INFLAMMATION, ANTIOXIDANT DEFENSE, STEROIDOGENESIS, AND STEROID METABOLISM: MECHANISMS FOR PREGNANCY COMPLICATIONS ASSOCIATED WITH ASSISTED REPRODUCTION

by

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BMSc, The University of Western Ontario, 2016

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF

THE REQUIREMENTS FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

in

THE FACULTY OF GRADUATE AND POSTDOCTORAL STUDIES

(Pharmaceutical Sciences)

THE UNIVERSITY OF BRITISH COLUMBIA

(Vancouver)

December 2021

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INFLAMMATION, ANTIOXIDANT DEFENSE, STEROIDOGENESIS, AND STEROID METABOLISM: MECHANISMS FOR PREGNANCY COMPLICATIONS ASSOCIATED WITH ASSISTED REPRODUCTION

submitted by Hayley Price in partial fulfilment of the requirements for

the degree of Doctor of Philosophy

in Pharmaceutical Sciences

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Abstract

The precise coordination of inflammatory signaling, oxidative stress responses, steroidogenesis, and steroid metabolism are required in all aspects of pregnancy, from fertilization and implantation to parturition. Dysfunction in any of these processes can lead to unfavourable pregnancy outcomes and complications requiring obstetric interventions. As the use of assisted reproductive technologies (ART) has increased in recent decades, it has become evident that these pregnancies are associated with increased risks compared to unassisted pregnancies. However, the underlying reason for these increased risks is unknown. Preliminary studies from our laboratory using a mouse model of ART provide evidence that inflammatory, antioxidant, or steroid pathways could be dysregulated in ART. In this thesis, we investigated the levels inflammatory mediators, measures of antioxidant defense, and steroid hormones in pregnancy to determine any associations with pregnancy outcome. Beginning with an investigation of non-steroidal anti-inflammatory drug (NSAID) use in pregnancy (Chapter 2), we determined the prevalence of NSAID use close to labour. Next, to investigate the differences between ART and unassisted pregnancies, we used a cohort of placentas from ART pregnancies and unassisted pregnancies matched for gestational age, maternal age, ethnicity, and singleton/twin pregnancy. In this cohort, we evaluated the levels of cytokines and vascular endothelial growth factors and identified associations with pregnancy outcome using the accompanying clinical chart profiles (Chapter 3). Further, the activity of antioxidant enzymes and levels of antioxidants were measured in these placentas (Chapter 4). In Chapter 5, we measured the levels of steroid hormones and steroid sulfates in maternal, placental, and fetal compartments, again comparing these results between ART and unassisted pregnancy and identifying associations with pregnancy outcome. No widespread dysregulation of these processes was found between ART

and unassisted pregnancies. However, when stratified for pregnancy outcome, two significant associations were found. In pregnancies complicated by chorioamnionitis and in twin pregnancy, there was dysregulation across all three signalling processes. These signalling pathways are complex and interconnected, but the data generated here adds novel findings to the ART field, and identifies potential pathways for future investigation to resolve the etiology of increased pregnancy complications in ART.

Lay Summary

Each year, more people are using assisted reproductive technologies to start a family. While most of these pregnancies result in healthy infants, they are associated with increased risks of complications compared to unassisted pregnancies. To understand why these complications are happening, we investigated inflammatory and antioxidant signaling processes and steroid levels within the placenta, the main organ of communication between the mother and developing fetus. These studies were performed in a cohort of placentas from pregnancies conceived using assisted reproductive technologies and unassisted pregnancies. While we did not find differences between the two groups overall, once we compared pregnancy outcomes two main differences were noted. Inflammatory, antioxidant, and steroid signalling were disrupted in pregnancies complicated by infection and in twin pregnancies. These studies will help identify potential pathways involved in pregnancy complications when assisted reproductive technologies are used, which can be manipulated in the long term to improve pregnancy outcomes.

Preface

The work presented in this thesis was conducted at the Faculty of Pharmaceutical Sciences at the University of British Columbia, with the exception of the cytokine and VEGF screening which was performed at the Centre for Blood Research at the University of British Columbia (Chapter 3) and the steroid LC-MS screening which was performed at the Centre for Brain Health the University of British Columbia (Chapter 5). I, Hayley R. Price, was responsible for developing the research questions, performing the experiments, analysing the results, and writing the chapters. Assistance from undergraduate summer students with specific projects are noted within the thesis. My supervisors, Drs. Abby Collier and Michael Coughtrie, my PhD committee members, Drs. Hugh Kim, Kiran Soma, and Karla Williams, and my past committee member Dr. Emma Guns provided feedback on the research design and interpretation of results. This study was conducted under approval from the Clinical Research Ethics Board at The University of British Columbia (H14-00092) and The University of Hawaii IRB for Human Subjects (CHS 15080).

The following manuscripts resulted from, or are related to, this thesis:

Published Papers:

1. Hayley R Price, Abby C Collier. Analgesics in pregnancy: An update on use, safety and pharmacokinetic changes in drug disposition. *Current Pharmaceutical Design* **(2017)** 23(40): 6098-6114. ACC provided an outline. HRP wrote the first draft of the manuscript. HRP and ACC edited and approved the final version for submission.

2. Hayley R Price, Tricia E Wright, Abby C Collier. Screening pregnant women and their neonates for illicit drug use: Consideration of the integrated technical, medical, legal,

ethical and social issues. *Frontiers in Pharmacology* **(2018)** 9: 961. HRP wrote the first draft of the manuscript. TEW and ACC provided feedback professional opinions. All authors edited and approved the final version for submission.

3. Hayley R Price, Camron Chehroudi, Stuart J Knight, Alexander D Smith, Dickson Lai, Hugh Kim, Tricia E Wright, Michael WH Coughtrie, Abby C Collier. Umbilical cord as an analytical matrix – a technical note. *Placenta* **(2020)** 90: 42-44. HRP, MWHC, and ACC conceptualized the study idea. HRP wrote the first draft of the manuscript. All authors were involved in generating data discussed in the manuscript. All authors provided feedback, edited, and approved the final version for submission.

4. Hayley R Price, Dickson Lai, Hugh Kim, Tricia E Wright, Michael WH Coughtrie, Abby C Collier. Detection and quantitation of non-steroidal anti-inflammatory drug use close to the time of birth using umbilical cord tissue. *Toxicology Reports* **(2020)** 8;7:1311-1318. HRP, MWHC, and ACC formulated the study idea. HRP conducted experiments. DL provided technical expertise. TEW was involved in tissue collection. HK and ACC received funding for the study. HRP wrote the first draft of the manuscript. All authors edited and approved the final version for submission.

5. Hayley R Price, Nick Pang, Hugh Kim, Michael W H Coughtrie, Abby C Collier. Protective placental inflammatory and oxidative stress responses are attenuated in the context of twin pregnancy and chorioamnionitis in assisted reproduction. *Journal of Assisted Reproduction and Genetics* **(2021)** Accepted. HRP, MWHC, and ACC formulated the study design. HRP and NP conducted experiments. HK and ACC received funding for the study. HRP wrote the first draft of the manuscript. All authors edited and approved the final version for submission.

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List of Abbreviations

3β-HSD	3B-hydroxysteroid dehydrogenase
°C	degrees Celsius
ε	extinction coefficient
µg	micrograms
µL	microlitres
µm	microns
µM	micromoles per litre
ACTH	adreno-corticotropic hormone
ANOVA	one-way analysis of variance
ART	assisted reproduction
BCA	bicinchoninic acid
BMI	body mass index
BSA	bovine serum albumin
CAPs	contraction-associated proteins
CDNB	1-chloro-2,4-dinitrobenzene
cm	centimetre
CNS	central nervous system
COX	cyclooxygenase
CRH	corticotropin-releasing hormone
CV	coefficient of variation
CYP	cytochrome P450
DHEA	dehydroepiandrosterone
DHEAS	dehydroepiandrosterone sulfate
DTNB	5,5'-dithio-*bis*-2-(nitrobenzoic acid)
E1S	Estrone sulfate
ELISA	enzyme-linked immunosorbent assay
F	bioavailability
FAD	flavine adenine dinucleotide
FDA	United States Food and Drug Administration
GPCR	G protein-coupled receptor
GPx	glutathione peroxidase
GR	glutathione reductase
GSH	reduced glutathione
GSSG	glutathione disulfide (oxidized glutathione)
GST	glutathione S-transferases
HPA	hypothalamic-pituitary-adrenal
HPLC	high-performance liquid chromatography
ICSI	intracytoplasmic sperm injection
IL	interleukin
IUGR	intra-uterine growth restriction
IVF	*in vitro* fertilization
JAK	Janus kinases
k_{el}	elimination rate constant
L	litres

M	molarity
m/z	mass-to-charge ratio
MAPK	mitogen-activated protein kinase
mg	milligram
min	minute
mL	millilitres
mM	millimolar
mmol	millimole
MS/MS	tandem mass spectrometry
MSD	MesoScale Diagnostics
NAD^+	nicotinamide adenine dinucleotide
$NADP^+$	nicotinamide adenine dinucleotide phosphate
NADPH	reduced nicotinamide adenine dinucleotide phosphate
NICU	neonatal intensive care unit
nm	nanometre
nmol	nanomole
NSAID	nonsteroidal anti-inflammatory drug
NQO	NADPH quinone oxidoreductases
OTC	over-the-counter
PCO_2	partial pressure of carbon dioxide
PG	prostaglandin
PGD	preimplantation genetic diagnosis
pH	potential of hydrogen
PlGF	placenta growth factor
PR	progesterone receptor
PROM	premature rupture of the membranes
QC	quality control
RAAS	renin-angiotensin-aldosterone system
S9	supernatant fraction obtained from organ homogenate
SEM	standard error of the mean
SOCS	suppressors of cytokine signaling
SOD	superoxide dismutase
SOD1	superoxide dismutase 1 / superoxide dismutase [Cu-Zn]
SOD2	superoxide dismutase 2 / Mn dependent superoxide dismutase
SOD3	superoxide dismutase 3 / extracellular superoxide dismutase [Cu-Zn]
StAR	steroidogenic acute regulatory protein
STARD3	StAR related lipid transfer domain containing 3
STAT	signal transducers and activators of transcription
STS	steroid sulfatase
SULT	sulfotransferase
$T_{1/2}$	half-life
TNB	5-thio-2-nitrobenzoic acid
TNF	tumor necrosis factor
TXA	thromboxane
UC	umbilical cord
UDP	uridine diphosphate

UDPGA	uridine diphosphate glucuronic acid
UGT	UDP glucuronosyl transferase
UHPLC	ultra-high performance liquid chromatography
UPLC	ultra-performance liquid chromatography
V	volt
V_d	volume of distribution
VEGF	vascular endothelial growth factor
VEGF-A	vascular endothelial growth factor A
VEGF-B	vascular endothelial growth factor B
VEGF-C	vascular endothelial growth factor C
VEGF-D	vascular endothelial growth factor D
VEGFR	VEGF receptor
VEGFR-1/Flt-1	VEGF receptor 1
VEGFR-2/KDR	VEGF receptor 2
VEGFR-3/Flt-4	VEGF receptor 3
XO	xanthine oxidase

Acknowledgements

I wish to express my appreciation and gratitude to my supervisors, Dr. Abby C. Collier and Dr. Michael W. H. Coughtrie for their continued support and wisdom in the last five years, and for the opportunity to do this work.

I would also like to thank the Collier and Coughtrie lab members, past and present, who have been an amazing support system: Michael Doerksen, Brandon Haefling, Dickson Lai, Dr. Yuejian Liu, and Dr. Alexander Smith. Alex, I'm eternally grateful for your advice and friendship. Thank you for putting up with me continuously knocking on your office door to discuss my struggles, both lab-related and not. Michael, I definitely laughed harder once you joined the lab, thank you for getting me through the last two years. A sincere thank-you to the undergraduate and graduate students I worked with on specific projects within this thesis. Camron Chehroudi, for his assistance with the multiplex ELISAs. Nick Pang, for his work on the GST assay. Anh Khoa Vo, for teaching me all things R. And Cecilia Jalabert, for her assistance with the steroid method, the introduction to Spanish music, and sharing my paranoia for contaminating mass spec samples.

I gratefully acknowledge the financial support from the Canadian Institutes of Health Research and the University of British Columbia for funding this work. Much appreciation for the University of Hawaii Reproductive Biorepository for providing the valuable samples used in this thesis.

To Doug: You are the most patient, supportive, and understanding partner. Thank you for your help in getting me to this point. Finally, to my family: Mom, Dad, and Alanna. You have supported me through it all. Thank you for dealing with the tears, celebrating the successes, and pretending to understand exactly what it is I do. I hope I made you proud.

To Mom and Dad,

for the unconditional love and support.

Chapter 1: General Introduction

1.1 Introduction

Pregnancy is a complex physiological state. From fertilization and implantation, through to parturition there are several important signalling molecules, enzymes, and cellular processes that must be tightly controlled for a successful pregnancy to occur[1,2]. Additionally, pregnancy is marked by both inflammatory and oxidative processes, resulting in a delicate balance between signalling that results in healthy growth and development, and dysregulation causing harmful outcomes for both the mother and the fetus[1,2].

The source and location of action of many of these signalling mediators is the placenta. The placenta is a unique organ which was originally believed to be a protective barrier for the fetus, but the understanding of its role has changed over the years. While it is the primary organ of communication between the mother and fetus and does provide fetal protection, the placenta also has a number of important synthesis, transfer, and immunologic functions[3]. Transfer of compounds across the placenta from mother to fetus is now understood to be an important process, and one that must be considered in terms of drug treatment and maternal exposure to xenobiotics[4,5]. Despite its vital role in pregnancy and its proximity to the fetus, the role of the placenta is often overlooked when investigating unfavourable pregnancy outcomes. However, it can provide an invaluable tool for studying the fetal environment and mechanisms underlying complications in pregnancy.

Pregnancies that are achieved using assisted reproductive technologies (ART) are at higher risk of experiencing pregnancy complications and requiring obstetric interventions[6,7]. In the 21st

century the use of ART has been increasing every year, both due to decreasing fertility rates in the developed world and with increasing access of LGBT2SQIA+ individuals to start a family[8,9]. Pregnancies achieved using ART are primarily of two technical types: *in vitro* fertilization (IVF), the cavitation of gametes in a dish, and intracytoplasmic sperm injection (ICSI), the injection of a single sperm into an egg using a needle. While these procedures are considered safe and effective means of conception, it is well documented that there are higher risks of complications including small-for-gestational age infants, premature births, and congenital abnormalities, as well as increased need for obstetric interventions such as induced labour and emergency caesarian[6,7,10-12]. The reasons for these complications are not completely understood, but abnormalities of the placenta have been noted in ART pregnancies. As such, the placenta, which is readily available following birth, provides a useful tissue for studying pregnancy complications associated with ART.

The underlying reason for infertility, the drugs involved in fertility treatment, and the ART procedures themselves (fertilization outside the body) have all been proposed as explanations for these increased complications[13]. However, the underlying physiological effects observed in pregnancy complications are complex and multifactorial, unlikely to have a single explanation. It is well recognized that there is a need for the basic and clinical research to determine the extent and long-term effects of negative pregnancy outcomes involved in ART, and how they can be mitigated. Treatment and manipulation in pregnancy has a chequered past in pharmacology and medicine, but careful evaluation of inflammatory, oxidative, and steroidogenic pathways in the placenta is important in understanding the etiology, underlying mechanisms and pathophysiology of negative pregnancy outcomes[14].

1.2 Human Placental Development and Function

1.2.1 Early Embryogenesis and Implantation

Fertilization of the oocyte in the fallopian tube signals the onset of embryogenesis and subsequent placentogenesis[15]. An overview of this process is presented in Figure 1.1. The fertilized egg, surrounded by the zona pellucida, is now called a zygote which grows and divides as it travels down the fallopian tube toward the uterus[16]. The zygote divides to become a 16-cell morula by day 3 post-fertilization. In the following days the outer cells, which are destined to develop into the early trophoblast, become tightly bound by cell-cell adhesions and transport fluid inside the cell ball. The resulting structure with a fluid filled cavity and an inner-cell mass at one pole is known as a blastocyst. The trophoblast is destined to become the placenta and the extraembryonic membranes, while the inner cell mass will give rise to the embryo proper. At approximately day 5, the zona pellucida is lost in a process known as blastocyst "hatching"[17].

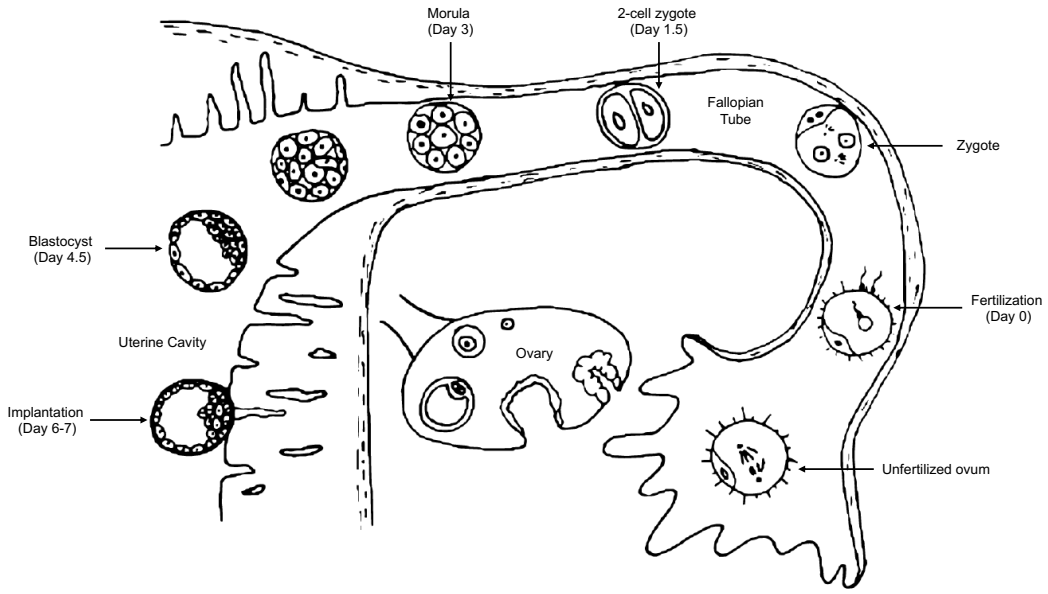

Figure 1.1 Stages of early embryonic development in humans.
Approximate location in the reproductive tract and day of each stage are noted. Original figure, created in Adobe Illustrator (2021).

This allows increased growth of the blastocyst and access to the uterine lining. Implantation occurs on day six or seven post-fertilization, thus, the first week of embryogenesis occurs in the fallopian tube prior to implantation[15]. Two processes must occur for successful implantation: differentiation of the uterine lining in preparation for implantation, and the ability of the embryo to interact with the endometrium[18].

Implantation is a critical period in reproduction. Approximately 50% of conceptions fail at this stage[19]. Additionally, the use of ART has a success rate of 25% in Canada, and most failures are thought to be due to implantation failure[8]. The trophoblast cells express the carbohydrate binding protein L-selectin which can bind to oligosaccharide molecules on the uterine epithelium[20]. A mucin-like protein is also secreted by the endometrium, which assists the blastocyte in "rolling" along the uterine lining[21]. At the implantation site, more secure attachments are formed by integrins, which occurs at approximately day six/seven post-fertilization[15].

Implantation most often occurs in the upper and posterior wall of the uterus. How the implantation site is chosen is not well understood, but certain signalling molecules, both from the mother and the blastocyst, are known to be involved in the process. During the 48-hour window of implantation, prostaglandin E2 and prostaglandin F2α have been shown to peak in maternal plasma[22].

1.2.2 Placentogenesis

The placenta is a remarkable and unique organ which has a lifespan limited to the gestation period. While critical to the growth and health of the fetus, it is situated outside the fetal body.

Following implantation, the trophoblast cells undergo rapid morphological and functional changes to form the placenta and extraembryonic membranes[15]. As the trophoblast invades the endometrium, it now consists of two distinct layers: an inner layer of mitotically active cells known as cytotrophoblasts, and an outer multinucleated structure called the syncytiotrophoblast[15]. Initial invasion of cytotrophoblast columns into the uterine lining around 13 days post-fertilization results in the formation of primary villi[23]. The villi will become the circulatory units of the placenta. At day 21, a fetal capillary network develops within the mesenchyme, establishing the feto-placental circulation and forming tertiary villi[15,24]. Simultaneously, extravillous cytotrophoblast cells have invaded the uterus and endothelium of the spiral arteries, forming trophoblastic plugs. As such, the placenta develops under low oxygen conditions until 10 weeks' gestation when the plugs are removed, and maternal blood can flow into the intervillous space[25].

At term, the human placenta is discoid and has an average diameter of 15-22 cm, a thickness of 2-3 cm and weighs approximately 470-500 g[26]. The placenta can be separated into three sections: the fetal-facing chorionic plate, the basal plate (maternal), and the intervillous space between the two which contains the vasculature of the placenta[26]. Figure 1.2 illustrates a cross-section of a normal human placenta. In the intervillous space are 30-40 villous trees, each an individual vascular unit of the placenta termed a cotyledon. Cotyledons are separated by maternally derived decidual septa which project into the intervillous space. The villi arise from the chorionic plate and are covered by the trophoblast[26]. The barrier between the maternal blood and fetal circulation consists of the trophoblast, connective tissue, and the fetal capillary endothelium, making the distance between the circulations approximately 3.5 μm[16]. Extensive branching of

the villi increases the surface area of the placenta to 11-14 m^2, which has a positive correlation with fetal weight at birth[27].

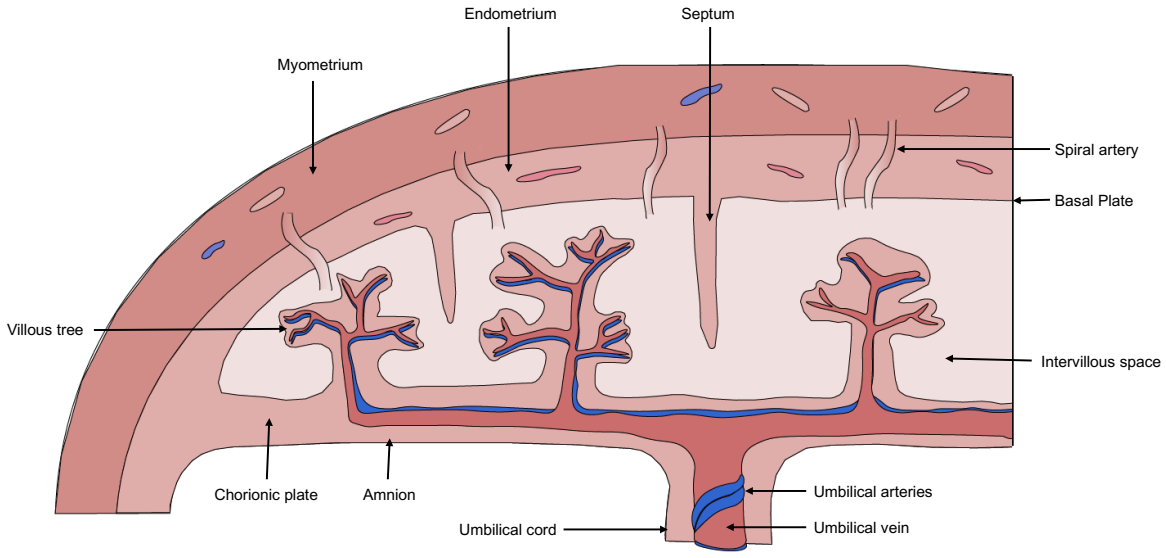

Figure 1.2 Schematic representation of a cross section of a normal human placenta.
Original figure, created in Adobe Illustrator (2021).

1.2.3 Villi

The villous trees are the main structure of the placenta. In the second half of pregnancy the villi are separated into different classifications based on characteristics and function. Arising from the chorionic plate are the stem villi, which are the main supporting structure of the central arteries and veins[28,29]. They are covered in a thick layer of trophoblast and little exchange of nutrients and gases occurs across these areas[29].

Mature intermediate villi are formed from repeated branching of the stem villi and make up the majority of the villous tree growth[28]. It is characterised by a more extensive capillary network

and thinner syncytiotrophoblast layer. There is some exchange of gasses, waste, and nutrients across the membranes in this area[28].

Arising from mature intermediate villi are the terminal villi. Terminal villi are the final branches of the villous trees and the functional units of maternal-fetal material exchange[30]. The trophoblast layer is thin with occasional nuclei and an extensive capillary network is present. Often described as "grape-like" extensions, these villi are highly vascularized. They appear around 27 weeks' gestation and account for 50% of the villous surface area (11-14 m^2)[28,31]. A diagram of the cross sections of chorionic villi are presented in Figure 1.3, illustrating the differences in the maternal-fetal interface between early pregnancy and term.

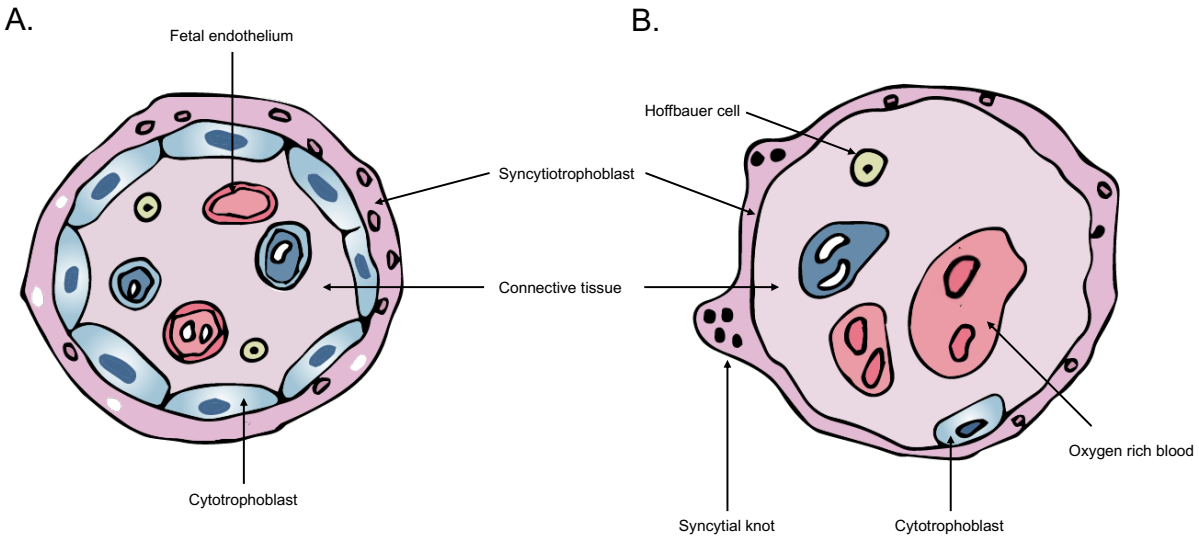

Figure 1.3 Cross section of chorionic villi illustrating the placental membrane.
A. Early pregnancy **B.** Term pregnancy. Original figure, created in Adobe Illustrator (2021).

1.2.4 Trophoblast

The specialized cells of the placenta, known as the trophoblast, perform the diverse actions of the organ, functioning as the location of gas exchange, waste/nutrient transfer, endocrine, and immunologic tissue[32]. Trophoblasts are involved in blastocyst invasion, and subsequently differentiate into cytotrophoblasts and the syncytiotrophoblast[28].

Cytotrophoblasts are involved in the early formation of the placenta[32]. They initially form a continuous layer around the embryo and develop into the primary and immature intermediate villi[15,33]. Further development and maturation of villous cytotrophoblasts results in mature intermediate villi and tertiary villi. These cells are mononucleate and mitotically active throughout pregnancy[33]. Undifferentiated cytotrophoblasts are cuboidal with low numbers of organelles[33]. They are also the progenitor cells for the syncytiotrophoblast and maintain the ability to replicate throughout pregnancy. Later in pregnancy, the cytotrophoblasts underlying the syncytiotrophoblast become sparse, no longer forming a continuous layer (illustrated in Figure 1.3).

In contrast, the syncytiotrophoblast is a continuous layer which covers the villous trees lining the intervillous space[32]. The structure is formed by the fusion of progenitor cytotrophoblasts, resulting in a multinucleated structure. In early implantation and placentogenesis, it has invasive properties and is involved in hollowing out spaces within the stroma to provide a space for nutrients to reach the developing placenta, and later the intervillous space where maternal blood flows[32,34]. As the villi develop, the syncytiotrophoblast loses its invasive capacity and becomes an epithelial-like surface covering the villi, becoming the primary structure involved in the

exchange of gases, nutrients, and waste between the maternal and fetal circulation[34]. It also carries out endocrine and immunologic functions[35].

In addition to the villous trophoblast are the extravillous trophoblast. These cells have extensive invasive properties and detach from the trophoblast columns, migrating deeper into the uterus than the villous placenta[36]. They are involved in the remodeling of spiral arteries to facilitate low resistance blood flow to the placenta[36]. However, they first form "plugs" in the spiral arteries, to prevent damage by oxidative stress and allow the placenta to develop under hypoxic conditions. Subsequently, they replace the endothelial layer of the spiral arteries, increasing elasticity and inducing apoptosis of smooth muscle, resulting in flaccid, sac-like vessels[37].

1.2.5 Extraembryonic Membranes

The extraembryonic membranes include the amnion, chorion, allantois, and yolk sac[38]. The inner cell mass differentiates into the epiblast and hypoblast on day 6 post conception[39]. The epiblast contains the cells destined to become the amnion, which is the fluid filled sac that surrounds the entire fetus[40]. The hypoblast layer will spread to form in the primary yolk sac and line the inner surface of the cytotrophoblast[41]. Another cellular layer called the extraembryonic mesoderm forms between the cytotrophoblast and the yolk sac. The extraembryonic mesoderm in combination with the cytotrophoblast will go on the form the chorion and gives rise to the stroma, which underlies the trophoblast in the developing placenta[39,40]. The primary yolk sac will develop into the secondary yolk sac at the wall of the developing gut, serving as a hematopoetic tissue until fetal organs take over at six weeks gestation and the yolk sac is incorporated into the umbilical cord[42].

1.2.6 Decidua

The decidua is the maternally derived portion of the placenta. It is formed from the endometrium during the luteal phase of the menstrual cycle in a process called decidualization[43]. Morphological changes of the endometrial cells must occur, including increased cell size, change in shape from fibroblastic to polyhedral, and the formation of intercellular junctions[43]. The decidua is important for the establishment of pregnancy and provides nutrition to the developing embryo before the placenta is developed. It also has important immunoregulatory roles, protecting the embryo from the maternal immune system[43]. Throughout pregnancy, it produces hormones, cytokines, and growth factors important for pregnancy maintenance. It also expresses receptors for these signalling molecules.

1.2.7 Development and Anatomy of the Umbilical Cord

The umbilical cord connects the developing fetus from the abdomen, or umbilicus, to the placenta. At approximately embryonic day 18, a mesenchymal structure called the connecting stalk forms, which connects the developing fetus to its surrounding membranes[44]. Within this stalk, the yolk sac extends to form the allantois. As the amniotic sac expands around the growing fetus, the connecting stalk containing the allantois and yolk sac are compressed to form the umbilical cord[44].

Enclosed within the umbilical cord are the umbilical vessels. Normally, one umbilical vein and two umbilical arteries are present[44]. The vein carries oxygen-rich blood from the placenta to the fetus, while the arteries return deoxygenated blood and waste to the placenta. The endothelium of these vessels differs from other vessels in the body and the villous of the placenta in that they are

incredibly rich in organelles[44]. Collagen fibers surround each of the vessels to form an adventitia, protecting the vessels and providing structure[44].

The vessels are surrounded by Wharton's jelly, a mucoid connective tissue derived from the extraembryonic mesoblast[44,45]. It is mainly comprised of the glycosaminoglycans, hyaluronic acid, chondroitin sulfate, proteoglycans, and collagens[45]. Myofibroblasts and mast cells are present within the Wharton's jelly, but cell distribution is uneven, being densest in the perivascular area and decreasing toward the perimeter of the cord[44]. This uneven distribution is paralleled by degree of differentiation of cells to myofibroblasts, which are more highly differentiated closer to the umbilical vessels. Wharton's jelly functions to support the umbilical vessels as they expand and contract and prevent kinking of the vessels to maintain blood flow[44,45].

The cord is enclosed by the amniotic epithelium which is firmly attached to the connective tissue beneath it[46]. At the attachment to the fetus' abdomen, a stratified squamous epithelium is observed, which transitions to a stratified columnar then simple columnar epithelium as it reaches the placenta[46]. It is generally agreed upon that the umbilical cord does not contain other blood or lymph vessels, and the tissue is not innervated[44]. At term, the average umbilical cord is approximately 50-60 cm in length and 1-2 cm in diameter[44].

1.3 Process of Human Parturition

Despite intensive investigative and research efforts due to the high incidence of premature labour and delivery, which accounts for nearly 85% of perinatal deaths, the process of parturition in humans is not completely understood[47]. What determines gestation length and the onset of

parturition has long been a major question for physicians and researchers, considering the natural variation of gestation length observed[48]. There is evidence that the growing fetus may outstrip the ability of the maternal/placental blood flow to supply oxygen and essential nutrients[49]. Umbilical blood flow increases throughout pregnancy, however, normalized to fetal body weight the blood flow declines[49]. As oxygen delivery decreases in late gestation, the maternal-placental supply becomes inadequate, which may play a role in the initiation of parturition. Two main changes must take place for parturition to proceed effectively: 1) Conversion of the uterus from a state of myometrial quiescence to active muscular contractions, and 2) dilation and effacement of the cervix to allow vaginal delivery of the fetus[50,51]. The myometrium is stimulated to induce myometrial contractions in a transition which is called "uterine activation". Activation of the myometrium occurs through the induced expression of contraction associated proteins (CAPs) which includes actin, myosin, oxytocin receptors, connexin-43, and prostaglandin receptors[51]. While it is impossible to completely study human parturition *in vivo*, the results of animal studies have identified hormones and signaling molecules involved in labour initiation, and their roles in humans are discussed below.

1.3.1 Placental Corticotropin-Releasing Hormone and Fetal Adrenal Axis

By the end of gestation, the fetal hypothalamus-pituitary-adrenal (HPA) axis is functional and plays important roles in the onset of parturition[50]. Increases in fetal HPA activity have been detected in many species at the end of gestation[50]. Late in pregnancy, there are high levels of corticotropin-releasing hormone (CRH) released from the fetus in addition to placental CRH, which is unique to humans[52]. This in turn leads to release of adreno-corticotropic hormone (ACTH) by the fetal pituitary, and subsequent cortisol release from the fetal adrenal.

Cortisol is one of the most important mediators in fetal maturation and preparation of parturition. Late pregnancy is characterized by progressively higher cortisol levels in maternal and fetal circulation[50]. Cortisol further stimulates the release of placental CRH and placental COX-2 expression, which leads to formation of prostaglandins and thromboxane, important mediators in parturition[52]. Additionally, cortisol plays important roles in the development of the fetal lungs, gastrointestinal tract, liver, and heart which should occur prior to parturition for fetal well-being[50].

In the fetal compartment, CRH and prostaglandin synthesis can further stimulate fetal ACTH by a feed-forward mechanism. In response, production of C19 steroids within the fetal adrenal are markedly increased leading up to parturition[50]. Once synthesized, dehydroepiandrosterone (DHEA) and it's sulfated form (DHEAS) are transferred to the placenta where they are converted to estrogens.

1.3.2 Estrogens

Estrogen levels increase towards the end of gestation due to high levels of DHEAS from the fetal adrenal which are required for estrogen synthesis in the placenta[50]. Estrogen stimulates the expression of CAPs, stimulates myometrial activity, and functions in cervical maturation[50]. Increases in oxytocin receptors, prostaglandin receptors, ion channels, and connexin-43 stimulated by estrogen are just part of the onset in labour in humans. Connexin-43, which forms gap junctions, are rare prior to the onset of labour and increases the cell-cell connections to more effectively transit the excitatory signals required for muscular contractions of the uterus[50].

1.3.3 Progesterone

The role of progesterone in human parturition is less well understood than in other mammals. In sheep, an abrupt decline in progesterone is observed in conjunction with a steep rise in estrogen at the end of pregnancy[53]. Parturition in sheep is particularly well studied due to the ability to insert catheters in the fetal vasculature and uterus, which doesn't respond to surgery by contracting like the human uterus[54]. It is well documented that high progesterone levels are required for the maintenance of pregnancy, and progesterone opposes the actions of estrogen, but a decline in progesterone is not involved in labour onset in humans. To support these claims, studies have shown that labour is not inhibited by exogenous progesterone, but labour can be initiated by the administration of a progesterone receptor blocker[55]. To account for the lack of progesterone withdrawal at labour in humans, some researchers have proposed a "functional" withdrawal of progesterone[56,57]. There are two forms of the progesterone receptor (PR): PR-B mediates the action of progesterone, while PR-A represses the transcription of PR-B[57]. Mesiano *et al.* proposed that relative ratios of progesterone receptors may change towards labour, and an increase in PR-A could inhibit progesterone induced relaxation of the myometrium[57].

1.3.4 Prostaglandins

As previously discussed, the fetal adrenal axis and resulting estrogen synthesis leads to synthesis of prostaglandins (PG) and prostaglandin receptors. Synthesis of prostaglandins occurs in the myometrium, endometrium, and fetal membranes[50]. Prostaglandins are lipid mediators of inflammation and blood flow. Labour itself is classified as in inflammatory state, and prostaglandins are essential for uterine activation and contractions, as they are involved in contraction of the smooth muscle of the uterus[50]. Around the onset of labour, there is a spike in

level of PGF2α[50]. The importance of prostaglandins in labour has been shown by the ability of exogenous PGF2α to induce labour, and conversely the inhibition of prostaglandin synthesis by NSAIDs, which prolongs labour[58].

1.3.5 Oxytocin

Oxytocin is perhaps the most well-known mediator in labour onset. It is the most frequently used medication to induce labour in a medical setting. It has been documented to increase the strength of contractions by increasing calcium levels and other contractile factors in the myometrium[59]. However, patients must be closely monitored following oxytocin administration, as labour often proceeds faster and more strongly, and the fetal heart rate may increase[59].

1.3.6 Uterine Stretch

There is evidence that fetal size and the resulting uterine stretch plays a role in the initiation of parturition[60]. In both animal models and *in vitro* human models, stretch imposed on myometrial cells resulted in increased expression of CAPs including COX-2, connexin-43, and the oxytocin receptor[60]. The effect of uterine stretch can be observed in multiple pregnancy and polyhydramnios, where preterm labour is more prevalent. There is also evidence that uterine stretch increases interleukin 8 (IL-8) levels[61]. Interleukin-8, a chemokine and pro-inflammatory cytokine, plays important roles in the inflammatory state observed during parturition. Uterine stretch alone, however, is not enough to overcome effects of progesterone in maintaining pregnancy[61].

1.4 Physiological Changes Occurring in Pregnancy

A wide range of maternal physiologic changes occur in pregnancy as the body anatomically adapts to the growing fetus and the introduction of the placental-fetal unit[62]. Rapid changes in endocrine function cause several changes to cardiac, renal, respiratory, and gastrointestinal systems, which may return to normal function following parturition or be permanently altered[62]. Importantly, these changes can affect the pharmacokinetics of certain drugs by influencing drug absorption, distribution, metabolism, and elimination[62]. A summary of the physiological changes that occur in pregnancy and their effects on drug pharmacokinetics is discussed below.

1.4.1 Cardiac Function

Perhaps the most dramatic are the cardiovascular changes occurring in pregnancy[63-65]. The body must adapt to meet fetal and placental cardiovascular demands. If inadequate changes occur, harmful conditions including preeclampsia and intra-uterine growth restriction (IUGR) can arise. A steep rise in cardiac output occurs in the first trimester, increasing 30-40% by the 8th week of gestation[63]. Cardiac output peaks around 10 weeks' gestation at 50% of non-pregnant cardiac output[65]. Notably, in twin pregnancy cardiac output is up to 15% higher than in singleton[64]. Along with increased cardiac output, heart rate increases throughout pregnancy by 10-20 bpm[64]. Peripheral resistance is decreased due to a number of factors, including the effect of progesterone on vascular tone and the remodelling of spiral arteries, which results in sac-like arteries with very little resistance[64].

1.4.2 Renal Function

Decreased vascular resistance also occurs within the kidneys, where vasodilation of arteries occurs. This results in a 50% increase in renal blood flow and glomerular filtration rate, which occurs in the first trimester[62,66]. Serum creatine, urea, and uric acid levels are decreased as they are filtered out of the blood. Also, at the beginning of pregnancy, the components of the renin-angiotensin-aldosterone system (RAAS) are increased, which can be attributed to increased vasodilation and decreased blood pressure[67]. Control of the RAAS is important for salt balance, which can be dangerous to the mother and fetus if dysregulated. In addition to the renin released by the kidneys, a further increase in renin is seen due to release by the ovaries and decidua[67]. These alterations can affect the exposure and distribution of drugs.

1.4.3 Respiratory Changes

Due to the increase in cardiac output, pulmonary blood flow and hyperventilation increase[68]. Additionally, tidal volume increases. These changes are driven by a variety of hormonal and chemical signals[62,68]. Progesterone increases sensitivity to carbon dioxide within the respiratory centres of the brain and relaxes the muscles in the airway causing bronchodilation[68]. As mentioned previously, pregnancy is classified as an inflammatory state, with cytokine profiles shifting throughout pregnancy. Important mediators of cytokine signalling include prostaglandins. Prostaglandins E_1 and E_2 can also contribute to bronchodilation[68]. However, as pregnancy progresses PCO_2 and residual volume decreases. The growing uterus causes the resting position of the diaphragm to move up approximately 5 cm, adding pressure to the lungs[69]. Pregnancy-related respiratory changes can affect the delivery of inhaled drugs.

1.4.4 Gastrointestinal System

Increasing levels of progesterone are believed to be responsible for changes to the gastrointestinal system[70]. The rate of gastric emptying and intestinal motility can be decreased by upwards of 50%, mainly due to the effects of progesterone on smooth muscle contractility[70]. This may affect the onset of action for drugs which are primarily absorbed in the intestine but can lead to increased drug exposure due to time spent in the intestinal tract[62]. Additionally, acidic gastric secretions decrease by approximately 40% while mucous secretions increase, raising the pH of the stomach[62]. This is coupled to increased gastric pressure, which may result in reflux into the esophagus. Increasing pH leads to changes in ionization of drugs that are weak acids or bases and may affect absorption.

1.4.5 Blood Volume and Plasma Proteins

By the end of pregnancy, total body water has increased by 8 L which includes increases in blood volume and extracellular fluid, as well as the addition of the fetal circulation and the fluid filled amniotic sac[71]. The addition of the fetal-placental unit and increase in body fluids and fat affects the disposition of many drugs. A such, the apparent volume of distribution (V_d) will be altered for these drugs[62,71]. Plasma volume increases by 50% by term, however, plasma protein production does not increase at the same rate, resulting in hypoalbuminemia[72]. For drugs which are highly protein bound, this will result in a greater fraction of unbound drug. Therefore, a higher proportion of drug may be free to leave the plasma and distribute into tissues[73]. While rare, this may result in toxicity for highly protein bound drugs.

1.4.6 Hepatic Metabolism

The changes occurring in maternal hepatic metabolism are not fully elucidated, but a number of important drug metabolising enzymes are known to have altered activity. In pregnancy, the activity of most hepatic enzymes increases, resulting in faster elimination[74]. Both phase I (reduction, oxidation, and hydrolysis) and phase II (conjugation) are affected. With respect to phase I, the cytochrome P450 (CYP) enzymes are reasonably well characterised in pregnancy. CYP1A2, CYP2B6, CYP2C9, and CYP2D6 are induced, but CYP2C19 activity is suppressed[74]. Activity of these enzymes may change at different times throughout pregnancy, and in the case of CYP2D6, the activity is 200% higher by the end of the third trimester[74]. The phase II metabolism enzyme family of UDP glucuronosyltransferases (UGTs) generally shows increased activity in pregnancy[75]. This can have significant effects on the metabolism and clearance of drugs metabolised by UGTs. Lamotrigine clearance is significantly increased in pregnancy, as well as morphine, which is metabolised into glucuronide conjugates[75]. Overall, for drugs metabolized through these pathways, pharmacokinetic changes are expected to occur, and the dose of drug may need to be adjusted in order to achieve the desired therapeutic effect.

1.4.7 Placental-Fetal Compartment

The effects of the placental-fetal compartment are also an important consideration for the ADME of certain drugs. The placenta, fetus, and amniotic sac present an entirely new compartment for drug distribution. Additionally, the difference in environment on the maternal and fetal sides of the placenta present the possibility of differential drug partitioning or “drug-trapping” on either side of the placenta[76]. The fetal compartment is slightly more acidic, and ion-trapping can occur as a result of the drugs acid:base properties[76]. This is especially relevant for weakly acidic or

basic drugs, particularly the NSAIDs aspirin, ibuprofen, and diclofenac which are commonly used OTC drugs[76].

1.4.8 Placental Metabolism

In addition to creating a new compartment for drug distribution, the placenta is an active metabolic organ, involved in both phase I and phase II metabolism[77]. Placental metabolism of drugs and other xenobiotics may limit fetal exposure but can also increase toxicity of molecules. Indeed, the thalidomide disaster of the 1960s changed our perception of the placenta as a protective barrier that prevents exposure of maternally circulating xenobiotics[78]. It is now well understood that xenobiotics can cross the placenta and undergo metabolism within the placenta before reaching the fetus. The major xenobiotic and drug metabolizing enzymes in the placenta are discussed below.

1.4.8.1 Cytochrome P-450 Family

The cytochrome P-450 (CYP) enzymes are a superfamily of monooxygenases containing a heme cofactor that are primarily involved in phase I metabolism. They are involved in metabolic processes throughout the entire body and use oxygen and electrons to oxidize substrates. In addition to the CYP isozymes CYP19 and CYP17 which are involved in steroid biosynthesis, the placenta expressed CYP isoforms involved in the metabolism of drugs and xenobiotics[79,80]. The expression of CYP enzymes in the placenta change throughout pregnancy and decline towards the end of gestation[80]. This is believed to be a protective mechanism as the fetus is most susceptible to teratogens in early pregnancy. In the first trimester placenta, CYP1A1, 1A2, 1B1, 2C, 2D6, 2E1, 2F1, 3A4, 3A5, 3A6, 3A7, and 4B1 have been identified, though not all have been

found to be active[81]. At term, CYP1A1, 1B1, 2E1, 2F1, 3A3, 3A4, 3A5, 3A5, and 4B1 have been identified[79]. Interestingly, maternal drug use and smoking has been shown to maintain the expression of specific CYP isoforms that would normally not be expressed in late pregnancy, so these trends of expression may not always be true[79].

1.4.8.2 Uridine-diphosphate glucuronosyltransferases (UGTs)

Phase II metabolism involves the conjugation of the substrate to another molecule, usually to increase polarity. While phase I metabolism (oxidative, reduction, and hydrolysis reactions) tends to introduce or expose functional groups on the substrate to increase polarity of the compound, it also reveals potential sites for conjugation reactions that occur in phase II. Uridine-diphosphate glucuronosyltransferases (UGTs) are membrane bound proteins and conjugate a uridine diphosphate glucuronic acid (UDPGA) moiety to xenobiotics[82,83]. The UGTs are present in the villous placenta throughout pregnancy. In the first trimester placenta, UGT1A and UGT2B isoforms (UGT2B4, 2B7, 2B10, 2B11, 2B15, and 2B17) have been identified[83]. The level of interindividual variability of these enzymes in high and can be affected by maternal drug use or smoking[83]. At term, UGT1A1, 1A4, 1A6, and 1A9 are detected, and the UGT2B isoforms remain active[82,84].

1.4.8.3 Sulfotransferases (SULTs)

Sulfotransferases (SULTs) catalyze the conjugation of a sulfo group to a substrate. This reaction produces a polar ester of sulfuric acid which promotes excretion. Many substrates of sulfation can also be glucuronidated, but SULTs also play an important role in the sulfation of steroids and catecholamines in the placenta and other tissues[85]. Of the 13 known sulfotransferases in humans,

several have been shown to be present in the placenta, including SULTs 1A1, 1A3, 2A1 and 2B1b isoforms[86,87].

1.5 Assisted Reproductive Technologies (ART)

Assisted reproductive technologies (ART) include procedures in which both oocytes and sperm are handled outside of the uterus with the aim of achieving a successful pregnancy[88]. Therefore, ART does not include procedures such as intrauterine insemination where only male gametes are handled. There are two technical types of ART that are primarily used: *in vitro* fertilization (IVF) and intracytoplasmic sperm injection (ICSI)[88]. While the increases observed in use of ART can partially be attributed to declining fertility in developed countries over the past three decades, it is important to note there are other reasons for individuals accessing ART. Increasingly, ART is being accessed by LGBTQIA+ individuals (with and without surrogacy), as well as single women looking to start a family[9]. Since the first IVF baby was born in 1978[89,90], ART has allowed thousands of people to have children, now accounting for 2% of all births and 18% of multiple births in North America[8].

When used to achieve pregnancy ART requires a number of clinical interventions, with treatment spanning weeks or months until pregnancy is established. A single ART cycle includes multiple steps, as follows. First, drugs are administered to suppress the natural menstrual cycle and initiate the growth of multiple follicles. After monitoring follicle development and determining they have reached an appropriate stage, a drug is given to induce ovulation and the oocytes are collected with ultrasound guidance and prepared for fertilization[88]. Following one of the fertilization methods described below, embryos can be transferred fresh or cryopreserved.

1.5.1 *In vitro* Fertilization

Conventional IVF involves the spontaneous cavitation of gametes in a dish. The oocyte is incubated in a dish with sperm at a ratio of approximately 75,000:1 and left to fertilize on its own[91]. Initial techniques in the 1970s and 1980s were less successful, but over the decades with improvement in equipment and technique, IVF provided favourable results[88,91]. This procedure has been recommended for use in female factor infertility[88]. Some favour this technique because it involves less outside manipulation of the gametes and resulting embryo. Compared to ICSI (discussed below), the zona pellucida is not manipulated in the process. Fertilized embryos are then cultured from 3-5 days and subsequently transferred to the uterus, or frozen to be transferred at a later date.

1.5.2 Intracytoplasmic Sperm Injection

In ICSI, a single sperm is injected into an oocyte using a needle[92]. Traditional IVF was found to be not as effective for people who had a poorer quality sperm sample, thus ICSI was developed in the 1990s[92]. ICSI was recommended in the case of a sperm related infertility, for example, a low sperm count or abnormal shape or motility, when eggs were not fertilized by traditional IVF, or previously frozen eggs were being used. However, use of ICSI in IVF cycles has increased dramatically since it led to the first successful birth in 1992[93]. Initially only used for male factor infertility, the utilization of ICSI has increased to around 75% of IVF cycles in the United States, but the justification of this practice has been debated[94]. Few studies provide evidence that ICSI is more effective than traditional IVF for non-male factor infertility. The benefits of ICSI in older patients (older than 37 years of age), is often debated, but in women below 37 randomized controlled studies have shown no benefit. One of the largest concerns over the prevalence of

ICSI usage is that it prevents natural sperm selection, acrosome reaction, and zona pellucida penetration, the outcomes of which remain unknown. Questions have also been raised over the effect of ICSI on the epigenetics of the embryo. However, until further studies can provide stronger evidence that conventional IVF should be used instead of ICSI, it will likely remain a prevalent procedure in fertility treatment.

1.5.3 Adverse Pregnancy Outcomes in ART

Assisted reproductive technologies are considered safe and effective methods to conceive. While ART has helped thousands of people conceive and have healthy children, it is well documented that both technical types of ART are associated with increased risks and more often require obstetric interventions than naturally conceived pregnancies[11]. Initially, one of the major concerns surrounding ART procedures was the frequency of twin, triplet, and higher order pregnancies that accompany the transfer of multiple embryos in the uterus. Despite recommendations to limit the number of embryos transferred, in many countries there are no guidelines regarding or monitoring the number, and physicians are free to decide how many embryos are transferred[95]. Twin and higher order pregnancies are accompanied with several increased risks in addition to the risks associated with ART[8]. A summary of the relative risks for pregnancy complications associated in ART is presented in Table 1.1 and discussed further below.

1.5.3.1. Placental Pathologies

When considering the underlying reasons for birth complications, the role of the placenta is often overlooked by physicians and researchers alike. However, several placental abnormalities have

been noted in ART[6,7,10]. Rates of placenta previa, placental abruption, placental accreta, and premature rupture of the membranes (PROM) are higher in ART pregnancies compared to unassisted pregnancies[10]. Additionally, the size and weight of the placenta is increased in ART pregnancies and there is a higher incidence of abnormal cord insertion[96]. In both livestock and mice, increased placental weight has been associated with neonatal mortality. Previously in the Collier laboratory, overweight and overgrowth of placentas was observed in a mouse model of ART, providing the first evidence of increased placental weight in ART[97]. Results in humans were confirmed by a population-based study in Norway which showed that placentas from ART pregnancies had significantly higher weights than normal pregnancies[96]. Additionally, there have been reports of increased incidence of hematomas and a reduction of villous trees in ART pregnancies[98]. It has been suggested that placental overgrowth may be a result of aberrant expression of imprinted genes involved in the process of placentation, but the mechanisms of how ART affects placentation remain unknown[99]. Epigenetic changes influencing methylation and gene expression have also been proposed. In ART pregnancies, differential DNA methylation was observed following IVF, both on imprinted and non-imprinted genes[99]. More work is required to understand the epigenetic changes observed in the placenta, and if the culturing and fertilization conditions are responsible.

1.5.3.2. Maternal Complications in ART

People who conceive using ART are more often faced with the need for obstetric interventions including induced labour and the need for emergency caesarian section[6,7]. Additionally, ART pregnancies have higher rates of pre-eclampsia and gestational diabetes, posing significant risks to the mother. However, ART is more commonly used in older women, who are more likely to

develop these complications in pregnancy. Maternal effects of preeclampsia can continue long after giving birth, with complications such as continued high blood pressure, increased risks of cardiovascular disease, and stroke[100]. During labour, higher rates of blood loss have been observed in ART pregnancies as well, which may occur as a result of higher preterm birth rates or preeclampsia[7].

1.5.3.3 Fetal Complications in ART

Fetal specific risks have also been noted in ART. This includes rates of premature births, small-for-gestational age babies, congenital abnormalities, and perinatal mortality[6,12,101]. Premature births are classified as births that take place more than three weeks before the expected due date. Premature infants have higher rates of admittance to the neonatal intensive care unit (NICU) and there are dramatic increases in perinatal morbidity and mortality due to developmental delays[12]. In addition to negative effects on the mother, preeclampsia can cause oxygen and nutrient deficiencies due to placental insufficiencies. This in turn leads to the fetus altering its blood flow to maintain adequate blood supply to vital organs such as the brain and adrenals[100]. However, these adaptations cannot be sustained indefinitely and unless oxygen and nutrient delivery returns to normal levels, it will lead to permanent changes in structure and metabolism of the fetus. These complications can increase risks later in life for cardiovascular disease.

There is also evidence of increased rates of congenital birth defects (non-chromosomal defects), including cardiac abnormalities, urogenital system abnormalities, and musculoskeletal disorders[101,102]. For example, in males conceived using ICSI there were higher rates of urogenital defects and hypospadias[102]. A systematic review of IVF/ICSI births found a 30-40% increased

risk in birth defects compared to natural conception[103]. Lastly, emerging evidence shows increased incidence of imprinting disorders and cancers associated with imprinted genes, including Beckwith-Wiedemann syndrome and retinoblastoma[104]. However, the results of these studies, and other studies investigating birth defects, are not always consistent and more follow up is needed.

Pregnancy Complication	Odds Ratio (95% Confidence Interval)
Bleeding in early pregnancy	4.59 (4.08-5.15)[6]
Preeclampsia	1.63 (1.53-1.74)[6]
Placental abruption	2.17 (1.74-2.72)[6]
Placental previa	3.65 (3.15-4.23)[6]
Premature rupture of the membranes	2.54 (2.34-2.76)[6]
Caesarian section	1.38 (1.32-1.43)[6]
Labour induction	1.37 (1.29-1.46)[6]
Congenital abnormalities	1.21 (0.63-2.62)[12]

Table 1.1 Risks of pregnancy complications associated with ART.

