Lipidomics of fetal membranes in labour and the initiation of parturition by fetal lung maturation markers

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Abbreviations

-d	Deuterated
A	α-hydroxy
AA	Arachidonic acid
AEA	N-arachidonoyl ethanolamine (anandamide)
AG	Arachidonoylglycerol
ALA	α-linoleic acid
ALEA	α-linolenoyl EA
BHT	Butylated hydroxytoluene
BMI	Body mass index
BSA	Bovine serum albumin
C	Carbon
cAMP	Cyclic adenosine monophosphate
CB	Cannabinoid
CDP	Cytidine diphosphate
CE	Cholesteryl ester
CER	Ceramide
CER-DIS	Ceramide deuterated internal standards
CHL	Cholesterol
CL-DIS	Complex lipid deuterated internal standards
CoA	Coenzyme A
COX	Cyclooxygenase
CYP450	Cytochrome P450
DAG	Diacylglycerol
DAGL	Diacylglycerol lipase
DAPI	Diamidino-2-phenylindole
DEA	Docosanoyl EA
DGLA	Dihomo-g-linolenic acid
DGLEA	Dihomo-ɣ-linoleoyl EA
DHA	Docosahexaenoic acid
DHEA	Docosahexaenoyl EA
DHET	Dihydro-eicosatetraenoic acid
DiHDPA	Dihydroxydocosapentaenoic acid
DiHETE	Dihydroxyeicosatetraenoic acid
DPA	Docosapentaenoic acid
DPEA	Docosapentaenoyl EA
DPPC	Dipalmitoylphosphatidylcholine
DS	Dihydrosphingosine
DTA	Docosatetraenoic acid
EA	Dthanolamide
EET	Epoxyeicosatetraenoic acid
EGF	Epidermal Growth Factor
ELOVL	Elongase of very long chain fatty acid
EO	Ester-linked omega-hydroxy
EPA	Eicosapentaenoic acid
EPEA	Eicosapentaenoyl EA
ESI	Electrospray ionisation
FAAH	Fatty acid amide hydrolase

FAME	Fatty acid methyl ester
FBS	Fetal bovine serum
FFA	Free fatty acid
FID	Flame ionization detector
FPP	Farnesyl pyrophosphate
GA	Gestational age
GC	Gas chromatography
GLA	γ-linoleic acid
HBSS	Hanks' Balanced Salt Solution
HDHA	Hydroxydocosahexaenoic acid
HEA	Heptadecanoyl EA
HEPE	Hydroxyeicosapentaenoic acid
HETE	Hydroxyeicosatetraenoic acid
HETrE	Hydroxyeicosatrienoic acid
HODE	Hydroxyl octadecadienoic acid
IFN-	Interferon
IL-	interleukin
IPP	isopentenyl pyrophosphate
LA	linoleic acid
LEA	Lineoleoyl EA
LGEA	Lignoceroyl EA
LOX	Lipoxygenase
LPA	Lysophosphatidic acid
LPC	Lysophosphatidylcholine
LPE	Lysophosphatidylethanolamine
m/z	Mass-to-charge
MA	Maternal age
MAG	Monoacylglycerol
MaR	Maresin
MEA	Myristoyl ethanolamide
MMP	Matrix metalloproteinase
MRM	Multiple reacton monitoring
MS	Mass spectrometer
MTT	3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
MUFA	Monounsaturated fatty acids
N	Non-hydroxy
NAE	N-acyl ethanolamine
NAPE	N-arachidonoyl PE
NAT	N-arachidonoyl taurine
NEA	Nervonoyl EA
NF-κB	Nuclear factor-κ B
NMR	Nuclear mgnetic resonance
OEA	Oleoyl EA
OxoODE	Oxooctadecadienoic acid
P	Phytosphingosine
PA	Palmitic acid
PC	Phosphatidylcholine
PDEA	Pentadecanoyl EA
PDX	Protectin
PE	Phosphatidylethanolamine

PEA	Palmitoyl EA
PG	phosphatidylglycerol
PGD_2	Prostaglandin D_2
PGE_2	Prostaglandin E_2
$PGF_{2\alpha}$	Prostaglandin $F_{2\alpha}$
PGI_2	Prostaglandin I_2 (prostacyclin)
PhA	Phosphatidic acid
PI	Phosphoinositol
PL	Phospholipase
PLA_2	Phospholipase A2
POEA	Palmitoleoyl EA
PPAR-α	Peroxisome proliferator-activated receptor α
PS	Phosphatidylserine
PUFA	Polyunsaturated fatty acids
Q	Quadrupole
R	Acyl chain
ROS	Reactive oxygen species
Rv	Resolvin
S	Sphingosine
S1P	Sphingosine-1-phospahte
sCO2	Supercritical phase carbon dioxide
SDA	Stearidonic acid
SFA	Saturated fatty acids
SM	Sphingomyelin
STEA	Stearoyl EA
TG	Triacylglyceride
TNF	Tumour necrosis factor
ToF	Time-of-flight
trans-EKODE	12,13-epoxy-9-keto-10(trans)-octadecenoic acid
TX	Thromboxane
UHPSFC	Ultra-high performance supercritical fluid
UPLC	Ultra-high pressure liquid chromatography
VEA	Vaccenoyl EA

Abstract

Introduction: The timing of human labour is key to pregnancy success. Although the stages of parturition are well defined, its initiation and point of origin remain elusive. Fetal membranes are tissues that encapsulate the fetus and amniotic fluid in the intrauterine cavity, and are considered a site for conveying maturation signals from the fetus to the maternal uterus. Reports point strongly to lipid mediators in instigating parturition; supported by the clinical use of Arachidonic Acid (AA)-derived prostanoids; prostaglandin E_2 (PGE_2) and $F_{2\alpha}$ ($PGF_{2\alpha}$), to induce labour onset. In pregnancy, both sides of the fetal membrane (amnion and choriodecidua) show upregulation of cyclooxygenase-2 (COX-2), a rate-limiting enzyme in prostanoid synthesis, as labour begins. Endocannabinoids and ceramides are additional bioactive lipid mediators altered in maternal blood and/or intrauterine tissues at this time. Near to term, fetal lung maturation is achieved by the synthesis of lung surfactants, with the main components being amniotic fluid DPPC (Dipalmitoylphosphatidylcholine) and LPC (lyso-form lysophosphatidylcholine), both palmitic acid (PA)-enriched compounds. This study explores lipidomes within the amnion and choriodecidua of the fetal membranes in various types of labour; term and preterm, compared to women at term whose labour is yet to begin. The influence of DPPC/LPC on fetal membrane lipid changes, prostanoid production and pro-inflammatory cytokine liberation are also considered; to pinpoint (for the first time) the epicentre for induction of normal human parturition, and the role of fetal lung surfactant in instigating/propagating this physiological chain-reaction.

Method: Amnion and choriodecidua were collected post-delivery from (i) term non-labouring women (TNL), (ii) those in spontaneous term human labour (STL), (iii) those with drug-induced term labour (ITL), and (iv) those in spontaneous preterm labour (PTL) without identified infection. Eicosanoids and related mediators, endocannabinoids, ceramides and structural lipids, were profiled using state-of-art mass spectrometry lipidomics, and relative abundances of fatty acids determined with gas chromatography. For *in vitro* experiments with DPPC and LPC, primary amnion and decidual mesenchymal cells were isolated and propagated from human fetal membranes. Resultant lipidomes were observed and matched to secreted pro-inflammatory cytokines by multiplex immunoassays.

Result: The choriodecidua actively produced contractile prostanoids in all labouring women, compared to those with no overt signs of labour at term. The amnion likewise produced labour associated prostanoids, but only in term labouring conditions (TNL) and more noticeably following induction (ITL). Physiological (i.e. non-induced) spontaneous labour enhanced LOX- and CYP450-derived lipid mediators, but this was more apparent in the choriodecidua than amnion.

For classical endocannabinoids, the amnion and choriodecidua both expressed 2-arachidonoylglycerol (2-AG), whilst N-arachidonoylethanolamine (AEA) was only detected in the choriodecidua. In both tissues, AEA was upregulated in spontaneous term and preterm labour, with a further increase in 2-AG with labour induction. N-acylethanolamine (NEA) profiles varied under different labour conditions, but linoleoyl-ethanolamide was consistently increased in spontaneous term and preterm parturition.

Total ceramide (CER) content was also elevated in amnion and choriodecidua under both term labouring scenarios. The CER classes showed variability across study groups, but generally the amnion was most active following induction, and choriodecidua in spontaneous term labour. Notably, CER(NS) and CER(ADS) were the only classes enhanced during preterm labour.

For glycerophospholipids, phosphatidylcholine (PC) and phosphatidylethanolamine (PE) were elevated within the choriodecidua under spontaneous term labour. In addition, AA-containing species were exaggerated within these glycerophospholipids in both spontaneous and preterm labour. The amnion conversely showed a depletion of imbedded-AA solely under term labour conditions, and its total AA was decreased relative to other fatty acids. Interestingly, AA was correlated inversely with PA and γ-linolenic acid within the amnion at term.

Within the amnion, regardless of onset, PA was the most dominant fatty acid; with a potential role in liberating AA. Given that DPPC/LPC(16:0) are rich in PA, these may conceivably enhance amnion PA, thereby liberating AA within the amnion at term. *In vitro*, LPC treated amnion epithelium, sufficiently increased PA within the cultured cells. Nevertheless, this failed to alter AA levels *per se*. Unlike DPPC, LPC-treated cells did show enhanced production of IL-8 secretion, a known leucocyte chemoattractant, above all other measured cytokines. In considering the direct inflammatory stimulation of isolated decidual mesenchymal cells, IL-8 exposed cells were investigated for PGE_2 production. However, this unfortunately showed no stimulatory effect.

Conclusion: These data confirm that the fetal membrane is a key source of lipid mediators involved in human parturition, but with the choriodecidua more responsive over amnion. Although choriodecidual activation was present in all labour conditions, the amnion was only reactive at term, possibly implying a response to fetal maturation. For the choriodecidua, upregulation of major glycerophospholipids implied a fundamental alteration in cellular composition, likely supporting reports of invading leukocytes to propagate the response. In spontaneous term labour, AA was mostly reduced in the amnion, a reduction inversely correlated with PA. With DPPC and LPC being suggested signals of fetal maturation and potential participants in triggering human labour; the liberation of AA from amnion epithelial by these compounds was considered. Although this hypothesis was not correct, lung surfactant was shown to stimulate IL-8 production from amnion cells and in doing so, could theoretically cascade inflammation to the choriodecidua and uterus/cervix.

Overall, this study has highlighted the fundamental mechanistic differences across different types of human labour, pinpointing its inflammatory onset within the choriodecidua, over amnion. For the first time, this study directly implicates fetal lung surfactant within the amniotic fluid at term in the propagation of labour signals from amnion to choriodecidua, specifically through the liberation of IL-8. It is hoped this knowledge furthers the understanding of human parturition and expedites the discovery of novel tocolytics with which to control it.

Declaration

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Chapter 1: Introduction

1.1. Human labour

1.1.1. Definition of labour

Labour is a physiological process that aims to end fetal occupation of the uterus. At the time of labour, the uterus changes from a relaxed extendable (quiescent) sac - that has carried the fetus during pregnancy - into a regularly contracting organ to assisting in the expulsion and delivery of the baby. Along with uterine contractions, the cervix also changes, becoming softer, shorter and more dilated; in a process termed “ripening”, to facilitate the fetus's passage through the birth canal.

1.1.2. Timing of labour

The timing of labour is crucial for fetal and maternal well-being. Term human pregnancy is defined between 36 weeks plus 6 days gestation and 42 weeks of pregnancy (1). This definition is based on statistical observations, when mortality and morbidity of the fetus are at their lowest (2). Preterm labour, when delivery occurs before 37 weeks gestation, shows a higher incidence of adverse outcomes, mostly due to fetal immaturity of organs, especially the fetal lungs (3).

For humans, the incidence of preterm delivery is around 5-10 % of all human pregnancies and is a major burden for parents and healthcare systems, costing more than 25 billion dollars annually in the USA alone (4). In England and Wales, it has been estimated that the long-term costs of supporting preterm infants until the age of 18 are around £2.9 billion to the public sector (5). On the other hand, delaying delivery beyond 42 weeks gestation, post-term, also holds adverse risks for maternal and fetal health; including (but not limited to) intrauterine fetal demise (IUFD), fetal meconium aspiration, increased risk of caesarean section and labour dystocia (6,7). Consequently, the mistiming of parturition and delivery can have marked maternal and fetal consequences, and are therefore seen as significant obstetric concerns.

Preterm birth causes can be classified into medically induced (iatrogenic) and spontaneous. One-third of total preterm births are due to medical interventions due to maternal or/and

fetal indications such as preeclampsia, placenta previa, and severe intrauterine growth restriction (8). Spontaneous preterm birth can be triggered spontaneously (most common), by premature rupture of the membrane (PPROM), or with signs of intrauterine infection (9). All the previous causes can overlap with each other; therefore, spontaneous preterm birth is referred to as a "syndrome." Globally, preterm birth complications count as the leading cause of death in children under five years old (10). The incidence of preterm birth varies around the world, for example, 8.6% in Europe, 11.2% in North America, and 13.4% in Africa (11). Such differences could be related to antenatal care quality and demographic diversity. For instance, in the USA, the risk of preterm birth in African-American women is higher than in white women, at 13.8% and 9%, respectively (12). Furthermore, overweight women were reported to have an increased risk of preterm birth (13). Given the range of factors that could alter the timing of labour, preterm studies are very challenging.

Over decades research has been conducted to identify factors that control the timing and onset of labour, but in many ways this process, particularly in humans, remains ill-defined. In animals (at least some) the steps to labour and birth are better established. For example, within the literature there are two discernible instances for the initiation of spontaneous labour in animals. In sheep, the fetus has the controlling hand; as disturbances/ablation of the fetal hypothalamic-pituitary (HPA) axis forces the pregnancy into a post-term stage, with the fetus continuing to grow (14). On the other hand, mice show an opposite reliance, with timing of labour mostly controlled by maternal signals, predominately through a decrease in serum progesterone (15). In humans, both endocrine systems may share a role, but although studied extensively, their controlling influence in determining parturition remains disputed (16–18).

Arguably, what makes these studies so difficult in humans is that *in vitro* and *in vivo* animal models of labour fail to truly reflect the human condition. Undoubtedly, there are contemporaneous mechanisms, including the aforementioned endocrine determinants, but more recent evidence elevates the importance of a pro-inflammatory intrauterine environment, as key to promoting cervical ripening and uterine contractions, which underscore human delivery. In this context, spontaneous human labour is marked by leukocyte infiltration and associated elevations in pro-inflammatory cytokines in the uterus,

cervix and fetal membranes prior to and during the labouring process (19). These cytokines, and other inflammatory mediators, such as prostaglandins, nitric oxide (NO) and matrix metalloproteinases (MMPs), are induced to facilitate contractions, cervical ripening and rupture of fetal membranes (20). This subsequent pro-inflammatory environment is not only linked to spontaneous labour, but also pathologic labour; as evidenced by the initiation of pre-term labour, through an intrauterine haemorrhage, infection, preterm rupture of membrane (PROM), and over distention of the intrauterine cavity (i.e. multiple gestations and polyhydramnios). All of which show an upsurge in pro-inflammatory status in reproductive tissues (21–23).

1.1.3. Inflammation and parturition

As biological micro-molecules, cytokines (such as the interleukins (IL-), interferons (IFN-) and tumor necrosis factor (TNF-) family members) are secreted from a range of nucleated cells to regulate tissue homeostasis. From an inflammatory perspective, cytokines are often classified into pro- and anti-inflammatory agents; pro-inflammatory attracting white blood cells and stimulating synthesis of additional pro-inflammatory proteins; anti-inflammatory interfering with their pro-inflammatory counterparts, inhibiting their production, secretion, or decreasing their impact (24). The suggestion that pro-inflammatory cytokines play a role in the normal physiology of labour is now long-held. mRNA of IL-1β and IL-8 in maternal leukocytes not only increases during pathological preterm labour, but are also elevated in normal term labour, compared to non-labouring controls (25). Indeed, IL-1β and inflammatory TNF-α are known to induce synthesis of other substrates which undoubtedly regulate the physiology of parturition, including prostaglandins, MMPs and oxytocin receptors (26,27). Moreover, cervical administration of IL-8, TNF-α and IL-1β can promote cervical ripening and initiate the labour process (28).

Prostaglandins are arguably the most important mediators linked to the type of sterile (non-infectious) inflammation which purportedly contributes to human labour. Like cytokines, they have autocrine and paracrine affects; with actions controlled by cellular origin and the expression of accepting receptors. As an example, prostaglandin E_2 (PGE_2) acts on

prostaglandin receptor 4 (EP4) in bone cells to activate resorption, whilst in the myometrium the same prostanoid initiates contractions via activation of EP3 (29,30).

Of the myriad of potential prostaglandins, PGE_2 and $PGF_{2\alpha}$ have a known pro-inflammatory role in the uterus and are potent uterotonic factors, stimulating uterine contractions for delivery. Their administration as pharmacological agents can induce cervical ripening and uterine contractions and, as such, they are approved and most often used for the pharmacological induction of labour (31).

During parturition, the main sources of PGE_2 and $PGF_{2\alpha}$ are the decidua and fetal membranes (amnion and chorion) (21), but it is clear that cytokines and the maternal/fetal endocrine system both impact upon the localised production of these prostanoids and other labour-inducing mediators (**Figure 1.1**). As evidence, IL-1β, cortisol and oestrogen are capable of stimulating PGE_2 from isolated fetal amnion or choriodecidual cells (21,32,33), along with IL-6 and acute phase reactant proteins resulting from infections, trauma, physiological stress and other inflammatory scenarios (25,26). Although intra-amniotic PGE_2 and $PGF_{2\alpha}$ remain low during pregnancy, it is intriguing to note a sharp surge in both prostanoids beyond 36 weeks gestation, i.e. before any outward sign of active labour (34). Not only does this imply a shift in the inflammatory status of the fetal unit prior to parturition, but also raises the question of its cause and role in the timing of labour.

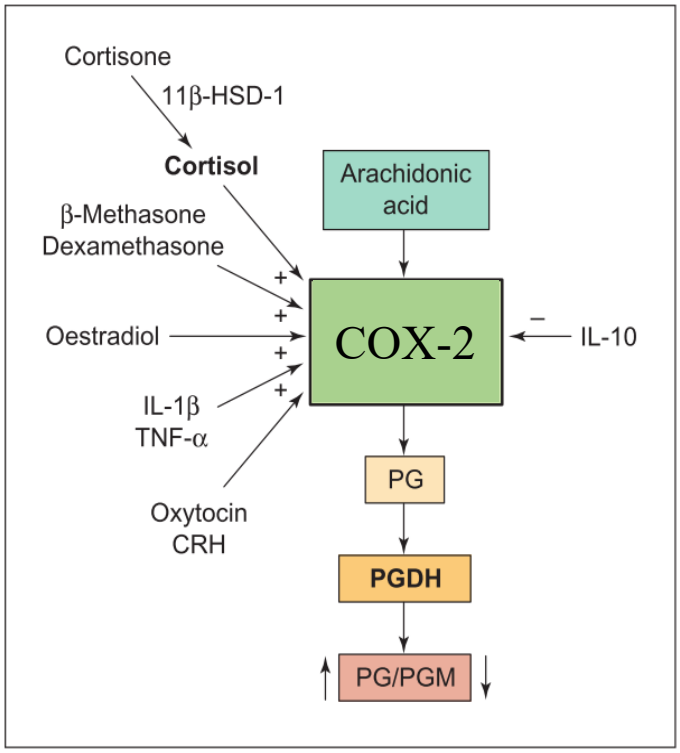


Figure 1.1. Suggested pregnancy-associated mediators involved in the regulation of cyclooxygenase-2 (COX-2). *COX-2 converts Arachidonic Acid into prostaglandins (PG), and 15-hydroxy prostaglandin dehydrogenase (PGDH) degenerate PG into PG metabolites (PGM). The ratio between PG and PG metabolites (PG/PGM) indicates active action within tissues. Abbreviations: 11β-hydroxysteroid dehydrogenase (11β-HSD), IL-1β: interleukin (IL-) IL-1β, IL-10, tumour necrosis factor α (TNF-α); corticotrophin-releasing hormone (CRH). Adopted from* (33).

Prostaglandins and other lipid mediators such as endocannabinoids and ceramides are known to modulate the inflammatory environment in physiological and pathological conditions (35–37). Given that human parturition is an inflammatory-driven condition, not surprisingly, some of these mediators were instigated in human parturition.

1.2. Lipid biology and mediators

Lipids are essential biological molecules that provide and store energy in the human body and are crucial for cellular structure (38,39). Structurally, lipids can be classified into; fatty acids, glycerolipids, glycerophospholipids, sphingolipids, sterol lipids, saccharolipids, polyketides, and prenol lipids (40). Moreover, lipids may act as signalling molecules, such as eicosanoids, endocannabinoids and ceramides, which have a noticeable influence in the inflammatory milieu within associated tissues (35,41,42).

1.2.1. Fatty acids

The fatty acid is the primary and simplest structure in the lipid classes and is chemically built by a hydrocarbon chain attached to carboxylic groups. The number of the double bonds can classify fatty acids into saturated (no double bonds), monosaturated (one double bond), and

polyunsaturated fatty acids (PUFA) (two and more double bonds) (43,44). Mammalian *de novo* fatty acid synthesis is a multi-step process involving different cellular compartments including, mitochondria, endoplasmic reticulum, and cytosol. Acetyl-CoA is converted into citrate in the mitochondria, and through citrate shuttle is transferred to the cytosol. Then citrate is converted back to acetyl-CoA and then into malonyl-CoA by acetyl-CoA carboxylase. Palmitic acid (C16:0) is a primary product of fatty acid synthase using acetyl-CoA and malonyl-CoA as substrates. Then with the action of fatty acid elongation and desaturation, more carbons and double bonds are added, respectively (45). The elongation process carries over in the smooth endoplasmic reticulum and has seven elongase enzymes (ELOVL). ELOVLs 1, 3, 6 and 7 act on saturated and monounsaturated fatty acids, whilst the others act on PUFAs (46).

Linoleic acid (LA; C18:2 n-6) and α-linolenic acid (ALA; C18:3 n-3) are termed essential fatty acids, as they need to be consumed from the diet, and in the fetal situation from the maternal blood. The reason behind this is that the human body lacks Δ12 and Δ15 desaturase enzymes, which are needed to generate LA and ALA, respectively (**Figure 1.2**). PUFAs can be classified according to the position of the last double bond into omega 3 (n-3), omega 6 (n-6), and omega 9 (n-9) fatty acids (44,47,48). N-3 and n-6 fatty acids have been studied extensively because of their essential roles in human physiology and pathology, especially related to inflammatory conditions. For many years, n-6 fatty acids were referred to as pro-inflammatory and n-3 as anti-inflammatory mediators. However, AA was then found to have both anti- and pro-inflammatory actions (49). Recently, AA (n-6) has also been shown to played a crucial role in fetal CNS development and is now a recommended additive in milk formula (50,51). Not surprisingly, n-3 fatty acid supplementation has been studied in relation to abnormal pregnancy conditions, such as preterm labour and preeclampsia (52,53). However, the outcome of these result has been questioned and remains open to debate.

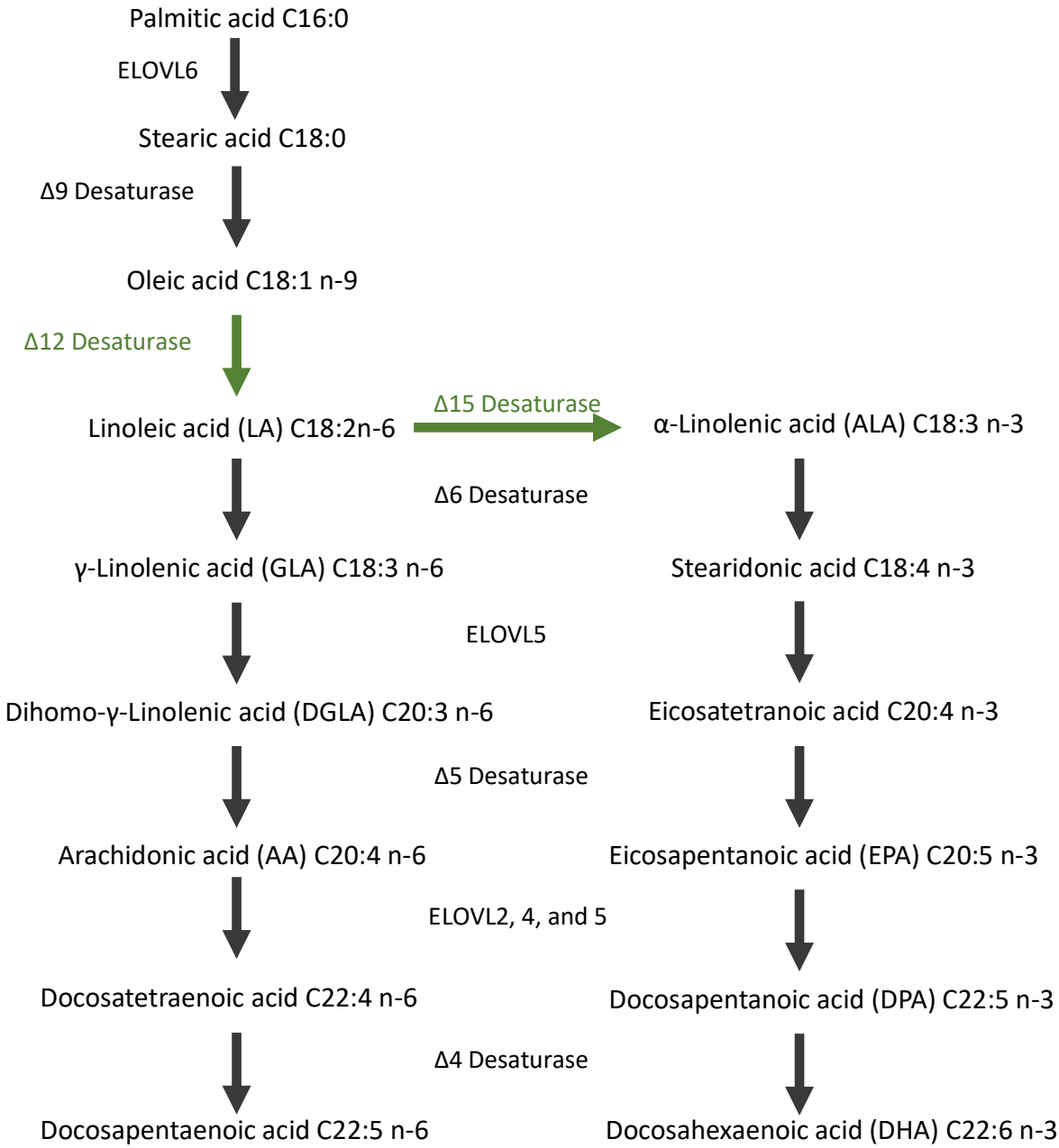


Figure 1.2. Schematic showing the PUFAs synthesis pathway, starting from palmitic acid (C16:0). Green arrow and test indicate enzymes that exist in plants but not in the human system. *ELOVL: Elongase of very long chain fatty acid.*

1.2.2. Glycerolipids

Glycerolipids are noteworthy in storing excessive fatty acids, as well as energy sources to warm up body temperature and prolong starvation (54). They are also involved in cellular structures and can act as signalling molecules (55). Glycerolipids have a glycerol backbone attached to a single fatty acid (monoacylglycerol), two fatty acids (diacylglycerol) (DG) or three fatty acids (triacylglycerol or triglyceride (TG)). The liver is the leading site for TG

synthesis, where it can be produced by a number of pathways, most commonly the Kennedy pathway (56,57) (see **Figure 1.3**).

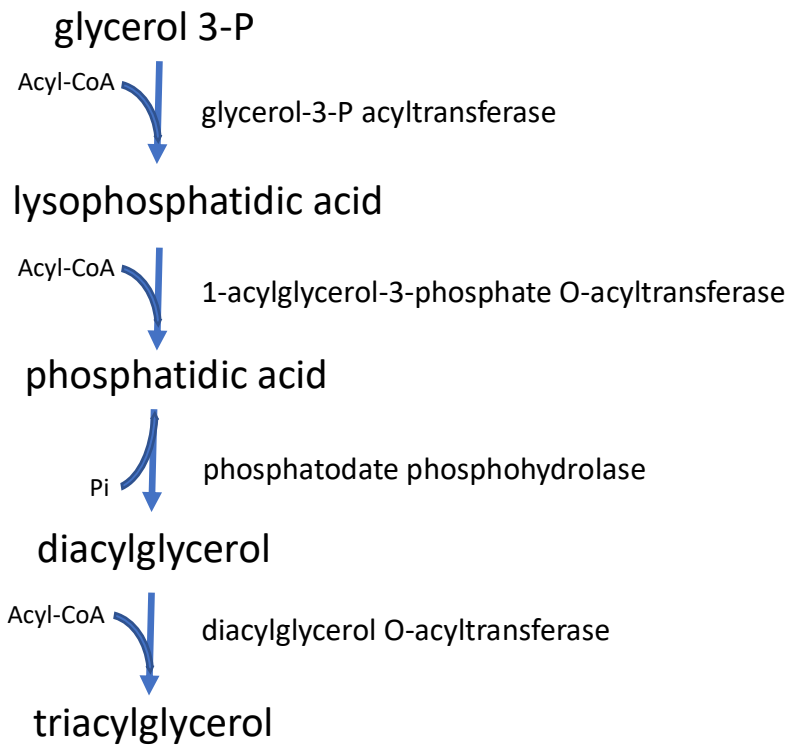


Figure 1.3. Diagram showing diacylglycerol (DG) and triacylglycerol (TG) synthesis by the Kennedy pathway. *Pi: Inorganic phosphate.*

TG is used to store excessive fatty acids as lipid droplets with cells to prevent them from lipotoxicity (58). From a maternal perspective, pregnancy is a hypertriacylglycerolemic state, with which to provide fatty acid supplies to the fetus, especially in the third trimester (59). These changes could provide a good FA supply to the fetus to fulfil the increasing demand. Also, DGs are elevated in cord blood and in the maternal plasma at term labour (60).

1.2.3. Glycerophospholipids

Like the glycerolipids, the glycerophospholipids have a glycerol backbone with two fatty acid groups attached on sn-1 and sn-2, but with the addition of phosphate on sn-3. At the phosphate site, different head groups such as choline, ethanolamine, inositol and serine can attach to phosphatidic acid to form phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylinositol (PI), and phosphatidylserine (PS), respectively (61). Also, glycerol can be attached to the phosphate group to form phosphatidylglycerol (PG) or hydroxy groups

for phosphatidic acid (PA)(62,63). In the human body, PC and PE are the most abundant glycerophospholipids, with the other in less abundance (64).

Glycerophospholipid synthesis is a complex process that crosses between its species and other lipid classes, such as glycerolipids and sphingolipids. The majority of glycerophospholipids are synthesised in the ER, and there are two common pathways involved in mammalian cells. Both require either activation of the head group or DG by adding cytidine diphosphate (CDP). For phosphatidylcholine and phosphatidylethanolamine synthesis, the head groups, choline and ethanolamine, undergo phosphorylation followed by the addition of CDP by the action of cytidyl transferases, to yield CDP-choline/CDP-ethanolamine. These then activated head groups react with DG to form PC or PE. Also, the PE head can be exchanged with serine to form PS. In a second method, CDP-DG (activated DG) reacts with the inositol head group or another CDP-DG to form PI and PG, respectively (61,65). It worth mentioning that the sn-1 of PC and PE can contain acyl-, ether-, or vinyl-ether bonds, with the latter referred to as a plasmalogen. Although plasmalogen function is not well explored, they are considered to have a protective action against oxidative stress (66). Glycerophospholipids can be degenerated or remodelled by phospholipase enzymes, as shown in **Figure 1.4** (67).

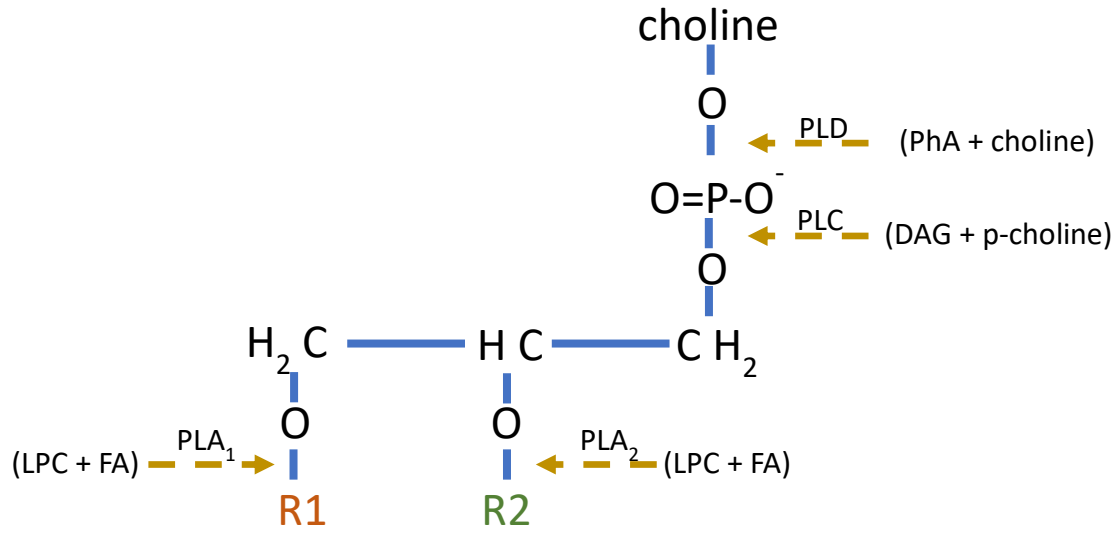


Figure 1.4.Phosphatidylcholine cleavage sites by different phospholipase enzymes; PLA_1, PLA_2, PLC, and PLD. Outcome of hydrolysis indicated between brackets. *Abbreviations: phosphatic acid (PhA), diacylglycerol (DAG), phosphocholine (p-choline), lysophosphatidylcholine (LPC), free fatty acid (FA), and position of fatty acid side chain (R).*

Glycerophospholipids are bipolar compounds and an essential structure for cellular and organelle membranes (61). As discussed earlier, glycerophospholipids are the primary component in lung surfactant, which adds tension pressure to alveoli to prevent them from collapsing (68). They also participate in cellular signalling, following the action of different phospholipases. This is especially true of PLA_2, which releases fatty acids at sn-2; a favourite site for AA and other PUFAs (69,70).

1.2.4. Sphingolipids

Sphingolipids are structural and signalling molecules for eukaryotic cells. Ceramides are the parent or precursor of other sphingolipids, such as glycosphingolipids and sphingomyelins (SM). For instance, MS is composed of ceramides attached to phospho-alcohol group donated from glycerophospholipids, such as PC or PE (71). Many studies have implicated ceramide in pro-inflammatory signalling, apoptosis and in stress-related conditions (72–74).

The sphingoid base is what makes sphingolipid standout from other lipid classes. The sphingoid base has four different forms sphingosine (S), dihydrosphingosine (DS), phytosphingosine (P), and 6-hydroxy-sphingosine (H). Also, each sphingoid base is attached to a fatty acid; non-hydroxy (N) or alpha-hydroxy (A), to create eight possible ceramide structures; CER(NS), CER(AS), CER(NDS), CER(ADS), CER(NP), CER(AP), CER(NH), and CER(AH). Also, there are four more that have LA attached to the omega carbon of omega-hydroxy fatty acids (CER(EOS), CER(EODS), CER(EOP), and CER(EOH)). These ceramides exist exclusively in the skin (see **Figure 1.5**) (75,76).

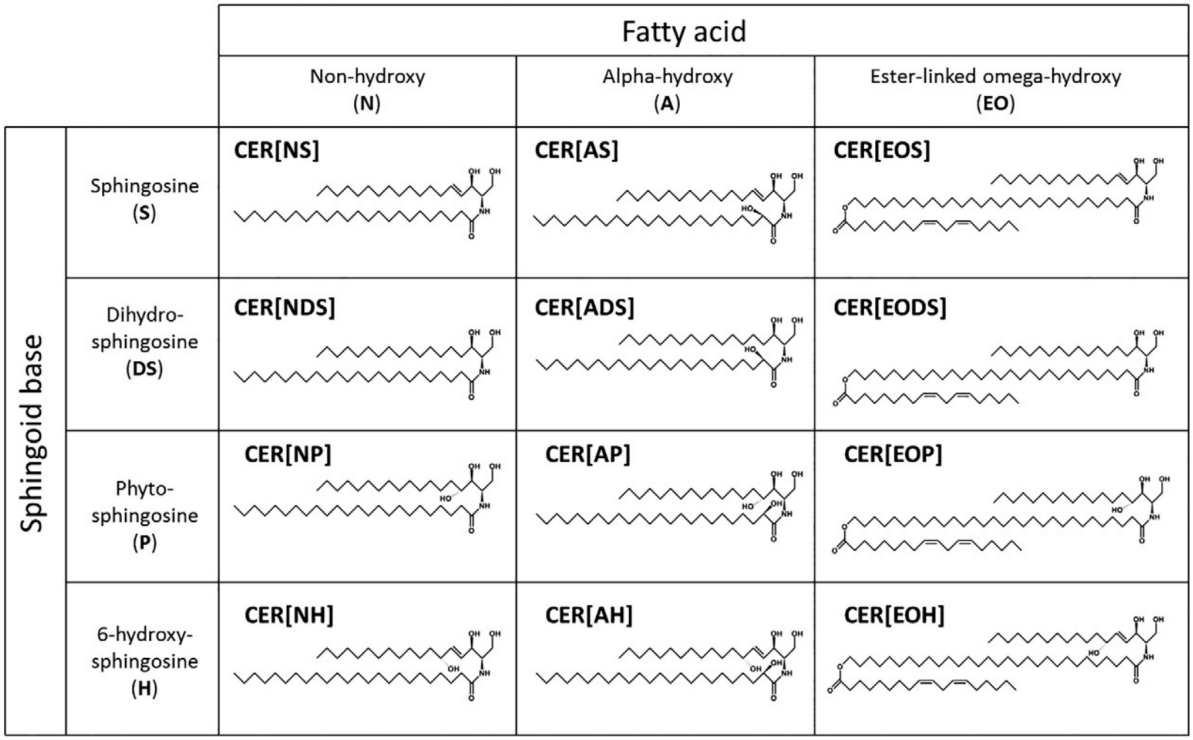


Figure 1.5. Ceramide family nomenclature, according to component fatty acid and sphingoid bases. *Adopted from* (75).

Ceramide synthesis is complex, and they can be generated from three pathways *de novo*, SM hydrolysis, and the salvage pathway. In the *de novo* pathway, serine palmitoyltransferase (SPT) is the rate-limiting enzyme that utilises L-serine and palmitoyl CoA to create 3-keto dihydrosphingosine. Then dihydrosphingosine is reduced into CER(DS) and then into CER(NDS) by the action of dihydroceramide synthase. Ceramide CER(NS) is generated from CER(NDS) by the action of 4Δ-desaturase (**Figure 1.6**). In the salvage pathway, sphingosine is created from Sphingosine-1-phosphate (S1P) by S1P phosphatase-1, and then ceramide synthase converts sphingosine into CER(NS). Lastly, CER(NS) can be created by hydrolysing MS by sphingomyelinase (71,72,77) (see **Figure 1.6**).

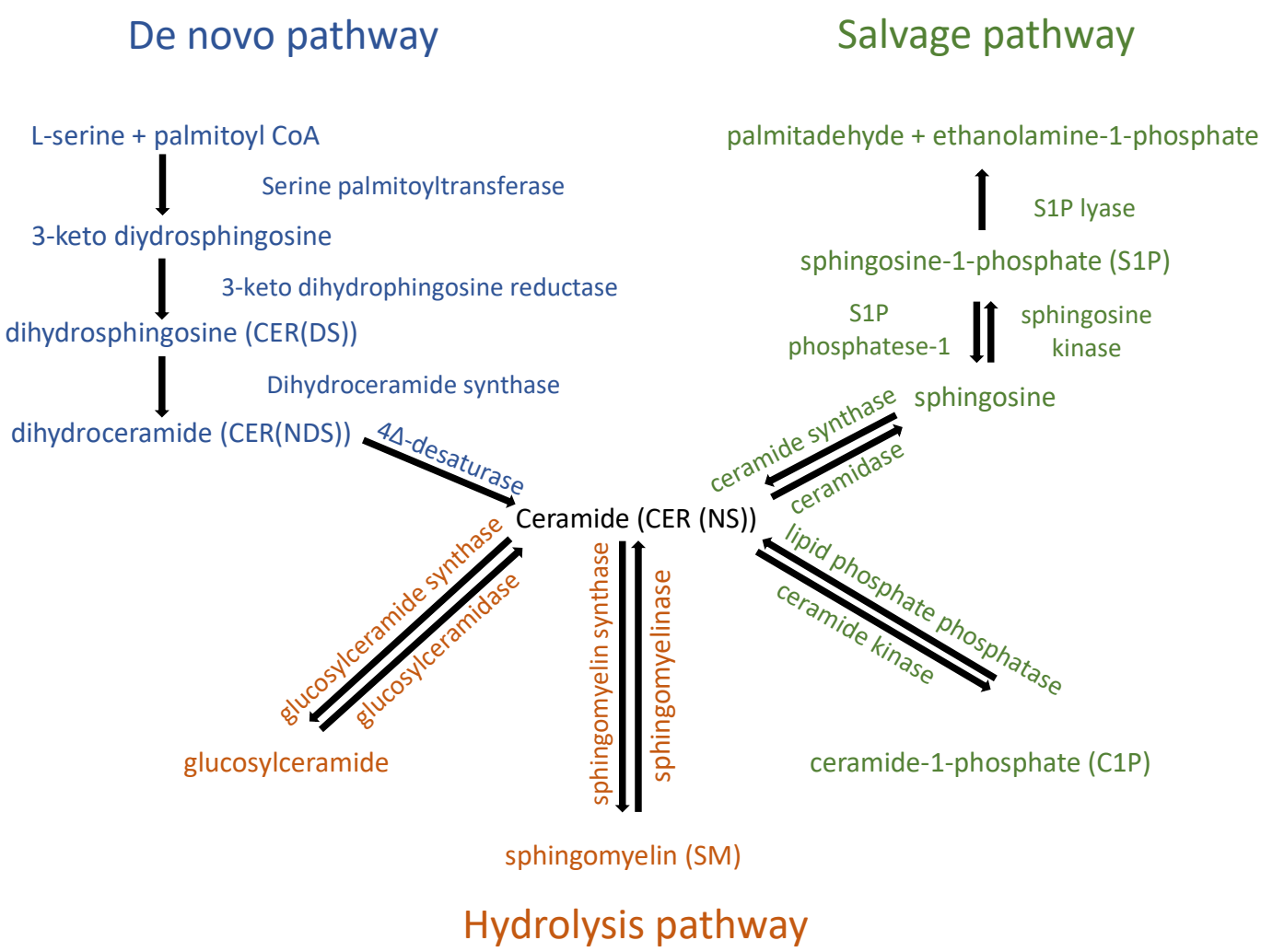


Figure 1.6. Schematic of ceramide (CER(NS)) synthesis pathways; de novo pathway (blue), salvage pathway (green) and hydrolysis pathway (orange). *Abbreviations: ceramide-1-phosphate (C1P) and sphingosine-1-phosphate (S1P).*

From a parturition perspective, ceramide was observed elevated in maternal plasma collected from the placental intervillous space at term spontaneous labour (78). Also, Signorelli et al. (2016) reported the upregulation of SPT (at the protein and mRNA placental level) in labouring term placentas; being inversely correlated with labour duration (42). In another study, true preterm labour showed upregulation of maternal plasma C16-ceramide over false preterm labour (79). Moreover, ceramide is known to enhance NF-κB (80), which is known to stimulate the production of labour associated mediators (81).

1.2.5. Sterol lipids

The placenta is considered an endocrine organ that secretes different types of hormones to maintain a normal pregnancy (82). Cholesterol is a precursor of steroid hormones such as progesterone, oestrogen and cortisol (83). The placenta transports maternal cholesterol to fulfil fetal demands of cholesterol(84). Furthermore, cholesterol is involved in cellular membrane structure, integrity, and enhances its fluidity (85). Cholesterol is a hydrophobic structure with four non-aromatic joined rings. Cholesterol synthesis is a lengthy and complicated mechanism that mostly takes place in the ER. Acetyl CoA and acetoacetyl CoA are the primary precursors that are converted by the action of 3-hydroxy-3-methyl-glutaryl-CoA (HMG CoA) synthase into HMG CoA. Then by HMG reductase, the rate liming enzyme, HMG CoA is converted into mevalonic acid. Melvalonic acid undergoes serial reactions to yield Isopentenylpyrophosphate (IPP). Then six IPP molecules are needed to create squalene. Squalene undergoes various reactions to form lanosterol, which has the four non-aromatic rings, then into cholesterol (86,87). Cholesterol can be stored intracellularly as a lipid droplet as cholesterol ester (CE) by the action of acyl-CoA cholesterol acyltransferase (ACAT) enzymes (88).

1.2.6. Eicosanoids and other related mediators

Eicosanoids are active lipid mediators that act locally due to their short half-life. AA and other 20-carbon containing PUFAs (DGLA and EPA) are the precursors of eicosanoids metabolites. Also, other PUFAs; LA and DHA are the precursors of octadecanoids and docosanoids, respectively. Among PUFAs, AA-derived mediators are the most dominant product explored in physiological and pathological conditions. Three common enzyme groups produce eicosanoids; cyclooxygenase (COX), which generate prostanoids (prostaglandins (PG) and thromboxanes (TX)), lipoxygenase (LOX), which produce leukotriene, and cytochrome P450 (CYP450), which produce epoxygenases (**Figure 1.7**)(41,75,89).

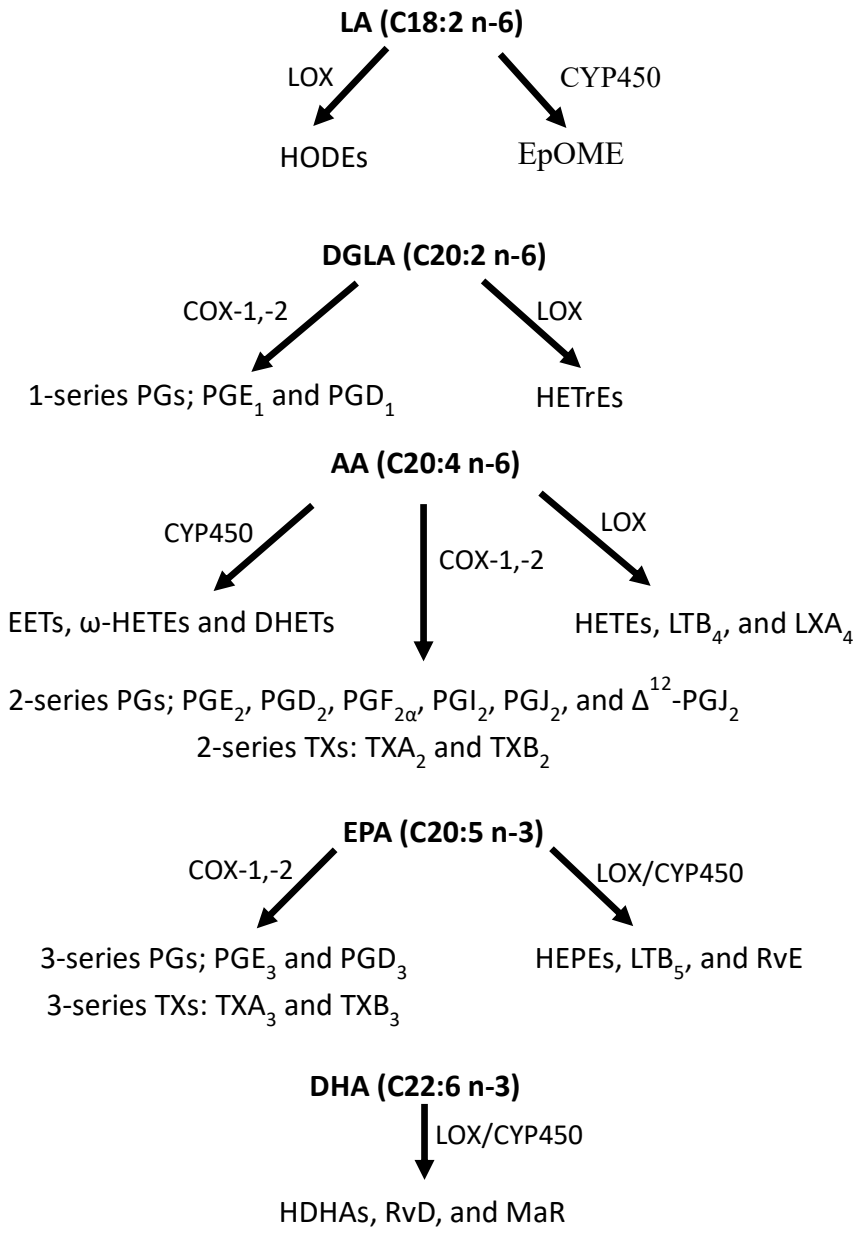


Figure 1.7. Schematic showing linoleic acid (LA), dihomo-ɣ-linolenic acid (DGLA), arachidonic acid (AA), αlinolenic acid (ALA), eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) oxidative mediators, driven through the cyclooxygenase (COX), lipoxygenase (LOX) and cytochrome P450 (CYP450) pathways. *Abbreviations: dihydroxyeicosatetraienoic acid (DHET), epoxyeicosatetraenoic acid (EET), epoxyoctadecenoic acid (EpOME), hydroeicosatetraenoic acid (HETE), hydroxydocosahexaenoic acid (HDHA), hydroxyeicosapentaenoic acid (HEPE), hydroxyeicosatrienoic acid (HETrE), hydroxyoctadecadienoic acid (HODE), Leukotriene B (LTB), Lipoxin (LX), maresin (MaR), prostaglandin (PG), resolvin (Rv), and thromboxane (TX).*

The eicosanoid production cascade commences with the liberation of AA. As discussed (**section 1.2.3**), the sn-2 position in glycerophospholipids is the favourable site for AA storage and other PUFAs, with the action of PLA_2 liberating them. There are numerous PLA_2 isoforms including secretory PLA_2 ($sPLA_2$), calcium-independent PLA_2 ($iPLA_2$), and cytosolic PLA_2

($cPLA_2$). $iPLA_2$ liberates low levels of AA and is involved in cellular homeostatic and membrane remodelling activities. $cPLA_2$ activation releases high quantities of AA and is upregulated under inflammatory conditions. $sPLA_2$ enhances the action of $cPLA_2$ and prolongs the availability of non-esterified AA (90–92). $cPLA_2$ also plays a crucial role in parturition; as $cPLA_2$ mutant mice are unable to undergo spontaneous labour (93).

For prostanoid synthesis, non-esterified AA and other PUFAs undergo oxidation by COX enzymes, which have two isoforms; COX-1 and COX-2; the latter being inducible and upregulated in inflammatory conditions (94). COX enzymes convert AA into PGH_2, which is an unstable metabolite, then into active biological PGs by the action of prostaglandin synthases. In pregnancy, prostanoid actions are specific to the tissue types; for instance, PGE_2 enhances vascular relaxation; likewise, it promotes myometrium contraction (41,95,96). Along with PGE_2, $PGF_{2\alpha}$ and TXA_2 are also known to stimulate myometrium contractions (97), and a recent systemic review reported that COX-2 expression was elevated in all gestational tissues (amnion, chorion, decidua and myometrium) at term during labour (98). Besides this labour inducing role of prostanoids, indomethacin, a COX inhibitor, can be used as a tocolytic to prolong gestation in cases of preterm labour onset (99). It is therefore apparent that prostanoids have an indisputable role in human parturition, regardless of gestational age.

In addition to COX pathways, PUFAs can be oxidised by the LOX pathway to produce a wide array of mediators, i.e. hydroeicosatetraenoic acids (HETEs) derived from AA, hydroxyoctadecadienoic acids (HODEs) from LA, and hydroxyeicosapentaenoic acids (HEPEs) and resolvins (RvE) from EPA (listed in **Figure 1.7**). There are three LOX isoforms; 5-, 12- and 15-LOX; according to the carbon position on which they oxygenate AA (100). Free PUFAs can also be utilised by CYP450, with mostly AA used form epoxyeicosatrienoic acids (EETs) and ω-HETEs; both mediators of reported anti-inflammatory actions and modify vascular and uterine muscle tone (41,101,102). As well as these mediators, EETs can be converted into less bioactive metabolites, dihydroxyeicosatetraienoic acids (DHETs), by the action of soluble epoxide hydrolase (sEH). Although the role of LOX- and CYP450-driven mediators' in parturition is not fully appreciated, some are elevated in amniotic fluid at term in labour, such as 5-HETE, 15-HETE, 8(9)EET and 11(12)EET(103,104). Of these, 5-HETE is one of the few

hydroxy fatty acids to be studied in reproductive tissues and found to stimulate uterine muscle strip contractions *ex vivo* (105).

Throughout the literature, papers have reported the production of prostaglandins from gestational tissues, including: amnion, chorion, decidua, placenta and myometrium. The vast majority of these have indirectly assessed these mediators, either from tissue cultures (106–108) or other *in vitro* models under sterile inflammation conditions to mimic parturition (109–112). Immunoassays are used commonly to analyse liberated prostanoids. However, these are typically limited to one analyte per run, with low specificity and issues of cross-reactivity. In rectifying these limitations, analytical methods, such as mass spectrometry, are arguably more robust, offering a much wider range of analytes and far greater sensitivity (113). These analytic methods have been seldom used before in this context, and therefore form the focus of this thesis.

1.2.7. Endocannabinoids

Endocannabinoids are molecules synthesised in the body that share reactivity with Δ^9-tetrahydrocannabinol (THC) (a psychoactive product of marijuana) towards cannabinoid receptors (CBs)(114). Initially, CBs were explored in the CNS but were later found expressed in other peripheral tissues (35). Anandamide and 2-arachidonoylglycerol (2-AG) are typical examples of endocannabinoid mediators. Both AEA and 2-AG are AA-containing compounds but from different lipid classes; ethanolamines and monoacylglycerols. AEA synthesis is unique as AA is mobilised into sn-1 PC in order to hydrolyse by N-acyl transferase (NAT) to form phospholipid *N*-arachidonoyl PE (NAPE) then into AEA by the action of NAPE-PLD (**Figure 1.10**). AEA is also catabolised by fatty acid amide hydrolase (FAAH) into AA and ethanolamine. For 2-AG synthesis, phosphatidylinositol (4,5)-biphosphate is converted by PLC into DG, then into 2-AG by the action of diacylglycerol lipase (DAGL). Also, AA can be regenerated from 2-AG by the action of monoacylglycerol lipase (MAGL) (35,115,116). Both AEA and 2-AG can be oxidised by COX and LOX enzymes to create PG-EA and PG glycerol, and HETE-EA and HETE-glycerol esters (respectively); although their biological function is not well-defined, they could compete with AA in prostanoid synthesis (**Figure 1.8**)(117).

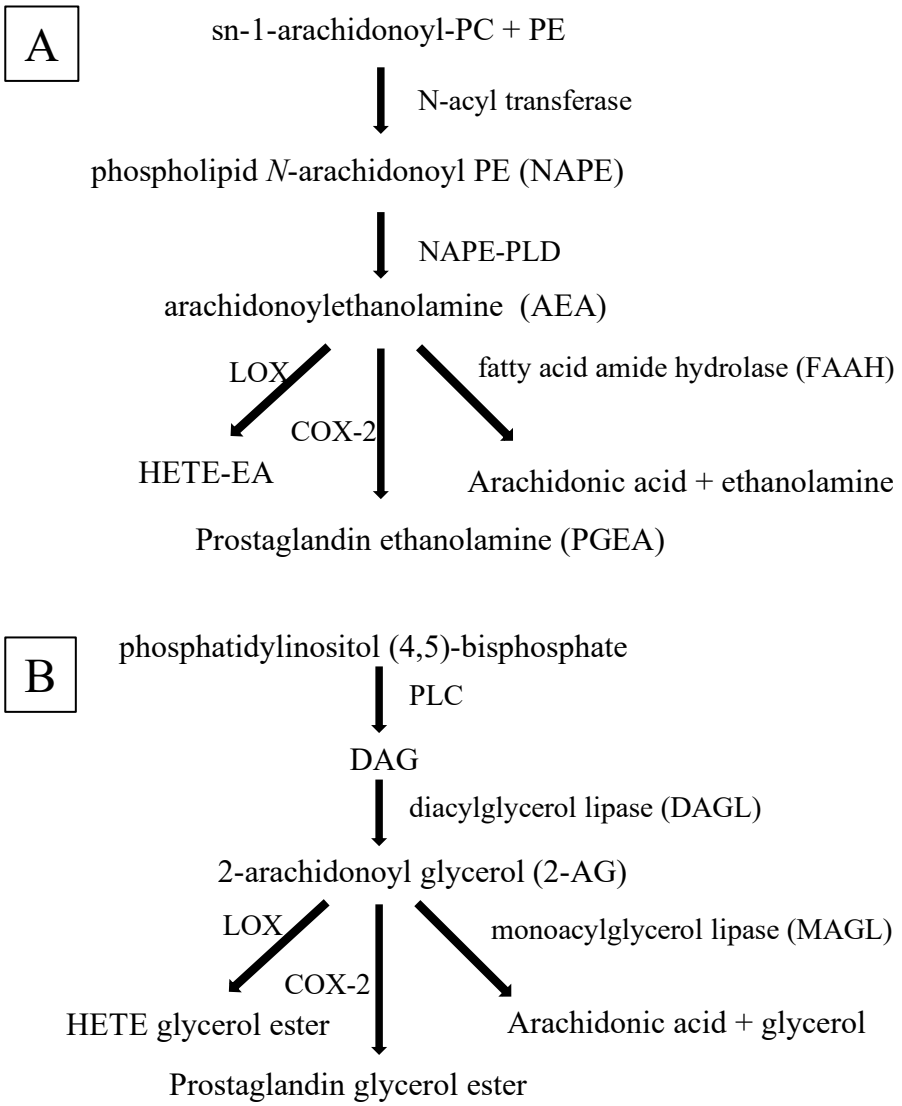


Figure 1.8. Diagram of classical endocannabinoid (AEA and 2-AG) synthesis. *Abbreviations: cyclooxygenase (COX), diacylglycerol (DAG), hydroeicosatetraenoic acid (HETE) -ethanolamine (HETE-EA), lipoxygenase (LOX), NAPE-specific phospholipase D (NAPE-PLD), phosphatidylcholine (PC), phosphatidylethanolamine (PE) and phospholipase C (PLC).*

From a pregnancy view, cannabis consumption is linked to adverse pregnancy outcomes such as low birth weight, preterm labour and stillbirth (118–120). However, it is difficult to conclusively link this observation to cannabis use, as the majority of women in these studies are also smokers and alcohol consumers. THC infusion increased the gestational length in lipopolysaccharide (LPS)-induced preterm labour in mice (121). This observation could suggest a tocolytic role of cannabis, at least in animal models. Endocannabinoids are tightly regulated from the early stages of implantation, with elevations in maternal plasma AEA and

defects in FAAH associated with abortion and implantation failure (122,123). Moreover, reducing the bioactivity of endocannabinoids by CB1 deletion is associated with preterm labour (124). In term labour, AEA and other endocannabinoid-like molecules, oleoyl ethanolamine (OEA) and palmitoyl ethanolamine (PEA), are elevated in the fetal system as identified in cord blood (78). With the induction of labour, maternal plasma AEA is increased and inversely correlated to duration (125). Although stimulation of CB receptors *in vitro* is enough to enhance uterine relaxation (126). It is more likely that 2-AG and AEA facilitate prostanoid production to regulate tone, with PGE_2 liberated from fetal membranes through CB1 (127) and AEA inducing uterine PGE_2 and $PGF_{2\alpha}$ through CB2 activation (128). With little more information than this, it could be tentatively suggested that endocannabinoids may perform a physiologic role in human parturition. However, this needs further support and experimentation.

1.2.8. Lipidomics

Lipidomics is the study of the lipid composition within a cell or tissue using analytical chemistry tools such as thin-layer chromatography (TLC), gas chromatography-flame ionisation detector (GC-FID), nuclear magnetic resonance (NMR) spectroscopy and mass spectrometry (MS) (129). Studying changes in the lipidome within tissues would improve our understanding of energy haemostasis, cellular membrane structure and cell signalling between physiological and pathological conditions.

TLC is a simple and inexpensive technique, but it provides only a qualitative analysis with poor reproducibility (130,131). Regarding GC-FID, the lipid extraction process is a lengthy procedure that includes a derivatisation step to extract in a volatile form. Also, GC-FID analysis has a limited number of analytes per run (131). NMR spectroscopy is a sensitive technique that offers chemical structure information of the analyte; however, it sensitivity decreases with complex matrices (132). The MS-based analysis is a gold-standard method for lipidomic profiling that provides high sensitivity and reports a wide range of metabolites(133).

The innovation of electrospray ionisation (ESI), which acts as a soft ionisation method, added a higher sensitivity to MS analysis (134). Nowadays, the ESI-MS technique is a standard

method to analyse lipidomics (135). There are two common MS approaches used to study lipidomics: targeted and untargeted methods. The targeted method is used with pre-knowledge of specific lipid species of interest, such as eicosanoid and endocannabinoid mediators. In this method, the analytes pass through two stages of MS (tandem MS). At the first stage, quadrupole (Q1), the analytes are filtered according to desired mass to charge ration (*m/z*) (parent ion), then the selected species are fragment with collision energy in Q2, then the fragment ions (daughter ions) are filtered again according to *m/z* in Q3 (134). As stated earlier, the pre-knowledge of desired species (parent and daughter ions for each species) is required to optimise multiple reaction monitoring (MRM). On the other hand, the untargeted method is a valuable tool to scan lipidomic species and classes in a wide spectrum analysis. Time of flight (ToF) MS is a simple form of MS commonly used in untargeted lipidomic profiling, which reports the analytes according to *m/z* after applying high and low collision energy, but without pre-selection (136). Given the previous fact, it acquires a massive amount of data which becomes challenging to be analysed. Therefore, the lipid species are usually reported as a total number of carbon and double bonds.

To conclude, lipid analysis is challenging due to the diversity of lipid classes. The MS-based analysis adds a new edge for exploring the lipidome by providing high sensitivity and reporting a wide range of species in a short time. Exploring the intrauterine lipidome could provide a better recognition of normal and abnormal parturition, leading to identification of therapeutic targets and biomarkers.

1.3. Parturition clocks

For decades, human parturition has been studied. Many hypotheses have tried to point out whether fetal maturation, gestational tissues (fetal membrane), or the maternal system has the upper hand to stimulate parturition. However, no clear answer is defined yet. In an attempt to rationalise the complexities of normal human parturition and understand irregularities in its induction and timing, contemporary thinking has proposed a system of "clocks" in which to define the fetal maturation events that culminate in localised fetal membrane inflammation, which then awakening the uterus as starting regular contraction and cervical dilatation to facilitate delivering of the fetus (15,21).

1.3.1. Fetal maturation clock

As a class of corticosteroids, glucocorticoids are hormones strongly linked to the timing of parturition and studied intensively as markers of human/animal fetal maturation. Within the adult and fetus, the Hypothalamic Pituitary Adrenal (HPA) axis controls its secretion, in a process which starts with release of corticotropin-releasing hormone (CRH) from the hypothalamus, which then stimulates adrenocorticotropic hormone (ACTH) from the anterior pituitary. In turn, ACTH stimulates glucocorticoid release, typically cortisol, in a circadian rhythm from the adrenal glands (**Figure 1.9**) (137). In the adult and maternal system, cortisol has a negative feedback on the HPA axis, whilst in the fetus, the placenta acts as a biological barrier to control exposure to maternal-derived cortisol. It performs this role by metabolising maternal cortisol to its inactive form, cortisone, by an abundance of placental 11-β hydroxysteroid dehydrogenase-2 (11-β HSD-2)(33). As such, it has a strong protective and quiescent effect.

With advancing gestation, another form 11-β HSD-1 is expressed in the fetal membrane (138). Unlike 11-β HSD-2, 11-β HSD-1 is responsible for reconversion of cortisone back to cortisol, as a potential means of elevating membrane PGs and cytokines (138). This newly derived cortisol would have capacity to upregulate COX-2 and $cPLA_2$ in the amnion (139), whilst inhibiting prostaglandin dehydrogenase (PGDH) in the chorion. By creating a feedback loop, this conversion to cortisol to pro-inflammatory mediators (**Figure 1.2**) (18,140), is considered to assist in the conditioning of fetal membranes for parturition. Certainly in the sheep, intrauterine infusions of ACTH and glucocorticoids induced preterm labour within days (141). Moreover, damage to the fetal HPA-axis can encourage ovine pregnancies to go post-term, even by up to 2 gestational months (14). In humans, intra-amniotic cortisol injections have similarly forced labour at varying gestations (142,143), again implying mechanistic similarities. Moreover, in humans at term, increasingly unchecked 11-β HSD-2, allows maternal cortisol to stimulate placental isoforms of CRH leading, to increased ACTH and cortisol for active labour-associated prostanoids release from the fetal membrane (144,145).

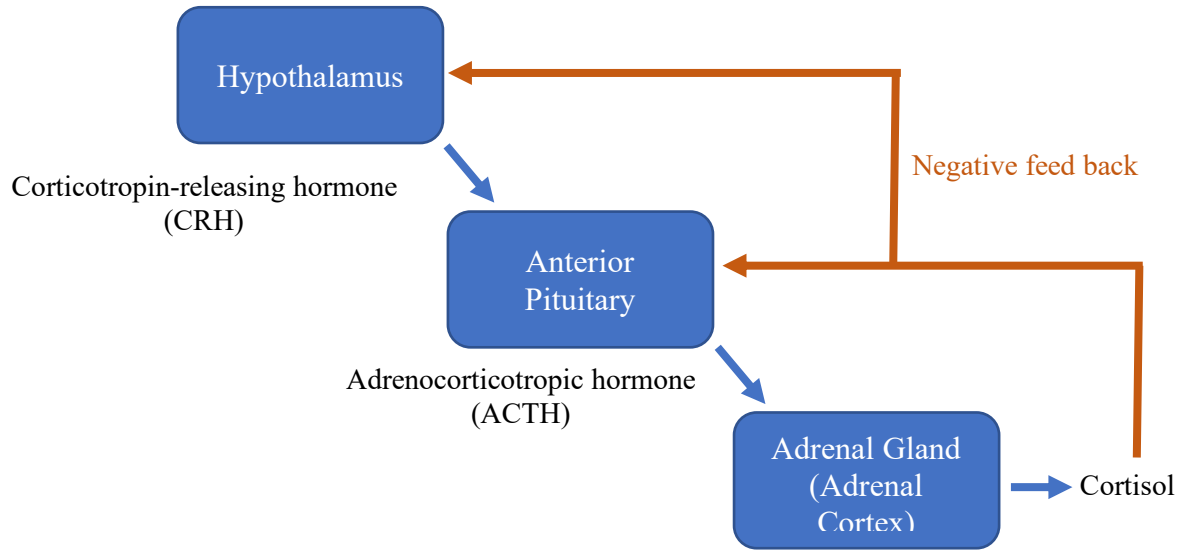


Figure 1.9. The adult hypothalamic pituitary adrenal (HPA) axis and negative feedback of cortisol.

In addition to the fetal membranes, cortisol has a fundamental role in maturing the fetal lungs, readying the fetus for delivery (146). Synonymous with lung maturation is the fetal ability to produce surfactant, thereby decreasing alveoli tension in new-borns (20). The primary composition of pulmonary surfactants are lipids, which account for 90% of the content, with more than 70% of which is PC (147). This surfactant increases in the amniotic fluid with advancing lung maturation in the third trimester, and with it the lecithin (a mixture glycerophospholipids)/SM ratio has been used clinically to assess fetal readiness for birth (148). Of the PC compounds which compose fetal surfactant, dipalmitoylphosphatidylcholine (DPPC) is the dominant and counts for up to 70% of its total (68). With regard to any possible impact on the fetal membrane, Ohtsuka et al. (1990) proposed DPPC acted to stimulate explanted amnion to release AA in the absence of concomitant prostaglandin (149). However, they were unable to confirm uptake of DPPC by the tissue and failed to report the labouring status of the membranes, which could have critically affected their outcome.

In addition to DPPC, platelet-activating factor (PAF), has a similar structure to PC, but with an alcohol saturated fatty acid at sn-1 and acetic acid at the sn-2 position. It is also suggested to be secreted from the fetal lungs and is shown increased in amniotic fluid in term and preterm labours (150,151). *In vitro*, PAF has a purported stimulatory effect on myometrium contraction (152) and is capable of encouraging PGE_2 secretion from exposed amnion (153). Surfactant protein-A (SP-A) is additionally found to increase in amniotic fluid with advancing

fetal lung maturation. Like other compounds, it is shown to have a pro-inflammatory effect, increasing IL-1β expression in macrophages (154) and stimulating PG synthesis in isolated and cultured amnion cells (155). In experimental mice, SP-A injections induce preterm delivery. Likewise, in knockout mice with the SP-A gene deleted, a delay in the timing of the delivery is noted, with decreasing expression of contraction-associated proteins (CAPs) in the affected myometrium (156). In human amniotic fluid, SP-A is lower in term labour than non-labouring pregnancies (157), but the authors attribute this unexpected finding to the breakdown of SP-A, as opposed to a true gestational reduction.

With most women delivering at term, when fetal maturation is fully achieved, the idea of related molecules signalling this maturity and directly or indirectly influencing a maternal inflammatory response is a promising one. Although candidates for this are highlighted above, the precise mechanisms for this cross-talk remain ill-defined. Routes to the uterus or maternal system are restricted, and therefore any such molecules, or their signals, would need to traverse the placenta or fetal membranes before instilling a maternal inflammatory/ parturition-type response in the uterus and cervix.

1.3.2. Maternal clock

1.3.2.1. Functional progesterone withdrawal

Progesterone maintains pregnancy from implantation to term (15). It has an important role in establishing the uterine irresponsive phase by inhibiting PGs synthesis and decreasing its sensitivity to stimulatory factors (158). It has the capacity to modify the immune system, for instance inhibiting lymphocyte proliferation and activation in a hamster implantation model (159), reducing pro-inflammatory cytokines, such as IL-1β and IL-8, and increasing anti-inflammatory IL-10 (25,160,161). In curtailing cervical ripening and fetal membrane separation, progesterone also has the reported ability to reduce the tissue remodelling impact of MMP-1 and MMP-3, induced by these pro-inflammatory cytokines (161).

As espoused already, a decline in systemic progesterone is related to uterine excitations and parturition in most animals (141). Similarly, administered progesterone is protective against inflammatory-induced preterm labour, through a reduction in uterine and cervical Toll-like

receptor 2 (TLR-2) expression; the membrane protein that propagates an infectious response (162). In humans, its suggested that progesterone withdrawal is functional rather than tangible near labour (158). An action replicated pharmacologically with progesterone receptor antagonists, such as mifepristone, which is used clinically for labour induction (163).

In determining the anti-inflammatory actions of progesterone; two isoforms of its receptor (PR) are noted: PR-A and PR-B. In human parturition, simulated progesterone withdrawal is proposed. In addition to pro-inflammatory cytokines, elevations in oxytocin receptor and prostaglandins (15,17), create a negative feedback loop in which this generated inflammatory environment is actually inhibitory for PR function (164). Thus, the inflammatory mediators themselves may lead to the functional (but not actual) withdrawal of progesterone within the reproductive tissues, removing its damping effect and encouraging prostaglandin production, and with it, the initiating steps of labour onset.

1.3.2.2. Uterine awakening

Naturally, the uterus is a contractile organ, but throughout pregnancy it develops a quiescent phase which makes it unresponsive to uterotonic mediators, at least until the onset of labour. Some mechanisms for this quiescence, can be placed in context with uterotonic stimulation, as considered already above, including the down regulation of oxytocin and PG receptors within the myometrium (165–167).

To understand uterine contractions at a molecular level, myometrial relaxation is achieved by an increase of intracellular cyclic adenosine monophosphate (cAMP) and/or a decrease in intracellular calcium level. For mechanisms and tocolytics see **Figure 1.10**. In labour, oxytocin and $PGF_{2\alpha}$ increase intracellular calcium within the myocytes of the myometrium (141), instigating a contractile response. In addition to the aforementioned role of the contraction-associated proteins (CAPs) (oxytocin receptor, COX-2, and PG receptors) (168) connexin 43 is implicated, as a gap junction protein that facilitates electronic signals between myocytes and uterine stretch, with direct effects on smooth muscleor indirect effects through leucocyte-dependent inflammation (15); both of which may result in contractile propagation (“awakening”).

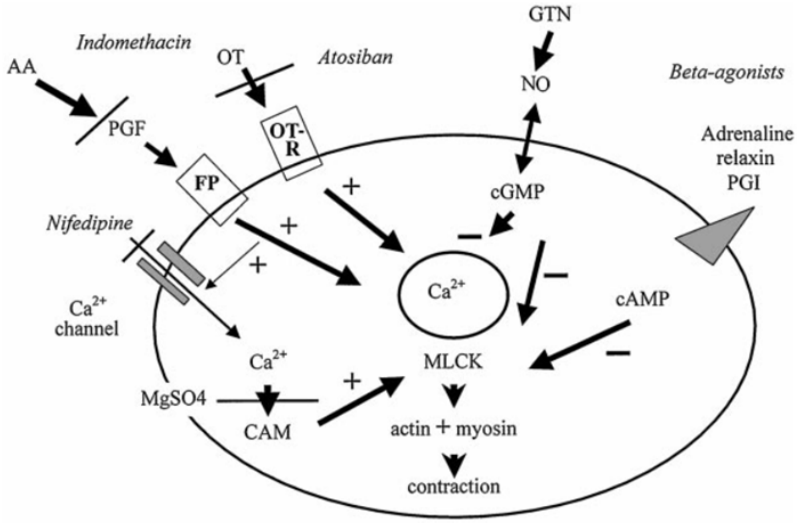


Figure 1.10. Schematic for myometrial contractions and regulatory factors. *(+) indicates stimulatory effect of calcium ions (Ca^{+2}), oxytocin (OT) and prostaglandin $F_{2\alpha}$ (PGF) acting through receptors, oxytocin receptor (OTR) and PGF receptor (FP). The calcium ion binds to calmodulin (CAM) which activates myosin light chain kinase (MLCK) which promotes uterine contractions. While the inhibitory factors (-); beta-agonists, adrenaline, relaxin, prostacyclin (PGI) stimulate uterine relaxation by increasing the intra-colonic concentration of cyclic adenosine monophosphate (cAMP) and cyclic guanosine monophosphate (cGMP). Additionally, uterine relaxation can be achieved by blocking the stimulatory pathway, i.e. using atosiban (which blocks OT receptors), indomethacin (which inhibits PG synthesis) and nifedipine (which blocks calcium channels). Source* (141).

There are two clinical examples when uterine stretch accelerates the timing of labour; polyhydramnios and twin pregnancies (169,170). In a pigtail macaque experiment, a balloon was introduced into the intra-amniotic cavity and inflated to mimic over-stretching of the uterine tissues. This showed a significant increase of pro-inflammatory cytokines, such as IL-1 β, IL-6, IL-8 and TNF-α, and PGE_2 and $PGF_{2\alpha}$ in the amniotic fluid (171). However, in this experiment, overstretching was applied simultaneously to all intra-uterine tissues, making it difficult to define its target (amnion, decidua or myometrium). In *in vitro* experiments, in which mechanical stretch is introduced to the myometrium, decidual tissues and fetal membranes, all of these tissues showed an increase in pro-inflammatory cytokines and MMPs (172–174). A recent study by Durn et al. profiled myometrium prostanoids directly from harvested intrauterine tissues using mass spectrometry (MS) analysis, and showed PGE_2 was the only prostanoid elevated with term labour. However, prostacyclin (PGI_2), a uterine relaxant, was always maintained at more than 3 times this raised level of PGE_2, perhaps

suggesting that the uterine muscles not a primary source of uterotonic prostanoids (175), and thus could the adjust tissue i.e. fetal membrane is the source of contractile prostanoids.

1.3.2.3. Decidual Clock

Anatomically, the decidua lies between the fetal membrane and myometrium. As a temporary layer, formed from the differentiation of the endometrium, the decidua is crucial in providing a favourable setting for early embryo attachment and development (176). In humans, decidualisation is unique amongst most animals as it starts after ovulation and is controlled by ovarian secretions, including progesterone and oestrogen (177). In mice, decidualisation is controlled by the implanted embryo (178), a difference that may ultimately contribute to the variable methods of parturition amongst mammals.

Histologically, the decidual layer contains newly formed blood vessels, glands, invading maternal leukocytes and decidual mesenchymal cells (179). The main populations of leukocytes are NK cells and monocytes located within the choriodecidua (180). Unlike local monocytes, decidual natural killer cells (NK) are unique in secreting disparate cytokines to their peripheral counterparts (181). Distinct from even placental white blood cells, choriodecidual leukocytes have a higher concentration of pro-inflammatory cytokines IL-6 and TNF-α, and lower anti-inflammatory cytokine and MMP-9 activity (**Figure 1.11**) (180). Certainly an upregulation of their pro-inflammatory markers, NF-kB and high-mobility group box 1 (HMGB1), would support their role in parturition (182). Furthermore, decidual tissues COX-2 expression is upregulated in term labour (98). Previous observations could implement decidua as a key source of labour inducer signalling.

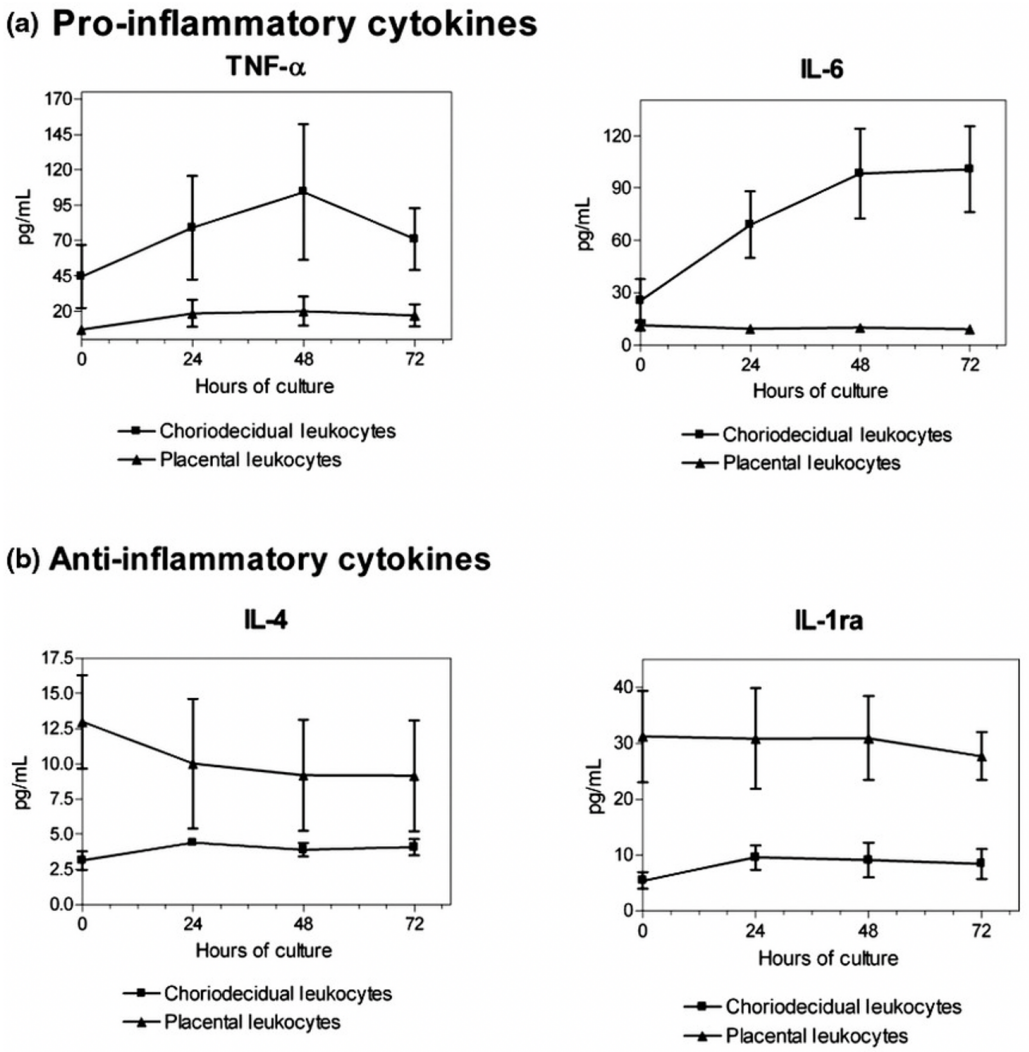


Figure 1.11. Comparisons between pro-inflammatory and anti-inflammatory cytokine production between choriodecidual and placental leukocytes. *(a) Shows pro-inflammatory cytokines, TNF-α and IL-6, against hours of culture. (b) Shows the relationship between IL-4 and IL-1ra (anti-inflammatory cytokines) and length of incubation. Source* (180)*.*

1.3.3. Fetal membrane clock

As already mentioned, the fetal membrane is a temporary avascular sac, composed of amnion and chorion, that seals the fetus and amniotic fluid inside the uterine cavity (183). The inner layer (closest to the fetus) is the amnion, composed of epithelial cells attached to a collagen matrix and fibroblast cells. The outer layer, attached to the decidua, is the chorion, and is composed of cytotrophoblast cells of placental origin (184). Of all possible sites for the signalling of parturition, the fetal membrane is perhaps the most logical, with amniotic fluid in direct contact with the amnion throughout the majority of gestation. Moreover, at term the fetal membrane has around twice the surface area of the placenta (138) and clinical

induction of labour is routinely performed by its irritation during a cervical “sweep” or insertion and expansion of a Foley blub (balloon) (185,186). Another evidence showing the crucial role of the fetal membrane in the parturition process is that preterm rupture of membrane and infection (chorioamnionitis) are well-recognised risk factors for preterm labour (187).

Despite likely endocrine involvement and inflammation, a more direct role for the fetal membrane in the timing of labour has been proposed. In one concept, biological ageing of the fetal membranes becomes a source of inflammation itself (15). More precisely, histological findings of swollen ER and mitochondria, are crude indictors for senescence and an age-driven process within the membranes (188). It is also implied that the natural phenomenon of telomere shortening is important (189). During early embryogenesis fetal leukocyte telomere length is high, but this shortens as pregnancy progresses (15). Similarly, it is reported that fetal membrane telomere length is shorter at term than preterm (190) and that this length is significantly lower in labour than in in non-labouring samples (191) (see **Figure 1.12**). This idea is logical; however, the telomere length could be a sequence of labouring process by itself.

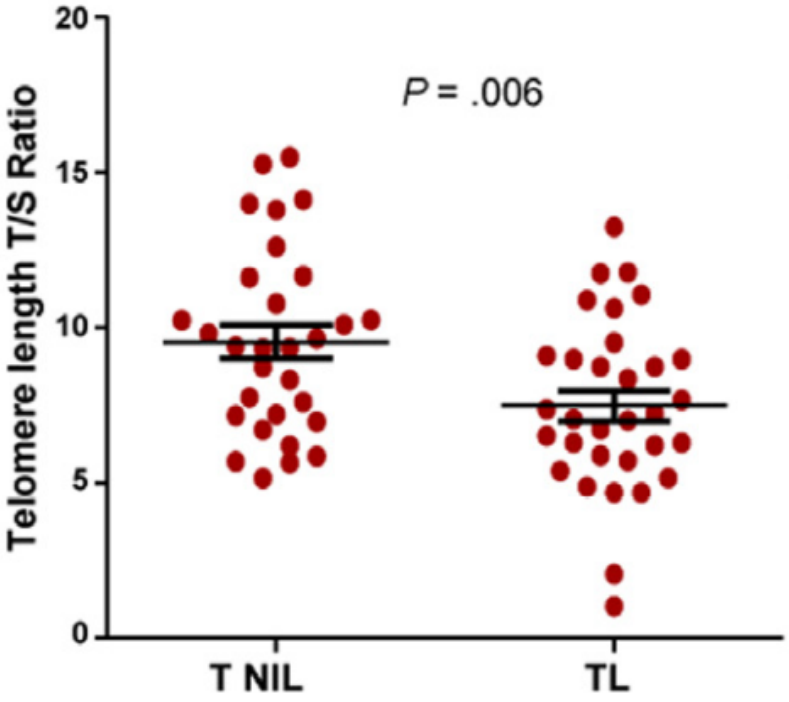


Figure 1.12. Telomere length between term labour (TL) and term not-in-labour (TNIL) fetal membrane samples. *Source* (191).

Perhaps another cause of telomere shorting in the fetal membranes is oxidative stress (OS). Naturally reactive oxygen species (ROS) are known to build up in the fetal system in the third trimester, presumably through an imbalance in fetal growth and oxygen supply (192,193). In defining the potential impact of ROS on fetal membranes, cigarette smoke extract has been used against amnion cells *in vitro*. Within these experiments, the amnion showed an increase in ROS production and associated shortening of telomeres (194), akin to that defined in the term fetal membrane.

As well as telomere shortening, aging cells produce an array of senescence-associated secretory phenotypes (SASPs). SASPs are a wide range of biological markers including pro-inflammatory cytokines; IL-1β, IL-6 and IL-8, chemokines, PGE_2,and MMPs (195). However, these SASP markers are not unique to the senescence, as they are also attenuated in response to infection, endocrine changes, and human labour itself (15). Thus, SASP could add a novel explanation as to how the fetal membrane upregulates an intrauterine pro-inflammatory setting, which ultimately begets labour onset. Notwithstanding though, it should be remembered that these ageing theories of inflammation, although logical, remain as conjecture, with limited supporting evidence. It should also be emphasised that human labour *per se* is a stressful biological event, which exposes intrauterine tissues to both prolonged mechanical forces and intermittent blood flow with each contraction.

Other possible pro-inflammatory stimulators within the fetal membranes are damage-associated molecular pattern*s* (DAMPs). These are cellular components that trigger the immune system and are particularly associated with cellular injury (196). Two good examples are the aforementioned telomere fragments and intra-amniotic HMGB1, both of which are linked to intra-amniotic sterile inflammation (**Figure 1.13**) (15). In support of DAMPs involved, amniotic fluid from term labouring pregnancies, contains exaggerated numbers of telomere fragments over their non-labouring counterparts (197) and HMGB1 can induce preterm labour in experimental mice, when injected intra-amniotically. Moreover, in experiments with fetal membranes, HMGB1 and telomere fragments stimulate the secretion of inflammatory mediators; IL-6 and IL-8 (197,198). These cytokines were reported to be upregulated in the fetal membrane and other gestational tissues at term labour(98). Besides the chemotactic effect, vaginal administration of IL-8 enhanced cervical changes and stimulated labouring in

animal models (28,199). Also, cervical secretion showed an increase of IL-8 with infection-free preterm labour (200). These observations could implement the importance of IL-8 in physiological and pathological labouring.

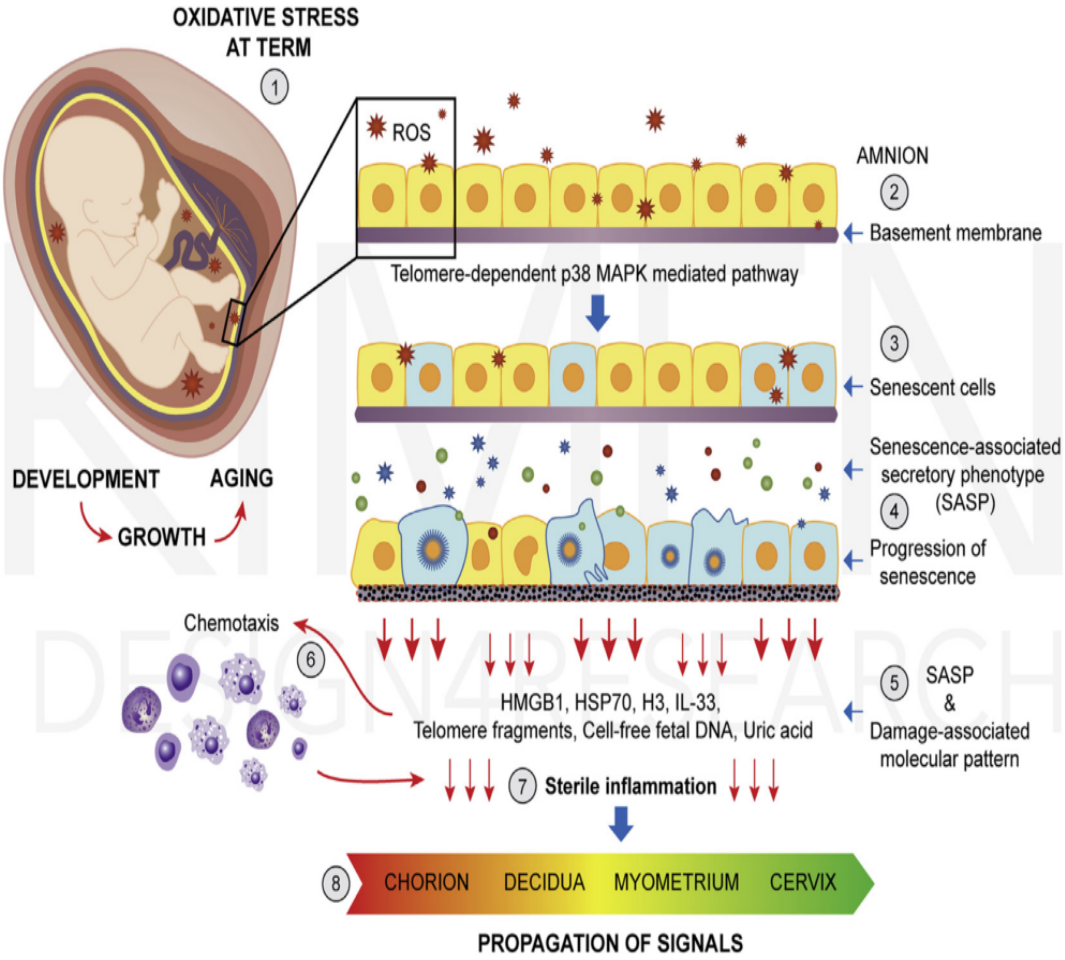


Figure 1.13. A proposed pathway whereby reactive oxygen species (ROS) enhance senescence in an amniotic layer of the fetal membranes to produce sterile inflammation in the intrauterine tissues. *Reactive oxygen species (ROS) accelerate the senescence process in amnion cells by shortening the telomere-dependent p38 MAPK pathway. Senescent cells subsequently produce senescence-associated secretory phenotype (SASP) which leads to cell death (apoptosis) and damage-associated molecular patterns (DAMPs). The liberation of telomere fragments, uric acid, HMGB1, HSP70, HS, IL-33, which attract leukocytes and propagate a sterile inflammatory environment which propagates labour onset. Source* (201).

Although many theories have tried to explain how the fetal membrane initiates the inflammatory signals that kick-start human parturition, a definitive answer is still wanting. There is a general agreement that the fetal membrane is the site that responds to fetal maturation signals and is an essential source of labour-associated mediators, specifically the

prostaglandins. Both fetal membrane tissues (amnion and choriodecidua) show an upregulation of COX-2 in association with term labour (98). Moreover, increased AA-derived prostanoids in the amniotic fluid and exaggerated $cPLA_2$ activity nearing term, prior to labour onset, both strongly indicate a role for the amnion in the early initiation and/or propagation of the intrauterine pro-inflammatory milieu, necessary for successful labour induction (104,202).

1.4. General hypothesis, aim and objectives

The timing of parturition and the awakening of the uterus is crucial for favourable maternal and fetal outcomes (1,3,203). For decades human parturition has been studied, however, there is no definitive answer as to how its initiated. There is a general agreement in the literature that parturition is an inflammatory driven process (19). Amongst the suggested inflammatory mediators, prostanoids have the most proven experimental and clinical efficacy in the induction of labour (31). Although endocannabinoids and ceramides are less well characterised in this role, their inflammatory influence in many other tissues, would also prompt their involvement. However, this has yet to be fully tested (42,60).

For such studies, the delivered fetal membrane (amnion and choriodecidua) is a valuable tissue to study the intrauterine inflammatory environment associated with human labour. By exploring these lipid mediators within the membranes under different types of labour (ie spontaneous term, preterm and term induced) it could be envisaged that a greater understanding of inflammation and its signals in human parturition would be forthcoming. In this regard, mass spectrometry-based lipidomics has seldom been used in the past, even though it provides a much broader spectrum of metabolites and far greater sensitivity. The hypothesis of this thesis is, therefore, that fetal membrane lipidomic composition alters with different types of labour and that comparisons across the two membrane components (amnion and choriodecidua), will help define the centre of the natural and pathological induction of labour and also the crosstalk (if any) between fetus and maternal uterus, in conveying biochemical signals of fetal maturation.

The main aim of this thesis is to explore a comprehensive lipidome profile of the fetal membrane, using state-of-the-art mass spectrometry lipidomics, with spontaneous (term and preterm) and induction of labour, and compare the finding to the not-in labour condition. Understanding the lipidome changes within amnion and choriodecidua with these labouring conditions could help us better understand the inflammatory role of each side of the membrane in physiological, pathological, and drug-induced labour.

Objectives:

- To investigate amnion and choriodecidual tissue lipid mediators, eicosanoids, endocannabinoids and ceramides, under different labouring and non-labouring conditions, using targeted MS based analysis.
- To explore structural lipids of the fetal membranes in different labouring groups compared to term non-labouring control pregnancies, using an untargeted MS based method.
- To create *in vitro* models of both fetal membrane components (amnion and choriodecidua).
- Mimic lipidomic alternations observed in the biological tissues and identify the likely signals which define fetal maturation and instigate and propagate the inflammatory-driven events in human parturition.

Chapter 2: Materials and methods

2.1. Human fetal membrane collection and tissue sampling

Written informed consent was taken from women prior to delivery at St Mary's Hospital, Manchester, UK. Ethical approvals were granted by the North West Local Research Ethics Committee (Reference Number: 08/H1010/55 and 08/H1010/55 (+55)). For tissue experiments, four groups of patients were identified: elective Caesarean section at term not in labour (TNL), spontaneous term in labour (STL), induced term labour (ITL), and preterm in labour (less than 37 weeks gestation) (PTL). Pregnancies complicated with proteinuria, hypertension, diabetes and congenital fetal abnormalities were excluded, along with women with signs of infection. The in-labour condition was defined by the presence of regular uterine contractions with ≥3cm cervical dilatation. The ITL group, were all low-risk late-term pregnancies (41-42 weeks gestation) receiving prostaglandin-derived medications for induction of labour (participant demographics shown in **Table 2.1**). Placentae were collected within 30 mins of delivery on all occasions. Fetal membranes were sampled from the mid-zone, at least 5 cm distant to the rupture site. Choriodecidua was removed from amnion by manual separation, and both tissues washed thoroughly with phosphate-buffered saline (PBS, 3.2 mM Na_2HPO_4, 0.5 mM KH_2PO_4, 1.3 mM KCl, 135 mM NaCl, pH 7.4) (Sigma-Aldrich, Poole, UK) to remove maternal blood. Each was cut to 3-4 cm length and stored separately at -80°C for future processing. For *in vitro* primary cell isolations, only tissues from the TNL group were used; with same general exclusion criteria applied.

