absence of contraindications (14). It is also recommended that these 150 minutes be accumulated over at least 3 days using a variety of aerobic and resistance training activities (14).

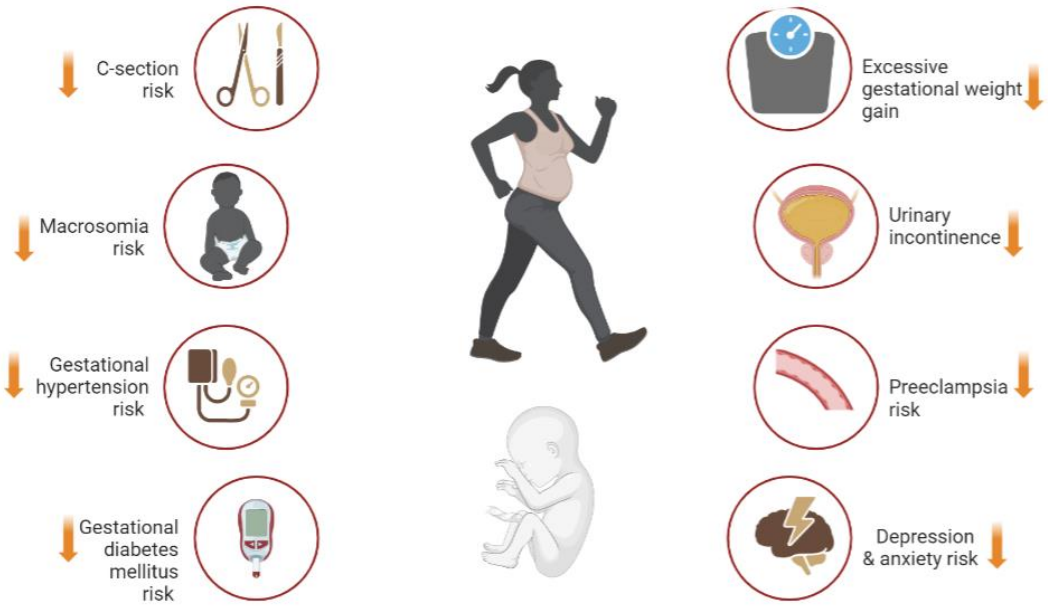

Figure 1. Gestational Physical Activity Associations with Pregnancy and Birth Outcomes. Created with biorender.com

Gestational PA supports gestational weight gain management and protects against exceeding the recommended weight gain targets in pregnancy (7). Excessive gestational weight gain and obesity increase the risk of GDM (15), gestational hypertension, and preeclampsia (16), and the risk of large for gestational-age babies (17). Prenatal PA can reduce the odds of macrosomia (birth weight >4000g) by 39 % without increasing the risk of preterm birth, low birth weight (<2500g), small for gestational age (<10th percentile), intrauterine growth restriction (10), miscarriage, prenatal mortality (18), or congenital disabilities (19).

As most pregnant individuals engage with healthcare professionals frequently, pregnancy is a valuable window of opportunity to make positive lifestyle changes (20). Despite the increased number of touchpoints with the healthcare system, less than 15% of pregnant individuals meet these minimum recommendations (21). Opportunities are missed because of several factors such as the misconception that PA can be harmful to the growing fetus and or the lack of information delivery by healthcare professionals

regarding PA guidelines (20). Better guideline dissemination can be achieved by gaining a better comprehension of two important questions; 'why' gestational PA is imperative and 'how' PA during pregnancy delivers its health benefits to the gestational parent (gesP) and growing fetus. While the literature has repeatedly answered 'why' gestational PA is important by underscoring its protective effects against birth and pregnancy complications, the question of 'how' these health benefits occur and their underlying mechanisms remains largely unexplored. One possible scenario is that the health impacts of PA are communicated to the developing fetus through the placental bridge by molecules such as metabolites. Thus, studying the fingerprint that PA leaves on the gesP, fetal, and placental metabolome may unravel some of the mechanisms driving these health benefits.

1.1.3. Metabolome

1.1.3.1. Metabolomics

Systemic biological processes encompass several dynamic molecular layers including the genome, transcriptome, proteome, and metabolome (Figure 2). The different layers of this communication corridor are highly interactive and are affected by environmental factors (22). The technological advancements in *omics* sciences have made it possible to explore the chemistry of biological systems deeper than ever. While all these layers offer a rich set of biological information, the metabolome is the cumulative downstream result of all other layers and so is the closest proxy of the phenotypic characteristics of an organism (23). The term 'metabolome' refers to the global collection of low-weight molecules (<1500 Da) that are called metabolites (24). The human metabolome consists of thousands of endogenous and exogenous compounds with different physiochemical properties (e.g., amino acids, carbohydrates, organic acids, fatty acids, and membrane lipids) (25). Endogenous metabolites are naturally produced within a biological system as a result of genetic and cellular processes, while exogenous metabolites originate from external sources such as food, drugs, or environmental exposures (26). Metabolites are the intermediates or end products of metabolism (23). Studying the metabolome can provide a readout of cellular metabolism and biochemical pathways, offering insights into the underlying mechanisms of health and disease (23). These molecules are highly responsive

to changes in genetics and environment, thereby they can be utilized to better understand the genotype x phenotype x environment interactions (27, 28). Metabolomics has a wide range of other applications, including but not limited to disease diagnosis and biomarker discovery, unraveling complex biological mechanisms, and advancing drug discovery and development (29).

Depending on the nature of the research question, metabolomic studies can take two main approaches, targeted and untargeted. Targeted assays are used where the objective is to investigate and quantify a small group of predefined metabolites with known chemical identities (30). In contrast, untargeted assays offer a more holistic approach and are used when the goal is to identify as many metabolites as possible in a sample. Untargeted assays can be employed to generate hypotheses for targeted assays (31). In untargeted assays, the identity of the metabolites is not necessarily known before the analysis (32), and as the metabolites in the sample are not predetermined, these assays typically provide relative rather than absolute quantifications (32).

Different analytical platforms are available for metabolomic data acquisition. The most frequently used platforms are nuclear magnetic resonance (NMR) spectroscopy and mass spectrometry (MS) coupled with liquid chromatography (LC-MS) and gas chromatography (GC-MS) (33). While each platform has its advantages and disadvantages, MS platforms offer the broadest metabolite coverage, and of the two MS platforms, LC-MS is the more versatile option for untargeted metabolomics (34). LC-MS instruments have broader metabolite coverage compared to GC-MS (35). LC-MS can detect and analyze a wide range of metabolites with different polarities, with less complicated sample extraction procedures (35). In untargeted metabolomic studies, the output of the instrument is a large complex dataset, even if the sample size is not high. This necessitates metabolomics-specific software for the post-data acquisition step to process and analyze the datasets (36). Prior to statistical analysis, the data need to go through a series of processing steps including baseline correction, peak detection, peak alignment, and ion annotation (36). Due to the complexity of the output and the high number of data points that are highly correlated with each other, both univariate and multivariate stats are required to analyze the dataset (36)

While metabolomic studies can be a powerful tool in understanding system biology, they have some inherent challenges. Unlike DNA, RNA, and proteins, metabolites exhibit a wide range of chemical diversity and lack a specific structural template. This diversity makes it impossible to measure all metabolites using a single extraction method or analytical platform (34). Additionally, our understanding of metabolic networks remains incomplete, complicating the interpretation of metabolomic data due to the unknown biological significance of many metabolites (34). Nevertheless, metabolomics can be a promising approach to unravel the metabolism of a biological system and its response to different stimuli.

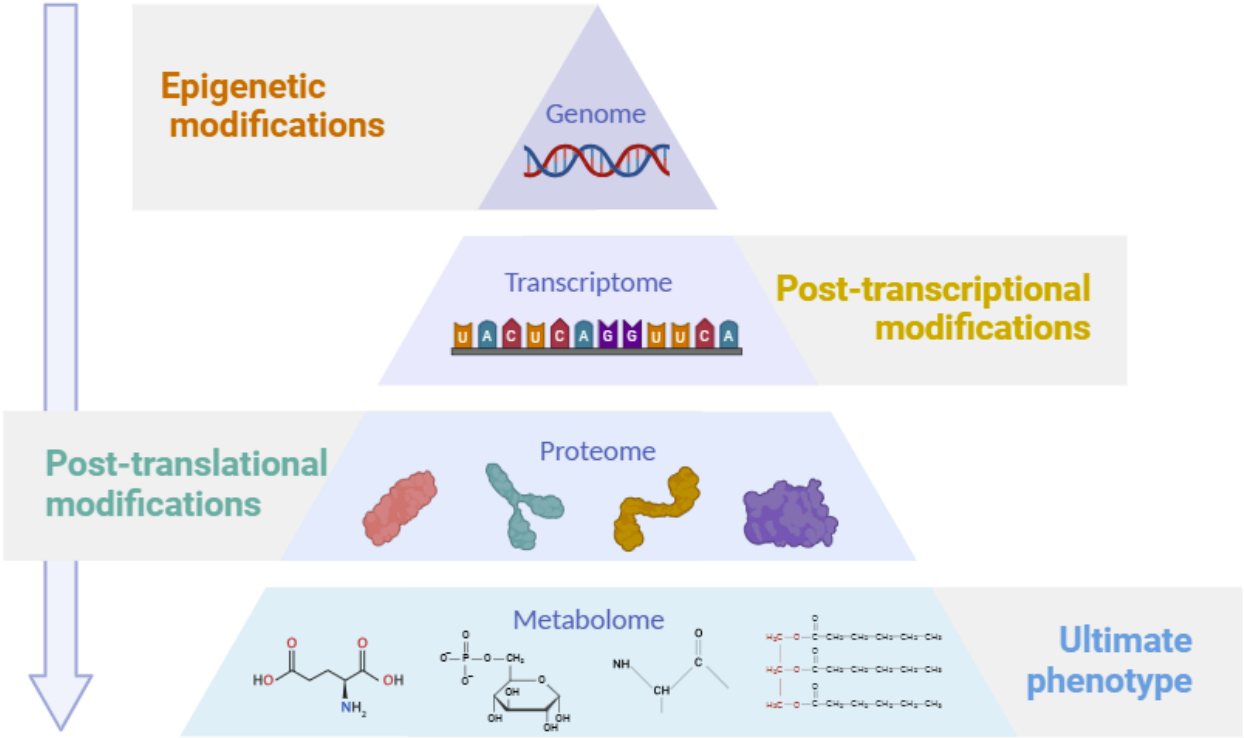

Figure 2. Information Flow in Biological Systems. Created with biorender.com

1.1.3.2. Metabolome and Pregnancy

Pregnancy is a time of vast and dynamic physiological, hormonal, and humoral adaptations (37). In each stage of the pregnancy, the fetus and the gesP have different physiological needs and so the metabolism of the birthing parent adapts to meet these demands (37). Therefore, a healthy pregnant individual has a

signature metabolism and a signature metabolome unique from their non-pregnant counterparts (Figure 3) (38-41). Considering that pregnancy itself acts as a stimulus to distinctively influence metabolome, when it comes to investigating the potential response of metabolome to exposures such as diet, or PA it is important to look at pregnant populations separately from non-pregnant populations.

For instance, many amino acids (AAs) experience a steady decline as gestation progresses. This is potentially due to high placental uptake and transfer to the fetus for protein synthesis (38, 39). This decrease can also be due to an increase in ketogenesis in pregnancy as AAs can be utilized as substrate for this metabolic pathway (42). A balance in the gesP AA levels is critical to a healthy pregnancy as many of these compounds play imperative roles in fetal development (43). Branched-chain AAs (BCAAs), specifically leucine, are a major activator of the mTOR signaling pathway which can enhance blastocyst quality, crucial for embryo implantation and development (43). Other AAs like arginine, ornithine, and proline are necessary for angiogenesis and gene expression, while methionine and choline are required for DNA and RNA synthesis, processes that are fundamental requirements for placental and fetal development (44, 45).

Lipid metabolism also changes during pregnancy mainly in response to higher gesP and fetal energy demands and insulin resistance (IR) secondary to placental growth hormone secretion (41). Efficient growth and development of the embryo, placenta, and fetus requires a well-balanced lipid metabolism and can affect the long-term programming of offspring health (41). Many compounds in the lipid class are bioactive metabolites that influence fetal health and development. For instance, long-chain polyunsaturated fatty acids (PUFAs), are essential for fetal neurodevelopment and growth. Specifically, in late gestation, their transfer to the fetus increases leading to a decline in circulating levels in the gesP (41). Fatty acids (FAs) need to be converted to triglycerides (TGs) and/or phospholipids to cross the placenta, as a result, gesPs experience an increase in phosphatidylcholines (PCs) and sphingomyelins (SMs) in late gestation (46). Circulating levels of other lipids such as cholesterol, total TG, and low-density lipoprotein (LDL) increase across gestation to meet the high energy demands (41). Cholesterol also is required for placental membrane formation (47). With

the progression of pregnancy, cholesterol production and utilization increase in response to hormonal changes, such as increased estrogen levels, to support the birthing parent and the fetus (47).

While the gesP metabolome can offer valuable biological information into the effects of different exposures on the birthing parent and the offspring, it alone does not offer a comprehensive perspective. The analysis of cord blood (CB) and placental metabolomes represent additional pivotal components. CB is reflective of fetal metabolism; therefore its metabolome can provide important information into how exposures of the gesP affect fetal metabolism (48). The placenta is a metabolically active tissue with a central role in supporting the growth and development of the fetus (49, 50). This temporary organ is responsible for gas and nutrient exchange, removal of the waste product from the fetus circulation, and production of several hormones. A well-functioning placenta is necessary for a successful pregnancy (51). Applying metabolomics to studying the gesP, CB, and placenta in uncomplicated pregnancies can highlight and identify the metabolic pathways that are involved in maintaining a healthy pregnancy. Metabolomics has the potential to reveal how exposures such as PA can contribute to a positive *in-utero* environment, affect the gesP, and the fetus, and protect against adverse pregnancy and birth outcomes.

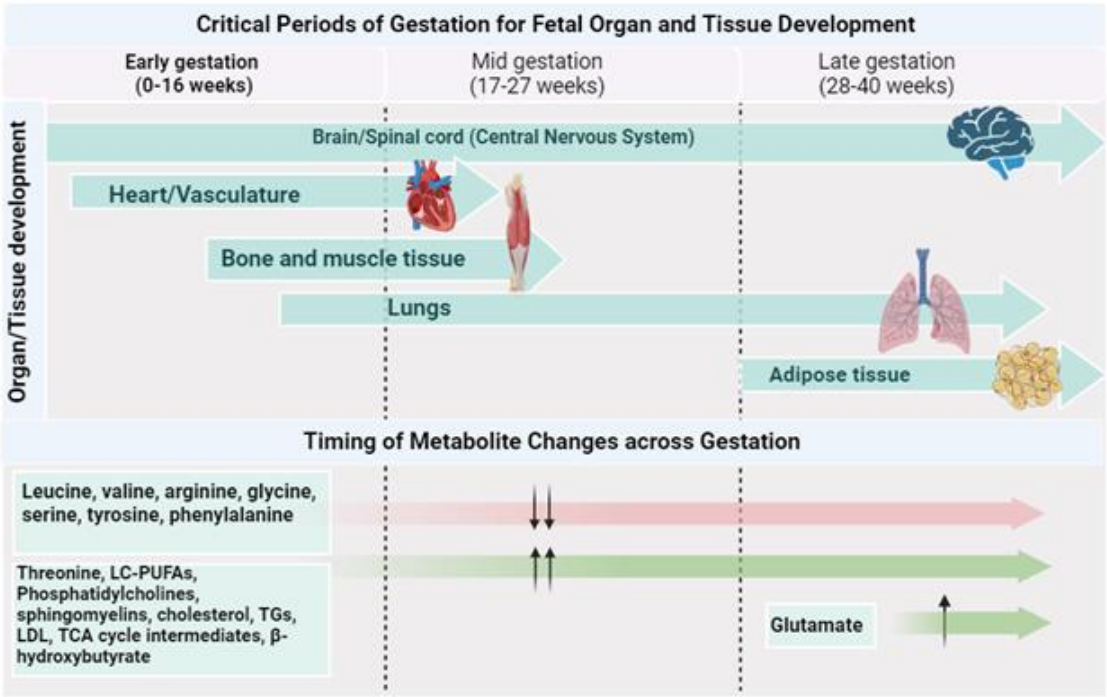

Figure 3. Changes in The Gestational Parent's Blood Metabolome During a Healthy Pregnancy. LDL (Low-density lipoprotein), LC-PUFA (Long chain-polyunsaturated fatty acid), TCA (Tricarboxylic acid cycle), TGs (Triglyceride). Created with biorender.com

1.1.3.3. Metabolome and Physical Activity: Non-pregnant Female Populations

A sedentary lifestyle and physical inactivity, are major global health concerns, ranking as the fourth leading behavioural risk factor for worldwide mortality (52). Engaging in regular PA, independent of dietary habits, offers significant benefits for metabolic health and serves as an effective protective measure against various physical and mental health disorders, including obesity, metabolic syndrome, diabetes, cardiovascular diseases, depression, and dementia (53). Despite advancements in understanding the physiological adaptations induced by PA, comprehensively grasping how PA enhances health and prevents these disorders remains challenging due to its multifaceted nature and complexity (different modes, durations, intensities, etc.). Consequently, substantial gaps persist in our understanding of how PA impacts cellular and molecular mechanisms across various physiological and pathophysiological conditions.

When the body is challenged with PA, its energy demands increase compared to the resting levels. To ensure energy homeostasis is maintained most, if not all, of the physiologic systems, including musculoskeletal, cardiovascular, respiratory, endocrine, and immune systems experience specific adaptations enhancing the body's efficiency and capacity to endure the activity (54). These adaptations can lead to changes in cellular and whole-body metabolism, leaving a distinct fingerprint on the metabolome. Numerous studies in non-pregnant female populations have highlighted the significant modifications in metabolic profiles as a result of PA and exercise (55-60). AAs, lipids, organic acids, and carbohydrates are among the main metabolite groups reported to be modulated by PA in non-pregnant female populations, as summarized in Table 1. These metabolites play vital roles in the body, and maintaining their ideal levels can protect against pathophysiological conditions, while deviations may disrupt homeostasis. Therefore, investigating the regulatory effects of PA on these metabolites can highlight the processes and the mechanisms through which PA delivers its health benefits. Herein, a brief review of the literature regarding the effects of PA on the metabolome of non-pregnant female individuals is provided.

AAs are essential to fundamental biological processes such as protein synthesis, cell division, redox status, gluconeogenesis, glycogenolysis, and lipolysis (61). One of the main AA groups inversely associated

with PA is BCAAs. Hamaya *et al*. found that women engaging in higher levels of leisure-time PA exhibited approximately 1% lower plasma BCAAs, even after adjusting for body mass index (BMI), diet quality scores, and caloric intake (59). BCAAs are vital for glucose, lipid, and protein metabolism, immune system functions, and gut health. However, their accumulation can disrupt normal metabolic processes (62-64). Chronic elevation of BCAAs may result from an imbalance between their appearance and disappearance. BCAAs can originate from tissue protein breakdown (inhibited by insulin), dietary intake, and gut microbial synthesis, while pathways for their disappearance include protein synthesis, excretion, and BCAA catabolism within mitochondria. Lower BCAA catabolism, often due to reduced mitochondrial capacity, is proposed as a primary contributor to their accumulation (65, 66). The elevation of these AAs can lead to overstimulation of the mTOR/p70S6K pathway and cause IR (65). Consistently, higher BCAA levels have been associated with overweight, IR, obesity, and diabetes (62). Habitual PA has been shown to enhance mitochondrial capacity, potentially explaining the observed inverse association between PA and BCAA levels (67).

Aromatic AAs (AAAs), such as tyrosine, tryptophan, phenylalanine, and sulfur-containing AAs (methionine, cysteine, and cystine) are two other sub-groups negatively associated with PA. Grapov *et al.* reported a reduction in plasma methionine, cysteine, cystine, tryptophan, and phenylalanine in response to an acute bout of exercise (57). Similarly, in a study by Kuehnbaum *et al.*, (2014) female adults with overweight showed decreased levels of L-Cystine after a 6-week high-intensity interval training (HIIT) intervention, despite no significant changes in weight or insulin sensitivity (55). Higher levels of AAAs have been tied to IR, reduced insulin secretion, oxidative stress, obesity, overweight, and type 2 diabetes (T2D) (68, 69). Accumulation of sulfur-containing AAs has also been associated with suboptimal insulin sensitivity, obesity, metabolic syndrome, inflammation, and endothelial dysfunction (70, 71). Grapov *et al.* also reported a decrease in aspartic acid, another AA positively linked to higher IR, and serine levels post-intervention (57, 72). An increase in ornithine levels, an AA involved in the uric acid cycle, was another observation of the Kuehnbaum *et al*. study (55). Uric acid cycle AAs act as precursors for nitric oxide synthesis and are necessary for endothelial functions (73). Dysregulation in the levels of the discussed AAs has been linked to

obesity, impaired insulin function, and various metabolic diseases. Therefore, it can be proposed that by regulating these AAs, PA can help restore the metabolic imbalance associated with these disorders, mitigate the risk factors contributing to their pathogenesis, and subsequently promote better metabolic health.

Another major trend in the literature is the effects of PA on lipid metabolites. Lipids include several species, whose physiological levels can promote metabolic health. Imbalances in their levels though, can be detrimental to essential cellular processes and normal metabolism (74). FAs are one of these species closely related to metabolic health. San Martin *et al*. reported an increase in arachidonic acid and a reduction in stearic acid in plasma after an 8-week aerobic and strength exercise intervention in women with obesity. While dietary intakes were not reported, participants were asked to maintain their usual dietary habits throughout the study (60). Arachidonic acid, a major circulating PUFA, has been previously associated with a lower risk of coronary heart disease (75). Elevated levels of stearic acid, a saturated long-chain FA, have been linked to an increased risk of coronary heart disease (75). Another study by Campbell *et al.* found a 30% reduction in behenic acid, a very long-chain saturated FA, in response to a 14–17-week weight loss and exercise intervention in women who were sedentary, obese, and insulin-resistant. To control for diet as a potential confounder, their sampling was done during a tightly controlled feeding test week (58). Behenic acid can raise total and LDL cholesterol levels, contributing to an unfavourable lipid profile (76). Generally, higher levels of long-chain saturated FAs are associated with metabolic diseases such as diabetes, cardiovascular diseases, and metabolic syndrome (77). While saturated and unsaturated FAs can originate from dietary sources, endogenous metabolic processes such as synthesis, elongation, and desaturation reactions also affect their tissue or circulating levels (78). Through modulating these metabolic processes PA can potentially induce the reported change in the FAs levels independent of their dietary intake and contribute to a healthier FA profile.

