# INCREASED OXIDATIVE DAMAGE AND PREMATURE PLACENTAL AGING CONTRIBUTE TO THE AETIOLOGY OF STILLBIRTH

by

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A thesis submitted in fulfilment of the requirements for the degree of Doctor of Philosophy in Reproductive Medicine

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> > August, 2018

### **Statement of Originality**

I hereby certify that the work embodied in the thesis is my own work, conducted under normal supervision. This thesis contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text. I give consent to the final version of my thesis being made available worldwide for loan and photocopying when deposited in the University's Digital Repository, subject to the provisions of the Copyright ACT 1968.

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I hereby certify that the work embodied in this thesis contains published papers/scholarly work of which I am a joint author. I have included as part of the thesis a written declaration, endorsed by my supervisor, attesting to my contribution to the joint publications/scholarly work.

#### Candidate:

Date: 01/08/18

By signing below, I confirm that Zakia Sultana has made a primary and original contribution to the publications, and manuscripts submitted and in preparation for publication, included in this thesis as detailed below.

Supervisor:

Date: 3/8/2018

Chapter	Title	Status	Contribution
2	Oxidative stress, placental	Published	Conception and designing of the
	ageing-related pathologies		review, research to find the
	and adverse pregnancy		publications referred to, manuscript
	outcomes		writing, designing and preparation
			of the figures.

3	Is there a role for placental	Published	Conception and designing of the
	senescence in the genesis of		review, research to find the
	obstetrical complications		publications referred to, manuscript
	and fetal growth restriction?		writing and designing and
			preparation of the figures.
5	Evidence that fetal death is	Published	Designing and executing in vitro
	associated with placental		experiments, data analysis, result
	aging		interpretation, preparation of the
			figures and manuscript writing.
6	Effect of serum-starvation	Prepared for	Designing and performing
	on lipid peroxidation and	publication	experiments, data analysis, result
	expression of sirtuins in		interpretation, preparation of the
	human placental explants:		figures and manuscript writing.
	implication for aldehyde		
	oxidase 1 and G-protein		
	coupled estrogen receptor 1		
	in placental oxidative		
	damage and aging		
7	Growth factor depletion in	Prepared for	Designing and executing
	placental trophoblast cells	publication	experiments, data analysis, result
	increases lipid peroxidation,		interpretation, preparation of the
	reduces mTORC1 activity		figures and manuscript writing.
	and alters mitochondrial		
	function via aldehyde		
	oxidase and GPER1		
	mediated pathways		

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### **Publication List**

#### **Journal Publications (full text)**

The work presented in this thesis has directly resulted in the following publications:

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- Maiti K, Sultana Z, Aitken RJ, Morris J, Park F, Andrew B, Riley SC, Smith R. Evidence that fetal death is associated with placental aging. American Journal of Obstetrics and Gynecology, 2017;217: 441.e1-14.
- Sultana Z, Maiti K, Aitken J, Morris J, Dedman L, Smith R. Oxidative stress, placental ageing related pathologies, and adverse pregnancy outcomes. American Journal of Reproductive Immunology, 2017;77:e12653.

#### **Abstract Publications**

The work presented in this thesis has directly resulted in the following abstract publications as part of conference presentations:

- 1. **Sultana Z**, Maiti K, Smith R. Use of an in vitro human placental explant culture model to interrogate the effects of starvation on placental function. Placenta, 2015;36:A49.
- Maiti K, Sultana Z, Aitken J, Smith R. The human placenta at 41 weeks of gestation shows evidence of aging with shortened telomeres, DNA oxidation and changes in IGFR2, autophagy and mTOR. Placenta, 2015;36:A49.

#### **Conference Presentations**

The work presented in this thesis has directly resulted in the following conference presentations:

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- Maiti K, Sultana Z, Aitken J, Smith R. Unexplained Antepartum Stillbirth Is Associated with Biochemical Evidence of Placental Aging. Reproductive Sciences, 2017;24:216A. Poster presentation delivered at the 64th Annual Scientific Meeting, Society for Reproductive Investigation, March 15-18, 2017, Orlando, Florida.
- 3. Maiti K, **Sultana Z**, Aitken J, Smith R. Evidence That Stillbirth Is Linked to Placental Aging. Reproductive Sciences, 2016;23:76A. Poster presentation delivered at the 63rd Annual Scientific Meeting, Society for Reproductive Investigation, March 16-19, 2016, Montreal, Canada.
- Sultana Z, Maiti K, Smith R. Use of an in vitro human placental explant culture model to interrogate the effects of starvation on placental function. Placenta, 2015;36:A49. Poster presentation delivered at the International Federation of Placenta Association (IFPA), September 8-11, 2015, Brisbane, Australia.
- Maiti K, Sultana Z, Aitken J, Smith R, The human placenta at 41 weeks of gestation shows evidence of aging with shortened telomeres, DNA oxidation and changes in IGFR2, autophagy and mTOR. Placenta, 2015;36:A49. Poster presentation delivered

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#### **Manuscripts Prepared for Publication**

- Sultana Z et al. Effect of serum-starvation on lipid peroxidation and expression of sirtuins in human placental explants: implication for aldehyde oxidase 1 and G-protein coupled estrogen receptor 1 in placental oxidative damage and aging.
- 2. **Sultana Z** et al. Growth factor depletion in placental trophoblast cells increases lipid peroxidation, reduces mTORC1 activity and alters mitochondrial function via aldehyde oxidase and GPER1 mediated pathways.

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## List of Abbreviations

#### General

•OH	hydroxyl radicals
4E-BPs	eukaryotic translation initiation factor 4E-binding proteins
4HNE	4-hydroxynonenal
8OHdG	8-hydroxydeoxy-guanosine
AChE	acetylcholinesterase
ADP	5'-adenosine di-phosphate
AMP	5'-adenosine mono-phosphate
АМРК	5' AMP-activated protein kinase
AOX1	aldehyde oxidase 1
ATP	5'-adenosine tri-phosphate
AUC	area under the curve
BCA	bicinchoninic acid assay
BMI	body-mass index
BSA	bovine serum albumin
Ca <sup>2+</sup>	Calcium ion
cAMP	cyclic 3',5'-adenosine monophosphate
CAT	catalase
CDK	cyclin-dependent kinases
cDNA	complimentary deoxyribonucleic acid

Ct	cycle threshold
DAPI	4,6-diamidino-2-phenylindole
DMEM	Dulbecco's modified eagle's medium
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleoside 5'-triphosphates
ECAR	extra-cellular acidification rate
ECM	extracellular matrix
EDTA	ethylene diamine tetraacetic acid
ELISA	enzyme linked immunosorbent assay
ETC	electron transport chain
FADH	flavin adenine dinucleotide
FBS	fetal bovine serum
FCCP	carbonyl cyanide-4 (tri-fluoromethoxy) phenylhydrazone
FGR	fetal growth restriction
FOXO	forkhead box class O
GPER1	G-protein coupled estrogen receptor 1
GSH-Ps	glutathione peroxidases
GSH-T	glutathione s-transferase
$H_2O_2$	hydrogen peroxide
HRP	horseradish peroxidase
ICD	international classification of disease
ICM	inner cell mass
IGF	insulin-like growth factor

IgG	immunoglobulin G
IHC	immunohistochemistry
IL	interleukin
IUGR	intrauterine growth restriction
LAMP2	lysosomal activated membrane protein 2
LC3B	microtubule-associated proteins 1A/1B light chain 3 B
LDS	lithium dodecyl sulphate
МАРК	mitogen activated protein kinase
MDA	malondialdehyde
mRNA	messenger ribonucleic acid
mtDNA	mitochondrial DNA
mTOR	mammalian/mechanistic target of rapamycin
mTORC1/mTORC2	mTOR complex 1/complex 2
NADH/NAD <sup>+</sup>	nicotinamide adenine dinucleotide
NADPH	nicotinamide adenine dinucleotide phosphate
$O_2^-$	superoxide anion radical
OCR	oxygen consumption rate
OD	optical density
OS	oxidative stress
p70S6K	phosphoprotein 70 ribosomal protein S6 Kinase
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PCR	polymerase chain reaction

РІЗК	phosphoinositide 3-kinase
pPROM	preterm premature rupture of membranes
pRB	retinoblastoma tumor suppressor protein
PVDF	polyvinylidene fluoride
RAS	renin-angiotensin system
RNA	ribonucleic acid
ROS	reactive oxygen species
RT	reverse transcription
RT	room temperature
RT-qPCR	real-time quantitative polymerase chain reaction
SAHF	senescence-associated heterochromatin foci
SASP	senescence-associated secretory phenotype
SA-β-gal	senescence-associated $\beta$ -galactosidase
SDS	sodium dodecyl sulfate
SEM	standard error of the mean
Ser	serine
SGA	small for gestational age
SIDS	sudden infant death syndrome
Sir2	silent information regulator 2
SIRT	sirtuins
SOD	superoxide dismutase
SQSTM1	sequestosome 1
STBMs	syncytiotrophoblast microparticles

TAE	tris-acetate EDTA
TBS	tris-buffered saline
TBST	tris-buffered saline with 0.1% tween-20
TCA	tri-carboxylic acid
TERC	telomerase RNA component
TERT	telomerase reverse transcriptase
Thr	threonine
Tris	trisaminomethane (2-amino-2-hydroxymethyl-propane-1,3-diol)
TSC1/2	tuberous sclerosis complex 1/2
ULK1	Unc-51 like autophagy activating kinase 1
WHO	world health organization
XF	extracellular flux
XO	xanthine oxidase

#### Units

%	percent
°C	degrees Celsius
g	grams
hrs	hours
kDa	kilo Dalton
L	litre
М	molar
mg	milligrams

min	minutes
mL	millilitres
mm	millimitres
mM	millimolar
nM	nanomolar
sec	seconds
V	volts
U	unit
μg	micrograms
μm	micromitres
μΜ	micromolar
g	gravity

#### Prefixes

m	milli (10 <sup>-3</sup> )
μ	micro (10 <sup>-6</sup> )
n	nano (10 <sup>-9</sup> )
р	pico (10 <sup>-12</sup> )

### Symbol

α	alpha
β	beta
Δ	delta

### Abstract

Stillbirth is a neglected public health problem affecting more than two million women and families globally each year with devastating and long-lasting psychosocial and financial impact. Rates of stillbirth, even in high-income countries with access to optimal obstetric care, have remained static in the past two decades. The causes of, or associations with, stillbirth that have been identified clinically include fetal factors such as genetic/structural abnormalities and growth restriction, maternal factors such as preeclampsia and infections and placental factors such as abruption and placenta previa. However, no specific cause has been established for the majority of stillbirths at term, and the rate of this category of death rises drammatically as gestation progresses beyond 38 weeks. Taking into account the functional definition of aging that is an increase in the risk of death with time, and the existence of placental pathologies in the unexplained stillbirth pregnancies resembling aging in other organs, we hypothesise that premature placental aging may be the primary factor in the aetiology of unexplained stillbirth. Premature aging may occur when cells experience increased oxidative stress that causes damage to cellular macromolecules, including DNA, RNA and lipids, and alters protein expression patterns, especially those that are crucial for cellular survival and function.

Therefore, the primary aim of this thesis was to investigate evidence that the placenta from late-gestation shows biochemical signs of oxidative damage and aging that would also be present in placentas associated with stillbirths. A further aim was to investigate the pathways that mediate the oxidative damage and aging in the placenta in pathologic pregnancies. We have shown that placentas from both late-term and stillbirth pregnancies show biochemical signs of aging in the form of increased DNA and lipid oxidation. Also, the expression of aldehyde oxidase 1 (AOX1), which is known to be involved in reactive oxygen species (ROS) generation and oxidative stress, is increased in placental tissues obtained from both late-gestation and stillbirth pregnancies. We tested the association of AOX1 in stillbirth pregnancy as an RNA sequencing study performed in our laboratory identified a significant increase in AOX1 mRNA in late-term placentas compared to term healthy placentas (unpublished). The demonstration of G-protein coupled estrogen receptor 1 (GPER1), a cell surface estrogen receptor, localisation on the apical surface of the normal placental syncytiotrophoblast and its role in the reduction of ROS generation and oxidative damage indicate that this receptor may be a critical step in the pathway of placental ROS induced oxidative damage.

Using a placental explant and a cell line culture model, we then tested the pathways that regulate placental oxidative damage and aging. Results presented in this thesis revealed that growth factor removal resulted in placental oxidative damage, with impaired mitochondrial function, decreased expression of sirtuins (proteins that control aging), alteration of nutrient sensing mammalianTORC1, and energy sensing AMP activated protein kinase pathways, all the changes are known to be associated with oxidative damage and aging in other tissues. Inhibition of AOX1 or stimulation of estrogen activation at GPER1 resulted in the blocking of all the changes observed after removal of growth factors. Together, these findings support the hypothesis that placental oxidation is regulated by estrogen activation at the GPER1 and inhibition of AOX1 leading to the inhibition of ROS generation and oxidative stress. Our study identifies potential biomarkers of oxidative damage and aging in stillbirth placentas that raise the possibility that these biomarkers of placental oxidative damage and aging may

be released into the maternal blood where they may have diagnostic value in predicting the fetus at risk for stillbirth. Treatment targeting AOX1 and/or GPER1 may arrest the oxidative damage in the placenta in pregnancies identified at risk and may lead to novel therapeutic strategies for delaying placental aging, as well as preventing stillbirth and other age-related adverse pregnancy outcomes.

Introduction

#### 1.1 Background

Stillbirth is an unaddressed global public health problem [1] with virtually no reduction in these deaths in more than 20 years [2]. Stillbirth is devastating for families who suffer longlasting psychosocial and financial impact [3]. In 2015, approximately 2.6 million babies were stillborn worldwide: almost all of them (98%) occurred in developing countries [4], with an estimated rate of stillbirth is 18.4 per 1000 total birth (worldwide), while in developed countries the rate is 3.4 per 1000 total birth [4] (Table 1.1). Although rates for developed countries are relatively lower than the developing and underdeveloped countries, stillbirth is a major health burden in developed countries, occurring in approximately 1 in 200 pregnancies and contribute substantially to perinatal mortality [5]. In high-income countries, stillbirth accounts for 60% of all perinatal deaths and 75% of all potentially preventable losses (defined as the perinatal death of a normally formed infant weighing 1000 g or more) [6-8]. Globally, stillbirth is ten times more common than Sudden Infant Death Syndrome (SIDS) and is nearly five times more common than infant deaths related to congenital anomalies [9].

Many improvements have been made in developed countries in maternal and neonatal outcomes over the last 50 years. Globally, maternal mortality has fallen from 422 per 100,000 pregnancies in 1980 to 251 in 2008 [10], and neonatal mortality has dropped from 33 per 1,000 live births in 1990 to 20 in 2013 [11]. However, the rates of stillbirth have not changed significantly in the last 20 years in many high-income countries including Australia (Figure 1.1) (Figure adapted from Flenady et al. [5]) and have increased in some regions [12]. In

Australia, stillbirth occurs in one in 137 women who reach 20 weeks gestation, and accounts for around two-thirds of all perinatal deaths at a rate of 7.4 per 1000 births [13], making it nearly 40 times more common than SIDS [14]. Figure 1.2 depicts the trends in stillbirth, neonatal and perinatal mortality rates and Figure 1.3 shows the trends in stillbirth rates by gestational ages in Australia from 1991 to 2009 (Figures adapted from Li et al. [13]).
	2000		2015		Annual
	Stillbirth rates per 1000 deliveries (uncertainty range)	Number of stillbirths (uncertainty range)	Stillbirth rates per 1000 deliveries (uncertainty range)	Number of stillbirths (uncertainty range)	rate of reduction in stillbirth rate 2000- 2015
Worldwide	24.7 (22.4-28.4)	3250 000 (2931 000-3740 000)	18.4 (16.6-21.0)	2620 000 (2359 000-2984 000)	2.0
Developed Countries	4.5 (4.4-4.6)	59 000 (58 000-61 000)	3.4 (3.4-3.5)	47 000 (46 000-48 000)	1.8
Southern Asia	35.5 (31.3-41.2)	1443 000 (1266 000-1684 000)	25.5 (22.5-29.1)	967 000 (847 000-1104 000)	2.2
Caucasus and Central Asia	16.8 (13.9-23.6)	23 000 (19 000-33 000)	11.9 (9.8-15.6)	23 000 (19 000-31 000)	2.3
Eastern Asia	14.3 (10.6-19.6)	240 000 (177 000-331 000)	7.2 (5.6-9.7)	129 000 (100 000-175 000)	4.5
Latin America	11.3 (10.3-12.8)	135 000 (123000-153 000)	8.2 (7.5-9.2)	91 000 (83 000-103 000)	2.1
North America and Middle East	19.9 (17.7-23.6)	156 000 (139 000-185 000)	14.5 (12.9-17.5)	148 000 (131 000-180 000)	2.1
South-eastern Asia	17.0 (14.6-21.5)	194 000 (166 000-246 000)	12.2 (10.7-14.6)	155 000 (135 000-186 000)	2.2
Sub-Saharan Africa	24.7 (22.4-28.4)	1000 000 (879 000-1194 000)	28.7 (25.1-34.20)	1060 000 (923 000-1271 000)	1.4
Table reproduced from Blencowe et al. [4] with permission.					

# **Table 1.1**Estimated stillbirth rates and number of stillbirths for 2000 and 2015 by Blencowe et al. [4].



Figure 1.1 Stillbirth rates at 28 or more weeks of gestation in some selected developed countries between 1990 and 2009. Figure adapted from Flenady et al. [5].



Figure 1.2 Trends in stillbirth (gestational age 20 weeks or more), neonatal and perinatal mortality rates, Australia, 1991–2009. Note: (a) Stillbirth and perinatal mortality are presented per 1,000 total births, and neonatal mortality is presented per 1,000 live births. (b) Perinatal and neonatal mortality rates were not available for Australia in 2009. Figure adapted from Li et al. [13].



Figure 1.3 Trends in stillbirth rates by gestational ages in Australia from 1991 to 2009. Stillbirth for specified gestational ages is presented per 1,000 babies at risk of stillbirth, those that are not yet born at the beginning of each period of gestation, and total stillbirth rate is presented per 1,000 births (both live and dead). Figure adapted from Li et al. [13].

### **1.1.1 Definition of stillbirth**

The definition of stillbirth varies making comparisons across different countries difficult. World Health Organization (WHO) defines "stillbirth is death prior to the complete expulsion or extraction from its mother of a product of conception, irrespective of the duration of pregnancy; the death is indicated by the fact that after such separation the fetus does not breathe or show any other evidence of life, such as beating of the heart, pulsation of the umbilical cord or definite movement of voluntary muscles"[15]. The term 'fetal death' is used as synonymous with 'stillbirth', but fetal death is a broader term that also applies to the death of a fetus at earlier stages of pregnancy and does not recognise the birth. Stillbirth is, therefore, the preferred term [16].

The International Classification of Disease-10th revision (ICD-10), which was developed several decades ago when gestational age assessment was not standard, gives birthweight as the first preference in the definition of stillbirth, with gestational age second. According to the ICD-10, stillbirth is fetal death when birth weight is  $\geq$ 500 g or death at 22 completed weeks of gestation or more if birth weight is unknown, or body length is  $\geq$ 25 centimetres if neither birthweight nor gestational age is known [17]. For international comparison, WHO recommends stillbirths be defined according to the ICD-10 definition of late fetal death, i.e., fetal death when birth weight is  $\geq$ 1000 g or at gestational age is 28 weeks or more, or body length is  $\geq$ 35 centimetres [18].

However, variability in definitions occurs among developed countries; many high-income countries register stillbirth at earlier weeks of gestation, some as early as 16 weeks [19]. In Australia, stillbirth is defined as death of a fetus before or during birth at more than or equal to 20 weeks gestation or of more than or equal to 400 g birthweight (Perinatal Society of Australia and New Zealand Clinical Practice Guideline for Perinatal Mortality; Second Edition, version 2.2, April 2009) [20]. New Zealand defines stillbirths to include all fetal deaths from 20 weeks gestational age or 400 g birthweight, while in Canada, the definition of a stillbirth includes fetal deaths from 20 weeks gestational age or 500 g birthweight. The United States requires states to report fetal deaths of 20 or more weeks gestation, but the definition of a 'fetal death' specifically excludes deaths that result from induced termination of pregnancy, however the UK applies a lower limit of 24 weeks gestational age to define 'stillbirths', but includes all fetal deaths that meet this criterion [16]. As the birthweight and gestational age threshold do not give equivalent results, therefore only the gestational age threshold has been proposed to be the most appropriate [2].

### 1.1.2 Classification systems for perinatal deaths and stillbirth

As highlighted in the Lancet series on stillbirth, the classification system for stillbirth varies globally as do the terms used to define it, making the collation of data more difficult [5]. Stillbirth can be classified depending on whether death occurred before or after the onset of labour; (i) antepartum stillbirth – which is an intrauterine death before the onset of labour or in which there is no evidence of life during labour, and (ii) intrapartum stillbirth – in which the fetus dies during labour or within a short period following live birth. In most developed

countries, intrapartum stillbirth rates are typically low, whereas antepartum stillbirth is relatively more common, and the aetiology remains unexplained in the majority of cases [21]. Stillbirth can be sub-classified according to the gestational age at birth; early stillbirths (20-28 weeks) and late stillbirths (28 weeks or more) [7]. This classification system is useful for international comparison and also allows stillbirths to be divided into those that are difficult to prevent, i.e., early losses and those that are potentially preventable, i.e., late losses. The primary method for classification of stillbirth is based on the underlying causes or associated obstetric disorders [7]. Panel 1.1 shows a classification system based on obstetric conditions that focused on eight major groups [8].

Panel 1.1: Classification of fetal deaths by obstetric causes (modified version of the				
Wigglesworth system)				
Congenital anomalies*				
1. Central nervous system				
2. Cardiovascular system				
3. Renal				
4. Alimentary (excluding diaphragmatic hernia)				
5. Chromosomal				
6. Biochemical				
7. Others (including musculoskeletal)				
Isoimmunisation				
8. Rhesus incompatibility				
9. Non-rhesus incompatibility				
Toxaemia				
10. Severe - diastolic blood pressure of $\geq 110$ mm Hg on two or more occasions and				
>20 weeks with proteinuria of ≥300 mg/24 h				
11 Other toxaemia				

# Antepartum haemorrhage†

- 12. Abruptio placentae
- 13. Placenta praevia
- 14. Other (with evidence of recurrent bleeding after the first trimester)

# Mechanical<sup>‡</sup>

- 15. Breech
- 16. Cord prolapse
- 17. Other mechanical

# Maternal disorder

- 18. Maternal trauma
- 19. Essential hypertension
- 20. Diabetes
- 21. Abdominal operations in pregnancy
- 22. Other (including maternal infection)

# Miscellaneous

23. (specify)

# Unexplained

- 24. Birthweight <2500 g and <37 weeks
- 25. Birthweight  $\geq$ 2500 g and <37 weeks
- 26. Birthweight <2500 g and  $\geq37$  weeks
- 27. Birthweight  $\geq$ 2500 g and  $\geq$ 37 weeks

\*Any structural or genetic defect incompatible with life or potentially treatable but causing death.

<sup>†</sup>In deaths with antepartum haemorrhage (APH) secondary to toxaemia, toxaemia is classified first and antepartum haemorrhage second.

‡Any death from uterine rupture, cord compression, birth trauma, or intrapartum asphyxia that is associated with disproportion, malpresentation, or breech delivery of babies weighing 1000 g or more. Deaths from anoxia or cerebral trauma should be classified as unexplained (codes 24–27) if there is no evidence of difficulty in labour. Antepartum deaths associated with cord entanglement in the absence of substantial circumstantial evidence that cord compression caused death (e.g., fetal death soon after external version) should be classified as unexplained (codes 24–27)).

### 1.1.3 Causes of stillbirths

The known causes or associations of stillbirths include congenital anomalies, fetal genetic/structural abnormalities, prematurity (preterm birth), fetal growth restriction, abruption associated with placental pathologies, maternal complications during pregnancy such as maternal diabetes, hypertension, preeclampsia, and infections [21,22]. However, the causes of stillbirth and attributable risk or importance vary in developed and developing countries (Panel 1.2). Placental pathologies are undoubtedly important, accounting for about 40% of stillbirths in systems designed to capture these pathologies, while the contribution of other important factors varies widely. Congenital abnormalities are noted in 6-27% of stillbirths, infection in 5-22% of stillbirths, and spontaneous preterm birth or preterm prelabour rupture of the membranes in 1-15% of stillbirths [23].

Among other causes of stillbirth, the small for gestational age (SGA, defined as fetal weight that is below the 10th percentile for gestational age, however, the definitions for SGA are highly variable among studies), accounts for 46 per 1000 compared to 4 per 1000 in the appropriate for gestational age fetus [24]. After 28 weeks gestation, the most common category of a stillbirth is 'unexplained' that have no known cause after complete pathologic evaluation, and the rate increases as gestational age advances [24]. A stillbirth that is unexplained by fetal, placental, maternal, or obstetric factors is the most frequent type of fetal demise, representing between 25% and 60% of all fetal deaths [25-29]. Stillbirths occurring antepartum and with an unexplained aetiology in the majority of cases are more common in

developed countries [21]. The estimated causes of stillbirth in Australia between 1990 and

2009 is shown in Figure 1.4 (data obtained from Hilder et al. [16]).

Panel 1.2: Commonly reported maternal risk factors for and causes of stillbirth in developing and developed countries

# **Developing countries**

- Asphyxia, infection and birth injury associated with obstructed or prolonged labour (low availability of caesarean-section)
- Congenitally acquired infections, especially syphilis, and gram-negative infections
- Hypertensive disease, preeclampsia and eclampsia
- Poor nutritional status
- Previous stillbirth
- Congenital anomalies
- Malaria
- Sickle-cell disease

# **Developed countries**

- Congenital anomalies
- Intrauterine growth restriction
- Placental thrombosis
- Diseases such as diabetes, systemic lupus erythematosus, renal disease, thyroid disorders, thrombophilia, cholestasis of pregnancy
- Hypertensive disease or preeclampsia
- Congenitally acquired infections such as Group B streptococcus and parvovirus B19
- Smoking
- Multiple gestation

Adapted from Smith and Fretts [7] and McClure et al. [30] with permission.





### 1.1.4 Risk factors for stillbirth

A number of risk factors for stillbirth have been identified including: ethnicity or demographic location, advanced maternal age (mothers aged >35 years), obesity (body-mass index, BMI  $\geq$ 25 kg/m<sup>2</sup>), smoking, hypertension, diabetes, multiple gestations, late gestational age and fetal growth restriction [14,26,27,31]. Studies have shown increased losses late in pregnancy, with the rate rising significantly after 37–39 weeks of gestation (Figure 1.5) [7,25-27,31]. Maternal age is a risk factor for fetal death with women aged 35 years or older

having a higher risk of fetal death compared to their younger counterparts [32]. Primiparity is another critical risk factor for stillbirth, women having their first birth face a greater risk of stillbirth as do women with multifetal pregnancies, contributing to about 15% of stillbirths in high-income countries [33]. Most stillbirths, even in high-income countries, in particular, those cases are classified as unexplained are frequently associated with fetal growth restriction (defined as a weight below the 10th percentile for the gestational age), around 52% of sudden unexplained stillbirths are growth restricted [33,34], suggesting that some unexplained fetal deaths could be avoided if fetal growth disturbances can be detected and interventions performed. However, most fetuses (75%) with intrauterine growth restriction are unrecognised until after birth, among them 85% of low-risk pregnancies [35].

Hypertension and diabetes are two of the most common medical conditions to complicate pregnancy, affecting 7–10% and 3–5% of women, respectively [36,37]. Population-based studies showed a two- to four-fold risk of stillbirth in women with diabetes [38,39]. Women with pre-pregnancy obesity and a BMI of greater than 25 have a higher risk of stillbirth [27]. Smoking is also associated with unexplained growth restricted stillbirth [40,41]. Studies have also highlighted the role of ethnicity and stillbirth, reporting that black and Asian women have a greater risk of stillbirth compared to white women even after adjusting for other risk factors [22,42,43]. In the Australian population, even after adjusting for potential confounding risk factors, mothers who were born in South Asia have an increased risk of antepartum stillbirth in late pregnancy compared with other women [44]. Social factors such as educational attainment, and access to and quality of care are all significantly associated with risk of stillbirth [7].



Figure 1.5 Relationship between gestational age and stillbirth in a Scottish cohort [31].

### 1.1.5 Stillbirth and the placenta

The placenta is essential for intrauterine life and therefore many of the broad categories of death, including preeclampsia, abruption and unexplained stillbirth, are thought to be associated with or caused by abnormalities of the placenta [45]. Placental pathology has been recognised as an important contributor in antepartum stillbirths, and particularly those that would have been considered as unexplained. Placental pathology (cause of death) has been associated with 11 to 65% of intrauterine fetal deaths, depending on the system used to

classify cause of death [46,47]. A high-quality research conducted in the United States reported that placental pathology is the leading cause (26%) of antepartum stillbirth [22]. A similar finding was also observed in a Swedish stillbirth cohort; reporting that 65% of the deaths can be attributed to placental insufficiency [48]. In a Dutch cohort of antepartum stillbirth, placental pathology was identified as the primary cause for 65% of stillbirth cases [49]. Abnormalities at a macroscopic, microscopic and/or molecular level within the placental villi, arteries, and veins or umbilical cord, can fatally disrupt blood and nutrient flow to the developing fetus resulting in fetal demise [45,50-54].

### **1.2** The importance of the placenta

The placenta is a highly specialised, extraordinary, but short-lived organ, developed during pregnancy, which allows the normal growth and development of the fetus and is discarded after birth. It typically weighs approximately 300–900 grams at term [55]. Placental growth and function are precisely regulated and coordinated to ensure the growth and development of the fetus. The main functions of the placenta are transportation of nutrients and metabolites, metabolism, protection from infection and endocrine influence on the mother. The placenta exchanges nutrients, e.g., amino acids, carbohydrates, lipids, vitamins, minerals, oxygen and other nutrients, and carbon dioxide and other waste products between the maternal and fetal circulatory systems [56]. Apart from transportation of nutrients, the syncytiotrophoblast secretes hormones into the maternal circulation and modulates the maternal immune response, allowing the fetal allograft to persist [57]. It also metabolises a number of substances and can release metabolic products into both fetal and maternal

circulations. The placenta can help to protect the fetus against certain xenobiotic molecules, infections and maternal diseases. Therefore, the function of this organ is crucial for a normal physiologic gestational process and a healthy baby as a final outcome. However, altered maternal nutrition and metabolism, restricted utero-placental blood flow and other perturbations in the feto-maternal compartment may disturb critical periods of fetal development. In response to these perturbations, placental structure and function changes, which influence the supply of nutrients and oxygen and alter the secretion of hormones and other signalling molecules into the fetal circulation. Thus, the placenta plays a critical role in modulating maternal-fetal resource allocation, thereby affecting fetal growth and the long-term health of the offspring.

### **1.2.1** Human placental development

In human embryonic development, the blastocyst is formed by 5–6 days after fertilization [58]. The blastocyst is composed of the trophectoderm layer and the inner cell mass (ICM). The blastocyst is surrounded by an extracellular matrix called the zona pellucida. The zona pellucida appears to be necessary at early cleavage stages of mammalian embryonic development to maintain the integrity of the ICM and to protect the ICM from the uterine environment [59]. However, it is shed or ruptures during blastocyst development by both physical expansion of the blastocyst due to increase in trophectoderm cell numbers and the action of proteolytic enzymes [60,61]. After the disappearance of the zona pellucida, the blastocyst makes contact with the endometrium and invades into the decidua of the endometrium approximately 6–7 days after fertilization [62]. The inner cell mass

differentiates into three cell layers: endodermal (inner), ectodermal (outer) and mesodermal (middle) layers. The endoderm and mesoderm cell layers are stretched between them forming the yolk sac and the amniotic sac, respectively [58]. Together the three cell layers give rise to the structures and organs of the body. Whereas the trophectoderm differentiates into trophoblast cells which form the chorion [58].

Immediately after attachment to the endometrium, the trophectoderm layer proliferates rapidly and differentiates into an inner layer of mononuclear cytotrophoblasts and a multinucleated outer epithelial layer known as the syncytiotrophoblast [56]. The syncytiotrophoblast is a terminally differentiated cell layer which is formed by the fusion of multiple cytotrophoblasts, a process called syncytialization. The combination of inner cytotrophoblasts and outer syncytiotrophoblast form finger-like structures called primary chorionic villi [56]. At the initial phase of differentiation, these villi are distributed symmetrically over the chorion. As gestation progresses the chorionic villi grow like branches of a tree (arborisation) and accumulate asymmetrically towards the uterine wall where the embryo is attached [56]. After the invasion of mesenchymal cells into the centre of the primary villi forming secondary villi, fetoplacental blood vessels arise inside the villi at the 5th week of gestation to form tertiary villi [63]. The placental vasculature system is essential for transferring nutrients, gases and hormones to the growing fetus. The proper branching of placental blood vessels (angiogenesis) is part of a successful pregnancy. Inadequate placental development, trophoblast invasion and vascular remodelling, as well as abnormal placental angiogenesis, have been reported in pathological pregnancies such as intrauterine growth restriction and preeclampsia [62,64-69].

In the first-trimester, the chorionic villi of the placenta are large, and the blood vessels in the villi are not prominent. In addition to villous trophoblast, an additional set of mononuclear trophoblasts, termed the extravillous trophoblast, grows outside the villi and extends into the decidualised endometrium [56,70]. During the first trimester of differentiation (up to 11–12 weeks) these extravillous trophoblasts erode into and plug the uterine spiral arteries and restrict the ability of the oxygenated maternal blood to access the placenta [71]. Consequently, the early stages of human embryonic development occur in an environment of low oxygen tension [72,73]. The hypoxic environment is thought to be necessary for the initial differentiation of the trophoblasts [74], in fact, miscarriage has been reported in the presence of the early arrival of oxygenated blood in the intervillous space [75].

As the placenta matures and increases in size in the second-trimester, the villi become smaller and more vascular. The syncytiotrophoblast cell layer draws up into 'syncytial knots' which are small clusters of nuclei, leaving a single syncytotrophoblast layer. Later the extravillous trophoblasts replace the endothelial layer covering the smooth muscle of the spiral arteries, rendering them flaccid and noncontractile, and are then called endovascular cytotrophoblasts [76]. The trophoblast plugs are gradually dislodged from the spiral arteries after 11–12 weeks of gestation, and maternal blood flows from the maternal spiral arteries into the intervillous spaces [72,73,77]. This process is associated with a sharp rise in oxygen tension, increased free radical generation and a burst of oxidative stress within the placental tissues. However, this oxidative stress returns to baseline upon a surge of antioxidant activity, as placental trophoblast cells gradually acclimate to the new oxidative surroundings [78]. The nutrients, gases and growth factors carried by maternal blood are readily taken up by the large surface of the syncytiotrophoblast allowing the fetus to grow in an oxygen and nutrient-rich environment.

A mature placenta in the third-trimester has small and highly vascularized chorionic villi to support the blood gas and nutrient exchange of the maternal-fetal circulation required by the growing fetus approaching term gestation. Syncytial knots are prominent in the third-trimester chorionic villi. Figure 1.6 illustrates the development of human placental chorionic villi at different stages of gestation.



# Figure 1.6 Development of human placental chorionic villi. Cross sections of (A) primary villi arborisation, (B) first-trimester, (C) second-trimester and (D) third-trimester villi. The chorionic villi in the first-trimester (B) are large, covered by two layers of cells, cytotrophoblasts and syncytiotrophoblasts and the blood vessels in the villi are not prominent. Second-trimester placental villi (C) are more vascular with a single cytotrophoblasts layer. The third-trimester (D) has highly vascularized chorionic villi, and syncytial knots are prominent. (Original artwork drawn by Lee Dedman, School of Creative Industries, University of Newcastle).

## 1.2.2 Placental shedding

Normal pregnancy is associated with the continuous release of trophoblast materials from the syncytiotrophoblast apical membrane into the maternal circulation. The syncytiotrophoblast that is formed by continuous fusion of cytotrophoblasts, when damaged or aged is extruded as membrane-enclosed multinucleated structures called syncytial nuclear aggregates or syncytial knots [79]. The extrusion of these materials into the maternal circulation counter balances the continuous input of cytotrophoblasts via syncytial fusion into the syncytiotrophoblast [80]. In 1893, Schmorl [81] first reported the presence of multinucleated fragments of the syncytiotrophoblast, referred to as 'Placentarzellen' or 'placental trophoblast cells', in the small pulmonary vessels of women who had died of eclampsia (reviewed in reference [82]).

In addition to syncytial knots, a large variety of other smaller syncytiotrophoblast derived material is released into the maternal blood and has been shown to be a feature of healthy pregnancy. As maternal blood only enters into the placenta after the first-trimester, it is not until about 12 weeks of gestation that the placental material appears in the maternal circulation. This material comprises syncytiotrophoblast cell fragments [83], syncytiotrophoblast microparticles (STBMs) [84,85], placenta-derived exosomes [86] and both cell-free DNA [87] and cell-free mRNA [88,89], all of which have been isolated from the plasma of healthy pregnant women and are significantly increased in preeclampsia. Aberrant appearance of the placental trophoblast material that arises from dysregulated differentiation and release of syncytiotrophoblast materials into the maternal circulation is accelerated in pregnancy-specific disorders including preeclampsia, new-onset hypertension, proteinuria and intrauterine growth retardation (IUGR) [90-94].

Moreover, there is substantial evidence that shedding of syncytiotrophoblast material into the maternal circulation contributes to the normal maternal physiological adaptation and immune response to pregnancy [91]. STBMs interact with both immune cells (particularly circulating neutrophils) and endothelial cells, thus modulating the maternal innate immune system [95] in a way that may contribute to the systemic inflammatory response of both normotensive and preeclamptic pregnancies [96]. Notably, placental exosomes in the maternal circulation suppress T-cell expansion [86,97]. Additionally, the circulating material of trophoblastic origin can be used to diagnose fetal genetic or cytogenetic abnormalities, as they contain the fetal genome, without the need for invasive procedures such as amniocentesis and chorionic villus sampling [79].

### **1.3** Apoptosis and its role in trophoblasts function

Apoptosis, or programmed cell death, is crucial to the development and homeostasis of all multi-cellular organisms and for many organs including the placenta. Apoptosis is known to occur in a number of biologic processes, both physiologic and pathologic. Trophoblast apoptosis is a physiologic event in healthy pregnancy, increases with advancing gestational age and is higher in post-term pregnancies, and therefore, is considered as a normal process in the development and aging of the placenta [98,99]. Apoptosis is proposed to occur as a regular event during the formation of the villous trophoblast bi-layer and syncytiotrophoblast formation from cytotrophoblasts (trophoblast differentiation) [100]. However, it is likely that placental insults can alter the regulation of apoptosis in the trophoblasts, possibly by modulating trophoblast cell turnover [100]. Cultured trophoblasts exposed to hypoxia show marked up-regulation of activity of tumour suppressor protein p53, enhanced expression of the pro-apoptotic Mtd-1 and decreased expression of the anti-apoptotic Bcl-2, all of which promote apoptosis [101-103], and the apoptosis is more marked in hypoxia/re-oxygenation [104]. Additionally, an up-regulated p53 and decreased Bcl-2 mediated increased apoptosis in placental syncytiotrophoblast is associated with some pregnancy pathologies, including IUGR and preeclampsia [94,105]. Syncytial knots, a characteristic feature of syncytiotrophoblast apoptosis, increase in placentas associated with preeclampsia and IUGR [90, 105]. In contrast, apoptosis decreases in extravillous trophoblasts in pregnancies complicated by preeclampsia and is associated with reduced trophoblast invasion [106]. Thus, apoptosis is differently regulated in villous and extravillous trophoblasts in normal placental development.