1.6 Analgesic Use in Pregnancy

The general consensus is that all drugs should be avoided in pregnancy unless it is known that the benefits to the mother outweigh the risks. However, the reality is that drug use in pregnancy is very common, both from prescribed and over-the-counter (OTC) drugs, and the majority of women will take at least one drug while pregnant[105,106]. Among the most commonly used groups of drugs are analgesics. A multicentre study from the United States reported analgesic use in first trimester is incredibly common, with 56% of people reporting taking acetaminophen, 24% taking ibuprofen, 5.3% taking aspirin, and 4% taking naproxen[106]. Prescription opioids were also reported, with approximately 1% of people reporting having used hydrocodone, oxycodone, or codeine[106]. Analgesic use in pregnancy is common world-wide, as a subsequent study with records from Europe, North America, South America, and Australia found 81% of women take medications in pregnancy and 50% reported OTC analgesic use[105]. Analgesics can be divided

into different classes with differing guidelines for use in pregnancy. The main classes are outlined below. We have published a more detailed review of analgesic use in pregnancy, what follows is a brief summary[107].

1.6.1 Nonsteroidal Anti-inflammatory Drugs

Nonsteroidal anti-inflammatory drugs (NSAIDs) include some of the most commonly used OTC drugs, including ibuprofen, naproxen, indomethacin, diclofenac, and aspirin. NSAIDs relieve pain and inflammation through inhibition of the cyclooxygenase (COX) enzymes, thereby reducing the production of prostaglandins and leukotrienes[108]. NSAIDs act on both the constitutively expressed COX-1 and the inducible form COX-2[109].

NSAIDs are contraindicated in the first and third trimesters[110]. In early pregnancy, it has been suggested that they can affect implantation and placentation due to an alteration in blood flow to the uterus[111]. Additionally, prostaglandins are included in the group of mediators required for successful implantation. In the third trimester when parturition is approaching, NSAIDs have been shown to have detrimental effects including alteration of uteroplacental blood flow, fetal pulmonary hypertension, nephrotoxicity, and premature closure of the *ductus arteriosus,* which connects the two sides of the fetal heart as the lungs do not function in gas exchange in utero[111-114].

1.6.2 Non-Opioid Agents

Acetaminophen and metamizole are analgesics that fall into the category of non-narcotic agents. Their mechanisms of action are not fully understood; however, they exhibit analgesic and

antipyretic effects with minimal anti-inflammatory effects. There is evidence that acetaminophen interacts with COX enzymes, thereby affecting thromboxane synthesis[115,116]. Additionally, studies have shown metabolites of acetaminophen may interact with the transient receptor potential cation channel or the endogenous cannabinoid system[117,118]. Metamizole may act similarly, through COX-3 and the endogenous cannabinoid system[119].

Metamizole has been banned in many countries due to increased risk of blood disorders, which may affect the fetus[120]. As such, it is not recommended for use in pregnancy. Acetaminophen is the first-line analgesic drug, with little to no evidence of adverse obstetric outcomes[121,122]. There have been no recommendations on adjustments to acetaminophen dosage or dosing interval. The drug does cross the placenta and can be measured in the fetal compartment[76]. Despite initial fears it may cause liver toxicity to the fetus, it does not appear to be the case. High levels of sulfotransferase have been proposed as a mechanism to compensate for lack of glucuronidation in the fetal liver[123]. More recently, attention has been paid to potential germline effects of acetaminophen use in pregnancy. In a study by Hurtado-Gonzales *et al.* using both rodent and human xenograft methodologies, acetaminophen usage led to reduced gonocyte number in the testes or ovaries of the offspring[124]. The extent of the effects in humans needs to be determined but raises concern surrounding the frequency with which acetaminophen is used in pregnancy.

1.6.3 Opioids

Opioids act on receptors in the central nervous system (CNS), namely mu, kappa, and delta opioid receptor subtypes, to reduce the perception of pain[125]. Opioids are powerful pain relievers and play crucial roles in pain management, however their addictive nature and withdrawal

potential are particularly important to consider in pregnancy. Opioid use is generally short term, and physicians will review the use of opioids in pregnant patients and evaluate benefits vs. risks[126]. A review of pregnant patients in the United States found 14% of the cohort was prescribed an opioid[126]. Opioids pose a risk of neonatal abstinence syndrome following birth, and infants of mothers using opioids must be monitored for withdrawal symptoms, which often require treatment and extensive monitoring. Methadone and buprenorphine, partial agonists of opioid receptors, are effective treatments for opioid withdrawal, and can be used to treat withdrawal in pregnancy[127]. Opioids also play a specific role in pregnancy in that they are commonly used during labour to manage pain[128]. Morphine is most commonly used for early labour analgesia and fentanyl during active labour, replacing remifentanil and meperidine which are not recommended due to toxicity concerns[128-130]. Use during labour may increase the risk of fetal bradycardia or apnea of the infant, however these drugs are generally considered safe[131].

1.6.4 Centrally Acting Analgesics

While opioid analgesics are considered centrally acting, they are generally considered separately from others based on their structure and mechanism of action. Tapentadol and tramadol are also considered centrally acting analgesics[132]. Both act primarily in combination as agonists of the mu opioid receptor and antagonists of serotonin and norepinephrine reuptake, with minimal inhibitory effects on muscarine, N-methyl-D-aspartate, and serotonin 2C receptors[132-134]. Neither drug has been extensively studied in pregnancy and are usually avoided in favour of other analgesics. Tapentadol has the potential to cause dependence and neonatal abstinence syndrome, and tramadol freely crosses the placenta which can lead to fetal complications[135,136].

1.6.5 Combination Analgesics

Combination analgesics are formulations which include more than one analgesic, or one analgesic in combination with a non-analgesic compound[137]. The non-analgesic compound is often caffeine, which is believed to enhance analgesic efficacy[137,138]. However, the evidence of these claims is not fully supported. As with most clinical trials, pregnant women are not included, providing limited information on how the drug should be used in pregnant patients. Because of this, the analgesic in its individual formulation is used in favour, if its safety profile is defined.

1.6.6 Triptans

Triptans act on 5HT1B, D, and F receptors, which are found on neurons[139,140]. Originally developed to constrict cerebral blood vessels, they were found to be effective in the treatment of migraines. Women are more affected by migraines than men, however the hormonal changes that occur in pregnancy can decrease both the frequency and intensity of migraines[141]. Some women, however, will still experience migraines or cluster headaches while pregnancy and may use triptans as treatment[142]. Evidence on the safety profile of triptans in pregnancy is lacking, but there are reports on sumatriptan use in pregnancy. Sumatriptan was the first triptan to be approved for migraine treatment. No significant effects of triptans were found in reference to malformation or miscarriage, but there may be increased risk of low birth weight or pre-term delivery[143,144]. Overall, most studies do not indicate high risk or toxicity from triptan use in pregnancy[144].

1.7 Summary and Research Aims

In summary, pregnancy is a complex physiological state which requires the precise control of inflammatory responses, oxidative stress responses, steroidogenesis, and steroid metabolism. A dysfunction or alteration is any of these responses can lead to complications in pregnancy and parturition. Additionally, pregnancies which utilize assisted reproductive technologies to conceive are at increased risk for several obstetric, maternal, and neonatal complications, but how ART affects these systems is currently unknown. The placenta, which connects the maternal and fetal circulations, is a vital organ in pregnancy, and has inflammatory, antioxidant, and steroidogenic functions. As a disposable organ, it provides an easily accessible and valuable tissue for studying the fetal environment and how it relates to pregnancy outcomes. As ART becomes increasingly common, a better understanding of adverse pregnancy outcomes and how they can be mitigated will be clinically important. Following the results of previously performed murine ART studies in our laboratory[97,145,146], we hypothesized that ART affects placental inflammatory, oxidative stress, and steroidogenic/steroid metabolism processes within the placenta. Additionally, these responses are intricately linked and can be altered by environmental factors or drug use in pregnancy. Therefore, we incorporated a study on the use of NSAIDs in pregnancy and their effect on inflammation and pregnancy outcome. As one of the most commonly used drug classes in pregnancy and a drug class being increasingly used off-label in ART procedures, without concrete evidence of safety and effectiveness, we aimed to determine their prevalence of use and safety in pregnancy.

The specific aims of this work were:

Aim 1 A) To develop and validate a novel UHPLC-MS/MS method to detect NSAIDs in human samples, and **B**) To use this method to determine the prevalence of NSAID use in the third trimester and identify associations with inflammatory markers and pregnancy outcomes.

Aim 2) To determine the effects of ART on placental inflammatory and angiogenic markers in terms of pregnancy outcome.

Aim 3) To determine the effects of ART on the placental antioxidant defense network and how it affects pregnancy outcome.

Aim 4) To investigate the effects of ART on steroid levels, steroidogenesis, and steroid metabolism with respect to pregnancy outcome.

Chapter 2: Development and Validation of a Novel UHPLC-MS/MS Method to Detect Nonsteroidal Anti-inflammatory Drugs (NSAID) in Human Samples, and Evaluation of the Prevalence of NSAID Use Close to Time of Birth in Pregnant Women

2.1 Introduction

It is a generally held view by clinicians and researchers that medication should be avoided in pregnancy unless the benefits outweigh the risks. Despite this view, drug use in pregnancy is quite common, both accidental usage before a person knows they are pregnant and intentional usage during pregnancy. Further, many women are prescribed drugs during pregnancy by their physicians[147]. Most drugs will cross the placenta to some extent, thereby exposing the fetus to the drug and leading to the potential for adverse effects[148]. Because pregnant patients are excluded from drug trials, they, and their physicians, are left with little guidance on the safety of specific drugs in pregnancy and this information is constantly changing as new information emerges[149].

One of the most common drug classes used in pregnancy are analgesics, both over-the-counter (OTC) and prescription[106]. Data from the United States reported first trimester drug use in over 5000 participants, and found 56% of respondents used acetaminophen, 24% used ibuprofen, 5.3% used aspirin, and 4% used naproxen[106]. Three out of these four common OTC analgesics are nonsteroidal anti-inflammatory drugs (NSAIDs). Outside of North America, the profile changes slightly, likely due to differing availability and the need to speak to a pharmacist before purchasing NSAIDs in certain countries[105]. A multinational study of nearly 10,000 records found that about half of pregnant people used OTC analgesics, with acetaminophen accounting for 47% and NSAIDs accounting for 4.5%[105].

2.1.1 NSAIDs

Nonsteroidal anti-inflammatory drugs are analgesics with anti-inflammatory and antipyretic effects[109]. The first NSAID, salicylic acid, is one of the oldest analgesics in the world. Originally obtained from natural sources such as willow bark, it was used for centuries to relieve pain, dating back to the ancient Egyptians[150]. The identification of the active compounds in willow bark led to the synthesis of acetylsalicylic acid (aspirin) in 1853 and again in 1897 by the Bayer Company[151]. The synthesis and formulation of aspirin led to the development of other drugs with similar structures, including ibuprofen, naproxen, and indomethacin, which became known collectively as NSAIDs. These drugs became commonly used for pain, inflammation and fever, eventually becoming one of the most used drug classes in the world[152]. For decades, NSAIDs were used without a clear understanding of their mechanism of action. However, in 1971 John Vane proposed a mechanism of action for NSAIDs[109]. He noted a decrease in the synthesis of prostaglandins and hypothesized that NSAIDs function by inhibiting the action of the cyclooxygenase enzymes[109]. Subsequent studies confirmed these findings, making this the accepted mechanism of action[108].

2.1.2 Mechanism of Action of NSAIDs

NSAIDs function by inhibiting the enzymes cyclooxygenase-1 (COX-1) and cyclooxygenase-2 (COX-2), blocking the formation of prostaglandins and thromboxanes which mediate the process of inflammation[108] (Figure 2.1). These enzymes catalyze the reduction of arachidonic acid to the intermediate prostaglandin G_2 and more stable prostaglandin H_2 (Figure 2.1). Prostaglandin H_2 can be further modified to form other prostaglandins including PGI_2, PGE_2, PGF_{2a}, and thromboxane A2 which go on to exert a wide range of effects through G protein-coupled

receptors (GPCRs)[153]. The COX enzymes have two separate but linked active sites. They share 60% sequence identity, but their main difference lies in their expression[154,155]. Cyclooxygenase-1 is constitutively expressed and widely distributed in most tissues throughout the body[154]. On the other hand, COX-2 is inducible, activated by inflammatory stimuli and is well characterized in the brain, kidney, female reproductive tract, and the placenta[154]. This discovery led to the hypothesis that COX-2 is involved in pathophysiologic states, and COX-1 is required for homeostasis[156]. The majority of NSAIDs are nonselective and inhibit both COX-1 and COX-2, but selective COX-2 inhibitors exist[157]. Although they were initially developed to prevent the gastrointestinal side effects of non-specific NSAIDs, selective COX-2 inhibitors did not prove to be as valuable as once thought, instead causing cardiotoxic effects leading to most being withdrawn from the market[154,157]. Certain selective COX-2 inhibitors are still prescribed, mainly for treatment of osteoarthritis and rheumatoid arthritis[157].

2.1.3 NSAID Use in Pregnancy

Despite the high rates of NSAID use known in pregnancy, non-Aspirin NSAIDs are not recommended for use throughout most of pregnancy[105,106]. At different stages of pregnancy and parturition, they can have specific detrimental effects on the fetus and placenta. Despite risks being recognized by medical professionals, many women are unaware of these risks and the widespread availability of NSAIDs OTC may play a role in this false perception of safety. Aspirin is typically a prescribed medication in pregnancy and should be considered separately from non-Aspirin NSAIDs.

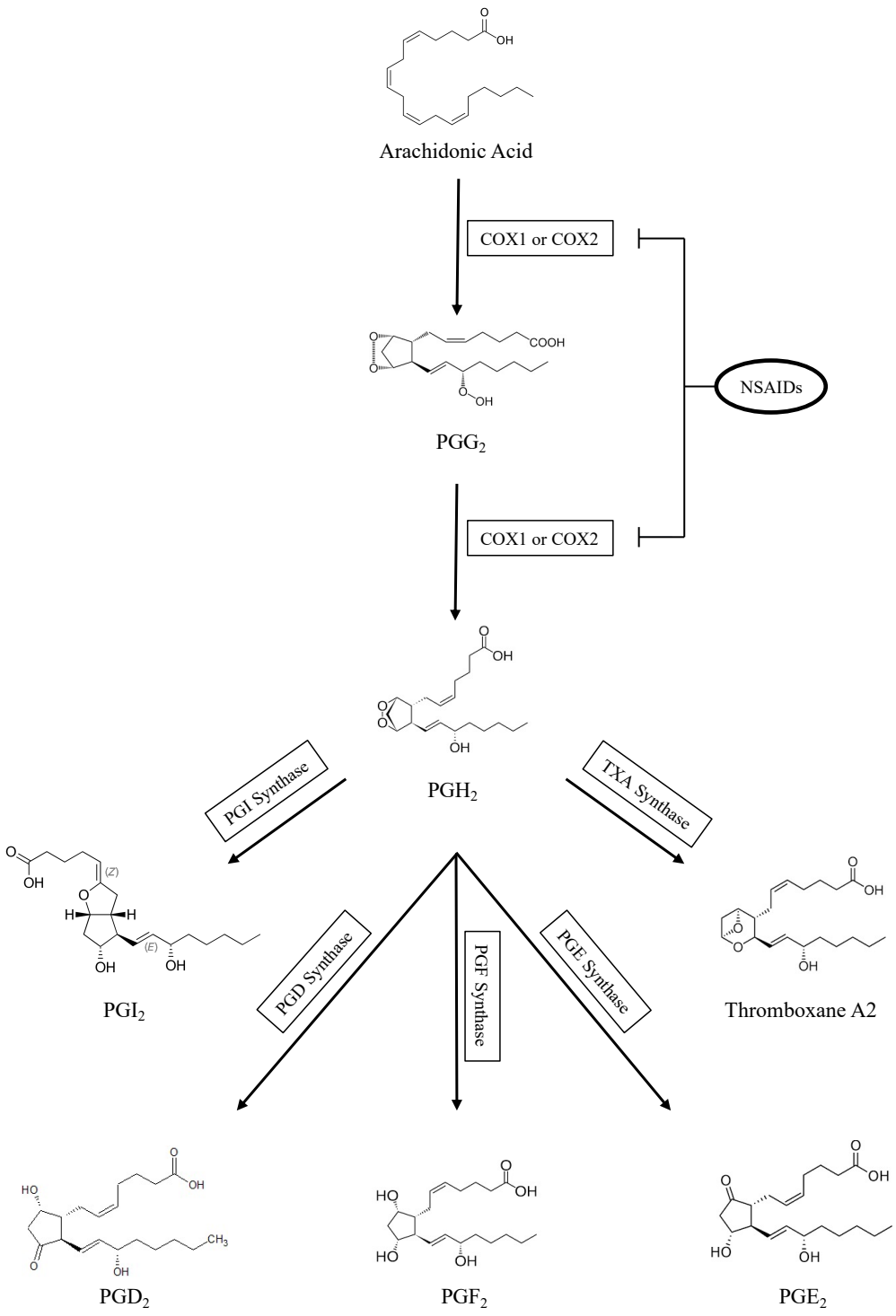

Figure 2.1 Overview of prostaglandin and thromboxane synthesis from arachidonic acid, illustrating NSAID mechanism of action.

Enzymes outlined in boxes. Original figure, created in Microsoft PowerPoint (2021).

2.1.3.1 NSAID Use in the First Trimester

In the first trimester of pregnancy, NSAID use is generally cautioned. At the time of implantation and placentation, it was believed that the effect of NSAIDs on uterine blood flow may cause complications and increase the risk of miscarriage[106]. Prostaglandins are required for successful implantation; therefore, the inhibition of their synthesis can have negative effects on implantation. Reviews of the current data suggest that NSAID exposure in first trimester is associated with increased risks of gastroschisis (Odds ratio: 2.37; 95% confidence interval: 1.44-3.88)[158] and cardiac malformations (Odds ratio: 1.86; 95% confidence interval: 1.32-2.62)[159,160]. Additionally, a dose-response relationship was observed for NSAID use around the time of conception and risk of miscarriage[161]. However, conflicting evidence exists in the literature and these claims are not always supported[159].

2.1.3.2 NSAID Use in the Second Trimester

In late first trimester and the second trimester, low dose aspirin has been proposed as a treatment for preventing or delaying the onset of preeclampsia[162,163]. This treatment has been gaining in popularity since evidence was first shown in 1985[163]. The American College of Obstetricians and Gynecologists recommends the use of low dose aspirin (81 mg/day) for women at a high risk of developing preeclampsia. Treatment should begin between 12-28 weeks' gestation and continue until delivery[162]. However, prophylactic use of low-dose aspirin is not recommended for other uses that have been proposed, including prevention of IUGR, prevention of spontaneous preterm birth, or prevention of early pregnancy loss[162]. For short duration treatment of moderate pain or inflammation, aspirin and other NSAIDs are generally believed to be safe in the second trimester and OTC use is not advised against[164].

2.1.3.3 NSAID Use in the Third Trimester

In the third trimester of pregnancy, when the placenta and fetus are much more developed, NSAID use is cautioned after 32 weeks' gestation because they can affect blood flow to the uterus and placenta, limiting oxygen and nutrient delivery to the fetus[111]. Other risks include increased incidence of oligohydramnios and persistent pulmonary hypertension of the neonate[112,165]. A further prominent risk includes premature closure of the ductus arteriosus[166]. The ductus arteriosus is a shunt present in the fetal circulation that diverts about 90% of the right ventricular output into the thoracic aorta, bypassing the lungs as they are not the location of gas exchange *in utero*. Closure of this duct can lead to progressive right heart dysfunction or heart failure[114]. This risk of this is highlighted by the use of indomethacin as a treatment for patent ductus arteriosus in neonates[167]. Prostaglandins play a significant role in the onset and progression of natural labour, and the inhibition of their formation by NSAIDs in late pregnancy can cause prolonged gestation or prolonged labour; under clinical supervision, non-aspirin NSAIDs may be used as tocolytics in the event of premature labour[110]. Finally, more recent evidence has highlighted the risks of nephrotoxicity to the fetus when NSAIDs are used close to birth, and severity increased the closer ingestion was to labour[113,165,168-170] . Fetal renal impairment can lead to oligohydramnios, which can increase the risks of miscarriage or premature birth and increase the risk of damage to fetal organs, but more data is required to define relative risks of these claims[113,170]. Overall, the use of NSAIDs in the third trimester should be cautioned, weighing benefits of treatment vs. risks of adverse events.

2.1.3.4 Off-label Aspirin Use in Assisted Reproduction

In the past 20 years, aspirin has been used off-label in pregnancy and assisted reproduction[171]. In contrast to the first trimester risks discussed above, it was proposed that low-dose aspirin could actually improve uterine blood flow, thickening the endometrium and improving rates of implantation[172]. Positive results were found in women with antiphospholipid syndrome who experienced recurrent pregnancy loss, leading to its use in ART to promote implantation and improve pregnancy rates[172]. Despite inconclusive results, the treatment has become more widespread during the embryo transfer stage but is considered a controversial treatment amongst fertility specialists[173,174]. Overall, this use of low-dose aspirin in pregnancy cannot be definitively recommended due to lack of evidence[174].

Considering the prevalence of NSAID use in pregnancy, the aim of this project was to develop a screening method for five of the most common NSAIDs which could be applied to human reproductive tissues. This method would be used to determine a more realistic insight into the prevalence of NSAID use in human pregnancy, as self-reported studies can be unreliable, especially in pregnancy[175]. With the number of tissues available for screening and the accompanying clinical chart information, we also hoped to uncover association of NSAID use with adverse pregnancy outcomes that have not been previously reported. With the emerging use of NSAIDs for treatment of preeclampsia and aiding with implantation in ART, it is important to revisit the safety profiles of these drugs to ensure unexpected side effects do not occur. A summary of the following project was published as an article in Toxicology Reports, titled "Detection and quantitation of non-steroidal anti-inflammatory drug use close to the time of birth using umbilical cord tissue"[176].

2.2 Materials and Methods

The following chemicals, solvents, and materials were used: diclofenac, ibuprofen, indomethacin, naproxen, salicylic acid, and aceclofenac (Sigma-Aldrich, Oakville, Ontario, Canada), Bio-Rad "drug-free" lyophilized pooled serum (Bio-Rad Clinical Diagnostics, Irvine, CA, USA), single donor human serum (Innovative Research, Novi, MI, USA), HPLC-grade methanol (Fisher Scientific, Ottawa, Ontario, Canada), HPLC-grade water (Fisher Scientific, Ottawa, Ontario, Canada), Tris base (Amresco, Solon, Ohio, USA 12 x 75 mm borosilicate glass tubes (Fisher Scientific, Ottawa, Ontario, Canada), Tissue Tearor^{TM} (Daigger Scientific, Vernon Hills, IL, USA).

2.2.1 Pharmacokinetic Analysis

A pharmacokinetic analysis was first performed to determine the expected concentrations of the five NSAIDs of interest in maternal plasma. A direct pharmacokinetic analysis for the concentration of each NSAID in umbilical cord cannot be performed due to the lack of information on the transfer of NSAIDs across the placenta and distribution into the umbilical cord. However, we hoped that establishing an estimate of expected concentrations in maternal plasma would provide some context to the levels found in umbilical cord lysate. The pharmacokinetic parameters for the NSAIDs described in the literature are presented in Table 2.1. The analysis was performed to determine the levels of NSAIDs expected to be present in maternal blood after a single oral dose (Equation 2.1) and after repeated dosing such that the drug reached steady state (Equation 2.2). The concentration of drug in the blood expected following five half-lives (representing elimination) was also calculated for each of these dosing situations. Additional analysis for up to ten half-lives was performed to determine the window of

detection using our UHPLC-MS/MS method. Analyses were performed for both low and high dosages recommended for analgesia of each drug, at both low and high average weight estimates in pregnancy.

Drug	V_d (L/kg)	F	Low Dose (mg)	High Dose (mg)	$T_{1/2}$ short (hr)	$T_{1/2}$ long (hr)
Salicylate	0.2	0.68	60	1200	2	20
Diclofenac	1.4	0.65	25	100	1	2
Ibuprofen	0.12	1	50	800	1.8	2
Indomethacin	0.955	1	25	75	4.5	6
Naproxen	0.16	0.95	250	500	12	15

Table 2.1 Pharmacokinetic parameters for NSAIDs.
Parameters in non-pregnant individuals.[177-179] Volume of distribution (V_d), Bioavailability (F), Half-life ($T_{1/2}$), Elimination rate constant (k_{el}).

$$Cp = \frac{Dose \times F}{V_d}$$

Equation 2.1 Plasma concentration achieved following a single oral dose.
Plasma concentration (Cp), Bioavailability (F), Volume of distribution (V_d).

$$Cp_{ss} = \frac{Dose \times F}{V_d \times k_{el} \times T_{1/2}}$$

Equation 2.2 Plasma concentration achieved following multiple oral doses.
Plasma concentration at steady state (Cp_{ss}), Bioavailability (F), Volume of distribution (V_d), Elimination rate constant (k_{el}), Half-life ($T_{1/2}$)

2.2.2 Sample Collection and Processing

Human umbilical cord samples were collected at birth with informed consent from patients for inclusion to the University of Hawaii Reproductive Biorepository, including consent for future

investigation. Umbilical cords were collected, washed, and snap-frozen in liquid nitrogen then stored at -80°C until use. Upon request, samples were cut from frozen and shipped on dry ice to the University of British Columbia. All samples were processed into lysates by lab technicians using the following protocol. Tissue pieces are thawed at room temperature, wet-weight recorded, then homogenized 1:3 (w:v) in Tris-HCl buffer containing 5 mM $MgCl_2$ (pH 7.4) using a Tissue Tearor^{TM}. Lysates were divided into 50 µL aliquots and frozen until use. This study was conducted under approval from the Clinical Research Ethics Board at The University of British Columbia (H14-00092) and The University of Hawaii IRB for Human Subjects (CHS 15080).

"NSAID-free" whole blood was collected from an anonymous donor free of NSAIDs for a minimum of six weeks. Blood was collected in sodium citrate tubes and delivered to our laboratory within 15 minutes of collection. Blood was centrifuged at 2000 x g for 20 minutes at 4°C to separate the plasma, which was then aliquoted and frozen at -80°C until use. The University of British Columbia's Ethics Review Board approved blood collection and use from the anonymous donor under the approval number H13-01805.

2.2.3 Instrumentation

The UHPLC-MS/MS system consisted of an Agilent 1290 Binary Pump, a 1290 Infinity Sample, a 1290 Infinity Thermostat, and a 1290 Infinity Thermostatted Column Compartment (Agilent, Mississauga, Ontario, Canada) interfaced with an AB Sciex QTrap® 5500 hybrid linear ion-trap triple quadrupole mass spectrometer by a Turbo Spray ion source (AB Sciex, Concord, Ontario, Canada). Data were acquired using Analyst 1.5.2 software on a computer operating Microsoft Windows XP Professional.

2.2.4 Chromatography Conditions

For this project, a Waters Acquity UPLC BEH C18, 1.7 µm, 2.1 x 50 mm column was used, protected by a Waters Acquity UPLC BEH C18 VanGuard, 1.7 µm, 2.1 x 5 mm guard column (Waters Corporation, Milford, MA, USA). Mobile phase A was HPLC-grade water with 0.1% formic acid and mobile phase B was HPLC-grade methanol with 0.1% formic acid. Mobile phase gradient programming is presented in Table 2.2. Injection volume per sample was 15 µL and the flow rate was set at 0.3 mL/min. The total run time was 10.0 minutes; between 2.0 minutes and 7.0 minutes the mobile phase was directed into the mass spectrometer, and outside this time frame flow was diverted to waste. These chromatographic conditions were decided upon based on balancing optimal separation of analytes with shortening the length of the run time.

Time (min)	Flow Rate (mL/min)	Mobile Phase A (%)	Mobile Phase B (%)
0.0	0.3	85	15
5.0	0.3	2	98
7.0	0.3	2	98
7.1	0.3	85	15
10.0	0.3	85	15

Table 2.2 Gradient programming for chromatographic separation of analytes.

2.2.5 Mass Spectrometry Conditions

The mass spectrometer's Turbo Spray ion source was operated in negative mode for the duration of the run. Nitrogen gas was used for curtain gas, collision gas, ion source gas 2 (vaporizing gas); Zero air was used for ion source gas 1 (nebulizing gas). Conditions of the machine were as follows: Curtain gas: 30 units, Collision gas: high, ion spray -4500 V, temperature: 450°C, ion source gas 1: 40 units, ion source gas 2: 60 units. Entrance potential was -10 units, and the resolution of Q1 unit, resolution of Q3 Unit, and dwell time was 150 msec. Diclofenac,

ibuprofen, indomethacin, naproxen, and salicylic acid were quantitated using multiple reaction monitoring. Transitions were: diclofenac *m/z* 294.0 → 250.0, *m/z* 294.0 → 214.0; ibuprofen *m/z* 205.1 → 161.0; indomethacin *m/z* 356.1 → 312.1, *m/z* 356.1 → 297.0 *m/z* 356.1 → 270; naproxen *m/z* 229.0 → 185.0, *m/z* 229.0 → 170.1; salicylic acid *m/z* 136.5 → 93.0. Chromatographic retention times were 4.99 min for diclofenac, 5.10 for ibuprofen, 4.99 for indomethacin, 4.46 for naproxen, and 3.37 for salicylic acid (Figure 2.2).

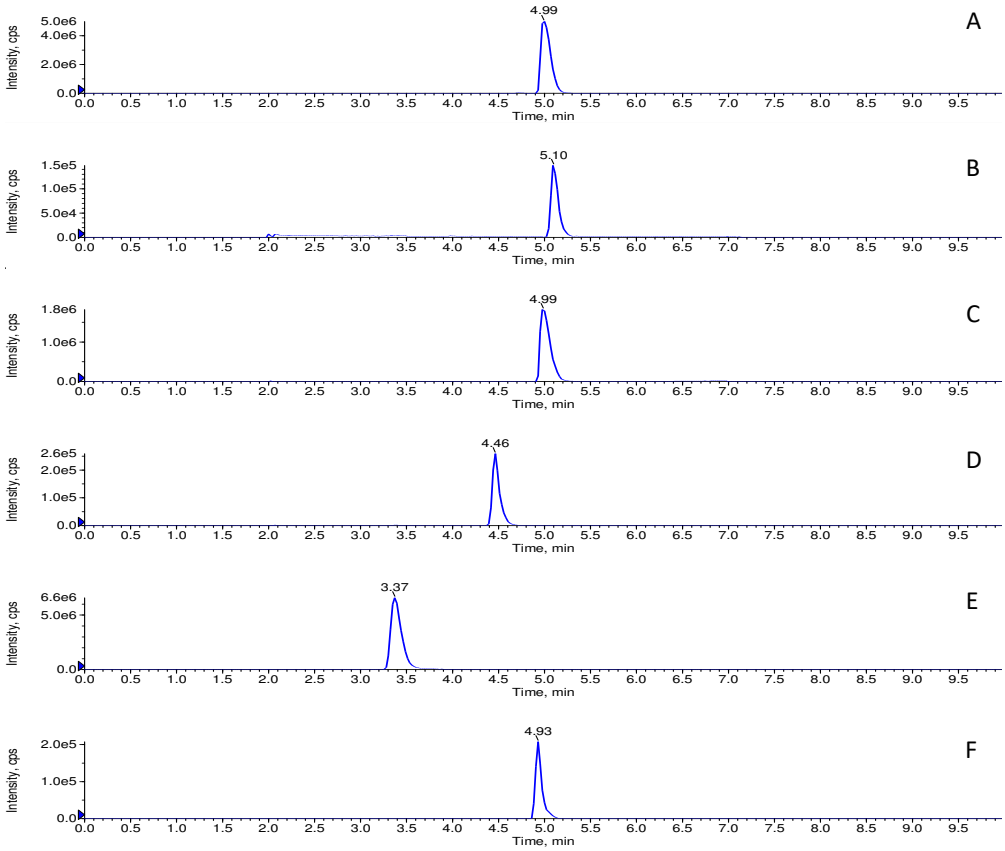

Figure 2.2 Chromatograms of NSAIDs analyzed in multiple reaction monitoring modes. **A.** Diclofenac (294.0/250.0) **B.** Ibuprofen (205.1/161.0) **C.** Indomethacin (356.1/312.1) **D.** Naproxen (229.0/185.0) **E.** Salicylic acid (136.5/93.0) **F.** Aceclofenac (352.0/75.0).

2.2.6 Preparation of Standard Solutions, Calibration Standards, and Quality Controls

Stock solutions of diclofenac, ibuprofen, indomethacin, naproxen, and salicylic acid were prepared in methanol to a concentration of 1 mg/mL. Intermediate solutions containing all five

NSAIDs were made at concentrations of 200 μg/mL, 50 μg/mL and 1 μg/mL by combining equal parts of stock solutions of each NSAID and diluting in methanol to the appropriate concentration. These intermediates were used for all further dilutions. Calibration standard solutions containing all NSAIDs were diluted in HPLC-grade water to 10, 25, 50, 100, 200, 300, 600, 1000, 2500, 5000 and 10000 ng/mL for standard curve calibration, so when 10 μL is spiked into 90 μL blank matrix the standard curve spans a range of 1-1000 ng/mL. The internal standard was prepared by dissolving aceclofenac in methanol to a concentration of 1 μg/mL, and further diluting in water to a working concentration of 100 ng/mL.

Quality control (QC) standards were prepared from separately weighed and diluted standard solutions of NSAIDs in methanol. Three QC standards were prepared by further dilution in HPLC-grade water to the following concentrations: QC_{low} at 35 ng/mL, QC_{mid} at 200 ng/mL, and QC_{high} at 800 ng/mL. All calibration standards and QCs were stored in glass vials, parafilmed, and stored at -20°C until days of analysis.

Calibration standards in solvent were prepared on the day of analysis by diluting the appropriate working standard directly in a 96-well plate with HPLC-grade methanol and adding 40 μL of internal standard to each well, with the exception of the double blank sample (50% HPLC water + 50% methanol). A single blank sample was prepared by combining 10 μL HPLC grade water with 40 μL of internal standard and 50 μL methanol.

2.2.7 Preparation of Calibration Standards in Human Plasma

Two different commercial serum samples were purchased for use as a blank matrix, but both were found to contain NSAIDs and were therefore inappropriate for use. In order to obtain an NSAID-free human blood sample, we contacted our collaborator at the Centre for Blood Research, who identified a donor self-reporting as not having taken NSAIDs in the past 6 weeks. This report was verified using the LC-MS method.

Calibration standards in human plasma were prepared by spiking 90 µL of the blank plasma matrix with the appropriately diluted standard solution. To all samples except the double blank, 40 µL of IS and 100 µL of 1 M hydrochloric acid were added and samples were vortex mixed. One molar HCl was used as it was determined that the compounds were extracted best at an acidic pH. Liquid-liquid extraction was performed using 2 mL MTBE. Samples were vortex mixed for 30 seconds then frozen at -80°C for 15 minutes. The organic layer was transferred to a clean set of tubes and samples were brought to dryness under nitrogen at 35°C. Dried residues were reconstituted in 100 µL of methanol/HPLC grade water 50:50 (v/v) for injection into the LC/MS system.

2.2.8 Method Application for Screening Human Plasma

The applicability of this method was determined in human plasma and serum samples. The two commercially purchased samples which were determined to contain NSAIDs were screened using the method to determine NSAID concentration. In addition, we screened 20 individual plasma samples collected by the Hawaii Biorepository (Honolulu, HI, USA) from non-surviving, non-transplantable organ donors with cardiovascular disease who had donated heart samples to

the biorepository. Consent for sample collection was provided to the biorepository, with approval for future study of drugs, diseases, and genetics. Unlike the commercial serum, these samples all showed varying degrees of hemolysis. These samples were anonymized and de-identified and the only clinical information available is as follows: Age: mean age (± SD) 54 ± 10 years, all > 18; Sex: n = 10 males and n = 10 females; Ethnicity: 25% Asian (1: Chinese, 1: Japanese, 1: Korean, 2 Asian Oriental); 50% Caucasian, and 25% other (2: Indian, 3: Native Hawaiian or Pacific Islander). Only limited patient history is available for medication, and no chart information for preparation as organ donors (such as drugs or other treatments administered) is available. Known medications in this cohort are as follows: Low dose aspirin 100%; angiotensin converting enzyme inhibitors 60%; angiotensin receptor inhibitors 15 %; "other" diuretics = 25%; beta blockers = 25%. These archival human plasma samples were used under exempt approval from the Institutional Review Board for Human Ethics at the University of Hawaii and the Review Ethics Board at the University of British Columbia (H14-00092). The bench scientist was blinded to the above sample information until screening was complete.

For screening of unknown samples, 100 µL of serum or plasma was used. 40 µL of IS and 100 µL of 1 M hydrochloric acid were added and samples were vortex mixed. Liquid-liquid extraction using MTBE was performed as described above. Samples were reconstituted in 100 µL of methanol/HPLC grade water 50:50 (v/v) for injection into the LC-MS.

2.2.9 Preparation of Calibration Standards in Human Umbilical Cord Lysate

A pooled, blank umbilical cord matrix for calibration curve preparation was made by pre-screening NSAID samples from our archive for which no clinical information was available

using the LC-MS method. Samples in which no NSAIDs were detected were pooled together and aliquoted for future curve preparation for validation and future screening. Calibration standards in blank UC lysate were prepared by spiking 10 μL of appropriately diluted calibrations standards and quality controls with 90 μL of lysate into disposable borosilicate glass tubes. Calibration curves were prepared fresh on the day of analysis in the concentrations 1.0, 2.5, 5.0, 10, 20, 30, 60, 100, 250, and 500 ng/mL, as the curve was not linear to 1000 ng/mL. Quality control concentrations were changed to: QC_{low} at 35 ng/mL, QC_{mid} at 200 ng/mL, and QC_{high} at 400 ng/mL. Due to issues with the stability of aceclofenac in UC lysate, the IS was changed to carprofen at 200 ng/mL. Carprofen was monitored at *m/z* 272.0 → 226.0, m/z 272.0 → 228.0.

2.2.10 Method Application for Screening Human Umbilical Cord Lysate

The method was designed in order to screen our archive of umbilical cord samples from the University of Hawaii Reproductive Biorepository, discussed above. Our cohort consisted of 380 umbilical cord lysates for which clinical information was available. For screening of these unknown samples, 100 μL of UC lysate was used. As above, 40 μL of IS and 100 μL of 1 M hydrochloric acid were added and samples were vortex mixed. The protocol for liquid-liquid extraction using MTBE was the same as described above for plasma samples. Dried residues were reconstituted in 100 μL of methanol/HPLC grade water 50:50 (v/v) for injection into the LC/MS system.

2.2.11 Method Validation

The novel UHPLC-MS/MS method described here was validated according to the United States Food and Drug Administration's Guidelines for Bioanalytical Method Validation to evaluate

linearity, limits of sensitivity, accuracy and precision, stability, matrix effects, and recovery[180]. The method was fully validated for use in solvent and plasma, and a partial validation was performed to apply the method to umbilical cord lysate. According to the FDA guidelines on bioanalytical method validation, a partial validation can be performed when there is a change of matrix within the same species. A full validation was performed in plasma because it is a more common matrix used in drug screening, therefore this method could be utilized by other laboratories for drug screening. We recognize that umbilical cord lysate is a highly specific matrix, not used by many researchers, therefore we aimed to make the method more accessible. Considering the widespread use of NSAIDs worldwide, this method could be adapted for use in other human samples, or for environmental screening which has become a concern with NSAIDs being detected in surface and ground water.

2.2.11.1 Linearity and Limits of Sensitivity

Linearity was determined by linear regression of the standard curve, using $1/x^2$ least squares weighting, with a minimum acceptable linearity of $r^2=0.985$. Standard curves were generated over the linear range, where the coefficient of variation (CV) of each point of the curve was <15% of the nominal concentration, with the exception of the lower limit of quantitation (LLOQ) where acceptable CV was ≤20%. The limit of sensitivity was determined by calculating 3 times the background signal-to-noise ratio in the blank sample at the elution time of each compound.

2.2.11.2 Accuracy and Precision

Intra- and inter-assay accuracy and precision were determined using the three QC samples (QC_{low}, QC_{mid}, QC_{high}). Intra-assay accuracy is expressed as a percentage of the nominal

concentration for each QC concentration, which was evaluated by triplicate analyses of each concentration in every validation run and screening run. Intra-assay precision was calculated using the same QC samples evaluated in triplicate and expressed as the coefficient of variation of these samples. Inter-assay accuracy and precision were evaluated by triplicate analysis of each QC concentration, over a minimum of three different validation runs.

2.2.11.3 Recovery

Recovery was determined in plasma by comparing the peak area ratios of samples spiked prior to extraction using the liquid-liquid extraction protocol with samples spiked after plasma extraction. Recovery was determined at all three QC concentrations.

2.2.11.4 Matrix Effects

The matrix effects of human plasma and umbilical cord lysate on the analytes and internal standard was determined by comparing the mean peak areas of the analytes in extracted plasma to the mean peak areas of the standard solutions spiked directly into a 96-well plate in solvent. Matrix effect was determined at the QC concentrations, each run in triplicate. The internal standard normalized matrix effect was calculated by the matrix effect of each analyte over the matrix effect of the internal standard.

2.2.11.5 Stability

The stabilities of each NSAID in plasma were also evaluated at the QC concentrations. Stock solutions of quality controls were spiked into blank plasma, in triplicate, and left on the benchtop at 20°C (to reflect standard Canadian laboratory temperatures) for 24 hours before being spiked

with the internal standard and extracted according to the extraction protocol. QC standards were also spiked into blank plasma and frozen at -80 °C for 24 hours and then thawed to room temperature before internal standard spiking and extraction, to determine the effects of freeze-thawing on samples. The stabilities of each compound were determined by comparing the mean peak area ratios from stability quality control samples to freshly prepared quality control samples in plasma and expressed as percent recovery. Additionally, samples were commonly queued in 96-well plates, covered by silicone plate covers, for up to 24 hours in the autosampler. Therefore, standard curves were re-injected at the end of the injection period to determine stability of the autosampler.

2.3 Results

2.3.1 Pharmacokinetic Analysis

Pharmacokinetic analysis was performed to determine expected maternal blood concentration of NSAIDs following a single oral dose and from steady state. Both low (81 kg) and high (86 kg) weight boundaries for pregnant women and low and high doses of each NSAID were used in calculations to provide a more detailed picture of plasma NSAID concentrations in pregnancy. Pharmacokinetic parameters for NSAIDs, volume of distribution (V_d), bioavailability (F), and half-life ($T_{1/2}$) were presented in Table 2.1. Following a low single oral dose, after five half-lives, the expected plasma concentration of NSAIDs ranges between 4.3 – 556 ng/mL, and for a high single oral dose expected plasma concentrations range between 11.1 – 1361.7 ng/mL (Table 2.3). Following ten half-lives of a single oral dose, the low dose concentration in plasma ranges from 0.1 – 17.4 ng/mL, and the high dose concentration in plasma would range between 0.3 – 42.6 ng/mL. Expected plasma concentrations following repeated dosing such that the drug reached steady state are presented in Table 2.4. After five half-lives, all NSAIDs would be quantitated

using the method, with the exception of diclofenac which fell slightly below the LLOQ. However, the LOS for the UHPLC-MS/MS method are below this window, allowing us to potentially detect active ingestion, giving us reliable detection windows. The exception to this statement is salicylic acid, which can be ingested from dietary sources in amounts that mimic low dose Aspirin use. It is therefore possible that false positives for salicylic acid could occur at concentrations below 2.47 μmol/L in plasma, the maximum concentration acquired from dietary ingestion.

Drug	**5×$T_{1/2}$ (days)**	**Low dose plasma conc after 5×$T_{1/2}$ (ng/mL)**	**High dose plasma conc after 5×$T_{1/2}$ (ng/mL)**	**10×$T_{1/2}$ (days)**	**Low dose plasma conc after 10×$T_{1/2}$ (ng/mL)**	**High dose plasma conc after 10×$T_{1/2}$ (ng/mL)**
Salicylate	4.2	409.9	1157.7	8.3	12.8	36.2
Diclofenac	0.4	4.3	11.1	0.8	0.1	0.3
Ibuprofen	0.4	156.0	1361.7	0.8	4.9	42.6
Indomethacin	0.9	9.8	19.9	1.9	0.3	0.6
Naproxen	3.5	556.0	842.4	7.1	17.4	26.3

Table 2.3 Plasma concentrations of NSAIDs following a single oral dose for low and high doses of drug.

		After 5×$T_{1/2}$		**After 10×$T_{1/2}$**	
Drug	**Average C_{ss} (Low dose, high dose) (mg/L)**	**Low dose plasma conc (ng/mL)**	**High dose plasma conc (ng/mL)**	**Low dose plasma conc (ng/mL)**	**High dose plasma conc (ng/mL)**
Salicylate	3.5, 70.6	110.3	2205.4	34.5	689.1
Diclofenac	0.2, 0.8	6.27	25.1	1.96	7.84
Ibuprofen	7.2, 115.3	225.2	3603.5	70.4	1126.1
Indomethacin	0.5, 1.4	14.1	42.4	4.4	13.3
Naproxen	25.7, 51.4	802.3	1604.17	250.7	501.5

Table 2.4 Plasma concentrations of NSAIDs for low and high doses of drug from steady state.

2.3.2 Optimization of Chromatography and Mass Spectrometry Conditions

After repeated trials of varying LC-MS/MS conditions, the conditions presented here were chosen, as they provided narrow and symmetrical peaks with the shortest run time of 10 minutes. Mobile phase B (methanol) give higher abundance for compounds with late retention times as compared to results when acetonitrile was used as mobile phase B. Additionally, 0.1% v/v formic acid was added to both mobile phases because it gave sharper peaks for compounds with earlier retention times compared to 2.5 mM ammonium formate, likely due to the higher acidity. During method development, calibration curves were constructed after extraction at acidic, basic, and neutral pH. All NSAIDs extracted better at an acidic pH, hence 100 µL of 1 M HCl was added to each sample prior to liquid-liquid extraction. The *m/z* transitions used for quantification of each compound were chosen based on the abundance of their daughter ions. A flow rate of 300 µL/min resulted in superior peak shape than other flow rates that were tested.

2.3.3 Method Validation

2.3.3.1 Linearity and Limits of Sensitivity

To evaluate the linear ranges of each compound, calibration curves were prepared and evaluated at least three times in each matrix (solvent, plasma, and umbilical cord lysate). The measured concentration for each point on the curve was within 15% of the actual concentration, with the exception of the bottom point of the curve, the LLOQ, for which 20% was deemed acceptable. The limit of sensitivity, which was determined by calculating 3X the background noise at the elution time for each compound, was below 1 ng/mL for all analytes (Table 2.5).

	Solvent		Plasma		UC Lysate	
	Limit of Sensitivity (ng/mL)	**Linear Range (ng/mL)**	**Limit of Sensitivity (ng/mL)**	**Linear Range (ng/mL)**	**Limit of Sensitivity (ng/mL)**	**Linear Range (ng/mL)**
Diclofenac	<1	10-1000	<1	10-1000	<1	5-500
Ibuprofen	<1	20-500	<1	20-1000	<1	10-500
Indomethacin	<1	5-1000	<1	5-1000	<1	5-500
Naproxen	<1	20-500	<1	20-500	<1	20-500
Salicylic Acid	<1	10-1000	<1	20-1000	<1	20-500

Table 2.5 Linearity and limits of sensitivity of the UHPLC-MS/MS method in solvent, plasma, and UC lysate.

2.3.3.2 Accuracy and Precision

Accuracy and precision were determined in solvent, plasma, and umbilical cord lysate using the three QC concentrations. Results for intra-day and inter-day accuracy and precision are presented below for solvent (Table 2.6), plasma (Table 2.7), and umbilical cord lysate (Table 2.8). Values were within acceptable limits defined by the FDA (±15% of the nominal concentration) for accuracy, and a CV less than 15% for precision.

	Nominal Concentration (ng/mL)	Accuracy (% nominal)		Precision (%CV)	
		Intra-day	Inter-day	Intra-day	Inter-day
Diclofenac					
QC_{low}	25	101.73	96.00	1.20	4.62
QC_{mid}	200	106.17	100.67	2.12	5.07
QC_{high}	800	97.63	99.81	3.39	4.40
Ibuprofen					
QC_{low}	25	95.07	99.60	3.27	4.53
QC_{mid}	200	93.00	89.50	0.93	3.80
QC_{high}	400	110.83	109.94	3.16	4.67
Indomethacin					
QC_{low}	25	95.60	99.38	11.72	6.34
QC_{mid}	200	103.00	101.83	1.28	3.07
QC_{high}	800	89.21	85.76	1.41	4.51
Naproxen					
QC_{low}	25	102.27	102.44	1.92	2.81
QC_{mid}	200	90.17	88.5	0.64	3.88
QC_{high}	400	111.33	113.78	3.19	3.84
Salicylic Acid					
QC_{low}	25	97.73	95.47	3.71	3.52
QC_{mid}	200	105.33	102.44	2.69	3.95
QC_{high}	800	92.42	91.79	3.21	2.34

Table 2.6 Intra-day and inter-day accuracy and precision for NSAIDs in solvent.

	Nominal Concentration (ng/mL)	Accuracy (% nominal)		Precision (%CV)	
		Intra-day	Inter-day	Intra-day	Inter-day
Diclofenac					
QC_{low}	35	96.28	91.43	0.79	4.88
QC_{mid}	200	92.33	88.94	2.19	4.29
QC_{high}	800	107.33	108.32	2.34	2.09
Ibuprofen					
QC_{low}	35	96.19	90.63	8.78	13.72
QC_{mid}	200	94.33	94.72	1.70	10.64
QC_{high}	400	113.00	110.02	1.81	5.71
Indomethacin					
QC_{low}	35	97.81	96.98	4.34	3.82
QC_{mid}	200	98.33	96.44	2.05	3.28
QC_{high}	800	91.42	92.51	4.64	2.53
Naproxen					
QC_{low}	35	96.95	99.65	6.02	10.7
QC_{mid}	200	89.33	87.83	5.17	4.59
QC_{high}	400	106.00	107.13	1.18	2.37
Salicylic Acid					
QC_{low}	35	102.29	97.65	2.66	13.06
QC_{mid}	200	97.00	92.16	10.80	8.35
QC_{high}	800	97.38	95.01	8.91	12.93

Table 2.7 Intra-day and inter-day accuracy and precision for NSAIDs in plasma.

	Nominal Concentration (ng/mL)	Accuracy (% nominal)		Precision (%CV)	
		Intra-day	Inter-day	Intra-day	Inter-day
Diclofenac					
QC_{low}	35	100.76	101.30	3.41	9.39
QC_{mid}	200	108.67	102.28	5.29	8.18
QC_{high}	800	107.83	103.11	7.42	10.09
Ibuprofen					
QC_{low}	35	96.86	95.33	7.92	7.63
QC_{mid}	200	103.16	114.18	4.12	9.29
QC_{high}	400	93.42	96.33	1.61	5.89
Indomethacin					
QC_{low}	35	92.10	101.75	7.04	10.24
QC_{mid}	200	106.17	100.94	6.86	5.64
QC_{high}	800	101.12	94.67	5.01	9.47
Naproxen					
QC_{low}	35	101.62	98.95	3.77	6.49
QC_{mid}	200	105.50	99.11	6.72	6.51
QC_{high}	400	102.17	103.00	7.57	11.85
Salicylic Acid					
QC_{low}	35	85.90	92.67	9.87	10.21
QC_{mid}	200	106.83	106.83	4.78	12.91
QC_{high}	800	102.17	104.81	7.07	9.90

Table 2.8 Intra-day and inter-day accuracy and precision for NSAIDs in umbilical cord lysate.

2.3.3.3 Recovery in Plasma

Recovery was determined by comparing the peak area ratios of pre-extraction and post-extraction spiked plasma. Results are presented in Table 2.9. Most compounds showed good values for recovery; however, the recovery of ibuprofen was lower at the lower quality control concentrations. The internal standard, aceclofenac, had a low recovery in plasma, leading to a

larger area ratio and therefore a recovery in excess of 100% for indomethacin, naproxen, and salicylic acid.

	Nominal Concentration (ng/mL)	Recovery (%)
Diclofenac		
QC_{low}	35	73.86
QC_{mid}	200	78.78
QC_{high}	800	90.59
Ibuprofen		
QC_{low}	35	76.95
QC_{mid}	200	67.20
QC_{high}	400	87.67
Indomethacin		
QC_{low}	35	141.74
QC_{mid}	200	132.22
QC_{high}	800	128.76
Naproxen		
QC_{low}	35	124.76
QC_{mid}	200	119.66
QC_{high}	400	105.37
Salicylic Acid		
QC_{low}	35	127.25
QC_{mid}	200	153.75
QC_{high}	800	165.06

Table 2.9 Recovery of analytes in plasma.
Calculated by the peak area ratio in spiked plasma over the peak area ratio of in plasma spiked post-extraction.

2.3.3.4 Matrix Effects of Plasma

The matrix effect of human plasma was evaluated by comparing the AUC of the analytes extracted from plasma to the AUC of the analytes in solvent. The matrix effect of the internal standard was also calculated, to determine the IS-normalized matrix effect, which can provide a clearer picture of quantifying the analytes in a different matrix. The mean area counts of IS in solvent were compared to the mean area counts of IS in plasma to determine this effect. Results

are presented in Table 2.10. For certain analytes, the matrix effects differed at different concentrations. This highlights the importance of preparing the calibration curve in the same matrix as the unknown samples, rather than solvent when using this method.

	Nominal Concentration (ng/mL)	**IS-normalized Matrix Effect (%)**
Diclofenac		
QC_{low}	35	123.23
QC_{mid}	200	112.63
QC_{high}	800	115.49
Ibuprofen		
QC_{low}	35	118.43
QC_{mid}	200	71.17
QC_{high}	400	56.95
Indomethacin		
QC_{low}	35	86.90
QC_{mid}	200	84.66
QC_{high}	800	91.19
Naproxen		
QC_{low}	35	105.65
QC_{mid}	200	99.38
QC_{high}	400	75.05
Salicylic Acid		
QC_{low}	35	83.67
QC_{mid}	200	82.22
QC_{high}	800	101.06

Table 2.10 Matrix effect of plasma compared to solvent spiked standards.

2.3.3.5 Matrix Effects of Umbilical Cord Lysate

During the partial validation of the method in umbilical cord lysate, we encountered problems with the internal standard aceclofenac. Over the course of calibration curve injection, the area count of aceclofenac continuously decreased. Initially, it was suspected that increasing concentration of another analyte led to a decrease in the ionization of aceclofenac. However, if the order of calibration curve was reversed, or a sample of the same concentration was repeatedly injected over this time period of a normal injection queue, the same results occurred.

Our laboratory chemical store contained phenylbutazone, another NSAID which is only used in veterinary medicine, and we tested this compound as an internal standard. When not using deuterated compounds as an IS, veterinary medications are good choices as they will not be found in any human samples. Phenylbutazone also proved to be unstable in umbilical cord lysate. Next, we tested another NSAID from our chemical store: carprofen, which is also used exclusively in the treatment of animals. We tested the matrix effect of this compound and confirmed that it was stable in the umbilical cord lysate matrix. The area counts remained stable over a 12-hour run time and extraction was sufficient. Therefore, we confirmed that carprofen was a suitable IS for use in umbilical cord lysate.