Table 2.1. Demographics and clinical characteristics of the study groups. *The numbers are expressed as mean and range. Term not in labour (TNL), spontaneous term labour (STL), induction term labour (ITL), and preterm labour (PTL), body mass index (BMI) at booking visit to antenatal clinic, and gestational age (GA). Statistical analysis= One-way ANOVA with Holm-Šídák's multiple comparisons test unless indicated.* [t] *Kruskal-Wallis test with Dunn's multiple comparisons test.* [a b c] *p≥ 0.05,* [a] *vs TNL,* [b] *vs STL,* [c] *vs ITL.*

Amnion				
	Eicosanoids and related mediators study			
	TNL (n=13)	STL (n=7)	ITL (n=9)	PTL (n=5)
MA	31.3 (23-40)	33 (26-40)	31.3 (26-38)	25.3 (18-35)
BMI	26.2 (20-31)	28.7 (21-43)	29.6 (22-36)	21 (18-24)
GA[t]	38.4 (37-39)	39.5 (38-41)	41.1 (41-42) [a]	34.2 (32-35) [a b c]
Parity	0.8 (0-2)	2 (0-6)	0.6 (0-3)	1 (0-4)

	Endocannabinoid and ceramide study			
	TNL (n=12)	STL (n=8)	ITL (n=9)	PTL (n=5)
MA	31.9 (23-40)	33 (26-40)	31.3 (22-38)	25.3 (18-35)
BMI	26 (20-34)	28.7 (21-43)	29.6 (22-36)	21 (18-24)
GA[t]	38.4 (37-39)	39.7 (38-41)	41.5 (41-42) [a]	34.2 (32-35) [a b c]
Parity	0.8 (0-2)	2 (0-6)	0.6 (0-3)	1 (0-4)
	Complex lipid study			
	TNL (n=12)	STL (n=5)	ITL (n=9)	PTL (n=5)
MA	31.6 (23-40)	33 (32-35)	32 (22-38)	25.3 (18-35)
BMI	26.1 (20-34)	31.5 (25-43)	29 (22-36)	21 (18-24)
GA[t]	38.4 (37-39)	39.7 (38-41)	41.1 (41-42) [a]	34. (32-35) [a b c]
Parity	0.7 (0-2)	1.6 (0-6)	0.5 (0-3)	1 (0-4)
	Total fatty acid study			
	TNL (n=5)	STL (n=4)	ITL (n=6)	PTL (n=4)
MA	33.6 (30-37)	32.33 (32-33)	31.33 (22-38)	25.25 (18-35)
BMI	23.4 (17-26)	31.75 (26-43)	29.67 (22-36)	21 (18-24)
GA[t]	37.86 (37-39)	39.86 (39-41) [a]	41 (41-42) [a]	32.7 (32-35) [a b c]
Parity	0.8 (0-1)	2.7 (0-6)	0.17 (0-1)	0.8 (0-4)
Choriodecidua				
	Eicosanoids and related mediators study			
	TNL (n=12)	STL (n=9)	ITL (n=9)	PTL (n=5)
MA	32.4 (23-40)	33 (26-40)	31.3 (22-36)	25.3 (24-35)
BMI	25.3 (16-34)	28.3 (21-43)	29.6 (22-36)	21 (18-24)
GA[t]	38.6 (37-39)	39.7 (38-41)	41.6 (41-42) [a]	34.2(32-35) [a b c]
Parity	0.9 (0-2)	1.7 (0-6)	0.6 (0-3)	1 (0-4)
	Endocannabinoid and ceramide study			
	TNL (n=13)	STL (n=11)	ITL (n=9)	PTL (n=6)
MA	32.5 (23-40)	33 (26-40)	31.3 (22-38)	25.3 (18-35)
BMI	26 (20-34)	28.3 (21-43)	29.5 (22-36)	21 (18-24)
GA[t]	38.6 (37-39)	39.7 (38-41)	41.2 (41-42) [a]	34.2 (32-35) [a b c]
Parity	0.9 (0-2)	1.7 (0-6)	0.6 (0-3)	1 (0-4)
	Complex lipid study			
	TNL (n=13)	STL (n=9)	ITL (n=9)	PTL (n=6)
MA	33.1 (23-40)	33 (26-40)	31.3 (22-36)	25.3 (18-35)
BMI	25.8 (20-34)	28.3 (21-43)	29.6 (22-36)	21 (18-24)
GA[t]	38.2 (37-39)	39.7 (38-41)	41.6 (41-42) [a]	34.2 (32-35) [a b c]
Parity	0.8 (0-2)	1.7 (0-6)	0.6 (0-3)	1 (0-4)
	Total fatty acid study			
	TNL (n=7)	STL (n=6)	ITL (n=7)	PTL (n=6)

MA	30.3 (21-38)	32.3 (32-33)	31.9 (22-38)	22.2 (18-26)
BMI	25 (17-34)	31.8 (26-43)	28.7 (22-36)	23 (18-28)
GA[t]	39.4 (39-40)	40.5 (39-41)	41.6 (41-42) [a]	33.8 (32-35) [a b c]
Parity	0.8 (0-1)	2.7 (0-6)	0.1 (0-1)	1 (0-4)

2.2. Analysis of fetal membrane lipid mediators using Liquid Chromatography Coupled Electrospray Tandem Mass Spectrometry (LC/ESI-MS/MS)

Mass spectrometry (MS) is a powerful tool for profiling and quantifying spectral analysis of metabolites, including lipids. Although lipid mediators can be analysed by enzyme immunoassay, the lack of sensitivity, through antibody cross-reactivity, limits analyses to single analytes. Liquid chromatography coupled electrospray tandem mass spectrometry (LC/ESI-MS/MS) affords far higher sensitivity, even at very low lipid concentrations, and is more time efficient; considering many lipids in a single run, and also less hazardous than other methods, such as gas chromatography (GC) GC/MS (204).

LC/ESI-MS/MS works by separating sample analytes after injection through a column, subsequent to them being ionised by electrospray ionization (ESI) and evaporated. The analytes are then filtered according to their mass-to-charge ratio (m/z) using a quadruple; then fragmented in a collision cell before undergoing filtration (**Figure 2.1**). Specifically for this lipid analysis, Acquity ultra-high-performance liquid chromatography (UPLC) was coupled to an ESI triple quadrupole Xevo TQ-S MS (Waters, Wilmslow, UK). The methods for detection of lipid mediators considered here, i.e. eicosanoids, endocannabinoids, endocannabinoid-like lipids and ceramides, have all been published previously (89,205).

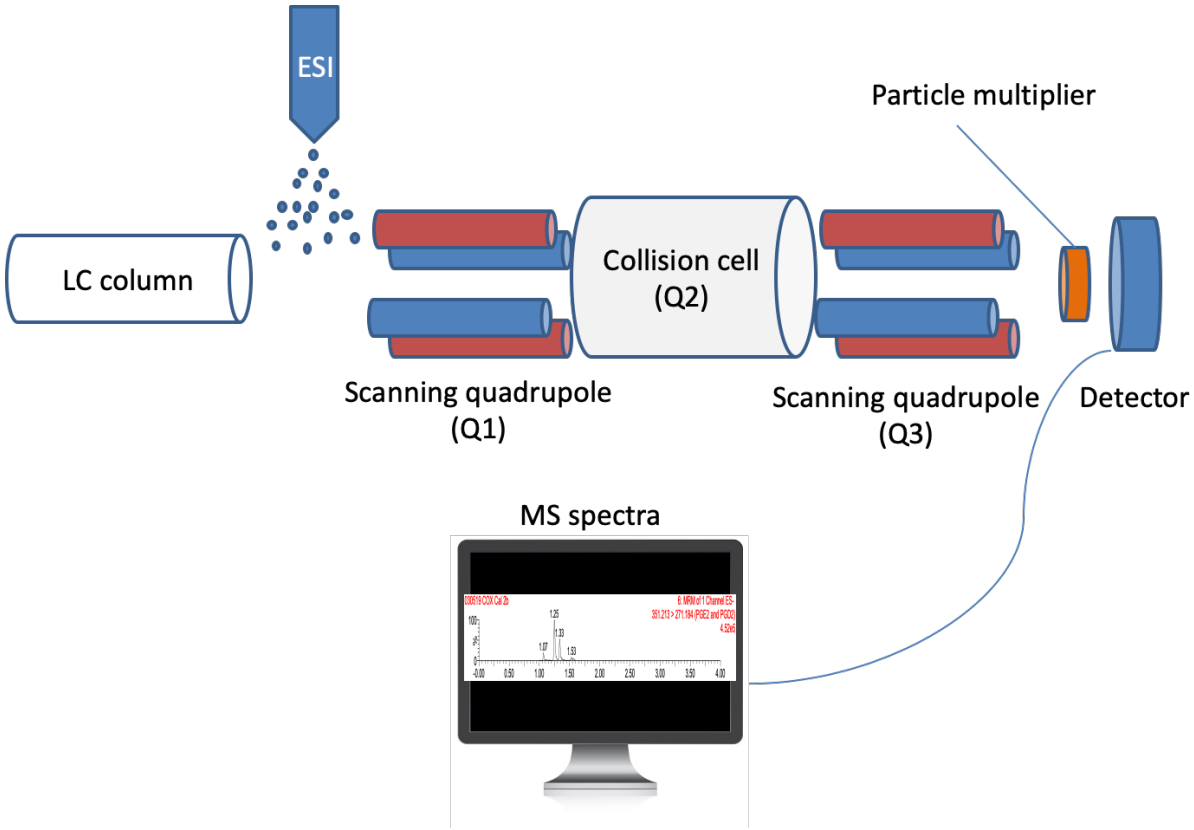


Figure 2.1. Liquid chromatography coupled electrospray tandem mass spectrometry (LC/ESI-MS/MS). Sample analytes are separated through an LC column and ionised and evaporated by ESI. *The ionised molecules are then filtered according to desired mass-to-charge ratio (m/z) (Q1), then fragmented in the collision cells (Q2), and filtered again using m/z (Q3). The desired analytes are then detected and reported as MS spectra.*

2.2.1. Analysis of eicosanoids and related mediators

The eicosanoid assay aims to profile oxidative polyunsaturated fatty acids (PUFA) mediators through various metabolic pathways; cyclooxygenase (COX), lipoxygenase (LOX) and cytochrome P450 monooxygenase (CYP450). The most common precursors of eicosanoids are: AA, dihomo-gamma-linolenic acid (DGLA), linoleic acid (LA), eicosapentaenoic acid (EPA), and docosahexaenoic acid (DHA); with their detected derivatives listed below (**Table 2.2**).

Table 2.2. PUFA-derived eicosanoids, as recognised by UPLC/ESI-MS/MS

DGLA/LA/ALA-derived	EPA-derived	DHA-derived	AA-derived
PGD_1	5-HEPE	RvD_1	PGD_2
PGE_1	8-HEPE	RvD_2	PGE_2
$PGF_{1\alpha}$	9-HEPE	RvD_3	15-keto PGE_2

13,14-dihydro-15-keto PGE_1	11-HEPE	RvD_4	13,14-dihydro-15-keto PGE_2
13,14-dihydro-15-keto $PGF_{1\alpha}$	12-HEPE	RvD_5	PGI_2 (as 6-keto $PGF_{1\alpha}$)
13,14-dihydro $PGF_{1\alpha}$	15-HEPE	MaR1	$PGF_{2\alpha}$
13,14-dihydro PGE_1	18-HEPE	MaR2	8-iso $PGF_{2\alpha}$
9-HODE	TXB_3	10(S)17(S)-DiHDPA (PDX)	13,14-dihydro $PGF_{2\alpha}$
13-HODE	PGD_3	4-HDHA	13,14-dihydro-15-keto $PGF_{2\alpha}$
9,10-DiHOME	RvE_1	7-HDHA	5-HETE
12,13-DiHOME	PGE_3	8-HDHA	8-HETE
9-OxoODE	$PGF_{3\alpha}$	10-HDHA	9-HETE
13-OxoODE	LTB_5	11-HDHA	11-HETE
9(10)-EpOME	LxA_5	13-HDHA	12-HETE
12(13)-EpOME	8(9)-EpETE	14-HDHA	15-HETE
Trans-EKODE	11(12)-EpETE	17-HDHA	20-HETE
9-HOTrE	14(15)-EpETE	20-HDHA	LTB_4
13-HOTrE	17(18)-EpETE	10(11)-EpDPE	PGJ_2
5-HETrE	8,9-DiHETE	13(14)-EpDPE	Δ^{12}-PGJ_2
15-HETrE	11,12-DiHETE	16(17)-EpDPE	15-deoxy-Δ^{12},14-PGJ_2
	14,15-DiHETE	19(20)-EpDPE	14,15-DiHET
	17,18-DiHETE	10,11-DiHDPA	11,12-DiHET
		13,14-DiHDPA	8,9-DiHET
		16,17-DiHDPA	5,6-DiHET
		19,20-DiHDPA	5(6)-EET
			11(12)-EET
			14(15)-EET
			8(9)-EET

			5-oxo-ETE
			15-oxo-ETE
			TXB_2
			5(S)6(R)15(S)-LxA_4
			5,15-DiHETE
			8,15-DiHETE

2.2.2. Materials

Methanol (LC-MS grade, ≥99.9%), methyl formate (LC-MS grade, ≥99.9%), acetonitrile (LC-MS grade, ≥99.9%), hydrochloric acid (HCl) (ACS grade, 36.6-38%), acetic acid (HPLC grade, ≥99.7%) were supplied by Sigma-Aldrich (Poole, UK). Ethanol (HPLC grade, ≥99.8%) and hexane (HPLC grade, 97.0%) from Fisher Scientific (Loughborough, UK). HCl was diluted in ultra-pure water to a concentration of 1M before use. The deuterated eicosanoid internal standards; prostaglandin B_2-d_4 (PGB2-d_4), 12-hydroxyeicosatetraenoic acid-d_8 (12-HETE-d_8), 15-HETE-d8, 8,9-epoxyeicosatrienoic acid-d11 (8(9)EET-d_{11}), 8,9-dihydroxyeicosatrienoic acid-d_{11} (8,9-DHET-d_{11}) and 12,13-dihydroxyoctadecanoic acid-d4 (12,13-DiHOME-d_4), were obtained in powder form from Cayman Chemicals (Ann Arbor, MI, USA) and dissolved individually in ethanol to a final concentration of 10ng/mL and stored at -80°C for up to a year. The prostanoid standards; PGE_1, PGD_1, 6-keto $PGF_{1\alpha}$, 13,14-dihydro-15-keto-PGE_1, 13,14-dihydro-15-keto PGF_1, $PGF_{1\alpha}$, PGD_2, PGE_2, 15-keto PGE_2, 13,14-dihydro PGE_1, 13,14-dihydro $PGF_{2\alpha}$, 13,14-dihydro $PGF_{1\alpha}$, 13,14-dihydro-15-keto $PGF_{2\alpha}$, $PGF_{2\alpha}$, 8-isoprostane $PGF_{2\alpha}$, $PGF_{3\alpha}$, Δ^{12}-PGJ_2, PGJ_2, thromboxane B_2 (TXB_2), TXB_3, 15-deoxy $\Delta^{12,14}$-PGJ_2, 13,14-dihydro-15-keto PGE_2, PGD_3, and PGE_3 were purchased from Cayman Chemicals.

The hydroxy fatty acid standards; 9-HODE, 13-HODE, resolvin D1 (RvD1), RvD2, RvE1, maresin1 (MaR1), protectin DX (PDX), 11-HDHA, 4-HDHA, 7-HDHA, 10- HDHA, 13-HDHA, 14-HDHA, 17-HDHA, 20-HDHA, leukotriene B4, 14,15-DHET, 11,12-DHET, 8,9-DHET, 5,6-DHET, 5(6)-EET, 11(12)-EET, 14(15)-EET, 8(9)-EET, 5-oxo-ETE, 15-HETrE, 5-HETE, 8-HETE, 9-HETE, 11-HETE, 12-HETE, 15-HETE, 20-HETE, 5-HEPE, 8-HEPE, 9-HEPE, 11-HEPE, 12-HEPE, 15-HEPE, 18-HEPE, 9-HOTrE, 13-HOTrE, 19,20-dihydroxydocosapentaenoic acid (19(20)-DiHDPA), 9(10)-

EpOME, 12(13)-EpOME, 9-OxoODE, 13-OxoODE, 5,15-dihydroxyeicosatetraenoic acid (5(15)-DiHETE), 8(15)-DiHETE, 19(20)- epoxydocosapentaenoic acid (19(20)-EpDPE), 16(17)-EpDPE, and 12,13-epoxy-9-keto-10(*trans*)-octadecenoic acid (*trans*-EKODE) were also obtained from Cayman Chemicals. Both prostanoid and hydroxy fatty acid standards were individually dissolved in ethanol to a concentration of 10ng/µL and stored at -80°C for up to a year before use.

2.2.3. Equipment and reagents

Fetal membrane tissues ≈30mg (wet weight) were homogenised by Dounce tissue grinder kit from DWK Life Sciences (Wheaton, USA). The following equipment were used for the eicosanoid extractions; deionised ultra-pure water, obtained from Elga Water Purelab flex purification system (High Wycombe, UK), a refrigerated benchtop centrifuge (3-16KL, Sigma-Aldrich), a Vortex (Fisons Whirlimixer) from Fisher Scientific, a custom made nitrogen drying cabinet (University of Bradford). The prepared samples were "cleaned" using STRATA™ solid phase extraction (SPE) cartridges C18-E, 500 mg, 6 mL, from Phenomenex (Macclesfield, UK). A SPE vacuum manifold was purchased from Phenomenex coupled with a vacuum pump (1c Vacuubrand, Wertheim, Germany). Other items included; 150mm unplugged glass Pasteur pipettes, Parafilm® from Fisher Scientific, Hamilton glass syringes (volumes: 10µL, 50µL, 100µL, 250µL) from SGE (Melbourne, Australia), round-bottomed and flat-bottomed 10mL glass tubes, amber glass vials (2mL), conical glass inserts (200µL), screw caps (8mm), and PTFE septa obtained from Phenomenex. Narrow range (2.5-4.5) pH indicator paper strips were also used supplied by MSD (Hoddesdon, UK).

The eicosanoid analyses were conducted with MS system purchased from Waters, consisting of Acquity UPLC coupled with a VanGuard pre-column filter (Acquity UPLC BEH, 1.7µm, 2.1 x 5mm) and C_{18} column (Acquity UPLC BEH, 1.7µm, 2.1 x 50mm) and electrospray ionisation triple quadrupole MS Xevo TQ-S.

2.2.4. Eicosanoid internal standards and calibration cocktails

Six deuterated internal standards were used to form the cocktail; PGB2-d_4, 12-HETE-d_8, 15-HETE-d_8, 12,13-DiHOME-d_4, 8(9)EET-d_{11} and 8,9 DiHET-d_{11}. 100µL of each stock solution (10ng/mL, as described in **section 2.2.2**) were mixed and diluted in 400µL of ethanol to a final

concentration of 1 pg/mL. The internal standard cocktail was then stored in -20°C for up to three months.

The calibration line standard for COX assays was prepared by adding 10µL of the following at 10ng/µL: PGE_1, PGD_1, 6-keto $PGF_{1\alpha}$, 13,14-dihydro-15-keto PGE_1, 13,14-dihydro-15-keto $PGF_{1\alpha}$, $PGF_{1\alpha}$, PGD_2, PGE_2, 15-keto PGE_2, 13,14-dihydro PGE_1, 13,14-dihydro $PGF_{2\alpha}$, 13,14-dihydro $PGF_{1\alpha}$, 13,14-dihydro-15-keto $PGF_{2\alpha}$, $PGF_{2\alpha}$, 8-iso $PGF_{2\alpha}$, $PGF_{3\alpha}$, Δ^{12}-PGJ_2, PGJ_2, TXB_2, TXB_3, 15-deoxy-Δ^{12},14-PGJ_2, 13,14-dihydro-15-keto PGE_2, PGD_3 and PGE_3. A total volume of 1mL was achieved by adding 760µL ethanol (100%) to give a final concentration of 100pg/µL. For LOX/CYP450 assays, the calibration line standard was likewise derived by adding 10µL of the following at 10ng/µL; 9-HODE, 13-HODE, RvD_1, RvD_2, MaR_1, PDX, 11-HDHA, 4-HDHA, 7-HDHA, 10-HDHA, 13-HDHA, 14-HDHA, 17-HDHA, 20-HDHA, RvE_1, TBX_3, LTB_4, 14,15-DHET, 11,12-DHET, 8,9-DHET, 5,6-DHET, 5(6)-EET, 11(12)-EET, 14(15)-EET, 8(9)-EET, 5-oxo-ETE, 15-HETrE, 5-HETE, 8-HETE, 9-HETE, 11-HETE, 12-HETE, 15-HETE, 20-HETE, 5-HEPE, 8-HEPE, 9-HEPE, 11-HEPE, 15-HEPE, 18-HEPE, 12-HEPE, 9-HOTrE, 13-HOTrE, 19(20)-DiHDPA, 9(10)-EpOME, 12(13)-EpOME, 9(10)-DiHOME, 9-OxoODE, 13-OxoODE, 5(15)-DiHETE, 8(15)-DiHETE, 19(20)-EpDPE, 16(17)-EpDPE, and *Trans*-EKODE. A total volume of 1mL was achieved by adding 460µL ethanol (100%) to a final concentration of 100pg/µL. Both calibration line standards were moved into amber vials, sealed with Parafilm® and stored at 80°C for up to three months.

2.2.5. Fetal membrane extractions for eicosanoid determination

Fetal membrane amnion and choriodecidua were weighed ≈30mg (wet weight) and fully homogenised on ice using a Dounce homogeniser (DWK Life Sciences) with 4mL ice-cold 15% (v/v) methanol-water solution. The resultant homogenate was transferred using a glass Pasteur pipette into a flat-bottled glass tube and 1ng/µL internal standard cocktail added. Samples were incubated for 30min on ice in the dark, with homogeniser carefully washed with ethanol. Following this incubation, homogenates and standards were centrifuged for 10 min at 4°C 1500x g. The upper layer (supernatant) was then transferred to a round-bottomed glass extraction tube, using glass Pasteur pipette and protein pellet stored at -20°C for future protein content analysis. In order to “clean-up” samples, a solid phase extraction was performed, using a pre-conditioned C18 cartridge (pre-treated with 100% methanol, then

ultra-pure water). Samples were further acidified to pH3 by addition of 0.1M HCl; as confirmed by pH indicator strip. Following acidification, samples were added to the SPE cartridges and left to drain spontaneously, without use of vacuum. Under low vacuum pressure, cartridges were subsequently washed with 6mL methanol (15% v/v) and deionised water, followed by 6mL hexane under high vacuum. 6mL methyl formate was then added to each cartridge to elute samples into round-bottomed collection tubes. These eluted samples were dried under gentle nitrogen (NO_2) and lipid residues re-suspended in 100µL neat ethanol. Samples were further centrifuged at 1500x g 4°C for 15 seconds, then divided into two closed-cap amber sample vials (50µL each) using a Hamilton syringe and stored at -20°C.

2.2.6. Eicosanoid assay UPLC/ESI-MS/MS settings

COX and LOX/CYP450 assays were run separately. Extracted samples and blanks (HPLC graded ethanol) were placed on trays at 8°C. The autosampler was set to 3µL injection volume. Chromatographic analysis was performed on a C_{18} column (Acquity UPLC BEH, 1.7µm, 2.1 x 50mm; Waters) at 60°C with a 0.6 mL/min flow rate. Two mobile phases were used; mobile phase A, ultra-pure water:acetic acid (1:0.002%, v/v) and mobile phase B, acetonitrile:acetic acid (1:0.002%, v/v). The solvent flow settings for both assays are described in **Tables 2.3** and **2.4**.

Table 2.3. Solvent gradient for prostanoid (COX) analysis. *Mobile phase A (ultra-pure water with 0.02% acetic acid); mobile phase B (acetonitrile with 0.02% acetic acid).*

Time (min)	Mobile phase A (%)	Mobile phase B (%)
0.0	80	20
0.5	80	20
0.6	60	40
2.5	60	40
4.0	35	65
4.1	80	20
5.8	80	20

Table 2.4. Solvent gradient for hydroxy fatty acid (LOX/CYP450) analysis. *Mobile phase A (methanol with 0.02% acetic acid), mobile phase B (acetonitrile with 0.02% acetic acid).*

Time (min)	Mobile phase A (%)	Mobile phase B (%)

0.0	75	25
3.0	20	80
3.2	75	25
5.0	75	25

For both assays, ESI negative mode was performed with tri-quadruples MS settings (capillary voltage, source temperature, dwell time, desolvation temperature) as described in **Table 2.5**. Argon gas was used in the collision cells. For each eicosanoid, multiple reaction monitoring (MRM), cone voltage, collision energy, and indicative retention times were determined "in-house" (**Tables 2.6 and 2.7**). These settings have been published previously (89,113,206). Samples were run in duplicate, with a blank injection in-between runs to reduce carry-over.

Table 2.5 COX and LOX/CYP450 MS/MS settings

Assay	Capillary voltage (kV)	Source temperature (°C)	Desolvation temperature (°C)	Dwell time (s)
COX	3.1	150	500	0.007
LOX/CYP450	1.5	150	600	0.003

Table 2.6 Multiple reaction monitoring (MRM) transition settings for COX assay.

Compound	MRM	Cone voltage	Collision energy (eV)	Retention time (min)
PGD_1	353>317	12	12	1.31
PGE_1	353>317	12	12	1.26
6-keto $PGF_{1\alpha}$	369>163	2	26	0.99
13,14-dihydro-15-keto $PGF_{1\alpha}$	355>113	48	28	1.60
$PGF_{1\alpha}$	355>311	14	24	1.16
13,14-dihydro PGE_1	355>337	18	16	1.38
13,14-dihydro-15-keto PGE_1	353>335	12	14	1.67
PGB_2-d_4	337>179	12	20	2.02
PGD_2	351>271	32	16	1.32
PGE_2	351>271	32	16	1.24
15-keto PGE_2	349>287	2	14	1.38
13,14-dihydro $PGF_{2\alpha}$	355>311	14	24	1.30
13,14-dihydro-15-keto $PGF_{2\alpha}$	353>113	2	28	1.48
$PGF_{2\alpha}$	353>193	2	24	1.17
8-iso $PGF_{2\alpha}$	353>193	2	24	1.09
PGJ_2	333>271	22	16	1.99
Δ^{12}-PGJ_2	333>271	22	16	2.02

15-deoxy-$\Delta^{12,14}$-PGJ_2	315>203	32	20	3.87
TXB_2	369>169	2	16	1.09
13,14-dihydro $PGF_{1\alpha}$	357>113	2	22	1.35
13,14-dihydro-15-keto PGE_2	351>333	12	12	1.55
TXB_3	367>169	30	16	1.02
PGD_3	349>269	2	16	1.16
PGE_3	349>269	4	14	1.11
$PGF_{3\alpha}$	351>193	2	22	1.06

Table 2.7. Multiple reaction monitoring (MRM) transition settings for LOX/CYP450 assay.

Compound	MRM	Cone voltage (V)	Collision energy (eV)	Retention time (min)
9-HODE	295>171	16	16	2.49
13-HODE	295>195	2	18	2.46
5-HETrE	321>115	24	14	3.07
15-HETrE	321>221	30	14	2.68
5-HETE	319>115	14	14	2.74
8-HETE	319>155	10	14	2.65
9-HETE	319>123	16	14	2.70
11-HETE	319>167	14	14	2.61
12-HETE	319>179	20	14	2.65
12-HETE-d_8	327>184	20	16	2.63
15-HETE	319>175	4	14	2.54
15-HETE-d_8	327>226	4	16	2.51
20 HETE	319>245	4	14	2.30
5(6)-EET	319>191	4	10	3.02
8(9)-EET	319>155	10	14	2.98
11(12)-EET	319>167	14	14	2.94
14(15)-EET	319>175	4	14	2.84
5-HEPE	317>115	16	12	2.45
8-HEPE	317>155	26	12	2.38
9-HEPE	317>149	20	14	2.42
11-HEPE	317>167	12	12	2.34
12-HEPE	317>179	28	12	2.39
15-HEPE	317>175	8	14	2.33
18-HEPE	317>215	12	14	2.24
8(9)-EpETE	317>155	22	10	2.69
11(12)-EpETE	317>179	34	12	2.66
14(15)-EpETE	317>207	26	14	2.63
17(18)-EpETE	317>255	6	12	2.55
5,6-DiHET	337>145	8	16	2.32
8,9-DiHET	337>127	4	18	2.22
11,12-DiHET	337>167	2	18	2.14
14,15-DiHET	337>207	18	16	2.04

5-oxo-ETE	317>203	14	18	2.93
15-oxo-ETE	317>113	38	16	2.62
LTB_4	335>195	12	14	1.86
LTB_5	333>195	32	16	1.62
LXA_5	349>115	22	14	1.18
LXA_4	351>115	18	16	1.37
RvE_1	349>195	14	16	0.79
RvD_1	375>141	18	12	1.37
RvD_2	375>175	2	22	1.24
RvD_3	375>147	50	20	1.20
RvD_4	375>101	46	18	1.52
RvD_5	359>199	22	16	1.82
4-HDHA	343>101	8	12	2.79
7-HDHA	343>141	6	14	2.67
8-HDHA	343>189	18	12	2.70
10-HDHA	343>153	2	16	2.61
11-HDHA	343>193	2	12	2.65
13-HDHA	343>193	2	12	2.57
14-HDHA	343>161	12	14	2.61
17-HDHA	343>201	14	14	2.55
20-HDHA	343>241	2	12	2.47
PDX	359>153	16	16	1.81
MaR_1	359>177	22	16	1.82
MaR_2	359>221	28	12	1.99
9 OxoODE	293>185	14	18	2.68
13 OxoODE	293>113	16	20	2.58
9 HOTrE	293>171	20	16	2.22
13 HOTrE	293>195	12	16	2.25
9(10) EpOME	295>171	16	16	2.88
12(13)EpOME	295>195	2	18	2.84
Trans EKODE	309>209	16	12	2.29
9,10 DiHOME	313>201	16	20	1.97
12,13 DiHOME	313>129	44	22	1.90
12,13 DiHOME-d_4	317>185	44	24	1.89
8(9) EET-d_{11}	330>155	14	12	2.96
8,9 DiHET-d_{11}	348>127	16	24	2.20
8,9-DiHETE	335>185	40	16	1.95
11,12-DiHETE	335>167	22	18	1.89
14,15-DiHETE	335>207	28	16	1.86
17,18-DiHETE	335>247	24	16	1.78
5,15 DiHETE	335>115	12	12	1.80
8,15 DiHETE	335>155	22	16	1.74
10(11)-EpDPE	343>153	32	12	2.91
13(14)-EpDPE	343>193	32	12	2.89
16(17) EpDPE	343>233	14	12	2.87
19(20) EpDPE	343>285	18	12	2.79
10,11-DiHDPA	361>153	26	18	2.19

13,14-DiHDPA	361>193	24	16	2.14
16,17-DiHDPA	361>233	48	16	2.11
19,20 DiHDPA	361>273	50	16	2.03

2.2.7. Eicosanoid data processing

Ion peaks in the mass spectra were identified using Masslynx - an extension of the TargetLynx programme (v 4.0, Waters) and quantified to matched compound calibration line and normalised to the class deuterated internal standard e.g. PGE_2 to PGB_2-d_4, and 12-HETE to 12-HETE-d_8. Analytes were reported by MassLynx as pg/µL, then multiplied by 100 to correct for sample volume (100µL) and divided by protein content. The lower limit of detection was set as a signal-to-noise ratio of 3; upper limited was set to 10.

2.3. Analysis of endocannabinoids, *N*-acyl ethanolamines and monoacylglycerols

A combined endocannabinoid and ceramide extraction was performed for the fetal membranes. The extracted samples were investigated by UPLC/ESI-MS/MS using an Acquity UPLC pump (Waters) coupled to an electrospray ionization triple quadrupole mass spectrometer (Xevo TQ-S; Waters).

2.3.1. Materials

Ethanol (HPLC grade, ≥99.8%) and hexane (HPLC grade, 97.0%) were obtained from Fisher Scientific (Loughborough, UK). Methanol (LC-MS grade, ≥99.9%), acetonitrile (LC-MS grade, ≥99.9%) and chloroform (LC-MS grade, ≥99.9%) were purchased from Sigma-Aldrich. For the endocannabinoid assay the deuterated internal standards were: AEA-d_8 and 2-AG-d_8 from Cayman Chemicals.

The following standards were used in the endocannabinoid assay calibration line: 2-AG, AEA, myristoyl ethanolamide (MEA), palmitoleoyl EA (POEA), pentadecanoyl EA (PDEA), palmitoyl EA (PEA), heptadecanoyl EA (HEA), stearoyl EA (STEA), oleoyl EA (OEA), vaccenoyl EA (VEA), lineoleoyl EA (LEA), α-linolenoyl EA (ALEA), dihomo-ɣ-linoleoyl EA (DGLEA), eicosapentaenoyl EA (EPEA), docosapentaenoyl EA (DPEA), docosahexaenoyl EA (DHEA), docosanoyl EA (DEA), nervonoyl EA (NEA), lignoceroyl EA (LGEA), N-arachidonoyl taurine (NAT), $PGF_{2\alpha}$-EA, PGE_2-EA,

PGD_2-EA, 15-HETE-EA, 5,(6)-EET-EA, 8,(9)-EET-EA, 11,(12)-EET-EA, 14,(15)-EET-EA, 2-palmitoyl glycerol (2-PG) and 2-lineoleoyl glycerol (2-LG); all supplied again by Cayman Chemicals.

2.3.2. Equipment

The equipment used as listed in **section 2.2.3**, with additional STRATA^{TM} SPE cartridges C18-E (500mg, 1mL) purchased from Phenomenex.

2.3.3. Preparation of endocannabinoid internal standards

The deuterated endocannabinoid internal standards were made by adding 100µL of AEA-d_8 and 200µL of 2-AG-d_8 with 700µL neat ethanol to a final concentration of 1ng/µL AEA-d_8 and 2ng/µL 2-AG-d_8. This solution was prepared freshly prior to each tissue extraction and stored at -20 °C.

2.3.4. Preparation of endocannabinoid standard stock solutions and quantitative calibration line

Lipid standards (as listed in **section 2.3.1**) were individually dissolved in ethanol to a final concentration of 10ng/µL and stored at -80°C for up to a year. Prior to each run, the working standard solution was made by diluting 10µL stock solution in 760µL ethanol, to give a final concentration of 100pg/µL. This standard cocktail was then stored in -20°C for up to 3 months. In order to make a quantitative calibration line; the working standard cocktail (100pg/µL) was further diluted in 1:2 serial dilutions to 20, 10, 5, 2.5, 1.25, 0.60, 0.30, 0.15, 0.075 and 0.04 pg/µL. Deuterated internal standards (1ng/µL AEA-d_8 and 2ng/µL 2-AG-d_8) were added to each vial; and vials were placed under a gentle nitrogen stream to evaporate the solvent. The resultant samples were reconstituted in 100µL ethanol and each vial capped and sealed with Parafilm® (Fisher Scientific, Loughborough, UK), and stored at -20°C for up to a week.

2.3.5. Fetal membrane endocannabinoid and ceramide extractions

Combined endocannabinoid and ceramide extractions were performed by adding both internal standards during the extraction process. Amnion and choriodecidua (approximately 40-30mg) were homogenised using 3mL Dounce homogeniser (DWK Life Sciences) with three changes of 1mL ice-cold chloroform:methanol (2:1). The homogenate was then transferred

to a clean flat-bottomed glass tube, using a glass pipette. Each tube was then spiked with the internal standards; 40µL deuterated ceramide cocktail (100ng/mL) and 20µL endocannabinoid cocktail (1ng/µL AEA-d_8 and 2ng/µL 2-AG-d_8). 1.5mL ice-cold ultra-pure water was then added, with an incubation for 30min in the dark (vortexed every 10min). Samples were then centrifuged at 1500x g for 5min at 4°C. Once centrifuged, the organic layer was aspirated carefully using a glass pipette into a clean round-bottomed glass tube and dried completely under nitrogen. Each sample was then reconstituted with 200µL ice-cold chloroform and kept on ice, ready for the next SPE cartridge step.

The SPE cartridges were pre-conditioned twice with 1mL ice-cold hexane. Samples were then transferred onto the cartridges and eluted passively without use of vacuum. The cartridges were then washed twice with 0.75mL ice-cold chloroform and eluted in two steps: twice with 1.5mL chloroform:methanol (2:1, v/v) followed by two washes with 1.5mL chloroform:methanol with formic acid (2:1:0.1, v/v/v). A vacuum was then used at higher pressure to elute the final drops into round-bottom glass collection tubes. The sample extracts were dried under gentle nitrogen and lipid residues reconstituted with 200µL methanol:formic acid (1:0.1, v/v). Samples were then centrifuged in a pulsatile matter to ensure lipid residues were dissolved completely. Samples were divided evenly (100µL) into two corneal inserts and vials closed and sealed with Parafilm®. Generated samples were stored at -20°C for up to a week.

2.3.6. Endocannabinoid assay UPLC/ESI-MS/MS settings

The extracted samples and blanks (HPLC grade methanol) were placed on sample trays at 8°C with injection volume set at 3µL. Chromatographic analysis was performed on a C_{18} column (Acquity UPLC BEH, 1.7 µm, 2.1 x 50mm) at 25°C with two mobile phases and 0.6 mL/min flow rate. The two mobile phases were; mobile phase A; ultra-pure water:acetic acid (1:0.02%, v/v) and mobile phase B; acetonitrile:acetic acid (1:0.02%, v/v). The protocol for each assay is described in **Table 2.8**. In accordance with Kendall et al. (205), the individual MRM settings for endocannabinoid species were as outlined in **Table 2.9**. A ESI positive mode was used with tandem MS settings: capillary voltage 1.8kV, source temperature 150°C, and desolvation

temperature 400°C. Argon gas was used in the collision cells. Samples were run in duplicate with blank injections between to reduce carry-over.

Table 2.8. Endocannabinoid mobile phase protocol. *Mobile phase A; ultra-pure water:acetic acid (1:0.02%, v/v) and mobile phase B; acetonitrile:acetic acid (1:0.02%, v/v).*

Time (min)	Mobile phase A (%)	Mobile phase B (%)
0.0	78	22
3.0	72	28
3.1	46	55
11.0	20	80
14.5	20	80
14.5	78	22

Table 2.9. Multiple reaction monitoring (MRM) transition settings for endocannabinoid assessments.

Compound	**MRM (*m/z*)**	**Cone voltage (V)**	**Collision energy (eV)**	**Indicative RT (min)**
2-AG-d8	387>293	35	20	6.19
11(12)-EET EA	364>346	35	20	4.04
14(15)-EET EA	364>346	35	20	3.79
15-HETE EA	364>62	35	20	3.61
2-AG	379>287	35	20	6.24
2-LG	355>263	35	20	6.29
2-OG	357>265	35	20	7.71
2-PG	331>239	35	20	7.30
2-STG	359>341	35	20	9.63
5(6)-EET EA	364>346	35	20	4.29
8(9)-EET EA	364>346	35	20	4.20
AEA	348>62	35	20	5.48
AEA-d8	356>63	35	20	5.41
ALEA	322>62	35	20	4.68
DEA	384>62	35	20	13.63
DGLEA	350>62	35	20	6.00
DHEA	372>62	35	20	5.38
DPEA	374>62	35	20	5.69
EPEA	346>62	35	20	4.74
HEA	314>62	35	20	7.15
LEA	324>62	35	20	5.41
LGEA	412>62	35	20	12.70

MEA	272>62	35	20	4.66
NAT	412>126	35	20	4.19
NEA	410>62	35	20	13.69
OEA	326>62	35	20	6.58
PDEA	286>62	35	20	5.32
PEA	300>62	35	20	6.15
PGD2-EA	378>360	35	20	2.50
PGE2-EA	378>360	35	20	2.16
PGF2a-EA	380>344	35	20	2.10
POEA	298>62	35	20	4.97
STEA	328>62	35	20	8.27
VEA	326>62	35	20	6.50

2.3.7. Endocannabinoid data processing

Similar to the eicosanoid data processing above (**section 2.4.7**), endocannabinoid peaks, samples, calibration lines, and deuterated internal standards, were identified with the MassLynx programme (v4.1). Endocannabinoid peaks were normalised according to the internal standards; AEA-*d* for N-acylethanolamines and 2-AG-*d* for monoacylglycerols. Endocannabinoid species were quantified against matched calibration lines, with generated concentrations multiplied by 200 (total extract volume) and finally normalised to protein content (pg/mL).

2.4. Analysis of ceramides

Combined ceramide and endocannabinoid extractions were done from the same biological sample. The lipid extract was spiked with the two deuterated internal standard assays and divided into two vials (**section 2.3.5**). However, each assay was analysed by a different ULC/ESI-MS/MS run.

2.4.1. Materials

Ethanol (HPLC grade, ≥99.8%) and hexane (HPLC grade, 97.0%) were obtained from Fisher Scientific. Methanol (LC-MS grade, ≥99.9%), acetonitrile (LC-MS grade, ≥99.9%) and chloroform (LC-MS grade, ≥99.9%) were purchased from Sigma-Aldrich. The following deuterated internal stands; CER[N(16)S(18)]-d_9, CER[N(16)DS(18)]-d_9, CER[N(16)H(18)]-d_9,

CER[N(16)P(18)]-d_9, CER[A(16)S(18)]-d_9, CER[A(16)DS(18)]-d_9, CER[A(16)H(18)]-d_9, and CER[A(16)P(18)]-d_9 were purchased from Avanti Polar Lipids .

2.4.2. Equipment

The list of equipment used is listed in **section 2.2.3.** In addition**,** STRATA^{TM} SPE cartridges (C18-E, 500 mg, 1mL) were purchased from Phenomenex.

2.4.3. Preparation of deuterated ceramide internal standards

The following deuterated ceramides; CER[A(16)S(18)]-d_9, CER[A(16)DS(18)]-d_9, CER[A(16)H(18)]-d_9, and CER[A(16)P(18)]-d_9 were dissolved in ethanol to a final concentration of 1 mg/mL; and CER[N(16)S(18)]-d_9, CER[N(16)DS(18)]-d_9, CER[N(16)H(18)]-d_9, CER[N(16)P(18)]-d_9 at 5mg/mL. To make the first master cocktail, 100µL of CER[A(16)S(18)]-d_9, CER[A(16)DS(18)]-d_9, CER[A(16)H(18)]-d_9, and CER[A(16)P(18)]-d_9 (1 mg/mL) and 20 µL of CER[N(16)S(18)]-d_9, CER[N(16)DS(18)]-d_9, CER[N(16)H(18)]-d_9, CER[N(16)P(18)]-d_9 (5 mg/mL) deuterated standards were mixed in a new vial and diluted with ethanol to achieve 100µg/mL. 10µL of this master standard cocktail was then diluted in 990µL to make a second cocktail of 1 µg/mL. Both standard cocktails were stored at -80°C for up to 6 months.

To prepare the working ceramide deuterated standard cocktail, 100µL of second master mix (1µg/mL) was diluted with 900µL ethanol to give a final concentration of 100ng/mL. This was then stored at -80°C for up to 3 months.

2.4.4. Fetal membrane ceramide extraction

A combined ceramide and endocannabinoid extraction was performed on the same biological samples (**section 2.3.5**).

2.4.5. Ceramide assay UPLC/ESI-MS/MS settings

Sample vials were placed in autosampler at 8°C. Samples were analysed as duplicates with blank (HPLC graded methanol) injection (3µL) in-between runs. A binary mobile phase was used to separate the sample through a C_{18} column (Acquity UPLC BEH, 1.7 µm, 2.1 x 50mm) at 30°C with 0.30 mL/min flow rate. **Table 2.10** gives the infusion settings for mobile phase A;

ultra-pure water:formic acid (1:0.1%, v/v), and mobile phase B; methanol:formic acid (1:0.1%, v/v). The MS was set as follows: source temperature 100°C, capillary voltage 3.5kV and desolvation temperature 450°C. **Table 2.11** shows individual MRM, cone voltage and collision energies for each ceramide species.

Table 2.10. Ceramide MS mobile phase protocol. *Mobile phase A; ultra-pure water: formic acid (1:0.1%, v/v), mobile phase B; methanol:formic acid (1:0.1%, v/v).*

Time (min)	Mobile phase A	Mobile phase B (%)
0	40	60
6	40	60
9	4	96
20	0	100
30	0	100
32	40	60
40	40	60

Table 2.11. Multiple reaction monitoring (MRM) transition settings for the ceramide assay.

Compound	MRM (*m/z*)	Cone voltage (V)	Collision energy (eV)	Indicative RT (min)
CER[ADS]				
CER[A(16)DS(18)]-d_9	565>284	50	30	10.78
CER[A(22)DS(18)]	640>284	50	30	9.73
CER[A(20)DS(18)]	612>284	50	30	9.92
CER[A(16)DS(22)]	612>340	50	30	11.19
CER[A(18)DS(20)]	612>312	50	30	11.20
CER[A(20)DS(20)]	640>312	50	30	11.50
CER[A(18)DS(22)]	640>340	50	30	11.51
CER[A(16)DS(24)]	640>368	50	30	11.60
CER[A(22)DS(19)]	654>284	50	30	11.65
CER[A(18)DS(23)]	654>354	50	30	11.68
CER[A(24)DS(16)]	640>256	50	30	11.75
CER[A(26)DS(16)]	668>256	50	30	11.91
CER[A(18)DS(24)]	668>368	50	30	11.91
CER[A(16)DS(25)]	654>382	50	30	11.96
CER[A(24)DS(17)]	654>270	50	30	11.97
CER[A(16)DS(26)]	668>396	50	30	11.98
CER[A(24)DS(18)]	668>284	50	30	12.12
CER[A(27)DS(16)]	682>256	50	30	12.16
CER[A(22)DS(22)]	696>340	50	30	12.28
CER[A(23)DS(18)]	654>284	50	30	12.32

CER[A(24)DS(19)]	682>298	50	30	12.34
CER[A(25)DS(18)]	682>284	50	30	12.41
CER[A(24)DS(20)]	696>312	50	30	12.60
CER[A(26)DS(18)]	696>284	50	30	12.65
CER[A(23)DS(20)]	682>312	50	30	12.81
CER[A(26)DS(19)]	710>298	50	30	12.87
CER[A(24)DS(22)]	724>340	50	30	13.11
CER[A(26)DS(20)]	724>312	50	30	13.13
CER[A(24)DS(21)]	710>326	50	30	13.34
CER[A(23)DS(22)]	710>340	50	30	13.34
CER[A(25)DS(20)]	710>312	50	30	13.40
CER[AH]				
CER[A(16)H(18)]-d_9	561>280	50	30	10.31
CER[A(20)H(18)]	608>280	30	30	10.78
CER[A(22)H(16)]	608>254	30	30	10.82
CER[A(22)H(17)]	622>266	30	30	10.90
CER[A(23)H(16)]	622>252	30	30	10.93
CER[A(22)H(18)]	636>280	30	30	11.01
CER[A(24)H(16)]	636>252	30	30	11.07
CER[A(26)H(14)]	636>224	30	30	11.12
CER[A(26)H(15)]	650>238	30	30	11.19
CER[A(24)H(17)]	650>266	30	30	11.19
CER[A(25)H(16)]	650>252	30	30	11.21
CER[A(26)H(16)]	664>252	30	30	11.32
CER[A(24)H(18)]	664>280	30	30	11.32
CER[A(28)H(14)]	664>224	30	30	11.35
CER[A(24)H(19)]	678>294	30	30	11.47
CER[A(28)H(15)]	678<238	30	30	11.50
CER[A(27)H(16)]	678<252	30	30	11.50
CER[A(25)H(18)]	678>280	30	30	11.50
CER[A(26)H(17)]	678>266	30	30	11.53
CER[A(24)H(20)]	692>308	30	30	11.67
CER[A(28)H(16)]	692>252	30	30	11.68
CER[A(26)H(18)]	692>280	30	30	11.69
CER[A(25)H(20)]	706>308	30	30	11.83
CER[A(26)H(19)]	706>294	30	30	11.85
CER[A(27)H(18)]	706>280	30	30	11.90
CER[A(28)H(17)]	706>266	30	30	11.94
CER[A(24)H(22)]	720>336	30	30	11.99
CER[A(26)H(20)]	720>308	30	30	12.04
CER[A(28)H(18)]	720>280	30	30	12.13

CER[A(26)H(21)]	734>322	50	30	12.24
CER[A(27)H(20)]	734>308	50	30	12.27
CER[A(28)H(19)]	734>294	50	30	12.30
CER[A(29)H(18)]	734>280	50	30	12.34
CER[A(26)H(22)]	748>336	50	30	12.46
CER[A(28)H(20)]	748>308	50	30	12.52
CER[A(30)H(18)]	748>280	50	30	12.60
CER[AP]				
CER[A(16)P(18)]-d_9	581>282	50	30	10.52
CER[A(22)P(16)]	628>254	50	30	11.05
CER[A(20)P(20)]	656>310	50	30	11.31
CER[A(22)P(18)]	656>282	50	30	11.35
CER[A(24)P(16)]	656>254	50	30	11.39
CER[A(22)P(19)]	670>296	50	30	11.49
CER[A(24)P(17)]	670>269	50	30	11.55
CER[A(22)P(20)]	684>310	50	30	11.67
CER[A(24)P(18)]	684>282	50	30	11.72
CER[A(26)P(16)]	684>254	50	30	11.77
CER[A(24)P(19)]	698>296	50	30	11.90
CER[A(25)P(18)]	698>282	50	30	11.92
CER[A(26)P(17)]	698>268	50	30	11.96
CER[A(24)P(20)]	712>310	50	30	12.08
CER[A(26)P(18)]	712>282	50	30	12.14
CER[A(24)P(21)]	726>310	50	30	12.30
CER[A(25)P(20)]	726>296	50	30	12.31
CER[A(26)P(19)]	726>324	50	30	12.35
CER[A(24)P(22)]	740>338	50	30	12.53
CER[A(26)P(20)]	740>310	50	30	12.57
CER[A(26)P(22)]	754>264	50	30	12.80
CER[AS]				
CER[A(16)S(18)]-d_9	545>264	30	30	10.68
CER[A(18)S(18)]	582>264	30	30	10.42
CER[A(20)S(18)]	610>264	30	30	10.51
CER[A(24)S(16)]	638>236	30	30	11.60
CER[A(23)S(18)]	652>264	30	30	11.74
CER[A(24)S(17)]	652<250	30	30	11.77
CER[A(25)S(16)]	652>236	30	30	11.82
CER[A(24)S(18)]	666>264	30	30	11.95
CER[A(22)S(18)]	638>264	30	30	11.98
CER[A(26)S(16)]	666>236	30	30	12.02

CER[A(23)S(20)]	680>292	30	30	12.13
CER[A(22)S(21)]	680>306	30	30	12.13
CER[A(24)S(19)]	680>278	30	30	12.15
CER[A(25)S(18)]	680>264	30	30	12.18
CER[A(26)S(17)]	680>250	30	30	12.20
CER[A(27)S(16)]	680>236	30	30	12.26
CER[A(24)S(20)]	694>292	30	30	12.37
CER[A(26)S(18)]	694>264	30	30	12.43
CER[A(23)S(22)]	708>320	30	30	12.57
CER[A(24)S(21)]	708>306	30	30	12.61
CER[A(26)S(19)]	708>278	30	30	12.66
CER[A(27)S(18)]	708>264	30	30	12.69
CER[A(24)S(22)]	722>320	30	30	12.87
CER[A(26)S(20)]	722>292	30	30	12.91
CER[A(25)S(22)]	736>320	30	30	13.16
CER[A(28)S(19)]	736>278	30	30	13.26
CER[A(26)S(22)]	750>320	30	30	13.47
CER[NDS]				
CER[N(16)DS(18)]-d_9	549>284	50	30	10.85
CER[N(20)DS(20)]	624>312	50	30	11.85
CER[N(18)DS(22)]	624>340	50	30	11.85
CER[N(22)DS(18)]	624>284	50	30	11.86
CER[N(24)DS(16)]	624>256	50	30	11.88
CER[N(16)DS(24)]	624>368	50	30	11.89
CER[N(22)DS(19)]	638>298	50	30	12.07
CER[N(23)DS(18)]	638>284	50	30	12.08
CER[N(25)DS(16)]	638>256	50	30	12.10
CER[N(24)DS(17)]	638>270	50	30	12.12
CER[N(20)DS(22)]	652>340	50	30	12.29
CER[N(24)DS(18)]	652>284	50	30	12.33
CER[N(18)DS(24)]	652>368	50	30	12.33
CER[N(26)DS(16)]	652>256	50	30	12.34
CER[N(16)DS(26)]	652>396	50	30	12.34
CER[N(23)DS(20)]	666>312	50	30	12.54
CER[N(24)DS(19)]	666>298	50	30	12.56
CER[N(25)DS(18)]	666>284	50	30	12.59
CER[N(26)DS(17)]	666>270	50	30	12.63
CER[N(22)DS(22)]	680>340	50	30	12.79
CER[N(24)DS(20)]	680>312	50	30	12.81
CER[N(20)DS(24)]	680>368	50	30	12.81
CER[N(26)DS(18)]	680>284	50	30	12.86

CER[N(18)DS(26)]	680>396	50	30	12.87
CER[N(23)DS(22)]	694>340	50	30	13.07
CER[N(22)DS(23)]	694>354	50	30	13.08
CER[N(24)DS(21)]	694>326	50	30	13.09
CER[N(25)DS(20)]	694>312	50	30	13.11
CER[N(26)DS(19)]	694>298	50	30	13.13
CER[N(27)DS(18)]	694>284	50	30	13.18
CER[N(28)DS(17)]	694>270	50	30	13.22
CER[N(24)DS(22)]	708>340	50	30	13.38
CER[N(22)DS(24)]	708>368	50	30	13.38
CER[N(20)DS(26)]	708>396	50	30	13.42
CER[N(26)DS(20)]	708>312	50	30	13.44
CER[N(28)DS(18)]	708>284	50	30	13.49
CER[N(25)DS(22)]	722>340	50	30	13.71
CER[N(24)DS(23)]	722>354	50	30	13.71
CER[N(23)DS(24)]	722>368	50	30	13.72
CER[N(22)DS(25)]	722>382	50	30	13.72
CER[N(26)DS(21)]	722>326	50	30	13.74
CER[N(27)DS(20)]	722>312	50	30	13.77
CER[N(28)DS(19)]	722>298	50	30	13.78
CER[N(24)DS(24)]	736>368	50	30	14.04
CER[N(26)DS(22)]	736>340	50	30	14.08
CER[N(22)DS(26)]	736>396	50	30	14.09
CER[N(28)DS(20)]	736>312	50	30	14.13
CER[N(27)DS(22)]	750>340	50	35	14.46
CER[N(28)DS(21)]	750>326	50	35	14.48
CER[N(29)DS(20)]	750>312	50	35	14.50
CER[NH]				
CER[N(16)H(18)]-d_9	545>280	30	30	10.45
CER[N(26)H(14)]	620>252	30	30	11.27
CER[N(23)H(18)]	634>280	30	30	11.38
CER[N(24)H(17)]	634>266	30	30	11.41
CER[N(25)H(16)]	634>252	30	30	11.45
CER[N(24)H(18)]	648>280	30	30	11.55
CER[N(26)H(16)]	648>252	30	30	11.64
CER[N(25)H(18)]	662>280	30	30	11.75
CER[N(26)H(17)]	662>266	30	30	11.79
CER[N(24)H(20)]	676>308	30	30	11.88
CER[N(26)H(18)]	676>280	30	30	11.96
CER[N(28)H(16)]	676>252	30	30	12.06
CER[N(25)H(20)]	690>308	30	30	12.12

CER[N(26)H(19)]	690>294	30	30	12.14
CER[N(27)H(18)]	690>280	30	30	12.18
CER[N(28)H(17)]	690>266	30	30	12.24
CER[N(26)H(20)]	704>308	30	30	12.35
CER[N(28)H(18)]	704>280	30	30	12.43
CER[N(26)H(21)]	718>322	30	30	12.58
CER[N(27)H(20)]	718>308	30	30	12.60
CER[N(28)H(19)]	718>294	30	30	12.66
CER[N(26)H(22)]	732>336	30	30	12.83
CER[N(28)H(20)]	732>308	30	30	12.89
CER[N(30)H(19)]	732>280	30	30	12.99
CER[N(28)H(21)]	746>322	30	35	13.15
CER[N(29)H(20)]	746>308	30	35	13.21
CER[N(30)H(19)]	746>294	30	35	13.23
CER[N(28)H(22)]	760>336	30	35	13.43
CER[N(30)H(20)]	760>308	30	35	13.51
CER[NP]				
CER[N(16)P(18)]-d_9	565>282	50	30	11.23
CER[N(24)P(16)]	640>254	50	30	11.55
CER[N(24)P(17)]	654>268	50	30	11.73
CER[N(24)P(18)]	668>282	50	30	11.90
CER[N(26)P(16)]	668>254	50	30	11.97
CER[N(24)P(19)]	682>296	50	30	12.04
CER[N(23)P(20)]	682>310	50	30	12.05
CER[N(25)P(18)]	682>282	50	30	12.13
CER[N(26)P(17)]	682>268	50	30	12.15
CER[N(24)P(20)]	696>310	50	30	12.30
CER[N(26)P(18)]	696>282	50	30	12.37
CER[N(28)P(16)]	696>254	50	30	12.45
CER[N(24)P(21)]	710>324	50	30	12.53
CER[N(25)P(20)]	710>310	50	30	12.55
CER[N(26)P(19)]	710>296	50	30	12.58
CER[N(27)P(18)]	710>282	50	30	12.63
CER[N(28)P(17)]	710>268	50	30	12.67
CER[N(24)P(22)]	724>338	50	30	12.78
CER[N(26)P(20)]	724>310	50	30	12.83
CER[N(28)P(18)]	724>282	50	30	12.90
CER[N(25)P(22)]	738>338	50	30	13.07
CER[N(26)P(21)]	738>324	50	30	13.09
CER[N(24)P(24)]	752>366	50	35	13.34
CER[N(26)P(22)]	752>310	50	35	13.40

CER[N(28)P(20)]	752>310	50	35	13.44
CER[N(26)P(23)]	766>352	50	35	13.69
CER[N(27)P(22)]	766>338	50	35	13.72
CER[N(28)P(21)]	766>324	50	35	13.76
CER[N(24)P(26)]	780>394	50	35	14.02
CER[N(26)P(24)]	780>366	50	35	14.03
CER[N(28)P(22)]	780>338	50	35	14.08
CER[N(30)P(20)]	780>310	50	35	14.16
CER[N(27)P(24)]	794>366	50	35	14.40
CER[N(28)P(23)]	794>352	50	35	14.42
CER[N(26)P(25)]	794>380	50	35	14.42
CER[N(29)P(22)]	794>338	50	35	14.47
CER[N(30)P(21)]	794>324	50	35	1 4.50
CER[N(26)P(26)]	808>394	50	35	14.77
CER[N(28)P(24)]	808>366	50	35	14.8
CER[N(30)P(22)]	808>338	50	35	14.88
CER[NS]				
CER[N(16)S(18)]-d_9	529>264	30	30	10.78
CER[N(22)S(18)]	622>264	30	30	11.75
CER[N(24)S(16)]	622>236	30	30	11.81
CER[N(21)S(20)]	636>292	30	30	11.91
CER[N(22)S(19)]	636>278	30	30	11.93
CER[N(23)S(18)]	636>264	30	30	11.95
CER[N(24)S(17)]	636>250	30	30	11.98
CER[N(25)S(16)]	636>236	30	30	12.02
CER[N(20)S(22)]	650>320	30	30	12.11
CER[N(22)S(20)]	650>292	30	30	12.14
CER[N(24)S(18)]	650>264	30	30	12.19
CER[N(26)S(16)]	650>236	30	30	12.25
CER[N(22)S(21)]	664>306	30	30	12.34
CER[N(23)S(20)]	664>292	30	30	12.37
CER[N(24)S(19)]	664>278	30	30	12.39
CER[N(25)S(18)]	664>264	30	30	12.43
CER[N(26)S(17)]	664>250	30	30	12.46
CER[N(27)S(16)]	664>236	30	30	12.49
CER[N(24)S(20)]	678>292	30	30	12.64
CER[N(26)S(18)]	678>264	30	30	12.70
CER[N(28)S(16)]	678>236	30	30	12.78
CER[N(24)S(21)]	692>306	30	30	12.87
CER[N(23)S(22)]	692>320	30	30	12.87
CER[N(25)S(20)]	692>292	30	30	12.91

CER[N(26)S(19)]	692>278	30	30	12.93
CER[N(27)S(18)]	692>264	30	30	12.97
CER[N(28)S(17)]	692>250	30	30	13.02
CER[N(22)S(24)]	706>348	30	30	13.14
CER[N(24)S(22)]	706>320	30	30	13.16
CER[N(26)S(20)]	706>292	30	30	13.20
CER[N(28)S(18)]	706>264	30	30	13.30
CER[N(24)S(23)]	720>334	30	30	13.46
CER[N(23)S(24)]	720>348	30	30	13.46
CER[N(25)S(22)]	720>320	30	30	13.49
CER[N(26)S(21)]	720>306	30	30	13.50
CER[N(27)S(20)]	720>292	30	30	13.53
CER[N(28)S(19)]	720>278	30	30	13.57
CER[N(29)S(18)]	720>264	30	30	13.61
CER[N(24)S(24)]	734>348	30	30	13.80
CER[N(26)S(22)]	734>320	30	30	13.82
CER[N(28)S(20)]	734>292	30	30	13.88
CER[N(24)S(25)]	748>362	30	35	14.14
CER[N(26)S(23)]	748>334	30	35	14.16
CER[N(25)S(24)]	748>348	30	35	14.16
CER[N(27)S(22)]	748>320	30	35	14.18
CER[N(28)S(21)]	748>306	30	35	14.21
CER[N(29)S(20)]	748>292	30	35	14.25
CER[N(24)S(26)]	762>376	30	35	14.52
CER[N(26)S(24)]	762>348	30	35	14.53
CER[N(28)S(22)]	762>320	30	35	14.57
CER[N(30)S(20)]	762>292	30	35	14.65
CER[N(25)S(26)]	776>376	30	35	14.91
CER[N(26)S(25)]	776>362	30	35	14.92
CER[N(28)S(23)]	776>334	30	35	14.96
CER[N(29)S(22)]	776>320	30	35	14.98
CER[N(30)S(21)]	776>306	30	35	15.04
CER[N(28)S(24)]	790>348	30	35	15.36
CER[N(26)S(26)]	790>376	30	35	15.36
CER[N(30)S(22)]	790>320	30	35	15.41

2.4.6. Ceramides data analysis

MassLynx (v4.1) was used to identify MS generated ceramide peaks. Again, peaks were normalised to deuterated internal standards. i.e. CER[N(16)S(18)]-d_9 and CER[N(16)DS(18)]-

d_9 for CER[NS] and CER [NDS] species, respectively. Resulting concentrations were normalised to protein content and reported as pg/mg of protein.

2.5. Complex lipid analysis using UHPSFC-ESI-MS/MS

Ultra-high performance supercritical fluid chromatography (UHPSFC), a mixture of liquid gas and liquid chromatography, provides superior separation of polar and non-polar compounds in comparison to more traditional LC or GC (207). Therefore, UHPSFC-ESI-MS offers sensitive profiling of a wide range of lipid classes including; PC, PE, cholesterol, cholesterol esters, Triglycerides (TG), diacylglycerol(DAG), free fatty acids (FFA) and sphingomyelin (SM) (208). UHPSFC coupled ESI-time to flight (ToF) MS (Waters) was used to profile lipid components in the fetal membranes (see **Figure 2.2**).

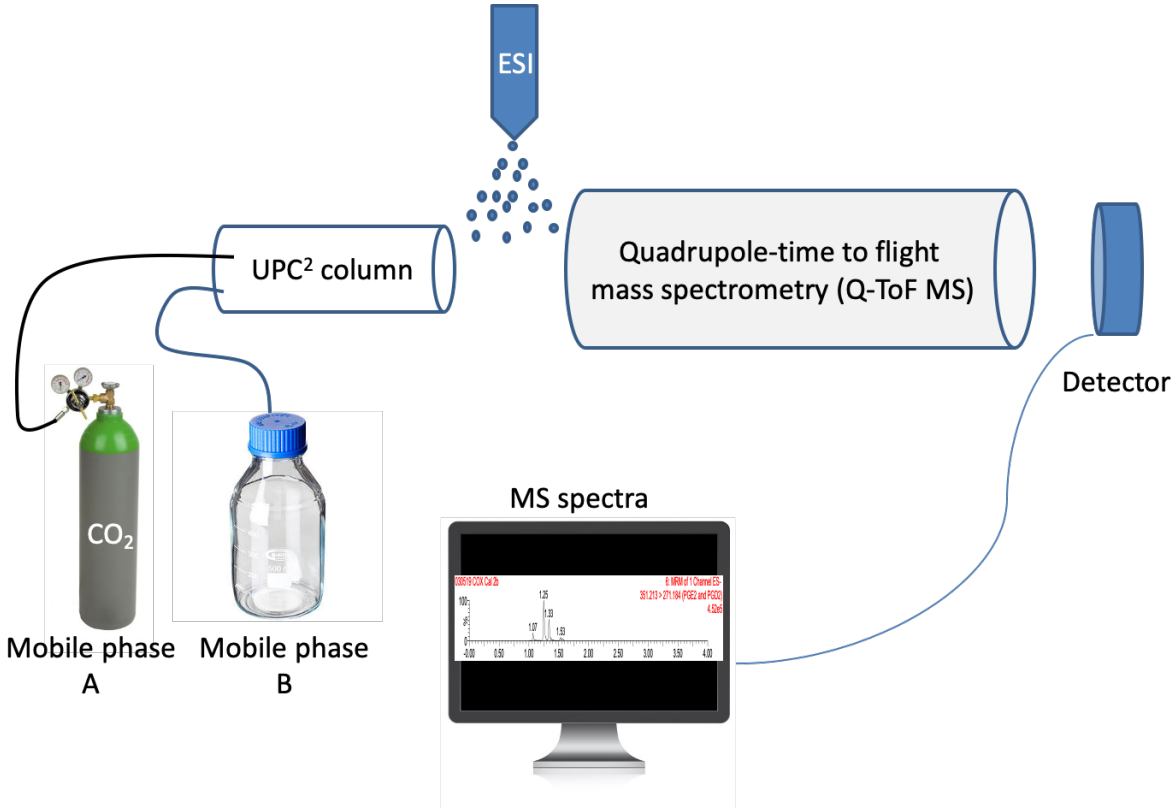


Figure 2.2. Ultra-high performance supercritical fluid chromatography (UHPSFC) coupled ESI-quadrupole Time to Flight (Q-ToF) MS. *Mobile phase A (compressed liquid CO_2) and mobile phase B (methanol:pure-water (99:1, v/v) with 30 mM Ammonium Acetate) were used to elute the sample through a UPC² column to separate the lipid analytes. ESI ionised separated compounds for subsequent Q-ToF MS analysis.*

2.5.1. Materials

Acetic acid (HPLC grade, ≥99,7%), ammonium acetate (>98%), methanol (HPLC grade, ≥99.0%), chloroform (HPLC grade, 99.0%), and isopropanol (HPLC grade, 99.0%) were supplied by Sigma-Aldrich. Liquid CO_2 cylinder CP grade (99.995%) was obtained from BOC (Guildford, UK).

Thirteen deuterated internal standards were used in this complex lipid assay: 15:0 cholesteryl (d7) ester (CE-d), cholesterol-d7 (Chl-d), 16:0-d31 sphingomyelin (SM-d), 16:0-d31-18:1 phosphatidylcholine (PC-d), 26:0-d4 lysophosphatidylcholine (LPC-d), 16:0-d31 phosphatidtylethanolamine (PE-d), 18:1 diacylglycerol-d5 (DAG-d), 16:0-d31-18:1 phosphatidylglycerol (PG-d), 16:0-d31-18:1 phosphatidic acid (PA-d), 18:1-d4 lysophosphatidylethanolamine (LPE-d), 16:0-d31-18:1 phosphatidylserine (PS-d) and C16:0-d31 (FFA-d) were purchased from Avanti Polar Lipids (Alabaster, Alabama, US).

2.5.2. Equipment

The equipment used for the lipid extractions of fetal membranes is described in **section 2.2.3.** Lipids were separated using a UHPSFC system (Acquity UPC^2 instrument) equipped with VanGuard pre-column filter (Acquity UPLC BEH, 1.7µm, 2.1 x 5mm) and C_{18} column (The Torus 2-PIC column,100mm x 2.1mm x 1.7µm) (Waters). The UHPSFC system was connected to an ESI quadrupole time-of-flight MS Synapt^{TM} G2 (Waters).

2.5.3. Preparation of internal standards for the complex lipid assay

The internal standard was a mixture of deuterated lipids, representative of 13 different classes (**section 2.5.1**). Stock solutions were prepared individually at 10mg/mL for CE-*d*, Chol-*d*, PC-*d*, PE-*d*, PS-*d*, phosphatidylglycerol-*d*, PA-*d*, 1mg/mL for SM-*d*, TAG-*d*, DAG-*d*, LPE-*d*, and 100mg/mL for C16:0-*d* (FFA-*d*). To prepare the working standard cocktail; 200µL each of deuterated TAG, LPC, SM, DAG and LPE, 60µL of PG, PA, PS, 40µL Chol, and 20µL CE, PC and PE, were combined with 2µL C16:0. The solvent was then dried under nitrogen, and lipid residue reconstituted with 2mL chloroform:isopropanol (1:1, v/v) to final concentration of 300µg/mL PG-*d*, PS-*d* and PA-*d*, 200 µg/mL Chol-*d*, and 100µg/mL C16:0-*d*, CE-*d*, PC-*d*, PE-*d*, TAG-*d*, LPC-*d*, SM-*d*, and DAG-*d*.

2.5.4. Fetal membrane lipid extraction for UHPSFC-ESI-MS/MS

Lipids were extracted from amnion and choriodecidual with chloroform:methanol (2:1,v/v) using the method of Bligh and Dyer (209). Approximately 30mg tissue was homogenised using a Dounce homogeniser (DWK Life Sciences) with ice-cold methanol. The homogenate was then transferred to a flat-bottomed glass tube; followed by addition of ice-cold chloroform to a final volume of 6mL and chloroform:methanol ratio of 2:1 (v/v). 25µl deuterated internal standard lipid cocktail was then added to each sample, followed by incubation on ice in the dark for 20 min. 1mL ice-cold ultra-pure water was the added to form a biphasic layer, which was centrifuged for 5 min at 1500x g 4°C. The lower organic layers were collected with a glass pipette and dried under gentle nitrogen. Dried samples were reconstituted in 500µL chloroform:isopropanol (1:1, v/v) and divided into two clean glass inserts and placed in vials (positive and negative modes). These were stored at -20°C for MS analysis.

2.5.5. UHPSFC-ESI-MS/MS settings

Each sample was run in duplicate in both positive and negative mode (for ESI), with an injection volume of 5µL and 7µL, respectively. The classes detected in positive mode were; Chol, CE, PC, PE, LPC, LPE, TG, DAG and SM; and those for negative mode were; FFA, PG, PhA and PS.

UHPSFC lipid separation was performed with UPC^2 Torus 2-PIC column (100mm x 3mm x 1/7µm) on the Acquity UPC^2 instrument. The mobile phases; mobile phase A; liquid CO_2, and mobile phase B; methanol:ultra-pure water (99:1 v/v) with 30 mM ammonium acetate, provided the gradient elution at a flow rate of 1.5 mL/min (**Table 2.11)**. The column temperature and back pressure remained at 60°C and 1800psi throughout.

For the ESI coupled Q-ToF-MS (Synapt^{TM} G2 High Definition mass spectrometer, Waters), detection ranges for positive and negative modes were set between 50 and 1100 *m/z*. For both modes, the desolvation temperature was 500°C, source temperature 150°C, capillary voltage 3kV, source offset 90V, and sampling cone 40V. The drying gas flow was 17 L/min, and cone gas flow 0.8 L/min. Argon gas was used in the collision cell. A makeup fluid

(methanol:pure-water (99:1, v/v) was introduced to the system at a flow rate of 0.25 mL/min. To lock mass settings, Leucine enkephalin (lock spray) was used at 556.6 *m/z* and 554.6 *m/z*, for positive and negative modes, respectively. Sodium formate was used as reference celebrant. Collision energy was set to 4V for low collision energy and 25 ramping to 40V for high collision energy. Data were acquired in MS^E mode.

Table 2.12. UHPSFC mobile phase settings. *Mobile phase A, liquid CO_2; mobile phase B, methanol:ultra-pure water (99:1 v/v) with 30mM ammonium acetate.*

Time (min)	Mobile phase A (%)	Mobile phase B (%)
0.0	99	1
0.1	99	1
0.5	90	10
1.5	90	10
7	55	45
8	55	45
9	99	1
10	99	1

2.5.6. Data processing for UHPSFC-ESI-MS/MS lipid analysis

Raw MS^E data were imported to Progenesis QI software (v2.3, NonLinear Dynamics, Newcastle, UK). Lipids were selected according to their retention times and *m/z* ratios, in accordance with an "in-house" lipid database and matched class deuterated internal standards. Lipids were reported as total carbon and total double bonds (although their positions could not be defined). Lipid concentrations were generated from the following calculation: Lipid (Peak area) x deuterated internal standard concentration / internal standard (PA) x protein concentration (mg/mL).

2.6. Protein content assay

2.6.1. Reagents and equipment

Sodium hydroxide (NaOH) (pellets, ≥98%) was purchased from Sigma-Aldrich and dissolved in deionised water to a final concentration of 1M. A DC Protein Assay Kit II including reagent A and B (Bio-Rad, Hercules, California, USA), was used to perform the colorimetric protein reaction. Bovine serum albumin (BSA) (1.5mg/mL) was obtained from (Bio-Rad).

The equipment used in the protein assay included: a water bath (SBS40 Stuart Equipment, Stone, UK), Multiskan^{TM} FC plate reader Labsystems (Hull, UK) and Corning® Costar®flat bottom 96-well plates Sigma-Aldrich. Data were analysed using Prism (v.8) (GraphPad, California, USA).

2.6.2. Protein content protocol

The DC protein content assay used a colorimetric method adapted from Lowry et al. (210). Frozen (-20^{0}C) protein pellets from lipid mediator and lipid content assays were used, with solvents aspirated carefully from their flat-bottomed glass storage tubes. 1mL of 1M NaOH was added, and samples incubated in the water bath for 90 min at 60°C. A calibration line was prepared with serial dilutions of BSA standard, giving 1.5, 1.0, 0,75, 0.50, 0.25, and 0 mg/mL. Samples were diluted in 1:2 (v/v) with NaOH and 5µL of diluted samples and standards added to 25µL Reagent A and 200µL Reagent B. The 96-well plates were incubated in the dark for 15 min, then read by Multiskan^{TM} FC plate reader at 650nm. Absorbance readings were plotted against standards using Prism (v.8) (GraphPad) and sample concentrations reported as mg/mL.

2.7. Total fatty acid analysis using gas chromatography (GC)

Gas chromatography (GC) is a common tool to separate volatile compounds, according to their interaction with a column, i.e. stationary phase. Although the GC is an old analytical technique in comparison to MS, it is still more sensitive in identifying structures of similar molecular weights but differing double bonds, i.e. PUFAs (211). There are two main steps in the analysis of fatty acid content; fatty acid extraction followed by trans-esterification; in which fatty acids are freed and esterified through addition of a methyl group to form fatty acid methyl esters (FAMEs). As a stable volatile structure, FAME samples are then injected into the GC, evaporated in the heater, and carried by mobile phase through a long capillary column. At the column, the FAME compounds interact with the column lining and are separated according to their chemical and physical characteristics. At the end of the column, a hydrogen flame ionises the FAMEs, which are then detected by flame ionisation detector (FID) (**Figure 2.3**).

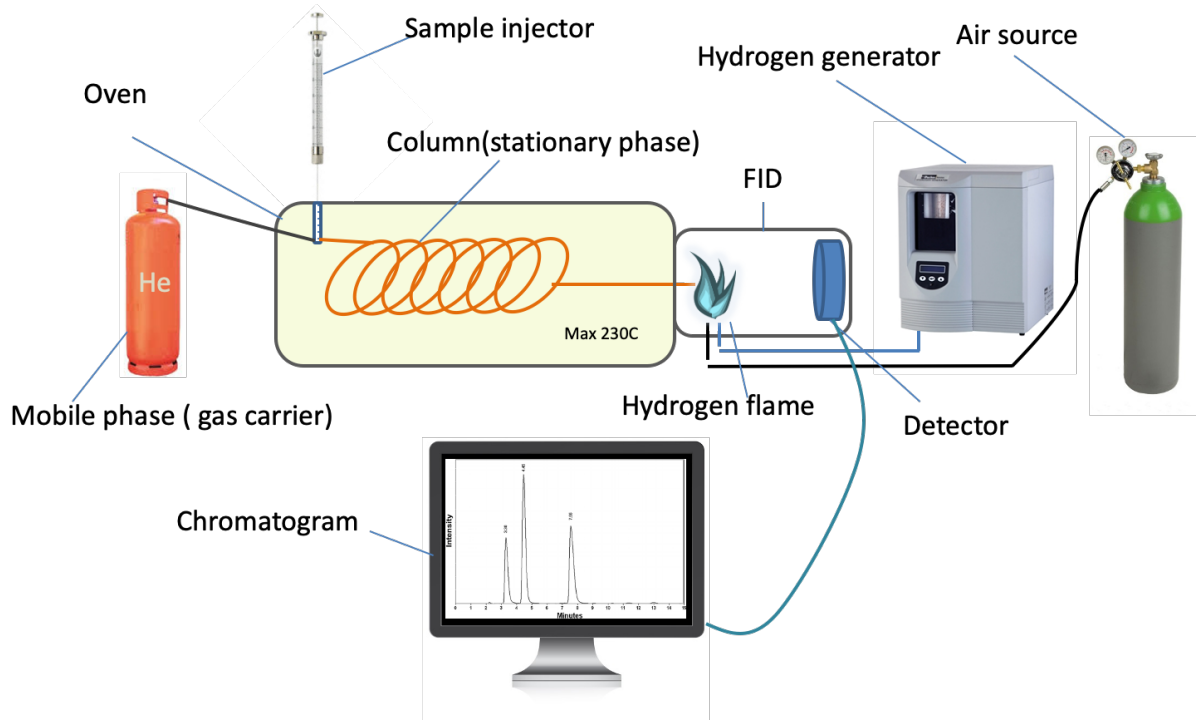


Figure 2.3. Gas chromatography flame ionisation detection (GC-FID). *An autosampler injects the sample through a heater, where it is evaporated and carried by gas carrier (Helium (He)) through the stationary phase (column) at various temperature settings. At the end of the column, the separated compounds are ionised by hydrogen flame, and detected by a flame ionisation detector (FID), and reported finally as a chromatogram.*

2.7.1. Materials

The following reagents were used for total fatty acid extraction and analysis; methanol (HPLC grade, >99.9%) and chloroform (HPLC grade, >99.8%) were purchased from Fisher Scientific. Toluene (HPLC grade, ≥99.5%), trimethylpentane (HPLC grade, ≥99.0%) and dichloromethane (DCM; HPLC grade, >99.8%) were obtained from ACROS organics. Sodium sulphate (ACS grade, ≥99.0%), potassium carbonate (ACS grade, >99.0%), 2,6-ditert-butyl-4-methylphenol (BHT)(≥99.0%), 14% boron trifluoride (BF_3)in methanol and heneicosaenoic acid (C21:0; ACS grade, ≥99.0%) were supplied by Sigma-Aldrich. FAME standards cocktails contained 37 components; C4:0, C:6:0, C8:0, C:10, C:11, C12:0, C13:0, C14:0, C14:1, C150, C15:1, C16:0, C16:1, C17:0, C17:1, C18:0, C18:1 n-9t, C18:1 n-9c, C18:1 n-7, C18:2 n-6t, C18:2 n-6c, C18:3 n-3, C18:3 n-6, C20:0, C20:1 n-9, C20:2 n-6, 20:4 n-6, C20:3 n-6, C20:3 n-3, C21:0, C22:0, C22:1 n-9, C20:5 n-3, C23:0, C22:2 n-6, C24:0, C24:1 n-9, C22:5 n-3, C22:6 n-3) (10 mg/mL in ethanol; used at varied concentrations), docosapentaenoic acid (DPA, C22:5n-3) methyl ester (1 mg/mL in ethanol) and vaccenic acid methyl ester (1 mg/mL in ethanol); all supplied by Supelco (Belleforte, USA).

2.7.2. Equipment

The tissues were homogenised with Dounce tissue grinder kit (DWK Life Sciences). The following equipment was used for fatty acid extraction and derivatisation methods; deionised ultra-pure water was obtained from Elga Water Purelab flex purification system, refrigerated benchtop centrifuge (Sigma 3-16KL) from Sigma-Aldrich, Vortex (Fisons Whirlimixer) from Fisher Scientific, Nitrogen drying cabinet Custom made (University of Bradford), Heating block from (Pierce Reacti-Therm, Rockford, UK), Hamilton glass syringes (volumes: 10µL, 50µL, 100µL, 250µL) from SGE, round-bottomed and flat-bottomed 10mL glass tubes , amber glass vials (2mL), conical glass inserts (200µL), screw caps (8mm), and septa were supplied by Phenomenex. 150mm unplugged glass Pasteur pipettes and Parafilm® from Fisher Scientific and glass wool pesticide grade from Sigma-Aldrich.

The prepared samples were analysed using auto-sampler 6850 Network GC systems coupled with GC-FID 6850 Series, obtained from Agilent technologies (Stockport, UK) and PX70 GC capillary column GC column (0.25mm ID, 0.25µm film, 60m) from SGE Europe (Milton Keynes, UK). The Parker Balston hydrogen generator was obtained from CLAIND (Lenno, Italy). The data were processed using ChemStation software (revision v.2.1.0433, Agilent technologies).

2.7.3. Preparation of heneicosaenoic acid (C21:0) as internal standard

Heneicosaenoic acid (C21:0) was used as the internal standard (IS) for the total FA analysis. C21:0 was chosen as its present in only trace amounts in human tissues (212). The aim of the internal standard is to correct for peak shifts and normalise changes imposed by trans-esterification. 25mg of C21:0 is dissolved in 25mL of 0.01% butylated hydroxytoluene (BHT) in 2:1 chloroform:methanol (v/v) to give a final concentration of 1mg/mL. This was then sealed with Parafilm® and stored at 4°C for up to three months.

2.7.4. Preparation of the FAME cocktail

The FAME cocktail aims to determine the retention time of each competent FAME. There are two analytical methods available in GC-FID; split and split-less injection. The split method is commonly used for high FA concentrations, such as oils; but for biological samples, as considered in this study, the split-less method is preferred. The commercial FAME mix (listed

in **2.7.1**) of Vaccenic and docosapentaenoic acid methyl esters were dried under gentle nitrogen and reconstituted with 1mL DCM to make the stock solutions. Working solutions were then derived by diluting stocks with DCM to a final concentration 200 ng/µL and stored in amber vials sealed with Parafilm® at -20°C for up to a year. The split FAME cocktail was made using a prepared stock solution; adding 20µL of FAME mix (1mg/mL), 20µL vaccenic acid methyl ester (200ng/µL) and 20µL docosapentaenoic acid methyl ester (200 ng/µL), with 140µL DCM. The split-less FAME cocktail was prepared by diluting 20µL of split cocktail with 40µL DCM. Both split and split-less FAME preparations were freshly made for each FA analysis and sealed with Parafilm® and stored (-20°C).

2.7.5. Sample preparation for fatty acid analysis using GC-FID

Fetal membrane samples (amnion and choriodecidua) were washed and stored as described (**section 2.1**). To extract fatty acids from stored frozen samples, tissues were defrosted on ice and homogenised with 3mL Dounce homogeniser using 1mL ice-cold 0.01% (w/v) BHT in 2:1 chloroform:methanol. This process was repeated three times and homogenate pooled into a flat-bottomed glass tube using glass Pasteur pipettes. 1mL ice-cold deionised water was then added to each 4mL homogenate. For cells lines, homogenised cell-pellets were reconstituted in 1mL ice-cold deionised water and also transferred to flat bottomed glass tubes containing 3mL of ice-cold 0.01% (w/v) BHT in 2:1 chloroform:methanol. All samples were vortexed and incubated on ice in the dark for at least 30 min. Following centrifugation (10min, 4°C, 1500x g) the organic layer (bottom layer) was transferred into a round-bottomed glass tube, again with glass Pasteur pipette. This was performed twice to obtain a higher extraction efficiency.

In order to dehydrate the fatty acid extract, 3 to 4 spatula tips of sodium sulphate were added to create a snowstorm appearance within the organic sample. This was then filtered through pre-conditioned glass wool into a fresh round-bottomed glass tube. 3mL of ice-cold 0.01%(w/v) BHT in 2:1 chloroform:methanol was passed through the glass-wool to ensure the full transfer of lipids. The extracted samples were then dried gently under nitrogen and reconstituted in 1mL of ice-cold 0.01% (w/v) BHT in 2:1 chloroform:methanol. These were stored at -20°C for up to a week until derivatisation could be conducted.

The aim of derivatisation is to trans-esterify extracted fatty acids into FAMEs. This was performed as described by Morrison and Smith (213). Extracted samples were dried under nitrogen, then 15µL of internal standard (C21:0) added before further drying to completion. The lipid residue was diluted in 250µl of toluene:methanol (1:1 v/v) and 250µl of 14%(v/v) BF_3-methanol. Tubes were closed tightly with lids and sealed with two layers of Parafilm® before warming to 100°C in heating blocks for 90 mins. After this tubes were checked for reduction of volume or change in colour (water contamination turns samples red) and placed on ice for 10 mins. The trans-esterification reaction was stopped by adding 1.5mL of 10%(w/v) K_2CO_3 solution and 2mL Trimethylpentane. Resulting samples were vortexed and centrifuged for 10min at 4°C, 1500x g. The upper layer (FAME containing) was then transferred carefully to a clean round-bottomed tube (and last step repeated). Within the round-bottom tubes, the FAME extracts were dried under nitrogen and reconstituted with 60µL dichloromethane. These were then transferred into vials with corneal glass inserts, sealed with Parafilm®, and stored at -20°C for no more than a week, before a GC run.

2.7.6. GC-FID protocol

The autosampler was set to inject 1µL of sample into the heating chamber at 220°C using splitless injection. Helium (HE) was used as a carrier-gas to transfer the evaporate though the solid phase (column) at different temperatures; starting with 70°C for 2 mins, followed by ramping the temperature at 20°C/min until reaching 150°C and held for 5 min. The temperature was then elevated to 218°C at 2.5°C/min, then ramped up to 225°C at rate 30.6°C/min and kept for 10 min. Lastly, the column temperature was again ramped to 230°C at a pace of 2.5°C/min and held for 3 min. The FID hydrogen flame was generated with a mixture of hydrogen and mixed air (1:10), and FID kept at a constant temperature of 225°C. The duration of the GC-FID run for each sample was 65 min followed by 10 min post-run.

To obtain good resolution and minimise carry-over, prior to each study, multiple DCM (blank) injections were performed, until a consistent and clear baseline was achieved. The splitless FAME cocktail was then injected to determine retention times for each FAME component (**Table 2.13**). The FAME samples were run in duplicate with blank in between.

Table 2.13. Fatty acid methyl ester (FAME) cocktail composition and retention times for GC-FID.

Common name	Lipid numbers	Fatty acid Systematic name	Concentration (µg/mL)	RT (min)
Butyric acid	C4:0	Butanoic acid	400	6.56
Caproic acid	C6:0	Hexanoic acid	400	8.30
Caprylic acid	C8:0	Octanoic acid	400	10.30
Capric acid	C10:0	Decanoic acid	400	12.62
Undecylic acid	C11:0	Undecanoic acid	200	14.07
Lauric acid	C12:0	Dodecanoic acid	400	15.79
Tridecylic acid	C13:0	Tridecanoic acid	200	17.83
Myristic acid	C14:0	Tetradecanoic acid	400	20.16
Myristoleic acid	C14:1	Cis-9-tetradecenoic acid	200	21.57
Pentadecylic acid	C15:0	Pentadecanoic acid	200	22.69
Pentadecenoic acid	C15:1	Cis-10-pentadecenoic acid	200	24.21
Palmitic acid	C16:0	Hexadecanoic acid	600	25.37
Palmitoleic acid	C16:1	Cis-9-hexadecenoic acid	200	26.60
Margaric acid	C17:0	Heptadecanoic acid	200	28.09
Heptadecenoic acid	C17:1	Cis-10-heptadecenoic acid	200	29.37
Stearic acid	C18:0	Octadecanoic acid	400	30.85
Vaccenic acid	C18:1n-7	Trans-11-octadecenoic acid	200	31.47
Oleic acid	C18:1 n-9c	Cis-9-octadecenoic acid	400	31.90
Elaidic acid	C18:1 n-9t	Trans-9-octadecenoic acid	200	32.16
Linoleic acid	C18:2 n-6c	Cis,cis-9,12-octadecadienoic acid	200	32.67
Linolelaidic acid	C18:2 n-6t	Trans,trans-9,12-octadecadienoic acid	200	33.69
α-linolenic acid	C18:3 n-3	All-cis-9,12,15-octadecatrienoic acid	200	34.86
Γ-linolenic acid	C18:3 n-6	All-cis-6,9,12-octadecatrienoic acid	200	35.83
Arachidic acid	C20:0	Eicosanoic acid	200	36.19
Gondoic acid	C20:1 n:9	Cis-11-eicosenoic acid	200	37.23
Heneicosylic acid	C21:0	Heneicosanoic acid	200	38.72
Eicosadienoic acid	C20:2	Cis-11,14-eicosadienoic acid	200	39.01
Eicosapentaenoic acid (EPA)	C20:5 n-3	All-cis-5,8,11,14,17-icosapentaenoic acid	200	39.01
Dihomo-γ-linolenic acid (DGLA)	C20:3 n-6	Cis,cis,cis-8,11,14-Eicosatrienoic acid	200	40.22
Eicosatrienoic acid	C20:3 n-3	All-cis-11,14,17-eicosatrienoic acid	200	41.06
Arachidonic acid (AA)	C20:4 n-6	All-cis-5,8,11,14-eicosatetraenoic acid	200	41.21
Behenic acid	C22:0	Docosanoic acid	400	41.39
Erucic acid	C22:1 n-9	Cis-13-docosenoic acid	200	43.45
Tricosylic acid	C23:0	Tricosanoic acid	200	44.17
Docosadienoic acid	C22:2		200	44.65

		All-cis-13,16-docosadienoic acid		
Lignoceric acid	C24:0	Tetracosanoic acid	400	47.21
Nervonic acid	C24:1	Cis-15-tetracosenoic acid	200	48.69
Docosapentaenoic acid (DPA)	C22:5 n-3	All-cis-7,10,13,16,19-docosapentaenoic acid	200	50.26
Docosahexaenoic acid (DHA)	C22:6 n-3	All-cis-4,7,10,13,16,19-docosahexaenoic acid	200	51.20

2.7.7. Processing FAME GC-FID data

Sample FAME peaks were identified according to their retention times using ChemStation software (v.2.1.0433) (Agilent technologies, Cheadle, UK). The peak area for each FAME was integrated and normalised to the internal standard. Data were presented as percentage of total fatty acid weight in each sample, as described by Hodson et al. (2008) (212).

2.8. Amnion epithelial cell treatment with lipid surfactant

2.8.1. Isolation of amnion cells

The amnion was peeled from the choriodecidua and membrane washed thoroughly in sterile Hanks balanced salt solution (HBSS) (without Calcium and Magnesium) (Sigma-Aldrich) 3-4 times, to remove excess blood. The membrane was then cut into 2-3 cm length pieces (bloody stained pieces discarded) and incubated in 50mL HBSS with 0.25% (v/v) trypsin-EDTA, in a water-bath at 37°C for 15 mins with mechanical stirring. The first digestion was discarded and membrane pieces further incubated (at 37°C) two more times in 100mL 0.25% trypsin-EDTA/HBSS for 30mins. The collected digests where then filtered through a 40um sterile filter, before fetal bovine serum (FBS) (Gibco, UK) was added to 10% (w/v) final concentration. Each digestion was centrifugated at 700x g for 10 min at room temperature. Supernatants were discarded and remaining cells resuspend in HBSS 10%(v/v) FBS. Cell viability and counting were then performed by haemocytometer and trypan blue exclusion, and the accepted cell isolate viability was > 80%. Cells were subsequently diluted in freezing solution FBS with 10% (v/v) dimethyl sulfoxide (DMSO) (Sigma-Aldrich) and stored at -80°C for 24 hours, before transfer to liquid nitrogen. The amnion epithelial cell isolations were guided by Motedayyen et al. (2017) (214).

2.8.2. Amnion epithelial cell characterisation

For each investigation, approximately 100,000 cells were used. Once cells were pelleted (5 min at 1500x g), 200µl HBSS/0.5% (w/v) BSA (Sigma-Aldrich) was added. To reduce non-specific antibody binding, Fc Receptor Blocking Solution (BioLegend, London, UK) was added (20µl/100µl) with incubation at room temperature for 10 mins. Unlabelled primary (mouse anti-human) antibodies were then added according to **Table.2.14**, with further 30 min incubation. After two washes with 1mL HBSS, a secondary antibody (Goat anti-mouse FITC) was added to each sample, followed by incubation in the dark at room temperature. Again, cells were washed twice with HBSS, before a final resuspension in HBSS/0.5% BSA for flow cytometric analysis. A BD ACCuri^{TM} C6 flow cytometry (BD Biosciences, San Jose, CA, USA) was used for both acquisition and analysis of data.

Table 2.14. Antibodies used for amnion epithelial cell characterisation.

Primary antibody	Target	Dilution	supplier
CD326 (EpCAM) clone 1B7 (0.5mg/mL)	Epithelial cells	1:125	Fisher Scientific (UK)
CD166 (25 µg) clone 3A6 (1mg/mL)	Mesenchymal cells	1/50	Bio-Rad (Deeside, UK)
CD 45 clone F10-89-4 (1mg/mL)	Leukocytes	1:400	Bio-Rad (Deeside, UK)
IgG control		1:150	Generon (Slough, UK)
Secondary antibody			
Goat Anti-mouse IgG FITC		1:150	Generon (Slough, UK)

2.8.3. Preparation of lipid surfactants for *in vitro* experimentation

LPC (C16:0) (Avant Polar Lipids, Alabaster, Alabama, US) was prepared freshly and dissolved directly in amnion cell medium. DPPC (Avant Polar Lipids, US) stock solution was prepared by adding 5mg of DPPC in 5mL HBSS. In order to help DPPC dissolve, the solution was sonicated in a water-bath at 65°C at a frequency of 45kHz (ultrasonic water bath, VWR, Malaysia).

2.8.4. MTT (tetrazolium salt 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay

The MTT assay is a semi-quantitative colorimetric assay that measures cell viability and metabolic activity. It relies on the ability of live cells (and more specifically their mitochondria) to react with tetrazolium salt 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to form purple coloured crystals (215). Under usual circumstances, the MTT directly

reflects the number of live cells present. For measuring the impact of agents on cell growth, amnion epithelial cells were seeded in 96-well plates in amnion culture medium; DMEM/F12 medium with penicillin (0.5mg/mL), streptomycin (0.5mg/mL), gentamycin (100µg/mL), 10% FBS (w/v), and 10ng/mL Epidermal Growth Factor (EGF) (Sigma-Aldrich). After incubation and experimental exposures, 20µL MTT (Sigma-Aldrich) (5mg/mL in HBSS) was added to each well and cells returned to culture for a further 3 hours at 37°C. Media was then removed gently to avoid disturbing the crystals and DMSO (100µL) added to solubilise the MTT for colorimetric detection. Absorbance's were recorded at 540nm and corrected at 690nm (FLUOstar ® Omega, BMG-LABTECH, Aylesbury, UK).