### **1.4** The role of autophagy in normal placentation

Autophagy is a dynamic, catabolic process by which cytoplasmic components such as macromolecules (e.g., damaged or long-lived proteins), injured organelles and invading organisms are delivered to lysosomes for degradation by lysosomal hydrolases [107]. The autophagic process is increased under certain environmental stresses such as starvation, growth factor depletion, hypoxia, endoplasmic reticulum stress, microbial infection and diseases that involve accumulation of protein aggregates [108,109]. This process involves sequestration of intracellular components into double-membrane vesicles called autophagosomes. Autophagosomes are then fused with lysosomes to form single-membrane autophagolysosomes or autolysosomes. Autolysosomes permit the degradation and recycling of sequestered components by lysosomal hydrolases to generate energy and to provide building blocks (such as, nucleotides, amino acids, free fatty acids) for new macromolecule synthesis and organelle biogenesis [110]. These autophagic functions are necessary for cellular homeostasis and cell survival during aging, tissue remodelling, growth control, adaptation to adverse environments, cellular immunity and neurodegenerative processes [111]. A schematic of the autophagic process is illustrated in Figure 1.7.



Figure 1.7 Schematic of autophagic progression. The autophagic process is initiated by appropriate autophagy induction signals to generate a sequestering membrane called the isolation membrane. Microtubule-associated proteins 1A/1B light chain 3 (LC3), a protein then conjugates to the membrane and controls the elongation of the membrane to form a phagophore. Cytoplasmic components, including misfolded proteins, damaged organelles such as mitochondria and endoplasmic reticulum are enclosed, as the phagophore expands, resulting in the formation of autophagosomes. The autophagosome then fuses with the lysosome to form an autolysosome to allow the degradation of the contents by lysosomal hydrolases. Entrapped cytoplasmic components including the inner membrane of the autophagosome and LC3 are then degraded, and LC3 from outer membrane and autophagy derived nutrients are recycled [112].

In mammals, autophagy has been reported to be crucial for pre-implantation development and in the regulation of fetal growth and survival [110,113]. In the human placenta, autophagic function seems to be increased under physiologic hypoxia in early placental tissue [114] and in pregnancies complicated by IUGR [115] and preeclampsia [116]. Hypoxiainduced autophagy enhances the invasion and vascular remodelling capacity of extravillous trophoblast cells up to 12 weeks of gestation. Disturbed activation of hypoxia-induced autophagy at this gestational stage can lead to abnormal placental development and preeclampsia [117]. Deficient invasion of the endometrium by extravillous trophoblasts, followed by incomplete conversion of the spiral arteries during the first-trimester of pregnancy are the most widely recognised predisposing factors for IUGR and preeclampsia [118]. As trophoblastic invasion and vascular remodelling are essential for maternal blood supply into the placenta, so autophagy plays an important role in extravillous trophoblast function under physiologic hypoxia. On the other hand, increased activation of autophagy induced by hypoxia in villous trophoblast cells is associated with an increased risk of IUGR and preeclampsia [115,116].

Additionally, sequestosome 1 (SQSTM1), also known as p62, a protein that interacts with polyubiquitinated proteins, may direct proteins to the autophagosome where the SQSTM1protein complex is selectively digested by autophagy [119]. Decreased levels of SQSTM1 in extravillous trophoblast suggests activation of autophagy, whereas increased levels of SQSTM1 reflects inhibition of autophagy [117]. Accumulation of SQSTM1 in extravillous trophoblasts is observed in preeclamptic placentas, showing the disruption of autophagy in extravillous trophoblast cells in preeclampsia, whereas a decline in expression of SQSTM1 in villous trophoblasts in preeclamptic placentas is consistent with an activation of autophagy in syncytiotrophoblasts in preeclampsia [117]. These two observations indicate that autophagy has different levels of activity in syncytiotrophoblasts and extravillous trophoblasts in preeclamptic placentas. These results may indicate a difference in susceptibility to hypoxia-dependent autophagic activation, that is, hypoxia induced-autophagy activation maintains cellular homeostasis in syncytiotrophoblasts in preeclamptic placentas, and the disruption of autophagy in extravillous trophoblasts might contribute to the hypoplastic placentation observed in this condition.

### **1.5** The mTOR and the placenta

### **1.5.1** The mTOR signalling pathway

Mammalian target of rapamycin (mTOR), also known as mechanistic TOR, is a master regulator of protein translation, thereby controlling the signalling network of cell growth, metabolism, proliferation and cell survival in response to a large number of upstream regulators, including growth factors, nutrients, oxygen, and energy within the cell [120,121]. This pathway is also a major regulator of many pathological conditions including cancer, obesity, type 2 diabetes, cardiovascular and neurodegenerative disorders [122].

mTOR is a conserved serine/threonine protein kinase that belongs to the phospho-inositide 3-kinase (PI3K) family. mTOR exists as two distinct multi-protein complexes: mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2) [123]. mTORC1 activity is highly sensitive to inhibition by rapamycin, whereas mTORC2 is insensitive to acute treatment with rapamycin, although long-term rapamycin treatment can indirectly inhibit mTORC2 in some cell types [124]. mTORC1 comprises the catalytic subunit of the complex, mTOR, and four associated proteins: regulatory-associated protein of mTOR (Raptor), mammalian lethal with Sec13 protein 8 (mLST8, also known as GβL), 40 kDa proline-rich AKT substrate (PRAS40; also known as AKT1s1) and DEP-domain-containing mTOR-interacting protein (Deptor) [125]. In addition to mTOR, mLST8 and Deptor, mTORC2 also consists of rapamycininsensitive companion of mTOR (Rictor), mammalian stress-activated protein kinase interacting protein (mSin1) and protein observed with Rictor-1 (Protor-1) [120]. mTORC1 activity is regulated by a range of factors including growth factors, nutrients, and stressors, such as DNA damage, whereas mTORC2 is regulated only by growth factors, such as insulin [126].

When activated, mTORC1 regulates many processes involved in anabolism including mRNA translation, protein synthesis and organelle biogenesis. mTOR activity promotes cell growth, metabolism, and proliferation by phosphorylating effector proteins and/or by limiting catabolic processes [120]. This complex also induces lipid biogenesis, affects glucose metabolism and mitochondrial function [127], and inhibits self-digestion by blocking autophagy [128]. Growth factors regulate mTORC1 activity by integrating multiple inputs such as PI3K, Akt, Tuberous Sclerosis Complex 1 and 2 (TSC1 and TSC2), a group of small

GTPases the Rag GTPases and a small GTP-binding protein Rheb (Ras homology enriched in brain) [129]. Growth factors trigger activation of a series of upstream kinases, which integrate to directly phosphorylate the TSC1-TSC2 complex, Rag GTPases and Rheb. The GTP-binding form of Rag-Ragulator (LAMTOR1-3) complex enhances translocation of mTORC1 to the surface of lysosomes, whereas GTP-bound Rheb is located on the lysosome surface where it binds to and activates mTORC1 [128,130]. Whereas nutrients, mainly amino acids, stimulate mTORC1 by modulating lysosomal translocation of mTORC1 by the v-ATPase-Rag GTPase-Ragulator complex [130]. The non-phosphorylated form of the TSC1-TSC2 complex is active and negatively regulates Rheb, thus reducing mTORC1 activity. Many growth factors phosphorylate and inactivate TSC1/TSC2 via Akt and mitogenactivated protein kinase 1 (MAPK1, also known as extracellular signal-regulated kinase 1/2 (ERK1/2) kinases), resulting in activation of the Rheb-mTORC1 pathway [126]. On the other hand, in response to energy depletion, 5' adenosine monophosphate (AMP) activated protein kinase (AMPK) inhibits mTORC1 indirectly by phosphorylation and activation of TSC1/TSC2 leading to inhibition of Rheb phosphorylation, and directly by phosphorylation of raptor.

Activated mTORC1 phosphorylates several downstream target proteins including the p-70 ribosomal protein S6 kinase 1 (S6K1), eukaryotic translation initiation factor 4E (eIF4E)binding protein 1 (4E-BP1), the autophagy regulator, Unc-51 like autophagy activating kinase (ULK1) and the transcription factor EB (TFEB) [131]. mTORC1 activation signals to the translation initiation machinery to initiate mRNA translation and protein synthesis via phosphorylation and activation of the protein kinase S6K1 and inhibition of the eIF4E inhibitor 4E-BP1 [132]. The protein levels of the phosphorylated forms of these downstream effectors is often used as a measure of the activity of the mTOR signalling pathway. Phosphorylation of ULK1 blocks autophagosome formation, whereas phosphorylation of TFEB prevents it from entering the nucleus and activating a catabolic transcription process [131]. In contrast, nutrient deprivation inactivates mTORC1, thus inhibiting anabolism and inducing catabolism to restore the nutrient reserves of the cells through the activation of autophagy and allowing the maintenance of normal cellular function. In fact, nutrient deprivation releases mTORC1 from lysosomes leading to its inactivation, whereas nutrition replenishment restores the lysosomal localization of mTORC1 and reactivation [130], in this manner mTORC1 acts as a nutrient sensor.

mTORC2, in contrast, regulates cell survival, metabolism, proliferation, cytoskeletal organization and cell polarization by phosphorylating many AGC kinases, including AKT, serum- and glucocorticoid-regulated kinase 1 (SGK1), and protein kinase C $\alpha$  (PKC $\alpha$ ) [120,123]. mTORC2 is activated by interaction with ribosomes induced by insulin-stimulated PI3K signalling [133]. Since, mTORC2 activates AKT by phosphorylation, which in turn enhances mTORC1 activity through inactivation of the TSC1-TSC2 regulator complex, mTORC2 is an upstream regulator of mTORC1 [126,134,135]. The mTOR signalling pathway is illustrated in Figure 1.8.



Figure 1.8The mTOR signalling pathway. Figure adapted from Laplante and Sabatini[128].

# 1.5.2 mTOR signalling regulates autophagy activation and vice versa

Evidence from previous studies indicates that mTORC1 plays a key role in regulating the autophagic process. Autophagy is induced by inactivation of mTORC1 via the AMPK

mediated pathway by the interaction between ULK1 and AMPK [136-138]. ULK1 is an inducer of autophagy that participates in autophagosome formation [131]. During starvation or stress, cellular energy level depletion activates the AMPK pathway and prevents mTORC1 activation by activating the TSC1-TSC2 complex or by inhibiting its regulator protein, Raptor. Activated AMPK also stimulates the kinase activity of ULK1 by directly phosphorylating ULK1 on multiple sites except Ser 757, thereby inducing autophagic function [136,137]. Under nutrient-rich conditions, high mTORC1 activity blocks the interaction between ULK1 and AMPK by phosphorylating ULK1 at Ser 757, thus inhibiting autophagy [136, 137]. Therefore, phosphorylation of ULK1 either by mTORC1 or AMPK initiates appropriate cellular responses to nutritional signals by inhibiting or inducing autophagy.

Although nutrient restriction induces autophagy and inhibits mTORC1 signalling, extended starvation reactivates mTORC1 and terminates autophagy in experimental models [139]. This observation indicates that autophagy may stimulate mTORC1 reactivation, thereby providing a negative feedback control to terminate excessive autophagy. The reactivation of mTOR after long term starvation could also be due to autophagy induced re-generation of intracellular nutrients and mTOR sensing those nutrients and consequent reactivation. So, sequential mTOR inactivation/activation control both the initiation and termination of protein degradation by autophagy during prolonged nutrient deprivation. Thus, autophagy and mTOR signalling self-regulate through feedback control mechanisms to maintain cellular functions in diverse environmental conditions.

### 1.5.3 The role of mTORC1 in placental nutrient-sensing

Nutrient-sensing signalling pathways control cellular function and growth in response to changes in local and systemic nutrient availability and growth factor signalling. The placenta mediates maternal-fetal exchange and has historically been regarded as a passive conduit for nutrients. However, emerging evidence suggests that the placenta directly controls maternalfetal resource allocation by influencing maternal nutrient delivery to the fetus [140]. The placenta regulates maternal supply by altering the secretion of hormones and signalling factors into the maternal circulation. It has been proposed that the placenta regulates maternal supply by integrating a multitude of maternal and fetal nutritional cues (including, uteroplacental blood flow and oxygenation, hormones such as insulin, leptin, and insulin-like growth factors (IGF-I and II)) with information from intrinsic nutrient-sensing signalling pathways to match fetal demand with maternal supply by regulating maternal physiology, placental growth, and nutrient transport [140]. This process, which is called placental nutrient sensing, ensures optimal allocation of resources between the mother and the fetus to maximize the chances for the propagation of parental genes without jeopardizing maternal health.

Nutrient transport is a primary function of the syncytiotrophoblast, and nutrient uptake by this cell determines not only placental growth but also has a major influence on fetal nutrient availability and growth. Therefore, placental trophoblast nutrient sensors may have a unique role in regulating fetal growth. Among different classes of nutrients, amino acid availability is a key determinant of fetal growth [141] and fetal concentrations of amino acids are

generally higher compared with maternal levels [142]. The placenta expresses 25 different amino acid transporter systems, but only a few have been studied in detail. Among them, 'System A', which is a Na<sup>+</sup>-dependent amino acid transporter, mediates the uptake of nonessential neutral amino acids against their concentration gradient into the syncytiotrophoblast, while 'System L', which is a Na<sup>+</sup>-independent exchanger, transports essential amino acids. The high intracellular concentration of nonessential amino acids that is generated serves as a driving force for the exchange of extracellular essential amino acids through System L [140]. Placental amino acid transporters are regulated by hormones, such as insulin, IGF-I and IGF-II, cytokines, and nutrients.

Trophoblast cells have an array of nutrient-sensing signalling pathways, including AMPK, amino acid response signal transduction pathway, and mTORC1, which regulate cell metabolism in response to altered nutrient levels [143]. Of these nutrient sensors, the evidence supporting a critical role for mTORC1 in placental nutrient sensing is particularly compelling [143]. The nutrient-sensing mTORC1 is highly expressed in the human placental syncytiotrophoblast [144] and the mTORC1 in cultured primary human trophoblast cells is regulated by glucose, and amino acids concentrations as well as by growth factor signalling [145]. mTORC1 is a positive regulator of the placental 'System A' and 'System L' amino acid transporters, which are critical for the transport of essential amino acids to the fetus [144,146,147]. Activation of placental mTORC1 increases, while inhibition of placental mTORC1 decreases the cell surface abundance of amino acids transporters in the trophoblast [148]. It is, therefore, possible that trophoblast mTORC1 signalling links maternal nutrient availability to fetal growth by modulating the flux of amino acids across the placenta.

mTORC1 has a multitude of upstream regulators, including free fatty acids, amino acids, glucose, adenosine triphosphate (ATP) and oxygen. It is likely that the placental concentrations of some of these nutrients are changed in conditions such as placental insufficiency, maternal under-nutrition or obesity [143]. For example, phosphorylation of placental AMPK is markedly decreased, indicating high ATP levels, in maternal obesity [149]. Insulin-like growth factor 2 (IGF-II) is abundantly expressed by the placenta in many species, including humans and rodents, and deletion of the placenta-specific promoter (P0 of the *Igf2* gene, Igf2P0; not expressed in humans), markedly affects maternal physiology and these changes may contribute to the placental and fetal phenotype [150,151]. mTORC1 is activated by insulin and IGF-I and placental insulin/IGF-I signalling is decreased in IUGR [152,153].

The IUGR fetus is often hypoxemic and hypoglycaemic and has low circulating levels of essential amino acids [154,155], consistent with the possibility that nutrient and oxygen levels are also low in the trophoblast. It is therefore predicted that these changes would inhibit placental mTORC1 activity in IUGR. Indeed, the majority of cases of idiopathic normotensive IUGR have been reported to have placental hypoxic-ischemic histological lesions [156] consistent with placental hypoxia. In hypoxia, mitochondrial respiration is impaired, leading to low ATP levels and activation of AMPK, which inhibits mTORC1. Furthermore, hypoxia also affects mTORC1 in AMPK-independent ways by inducing the expression of REDD1, which inhibits mTORC1 by promoting the assembly of TSC1/TSC2 [157]. On the other hand, diabetes with suboptimal control may also be associated with

increased placental ATP and nutrient levels, which would tend to activate mTORC1 signalling. Thus, local levels of nutrients and growth factors, well-established stimuli of mTORC1, are almost certainly altered in the placenta in association with common pregnancy complications such as IUGR.

Placental mTORC1 signalling is altered in pregnancy complications associated with abnormal fetal growth in human and also in animal models where maternal nutrient availability has been altered experimentally [148]. In the human, placental mTORC1 activity is reduced in pregnancies complicated by IUGR [144,152]. An activation of placental mTORC1 signalling in association with maternal obesity in human [149] and in obese mice [158] associated with fetal overgrowth, suggesting that mTORC1 activation stimulates placental amino acid transport and contributes to increased fetal growth. Moreover, placental mTORC1 activity has been reported to be decreased in hyperthermia-induced IUGR in sheep [159], in response to a maternal low protein diet in rats [160] and to maternal calorie restriction in the baboon [161]. It is well-established that circulating adiponectin is inversely correlated to BMI, thereby representing an important nutritional cue carrying information on maternal nutritional status. Interestingly, maternal adiponectin inhibits placental mTORC1 signalling, which may represent an endocrine link between maternal adipose tissue, placental function and fetal growth [162]. Collectively, this emerging evidence is consistent with a critical role for mTORC1 in placental nutrient-sensing. Therefore, understanding the mechanisms by which this sensing occurs may help identify novel approaches to modulate placental function in pregnancy complications.

### 1.6 Aging, oxidative stress and placental aging

### **1.6.1** Cellular and tissue aging

Aging is associated with the development of a large number of metabolic disorders, including cancer, type 2 diabetes, cardiovascular and neurodegenerative diseases. Aging can be defined as an age-dependent decline and deterioration of functional properties at the cellular, tissue and organ level, leading to a decreased adaptability to internal and external stress and an increased vulnerability to disease and mortality [163]. In mitotic tissues, the progressive accumulation of senescent cells is thought to be one of the causal factors for aging [164].

Cellular senescence, which is defined as a state of irreversible cell cycle arrest, triggered by a plethora of intrinsic and extrinsic stimuli or stressors including: short or dysfunctional telomeres, DNA damage and DNA damage response, epigenomic disruption, over expression of certain oncogenes, altered mitochondrial function and oxidative stress created by reactive oxygen species (ROS) [165-167]. Senescent cells within tissues contribute to the aging process and disease development by altering normal cellular function, changing the behaviour of neighbouring cells, degrading structural components such as the extracellular matrix and accelerating the loss of tissue regeneration capacity by reducing stem and progenitor cells [164]. Removal of senescent cells can delay aging-associated disorders in mice [168]. A detailed discussion of cellular senescence has been presented in Chapter 3 of this thesis.

### **1.6.2** Oxidative stress and placental aging

Oxidative stress, which is described as an imbalance in the generation of ROS and the ability of antioxidant defences to scavenge them, is an important contributing factor in the pathophysiology of complicated pregnancies. Oxidative stress can arise from increased ROS production and/or defects in antioxidant defence mechanisms [169]. These ROS are oxygen free radicals that contain one or more unpaired electrons, produced from the reduction of molecular oxygen and generated as by-products of aerobic respiration and metabolism. These molecules have diverse chemical properties and are capable of activating and modulating various signalling pathways, including those involved in cell growth, differentiation, and metabolism [170]. They can also induce cellular oxidative damage by interacting with DNA and intracellular macromolecules such as proteins and membrane lipids, leading to cellular malfunction that may initiate pathological processes.

The free radical theory of aging [171] postulates that aging and degenerative diseases associated with aging are due to the oxidative damage by ROS on cellular components. Moreover, the mitochondrial free radical theory of aging [172] proposes that ROS damage mitochondrial DNA (mtDNA), proteins and other macromolecules that lead to respiratory chain dysfunction. Mutant mtDNA induces an increased production of ROS, further facilitating mtDNA damage and creates a self-amplifying deterioration [172]. The increased generation of ROS can cause lipid peroxidation, protein damage, and several types of DNA lesions in cells, which may result in altered or complete loss of cellular function, compromised tissue and organ function, and aging.
Mechanistically, oxidative stress induces activation of processes, including repair pathways, inhibition of cell proliferation (transient cell-cycle arrest or senescence), or apoptosis [173]. Oxidative stress activates a specific p53 transcriptional response, mediated by p21/p53 and p16, which regulates the cellular response to DNA damage, leading to a halt in proliferation via senescence and contributes to aging [174]. To counterbalance the ROS, cells have endogenous antioxidant systems, including non-enzymes, e.g., vitamin C and E, and glutathione, enzymes, e.g., superoxide dismutase, glutathione peroxidases, glutathione S-transferase and catalase, and trace elements, e.g., copper, zinc, manganese and selenium [175].

Pregnancy itself is a state of oxidative stress, arising from the increased metabolic activity in placental mitochondria and an increased ROS production due to the higher metabolic demand of the growing fetus [176,177]. Superoxide anions produced by placental mitochondria appear to be a major source of ROS and lipid peroxidation that contribute to the oxidative stress in the placenta [178]. Although a physiological balance between ROS and antioxidant activity is maintained in normal pregnancies [179], an imbalance may increase oxidative stress.

The placenta experiences a heightened level of oxidative stress in certain pathologic pregnancies, especially, those that are complicated by maternal smoking, gestational diabetes, fetal growth restriction, preeclampsia, and miscarriage [50,180,181]. Often antioxidant activity is upregulated in response to oxidative stress. However, persistent,

overwhelming oxidative stress leads to consumption and decline of antioxidants, and affects placental antioxidant capacity and reducing systems [177].

In the post-mature placenta, the accumulation of oxidative damage to lipids, proteins, and DNA in the placental tissue may induce a form of advanced aging [50], termed premature aging. Premature aging can occur when the intrauterine environment is affected by conditions that increase oxidative stress, causing irreversible changes in placental tissue [65,182]. It has been hypothesised that aging of the placenta is usually associated with placental insufficiency, preventing this organ from meeting the needs of the fetus, and as a consequence, the viability of the fetus is compromised [50]. Table 1.2 summarises the effect of oxidative stress on placental function and pathological events at different stages during pregnancy.

**Table 1. 2**Effect of oxidative stress on placental function and pathological events inpregnancy.

Oxidative Stress		
Gestation	Effects and pathologies	Outcomes
First- trimester	<ul> <li>Arrival of oxygenated blood before 10-11weeks leads to rise of oxygen tension and oxidative stress</li> <li>Inadequate trophoblastic invasion leads to incompletely developed spiral arteries</li> <li>Increased vascular resistance in the placenta and reduced utero-placental perfusion</li> <li>Ischemia-reperfusion injury</li> </ul>	Early pregnancy loss, Recurrent miscarriage, Preeclampsia, Preterm birth, IUGR
Second- trimester	<ul> <li>A sharp rise in oxygen tension, increased ROS generation and a burst of oxidative stress</li> <li>Impairment of uterine perfusion</li> <li>Persistent, overwhelming oxidative stress leads to consumption and decline of antioxidants</li> <li>Reduction of antioxidant capacity and reducing systems</li> </ul>	Preeclampsia, IUGR
Third- trimester	<ul> <li>Increased oxidative stress causes damage to cellular components, e.g., lipids, proteins, and DNA</li> <li>Induces DNA damage and telomere shortening accelerates telomere-dependent senescence of the fetal membranes</li> <li>Induces a form of advanced aging leading to placental insufficiency</li> </ul>	Preterm birth, IUGR, Stillbirth

#### 1.7 Cellular generation of reactive oxygen species

Reactive oxygen species (ROS) are integral components of multiple cellular signalling pathways, derived from the metabolism of oxygen as by-products of cell respiration, and are continuously produced in all aerobic organisms. Examples include peroxides, superoxide anions, hydrogen peroxides, hydroxyl radical, hydroxyl ions, molecular and singlet oxygen (Figure 1.9). ROS form part of the redox system contributing to cell metabolism, morphology and signalling pathways, however, excessive or inappropriately localised ROS can damage cells. The difference between physiological function and pathological damage of ROS is fundamentally due to the disturbance of the exquisite cellular redox balance. Generally, the ROS that have low reactivity are more likely to participate in cell signalling than in cellular damage.

There are numerous sites of ROS generation within the cell that can be divided in mitochondrial and non-mitochondrial sources, such as (nicotinamide adenine dinucleotide phosphate (NADPH) oxidases, uncoupled nitric oxide synthases, xanthine oxidase and aldehyde oxidase.



Figure 1.9 Reactive oxygen species.

#### 1.7.1 Mitochondrial generation of ROS

Mitochondria are a primary source of ROS within most mammalian cells. Mitochondrial ROS contributes to various cellular signalling pathways, including cellular differentiation [183], cellular adaptation to hypoxia [184], apoptosis [185], autophagy [186], cellular [187] and tissue [188] inflammation, and immune responses [189], depending on the context and triggering stimuli [190]. ROS are produced mostly as by-products of mitochondrial respiration and metabolism. Nutrients, such as glucose, amino and fatty acids, are converted

into metabolic intermediates (e.g., pyruvate, acetyl-CoA, oxaloacetate, 2-oxoglutarate) which are then systematically metabolised and decarboxylated by eight different enzymes in the tricarboxylic acid (TCA) cycle into the mitochondrial matrix (Figure 1.10). The decarboxylation steps are coupled to the transfer of electrons to NAD<sup>+</sup> (nicotinamide adenine dinucleotide) producing NADH [191].

In the mitochondrial inner membrane, there is an enzymatic machinery composed of the electron transport chain (ETC) that transports electrons from reduced metabolic compounds (such as NADH) through four protein complexes (Complex I to IV) in which the molecular oxygen (O<sub>2</sub>) serves as the terminal electron acceptor. Complex I (NADH:ubiquinone oxidoreductase) oxidises NADH and the liberated electrons are passed to ubiquinone (Coenzyme 'Q') generating ubiquinol (QH<sub>2</sub>). Complex II (succinate dehydrogenase; SDH), can also feed electrons (electrons from TCA cycle) into the Q pool. The electrons are then passed to Complex III through cytochrome C and on to Complex IV which then reduces the terminal electron acceptor O<sub>2</sub>, resulting in the generation of water (H<sub>2</sub>O) (Figure 1.10) [191].



Figure 1.10 Schematic representation of ROS generation in the mitochondrial respiratory chain. Nutrients in the form of pyruvate (generated from glucose via glycolysis in the cytoplasm), acetyl-CoA (produced from pyruvate or generated by fatty acid oxidation), or amino acids, enter the tricarboxylic acid (TCA) cycle and are systematically oxidized by the concerted action of 8 different enzymes. Carbon oxidation is coupled to decarboxylation reactions which yield the necessary electrons required to drive oxidative phosphorylation. Electrons from the TCA cycle are transferred to NAD<sup>+</sup> generating the electron carrier NADH which is then oxidised by Complex I.

Electrons can also be provided by Complex II which oxidises succinate to fumarate in the TCA cycle. Electrons then flow through a series of redoxactive prosthetic groups and molecules to the terminal electron acceptor O<sub>2</sub> at Complex IV. The favourable energy change associated with electron flow is coupled to the pumping of protons through Complexes I, III, and IV into the intermembrane space. The protons are then transported back into the matrix by ATP synthase which couples the favourable energy change of proton transfer to the production of ATP from ADP and inorganic phosphate  $(P_i)$ . In this process, various respiratory complexes leak electrons to oxygen producing superoxide anion radical  $(O_2^{-})$ . Superoxide radical may reduce cytochrome c, in the intermembrane space, or maybe dismutated to hydrogen peroxide  $(H_2O_2)$  and oxygen in the matrix and the intermembrane space. NADH, nicotinamide adenine dinucleotide; MAO, monoamine oxidase; SOD, superoxide dismutase; cyt c, Cytochrome c;  $e^{-}$ , electrons;  $\Delta pH$ , proton gradient across inner membrane; Pdh, pyruvate dehydrogenase; PC, pyruvate carboxylase; CS, citrate synthase; Can, aconitase; Idh, isocitrate dehydrogenase; Odh, 2-oxoglutarate dehydrogenase; SCS, succinyl-CoA synthase; Fum, fumarase; Mdh, malate dehydrogenase; Q; ubiquinone. Figure adapted from references [192] and [191].

Due to this redox difference, electron flow from NADH to  $O_2$  through the respiratory complexes is an energetically favourable process. This process is coupled to the pumping of protons through Complexes I, III, and IV into the intermembrane space and establishes a

proton gradient ( $\Delta$ pH) across the mitochondrial inner membrane. Together with the electrical gradient,  $\Delta$ pH creates a temporary form of stored energy, termed the proton motive force (PMF) which provides the driving force for ATP production at the mitochondrial Complex V (ATP synthase). Oxygen (O<sub>2</sub>) can only accept one electron at a time during its reduction to H<sub>2</sub>O. Some electrons can 'spin-off' prematurely from Complexes I and III, which univalently reduce O<sub>2</sub> resulting in the production of the proximal ROS superoxide anion radical (•O<sub>2</sub><sup>-</sup>), either in the matrix or in the intermembrane space of the mitochondria (Figure 1.10).

Superoxide anion radical is quickly dismutated to hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>). Hydrogen peroxide, which is considered to be a key ROS signalling molecule, is a mild oxidant and has the longest cellular half-life (1ms) allowing its diffusion through membranes [191,193,194]. The control of mitochondrial superoxide anion radical and hydrogen peroxide is carried out through the regulation of the flux of the metabolic pathways that regulate the flow of electrons into the electron transport chain [195,196]. Cellular levels and half-lives for these ROS can vary considerably depending on the cell type, nutritional and environmental conditions [191].

Accumulation of hydrogen peroxides and superoxide anion radicals into the cell can lead to the generation of hydroxyl radicals (•OH). Hydroxyl radicals are highly reactive species that indiscriminately oxidize biological macromolecules such as proteins, lipids and nucleic acids [192]. Similarly, increased steady state concentrations of superoxide radicals may reduce transition metals, such as iron (Fe), thereby disassembling Fe-S clusters in various TCA cycle enzymes and in respiratory complexes. Superoxide radical can also react with nitric oxide to form peroxynitrite by-products, which are also highly reactive oxidants [192]. Although under normal conditions, mitochondria trigger intra- and inter-cellular signals through the release of ROS, ROS overproduction is associated with a number of pathologies including aging, neurodegenerative and metabolic diseases (Figure 1.11). The balance of ATP and ROS production in the mitochondria has a central role in cellular homeostasis [192].



Figure 1.11 Mitochondrial ROS induced aging and disease. Increased Mitochondrial ROS generation can contribute to the initiation of aging and disease processes. ROS overproduction by mitochondria can lead to oxidative damage to mitochondrial proteins, membranes and DNA (mtDNA), impairing the ability of mitochondria to synthesize ATP and to carry out their wide range of metabolic functions, including the TCA cycle, fatty acid  $\beta$ -oxidation, amino acid metabolism, haem synthesis and Fe-S centre assembly that are central to the normal operation of most cells. Mitochondrial oxidative damage can also increase the tendency of mitochondria to release intermembrane space proteins such as cytochrome c (cyt c) to the cytosol by mitochondrial outer membrane permeabilization (MOMP) and thereby activate the cell's apoptotic machinery. In addition, mitochondrial ROS production leads to induction of the mitochondrial permeability transition pore (PTP), which renders the inner permeable small molecules in situations membrane to such as ischaemia/reperfusion injury. Consequently, mitochondrial oxidative damage contributes to a wide range of pathologies. In addition, mitochondrial ROS may act as a modulatable redox signal, reversibly affecting the activity of a range of functions in the mitochondria, cytosol and nucleus. Figure adapted from Murphy [197].

#### 1.7.2 Non-mitochondrial generation of ROS

ROS can be generated during the catalytic action of multiple enzymes, such as peroxidases, xanthine oxidase (XO), cyclooxygenases and aldehyde oxidases (AOX) [198]. Cytochrome c reductase, also known as cytochrome P-450 can also produce ROS during the metabolism of xenobiotics in the endoplasmic reticulum [199]. Hydrogen peroxide, which can readily be decomposed into a highly reactive hydroxyl radical, is a key ROS signalling factor implicated in the free-radical theory of aging. Hydrogen peroxide is produced by many enzymes including XO, monoamine and D-amino acid oxidase and AOX, as well as by the peroxisomal pathway for  $\beta$ -oxidation of fatty acids [200]. In addition, multiple extracellular signals such as growth factors, cytokines, G-protein-coupled receptor antagonists and mechanical distortion of cells can generate ROS through the activation of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase complex [201].

The NADPH oxidases are a family of membrane-bound multimeric enzymes that are widely expressed and evolutionarily conserved and specifically designed to translocate electrons (from NADPH) across a membrane in a manner that results in the formation of ROS (Figure 1.12). The NADPH oxidase consists of six transmembrane helices, binding sites for NADPH and flavin adenine dinucleotide (FAD), and heme-coordinating histidine residues [202]. ROS production by NADPH oxidase complex is mediated by one of seven enzymatic systems (NOX1 to 5, Duox1, and Duox2) that have differential cellular and tissue-specific expressions [192]. Moreover, NADPH oxidase complex activation requires the association with FAD cofactor, distinct membrane and cytoplasmic co-activator proteins and the binding

of Ca<sup>2+</sup> to some intracellular domains (Nox 5) allowing the pathway and isoform specificities [202,203]. Activation of NADPH oxidase complex results in the transport of electrons, from cytoplasmic NADPH through FAD and heme cofactors, across plasma and intracellular membranes to produce superoxide anion radical on the extracellular surface, which is readily dismutated to hydrogen peroxide and molecular oxygen via extracellular superoxide dismutase [204].



Figure 1.12 NADPH oxidase complex translocates electrons (from NADPH) across a membrane, which results in the formation of ROS (mainly superoxide anion (O<sub>2</sub><sup>-</sup>)). The seven NADPH oxidase family members (Nox 1 to 5, Duox 1 and 2) share conserved features, including six transmembrane domains, which contain four heme-binding histidines. The carboxy terminus consists

of an FAD-binding domain followed by an NADPH-binding domain. Nox 5 includes an additional N-terminal calmodulin-like Ca<sup>2+</sup>-binding domain. Duox 1 and 2 also include peroxidase homology domains. Figure adapted from Chen et al. [202].

#### 1.7.3 Aldehyde oxidase mediated ROS generation

Human aldehyde oxidase 1 (AOX1, EC 1.2.3.1) is a cytosolic enzyme predominantly expressed in liver, but detectable amounts are also found in other tissues, such as lung, blood vessels, heart, kidney and skin [205,206]. Although AOX1 is mostly known for its role in the biotransformation of certain drugs and detoxification of xenobiotics, it also serves as an important source of ROS in biological tissues and may play a vital role in ROS-mediated redox signalling and tissue injury [207].

AOX1 belongs to the molybdenum hydroxylase family along with xanthine dehydrogenase (XDH) and xanthine oxidase (XO), collectively known as xanthine oxidoreductase (XOR) [207]. AOX1 and XO share a similar structure and amino acid sequence, with ~86% homology. AOX1, in its catalytically active form, exists as a homodimer. Each monomer consists of four discrete regions: an N-terminal domain containing two spectroscopically distinct 2Fe/2S clusters, an intermediate domain containing a flavin adenine dinucleotide (FAD) binding site, and a C-terminal catalytically active domain contains the molybdenum cofactor (Moco) binding site and the substrate binding pocket (Figure 1.13) [208].



Figure 1.13Structure of human aldehyde oxidase 1 (AOX1). Figure obtained fromProtein Data Bank (NGL view of human aldehyde oxidase (4UHW) [209]).

AOX1 is characterized by broad substrate specificity. AOX1 primarily catalyses two metabolic pathways: (i) oxidative hydroxylation of aromatic *N*-heterocycles, and (ii) oxidation of aldehydes (aliphatic and aromatic aldehydes) and NADH [210-212]. Nitrogen-containing aromatic heterocycles are key scaffolds to build pharmacophores in medicinal chemistry, and a number of these compounds are putative substrates of AOX1. It also metabolises certain drugs of pharmacological interest, especially those which contain 54

aromatic azaheterocyclic substituents (e.g., zaleplon, brimonidine) [205]. Moreover, AOX1 catalyses the reduction of different functional groups including S-oxides, N-oxides, nitro groups and heterocycles [212,213]. Abundant amounts of AOX1 have been observed in adipose tissue and are proposed to play a critical role in adipogenesis and lipid metabolism [214,215]. Although AOX1 and XO have a similar structure their substrate specificity and inhibitor susceptibility are different [211,216]. Both XO and AOX1 exhibit broad specificity, accepting a wide variety of reducing substrates, however AOX1 catalyses the oxidation of aldehydes and NADH more efficiently, whereas XOR has higher affinity for xanthine, hypoxanthine, purines, and pyrimidines [206,207].

Aldehydes and NADH are different site-specific electron donors for AOX1. Aldehydes donate electrons to AOX1 at the molybdenum site, while NADH reduces AOX1 at the FAD site (Figure 1.14) [216]. In addition to its drug metabolism function, AOX1 is also involved in regulation of ROS homeostasis [206]. Both aldehydes and NADH serve as good substrates for ROS formation by AOX1. Studies demonstrated that metabolism of aldehydes by AOX1 in the presence of oxygen produce significant amounts of ROS, such as superoxides ( $\cdot$ O<sub>2</sub><sup>-</sup>) and hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) (Figure 1.14) [217]. NADH can also be oxidised by AOX1, with  $\geq$ 65% of the total electron flux from NADH oxidation leading to generation of  $\cdot$ O<sub>2</sub><sup>-</sup> [207]. Because of its wider substrate specificity and greater level of activity, this enzyme is considered to be an important basal source of ROS under normal physiological conditions. This basal level of ROS generation may be greatly enhanced in disease settings where cellular aldehyde levels are increased.



**Figure 1.14** Substrate binding sites at aldehyde oxidase 1. R-COH denotes an aliphatic or aromatic aldehyde and R-COOH represents the corresponding acids.

Free radicals generated during the metabolism of aldehydes (such as acetaldehyde) [218]or oxidation of NADH [219] by AOX1 in the presence of catalytic iron have been suggested to initiate alcohol-induced liver injury. Metabolism of alcohol produces acetaldehyde and NADH that can both be substrates for AOX1 and thereby results in ROS formation. Alcoholinduced ROS generation by AOX1, can also induce carcinogenic mutations and DNA damage leading to breast cancer [220]. Lipid peroxidation, glycation and amino acid oxidation are major pathways for the generation of endogenous aldehydes. Lipid peroxidation produces more than 200 different aldehydes: among them, 4-hydroxynonenal (4HNE) and malondialdehyde (MDA) are two major aldehydes produced in membrane lipid peroxidation [221]. Levels of MDA in humans are  $0.1-35 \mu$ M, and because of its lipophilic nature, the concentration of 4HNE can accumulate up to millimolar levels within microsomal membranes [221]. In certain disease states the levels of these aldehydes are even higher [222]. Under these circumstances AOX1 is the major cytosolic enzyme responsible for the metabolism of endogenous aldehydes and this leads to production of superoxide ( $\bullet$ O<sub>2</sub><sup>-</sup>) and hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) and may explain the ROS mediated oxidative liver injury observed in chronic alcoholics [217].

Owing to its ability to generate ROS, such as  $\cdot O_2^-$  and H<sub>2</sub>O<sub>2</sub>, and its suggested role in ROS induced oxidative liver injury in chronic alcoholics [218,219], ischemia-reperfusion injury [223], inflammation and various inflammatory diseases [217], and cardiovascular diseases [224], AOX1 may be an important target for studying ROS induced placental oxidative damage in pathological pregnancies. A number of AOX1 inhibitors have been identified, including raloxifene,  $\beta$ -estradiol, menadione, amidone and diphenyleneiodonium [207]. Among them, raloxifene, which is a specific and potent inhibitor of human liver AOX1, has been shown to reduce oxidative damage that is achieved by a reduction in the release of ROS from vascular endothelial cells [225] and to reduce lipid peroxidation by blocking AOX1 activation in renal tissue [226].

