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PLACENTAL UNTARGETED LIPIDOMICS UNVEILS SPHINGOLIPID AND CARNITNE CHANGES IMPLICATED IN ER AND MITOCHONDRIAL DYSFUNCTION IN EARLY-ONSET PREECLAMPSIA

Scientific Disciplinary Sector

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PREFACE

This dissertation entitled "Placental untargeted lipidomics unveils sphingolipid and carnitine changes implicated in ER and mitochondrial dysfunction in early-onset Preeclampsia" is the result of three academic years of PhD in Molecular and Translational Medicine, from 2016 to 2019. This project is part of a wider project made in collaboration with:

-the Department of Biochemistry and the Cambridge Systems Biology Centre of University of Cambridge (United Kingdom),

-the Centre for Trophoblast Research at the Department of Physiology, Development and Neuroscience of University of Cambridge (United Kingdom),

-the Institute of Nutrition of the Federal University of Rio de Janeiro (Brazil),

-the Department of Obstetrics and Gynaecology of the University of Toronto (Canada).

The aim of this project was to investigate the metabolomics profiles of placental tissues of women affected by preeclampsia compared with those of normotensive women.

To whom it may concerns, during my PhD, I studied metabolomics analysis, setting up new analytical methods for the extraction of metabolites and lipids and subsequent mass spectrometry analyses. I also performed the multivariate and univariate data statistical analysis.

The first two years, I worked in Cagliari, Italy, at the Department of Life and Environmental Science where I improved my knowledge in mass spectrometry.

In September 2018, I started an internship at the Department of Biochemistry, University of Cambridge in England where I performed the analysis of lipid and polar profile of placental tissues. The research was challenging, but conducting extensive investigations allowed me to highlight interesting metabolic alterations involved in the pathology. Although these results need more studies to clarify the most important metabolic pathways correlated with the disease, this project, in its current format, can be considered valuable for the investigation of preeclampsia.

I would like to thank my supervisors Professor Alberto Angioni and Professor Jules Griffin for their excellent guidance and support during this process.

I, also, wish to thank Dr. Antonio Murgia and Professor Tatiana El Bacha for the support and the precious help and advice during each phase of development of the project without whose cooperation I would not have been able to conduct this analysis.

I hope that this work would be a valid help for people that are studying pathologies with a metabolomics approach and I hope you will enjoy this thesis.

Carla Lai

ABBREVIATIONS

AA: arachidonic acid

ACVR2: activin A receptor type 2A

ADMA: asymmetric dimethyl arginine

ADP: adenosine diphosphate

AF: asymmetric field

AMP: adenosine monophosphate

Ang II: Angiotensin II

APCI: atmospheric pressure chemical ionization

APPI: atmospheric pressure photo ionization

CCS: collisional cross section

CDP: cytidine diphosphate

CE: cholesteryl esters

Cer: ceramides

CH: cholesterol

CMP: cytidine monophosphate

CSF1: macrophage colony stimulating factor 1

DAG: diacylglycerol

DBP: diastolic blood pressure

DT: drift time

DTIMS: drift time Ion mobility spectrometry

Eng: endoglin

EOPE: early-onset preeclampsia

ER: endoplasmic reticulum

ERAP1: endoplasmic reticulum aminopeptidase1

ERAP2: endoplasmic reticulum aminopeptidase 2

ESI: electro spray ionization

FAD: flavin adenine dinucleotide

FA: fatty acyls

FFA: free fatty acids

GC-MS: gas chromatography-mass spectrometry

GDP: guanosine diphosphate

GL: glycerolipids

GMP: guanosine monophosphate

GNB3: guanine nucleotide binding protein

GP: glycerophospholipids

HELLP: haemolysis, elevated liver enzymes, and low platelet syndrome

HO-2: heme oxygenase-2

HPLC: high-performance liquid chromatography

IFN-ɣ: interferon-ɣ

IGF1: insulin-like growth factor 1

IGF2R: insulin –like growth factor 2 receptor

IL4R: interleukin-4 receptor

IMS: ion mobility spectrometry

IUGR: intra uterine growth restriction

KNN: Key-nearest neighbour

LC: liquid chromatography

LOPE: late-onset preeclampsia

m/z: mass-to-charge ratio

MHC: major histocompatibility complex

MS: mass spectrometry

MS/MS: tandem mass spectrometry

MUFA: monounsaturated fatty acids

NK: natural killer

NMR: nuclear magnetic resonance

NO: nitric oxide

NPTC: normotensive preterm control group

NTC: normotensive term control group

PA: phosphatidic acid

PC: phosphatidylcholines

PCA: principal components analysis

PE: preeclampsia

PEt: phosphoethanolamine

PG: glycerophospholipids

PI: phosphatidylinositols

PL: phospholipids

PlGF: placental growth factor

PLS-DA: partial least square discriminant analysis

ProOH: hydroxyproline

PS: phosphatidylserine

PUFA: polyunsaturated fatty acids

QC: quality control

QqQ-MS: triple quadrupole mass spectrometer

QTOF-MS: Quadrupole Time-of-flight mass spectrometer

ROCK2: rho associated coiled-coil containing protein kinase 2

RT: retention time

SAM: s-adenosyl methionine

SBP: systolic blood pressure

sEng: short form of Endoglin

SFA: saturated fatty acids

sFlt-1: soluble fms-like tyrosine kinase 1

SM: sphingomyelins

SP: sphingolipids

TG: triacylglycerols

TGF-β transforming growth factor Beta

THBS4: trombospondin 4

TLR4: Toll-like receptor 4

TNF-α: tumor necrosis factor α

TW: traveling wave

UDP: uridine diphosphate

UHPLC: Ultra high-performance liquid chromatography

UMP: uridine monophosphate

uNK: uterine natural killer

UPR: unfolded protein response

VEGF: vascular endothelial growth factor

VIP: variable influence on projection

WHO: World Health Organization

ABSTRACT

Preeclampsia (PE) is a one of the most severe obstetrical complications characterized by the presence of hypertension in a previously normotensive woman, along with a variety of damages to specific organs in addition to the hypertension. While documented for a long time, PE represents one of the leading causes of maternal and perinatal morbidity and mortality and its aetiology and pathogenesis are still unclear. This needs to be addressed due to the serious and lifelong consequences that can affect the mother and her baby. In the past decades, metabolomics has proved to be particularly useful in understanding the biochemical mechanisms involved in the pathophysiology of PE and in the identification of biomarkers for its diagnosis. In this work, the polar and the lipid metabolite profile of 22 placental tissues of patients affected by PE compared with those of normotensive patients has been studied by high resolution liquid chromatography coupled with several technological platforms based on mass spectrometry including a triple quadrupole, a quadrupole time of flight mass spectrometer and ion mobility mass spectrometry prior to multivariate statistical data analysis. The results highlight differences in the levels of numerous compounds related to the metabolism of lipids, nucleotides and amino acids and these metabolites discriminate PE samples from samples from normotensive women. Decreased levels of several sphingolipid species belonging to the classes of ceramides and sphingomyelins as well as different glycerophospholipids such as lysoPC and PC in PE samples reflect the structural alteration of the placenta and the dysregulation of different cellular compartments. Impaired mitochondrial function with reduced fatty acid oxidation in PE was demonstrated by decreased levels of long-chain acylcarnitines while increased levels of those with short and middle chain suggest peroxisome fatty acid impairment. Decreased levels of several amino acids and nucleotides support the hypothesis of the activation of unfolded protein response of endoplasmic reticulum resulting in the inhibition of the synthesis of proteins, RNA and DNA. However, despite the significant reduction of the levels of several metabolites, the placenta might activate an adaptive response under stress conditions to modulate the cellular activity and ensure the essential functions for foetal growth including the oxidation of long-chain fatty acids and the creatine-phosphocreatine system. In conclusion, the application of metabolomics to placental samples represent a useful

approach to understand possible pathological mechanisms and a valuable tool for the investigation of the molecular aspects of PE pathogenesis.

1. INTRODUCTION

1.1 Preeclampsia

Preeclampsia (PE) is a heterogeneous, multisystem pregnancy-specific syndrome characterized by the presence of new-onset hypertension in a previously normotensive woman, along with a variety of organ-specific pathologies including malfunction of kidneys, liver and lungs (1). PE is often associated with the onset of proteinuria after 20 weeks of gestation, although it is no longer considered a criteria for diagnosis (1). PE is one of the most severe obstetrical complications and represents one of the leading causes of maternal and perinatal morbidity and mortality (2). It affects 2- 5 % of pregnancies in Europe and North America, whilst its incidence can reach up to 10 % in developing countries due to the inadequacy of prenatal care (3). PE has been strongly associated with an increased risk of later-life death due to cardiovascular disease, independently from other risk factors (4). The most common symptoms of this condition include maternal multi-organ dysfunction, such as renal insufficiency, liver damage, neurological and haematological complications, lung malfunction, utero-placental dysfunction, foetal growth restriction and premature birth, with nearly 40 % of deliveries before the 35th week of gestation (5). Moreover, PE is characterised by abnormal vascular response to placentation that is associated with increased systemic vascular resistance, higher platelet aggregation, activation of the coagulation system, and endothelial cell dysfunction (6). Given the severity of the condition and the associated complications, early diagnostic tools for PE is of fundamental importance. However, to date the delivery of the baby and the removal of the placenta is the only medical remedy (7).

1.2 Historical background

The word eclampsia, which is defined as the manifestation of seizures in women with preeclampsia (1), originates from the Greek "*èklampsis"* and means "lightning", in relation to the unexpected onset of convulsions in pregnant women (8). The first report of eclampsia dates back to 2200 BC in the papyri of ancient Egypt (9), and, despite the

fact that the term eclampsia was not yet specified, it was Mauriceau at the end of the 17th century, which observed that "primiparous women are at far more risk to develop convulsions than multiparous ones" (10). In 1739, enlightened by the disappearance of symptoms after delivery, Bossier de Sauvage made for the first time the distinction between seizure during pregnancy and epilepsy (10).

At the end of the 18th century and through the 19th century, the classifications of preeclampsia and eclampsia became more refined as its classic signs and symptoms became better understood (11). In 1797, Demanet noted a connection between women affected with oedema and eclampsia, while it was P. Rayer in 1840 who described the presence of proteinuria in women who developed eclampsia (11). Three years later, J. Lever recognized the reversibility of the syndrome, specifying that it was a different disease from the glomerulonephritis (12). In the same year, R. Johns reported the connection between predictive symptoms during the later months of pregnancy and the development of convulsions (13). These premonitory symptoms, such as headache, temporary loss of vision, severe stomach pain, and oedema of hands, arms, neck, and face, are the same, which are taught to medical students nowadays. In 1897, the concept of preeclampsia was officially recognised, with the first diagnosis of eclamptic hypertension by Vaquez and Nobecourt (12). In 1896, the 31-year-old Italian physician Scipione Riva Rocci developed the sphygmomanometer, an innovative device for the measurement of blood pressure which became part of the non-invasive clinical diagnosis of hypertension (13-14), and hence also of preeclampsia.

The 20th century was characterised by a widespread interest of the epidemiology of hypertensive disorder during pregnancy (15). The discovery of the differences in trophoblast invasion in pregnancies with pre-eclampsia compared to those without complications dates back to the 1970s, thanks to the discoveries of Brosens and Renaer (16) then confirmed by a number of other studies, including those by Pijnenborg and coworkers (17). In particular, they observed the "shallow implantation": in cases of PE, trophoblast invasion into the decidua is superficial and consequently cause an altered vascular remodelling, in particular when associated with intra uterine growth restriction (IUGR) (17-18). The later examination of placental bed biopsies better highlighted how trophoblast cells fail to deeply invade the decidua and this is thought to cause an incomplete remodeling of the maternal spiral arteries and, consequently, poor utero-placental perfusion and intermittent placental hypoxia. This results in

placental stress, e.g., endoplasmic reticulum, mitochondrial and cytoplasmic stress and impaired placental function, affecting the transfer of nutrients, oxygen to the growing foetus (17-20).

Towards the end of the 20th century, Roberts and colleagues focused on preeclampsia and eclampsia aetiology. They suggested that preeclampsia represented an endothelial disorder and assumed that the ischemic placenta released damaging factors into the maternal circulation. Although the identity of such factors was unknown, they hypothesized them to cause endothelial dysfunctions and activation of the coagulation cascade, blood pressure abnormalities, and loss of fluid from the intravascular space (21).

In 2001, Redamn *et al*. extended the concept of PE as a two-stage disease. Briefly, they suggested the failure of the trophoblastic invasion at the beginning of pregnancy as a predisposing condition of PE, while apoptosis and subsequent systemic inflammatory response due to the release of placental debris in the maternal circulation would trigger the onset of other symptoms. The clearance of these debris from the maternal circulation is usually observed in all pregnant women, but PE develops if their concentration is abnormally high and the systemic inflammatory response is not efficiently counteracted (22). Therefore, the first stage of the disease consists in reduced placental perfusion caused by abnormal implantation and subsequent vascular remodelling, which interacts with maternal genetic, behavioural, and environment factors to produce stage 2 of PE (13).

In 2013 Roberts *et al.*, described the differences between early-onset PE (EOPE) (< 34 weeks of gestation) and late-onset PE (LOPE) (>34 weeks of gestation) and the distinct outcomes related to neonatal morbidity/mortality. One of the most important is foetal growth restriction, often associated with EOPE but not with LOPE (7). At present, it is already known that EOPE is initiated by defective placentation, resulting in release of pro-inflammatory factors by the placenta and endothelial dysfunction, while LOPE syndrome is due to underlying chronic inflammatory conditions like obesity, diabetes or genetic predisposition (23). This distinction between LOPE and EOPE gained the attention of the scientific community, leading to a number of epidemiological studies reporting LOPE as the predominant phenotype with 90 % of cases in developed countries, and around 70 % in developing areas (24).

To date, despite all the available information, the pathogenesis and the aetiology of PE is yet to be fully understood. The most commonly accepted theories refer to mechanisms involving oxidative stress, immunologic intolerance between the foetal-placental and maternal tissue, and angiogenic imbalance (25), as well as the interaction between environmental factors and maternal, foetal and paternal genes appear to play a fundamental role on the occurrence of PE (26-28).

1.3 Epidemiology and risk factors

In 2012, PE and eclampsia ranked as second and third in the world ranking of maternal morbidity and mortality causes (29), confirming the results of an analysis implemented by the World Health Organization (WHO) evaluating the causes of maternal death between 2003 and 2009. This hypertensive disease was found to be responsible for 14 % of the cases in the WHO study, preceded only by haemorrhagic causes, which are responsible for 27.1 % of the maternal deaths (30). Moreover, the WHO estimates that the incidence of fatalities due to preeclampsia is seven times higher in developing countries (2.8 % of live births) than in developed countries (0.4 %) (31), with the absence of prenatal care and the lack of access to quality hospital care being the main cause of maternal mortality in low-income countries (32-33).

The prevalence of the disease differs depending on the country considered: in Europe for instance, the incidence of PE is 2.2 % in the Netherlands, 4.5 % in Norway, and reaches 9.4 % in Finnish nulliparous women aged 35 and over (34-36). Similar differences are seen in African countries: in South Africa, Egypt, Tanzania, and Ethiopia the incidence of PE varies from 1.8 % to 7.1 % (37-40) with the highest prevalence range in Nigeria between 2 % to 16.7 % (41).

The frequency of eclampsia in the developed countries of North America and Europe is lower and more homogenous when compared to preeclampsia, with about 5–7 cases per 10,000 deliveries, while the prevalence in developing nations varies widely, from one case per 100 pregnancies to one case per 1,700 pregnancies (42). In 2005, the highest frequency for eclampsia was described from Nigeria with 99/100 deliveries and the lowest in United Kingdom with 2.7/10000 (43). These geographical differences suggest that African American women have higher incidence of

hypertensive disorders in pregnancy and suffer from more severe complications than Caucasians (44-45).

Apart from the genetic component, several other risk factors for PE have been described, including nulliparity, advanced maternal age, poor socioeconomic status, high body mass index, family history of PE, occurrence of PE in previous pregnancies, multiple type of pregnancy, family history of diabetes mellitus and hypertension, and use of assisted reproductive techniques (46-49). In addition, pre-existing medical conditions increase the risk for hypertensive disorders of pregnancy including chronic hypertension, diabetes, renal diseases, vascular and connective tissue diseases, antiphospholipid antibody syndrome and autoimmune diseases such a systemic *lupus erythematosus* (47-52).

Although cigarette smoking is known to have adverse effects on pregnancy, it has been shown to be a protective factor in PE, possibly due to increased carbon monoxide levels in smokers, which has a vasodilatory effect. Nonetheless, smokers affected by PE have more severe symptoms and poorer outcomes than non-smokers (53-54).

Some researchers have investigated the association of PE with long inter-pregnancy interval, with contrasting results, which suggests the presence of confounding factors (55, 56). One of these is the change of partner, which is often strongly associated with the inter-pregnancy interval and with increased risk of PE (57-58). The role of the father, and especially the age, might be involved in the pathogenesis of PE and the risk rises with increasing age. The possible explanation for these findings is that the sperm can be damaged because of genetic mutations that occur with aging, or from environmental sources such as radiation, heat and pesticides (59-60).

Previous induced or spontaneous abortions in nulliparous and multiparous women have been described as protective factors against the risk of PE in the subsequent pregnancy (61).

Placentation, considered a trigger factor for the disease, has an immunological basis in the interaction between maternal, paternal and foetal genes, and the immune maladaptation on the foetal-maternal interface could be a central mechanism (62). Prolonged exposure to seminal fluid increase the maternal immune system tolerance to paternal antigens decreasing the risk to develop PE. For this reason, the use of condoms compared to the use of other contraceptive methods, which permit the

exposure of sperm with the uterus, is among the risk factors for for developing the disease. This also explains the increasing risk of PE in the case of artificial insemination (63).

Numerous epidemiologic studies show that the relative risk for preeclampsia is increased in patients affected by depression and anxiety as well as stressful situations (64) such as in the working environment, while low-stress conditions, on the contrary, are preventative (65-66).

All these data suggest that preeclampsia and eclampsia constitute a worldwide public health problem to be dealt with and special attention should be given to developing countries.

1.4 Aetiology

Despite several scientific studies conducted recently, the cause of PE is still unclear, but since delivery is the only definitive cure for this disease, the placenta is considered the focus for all manifestations of preeclampsia. Defective placentation and endothelial dysfunction caused by incomplete trophoblastic cell invasion with subsequent incomplete remodelling of maternal spiral arteries are recognised as playing an essential role. Cytotrophoblasts in women with preeclampsia seem to not express vascular-type adhesion molecules, which impair their ability to form sufficient connections with the uterine vessels and to reduce the placental blood perfusion, causing intermittent placental hypoxia (5-6).

Different studies investigating genetic and/or infectious agents as the cause of the pathology have been conducted. It is thought that the presence of bacteria in the placenta could start the release of antiangiogenic factors, like soluble fms-like tyrosine kinase 1 (sFlt-1), and decrease the release of proangiogenic factors, such as placental growth factor (PlGF) and vascular endothelial growth factor (VEGF). This would result in an antiangiogenic state causing endothelial dysfunction, possibly responsible for the clinical manifestations of PE (21, 62, 67-68). Different infectious agents have been found in the blood and serum of PE patients, such as *Chlamydia pneumonia*, *Sneathia amnii*, *Porphyromonas gingivalis*, *Tannerella forsythensis*, and *Eikenella corrodens* (68-71). The possible sites of initial infection reported in these studies are

the respiratory tract, the female reproductive tract and the periodontal cavity (68-71). The role of bacteria in the multifactorial cause of PE was further confirmed with the first characterisation of the microbiome of the placenta by Amarasekara and co-workers in 2014, where several infectious agents, with one or more predominant antigens, were identified in seven women with PE (72). In contrast to the above studies, the absence of infectious agents in venous blood and urine collected at the same time of the delivery suggests that they are confined to the foetal-maternal surface of the placenta.

Genetic studies across generations have demonstrated that the presence of family history of PE might increase the risk up to fivefold, and that a predominant genetic etiological component derives from maternal, followed by foetal or paternal genes (26-28, 73-75). Furthermore, strong associations between PE and gene variants involved in lipid metabolism, thrombophilia, inflammation, oxidative stress and the renin angiotensin system have been identified (75).

In 2007, Goddard *et al.* published a genetic study of PE where six genes, namely insulin-like growth factor 1 (IGF1), interleukin-4 receptor (IL4R), insulin –like growth factor 2 receptor (IGF2R), guanine nucleotide binding protein (GNB3), macrophage colony stimulating factor 1 (CSF1), and trombospondin 4 (THBS4), were found to be related to PE with a statistically significant maternal-foetal genotype interaction (76). Other studies based on large cohorts of affected women suggested that variations in activin A receptor type 2A (ACVR2), rho associated coiled-coil containing protein kinase 2 (ROCK2), endoplasmic reticulum aminopeptidase1 (ERAP1), and endoplasmic reticulum aminopeptidase 2 (ERAP2) genes are associated with PE (75, 77-79). However, these results were refuted by an explorative whole-exome sequencing study, which did not find association between the previously reported genes and PE and reported that genetic markers have a minor potential for the prediction of the disease (80). Nevertheless, at present, several candidate genes for PE involved in various pathophysiological paths have been proposed, but no universally accepted gene variants have been identified (75).

In addition to genetic mark up and placental microbiota, environmental factors such as the maternal diet are implicated in the aetiology of PE. Scholl *et al*. in their prospective study reported that a diet rich of polyunsaturated fat (such as linolenic and linoleic

acid), might be responsible for an increased excretion of the isoprostane 8-iso-prostaglandin F2α, derived from arachidonic acid, a pro-inflammatory compound that present vasoconstriction effect in both the placenta and maternal organs. Isoprostanes are prostaglandin-like compounds formed as a result of non-enzymatic free radical mediated peroxidation of fatty acids. They function as vasoconstrictors in the placenta, as well as in maternal organs and might stimulate the production of endothelin-1 from endothelial cells and initiate platelet activation (81).

1.5 Pathogenesis

To date, PE is considered a systemic syndrome of pregnancy originating in the placenta that disappears after delivery. Considering the moment of the manifestation, PE can be distinguish into the categories EOPE and LOPE but also in "preterm PE" when the onset occurs between the 34th week and the 37th weeks and "term PE" when it occurs after 37th week (82). Preterm PE has typically more severe clinical presentation and it is often accompanied by foetal growth restriction (83). Even though the cause of PE has not yet been fully elucidated, the pathogenesis is thought to occur in two steps related to abnormal placentation and maternal inflammatory response (84-85).

The most commonly accepted theory assumes that PE starts with impaired trophoblastic invasion and failure in the spiral artery remodelling. During normal pregnancy, extravillous cytotrophoblast derived from the foetus invade the decidua and the myometrium, remodelling the maternal uterine spiral arteries. Small diameter, high-resistance arteries are converted into high capacity, low-resistance vessels (17-20, 86). On the other hand, in PE, cytotrophoblast invasion is limited to the decidual portion and does not affect the myometrial segments: spiral arteries remain narrow, and the blood supply to the foetus is restricted (17). This seems to be linked to the lack of expression of vascular-type adhesion molecules in cytotrophoblasts of women with PE, which altered their ability to form sufficient connections with the uterine vessels (17). This defective remodelling and the relative reduction of utero-placental flow leads to placental ischemia (87) and subsequent placental structural damage, ischemic stress, villous trophoblast dysfunction and release of damaging placental factors into

the maternal circulation such as antiangiogenic factors, pro-inflammatory cytokines, and syncytiotrophoblast debris (88-92).