2.3.3.6 Stability in Plasma

The stability of the NSAIDs in human plasma were evaluated in two contexts: a 24-hour benchtop stability at 20°C to mimic laboratory conditions and a freeze-thaw cycle at -80°C. Results from the stability studies are outlined in Table 2.11. Analytes were stable at standard laboratory temperature of 20°C for 24 hours, quantitated as ±15% compared to freshly prepared QCs, with the exception of ibuprofen and naproxen at the lowest quality control. After one freeze-thaw cycle at -80°C analytes were observed to be stable except for ibuprofen which exhibited a deviation of 40% at the lowest concentration, and salicylic acid which had 30% loss at the lowest QC concentration. For both of these analytes, less loss was observed at higher QC concentrations. These results indicate that freeze-thaw in plasma is associated with degradation or extraction loss of ibuprofen and salicylic acid. In contrast to plasma, QCs in solvent undergoing freeze thaw did not exhibit loss. This was evaluated by preparing solvent QC

samples at the beginning of method development and freezing at -80°C and analyzing these samples in subsequent batches during method development, validation, and screening. Samples were compared to freshly prepared solvent QCs and were within 15%, as deemed acceptable by FDA standards. The maximum time samples were frozen was six months. Additionally, we evaluated the stability of samples in the autosampler, as screening was often done in large batches and samples were queued in the autosampler for a maximum of 24 hours. Standard curves and QCs were periodically re-injected at the end of the batch, to ensure acceptable accuracy, precision, and stability over the time frame. Results from these studies show that samples can be in the autosampler at 4°C for up to 24 hours without loss.

		Stability Test	
	Nominal Concentration (ng/mL)	**Benchtop; 24 h, 20°C % fresh prepared (%CV)**	**Freeze/thaw, -80 °C % fresh prepared (%CV)**
Diclofenac			
QC_{low}	35	102.07 (4.09)	96.01 (4.92)
QC_{mid}	200	104.45 (1.87)	105.98 (1.07)
QC_{high}	800	98.81 (0.72)	99.28 (0.42)
Ibuprofen			
QC_{low}	35	77.89 (7.08)	60.72 (8.67)
QC_{mid}	200	87.16 (6.51)	75.30 (3.39)
QC_{high}	400	88.33 (4.20)	86.25 (4.56)
Indomethacin			
QC_{low}	35	86.31 (4.75)	90.76 (1.05)
QC_{mid}	200	90.04 (2.16)	97.46 (1.36)
QC_{high}	800	86.69 (2.97)	92.21 (3.51)
Naproxen			
QC_{low}	35	81.46 (3.75)	82.79 (8.66)
QC_{mid}	200	94.58 (4.08)	97.44 (13.11)
QC_{high}	400	106.61 (1.82)	107.25 (1.82)
Salicylic Acid			
QC_{low}	35	90.932 (6.99)	70.34 (2.79)
QC_{mid}	200	94.10 (8.51)	68.72 (10.53)
QC_{high}	800	102.19 (10.01)	82.31 (11.94)

Table 2.11 Stability of NSAIDs in plasma.
Tested at room temperature for 24 hours or -80°C for one freeze/thaw.

2.3.3 Method Application in Human Plasma

Pooled human serum from Bio-Rad (Irvine, CA, USA) was positive for all five NSAIDs. Diclofenac and indomethacin were detected below the lower limit of quantitation, while ibuprofen (264 ng/mL), naproxen (1320 ng/mL), and salicylic acid (387 ng/mL) were quantifiable. In contrast, the serum sample from Innovative Research (Novi, MI USA) did not contain diclofenac or indomethacin, but ibuprofen (897 ng/mL), naproxen (1670 ng/mL) and salicylic acid (844 ng/mL) were quantified. Differential detection and quantitation of drugs were reported in respective samples, showing method selectivity and sensitivity.

The detection of NSAIDs from individual donors is presented in Table 2.12. Salicylic acid, as expected, was detected and quantified in all subjects, with the exception of S16 and S19 where Salicylic acid was detected but not quantified. Ibuprofen was detected but not quantifiable in 2/20 samples (S1 and S4, 10% prevalence) but no other drugs were detected. As these are archival samples, it is possible that NSAIDs have degraded, although the salicylic acid appears stable. Additionally, the window for detection would be within 3 – 10 days of last ingestion (depending on the NSAID) and as these were deaths where organ donation was considered, it is unknown if the blood collection was rapid, or if the patient had been ventilated for a period of time – allowing drug to wash out.

2.3.4 Method Application in Human Umbilical Cord Lysate

In order to reliably use the method to screen umbilical cord lysate, the IS had to be replaced. Aceclofenac was validated in plasma but was found to be unstable in umbilical cord. During the standard curve and QC injection, a run time of approximately five hours, the area count of

aceclofenac would drop to approximately 55% of starting values. Two tests were carried out to ensure that increasing concentration of another compound did not interfere with the ionization. First, the standard curve and QCs were injected in reverse order, from highest to lowest concentration and second, an extracted sample spiked with 100 ng/mL of NSAIDs was injected repeatedly over a five-hour period. In both tests, a similar decline of aceclofenac was observed.

The second-choice compound from our chemical stores was phenylbutazone, an NSAID which is only used in veterinary medicine. The same tests were completed for phenylbutazone, with an extended run time of tests because the expected run time during umbilical cord screening was expected to exceed eight hours. Over a run-time of eight hours, area counts of phenylbutazone counts also declined to approximately 45% of starting values.

The next compound tested for use as the IS was carprofen, another NSAID from our chemical inventory. Similar to phenylbutazone, carprofen is an NSAID used exclusively in veterinary medicine, and would therefore not be found naturally in any of the samples. Over an eight-hour test of standard curve and QC injection, area counts of carprofen remained above 85% of the starting values. Batches of umbilical cords during screening were routinely run for 12-16 hours, and carprofen was verified to be stable over the entire duration of the run, as determined by reinjection of the standard curve at the end of the run.

In total, 380 umbilical cord lysates were screened from our archives. Two samples were positive for ibuprofen, one sample was positive for indomethacin, and eight samples were positive for salicylic acid. All salicylic acid positive samples were quantified, but the samples positive for

ibuprofen and indomethacin were below the LLOQ for the curves. However, peak values were above the limit of sensitivity of the assay, and samples were classified as "positive". For salicylic acid positive samples, the concentrations detected were 27.0, 42.3, 45.4, 48.1, 81.0, 108.0, and 423.0 ng/mL. Chromatograms of positive samples are presented in Figure 2.3.

	NSAID levels (ng/mL)				
Sample #	**Diclofenac**	**Ibuprofen**	**Indomethacin**	**Naproxen**	**Salicylic Acid**
Pool 1 (Bio-Rad)	D	264	D	1320	387
Pool 2 (Innovative Research)	0	897	0	1670	844
1	0	D	0	0	21.7
2	0	0	0	0	48.9
3	0	0	0	0	168
4	0	D	0	0	222
5	0	0	0	0	2155
6	0	0	0	0	27.9
7	0	0	0	0	401
8	0	0	0	0	198
9	0	0	0	0	29.3
10	0	0	0	0	150
11	0	0	0	0	398
12	0	0	0	0	70.2
13	0	0	0	0	36.1
14	0	0	0	0	53.2
15	0	0	0	0	22.9
16	0	0	0	0	D
17	0	0	0	0	1995
18	0	0	0	0	132
19	0	0	0	0	D
20	0	0	0	0	29.3

Table 2.12 Pilot study for detection of NSAIDs.
Pooled commercial plasma from two sources, and from archival blood samples from a biobank. D = detected but below lower limit of quantitation.

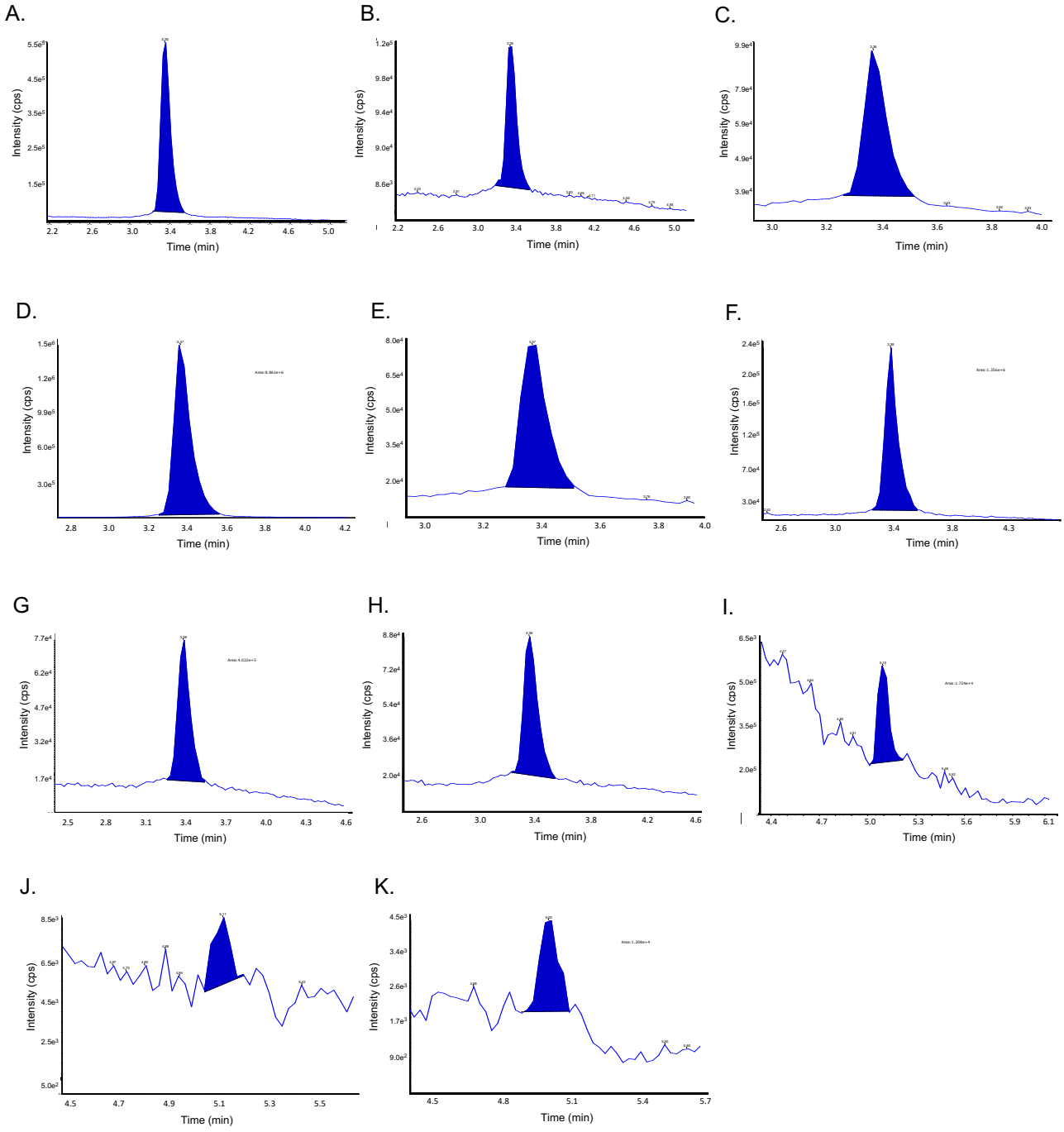

Figure 2.3 Chromatograms of UC samples positive for NSAIDs.
A. Sample 555, SA 99.1 ng/mL **B.** Sample 464, SA 27 ng/mL **C.** Sample 186, SA 48.1 ng/mL **D.** Sample 232, SA 432 ng/mL **E.** Sample 491, SA 42.3 ng/mL **F.** Sample 541, SA 45.4 ng/mL **G.** Sample 481, SA 108.0 ng/mL **H.** Sample 545, SA 81.0 ng/mL **I.** Sample 217, IBU below LLOQ **J.** Sample 508, IBU below LLOQ **K.** Sample 250, IND below LLOQ.

Comparisons were made between NSAID positive samples and NSAID negative samples based on maternal age, maternal BMI, gestational age, gestational diabetes mellitus, membrane rupture, premature rupture of the membranes, preterm labour, baby weight, and baby sex. No significant differences were observed. All pregnancies ended in vaginal delivery, with labour proceeding spontaneously and normally. Additionally, there were no NICU admissions, cases of IUGR, gestational hypertension, preeclampsia, HIV, cancer, or cardiac disease in the NSAID positive group. Data collected by self-report by the pregnant women to physicians indicates no ingestions of drugs including phencyclidine, benzodiazepines, cocaine, amphetamine, opiates, barbiturates, or alcohol use.

2.4 Discussion

2.4.1 Development of a LC-MS/MS Method for Quantitation of NSAIDs in Human Tissue

This novel method can accurately and precisely quantitate five NSAIDs in solvent and is also validated to the United States Food and Drug Administration (FDA) standards in human plasma and human umbilical cord lysate. It should be noted that salicylic acid, and not acetylsalicylic acid was included in the method because it is unstable in many organic solvents, and is rapidly hydrolyzed to salicylic acid in blood, therefore reflecting biological relevance[181]. We fully validated the method to FDA standards of bioanalytical method validation, evaluating linearity, limits of sensitivity, accuracy, precision, stability (benchtop, autosampler, and freeze-thaw stability), matrix effects, and recovery. For all parameters, we met the guidelines set out by the FDA[180].

The pilot application study demonstrated method selectivity and sensitivity. Using 20 individual donors, no NSAIDs were detected in most participants except for 100% detection (but only 90% quantitation) of the known positive control. All donors were taking low-dose aspirin which was blinded to the bench scientist, again showing selectivity and sensitivity. The overall prevalence of non-aspirin NSAID use in individual donors was 10% which is similar to what has been previously reported[182]. Based on our stability and freeze/thaw study, it is likely that ibuprofen and salicylic acid levels detected in archival samples were lower than original plasma, because the samples had been stored at -80°C and the freeze/thaw cycle shows that without a preservative these two analytes might degrade. Our method can be applied to a wide range of disciplines from human health and biomedical sciences; through aquatic, environmental, and forensic toxicology to veterinary medicine and we believe it will have excellent applications for future investigators.

The main purpose behind the creation and validation of the method was to screen human umbilical cord lysate for the presence of five of the most commonly used NSAIDs. The umbilical cord presents a unique tissue for screening of drugs and other compounds following birth[183]. It exists entirely on the fetal side of the placenta and is easy to collect as it is usually discarded as medical waste. When compared to meconium as a screening matrix, it has shown 90% agreement, but has the advantage of providing a more rapid turnaround time as it is available for 100% of neonates immediately following birth[183]. There has been a recent shift in the interest of screening umbilical cord compared to meconium for drug screening[184]. Despite this, there are relatively few screening methods that have been tested and validated in umbilical cord. Our UHPLC-MS/MS method is one of very few published screening methods in umbilical cord. While most drugs cross the placenta to some extent, how drugs distribute into the umbilical

cord and the window of detection is not completely understood. However, it has been proposed that drugs may distribute into the umbilical cord and be stored, providing a view of substances ingested in the second half of pregnancy. Our method found similar, if conservative, results to maternal self-reported values[185].

2.4.2 Limitations of the Study

The method presented has certain technical limitations that are relevant to the interpretation of the results. A limitation with respect to salicylic acid is that numerous foods commonly present in the diet contain high levels of salicylic acid[186]. Because of the sensitivity of our method, it is likely that false positives would occur in the very low ranges up to 2.5 μmol/L where, in the uncontrolled human population, we do not know if the salicylic acid present in blood is due to aspirin ingestion at low levels and/or a long time since the last dose, or whether diet-derived salicylic acid is being detected[186,187]. For public health screening and/or environmental detection at very low levels, it should always be considered that salicylic acid present in samples could come from a variety of sources. Another important consideration is the challenges presented by biological matrices. In validating both plasma and umbilical cord lysate, we encountered matrix effects that solidified the FDA suggestion that calibration curves and quality controls be prepared in a blank version of the matrix of interest, as we did in our validation. In switching matrices, we also observed decreases in sensitivity which may lead to false negatives in drug screening but could potentially be mitigated by a more extensive sample cleanup and extraction method.

While the use of archival samples is an advantage for increasing sample size, there are also limitations with archives, including storage time, which in these samples, exceeded our stability

studies. The umbilical cords used in this study were stored for up to one year at -80°C at the biobank before being cut, shipped, and processed into lysates. The lysates were stored for a further 3 years before screening, therefore analyte degradation during this time is possible. While this may affect our values measured and increase the possibility of false negatives, it means our results are likely conservative and NSAID ingestion in late pregnancy is likely higher than reported. Additionally, the distribution and accumulation of NSAIDs in the fetal compartment and umbilical cord are not well understood. Based on the hydrophilic nature of NSAIDs and the compounds, such as glycosaminoglycans, that make up the umbilical cord, we expect that NSAIDs would distribute into the umbilical cord[188]. While metabolite studies are more often performed, determining the parent concentration of NSAID in the fetal compartment is important, as certain NSAIDs readily cross the placenta and can equilibrate in maternal and fetal circulations, in addition it is the parent compounds that may have negative effects as metabolites are largely inactive[189]. Once in the fetal compartment, parent drug concentration is not expected to be rapidly metabolized due to lower levels of fetal metabolism enzymes. Lastly, we were limited by the information available in the clinical chart profiles corresponding to the samples. We did not have access to any information on drugs prescribed in pregnancy or administered during labour. As previously discussed, the administration of low-dose Aspirin in the prevention of preeclampsia has become a common clinical practice, and salicylic acid was drug most often detected and quantitated in our study[162]. However, these umbilical cords were collected in 2014-2015, which is before the indication gained traction among OB/GYNs, as reported by our collaborator, Dr. Tricia Wright (*pers. comm.)*.

2.5 Summary

A method for simultaneous detection and quantitation of diclofenac, ibuprofen, indomethacin, naproxen, and salicylic acid was developed and validated in solvent, human plasma, and human umbilical cord lysate. The method presented is the first UHPLC-MS/MS method, to our knowledge, that simultaneously quantifies five of the most common NSAIDs in a single analytical run in biological samples. Notably, a small amount of plasma or tissue lysate is required for sample screening with this method. Considering the extremely high prevalence of NSAID usage world-wide, our method provides a useful tool to screen human plasma and other biological matrices for these drugs and should therefore have broad applications in the biological and biomedical sciences.

In a moderately sized cohort of 380 umbilical cords, we report a prevalence of 3% for NSAID ingestion close to the time of labour. From a clinical epidemiology perspective, this is concerning, because the limitations of our method suggest our findings are conservative. In addition to the long-known dangers of NSAIDs in pregnancy, recent studies have highlighted additional risks may be present, including renal injury to the neonate[170]. This cohort size is more than most researchers have access to but was still hampered by a low percentage of positive detections. This highlights the size of cohort needed to discover risk factors and adverse effects on a population basis. As such, this cohort we did not observe any clinical outcomes associated with NSAID usage and a much larger cohort is required to confirm NSAID risks in the third trimester. With the widespread availability and usage of NSAIDs, in addition to the emerging use of Aspirin for implantation in ART and indomethacin as a tocolytic, safety guidelines must be reconsidered. Determination of the therapeutic window and safe dosages of NSAIDs are

important to prevent unexpected adverse outcomes and guide decision making in pregnant patients. As clinical practice and guidelines are constantly changing, the pharmacology and safety profiles of all drug classes must be evaluated and updated continuously as new evidence emerges.

Chapter 3: Dysregulation of Inflammatory Cytokine and Vascular Endothelial Growth Factors in Human Umbilical Cord and in the Context of Assisted Reproduction in Placenta

3.1 Introduction

Cytokines and vascular endothelial growth factors (VEGFs) play essential roles in all stages of pregnancy, from implantation and placentation to parturition[190]. Dysregulation in the signalling of either of these systems can contribute to complications or unfavourable pregnancy outcomes including gestational diabetes, fetal inflammatory response syndrome, hypertension, pre-eclampsia, or preterm birth[191-194]. VEGFs, while important for vasculogenesis and angiogenesis in placental development and growth, are also involved in the closely related process of inflammation[190].

Cytokines are involved in the regulation of immune and inflammatory responses. These small proteins, which include chemokines and interleukins, are produced mainly by helper T cells (Th) and macrophages[195]. Cytokines may act in an autocrine fashion but are also involved in paracrine and endocrine signalling to alter gene expression and initiate the action of other cells in immune responses[196]. Cytokine receptors are cell-surface transmembrane proteins which function as dimers[196]. Much of cytokine signalling occurs through Janus kinases (JAKs) and signal transducers and activators of transcription (STATs), or the JAK/STAT pathway[196]. Cytokine binding and dimerization of receptors results in two JAKs brought into close proximity to each other and subsequent transphosphorylation. Phosphorylated JAKs will recruit and phosphorylate STATs, transcription factors which will dimerize and translocate to the nucleus to regulate gene expression[196].

The VEGF family is comprised of 5 members: VEGF-A, VEGF-B, VEGF-C, VEGF-D, and placenta growth factor (PlGF)[197]. However, a number of isoforms of each family member exist due to alternative splicing. The VEGFs act through tyrosine kinase receptors VEGF receptor (VEGFR)-1/Flt-1, VEGFR-2/KDR, and VEGFR-3/Flt-4, and non-tyrosine kinase receptors neuropilin-1 and neuropilin-2[197]. The tyrosine kinase receptors can act as both homo- and hetero-dimers and bind VEGF family members with different affinities[197]. Upon VEGF binding and receptor dimerization, kinases are activated and autophosphorylate tyrosine residues of the intracellular domain. Phosphorylated tyrosines bind signalling molecules including Src and phospholipase C, activating a variety of cellular processes that can affect proliferation, permeability, and angiogenesis.

3.1.1 Cytokines and Pain

Pro-inflammatory cytokines, including IL-1β, IL-6, and TNFα, may play roles in pain, in addition to their inflammatory functions[198]. A growing body of literature supports the hypothesis that pro-inflammatory signalling is involved in pathological pain[195,198,199]. As described previously, NSAIDs exhibit their anti-inflammatory functions by inhibiting COX enzymes, blocking the production of prostaglandins and thromboxanes[108,109]. However, there is emerging evidence that NSAIDs can play a role in inflammatory signalling by cytokines[198]. NSAID treatment was found to decrease serum IL-6 and TNFα but increase the anti-inflammatory cytokine IL-10[198]. Additionally, cytokines can increase the expression of prostaglandins and COX, creating a feed-forward inflammation loop. The effects of NSAIDs on cytokine profiles in pregnancy has not been studied.

3.1.2 Cytokines in the Placenta

Throughout all stages of pregnancy, the placenta produces and responds to a wide range of cytokines, in fact, virtually all known cytokines have been found in the placenta[1,200,201]. While many aspects of pregnancy are classified by inflammatory processes, a precise balance of pro- and anti-inflammatory signalling is required for normal growth and development[1]. During implantation and early placentation, cytokines are released by the embryo and endometrial glands which are involved in the advancement of the trophoblast into the endometrium[201]. Of particular importance at this stage are the proinflammatory cytokines IL-6 and TNFα and the chemokine IL-8. The trophoblast is the primary location for cytokine production, however there is also evidence of cytokine production in the decidua, amniotic membrane, and stromal cells[200]. Interferons have been identified in the trophoblast, decidua, and amniotic membrane, as well as placental macrophages[201]. The trophoblast and decidua are responsible for production of both inflammatory cytokines (IL-1β, TNFα, IL-6, and IFN-γ) and anti-inflammatory cytokines (IL-4, -10, -13)[201]. These anti-inflammatory cytokines inhibit the production of pro-inflammatory cytokines and other inflammatory signals, and have been found in the trophoblast and decidua, but may not be produced in the fetal membranes[200].

The cytokine profile changes throughout pregnancy[1]. During implantation and placentation, a Th1, or primarily proinflammatory, phenotype is observed. In the second trimester as the fetus undergoes rapid growth and development, the cytokine profile shifts to Th2 phenotype[1]. As parturition approaches in the third trimester, Th1 cytokines are produced in advance of the inflammatory processes involved in labour[1,200]. Therefore, it is evident that cytokines play integral roles in the communication at the maternal-fetal interface for the entirety of pregnancy.

Dysregulation of cytokine signalling can have detrimental effects on pregnancy outcomes and fetal health, discussed below.

3.1.3 VEGFs in the Placenta

Similar to cytokines, VEGFs play important roles during implantation and placentation[193,202]. During this time, there is extensive vasculogenesis and angiogenesis to develop the vasculature of the placenta and remodel the uterine spiral arteries to facilitate low resistance blood flow[202]. In particular, VEGF-A is important for placental angiogenesis and influencing vascular permeability, therefore the levels of VEGF-A are highest during early placentation[202]. VEGF-C is involved in immune tolerance at the maternal-fetal interface, exhibiting feto-protective effects[202]. The production of VEGF-C is partially regulated by hypoxia, which explains its role in early pregnancy as the placenta develops under hypoxic conditions[25]. Further, VEGF-D stimulates the growth of vascular endothelia cells and is present in the syncytiotrophoblast and villous endothelium, and PlGF functions in the growth of the syncytiotrophoblast and invasion at the maternal decidua[202]. While VEGF-B is present in the placenta during early pregnancy, its function has not been fully elucidated[203]. The roles of VEGF-A, -C, -D, and PlGF in early pregnancy are essential for placental development as the vascular network develops, however VEGFs are present in the placenta throughout pregnancy[202]. In the third trimester, VEGF-A is localized mainly to the terminal villi and fetal endothelial cells[202]. Additionally, PlGF, which was first cloned from a human placental cDNA library, is highly expressed in the placenta throughout gestation, functioning in controlling growth and differentiation of the trophoblast[202,204]. Altogether, the VEGFs function to promote angiogenesis, growth of fetal endothelial cells, affect

permeability, and influence trophoblast function; interruption of any of these processes can lead to several placental and pregnancy complications, discussed below.

3.1.4 Pregnancy Complications Associated with Dysregulated Cytokine and VEGF Signalling

As described above, both cytokines and VEGFs have critical functions in all stages of pregnancy. Dysregulated cytokine and VEGF signalling together have been implicated in the pathophysiology of several pregnancy complications. These responses are well-characterized and understood in the contexts of gestational diabetes, preeclampsia, preterm birth, and recurrent pregnancy loss[194,205-207]. In gestational diabetes, placental pro-inflammatory cytokines are upregulated, particularly TNFα[208]. Additionally, placentas from pregnancies complicated with gestational diabetes are often hyper vascularized and have altered levels of VEGF-A, PlGF, and increased expression of VEGF receptors favouring a pro-angiogenic state[209-211]. Preeclampsia, which is characterized by decreased trophoblast invasion and minimal spiral artery remodelling, exhibits increased levels of cytokines produced in response to placental hypoxia, which disrupts the balance of pro- to anti-inflammatory signals[212,213]. Additionally, there is a decrease in the ratio of membrane/soluble VEGR receptors, thereby impairing angiogenesis and endothelial cell function[211]. Similarly, recurrent pregnancy loss and preterm birth are associated with a Th1 (pro-inflammatory) cytokine profile, inadequate spiral artery remodelling, and poor trophoblast invasion. Therefore, it has been proposed that high levels of pro-inflammatory cytokines activate processes normally involved in labour and can lead to preterm premature rupture of the membranes, cervical ripening, and preterm birth[214]. Increased levels of VEGF-A have also been

detected in preterm placentas[215]. VEGF-A is thought to be involved in the ripening of the cervix, which may define its role in preterm birth[215].

3.1.5 Cytokine and VEGF signalling in ART

While studies of pregnancy complications associated with dysregulated cytokine and VEGF signalling are relatively well defined, the effects on these signalling molecules specific to ART are understudied. However, conditions such as IUGR and preterm birth which are associated with dysregulated cytokine and VEGF signalling are more common in ART pregnancies[11]. It follows, that similar alterations in cytokines and VEGF signalling may be involved in the underlying pathophysiology of these increased risks of complications in ART. Previously, our laboratory has shown evidence of dysregulated inflammatory responses in a mouse model of assisted reproduction[145]. Placentas from murine ART pregnancies showed higher evidence of oxidative stress, increased levels of IL-6, and decreased levels of progesterone – a major anti-inflammatory steroid[145]. Additionally, our group has shown human placenta suppressors of cytokine signalling (SOCS) are dysregulated in assisted reproduction[216]. Signalling of IL-6 and SOCS are intricately linked with downstream signalling of the JAK/STAT and mitogen-activated protein kinase (MAPK) pathways which can influence further inflammatory signalling and tissue remodelling. SOCS3 is an upstream inhibitor of IL-6 secretion, opposes the actions of IL-6 by inhibiting JAK/STAT signalling, and preventing production of other inflammatory cytokines through MAPK pathway inhibition[217]. A diagram of this process is presented in Figure 3.1. Conversely, IL-6 activates JAK/STAT and MAPK signalling to influence inflammatory responses[217]. In ART, we found levels of SOCS3 were significantly lower, and association of SOCS/cytokines present in unassisted pregnancy were lost in ART[216]. More recently,

intracellular cytokine ratios were found to be elevated in over 40% of patients with a history of implantation failure in ART treatment[218]. Immunomodulation using an immunotherapy treatment regime of prednisolone, indralipid transfusions, and supplements led to improvements in implantation and miscarriage rates, however further trials are needed to confirm these results[218].

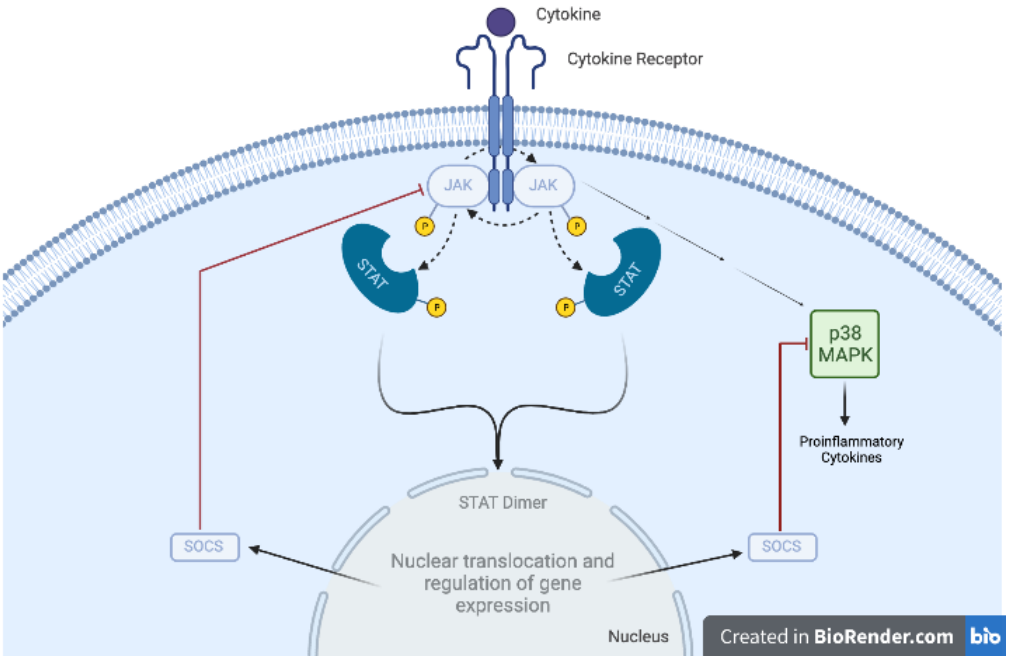

Figure 3.1 Cytokine signalling through the JAK/STAT pathway and SOCS inhibition of the JAK/STAT and p38 MAPK signalling pathways.
Figure created in Biorender.com.

With respect to VEGF signalling in ART, it has been demonstrated in sheep that expression of angiogenic factors are altered during early pregnancy in embryos fertilized using IVF[219]. Although there are no perfect animal models for human pregnancy, sheep have been studied extensively[54]. This is mainly due to the ability to implant catheters and monitors into the uterus and fetal vasculature without disrupting pregnancy[54]. Results from other animal models have confirmed altered expression of angiogenic factors and reduced vascularization. Johnson *et al.* identified decreased mRNA expression of VEGF, VEGFR-1 and PlGF in ART compared to natural mating in utero-placental tissues[220].

While it is well documented that pregnancy complications associated with altered VEGF signalling, such as preeclampsia and IUGR, are increased in ART, there are few efforts investigating these pathways in human ART and if they differ from unassisted pregnancy. In the following studies, we aimed to determine if cytokine and VEGF levels differ between placentas from unassisted pregnancies and ART pregnancies, and how these differences are related to pregnancy outcomes. The results add to a growing body of evidence of altered cytokine and VEGF signalling in ART, which can hopefully elucidate the etiology of pregnancy complications, and potentially in the longer term; aid in effective treatments to improve pregnancy outcomes.

3.2 Materials and Methods

Multiplex ELISA kits were purchased from Meso Scale Diagnostics (Rockville, MD, USA); all other chemicals were purchased from Sigma-Aldrich (Oakville, ON, Canada), unless specified.

3.2.1 Sample Collection

The umbilical cords (n = 380) used in this section are the tissues outlined in Chapter 2. The human placentas (n = 126) were collected immediately following birth for inclusion into the University of Hawaii Reproductive Biorepository (Honolulu, HI, United States of America). Prior to birth, written informed consent was provided for inclusion into the Biorepository and for use in future investigations. Samples were anonymized and deidentified prior to inclusion. Placentas were washed and snap frozen in liquid nitrogen and frozen at -80°C for future use. At The University of British Columbia, the primary project these tissues were to be used for was comparison of ART and unassisted pregnancy, and these studies were approved by the University's Clinical Research Ethics Board (H14-00092). Villous placenta samples, with

corresponding maternal plasma and cord serum, where available, were acquired from the biorepository based on matching criteria for ART and natural conception as outlined in Table 3.1. Matches were attempted to be made with 100% ethnicity match, gestational age +/- 5 days, and maternal age +/- 4 years, but criteria were loosened on subsequent rounds until 75 matches were made, of which only the top 56 matched cases were transferred to UBC from the Hawaii Biorepository.

Matching Round	Description	# of Cases
1	100% Match Ethnicity, Gestational Age +/- 5, Maternal Age +/- 4	37
2	100% Match Ethnicity, Gestational Age +/- 5, Maternal Age +/- 5	1
3	100% Match Ethnicity, Gestational Age +/- 6, Maternal Age +/- 5	3
4	100% Match One Race, Gestational Age +/- 6, Maternal Age +/- 5	11
5	100% Match All Races, Gestational Age +/- 6, Maternal Age +/- 5	6
6	80% Match All Races, Gestational Age +/- 6, Maternal Age +/- 5	2
7	75% Match All Races, Gestational Age +/- 6, Maternal Age +/- 5	0
8	50% Match All Races, Gestational Age +/- 6, Maternal Age +/- 5	3
9	0% Match Race, Gestational Age +/- 6, Maternal Age +/- 5	6
10a	50% Match All Races, Gestational Age +/- 6, No Maternal Age Match	2
10b	50% Match All Races, No Gestational Age Match, Maternal Age +/- 5	
11a	0% Match Race,Gestational Age +/- 6, No Maternal Age Match	4
11b	0% Match Race, No Gestational Age Match, Maternal Age +/- 5	

Table 3.1 Matching criteria and number of matches made on each round from The University of Hawaii Reproductive Biorepository.

3.2.2 Tissue Lysate Preparation

Placenta lysates were prepared in the same manner as the umbilical lysates, as previously discussed. Briefly, placenta pieces were thawed at room temperature and wet weight recorded. Tissues were mechanically homogenized in Tris-HCl buffer with 5 mM $MgCl_2$ and 2mM phenylmethylsulfonylfluoride (pH 7.4) 1:3 (w:v). Lysates were divided into 50 μL aliquots and frozen at -80°C until future use.

3.2.3 Bicinchoninic Acid Assay for Protein Content

The protein content in placental lysate samples was determined using the bicinchoninic acid (BCA) assay. The BCA assay is dependent on the formation of a protein-Cu^{2+} complex and subsequent reduction of the copper, which is proportional to the amount of protein present in the sample. A BCA Assay kit containing a BCA solution and a Copper (II) Sulfate Pentahydrate 4% solution was purchased from Sigma (Oakville, ON, Canada). The standard curve was made using bovine serum albumin (BSA), serially diluted in Tris-HCl, 10 mM $MgCl_2$ pH 7.4 to create a curve over the range of 0 -1000 μg/mL total protein. Placenta lysates were diluted 1/50 in buffer to place the concentration in the mid-range of the curve. BCA working reagent was made by combining eight parts of BCA solution and one-part Copper (II) Sulfate solution. To each well in a 96 well plate, 25 μL of diluted protein was combined with 200 μL of working reagent. The plate was incubated at 37°C for 30 minutes, allowed to cool to room temperature on the benchtop, and absorbance was measured at 562 nm using the Flex Station Plate Reader (Molecular Devices, San Jose, CA, The United States of America). All measurements were performed in triplicate and the mean of the readings was used to determine protein content. Precision was considered acceptable if the CV was <15%.

3.2.4 Validation of Commercial ELISA for Detection in Umbilical Cord Lysates

Cytokine and VEGF levels were measured using commercial multiplex ELISAs from Mesoscale Diagnostics (MSD) (Rockville, MD, The United States of America). Antibodies for the V-PLEX Proinflammatory Panel 1 Human Kit (Catalog No. 15049D-1) were selected to simultaneously detect and quantify IFN-γ, IL-1β, IL-6, IL-8, IL-10, and TNFα and the antibodies for the V-PLEX Angiogenesis Panel 1 Human Kit (Catalog No. K15190D-1) were selected to detect and quantify VEGF-A, VEGF-C, and VEGF-D. Standard curves using cytokine standards from the kit were made over the ranges outlined in Table 3.2 and ranges for standard curves for the VEGF panel are shown in Table 3.3. Kits were performed according to the manufacturer's instructions.

Analyte	LLOQ (pg/mL)	ULOQ (pg/mL)
IFN-γ	1.76	938
IL-1β	0.646	375
IL-6	0.633	488
IL-8	0.591	375
IL-10	0.298	233
TNFα	0.690	248

Table 3.2 Linear range of analytes for V-PLEX Proinflammatory Panel 1 Human Kit. Lower limit of quantitation (LLOQ), Upper limit of quantitation (ULOQ).

Analyte	LLOQ (pg/mL)	ULOQ (pg/mL)
VEGF-A	0.48	1510
VEGF-C	11.1	17500
VEGF-D	2.53	18800

Table 3.3 Linear range of analytes for V-PLEX Angiogenesis Panel 1 Human Kit. Lower limit of quantitation (LLOQ), Upper limit of quantitation (ULOQ).

The multiplex ELISAs were validated by the manufacturer for use in plasma and cell culture supernatant, however utility in human umbilical cord lysate needed to be assessed. To validate the specificity of the ELISA for detection of analytes in umbilical cord lysates, spiking studies

were performed along with unknown samples, blinded to the bench scientists. All samples were blinded by study number until screening and analysis was complete. Recovery of the spiked analytes was evaluated to determine any matrix effects.

3.2.5 Screening of Cytokines and VEGFs in Umbilical Cord Lysates

The umbilical cords screened in this chapter were also screened for NSAID exposure in Chapter 2. However, due to sample availability only 360/380 cords could be screened using the multiplex ELISAs. During method validation, correct dilution of umbilical cord lysates was also assessed. Dilutions of 4:5, 1:2, and 1:5 were evaluated. For umbilical cord lysate a 1:2 dilution with assay buffer showed the best values across the six cytokines in the panel, and a 4:5 dilution showed the best results for the angiogenesis panel. Due to costs association with the technology, samples were run in duplicate, but were rerun if the duplicates varied by more than 15%. These studies were performed with Camron Chehroudi, an undergraduate summer student working in the Centre for Blood Research under the Co-supervision of Dr. Hugh Kim and Dr. Abby C. Collier. The bench scientists alternated running the cytokine panel and the angiogenesis panel to avoid bias and human error.

3.2.6 Validation of Commercial ELISA for Detection in Placenta Lysates

Placental lysate also needed to be evaluated for use with the multiplex ELISAs. Similarly, spiking studies were performed to validate the assay and determine the specificity of the analytes. Positive control placentas were spiked with analytes from the kit and included in the screening, blinded to the bench scientist. Unblinding did not occur until all samples had been

screened. Selectivity and recovery of the analytes were evaluated from the results of these spiking studies.

3.2.7 Screening of Cytokines and VEGFs in Placenta Lysates

The placenta samples outlined above were also screened for cytokines and angiogenic factors using the multiplex ELISAs. Evaluation of lysate dilution revealed that a 1:5 dilution of placental lysate yielded the best results to put the samples in the mid-range of the curves for both the cytokine and the angiogenesis panels. Lysates were diluted in assay buffer. As with the umbilical cord samples, bench scientists alternated running the cytokine and angiogenesis panels to avoid researcher bias.

3.2.8 Demographic and Statistical Analyses for Association of Cytokine and VEGF levels in Umbilical Cord Lysate and Placenta Lysate with Pregnancy Outcome

Sourcing tissues from a biobank provides a number of benefits, mainly, a wide range of clinical information is collected along with the tissues. For both the umbilical cord samples and the placenta samples, we had access to approximately 35 ICD9/10 clinical chart codes covering maternal and fetal characteristics, obstetric, and neonatal outcomes. For umbilical cord lysates, we combined these results with the NSAID screening results outlined in Chapter 2. The levels of cytokines were compared in the NSAID positive and NSAID negative samples using a Student's t-test with Welch's correction for unequal variance. Additional analysis of the correlation and relationship of cytokines and VEGF molecules with continuous and discrete variables were performed by Camron Chehroudi and outlined in the results section.

For the placenta samples, we first compared the clinical chart codes in the unassisted pregnancy group to the ART group to establish any differences present between the two groups. Levels of cytokines and VEGF molecules were compared between ART and unassisted pregnancy groups using unpaired Student's t-test. This analysis was performed using GraphPad Prism 6.0 for Mac OSX (San Diego, CA, USA). The ART and natural conception groups were subsequently stratified using ICD9/10 clinical chart codes and reanalyzed for associations with analytes. Subgroups of ICD9/10 codes were only included in the analysis if there were a minimum of four samples in the group. Groups were assessed for normality and comparisons were made using a two-factor ANOVA and Bonferroni correction. Reorganization and analysis of data was performed using R and R Studio (Boston, MA, USA) and graphed using GraphPad Prism (San Diego, CA, USA).

3.3 Results

3.3.1 Multiplex ELISA Validation for Umbilical Cord Lysates

The multiplex platform has the advantage that very small pieces of tissue can be used for simultaneously detecting six cytokines or three angiogenic factors. Testing of different amounts of umbilical cord lysate revealed that between 10 and 25 μL of lysate was required for the cytokine panel (equivalent to approximately 2.5 – 6.25 mg of tissue) and 25-40 μL of umbilical cord lysate were required for the angiogenesis panel (approximately 6.25 – 10 mg of tissue).

Validation of the multiplex ELISA was performed by spiking umbilical cord lysates with analytes, which the bench scientist was blinded to until analysis was complete. Lysates were spiked with commercial standards of TNFα, IL-1β, and VEGF-D on two separate days. The bench scientist was able to detect the spiked samples every time, indicating good selectivity for

these analytes. The concentrations of spiked samples were subtracted from the concentration of non-spiked samples to determine the recovery for these analytes. The recovery of TNFα was 57 ± 5% of expected concentration (1150 ± 98 pg/mL compared to 2000 pg/mL expected). Recovery of IL-1β was 33 ± 10% (17 ± 5 pg/mL compared to 50 pg/mL expected) and recovery of VEGF-D was 7 ± 4% (2 ± 1 pg/mL compared to 30 ng/mL expected). Due to matrix effects of umbilical cord, detection is lower than absolute levels of analytes. Matrix effects are not unexpected with this tissue, as we have experienced problems in the past. Therefore, it is highly possible that concentrations of these analytes will be missed if they fall on the lower end of the curve. Increasing the amount of lysate up to 40 μL was helpful for some analytes that fell below the LLOQ, but some samples were still out of the detection range. Therefore, it is important to note that absolute values of cytokines and angiogenic factors are not reported, but relative comparisons between groups are valid for analysis as all samples were subject to the same level of matrix and/or storage effects.

Of the 380 umbilical cords screened, IFN-γ was detected in 34%, IL-10 was detected in 74%, and TNFα was detected in 99%; IL-1β, IL-6, and IL-8 were detected in all samples. For the angiogenesis panel, VEGF-A was detected in 48% of umbilical cord samples, VEGF-C was detected in 4% of samples, and VEGF-D was detected in 35% of samples.

3.3.2 Correlations of Cytokines and VEGF Molecules in Umbilical Cord with Continuous Variables

The following analyses were performed with Camron Chehroudi for his undergraduate summer student research project and summarized here for completeness. Correlation analyses were

performed using Pearson's or Spearman's tests, as appropriate, between cytokine and VEGF levels in the umbilical cord and the following continuous variables: maternal age (years), gestational age (days), baby weight (grams), baby head circumference (cm), baby chest circumference (cm), length of membrane rupture (cm), amount of blood loss (mL), and length of hospital stay (days). There were no biologically significant correlations observed between any of the analytes and these variables. The r^2 values were below 0.2 for all analyses, indicating poor biological significance.

3.3.3 Differences Between Cytokine and VEGF Molecules in Umbilical Cord with Discrete Variables

For clinical chart information with binary outcomes, unpaired t-tests were performed with Welch's correction for unequal variance. Only groups with four or more cases were considered for analysis. No differences were observed in relative cytokine and VEGF levels and the clinical outcomes as follows: β-streptococcus or chlamydia infection, fetal bradycardia, fetal distress, fetal heart trace monitoring, gestational hypertension, IUGR, maternal anaemia, mild pre-eclampsia, neonatal respiratory distress syndrome, and placental abruption. Additionally, there were no significant differences observed for the analytes in IFN-γ, VEGF-C, or VEGF-D with any of the variables described in the clinical charts of the women and their neonates.

Significant differences were observed for the analytes IL-1β, IL-6, IL-8, IL-10, TNFα, and VEGF-A. Levels of IL-1β were significantly decreased in severe pre-eclampsia and gonococcus infection and significantly increased in premature rupture of the membranes, chorioamnionitis, meconium passage in utero, and NICU admission. Interleukin-6 was decreased in cases of severe

pre-eclampsia, oligohydramnios, gonococcus infection, caesarean section, and repeat caesarean, but significantly increased in cases of chorioamnionitis and meconium passage in utero. The pro-inflammatory cytokine TNFα was decreased in severe pre-eclampsia and gonococcus infection but increased in chorioamnionitis.

Interleukin-8, which is classified as a chemokine but is involved in the pro-inflammatory response, showed significant decreases in several adverse maternal and fetal outcomes. Notably, levels were decreased in gestational diabetes, premature labour, premature birth, chromic maternal hypertension, oligohydramnios, caesarean section, and repeat caesarean section. As was observed with the classic pro-inflammatory cytokines, the levels of IL-8 were increased in chorioamnionitis and meconium passage in utero. In contrast to these results, IL-10, the only anti-inflammatory cytokine included in the panel, showed the opposite pattern for many of the clinical outcomes that were significant for the pro-inflammatory cytokines. Levels of IL-10 were decreased in gonococcus infection and repeat caesarean section, but increased in membrane rupture, premature rupture of the membranes, chorioamnionitis, meconium passage in utero, and admission to the NICU.

In the angiogenesis panel, VEGF-A, which was the most routinely detected VEGF isoform, was significantly decreased in 12 of the clinical outcomes investigated in discrete variable analysis. The outcomes affected include maternal variables, labour and delivery variables, placental abnormalities, and neonatal outcomes. More detailed results of this study can be found in Chehroudi *et al.* 2019[221].

3.3.4 Relationships Between NSAID Exposure and Levels of Cytokines in the Umbilical Cord

The results of the NSAID screening study in Chapter 2 were combined with the cytokine panel results to determine any relationships between NSAID exposure and inflammation. Due to low numbers of NSAID positive samples, analyses were not as robust as originally planned. Differences in cytokine levels could not be assessed between the different NSAIDs or levels of NSAID exposure. As such, the NSAID positive samples were grouped together and compared against NSAID negative samples. There were no differences observed in the levels of cytokines between NSAID positive and NSAID negative samples (Figure 3.2). Comparisons could not be made between NSAID exposure and clinical outcomes using ICD 9/10 codes due to low numbers (less than 4 samples per group).

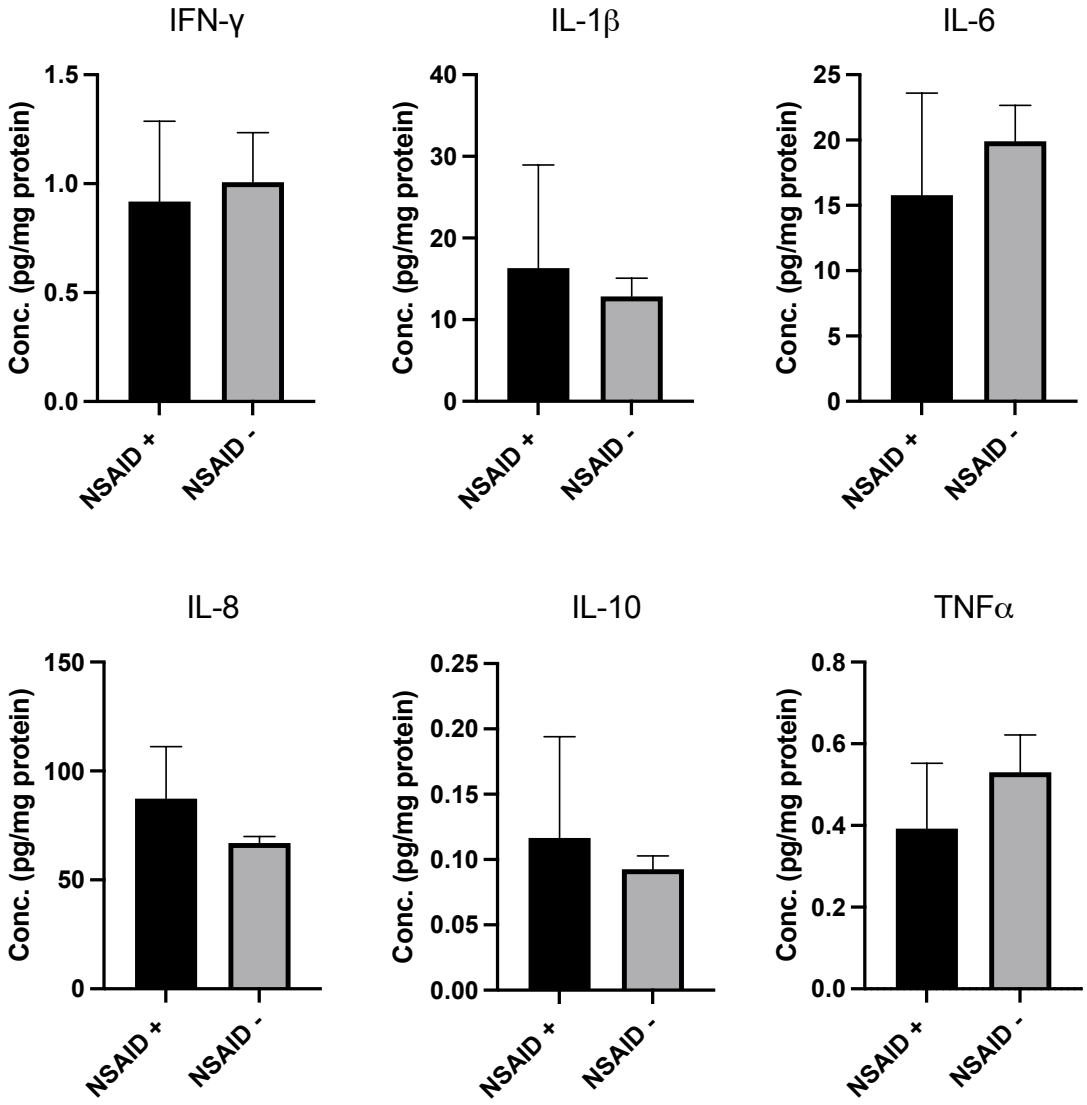

Figure 3.2 Levels of cytokines in NSAID positive and NSAID negative umbilical cord samples.
Bars are mean ± SEM.

3.3.5 Multiplex ELISA Validation for Placenta Lysates

As observed in the umbilical cord screening, the multiplex ELISA has the advantage of detecting multiple analytes with very small amounts of tissue required. For both the cytokine and angiogenesis panels, 25 μL of villous placental lysate are required, meaning approximately 6.25 mg of placental tissue are necessary for analyte detection.

Validation was performed by spiking known concentrations of calibration standards into placental samples, blinded to the bench scientist, to determine method selectivity and recoveries. The bench scientist accurately detected the spiked samples, and determined the concentration, except for VEGF-A which was undetectable even when spiked at the high end of the calibration curve. The method was selective for all other analytes investigated. The recoveries of each analyte are presented in Table 3.4. Due to the matrix effects of placental lysate, detection is lower than absolute levels of analytes with the exception of IL-8. Accordingly, low levels of analytes may be missed, increasing the rate of Type II/false negative error. Absolute quantitation is only possible for IL-8 using this method, but relative comparisons may be made between groups. For experimental villous placental lysates, IL-1β, IL-6, IL-8, TNFα, VEGF-C, and VEGF-D were detected in all placental lysates tested. IL-10 was detected in 77% of lysates, INF-γ was detected in 66% of lysates, and VEGF-A was never detected.

Analyte	**Recovery in Placental Lysates (Mean +/- SD)**
IFN-γ	45.6% +/- 3.3
IL-1β	84.4% +/- 3.2
IL-6	81.3% +/- 1.7
IL-8	109.9 % +/- 0.9
IL-10	47.2% +/- 2.0
TNFα	57.9% +/- 5.3
VEGF-A	-
VEGF-C	87.3% +/- 13.3
VEGF-D	66.4% +/- 5.2

Table 3.4 Analyte recoveries using the MSD Multiplex ELISA platform.
Assessed in duplicate over 3 days of analysis.

3.3.6 Correlations of Cytokines and VEGF Molecules in Villous Placenta with Continuous Variables

Correlation analyses were performed between relative cytokine and VEGF levels in the placenta and the continuous variables maternal age (years), gestational age (days), maternal weight gain in pregnancy (kg), maternal BMI (kg/m^2), baby weight (grams), baby length (cm), and baby head circumference (cm). There were no biologically significant differences observed with the continuous variables listed above. Biological significance was defined as a correlation coefficient (r^2) above 0.2.

3.3.7 Differences Between Cytokine and VEGF Molecules in Villous Placenta with Discrete Variables

The levels of cytokines and VEGF isoforms in the placenta were first compared between ART and unassisted pregnancies, however no statistically significant differences were found between the groups. Data were further stratified from ART and unassisted pregnancy into groups corresponding to ICD9/10 chart codes that are discrete variables. No significant differences were observed between ART and unassisted pregnancy stratified into groups for augmentation, delivery method, gestational diabetes, induction of labour, IUGR, membrane rupture, preterm birth, or small for gestational age infants (*data not shown*).

There were two situations in which dysregulated inflammatory signalling was observed in ART pregnancies: in pregnancies complicated by chorioamnionitis and in twin pregnancies. In cases of chorioamnionitis, ART attenuated placental signalling (Figure 3.3). Across all six cytokines included in the panel, there was a trend of increased cytokine levels in placentas from unassisted

pregnancies complicated by chorioamnionitis. The levels of IL-1β, IL-6, and IL-8 were significantly increased ($P < 0.05$). In contrast, the levels of IFN-γ were significantly decreased in ART placentas with chorioamnionitis compared to ART placentas without chorioamnionitis. The same trend was observed in ART placentas for levels of TNFα (Figure 3.3).

In twin compared to singleton pregnancies, ART also attenuated inflammatory signalling (Figure 3.4). The relative levels of IL-1β, IL-6 and TNFα were significantly decreased in naturally conceived twins compared to naturally conceived singletons, while the decrease in IL-8 was approaching significance ($p = 0.06$). In twins conceived as a result of ART, the dampening of inflammatory signalling in the placenta was not observed. While not significant, all six cytokines had a trend of increased concentration compared to singleton pregnancies, and ART twins had significantly higher levels of TNFα compared to naturally conceived twins (Figure 3.4).