San Martin *et al*. also reported that several plasma phospholipids and TGs are affected by PA intervention. They found positive associations for PC(20:2) and negative associations for PC(36:0), PC(40:6), PC(36:5p), and PC(42:1p) (60). As the main phospholipids in mammalian cells, PCs are necessary for

membrane integrity, cell growth, nerve myelination, and the secretion of very LDLs from hepatocytes. PCs are also fundamental components of pulmonary surfactant (79). While the exact roles of these specific PC species in health and disease are not fully understood, PCs generally play significant roles in various processes, including insulin signaling pathways and inflammation (80-82). Individual plasma lysophosphatidylcholine (LPC) (16:0p), LPC(18:0p), and LPC(20:2) were reported to be higher post-intervention (60). LPCs are another phospholipid species that originate from PCs. A dysregulation in LPC levels has been linked to impaired glucose tolerance, obesity, and T2D (83, 84). These reported associations for the PCs and LPCs align with the concept that PA enhances glucose tolerance and insulin sensitivity (85). A decrease in TG(54:8), TG(56:9), and TG(60:11) species and an increase in TG(48:0), and TG(50:0) species were also reported in this study (60). Higher circulating levels of TGs are characteristic of dyslipidemia (86). TG accumulation in the blood can lead to IR by inhibiting the insulin signaling pathway and activating the serine-threonine kinase pathway (86). This pathway could be another avenue through which PA improves the lipid profile and insulin function.

Other findings from the San Martin *et al*. study were an increase in SM(d18:1/26:1), and a decrease in SM(d18:1/20:0) post-intervention (60). SMs are another abundant phospholipid found in cell membranes and play central roles in cell signaling processes, including cell growth, differentiation, and apoptosis (87, 88). Although data on these specific SM species are limited, it is known that SMs impact cardiometabolic health by affecting cholesterol homeostasis and possessing atherogenic properties (87). SMs retain cholesterol within cells and arterial walls, and their levels in cell membranes directly correlate with cholesterol levels. Circulating SM levels have emerged as an independent risk factor for coronary heart disease (87, 89, 90). In animal models, decreasing plasma membrane SMs has been shown to enhance insulin sensitivity (90). Given their atherogenic properties and their effects on insulin function and cholesterol homeostasis, the regulatory effect of PA on SMs suggests another pathway through which PA contributes to cardiometabolic health.

Regarding other metabolites, Grapov *et al*. reported inverse associations between PA and carbohydrate-related metabolites and the organic acids involved in the tricarboxylic acid cycle (TCA). Post-

intervention, women who were sedentary, obese, and insulin-resistant showed reduced circulating levels of fructose, a monosaccharide; maltose, a disaccharide; and glycerol-3-galactoside (57). Both fructose and maltose have been previously linked to IR, indicating that PA's effects on carbohydrate metabolism extend beyond glucose regulation (91, 92). Additionally, PA was associated with decreased levels of citric acid and increased levels of succinic acid, two organic acids involved in the TCA cycle, suggesting an enhancement of energy utilization (57).

Glutathione-cysteine disulfide and cysteinyl glycine-cysteine, two oxidized thiols were also reported to reduce after 6 weeks of HIIT without any significant changes in weight or insulin sensitivity (55, 56). Thiols play important roles in metabolic processes, including enzymatic reactions, apoptosis, detoxification, and antioxidant protection. Maintaining a balance in oxidized thiol levels is essential for these processes, and disruptions can contribute to reproductive disorders, urinary system disorders, and metabolic diseases such as diabetes and cancer (93). The reduction in oxidized thiols by PA indicates improved antioxidant capacity (55). In another study, a decrease in circulating glutathione-L-cysteine mixed disulfide and plasma hypoxanthine concentrations after a six-week HIIT intervention was observed suggesting an increase in antioxidant capacity and reduced energetic stress induced by PA (56).

Table 1. Metabolite Profile of Physical Activity in Non-pregnant Female Population.

Authors	Study population and sample size	Sample	Study design	PA intervention/ measurement	Metabolite class	Altered metabolites
Kuehnbaum *et al.*, 2014	11 women with overweight/obesity	Plasma	Interventional	HIIT for 6-week	Amino acids	**Positive:** Ornithine **Negative:** L-Cystine
					Others	**Negative:** Glutathione-cysteine disulfide, cysteinyl glycine-cysteine disulfide
Kuehnbaum *et al.*, 2015	9 women with overweight/obesity	Plasma	Interventional	HIIT for 6-week	Others	**Negative:** Glutathione-L-cysteine mixed disulfide, hypoxanthine
Grapov *et al.*, 2019	Sedentary women with obesity and insulin resistance	Plasma	Interventional	Data from calorie-restricted diet + 14 weeks 4 day/week aerobic exercise at 60-75 0% of maximal HR combined with data from an acute bout of exercise	Amino acids	**Negative:** Methionine, cysteine, cystine, serine, tryptophan, phenylalanine, aspartic acid
					Others	**Positive:** Succinic acid, glycolic acid, 1-dodecanol **Negative:** Fructose, maltose, chlorogenic acid, glycerol-3-galactoside, citric acid, 2-hydroxybutannoic, 3-hydroxybutanoic
Hamaya *et al.*, 2022	18,897 women	Plasma	Cross-sectional	Self-reported questionnaire	Amino acids	**Negative:** Total BCAAs, Leucine
Campbell *et al.*, 2014	12-15 women with obesity	Plasma	Interventional	4 days/week for 30-40 minutes of aerobic exercise at an intensity of 60–75% of maximal HR for 9 weeks + calorie restriction	Lipids	**Negative:** Behenic acid
					Others	**Negative:** Alpha-ketoglutarate, pyruvate, uric acid
San Martin *et al.*, 2022	14 women with obesity	Plasma	Interventional	8 weeks of aerobic and strength exercises, alternately, 55 min at 75–90% of maximum HR, 3 times a week	Lipids	**Positive:** LPC(16:0p), LPC(18:0p), LPC(20:2), PC(20:2), TG(48:0), TG(50:0), SM(d18:1/26:1), arachidonic acid **Negative:** PC(36:0), PC(40:6), PC(36:5p), PC(42:1p), PE(40:4p), PE(36:5), PE(38:3p), TG(54:8), TG(56:9), TG(60:11), SM(d18:1/20:0), stearic acid

Note: AAA (Aromatic Amino Acid), BCAA (Branched-Chain Amino Acid), HIIT (High-Intensity Interval Training), HR (Heart Rate), LPC (Lysophosphatidylcholine), PA (Physical Activity), PC (Phosphatidylcholine), PE (Phosphatidylethanolamine), SM (Sphingomyelin), TG (Triglyceride).

1.1.3.4. Metabolome and Physical Activity: Pregnant Populations

Regarding the pregnant populations, most metabolomic studies to date have focused on pathophysiological conditions and adverse exposures. Limited studies have examined the relationship between the metabolome response of pregnant individuals and their PA status, as briefly reviewed in Table 2. An intervention study has reported lower gesP serum alanine levels, comparing the intervention group (diet and PA) to controls (94). Lower total serum AAs have been reported in response to another dietary and PA interventional study (95). In addition to total AA, this study also reported reductions, though insignificant, in leucine, tryptophan, and asparagine levels during late gestation in the intervention group compared to controls (95). Higher circulating alanine levels in gesPs have been linked to several pregnancy complications, including gestational IR, GDM, pre-eclampsia, preterm birth, and higher birth weight (96-99). Moreover, increased alanine levels have been detected in the CB of fetuses exposed to GDM (99).

Maitre *et al*. reported a negative association between PA and urinary BCAAs during late gestation (100). This study did not clearly specify the method used to collect their PA data. BCAAs, specifically leucine, are essential for embryo implantation and development, playing a central role in enhancing blastocyst quality through activation of the mTOR signaling pathway (43). While physiological BCAA levels play important roles, elevated BCAA levels in the circulation of birthing parents are associated with an increased risk of pregnancy complications such as GDM and gestational obesity (101, 102). Additionally, higher BCAA levels in CB have been observed in fetuses exposed to gestational IR, obesity, and GDM, as well as in cases of higher birth weight and preterm birth (99, 103). Similarly, increased placental concentrations of BCAAs have been reported in instances of preterm birth and gestational obesity (104, 105). These alterations in AA levels may result from disrupted gestational and/or placental metabolism, or they could be a contributing factor to these conditions (99). Regardless, PA's effect on BCAA levels can potentially protect against these pregnancy complications and related metabolic disruptions, promoting healthier pregnancy outcomes and fetal development.

Regarding lipid metabolism, observational data have reported a positive association between PA and total monounsaturated FAs in mid-pregnancy (106). Specifically, PA has been linked to higher levels of plasma eicosenoic acid and oleic acid in mid-pregnancy, and palmitoleic acid in mid to late-pregnancy (106). Additionally, a negative correlation has been observed between PA and pentadecanoic acid, an odd-chain saturated FA, in the serum of gesPs during late pregnancy (107). Intervention studies further support these findings. A combined dietary and PA intervention throughout pregnancy has been shown to result in a more favourable FA profile, characterized by higher concentrations of beneficial FAs such as linoleic acid, omega-6 FAs, and PUFAs, and lower concentrations of saturated FAs (94). In general populations, saturated FAs have been positively associated with inflammatory markers, including C-reactive protein and interleukin-6. A higher saturated FA to PUFA ratio has been also linked to increased levels of C-reactive protein (108). In pregnant populations, the PUFAs to total FAs ratio has been negatively correlated with the BMI of birthing parents (109). Insufficient availability and impaired transfer of long-chain PUFAs have been associated with several pregnancy complications, such as pre-eclampsia, fetal growth restriction, and GDM, which can adversely affect fetal and placental growth and development (110). Acylcarnitines, including myristoleoylcarnitine, and palmitoleoylcarnitine, are other members of the lipid class, significantly reduced in late pregnancy following dietary and PA interventions (95). As intermediates of FA metabolism, these compounds serve as important biomarkers of metabolic health. Higher gesP acylcarnitine levels were previously linked to GDM and childhood obesity (111). The regulatory effect of PA on lipid profile provides a plausible explanation for the health benefits associated with gestational PA. By promoting a healthier lipid profile, PA can reduce the risk of pregnancy complications, thereby supporting better outcomes for both the gesP and offspring.

As reviewed above, existing literature primarily attributes findings to lifestyle interventions or relies on self-reported PA questionnaires. In interventional studies, where changes in participant dietary habits and PA levels occur simultaneously, the observed alterations in metabolite levels may be influenced not only by increased PA but also by dietary changes. Consequently, these results cannot be solely interpreted as the

specific impact of PA on the metabolome. As for the observational studies, the PA data were obtained by self-reported questionnaires which tend to overestimate PA levels (112). Moreover, research has predominantly focused on late gestation, with limited data available for other stages of pregnancy. Additionally, the fetal and placental metabolome response to a physically active pregnancy remains largely unexplored. These factors collectively create a significant gap in our understanding of the effects of PA on the gesP, CB, and placental metabolomes. Further research is necessary to study the isolated effects of PA on metabolome while employing objective methods to collect PA data.

Table 2. Metabolite Profile of Physical Activity in Pregnant Population.

Authors	Study population and sample size	Sample	Study design	PA intervention/ measurement	Metabolite class	Metabolites	Gestational stage
Maitre *et al*., 2016	806 Pregnant individuals (early pregnancy), 886 pregnant women (late pregnancy)	Gestational parent urine	Cohort	-	Amino acid	**Negative:** BCAAs	Late gestation
Patel *et al*., 2018	343 Pregnant individuals with obesity and their offspring (intervention=169, control= 174)	CB serum	Randomized controlled trial	An initial session with a health trainer followed by eight weekly sessions + dietary advice recommending foods with a low dietary glycemic index, avoidance of sugar-sweetened beverages and reduced saturated fats	-	No detected associations	Post delivery
Mills *et al*., 2019	1158 Pregnant individuals with obesity (intervention=577, control= 581)	Gestational parent serum	Randomized controlled trial	An initial session with a health trainer followed by eight weekly sessions + dietary advice recommending foods with a low dietary glycemic index, avoidance of sugar-sweetened beverages and reduced saturated fats	Lipids	**Negative:** Extremely large, very large, large, and medium VLDL particles, specifically those containing triglycerides	Late gestation
Haslam *et al*, 2020	Pregnant individuals in mid and early pregnancy (intervention=13, control= 16)	Gestational parent plasma	Randomized controlled trial	A lifestyle modification intervention focused on improving PA levels and diet quality and calorie intake or standard care control group before 16 gestational weeks	Amino acids Lipids Others	**Negative:** Amino acids **Negative:** Acyl carnitines **Negative:** 3-Hydroxybutyrate, bile acids	Late gestation
Rafiq *et al*., 2022	600 Pregnant individuals (24-36 weeks)	Gestational parent serum	Cohort	Questionnaires	Lipids	**Negative:** 17:0 SFA	Late gestation
Xia *et al*., 2022	318 Pregnant individuals	Gestational parent plasma	Cohort	Self-administrated Pregnancy PA Questionnaire	Lipids	**At 15–26 weeks:** **Positive:** total MUFAs MVPA **At 15–26 weeks:** **Positive:** oleic acid and eicosenoic acid **At 23-31 weeks:** **Positive:** palmitoleic acid	Early, mid and late gestation

Note: BCAA, Branched-chain amino acid; MUFAs, Monounsaturated FA; MVPA, Moderate to vigorous physical activity; PA, Physical activity; SFA, Saturated fatty acid; VLDL, Very low-density lipoprotein.

1.2. Thesis Aims and Hypothesis

This project aims to explore the associations between gestational PA and metabolome by comparing the serum profiles of physically active gesPs (A-gesP) and inactive gesPs (I-gesP). We also aim to study the relationship between gesP PA and the fetal metabolomic profiles by examining the CB of babies born to active (AB) and inactive (IB) pregnancies. Furthermore, we aim to lay the groundwork for future analysis of placenta samples in active and inactive pregnancies by developing a metabolite extraction protocol for this specific sample matrix.

Aim 1: Identify the associations between the PA status and mid-pregnancy serum metabolome in A-gesP vs. I-gesP based on their mid-pregnancy PA levels.

Is there a difference in the serum metabolome profile of physically active vs. inactive pregnant individuals? If differences are observed, will those align with metabolite alterations previously associated with improved pregnancy outcomes and reduced risk of pregnancy complications?

Aim 2: Identify the associations between the gesP PA status and their offspring's CB metabolome based on mid to late-pregnancy PA levels.

Is there a difference in the CB serum metabolomes based on their gesP PA status? Will those differences be indicative of better cardiometabolic health and consistent with reduced risks of adverse birth outcomes?

Aim 3: Develop a protocol to perform metabolite extraction of placenta samples for future analysis of matched gesP, cord blood, and placenta samples.

1.3. Study Rationale

Pregnancy represents a critical period characterized by dynamic metabolic changes to support fetal growth and development, leading to a distinct metabolome profile compared to non-pregnant individuals (42). This difference in the baseline metabolism of this population makes it necessary to study pregnant individuals

separate from non-pregnant populations as pregnancy itself is a strong stimulus to induce changes in the metabolism. Gestational PA has been recognized as a modifiable factor that can improve the gesP and fetal health. However, the underlying mechanisms remain poorly understood. As metabolites are the intermediates or end products of metabolism, they can be a powerful tool to study the changes in the metabolism of a biological system in response to a stimulus such as PA (23). However, existing literature on the effects of PA on gestational and fetal metabolome remains limited and inconclusive.

By utilizing an objective assessment of PA, we aim to provide an accurate evaluation of PA status. Additionally, by controlling for as many confounding factors as possible (i.e., gesP diet, offspring sex, delivery mode), we seek to isolate the effect of PA on the metabolome. To accurately classify PA status for mid-pregnancy serum analysis (aim 1), we used PA data from the same stage to determine the activity status of the pregnant individuals. As for the CB data (aim 2), both mid- and late-gestation data were used for PA classification. Examining the CB of offspring whose gesPs were categorized as active across pregnancy is necessary as a drop in PA levels during late gestation, even if the gesP is active in mid-gestation, can potentially affect the CB metabolome. Pinpointing the metabolome changes induced by PA can uncover the underlying metabolic pathways influenced by PA, shedding light on potential mechanisms mediating the observed associations between PA and better gesP and fetal health outcomes.

Additionally, given the pivotal role the placenta plays throughout gestation, it is very plausible that the health benefits of gestational PA are delivered partly through enhancing placental functions. Therefore, the importance of studying the placental metabolome in the context of PA cannot be overlooked. Obtaining valid and reproducible metabolomic results requires a validated extraction protocol. Although validated extraction protocols are available for similar tissue samples, it is necessary to carefully design a protocol appropriate for the sample matrix, the analytical platform used, and the availability of different equipment and consumables involved in the extraction protocol for a reliable analysis of our placenta samples (113).

Chapter 2: Gestational Parent and Cord Blood Metabolome Associations with Physical Activity Status of Pregnant Individuals

Preamble to Manuscript 1

The manuscript "Gestational Parent and Cord Blood Metabolome Associations with Physical Activity Status of Pregnant Individuals" focuses on the first and second objectives of this thesis. Herein, we aimed to identify the associations between gesP PA and the gesP and CB serum metabolome profiles.

Gestational Parent and Cord Blood Metabolome Associations with Physical Activity Status of Pregnant Individuals

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A.Y. was involved in the project's conception, performed the data analysis, interpreted the results, and authored the manuscript. A.C.S. helped with the project's conception and data analysis. I.H. and J.S. were responsible for sample analysis and the acquisition of metabolomic data. K.A. contributed to the project's conception, helped interpret the results, and edited the manuscript.

Keywords: Pregnancy; Physical Activity; Metabolomics; Mass Spectrometry.

2.1. Abstract

Background: Gestational physical activity (PA) is well-documented for its ability to mitigate the risk of several pregnancy and birth complications, including gestational diabetes, gestational hypertension, and macrosomia. Despite the well-established protective effects of PA during pregnancy, the underlying mechanisms behind these benefits remain elusive. Studying the metabolome and how it responds to PA can be valuable in understanding these mechanisms. Despite the significance of the metabolome profiles in mapping out these mechanisms, there is limited data on the metabolomic profile of the gestational parent (gesP) and no data regarding cord blood metabolome associations with PA.

Method: The first aim of this project was to study the associations of PA with the gesP serum metabolome in mid-pregnancy. The second aim of the study was to look into the cord blood metabolome associations with the PA of participants who maintained their PA status from (mid 24-28 weeks) to late gestation (34-38 weeks). For the first aim, participants were classified as "active" or "inactive" based on their mid-pregnancy (24-28 weeks) PA data, objectively assessed using accelerometers. For the second aim, PA classification was done based on mid and late-gestation accelerometry data. An untargeted metabolomics approach was applied to analyze the global metabolic profile of our samples using Ultra-Performance Liquid Chromatography-Quadrupole Time-of-Flight Mass Spectrometry (Agilent Technologies Inc., Canada), a highly sensitive platform with broad metabolite coverage and high resolution. Data processing was performed in MS-DIAL and multivariate and univariate statistical analysis on MetaboAnalyst 6.0.

Results: Our gestational serum sample analysis revealed no major differences in the serum metabolome in physically active and inactive pregnancies based on the clustering of the samples in PCA and PLS-DA plots. We identified pipecolic acid, a lysine derivative, to be significantly higher in the inactive participants compared to the active group. Receiver Operating Characteristic (ROC) analysis also revealed that pipecolic acid and phosphatidylcholine (18:1e/8-HEPE) exhibited a fair ability to discriminate between the active and inactive groups (AUC>0.7). Regarding our cord blood results, the PLS-DA plot revealed a moderate separation between the cord blood metabolome of babies born to active vs. inactive pregnancies.

Babies born to active pregnancies showed significantly lower levels of phenylalanine, valine, threonine, lysophosphatidylcholine (LPC) 16:0, LPC 18:2, LPC 18:1, O-acetylcarnitine, and 5-hydroxyindole-3-acetic acid. ROC analysis revealed several metabolites with discriminatory power between the cord blood serum samples. Another important observation of the study was the strong separation between the gesP and cord blood metabolome profile. This indicates that the cord blood metabolome might correlate more closely with the placental metabolome rather than the gesP serum metabolome.

Conclusion: Gestational PA can affect the gesP and fetal metabolome, with the magnitude of the effect being much higher in the fetus. The protective effect of gestational PA against birth and pregnancy complications is likely through modulating pathways that the identified metabolites are involved in. As many of these metabolites have been previously linked to oxidative stress and inflammation, the main contributor to the observed differences is posited to be an enhanced antioxidant capacity and lower inflammation induced by higher PA status.

2.2. Introduction

A healthy pregnancy is accompanied by vast physiological, hormonal, and humoral adaptations and changes to accommodate and nurture the growing fetus, provide the necessary energy and substrates required by the fetus following birth, and also meet the altered physiological demands of the birthing parent during gestation, labour, and breastfeeding periods (37, 46). Different factors trigger these changes including gestational hormones such as estrogen, progesterone, prolactin, cortisol, placental growth hormone, and human placental lactogen, and the physiological needs of the fetus and the gestational parent (gesP) in each stage of the pregnancy (40). In response to these dynamic physiological changes, the protein, fat, and carbohydrate metabolism of the birthing parent gets affected, leading to a distinctive metabolome profile throughout the gestational journey (39).

As metabolites are the biochemical fingerprint of cellular and systemic metabolism, the gestational metabolome can highlight the health status of the gesP and predict the pregnancy and fetal health outcomes (114, 115). Metabolomic studies have been utilized to better understand the effects of adverse exposures such as gestational diabetes mellitus (GDM) (116), pre-eclampsia, gestational hypertension (117), fetal growth restriction (118) and preterm delivery (119) on the gesP metabolome. Yet the effect of positive exposures on the metabolome, such as being physically active, remains largely understudied.

The umbilical cord is the vital link between the developing fetus and the placenta. The umbilical cord vein and arteries carry the fetal blood from and to the placenta facilitating the exchange of oxygen and nutrients while eliminating carbon dioxide and other waste substances (Figure 4) (120). A once discarded material, its true biological potential in research and clinical practice is just being recognized. The composition of arterial umbilical cord blood (CB) resembles fetal metabolism and the venous CB resembles placental metabolism (121). Therefore it can be utilized in a variety of ways from enhancing our understanding of the intrauterine environment, fetal development, placental functions, assessment of neonatal condition at birth, and early biomarker detection of adverse perinatal conditions (122).