Different studies suggest that alterations in circulating angiogenic factors, which regulate the process of new blood vessels formation from pre-existing ones, play a pathogenic role in PE. In normal placental vasculogenesis, vascular endothelial growth factor (VEGF), its receptor (VEGFR), and PlGF are essential (93-94). The molecular mechanisms related to the altered regulation and signalling contributing to the inadequate cytotrophoblast invasion have been elucidated using *in vitro* models of spiral artery remodelling (95) and animal models, such as mice where deletions in these genes demonstrated to cause defective and early placental vasculogenesis (93-94). The altered vasculogenesis results in a persistent state of underperfusion, which produces placental hypoxia, local oxidative stress, and ultimately results in systemic inflammatory response and endothelial dysfunction, leading to the onset of the clinical symptoms of PE (96). Indeed, placental ischemia leads to excessive production of antiangiogenic factors such a sFlt-1, which binds VEGF and PlGF locally as well as in maternal circulation, acting as a scavenger that prevents the interaction with their membrane receptors placed in the endothelium (97). Increased levels of sFlt-1 and low VEGF/PlGF contribute to the development of hypertension and represent a reliable marker of placental component of PE, used in the clinical practice (98-99).

Other factors released by the placenta act synergistically with sFlt-1 to induce an antiangiogenic environment. Placental ischemia causes placental secretion of endoglin (Eng), resulting in increased levels of its short form (sEng) in the maternal blood which acts as an auxiliary receptor in the transforming growth factor Beta (TGF-β) pathway. In normal pregnancies, TGF-β decreases after the ninth week of gestation promoting cytotrophoblast invasion, while it has been found strongly increased in placentas in PE pregnancies (100). sFlt-1 and sEng levels rise in the serum of preeclamptic women weeks before the appearance of evident clinical manifestations of the disease, and they were found to be correlated with the severity of the syndrome (101-102). Although high levels of sFlt-1 and sEng are widely recognized to be responsible for maternal endothelial dysfunction and contributors to the pathogenesis, the upstream mechanisms of placental damage remain to be elucidated.

In addition, elevated levels of the vasoconstrictor endotelin released from the placenta and an imbalance between the vasoconstrictor thromboxane A_2 and the vasodilator prostacyclin are implicated in PE (103). All these findings suggest that dysregulation of angiogenic factors in the maternal-foetal interface is strictly associated with failed vasculogenesis and impaired placentation (104- 105).

Moreover, over recent decades the immune system, and in particular the defective function of uterine natural killer (uNK) cells, have been suggested to play an important role in the pathogenesis of PE (106). Before the implantation starts, uNK cells are the major constituent of maternal immune system in the endometrium, and are directly implicated with placentation, vascular remodelling of uterine arteries, and angiogenesis (106-107). In 2016, Cavalli *et al.* in their study showed that the treatment of immunocompromised mice with elevated uterine artery resistance with uNK reduced this resistance, suggesting improved placentation (108). Further implication of the immune response in the development of preeclampsia was reported by several genetic studies describing the interaction between maternal natural killer (NK) cells and foetal major histocompatibility complex (MHC) antigens as an initial step in the development of the disease. Particular combinations of maternal NK cells and foetal MHC genotypes are involved in impaired placental growth, and described as being associated with an increased risk of miscarriage and preeclampsia (109).

One more mechanism that seems to be implicated in the pathogenesis of PE is the renin–angiotensin-aldosterone system. Angiotensin II (Ang II) is a peptide mediator that increases blood pressure by signalling arterial vasoconstriction after binding to its receptor. Decidual expression of the gene encoding the angiotensin type 1 receptor has been found higher in PE than in normal pregnancies, which explains why preeclamptic women have increased vascular sensitivity to Ang II and other vasoconstrictive agents promoting utero-placental dysfunction. (75-110-111). Agonistic autoantibodies were described to be able to bind and activate the Ang II receptor (112) and they may be one of the contributors to poor cytotrophoblast placental invasion leading to the production of antiangiogenic factors and endothelial damage. However, the relevance of this finding remains to be elucidated (113).

Considering the oxidative stress that characterize the pathology, it has been reported that the nitric oxide synthase is reduced in PE (75), and two of the several

polymorphisms described, are strongly related with the disease (114). Nitric oxide is an active vasodilator in the foetal placental vasculature where it preserves basal vascular tone and attenuates the action of vasoconstrictors (115). Additionally, decreased levels of the antioxidant heme oxygenase-2 (HO-2) are also associated with placental ischemia (116) which contributes to the increased oxidative stress and the formation of micro-emboli (117).

Regarding the systemic inflammatory response, PE is associated with an increase in pro-inflammatory cytokines such as interferon-ɣ (IFN-ɣ), tumor necrosis factor ɑ (TNF-ɑ), and interleukins, as well as a decrease in anti-inflammatory cytokines (118). Over-expression of placental and renal Toll-like receptor 4 (TLR4) in PE, whose activation induces the production of inflammatory cytokines, is responsible for both placental and renal dysfunctions (119).

In summary, there is no single mechanism that has been identified as responsible for determining the disease, but certainly, the placenta plays the central role. Although the significant advances in understanding the pathogenesis, it remains unclear whether the pathways found to be involved are all interrelated, have synergistic effects, or act independently. We can certainly say that PE is a multifactorial disease, where defective placentation, immune maladaptation, genetic polymorphisms, vascular and endothelial dysfunction, oxidative stress and environmental factors all contribute to the development of this complicated heterogeneous condition.

1.6 Symptomatology

PE is often diagnosed during routine prenatal care, although the interpretation of the maternal symptoms may be challenging because of the wide spectrum of presentation and the time of onset, especially in the first asymptomatic step (120, 121). Depending on the severity of the symptoms, the disorder can be classified as mild or severe, and consequently requires different treatments, particularly at preterm gestations (122).

The key features of PE are new-onset hypertension and proteinuria (123), even though it has recently been reported that women with PE can have minimal or absent proteinuria (1,124). As a consequence of hypertension, fluid retention manifesting as

increasing maternal facial oedema and rapid weight gain is frequently associated with PE and, albeit not unique for this condition, it is prudent to survey affected patients for hypertension and proteinuria (125). Different symptoms might manifest in women with severe PE underlying multisystem morbidity, such as severe headache, visual disturbances (including blindness), right upper quadrant or epigastric pain, nausea or vomiting (126- 128).

Neurological involvement may occur with stroke or reversible ischaemic neurological deficit, cortical blindness, retinal detachment, and posterior reversible encephalopathy (129). Due to angiospasms in the brain and brain oedema, PE can progress into eclampsia, with new-onset of generalised convulsions (130).

Cardiovascular and respiratory complications can include peripartum cardiomyopathy, ischemic and coronary artery heart disease, pulmonary oedema, acute lung injury, and acute respiratory distress syndrome (131).

Liver dysfunction associated with the disease can manifest with haematoma or rupture, while renal involvement includes acute renal insufficiency that might require dialysis (132). One of the most severe variants of PE is represented by haemolysis, elevated liver enzymes, and low platelet (HELLP) syndrome, which is characterised by microangiopathic haemolytic anaemia, hepatic dysfunction, and thrombocytopenia (133). This syndrome is a specific liver-related disorder of pregnancy that was first described by Weinstein in 1982 (134) and refers to the set of clinical and laboratory abnormalities in pregnant women in the third trimester not associated with proteinuria or severe hypertension. HELLP syndrome often has an acute onset, with rapid deterioration of the maternal condition, and one third of the cases arises before 28 weeks of gestation (135).

1.7 Diagnosis

Given the variety and severity of the symptoms involved, the American College of Obstetricians and Gynecologists and the International Society for Studies in Gestational Hypertension suggested that every hypertensive pregnant woman should be investigated for multiple organ involvement (136-137). Recently, the diagnostic criteria for the definition of PE have been updated based on their association with the

adverse clinical outcomes and some pre-existing features have been modified (1). PE was frequently defined as new-onset hypertension in association with proteinuria. Hypertension is diagnosed when either the systolic blood pressure (SBP) or the diastolic blood pressure (DBP) are found to be higher than 140 mm Hg for the former or 90 mm Hg for the latter on two different measurements taken 6 hours apart. Proteinuria is the excretion of at least 300 mg of protein in 24-hour urine collection (122). Proteinuria can also be evaluated as the ratio protein/creatine ≥ 0.3 mg/dL or by urine dipstick test equal to or above 1, although recent studies related to the accuracy of the last method and its prevalence in clinical practice are heterogeneous (138, 139). Nonetheless, given the recently recognised lack of correlation between the magnitude and change in proteinuria and the severity of the disease, proteinuria is no longer considered a mandatory symptom for the diagnosis (1,140).

Severe forms of PE are diagnosed with SBP ≥ 160 mm Hg, DBP ≥ 110 mm Hg (both measured twice at least 15 min apart), and coexistence of at least one of the following conditions (136,137):

- haematological complications consisting of haemolysis or thrombocytopenia, defined as platelet counts less than 100.000/µL;
- either hepatic damage characterized by elevated blood concentration of transaminases (two times above normal levels), pain in the right hypochondrium, or epigastralgia;
- renal insufficiency detected as serum creatinine > 1.1 mg/dL or doubling of serum creatinine level in the absence of any other renal disease;
- pulmonary oedema or cyanosis;
- new-onset of visual or cerebral disturbances;
- neurological complications characterized by scotomas, persistent cephalgia accompanied by hyperreflexia, confused state or eclampsia, and cerebrovascular accident or amaurosis (136-137).

Moreover, PE is strongly related with utero-placental dysfunctions due to the impaired spiral arteries remodelling and it is often associated with foetal growth restriction. Due to these modifications, the spiral arteries maintain their muscular elastic structure, with abnormal blood-flow velocity and resistance in placental vessels (80-82). Non-invasive Doppler ultrasound evaluation can detect this pathological resistance and it is

widely used in clinical practice. As reported in the results of a recent comprehensive meta-analysis, uterine artery Doppler ultrasound preformed in the first trimester is a useful test for the prediction of early-onset PE and foetal growth restriction (141). Changes in the Doppler ultrasound measurements of the umbilical artery, especially when combined with altered measurements in uterine arteries, may be considered as tools for diagnosing PE (141-142). In 2018, Razik *et al*. reported that abnormal platelet parameters combined with abnormal Doppler results are predictors of the severity rather than the rate of development of PE (143). Nevertheless, given the moderate sensitivity of this technique, clinicians and mothers need to be aware of the possibility of not being able to identify women who develop PE later in pregnancy (144). Further studies in this direction may be clinically relevant for the development of prediction models combining Doppler studies with maternal risk factors and analysis of blood biomarkers.

1.8 Therapies and prevention

Considering the complexity of the disease and the results obtained so far, the ability to prevent PE is still limited. Early detection, careful monitoring, and treatment are crucial to reduce the mortality related to this disorder, and even though much progress has been made in the last decades, the decisive treatment remains the delivery of the baby and the placenta. Timing of delivery must be carefully planned based on gestational age, severity of the disease, as well as maternal and foetal conditions. If PE is diagnosed beyond 37 weeks of gestation, induction of labour is the treatment of choice to decrease both mother and foetal death (145). On the other hand, in cases of mild PE occurring between 34-37 weeks, immediate delivery may increase the risk of neonatal respiratory distress syndrome while not reducing occurrence of adverse maternal outcomes (146), hence monitoring of the condition is the safest option. When possible, vaginal delivery is preferable to avoid the added physiological distress caused by caesarean section; if not, regional anaesthesia is preferable, which is generally contraindicated in the presence of coagulopathy, because it entails fewer risks for the mother (147).

Nowadays, the mortality can be strongly reduced and several potential therapeutic agents have been studied and recognised, although worldwide consensus for the management of PE is still lacking. When treatment other than delivery is indicated, the management generally depends on the acuity and severity of the hypertension as well as comorbidities. At this time, the antihypertensive drugs most commonly recommended by international guidelines are hydralazine, labetalol, calcium channel blockers and clonidine (147-149). However, there are no randomized control trials to base the preference for the use of one antihypertensive agent over another. Hydralazine acts as a direct vasodilator of arterioles while labetalol reduces the blood pressure by blocking β- and α-adrenergic receptors. Labetalol has been reported to better preserve uteroplacental blood flow when compared with other β-blockers (150-151). In the acute setting of PE, short-acting parenteral antihypertensive agents like intravenous hydralazine or labetalol are recommended to treat severe hypertension; but because of the possible large shifts in blood pressure that can affect the foetus, patients require intensive monitoring (152). The results of the meta-analysis conducted by Shekhar *et al.* reported that oral nifedipine, a calcium channel blocker, is an appropriate antihypertensive for the treatment of severe pregnancy hypertension of any classification. These treatments are widely available and can be globally employed, even in developing countries (153). Other drugs acting on the cardiovascular system, such as heparin and dalteparin, show promising effects in women with high risk for PE, but the populations they were tested on were too small to draw definite conclusions (154,155).

In order to prevent eclamptic seizures in patients with severe PE, the WHO and the American College of Obstetricians and Gynaecologist supports the effectiveness of treatments with magnesium sulphate in preference to other anticonvulsants. Treatment with magnesium sulphate is usually given from labour to postpartum in both severe PE and eclampsia (156, 157).

A systematic review and meta-analysis conducted by Roberge *et al.* demonstrated that early use of low-dose aspirin reduces with moderate benefits the occurrence of PE, especially in case of severe forms, likely because of its inhibitory effects on thromboxane production (158). Given these significant effects, a low-dose aspirin therapy is considered as primary prevention and is initiated in the late first trimester in women with high risk (159).

In recent decades, several nutritional interventions and changes in life style have been investigated. A number of studies evaluated the effect of calcium supplementation in women with low dietary calcium intake (149), resulting in modest blood pressure reduction in pregnant women with above-average risk for hypertensive disorders when compared with both placebo and no treatment (160).

Considering the involvement of oxidative stress in the disease, antioxidants like vitamin C and E have been tested to evaluate their effect on maternal and foetal outcomes, vitamin C supplementation seems beneficial but vitamin E was shown to be harmful in PE (161). Similarly, administration of the antioxidant drug silymarin did not improve the abnormal cardiovascular parameters in patients with PE (162). Vitamin D deficiency seems to increase the risk to develop the pathology, however advantages of the supplementation is still unknown (163).

Restriction of salt dietary intake and use of diuretics have been studied but no statistically significant differences between controls and patients have been demonstrated. However, the moderation of excessive dietary salt intake remains a healthy dietary practice (164). Furthermore, neither bed rest during pregnancy nor restriction of physical activity have shown efficacy in preventing occurrence of the disease in high-risk women (165,166).

The data reported so far indicate that both behavioural and pharmacological approaches are unfortunately not very effective in prevention and further studies are needed; however, a healthy lifestyle aimed for example at monitored weight gain and reduction of stress during pregnancy is recommended.

1.9 Metabolomics

The word metabolism originates from the Greek "*μεταβολή*", which means "change". The importance of changes in biological fluids was already known by both Ancient Chinese and Ayurveda Hindus cultures around 1500–2000 BC which used urine as source of health-related information, such as its sweet taste as indication of the not-yet-identified diabetes mellitus (167). Even in modern times, changes in the smell or colour of urine are known to be related to changes in the concentration of chemical components and dysregulation of biochemical pathways that indicate certain metabolic

diseases. The first concept of metabolism dates back to the 13th century with Ibn al-Nafis, who stated: "the body and its parts are in a continuous state of dissolution and nourishment, so they are inevitably undergoing permanent change" (167). At the beginning of the 20th century, the discovery of enzymes by German chemist Eduard Buchner opened the focus on intracellular chemical reactions and inspired the development of the field of biochemistry. Thanks to several technological developments of that century, which yielded new insights into enzymatic reactions and intracellular biochemical pathways, the biochemical understanding of metabolism developed rapidly (168). Towards the mid-20th century, Williams and colleagues suggested that each individual might have a different biological fluid composition depending on their health condition, as exemplified with the use of paper chromatography on biological fluids of patients with schizophrenia and alcoholism (168). Horning and his co-workers coined the expression "metabolic profile" several decades later when they first demonstrated that chemical compounds present in human samples such as urine or extracts from tissues were detectable by gas chromatography coupled with mass spectrometry (169).

Metabolomics is a relatively recent science focused on endogenous and exogenous small molecules, and is one of the -omics sciences along genomics and proteomics (170). Genomics studies the entire genomes, including the complete set of genes, their nucleotide sequence and organization, and their interactions within a species and with other species. Proteomics refers to the global analysis of proteins with the aim of elucidating their roles made from combining the 22 genetically-encoded amino acids and also to investigate the differentially expressed proteins in a system (171). The term metabolomics was first coined in the end of the nineties to describe the science focusing on the use of analytical techniques to identify and quantify the entirety of metabolites (i.e. the metabolome) present in organisms, tissues, cells, or biofluids (170). *In vivo*, the quantitative measurement of the metabolic response to the physio-pathological stimuli or genetic modification was distinguished as metabonomics (172) but in fact, these two terms metabolomics and metabonomics are used indistinctly in the specialized scientific literature (170).

Metabolites are the intermediates or final products of all the chemical reactions taking place in the complex system of biological processes within an organism, hence representing its physiological and pathological state. Metabolites are a wide range of

compounds with high degree of diversity in their chemical properties, molecular weights (typically < 1500 Da) and large variations in concentration. They can be polar or nonpolar, as well as organic or inorganic molecules. Within metabolomics, lipidomics represents the subfield focusing on the least polar compounds, i.e. lipids. Because of their diversity, the chemical separation and detection of metabolites are challenging technical steps in metabolomics. For this reason, as well as for the diversity of the biological samples studied, a careful evaluation of the most appropriate sample preparation, extraction procedure and analytical tools to be used is required at the beginning of each analysis in order to obtain relevant and comprehensive information. The biological samples used to study human pathologies are very complex and include plasma, serum, urine, tissue, cerebrospinal fluid, hair, saliva, stool, and exhaled breath (173).

The major analytical platforms currently employed in metabolomics are mass spectrometry coupled with either gas or liquid chromatography (GC-MS or LC-MS, respectively), and nuclear magnetic resonance (NMR) spectroscopy. Although these methods are able to recognize hundreds of metabolites, characterisation of the complete metabolic profile is not possible even when using a combination of all (174). Each one of these methods has its advantages as well as it shortcomings, and the utilisation of one over the other needs to be evaluated considering a number of factors. NMR spectroscopy is an acclaimed technique thanks to both its high reproducibility, which allows comparisons between experiments performed in different laboratories around the globe, and its ability to provide structural information of small organic molecules, which enables metabolites identification (175). However, it is not as sensitive as MS-based techniques, requiring larger amounts and highly concentrated samples. On the other hand, MS analyses are very sensitive and permit the detection of metabolites present at low concentration in biological matrices (175). Despite its high resolution, which allows characterisation and identification of biomarkers, direct analysis by GC-MS is limited to nonpolar and volatile molecules. Chemical derivatization can overcome this limitation and allows the analysis of many interesting classes of compounds, including sugars, nucleosides, amino acids etc., but at the same time, it might introduce high variability and possible losses during sample preparation (175). Conversely, LC-MS can be directly applied to the identification of a vast number of compounds, being the most applied MS-based tool for the analysis of both

polar and nonpolar metabolites, as demonstrated by the rapid and continuing increase of publications using this technique.

The improvement in sensitivity and selectivity of these analytical techniques generates highly complex data, with an ever increasing number of signals detected in each sample. In the case of LC- and GC-MS analysis, data generated from each acquisition contains information regarding mass-to-charge ratio (m/z), retention time (RT) and intensity of each detected species. This massive amount of data is produced in the analysis of every sample, requiring specific and standardised approaches for their study and interpretation (176).

In general, metabolomic analyses can be classified as targeted or untargeted, each with their own advantages and limitations. Targeted analyses focus on a specific group of known metabolites usually allowing identification and quantification. In contrast, untargeted metabolomics focuses on all detectable metabolites in a sample, including chemical unknowns, in order to obtain patterns or fingerprints (177).

Because of the complexity of the data, both univariate and multivariate statistical analyses are normally used to extract relevant information and aid the biological interpretation of the condition and/or system studied. Univariate methods consider only one variable (metabolite) at a time, while multivariate methods comprise all variables at once, showing the relationship among metabolites. Among the multivariate statistical analysis methods, principal components analysis (PCA) and partial least square discriminant analysis (PLS-DA) are the most commonly used in metabolomics, mainly for untargeted analyses. These methods are dimension-reduction tools very useful to understand the chemical processes underlying biological changes in complex matrices in large datasets (178).

1.10 Preeclampsia metabolomics state of the art

In the past decades, metabolomics has proved to be particularly useful in understanding the biochemical mechanisms involved in the pathophysiology of PE and in the identification of biomarkers for its diagnosis. The literature published to date suggests that metabolomics approaches may help distinguish the metabolic profiles of

women with PE when compared with both healthy pregnancies and other pregnancy complications (179). As confirmed by Benton *et al.* in 2016, investigation of the metabolome during pregnancy has the potential to provide new insights into the physiology of healthy pregnancies across trimesters as well as the pathophysiology of pregnancy complications including not only of PE but also IUGR, preterm birth and gestational diabetes. Nonetheless, homogeneity during sampling is fundamental in order to avoid divergent metabolic profiles caused by potential confounders like differences in gestational age and pre-existing clinical conditions such as obesity, metabolic syndrome or diabetes (180).

The application of metabolomics to PE has the potential to obtain more information on the pathogenesis of the disease which will then eventually help its prevention, early diagnosis and distinction between the different subtypes. Over the past decades, several independent scientific studies have been conducted using different analytical platforms discussed above. Maternal plasma and serum are the most commonly analysed samples, followed by urine, placenta and hair. The use of hair as a biological sample during pregnancy has attracted international attention due to its non-invasive nature and because it provides an overview of the effects from long-term exposure of known exogenous teratogens such as tobacco, alcohol, and drugs (180-182).

These metabolic studies highlighted several metabolite classes to be involved in PE, including free fatty acids, phospholipids (PL), carnitines, amino acids and eicosanoids (180-183). Among the amino acid class, decreased taurine levels were found to be correlated with PE in two different metabolomics studies performed in both serum and placenta samples (183-184). Taurine is implicated in human trophoblast differentiation and survival in the healthy placenta and has important osmolyte, anti-oxidative and cytoprotective properties. It is hypothesised to play a regulatory role in trophoblast invasion and spiral artery remodelling, with vaso-protective and anti-hypertensive activities (185). Conversely, an increase in the amino acid histidine, along with a decrease in lactate, rise in triglycerides, lipids, phospholipids, and fatty acids in both serum and plasma were also associated with high risk of PE (183,185-188). Increased risk of PE was also associated with high levels of urinary hippurate, which has long been correlated with the microbial degradation of certain dietary components, hepatic function and toluene exposure, and is commonly used as a measure of renal clearance (189). However, two years later an inverse correlation between hippurate and PE was

found, leaving the implication of this molecule on the development of PE uncertain (186).

Alterations in lipid metabolism and signalling system in PE was highlighted by several studies. In 2016, Brown *et al.* published a comprehensive, quantitative lipidomics analysis of human placenta. The results showed a higher concentration of neutral lipid content (triacylglycerols and cholesteryl esters) in PE than in healthy placentae (190), with a different placental profile than what observed in the previous study published by Korkes *et al.* In their study, glycerophosphoserines was the prevalent lipid class in placental samples followed by macrolide and lactone polyketides, both increased when compared with control group (191). Decreased levels of fatty acids, isoprenoids, steroid, vitamin D and amino acids and their related metabolites, as well as increased levels of phospholipids were found on placental tissue by Dunn and colleagues (192). The same year, Schott *et al.* reported no differences in total amount of phospholipids, but decreased levels of 2-acyl-lysophosphatidylcholine and phosphatidylinositol in placenta from PE pregnancies when compared to healthy controls; however, the relationship between these changes and with malfunction in membrane and/or phospholipid metabolism remains unclear (193). Lipid peroxidation and altered lipoprotein concentrations have been linked to endothelial dysfunctions and oxidative stress and although with discordant results, accumulation and secretion of acylcarnitines in plasma and serum was reported to be associated with dysregulated mitochondrial fatty acid oxidation in PE (187,189,194).