Amnion epithelial cells were exposed to DPPC and LPC (C16:0) in duplicate in various concentrations (5, 10, 20, 50, and 100µg/mL) over 24 hours, to define their impact on cell viability and proliferation.

2.8.5. Amnion epithelial cell exposure to surfactant lipids

For the lipid surfactant experiments, amnion epithelial cells (passage 1) were seeded and cultured in T75 flasks in amnion medium (described in **2.8.4**). On reaching 60% confluence, the cells were exposed to fresh medium containing 20mg/mL DPPC or LPC (C16:0) for a further 24 hours. The previous concentration was chosen for DPPC and LPC (C16:0) based on MMT assay and incorporated fatty acid changes, as seen in cultured exposed cells. After 24 hours, the culture medium was collected and centrifuged at 700x g for 10 min at room temperature and aliquoted and stored at -80°C. The cells were washed three times with HBSS to remove remnants of surfactant lipids, before being harvested with 0.25% trypsin-EDTA and stored at -80°C ready for GC analysis (**section 2.7**) or secretory cytokine profiling (**section 2.8.6**).

2.8.6. Cytokines profiling

Secreted cytokines from surfactant-treated amnion epithelial cells were quantified by Luminex methodology using the LEGENDplex™ Human Inflammatory Panel 1 kit (BioLegend®, San Diego, CA, US), including; IL-1β, IFN-α2, IFN-γ, TNF-α, MCP-1 (CCL2), IL-6, IL-8 (CXCL8), IL-10, IL-12p70, IL-17A, IL-18, IL-23, and IL-33. Samples were processed in accordance with the

manufacturer's manual and analysed with BD FACSVers (BD Biosciences) within the Core Technology Facility, University of Manchester. Data analyses were performed with LEGENDplex™ data analysis software (v.8, BioLegend®), normalised to cell numbers in the original cultures, and expressed as ng/million cells.

2.9. Decidual mesenchymal cells exposure to IL-8

2.9.1. Decidual mesenchymal cell isolation

This protocol was based on the combined methods of Grimwood et al. and Pelekanos et al. (216,217). The choriodecidual layer was first separated from the fetal membranes. The maternal side was placed upward, and decidual tissues scraped way using a cell scraper. The collected decidua was then minced finely with sterile scissors and washed thoroughly 3-4 times with HBSS. Enzymatic digestion was then performed with Collagenase I (1mg/mL), Dispase (1mg/mL) and DNAase (6.25U/mL) for 1 hour at 37°C with continuous mechanical stirring (Sigma-Aldrich, UK). The resulting digest was passed through a 100µm filter twice, followed by a single 70µm filtration. Cells were counted using a haemocytometer and viability assessed by trypan blue. Cells were cultured in T75 flasks at density $1x10^6$ in DMEM/10% Ham's F-12 containing L-glutamine (1.5mg/mL) (Sigma-Aldrich, UK), penicillin and streptomycin (0.5mg/mL) (Sigma-Aldrich, UK), 20% (v/v) FBS and added β-estradiol at 10^{-8}M and progesterone at $5x10^{-7}$M (Sigma-Aldrich, UK), to enforce decidualisation (218). The cells were stored at passage 2 or experimented at passage 3.

2.9.2. Decidual mesenchymal cell characterisation

For Immunocytochemistry, cells were seeded at approximately 5000 cells per well in a 24-well tissue culture plate (Greiner bio-one, UK). Prior to seeding, glass coverslips were sterilised with 80% (v/v) ethanol and placed in the bottom of each well. Cells were covered with 500µL of culture media and necessary supplements for growth. After desired densities were reached, the culture media was discarded, and cells washed twice with HBSS. The cells were then fixed by adding 4% (w/v) formaldehyde solution for 20 minutes at room temperature. After a further two washes with HBSS, the fixed cells were covered and stored in HBSS/0.1% BSA at 2-8°C. After blocking and permeabilisation with (10% normal rabbit

serum (v/v), 0.3% Triton X-100(v/v) (Sigma-Aldrich) in HBSS for 45 minutes at room temperature, the blocking buffer was removed, and the fixed cells were covered in unconjugated primary antibodies (**Table.2.15**) overnight at 2-4 °C. Then the cells were washed two times with washing buffer and diluted with secondary antibody for 1 hour at room temperature in a dark place. Followed by two rinses with the washing buffers, then the coverslips were placed gently to microscope slides that pre-topped VECTASHIELD mounting medium with DNA stain solution (diamidino-2-phenylindole) (DAPI) (vector laboratories, UK). The slides were visualised using a fluorescence microscope with an appropriate filter sets Nikon ECLIPSE 80i (488nm and 358nm, FITC and DAPI respectively) and images were analysed and merged using NIS-Elements programme (Nikon UK, UK).

Table 2.15. Antibodies used for decidual mesenchymal cell characterisation:

Primary antibody	Target	Dilution	supplier
CD 45 clone F10-89-4 (1mg/mL)	Leukocytes	1:400	Bio-Rad (Deeside, UK)
Vimentin (V9) (0.2mg/mL)	Mesenchymal cells	1:100	Fisher Scientific (Loughborough, UK)
Cytokeratin7 (1mg/mL)	Trophoblast	1:200	Fisher Scientific (Loughborough, UK)
Secondary antibody			
Goat Anti-mouse IgG FITC		1:150	Generon (Slough, UK)

2.9.3. MTT assay

Decidual mesenchymal cells were exposed to AA in different concentration (1, 5, 10, 20, 30, 40, 50µM) (Sigma-Aldrich, UK) in duplicate to define effects on cell viability and proliferation over the culture period considered 48 hours exposure. MMT assay was described in detail on **section 2.8.4**.

2.9.4. PGE_2, $PGF_{2\alpha}$ and TXA_2 profiling using Enzyme-linked immunosorbent assays (ELISA)

For eicosanoid recognition (PGE_2, $PGF_{2\alpha}$ and TXA_2), decidual mesenchymal cells were seeded and cultured in T25 flasks and allowed to reach 60% confluency. The standard decidual mesenchymal media (see above) was then changed AA (5 and 10µM) containing medium and the cell were incubated for 72h (medium changed every 24h). Then the cells were washed

three times with HBSS and then the media were changed to serum-free medium for 24h, except 10µM Calcium Ionophore A23187 treated cells were incubated for 30min. All products purchased from Sigma-Aldrich, UK.

Once completed, culture media was collected and centrifuged at 700x g for 10 min at room temperature and aliquoted and stored at -80°C. The resultant supernatants were collected and aliquoted into pre-chilled tubes and stored at -80°C for further analysis. PGE_2, $PGF_{2\alpha}$ and TXA_2 ELISA kits (competitive assays), were purchased from Enzo Life Sciences (UK) and performed according to manufacturer's instructions. Resulting plates were measured by spectrophotometer (FLUOstar ® Omega, BMG-LABTECH) at wavelength 405nm corrected to 580nm.

2.9.5. IL-8 treatment

Decidual mesenchymal cells were culture to 60% confluence and loaded with AA (10µM) for 48 hours; then the cells washed three times with HBSS to washout AA remnant. Then the cells were exposed to recombinant human IL-8 (77 a.a) (Peprotech EC Ltd, London, UK) at concentration 30pg/mL, 50ng/mL and 100ng/mL for 24 hours in serum-free medium. The collected medium was prepared, stored and analysed using PGE_2 ELISA kit as described in **section 2.9.4**.

Chapter 3: Fetal membrane lipid mediator profiling

3.1. Introduction

The timing of human labour is crucial for maternal and fetal wellbeing, with preterm labour associated with increased fetal mortality (219). Throughout pregnancy, the uterine muscles are kept in a relaxed state, but at the time of labour the uterus becomes contractile to facilitate fetal and placental delivery. Although most women deliver at term, i.e. around 40 weeks gestation, the question regarding the timing of parturition remains enigmatic. Nevertheless, growing evidence supports the involvement of natural inflammatory mediators, particularly pro-inflammatory prostanoids such as PGE_2 and $PGF_{2\alpha}$, which have been commonly adopted to initiate labour in clinical settings (220).

The upscaling of inflammatory markers in the intrauterine milieu is a common observation in human parturition. For instance, a systemic review by Menon et.al. reported that the pro-inflammatory cytokines IL-1β, IL-6 and IL-8 were upregulated in fetal membrane tissues, placenta and myometrium after spontaneous labour at term (98). Also, the amniotic fluid showed an increase of PGE_2 with advancing gestational age, and even a surge at the time of labour (104); the latter supporting the importance of prostaglandins in the initiation of a parturition cascade, rather than a consequential element of it.

The fetal membrane is a thin structure that seals the amniotic fluid and conceptus within the intrauterine cavity. The membrane is composed of two layers, the amnion and chorion. The amnion being the inner layer, in-direct contact with the amniotic fluid, and chorion, the outer lay associated with the uterine decidua, which is shed in combination at delivery as the choriodecidua. In addition to containing the products of conception and amniotic fluid, these tissues are presumed to have barrier functions and fulfil paracrine signalling between the maternal and fetal compartments. Given its ideal location to receive and transmit signals between fetus and uterine myometrium, the fetal membrane is considered to have a prominent role in initiating and maintaining human parturition. Certainly this is supported by the simple fact that irritating the membranes by “sweeping” or their rupture can “kick-start”

labour (185,221) and that they are a known source of inflammatory mediators, including cytokines and prostaglandins (98,222).

Eicosanoids are a diverse family of lipid mediators with fundamental roles in physiology and disease (36). N-3 and n-6 PUFAs are the precursors of eicosanoids, octadecanoids and docosanoids, the latter two classes we will be referred to as 'related mediators' (89). The eicosanoids include a range of prostaglandins and thromboxanes (collectively known as prostanoids), leukotrienes and hydroxy fatty acids, and are known to alert the intrauterine inflammatory environment (223,224). The prostanoids are formed by oxidation of PUFAs by the enzyme cyclo-oxygenase (COX) and specific prostanoid synthases (225). COX has two isoforms, COX-1 and COX-2 and is therapeutic target for nonsteroidal anti-inflammatory drugs (NSAID), as the rate-limiting enzyme in prostanoid synthesis (90,224). Arachidonic acid (AA)-derived prostanoids are the most common class studied in intrauterine tissues, particularly in association with parturition.

At the time of labour, COX-2 is found to be upregulated in many of the intrauterine tissues, including amnion, chorion, decidua and myometrium (98). Also, AA-derivative prostanoids; PGE_2 and $PGF_{2\alpha}$ and PGJ_2 are raised in the amniotic fluid in term labour (104). AA, along with other PUFAs, can be utilised by lipoxygenases (LOX) and cytochrome P450 (CYP450) enzymes to form eicosanoids including mono- and poly-hydroxy fatty acids, and fatty acid epoxides (89). Some of these mediators are also increased in amniotic fluid with different types of labour (104,226). Unlike the prostanoids, the involvement of these lipids in human parturition has not been studied in detail. Equally, the importance of endocannabinoids in relation to labour, as lipid modulators of inflammation, is only poorly defined.

Endocannabinoids are lipid mediators that are known to bind the endocannabinoid receptors CB1 and CB2. The two classic endocannabinoids are the AA-derivatives N-arachidonoylethanolamide (anandamide; AEA) and 2-arachidonoylglycerol (2-AG) (227). AEA levels in maternal plasma have been shown to be increased in spontaneous labour (228) and also raised in women receiving induction of labour; inversely correlating with its duration. Both AEA and 2-AG can be sources of AA acid through their hydrolysis by fatty acid amide hydrolase (FAAH) and monoacylglycerol lipase (MAGL), respectively. Thus, AEA and 2-AG, may

also be another source of AA for prostanoid synthesis (227). Other NEAs that are synthesised by the same pathway as AEA, some act on CB receptors while others do not activate these receptors; therefore, not all *N*-acyl ethanolamines (NAEs) are considered endocannabinoids.

Ceramides are another class of lipid mediators, and members of the sphingolipid family, known to stimulate COX-2 and pro-inflammatory cytokine production in *in vitro* models of reproductive tissues (229,230). Labouring women have demonstrated increased plasma ceramide levels compared to their non-labour counterparts (60). Placental tissues show increased ceramide production and upregulation of serine palmitoyl transferase (SPT) the rate limiting step for *de novo* ceramide biosynthesis, in both spontaneous and induced labour (42). Moreover, ceramide can be generated from hydrolysis of SM, and by the so-called "salvage pathway" (**Chapter 1, Figure 1.8**)(231). An early study also showed an elevation of ceramide lactosides within the amniotic fluid of preterm labouring pregnancies associated with chorioamnionitis (232).

Given this evidence, it could be hypothesized that the fetal membrane would be good a source of lipid mediators, i.e. eicosanoids, endocannabinoids and ceramides, that could influence the intrauterine inflammatory environment, and that this lipidome profile may be different in different types of labour, according to how and when in gestation labour was induced; ie term vs preterm, spontaneous vs induction, as compared to non-labouring, quiescent tissues at term.

Given the significance of lipid mediators in parturition, it is important to understand their profile within fetal membranes as this will allow us to identify the underlying molecular mechanisms. Previous studies have shown altered enzyme activities, presence of lipid mediators in amniotic fluid, and their production in *in vitro* models. However, the aim here is to undertake the most comprehensive study to date, to explore lipid mediators within the fetal membranes directly, using state-of-the-art mass spectrometry lipidomics, with greater sensitivity and broader profiling than previously undertaken. The objective is to explore the prevalence of eicosanoids and related mediators, endocannabinoids and ceramides, in amnion and choriodecidual tissues under different labouring conditions, in comparison to a non-labouring group at term.

3.2. Material and methods

Fetal membrane tissue sampling and storage are described in **section 2.1**. The analysis of lipid mediators was performed by UPLC/ESI-MS/MS, as detailed in **sections 2.2, 2.3** and **2.4**. Protein content protocols can be found in **section 2.6.**

3.3. Statistical analysis

Lipid mediator data were analysed using Prism (v.8) (GraphPad, California, USA). Each lipid extract was injected in duplicate (technical repeats). The data were first tested for Gaussian distribution using the Shapiro-Wilk and Kolmogorov–Smirnov normality tests. With a mixture of normal and not-normal distributions, all data were subsequently log-transformed and normality verified before parametric one-way and two-way ANOVAs with Dunnett's multiple comparisons were performed. Datasets were subsequently expressed as means with standard error of the mean (±SEM). A similar statistical approach was published by Cicchi et al. (2020) and Durn et al. (2010) (175,233). Exceptions were for total CER(ADS), CER(AS), and CER(NH), and CYP450 (total and species profiling) and AEA data, where all study groups showed a normal distribution. For these, statistical testing was performed on the raw data. One sample from the term in labour group was excluded from the endocannabinoid analysis due to technical complications.

3.4. Results

3.4.1. Fetal membrane eicosanoids and related mediators

The eicosanoid profiling of fetal membranes included COX-, LOX- and CYP450-derived mediators of AA, DGLA and EPA. Also, octadecanoids and docosanoids, derivatives of LA and DHA respectively, were profiled. Both sides of the fetal membrane were separated, homogenised and extracted. The lipid mediators were analysed by UPLC/ESI-MS/MS. Lipid mediator concentrations were normalised to protein content (pg/mg).

The biological tissues were distributed into two study groups. Firstly a “term pregnancy study”, to understand lipid mediator changes in physiological spontaneous term labour (STL

n=7 and n=9, amnion and choriodecidua, respectively) and drug-induced term labour (ITL, n=9 and 9), as compared to a term not-labouring group (TNL, n=13 and 12, amnion and choriodecidua, respectively). Secondly, a "preterm pregnancy study" including comparison of a preterm labour cohort (PTL, n=5 and 5, amnion and choriodecidua, respectively) and STL against TNL.

3.4.1.1. COX-derived lipid mediators of term fetal membranes

A wide range of COX metabolites were analysed in the fetal tissues, including 1, 2, and 3 series prostaglandins and thromboxanes. In the amnion, seven COX-derived species were detected; PGE_1, PGE_2, 15-keto PGE_2, 13,14 dihydro 15-keto PGE_2, $PGF_{2\alpha}$, TXA_2, and $\Delta^{12}PGJ_2$. And ten species in choriodecidua, including; PGE_1, 13,14 dihydro PGE_1, 13,14 dihydro 15-keto PGE_1, PGE_2, 15-keto PGE_2, 13,14 dihydro 15-keto PGE_2, $PGF_{2\alpha}$, 13,14 dihydro 15-keto $PGF_{2\alpha}$, TXA_2, and $\Delta^{12}PGJ_2$. In both amnion and choriodecidua, the TNL group showed detectable baseline levels of total prostanoids with means (± SEM) of 2465±555 pg/mg of protein and 1728±267, respectively (**Figure 3.1**). In the amnion, total COX-derived metabolites were upregulated in the ITL group compared to those not-in-labour. In contrast, the spontaneously labouring STL group showed a similar baseline to TNL. Alternatively, the choriodecidua showed a significant increase in prostanoid production in both labouring groups (STL and ITL) compared to TNL. In both tissues, AA-derivative prostanoids were the most abundant compounds, followed by DGLA-derivatives. All EPA-derived prostanoids were below the methods of detection.

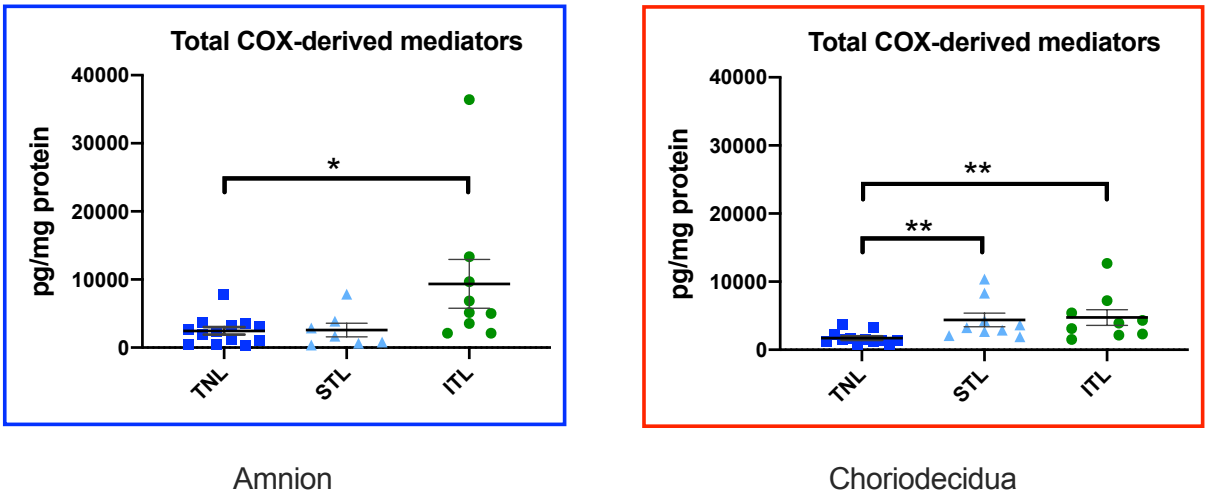


Figure 3.1. ***Term fetal membrane total COX-derived mediators. Amnion (left side, blue frame) and choriodecidua (right side, red frame).*** *Term not in labour (TNL), spontaneous term labour (STL) and induced term labour (ITL). Mean and SEM indicated. Statistical analysis = one-way ANOVA with Dunnett's multiple comparison test, *p≤0.05, **p≤0.01.*

Of all COX-mediators analysed, total AA-derived mediators were significantly upregulated in the choriodecidua in both labouring pregnancies, compared to TNL (**Figure 3.2**). With the amnion again showing a marked rise only in the ITL group compared to TNL controls (**Figure 3.2**). Regarding the total DGLA-mediators, both tissues showed similar production between TNL and STL groups, whilst those for ITL showed increased elevation, which failed to reach statistical significance (**Figure 3.2**). EPA mediators were below the limit of detection in both sides of the fetal membrane.

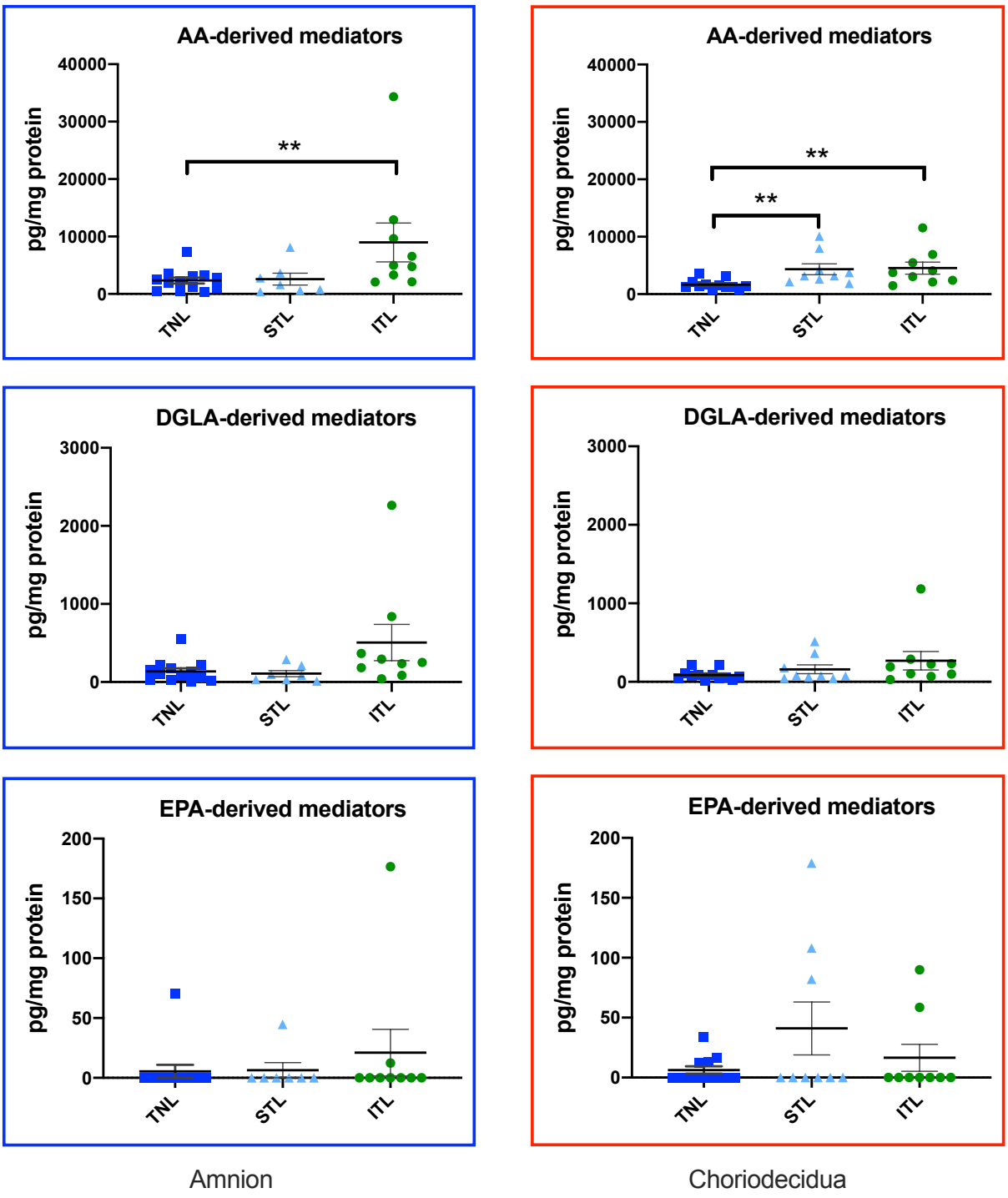


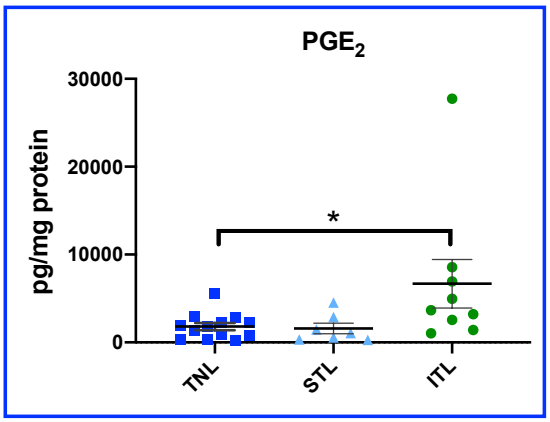
Figure 3.2 Term fetal membrane AA-, DGLA-, and EPA-derived mediators. Amnion (left side, blue frame) and choriodecidua (right side, red frame). *Term not in labour (TNL), spontaneous term labour (STL) and induced term labour (ITL). Mean and SEM indicated. Statistical analysis = one-way ANOVA with Dunnett's multiple comparisons test, *p≤0.05, **p≤0.01.*

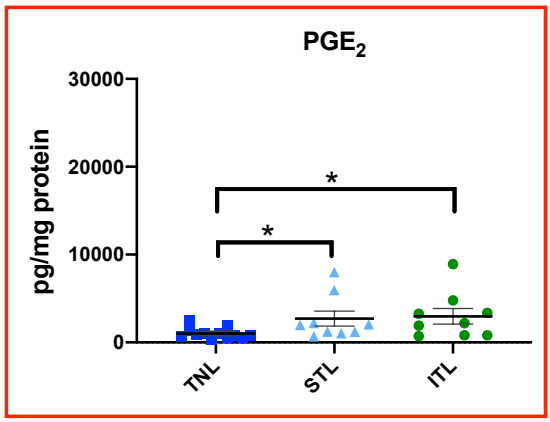
As shown in **Figure 3.3**, the labour-associated prostaglandins, PGE_2 and $PGF_{2\alpha}$, and their metabolites, along with TXA_2 (measured as TXB_2), were the most abundant AA-derivatives seen in the amnion and choriodecidua. The other AA metabolites PGI_2 (measured as 6-keto

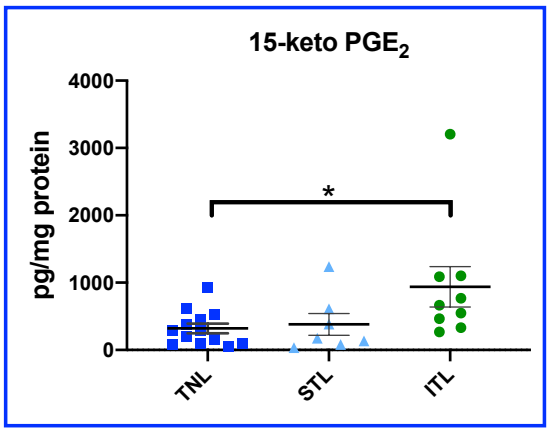
$PGF_{1\alpha}$), PGD_2 and PGJ_2, were all below the limits of detection in most samples. This was the same for $\Delta^{12}PGJ_2$ within the amnion; with only low levels also defined in the choriodecidua. For the amnion, PGE_2 and its metabolites were the most synthesised 2-series prostanoid, followed by $PGF_{2\alpha}$ and TXA_2. In the ITL group, PGE_2 levels were increased significantly compared to TNL, whilst in the STL group they were similar (see **Figure 3.3**). Although PGE_2 was not increased in STL, its inactive metabolite 13,14 dihydro 6-keto PGE_2 was significantly elevated, indicating the tissue was actively producing PGE_2 at an earlier time-point. Again, the ITL tissues expressed a significant increase in $PGF_{2\alpha}$, while STL levels were similar to TNL. TXA_2 levels failed to show any significant changes in term labour, compared to controls.

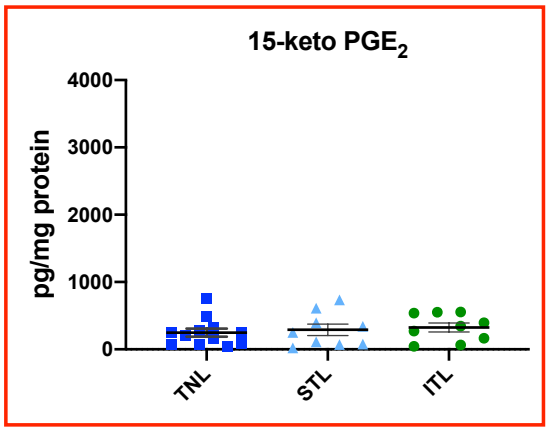
Choriodecidual tissues are an active site for the production of labour-associated prostanoids in both spontaneous and induced labour conditions (**Figure 3.3)**. Of the AA-drived prostanoids, PGE_2 was the most abundant, followed by TXA_2 and then $PGF_{2\alpha}$. PGE_2 and its metabolite (13,14 dihydro 15-keto PGE_2) were raised significantly in the ITL group, and STL likewise showed a marked increase in PGE_2. $PGF_{2\alpha}$ and its metabolite (13,14 dihydro 15-keto $PGF_{2\alpha}$) were upregulated in both STL and ITL, and reached significance in all but ITL, in which $PGF_{2\alpha}$ remained borderline ($p=0.0526$). TXA_2 was also elevated significantly in both labouring conditions. Overall, STL showed higher concentrations of $PGF_{2\alpha}$ and TXA_2 than ITL, perhaps indicating greater relative COX-2 activity. However, PGE_2 was found at similar levels between labouring conditions, suggesting PGE_2 production in both amnion and choriodecidua were raised in term parturition, regardless of labour stimulus.

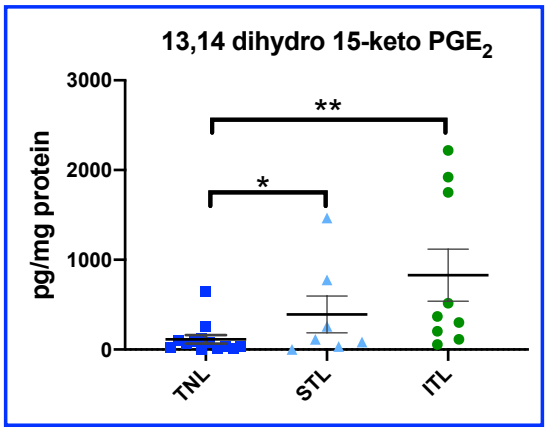
In both fetal membrane tissues, detection of DGLA-derivative prostanoids was limited to PGE_1 and its metabolites. In amnion, STL had a comparable level of PGE_1 to TNL, whilst the ITL group showed a significant elevation. For choriodecidua, both labouring conditions generated an increasing trend for PGE_1 (**Figure 3.3**).

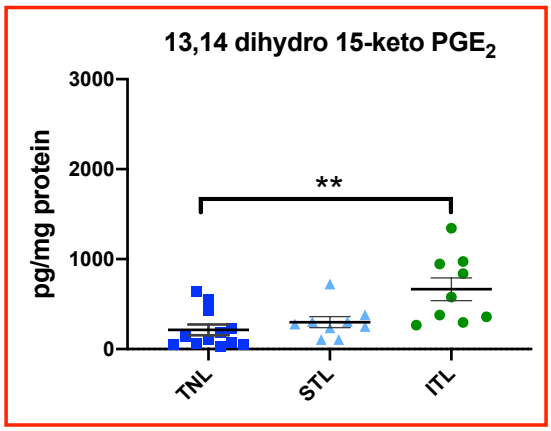


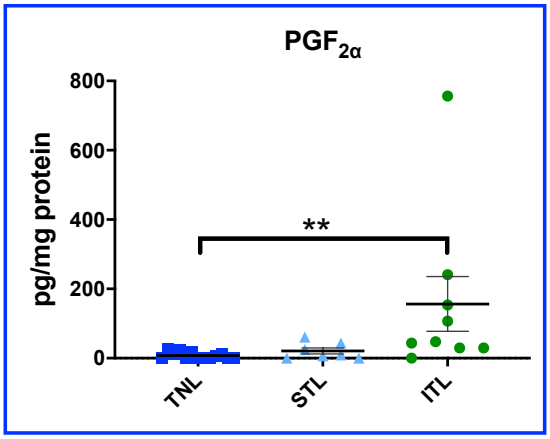


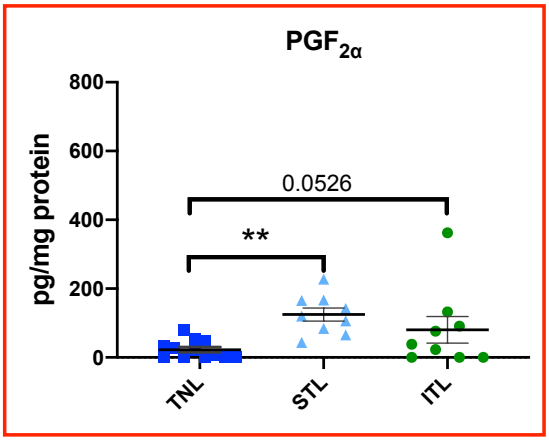






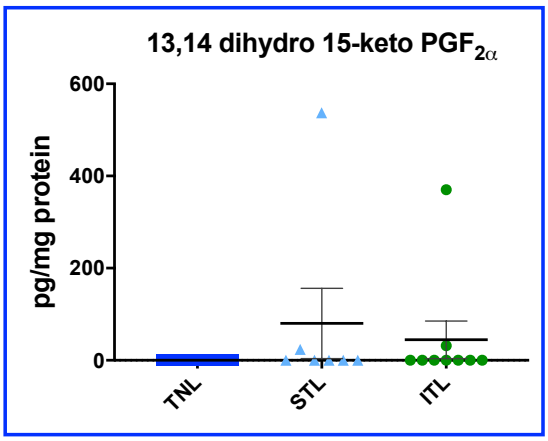


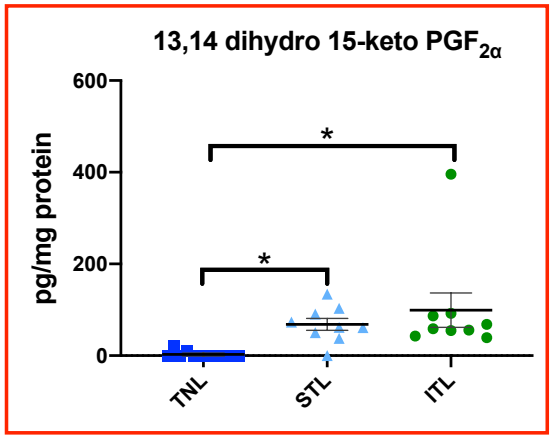


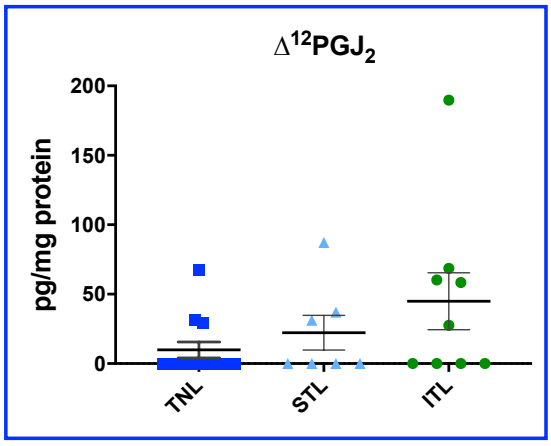


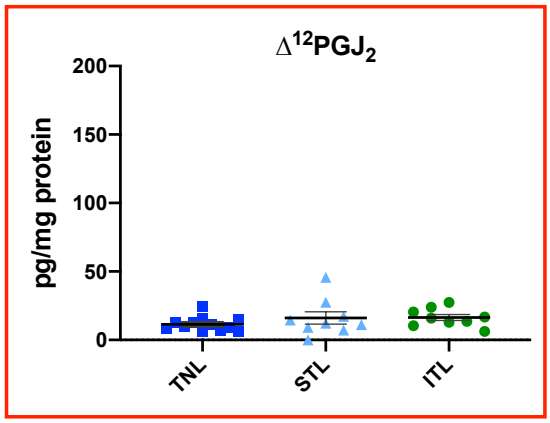
Amnion

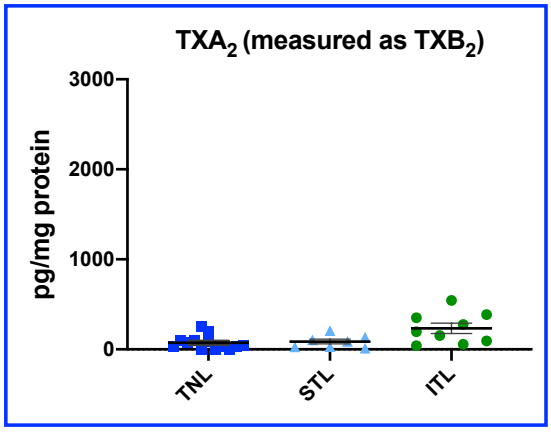
Choriodecidua

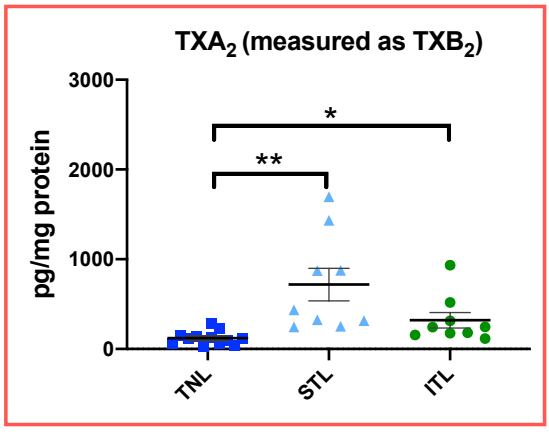


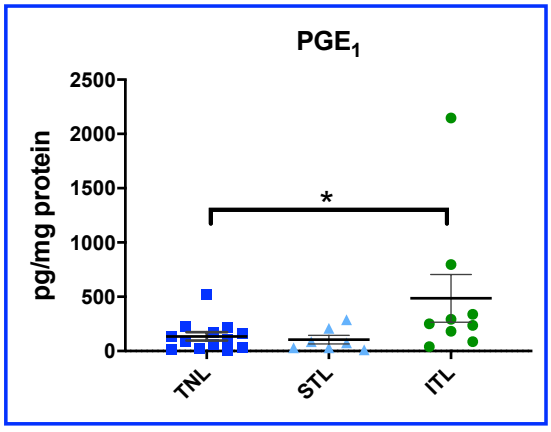


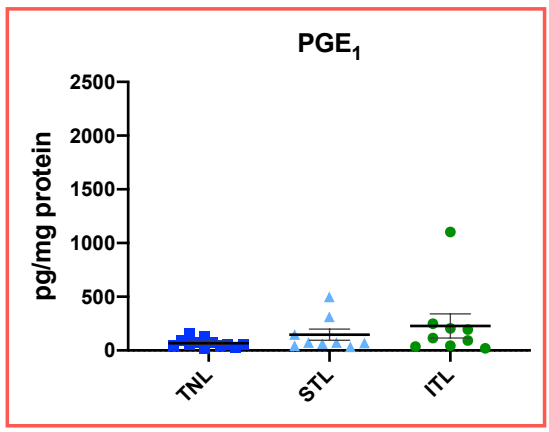












Amnion

Choriodecidua

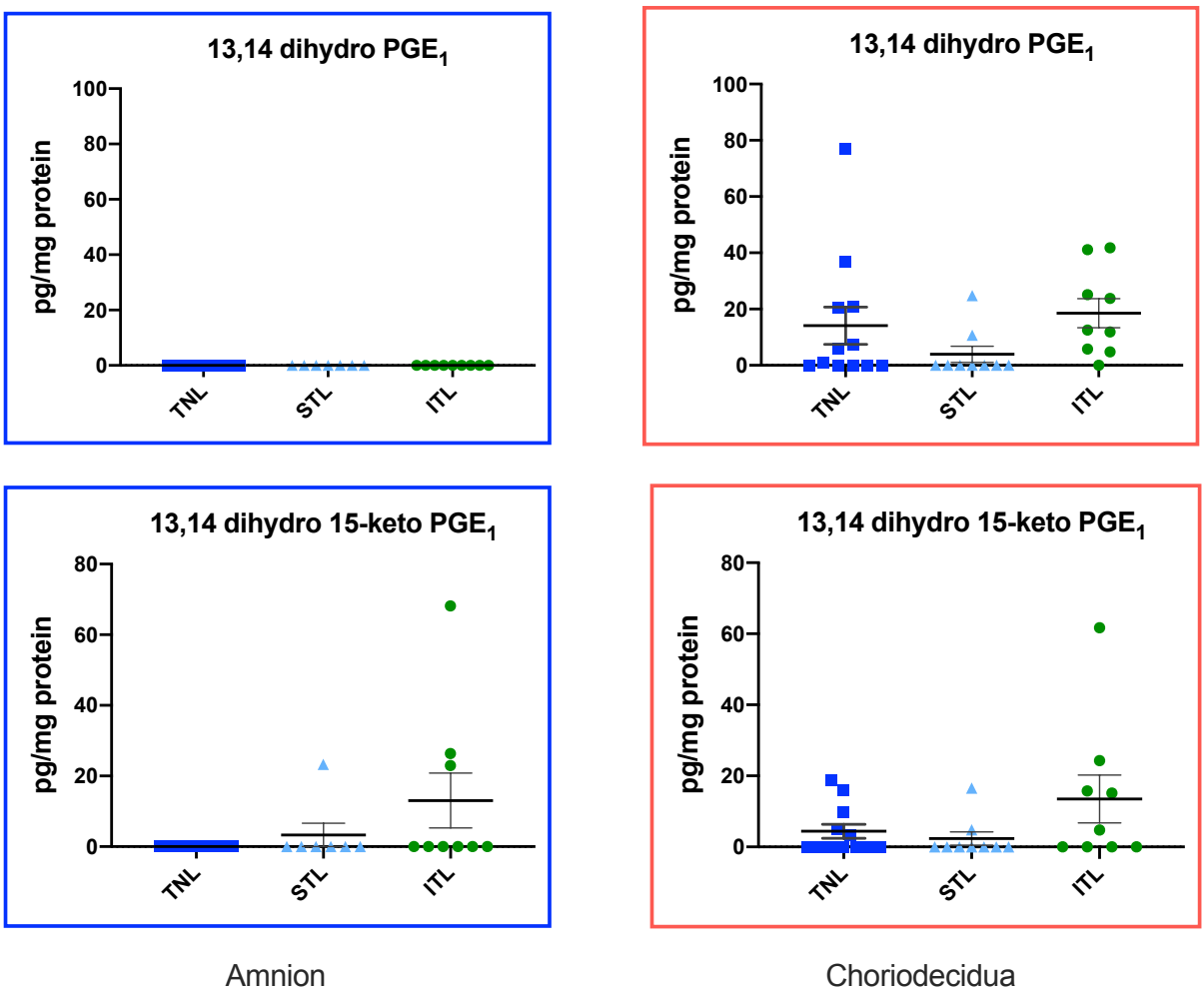


Figure 3.3. Term fetal membrane prostanoid species. Amnion (left side, blue frame) and choriodecidua (right side, red frame). *Term not in labour (TNL), spontaneous term labour (STL) and induced term labour (ITL). Mean and SEM indicated. Statistical analysis = two-way ANOVA with Dunnett's multiple comparison test, *p≤0.05, **p≤0.01.*

Overall, these results suggest that amnion tissues were more active in their production of prostanoids when labour was induced at term. Although not entirely replicated in spontaneous labour, raised levels of 13,14 dihydro 15-keto PGE_2, would also indicate the amnion had been actively producing PGE_2 in the early stages of parturition. In the choriodecidua, the AA-derived prostanoids, PGE_2, $PGF_{2\alpha}$, and TXA_2, were all actively produced in term labour, but arguably with greater abundance when labour occurred spontaneously, than when augmented.

3.4.1.2. Hydroxy fatty acid profiles of term fetal membranes

The production of hydroxy fatty acids formed by LOX and CYP450 pathways in fetal membranes were examined under spontaneous and induced-labour conditions, compared to term non-labouring controls. In amnion, 14 LOX-derived mediators were detected; 5-Oxo-ETE, 5-HETE, LTB_4, 11-HETE, 12-HETE, 15-HETE, 15-Oxo-ETE, 9-HODE, 9-OxoODE, 13-HODE, trans-EKODE, 15-HETrE, 12-HEPE and 14-HDHA, and 20 in choriodecidua; 5-Oxo-ETE, 5-HETE, LTB_4, 8-HETE, 11-HETE, 12-HETE, 15-HETE, 15-Oxo-ETE, 9-HODE, 9-OxoODE, 13-HODE, trans-EKODE, 15-HETrE, 12-HEPE, 15-HEPE, 18-HEPE, 10-HDHA, 13-HDHA, 14-HDHA and 17-HDHA. Also, in the amnion eight CYP450-derived species were detected: 5(6)EET, 8(9)EET, 11(12)EET, 11,12DHET, 9(12)EpOME, 12(13)EpOME, 9,10DiHOME, and 12(13)DiHOME, and nine in choriodecidua; 5(6)EET, 8(9)EET, 11(12)EET, 14(15)EET, 11,12DHET, 9(12)EpOME, 12(13)EpOME, 9,10DiHOME, and 12(13)DiHOME. Total LOX-derived lipid mediators were more than ten times higher than that of CYP450 mediators in both membrane tissues (**Figure 3.4**).

In the amnion, STL pregnancies showed a trend for increased hydroxy fatty acid production, compared to those not-in labour (TNL), but this failed to reach significance. For ITL, a substantial and significant increase in total LOX-derived mediators was recorded, but this was not repeated for the total CYP450-derived mediators. In the choriodecidua, STL production of hydroxy fatty acids was significantly raised for both LOX and CYP450 pathways; with the ITL group likewise non-significantly elevated compared to TNL.

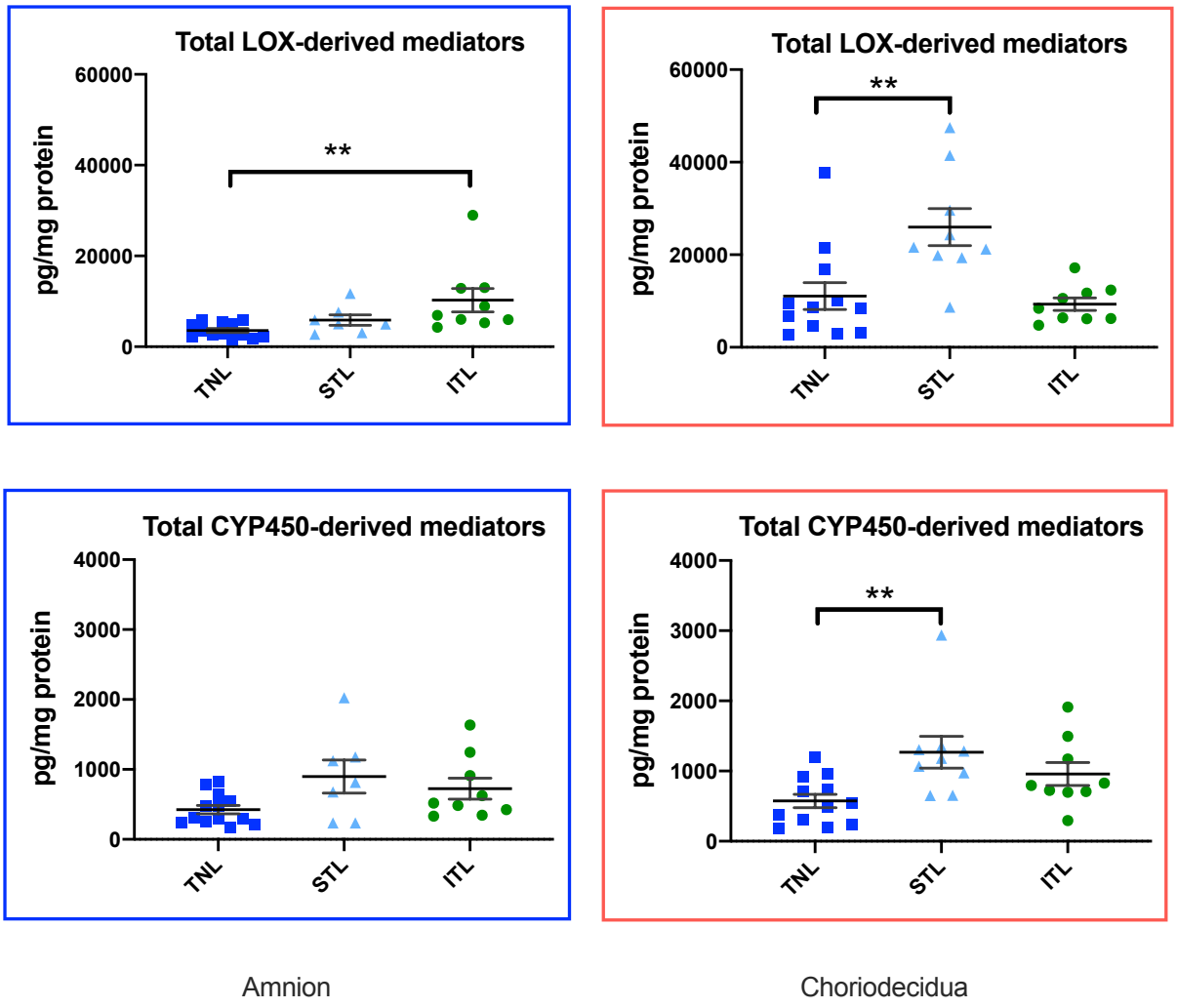
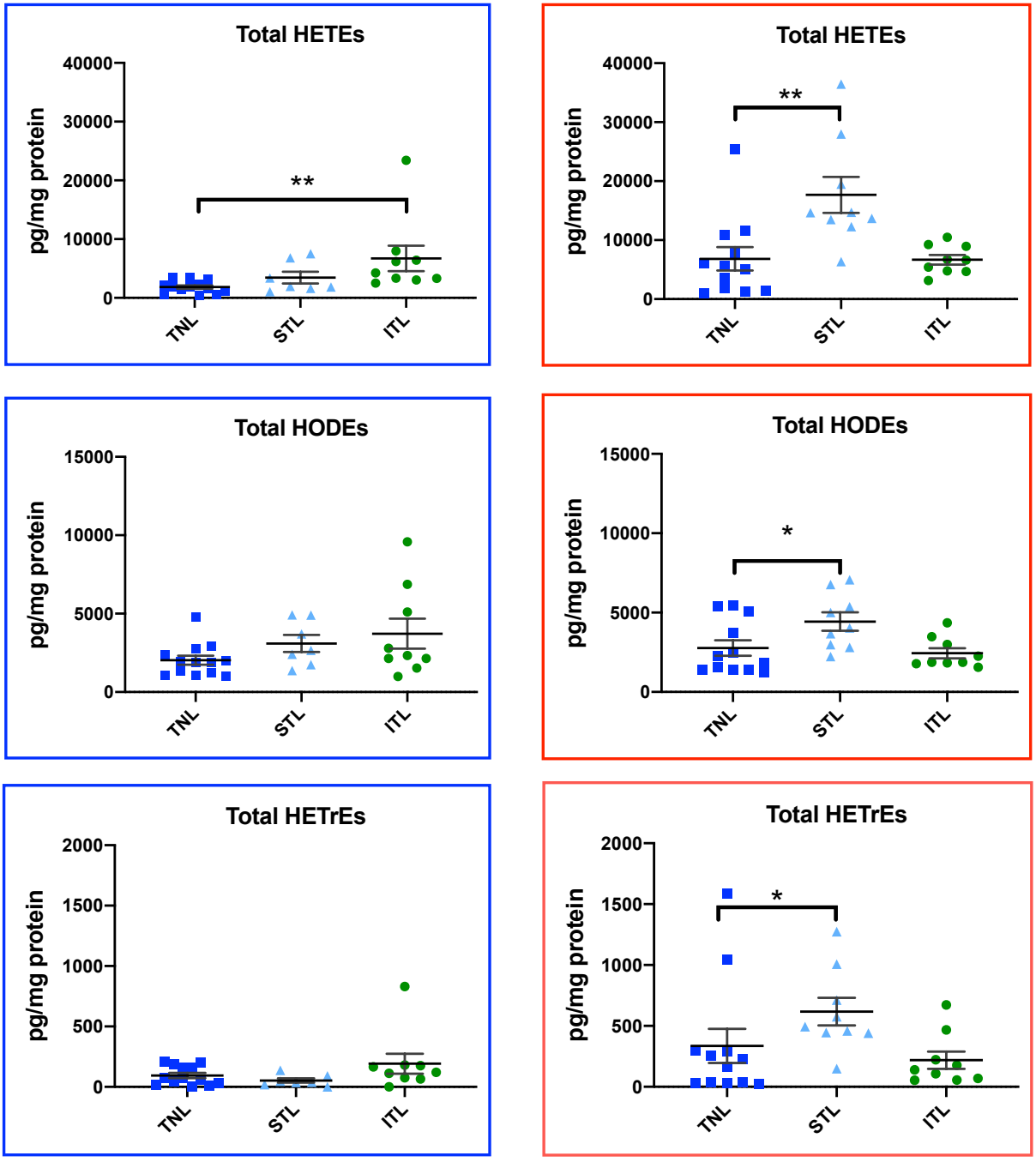


Figure 3.4. Term fetal membrane total LOX- and CYP450-derived mediators. *Amnion (left side, blue frame) and choriodecidua (right side, red frame). Term not in labour (TNL), spontaneous term labour (STL) and induced term labour (ITL). Mean and SEM indicated. Statistical analysis = one-way ANOVA with Dunnett's multiple comparisons test, *p≤0.05, **p≤0.01.*

Figure 3.5 shows the sum of the five PUFA-derived hydroxy fatty acids. In amnion and choriodecidua, HETEs (AA-derived) and HODEs (LA-derived) were the dominant metabolites, and HETrEs (DGLA-derived), HEPEs (EPA-derived) and HDHAs (DHA-derived) were at lower concentrations. The choriodecidua was more active in producing these mediators than amnion, with more prominence in the omega-3 PUFA-derived species (i.e. HDHAs and HEPEs). Notwithstanding, the choriodecidua base-line for HDHAs (TNL level) was 35 times higher than that of the amnion; with induced labour (ITL) producing a significant increase in both HETEs and HEPEs. While spontaneous labour showed an increasing trend in HETEs and HODEs levels, this failed to reach significance over their non-labouring counterparts. For the choriodecidua,

only spontaneous labour resulted in a significant increase in all five hydroxy fatty acid mediators considered (**Figure3.5**).



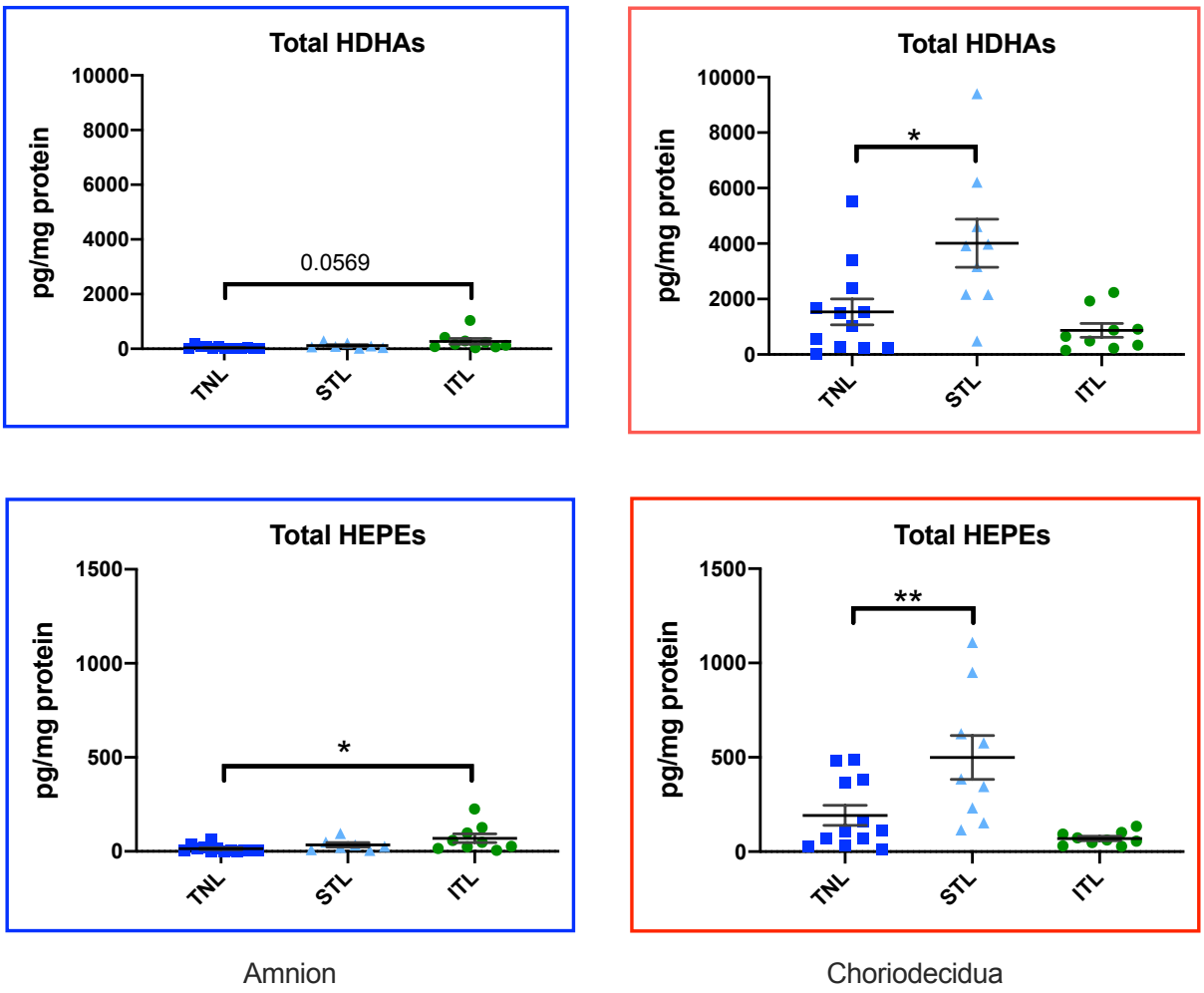


Figure 3.5. Term fetal membrane total HETEs, HODEs, HETrEs, HDHAs, and HEPEs. *Amnion (left side, blue frame) and choriodecidua (right side, red frame). Term not in labour (TNL), spontaneous term labour (STL) and induced term labour (ITL). Mean and SEM indicated. Statistical analysis = one-way ANOVA with Dunnett's multiple comparisons test, *p≤0.05, **p≤0.01.*

In both fetal membrane tissues, only the AA-derived 5-LOX mediators, 5-HETE, 5-Oxo-ETE and LTB_4, were above the limit of assay detection. 5-HETE, an *in vivo* inducer of uterine contractions (105), was significantly raised in both in-labour groups, with greater elevation in ITL over STL (**Figure 5.6**). 5-Oxo-ETE, a downstream metabolite of 5-HETE, showed the same trend, but only reached significance for ITL in the amnion (**Figure 5.6**). The 12-LOX metabolites 12-HETE, 12-HEPE, and 14-HDHA were also upregulated significantly in the amnion following labour induction (ITL), but only 12-HETE reached a significant level with spontaneous labour (STL). In choriodecidua, 12-HETE and 14-HADHA were upregulated only in the STL group. LTB_4 was detected in low concentrations in both membrane tissues, and without significant variation between groups.

The 15-LOX-derived metabolites 15-HETE, 15-Oxo-ETE, 15-HETrE, 13HODE, 15-HEPE and 17-HDHA, were all detected in choriodecidual tissue, with a statistically significant increase in STL pregnancies, except 15-Oxo-ETE and 13-HODE. 15-HETE, 15-Oxo-ETE, 15-HETrE and 13-HODE were measurable in the amnion and showed an increasing trend in ITL. 17-HDHA was only detected in the choriodecidua and was the most abundant HDHA-derived metabolite. 17-HDHA is the precursor of the D-series resolvins, which are known as anti-inflammatory mediators and enhance tissue repair (234,235). Trans-EKODE, a non-enzymatic derived mediator, was also detected in both sides of the membrane, but without significant change between groups.

Generally, the choriodecidua was more active in producing LOX-derived lipid mediators than the amnion. More specifically, the STL choriodecidua dominated the production of lipid mediators through 5-, 12- and 15-LOX enzymes. In the amnion, the STL group only showed significant elevations in 5-HETE (a uterine muscle contractor) and 12-HETE (a chemokine) through the LOX-dependent pathway (**Figure 5.6**).

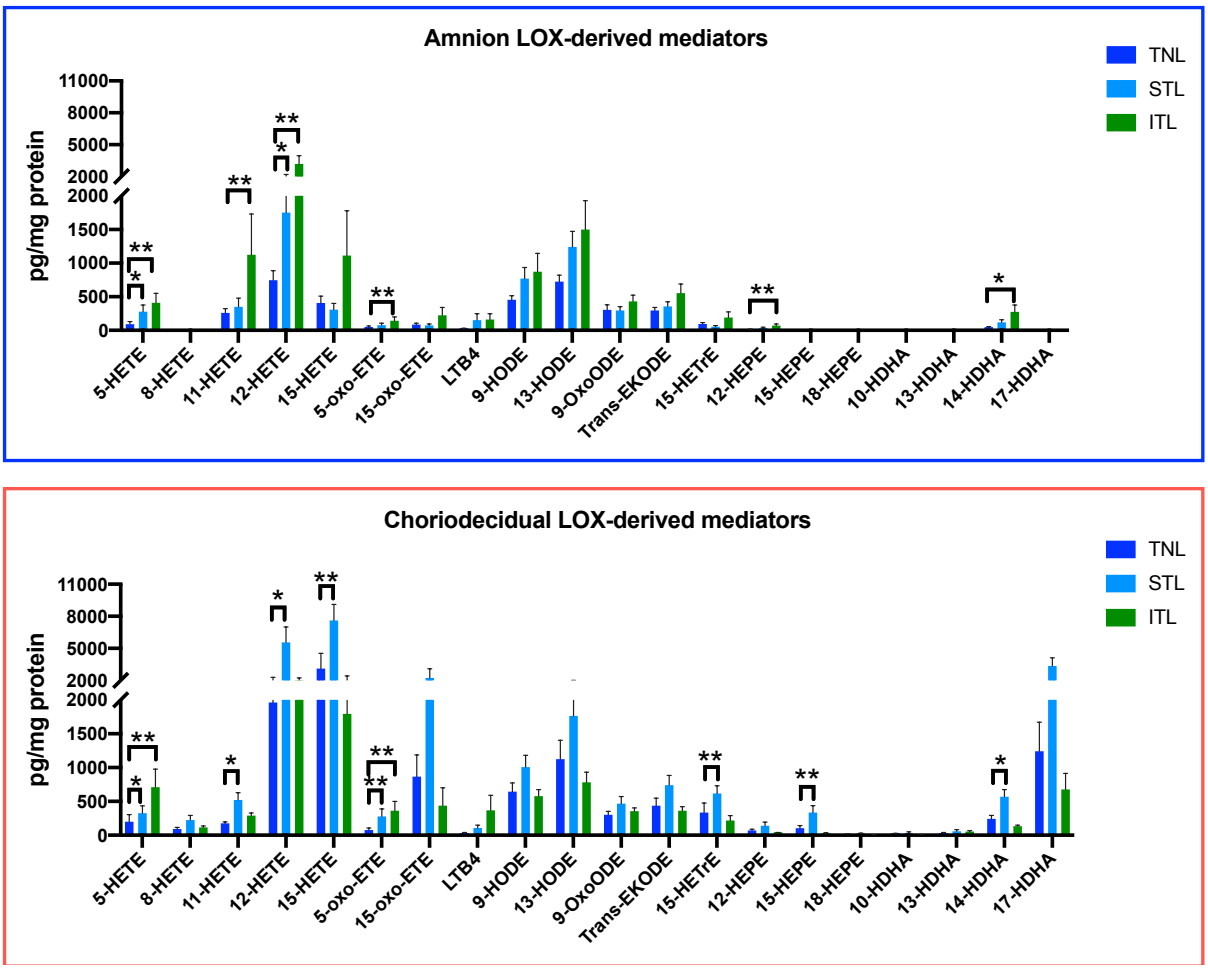


Figure 3.6. Term fetal membrane LOX-derived mediators. *Amnion (top, blue frame) and choriodecidua (bottom, red frame). Term not in labour (TNL), spontaneous term labour (STL) and induced term labour (ITL). Mean and SEM indicated. Statistical analysis = two-way ANOVA with Dunnett's multiple comparisons test, *p≤0.05, **p≤0.01.*

The CYP450 epoxygenase-derived metabolites of AA (EETs) and LA (EpOMEs) were the only lipid mediators detected in both membrane tissues (**Figure 3.7**). The CYP450 hydroxylase derivatives (e.g. 18-, 19- and 20-HETE) were all below the method detection limit. EETs are generally considered as anti-inflammatory mediators and play roles in vascular homeostasis. There is only limited knowledge of them in parturition (102). 14(15)-EET, an *in vitro* uterine relaxer, was quantifiable only in the choriodecidua and was raised significantly in the STL group (101). 5(6) EET, another *in vitro* uterine relaxant and vasodilator, was increased in both tissues under all labouring conditions, but this only reached significance in STL (101).

The other epoxygenase-AA derived mediators, 8(9) EET and 11(12) EET, were elevated in choriodecidual term conditions; and 8(9) EET in STL amnion (**Figure 3.7**). Moreover, in both

amnion and choriodecidua, the following LA-derived mediators were detected: 9(10)-and 12(13) EpOME and their soluble epoxide hydrolase (sEH) derived metabolites 12, 10-, and 12,13-DiHOME, with 12(13)EpOME production upregulated in STL (**Figure 3.7**). Soluble epoxide hydrolase (sEH) converts the epoxygenases to their corresponding diols, DHETs and DiHOMEs (236). For this study, the ratio of DiHOMEs:EpOMEs was used as an index of CYP/sEH activity. This ratio showed a trend for inhibition in STL in both membrane tissues, although this only reached significance within the amnion (**Figure 3.8)**.

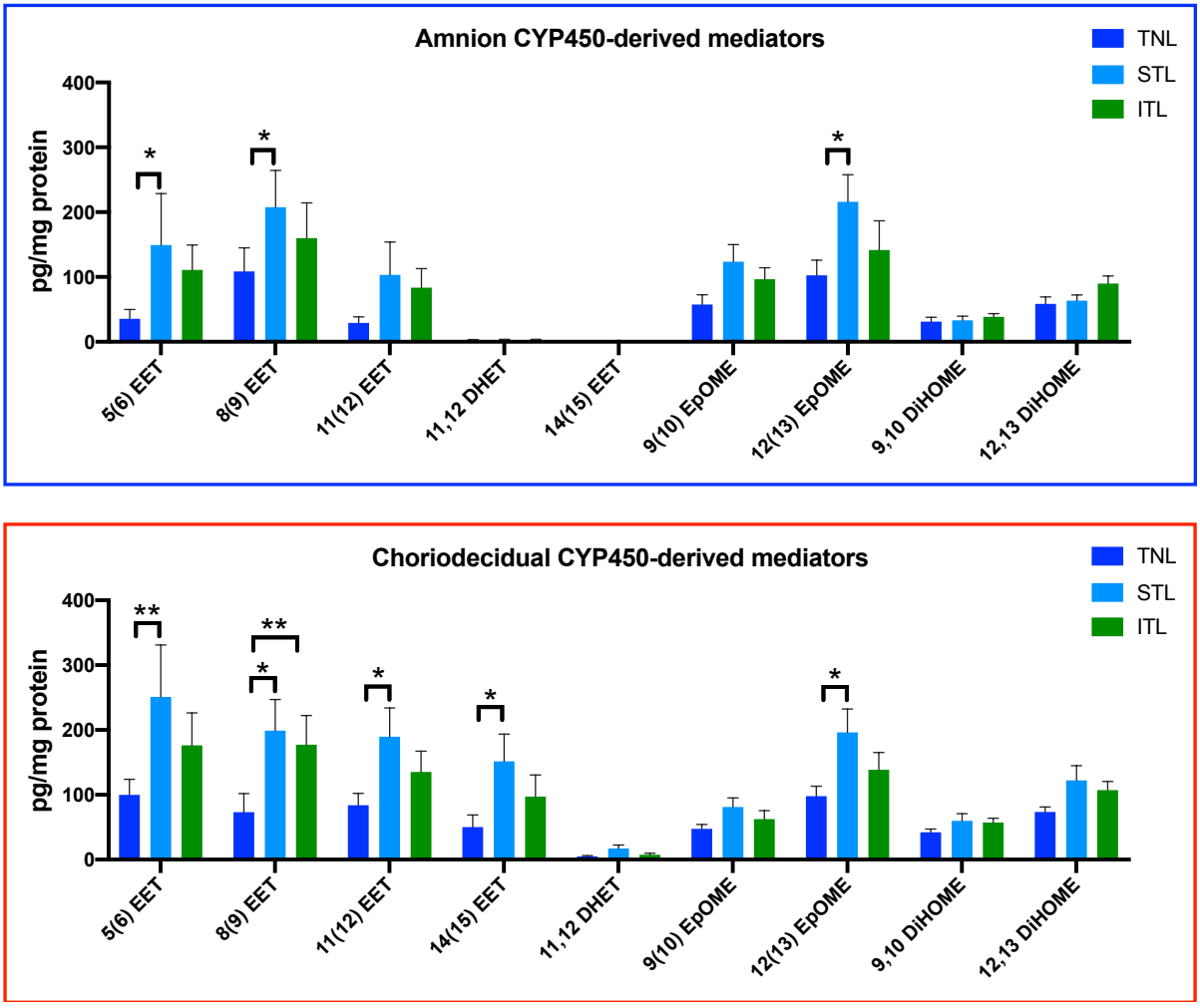


Figure 3.7 Term fetal membrane CYP450-derived mediators. Amnion (top, blue frame) and choriodecidua (bottom, red frame). *Term not in labour (TNL), spontaneous term labour (STL) and induced term labour (ITL). Mean and SEM indicated. Statistical analysis = two-way ANOVA with Dunnett's multiple comparisons test, *p≤0.05, **p≤0.01.*

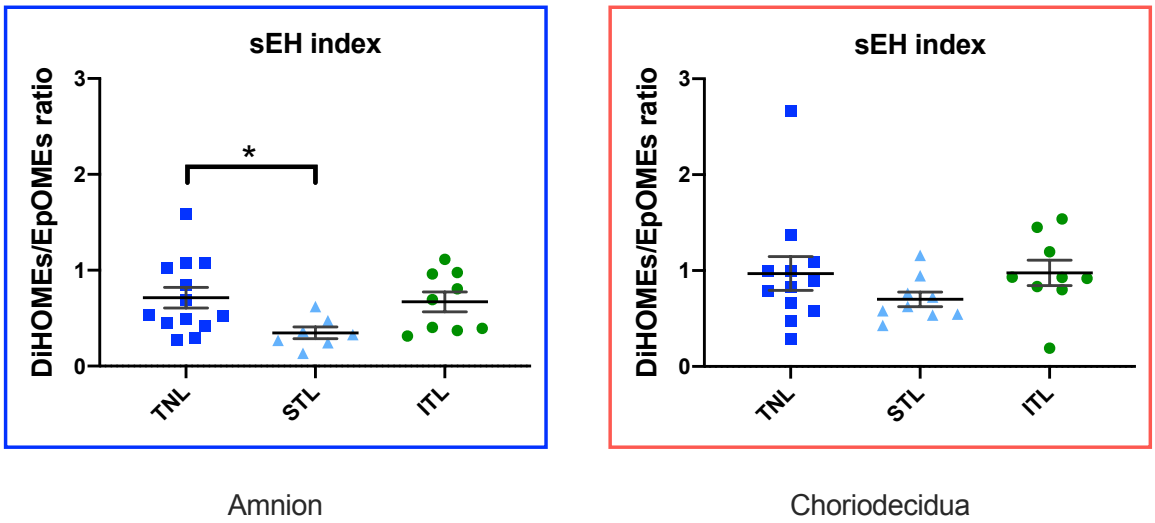


Figure 3.8 . Term fetal membrane CYP/sEH index. *Amnion (left side, blue frame) and choriodecidua (right side, red frame). Term not in labour (TNL), spontaneous term labour (STL) and induced term labour (ITL). Mean and SEM indicated. Statistical analysis = one-way ANOVA with Dunnett's multiple comparisons test, *p≤0.05, **p≤0.01.*

In concluding this part of the study, both amnion and choriodecidua are actively producing hydroxy fatty acids following the initiation of labour. For the induced labour group (ITL), this production was greatest in the amnion, whilst in spontaneous labour (STL) this applied to equally to the choriodecidua. Of all detected hydroxy fatty acids, HETEs and HODEs were most abundant throughout. 5-HETE, a uterotonic mediator, was actively produced in labour in both sides of the fetal membrane, and the chemotactic lipidome, 12-HETE, was significantly increased under spontaneous labouring conditions. The 12-LOX-derived meditators showed an increasing trend in labour in the amnion, regardless of induction. Moreover, both 12- and 15-LOX-derived meditators were elevated in the choriodecidua in spontaneous labour only. The epoxides and diols EETs and EpOMEs were elevated in labour, but their production was higher in spontaneous parturition over labour induction.

3.4.1.3. COX-derived lipid mediators of the preterm fetal membranes

Alongside the spontaneous term arm of the study, a preterm arm looked to profile prostanoids in amnion and choriodecidual tissues, but this time after labour before 37 weeks gestation. The preterm amnion showed a similar total COX-derived mediator level to the other term groups (**Figure 3.9**). However, the total COX-derived metabolites are shown higher in the choriodecidua in this preterm group (PTL). Although sample numbers were low (n=5), the PTL cohort produced comparable prostanoids to that of the STL group.

In amnion and choriodecidual tissues, AA-derived metabolites were the dominant prostanoids, followed by DGLA-derived mediators (**Figure 3.10**). Also, EPA-derived metabolites were below detection in most cases. Of the three FA-derived prostanoids in the amnion, all showed similar patterns between PTL and term pregnancy groups (TNL and STL). In preterm choriodecidual tissues, the AA-derived metabolites were significantly higher than TNL, whilst the DGLA-derived prostanoids were similar between pregnancies. Also, both amnion and choriodecidua produced EPA-mediators at trace amounts.

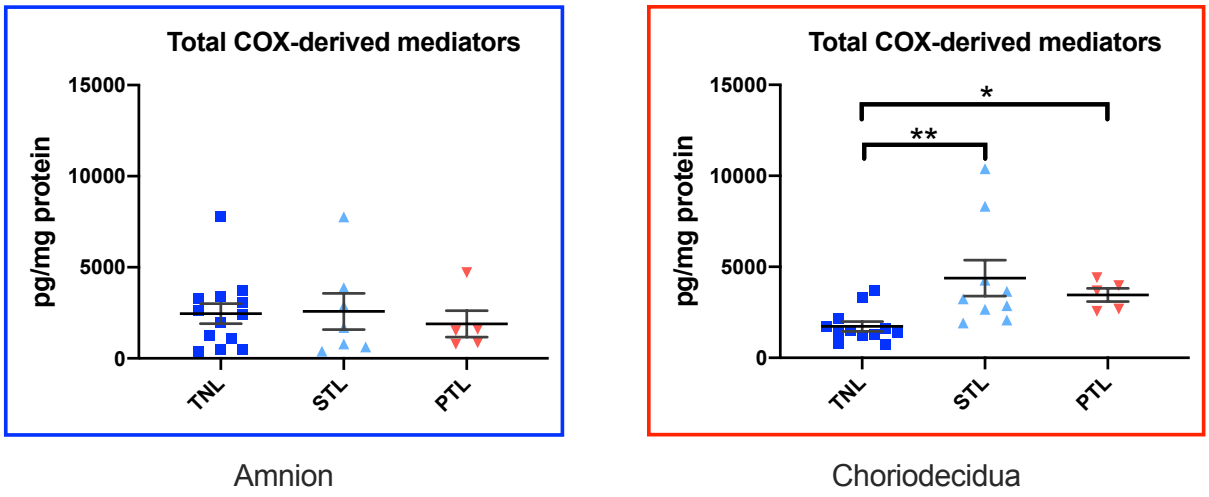


Figure 3.9. Preterm fetal membrane total COX-derived mediators. Amnion (left side, blue frame) and choriodecidua (right side, red frame). *Term not in labour (TNL), spontaneous term labour (STL) and preterm labour (PTL). Mean and SEM indicated. Statistical analysis = one-way ANOVA with Dunnett's multiple comparisons test, *p≤0.05, **p≤0.01.*

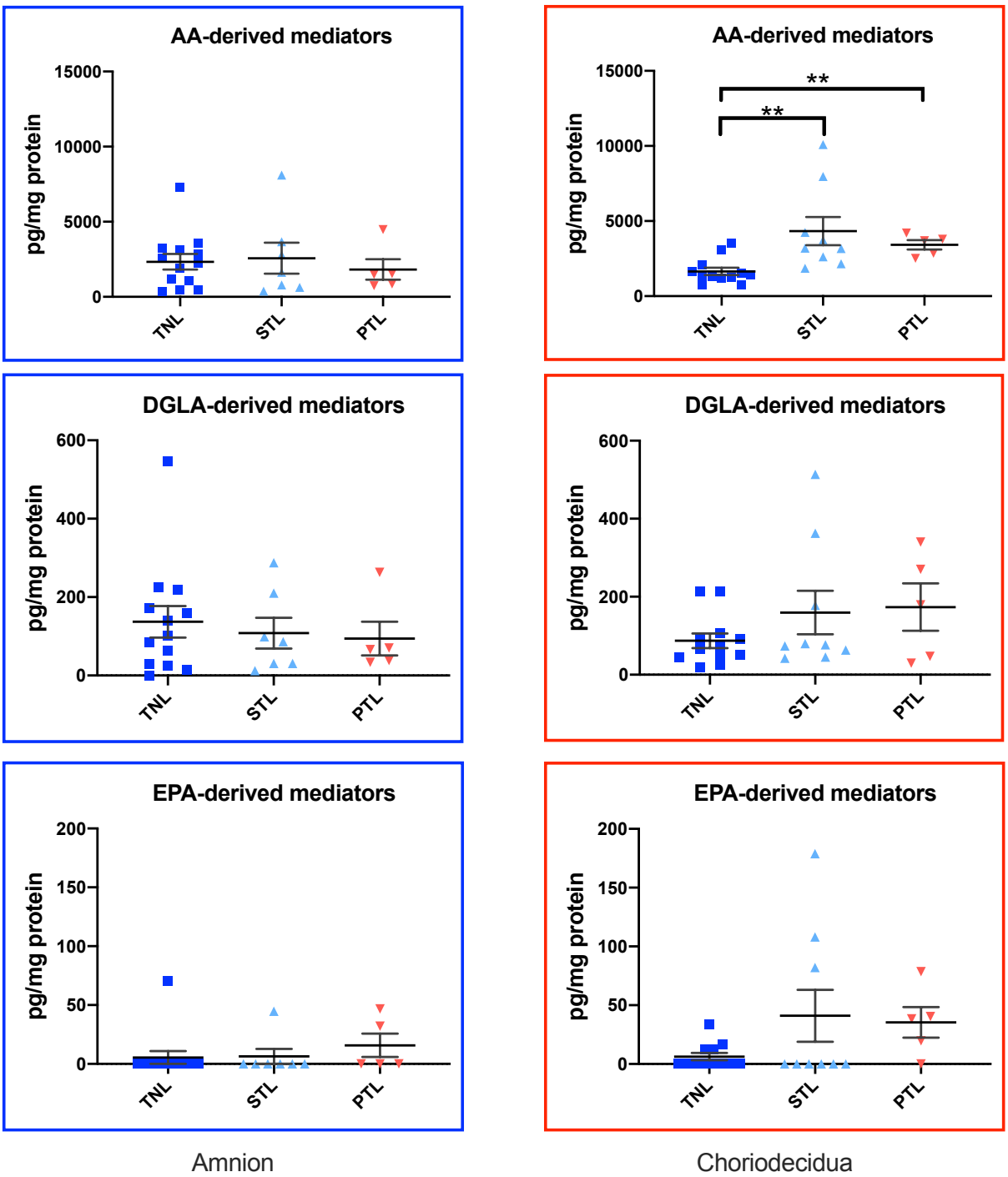
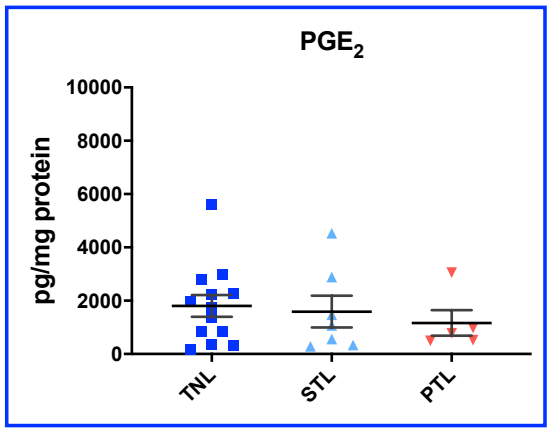


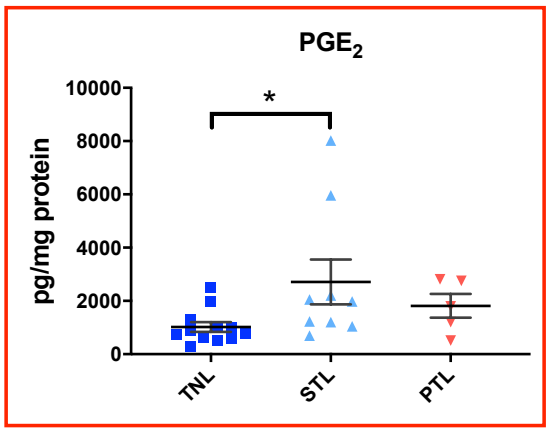
Figure 3.10. Preterm fetal membrane total AA-, DGLA-, and EPA-derived mediators. Amnion (left side, blue frame) and choriodecidua (right side, red frame). *Term not in labour (TNL), spontaneous term labour (STL) and preterm labour (PTL). Mean and SEM indicated. Statistical analysis = one-way ANOVA with Dunnett's multiple comparisons test, *p≤0.05, **p≤0.01.*

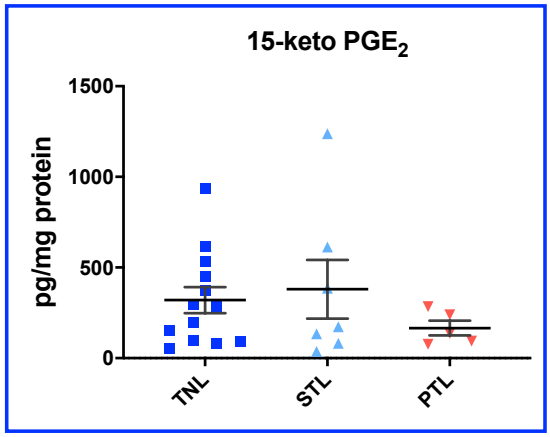
A detailed profile of each prostanoid is presented in **Figure 3.11**. PGE_2 was the most abundant prostanoid in the PTL group, with greater levels in choriodecidua than amnion (1166±481 vs 1815±445, pg/mg protein, respectively). PGE_2 values were similar in amnion and choriodecidua in PTL compared to TNL.

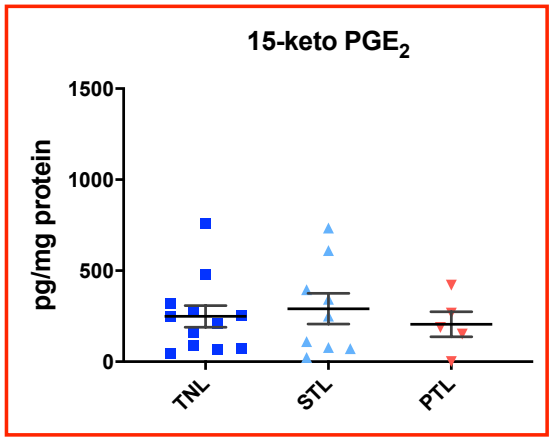
TXA_2 was the second most common prostanoid in PTL, found higher in both choriodecidua and amnion. Although $PGF_{2\alpha}$ showed a trend for increase in both fetal tissues in PTL, this only reached significance in the choriodecidua (**Figure 3.11**). PGE_1 and other DGLA-derived prostanoids were comparable between PTL and TNL, although with an elevated pattern in the choriodecidua.

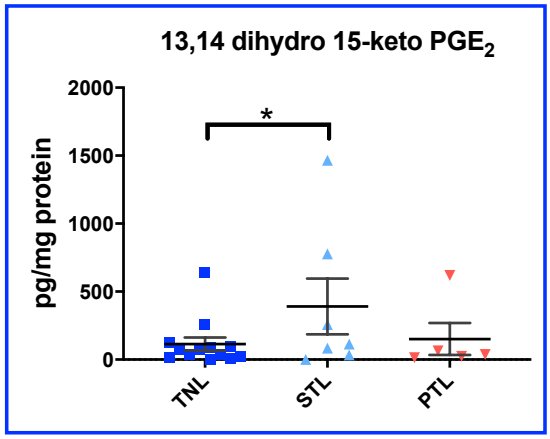
Arguably, the PTL group actively produced more prostanoids in the choriodecidua than the amnion. Of these, AA-derived mediators were the most prevalent on both sides of the fetal membranes. PTL was characterised by higher TXA_2 and $PGF_{2\alpha}$ than term non-labouring controls (TNL).

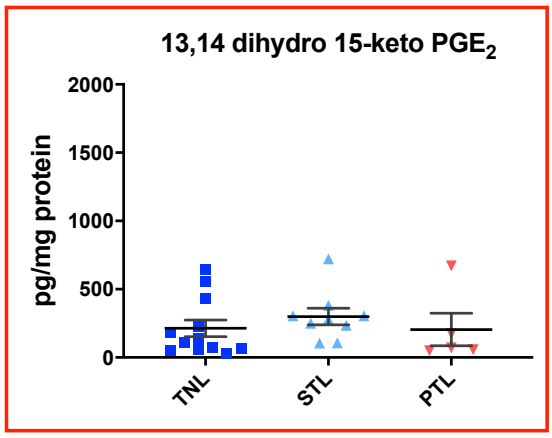


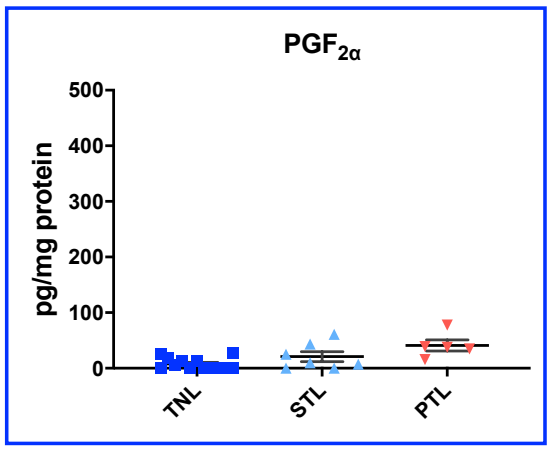


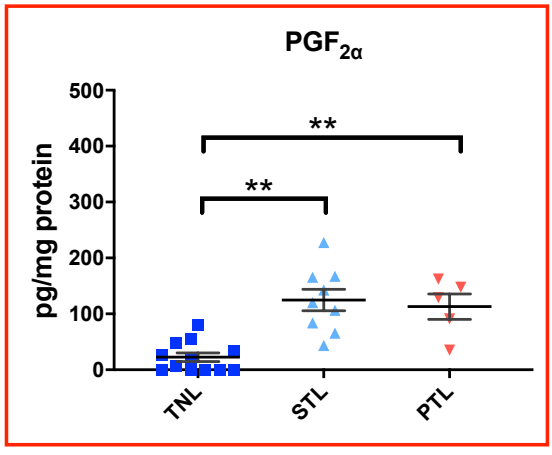






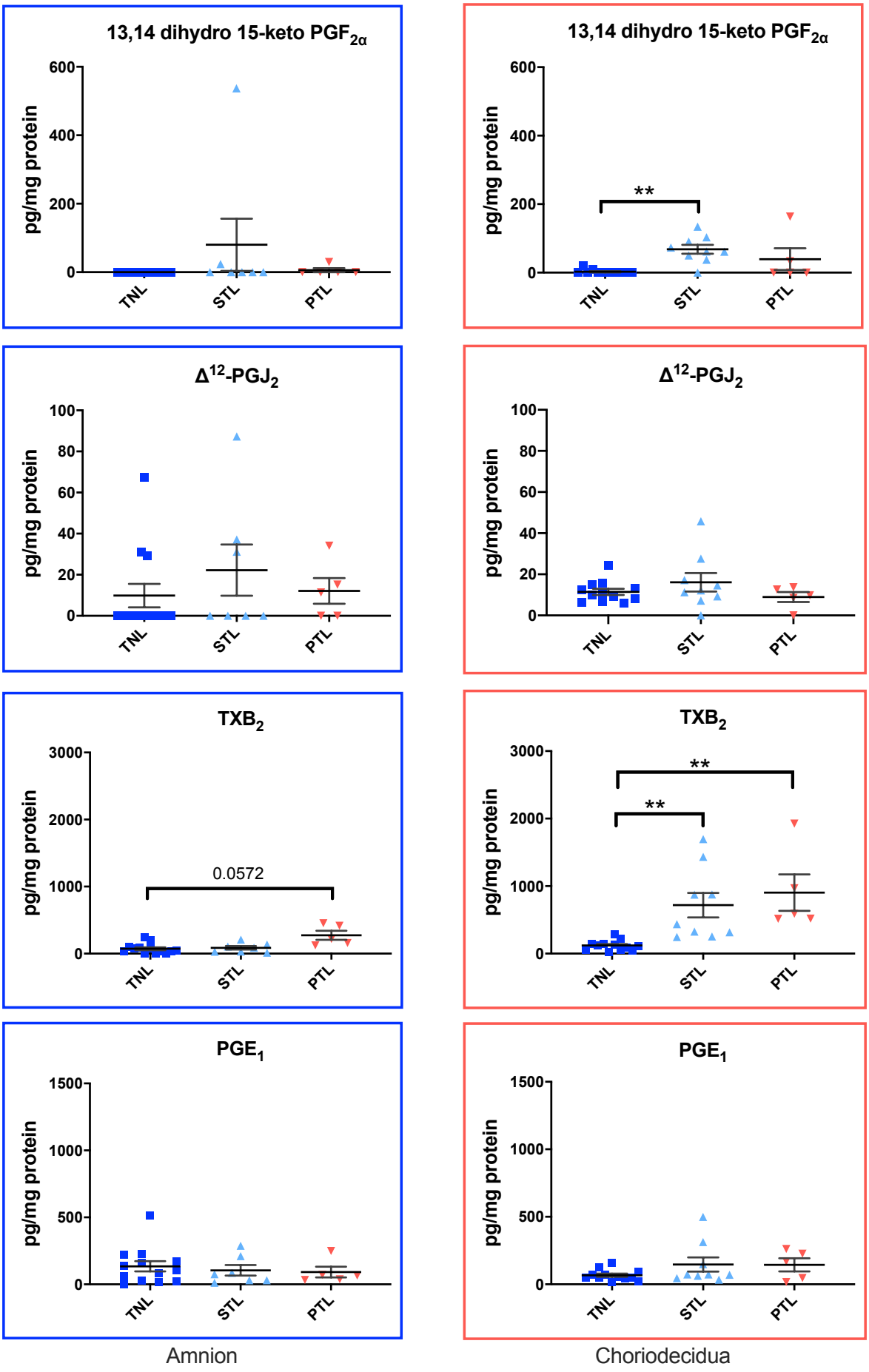






Amnion

Choriodecidua


13,14 dihydro 15-keto PGF2α
pg/mg protein
600
400
200
0
TNL
STL
PTL
13,14 dihydro 15-keto PGF2α
**
Δ12-PGJ2
100
80
60
40
20
TXB2
3000
2000
1000
0.0572
PGE1
1500
500
Amnion
Choriodecidua

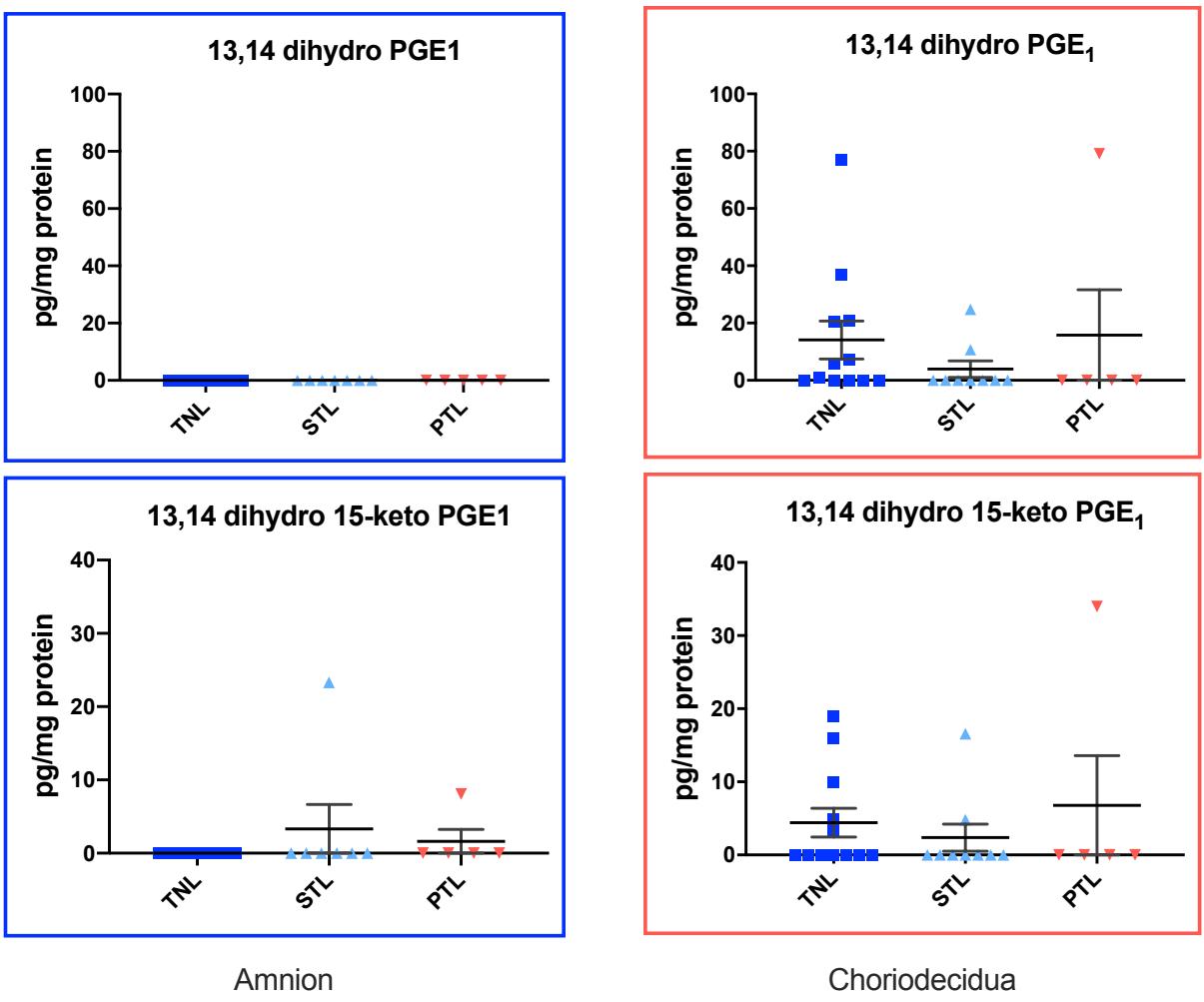


Figure 3.11. Preterm fetal membrane total COX-derived species. Amnion (left side, blue frame) and choriodecidua (right side, red frame). *Term not in labour (TNL), spontaneous term labour (STL) and preterm labour (PTL). Mean and SEM indicated. Statistical analysis = two-way ANOVA with Dunnett's multiple comparisons test, *p≤0.05, **p≤0.01.*

3.4.1.4. Hydroxy fatty acid profiles of preterm fetal membranes

LOX- and CYP450- derived mediators were profiled in PTL along with STL and compared to TNL (**Figure 3.12**). LOX mediators were higher than CYP450 metabolites in both membrane tissues. In the amnion, PTL showed comparable concentrations to TNL for LOX- and CYP-450 mediators, but with a non-significant trend in production equivalent to STL (**Figure 3.12**). In choriodecidua, the PTL showed an increased trend for the metabolites of both pathways but the differences failed to reach significance.