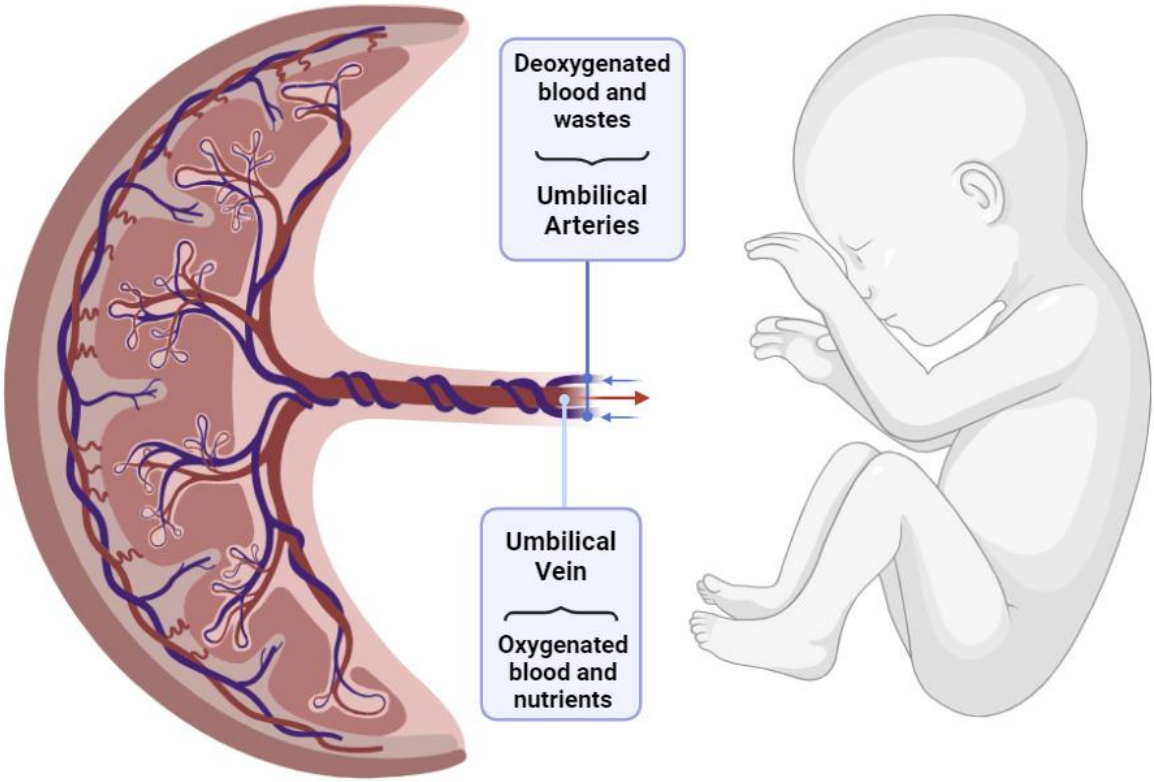


Figure 4. Umbilical Vein and Arteries Blood Flow. Created with biorender.com.

The *in-utero* conditions the developing fetus is exposed to can influence various parameters in CB. One such exposure, the physical activity (PA) status of the birthing parent can induce changes in CB anti-oxidative capacity, high-density lipoprotein (HDL) cholesterol levels, stem cells, C-peptide, and leptin levels (123-127). This suggests that gestational PA can impact fetal metabolism and hence induce changes in the CB metabolome. Despite the suggestive evidence, the impact of gestational PA on the CB metabolome is yet to be characterized.

Understanding the CB metabolome response to PA is of great importance as several studies have demonstrated that certain metabolites correlate with newborn birthweight, adiposity, and birth length (98, 103, 128, 129). More importantly, there are metabolites whose CB levels have been linked to birth, pregnancy complications, and even long-term offspring cardiometabolic health such as GDM, large for gestational age,

early childhood obesity (130), and elevated childhood blood pressure (131). The CB metabolome response to PA can reveal how PA can influence the *in-utero* environment, fetal development and protect against pathophysiological states in the birthing parent and the fetus (48).

2.3. Method

2.3.1. Participant Recruitment and Ethical Statement

Participants were recruited as a part of the CIHR-funded '**P**hysica**L** **AC**tivity and di**E**tary implicatio**N**s **T**hroughout pregn**A**ncy' (PLACENTA) study in Ottawa, Ontario. The inclusion criteria for participation were as follows: aged between 18 and 40 years, being able to communicate in either French or English, carrying a singleton fetus, having a gestational age lower than 28 weeks, having a stable weight for at least six months before conception (±5 kg), having a pre-pregnancy body mass index (BMI) between 18.5 – 29.9 kg/m^2, and delivering at term and locally in Ottawa. The exclusion criteria were being diagnosed with pre-pregnancy diabetes, untreated thyroid disease, hypertension, having contraindications to PA, delivering pre-term, or planning to give the child up for adoption. Pre-screening was performed on the interested individuals to assess their eligibility. Written consent forms were provided to those eligible to participate in the PLACENTA study. This study has been approved by the University of Ottawa's Research Ethics Board and adheres to all ethical considerations of the Declaration of Helsinki (CHEOREB# 16/68X, uOttawa H11-15-29, TOH #20160178-01H).

2.3.2. Physical Activity and Dietary Assessment

Participants were asked to visit our lab twice during their pregnancy: mid- (between 24-28 weeks gestation), and late- (between 34-38 weeks gestation) gestation. Each visit included performing sociodemographic, diet history, and depression questionnaires. Following each visit, participants were provided with a take-home package that included an omnidirectional Actical® accelerometer (Philips Respironics, Bend, OR, USA).

Dietary data were collected and analyzed using the Automated Self-Administered 24-hour (ASA24) Dietary Assessment Tool, version ASA24-Canada-2018 (National Cancer Institute, Bethesda, MD). To

evaluate free-living PA, participants were asked to wear the accelerometer around their waist for seven consecutive days during waking hours. At least three valid days with a minimum of 10 hours of wearing time were required to be eligible for any further analyses (132). Following the procedures outlined in the Canadian Health Measures Survey, minutes of PA per week were calculated from the accelerometer data output using SAS version 9.4, as previously described (133). Briefly, the accelerometer count thresholds were as follows: sedentary time was defined as less than 100 counts per minute (CPM), light-intensity PA (LPA) ranged from 100 to less than 1535 CPM, moderate-intensity physical activity (MPA) was from 1535 to less than 3962 CPM, and vigorous-intensity PA (VPA) was 3962 CPM or more (133). The PA classification of the participants used the *2019 Canadian guideline for PA throughout pregnancy* recommending at least 150 minutes of MPA a week, or an average of 21.4 minutes per day (14). To ensure the distinction in PA levels between groups, 1SD (2.5 minutes) above or below the recommended 21.4 minutes per day was defined as the cut-off point. For aim 1, participants who averaged more than 21.4 + 2.5 minutes of moderate to vigorous PA (MVPA) in their mid-pregnancy were classified as active and those below 21.4 – 2.5 minutes were classified as inactive. For aim 2, participants who averaged above or below the cut-off points in both mid- and late-gestation were classified as active and inactive, respectively.

2.3.3. Sample Collection

Participants were asked to fast overnight before each visit. Fasting blood samples were collected, according to best practices, at each gestational time point (24-28 weeks and 34-38 weeks gestation). CB samples were obtained at term delivery (37-42 weeks). After collection, the blood samples were centrifuged, and serum was collected and stored at -80℃ for subsequent metabolomic analysis (134).

2.3.4. Metabolite Extraction from Gestational Parent and Cord Blood Serum

Initially, 50 µL of each serum sample was moved to an Eppendorf tube. 200 µL of 100% ice-cold methanol (MeOH) containing 5 µM of purine as an internal standard was added to the samples. Tubes then were vortexed for 60 seconds and sonicated for 5 minutes. After sonication, the samples were allowed to rest for 2 minutes. Afterwards, the tubes were centrifugated at 14,000 xg for 10 minutes at 4ºC. The supernatant,

containing the extracted metabolites was collected in a new Eppendorf and dried under speedVac (37-45°C). The dried extract was reconstituted in 100 µl of MeOH:ddH_2O (1:1), then vortexed and sonicated for 2 minutes. The reconstituted extraction was then transferred into a new HPLC-grade centrifugal tube and centrifuged at 14,000 xg for 15 minutes. The final solutions were transferred into HPLC vials and stored at -80 for liquid chromatography- mass spectrometry (LC-MS) analysis, as previously described (135, 136).

2.3.5. Quality Control Samples

Blank controls were prepared by going over the same steps in the extraction protocol in the absence of any serum samples to control for solvent purity, instrument background noises and/or contaminations from the sample extraction steps and the consumables (137). Pooled quality controls (QCs) were prepared by transferring 5-10 µL of serum into an Eppendorf tube and vortexing them for 10 seconds to become homogenized. From there the mixed serum sample was split into 50 µL aliquots. Each aliquot was extracted following the same steps as the extraction protocol (137). Purine was spiked into samples by preparing a 10mM purine (0.006 g in 5 mL 100% MeOH) as a stock solution. The internal standard was used to normalize the data and control for random errors and drifts in the instrument function (137).

2.3.6. Data Acquisition by LC-MS

Ultra-Performance Liquid Chromatography-Quadrupole Time-of-Flight Mass Spectrometry was employed for data acquisition (LC-MS QTOF 6545i Agilent (Agilent Technologies Inc., Canada)). Samples were randomly injected into the instrument, starting with a blank sample and then a pooled QC. A blank and QC were injected between every 10 runs. LC was conducted utilizing a reversed-phase Kinetex C18 column (2.6 µm, 2.1 × 100 mm, Phenomenex). Metabolites were eluted employing a binary gradient system at a flow rate of 500 µL/minute, consisting of water (solvent A) and MeOH with 0.1% acetic acid (solvent B). The gradient profile was 0 minute 2% B; 13 minute 98%B; 15 minute 98%. The column temperature was set at 50°C, with a sample injection volume of 1 µL. Ionization was achieved through electrospray ionization in both positive and negative modes, utilizing an Agilent dual jet stream ion source. The MS settings were optimized as follows: Vcap 3000 V, nozzle voltage 0 V, Fragmentor 140 V, Oct 1 RF Vpp 750 V, gas

temperature 250°C, drying gas flow 16 L/minute, nebulizer gas pressure 45 psi, sheath gas temperature 385°C, and sheath gas flow 12 L/minute. Mass spectra were acquired over a range of m/z 25–1250. Tandem MS (MS/MS) analysis was performed with fixed collision energies set at 10, 20, and 40 V, enabling the fragmentation of precursor ions and the generation of product ions for further characterization and identification of metabolites.

2.3.7. Data Processing and Analysis

The raw data was converted into mzmL using MassHunter Data Acquisition (version 9.0, Agilent Technologies, Santa Clara, USA). MS-DIAL software (RIKEN, version 4.96) was utilized for data processing, such as peak selection, deconvolution, and peak alignment (138). The MS-DIAL parameters were set as follows: MS1 and MS2 tolerances 0.03 and 0.04 Da, respectively; minimum peak height: 1000; mass slice width: 0.1; linear-weighted moving average as the smoothing method using 3 scans and peak width 5 scans; sigma window value for deconvolution: 0.5; and 0.1 minute and 0.03 Da tolerances for peak alignment. For compound annotation the following criteria were required: an error < 20 ppm for m/z value and a similarity ≥ 65% for MS/MS spectra comparing the experimental values to the theoretical values available in the MS-DIAL library and a customized library (138).

The annotated metabolite table was exported to the MetaboAnalyst 6.0 to perform both univariate/multivariate analyses (139). Features with more than 50 percent missing values were removed from the dataset. Other missing values were replaced by 1/5 of the minimum positive value of each variable. Unsupervised Principal Component Analysis (PCA) and Supervised Partial Least Square-Discriminant Analysis (PLS-DA) models, volcano plots, and Receiver Operating Characteristic curves (ROC) were generated at a confidence level of 95%. Feature selection within the PLS-DA model was guided by variable importance projection (VIP) scores exceeding 1.4, thereby highlighting key variables contributing to group separation. PLS-DA model validation was assessed based on the goodness-of-fit R2 ($0 \leq R2 \leq 1$), and the predictive ability Q2 ($0 \leq Q2 \leq 1$) values. Volcano plots were used to identify independent changes in

metabolite levels between the two study groups. Classical and multivariate ROC curves were employed to identify metabolites with discriminatory power between the study groups.

Normality was assessed using the Shapiro–Wilk test. Based on normality, data were analyzed by Student's t-test or Mann–Whitney U test where appropriate. For multivariate analysis data was normalized by sum, log transformation, and auto-scaling. Regarding lifestyle variables, participants, and birth characteristics, statistical analyses were conducted using JASP (version 0.18.3).

2.4. Results

2.4.1. Participant Characteristics Aim 1

Based on the accelerometry data twenty-nine participants were included in this study, of which 13 were active and 16 inactive based on their mid-pregnancy PA data. Characteristics of participants are presented in Table 3. There were no significant differences in the study groups in terms of age, pre-pregnancy weight, pre-pregnancy BMI, gestational weight gain, or offspring sex. The mean gestational weight gains were within the normal ranges in both groups (140). The only significant differences between the two groups were their mid-pregnancy MVPA ($p < 0.001$), MPA ($p < 0.001$), VPA ($p = 0.02$) (minute/day) scores, and total steps (<0 .001) (count/day). The dietary intake data revealed no significant differences between groups regarding mid-pregnancy total calorie, fat, protein, carbohydrate, sugar, and fibre intakes. Although not significantly different, the A-gesP had a higher mean intake in all the macronutrient groups and total calories.

Table 3. Participant characteristics.

Characteristics	I-gesP (n= 16)	A-gesP (n=13)	P-value
Age (y)	33.13 (3.16)	31.92 (2.18)	0.26
Height (cm)	165.26 (6.59)	166.1 (6.36)	0.73
Pre-pregnancy weight (kg)	67.51 (12.04)	63.31 (9.16)	0.31
Pre-pregnancy BMI (kg/m^2)	24.62 (3.49)	22.9 (2.7)	0.16
GWG (kg)	12.9 (3.91)	13.29 (4.21)	0.94
Mid-pregnancy weight (kg)	74.85 (11.67)	72.15 (9.74)	0.51
Offspring sex (F:M)	5:11	6:7	0.41
Mid-pregnancy PA			
MVPA (min/day)	10.6 (4.46)	38.15 (6.32)	**< 0.001**
MPA (min/day)	10.39 (4.42)	33.86 (8.21)	**< 0.001**
VPA (min/day)	0.202 (0.54)	4.29 (7.04)	**0.02**
LPA (min/day)	170.54 (47.32)	169.91 (49.02)	0.97
Steps (count/day)	5469.26 (1002.84)	8682.77 (1532.12)	**<0 .001**
Mid-pregnancy dietary intakes			
Total caloric intake (kcal/day)	2204.16 (345.79)	2483.69 (626.44)	0.24
Protein (g/day)	96.02 (17.28)	98.73 (32.57)	0.82
Fats (g/day)	94.14 (21.18)	99.48 (27.32)	0.63
CHO (g/day)	252.2 (54.78)	313.75 (86.83)	0.07
Sugars (g/day)	110.12 (33.15)	136.32 (36.89)	0.11
Fibre (g/day)	24.43 (7.28)	28.64 (7.96)	0.23

Note: Values are presented as mean (SD). * p < 0.05 were considered as significant. BMI, body mass index; F, female; M, male; CHO, carbohydrates; LPA, light-intensity physical activity; MPA, moderate-intensity physical activity; MVPA, moderate-to-vigorous-intensity physical activity; VPA, vigorous-intensity physical activity.

2.4.2. Metabolomics Results Aim 1

Untargeted metabolomic analysis of gesP serum identified 3011 features of which 74 unique metabolites were annotated by retention time (RT) and mass-to-charge ratio (m/z) (Appendix Table 1). The identified metabolites mainly belonged to the classes of amino acids (AAs), organic acids, and phospholipids. Among these, 42 metabolites were consistently detected in all samples. The heat map in Figure 5A illustrates the relative abundance patterns of metabolites across the active and inactive participants. Each row represents a metabolite, and each column represents an individual sample. Figure 5B presents the relative abundance of metabolites between the two groups. The colour scale on the right side of the heat map represents the normalized abundance values, with red indicating higher abundance and blue indicating lower abundance. The majority of metabolites had a higher mean abundance in the inactive gesPs ((I-gesP) group compared to active gesPs (A-gesP).

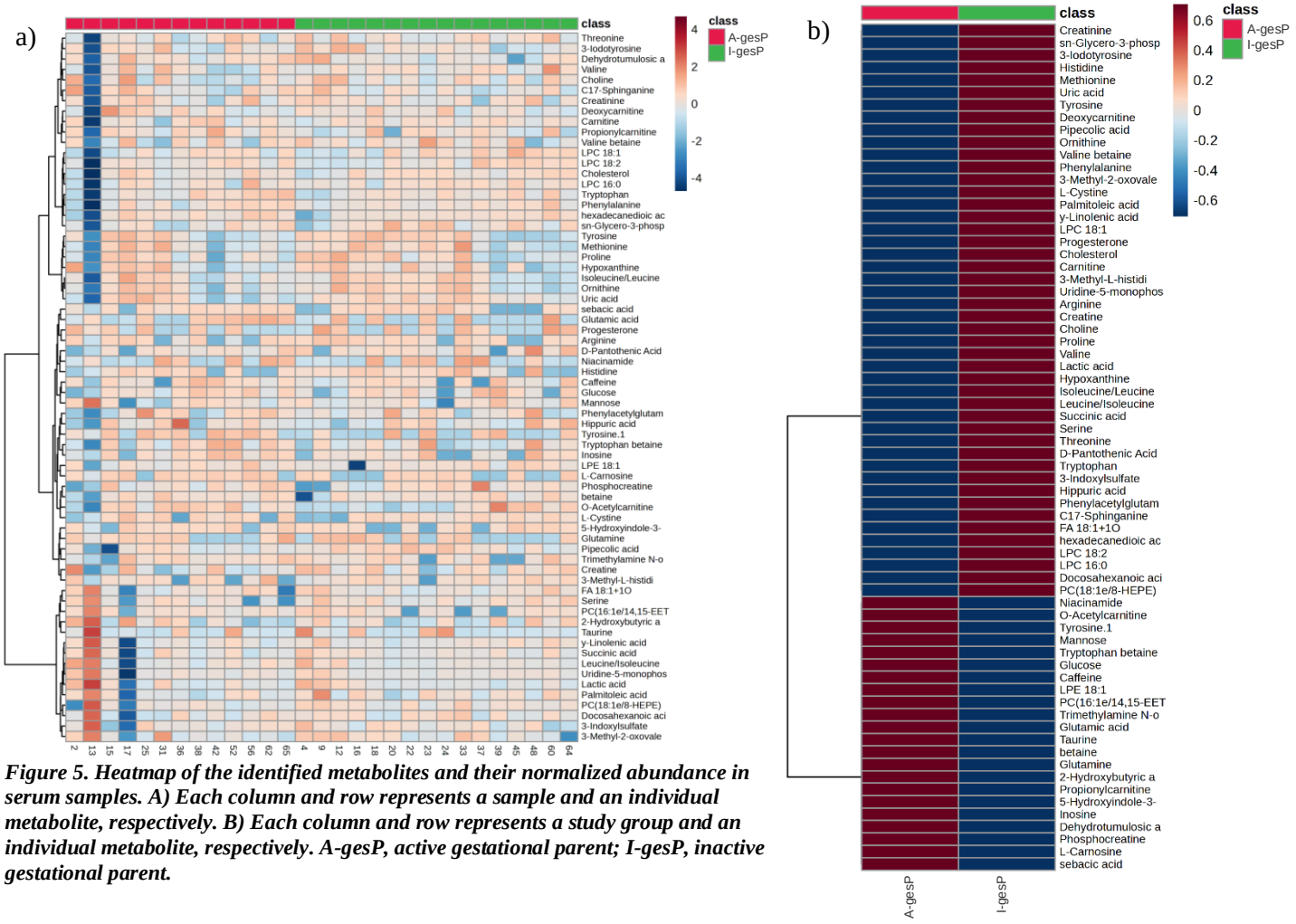


Figure 5. Heatmap of the identified metabolites and their normalized abundance in serum samples. A) Each column and row represents a sample and an individual metabolite, respectively. B) Each column and row represents a study group and an individual metabolite, respectively. A-gesP, active gestational parent; I-gesP, inactive gestational parent.

Unsupervised PCA analysis was conducted to visualize differences within the study group (Figure 6A). The analysis did not reveal a distinct separation between the active and inactive groups, suggesting no discernible differences in overall metabolome profiles between the study groups. PC1 was responsible for 19.4% and PC2 for 13.7 % of the observed variance. Supervised PLS-DA was performed to further evaluate the differences between the study groups. PLS-DA plot successfully distinguished between the groups (Figure 6B), however, cross-validation analyses indicated that the model lacked predictive value and exhibited overfitting tendencies (R^2= 0.5, Q^2= -0.17).

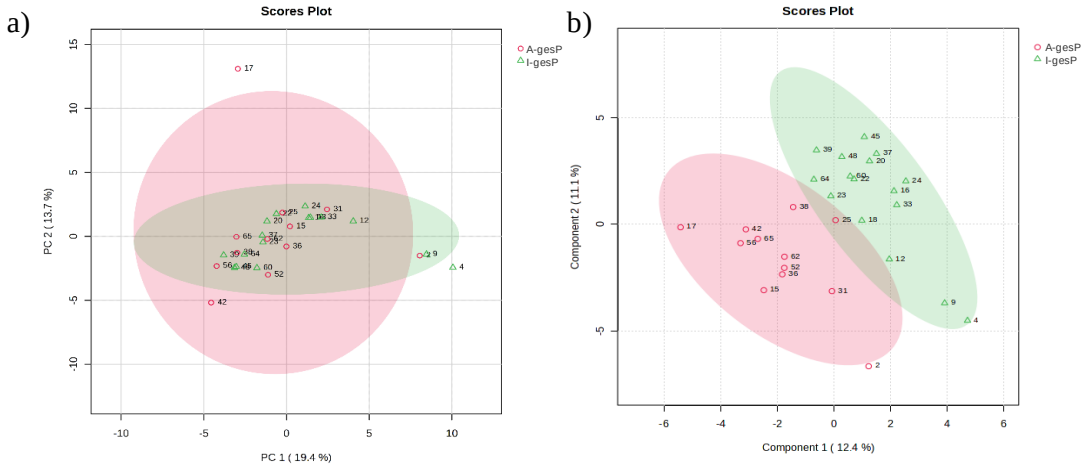


Figure 6. A) Principal Component Analysis (PCA) with 95% confidence of gestational parent serum in active vs. inactive pregnancies. B) Partial Least Squares-Discriminant Analysis (PLS-DA) with 95% confidence of gestational parent serum in active vs. inactive pregnancies (R^2= 0.5, Q^2= -0.17). A-gesP, active gestational parent; I-gesP, inactive gestational parent.