# **1.7.4** Role of G protein-coupled estrogen receptor 1 (GPER1) activation in attenuating ROS production and oxidative damage

Estrogens play a vital role in many areas of human physiology, including reproduction, immunity, and vascular and nervous system biology, as well as in diseases, such as cancer and reproductive disorders [227]. The membrane-associated G protein-coupled estrogen receptor 1 (GPER1), previously known as GPR30, is widely expressed in various tissues including the brain, heart, vasculature, pancreas, ovary, kidney and the placental syncytiotrophoblast membrane [228,229]. Activating GPER1 by estrogen results in intracellular calcium mobilization and synthesis of phosphatidylinositol 3,4,5-trisphosphate in the nucleus [230]. Additionally, the GPER1 signalling mechanisms include the rapid activation of mitogen-activated protein kinases (MAPKs), extracellular regulated MAPK1 (ERK-1), ERK-2, and phosphatidylinositol 3-kinase (PI3K), as well as increased cytosolic cAMP and calcium [231-233]. G1, a nonsteroidal, high-affinity, highly selective agonist of GPER1 [233], can selectively bind GPER1 in the same cell where other estrogen receptors are present. Identification of G1 has enabled the role of GPER1 in human physiology to be defined and opened the door to the generation of diagnostic and therapeutic strategies directed at individual estrogen receptors [234].

Pharmacological activation of GPER1 by its agonist G1 has been shown to induce antiinflammatory protective effects in multiple sclerosis [235] and cardioprotective effects against ischemia-reperfusion injury [236-238] in rodent models. The post-ischaemic GPER1 activation by  $\beta$ -estradiol induces cardioprotective effects against ischemia-reperfusion injury by attenuating mitochondrial dysfunction, preserving mitochondrial membrane potential, inhibition of mitochondrial permeability transition pore opening, resulting in the reduction of mitochondrial ROS generation in an ovariectomized-rat model [239]. Moreover, chronic G1 treatment has been shown to reduce lipid oxidation (measured using a lipid oxidation marker, 8-isoprostane) in renal tissue achieved by attenuated oxidative stress [226]. GPER1 activation reduces NADPH-stimulated superoxide generation in carotid and intracranial arteries of normotensive rats, achieved after chronic administration of the agonist G1 [240]. These studies suggested that GPER1 activation by its agonist G1 may block ROS generation or directly scavenge ROS, but the exact mechanism is unclear. The presence of the cell surface estrogen receptor GPER1 on the placental syncytiotrophoblast apical membrane, suggests that this receptor may play a role in modulating oxidative damage within the placenta [229]. Elucidation of the underlying mechanisms of GPER1 to attenuate ROS generation and oxidative damage in placenta needs future studies.

## **CHAPTER 2**

## Oxidative Stress, Placental Aging Related Pathologies, and Adverse Pregnancy Outcomes

This Chapter contains a review article published in the American Journal of Reproductive Immunology, and is reproduced with permission. This review article summarises the literature regarding the role of oxidative stress and premature placental aging in the pathophysiology of pregnancy complications. The format of the paper has been altered for the purposes of this thesis. The original manuscript is included in appendix A.

### Oxidative Stress, Placental Ageing Related Pathologies, and Adverse Pregnancy Outcomes

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### Declaration

I, Zakia Sultana, attest that I have made a primary and original contribution to the above publications as detailed below and endorsed by my supervisors. I was involved in the conception and designed of the review, I undertook the research to find the publications referred to in the review. I primarily wrote the review, designed and prepared the figures, and I edited and approved the final version of the review.

Zakia Sultana

Laureate Professor Roger Smith

#### 2.0 Abstract

Oxidative stress (OS), an imbalance between free radical generation and antioxidant defence, is recognised as a key factor in the pathogenesis of adverse pregnancy outcomes. Although OS is a common future of normal pregnancy, persistent, overwhelming OS leads to consumption and decline of antioxidants, affecting placental antioxidant capacity and reducing systems. The accumulation of OS causes damage to lipids, proteins, and DNA in the placental tissue that induces a form of accelerated aging. Premature aging of the placenta is associated with placental insufficiency that prevents the organ meeting the needs of the fetus, and as a consequence, the viability of the fetus is compromised. This review summarises the literature regarding the role of OS and premature placental aging in the pathophysiology of pregnancy complications.

#### 2.1 Introduction

All living organisms have limited life cycles, and aging is part of that life cycle. Each organ within an organism also exhibits aging related changes; the placenta is no exception. The placenta, a specialised organ formed during pregnancy, grows throughout gestation, performs multiple functions including, endocrine regulation and nourishment of the fetus [241], but also ages and is discarded at the end of pregnancy while the fetus may live for another hundred years. So, placental aging is a normal physiologic phenomenon [242]. However, there are likely to be some placentas which show signs of aging earlier than others, in the same way as some individual age more quickly than others. Premature aging and

degenerative changes in the placenta may reduce the functional capacity of the placenta and lead to abnormal pregnancy outcomes. The placenta is the primary organ for transferring nutrients from the mother to the fetus; so, growth and function of the placenta are precisely regulated and coordinated to ensure the optimal growth and development of the fetus. The placenta exchanges nutrients e.g., oxygen, amino acids, carbohydrates, minerals, and waste products e.g., carbon dioxide between the maternal and fetal circulatory systems [56]. It releases hormones into both the maternal and fetal circulations to affect uterine function, maternal metabolism, fetal growth, and development. Moreover, it metabolises some substances and can release metabolic products into both fetal and maternal circulations. The placenta can help to protect the fetus against certain xenobiotic molecules, infections, and maternal diseases. Therefore, the adequate function of this organ is crucial for a normal physiologic gestational process and a healthy baby as a final outcome.

In this review we focus on the role of OS in the pathophysiology of pregnancy complications, beginning with a brief overview of placental development at different stages of gestation. We then discuss the biochemical markers of aging and OS-induced placental aging. Finally, we discuss the studies indicating that OS and placental aging play a role in the pathophysiology of abnormal pregnancies, with a particular emphasis on pregnancy complicated by spontaneous preterm birth, intrauterine growth restriction, preeclampsia, pregnancy loss and stillbirth.

#### 2.2 Human placental development

In human embryonic development, the blastocyst is formed by 5–6 days after fertilisation and is composed of the outer trophectoderm layer and the inner cell mass [58]. The blastocyst makes contact with the endometrium and invades into the decidua of the endometrium at approximately 6-7 days after fertilisation [62]. Immediately after attachment to the endometrium, the trophectoderm layer proliferates rapidly and differentiates into an inner layer of mononuclear cytotrophoblasts and a multinucleated outer epithelial layer known as the syncytiotrophoblast [56]. The syncytiotrophoblast is a terminally differentiated cell layer which is formed by the fusion of multiple cytotrophoblasts, a process called syncytialization. The combination of inner cytotrophoblasts and outer syncytiotrophoblast form finger-like structures called primary chorionic villi [56]. At the initial phase of differentiation, these villi are distributed symmetrically over the chorion. As gestation progresses, the chorionic villi grow like branches of a tree (arborisation) and accumulate asymmetrically towards the uterine wall where the embryo is attached [56]. After the invasion of mesenchymal cells into the centre of the primary villi forming secondary villi, fetoplacental blood vessels arise inside the villi at the 5th week of gestation to form tertiary villi [63]. The placental vasculature system is essential for transferring nutrients, gases and hormones to the growing fetus. The proper branching of placental blood vessels (angiogenesis) is part of a successful pregnancy. Inadequate placental development, trophoblast invasion, and vascular remodelling, as well as abnormal placental angiogenesis, have been reported in pathological pregnancies such as intrauterine growth restriction and preeclampsia [62,64,65].

In the first-trimester, the chorionic villi of the placenta are large, and the blood vessels in the villi are not prominent. In addition to villous trophoblast, an additional set of mononuclear trophoblasts, termed the extravillous trophoblast, grows outside the villi and extends into the decidualised endometrium [56,70]. During the first-trimester of differentiation (up to 11–12 weeks) these extravillous trophoblasts erode into and plug the uterine spiral arteries and restrict the ability of the oxygenated maternal blood to access the placenta [71]. Consequently, the early stages of human embryonic development occur in an environment of low oxygen tension [72]. The hypoxic environment is thought to be necessary for the initial differentiation of the trophoblasts, in fact, miscarriage has been reported in cases of the early arrival of oxygenated blood in the intervillous space [75]. As the placenta matures and increases in size in the second-trimester, the villi become smaller and more vascular. The syncytiotrophoblast cell layer draws up into 'syncytial knots' which are small clusters of nuclei, leaving a single cytotrophoblast layer. Later the extravillous trophoblasts replace the endothelial layer covering the smooth muscle of the spiral arteries and render them flaccid and noncontractile [76]. The trophoblast plugs are gradually dislodged from the spiral arteries after 11–12 weeks of gestation, and maternal blood invades from the maternal spiral arteries into the intervillous spaces [72,77]. This process is associated with a sharp rise in oxygen tension, increased free radical generation and a burst of OS within the placental tissues, however, this OS returns to baseline upon a surge of antioxidant activity, as placental trophoblast cells gradually acclimate to the new oxidative surroundings [78]. The nutrients, gases and growth factors carried by maternal blood are readily taken up by the large surface of the syncytiotrophoblast allowing the fetus to grow in an oxygen and nutrient rich environment. A mature placenta in the third-trimester has small and highly vascularized chorionic villi to support the blood gas and nutrient exchange of the maternal-fetal circulation required by the growing fetus approaching term gestation. Syncytial knots are prominent in the third-trimester chorionic villi. Figure 2.1 illustrates the development of human placental chorionic villi at different stages of gestation.



Figure 2.1 Development of human placental chorionic villi. Cross sections of (A) primary villi arborisation, (B) first-trimester, (C) second-trimester and (D) third-trimester villi. The chorionic villi in the first-trimester (B) are large, covered by two layers of cells, cytotrophoblasts and syncytiotrophoblasts and the blood vessels in the villi are not prominent. Second-trimester placental villi (C) are more vascular with a single cytotrophoblasts layer. The third-trimester (D) has highly vascularized chorionic villi, and syncytial knots are prominent.

#### 2.3 Apoptosis and its role in the trophoblasts function

Apoptosis, or programmed cell death, is crucial to the development and homoeostasis of all multi-cellular organisms and for many organs including the placenta. Apoptosis is known to occur in a number of biologic processes, both physiologic and pathologic. Trophoblast apoptosis is a physiologic event in normal pregnancy, increases with advancing gestational age and is higher in post-term pregnancies, and therefore, is considered as a normal process in the development and aging of the placenta [98,99]. Apoptosis is proposed to occur as a normal event during the formation of the villous trophoblast bi-layer and syncytiotrophoblast formation from cytotrophoblasts (trophoblast differentiation) [100]. However, it is likely that placental insults can alter the regulation of apoptosis in the trophoblasts, possibly by modulating trophoblast cell turnover [100].

Cultured trophoblasts exposed to hypoxia show marked up-regulation of activity of tumour suppressor protein p53, enhanced expression of the pro-apoptotic Mtd-1 and decreased

expression of the anti-apoptotic Bcl-2, all of which promote apoptosis [101-103], and the apoptosis is more marked in hypoxia/re-oxygenation [104]. Additionally, an up-regulated p53 and decreased Bcl-2 mediated increased apoptosis in placental syncytiotrophoblast is associated with some pregnancy pathologies, including intrauterine growth restriction (IUGR) and preeclampsia [94,105]. Syncytial knots, a characteristic feature of syncytiotrophoblast apoptosis, increase in placentas associated with preeclampsia and IUGR [90,105]. In contrast, apoptosis decreases in extravillous trophoblasts in pregnancies complicated by preeclampsia and is associated with reduced trophoblast invasion [106]. Thus apoptosis is differently regulated in villous and extravillous trophoblasts in normal placental development.

#### 2.4 Aging, OS and placental aging

#### 2.4.1 Cellular senescence and aging

Aging can be defined as an age-dependent decline and deterioration of functional properties at the cellular, tissue and organ level, leading to a decreased adaptability to internal and external stress and an increased vulnerability to disease and mortality [163]. Age-related diseases and premature aging syndromes are often characterised by short telomeres and reduced or complete loss of telomerase activity [243].

Telomeres are nucleoprotein structures comprised of double-stranded DNA region of TTAGGG repeats which is typically 10–15 kb long in humans, located at the termini of the chromosomes and are essential for chromosomal stability and cell survival [244]. Telomeres

protect DNA ends from breaks, end-to-end fusion and degradation by forming a protective cap with a 150–200 nucleotides long G-rich single-stranded telomere overhang and telomere binding protein complexes [243]. Telomeres are progressively shortened with each cell division, and shortening is accelerated as a consequence of environmental stressors and insults, such as hyperglycaemia, hypoxia, and OS [245-248]. Once a critical shortening of telomeres is attained, cells enter a state of irreversible metabolic arrest known as senescence, which leads to a process of cellular or tissue aging [244,249].

Cell senescence is distinct from apoptotic cell death. Senescence is a biological aging process in which cells change morphologically, in gene and protein expression, and in the activation of key signalling constituents (such as p38 and p53) that determine the fate of a tissue [250]. Cellular senescence has been associated with a gradual deterioration of functional characteristic of the cell, although there is no evidence that senescent cells undergo a cell death pathway. Senescent cells are resistance to apoptosis or programmed cell death through the overexpression of Bcl-2 protein, leading to the accumulation of these cells within tissues [251]. The accumulation of senescent cells within tissues contribute to the aging process and generating age-related phenotypes by altering metabolic function, degrading structural components, reducing tissue renewal and repair, changing the behaviour of neighbouring cells or the extracellular environment, and reducing the pool of growth-competent mitotic cells [164]. Premature senescence can also occur, independent of telomere size, as a consequence of progressive DNA damage, telomere uncapping and telomere dysfunction caused by extrinsic or intrinsic stressors including OS, resulting in end-to-end fusion and aggregation of telomeric DNA [241,252,253].

Telomere length is regulated by the enzyme telomerase, a specific reverse transcriptase, capable of adding telomeric repeats to the ends of the chromosome [254]. Telomerase consists of a catalytic protein component, telomerase reverse transcriptase (TERT) and an RNA template component, telomerase RNA component (TERC). TERC is widely expressed, but TERT expression is tightly regulated and is considered to be the rate-limiting factor in telomerase activity [255]. The absence of functional telomerase or loss of telomerase activity leads to progressive telomere shortening during cell division [249,254]. Telomere shortening may also be associated with a lack of adequate damage repair mechanisms that protect DNA damage [253]. Due to their high oxidation potential, the guanine-rich residues in telomeres are extremely susceptible to free radical attack [50]. There is a clear relationship between OS and telomere length and telomerase activity, the indicators of cellular senescence and aging [256]. Therefore, measurement of telomere length and telomerase activity can be used as biological markers for tissues suffering OS and age [256-258].

#### 2.4.2 OS and placental aging

OS is an important contributing factor in the pathophysiology of complicated pregnancies. OS is described as an imbalance in the generation of reactive oxygen species (ROS) and the ability of antioxidant defences to scavenge them. OS can arise from increased ROS production and/or defects in antioxidant defence mechanisms [169]. These ROS are oxygen free radicals that contain one or more unpaired electrons, produced from the reduction of molecular oxygen and generated as by-products of aerobic respiration and metabolism. These molecules have diverse chemical properties and are capable of activating and modulating various signalling pathways, including those involved in cell growth, differentiation, and metabolism [170]. They can also induce cellular oxidative damage by interacting with DNA and intracellular macromolecules such as proteins and membrane lipids, leading to cellular malfunction that may initiate pathological processes.

The free radical theory of aging [171] postulates that aging and degenerative diseases associated with aging are due to the oxidative damage by ROS on cellular components. Moreover, the mitochondrial free radical theory of aging [172] proposes that ROS damage mitochondrial DNA (mtDNA), proteins and other macromolecules that lead to respiratory chain dysfunction. Mutant mtDNA induces an increased production of ROS, further facilitating mtDNA damage and creates a self-amplifying deterioration [172]. The increased generation of ROS can cause lipid peroxidation, protein damage, and several types of DNA lesions in cells, which may result in altered or complete loss of cellular function, compromised tissue and organ function, and aging. Mechanistically, OS induces activation of processes, including repair pathways, inhibition of cell proliferation (transient cell-cycle arrest or senescence), or apoptosis [173]. OS activates a specific p53 transcriptional response, mediated by p21/p53 and p16, which regulates the cellular response to DNA damage, leading to a halt in proliferation via senescence or apoptosis and contributes to aging [174]. To counterbalance the ROS, cells have endogenous antioxidant systems, including nonenzymes, e.g., vitamin C and E, and glutathione (GSH), enzymes, e.g., superoxide dismutase (SOD), glutathione peroxidases (GSH-Ps), glutathione S-transferase (GSH-T) and catalase (CAT), and trace elements, e.g., copper, zinc, manganese and selenium [175].

Pregnancy itself is a state of OS, arising from the increased metabolic activity in placental mitochondria and an increased ROS production due to the higher metabolic demand of the growing fetus [176,177]. Superoxide anions produced by placental mitochondria appear to be a major source of ROS and lipid peroxidation that contribute to the OS in the placenta [178]. Although a physiological balance between ROS and antioxidant activity is maintained in normal pregnancies [179], an imbalance may increase OS.

The placenta experiences a heightened level of OS in certain pathologic pregnancies, especially, those that are complicated by maternal smoking, gestational diabetes, fetal growth restriction, preeclampsia, and miscarriage [50,180,181]. Often antioxidant activity is upregulated in response to OS. However, persistent, overwhelming OS leads to consumption and decline of antioxidants, and affects placental antioxidant capacity and reducing systems [177]. In the post-mature placenta, the accumulation of OS damage to lipids, proteins, and DNA in the placental tissue may induce a form of advanced aging [50]. Premature aging can occur when the intrauterine environment is affected by conditions that increase OS, causing irreversible changes in placental tissue [65,182]. It has been hypothesised that aging of the placenta is usually associated with placental insufficiency, preventing this organ from meeting the needs of the fetus, and as a consequence, the viability of the fetus is compromised [50]. Figure 2.2 summarises the effect of oxidative stress on placental function and pathological events at different stages during pregnancy.





#### 2.5 OS, placental aging, and adverse pregnancy outcomes

#### 2.5.1 OS and spontaneous preterm birth

Preterm birth is defined as birth before 37 weeks of gestation, affects 5–18% of pregnancies and is a leading cause of infant morbidity and mortality. Most of the preterm births occur after the spontaneous onset of labour (with or without preterm premature rupture of the membrane, pPROM), but the precise mechanisms of onset of preterm labour remain unclear [259]. Labour induces changes of gene expression in chorioamniotic membranes that are consistent with the localised acute inflammatory response, despite the absence of histologically detectable inflammation [260]. It has been hypothesised that cellular apoptosis transmits an inflammatory signal that stimulates parturition [261]. Although they are resistance to apoptosis, senescent cells may transmit both inflammatory and senescencepromoting signals to induce labour [250]. It has also been suggested that labour is associated with senescence-associated changes in the placental membranes mediated by the p38 MAPK pathway, including telomere shortening, p38 MAPK activation, increased expression of p21 and SA-β-galactosidase [262]. OS at term induces DNA damage and telomere shortening, which accelerates telomere-dependent senescence of the fetal membranes, resulting in senescence-associated inflammatory activation that may contribute to parturition [263]. It has long been thought both term and preterm labour have similar processes that occur through a 'common pathway'. The activation of this common pathway through physiological signals results in term labour, while preterm labour is a 'syndrome', which occurs from spontaneous activation of one or more of the components of the common pathway induced by multiple pathologic processes or risk factors [259]. Spontaneous preterm labour or pPROM is likely to be triggered by premature placental aging caused by OS-induced damage and premature senescence of the intrauterine tissues, especially the fetal membranes of the placenta [264-266], and vascular, endocrine or immune system dysfunction [267]. ROS activates NF-kappa B, which stimulates COX-2 expression and systemic inflammation. Infection, inflammation or exogenous factors (e.g., lead) up-regulates ROS, resulting in OS-induced tissue injury and the consequent decrease in antioxidant defences are likely to increase the risk of preterm birth [268]. Preterm birth is also associated with ROS-mediated redox imbalance (balance between
pro- and anti-oxidants). In preterm birth, increased placental and maternal serum levels of oxidised metabolites (malondialdehyde) with reduced levels of antioxidant (GSH, selenium, GSH-T) are observed compared to term labour [269-272]. However, the expression of Mn-SOD in fetal membranes of women in preterm labour is increased, likely to constrain the inflammatory processes and OS [273].

#### 2.5.2 OS and IUGR

IUGR, also known as fetal growth retardation, is a failure of a fetus to reach its genetic growth potential. IUGR is a leading cause of fetal, neonatal and perinatal morbidity and mortality [274]. IUGR is defined as an estimated fetal weight of less than the 10th percentile for gestational age [100]. Most intrauterine deaths, in particular, those that are classified as unexplained, are associated with IUGR. Around 76% of intrauterine deaths are associated with IUGR [33,34]. IUGR also strongly affects the long-term health of survivors [275]. Some potential risk factors for IUGR include maternal smoking, infection, obesity, malnutrition, and chromosomal abnormalities, but the majority of cases remain unexplained [276]. The most common aetiology for IUGR is uteroplacental dysfunction, due to diminished maternal uteroplacental blood flow [277]. The placenta is the central organ for transporting nutrients and oxygen from the mother to the fetus. Inadequate function of this organ limits the supply of critical substrates to support the normal aerobic growth of the fetus [278]. Recently, it has been hypothesised that placental insufficiency originates in the early stage of gestation when the trophoblast invades spiral arteries in the placental bed [278]. This process requires high energy availability for cell growth, proliferation and metabolic activity that generates ROS

and OS. Inadequate trophoblastic invasion to the spiral arteries may occur when the chorioallantoic villi encounter an injury caused by stimuli or mediators [279]. Among the diverse number of stimuli or mediators, OS has the leading role [104]. Consequently, incompletely developed spiral arteries cause ischemia (hypoxia)-reperfusion that exacerbates the OS and contributes to damage of the placental tissue [100].

Damage resulting from OS predominantly occurs to membrane lipids, proteins, and nuclear and mitochondrial DNA. Plasma and tissue levels of malondialdehyde (MDA), an end product of fatty acid oxidation, is frequently measured as indicators of lipid peroxidation and OS. The levels of MDA and xanthine oxidase (XO, an enzyme that generates ROS) are higher in maternal plasma, umbilical cord plasma, and placental tissues of the patients with IUGR pregnancy compared to healthy pregnancies [280], which suggest that OS has a role in IUGR. In nuclear and mitochondrial DNA, 8-hydroxy-2'-deoxyguanosine (8OHdG, an oxidised derivative of deoxyguanosine) is one of the predominant forms of free radical-induced oxidative lesions, and has therefore been widely used as a biomarker for oxidative DNA damage, as well as OS. The levels of 8OHdG and redox factor-1 (Ref-1) are significantly higher in placentas from IUGR compared to normal pregnancies [281-283]. Ref-1 is a redox regulator that repairs oxidative DNA damage, and its concentration increases in response to oxidative damage. Placental antioxidant levels and antioxidant activity are also altered in pregnancies complicated by IUGR. In IUGR, the SOD and GSH-Px activities in maternal plasma, cord blood, and placental tissues are increased, while CAT activity is decreased [280]. The mRNA levels of the reducing systems, glutaredoxin and thioredoxin, are also depleted in placentas with IUGR [284]. Moreover, the IUGR placenta shows signs of aging markers, including shortening of telomere length and reduced telomerase activity. A significantly shorter telomere and/or an absent or reduced telomerase activity are observed in the placentas from IUGR pregnancies [255,285-287] with a reduced expression of hTERT, which is the rate-limiting factor in the telomerase activity [255]. Also, the expression of telomere-induced senescence markers p21 and p16 are elevated, and anti-apoptotic protein Bcl-2 is decreased in IUGR placentas [285]. Together with increased OS markers and reduced antioxidants capacity, the evidence of aging markers supports the concept of the role of OS in placental aging and IUGR.

#### 2.5.3 OS and preeclampsia

Preeclampsia is a hypertensive disorder of human pregnancy, and it frequently occurs in association with IUGR. Preeclampsia affects 5–7% of all pregnancies worldwide and remains a leading cause of fetal growth retardation, premature delivery and maternal death [177,288,289]. The main features of preeclampsia are new-onset maternal hypertension (blood pressure  $\geq$ 140/90 mm Hg), reduced uteroplacental blood flow, proteinuria ( $\geq$ 300 mg/24 hours), oedema, and occurrence primarily in nulliparous women in their third-trimester [177]. Among the two distinct subtypes, early-onset preeclampsia (occurs before 34 weeks) confers a higher risk of life-threatening maternal complications and fetal and perinatal death, than the late-onset (occurs at 34 weeks or later), and early delivery is the only treatment [290]. This disorder develops during pregnancy, and the rapid and complete recovery after childbirth indicate that the placenta has a pivotal role in the pathogenesis of this disease [92]. Although the aetiology of preeclampsia are still subject to debate, the basic

pathologic event in preeclampsia is an injury to the vascular endothelium [291] that is mediated by OS from increased placental ROS or decreased antioxidant activity [292]. Consequently, trophoblastic invasion to the spiral arteries is inhibited that limits the spiral artery remodelling to the decidual portions and the myometrial segments of the arteries remain narrow and contractile [293]. Therefore, in preeclampsia, increased vascular resistance in the placenta leads to reduced uteroplacental perfusion [293,294]. The resultant hypoxia or ischemia, together with intermittent perfusion, is associated with the conversion of xanthine dehydrogenase to XO and the increased XO activity provokes ROS synthesis in the placenta [295,296]. Both preeclampsia and IUGR share similar pathophysiology that is associated with defective placentation, but preeclampsia (with or without IUGR) is distinguished from IUGR (without preeclampsia) by extension of disturbances into the maternal vasculature [293,297].

In preeclampsia, both the circulating and placental tissue levels of markers of OS are elevated and antioxidant capacities are compromised [296,298]. Polyunsaturated fatty acids, which are found in abundance in the cell membrane and in circulating lipoproteins, are highly susceptible to oxidation by free radicals to form lipid peroxides, and the process is called lipid peroxidation [299]. When lipid peroxidation is initiated, it becomes self-propagating and continues until it is interrupted by an antioxidant. Normal pregnancy is associated with increased free-radical production, lipid peroxidation, and OS, however, antioxidant activity is also upregulated [179] that counterbalances free-radical generation and oxidative damage. In contrast, pre-eclampsia is associated with increased lipid peroxidation in the maternal circulation and the placenta and decreased antioxidant activity [299-301]. Superoxide anions produced by the enzyme XO in the placental mitochondria appear to be a major source of OS and contribute to an overall increase in maternal blood and placental lipid peroxidation in preeclamptic women [178,300]. Two major end-products of lipid peroxidation, MDA and 4hydroxynonenal (4HNE) are frequently measured as indicators of lipid peroxidation and OS. Increased placental and serum levels of MDA and 4HNE, and placental XO expression of preeclamptic observed compared with women is normotensive subjects [178,295,299,302,303], whereas, maternal circulating and placental levels of antioxidants, for example, CAT, GPX, and SOD are decreased in preeclampsia compared to healthy pregnancy [299,301,302,304]. Also, the expression of 8OHdG is increased in both maternal blood and the placental trophoblast in pregnancy complicated by preeclampsia with or without IUGR [281-283]. The level of Ref-1 that repairs oxidative DNA damage is also higher in the preeclamptic placenta [281-283]. Serum levels of derivatives of reactive oxygen metabolites (d-ROMs), for example, organic hyper oxides, are also increased in preeclamptic women [282,283], indicating increased ROS in maternal circulation from which they are produced. The increased ROS in the maternal circulation may originate from the placenta, as the d-ROMs decrease following delivery [282]. Additionally, in preeclamptic placentas with or without IUGR, telomeres are shorter, and telomerase activity is reduced compared to healthy placentas [252,255].

#### 2.5.4 OS and early pregnancy loss

OS has been implicated in early pregnancy loss. There is a sharp increase in oxygen tension when the maternal blood enters into the placenta, and this is associated with a burst of OS [78]. It is not until about 11–12 weeks of gestation that the maternal blood invades into the intervillous space. The arrival of oxygenated blood before 10–11 weeks leads to deterioration of the syncytiotrophoblast caused by OS, resulting in loss of pregnancy including, spontaneous miscarriage and recurrent pregnancy loss [75,268]. The high levels of OS markers, such as nitrotyrosine residues, 4HNE adducts and heat shock protein 70 in the placentas from early pregnancy loss [78], suggest that increased ROS generation is due to premature establishment of maternal-placental perfusion, resulting in oxidative damage to the trophoblasts with subsequent termination of the pregnancy [169], The expression of these markers is induced in vitro by exposing early placental villi to 21% oxygen and is associated with increased ROS production [305]. This OS in early stage of pregnancy can impair a number of cell functions including, matrix remodelling, angiogenesis, cytotrophoblasts proliferation, migration and fusion, and endocrine function [306], resulting in pregnancy loss.

# 2.5.5 OS, placental aging and stillbirth

Stillbirth, which is intrauterine fetal death at or after 20 weeks of gestation, is a major obstetric complication. Although a number of risk factors for stillbirth have been identified including advanced maternal age, obesity, smoking, late gestational age and IUGR [14,31], most cases remain unexplained. Recently studies on stillbirth have postulated an association between stillbirth and placental pathology, including infarction, vessel wall thickening and calcification, and dysfunction [50,307-309]. A 2016 study shows a significant reduction of telomere length in placentas associated with unexplained stillbirth indicating a telomere-dependent senescence in the placenta, and suggesting that this may cause premature placental

aging and placental dysfunction leading to fetal death [307]. We have hypothesised that OS causes changes in proteins, lipids and DNA in the placenta, which may induce a form of advanced aging, leading to placental insufficiency and an inability to meet the demands of the growing fetus that ultimately causes fetal demise [50].

# 2.6 Summary

There is accumulating evidence that demonstrates an association between OS and placental aging that contribute to poor pregnancy outcomes. Altered cellular metabolism is observed in several pathological situations, and these metabolic shifts that elevate ROS generation can increase telomere shortening or induce telomerase dysfunction leading to premature senescence and aging. Conversely, dysfunctional telomerase may itself induce altered metabolic and mitochondrial functions that may, in turn, cause further OS deregulation. OS also activates processes or mediators that cause inhibition of cellular proliferation or increased apoptosis. Premature placental aging is the consequences of OS-induced damage to lipids, proteins, and DNA in the placental tissue that may cause cellular senescence or cell death in the placenta, leading to placental dysfunction and insufficiency. OS-induced endothelial dysfunction contributes to the pathogenesis of pregnancy complications including, preeclampsia, IUGR, preterm birth, recurrent pregnancy loss. Alteration of antioxidant capacity or changes apoptosis regulation in the placenta are also significant factors that contribute to the pathophysiology of abnormal pregnancies.

# **CHAPTER 3**

# Is there a Role for Placental Senescence in the Genesis of Obstetrical Complications and Fetal Growth Restriction?

This Chapter contains a review article published in the American Journal of Obstetrics and Gynecology, and is reproduced with permission. The format of the paper has been altered for the purposes of this thesis. The original paper is included in appendix A.

# Is there a Role for Placental Senescence in the Genesis of Obstetrical Complications and Fetal Growth Restriction?

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# Declaration

I, Zakia Sultana, attest that I have made a primary and original contribution to the above publications as detailed below and endorsed by my supervisors. I was involved in the conception and designed of the review, I undertook the research to find the publications referred to in the review. I primarily wrote the review, involved in designing and preparing the figures and tables, and I edited and approved the final version of the review.

Zakia Sultana

Laureate Professor Roger Smith

#### 3.0 Abstract

The placenta ages as pregnancy advances yet, its continued function is required for a successful pregnancy outcome. Placental aging is a physiological phenomenon; however there are some placentas that show signs of aging earlier than others. Premature placental senescence and aging are implicated in a number of adverse pregnancy outcomes, including fetal growth restriction, preeclampsia, spontaneous preterm birth, and intrauterine fetal death. Here we discuss cellular senescence, a state of terminal proliferation arrest and how senescence is regulated. We also explore the role of physiologic placental senescence and how aberrant placental senescence alters placental function contributing to the pathophysiology of fetal growth restriction, preeclampsia, spontaneous preterm labour/birth and unexplained fetal death.

# 3.1 Cellular senescence and aging

A key feature of aging is a progressive loss of function at the cellular, tissue and organ level, resulting in a reduced adaptability to stress and an increased vulnerability to disease and mortality [163]. In mitotic tissues, the progressive accumulation of senescent cells is thought be one of the causal factors of aging [164]. Thus, the biomarkers of cellular senescence can be used as markers of tissue aging. Such biomarkers of cellular senescence have been summarized in a later section (see section '3.1.3 Biomarkers of senescence'). Senescent cells within tissues contribute to the aging process and disease development by altering normal cellular function, changing the behavior of neighbouring cells, degrading structural

components such as the extracellular matrix and accelerating the loss of tissue regeneration capacity by reducing stem and progenitor cells [164]. Elimination of senescent cells can delay aging-associated disorders in mice [168].

Cellular senescence is a state of irreversible, terminal arrest of cell proliferation (growth), triggered by a plethora of intrinsic and extrinsic stimuli or stressors. These stimuli or stressors include short or dysfunctional telomeres, DNA damage (telomeric or genomic DNA) and DNA damage response mediators, strong mitogenic signals (e.g., overexpression of oncogenic renin-angiotensin system (RAS), a mutant RAS-p21 protein, RAS involves transmitting signals and activating signalling cascades, including mitogen-activated protein kinase (MAPK) and phosphoinositide 3-kinase/mammalian target of rapamycin complex pathways), epigenomic disruption (chromatin disruption), over expression of certain oncogenes, deteriorating mitochondrial function and oxidative stress created by reactive oxygen species (ROS) (Figure 3.1) [165-167].



Figure 3.1 An overview of cellular senescence. Telomere-dependent replicative senescence and stress-induced premature senescence act through the modulation of proteins p53 and RB. Senescence stimuli, such as DNA damage, strong mitogenic signals, overexpression of oncogenes, epigenomic disruption, telomere dysfunction and ROS engage in cell signalling cascades that cause activation of one or both of the pathways that regulate cell senescence, the p53-p21 and p16-pRB pathways. Activation of p53 induces the expression of a CDK inhibitor, p21. Senescence stimuli, which involve the p16-pRB pathway upregulate the expression of another CDK inhibitor, p16. Both p21 and p16 suppress the phosphorylation and inactivation of pRB, and hereby maintain its hypophosphorylated and active state. Active pRB halts cell cycle progression by inhibiting gene transcription via downregulating transcription factor E2F. Senescent cells remain metabolically active, despite their terminal growth arrest, and secrete proinflammatory cytokines, chemokines, growth factors, and proteases, collectively termed the senescence-associated secretory phenotype.

The stressors that trigger senescence act by two major pathways controlled through stabilization of the tumor suppressor protein p53, and transcriptional inactivation of the cyclin-dependent kinases (CDKs). The suppression of CDKs is produced by transcriptional activation of the CDK inhibitor p21 (also termed p21<sup>Cip1</sup>), in concert with the CDK inhibitor, p16 (also known as p16<sup>INK4a</sup>) and pRB (retinoblastoma tumor suppressor protein) [310,311]) (Figure 3.1). When activated, p53 inhibits cell proliferation via activation of its transcriptional target p21 [311]. Both p21 and p16 maintain the protein pRB in its hypophosphorylated and active state [311,312]. Active pRB suppress the E2F1 (a member of E2F family of transcription factors which induce gene transcriptions that are essential for cell proliferation)-dependent expression of genes that regulate progression of G1/S phase of the cell cycle, and thereby irreversibly blocks cell cycle entry [313] (see Panel 3.1 for cell cycle). Silencing of E2F target genes is mediated by pRB-dependent reorganization of chromatin into distinct heterochromatin structures that accumulate in the nucleus of senescent cells termed senescence-associated heterochromatin foci (SAHF) [312]. Interestingly, a senescent cell can re-enter the cell cycle following inhibition of p53 if the cell senescence occurred due to activation of the p53-p21 pathway, however, cells that senesce solely via the p16–pRB pathway are unable to resume proliferation even after inhibition of p53, pRB or p16 [314].

# Panel 3.1 : Cell cycle

The cell cycle or cell-proliferation cycle is a series of events that take place in a mitotic cell in order to produce two daughter cells. In eukaryotic cells, the stages of the cell cycle are divided into two major phases: interphase and the M phase.

**Interphase:** During interphase the cell grows in size and makes a copy of the cell's DNA (called DNA replication) to prepare for the cell division. The interphase is comprised of three stages:  $G_1$ , S, and  $G_2$ .

- **G**<sub>1</sub>. In the first gap phase the cell increases in size, copies organelles, and makes the molecular building blocks it will need in later steps. The G<sub>1</sub> checkpoint control mechanism ensures that everything is ready for DNA synthesis.
- **S phase.** DNA synthesis occurs during this phase. It also duplicates a microtubuleorganizing structure called the centrosome. The centrosomes help separate DNA during M phase.
  - G<sub>2</sub>. Cell continues to grow in the second gap phase, synthesises proteins and organelles. During this phase microtubules begin to reorganize to form a spindle. The G<sub>2</sub> checkpoint control mechanism ensures that everything is ready to enter the M phase and divide.

**M phase:** During the M phase, cell growth stops and cellular energy is focused on the orderly division into two daughter cells. At this stage cell separates its DNA into two sets and divides its cytoplasm, forming two new cells.

G1, gap 1; G2, gap 2; M, mitotic; S, synthesis phase.

# 3.1.1 Causes of cellular senescence

A critically short telomere is thought to be one factor initiating cellular senescence. Telomeres are highly conserved repetitive DNA regions, consist of tandem arrays of the hexanucleotide sequence TTAGGG in the human, which is typically 10–15 kb long [315], located at the end of linear chromosomes, and are essential for chromosomal stability and cell survival [244,316]. Telomeres protect DNA ends from double-strand breaks, end-to-end fusion and degradation by forming a protective cap with a guanine-rich single-stranded telomere overhang and telomere binding protein complexes [243,317]. Because of an inability to replicate telomeric DNA at the ends of chromosomes (known as the end-replication problem of eukaryote DNA), telomeres are progressively shortened every time a cell divides [164]. When telomeres reach a critical minimum length, their protective structure is distorted, resulting in the exposure of DNA ends and a DNA damage response, which lead to the activation of the cellular senescence pathway [166,244,249,318]. This phenomenon is commonly known as replicative senescence. Telomere shortening is also accelerated as a consequence of environmental stressors and insults, such as hyperglycemia, hypoxia, and oxidative stress [245-248], which lead to the oxidation of the guanosine residues.

Telomere length is regulated by the enzyme telomerase, which is a specific reverse transcriptase capable of adding telomeric repeats to the ends of the chromosome [254]. Telomerase consists of a catalytic protein component, telomerase reverse transcriptase and an RNA template component, telomerase RNA component. Telomerase reverse transcriptase is considered to be the rate-limiting factor in the telomerase activity [255]. The absence of a functional telomerase or loss of telomerase activity leads to a progressive telomere shortening during cell division, resulting in telomere-dependent replicative senescence and an inability to further divide when a critically short telomere length is reached [249,254,319].