Different studies investigated the different subtypes of PE. Despite the recognised differences in the pathophysiology of EOPE and LOPE, a large number of metabolites appeared to be associated with both subtypes. Altered blood and urine levels of carnitines, glucose, pyruvate, 3-hydroxyisovalerate, and their related metabolites were associated with both subtypes when compared to normal pregnancy (181,195). Glycerol, isopropanol, and trimethylamine were also associated with both subtypes but with different effects depending on the classification, as also reported by Bahado-Singh and colleagues (195-196). Some authors linked also increased levels of creatinine, alanine, and phenylalanine with PE, while glycine, glutamate, and glutamine levels were inversely related (197,198). On the other hand, variation in the levels of different metabolites were identified as possible first-trimester biomarkers which could predict future development of EOPE, such as increased levels of

propylene glycol and 3-hydroxyisovalerate, and decreased levels of formate, choline, 3-hydroxyisovalerate, succinate, phenylalanine, glycerol, glycine, glucose, isopropanol, and acetate (196). Increased levels of 3-hydroxyisovalerate were also validated in three more studies based on serum (183,193,199).

Although the large number of scientific studies published so far has helped understand the pathogenesis of PE at the molecular level, a comprehensive profiling of the placenta, using both untargeted and targeted approaches to identify polar and lipid metabolites, has yet to be achieved. This comprehensive approach will certainly help the search for reliable biomarkers and their timely translation into the clinical setting.

2 AIM OF THE WORK

My Ph.D. study is part of a larger project aimed at improving the knowledge of the pathophysiology of PE through the use of multidisciplinary approaches such a metabolomics, immunohistochemistry and immunofluorescence. This thesis is focused on the metabolomics part of the project and is made in collaboration with:

-the Department of Biochemistry and the Cambridge Systems Biology Centre of University of Cambridge (United Kingdom);

-the Centre for Trophoblast Research, Department of Physiology, Development and Neuroscience of University of Cambridge (United Kingdom);

-the Institute of Nutrition of the Federal University of Rio de Janeiro (Brazil);

-the Department of Obstetrics and Gynaecology of the University of Toronto (Canada).

As previously reported, although PE has been documented for a long time and studied widely, its aetiology and pathogenesis are still unclear and there is a need to be improve our understanding due to the serious and lifelong consequences that can affect the mother and her baby.

In this thesis, placenta tissues of patients affected by early-onset PE were analysed and compared with those of healthy women, with the aim to characterize the metabolomic profiles of placenta in PE pregnancies and to deduce new possible metabolic pathways implicated in the pathogenesis. The placental samples were obtained from the Research Centre for Women's and Infants' Health BioBank at the Lunenfeld-Tanenbaum Research Institute at the University of Toronto. The targeted and untargeted metabolomic analyses for this project were performed at the Department of Biochemistry of the University of Cambridge using instruments with high sensitivity and reproducibility such as liquid chromatography coupled with mass-spectrometry and ion mobility. Resulting datasets were processed using both multivariate and univariate statistical analyses in order to fully understand the most significant metabolites involved in this disease.

3 MATERIALS AND METHODS

3.1 Patients

All samples came from the Research Centre for Women's and Infants' Health BioBank at the Lunenfeld-Tanenbaum Research Institute at the University of Toronto (Canada) and were collected at the Department of Obstetrics and Gynaecology of Mount Sinai Hospital in Toronto, (Canada). This study has been developed on placental tissues (45±5 mg wet weight) of patients affected by PE compared to normotensive women. Placental tissues were obtained with local ethical permission and written informed consent was obtained from each woman. Preeclampsia was diagnosed, using the criteria previous reported, with new-onset hypertension observed on at least two separate occasions combined with proteinuria. Only placentas from early-onset preeclampsia cases (<34 week) were used in the study.

For this study, the samples were divided into two groups as follows:

- 8 placental samples from PE pregnancies (7 samples from a non-laboured caesarean section and 1 sample obtained from a vaginal delivery)
- 14 placental samples from normotensive donors (4 term controls delivered by non-laboured caesarean section and 10 controls pre-term obtained from vaginal deliveries)

The term control group (NTC) was represented by healthy normotensive term patients that displayed no abnormalities on routine scans, while the normotensive preterm control group (NPTC) was delivered vaginally and for some of them acute conditions were reported such as chorionic vasculitits, acute chorioamnionitis and acute funisitis. Women with chronic hypertension, diabetes mellitus or pre-existing renal disease were excluded from this study. The selection of control group based on 2 different subgroups was driven by the similar number of gestational weeks between NTPC and PE and also by the absence of acute stress in NTC women due to the non-laboured caesarean section as well as the PE. Further reasons that led to consider the two control categories as a single class will be explained in the 4.1.2 paragraph.

The average age of mothers was 31±4 for PE, 30±5 for NPTC and 29±5 for NTC. The max average values measured of SBP and DBP were 157±14 and 110±11 mmHg,

respectively, for preeclamptic women, 114±9 and 73±10 for NPTC, and 123±6 and 70±8 for NTC. The result of proteinuria test was 4.0±4.2 g/day for PE and negative for both control groups. The gestational age expressed in weeks was 30.8±2.8 for PE, 29.4±2.9 for NPTC and 39.3±0.5 for NTC, while the weight in grams of placenta and babies was 149±34 and 853±243 respectively for PE, 262±68 and 1441±643 for NPTC, and 484±42 and 3598±381 for NTC. Details of placental collection are given in table 1.

For each placenta, four to six small pieces of tissue from separate lobules were rinsed three times in saline solution, blotted dry and snap-frozen in liquid nitrogen within 10 min of delivery; the samples were stored at –80°C until analyses.

3.2 Materials

All solvents were Chromasolv LC-MS grade. Methanol, chloroform, isopropanol, acetonitrile, ultrapure water and analytical standards were purchased from Sigma Aldrich (Merck KGaA, Darmstadt, Germany)

3.2.1 Internal Standards

Ten deuterated compounds consisting of amino acids, organic acids and nucleotides were used for the hydrophilic profile analysis. The standards were: proline-d3, valine-d8, leucine-d10, lysine U-13C, glutamic acid 13C, phenylalanine-d5, succinic acid-d3, serotonin-d4, adenosine-^{13}C-10,$^{15}N_5$ 5′-monophosphate and adenosine-$^{15}N_5$ 5′-triphosphate. Each standard was prepared at a 10 mM concentration while the concentration for ATP and ADP was 2 mM. Subsequently a mix solution of all the internal standard was prepared at the concentration of 10 µM and used as a reconstituting solution for samples.

A solution of eight labelled acylcarnitines used for the carnitine analysis was purchased from Cambridge Isotope Laboratories, Inc. (Massachusetts, USA). The mixture contained the following standards: $^{2}H_9$-carnitine, $^{2}H_3$-acetylcarnitine, $^{2}H_3$-propyonilcarnitine, $^{2}H_3$-butyrylcarnitine, $^{2}H_9$-isovalerylcarnitine, $^{2}H_3$-

octanoylcarnitine, $^{2}H_{9}$-myristoylcarnitine, $^{2}H_{3}$-palmitoylcarnitine. This solution was diluted 1:20 using methanol and used as reconstituting solution for samples.

A mixture of 26 deuterated lipids including representatives of the following classes: phosphatidic acid (PA), phosphatidylcholines (PC), phosphatidylethanolamines (PEt), glycerophospholipids (PG), phosphatidylinositols (PI), phosphatidylserine (PS), sphingomyelin (SM), ceramides (Cer), triacylglycerols (TG) and fatty acids (FA) was used for the placenta lipid profile analysis. The stock solutions were prepared as following: 10 mg of each standard was dissolved in one mL of methanol, apart from TG(45:0) and TG(48:0) that were dissolved in one mL of chloroform:methanol solution (1:1 v/v), and TG(54:0) which was solubilised in chloroform:methanol solution (2:1 v/v). Subsequently a mix solution of all the internal standard diluted 1:100 was prepared using methanol and used as a reconstituting solution for samples. A full list for the deuterated lipids used is reported in table 2.

3.3 Sample extraction

With the aim to investigate the metabolic profile of placenta samples, a modified Folch method (200) was used to extract and separate hydrophilic and lipophilic metabolites from the placenta tissues. Briefly, 0.35 mL of cold methanol and 0.35 mL of chloroform were added to plastic Eppendorf tubes (Eppendorf®, 022363204, USA) containing 45±5 mg of frozen placental fragments and one metallic bead. Samples were lysed with the tissue lyser (Qiagen, 2 × 2.5 min, 17 s^{-1}). After the samples were homogenized, 0.35 ml of chloroform and 0.15 mL of Chromasolv ultrapure water were added to the Eppendorf tubes before thoroughly vortexed for 30 seconds and centrifugation (17,000 × g, 10 min). The resulting two phases were separated into different glass vials, dried under a gentle stream of nitrogen and stored at −80 °C until further analysis.

3.4 Sample preparation for the hydrophilic phase analysis

The dried hydrophilic phase was subjected to two solvent reconstitutions due to different subsequent LC-MS analyses. The first analysis was conducted using a normal phase column, while the second analysis was conducted using a reversed phase column (see LC-MS method paragraph for further information). Initially, the samples were reconstituted in 0.1mL of acetonitrile: ammonium carbonate water solution (10mM) (7:3 v/v) containing the mixture of 10 polar internal standards at the concentration of 10µM. After reconstitution, the samples were sonicated for 5 minutes and centrifuged at the speed of 17,000 × g for 10 min. Centrifuged samples were then transferred into new glass vials and injected using the normal phase method. At the end of the analysis, samples were dried under a gentle stream of nitrogen and reconstituted with 0.1 mL of an aqueous solution containing ammonium acetate (10 mM) and injected using the reversed phase method.

3.5 Sample preparation for the lipid phase analysis

The dried chloroform phase was reconstituted with 50 µL methanol containing the mixtures of lipids internal standards previously described and 75 µL of a solution of isopropanol:acetonitrile:water (2:1:1 v/v). Quality control (QC) samples were prepared taking an aliquot of 10 µL of solution of each sample. All samples were analysed in positive and negative mode. For positive mode, the samples and QC were diluted 1:10 with the same reconstituted solution.

3.6 Sample preparation for the carnitine analysis

Each sample was prepared by mixing 50 µL of the lipid phase with the same amount of the hydrophilic phase to allow the analysis of carnitines that were soluble in both the aqeous and lipid phases. The mixed solution was first vortexed for 30 seconds and then dried under a gentle stream of nitrogen. Dried samples were reconstituted with 50 µL of a methanol:water solution (4:1 v/v) containing the labelled acylcarnitine internal standards at the concentration of 10µM. After reconstitution, samples were sonicated

for 5 minutes and centrifuged at the speed of 17,000 × g for 5 min. Centrifuged samples were transferred into new glass vials with insert and injected.

3.7 Analytical platforms

3.7.1 Ultra high-performance liquid chromatography (UHPLC)

High-performance liquid chromatography (HPLC) has been used in analytical chemistry and biochemistry for decades to separate, identify, and quantify compounds in both industry and in academia (201).

The principle on which the separation mechanism of HPLC is based depends on the affinity and interaction of sample analytes with the stationary phase (the packed column) and the mobile phase (solvents). In 2004, with the development of ultra high-performance liquid chromatograph (UHPLC), which can withstand extremely high pressures (up to 1000 bars), liquid chromatography (LC) technique has been considerably improved. The development of UHPLC enables the reduction of the time of the analysis and mobile phase consumption with increased sensitivity and separation performance (202).

In HPLC, there are four major separation modes: reversed-phase, normal-phase, ion exchange, and size exclusion, but only the first two are currently available for UHPLC. In the normal-phase method, the polar stationary phase is usually eluted with non-polar solvents such as acetonitrile, isopropanol and methanol. This means that polar molecules, because of the affinity with the stationary phase elute slowly compared with non-polar compounds, more analogous with the mobile phase, which elute quickly. On the other hand, the reversed-phase chromatography technique works with the use of a polar mobile phase and a non-polar stationary phase. The different polarity of the stationary phase depends on the packing material used and the presence of the long hydrocarbon chains in the column surface, usually C18, C8, C4, and phenyl modifications to the silica. The C18-bonded silica is the most popular type of reversed-phase mode. Today in metabolomics, the use of reversed-phase chromatography has achieved high reproducibility and applicability especially if combined with mass spectrometry (203).

3.7.2 Mass Spectrometry

Mass Spectrometry (MS) is a sensitive analytical technique widely used in metabolomics, which allows the detection and quantification of known analytes and to identify both organic and inorganic compounds present in very low concentration. This technique, conducted under vacuum, consists of the production of gas-phase ions and the measurement of charged particles according to their m/z ratio under the influence of an external electric and/or magnetic fields. The vacuum environment is a necessary condition to avoid the collisions of ions with other gaseous molecules that would increase the complexity of the spectrum. The mass spectrometer is generally constituted of three essential parts: the ion source, the mass analyser, which separates the ions according to their m/z ratio, and the detector that measures the abundance and converts the ions into electrical signals. Depending on the instrument, selected ions can even be fragmented and detected by a second analyser. Processed signals from the detector are then transmitted to the computer. The ion signals resulting in a mass spectrum provides information about the molecular weight, structure, identity, and quantity of the compound (203).

The samples can be introduced into the ion source using direct infusion, direct insertion methods or by coupling to chromatography. Direct infusion means that a capillary is employed to introduce the sample as a gas or a solution, while in the direct insertion, the sample is placed on a probe or a plate and then inserted into the source through a vacuum interlock (203).

Three major ionization sources are available: atmospheric pressure photo ionization (APPI), atmospheric pressure chemical ionization (APCI) and pneumatically assisted electro spray ionization (ESI). Nowadays, the use of ESI in LC-MS-metabolomics applications is predominant. Positive ESI ionization covers the widest range of compounds, while negative ESI ionization is commonly used to extend the coverage of metabolic information (203).

Since several ionization sources and mass analysers exist, only those that were used for our analysis are explained below.

3.7.3 Triple quadrupole mass spectrometer (QqQ-MS)

A triple quadrupole mass spectrometer (QqQ-MS) is a combination of two quadrupole mass filters separated by a collision cell. Charged ions generated in the ion source enter the mass analyser. The first quadrupole acts as a filter with the selection of a specific m/z ratio ion known as "precursor ion". Ions are fragmented in the gas-filled collision cell generating "product ions" which are then filtered according to their m/z ratio in the quadrupole mass analyser and recorded by the detector. QqQ-MS instruments are particularly useful for targeted and quantitative analysis, as it is possible to get high selectivity and sensitivity (203).

3.7.4 Quadrupole Time-of-flight mass spectrometer (QTOF-MS)

Time of flight mass spectrometer (TOF-MS) is one of the simplest ways to perform mass spectrometry, although it is capable of high resolution. QTOF-MS measures the time that ions take to travel from the beginning to the end of a field-free flight tube. After being expelled from the source, ions are accelerated towards the flight tube by a difference of potential applied between an electrode and the extraction grid. All the ions acquire the same kinetic energy and are separated depending on their m/z ratio. That means that low mass ions reach the detector positioned on the other extremity of the flight tube more rapidly than those with a high mass. The higher mass resolution and accuracy to detect a large number of compounds compared with single and triple quadrupole instruments has made these instruments suitable in many different fields such a clinical research, forensic toxicology, food safety, and environmental screening (203-204).

In metabolomics, and particularly in lipidomics, one of the most common challenges is the separation and identification of different compounds with the same m/z ratio. The development of tandem mass spectrometry has helped to overcome this challenge.

3.7.5 Tandem mass spectrometry (MS/MS)

Tandem mass spectrometry (MS/MS) is a technique where two or more mass analysers are coupled with a collision cell to increase the detection and reveal aspects of the chemical structure of compounds. It is a highly useful technique to identify unknown compounds, and for quantification. This technique is based on the selection of an ion called “precursor ion” in the first analyser, the fragmentation into one or more product ions in the collision cell and the mass detection of the products in the second mass analyser. The interpretation of fragments enables the identification of the precursor ion and gives information of the chemical structure of the ion detected (203,205). However, one of the most common problems in lipidomics is the presence of molecules having the same mass fragmentation pattern. The relatively new integration of ion mobility separation, based on molecular charge-size distribution, introduced an additional analytic dimension, which allows further discrimination of isomers in complex human matrices (206).

3.7.6 Ion mobility spectrometry (IMS)

Ion mobility spectrometry (IMS) is an analytical gas-phase technique that allows the separation of ions on the basis of their dimension and shape under the influence of an electric field. In literature, four commonly IM techniques are described: drift time (DT), traveling wave (TW), asymmetric field (AF), and differential ion mobility.

DTIMS is the oldest and theoretically simplest form of ion mobility. Packets of ions coming from the desolvation chamber are pulsed into the separation cell, known as the drift tube, which is filled with an inert gas (usually helium or nitrogen). The application of a static uniform electric field pushes these ions in the direction of the applied field and they are separated according to their charge, shape, and size. Due to the lower interactions with the gas, compact molecules travel faster than those with more extended topologies. In addition, ions with multiple charges have a higher mobility than those that are singly charged. The time required for an ion to cross the drift tube and reach the detector is called the drift time. Using the drift time value and the Mason-

Schamp equation reported below, it is possible to calculate the collisional cross section (CCS).

$$K = \frac{3}{16}\sqrt{\frac{2\pi}{\mu kT}}\frac{Q}{n\sigma}$$

Where Q is the ion charge, n is the drift gas number density, μ is the reduced mass of the ion and the drift gas molecules, k is Boltzmann constant, T is the drift gas temperature, and σ is the collision cross section between the ion and the drift gas molecules. The CCS is a physicochemical parameter specific for each compound and is related to the conformation assumed by the ions in the gaseous phase. It is proving very helpful for lipid annotations and identification (207).

One of the major advantages of IM analysis lies in its ability to differentiate some important classes of isomers that often co-elute using LC-MS methods. Isomers are chemical compounds that have the same molecular formula but different structures. The ability to resolve these molecules is an inherent strength of IMS (206).

3.8 LC/MS Analysis conditions

3.8.1 UHPLC/MS/MS normal-phase and reverse-phase polar profile analysis of placenta samples

A Thermo scientific UHPLC+ series coupled with a TSQ Quantiva mass spectrometer (Thermos Fisher Scientific, Waltham, Massachusetts, United States) was used with an electrospray ion source (ESI), operated simultaneously in positive and negative ion mode. The electrospray voltage was set to 3500 V (positive ion mode) and 2500 V (negative ion mode). Nitrogen was used as a drying gas. Hydrophilic compounds were analysed using two different methods. Initially, a BEH Amide (150 x 2.1 mm 1.7µm) column was used for the normal phase separation conditioned at 30°C. The mobile phase consisted of: (A) a 0.1M of an ammonium carbonate water solution and (B) acetonitrile. The mobile phase was pumped at a flow rate of 600 µL/min programmed as follows: initial conditions 20% of A for 1.50 min, then a linear increase from 20% to 60% of A in 2.5 min and kept at this percentage for one minute and then brought back to initial condition after 0.1 min.

Before the analysis, dried samples were reconstituted in 0.1mL of a acetonitrile:10mM ammonium carbonate water solution (7:3 v/v) containing a mixture of 10 hydrophilic internal standards at the concentration of 10µM (proline-d3, valine-d8, leucine-d10, lysine U13, glutamic acid C13, phenilanine-d5, succinic acid-d3, serotonine-d4, adenosine-13C10,15N5 5′-monophosphate and adenosine-15N5 5′-triphosphate).

Secondly, an ACE Excel 2 C18 PFP (100A. 150 x 2.1 mm 5µ) column was used for reverse phase separation. Before the analysis, dried samples were reconstituted in 0.1mL of a 10mM ammonium acetate water solution. The column was conditioned at 30°C. The mobile phase consisted of: (A) a 0.1% of formic acid water solution and (B) a 0.1% of formic acid acetonitrile solution. The mobile phase was pumped at a flow rate of 500 µL/min programmed as follows: initially kept at 100% of A for 1.60 min, then subjected to a linear decrease from 100% to 70% of A in 2.4 min and to 10% in 0.5 min, constant for 0.5 min and brought back to initial conditions after 0.1 min.

Xcalibur software (Thermos Fisher scientific, Waltham, Massachusetts, United States) was used for data acquisition. Putative recognition of all detected metabolites was performed using a targeted MS/MS analysis. Calculated masses and mass fragments of the detected compounds are reported in table 3.

Prior to statistical analysis all intensities were normalised for wet weight of each placenta sample.

3.8.2 UHPLC/MS/MS carnitines analysis of placenta samples

A Thermo Scientific UHPLC+ Vanquish series coupled with a TSQ Quantiva mass spectrometer (Thermos Fisher Scientific, Waltham, Massachusetts, United States) was used with an electrospray ion source (ESI), operated simultaneously in positive and negative ion mode. Before the analysis, samples were reconstituted in 50 µL of a methanol:water solution (4:1 v/v) containing the labelled acylcarnitine internal standards at the concentration of 10µM. The electrospray voltage was set to 3500 V (positive ion mode) and 2500 V (negative ion mode). Nitrogen at 48 mTorr and 420 °C was used as a drying gas for solvent evaporation. The organic phases were analysed

with an ACE Excel 2 C18 PFP (100A. 150 x 2.1 mm 5µ) column conditioned at 40°C. The mobile phase consisted of: (A) a 0.1% of formic acid water solution and (B) a 0.1% of formic acid methanol solution. The mobile phase was pumped at a flow rate of 500 µL/min programmed as follows: initially at 99.5% of A for 1 min, then subjected to a linear decrease from 99.5% to 0% of A in 9 min and kept at these conditions for 2 minutes and brought back to initial conditions after 0.1 min. Xcalibur software (Thermos Fisher scientific, Waltham, Massachusetts, United States) was used for data acquisition. Putative identification of all detected metabolites was performed using a targeted MS/MS analysis. Calculated masses and mass fragments of the detected compounds are reported in table 4.

Prior to statistical analysis, all intensities were normalised to the wet weight of each placenta sample.

3.8.3 Ion Mobility QTOF LC/MS lipid profile analysis of placenta samples

An Agilent 6560 Ion mobility Quadrupole Time-of-Flight (DTIM-QTOF) LC-MS system coupled with the Agilent 1290 UHPLC, was used to combine separation power and selectivity of LC, DTIM, and MS techniques. The Dual Agilent Jet Stream electrospray ionization source was operated separately in positive and negative ion mode.

3.8.3.1 Positive ion mode

The electrospray capillary potential was set to 60 V, the needle at 20 kV. Nitrogen (5L/min) at 48 mTorr and 375 °C was used as a drying gas for solvent evaporation; sheath gas temperature of 275 °C and flow of 12 L/min. Full-scan spectra were obtained in the ranges of 50 - 1200 amu, scan time of 0.20 amu, scan width of 0.70 amu, and detector at 2950 V. The lipid phase was analysed by an ACQUITY CSH C18 column (100 x 2.1 mm 1.7 µm, Waters) conditioned at 55°C. The mobile phase consisted of: (A) 10 mM ammonium formate solution in 40 % of ultrapure water and 60 % of acetonitrile and (B) 10 mM ammonium formate solution containing 90 % of

isopropanol, 10 % of acetonitrile (v/v). The mobile phase was pumped at a flow rate of 400 µL/min with initial condition equal to 60 % of A and 40 % of B. Solvent B was increased linearly to 43 % in 2 min, to 50 % at 2.1 min, to 54 % at 12 min and then to 99 % in 18 min. At 18.1 min solvent B was brought back to the initial conditions and remained at this percentage for 1.9 minutes. The column was re-equilibrated for 3 min at 40 % solvent B.

An Agilent tuning solution was injected before the analysis to tune the instrument in the m/z range 100-1700, and before every 10 samples to perform CCS re-calibration. During samples acquisition, an Agilent reference mix was constantly injected for mass re-calibration.

The Agilent Mass Hunter LC-MS acquisition console was used for data acquisition.