Predictive abilities of the potential biomarkers were assessed by ROC curve analysis according to the following criteria: the area under the curve (AUC) of 0.9–1.0 indicated excellent performance, 0.8–0.89 good performance, 0.7–0.79 fair performance, 0.6–0.69 poor performance, and <0.6 insignificant value (141). Among all the identified compounds, only two metabolites demonstrated an AUC exceeding 0.7, suggesting potential predictive capability for PA status (Table 4). Pipecolic acid exhibited an AUC of 0.78

(Specificity=0.7, Sensitivity=0.8), and phosphatidylcholine (PC) (18:1e/8-HEPE) showed an AUC of 0.73 (Specificity=0.8, Sensitivity=0.6), with higher levels in I-gesP compared to A-gesP (Figure 7).

Table 4. Predictive value of two metabolites with AUC > 0.7 by ROC analysis in gesP serum.

Metabolite	AUC	Specificity	Sensitivity
Pipecolic acid	0.78	0.7	0.8
PC(18:1e/8-HEPE)	0.73	0.8	0.6

Note: AUC, area under the curve; ROC, Receiver Operating Characteristic.

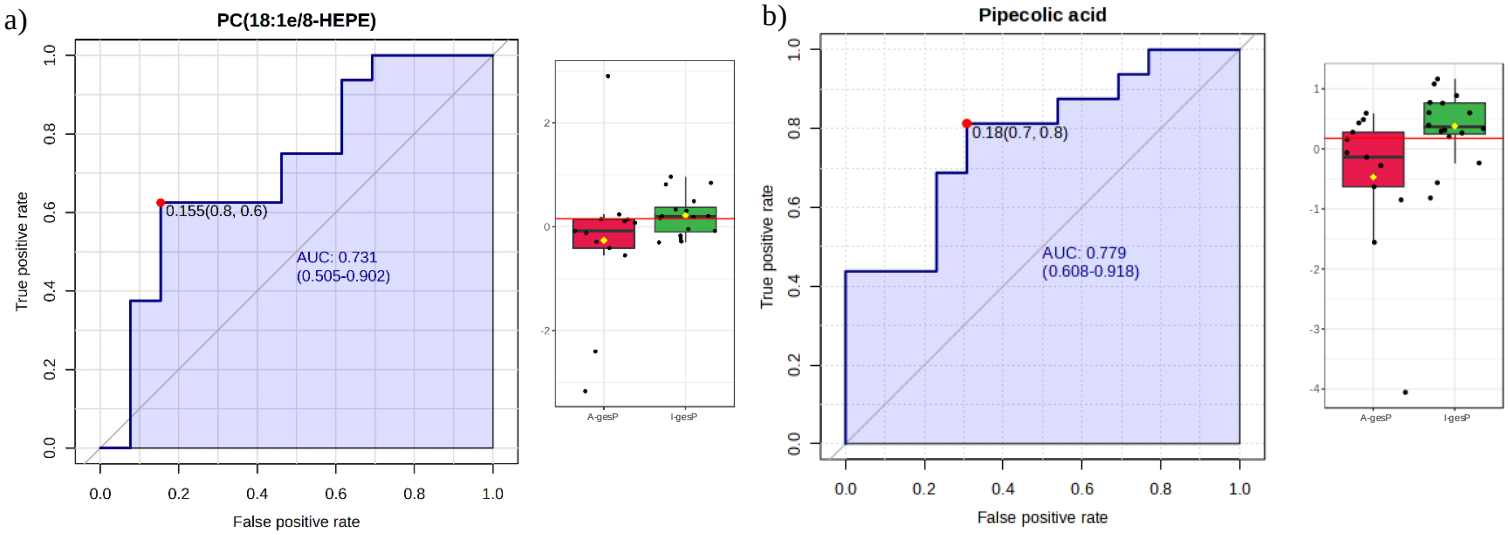


Figure 7. ROC curve graph and relative abundance of two metabolites with AUC > 0.7 in gestational parent serum. AUC, area under the curve; ROC, Receiver Operating Characteristic, A-gesP, active gestational parent; I-gesP, inactive gestational parent.

Furthermore, multivariate exploratory ROC analysis was performed using SVM as a classification and feature ranking approach. The ROC curves derived from the cross-validation performance of different models are presented in Figure 8A. Among the models evaluated, Model 34 demonstrated the best performance, achieving an AUC of 0.603 (95% CI: 0.175-0.956) which indicates a poor level of classification accuracy. Figure 8B illustrates the significant metabolites identified in the best-performing model (Model 34). The selection frequency of these metabolites, which indicates their importance in the classification

model, is shown. Pipecolic acid, docosahexaenoic acid, and tyrosine were among the top-ranked features, highlighting their potential relevance in the classification model.

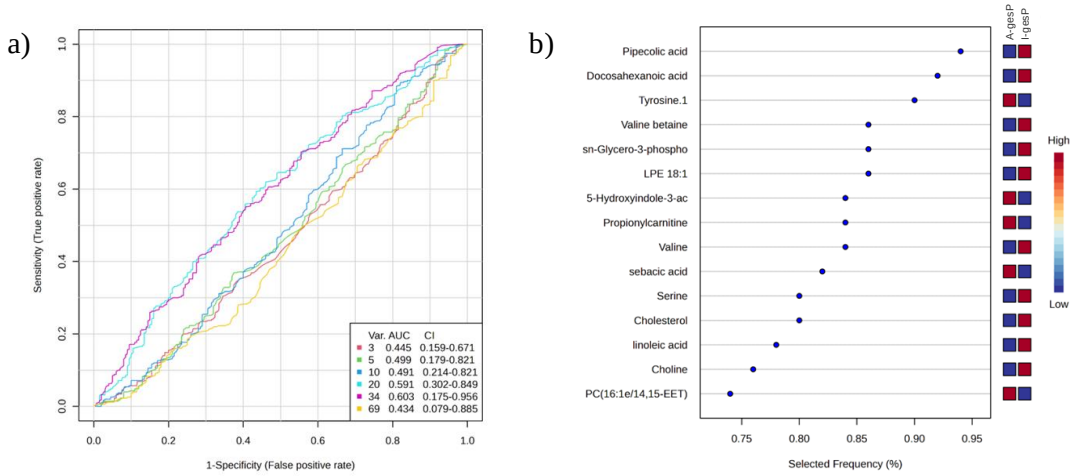


Figure 8. Predictive value of the gestational parent metabolites by multivariate ROC analysis. a) Multivariate Exploratory ROC Analysis models and their AUC. b) Significant features of metabolites and ratios in the best model of the multivariate Exploratory ROC (AUC=0.603). A-gesP, active gestational parent; AUC, area under the curve; CI, confidence interval; I-gesP, inactive gestational parent; ROC, Receiver Operating Characteristic.

To identify independent changes in metabolite levels, a volcano plot analysis was conducted. At a fold change (FC) threshold of > 1.2 and a p-value cutoff of < 0.05, one metabolite, pipecolic acid, was observed to be significantly higher in I-gesP compared to A-gesP (Figure 9). Upon applying a false discovery rate (FDR) adjusted p-value of < 0.05, this metabolite no longer remained significantly different between the groups.

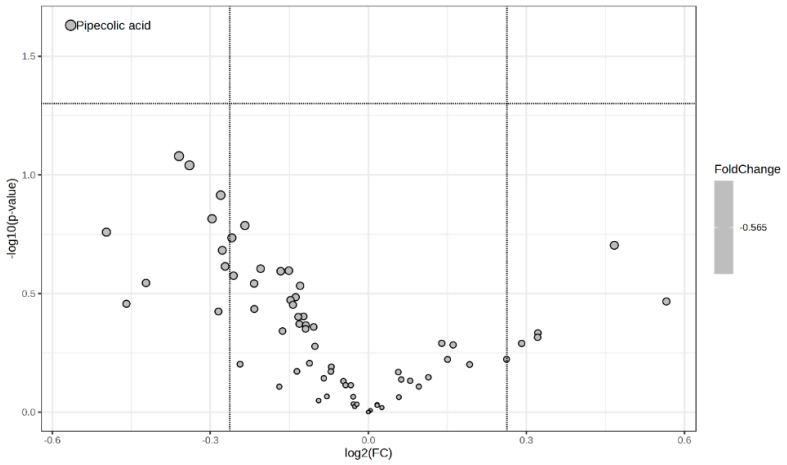


Figure 9. Volcano plot displaying metabolites with significantly higher levels in I-gesP compared to A-gesP. FC > 1.2 and P < 0.05 was considered as significant. FC, fold change; I-gesP, inactive gestational parent; A-gesP, active gestational parents.

2.4.3. Participant Characteristics Aim 2

Characteristics of participants are presented in Table 5. The cohort consisted of 17 venous CB samples, categorized into two groups based on gesP mid and late-pregnancy PA status: babies born to active pregnancies (ABs) (n=6) and babies born to inactive pregnancies (IBs) (n=11). A-gesP demonstrated significantly higher levels of MVPA at both mid- and late-gestation compared to the I-gesP (MVPA mid-gestation: $p < 0.001$; MVPA late-gestation: $p < 0.001$). Mid-pregnancy MPA and total step counts were also significantly higher in A-gesP. In late-pregnancy, MPA, LPA, and total step counts were significantly higher in the A-gesP. There were no statistically significant differences between the two groups in terms of gesP age, pre-pregnancy weight, pre-pregnancy BMI, mid-pregnancy weight, late-pregnancy weight, height, and gestational age at delivery. Birth length, birth weight, and weight-for-length z-score were not different between the AB and IB. ABs had a significantly lower placental weight compared to IBs ($p= 0.04$). The distribution of offspring sex and delivery mode did not significantly differ between the two groups. Regarding the gesP dietary intakes, no significant differences were observed in mid-pregnancy total caloric, fat, protein, or sugar intakes. Though insignificant, all the mentioned macronutrient groups had a higher mean intake in the active gesPs. Active gesPs had significantly higher levels of carbohydrate and fibre intake in mid-gestation. As for late pregnancy dietary intakes, protein and fibre intake were significantly higher in the active gesPs (Table 5). All the other macronutrient groups had a higher average in the active group compared to the inactive group in late gestation.

Table 5. Participant characteristics.

Gestational parent characteristics	**IBs (n= 11)**	**ABs (n=6)**	**P-value**
Age (y)	34.09 (3.21)	32.5 (2.81)	0.33
Height (cm)	166.81 (5.92)	165 (9.02)	0.62
Pre-pregnancy weight (kg)	69.63 (13.07)	59.833 (9.84)	0.13
Pre-pregnancy BMI (kg/m^2)	24.96 (4.12)	21.86 (1.74)	0.1
GWG (kg)	12.6 (3.8)	13.08 (6.26)	0.84
Mid-pregnancy weight (kg)	76.42 (11.94)	69.57 (12.02)	0.18
Late-pregnancy weight (kg)	81.63 (11.98)	74.23 (13.83)	0.12
Birth characteristics			
Gestational age at delivery (weeks)	39.87 (1.38)	40.13 (0.71)	0.67
Offspring sex (F:M)	4:7	2:4	0.9
Delivery mode (CS:V)	2:9	0:6	0.27
Placenta weight (g)	537.64 (91.75)	453 (42.35)	**0.04**
Birth length (cm)	50.58 (2.01)	50.13 (1.63)	0.65
Birth weight (g)	3406.36 (427.91)	3250 (322.49)	0.45
Fetal:placental weight ratio	6.44 (1)	7.23(1.02)	0.14
Weight-for-length z-score	-0.56 (1.04)	-0.58 (1.04)	0.96
Offspring body fat %	17.13 (2.17)	17.35 (2.95)	0.87
Gestational parent mid-pregnancy PA			
MVPA (min/day)	10.37 (4.92)	41.78 (4.74)	< **0.001**
MPA (min/day)	10.3 (4.94)	37.8 (7.05)	< **0.001**
VPA (min/day)	0.06 (0.11)	3.98 (7.18)	0.07
LPA (min/day)	178.23 (48.27)	163.36 (42.13)	0.54
Steps (count/day)	5601.76 (1132.79)	9559.33 (1140.63)	<**0 .001**
Gestational parent late-pregnancy PA			
MVPA (min/day)	7.54 (6)	31.48 (7.93)	< **0.001**
MPA (min/day)	7.46 (5.93)	30.73 (9.03)	< **0.001**
VPA (min/day)	0.06 (0.13)	0.77 (1.88)	0.84
LPA (min/day)	185.13 (56.92)	152.26 (35.66)	**0.02**
Steps (count/day)	5418.45 (2176.06)	7652.96 (1790.79)	< **0.001**
Gestational parent mid-pregnancy dietary intakes			
Total caloric intake (kcal/day)	2189.07 (299.62)	2604.11 (694.34)	0.24
Protein (g/day)	88.72 (16.45)	113.67 (34.79)	0.14
Fats (g/day)	99.92 (20.96)	99.73(27.52)	0.99
CHO (g/day)	241.69 (40)	330.23(104.47)	**0.03**
Sugars (g/day)	106.53 (33.94)	136.51 (28.24)	0.07
Fibre(g/day)	21.83 (4.06)	31.2 (6.96)	**0.02**
Gestational parent late-pregnancy dietary intakes			
Total caloric intake (kcal/day)	2345.78 (422.38)	2625.58 (604.56)	0.34
Protein (g/day)	93.18 (13.23)	114.56 (14.4)	**0.02**
Fats (g/day)	95.11 (29.79)	110.9 (26)	0.35
CHO (g/day)	288.21 (64.26)	313.86 (83.51)	0.54
Sugars (g/day)	134.45 (4.4)	136.36 (35.35)	0.83
Fiber (g/day)	23.32 (4.05)	32.37 (3.6)	**0.002**

Note: Values are presented as mean (SD). p < 0.05 were considered as significant. BMI, body mass index; CS, cesarian section; F, female; M, male; CHO, carbohydrates; LPA, light-intensity physical activity; MPA, moderate-intensity physical activity; MVPA, moderate-to-vigorous-intensity physical activity; VPA, vigorous-intensity physical activity; V, vaginal.

2.4.4. Metabolomic Results Aim 2

Untargeted metabolomic analysis of CB serum identified 3011 unique features of which 75 metabolites were annotated by RT and m/z (Appendix Table 1). Figure 10A illustrates the normalized average metabolite levels in the CB of AB, IB, and their corresponding gesP mid-pregnancy serum samples. Metabolites are represented along the y-axis, while the x-axis represents the different study groups (I-gesP, A-gesP, IB, and AB). Figure 10A represents the normalized levels of metabolites in each sample. The majority of metabolites exhibited higher averages in the IBs compared to ABs. Metabolites such as hexadecanedioic acid, glucose, mannose, betaine, lysophosphatidylcholine (LPCs), tryptophan, phenylalanine, o-acetylcarnitine, and hexadecanedioic acid were higher on average in IB. Metabolites such

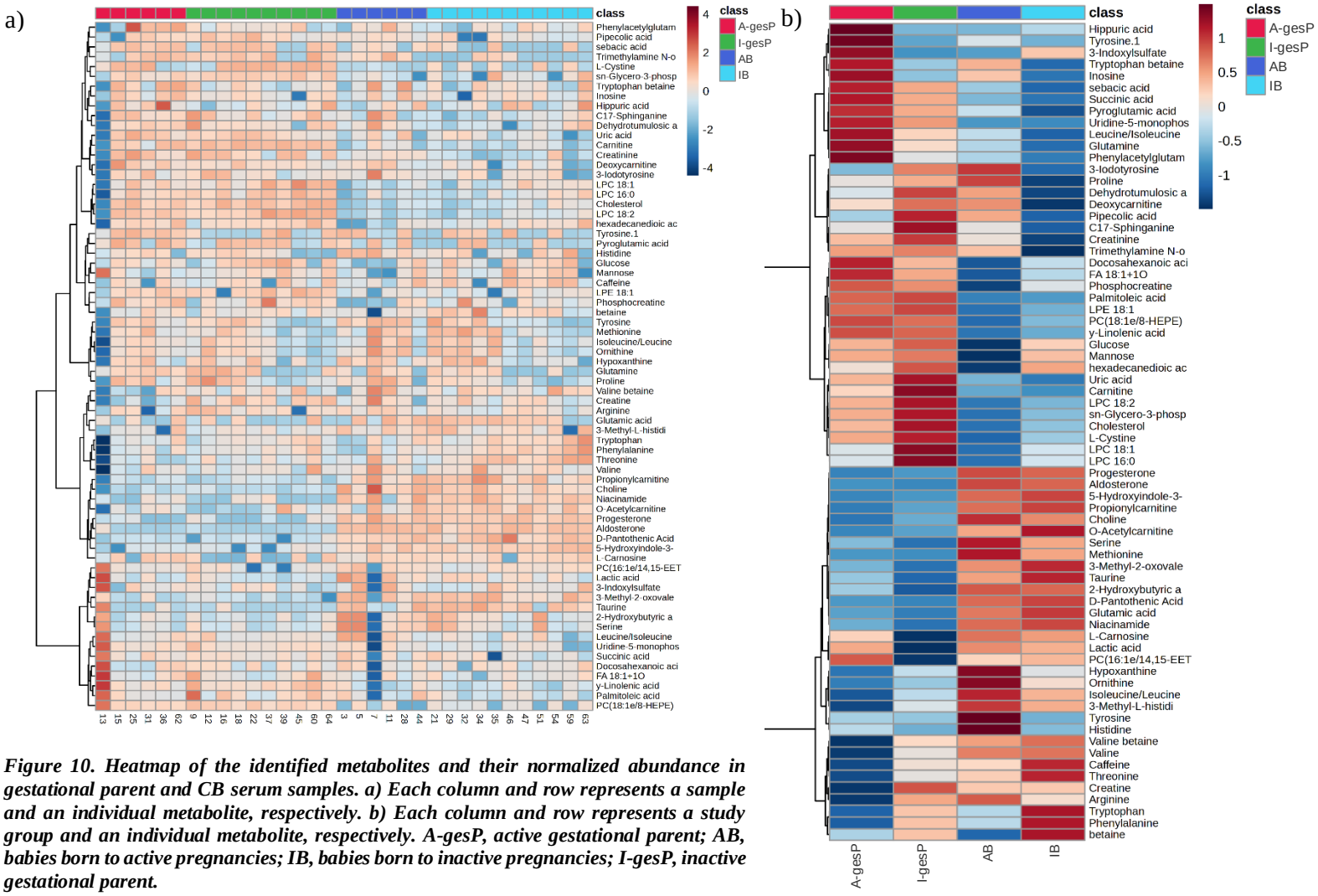


Figure 10. Heatmap of the identified metabolites and their normalized abundance in gestational parent and CB serum samples. a) Each column and row represents a sample and an individual metabolite, respectively. b) Each column and row represents a study group and an individual metabolite, respectively. A-gesP, active gestational parent; AB, babies born to active pregnancies; IB, babies born to inactive pregnancies; I-gesP, inactive gestational parent.

as histidine, tyrosine, methionine, arginine, ornithine, hypoxanthine, and deoxycarnitine were found to be higher in the CB of the ABs.

The PCA was not able to distinguish between the AB and IB CB serum and the A-gesP and I-gesP mid-pregnancy serum. The CB samples clustered together and the gestational serum samples clustered together regardless of the PA status (Figure 11). To further analyze the differences between groups, PLS-DA was employed. The PLS-DA model provided better separation, specifically for the CB samples as presented in Figure 12A. However, clustering persisted among gestational serum samples, irrespective of activity levels. The model was further cross-validated to ensure that we were not overfitting (R2= 0.84, Q2= 0.77). Figure 12B represents Variable Importance in Projection (VIP) scores from the PLS-DA model. Metabolites with

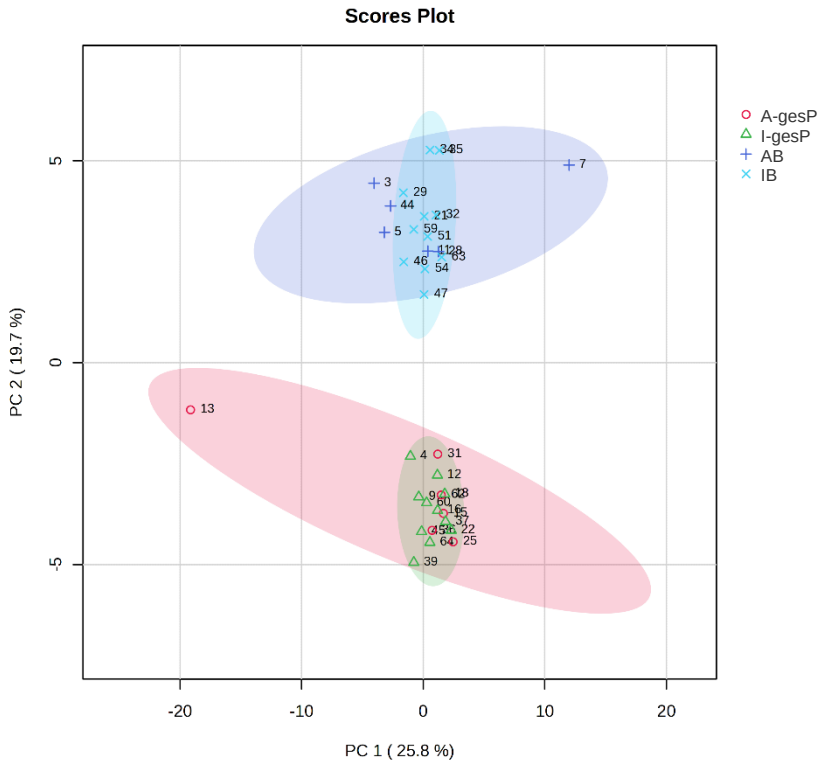


Figure 11. Principal Component Analysis (PCA) score with 95% confidence of CB and gestational parent serum in active vs. inactive pregnancies. A-gesP, active gestational parent; AB, babies born to active pregnancies; IB, babies born to inactive pregnancies; I-gesP, inactive gestational parent.

high VIP scores are more important in driving class separation, while those with lower VIPs contribute less to the separation.