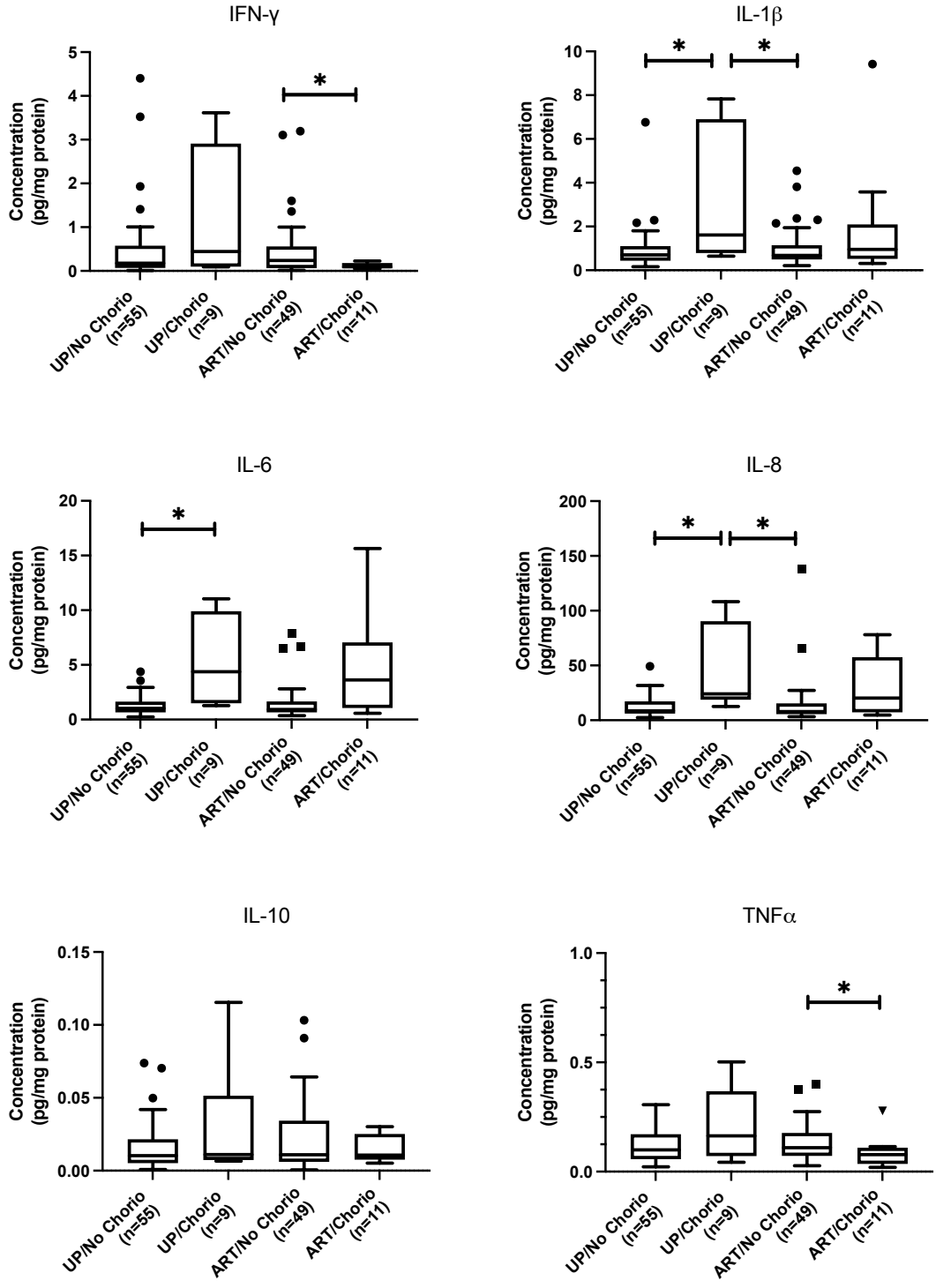

Figure 3.3 Levels of cytokines in human placenta. Unassisted pregnancies, without and with chorioamnionitis vs. assisted reproduction pregnancies, with and without chorioamnionitis.

Tukey method used to plot whiskers and outliers. Unassisted pregnancy (UP), Assisted reproduction pregnancy (ART), Chorioamnionitis (Chorio), No chorioamnionitis (No Chorio). * $p < 0.05$.

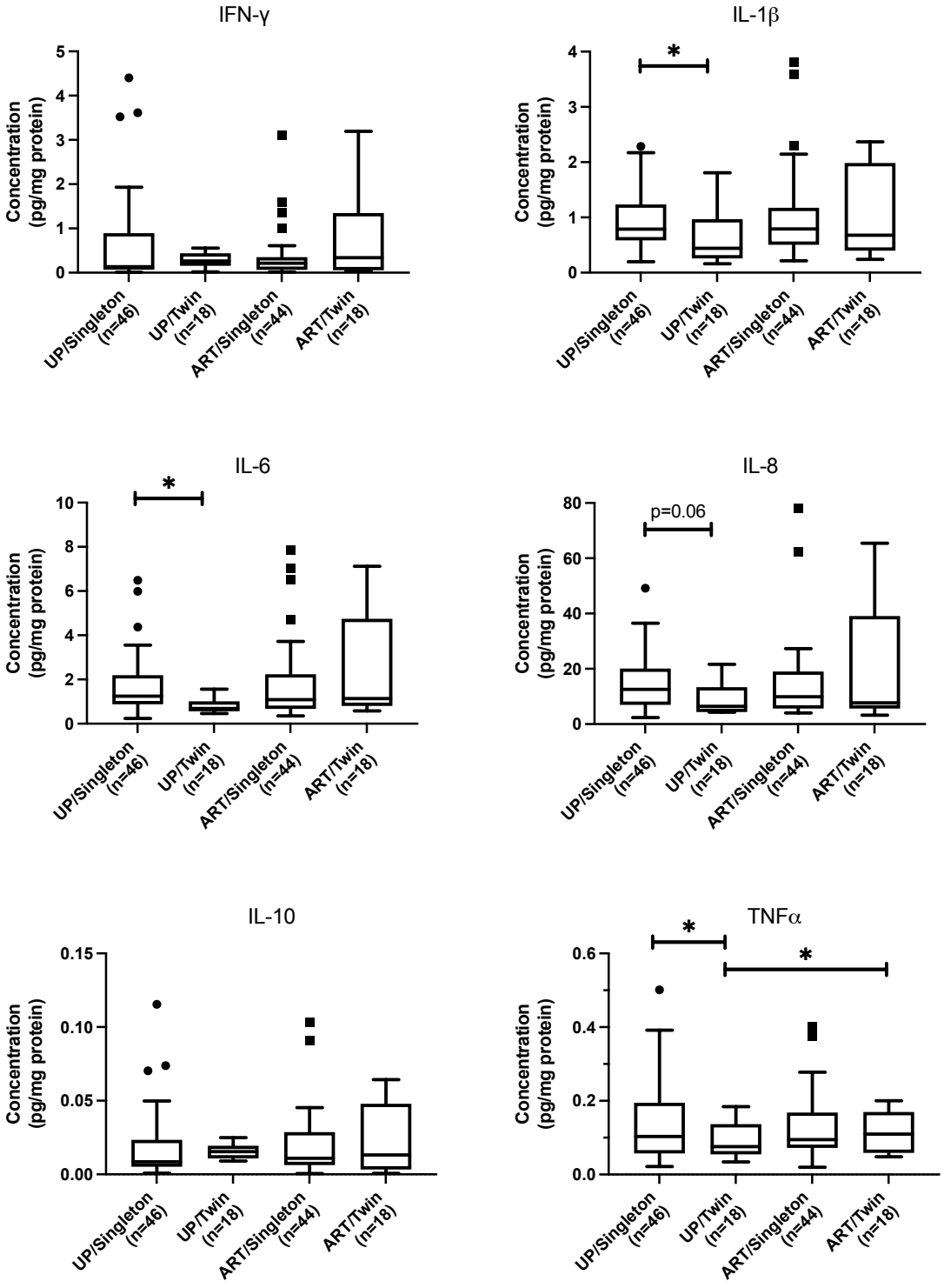

Figure 3.4 Levels of cytokines in human placenta. Unassisted pregnancies, singleton and twin vs. assisted reproduction pregnancies, singleton and twin.

Tukey method used to plot whiskers and outliers. Unassisted pregnancy (UP), Assisted reproduction pregnancy (ART). * $p < 0.05$.

3.4 Discussion

3.4.1 Use of Multiplex ELISA Platform for Measuring Cytokines and VEGFs in Human Umbilical Cord and Placental Tissue

In this study, we determined the utility and validated the use of commercial multiplex ELISAs for use in reproductive tissues (umbilical cord and placenta). The methods for both cytokine and VEGF platforms were reliable, as a blinded bench scientist was always able to detect spiked samples. While only relative quantitation is possible due to matrix effects of these tissues, in case-control studies such as these, comparison using relative quantitation is valid. Samples were prepared and run under the same conditions, with positive and negative controls making these analyses valid. However, most of the analytes had below 100% recovery due to matrix effects, meaning the rate of Type II error (false negative) is increased in our dataset. Another implication of this is our results are conservative, as we are missing data on the lower end of the linear range. An advantage of the multiplex platform is the ability to simultaneously detect analytes in a single run. This not only saves time but has the advantage of requiring very small sample volumes to generate a large amount of data. While human umbilical cord and placentas are large and generally easy to collect since they are regarded as medical waste, a matched unassisted and ART pregnancy cohort such as this one is precious and takes years to collect. Accordingly, care should be taken to avoid sample waste. Additionally, for murine models of ART sample amounts are much smaller and the MSD platform could provide added benefit.

An additional finding in this study is the detection and quantitation of specific analytes differs between umbilical cord and placenta. Umbilical cords and placentas used in these studies were collected in the same time period and processed to lysates using identical methods. In umbilical

cord lysates, VEGF-A was the predominant isoform detected while VEGF-C and VEGF-D were rarely detected[221]. However, in placenta the opposite pattern was observed, with VEGF-A never detected and VEGF-C and VEGF-D were detected in 100% of samples. In other studies, the VEGF-A isoform has been detected in the placenta by measuring mRNA or in supernatant of cultured placental cells[222-224], therefore the MSD multiplex ELISA platform may not be appropriate for evaluating VEGF-A levels in placenta, where recovery was only 57.9%. VEGFs are critical throughout decidualization, placentation, and pregnancy. Specifically, VEGF-A is involved in early angiogenesis and VEGF-D is more involved in trophoblast function, mitogenesis, and later vessel development in the placenta[202]. Dysregulation of VEGF is involved in the pathophysiology of maternal hypertensive disorders, gestational diabetes, and IUGR, therefore other methods such as PCR or Western blot may be more appropriate to study VEGF expression in studying these complications in relation to ART.

3.4.2 The Association of Pregnancy Outcome with Cytokine Levels

We identified that villous placental cytokine levels are altered in ART pregnancies, in the context of pregnancies complicated by chorioamnionitis and twin pregnancies. In contrast to the results of studies from our laboratory using a mouse model of ART, we did not observe any differences between ART and unassisted pregnancy groups, until stratified analysis[145]. Only 1-4% of births are complicated by chorioamnionitis, but the rate in preterm births is 40-70%[225]. Prematurity is the most significant cause of perinatal morbidity and mortality worldwide. Risk factors for chorioamnionitis include PROM, prolonged labour, African American ethnicity, meconium passage in utero, smoking, and bacterial vaginosis, however, ART has not been shown to be associated with chorioamnionitis[226-229]. Chorioamnionitis is characterized clinically

by the invasion of neutrophils and inflammation at the placenta and fetal membranes[225]. Chorioamnionitis most often occurs by an ascending infection of the genital tract, but there can be cases of "sterile" inflammation, where cytokines and chemokines are present and characteristic of chorioamnionitis, but no infectious microorganisms can be detected[230,231]. Studies have found significant increases in IL-1β, IL-6, IL-8 and TNFα in the amniotic fluid of women with ascending infections[232]. The maternal and placental response to infection, mainly release of inflammatory markers and chemokines, causes an inflammatory response that mimics the natural progression of labour[233,234]. Both IL-1β and TNFα stimulate synthesis of prostaglandins in the amnion, chorion, and decidua by increasing expression of COX-2[234]. Cytokines may also increase the expression of prostaglandin receptors to perpetuate their actions. As such, chorioamnionitis often results in preterm birth[235].

In the cohort described here, 5/8 placentas from the unassisted pregnancy group and 4/10 placentas from the ART group complicated by chorioamnionitis were also preterm. One set of twins were in each subgroup. The activation of labour processes and preterm birth is feto-protective, to activate the inflammatory responses of the immune system and remove the fetus from potential harmful stimuli[236]. It should also be noted that the incidence of chorioamnionitis in this cohort is much higher than the overall incidence of 1-4%. The reasons for this are unknown, but importantly rates were higher in both ART and unassisted pregnancy, meaning our results are not confounded by higher rates of chorioamnionitis in one group.

Chorioamnionitis is a serious condition and increases risks for maternal and/or fetal sepsis, fetal brain injury, respiratory distress, and fetal illness following birth[235]. Antibiotics are the first-line

treatment for chorioamnionitis but are not effective in preventing the inflammation-associated morbidities[225]. There is emerging work into the development of therapeutic approaches to treat and prevent the potentially dangerous effects of chorioamnionitis[237]. Blockade of TNFα signalling has been investigated and showed improved pregnancy outcomes in murine models[238,239]. Further studies in humans found treatment may prevent spontaneous abortion, but the antibodies crossed the placenta and were detectable after birth, raising concerns of immunosuppression[240]. Similar research has been done on IL-1 inhibitors, but evidence in humans is sparse and the effectiveness of preventing preterm birth debated[241]. As novel treatments emerge, there should be consideration of how ART affects inflammatory processes in response to chorioamnionitis, as it may present differently than in unassisted pregnancies.

Secondly, we identified a differential inflammatory response between twins and singletons in unassisted pregnancies that is not present in ART. Across all cytokines in the multiplex panel, placental levels were similar or lower in twins compared to singletons in unassisted pregnancies, indicating the immune system appears to be dampened. While this has not been noted in the literature previously, it could indicate an adaptive response to protect the multiple fetuses from the maternal immune system[242]. Pregnancy is a unique immunological state, where the immune system must be active to protect the mother and fetus from infection, but modulated to prevent the identification of the fetus as "not-self". This is especially important at the maternal-fetal interface, where is has been demonstrated that the levels of pro-inflammatory IL-10 and the ratio to uNK cells is critical in maintaining pregnancy[243-245]. In ART pregnancies, cytokine levels were not statistically different between singletons and twins, and levels of TNFα were significantly higher in ART twins compared to naturally conceived twins. This reversal in pattern

of placental cytokine levels could be contributing to the increased incidence of complications in twin ART pregnancies. Risks associated with multiple pregnancy affect both the mother and fetus and include preterm birth, pre-eclampsia, embolism, and heart failure[246]. The frequency of multiple pregnancy in ART has decreased as techniques have improved upon implantation rates and physicians favoured single embryo transfer when the underlying cause of fertility was not expected to affect implantation[95]. However, in most countries where ART is commonly used there are no legal regulations on the number of embryos that can be transferred. In Canada in 2006, the Canadian Fertility and Andrology Society and the Society of Obstetricians and Gynecologists published a set of joint guidelines to minimize multiple pregnancy and improve birth outcomes, and 89% of IVF cycles were compliant with these recommendations[247]. However, in older patients or patients with a poor prognosis, up to three embryos may be transferred in specific situations, therefore with increasing use of ART it is important to understand the pathophysiology of complications in twin pregnancies to improve outcomes[247].

Inter-individual variation is important to discuss with respect to the results of this study. The number of placentas to be included in each cohort were powered *a priori* using studies with a cohort of placentas from a mouse model of ART in our laboratory. Mice were inbred and housed in facilities with consistent diet, temperature, and light conditions and therefore not representative of the inter-individual variation observed in humans. To partially mitigate the effects of variation in humans, the groups were baseline matched for gestational age, maternal age, ethnicity, and singleton vs. twin pregnancy.

Secondly, the underlying causes of infertility or reasons for utilizing ART vary widely, and were unknown for this cohort, which may have an effect on the outcomes measured. While we cannot account for all environmental factors, all placentas were collected from Kapi‘olani Medical Centre in Honolulu, an environment distinctly different from the continental US or Canada. The preliminary matching was effective for mitigating variation in the primary analysis between ART and unassisted pregnancy, however matching was lost in secondary stratification for pregnancy outcome using ICD9/10 clinical chart codes. The consequences of this are two-fold. First, power may be lost in secondary analysis and second, confounding of variables in stratified groups can occur. However, significant results for both chorioamnionitis and twin pregnancy show strong signal-to-noise indicating power was not lost upon stratification. We also evaluated the means for gestational age and maternal age in stratified chorioamnionitis and twin groups and did not find any significant differences, with the exception of gestational age in twin pregnancy, which was shorter, as expected. Additionally, a well-documented difference between ART and natural conception is the number of vaginal births vs Caesarian sections, with Caesarian being significantly higher in ART pregnancies. While this trend was observed in our sample set, there were no significant differences in the levels of cytokines or VEGFs between delivery method in either group. Other significant differences between the natural conception and ART groups were the rates of gestational diabetes and high blood pressure, which were higher in the ART group, but no significant associations were found between cytokine levels and either clinical outcome.

3.4.3 Limitations of the Study

Certain limitations should be discussed with respect to the findings of this study. As discussed in Chapter 2, the umbilical cords used in this study were archival samples, which had been stored

for approximately 5 years at -80 ºC before screening occurred. The placentas were archived as well, having been collected in Hawaii prior to being shipped to The University of British Columbia and processed into lysates in 2018. The stability of cytokines and VEGFs in placenta has not been extensively studied, but it is well documented that sample collection, handling, and storage in general can affect cytokine measurements in biological samples. In blood samples, cytokine levels will increase more rapidly at increasing storage temperature[248]. There is evidence that cytokines will remain stable for two years at -80 ºC but may decrease 10-20% in years following[248]. Additionally, repeated freeze-thawing decreases overall cytokine levels. Efforts were made to minimize the negative effects of storage and processing on sample stability. Original placenta samples were collected and immediately frozen in liquid nitrogen before freezing at -80 ºC. Samples were kept frozen when pieces were cut and shipped to The University of British Columbia on dry ice. Importantly, both the unassisted and ART placentas were collected over the same time period and treated in the same manner. Samples were only thawed once when processed to lysate which was immediately aliquoted and stored at -80 ºC; lysates used for assays were never freeze-thawed. Concern has also been raised over potential for interaction between multiple antibodies and cytokines in multiplex assays, and direct comparison between ELISA and multiplex platforms are rare[249]. Here, we have noted that absolute quantitation cannot be performed due to matrix effects, but our comparison analysis is valid because the matrix effect would similarly affect all analytes allowing relative comparisons, and confidence in our studies increased due to the cautious and equal treatment of samples from collection to performing the assay.

3.5 Summary

In summary, this is the first report in humans that inflammatory signaling in the placenta is attenuated in the contexts of chorioamnionitis and multiple birth when ART is the method of conception. The processes of inflammation and inflammatory signaling are complex and cover more pathways than discussed here. However, these data provide direction for further investigation of the effects of chorioamnionitis and twin pregnancy in ART. A concurrent study in our laboratory identified lower SOCS3 in syncytiotrophoblast of ART placentas compared to unassisted conception[216]. This was a pilot study, which used a subset of the placentas included in this study and investigated the differences of SOCS 1, 2, and 3. In addition to altered SOCS3 expression, differential expression patterns were identified between ART and natural conception. Positive correlations were observed between SOCS1/IL-10, SOCS2/IFN-γ and SOCS3/IFN-γ in unassisted conception pregnancies, but like the patterns observed here, these patterns were lost in ART[216]. Future studies should investigate up-stream and down-stream mechanistic signaling pathways of cytokines, including SOCS and Jak/STAT.

This was the first study, to our knowledge, to identify differences in the cytokine profiles of the placenta between singleton and twin pregnancies. Evidence that these differences may be lost in ART provides context for the increased complications observed in multiple pregnancies achieved using ART. Additionally, we identified differences in the levels of immunomodulatory cytokines in chorioamnionitis, which likely affects downstream signaling of inflammatory mediators and cellular function. While these results are novel to the ART field, the impact of the results presented in this chapter extends beyond ART to all singleton vs. twin pregnancies and immunology studies of the maternal-fetal interface.

Chapter 4: Dysregulation of the Antioxidant Defense Network in the Human Placenta in the Context of Assisted Reproduction

4.1 Introduction

Within living organisms, a balance between the production and scavenging of reactive oxygen species (ROS) is critical for proper cell function and survival. In mammalian cells, ROS including superoxide anion (O_2^-), hydroxyl anion (OH^-), and hydrogen peroxide (H_2O_2) are products of normal cellular metabolism and function[250]. While low levels are essential for normal cellular process, when levels are too high damage can result as they modify proteins, nucleic acids, and other cellular structures[250,251]. The mitochondria are the main organelles involved in ROS production, as respiratory chain oxidative phosphorylation produces high levels of ROS[250]. Despite mitochondrial mechanisms present to manage ROS, levels produced are higher than can be dealt with within the organelle. Additionally, ROS are produced during the metabolism of arachidonic acid to prostaglandins and leukotrienes by lipoxygenases and COX enzymes[252,253].

In order to scavenge excess free radicals and prevent cellular damage, an enzymatic antioxidant defense network in present in cells. This network is made up of important enzymes including superoxide dismutase (SOD), glutathione peroxidase (GPx), catalase, glutathione reductase (GR), and glutathione S-transferase (GST)[254]. Certain vitamins and minerals are also antioxidants, providing a non-enzymatic method of antioxidant defense[254]. Important antioxidant vitamins are vitamins A, C, and E. Vitamin A is capable of quenching a singlet oxygen radical, forming a more stable compound that does not confer cellular damage[255]. Both vitamin C and E stop lipid peroxidation in membranes, forming tocopheroxyl radicals and ascorbyl radicals which are non-damaging and can be reduced by glutathione dependent enzymes[256]. An abbreviated diagram of the antioxidant defense network is presented in Figure 4.1.

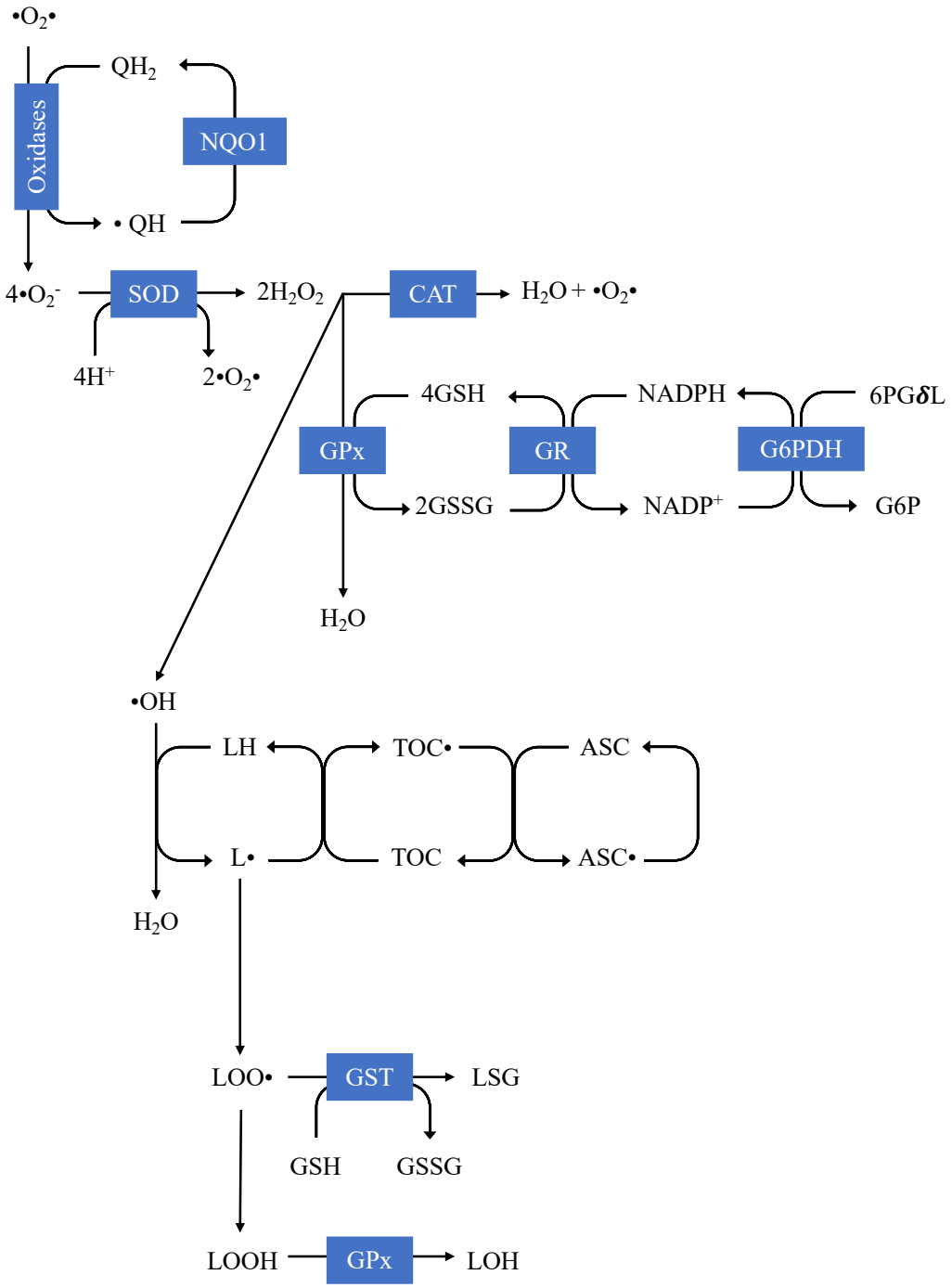

Figure 4.1 Abbreviated diagram of the antioxidant defense network.
Enzymes in blue boxes. 6-phosphoglucono-*δ*-lactone (6PG*δ*L), Ascorbic acid/Vitamin C (ASC), Catalase (CAT), Glucuse-6-phosphate (G6P), Glucose-6-phosphate dehydrogenase (G6PDH), Glutathione peroxidase (GPx), Glutathione reductase (GR), Glutathione (GSH/GSSH), Glutathione-S transferase (GST), Linoleic acid (LH), Lipid hydroperoxides (LOOH/LOO), Inert lipid alcohol (LOH), Glutathione conjugated lipid (LSG), NADPH dehydrogenase quinone 1 (NQO1), Superoxide dismutase (SOD), Tocopherol/Vitamin E (TOC), Ubiquinol (QH/QH_2). Original figure.

In the placenta, an antioxidant defense network is present within the trophoblast[257]. Pregnancy is categorized as a state of oxidative stress, and increased metabolism by mitochondria in the placenta outstrips the ROS scavenging ability in the trophoblast[257]. A balance between ROS production and scavenging is critical for successful implantation, placentation, and embryogenesis[258,259]. In different stages of pregnancy and placentation, excess ROS production can lead to specific pathologies[258]. As in other cells, the mitochondrial electron transport chain is responsible for the majority of ROS production in the placenta. It is estimated that in a 60 kg woman, 160-320 mmol of superoxide anion are produced each day[260]. ROS are also produced in the placenta by xanthine oxidoreductase, NADPH oxidase, nitric oxide synthase, and heme oxygenase[261-264].

The placenta contains the major antioxidant systems: glutathione and glutathione associated enzymes (GST, GR, GPx), SOD, catalase, oxidoreductases, and the antioxidants vitamin C and E. There may be regional difference in the expression of placental antioxidant enzymes related to the degree of oxidation as it relates to maternal blood flow; expression of catalase and GPx were higher in the trophoblast more central to the placenta[265]. As discussed in Chapter 1, the placenta develops under hypoxic conditions in early pregnancy, as the EVT form trophoblastic plugs which block maternal blood flow to the placenta[25]. At the end of the first trimester, there is a sharp rise in oxygenation when the spiral arteries open, which can potentially cause oxidative stress and cellular damage[69]. Placental antioxidant defenses are present by this point to combat ROS production. Increasing oxygenation of the placenta is associated with increased antioxidant enzyme mRNA, and reperfusion of the placenta leads to increased expression of these enzymes[266].

4.1.1 Glutathione S-Transferase

There are three GST families: cytosolic, mitochondrial, and microsomal. Cytosolic and mitochondrial GSTs are distantly related soluble enzymes with some structural similarities, while microsomal GSTs are structurally different membrane-bound proteins, also referred to as membrane-associated proteins in eicosanoid and glutathione metabolism (MAPEG)[267]. These enzymes catalyze the addition of glutathione (GSH) to compounds with an electrophilic carbon, nitrogen, or sulfur. GSH is a tripeptide (cysteine, glycine, and glutamic acid) found in essentially all cells which can be present in reduced (GSH) form and or the dimerized/oxidized form (GSSG). Reduced glutathione serves as the nucleophilic co-substrate for GSTs.

Of the cytosolic GSTs, which exist as dimers and are named based on their subunit composition, only GSTP1 expression and activity is well characterized in human placenta[268]. GSTs are considered protective enzymes because their action almost always leads to less active products which are more easily excreted, and they are involved in detoxification of oxidative stress products. Exogenous substrates include pesticides, insecticides, chemotherapeutic agents, and epoxides generated from environmental carcinogens[269]. GSTs are also involved in scavenging of free radicals produced through cellular respiration. Further, peroxidation products of membrane lipids by free radicals may also be substrates of GSTs, whereby the conjugation with GSH prevents action of these harmful electrophiles within membranes[268]. GST activity is partially dependent on the oxidative state of the cell, activity of GR, levels of cellular thiols, and ratio of GSH/GSSH[270].

4.1.2 Glutathione Peroxidase

Glutathione peroxidases are another family of cytosolic, glutathione dependent enzymes involved in detoxification of ROS[271]. These enzymes catalyze the reduction of H_2O_2 into water using reduced glutathione as an electron donor. There are currently eight mammalian GPx enzymes described, which exhibit tissue specific expression. Most GPx enzymes identified in humans are selenoproteins, and GSH regenerates the reduced from of selenocysteine within the protein complex[271]. With the exception of monomeric GPx-4, 7, and 8, these proteins exist as tetramers and can are important for the reduction of lipid hydroperoxides to their unreactive alcohols in addition to free H_2O_2[271].

$$H_2O_2 + 2\ GSH \rightarrow GSSG + 2H_2O$$

Equation 4.1 Reaction catalyzed by glutathione peroxidase.
Reduction of hydrogen peroxide to water.

GPx1, 3, and 4 are all expressed in cytotrophoblast and Hofbauer cells within the placenta[272]. Comparison of expression across the placenta has identified differential localization for GPx3 and 4, which may relate to levels of oxygenation within the placenta, but distribution of GPx1 appears to be uniform[272]. Additionally, GPx3 is the predominant GPx isoform in plasma, and the placenta produces this extracellular form of GPx and secretes it into the maternal circulation[273]. Hypoxia is thought to up-regulate GPx3 expression, which may help prevent damage from oxidative stress when high levels of oxygen enter the placenta after it develops under hypoxic conditions[273].

4.1.3 Glutathione Reductase

While not directly involved in the scavenging of free radicals, GR is an important contributor to the antioxidant defense network because it catalyzes the reduction of glutathione disulphide (oxidized glutathione, GSSG) to GSH. It is therefore essential for restoring the intracellular GSH levels required for GST and GPx[270]. A high GSH/GSSG within cells is essential for protection against oxidative stress[270]. Cysteine containing structures like GSH are readily oxidized, accepting an electron from a ROS which leads to formation of a disulphide bridge between two GSH molecules[274]. The reduction reaction to regenerate GSH is dependent on the presence of NADPH and flavine adenine dinucleotide (FAD). In fact, across species GR contains two highly conserved domains: an FAD binding-domain and NADPH binding site[274]. GR exists in both the cytosol and the mitochondria, and evidence shows that a single gene expresses both forms of the enzyme[274]. GR activity has been measured in first trimester placental lysate at a level higher than that of blood cells, and is sufficient to maintain almost all GSH in reduced form in the organ, indicating the importance of this enzyme from early in pregnancy[275].

4.1.4 Superoxide Dismutase

In contrast to the other enzymes discussed here, SOD is a non-glutathione dependent enzyme involved in the antioxidant defense network. As its name suggests, SOD catalyzes the dismutation of superoxide into oxygen and hydrogen peroxide. Dismutation, or disproportionation, is the conversion of one compound into two compounds, one of higher and one of lower oxidation states. While hydrogen peroxide is still classified as a ROS, it is less damaging than superoxide and can subsequently be converted by GPx into non-reactive compounds. SOD is another highly conserved enzyme present in aerobic organisms[276]. Different

isoforms include Cu/Zn SOD (SOD1), Mn SOD (SOD2), and Cu/Zn extracellular SOD (SOD3). SOD1 is primarily found in the cytosol while SOD2 is present in the mitochondrial matrix[276]. Expression and activity of SOD is influenced by cellular oxidative state. When high levels of hydrogen peroxide are generated by oxidative stress, SOD translocates to the nucleus acts as a transcription factor[276].

$$2O_2^- + 2H^+ \rightarrow O_2 + H_2O_2$$

Equation 4.2 Reaction catalyzed by superoxide dismutase.
Dismutation of superoxide into oxygen and hydrogen peroxide.

In the placenta, SOD is the primary antioxidant enzyme present. In comparison to other lipid peroxidases, the activity of SOD increases across gestation[277]. Both Cu/Zn and Mn SOD isoforms exist in the human placenta, however there is evidence that they exhibit specific localization. Mn SOD is primarily located in the fetal vascular endothelium and to a lesser extent in the villous placenta[277]. The Cu/Zn isoform is found mainly in stromal tissue of the placenta, with lower expression in syncytiotrophoblast.

4.1.5 Antioxidant Defense Dysfunction in Pregnancy Complications

Oxidative stress and dysregulated antioxidant defense have been identified in the pathophysiology of preeclampsia, gestational diabetes, and IUGR[278-280]. Preeclampsia, which is characterized by hypertension and proteinuria, is associated with an imbalance of oxidative stress and antioxidants, evident throughout gestation and at delivery. Blood samples from preeclamptic women at three time points in pregnancy found higher levels of oxidative stress markers than patients without preeclampsia[278]. At birth, placentas had lower expression of GPx and lower

GSH levels[278]. Serum, placenta, and decidua of preeclamptic patients had higher levels of lipid peroxides and lower levels of the antioxidant vitamin E[281]. Additionally, the expression and localization of SOD in placenta differs in preeclampsia. Similarly, gestational diabetes is thought to develop due to increased oxidative stress in pregnancy. Total antioxidant capacity is lower in gestational diabetes compared to healthy pregnancy[279]. Across the antioxidant defense network, gestational diabetes was associated with decreased levels of GSH and SOD activity in maternal blood and placentas[280]. Further, while preeclampsia can lead to IUGR, when IUGR occurs without other complications, the antioxidant defense network is decreased and oxidative stress in increased[280].

4.1.6 Antioxidant Defense Dysfunction in ART

Previous work in our laboratory using a mouse model of assisted reproduction found that placental oxidative stress was raised in ART[145]. These processes were investigated based on the higher rates of low birth weight and prematurity observed in ART pregnancies, both of which are associated with placental inflammation and oxidative stress[145]. The mouse model showed no evidence of structural abnormalities, however there were significantly lower levels of lipids, lower RNA levels, higher DNA damage, and increased apoptosis in the ART placentas, all of which are markers of oxidative stress[145]. Notably, evidence of increased oxidative stress and inflammation were more apparent in ICSI placentas compared to traditional IVF placentas, an effect that has not been extensively studied in human pregnancy but is concerning as ICSI is becoming standard of practice[94]**.** Within the antioxidant defense network, all ART pregnancies (IVF and ICSI) had lower activities of SOD, thioredoxin reductase, and xanthine oxidase in the mouse. ICSI placentas showed further effects, with lower activities of GST, GR, GPx[145]. The

activities of some of these enzymes were also lower in murine fetal livers from ICSI pregnancies, indicating that oxidative stress in ART pregnancies may affect fetal development and exacerbate the effects of oxidative stress. Whether these effects are also occurring in human ART pregnancies is currently unknown. However, higher risks of complications where placental oxidative stress has been implicated in the underlying pathophysiology provides evidence that the same dysfunction may be occurring in ART.

4.2 Materials and Methods

The following are chemicals and suppliers used: Cibacron blue (Biovision, Milpitas, CA, USA), 1-chloro-2,4-dinitro benzene (CDNB, Sigma, St. Louis, MI, USA), glutathione (GSH, Sigma, St. Louis, MI, USA), Optically clear microplates (Corning Inc., Corning, NY, USA), $CuCl_2 \cdot 2H_2O$ (Sigma, St. Louis, MI, USA), neocuproine (Tokyo Chemical Industry, Tokyo, Japan), DL- α-tocopherol (Sigma, St. Louis, MI, USA), absolute ethanol (Sigma, St. Louis, MI, USA), ammonium acetate (EMD Millipore, Burlington, MA, USA). Additionally, the following kits were used: Cayman Chemical Glutathione reductase assay kit (Item No. 703202), Cayman Chemical glutathione peroxidase assay kit (Item No. 703102), and Cayman Chemical superoxide dismutase assay kit (Item No. 706002, Cayman Chemical Company, Ann Arbor MI, USA).

4.2.1 Placenta Cellular Protein Extract (S9) Preparation

The placental tissue lysates prepared as outlined in Chapter 3 were further processed to placenta S9 fraction to provide a cleaner sample matrix for enzyme activity assays. Tissue lysate was centrifuged at 10,000 x g for 20 minutes at 4°C. The resulting supernatant was removed, assessed for protein content, and aliquoted and stored at -80°C until assaying for enzyme

activity. Protein content was determined using the BCA assay with BSA as a protein standard, as described in Chapter 3.

4.2.2 Assay for Determination of General GST Activity

General GST activity was measured as previously described by Gonzales *et al*.[282], with modifications for microplate technology. The substrate used was CDNB and Cibacron blue was used as an inhibitor. Cibacron blue strongly absorbs in the blue spectrum, resulting in high absorbance readings for negative controls, but the slope of these samples should be flat to indicate inhibition. Pooled human liver S9 and pooled human placenta S9 were included as positive controls. The following was added to each well in an optically clear plate: 1 µL of 50 mM CDNB, 10 µL of sample diluted to 2 mg/mL total protein, 10 µL of 20 mM Cibacron blue in 0.1 M K_xPO_4 buffer at pH 6.5 (to negative control samples), and 0.1 M K_xPO_4 to a final volume of 90 µL. Samples were incubated for 2 minutes at 37°C, then 10 µL of 10 mM glutathione was added to each well, with exception to the no cofactor negative control, to initiate the reaction. Samples were vigorously mixed for 5 seconds, and absorbance was measured every 5 seconds for 20 minutes at 340 nm. All samples, positive controls, and negative controls were run in triplicate.

Specific activity (mmol/min/ mg protein) was calculated using the Beer-Lambert Law with the slope of the line and an extinction coefficient (ε) = 9.6 mM/cm according to the molar extinction coefficient described by Habig, Pabst, and Jakoby[283]. The mean of the samples assayed in triplicate was used to determine total GST activity. Acceptable precision was considered to be a CV < 15%. Inter-day accuracy and precision were evaluated using the positive and negative

control samples. This work was performed with the help of an undergraduate summer student, Nick Pang.

4.2.3 Assay for Determination of Glutathione Reductase Activity

The activity of glutathione reductase was measured in placental S9 samples using a commercially available assay kit from Cayman Chemical (Cayman Chemical Company, Ann Arbor MI, USA). The kit contains potassium phosphate buffers, a glutathione reductase control from Baker's Yeast, a GSSG solution, and NADPH. Controls and placental samples were run in triplicate and only accepted if CV < 15%. The background of the assay was determined by adding only buffer and GSSG to the wells. The activity of GR in Baker's Yeast was determined by adding buffer, GSSG, and yeast control to the wells. To the sample wells, 100 µL of buffer, 20 µL of GSSG, and 20 µL of S9 at 2 mg/mL total protein were added to the plate. The reaction was initiated by the addition of 50 µL of NADPH to all wells. The plate was mixed on the plate reader and read immediately. Absorbance was read at 340 nm every 20 seconds for 5 minutes. Glutathione reductase activity was determined by calculating the change in absorbance over time and subtracting the background from the plate, then calculated using Beer Lambert Law with an extinction coefficient of 0.00622 $\mu M^{-1}cm^{-1}$ for NADPH and correcting for sample dilution to obtain activity in nmol/min/mg protein.

4.2.4 Assay for Determination of Glutathione Peroxidase Activity

Activity of glutathione peroxidase was determined using a commercially available kit from Cayman Chemical (Cayman Chemical Company, Ann Arbor MI, USA) which measures the activity of glutathione peroxidase indirectly as a coupled reaction to glutathione reductase. The

kit contains appropriate buffers, bovine erythrocyte GPx as a positive control, a co-substrate mixture containing glutathione and glutathione reductase, NADPH, and cumene hydroperoxide as the substrate. Placenta S9 samples were diluted to 1 mg/mL in order to minimize interference at 340 nm and to dilute activity values onto the curve. Buffer, co-substrate mixture, and NADPH were added to each well. Twenty microlitres of either positive control sample or placenta S9 were added to triplicate wells. Assay background was determined by performing the assay in the absence of sample or control. The reaction is initiated by addition of 20 µL of cumene hydroperoxide and vigorous mixing of the plate. Absorbance was measured at 340 nm every 20 seconds for 5 minutes. The mean of the triplicate values vs. time was plotted and the change in absorbance over time determined. Glutathione peroxidase activity is calculating using Beer Lambert law and the extinction coefficient of 0.00622 $\mu M^{-1}cm^{-1}$ for NADPH. Values were corrected for sample dilution to obtain the activity in nmol/min/mg protein.

4.2.5 Assay for Determination of Superoxide Dismutase Activity

Activity of superoxide dismutase was measured using a commercially available kit from Cayman Chemical (Cayman Chemical Company, Ann Arbor, MI, USA) which utilizes a tetrazolium salt to detect superoxide radicals generated by xanthine oxidase and hypoxanthine. The kit contains buffers containing 0.1 mM diethylenetriaminepentaacetic acid and 0.1 mM hypoxanthine, a tetrazolium salt as the radical detector erythrocyte SOD (Cu/Zn) standard, and xanthine oxidase. The standard curve was prepared in the range of final SOD activity of 0, 0.005, 0.01, 0.02, 0.03, 0.04, and 0.05 U/mL, where one unit is defined as the amount of enzyme needed to exhibit 50% dismutation of the superoxide radical. The assay was performed by adding 200 µL of radical detector and 10 µL of sample or standard in triplicate to the wells of a 96 well plate. Placental S9

samples were diluted to 0.05 mg/mL total protein to fall in the range of the standard curve. The reaction was initiated by the addition of xanthine oxidase to the wells. The plate was placed in the plate reader, carefully mixed, and incubated for 30 minutes at room temperature. The absorbance was read at 450 nm on the plate reader following incubation. A linearized rate of SOD activity was calculated by dividing the average absorbance of all standards and samples by the average of the zero-standard sample. The linearized rate was plotted as a function of final SOD activity and final SOD activity of samples was calculated using their linearized rate and the equation of the line.

4.2.6 Biochemical Assay for Detection and Quantitation of Vitamin E

Levels of vitamin E were measured in placenta S9 samples using an in-house plate-based method based off the method by Tütem *et al.*[284] using DL-α-tocopherol as a standard. Standards of DL-α-tocopherol were made in buffer at concentrations of 1, 5, 10, 25, 50, 100, 250, and 500 µM. To clean borosilicate glass tubes the following were added: 100 µL of 10 mM $CuCl_2 \cdot 2H_2O$, 250 µL of 3 mM neocuproine (prepared in absolute ethanol), 300 µL of absolute ethanol, 100 µL of 1 M ammonium acetate, and 50 µL of S9 sample at 2 mg/mL total protein or DL-α-tocopherol standard. Tubes were covered in foil and incubated at room temperature on the lab bench for 30 minutes. Following incubation, tubes were carefully mixed and 100 µL of product was transferred into three wells in a clear 96 well microtiter plate. Absorbance is measured at 450 nm and the average of triplicate readings calculated. Quantitation of vitamin E in placenta S9 samples was calculated by comparison to the standard curve. Values were accepted if the percent coefficient of variation for the triplicate values was < 15%.

4.2.7 Biochemical Assay for Detection of Glutathione

Levels of glutathione in placental S9 samples were measured using a commercial kit from Cayman Chemicals (Cayman Chemical Company, Ann Arbor, MI, USA). The Cayman glutathione kit functions in quantification of GSH using an optimized enzymatic recycling method[285]. Reaction of the sulfhydryl group of GSH with 5,5'-dithio-*bis*-2-(nitrobenzoic acid) (DTNB) to produce a yellow 5-thio-2-nitrobenzoic acid (TNB) which is reduced by glutathione reductase to recycle GSH and TNB. TNB production is therefore directly proportional to the concentration of GSH. Of note, GSH is easily oxidized to the disulfide dimer GSSG. In turn, GSSG is reduced to GSH by glutathione reductase. This kit utilizes glutathione reductase meaning both GSH and GSSG are measured, which indicates total glutathione. Components of the kit include a 2-(N-morpholine) ethane sulphonic acid buffer, GSH cofactor mixture containing $NADP^+$ and glucose-6-phosphate, and an enzyme mixture containing glutathione reductase and glucose-6-phosphate dehydrogenase. GSSG is used as the standard at the concentrations equivalent to 0, 0.5, 1, 2, 4, 8, 12, and 16 μM total GSH.

To perform the assay, 50 μL of GSSG standard or placenta S9 sample at 1 mg/mL total protein are added in triplicate to a clear 96-well plate and the plate is covered. An assay cocktail containing buffer, cofactor mixture, enzyme mixture, water and DTNB is prepared immediately before use. 150 μL of assay cocktail was added to all wells and the plate was mixed in the dark on the plate reader and incubated. The end point method was deemed acceptable for these samples, and the absorbance was measured at 412 nm after 25 minutes of incubation. The average absorbance of the triplicate values was calculated and total glutathione concentration in

the samples was determined by comparison to the standard curve. For a sample to be accepted, the % CV of triplicate values must be < 15%.

4.2.8 Statistical Analyses

Differences in the activities of antioxidant enzymes and the levels of antioxidants were first evaluated between placentas from unassisted and ART groups as a whole. For continuous variables, relationships with outcomes were plotted and assessed using Pearson's test. For discrete variables, a Student's t-test was used for each outcome variable. Unassisted and ART groups were subsequently stratified based on ICD9/10 clinical chart codes, assessed for normality, and reanalysed using a two-factor ANOVA with appropriate post-hoc analysis. Only case groups with a minimum of four samples were included in the stratified analysis. Statistical analyses were performed using R and R Studio (Boston, MA, USA) and visualized using GraphPad Prism (San Diego, CA, USA).

4.3 Results

4.3.1 Biochemical Assays for Antioxidant Enzyme Activities and Antioxidant Levels

The assays for GST, GR, GPx, SOD, Vitamin E, and GSH were successfully applied to human villous placenta samples. Intra- and inter-day accuracy and precision for each assay are presented in Table 4.1. All assays had acceptable precision (% CV < 15%). Enzyme activities and antioxidant levels were measured in 100% of placental samples and distributions of activity level or concentration are shown in Figure 4.2.

Assay	Intra-day Precision (%CV)	Inter-day Precision (%CV)
GST	5.2	12.4
GR	1.4	8.6
GPx	0.7	5.7
SOD	5.4	5.6
Vitamin E	1.3	9.2
GSH	1.6	9.4

Table 4.1 Intra-day and inter-day precision of antioxidant enzyme and antioxidant assays.

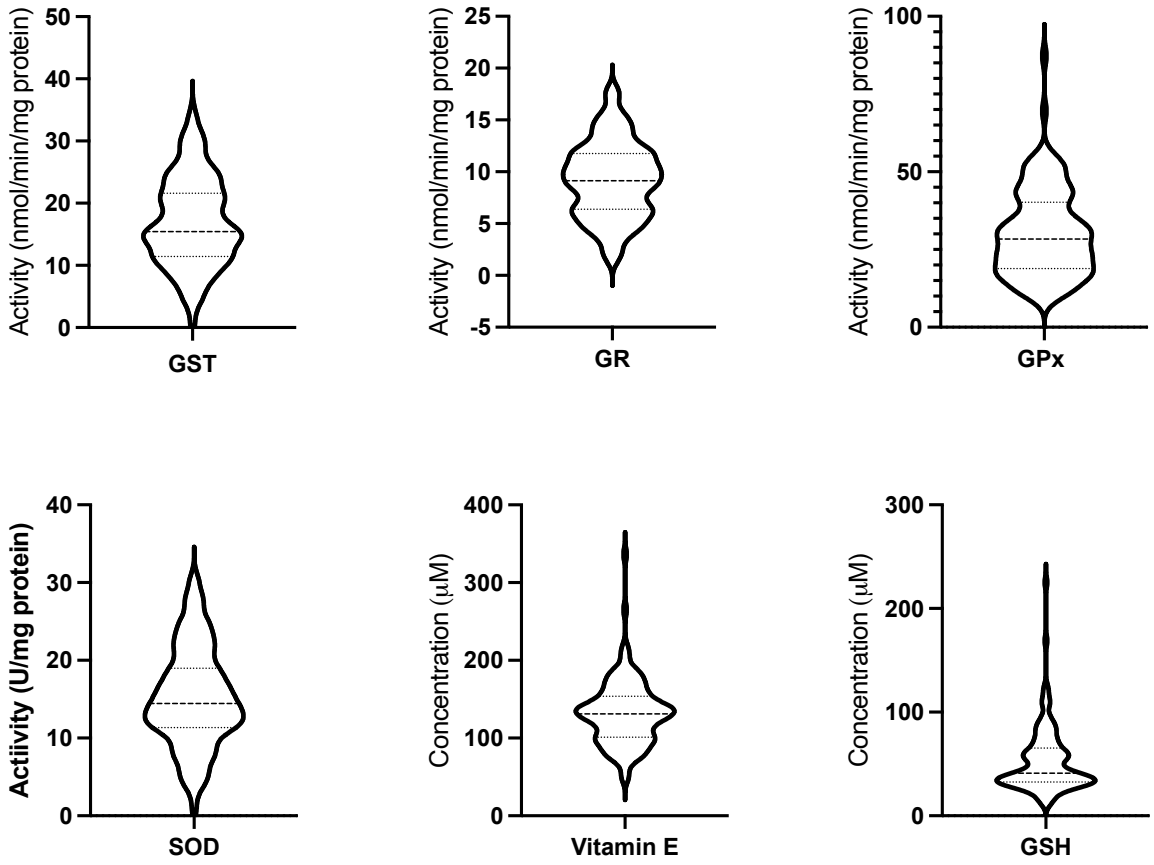

Figure 4.2 Antioxidant enzyme activities and antioxidant levels for all placentas screened showing frequency distribution of the data.

Dotted lines represent median, 1st quartile, and 3rd quartile.

4.3.2 Correlations of Antioxidant Enzyme Activities and Antioxidant Levels in Placenta with Continuous Variables

Antioxidant enzyme activity and antioxidant levels were evaluated against the continuous variables maternal age (years), gestational age (days), maternal weight gain in pregnancy (kg), maternal BMI (kg/m^2), baby weight (g), baby length (cm), and baby head circumference (cm). However, no biologically significant correlations were observed (*data not shown*).

4.3.3 Differences Between Antioxidant Enzyme Activities and Antioxidant Levels in Placentas with Discrete Variables

The activities of antioxidant enzymes and levels of antioxidant were compared against unassisted pregnancy and ART groups as a whole, however no statistically significant differences were found (Figure 4.3). The groups were subsequently stratified based on the following discrete variables: augmentation, delivery method, gestational diabetes, induction, IUGR, spontaneous membrane rupture, preterm birth, small for gestational age infants, chorioamnionitis, and twin pregnancy. Outcomes were assessed for normality and analyzed using a two-factor ANOVA with a Bonferroni correction. No significant differences were found with most discrete variables; however, significant differences were found in pregnancies complicated by chorioamnionitis and twin pregnancy.

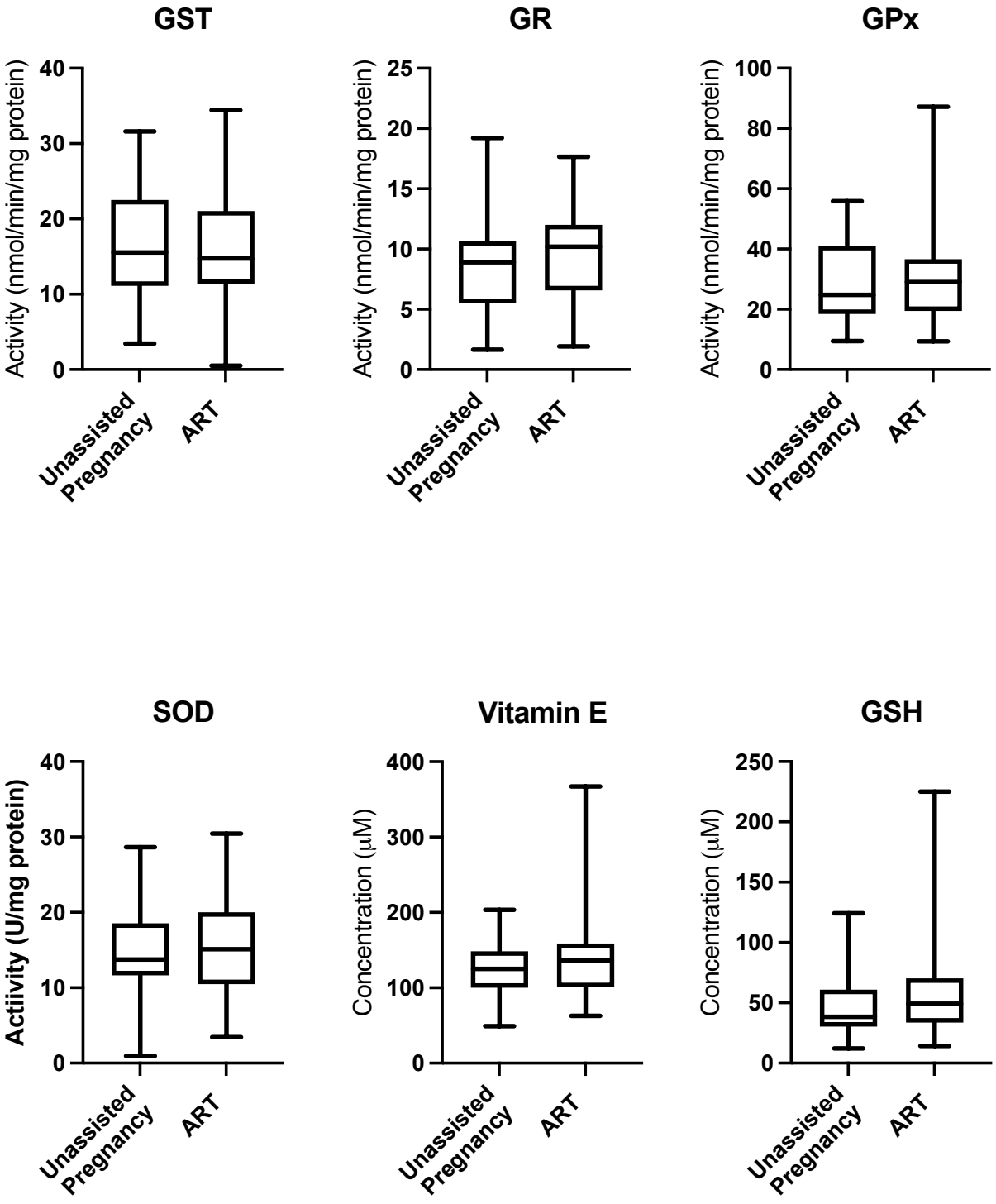

Figure 4.3 Antioxidant enzyme activities and antioxidant levels for placentas from unassisted pregnancies and ART pregnancies.

Box plots represent minimum, Q1, median, Q3, and maximum. No significant differences noted between unassisted pregnancies and ART.