Senescence can also be induced independently of telomere length by a process termed premature senescence. Premature senescence leads to premature aging and is linked to several disease process [181]. Premature senescence occurs as a consequence of progressive DNA damage and the DNA damage response, telomere uncapping and telomere dysfunction caused by extrinsic or intrinsic stressors including oxidative stress by ROS, resulting in end-to-end fusion and aggregation of telomeric DNA [241,252,253]. ROS stimulates senescence by inducing DNA damage, and by engaging p53-p21 and p16-pRB signal transduction cascades, either directly or indirectly [166]. Genomic damage or epigenomic perturbation, including dysfunctional telomeres and DNA double strand breaks, activates the DNA damage response. The signal transduction pathways then lead to arrest of the cell cycle [320].

### **3.1.2** Features of cellular senescence

Senescent cells are distinct from their proliferation-competent counterparts; the former display altered characteristics, morphologically, in gene and protein expression, and in the activation of key signalling constituents [164]. Morphologically, senescence cells are enlarged, multinucleated, often double in volume and adopt a flattened or more spindle-shaped morphology, depending on the type of senescence inducer [166]. Senescent cells are resistant to apoptosis or programmed cell death through the overexpression of the anti-apoptotic Bcl-2 protein, leading to the accumulation of these cells within tissues [251].

Senescent cells display significant changes in their secretory phenotype. Senescent cells remain metabolically active, despite their terminal growth arrest, and secrete proinflammatory cytokines, chemokines, growth factors, and proteases, collectively termed the senescence-associated secretory phenotype [321]. The expression of interleukin (IL)-1*a*, IL-1 $\beta$ , IL-6, IL-8 and tumor necrosis factor- $\alpha$  have been shown to increase in senescent cells [167,322]. Increased expression of matrix metalloproteinases (enzymes that degrade extracellular matrix proteins such as collagen and elastin) is also common [323]. The senescence-associated secretory phenotype in senescent cells can induce senescence in neighbouring cells [324], alter the behaviour of surrounding cells and tissue homeostasis by activating various cell-surface receptors and their signal transduction pathways, and induce tumorigenesis and malignant progression of nearby premalignant cells [167,311,320,325]. Senescence-associated  $\beta$ -galactosidase (SA- $\beta$ -gal) activity is increased in senescent cells and has been widely used as a biomarker for cellular senescence [326]. The SA- $\beta$ -gal most likely derives from increased lysosomal beta-galactosidase, associated with the increased lysosomal biogenesis that occurs in senescent cells[327]. Despite its apparent specificity for senescent cells, SA- $\beta$ -gal is not required for senescence [327].

Perturbation of mitochondrial homeostasis is also an important characteristic feature of cellular senescence. Aging is generally linked to a progressive mitochondrial dysfunction [328]. Mitochondrial dysfunction is characterised by increased ROS generation, impaired mitochondrial dynamics (imbalance in fission and fusion; typically more fusion, resulting in the formation of abnormally enlarged mitochondria); depolarization of the inner membrane, which stalls the mitochondrial electron transport chain; reduced 5' adenosine triphosphate (ATP) generation and increased 5' adenosine monophosphate-activated protein kinase (AMPK) activation, reduced nicotinamide adenine dinucleotide oxidase/nicotinamide

adenine dinucleotide hydroxide (NAD<sup>+</sup>/NADH) ratio and altered metabolism, and mitochondrial Ca<sup>2+</sup> accumulation [329]. These changes in mitochondrial function can induce the activation of p53-p21 and/or p16-pRB signalling pathways that eventually lead to cellular senescence (Figure 3.2) [329].



**Figure 3.2 Perturbation of mitochondrial homeostasis.** Changes in mitochondrial function trigger cellular senescence via activation of p53-p21 and/or p16-pRB signal transduction cascades.

Increased mammalian target of rapamycin complex 1 (mTORC1) kinase activity is a common feature of senescent cells. mTORC1 is a conserved serine/threonine kinase, belonging to the phosphoinositide 3-kinase family that induce anabolism by regulating protein translation, nucleotide and lipid biogenesis, and inhibit the catabolic process by blocking autophagy (a process that involves fusion of acid and proteolytic enzyme containing lysosomes with autophagosomes that contain damaged organelles and misfolded proteins that is central to the cell recycling system) [330]. Persistent mTORC1 signalling in senescent cells may result from defects in sensing of amino acids and growth factor starvation [331]. In senescent cells increased mTORC1 activity promotes protein synthesis while inhibiting cellular proliferation [325,332]. mTORC1 activation activates intracellular signalling cascades that regulate mitochondrial function and apoptosis [333] while concurrently inhibiting autophagy [334], which leads to the accumulation of damaged cellular contents including misfolded proteins, as well as lipid droplets that can be seen by light microscopy as granular cytoplasmic inclusions surrounding the nucleus of senescent cells [335,336]. Interestingly, mTORC1 inhibition by rapamycin not only delays the progression of cellular senescence but also prevents the permanent loss of proliferative capacity and allows the arrested cells to re-enter the cell cycle [325,337-339]. Rapamycin can also prolong life span of various species including yeast, flies and mice [333,340,341] by blocking the effects of mTORC1 [325,333]. In addition, metformin, a popular hypoglycaemic agent, has recently

been shown to extend longevity in worms [342] and mice [343], possibly by modulating several age-related pathways, including, mitochondrial function and AMPK activity and the nutrient-sensing mTORC1 pathway [340].

#### **3.1.3** Biomarkers of senescence

The importance of senescence in aging and several age-related pathologic conditions has led to the identification of several senescence biomarkers (Table 3.1). The current methods to assess biomarkers of cell and tissue senescence have been reviewed by Bernardes et al. [344]. Expression of  $\beta$ -galactosidase (SA- $\beta$ -gal) is known to be one of the well-characterized and simplified methods to detect senescence *in vitro* culture cells as well as for aged tissues *in vivo*. The assay which measures SA- $\beta$ -gal activity expressed by senescent cells that can be detectable at pH 6.0 by immunohistochemistry [326]. SA- $\beta$ -gal is expressed in senescent cells, and but not in other cell types and is shown to increase in an age-dependent manner in human skin samples [326] and therefore is a widely used and reliable marker for detection of senescent cells in a variety of species and pathologic conditions [345-350].

Another important biomarker of senescence is senescence-associated heterochromatin foci (SAHF), both in cultured cells and *in vivo*. In the senescent cell nucleus, the chromatin undergoes dramatic remodelling through the formation of domains of facultative heterochromatin foci, called SAHF, [351], which can be visualised under microscopy as 4,6-diamidino-2-phenylindole (DAPI)-stained punctate areas. SAHF irreversibly silence and repress several E2F-target genes, (e.g., Cyclin A) [351] and are triggered by p16 or p53

pathway activation [352]. Transcription starting sites are absent in SAHF regions, which are enriched in transcription-silencing histone, for example, HP1, macroH2A, H3Lys9me3 (trimethylation of lysine 9 in histone 3) [352]. Other protein complexes that have shown to be accumulated at SAHF include chromatin regulators HIRA, Asf1, and HMGA, which are considered as valuable biomarkers of senescence [344].

The senescence-associated secretory phenotype, which is characterized by the secretion of inflammatory signals that resembles a local immune response, is a hallmark of senescent cells. The expression of inflammatory cytokines (IL-6) or chemokines (IL-8) has been extensively used as biomarkers for measuring senescence in cells and in tissue [353]. p16-pRB and p53-p21 are two major cellular pathways that are involved in induction of cellular senescence as described in previous section. Increased levels and/or activity of p16, p53 and p21 have been shown to be associated with cell senescence and are considered as important biomarkers of cell senescence and tissue aging [348,354-357]. Other cellular senescence markers include, telomere shortening and dysfunction [358,359], and activated and persistent DNA-damage response [344].

Biomarkers	Trend	References
SA-β-gal	+	[326,345-
		350]
SAHF		
H3Lyn9me3, H1, macroH2A	+	[360-362]
HMGA, HP1	+	[361,363]
HIRA, Asf1	+	[360]
SASP		
IL-6, IL-8	+	[353]
Senescence inducers		
p16	+	[348,354]
p53/p21	+	[355]
		[356,357]
		[354]
Telomere length and DDR	_	[344,358]

DDR, DNA damage response; IL, interleukin; SA- $\beta$ -gal, senescence-associated  $\beta$ -galactosidase; SAHF, senescence- heterochromatin foci; SASP, senescence-associated secretory phenotype.

# 3.2 Cellular senescence and placental aging in pathological pregnancies

# 3.2.1 Physiologic and pathologic placental senescence and aging

The placental syncytiotrophoblast is a multinucleated, single layer of terminally differentiated cells covering the chorionic villi. The layer is replenished by fusion of cytotrophoblasts with the overlying layer of syncytiotrophoblast, resulting in a huge syncytium with multiple nuclei. Mature (term) placental syncytiotrophoblast displays molecular markers of cellular senescence, for example, SA-β-gal, and an increased expression of the CDK inhibitors p16 and p21, and tumor suppressor p53 [364]. Heterochromatin foci can be seen within the nuclei resulting from reorganisation chromatin structures [336]. Evidence of oxidative damage and aging in the syncytiotrophoblast increases as gestation advances [229] and is associated with mammalian target of rapamycin complex activation and telomere shortening.

Fusion of cytotrophoblasts with the syncytiotrophoblast is a physiologic process by which differentiated cytotrophoblast cells are incorporated into the syncytiotrophoblast that starts at around 12 weeks and continues until term [365]. This process is essential to achieve the rapid and extensive expansion of the placental villi, contributing to the overall growth of the placenta and constant damage repair of the chorionic villi which is accomplished through further fusion with underlying cytotrophoblasts. This process requires an endogenous human defective retroviral element encoding ERVWE1, also known as syncytin-1 (syncytin-A in mice), which is expressed in all trophoblast cell lineages. Expression of ERVWE1 causes cell fusion a process that induces cellular senescence in normal and cancer cells [364]. In syncytin-A knockout mouse embryos, failure of cytotrophoblast cell fusion results in intrauterine growth restriction (IUGR) and fetal demise in mid-gestation [366]. In humans, a reduced expression of syncytin-1 has been observed in placentas associated with IUGR and preeclampsia [367].

Trophoblast senescence is a physiological phenomenon and is expected to progress gradually as pregnancy advances to term, that is with placental aging. However, premature or accelerated senescence and aging can occur as a result of placental stress that can lead to placental and clinical pathology. Premature or accelerated senescence happens when the placenta encounters stressors including, oxidative, mitochondrial or endoplasmic reticulum stress, which therefore contribute to the pathophysiology of pregnancy complications, such as preeclampsia and fetal growth restriction (FGR). Low levels of stress can induce adaptive responses, including upregulation of antioxidant capacities and cell turnover by autophagy, moderate levels may interfere with stem cell behavior and reduce cell proliferation, while elevated levels of stress can cause the release of proinflammatory cytokines and antiangiogenic factors, and may contribute to the pathophysiology of preeclampsia, while chronic stress may accelerate senescence of the trophoblast [368]. The consequences of accelerated senescence in the cytotrophoblast/syncytiotrophoblast are potentially compromised placental nutrient transport that can cause compromised fetal growth, with or without preeclampsia.

Likewise, maternal decidual cells and fetoplacental membranes display features of senescence as pregnancy approaches term [250,336]. A progressive natural physiologic senescence and aging of decidual cells and placental membranes maybe important for modulating the cell signalling pathways that are required for the onset of labour at term. Increased expression of cellular senescence signals, including p53, p21, senescence-associated secretory phenotype (IL-6 and IL-8) and SA- $\beta$ -gal from both the maternal decidua and fetal membranes has been found to be associated with labour at term [250,262], which may contribute to human parturition. Early secretion of the senescence-associated inflammatory signals (such as IL-1 $\beta$ , IL-6 and IL-8) due to senescence of the chorioamniotic

membranes triggered by pathologic processes may promote premature membrane rupture and spontaneous preterm labour [336,369]. It is likely that placental aging determines pregnancy duration and parturition [370], and premature aging may lead to early onset of labour.

## 3.2.2 Placental senescence in small for gestational age fetuses and neonates

FGR, also called small for gestational age is defined as an estimated fetal weight below the 10th percentile for gestational age [371] and affects more than 15% of pregnancies worldwide [372]. Poor placentation and placental dysfunction are known to predispose to FGR. Placental dysfunction due to the failure of trophoblast invasion and maternal spiral artery transformation, caused by ROS-mediated oxidative stress [104,279,373], has been reported in FGR [374]. ROS-induced oxidative damage affects membrane lipids, proteins, and nucleic acids (both DNA and RNA) [373]. In genomic and mitochondrial DNA, 8-hydroxy-2'-deoxyguanosine (80HdG, an oxidized derivative of deoxyguanosine) is one of the predominant forms of ROS-induced oxidative DNA lesions and has therefore been widely used as a biomarker for oxidative DNA damage. The level of 80HdG is reported to be significantly higher in placentas associated with FGR [281-283].

Increased trophoblast senescence has been observed in FGR. FGR placentas display senescence markers, including short telomeres, telomere aggregation or dysfunction and a reduction of telomerase activity [252,255,285-287,375-377]. Specifically a strong association between reduction of placental trophoblast telomere length and FGR pregnancies

has been reported [255,285,376-378]. An absent or a decrease in telomerase activity is also observed in the placentas from FGR pregnancies [255,285-287]. FGR placentas display upregulation of the senescence markers p21 and p16, tumor suppressor protein p53, IL-6 and a reduced expression of anti-apoptotic protein Bcl-2 [241,285]. There is also an elevated level of SAHF in FGR placenta [378]. The presence of oxidized DNA as 80HdG, is increased in placental trophoblast complicated by FGR [241]. Overall, there is a strong association between reduction of telomere length in placental trophoblast and DNA damage and FGR, suggesting that senescence in trophoblast cells may contribute to the etiology of FGR.

#### 3.2.3 Preeclampsia and placental senescence

Preeclampsia is a hypertensive disorder of pregnant women and often occurs in association with FGR. Preeclampsia is the leading cause of maternal and neonatal death and preterm birth, affecting 5–7% pregnancies worldwide [177,288,289]. Preeclampsia is characterised by new-onset maternal hypertension (blood pressure  $\geq$ 140/90 mm Hg), diminished uteroplacental blood flow, proteinuria ( $\geq$ 300 mg/24 hours), and oedema[177]. An injury to the vascular endothelium is the basic pathologic event in preeclampsia [291,373], caused by placental oxidative and endoplasmic reticulum stress [292,368], which are known to trigger cellular senescence and may therefore contribute to the clinical features of this pregnancy complication. Increased placental or trophoblast senescence has been demonstrated in preeclampsia in terms of senescence biomarkers, including short telomere, telomere aggregation and dysfunction and telomerase activity, senescence-associated secretory phenotype, and expression of tumor suppressor p53, and CDK inhibitors P16 and p21. In preeclamptic placentas the formation of telomere (or nuclear) aggregate (the SAHF) is increased compared with placentas from normotensive women [252,255,379]. The expression of senescence inducers p53, p21 and p16 are higher in pregnancy complicated by preeclampsia [241,380-382]. Moreover, a high level of proinflammatory cytokines (IL-1 $\beta$ and IL-6) profile can be demonstrated in preeclampsia [241,383]. DNA oxidation as measured by expression of 80HdG in preeclamptic placenta is higher than in the healthy placentas [241].

#### 3.2.4 Placental senescence in spontaneous preterm labour/birth

Preterm birth is the leading cause of neonatal death and the second leading cause of infant mortality [384]. Spontaneous preterm birth may occur after the spontaneous onset of labour with or without preterm premature rupture of the membrane (pPROM). Both term and preterm labour occur through activation of a 'common pathway' characterised by increased myometrial contractility, cervical ripening (dilatation) and decidua/chorioamniotic membrane activation, and chorioamniotic membranes rupture [259,385], and is likened to an inflammatory activation, particularly of cytokines and chemokines, in the gestational membranes [386]. In term delivery, physiological signals activate the pathway to labour, while in preterm labour several pathologic processes or conditions induce labour by activating one or more of the components of this pathway [259]. Labour promotes alterations of gene expression in placental membranes, which are compatible with the localised acute inflammatory response, without evidence of histologically observable inflammation [260]. Labour is also associated with expression of senescence-associated signals in the placental chorioamniotic membranes, for example, telomere length reduction, and increased expression of p53, p21, senescence-associated secretory phenotype (IL-6 and IL-8), and SA- $\beta$ -gal, mediated through the activation of the p38 MAPK pathway [262,387].

Senescent cells may transmit inflammatory (cytokines and chemokines, the senescenceassociated secretory phenotype) and senescence-promoting signals, which may cause changes in gene expression patterns in chorioamniotic membranes (overexpression of IL-8, IL-6, toll-like receptor 2 and superoxide dismutase) and in amniotic fluid (IL-1 $\alpha$ , IL-1 $\beta$ , IL-6, IL-8) that stimulate labour [260]. Increased levels of anti-inflammatory cytokines and chemokines, for example, tumor necrosis factor- $\alpha$ , IL-1 $\beta$ , IL-6, and IL-8, have been found in cervicovaginal secretions in women who deliver preterm, that are associated with early-onset labour [369], and early initiation of these inflammatory signals is likely to promote premature labour. Chorioamniotic membranes from spontaneous preterm labour without acute histologic chorioamnionitis (inflammation of the fetal membranes) show signs of cellular senescence, for examples, increased levels of CDKN1A (the gene that encodes p21) and SAβ-gal, and downregulated CDK and cyclins (CCNA2, CCNB1, and CCNE1) compared with preterm not-in-labor membranes [388]. Telomeric DNA fragments released from senescent fetal cells into the amniotic fluid may induce amniotic cell senescence via p38 MAPK activation and stimulate sterile inflammatory signals that promote parturition [263]. Although there is a strong association between inflammatory activation and labour (both term and preterm), whether these inflammatory signals result in the induction of labour remains unclear.

Premature senescence of the intrauterine tissues, especially of the fetal membranes, triggered by senescence stimuli such as oxidative DNA damage by ROS, may contribute to spontaneous preterm labour or pPROM [264-266], possibly via inflammatory signals (the senescence-associated secretory phenotype). Increased expression of the biomarkers of the senescence phenotype, for example, p53, p21 and p38 MAPK were observed in the fetal membranes in preterm births with pPROM compared with spontaneous preterm and term deliveries [266]. The senescence phenotype could be induced in vitro in term fetal membranes by exposure to cigarette smoke extract. As smoke causes oxidative stress these data suggest that ROS-mediated damage to the fetal membranes may result in premature senescence in fetal membranes in pPROM [266]. Significantly shorter telomeres are also found in fetal membranes in pPROM compared with spontaneous preterm births with intact membranes, indicating that premature senescence and aging of the placental membranes may lead to pPROM [389]. Studies using a mouse model suggest that in normal mouse pregnancy, progressive uterine decidual and fetal membrane senescence occur as term approaches [250], while uterine p53 deficient transgenic mice show premature and accelerated decidual senescence, with increased levels of p21, IL-8, and other cytokines and this is associated with spontaneous preterm birth [390-392]. Interestingly, an additional deletion of the p21 gene can prevent spontaneous preterm birth, indicating that p21-dependent senescence in the decidua causes preterm birth in mouse [391].

#### 3.2.5 Placental senescence and aging in late gestation and fetal death

There is evidence of oxidative damage and aging in late gestational tissues [229]. It has been hypothesised that in late pregnancy, fetal needs for nutrients and oxygen rises, if the demands

exceed the placenta's ability to transfer, the placenta experiences stress that stimulates ROS generation and oxidative stress, and the resulting oxidative damage leads to aging in the placental tissue [50,306]. The risk of fetal death increases exponentially late in pregnancy, especially after 41 weeks of gestation [393,394], suggesting that placental aging plays a key role in the clinical features of this complication.

A recent study by Maiti et al. reported that placentas from unexplained intrauterine fetal death display evidence of oxidative damage and aging [229]. Increased expression of 8OHdG (a marker of DNA oxidation) and 4-hydroxynonenal (a marker of lipid peroxidation) have been observed in fetal death-associated placentas [229], compared with term placentas; expression of both these markers have also been described to increase in aging tissues [395], such as the brain in Alzheimer's disease [396,397]. Also, a dysregulated lysosomal distribution and an increased autophagosome size with failure autophagosome-lysosome fusion have also been noted in placentas associated with fetal death, suggesting an overall inhibition of autophagy. Placentas from late-term pregnancies show similar changes in oxidation of DNA and lipid, lysosomal distribution, and larger autophagosomes compared with placentas from women delivered at term [229]. Increase expression of aldehyde oxidase 1 (AOX1, an enzyme that is known to involve in ROS generation [207]), is observed in placentas from both fetal death and late-term pregnancies. In vitro placental explants deprived of growth factors show similar changes in oxidation of lipid, lysosomal distribution and autophagosome size, which can be blocked by inhibitors of AOX1, suggesting that this enzyme plays a key role in placental aging [229].

Ferrari et al. [398] demonstrated that unexplained fetal death–associated placentas exhibit shortened telomeres. The authors observed an overall 2-fold reduction of telomere length in placentas from fetal death (both early and late term) with or without growth restriction compared with term live birth placentas. They also reported that the telomere length in fetal death placentas is comparable to those of pPROM, while telomeres are shorter in fetal death compared with spontaneous preterm birth [398]. Taken together, reduced telomere length, increased DNA and lipid oxidation, and inhibition of autophagy, changes that are consistent with cellular senescence and aging, indicate that placental senescence and aging is an etiological factor in fetal death.

# 3.3 Concluding remarks

Senescence has both beneficial and detrimental effects on gestational tissue, depending on the cell type and timing of onset. While physiological senescence in placental trophoblasts appears to be necessary for the formation of the syncytium, and growth and function of the placenta, it is likely that placental cell senescence plays a key role in pathogenesis of a number of adverse pregnancy outcomes, including FGR, preeclampsia, spontaneous preterm birth, and intrauterine fetal death. The senescence-associated secretory phenotype, especially matrix metalloproteinase that is released by the syncytiotrophoblast in early gestational tissue may be necessary for trophoblast penetration during the lacunar stage of very early placentation [336]. There is also a link between placental senescence and the onset of labour. Spontaneous preterm labour and pPROM may be promoted by premature and accelerated senescence of placental membranes and decidua that can be induced by several endogenous and exogenous factors, such as ROS. The physiological programming of senescence may be essential in determining the timing of labour onset. In FGR the increased expression of biomarkers of DNA damage, reduction of telomere length and telomerase activity, upregulation of senescence inducing p53 and p16, and elevated levels of senescenceassociated secretory phenotype and SAHF support the concept that placental senescence and aging contribute to FGR. There is also evidence of placental oxidative DNA damage, and premature senescence in late gestational tissues. Therefore, it would appear that aging is a key factor that may affect function in the short but important life span of the placenta.

# **3.4** Glossary of terms

**Telomeres**: highly conserved repetitive DNA regions, consist of tandem arrays of the hexanucleotide sequence 'TTAGGG' in the human. Telomeres protect DNA ends from breaks, end-to-end fusion and degradation by forming a protective cap with a guanine-rich single-stranded telomere overhang.

**Telomerase:** a reverse transcriptase enzyme, which regulates telomere length by adding telomeric repeats to the ends of chromosomes.

**Replicative senescence**: dependent on telomere length, occurs as a result of progressive telomere shortening during mitotic cell division. DNA polymerases are unable to replicate DNA at the ends of chromosomes (known as the 'end-replication problem' of eukaryote DNA) leaving ~50-200 bp of un-replicated telomeric DNA in each round of DNA replication. When telomeres reach a critical minimum length, their protective structure is distorted (leads

to dysfunctional telomeres), resulting in the exposure of DNA ends and a DNA damage response, which leads to the activation of the cellular senescence pathway.

**Premature senescence**: is independent of telomere length and occurs as a consequence of DNA damage and the DNA damage response caused by stress such as elevated reactive oxygen species, activation of oncogenes, telomere dysfunction and cell-cell fusion.

**RAS**: a mutant RAS-p21 protein, RAS involves transmitting signals and activating signalling cascades, including mitogen-activated protein kinase and phosphoinositide 3-kinase/mammalian target of rapamycin complex pathways

**Chromatin**: Chromatin is a mass of genetic material composed of DNA and proteins, primarily histones, which condenses to form chromosomes during eukaryotic cell division. Chromatin compresses the DNA into a compact unit that will be less voluminous and can fit within the nucleus. Histones help to organize DNA into structures called nucleosomes by providing a base on which the DNA can be wrapped around. Post-translational modification to histone proteins which includes methylation, phosphorylation, and acetylation, can cause disruption in chromatin structure.

**Heterochromatin**: is a chromatin variety in which DNA, which codes inactive genes ("turned off"), is more condensed and associated with structural proteins. Heterochromatin protects chromosome integrity and gene regulation. While DNA, which codes genes that are actively transcribed ("turned on"), is more loosely packaged and associated with RNA polymerases, referred to as euchromatin.

**Cyclin-dependent kinases**: a family of multifunctional enzymes that can phosphorylate various protein substrates involved in cell cycle progression.

**Cyclin-dependent kinase inhibitors, p16 and p21:** proteins which inhibit cyclin-dependent kinase and are involved in cell cycle arrest at the G1 phase.

**p53:** a tumor suppressor gene.

**pRB**: retinoblastoma protein is a tumor suppressor, which plays a pivotal role in the negative control of the cell cycle and in tumor progression. The retinoblastoma protein represses gene transcription, by directly binding to the transactivation domain of E2F genes and by binding to the promoter of these genes as a complex with E2F.

**E2F:** is a group of genes that code transcription factors, such as E2F1 and E2F2, in higher eukaryotes. The E2F family plays a crucial role in the control of cell cycle and action of tumor suppressor proteins. E2F proteins can mediate both cell proliferation and p53-dependent/independent apoptosis. The retinoblastoma protein binds to the E2F1 transcription factor that preventing it from interacting with the cell's transcription machinery.

Antiapoptotic Bcl-2: is regulator proteins that regulate cell death via apoptosis, by inhibiting apoptosis (antiapoptotic).

**ERVWE1:** ERVWE-1 gene (endogenous retrovirus group W envelope member 1) is an human defective retroviral fusogen found in humans and other primates that encodes the protein syncytin-1. Syncytin-1 is a cell-cell fusion protein, highly expressed in normal placental tissue whose function is most well characterized in placental development.

**p38 MAPK:** a member of mitogen-activated protein kinase, which mediates a wide variety of cellular behaviours in response to extracellular stimuli.

**mTORC1**: a conserved serine/threonine kinase that induces anabolism by regulating protein translation, nucleotide, and lipid biogenesis and inhibits the catabolic process by blocking autophagy.

**AMPK:** 5' adenosine monophosphate-activated protein kinase plays a key role as a master regulator of cellular energy homeostasis. The kinase is activated in response to stresses that deplete cellular ATP supplies such as low glucose, hypoxia. Cellular stresses that inhibit ATP production or increase its consumption change the AMP:ATP ratio and activate the pathway. 5' adenosine monophosphate activation positively regulates signalling pathways that replenish cellular ATP supplies, including fatty acid oxidation and autophagy.

**Reactive oxygen species:** oxygen free radicals that contain one or more unpaired electrons, produced as by-products of mitochondrial respiration and metabolism, and are capable of activating and modulating various signalling pathways, including those involved in cell growth, differentiation, and metabolism. Examples include peroxides, superoxide, hydroxyl radical, and singlet oxygen.

**SA-\beta-gal:** senescence-associated beta-galactosidase is a hydrolase enzyme that catalyzes the hydrolysis of  $\beta$ -galactosides into monosaccharides only in senescent cells. Therefore, expression of SA- $\beta$ -gal is considered to be a biomarker of cellular senescence.

**8OHdG:** 8-hydroxy-2' -deoxyguanosine is an oxidized derivative of deoxyguanosine. In genomic and mitochondrial DNA, 8-hydroxy-2' -deoxyguanosine is one of the major products of free radical-induced oxidative lesions and has therefore been widely used as a biomarker for DNA damage and oxidative stress.

**Mitochondrial fusion and fission:** Mitochondria are dynamic organelles that constantly fuse (fusion) and divide (fission) and are termed mitochondrial dynamics. Mitochondria fusion and fission are important for mitochondrial inheritance and for the maintenance of mitochondrial functions. Fusion helps mitigate stress by mixing the contents of partially damaged mitochondria as a form of complementation. Fission is needed to create new mitochondria, but it also contributes to quality control by enabling the removal of damaged mitochondria and can facilitate apoptosis during high levels of cellular stress.
### **CHAPTER 4**

# Hypothesis, Aims and Research Methodologies

#### 4.0 Summary

The first part of this chapter outlines the hypothesis based on the literature discussed in the previous chapters and the aims of this thesis. The second part of this chapter provides the methods for the studies in extended detail to that provided briefly in individual data chapters due to the complexities in the different study protocols as well as the word restrictions in published manuscripts or manuscripts prepared for publication. It is to be noted that as part of this thesis, initial experiments included studies on oxidative damage and aging of the placenta in pathological pregnancies, with later experiments investigated the pathways that mediate the oxidative damage and aging in the placenta. As such, chapters in this thesis have been presented in that order. The methods detailed in this chapter however have been presented as a cohesive protocol including all study groups.

#### 4.1 Hypothesis and aims

#### 4.1.1 Hypothesis

There is accumulating evidence that demonstrates an association between oxidative stress and placental aging that contribute to poor pregnancy outcomes, including fetal growth restriction [241,255,285,286], preeclampsia [241,252,255], preterm labour [264,388], preterm premature rupture of membranes [264,266], and fetal death [229,307]. In light of the literature discussed in the Chapter 1 (Introduction), and Chapter 2 and 3 (literature review), the general thesis hypothesis is that: Oxidative stress induced damage causes changes in protein expression, increased oxidation of lipids and DNA/RNA in the placenta, which may induce a form of advanced aging, leading to placental dysfunction and an inability to meet the demands of the growing fetus that ultimately causes fetal demise.

We have also hypothesised that: the placental oxidation is regulated by estrogen activation at the G-protein coupled estrogen receptor 1 (GPER1) leading to inhibition of aldehyde oxidase 1 (AOX1) and reactive oxygen species (ROS) generation. We proposed that loss of GPER1 activation may lead to an increase in AOX1 activation and ROS generation, which increases DNA oxidation and telomere shortening, lipid peroxidation, alters lysosomesautophagosomes function, and causes changes in protein expression and mitochondrial function. All these changes may accelerate the aging process in the placenta. The diagrammatic representation of the proposed hypothesis is shown in Figure 4.1.



**Figure 4.1** Regulation of placental oxidative damage and aging via AOX1 and GPER1 mediated pathways.

#### 4.1.2 Aims

The aims of this thesis are:

Aim 1: To measure biochemical markers of oxidative damage and aging in placentas associated with late-term and unexplained stillbirth.

**Aim 2:** To develop an *in vitro* human placental explant and a placental cell line culture model to test the pathways involved in oxidative damage and aging in the placenta.

**Aim 3:** To evaluate mitochondrial function in placental trophoblast cells under oxidative stress using the Seahorse extracellular flux analyser.

#### 4.1.3 Study design

Aim 1: Aim 1 determines if the oxidative damage and aging related changes in DNA and lipid oxidation, lysosome and autophagosome function, mRNA and protein expression for aldehyde oxidase AOX1, are altered in placentas from late-term and stillbirth associated pregnancies compared with placentas from early term pregnancies. We test the association of AOX1 in stillbirth pregnancy as an RNA sequencing study performed in our laboratory identified a significant increase in AOX1 mRNA in late-term placentas compared to term healthy placentas (unpublished work). For this, human placentas from normal term (37–39 completed weeks of gestation), late-term (>41 weeks of gestation), and stillbirth pregnancies will be collected. Villous tissues will be randomly sampled from different regions of the placentas. Placental samples will be analysed for expression of AOX1 by immunohistochemistry and RT-PCR, oxidation of DNA by immunohistochemistry for 8-hydroxy, 2'- deoxy-guanosine (80HdG), oxidation of lipid by immunohistochemistry for 4-hydroxynonenal (4HNE), lysosome location by immunohistochemistry for lysosome associated membrane protein 2 (LAMP2), autophagosome size by immunohistochemistry for microtubule-associated proteins 1A/1B light chain 3 B (LC3B). Results will be analysed to determine if there is a statistically significant difference among the groups.

The results from Aim 1 will determine whether there is evidence for oxidative damage and aging related changes in biochemical parameters in placentas from late-term and stillbirth associated pregnancy, and also confirm if there is an association between placental aging and AOX1 expression.

Aim 2: Placental explants (small villous tissue sections) and placental first-trimester extra villous trophoblast cell line (HTR8/SVneo cell line) will be used to test the proposed hypothesis that AOX1 and GPER1 mediate oxidative damage and aging in the placenta. Oxidative stress will be induced in placental explants and the HTR8/SVneo cell line and the sample will be analysed to measure the oxidative stress and aging associated biochemical parameters. Oxidative stress will be induced by culturing both placental explants and placental HTR8/SVneo cell line in serum-free (growth factors deprived) medium. To investigate whether AOX1 and GPER1 regulate the aging process in placenta, the explant and cell line will be treated with an AOX1 inhibitor, raloxifene, and a GPER1 agonist, G1 in serum-free medium. The explants will then be subjected analysis to by immunohistochemistry, ELISA and western blotting to measure changes in lipid peroxidation, autophagosome size and lysosomal location and protein expression. The cells will be tested for DNA oxidation, lipid peroxidation, and protein expression by ELISA and western blotting.

The results generated from Aim 2 will determine the role of AOX1 and GPER1 in regulating the pathways to oxidative damage in the placenta. This will provide a mechanistic basis for

understanding the aetiology of the aging phenotype of the placenta in stillbirth and other pregnancy complications. This will also confirm if AOX1 and GPER1 are appropriate targets for the development of therapeutics to slow aging in the placenta.

Aim 3: Placental first-trimester extra villous trophoblast cell line (HTR8/SVneo cell line) will be used to evaluate mitochondrial function. Oxidative stress will be induced by culturing cells of the HTR8/SVneo cell line in serum-free medium. The cells will also be pre-treated either with raloxifene, an AOX1 inhibitor, or G1, a GPER1 agonist in serum free-medium before being assessed with a Seahorse XF analyser. The assay uses the XF Cell Mito Stress Test kit that can measure real-time changes in the mitochondrial function and bioenergetics of cells. Cells will be cultured at an optimum density in cell culture microplates. In the assay, cells will be metabolically disturbed by the sequential addition of three different compounds oligomycin, carbonyl cyanide-4 (trifluoromethoxy) phenylhydrazone (FCCP), and a mix of rotenone/antimycin, that shift the metabolic profiles of the cell. The Oxygen Consumption Rate (OCR) of the cells, which is an indicator of mitochondrial oxidative phosphorylation, and the Extra Cellular Acidification Rate (ECAR), an indicator of cellular glycolytic capacity, will be measured. From the value of OCR, a variety of mitochondrial bioenergetic parameters, including basal OCR, ATP-linked OCR, maximal respiration capacity, spare/reserve capacity, proton leak and non-mitochondrial OCR will be estimated. The value of ECAR will provide an estimation of cellular glycolytic function.

The results from aim 3 will be used to determine if oxidative stress causes changes in the mitochondrial function of the placental trophoblast cells. The results will also confirm if

inhibition of AOX1 and/or GPER1 activation alter the oxidative stress induced mitochondrial dysfunction in the placental trophoblast cells.

#### 4.2 Extended methodologies

#### 4.2.1 Ethics

This study was approved by the human research ethics committee of the Hunter New England Health Services and the University of Newcastle, NSW, Australia (Ethics approval ref. number is H-2010-1210). Human placentas were collected from the delivery suite at John Hunter Hospital (New Lambton Heights, NSW) after written informed consent was obtained from the patients by midwives.

#### 4.2.2 Collection and processing of placental tissues

For Aim 1, placentas were collected from women at 38–39 weeks (term) gestation undergoing elective caesarean section (for previous caesarean section) and following normal vaginal delivery, women at 41+ weeks (late-term) gestation undergoing caesarean section or following normal vaginal delivery, and women who had stillborn infants undergoing vaginal delivery. Placentas were collected immediately after delivery and processed without further delay. Villous tissues were randomly sampled from multiple sites of the placenta and were prepared for histology, protein analysis and western blotting, and RNA extraction. For each placenta tissues were obtained from at least 5 different regions of the placenta and 4–5mm beneath the chorionic plate. Samples from each individual placenta were immediately frozen under liquid nitrogen and stored at -80 °C until subsequent experiments. For histology experiments, tissues were fixed in 2% formaldehyde for 24 hours, stored in 50% ethanol at room temperature and embedded in paraffin.

#### 4.2.3 Placental explant culture

Explants are small tissue sections, which can be cultured in a culture dish or flask with standard culture conditions. Placental explants are prepared by dissecting and mincing placental tissue into small pieces, followed by plating in a tissue culture plate and incubation in appropriate culture condition for a definite time period [399]. The explant method possesses several advantages over the use of primary cells. Firstly, the presence of the extracellular matrix (ECM) remains functional during explant culture *in vitro* and provides crucial biochemical and biomechanical signals that are essential for tissue morphogenesis, differentiation and homeostasis [400]. ECM also acts as a localised reservoir of growth factors and cytokines and controls their bioavailability. Secondly, cytokines, enzymes and growth factors are released from the ECM and the cellular components into the culture medium that are required for the growth, proliferation and function of the cells [400]. Thirdly, the explants provide a model in which a heterogeneous population of placental trophoblast cells are retained in anatomical structure with preservation of some cell-cell and cell-matrix interactions [401]. Thus, explants are useful for studying placental biochemistry and function *in vitro* as they retain some level of tissue integrity and are physiologically more relevant than a cell line.

Placental explants were used to perform part of the experiments outlined in Aim 2. For explant cultures, human term placentas (37–39 weeks of gestation) were obtained from women with normal singleton pregnancies without any symptoms of labour after an elective (a scheduled repeat) caesarean section. Placentas were collected immediately after delivery and prepared for explant culture. Placentas from patients with infection, smoking, obesity, diabetes, preeclampsia, placenta praevia, intra-uterine growth restriction or abruption were excluded.

Villous tissues of placentas were randomly sampled from different regions of placenta and 4–5 mm beneath the chorionic plate. Tissues were washed several times with Dulbecco's phosphate buffered saline (PBS) (Cat. No. 21600010, Thermo Fisher Scientific) under sterile condition to remove excess blood. Villous explants of ~2 mm<sup>3</sup> were dissected and placed into 100 mm culture dishes (30 pieces /dishes) containing 30 mL of growth medium (Dulbecco's modified Eagle's medium (DMEM, Cat. No. 31053028, Thermo Fisher Scientific) supplemented with 2 mM L-glutamine (Cat. No. 25030081, Thermo Fisher Scientific), 1% sodium pyruvate (Cat. No. 1360070, Thermo Fisher Scientific), 1% antibiotic-antimycotic solution (10,000 units/mL of penicillin, 10,000 µg/mL of streptomycin, and 25 µg/mL of Amphotericin B, Cat. No. 15240062, Thermo Fisher Scientific) solution with the addition of 10% (v/v) fetal bovine serum (FBS, Cat. No. SFBS-F, Bovogen Biologicals, Australia). Explants were cultured in a cell culture chamber at 37 °C temperature and maintaining 95% air (20% oxygen) and 5% CO<sub>2</sub> for 24 hours.

At day 2, villous explants were transferred to fresh 30 mL growth medium and incubated in a cell culture chamber for 90 min. After this the tissues were washed in serum-free medium (DMEM medium supplemented with 2 mM L-glutamine, 1% Na-pyruvate, 1% Antibiotic-Antimycotic solution without the addition of FBS). Next 6-7 pieces of villous tissue weighing approximately 400 mg were transferred to a 60 mm culture dish containing 6 mL serum-free medium with or without the addition of pharmacological agents, for example, an aldehyde oxidase 1 (AOX1) inhibitor, raloxifene (at a concentration of 100 nM) (Cat. No. R1402-500MG, Sigma-Aldrich) and G-protein coupled estrogen receptor 1 (GPER1) agonist, G1 (at a concentration 1 µM) (Cat. No. 3577, Tocris-Bioscience), for subsequent incubation for up to 24 hours. At the end of each incubation time some tissues were fixed in 2% formaldehyde, subjected to routine histological processing and embedded in paraffin wax, and some tissues were snap frozen in liquid nitrogen and stored at -80 °C until subsequent experiments (ELISA and western blotting). For each placental explant culture, samples were also collected at time '0 (zero)' hour i.e., before incubation in serum-free medium, and were formalin fixed and stored frozen at -80 °C until further experiments. A schematic representation of placental explant culture is presented in Figure 4.2.