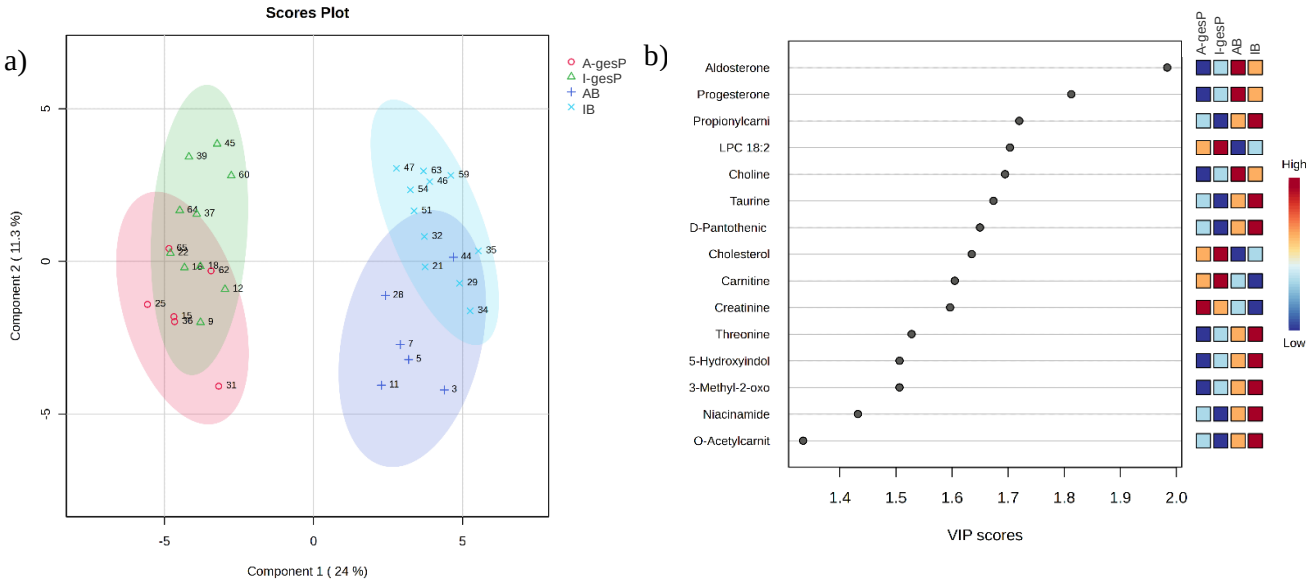


Figure 12. a) Partial Least Squares-Discriminant Analysis (PLS-DA) with 95% confidence of gestational parent and CB serum in active vs. inactive pregnancies (R^2= 0.84, Q^2= 0.77). b) PLS-DA VIP scores. A-gesP, active gestational parent; AB, babies born to active pregnancies; IB, babies born to inactive pregnancies; I-gesP, inactive gestational parent, VIP; variable importance projection.

ROC curve analysis revealed 4 metabolites with an AUC <0.8. Of these metabolites creatinine, and deoxycarnitine were higher in AB, and hexadecanedioic acid, and phenylalanine were higher in IB (Table 6 and Figure 13).

Table 6. Predictive value of metabolites with AUC > 0.8 by ROC analysis in CB serum.

Metabolite	AUC	Specificity	Sensitivity
Creatinine	0.91	1	0.9
Hexadecanedioic acid	0.86	0.7	0.9
Phenylalanine	0.85	0.8	0.8
Deoxycarnitine	0.85	0.7	0.9

Note: AUC, area under the curve; ROC, Receiver Operating Characteristic.

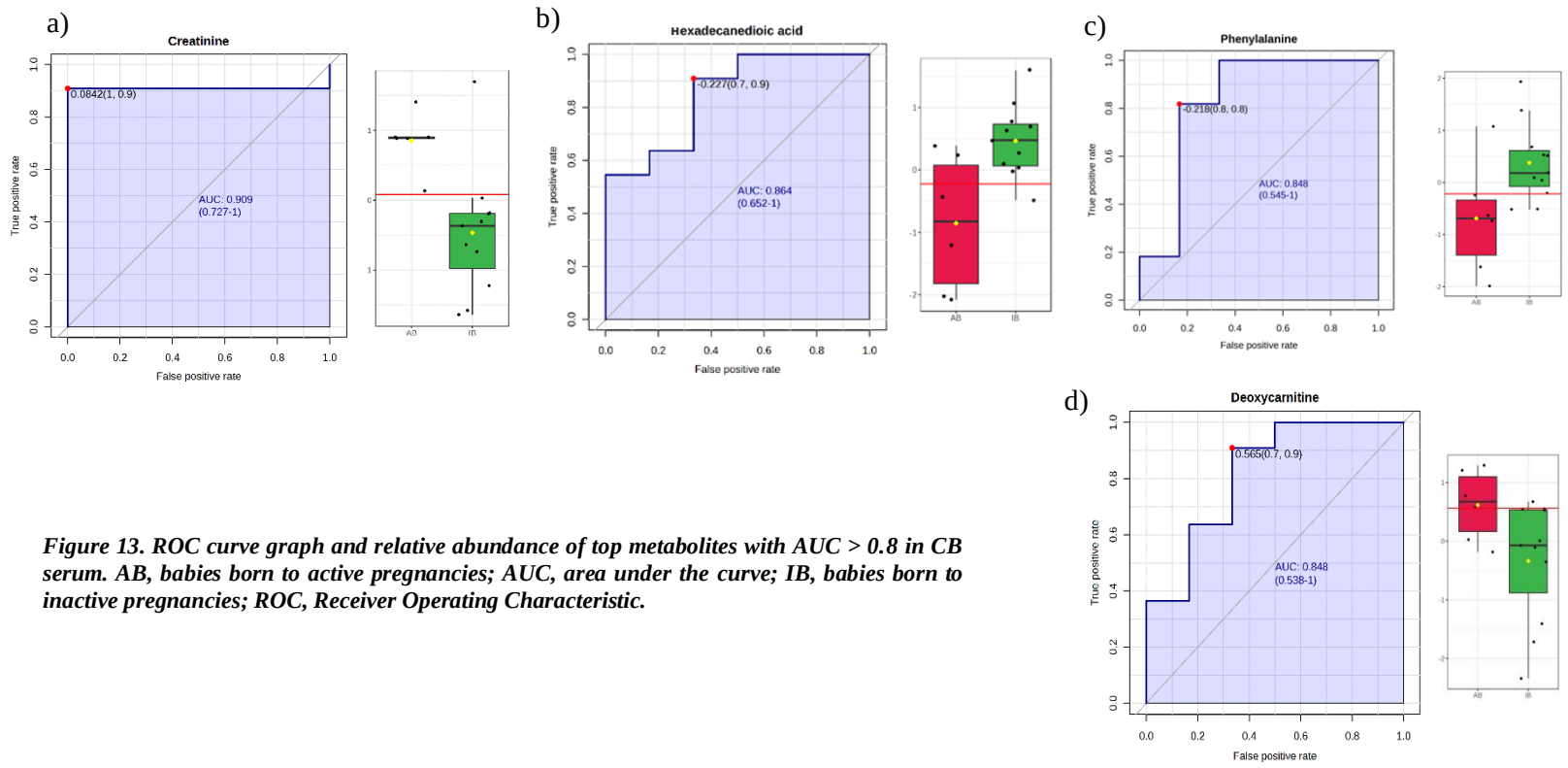


Figure 13. ROC curve graph and relative abundance of top metabolites with AUC > 0.8 in CB serum. AB, babies born to active pregnancies; AUC, area under the curve; IB, babies born to inactive pregnancies; ROC, Receiver Operating Characteristic.

Volcano plots were performed to identify different metabolites in the CB of IB and AB groups. Parametric tests were used for normally distributed metabolites (Figure 14A) and non-parametric tests were used for those without a normal distribution (Figure 14C). At the > 1.2 FC threshold and P value <0.05, 8 metabolites including phenylalanine, threonine, valine, LPC 16:0, LPC 18:2, LPC 18:1, O-Acetylcarnitine, and 5-hydroxyindole-3-acetic acid were identified to be significantly lower in AB compared to IB (Table 7 and Figure 14A and C). Upon employing FDR-adjusted p-value <0.05, threonine remained significantly higher in the IB group (Table 7 and Figure 14B).

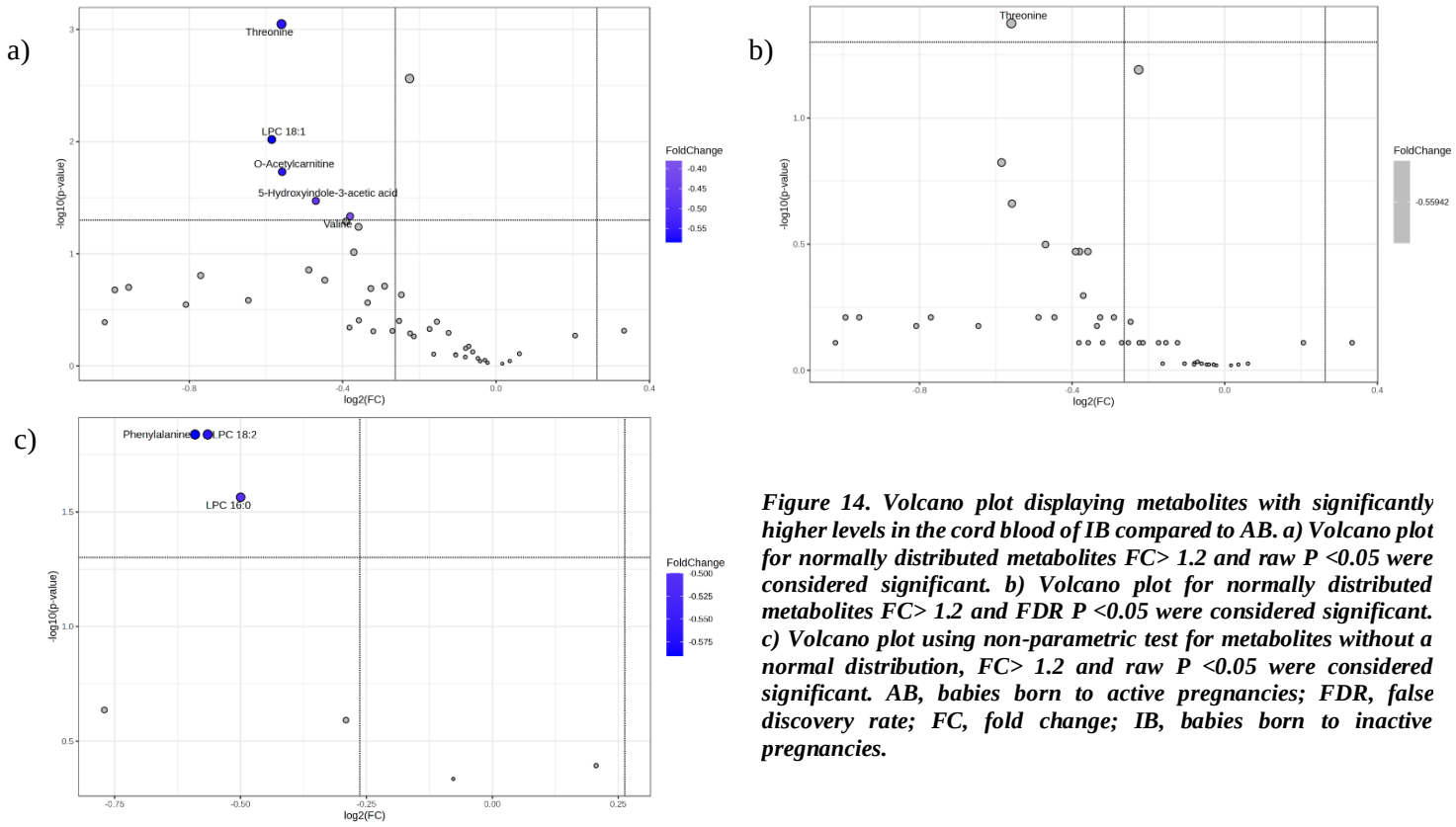


Figure 14. Volcano plot displaying metabolites with significantly higher levels in the cord blood of IB compared to AB. a) Volcano plot for normally distributed metabolites FC> 1.2 and raw P <0.05 were considered significant. b) Volcano plot for normally distributed metabolites FC> 1.2 and FDR P <0.05 were considered significant. c) Volcano plot using non-parametric test for metabolites without a normal distribution, FC> 1.2 and raw P <0.05 were considered significant. AB, babies born to active pregnancies; FDR, false discovery rate; FC, fold change; IB, babies born to inactive pregnancies.

Table 7. The raw and FDR p-values and fold changes used to generate the volcano plot of the significantly higher metabolites in IB compared to AB.

Metabolite	FC	log2(FC)	Raw p value	-log10(raw.p)	FDR p value	-log10(FDR p)
Phenylalanine	0.66	-0.59	**<0.001****	3.38	> 0.05	
Threonine	0.68	-0.56	**<0.001***	3.05	**0.03***	1.5
LPC 16:0	0.71	-0.5	**<0.001****	3.01	> 0.05	
LPC 18:2	0.68	-0.57	**0.01****	2.16	> 0.05	
LPC 18:1	0.67	-0.58	**0.01***	2.02	> 0.05	
O-Acetylcarnitine	0.68	-0.56	**0.02***	1.73	> 0.05	
5-Hydroxyindole-3-acetic acid	0.72	-0.47	**0.03***	1.47	> 0.05	
Valine	0.77	-0.38	**0.04***	1.33	> 0.05	

Note: AB, babies born to active pregnancies; AB, babies born to active pregnancies; FC, fold change; FC, fold change; FDR, false discovery rate; FDR, false discovery rate; IB, babies born to inactive pregnancies; IB, babies born to inactive pregnancies.* p value from parametric test; ** p value from non-parametric test.

2.5. Discussion

The protective effects of PA against birth and pregnancy complications are well documented. Despite this, little investigation has focused on the mechanisms underlying this protective effect. Previous studies have demonstrated that dietary and exercise interventions can lead to metabolome changes in late pregnancy (94, 95). Observational studies too have reported differences in late gestation metabolome profile based on self-reported PA status. To the best of our knowledge, this is the first study looking at the associations between mid-pregnancy metabolome and an objective assessment of the PA status of pregnant individuals (aim 1). This is also the first study investigating the effects of maintaining PA levels from mid to late gestation on the CB metabolome (aim 2).

2.5.1. Gestational Parent Serum Metabolome and Physical Activity

This study did not find a significant difference in the overall serum metabolome profile between A-gesP and I-gesP. This could be attributed to the uncomplicated nature of pregnancies in both groups. Healthy pregnancies tend to exhibit more stable and regulated metabolic profiles, with fewer extreme fluctuations that might be observed in high-risk or complicated pregnancies (142). Without the presence of pregnancy-related complications, the metabolic changes driven by PA might be subtler and less discernible within the overall metabolome profile. To control for possible confounders and isolate the effects of PA, we compared the dietary intakes and different clinical and sociodemographic characteristics between our study groups and found no significant differences other than a higher mid-pregnancy total MVPA, MPA, VPA, and total steps in our A-gesP group. This homogeneity in our cohort and the healthy nature of the pregnancies could explain why an observable change in the overall metabolome was not detected based solely on PA status. Additionally, though not statistically significant, the A-gesP had higher mean intakes for all the macronutrient groups potentially explaining the similarity of BMI and gestational weight gain despite the difference in PA status. While no differences in the overall metabolome were observed, ROC analysis identified two metabolites, pipecolic acid and PC(18:1e/8-HEPE), as potential biomarkers of PA status. Notably, pipecolic acid levels were significantly higher in I-gesP compared to A-gesP.

Pipecolic acid is a by-product of lysine degradation, present in human biofluids (143, 144). While this metabolite is relatively understudied in the context of PA and/or pregnancy, our data is consistent with a previous serum LC-MS analysis conducted on rats subjected to chronic unpredictable mild stress, which found a decrease in pipecolic acid levels following aerobic exercise (145). Conversely, another study reported higher pipecolic acid levels in Caucasian women with greater muscle mass and strength, though the participants' health and PA status were not specified which might explain the conflicting results (146).

While the exact biological roles of pipecolic acid in humans remain unclear, this metabolite has been regarded as a possible neurotransmitter and its accumulation in the brain during development can be potentially neurotoxic. A previous study has shown that induced maternal hyperpipecolatemia in animal models can lead to altered fetal brain growth and reduced postnatal survival (147). Therefore, the lower pipecolic acid levels found in A-gesP may mitigate the potential adverse effects that exposure to high levels of this metabolite can have on fetal brain development.

As pipecolic acid is metabolized within the peroxisomes, its accumulation is also associated with genetic disorders involving impaired peroxisomal function, such as Zellweger spectrum disorders and liver dysfunction (148). Higher circulating pipecolic acid has been also linked to an increase in type 2 diabetes (T2D) risk (149). Peroxisomes are single membrane-bound organelles that are fundamental regulators of energy homeostasis and play essential roles in lipid, and reactive oxygen species (ROS) metabolism. Dysfunction in peroxisomes has been associated with an elevated risk of obesity, T2D, and hepatic steatosis (150).

Given that pipecolic acid metabolism occurs within peroxisomes, our observation of significantly lower pipecolic acid levels in the A-gesP tentatively suggests a potential role of PA in enhancing peroxisomal functions. In support of this hypothesis, several studies have reported a positive association between PA and the expression of peroxisome proliferator-activated receptor gamma (PPARγ), a group of nuclear receptor proteins closely linked to peroxisomal functions (151).

While further investigation of other markers of peroxisomal functions is warranted to conclude a direct causal link, our observation of decreased pipecolic acid levels among A-gesP hints at the possibility that PA might stimulate increased expression of PPARγ. This potential upregulation of PPARγ could subsequently enhance peroxisomal functions, and hence, lead to the observed lower levels of pipecolic acid within our A-gesP group.

PC(18:1e/8-HEPE) emerged as another metabolite with potential discriminatory power between the I-gesP and A-gesP groups, exhibiting a higher, though statistically insignificant, average in I-gesP. This compound is an oxidized phospholipid, a class of molecules produced when cellular phospholipids containing polyunsaturated fatty acids undergo oxidative stress (152). Oxidized phospholipids play several roles in various biological processes, including cell signaling, inflammation, and the pathogenesis of several diseases, particularly cardiovascular conditions like atherosclerosis and thrombosis (153, 154). Elevated plasma oxidized phospholipids are associated with lower levels of HDL cholesterol and have been observed in animal models of T2D (153, 155). In pregnant individuals, higher plasma oxidized phospholipids have been reported in those with preeclampsia compared to healthy controls (156, 157).

The production of oxidized phospholipids can be facilitated by several enzymes, including cyclooxygenase and lipoxygenase, and through exposure to ROS (152). These compounds in general are closely related to oxidative stress and oxidized PCs in particular have been proposed as markers of oxidative stress (158). In non-pregnant populations, habitual PA is known to enhance the antioxidant capacity of the body (159). Regular PA promotes the enzymatic antioxidant defenses such as superoxide dismutase, and glutathione peroxidase, which in turn enhances the body's capacity to scavenge ROS and mitigate oxidative damage (160). Therefore, considering that oxidized PCs can increase due to higher oxidative stress, our results indicating lower levels of PC(18:1e/8-HEPE) as a potential marker of higher PA status likely reflect a greater antioxidant capacity in A-gesP.

2.5.2. Cord Blood Serum Metabolome and Physical Activity

The moderate separation observed between our CB samples of gestational individuals who maintained their PA levels from mid to late gestation can be indicative of a distinct overall CB metabolome profile. Importantly, this separation in CB samples was not attained when classifying the participant only based on their mid-pregnancy PA status (Appendix Figure 1). This result highlights the importance of the maintenance of PA across the pregnancy on fetal metabolism. However, our gestational serum samples of those who maintained their PA levels still clustered together regardless of PA status. To control for possible confounders and isolate the effects of PA, we compared the dietary intakes and different clinical and sociodemographic characteristics of our study groups. We found no significant differences other than a lower placenta weight in ABs. The lack of difference in the birth characteristics of the two groups is in line with a previous study in our lab reporting no differences in the traditional clinical metric such as birth weight, length, and body fat percentage in the babies based on the PA status of their gesP (161). The lower placental weight in ABs is consistent with the literature reporting lower placental weight in pregnancies with higher PA levels (162). A significantly higher mid-gestation carbohydrate, mid- and late-gestation fibre , and late-gestation protein intake was observed in A-gesPs, potentially explaining the similarity in gestational weight gain despite higher PA levels. Although minor, the differences in diet during mid and late gestation, as well as variations in gestational parent BMI, could mask or amplify CB metabolome differences related to the PA status. For instance, higher gestational parent fibre intake can potentially impact the gestational parent gut microbiome and lipid metabolism, which can affect the offspring's metabolism (31). Higher gesP protein intakes have also been reported to be associated with lower levels of hormones and adipokines in the CB such as growth-promoting hormones, leptin, and adiponectin (163, 164). Therefore, it must be noted that these differences in dietary intake can be a source contributing to variation in the CB metabolome profiles.

One notable observation in our PCA and PLS-DA plots was a strong separation between our gesP serum and our CB serum which has been previously reported in the literature (165). This separation may indicate that the CB metabolome is more correlated to the placental metabolism than the gesP. Another

possible explanation for the lack of similarity between our gesP metabolome and our CB metabolome can be the timing of sampling. Given that CB samples were collected at birth the composition might be more comparable to late-gestation serum as the gesP circulating metabolome changes from mid- to late-gestation (38).

Furthermore, several CB metabolites were significantly different between AB and IB. Several metabolites had a high level of discriminatory power in distinguishing between the two groups (IB and AB). Creatinine exhibited the highest AUC from our ROC analysis, with a higher average in AB serum samples. Creatinine is a byproduct of creatine phosphate breakdown in muscle tissue and it is closely related to muscle mass and PA (166). As creatinine crosses the placental barrier and transfers to the fetus (167), the higher serum creatinine in the CB of AB likely originates from the higher PA levels of their birthing parent, and potentially their greater muscle mass compared to inactive individuals (166).

Hexadecanedioic acid, a long-chain dicarboxylic acid, had the second-highest AUC, with a lower average in the AB compared to the IB group. This metabolite is a by-product of fatty acid (FA) ω-oxidation and is metabolized via β-oxidation in peroxisomes (168). ω-Oxidation is a minor metabolic pathway responsible for 5% to 10% of total hepatic FA metabolism. An elevation in this pathway is observed in the cases of increased mono-carboxylic free FA, a characteristic of conditions such as obesity and diabetes or altered β-oxidation (169). The lower level of this metabolite in our AB can be indicative of an ameliorative effect of PA on mitochondrial capacities and FA metabolism.