4.3.4 Antioxidant enzyme activities and antioxidant levels in pregnancies complicated by chorioamnionitis

In unassisted pregnancies and ART pregnancies, the activities of GST and GPx were different in pregnancies with/without chorioamnionitis. Placental GST activity was lower in ART pregnancies with chorioamnionitis compared to ART pregnancies without chorioamnionitis and unassisted pregnancies with chorioamnionitis (Figure 4.4). There was no difference in GST activity in placentas of unassisted pregnancies with and without chorioamnionitis. With respect to the activity of placental GPx, activities were higher in unassisted pregnancies with chorioamnionitis compared to unassisted pregnancies without chorioamnionitis. However, an increase in GPx activity was not observed in ART, and the activity of GPx in ART pregnancies with chorioamnionitis were significantly lower than in unassisted pregnancies with chorioamnionitis (Figure 4.4). There were no differences in GR and SOD activity associated with chorioamnionitis, and no differences in the levels of antioxidants vitamin E or GSH associated with chorioamnionitis.

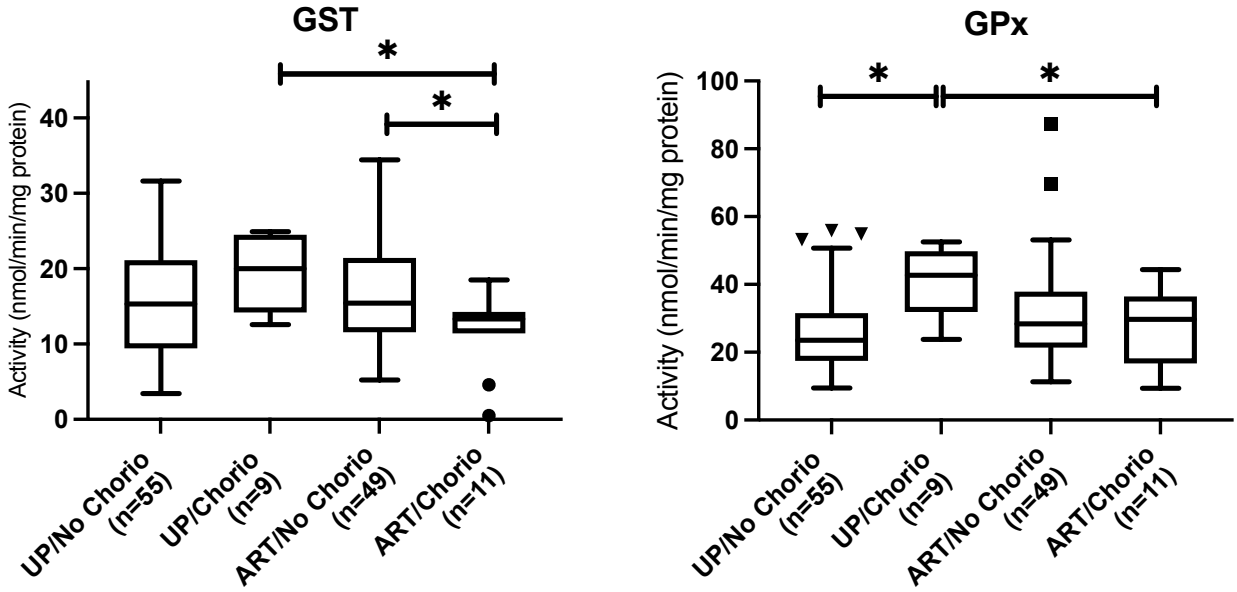

Figure 4.4 Activities of GST and GPx in placentas from unassisted pregnancies and ART pregnancies, with and without chorioamnionitis.
Box plots represent minimum, Q1, median, Q3, and maximum. Unassisted pregnancy (UP), Assisted reproduction pregnancy (ART) Chorioamnionitis (Chorio), No chorioamnionitis (No Chorio). * $P < 0.05$.

4.3.5 Antioxidant Enzyme Activities and Antioxidant Levels in Twin Pregnancies

In naturally conceived twin pregnancies, we observed a decrease in the activities of placental GST and GPx compared to naturally conceived singletons (Figure 4.5). Placental GST activity was higher in ART singletons compared to naturally conceived twins, but no differences were observed between ART singletons and twins. In contrast, vitamin E levels were significantly higher in ART twins compared to naturally conceived twins (Figure 4.5). There were no differences in the activities of GR or SOD, or with the levels of GSH associated with twin pregnancies.

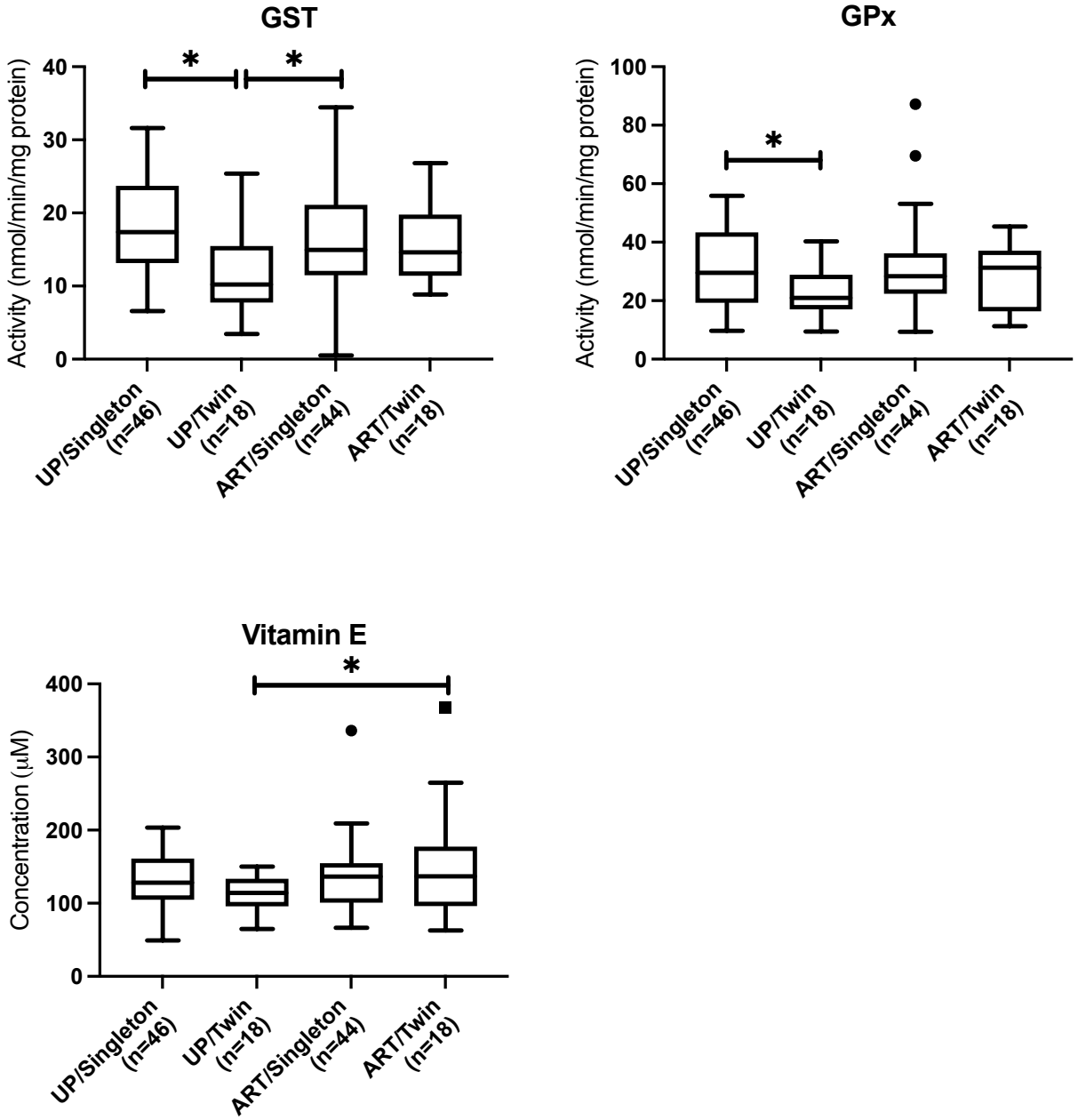

Figure 4.5 Activities of glutathione S-transferase (GST) and glutathione peroxidase (GPx) and levels of vitamin E in placentas of naturally conceived and ART singletons and twins. Box plots represent minimum, Q1, median, Q3, and maximum. Unassisted pregnancy (UP), Assisted reproduction pregnancy (ART) * $P < 0.05$.

4.4 Discussion

4.4.1 The Association of Pregnancy Outcome with Antioxidant Defense Enzyme Activity in Assisted Reproduction

Following, the previous investigation into cytokine levels in the placenta (Chapter 3), there were no differences observed in the antioxidant defense network between ART and natural conception. As shown in Figure 4.3, activities of antioxidant defense enzymes and the levels of antioxidants were very similar in terms of median level and distribution between the two groups. These results contrast with the mouse model of ART, where the levels of catalase, GPx, GR, GST, SOD, TRx, and XO were significantly decreased in ART[145]. Additionally, the mouse model showed more pronounced effects when ICSI was utilized compared to traditional IVF. While we do not know whether the ART pregnancies in this cohort used ICSI or traditional IVF, a systematic review of ART practices in North America found that ICSI was used in approximately 70% of ART cycles, so it is likely that most patients in our ART cohort utilized ICSI[8]. A consideration of this for future murine studies is the fertilization method used in generating the ART model is important for the interpretation of the results. While we know there are differences between IVF and ICSI in terms of inflammation and oxidative stress, if these effects extend to cellular functions is unknown.

Similar to the caveat in the previous chapter, significance may be lost in our human cohort due to inter-individual variation and a relatively small sample size which was powered off the mouse studies. While the sample sizes in the ART and unassisted conception groups may be regarded as small, gathering samples with this level of matching (for gestational age, maternal age, ethnicity) is incredibly difficult; from the University of Hawaii Reproductive Biorepository, only 75

matches could be made from a collection of over 10, 000 samples, which was reduced to 56 assayable samples when highest matching stringency was required. This is one of the reasons studying these pregnancy complications is so difficult. To detect differences between ART and natural conception that can account for the known increase of obstetric risks and complications in ART, much larger sample groups are required which is not often feasible in practice. However, an interesting outcome of this study is the results align so closely with the results from the cytokine studies after stratified analysis, specifically with respect to chorioamnionitis and twin pregnancies. Observing significant results indicates high signal-to-noise, which means these results are likely significant from a biological perspective. A more in-depth discussion of the effects on chorioamnionitis and twin pregnancy follow below.

4.4.2 Effects of Chorioamnionitis on the Antioxidant Defense Network

In ART pregnancies with chorioamnionitis, we observed lower activities of GST and GPx compared to unassisted pregnancies with chorioamnionitis. Additionally, there was an increased activity of GPx in unassisted pregnancies with chorioamnionitis, but the opposite trend was observed in ART. This was identical to the patterns we observed in cytokine levels in Chapter 3. Recall unassisted pregnancies with chorioamnionitis had increased levels of cytokines, but this pattern was lost or opposite for certain cytokines in ART. The decreased levels of cytokines were concerning, as we would expect an increase in response to infection and the similar effects on antioxidant enzymes adds to stress within the placenta and the ability to fight the infection and protect the fetus from noxious stimuli.

The processes of inflammation, cytokine release, and oxidative stress are intricately linked. In nephropathy, pancreatitis, and lung pathologies interactions between cytokines and oxidative stress have been reported in the literature and similar responses are to be expected in the placenta[286-288]. Evidence of placental oxidative stress and inflammation are most well studied in preeclampsia[289]. Researchers have noted an imbalance between ROS and antioxidant defense, which was accompanied with increased levels of pro-inflammatory cytokines. These interactions are not linear; inflammatory and oxidative responses perpetuate each other leading to more cell stress and damage. In the placenta, it has been noted that expression of inflammatory cytokines and antioxidant enzymes are impaired in the placenta in response to oxidative stress[290]. Further, oxidative stress increases release of prostaglandins, increasing activity of signalling cascades and risk of tissue damage. These effects can have detrimental effects on nutrient availability to the fetus, as they have direct effects on endothelial cell function, both placental and maternal[291].

Oxidative stress is also known to increase apoptosis of trophoblast cells[257]. While not studied here, the mouse model showed increased apoptosis in ART[145]. In a chorioamnionitis model in sheep, effects on antioxidant defense were seen throughout fetal tissues, and thought to be a host response to reduce oxidative injury[292]. Following a dose of intra-amniotic endotoxin, there was upregulation of antioxidant enzymes in the lungs to prevent damage. In human placenta, chorioamnionitis also increased detection of mitochondrial SOD[293]. The upregulation of antioxidant enzymes in response to intra-amniotic infection functions as a protective mechanism to maintain cellular function. If these responses are muted in ART pregnancies with chorioamnionitis, as results here suggest, that could result in dangerous outcomes to the fetus including preterm birth or serious infection of the fetus. While chorioamnionitis is relatively rare,

it is a serious condition that increases fetal morbidity and mortality. If both inflammatory and antioxidant defense responses are muted in ART pregnancies complicated with chorioamnionitis, special care and monitoring may be needed to prevent pregnancy complications and improve outcomes overall.

4.4.3 Effects of Twin Pregnancy on the Antioxidant Defense Network

The second clinical situation where we found differences in the antioxidant defense network was in twin pregnancies compared to singleton. Activities of GST and GPx were lower in unassisted twin pregnancies, but this pattern was not observed in ART. This followed up on our studies that cytokine levels were lower in naturally conceived twins but not in ART twins. There is evidence in the literature that twin pregnancies are associated with more oxidative stress, which is involved in IUGR observed in twin pregnancy[294-296]. Measurements of oxidative stress markers such as delta-aminolevulinate dehydratase and thiobarbituric acid reactive substances showed increased oxidative stress and reduced antioxidant defense in twins[297]. In sheep, twin pregnancies exhibited higher levels of oxidative stress, which differed over time and became more pronounced in late gestation[298].

In our unassisted pregnancy group, results followed what is seen in the literature, with reduced antioxidant capacity in twins compared to singletons. Earlier, we proposed that the lower levels of cytokines in naturally conceived twins was an adaptive mechanism to lower immune responses and protect the twins from the maternal immune system recognizing it as “not-self”. Considering the links of pro-inflammatory cytokines and oxidative stress, the lower levels of antioxidant enzymes in naturally conceived twins fit with evidence in the literature. While there

was no decreased activity of antioxidant enzymes in ART twins, this is not necessarily a protective mechanism to prevent oxidative stress but could instead be a downstream effect of dysregulated cytokines and immune responses. Evidently, more work is needed to unravel the complex mechanisms involved in increased pregnancy complications for both singletons and multiple births in ART, but our data which provides evidence of differences in both inflammation and oxidative stress may provide a starting point.

4.4.4 Limitations of the Study

In addition to the limitations of the placental samples discussed in Chapter 3 (use of archival samples, stability of antioxidant and enzyme activity, and effects of freeze-thaw) some additional limitations should be discussed. As mentioned in the introduction of this chapter, many of the enzymes studied here exhibit regional expression within the placenta. The placenta punches taken here were requested to not be from the periphery or too close to the umbilical cord, as the structure varies more widely in these locations. There may, however, have been some variation in sampling site due to sample availability, and we were not able to account for this in our analysis since we do not know the precise location the punches were taken from. Additionally, it may be possible that we would have seen differences in enzyme activity or antioxidant levels between ART and natural conception between different regions of the placenta, if we had been able to get samples from the periphery, middle, and close to the cord insertion.

The antioxidant enzymes assayed here also have different isoforms and subcellular locations, discussed above. With GSTs for example, we performed a general GST assay to get a measure of the overall GST activity within the placenta, without focussing on specific isoforms. The assays

could have been performed with specific substrates that are selective for different isoforms (ethacrynic acid for GST-P, 3,4-dichloronitrobenzene for GST-M, and 4-nitro-2-benzyl chloride for GST-T)[299]. For GST and the other enzymes assayed, we could also have separated the subcellular fractions to target specific isoforms of each enzyme. However, we believed it would be more valuable to assay the general activity of a broader range of antioxidant enzymes. By taking this approach it is possible that we may have missed variation within specific isoforms of enzymes, which were covered up by performing total enzyme activity assays. In contrast to the analytes measured in Chapter 3, these enzymes are very stable at -80 ºC and loss due to archival time is expected to be minimal.

4.5 Summary

This is the first report in humans to show altered antioxidant defense in chorioamnionitis and twin pregnancies when ART is used. These results build on our reports of attenuated placental inflammatory signalling in chorioamnionitis and twin pregnancy in ART, and on prior murine work where antioxidant defenses was altered in ART. The mechanisms underlying the interactions of these systems in the placenta are not completely understood. A potential route for future investigation is the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kB). This transcription factor is involved in the production of cytokines, but also responds to inflammatory stress and free radicals within the cell[300]. The NF-κB pathway is complex; whether it acts in a pro- or anti-inflammatory, or pro- or antioxidant is context dependent[300]. NF-κB is particularly well studied in preeclampsia, which shares many of the same complications increased in ART pregnancies overall[301]. Past studies have shown placental NF-κB activation is 10-fold higher in preeclampsia, and oxidative stress in combination with TNFα drives its

activity. Investigation of NF-κB in combination with SOCS signalling is a promising pathway to better understand our results in a mechanistic fashion.

Overall, the results of Chapters 3 and 4 identify dysregulation of inflammatory and antioxidant defense responses which have not been previously reported in the ART literature. Inflammation and oxidative stress are complex processes but are intricately linked through a number of cellular signalling pathways. Using our studies as a starting point, future investigations may elucidate the mechanisms underlying the associations observed here with unfavourable pregnancy outcomes. In the long term, a greater understanding of dysregulated inflammation and oxidative stress responses within the placenta may help physicians understand the risks associated with ART pregnancy and provide therapeutic targets to minimize these risks.

Chapter 5: Association of Maternal-Placental-Fetal Steroids and Steroid Sulfates with Pregnancy Outcome in Assisted Reproduction

5.1 Introduction

The steroid hormones progestogens, androgens, and estrogens are required from fertilization to establish and maintain a successful pregnancy. Much like the roles of cytokines, VEGFs, and antioxidants discussed previously, steroid hormones are involved in all aspects of pregnancy from placentation to parturition[3]. One of the major functional roles of the placenta is as an endocrine organ. It is involved in the biosynthesis and metabolism of steroids as well as the exchange of steroid hormones between the maternal and fetal compartments[3]. The correct balance of steroid hormone production and metabolism is vital for correct placental function and growth and development of the fetus. In early pregnancy, before the placenta has developed, the ovaries are responsible for producing adequate levels of progesterone to maintain pregnancy. Around eight-nine weeks gestation, the placenta takes over as the main source of progesterone and begins synthesizing other steroid hormones required for pregnancy within the syncytiotrophoblast[3]. However, a coordination between the placenta, fetal adrenal, and maternal endocrine tissues is required.

Progestogens, androgens, and estrogens have distinct functions, which will be discussed below. Additionally, the placenta expresses the major steroidogenesis and steroid metabolism enzymes throughout pregnancy[302]. A diagram of the steroidogenesis pathway is presented in Figure 5.1. Steroids are active in their free, unconjugated forms, and mainly function to modulate gene expression in combination with their respective receptors which act as transcription factors[303]. When transported throughout the body, steroids may be bound to carrier proteins such as sex

hormone-binding globulin, or they can be metabolized to steroid conjugates which have increased water solubility[303]. Dysregulation of steroidogenesis or steroid metabolizing enzymes within the placenta can lead to pregnancy loss, preeclampsia, or preterm birth[304,305].

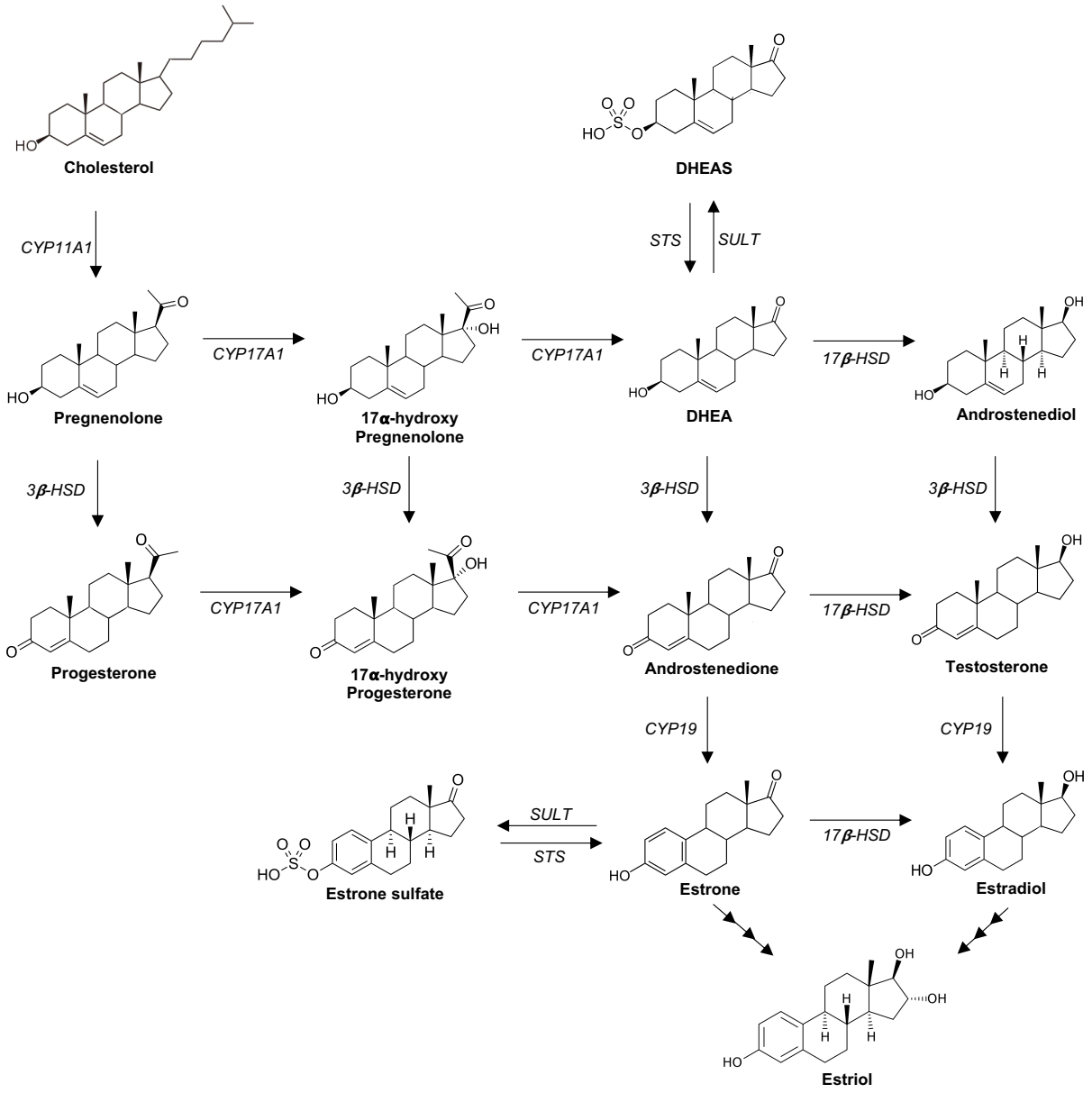

Figure 5.1 Summarized steroidogenesis pathway.
Enzymes labelled next to arrows.

5.1.1 Progesterone

Progesterone is the main steroid hormone for the maintenance of pregnancy. The corpus luteum secretes progesterone in advance of implantation, which is involved in the decidualization of the endometrium[3]. The ovary continues to secrete progesterone until approximately the eighth week of gestation when the developing placenta takes over[3]. Within the placenta, the syncytiotrophoblast is the main source of progesterone, secreting around 250 mg/day by the end of the third trimester[302]. Progesterone functions to maintain pregnancy by influencing the maternal immune system to increase tolerance toward the fetus[306]. It also exhibits anti-inflammatory effects by inhibiting the production of pro-inflammatory cytokines[302,306]. If progesterone levels are insufficient, placental inflammation and oxidative stress can occur which is believed to be involved in the pathophysiology of miscarriage and preterm birth.

5.1.2 Estrogens

The estrogens include estrone (E1), estradiol (E2), and estriol (E3). Mainly produced in the placenta, production increases throughout pregnancy and functions to promote endometrial and mammary cell proliferation, promote placental angiogenesis, and promote labour by stimulating myometrial activity at the end of the third trimester[50,307]. The majority of the estrogens produced by the placenta are converted from C19 steroids taken up from the maternal and fetal circulations. The placenta expresses high levels of aromatase, which is responsible for the conversion of C19 steroids to estrogens; this process is described in more detail below. Estrogens are intricately involved in the process of angiogenesis, and have therefore been implicated in the development of preeclampsia[308]. Correct estrogen levels are important for regulating processes of

placentation and development throughout pregnancy, and have been proposed as a potential biomarker for pregnancy complications[308].

5.1.3 Cholesterol Uptake and Lack of Steroidogenic Acute Regulatory Protein (StAR)

The synthesis of steroid hormones begins with cholesterol[303]. The placenta requires high levels of cholesterol for growth and function which must be taken up from the maternal circulation. Cholesterol circulates in the blood associated with lipoprotein particles[303], of which low-density lipoproteins (LDLs) are associated with approximately 70% of circulating cholesterol[309]. The syncytiotrophoblast expresses LDL receptors which bind LDL and endocytose the particle into the cell[310]. Cholesterol is then transported to the mitochondria where the process of steroidogenesis begins[311]. The process of placental intracellular cholesterol transport is not completely elucidated, but there is evidence that heat-shock protein 60 (HSP60) is involved[311].

In contrast to other steroidogenic tissues, the human placenta does not express steroidogenic acute regulatory protein (StAR)[312]. Instead, the placenta expresses a StAR-like protein, STARD3 (or MLN64) to transport cholesterol from the outer to the inner mitochondrial membrane[312]. Here, the cholesterol side-chain cleavage enzyme, CYP11A1 or P450scc, is present.

5.1.4 Cholesterol side-chain Cleavage (CYP11A1)

The enzyme CYP11A1 is the rate-limiting step in steroid biosynthesis[313]. To convert cholesterol to pregnenolone, CYP11A1 catalyses three oxidative reactions requiring O_2, NADPH, and electrons[313]. First is a hydroxylation at C-22, followed by a hydroxylation at C-20. Lastly, the side chain of cholesterol is cleaved at the C20-C22 bond to yield pregnenolone. The

intermediates of this reaction do not accumulate in cells, likely remaining in the active site until the third reaction is complete. In the placenta, CYP11A1 is found only in the syncytiotrophoblast and is expressed from early pregnancy[314].

5.1.5 3β-hydroxysteroid Dehydrogenase (3β-HSD)

The enzyme 3β-HSD, also known as Δ^5-Δ^4 isomerase, is responsible for the conversions of pregnenolone to progesterone, 17α-hydroxypregnenolone to 17α-hydroxyprogesterone, and DHEA to androstenedione. Belonging to a family of oxidoreductases, humans express two isoforms of 3β-HSD: 3β-HSD I and 3β-HSD II[315]. Type I is expressed in placenta while type II is expressed in the ovary, testis, and adrenal gland. In the placenta, 3β-HSD I is expressed in the endoplasmic reticulum of the trophoblast[315]. In pregnant women with polycystic ovarian syndrome (PCOS), there is evidence of higher activity of 3β-HSD in the placenta, which may contribute to increased levels of androgens observed in women with PCOS and affect placental and fetal weight[316].

5.1.6 17α-hydroxylase/C17,20-lyase (CYP17)

Cytochrome P450 17A1 is also known as 17,20-lyase or 17α-hydroxylase because it has both lyase and hydroxylase activity. This dual action allows for the conversion of pregnenolone to 17α-hydroxypregnenolone to DHEA, and progesterone to 17α-hydroxyprogesterone to androstenedione. Early studies of the human placenta found no evidence of expression or activity of CYP17 in the human placenta[317,318]. This led to the hypothesis that the human placenta was unable to convert estrogens directly from cholesterol and required the uptake of C19 steroids from the maternal and fetal circulations in order to synthesize estrogens. More recent studies

have identified CYP17 expression in the human placenta and human placental cell lines, however they are present at much lower levels than the other steroidogenesis enzymes, and much lower than other species investigated including mice, rats, and sheep[319-321]. The level of estrogens produced by the placenta is around 100-120 mg/day at term, which cannot be accounted for by the activity level of CYP17 observed[302]. Therefore, while it may be expressed at low levels in the placenta, uptake of C19 steroids still accounts for the majority of estrogen synthesis. CYP17 is localized to the endoplasmic reticulum within the trophoblast[321].

5.1.7 Aromatase (CYP19)

Aromatase is the key enzyme involved in the synthesis of estrogens[322]. It catalyzes the aromatization of the androgens androstenedione and testosterone to estrone and estradiol, respectively. This is carried out by three successive hydroxylations of C19 followed by elimination of the methyl group leading to aromatization of the ring[323]. The affinity of androstenedione for CYP19 is higher than that of testosterone, making the conversion to estrone more favourable[323]. Like 3β-HSD and CYP17, aromatase is localized to the endoplasmic reticulum[322]. The activity of aromatase within the placenta is important for a successful pregnancy to occur. There is evidence of decreased expression and activity of aromatase in pregnancies complicated by preeclampsia[324]. Additionally, female pseudohermaphoditism may be caused by a placental aromatase deficiency[325].

5.1.8 Steroid Sulfatase

The placenta must use C19 precursors from maternal and fetal sources in order to produce adequate levels of estrogens[326]. The main sources of C19 steroids are circulating DHEAS and

16α-OH-DHEAS produced in the maternal and fetal adrenal glands[326]. Before converting DHEAS to downstream estrogens, the sulfate group must be removed. This is carried out by the enzyme steroid sulfatase (STS)[327]. The placenta is the richest source of STS, with expression shown to be 34 times higher than other tissues, highlighting the importance of this enzyme in estrogen synthesis in the placenta[328]. STS is also localized to the syncytiotrophoblast and associated with the endoplasmic reticulum[328]. Dysregulation of STS in the placenta has been shown in the placentas of women with early onset preeclampsia[328]. Additionally, the gene for STS is located on the X chromosome, and males with an inactivation of the STS gene develop a condition called X-linked ichthyosis, which manifests as a scaly skin condition at birth[329]. The placentas of these male fetuses will also have an STS deficiency, which has been associated with delayed or prolonged labour due to lower levels of estrogen production[330,331].

5.1.9 Steroid Levels in Assisted Reproduction Technologies

Because of the important roles of steroids in the maternal-placental-fetal unit, the steroid metabolome in these compartments is an important source of information to investigate and predict pregnancy complications. How ART affects steroid hormone levels, production, and metabolism in humans is not well understood. However, our laboratory has extensively studied the effects of ART on steroid hormone delivery and metabolism in a murine model of ART. It was first noted that ART impairs placental steroid metabolism[97]. Levels of placental estrone were significantly decreased, and increased activity of conjugation enzymes was coupled with decreased activity of the opposing regeneration enzymes, skewing the ratio of active:conjugated steroids[97]. Further, levels of estradiol were increased in ART and progesterone levels were decreased[332]. ART was also associated with lower levels of steroid hormones being transferred

from the placenta to the fetal circulation. Notably, for many of these measured outcomes, the effects were more severe when ICSI was used in comparison to traditional IVF. In a further investigation of preimplantation genetic diagnosis (PGD), a common practice in ART, altered steroid levels in the placenta and fetal compartment[333]. The activities of UGTs and SULTs were also decreased in placentas and fetal livers[333].

Here, we aimed to determine if the dysregulation of steroid hormone levels, delivery, and metabolism observed in the murine model of ART are also observed in humans. The purpose of this aim is to provide an overview of maternal-placental-fetal steroid hormone production and metabolism and determine if steroid balance is affected by ART. If the same patterns are observed, it may contribute to our understanding of the pathophysiology underlying the increased risks of pregnancy complications associated with ART.

5.2 Materials and Methods

Deuterated internal standards pregnenolone-d4, progesterone-d9, DHEA-d6, testosterone-d5, 17β-estradiol-d4, and cortisol-d8 were purchased from C/D/N Isotopes Inc (Pointe-Claire, Quebec, Canada). Deuterated steroid sulfate internal standards DHEAS-d5 and E1S-d4 were purchased from Sigma-Aldrich (St. Louis MI, USA) and CDN Isotopes (Pointe-Claire, Quebec, Canada). Certified reference standards for progesterone, pregnenolone, DHEA, androstenedione, testosterone, estrone, 17β-estradiol, estriol, and cortisol were purchased from Cerillant Co (Round Rock, Texas, United States of America). Steroid sulfate standards DHEAS and E1S were purchased from Cayman Chemicals (Ann Arbor, MI, USA) and Sigma-Aldrich (St. Louis MI, USA), respectively. HPLC grade methanol, acetonitrile, and hexanes were purchased from

Fisher Scientific (Ottawa, Ontario, Canada). Borosilicate glass culture tubes were purchased from VWR International (Mississauga, Ontario, Canada), zirconium ceramic oxide beads (1.4-mm diameter) and microcentrifuge tubes were purchased from Fischer Scientific (Ottawa, Ontario, Canada), polypropylene vials were purchased from Sarstedt AG & Co. (Numbrecht, Germany), and LC glass vial inserts were purchased from Agilent (Santa Clara, California, United States of America). Pooled human charcoal stripped serum was purchased from Innovative Research (Novi, MI, USA).

5.2.1 Steroid LC-MS/MS Method

5.2.2.1 Sample Processing

The placenta samples used in this section were previously described in Chapter 2. For this LC-MS method, whole tissue was used. Whole tissue shipped from the University of Hawaii Reproductive Biorepository (Honolulu, HI, United States of America) was never thawed, and kept cold on dry ice during slicing. Thin slices of tissue were taken through the entirely of the tissue punch to get a more representative sample, and frozen weight recorded. Approximately 15 mg of tissue per sample was weighed, because a 10-fold dilution of sample placed the steroid levels in the linear range of the curve but cutting 1.5 mg of tissue led to more variable results, discussed below. Maternal plasma and cord serum samples were shipped on dry ice and were thawed once to aliquot to smaller sample volumes.

5.2.1.2 Instrumentation

Steroids were separated and quantified with a Nexera X2 UHPLC system (Shimadzu Corp., Kyoto, Japan) with a KrudKatcher ULTA HPLC In-Line filter (Phenomenex, Torrance, CA,

USA). The system was interfaced with an AB Sciex QTrap® 6500 triple quadrupole tandem mass spectrometer (AB Sciex, Concord, ON, Canada). Data were acquired using Analyst 1.5.2 software and analyzed using MultiQuant™ Software (AB Sciex, Concord, ON, Canada).

5.2.1.3 Chromatography Conditions

For this project, compounds were separated by a Poroshell 120 HPH C18, 2.7 µm, 2.1 x 50 mm column, protected by a Poroshell 120 HPH C18 guard column (Agilent, Mississauga, ON, Canada). Mobile phase A was MilliQ water with 0.1 mM ammonium fluoride and mobile phase B was HPLC-grade methanol. Mobile phase gradient programming was as follows: Mobile phase B was held at 10% for the first 0.5 minutes, then upped to 42% from 0.6 to 4.0 minutes. From 4.1 minutes to 9.4, mobile phase B was ramped to 60%, then to 70% between 9.4 to 9.5 minutes. Finally, mobile phase B was ramped to 98% until 11.9 minutes and held until 13.4 minutes before returning to starting conditions of 10% for one minute. The total run time was 14.9 minutes, and the flow rate was 0.4 mL/min. Of the 50 µL of sample in the vial, 10 µL was injected into the system.

5.2.1.4 Mass Spectrometry Conditions

The mass spectrometers' electrospray ionization source was operated in positive mode for all steroids except the estrogens (estrone, estradiol, and estriol) which were acquired in negative ionization mode. Steroids were detected using two multiple reaction monitoring (MRM) transitions and deuterated internal standards were measured using one MRM transition. Transitions and retention times are shown in Table 5.1. The deuterated form of each compound was used as the IS, except testosterone-d5 was used as the IS for both testosterone and

androstenedione, and 17β-Estradiol-d4 used as the IS for estrone, 17β-estradiol, and estriol. A representative chromatogram from a standard curve calibrator is presented in Figure 5.2.

Steroid	**Retention Time (min)**	**Quantifier m/z**	**Qualifier m/z**
Pregnenolone	10.6	299.1→159.1	299.1→105.1
Progesterone	10.27	315.2→97.0	315.2→109.1
DHEA	8.3	271.1→253.0	271.1→213.2
Androstenedione	6.92	287.2→97.2	287.2→109.1
Testosterone	7.77	289.0→97.0	289.0→109.1
Estrone	7.07	269.0→145.0	269.0→143.0
17β-Estradiol	7.20	271.0→145.0	271.0→143.0
Estriol	2.46	287.1→171.0	287.1→144.9
Cortisol	3.70	363.3→121.2	363.3→327.1
Pregnenolone-d4	10.57	303.0→159.1	-
Progesterone-d9	10.23	324.2→100.0	-
DHEA-d6	8.23	277.1→219.2	-
Testosterone-d5	7.72	294.0→100.0	-
17β -Estradiol-d4	7.39	275.0→147.0	-
Cortisol-d4	3.70	367.2→121.1	-

Table 5.1 Retention times and mass transitions for analytes and internal standards.

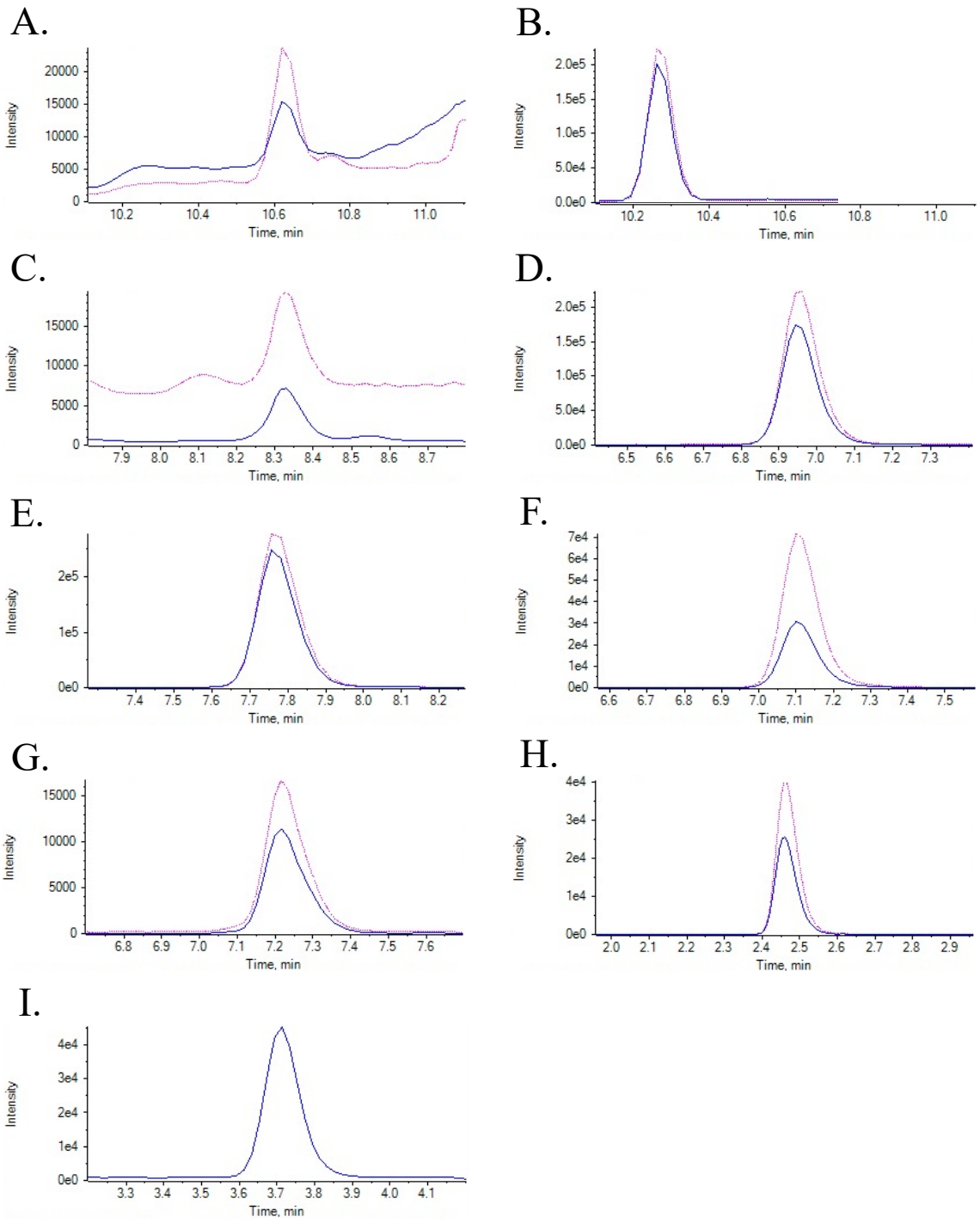

Figure 5.2 Quantifier and qualifier ions for steroid analytes.
Standard at 50 pg. A. Pregnenolone 10.60 min B. Progesterone 10.27 min C. DHEA 8.3 min D. Androstenedione 6.92 min E. Testosterone 7.77 min F. Estrone 7.07 min G. 17β-Estradiol 7.20 min H. Estriol 2.46 min I. Cortisol 3.70 min

5.2.1.5 Preparation of Standard Solutions, Calibration Standards, and Quality Controls

Stock solutions of steroids (pregnenolone, progesterone, DHEA, androstenedione, testosterone, estrone, 17β-estradiol, and estriol) were prepared at 1 mg/mL in HPLC-grade methanol and diluted further in methanol to a concentration of 20 μg/mL. Standard stock mix was prepared by combining 20 μL of each stock solution, except cortisol, in a glass vial and adding an additional 40 μL of methanol to a final volume of 200 μL. This standard stock mix was used to prepare calibration standards at 2, 4, 8, 20, 50, 125, 500, 1000, 2000, 5000, and 10000 pg/10 μL, diluting with 50% methanol. A curve for cortisol was prepared in the same manner, but separately because it was added to the method just prior to tissue screening.

Quality control (QC) standards were prepared from separate stocks of steroids in methanol. The three QC standards, designated QC_{low}, QC_{mid}, and QC_{high} were diluted in 50% methanol to final concentrations of 20, 200, and 2000 pg/10μL, respectively. All calibration standards were stored in glass vials, parafilmed, and stored at -20°C until days of analysis.

Stock solutions of deuterated steroids (pregnenolone-d4, progesterone-d9, DHEA-d6, testosterone-d5, 17β-estradiol-d4, and cortisol-d4) were prepared in methanol and diluted to final concentrations between 100-10000 ng/mL. Stock solutions were combined and diluted in 50% methanol to yield the final concentrations: pregnenolone-d4 at 10 ng/mL, progesterone-d9 at 0.2 ng/mL, DHEA-d6 at 6 ng/mL, testosterone-d5 at 0.2 ng/mL, 17β-estradiol-d4 at 2 ng/mL, and cortisol-d4 at 2 ng/mL.

5.2.1.6 Steroid Extraction

Steroids were extracted from standards, tissues, plasma, and serum using mechanical homogenization followed by hexane wash, as follows. For placenta samples, 1 mL of acetonitrile was added to the pre-weighed placenta slices in polypropylene tubes with five zirconium oxide beads. Samples were homogenized using a bead mill homogenizer (Omni International Inc., Kennesaw, GA, United States of America) at 4 m/s for four intervals of 30 seconds, separated by break periods of 30 seconds to homogenize the tissue and release the steroids into solution. Samples were then centrifuged at 16,100g for 5 minutes. Five zirconium oxide beads were added to a new set of polypropylene vials for calibration standards, blanks, double blanks, and samples. To vials corresponding to calibration standards, blanks, double blanks, and plasma or serum samples, 1000 μL of acetonitrile was added, and 900 μL of acetonitrile were added to vials corresponding to placenta samples.

From the centrifuged placenta samples, 100 μL of supernatant was added to bring the volume to 1000 μL as with the other tubes. These additional homogenizing and dilution steps were necessary for placenta samples because preliminary testing revealed the concentration of certain steroids in the tissue was very high. Additionally, results were more consistent when larger pieces (13-18 mg of tissue) were used and diluted compared to slicing 1-2 mg.

To all vials except the double blanks, 50 μL of the deuterated internal standard mixture were added. The calibration curve and QCs were prepared by adding 10 μL of the appropriately diluted standard solution to vials. For plasma and serum samples, 10 μL of sample was added to the appropriate polypropylene tube. All standards and samples were homogenized at 4 m/s for 30

seconds in the bead mill homogenizer, then centrifuged at 16,100g for 5 minutes. One millilitre of supernatant was transferred to pre-methanol-rinsed borosilicate glass culture tubes and 500 μL of HPLC-grade hexanes was added to each tube. Samples were vortex mixed and centrifuged as 3200g for 2 minutes. The hexane layer was pipetted off and discarded, and the remaining acetonitrile was dried in a vacuum centrifuge at 60°C for 45 minutes. Samples were reconstituted in 55 μL of HPLC-grade methanol/water 25:75 (v/v), vortexed, and transferred to 0.6 mL polypropylene microcentrifuge tubes using gel loading pipette tips. Samples were then centrifuged at 16,100g for 2 minutes and 50 μL of supernatant was transferred to a glass LC vial insert and stored at -20°C until injection and analysis.

5.2.1.7 Method Validation in Human Placenta, Maternal Plasma, and Cord Serum

The UHPLC-MS/MS method for steroid screening was developed and fully validated by members of Dr. Kiran Soma's laboratory for use in plasma, serum, whole blood, brain tissue, adrenal tissue, and other animal tissues[334,335]. A partial validation was performed with the assistance of Cecilia Jalabert and Cathy Ma for use in human placental tissue. As new standards were prepared and the linear range of the curve was extended, the linearity, limits of quantitation, accuracy, and precision of the new curve was evaluated. Additionally, the recovery and matrix effects of human placenta were evaluated, outlined below.

5.2.1.8 Linearity and Limits of Quantitation

The linearity of the method was originally evaluated over the range of 0.2-1000 ng/mL. Preliminary screening of human placenta tissue revealed high levels of steroids, as expected. Therefore, the lower end of the curve was deemed unnecessary, and the decision was made to

extend the curve so the levels of all steroids could be detected simultaneously. The range of the curve was extended to 2-10000 ng/mL. Linearity was determined by linear regression of the standard curve, using $1/x^2$ least squares weighting. The minimum accepted linearity was r^2=0.985, and the coefficient of variation (CV) of each point was <15% of the nominal concentration or ≤20% for the lowest point on the curve, the lower limit of quantitation (LLOQ).

5.2.1.9 Accuracy and Precision

The intra- and inter-assay accuracy and precision were evaluated using the QCs (QC_{low}, QC_{mid}, QC_{high}) which were run in triplicate in each batch and injected in decreasing order of concentration following two blank samples after the injection of the standard curve. Accuracy was calculated and expressed as a percentage of the nominal concentration of the QC and precision was calculated as the CV of the samples. Inter-assay accuracy and precision were calculated using the QC samples over a minimum of three different validation and sample batches.

5.2.1.10 Recovery in Human Placenta

Recovery was determined by comparing the calculated concentration of pooled placenta tissue spiked with QC_{low} and QC_{high} stock standards to the concentration of unspiked pooled placenta tissue. Previous members of the Soma laboratory had evaluated the recovery in plasma, serum, and whole blood and excellent recovery was observed.

5.2.1.11 Matrix Effects of Human Placenta, Maternal Plasma, and Cord Serum

The matrix effects of human placenta were evaluated in pooled placenta tissue that was homogenized using the bead mill homogenizer. Volumes from the pooled homogenate were taken from the pool and diluted to test the effects of increasing amounts of placenta tissue. Amounts of placenta tested were 0.1, 0.5, 1.0, and 5.0 milligrams. Calculated concentrations were compared to the standard curve and should increase in the correct ratios if placenta tissue is not affecting ionization of the analytes. Additionally, the effect of the tissue on the level of internal standards detected was evaluated.

The matrix effects of maternal blood and cord serum were evaluated by homogenizing 5, 10, and 20 µL of the same sample. Similar to placenta, the calculated concentrations should be the same when corrected for plasma/serum volume. The level of the internal standards was evaluated to determine if increasing plasma/serum volume affected ionization.

5.2.2 Steroid Sulfate LC-MS/MS Method

5.2.2.1 Sample Processing

The maternal plasma and serum samples screened with this method are the same samples screened using the steroid method described above. Samples were only thawed once prior to analysis to give smaller sample volumes and avoid unnecessary freeze-thaws. To prepare the standard curve in the most similar matrix to the samples being screened, charcoal stripped serum was used. The charcoal stripped pooled human serum purchased from Innovative Research (Novi, MI, USA) had detectable levels of DHEAS and E1S and required further stripping before use. The serum was stripped according to a method adapted from Carter, 1978[336]. Briefly, 6 mL

aliquots of serum were dispensed into 15 mL conical tubes and mixed with 800 mg of activated charcoal. Tubes were vortex mixed and shaken for 8 hours at 4°C. Following incubation, tubes were centrifuged twice for 20 minutes at 2000 x g. The supernatant was removed and mixed with 400 mg of kaolin, vortexed, and centrifuged for a further 15 minutes. The resulting supernatant was poured off and filtered through a 0.22 µm syringe filter. This process was repeated to yield 3X charcoal stripped serum, which had no detectable E1S and levels of DHEAS that were less than 1% of the LLOQ.

5.2.2.2 Instrumentation

The UHPLC-MS/MS system used for the steroid sulfate method differs from the system for the steroid method. This system consisted of an Agilent 1290 UHPLC system (Agilent, Mississauga, ON, Canada) interfaced with a Turbo Spray ion source to an AB Sciex QTrap® 5500 hybrid linear ion-trap triple quadrupole mass spectrometer (AB Sciex, Concord, ON, Canada). Data were acquired using Analyst 1.5.2 software.

5.2.2.3 Chromatography Conditions

Compounds were separated using a Waters Acquity UPLC BEH C18 1.7 µm, 2.1 x 50 mm column protected by a Waters Acquity UPLC BEH C18 VanGuard Pre-column, 130Å 1.7µm 2.1 x 5mm (Waters Corporation, Milford, MA, USA). Mobile phase A was 10mM ammonium acetate pH 7.8 in deionized water and mobile phase B was HPLC-grade acetonitrile. Mobile phase gradient programming is presented in Table 5.2. Total run times was 10 minutes, flow rate was 300 µL/min, and injection volume was 5 µL. The autosampler temperature was set to 4°C

and column oven temperature set to 30°C. Between each injection the needle was washed for 5 seconds with deionized water/HPLC-grade acetonitrile (50:50 v/v).

Time (min)	Mobile Phase A (%)	Mobile Phase B (%)	Flow rate (µL/min)
0	90	10	300
1	90	10	300
6	10	90	300
8	10	90	300
8.1	90	10	300
10	90	10	300

Table 5.2 Mobile phase gradient programming for the steroid sulfate LC-MS/MS method. Mobile phase A: 10mM Ammonium Acetate pH 7.8 in Deionized water Mobile phase B: HPLC-grade acetonitrile.

5.2.2.4 Mass Spectrometry Conditions

The mass spectrometers' ion source was operated in negative mode for the duration of the run. Steroids were detected using the most abundant transition as determined by direct infusion of standards. Ion source temperature was 450°C, IonSpray voltage was -4500 V, and dwell time was 150 msec. Transitions, retention times, and spectrometry parameters are outlines in Table 5.3. Steroids were calculated using the deuterated form of the compound as the IS. Analyte traces from a standard curve calibrator is presented in Figure 5.3.

	Q1 Mass (Da)	Q3 Mass (Da)	Retention Time (min)	EP	DP	CE	CXP
d5-DHEAS	372.1	98.0	3.83	-10	-90	-44	-11
DHEAS	367.1	97.0	3.83	-10	-90	-40	-11
d4-E1S	353.1	273.0	3.76	-10	-70	-44	-17
E1S	349.1	269.0	3.76	-10	-85	-44	-9

Table 5.3 Mass spectrometry parameters for steroid sulfates. Entrance potential (EP), Declustering potential (DP), Collision energy (CE), Collision cell exit potential (CXP).

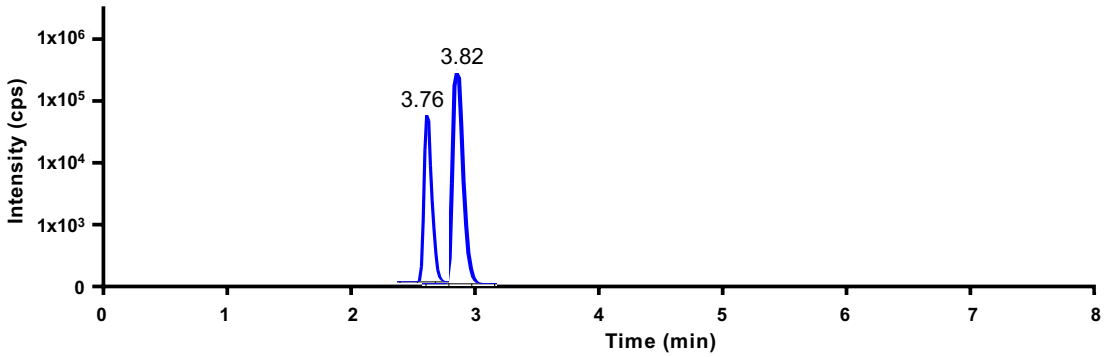

Figure 5.3 Representative chromatogram of standard curve calibrator for DHEAS (3.82 s) and E1S (3.76 s).
Intensity in counts per second (cps).

5.2.2.5 Preparation of Standard Solutions, Calibration Standards, and Quality Controls

Stock standards of DHEAS and E1S were prepared in HPLC-grade methanol to a concentration of 1 mg/mL. Standard stock mix was prepared by combining 90 µL of DHEAS and 36 µL of E1S with 2874 µL methanol in a glass vial. This stock standard mix was used to prepare calibration curve standards at 5, 10, 25, 50, 100, 250, 500, 1000, 1500 ng/mL for DHEAS and 2, 4, 10, 20, 40, 100, 200, 400, 600 ng/mL for E1S, diluting with methanol. Quality control standards were prepared from separate steroid stocks. For DHEAS, QC_{low} and QC_{high} were prepared at 75 and 750 ng/mL, respectively. For E1S, QC_{low} was 30 ng/mL and QC_{high} was 300 ng/mL. All calibration standards and QCs were stored in glass vials at -20°C until use.

The internal standards DHEAS-d5 and E1S-d4 were prepared in HPLC-grade methanol and HPLC-grade methanol/MilliQ H_2O (50:50 v/v), respectively, to 10 µg/mL. The stock solutions were combined and diluted in methanol to 100 ng/mL for DHEAS-d5 and 5 ng/mL for E1S-d4. Enough working solution was made for the duration of the steroid sulfate study.

5.2.2.6 Steroid Sulfate Extraction

Steroid sulfates were extracted from maternal plasma, cord serum, and calibration curve samples by protein precipitation, as follows. The calibration curve by spiking 10 μL of appropriate standard into 90 μL 3X charcoal stripped plasma in a microfuge tube. For maternal plasma and cord serum, 100 μL sample was transferred to a microfuge tube. To all tubes, 15 μL of IS mixture was added, followed by 300 μL of HPLC-grade acetonitrile. Samples were vortexed then centrifuged at 13 000 x g for 10 minutes at 4°C. Two hundred and fifty microliters of supernatant were removed and dried under nitrogen at 30°C until all solvent was evaporated. Samples were reconstituted in ammonium hydroxide pH 7.8/acetonitrile (90:10 v/v) and transferred to glass LC vial inserts and queued for injection to the LC-MS system immediately.

5.2.2.7 Linearity and Limits of Quantitation

Linearity was determined over the range of the calibration curve for each steroid sulfate, 5-1500 ng/mL for DHEAS and 2-600 ng/mL for E1S. The standard curve was accepted if r^2 was greater or equal to 0.985 and each point was within 15% of the nominal concentration or 20% for the lowest point on the curve, which was used as the LLOQ.

5.2.2.8 Accuracy and Precision

Intra- and inter-assay accuracy and precision were evaluated using the QC samples injected with each run, which were run in triplicate following injection of methanol and a blank sample after injection of the standard curve. Accuracy was calculated and expressed as a percentage of the nominal concentration of the QC and precision was calculated as the CV of the samples. Inter-

assay accuracy and precision were calculated using the QC samples over the four days of sample injection.