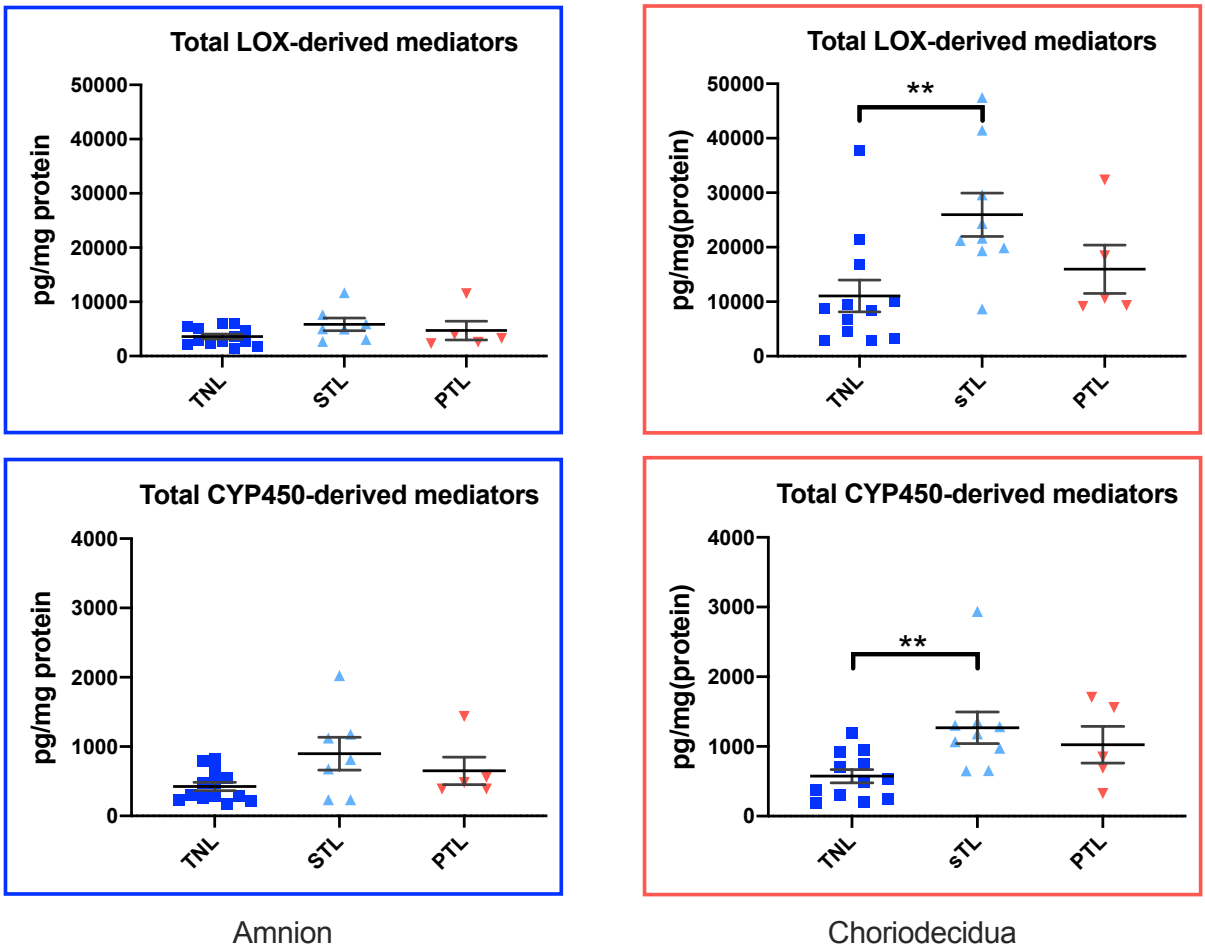
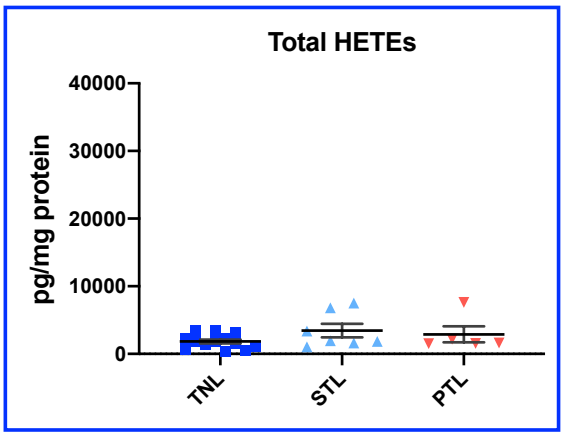
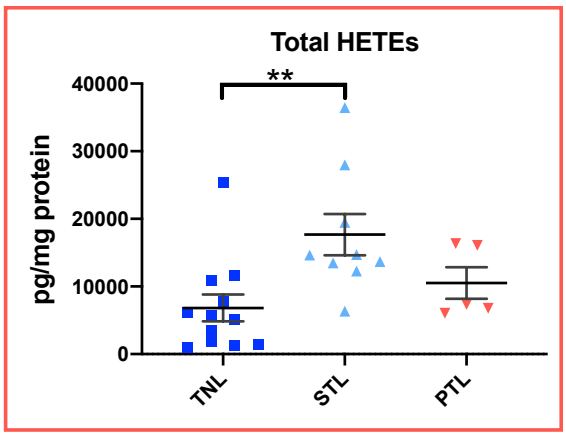
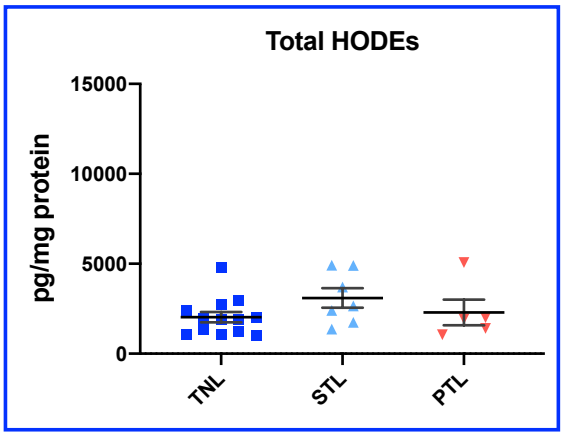


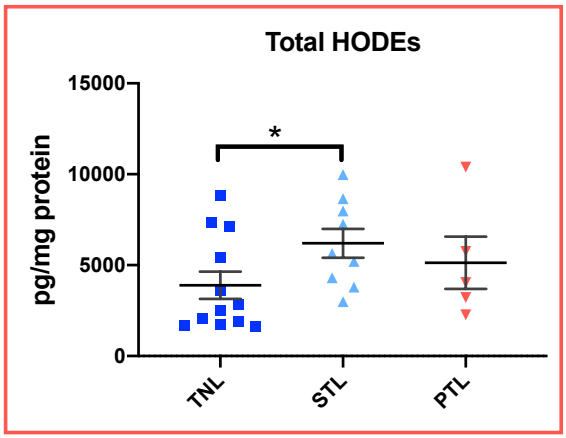
Figure 3.12. Preterm fetal membrane total LOX-, CYP450-derived mediators. *Amnion (left side, blue frame) and choriodecidua (right side, red frame). Term not in labour (TNL), spontaneous term labour (STL) and preterm labour (PTL). Mean and SEM indicated. Statistical analysis = one-way ANOVA with Dunnett's multiple comparisons test, *p≤0.05, **p≤0.01.*

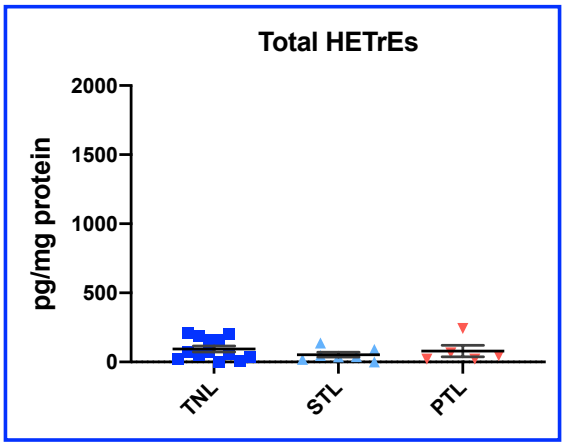
For total hydroxy fatty acids, there was no change in the amnion for the five hydroxy PUFA-derived lipidomes between PTL and TNL (**Figure 3.13**). In the choriodecidua, total HETEs and HODEs were the highest detectable hydroxy fatty acids, and both showed an increasing trend in PTL over TNL. All other hydroxy fatty acids, HETrEs, HDHAs and HEPE, were similar to TNL (**Figure 3.13**).

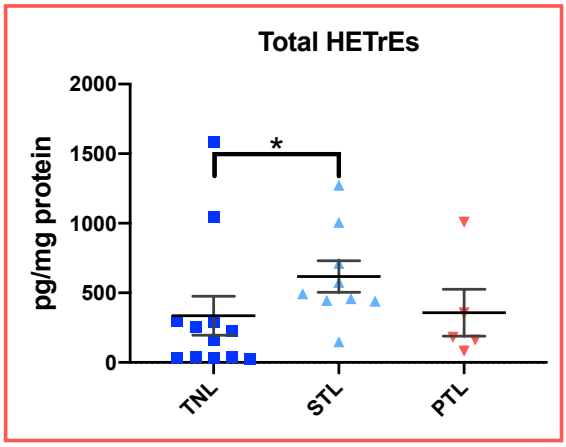


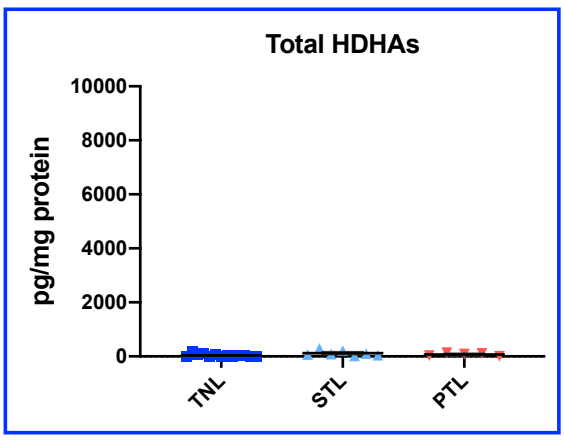


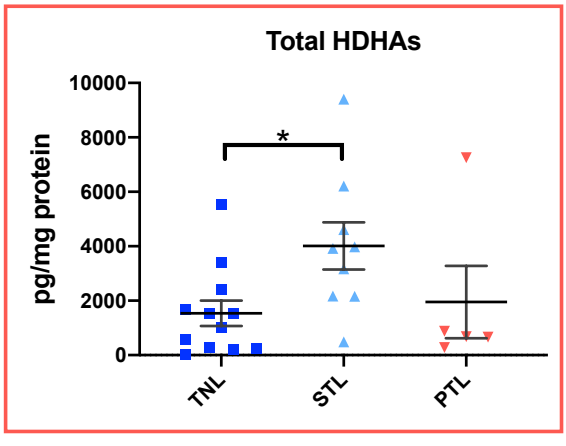












Amnion

Choriodecidua

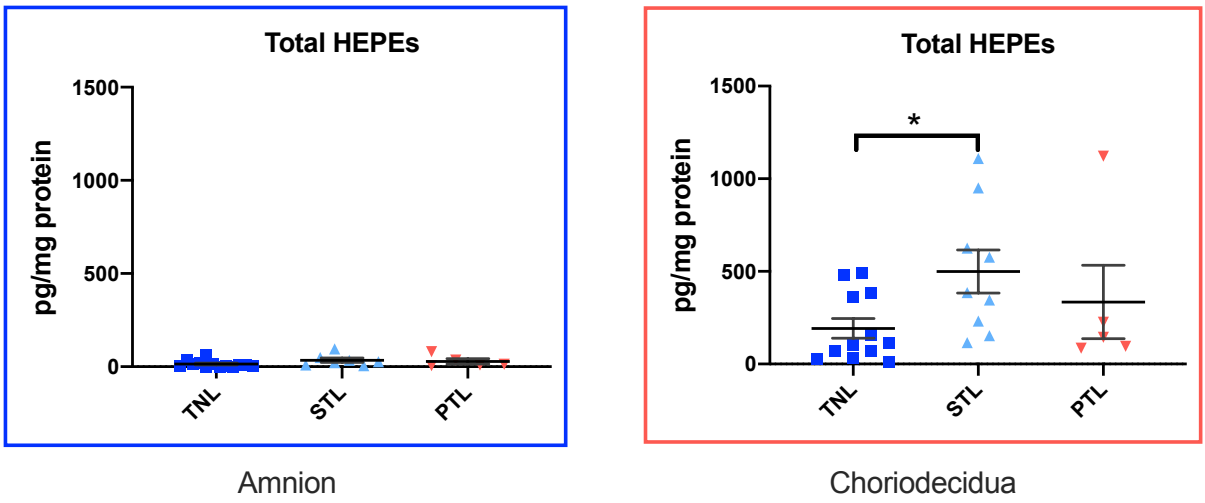


Figure 3.13. Preterm fetal membrane total HETEs, HODEs, HETrEs, HDHAs, and HEPEs mediators. Amnion (left side, blue frame) and choriodecidua (right side, red frame). *Term not in labour (TNL), spontaneous term labour (STL) and preterm labour (PTL). Mean and SEM indicated. Statistical analysis = one-way ANOVA with Dunnett's multiple comparisons test, *p≤0.05, **p≤0.01.*

The PTL group showed increased production of 12-LOX mediators, with 12-HETE the most abundant hydroxy fatty acid in both amnion and choriodecidua (**Figure 3.14**). 12-HETE production was significantly raised in the choriodecidua in PTL, with an increased trend in the amnion. The other 12-LOX mediators; 12-HEPE and 14HDHA, showed a similar pattern of increase, but this failed to reach significance. The remaining LOX-meditators were equal between PTL and TNL (**Figure 3.14**).

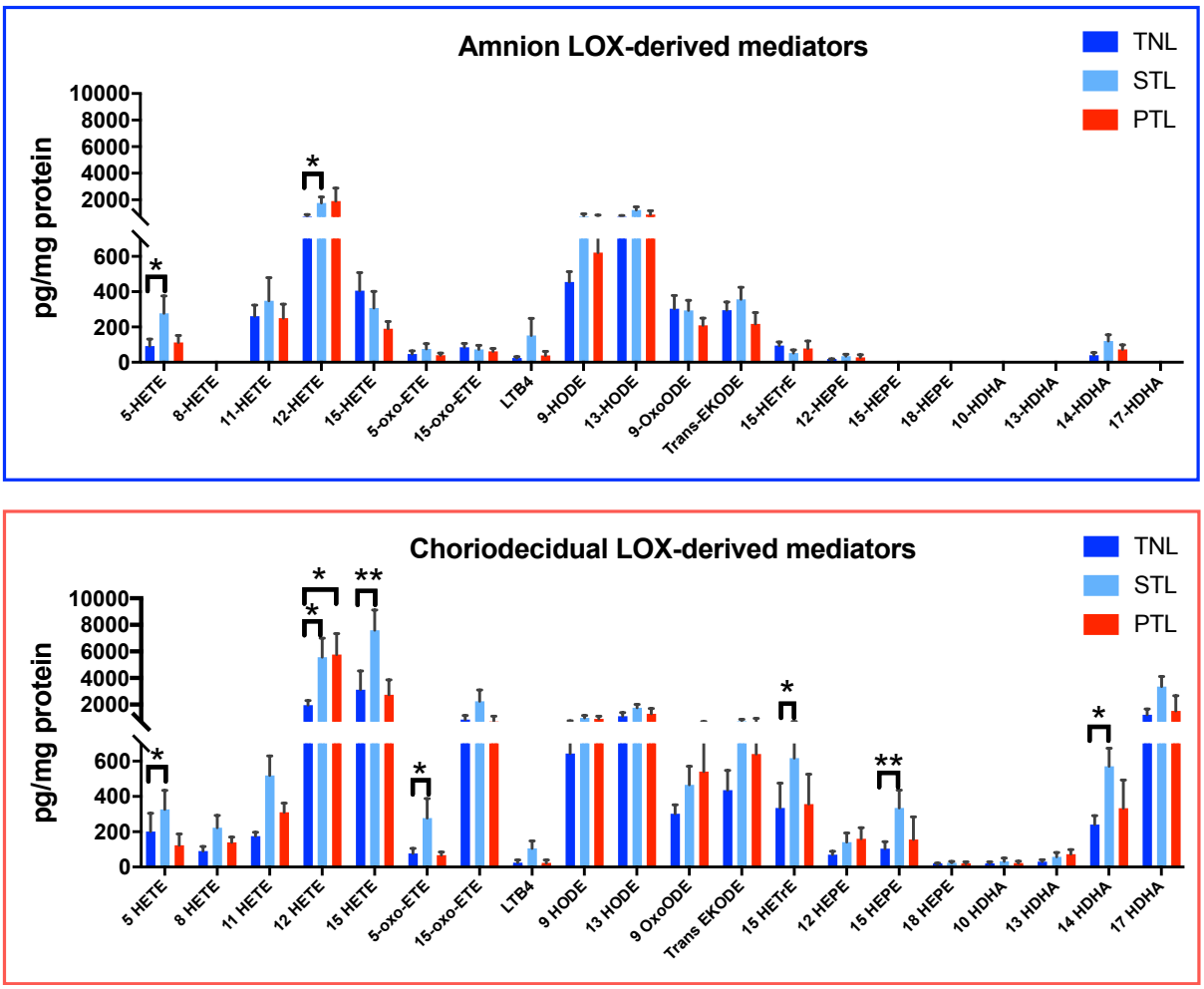


Figure 3.14. Preterm fetal membrane LOX-derived mediators. Amnion (top, blue frame) and choriodecidua (bottom, red frame). *Term not in labour (TNL), spontaneous term labour (STL) and preterm labour (PTL). Mean and SEM indicated. Statistical analysis = two-way ANOVA with Dunnett's multiple comparisons test, *p≤0.05, **p≤0.01.*

In general, the PTL group showed no significant changes in amnion and choriodecidua in comparison to the TNL group, although a pattern for increased production of the CYP450-derived mediators, EETs and EpOMEs, was noted (**Figure 3.15**). As observed in term pregnancies, the CYP/sEH ratio was unresponsive in the choriodecidua, but was significantly reduced in amnion in the PTL group verses TNL (**Figure 3.16**).

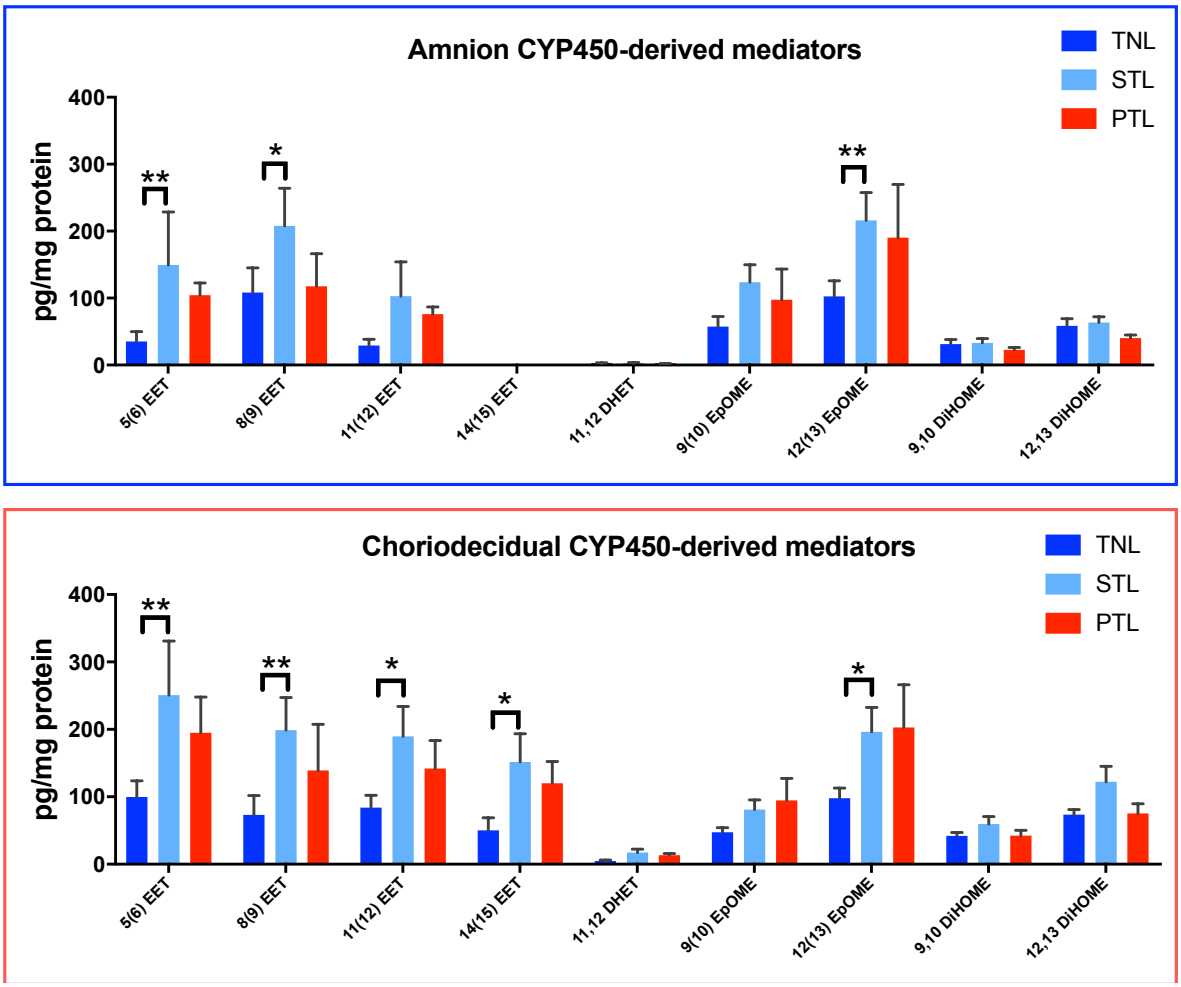


Figure 3.15. Preterm fetal membrane CYP450-derived mediators. Amnion (top, blue frame) and choriodecidua (bottom, red frame). *Term not in labour (TNL), spontaneous term labour (STL) and preterm labour (PTL). Mean and SEM indicated. Statistical analysis = two-way ANOVA with Dunnett's multiple comparisons test, *p≤0.05, **p≤0.01.*

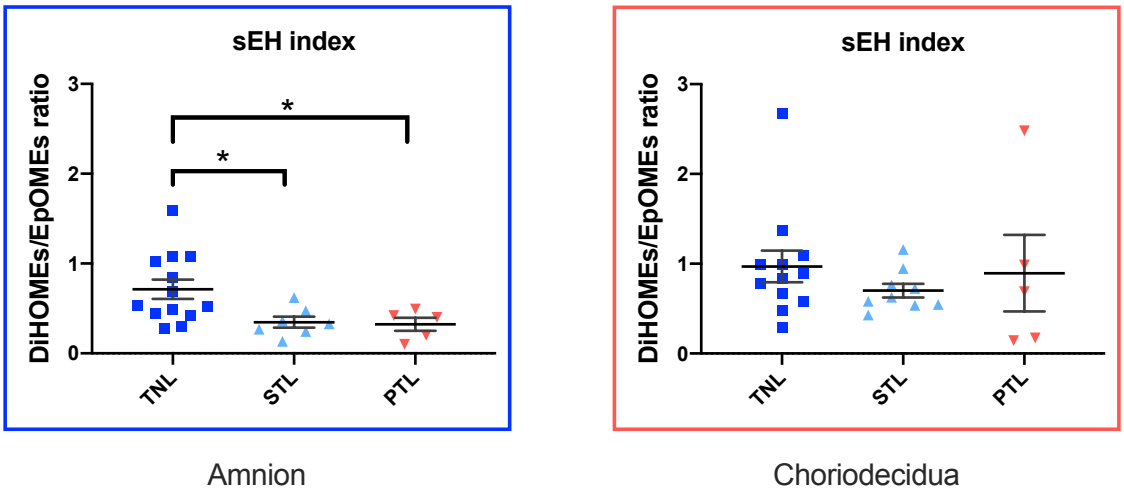


Figure 3.16. Preterm fetal membrane CYP/sEH index. Amnion (left side, blue frame) and choriodecidua (right side, red frame). *Term not in labour (TNL), spontaneous term labour (STL) and preterm labour (PTL). Mean and SEM indicated. Statistical analysis = one-way ANOVA with Dunnett's multiple comparisons test, *p≤0.05, **p≤0.01.*

In summary, the PTL group showed a trend towards increased production of hydroxy fatty acids compared to term pregnancies not-in-labour (TNL). Of the species considered, HETEs and HODEs were dominant in PTL, with greater production in choriodecidua over amnion. The 12-LOX-derived lipidomes showed an increasing trend in both tissues in PTL, with 12-HETE, a chemokine, the highest detected hydroxy fatty acid throughout the fetal membranes. Interestingly, this was the only LOX mediator to be significantly higher in the PTL choriodecidua. Also, the CYP-derived mediator level was comparable to the TNL group.

3.4.2. Endocannabinoids, N-acyl ethanolamines and monoacylglycerols in fetal membranes

Amnion and choriodecidua profiling of endocannabinoids was performed with UPLC/ESI-MS/MS (**section 2.3**). The analysis included the classical endocannabinoids, AEA and 2-AG, and a series of endocannabinoid-like N-acyl ethanolamines and monoacylglycerols. The data were organised into two study themes: term and preterm observations, addressing differences elicited by labour *per se*, and similarities or irregularities during preterm, pathological onset. The term study included STL (n=8 and 11, amnion and choriodecidua, respectively) and ITL (n=9 and 9) compared to TNL (n= 12 and 13). The preterm study incorporated a preterm group PTL (n=5 and 6) with STL and TNL for comparison.

3.4.2.1. Endocannabinoid, N-acyl ethanolamine and monoacylglycerol profiles of term fetal membranes

The profiles of endocannabinoids found produced by term fetal membrane tissues are presented in **Figure 3.17**. 2-AG was the most abundant species in both amnion and choriodecidua at term, with higher levels in the former. 2-AG was not affected by labour in the amnion, either in STL or ITL, but was raised in the choriodecidua in ITL. AEA concentrations were under the limit of detection in most amnion tissues, but where more prevalent in choriodecidua in STL vs TNL.

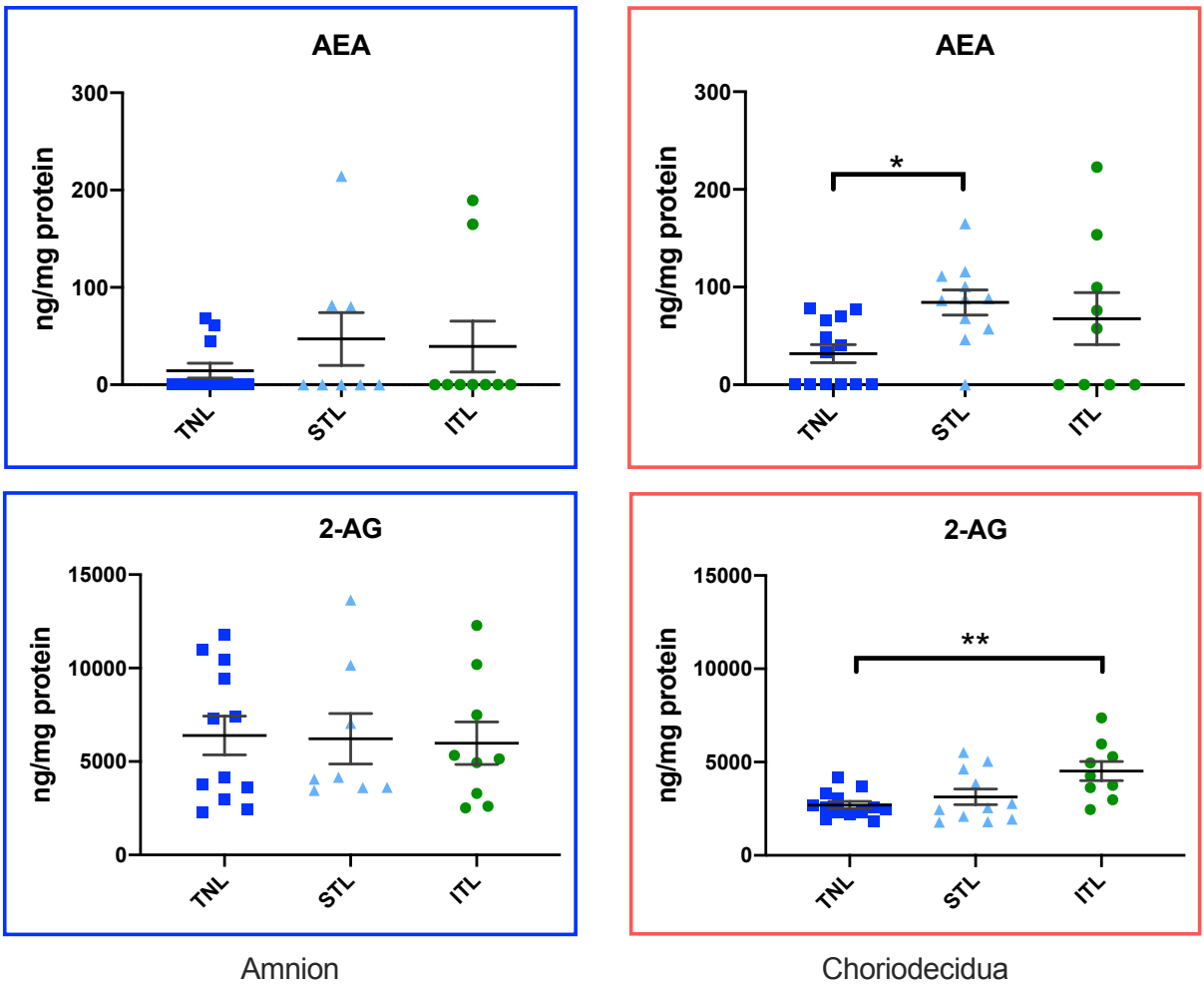


Figure 3.17. Term fetal membrane classical endocannabinoids (AEA and 2-AG). Amnion (left side, blue frame) and choriodecidua (right side, red frame). *Term not in labour (TNL), spontaneous term labour (STL) and induced term labour (ITL). Mean and SEM indicated. Statistical analysis = one-way ANOVA with Dunnett's multiple comparisons test, *p≤0.05, **p≤0.01.*

Figure 3.18 shows the profiles of NAEs found in term fetal membrane tissues. In both amnion and choriodecidua, the STL group showed increased production of LEA over TNL, with DHEA raised non-significantly in the choriodecidua under labouring conditions (STL and ITL); but conversely below detection in the amnion. PEA, an anti-inflammatory and pain relief mediator (237,238), was the most prominent N-acylethanolamine produced in both tissues. In the amnion, in both labouring groups, PEA was raised, but this only reached statistical significance in ITL. Likewise, this trend was replicated in the choriodecidua. OAE showed a pattern of increased production in both membrane tissues in labouring groups, but this only reached statistical significance in the ITL amnion (**Figure 3.18**).

The following N-acylethanolamines; MEA, PDEA, POEA, HEA, and VEA were detected in both sides of the fetal membrane tissues with varying patterns between labouring groups (**Figure 3.18**). MEA did not show any change in neither group nor tissues. PDEA in amnion tissues was unchanged between study groups; however, its level increased significantly in choriodecidual STL tissues. POEA were detected at lower levels in amnion and choriodecidua, but were raised significantly in both labouring conditions. Palmitoleic acid (the precursor of POEA) is referred to as a "lipokine" and showed an anti-inflammatory action, which could have a role to balance the intrauterine inflammatory environment (239). In amnion tissues, HEA was upregulated in the ITL group, while VEA failed to show a significant difference in term labour. Nevertheless, in choriodecidua, HEA and VEA were significantly increased in both labour groups (**Figure 3.18**).

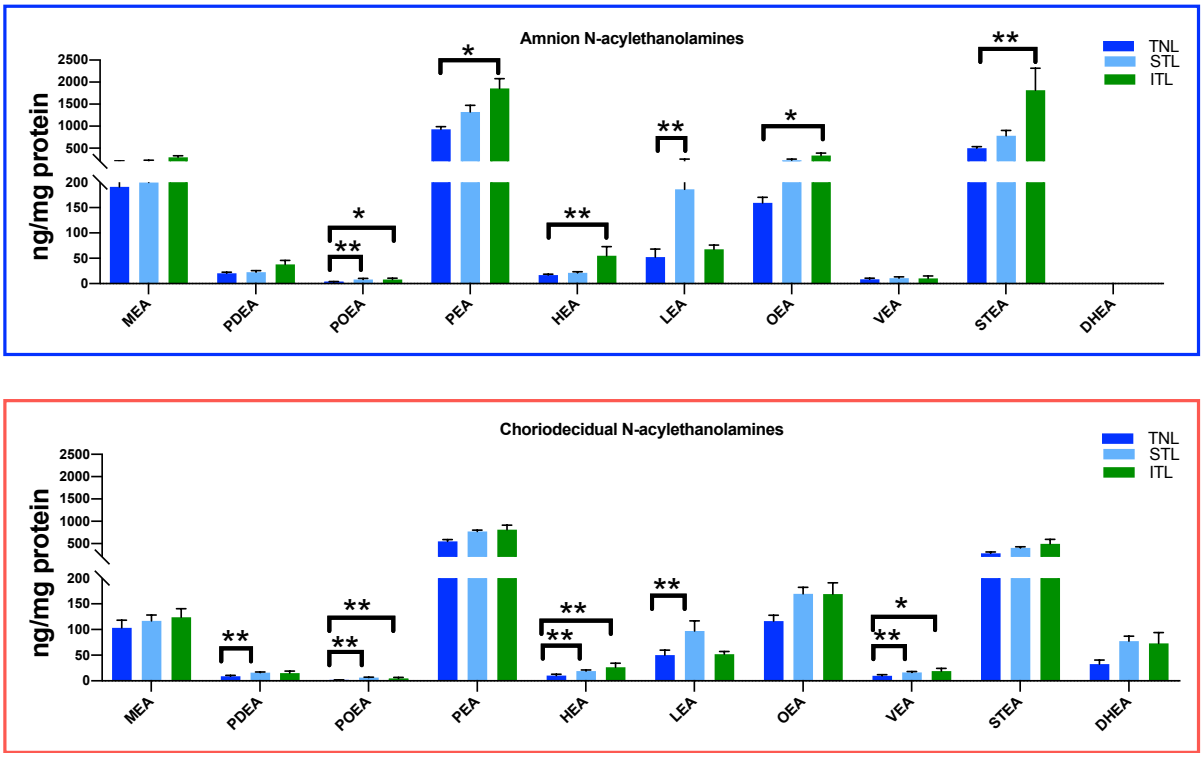


Figure 3.18. Term fetal membrane N-acylethanolamines. Amnion (top, blue frame) and choriodecidua (bottom, red frame). *Term not in labour (TNL), spontaneous term labour (STL) and induced term labour (ITL). Mean and SEM indicated. Statistical analysis = two-way ANOVA with Dunnett's multiple comparisons test, *p≤0.05, **p≤0.01.*

MAGs are another class of lipids structurally similar to 2-AG, but again have physiological functions not fully explored in pregnancy and other situations**. Figure 3.19** shows palmitoyl glycerol (PAG) and stearoyl glycerol (STG) were the most abundant MAGs detected in the fetal

membranes (**Figure 3.19**). Also, both reached a statistically significant increase only in ITL, not STL, in amnion and choriodecidua compared to TNL controls. The choriodecidua also produced more LG and OG in STL over TNL. However, both these lipid mediators failed to show a significant change in amnion tissues (**Figure 3.19**).

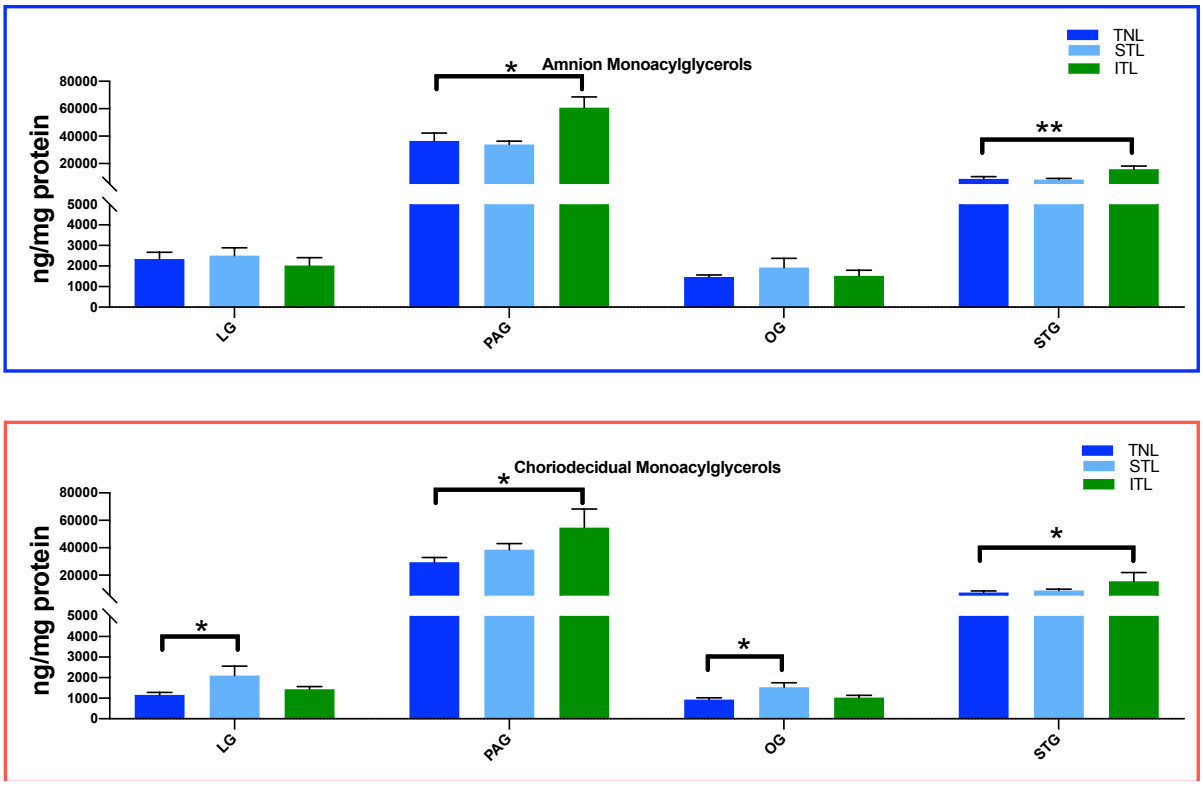


Figure 3.19. Term fetal membrane monoacylglycerols. Amnion (top, blue frame) and choriodecidua (bottom, red frame). *Term not in labour (TNL), spontaneous term labour (STL) and induced term labour (ITL). Mean and SEM indicated. Statistical analysis = two-way ANOVA with Dunnett's multiple comparisons test, *p≤0.05, **p≤0.01.*

To summarise, the profiles of AEA and 2-AG were different between amnion and choriodecidua. The amnion was not a major site of AEA production, as most samples had levels below the method detection limits. However, the choriodecidua did produce detectable concentrations of AEA, which were further increased during labouring at term. The amnion base-line for 2-AG was higher than choriodecidua, but this was not affected by labour. A similar pattern was observed for the N-acylethanolamines and monoacylglycerols found in both sides of the fetal membrane, with a trend towards increased production with term labour instigated.

3.4.2.2. Endocannabinoids, N-acyl ethanolamines and monoacylglycerols profiles of preterm fetal membranes

For this arm of the study, PTL (n=5 and 6, amnion and choriodecidua) and STL (n=8 and 11) patients were tested against TNL (n=12 and 13). The levels of AEA and 2AG found in preterm labour tissues are summarised in **Figure 3.20**; amnion showed a similar profile for AEA to that at term, with most samples having AEA levels below the method limit of detection. However, the preterm choriodecidual tissue produced detectable levels of AEA and 2AG, with a significant increase compared to STL. Similar to the term study, 2-AG was the most dominant classic endocannabinoid detected in both sides of the membrane. The preterm labour tissue produced higher 2-AG in the amnion than choriodecidual tissue, but again this failed to differ significantly from TNL. However, PTL choriodecidua 2-AG levels were markedly lower than TNL.

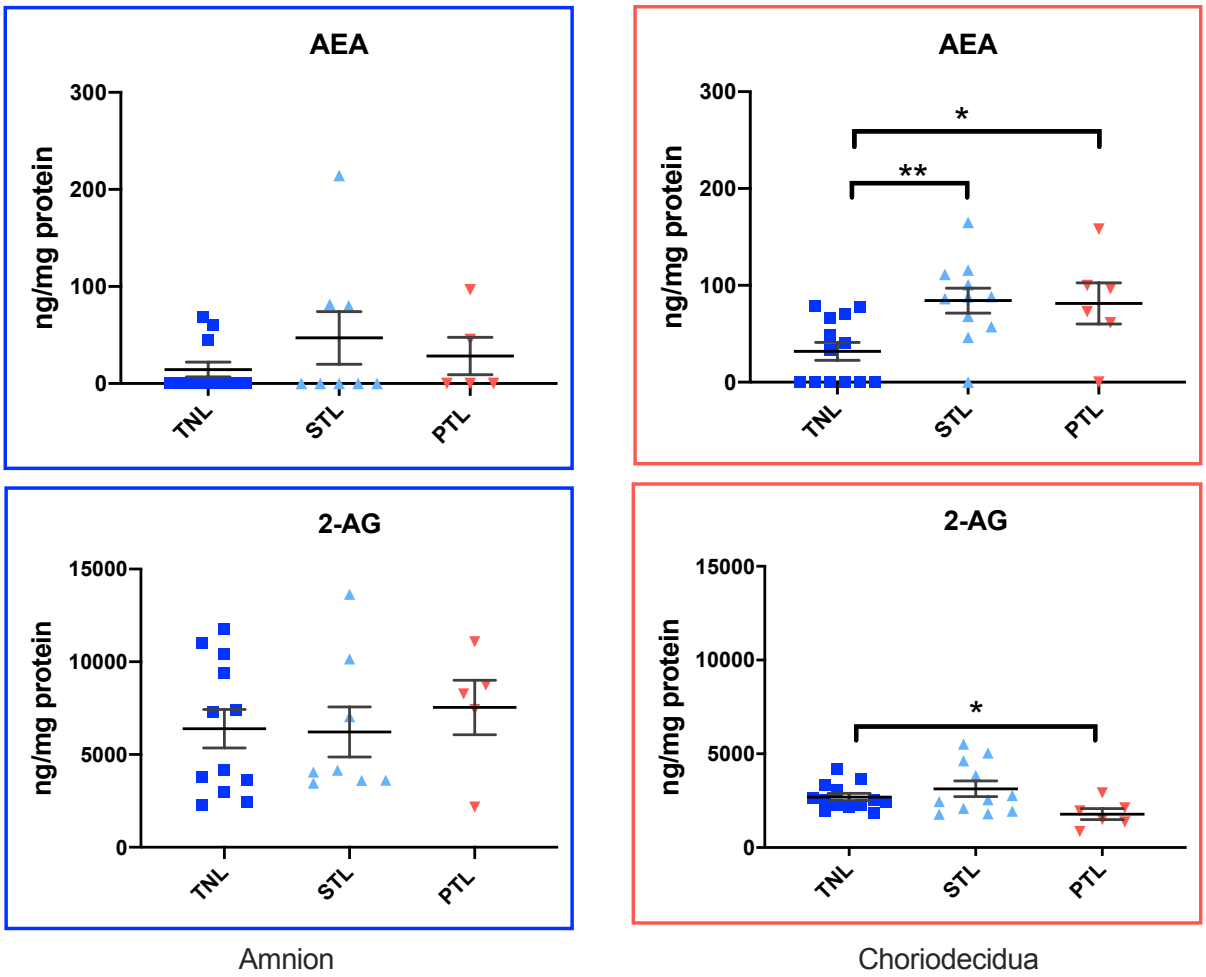


Figure 3.20. Preterm fetal membrane classical endocannabinoids. Amnion (left side, blue frame) and choriodecidua (right side, red frame). *Term not in labour (TNL), spontaneous term labour (STL) and preterm labour (PTL). Mean and SEM indicated. Statistical analysis = one-way ANOVA with Dunnett's multiple comparisons test, *p≤0.05, **p≤0.01.*

PEA and STEA were the highest N-acylethanolamines detected in amnion and choriodecidua (**Figure 3.21**). Both mediators showed an increasing trend in amnion and choriodecidua. Both tissues showed a significant upregulation of LEA in preterm tissues, even higher than that for STL. In the choriodecidua, DHEA was higher than the amnion, with POEA, HEA and VEA, all showing a significant increase with PTL (**Figure 3.21**).

Regarding monoacylglycerols, both tissues had similar profiles (**Figure 3.22**). PAG and STG were the dominant mediators detected. In amnion, these were similar between PTL and TNL. In the choriodecidua, only OG was increased.

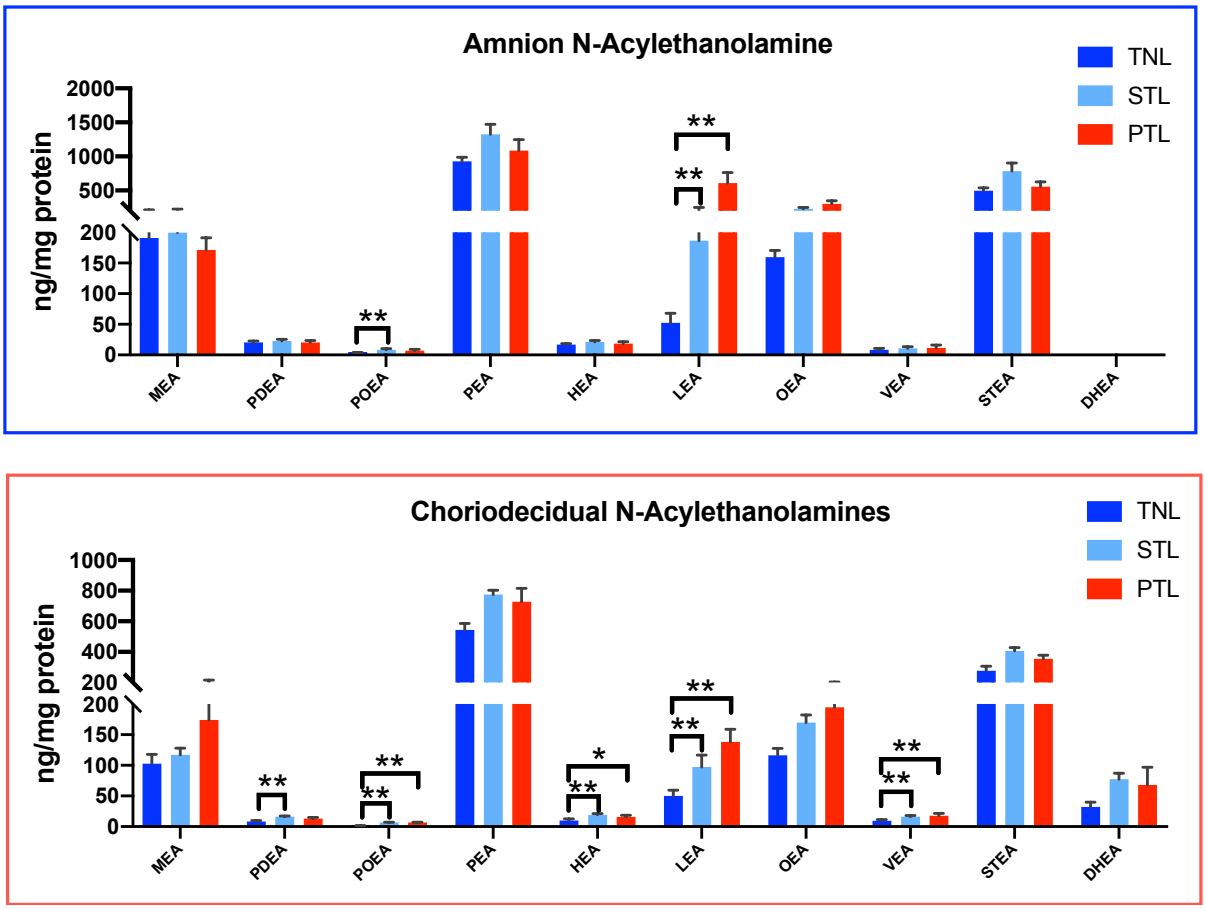


Figure 3.21. Preterm fetal membrane N-acylethanolamines. Amnion (top, blue frame) and choriodecidua (bottom, red frame). *Term not in labour (TNL), spontaneous term labour (STL) and preterm labour (PTL). Mean and SEM indicated. Statistical analysis = two-way ANOVA with Dunnett's multiple comparisons test, *p≤0.05, **p≤0.01.*

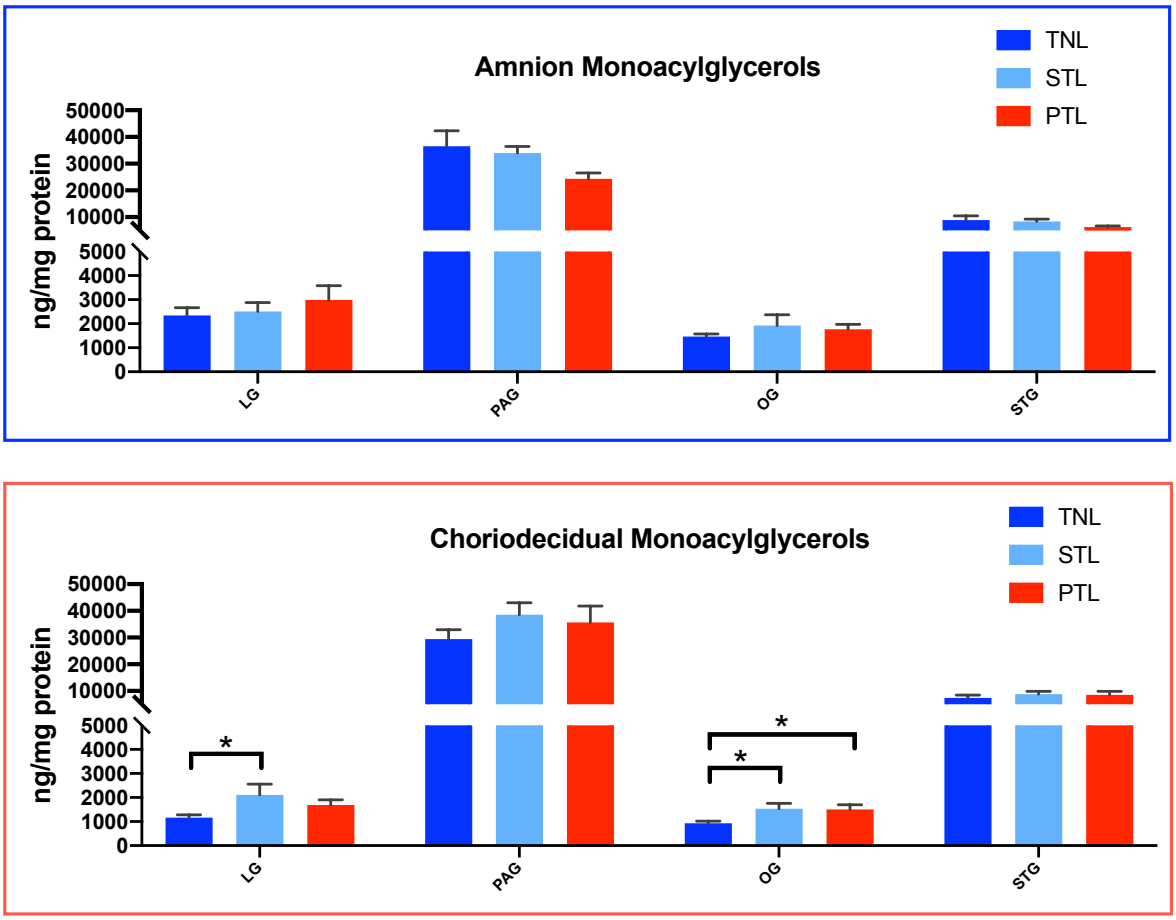


Figure 3.22. Preterm fetal membrane monoacylglycerols. Amnion (top, blue frame) and choriodecidua (bottom, red frame). *Term not in labour (TNL), spontaneous term labour (STL) and preterm labour (PTL). Mean and SEM indicated. Statistical analysis = two-way ANOVA with Dunnett's multiple comparisons test, *p≤0.05, **p≤0.01.*

To summarise, in preterm labour conditions AEA and 2-AG were produced at different concentrations. The amnion was not a source of AEA and 2-AG production was similar to term conditions. However, the preterm choriodecidua was able to produce AEA and 2-AG at detectable levels and AEA showed an increase in production while 2-AG was lower than the TNL group. PEA and SEA, as well as PAG and STG, were the most abundant lipid species detected in the preterm tissues. LEA was raised in amnion and choriodecidua in preterm labour and exceeded STL levels in both tissues.

3.4.3. Fetal membrane ceramide profiles

3.4.3.1. Ceramide profiles of term fetal membranes

This study aimed to explore ceramide production in the fetal membranes of labouring and non-labouring pregnancies at term. In amnion and choriodecidua, the overall ceramide production, which includes eight ceramide classes CER(NDS), CER(NS), CER(ADS), CER(AS), CER(AH), CER(NH), CER(AP), and CER(NP), was upregulated in STL and ITL in comparison to the TNL group (**Figure 3.23**).

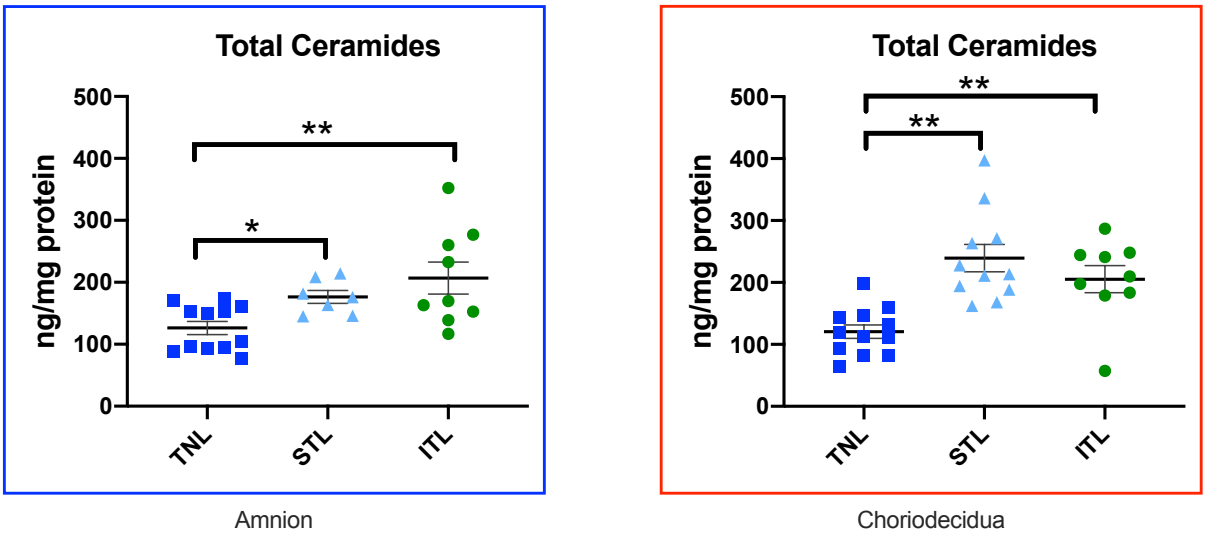
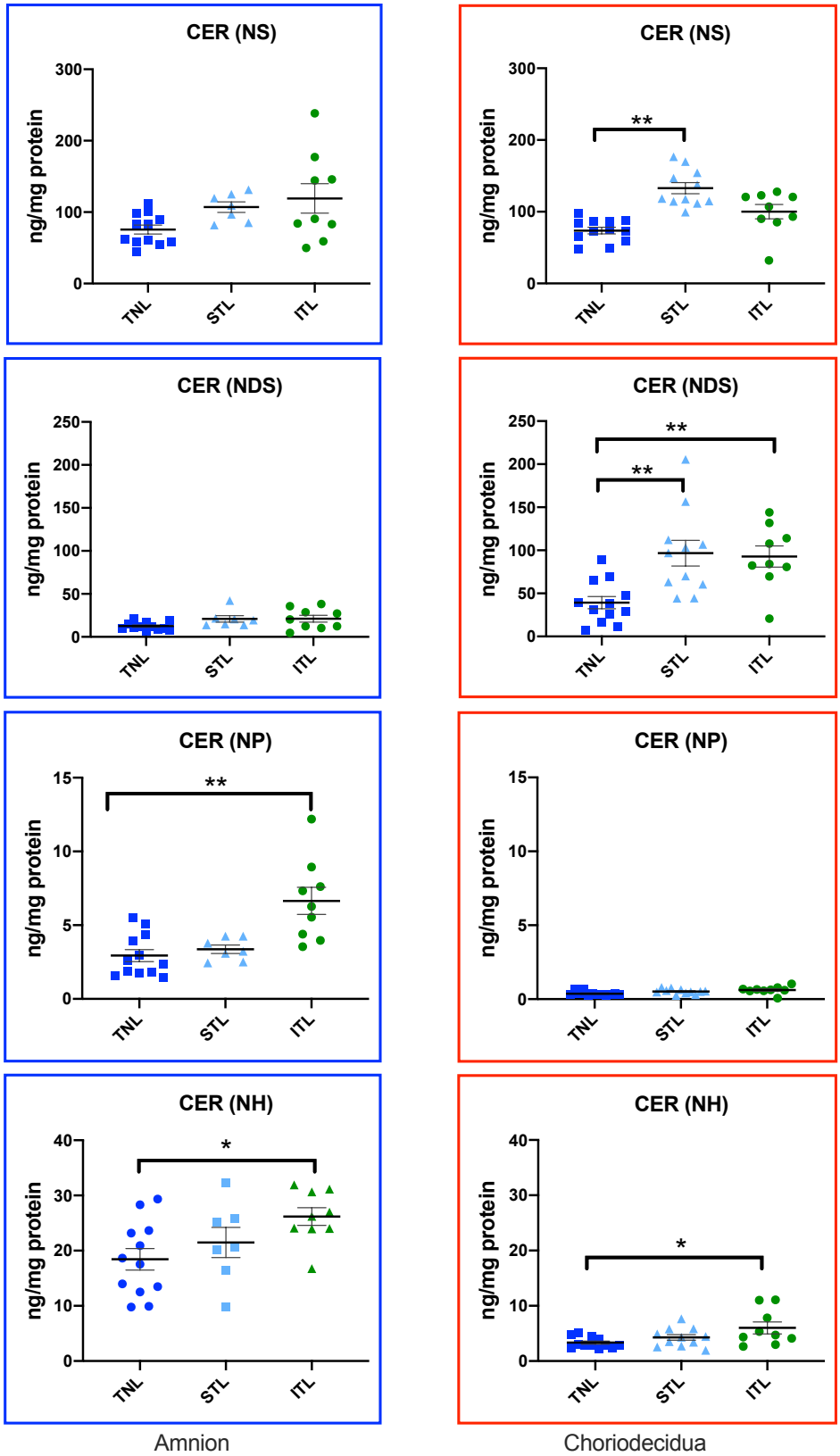


Figure 3.23. Term fetal membrane total ceramides. Amnion (left side, blue frame) and choriodecidua (right side, red frame). *Term not in labour (TNL), spontaneous term labour (STL) and induced term labour (ITL). Mean and SEM indicated. Statistical analysis = one-way ANOVA with Dunnett's multiple comparisons test, *p≤0.05, **p≤0.01.*

Ceramides of the CER(NS) class were the dominant species in both membrane tissues (**Figure 3.24**). Also, CER(NP), CER(NH), and CER(ADS) were much higher in the amnion than choriodecidua. The CER(NDS) class was more prominent in the choriodecidua than amnion, whilst only one species of the CER(AH) class was detected in both tissues, but at very low levels.

In the amnion, spontaneous labour showed a trend of increasing expression of most ceramide classes. ITL induced a significant increase in production CER(NP), CER(NH), CER(ADS), CER(AS), and CER(AP), compared to TNL. For the choriodecidua, CER(NDS) and CER(NS) were significantly elevated in STL, whilst in ITL, CER(NDS) and CER(NH) were raised significantly

(**Figure 3.24**). The CER(ADS) class showed a statistical increase in both labouring term groups, but the levels of phytoceramides CER(NP) and CER(AP) did not change.



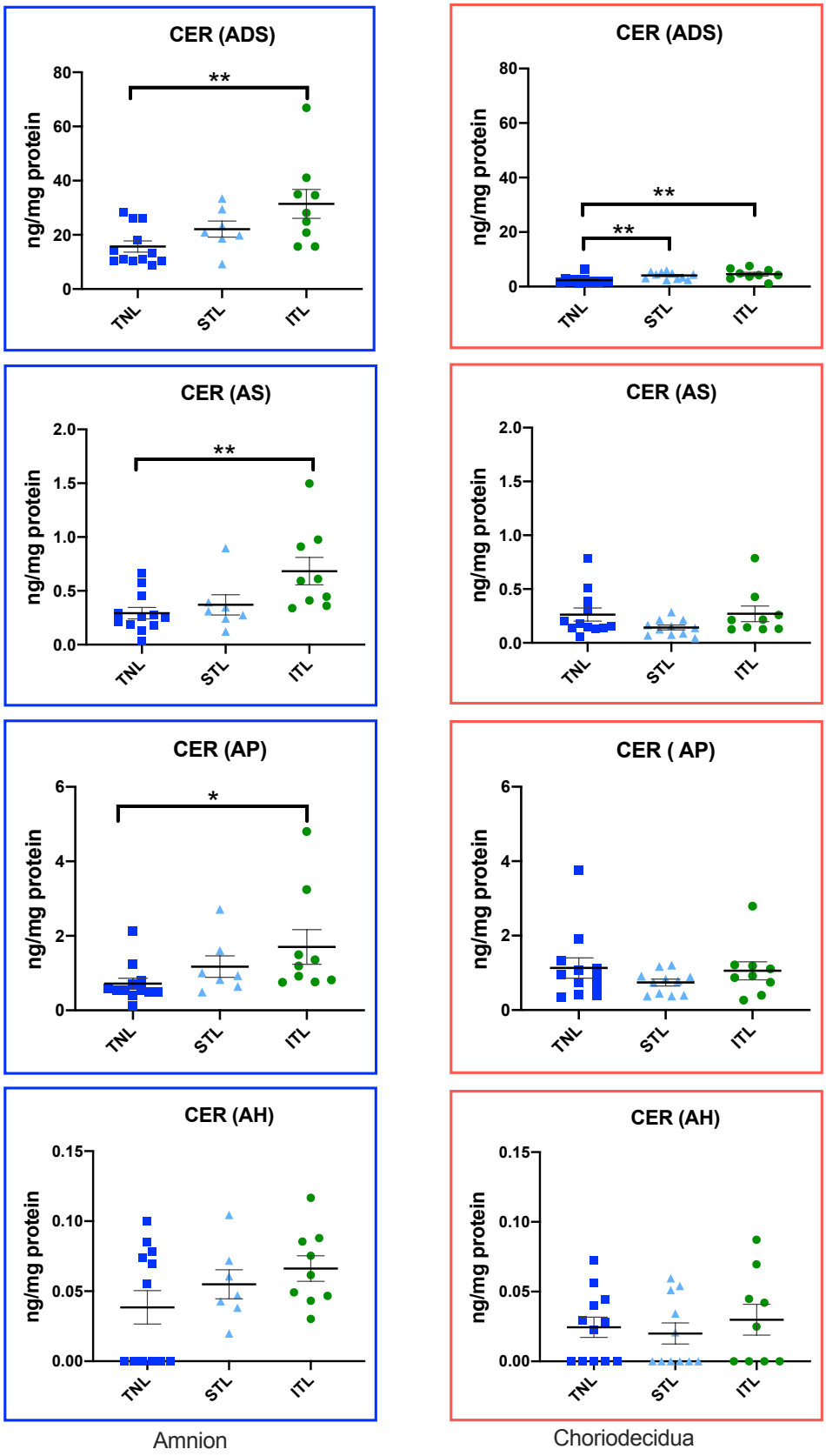


Figure 3.24. Term fetal membrane ceramides classes. Amnion (left side, blue frame) and choriodecidua (right side, red frame). *Term not in labour (TNL), spontaneous term labour (STL) and induced term labour (ITL). Mean and SEM indicated. Statistical analysis = one-way ANOVA with Dunnett's multiple comparisons test, *p≤0.05, **p≤0.01.*

Ceramide biosynthesis is driven through three different pathways: *de novo* biosynthesis, the salvage pathway and the sphingomyelin hydrolysis pathway (37,231). For *de novo* biosynthesis, dihydroceramides e.g. CER(NDS) and CER(ADS), are converted into CER(NS) and CER(AS), respectively; through the enzyme DEGS1. Dihydroceramides are also converted to the phytoceramides; CER(NP) and CER(AP) by the enzyme DEGS2 (240). Indicative indexes for DEGS can be estimated using the ratio end-products to substrates, e.g. CER(AS) and CER(NS) to CER(ADS) and CER(NDS) ratio to estimate DEGS1, and CER(NP) and CER(AP) to CER(ADS) and CER(NDS) ratio for DEGS2 (**Figure 3.25**). In the amnion, DEGS1 and DEGS2 activities were unchanged by labour at term. Whilst in the choriodecidua, DEGS1 index was decreased in ITL, and DEGS2 index were significantly reduced and STL and ITL (**Figure 3.25**).

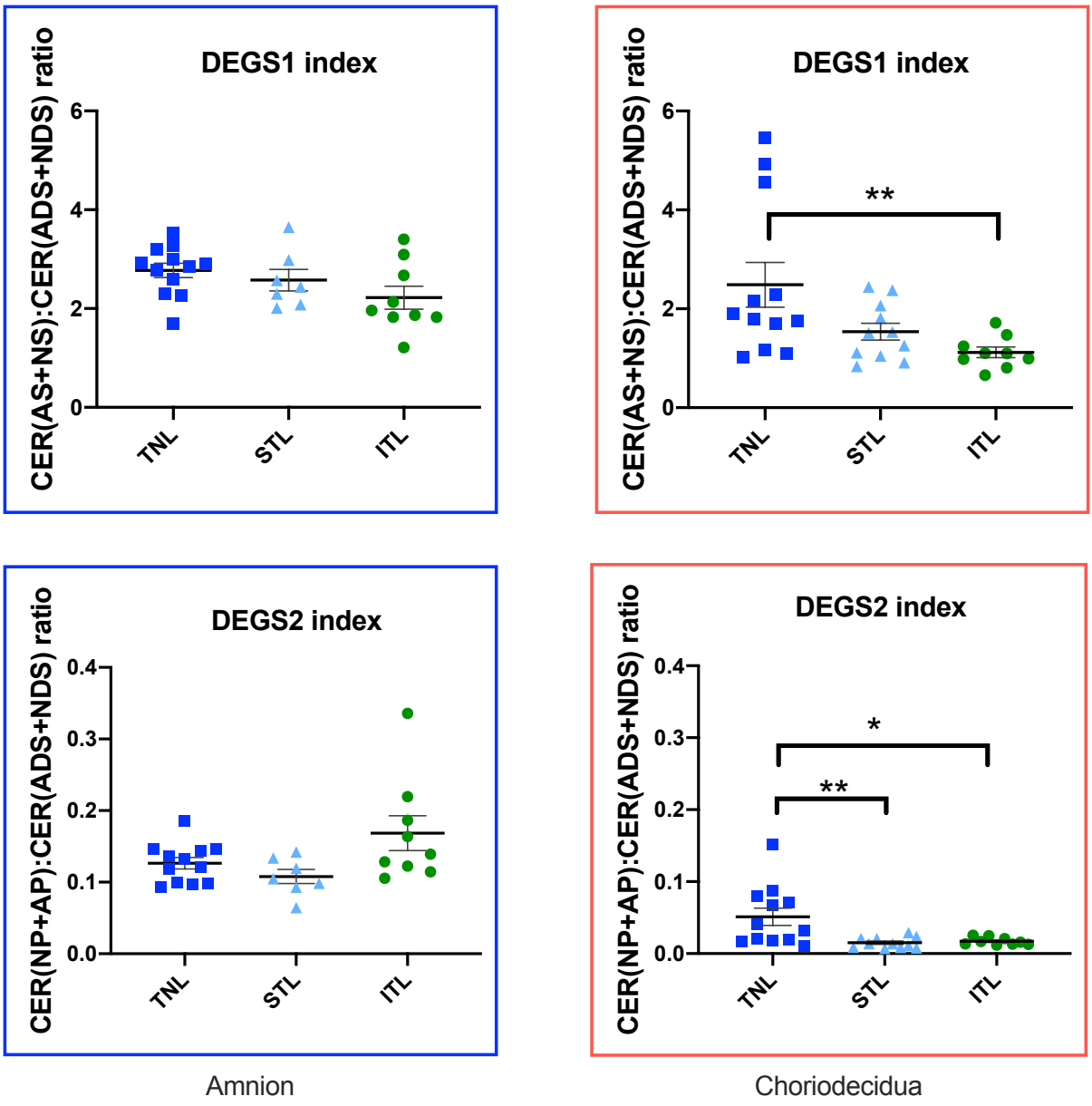


Figure 3.25. Term fetal membrane DEGS1 and DEGS2 index. Amnion (left side, blue frame) and choriodecidua (right side, red frame). *Term not in labour (TNL), spontaneous term labour (STL) and induced term labour (ITL). Mean and SEM indicated. Statistical analysis = one-way ANOVA with Dunnett's multiple comparisons test, *p≤0.05, **p≤0.01.*

Figures 3.26-3.29 show the non-hydroxy fatty acid ceramides detected in both amnion and choriodecidua, grouped according to ceramide class. Most ceramide species found in fetal membranes, were of the CER(NS) and CER(NDS) classes (34 and 22 species, respectively). The non-hydroxy fatty acid ceramides, CER(NS) CER(NDS), CER(NH), CER(NP) were mostly derivatives of 18 carbon sphingoid bases with a 24 carbon acyl chain.

In fetal membranes, the sphingoid bases of the CER(NDS) species ranged between 17 to 26 carbon lengths, and the acyl chain lengths were between 18-26 carbons (**Figure 3.26**). In choriodecidua, the levels of CER(NDS) species were greater than in the amnion. In the amnion, both in-labour groups showed higher levels of CER(NDS) species than TNL, but ITL had higher significant species than STL, with 9 and 3 species, respectively. (**Figure 3.26**). In the choriodecidua, both STL and ITL groups had higher levels of CER(NDS) species than TNL (11 and 10 species, respectively) (**Figure 3.26**).

In amnion and choriodecidua, the sphingoid bases of the CER(NS) species were ranging between 16 to 26 carbon lengths, and the acyl chain lengths were between 22 to 30 carbon (**Figure 3.27**). The choriodecidua produced higher levels CER(NS) species with 18 carbon length sphingoid bases than amnion, but with amnion liberating longer CER(NS) species with sphingoid base ranged between 19 to 24 carbon length. Labouring conditions at term showed a trend for increased CER(NS) species within the amnion. However, this only reached statistical significance in ITL, with 18 CER(NS) species in total (**Figure 3.27**). Within the choriodecidua, term labour increased most of the CER(NS) species, reaching significance in 10 and 2 species in STL and ITL, respectively (**Figure 3.27**).

In both sides of the fetal membrane, the acyl chain of CER(NH) species ranged between 23 and 28 carbon length, and 15 to 20 carbon length for sphingoid bases **(Figure 3.28).** The amnion expressed higher levels of CER(NH) species than choriodecidua. For the amnion, ITL showed a significant increase in seven species of CER(NH). Whilst in the choriodecidua, two species, N(26)H(15) and N(24)H(18), were significantly elevated in both term labouring scenarios. The CER(NP) species' sphingoid bases ranged between 16 and 22 carbon length and 23 to 28 carbon for acyl chain, in amnion and choriodecidua. The CER(NP) species showed a particular rise in amnion in all 22 species in the ITL group, but none in STL. In choriodecidua, two and five CER(NP) species were increased in STL and ITL, respectively (**Figure 3.29**). However, amnion produced more CER(NP) species than choriodecidua overall.

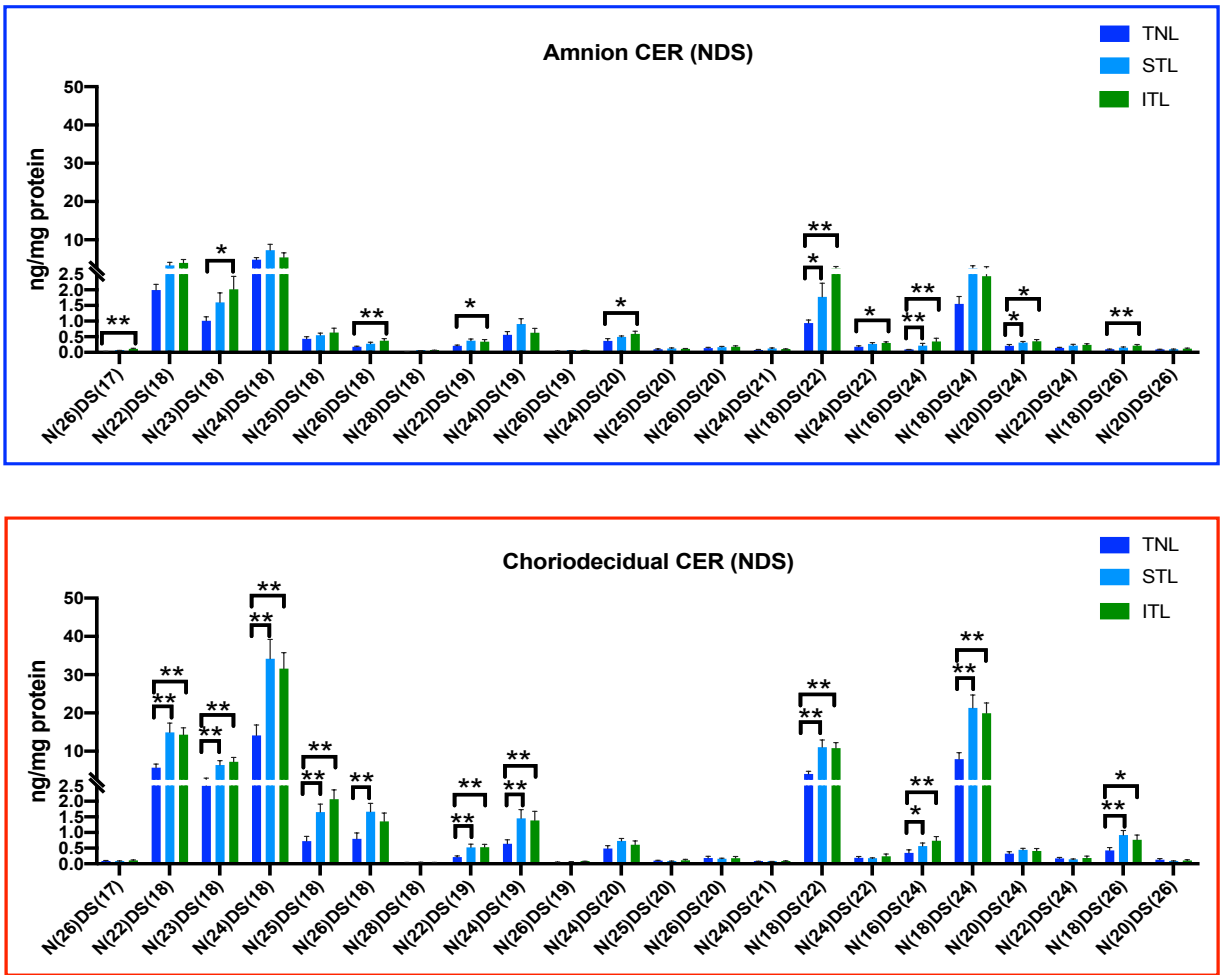


Figure 3.26. Term fetal membrane CER (NDS) species. Amnion (top, blue frame) and choriodecidua (bottom, red frame). *Term not in labour (TNL), spontaneous term labour (STL) and induced term labour (ITL). Mean and SEM indicated. Statistical analysis = two-way ANOVA with Dunnett's multiple comparisons test, *p≤0.05, **p≤0.01.*

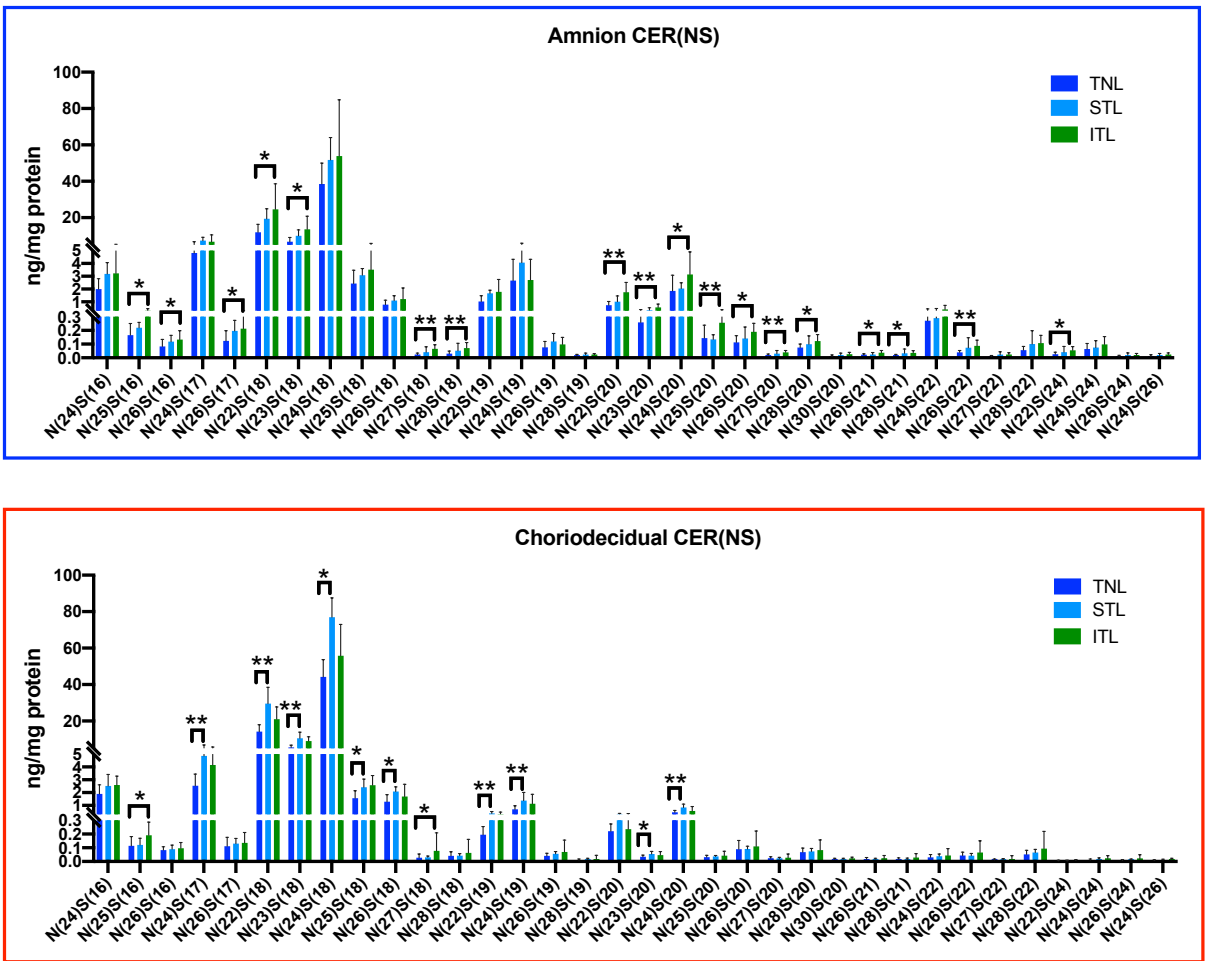


Figure 3.27. Term fetal membrane CER (NS) species. Amnion (top, blue frame) and choriodecidua (bottom, red frame). *Term not in labour (TNL), spontaneous term labour (STL) and induced term labour (ITL). Mean and SEM indicated. Statistical analysis = two-way ANOVA with Dunnett's multiple comparisons test, *p≤0.05, **p≤0.01.*

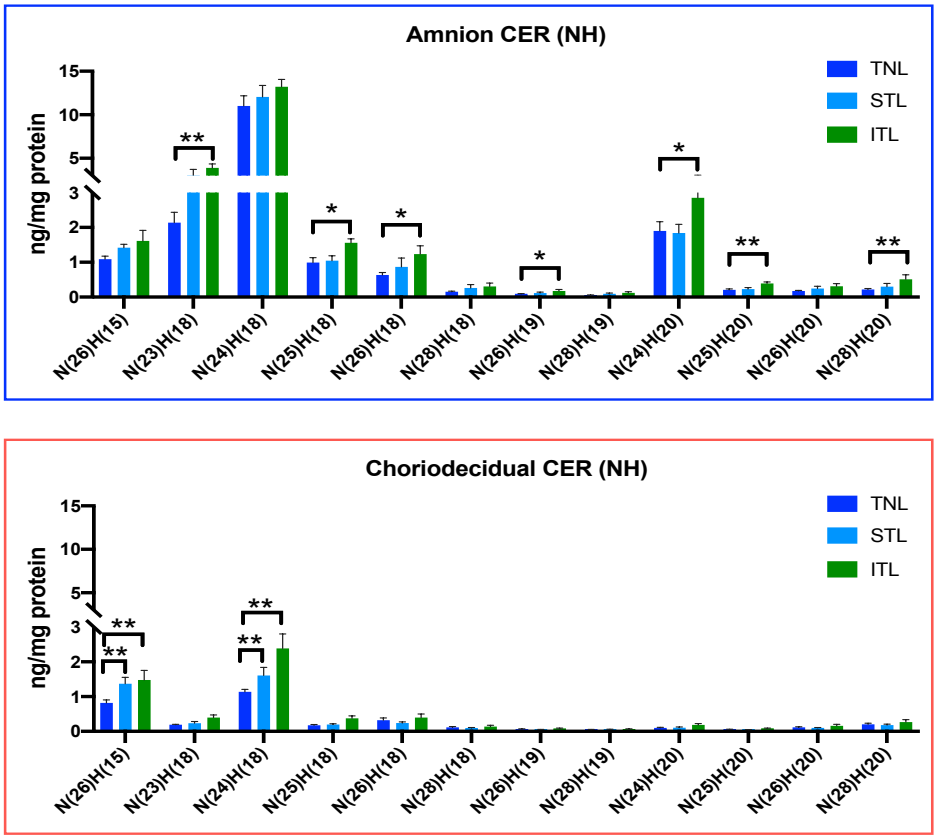


Figure 3.28. Term fetal membrane CER (NH) species. Amnion (top, blue frame) and choriodecidua (bottom, red frame). *Term not in labour (TNL), spontaneous term labour (STL) and induced term labour (ITL). Mean and SEM indicated. Statistical analysis = two-way ANOVA with Dunnett's multiple comparisons test, *p≤0.05, **p≤0.01.*

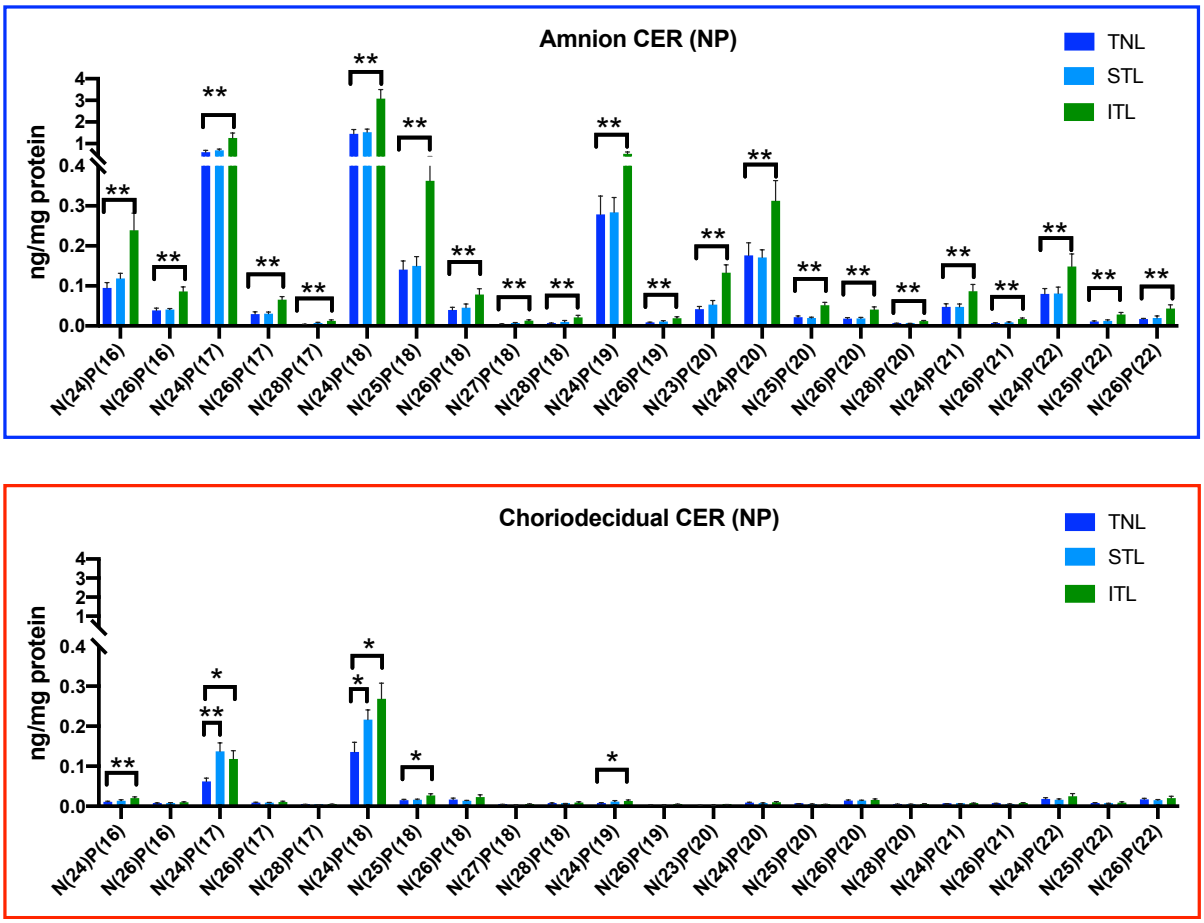


Figure 3.29. Term fetal membrane CER (NP) species. Amnion (top, blue frame) and choriodecidua (bottom, red frame). *Term not in labour (TNL), spontaneous term labour (STL) and induced term labour (ITL). Mean and SEM indicated. Statistical analysis = two-way ANOVA with Dunnett's multiple comparisons test, *p≤0.05, **p≤0.01.*

In amnion and choriodecidua, only three species were detected from the CER(ADS) class; all had 18 carbon length acyl chains and 22, 23, and 24 carbon length sphingoid bases, with amnion higher than choriodecidua (**Figure 3.30**). With term labour, CER(ADS) species showed an increasing trend in the amnion, but with A(18)DS(22) and A(18)DS(24) only reaching significance in the ITL group (**Figure 3.30**). In choriodecidua, both species were more expressed in both labouring conditions, as compared to TNL (see **Figure 3.30**).

Four CER(AS) species were detected with low abundance in fetal membranes, with 24 and 26 carbon length acyl chains and 19, 20 and 22 carbon length sphingoid bases **(Figure 3.31)**. Within the amnion, only ITL showed enhanced CER(AS) ceramide production, namely A(24)S(20) and A(26)S(20). On the other hand, the choriodecidua show no differential

response in term labour, except the A(24)S(20) level was significantly reduced in STL (**Figure 3.31**).

The CER(AP) species were the highest of the alpha-hydroxy ceramides detected in the fetal membranes, with 11 species recognised in total (see **Figure 3.32**). These species had sphingoid bases of 16 to 22 carbon length and acyl chains with 24, 25 and 26 carbon length. Similar to other alpha-hydroxy ceramides, the amnion expressed a greater CER(AP) than choriodecidua, with labour encouraging greater numbers; 2 and 7 species in the amnion for STL and ITL, respectively (**Figure 3.32**). For the choriodecidua, the ITL group showed comparable numbers to controls. However, the STL group showed a subtle decline, with only one species, A(24)P(21), reduced significantly (**Figure 3.32**).

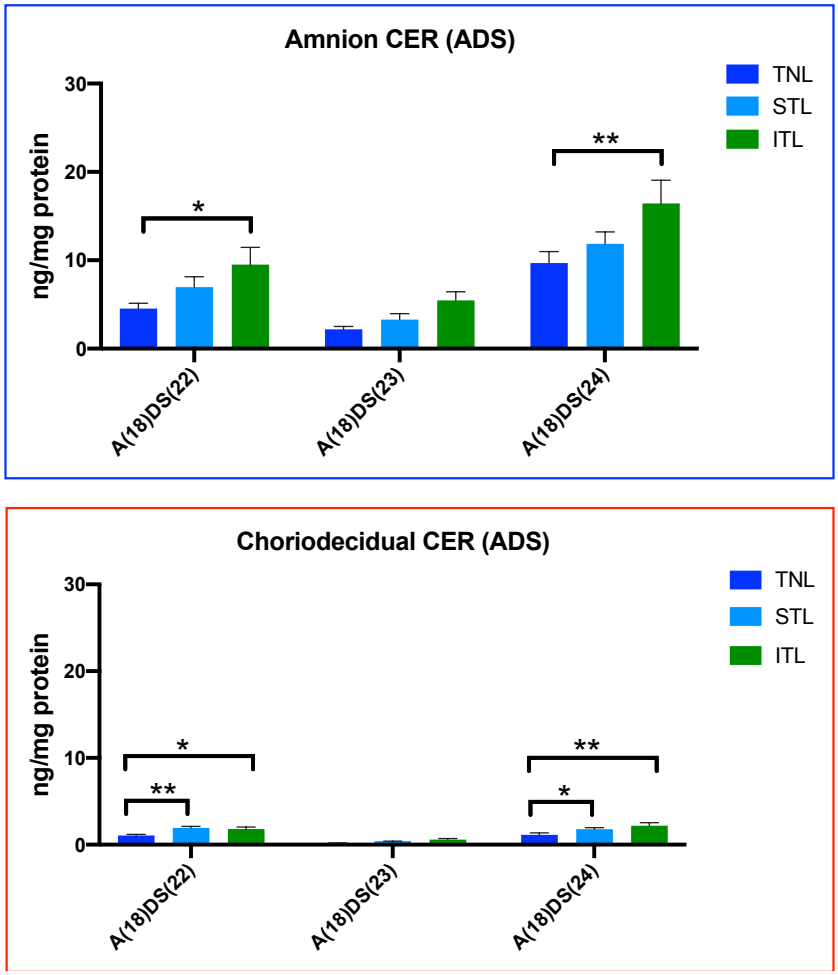


Figure 3.30. Term fetal membrane CER (ADS) species. Amnion (top, blue frame) and choriodecidua (bottom, red frame). *Term not in labour (TNL), spontaneous term labour (STL) and induced term labour (ITL). Mean and SEM indicated. Statistical analysis = two-way ANOVA with Dunnett's multiple comparisons test, *p≤0.05, **p≤0.01.*

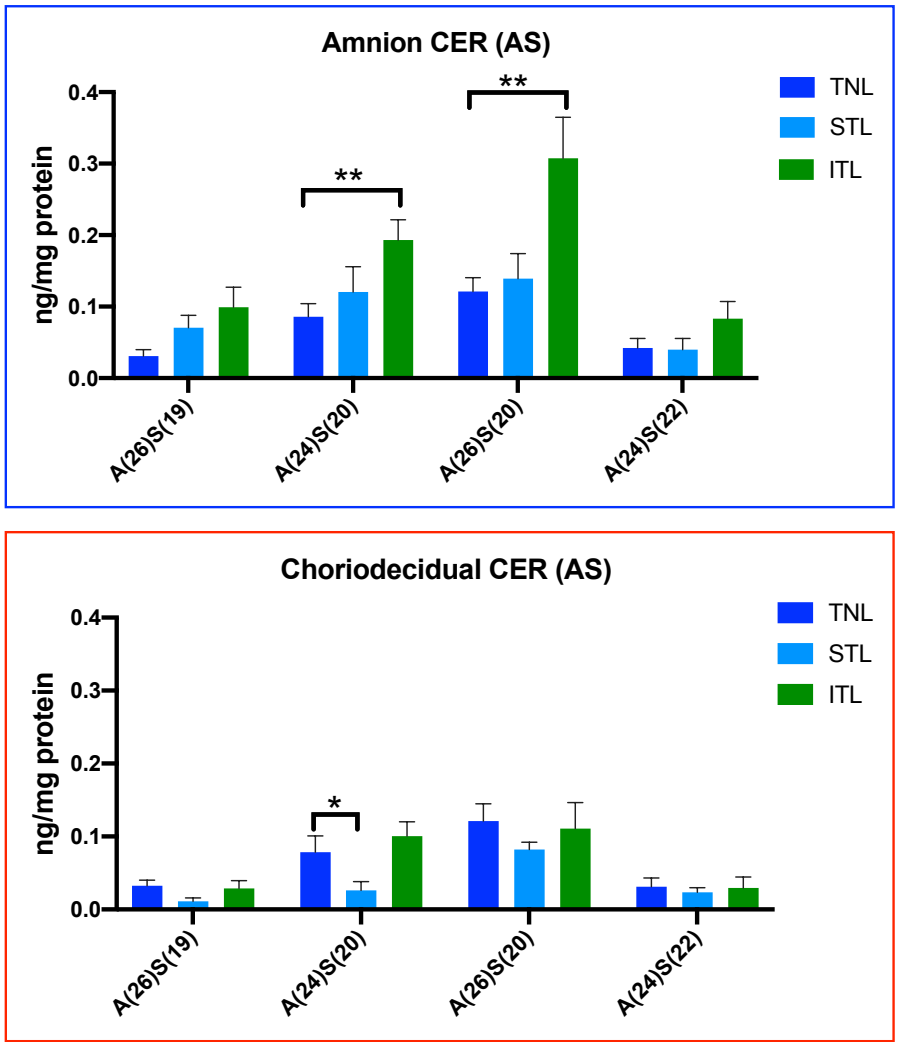


Figure 3.31 Term fetal membrane CER (AS) species. Amnion (top, blue frame) and choriodecidua (bottom, red frame). *Term not in labour (TNL), spontaneous term labour (STL) and induced term labour (ITL). Mean and SEM indicated. Statistical analysis = two-way ANOVA with Dunnett's multiple comparisons test, *p≤0.05, **p≤0.01.*

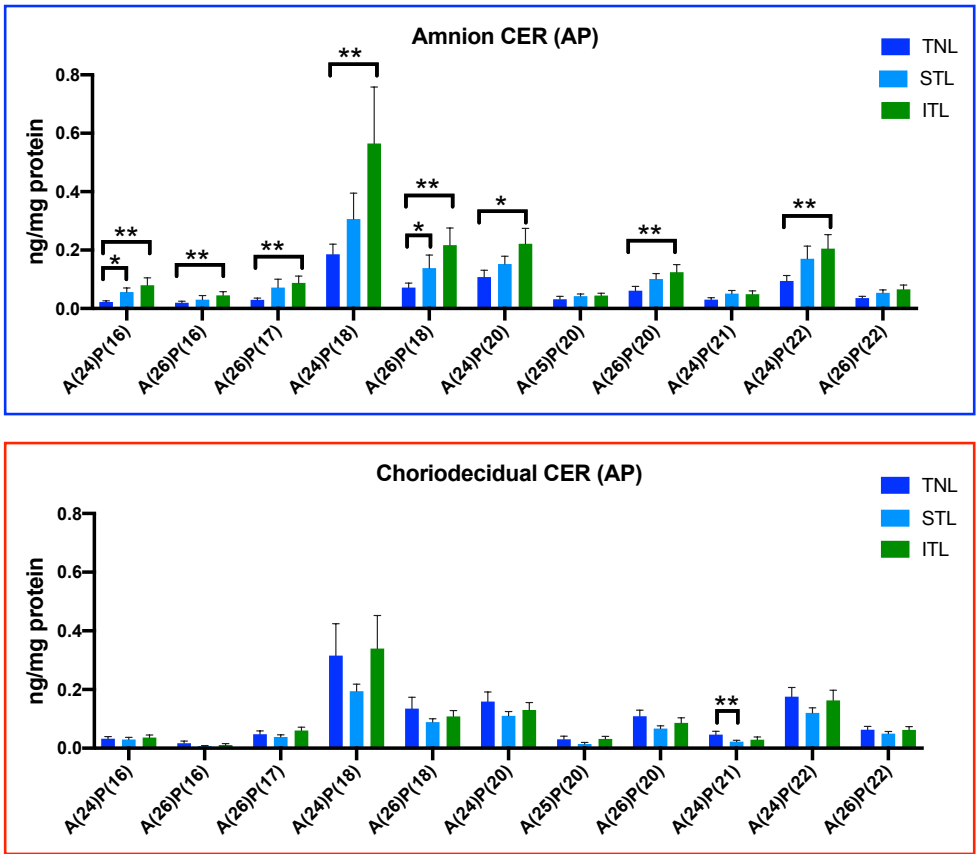


Figure 3.32. Term fetal membrane CER (AP) species. Amnion (top, blue frame) and choriodecidua (bottom, red frame). *Term not in labour (TNL), spontaneous term labour (STL) and induced term labour (ITL). Mean and SEM indicated. Statistical analysis = two-way ANOVA with Dunnett's multiple comparisons test, *p≤0.05, **p≤0.01.*

In summary, the term study showed that both amnion and choriodecidua produce and upregulate ceramides during spontaneous labour and following labour induction. CER(ADS), CER(NP) and CER(NH) were found at higher concentrations in the amnion than the choriodecidua, with CER(NDS) also more abundant in the latter. Within the amnion, ceramides were more actively produced following induction compared to spontaneous labour. Whereas in the choriodecidua, this situation was reversed. In both tissues, the 18 carbon sphingoid base ceramides were the dominant species, and CER(NS) was the most abundant ceramide class, increasing significantly in labour in the choriodecidua.