From the AA class, an aromatic amino acid (AAA), phenylalanine, a branched chain amino acid (BCAA), valine, and threonine were significantly lower in ABs. Phenylalanine also had an AUC > 0.8 indicating potential predictive power in differentiating the IB and AB groups. While physiological levels of AAAs and BCAAs are essential for many biological processes, elevated levels of both groups have a well-established association with obesity, higher insulin resistance (IR), and T2D risk in non-pregnant individuals (170, 171). There is also evidence that higher levels of these AAs can be predictive of future impairment in insulin functions (171, 172). In the context of pregnancy, higher CB phenylalanine and valine have been

positively associated with obesity in birthing parents (103). Higher CB threonine levels have also been reported in babies born from gesPs with GDM (173). Interestingly, higher CB threonine levels have also been linked to higher neonatal adiposity (174). While there was no significant difference in the body fat % between our groups (AB and IB), the reduced threonine levels observed in CB samples could suggest a possible metabolic pathway underlying the established negative correlation between gestational PA and fetal adiposity (126). As previous findings have associated phenylalanine, valine, and threonine with glycemia, insulin function, obesity, and GDM, our data showing lower CB levels of these metabolites can indicate that PA fosters a favourable intrauterine environment, improving pregnancy outcomes for both the offspring.

5-Hydroxyindole-3-acetic acid is a tryptophan-related metabolite presented in significantly lower levels in the AB group. This metabolite is the end product of the enzymatic degradation of serotonin. Previous studies have reported a positive association between this metabolite and c-reactive protein, linking this metabolite to systemic inflammation (175, 176). Higher concentrations of this metabolite were also reported in those with metabolic syndrome (176, 177). Our findings of the lower levels of this metabolite in our AB group might link higher gestational PA to a more regulated fetal inflammatory profile.

From the lipid class, LPC 16:0, LPC 18:2, LPC 18:1, and O-acetylcarnitine were significantly lower in the CB of AB compared to IB. The lower levels of LPCs are noteworthy as a positive association between CB LPCs and birthweight has been previously reported in the literature (128). In addition to birth weight, higher levels of CB LPC 18:1 have been correlated with increased weight at 6 months old (178), suggesting that the PA status of the gesP may influence early growth trajectories and childhood obesity. Additionally, elevated CB LPC 18:2 levels have been linked to higher childhood diastolic blood pressure (131). We theorize that the lower levels of LPC 18:2 in AB is a potential link between PA during gestation and downstream cardiometabolic health in offspring.

O-acetylcarnitine is a short-chain acylcarnitine, a by-product of FA β-oxidation (179). Higher levels of short-chain acylcarnitine can be indicative of suboptimal FA β-oxidation. In non-pregnant populations, higher levels of this metabolite are linked to higher IR (179). Acetylcarnitine has been reported to be

positively associated with CB leptin, an indicator of newborn higher adiposity (174). Consistently, gesP MVPA scores have been previously reported to be inversely associated with CB leptin levels (180). Another study has reported significantly higher levels of acetylcarnitine in the CB of infants with macrosomia compared to normal-weight babies (181). Higher levels of this metabolite in the dried blood spot of macrosomic infants post-birth have also been observed (182). The lower O-acetylcarnitine level in our AB hints at better fetal mitochondrial capacity and hence enhanced FA β-oxidation as a result of the higher PA status of the gesP. This finding also suggests a potential pathway through which PA can protect against macrosomia and affect neonatal adiposity.

2.6. Limitations

The present study is the first of its kind to study associations between objectively measured gestational PA and the serum and CB metabolome in a healthy pregnant population. Nevertheless, several limitations need to be considered in this project. Firstly, the relatively small sample size of the study limits the statistical power, making it challenging to detect small but potentially significant differences in metabolomic profiles between active and inactive individuals and their offspring. A larger cohort would enhance the reliability and generalizability of the findings (183). Another important consideration when interpreting the results from this project is that many of the discussed metabolites lost their statistical significance upon FDR adjustment. Therefore, there is a possibility that the identified differences may include false positives. Given the exploratory nature of our study, these results should be considered as potential metabolites that could serve as a basis for future research and need to be validated in upcoming studies. Consequently, these findings should be interpreted as preliminary and hypothesis-generating rather than definitive evidence of metabolite changes.

Although we compared macronutrient dietary intakes and used fasting serum samples to control for dietary variations, we did not control for dietary patterns or specific food groups that could influence the metabolome (184-186). Given the limited sample size and exploratory nature of this study, we did not control for variations in dietary intake or BMI of gestational parents as covariates in our analysis. Future targeted

studies with larger sample sizes are necessary to further investigate the confounding effects of covariates such as these on the associations between the metabolome and PA. The study focuses on mid-pregnancy serum, which might not provide a complete picture of how PA influences the metabolome throughout the entire gestational period as previous studies have suggested that the gestational metabolome undergoes dynamic changes throughout gestation (187). Including additional time points, such as early and late pregnancy, could offer a more comprehensive understanding of PA's effects over time. Furthermore, the study population lacks diversity in terms of ethnicity, with most participants being Caucasian. Differences in the metabolome based on ethnicity have been noted both in adult populations (188) and CB samples (189). This homogeneity limits the applicability of the findings to broader populations.

It is also important to note that as our study does not provide absolute quantification for the reported metabolites, it cannot be concluded that their levels in our inactive groups (IB and I-gesP) exceed the physiological levels. Lastly, the metabolomic workflow used for this project has its limitations in terms of sensitivity and coverage of the entire metabolome (166). Although great care was taken to ensure precise data measurement, some metabolites might not be extracted, detected, and annotated, which can restrict the comprehensiveness of the study as an untargeted metabolomic study. Additionally, we were able to identify only about 3% of the detected features in the sample using the available libraries. It is important to consider that all comparisons between study groups were based solely on these identified metabolites, rather than the full spectrum of features present in the samples. This limited metabolite identification could influence the interpretation of the results, as potentially important, unidentified metabolites may contribute to differences between the groups. Collectively, while this study provides some of the first data examining the association between PA and the metabolome during pregnancy, these limitations highlight the need for a cautious interpretation of the results and the need for future adequately powered studies to fully elucidate the effects of PA on gesP and fetal health.

2.7. Conclusion

In conclusion, while our study did not show an overall different metabolome profile in gestational individuals with higher PA status based on the identified metabolites, individual metabolites including pipecolic acid and an oxidized PC were identified to be associated with PA status, potentially indicating better antioxidant capacities and peroxisomal functions in our A-gesP. The lack of difference in the overall gesP metabolome profiles can be due to several factors including the homogeneity of our participants, the uncomplicated nature of the pregnancies in our cohort, and our small sample size. The second aim of our study revealed several significantly different metabolites in the CB samples between AB and IB, underlining the potential impact of gestational PA on fetal metabolism. We identified distinct metabolite signatures associated with PA, including alterations in AAs (AAAs and BCAAs), and different lipid species, previously linked to pathways involved in childhood obesity, macrosomia, IR, GDM, and cardiovascular health. These findings underscore the metabolic benefits of gestational PA to the fetus and suggest different mechanisms through which PA can contribute to optimal pregnancy outcomes, gesP, and fetal health.

Chapter 3: Development of an Extraction Protocol for Placenta Tissue Metabolomics

3.1. Introduction

The placenta plays a central role in sustaining new life. This organ of pregnancy is the key regulator of fetal and gesP interactions. As the interface between the growing fetus and the gesP, the placenta is responsible for exchanging nutrients and gases while removing waste from the fetal blood without mixing the gesP and fetal blood (51). Placenta is also metabolically active tissue rather than a simple permeable barrier and it can metabolize different substances such as nutrients, drugs, and other xenobiotics (190). Endocrine capacities are another characteristic of this specialized organ. The placenta produces various hormones and cytokines that are decisive for growth, and immune regulations throughout gestation such as human chorionic gonadotropin, progesterone, estrogen, placental lactogen, growth hormone, and leptin (191).

The success of the pregnancy and the healthy development of the fetus is dependent on placental functions. Placental function is dependent on the formation of its vascular system through vasculogenesis and angiogenesis (190). Placental structure and function such as placental size, villous architecture, uteroplacental blood flow, cytokine production, and nutrient transport efficiency, can be linked to pregnancy and fetal complications such as fetal growth restriction, preeclampsia, and GDM (192, 193).

Many metabolites play a pivotal role in placental formation and development. For instance, BCAAs are essential for embryo implantation and development as they play an important role in enhancing blastocyst quality via the mTOR signaling pathway activation (43). Other AAs such as arginine, its related AA and metabolites, ornithine, proline, and nitric oxide play essential roles in angiogenesis and gene expression essential for placental development (44). Meanwhile, methionine and choline are critical for DNA and RNA synthesis and facilitate essential epigenetic modifications (45).

Several factors can impact the placental metabolome in healthy pregnancies, including but not limited to the sex of the fetus and delivery mode (194). Embryonic and fetal adaptations and their response to environmental stimuli *in utero* are distinct between male and female fetuses. These differences can give rise to sex-specific distinctions in the placental metabolome (194). The delivery mode can also affect the placental metabolome as vaginal delivery is accompanied by higher placental oxidative stress than caesarian section delivery (195). Furthermore, as the placenta has a maternal-facing and fetal-facing side, these different locations also have distinct metabolite profiles (196). Therefore, it is necessary to control these factors when investigating the placental metabolome.

Considering the significant role the placenta plays in fetal health and pregnancy outcomes, it is highly plausible that many of the health benefits of gestational PA are bestowed by enhancing placental functions. Despite the suggestive evidence, most placenta-related studies have concentrated on adverse *in-utero* environments (e.g. GDM, preeclampsia, birthing parent obesity (197-199)), with limited exploration of positive exposures such as higher PA of the gesP that can promote health and reduce disease predisposition (191). Unlike other organs, the adaptive responses of the placenta to PA and exercise remain poorly understood (54). Studies from our lab as well as others, have suggested that PA can enhance placental functions by improving placental vascularization and angiogenesis (200, 201), circulation (202, 203), and nutrient transport (204). The data from these studies suggests that the placenta is indeed affected by the PA status of the gesP. Yet, many aspects of potential placental adaptations to PA, such as the metabolome response, remain unstudied.

The metabolome is the biochemical fingerprint of molecular and cellular metabolism, and it is strongly influenced by external stimuli and environmental exposures (27, 28). One such exposure, gestational PA can potentially alter the placental metabolome through inducing changes in the metabolism of this organ. Mapping out the placenta-specific metabolome changes in response to PA can provide insight into affected metabolic pathways and subsequently contribute to a better understanding of the mechanisms underlying gestational PA health benefits (205).

3.2. Aim and Significance

In the second chapter of this thesis, a gap was observed between the metabolome profiles of the gesP and the CB. None of the metabolites significantly different in our CB groups (AB vs. IB) demonstrated a similar pattern in the gesP serum (A-gesP vs. I-gesP). There was also a strong separation between the gesP and CB overall metabolome profiles. One possible explanation is that the CB metabolome more closely resembles the placental metabolome rather than the gesP metabolome. We hypothesize that the gesP's PA modulates the CB metabolome by inducing changes in the placental metabolism. This hypothesis is supported by literature indicating the placenta of physically active pregnant individuals exhibits reduced placental oxidative stress, lower inflammatory profile potentially linked to variations in Hofbauer cell polarization and CD206 protein expression, and higher levels of vascular endothelial growth factor, an anti-inflammatory protein (206-208). This underscores the necessity of placental metabolome analysis to understand how the gestational PA is communicated to the fetus.

Having access to a cohort that includes matched placental and CB samples, we plan to analyze the placental metabolome profile. Additionally, to further expand the comprehensiveness of our results, we aim to incorporate simultaneous lipidomics (the study of lipid metabolites) and metabolomics analysis within the same samples using a single extraction. Using a single extraction protocol ensures that the metabolomic and lipidomic profiles are derived from the same biological context enhancing the reliability of our comparative analyses between the two datasets.

Although validated extraction protocols are available for similar tissue samples, it is necessary to carefully design a protocol specific to the sample matrix used to obtain robust and reproducible results (113). In this chapter, we present the placenta extraction protocol for a concurrent extraction of lipids and metabolites developed in our lab and discuss the associated challenges.

3.3. Protocol Development

To extract lipids and metabolites simultaneously, I needed to ensure that our protocol used a liquid-liquid extraction method incorporating an organic and an aqueous phase that are immiscible to each other (209). The basis of this method is separating the compounds based on their different solvent solubility and the immiscibility of the two phases. The metabolites with lower polarity, primarily lipids, are more soluble in the organic phase and the more polar metabolites are separated in the aqueous phase (209). The solvent used for the organic phase is commonly chloroform and the solvents for the aqueous phase are usually water and MeOH (209).

One important consideration when using a protocol involving chloroform is the incompatibility of the chloroform and plastic materials. Chloroform can interact with certain plastics, leading to leaching of chemicals or degradation of the plastic (210). This can compromise the integrity of the extraction process and introduce contaminants into the samples and the equipment (211). To address this, we utilized Kimble tubes instead of an Eppendorf and we avoided the use of plastic pipette tips in the instances where chloroform was present. Additionally, we ensured that all chloroform-containing solutions were handled and stored in glass containers. Serological pipette tips were used for adding chloroform to the samples. As for the collection of the aqueous and organic phases, as more delicacy was required pasture pipettes were replaced (Figure 15).

Figure 15. Appropriate Consumables in Protocols using Chloroform. Created with biorender.com

The first version of our liquid-liquid extraction protocol was a modified version of the Bligh and Dyer extraction method (212). Briefly, 50±5 mg of the powdered tissue was separated into an extraction tube, 1.5 mL of ddH_2O was added to the samples. Next, 2 mL of acidified MeOH (1% acetic acid) was added to the extraction tube, and the samples were placed on ice for 30 seconds. Afterwards, the samples were vortexed for 15 seconds and then placed on ice for 10 seconds. This procedure was repeated three times. The samples were then mixed in a water bath at 25°C for 3 minutes at 50 rpm while being kept in a container with ice. Following this, 1.5 mL of chloroform was added using glass pipette tips, and the tubes were vortexed for 30 seconds. The tubes were placed on ice for 1 minute and then centrifuged at 528 x g (2000 rpm) for 2 minutes at 4°C. After centrifugation, the biphasic solution formed has chloroform on the bottom and water/MeOH on the top. The top (aqueous) and bottom (chloroform) phases were carefully collected and transferred into specific collection tubes. The samples were recorded and stored appropriately for subsequent analysis.

One important factor during metabolite extraction is achieving an optimal concentration of metabolites within the sample (213). The main indicator of this dilution is the amount of sample used relative to the final volume of the extraction. This balance is for successful analysis using LC-MS. If the sample is too concentrated, it can overwhelm the LC-MS detector, leading to saturation or detector overload. This saturation can result in distorted peak shapes, reduced sensitivity, and inaccurate measurement of metabolites (213). Additionally, high concentrations of certain metabolites may cause ion suppression or enhancement effects, further reducing the reliability of the data (213). On the other hand, if the sample is too diluted, metabolites may fall below the detection limit, resulting in poor sensitivity and decreased analyte detection. Dilution beyond the desired range can also lead to decreased chromatographic resolution, peak broadening, and reduced signal-to-noise ratios, all of which compromise the quality and reliability of the metabolomic data (213). As different biological samples have different metabolite contents the best dilution for the extracts based on the amount of sample used can only be tested by running the samples in the LC-MS (113). To avoid harm to the LC-MS equipment I started with higher dilutions. An initial analysis of the extracted metabolites without concentrating the extracts (50mg of sample in 3.5 mL of solvent) was performed.

To test this protocol, a pilot extraction and analysis of a placenta sample was performed. MS parameters were set as previously described (2.3.6). The biggest issue encountered was a large amount of protein contamination in the extracted sample (Figure 16). The presence of proteins in the extraction can harm the equipment as the sizes of these compounds are large and can block the column and the lines of the LC-MS and disrupt the instrument's accuracy, precision, and lifetime (214). Additionally, in the presence of proteins, the metabolite(s) will be masked and the LC-MS will not be able to detect them. It was suspected that the use of acidified MeOH was the underlying reason as in the instances of using acidified MeOH, an increase in our centrifuge time to up to 20 minutes was necessary to see the phase separation, and even then, the separation obtained was not ideal. A previous study optimizing an extraction protocol for combined metabolomics, peptidomes, and proteomics reported that an acidified MeOH/water/acetic acid compared to a MeOH/chloroform/water extraction had a higher number of proteins present in the final solvents (215). Therefore, a decision was made to replace the acidified MeOH with regular MeOH and as an additional cautionary measure, a deproteinization step was included in the protocol using an Amicon filter with a 3kDA cut-off point (MilliporeSigma, Burlington, MA USA). This filter would capture the metabolites while removing any particle larger than the 3 kDA cut-off point.

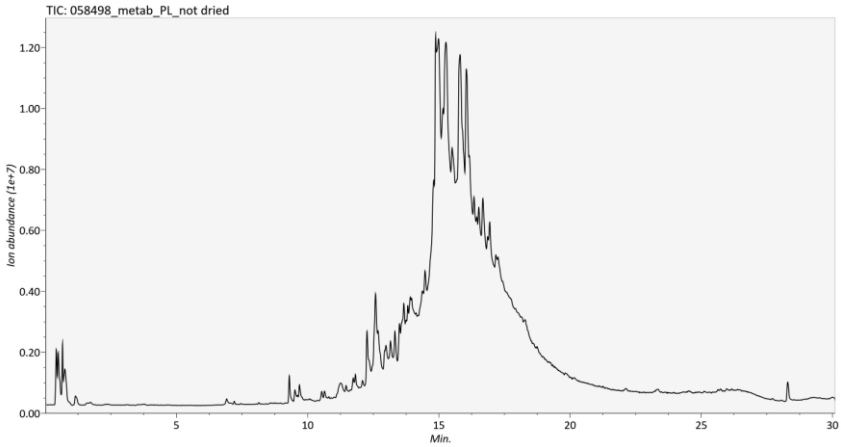


Figure 16. Pilot placenta sample total ion chromatogram using the unmodified extraction protocol.

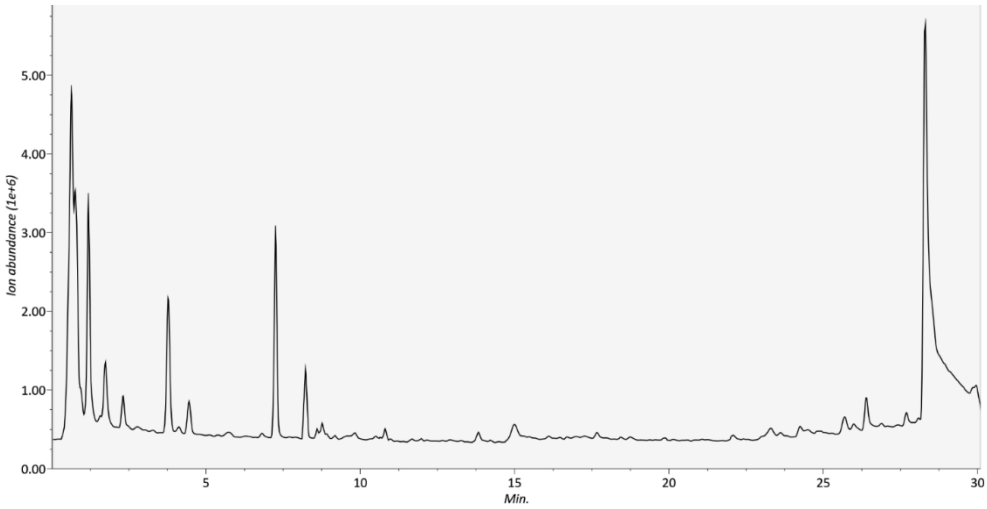
In the next run, the same steps in the initial extraction protocol were repeated, but acidified MeOH was replaced with regular MeOH. Additionally, prior to LC-MS analysis, the samples were filtered by adding 500 µL of the extraction to the filter, followed by centrifugation for 30 minutes at 14000 rpm. The filtered extracts were then transferred to HPLC vials and analyzed by the LC-MS. As a quality control measure, a blank sample was prepared using the same ratio of MeOH and water in the final extraction in the absence of the placenta sample (2:1.5 mL). The blank sample was also filtered down, to control possible contamination from the filters.

The ion chromatogram from the sample included some peaks that could have been representative of the metabolites. Upon analysis of the blank, the same peaks were present in the blank. This pointed to sample contamination related to the filter, potentially due to the incompatibility of the filters with the ratio of the MeOH (57%) in the extraction. These filters are reported to be compatible with ≤60% MeOH levels (UFC500324). Even though the ratio used in the extraction was less than the compatible ratios, the MeOH was still reacting with the material in the filter and causing it to leach into the extraction. Another observation from this run was that the blank and the sample were almost identical to each other, and other than the compounds originating from the filter no other peaks were identified. This indicated a higher-than-optimal dilution of the extraction and the need to concentrate the samples.

To fix the above issues, a drying step was added to the protocol using a nitrogen gas flow. Briefly, after the collection of the aqueous phase, the solvent was dried under a flow rate of approximately 2 psi Nitrogen gas, while placed in a dry bath at 30 degrees to accelerate the evaporation (215). The total drying time for the sample was approximately 3 hours for the solvent to entirely evaporate. After this step, the dried extracts were reconstituted in 500 µL of MS-grade water and then filtered as previously described. These two additional steps resolved the low dilution issue and the incompatibility of the solvent and the filters as the composition of the final extraction was 100% water. Once analyzed, the contamination from the filter was no

longer present in the blanks and we were able to identify up to 93 metabolites in the sample (Figure 17 A and B, Appendix Table 2).

a)



b)

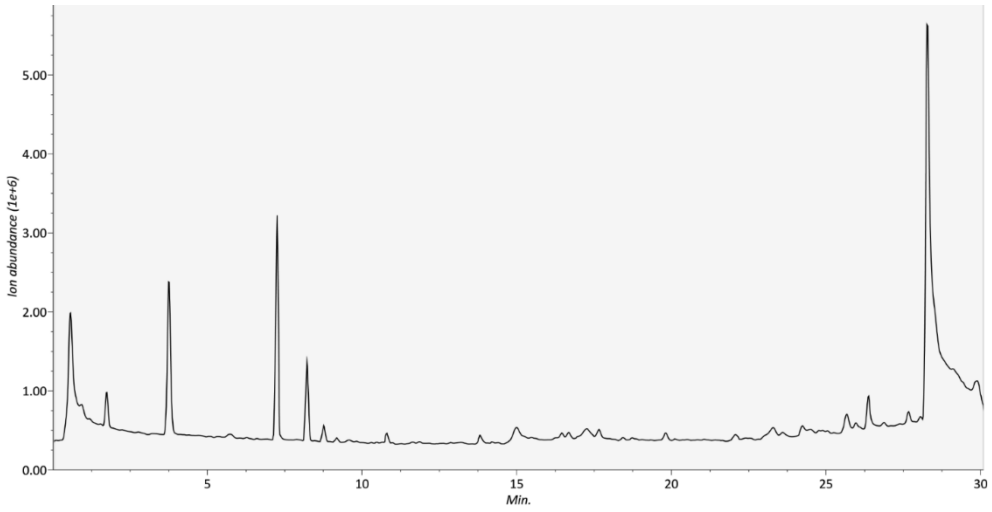


Figure 17. a) Pilot placenta sample total ion chromatogram using the modified extraction protocol. b) Blank sample using the modified extraction protocol.