3.8.3.2 Negative ion mode

The electrospray capillary potential was set to 60 V, the needle at 20 kV. Nitrogen (5L/min) at 48 mTorr and 375 °C was used as a drying gas for solvent evaporation, sheath gas temperature of 275 °C and flow of 12 L/min. Full-scan spectra were obtained in the ranges of 50 - 1200 amu, scan time of 0.20 amu, scan width of 0.70 amu, and detector at 2950 V. The organic layers were analysed using a reverse-phase ACQUITY CSH C18 column (100 x 2.1 mm 1.7 µm, Waters, UK) conditioned at 55°C. The mobile phase consisted of: (A) 10 mM ammonium acetate solution in 40% of ultrapure water and 60 % of acetonitrile and (B) 10 mM ammonium acetate solution containing 90% of isopropanol, 10 % of acetonitrile with 0.1% of formic acid (v/v). The mobile phase was pumped at a flow rate of 400 µL/min with initial condition of 60% of A and 40% of B. Solvent B was increased linearly to 43 % in 2 min, to 50 % at 2.1 min and to 54 % at 12 min, reaching 99% in 18 min. At 18.1 min solvent B was brought back to the initial conditions and kept at this percentage for 1.9 minutes. The column was re-equilibrated for 3 min at 40 % solvent B.

An Agilent tuning solution was injected both before the analysis to tune the instrument in the m/z range 100-1700, and every 10 samples to perform CCS re-calibration. During samples acquisition, an Agilent reference mix was constantly injected for mass re-calibration.

The Agilent Mass Hunter LC-MS acquisition console was used for data acquisition.

3.8.3.3 MS/MS analysis

Data were collected using the same m/z ratio range of the MS scan mode and injected with three different collision energy: 20, 30 and 40 V. The analysis was conducted in a targeted MS/MS mode, choosing the parent masses and the respective retention time. All the information about the MS/MS data is reported in table 5**.** Examples of a MS/MS experiments are reported in figure 1**.**

3.8.3.4 Chromatogram pre-processing.

Data pre-processing, including mass and CCS re-calibration and feature finding, was carried out using the packages IM-MS Reprocessor, IM-MS Browser and Mass Profiler from the MassHunter Suite (version B.08.00, Agilent Technologies, Santa Clara, USA).

The resulting matrices were processed using a KNIME pipeline comprising both KNIME native nodes and integrated R scripts. QC-based feature filtering with missing value 50 and relative standard deviation threshold 0.2 was performed to eliminate noise/background signals and to remove features with poor repeatability. Imputation of missing values was performed using a Key-nearest neighbour (KNN) approach based on the R library impute. Feature annotation was performed based on the AccurateMassSearch node of the OpenMS library (208).

3.9 Chemometric analysis

The matrix of data created by the chromatogram pre-processing was processed using the SIMCA Software package (14.0, Umetrics, Umeå, Sweden). Prior to multivariate analysis, all intensities were normalised for the wet weight of each placenta sample. Principal components analysis (PCA) and partial least square-discriminant analysis (PLS-DA) were employed.

3.9.1 Chemometrics

The development of analytical platforms has generated huge and complex data matrices, which are difficult to summarise and interpret without appropriate tools. Chemometrics is the science that allows information to be extracted from multiple measurements made on chemical systems with the use of mathematical and statistical procedures. The measurements can have many different origins: spectroscopic (near infrared, fluorescence), chromatographic (GC, HPLC) and physical properties (temperature, pressure, concentration). In metabolomics, the characterisation of the samples (observations) take place through the study of different properties (variables). The multivariable system is usually organised into a data table, where each row constitutes an observation and the columns represent the variables (209). In this thesis, the extracts of placenta samples were used as observations, while spectral peak intensities were used as variables.

3.9.2 Scaling

The following step after pre-processing and extracting features to a suitable form, consists of pre-treatment of data through normalization, scaling, centring and other processes to maximise the contribution of variables to the multivariate models. Each of these mathematical transformations are used to put all samples and variables on a comparable scale and to alter the importance of each one to the multivariate model (one can make all equal or scale so that features less affected by noise have a larger role). These methods involve several statistical calculations: scaling factors correspond to the mean measured for each variable (metabolite), standard deviation, and maximum and minimum values. In this study, Pareto scaling was chosen as a pre-treatment method which consists of the use of the square root of the standard deviation as scaling factor. This approach helps to reduce the variances in intensity between the different variables, which would otherwise prejudice the results of the model by conferring higher importance to the most abundant variables (210).

3.9.3 Principal components analysis (PCA)

Principal components analysis (PCA) is a common tool in metabolomics, and is usually the first step in a study and analysis of multivariate data. PCA is an explorative method that projects the original data on to a lower dimensional space showing the distribution of observations and reducing their dimensions into a smaller number of new latent variables called principal components. Principal components highlight the variability among the samples, the presence of groups with similar properties or abnormal observations (outliers), the relations between the observations and the variables and even between the variables. Using a system of Cartesian axes, a matrix X with N observations and K variables is a space with as many dimensions as the number of variables. Each variable represents a co-ordinate axis where its length is the result of a previous normalisation according to the scaling criterion. The first component is the line that best estimates the data in the least squares sense, describes the highest variation within the data and goes through the average point (origin). The number of principal components explain how much information is described by the model. Usually one principal component is not enough to represent the variability of a dataset. When a second is calculated, it passes orthogonally to the first.

In a PCA model, different plots can be interpreted. The projection of the samples into the model space is called a scores plot and describes if there are any groupings, trends, or outliers while the loadings plot reports the projection of variables, describing the influence of the variables, and the relation among them. The loadings analysis is required to interpret the scores plot and describe the data structure based on the variable correlations. The interpretation of distance from the origin provides useful information, as for example the scores that are closer to the origin have average properties and the more a variable is far away from the origin the greater is the impact on the model. When two or more variables are negatively correlated, they are positioned in the diagonally opposed quadrants.

Different parameters or diagnostic tools can be used to verify the quality of the model. The R^2 parameter express a quantitative measure of how well the model is able to reproduce mathematically the dataset. Q^2 is an estimate value of the predictive ability of the model. The value of R^2 varies between 1 and 0 and the closer is to 1 the more the model is close to the referred experimental models. Similar values of R^2 and Q^2

indicate that the system is homogenous, whereas if the system is heterogeneous Q^2 will be lower than R^2. The presence of possible outliers can be defined calculating their leverage. The leverage is the influence of an observation on the model and is proportional to its distance to the centre of the data, the higher the leverage the stronger is the outlier. Another diagnostic tool to identify strong outliers is the Hotelling's T^2. It is a multivariate generalisation of the Student's t-test, which provides a check for observations adhering to the multivariate normality. In SIMCA, moderate outliers can be identified by the residual of each observation and is called DModX (211).

A further tool to study the similarities and dissimilarities among samples is the Cooman's plot. It is based on DModX and Hotelling's T2 analysis and shows the orthogonal distance of new objects to two selected groups of samples at the same time. Briefly, if an object belongs to a group, it is within the limits, which are represented by the left vertical line or below the horizontal line. These limits may be changed for exploratory purpose and the higher the significance level, the more strictly the new object will be judged with respect to its true membership. Objects that are within both limits, near the origin, must be classified as belonging to both groups (212).

3.9.4 Partial least square-discriminant analysis (PLS-DA)

Partial least square-discriminant analysis (PLS-DA) describes a set of latent variables measured on a set of observations in a supervised fashion. PLS-DA classifies observations on the basis of their class membership, and can be used for predictive and descriptive models as well as for the selection of discriminative variable. PLS-DA relates two data matrices, usually called X and Y. The matrix X generally is constituted by the observations and their variables, while matrix Y contains qualitative values, for example, class membership, gender, or treatment of the samples. This analysis is often performed to improve the separation between groups of observations by rotating PCA components in order to obtain the maximum separation among classes, and to understand which variables are involved. In the same way as PCA, it is possible to verify the quality of the model using mathematical parameters or diagnostic tools. In the context of PLS-DA, R^2 and Q^2 refers to the performance of Y data. The generated R^2Y value describes the classificatory power of the model and Q^2Y the predictive

ability. When the Q^2Y value reaches a plateau or decreases, this indicates that the model is losing the predictive ability (211).

Another statistical method to validate the classificatory power of the model is the permutation test. It is a procedure able to test the null hypothesis of two different groups by mixing the observed data and to check the validity and the grade of the overfit. The test checks the exchangeability of data and verifies if mixing the data keeps the same result of the original model. It is a non-parametric test that provides an assessment of the false positives in the model. Three parameters are used for this analysis: the null hypothesis, which determines what we permute, the test statistic that affects the power and the number of permutations, which affects precision of the estimated p-value (211).

To interpret and summarize the importance of the variables and also to find the possible discriminants, the variable influence on projection (VIP) is very helpful. The VIP score is the result of a squared function of PLS-DA components and original variable; this means that only positive values are possible. Variables with a VIP value larger than 1 affect the studied model with more strength and are usually deemed important variables for the model (211).

3.10 Univariate statistical analysis.

In univariate statistical analysis, only one variable is examined independently and can be used, for example, for determining the significant difference between two groups of samples such as patients versus controls or to test the efficiency of different treatments. When two groups are examined, tests such as the t test are used for a two-wise comparison, while where studies concern three or more groups, analysis of variance (ANOVA) is often used, followed by corrections for multiple testing such as the least-square test, Dunnett's or Bonferroni's t test, and Scheffé's F test. The difference lies in the "conservatism" that means that more replications and stronger responses are required to highlight the statistically significant differences between the groups (213).

In this thesis, GraphPad Prism software (version 8.01, GraphPad Software, Inc., CA, USA) was used to perform univariate statistical analysis. To verify the statistical

significance of discriminant metabolites obtained using multivariate statistical analysis and to find the differences in the results of the targeted hydrophilic phase analysis a Mann Whitney U test was performed. It is a nonparametric test able to compare outcomes between two independent groups. It is used to test the null hypothesis that two samples come from the same population or, alternatively, whether observations in one sample tend to be statistically different from observations in the other. Although it is a non-parametric test, it does assume that the two distributions are similar in distribution (213).

4 RESULTS

The metabolomics profile of placenta samples obtained from pregnancies complicated by preeclampsia and healthy pregnancies was analysed through the use of hyphenated mass spectrometry platforms and statistical analyses.

4.1 Lipid analysis of placenta samples

The lipid profiles of placental samples from preeclamptic and normotensive pregnancies were analysed by DTIM-QTOF-LC/MS. After processing the data, a matrix with 4037 mass spectral features composed of all the positive and negative signals were selected and statistically analysed. Examples of lipid extract chromatograms are shown in figure 2. The obtained matrix was analysed by both univariate and multivariate statistics.

4.1.1 Lipid profile composition of placenta samples

The dataset was composed by 930 mass spectral features annotated as glycerophospholipids (GP), 239 glycerolipids (GL), 417 sphingolipids (SP), 35 fatty acyls and 29 as sterols. GP was the prevalent lipid class representing 75.8 % of total annotated lipid followed by SP with 16.6 %, GL with 3.6 % and fatty acyls and sterols with 3.6 % and 0.4 % respectively. The relative abundance of each lipid class for each sample was calculated and compared between the diseased and control groups using the Mann Whitney *U* test. However, the analysis did not show any significant difference between the pathological and control group (figure 3). Using their m/z ratio, RT and CCS, and after the comparison with analytical standards, the annotated fatty acids were then divided into three different categories based on saturated fatty acids (SFA), monounsaturated fatty acids (MUFA) and polyunsaturated fatty acids (PUFA). Total FFA content in placentas from PE pregnancy was constituted of 78.0 % of SFA, 8.3 % of MUFA and 13.8 % of PUFA while in control samples resulted of 76.2 %, 8.3 % and 15.5 %, respectively (table 6). The most abundant SFA in both groups was palmitic acid, oleic acid in the category of MUFA and arachidonic acid for PUFA

(figure 4). Examining single species we observed a significant decrease of the levels of lignoceric acid and erucic acid in PE compared with the control group (figure 5).

Examining cholesterol and cholesterol esters, there was a significant decrease of CE18:2 and a trend for other CE species decreasing in PE samples compared with the control group and no significant changes were found for free cholesterol (figure 6). SM and Cer are shown in figure 7, although no changes were statistically significant.

4.1.2 Multivariate statistical analysis of lipid profile

To investigate the correlations between compositional and analytical data, and to observe sample distribution into a multivariate space, a PCA was performed using the SIMCA 14.0 software. The dataset used in the model was the total annotated signals. The Hotelling's T^2 and the DmodX analyses did not show any outliers and all samples were kept for the analysis. Using two principal components, the model reported the following validation parameters: an R^2X and a Q^2 of 0.36 and 0.08, respectively. The two principal components explained 23 % and 13 % of the total variance, respectively. Using PCA, visual analysis of the score plots showed no clusters based on pathology, delivery mode or considering the gender of the babies (figure 8). QC samples were tightly clustered in relation to the clustering of the placental samples showing good technical and instrumental reproducibility. To find possible discriminant metabolites between the PE and the control group (C), PLS-DA was performed and a clear separation between the two groups was observed. The R^2Y and Q^2 resulting from the PLS-DA model were 0.92 and 0.68, respectively. The permutation test demonstrated good predictability of the model (figure 9). Considering the impact on the classification because of delivery mode, we decided to verify a possible influence of the model due to the delivery classification instead of the pathology. Performing PLS-DA using the delivery mode as a classification, comparing caesarean section placenta samples *vs* vaginally delivered placenta samples, there was good classification, with R^2Y and Q^2 values of 0.95 and 0.69, respectively. However, the permutation test was not able to validate the model (Q^2 of 0.08) (figure 10 and table 9). To further discriminate the effects of delivery mode and pathology, we also prepared the Cooman's plot. The plot, reported in figure 11, indicates the two subgroups of control, NTC and NPTC, as

similar clusters even if subjected to a different delivery mode, confirming our hypothesis that the pathology has a stronger classificatory power than the delivery mode overall.

Given these results, the classification based on lipids are largely based on the presence of preeclampsia as it presented the most discriminant power among all the variables tested. Therefore, PLS-DA of PE *vs* C (term and pre-term control) samples was performed, and twenty-five metabolites were interpreted as different between PE and C after the comparison between VIP analysis, the coefficients analysis and after the confirmation with Mann–Whitney *U* test. We identified all the discriminant lipid species comparing the annotations with the Lipidomics libraries and using tandem MS/MS analysis. Along the discriminant compounds, PE samples were characterised by a decrease in sphingolipids and especially in ceramides (Cer) and sphingomyelins (SM) species, in particular: Cer(18:2/16:0), Cer(18:2/22:0), Cer(18:1/24:1), Cer(18:2/24:1), SM(18:2/16:0), SM(18:1/22:1), SM(18:2/22:0), SM(17:1/24:1), SM(18:2/24:1), SM(18:1/24:1), SM(18:1/22:0), SM(18:1/24:0) and SM(18:2/24:0). On the other hand, only SM(18:1/18:0) was increased. Moreover, in the glycerophospholipids class, several species were decreased in the PE samples when compared with C samples, as LysoPC16:0, LysoPC18:1, PC(16:0/20:4), PC(18:1/20:4), PC(14:0/18:0), PC(18:0/20:4). Additionally, PC(16:0/20:3), PC(18:1/20:3) and PC(18:0/20:3) levels were increased in PE compared to C samples. Furthermore, stearoylcarnitine and one phosphoetanolamine (PEt) plasmalogen species PEt(P-18:0/20:4) were found decreased in PE samples (Figure 9 and Table 8).

4.2 Targeted acylcarnitines analysis of placenta samples

Using LC/MS-MS QqQ, 37 acylcarnitines from 22 placenta samples were measured (an example of a LC/MS-MS QqQ chromatogram is reported in figure 14 and the list of detected compounds in table 4). The obtained dataset before being submitted to the Mann Whitney *U* test was normalised dividing the detected area of the most abundant fragment by the weight of each placenta sample. Overall, the abundance of 11 acycarnitines was significantly different in PE when compared to the control group. The results showed that 3 short- and middle-chain acylcarnitines increased, C3, C8:1

and C10:2, while C8DC (suberylcarnitine) significantly decreased in PE when compared with normotensive control group. In addition, the levels of 7 long chain acylcarnitines significantly decreased in PE when compared with control group: C17, C18, C18OH, C18:2OH, C20, C20:1 and C20:2 (figure 15).

4.3 Polar metabolite profile of plasma samples

Using LC/MS-MS QqQ, 102 polar compounds from 22 placenta samples were measured (examples of an LC/MS QqQ chromatogram is reported in figure 16 and the list of detected compounds in table 3). The obtained dataset before being submitted to the Mann Whitney *U* test was normalised dividing the detected area of the most abundant fragment by the weight of each placenta sample. Overall, the levels of 42 polar metabolites were found to be statistically significantly changed in PE when compared with the control group. 37 discriminant metabolites reported below were found decreased in PE: adenine, adenosine diphosphate (ADP), adenosine monophosphate (AMP), alanine, aminoadipic acid, aminoisobutyric acid, arginine, asparagine, aspartic acid, asymmetric dimethyl arginine (ADMA), citrulline, cytidine diphosphate (CDP)- choline, cytidine monophosphate (CMP), dimethylallyl pyrophosphate, flavin adenine dinucleotide (FAD), glutamic acid, guanosine monophosphate (GMP), guanosine diphosphate (GDP), histidine, hydroxymethylglutaryl-CoA, hypoxanthine, isoleucine, leucine, methylarginine, methionine sulfoxide, ornithine, phenylalanine, proline, S-adenosyl methionine, S-adenosyl homocysteine , serine, serotonine , taurine, threonine, uridine diphosphate (UDP) N-acetylglucosamine, uridine monophosphate (UMP), and valine. On the other hand levels of 2/3-phosphoglyceric acid, creatine, folic acid, hydroxyproline and phosphocreatine were significantly increased in PE when compared with normotensive controls (table 11 and figure 17, 18 and 19).

5 DISCUSSION

The aim of this Ph.D. project was to better understand PE using a metabolomics approach. Comparing placental metabolic profiles of patients affected by PE with those of normotensive women, we observed significant changes in the levels of numerous compounds that can be related to several metabolic pathways such as the metabolism of lipids, nucleotides and amino acids. The overall alteration in metabolites might reflect the structural damage of the placenta and a dysregulation of different cellular compartments such as the endoplasmic reticulum and mitochondria, due to oxidative stress, resulting from altered maternal spiral arteries remodelling at the genesis of the pathology and therefore subsequent malperfusion of placental and foetal tissues.

In recent decades, the interest in lipidomics and in particular the research on bioactive lipids as a diagnostic tool in translational and molecular medicine has increased the attention of the scientific community (214-215). Lipids are a wide class of nonpolar molecules involved in different metabolic pathways with several functions. On the basis of their structure, they can take part in the composition of the cellular membranes, used as important sources of energy or act as precursors for the biosynthesis of other molecules (216).

SP are a class of lipids consisting of a sphingosine backbone bound with a serine binding to the acyl group of a fatty acid (217). The biosynthesis of SP starts in the endoplasmic reticulum (ER) with the condensation of serine and palmitoyl-CoA into 3-ketosphinganine catalysed by serine palmitoyltransferase, while the conversion into Cer, SM and other derivative products can occur in different cellular compartments such as the Golgi apparatus and mitochondria. Cer species can be synthesized *de novo* or originate from the catabolism of other SP species, while SM originate from Cer conversion (217). These lipids categories are biologically active molecules involved in many metabolic processes in the human body including proliferation, cell growth and survival, cell migration, inflammation, angiogenesis and resistance to apoptotic cell death (217-220). In our study, the levels of different species of Cer and SM were altered, as well as a strong down-regulation of serine in PE samples when compared with healthy controls. These results confirm the presence of a dysregulation of SP metabolism in PE as reported by other authors not only in studies conducted in

placental samples, but also in plasma and in the umbilical cord veins, linking SP signalling to impaired trophoblast differentiation and a high cell turnover, which are typical of PE (193,221-222). Almost all discriminant SP species were decreased in PE, except for the SM(18:1/18:0), which was significantly increased. In the literature, this specie has been correlated with the promotion of exosomal release of the antiangiogenic factor Eng, affecting vascular homeostasis and inducing hypertension *in vivo*, characteristic symptoms of PE (223). Ermini *et al.* demonstrated how lipid composition in placental tissues is influenced by oxidative stress and that the hypoxic environment stimulated, in the Golgi apparatus, an increase in the synthesis of SM containing 18:0 as a fatty acyl chain (223). Increased levels of these SM promoted the formation of lipids rafts containing End and metalloproteinase-14, which were first transferred to the surface of the syncytiotrophoblast, where Eng is cleaved into its short active form (sEng), and then secreted into the maternal circulation as exosomes (223-224). These exosomes may also contain TGF-β receptors 1 and 2, which in the blood circulation of women with PE prevent the vascular effects of TGF-β (99,223) affecting vasculogenesis and angiogenesis (100-102,225). In pregnancies without complications, the secretion of micro and nano-vesicles from the syncytiotrophoblast into the maternal circulation is crucial to maternal-foetus cross-talk regarding the transport of proteins lipids and nucleic acids (226). On the other hand, in early-onset PE, due to the oxidative and inflammatory stress, the release of these signalling vesicles, and hence the concentration of sEng and other antiangiogenic factors like sFt1, is abnormally high which stimulates aberrant binding to circulating PlGF and VEGF, impairing angiogenesis and the signalling in the vasculature which results in endothelial cell dysfunction (3, 97, 100-102, 227). This connection has also been confirmed in animal models where an over-expression of sEng and sFlt1 causes a severe preeclampsia-like syndrome with focal vasospasm, hypertension, choroid plexus endotheliosis, and increased vascular permeability with brain edema (228). These findings, together with the above-cited references demonstrate the strong impact of SM and Cer in the pathophysiology of PE.

The GP class consists of phospholipids species, which are the main components of cellular membranes and which were the predominant species in our open profile lipid analysis. Even though we did not find significant changes in the total amount of phospholipids comparing pathological and control groups, several compounds

belonging to this class were found to be discriminatory and in particular: PC, LysoPC and PEt. Related to the synthesis of PC and hence to the membrane composition, we also found decreased levels of CDP-choline in PE samples, suggestive of alterations in the metabolism of phosphocholine into PC catalysed by phosphocholine cytydiltranferase (229). On the other hand, no differences were found in the levels of other compounds involved in the synthesis of PC, such as DAG, uridine, choline or cytidine triphosphate, suggesting that the previous enzymatic steps of the PC synthesis were not affected in the PE syndrome (229). Further enzymatic studies are needed to understand the decrease in CDP-choline content in PE samples. Moreover, alterations in the structural lipids profile have been related to abnormal placental architecture in PE (190) and changes in the content of placental PC species and cholesterol, recognised as key membrane lipids, have been linked to a reduced numbers of placental villi and abnormal syncytialmembrane structures associated with the pathological state (230). Among the PC species, we observed an interesting trend, where the levels of the majority of discriminants were decreased in PE samples except for PC(16:0/20:3), PC(18:1/20:3) and PC(18:0/20:3) which were all characterised by the presence of a 20:3 long-chain fatty acid. This fatty acid, known as dihomo-γ-linolenic acid, derives from linoleic acid and can be converted into arachidonic acid (AA) by a desaturase or converted via cyclooxygenase and lipoxygenase pathways in different bioactive metabolites known as eicosanoids, which regulate inflammation and proliferation of cells (231). Altered PC content in placenta samples from PE pregnancies compared to normotensive ones has been suggested to be directly responsible for maternal endothelial dysfunction (192,229,232), acting as precursors of several molecules, including second messengers involved in cell proliferation, inflammation and oxidative stress (191). Alterations in the composition of the cellular membrane have been also correlated with different tumour behaviours and cancer progression (233). In our study, structural membrane modifications during preeclampsia may also be confirmed by the significant increase of hydroxyproline (ProOH) in placental tissue from PE pregnancies when compared to controls. ProOH is a non-essential amino acid and the major component of the protein collagen. Anomalies in ProOH metabolism have been associated to different disorders such as graft *versus* host disease, keloids, and vitiligo (234). Uzun and co-workers in their study have shown the correlation between elevated placental levels of ProOH and

hyaluronic acid with increased fibronectin in maternal serum and cord blood of preeclamptic patients reflecting placental structural damage (235).