Figure 4.2 Schematic of placental explant culture.

#### 4.2.4 Placental cell line culture

HTR8/SVneo cell line, an immortalized human trophoblast cell line, was used to perform the placental cell line experiments outlined in Aim 2. The HTR8/SVneo is originated from first-trimester placental extravillous cytotrophoblast [402]. We have chosen HTR8/SVneo cells as these cells are often considered a closer model of trophoblast cells, because they were established by immortalizing a physiologic extravillous trophoblast cell via transfection with a gene encoding the simian virus 40 large T antigen (SV40) [403].

Cell lines offer several advantages over, primary cells or explants, for example, they are cost effective, grow comparatively faster and need minimal care, can be cryopreserved for future

use, and bypass ethical concerns associated with the use of animal and human tissue [404]. Cell lines also provide a uniform population of cells, which is valuable since it provides a consistent sample and reproducible results. Moreover, in a cell line culture the micro-environment of the cells such as regulation of matrix, cell-cell interactions, can be controlled and the cells can be genetically manipulated to alter their gene expression pattern. However, the main disadvantage of using a cell line is that, serial passage of cells over an extended period of time can cause genotypic and phenotypic variation and the resulting genetic drift can also cause heterogeneity in cultures over time [404].

In this study, the HTR8/SVneo cells were maintained at 37 °C in RPMI growth medium (RPMI-1640 medium (Cat No. 11835055, Thermo Fisher Scientific) supplemented with 2 mM L-glutamine, 1% Na-pyruvate, 1% antibiotic-antimycotic solution with the addition of 10% (v/v) FBS) in a humidified atmosphere of 5% CO<sub>2</sub> and 95% air. Cells were subcultured and grown in T75 flasks to the stage of 70-80% confluence. To passage the cells, flasks were rinsed with PBS, a dilution of 1:10 of trypsin EDTA (Cat No. 15400054, Thermo Fisher Scientific) and PBS were added, and cells were incubated at 37 °C in a humidified atmosphere of 5% CO<sub>2</sub> for 5 min. Once cells were detached, 5 mL of RPMI growth medium was added to inactivate the trypsin. The cell suspension was taken in a tube and centrifuged at 125g for 5 min. The supernatant was discarded, and the cell precipitate was resuspended in 5 mL growth medium.

Cells were counted using a haemocytometer, and 7 x  $10^5$  cells were seeded in each 60 mm culture dish and cultured in 6 mL of growth medium for 24 hours, in a humidified incubator

at 37 °C at 5% CO<sub>2</sub>. The medium was then discarded, and the cells were rinsed with 6 mL of serum-free medium (RPMI medium supplemented with 2 mM L-glutamine, 1% Na-pyruvate, 1% Antibiotic-Antimycotic solution without the addition of FBS). Six mL of serum-free medium with or without the pharmacologic agent (raloxifene and G1, both at 100 nM concentrations) were added. In Control plates, growth medium was replaced with 6 mL fresh RPMI growth medium. The plates were then returned to an incubator at 37 °C with 5% CO<sub>2</sub> and cultured for 24 hours. At the end of the incubation, medium was discarded, 300 µL either PBS, or protein extraction buffer (in-house lysis buffer, composed of PBS, 1% Triton-X-100 (Cat. No. 161-0407, Bio-Rad Laboratories), 0.1 % Brij-35 (Cat. No. 20150, Thermo Fisher Scientific), complete mini protease inhibitor cocktail tablets (Cat. No. 4693124001, Roche) and PhosSTOP phosphatase inhibitor tablets (Cat. No. 4906837001, Roche), pH 7.4) was added in each culture dish for DNA and protein extraction, respectively. Cells were scrubbed from the culture dish, taken in a microfuge tube, snap frozen in liquid nitrogen and then stored at -80 °C for subsequent experiments. A schematic representation of the cell culture procedures is presented in Figure 4.3.



Figure 4.3 Schematic of placental cell line culture

#### 4.2.5 Protein analysis and western blotting

Western blotting is a widely used technique to separate and detect specific proteins from a complex mixture of proteins in cell lysates or tissue homogenates. This technique uses gel electrophoresis to separate denatured proteins based on molecular weight. The proteins are then transferred to a nitrocellulose or a polyvinylidene difluoride (PVDF) membrane producing a band for each protein. The membrane is then treated with a blocking solution (usually bovine serum albumin (BSA) or skim milk) to prevent from non-specific binding of proteins to the membrane. The membrane is then incubated with primary antibodies specific to the protein of interest, followed by secondary antibodies that bind with the primary antibodies. The unbound antibody is washed off leaving only the bound antibody to the protein of interest. The bound antibodies are then detected by developing the membrane and then it is visualised using an imaging system. As the antibodies only bind to the protein of

interest, only one band should be visible. The density of the band corresponds to the amount of protein present; the intensity of the band can be semi-quantitatively compared to a standard of known concentration.

#### 4.2.5.1 Tissue and cell lysate preparation

Samples of placental explant were crushed under liquid nitrogen. Aliquots of 50 mg of placental tissues were homogenised in 300  $\mu$ L of in house lysis buffer using a Precellyse Lysing Kit (Sapphire Bioscience) in a Precellyse 24 homogeniser (Bertin Technologies) at 5500 rpm using 3 × 30 sec homogenisation intervals with a 20 sec break between homogenisations. The lysate was centrifuged at 13,000g for 10 min at 4 °C to pellet insoluble cellular debris, and the supernatant containing protein extract was separated and stored at - 80 °C until further experiment.

For cell lysate extraction, cells with lysis buffer was sonicated using an ultrasonicator (Misonix XL-2000, Misonix, Inc.), centrifuged at 13,000g for 10 min at 4 °C and the supernatant containing cell extract was separated.

#### 4.2.5.2 Total protein concentration quantification

The total protein concentration of each tissue and cell extract was measured spectrophotometrically using a Pierce Bicinchoninic Acid (BCA) Protein Assay Kit (Cat. No. 23227, Thermo Fisher Scientific). Ten  $\mu$ L of standards and samples were loaded in duplicate

in a flat-bottomed 96 well plate. Buffer A and buffer B at a ratio of 50:1 were mixed and 200  $\mu$ L of this mixture was added into each well. The plate was placed on a plate shaker and mixed for 30 seconds, and then incubated at 37 °C for 30 min. The plate was then read at absorbance of 562 nm in a SPECTROstar Nano Microplate Reader (BMG LABTECH) and the protein concentration of samples were calculated from the standard curve using MARS Data Analysis Software (BMG LABTECH). The results of this analysis were used to calculate the volume of sample required to load a certain amount of protein onto each well of a gel.

#### 4.2.5.3 Western blotting

The expression of proteins, SIRT1, SIRT2, SIRT6 and GPER1 in the placental tissues and P-p70S6K, P-AKT and P-AMPK $\alpha$  in the placental HTR8/SVneo cell line were quantified using western blotting. To perform western blotting, protein samples (30 µg of proteins for tissue and 20 µg of proteins for cell lysates) were mixed with 5 µL of LDS sample loading buffer (Cat. No. NP0007, Thermo Fisher Scientific) containing sample reducing agent Cat. No. NP0009, Thermo Fisher Scientific). Samples loaded into each well of a gel were made up to the same final volume of approximately 20 µL by adding necessary volumes of milli Q water and then heated in a 70 °C heating block for 10 min. Samples were then centrifuged for 30 sec on a microfuge at 10,000g. Samples were then loaded in to Nu-PAGE (polyacrylamide gel electrophoresis) 4-12% Bis-Tris precast 12 well gels (Cat. No. NP0322BOX, Thermo Fisher Scientific), and proteins were then separated by electrophoresis using an electrophoresis power supply unit (Bio-Rad, CA, USA) for 50-60 min at constant

potential of 200 V using the 1X Nu-PAGE SDS MOPS running buffer (Cat No. NP000102, Thermo Fisher Scientific). MagicMark<sup>™</sup> XP Western Protein Standard (Cat. No. LC5602, Thermo Fisher Scientific) was used as a standard protein ladder. Separated proteins were then transferred to a nitrocellulose membrane (iBlot<sup>™</sup> Gel Transfer Stack, nitrocellulose, mini, Cat. No. IB301032, Thermo Fisher Scientific) for 7 min using an iBlot® Gel Transfer Device (Thermo Fisher Scientific).

After the transfer step, membranes were dried between filter papers for 30 min. The nitrocellulose membranes were rehydrated in MilliQ water for 1 min. After that the membranes were incubated in a blocking buffer containing 1% bovine serum albumin (BSA) (Cat. No. A4737-100G, Sigma-Aldrich) in TBS-T (tris-buffered saline containing 0.1 % tween-20) for an hour at room temperature (RT). The membranes were then incubated with primary antibodies (SIRT1, SIRT2, SIRT6, GPER1, P-p70S6K (Thr389), P-AKT (Ser479), P-AMPKa (Thr172)) in 1% BSA in TBS-T at a dilution recommended by the manufacturer overnight at 4 °C. The next day membranes were washed three times for 5 min each in TBS-T. The membranes were then incubated with the appropriate HRP conjugated secondary antibody diluted in 1% BSA in TBS-T for an hour at RT. After 3 further washings in TBS-T, the immunoreactive bands were developed in Luminata reagent (Cat No. 34580, Thermo Fisher Scientific) and enhanced chemiluminescence was used for protein detection using an Amersham Imager 600 (GE Healthcare). Quantification of the photographs was performed by measuring the optical density (OD) of the target bands of the blot using an Amersham Imager 600 Analyser. The loading was verified by staining the individual blots with Ponceau S solution for 5 min (0.1% w/v Ponceau S (Cat. No. P3504-50G, Sigma-Aldrich) in 5% acetic acid). Ponceau S stained blots were ringed using milliQ water and colorimetric photographs were taken using an Amersham Imager 600 (GE Healthcare). Target band densities were normalized by dividing the OD values of target proteins with the OD values of Ponceau S stained band of the same blots. For each protein target, the band with the lowest mean density was assigned an arbitrary value of 1. All individual densitometry values were expressed relative to this mean. The results were graphed using GraphPad Prism software version 7 (GraphPad Prism, CA, USA).

**Table 4.1**The antibodies, and antibody incubation conditions used for western blottingexperiment.

Antibody	Supplier	Catalogue	Species, Clonality	Antibody	Diluting
		No.	and Isotype	Dilution	Medium
SIRT1	Cell	8469	Mouse	1:500	1% BSA
(IF3)	Signaling		monoclonal IgG		
SIRT2	Cell	12672	Rabbit	1:500	1% BSA
(D4S6J)	Signaling		monoclonal IgG		
SIRT6	Cell	12486	Rabbit	1:500	1% BSA
(D8D12)	Signaling		monoclonal IgG		
Phospho-	Cell	9234	Rabbit	1:1000	1% BSA
p70S6K	Signaling		monoclonal IgG		
(Thr389)					
Phospho-	Cell	4060	Rabbit	1:1000	1% BSA
Akt	Signaling		monoclonal IgG		
(Ser473)					

Phospho-	Cell	50081	Rabbit	1:1000	1% BSA
ΑΜΡΚα	Signaling		monoclonal IgG		
(Thr172)					
GPER1	Novus	NLS1183	Rabbit	1:1000	1% BSA
	Biologicals		polyclonal IgG		
Secondary	Cell	7074	Goat	1:2000	1% BSA
Anti-Rabbit	Signaling				
IgG HRP-					
Conjugate					
Secondary	Cell	7076	Horse	1:2000	1% BSA
Anti-Mouse	Signaling				
IgG HRP-					
Conjugate					

#### 4.2.6 Confocal microscopy

Confocal microscopy is one of the methods to detect endogenous proteins and to visualize intracellular localization of proteins in intact cells and tissue section. The technology uses a laser beam with a precise wavelength selectivity and high illumination power. In confocal microscopy, the specimen is scanned by a point light source and a point detector. Because of the point scan/pinhole detection system, light which comes from the focal point are detected, while light contribution from the neighbourhood of the scanning spot in the specimen can be eliminated, allowing high Z-axis resolution. Fluorescence detection by sensitive photomultiplier tubes allows the usage of filters with a narrow band width, resulting in minimal cross-talk (overlap) between two spectra. This is particularly important in demonstrating co-localization of proteins with multicolour labelling.

#### 4.2.6.1 Preparation of slides

Fluorescent immunohistochemistry (IHC) was performed on formalin-fixed, paraffinembedded tissue sections collected from fresh placental tissues and placental explants. Representative paraffin-embedded tissue specimens were sliced to 6 µm thick sections using a Microtome (RM2265 Leica Biosystems, Germany). Each individual section was attached onto a glass slide using a water bath that was pre-warmed at 40 °C, air dried and stored at RT in a microscope slide storage box. The specimen slides were deparaffinised by placing tissue slides in 3 changes of xylene (5 min each), rehydrated in descending grades of ethanol to distilled water. Heat-induced epitope retrieval was performed in a microwave oven maintaining a sub-boiling temperature for 15 min in citrate buffer at pH 6 or tris-EDTA buffer at pH 9.5 as recommended by supplier and then allowed to cool at RT for 20 min. Liquid Blocker Super Pap Pen, (Cat. No. ID300, Pro Sci Tech) was used to encircle the tissue section to ensure that reagents would remain on the section during incubations. To block any nonspecific binding, the slides were incubated with 1% BSA in TBS-T for an hour at RT in the dark in a humidified slide chamber. Afterward, the slides were incubated overnight at RT in a humidified chamber with primary antibodies (80HdG, Lamp2, 4HNE and LC3B, AOX1, GPER1) at a dilution recommended by suppliers. As a negative control, the primary antibody was replaced with a corresponding IgG Isotype control (mouse or rabbit) in order to confirm its specificity. The sections were washed three times 5 min each with TBS-T before incubating with Alexa-conjugated corresponding secondary antibodies (anti-rabbit or antimouse) for 2 hours. The slides were washed further for 3 times 5 min each in TBS-T. Following incubation with the first set of primary and secondary antibodies, double staining by a second set of primary and secondary antibodies was also performed using the same procedures in order to detect the co-localisation of the two different proteins. The slides were mounted with ProLong<sup>™</sup> Gold Antifade Mountant with DAPI (Cat. No. P36962, Thermo Fisher Scientific), covered with glass coverslips, allowed to dry at RT in the dark and stored in the dark until imaged.

#### 4.2.6.2 Imaging

The fluorescent photographs were captured from six representative fields on a prepared slide with a Nikon eclipse 90i confocal microscope or a Nikon eclipse Ti fluorescence microscope (Nikon Instruments Inc.) equipped with 405 nm, 488 nm and 561 nm laser lines. Images were digitally recorded by Nikon NIS Element Advanced Research Microscope Imaging Software (Nikon Instruments Inc.). Within the program, the red colour channel with the wavelength of 555 nm was used for the detection of 8OHdG, 4HNE and LAMP2, the green channel with the wavelength of 488 nm for LC3B, AOX1, and GPER1, while the blue channel with the wavelength of 405 nm was used for the detection of DAPI (nucleus). Slides were first examined at 10X magnification and then the images were recorded at 20X, 40X, or 100X magnification as specified in the individual figure legend. Quantification of protein expression was performed by measuring the integrated optical density of all positive staining for the antibodies and its ratio to the total tissue area of each photograph was calculated as the intensity of the proteins using Nikon NIS Element Advanced Research Microscope Imaging Software (Nikon Instruments Inc.). To assess proteins staining, a uniform setting was maintained for all slides.

#### **Table 4. 2**The antibodies, and antibody incubation conditions used for

immunohistochemistry.

Antibody	Supplier	Catalogue	Species,	Antibody	Diluting
		No.	Clonality	Dilution	Medium
			and Isotype		
80HdG	Abcam	ab62623	Mouse	1:3000	1% BSA
			monoclonal IgG		
4HNE	Abcam	ab48506	Mouse	1:200	1% BSA
			monoclonal IgG		
LC3B	Novus	NB600-	Rabbit	1:200	1% BSA
	Biologicals	1384	polyclonal IgG		
Lamp2	BD	555803	Mouse	1:200	1% BSA
	Biosciences		monoclonal IgG		
AOX1	Proteintech	19495-1-	Rabbit	1:200	1% BSA
		AP	polyclonal IgG		
GPER1	Novus	NLS1183	Rabbit	1:200	1% BSA
	Biologicals		polyclonal IgG		
Rabbit	Cell	3900	Rabbit	1:1000	1% BSA
IgG	Signaling		monoclonal IgG		
Isotype					
Control					
Mouse	Cell	5415	Mouse	1:1000	1% BSA
IgG	Signaling		monoclonal IgG		
Isotype					
Control					
AlexaFLuor-	Thermo	A-11008	Goat	1:1000	1% BSA
488 Anti-	Fisher				
Rabbit IgG	Scientific				

AlexaFluor-	Thermo	A-21422	Goat	1:1000	1% BSA
555 Anti-	Fisher				
Mouse IgG	Scientific				

#### 4.2.7 RNA extraction and real-time PCR

In the real-time polymerase chain reaction (RT-PCR), also called quantitative PCR, the progress of the PCR reaction is detected as it occurs in real time. The advantages of the technique include sensitivity, large dynamic range, accurate quantification, enhanced specificity, and the ability to measure the expression of low abundance transcripts [405]. During RT-PCR assay, the amount of product generated is measured throughout the reaction cycles by monitoring the fluorescence of the dyes added to the reaction. The fluorescent signal in the reaction is directly proportion to the amount of the amplified DNA. The number of amplification cycles (cycle threshold, Ct) required for the fluorescent signal to reach the threshold is recorded. The Ct values are inversely proportional to the amount of target gene in the sample. The data obtained can be quantified by normalisation to a housekeeping gene. The basic steps involved in generating an RT-PCR result include RNA extraction, the generation of cDNA by reverse transcription, and measurement of the cDNA of the gene of interest by RT-PCR [405].

#### 4.2.7.1 RNA extraction

Placental tissues were crushed under liquid nitrogen. Approximately 100 mg of crushed placental tissues were homogenised in 2 mL of Trizol reagent (Cat. No. 15596018, Thermo

fisher Scientific) by Ultra Turrax homogenizer. Total RNA was extracted from Trizol-extract by Direct-zol<sup>™</sup> RNA MiniPrep (Cat No. R2052, Zymo Research) following the manufacturer instructions. The RNA was treated with DNAse 1 (Cat. No. AMPD1-1KT, Sigma Aldrich) to remove potentially contaminating DNA and was purified by a RNA Clean & Concentrator<sup>™</sup>-5 kit (Cat. No. R1016, Zymo Research). RNA concentrations were measured by a spectrophotometer using a NanoDrop 2000 spectrophotometer (NanoDrop Technologies). The RNA quality was observed by running the DNAse 1 treated sample in agarose gel with ethidium bromide in 1X TAE buffer (Cat. No. AM9869, Thermo Fisher Scientific).

#### 4.2.7.2 Reverse transcription of RNA to cDNA

For each sample, 1  $\mu$ g of purified RNA was reverse-transcribed to cDNA using a SuperScript® III First-Strand Synthesis System kit (Cat. No. 18080051, Thermo Fisher Scientific) following the manufacturer's protocol. Briefly, 1  $\mu$ g of the RNA was mixed with 1  $\mu$ L of random primer and 1  $\mu$ L dNTP mix (10 mM), adjusted the volume to 10  $\mu$ L with ultrapure (nuclease free) water, incubated at 65 °C for 5 min and placed on ice for 1 min. Ten  $\mu$ L of cDNA master mix containing 2  $\mu$ L of 10X RT buffer, 4  $\mu$ L MgCl<sub>2</sub> (25 mM), 2 $\mu$ l of 0.1 MDTT, 1 $\mu$ l of RNase inhibitor (49 U/ $\mu$ L) and 1  $\mu$ L of Reverse transcriptase was added. The samples were incubated at 25 °C for 10 min, followed by 50 °C for 50 min, and then 85 °C for 5 min to terminate the reactions. The sample was then chilled on ice and stored at -30 °C. The obtained cDNA was used as template to run quantitative RT-PCR.

#### 4.2.7.3 Real-time quantitative polymerase chain reaction (RT-qPCR)

To determine the mRNA levels of the genes of interest in the cDNA sample, quantitative RT-PCR was conducted using a 7500 PCR system (Applied Biosystem). To quantify mRNA expression for aldehyde oxidase 1 (AOX1) we used a Taqman gene expression assay for AOX1 (Assay ID: Hs00154079\_m1, Cat. No. 4331182, Thermo Fisher Scientific) and Taqman gene expression master mix (Cat. No. 4369016, Thermo Fisher Scientific) with 18s ribosomal RNA (Cat. No. 4319413E, Thermo Fisher Scientific) as a housekeeping gene. To quantify mRNA expression for G-protein coupled receptor 1 (GPER1) we used SyBr green master mix to (Cat. No. 4368708, Thermo Fisher Scientific) and a GPER1 primer (Forward primer 5'-CGTCCTGTGCACCTTCATGT-3' Backward primer 5'-AGCTCATCCAGGTGAGGAAGAA-3') with beta-actin as the housekeeping gene.

In this reaction, PCR products are quantitatively synthesized using the gene expression master mix with cDNA from the reverse transcription step as template. In each reaction,  $4 \mu$ L of the cDNA template was mixed with 10  $\mu$ L of 2X master mix, 1  $\mu$ L of gene expression assay (or primer) and 5  $\mu$ L of nuclease free water to a total volume of 20  $\mu$ L. Then the PCR reaction mix was transferred into each well of a 96 well PCR plate, centrifuged briefly and the plate loaded into the instrument to run the PCR reaction using different temperature cycles. Gene expression levels were calculated by the comparative Ct method, relative to the housekeeping genes. The relative abundance of the target gene was determined using the formula  $2^{\Delta\Delta Ct}$ . All samples were reverse-transcribed in duplicate, and cDNA was run in triplicate to allow assessment of sample homogeneity and technical variability.

#### 4.2.8 Enzyme-linked immunosorbent assay (ELISA)

ELISA is a sensitive and specific assay for the detection and quantitation of antigens or antibodies in a biological fluid or a test solution. In an ELISA, antibody or antigen is passively adsorbed to solid surfaces such as the well of a plastic 96 well plate [406]. Since the antibody or antigen is bound to the plate, bound and free reagents are easily separated by washing procedures [212]. The final result is a colour reaction that can be observed by eye, and read accurately using a spectrophotometer [406]. In a competitive ELISA, the well of an ELISA plate is coated with a known quantity of enzyme-labelled antigen as reference antigen, the antibody and the unknown sample are added [407]. The enzyme-labelled antigen competes for antibody binding sites with antigen in the sample. The more antigen in the sample the less enzyme-labelled antigen is retained in the well and the weaker the florescence signal. Therefore, the decrease in reaction product is proportional to the antigen present in the test solution. In some competitive ELISAs, the plate is coated an antibody instead of an antigen. The advantages of a competitive ELISA include high specificity and the ability to use complex or impure samples.

#### 4.2.8.1 HNE adduct competitive ELISA

For detection and quantitation of 4HNE protein adduct in placental explant and cell extracts, we used OxiSelect<sup>™</sup> HNE Adduct Competitive ELISA Kit (Cat. No. STA- 838, Cell Biolabs Inc.). This assay is based on the competition between HNE conjugate and the anti-HNE antibody for HNE-protein. In this assay, at first an HNE conjugate is coated on an ELISA plate. The unknown HNE protein samples or HNE-BSA standards are then added to the HNE conjugate adsorbed ELISA plate. After a brief incubation, an anti-HNE polyclonal antibody is added, followed by an HRP conjugated secondary antibody. The amount of HNE protein adducts in unknown samples is quantified by comparison with a predetermined HNE-BSA standard curve. A representative HNE-BSA standard curve and an ELISA plate are shown in Figure 4.4.



# **Figure 4.4** HNE-BSA competitive ELISA standard curve (A) and a representative ELISA plate (B).

Tissue and cell lysates were prepared by homogenising/sonicating samples in ultrapure water and then centrifuged at 13,000g for 10 min at 4 °C. The supernatant containing tissue or cell extract were separated. Protein concentration was estimated using a BCA protein assay kit as described in Section 4.2.5.2.

In this assay, at first, an HNE conjugate (composition was not disclosed by the supplier) was coated on a 96-well plate and incubated overnight at 4 °C. HNE conjugate was removed, and the wells were rinsed twice with 1X PBS. Assay Diluent (200 µL) was added to each well and blocked for an hour at RT and then transferred to 4 °C until use. Immediately before use, Assay Diluent was removed, 50 µL unknown samples (for 40 µg protein in each well) or HNE-BSA standards diluted in Assay Diluent were added. The plate was then incubated for 10 min at RT on an orbital shaker. The diluted anti-HNE antibody (50 µL) was added to each well and incubated at RT for 2 hours on an orbital shaker. The plate was then washed 3 times with 1X wash buffer with thorough aspiration between each wash. The diluted HRP Conjugated Secondary Antibody (100 µL) was added to all wells and incubated for an hour at RT on an orbital shaker. The strip wells were then washed further 3 times with 1X wash buffer. The substrate solution (100  $\mu$ L) was added to each well and incubated at RT for 2–5 min on an orbital shaker and the changes of colour were monitored. The stop solution (100  $\mu$ L) was added to each well to stop the enzyme reaction. The absorbance of each well was read on a microplate reader (SPECTROstar Nano, BMG LABTECH) using 450 nm as the primary wave length. The content of HNE protein adducts in unknown samples was determined by comparison with the predetermined HNE-BSA standard curve using Graphpad software. The results were graphed, and the statistical analysis was performed using Graphpad software.

#### 4.2.8.2 DNA/RNA oxidative damage ELISA

For detection and quantitation of DNA and RNA oxidation of placental trophoblast cells we used DNA/RNA Oxidative Damage (Clone 7E6.9) ELISA Kit (Cat No. 501130, Cayman Chemicals). This assay is based on the competition between oxidative damaged DNA and RNA guanine species (8-hydroxy-2-deoxyguanosine (8OHdG) and 8-hydroxyguanosine (80HG)), in samples and an 80HG-acetylcholinesterase conjugate (DNA/RNA oxidative damage AChE Tracer) for a primary antibody specific to 80HdG/80HG (DNA/RNA oxidative damage monoclonal antibody). The amount of tracer is held constant while the concentration of 8OHdG/8OHG varies, so the amount of tracer that is able to bind to the monoclonal antibody is inversely proportional to the concentration of 8OHdG/8OHG in the well. The antibody/8OHdG/8OHG complex binds to the goat polyclonal anti-mouse IgG that has been previously attached to the well. The plate is washed to remove any unbound reagents and then substrate (Ellman's reagent, which contains the substrate to AChE) is added to the well. A schematic of this technique is shown in Figure 4.5 (A). The product of this enzymatic reaction has a distinct yellow colour and absorbs strongly at 412 nm. The intensity of the colour, determined spectrophotometrically, is proportional to the amount of AChE tracer bound to the well, which is inversely proportional to the amount of free 80HdG and 80HG present in the well during the incubation. The amount of 8OHdG and 8OHG in unknown samples is estimated by comparison with a predetermined 8OHdG/8OHG ELISA standard curve. A representative 8OHdG/8OHG ELISA standard curve and an ELISA plate are shown in Figure 4.5 (B and C).



**Figure 4.5** (A) Schematic of the DNA/RNA oxidative damage AChE ELISA, (B) a representative DNA/RNA oxidative damage ELISA standard curve and (C) an ELISA plate after development. B/B<sub>0</sub>, ratio of the absorbance of a sample/standard well (B) to that of the maximum tracer binding (B<sub>0</sub>) well.

In this experiment, at first total DNA was extracted from placental HTR8/SVneo cells in PBS using DNeasy MiniPrep Blood & Tissue Kit (Cat. No. 69504, QIAGEN Pty Ltd, VIC,

Australia) following the manufacturer's protocols. Cleavage of extracted DNA into smaller components was done by treatment with deoxyribonuclease 1 (DNAse 1). DNA concentration was measured by a spectrophotometer using a NanoDrop 2000 spectrophotometer (NanoDrop Technologies). 2.5 µg of unknown DNA samples or appropriate amount of DNA/RNA oxidative damage ELISA standards diluted in ELISA buffer to a total volume of 50 µL were loaded in duplicate in each well of a 96 well plate. DNA/RNA oxidative damage AChE Tracer (50 µL) and appropriate dilution of ELISA monoclonal antibody (50  $\mu$ L) were added to each sample and standard wells. The plate was then covered with plastic film and incubated for 18 hours at 4 °C. Then the materials from each well were discarded and rinsed five times with 1X wash buffer with thorough aspiration between each wash. 200 µL of the Ellman's reagent (substrate solution) was added to each well and incubated at RT for 90 min on an orbital shaker. The absorbance was read on a microplate reader (SPECTROstar Nano, BMG LABTECH) using 420 nm as the primary wavelength. The content of 8OHdG in unknown DNA samples was determined using the equation obtained from the standard curve using GraphPad Prism software. The results were graphed, and the statistical analysis was performed using GraphPad Prism software.

## 4.2.9 Assessment of mitochondrial respiration function using the Seahorse extracellular flux analyser

Assessment of cellular energy metabolism (mitochondrial oxidative phosphorylation and glycolytic activity) are essential for investigating cell functions in a variety of cellular situations and pathological conditions. Recent technological advances in the ability to monitor and to assess mitochondrial metabolic function and cellular glycolytic capacity have

enabled researchers to understand the role of mitochondrial dysfunction in the pathogenesis of these diseases. Seahorse Bioscience has developed a technique, "the XF Cell Mito Stress Test Assay" that can measure real-time changes in the bioenergetics of cell monolayers. In this assay, cells are cultured at an optimum density in cell culture microplates. The assay measures the Oxygen Consumption Rate (OCR) of the cells, which is an indicator of mitochondrial oxidative phosphorylation, and the ExtraCellular Acidification Rate (ECAR), an indicator of cellular glycolytic capacity. In the assay, cells are metabolically disturbed by the sequential addition of three different compounds (oligomycin, carbonyl cyanide-4 (trifluoromethoxy) phenylhydrazone (FCCP), and a mix of rotenone/antimycin) that shift the metabolic profiles of the cell. The sequential injections of these compounds aid in the estimation of a variety of mitochondrial bioenergetic parameters, including basal OCR, ATP-linked OCR, maximal respiration capacity, spare/reserve capacity, proton leak and non-mitochondrial OCR, as well as glycolytic function of the cells. A schematic of this technique is shown in Figure 4.6.



Figure 4.6 Schematic of the mitochondrial stress test. Parameters of (A) mitochondrial oxidative phosphorylation and (B) cellular glycolytic capacity. Oxygen Consumption Rate (OCR) was measured under basal conditions followed by sequential injections of oligomycin, FCCP, and rotenone/antimycin A, which estimate basal respiration, ATP-linked respiration, proton leak, maximal respiration, spare respiratory capacity, and non-mitochondrial respiration. The target of action of the inhibitors of mitochondrial ETC used in the Seahorse XF Cell Mito Stress Test assay (C). Oligomycin inhibits ATP synthase (complex V), FCCP transports protons across the mitochondrial inner membrane, and interrupts electron flow through the membrane, thereby

inhibiting oxygen consumption and the production of ATP, and rotenone and antimycin A inhibit complexes I and III, respectively, which prevents the electron flow from Complex I and III to ubiquinone. NADH, nicotinamide adenine dinucleotide; FADH<sub>2</sub>, Flavin adenine dinucleotide; Q, Coenzyme Q (ubiquinone).

The sequence of addition and the functions of these compounds in the cell mito stress test are as follows:

**Oligomycin:** this blocks ATP production by inhibiting the proton channel of the Complex V (ATP synthase) (Figure 4.6 C). It is used to estimate the percentage of consumed oxygen that is devoted to generate ATP and the percentage of consumed oxygen which is needed to overcome the natural proton leak through the mitochondrial inner membrane. The OCR is decreased after injection of oligomycin. The decrease in OCR is associated with the mitochondrial oxygen consumption that is used to produce ATP and is therefore a direct measure of cellular ATP level. However, the ECAR is increased after addition of oligomycin, because the cells use glycolytic pathway to generate ATP in response to reduced ATP level.

**FCCP**: is an uncoupling agent and a proton ionophore, which allows the transport of protons across the mitochondria inner membrane, but inhibits proton transfer via ATP synthase, resulting in the inhibition of ATP production (Figure 4.6 C). FCCP disrupts the mitochondrial proton gradient and the membrane potential and allows the maximum electron flux through the ETC, which causes a rapid oxygen uptake by Complex IV without generating ATP. This

increases both OCR and ECAR. The FCCP-stimulated OCR is used to quantify the reserve (spare) respiratory capacity of cells, which is the difference between maximal OCR (after injection of FCCP) and the basal OCR (before injection of a compound). Reserve respiratory capacity is a measure of the cells ability to respond to increased energy demand.

**Rotenone/Antimycin A:** Rotenone is an inhibitor of Complex I and antimycin is a Complex III inhibitor (Figure 4.6 C). Rotenone prevents the electron flow from Complex I to ubiquinone (Coenzyme Q), which blocks the potential energy in NADH from being transformed to usable energy in the form of ATP. This combination halts mitochondrial oxygen consumption and enables the estimation of both the mitochondrial and non-mitochondrial fractions that contribute to respiration. After addition of rotenone/antimycin A, a reduction in OCR is observed, which is due to the reduction of mitochondrial respiration, while a concomitant increase in ECAR is also observed, as the cell shifts to a more glycolytic state in order to maintain its energy balance.

Seahorse XF analyser offers a rapid method to monitor the two major metabolic pathways of the cell in real time: oxidative phosphorylation, measured by OCR and glycolysis measured by ECAR in a single well. In addition, the assay requires a smaller number of cells with relatively high throughput, which is particularly useful for slow growing cells or precious samples. It has however some limitations. First, the cost associated with reagents, injectable compounds, new fluorescent plate for each assay, and the cost of optimizing compounds and cell number prior to any assay, are higher in comparison to conventional techniques. Secondly, the technique is able to use only four injectable compounds to assess mitochondrial
dysfunction in any single assay. As the assay itself requires three inhibitors of ETC to be injected, so there is only one type of injectable therapeutic agent can be used in a single assay. Therefore, it will be necessary to use multiple plates if requires testing of more than four injectable compounds, which again increases cost. A third drawback is that injectable compounds may potentially interfere with sensor fluorescence that may produce misleading data [408].

To assess mitochondrial function and bioenergetics in the placental trophoblast HTR8/SVneo cell line, we used the Seahorse XF96 Extracellular Flux Analyser and Seahorse XF Cell Mito Stress Test Kit (Cat. No. 103015-100, Agilent Technologies, Inc. CA, USA) as described previously [409]. In preliminary experiments, the optimal number of cells was estimated to ensure an optimal and measurable baseline OCR (oxygen consumption rate); 1, 2, 4 and 8 X10<sup>4</sup> cells/well were loaded onto Seahorse 96-well plates, and proportional responses were measured accordingly (data not shown). Based on the optimal baseline readings recommended by the manufacturer, 20,000 cells/well was selected to load in all experiments. Accordingly, FCCP concentration was also optimized to obtain maximal effects and readings. Sequential injections of oligomycin, FCCP, and a mix of rotenone/antimycin A was performed to determine basal OCR, ATP-linked OCR, maximal respiration, proton leak, non-mitochondrial OCR, and spare respiratory capacity.

#### 4.2.9.1 Cell preparation

In the preceding day before the assay, 20,000 cells were seeded in each well onto a Seahorse XF96 Cell Culture Microplate (Cat. No. 101085-004, Agilent Technologies, Inc.) in 100 μL Chapter 4

RPMI growth medium (RPMI-1640 medium supplemented with 2 mM L-glutamine, 1% Napyruvate, 1% antibiotic-antimycotic solution with the addition of 10% (v/v) FBS). The cells were incubated in humidified 37 °C incubator with 5% CO<sub>2</sub> for 20–24 hours. After that growth medium from each well was removed, and 100  $\mu$ L of serum-free medium (RPMI-1640 medium supplemented with 2 mM L-glutamine, 1% Na-pyruvate, 1% antibioticantimycotic solution) with or without the addition of AOX1 inhibitor raloxifene (1, 10, and 100 nM) or G1 (1, 10 and 100 nM) was added. In control wells, growth medium was replaced with fresh growth medium. The plates were then returned to a 37 °C incubator with 5% CO<sub>2</sub> and cultured for 24 hours.

#### 4.2.9.2 Measurement of oxygen consumption rate and extracellular acidification rates

Prior to performing an assay, the medium from each well was removed, and 175  $\mu$ L of prewarmed assay medium (Seahorse XF base medium (DMEM, Cat. No. 102353-100, Agilent Technologies, Inc.) supplemented with 25 mM glucose, 2 mM L-glutamine, and 1 mM sodium pyruvate; pH 7.4) was added. Cell culture plates were then placed into a 37 °C non-CO<sub>2</sub> incubator for 1 hour to allow pre-equilibration with the assay medium. Appropriate dilution of pre-warmed oligomycin, FCCP, and rotenone/antimycin A solution (prepared in assay medium and adjusted to pH 7.4) were loaded into the injector ports A, B and C of sensor cartridge, respectively, in order to achieve the final concentrations of compounds which were as follows: 1  $\mu$ M oligomycin, 0.5  $\mu$ M FCCP, 0.5 uM rotenone/antimycin A.

During this time the cartridge was calibrated prior to the start of an assay. Oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) were measured using

the XF96 Extracellular Flux analyser (Seahorse Bioscience, Billerica, MA, USA) under basal conditions (prior to the addition of an agent) followed by the sequential addition of oligomycin, FCCP, as well as rotenone/antimycin A, according to the described protocol by Nicholls et al [409]. OCR (pmol/min) and ECAR (mpH/min) data points refer to the average rates during the measurement cycles, which were then normalized against cell counts. In this assay, baseline OCR or ECAR (refers to the starting rates prior to the addition of an agent), which can be used for comparisons with those rates after the addition of compounds. This allows for an estimation of the contribution of individual parameters for basal respiration, proton leak, maximal respiration, spare respiratory capacity, non-mitochondrial respiration and ATP production [410].

#### 4.2.9.3 Cell counts

After the assay, cell culture plates were retrieved, and cells were fixed in 4% paraformaldehyde (Cat. No. P6148-500G, Sigma Aldrich). Automated cell counting was performed with Hoechst (Hoechst 33342, Cat. No. H3570, Thermo Fisher Scientific) staining of nuclear DNA with fluorescent imaging using the Citation 3 image reader (BioTek, Winooski, VT, USA). These cell counts were used to normalize both OCR and ECAR data to account for variable cell densities in different wells or with different pre-treatments etc.

#### **4.2.10** Data presentation and statistical analysis

Sample numbers are shown in the legends to individual figures. The results are presented as scatter plots showing the median or mean  $\pm$  standard error of the mean (S.E.M) as indicated

in the individual figure legends. Statistical differences were analysed by a standard two-tailed t-test using GraphPad Prism software (version 7, Graph Pad Software, Inc., San Diego, California) with either Mann-Whitney test (unpaired, non-parametric) for comparison between two groups or Wilcoxon matched-pairs signed rank test (paired, non-parametric) for repetitive measurement between treatment and control group as indicated in the figure legends. A  $\rho$  value of less than 0.05 was considered statistically significant. All the graphs were prepared using GraphPad Prism software.