3.4.3.2. Ceramide profiling of preterm fetal membranes

Similar to the term study, eight ceramides classes CER(NDS), CER(NS), CER(ADS), CER(AS), CER(AH), CER(NH), CER(AP), and CER(NP) were detected in the preterm fetal membranes (**Figure 3.33)**. In both tissues, the total ceramides in the preterm labour group showed no

significant differences to the TNL controls. However, in the choriodecidua, the PTL showed a trend towards increased total ceramide production. On inspection, it is clear that lower ceramide levels are detected in the amnion in PTL as compared to the choriodecidua.

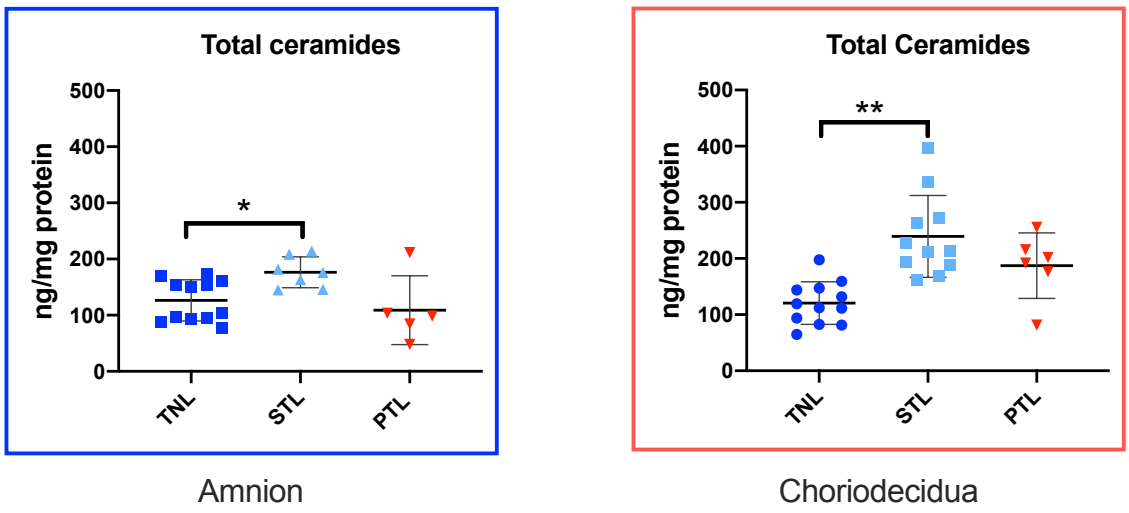
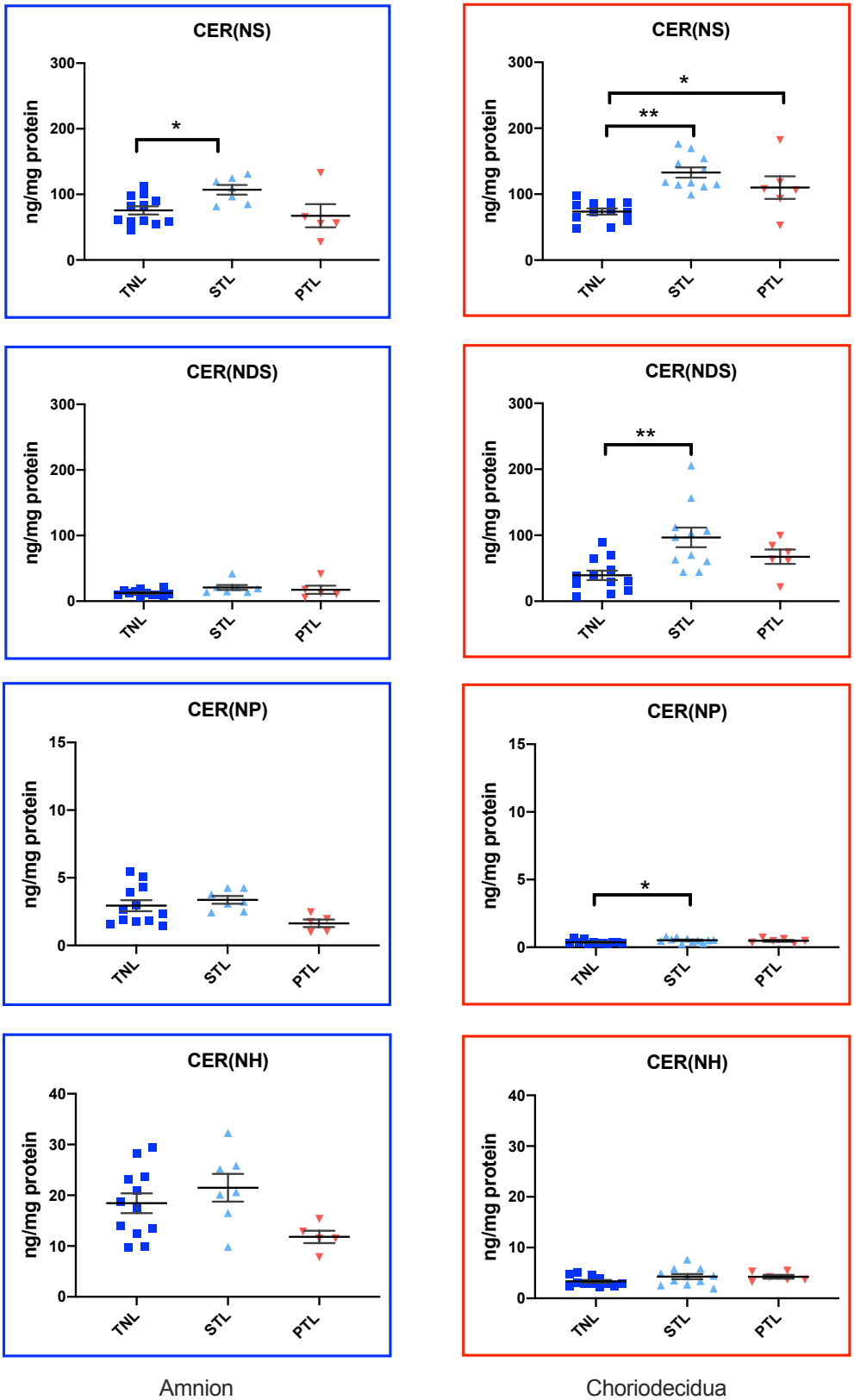


Figure 3.33. Preterm fetal membrane total ceramides. Amnion (left side, blue frame) and choriodecidua (right side, red frame). *Term not in labour (TNL), spontaneous term labour (STL) and preterm labour (PTL). Mean and SEM indicated. Statistical analysis = one-way ANOVA with Dunnett's multiple comparisons test, *p≤0.05, **p≤0.01.*

In PTL, in both tissues, the most abundant ceramide classes were CER(NS) and CER(NDS) (**Figure 3.34**). In the amnion, these showed a decreasing pattern of expression over TNL, but this failed to reach significance. In the choriodecidua, the PTL group showed a significant increase in CER(NS) and CER(ADS) expression, and a trend towards increasing production in most other ceramide classes but with less abundance than in STL (**Figure 3.34**). Similar to the term study, only one CER(AH) species was detected in preterm labour, but again this was at a low level of expression.

In both membrane tissues, the indicative DEGS1 activity, which was similar between PTL and TNL in both membrane tissues (**Figure 3.35**). Also, in **Figure 3.35**, the DEGS2 index is seen comparable between groups in the amnion but significantly reduced (over TNL) in the choriodecidua in PTL, similar to that in STL.



Amnion

Choriodecidua

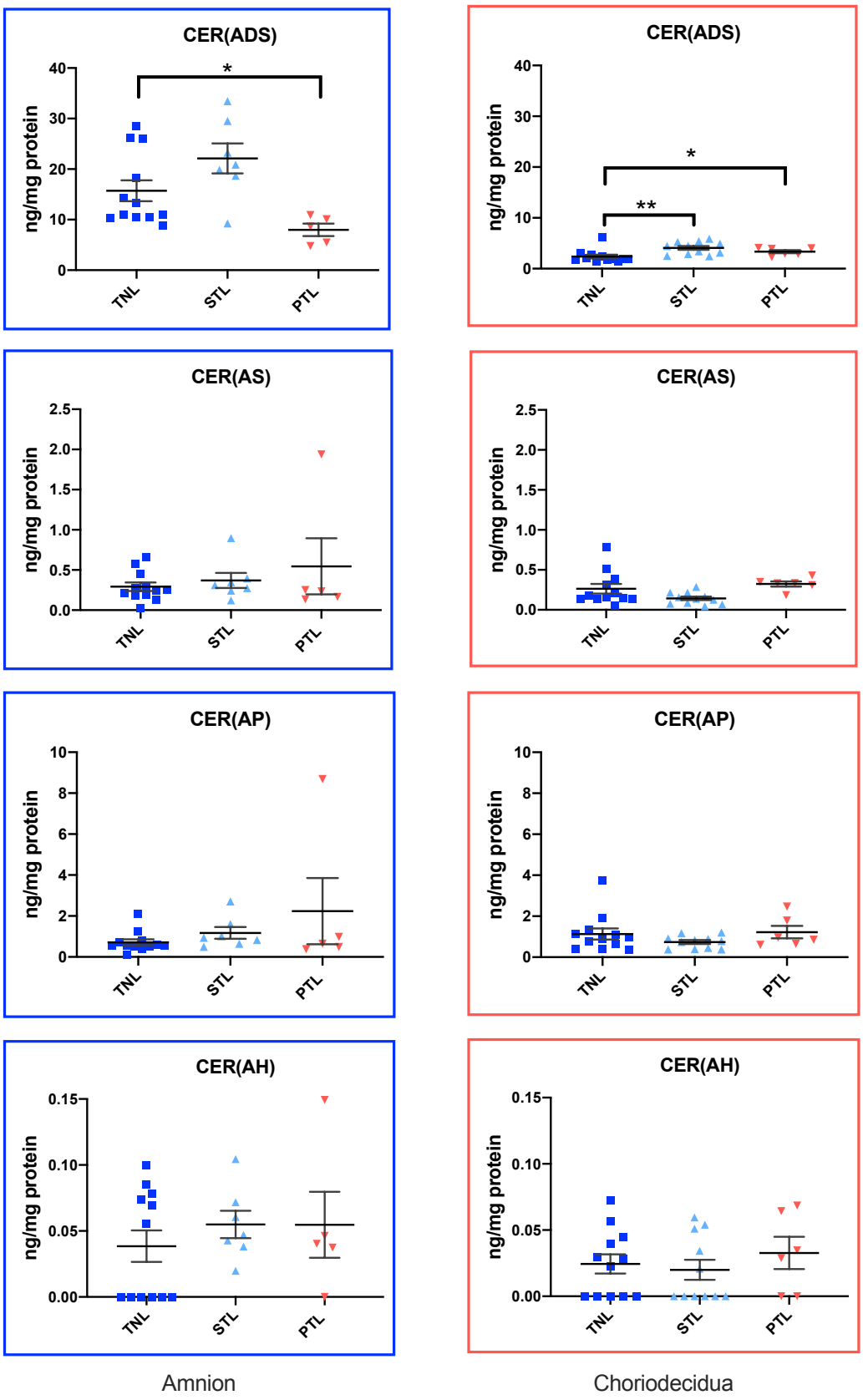


Figure 3.34. Preterm fetal membrane ceramides classes. Amnion (left side, blue frame) and choriodecidua (right side, red frame). *Term not in labour (TNL), spontaneous term labour (STL) and preterm labour (PTL). Mean and SEM indicated. Statistical analysis = one-way ANOVA with Dunnett's multiple comparisons test, *p≤0.05, **p≤0.01.*

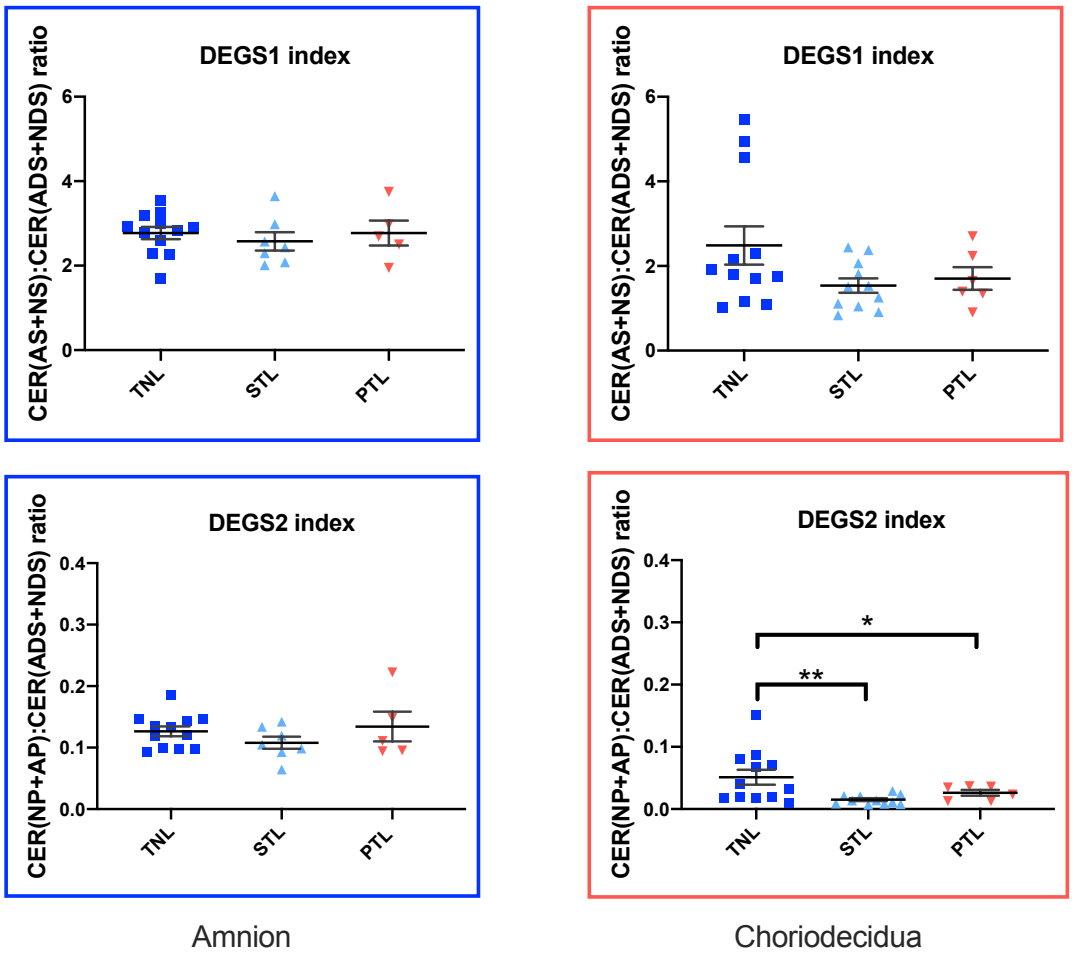


Figure 3.35. Preterm fetal membrane DEGS1 and DEGS2. Amnion (left side, blue frame) and choriodecidua (right side, red frame). *Term not in labour (TNL), spontaneous term labour (STL) and preterm labour (PTL). Mean and SEM indicated. Statistical analysis = one-way ANOVA with Dunnett's multiple comparisons test, *p≤0.05, **p≤0.01.*

Similar to the term study, the 18 carbon length sphingoid bases CER(NDS), CER(NS), CER(NH), and CER(NP) were the dominant species in preterm amnion and choriodecidual tissues (**Figures 3.36-3.39**). As seen in **Figure 3.36**, the preterm amnion tissue showed a similar pattern in CER(NDS) species to TNL. For the choriodecidual tissues, PTL expressed a high concentration of CER(NDS) species but not as increased as the STL level, with six species reaching statistical significance.

The 18 carbon sphingoid bases CER(NS) species were more abundant in both tissues, while the more extended carbon length sphingoid bases were higher in the amnion. In amnion tissues, the PTL group showed a similar expression of CER(NS) species to the TNL group.

However, preterm choriodecidual tissues showed an increasing trend, with three species reaching statistical significance (**Figure 3.37**).

For the CER(NH) and CER(NP) families, the amnion expressed higher levels than the choriodecidual tissues. As seen in **Figure 3.38**, none of CER(NH) species showed a significant change in amnion nor choriodecidua. In **Figure 3.39**, the preterm amnion expressed a trend of lower concentrations in the dominant CER(NP) species than TNL but without reaching statistical significance. The preterm choriodecidual tissues showed a significant increase in three long-chain sphingoid base CER(NP) species; N(26)P(20), N(28)P(20) and N(24)P(21) (see **Figure 3.39**).

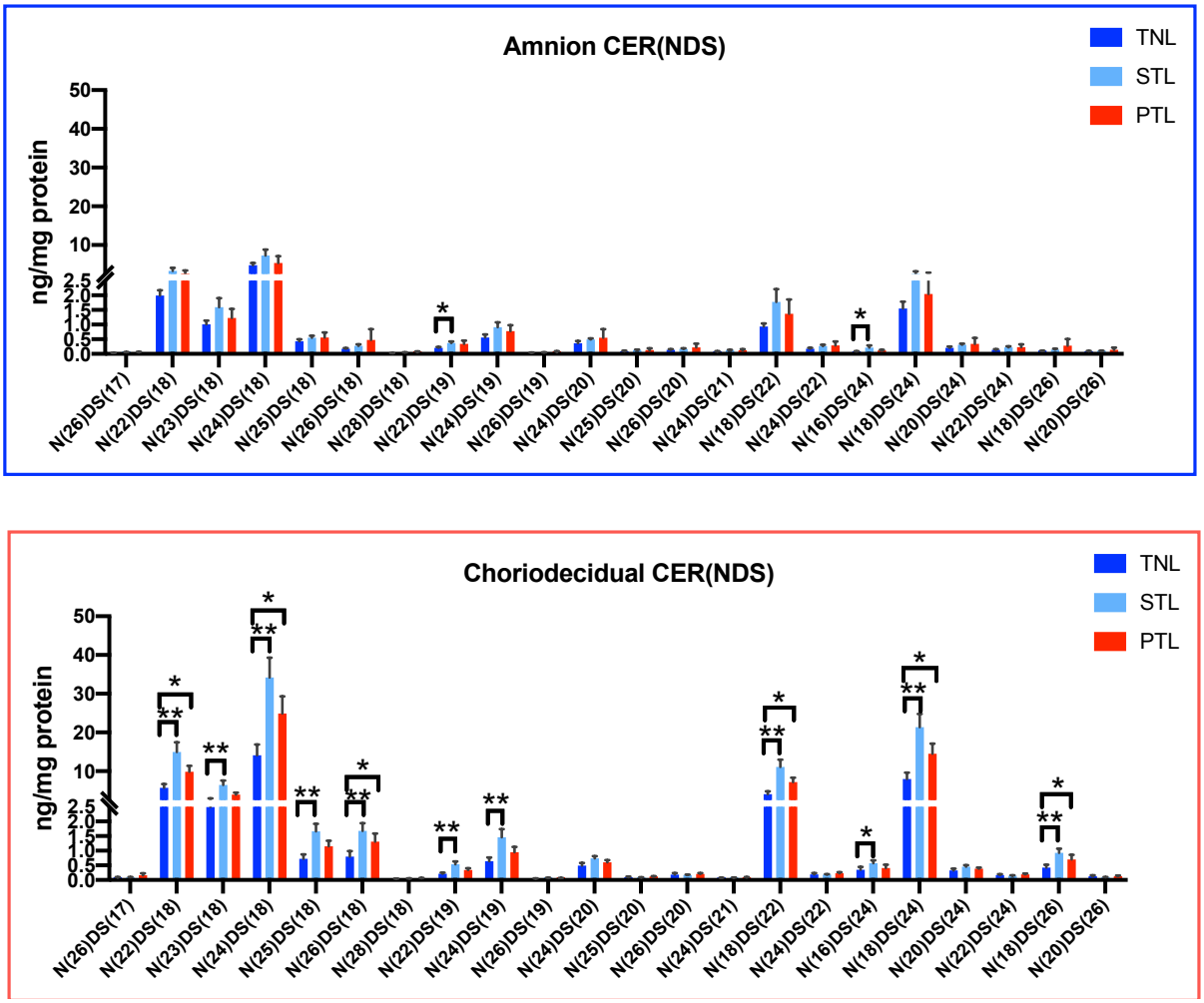


Figure 3.36. Preterm fetal membrane CER (NDS). Amnion (top, blue frame) and choriodecidua (bottom, red frame). *Term not in labour (TNL), spontaneous term labour (STL) and preterm labour (PTL). Mean and SEM indicated. Statistical analysis = two-way ANOVA with Dunnett's multiple comparisons test, *p≤0.05, **p≤0.01.*

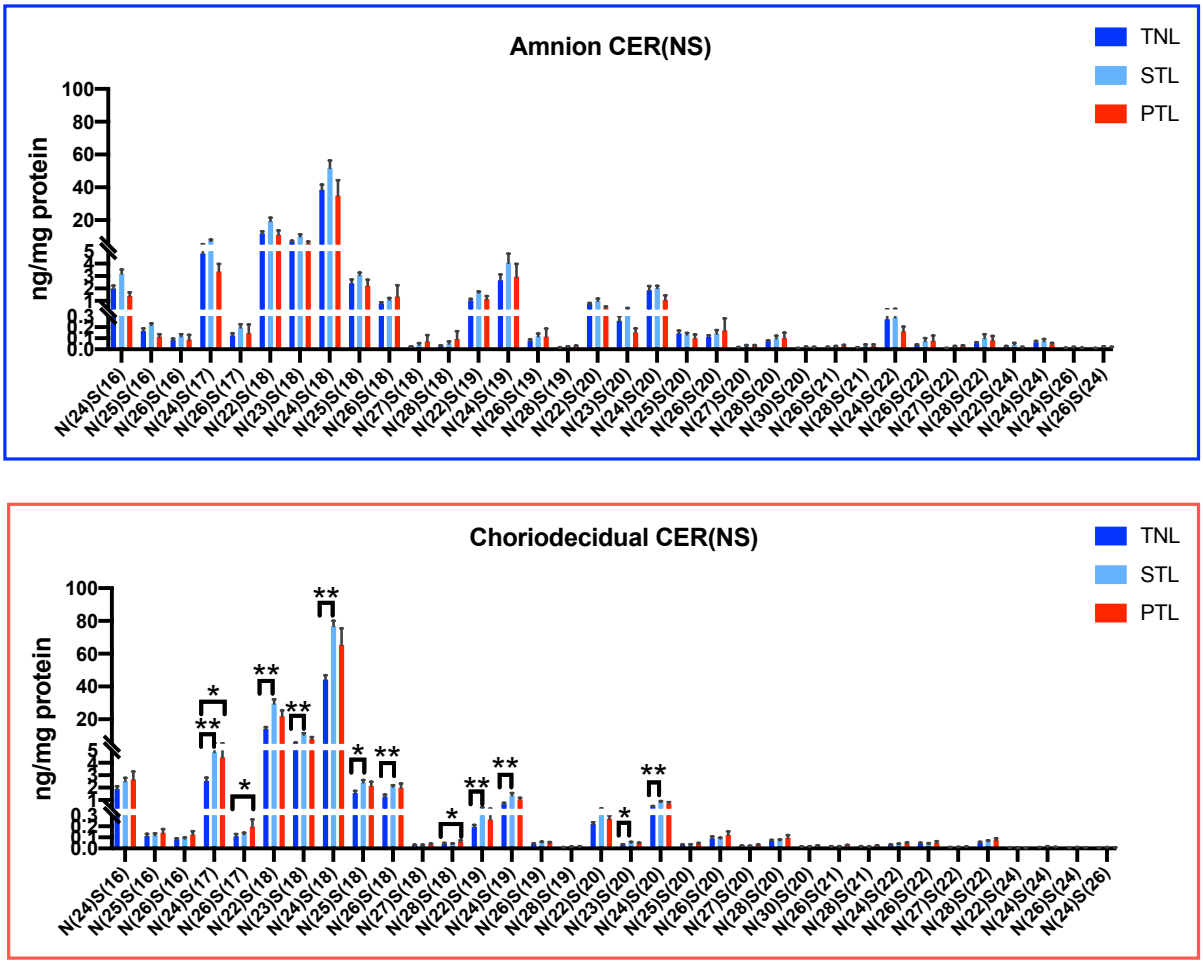


Figure 3.37. Preterm fetal membrane CER (NS). Amnion (top, blue frame) and choriodecidua (bottom, red frame). *Term not in labour (TNL), spontaneous term labour (STL) and preterm labour (PTL). Mean and SEM indicated. Statistical analysis = two-way ANOVA with Dunnett's multiple comparisons test, *p≤0.05, **p≤0.01.*

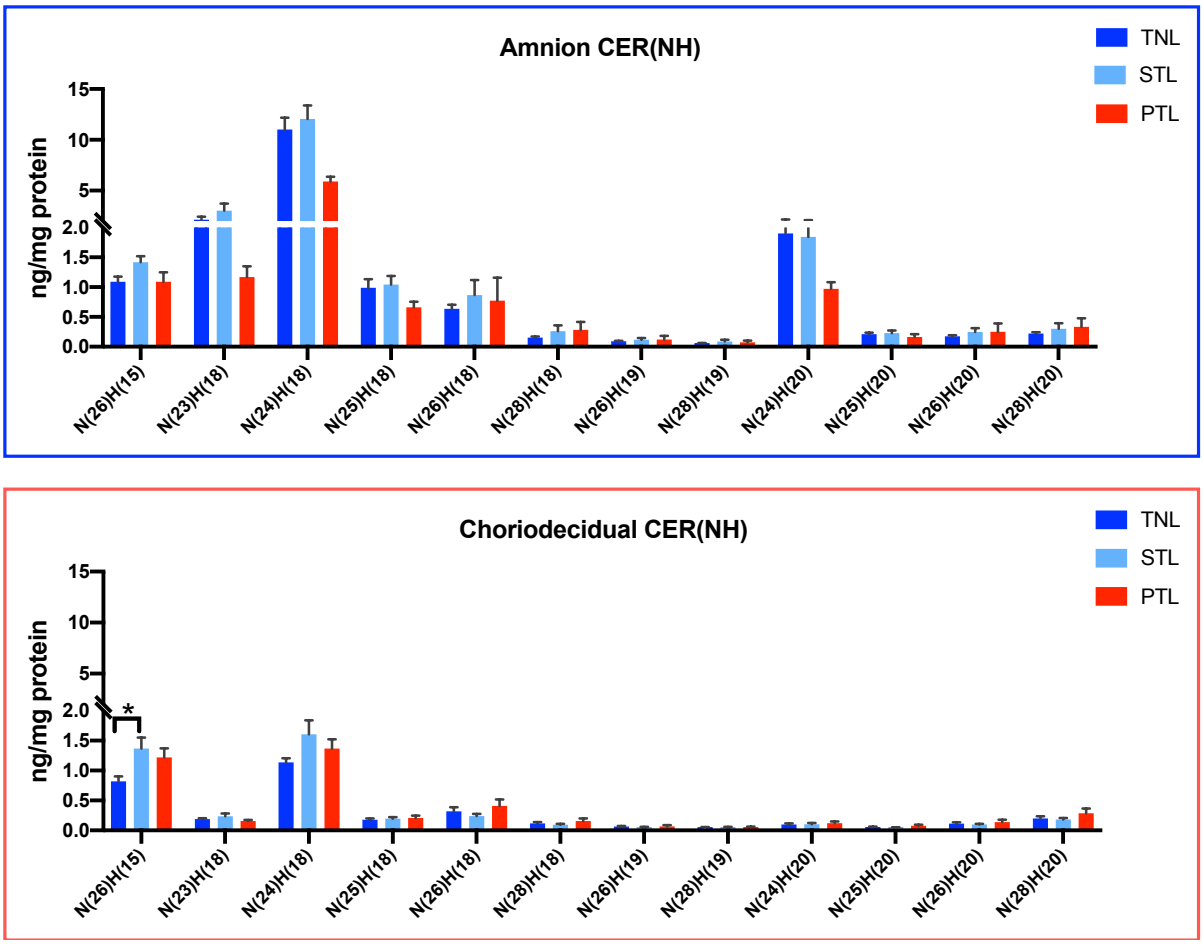


Figure 3.38. Preterm fetal membrane CER (NH). Amnion (top, blue frame) and choriodecidua (bottom, red frame). *Term not in labour (TNL), spontaneous term labour (STL) and preterm labour (PTL). Mean and SEM indicated. Statistical analysis = two-way ANOVA with Dunnett's multiple comparisons test, *p≤0.05, **p≤0.01.*

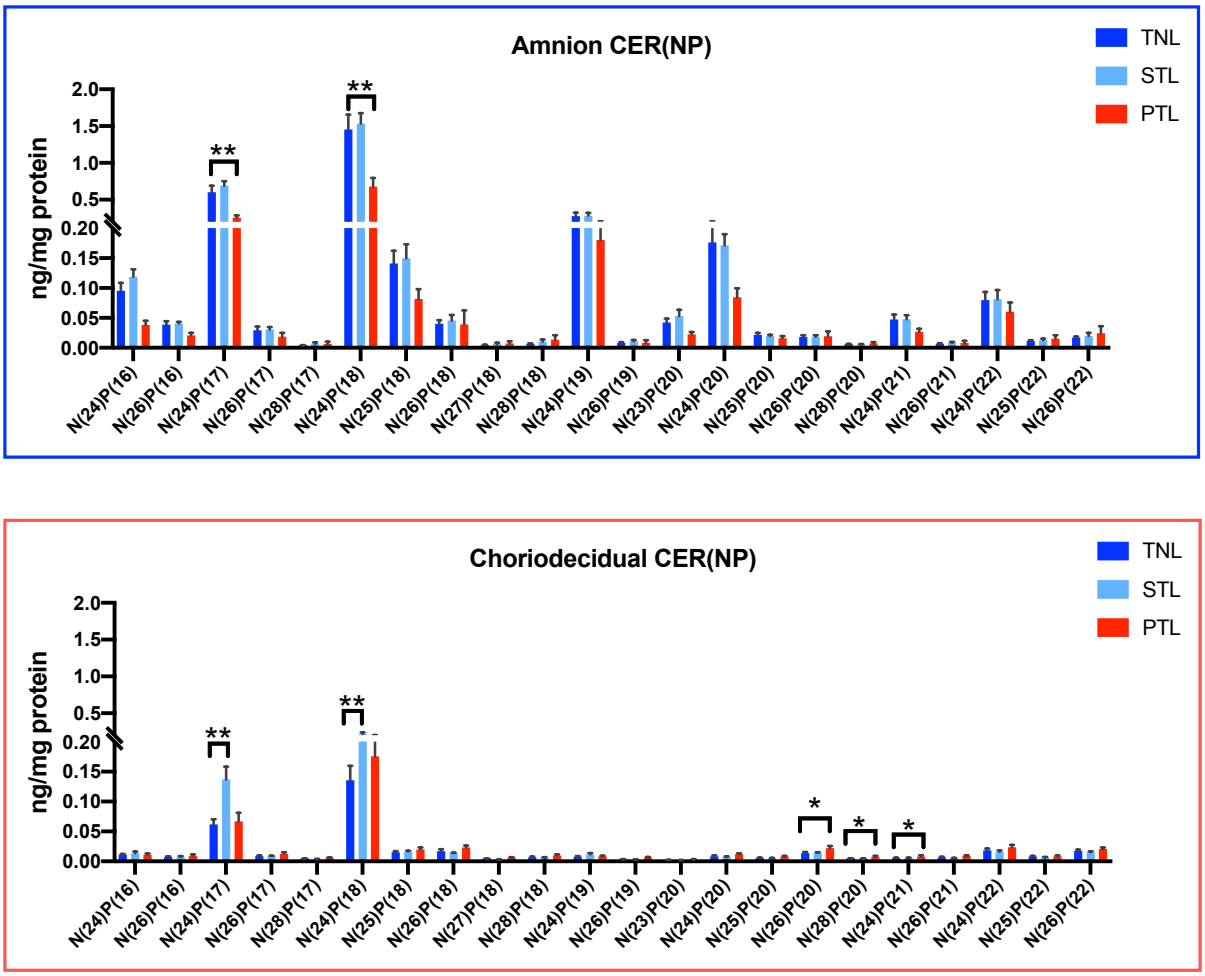


Figure 3.39 Preterm fetal membrane CER (NP). Amnion (top, blue frame) and choriodecidua (bottom, red frame). *Term not in labour (TNL), spontaneous term labour (STL) and preterm labour (PTL). Mean and SEM indicated. Statistical analysis = two-way ANOVA with Dunnett's multiple comparisons test, *p≤0.05, **p≤0.01.*

The preterm labour group expressed a higher level of alpha-hydroxy acyl ceramide classes CER(ADS), CER(AS) and CER(AP) in amnion than choriodecidua (**Figures 3.40**, **3.41**, and **3.42**). In **Figure 3.40**, preterm amnion tissues showed a trend for lower expression of CER(ASD) species than the TNL group; whilst A(18)DS(22) was significantly increased in the preterm choriodecidua. In **Figures 3.41** and **3.42**, the PTL group showed a non-significant change in CER(AS) and CER(AP) species compared to controls.

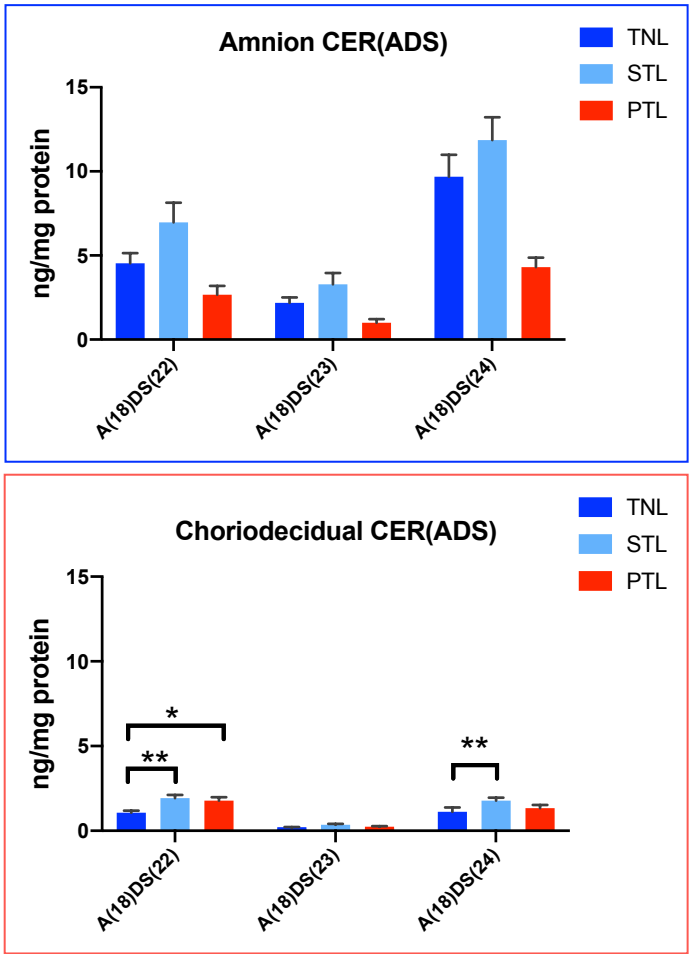


Figure 3.40 Preterm fetal membrane CER (ADS). Amnion (top, blue frame) and choriodecidua (bottom, red frame). *Term not in labour (TNL), spontaneous term labour (STL) and preterm labour (PTL). Mean and SEM indicated. Statistical analysis = two-way ANOVA with Dunnett's multiple comparisons test, *p≤0.05, **p≤0.01.*

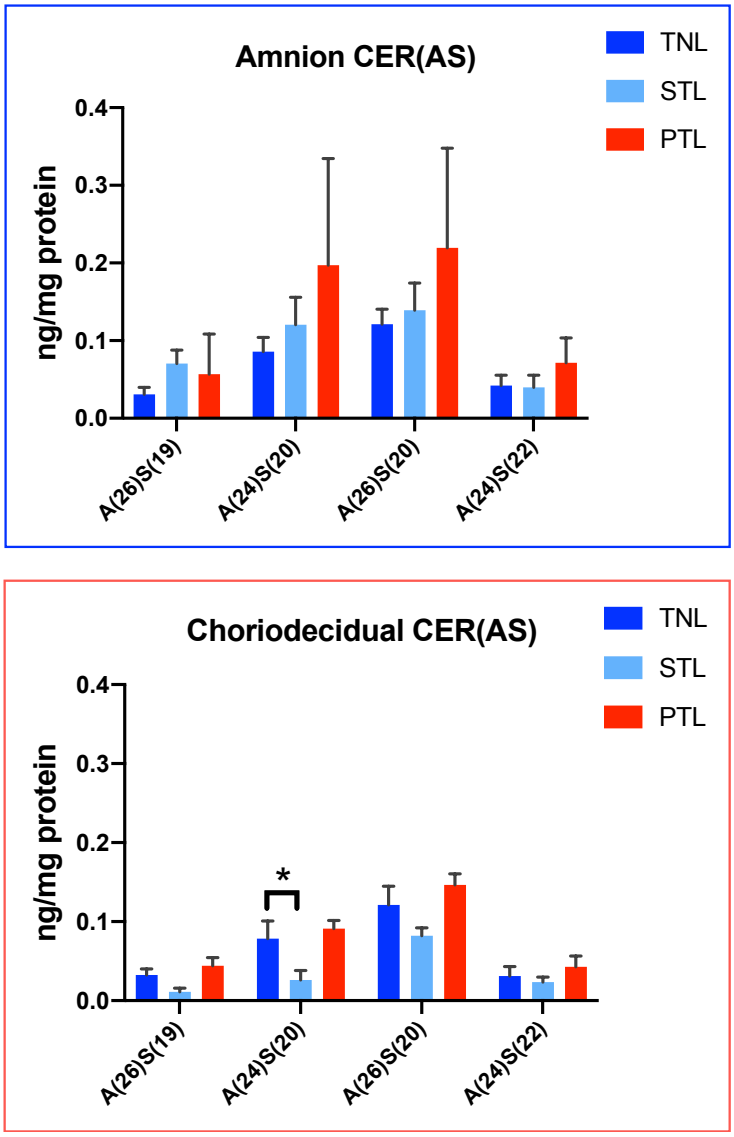


Figure 3.41. Preterm fetal membrane CER (AS). Amnion (top, blue frame) and choriodecidua (bottom, red frame). *Term not in labour (TNL), spontaneous term labour (STL) and preterm labour (PTL). Mean and SEM indicated. Statistical analysis = two-way ANOVA with Dunnett's multiple comparisons test, *p≤0.05, **p≤0.01.*

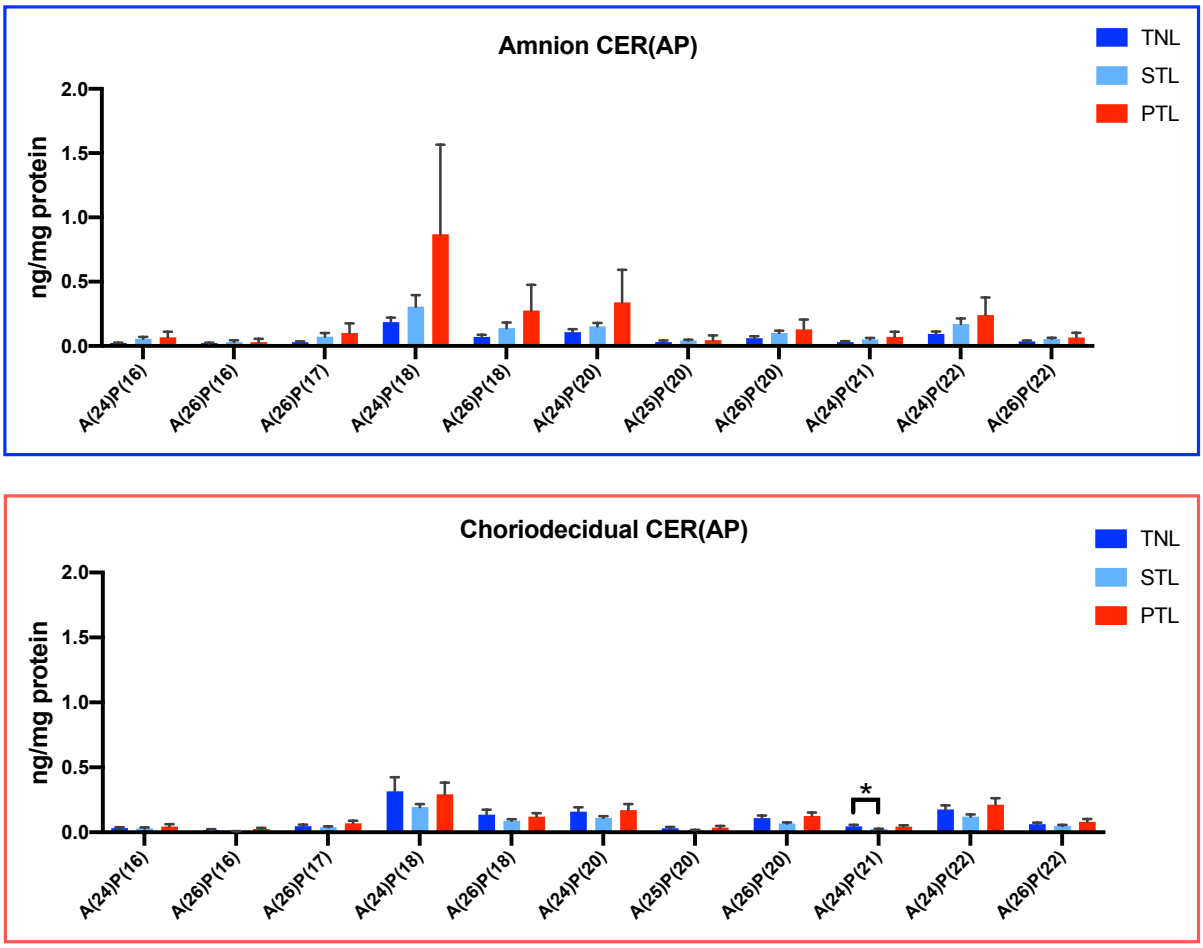


Figure 3.42. Preterm fetal membrane CER (AP). Amnion (top, blue frame) and choriodecidua (bottom, red frame). *Term not in labour (TNL), spontaneous term labour (STL) and preterm labour (PTL). Mean and SEM indicated. Statistical analysis = two-way ANOVA with Dunnett's multiple comparisons test, *p≤0.05, **p≤0.01.*

In summary, the preterm fetal membrane tissues produce ceramides from CER(NDS), CER(NS), CER(ADS), CER(AS), CER(AH), CER(NH), CER(AP) and CER(NP). Similar to the term study, CER(NS) was the dominant class in the preterm amnion and choriodecidua. In comparison to the TNL group, the PTL group showed an increasing trend of total ceramide production in the choriodecidua and lower levels in the amnion tissues. The PTL group showed a comparable or lower trend in most ceramide classes in amnion compared to TNL. However, in the choriodecidua, the preterm labour tissues expressed higher production of CER(NS) and CER(ADS), with other ceramide classes showing an increasing trend. Similar to the term study, the 18 carbon length sphingoid bases were the dominant CER(NS) species. Although an upregulation CER(NS) was observed in the preterm choriodecidua, the DEGS1 index failed to reflect this increase.

3.5. Discussion

To our knowledge, this is the first study to report a detailed profile of eicosanoids and related mediators, endocannabinoids and ceramides, using MS-based techniques, within fetal membranes under spontaneous term, preterm and drug-induced labour. In stimulating prostanoid production, drug-induced labour appeared most active within the amnion; whilst greater activity was afforded to the choriodecidua for all other "in labour" conditions. In addition to this distinction, total LOX- and CYP-mediators were selectively enhanced in the choriodecidua under term spontaneous labour, as opposed to preterm and induced labour equivalents. For classical endocannabinoids, both tissues were found capable of producing AEA and 2-AG. However, only spontaneous term and preterm labour stimulated AEA within the choriodecidua. Alongside elevations in total ceramide in all term labours, these collective findings support the hypothesis that the fetal membrane is an essential source of inflammation within human intrauterine tissues; and is therefore likely responsible for human parturition.

The amnion is the proximal half of the fetal membrane, adjacent to the amniotic fluid. As such, it is arguably the site where maturation signals from the fetus could initiate human parturition. Amnion baseline production of total prostanoids was greatest in the choriodecidua at term in pregnancies not yet in active labour. This finding agrees with a previous report where COX-2 expression was higher in the amnion than choriodecidua (241). PGE_2 was the dominant prostanoid produced by the amnion in this study. This aligns with previously reported exaggerations in amniotic fluid at this stage (242) and an increase in COX-2 and PGE_2 in the amnion following delivery (98). However, unlike this latter finding, the presented data here implies that PGE_2 levels are comparable between term spontaneous labour and those not-in labour. This dichotomy can be explained by the observed elevations in its metabolite (13,14, dihydro 15-keto PGE_2); which likewise confirms the amnion as a source of PGE_2, even before the clinical signs of labour have begun; a suggested also supported elsewhere (34). Although $PGF_{2\alpha}$ has been shown to increase in amniotic fluid in term labour (104), our data failed to yield a significant increase in spontaneous term labour. It is, therefore, possible that this rise in $PGF_{2\alpha}$ could be secreted by the amnion before the start of uterine contraction limits of cervical dilatation and amniotic pooling over the more direct measures of the fetal membranes considered here. In this chapter, preterm amnion

prostanoid production was comparable to term non-labouring tissues, perhaps implying that the amnion does not participate in preterm parturition; at least not from a prostanoid perspective. This finding agrees with Lee et al. (2010), who reported that COX-2 and PGE_2 were never raised in preterm labour (241). Within this study the preterm amnion didn't show an increase in $PGF_{2\alpha}$ production which agrees with an amnion study which reported no increase in amniotic fluid in infection-free preterm labour (243). To our knowledge, this first detailed prostanoids profile shows the effect of induction of labour on the fetal membrane prostanoid production. The drug-induced labour (the ITL group) obviously enhanced labour-associated prostanoids; PGE_2, $PGF_{2\alpha}$, and TXA_2 production in amnion.

The choriodecidua abuts the uterine muscles, making it a likely source of myometrial-derived inducers of contraction. Within this study, contractile prostanoids were detected within the choriodecidua of all in-labour groups, strongly indicative of its role in clinical onset. In term labour, PGE_2, $PGF_{2\alpha}$, and TXA_2 were all elevated in the choriodecidua; a result supported by other previous term labour findings, including enhanced PGE_2, COX-2 and PGF synthase (AKR1B1) expression (244–246). Within this study, the preterm choriodecidua selectively enhanced $PGF_{2\alpha}$ and TXA_2 (but not PGE_2), perhaps reflecting a different underlying mechanism to spontaneous labour at term. However, with both amnion and choriodecidua showing elevations in tocolytic prostanoids with induction of labour, it would appear fetal membrane is a key source of contractile prostaglandins post drug induction. In general, this emphasis across the fetal membrane, after induction, is supported by a previous report of raised PGF synthase (AKR1B1) (245).

PGI_2 is the dominant prostanoid detected previously within the human myometrium (175), where it is known to enhance uterine relaxation (247). In our study, PGI_2 was not detected in either half of the fetal membrane, which is also supported by its absence in the amniotic fluid at term in labour (104). In theory, this could imply that the fetal membranes and not the myometrium are the dominant source uterotonic mediators in the intrauterine cavity. Many reports have concluded that PGDH is expressed in choriodecidua and not amnion (248,249). However, in our own analysis, prostaglandin metabolites, such as 15-ket PGE2, 13,14 dihydro 15-keto PGE_2, were all detected in both amnion and choriodecidua, strongly indicating PGDH activity in both tissues.

The hydroxy fatty acid profiles of fetal membranes showed a wide range of metabolites, driven by the LOX and CYP450 pathways. Although many hydroxy fatty acids have been detected in amniotic fluid in earlier studies (104,250–252), their role in parturition remains ill-defined. In other tissues, LOX and CYP450-derived mediators are known to modulate the inflammatory environment and influence the vascular tone in physiological and pathological conditions (253–255). In gestational tissues, they could therefore help maintain a blood supply and sustain uterine contractibility during labour. Of the hydroxyl fatty acids detected, the LOX-derived metabolites were the most prevalent in both membrane tissues, with a dominance of HETEs and HODEs. Overall, a different pattern in total LOX mediators was noted between amnion and choriodecidua across the types of labour, which in many ways was similar to the prostanoids. Induction of labour elicited the greatest levels of LOX-derivatives, but there was a specific elevation in the choriodecidua with spontaneous labour; imply an increase in availability of the substrates (free PUFAs) within these tissues, and perhaps a richer supply of maternal blood to the choriodecidua over amnion.

Of the few hydroxy fatty acids studied in detail in reproductive tissues, 5-HETE has been found to stimulate uterine muscle (strip) contractions *ex vivo* (105). Also, a previous report defined an increase in 5-LOX protein expression in choriodecidua but not amnion with term labour (256). For this study, 5-HETE was significantly increased in both amnion and choriodecidua with term labour, suggesting elevations in 5-LOX in both tissues. In *in vitro* models, 12-HETE fails to show a contractile effect on the myometrium (105). However, it is reported to have a chemoattractant effect on human leukocytes (257), and therefore a possible active role. For both spontaneous and induced labour at term, amnion 12-HETE was significantly raised compared to non-labouring controls. This was replicated in the choriodecidua only in spontaneous labour, preterm or otherwise. An observation which again may be reflective of leukocyte invasion in choriodecidua, under both these conditions (258–260).

15-HETE was the most abundant lipoxygenase elevated in the choriodecidua with spontaneous term labour. Its function in parturition is unclear, but it has reported anti-inflammatory properties (261). In a previous observation by Maddipati et al. (2014), 5-HETE and 15 HETE were raised at term in amniotic fluid, but not 12-HETE (104). The data presented

here on the amnion, obtained from spontaneous term labour, has shown an increase of 5-HETE and 12-HETE but not 15-HETE, which may be reflective of different in-labour definitions. Overall, the choriodecidua seemed to have more polyunsaturated fatty acid resources than amnion, with more detectable levels of HETrEs, HDHAs and HEPEs within the spontaneous term labour group. This perhaps implies increased bioavailability of PUFA precursors from maternal blood and/or higher PLA_2 activity within the choriodecidua in these cases. In both tissues, LTB_4 was under the limit of detection in all groups. This finding supports the assumption that the tissues were infection-free, as an elevation in amniotic fluid LTB_4 is strongly associated with chorioamnionitis (226).

In this study, the fetal membrane was found to produce CYP450-derived lipid mediators, including fatty acyl epoxides and diols. A specific role for the epoxides and diols in parturition has not been defined. However, the AA-derived EETs are considered anti-inflammatory and modify vascular tone in other tissues (261). There are few *in vitro* studies that have examined epoxygenase mediators in gestational tissues. For example, a report by Pearson et al. (2009) explored 5(6)EET in myometrium and uterine arteries and found it enhanced relaxation in both tissues(101). Another study by Gonzalez et al. (1997) reported the uterine contractile effect of 14(15)EET on rat uterine muscles (262). Within this study, the amnion has shown the ability to produce three EETs: 5(6)EET, 8(9)EET and 11(12)EET; whilst choriodecidua four: 5(6)EET, 8(9)EET, 11(12)EET, and 14(15)EET. In general, these data agree with an amniotic fluid profile published by Maddipati et al. (2014), which highlighted an increase in 8(9)EET and 11(12)EET with term spontaneous labour(104). However, unlike these fluid assessments, the membrane data reported here adds a further increase in 5(6)EET and 8(9)EET in the amnion, without 14(15)EET and 20-HETE detection.

For the fetal tissues considered here, both showed an ability to produce LA-derived CYP450 mediators, predominantly 9(10)EpOME and 12(13)EpOME, and also their metabolites 9,10 and 12,13 DiHOME. Likewise, 12(13)EpOME was significantly raised in spontaneous term labour in both sides of the membrane. These data suggest that upregulation of the epoxides is unique for spontaneous term labour, and given what is reported about their function, a possible role could be envisaged for them to enhance myometrial contractility and to increase blood flow to gestational tissues during labour. The CYP/sEH index showed a trend for

inhibition in spontaneous term labour in both membranes, although this only reached significance in the amnion. This finding may indicate that both amnion and choriodecidua actively try to maintain epoxides to stimulate uterine contractions and/or remodel the uterine vasculature to increase blood flow to the gestational tissues.

This study has also considered the production of endocannabinoids and endocannabinoid-like lipids, including NAEs and MAGs, by the fetal membranes. The endocannabinoids, AEA and 2-AG have been reported to stimulate PGE_2 production in fetal membrane explants through CB1 receptors (127). The data presented shows that AEA is detected only in the choriodecidua, while 2-AG is found in both membranes. For the choriodecidua, AEA was increased in spontaneous and preterm labouring groups, as supported by previous reports of increased AEA in maternal plasma during spontaneous term delivery (60) and in women with a high risk of preterm labour (263). Although another study reported an increase in maternal plasma AEA after labour induction (125), our data would imply this rise is not from fetal membranes. 2-AG expression was higher in the amnion than choriodecidua, but no alterations were associated with the types of labour. In choriodecidua, 2-AG was raised only after induction, indicating that neither tissue is likely a source of 2-AG in physiological labour. In preterm labour, 2-AG was significantly lower than at term, perhaps suggesting an increase with advancing gestation. Another possible explanation is increasing 2-AG breakdown by diacylglycerol lipase (DAGL), which releases incorporated AA to be metabolised in other pathways. LEA showed a significant increase in both spontaneous labouring conditions (STL and PTL) in amnion and choriodecidua. The importance of this again is unclear, as the action of LEA on reproductive tissues is not well studied. Nevertheless, it reportedly binds CB receptors (264) and has anti-inflammatory effects, inhibiting FAAH activity and increasing the bioavailability of NEAs, including AEA (265,266). Given the upregulation of LEA in all spontaneous term and preterm conditions, both in amnion and choriodecidua, this could flag a previously unrecognised role in human parturition.

PEA is a potent PPAR-α agonist that enhances anti-inflammatory actions, and acts as an analgesic compound (237). Birchenall et al. (2019) reported an increase of PEA in maternal plasma after term delivery (78). However, the choriodecidual analysis here has shown an increasing trend in PEA, which failed to reach statistical significance. Low POEA was also

defined and also elevated significantly in the amnion in spontaneous labour and induction, and in the choriodecidua under all labouring conditions. The role of palmitoleic acid, the precursor of POEA, in reproductive tissues is unknown. However, palmitoleic acid has been shown to suppress pro-inflammatory cytokines and enhances insulin sensitivity in other conditions (267). DHEA was also at a measurable level, but only in the choriodecidua. Along with the eicosanoid observations, these findings confirm that the choriodecidua is richer in omega-3 PUFA-derived lipids than amnion.

PDEA, HEA and VEA were all upregulated with term labour in choriodecidua. Bachkangi et al. (2019) have shown that increased AEA in maternal plasma may predict preterm labour (263). From observations here, the rise in AEA and LEA, POEA, HEA, and VEA in choriodecidua would likely reflect an increase in maternal blood within the tissue and, as such may well be used to improve prediction of labour onset. The upregulation of NAEs in choriodecidua could indicate an upregulation of its synthesis pathway through increased NAPE-PLD activity. Also, these lipids could compete with the FAAH enzyme, which leads to an increase in AEA's bioactivity in intrauterine tissues. Again, exploring the biological action of these NEAs across gestation could provide a better understanding of their physiological and pathological roles in human parturition.

Ceramides are sphingolipids known to stimulate NF-κB (268) and are known to upregulate COX-2 and oxytocin receptor expression (81). In a previous study, non-hydroxy acyl ceramide (extracted from the bovine brain) enhanced PGE_2 secretion in amnion and decidual cells (269). In this study, non-hydroxy fatty acid ceramides (CER(NDS) and CER(NS)) classes were significantly elevated in the choriodecidual with spontaneous term labour. Also, the preterm choriodecidua produced high CER(NS); data which perhaps indicate that ceramides could enhance contractile prostanoids and other inflammatory mediators in choriodecidua in physiological and pathological scenarios. In amnion, an increase in total ceramide production was observed only in the term labour group. The spontaneous labour cohort showed a trend for increased ceramide classes (CER(NDS), CER(NS), and CER(ADS)). Whilst post-induction: CER(NP), CER(NH), CER(ADS), CER(AS), and CER(AP) classes were raised against controls, perhaps indicating that the amnion produces more ceramide classes under drug-induced conditions than physiological labour. Again, these data may also indicate that choriodecidua

is more reactive than amnion with regard to ceramide production in physiological and pathological parturition.

Signorelli et al. (2016) reported placental production of total CER(NS), and C16:0, 24:0, and 24:1 acyl containing CER(NS) were significantly elevated in spontaneous and induced term labour (42). We have observed a similar increase in 24 and 22 carbon length acyl chain CER(NS) species within the choriodecidua. However, the 16 carbon length acyl chain CER(NS) species were below detection limits, and C24:1 was not included in this analysis. Laudanski et al. (2016) reported a greater increase in plasma C16:0 acyl bearing CER(NS) in preterm labour over false preterm labour (79). Within this study, the 16 carbon length acyl chain CER(NS) species were below the limit of detection within the preterm choriodecidua; but the following species: N(24)S(17) and N(26)S(17) and N(28)S(18) were also significantly elevated. Here the ceramides are reported in a more detailed way, including the acyl chain's carbon length and sphingoid bases. Thus, a possible involvement of these species with either inflammatory roles in gestational tissues or uterine contractility would be more specific to human parturition. Feasibly, if the upregulation of ceramides in the preterm choriodecidua is matched or reflected to maternal blood, this finding could also have possible use in improving current preterm labour prediction. However, this possibility needs to be further explored.

In choriodecidua, dihydroceramide CER(NDS) and CER(ADS) were observed upregulated in the term labouring groups; and CER(ADS) also in preterm labour. In the round, this could indicate an upregulation of SPT, the rate-liming enzyme in the *de novo* pathway. This observation is supported by a published report where placental SPT was upregulated in term labour (42). The amnion showed an increasing trend for CER(NS) in spontaneous term labour. However, in both tissues, the DEGS1 index failed to match the analytical finding of raised CER(NS). Although paradoxical, this may be explained by a shift away from the *de novo* pathway towards either sphingomyelin hydrolysis and/or salvage pathways, and this possibility could be addressed by measuring the protein expression of the enzymes. The endocannabinoids with the activation of CB1 are reported to stimulate ceramide production by *de novo* and SMase pathways (270). Given the choriodecidual observations of increased AEA in tandem with CER(NS), in the spontaneous term and preterm labour, a possible role of AEA in

enhancing ceramide generation may be possible. However, here again, further studies are necessary.

This study has also considered the more detailed species of ceramides. In amnion and choriodecidua, 18-carbon sphingoid bases were the most abundant species in the non-hydroxy ceramides. This analysis also showed the presence of very-long-chain fatty acids. For instance, the dominant fatty acyl chain lengths in CER(NS) species ranged between 22 to 24 carbons. Any such increase in very-long-chain fatty acids could indicate an upregulation of fatty acid elongases and concomitant elongation of very-long-chain fatty acids (ELOVLs) (271). Also, the length of the fatty acyl chains in each ceramide species could be used to signpost the ceramide synthase (CerS) isoforms involved, as these are fatty acid chain length become specific (37). Notwithstanding, this suggestion could prove challenging to achieve in this study, with such a wide range of carbon chain lengths associated with the fetal membranes.

3.6. Conclusion

In conclusion, both sides of the fetal membrane showed unique lipid mediator profiles. Only at term, the amnion more actively produces prostanoids, LOX-derived mediators, and ceramides following drug-induced labour, whilst spontaneous labour showed upregulation of total ceramide production. Perhaps given the location and richness of maceral blood, the choriodecidua was more involved (than amnion) and more active in prostanoid production in all in-labour study groups. In spontaneous term and preterm labour, endocannabinoid activity was increased in the choriodecidua. Also, choriodecidual ceramide production was elevated in both term in-labour groups. Such differences clearly suggest the active involvement of eicosanoids, endocannabinoids and ceramides in the process of human parturition. These data suggest that of the two, the choriodecidua is more active in lipid mediator production than amnion when active labour is manifest. Also, that preterm labour doesn't seem to activate the amnion, perhaps implying that fetal maturation is always necessary for this part of spontaneous human parturition.

In general, bioactive lipids are known to contribute to inflammatory signalling pathways. However, besides prostanoids, there is little known about the role of hydroxy fatty acids,

endocannabinoids or ceramides in the process of human labour. These data certainly strengthen the need for further exploration of their roles in either: myometrial contractility, the weakening fetal membranes, and the inflammatory signals which kick start and propagate the labour cascade. It is also envisaged that the further study of these molecules and underlying mechanisms could expedite development of new drug targets and therapeutics to control and mitigate human labour mistiming.

Chapter 4: Fetal membrane complex lipids and fatty acid abundance

4.1. Introduction

How parturition is initiated in humans remains a conundrum. What is known is that upscaling of intrauterine inflammation is a key element and that lipid mediators play a crucial role. In humans, arachidonic acid (AA)-derived prostaglandins have proven clinical efficacy in initiating labour, as signalled by cervical ripening and uterine contractions (220). Thus, at the heart of these physiological events are lipids, and a greater understanding of their role in human partition is required.

Across all human physiology, lipids are essential as a source of energy (38), as cellular membrane components, and as signalling molecules (39). One group, the glycerophospholipids, which includes phosphatidylcholine (PC) and phosphatidylethanolamine (PE), are integral to cell membranes and contain two fatty acids, with second position (sn-2) favourable for AA and other polyunsaturated FAs (PUFAs). Phospholipase A_2 (PLA_2) is an enzyme that releases these incorporated PUFAs into a free form, which can then be utilised as cell and tissue signalling mediators, such as the eicosanoids (92). Cytosolic PLA_2 ($cPLA_2$) is a subtype of PLA_2 that is known to be upregulated within the gestational tissues with advancing gestational age and at labour (182,202). Moreover, $cPLA_2$ has a suggested distinct role in initiating labour, as $cPLA_2$ mutant mice failed to enter spontaneous labour (93).

In addition to glycerophospholipids, sphingomyelins (SM) are a subclass of sphingolipids that maintain the cell membrane, but also are a source of ceramides when hydrolysed by sphingomyelinases (SMase) (272). Ceramides are well-recognised second messengers regulating the inflammatory milieu, by stimulating cytokine production and NF-κB expression, and controlling cellular homeostasis, through regulation of proliferation, differentiation and apoptosis (273). Recent studies have shown ceramide to be upregulate in placenta and maternal blood during physiological labour (42,78). However, gestational tissue SM profile

and changes under different types of labour have not been reported yet. Understanding SM changes within these tissues could give a hint about the ceramide synthesis pathway.

Like lipids in general, cholesterol itself is crucial for cell membrane structure and fluidity (274) and precursor for steroid hormones, including progesterone and cortisol (83), which are essential for maintaining pregnancy and fetal development, i.e. stimulation of fetal lung maturation at term (275). Of the glucocorticoids, cortisol is produced by gestational tissues (276) and has capacity to propagate parturition by upregulation of uterotonic prostaglandin from the fetal membranes (277). Within cells and the circulation, cholesterol is used to store excess fatty acids in the form of cholesterol esters (CE), as governed by the actions of lecithin cholesterol acyltransferase (LCAT) enzyme. Akin to CE, triglycerides (TG) are another class of lipids capable of storing fatty acids as intracellular lipid droplets (278). Diacylglyceride (DG) is the precursor for TG synthesis (38), and is generated from hydrolysing glycerophospholipid via the action of phospholipase C (PLC) (67). DG is also a source of 2-AG and other monoacylglycerols (MAG) by the action of diacylglycerol lipase (DAGL) (115). This classical endocannabinoid can also be a source of free AA by the action of monoacylglycerol lipase (MAGL)(279).

Following spontaneous human labour, lipidomic profiles, such as glycerophospholipids, sphingolipids, cholesterol and free fatty acids, are altered in both the maternal circulation and fetal cord blood (78); implying that parturition either influences lipidomic compositions in maternal and fetal compartments or precedes it. In **Chapter 3** (Fetal membrane lipid mediator profiling), it was shown that both sides of the fetal membrane, the amnion and choriodecidua, are capable of producing a wide range of inflammatory lipid mediators, including eicosanoids, ceramides and endocannabinoids, indicating stimulation of the inflammatory milieu at the fetal-maternal border. Amnion was producing more AA-derived COX and LOX mediators and ceramide in term labour. Choriodecidua actively produced eicosanoids, endocannabinoids and ceramides in term and preterm conditions, but more pronounced in spontaneous term labour. These lipid mediators are generated from different complex lipid structures and pathways. Therefore, understanding precise changes in the fetal membrane lipidome within these types of labour, may give stronger clues to the timing and origins of these mediators and how they were generated.

This chapter will test the hypothesis that fetal membrane lipid composition is altered fundamentally in different types of human labour and that these unique profiles are in direct contrast to non-labouring pregnancies at term. The aim was to profile structural lipid composition within the fetal membranes using UHPSFC-ESI-MS/MS and GC-FID analysis, to provide high sensitivity and a wide range of lipid species. The objectives were to discover (a) the complex lipid profiles and (b) total abundance of fatty acids within both amnion and choriodecidua under various labour scenarios: spontaneous term (STL), induction at term (ITL) and preterm labour (PTL) onset.

4.2. Material and methods

Amnion and choriodecidua were sampled and stored are described in **section 2.1**. The extraction of complex lipids; along with equipment, materials, methods and data processing are also explained in **section 2.5**. Complex-lipid analysis was performed with UHPSFC-ESI-MS/MS, provided by Waters (Wilmslow, UK). Fetal membrane lipid extraction for total fatty acids was performed by GC-FID (Agilent Technologies, Stockport, UK) as described in **section 2.7**.

4.3. Statistical analysis

Fetal membrane complex lipids and fatty acid abundance were analysed with Prism (v.8) (GraphPad, California, USA). Each sample was analysed in duplicate (technical repeats) Complex lipid data were corrected to tissue protein content (see **section 2.6**) and expressed as means and standard error of the mean (SEM), after confirming normality with Shapiro-Wilk and Kolmogorov–Smirnov tests for normal distribution. The statistical approach for complex lipids was similar to that of **Chapter 3**, i.e. one-way ANOVA for total lipid classes, and two-way ANOVA for the detailed profiling, following initial log-transformations. Only total PE values gave confirmed normality, and raw data was therefore used without transformation. Dunnett's multiple comparisons where used to compare pregnancy groups against term non-labour controls (TNL). For all fatty acid abundances, normally distribution was achieved and results presented as means and standard deviations (SD). One-way ANOVA with Dunnett's post hoc correction was used comparing labouring groups to TNL, and a Pearson correlation coefficient investigated relationships between individual fatty acids to AA.

4.4. Results

4.4.1. Fetal membrane complex lipid profiles

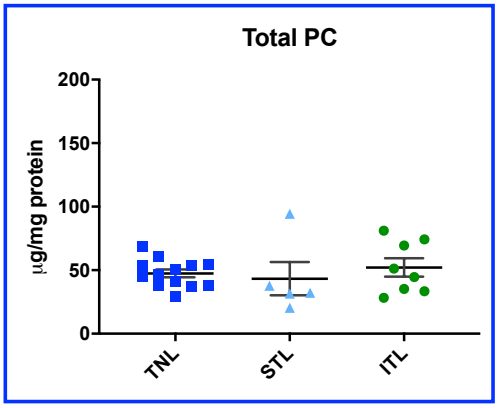
Similar to the previous chapter, fetal membrane tissues (amnion and choriodecidua) were grouped into “term” and “preterm” pregnancies. For the term cohorts, spontaneous term labour pregnancies (STL, n=5 amnion and n=9 choriodecidua), along with drug-induced term labour (ITL, n=9 amnion and n=9 choriodecidua) were compared to term pregnancies which were not yet in labour (TNL, n=12 amnion and n=13 choriodecidua). For the “preterm study” comparisons were drawn between preterm labour (PTL, n=5 and 6, amnion and choriodecidua, respectively) and STL and TNL. Lipid profiles included PC, PE, PG, LPC, LPE, TG, DG, SM, cholesterol, CE and FFAs. Using UHPSFC-ESI-MS/MS, lipid compounds were determined as total carbon atoms and double bonds; however, the positions of these double bonds could not be distinguished. Under this analysis, PC and PE species with shared mass could contain acyl, ether, or vinyl-ether-bonds at the sn-1 position. Without further distinction between these isobaric species, these three possibilities were reported as follows; using PC as an example: 35:5/36:4p/36:5e.

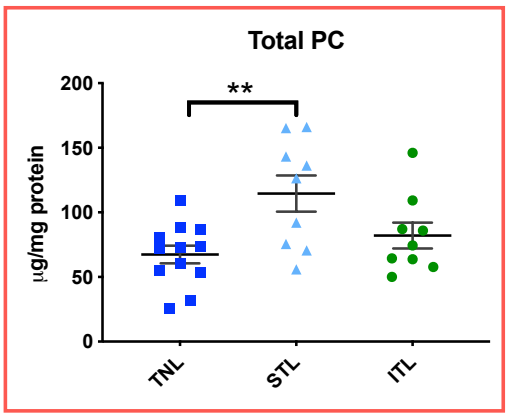
4.4.1.1. Term fetal membrane lipid profiles

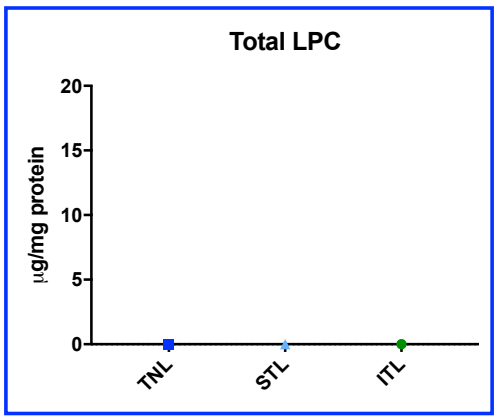
Figure 4.1 shows the lipid classes in term fetal membranes. In the amnion, nine classes were detected, from highest to lowest concentrations: PC (47.466 μg/mg protein), PE (26.565 μg/mg), TG (15.149 μg/mg), free fatty acids (FFA) (8.01 μg/mg), cholesterol (7.29 μg/mg), SM (2.09 μg/mg), DG (1.27 μg/mg), PG (0.11 μg/mg) and CE (0.04 μg/mg). Ten lipid classes were defined within the choriodecidua; highest to lowest; PC (67.45 μg/mg), FFA (40.06 μg/mg), PE (36.68 μg/mg), SM (13.9 μg/mg), CE (7.57 μg/mg), TG (5.2 μg/mg), LPC (1.79 μg/mg), DG (1.5 μg/mg), PG (0.4 μg/mg) and cholesterol (0.26 μg/mg). Within these studies, PS and LPE levels were below the limit of detection. Comparing membranes, the choriodecidua showed higher lipid content for SM, CE, LPC, and FFA; whilst amnion was higher for TG and cholesterol only.

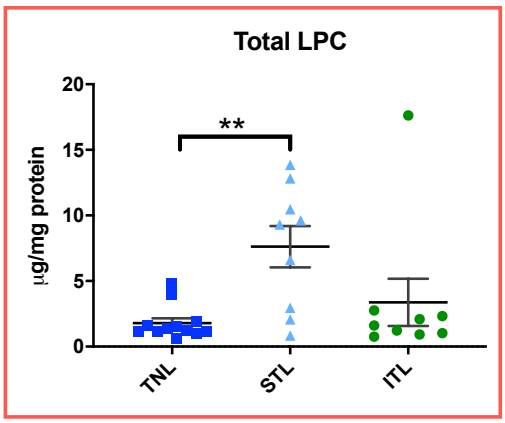
For amnion, labouring showed a comparable pattern of lipid distribution to TNL, except for FFA, PG and TG, which all rose significantly. For choriodecidua, the STL group showed higher levels of all lipids, except for PE, PG, and DG which failed to statistically diverge. The ITL group

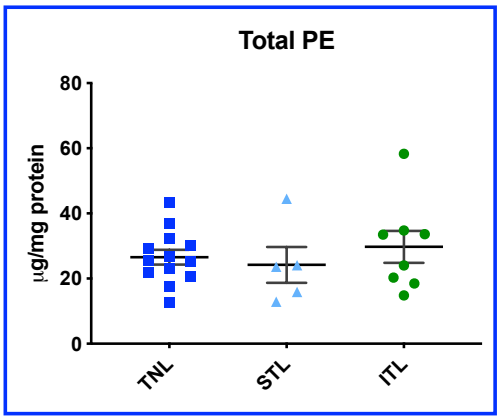
showed comparable values to TNL for all lipid classes, except for TG, which was markedly increased (see **Figure 4.1**).

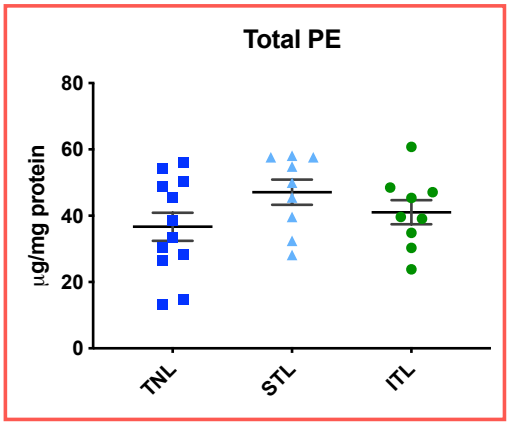


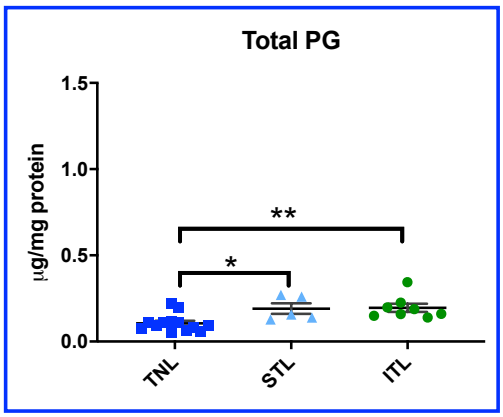


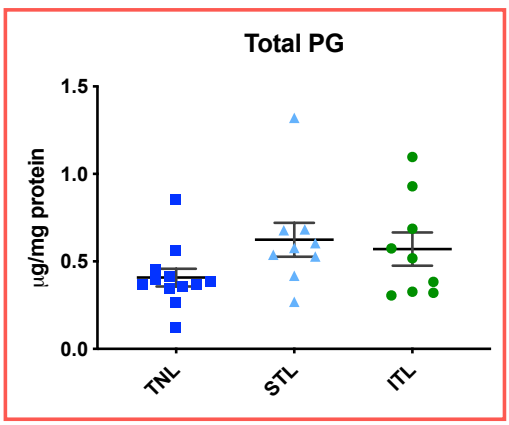






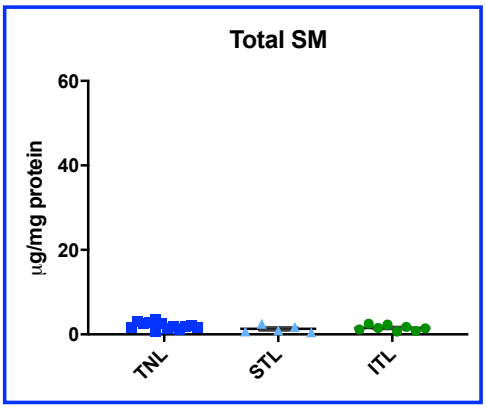


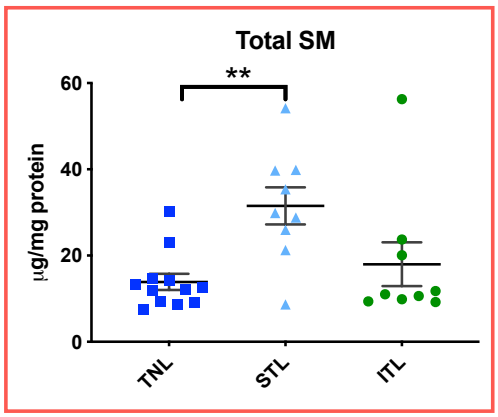


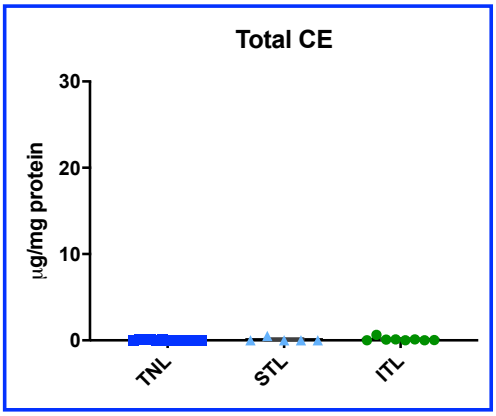


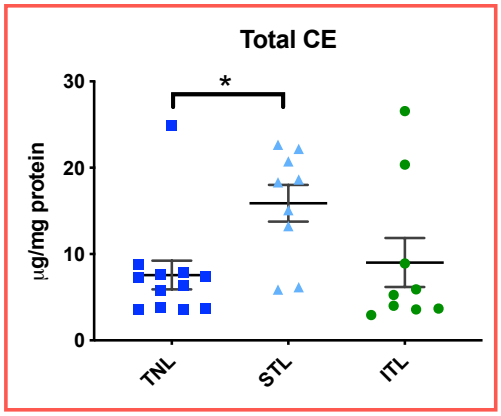
Amnion

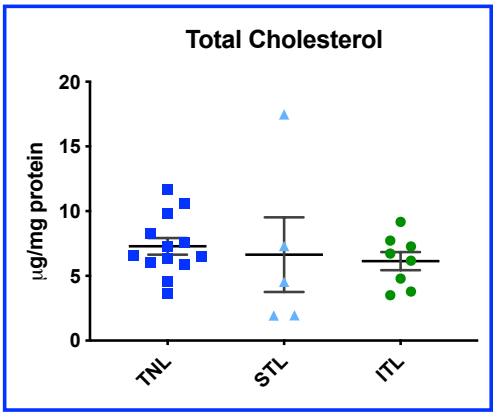
Choriodecidua

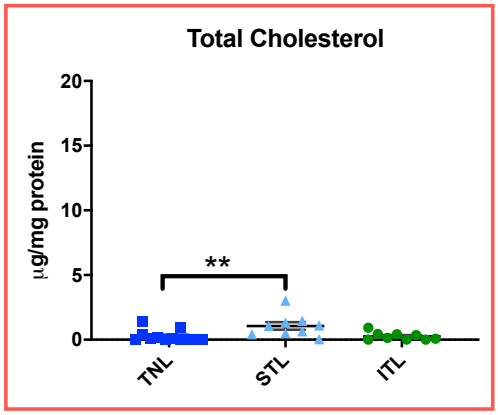


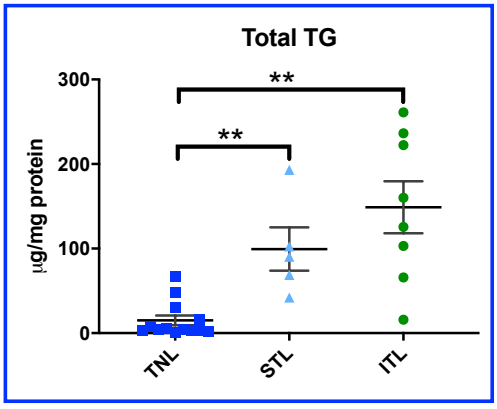


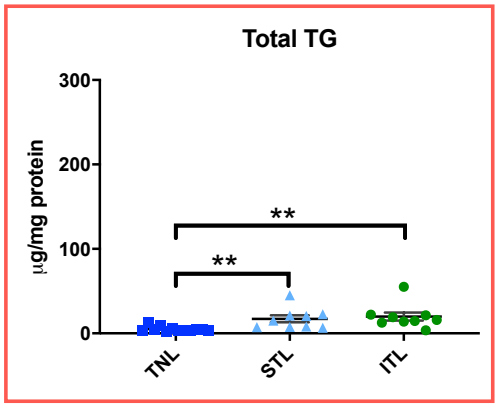












Amnion

Choriodecidua

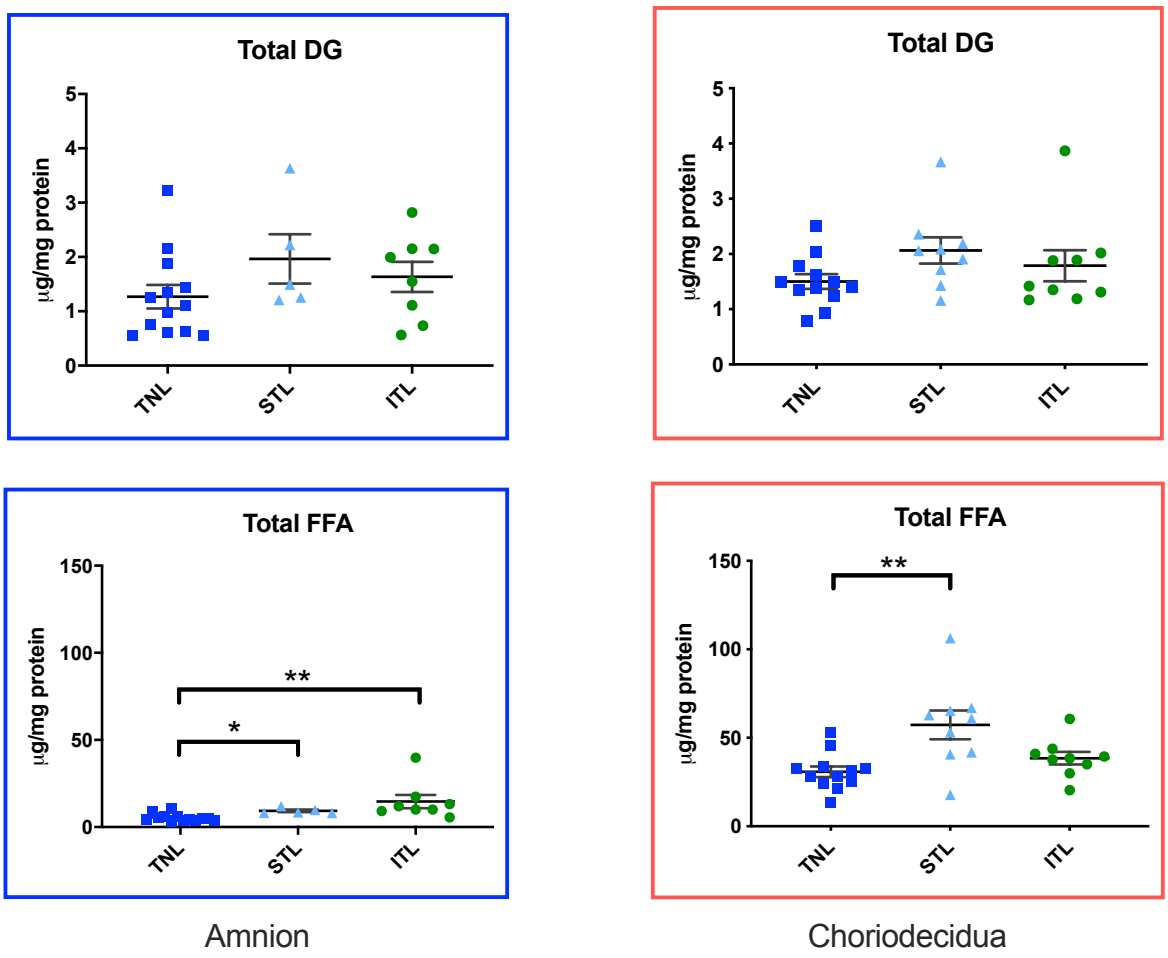
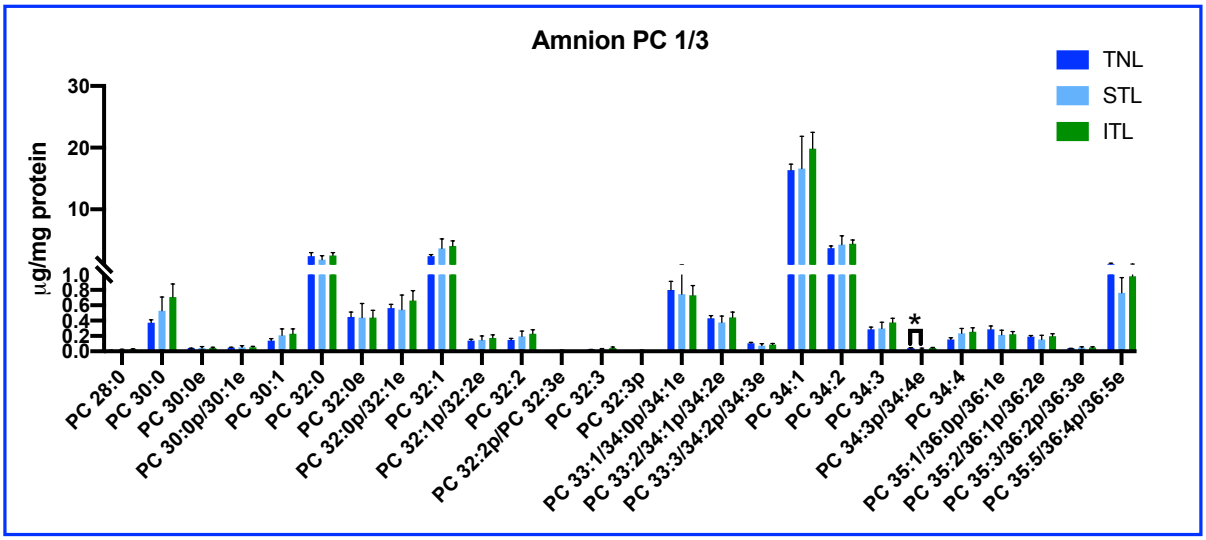
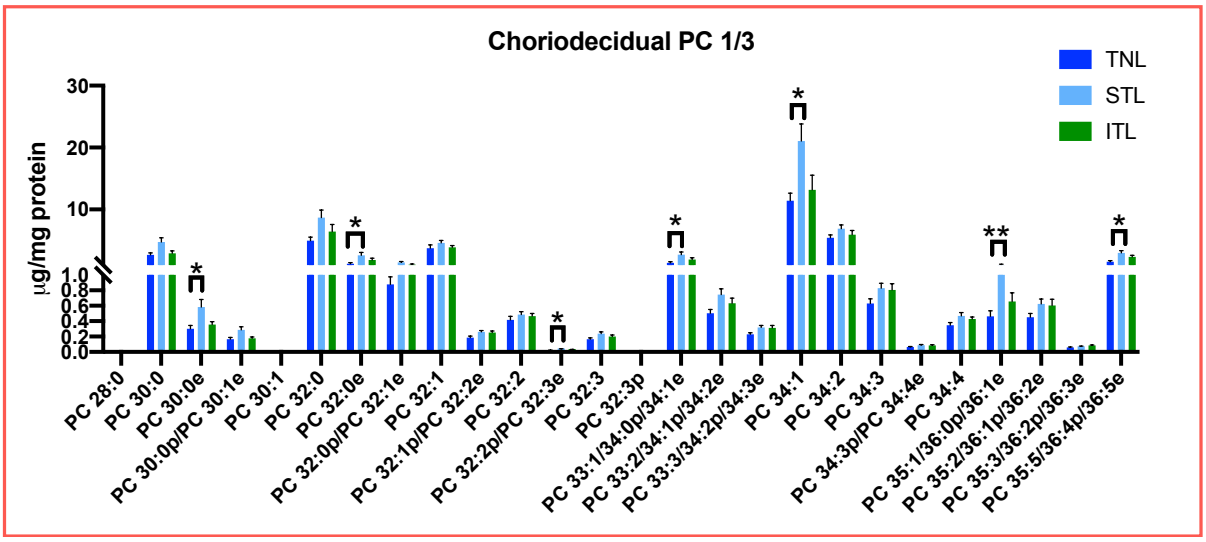


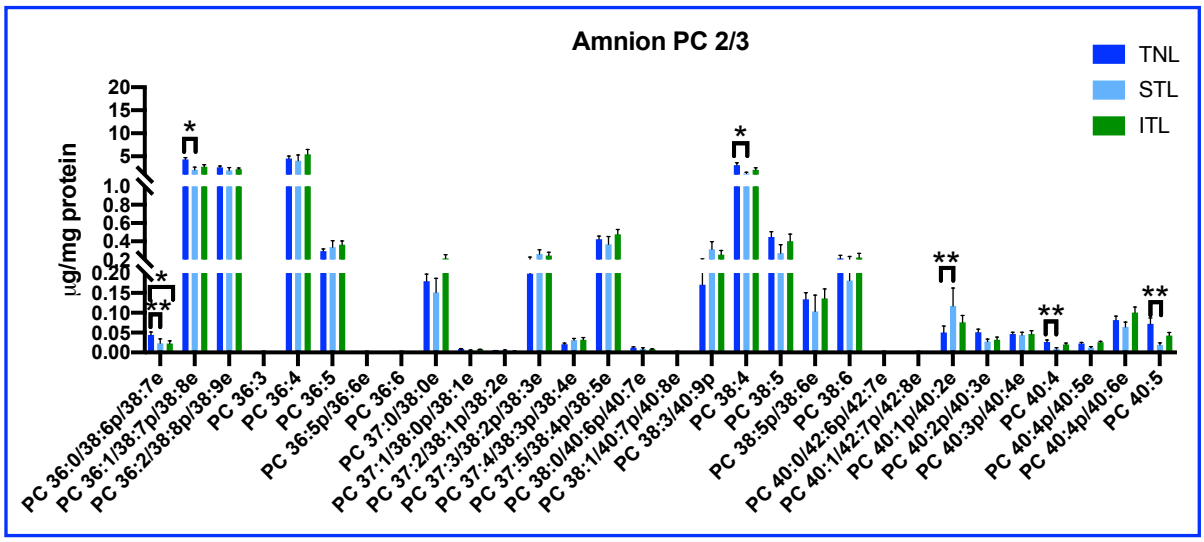
Figure 4.1. Term fetal membrane complex lipids. *Amnion (left, blue frame) and choriodecidua (right, red frame). Term not in labour (TNL), spontaneous term labour (STL) and induced term labour (ITL). Mean and SEM indicated. Statistical analysis = one-way ANOVA with Dunnett's multiple comparisons test, *p≤0.05, **p≤0.01.*

Figure 4.2 shows detailed PC species within the term fetal membranes; 50 and 72 PC species were detected in the amnion and choriodecidua, respectively. In both tissues, PC 34:1, 36:4, 36:1/38:7p/38:8e, 34:2, and 38:4 were most dominant. For the amnion, STL and ITL groups showed comparable expression to TNL for most PC species. However, a few - possibly containing AA (20:4) - were significantly reduced in STL, specifically: PC 34:3p/34:4e, 36:0/38:6p/38:7e, 36:1/38:7p/38:8e, 38:4, 40:4, 40:5, 42:5p/42:6e. Alternatively, a single species, PC 40:1p/40:2e was markedly elevated in STL vs. TNL….For the ITL group, a trend for decreased AA-containing species was evident; with one species in particular, PC36:0/38:6p/38:7e, reaching statistical significance.