Pregnancy is considered a physiological state associated with oxidative stress, described by the imbalance between the production of reactive oxygen species (ROS) and reduced antioxidant defence mechanisms (236). However, in pregnancy complications such a PE, IUGR or maternal diabetes the production of these reactive species can be even higher, affecting placental functions (237). In our samples, a significant decrease of the PEt (P-18:0/20:4), a plasmalogen species, was found in PE samples compared to controls. This lipid species is a special type of phospholipid produced in the peroxisomes that due to a vinyl-ether linked in sn-1 and typically a PUFA at the sn-2 position acts as endogenous antioxidants, preventing lipid oxidation (238). We suggest the depletion of this PEt species as a consequence of a protective mechanism of the placental tissue against oxidative stress during intermittent hypoxia, experienced by the placenta in PE pregnancies. Decreased levels of PEt species were also found by Korkes and co-workers in plasma samples of preeclamptic patients. They associated this reduction with the release of arachidonic acid, the same FA bonded in our PEt species, used in the synthesis of prostaglandins, thromboxanes, prostacyclins and leukotrienes and therefore implicated with vasodilation in the case of prostaglandin synthesis or platelet aggregation and vasoconstriction in the case of the thromboxanes (191). Considering oxidative stress-related metabolites, we observed a significant decrease in several compounds involved in the arginine-nitric oxide pathway in PE. Nitric oxide (NO) is produced in endothelial cells by a family of nitric oxide synthases with arginine as a precursor, in presence of oxygen and the cofactor tetrahydrobiopterin (239). NO acts as a vasodilator preventing platelet aggregation and its efficiency depends on the ability of placental membrane transports to uptake arginine as well as NO synthases activity (239). NO synthases and NO can be inhibited by asymmetric dimethylarginine (ADMA), whose increased plasma levels is recognized as potential biomarker of endothelial and cardiovascular disorders (239-240) and also in PE (241). Several studies have reported increased ADMA concentration in plasma of patients with preeclampsia when compared to normal pregnancies suggesting its availability as a biomarker for the prediction of the pathology (241-242). In agreement with the reports of Kim *et al.,* we found a strong decrease not only in arginine levels in PE (243) but also in ADMA, s-adenosyl

methionine and s-adenosyl homocysteine which are substrates that take part in the synthesis of ADMA as methyl donors (240). Unfortunately, having no data related to the plasma of our patients, we merely speculate that the strong down-regulation of ADMA in placental tissues can be explained by its release into the maternal circulation (although local changes in vasoconstriction could still impact systemic blood pressure). The oxidative stress and the increased production of free radicals as a result of mitochondrial dysfunction might reflect an impairment in oxidative phosphorylation and ATP synthesis possibly related to changes in the fatty acid oxidation (236). During the last trimester of normal pregnancy, maternal lipid metabolism switches to a catabolic state using long-chain fatty acids as a source of energy (244). However, impaired mitochondrial function and fatty acid oxidation in PE have been previously documented. In a recent study conducted with umbilical endothelial cells of patients affected by PE, a reduction in overall fatty acid oxidation along with a depletion in the respiratory chain enzymes and the mitochondrial content marker citrate synthase have been demonstrated and also confirmed by electron microscopy (245). Other groups reported decreased expression and activity of placental cytochrome *c* oxidase in PE (246-247), increased levels of carnitine and acylcarnitines in blood samples of patients affected by PE (248) and also of carnitine precursors in the umbilical cord plasma of newborns (249) while reduced levels of acylcarnitines were found in placental tissue in PE (192). The mitochondrial alterations may also explain the altered levels of acylcarnitines found in our samples from PE when compared with controls. Our results showed a statistically significant increase of some short and middle-chain acylcarnitines in PE (C:3, C8:1 and C10:2) while the levels of the long-chain ones, including C17, C18, C18OH, C18:1, C18:2 OH, C20, C20:1 and C20:2, were found significantly decreased. Carnitines are hydrophilic molecules responsible for both energy metabolism, acting as a shuttle for lipids from the cytoplasm to the intracellular organelles such as mitochondria peroxisomes and microsomes, but also for the elimination of excess toxic acyl groups (250) which if not efficient may contribute to the development of the inflammatory state of PE. The binding of carnitines with the acyl residues forms acylcarnitines whose levels can be used as diagnostic tools of inborn errors of metabolism (250). The carnitine palmitoyl transferase 1 (CPT-1) catalyses the conjugation of fatty acids to carnitine and the subsequent transfer to the mitochondria. The deficiency of this enzyme was described in the case of elevated free and short chain acyl carnitine and low levels of long-chain

ones and the diagnoses is usually confirmed by CPT-1 assay (251). Considering our results, this suggests mitochondrial oxidation of long-acylcarnitines is increased, which explains their decrease in the placenta. Since glucose utilization has been suggested to be decreased in PE, the oxidation of long-chain fatty acids may be required to maintain ATP concentrations. The increase in middle and short-chain acyl carnitines, on the other hand, suggest peroxisome FA impairment (252).

The overall oxidative stress state due to placental malperfusion also affects other cell compartments such as the ER (253). This organelle plays essential roles in vital functions of the cells including synthesis, post-translational modification and secretion of proteins, particularly those bound to the plasma membrane (254). The success of these activities is strongly connected to the adequate concentration of oxygen and nutrients which in the case of stress conditions such as hypoxia and amino acids depletion can lead to a homeostatic mechanism known as the unfolded protein response (UPR) aimed to conserve resources, restore cellular functions and/or remove damaged cells (255). On the other hand, alterations in ER-UPR may contribute to increased inflammatory state disturbing several other cellular pathways, including apoptosis, and lipid synthesis (256). The activation of this pathway occurs through three different transmembrane protein sensors localized in the ER: the protein inositol-requiring enzyme 1, the activating transcription factor 6, and the protein kinase RNA−like ER kinase. These proteins regulate gene expression as a result of various stimuli such as the accumulation of unfolded or misfolded proteins, the perturbation of Ca^{2+} levels and also by mitochondrial ROS production. (255,257). UPR has been previously documented in the case of IUGR, which is often related to the early-onset PE, but not in the late-onset (258-259). Protein and nucleotide syntheses are an energy-demanding processes that can be temporarily downregulated or even blocked under stress. The significant decrease in the concentration of several amino acids and nucleotides including adenine, AMP, CMP, GMP, and UMP found in PE samples when compared to controls, supports the hypothesis of inhibition of protein and nucleotide synthesis in PE also connected to UPR. This aspect not only decreased RNA and DNA synthesis (for proliferation) but also ATP, NAD and other molecules (260-261). However, despite a significant reduction in AMP and ADP, placental energetics was not affected as demonstrated by the unchanged content of ATP, and also by the metabolites involved in the glycolytic pathway. These results suggest that other

pathways may provide ATP production, including fatty acid oxidation as previously mentioned and the creatine-phosphocreatine system, known to be involved in the buffering of energy homeostasis (262). As a confirmation to this hypothesis, we found a significant increase in the levels of creatine and phosphocreatine in PE compared with normotensive controls. These findings suggest that the placenta might activate an adaptive response under stress conditions to modulate the cellular activity and ensure the essential functions for foetal growth (263). Indeed, higher amounts of P-creatine was described in placentas from the 1st trimester compared to 2nd and term, indicating the importance of the creatine-phosphocreatine system when mitochondrial activity is decreased and oxygen availability is low (264).

The origin of placental stress and its contribution to the onset of PE remains to be fully described, however, new insights into the molecular mechanisms of this disease suggest that activation not only of the ER-UPR but also mitochondrial UPR can initiate inflammation and proapoptotic responses, which are in the epicentre of the pathogenesis of PE (265).

6 CONCLUSIONS

The aim of this PhD thesis was to investigate the metabolomic profiles of placental tissues of women affected by preeclampsia compared with those of normotensive controls. The analyses were performed with the use of different mass spectrometric platforms. The hydrophilic phase was analysed with targeted methods using a Thermo LC/MS QqQ while the lipid phase analysis was performed with an untargeted method by an Agilent DTIM-QTOF-LC/MS. The levels of numerous compounds related to the metabolism of lipids, nucleotides and amino acids were found altered and recognised as discriminatory for PE samples when compared with normotensive women. These changes reflect the structural damage of the placenta and the dysregulation of different cellular compartments such as the endoplasmic reticulum and the mitochondria, due to oxidative stress, resulting from altered maternal spiral arteries remodelling at the genesis of the pathology and therefore subsequent to malperfusion of placental and foetal tissues.

Decreased levels of several sphingolipid species belonging to the classes of ceramides and sphingomyelins in PE samples were observed when compared with controls. On the other hand, the levels of SM (18:0/18:1) were increased which has been previously described to be correlated with the exosomal release of anti-angiogenic factor endoglin, affecting vascular homeostasis and inducing hypertension *in vivo*, symptoms typical of PE. As well as SP, also glycerophospholipids such as LysoPC and PC were decreased in PE samples suggesting placental structural damage further confirmed by decreased levels of CDP-choline, involved in the metabolism of PC and increased levels of hydroxyproline, which is correlated to abnormal placental architecture in PE.

The overall oxidative stress state due to placental malperfusion in PE pregnancies was reflected in different cellular compartments and confirmed by decreased levels of a phosphoethanolamine plasmalogen species and by several compounds involved in the arginine-nitric oxide pathway. Distressed mitochondrial function with impaired fatty acid oxidation in PE was indicated by decreased levels of long-chain acylcarnitines while increased levels of those with short and middle chain, suggesting peroxisome fatty acid impairment.

Decrease levels of several amino acids and nucleotides suggest the activation of ER-UPR resulting in the inhibition of the synthesis of proteins, RNA and DNA that are energy-demanding processes during stress. However, despite the significant reduction of the levels of AMP and ADP, the content of ATP was unaffected. On the other hand, increased levels of creatine and phosphocreatine suggest that other pathways may provide ATP production, including the oxidation of long-chain fatty acids and the creatine-phosphocreatine system. These findings suggest that the placenta might activate an adaptive response under conditions of stress to modulate cellular activity and ensure essential functions for the foetal growth.

A limitation of the present study is that a possible confounding variable might contribute to the differences between PE and normotensive pre-term placental samples such as the impact of delivery mode. The difficulties consisted in having access to pre-term control placental samples derived from caesarean sections at gestational ages comparable to those in early-onset preeclampsia, as non-laboured caesarean-delivered pre-term placentas from healthy pregnancies are clinically difficult to obtain. Those available samples used in this study are not stress-free owing to the clinical conditions induced from spontaneous delivery and the ischemia-reperfusion stress imposed by vaginal delivery. On the other hand, despite this limitation, we are confident that the observed changes in the profile of polar metabolites, e.g., related to amino acid and nucleotide metabolism, truly reflects the altered pathways as a consequence of the pathology itself as it contributes to the discussion about ER and mitochondrial distress in PE, already well established in the literature.

In conclusion, the metabolomics study based on the analysis of both polar and lipid metabolite profiles though the use of LC/MS/MS and IM platforms can be considered a valuable tool for the investigation of the molecular aspects of PE pathogenesis. However, further studies are strongly recommended to understand the role of the several bioactive lipid species involved in the pathology, the genesis of oxidative stress and the possible differences resulting from the delivery mode.

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8 TABLES

Table 1. *Samples clinical records. Values are expressed as mean ± standard deviation.*

	Preeclampsia (n=8)	Controls (n=14)	
		Term (n=4)	**Preterm** (n=10)
Maternal age (years)	31 ± 4	30 ± 5	29 ± 5
Gestational age of delivery (weeks)	30.8 ± 2.8	39.3 ± 0.5	29.4 ± 2.9
Mode of Delivery	7 c/s no labour 1 vaginal	4 c/s no labour	10 vaginal
Gender of baby	2 M- 6 F	3 M- 1 F	6 M- 4 F
New-born weight (g)	853 ± 243	3598 ± 381	1441 ± 643
Placental weight (g)	149 ± 34	484 ± 42	262 ± 68
Max SBP (mmHg)	157 ± 14	123 ± 6	114 ± 9
Max DBP (mmHg)	101 ± 11	70 ± 8	73 ± 10
Proteinuria (g/day)	4.0 ± 4.2	negative	negative

SBP= systolic blood pressure, DBP= diastolic blood pressure, c/s= caesarean section

Table 2. *List of lipids used for the placenta internal standard mixture.*

Name	trivial name	class	store
N-palmitoyl-d31-D-erythro-sphingosine (C16-d31 Ceramide)	C16-d31 Ceramide	Cer	Avanti
1-palmitoyl-d31-2-oleoyl-sn-glycero-3-phosphate (sodium salt) (16:0-d31-18:1 PA)	16:0-d31-18:1 PA	PA	Avanti
1-palmitoyl(D31)-2-oleyl-sn-glycero-3-phosphocholine (16:0-d31-18:1 PC)	16:0-d31-18:1 PC	PC	Avanti
1-palmitoyl(D31)-2-oleyl-sn-glycero-3-phosphoethanolamine (16:0-d31-18:1 PE)	16:0-d31-18:1 PEt	PEt	Avanti
1-palmitoyl-d31-2-oleoyl-sn-glycero-3-[phospho-rac-(1-glycerol)] (sodium salt) (16:0-d31-18:1 PG)	16:0-d31-18:1 PG	PG	Avanti
1-palmitoyl-d31-2-oleoyl-sn-glycero-3-phosphoinositol (ammonium salt) (16:0-d31-18:1 PI)	16:0-d31-18:1 PI	PI	Avanti
1,2-dimyristoyl-d54-sn-glycero-3[phospho-L-serine] (sodium salt)(14:0 PS-d54)	14:0 PS-d54	PS	Avanti
N-palmitoyl(D31)-d-erythro-sphingosylphosphorylcholine (16:0-d31 SM)	16:0-d31 SM	SM	Avanti
1,2-dipalmitoyl-d62-sn-glycero-3-[phospho-L-serine] (sodium salt)	16:0 PS-d62	PS	Avanti
cholesteryl-2,2,3,4,4,6-d6-octadecanoate (18:0-d6 CE)	18:0-d6 CE	Cer	Qmx
Pentadecanoic-d29 Acid (15:0-d29 FA)	15:0-d29 FA	FA	Qmx
Heptadecanoic-d33-acid (17:0-d33 FA)	17:0-d33 FA	FA	Qmx
Eicosanoic-d39 Acid (20:0-d39 FA)	20:0-d39 FA	FA	Qmx
Tetradecylphosphocholine-d42 (14:0-d29 LPC-d13)	14:0-d29 LPC-d13	LysoPC	Qmx
Glyceryl Tri(pentadecanoate-d29) (45:0-d29 TG)	45:0-d29 TG	TAG	Qmx
glyceryl-tri(hexadecanoate-d31) (48:0-d31 TG)	48:0-d31 TG	TAG	Qmx
Glyceryl Tri(octadecanoate-d35) (54:0-d35 TG)	54:0-d35 TG	TAG	Qmx
arachidonic acid d8	arachidonic acid d8	PUFA	Cayman Chemicals
linoleic acid d4	linoleic acid d4	PUFA	Cayman Chemicals
11(12)-EET-d11	11(12)-EET-d11	oxFA	Cayman Chemicals

Prostaglandin E2-d4	Prostaglandin E2-d4	oxFA	Cayman Chemicals
12-HETE-d8	12-HETE-d8	oxFA	Cayman Chemicals
TxB2-d4	TxB2-d4	oxFA	Cayman Chemicals
LTB2-d4	LTB2-d4	oxFA	Cayman Chemicals

oxFA = oxidated fatty acid

Table 3. *UHPLC/MS QqQ detected metabolites from the MS/MS analysis of placenta samples.*

compound name	**t_R (min)**	**Precursor ion (m/z)**	**Product ion (m/z)**	**Ionisation**	**CE (V)**	**RF Lens (V)**	**Method**
2/3-phosphoglyceric acid	4.0	185.0	97.1	Negative	16	53	normal phase analysis
2 hydroxybutyric acid	1.9	103.0	57.2	Negative	12	44	reversed phase analysis
5 hydroxytryptophan	4.1	221.2	204.1	Positive	11	58	reversed phase analysis
acetylCoA	3.3	810.2	303.0	Positive	33	147	normal phase analysis
aconitic acid	3.9	173.1	85.1	Negative	15	39	normal phase analysis
adenine	3.3	136.1	119.2	Positive	22	88	reversed phase analysis
adenosine	1.2	268.1	136.1	Positive	18	68	normal phase analysis
adenosine diphosphate	3.9	428.0	136.2	Positive	24	91	normal phase analysis
adenosine monophosphate	3.8	346.1	134.1	Negative	34	108	normal phase analysis
adenosine triphosphate	4.1	506.0	159.0	Negative	32	123	normal phase analysis
adenosine-$^{13}C_{10}$,$^{15}N_5$ 5′-monophosphate	3.8	363.1	146.1	Positive	20	82	normal phase analysis
adenosine-$^{15}N_5$ 5′-triphosphate	4.1	513.0	141.2	Positive	32	102	normal phase analysis
alanine	0.7	90.1	44.3	Positive	11	30	reversed phase analysis
alphaketobutyric acid	2.9	103.6	57.9	Positive	14	56	normal phase analysis
alpha ketoglutaric acid	3.4	145.0	101.1	Negative	8	42	normal phase analysis
aminoadipic acid	0.8	162.1	116.2	Positive	11	57	reversed phase analysis
aminoisobutyric acid	0.8	104.0	85.9	Positive	5	36	reversed phase analysis
arginine	0.7	175.2	70.2	Positive	23	52	reversed phase analysis
asparagine	0.6	133.1	116.0	Positive	8	42	reversed phase analysis
aspartic acid	0.7	134.2	74.2	Positive	15	31	reversed phase analysis
asymetric dimethyl arginine	1.0	189.3	70.2	Positive	23	52	reversed phase analysis
betaine	1.1	118.1	58.2	Positive	26	67	reversed phase analysis
biphosphoglyceric acid	0.5	265.0	97.1	Negative	22	80	normal phase analysis
choline	0.8	104.1	60.1	Positive	15	60	reversed phase analysis

citric acid	1.4	191.0	111.0	Negative	15	53	reversed phase analysis
citrulline	0.8	176.2	159.1	Positive	8	59	reversed phase analysis
creatine	0.9	132.0	43.1	Positive	30	30	reversed phase analysis
creatinine	0.9	114.1	86.1	Positive	11	49	reversed phase analysis
cyclic adenosine monophosphate	1.8	330.1	136.2	Positive	25	91	normal phase analysis
cyclic guanosine monophosphate	2.8	346.0	152.1	Positive	21	86	normal phase analysis
cytidine	1.2	244.1	112.1	Positive	14	39	reversed phase analysis
cytidine 5'-diphosphate	4.0	402.0	158.9	Negative	27	115	normal phase analysis
cytidine 5'-diphosphocholine	3.8	489.1	184.1	Positive	38	89	normal phase analysis
cytidine 5'-monophosphate	4.0	324.1	112.2	Positive	15	62	normal phase analysis
cytidine triphosphate	4.2	484.0	112.2	Positive	21	91	normal phase analysis
cytosine	1.1	112.0	95.1	Positive	18	84	reversed phase analysis
dihydroxyacetonephosphate	3.9	169.0	97.1	Negative	12	48	normal phase analysis
dimethylallylpyrophosphate	3.1	244.9	79.0	Negative	22	124	normal phase analysis
dimethylglycine	3.5	104.0	62.9	Positive	10	54	normal phase analysis
flavin adenine dinucleotide	3.5	786.2	348.2	Positive	20	146	normal phase analysis
fructose biphosphate	4.2	339.0	97.1	Negative	22	83	normal phase analysis
fumaric acid	3.6	115.0	71.2	Negative	7	50	normal phase analysis
gamma amino butyric acid	0.8	104.0	41.0	Positive	10	30	reversed phase analysis
glucose	2.5	179.2	89.1	Negative	10	0	normal phase analysis
glucose/fructose-6phosphate	4.1	259.0	97.1	Negative	17	60	normal phase analysis
glutamic acid	0.7	148.0	84.2	Positive	16	46	reversed phase analysis
glutamic acid $^{13}C_5$ ^{15}N	0.7	154.1	89.1	Negative	13	55	reversed phase analysis
glyceraldehyde 3-phosphate	3.8	169.0	97.0	Negative	10	54	normal phase analysis
guanine	3.9	152.1	135.1	Positive	19	84	normal phase analysis
guanosine	2.0	284.1	152.1	Positive	15	80	normal phase analysis
guanosine monophosphate	4.0	362.1	211.1	Negative	20	98	normal phase analysis
guanosine triphosphate	4.2	524.0	152.1	Positive	24	95	normal phase analysis

guanosine diphosphate	4.1	442.0	150.1	Negative	27	120	normal phase analysis
histidine	0.7	156.1	110.2	Positive	13	45	reversed phase analysis
homocysteine	0.9	136.0	90.1	Positive	10	52	reversed phase analysis
hydroxyl methyl glutaryl CoA	3.9	912.2	405.2	Positive	33	249	normal phase analysis
hydroxyproline	0.9	132	86	Positive	8	35	reversed phase analysis
hydroxytirosine	1.1	198.1	152.0	Positive	12	57	reversed phase analysis
inosine	1.6	269.2	137.1	Positive	10	104	normal phase analysis
hypotaurine	0.8	110.1	91.9	Positive	10	51	normal phase analysis
hypoxanthine	1.2	137.1	119.1	Positive	20	249	reversed phase analysis
isoleucine	1.7	132.0	86.2	Positive	8	35	reversed phase analysis
isopentenyl pyrophosphate	3.1	245.3	79.1	Negative	22	135	normal phase analysis
lattic acid	1.6	89.0	43.1	Negative	10	33	normal phase analysis
leucine	2.0	132.0	86.2	Positive	8	35	reversed phase analysis
leucine d_{10}	2.0	142.0	96.2	Positive	8	35	reversed phase analysis
malic acid	3.7	133.0	115.1	Negative	12	51	normal phase analysis
methyl arginine	0.9	189.3	70.2	Positive	23	52	reversed phase analysis
methyl cytosine	1.4	126.1	109.1	Positive	19	78	reversed phase analysis
methionine	1.2	150.1	61.2	Positive	23	36	reversed phase analysis
methionine sulfoxide	3.6	166.0	149.1	Positive	12	48	reversed phase analysis
methyl histidine	0.7	170.2	95.2	Positive	30	59	reversed phase analysis
nicotinamide adenine dinucleotide	3.8	664.1	136.2	Positive	43	103	normal phase analysis
nicotinamide adenine dinucleotide hydrate	3.6	666.2	301.9	Positive	38	100	normal phase analysis
nicotinamide adenine dinucleotide phosphate	4.2	742.1	408.1	Negative	35	97	normal phase analysis
nicotinamide adenine dinucleotide phosphate hydrate	3.9	746.1	728.9	Positive	17	126	normal phase analysis
nicotinamide	1.2	123.2	80.2	Positive	20	30	reversed phase analysis

nicotinic acid	1.2	124.1	80.1	Positive	21	168	reversed phase analysis
ornithine	0.6	133.2	70.2	Positive	16	44	reversed phase analysis
phenyl-d_5-alanine	3.6	171.1	125.2	Positive	13	41	reversed phase analysis
phenilalanine	3.6	166.1	120.2	Positive	13	41	reversed phase analysis
phosphocreatine	3.9	210.0	79.0	Negative	16	50	normal phase analysis
phosphoenolpyruvate	3.9	167.0	79.1	Negative	14	45	normal phase analysis
proline	0.8	116.0	70.2	Positive	15	36	reversed phase analysis
proline-2,5,5-d_3	0.8	121.2	74.2	Positive	17	46	reversed phase analysis
pyruvic acid	0.6	87.0	42.9	Negative	10	43	normal phase analysis
riboflavin	4.4	377.2	243.1	Positive	22	249	reversed phase analysis
ribulose 5phosphate	3.9	229.3	97.0	Negative	10	121	normal phase analysis
adenosyl methionine	3.9	399.1	250.1	Positive	16	79	normal phase analysis
adenosyl-homocysteine	3.5	385.1	136.1	Positive	20	73	normal phase analysis
sarcosine	0.7	90.1	44.3	Positive	11	30	reversed phase analysis
serine	0.7	106.1	60.2	Positive	10	39	reversed phase analysis
serotonine d_4	3.8	181.2	164.1	Positive	10	61	reversed phase analysis
succinic acid	1.8	117.0	73.0	Negative	10	45	reversed phase analysis
succinic acid-1,4-$13C_2$	1.8	119.0	74.0	Negative	10	45	reversed phase analysis
taurine	0.7	126.1	44.1	Positive	14	93	reversed phase analysis
threonine	0.7	120.1	74.1	Positive	9	66	reversed phase analysis
tryptophan	4.2	205.2	188.1	Positive	11	58	reversed phase analysis
tyrosine	2.3	182.1	136.1	Positive	12	46	reversed phase analysis
uracil	1.1	113.0	96.1	Positive	17	87	reversed phase analysis
uridine	1.2	245.1	112.9	Positive	12	46	reversed phase analysis
uridine diphosphate	4.1	403.0	159.0	Negative	28	103	normal phase analysis
uridine diphosphate glucose	3.9	565.1	323.0	Negative	23	249	normal phase analysis
uridine diphosphate N-acetyl glucosamine	3.8	606.1	159.0	Negative	28	103	normal phase analysis

uridine monophosphate	3.9	325.0	97.2	Positive	15	64	normal phase analysis
uridine triphosphate	4.2	483.0	159.0	Negative	31	117	normal phase analysis
valine	1.1	118.1	72.2	Positive	10	67	reversed phase analysis
valine d_8	1.1	126.1	80.2	Positive	10	41	reversed phase analysis

tR=retention time, CE= collision energy, RF=radio frequency, V=voltage

Table 4.*UHPLC/MS QqQ detected metabolites from the MS/MS analysis of placenta samples.*