## **CHAPTER 5**

## **Evidence that Fetal Death is Associated** with Placental Aging

This Chapter contains an original research article published in the American Journal of Obstetrics and Gynaecology, and is reproduced with permission. In this paper we report a novel evidence that unexplained stillbirth is linked to placental aging. The format of the paper has been altered for the purposes of this thesis. The original paper is included in appendix A.

#### Evidence that Fetal Death is Associated with Placental Aging

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pages. 441.e1-14.

### Declaration

I, Zakia Sultana, attest that I have made a primary and original contribution to the above publications as detailed below and endorsed by my supervisors. I was involved in the designing and performing the *in vitro* experiments, data analysis, and result interpretation. I also involved in preparation of the figures and manuscript writing.

Zakia Sultana

Laureate Professor Roger Smith

#### 5.0 Abstract

#### **Background:**

The risk of unexplained fetal death or stillbirth increases late in pregnancy suggesting that placental aging is an etiological factor. Aging is associated with oxidative damage to DNA, RNA and lipids. We hypothesized that placentas at more than 41 completed weeks of gestation (late-term) would show changes consistent with aging that would also be present in placentas associated with stillbirths.

#### **Objective:**

We sought to determine whether placentas from late-term pregnancies and unexplained stillbirth show oxidative damage and other biochemical signs of aging. We also aimed to develop an *in vitro* term placental explant culture model to test the aging pathways.

#### Study design:

We collected placentas from women at 37–39 weeks gestation (early-term and term), lateterm and with unexplained stillbirth. We used immunohistochemistry to compare the three groups for: DNA/RNA oxidation (8-hydroxy-deoxyguanosine, 8OHdG), lysosomal distribution (Lysosome-associated membrane protein 2, LAMP2), lipid oxidation (4-hydroxynonenal, 4HNE), and autophagosome size (Microtubule-associated proteins 1A/1B light chain 3 B, LC3B). The expression of aldehyde oxidase 1 (AOX1) was measured by real-time PCR. Using a placental explant culture model, we tested the hypothesis that AOX1 mediates oxidative damage to lipids in the placenta.

#### **Results:**

Placentas from late-term pregnancies show increased AOX1 expression, oxidation of DNA/RNA and lipid, perinuclear location of lysosomes and larger autophagosomes

compared to placentas from women delivered at 37–39 weeks. Stillbirth associated placentas showed similar changes in oxidation of DNA/RNA and lipid, lysosomal location and autophagosome size to placentas from late-term. Placental explants from term deliveries cultured in serum free medium also showed evidence of oxidation of lipid, perinuclear lysosomes and larger autophagosomes, changes that were blocked by the G protein-coupled estrogen receptor 1 (GPER1) agonist G1, while the oxidation of lipid was blocked by the AOX1 inhibitor raloxifene.

#### **Conclusions:**

Our data are consistent with a role for AOX1 and GPER1 in mediating aging of the placenta that may contribute to stillbirth. The placenta is a tractable model of aging in human tissue.

#### 5.1 Introduction

Unexplained fetal death is a common complication of pregnancy occurring in approximately 1 in 200 pregnancies in developed countries [5] and more frequently in the developing world. While no cause has been established, the rate of fetal death rises rapidly as gestation progresses beyond 38 weeks [411]. Johnson et al.[412] have proposed the operational definition of aging as an increase in risk of mortality with time, which is consistent with a role for aging in the aetiology of stillbirth (Figure 5.1) [50]. Supporting this view a histopathological study of placentas associated with cases of unexplained intrauterine death at term revealed that 91% showed thickening of the maternal spiral artery walls, 54% contained placental infarcts, 10% had calcified areas and 13% demonstrated vascular occlusion [413], another reported increased atherosclerosis [414]; changes that are associated

with aging in other organs. Supporting a link between placental aging and stillbirth, Ferrari at al. have recently reported that telomere length is reduced in placentas associated with stillbirth [398]. Fetal growth restriction is also associated with both stillbirth and telomere shortening [415]. We therefore sought to determine whether placentas from women who delivered after 41 completed weeks (late-term) or had stillbirth had biochemical evidence of aging. As markers of aging we chose to measure 8-hydroxy-deoxyguanosine (a marker of DNA oxidation) and 4-hydroxynonenal (a marker of lipid oxidation) as both have been described to increase in the brain with aging, and the enzyme aldehyde oxidase which is known to generate oxidative damage in the kidney. Aging is also known to affect the effectiveness of the intracellular recycling process that involves fusion of acidic hydrolase containing lysosomes with autophagosomes, we therefore sought changes in these intracellular organelles in the late-term placentas and those associated with stillbirth.

#### 5.2 Materials and methods

#### 5.2.1 Ethics, collection and processing of tissues

This study was approved by the human research ethics committee of the Hunter New England Health Services and the University of Newcastle, NSW, Australia. Human placentas were collected after written informed consent was obtained from the patients by midwives. Placentas were collected from women at 37–39 weeks gestation undergoing caesarean section for previous caesarean section or normal vaginal delivery, women at 41+ weeks gestation undergoing caesarean section or normal vaginal delivery, and women who had stillborn infants undergoing vaginal delivery. Placentas were collected immediately after delivery and processed without further delay. Villous tissues were sampled from multiple sites and prepared for histology and RNA extraction. For each placenta, tissues were obtained from at least 5 different regions of the placenta and 4–5 mm beneath the chorionic plate. Samples from each individual placenta were immediately frozen under liquid nitrogen and stored at -80 °C until subsequent experiments. For histology experiments, tissues were fixed in 2% formaldehyde for 24 hours, stored in 50% ethanol at room temperature (RT) and embedded in paraffin. To create a placental roll a 2 cm strip of chorioamniotic membrane was cut from the periphery of the placenta keeping a small amount of placenta attached to the membrane. The strip was rolled around forceps leaving residual placenta at the centre of the cylindrical roll. The cylindrical roll was then cut perpendicular to the cylindrical axis to obtain 4 mm thick sections and fixed in formalin. Placentas from patients with infection, diabetes, preeclampsia, placenta praevia, intra-uterine growth restriction or abruption were excluded.

#### 5.2.2 Reagents and antibodies

Antibodies against LAMP2 and AOX1 were obtained from BD Biosciences (North Ryde, Australia) and Proteintech (Rosemont, USA), respectively. Antibody against LC3B and GPER1 were obtained from Novus Biologicals (Littleton, USA). Antibodies against 80HdG and 4HNE were purchased from Abcam (Melbourne, Australia). Dulbecco's modified Eagle's medium (DMEM), antibiotic-antimycotic (anti-anti), Nupage precast 12 well protein gel and prolong gold antifade mounting media with DAPI, Alexa conjugated secondary antibodies were obtained from Thermo Fisher Scientific Australia Pty (Scoresby, Australia).

The horse radish peroxide (HRP) conjugated secondary antibodies were purchased from Cell Signalling Technologies (Beverly, MA, USA). Fetal bovine serum was obtained from Bovogen Biologicals Pty Ltd (VIC, Australia). Protease inhibitor and phosphatase inhibitor were supplied by Roche (Castle Hill, Australia). Raloxifene was purchased from Sigma-Aldrich (Sydney, Australia) and G1 was supplied by Tocris-bioscience (Bristol, UK). The BCA protein assay kit was obtained from Thermo Fisher Scientific (Scoresby, Australia). All other chemicals were purchased from Ajax Finechem Pty Ltd and Sigma-Aldrich (Sydney, Australia).

#### 5.2.3 Placental explant culture

For *in vitro* experiments, human term placentas (all at 39 weeks of gestation) were obtained from women with normal singleton pregnancies without any symptoms of labour after an elective (a scheduled repeat) caesarean section. Placentas were collected immediately after delivery and prepared for explant culture. Villous tissues of placentas were randomly sampled from different regions of the placenta 4–5 mm beneath the chorionic plate. Tissues were washed several times with Dulbecco's phosphate-buffered saline (PBS) under sterile conditions to remove excess blood. Villous explants of ~2 mm<sup>3</sup> were dissected and placed into 100 mm culture dishes (30 pieces/dish) containing 25 mL of DMEM supplemented with 2 mM L-glutamine, 1% Na-pyruvate, 1% penicillin/streptomycin (100X) solution with the addition of 10% (v/v) fetal bovine serum (FBS) and cultured in a cell culture chamber at 37 °C temperature under 95% air (20% oxygen) and 5% CO<sub>2</sub> for 24 hours. At day 2, villous explants were transferred to fresh 30 mL growth medium and incubated in a cell culture chamber for 90 min and washed in DMEM without FBS (referred to as 'serum-free medium' or 'growth factor deficient medium'). Next 6–7 pieces of villous tissue weighing approximately 400 mg were transferred to a culture dish (60 mm) containing 6 mL serum-free medium with or without the addition of pharmacological agents, for example, raloxifene (1 nM) or the GPER1 agonist G1 (1 nM), for subsequent incubation for 24 hours. At the end of 24 hours some tissues were fixed in 2% formaldehyde, subjected to routine histological processing and embedded in paraffin wax, and some tissues were immediately frozen in liquid nitrogen and stored at -80 °C until subsequent experiments. For each placental explant culture, samples were also collected at time '0 (zero)' hour i.e., before incubation in serum free medium, and were formalin fixed and stored frozen at -80 °C until further experiments.

#### 5.2.4 Western blotting

The western blotting was performed as previously described [416]. Samples of placenta (1gm) were crushed under liquid nitrogen. Aliquots of 100 mg of placental tissues were homogenised in 1 mL of lysis buffer (PBS, 1% Triton-X-100, 0.1 % Brij-35, 1 X protease inhibitor, 1 X phosphatase inhibitor, pH 7.4). The protein concentration of each placental extract was measured using a BCA protein assay kit and 40 µg of placental extract was separated by electrophoresis in NuPage bis-tris precast 12 well gels for 50 min at a constant 200 V. Separated proteins were then transferred to nitrocellulose membrane using a Novex transfer system for 70 min and blocked overnight at 4 °C with 1% bovine serum albumin (BSA) in tris buffered saline with 0.1 % tween-20 (TBST). The membranes were then incubated with primary antibody in 1% BSA in TBST for 2 hours at RT, then washed three

times with TBST, followed by incubating with HRP conjugated secondary antibodies in 1% BSA in TBST for an hour. After three further washes with TBST, the immuno-reactive bands were developed in Luminata reagent (Merck Millipore) and detected using an Intelligent Dark Box LAS-3000 Imager (Fuji Photo Film, Tokyo, Japan).

#### 5.2.5 Immunohistochemistry

Fluorescent immunohistochemistry (IHC) was performed according to previously published methods [416]. Six µm paraffin placental sections were deparaffinised and hydrated, then heated with tris-EDTA buffer (pH 9) in a microwave oven for antigen retrieval. The sections were blocked with 1 % BSA in TBST for an hour at RT. The sections were incubated with primary antibodies overnight and washed three times with TBST, before incubation with Alexa-conjugated secondary antibodies for 90 mins. The sections were mounted with prolong gold antifade mounting media with DAPI. The fluorescent photographs for Figures 5.2, 5.3, 5.4, 5.5, 5.6, 5.7, 5.S1, 5.S2 and 5.S3 were taken on a Nikon eclipse 90i confocal microscope (Nikon Instruments Inc.). The fluorescente photographs for Figure 5.8 were taken on Nikon eclipse Ti fluorescence microscope (Nikon Instruments Inc.).

#### 5.2.6 RNA isolation and real time PCR

Placental tissues were crushed under liquid nitrogen. Approximately 100 mg of crushed placental tissues were homogenised in 2 mL of Trizol reagent (Thermo fisher Scientific) with an Ultra Turrax homogenizer. Total RNA was extracted from the Trizol-extract by Direct-

zol<sup>™</sup> RNA MiniPrep (Zymo Research). The RNA was treated with DNAse 1 (Sigma Aldrich) and purified with a RNA Clean & Concentrator<sup>TM</sup>-5 kit (Zymo Research). The RNA quality was checked by running the DNAse treated sample in agarose gel with ethidium bromide in 1X TAE buffer. The purified RNA was used to make cDNA using a SuperScript® III First-Strand Synthesis System kit (Thermo Fisher Scientific). The cDNA was used to run real-time PCR by Taqman primers for aldehyde oxidase 1 (AOX1) (Thermo Fisher Scientific, Assay ID: Hs00154079\_m1) and Taqman gene expression master mix (Thermo Fisher Scientific) with an internal control of 18s ribosomal RNA (Thermo Fisher Scientific) to quantify mRNA for AOX1. We used a SyBr green master mix (Thermo Fisher Scientific) to quantify mRNA for G-protein coupled receptor 1 (GPER1) (Invitrogen, Forward primer 5'-CGTCCTGTGCACCTTCATGT-3' Backward 5'primer AGCTCATCCAGGTGAGGAAGAA-3') with respect to beta-actin (Thermo Fisher Scientific) as an internal control using an Applied Biosystem 7500 PCR system.

#### 5.2.7 Statistical analysis

Sample numbers are shown in the legends to individual figures. The data in Figures 5.2, 5.4, 5.5, 5.6 and 5.8 were analysed using the Mann-Whitney test (two way) and results are presented as scatter plots showing the median. The data in Figure 5.7, 5.S2 and 5.S3 were analysed using the Wilcoxon matched-pairs signed rank test and results are presented as mean showing the standard error of the mean (S.E.M.). All the  $\rho$ -values were calculated using the Graphpad Prism software (Version 7, Graph Pad Software Inc., San Diego, California). A  $\rho$  value of  $\leq 0.05$  was considered statistically significant.

#### 5.3 Results

#### 5.3.1 Subject characteristics

Demographic and clinical characteristics of the study participants are reported in table 5.1.

#### 5.3.2 Relationship between stillbirth risk and length of gestation

To illustrate the relationship between stillbirth risk and length of gestation we created a Kaplan Myer plot of the data on human gestational length in a population with relatively low levels of medical intervention from Omigbodun and Adewuyi [417] and combined it with the data on risk of stillbirth per 1000 continuing pregnancies from Sutan et al. [411] (Figure 5.1 reproduced with permission [50]). The data illustrate that stillbirth is consistent with an aging etiology as defined by Johnson et al. [412].

Characteristic	37–39 Weeks	Late-term	Stillbirth
Number of cases	34	28	4
Gestational ages (weeks)	$38.57\pm0.15$	$41.46\pm0.06$	32 32.57 39 40.14
Fetal growth restriction (number of cases)	0	0	No Yes No Yes
Maternal age (years)	$31.03\pm0.88$	$28.81 \pm 1.15$	$30.21 \pm 2.68$
Vaginal birth (%)	41.20 %	64.30 %	100.00 %
BMI (kg/m <sup>2</sup> ) at second trimester or at birth	$29.10 \pm 1.50$	$28.52 \pm 1.10$	$27.40 \pm 2.40$
Ethnicity			
Caucasian (%)	82.35 %	96.42 %	75.00 %
Smoker (%)	17.64 %	17.85 %	0.00 %

### **Table 5.1**Demographic and clinical characteristics of the study subjects

Data are presented as (Mean  $\pm$  SEM) or percentage. BMI, Body Mass Index



Figure 5.1 Relationship between stillbirth and number of continuing pregnancies. Kaplan Myer plot of number of continuing pregnancies as a function of gestational age and plot of unexplained stillbirth per 1000 continuing pregnancies, data from Omigbodun and Adewuyi [417] and Sutan et al. [411]. Plot shows the increase in risk of stillbirth with time consistent with the operational definition of aging proposed by Johnson et al. [412] and the relatively small number of pregnancies at risk of stillbirth by 41 weeks because of prior delivery. Figure reproduced with permission from Smith et al. [50].

#### 5.3.3 DNA/RNA oxidation

We sought evidence of placental DNA/RNA oxidation as measured by 8-hydroxydeoxyguanosine (8OHdG), as a marker of DNA/RNA oxidation that has previously been observed in aging tissues [395] such as the brain in Alzheimer's disease [396]. Immunohistochemistry (IHC) was performed for 8OHdG and the average intensity of 8OHdG staining in nuclei/frame demonstrated a significant increase in DNA/RNA oxidation in late-term and stillbirth associated placentas (Figure 5.2).



### Figure 5.2 DNA/RNA oxidation in late-term and stillbirth placentas. Confocal microscopy showed increased 8OHdG staining (red) in nuclei from late-term (B) and stillbirth placentas (C) compared to 37–39 week placentas (A). DAPI (blue) staining identifies the nuclei. The graph (D) illustrates that late-term and stillbirth placentas have increased intensity of nuclear 80HdG staining $(\rho < 0.0001$ for late-term placentas, $\rho = 0.0005$ for stillbirth placentas, Mann Whitney test) compared to 37–39 week placentas. In Figure 5.2 D open circles and filled circles represent 37-39 week caesarean non-labouring placentas (n=10) and vaginal delivery labouring placentas (n=8) respectively, and open squares and filled squares represent late-term labouring caesarean placentas (n=5) and labouring vaginal delivery placentas (n=13) respectively, and filled triangles represent third trimester labouring vaginal delivery unexplained stillbirth placentas (n=4). Each point in the graph represents the average intensity of 80HdG of 60 nuclei in 6 images per placenta photographed at 100X magnification and 1.4 optical resolution. Scale bar, 20 µm. The microscopy also indicates increased staining in the cytosol of late-term and stillbirth placentas representing oxidised RNA (8-hydroxyguanosine) that is also detected by the antibody.

#### 5.3.4 Movement and clustering of lysosomes in late-term and stillbirth placentas

Misfolded proteins and damaged mitochondria are normally recycled in autophagosomes in a process that involves autophagosome fusion with proteolytic enzyme containing lysosomes. Accumulation of abnormal protein is thought to play a role in aging particularly in the brain, for instance the accumulation of tau and amyloid protein in Alzheimer's disease [418,419] and mutant huntin in Huntington's disease [420]. In Huntington's disease, the distribution of the lysosomes within neurones is altered with increased perinuclear accumulation of lysosomes [421]. We used a lysosomal marker, lysosome-associated membrane protein-2 (LAMP2) to analyse the distribution of lysosomes in the placenta by IHC. IHC showed lysosomes positioned on the apical surface of early-term placental syncytiotrophoblast (Figure 5.3 A, D and E), whereas lysosomes relocated to the perinuclear and the basal surface in late-term and stillbirth placentas (Figure 5.3 B, C, F and G).



Figure 5.3 Changes in lysosomal distribution in late-term and stillbirth placentas. IHC of LAMP2 (red), a lysosomal marker showed that lysosomes predominantly localise to the apical surface of 37–39 week placentas (A),

whereas lysosome distribution extends to the perinuclear and basal surface of syncytiotrophoblast in late-term (B) and stillbirth placentas (C). Intensity calculation across the syncytiotrophoblast showed that the distribution of LAMP2 in late-term (n=5, Figure 5.3 F) and unexplained stillbirth placentas (n=4, Figure 5.3 G) shifts to the perinuclear and basal surface whereas lysosome distribution in 37–39 week caesarean placentas (n=5, Figure 5.3 D) and vaginal delivery placentas (n=5, Figure 5.3 E) remained in the apical region of the syncytiotrophoblast. DAPI (blue) staining identifies the nuclei. In Figure 5.3 D–G, each coloured line represents results on an individual placenta, and shows the mean intensity of LAMP2 across the syncytiotrophoblast at 5 random sites per image (example represented by light green line in 5.3 A, B and C) for 6 separate images per placenta. Images were photographed at 100X magnification; scale bar, 20 µm.

#### 5.3.5 Lipid oxidation in placental tissue

The increase in DNA oxidation which we had demonstrated suggested free radical damage that might also lead to lipid peroxidation. Lipid peroxidation has been observed to increase in Alzheimer's disease as measured by the formation of 4-hydroxynonenal (4HNE) [397]. We therefore performed IHC for 4HNE in late-term, stillbirth and 37–39 weeks placental tissue. This revealed a marked, statistically significant increase in 4HNE staining in late-term syncytiotrophoblast that we also observed in placentas associated with stillbirth shown in Figure 5.4 A–D.



Figure 5.4 Lipid peroxidation is increased in late-term and stillbirth placentas. 4HNE (red) immunostaining in 37–39 week (A), late-term (B), and stillbirth (C) placentas. DAPI (blue) staining identifies nuclei. The intensity of 4HNE is significantly increased in late-term placentas (D) (ρ<0.0001, Mann Whitney test) and stillbirth placentas (ρ=0.0014, Mann Whitney test). In Figure 5.4 D open circles and filled circles represent 37–39 week caesarean non-labouring placentas (n=20) and vaginal delivery labouring placentas (n=14) respectively, and open squares and filled squares represent late-term labouring caesarean placentas (n=10) and vaginal delivery placentas (n=18) respectively, while filled triangles represent third trimester labouring vaginal delivery unexplained stillbirth placentas (n=4). Each point in 5.4 D represents the mean intensity per unit area for 6 images taken for each individual placenta. Images were photographed at 100X magnification; scale bar, 20  $\mu$ m.

# 5.3.6 Larger autophagosomes containing 4HNE occur in late-term and stillbirth associated placentas

Inhibition of autophagosome function with failure of fusion with lysosomes leads to an increase in autophagosome size [422,423]. This process leads to inhibition of overall autophagic function that is seen in Alzheimer's disease [422], Danon's disease [423] and neurodegeneration [424]. We detected autophagosomes using IHC with an antibody against LC3B. We observed a significant increase in the size of autophagosomes (Figure 5.5 D) in both late-term (Figure 5. 5 B) and stillbirth (Figure 5.5 C) associated placentas compared to 37–39 week placentas (Figure 5.5 A). Dual labelled fluorescence immunostaining showed that the larger autophagosomes of late-term and stillbirth placentas contained 4HNE, a product of lipid peroxidation (Supplementary Figure 5.S1).



Figure 5.5 Larger autophagosomes occur in late-term and stillbirth placentas. Immunofluorescence staining of LC3B (green) in the 37–39 week (A), late-term (B), and unexplained stillbirth (C) placentas. DAPI (blue) staining indicates the nuclei. Autophagosome size was quantified using NIS element software and the diameter was measured at an arbitrary intensity range of 1000–3000, diameter range 0.2–1 µm and circularity range 0.5–1. Analysis (D) showed that late-term and stillbirth placentas have significantly larger (ρ=0.012 and ρ=0.0019, respectively, Mann Whitney test) autophagosomes than 37–39 week placentas. In 'D' open circles and filled circles represent 37–39 week caesarean non-labouring placentas (n=11) and vaginal delivery

labouring placentas (n=10) respectively, and open squares and filled squares represent late-term labouring caesarean placentas (n=8) and labouring vaginal delivery placentas (n=15) respectively, while filled triangles represents unexplained stillbirth placentas (n=4). Each point in the graph represents the average diameter of LC3B particles in six images taken for each placenta. Original magnification, 100X; scale bar, 20  $\mu$ m. Arrow heads indicate autophagosomes (LC3B positive particles).

#### 5.3.7 Role of aldehyde oxidase 1 (AOX1) in placental oxidative damage

Aldehyde oxidase 1 (AOX1) is a molybdoflavoenzyme, which oxidises a range of aldehydes including 4HNE into corresponding acids and peroxides [425]. We provide evidence that AOX1 is involved in the generation of the increased 4HNE observed in late-term and stillbirth associated placentas using co-localisation. Dual labelled fluorescence IHC showed that AOX1 co-localises to 4HNE positive particles in late-term (Figure 5.6 A–C) and stillbirth placentas (Figure 5.6 D–F). Additionally, real-time qPCR showed that late-term and stillbirth placentas expressed significantly higher mRNA for AOX1 than 37–39 week placentas (5.6 G). These data support the concept that AOX1 plays a role in the oxidative damage that occurs in the late-term and stillbirth associated placentas.



Figure 5.6Co-localisation of aldehyde oxidase (AOX1) and 4HNE, and increased<br/>expression of AOX1 mRNA in late-term and stillbirth placentas.<br/>Representative dual labelled fluorescence immunostaining in late-term (A–C)<br/>and stillbirth (D–F) placentas showed that AOX1 positive particles (green) are<br/>co-localized with 4HNE (red). Orange dots (pointed by arrow heads in C and<br/>F) indicate co-localization. Nuclei are stained with DAPI (blue). Real-time

PCR showed that expression of AOX1 mRNA is increased in late-term ( $\rho$ =0.0097) and stillbirth ( $\rho$ =0.012) placentas compared to early-term placentas (G). Original magnification100X; scale bar 20  $\mu$ m.

#### 5.3.8 Pharmacological inhibition of AOX1 using placental explant culture

Our data provide clear evidence for increased lipid oxidation, disordered lysosomeautophagosome interactions and increased AOX1 expression in the late-term and stillbirth placental syncytiotrophoblast. To determine if these events were causally linked we developed a placental explant culture system using term placental tissue cultured in serumfree (growth factor deficient) medium. IHC showed that serum deprivation significantly increased production of 4HNE at 24 hours after incubation (Figure 5.7 A-C, F and G). We also found a significant increase in the size of autophagosomes (Supplementary Figure 5.S2) and a change in lysosomal distribution to a perinuclear location after 24 hours incubation in serum-free medium (Supplementary Figure 5.S3). We sought to determine cause and effect relationships between the development of lipid oxidation observed when placental explants were cultured in the absence of serum, and AOX1. To achieve this we used a potent AOX1 inhibitor, raloxifene [426] and a GPER1 agonist, G1. We used the GPER1 agonist G1 as we had detected GPER1 expression on the apical surface of syncytiotrophoblast (Figure 5.8 A and B) and the GPER1 agonist has been shown to inhibit production of 4HNE in the kidney [226]. Both raloxifene and G1 inhibited the production of 4HNE in the serum starved placental explants after 24 hours of treatment (Figure 5.7 D-G). G1 also prevented the changes in lysosomal distribution within the syncytiotrophoblast (Supplementary Figure 5.S3).



Figure 5.7 Pharmacologic inhibition production. of **4HNE** Fluorescence immunostaining with antibody against 4HNE (red) in serum starved placental explant (A) at time 0 (just before starvation) (B) at 24 hours after culturing in medium containing FBS (control treatment), (C) at 24 hours after starvation (culturing in medium without FBS), (D) 24 hours after treatment with an AOX1 inhibitor, raloxifene (RLX) and (E) 24 hours after treatment with a membrane estrogen receptor GPER1 agonist, G1. Intensity calculation showed that the production of 4HNE (induced by serum starvation) is significantly reduced after treating placental explants with raloxifene (F) and G1 (G). Data are mean  $\pm$  S.E.M., \*  $\rho$ <0.05 (n=6). Original magnification, 20X; scale bar, 100 µm. DAPI (blue) staining indicates the nuclei.

# **5.3.9** Presence of the cell surface estrogen receptor GPER1 on the apical surface of the syncytiotrophoblast

As the GPER1 agonist had evident effects in placental explant cultures we undertook characterisation of GPER1 expression in placental tissue. The expression of GPER1 in a section of placenta roll (described in the Method section) detected by fluorescent IHC showed that GPER1 in expressed in placental villi (Figure 5.8 A), which at higher magnification (100X), was localised to the apical surface of placental villi (Figure 5.8 B). Real time PCR for GPER1 showed that placental villi have significantly higher expression of GPER1 than amnion, chorion or decidua (Figure 5.8 C). Western-blot for GPER1 also confirmed higher protein levels of GPER1 in placental villous tissue than amnion, chorion or decidua (Figure 5.8 C). The demonstration of GPER1 localisation on the apical surface of the syncytiotrophoblast indicates the plausibility of estrogen inhibition of AOX1 activity in the placenta.



Figure 5.8 Expression of GPER1 in placenta and myometrium, but not in membranes by IHC, real-time PCR and western-blotting. Fluorescence IHC showed that GPER1 (green) is localized predominantly in the placental in a section of a term placental roll photographed at 10X magnification (A). GPER1 (green) was shown to localize in the apical layer of syncytiotrophoblast of placental villi (B), when photographed at 100X magnification. Scale bar in 'A' and 'B' represent 100 μM and 20 μM, respectively. The real-time qPCR data showed that the mRNA for GPER1 is

expressed in higher amounts in term placenta, whereas amnion, chorion and decidua show very low expression of GPER1 (C). The expression of mRNA for GPER1 follows the order: decidua<chorion<amnion<placenta. The western blot of protein extract from the breast cancer cell line MCF-7, term placenta, myometrium, amnion, chorion and decidua are presented in 'D'. Placenta, myometrium and MCF-7 cell lines expressed higher amounts of GPER1 than amnion, chorion or decidua (D). Western-blotting data showed that all the tissues expressed glycosylated GPER1 (denoted by \*\* or by \*\*\*) and non-glycosylated nascent GPER1 (denoted by \*). The sypro-ruby stain of the same PVDF membrane is used as internal loading control (E).

#### 5.4 Comment

Our data indicate that between 37–39 and 41 weeks of gestation dramatic changes occur in the biochemistry and physiology of the placenta. In particular there is increased oxidative damage to DNA/RNA and lipid, a change in position of lysosomes which accumulate at the perinuclear and basal surface of the syncytiotrophoblast, the formation of larger autophagosomes which are associated with oxidised lipid, and there is increased expression of the enzyme AOX1. The same changes are observed in placentas associated with stillbirth. Some of our results are semi-quantitative as this is the nature of western analysis, nevertheless the robustness of our results is supported by the use of multiple end points for aging, and the biological plausibility of the reported links. Further supporting our hypothesis, similar changes in oxidation of lipid, localisation of lysosomes and size of autophagosomes

occurred in placental explants deprived of growth factors, and these changes were blocked by inhibition of AOX1.

Stillbirth occurs in approximately 1 in 200 pregnancies in developed countries [5]. The Lancet [5] and the BMJ [427] have recently highlighted gaps in our knowledge of this condition. Stillbirth frequently occurs in the setting of fetal growth restriction and in this setting telomere shortening and oxidative damage have been observed in associated placentas [428]. The risk of stillbirth per 1000 continuing pregnancies rises dramatically after 38 weeks of gestation. We have suggested [50] that stillbirths in late gestation are a consequence of placental aging. More than 90% of pregnancies have delivered by the end of the 40th week of gestation [417], consequently changes that occur in the placenta in pregnancies that have gone past the usual term have little effect on population level infant survival, since most have already delivered. Such late gestation changes may exist in a kind of Medawar's Shadow [429] that allows deleterious genes to persist in the population if their damaging effects occur after reproduction, especially if the same genes exert positive actions earlier in pregnancy. This Medawar's Shadow effect has been proposed to explain the high prevalence of Huntington's disease that is associated with increased fertility in early life but disastrous neurological deterioration after reproduction has occurred [430]. Our immunofluorescence data show high levels of 8OHdG and 4HNE in late-term and stillbirth placentas supporting this postulated pathway to placental aging. Increases in oxidative damages to DNA and lipid have also been reported in Alzheimer's disease [397,431].

We have also seen marked accumulation of particles positive for the lysosomal marker LAMP2 in the perinuclear and basal side of the syncytiotrophoblast of late-term placentas and placentas associated with stillbirth. This phenomenon closely resembles 'lysosomal positioning' which occurs in cells under nutritional stress [432]. Autophagy is an important cellular recycling process that involves fusion of acidic lysosomes with the autophagosomes. Our data show that stillbirth and late-term placentas contain larger autophagosomes than 37–39 week placentas indicating inhibition of the autophagic process in these placentas. Our data further indicate that the autophagosomes are coated with oxidised lipid in the form of 4HNE which may play a role in the failure of lysosomal-autophagosome fusion. Such disturbances in the function of autophagosomes may lead to the accumulation of abnormal protein and deterioration in the function of the syncytiotrophoblast.

Stillbirth is not restricted to the late-term setting and is known to be associated with cigarette smoking and growth restriction. It seems likely that smoking accelerates aging related pathways. Evidence for this is the finding that telomere length is reduced in the fetuses of women who actively smoke during pregnancy [433], and similar changes are to be expected in the placentas of smokers. Down's syndrome is associated with advanced aging or progeria [434,435] and also with increased rates of stillbirth [436,437], raising the possibility that accelerated placental aging may play a part in stillbirth related to Down's and some other congenital anomalies. Similarly, placental abruption is associated with growth restriction, maternal smoking and stillbirth, and placental aging may play a part in this condition [438,439].

We have used cultured term placental explants to interrogate the pathways leading to the lipid oxidation and disturbed autophagosome function. We measured production of 4HNE and the diameter of autophagosomes following serum depletion. We observed a significant increase in 4HNE and a significant increase in autophagosome size suggesting inhibition of autophagy by oxidative damage as we had previous observed in the stillbirth and late-term placentas. Raloxifene a potent inhibitor of AOX1 has been shown to reduce oxidative damage in endothelial cells [225]. We have demonstrated that the AOX1 inhibitor raloxifene is also able to block the oxidative damage to the lipid in placental explants. The role of AOX1 was confirmed using the GPER1 agonist G1 that has been shown to block AOX1 activation and reduce 4HNE in renal tissue [226]. The G1 also blocked the changes in lysosomal positioning within the explants. We report the novel finding of the presence of the cell surface estrogen receptor GPER1 on the syncytiotrophoblast apical membrane, suggesting that this receptor may play a role in modulating oxidative damage within the placenta. It has been shown that urine from pregnant women carrying a fetus with post-maturity syndrome have lower estrogen:creatinine ratios than women carrying normal foetuses [440]. These data support the possibility that low estrogen concentrations may lead to loss of the cell surface estrogen receptor (GPER1) mediated inhibition of AOX1 and consequently placental oxidative damage and impaired function.

The changes in the late-term placenta occur as the fetus continues to grow and to require additional supplies of nutrients. Post-maturity syndrome is a condition seen in post-dates infants who show evidence of late gestation failure of nutrition [441]. Normal human infants born at term have 12–14% body fat whereas post maturity syndrome is associated with the

birth of a baby with severe wrinkling of the skin due to loss of subcutaneous fat. Post-maturity syndrome is rarely seen in modern obstetric practice where delivery is usually effected before 42 weeks of gestation using induction of labour or caesarean section if labour has not occurred spontaneously. While none of the infants born to mothers in our study exhibited post-maturity syndrome, our data suggest that the physiological function of the placenta after 41 completed weeks is showing evidence of decline that has many features in common with aging in other tissues. The known exponential increase in unexplained intrauterine death that occurs after 38 weeks of gestation may therefore be a consequence of aging of the placenta and decreasing ability to adequately supply the increasing needs of the growing fetus. This knowledge may impact on obstetric practice to ensure infants are born before the placenta ages to the point of critical failure [442]. However, it is notable that not all placentas in our late-term cohort exhibited evidence of aging and it is known that infants born later in gestation have lower rates of special school needs, with those born at 41 weeks having the lowest rates [443]. The conflicting pressures of late gestation increases in stillbirth and falling rates of intellectual disability make obstetric care at this time very challenging, diagnostics that can predict pregnancies at increased risk of stillbirth would be useful and some progress in their development has been made [444]. Our data also indicate that the placenta may provide a tractable model of aging in a human tissue that uniquely ages in a 9 months period of time. The results suggest that the rate of aging of the placenta varies in different pregnancies and raises the possibility that the rate of aging of the placenta may parallel the rate of aging of the associated fetus carrying the same genome. Our work identifies potential therapeutic targets such as AOX1 that may arrest the oxidative damage to placentas in pregnancies identified at high risk of stillbirth when extreme prematurity precludes delivery. Finally, our data raise the possibility that markers of placental oxidative damage and AOX1 mRNA may be released into maternal blood where they may have diagnostic value in predicting the fetus at risk for stillbirth.

#### 5.5 Supplementary information





Figure 5.S1 Oxidised lipids within autophagosomes of late-term placentas. Representative dual labelled fluorescence immunostaining showed that LC3B, an autophagosome marker (green) is co-localised with 4HNE, a marker of lipid peroxidation (red). Orange dots (pointed by arrow heads in C) indicate the co-localization. DAPI (blue) staining indicates the nuclei. Original magnifications 100X; scale bar 20 μm.


Figure 5.S2 Changes in autophagosome size in placental explants cultured in serum deprived medium. Fluorescence immunostaining with antibody against LC3B (green) in serum starved placental explant (A) at time 0 (just before starvation) and (B) at 24 hours after starvation. DAPI (blue) staining indicates nuclei. (C) Immunohistochemical analysis showed that the size of autophagosomes (LC3B positive particles) increased 24 hours after serum

starvation compared to 0 hour. Data presented as mean  $\pm$  S.E.M., \*\*\*  $\rho$ =0.0002 (n=13). Scale bar, 20  $\mu$ M.



Figure 5.S3 GPER1 regulates lysosomal distribution in placental explants cultured in serum deprived medium. Fluorescence immunostaining with antibody against LAMP2 (red) in serum starved placental explant (A) at time 0 (just before starvation), (B) at 24 hours after culturing in medium containing FBS, (C) at 24 after starvation (culturing in medium without) FBS, and (D) 24 hours after treatment with GPER agonist, G1. DAPI (blue) staining indicates nuclei. Intensity calculation (E) across the syncytiotrophoblast showed that the distribution of LAMP2 at 24 hours after starvation shifts to the perinuclear and basal surface compared to control treatment (n=7). Each coloured line in 'E' represents the mean intensity of LAMP2 across the syncytiotrophoblast at

5 random sites per image for 6 separate images per experiment. In 'F', each coloured bar indicates mean of the area under the curve (AUC) of the corresponding coloured line presented in 'E' and statistical differences were calculated. Original magnifications, 40X; scale bar, 20  $\mu$ m. Data presented as mean  $\pm$  S.E.M.; \*  $\rho$ <0.05 (n=7).

#### 5.6 Glossary of terms

Aldehyde Oxidase 1(AOX1) — an oxidizing enzyme with a wide range of substrates, that generates peroxides.

Autophagosome — an intracellular organelle that collects damaged proteins and old mitochondria.

G protein-coupled estrogen receptor 1 (GPER1) — a cell surface estrogen receptor distinct from nuclear estrogen receptors.

8-hydroxy-deoxyguanosine (8OHdG) — a product of DNA oxidation.

4-hydroxynonenal (4HNE) — a product of lipid peroxidation.

Lipid peroxidation — the oxidative degradation of lipids.

Lysosome — an intracellular organelle that contains proteolytic enzymes in an acid environment.

## **CHAPTER 6**

Effect of Serum-Starvation on Lipid Peroxidation and Expression of Sirtuins in Human Placental Explants: Implication for Aldehyde Oxidase 1 and G-Protein Coupled Estrogen Receptor 1 in Placental Oxidative Damage and Aging

#### 6.0 Abstract

**Background:** Placental aging is associated with major obstetric complications, including fetal growth restriction, preeclampsia, preterm labour, and fetal death. Increased placental oxidative stress and premature aging are considered to be the key in the genesis of these complications. Also, changes in placental protein expression, especially those that are crucial for cell survival and metabolic function, appears to be associated with these complications.

**Objective:** We have proposed a novel hypothesis that human aldehyde oxidase 1 (AOX1) and G-protein coupled estrogen receptor 1 (GPER1) regulate placental oxidation and aging. We aimed to develop an *in vitro* human placental explant model to test our hypothesis. In this study, using the placental explant model, at first, we investigated if growth factor starvation induces placental oxidative damage and how this is mediated. We also investigated whether the expression of placenta sirtuins is altered in placenta under oxidative stress. Sirtuins are a group of proteins that regulate longevity and aging, and decreased expression of sirtuins has been shown to be associated with shorter life span in experimental organism. We then explored the effect of inhibition of AOX1 or activation of GPER1 on their protein expression.

**Methods:** To induce oxidative damage we cultured the placental explants in serum-free medium. We blocked AOX1 activity and activated GPER1 by treating the explants with an AOX1 inhibitor, raloxifene, and a GPER1 agonist G1. We then tested our hypothesis that AOX1 and GPER1 mediate oxidative damage to lipids in placenta using an ELISA for 4-hydroxynonenal (4HNE, a product of lipid peroxidation). Using western blotting, we also measured the expression of the sirtuins SIRT1, SIRT2, and SIRT6 after serum-starvation and after treatment with both raloxifene and G1.