5.2.3 Statistical Analyses

The levels of each steroid and steroid sulfate in placenta, maternal plasma, and cord serum were compared between unassisted pregnancy and ART groups using a Student's t-test. Unassisted pregnancy and ART groups were then stratified based on ICD9/10 clinical chart codes and steroid/steroid sulfate levels compared between the four resulting groups. Multiple linear regression analysis was performed to determine association between steroid levels and the maternal, obstetric, and neonatal outcomes described in the clinical charts. Because maternal age, maternal BMI, and fetal sex have been previously shown to affect steroid hormone levels, these variables were built into the linear regression models to account for the variation they may cause. Statistical analysis was performed using R Software and R Studio (Boston, MA, USA) and visualized using GraphPad Prism (San Diego, CA, USA).

5.2.4 Steroid Ratios for Investigating Activities of 3β-HSD and CYP19

To investigate the activities of 3β-HSD and CYP19 within the placenta, we used the ratios of product/substrate as a proxy for enzyme activity. For 3β-HSD, the ratio of progesterone/pregnenolone (P4/P5) was calculated based of the results of the steroid LC-MS/MS method and results were compared between unassisted pregnancy and ART groups as before. Groups were then stratified based on ICD9/10 clinical chart codes and compared again. For CYP19 activity, the ratio of estradiol/testosterone (E2/T) was calculated, and comparisons were

made as above. Statistical analysis was performed using R Software and R Studio (Boston, MA, USA) and visualized using GraphPad Prism (San Diego, CA, USA).

5.3 Results

5.3.1 Method Validation for Steroid LC-MS/MS Method

As previously mentioned, the steroid screening method had been previously developed and fully validated by members of Dr. Kiran Soma's laboratory. As per the FDA guidelines on bioanalytical method validation, a partial validation was performed to determine linearity, limits of quantitation, accuracy, precision, recovery, and matrix effects of human placenta. A representative chromatogram is shown in Figure 5.4.

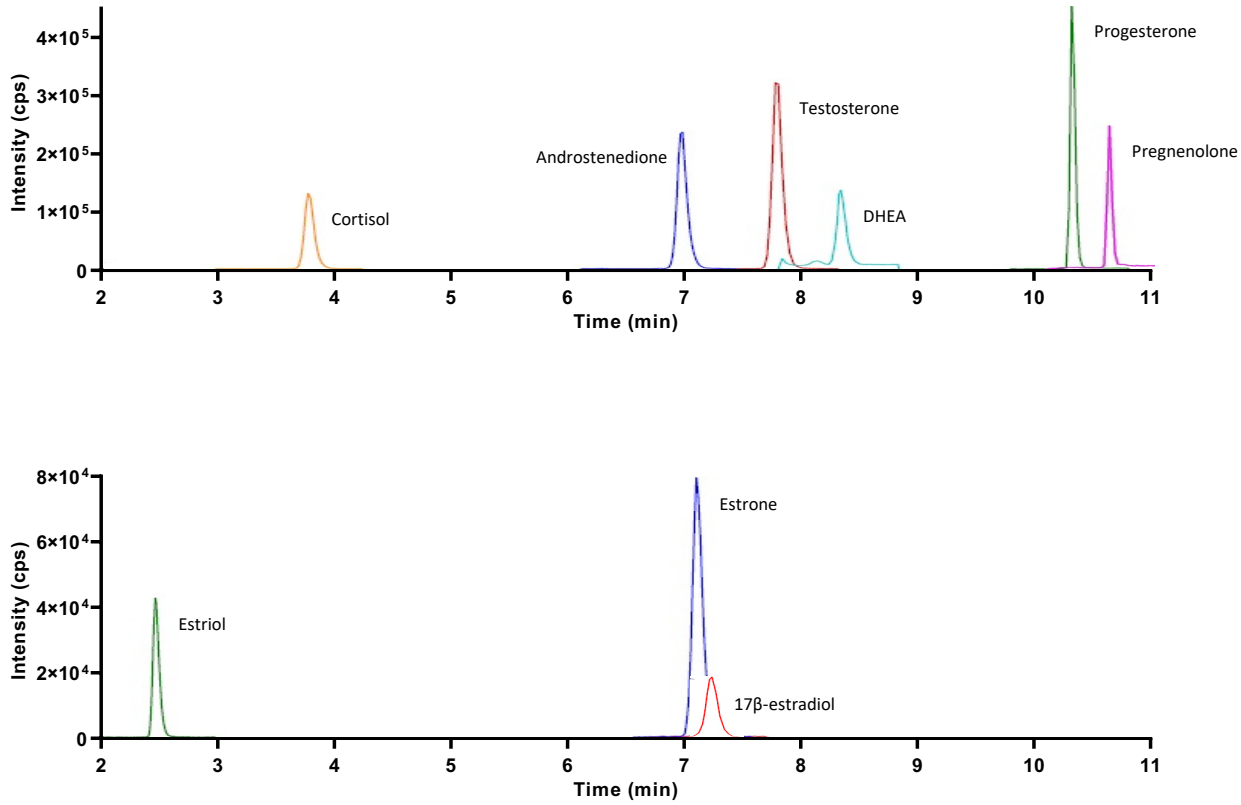

Figure 5.4 Representative chromatogram of the quantifier ion of steroid analytes. This sample contains reference standards all at 50 pg, except DHEA and pregnenolone at 500 pg. Upper panel shows steroids quantified in positive mode, bottom panel shows steroids quantified in negative mode. Counts per second (cps).

5.3.1.1. Linearity and Limits of Quantitation

The calibration curves were prepared on day of analysis in solvent and steroids were extracted using the same protocol as the placenta tissue, maternal plasma, and cord serum. As previously mentioned, the curves were extended past their previously validated range of 0.2-1000 ng/mL and changed to 2-10000 ng/mL. The curves for all steroids were linear to 10000 pg with the exception of androstenedione, which was terminated at 5000 pg (Table 5.4). The LLOQ is the lowest point of the curve, and the calculated value was acceptable if it fell within 20% of the nominal concentration, for all other points the values fell within 15% of the nominal concentration. Most calculated values were much closer than 15%. The linear ranges for all compounds are presented in Table 5.4.

Analyte	Linear Range (ng/mL)	Linearity (r^2 value)
Pregnenolone	4-10000	0.994
Progesterone	2-10000	0.999
DHEA	4-10000	0.996
Androstenedione	2-5000	0.998
Testosterone	2-10000	0.999
Estrone	2-10000	0.997
17β-Estradiol	2-10000	0.998
Estriol	2-10000	0.993
Cortisol	2-10000	0.999

Table 5.4 Linear ranges and r2 values for the steroids included in the LC-MS/MS method.

5.3.1.2 Accuracy and Precision

Accuracy and precision were evaluated following each injection queue using the QCs run in triplicate at three different concentrations. Acceptable values defined by the FDA are accuracy within 15% of the nominal concentration and a CV less than 15% for precision. Intra-day and inter-day accuracy and precision results are outlined in Table 5.5. Values fell within acceptable limits.

Analyte	Nominal Concentration	Accuracy (% Nominal)		Precision (%CV)	
		Intra-day	Inter-day	Intra-day	Inter-day
Pregnenolone					
QC_{low}	20	105.95	96.28	6.25	7.84
QC_{mid}	200	100.13	104.57	1.14	5.83
QC_{high}	2000	92.65	100.05	2.14	5.67
Progesterone					
QC_{low}	20	104.96	105.75	6.25	4.36
QC_{mid}	200	102.05	103.39	3.93	2.84
QC_{high}	2000	96.55	95.99	1.87	2.20
DHEA					
QC_{low}	20	91.32	90.96	2.37	4.25
QC_{mid}	200	97.90	97.25	1.28	3.55
QC_{high}	2000	101.07	100.92	1.70	2.71
Androstenedione					
QC_{low}	20	100.95	99.33	1.49	5.12
QC_{mid}	200	99.20	97.91	1.22	3.43
QC_{high}	2000	101.55	96.26	2.03	5.13
Testosterone					
QC_{low}	20	105.46	104.98	0.68	2.18
QC_{mid}	200	105.00	104.10	1.22	1.15
QC_{high}	2000	98.76	97.65	1.99	1.42
Estrone					
QC_{low}	20	101.68	101.70	1.00	2.43
QC_{mid}	200	98.41	100.39	1.01	2.26
QC_{high}	2000	104.11	100.03	6.24	4.18
17β-Estradiol					
QC_{low}	20	101.74	101.50	2.15	3.10
QC_{mid}	200	101.51	101.27	1.47	2.12
QC_{high}	2000	97.00	98.40	1.63	2.51
Estriol					
QC_{low}	20	96.74	99.33	0.89	2.75
QC_{mid}	200	96.10	100.12	1.25	3.27
QC_{high}	2000	97.31	98.85	2.41	3.61

Table 5.5 Intra-day and inter-day accuracy and precision for the steroid analytes included in the LC-MS method.

Accuracy and precision calculated using the three quality control (QC) concentrations.

5.3.1.3 Recovery in Human Placenta

Recovery was determined by comparing the calculated concentration in unspiked placenta samples to placenta spiked with QC low and QC high calibration standards. Results are

presented in Table 5.6. Recovery for the steroids yielded good results, within 15% of the unspiked concentration. The QC_{low} spike for progesterone was not detectable because the unspiked placenta was above the highest point of the curve (1000 pg, before the calibration curve was extended) and the spike of 20 pg was not detectable for this sample. These results helped lead to the decision of extending the curve. Additionally, DHEA was not detectable in the human placenta. Recovery results show that DHEA can be accurately and reliably extracted from the placenta (103.2-108.7%), therefore DHEA was not present in the tissue, or amounts are below the LLOQ of 2 pg.

Analyte	**Recovery (%)**
Pregnenolone	
QC_{low}	87.3%
QC_{high}	94.8%
Progesterone	
QC_{low}	-
QC_{high}	121%
DHEA	
QC_{low}	103.2%
QC_{high}	108.7%
Androstenedione	
QC_{low}	115.6%
QC_{high}	111.6%
Testosterone	
QC_{low}	98.9%
QC_{high}	101.6%
Estrone	
QC_{low}	109.7%
QC_{high}	101.9%
17β-Estradiol	
QC_{low}	100.7%
QC_{high}	100.5%
Estriol	
QC_{low}	115.2%
QC_{high}	109.5%

Table 5.6 Recovery of the steroid analytes included in the LC-MS/MS method. Recovery was calculated at two concentrations: QC_{low} (20 ng/mL) and QC_{high} (2000 ng/mL).

5.3.1.4 Matrix Effects of Human Placenta

The matrix effects of human placenta were evaluated to determine if increasing amounts of tissue affected the levels of steroids measured. Placenta tissue was pooled and homogenized and supernatant corresponding to 0.1, 0.5, 1.0, and 5.0 mg of tissue were tested. Area ratios were plotted against calculated concentration (Figure 5.5). Area ratios increased linearly with amount of tissue, showing minimal matrix effects for placenta tissue. Using the linear ranges of the curves and the amounts measured in pooled placenta tissue, the decision to use 1.5 mg of placenta tissue for screening was made in order to measure all steroids simultaneously using one dilution of tissue lysate.

The effect of increasing placenta tissue on the ionization of the internal standards was also evaluated. The peak area of each internal standard was compared between QC's, calibration standards, and 0.1, 0.5, 1.0, and 5.0 mg of placenta tissue (Figure 5.6). There were no differences in peak area until 5.0 mg of placenta, which decreased the area of pregnenolone-d9 and progesterone-d9 (Figure 5.6).

To determine the matrix effects of maternal blood and cord serum, steroids were extracted from 5, 10, and 20 μL of the same sample. For maternal plasma, there were no matrix effects for 5 or 10 μL of sample, but 20 μL led to an ion ratio issue with the quantifier and qualifier for pregnenolone and DHEA. Additionally, pregnenolone was not detectable in maternal plasma. For cord serum, the 5 μL of sample showed an ion ratio issue, but 10 μL and 20 μL had no matrix effects. All steroids were detectable in cord serum. Based on the concentrations of

steroids measured and the matrix effects observed, 10 µL was determined to be the optimal sample amount for both maternal plasma and cord serum.

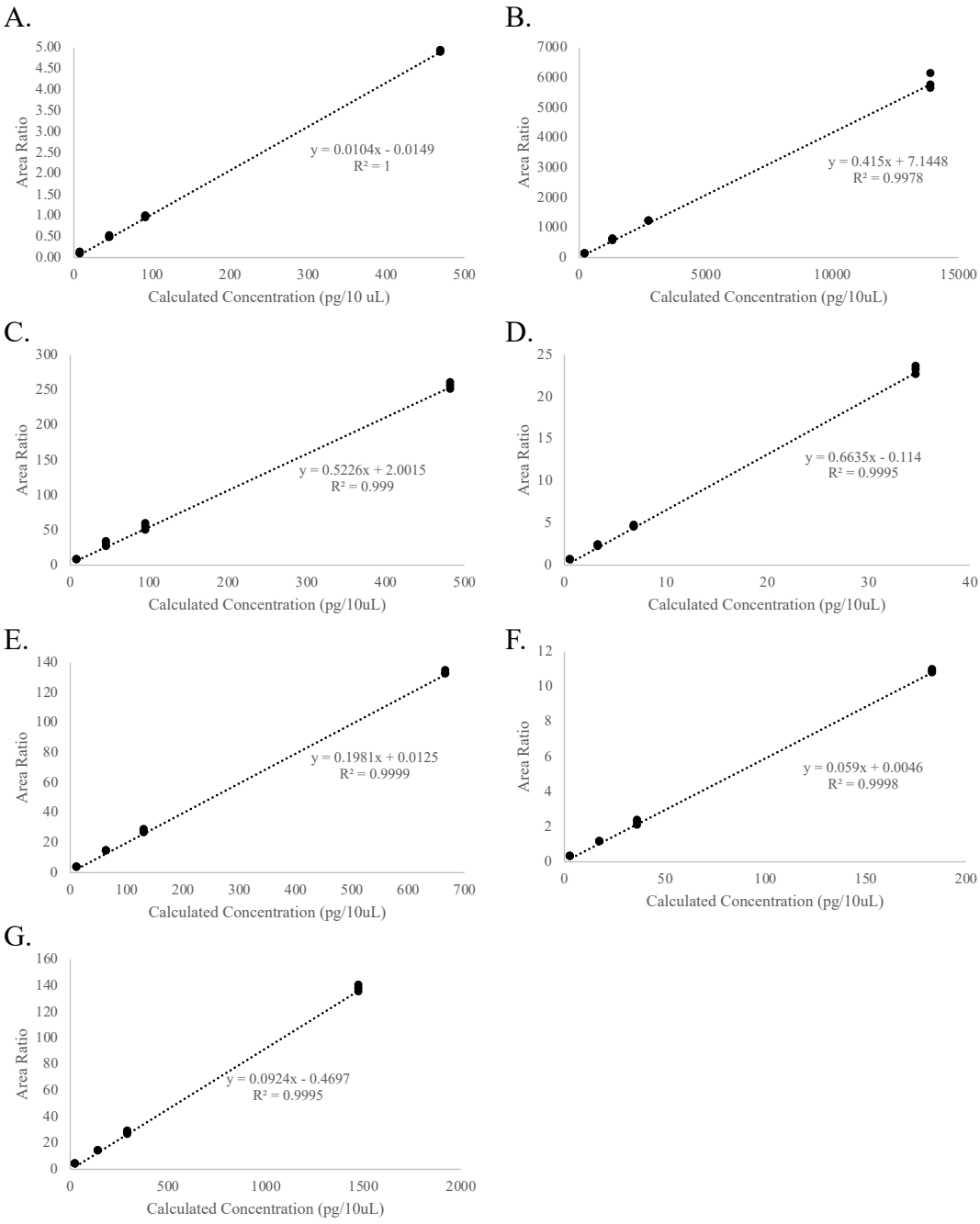

Figure 5.5 Plots evaluating the matrix effect of increasing amount of placenta tissue. Linearity indicates minimal matrix effect. A. Pregnenolone B. Progesterone C. Androstenedione D. Testosterone E. Estrone F. 17β-Estradiol G. Estriol.

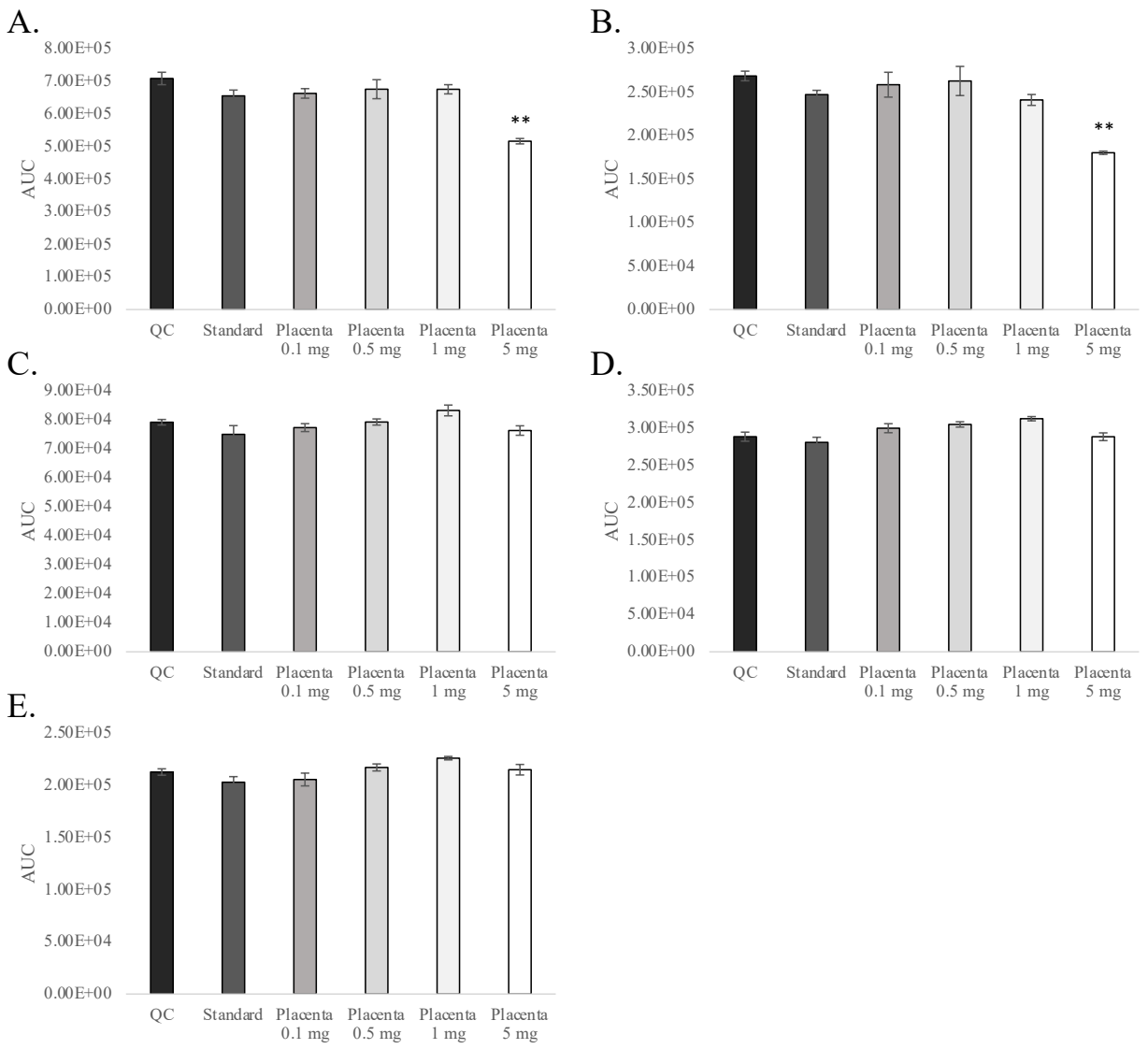

Figure 5.6 Matrix effect of increasing concentration of placental tissue on the ionization of internal standards.

A. Pregnenolone-d4 B. Progesterone-d9 C. DHEA-d6 D. Testosterone-d5 E. 17β-Estradiol-d4. **p<0.01.

5.3.2 Method Parameters for Steroid Sulfate LC-MS/MS Method

5.3.2.1 Steroid Sulfate Extraction

The UHPLC-MS/MS method was originally intended for screening maternal plasma, cord serum, and placenta. A protein precipitation with acetonitrile was an effective extraction method for both maternal plasma and cord serum but did not work for either placenta lysate or S9

fraction. There were no measurable amounts of DHEAS or E1S in either tissue preparation. Multiple liquid-liquid extraction techniques were tested using MTBE and ethyl acetate, and different pH values, but steroid sulfates could still not be detected. To determine if the analytes were remaining in the buffer/tissue after extraction, tissue slices were made from remaining frozen tissue punches and lysed directly in acetonitrile, followed by a hexane wash using the method described above for the steroid LC-MS/MS method. Increasing amounts of tissue were tested, however no steroid sulfates were detected using the LC-MS/MS method following extraction. To ensure the lysing directly in acetonitrile/hexane wash preparation could extract steroid sulfates, it was tested using a slice of adrenal gland from our archives (where high levels of DHEAS are expected). The extraction method worked well for adrenal gland, producing a clean sample with DHEAS levels on the high end of the calibration curve. It is possible that low levels of DHEAS and E1S are present in placental tissue, but at levels lower than we are capable of extracting and measuring with the methods available to us. A solid-phase extraction technique may be a useful technique for extracting steroid sulfates from placental tissue, but this was not evaluated here. Therefore, only the circulating levels of DHEAS and E1S were evaluated in maternal and fetal plasma.

5.3.2.2 Linearity and Limits of Sensitivity

The calibration curves were prepared on day of analysis in 3X charcoal stripped plasma and extracted by acetonitrile protein precipitation alongside the patient samples. Both curves were linear over the range of the calibration standards, which were chosen to cover the concentrations of DHEAS and E1S expected in maternal plasma and fetal serum. Linear ranges and linearity measures are presented in Table 5.7. The LLOQ is the lowest point of the curve, and the

calculated value was acceptable if it fell within 20% of the nominal concentration, for all other points the values fell within 15% of the nominal concentration.

Analyte	Linear Range (ng/mL)	Linearity (r^2 value)
DHEAS	5-1500	0.995
E1S	2-600	0.999

Table 5.7 Linear ranges and r2 values for the steroid sulfates included in the LC-MS/MS method.

5.3.2.3 Accuracy and Precision

Accuracy and precision were evaluated for each run using the QCs run in triplicate at two concentrations, QC_{low} and QC_{high}. Acceptable values defined by the FDA are accuracy within 15% of the nominal concentration and a CV less than 15% for precision. Intra-day and inter-day accuracy and precision results are outlined in Table 5.8. Values were within the acceptable limits.

Analyte	Nominal Concentration	Accuracy (% Nominal)		Precision (%CV)	
		Intra-day	Inter-day	Intra-day	Inter-day
DHEAS					
QC_{low}	75	89.43	88.75	8.31	7.30
QC_{high}	750	105.28	107.30	2.32	2.27
E1S					
QC_{low}	30	104.76	109.20	5.69	5.14
QC_{high}	300	97.17	101.12	10.24	10.71

Table 5.8 Intra-day and inter-day accuracy and precision for the steroid sulfates included in the LC-MS method.

Accuracy and precision calculated using the two quality control (QC) concentrations.

5.3.3 Association of Maternal, Placental, and Fetal Steroid Hormone Levels with Pregnancy Outcome

The levels of steroid hormones in the placenta, maternal plasma, and cord serum were first compared between unassisted pregnancies and ART pregnancies. Plasma and serum samples corresponding to all placenta samples were not available from the biorepository, only a subset

existed from both unassisted and ART pregnancy. For unassisted pregnancies, 35 maternal plasma samples were available, and 24 cord serum samples were available. For ART pregnancies, we had access to 33 maternal plasma samples and 27 cord serum samples. The only statistically significant difference found was in the levels of estrone in the placenta, which were higher in assisted reproduction. Graphs comparing the levels in maternal plasma, placenta, and cord serum are shown in Figure 5.7.

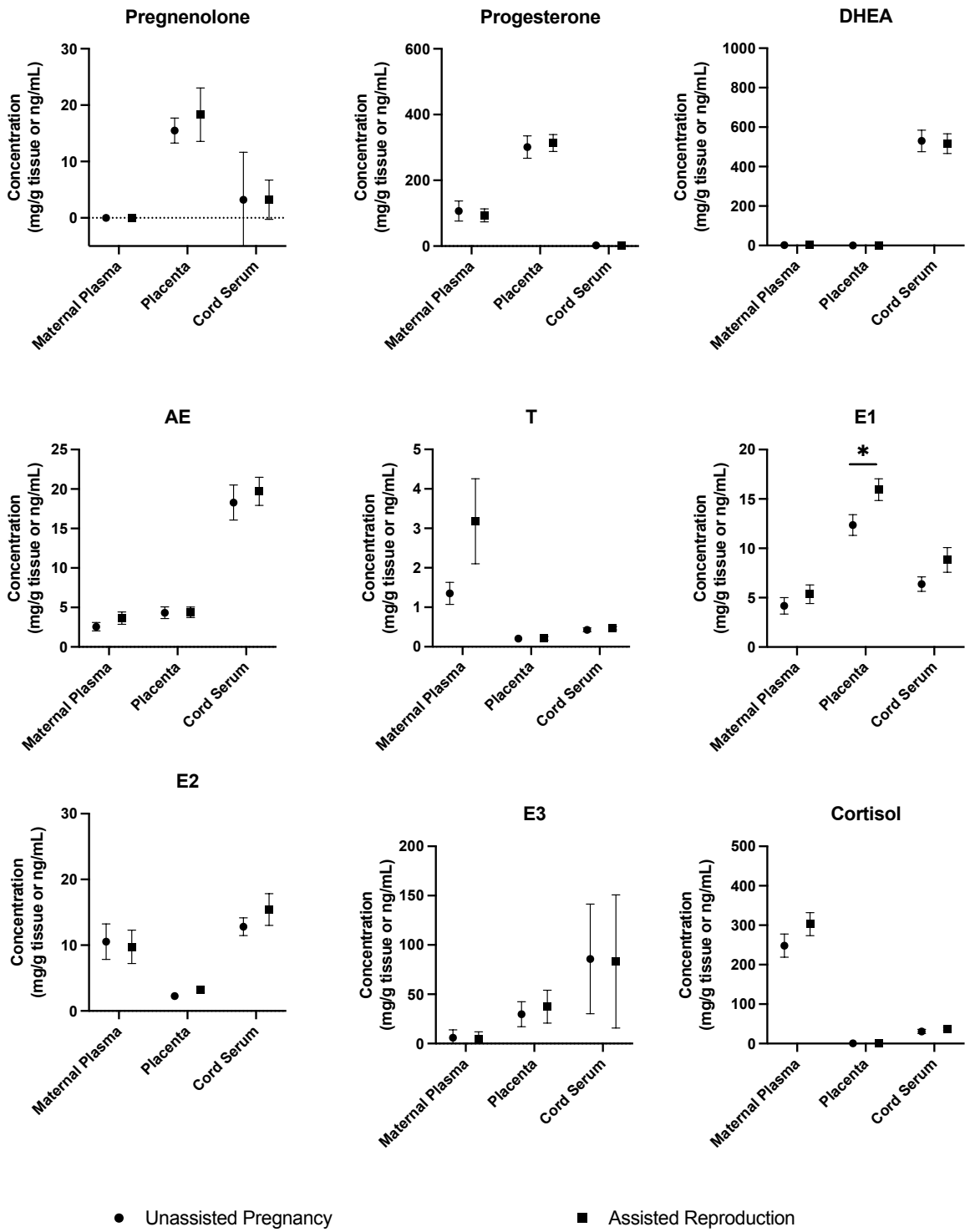

Figure 5.7 Levels of steroids in maternal plasma or cord serum (ng/mL) and placental tissue (mg/g) comparing levels across the maternal-placental-fetal interface.
Steroids detected in all tissues, except pregnenolone in maternal plasma (detected in 4/35 for unassisted and 2/33 for ART) and DHEA in maternal plasma (detected in 29/35 for unassisted and 28/32 for ART). DHEA never detected in placental tissue. *$p<0.05$

Data for placental steroid levels were further stratified from unassisted pregnancy and ART into groups using ICD9/10 clinical chart codes. In the stratified analysis, multiple linear regression models were built using R Software in order to incorporate the effects of maternal age, maternal BMI, and fetal sex which are known to affect steroid hormone levels. No significant differences were observed between ART and unassisted pregnancy stratified into groups for augmentation, induction, IUGR, or membrane rupture, or small for gestational age infants. When unassisted pregnancy and ART were stratified based on delivery method (vaginal delivery vs. Caesarean section) the levels of cortisol were significantly lower in Caesarian delivery of unassisted pregnancies. No other differences existed with respect to delivery method.

There were two clinical situations where the levels of more than one steroid were altered after multiple linear regression of stratified groups. In both unassisted and ART pregnancies complicated by chorioamnionitis, levels of estrone were increased, but the levels of estradiol were decreased (Figure 5.8). Pregnenolone levels were higher in unassisted pregnancies with chorioamnionitis but unchanged in ART, and the opposite pattern was observed for androstenedione. Additionally, cortisol levels were significantly lower in unassisted pregnancies with chorioamnionitis compared to unassisted pregnancies without chorioamnionitis.

Second, differences were observed in steroid levels between unassisted pregnancy and ART pregnancy singleton and twins (Figure 5.9). The changes observed occurred in both unassisted pregnancy and ART pregnancy. The levels of progesterone were increased in twins, but levels of androstenedione, testosterone, and estrone were decreased in twins. Additionally, the levels of cortisol were lower in twins of unassisted pregnancy compared to singletons (Figure 5.9).

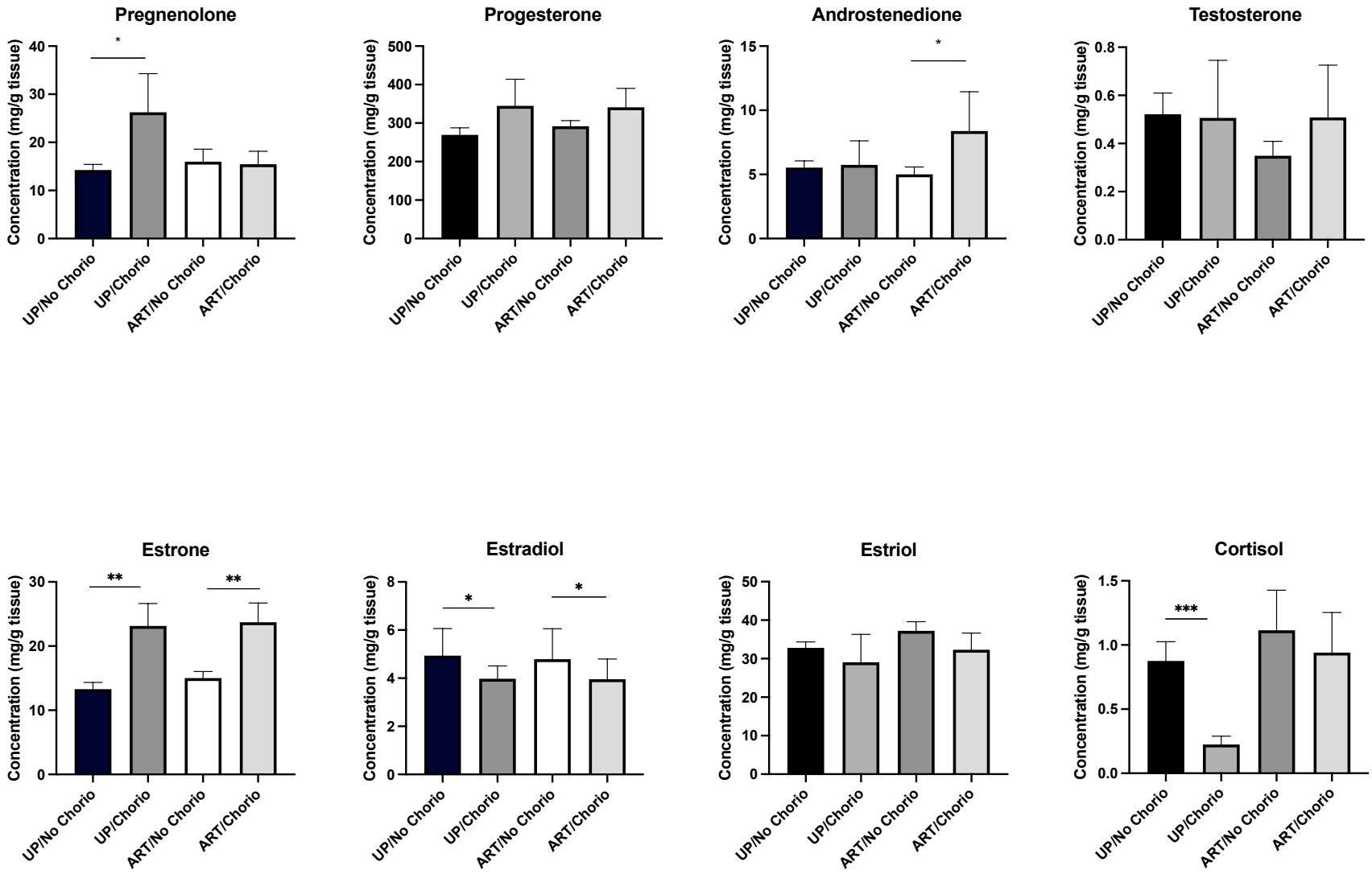

Figure 5.8 Placental steroid levels in unassisted pregnancies and ART pregnancies with and without chorioamnionitis.
Bars are mean ± SEM. Unassisted pregnancy (UP), Assisted reproduction pregnancy (ART) Chorioamnionitis (Chorio), No chorioamnionitis (No Chorio). *p<0.05, **p<0.01, ***p<0.001.

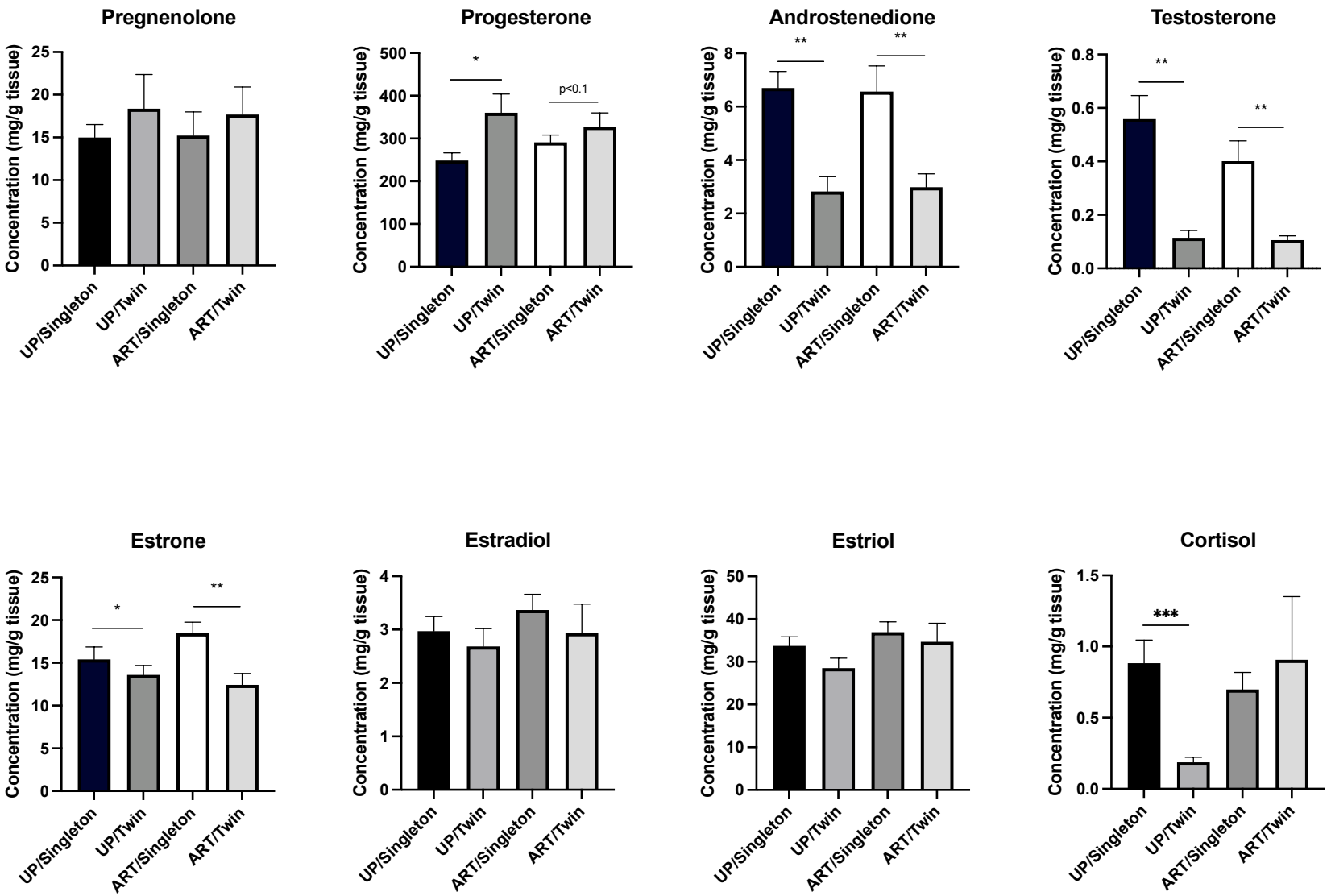

Figure 5.9 Placental steroid levels in singleton and twin unassisted pregnancies and ART pregnancies.
Unassisted pregnancy (UP), Assisted reproduction pregnancy (ART) Bars are mean ± SEM. *p<0.05, **p<0.01

5.3.4 Association of Maternal and Fetal Circulating Steroid Sulfate Levels with Pregnancy Outcome

The levels of steroid sulfates were measured in maternal plasma and cord serum samples of both unassisted pregnancies and ART pregnancies. There were no differences in the levels of maternal DHEAS or E1S between unassisted pregnancy and ART (Figure 5.10). In the fetal circulation, levels of E1S were significantly higher in ART (Figure 5.11). Because of the lower numbers of samples, we could not conduct the full stratified analysis performed in the placenta; only subgroups with a minimum of four samples were included in the analysis. Accordingly, no significant differences were observed in the subgroup analysis.

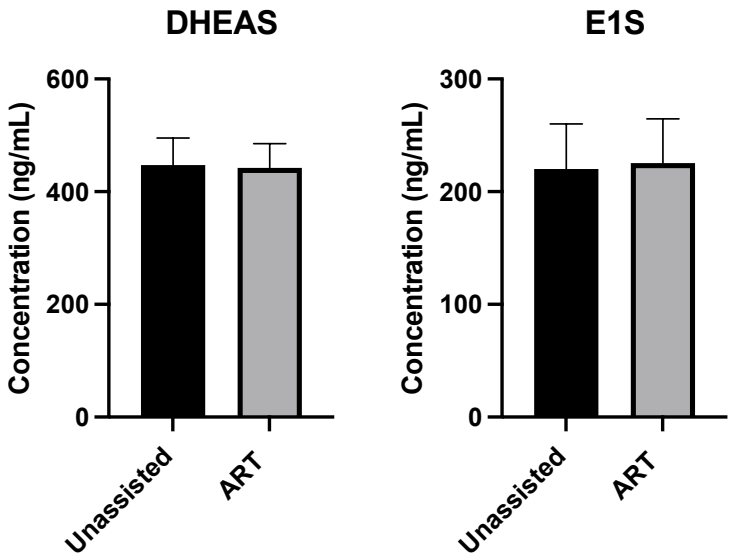

Figure 5.10 Levels of DHEAS and E1S in maternal plasma, comparing unassisted and ART pregnancies.
Bars are mean ± SEM. No significant differences observed.

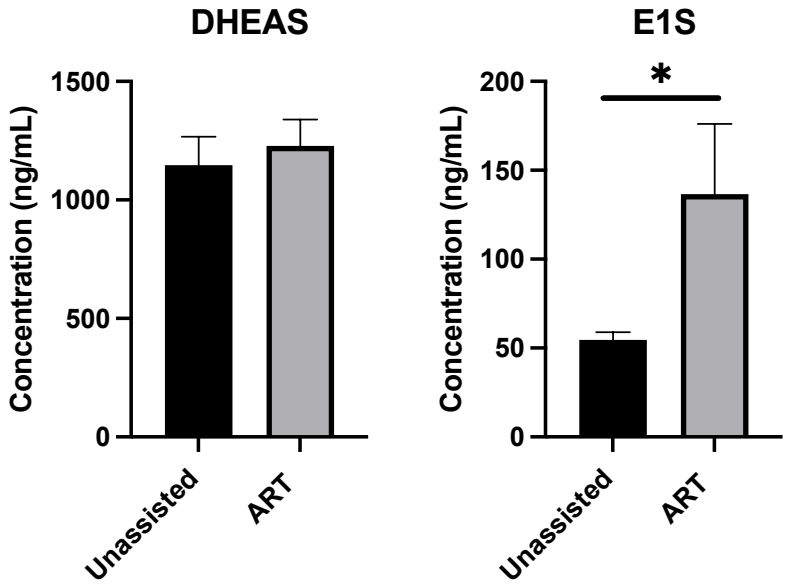

Figure 5.11 Levels of DHEAS and E1S in cord serum, comparing unassisted and ART pregnancies.
Bars are mean ± SEM. *p<0.05.

5.3.5 Steroid Ratios as a Measure of 3β-HSD and CYP19 Activities and Association with Pregnancy Outcome

As a measure of steroidogenic enzyme activity, the ratio of product: substrate was calculated for the reactions of 3β-HSD and CYP19. For 3β-HSD, the ratio of progesterone to pregnenolone (P4/P5) was used and for CYP19 the ratio of estradiol to testosterone (E2/T) was used. The ratios were first compared between unassisted pregnancy and ART, building a regression model to incorporate the effects of gestational age, maternal age, and fetal sex on steroid levels. There were no differences in the ratio of progesterone/pregnenolone between unassisted pregnancies and assisted reproduction (Figure 5.12). The ratio of estradiol/testosterone was significantly higher in ART compared to unassisted pregnancy (Figure 5.12).

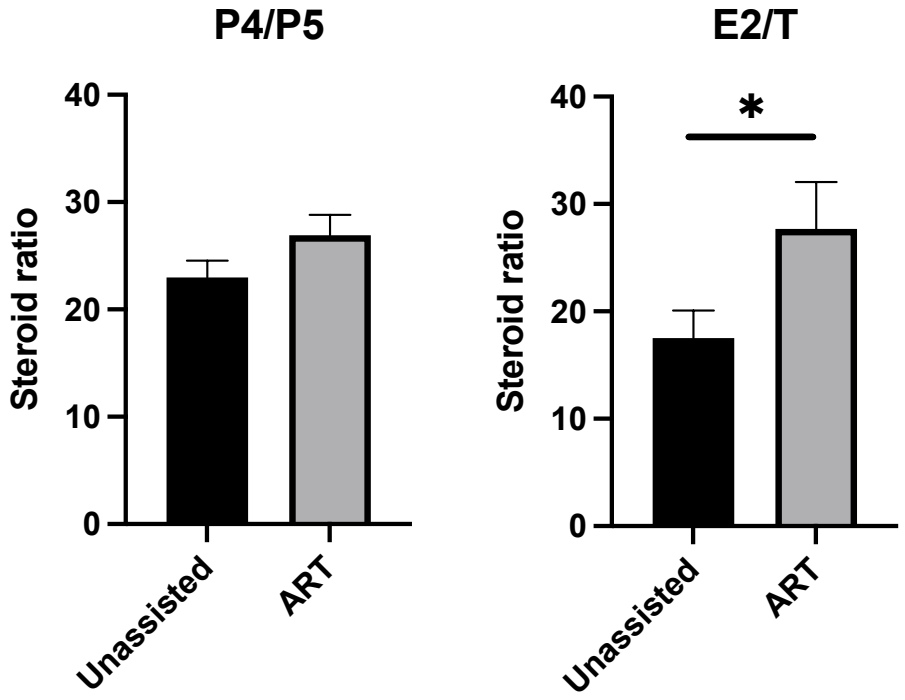

Figure 5.12 Ratio of progesterone to pregnenolone (P4/P5) in placentas as a measure of 3β-HSD activity and ratio of estradiol to testosterone (E2/T) in placentas as a measure of CYP19 activity, comparing unassisted pregnancy to assisted reproduction.
Bars are mean ± SEM. *p<0.05.

Unassisted pregnancy and ART groups were further stratified based on pregnancy outcome using clinical chart codes. No significant differences were observed between ART and unassisted pregnancy stratified into groups for augmentation, chorioamnionitis, delivery method, induction, IUGR, or membrane rupture, or small for gestational age infants. When stratified for singleton vs. twin pregnancy, a significant difference was observed between the ratio of estradiol/testosterone in unassisted pregnancies (Figure 5.13). The ratio of estradiol/testosterone was significantly higher in unassisted twins compared to unassisted singletons, however no difference was observed in ART. No differences were observed in the ratio of progesterone/pregnenolone in singleton vs. twins, but results are shown for completeness (Figure 5.13)

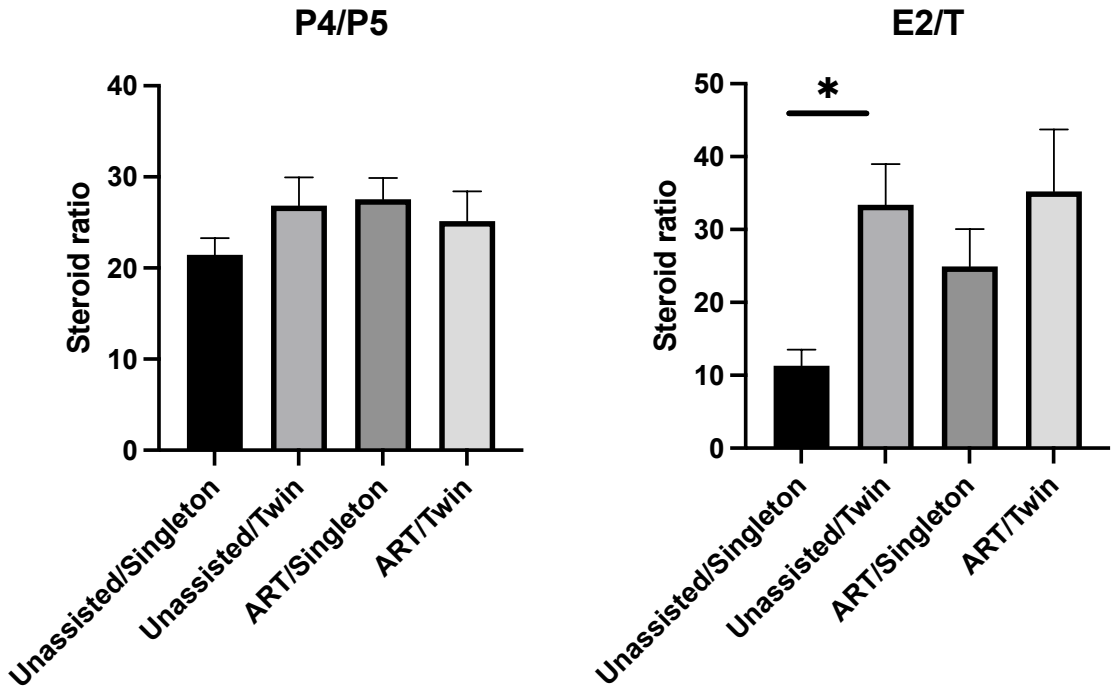

Figure 5.13 Ratio of progesterone to pregnenolone (P4/P5) in placentas as a measure of 3β-HSD activity and ratio of estradiol to testosterone (E2/T) in placentas as a measure of CYP19 activity, comparing singletons and twins of unassisted pregnancy to assisted repr
Bars are mean ± SEM. *p<0.05.

5.4 Discussion

5.4.1 Comparison of Steroid Hormone Levels in Assisted Reproduction and Unassisted Pregnancy

In this study we measured the levels of the steroid hormones pregnenolone, progesterone, androstenedione, DHEA, testosterone, estrone, estradiol, estriol, and cortisol in placentas, maternal plasma, and cord serum from a matched cohort of unassisted and ART pregnancies. The UHPLC-MS/MS method used was highly sensitive, accurate, and precise and was successfully adapted for use in human placental tissue. Because the method was originally designed for steroid screening in brain and whole blood from birds, where levels are expected to be low, the method had the benefit of requiring very small amounts of tissue or plasma for

screening. However, to apply the method to pregnant human samples, where steroid levels are much higher, the method needed to be adapted. Extension of the calibration curves by 10 times their original ranges yielded excellent results for linearity, showing the method was robust over a wide range of concentrations. Further, the addition of a dilution step in the extraction method for placental tissue samples resulted in more accurate results with a minimal matrix effects.

Some differences were noted between maternal plasma, placental tissue, and cord serum in the steroid analysis. Pregnenolone was only detectable in 7% of maternal plasma samples but was quantified in 100% of cord serum and placental tissue samples. In placenta, all steroids in the method were measured except DHEA, which was never detected in the tissue but quantified in 87% of maternal plasma samples and 100% of cord serum samples. Notably, the levels of DHEA in cord serum were 210-220 times higher than in maternal plasma. In adults, DHEA circulates predominantly in its sulfated from, DHEAS, which is the most abundant circulating steroid. Reference ranges are 20-90 ng/mL for DHEA and 500-2500 ng/mL for DHEAS, which is highly variable based on sex and age[303]. Pregnancy is known to affect circulating levels of DHEA and DHEAS, and our results align with previously published results in pregnancy[337]. The fetal adrenal produces high levels of DHEA and DHEAS in pregnancy, which increase as gestation progresses and accounts for the high levels measured here[338]. The lack of DHEA in the placenta was surprising, as the uptake of DHEAS from fetal circulation and subsequent removal of the sulfate group by STS is well characterized in the placenta[326,327,339]. However, the production of estrogens increases during pregnancy and peaks at parturition. The activities of steroidogenesis enzymes remain active in the placenta, and it is possible the DHEA in the placenta had been converted to estrogens or is present at a level too low to detect using the UHPLC-MS/MS

method. The utility of the method for extracting and measuring DHEA in tissue was tested in a human adrenal from our archives, and high levels of DHEA were quantified. Additionally, it has been reported that 16α-OH-DHEA is the most abundant C19 steroid precursor for estrogen synthesis in the placenta, contributing to 90% of placental estriol produced[340,341]. Therefore, it may have been beneficial to screen for 16α-OH-DHEA in the placenta, but it was not available at the time of screening.

Previous studies from a murine model of ART in our laboratory identified differences in steroid hormone levels, metabolism, and delivery between unassisted pregnancy and ART[97,332,333]. In human samples, we did not identify differences in steroid hormone levels in the maternal, placental, or fetal compartments, with the exception of estrone levels in the placenta which were increased in ART. However, this remains important as any dysregulation of the steroidogenesis pathway has the potential to impact pregnancy outcomes. Due to the differences in steroidogenesis in humans and mice, we did not expect the results here to be the same as observed in mice. In mice, the ovary is responsible for producing steroids throughout pregnancy, while in humans the corpus luteum regresses. The mouse placenta also expresses higher levels of CYP17 and can therefore synthesize estrogens on its own. While mice provide a convenient and cost-effective model, they are far from perfect models of human pregnancy.

To our knowledge, this is the largest study to compare steroid levels in all three compartments between ART and unassisted pregnancy. While the results did not match the murine studies, it is encouraging that steroid levels do not appear to be altered in ART overall, as correct steroid levels are important in all aspects of pregnancy. However, once ART and unassisted pregnancy

groups were stratified, differences were noted in steroid hormone levels in pregnancies complicated by chorioamnionitis and twin pregnancies. In contrast to the differences observed in cytokine levels and the antioxidant defense network in these two clinical situations, both unassisted pregnancy and ART exhibited the same patterns in altered steroid levels.

In pregnancies complicated by chorioamnionitis, there were changes in the levels of estrone and estradiol observed in both ART and unassisted pregnancy, but changes in pregnenolone, androstenedione, and cortisol were specific to the method of conception. This is the first study to directly compare placental steroid levels between pregnancies with and without chorioamnionitis, however the results may be partially explained by studies investigating the effects of infection on placental transporters[342,343]. In both rats and humans, maternal infection leads to decreased expression and activity of placental transporters ABCG2 and SLCO2B1[342,343]. In placentas of preterm births with infection, mRNA and expression of SLCO2B1 and ABCG2 were decreased[343]. At term, placentas of women with infection had lower levels of mRNA expression. Downregulation of SLCO2B1 could directly affect the levels of placental steroids as it is responsible for the uptake of DHEAS from the fetal circulation[326]. As previously described, the placenta requires the uptake of DHEAS to synthesize the high levels of estrogens required in pregnancy. The expression of these transporters also correlated with expression of pro-inflammatory cytokines[343], which we have shown are also dysregulated in chorioamnionitis. Further, there is evidence that hypoxia signalling can result in downregulation of placental ABCG2[344]. This may also affect transfer of steroids to the fetus in hypoxia-related pregnancy complications, of which chorioamnionitis is related[344]. Complex interactions of positive and negative feedback exist between inflammatory cytokines, prostaglandins, and steroid hormones

which requires more work to decipher in this context, but further investigation may help determine where the dysregulation is occurring.

In twin vs. singleton pregnancies, progesterone was increased while androstenedione, testosterone, and estrone were decreased in twins of both unassisted and ART pregnancies. Few studies investigating placental steroid levels between singletons and twins exist, however there is evidence of altered circulating steroids in twins compared to singletons[345-347]. By mid-gestation, serum measurements of estradiol and testosterone show increased levels in twins[345]. Similar results were shown in the third trimester by Houghton *et al.,* where maternal steroid levels were modestly higher in twins, but similar in cord serum at birth[346]. Further, in a sample of ART pregnancies, levels of estradiol and β-hCG were higher in the first trimester, suggesting the potentially role of these hormones in support of early twin pregnancy[347]. In contrast, the levels of androstenedione, testosterone, and estrone were lower in the placentas of twin pregnancies. This pattern is consistent with changes in placental uptake and transport of steroids. The placenta can synthesize progesterone on its own, without necessary uptake of precursors, and higher levels of progesterone may be expected in twins, to maintain the pregnancy of two fetuses. Lower levels of downstream steroids which require the uptake of C19 steroids from the fetal circulation may indicate dysregulation in steroid transport in twins, however more work in needed to investigate transporter and enzyme function to confirm this.

5.4.2 Comparison of Steroid Sulfate Levels in Assisted Reproduction and Unassisted Pregnancy

In addition to the free steroids measured, the levels of circulating steroid sulfates DHEAS and E1S were measured in the maternal plasma and cord serum samples in the cohort. The measurement of steroid sulfates was originally intended to be performed in placental tissue, but they were never detected using the LC-MS/MS method despite multiple preparations and extraction methods tested. This contrasts with the documented uptake of both DHEAS and E1S in placental tissue[326,348]. However, as discussed above, 16α-OH-DHEAS is more abundant in the fetal circulation and may have been a better compound to include in the LC-MS/MS method.

The only significant difference observed in circulating steroid sulfate levels was the level of E1S in cord serum, which was higher in ART compared to unassisted pregnancy. This is the first evidence of altered levels of steroid sulfates in the fetal circulation in humans. In fact, there is little research until the levels of conjugated estrogen in humans. Most studies of circulating estrone sulfate define the levels in bovine, ovine, and goat pregnancy with respect to fetal number and growth[152,349-351]. Estrone sulfate is produced by the feto-placental unit[351], but it's roles outside of a circulating form of estrogen and any associations with adverse pregnancy outcomes are not well defined.

5.3.4 Association of Steroid Ratios and Pregnancy Outcome

The ratios of progesterone/pregnenolone and estradiol/testosterone were used as a proxy for the activities of the steroidogenesis enzymes 3β-HSD and CYP19, respectively. While not a perfect measure of enzyme activity, this analysis was performed as a preliminary measure of

steroidogenesis enzyme activity, with plans to perform enzyme activity assays in the future. ART pregnancies had a higher ratio of estradiol/testosterone than unassisted pregnancy, indicating increased activity of CYP19.