Acylcarnitines	**t_R (min)**	**Precursor ion (m/z)**	**Product ion (m/z)**	**CE(V)**	**RF Lens (V)**	**MW *p* value**
C0	0.89	162.1	85.1	23	71	0.4411
C2	2.51	204.1	85.2	20	72	0.3650
C3	3.25	218.1	85.0	20	78	0.0352
C4	4.01	232.1	85.2	23	78	0.5699
C5-OH	3.27	262.1	85.1	21	81	0.1266
C5:1	4.62	244.1	85.2	21	81	0.4822
C5	4.94	246.2	85.2	21	81	0.2119
C6	5.88	260.2	85.2	21	81	0.0594
C8:1	6.5	286.2	85.2	23	94	0.0006
C8	7.37	288.2	85.2	23	94	0.8676
C10:2	7.5	312.2	85.2	23	94	0.0240
C10	8.17	316.2	85.2	23	94	0.9734
C5-DC	7.46	332.2	85.2	23	94	>0.9999
C12:1	8.4	342.2	85.1	26	122	0.8676
C12	9.01	344.2	85.1	26	122	0.2119
C14-OH	9.04	388.3	85.1	26	122	0.2382
C14:2	8.17	368.2	85.1	26	122	0.5252
C14:1	9.2	370.3	85.1	26	122	0.1100
C14	9.46	372.3	85.1	26	122	0.0817
C8-DC	9.46	374.3	85.1	26	122	0.0421
C15	9.64	386.3	85.1	27	123	0.2667
C16:1-OH	9.01	414.3	85.1	27	123	0.1100
C16:2	9.35	396.3	85.1	27	123	0.1100
C16:1	9.55	398.3	85.1	27	123	0.1876
C16-OH	9.51	416.3	85.1	27	123	0.1100
C16	9.77	400.3	85.1	27	123	0.1450
C17	9.61	414.3	85.1	27	123	0.0292
C18:2-OH	9.36	440.3	85.1	27	123	0.0421
C18:1-OH	9.59	442.3	85.1	27	123	0.0950
C18-OH	9.82	444.3	85.1	27	123	0.0292
C18:2	9.66	424.3	85.1	27	123	0.1100
C18:1	9.81	426.3	85.1	27	123	0.0502
C18	9.98	428.3	85.1	27	123	0.0292
C20:4	9.63	448.3	85.1	27	123	0.2667
C20:2	9.88	452.3	85.1	27	123	0.0103
C20:1	10	454.3	85.1	27	123	0.0128
C20	10	456.3	85.1	27	123	0.0081

tR=retention time, CE= collision energy, RF=radio frequency, V=voltage

Table 5. Discriminant lipids of preeclamptic placenta samples identified by DTIM-QTOF-LC/MS-MS.

Lipid	Adduct	m/z experimental	m/z theoretical	Δppm	Fatty acid composition	t_R (min)	$^{DT}CCS_{N2}$ (Å^2)	Product ion (m/z)
LysoPC 16:0	$+H^+$	496.3375	496.3398	-5.7	16:0	1.37	228.45	313.3, 258.1, 184.1, 104.1
LysoPC 18:1	$+H^+$	522.3548	522.3554	-2.2	18:1	1.43	231.31	339.3, 237.3, 184.1, 104.1
Stearoylcarnitine	$+H^+$	428.3720	428.3734	-4.6	18:0	1.86	219.3	144.1, 85.0, 60.1
SM 34:2	$+H^+$	701.5569	701.5592	-4.1	18:2, 16:0	5.26	278.99	518.5, 262.2, 184.7
SM 34:2	$+Na^+$	723.5390	723.5411	-3.7	18:2, 16:0	5.26	279.66	701.5, 518.5, 262.2, 184.7
PC 36:3	$+H^+$	784.5822	784.5851	-4.4	16:0, 20:3	8.21	289.03	601.5, 528.3, 496.3, 363.3, 313.3,184.1
PC 38:4	$+H^+$	810.5989	810.6007	-2.9	18:1, 20:3	8.44	292.81	522.3, 339.2, 289.2, 184.1
PC 36:4	$+H^+$	768.5879	768.5902	-3.7	16:0, 20:4	8.65	288.23	585.3, 485.1, 287.3, 184.7
PC 38:5	$+H^+$	794.6041	794.6058	-2.8	18:1, 20:4	8.80	292.41	611.3, 508.2, 455.1, 362.8, 184.7
SM 36:1	$+Na^+$	753.5875	753.5881	-1.5	18:1, 18:0	8.99	289.39	731.6, 450.2, 322.9, 264.4, 184.7
SM 36:1	$+H^+$	731.6043	731.6062	-3.3	18:1, 18:0	8.99	288.36	450.2, 322.9, 264.4, 184.7
PC 38:3	$+H^+$	812.6135	812.6164	-4.2	18:0, 20:3	10.96	294.76	629.5, 528.3, 449.9, 341.3, 184.1
PC 32:0	$+H^+$	720.5877	720.5902	-4.2	14:0, 18:0	11.00	284.69	509.8, 227.8, 184.7
PC 38:4	$+H^+$	796.6186	796.6215	-4.3	18:0, 20:4	11.59	294.91	612.6, 361.2, 341.2, 226.1, 184.7
PEt P 38:4	$+H^+$	752.5557	752.5589	-4.9	18:0, 20:4	11.73	279.9	611.5, 466.3, 392.3, 361.3

SM 42:3	$+H^+$	811.6664	811.6688	-3.6	18:2, 24:1	12.51	301.27	628.4, 262.4, 184.7
SM 30:2	$+H^+$	785.6495	785.6531	-5.3	18:2, 22:0	12.64	297.87	601.6, 369.9, 322.2, 184.7
SM 41:2	$+H^+$	799.6664	799.6688	-3.6	17:1, 24:1	13.37	300.48	758.4, 344.1, 250.5, 184.7
SM 42:2	$+H^+$	813.6813	813.6844	-4.5	18:2, 24:0	13.58	304.48	612.5, 309.2, 262.5, 184.7
SM 42:2	$+Na^+$	835.6651	835.6663	-2.1	18:1, 24:1	13.72	302.53	813.7, 612.5, 366.4, 264.4, 184.7
SM 42:2	$+H^+$	813.6821	813.6844	-3.5	18:1, 24:1	13.72	304.27	612.5, 366.4, 264.4, 184.7
SM 42:1	$+Na^+$	837.6807	837.6820	-2.2	18:1, 24:0	14.25	307.36	815.7, 426.2, 264.4, 184.7
SM 42:1	$+H^+$	815.6979	815.7001	-3.3	18:1, 24:0	14.25	306.72	426.2, 264.4, 184.7
SM 34:2	$+OAc^-$	759.5642	759.5658	-2.8	18:2, 16:0	5.26	283.53	685.3, 255.2, 168.1
Cer 34:2	$+OAc^-$	594.5087	594.5103	-3.6	18:2, 16:0	7.29	251.99	534.5, 516.5, 296.2, 280.3, 261.2
Cer 34:2	$-H^-$	534.4870	534.4892	-5.1	18:2, 16:0	7.29	252.28	516.5, 296.2, 280.3, 261.2
PE P 38:4	$-H^-$	750.5427	750.5443	-2.9	18:0, 20:4	11.52	272.83	464.3, 303.2, 267.3, 259.2
SM 42:3	$+OAc^-$	869.6737	869.6753	-2.5	18:2, 24:1	12.35	303.72	795.8, 365.3, 168.0
SM 42:2	$+OAc^-$	843.6575	843.6597	-3.2	18:1, 22:1	12.49	300.05	769.6, 337.3 , 168.0
SM 42:2	$-H^-$	871.6894	871.6910	-2.4	18:1, 24:1	13.55	305.26	685.5, 255.2, 168.0
SM 40:1	$+OAc^-$	845.6737	845.6753	-2.6	18:1, 22:0	13.59	301.50	771.6, 339.3, 168.0
SM 42:2	+OAc-	871.6895	871.6910	-2.3	18:2, 24:0	13.69	305.29	797.6, 367.5, 168.0

Cer 42:3	+OAc-	704.6181	704.6163	-3.3	18:2, 24:1	13.76	276.1	644.6, 614.6, 390.4, 364.6, 347.3, 261.2, 235.2
Cer 40:0	+OAc-	678.6026	678.6042	-3.2	18:2, 22:0	13.79	271.47	618.6, 380.5, 364.3, 338.3, 321.3, 261.2, 249.2
SM 42:1	$+OAc^{-}$	873.7051	873.7066	-2.4	18:1, 24:0	14.21	306.76	799.8, 367.6, 168.0, 122.9
Cer 42:2	+OAc-	706.6338	706.6355	-3.2	18:1, 24:1	14.24	277.48	646.6, 616.6, 390.4, 364.6, 347.3 237.2, 263.2

Table 6. *Relative abundance of fatty acids content in Preeclampsia vs normotensive controls Group. Results are expressed as mean ± standard deviation. Mann Whitney U test was used to test for differences among groups.*

Lipid class	**Controls**	**PE**	**MW *p* values**
FFA (% lipid annotated)	3.2±0.9	3.4±0.6	0.2973
SFA (% lipid annotated)	2.4±0.8	2.7±0.6	0.6163
MUFA (% lipid annotated)	0.3±0.1	0.3±0.1	0.6335
PUFA (% lipid annotated)	0.5±0.3	0.5±0.3	0.9335

Table 7.*Lipid profile composition of placental samples obtained from the open profile lipid analysis by IM-QTOF-LC/MS-MS.*

Lipid class	Number of features of lipid annotated for each lipid class
Glycerolipids	169 triacylglycerols
	81 diacylglycerols
	5 monoacylglycerols
	1 monogalatosyldiacyglycerol
	1 sulfoquinovosyldiacylglycerol
Glycerophospholipids	42 cardiolipins
	6 lysoPC
	7 lysoPE
	45 glycerophosphates
	434 glycerophosphocoline
	51 glycerophosphoetanolamines
	109 glycerophosphoglycerols
	93 glycerophosphoinositols
	142 glycerophosphoserines
Sphingolipids	101 ceramides
	34 glyceroceramides
	26 lactosylceramides
	16 ceramide phosphoethanolamines
	12 ceramide phosphoinositols
	116 sphingomyelins
Sterols	12 cholesteryl esters
	17 glycerocholesterols
Fatty acyls	20 fatty acids
	15 acyl carnitines

Table 8. *Relative abundance of major lipid classes in control group and preeclampsia samples.*

Lipid class	**Controls (%)**	**Preeclampsia (%)**	**MW *p* value**
Glycerophospholipids	75.5±1.4	76.0±1.1	0.4020
Glycerolipids	3.6±0.6	3.7±0.6	0.9598
Sphingolipids	16.9±0.8	16.3±0.6	0.0594
Sterols	0.5±0.1	0.4±0.2	0.4015
Fatty acyls	3.5±0.9	3.6±0.6	0.5140

Table 9.*Validation parameters of multivariate statistical data analysis of placenta samples by DTIM-QTOF-LCMS. Ptest= permutation test.*

ANALYSIS	**PE *vs* C**			
	R^2X	**R^2Y**	**Q^2**	**Ptest Q^2**
PCA	0,4		0,1	
PLS-DA (based on the pathology)	0,3	0,9	0,7	-0.1
PLS-DA (based on delivery mode)	0,4	0,9	0,7	0.1

Table 10. *Discriminant metabolites from the Mann Whitney U test of PE. The relative analysis was the open profile analysis by DTIM-QTOF -LC/MS. MWp= Mann Whitney p value*

Preeclampsia vs Control group					
Compound	**MW*p***	**Levels in PE**	**Compound**	**MW*p***	**Levels in PE**
LysoPC(16:0)	0.0002	↓	SM(18:2/16:0)	0.0017	↓
LysoPC(18:1)	<0.0001	↓	SM(18:1/18:0)	0.0084	↑
PC(16:0/20:3)	0.0446	↑	SM(18:1/22:1)	<0.0001	↓
PC(18:1/20:3)	0.0193	↑	SM(18:2/22:0)	<0.0001	↓
PC(16:0/20:4)	0.0159	↓	SM(17:1/24:1)	<0.0001	↓
PC(18:1/20:4)	0.0252	↓	SM(18:2/24:1)	<0.0001	↓
PC(18:0/20:3)	0.0168	↑	SM(18:1/24:1)	0.0004	↓
PC(14:0/18:0)	0.0197	↓	SM(18:1/22:0)	0.0050	↓
PC(O-18:0/20:4)	0.0064	↓	SM(18:1/24:0)	0.0061	↓
PEt(P-18:0/20:4)	0.0305	↓	SM(18:2/24:0)	<0.0001	↓
stearoylcarnitine	0.0159	↓	Cer(18:2/16:0)	0.0206	↓
erucic acid	0.0461	↓	Cer(18:2/22:0)	<0.0001	↓
lignoceric acid	0.0081	↓	Cer(18:1/24:1)	0.0187	↓
CE 18:2	0.0441	↓	Cer(18:2/24:1)	0.0001	↓

Table 11.*Discriminant metabolites from the Mann-Whitney U test of placenta polar metabolite profile samples. The relative analysis was the targeted MS/MS analysis by UHPLC/MS QqQ. MWp value= Mann-Whitney p value*

compound	**Mw *p* value**	**Levels in PE**
2/3-phosphoglyceric acid	0.0159	↑
adenine	0.0199	↓
adenosine diphosphate	0.0016	↓
adenosine monophosphate	0.0064	↓
alanine	0.0197	↓
aminoadipic acid	0.0186	↓
aminoisobutyric acid	0.0126	↓
arginine	0.0002	↓
asparagine	0.0016	↓
aspartic acid	0.0003	↓
asymetric dimethyl arginine	<0.0001	↓
citrulline	0.0128	↓
creatine	0.0292	↑
cytidine diphosphocholine	0.0002	↓
cytidine monophosphate	0.0240	↓
dimethylallylpyrophosphate	0.0197	↓
flavin adenine dinucleotide	0.0016	↓
folic acid	0.0368	↑
glutamic acid	0.0029	↓
guanosine monophosphate	0.0022	↓
guanosine diphosphate	0.0352	↓
histidine	0.0103	↓
hydroxymethylglutaryl-CoA	0.0072	↓
hydroxyproline	0.0096	↑
hypoxanthine	0.0034	↓
isoleucine	0.0246	↓
leucine	0.0159	↓
methyl arginine	0.0018	↓
methionine sulfoxide	0.0159	↓
ornithine	0.0126	↓
phenilalanine	0.0368	↓
phosphocreatine	0.0473	↑
proline	0.0103	↓
s-adenosyl-methionine	0.0016	↓
s-adenosyl-homocysteine	0.0159	↓
serine	<0.0001	↓
serotonine	0.0352	↓
taurine	0.0236	↓
threonine	0.0004	↓
uridine diphosphate N-acetyl glucosamine	0.0103	↓
uridine monophosphate	0.0103	↓
valine	0.0246	↓

9 FIGURES

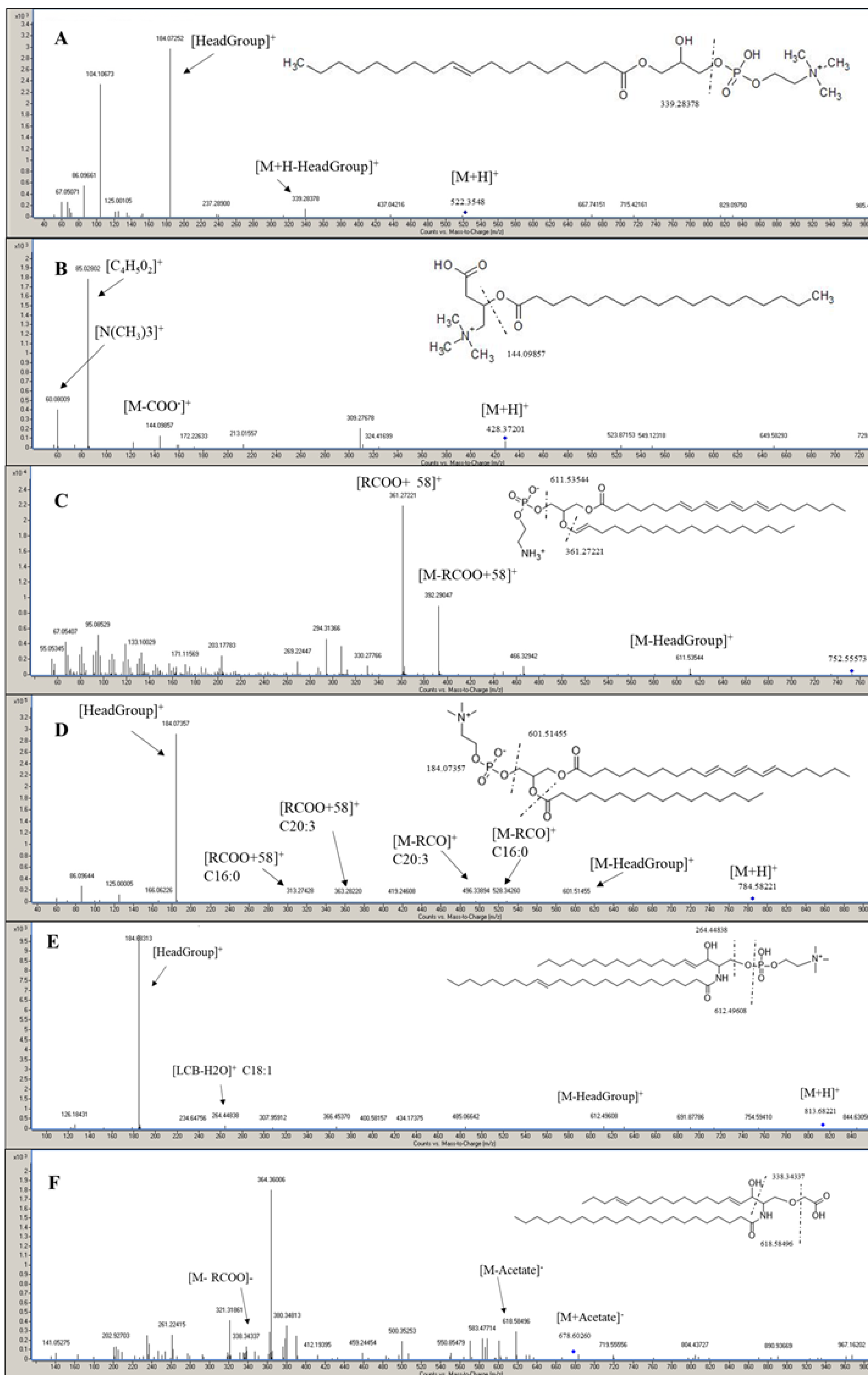

Figure 1. *MS/MS fragmentation of different lipid categories: A LysoPC 18:1; B) Acylcarnitine C18:0; C)PEt (P-18:0/20:4); D)PC (16:0/20:3); E) SM (18:1/24:1); F); Cer (18:2/22:0)*

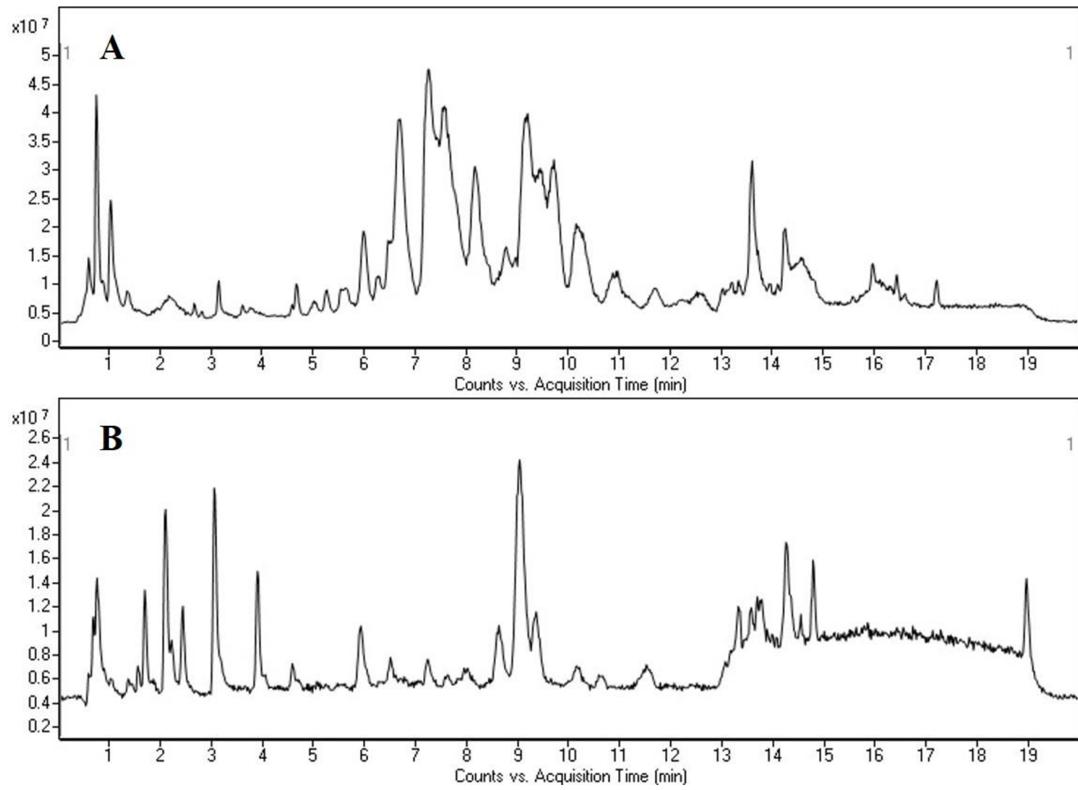

Figure 2. *Examples of DTIM LC/MS chromatograms from the placenta tissue extract. A=positive mode B= negative mode*

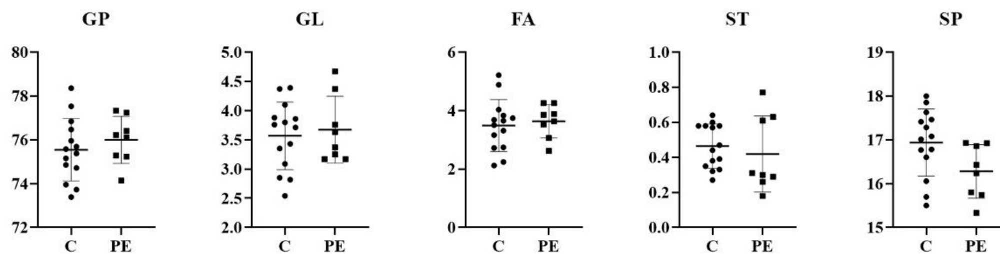

Figure 3.*Scatter plots related to the relative abundance of major lipid categories of placenta samples underwent from the Mann-Whitney U test. The relative analysis was the open profile analysis by DTIM-QTOF -LC/MS. GP=glycerophospholipids, GL=glycerolipids, SP= sphingolipids, FA=fatty acyls and ST=sterols*