In the choriodecidua, both STL and ITL showed a trend for upregulated PC species against TNL, but with greater propensity in STL over ITL. Overall, STL produced a significant increase in 31 PC species; including the possible AA-containing PC 30:0e, 32:0e, 32:2p/32:3e, 33:1/34:0p/34:1e,34:1, 35:1/36:0p/36:1e, 35:5/36:4p/36:5e, 36:5p/36:6e, 36:6, 37:1/38:0p/38:1e, 37:4/38:3p/38:4e, 37:5/38:4p/38:5e, 38:0/40:6p/40:7e, 38:4, 38:5p/38:6e, 38:6, 40:0/42:6p/42:7e, 40:1/42:7p/42:8e, 40:1p/40:2e, 40:6, 42:0/44:6p/44:7e, 42:1/44:7p/44:8e, 42:1p/42:2e, 42:2/44:8p/44:9e, 42:3p/42:4e, 42:4p/42:5e, 42:5p/42:6e, 44:0/46:6p/46:7e, 44:1/46:7p/46:8e and 44:5p/44:6e. Only 6 species reached a significant increase in the ITL group, namely PC 37:1/38:0p/38:1e, 42:0/44:6p/44:7e, 42:3p/42:4e, 40:0/42:6p/42:7e, 40:1/42:7p/42:8e and 42:5p/42:6e.






Amnion PC 2/3
TNL
STL
ITL
µg/mg protein
PC 36:0/38:6p/38:7e
PC 36:1/38:7p/38:8e
PC 36:2/38:8p/38:9e
PC 36:3
PC 36:4
PC 36:5
PC 36:5p/36:6e
PC 36:6
PC 37:0/38:0e
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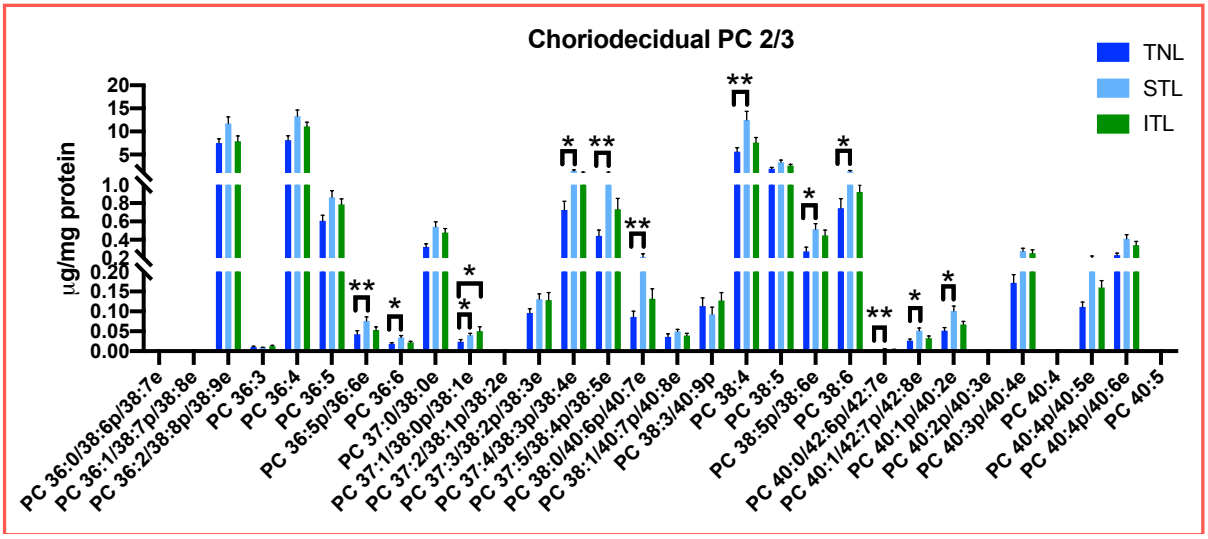
PC 40:5


Choriodecidual PC 2/3
TNL
STL
ITL
µg/mg protein
PC 36:0/38:6p/38:7e
PC 36:1/38:7p/38:8e
PC 36:2/38:8p/38:9e
PC 36:3
PC 36:4
PC 36:5
PC 36:5p/36:6e
PC 36:6
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PC 38:4
PC 38:5
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PC 40:1/42:7p/42:8e
PC 40:1p/40:2e
PC 40:2p/40:3e
PC 40:3p/40:4e
PC 40:4
PC 40:4p/40:5e
PC 40:4p/40:6e
PC 40:5

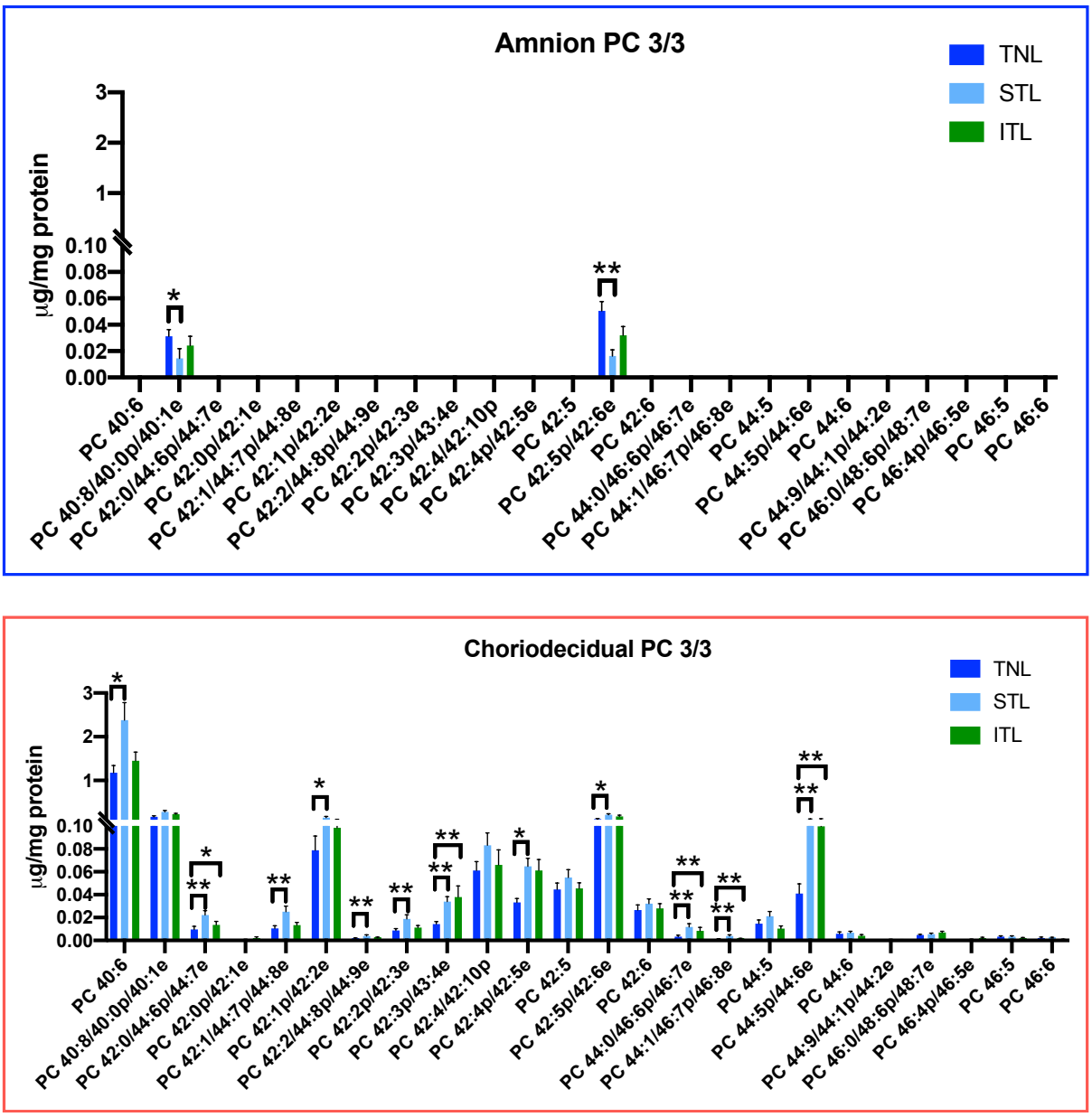
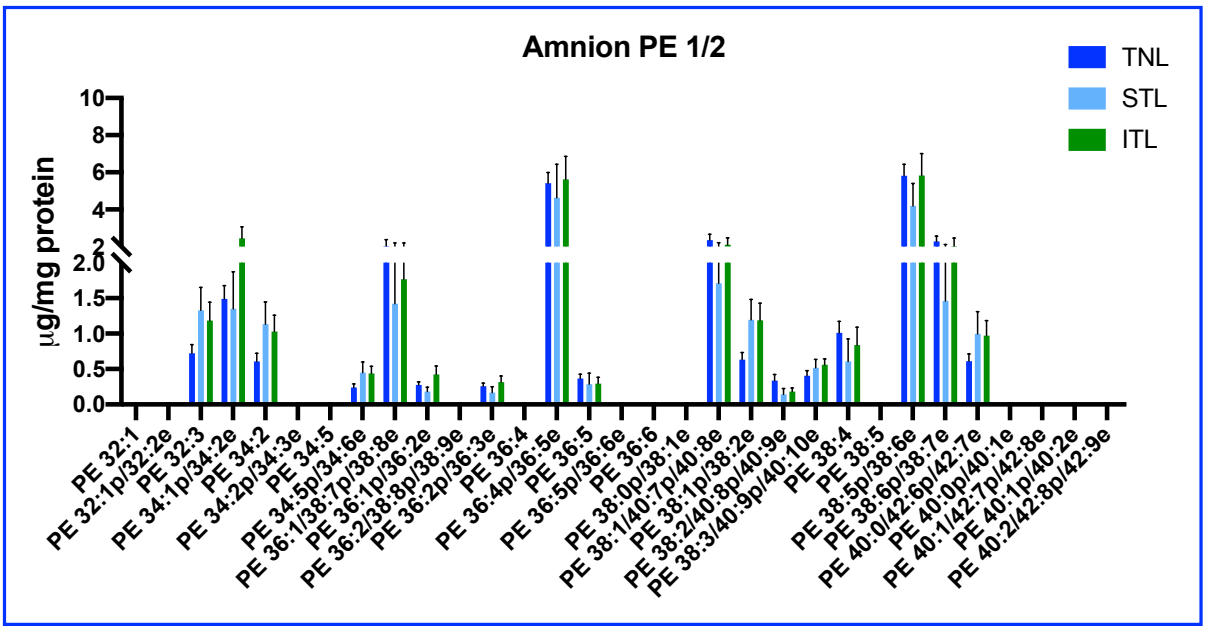


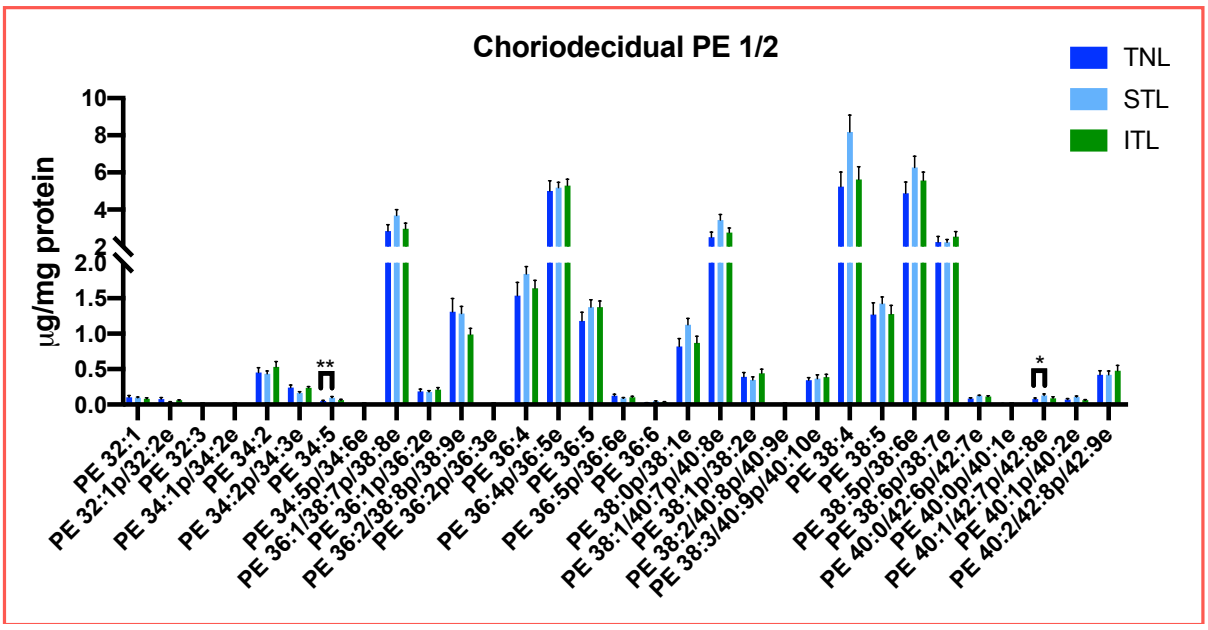
Figure 4.2. Term fetal membrane PC species. *Amnion (top, blue frame) and choriodecidua (bottom, red frame). Term not in labour (TNL), spontaneous term labour (STL) and induced term labour (ITL). Mean and SEM indicated. Statistical analysis = two-way ANOVA with Dunnett's multiple comparisons test, *p≤0.05, **p≤0.01.*

Figure 4.3 shows a detailed profile of PE species in the amnion and choriodecidua. Overall, the choriodecidua profiled a higher number than amnion, with 57 vs. 21 species, respectively. Similar to the PC profiling above, the dominant PE species were potentially AA-containing. The most abundant in the amnion were PE 38:5p/38:6e, 36:4p/36:5e, 38:1/40:7p/40:8e, 38:6p/38:7e, 36:1/38:7p/38:8e; and for the choriodecidua; PE 38:4, 36:4p/36:5e, 38:5p/38:6e, 36:1/38:7p/38:8e, 38:1/40:7p/40:8e.

In the amnion, the STL group showed a trend for decreased concentrations of most PE species, especially those of AA-containing species, potentially: 38:1/40:7p/40:8e, 38:2/40:8p/40:9e,38:5p/38:6e, 40:0/42:6p/42:7e. Nevertheless, of these only PE 40:7/40:0e reached statistical significance. ITL values for PE were similar to TNL for all recorded species.

Term labour showed an increase in PE species within the choriodecidua, but this was most noticeable for STL over ITL. More specifically, the following 12 species with were markedly raised in the STL group: PE 34:5, 40:1/42:7p/42:8e, 42:0/44:6p/44:7e, 42:2/44:8p/44:9e, 42:2p/42:3e, 43:3p/43:4e, 42:7/42:0e, 42:8/42:0p/42:1e, 44:5p/44:6e, 44:8/44:0p/44:1e, 46:2/48:8p/48:9e and 46:5p/46:6e. Alternatively, 6 were elevated in the ITL group; 44:8/44:0p/44:1e, 46:1/48:7p/48:8e, 46:2/48:8p/48:9e, 46:5p/46:6e, 48:5 and 48:8/48:0p/48:1e; all with potential for being AA-containing species.


Amnion PE 1/2
TNL
STL
ITL
µg/mg protein
10
8
6
4
2
2.0
1.5
1.0
0.5
0.0
PE 32:1
PE 32:1p/32:2e
PE 32:3
PE 34:1p/34:2e
PE 34:2
PE 34:2p/34:3e
PE 34:5
PE 34:5p/34:6e
PE 36:1/38:7p/38:8e
PE 36:1p/36:2e
PE 36:2/38:8p/38:9e
PE 36:2p/36:3e
PE 36:4
PE 36:4p/36:5e
PE 36:5
PE 36:5p/36:6e
PE 36:6
PE 38:0p/38:1e
PE 38:1/40:7p/40:8e
PE 38:1p/38:2e
PE 38:2/40:8p/40:9e
PE 38:3/40:9p/40:10e
PE 38:4
PE 38:5
PE 38:5p/38:6e
PE 38:6p/38:7e
PE 40:0/42:6p/42:7e
PE 40:0p/40:1e
PE 40:1/42:7p/42:8e
PE 40:1p/40:2e
PE 40:2/42:8p/42:9e


Choriodecidual PE 1/2
TNL
STL
ITL
µg/mg protein
10
8
6
4
2
2.0
1.5
1.0
0.5
0.0
**
*
PE 32:1
PE 32:1p/32:2e
PE 32:3
PE 34:1p/34:2e
PE 34:2
PE 34:2p/34:3e
PE 34:5
PE 34:5p/34:6e
PE 36:1/38:7p/38:8e
PE 36:1p/36:2e
PE 36:2/38:8p/38:9e
PE 36:2p/36:3e
PE 36:4
PE 36:4p/36:5e
PE 36:5
PE 36:5p/36:6e
PE 36:6
PE 38:0p/38:1e
PE 38:1/40:7p/40:8e
PE 38:1p/38:2e
PE 38:2/40:8p/40:9e
PE 38:3/40:9p/40:10e
PE 38:4
PE 38:5
PE 38:5p/38:6e
PE 38:6p/38:7e
PE 40:0/42:6p/42:7e
PE 40:0p/40:1e
PE 40:1/42:7p/42:8e
PE 40:1p/40:2e
PE 40:2/42:8p/42:9e

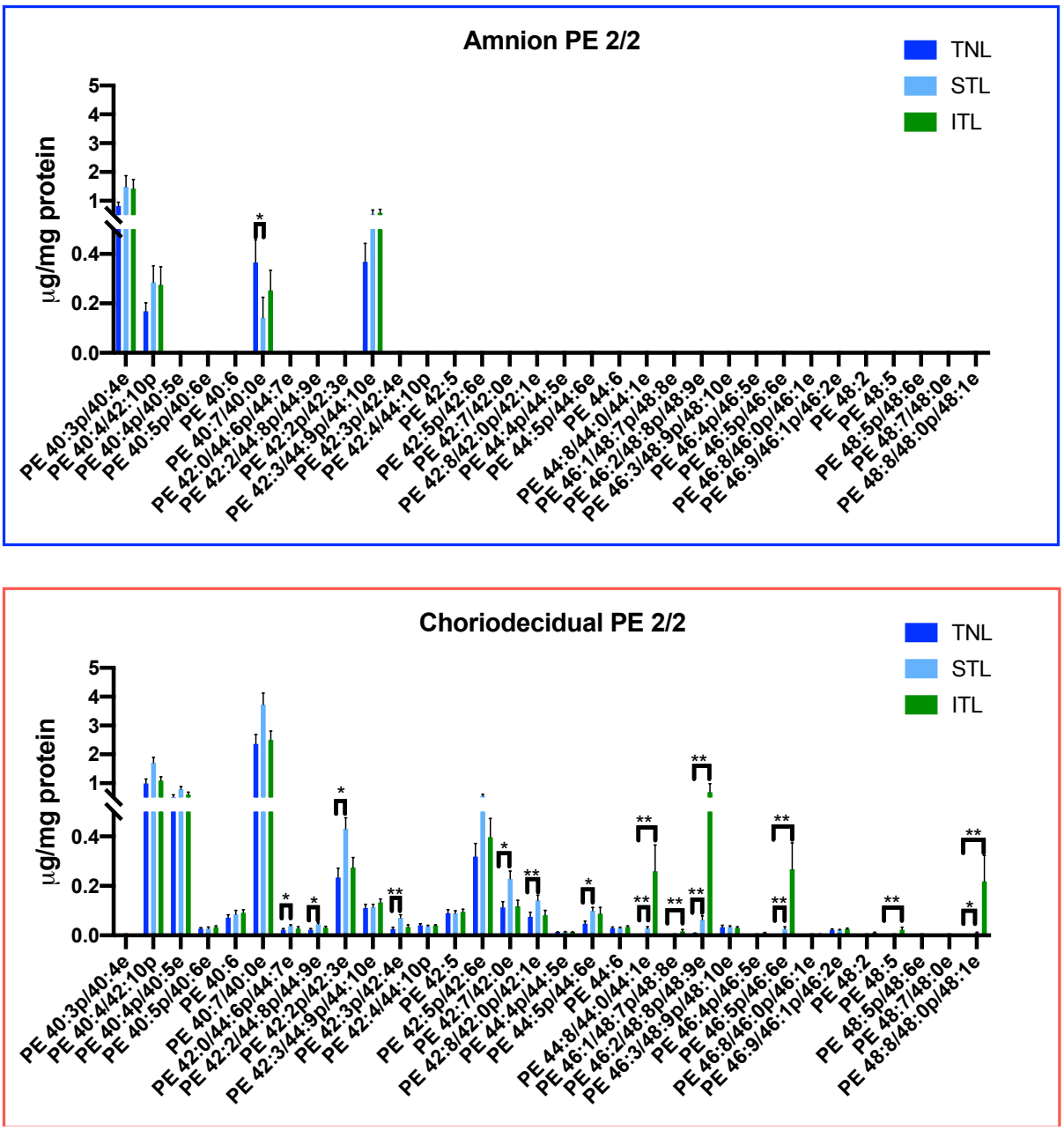


Figure 4.3. Term fetal membrane PE species. *Amnion (top, blue frame) and choriodecidua (bottom, red frame). Term not in labour (TNL), spontaneous term labour (STL) and induced term labour (ITL). Mean and SEM indicated. Statistical analysis = two-way ANOVA with Dunnett's multiple comparisons test, *p≤0.05, **p≤0.01.*

Although there was low abundance of PG within the membranes, an interesting observation was revealed under labouring conditions (**Figure 4.4**). Within the amnion, 8 PG species were detected, and each elevated in the term labouring groups, with PG 36:4 and PG 48:4 reaching significance. In the choriodecidua, 13 PG were detected with variable patterns of expression between study groups; PG 36:1 was higher in STL and ITL, whilst PG 34:1 and 36:2 were lower in STL and ITL, respectively (**Figure 4.4**).

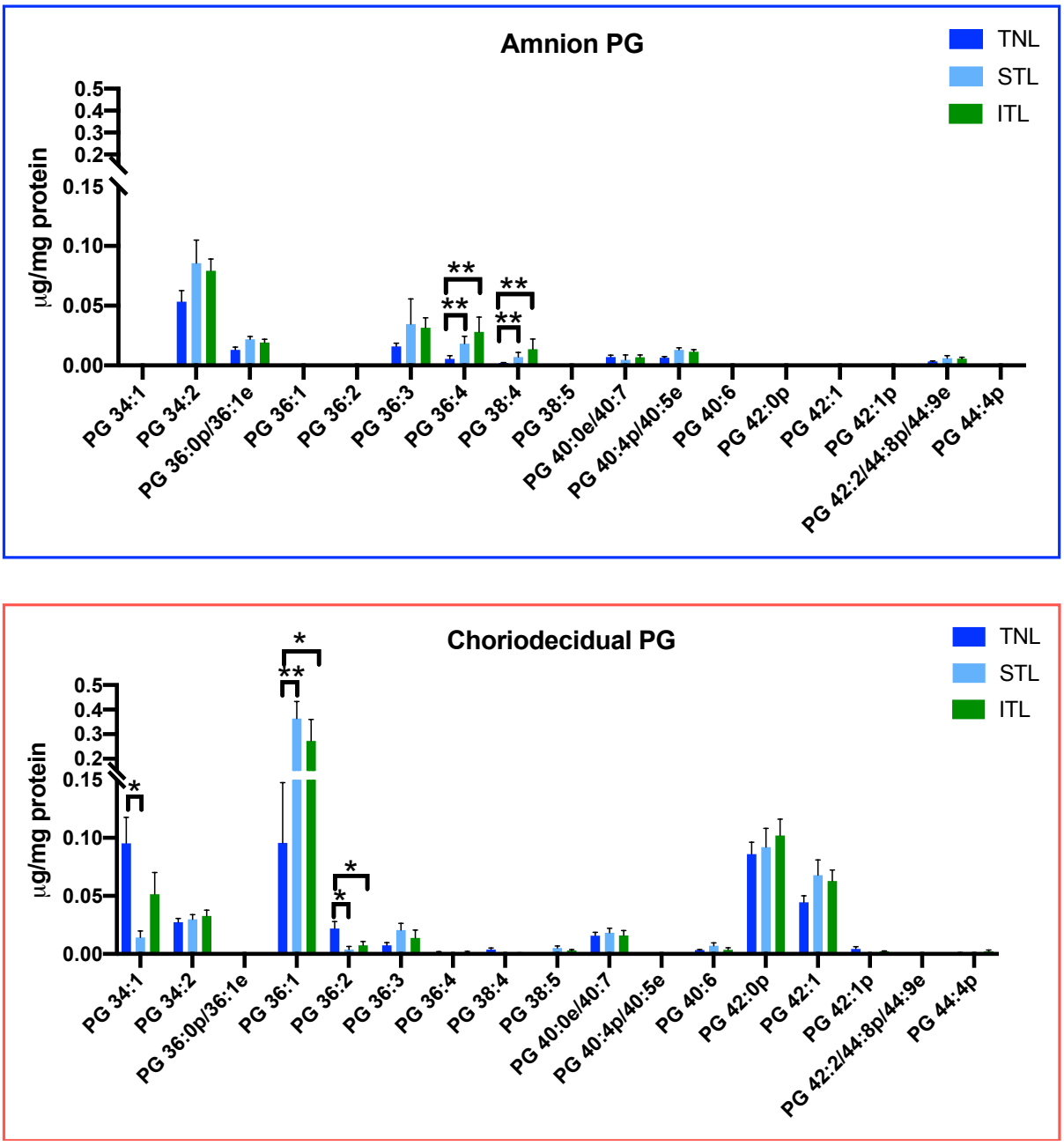


Figure 4.4. Term fetal membrane PG species. *Amnion (top, blue frame) and choriodecidua (bottom, red frame). Term not in labour (TNL), spontaneous term labour (STL) and induced term labour (ITL). Mean and SEM indicated. Statistical analysis = two-way ANOVA with Dunnett's multiple comparisons test, *p≤0.05, **p≤0.01.*

LPC species were detected solely within the choriodecidua; with 13 species defined (**Figure 4.5**). The most prominent being LPC 16:0, 24:1, 18:0, and 18:1. Across pregnancies, STL and ITL produced higher levels of LPC than TNL, but this was most evident in STL, where ten LPC were raised significantly.

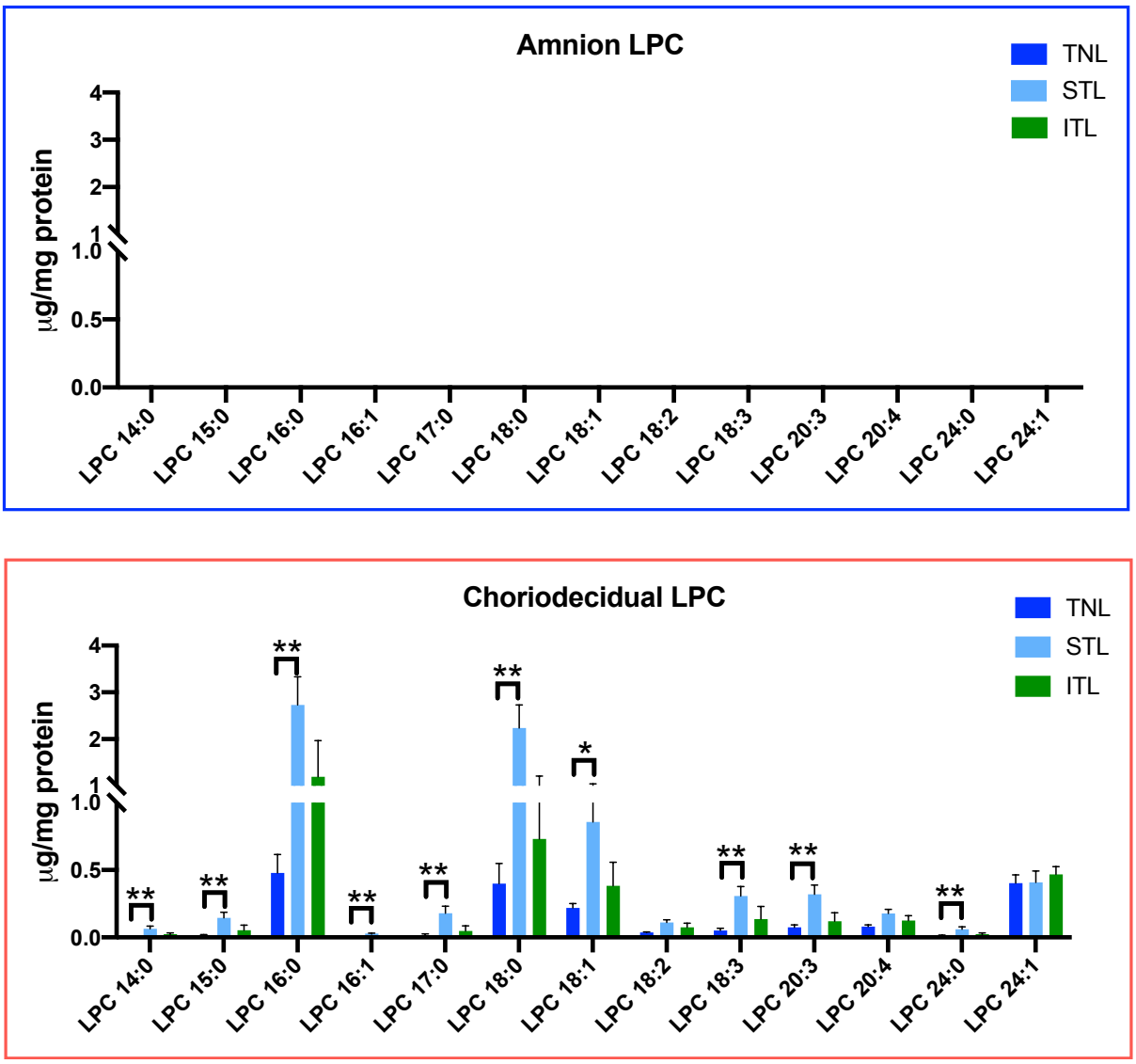
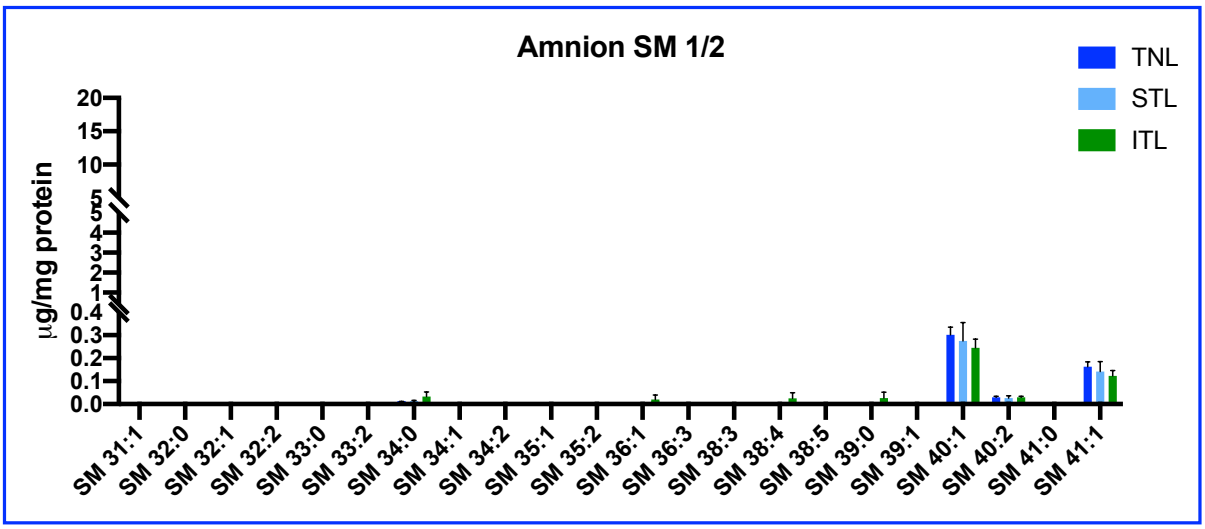
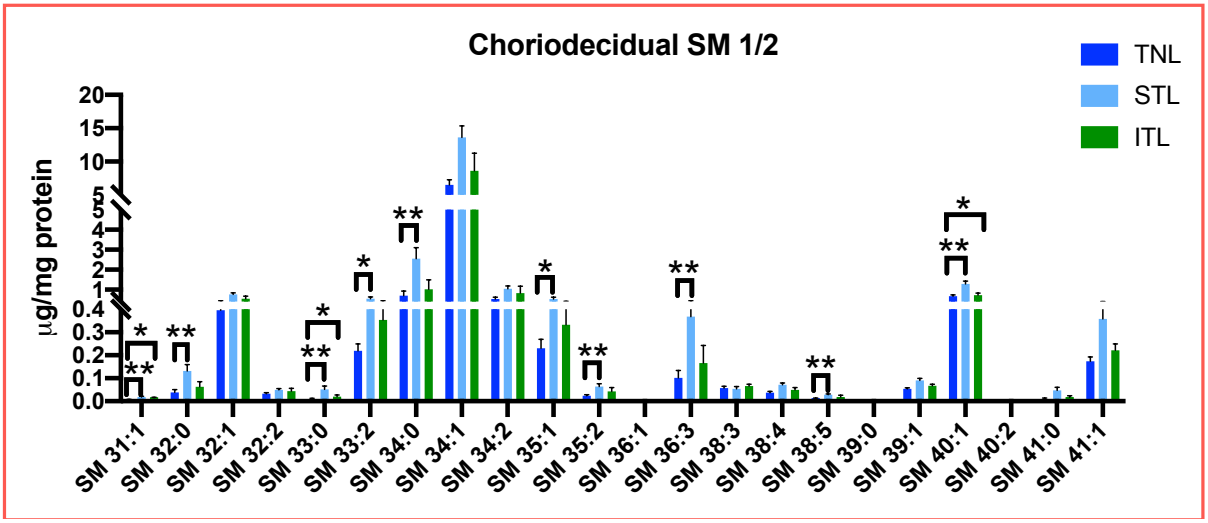


Figure 4.5. Term fetal membrane LPC species*. Amnion (top, blue frame) and choriodecidua (bottom, red frame). Term not in labour (TNL), spontaneous term labour (STL) and induced term labour (ITL). Mean and SEM indicated. Statistical analysis = two-way ANOVA with Dunnett's multiple comparisons test, *p≤0.05, **p≤0.01.*

The amnion expresses fewer SM species than choriodecidua; 23 and 38, respectively (see **Figure 4.6**). Within the amnion, dominate SM are: 42:1, 42:2, 40:1, 41:1 and 44:4; whilst choriodecidua is rich in SM 34:1, 42:2, 34:0, 40:1 and 42:3. Labouring deceases the majority of SM in the amnion, with SM 44:1 and SM 43:2 falling significantly in STL and ITL, respectively; but increases MS species in the choriodecidua, with 20 actively raised in STL and 2 elevated in ITL, significantly.


Amnion SM 1/2
TNL
STL
ITL
µg/mg protein
20
15
10
5
4
3
2
1
0.4
0.3
0.2
0.1
0.0
SM 31:1
SM 32:0
SM 32:1
SM 32:2
SM 33:0
SM 33:2
SM 34:0
SM 34:1
SM 34:2
SM 35:1
SM 35:2
SM 36:1
SM 36:3
SM 38:3
SM 38:4
SM 38:5
SM 39:0
SM 39:1
SM 40:1
SM 40:2
SM 41:0
SM 41:1


Choriodecidual SM 1/2
TNL
STL
ITL
µg/mg protein
20
15
10
5
4
3
2
1
0.4
0.3
0.2
0.1
0.0
*
**
SM 31:1
SM 32:0
SM 32:1
SM 32:2
SM 33:0
SM 33:2
SM 34:0
SM 34:1
SM 34:2
SM 35:1
SM 35:2
SM 36:1
SM 36:3
SM 38:3
SM 38:4
SM 38:5
SM 39:0
SM 39:1
SM 40:1
SM 40:2
SM 41:0
SM 41:1

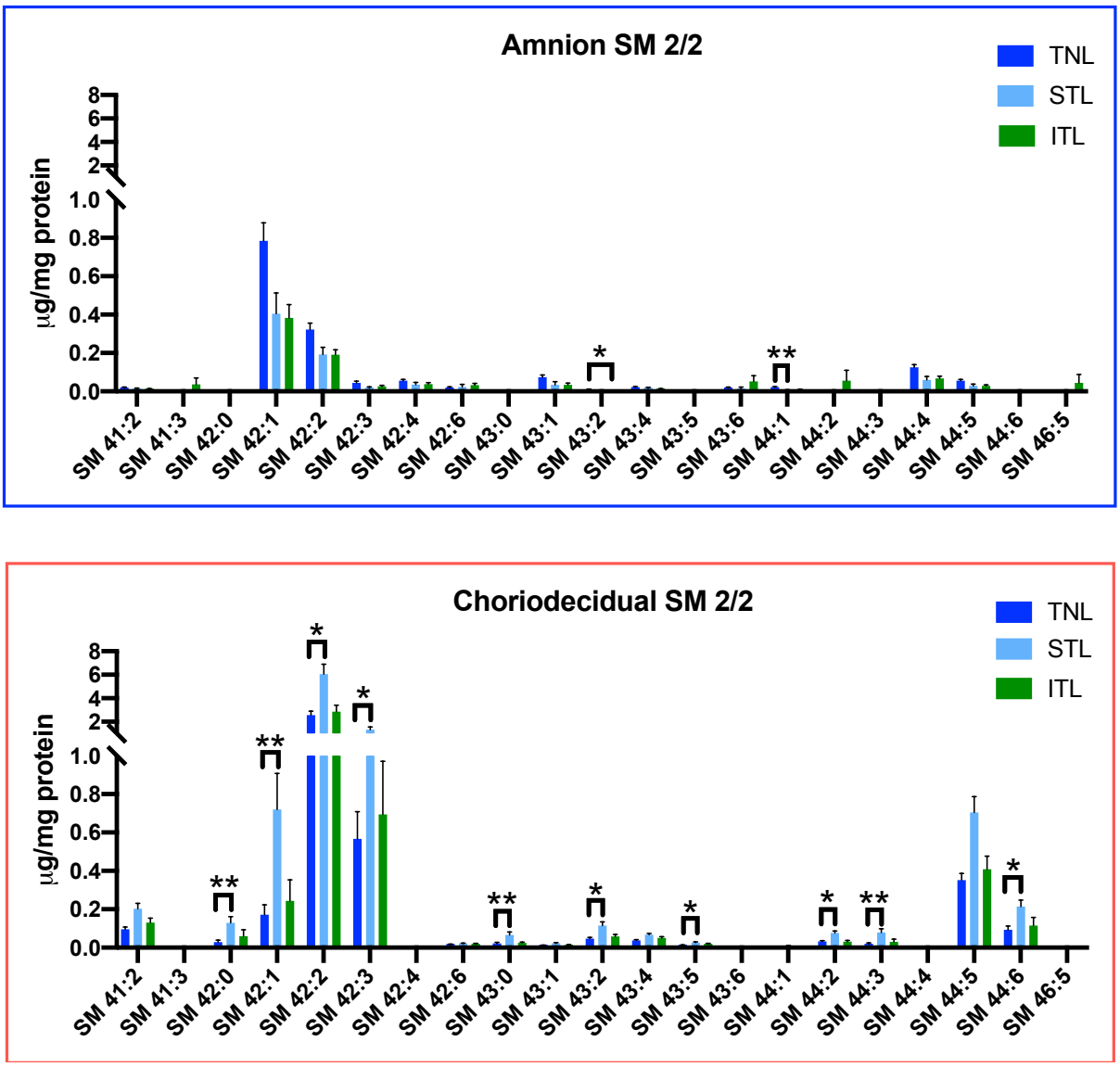


Figure 4.6. Term fetal membrane SM species. *Amnion (top, blue frame) and choriodecidua (bottom, red frame). Term not in labour (TNL), spontaneous term labour (STL) and induced term labour (ITL). Mean and SEM indicated. Statistical analysis = two-way ANOVA with Dunnett's multiple comparisons test, *p≤0.05, **p≤0.01.*

The amnion showed lower abundance of CE, with only two species detected; CE 18:1 and CE 20:2. 12 species were recognised in the choriodecidua, the highest being CE 16:0, 18:2, 22.6, 20:4 (**Figure 4.7**). Labouring generally increased these CE profiles; most notably in STL, where 10 recorded species were elevated.

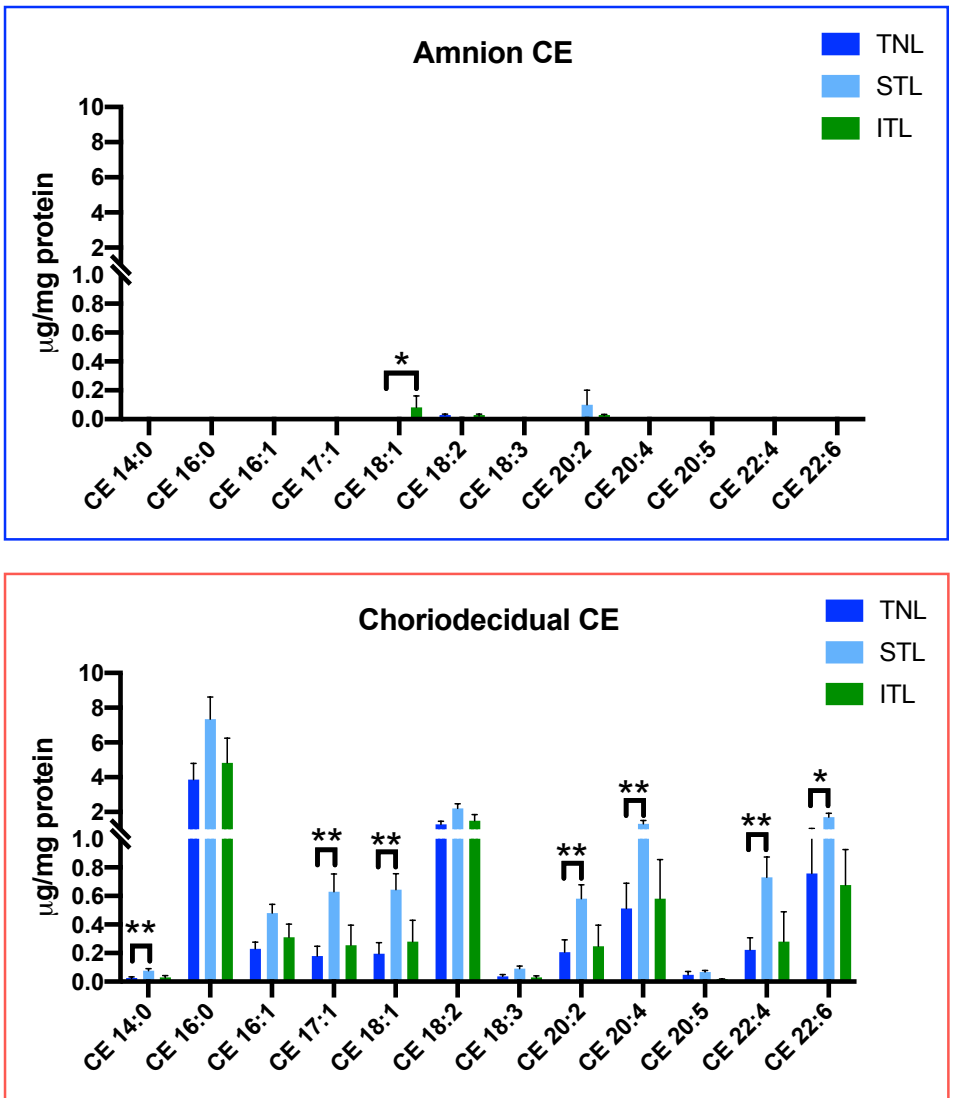
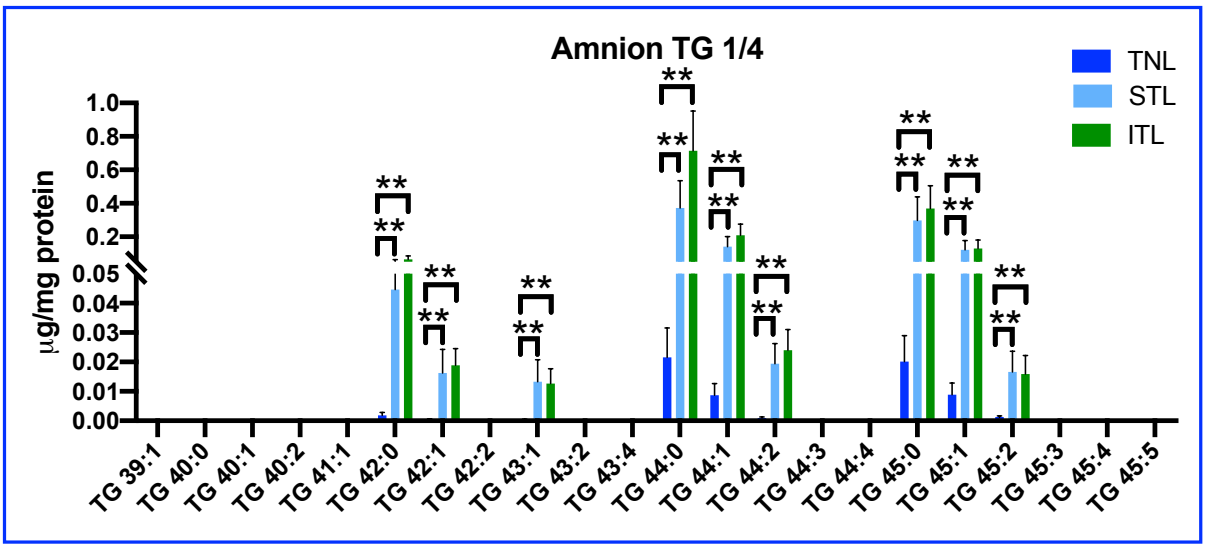
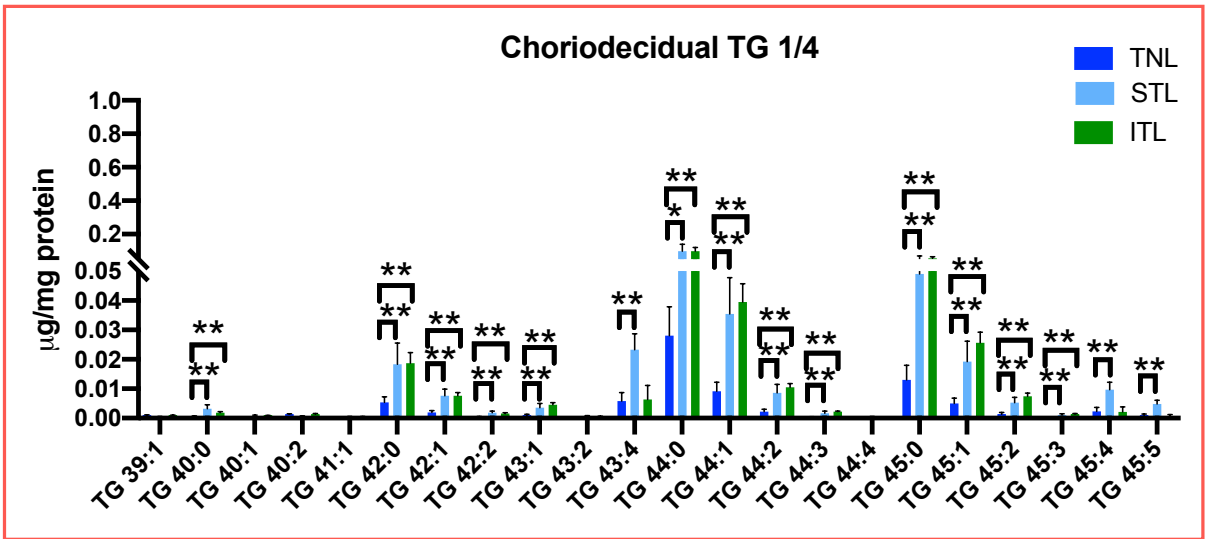
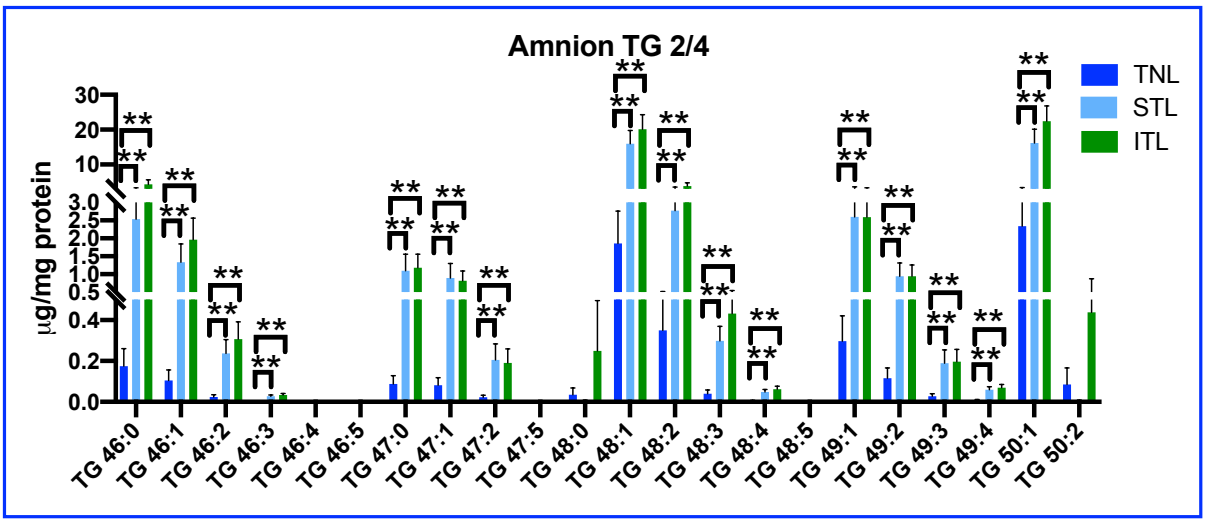


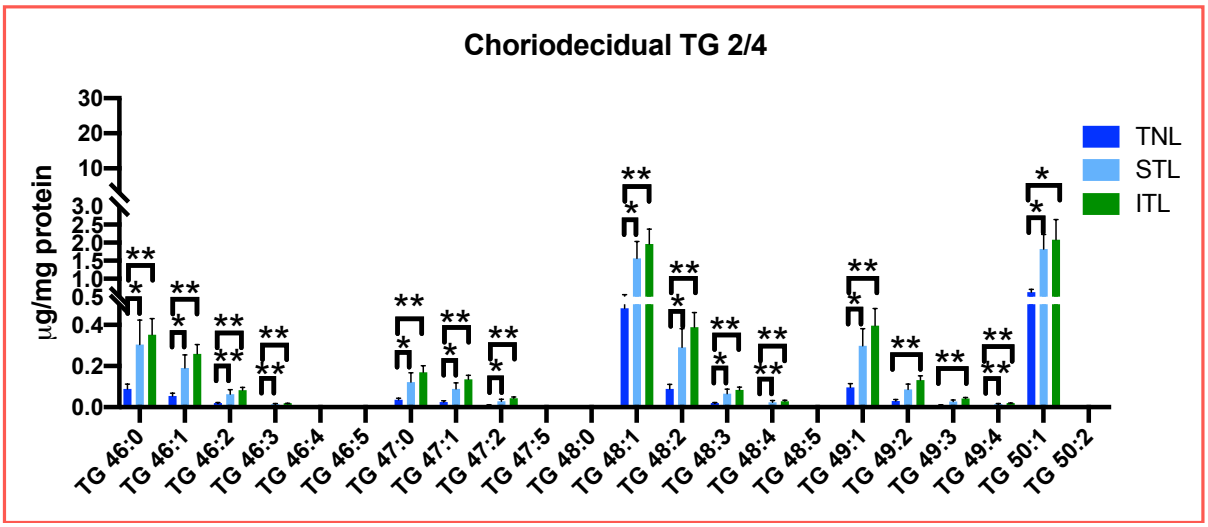
Figure 4.7. Term fetal membrane CE species. *Amnion (top, blue frame) and choriodecidua (bottom, red frame). Term not in labour (TNL), spontaneous term labour (STL) and induced term labour (ITL). Mean and SEM indicated. Statistical analysis = two-way ANOVA with Dunnett's multiple comparisons test, *p≤0.05, **p≤0.01.*

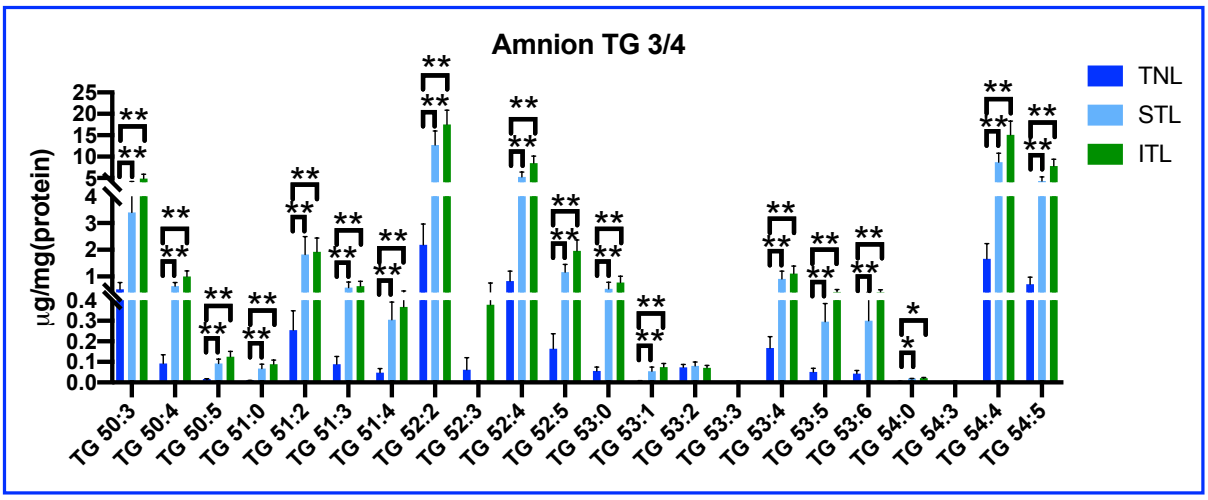
The choriodecidua gave a greater number of TG species than amnion, with 81 and 68, respectively (**Figure 4.8**). The dominant species in amnion were TG 50:1, 52:2, 48:1, 54:4, 52:4, whilst TG 52:2, 54:4, 50:1, 48:1, 56:5 were more abundant in choriodecidua. STL and ITL significantly increased TG in the amnion by 57 and 63 species, respectively. Similarly, term labour (regardless of induction) showed increased TG levels in 62 and 60 species for STL and ITL, respectively (**Figure 4.8**).

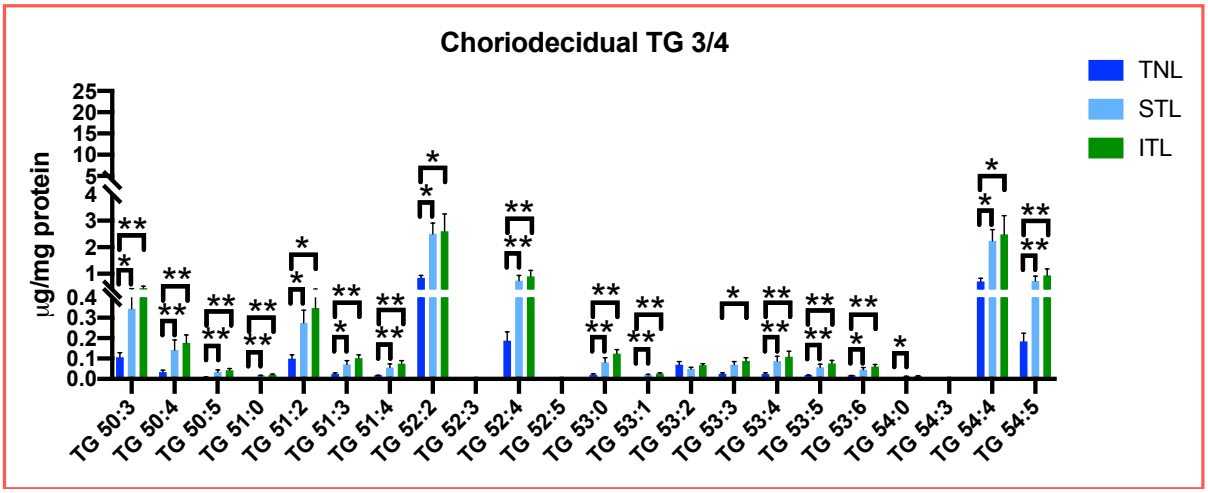

Amnion TG 1/4
TNL
STL
ITL
µg/mg protein
1.0
0.8
0.6
0.4
0.2
0.05
0.04
0.03
0.02
0.01
0.00
TG 39:1
TG 40:0
TG 40:1
TG 40:2
TG 41:1
TG 42:0
TG 42:1
TG 42:2
TG 43:1
TG 43:2
TG 43:4
TG 44:0
TG 44:1
TG 44:2
TG 44:3
TG 44:4
TG 45:0
TG 45:1
TG 45:2
TG 45:3
TG 45:4
TG 45:5


Choriodecidual TG 1/4
TNL
STL
ITL
µg/mg protein
1.0
0.8
0.6
0.4
0.2
0.05
0.04
0.03
0.02
0.01
0.00
TG 39:1
TG 40:0
TG 40:1
TG 40:2
TG 41:1
TG 42:0
TG 42:1
TG 42:2
TG 43:1
TG 43:2
TG 43:4
TG 44:0
TG 44:1
TG 44:2
TG 44:3
TG 44:4
TG 45:0
TG 45:1
TG 45:2
TG 45:3
TG 45:4
TG 45:5


Amnion TG 2/4
TNL
STL
ITL
µg/mg protein
TG 46:0
TG 46:1
TG 46:2
TG 46:3
TG 46:4
TG 46:5
TG 47:0
TG 47:1
TG 47:2
TG 47:5
TG 48:0
TG 48:1
TG 48:2
TG 48:3
TG 48:4
TG 48:5
TG 49:1
TG 49:2
TG 49:3
TG 49:4
TG 50:1
TG 50:2


Choriodecidual TG 2/4
TNL
STL
ITL
µg/mg protein
TG 46:0
TG 46:1
TG 46:2
TG 46:3
TG 46:4
TG 46:5
TG 47:0
TG 47:1
TG 47:2
TG 47:5
TG 48:0
TG 48:1
TG 48:2
TG 48:3
TG 48:4
TG 48:5
TG 49:1
TG 49:2
TG 49:3
TG 49:4
TG 50:1
TG 50:2


Amnion TG 3/4
µg/mg(protein)
TNL
STL
ITL
TG 50:3
TG 50:4
TG 50:5
TG 51:0
TG 51:2
TG 51:3
TG 51:4
TG 52:2
TG 52:3
TG 52:4
TG 52:5
TG 53:0
TG 53:1
TG 53:2
TG 53:3
TG 53:4
TG 53:5
TG 53:6
TG 54:0
TG 54:3
TG 54:4
TG 54:5


Choriodecidual TG 3/4
µg/mg protein
TNL
STL
ITL
TG 50:3
TG 50:4
TG 50:5
TG 51:0
TG 51:2
TG 51:3
TG 51:4
TG 52:2
TG 52:3
TG 52:4
TG 52:5
TG 53:0
TG 53:1
TG 53:2
TG 53:3
TG 53:4
TG 53:5
TG 53:6
TG 54:0
TG 54:3
TG 54:4
TG 54:5

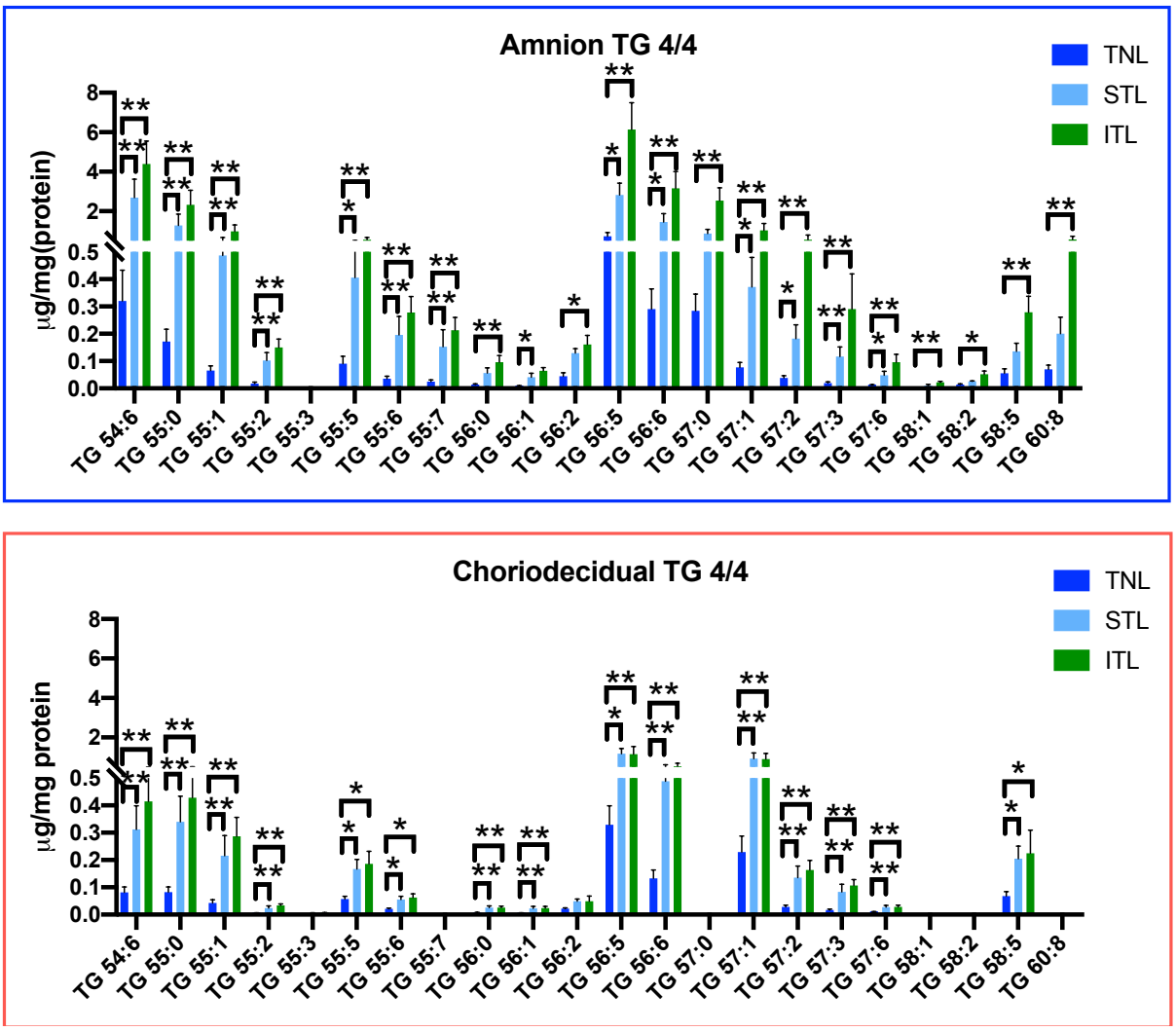


Figure 4.8. Term fetal membrane TG species*. Amnion (top, blue frame) and choriodecidua (bottom, red frame). Term not in labour (TNL), spontaneous term labour (STL) and induced term labour (ITL). Mean and SEM indicated. Statistical analysis = two-way ANOVA with Dunnett's multiple comparisons test, *p≤0.05, **p≤0.01.*

The choriodecidua was higher in DG than equivalent amnion (**Figure 4.9**) with 21 and 7 species, respectively. DG 37:4 and DG 38:3 were highest in both tissues. Within the amnion, STL and ITL increased TG concentrations over TNL samples, but this only reached significance for DG 30:3, in the ITL group. For the choriodecidua, both "in-labour" groups generated higher levels of DG, but again this reached significance in only 8 low abundant species in the STL group, and none at all in ITL.

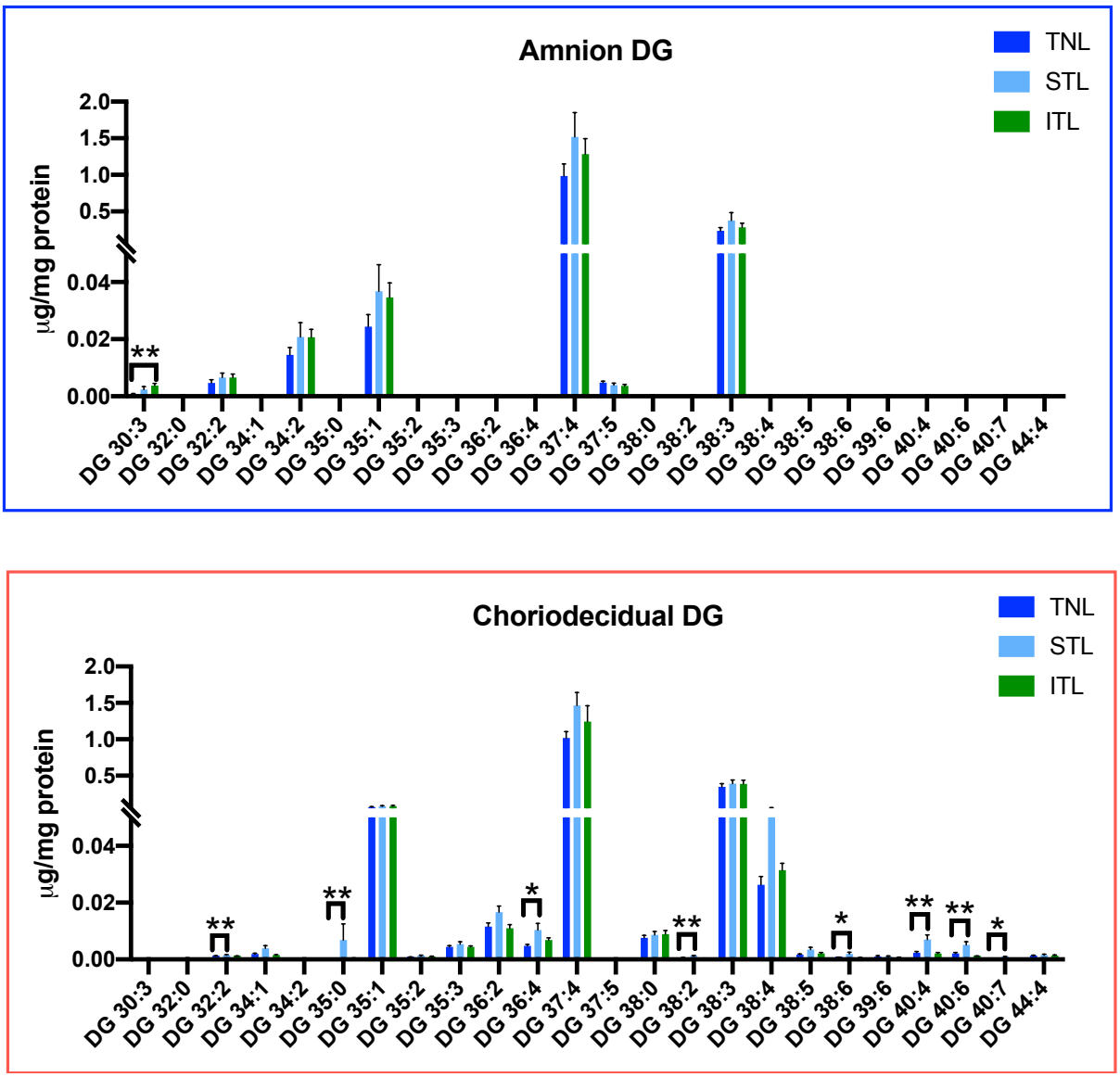


Figure 4.9. Term fetal membrane DG species. *Amnion (top, blue frame) and choriodecidua (bottom, red frame). Term not in labour (TNL), spontaneous term labour (STL) and induced term labour (ITL). Mean and SEM indicated. Statistical analysis = two-way ANOVA with Dunnett's multiple comparisons test, *p≤0.05, **p≤0.01.*

Figure 4.10 shows the FFA composition of isolated fetal membranes. There were 21 and 19 FFA species detected in the amnion and choriodecidua, respectively. In both tissues, a wide range of FFA were noted, including saturated, monounsaturated and polyunsaturated forms. Palmitic acid (C16:0) was the dominant saturated fatty acid, oleic acid (18:1) the dominated monounsaturated FA, and AA (C20:4) the polyunsaturated FA of greatest abundance. Significantly, free AA was more than 10 times richer in the choriodecidua than amnion.

In the amnion, both labouring conditions (STL and ITL) gave an increased FFA profile over TNL; with ITL proffering greater elevations than STL in 13 and 7 FFA, respectively. Although, free AA didn't show an increase in labour, the omega-3 precursor FFA, 18:2 (LA), was significantly elevated in both labouring conditions. Also, long-chain fatty acids i.e. FFA 22:0, 23:0, and 24:1 were upregulated in term labour over non-labour controls.

In the choriodecidua, the STL group showed a variable pattern in FFA profile, as compared to TNL. Increased production of ALA/GLA (18:3), AA (20:4), 24:0, 24:2 and 26:1 were noted, but two FFAs; 14:0 and 19:0 were concomitantly reduced. ITL profiles showed a similar level of FFA to TNLs. However, only two species were significantly raised; ALA/GLA (18:3) and 24:0.

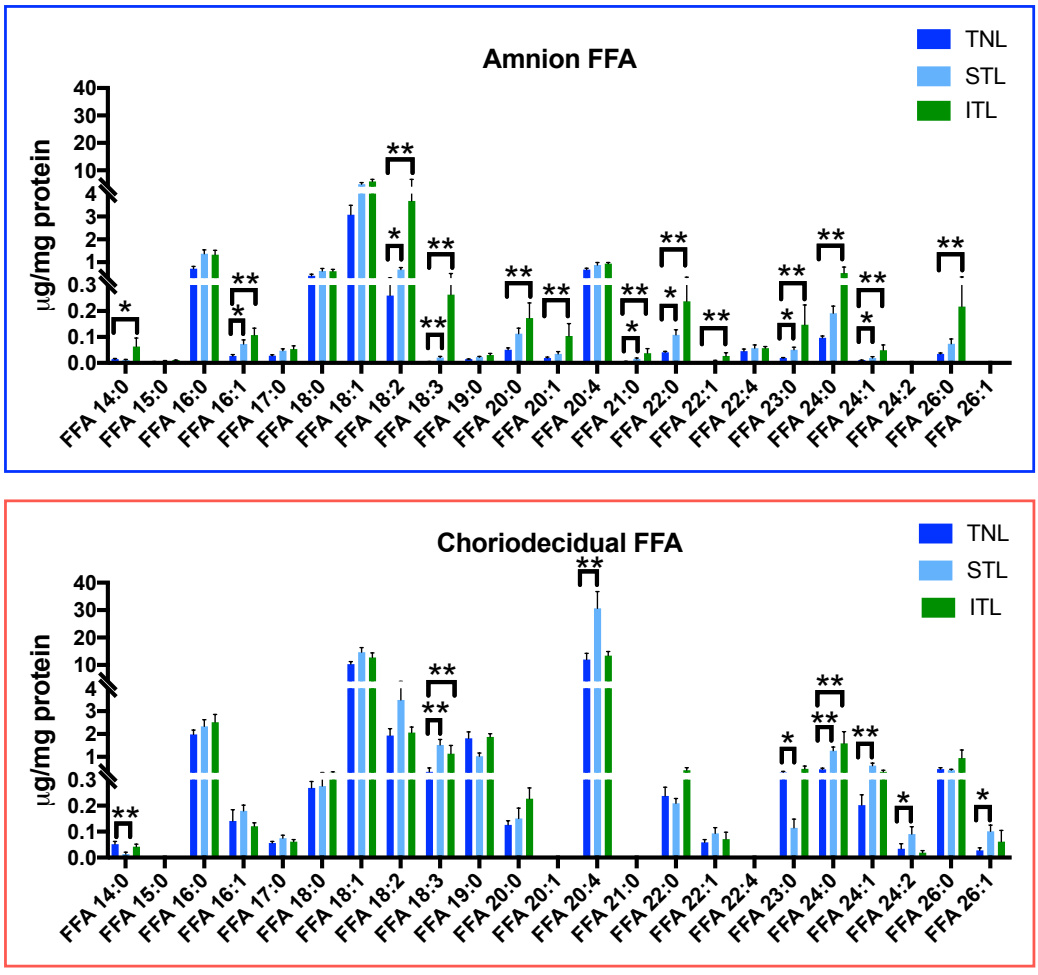


Figure 4.10. Term fetal membrane FFA species. *Amnion (top, blue frame) and choriodecidua (bottom, red frame). Term not in labour (TNL), spontaneous term labour (STL) and induced term labour (ITL). Mean and SEM indicated. Statistical analysis = two-way ANOVA with Dunnett's multiple comparisons test, *p≤0.05, **p≤0.01.*

In summary, term amnion and choriodecidua expressed different lipidomic profiles. The amnion was higher in cholesterol and TG than choriodecidua. In comparison, the choriodecidua was richer in glycerophospholipids, SM, CE, and FFAs (including AA). Also, LPC species were detected in choriodecidua, potentially indicating higher lipid mobilisation than the amnion.

In the amnion, in-labour tissues showed similar composition in lipid classes to non-labouring samples, but PG, TG and FFA were evidently raised. Also, STL amnion showed a reduction in some of the AA-containing PC and PE species. Again in STL, most SM species showed a decreasing trend and two species reached statistical significance. Free AA within in-labour tissues was comparable to controls; and free LA and long-chain FFAs were upregulated.

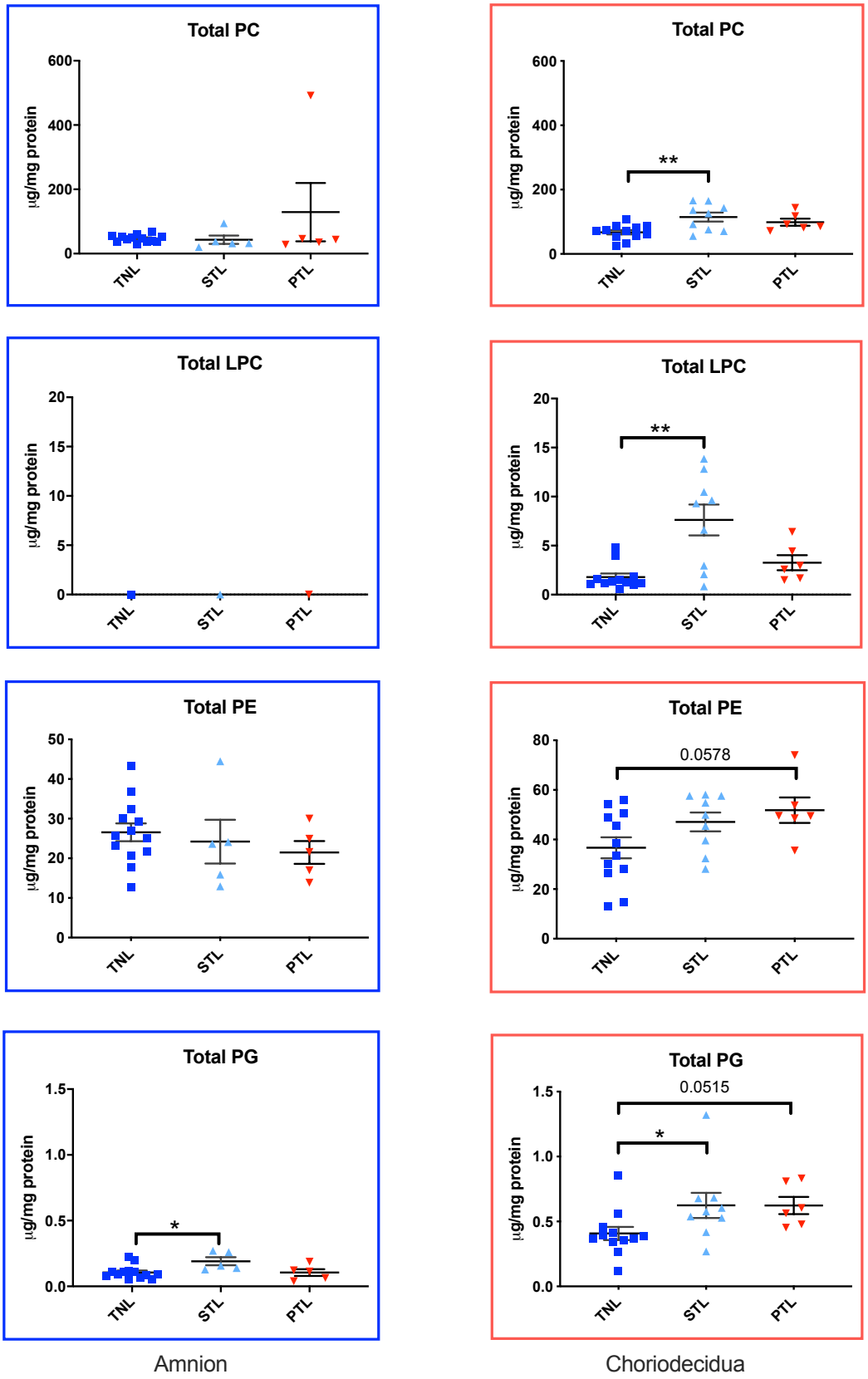
In the choriodecidua, spontaneous labour showed an increase in PC, LPC, SM, TG, cholesterol and FFA; whilst TG was specifically upregulated following induction. AA-containing PC and PE species and plasmalogens were upregulated with STL, with LPC also elevated indicating raised PLA_2 activity. Free AA were significantly elevated in STL, and long-chain fatty acids were upregulated in both term labouring conditions. The STL group is notably rich in glycerophospholipids and in AA (free and imbedded forms) than the control.

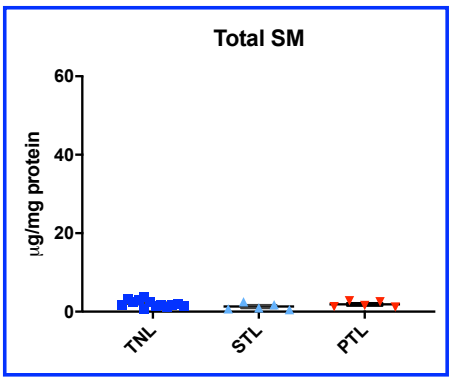
4.4.2. Preterm fetal membrane complex lipid profiles

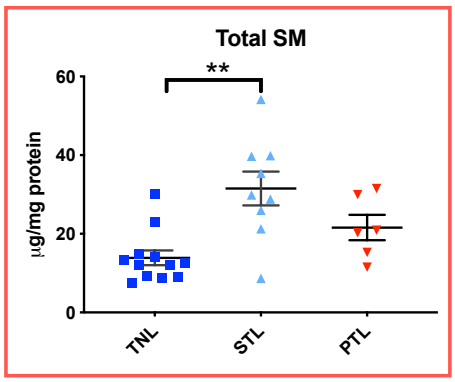
Figure 4.11 indicates lipid classes for fetal membranes in the preterm study, i.e., STL, PTL and TNL groups. For the amnion from pre-term pregnancies, PC and PE were the most abundant classes, with concentrations of 129.16 and 21.40µg/mg protein, respectively. These were followed by FFA (9.17 µg/mg), free cholesterol (6.69 µg/mg) and TG (5.34 µg/mg); with no variations at all to non-labouring term tissues (TNL).

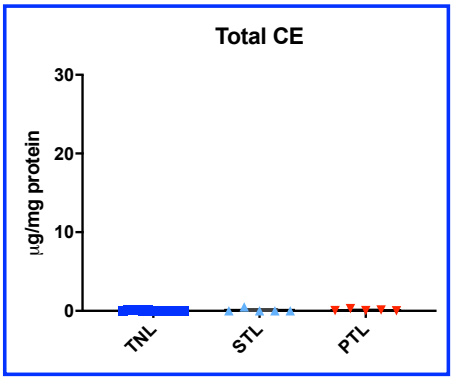
For the choriodecidua, the most abundant lipid classes were PC (98.75 µg/mg), FFA (61.57 µg/mg), PE (51.84 µg/mg) and SM (21.59 µg/mg); with other classes detected as follows; CE (13.24 µg/mg) and TG (6.89 µg/mg). Overall, the choriodecidua showed increased expression

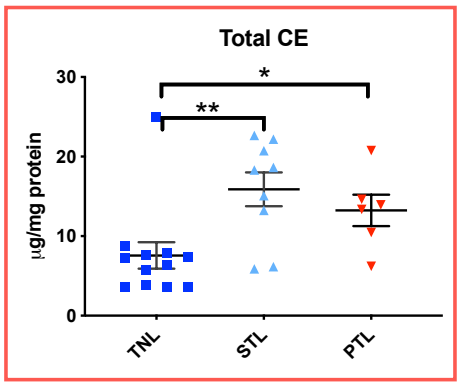
of most lipid classes, with CE and FFA reaching significance; and PE and PG nearing significance.

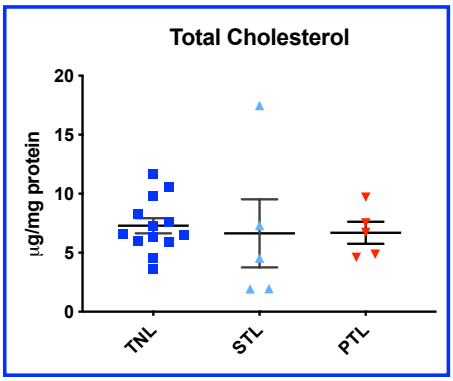


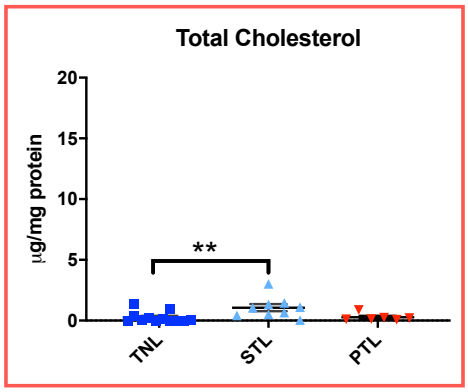


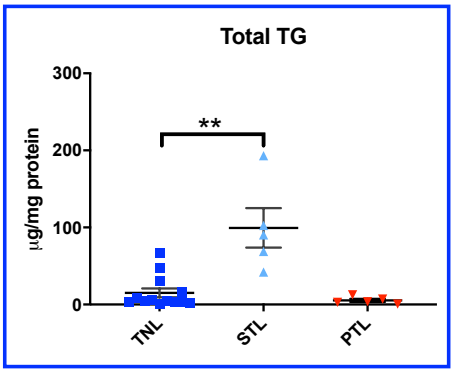


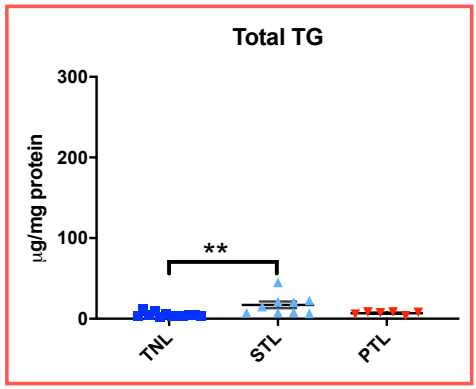












Amnion

Choriodecidua

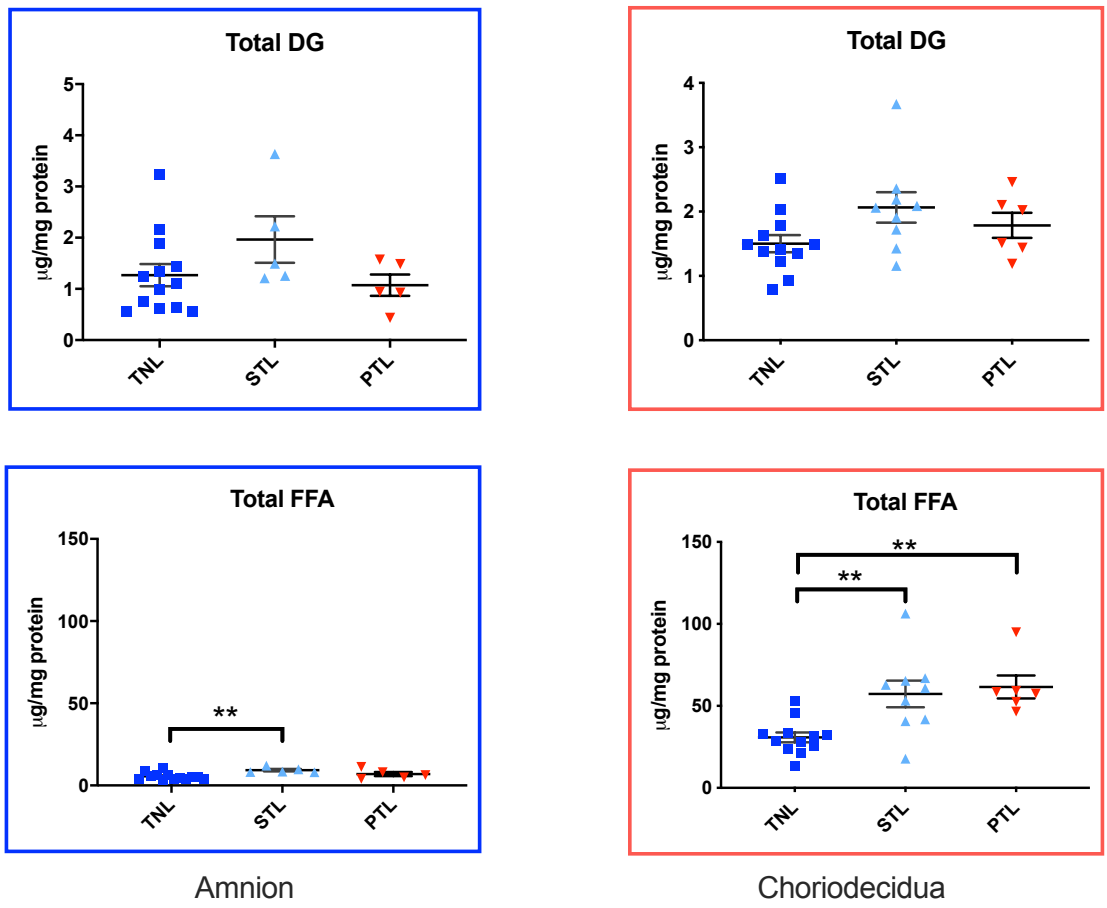
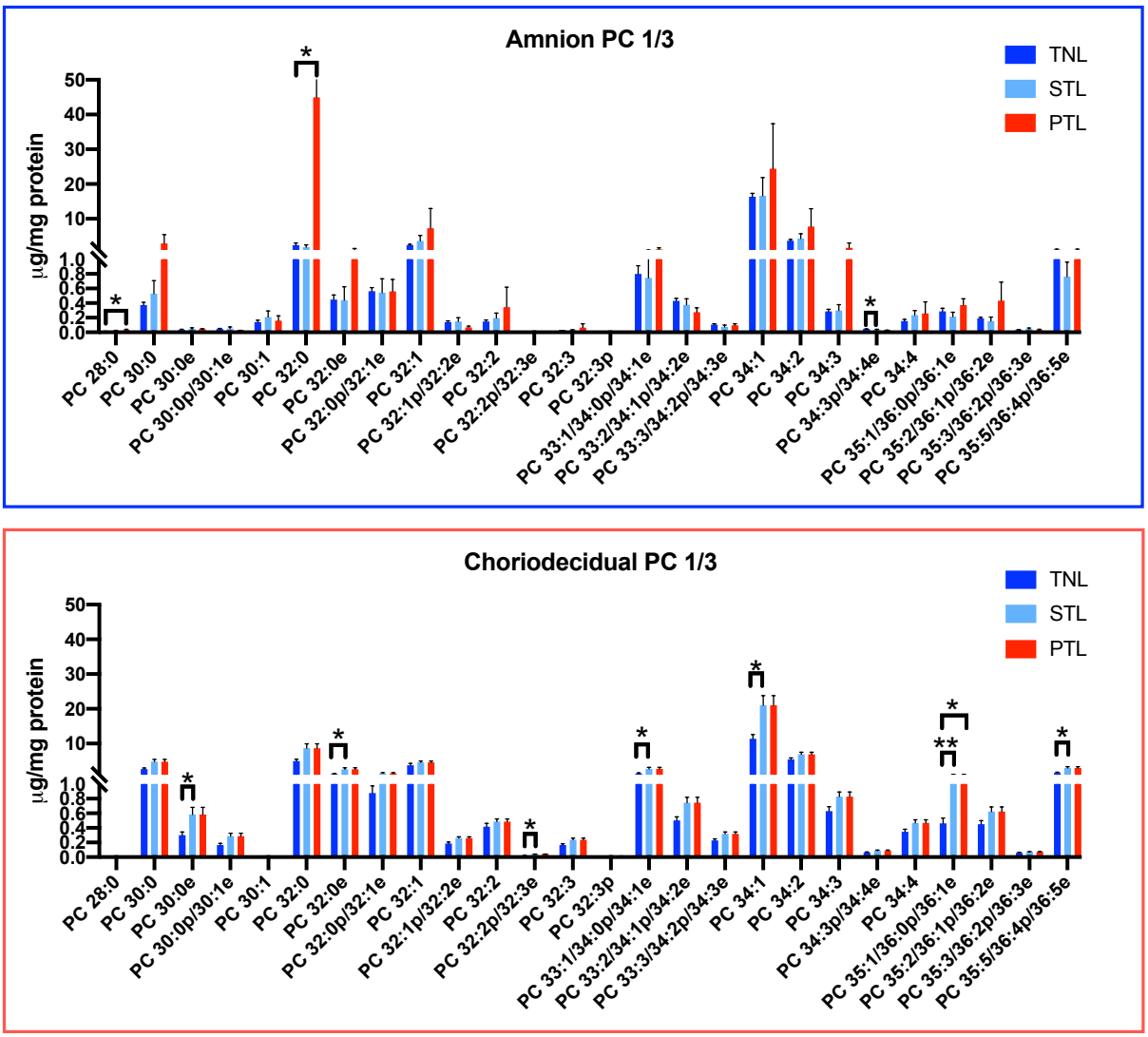


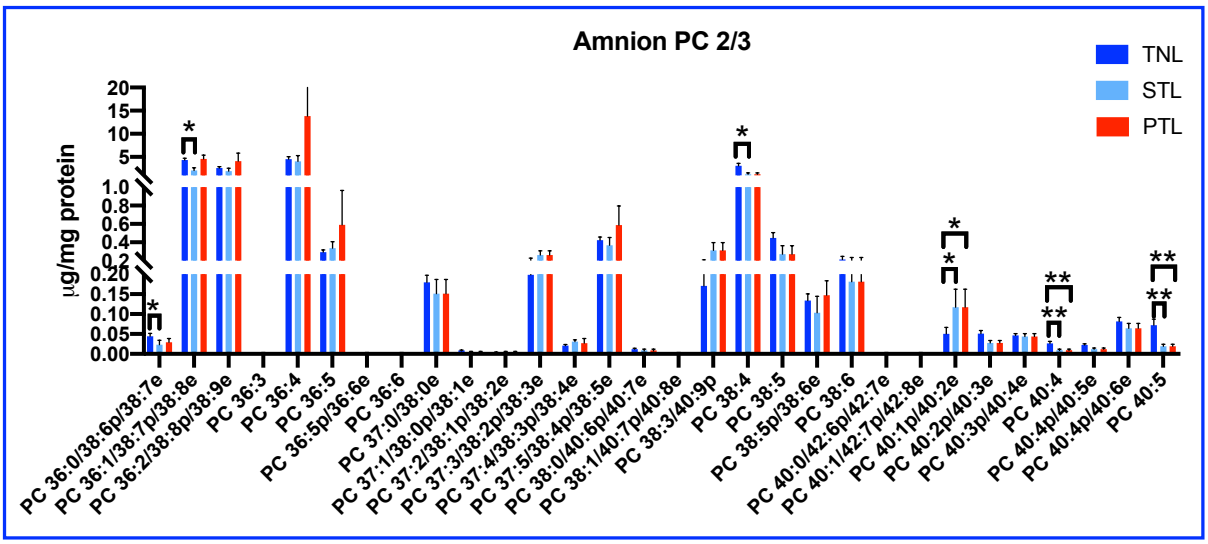
Figure 4.11. Preterm fetal membrane complex lipid profiles. *Amnion (left side, blue frame) and choriodecidua (right side, red frame). Term not in labour (TNL), spontaneous term labour (STL) and preterm labour (PTL). Mean and SEM indicated. Statistical analysis = one-way ANOVA with Dunnett's multiple comparisons test, *p≤0.05, **p≤0.01.*

Figure 4.12 shows PC species in amnion and choriodecidua for the preterm study. In the amnion, PTL showed variation across PC species with a significant increase reached for PC 28:0, 30:1 and 40:1p/40:2e against the not in-labour group. Of note, a number of species of AA-containing potential, were decreased with PC 40:4, 40:5, 40:8/40:0p/40:1e and 42:5p/42:6e reaching statistical significance in comparison to the TNL group.

With similarities to spontaneous term labour (STL), the PTL choriodecidua gave an increased pattern of expression across PCs, with the following reaching significance; PC 35:1/36:0p/36:1e, 36:5p/36:6e, 37:1/38:0p/38:1e, 38:4, 40:0/42:6p/42:7e, 42:0/44:6p/44:6e, 42:1/44:7p/448e, 42:1p/42:2e, 42:2/44:8p/44:9e, 42:2p/42:3e, 42:3p/42:4e, 44:0/46:6p/46:7e, 44:1/46:7p/46:8e, and 46:6 (**Figure 4.12**).