The final version of the protocol after all the modifications was set as follows: Briefly, 50±5 mg of the powdered tissue will be separated into an extraction tube, and 1.5 ml of MS-grade H_2O will be added to the samples. Next, 2 ml of MS grade MeOH will be added to the extraction tube, and the samples will be placed on ice for 30 seconds. Afterward, the samples will be vortexed for 15 seconds and then placed on ice for 10 seconds. This procedure will be repeated three times. The samples will then be mixed in a cold bath at

25℃ for 3 minutes at 50 rpm while being kept in a container with ice. Following this, 1.5 ml of chloroform will be added using glass pipette tips, and the tubes will be vortexed for 30 seconds. The tubes will be placed on ice for 1 minute and then centrifuged at 528 x g (2000 rpm) for 2 minutes at 4°C. After centrifugation, the biphasic solution formed will have chloroform on the bottom and water/MeOH on the top. The top (aqueous) and bottom (chloroform) phases will be carefully collected and transferred into specific collection tubes. The top phase will be dried under a stream of Nitrogen gas set at 2 psi. The dried extracts will be resuspended in solvent by adding 500 µL of MS-grade water followed by a 10-minute incubation at 30°C and a 1-minute certification at 2000 rpm. Following resuspension, the 500 µL will be filtered down using Amicon Ultra – 0.5mL 3K centrifugal filters by being centrifuged at 14000 rpm. The final filtered extractions will be transferred in an amber glass HPLC vial with 250 µL insert and stored at -80°C until LC-MS analysis.

3.4. Conclusion

In conclusion, this chapter details the development and refinement of a placenta tissue metabolite extraction protocol, tailored to address the unique challenges of our sample matrix. Our initial protocol, a modified version of the Bligh and Dyer extraction method, underwent several critical adjustments to ensure the reliability and quality of our metabolomic data. The importance of achieving an ideal concentration of metabolites, as well as controlling contamination from both the consumables and the sample matrix itself (e.g., proteins), was highlighted. Ensuring the compatibility of the solvent with the consumables was crucial, as it significantly affected the metabolomic data. Likewise, the importance of blank samples throughout the process for identifying potential sources of contamination was highlighted. These refinements were necessary to maximize sample utility, ensure data accuracy, and protect the analytical equipment, ultimately enabling us to produce reliable, metabolomic data.

Chapter 4: Discussion and General Conclusions

4.1. Key Findings

This study provides new information into the associations between gestational PA and the gesP and CB serum metabolome. No significant differences were observed in gesP and birth characteristics in both aims. Regarding dietary intakes, no significant differences were observed in the first aim of the project. As for the second aim, A-gesP only had a higher mid-gestation carbohydrate and fibre intake and a higher late-gestation protein and fibre intake. These similarities in the dietary intakes between groups can indicate that the observed differences are most likely due to the differences in the PA status of the study groups.

While the overall serum metabolome profile did not show significant differences between A-gesP and I-gesP, pipecolic acid and PC(18:1e/8-HEPE) emerged as potential biomarkers of PA status. Pipecolic acid was also significantly higher in I-gesP.

A moderately distinct CB metabolome profile was observed based on the mid- to late- gestation PA status, underscoring the importance of continuous PA throughout pregnancy for influencing fetal metabolism. AB also exhibited significant differences in several key AAs compared to IB. Notably, phenylalanine, valine, and threonine were lower in AB. A metabolite involved in tryptophan catabolism, 5-hydroxyindole-3-acetic acid was also lower in AB compared to IB. From the lipid class, LPC 16:0, LPC 18:2, LPC 18:1, and O-acetylcarnitine exhibited significantly lower levels in the AB compared to IB. Additionally, several metabolites including creatinine, hexadecanedioic acid, phenylalanine, and deoxycarnitine, exhibited a high predictive power for differentiating the CB groups.

This project confirmed a strong separation between the metabolomic profiles of gesP serum and CB, aligning with previous research indicating that the CB metabolome is likely more closely linked to placental metabolism than the gesP serum metabolome (216). This finding underlines the need for analysis of placenta samples in parallel with gesP and CB metabolome to better understand how gestational PA is transferred to

the fetus. To provide the groundwork for this future analysis, this project developed a protocol for placenta sample metabolite extraction. The pilot runs using our extraction protocol highlighted the value of solvent and consumables compatibility, the importance of concentrating the samples, and the potential need for filtration steps while extracting the metabolites from the placenta samples.

4.2. Linking Physical Activity, Gestational and Fetal Health and the Differences in Metabolites: A Possible Role for Higher Antioxidant Capacity and Lower Inflammation?

It is well-established in the literature that following gestational PA guidelines is an effective strategy to protect the birthing parent and fetus against pregnancy and birth complications such as GDM, gestational hypertension, and macrosomia (8). However, the underlying mechanisms through which PA confers these protective effects remain largely unexplored. Metabolites, which serve as the biochemical fingerprint of cellular and systemic metabolism, offer an avenue for uncovering these mechanisms (24).

Herein, we aimed to provide a comprehensive overview of the differences in the metabolome of the birthing parent and the offspring associated with PA to unravel the underlying metabolic responses. The first aim of the study was to examine the relationship between mid-pregnancy gestational PA status and the mid-gestation circulating metabolome of the gesP. The second aim was to investigate how CB metabolites are related to gesP PA status, based on PA data from mid and late gestation.

Contrary to our hypothesis expecting that metabolomic profiles of A-gesP and I-gesP would be distinguishable from each other, the magnitude of the differences was rather small. We only observed one significantly lower metabolite, pipecolic acid, in our A-gesP group. Additionally, PC(18:1e/8-HEPE) and pipecolic acids emerged as potential biomarkers of PA status with higher levels in I-gesP. The results from our CB were more consistent with our hypothesis and we observed several key metabolites were lower in the AB group including phenylalanine, valine, threonine, LPCs, O-acetylcarnitine, and 5-hydroxyindole-3-acetic acid. One possible explanation for the smaller effect of PA on the gesPs' metabolome compared to CB can be

the greater plasticity of the developing fetus's metabolism compared to that of an adult (217). This high plasticity in fetal metabolism can lead to more pronounced metabolic changes upon exposure to PA. While the same intensity of exposure might not be strong or long enough to induce detectable changes in the more stable and tightly regulated metabolome of a healthy pregnant individual.

Many of the significantly different metabolites identified in our study have been previously linked to oxidative stress, inflammation, and mitochondrial capacities. The definition of oxidative stress is "an imbalance between oxidants and antioxidants in favour of the oxidants, leading to a disruption of redox signaling and control and/or molecular damage" (218). Oxidative stress has a dual nature. At low levels, it can have a regulatory effect and act as a mediator in several cellular processes and signaling pathways (219). At high levels though, it can contribute to disease development. High oxidative stress can dysregulate redox-sensitive metabolic and signaling pathways and have toxic effects on biomolecules such as DNA, proteins, and lipids (219). The term inflammation describes the body's defensive reaction involving the activation of the immune system (220). While inflammation is necessary for the immune system, if chronically or incorrectly activated it can lead to an elevation in pro-inflammatory cytokines and interfere with normal metabolism (220). Oxidative stress, inflammation, and mitochondrial functions are interconnected processes. Mitochondria can contribute to ROS generation through the respiratory chain complexes I and III and can also activate and modulate the immune responses (221). ROS from both mitochondrial and non-mitochondrial sources can act as a signaling molecule activating inflammatory responses (222). We propose that our results are indicative of better antioxidant and mitochondrial capacities, and lower inflammation as a result of higher PA status. Therefore, the reported associations can be the underlying factor for PA's contribution to the health of the gesP and fetus.

Regarding the lower pipecolic acid levels in our A-gesP group, we hypothesized that this finding implies a role for PA in enhancing peroxisomal functions, as the catabolism of this metabolite takes place within peroxisomes (148). Better peroxisomal function can likely contribute to lower oxidative stress and inflammation (223, 224). Peroxisomes are one of the main cellular sites of oxygen consumption. Their

involvement in oxygen metabolism was first discovered by De Duve *et al.*, describing a respiratory pathway through which electrons originating from various metabolites reduce O_2 to H_2O_2 and further to H_2O (225). This demonstration of H_2O_2, $O_2^{\cdot-}$, $^{\cdot}OH$ production and the later discovery of various enzymes within the peroxisomes involved in ROS metabolism such as catalase and glutathione peroxidase, suggest that these organelles are involved in both the production and scavenging of ROS (219). PPARs are one of the main peroxisomal protein regulators at the transcriptional level (223). The link between peroxisomal functions and PA can be through the effects of PA on increasing PPARγ expression as reported by previous studies (151). In an animal cell culture model when PPARγ was inhibited a decrease in the activity and expression of catalase, one of the main antioxidant defenses in the peroxisomes was observed (226). PPARγ agonists have been previously reported to inhibit inflammatory gene expression (224). We suggest that the observed decrease in pipecolic acid levels reflects improved peroxisomal functions as a result of PA inducing higher PPARγ expression, ultimately contributing to higher antioxidant capacity and reduced inflammation.

Regarding our observation of PC(18:1e/8-HEPE) as a potential biomarker of PA status with higher albeit non-significant levels in I-gesP, the same hypothesis can be proposed. As oxidized PCs can increase due to higher oxidative stress and under the effects of several enzymes such as cyclooxygenase and lipoxygenase, the lower observed levels can be reflective of a greater antioxidant capacity in A-gesP. Supporting this notion, exercise has been reported to regulate the enzymes involved in the production of oxidized PCs, cyclooxygenase, and lipoxygenase, in animal models. A reduction in the expression of cyclooxygenase-2 enzyme was reported in response to training in rats (227). In another animal study, rats with a low aerobic running capacity exhibited higher cyclooxygenase and lipoxygenase-derived pro-inflammatory factors (228). These data again support the notion that PA can enhance the antioxidant capacity of the gesP and possibly regulate the inflammatory profile.

Moving on to the CB results, the lower levels of valine, a BCAA, in the CB of AB can point to a higher mitochondrial capacity in this group. Many studies have reported that elevated BCAA levels are linked to conditions associated with altered metabolism such as obesity, IR, and T2D (66). A balance between BCAA

appearance and disappearance determines circulating BCAA levels. The origins of BCAA appearance can be from protein breakdown in tissues, food intake, and gut microbial synthesis. The main contributors to BCAA disappearance are protein synthesis, excretion, and BCAA catabolism (66). This diversity in the pathways involved in their balance complicates the identification of specific mechanisms underlying their elevation. While it is possible that an interplay of these pathways defines the BCAA levels in circulation, a loss of catabolic capacity is one of the main proposed contributors to elevated levels (66). Lower mitochondrial capacity can lower BCAA catabolism and lead to higher levels of BCAA in circulation (66). BCAAs are catabolized within the mitochondria under the effects of two important enzymes, BCAA aminotransferases, and branched-chain α-keto acid dehydrogenase (BCKD) complex, with the latter being the rate-limiting enzyme complex. Our findings of lower valine levels in CB serum of AB compared to IB, are consistent with literature suggesting that PA can accelerate BCAA metabolism possibly through an increase in BCKD complex activity (229). Exercise has been reported to also increase the mRNA expression of this enzyme (230). The observed lower valine levels in our ABs propose a higher mitochondrial capacity in the this group which favours lower oxidative stress and inflammation.

As for our observations of lower phenylalanine in ABs, phenylalanine has been linked to mitochondrial functions, oxidative stress, and inflammation. In animal models of hyperphenylalaninemia alteration in bioenergetics, apoptosis, TCA cycle, respiratory chain electron flow, and decreased mitochondrial mass were some of the disruptions induced by phenylalanine (231). Oxidative stress-related markers such as increased lipoperoxidation, disturbances in non-enzymatic and enzymatic antioxidant defenses, ROS generation, and upregulations in gene expression of several proteins involved in inflammation have also been reported in association with induced hyperphenylalaninemia (231). Considering these, the lower CB levels of this AA in our ABs can be indicative of better mitochondrial functions, lower oxidative stress, and a more regulated inflammatory state.

Another important observation in our study was the lower LPCs in the AB group compared to IB. While physiological levels of LPC serve as important signaling molecules, elevated levels play a critical role

in the pathogenesis of atherosclerosis (232). This lipid group has been recognized to have pro-inflammatory properties. LPCs are produced via the action of phospholipase A(2) on PCs. Overexpression and/or enhanced activity of enzymes such as lipoprotein-associated phospholipase A2 (LP-PLA2) can result in elevated levels of LPCs (233, 234). PA can have regulatory effects on this enzyme. A previous study has reported a reduction in LP-PLA2 levels induced by a 12-week exercise rehabilitation intervention in coronary heart disease patients (235). Physical inactivity and sedentary time have been positively associated with LP-PLA2 levels in individuals with obesity (236). These findings are consistent with the observed higher LPC levels in the CB serum of our IB group which can be due to the regulatory effects of PA on the enzymes involved in LPC generation.

Higher LPC levels can disrupt several metabolic pathways in different cell types. In endothelial cells, it can produce prolonged endothelial cell activation, alter chemokine expression, and induce oxidative stress (237). In the liver, elevated levels of LPC can downregulate genes responsible for hepatic FA oxidation (237). The connection between elevated levels of LPCs and altered FA oxidation would be in line with the observed higher levels of O-acetylcarnitine and hexadecanedioic acid in our IB group. As previously discussed, hexadecanedioic acid is a byproduct of FA ω-oxidation, a metabolic pathway that is upregulated in the cases of altered FA β-oxidation. O-acetylcarnitine is also a short-chain acetylcarnitine elevated levels of which have been linked to incomplete FA β-oxidation (179). The lower levels of these metabolites in ABs can suggest enhanced FA β-oxidation. These data are consistent with previous studies demonstrating that PA can promote more efficient mitochondrial FA β-oxidation (238).

The hypothesis suggesting that the observed group differences in our study stem from improved antioxidant status and inflammatory regulation aligns with prior research associating gestational MVPA levels with higher CB serum antioxidant capacity, measured by advanced oxidation protein products and higher paraoxonase-1 activity (239). Paraoxonase-1 activity also has anti-inflammatory properties (240). Additionally, exercise interventions have been shown to result in lower levels of CB pro-inflammatory cytokines such as TNF-α and interleukin-6, providing further support for the proposed link between

gestational PA and improved fetal inflammatory regulation (241). Interestingly, in both of the aforementioned studies, the gesP markers were not different between active and inactive groups (239, 241). This echoes our findings, where we did not observe the same degree of change in the metabolome profile of the gesPs as we did in our CB results. The similar observed results from these studies further reinforce the idea that the differential plasticity between fetal and adult metabolism makes the fetus more responsive to environmental exposures such as PA status of the gesP (217).

We also propose that the CB metabolome might correlate more closely with placental metabolism than the gesP (216). Supporting this notion, higher MVPA has been reported to attenuate the placental oxidative stress in obese pregnant individuals (206). A previous study in our lab also demonstrated a reduced inflammatory profile potentially due to differences in Hofbauer cell polarization and CD206 protein expression in the placenta of physically active individuals (207). Furthermore, our lab reported higher levels of vascular endothelial growth factor protein expression, a protein with anti-inflammatory properties, in the placenta of physically active pregnant individuals (208). A higher antioxidant capacity and lower inflammation of the placenta induced by PA could be the underlying contributors to the observed metabolite differences in our CB samples. However, no study to date has looked into the gesP PA status and its effects on the placental metabolome. These findings highlight the necessity of analyzing placental metabolome in addition to gesP and CB samples to better understand how gestational PA is communicated to the fetus.

It is possible that gestational PA can lower oxidative stress and inflammation in the fetus by upregulating antioxidant defenses and promoting anti-inflammatory responses, as reported in adult populations (242). Contracting muscles produce ROS, as their oxygen consumption increases (243). This increase in O2 consumption leads to an acute increase in ROS levels which acts as a signaling molecule activating antioxidant defenses and other protective mechanisms. Over time, regular PA results in cellular adaptations that include activation of pathways that improve the body's endogenous antioxidant defenses. In non-pregnant populations, it has been observed that habitual PA can increase antioxidant defenses and anti-inflammatory responses (242). The potentially enhanced antioxidant capacity and better inflammatory profile

observed in the AB and A-gesP group may reflect a compensatory response to the acute oxidative stress induced by PA.

Oxidative stress and inflammation can affect neonatal and birth health outcomes. Higher levels of CB oxidative markers and lower antioxidant biomarkers have been repeatedly reported in preterm birth (244). Elevated inflammatory markers in the CB have been linked to higher birth weight z score, body fat percentage, and the risk for large for gestational age babies (245). In the gesP, higher oxidative stress and inflammation are features associated with many pregnancy complications such as obesity, GDM, gestational hypertension, and pre-eclampsia (246). Therefore, by reducing oxidative stress and inflammation, PA protects the gesP and the offspring against these pregnancy complications.

In conclusion, we suggest that following PA guidelines can contribute to a healthier fetal metabolome profile indicative of better antioxidant capacity, lower inflammatory markers, and better mitochondrial function in the offspring. Regarding the gesP, the same intensity of PA exposure might not be sufficient to induce a high magnitude of change in the gesP metabolome. Regardless, the differences in the serum metabolome of gesPs, while minimal, can still suggest a better antioxidant capacity and lower inflammation. While it remains to be fully elucidated, we suggest improvements to antioxidant defenses and a lower inflammatory profile as potential mechanisms underlying the protective effect of PA against pregnancy and birth complications.

4.3. Significance

To better understand the effects of PA on gesP and fetal health, it is crucial to examine their metabolic profiles. Since metabolites are the end products of metabolism, identifying the metabolite profile associated with higher PA levels reveals the metabolic response of the birthing parent and the offspring to PA. This study is among the first to demonstrate differences in the CB metabolome profiles in babies born to active and inactive healthy pregnancies. We also identified a metabolite, pipecolic acid, with significantly lower levels in inactive gesPs. This project highlights the potential for gestational PA to significantly influence fetal

metabolic health and promote a healthier metabolism that can extend into childhood and adulthood and thus underscores the importance of promoting PA during pregnancy as an effective intervention to enhance gestational and fetal health. Our findings lay the groundwork for future targeted metabolomics and multi-omics studies aimed at providing absolute quantification of metabolites and elucidating a comprehensive view of the regulated network of molecular pathways affected by gestational PA. Comprehension of the network of metabolites affected by gestational PA would ultimately guide the development of targeted strategies to improve gestational and fetal health outcomes. As the plausible next step is to characterize the placenta metabolome and compare it with CB, there was a need to develop and test an appropriate placenta extraction method. Subsequently, as part of this set of thesis projects, an extraction protocol has been developed that can be utilized to study placental metabolome response to PA contributing to our understanding of how gestational PA is communicated to the fetus.

4.4. Future Directions

While this study illustrates the association between PA and the metabolome during pregnancy there remains a need for future studies to fully elucidate the effects of PA on gesP and fetal health. It is also essential to explore the placental metabolome response to gesP's PA. The placenta plays a central role in sustaining new life. As the interface between the growing fetus and the gesP, this organ is the key regulator of fetal and gesP interactions (51). Previous studies have reported that gestational PA can enhance placental functions by improving vascularization and angiogenesis (191, 201), circulation (202, 203), and nutrient transport (204). These published findings suggest that the placenta responds to gestational PA, but the metabolomic aspect of this response remains unexplored.

Another critical aspect of the metabolome response to gestational PA is providing a longitudinal, stage-specific metabolome profiling, ideally in the same cohort. Pregnancy is a dynamic period with different gestational and fetal needs in each stage. The metabolism of the gesP undergoes different adaptations to match the supply to the demands in each stage of the gestation (205). These trimester-specific adaptations can lead

to distinct metabolome profiles in each stage, necessitating studying the effects of other stimuli such as PA independently in each stage (205).

Targeted metabolomic studies designed to validate and quantify the identified metabolites and their by-products can be another critical research avenue. Providing quantification of these metabolites would increase the comparability of our results with the literature. Future studies with larger sample sizes should also move beyond the binary classification of active versus inactive, focusing on how varying modes, intensities, and durations of exercise influence gestational metabolome profiles. These targeted studies will enhance the ability to utilize these findings to design practical interventions (e.g., exercise mimetics, personalized PA interventions) for improving gesP and fetal health. This quantification will also make it feasible to study whether the observed changes in different metabolites are within the physiological levels or not, which is an important factor to consider when interpreting the results.

Last but not least, applying a multi-omic approach to investigate the molecular effects of PA on gestational health on a systemic level can provide a more comprehensive understanding of the underlying biological mechanisms of PA health benefits. Multi-omics integrates various omics technologies such as genomics, transcriptomics, proteomics, and metabolomics, allowing for a holistic view of how PA influences gestational health (247). By examining these different layers of biological information simultaneously, uncovering complex interactions and regulatory networks that single-omics studies might miss becomes possible.