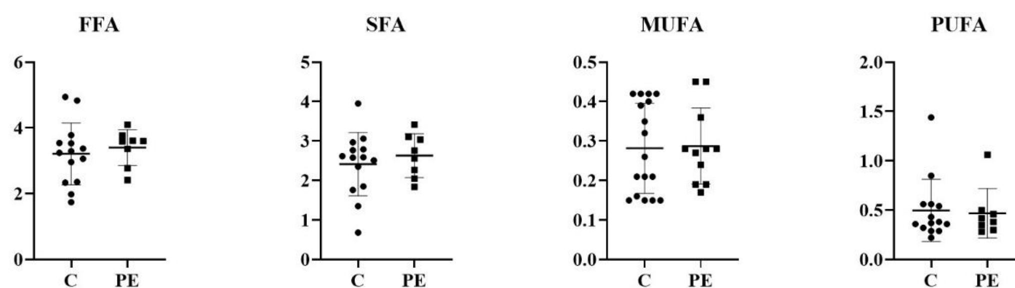

Figure 4. *Scatter plots related to the relative abundance of free fatty acids content of placenta samples underwent from the Mann Whitney U test. The relative analysis was the open profile analysis by DTIM-QTOF-LC/MS in negative mode. % of lipid annotated is shown in the y axis. FFA=free fatty acids, SFA= saturated fatty acids, MUFA= monounsaturated fatty acids, PUFA= polyunsaturated fatty acid.*

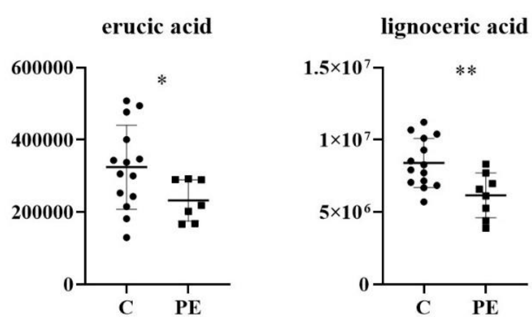

Figure 5. *Scatter plots related to the free fatty acids discriminant underwent from the Mann Whitney U test. The relative analysis was the open profile analysis by DTIM-QTOF-LC/MS in negative mode. Variable intensities are shown in the y axis. *,** indicates levels of significance with a p value<0.05, <0.01 respectively.*

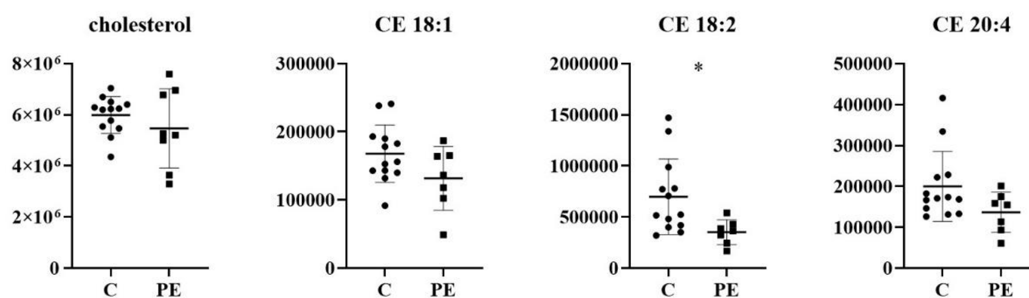

Figure 6*. Scatter plots related to the cholesterol and cholesteryl esters (CE) analysis from the Mann-Whitney U test. The relative analysis was the open profile analysis by DTIM-QTOF-LC/MS in positive mode. Variable intensities are shown in the y axis. * indicates levels of significance with a p value <0.0.*

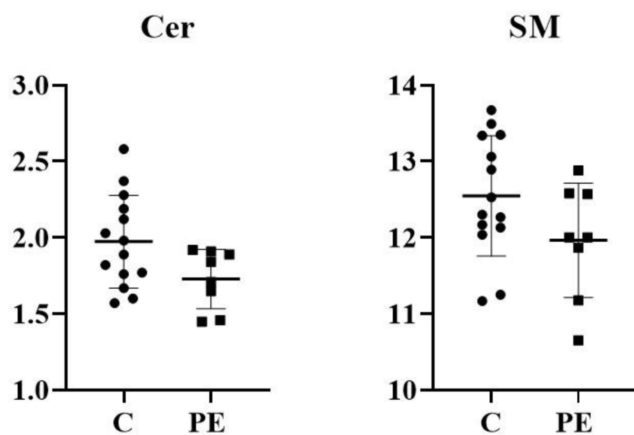

Figure 7. *Scatter plots related to ceramides and sphingomyelins content in placenta samples submitted to the Mann-Whitney U test. The relative analysis was the open profile analysis by DTIM-QTOF -LC/MS. Relative abundance of lipid class in comparison to annotated lipids is shown in the y axis.*

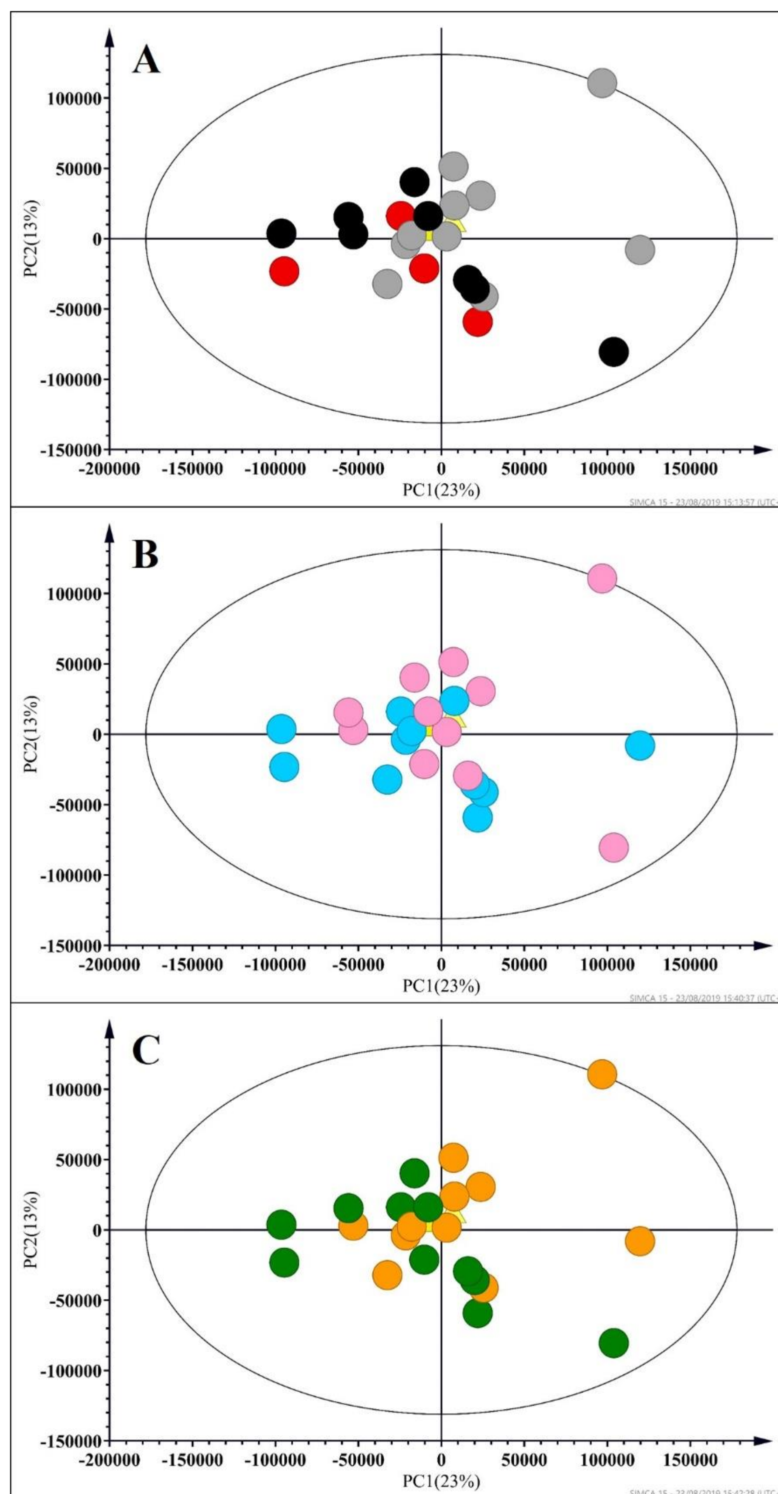

Figure 8. *Score plots from the Agilent Ion mobility-Q-TOF open profile lipid analysis. A) Scores plot from the PCA coloured on the bases of pathology. QC: yellow filled triangles, PE samples: black filled circles, Preterm controls: dark grey filled circles, Term controls: red filled circles. B) Scores plot from PCA based on the gender of the babies QC: yellow filled triangles, Female: pink filled circles, Male: blue filled circles. C) Scores plot from PCA based on delivery mode QC: yellow filled triangles, vaginal delivery: orange filled circles, caesarean section: green filled circles. R2X and a Q2 of 0.36 and 0.08, respectively. The two principal components described 23% and 13% of the total variance, respectively.*

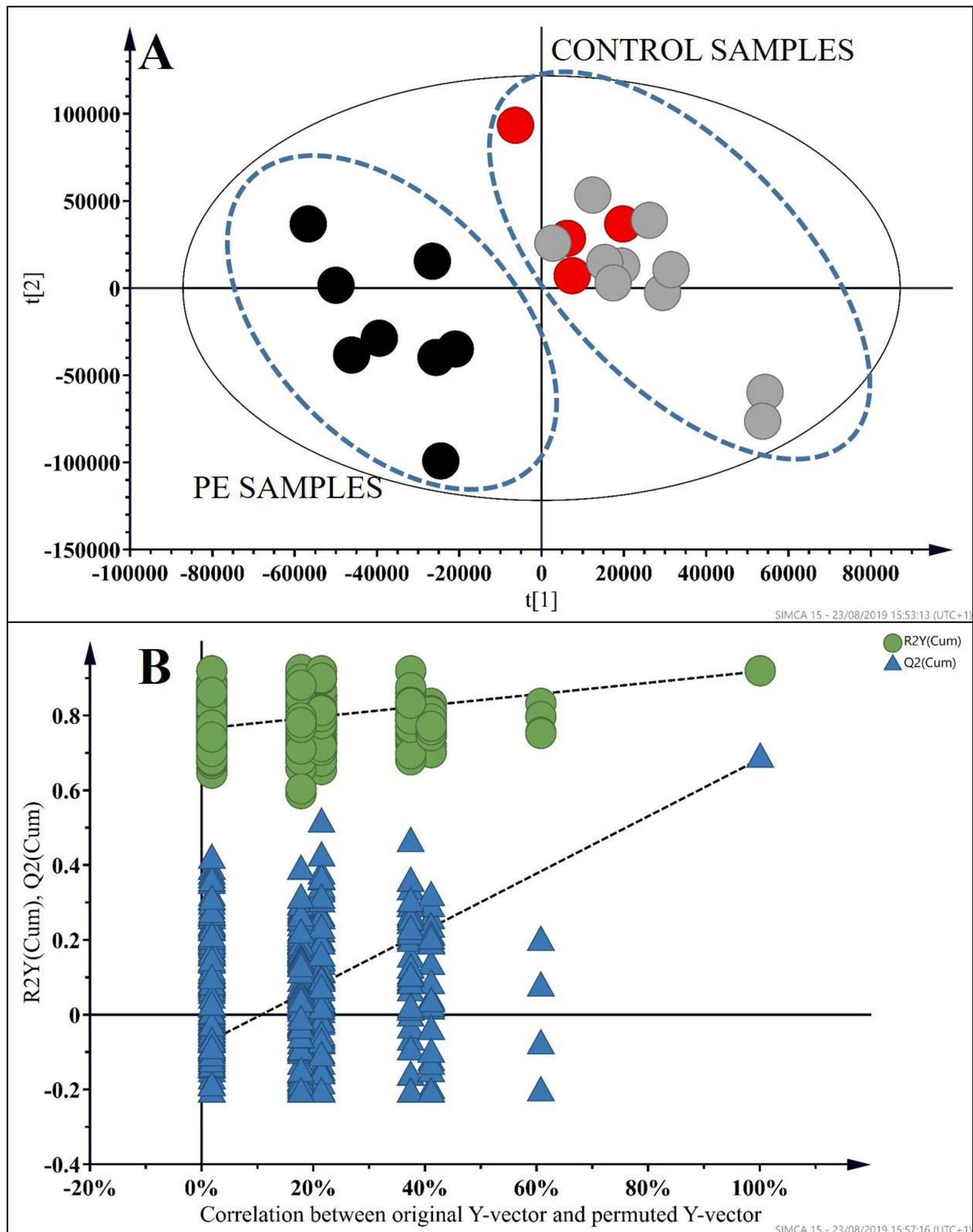

Figure 9. *Scores plot from the Agilent Ion mobility-Q-TOF open profile lipid analysis. A) Scores plot from PLS-DA based on the pathology: PE samples (black filled circles) vs normotensive control samples (term controls: red filled circles and preterm controls: grey filled circles).* $R^2Y=0.92$ *and* $Q^2=0.68$ *B) Permutation test from the PLS-DA of PE samples vs normotensive samples (Q2=-0.07). Green circles represent* R^2Y *and blue triangles the* Q^2*.*

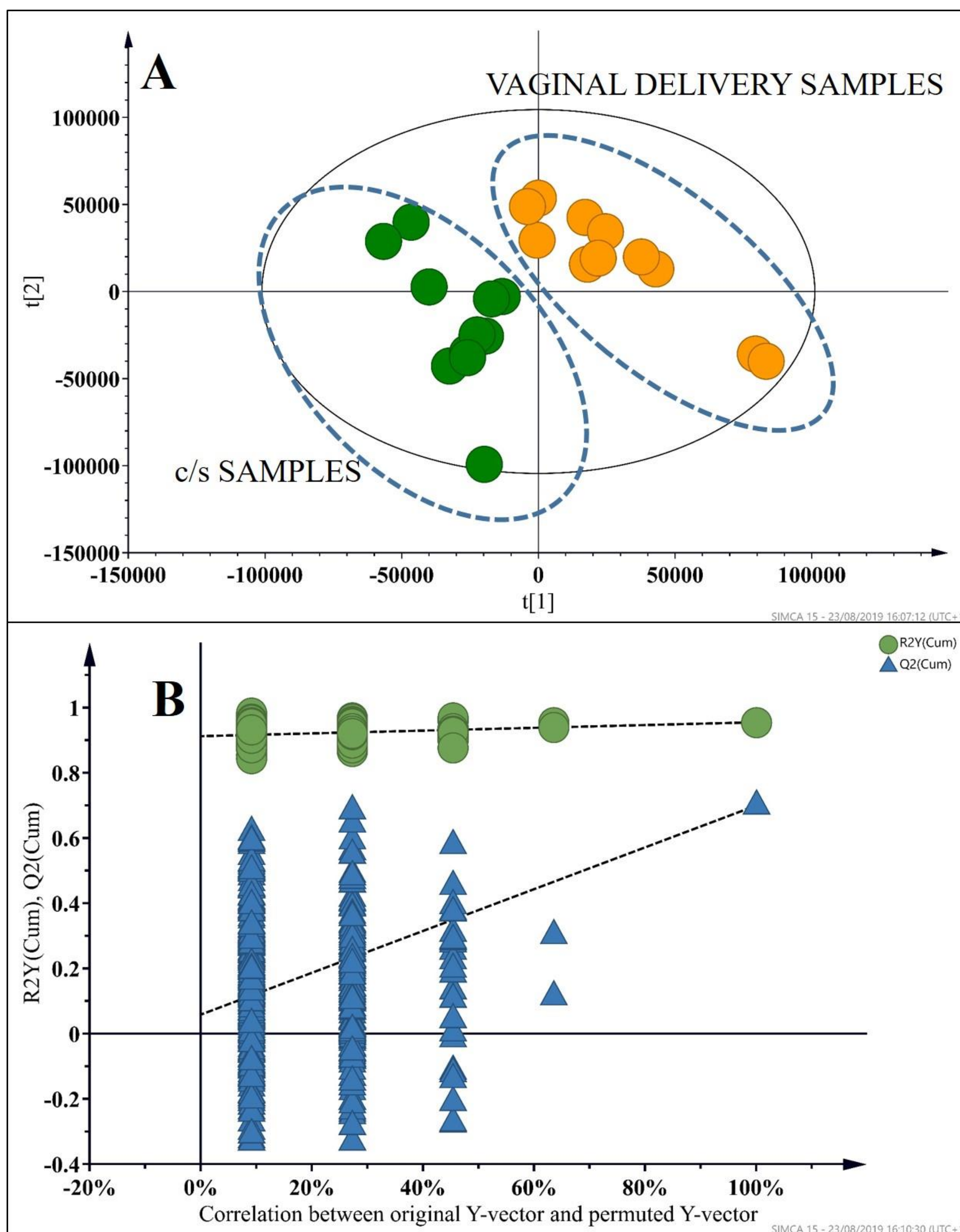

Figure 10. *Scores plot from the Agilent Ion mobility-Q-TOF open profile lipid analysis. A) Score plot from the PLS-DA based on the delivery mode: vaginal delivery (orange filled circles) vs caesarean section (c/s) samples (green filled circles).* R^2Y*= 0.95 and* Q^2*= 0.69. B) Permutation test from PLS-DA based on the delivery mode (Q2=0,08). Green circles represent* R^2Y *and blue triangles the* Q^2*.*

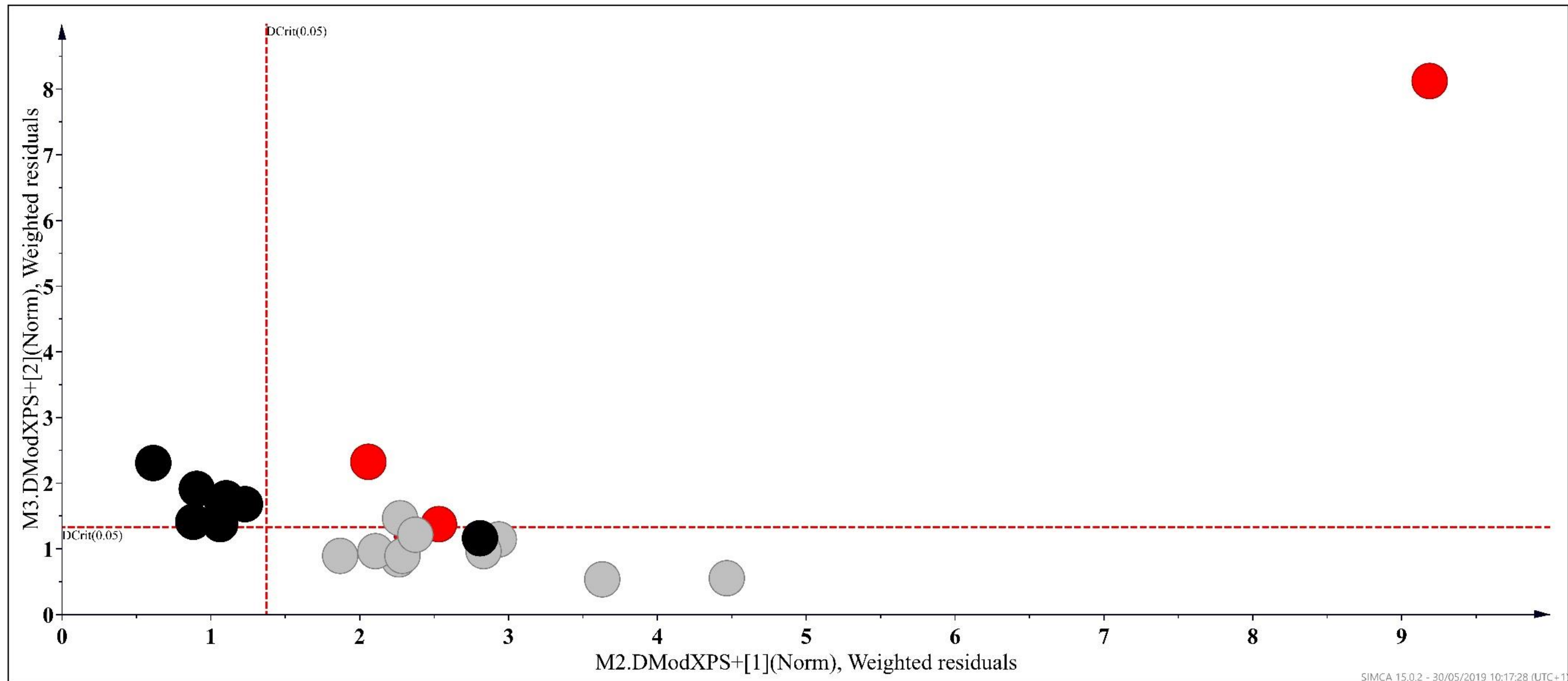

Figure 11.*Cooman's Plot. PE samples: black filled circles, Normotensive Preterm controls: grey filled circles, Normotensive Term controls: red filled circles*

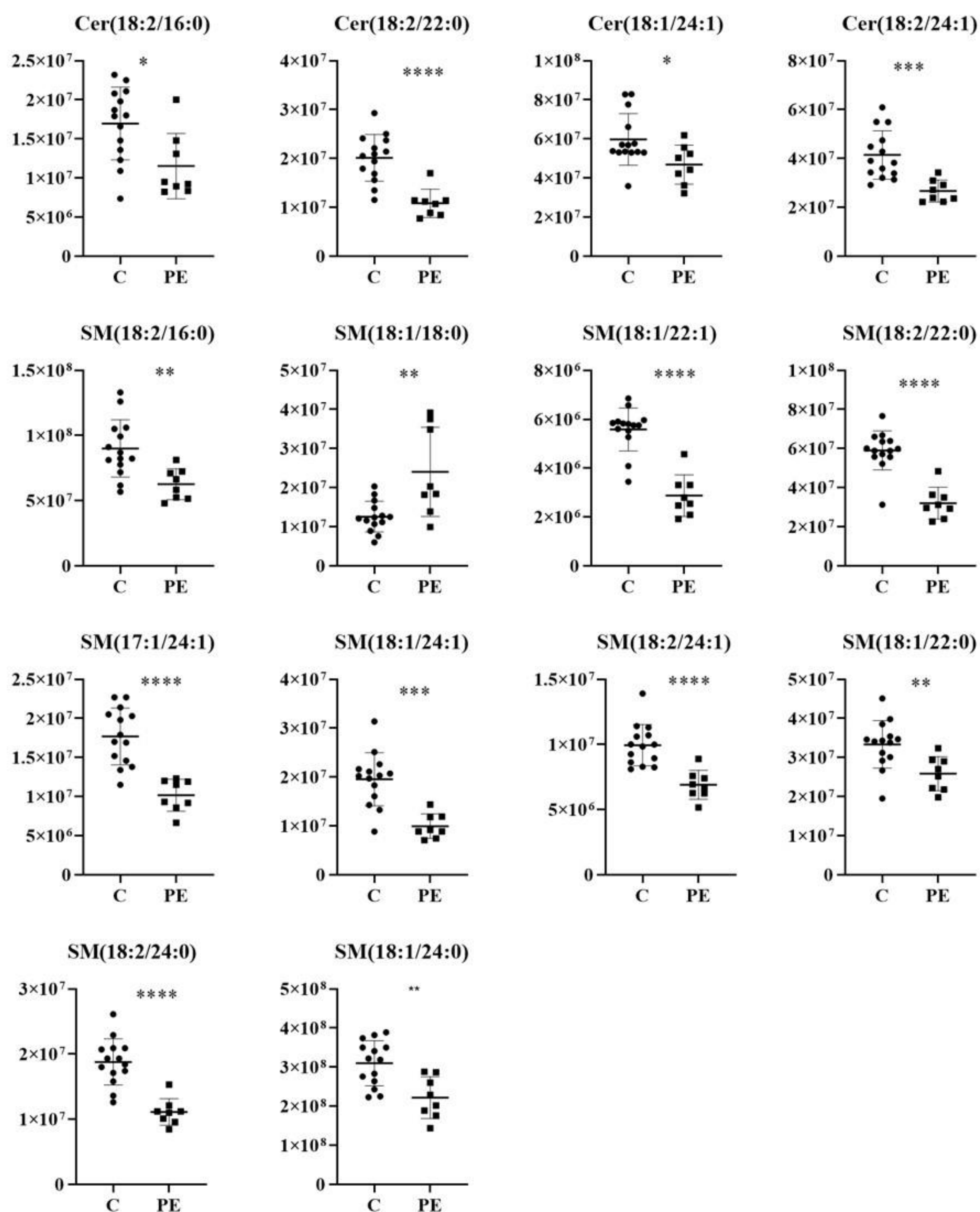

Figure 12. *Scatter plots related to discriminant metabolites analysed by the Mann-Whitney U test. The relative analysis was the open profile assay by DTIM-QTOF-LC/MS. Variable intensities are shown in the y axis. *, **, *** and **** indicates levels of significance with a p value <0.05, <0.01 <0.005 and <0.0001, respectively.*