Amnion PC 1/3
TNL
STL
PTL
µg/mg protein
50
40
30
20
10
1.0
0.8
0.6
0.4
0.2
0.0
PC 28:0
PC 30:0
PC 30:0e
PC 30:0p/30:1e
PC 30:1
PC 32:0
PC 32:0e
PC 32:0p/32:1e
PC 32:1
PC 32:1p/32:2e
PC 32:2
PC 32:2p/32:3e
PC 32:3
PC 32:3p
PC 33:1/34:0p/34:1e
PC 33:2/34:1p/34:2e
PC 33:3/34:2p/34:3e
PC 34:1
PC 34:2
PC 34:3
PC 34:3p/34:4e
PC 34:4
PC 35:1/36:0p/36:1e
PC 35:2/36:1p/36:2e
PC 35:3/36:2p/36:3e
PC 35:5/36:4p/36:5e

Choriodecidual PC 1/3
TNL
STL
PTL
µg/mg protein
50
40
30
20
10
1.0
0.8
0.6
0.4
0.2
0.0
PC 28:0
PC 30:0
PC 30:0e
PC 30:0p/30:1e
PC 30:1
PC 32:0
PC 32:0e
PC 32:0p/32:1e
PC 32:1
PC 32:1p/32:2e
PC 32:2
PC 32:2p/32:3e
PC 32:3
PC 32:3p
PC 33:1/34:0p/34:1e
PC 33:2/34:1p/34:2e
PC 33:3/34:2p/34:3e
PC 34:1
PC 34:2
PC 34:3
PC 34:3p/34:4e
PC 34:4
PC 35:1/36:0p/36:1e
PC 35:2/36:1p/36:2e
PC 35:3/36:2p/36:3e
PC 35:5/36:4p/36:5e


Amnion PC 2/3
TNL
STL
PTL
µg/mg protein
PC 36:0/38:6p/38:7e
PC 36:1/38:7p/38:8e
PC 36:2/38:8p/38:9e
PC 36:3
PC 36:4
PC 36:5
PC 36:5p/36:6e
PC 36:6
PC 37:0/38:0e
PC 37:1/38:0p/38:1e
PC 37:2/38:1p/38:2e
PC 37:3/38:2p/38:3e
PC 37:4/38:3p/38:4e
PC 37:5/38:4p/38:5e
PC 38:0/40:6p/40:7e
PC 38:1/40:7p/40:8e
PC 38:3/40:9p
PC 38:4
PC 38:5
PC 38:5p/38:6e
PC 38:6
PC 40:0/42:6p/42:7e
PC 40:1/42:7p/42:8e
PC 40:1p/40:2e
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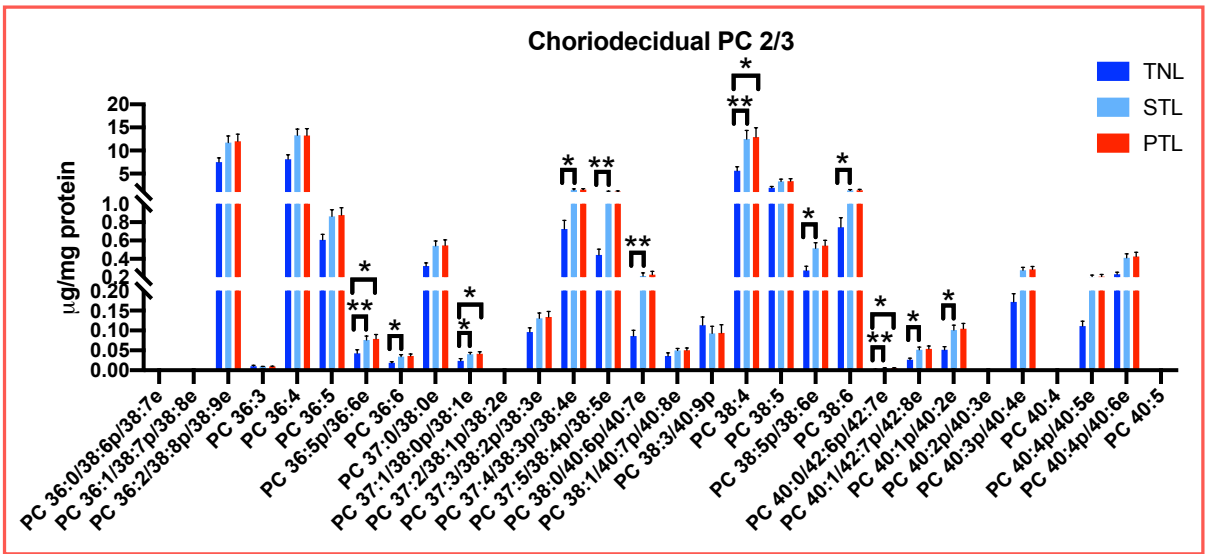
PC 40:4p/40:5e
PC 40:4p/40:6e
PC 40:5


Choriodecidual PC 2/3
TNL
STL
PTL
µg/mg protein
PC 36:0/38:6p/38:7e
PC 36:1/38:7p/38:8e
PC 36:2/38:8p/38:9e
PC 36:3
PC 36:4
PC 36:5
PC 36:5p/36:6e
PC 36:6
PC 37:0/38:0e
PC 37:1/38:0p/38:1e
PC 37:2/38:1p/38:2e
PC 37:3/38:2p/38:3e
PC 37:4/38:3p/38:4e
PC 37:5/38:4p/38:5e
PC 38:0/40:6p/40:7e
PC 38:1/40:7p/40:8e
PC 38:3/40:9p
PC 38:4
PC 38:5
PC 38:5p/38:6e
PC 38:6
PC 40:0/42:6p/42:7e
PC 40:1/42:7p/42:8e
PC 40:1p/40:2e
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PC 40:3p/40:4e
PC 40:4
PC 40:4p/40:5e
PC 40:4p/40:6e
PC 40:5

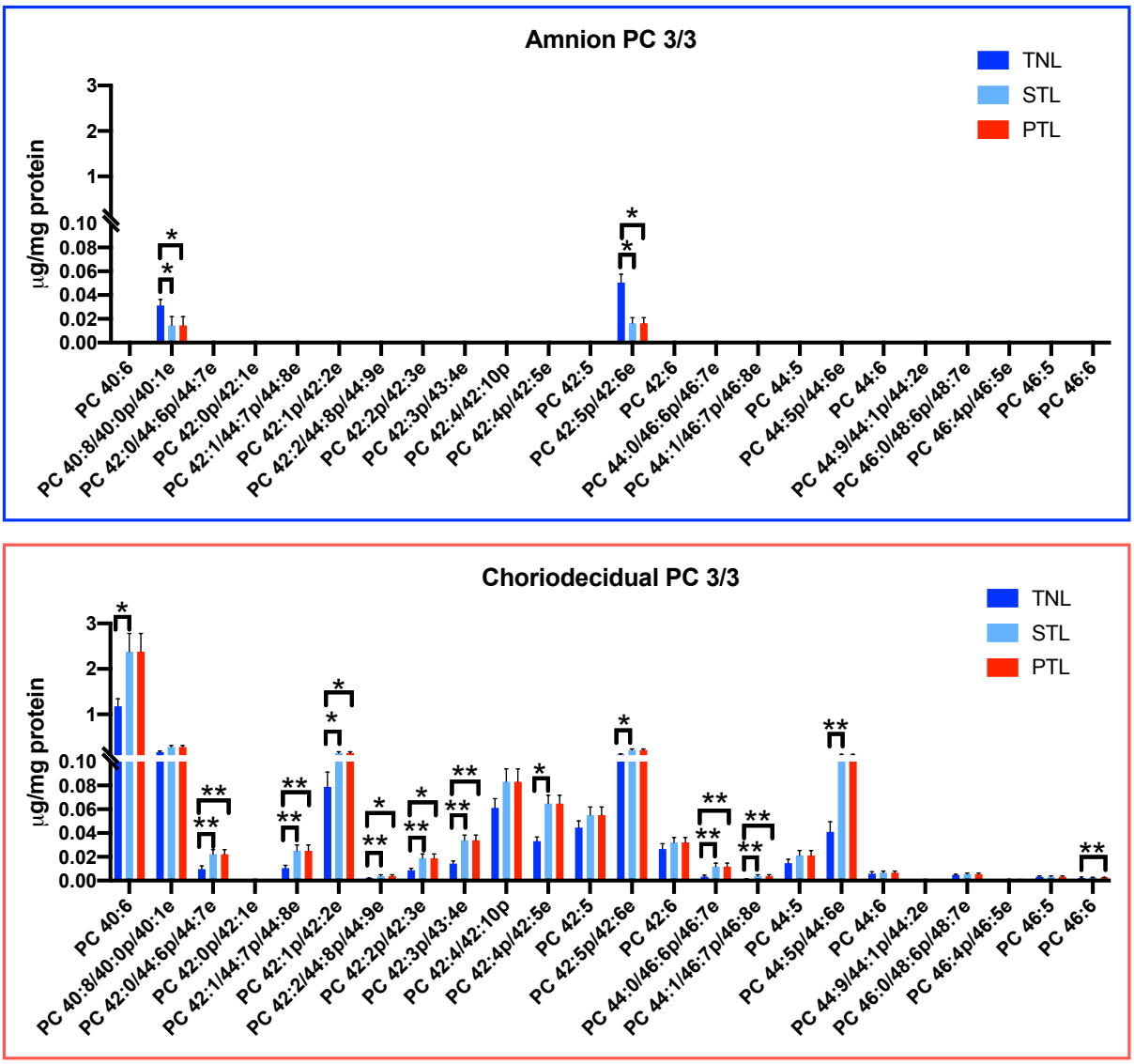
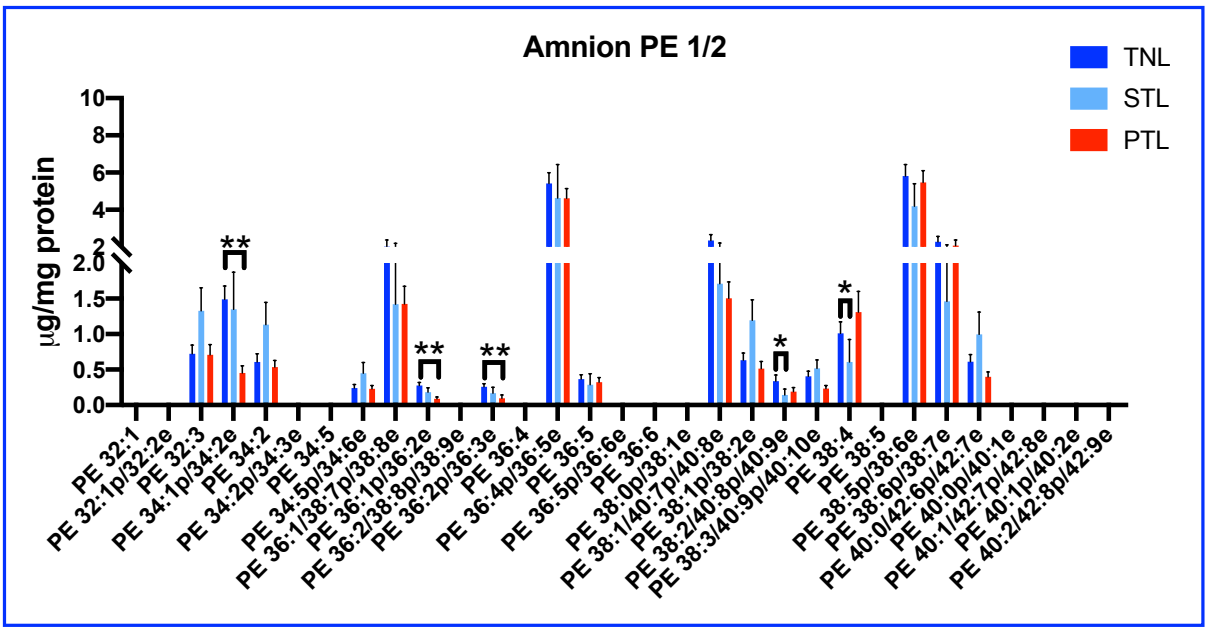
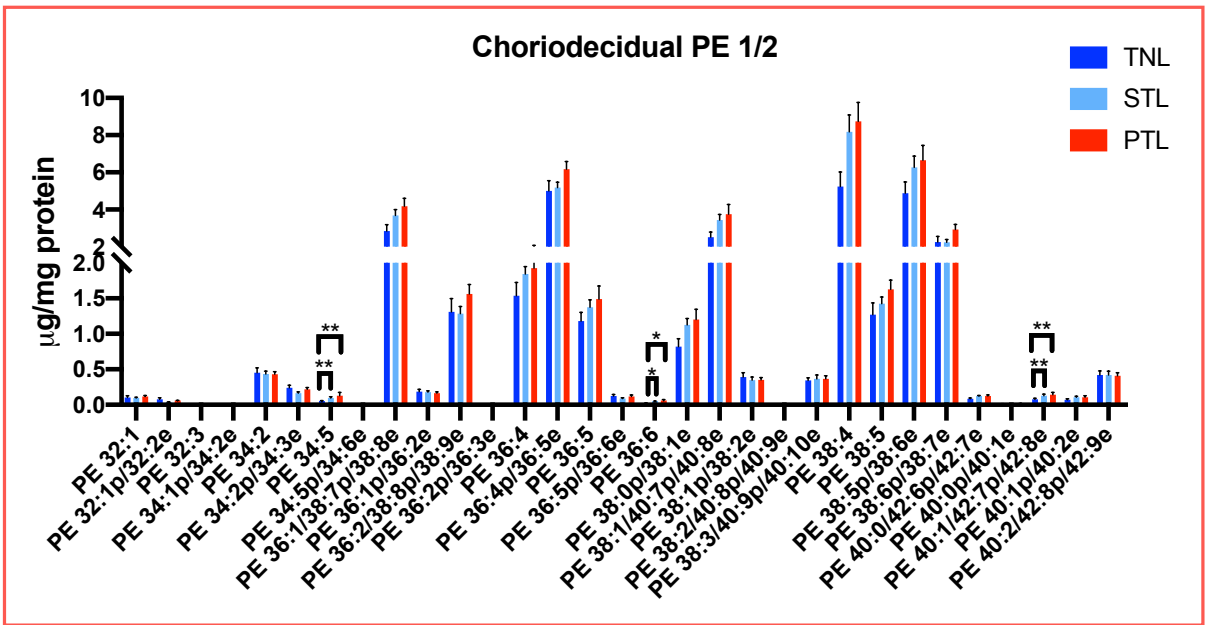


Figure 4.12. PC species in preterm fetal membranes. *Amnion (top, blue frame) and choriodecidua (bottom, red frame). Term not in labour (TNL), spontaneous term labour (STL) and preterm labour (PTL). Mean and SEM indicated. Statistical analysis = two-way ANOVA with Dunnett's multiple comparisons test, *p≤0.05, **p≤0.01.*

Figure 4.13 shows the PE profiles for fetal membranes in the preterm study. For the amnion, the preterm group (PTL) gave a mostly similar profile to TNL, with a few species showing a significant reduction: PE 34:1p/34:2e, 36:1p/36:2e and 36:2p/36:3e. In the choriodecidua, PTL showed an increase in the majority of PE species, with following species, especially those with four or more double bonds, reaching significance; PE 34:5, 36:6, 40:1/42:7p/42:8e, 42:2/44:8p/44:9e, 42:2p/42:3e, 42:3p/42:4e, 42:5p/42:6e, 42:6/42:0e, 42:8/42:0p/42:1e, 44:5p/44:6e, 44:8/44:0p/44:1e, 46:2/48:8p/48:9e and 46:5p/46:6e.


Amnion PE 1/2
TNL
STL
PTL
µg/mg protein
PE 32:1
PE 32:1p/32:2e
PE 32:3
PE 34:1p/34:2e
PE 34:2
PE 34:2p/34:3e
PE 34:5
PE 34:5p/34:6e
PE 36:1/38:7p/38:8e
PE 36:1p/36:2e
PE 36:2/38:8p/38:9e
PE 36:2p/36:3e
PE 36:4
PE 36:4p/36:5e
PE 36:5
PE 36:5p/36:6e
PE 36:6
PE 38:0p/38:1e
PE 38:1/40:7p/40:8e
PE 38:1p/38:2e
PE 38:2/40:8p/40:9e
PE 38:3/40:9p/40:10e
PE 38:4
PE 38:5
PE 38:5p/38:6e
PE 38:6p/38:7e
PE 40:0/42:6p/42:7e
PE 40:0p/40:1e
PE 40:1/42:7p/42:8e
PE 40:1p/40:2e
PE 40:2/42:8p/42:9e


Choriodecidual PE 1/2
TNL
STL
PTL
µg/mg protein
PE 32:1
PE 32:1p/32:2e
PE 32:3
PE 34:1p/34:2e
PE 34:2
PE 34:2p/34:3e
PE 34:5
PE 34:5p/34:6e
PE 36:1/38:7p/38:8e
PE 36:1p/36:2e
PE 36:2/38:8p/38:9e
PE 36:2p/36:3e
PE 36:4
PE 36:4p/36:5e
PE 36:5
PE 36:5p/36:6e
PE 36:6
PE 38:0p/38:1e
PE 38:1/40:7p/40:8e
PE 38:1p/38:2e
PE 38:2/40:8p/40:9e
PE 38:3/40:9p/40:10e
PE 38:4
PE 38:5
PE 38:5p/38:6e
PE 38:6p/38:7e
PE 40:0/42:6p/42:7e
PE 40:0p/40:1e
PE 40:1/42:7p/42:8e
PE 40:1p/40:2e
PE 40:2/42:8p/42:9e

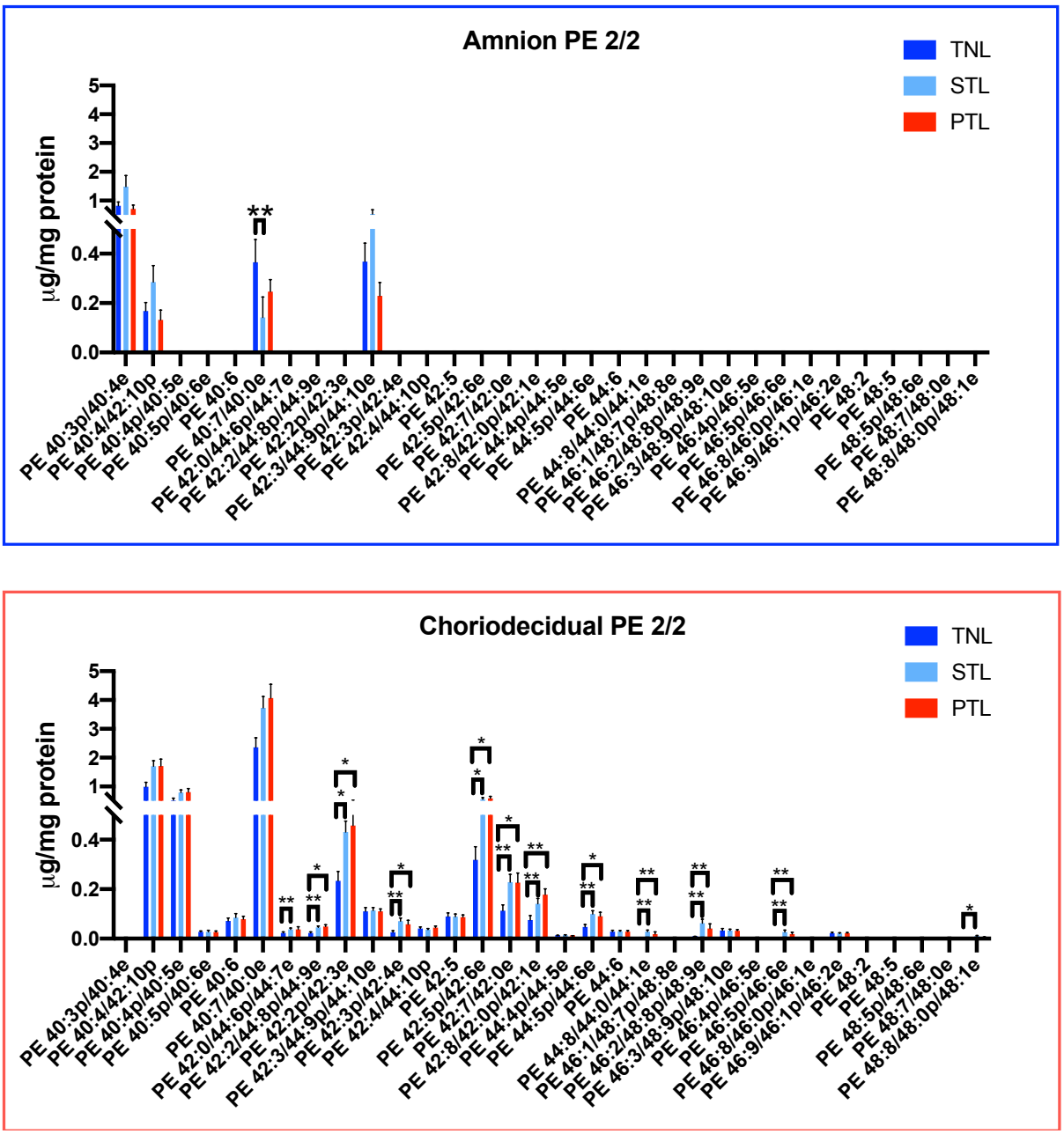


Figure 4.13. Preterm fetal membrane PE species. *Amnion (top, blue frame) and choriodecidua (bottom, red frame). Term not in labour (TNL), spontaneous term labour (STL) and preterm labour (PTL). Mean and SEM indicated. Statistical analysis = two-way ANOVA with Dunnett's multiple comparisons test, *p≤0.05, **p≤0.01.*

Figure 4.14 illustrates fetal membrane PG composition in the preterm study. In the amnion, preterm tissues expressed trace amounts of PGs and failed to show any difference compared to TNL. In choriodecidua, preterm tissues showed a similar pattern of PG species to term spontaneous labour (STL) and only PG 34:1 and PG 36:1 increased and decreased, respectively, in comparison to TNL.

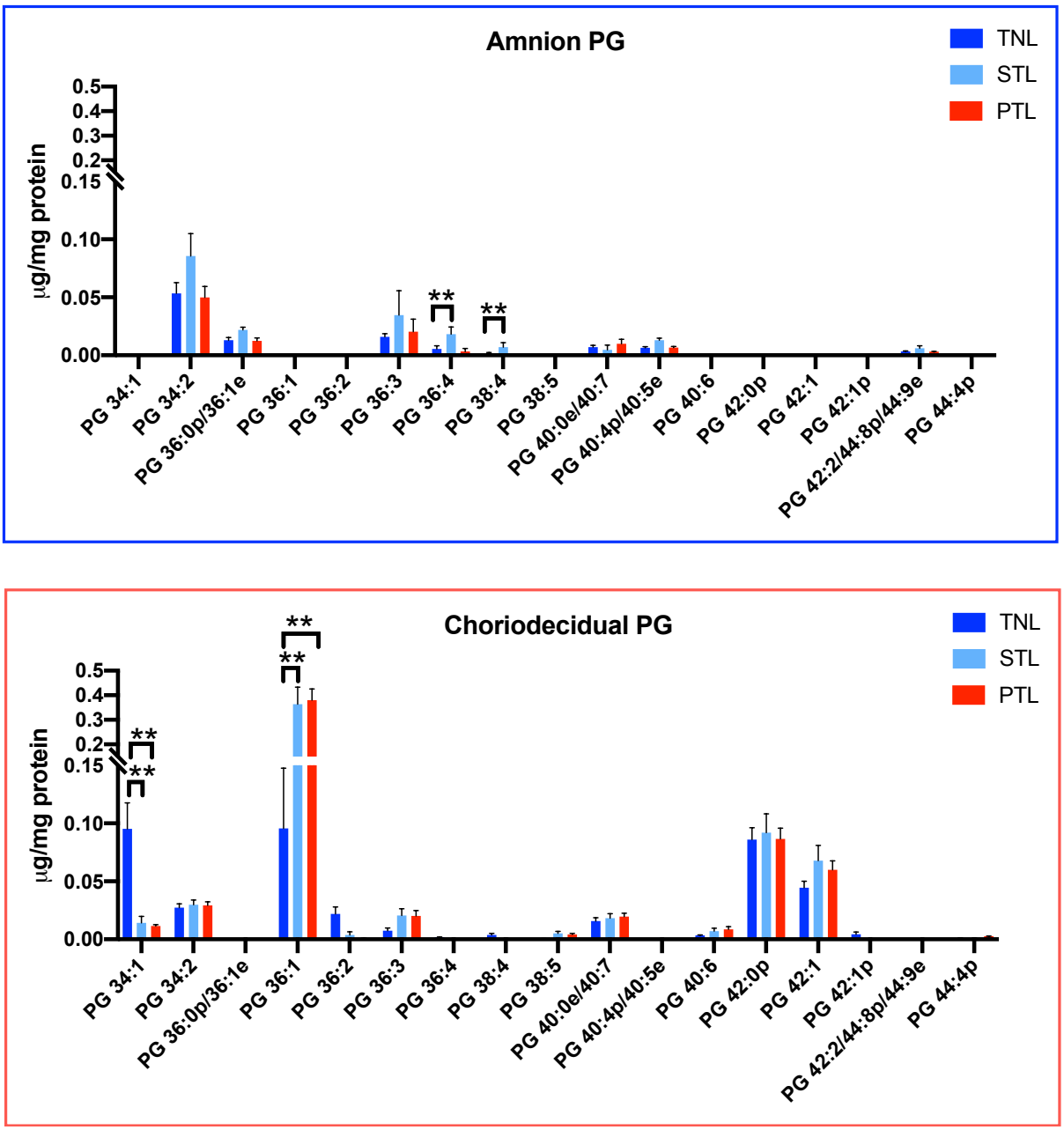


Figure 4.14. Preterm fetal membrane PG species. *Amnion (top, blue frame) and choriodecidua (bottom, red frame). Term not in labour (TNL), spontaneous term labour (STL) and preterm labour (PTL). Mean and SEM indicated. Statistical analysis = two-way ANOVA with Dunnett's multiple comparisons test, *p≤0.05, **p≤0.01.*

In the amnion, LPCs were below the detection limit (**Figure 4.15**). In the choriodecidua, the preterm group showed a trend for increases in most LPC, but this only achieved significance in the lowest abundant species; LPC 14:0, 15:0, and 17:0 (**Figure 4.15**).

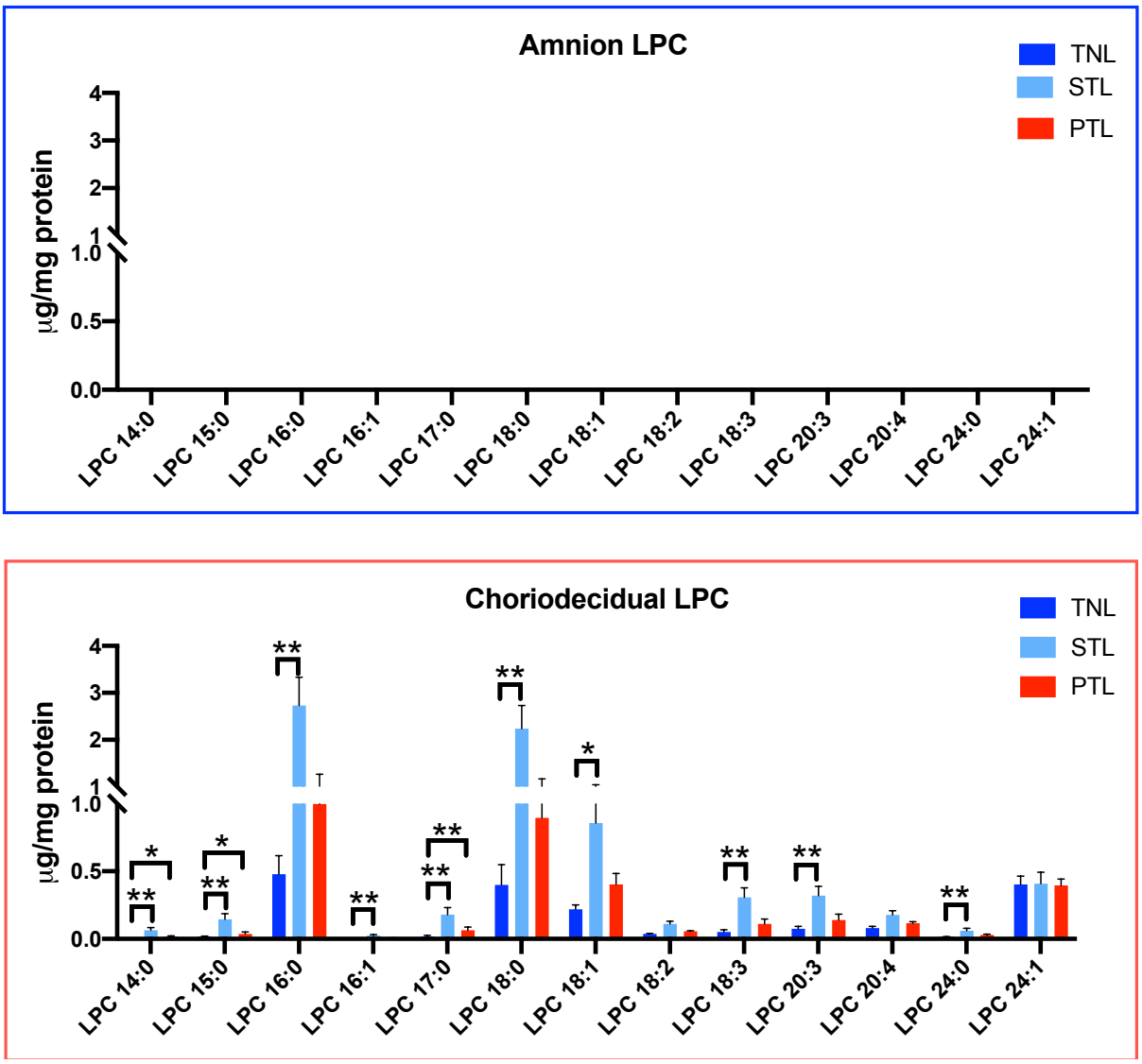
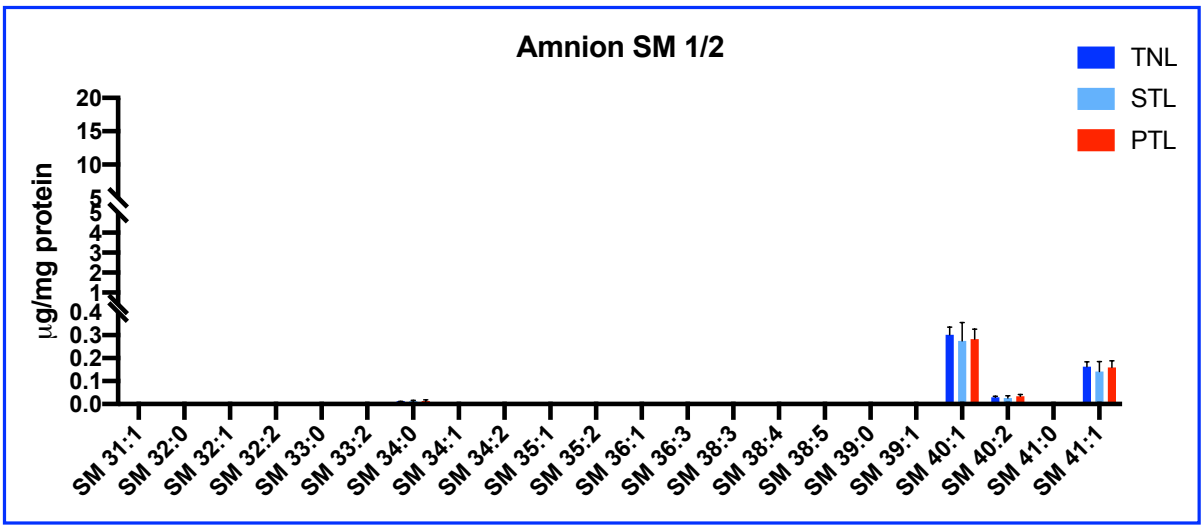
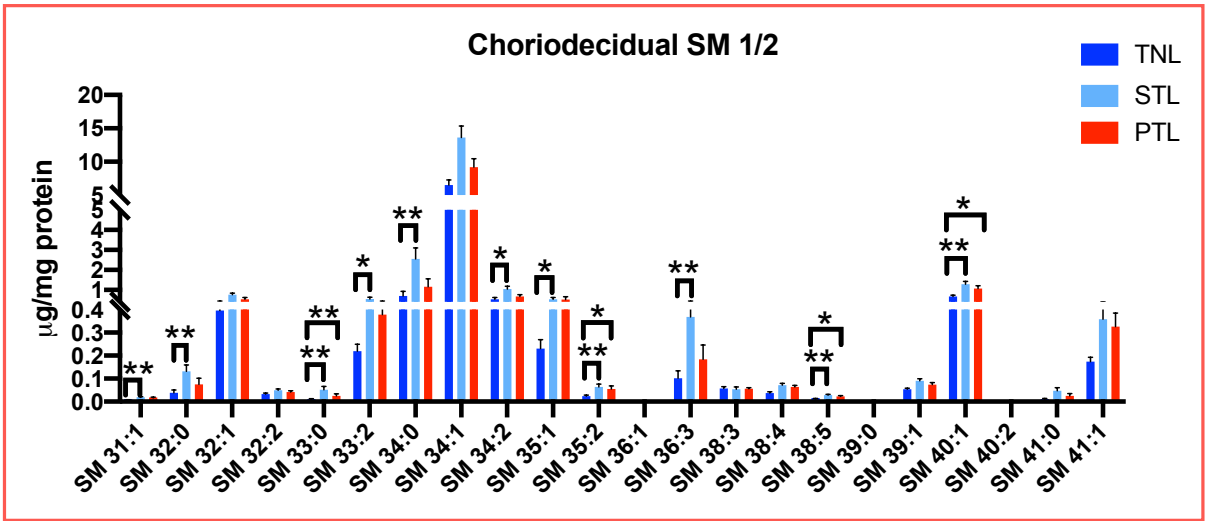


Figure 4.15. Preterm fetal membrane LPC species. Amnion (top, blue frame) and choriodecidua (bottom, red frame). *Term not in labour (TNL), spontaneous term labour (STL) and preterm labour (PTL). Mean and SEM indicated. Statistical analysis = two-way ANOVA with Dunnett's multiple comparisons test, *p≤0.05, **p≤0.01.*

Figure 4.16 shows SM species for the preterm labour study. In amnion, the preterm group gave variant expression of SMs, with a significant increase only in the lowest abundant species, SM 43:2. In choriodecidua, the preterm group showed a general trend for increased SM in PTL compared to STL, but here again predominantly in those of lowest abundance; reaching significance for SM33:0, 35:2, 38:5, 40:1, 42:0, 43:0, 43:5 and 44:3.


Amnion SM 1/2
TNL
STL
PTL
µg/mg protein
SM 31:1
SM 32:0
SM 32:1
SM 32:2
SM 33:0
SM 33:2
SM 34:0
SM 34:1
SM 34:2
SM 35:1
SM 35:2
SM 36:1
SM 36:3
SM 38:3
SM 38:4
SM 38:5
SM 39:0
SM 39:1
SM 40:1
SM 40:2
SM 41:0
SM 41:1


Choriodecidual SM 1/2
TNL
STL
PTL
µg/mg protein
SM 31:1
SM 32:0
SM 32:1
SM 32:2
SM 33:0
SM 33:2
SM 34:0
SM 34:1
SM 34:2
SM 35:1
SM 35:2
SM 36:1
SM 36:3
SM 38:3
SM 38:4
SM 38:5
SM 39:0
SM 39:1
SM 40:1
SM 40:2
SM 41:0
SM 41:1

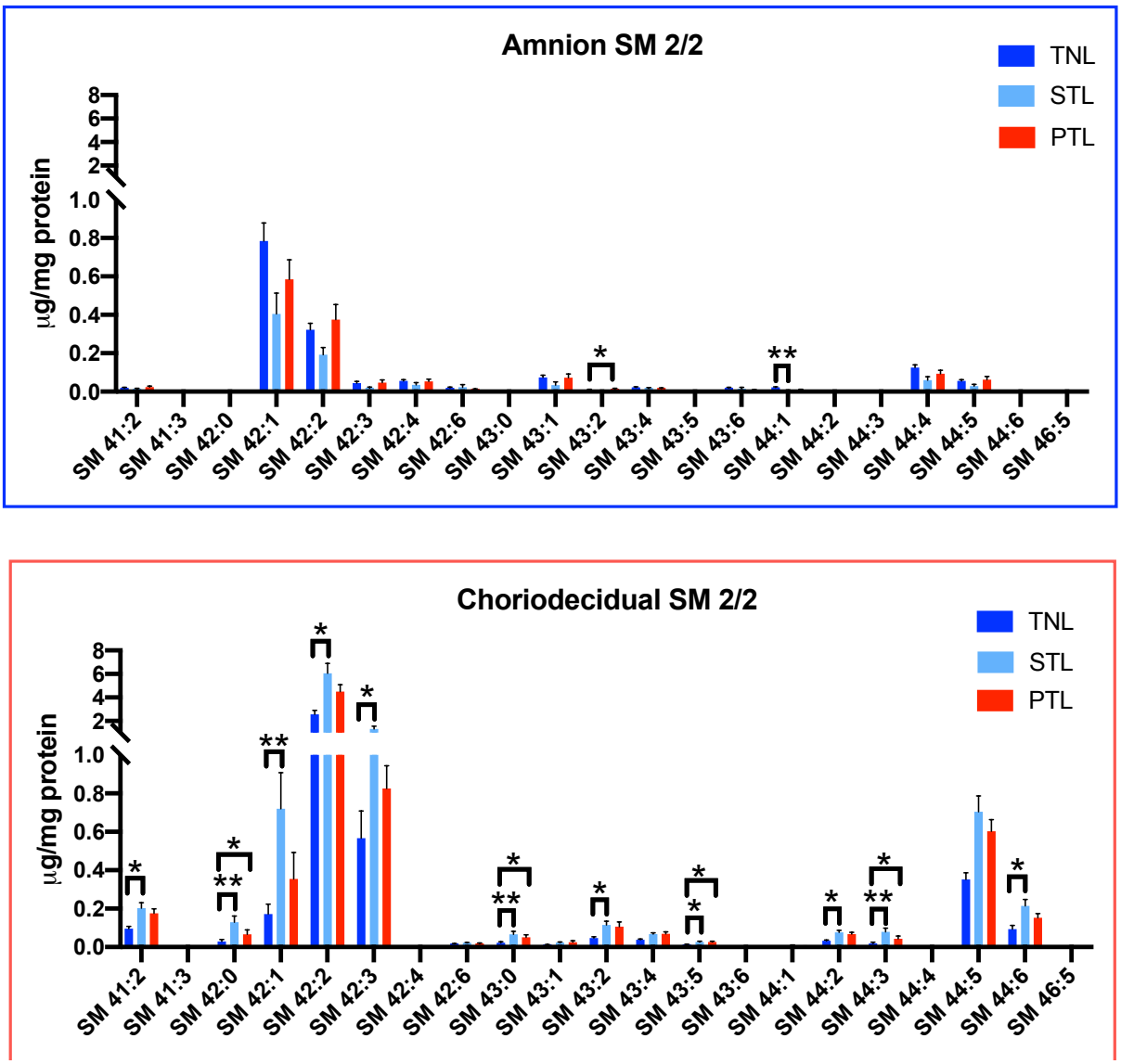


Figure 4.16. Preterm fetal membrane SM species. Amnion (top, blue frame) and choriodecidua (bottom, red frame). *Term not in labour (TNL), spontaneous term labour (STL) and preterm labour (PTL). Mean and SEM indicated. Statistical analysis = two-way ANOVA with Dunnett's multiple comparisons test, *p≤0.05, **p≤0.01.*

Figure 4.17 gives the CE profiles of fetal membranes across the three study groups; TNL, STL and PTL. Within these data, the amnion showed low concentrations for all CE species, whilst choriodecidua, showed a similar pattern in PLT to TNL.

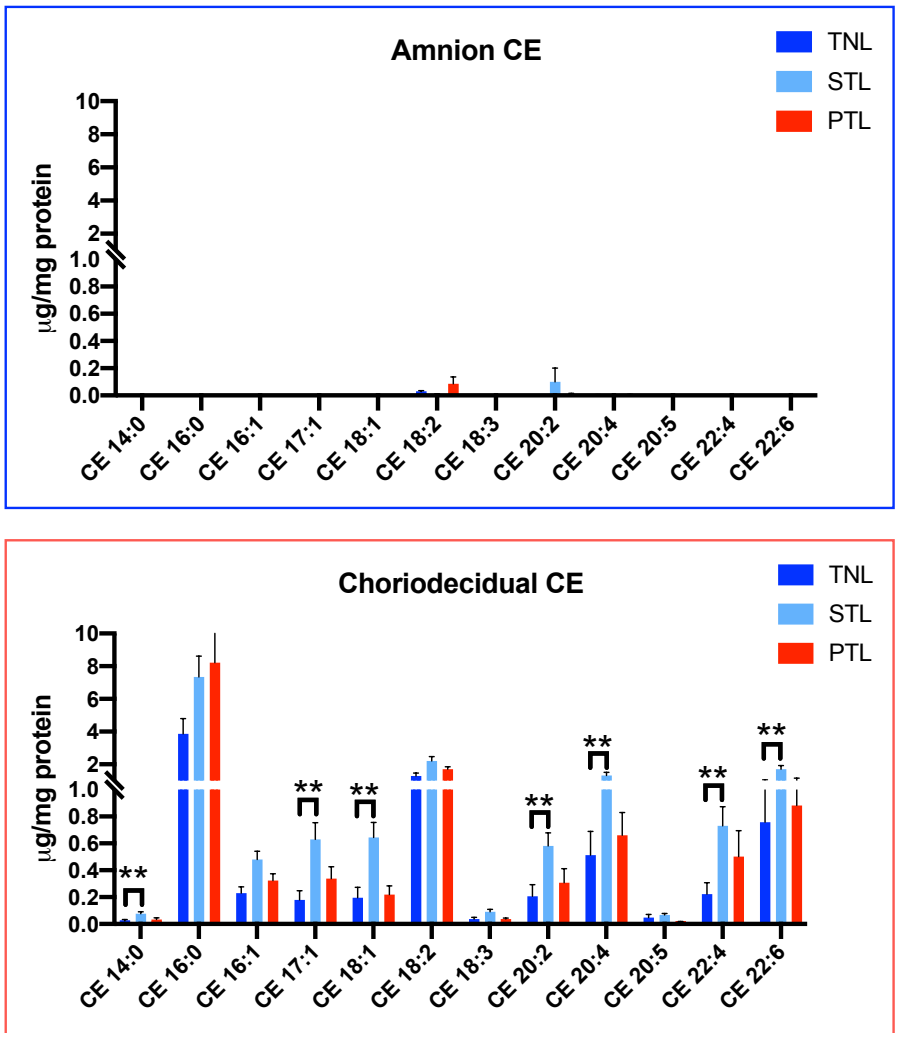
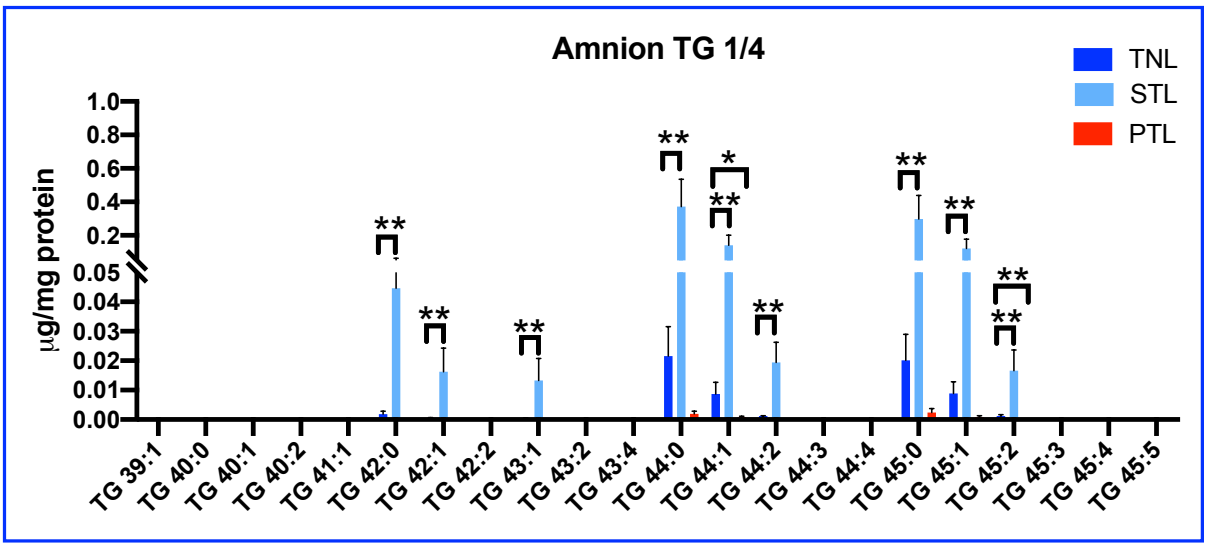
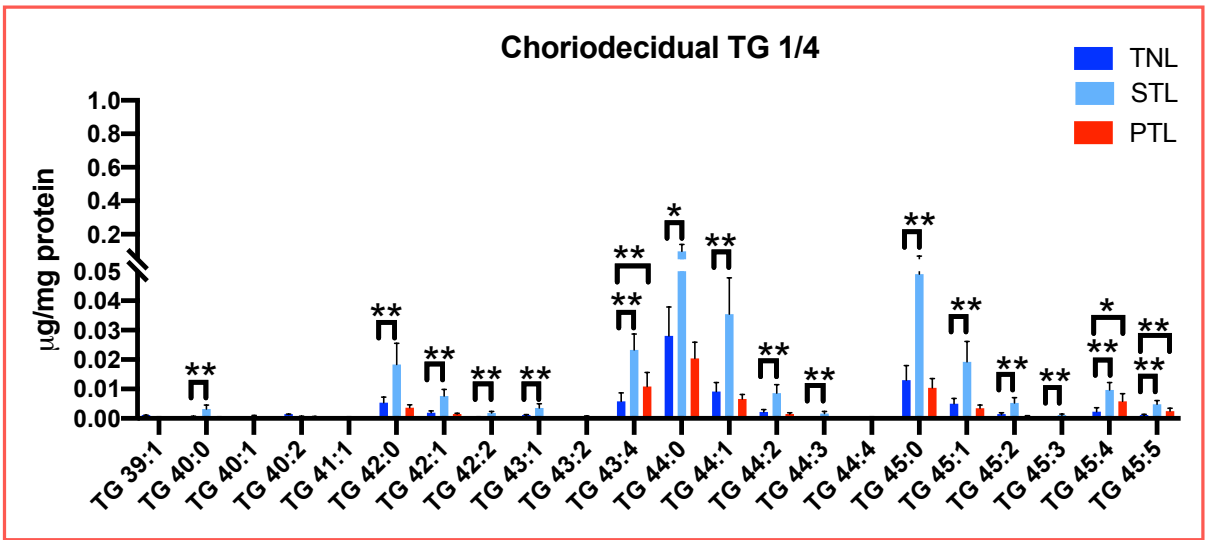
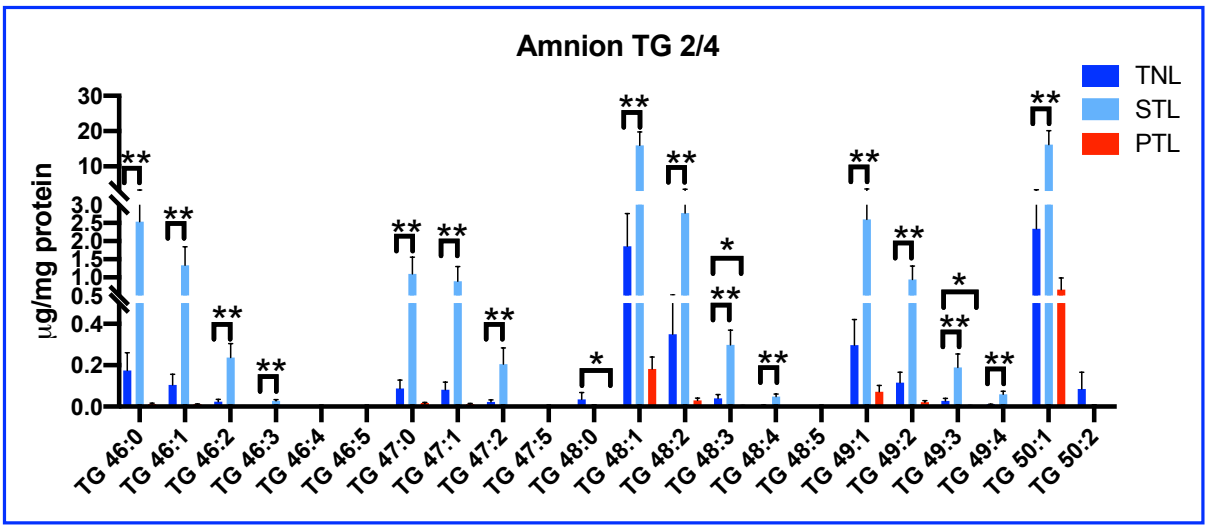


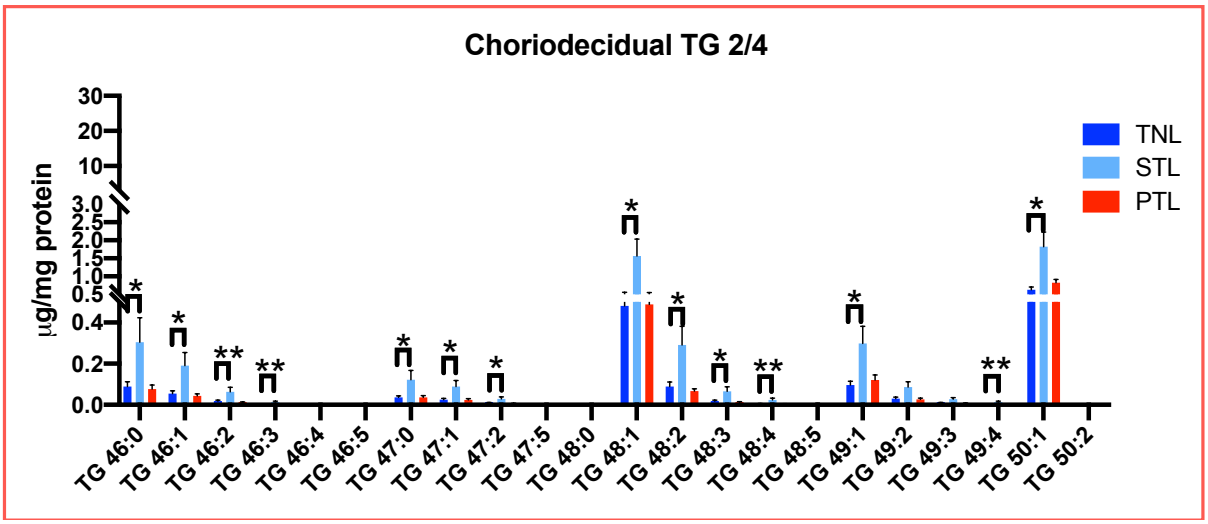
Figure 4.17. Preterm fetal membrane CE species. *Amnion (top, blue frame) and choriodecidua (bottom, red frame). Term not in labour (TNL), spontaneous term labour (STL) and preterm labour (PTL). Mean and SEM indicated. Statistical analysis = two-way ANOVA with Dunnett's multiple comparisons test, *p≤0.05, **p≤0.01.*

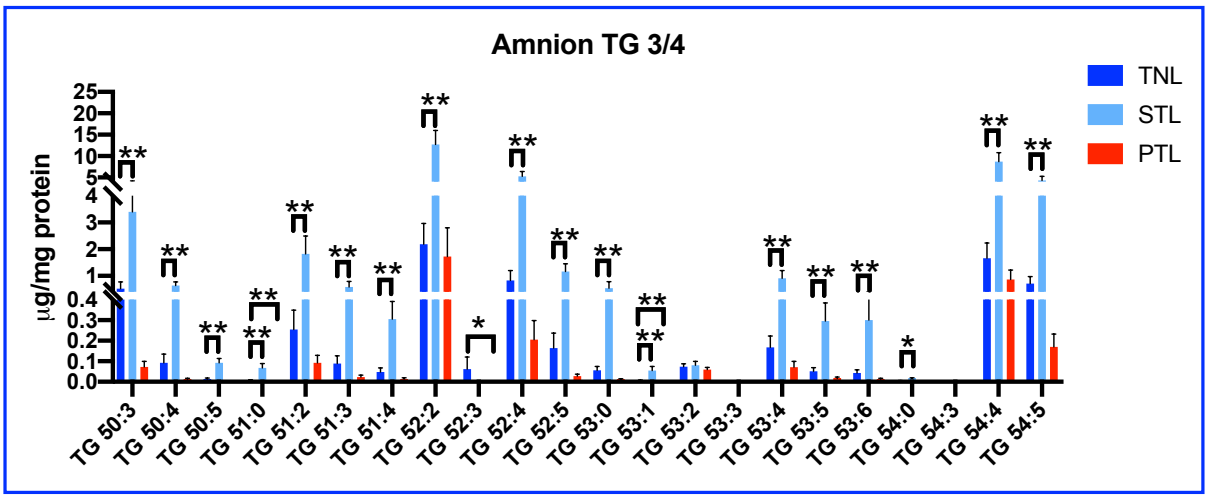
The preterm amnion showed a general reduction in TG species compared to TNL; reaching significance for TG 44:1, 45:2, 48:0, 48:3, 49:3, 51:0, 52:3 and 53:1 (**Figure 4.18**). In the choriodecidua, PTL tissue gave a variant pattern in TG species compared to TNL, with the following significantly increased; TG 34:4, 45:4 and 45:5 (**Figure 4.18**).

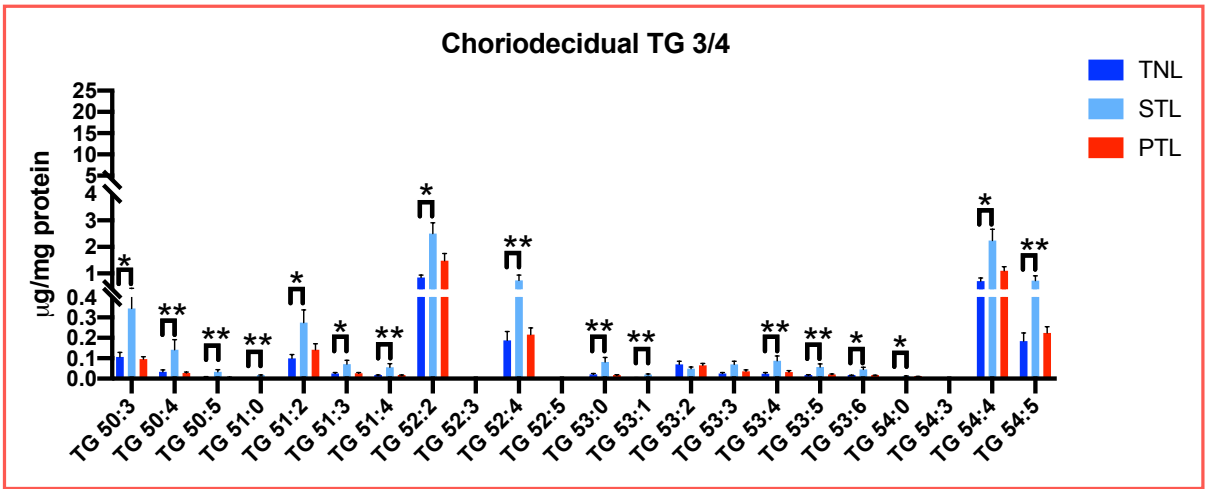

Amnion TG 1/4
TNL
STL
PTL
µg/mg protein
1.0
0.8
0.6
0.4
0.2
0.05
0.04
0.03
0.02
0.01
0.00
TG 39:1
TG 40:0
TG 40:1
TG 40:2
TG 41:1
TG 42:0
TG 42:1
TG 42:2
TG 43:1
TG 43:2
TG 43:4
TG 44:0
TG 44:1
TG 44:2
TG 44:3
TG 44:4
TG 45:0
TG 45:1
TG 45:2
TG 45:3
TG 45:4
TG 45:5


Choriodecidual TG 1/4
TNL
STL
PTL
µg/mg protein
1.0
0.8
0.6
0.4
0.2
0.05
0.04
0.03
0.02
0.01
0.00
TG 39:1
TG 40:0
TG 40:1
TG 40:2
TG 41:1
TG 42:0
TG 42:1
TG 42:2
TG 43:1
TG 43:2
TG 43:4
TG 44:0
TG 44:1
TG 44:2
TG 44:3
TG 44:4
TG 45:0
TG 45:1
TG 45:2
TG 45:3
TG 45:4
TG 45:5


Amnion TG 2/4
TNL
STL
PTL
µg/mg protein
30
20
10
3.0
2.5
2.0
1.5
1.0
0.5
0.4
0.2
0.0
TG 46:0
TG 46:1
TG 46:2
TG 46:3
TG 46:4
TG 46:5
TG 47:0
TG 47:1
TG 47:2
TG 47:5
TG 48:0
TG 48:1
TG 48:2
TG 48:3
TG 48:4
TG 48:5
TG 49:1
TG 49:2
TG 49:3
TG 49:4
TG 50:1
TG 50:2


Choriodecidual TG 2/4
TNL
STL
PTL
µg/mg protein
30
20
10
3.0
2.5
2.0
1.5
1.0
0.5
0.4
0.2
0.0
TG 46:0
TG 46:1
TG 46:2
TG 46:3
TG 46:4
TG 46:5
TG 47:0
TG 47:1
TG 47:2
TG 47:5
TG 48:0
TG 48:1
TG 48:2
TG 48:3
TG 48:4
TG 48:5
TG 49:1
TG 49:2
TG 49:3
TG 49:4
TG 50:1
TG 50:2


Amnion TG 3/4
µg/mg protein
TNL
STL
PTL
TG 50:3
TG 50:4
TG 50:5
TG 51:0
TG 51:2
TG 51:3
TG 51:4
TG 52:2
TG 52:3
TG 52:4
TG 52:5
TG 53:0
TG 53:1
TG 53:2
TG 53:3
TG 53:4
TG 53:5
TG 53:6
TG 54:0
TG 54:3
TG 54:4
TG 54:5


Choriodecidual TG 3/4
µg/mg protein
TNL
STL
PTL
TG 50:3
TG 50:4
TG 50:5
TG 51:0
TG 51:2
TG 51:3
TG 51:4
TG 52:2
TG 52:3
TG 52:4
TG 52:5
TG 53:0
TG 53:1
TG 53:2
TG 53:3
TG 53:4
TG 53:5
TG 53:6
TG 54:0
TG 54:3
TG 54:4
TG 54:5

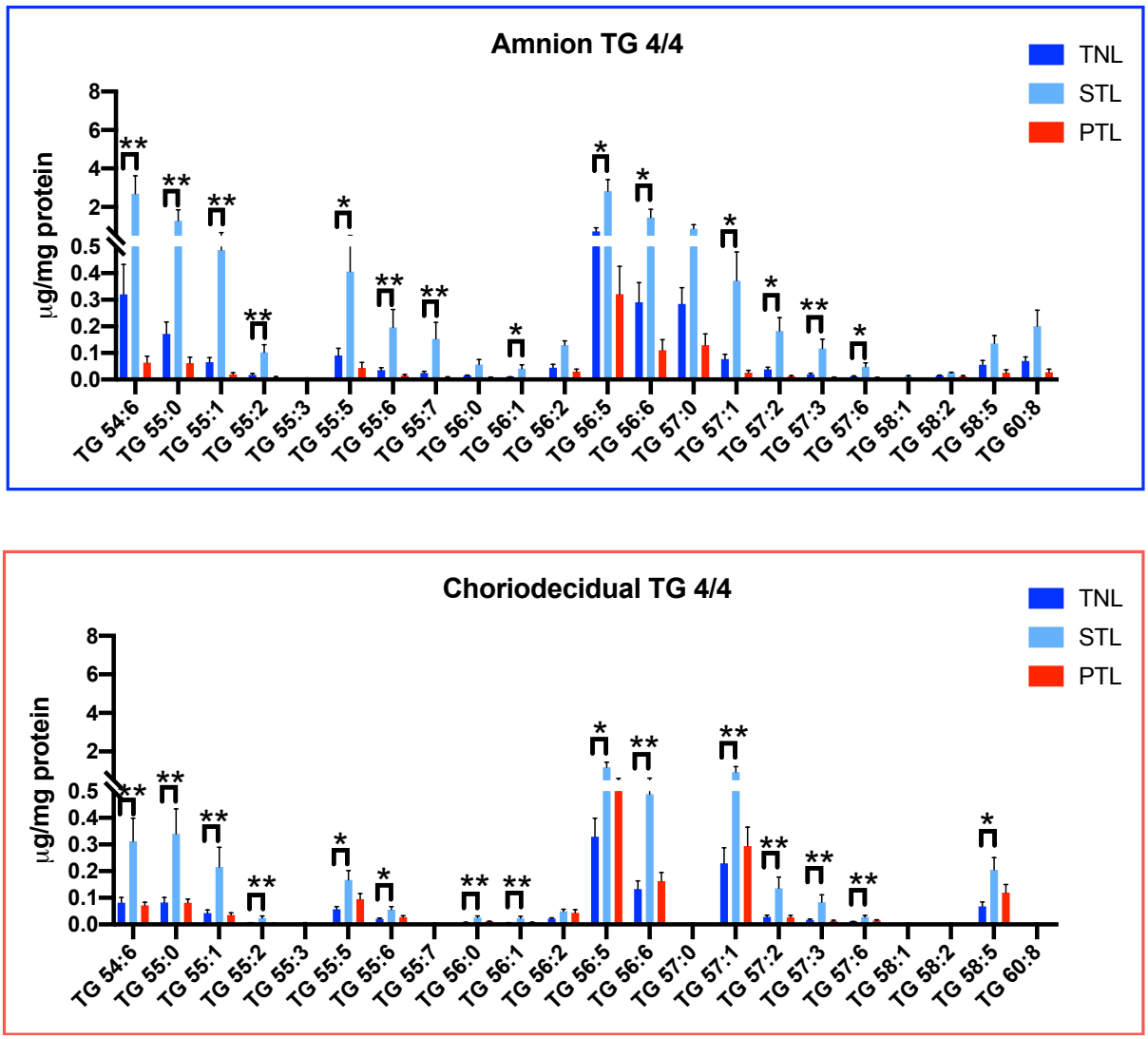


Figure 4.18. Preterm fetal membrane TG species. *Amnion (top, blue frame) and choriodecidua (bottom, red frame). Term not in labour (TNL), spontaneous term labour (STL) and preterm labour (PTL). Mean and SEM indicated. Statistical analysis = two-way ANOVA with Dunnett's multiple comparisons test, *p≤0.05, **p≤0.01.*

The PTL amnion gave similar DG species levels to TNL (**Figure 4.19**). However, the choriodecidua was increased in preterm labour (PTL) across DG, most significantly DG 48:2, 39:6 and 40:4; but this was not as extensive as that seen with STL (**Figure 4.19**).

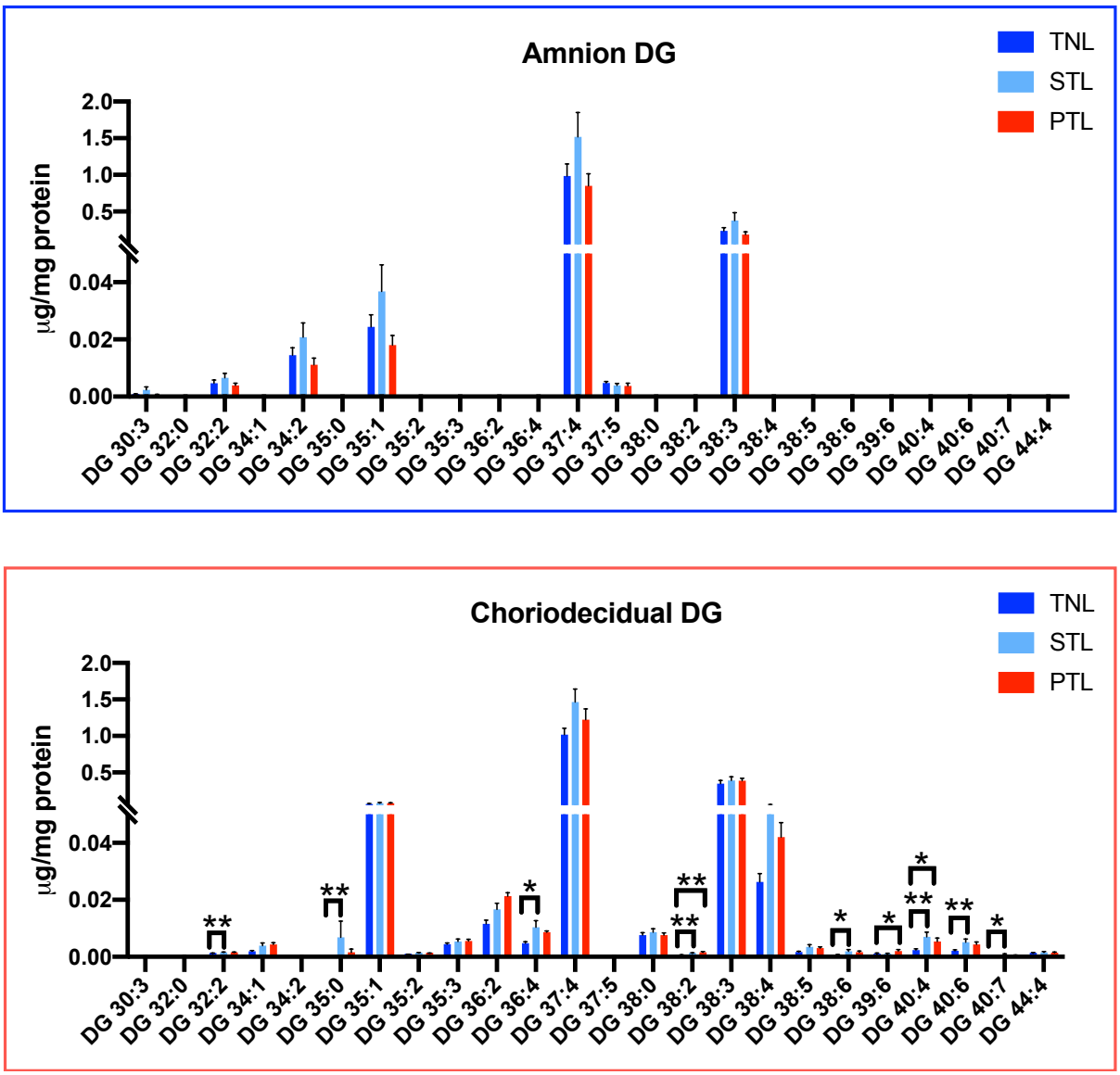


Figure 4.19. Preterm fetal membrane DG species. *Amnion (top, blue frame) and choriodecidua (bottom, red frame). Term not in labour (TNL), spontaneous term labour (STL) and preterm labour (PTL). Mean and SEM indicated. Statistical analysis = two-way ANOVA with Dunnett's multiple comparisons test, *p≤0.05, **p≤0.01.*

Figure 4.20 indicates detected FFA in the preterm study. In the amnion, the preterm group showed a significant increase in expression of the following mono- and poly-unsaturated FFAs; 18:2, 18:3, 20:1, 22:1, and 24:1. In the choriodecidua, PTL showed a similar pattern to STL; with significant reductions reached in the saturated FFAs, 14:0 and 23:0; and significant increases recorded in the mono- and poly-unsaturated FFAs; 16:1, 18:2, 18:3, 20:4, 22:1, 24:0, 24:1 and 26:1.

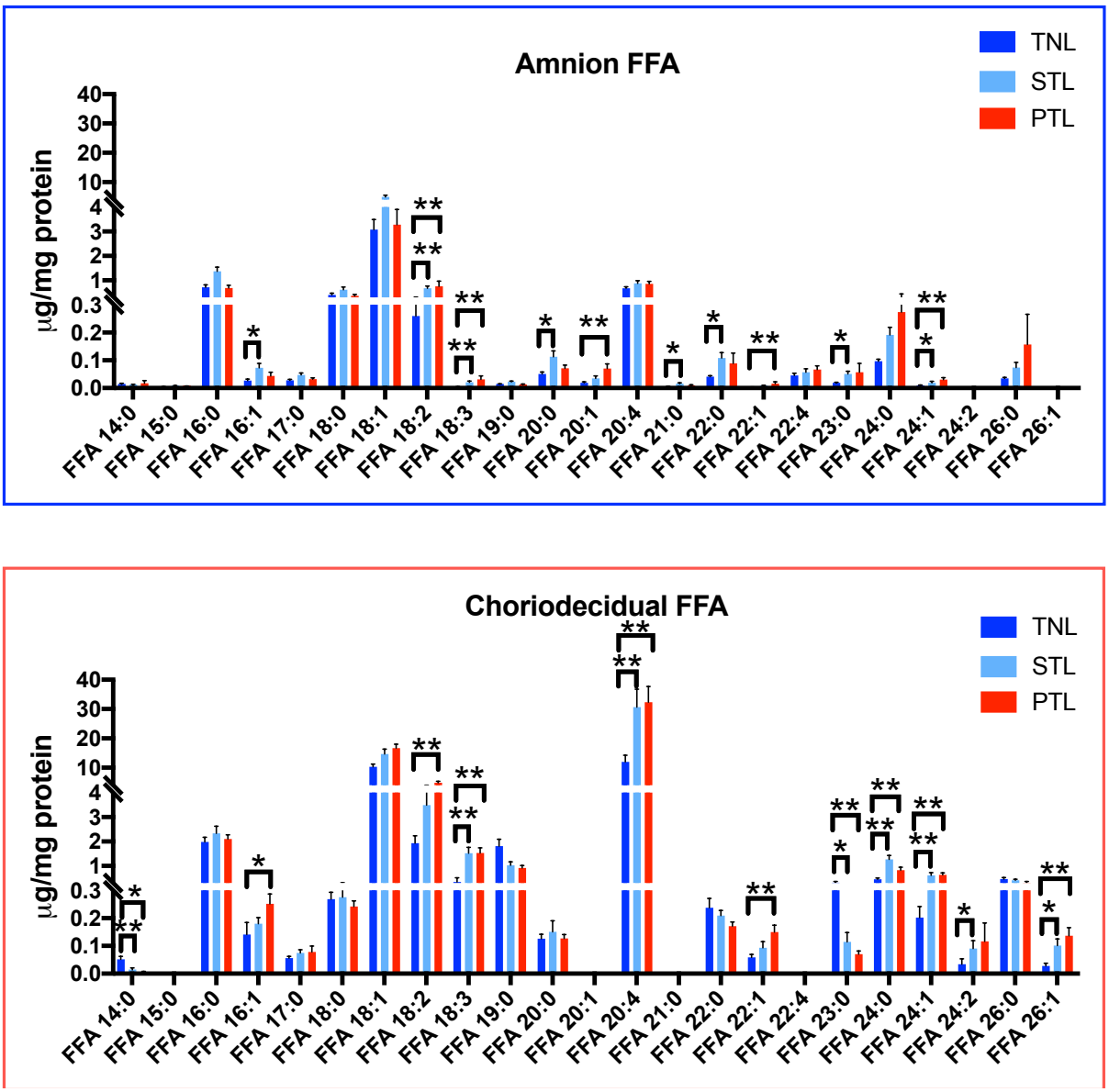


Figure 4.20. Preterm fetal membrane FFA species. *Amnion (top, blue frame) and choriodecidua (bottom, red frame). Term not in labour (TNL), spontaneous term labour (STL) and preterm labour (PTL). Mean and SEM indicated. Statistical analysis = two-way ANOVA with Dunnett's multiple comparisons test, *p≤0.05, **p≤0.01.*

In conclusion, preterm amnion showed a similar lipid composition to that already defined above TNL. On the other hand, the preterm choriodecidua showed an increased trend in glycerophospholipids, LPC and SM, compared to non-labouring tissue at term, with arguably greater availability of free AA.

4.4.3. Total fatty acid profiles in fetal membranes

Fetal membrane total fatty acid abundance was measured using GC-FID analysis. This analysis provides a sensitive way to report total fatty acids; carbon and double bond numbers, and also double bond positions. The term study included STL (n=4 and n=6, amnion and choriodecidua, respectively) and ITL (n=6 and n=7) groups. Both were statistically challenged with a TNL group (n=5 and n=7). For the preterm study, a PTL group was included (n=4 and n=6 amnion and choriodecidua, respectively). The data were expressed throughout as a percentage of the total fatty acid weight.

4.4.4. Total fatty acid profiles in term fetal membranes

Figure 4.21 shows fatty acid abundances in term fetal membrane. Palmitic acid (C16:0), oleic acid (C18:1), stearic acid (C18:0) and AA (C20:4n-6) were the most plentiful fatty acids in amnion and choriodecidua. The STL amnion showed a significant increase in C14:0, C16:0, and C18:3n-3, and significant decrease in C17:1, C18:0, C20:4n-6, C22:0, C22:1n-9 and C24:1, respectively. Likewise, ITL showed an increase in C14:0, C16:0, and C18:3n-3, and decrease in C17:1, C20:4n-6, C22:0, C22:1n-9, C22:6n-3, C23:0 and C24:1. In the choriodecidua, no significant changes were evident in fatty acids between STL and TNL tissues. However, for ITL, a significant elevation in C16:0, C16:1, C20:0, and C20:5n-3 were reported, along with a marked reduction in C18:0 and C20:1n-9.

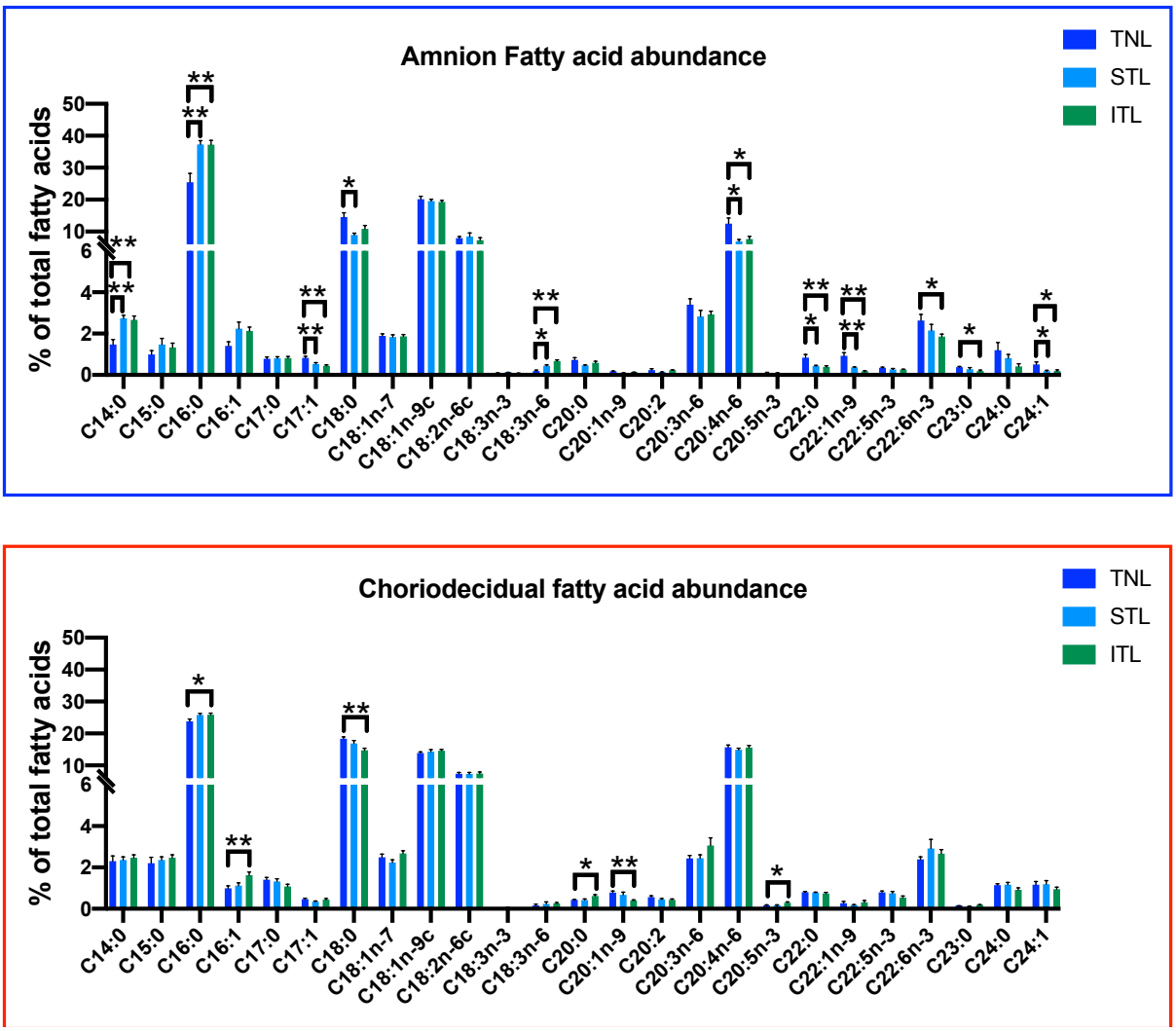
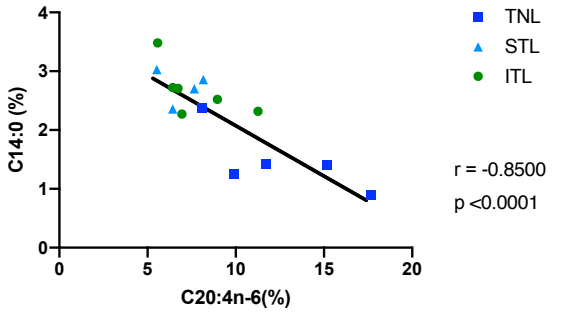
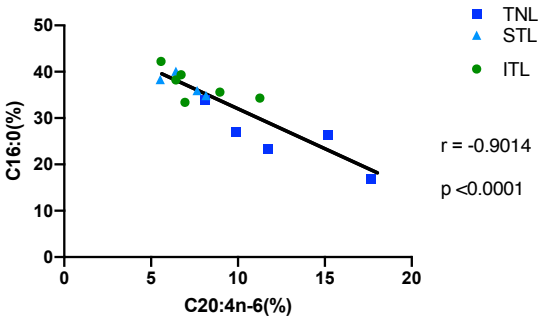
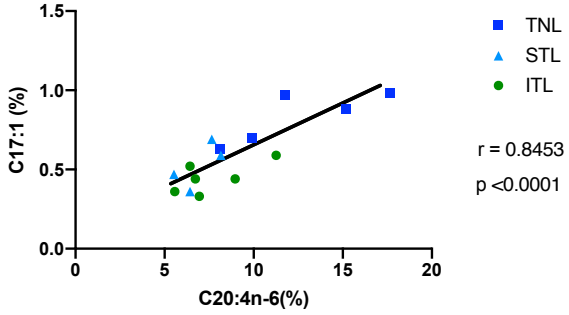


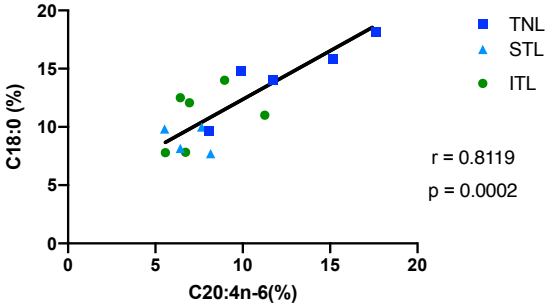
Figure 4.21. Term fetal membrane fatty acid profile*. Amnion (top, blue frame) and choriodecidua (bottom, red frame). Term not in labour (TNL), spontaneous term labour (STL) and induced term labour (ITL). Mean and SD indicated. Statistical analysis = one-way ANOVA with Dunnett's multiple comparisons test, *p≤0.05, **p≤0.01.*

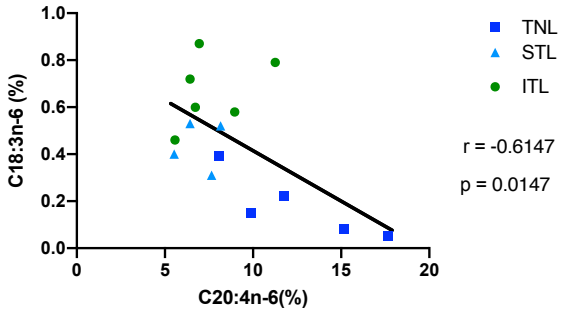
For the amnion, AA abundance was significantly altered under labouring conditions. In studying correlations between these changes and those in other FA (**Figure 4.22**), an inverse relationship was recorded between AA levels and C14:0, C16:0 and C18:3n-3, specifically; and positive correlation defined with C17:1, C18:0, C22:0, C22:1-9, C22:6n-3, C23:0 and C24:1.

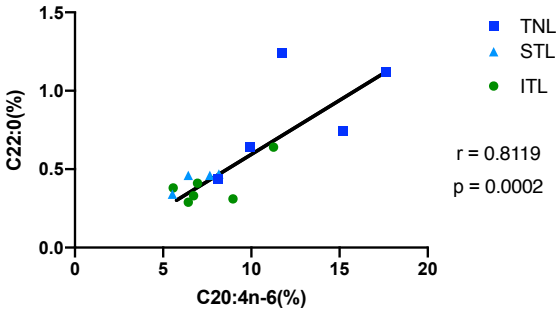

C14:0 (%)
C20:4n-6(%)
TNL
STL
ITL
r = -0.8500
p <0.0001

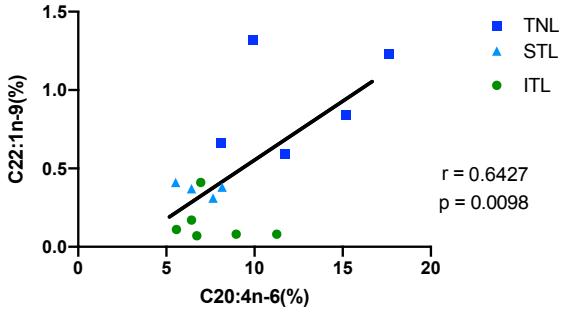

C16:0(%)
C20:4n-6(%)
TNL
STL
ITL
r = -0.9014
p <0.0001

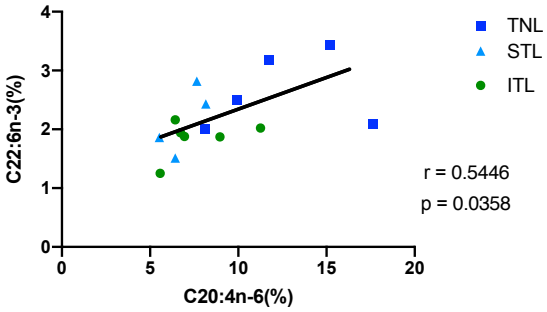

C17:1 (%)
C20:4n-6(%)
TNL
STL
ITL
r = 0.8453
p <0.0001


C18:0 (%)
C20:4n-6(%)
TNL
STL
ITL
r = 0.8119
p = 0.0002


C18:3n-6 (%)
C20:4n-6(%)
TNL
STL
ITL
r = -0.6147
p = 0.0147


C22:0(%)
C20:4n-6(%)
TNL
STL
ITL
r = 0.8119
p = 0.0002


C22:1n-9(%)
C20:4n-6(%)
TNL
STL
ITL
r = 0.6427
p = 0.0098


C22:6n-3(%)
C20:4n-6(%)
TNL
STL
ITL
r = 0.5446
p = 0.0358

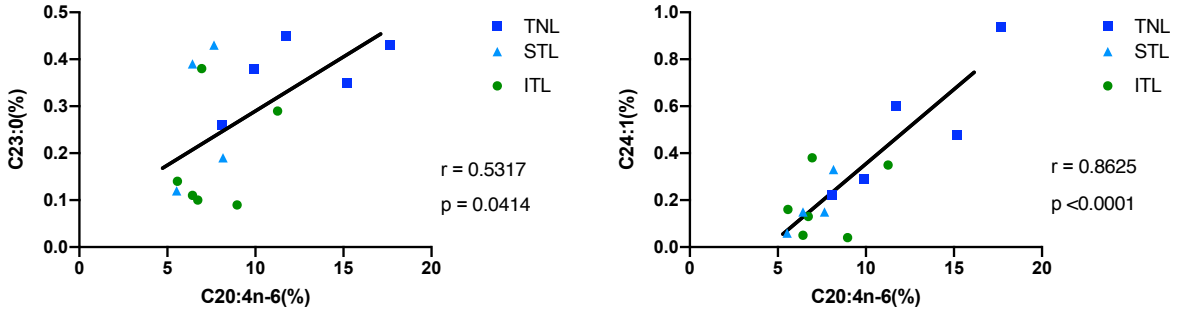


Figure 4.22. Arachidonic acid (C20:4n-6) correlation with other fatty acids in term amnion tissues. *Term not in labour (TNL), spontaneous term in labour (STL), and induction of labour (ITL). The data were expressed as percentage of total weight of fatty acids. P-value and correlation coefficient (r) indicated. The statistical analysis = two-tailed Pearson correlation test.*

With AA as the dominant FA in amnion and choriodecidua, it is intriguing to note a strong depletion in AA in the amnion under all term labouring conditions, but sustained, perhaps unexpected, level of AA in the choriodecidua regardless of labour and type of onset.

4.4.5. Profiling fatty acids in preterm fetal membranes

Figure 4.23 illustrates total fatty acid composition in the preterm study. In the amnion, the PTL group showed similarities to TNL, except for C20:5n-3, which was in any case detected at only trace levels. In choriodecidua, the PTL showed significant reductions in C17:0, C17:1 and C20:4n-6, and an increase in C18:1n-9 compared to TNL, whilst all other FA remained unaltered.

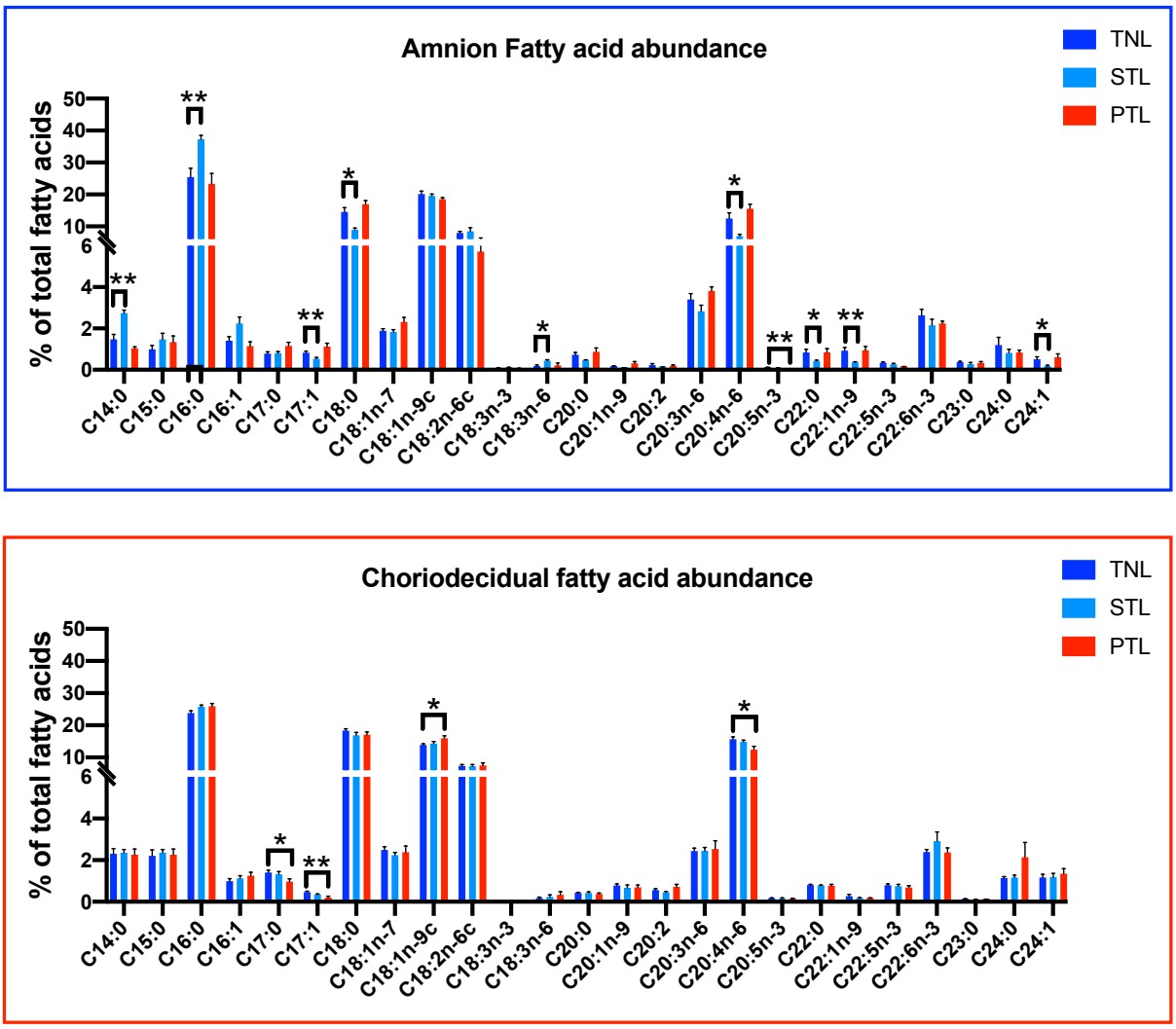


Figure 4.23. Preterm fetal membrane fatty acid profile. *Amnion (top, blue frame) and choriodecidua (bottom, red frame). Term not in labour (TNL), spontaneous term labour (STL) and preterm labour (PTL). Mean and SD indicated. Statistical analysis = one-way ANOVA with Dunnett's multiple comparisons test, *p≤0.05, **p≤0.01.*

In conclusion, labouring preterm amnion maintained its AA levels compared to non-labour pregnancies at term. Whereas preterm choriodecidua was lower in AA than its term non-labouring counterpart.

4.5. Discussion

To our knowledge, this the first comprehensive study to explore lipid composition changes in human fetal membranes under different labouring conditions. The amnion and choriodecidua showed a distinct lipid distribution, similar to that observed in the previous chapter; with choriodecidua showing greater alterations in lipid classes over amnion in all labouring groups, and more noticeable with the spontaneous term labour. Notably, the amnion showed

depletion of arachidonic acid, which may support its proposed role as a source of contractile prostanoids; at least in the early stages of physiological parturition (34,104).

Within the amnion, the major membrane glycerophospholipids, PC and PE, failed to be modified across labour, but choriodecidua showed increased total PC, and a trend towards increased total PE in spontaneous term tissues, over non-labouring membranes at term. The preterm term choriodecidua also showed increased total PC and PE, but this failed to reach statistical significance under tested numbers. This increase could have a number of explanations. Firstly, a reflection of previously reported maternal plasma PC and PE in term labour (78). Secondly, a result of excess free polyunsaturated fatty acids, also observed. Thirdly, a distinct change in cellular populations present within the tissues as parturition is propagated; most likely leukocytes, as proposed in the literature (19,258,280) through active recruitment.

Leukocyte invasion into gestational tissues at term and preterm labour has been observed by many researchers (19,258,280,281). Within these studies, the infiltrated leukocytes are predominantly localised to the choriodecidua over amnion (258,280). Although difficult to pinpoint the timing of this leukocyte invasion, Hamilton et al. (2012) reported increased macrophage infiltrate in rat decidua, 12 hours prior to the start of uterine contractions (258). This same study showed a predominance of invasive macrophages in the choriodecidua at term, but higher numbers of NK and T-cells in preterm labouring tissues. This difference could in part explain the lipid data presented here; particularly, the greater abundance of glycerophospholipid in the choriodecidua. It is foreseen, that infiltrating leukocytes at this location could have the role of priming the inflammatory environment of the choriodecidua; presumably through secretion of pro-inflammatory cytokines, such as IL-1β, IL-6, TNFα, and prostaglandins to enhance local, constituent production of other more key labour-associated mediators (282,283).

AA and other PUFAs tend to be stored esterified at the sn-2 position in PC and PE (284). In this study, free PUFAs were higher in the choriodecidua than amnion, with free AA (C20:4) and GLA/ALA (C18:3) over 10-fold more abundant. Also, in choriodecidua, free AA levels were significantly elevated in spontaneous term and preterm labour in comparison to the control,

and PC and PE species (with potential incorporated AA and PUFAs) were significantly elevated. Of these elevated species, plasmalogens were the dominant structures; ie PC36:5p/36:6e, PC37:4/38:3p/38:4e, PE 44:5p/44:6e and PE46:5p/46:6e. Plasmalogens are a subclass of PC and PE that contain a vinyl ether bond at the sn-1 position, and are known to be enriched in AA and DHA at sn-2 (70). In general, plasmalogens are referred as having an anti-inflammatory role in different tissues (70,285). Nevertheless, their importance in gestational tissues and parturition have not been defined.

The rising of choriodecidual plasmalogens under spontaneous term and preterm conditions, could theoretically provide a store for excess free AA, and a source to release AA though the action of PLA_2 enzymes (70).In drug-induced labour, choriodecidual plasmalogens showed fewer elevated species than spontaneous labour at term, perhaps reflected by a concomitant lack of free AA, implying that mechanistic difference between spontaneous and induced labour. In **Chapter 3**, increased AA-derived prostanoids and AEA production were observed in the choriodecidua under spontaneous labour; but these same tissues now show an upregulation of PC and PE with additional storage of AA. Rationalising these two seemingly conflicting findings, would mean an increase in maternal derived–AA as a source to maintain prostanoid production in labour, such as that implied above from the maternal circulation or active leucocyte infiltration. Although the latter has more consistent support (see above), the former is also supported by a report of increased free AA within maternal plasma at the time of labour (286), as a reversal to maternal AA decline throughout the preceding weeks of pregnancy (287), through unprecedented fetal and placental sequestration. For induced labour, these physiological increases in free AA and AA-containing PC and PE, were less prevalent than those seen in the spontaneous group; perhaps reinforcing the requirement for free and stored AA in the choriodecidua for natural parturition.

In amnion, in direct opposition to the choriodecidua, PC and PE profiles showed a selective reduction in AA-containing species in spontaneous term labour, i.e. PC 34:3p/34:4e, 36:0/38:6p/38:7e, 36:1/38:7p/38:8e, 38:4, 40:4, 40:5, 42:5p/42:6e and single PE 40:7/40:0e; perhaps indicative of amnion depletion in AA storage. To some extent, these data agree with a previous report of observed AA depletion in amnion PE and PI in term labour, although analytical differences and definitions of labour prevent direct comparison (288).

Overall, these data are in line with the amnion being a prominent source of prostanoids during the earlier stages of parturition, i.e. before overt signs of cervical ripening and uterine contractions; to be eventually superseded by the more prolonged prostanoid production within the choriodecidua. For induced pregnancies, a trend for a reduction in the amnion for AA-containing species was also evident. Given we observed a significant increase of AA-derived prostanoids, we suggest that uterotonic drugs stimulated AA releasing from amnion glycerophospholipids. Although the preterm amnion showed a reduction in AA-containing PC and PE species, no significant increase in AA-derived prostanoids or classical endocannabinoids (AA-derived) were concomitantly observed. Although difficult to square with the above suggestions, there remains the possibility that AA increases in amnion when reaching term. We couldn't detect PI, PS and PhA in our study; but this could be due to their low biological concentrations.

LPC species were only detected in the choriodecidua with labour, indicative of resident and timely PLA_2 activity. In spontaneous labours, the choriodecidua showed a significant increase in LPCs, but this was more noticeably at term; a finding supportive of the elevations in eicosanoids as shown in Chapter 3 and in accord with reports of upregulation of $cPLA_2$ gene expression (PLA2G4A) in the labouring term choriodecidua (182). Alongside cellular cPLA2, extracellular $sPLA_2$ within maternal plasma, may also play a role in term and preterm labour (289), by hydrolysing extracellular glycerophospholipids in the choriodecidua and mobilising FAs (including AA) for the production of lipid mediators. *In vitro* experimentation, shows an attraction for macrophages towards LPC (290), which in theory could provoke macrophage invasion of the choriodecidua and parturition cascade. In a previous report, the amnion has shown higher expression of mRNA $cPLA_2$ than the choriodecidua (291). Amnion $cPLA_2$ activity was also reportedly increased with advancing gestation. However, its activity supposedly declines with labour (202); possibly explaining the undetected amnion LPCs in these collected data. Other lyso-glycerophospholipids, such as LPE and LPA, were also undetected in the fetal membranes here, either through low biological abundance or low assay sensitivity.

Of the recorded glycerophospholipid, PG was considered exceptional, as it was found significantly elevated in the amnion in both spontaneous labour and after induction. PG

makes up about 10-15% composition of lung surfactant and is found elevated in the amniotic fluid at term (292). Given PG was observed in elevated in the amnion in all labouring conditions at term, it may conceivably have a physiological role in human parturition. In the choriodecidua, spontaneous term and preterm labour showed upregulation of total PG, again this could be explained by changes in cellular populations and leukocyte immigration.