Once the unassisted and ART groups were stratified based on singleton vs. twin pregnancy, the difference in ratio of estradiol/testosterone was lost in ART but a significant difference was observed in unassisted pregnancies. The conversion to estrogens was more strongly favoured in unassisted twin pregnancies compared to unassisted singletons, but no significant differences existed between ART singletons and twins. Placental metabolism in favour of estrogen in twins supports previous research showing higher circulating estrogens in twins[345], however this was not reflected in our measurements of estrone, estradiol, and estriol in tissue. More research into the enzymes involved in converting between estrone, estradiol, and estriol may make the changes observed in the steroidogenesis pathway clearer.

5.4.4 Limitations of the Study

Certain limitations exist with respect to the interpretation of these findings. First, when sample matching was done for ART vs. unassisted pregnancies, the number (n=56) was based on available placenta samples which were the main tissue of interest for the investigation. Maternal plasma and cord serum samples were not available for all placenta samples. As such, when analysing differences between maternal plasma and cord serum, there are less samples included meaning the analysis is underpowered. Initial matching based on maternal age, gestational age, and ethnicity was also lost for this subset.

In the steroid LC-MS/MS method, very small pieces of tissue are required (1.5 mg) which could result in high variability depending on where the sample was taken from the placenta, a 500-600 g organ. To mitigate this variability, we incorporated an additional dilution step so that larger pieces of tissue could be sliced from the punches and diluted into the range of the curve. During initial testing of the method in placenta, it was noted that variability was high if only 1-2 mg of tissue were sliced from the original punch, however, slicing a thin piece across the entire punch and diluting the sample yielded consistent results. While everything was done to minimize sampling bias during tissue slicing, there could still be differences based on tissue slicing that could not be avoided.

For the steroid sulfate LC-MS/MS method, we were not able to detect the levels of either DHEAS or E1S in the placenta. Measurement of DHEAS in tissue has been previously reported, however radioimmuno assays were used, which may be confounded by cross-reactivity of DHEA or other steroids that are similar in structure[352,353]. To measure the levels of steroid sulfates in our placental samples, a better extraction method such as solid-phase extraction could be employed. Additionally, the method could be altered to include quantitation of 16α-OH-DHEAS, which is the most abundant sulfated form of DHEAS taken up from the fetal circulation, as well as other steroid sulfates such as progesterone-sulfate, cholesterol-sulfate, or estriol-sulfate.

5.5 Summary

In summary, this is the largest study comparing steroid levels between unassisted pregnancy and ART in humans in maternal, placental, and fetal compartments. The system of steroid hormone

regulation and action involves complex interactions between production, metabolism, and transport, which were not completely covered here. Ongoing future studies will investigate the action of steroid metabolism enzymes, including activity assays for 3β-HSD and CYP19 to build on the preliminary work using steroid ratios presented in this chapter. Further, the activity of steroid metabolism enzymes SULTs and UGTs, as well as their opposing regeneration enzymes STS and β-glucuronidase would greatly expand on these results. In the murine model of ART, the ratio of steroid metabolism to regeneration was dysregulated, with effects more strongly observed in ICSI[97]. Altered metabolism and clearance of steroids in the placenta affects the levels of active steroid and the passage of steroids across the placenta. Understanding the roles of the enzymes in human ART would provide a deeper understanding of the processes at play.

The widespread dysregulation of steroid levels, steroidogenesis, steroid metabolism, and delivery observed in the murine model of ART was not observed our cohort of human samples. Importantly, this provides evidence against altered steroid levels as an underlying cause of the increased pregnancy complications observed in ART. An interesting result of the steroid studies is the continued theme of dysregulation in pregnancies with chorioamnionitis and twin pregnancies. However, the effects on steroids were the same in ART and unassisted pregnancy, in contrast to the loss of patterns in ART for cytokines and antioxidant defense systems. Cytokines, antioxidant defense, and steroids interact in complex feed-forward and feedback systems to affect gene expression and cellular function. Together, the results of all three systems investigated in this thesis may help elucidate where dysregulation is occurring in chorioamnionitis and twin pregnancies in ART.

Chapter 6: General Conclusions and Summary

6.1 General Discussion

Our understanding of human pregnancy with respect to fertility, pregnancy complications, and parturition remains a continuously evolving aspect of reproductive biology. Pregnancy is a unique state with respect to immunology and physiology and requires precise control of inflammatory and immune responses to result in a healthy birth. Additionally, declining fertility has been well-documented in the late 20th and 21st centuries, resulting in increased access of fertility treatment and ART[8]. Prior to the efforts described in this thesis, it was well-documented that pregnancies achieved using ART were associated with increased risks and complications compared to unassisted pregnancies[7,10,11]. These complications encompass maternal, placental, and fetal risks, including preeclampsia, placenta previa, premature births, and small-for-gestational age infants. However, there was much debate over the etiology of these complications, which could result from the underlying infertility, the drugs involved in manipulation of the natural menstrual cycle, or the ART procedures themselves. Many of these complications are well characterized in pregnancy, but the molecular and cellular changes occurring in these complications specific to ART, and why they are increased in ART, are not well understood.

Beginning with a screening method for NSAIDs in reproductive tissues, we reported a conservative estimate of 3% rate of NSAID use close to the time of birth[176]. This was consistent with rates of NSAID use by self-report. Despite guidelines against the use of NSAIDs at specific times of pregnancy their use is relatively common, as highlighted in this study. Further, analgesics are among the most common drugs in pregnancy and with emerging evidence of

adverse effects to the fetus, it is important to monitor their use[106]. Since this screening study was conducted, a call for precaution of acetaminophen usage in pregnancy has been made[354]. However, further investigation is required to support these claims. Acetaminophen has long been recommended as the analgesic of choice during pregnancy. Negative effects on fetal endocrine development, urogenital and reproductive effects, and neurodevelopment have been identified, and there is evidence that effects on prostaglandin synthesis are involved in these complications[354-357]. New adverse effects of NSAIDs, which also function through inhibition of prostaglandin synthesis, have also been identified[170]. This highlights the importance of continued monitoring of drug safety in pregnancy and educating pregnant people on the effects of drug use in pregnancy.

In this thesis, we built on previous studies from our laboratory using a murine model of ART[97,145,146,332,333]. These studies identified differences in inflammatory responses, oxidative stress, and steroidogenesis pathways, where dysregulation in IVF and ICSI could be contributing to increased pregnancy complications. Here, we aimed to determine if the same effects were found in human pregnancy. By investigating several inflammatory, oxidative, and steroidal processes in the placenta, we hoped to unravel some of the mechanisms involved in the increased pregnancy complications observed.

In Chapter 3, we identified dysregulated cytokine levels in ART, in the context of pregnancies complicated by chorioamnionitis and in twin pregnancy. We proposed that protective signalling was attenuated in both clinical situations, which may play a role in the complications observed in chorioamnionitis and twin pregnancy, where there are increases in preterm birth, fetal distress,

and admission to the NICU. Differing cytokine profiles are expected between pregnancies with an infection present and those without, but this study provided the first evidence of dysregulated cytokines in ART pregnancies. In twins, a novel study from 2021 documented differential cytokine profiles between singleton and twin ART pregnancies at implantation, but no comparisons were made to unassisted pregnancy[358]. This highlights the interest and importance of studying inflammatory processes in singletons and twins. We add to this study, by documenting altered cytokine profiles in term placentas and the existence of differences in ART compared to unassisted pregnancy.

In Chapter 4, we built upon the results of the previous chapter by studying the antioxidant defense network, which is intricately associated with cytokines and inflammation. Again, there were no differences between ART and natural conception as indicated by murine studies, but differences were found when groups were stratified based on pregnancy outcome. The results of this chapter provided further support of dysregulation occurring in pregnancies complicated by chorioamnionitis and in twin pregnancies. The processes of inflammation and oxidative stress are intricately linked through signalling cascades affecting gene expression and cellular function (Figure 6.1). These systems feed into each other, further driving the processes of inflammation and oxidative stress, presenting promising pathways of future study in this subject area.

Finally, in Chapter 5 we evaluated the processes of steroidogenesis and steroid metabolism in the maternal-fetal-placental unit. We detailed differences in placental steroid levels in chorioamnionitis and twin pregnancy, both in ART and unassisted pregnancy. Prior to this, a large-scale study investigating steroid levels in maternal, placental, and fetal compartments in

ART had never been performed. Steroids, particularly progesterone, also play into the processes of inflammation and oxidative stress. Overall, these data suggest changes in steroidogenesis and steroid metabolism in response to infection and multiple birth. However, where the differences exist between ART and unassisted pregnancy may still be involved within the complex interactions of these three processes.

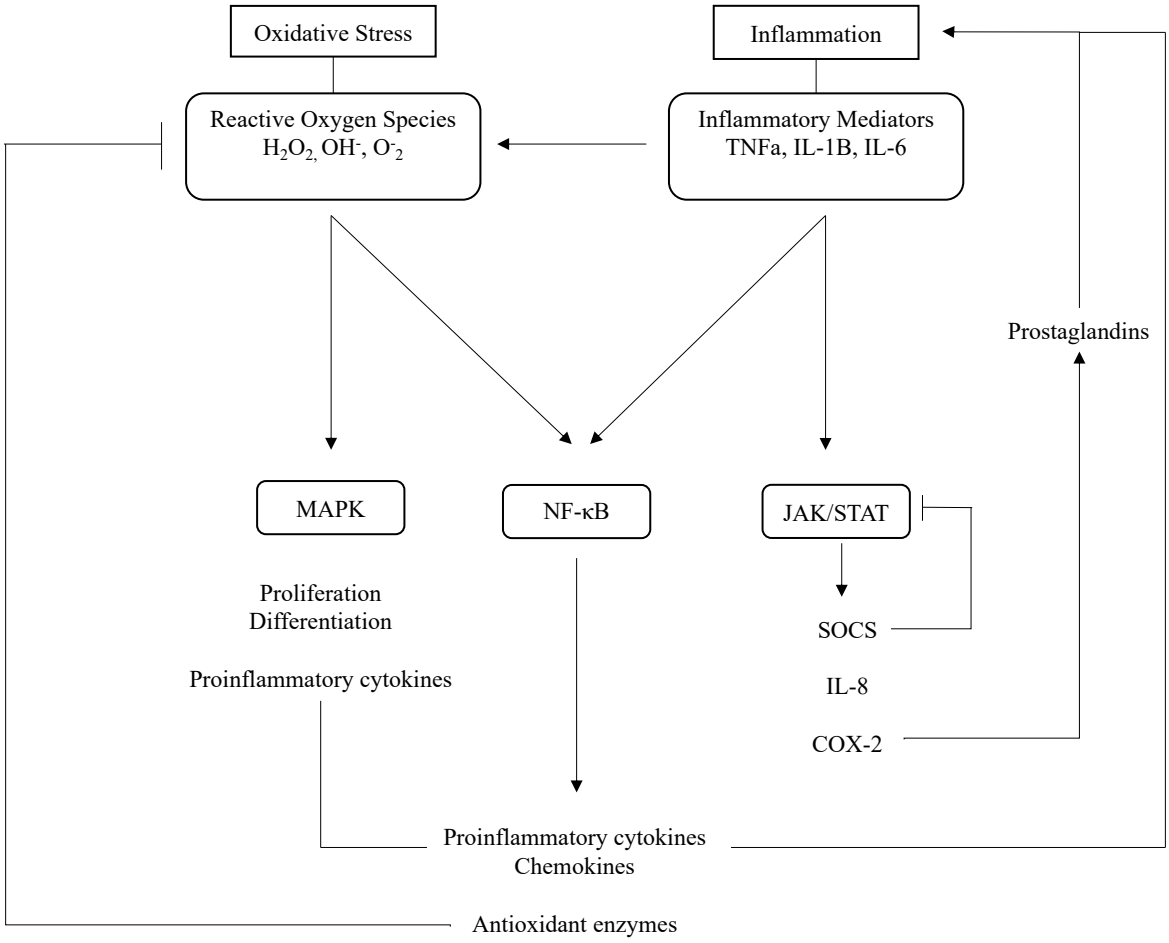

Figure 6.1 Interactions between cellular inflammation and oxidative stress.
Original figure, created in Microsoft PowerPoint.

In this cohort, a wide range of methodologies were used to study inflammation, antioxidant defense, and steroidogenesis. While no differences were observed between ART and unassisted

pregnancy groups overall, as observed in murine studies, it was encouraging to see similar themes emerge based on pregnancy outcome. The continued observations of differences in chorioamnionitis and twin pregnancy across the range of studies performed indicates strong signal-to-noise and biological significance. These results are novel in the ART field and present a multitude of future directions for the field to progress. These future directions, in addition to strengths and limitations, are discussed below.

6.2 Strengths and Limitations

A number of the limitations specific to each study are discussed in the corresponding chapters. However, it is important again to discuss the size of the cohort. It can be argued the cohort is small and underpowered, but this is one of the largest matched cohorts of unassisted and ART pregnancy in the world. A cohort such as this is very difficult to collect. The reproductive biorepository at the University of Hawaii contained over 10,000 placentas with corresponding clinical chart information at the time the samples were requested. Only 56 matches could be made with the level of matching we requested. The matching based on gestational age, maternal age, ethnicity, and singleton vs. twin makes it a very powerful and unique cohort in the ART field. It also highlights the importance of Biobanks for studies such as these, which would have required years of prospective sample collection to achieve this level of matching and number of samples.

One of the clinical situations that continuously arose in analysis was twin pregnancy. A limitation of our clinical chart profiles was that we did not know the type of twin pregnancy, identical or fraternal. Zygotic splitting can occur following embryo transfer, resulting in a twin

pregnancy even if a single embryo is transferred[359]. The largest study to investigate zygotic splitting after embryo transfer included nearly 1 million cases of single embryo transfer and concluded 1.36% of cases resulted in multiple pregnancy following zygotic splitting[360]. Factors including ICSI, frozen-thawed embryo transfer, and assisted hatching have been proposed to increase the chances of embryo splitting[360]. Additionally, twins may be dichorionic-diamniotic, monochorionic-diamniotic, or monochorionic-monoamniotic. Because there were two placentas associated with the twins in this cohort, we can confer they are dichorionic, but we did not have information on other characteristics of the twin pregnancy. Therefore, there may be significant differences between the types of twins in the ART and unassisted pregnancy groups, which is a limitation in the interpretation of the results with respect to twin pregnancy.

6.3 Future Directions

The results outlined above add to a growing body of literature investigating the mechanisms of pregnancy complications in ART. The complex nature of the processes investigated mean there is still much to be done to improve pregnancy outcomes when ART is used as the method of conception. The experiments performed here have resulted in a large dataset which presents the opportunity for future investigations. A matched cohort of ART and unassisted pregnancies such as this one is difficult to collect and takes many years to process and match. The data generated from the laboratory experiments, combined with the clinical charts that accompany the samples, resulted in a dataset for 126 placentas covering maternal and fetal characteristics, over 20 ICD9/10 clinical chart codes for pregnancy, the levels of six cytokines, three VEGFs, the activities of four antioxidant enzyme activities, levels of two antioxidants, nine steroids, and two steroid sulfates. Accordingly, this dataset could be used in countless future studies. An important

avenue for research is the relationships that may exist between the measured variables. Steroid hormones can affect the expression of cytokines, and pregnancy itself is a good example of this, as altered steroid levels influence the maternal immune system. How steroid levels affect immunoregulatory cytokines specifically within the placenta is currently undefined but presents an interesting direction for research, as it is the anatomical barrier between two genetically distinct organisms.

Specific to Chapter 5, experiments are currently ongoing to establish reliable measurements of the activities of 3β-HSD and CYP19. This will provide a comprehensive view of steroidogenesis within the placenta. Due to the pitfalls of the steroid sulfate measurements in placental tissue, another valuable investigation would be the activities of steroid metabolism and regeneration enzymes, which were dysregulated in the murine model of ART and impact the levels of active steroids delivered across the placenta[97].

An additional area of research is the investigation of pathways closely related to the systems measured here, such as prostaglandins, NF-κB, SOCS, and JAK-STAT. By investigating these downstream signalling cascades and effector molecules, we should be able to gain a more precise mechanistic insight into the dysregulation occurring. Careful evaluation of these pathways may present future therapeutic targets for preventing complications in pregnancy and complications associated with multiple births and infection.

Finally, a potential avenue for research which has proven valuable in investigating pregnancy complications is the use of genome-wide association studies (GWAS) and global gene

expression datasets. Comparisons of gene expression in complicated vs. uncomplicated pregnancies have identified genes of interest involved in preterm birth, preeclampsia, and gestational diabetes[361-364]. A past study combined whole genome sequencing, RNA sequencing, and DNA methylation from preterm and term births, and identified 72 candidate biomarker genes altered in preterm birth[362]. Affected pathways included growth factors, prolactin signalling pathways, inflammatory pathways, chemokine signalling, and IFN-γ signaling. Importantly, both chorioamnionitis and twin pregnancies have high rates of preterm birth. Additionally, there is recent evidence that monozygotic twins are associated with epigenetic changes that persist to adulthood[365]. These changes occurred at specific regions of the genome, and were associated with genes involved in cell adhesion, WNT signaling, and cell fate[365]. The relationship between epigenetic changes in monozygotic twins and pregnancy outcomes is a unique area for future research. To our knowledge there has not been a GWAS or gene expression study comparing ART and unassisted pregnancy, but several of the genes identified in the preterm birth study are related to the outcomes measured in this thesis.

Our studies have helped to identify potential pathways of interest involved in pregnancy complications in ART and are a first step in resolving the pathophysiology of these adverse outcomes. By investigating the downstream pathways outlined above, the long-term goal is to identify treatment targets and improve pregnancy outcomes in ART.

References

1. Mor G, Cardenas I, Abrahams V, Guller S. Inflammation and pregnancy: the role of the immune system at the implantation site. Ann N Y Acad Sci 2011;1221:80-7. DOI: 10.1111/j.1749-6632.2010.05938.x.
2. Duhig K, Chappell LC, Shennan AH. Oxidative stress in pregnancy and reproduction. Obstet Med 2016;9(3):113-6. DOI: 10.1177/1753495X16648495.
3. Gude NM, Roberts CT, Kalionis B, King RG. Growth and function of the normal human placenta. Thromb Res 2004;114(5-6):397-407. DOI: 10.1016/j.thromres.2004.06.038.
4. Villee CA. Placental Transfer of Drugs. Ann N Y Acad Sci 1965;123:237-44. DOI: 10.1111/j.1749-6632.1965.tb12262.x.
5. Farrar HC, Blumer JL. Fetal effects of maternal drug exposure. Annu Rev Pharmacol Toxicol 1991;31:525-47. DOI: 10.1146/annurev.pa.31.040191.002521.
6. Kallen B, Finnstrom O, Nygren KG, Otterblad Olausson P, Wennerholm UB. In vitro fertilisation in Sweden: obstetric characteristics, maternal morbidity and mortality. BJOG 2005;112(11):1529-35. DOI: 10.1111/j.1471-0528.2005.00745.x.
7. Reddy UM, Wapner RJ, Rebar RW, Tasca RJ. Infertility, assisted reproductive technology, and adverse pregnancy outcomes: executive summary of a National Institute of Child Health and Human Development workshop. Obstet Gynecol 2007;109(4):967-77. DOI: 10.1097/01.AOG.0000259316.04136.30.
8. Kushnir VA, Barad DH, Albertini DF, Darmon SK, Gleicher N. Systematic review of worldwide trends in assisted reproductive technology 2004-2013. Reprod Biol Endocrinol 2017;15(1):6. DOI: 10.1186/s12958-016-0225-2.
9. Jin H, Dasgupta S. Disparities between online assisted reproduction patient education for same-sex and heterosexual couples. Hum Reprod 2016;31(10):2280-4. DOI: 10.1093/humrep/dew182.
10. Romundstad LB, Romundstad PR, Sunde A, von During V, Skjaerven R, Vatten LJ. Increased risk of placenta previa in pregnancies following IVF/ICSI; a comparison of ART and non-ART pregnancies in the same mother. Hum Reprod 2006;21(9):2353-8. DOI: 10.1093/humrep/del153.
11. Okun N, Sierra S, Genetics C, Special C. Pregnancy outcomes after assisted human reproduction. J Obstet Gynaecol Can 2014;36(1):64-83. DOI: 10.1016/S1701-2163(15)30685-X.
12. Buckett WM, Chian RC, Holzer H, Dean N, Usher R, Tan SL. Obstetric outcomes and congenital abnormalities after in vitro maturation, in vitro fertilization, and intracytoplasmic sperm injection. Obstet Gynecol 2007;110(4):885-91. DOI: 10.1097/01.AOG.0000284627.38540.80.
13. Shevell T, Malone FD, Vidaver J, et al. Assisted reproductive technology and pregnancy outcome. Obstet Gynecol 2005;106(5 Pt 1):1039-45. (In eng). DOI: 10.1097/01.AOG.0000183593.24583.7c.
14. Herbst AL. Diethylstilbestrol and other sex hormones during pregnancy. Obstet Gynecol 1981;58(5 Suppl):35S-40S. (https://www.ncbi.nlm.nih.gov/pubmed/7031540).
15. Cunningham FG, Leveno KJ, Bloom SL, et al. Implantation and Placental Development. Williams Obstetrics, 24e. New York, NY: McGraw-Hill Education; 2013.
16. Cunningham FG, Leveno KJ, Bloom SL, et al. Embryogenesis and Fetal Morphological Development. Williams Obstetrics, 24e. New York, NY: McGraw-Hill Education; 2013.

17. Sathananthan H, Menezes J, Gunasheela S. Mechanics of human blastocyst hatching in vitro. Reprod Biomed Online 2003;7(2):228-34. DOI: 10.1016/s1472-6483(10)61757-9.
18. Ashary N, Tiwari A, Modi D. Embryo Implantation: War in Times of Love. Endocrinology 2018;159(2):1188-1198. DOI: 10.1210/en.2017-03082.
19. Timeva T, Shterev A, Kyurkchiev S. Recurrent implantation failure: the role of the endometrium. J Reprod Infertil 2014;15(4):173-83. (https://www.ncbi.nlm.nih.gov/pubmed/25473625).
20. Feng Y, Ma X, Deng L, et al. Role of selectins and their ligands in human implantation stage. Glycobiology 2017;27(5):385-391. DOI: 10.1093/glycob/cwx009.
21. Sharma A, Kumar P. Understanding implantation window, a crucial phenomenon. J Hum Reprod Sci 2012;5(1):2-6. DOI: 10.4103/0974-1208.97777.
22. Kim SM, Kim JS. A Review of Mechanisms of Implantation. Dev Reprod 2017;21(4):351-359. DOI: 10.12717/DR.2017.21.4.351.
23. Fisher SJ, Damsky CH. Human cytotrophoblast invasion. Semin Cell Biol 1993;4(3):183-8. DOI: 10.1006/scel.1993.1022.
24. Burton GJ, Charnock-Jones DS, Jauniaux E. Regulation of vascular growth and function in the human placenta. Reproduction 2009;138(6):895-902. DOI: 10.1530/REP-09-0092.
25. Weiss G, Sundl M, Glasner A, Huppertz B, Moser G. The trophoblast plug during early pregnancy: a deeper insight. Histochem Cell Biol 2016;146(6):749-756. DOI: 10.1007/s00418-016-1474-z.
26. Cunningham FG, Leveno KJ, Bloom SL, et al. Placental Abnormalities. Williams Obstetrics, 24e. New York, NY: McGraw-Hill Education; 2013.
27. Barker DJ, Thornburg KL, Osmond C, Kajantie E, Eriksson JG. The surface area of the placenta and hypertension in the offspring in later life. Int J Dev Biol 2010;54(2-3):525-30. (In eng). DOI: 10.1387/ijdb.082760db.
28. Benirschke K, Burton GJ, Baergen RN. Architecture of Normal Villous Trees. Pathology of the Human Placenta. Berlin, Heidelberg: Springer Berlin Heidelberg; 2012:101-144.
29. Demir R, Kosanke G, Kohnen G, Kertschanska S, Kaufmann P. Classification of human placental stem villi: review of structural and functional aspects. Microsc Res Tech 1997;38(1-2):29-41. (In eng). DOI: 10.1002/(sici)1097-0029(19970701/15)38:1/2<29::Aid-jemt5>3.0.Co;2-p.
30. Plitman Mayo R, Charnock-Jones DS, Burton GJ, Oyen ML. Three-dimensional modeling of human placental terminal villi. Placenta 2016;43:54-60. (In eng). DOI: 10.1016/j.placenta.2016.05.001.
31. Wang YZ, Z. Structure of the Placenta. Vascular Biology of the Placenta: Morgan & Claypool Publishers; 2010.
32. Benirschke K, Burton GJ, Baergen RN. Basic Structure of the Villous Trees. Pathology of the Human Placenta. Berlin, Heidelberg: Springer Berlin Heidelberg; 2012:55-100.
33. Mori M, Ishikawa G, Luo SS, et al. The cytotrophoblast layer of human chorionic villi becomes thinner but maintains its structural integrity during gestation. Biol Reprod 2007;76(1):164-72. DOI: 10.1095/biolreprod.106.056127.
34. Yabe S, Alexenko AP, Amita M, et al. Comparison of syncytiotrophoblast generated from human embryonic stem cells and from term placentas. Proc Natl Acad Sci U S A 2016;113(19):E2598-607. DOI: 10.1073/pnas.1601630113.

35. Staud F, Karahoda R. Trophoblast: The central unit of fetal growth, protection and programming. Int J Biochem Cell Biol 2018;105:35-40. DOI: 10.1016/j.biocel.2018.09.016.
36. Kaufmann P, Castellucci M. Extravillous trophoblast in the human placenta: A review. Placenta 1997;18:21-65. DOI: https://doi.org/10.1016/S0143-4004(97)80079-3.
37. Pollheimer J, Vondra S, Baltayeva J, Beristain AG, Knofler M. Regulation of Placental Extravillous Trophoblasts by the Maternal Uterine Environment. Front Immunol 2018;9:2597. DOI: 10.3389/fimmu.2018.02597.
38. Cross JC. Formation of the placenta and extraembryonic membranes. Ann N Y Acad Sci 1998;857:23-32. DOI: 10.1111/j.1749-6632.1998.tb10104.x.
39. Carlson B. Human embryology and developmental biology: Elsevier, 2013.
40. Benirschke K, Burton GJ, Baergen RN. Anatomy and Pathology of the Placental Membranes. Pathology of the Human Placenta. Berlin, Heidelberg: Springer Berlin Heidelberg; 2012:249-307.
41. Carlson B. Human embryology and developmental biology: Elsevier, 2013.
42. Huyhn A, Dommergues M, Izac B, et al. Characterization of hematopoietic progenitors from human yolk sacs and embryos. Blood 1995;86(12):4474-85. (In eng).
43. Mori M, Bogdan A, Balassa T, Csabai T, Szekeres-Bartho J. The decidua-the maternal bed embracing the embryo-maintains the pregnancy. Semin Immunopathol 2016;38(6):635-649. DOI: 10.1007/s00281-016-0574-0.
44. Benirschke K, Burton GJ, Baergen RN. Anatomy and Pathology of the Umbilical Cord. Pathology of the Human Placenta. Berlin, Heidelberg: Springer Berlin Heidelberg; 2012:309-375.
45. Davies JE, Walker JT, Keating A. Concise Review: Wharton's Jelly: The Rich, but Enigmatic, Source of Mesenchymal Stromal Cells. Stem Cells Transl Med 2017;6(7):1620-1630. (In eng). DOI: 10.1002/sctm.16-0492.
46. Saleh R, Reza HM. Short review on human umbilical cord lining epithelial cells and their potential clinical applications. Stem Cell Res Ther 2017;8(1):222. DOI: 10.1186/s13287-017-0679-y.
47. Rush RW, Keirse MJ, Howat P, Baum JD, Anderson AB, Turnbull AC. Contribution of preterm delivery to perinatal mortality. Br Med J 1976;2(6042):965-8. (In eng). DOI: 10.1136/bmj.2.6042.965.
48. Jukic AM, Baird DD, Weinberg CR, McConnaughey DR, Wilcox AJ. Length of human pregnancy and contributors to its natural variation. Hum Reprod 2013;28(10):2848-55. DOI: 10.1093/humrep/det297.
49. Link G, Clark KE, Lang U. Umbilical blood flow during pregnancy: evidence for decreasing placental perfusion. Am J Obstet Gynecol 2007;196(5):489.e1-7. (In eng). DOI: 10.1016/j.ajog.2006.11.017.
50. Kota SK, Gayatri K, Jammula S, et al. Endocrinology of parturition. Indian J Endocrinol Metab 2013;17(1):50-59. (In eng). DOI: 10.4103/2230-8210.107841.
51. Cook JL, Zaragoza DB, Sung DH, Olson DM. Expression of myometrial activation and stimulation genes in a mouse model of preterm labor: myometrial activation, stimulation, and preterm labor. Endocrinology 2000;141(5):1718-28. (In eng). DOI: 10.1210/endo.141.5.7474.

52. Zoumakis E, Makrigiannakis A, Margioris AN, Stournaras C, Gravanis A. Endometrial corticotropin-releasing hormone. Its potential autocrine and paracrine actions. Ann N Y Acad Sci 1997;828:84-94. (In eng). DOI: 10.1111/j.1749-6632.1997.tb48525.x.
53. Taylor MJ, Webb R, Mitchell MD, Robinson JS. Effect of progesterone withdrawal in sheep during late pregnancy. J Endocrinol 1982;92(1):85-93. (In eng). DOI: 10.1677/joe.0.0920085.
54. Barry JS, Anthony RV. The pregnant sheep as a model for human pregnancy. Theriogenology 2008;69(1):55-67. (In eng). DOI: 10.1016/j.theriogenology.2007.09.021.
55. Chwalisz K. The use of progesterone antagonists for cervical ripening and as an adjunct to labour and delivery. Hum Reprod 1994;9 Suppl 1:131-61. (In eng). DOI: 10.1093/humrep/9.suppl_1.131.
56. Brown AG, Leite RS, Strauss JF, 3rd. Mechanisms underlying "functional" progesterone withdrawal at parturition. Ann N Y Acad Sci 2004;1034:36-49. (In eng). DOI: 10.1196/annals.1335.004.
57. Mesiano S, Chan EC, Fitter JT, Kwek K, Yeo G, Smith R. Progesterone withdrawal and estrogen activation in human parturition are coordinated by progesterone receptor A expression in the myometrium. J Clin Endocrinol Metab 2002;87(6):2924-30. (In eng). DOI: 10.1210/jcem.87.6.8609.
58. Olson DM, Ammann C. Role of the prostaglandins in labour and prostaglandin receptor inhibitors in the prevention of preterm labour. Front Biosci 2007;12:1329-43. (In eng). DOI: 10.2741/2151.
59. Uvnäs-Moberg K, Ekström-Bergström A, Berg M, et al. Maternal plasma levels of oxytocin during physiological childbirth - a systematic review with implications for uterine contractions and central actions of oxytocin. BMC Pregnancy Childbirth 2019;19(1):285. (In eng). DOI: 10.1186/s12884-019-2365-9.
60. Lei K, Chen L, Cryar BJ, et al. Uterine Stretch and Progesterone Action. The Journal of Clinical Endocrinology & Metabolism 2011;96(6):E1013-E1024. DOI: 10.1210/jc.2010-2310.
61. Loudon JA, Sooranna SR, Bennett PR, Johnson MR. Mechanical stretch of human uterine smooth muscle cells increases IL-8 mRNA expression and peptide synthesis. Mol Hum Reprod 2004;10(12):895-9. (In eng). DOI: 10.1093/molehr/gah112.
62. Loebstein R, Lalkin A, Koren G. Pharmacokinetic changes during pregnancy and their clinical relevance. Clin Pharmacokinet 1997;33(5):328-43. DOI: 10.2165/00003088-199733050-00002.
63. Kerr MG. Cardiovascular dynamics in pregnancy and labour. Br Med Bull 1968;24:19-24.
64. Sanghavi M, Rutherford JD. Cardiovascular physiology of pregnancy. Circulation 2014;130(12):1003-8. DOI: 10.1161/CIRCULATIONAHA.114.009029.
65. Troiano NH. Physiologic and Hemodynamic Changes During Pregnancy. AACN Adv Crit Care 2018;29(3):273-283. (In eng). DOI: 10.4037/aacnacc2018911.
66. Davison JM, Hytten FE. Glomerular filtration during and after pregnancy. J Obstet Gynaecol Br Commonw 1974;81(8):588-95. (https://www.ncbi.nlm.nih.gov/pubmed/4420303).
67. Malha L, Sison CP, Helseth G, Sealey JE, August P. Renin-Angiotensin-Aldosterone Profiles in Pregnant Women With Chronic Hypertension. Hypertension 2018;72(2):417-424. DOI: 10.1161/HYPERTENSIONAHA.118.10854.

68. LoMauro A, Aliverti A. Respiratory physiology of pregnancy: Physiology masterclass. Breathe (Sheff) 2015;11(4):297-301. DOI: 10.1183/20734735.008615.
69. Weinberger SE, Weiss ST, Cohen WR, Weiss JW, Johnson TS. Pregnancy and the lung. Am Rev Respir Dis 1980;121(3):559-81. DOI: 10.1164/arrd.1980.121.3.559.
70. Parry E, Shields R, Turnbull AC. Transit time in the small intestine in pregnancy. J Obstet Gynaecol Br Commonw 1970;77(10):900-1. DOI: 10.1111/j.1471-0528.1970.tb03423.x.
71. Pirani BB, Campbell DM, MacGillivray I. Plasma volume in normal first pregnancy. J Obstet Gynaecol Br Commonw 1973;80(10):884-7. DOI: 10.1111/j.1471-0528.1973.tb02146.x.
72. Dean M, Stock B, Patterson RJ, Levy G. Serum protein binding of drugs during and after pregnancy in humans. Clin Pharmacol Ther 1980;28(2):253-61. DOI: 10.1038/clpt.1980.158.
73. Dean M. Opioids in renal failure and dialysis patients. J Pain Symptom Manage 2004;28(5):497-504. DOI: 10.1016/j.jpainsymman.2004.02.021.
74. Koren G, Pariente G. Pregnancy- Associated Changes in Pharmacokinetics and their Clinical Implications. Pharm Res 2018;35(3):61. (In eng). DOI: 10.1007/s11095-018-2352-2.
75. Jeong H. Altered drug metabolism during pregnancy: hormonal regulation of drug-metabolizing enzymes. Expert Opin Drug Metab Toxicol 2010;6(6):689-699. (In eng). DOI: 10.1517/17425251003677755.
76. Levy G, Procknal JA, Garrettson LK. Distribution of salicylate between neonatal and maternal serum at diffusion equilibrium. Clin Pharmacol Ther 1975;18(2):210-4. DOI: 10.1002/cpt1975182210.
77. Syme MR, Paxton JW, Keelan JA. Drug transfer and metabolism by the human placenta. Clin Pharmacokinet 2004;43(8):487-514. DOI: 10.2165/00003088-200443080-00001.
78. Kim JH, Scialli AR. Thalidomide: the tragedy of birth defects and the effective treatment of disease. Toxicol Sci 2011;122(1):1-6. DOI: 10.1093/toxsci/kfr088.
79. Hakkola J, Pasanen M, Hukkanen J, et al. Expression of xenobiotic-metabolizing cytochrome P450 Forms in human full-term placenta. Biochemical Pharmacology 1996;51(4):403-411. DOI: https://doi.org/10.1016/0006-2952(95)02184-1.
80. Cizkova K, Tauber Z. Time-dependent expression pattern of cytochrome P450 epoxygenases and soluble epoxide hydrolase in normal human placenta. Acta Histochemica 2018;120(6):513-519. DOI: https://doi.org/10.1016/j.acthis.2018.06.002.
81. Hakkola J, Raunio H, Purkunen R, et al. Detection of cytochrome P450 gene expression in human placenta in first trimester of pregnancy. Biochemical Pharmacology 1996;52(2):379-383. DOI: https://doi.org/10.1016/0006-2952(96)00216-X.
82. Collier AC, Ganley NA, Tingle MD, et al. UDP-glucuronosyltransferase activity, expression and cellular localization in human placenta at term. Biochem Pharmacol 2002;63(3):409-19. (In eng). DOI: 10.1016/s0006-2952(01)00890-5.
83. Collier AC, Tingle MD, Paxton JW, Mitchell MD, Keelan JA. Metabolizing enzyme localization and activities in the first trimester human placenta: the effect of maternal and gestational age, smoking and alcohol consumption. Hum Reprod 2002;17(10):2564-72. (In eng). DOI: 10.1093/humrep/17.10.2564.
84. Collier AC, Thévenon AD, Goh W, Hiraoka M, Kendal-Wright CE. Placental profiling of UGT1A enzyme expression and activity and interactions with preeclampsia at term. Eur J

Drug Metab Pharmacokinet 2015;40(4):471-480. (In eng). DOI: 10.1007/s13318-014-0243-4.
85. Bernier F, Leblanc G, Labrie F, Luu-The V. Structure of human estrogen and aryl sulfotransferase gene. Two mRNA species issued from a single gene. J Biol Chem 1994;269(45):28200-5. (In eng).
86. Stanley EL, Hume R, Visser TJ, Coughtrie MW. Differential expression of sulfotransferase enzymes involved in thyroid hormone metabolism during human placental development. J Clin Endocrinol Metab 2001;86(12):5944-55. (In eng). DOI: 10.1210/jcem.86.12.8081.
87. He D, Meloche CA, Dumas NA, Frost AR, Falany CN. Different subcellular localization of sulphotransferase 2B1b in human placenta and prostate. Biochem J 2004;379(Pt 3):533-540. (In eng). DOI: 10.1042/BJ20031524.
88. Wang J, Sauer MV. In vitro fertilization (IVF): a review of 3 decades of clinical innovation and technological advancement. Ther Clin Risk Manag 2006;2(4):355-364. (In eng). DOI: 10.2147/tcrm.2006.2.4.355.
89. Steptoe PC, Edwards RG. Reimplantation of a human embryo with subsequent tubal pregnancy. Lancet 1976;1(7965):880-2. (In eng). DOI: 10.1016/s0140-6736(76)92096-1.
90. Steptoe PC, Edwards RG. Birth after the reimplantation of a human embryo. Lancet 1978;2(8085):366. (In eng). DOI: 10.1016/s0140-6736(78)92957-4.
91. Eftekhar M, Mohammadian F, Yousefnejad F, Molaei B, Aflatoonian A. Comparison of conventional IVF versus ICSI in non-male factor, normoresponder patients. Iran J Reprod Med 2012;10(2):131-136. (In eng) (https://pubmed.ncbi.nlm.nih.gov/25242986 https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4163275/).
92. Palermo G, Joris H, Derde M-P, Camus M, Devroey P, Van Steirteghem A. Sperm characteristics and outcome of human assisted fertilization by subzonal insemination and intracytoplasmic sperm injection**Supported by the Belgian Fund for Medical Research (grants 3.0036.85 and 3.0018.92), Brussels, Belgium. Fertility and Sterility 1993;59(4):826-835. DOI: https://doi.org/10.1016/S0015-0282(16)55867-1.
93. Palermo G, Joris H, Devroey P, Van Steirteghem AC. Pregnancies after intracytoplasmic injection of single spermatozoon into an oocyte. Lancet 1992;340(8810):17-8. (In eng). DOI: 10.1016/0140-6736(92)92425-f.
94. Haas J, Miller TE, Nahum R, et al. The role of ICSI vs. conventional IVF for patients with advanced maternal age—a randomized controlled trial. Journal of Assisted Reproduction and Genetics 2021;38(1):95-100. DOI: 10.1007/s10815-020-01990-5.
95. Klitzman R. Deciding how many embryos to transfer: ongoing challenges and dilemmas. Reprod Biomed Soc Online 2016;3:1-15. (In eng). DOI: 10.1016/j.rbms.2016.07.001.
96. Haavaldsen C, Tanbo T, Eskild A. Placental weight in singleton pregnancies with and without assisted reproductive technology: a population study of 536,567 pregnancies. Hum Reprod 2012;27(2):576-82. DOI: 10.1093/humrep/der428.
97. Collier AC, Miyagi SJ, Yamauchi Y, Ward MA. Assisted reproduction technologies impair placental steroid metabolism. J Steroid Biochem Mol Biol 2009;116(1-2):21-28. (In eng). DOI: 10.1016/j.jsbmb.2009.04.005.
98. Sundheimer LW, Pisarska MD. Abnormal Placentation Associated with Infertility as a Marker of Overall Health. Semin Reprod Med 2017;35(3):205-216. (In eng). DOI: 10.1055/s-0037-1603570.

99. Choux C, Carmignac V, Bruno C, Sagot P, Vaiman D, Fauque P. The placenta: phenotypic and epigenetic modifications induced by Assisted Reproductive Technologies throughout pregnancy. Clinical Epigenetics 2015;7(1):87. DOI: 10.1186/s13148-015-0120-2.
100. Almasi-Hashiani A, Omani-Samani R, Mohammadi M, et al. Assisted reproductive technology and the risk of preeclampsia: an updated systematic review and meta-analysis. BMC Pregnancy and Childbirth 2019;19(1):149. DOI: 10.1186/s12884-019-2291-x.
101. Allen VM, Wilson RD, Cheung A. Pregnancy outcomes after assisted reproductive technology. J Obstet Gynaecol Can 2006;28(3):220-233. (In eng fre). DOI: 10.1016/s1701-2163(16)32112-0.
102. Lv H, Diao F, Du J, et al. Assisted reproductive technology and birth defects in a Chinese birth cohort study. Lancet Reg Health West Pac 2021;7:100090. (In eng). DOI: 10.1016/j.lanwpc.2020.100090.
103. Hansen M, Bower C, Milne E, de Klerk N, Kurinczuk JJ. Assisted reproductive technologies and the risk of birth defects--a systematic review. Hum Reprod 2005;20(2):328-38. (In eng). DOI: 10.1093/humrep/deh593.
104. Eroglu A, Layman LC. Role of ART in imprinting disorders. Semin Reprod Med 2012;30(2):92-104. (In eng). DOI: 10.1055/s-0032-1307417.
105. Lupattelli A, Spigset O, Twigg M, et al. Medication use in pregnancy: a cross-sectional, multinational web-based study. . BMJ Open 2014;4(e004365.).
106. Thorpe P, Gilboa S, Hernandez-Diaz S, et al. Medications in the first trimester of pregnancy: most common exposures and critical gaps in understanding fetal risk. . Pharmacoepidemiology and Drug Safety 2013;22:1013–1018.
107. Price HR, Collier AC. Analgesics in Pregnancy: An Update on Use, Safety and Pharmacokinetic Changes in Drug Disposition. Curr Pharm Des 2017;23(40):6098-6114. (In eng). DOI: 10.2174/1381612823666170825123754.
108. Vane JR, Botting RM. Mechanism of action of anti-inflammatory drugs. Scand J Rheumatol Suppl 1996;102:9-21. (https://www.ncbi.nlm.nih.gov/pubmed/8628981).
109. Vane JR. Inhibition of prostaglandin synthesis as a mechanism of action for aspirin-like drugs. Nat New Biol 1971;231(25):232-5. (https://www.ncbi.nlm.nih.gov/pubmed/5284360).
110. Risser A, Donovan D, Heintzman J, Page T. NSAID prescribing precautions. Am Fam Physician 2009;80(12):1371-8. (https://www.ncbi.nlm.nih.gov/pubmed/20000300).
111. Venuto RC, O'Dorisio T, Stein JH, Ferris TF. Uterine prostaglandin E secretion and uterine blood flow in the pregnant rabbit. J Clin Invest 1975;55(1):193-7. DOI: 10.1172/JCI107911.
112. Van Marter LJ, Leviton A, Allred EN, et al. Persistent pulmonary hypertension of the newborn and smoking and aspirin and nonsteroidal antiinflammatory drug consumption during pregnancy. Pediatrics 1996;97(5):658-63. (https://www.ncbi.nlm.nih.gov/pubmed/8628603).
113. Musu M, Finco G, Antonucci R, et al. Acute nephrotoxicity of NSAID from the foetus to the adult. Eur Rev Med Pharmacol Sci 2011;15(12):1461-72. (https://www.ncbi.nlm.nih.gov/pubmed/22288307).
114. Koren G, Florescu A, Costei AM, Boskovic R, Moretti ME. Nonsteroidal antiinflammatory drugs during third trimester and the risk of premature closure of the

ductus arteriosus: a meta-analysis. Ann Pharmacother 2006;40(5):824-9. DOI: 10.1345/aph.1G428.
115. Hinz BC, O.; Brune, K. . Acetaminophen (paracetamol) is a selective cyclooxygenase-2 inhibitor in man. The FASEB Journal 2008;22(2):383–390.
116. Crunfli F, Vilela F, Giusti-Paiva A. Cannabinoid CB1 receptors mediate the effects of dipyrone. Clinical and Experimental Pharmacology and Physiology 2015;42(3):246-55.
117. Andersson D, Gentry C, Alenmyr L, et al. TRPA1 mediates spinal antinociception induced by acetaminophen and the cannabinoid Δ(9)-tetrahydrocannabiorcol. . Nature Communications 2011;2:551.
118. Högestätt E, Jönsson B, Ermund A, et al. Conversion of acetaminophen to the bioactive N-acylphenolamine AM404 via fatty acid amide hydrolase-dependent arachidonic acid conjugation in the nervous system. . Journal of Biological Chemistry 2005;280(36): 31405–12.
119. Chandrasekharan NV, Dai H, Roos KLT, et al. COX-3, a cyclooxygenase-1 variant inhibited by acetaminophen and other analgesic/antipyretic drugs: cloning, structure, and expression. Proceedings of the National Academy of Sciences of the United States of America 2002;99(21):13926-13931. (In eng). DOI: 10.1073/pnas.162468699.
120. Hedenmalm K, Spigset O. Agranulocytosis and other blood dyscrasias associated with dipyrone (metamizole). Eur J Clin Pharmacol 2002;58(4):265-74. DOI: 10.1007/s00228-002-0465-2.
121. Headley J, Northstone K, Simmons H, Golding J, Team AS. Medication use during pregnancy: data from the Avon Longitudinal Study of Parents and Children. Eur J Clin Pharmacol 2004;60(5):355-61. DOI: 10.1007/s00228-004-0775-7.
122. Werler MM, Mitchell AA, Hernandez-Diaz S, Honein MA. Use of over-the-counter medications during pregnancy. Am J Obstet Gynecol 2005;193(3 Pt 1):771-7. DOI: 10.1016/j.ajog.2005.02.100.
123. Richard K, Hume R, Kaptein E, Stanley EL, Visser TJ, Coughtrie MW. Sulfation of thyroid hormone and dopamine during human development: ontogeny of phenol sulfotransferases and arylsulfatase in liver, lung, and brain. J Clin Endocrinol Metab 2001;86(6):2734-42. DOI: 10.1210/jcem.86.6.7569.
124. Hurtado-Gonzalez P, Anderson RA, Macdonald J, et al. Effects of Exposure to Acetaminophen and Ibuprofen on Fetal Germ Cell Development in Both Sexes in Rodent and Human Using Multiple Experimental Systems. Environ Health Perspect 2018;126(4):047006. (In eng). DOI: 10.1289/ehp2307.
125. Waldhoer M, Bartlett SE, Whistler JL. Opioid receptors. Annu Rev Biochem 2004;73:953-90. DOI: 10.1146/annurev.biochem.73.011303.073940.
126. Bateman BT, Hernandez-Diaz S, Rathmell JP, et al. Patterns of opioid utilization in pregnancy in a large cohort of commercial insurance beneficiaries in the United States. Anesthesiology 2014;120(5):1216-24. DOI: 10.1097/ALN.0000000000000172.
127. Noormohammadi A, Forinash A, Yancey A, Crannage E, Campbell K, Shyken J. Buprenorphine Versus Methadone for Opioid Dependence in Pregnancy. Ann Pharmacother 2016;50(8):666-72. DOI: 10.1177/1060028016648367.
128. Halpern SH, Leighton BL, Ohlsson A, Barrett JF, Rice A. Effect of epidural vs parenteral opioid analgesia on the progress of labor: a meta-analysis. JAMA 1998;280(24):2105-10. (https://www.ncbi.nlm.nih.gov/pubmed/9875879).

129. Soontrapa S, Somboonporn W, Komwilaisak R, Sookpanya S. Effectiveness of intravenous meperidine for pain relief in the first stage of labour. J Med Assoc Thai 2002;85(11):1169-75. (https://www.ncbi.nlm.nih.gov/pubmed/12546313).
130. Tsui MH, Ngan Kee WD, Ng FF, Lau TK. A double blinded randomised placebo-controlled study of intramuscular pethidine for pain relief in the first stage of labour. BJOG 2004;111(7):648-55. DOI: 10.1111/j.1471-0528.2004.00160.x.
131. Mardirosoff C, Dumont L, Boulvain M, Tramer MR. Fetal bradycardia due to intrathecal opioids for labour analgesia: a systematic review. BJOG 2002;109(3):274-81. (https://www.ncbi.nlm.nih.gov/pubmed/11950182).
132. Guay J. Methemoglobinemia related to local anesthetics: a summary of 242 episodes. Anesth Analg 2009;108(3):837-45. DOI: 10.1213/ane.0b013e318187c4b1.
133. Hara K, Minami K, Sata T. The effects of tramadol and its metabolite on glycine, gamma-aminobutyric acidA, and N-methyl-D-aspartate receptors expressed in Xenopus oocytes. Anesthesia and Analgesia 2005;100(5):1400–05.
134. Ogata J, Minami K, Uezono Y, et al. The inhibitory effects of tramadol on 5-hydroxytryptamine type 2C receptors expressed in Xenopus oocytes. Anesthesia and Analgesia 2004;98(5):1401–06.
135. Bloor M, Paech MJ, Kaye R. Tramadol in pregnancy and lactation. Int J Obstet Anesth 2012;21(2):163-7. DOI: 10.1016/j.ijoa.2011.10.008.
136. Chang EJ, Choi EJ, Kim KH. Tapentadol: Can It Kill Two Birds with One Stone without Breaking Windows? Korean J Pain 2016;29(3):153-7. DOI: 10.3344/kjp.2016.29.3.153.
137. Beaver WT. Combination analgesics. Am J Med 1984;77(3A):38-53. (https://www.ncbi.nlm.nih.gov/pubmed/6486130).
138. Raffa RB. Pharmacology of oral combination analgesics: rational therapy for pain. J Clin Pharm Ther 2001;26(4):257-64. (https://www.ncbi.nlm.nih.gov/pubmed/11493367).
139. Tepper SJ, Rapoport AM, Sheftell FD. Mechanisms of action of the 5-HT1B/1D receptor agonists. Arch Neurol 2002;59(7):1084-8. (https://www.ncbi.nlm.nih.gov/pubmed/12117355).
140. Tfelt-Hansen PC, Olesen J. The 5-HT1F receptor agonist lasmiditan as a potential treatment of migraine attacks: a review of two placebo-controlled phase II trials. J Headache Pain 2012;13(4):271-5. DOI: 10.1007/s10194-012-0428-7.
141. Sances G, Granella F, Nappi RE, et al. Course of migraine during pregnancy and postpartum: a prospective study. Cephalalgia 2003;23(3):197-205. (https://www.ncbi.nlm.nih.gov/pubmed/12662187).
142. van Vliet JA, Favier I, Helmerhorst FM, Haan J, Ferrari MD. Cluster headache in women: relation with menstruation, use of oral contraceptives, pregnancy, and menopause. J Neurol Neurosurg Psychiatry 2006;77(5):690-2. DOI: 10.1136/jnnp.2005.081158.
143. Wojnar-Horton RE, Hackett LP, Yapp P, Dusci LJ, Paech M, Ilett KF. Distribution and excretion of sumatriptan in human milk. Br J Clin Pharmacol 1996;41(3):217-21. (https://www.ncbi.nlm.nih.gov/pubmed/8866921).
144. Soldin OP, Dahlin J, O'Mara DM. Triptans in pregnancy. Ther Drug Monit 2008;30(1):5-9. DOI: 10.1097/FTD.0b013e318162c89b.
145. Raunig JM, Yamauchi Y, Ward MA, Collier AC. Placental inflammation and oxidative stress in the mouse model of assisted reproduction. Placenta 2011;32(11):852-8. (In eng). DOI: 10.1016/j.placenta.2011.08.003.

146. Sato BLM, Sugawara A, Ward MA, Collier AC. Single blastomere removal from murine embryos is associated with activation of matrix metalloproteinases and Janus kinase/signal transducers and activators of transcription pathways of placental inflammation. Molecular human reproduction 2014;20(12):1247-1257. (In eng). DOI: 10.1093/molehr/gau072.
147. Andrade SE, Gurwitz JH, Davis RL, et al. Prescription drug use in pregnancy. American Journal of Obstetrics and Gynecology 2004;191(2):398-407. DOI: https://doi.org/10.1016/j.ajog.2004.04.025.
148. Morgan DJ. Drug disposition in mother and foetus. Clin Exp Pharmacol Physiol 1997;24(11):869-73. (In eng). DOI: 10.1111/j.1440-1681.1997.tb02707.x.
149. van der Graaf R, van der Zande ISE, den Ruijter HM, et al. Fair inclusion of pregnant women in clinical trials: an integrated scientific and ethical approach. Trials 2018;19(1):78. (In eng). DOI: 10.1186/s13063-017-2402-9.
150. Desborough MJR, Keeling DM. The aspirin story - from willow to wonder drug. Br J Haematol 2017;177(5):674-683. (In eng). DOI: 10.1111/bjh.14520.
151. Norn S, Permin H, Kruse PR, Kruse E. [From willow bark to acetylsalicylic acid]. Dan Medicinhist Arbog 2009;37:79-98. (In dan).
152. Singh G. Gastrointestinal complications of prescription and over-the-counter nonsteroidal anti-inflammatory drugs: a view from the ARAMIS database. Arthritis, Rheumatism, and Aging Medical Information System. Am J Ther 2000;7(2):115-21. (In eng). DOI: 10.1097/00045391-200007020-00008.
153. Poole EM, Hsu L, Xiao L, et al. Genetic variation in prostaglandin E2 synthesis and signaling, prostaglandin dehydrogenase, and the risk of colorectal adenoma. Cancer Epidemiol Biomarkers Prev 2010;19(2):547-57. (In eng). DOI: 10.1158/1055-9965.Epi-09-0869.
154. Rouzer CA, Marnett LJ. Cyclooxygenases: structural and functional insights. J Lipid Res 2009;50 Suppl(Suppl):S29-34. (In eng). DOI: 10.1194/jlr.R800042-JLR200.
155. Fitzpatrick FA. Cyclooxygenase enzymes: regulation and function. Curr Pharm Des 2004;10(6):577-88. (In eng). DOI: 10.2174/1381612043453144.
156. Xie WL, Chipman JG, Robertson DL, Erikson RL, Simmons DL. Expression of a mitogen-responsive gene encoding prostaglandin synthase is regulated by mRNA splicing. Proc Natl Acad Sci U S A 1991;88(7):2692-6. (In eng). DOI: 10.1073/pnas.88.7.2692.
157. Rahme E, Nedjar H. Risks and benefits of COX-2 inhibitors vs non-selective NSAIDs: does their cardiovascular risk exceed their gastrointestinal benefit? A retrospective cohort study. Rheumatology (Oxford) 2007;46(3):435-8. (In eng). DOI: 10.1093/rheumatology/kel428.
158. Kozer E, Nikfar S, Costei A, Boskovic R, Nulman I, Koren G. Aspirin consumption during the first trimester of pregnancy and congenital anomalies: a meta-analysis. Am J Obstet Gynecol 2002;187(6):1623-30. (In eng). DOI: 10.1067/mob.2002.127376.
159. Nakhai-Pour HR, Bérard A. Major malformations after first trimester exposure to aspirin and NSAIDs. Expert Rev Clin Pharmacol 2008;1(5):605-16. (In eng). DOI: 10.1586/17512433.1.5.605.
160. Ericson A, Källén BA. Nonsteroidal anti-inflammatory drugs in early pregnancy. Reprod Toxicol 2001;15(4):371-5. (In eng). DOI: 10.1016/s0890-6238(01)00137-x.