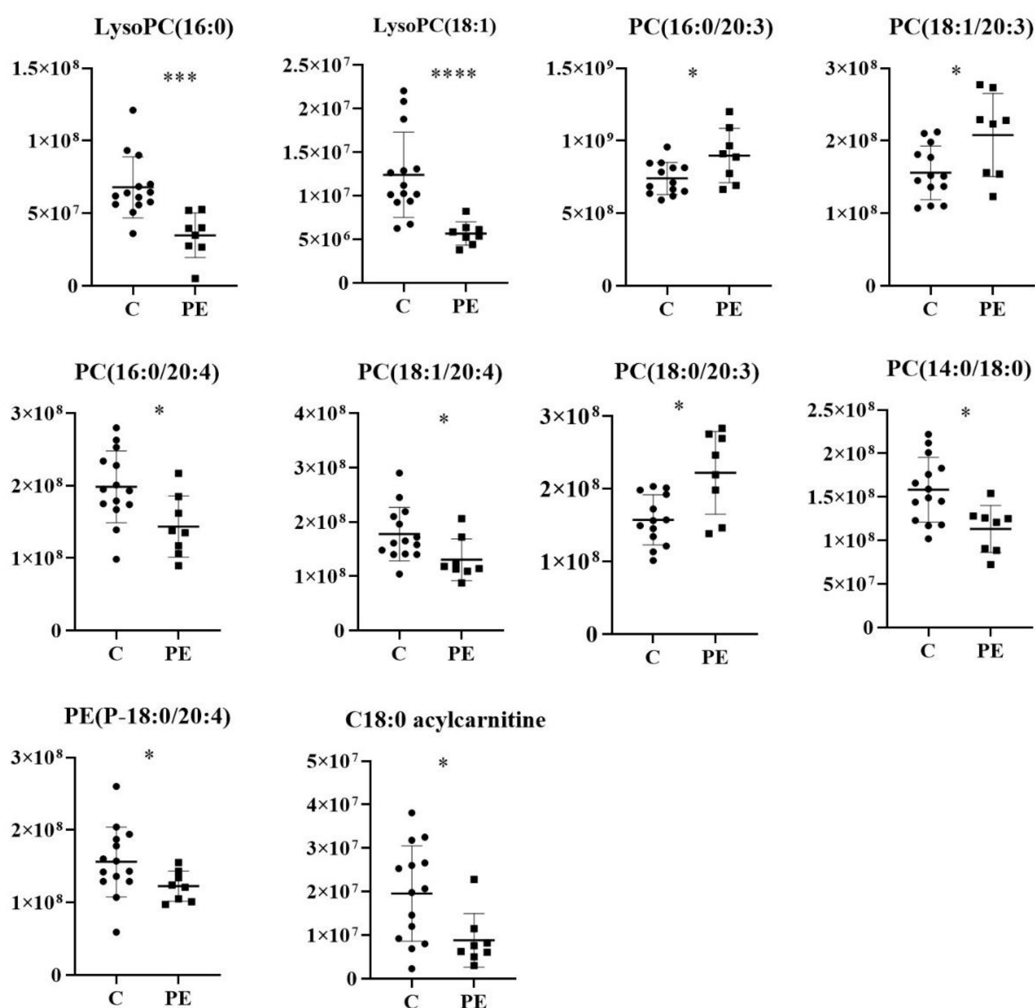

Figure 13. *Scatter plots related to discriminant metabolites submitted to the Mann-Whitney U test from the open profile analysis by DTIM-QTOF-LC/MS. Variable intensities are shown in the y axis. *, **, *** and **** indicates levels of significance with a p value <0.05, <0.01 <0.005 and <0.0001, respectively.*

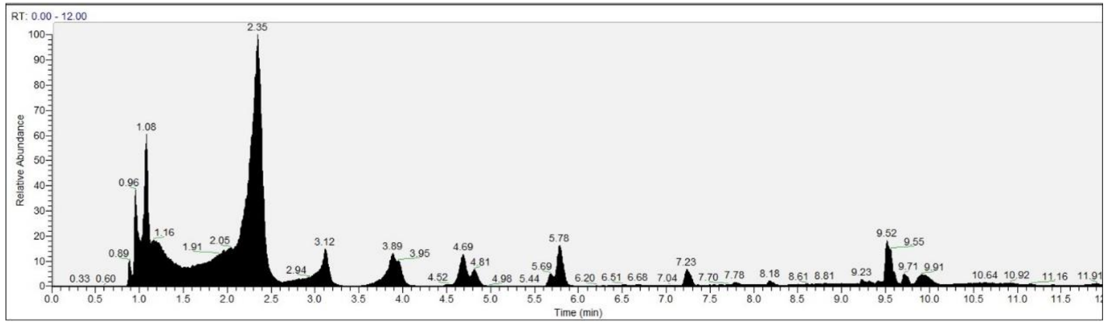

Figure 14. *Example of LC/MS QqQ chromatogram from the placenta acylcarnitines profile analysis.*

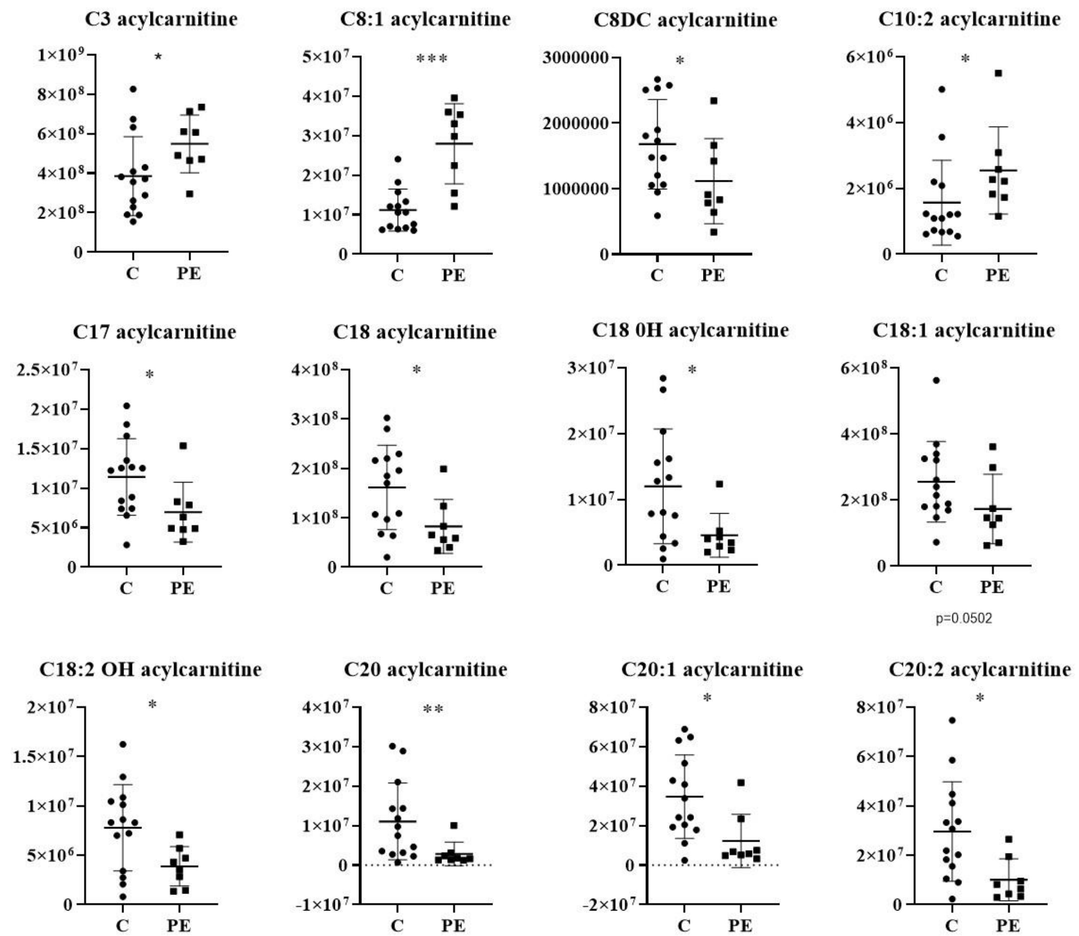

Figure 15. *Scatter plots related to the acylcarnitines assay analysed by the Mann-Whitney test. Variable intensities are shown in the y-axis. *, **, and *** indicates levels of significance with a p value<0.05, <0.01, <0.001, respectively.*

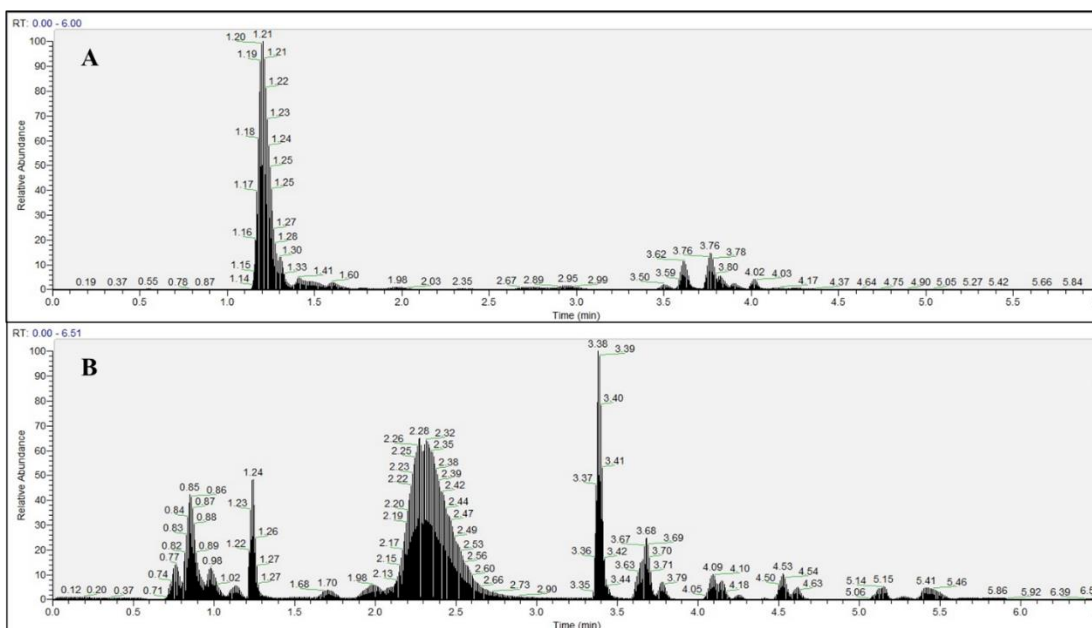

Figure 16. *Example of LC/MS QqQ chromatograms from the placenta polar phase analyses. A=normal phase analysis, B=reversed phase analysis*

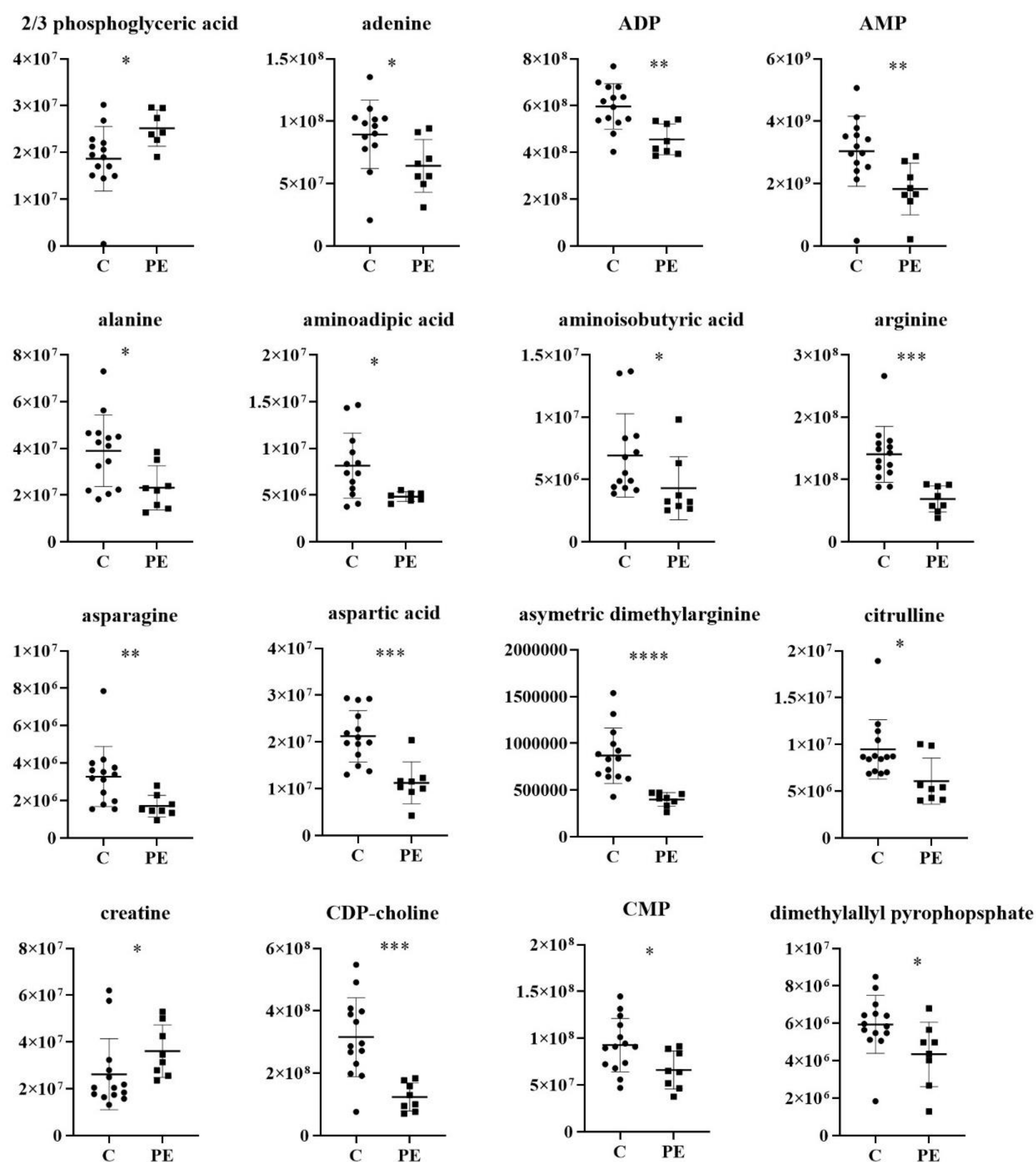

Figure 17. *Scatter plots related to the most discriminant metabolites of placenta samples from the Mann Whitney U test. The assay was the targeted MS/MS analysis by LC/MS QqQ. Variable intensities are shown in the y-axis. *, **, ***and **** indicates levels of significance with a p value<0.05, <0.01, <0.001 and <0.0001, respectively.*

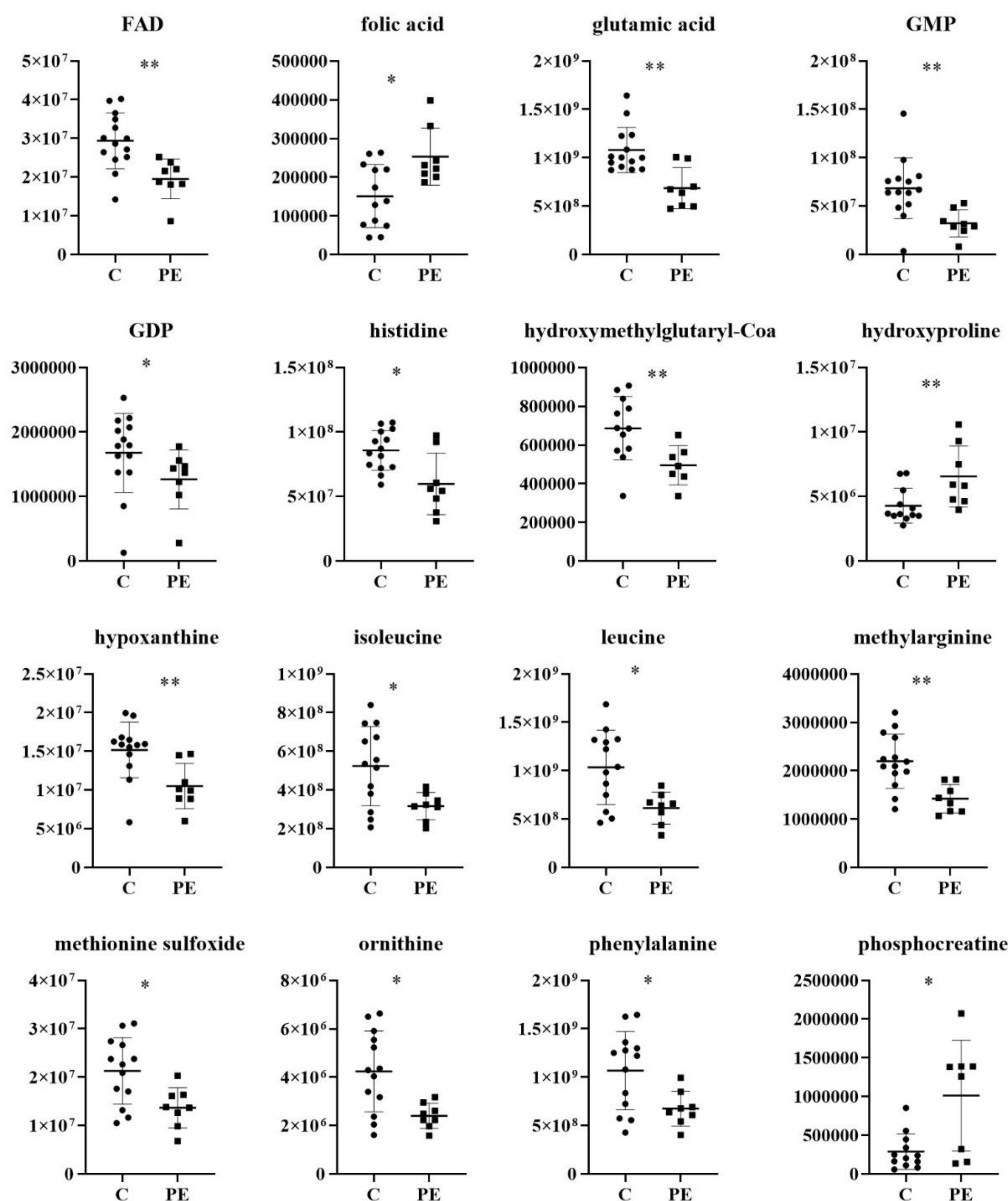

Figure 18. *Scatter plots related to the most discriminant metabolites of placenta samples processed by the Mann-Whitney test. The relative analysis was the targeted MS/MS analysis by LC/MS QqQ. Variable intensities are shown in the y-axis. *, **, *** and **** indicates levels of significance with a p value<0.05, <0.01, <0.001 and <0.0001, respectively.*

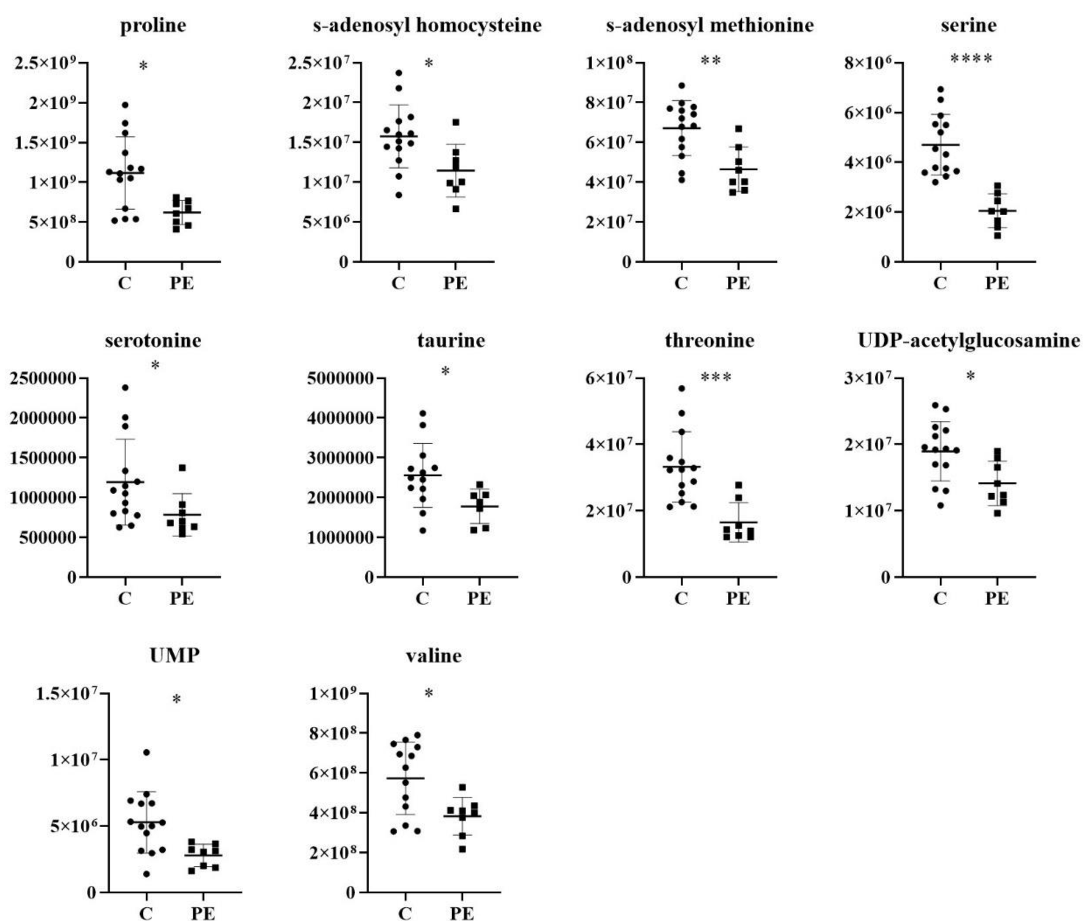

Figure 19. *Scatter plots related to the most discriminant metabolites of placenta samples processed by the Mann-Whitney test. The relative analysis was the targeted MS/MS analysis by LC/MS QqQ. Variable intensities are shown in the y-axis. *, **, *** and **** indicates levels of significance with a p value<0.05, <0.01, <0.001 and <0.0001, respectively.*