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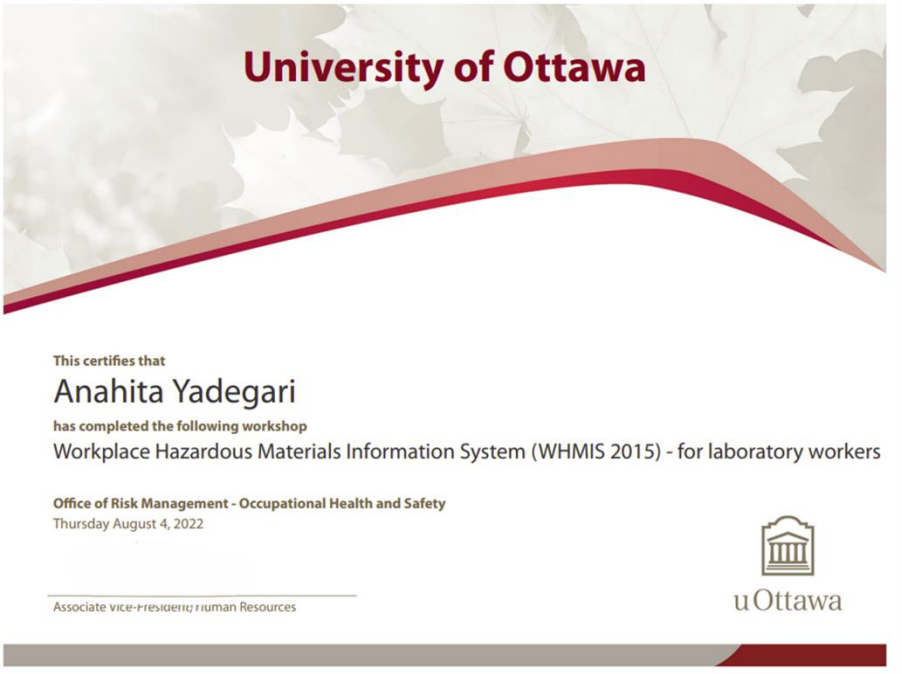
APPENDICES

APPENDIX A: TRAINING CERTIFICATION

A. 1. Lab Safety Training

A. 2. Workplace Hazardous Materials Information System (WHMIS 2015) - for laboratory workers



A. 3. Ethical Conduct for Research Involving Humans (TCPS 2: CORE 2022)

PANEL ON RESEARCH ETHICS
Navigating the ethics of human research

TCPS 2: CORE 2022

Certificate of Completion

This document certifies that

Anahita yadegari

successfully completed the Course on Research Ethics based on the Tri-Council Policy Statement: Ethical Conduct for Research Involving Humans (TCPS 2: CORE 2022)

Certificate # 0000834931 **14 August, 2022**

APPENDIX B: SCHOLARLY ACHIEVEMENTS

B.1. Peer-reviewed articles:

1. da Silva, A. C. R., **Yadegari, A.**, Tzaneva, V., Vasanthan, T., Laketic, K., Shearer, J., Bainbridge, S. A., Harris, C., & Adamo, K. B. (2023). **Metabolomics to Understand Alterations Induced by Physical Activity during Pregnancy.** Metabolites, 13(12), 1178. https://doi.org/10.3390/metabo13121178
2. Mirmiran, P., Estaki, S., **Yadegari, A.**, Golzarand, M., & Azizi, F. (2023). **Adherence to a modified nordic diet and the risk of cardiovascular events in a non-nordic population: a prospective cohort study.** European journal of clinical nutrition, 77(9), 919–924. https://doi.org/10.1038/s41430-023-01325-4
3. Daneshvar, M., **Yadegari, A.**, Hasanzadeh, M., & Djafarian, K. (2023). **Evaluation of Selenium Status among Iranian Pregnant Women: A Systematic Review and Met-Analysis.** 8. https://doi.org/10.18502/jnfs.v8i2.12604
4. Jabbari, M., Pourmoradian, S., Eini-Zinab, H., Mosharkesh, E., Hosseini Balam, F., Yaghmaei, Y., **Yadegari, A.**, Amini, B., Arman Moghadam, D., Barati, M., & Hekmatdoost, A. (2022). **Levels of evidence for the association between different food groups/items consumption and the risk of various cancer sites: an umbrella review.** International journal of food sciences and nutrition, 73(7), 861–874. https://doi.org/10.1080/09637486.2022.2103523
5. Daneshvar, M., **Yadegari, A.**, Ribaldone, D. G., Hasanzadeh, M., & Djafarian, K. (2022). **Zonulin levels in complicated pregnancy: a systematic review and meta-analysis.** Journal of obstetrics and gynaecology: the journal of the Institute of Obstetrics and Gynaecology, 42(7), 2621–2628. https://doi.org/10.1080/01443615.2022.2114822

B.2. Articles under submission:

1. Ana Carolina R. da Silva, **Anahita Yadegari**, Velislava Tzaneva, Ian Han, Cory Harris, Jane Shearer, Shannon A. Bainbridge, Kristi Adamo*. **Gestational Parent and Cord Blood Metabolite Associations with Physical Activity Status.**

B.3. Conferences:

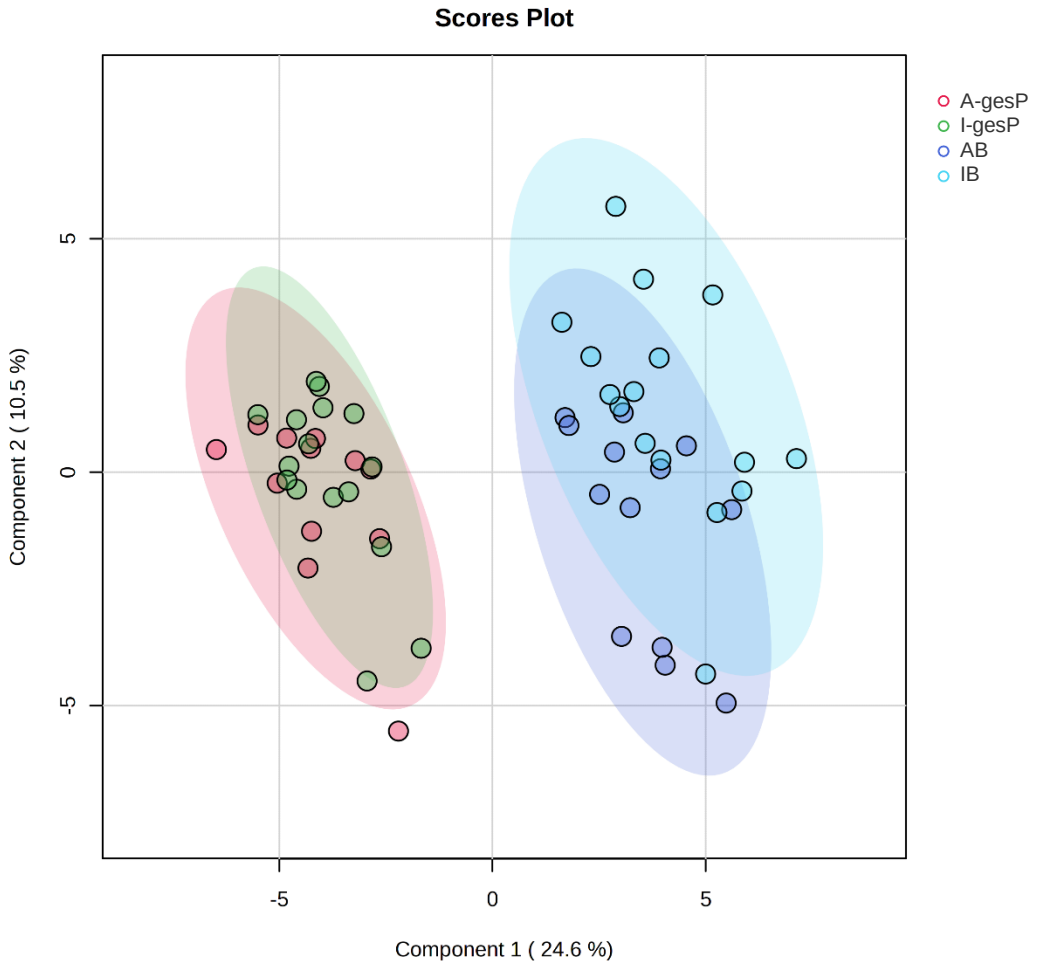
1. **Yadegari A**, da Silva ACR, Tzaneva V, Adamo KB. **A Proposal for Exploring Gestational Parent, Cord Blood, and Placental Metabolite Associations with Physical Activity Status of Pregnant Individuals.** Minds in Motion 2024. McGill University, Montreal, Canada.
2. Ana Carolina R. da Silvaa*, **Anahita Yadegari***, Tarushika Vasanthan, Katarina Laketic, Velislava Tzaneva, Cory Harris, Jane Shearer, Kristi Adamo. **Maternal and Cord Blood Metabolite Associations with Physical Activity Status.** DOHaD Canada May 2023.
3. **Anahita Yadegari**, Mojtaba Daneshvar. **"Asymmetric Dimethylarginine Concentrations in Gestational Diabetes: A Systematic Review and Meta-analysis"**, 3rd National Conference on Interdisciplinary Research in Management & Medical Sciences, 25 February 2022**.**

APPENDIX C: SUPPLEMENTARY DATA.

Appendix Table 1. Annotated metabolites in gestational parents and cord blood serum.

Retention time (min)	m/z	Metabolite name	Adduct	Metabolite Class HMDB
0.671	305.02356	Uridine-5-monophosphate	[M-H2O-H]-	Pyrimidine ribonucleoside monophosphate
0.748	170.09146	3-Methyl-L-histidine	[M+H]+	Histidine and derivative
0.753	175.11887	Arginine	[M+H]+	L-alpha-amino acid
0.77	203.05299	Theobromine	[M+H]+	Xanthine
0.785	132.07761	Creatine	[M+H]+	Alpha amino acid and derivative
0.786	124.0075	Taurine	[M-H]-	Organosulfonic acid
0.827	76.07557	Trimethylamine N-oxide	[M+H]+	Trialkyl amine oxide
0.827	104.1065	Choline	[M+H]+	Choline
0.828	116.07107	Proline	[M+H]+	L-alpha-amino acid
0.83	258.1091	sn-Glycero-3-phosphocholine	[M+H]+	Phosphatidylcholine
0.862	146.04541	Glutamic acid	[M-H]-	L-alpha-amino acid
0.872	162.11525	Carnitine	[M+H]+	carnitine
0.878	118.08672	Valine	[M+H]+	Branched amino acid
0.88	140.06731	Betaine	[M+H]+	Alpha-amino acid
0.882	114.06599	Creatinine	[M+H]+	Alpha-amino acid derivative
0.909	135.00246	3-Iodotyrosine	[M+H]+	Tyrosine and derivatives
0.922	89.02621	Lactic acid	[M-H]-	Alpha hydroxy acids and derivative
0.946	156.07635	Histidine	[M+H]+	Alpha-amino acid
0.954	150.05763	Methionine	[M+H]+	Alpha-amino acid
0.959	169.03503	Uric acid	[M+H]+	Xanthine
0.979	182.08163	Tyrosine	[M+H]+	Tyrosine and derivative
0.98	146.11699	Deoxycarnitine	[M+H]+	straight chain fatty acid
0.983	147.07578	Glutamine	[M+H]+	Alpha-amino acid
1.005	130.08574	Pipecolic acid	[M+H]+	Alpha-amino acid
1.059	133.09976	Ornithine	[M+H]+	Alpha-amino acid
1.059	137.04065	Hypoxanthine	[M+H]+	Hypoxanthine
1.062	132.09938	Isoleucine/Leucine	[M+H]+	Branched amino acid
1.123	130.0451	Pyroglutamic acid	[M+H]+	Alpha-amino acid
1.163	117.01956	Succinic acid	[M+H]+	Dicarboxylic acids and derivative
1.262	204.13255	O-Acetylcarnitine	[M+H]+	Acyl carnitine
1.267	104.0434	Serine	[M-H]-	Alpha-amino acid
1.267	103.04023	2-Hydroxybutyric acid	[M-H]-	Alpha hydroxy acids and derivative
1.272	61.02814	Urea	[M+H]+	Alpha-amino acid derivative
1.34	160.13374	Valine betaine	[M+H]+	Alpha-amino acid derivative
1.492	123.05575	Niacinamide	[M+H]+	Nicotinamide
1.822	120.08244	Threonine	[M+H]+	Alpha-amino acid
1.831	166.09291	Phenylalanine	[M+H]+	Aromatic amino acid
1.887	218.13779	Propionylcarnitine	[M+H]+	Acyl carnitine
2.047	220.10664	D-Pantothenic Acid	[M+H]+	Secondary alcohols
2.351	192.06595	5-Hydroxyindole-3-acetic acid	[M+H]+	Indole-3-acetic acid derivative
2.502	129.05722	3-Methyl-2-oxovaleric acid	[M-H]-	Short-chain keto acids and derivative
2.506	205.10229	Tryptophan	[M+H]+	Aromatic amino acid
2.793	212.00194	3-Indoxylsulfate	[M-H]-	Arylsulfates
3.608	182.07491	Tyrosine	[M+H]+	Aromatic amino acid
3.73	179.05699	Mannose	[M-H]-	Hexose
3.865	180.06572	Hippuric acid	[M+H]+	Hippuric acid
3.954	265.11948	Phenylacetylglutamine	[M+H]+	N-acyl-alpha amino acid
4.092	269.11224	Inosine	[M+H]+	Purine nucleosides
4.092	247.14662	Tryptophan betaine	[M+H]+	L-alpha-amino acid
4.107	181.07314	Glucose	[M+H]+	Hexose
4.576	485.35892	Dehydrotumulosic acid	[M+H]+	

5.571	195.09009	Caffeine	[M+H]+	Xanthine
6.053	241.06813	Triacetin	[M+H]+	Triacylglycerol
6.802	212.0696	Phosphocreatine	[M+H]+	Alpha-amino acid derivative
8.255	227.12566	L-Carnosine	[M+H]+	Hybrid peptides
8.269	225.10207	Sebacic acid	[M+H]+	Medium-chain fatty acid
9.071	241.02831	L-Cystine	[M+H]+	L-cysteine-S-conjugate
9.081	361.20099	Aldosterone	[M+H]+	21-hydroxysteroid
9.522	288.29047	C17-Sphinganine	[M+H]+	Sphingolipid
10.762	325.16287	Tributyrin (C4:0)	[M+H]+	Fatty alcohol
11.159	297.24316	FA 18:1+1O	[M-H]-	Lineolic acids and derivative
11.278	309.1962	Hexadecanedioic acid	[M+H]+	Long-chain fatty acids
11.789	480.30444	LPE 18:1	[M+H]+	Lysophosphatidylethanolamine
11.913	520.336	LPC 18:2	[M+H]+	lysophosphatidylcholine
11.92	496.34039	LPC 16:0	[M+H]+	lysophosphatidylcholine
11.928	253.2166	Palmitoleic acid	[M-H]-	Long-chain fatty acids
12.012	277.21649	y-Linolenic acid	[M-H]-	Long-chain fatty acids
12.184	522.35132	LPC 18:1	[M+H]+	lysophosphatidylcholine
12.368	327.23212	Docosahexanoic acid	[M+H]+	Long-chain fatty acid
12.372	315.23193	Progesterone	[M+H]+	Steroids and steroid derivative
12.829	281.24927	Linoleic acid	[M+H]+	Long-chain fatty acid
13.348	369.35141	Cholesterol	[M+H]+	Steroids and steroid derivative
14.053	840.57446	PC(16:1e/14,15-EET)	[M+Hac-H]-	Oxidized phosphatodylcholine
14.194	866.59064	PC(18:1e/8-HEPE)	[M+Hac-H]-	Oxidized phosphatodylcholine



Appendix Figure 1. Partial Least Squares-Discriminant Analysis (PLS-DA) with 95% confidence of gestational parent serum in active vs. inactive pregnancies (R^2= 0.76766, Q^2= 0.72748). A-gesP, active gestational parent; I-gesP, inactive gestational parent; AB, babies born to active pregnancies; IB, babies born to inactive pregnancies.

Appendix Table 2. Annotated metabolites in the pilot placenta sample.

Metabolite	Retention time (min)	m/z	Adduct	S/N
Histamine	0.418667	112.0869	[M+H]+	98.06255
N1-acetylspermine	0.42775	245.2341	[M+H]+	65.07326
Choline	0.53085	104.1073	[M]+	1884.234
Acetylcholine	0.53085	146.1178	[M]+	599.9579
1-Methyl-hydantoin	0.530933	115.0366	[M+H]+	24.083
Carnitine	0.5645	162.1126	[M+H]+	615.587
5-Aminovaleric acid betaine	0.5645	160.1334	[M+H]+	629.5269
Glutamic acid	0.5681	148.0605	[M+H]+	71.80949
Proline	0.57785	116.071	[M+H]+	52.86246
L-Valine	0.580233	118.0865	[M+H]+	40.12787
Creatine	0.580233	132.077	[M+H]+	57.10519
2-(4-Hydroxyphenyl) Propionic acid	0.580233	189.0737	[M+Na]+	370.359
Sn-glycero-3-phosphocholine	0.594067	258.1104	[M+H]+	1075.347
Adenosine monophosphate	0.608533	348.0709	[M+H]+	2436.075
Assymetrical dimethylarginine	0.611817	203.1393	[M+H]+	265.3517
L-Pipecolic acid	0.6119	130.0865	[M+H]+	45.37614
Acetylcarnitine	0.6242	204.125	[M+H]+	28673.1
Uric acid	0.636817	169.0358	[M+H]+	85.1647
Proline betaine	0.636817	144.1022	[M+H]+	136.3696
Crotonic acid	0.636817	85.02832	[M-H]-	31.31934
NAD	0.637967	664.1172	[M]+	748.8308
Hypoxanthine	0.651433	137.0461	[M+H]+	1818.559
Glutathione oxidized	0.651433	613.1604	[M+H]+	4274.063
Ophthalmic acid	0.654567	290.1353	[M+H]+	95.24649
Methionine	0.654567	150.0587	[M+H]+	62.91357
Nicotinamide	0.672217	123.0554	[M+H]+	41.58239
5-Oxo-D-Proline	0.672217	130.05	[M+H]+	83.05054
Xanthine	0.679117	153.0409	[M+H]+	94.63319
Uracil	0.679117	113.0347	[M+H]+	46.99334
Tyrosine	0.694083	182.0814	[M+H]+	2637.571
3-Hydroxycinnamic acid	0.694083	165.0548	[M+H]+	545.8886
Purine	0.749917	119.0355	[M-H]-	60.62143
Inosine	0.76385	269.0883	[M+H]+	1857.364
Adenosine	0.76385	268.1054	[M+H]+	7419.664
Adenine hydrochloride	0.76385	136.0621	[M+H]+	2492.332
Norleucine	0.780033	132.1022	[M+H]+	1010.364
2-Methylpyrrolidine	0.780033	86.09647	[M+H]+	662.9945
Guanosine monophosphate (GMP)	0.796433	364.0654	[M+H]+	80.62706
Guanosine	0.812767	284.0992	[M+H]+	232.5859
Guanine	0.812767	152.0568	[M+H]+	391.6456
Propionyl-carnitine	0.82645	218.1392	[M+H]+	6120.016
3-Aminophenol	0.827417	110.035	[M+H]+	23.75616
Pro-Gly	0.956483	173.0786	[M+H]+	1070.839
Cyclohexylamine	0.966767	100.112	[M+H]+	11.09316

Quinaldic acid	1.13355	174.0549	[M+H]+	86.09599
Indole	1.139117	118.0651	[M+H]+	30.32923
Kynurenine	1.142433	209.0922	[M+H]+	248.1143
Aniline	1.142433	94.06503	[M+H]+	98.3923
5-Hydroxyindoleacetic acid	1.142433	192.0656	[M+H]+	258.3102
3-Methyl-2-oxindole	1.142433	146.0601	[M-H]-	41.93988
Phenylalanine	1.187983	166.0868	[M+H]+	8227.719
Cinnamic acid	1.187983	149.06	[M+H]+	89.4884
4-Amino-3-hydroxybutyric acid	1.187983	120.0814	[M+H]+	7333.342
2-Hydroxybutyric acid	1.187983	103.0543	[M-H]-	1424.193
3-Hydroxybutyric acid	1.192033	105.0448	[M+H]+	70.26236
2-Piperidone	1.25025	100.0757	[M+H]+	685.7413
Thiabendazole	1.278733	202.0498	[M+H]+	50.0028
Alanyl-Leucine	1.4478	203.1391	[M+H]+	49.95863
L-Leucine	1.452667	132.1025	[M+H]+	35.29208
Pantothenic acid	1.68135	220.1181	[M+H]+	81.67323
Butyryl-carnitine	1.699883	232.1547	[M+H]+	3955.408
N-Acetylarginine	1.745333	217.1052	[M+H]+	2323.013
N6-Succinyladenosine	2.136317	384.1155	[M+H]+	97.14571
Tryptamine	2.296783	159.0916	[M-H]-	25.79475
Creatine	2.296783	132.0805	[M+H]+	12.21263
Betaine	2.296783	118.0654	[M+H]+	7.058352
1,5,5-Trimethylhydantoin	2.296783	143.0732	[M+H]+	16.98413
3-Indoleacrylic acid	2.304133	170.0598	[M+H-H2O]+	68.43515
Tryptophan	2.3193	205.0974	[M+H]+	590.6335
Creatinine	2.787367	114.0915	[M+H]+	64.35252
Theophylline	2.869417	181.0724	[M+H]+	66.29762
5-S-Methylthioadenosine	3.199517	298.0973	[M+H]+	86.63643
Tryptophan betaine	3.844917	247.1446	[M+H]+	71.92744
Valeryl-carnitine	4.117333	246.1704	[M+H]+	369.633
Caffeine	6.782817	195.0882	[M+H]+	61.96675
Leucyl-leucine	6.926084	245.1858	[M+H]+	27.88835
trans-Urocanic acid	7.252017	139.052	[M+H]+	52.60219
Ajmaline	8.222716	327.2019	[M+H]+	994.5674
3-HYDROXY-4-(SUCCIN-2-YL)-CARYOLANE delta-LACTONE	8.450566	319.2024	[M-H]-	24.64997
Aspidocarpine	8.765266	371.2284	[M+H]+	106.5233
Hexanoyl-carnitine	8.916017	260.1861	[M+H]+	590.561
HEPES	9.1031	239.0892	[M+H]+	23.31386
Glutaryl-carnitine	9.4484	276.1265	[M+H]+	38.68567
Octenoyl-carnitine	10.63505	286.2021	[M+H]+	193.1343
Estrone	11.87465	269.1363	[M-H]-	37.63987
Octanoyl-carnitine	11.9805	288.2174	[M+H]+	242.3199
4',7-Dimethoxy-3-hydroxyflavone	12.65082	299.1107	[M+H]+	31.93936
Diethyltoluamide	14.00932	192.1382	[M+H]+	30.1831

Estradiol-17alpha	14.82503	271.188	[M-H]-	37.31852
Thiamine	14.93558	265.1044	[M+H]+	46.86948
3-(5,7-dimethoxy-4-oxochromen-2-yl) propanoic acid	17.57978	279.0993	[M+H]+	23.52887
Asiatic Acid	23.26865	487.3612	[M-H]-	116.4807
Erucamide	28.31082	338.3443	[M+H]+	20268.14