**Results:** We found that serum free medium causes an increase in 4HNE production compared to the serum control ( $\rho = 0.0005$ , N=6). Significant decreases in the expression of SIRT1 ( $\rho=0.011$ , N=5), SIRT2 ( $\rho=0.003$ , N=5) and SIRT6 ( $\rho=0.002$ , N=5) were also observed after serum deprivation. Both raloxifene and G1 significantly lowered the serum-starvation induced production of 4HNE (raloxifene,  $\rho=0.0005$ ; G1,  $\rho=0.0005$ ). Reduction of oxidative stress by either blocking AOX1, or by inducing the activity of GPER1 upregulated the expression of the three sirtuins, SIRT1 (raloxifene,  $\rho=0.0195$ ; G1,  $\rho=0.0078$ ), SIRT2 (raloxifene,  $\rho=0.0273$ ; G1,  $\rho=0.0059$ ) and SIRT6 (raloxifene,  $\rho=0.0117$ ; G1,  $\rho=0.0371$ ).

**Conclusion:** There is evidence for a role for both AOX1 and GPER1 in mediating placental oxidation and aging that may contribute to the pathogenesis of obstetric complications. Inhibition of AOX1 or activation of GPER1 reduces placental oxidative damage and increases the activity of SIRT1, SIRT2 and SIRT6, and thus rescues the placenta from oxidative damage and aging. Treatment targeting AOX1 and/or GPER1 in the placenta may be novel therapeutic strategies for delaying placental aging and preventing pregnancy complications.

#### 6.1 Introduction

Placental aging causes major obstetric complications, including fetal growth restriction [241,255,285,286], preeclampsia [241,252,255], preterm labour [264,388], preterm premature rupture of membranes (pPROM) [264,266], and fetal death [229,307]. Premature placental aging is considered to be the key in the pathogenesis of unexplained stillbirth [229,307]. There is also strong evidence of aging in late gestational tissues [229]. Premature

aging may be triggered by stress that may affect the functions of this vital organ. Therefore, premature aging of the placenta may be a key component in determining the outcome of pregnancy.

Intrauterine fetal death, also known as stillbirth, is a major health burden, occurring in approximately 1 in 200 pregnancies. In 2015, there were approximately 2.6 million stillborn babies worldwide [4]. There is an increased risk of fetal death late in pregnancy [393,394]. Recently, Ferrari et al. demonstrated that placentas from unexplained fetal death exhibit short telomeres (a marker of cellular senescence and aging), with an overall 2-fold reduction of telomere length in placentas from fetal death (both early and late term) compared to term live birth placentas [398], suggesting that premature placental aging may play a significant role in the genesis of this complication. In we recently reported that placentas from unexplained stillbirth show evidence of oxidative damage and premature aging [229]. Increased expression of 8-hydroxy-deoxyguanosine (80HdG, an oxidised derivative of deoxyguanosine, used as a marker of DNA oxidation) and 4-hydroxynonenal (4HNE, a product of lipid peroxidation) have been observed in fetal death associated placentas compared to term placentas [229]. Also, a dysregulated lysosomal distribution, an increased autophagosome size with failure of autophagosome-lysosomes fusion were all associated with fetal death [229], suggesting an overall inhibition of autophagy. Autophagy, a lysosomemediated catabolic process of eukaryotic cells, is central to the cell recycling system that digests cellular constituents. A reduced autophagic potential is linked to normal or pathological aging [445]. Late-term (after 41 weeks of gestation) placentas show increased oxidation of DNA and lipids, and inhibition of autophagy compared to term placentas [229].

Moreover, an upregulation of aldehyde oxidase 1 (AOX1) expression, an enzyme that is known to be involved in production of reactive oxygen species (ROS) [207], is observed in placentas from both fetal death and late-term pregnancies compared to term pregnancies [229]. We then tested the hypothesis that AOX1 mediates oxidative damage in placenta using small villous tissue sections, known as placental explants. We have chosen to study explants, because it provides a model in which multiple cell types are retained in anatomical structure and is physiologically more relevant. Similar to late-term and stillbirth placentas, human placental explants cultured in serum-free (growth factors deficient) medium showed changes in oxidation of lipid, lysosomal distribution and autophagosomes size, which were blocked by inhibitors of AOX1 [229], suggesting that this enzymes plays a key role in placental oxidation and aging.

AOX1, an enzyme of the molybdenum hydroxylase family, catalyses the oxidation of a range of substrates including aldehydes, nicotinamide adenine dinucleotide (NADH) and nitrogencontaining aromatic heterocycles [210-212]. AOX1 is mostly known for its role in the biotransformation of certain drugs and detoxification of xenobiotics, however, it also serves as an important source of ROS in biological tissues and may play a vital role in ROSmediated redox signalling and tissue injury [206,207]. In the presence of molecular oxygen, AOX1 oxidises aldehydes into corresponding acids and ROS, mainly superoxides ( $\cdot$ O<sub>2</sub><sup>-</sup>) and hydrogen peroxides (H<sub>2</sub>O<sub>2</sub>), which contribute to the basal level of ROS under normal physiological conditions [217]. This basal level of ROS may be greatly enhanced in certain disease settings where cellular aldehyde levels are increased [222]. The ROS generated during the metabolism of aldehydes by AOX1 has been suggested to promote oxidative stress-induced liver injury in chronic alcoholics [218,219], ischemia-reperfusion injury [223], inflammation and various inflammatory diseases [217], and cardiovascular diseases [224]. Thus, AOX1 may have an important role in ROS induced placental oxidative damage. A number of AOX1 inhibitors have been identified, including raloxifene,  $\beta$ -estradiol, menadione and amidone [207]. Among them, raloxifene, which is a potent inhibitor of human liver AOX1, has been shown to reduce ROS generation and oxidative damage in vascular endothelial cells [225] and reduce lipid peroxidation in renal tissue [226]. In Addition, GPER1, an estrogen receptor that is widely expressed in various tissue types including the brain, heart, vasculature, pancreas, ovary, kidney and the placental syncytiotrophoblast membrane, is known to regulate ROS production and oxidative stress [228,229]. Estrogen activation at GPER1 by its agonist G1 or E2 (\beta-estradiol) has been shown to reduce NADPHstimulated ROS generation in rat heart [239,240] and in human umbilical vein endothelial cells [446], while GPER1 inactivation by its antagonist G15 increases ROS generation in human umbilical vein endothelial cells [446]. G15 also blocks the effect of GPER1 activation by E2 in reducing ROS production in rat heart [239].

Lipids, particularly the polyunsaturated fatty acids of cellular membranes, are susceptible to non-enzymatic oxidation by ROS, a process commonly referred to lipid peroxidation. Lipid peroxidation is a chain reaction that produces multiple breakdown molecules, and the levels of these molecules in tissues and in biological fluids are considered potential biomarkers for oxidative stress and oxidative stress-related diseases *in vivo* [447]. Among the products of lipid peroxidation, 4-hydroxynonenal (4HNE) is most intensively studied [448]. Due to its high electrophilicity, 4HNE readily reacts with cellular macromolecules, such as proteins and

lipids, and at higher concentration, with DNA, that contributes further to oxidative stress [221,449]. A low concentration of 4HNE can be detected in human tissue and biological fluid under physiologic conditions, however, in certain disease states the levels of this marker increase significantly [222]. Increased production of 4HNE has been shown to be associated with age-related disorders such as Alzheimer's disease [397], aging tissue, for example, late-term placentas and placentas associated with stillbirth [229].

Alteration in placental protein expression, especially those that are crucial for development and function of placenta, appears to be associated with various pathologic situations including preeclampsia [450-454], fetal growth restriction [455-459], gestational diabetes [460], early pregnancy loss [461], and fetal death [462,463]. These pregnancy complications have been shown to be associated with increased placental oxidative stress and aging [464,465]. However, it is unknown how the placenta utilises its energy to protect itself from oxidative damage and aging [466]. We hypothesized that the placenta might use similar mechanisms that most of the organisms employ to control physiological aging. Here, we investigated a family of proteins, called sirtuins, in the placenta that control life span in multiple organisms, and attempted to link these proteins to the pathways that mediate oxidative damage and aging in the placenta.

The sirtuins (SIRT) are a conserved family of nicotinamide adenine dinucleotide (NAD)dependent histone/protein deacetylases that modulate distinct metabolic, cell proliferation and stress response pathways [467,468]. Sirtuins have the ability to directly linked the cellular energetic status (reflected by NAD<sup>+</sup> / NADH ratio) to protein post-translational modifications through catalysing the removal of acetyl groups from the *N*-acetyl-lysine of histones and non-histone proteins [469]. The deacetylation reaction consumes NAD<sup>+</sup> and generates deacetylated protein, nicotinamide and the unique metabolite 2'-*O*-acetyl-ADP-ribose [470]. The 2'-*O*-acetyl-ADP-ribose may act as a secondary messenger, a cofactor, or as a regulator of other enzymes/proteins to elicit the appropriate cellular response [471].

In mammals, seven sirtuin members (SIRT1 to SIRT7) have been identified that have distinct subcellular localizations: SIRT1, SIRT6, and SIRT7 are predominantly found in the nucleus, SIRT2 is cytosolic, and SIRT3, SIRT4, and SIRT5 are primarily located in the mitochondria [468]. Sirtuins are important sensors of metabolic activity and energy (NAD<sup>+</sup>) depletion and thus critical for cell survival. SIRT1 is the primary deacetylase with many down-stream targets including transcription factors, and controls angiogenesis, DNA repair, stress resistance, inflammation, lipid metabolism, and gluconeogenesis [472-474]. SIRT1 promotes cell survival by protecting against apoptosis in response to oxidative stress and DNA damage [475]. SIRT2 has been shown to be implicated in aging, cell survival, metabolism, and stress response signalling [475]. Both SIRT1 and SIRT2 are seen in placental syncytiotrophoblast, cytotrophoblasts and in the membranes, where they function as anti-inflammatory proteins [474]. SIRT3 controls fatty acid oxidation in the mitochondria, increases mitochondrial respiration and reduces ROS generation, and prevents hypoxia-induced apoptosis. SIRT6 plays a crucial role in DNA repair processes [475]. Therefore, the functions of these proteins are essential in protecting cells from extreme stress.

There is also evidence linking aging and oxidative damage with sirtuins. During oxidative damage and aging process, cellular energy levels are reduced [476] and sirtuins are downregulated [476-478]. In human pelvic skin samples, SIRT1 [476], and peripheral blood mononuclear cells, SIRT2 [479] activities are reduced with donor age, while in primary dermal fibroblasts, SIRT1 and SIRT6 are downregulated through passaging [480]. Likewise, levels of SIRT1, SIRT3, and SIRT6 were shown to be significantly decreased in ovaries of aged mice [481]. Moreover, evidence from studies showed a strong link between sirtuins and life span in experimental organisms. Genetic deletion of Sir2 (silent information regulator 2, that encode mammalian homolog of sirtuins in yeast) gene severely shortens life span in Saccharomyces cerevisiae, while its overexpression increases life span beyond that for wildtype cells [482,483]. Similarly, overexpression of Sir2.1 gene, a homolog of yeast Sir2, increases life span in C. elegans [484] and Drosophila [485]. Moreover, overexpression of Sir2 rescues the oxidative stress-induce shortened life span phenotype observed when S. *cerevisiae* are treated with hydrogen peroxide [486], suggesting that Sir2 may influence life span extension by diminishing oxidative stress [487,488].

In mammals, substantial evidence exists that sirtuins contribute to the cellular response to oxidative stress by directly deacetylating and activating several transcription factors and enzymes that are critical in maintaining cellular ROS homeostasis and antioxidant levels [467]. For example, SIRT1 and SIRT2 deacetylate and activate several members of the redox-sensitive transcription factors forkhead box class O (FOXO) family, such as FOXO3, which promote cellular resistance to ROS by increasing the expression of stress response genes, including superoxide dismutase 2 (SOD2) [489-492]. SIRT6 regulates apoptosis and

cellular senescence via deacetylation of histone H3 lysine 9 (H3K9) at nuclear factor-κB signalling (NF- κB) target gene promoters that controls aging-associated changes in gene expression [493] or at telomeric chromatin [494]. SIRT6 deficient cells display sensitivity to oxidative stress and a reduced capacity for DNA damage repair [495]. SIRT6 knockout mice show multiple hallmarks of premature aging, such as, osteoporosis, spinal curvature disorders, subcutaneous fat atrophy, lymphocyte telomere attrition, and life-threatening metabolic defects with steep falls in serum levels of glucose and growth factors that ultimately led to premature death in very early life [493,495]. In support of the anti-aging effects of SIRT6, male mice overexpressing SIRT6 have a significantly longer life span than their wild-type counterparts [496]. Mechanistically, in response to oxidative stress, SIRT6 stimulates DNA double-strand break repair by stabilising the DNA double-strand break repair factor, DNA-dependent protein kinase (DNA-PK) or activating ADP-ribosylating enzyme, poly (ADP-ribose) polymerase 1 (PARP1) [497-499].

Given the intriguing links of both oxidative damage in lipids and sirtuins to oxidative stress and aging-associated processes, and a potential link of oxidative stress and AOX1 and GPER1 pathways, we hypothesized that sirtuins and AOX1 and/or GPER1 might functionally interact. In this study, to confirm our previous finding, at first, we investigated if growth factor removal induces oxidative stress in the placenta. We then aimed to investigate whether the expression of sirtuins in the placenta is altered in response to oxidative stress, and to explore if modulating the pathways that cause placental oxidation can attenuate the changes in the expression of these proteins.

#### 6.2 Experimental

#### 6.2.1 Human placental explant culture

The study was performed in accordance with the Hunter New England Health Services and the University of Newcastle Research Ethics Committee (Ethics approval ref. number is H-2010-1210). Human term placentas (37-39 weeks of gestation) were obtained from women with uncomplicated singleton pregnancies without any symptoms of labour after an elective (a scheduled repeat) caesarean section. Written informed consent was obtained from all patients before collecting a sample. Placentas from patients with infection, smoking, obesity, diabetes, preeclampsia, placenta praevia, intrauterine growth restriction or abruption were excluded. Placentas were collected immediately after delivery and prepared for explant culture. Villous tissues of placentas were randomly sampled from different regions of the placenta and 4-5 mm beneath the chorionic plate. Tissue biopsies were washed several times with sterile Dulbecco's phosphate buffered saline (PBS) to remove excess blood and dissected into approximately 2 mm<sup>3</sup> explants under sterile condition. Explants (30 per 100 mm culture dish) were submerged in 30 mL of culture medium containing Dulbecco's modified Eagle's medium (DMEM) supplemented with 2 mM L-glutamine, 1% Na-pyruvate, antibiotic-antimycotic solution (10,000 units/mL of penicillin, 10,000 µg/mL of streptomycin, and 25  $\mu$ g/mL of Amphotericin B) with the addition of 10% (v/v) fetal bovine serum (FBS) (referred to as 'growth medium' hereafter). Explants were cultured in a cell culture chamber at 37 °C temperature and maintaining 95% air (20% oxygen) and 5% CO<sub>2</sub> for up to 24 hours, as previously described [229]. At day 2, villous explants were transferred to fresh 30 mL growth medium and incubated in a cell culture chamber for 90 min. After this, the tissues were washed in DMEM without FBS (referred to as 'serum-free medium' thereafter). Next 6–7 pieces of villous explants weighing approximately 400 mg were transferred to a 60 mm culture dishes, each containing 6 mL growth medium, serum-free medium, raloxifene (100 nM) in serum-free medium and GPER1 agonist, or G1 (1  $\mu$ M) in serum-free medium, for subsequent incubation for 24 hours. At the end of incubation time tissues were snap frozen in liquid nitrogen and stored at -80 °C until subsequent experiments (ELISA, and western blotting). For each placental explant culture, samples were also collected at time '0 (zero)' hour, i.e., before incubation in serum-free medium, and were stored frozen at -80 °C until further experiments.

#### 6.2.2 Enzyme-linked immunosorbent assay (ELISA)

For detection and quantitation of 4HNE protein adducts in placental explants, we used OxiSelect<sup>TM</sup> HNE Adduct Competitive ELISA Kit (Cell Biolabs Inc, STA- 838). This assay is based on the competition between 4HNE conjugate and the anti-HNE antibody for HNE-protein. In this assay, first, an HNE conjugate is coated on an ELISA plate. The unknown HNE-protein samples or HNE-BSA standards are then added to the HNE conjugate adsorbed ELISA plate. After a brief incubation, an anti-HNE polyclonal antibody is added, followed by an HRP conjugated secondary antibody. The amount of HNE protein adducts in unknown samples is determined by comparison with a predetermined HNE-BSA standard curve.

Tissue extract was prepared by homogenising 50 mg of crushed placental tissues in 300  $\mu$ L of deionised water using a Precellyse Lysing Kit (Sapphire Bioscience) in a Precellyse 24

homogeniser (Bertin Technologies). The lysate was centrifuged at 13,000g for 10 min at 4 °C to pellet insoluble cellular debris, and the supernatant containing protein extract was separated. The total protein concentration of each tissue extract was measured spectrophotometrically using a Pierce Bicinchoninic Acid (BCA) Protein Assay Kit (Thermo Fisher Scientific) in a SPECTROstar Nano Microplate Reader (BMG LABTECH).

In this assay, at first, an HNE conjugate was coated on a 96-well plate and incubated overnight at 4 °C. HNE conjugate was removed, and the wells were rinsed twice with 1X PBS. Assay diluent was added to each well and blocked for an hour at RT and then transferred to 4 °C until use. Immediately before use, assay diluent was removed, appropriate dilution of unknown samples (for 40 µg protein in each well) or HNE-BSA standards diluted in assay diluent were added. The plate was incubated for 10 min at RT on an orbital shaker. The diluted anti-HNE antibody was added to each well and incubated at RT for 2 hours on an orbital shaker. The plate was then washed 3 times with 1X wash buffer with thorough aspiration between each wash. The diluted HRP conjugated secondary antibody was added to all wells and incubated for 1 hour at RT on an orbital shaker. The strip wells were then washed a further 3 times with 1X wash buffer. The substrate solution was added to each well, incubated at RT for 2–5 min on an orbital shaker and the changes of colour were monitored. The stop solution was added to each well to stop the enzyme reaction. The absorbance of each well was read on a microplate reader (SPECTROstar Nano, BMG LABTECH) using 450 nm as the primary wavelength. The content of HNE protein adducts in unknown samples was determined by comparison with the predetermined HNE-BSA standard curve using

GraphPad software. The results were graphed, and the statistical analysis was performed using GraphPad software version 7 (GraphPad Prism, CA, USA).

#### 6.2.3 Protein extraction and western blotting

Samples of placental explant were crushed under liquid nitrogen. Aliquots of 50 mg of the ground of placental tissues were homogenised in 300 µL of in-house lysis buffer (1% Triton-X-100, 0.1 % Brij-35, 1X protease inhibitor cocktail tablet (Roche) and 1X phosphatase inhibitor tablet (Roche) in PBS at pH 7.4) using a Precellyse Lysing Kit in a Precellyse 24 homogeniser. The lysate was centrifuged, the supernatant containing tissue extract was separated, and the total protein concentration of each tissue extract was measured spectrophotometrically using a Pierce BCA Protein Assay Kit as described in the Section 6.2.2 in this Chapter. Thirty  $\mu$ g of protein samples were mixed with 5  $\mu$ L of LDS loading buffer containing sample reducing agent (Thermo Fisher Scientific) and then heated in a 70 °C heating block for 10 min. Proteins were then loaded into Nu-PAGE 4–12% Bris-Tris precast 12 well gels (Thermo Fisher Scientific) and were separated by electrophoresis for 60 min at a constant potential of 200 V. MagicMark<sup>™</sup> XP Western Protein Standard (Thermo Fisher Scientific) was used as a standard protein ladder. Separated proteins were then transferred to a nitrocellulose membrane (iBlot<sup>TM</sup> Gel Transfer Stack, nitrocellulose, mini, Thermo Fisher Scientific) for 7 min using an iBlot® Gel Transfer Device (Thermo Fisher Scientific). The membranes were incubated in a blocking buffer containing 1% bovine serum albumin (BSA) in TBS-T (Tris-buffered saline containing 0.1 % tween-20) for an hour at room temperature (RT). The membranes were then incubated with primary antibodies (SIRT1, SIRT2 and SIRT6) in 1% BSA in TBS-T at a dilution recommended by manufacturer overnight at 4 °C. The next day membranes were washed three times for 5 min each in TBS-T. The membranes were then incubated with the appropriate HRP conjugated secondary antibody diluted in 1% BSA in TBST for an hour at RT. After 3 further washing in TBS-T, the immunoreactive bands were developed in Luminata reagent (Thermo Fisher Scientific) and enhanced chemiluminescence was used for protein detection using an Amersham Imager 600 (GE Healthcare). Quantification of the photographs was performed by measuring the optical density (OD) of the target bands of the blot using an Amersham Imager 600 Analyser. The loading was verified by Ponceau S staining of the individual blot. Target band densities were normalized by dividing the OD values of target proteins with the OD values of Ponceau S stained band of the same blots. For each protein target, the band with a lowest mean density was assigned an arbitrary value of 1. All individual densitometry values were expressed relative to this mean. The results were graphed using GraphPad Prism software.

#### 6.2.4 Data presentation and statistical analysis

Sample numbers are shown in the legends to individual figures. The quantitative data were presented as mean  $\pm$  standard error of the mean (S.E.M). Statistical differences were analysed by *t*-test with Wilcoxon matched-pairs signed rank test (paired, non-parametric) using GraphPad Prism software for repetitive measurement between treatment and control group. A  $\rho$  value of less than 0.05 was considered statistically significant. All the graphs were prepared using GraphPad Prism software.

#### 6.3 Results

### 6.3.1 Serum-starvation increases lipid peroxidation in placental explants via AOX1 and GPER1 mediated pathways

In our previous study, we have shown that serum starvation increases lipid peroxidation, measured as production of 4HNE in placental explants, via AOX1 and or GPER1 mediated pathways [229]. To confirm our previous observation, here we further investigate whether the AOX1 and GPER1 are involved in mediating placental oxidation and aging using a quantitative assay. To induce in vitro oxidative stress damage, we starved the placental explants by culturing in serum-free (growth factor deficient) medium for 24 hours. Serum starvation is shown to increase oxidative stress in cultured cells [500]. We also treated the explants with either raloxifene, a potent AOX1 inhibitor or with G1, a GPER1 agonist, in serum-free medium for 24 hours. We then quantitatively measured the concentration of 4HNE in placental explants by ELISA. We observed a significantly higher amount of 4HNE  $(\rho=0.0005)$  in placental explants cultured in the serum-free medium compared with those cultured in growth medium (Figure 6.1). A sharp reduction of 4HNE production was noted when placental explants were cultured in serum-free medium with raloxifene ( $\rho=0.0005$ ), or G1 ( $\rho$ =0.0005) (Figure 6.1), further confirming that both AOX1 and GPER1 regulate the oxidative stress induced lipid peroxidation in the placenta.



Figure 6.1 Serum-starvation increases lipid peroxidation in placental explants via activation of AOX1 and loss of GPER1 function. Quantitative measurement of 4HNE in placental explants by ELISA. Serum starvation (serum-free) for 24 hours increases 4HNE production compared to controls (Cont.) at both '0 hr' (just before starvation) and at '24 hrs' after culturing in serum-containing medium. Both raloxifene (100 nM concentration) and G1 (1  $\mu$ M concentration) treatments for 24 hours in serum-free medium inhibited 4HNE production via blocking of AOX1. Data are presented as mean ± S.E.M (N=6 independent experiments, n=12 individual data points). Statistical differences ( $\rho$  values) were calculated using *t*-test (paired, non-parametric) using GraphPad Prism software and a  $\rho$  value of less than 0.05 was considered statistically significant, \*\*  $\rho$ <0.001.

# 6.3.2 Growth factor removal downregulates SIRT1, SIRT2 and SIRT6 mediated by AOX1 and GPER1

To study a potential connection between sirtuins and oxidative stress, we compared the expression of three members of the sirtuin family of longevity proteins, SIRT1, SIRT2 and SIRT6, in placental explants cultured in serum-free and serum control medium using western blotting. All the three SIRT1, SIRT2 and SIRT6 were highly expressed in control samples. However, the expression of SIRT1 ( $\rho$ =0.0117), SIRT2 ( $\rho$ =0.0039) and SIRT6 ( $\rho$ =0.002) were significantly reduced in explants cultured in serum-free medium (Figure 6.2). Therefore, we next examined whether modulation of pathways that increase oxidative stress can attenuate the changes in expressions of these sirtuin members. To inhibit AOX1 we cultured explants in serum-free medium either with its inhibitor raloxifene, and to induce GPER1 activation we used G1, a GPER1 agonist. Our western blotting data showed that treatment with either raloxifene or G1 significantly upregulated the expression of all three sirtuins, SIRT1 (raloxifene,  $\rho=0.0195$ ; G1,  $\rho=0.0078$ ), SIRT2 (raloxifene,  $\rho=0.0273$ ; G1,  $\rho$ =0.0059) and SIRT6 (raloxifene,  $\rho$ =0.0117; G1,  $\rho$ =0.0371) (Figure 6.2). This data suggests that AOX1 and GPER1 may mediate the activity of these proteins possibly by regulating ROS generation of oxidative stress.



Figure 6.2 Removal of growth factors downregulates SIRT1, SIRT2 and SIRT6 mediated by AOX1 activation and loss of GPER1 function. Western blots of serum-starved placental explants and after treatment with G1 and raloxifene for 24 hours, against antibody as shown (A), and quantification of the protein expression (B–D). Serum starvation (serum-free) for 24 hours decreases the protein expression for SIRT1 (B), SIRT2 (C) and SIRT6 (D) compared to controls (Cont.) at both '0 hr' (just before starvation) and at '24 hrs'. Both Raloxifene (100 nM concentration) and G1 (1 μM concentration) treatment for 24 hours in serum-free medium upregulates the expression of

SIRT1, 2 and 6. Data are presented as mean  $\pm$  S.E.M of N=5 independent experiments, n=10 individual data points. Statistical difference ( $\rho$ ) values were calculated using *t*-test (paired, non-parametric) using GraphPad Prism software and a  $\rho$  value of less than 0.05 was considered statistically significant, \*  $\rho$ <0.05, \*\*  $\rho$ <0.01.

#### 6.4 Discussion

In this study, we demonstrate that placental oxidative stress induced by growth factor removal causes lipid peroxidation and downregulates the expression of three members of sirtuins, SIRT1, SIRT2 and SIRT6, in human placental explants. We then tested our hypothesis that AOX1 activation and/or loss of GPER1 function mediate the oxidation of the placenta. Our results show that inhibition of AOX1 with the potent AOX1 inhibitor raloxifene and activation of GPER1 by its agonist G1 can attenuate lipid peroxidation in placental explants. Biochemical analysis of placental explants further reveals that reduction of placental oxidative damage by blocking of AOX1 or by inducing GPER1 can rescue the expression of SIRT1, SIRT2 and SIRT6.

Oxidative stress causes changes in the cellular macromolecules including proteins, lipids and DNA [501], which promotes premature cellular senescence and aging, leading to multiple pathologic conditions, such as Alzheimer disease, Parkinson disease, cardiovascular diseases, musculoskeletal weaknesses, and bone and muscle loss [502]. Placental oxidative stress is thought to be the primary cause of pregnancy-related complications including

spontaneous abortion, pre-term birth, fetal growth restriction, preeclampsia and fetal death [464].

In our previous study, we showed a significant difference in levels of placental 4HNE, a biomarker of lipid peroxidation and 8OHdG, a marker of DNA oxidation, between cases of late-term and unexplained stillbirth, with those of term pregnancies, suggesting an increased placental oxidative damage [229]. We have also shown that induction of oxidative stress in placental explants stimulates the production of 4HNE [229]. The present study confirms that similar change of 4HNE production is induced by oxidative stress in placental explants using a quantitative measurement for 4HNE. In accordance with our results, the level of 4HNE has been shown to be elevated in severe pregnancy complications associated with oxidative damage, such as preeclampsia [503,504]. Increased levels of placental 8OHdG are associated with pregnancies complicated by both preeclampsia and fetal growth restriction [281]. The markers of oxidative damage including production of 4HNE and 8OHdG have been shown to be increased in placentas from women who smoked during pregnancy [180].

We observed in our previous study that the levels of AOX1 is increased in placentas from women who gave birth after 41 weeks and in those who had had a stillborn baby [229], indicating that this enzyme may mediate the placental oxidation. The presence of the cell surface estrogen receptor GPER1 on the normal placental syncytiotrophoblast membrane [229], and its role in reducing ROS generation and oxidative stress [226,239,240], suggests that this receptor may play a role in modulating oxidative damage within the placenta. To test our hypothesis that the placental oxidation is controlled through both the activation of

AOX1 and loss of GPER1 function, we used a potent AOX1 inhibitor, raloxifene and a GPER1 agonist, G1. Raloxifene has previously been shown to reduce oxidative damage by lowering 4HNE production in renal tissue [226] and reducing ROS generation in vascular endothelial cells in rats [225] and in humans [505]. Estrogen activation at GPER1 by G1 has been shown to reduce lipid peroxidation in renal tissue [226], and rescue oxidative stress-induced damage in human renal epithelial cells [506], by blocking superoxide generation [239,240]. Consistent with the previous study, our results showed that both raloxifene and G1 prevented serum-starvation induced lipid peroxidation (4HNE production) in placental explants. These results indicate that activation of AOX1 or low estrogen level, which causes a reduction of GPER1 function, may lead to increased placental ROS generation, resulting in placental oxidative damage. These findings also suggest that the placental explant is a suitable model of to study placental aging in pathologic pregnancies.

In this study, a group of longevity proteins, known as sirtuins, have been investigated; SIRT1, SIRT2, and SIRT6 [507]. All these proteins are controlled by intracellular NAD<sup>+</sup> levels and thus, available cellular energy [466]. These proteins regulate cell survival and aging [475], and therefore, may be crucial for placental maintenance and successful pregnancy. Our results showed a significant decrease in the expression of all the three sirtuins, SIRT1, SIRT2 and SIRT6 in the serum-starved placental explants. Consistent with our results, previous studies showed an association with reduced expression of SIRT1, SIRT2 and SIRT6 with pathological pregnancies. SIRT1 has been shown to be downregulated in placentas associated with preeclampsia [466], while a significant reduction in SIRT2 protein expression in placentas is associated with both preeclampsia and fetal growth restriction [508]. Another

study reported that SIRT6 expression is significantly decreased in placental membranes in spontaneous preterm labour, suggesting that SIRT6 may control the terminal effector pathways of human labour [509].

SIRT1, which is the key NAD<sup>+</sup>-dependent protein deacetylase, regulates many cellular processes, including cellular maintenance, differentiation, stress response and cell survival [510], and thus has been implicated in various pathological conditions including aging, cancer, diabetes, obesity, cardiovascular and neurodegenerative diseases [511]. Increased protein acetylation is associated with the development of numerous disease conditions including aging and neurodegenerative disorders [512,513]. In the brains of Alzheimer's patients, SIRT1 level is reduced [514], and is associated with acetylation of proteins, such as tau and amyloid  $\beta$ , that prevents degradation of phosphorylated forms of these proteins [513,515,516]. The accumulation of hyper phosphorylated tau is a common feature of Parkinsonism and Alzheimer's disease [513,514]. Overexpressing SIRT1 protects against neurodegeneration in a mouse model of Alzheimer's disease [517].

Studies in an animal model showed that SIRT1 defective mice are growth restricted with developmental defects of the retina and heart, and often died postnatally [518]. In addition, both embryos and placentas from SIRT1 deficient mice are smaller, with abnormalities in trophoblast differentiation and placental development. Overexpression of cardiac-specific SIRT1 in mice can delay aging and protect from oxidative stress [519]. Also, upregulation of SIRT1 attenuates oxidative stress via upregulation of superoxide dismutase 2 and catalase in human astrocytes [520]. Evidence suggests that SIRT2 plays crucial roles in regulating

important cellular pathways, including proliferation and differentiation, inflammation, cellular metabolic regulation, energy homeostasis and the oxidative stress response [521]. SIRT2 is downregulated in obese human adipose tissue [522], and insulin-resistant human hepatocytes and mouse livers [521], and is associated with increased generation of ROS. SIRT6 is known to regulate both longevity and aging; SIRT6 overexpression leads to enhanced longevity in male mice [496], while mice lacking SIRT6 exhibit progeroid phenotypes (a genetic disorder resembling accelerated aging) [495]. Functionally, SIRT6 plays an important role in DNA repair, telomere and telomerase function, genomic stability, and cellular senescence [523,524]. The activity of SIRT6 is linked to redox homeostasis in human embryonic stem cells; the SIRT6 deficient cells display premature and progressively accelerated cellular senescence, exhibit increased ROS generation, dysregulated redox metabolism, and increased sensitivity to oxidative stress [525].

Together with the observation of previous studies and based on our results, we hypothesised that increased ROS generation and oxidative stress may change the expression of these proteins, and modulation of ROS production might alter this response. Thus, we attempted to reduce oxidative stress by inhibiting AOX1 or activating GPER1, both of which are known to regulate ROS generation [206,207,240], in placental explants. Our results indicate that reduction of ROS generation either by blocking AOX1, or by activating GPER1 upregulates the expression of the sirtuins, SIRT1, SIRT2 and SIRT6, and thus rescues the cells from oxidative damage and aging (Figure 6.3).



Figure 6.3 Effect of AOX1 inhibition or GPER1 activation on sirtuins. Diagram showing how ROS, and oxidative stress downregulate the activity of sirtuins. Reduction of ROS generation by AOX1 inhibition or GPER1 activation may activate the sirtuins. Upon activation, SIRT1, SIRT2 and SIRT6 deacetylate several proteins that promote resistance to oxidative stress, DNA damage

repair and increase cell survival. Ac, acetyl group; FOXO, forkhead box class O; SOD2, superoxide dismutase 2; H3K9, histone H3 lysine 9; NF-  $\kappa$ B, nuclear factor- $\kappa$ B.

Our study confirms a strong link between both AOX1 and GPER1 in mediating placental oxidative damage, and indicates an association between sirtuins and oxidative damage and aging in the placenta. There could also be an interlinking between AOX1 or GPER1 mediated pathways in controlling sirtuins. Treatment targeting AOX1 and/or GPER1 in the placenta may lead to novel therapeutic strategies for delaying placental aging. Further studies are now underway in our laboratory to determine the cell signalling events that occur in the placenta under oxidative stress that contribute to the development of an aging phenotype.

## **CHAPTER 7**

Growth Factor Depletion in Placental Trophoblast Cells Increases Lipid Peroxidation, Reduces mTORC1 Activity and Alters Mitochondrial Function via Aldehyde Oxidase 1 and GPER1 Mediated Pathways

#### 7.0 Abstract

**Background:** Biochemical evidence of placental aging in the form of increased oxidised lipid (4-hydroxynonenal, 4HNE) and oxidised DNA (8-hydroxy-2-deoxyguanosine, 8OHdG) have been demonstrated in association with fetal death. The increased production of 4HNE was replicated in serum-deprived placental explants and shown to depend on aldehyde oxidase (AOX1) activity. It was unclear how serum deprivation impacted upon nutrient sensing pathways and mitochondrial function and the relationship to AOX1 activity.

**Objective:** We have used the HTR8/SVneo placental cell line to test the hypothesis that serum deprivation-induced changes in nutrient sensing pathways, and mitochondrial function were downstream from AOX1 and G-protein-coupled estrogen receptor (GPER1).

**Methods:** To induce oxidative damage we removed growth factors from HTR8/SVneo cells by culturing them in serum-free medium for 24 hours. To inhibit AOX1 activity the cells were treated with raloxifene, a potent AOX1 inhibitor, or G1, a GPER1 agonist, that is also known to inhibit AOX1 activity. Lipid peroxidation and DNA oxidation were quantified using ELISA for 4HNE and 8OHdG, respectively. Western blotting was used to measure phosphorylation of p70S6K that is a downstream target of mTORC1, AKT, an upstream regulator of mTORC1, and AMPK, a cellular energy sensor that promotes energy generation and inhibits mTORC1. A Seahorse Extracellular Flux (XF) Analyser was used to evaluate mitochondrial function including oxygen consumption rate and ATP (adenosine triphosphate) production.

**Results:** Serum-starved HTR8/SVneo cells showed evidence of increased lipid oxidation as 4HNE production ( $\rho$ =0.015, N=4) and DNA oxidation as 8OHdG ( $\rho$ =0.0078, N=4). Both raloxifene and G1 treatment blocked the serum-starvation induced 4HNE production, while production of 8OHdG was blocked by raloxifene. A significant decrease in phosphorylation of both p70S6K ( $\rho$ =0.0005, N=6 and AKT ( $\rho$ =0.0078, N=4) was observed after serum deprivation. Serum deprivation increased AMPK phosphorylation ( $\rho$ =0.0195, N=4). Following serum starvation, the XF assay (N=7) showed a significant decrease in mitochondrial respiration, with a 42% depletion of mitochondrial basal respiration rate, a 44% decrease in ATP production (ATP-linked respiration), a 34% drop of proton leak, a 41% decline of respiration capacity (maximum oxygen consumption rate), and a 47% reduction of reserve (spare) respiration capacity. The increase in AMPK phosphorylation and the decreases in p70S6K and AKT phosphorylation, mitochondrial ATP generation and oxygen consumption were all blocked by inhibition of AOX1 using either raloxifene or G1.

**Conclusion:** Serum-depletion that removes growth factors increases lipid peroxidation and DNA oxidation, while decreasing mTORC1 activity and mitochondrial energy generation, all these changes are downstream from the enzyme AOX1. AOX1 plays a central role in controlling placental cell responses to growth factor deprivation, AOX1 may play a similar role in the aging placental phenotype that is associated with fetal death.

#### 7.1 Introduction

Pregnancy is associated with reactive oxygen species (ROS) production by placental mitochondria [176,177]. The placental oxidative stress becomes exaggerated under certain obstetric conditions [178]. Increased placental oxidative damage and premature aging are demonstrated in major obstetric complications, including intrauterine fetal growth restriction (IUGR) [255,280-283,285-287], preeclampsia [178,252,255,281-283,295,299,302,303], preterm labour and preterm premature rupture of membranes

[264-266,269-272], and fetal death [229,307]. Biochemical evidence of placental aging in the form of increased oxidised lipid (4-hydroxynonenal, 4HNE) and DNA (8-hydroxy-2-deoxyguanosine, 8OHdG) [229], and telomere shortening (an indicator of cellular senescence and aging) [307] have been demonstrated in association with fetal death. Increased lipid peroxidation (production of 4HNE) has been observed in placental explants cultured in serum-deprived medium, which were then shown to be blocked by inhibiting aldehyde oxidase 1 (AOX1) activity using its inhibitor raloxifene, and the Gprotein-coupled estrogen receptor (GPER1) agonist G1 that is thought to act through inhibition of AOX1 activity [229]. In this study, we aimed to generate an aging phenotype in a first-trimester placental trophoblast cell line, the HTR8/SVneo, in which oxidative stress associated changes were induced by removing growth factors (serum) by culturing them in serum-free medium for 24 hours. The HTR8/SVneo cells are shown to express both GPER1 [526] and AOX1 [527]. Oxidative stress increases AOX1 expression in placental HTR8/SVneo cells [527,528], while GPER1 activation by its agonist G1 reduces ROS generation and oxidative stress in rat's kidney [226] and heart [529] cells. To inhibit AOX1 activity and to induce GPER1 activation, we treated the HTR8/SVneo cells with raloxifene, a potent AOX1 inhibitor, and G1, a GPER1 agonist. We have chosen the HTR8/SVneo cell line to test our hypothesis as the cell line is uniform and easily modifiable, i.e., genetic manipulation is easier.