161. Li DK, Ferber JR, Odouli R, Quesenberry C. Use of nonsteroidal antiinflammatory drugs during pregnancy and the risk of miscarriage. Am J Obstet Gynecol 2018;219(3):275.e1-275.e8. (In eng). DOI: 10.1016/j.ajog.2018.06.002.
162. ACOG Committee Opinion No. 743: Low-Dose Aspirin Use During Pregnancy. Obstet Gynecol 2018;132(1):e44-e52. (In eng). DOI: 10.1097/aog.0000000000002708.
163. Beaufils M, Uzan S, Donsimoni R, Colau JC. Prevention of pre-eclampsia by early antiplatelet therapy. Lancet 1985;1(8433):840-2. (In eng). DOI: 10.1016/s0140-6736(85)92207-x.
164. Bloor M, Paech M. Nonsteroidal anti-inflammatory drugs during pregnancy and the initiation of lactation. Anesth Analg 2013;116(5):1063-75. (In eng). DOI: 10.1213/ANE.0b013e31828a4b54.
165. Antonucci R, Zaffanello M, Puxeddu E, et al. Use of non-steroidal anti-inflammatory drugs in pregnancy: impact on the fetus and newborn. Curr Drug Metab 2012;13(4):474-90. (In eng). DOI: 10.2174/138920012800166607.
166. Aker K, Brantberg A, Nyrnes SA. Prenatal constriction of the ductus arteriosus following maternal diclofenac medication in the third trimester. BMJ Case Rep 2015;2015:bcr2015210473. (In eng). DOI: 10.1136/bcr-2015-210473.
167. Rathi P, Messina C, Mintzer JP. Indomethacin dosing strategy and neonatal patent ductus arteriosus closure. J Neonatal Perinatal Med 2019;12(4):411-417. (In eng). DOI: 10.3233/npm-180148.
168. Ejaz P, Bhojani K, Joshi VR. NSAIDs and kidney. J Assoc Physicians India 2004;52:632-40. (In eng).
169. Boubred F, Vendemmia M, Garcia-Meric P, Buffat C, Millet V, Simeoni U. Effects of maternally administered drugs on the fetal and neonatal kidney. Drug Saf 2006;29(5):397-419. (In eng). DOI: 10.2165/00002018-200629050-00004.
170. Kirpalani A, Rieder M. Is NSAID use in children associated with the risk of renal injury? Paediatr Child Health 2019;24(2):119-121. (In eng). DOI: 10.1093/pch/pxy183.
171. Usadi RS, Merriam KS. On-label and off-label drug use in the treatment of female infertility. Fertil Steril 2015;103(3):583-94. (In eng). DOI: 10.1016/j.fertnstert.2015.01.011.
172. Empson M, Lassere M, Craig J, Scott J. Prevention of recurrent miscarriage for women with antiphospholipid antibody or lupus anticoagulant. Cochrane Database Syst Rev 2005;2005(2):Cd002859. (In eng). DOI: 10.1002/14651858.CD002859.pub2.
173. Evaluation and treatment of recurrent pregnancy loss: a committee opinion. Fertil Steril 2012;98(5):1103-11. (In eng). DOI: 10.1016/j.fertnstert.2012.06.048.
174. Siristatidis CS, Dodd SR, Drakeley AJ. Aspirin for in vitro fertilisation. Cochrane Database Syst Rev 2011(8):Cd004832. (In eng). DOI: 10.1002/14651858.CD004832.pub3.
175. Grekin ER, Svikis DS, Lam P, et al. Drug use during pregnancy: validating the Drug Abuse Screening Test against physiological measures. Psychol Addict Behav 2010;24(4):719-23. (In eng). DOI: 10.1037/a0021741.
176. Price HR, Lai D, Kim H, Wright TE, Coughtrie MWH, Collier AC. Detection and quantitation of non-steroidal anti-inflammatory drug use close to the time of birth using umbilical cord tissue. Toxicol Rep 2020;7:1311-1318. (In eng). DOI: 10.1016/j.toxrep.2020.09.003.

177. Davies NM, Skjodt NM. Choosing the right nonsteroidal anti-inflammatory drug for the right patient: a pharmacokinetic approach. Clin Pharmacokinet 2000;38(5):377-92. (In eng). DOI: 10.2165/00003088-200038050-00001.
178. Chyka PA, Erdman AR, Christianson G, et al. Salicylate poisoning: an evidence-based consensus guideline for out-of-hospital management. Clin Toxicol (Phila) 2007;45(2):95-131. (In eng). DOI: 10.1080/15563650600907140.
179. Willis JV, Kendall MJ, Flinn RM, Thornhill DP, Welling PG. The pharmacokinetics of diclofenac sodium following intravenous and oral administration. Eur J Clin Pharmacol 1979;16(6):405-10. (In eng). DOI: 10.1007/bf00568201.
180. Bioanalytical Method Validation Guidance for Industry. In: Research CfDEa, ed. Silver Spring MD2018.
181. Rumble RH, Roberts MS, Wanwimolruk S. Determination of aspirin and its major metabolites in plasma by high-performance liquid chromatography without solvent extraction. J Chromatogr 1981;225(1):252-60. (In eng). DOI: 10.1016/s0378-4347(00)80270-4.
182. Zhou Y, Boudreau DM, Freedman AN. Trends in the use of aspirin and nonsteroidal anti-inflammatory drugs in the general U.S. population. Pharmacoepidemiol Drug Saf 2014;23(1):43-50. (In eng). DOI: 10.1002/pds.3463.
183. Montgomery D, Plate C, Alder SC, Jones M, Jones J, Christensen RD. Testing for fetal exposure to illicit drugs using umbilical cord tissue vs meconium. J Perinatol 2006;26(1):11-4. (In eng). DOI: 10.1038/sj.jp.7211416.
184. Wright TE, Milam KA, Rougee L, Tanaka MD, Collier AC. Agreement of umbilical cord drug and cotinine levels with maternal self-report of drug use and smoking during pregnancy. Journal of perinatology : official journal of the California Perinatal Association 2011;31(5):324-329. (In eng). DOI: 10.1038/jp.2010.132.
185. Stosic R, Dunagan F, Palmer H, Fowler T, Adams I. Responsible self-medication: perceived risks and benefits of over-the-counter analgesic use. International Journal of Pharmacy Practice 2011;19(4):236-245. DOI: 10.1111/j.2042-7174.2011.00097.x.
186. Duthie GG, Wood AD. Natural salicylates: foods, functions and disease prevention. Food Funct 2011;2(9):515-20. (In eng). DOI: 10.1039/c1fo10128e.
187. Blacklock CJ, Lawrence JR, Wiles D, et al. Salicylic acid in the serum of subjects not taking aspirin. Comparison of salicylic acid concentrations in the serum of vegetarians, non-vegetarians, and patients taking low dose aspirin. J Clin Pathol 2001;54(7):553-5. (In eng). DOI: 10.1136/jcp.54.7.553.
188. Damasceno EB, de Lima PP. Wharton's jelly absence: a possible cause of stillbirth. Autops Case Rep 2013;3(4):43-47. (In eng). DOI: 10.4322/acr.2013.038.
189. Keelan JA, Pugazhenthi K. Trans-placental passage and anti-inflammatory effects of solithromycin in the human placenta. Placenta 2014;35(12):1043-8. (In eng). DOI: 10.1016/j.placenta.2014.09.009.
190. Kalagiri RR, Carder T, Choudhury S, et al. Inflammation in Complicated Pregnancy and Its Outcome. Am J Perinatol 2016;33(14):1337-1356. (In eng). DOI: 10.1055/s-0036-1582397.
191. El-Shazly S, Makhseed M, Azizieh F, Raghupathy R. Increased expression of pro-inflammatory cytokines in placentas of women undergoing spontaneous preterm delivery or premature rupture of membranes. Am J Reprod Immunol 2004;52(1):45-52. (In eng). DOI: 10.1111/j.1600-0897.2004.00181.x.

192. Hagberg H, Mallard C, Jacobsson B. Role of cytokines in preterm labour and brain injury. Bjog 2005;112 Suppl 1:16-8. (In eng). DOI: 10.1111/j.1471-0528.2005.00578.x.
193. Padavala S, Pope N, Baker P, Crocker I. An imbalance between vascular endothelial growth factor and its soluble receptor in placental villous explants of intrauterine growth-restricted pregnancies. J Soc Gynecol Investig 2006;13(1):40-7. (In eng). DOI: 10.1016/j.jsgi.2005.09.010.
194. Leach L. Placental vascular dysfunction in diabetic pregnancies: intimations of fetal cardiovascular disease? Microcirculation 2011;18(4):263-9. (In eng). DOI: 10.1111/j.1549-8719.2011.00091.x.
195. Zhang J-M, An J. Cytokines, inflammation, and pain. Int Anesthesiol Clin 2007;45(2):27-37. (In eng). DOI: 10.1097/AIA.0b013e318034194e.
196. O'Shea JJ, Gadina M, Schreiber RD. Cytokine Signaling in 2002: New Surprises in the Jak/Stat Pathway. Cell 2002;109(2, Supplement 1):S121-S131. DOI: https://doi.org/10.1016/S0092-8674(02)00701-8.
197. Holmes DIR, Zachary I. The vascular endothelial growth factor (VEGF) family: angiogenic factors in health and disease. Genome Biol 2005;6(2):209-209. (In eng). DOI: 10.1186/gb-2005-6-2-209.
198. Yan Y, Guo TM, Zhu C. Effects of nonsteroidal anti-inflammatory drugs on serum proinflammatory cytokines in the treatment of ankylosing spondylitis. Biochem Cell Biol 2018;96(4):450-456. (In eng). DOI: 10.1139/bcb-2017-0267.
199. Summer GJ, Romero-Sandoval EA, Bogen O, Dina OA, Khasar SG, Levine JD. Proinflammatory cytokines mediating burn-injury pain. PAIN® 2008;135(1):98-107. DOI: https://doi.org/10.1016/j.pain.2007.05.012.
200. Bowen JM, Chamley L, Keelan JA, Mitchell MD. Cytokines of the placenta and extra-placental membranes: roles and regulation during human pregnancy and parturition. Placenta 2002;23(4):257-73. (In eng). DOI: 10.1053/plac.2001.0782.
201. Bowen JM, Chamley L, Mitchell MD, Keelan JA. Cytokines of the placenta and extra-placental membranes: biosynthesis, secretion and roles in establishment of pregnancy in women. Placenta 2002;23(4):239-56. (In eng). DOI: 10.1053/plac.2001.0781.
202. Wang YZ, Z. Angiogenic Factors. In: Publishers MaC, ed. Vascular Biology of the Placenta2010.
203. Vuorela P, Hatva E, Lymboussaki A, et al. Expression of vascular endothelial growth factor and placenta growth factor in human placenta. Biol Reprod 1997;56(2):489-94. DOI: 10.1095/biolreprod56.2.489.
204. Maglione D, Guerriero V, Viglietto G, Delli-Bovi P, Persico MG. Isolation of a human placenta cDNA coding for a protein related to the vascular permeability factor. Proc Natl Acad Sci U S A 1991;88(20):9267-71. (In eng). DOI: 10.1073/pnas.88.20.9267.
205. Rao R, Sen S, Han B, Ramadoss S, Chaudhuri G. Gestational diabetes, preeclampsia and cytokine release: similarities and differences in endothelial cell function. Adv Exp Med Biol 2014;814:69-75. (In eng). DOI: 10.1007/978-1-4939-1031-1_6.
206. Krukier, II, Pogorelova TN. Production of vascular endothelial growth factor and endothelin in the placenta and umbilical cord during normal and complicated pregnancy. Bull Exp Biol Med 2006;141(2):216-8. (In eng). DOI: 10.1007/s10517-006-0131-2.
207. Sun Y, Chen M, Mao B, Cheng X, Zhang X, Xu C. Association between vascular endothelial growth factor polymorphism and recurrent pregnancy loss: A systematic

review and meta-analysis. Eur J Obstet Gynecol Reprod Biol 2017;211:169-176. (In eng). DOI: 10.1016/j.ejogrb.2017.03.003.
208. Wedekind L, Belkacemi L. Altered cytokine network in gestational diabetes mellitus affects maternal insulin and placental-fetal development. J Diabetes Complications 2016;30(7):1393-400. (In eng). DOI: 10.1016/j.jdiacomp.2016.05.011.
209. Nuzzo AM, Giuffrida D, Moretti L, et al. Placental and maternal sFlt1/PlGF expression in gestational diabetes mellitus. Sci Rep 2021;11(1):2312. (In eng). DOI: 10.1038/s41598-021-81785-5.
210. Troncoso F, Acurio J, Herlitz K, et al. Gestational diabetes mellitus is associated with increased pro-migratory activation of vascular endothelial growth factor receptor 2 and reduced expression of vascular endothelial growth factor receptor 1. PLoS One 2017;12(8):e0182509. (In eng). DOI: 10.1371/journal.pone.0182509.
211. Meng Q, Shao L, Luo X, et al. Expressions of VEGF-A and VEGFR-2 in placentae from GDM pregnancies. Reproductive biology and endocrinology : RB&E 2016;14(1):61-61. (In eng). DOI: 10.1186/s12958-016-0191-8.
212. Conrad KP, Benyo DF. Placental cytokines and the pathogenesis of preeclampsia. Am J Reprod Immunol 1997;37(3):240-9. (In eng). DOI: 10.1111/j.1600-0897.1997.tb00222.x.
213. Raghupathy R. Cytokines as key players in the pathophysiology of preeclampsia. Med Princ Pract 2013;22 Suppl 1(Suppl 1):8-19. (In eng). DOI: 10.1159/000354200.
214. Smith GCS, Crossley JA, Aitken DA, et al. Circulating Angiogenic Factors in Early Pregnancy and the Risk of Preeclampsia, Intrauterine Growth Restriction, Spontaneous Preterm Birth, and Stillbirth. Obstetrics & Gynecology 2007;109(6) (https://journals.lww.com/greenjournal/Fulltext/2007/06000/Circulating_Angiogenic_Factors_in_Early_Pregnancy.11.aspx).
215. Mowa CN, Jesmin S, Sakuma I, et al. Characterization of vascular endothelial growth factor (VEGF) in the uterine cervix over pregnancy: effects of denervation and implications for cervical ripening. J Histochem Cytochem 2004;52(12):1665-74. (In eng). DOI: 10.1369/jhc.4A6455.2004.
216. S. J. Knight ADS, H. Kim, A. C. Collier. Human placental suppressors of cytokine signalling (SOCS) and inflammatory cytokines are dysregulated in assisted reproduction, advanced maternal age and pre-term birth. Clinical and Experimental Obstetrics & Gynecology 2020;47(2):277-286. DOI: 10.31083/j.ceog.2020.02.5299.
217. Babon JJ, Varghese LN, Nicola NA. Inhibition of IL-6 family cytokines by SOCS3. Semin Immunol 2014;26(1):13-19. (In eng). DOI: 10.1016/j.smim.2013.12.004.
218. Harrity C, Shkrobot L, Walsh D, Marron K. ART implantation failure and miscarriage in patients with elevated intracellular cytokine ratios: response to immune support therapy. Fertility Research and Practice 2018;4(1):7. DOI: 10.1186/s40738-018-0052-6.
219. Grazul-Bilska AT, Johnson ML, Borowicz PP, et al. Placental development during early pregnancy in sheep: effects of embryo origin on vascularization. Reproduction (Cambridge, England) 2014;147(5):639-648. (In eng). DOI: 10.1530/REP-13-0663.
220. Johnson ML, Reynolds LP, Borowicz PP, Redmer DA, Grazul-Bilska AT. Expression of mRNA for Factors that Influence Angiogenesis in Ovine Utero-Placental Tissues During Early Pregnancy: Effects of Assisted Reproductive Technology. Biology of Reproduction 2011;85(Suppl_1):352-352. DOI: 10.1093/biolreprod/85.s1.352.
221. Chehroudi C, Kim H, Wright TE, Collier AC. Dysregulation of inflammatory cytokines and inhibition of VEGFA in the human umbilical cord are associated with negative

pregnancy outcomes. Placenta 2019;87:16-22. (In eng). DOI: 10.1016/j.placenta.2019.09.002.
222. Agarwal R, Kumari N, Kar R, et al. Evaluation of Placental VEGFA mRNA Expression in Preeclampsia: A Case Control Study. J Obstet Gynaecol India 2019;69(2):142-148. (In eng). DOI: 10.1007/s13224-018-1128-2.
223. Szentpéteri I, Rab A, Kornya L, Kovács P, Joó JG. Gene expression patterns of vascular endothelial growth factor (VEGF-A) in human placenta from pregnancies with intrauterine growth restriction. J Matern Fetal Neonatal Med 2013;26(10):984-9. (In eng). DOI: 10.3109/14767058.2013.766702.
224. Tanaka K, Watanabe M, Tanigaki S, Iwashita M, Kobayashi Y. Tumor necrosis factor-α regulates angiogenesis of BeWo cells via synergy of PlGF/VEGFR1 and VEGF-A/VEGFR2 axes. Placenta 2018;74:20-27. DOI: https://doi.org/10.1016/j.placenta.2018.12.009.
225. Tita ATN, Andrews WW. Diagnosis and management of clinical chorioamnionitis. Clin Perinatol 2010;37(2):339-354. (In eng). DOI: 10.1016/j.clp.2010.02.003.
226. Soper DE, Mayhall CG, Dalton HP. Risk factors for intraamniotic infection: a prospective epidemiologic study. Am J Obstet Gynecol 1989;161(3):562-6; discussion 566-8. (In eng). DOI: 10.1016/0002-9378(89)90356-6.
227. Newton ER, Prihoda TJ, Gibbs RS. Logistic regression analysis of risk factors for intra-amniotic infection. Obstet Gynecol 1989;73(4):571-5. (In eng).
228. Piper JM, Newton ER, Berkus MD, Peairs WA. Meconium: a marker for peripartum infection. Obstet Gynecol 1998;91(5 Pt 1):741-5. (In eng). DOI: 10.1016/s0029-7844(98)00048-9.
229. Rouse DJ, Landon M, Leveno KJ, et al. The Maternal-Fetal Medicine Units cesarean registry: chorioamnionitis at term and its duration-relationship to outcomes. Am J Obstet Gynecol 2004;191(1):211-6. (In eng). DOI: 10.1016/j.ajog.2004.03.003.
230. Romero R, Miranda J, Chaiworapongsa T, et al. Prevalence and clinical significance of sterile intra-amniotic inflammation in patients with preterm labor and intact membranes. Am J Reprod Immunol 2014;72(5):458-74. (In eng). DOI: 10.1111/aji.12296.
231. Kim CJ, Romero R, Chaemsaithong P, Chaiyasit N, Yoon BH, Kim YM. Acute chorioamnionitis and funisitis: definition, pathologic features, and clinical significance. American Journal of Obstetrics and Gynecology 2015;213(4, Supplement):S29-S52. DOI: https://doi.org/10.1016/j.ajog.2015.08.040.
232. Arntzen KJ, Kjøllesdal AM, Halgunset J, Vatten L, Austgulen R. TNF, IL-1, IL-6, IL-8 and soluble TNF receptors in relation to chorioamnionitis and premature labor. J Perinat Med 1998;26(1):17-26. (In eng). DOI: 10.1515/jpme.1998.26.1.17.
233. Kniss DA, Zimmerman PD, Garver CL, Fertel RH. Interleukin-1 receptor antagonist blocks interleukin-1-induced expression of cyclooxygenase-2 in endometrium. Am J Obstet Gynecol 1997;177(3):559-67. (In eng). DOI: 10.1016/s0002-9378(97)70146-7.
234. Molnár M, Romero R, Hertelendy F. Interleukin-1 and tumor necrosis factor stimulate arachidonic acid release and phospholipid metabolism in human myometrial cells. Am J Obstet Gynecol 1993;169(4):825-9. (In eng). DOI: 10.1016/0002-9378(93)90011-7.
235. Jain VG, Willis KA, Jobe A, Ambalavanan N. Chorioamnionitis and neonatal outcomes. Pediatric Research 2021. DOI: 10.1038/s41390-021-01633-0.

236. Kallapur SG, Presicce P, Rueda CM, Jobe AH, Chougnet CA. Fetal immune response to chorioamnionitis. Semin Reprod Med 2014;32(1):56-67. (In eng). DOI: 10.1055/s-0033-1361823.
237. Cappelletti M, Presicce P, Kallapur SG. Immunobiology of Acute Chorioamnionitis. Frontiers in Immunology 2020;11(649) (Review) (In English). DOI: 10.3389/fimmu.2020.00649.
238. Renaud SJ, Cotechini T, Quirt JS, Macdonald-Goodfellow SK, Othman M, Graham CH. Spontaneous pregnancy loss mediated by abnormal maternal inflammation in rats is linked to deficient uteroplacental perfusion. J Immunol 2011;186(3):1799-808. (In eng). DOI: 10.4049/jimmunol.1002679.
239. Gelber SE, Brent E, Redecha P, et al. Prevention of Defective Placentation and Pregnancy Loss by Blocking Innate Immune Pathways in a Syngeneic Model of Placental Insufficiency. J Immunol 2015;195(3):1129-38. (In eng). DOI: 10.4049/jimmunol.1402220.
240. Winger EE, Reed JL. Treatment with tumor necrosis factor inhibitors and intravenous immunoglobulin improves live birth rates in women with recurrent spontaneous abortion. Am J Reprod Immunol 2008;60(1):8-16. (In eng). DOI: 10.1111/j.1600-0897.2008.00585.x.
241. Leitner K, Al Shammary M, McLane M, Johnston MV, Elovitz MA, Burd I. IL-1 receptor blockade prevents fetal cortical brain injury but not preterm birth in a mouse model of inflammation-induced preterm birth and perinatal brain injury. Am J Reprod Immunol 2014;71(5):418-26. (In eng). DOI: 10.1111/aji.12216.
242. Racicot K, Kwon JY, Aldo P, Silasi M, Mor G. Understanding the complexity of the immune system during pregnancy. Am J Reprod Immunol 2014;72(2):107-16. (In eng). DOI: 10.1111/aji.12289.
243. Blois SM, Freitag N, Tirado-González I, et al. NK cell-derived IL-10 is critical for DC-NK cell dialogue at the maternal-fetal interface. Scientific Reports 2017;7(1):2189. DOI: 10.1038/s41598-017-02333-8.
244. Murphy SP, Fast LD, Hanna NN, Sharma S. Uterine NK cells mediate inflammation-induced fetal demise in IL-10-null mice. J Immunol 2005;175(6):4084-90. (In eng). DOI: 10.4049/jimmunol.175.6.4084.
245. Plevyak M, Hanna N, Mayer S, et al. Deficiency of decidual IL-10 in first trimester missed abortion: a lack of correlation with the decidual immune cell profile. Am J Reprod Immunol 2002;47(4):242-50. (In eng). DOI: 10.1034/j.1600-0897.2002.01060.x.
246. Cheong-See F, Schuit E, Arroyo-Manzano D, et al. Prospective risk of stillbirth and neonatal complications in twin pregnancies: systematic review and meta-analysis. Bmj 2016;354:i4353. (In eng). DOI: 10.1136/bmj.i4353.
247. Guidelines for the number of embryos to transfer following in vitro fertilization No. 182, September 2006. Int J Gynaecol Obstet 2008;102(2):203-16. (In eng). DOI: 10.1016/j.ijgo.2008.01.007.
248. Keustermans GC, Hoeks SB, Meerding JM, Prakken BJ, de Jager W. Cytokine assays: an assessment of the preparation and treatment of blood and tissue samples. Methods 2013;61(1):10-7. (In eng). DOI: 10.1016/j.ymeth.2013.04.005.
249. Zhou X, Fragala MS, McElhaney JE, Kuchel GA. Conceptual and methodological issues relevant to cytokine and inflammatory marker measurements in clinical research. Curr

Opin Clin Nutr Metab Care 2010;13(5):541-7. (In eng). DOI: 10.1097/MCO.0b013e32833cf3bc.
250. Nohl H, Kozlov AV, Gille L, Staniek K. Endogenous Oxidant-Generating Systems. In: Grune T, ed. Reactions, Processes: Oxidants and Antioxidant Defense Systems. Berlin, Heidelberg: Springer Berlin Heidelberg; 2005:1-18.
251. Griffiths HR. Chemical Modifications of Biomolecules by Oxidants. In: Grune T, ed. Reactions, Processes: Oxidants and Antioxidant Defense Systems. Berlin, Heidelberg: Springer Berlin Heidelberg; 2005:33-62.
252. Cho K-J, Seo J-M, Kim J-H. Bioactive lipoxygenase metabolites stimulation of NADPH oxidases and reactive oxygen species. Mol Cells 2011;32(1):1-5. (In eng). DOI: 10.1007/s10059-011-1021-7.
253. Ostuni MA, Gelinotte M, Bizouarn T, Baciou L, Houée-Levin C. Targeting NADPH-oxidase by reactive oxygen species reveals an initial sensitive step in the assembly process. Free Radic Biol Med 2010;49(5):900-7. (In eng). DOI: 10.1016/j.freeradbiomed.2010.06.021.
254. Davies KJA. The Broad Spectrum of Responses to Oxidative Stress in Proliferating Cells. In: Grune T, ed. Reactions, Processes: Oxidants and Antioxidant Defense Systems. Berlin, Heidelberg: Springer Berlin Heidelberg; 2005:63-75.
255. Palace VP, Khaper N, Qin Q, Singal PK. Antioxidant potentials of vitamin A and carotenoids and their relevance to heart disease. Free Radic Biol Med 1999;26(5-6):746-61. (In eng). DOI: 10.1016/s0891-5849(98)00266-4.
256. Traber MG, Stevens JF. Vitamins C and E: beneficial effects from a mechanistic perspective. Free radical biology & medicine 2011;51(5):1000-1013. (In eng). DOI: 10.1016/j.freeradbiomed.2011.05.017.
257. Myatt L, Cui X. Oxidative stress in the placenta. Histochem Cell Biol 2004;122(4):369-82. (In eng). DOI: 10.1007/s00418-004-0677-x.
258. Rani N, Dhingra R, Arya DS, Kalaivani M, Bhatla N, Kumar R. Role of oxidative stress markers and antioxidants in the placenta of preeclamptic patients. J Obstet Gynaecol Res 2010;36(6):1189-94. (In eng). DOI: 10.1111/j.1447-0756.2010.01303.x.
259. Guérin P, El Mouatassim S, Ménézo Y. Oxidative stress and protection against reactive oxygen species in the pre-implantation embryo and its surroundings. Hum Reprod Update 2001;7(2):175-89. (In eng). DOI: 10.1093/humupd/7.2.175.
260. Cadenas E, Davies KJA. Mitochondrial free radical generation, oxidative stress, and aging11This article is dedicated to the memory of our dear friend, colleague, and mentor Lars Ernster (1920–1998), in gratitude for all he gave to us. Free Radical Biology and Medicine 2000;29(3):222-230. DOI: https://doi.org/10.1016/S0891-5849(00)00317-8.
261. Many A, Hubel CA, Fisher SJ, Roberts JM, Zhou Y. Invasive cytotrophoblasts manifest evidence of oxidative stress in preeclampsia. Am J Pathol 2000;156(1):321-31. (In eng). DOI: 10.1016/s0002-9440(10)64733-5.
262. Matsubara S, Sato I. Enzyme histochemically detectable NAD(P)H oxidase in human placental trophoblasts: normal, preeclamptic, and fetal growth restriction-complicated pregnancy. Histochem Cell Biol 2001;116(1):1-7. (In eng). DOI: 10.1007/s004180100301.
263. Eis AL, Brockman DE, Pollock JS, Myatt L. Immunohistochemical localization of endothelial nitric oxide synthase in human villous and extravillous trophoblast

populations and expression during syncytiotrophoblast formation in vitro. Placenta 1995;16(2):113-26. (In eng). DOI: 10.1016/0143-4004(95)90000-4.
264. Lyall F, Barber A, Myatt L, Bulmer JN, Robson SC. Hemeoxygenase expression in human placenta and placental bed implies a role in regulation of trophoblast invasion and placental function. Faseb j 2000;14(1):208-19. (In eng). DOI: 10.1096/fasebj.14.1.208.
265. Hempstock J, Bao YP, Bar-Issac M, et al. Intralobular Differences in Antioxidant Enzyme Expression and Activity Reflect the Pattern of Maternal Arterial Bloodflow Within the Human Placenta. Placenta 2003;24(5):517-523. DOI: https://doi.org/10.1053/plac.2002.0955.
266. Nuzzo AM, Camm EJ, Sferruzzi-Perri AN, et al. Placental Adaptation to Early-Onset Hypoxic Pregnancy and Mitochondria-Targeted Antioxidant Therapy in a Rodent Model. The American Journal of Pathology 2018;188(12):2704-2716. DOI: https://doi.org/10.1016/j.ajpath.2018.07.027.
267. Nebert DW, Vasiliou V. Analysis of the glutathione S-transferase (GST) gene family. Hum Genomics 2004;1(6):460-464. (In eng). DOI: 10.1186/1479-7364-1-6-460.
268. Raijmakers MTM, Steegers EAP, Peters WHM. Glutathione S-transferases and thiol concentrations in embryonic and early fetal tissues. Human Reproduction 2001;16(11):2445-2450. DOI: 10.1093/humrep/16.11.2445.
269. Allocati N, Masulli M, Di Ilio C, Federici L. Glutathione transferases: substrates, inihibitors and pro-drugs in cancer and neurodegenerative diseases. Oncogenesis 2018;7(1):8. DOI: 10.1038/s41389-017-0025-3.
270. Forman HJ, Dickinson DA. Oxidative signaling and glutathione synthesis. Biofactors 2003;17(1-4):1-12. (In eng). DOI: 10.1002/biof.5520170101.
271. Ursini F, Maiorino M. Glutathione Peroxidases. In: Lennarz WJ, Lane MD, eds. Encyclopedia of Biological Chemistry (Second Edition). Waltham: Academic Press; 2013:399-404.
272. Mistry HD, Kurlak LO, Williams PJ, Ramsay MM, Symonds ME, Broughton Pipkin F. Differential expression and distribution of placental glutathione peroxidases 1, 3 and 4 in normal and preeclamptic pregnancy. Placenta 2010;31(5):401-8. (In eng). DOI: 10.1016/j.placenta.2010.02.011.
273. Avissar N, Eisenmann C, Breen JG, Horowitz S, Miller RK, Cohen HJ. Human placenta makes extracellular glutathione peroxidase and secretes it into maternal circulation. Am J Physiol 1994;267(1 Pt 1):E68-76. (In eng). DOI: 10.1152/ajpendo.1994.267.1.E68.
274. Couto N, Wood J, Barber J. The role of glutathione reductase and related enzymes on cellular redox homoeostasis network. Free Radical Biology and Medicine 2016;95:27-42. DOI: https://doi.org/10.1016/j.freeradbiomed.2016.02.028.
275. Di Ilio C, Polidoro G, Arduini A, Muccini A, Federici G. Glutathione peroxidase, glutathione reductase, glutathione S-transferase, and gamma-glutamyltranspeptidase activities in the human early pregnancy placenta. Biochem Med 1983;29(2):143-8. (In eng). DOI: 10.1016/0006-2944(83)90034-0.
276. Altobelli GG, Van Noorden S, Balato A, Cimini V. Copper/Zinc Superoxide Dismutase in Human Skin: Current Knowledge. Frontiers in Medicine 2020;7(183) (Mini Review) (In English). DOI: 10.3389/fmed.2020.00183.
277. Myatt L, Eis AL, Brockman DE, Kossenjans W, Greer IA, Lyall F. Differential localization of superoxide dismutase isoforms in placental villous tissue of normotensive,

pre-eclamptic, and intrauterine growth-restricted pregnancies. J Histochem Cytochem 1997;45(10):1433-8. (In eng). DOI: 10.1177/002215549704501012.
278. D'Souza V, Rani A, Patil V, et al. Increased oxidative stress from early pregnancy in women who develop preeclampsia. Clin Exp Hypertens 2016;38(2):225-32. (In eng). DOI: 10.3109/10641963.2015.1081226.
279. Parast VM, Paknahad Z. Antioxidant Status and Risk of Gestational Diabetes Mellitus: a Case-Control Study. Clin Nutr Res 2017;6(2):81-88. (In eng). DOI: 10.7762/cnr.2017.6.2.81.
280. Zhang C, Yang Y, Chen R, et al. Aberrant expression of oxidative stress related proteins affects the pregnancy outcome of gestational diabetes mellitus patients. Am J Transl Res 2019;11(1):269-279. (In eng) (https://pubmed.ncbi.nlm.nih.gov/30787985 https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6357329/).
281. Serdar Z, Gür E, Develioğlu O, Colakoğullari M, Dirican M. Placental and decidual lipid peroxidation and antioxidant defenses in preeclampsia. Lipid peroxidation in preeclampsia. Pathophysiology 2002;9(1):21. (In eng). DOI: 10.1016/s0928-4680(02)00052-4.
282. González P, Tuñón MJ, Manrique V, Garcia-Pardo LA, González J. Changes in hepatic cytosolic glutathione S-transferase enzymes induced by clotrimazole treatment in rats. Clin Exp Pharmacol Physiol 1989;16(11):867-71. (In eng). DOI: 10.1111/j.1440-1681.1989.tb01526.x.
283. Pabst MJ, Habig WH, Jakoby WB. Glutathione S-transferase A. A novel kinetic mechanism in which the major reaction pathway depends on substrate concentration. J Biol Chem 1974;249(22):7140-7. (In eng).
284. Tütem E, Apak R, Günaydı E, Sözgen Başkan K. Spectrophotometric determination of vitamin E (??-tocopherol) using copper(II)-neocuproine reagent. Talanta 1997;44:249-255. DOI: 10.1016/S0039-9140(96)02041-3.
285. Tietze F. Enzymic method for quantitative determination of nanogram amounts of total and oxidized glutathione: applications to mammalian blood and other tissues. Anal Biochem 1969;27(3):502-22. (In eng). DOI: 10.1016/0003-2697(69)90064-5.
286. Elmarakby AA, Sullivan JC. Relationship between oxidative stress and inflammatory cytokines in diabetic nephropathy. Cardiovasc Ther 2012;30(1):49-59. (In eng). DOI: 10.1111/j.1755-5922.2010.00218.x.
287. Pereda J, Sabater L, Aparisi L, et al. Interaction between cytokines and oxidative stress in acute pancreatitis. Current medicinal chemistry 2006;13 23:2775-87.
288. Crapo JD. Oxidative stress as an initiator of cytokine release and cell damage. Eur Respir J Suppl 2003;44:4s-6s. (In eng). DOI: 10.1183/09031936.03.00000203a.
289. Tenório MB, Ferreira RC, Moura FA, Bueno NB, de Oliveira ACM, Goulart MOF. Cross-Talk between Oxidative Stress and Inflammation in Preeclampsia. Oxidative Medicine and Cellular Longevity 2019;2019:8238727. DOI: 10.1155/2019/8238727.
290. Lappas M, Mitton A, Permezel M. In response to oxidative stress, the expression of inflammatory cytokines and antioxidant enzymes are impaired in placenta, but not adipose tissue, of women with gestational diabetes. J Endocrinol 2010;204(1):75-84. (In eng). DOI: 10.1677/joe-09-0321.
291. Malek A, Sager R, Schneider H. Effect of Hypoxia, Oxidative Stress and Lipopolysaccharides on the Release of Prostaglandins and Cytokines from Human Term

Placental Explants. Placenta 2001;22:S45-S50. DOI: https://doi.org/10.1053/plac.2001.0635.
292. Nitsos I, Moss TJ, Cock ML, Harding R, Newnham JP. Fetal responses to intra-amniotic endotoxin in sheep. J Soc Gynecol Investig 2002;9(2):80-5. (In eng). DOI: 10.1016/s1071-5576(01)00155-1.
293. Than NG, Romero R, Tarca AL, et al. Mitochondrial manganese superoxide dismutase mRNA expression in human chorioamniotic membranes and its association with labor, inflammation, and infection. J Matern Fetal Neonatal Med 2009;22(11):1000-13. (In eng). DOI: 10.3109/14767050903019676.
294. Sales F, Peralta OA, Narbona E, et al. Hypoxia and Oxidative Stress Are Associated with Reduced Fetal Growth in Twin and Undernourished Sheep Pregnancies. Animals (Basel) 2018;8(11):217. (In eng). DOI: 10.3390/ani8110217.
295. Zhang GL, He ZM, Shi XM, Gou CY, Gao Y, Fang Q. Discordant HIF1A mRNA levels and oxidative stress in placental shares of monochorionic twins with selective intra-uterine growth restriction. Placenta 2015;36(3):297-303. DOI: https://doi.org/10.1016/j.placenta.2014.12.019.
296. Chakraborty P, Dugmonits KN, Orvos H, Hermesz E. Mature Twin Neonates Exhibit Oxidative Stress via Nitric Oxide Synthase Dysfunctionality: A Prognostic Stress Marker in the Red Blood Cells and Umbilical Cord Vessels. Antioxidants 2020;9(9):845. (https://www.mdpi.com/2076-3921/9/9/845).
297. Jantsch LB, de Lucca L, Dorneles BN, Konopka CK, Gonçalves TdL. Evaluation of oxidative stress and δ-aminolevulinate dehydratase activity in twin pregnancies. The Journal of Maternal-Fetal & Neonatal Medicine 2020;33(18):3071-3076. DOI: 10.1080/14767058.2019.1568980.
298. Santarosa BP, Dantas GN, Ferreira DOL, et al. Comparison of oxidative stress markers between single and twin gestations in Dorper ewes during pregnancy, delivery and postpartum. Small Ruminant Research 2021;197:106333. DOI: https://doi.org/10.1016/j.smallrumres.2021.106333.
299. Habig WH, Jakoby WB. Assays for differentiation of glutathione S-transferases. Methods Enzymol 1981;77:398-405. (In eng). DOI: 10.1016/s0076-6879(81)77053-8.
300. Lingappan K. NF-κB in Oxidative Stress. Curr Opin Toxicol 2018;7:81-86. (In eng). DOI: 10.1016/j.cotox.2017.11.002.
301. Armistead B, Kadam L, Drewlo S, Kohan-Ghadr H-R. The Role of NFκB in Healthy and Preeclamptic Placenta: Trophoblasts in the Spotlight. Int J Mol Sci 2020;21(5):1775. (In eng). DOI: 10.3390/ijms21051775.
302. Chatuphonprasert W, Jarukamjorn K, Ellinger I. Physiology and Pathophysiology of Steroid Biosynthesis, Transport and Metabolism in the Human Placenta. Frontiers in Pharmacology 2018;9(1027) (Review) (In English). DOI: 10.3389/fphar.2018.01027.
303. White BA. Endocrine and reproductive physiology: Elsevier, 2019.
304. Konstantakou P, Mastorakos G, Vrachnis N, Tomlinson JW, Valsamakis G. Dysregulation of 11beta-hydroxysteroid dehydrogenases: implications during pregnancy and beyond. The Journal of Maternal-Fetal & Neonatal Medicine 2017;30(3):284-293. DOI: 10.3109/14767058.2016.1171308.
305. Sathishkumar K, Balakrishnan M, Chinnathambi V, Chauhan M, Hankins GD, Yallampalli C. Fetal sex-related dysregulation in testosterone production and their

receptor expression in the human placenta with preeclampsia. J Perinatol 2012;32(5):328-35. (In eng). DOI: 10.1038/jp.2011.101.
306. Halasz M, Szekeres-Bartho J. The role of progesterone in implantation and trophoblast invasion. Journal of Reproductive Immunology 2013;97(1):43-50. DOI: https://doi.org/10.1016/j.jri.2012.10.011.
307. Costa MA. The endocrine function of human placenta: an overview. Reproductive BioMedicine Online 2016;32(1):14-43. DOI: https://doi.org/10.1016/j.rbmo.2015.10.005.
308. Berkane N, Liere P, Oudinet J-P, et al. From Pregnancy to Preeclampsia: A Key Role for Estrogens. Endocrine Reviews 2017;38(2):123-144. DOI: 10.1210/er.2016-1065.
309. Brown AJ, Sharpe LJ. Chapter 11 - Cholesterol Synthesis. In: Ridgway ND, McLeod RS, eds. Biochemistry of Lipids, Lipoproteins and Membranes (Sixth Edition). Boston: Elsevier; 2016:327-358.
310. Shi W, Swan KF, Lear SR, O'Neil JS, Erickson SK, Henson MC. Regulation of Pathways Determining Cholesterol Availability in the Baboon Placenta with Advancing Gestation1. Biology of Reproduction 1999;61(6):1499-1505. DOI: 10.1095/biolreprod61.6.1499.
311. Olvera-Sanchez S, Espinosa-Garcia MT, Monreal J, Flores-Herrera O, Martinez F. Mitochondrial heat shock protein participates in placental steroidogenesis. Placenta 2011;32(3):222-9. (In eng). DOI: 10.1016/j.placenta.2010.12.018.
312. Tuckey RC, Bose HS, Czerwionka I, Miller WL. Molten globule structure and steroidogenic activity of N-218 MLN64 in human placental mitochondria. Endocrinology 2004;145(4):1700-7. (In eng). DOI: 10.1210/en.2003-1034.
313. Tuckey RC, Headlam MJ. Placental cytochrome P450scc (CYP11A1): comparison of catalytic properties between conditions of limiting and saturating adrenodoxin reductase. J Steroid Biochem Mol Biol 2002;81(2):153-8. (In eng). DOI: 10.1016/s0960-0760(02)00058-4.
314. Tuckey RC, Kostadinovic Z, Cameron KJ. Cytochrome P-450scc activity and substrate supply in human placental trophoblasts. Molecular and Cellular Endocrinology 1994;105(1):103-109. DOI: https://doi.org/10.1016/0303-7207(94)90041-8.
315. Mason JI, Ushijima K, Doody KM, et al. Regulation of expression of the 3 beta-hydroxysteroid dehydrogenases of human placenta and fetal adrenal. J Steroid Biochem Mol Biol 1993;47(1-6):151-9. (In eng). DOI: 10.1016/0960-0760(93)90069-9.
316. Maliqueo M, Lara HE, Sánchez F, Echiburú B, Crisosto N, Sir-Petermann T. Placental steroidogenesis in pregnant women with polycystic ovary syndrome. Eur J Obstet Gynecol Reprod Biol 2013;166(2):151-5. (In eng). DOI: 10.1016/j.ejogrb.2012.10.015.
317. Pion R, Jaffe R, Eriksson G, Wiqvist N, Diczfalusy E. STUDIES ON THE METABOLISM OF C-21 STEROIDS IN THE HUMAN FOETO-PLACENTAL UNIT. I. FORMATION OF A BETA-UNSATURATED 3-KETONES IN MIDTERM PLACENTAS PERFUSED IN SITU WITH PREGNENOLONE AND 17-ALPHA-HYDROXYPREGNENOLONE. Acta Endocrinol (Copenh) 1965;48:234-48. (In eng).
318. Voutilainen R, Miller WL. Developmental expression of genes for the stereoidogenic enzymes P450scc (20,22-desmolase), P450c17 (17 alpha-hydroxylase/17,20-lyase), and P450c21 (21-hydroxylase) in the human fetus. J Clin Endocrinol Metab 1986;63(5):1145-50. (In eng). DOI: 10.1210/jcem-63-5-1145.
319. Escobar JC, Patel SS, Beshay VE, Suzuki T, Carr BR. The human placenta expresses CYP17 and generates androgens de novo. J Clin Endocrinol Metab 2011;96(5):1385-92. (In eng). DOI: 10.1210/jc.2010-2504.

320. Pezzi V, Mathis JM, Rainey WE, Carr BR. Profiling transcript levels for steroidogenic enzymes in fetal tissues. J Steroid Biochem Mol Biol 2003;87(2):181-189. DOI: https://doi.org/10.1016/j.jsbmb.2003.07.006.
321. Noyola-Martínez N, Halhali A, Zaga-Clavellina V, Olmos-Ortiz A, Larrea F, Barrera D. A time-course regulatory and kinetic expression study of steroid metabolizing enzymes by calcitriol in primary cultured human placental cells. J Steroid Biochem Mol Biol 2017;167:98-105. DOI: https://doi.org/10.1016/j.jsbmb.2016.11.015.
322. Kamat A, Mendelson CR. Identification of the regulatory regions of the human aromatase P450 (CYP19) gene involved in placenta-specific expression. J Steroid Biochem Mol Biol 2001;79(1):173-180. DOI: https://doi.org/10.1016/S0960-0760(01)00156-X.
323. Reed KC, Ohno S. Kinetic properties of human placental aromatase. Application of an assay measuring 3H2O release from 1beta,2beta-3H-androgens. J Biol Chem 1976;251(6):1625-31. (In eng).
324. Jobe SO, Tyler CT, Magness RR. Aberrant synthesis, metabolism, and plasma accumulation of circulating estrogens and estrogen metabolites in preeclampsia implications for vascular dysfunction. Hypertension 2013;61(2):480-7. (In eng). DOI: 10.1161/hypertensionaha.111.201624.
325. Shozu M, Akasofu K, Harada T, Kubota Y. A new cause of female pseudohermaphroditism: placental aromatase deficiency. J Clin Endocrinol Metab 1991;72(3):560-6. (In eng). DOI: 10.1210/jcem-72-3-560.
326. Ugele B, St-Pierre MV, Pihusch M, Bahn A, Hantschmann P. Characterization and identification of steroid sulfate transporters of human placenta. Am J Physiol Endocrinol Metab 2003;284(2):E390-8. (In eng). DOI: 10.1152/ajpendo.00257.2002.
327. Salido EC, Yen PH, Barajas L, Shapiro LJ. Steroid sulfatase expression in human placenta: immunocytochemistry and in situ hybridization study. J Clin Endocrinol Metab 1990;70(6):1564-7. (In eng). DOI: 10.1210/jcem-70-6-1564.
328. Gratton AM, Ye L, Brownfoot FC, et al. Steroid sulfatase is increased in the placentas and whole blood of women with early-onset preeclampsia. Placenta 2016;48:72-79. (In eng). DOI: 10.1016/j.placenta.2016.10.008.
329. Reed MJ, Purohit A, Woo LW, Newman SP, Potter BV. Steroid sulfatase: molecular biology, regulation, and inhibition. Endocr Rev 2005;26(2):171-202. (In eng). DOI: 10.1210/er.2004-0003.
330. Rizk DE, Johansen KA. Placental sulfatase deficiency and congenital ichthyosis with intrauterine fetal death: case report. Am J Obstet Gynecol 1993;168(2):570-1. (In eng). DOI: 10.1016/0002-9378(93)90495-5.
331. Keren DF, Canick JA, Johnson MZ, Schaldenbrand JD, Haning RV, Jr., Hackett R. Low maternal serum unconjugated estriol during prenatal screening as an indication of placental steroid sulfatase deficiency and X-linked ichthyosis. Am J Clin Pathol 1995;103(4):400-3. (In eng). DOI: 10.1093/ajcp/103.4.400.
332. Raunig JM, Yamauchi Y, Ward MA, Collier AC. Assisted reproduction technologies alter steroid delivery to the mouse fetus during pregnancy. J Steroid Biochem Mol Biol 2011;126(1-2):26-34. (In eng). DOI: 10.1016/j.jsbmb.2010.12.012.
333. Sugawara A, Sato B, Bal E, Collier AC, Ward MA. Blastomere removal from cleavage-stage mouse embryos alters steroid metabolism during pregnancy. Biology of reproduction 2012;87(1):4-9. (In eng). DOI: 10.1095/biolreprod.111.097444.

334. Koren L, Ng ES, Soma KK, Wynne-Edwards KE. Sample preparation and liquid chromatography-tandem mass spectrometry for multiple steroids in mammalian and avian circulation. PLoS One 2012;7(2):e32496. (In eng). DOI: 10.1371/journal.pone.0032496.
335. Jalabert C, Ma C, Soma KK. Profiling of systemic and brain steroids in male songbirds: Seasonal changes in neurosteroids. J Neuroendocrinol 2021;33(1):e12922. (In eng). DOI: 10.1111/jne.12922.
336. Carter P. Preparation of ligand-free human serum for radioimmunoassay by adsorption on activated charcoal. Clin Chem 1978;24(2):362-4. (In eng).
337. Soldin OP, Guo T, Weiderpass E, Tractenberg RE, Hilakivi-Clarke L, Soldin SJ. Steroid hormone levels in pregnancy and 1 year postpartum using isotope dilution tandem mass spectrometry. Fertil Steril 2005;84(3):701-10. (In eng). DOI: 10.1016/j.fertnstert.2005.02.045.
338. Kaludjerovic J, Ward WE. The Interplay between Estrogen and Fetal Adrenal Cortex. J Nutr Metab 2012;2012:837901-837901. (In eng). DOI: 10.1155/2012/837901.
339. Tomi M, Eguchi H, Ozaki M, et al. Role of OAT4 in Uptake of Estriol Precursor 16α-Hydroxydehydroepiandrosterone Sulfate Into Human Placental Syncytiotrophoblasts From Fetus. Endocrinology 2015;156(7):2704-12. (In eng). DOI: 10.1210/en.2015-1130.
340. Schweigmann H, Sánchez-Guijo A, Ugele B, et al. Transport of the placental estriol precursor 16α-hydroxy-dehydroepiandrosterone sulfate (16α-OH-DHEAS) by stably transfected OAT4-, SOAT-, and NTCP-HEK293 cells. J Steroid Biochem Mol Biol 2014;143:259-265. DOI: https://doi.org/10.1016/j.jsbmb.2014.03.013.
341. Siiteri PK, MacDonald PC. The utilization of circulating dehydroisoandrosterone sulfate for estrogen synthesis during human pregnancy. Steroids 1963;2(6):713-730. DOI: https://doi.org/10.1016/0039-128X(63)90044-8.
342. Petrovic V, Wang J-H, Piquette-Miller M. Effect of Endotoxin on the Expression of Placental Drug Transporters and Glyburide Disposition in Pregnant Rats. Drug Metabolism and Disposition 2008;36(9):1944-1950. DOI: 10.1124/dmd.107.019851.
343. Petrovic V, Kojovic D, Cressman A, Piquette-Miller M. Maternal bacterial infections impact expression of drug transporters in human placenta. Int Immunopharmacol 2015;26(2):349-56. (In eng). DOI: 10.1016/j.intimp.2015.04.020.
344. Francois LN, Gorczyca L, Du J, et al. Down-regulation of the placental BCRP/ABCG2 transporter in response to hypoxia signaling. Placenta 2017;51:57-63. (In eng). DOI: 10.1016/j.placenta.2017.01.125.
345. Thomas HV, Murphy MF, Key TJ, Fentiman IS, Allen DS, Kinlen LJ. Pregnancy and menstrual hormone levels in mothers of twins compared to mothers of singletons. Ann Hum Biol 1998;25(1):69-75. (In eng). DOI: 10.1080/03014469800005432.
346. Houghton LC, Lauria M, Maas P, Stanczyk FZ, Hoover RN, Troisi R. Circulating maternal and umbilical cord steroid hormone and insulin-like growth factor concentrations in twin and singleton pregnancies. J Dev Orig Health Dis 2019;10(2):232-236. (In eng). DOI: 10.1017/S2040174418000697.
347. Póvoa A, Xavier P, Matias A, Blickstein I. First trimester β-hCG and estradiol levels in singleton and twin pregnancies after assisted reproduction. J Perinat Med 2018;46(8):853-856. (In eng). DOI: 10.1515/jpm-2017-0132.
348. Lofthouse EM, Cleal JK, O'Kelly IM, Sengers BG, Lewis RM. Estrone sulphate uptake by the microvillous membrane of placental syncytiotrophoblast is coupled to glutamate

efflux. Biochem Biophys Res Commun 2018;506(1):237-242. (In eng). DOI: 10.1016/j.bbrc.2018.10.074.
349. Isobe N, Nakao T, Uehara O, Yamashiro H, Kubota H. Plasma concentration of estrone sulfate during pregnancy in different breeds of Japanese beef cattle. J Reprod Dev 2003;49(5):369-74. (In eng). DOI: 10.1262/jrd.49.369.
350. Takahashi T, Hirako M, Takahashi H, Patel OV, Takenouchi N, Domeki I. Maternal plasma estrone sulfate profile during pregnancy in the cow; comparison between singleton and twin pregnancies. J Vet Med Sci 1997;59(4):287-8. (In eng). DOI: 10.1292/jvms.59.287.
351. Edmondson MA, Roberts JF, Baird AN, Bychawski S, Pugh DG. Chapter 8 - Theriogenology of Sheep and Goats. In: Pugh DG, Baird AN, eds. Sheep and Goat Medicine (Second Edition). Saint Louis: W.B. Saunders; 2012:150-230.
352. Kazihnitková H, Tejkalová H, Benešová O, Bičíková M, Hill M, Hampl R. Simultaneous determination of dehydroepiandrosterone, its 7-hydroxylated metabolites, and their sulfates in rat brain tissues. Steroids 2004;69(10):667-674. DOI: https://doi.org/10.1016/j.steroids.2004.06.002.
353. Szymczak J, Milewicz A, Thijssen JHH, Blankenstein MA, Daroszewski J. Concentration of Sex Steroids in Adipose Tissue after Menopause. Steroids 1998;63(5):319-321. DOI: https://doi.org/10.1016/S0039-128X(98)00019-1.
354. Bauer AZ, Swan SH, Kriebel D, et al. Paracetamol use during pregnancy — a call for precautionary action. Nature Reviews Endocrinology 2021. DOI: 10.1038/s41574-021-00553-7.
355. Graham GG, Davies MJ, Day RO, Mohamudally A, Scott KF. The modern pharmacology of paracetamol: therapeutic actions, mechanism of action, metabolism, toxicity and recent pharmacological findings. Inflammopharmacology 2013;21(3):201-32. (In eng). DOI: 10.1007/s10787-013-0172-x.
356. Amateau SK, McCarthy MM. Induction of PGE2 by estradiol mediates developmental masculinization of sex behavior. Nat Neurosci 2004;7(6):643-50. (In eng). DOI: 10.1038/nn1254.
357. Lim H, Paria BC, Das SK, et al. Multiple female reproductive failures in cyclooxygenase 2-deficient mice. Cell 1997;91(2):197-208. (In eng). DOI: 10.1016/s0092-8674(00)80402-x.
358. Simpson S, Kaislasuo J, Peng G, et al. Peri-implantation cytokine profile differs between singleton and twin IVF pregnancies. Am J Reprod Immunol 2021;85(3):e13348. (In eng). DOI: 10.1111/aji.13348.
359. Li H, Shen T, Sun X. Monozygotic dichorionic-diamniotic pregnancies following single frozen-thawed blastocyst transfer: a retrospective case series. BMC Pregnancy and Childbirth 2020;20(1):768. DOI: 10.1186/s12884-020-03450-5.
360. Ikemoto Y, Kuroda K, Ochiai A, et al. Prevalence and risk factors of zygotic splitting after 937 848 single embryo transfer cycles. Hum Reprod 2018;33(11):1984-1991. (In eng). DOI: 10.1093/humrep/dey294.
361. Dolan SM, Christiaens I. Genome-wide association studies in preterm birth: implications for the practicing obstetrician-gynaecologist. BMC pregnancy and childbirth 2013;13 Suppl 1(Suppl 1):S4-S4. (In eng). DOI: 10.1186/1471-2393-13-S1-S4.

362. Knijnenburg TA, Vockley JG, Chambwe N, et al. Genomic and molecular characterization of preterm birth. Proceedings of the National Academy of Sciences 2019;116(12):5819. DOI: 10.1073/pnas.1716314116.
363. Gray KJ, Saxena R, Karumanchi SA. Genetic predisposition to preeclampsia is conferred by fetal DNA variants near FLT1, a gene involved in the regulation of angiogenesis. American journal of obstetrics and gynecology 2018;218(2):211-218. (In eng). DOI: 10.1016/j.ajog.2017.11.562.
364. Lowe WL, Jr., Scholtens DM, Sandler V, Hayes MG. Genetics of Gestational Diabetes Mellitus and Maternal Metabolism. Curr Diab Rep 2016;16(2):15. (In eng). DOI: 10.1007/s11892-015-0709-z.
365. van Dongen J, Gordon SD, McRae AF, et al. Identical twins carry a persistent epigenetic signature of early genome programming. Nature Communications 2021;12(1):5618. DOI: 10.1038/s41467-021-25583-7.