DG can be generated from hydrolysing glycerophospholipids by the action of PLC (293), and with the action of DAGL; which hydrolyses DG to 2-AG (and other MAGs) (115). In the previous chapter, an increase in 2-AG and other MAG were observed in term in-labour fetal membranes, especially those after induction. In 1981, Okazaki et al. (1981) reported an increase of PLC activity in amnion and decidual tissues on reaching term (294). However, in these studies, no significant increase in total DG or DG species were observed in amnion or choriodecidua, to support their finding. Given DG is the precursor for TG synthesis, an increase DG level could (in theory) be masked by generating TGs within the fetal membranes. Such a notion is fully supported here, as TG was elevated in term labour both within the amnion and choriodecidua. Again, this accumulation of TG in labour at term might suggest an enhancement of fatty acid supplies to these tissues; perhaps in line with the ever-growing requirements of the foetus for energy and fat deposition (295).

As aforementioned, SM is a lipid involved in cellular membrane structure. It is synthesised from ceramides, but can also be a source of ceramides by the action of SMase (231). The previous chapter observed an increase in CER(NS) production within labouring fetal membranes, but this was not directed through the *de novo* pathway. As evidence by the DES1 index, which failed to correlate with the increase. In amnion, spontaneous term labour showed a decreasing in total MS, and fewer species reached statistical importance. These data suggest that the sphingomyelin hydrolysis pathway, orchestrates amnion ceramide production. Whilst, upregulation of SM in the choriodecidua in term and preterm labour, prompts ceramide production through the salvage-type pathway (see **Chapter 3 section 3.4.3**). However, for conclusion, direct measurement of enzyme expression and activity would be necessary.

In 2019, Birchenall et al. (2019) showed maternal plasma cholesterol to be increased in term labour (78). In agreement, these data pinpoint an increase in cholesterol in the choriodecidua with labour at term. Cholesterol is the precursor of steroid hormones, including cortisol (296), which is reported to be elevated in maternal and fetal plasma in term labour (78). Cortisol is believed to enhance contractile prostaglandin synthesis from the fetal membranes (297,298) and also enhance lipolysis (299), to exaggerate the free fatty acid profile during labour. In this study, no alternations in cholesterol were observed in the amnion under labouring conditions. Nevertheless, the amnion remained around 28 times richer in cholesterol than choriodecidua throughout pregnancy. A finding supported by Loganath et al. (2000), who found that amnion explants synthesised cholesterol far more efficiently than chorion (300,301), along with the generation of steroid hormones (300). In addition, cholesterol can help store excess free fatty acids in the CE form, with both CE and TG retained intracellularly as lipid droplets (278). In this study, total CE was more than 200 times greater in choriodecidua than amnion. A margin which strongly evokes greater activity of acyl-coenzyme A (CoA):cholesterol acyltransferases (ACATs) in the choriodecidua, which esterifies long-chain fatty acyl-CoA and cholesterol to CE(88). With increased expression of CE in the choriodecidua in STL and PTL, upregulation of ACATs would appear to be a feature of human labour, regardless of gestational age and onset.

The fetal membrane FFA profiles indicate that many saturated, mono- and poly-unsaturated FFA species were upregulated under all forms of labour, indicative of increased lipid hydrolysis. Labouring is a stressful condition that leads to consumption of energy, and the FFAs would be utilised by the fetal membrane and adjacent tissues, e.g., myometrium, for energy generation. In this study, an upregulation of long-chain FFA species was specifically observed, which could indicate increased activity of ELOVL enzymes in the fetal membranes. As discussed earlier, the observed rise in free PUFAs, i.e. AA (FFA 20:4), FFA 18:2 (trend increase) and FFA 18:3 in the choriodecidua in spontaneous labouring, would support the recognised increase in eicosanoids and related mediators, as observed in **Chapter 3**. In preterm labour and at term, an increase in FFA18:2 (LA) could reflect the increase in LEA in choriodecidua. Also, FFA 24:0 increased in the spontaneous term group, support increase of 24 carbon length acyl chain CER(NDS), CER(NS) and CER(NP) species in the same group in the previous chapter. Palmitoleic acid is a particular FA known to have anti-inflammatory actions in different tissues (239). In the preterm choriodecidua, free palmitoleic acid (FFA 16:1) was

increased compared to non-labouring samples, supporting the recorded increase in POEA, a product of palmitoleic acid, in the same group in the previous study. Conceivably, this increase in palmitoleic acid could provide a general protective measure, to restrict the onset of inflammatory labour, in the absence of full and complete fetal maturation. In the amnion, all labouring groups showed an increase in two free PUFAs: FFA 18:2 (LA) and FFA 18:3. Like the choriodecidua, under spontaneous labour the amnion demonstrated an increase in free LA which correlated with increase of LEA, a product of LA, as discussed in **Chapter 3**. In amnion, free palmitoleic acid increased in both term labouring conditions, again matching the increase in POEA, potentially providing haemostatic balance between anti- and pro-inflammatory signals in gestational tissues to ensure correct timing of parturition.

For the relative abundance of total fatty acids in amnion and choriodecidua, GC-FID was chosen to provide the best identification of double-bond positions within the fatty acids; thus more clearly distinguishing between omega-3 and -6 fatty acids within tissues. For the amnion, induced and spontaneous term labour showed a significant reduction in AA, supporting its suggested mobilisation for eicosanoid production. Palmitic acid (C16:0) was the highest fatty acid detected within the amnion and was inversely correlated to AA in all term groups. It is of particular interest then that amniotic fluid becomes rich in palmitic acid-containing lipids, such as dipalmitoylphosphatidylcholine (DPPC) at term, with fetal release of a lung surfactants, indicative of fetal maturation (302). The possibility that DPPC is a direct initiator of spontaneous human labour at term is therefore considered in **Chapter 5**.

4.6. Conclusion

Labour is a biologically stressful processes, which contributes to changes in lipidomic composition in gestational tissues, with fundamental differences between amnion and choriodecidua. Notwithstanding, some of these changes may have direct roles in initiating human labour itself.

The choriodecidua is richer in most profiled lipids than amnion, but TG and cholesterol are higher in the latter. In the amnion, only term labouring conditions showed signs of AA-depletion, supporting the previous chapter on increasing AA-derived eicosanoids in spontaneous and drug-induced labour.

In probability, fetal maturation and its increased secretion of palmitic acid-rich compounds, such DPPC, as a marker for lung maturity could influence liberation AA from amnion. As we observed, AA is negatively correlated with palmitic acid in term labouring conditions. In the previous chapter, spontaneous labour induced ceramide production in amnion tissues, and here we observed a decline in SM species, suggesting direct ceramide generation from SM hydrolysis. The amnion preterm lipid profiles were comparable to not-in labour, indicating fetal maturation could have a crucial role in influencing lipidomic composition in the amnion in labour.

In the choriodecidua, spontaneous term and preterm labour upregulated glycerophospholipid and SM composition, perhaps reflect the role of leukocyte invasion of this tissue, to propagate the human labour cascade. (19,258,280,281). Given that term labour had more noticeable changes than preterm labour in this regard, this could be reflective of the different underlying mechanisms, sources and types of invading leukocytes (258), which are undoubtedly different within these pregnancy scenarios.

Glycerophospholipid hydrolysis by PLA_2 was defined as a key component of spontaneous term and preterm labour, identified by exaggerated LPC and free PUFAs. In maintaining the necessary levels of AA for eicosanoid and AEA production, as defined in Chapter 3, it could be argued that additional and prolonged AA must be supplied from maternal sources, either from plasma or leucocytes, to retain uterotonic activity and progress labour beyond its initial stages (286). The further understanding of this process could inform more physiologic ways of controlling human labour, both its induction and suppression, to those already available to the obstetrician.

Chapter 5: Lung maturation marker dipalmitoylphosphatidylcholine (DPPC) and initiation the parturition

5.1. Introduction

Labour is a physiological process with sole aim of fetal delivery. At the time of labour, the uterus changes from a relaxed extendable (quiescent) vessel, into a regularly contractile organ, to facilitate expulsion of the baby. Along with uterine contractions, the cervix also changes; becoming softer, shorter and more dilate, in a process termed “ripening”, to further facilitate passage of the fetus through the birth canal.

In humans, the timing of these changes is crucial for fetal and maternal well-being. Term pregnancy is defined between 36 weeks plus 6 days gestation to 42 weeks of pregnancy (1); with a definition based on statistical observations, when mortality and morbidity of the fetus are at their lowest (2). Preterm labour, when delivery occurs before 37 weeks gestation, shows a greater incidence of poor pregnancy outcomes, expressly for the fetus, and mainly through immaturity of the fetal organs, especially the lungs (3).

As implied already, human parturition is characterised as an inflammatory process with pro-inflammatory mediators, such as cytokines and prostaglandins, upregulated in the intrauterine tissues (98) and local prostanoids liberated to initiate and enhance cervical ripening (220). Within this physiological sequence, fetal maturation has been long held as a consideration of timing, with the majority of women delivering at term, in line with optimal fetal development. In situations of preterm delivery and fetal immaturity, a common complication of the newborn is respiratory distress syndrome, which is mainly attributed to a lack of lung surfactant, and can be manifested with breathing difficulties, lung collapse and oxygen desaturation (303). These lung surfactants, which are so important for a baby’s first breath, work by decreasing surface tension over the alveoli, preventing them from collapsing and increasing the surface area (68). They are therefore crucial in both supporting and arguably initiating the key switch in a baby’s circulation, from amniotic sac to the air.

Dipalmitoylphosphatidylcholine (DPPC) is a major element in the human lung surfactant and importantly increases in the fetus in amniotic fluid near to term. Originating from the fetal lung, it is widely used to clinically assess fetal lung maturity (68,304,305). The concept of fetal lung maturation signals and initiating labour was first introduced in 1988 when extracted surfactant lipids from amniotic fluid were shown to stimulate prostaglandin secretion from amnion explants (306). However, the amniotic fluid samples were collected from labouring women, which could be contaminated with pro-inflammatory markers such as prostanoids. Another report by Ohtsuka et al. (1990) has shown increase PGE_2 production from amnion explants post-DPPC treatment (307). However, the assay was not sensitive enough to distinguish between PGE_2 and other prostanoids; also labouring status was not included in the method description. Although earlier studies speculated DPPC's role in human parturition, more detailed studies are needed, specifically those more detailed inflammatory panels and sensitive tools for prostanoid analysis. Moreover, surfactant proteins (SPs) account for about 10% of total surfactant content (308). SP-A is the most dominant surfactant protein and has been shown to propagate parturition in mice (309). However, Chaiworapongsa et al. observed SP-A level in amniotic fluid decreases at term in labour, making SP-A's role in human parturition questionable (310).

As already explained, the fetal membrane is an integral part of human placenta, which maintains amniotic fluid within the uterine cavity. Histologically divided into amnion (in direct contact with the amniotic fluid) and choriodecidua (the feto-maternal membrane which initiates uterine contractions) (**Figure 5.1**). In **Chapters 3** and **4**, the importance of the fetal membrane as a source of lipid mediators, including AA-derived prostanoids, was highlighted, under various labouring conditions. In term spontaneous labour, the amnion was defined as expressing high levels of the PGE_2 metabolite (13,14 dihydro 15-keto PGE_2), along with signs of total AA depletion within the tissues. In more precisely pinpointing the inflammatory spark for labour, the choriodecidua was shown to produce the AA-derived metabolites (PGE_2, PGF_{2a} and TXA_2), which are biologically (and clinically) responsible for initiating human labour.

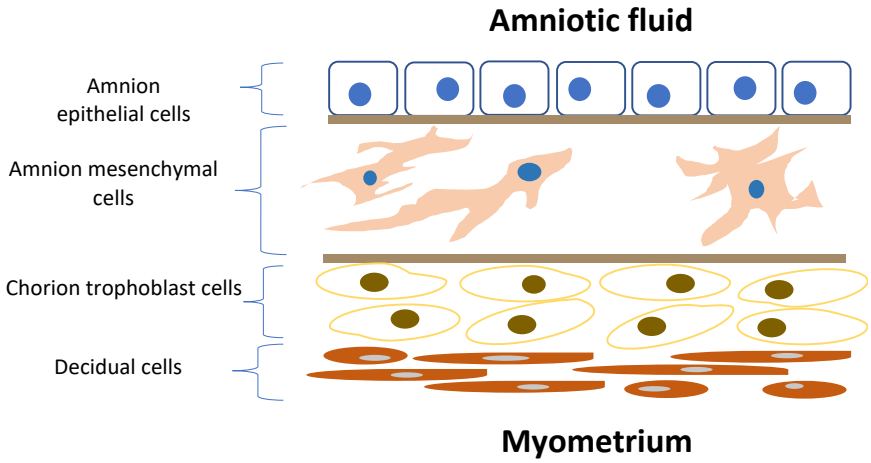


Figure 5.1. The human fetal membrane in cross-section with component cells illustrated.

At the end of the third trimester, fetal lung maturity is achieved by production of lung surfactant. Palmitic acid (PA) is its main constituent, making up more than 86% of its content (306). The preceding chapter identified an inverse correlation between amnion AA and palmitic acid (PA), which could perhaps be an early step in the crosstalk between amnion and choriodecidua in orchestrating inflammatory-driven parturition. In this regard, it is of potential importance that DPPC contains two molecules of PA, with direct contact to the amnion. LPC (16:0), a DPPC cleaved form with a single PA molecule, is also present and more readily soluble in water than DPPC (311), perhaps providing better cell uptake. Given its relative abundance within amniotic fluid at term, in-line with fetal maturation, it is interesting to hypothesise whether amniotic fluid DPPC or LPC (16:0) stimulates the liberation of AA within the amnion and thereby kick starts the parturition cascade; providing the first identifiable link between fetal lung maturation at term and the biological onset of labour in the most relevant intrauterine tissues.

Aim: To utilise *in vitro* cell cultures of fetal membrane components to examine the effects of DPPC/LPC (16:0) on known pro-inflammatory markers of human labour.

Objectives:

- To isolate and characterise primary cell lines from amnion and decidual tissues; and to optimise culture conditions to perform remaining studies.

- Measure total fatty acid abundance in isolated cells in culture and compare it to the not-in labour tissues.
- Prime the isolated cells with AA to have a comparable level to biological samples.
- Assess the influence of DPPC and LPC (16:0) on the fatty acid composition of amnion epithelial cells and resultant secretion of pro-inflammatory cytokines.
- Measure the potential influence of amnion epithelial secreted cytokines on decidual prostaglandin production.

5.2. Methods

Amnion epithelial and decidual mesenchymal cells were isolated from term placentae obtained from not-in labour elective caesarean sections. Fetal membrane tissues were digested as described in **sections 2.8.1 and 2.9.1**. Amnion epithelial cells were characterised at first passage using flow cytometry (**section 2.8.2**), and decidual mesenchymal cells at passage 3 using immunocytochemistry (**section 2.9.2**). The effect of DPPC, LPC(C16:0) and AA on the viability of cultured cells was tested using MMT assay, as described in **sections 2.8.4 and 2.9.3**. Total fatty acid extractions, derivatisations and analyses for treated and untreated cultured cells were performed by GC-FID as described in **section 2.7**. The secretory cytokine profiles of DPPC and LPC(C16:0) treated amnion epithelial cells were determined with LEGENDplex™ Human Inflammatory Panel 1 kit (BioLegend®, San Diego, CA, US) (**section 2.8.6**). Cultured decidual mesenchymal cells were treated with recombinant human IL-8 (77a.a, Peprotech EC Ltd, London, UK) in serum-free medium for 24 hours (**section 2.9.4**). Resultant secreted PGE_2, $PGF_{2\alpha}$ and TXA_2 (measured as TXB_2) were determined using commercial ELISAs (Life Sciences, UK) (**section 2.9.5**).

5.3. Statistics

All data were analysed by Prism (v.8) (GraphPad, California, USA). Fatty acids were expressed as percentage of total fatty acids, whilst secretory prostaglandins and cytokines were corrected to cell number at the time of media collection. All experiments were repeated at least three times (independent experiments, n=3), except for AA abundance of DMC post AA treatment (n=1) (**Figure 5.13**). Also, these experiments had at least two technical repeats. For the MTT assay, cell viability was presented as a percentage compared to untreated controls.

Normality testing was performed using Shapiro-Wilk test. All parametric data are expressed as means and standard deviations (±SD) from the mean. One-way ANOVA with Tukey's post hoc test was performed on all datasets, with the exception of tissue total fatty acids vs cells. Here, testing was a two-tailed unpaired t-test. Fatty acid relationships were defined by a Pearson correlation coefficient.

5.4. Results

5.4.1. Characterisation of isolated amnion epithelial cells

Human amnion tissues contain two cell types; amnion epithelial and mesenchymal cells (312). Amnion epithelial cells are the superficial layer in-direct contact with the amniotic fluid. They are also the dominant cellular phenotype (313,314). Following isolation and culture from collected fetal membranes, epithelial purity was established at first passage by flow cytometry and morphological examination (**Figure 5.2 and 5.3**). Amnion epithelial cells were defined by their common surface marker, EpCAM, and the isolated cells were highly positive to EpCAM of greater than 85%, and mesenchymal marker (CD166) and leukocytes marker (CD45) of less than 10% and 5%, respectively (315–317). Amnion epithelial populations were defined after flow cytometric exclusion of apoptotic cells by propidium iodide (PI) (**Figure 5.3**).

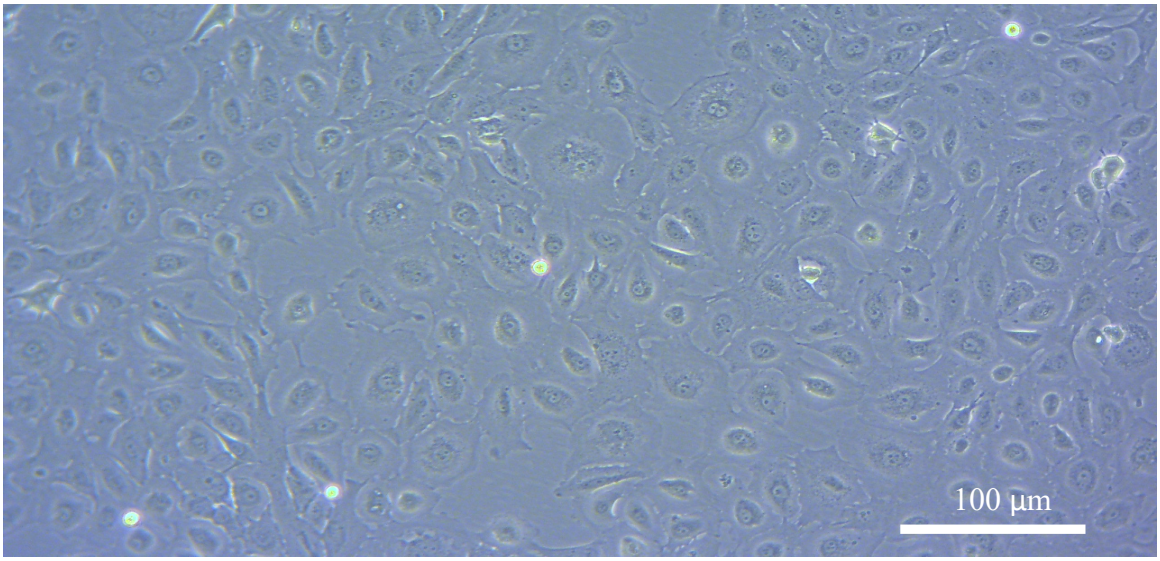


Figure 5.2 Representative image of cultured amnion epithelial cells (first passage).

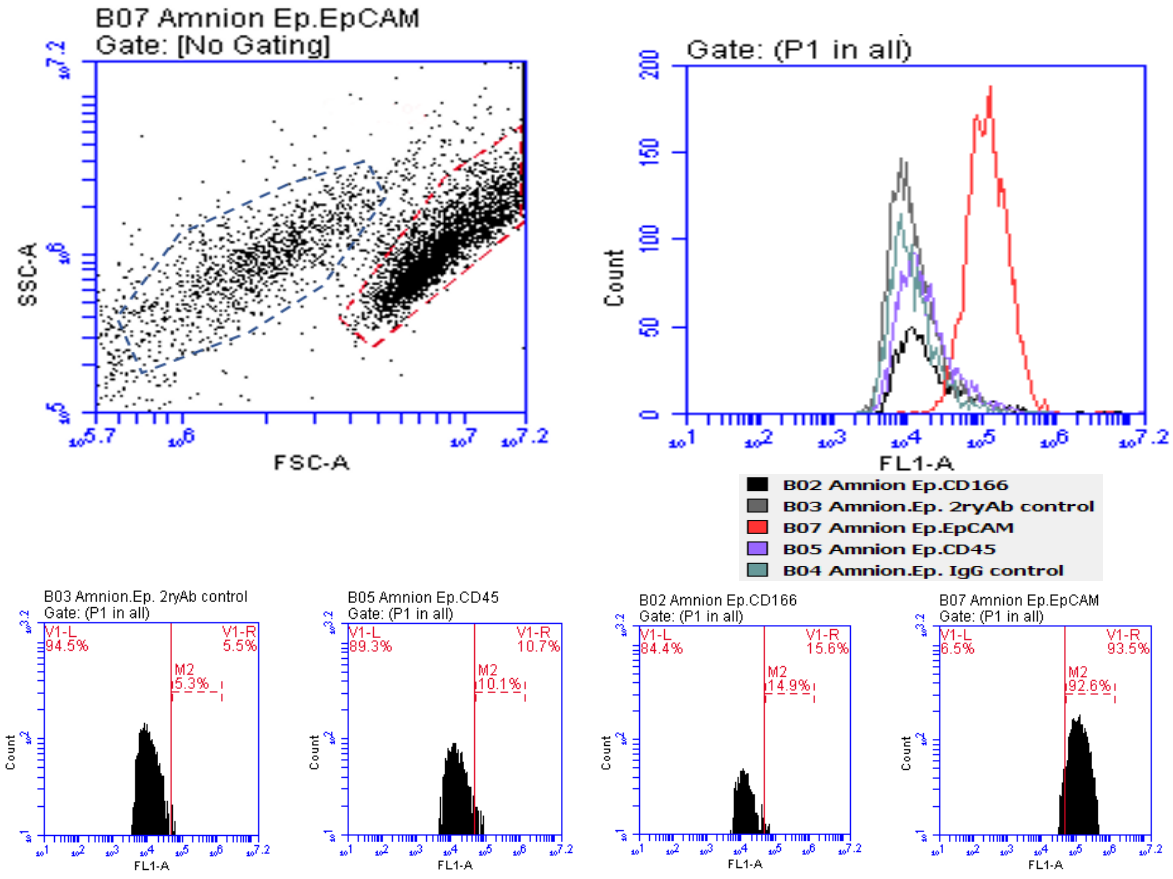


Figure 5.3. Representative purity of isolated amnion epithelial cells, defined by flow cytometry. The cell population (red dotted line) was selected after excluding propidium iodide-stained non-viable cells (blue dotted line). FL1-A was selected to detect green fluorescence (ie FITC-labelled secondary antibody). Removing isotype control values, 87% were positive for EpCAM, whilst 9.6% and 4.8% were positive for CD166 and CD45, respectively.

5.4.2. Effect of DPPC and LPC (C16:0) on amnion epithelial cell fatty acid composition

To examine the influence of surfactant**s** on amnion fatty acid composition; intact DPPC and LPC (C16:0), were chosen to treat cultured amnion epithelial cells at the first passage to reduce the risk of changing the cellular morphology (318). Unlike DPPC, which requires sonication for dispersal, LPC (C16:0) dissolves in culture medium spontaneously, perhaps providing better cellular uptake.

Before exposure, base-line fatty acid profiles of cultured amnion epithelial cells were compared to those of the not-in labour amnion tissues, as reported in **Chapter 4 section 4.4.4** (**Figure 5.4**). Cultured cells were richer in oleic acid (C18:1n-9), vaccenic acid (C18:1n-7) and omega 3 fatty acids; C22:5 and C22:6; whilst conversely, the amnion tissues themselves were

higher in C17:1, C18:2n-6, C20:0 and C23:0. Although higher levels of PA and AA were indicated within the amnion tissues over isolated epithelial cells, theses failed to reach statistical significance making them comparable for experimentation.

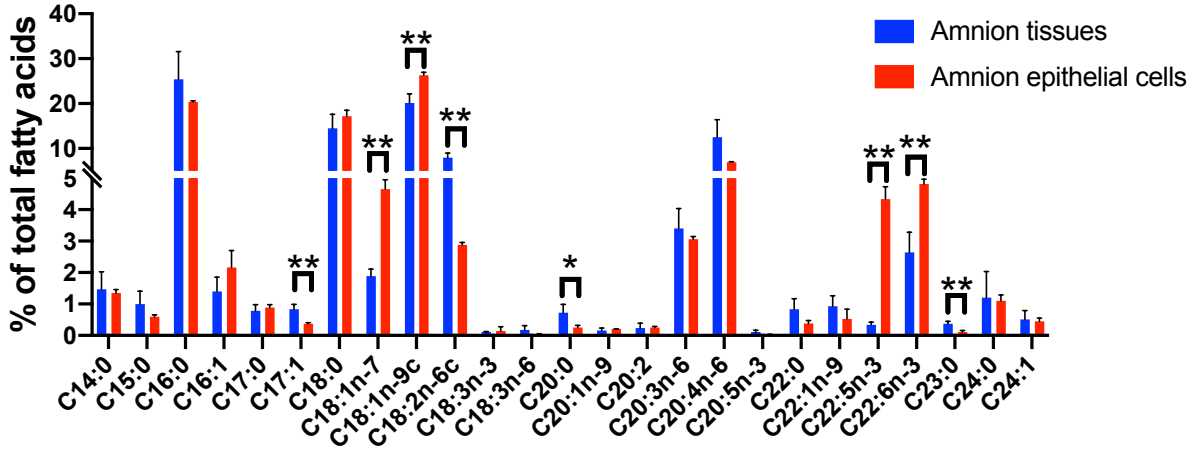


Figure 5.4. Fatty acid relative abundance of amnion tissues and isolated amnion epithelial cells (n=5 and 3, respectively). Mean and ±SD indicated. Statistical analysis = two-tailed unpaired t-test, *p≤0.05, **p≤0.01.

In order to optimise DPPC and LPC(C16:0) treatment, amnion epithelial cells were challenged with various concentrations. As seen in **Figure 5.5**, cells maintained their viability with all applied concentrations of DPPC. However, LPC(C16:0) reduced viability at 100µL/mL, at which the population was reduced to 10.0±1.2 %.

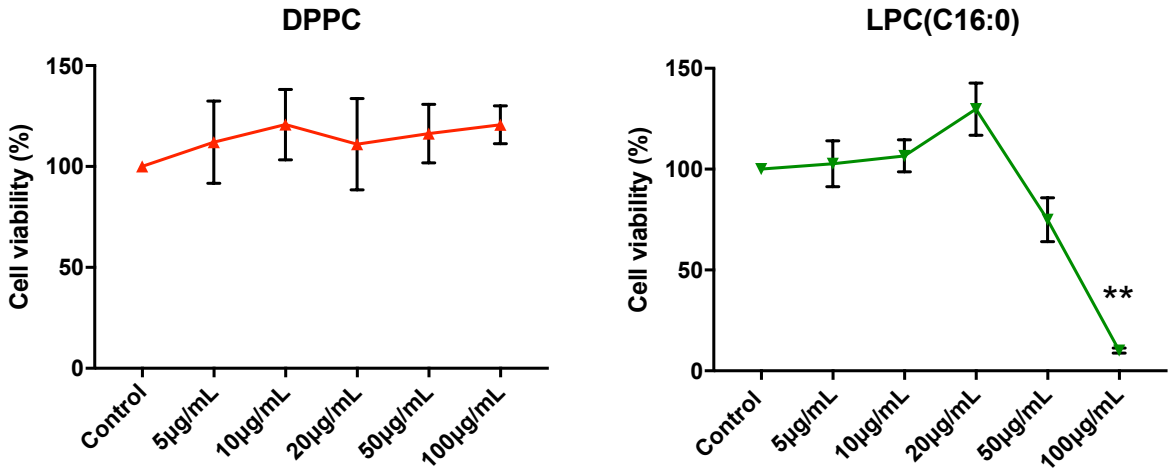


Figure 5.5. The effect of different concentrations of DPPC and LPC (C16:0) on cultured amnion epithelial cell viability. *Data presented as percentage viability compared to untreated controls (n=3) Mean and ±SD indicated. Statistical analysis = one-way ANOVA with Dunnett's multiple comparisons test, *p≤0.05, **p≤0.01.*

In order to examine the cellular uptake of PA post-DPPC and LPC (C16:0) treatment, the cells were exposed to different concentrations ranging from 5 to 20µM of both treatments. Increasing concentrations of DPPC, up to 20ug/ml, failed to influence the inherent levels of PA within these cells (**Figure 5.6**). However, upscaling of intracellular PA was evident with increasing LPC (C16:0) concentrations, with significance arising at 10 and 20µL/mL, with elevations of 26.3% and 30.0%, respectively (**Figure 5.6**).

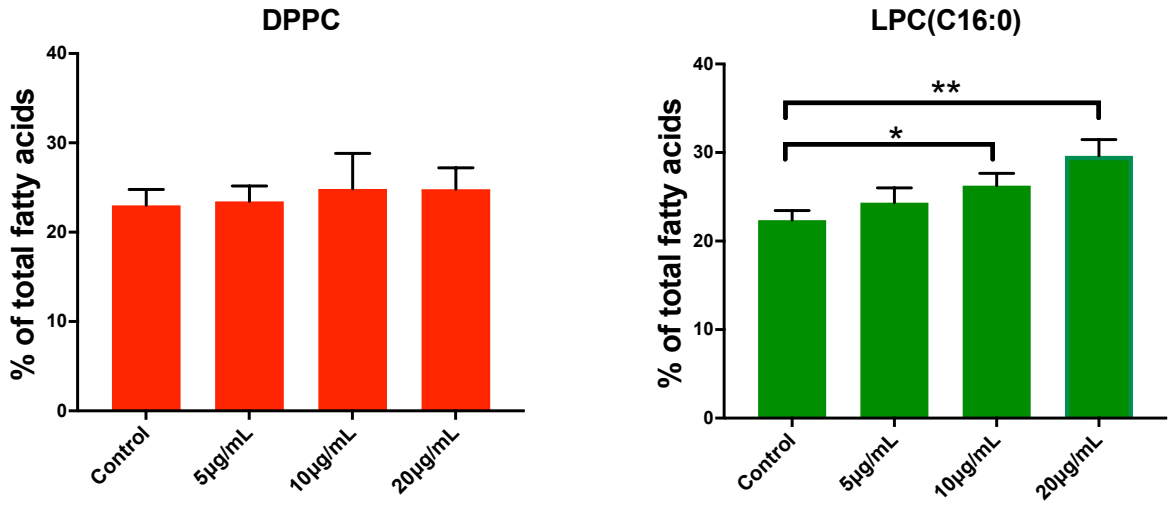


Figure 5.6. Palmitic acid (C16:0) relative abundance following DPPC and LPC (C16:0) treatment of amnion epithelial cells (n=3). Mean and ±SD indicated. Statistical analysis = one-way ANOVA with Dunnett's multiple comparisons test, *p≤0.05, **p≤0.01.

From previous biochemical observations, fetal lung maturation is determined from an amniotic fluid level of DPPC in excess 10µg/ml. This rises further to over 16µg/ml in cases of gestational diabetes (319). Given the *in vitro* observations of epithelial cell viability above, 20µg/mL DPPC and LPC, were chosen for subsequent experiments on alterations in cellular fatty acids for 24 hours. In these experiments (**Figure 5.7**), DPPC failed to influence the fatty acid profiles of cultured amnion epithelial cells, as compared to untreated controls. Significantly, this was not the case for LPC, which showed a marked elevation in PA (29.64 ± 1.84%) and concomitant reduction in C14:0, C15:0, C17:0, C17:1, C18:0n-7, C18:0n-9c, C18:2n-6c, and C20:3n-6; but no alteration in C20:4n-6, AA (**Figure 5.7**).

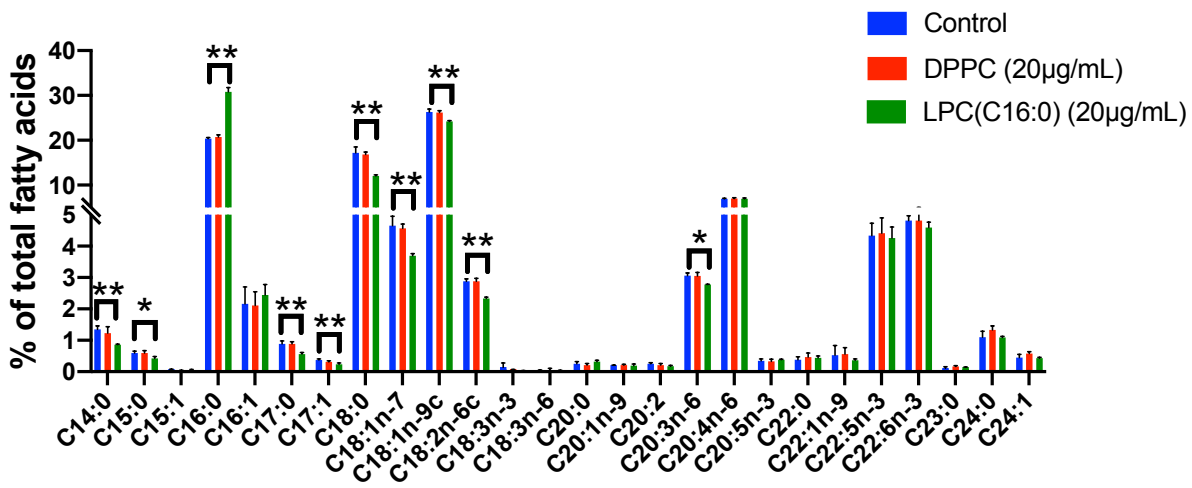
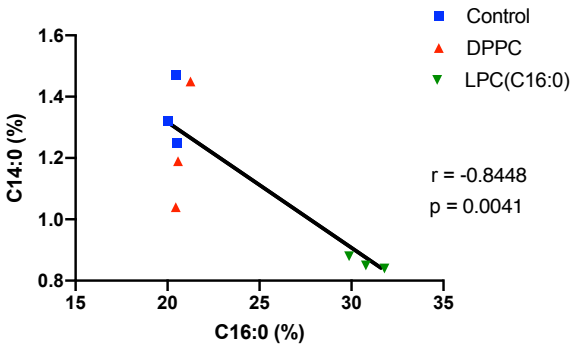
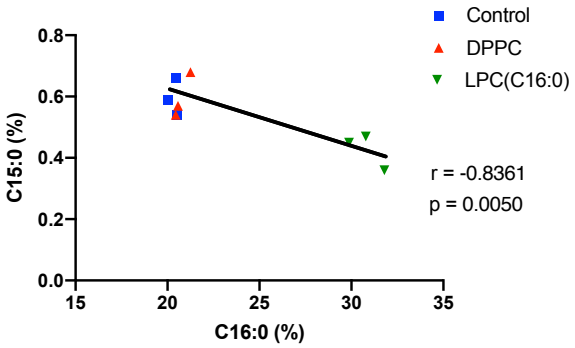
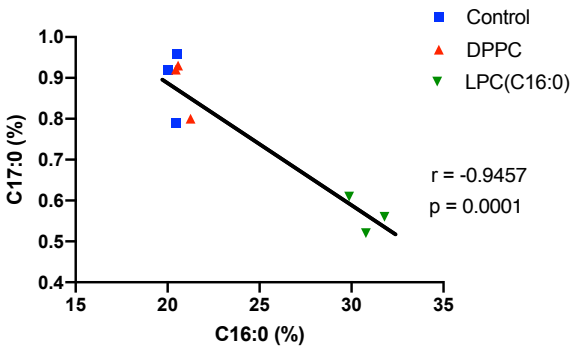


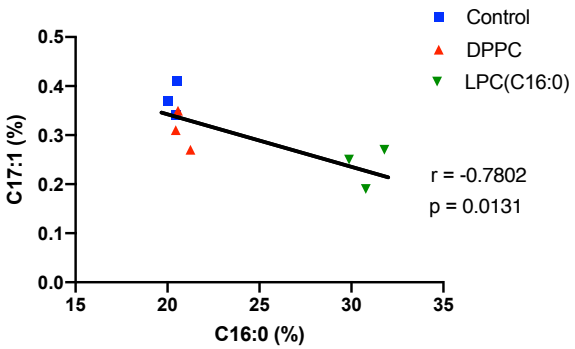
Figure 5.7. The effect of DPPC and LPC (C16:0) on the relative abundance fatty acids in amnion epithelial cells (n=3). *Mean and ± SD indicated. Statistical analysis = one-way ANOVA with Dunnett's multiple comparisons test, *p≤0.05, **p≤0.01.*

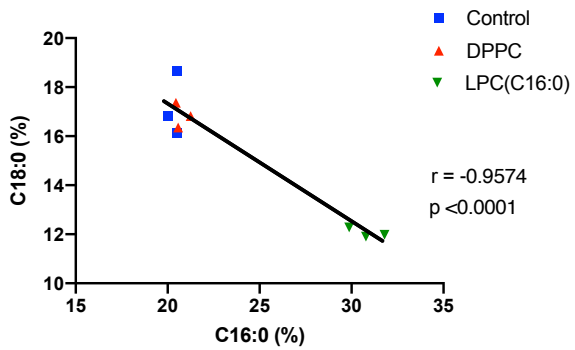
Figure 5.8 shows associations between PA and other fatty acids within all treated and untreated amnion epithelial cell cultures. Although combined to exaggerate experimental numbers, PA was inversely correlate with C14:0, C15:0, C17:0, C17:1, C18:0n-7, C18:0n-9c, C18:2n-6c and C20:3n-6.

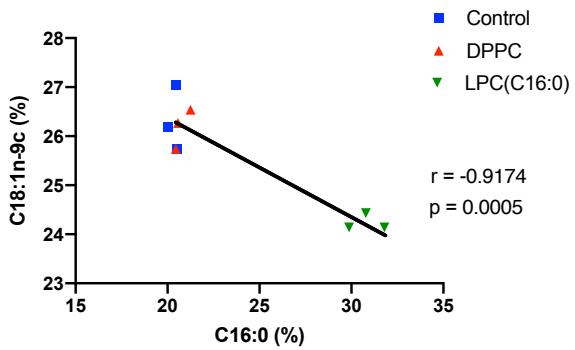

Control
DPPC
LPC(C16:0)
r = -0.8448
p = 0.0041
C14:0 (%)
C16:0 (%)

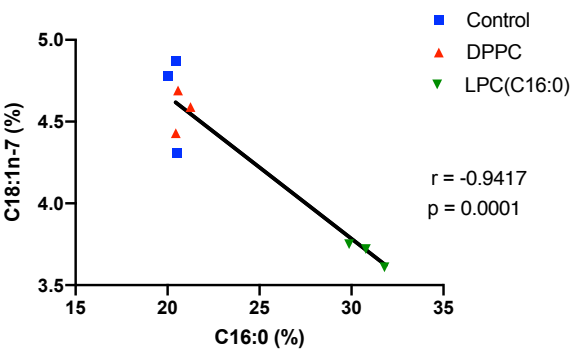

Control
DPPC
LPC(C16:0)
r = -0.8361
p = 0.0050
C15:0 (%)
C16:0 (%)

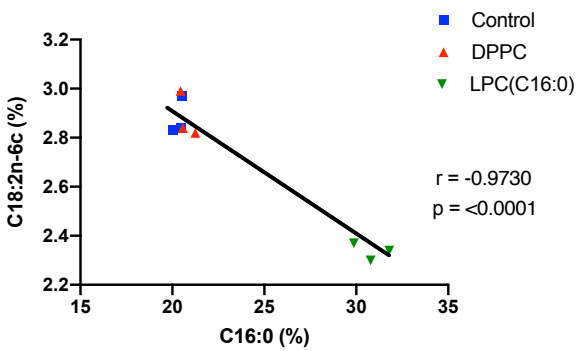

Control
DPPC
LPC(C16:0)
r = -0.9457
p = 0.0001
C17:0 (%)
C16:0 (%)


Control
DPPC
LPC(C16:0)
r = -0.7802
p = 0.0131
C17:1 (%)
C16:0 (%)


Control
DPPC
LPC(C16:0)
r = -0.9574
p <0.0001
C18:0 (%)
C16:0 (%)


Control
DPPC
LPC(C16:0)
r = -0.9174
p = 0.0005
C18:1n-9c (%)
C16:0 (%)


Control
DPPC
LPC(C16:0)
r = -0.9417
p = 0.0001
C18:1n-7 (%)
C16:0 (%)


Control
DPPC
LPC(C16:0)
r = -0.9730
p = <0.0001
C18:2n-6c (%)
C16:0 (%)

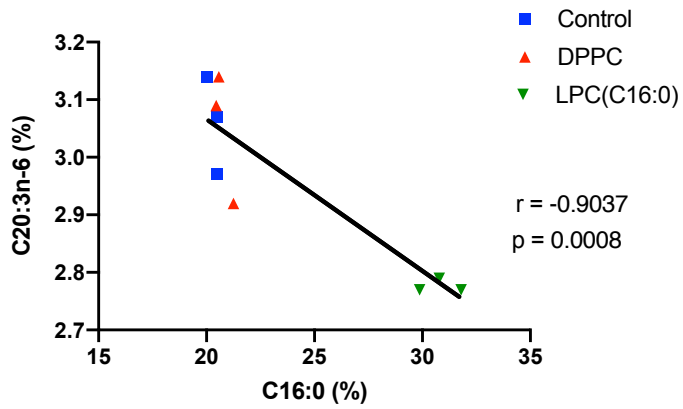
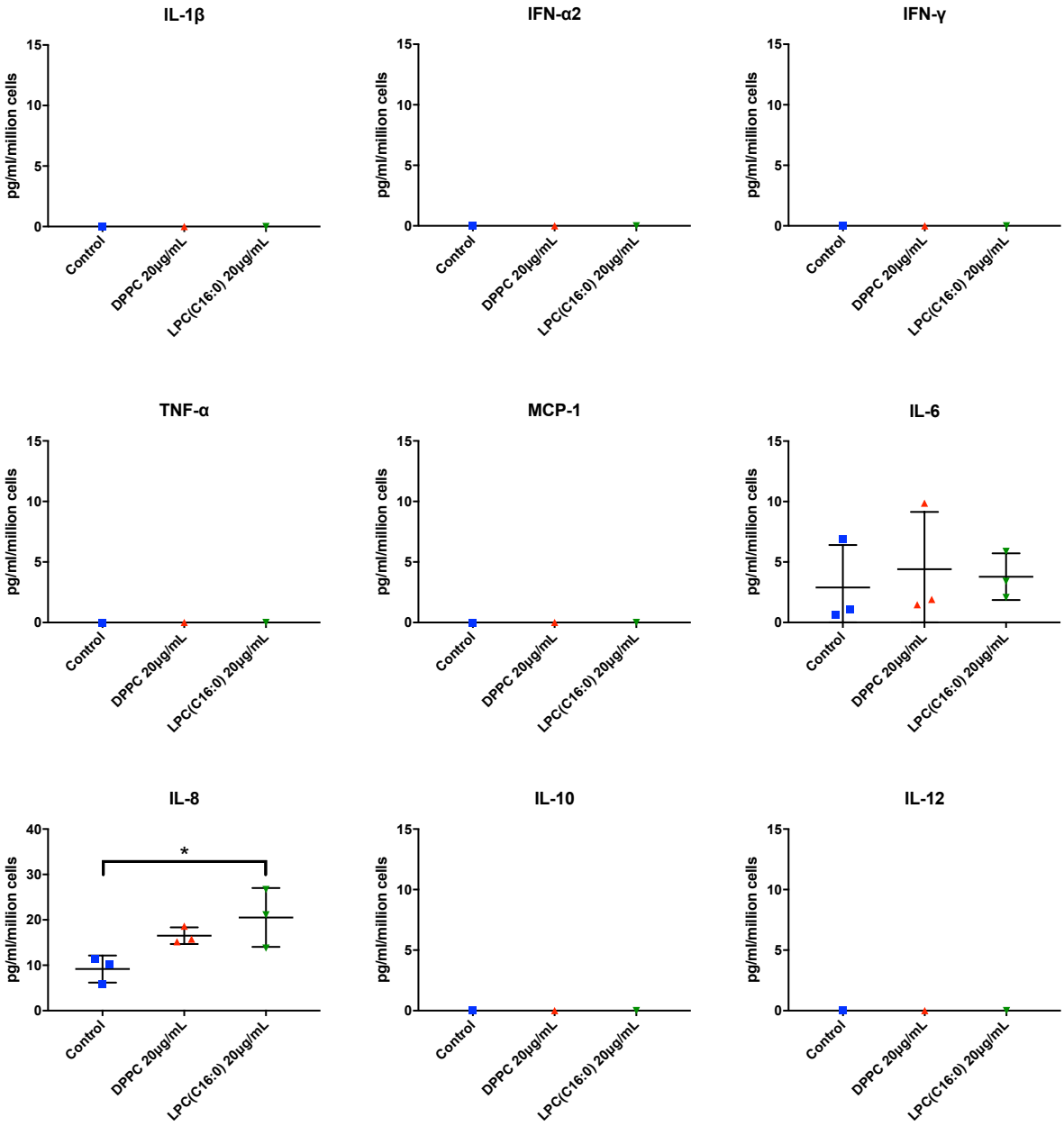


Figure 5.8. Palmitic acid (C16:0) correlations with other component fatty acids in cultured amnion epithelial cells, treated or untreated with DPPC and LPC (16:0). *Data expressed as percentage total fatty acid weight. P-values and correlation coefficients(r) indicated. The statistical analysis = two-tailed Pearson correlation test.*

5.4.3. The effect of DPPC and LPC on amnion epithelial cytokine secretions

To study the inflammatory influence of DPPC and LPC on the amnion epithelium, culture secreted pro-inflammatory cytokines were measured in post-exposure collected medium. **Figure 5.9** illustrates a bundle of pro-inflammatory cytokines tested for this purpose; IL-1β, IFN-α2, IFN-γ, TNF-α, MCP-1, IL-6, IL-8, IL-10, IL-12p70, IL-17A, IL-18, IL-23, and IL-33. Of these 13 cytokines, only IL-6 and IL-8 were detectable. IL-6 showed a similar level of liberation between study groups, whilst IL-8 was raised in both DPPC and LPC (C16:0) treated cells; but with only statistical significance achieved in the latter **(Figure 5.9)**.


IL-1β
IFN-α2
IFN-γ
TNF-α
MCP-1
IL-6
IL-8
IL-10
IL-12
pg/ml/million cells
Control
DPPC 20μg/mL
LPC(C16:0) 20μg/mL
*

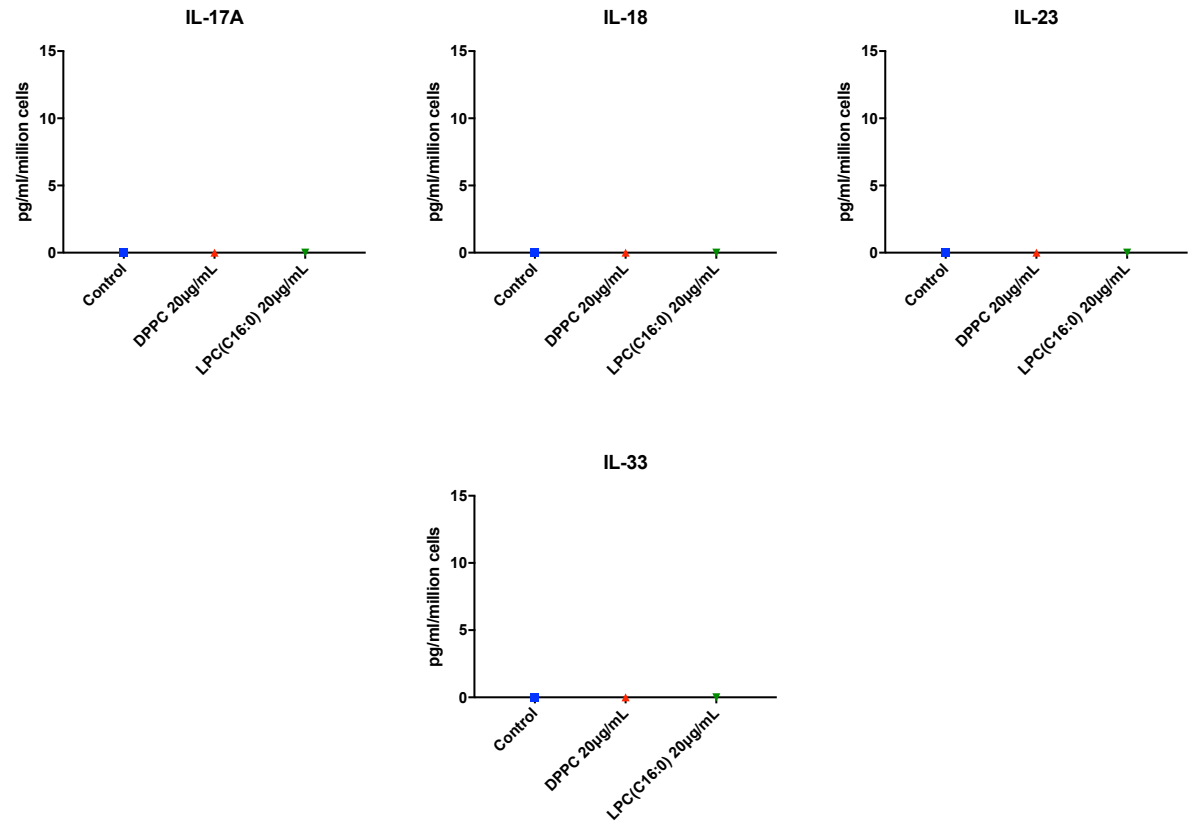


Figure 5.9. Secreted amnion epithelial cytokines following DPPC (20µg/mL) and LPC(C16:0) (20µg/mL) treatment in culture. *Mean and ±SD indicated. Statistical analysis = one-way ANOVA with Dunnett's multiple comparisons test, *p≤0.05, **p≤0.01.*

5.4.4. The characterisation of decidual mesenchymal cells and IL-8 treatment

The previous chapters have shown the choriodecidua to be the most active site for production of labour-related prostanoids in spontaneous parturition. Moreover, the choriodecidua lies adjacent to the myometrium, as a primary source of uterotonic mediators. In order to examine the possibility of amnion-choriodecidual crosstalk, in propagation of a fetal maturation signal, decidual mesenchymal cells were chosen as a simple but direct experimental model.

The decidua is composed of a mixed population of cells, including leukocytes, blood vessels and decidual mesenchymal cells (DMC) (320). Of these, DMCs are the major population with purported importance in attracting and stimulating resident and maternal immune cells (321). As seen in **Figure 5.10**, isolated DMCs were typically positive for Vimentin (common mesenchymal marker) after 3 passages, and invariably negative for cytokeratin 7 and CD45; markers of trophoblast/epithelial and leucocytes, respectively (322). To maintain

decidualisation in these cultures, reminiscent of the pregnant decidua, progesterone and oestrogen were included in the culture medium, at 1µM and 10nM respectively, as required by Michalski et al. (2018) (323). Following confirmation, these decidual mesenchymal cells were subsequently used for experimental investigations.

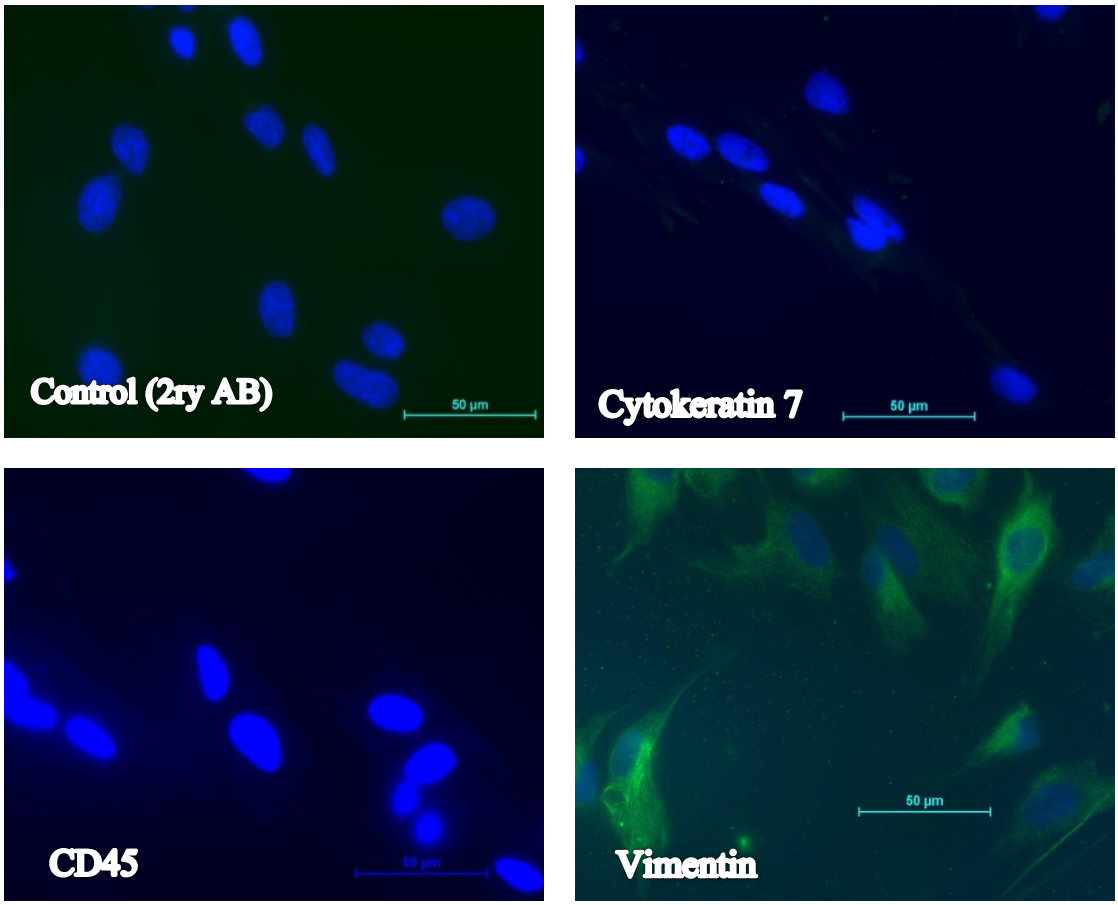


Figure 5.10. Representative immunocytochemistry for isolated decidual mesenchymal cells at passage 3 of culture*. Showing DAPI (blue) staining of nuclei, control slides (primary antibody removed), vimentin positivity (FITC green fluorescence) and undetected cytokeratin 7 or CD45 (again both FITC (green) conjugated). Scale bar= 50µm.*

5.4.5. The effect of IL-8 on DMC prostaglandin secretion:

To determine the possible importance of amnion liberated IL-8, subsequent to fetal maturation and DCCP/LPC(C16:0) exposure, DMCs were treated with known bioactive levels of human IL-8 in culture (324).

Before starting IL-8 treatment, the fatty acid profile was compared between isolated decidual cells and not-in labour choriodecidual tissues (see **Chapter 4 section 4.4.4**). The choriodecidual tissues were richer in AA than the DMCs, 15±1.8% and 7.8±0.25% respectively. In regard of other FAs, DMCs were higher in C18:0, C18:1n-7, C18:1n-9c, C22:1n-9, C22:5n-3, C22:6n-3 and C24:0, while choriodecidual tissues were higher in C14:0, C15:0, C15:1, C17:0, C18:2n-6c, C18:3n-3, C18:3n-6, C20:1n-9, C20:5n-3, C22:0, C22:2,and C24:1.

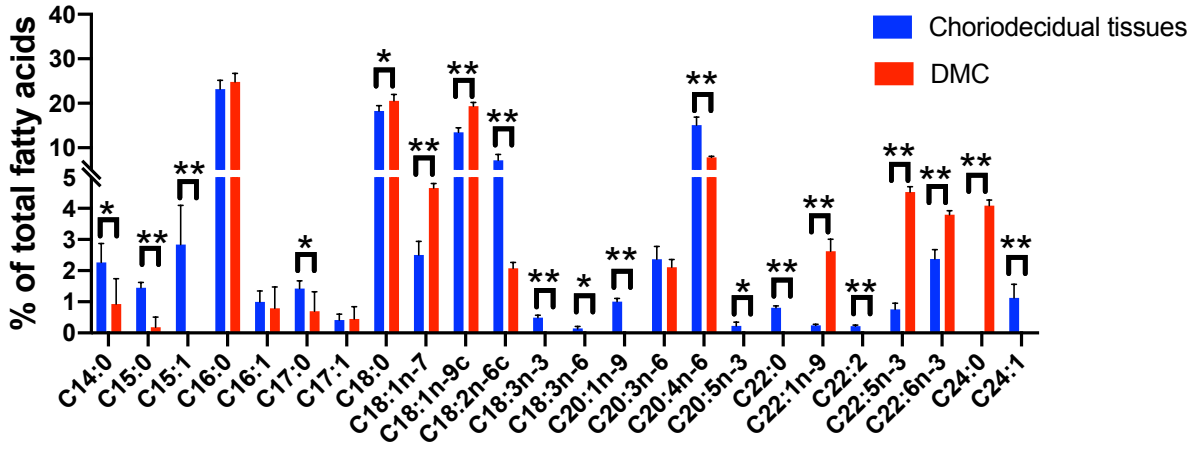


Figure 5.11. Fatty acid profile of choriodecidual tissues and isolated decidual mesenchymal cells (n=6 and 3, respectively). *Mean and ±SD indicated. Statistical analysis = two-tailed unpaired t-test, *p≤0.05, **p≤0.01.*

In order to upregulate AA levels of DMCs to match biological tissues, DMCs were challenged with different concentrations of AA (0-50µM) to test cytotoxicity. As seen in the **Figure 5.12**, DMCs viability was unchanged with different AA concentrations. A pilot study was made to assess the effect of the treatment duration (48 and 72h) on the cellular AA level at 10µM AA and showed that increasing treatment duration also affected the embedded AA levels (supplement **Figure 5.13**). DMCs were treated with low levels of AA (5 and 10µM) for 72h as seen in **Figure 5.14**, and the cellular AA levels increased to 11±0.42% and 13±1.78%, respectively.

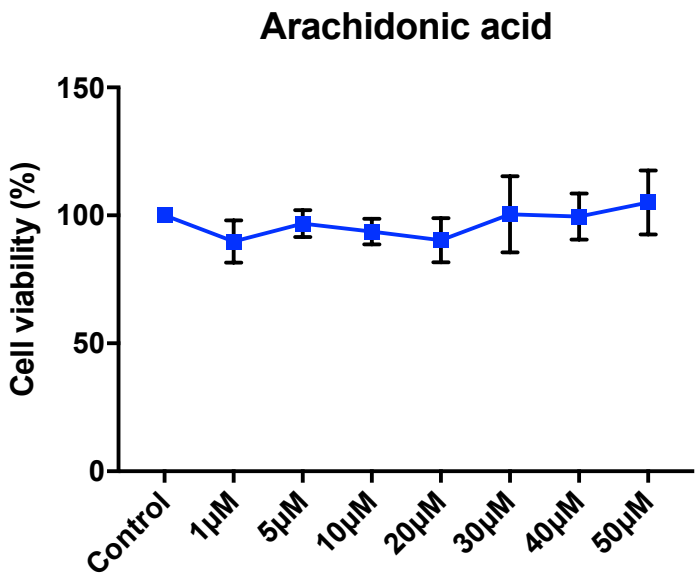


Figure 5.12. Effect of different treatment concentrations of arachidonic acid (AA) on decidual mesenchymal cell viability (n=3)*. Data were presented as percentage of viability to control (n=3) Mean and ±SD indicated. Statistical analysis = one-way ANOVA with Dunnett's multiple comparisons test, *p≤0.05, **p≤0.01.*

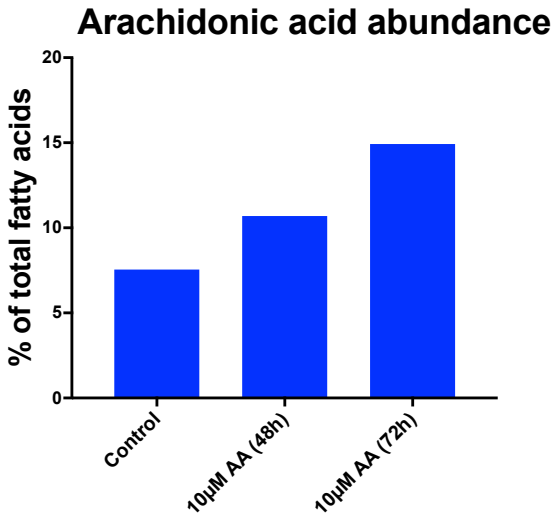


Figure 5.13 Arachidonic acid (C20:4n-6) relative abundance of decidual mesenchymal cells (DMC) post arachidonic acid treatment for 48 and 72 hours (n=1).

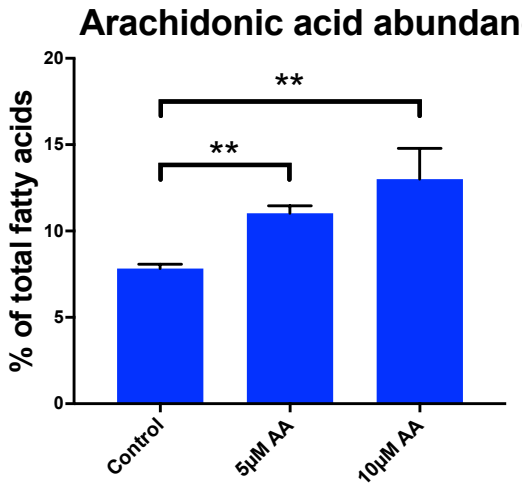


Figure 5.14. Arachidonic acid (C20:4n-6) relative abundance of decidual mesenchymal cells (DMC) post arachidonic acid treatment for 72 hours (n=3*). Mean and ±SD indicated. Statistical analysis = one-way ANOVA with Dunnett's multiple comparisons test, *p≤0.05, **p≤0.01.*

To have a better understanding of the labour-associated prostanoids DMCs are capable of secreting, AA pre-treated cells and untreated cells were cultured with serum-free medium with/without calcium ionophore (CI) A23187, a potent COX enhancer (325). **Figure 5.15** demonstrates that DMCs secreted $PGF_{2\alpha}$ and TXA_2 at trace amounts and were not stimulated with CI. However, PGE_2 secretory levels were increased in both AA pre-treated cells with CI which would indicate that DMCs were exclusively producing PGE_2 out of the labour associated prostanoids and that increasing embedded AA key for prostaglandin production.

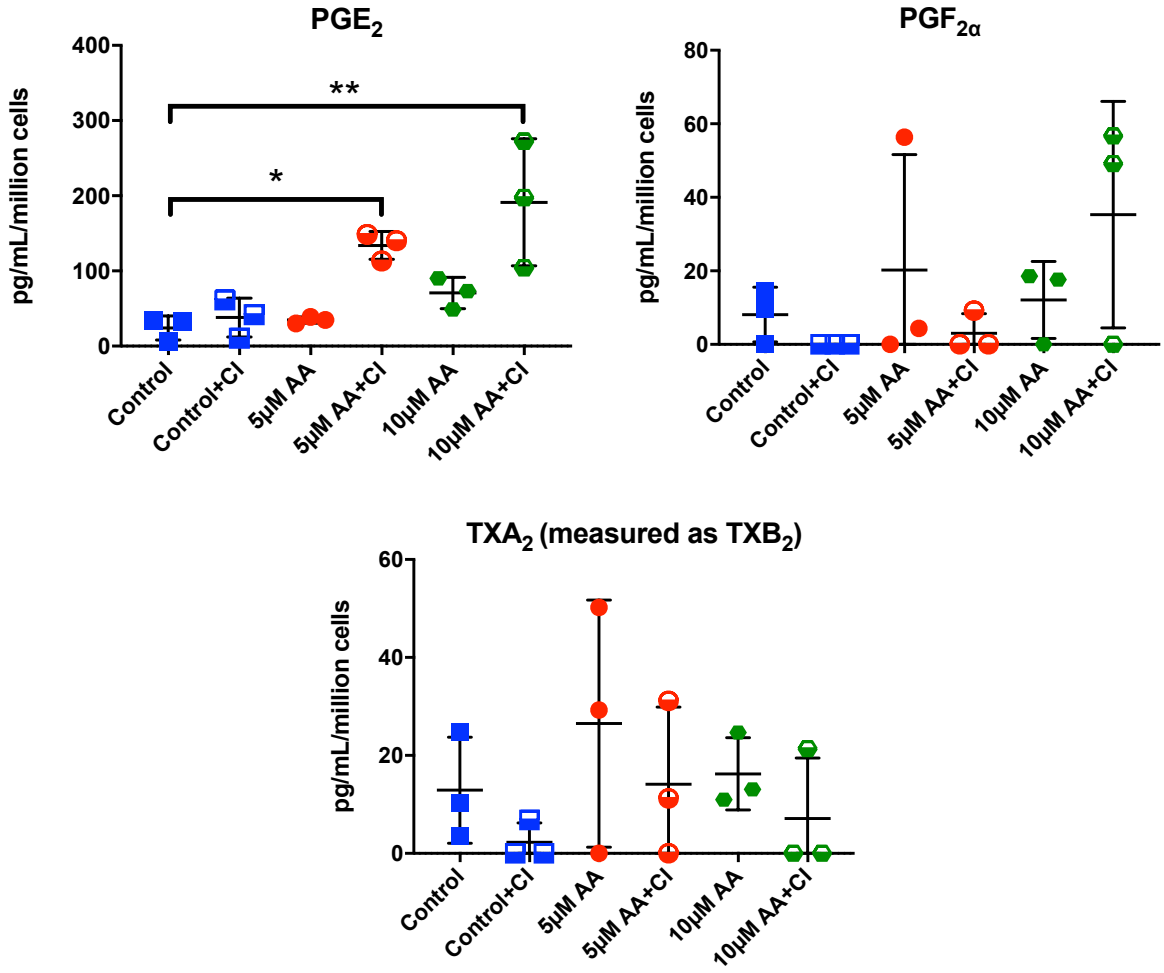


***Figure 5.15. Secretory PGE_2, $PGF_{2\alpha}$, and TXA_2 (measured as TXB_2) profiling of decidual mesenchymal cells (DMC)**. Control and AA pre-treated cells (5 and 10μM) for 72 hours with/without calcium ionophore (CI) included. Mean and ±SD indicated. Statistical analysis = one-way ANOVA with Dunnett's multiple comparisons test, *p≤0.05, **p≤0.01.*

To explore the IL-8 effect on PGE_2 secretion, the DMCs were pre-loaded with 10μM AA for 72h (to achieve physiological AA levels) then exposed to different concentrations of IL-8 (30pg/mL, 50ng/mL, and 100ng/mL) in serum-free medium for 24h. As seen in **Figure 5.16**, IL-8 did not stimulate DMC PGE_2 secretion, as expected.

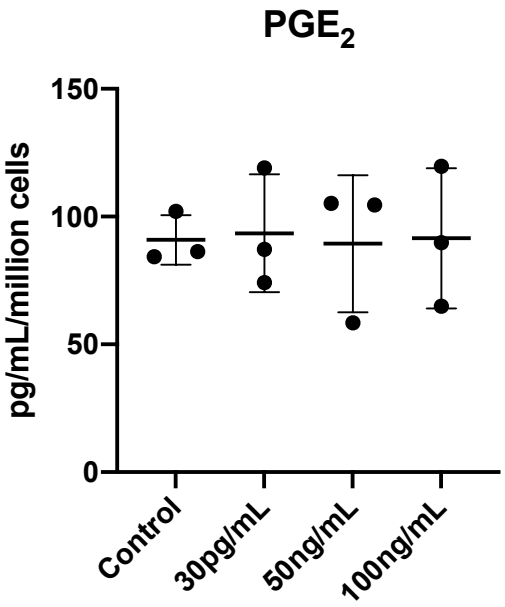


Figure 5.16. Secretory PGE_2 profiling of IL-8 treated decidual mesenchymal cells (DMC) (n=3). *Mean and ±SD indicated. Statistical analysis = two-tailed unpaired t-test, *p≤0.05, **p≤0.01.*

5.5. Discussion

This chapter has illustrated the successful isolation and culture of two primary cell lines harvested from the fetal membranes of uncomplicated human pregnancies at term; amnion epithelial cells and DMCs. Once maintained in culture, and where necessary manipulated to reflect the fatty content of their tissues of origin, experiments were performed to define the impact of DPPC and LPC (C16:0) in initiating a cascade in inflammatory signalling from amnion to decidua, as a reflection of pathway from fetal maturation to maternal labour induction in human pregnancy.

For these experiments, the major fetal lung surfactant was considered, intact DPPC and its hydrolysed form LPC (C16:0); both molecules with component PA at their heart. In replicating their first association with the fetal membranes, these surfactants, at term levels, were applied to amnion epithelial cells, isolated and maintained in culture. As shown above, only with hydrolysed LPC was PA incorporated and upregulated within the lipid content of the cells, impacting upon the general fatty acid content. In accordance with this thesis, it was expected that these PA elevations would preferentially liberate cellular AA, as a defined in the tissues observations in **Chapter 4**. Nevertheless, in isolated cells only an inverse

relationship between PA and other embedded fatty acids such as stearic acid (C18:0) and C17:1 were defined, which can also be matched with the tissues profile (**Figure 5.17**).

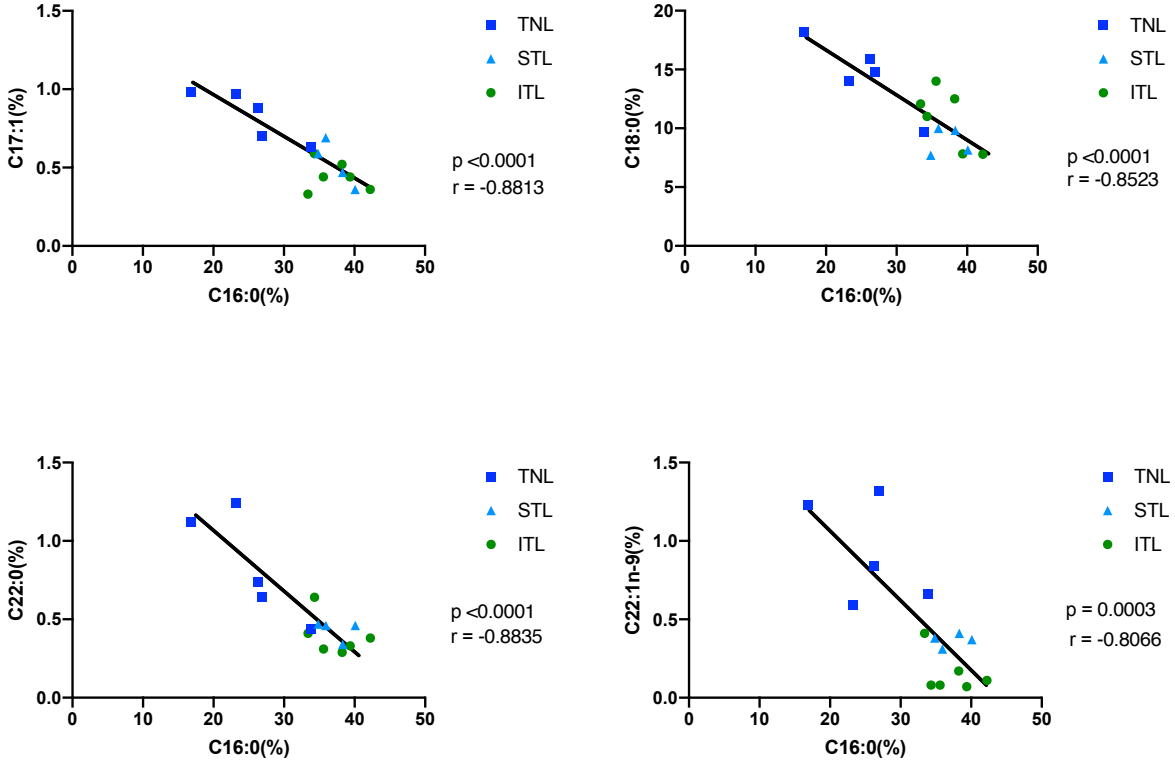


Figure 5.17. Negative correlation of palmitic acid (C18:0) with other fatty acids in term amnion tissues. *Data expressed as percentage total fatty acid weight. P-values and correlation coefficients(r) indicated. The statistical analysis = two-tailed Pearson correlation test.*

In the literature, Ohtsuka et al. (1990) reported that DPPC was able to stimulate prostaglandin production from cultured amnion tissues. However, their assay was not sensitive enough to determine the exact prostanoids; given their cross-reactivities for PGE_1 and PGB of 63% and 57%, respectively. For this study DPPC concentrations up to 800µg/mL where used; far greater than the physiological levels previously defined in human amniotic fluid (149,319). Moreover, they failed to report whether the placenta was collected from labouring or non-labouring women, which could greatly influence the inflammatory setting of the tissue.

Although DPPC and LPC failed to alter AA relative abundance of amnion cells, LPC (16:0) stimulated production of a key pro-inflammatory cytokine; IL-8. IL-8 is known as a chemotactic molecule which stimulates immune cell infiltration in many tissues and inflammatory scenarios (326,327). The infiltration of leukocytes within intrauterine tissues is a common observation with human parturition (19,258). IL-8 was reported to be elevated in

the amniotic fluid at term labour (328). However, this may not necessarily influence uterine tissues directly, given its distant location. IL-8 expression is also upregulated within the ripening cervix (329) and when administered vaginally and cervically, undoubtedly enhances cervical ripening within experimental animals (28,199). Its protection, therefore, from the amnion may well be a strong inflammatory signal, with which to propagate a labour cascade, perhaps across the fetal membranes from amnion to choriodecidua to myometrium and uterine muscular tissues.

To further establish the fetal-to-maternal signalling properties of IL-8, optimised and decidualised DMCs were exposed to bioactive levels of the cytokine, once embedded levels of AA had been artificially raised to match the values defined in the choriodecidua of not-in labour term tissues. In these studies, only AA pre-treated DMCs, post-CI and COX-enhanced, were able to produce the more dominant prostanoid (PGE_2) in labouring choriodecidual tissues. This observation suggests the importance of AA availability within the gestational tissues for prostanoid production, which supports our observation in the previous chapter where AA level was maintained in choriodecidua even with high production of prostanoids.

In **Chapter 4**, we observed an increase of $PGF_{2\alpha}$ and TXA_2 along with PGE_2 with labouring conditions. However, DMC production of PGE_2 was relatively stunted in comparison, perhaps reflecting decidual tissues containing a different population of cells (320). When exposed to IL-8, PGE_2 production from cultured DMCs was unresponsive, regardless of the initially raised AA content. Although disappointing, this should not preclude the importance of amnion generated IL-8, as a fundamental signal for the choriodecidua. IL-8 expression increases in all gestational tissues in physiological labour, which points to its important role in human parturition (98).

IL-8 is known to attract and activate neutrophils, and IL-8 correlates with increased MMPs secretion in the myometrium and fetal membranes (330), which would enhance cervical ripening and the rupture of fetal membranes (331,332). Also, neutrophils are known to further produce prostanoids to stimulate uterine contractions (333). The neutrophil is a part of the innate system, which is the first line of defence in fighting infections (334). Not surprisingly, neutrophil infiltration is identified in preterm fetal membranes complicated by

infection (258). Also, Keski-Nisula et al. (2000) reported neutrophil infiltration within decidua following spontaneous term labour (259). Additionally, neutrophils were the dominant leukocytes attracted to an extract obtained from an in-labour fetal membrane previously tested, and these attracted neutrophils were higher with the extract collected from in-labour than not-in labour (335). The previous observation could support that the neutrophil infiltration could have a role in physiological labour. For this thesis, IL-8 treatment was only exposed to DMCs due to time limitations. However, exploring the IL-8 effect on inflammatory markers on chorion (trophoblast) and other decidual cells would be highly recommended. Also, we are suggesting using a fetal membrane tissue model, with two chambers system (amnion and decidua), by exposing amnion to DPPC/LPC (16:0) and measuring labour-associated mediators from the decidual side. Also, using a similar model to instigate leukocyte chemotaxis study, could provide a broader understanding of how major lung surfactant (DPPC) crosstalk between fetal to maternal side of the fetal membrane to initiate labour.

5.6. Conclusion

For the first time, we report the influence of DPPC/LPC (16:0) on amnion epithelial cell fatty acid composition. These data illustrate a novel role for DPPC, a key marker of fetal maturation, in stimulating amnion IL-8 production, a well-recognised pro-inflammatory chemokine related to human parturition. Also, we have observed that priming the DMCs is essential for prostanoid production (post-CI exposure), which supports our previous observation that the choriodecidua remains rich in AA even with profound secretion of prostanoids under labour conditions (**Chapter 3**). Although we failed to observe a triggering effect of IL-8 on treated DMCs, we would encourage more detailed study in this regard, with further *in vitro* and more sophisticated *ex vivo* experimental models.

Chapter 6: General discussion

Human parturition is a natural event observed and studied for decades, with more recent reports in agreement with a necessary upregulation of the intrauterine inflammatory environment (15,26,98,223,336). The fetal membrane is a tissue that surrounds the fetus during gestation, which expands with pregnancy to contain the increasing amniotic fluid and fetal growth. Besides placental implantation, the fetal membrane directly connects with the uterine lining (decidua), making it a plausible site for "crosstalk" between fetal and maternal parturition "clocks". Moreover, the fetal membrane is a well-recognised site for clinically stimulating or accelerating labour through different forms of irritation, i.e. sweeping, balloon insertions or membrane rupture (185,186).

Active lipid mediators, such as eicosanoids, endocannabinoids and ceramides, are important molecules that alert the inflammatory environment in health and disease (35,71,94,337). Among these mediators, AA-derived prostanoids are well explored in human parturition. PGE_2 and $PGF_{2\alpha}$ and TXA_2 stimulate uterine contractions *in vitro* (97), and both PGE_2 and $PGF_{2\alpha}$ are used clinically to induce labour in a patient with an unfavourable cervix (31). Many reports have speculated as to the importance of the fetal membrane as a source of inflammatory mediators to kick start human parturition (98,222). However, the downside of these reports is that they measured and made indirect conclusions from amniotic fluid levels, or *in*/*ex vivo* experimentation, as aside from direct tissue assessments. In this regard, most of the reported measures were analysed using immunoassay methods, which are also known to cross-react with inter-metabolites and usually limited to one metabolite per run.

This thesis hypothesised the fetal membrane to be a key source of active lipid mediators within the intrauterine milieu, responsible for labour onset. It firstly aimed to explore eicosanoids, endocannabinoids and ceramides from both sides of the fetal membrane (amnion and choriodecidua) under different labouring settings; spontaneous term labour (STL), induction at term (ITL) and preterm labour (PTL), and compared these to a control non-labouring (TNL) group at term; using MS-based techniques of high sensitivity and a wide range of analytes. The second aim was to profile structural lipid composition and fatty acid relative abundance within the fetal membranes in these same study groups. For this, an upregulation

of palmitic acid (PA) was observed with concomitant loss of arachidonic acid (AA) within the term amnion. This finding led to the further idea that PA-containing fetal lung surfactant, DPPC/LPC (16:0), was the natural stimulus for AA liberation within the amnion, capable of "kick-starting" a parturition cascade, across the membrane and on into the uterus and cervix. For this final element, the aim was to utilise *in vitro* cell cultures of fetal membrane components to examine the effects of DPPC/LPC (16:0) on AA levels within the tissue and the secretion of known pro-inflammatory markers of human labour, which may be liberated in response.

These studies confirmed the fetal membrane as an important source of lipid mediators involved in human parturition, but with the choriodecidua being generally more responsive and active than the amnion, pinpointing the crucial role of the choriodecidua as the focal point for labour onset. Importantly, the amnion was only active (in regard to these mediators) with term labour, perhaps implying that this fetal maturation signal is a prerequisite for stimulating the inflammatory cascade which culminates in parturition.

In the lipidome study, the choriodecidua showed a significant upregulation of its cellular composition defined by its glycerophospholipids, under both spontaneous term and preterm conditions. Although speculative, this finding would support the many published reports of invading leukocytes into choriodecidua during active labour. In term group in labour, the amnion showed a hint of AA depletion, as observed by a reduction in stored AA within the major glycerophospholipids and total AA pool. Although fetal lung surfactant (DPPC/LPC 16:0) failed to reciprocate this reduction in AA *in vitro*, a novel effect was observed with the provoked production of IL-8 from amnion epithelial cells, exposed to physiological levels of surfactant. As IL-8 is a well-recognised labour-associated chemokine/cytokine, it is readily assumed that such a response would undoubtedly attract maternal leukocytes to the adjacent choriodecidua, and in doing so initiate further prostanoid and cytokine production to fully propagate the steps towards parturition.

6.1. Fetal membrane lipid mediator profiling

This study showed that the amnion and choriodecidua produced a wide range of mediators categorised under eicosanoids, endocannabinoids and ceramides. In the contractile prostanoid profile, the amnion was a source of the PGE_2 metabolite (13,14, dihydro 15-keto PGE_2) in spontaneous term labour, suggesting the amnion as a source of PGE_2 in the earlier stages of parturition. This finding is supported by a rise in amniotic fluid PGE_2 when fetal maturation is achieved (at term), even before clinical labour has started (34). The other contractile prostanoids ($PGF_{2\alpha}$ and TXA_2) were not elevated in the term amnion over the non-labouring group. In contrast to a previously reported rise of these in amniotic fluid (104,338), this may perhaps be explained by contamination from fetal secretions or their secretion before the time of uterine contractions. Moreover, pre-fetal maturation (i.e. preterm labour) also failed to show an upregulation of contractile prostanoids in the amnion, as previously reported in amniotic fluid (241,339).

In the amnion, PGE_2 and $PGF_{2\alpha}$ were upregulated only after labour induction, whereas the choriodecidua produced contractile prostanoids in all labour groups studied. This could perhaps reflect the position of the choriodecidua adjacent to the contractile myometrium. Under term conditions (physiological and induced labour), PGE_2, $PGF_{2\alpha}$ and TXA_2 were all upregulated. These data are supported by previously recorded elevations in of COX activity, and prostaglandin synthases at this time, and measured prostanoids in term and preterm tissues (244–246). Interestingly, preterm labour demonstrated a surge of $PGF_{2\alpha}$ and TXA_2 but not PGE_2, implying fundamental differences in underlying mechanisms of induction between term and preterm parturition.

For the eicosanoid profile, LOX- and CYP450-derived mediators were detected in both the fetal membranes. Generally, these mediators are known to modulate the inflammatory environment and regulate vascular tone (253–255). Similar to prostanoids, the term in-labour amnion showed LOX- and CYP450 activity, a finding to support the suggestion of the amnion is a source of inflammatory mediators at the time of fetal maturation. In the choriodecidua, physiological labour also proved an optimal stimulus for these pathways. Of the LOX-derived mediators, 5-HETE, a myometrium contraction enhancer (105), and 12-HETE, a chemoattractant (257), were upregulated in spontaneous term labour in the amnion and

choriodecidua. This is perhaps indicative of the fetal membranes as a source of uterotonic mediators, with which maternal and resident uterine leucocytes could be invited into the gestational tissues. To some extent this finding would agree with Maddipati et al. (2014), who reported 5-HETE (but not 12-HETE) as being raised in amniotic fluid in term labour (104). Moreover, the choriodecidua in the preterm study showed upregulation of 12-HETE, which would also coincide with repeated reports of leukocyte infiltration into labouring tissues at term and preterm, regardless of infections (19,258,280,281).

The exact function of EETs in human parturition is not well explored; however, they are known to modulate vascular tone in other tissues (253,340). In spontaneous term labour, these epoxygenase mediators were exaggerated in fetal membranes, as defined in this study. In previous *in vitro* experimentations using myometrium, 14(15)EET has stimulated uterine contractions (262) and 5(6)EET has been shown to enhance myometrium and uterine artery relaxation (101). This would imply that EETs have a possible role in physiological parturition, by enhancing uterine contractions and increasing the necessary blood supply to the gestational tissues during labour. In the amnion here, spontaneous term labour upregulated 5(6)EET and 8(9)EET compared to not-in labour tissues; data partially supportive of past amniotic fluid analysis, where 8(9)EET, 11(12)EET and 14(15)EET were elevated with labour at term (242). In choriodecidua, 5(6)EET, 8(9)EET, 11(12)EET, and 14(15)EET were upregulated in spontaneous labour. These findings could suggest that both sides of the fetal membrane are a source of EETs under physiological labouring condition.

For the endocannabinoid profile, the amnion mainly produced 2-AG, which was unchanged with different labour conditions. In opposition, the choriodecidua was capable of producing both classical endocannabinoids (AEA and 2-AG). In spontaneous term and preterm labour, AEA was significantly elevated compared to non-labour controls. Previous reports support this observation, showing increased in AEA in maternal plasma under both conditions (60,263). Although *in vitro* experiments suggest AEA enhances uterine relaxation (341), it could theoretically also have an indirect uterotonic effect by stimulating PGE_2 production, as demonstrated in fetal membrane explants (127). In choriodecidua, 2-AG was stimulated only post-drug induction, which could imply this tissue was not a source of 2-AG in spontaneous labouring. Like the other endocannabinoids, the NEA profile highlighted various differences

in the fetal membranes under disparate labour conditions. Some of these NEAs could activate CB receptors such as LEA and, but these have less affinity than the classical endocannabinoids (264). Nevertheless, further studies would be recommended to explore their otherwise unknown roles in human parturition.

In the ceramide profile, total ceramide content was increased in the amnion and choriodecidua under term labour. In the amnion, induction of labour was most notable for its stimulated ceramide expression, with five ceramide classes elevated significantly in comparison to controls, CER(NP), CER(NH), CER(ADS), CER(AS) and CER(AP). In the choriodecidua, ceramide classes showed a variable increase between study groups. In spontaneous term labour, ceramide showed a rise in CER(NS), CER(NDS) and CER(ADS) classes and post induction, CER(NDS), CER(NH) and CER(ADS) were elevated. These observations support the previously reported upregulation of ceramides in maternal blood and placenta with labour at term (42,78) and also the upregulation of CER(NS) and CER(ADS) classes, preterm. Laudanski et al. (2016) reported an increase in 16 carbon length acyl CER(NS) in maternal plasma with true preterm labour (79). However, this ceramide species was not defined in the fetal membranes here, perhaps due to differences in sample types and preparation. Although a part for ceramides in human parturition has not been recognised, it was reported to stimulate of PGE_2 and cytokines in gestational tissues *in vitro* (229,230), which would again indicate a potential supportive role.

6.2. Fetal membrane complex lipids and fatty acid abundance

Besides their signalling role, lipids are crucial for cell structure and are an important energy source, especially during starvation and stress. In this thesis, the fetal membrane complex lipid profile showed differences between the pregnant (labouring and non-labouring) study groups. In the amnion, the major glycerophospholipid classes (PC and PE) showed consistency between study groups. However, in the choriodecidua, under spontaneous term and preterm labour, PC and PE were upregulated. This may be explained by changes in the cellular populations within these tissues, i.e. from leukocyte invasion during labour (19,258,280,281), in support of the inflammatory environment. Perhaps importantly in this regard, PC and PE are a favourable source of AA (in the sn-2 position) (284), with which to maintain prostanoid

production. Although the depletion of AA-containing species was recognised within amnion under spontaneous term labour, our observations support our notion of amnion being a source of prostanoids at the earlier stages of labour only. Arguably, this would not sustain the choriodecidua. However, as PC and PE AA-containing species are upregulated in spontaneous labour. This may be enough to maintain production of labour-associated prostanoids throughout the membrane, as these tissues remain rich in embedded AA, over the course of parturition.

PLA_2 is a key enzyme that liberates AA from glycerophospholipids. The presented data detected LPC only in choriodecidua, with term labour containing higher levels than those not-in labour at the same gestation. This finding suggests higher PLA_2 activity within these tissues with physiological labour. This notion is supported by a previous report of upregulated choriodecidual $cPLA_2$ gene expression with labour at term (182). Interestingly, LPC , a product of $cPLA_2$, is reported to have a chemotactic function on macrophages (290), which could point to a signalling role of LPC to prime choriodecidual tissues in labour.

SM is an important lipid class involved in cellular membrane structure and signalling (231). In the amnion, physiological labour profiled a decreasing trend for total SM over the not-in labour group. Given a defined increase in CER(NS) in term labour, it could be implied that the amnion has generated CE(NS) predominantly by the MS hydrolysis pathway. However, in the term and preterm labour choriodecidua, the defined increase (trend in preterm) of total SM, would argue for a change in cellular composition, more akin to leukocyte invasion, than over direct any metabolic transformation of the tissue.

Fatty acids are a considerable energy source during starvation and stressful biological conditions. In this study, the total free fatty acid level increased in the amnion under labouring term conditions, and with spontaneous labour in the choriodecidua. Increasing total FFA could provide a key source of energy within the fetal membranes and other gestational tissues, with which to sustain labour. In the amnion, free AA was comparable between groups. However, in the choriodecidua, measured free AA was higher in spontaneous term and preterm conditions over non-labouring controls, perhaps suggesting greater PLA_2 activity, as previously reported (182,289). Several long-chain FFAs species also increase an increase in

both sides of the membrane under different labour conditions. This could plausibly offer ELOVL enzyme activity stimulation in these tissues to adapt to their demands, such as feeding ceramide synthesis pathway.

In order to report the position of fatty acid double bonds, fetal membrane fatty acid abundances were profiled using GC-FID analysis. At term, the amnion tissues showed depletion of AA with labour, which supports the role of the amnion as a source of AA-derived prostanoids, likely before uterine contractions. Interestingly, PA, the dominant FA, showed an inverse correlation with amnion AA. A finding led to the suggestion that DPPC, a fetal lung maturation marker, could stimulate AA release from AA and invoke inflammatory signals in amnion.

6.3. Lung maturation marker Dipalmitoylphosphatidylcholine (DPPC) and the initiation of parturition.

Fetal lung maturation is a well-recognised element that determines healthy pregnancy outcomes (303), DPPC , rich in palmitic acid, is a major surfactant component secreted into the amniotic fluid, which increases in the late third trimester and correlates with advancing fetal lung maturation (68,304,305). In this study, the effect of DPPC (and its lyso-form (LPC (16:0)) was observed on fatty acid abundance and secretory cytokine production in isolated primary cells of the fetal membrane tissues, which were not yet in labour. For amnion epithelial cells, LPC 16:0 treatment was able to upregulate PA abundance, similar to that seen in amnion tissues in labour. However, this increase failed to liberate AA from the treated cells, as anticipated. These data appear to contradict Ohtsuka et al. (1990), where they reported amnion explants stimulated PGE production post DPPC treatment (307). Nevertheless, their report failed to specify the labouring status of the collected tissues and the concentration of DPPC employed was supra-physiological. Although increases in prostanoid production, were not observed following our own DPPC/LPC stimulation, LPC (16:0) encouraged the release of IL-8, which is a strong chemotactic, upregulated in all gestational tissues at term labour (98). In animal models, induction of labour is successfully achieved after cervical administration of IL-8 (28,199), which perhaps points to its fundamental role in infection-related labour, but also now in spontaneous labour. Given its chemotactic influence, IL-8 is a likely early

inflammatory signal which is directed from fetal lung maturity to initiate the labour cascade from amnion to choriodecidua. To explore this suggested crosstalk between amnion and choriodecidua, we examined the effect of IL-8 on labour-associated prostanoids by isolated decidual mesenchymal cells. Under these in vitro conditions, IL-8 failed to stimulate secretory PGE_2 from treated cells, but it would be highly advantageous to now explore the wider effect of IL-8 on the labour-associated mediators of other choriodecidua cell populations, either resident or infiltrating, such as trophoblast and decidual leukocytes.

6.4. Summary

In conclusion, the amnion and choriodecidua showed a unique lipidomic profile. Our data suggest that amnion is only active after fetal maturation is achieved, whilst the choriodecidua has a profile dependent on labouring conditions. For the amnion, spontaneous term and induction of labour stimulated prostanoid production, LOX-derived mediators, and total ceramide production, but most notably, this was following induction. Also, term labour conditions showed signs of depletion of AA storage within amnion, suggesting previous prostanoid production, perhaps at the very earlier stages of parturition.

The choriodecidua was associated with significant contractile prostanoid production in both term and preterm labour; an observation that fits squarely with its location adjacent to the contractile myometrium. Physiological labour stimulated LOX- and CYP450-derived mediators from the choriodecidua, whilst for spontaneous labour, the classical endocannabinoid, AEA, upregulated. Total ceramide expression was also increased in choriodecidua with spontaneous labour at term and also the following induction. These data propose that these lipid mediators could have a previously unrecognised role in physiological and pathological labour; however, this requires further refining. For the complex lipid profiles, an increase in glycerophospholipids was observed in the choriodecidua in term and preterm labour. Like other observed changes in lipids in this tissue, it is conceivable that these data reflect increases in the cellular composition of the choriodecidua with active labour, most likely by the expansion of invading leukocytes, which are already known to more abundant once labour progresses.

The fatty acid abundance profile of term amnion tissues showed an inverse correlation between AA and PA, which led to the DPPC hypothesis. Although DPPC treatment didn't stimulate AA release from amnion cells, it nevertheless stimulated IL-8 secretion. This novel fetal maturation signal directed by DPPC across the amniotic fluid, may very well prove to be the pro-inflammatory stimulus that eventually attracts leukocytes to the choriodecidua to initiating and propagate labour at term; this suggestion certainly warrants greater investigation.

6.5. Limitations and future work

In the lipidomic profile of the fetal membrane (**Chapter 3** and **4**), increasing the number of samples within the groups, specifically the PTL and STL groups, would increase the statistical power. Also, we have used the clinical definition of labour of ≥3 cm cervical dilatation. Given that we observed increasing prostaglandin metabolites in the amnion, we suggest including a latent phase labour group in the study, to elucidate membrane lipidome changes during early parturition. Also, presenting the data according to cervical dilatation and length of labour would add another dimension as to how the fetal membrane lipidome alters with labour progression. Maternal demographic factors, i.e., GA, MA, ethnicity, and BMI, are well-recognised elements that influence the timing of labour(12,13,187). However, due to the limitation of time and the number of samples available, we were unable to study the data in relation to these factors.

Our study explored a wide range of mediators, including hydroxy fatty acids, ceramides, endocannabinoids, and NEAs, altered under different parturition types. However, their role is not well-recognised in the context of parturition. Therefore, speculating their roles in an inflammatory context, and in relation to vascular and myometrium tone alteration, using *in vitro* and *ex vivo* models, is highly recommended. The mediators raised in preterm labour could be screened in maternal blood as biological markers for predicting this condition, and as targeted therapy for tocolysis. Furthermore, choriodecidua contains several cell populations, using mass spectrometry imaging techniques would provide more detailed, cell-specific information on the cellular lipidome changes (342,343).

Lastly, this thesis has shown that DPPC in its lysed form (LPC16:0) stimulated IL-8 secretion from amnion epithelial cells. However, due to time limitations, we only managed to report n=3 experiments. Adding more repeats, including a positive control (LPS), would strengthen this finding. Also, we suggest using a more sophisticated model, i.e., a fetal membrane tissue culture model, which would give a better illustration of how LPC16:0 stimulates labour-associated mediators from the decidual side, and allow examination of the types of infiltrated leukocytes using a chemotactic assay (344). Moreover, investigating amniotic $sPLA_2$ levels in term pregnancy could add new knowledge as to how fetal lung maturation propagates the inflammatory environment in amnion.

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