The placenta is the primary organ that exchanges gases and nutrients between the mother and fetus. The transfer function of the placenta depends on the capacity of the placental syncytiotrophoblast in sensing nutrient availability [140]. Trophoblast cells have an array of nutrient-sensing signalling pathways, including mechanistic (mammalian) target of rapamycin complex 1 (mTORC1) and energy-sensing AMP-activated protein kinase (AMPK), which regulate cell proliferation and metabolism in response to altered cellular nutrients and energy levels [143]. The evidence supporting a critical role for mTORC1 (a detailed discussion about mTORC1 is presented in Chapter 1) in placental nutrient sensing is particularly compelling [143]. Under nutrient-rich conditions, mTORC1 activity is increased in response to amino acid, energy and growth factor availability or increases in upstream kinases including AKT that inhibit cellular degradation by autophagy, supporting cellular growth [530-532]. The nutrient-sensing mTORC1 is abundantly expressed in the human placental syncytiotrophoblast [144], and the mTORC1 signalling in cultured primary trophoblast cells is regulated by glucose, amino acids and growth factor signalling [145]. mTORC1 is a positive regulator of the placental amino acid transporters, which are crucial for the delivery of essential amino acids to the fetus [144,146,147]. Activation of placental mTORC1 increases, while inhibition of placental mTORC1 decreases the abundance of placental trophoblast amino acids transporters [148]. Consequently, trophoblast mTORC1 signalling connects nutrients availability to the growth of the fetus by regulating the flow of amino acids through the placental syncytiotrophoblast [148]. Changes in trophoblast mTORC1 signalling have been shown in complicated pregnancies especially those that are associated with abnormal fetal growth in humans and also in laboratory animals where maternal nutrient supply was altered experimentally [148]. In human placenta, mTORC1 signalling is reduced in pregnancies complicated by IUGR [144,152]. Increased activation of placental mTORC1 signalling in obese women [149] and in obese mice [158] is associated with fetal overgrowth, suggesting that mTORC1 activation triggers placental amino acid transportation and contributes to fetal overgrowth.

In addition, AMPK, is a key sensor for cellular energy that controls metabolic function [533]. AMPK is activated during nutritional starvation or stress, inhibits the mTORC1 pathway and positively regulates autophagy [137,534,535]. AMPK activity is regulated by the ratio of ATP to ADP and AMP, which is a measure of cellular energy status and is strictly controlled by the cell. Eukaryotic cells use ATP (adenosine triphosphate) as a direct energy source. ATP is generated via glycolysis and/or oxidative metabolism of cellular nutrients, for example, glucose, amino and fatty acids in the mitochondrial electron transport chain (ETC) [536]. ATP is converted to ADP (adenosine diphosphate) or AMP (adenosine monophosphate) and pyrophosphate molecules that supply energy for essential cellular processes [532]. AMPK exists as heterotrimeric complexes comprised of a catalytic  $\alpha$  subunit and two regulatory subunits,  $\beta$  and  $\gamma$ . AMPK senses the cells energy status by direct binding AMP, ADP or ATP in the adenine nucleotide-binding sites on its  $\gamma$  subunit [532,537]. Under low energy conditions, increased binding of AMP and ADP leads to a conformational change of the enzyme complexes that activates AMPK through the phosphorylation at Thr172 by upstream kinase LKB1, while replenishing cellular energy levels displaces AMP and ADP by ATP, which promotes AMPK dephosphorylation and inactivation [532,533,536,537]. Active AMPK regulates cellular metabolic activity by phosphorylating and activating key signalling proteins, including transcription factors and co-activators that stimulate ATP-producing pathways, and inhibits ATP-consuming biosynthetic pathways, as well as reducing cell growth and proliferation by triggering phosphorylation events that inhibit the mTORC1 pathway [533,537].

The goal of this study was to investigate the effect of growth factor deprivation on the nutrient-sensing mTORC1 and energy sensing AMPK pathways in placental trophoblast

cells and whether this pathway is mediated by AOX1 and GPER1. A further aim of this study was to assess mitochondrial function and bioenergetics in a placental HTR8/SVneo cell line to understand the potential role of mitochondrial dysfunction in aging of the placenta and the relationship between mitochondrial dysfunction and AOX1 or GPER1 activity.

In the human placenta the metabolic activity of mitochondria increases to meet the rising nutritional demands of the growing fetus as gestation advances, resulting in the excessive generation of ROS and oxidative stress [538]. Increased placental mitochondrial ROS production and oxidative stress have been associated with pregnancy complications, such as preeclampsia [178,539], IUGR [540], and gestational diabetes [541], and are suggested to be due to the dysfunctional mitochondria. In addition, to be a major source of ROS, mitochondria could become a target for ROS-induced damage, which may adversely affect mitochondrial metabolic function in sustained oxidative stress. Thus, mitochondrial dysfunction and ROS production could be part of vicious cellular signalling pathways that regulate the mechanism of placental dysfunction in these pregnancy pathologies.

Mitochondria are considered as the primary source of cellular energy that is generated in the form of ATP and used to maintain cell's biological function. Mitochondria metabolise cellular nutrient substrates including glucose, fatty acids, and amino acids by oxidative phosphorylation. Mitochondrial dysfunction has been linked with a number of disease conditions such as aging, cancer and cardiovascular diseases [542]. Mitochondrial dysfunction is characterised by increased ROS generation, impaired mitochondrial dynamics, depolarization of the inner membrane, which stalls the mitochondrial electron transport chain, reduced ATP generation and increased mitochondrial AMPK activation,
reduced NAD<sup>+</sup>/NADH ratio and mitochondrial Ca<sup>2+</sup> accumulation [329]. During oxidative phosphorylation, nutrients are converted into energy through a series of enzymatically controlled oxidation and reduction reactions via the tricarboxylic (TCA) cycle in the mitochondrial matrix. The TCA cycle uses an electron transport chain (ETC) in the mitochondrial inner membrane to transport electrons through four protein complexes (Complex I to IV), with molecular oxygen (O<sub>2</sub>) as a terminal electron acceptor, to generate ATP (see Figure 4.6C) [409]. The energy harvested from electron flow is used to pump protons from mitochondrial matrix through Complexes I, III, and IV into the intermembrane space. This process creates a proton gradient across the mitochondrial inner membrane, which then couples with the electrical gradient to create a proton motive force ( $\Delta p$ ). The  $\Delta p$  drives protons back into the matrix through the ATP synthase (Complex V) that provides the driving force for the conversion of ADP and inorganic phosphate to ATP. However, proton can return to the matrix independent of ATP synthase by other mechanism, termed proton leak. Proton leak occurs when  $\Delta p$  is depleted without catalysing ATP synthesis (for instance, by the action of ATP synthase inhibitor e.g., oligomycin) [543]. The mechanisms by which proton leak occurs, include direct movement of protons across the phospholipid membrane and diffusion through integral membrane proteins or uncoupling proteins (UCP1, UCPx) [543]. During this process, electrons can 'spin-off' prematurely from Complexes I and III prior to the reduction of oxygen to water at Complex IV, which univalently reduce oxygen (O<sub>2</sub>) resulting in the production of superoxide radical  $(O_2^{-})$  (a detailed description on electron leak and superoxide generation is presented in the Chapter 1 of this thesis) [191,543]. Superoxide radical is the primary ROS formed by the ETC, however it is quickly dismutated to hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) in the matrix or in the intermembrane space. Hydrogen peroxide has the longest cellular half-life (1ms) and is considered to be a key ROS

signalling molecule [191]. At low concentrations, superoxide radicals may be involved in cellular signal transduction, but at high concentrations the radicals cause oxidative damage due to their high reactivity towards other cellular compounds [197]. Proton leak and superoxide production are intricately linked; superoxide production is decreased with reduction in  $\Delta p$  due to higher proton leak [543,544]. Increasing proton leak, also termed as mild uncoupling, therefore provide an attractive therapeutic target for many disease conditions that are associated with increased mitochondrial ROS (superoxide) generation including, obesity, diabetes, aging and age-related diseases [543,545].

Cells also generate ATP, independent of oxygen, by the glycolytic pathway. Glycolysis converts glucose into pyruvate with the concurrent generation of ATP and NADH (Figure 7.1). Pyruvate, which is a metabolic intermediate has several potential fates. One fate is that, pyruvate enters into the mitochondria via the transport protein pyruvate translocase (also known as pyruvate carrier). In the mitochondrial matrix, pyruvate is converted into Acetyl CoA by the action of pyruvate dehydrogenase complex, which is then participate in the TCA cycle to produce NADH and FADH<sub>2</sub> that donate electrons in the ETC to generate ATP (Figure 7.1) [546,547]. A decrease in the activity of pyruvate translocase and/or pyruvate dehydrogenase complex lead to a reduction of pyruvate-dependent mitochondrial respiration and are shown to be implicated in aging process [547-550]. Alternatively, pyruvate can be converted into lactate in the cytosol by lactate dehydrogenase leading to the regeneration of NAD<sup>+</sup> from NADH and net protons (Figure 7.1) [542,546]. Although, glycolysis is less efficient in ATP production compared to oxidative phosphorylation in normal cells, in some cellular contexts particularly malignant cells shift to glycolysis for energy generation [546]. The protons that are produced during conversion of pyruvate to lactate are then pumped out from the cell into

the extracellular space or surrounding medium to balance the intracellular pH by several mechanisms [551], which cause extracellular acidification [542]. Extracellular acidification rate is directly linked to the glycolytic production of lactate, therefore is commonly used as a direct and quantitative measure of cellular glycolytic activity. Hypoxia and ischemia has been shown to induce a significant fall in both intracellular and extracellular pH in animal models [552]. The acidification of extracellular environment, termed 'extracellular or metabolic acidosis', which results from an increase in lactic acid production via glycolysis, causes reduction of intracellular pH and increase ROS production by mitochondria [553]. It also increases cytosolic calcium ion concentration [554]. Extracellular acidosis has been shown to modulate the function of immune cells [555] and alter important cell signalling pathways [556]. An increased glycolytic flux is associated with various pathological conditions such as inflammation and tumorigenesis [557].



Figure 7.1Schematic representation of the cellular glycolytic pathway.Nicotinamide adenine dinucleotide (NADH) is regenerated by enzymaticactions of Hexokinase and Lactate dehydrogenase in the cytosol as glucose

is converted to lactate via pyruvate intermediate. Pyruvate can also be translocated into the mitochondria via the pyruvate translocase and then enters into the TCA cycle in the mitochondrial matrix.

Assessment of cellular energy metabolism (mitochondrial oxidative phosphorylation and glycolytic activity) are essential for investigating cell functions in a variety of cellular situations and pathological conditions. Recent technological advances in the ability to monitor and to assess mitochondrial metabolic function and celular glycolytic capacity have enabled researchers to understand the role of mitochondrial dysfunction in the pathogenesis of these diseases. Seahorse Bioscience has developed a technique, "the XF Cell Mito Stress Test Assay" that can measure real-time changes in the bioenergetics of cell monolayers. In this assay, cells are cultured at an optimum density in cell culture microplates. The assay measures the Oxygen Consumption Rate (OCR) of the cells, which is an indicator of mitochondrial oxidative phosphorylation, and the Extra-Cellular Acidification Rate (ECAR), an indicator of cellular glycolytic capacity. In the assay, cells are metabolically disturbed by the sequential addition of three different compounds (oligomycin, carbonyl cyanide-4 (trifluoromethoxy) phenylhydrazone (FCCP), and a mix of rotenone/antimycin) that shift the metabolic profiles of the cell. The sequential injections of these compounds aid in the estimation of a variety of mitochondrial bioenergetic parameters, including basal OCR, ATP-linked OCR, maximal respiration capacity, spare/reserve capacity, proton leak and non-mitochondrial OCR, as well as glycolytic function of the cells. The sequence of addition and the functions of these compounds in the cell mito stress test assay have been discussed in the Section 4.2.9 of this thesis.

Seahorse XF analyser offers several advantages. Firstly, it is a rapid method to monitor the two major metabolic pathways of the cell, oxidative phosphorylation and glycolysis in a single well. Secondly, it requires a smaller number of cells with relatively high throughput. However, it has some limitations. First of all, the cost associated with reagents, injectable compounds, new fluorescent plate for each assay, and the cost of optimization of compounds and cell number prior to any assay, are higher in comparison to conventional techniques. In addition, the technique is able to use only four injectable compounds to assess mitochondrial dysfunction in any single assay. As the assay itself requires three inhibitors of ETC to be injected, so there is only one type of injectable therapeutic agent can be used in a single assay. Therefore, it will be necessary to use multiple plates if requires testing of more than four injectable compounds, which again increases cost. A final drawback is that injectable compounds may potentially interfere with sensor fluorescence that may produce misleading data [408].

## 7.2 Experimental

#### 7.2.1 Placental cell line culture

HTR8/SVneo cells (a first-trimester human placental extravillous trophoblast cell line) was used in this study. Cells were maintained at 37 °C in growth medium (RPMI-1640 medium supplemented with 2 mM L-glutamine, 1% Na-pyruvate, 1% antibioticantimycotic solution (10,000 units/mL of penicillin, 10,000  $\mu$ g/mL of streptomycin, and 25  $\mu$ g/mL of Amphotericin B) with the addition of 10% (v/v) fetal bovine serum (FBS)) in a humidified atmosphere of 5% CO<sub>2</sub> and 95% air. Cells were subcultured and grown in T75 flasks to the stage of 70-80% confluence. To passage the cells, flasks were rinsed with phosphate buffered saline (PBS), a dilution of 1:10 of trypsin EDTA (Invitrogen) and PBS were added, and cells were incubated at 37 °C in a humidified atmosphere of 5% CO<sub>2</sub> for 5 min. Once cells detached, 5 mL of growth medium was added to inactivate the trypsin. The cell suspension was taken in a tube and centrifuged at 125g for 5 min. The supernatant was discarded, and the cell precipitate was resuspended in 5 mL growth medium. Cells were counted using a haemocytometer, and  $7 \ge 10^5$  cells were seeded in each 60 mm culture dish and cultured in 6 mL of growth medium for 24 hours, in a humidified incubator at 37 °C at 5% CO<sub>2</sub>. The medium was then discarded, and the cells were rinsed with 6 mL of serum-free medium (RPMI medium supplemented with 2 mM L-glutamine, 1% Na-pyruvate, 1% Antibiotic-Antimycotic solution without the addition of FBS). Six mL of serum-free medium with or without the pharmacologic agent (raloxifene and G1, both at 100 nM concentrations) were added. In Control plates, growth medium was replaced with 6 mL fresh growth medium. The plates were then returned to an incubator at 37 °C with 5% CO<sub>2</sub> and cultured for 24 hours. At the end of the incubation, medium was discarded, 300 µl either PBS, or protein extraction buffer (in-house lysis buffer, composed of PBS, 1% Triton-X-100, 0.1 % Brij-35, 1X protease inhibitor, 1X phosphatase inhibitor, pH 7.4) was added in each culture dish for DNA and protein extraction, respectively. Cells were scrubbed from the culture dish, taken in a microfuge tube, snap frozen in liquid nitrogen and then stored at -80 °C for subsequent experiments.

#### 7.2.2 Monitoring cell morphology using phase contrast imaging

Cells were seeded in 6 well culture plates (3 x  $10^{5}$ /well) and cultured in 3 mL of growth medium for 24 hours, in a humidified incubator at 37 °C at 5% CO<sub>2</sub>. Medium from each well was removed, and the cells were rinsed with 3 mL of serum-free medium. Three mL of serum-free medium with or without the addition of AOX1 inhibitor raloxifene (10, and

100 nM) and G1 (10 and 100 nM) were added. In Control wells, growth medium was replaced with fresh growth medium. The plates were then returned to a 37 °C incubator with 5%  $CO_2$  and cultured for 24 hours. The morphology of cells was monitored using a Nikon eclipse Ti fluorescence microscope imaging system using phase contrast function (Nikon Instruments Inc.).

#### 7.2.3 Protein analysis and western blotting

For cell lysate extraction, cell lysis buffer was added, and the cells was sonicated using an ultrasonicator, centrifuged at 13,000g for 10 min at 4 °C and the supernatant was The total protein concentration of cell extract was separated. measured spectrophotometrically using a Pierce BCA Protein Assay Kit (Thermo Fisher Scientific, Australia) in a SPECTROstar Nano Microplate Reader (BMG LABTECH). Twenty µg of protein samples were mixed with 5 µL of LDS sample loading buffer (Thermo Fisher Scientific) containing sample reducing agent and then heated in a 70 °C heating block for 10 min. Proteins were then separated by electrophoresis using Nupage 4-12% SDS-PAGE (sodium dodecyl sulphate polyacrylamide gel electrophoresis) precast 12 well gels (Thermo Fisher Scientific) for 50–60 min at a constant potential of 200 V. MagicMark<sup>™</sup> XP Western Protein Standard (Thermo Fisher Scientific) was used as a standard protein ladder. Separated proteins were then transferred to a nitrocellulose membrane (iBlot<sup>™</sup> Gel Transfer Stack, nitrocellulose, mini) for 7 min using an iBlot® Gel Transfer Device (Thermo Fisher Scientific). The membranes were incubated in blocking buffer containing 1% bovine serum albumin (BSA) in TBS-T (Tris-buffered saline containing 0.1 % tween-20) for an hour at room temperature (RT). The membranes were then incubated with primary antibodies (P-p70S6K (Thr389), P-AKT (Ser479), P-AMPKa (Thr172)) in 1% BSA in TBS-T at a dilution recommended by the manufacturer overnight at 4 °C. The next day membranes were washed three times, 5 min each in TBS-T. The membranes were then incubated with the appropriate HRP conjugated secondary antibody diluted in 1% BSA in TBST for 2 hours at RT. After 3 further washings in TBS-T, the immunoreactive bands were developed in Luminata reagent (Thermo Fisher Scientific) and enhanced chemiluminescence was used for protein detection using an Amersham Imager 600 (GE Healthcare). Quantification of the photographs was performed by measuring the band intensity of the blots using an Amersham Imager 600 Analyser. The loading was verified by Ponceau S staining of the individual blots. Target band densities were normalized to loading using Ponceau S staining. For each protein target, the band with the lowest mean density was assigned an arbitrary value of 1. All individual densitometry values were expressed relative to this mean. The results were graphed using GraphPad Prism software version 7 (GraphPad Prism, CA, USA).

#### 7.2.4 Enzyme-linked immunosorbent assay (ELISA)

#### 7.2.4.1 HNE adduct competitive ELISA

For detection and quantitation of 4HNE protein adduct in placental cell extract, we used an OxiSelect<sup>™</sup> HNE Adduct Competitive ELISA Kit (Cell Biolabs Inc, STA- 838). This assay is based on the competition between HNE conjugate and the anti-HNE antibody for HNE-protein. In this assay, at first, an HNE conjugate is coated on an ELISA plate. The unknown HNE protein samples or HNE-BSA standards are then added to the HNE conjugate reabsorbed ELISA plate. After a brief incubation, an anti-HNE antibody is added, followed by a secondary antibody (HRP conjugated). The amount of HNE protein adducts in unknown samples is estimated from a predetermined HNE-BSA standard curve.

In this experiment, cell lysate was prepared by sonicating in ultrapure water and then centrifuged at 13,000g for 10 min. The supernatant containing tissue or cell extract were separated. Protein concentration was estimated using the Pierce BCA protein assay kit.

In this assay, at first, an HNE conjugate was coated on a 96-well plate and incubated overnight at 4 °C. HNE conjugate was removed, and the wells were rinsed twice with 1X PBS. Assay Diluent was added to each well and blocked for an hour at RT and then transferred to 4 °C until use. Immediately before use, Assay Diluent was removed, appropriate dilution of unknown samples (for 40 µg protein in each well) and HNE-BSA standards were added. The plate was then incubated for 10 min at RT on an orbital shaker. The diluted anti-HNE antibody was added to each well and incubated at RT for 2 hours on an orbital shaker. The plate was then washed 3 times with 1X wash buffer with thorough aspiration between each wash. The diluted HRP conjugated secondary antibody was added to all wells and incubated for an hour at RT on an orbital shaker. The strip wells were then washed a further 3 times with 1X wash buffer. The substrate solution was added to each well, incubated at RT for 2–5 min on an orbital shaker and the changes of colour were monitored. The stop solution was added to each well to stop the enzyme reaction. The absorbance of each well was read on a microplate reader (SPECTROstar Nano, BMG LABTECH) using 450 nm as the primary wavelength. The content of HNE protein adducts in unknown samples was determined by comparison with the predetermined HNE-BSA standard curve using GraphPad Prism software. The results were graphed, and the statistical analysis was performed using GraphPad Prism software.

For detection and quantitation of DNA and RNA oxidation of placental trophoblast cells, we used a DNA/RNA Oxidative Damage (Clone 7E6.9) ELISA Kit (Cayman Chemicals, Cat No. 501130). This assay is based on the competition between oxidatively damaged DNA and RNA guanine species, 8-hydroxy-2-deoxyguanosine (8OHdG) and 8hydroxyguanosine (80HG), respectively, in samples and an 80HdG/80HGacetylcholinesterase conjugate (DNA/RNA oxidative damage AChE Tracer) for a limited about of DNA/RNA oxidative damage monoclonal antibody. The amount of tracer is held constant while the concentration of 8OHdG/8OHG varies, so the amount of tracer that is able to bind to the monoclonal antibody is inversely proportional to the concentration of 8OHdG/8OHG in the well. The antibody/8OHdG/8OHG complex binds to the goat polyclonal anti-mouse IgG that has been previously attached to the well. The plate is washed to remove any unbound reagents and then the substrate (Ellman's reagent, which contains the substrate for AChE) is added to the well. The product of this enzymatic reaction has a distinct yellow colour and absorbs strongly at 405–420 nm. The intensity of the colour, determined spectrophotometrically, is proportional to the amount of AChE tracer bound to the well, which is inversely proportional to the amount of free 8OHdG and 80HG present in the well during the incubation.

In this experiment, at first total DNA was extracted from placental HTR8/SVneo cells in PBS using DNeasy MiniPrep (QIAGEN Pty Ltd, VIC, Australia) following the manufacturer's instructions. Cleavage of DNA into smaller components was done by treatment with deoxyribonuclease 1 (DNAse 1). DNA concentration was measured by a spectrophotometer using a NanoDrop 2000 spectrophotometer (NanoDrop

Technologies). In this assay, 2.5 µg of unknown DNA and DNA/RNA oxidative damage ELISA standards were loaded in duplicate in a 96 well plate. DNA/RNA oxidative damage AChE Tracer and ELISA monoclonal antibody were added to each sample and standard wells. The plate was then covered with plastic film and incubated for 18 hours at 4 °C. Then the materials from each well were discarded and rinsed five times with 1X wash buffer with thorough aspiration between each wash. The substrate solution (Ellman's reagent) was added to each well and incubated at RT for 90 min on an orbital shaker. The absorbance was read on a microplate reader (SPECTROstar Nano, BMG LABTECH) using 420 nm as the primary wavelength. The content of 80HdG in unknown DNA samples was determined using the equation obtained from the standard curve using GraphPad Prism software. The results were graphed, and the statistical analysis was performed using GraphPad Prism software.

#### 7.2.5 Assessment of mitochondrial respiration function

To assess mitochondrial function and bioenergetics in the placental trophoblast HTR8/SVneo cell line, we used the Seahorse XF96 Extracellular Flux Analyser and XF Cell Mito Stress Test Kit (Seahorse Bioscience, North Billerica, MA) as described above [409].

In preliminary experiments, the optimal number of cells was estimated to ensure an optimal and measurable baseline OCR (oxygen consumption rate); 1, 2, 4 and 8 X10<sup>4</sup> cells/well were loaded onto Seahorse 96-well plates, and proportional responses were measured accordingly (data not shown). Based on the optimal baseline readings recommended by the manufacturer, 20,000 cells/well was selected to load in all

experiments. Accordingly, FCCP concentration was also optimized to obtain maximal effects and readings. Sequential injections of oligomycin, FCCP, and a mix of rotenone/antimycin A was performed to determine basal OCR, ATP-linked OCR, maximal respiration, proton leak, non-mitochondrial OCR, and spare respiratory capacity.

## 7.2.5.1 Cell preparation

In the preceding day before the assay, 20,000 cells were seeded in each well onto a 96well plate (Seahorse Bioscience, North Billerica, MA) in 100  $\mu$ L growth medium (RPMI-1640 medium supplemented with 2 mM L-glutamine, 1% Na-pyruvate, 1% antibioticantimycotic solution with the addition of 10% (v/v) FBS). The cells were incubated in humidified 37 °C incubator with 5% CO<sub>2</sub> for 20–24 hours. After that growth medium from each well were removed and added 100  $\mu$ L of serum-free medium (RPMI-1640 medium supplemented with 2 mM L-glutamine, 1% Na-pyruvate, 1% antibioticantimycotic solution) with or without the addition of AOX1 inhibitor raloxifene (1, 10, and 100 nM) and G1 (1, 10 and 100 nM). In control wells, growth medium was replaced with fresh growth medium. The plates were then returned to a 37 °C incubator with 5% CO<sub>2</sub> and cultured for 24 hours.

#### 7.2.5.2 Measurement of oxygen consumption rate and extracellular acidification rates

Prior to performing an assay, the medium from each well was removed, and 175  $\mu$ L of pre-warmed assay medium (XF base medium (DMEM) supplemented with 25 mM glucose, 2 mM L-glutamine, and 1 mM sodium pyruvate; pH 7.4) was added. Cell culture plates were then placed into a 37 °C non- CO<sub>2</sub> incubator for 1 hour to allow pre-

equilibration with the assay medium. Appropriate dilution of pre-warmed oligomycin, FCCP, and rotenone/antimycin A solution (prepared in assay medium and adjusted to pH 7.4) were loaded into the injector ports A, B and C of sensor cartridge, respectively, in order to achieve the final concentrations of compounds which were as follows: 1  $\mu$ M oligomycin, 0.5  $\mu$ M FCCP, 0.5 uM rotenone/antimycin A.

During this time the cartridge was calibrated prior to the start of an assay. Oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) were measured using the XF96 Extracellular Flux analyser (Seahorse Bioscience, Billerica, MA, USA) under basal conditions (prior to the addition of an agent) followed by the sequential addition of oligomycin, FCCP, as well as rotenone/antimycin A, according to the described protocol by Nicholls et al [409]. OCR (pmol/min) and ECAR (mpH/min) data points refer to the average rates during the measurement cycles, which were then normalized against cell counts. In this assay, baseline OCR or ECAR (refers to the starting rates prior to the addition of an agent), which can be used for comparisons with those rates after the addition of compounds. This allows for an estimation of the contribution of individual parameters for basal respiration, proton leak, maximal respiration, spare respiratory capacity, non-mitochondrial respiration and ATP production [410].

## 7.2.5.3 Cell counts

After the assay, cell culture plates were retrieved, and cells were fixed in 4% paraformaldehyde. Automated cell counting was performed with Hoechst (Hoechst 33342, Thermo Fisher Scientific) staining of nuclear DNA with fluorescent imaging using the Citation 3 image reader (BioTek, Winooski, VT, USA). These cell counts were used

to normalize both OCR and ECAR data to account for variable cell densities in different wells or with different pre-treatments etc.

## 7.2.6 Data presentation and statistical analysis

Sample numbers are shown in the legends to individual figures. The quantitative data were presented as mean  $\pm$  standard error of the mean (S.E.M). Statistical differences were analysed by Student's *t*-test (paired, non-parametric) with Wilcoxon matched-pairs signed rank testing using GraphPad Prism software (version 7, Graph Pad Software, Inc., San Diego, California) for repetitive measurement between treatment and control group. A  $\rho$  value of less than 0.05 was considered statistically significant. All the graphs were prepared using GraphPad Prism software.

For the mitochondrial respiration assay, the XF mito stress test report generator automatically calculates the mitochondrial bioenergetic parameters from Wave data that has been exported to GraphPad. The OCR and ECAR are presented as the mean  $\pm$  standard error of the mean (S.E.M) of 7 independent experiments, with 3–10 replicate wells for each group (control and pre-treatment groups) in each experiment. The mitochondrial bioenergetic parameters of each group were estimated based on the percent (%) of the control group. A paired Student's *t*-test was performed for each experimental group to assess the statistical significance against their respective controls using GraphPad Prism software, and a  $\rho$  value of less than 0.05 was considered statistically significant.

#### 7.3 Results

# 7.3.1 Characterization of placental HTR8/SVneo cell line cultured in growth factor deficient medium.

In this study, we have used an immortalized human trophoblast cell line, the HTR8/SVneo, which originated from placental extravillous cytotrophoblast [402]. HTR8/SVneo was established by transfection of human placental primary trophoblasts, extracted from first trimester villous explants, with a gene encoding simian virus 40 large T antigen to immortalize them [403]. Here, we cultured placental HTR8/SVneo cell line in serum-free medium (growth factor deficient medium) for 24 hours to induce oxidative stress associated changes. Serum starvation is known to induce oxidative stress in cultured cells [500]. To inhibit AOX1 activity the cells were treated with raloxifene, a potent AOX1 inhibitor, or G1, a GPER1 agonist, that is also known to inhibit AOX1 activity, in serum-free medium for 24 hours. The morphology of the control and treated cells was monitored using phase contrast microscopy. Morphologically, control cells were small, relatively round and showed an epithelial-like morphology (Figure 7.2A). However, serum-free treated cells were elongated, spindle-shaped and fibroblast-like morphology (Figure 7.2B). The cell proliferation was also higher in control medium than the serum-free medium. While, the morphology of raloxifene and G1 treated cells were comparable to control cells with small, relatively round and epithelial-like morphology (Figure 7.2 C-F).



**Figure 7.2 Phase contrast images of human placental HTR8/SVneo cells**. (A) Control (growth medium), (B) Serum-free, (C) G1 (10 nM), (D) G1 (100 nM), (E) Raloxifene (10 nM) and (F) raloxifene (100 nM). Original image magnification is 20X; scale bar represents 50 μm.

## 7.3.2 Lipid peroxidation in placental trophoblast cells – an indicator of cellular oxidative damage

Lipid peroxidation is a well-defined mechanism of cellular oxidative damage and tissue aging. In cells, fatty acids (lipid) are oxidised by peroxides (highly reactive oxygen free radicals), known as lipid peroxidation, which produces 4-hydroxynonenal (4HNE) as a by-product. The cellular levels of 4HNE are an indicator of lipid peroxidation and oxidative damage as well. Increased production of 4HNE has been observed in agingassociated diseases such as Alzheimer's disease [397]. We, therefore, performed a quantitative measurement of 4HNE by ELISA, which estimates the amount of 4HNEadduct, in cell extracts. We observed a significant increase in the amount of 4HNE  $(\rho=0.0078)$  in placental trophoblast cells cultured in the serum-free medium compared with those cultured in serum-containing medium (Figure 7.3). We then sought to determine the relationships between the production of 4HNE, and AOX1 or GPER1 activity. For this, we cultured the cells in serum-free medium with a potent AOX1 inhibitor, raloxifene [426], or a GPER1 agonist, G1, which has been shown to inhibit production of 4HNE in the kidney [226]. As predicted, both raloxifene and G1 blocked the serum starvation-induced production of 4HNE in placental trophoblast cells after 24 hours of treatment (Figure 7.3).



Figure 7.3 Serum-starvation increases lipid peroxidation in placental trophoblast cells. Quantitative measurement of 4HNE, a product of lipid peroxidation, in placental HTR8/SVneo cells by ELISA. Serum starvation (serum-free) for 24 hours increases production of 4HNE compared to controls (Cont.) at both '0 hr' (just before starvation) and at '24 hrs' after culturing in serum-containing medium. Both raloxifene (100 nM concentration) and G1 (100 nM concentration) treatments for 24 hours in serum-free condition blocked 4HNE production through inhibition of AOX1. Data are presented as mean ± S.E.M (N=4 independent experiments, n=8 individual data points). Statistical differences (p values) were calculated using t-test (paired, non-parametric) using GraphPad

Prism software and a  $\rho$  value of less than 0.05 was considered statistically significant, \*\*  $\rho$ <0.01.

## 7.3.3 DNA oxidation in placental trophoblast cells

In mitochondrial or genomic DNA, 8-hydroxy-2'-deoxyguanosine (8OHdG), which is an oxidised derivative of deoxyguanosine, is one of the major products of ROS-induced oxidative lesions and has therefore been widely used as an indicator of cellular oxidative DNA damage. The expression of 8OHdG has been shown to increase in aging tissues, such as the brain in Alzheimer's disease [395,396]. We, therefore, performed an ELISA for 8OHdG, which can quantitatively estimate the amount of 8OHdG in samples. As presented in Figure 7.4, the amount of 8OHdG was significantly higher in cells starved in serum-free condition compared to control ( $\rho$ =0.0078), which was later blocked (a certain extent) by treatment with AOX1 inhibitor raloxifene ( $\rho$ =0.0391).



Figure 7.4 Serum-starvation increases DNA oxidation in placental trophoblast cells. Quantitative measurement of 80HdG in placenta HTR8/SVneo cells by ELISA. Serum starvation (serum-free) for 24 hours increases production of 80HdG compared to controls (Cont.) at both '0 hr' (just before starvation) and at '24 hrs' after culturing in medium containing serum. Raloxifene (100 nM concentration) treatment for 24 hours in serum-free condition blocked 80HdG production through inhibition of AOX1. Data are presented as mean  $\pm$  S.E.M (N=4 independent experiment, n=8 individual data points). Statistical differences ( $\rho$  values) were calculated using *t*-test (paired, non-parametric) using GraphPad Prism software and a  $\rho$  value of less than 0.05 was considered statistically

significant, \*  $\rho < 0.05$ , \*\*  $\rho < 0.01$ .

# 7.3.4 Growth factors deprivation decreases the activity of nutrient sensing mTORC1 mediated by AOX1 and GPER1

mTORC1 serves as a key molecule that regulates cell growth by monitoring cellular levels of amino acids and growth-stimulating signals. To examine mTORC1 activity, phosphorylation of the mTORC1 substrate p70S6K, a downstream target of mTORC1, and AKT, an upstream regulator of mTORC1, was assessed by western blotting. Phosphorylation of p70S6K at Thr389 and AKT at Ser473 was significantly decreased by serum starvation (growth factors deprivation) (Figure 7.5 A, B and C). This inhibition of p70S6K and AKT phosphorylation was prevented by treatment with both raloxifene (100 nM concentration) and the GPER1 agonist, G1 (100 nM concentration) in serum-free medium (Figure 7.5 A, B and C). These results suggested that mTORC1 activity is regulated by both AOX1 and GPER1 during growth factors deprivation.



Figure 7.5 Serum starvation decreases the activity of nutrient sensing mTORC1 via AOX1. Western blots of serum-starved placental HTR8/SVneo cells and after treatment with G1 and raloxifene for 24 hours, against antibody as shown (A), and quantification of the protein expression (B and C). Serum starvation (serum-free) for 24 hours decreases the activity of mTORC1 measured by phosphorylation of p70S6K and AKT compared to controls (Cont.) at both '0 hr' (just before starvation) and at '24 hrs' (B and C). Both Raloxifene (100 nM concentration) and G1 (100 nM concentration) treatment for 24 hours in serum-free medium reactivate mTORC1 activity (B and C). Data are presented as mean ± S.E.M. For

P-p70S6K, N=6 independent experiment, n=12 individual data points and for P-AKT, N=4 independent experiment, n=8 individual data points. Statistical difference ( $\rho$ ) values were calculated using *t*-test (paired, nonparametric) using GraphPad Prism software and a  $\rho$  value of less than 0.05 was considered statistically significant, \*  $\rho$ <0.05, \*\*  $\rho$ <0.01, \*\*  $\rho$ <0.001. M, molecular weight marker.

## 7.3.5 Serum starvation increases the activity of AMPK via GPER1

AMP-activated kinase (AMPK) appears as a major energy sensor to modulate cellular activities in response to energy stress [532]. When cellular energy is depleted, activation of AMPK triggers the activation of pathways that promote ATP-synthesis and cellular energy generation such as autophagy, and inhibits energy-consuming biosynthetic pathways, for example, mTORC1 [533,537]. To investigate the AMPK activity in placental HTR8/SVneo cells under growth factor depletion, we measured the phosphorylation of AMPK by western blotting. Serum starvation significantly increased the phosphorylation of AMPK ( $\rho$ =0.0195) (Figure 7.6 A and B). Treatment of cells with the GPER1 agonist, G1 (100 nM concentration) in serum-free medium significantly attenuated the serum deprivation-induced increase in AMPK phosphorylation ( $\rho$ =0.0020) (Figure 7.6 A and B). However, raloxifene treatment did not inhibit AMPK phosphorylation.



Figure 7.6 Serum starvation increases the activity of AMPK. (A) Western blot of serum-starved placental HTR8/SVneo cells and after treatment with G1 and raloxifene for 24 hours with an antibody against P-AMPKα (Thr172), and (B) quantification of protein expression. Serum starvation (serum-free) for 24 hours increases AMPK phosphorylation compared to controls (Cont.) at both '0 hr' (just before starvation) and at '24 hrs' (B). G1 (100 nM concentration) treatment for 24 hours in serum-free medium inhibited the AMPK phosphorylation. Data are presented as mean ± S.E.M (N=4 independent experiment, n=8 individual data points). Statistical differences (ρ values) were calculated with a *t*-test (paired, non-parametric) using GraphPad Prism software and a ρ value of less than 0.05

was considered statistically significant. \*  $\rho$ <0.05, \*\*  $\rho$ <0.01. M, molecular weight marker.

## 7.3.6 Mitochondrial respiration activity and glycolytic capacity of placental trophoblast cells

#### 7.3.6.1 Mitochondrial oxygen consumption and ATP production

The general scheme of the mitochondrial stress test and the mitochondrial respiration parameters are shown in Figure 7.7 A. Sequential injections of oligomycin, FCCP, rotenone/antimycin A measure basal respiration, ATP-linked respiration (a measure of ATP production), proton leak, maximal respiration, spare respiratory capacity, and non-mitochondrial respiration. To assess the effect of serum starvation, and AOX1 inhibition and GPER1 activation on mitochondrial function, the mitochondrial stress test was performed on pre-treated cells in serum-free medium and with the addition of raloxifene (1, 10 and 100 nM) and G1 (1, 10 and 100 nM) and individual parameters were calculated. The oxygen consumption rate (OCR), and the individual parameters of mitochondrial respiration are shown in Figure 7.7 B–H.

As shown in Figure 7.7 B, OCR was reduced after injection of oligomycin (an inhibitor of ATP synthase (Complex V)), which inhibited the generation of ATP and reduced mitochondrial oxygen consumption in all groups (control and pre-treated). The decrease in OCR following injection of oligomycin correlates with the mitochondrial respiration associated with ATP production. Addition of FCCP (an uncoupling agent that disrupts the mitochondrial membrane potential and ETC and inhibits ATP synthesis) caused a sharp rise of OCR (Figure 7.7 B). The increase in OCR reflects the maximal respiratory

activity that the cells can sustain in energetically unfavourable conditions. Spare respiratory capacity, which is a measure of the cells' ability to respond to increased energy demand, can be estimated from the difference between maximal respiration and basal respiration. After addition of rotenone/antimycin A (Complex I and Complex III inhibitors, respectively), a decrease in OCR was observed (Figure 7.7 B). The decrease in OCR is due to inhibition of mitochondrial respiration caused by blocking of electron transfer from complex 1 to ubiquinone, which blocks the potential energy being converted to usable energy in the form of ATP. This OCR was used to calculate both the mitochondrial and non-mitochondrial fraction contributions to respiration.

The placental HTR8/SVneo cells cultured in serum-free medium showed decreases in basal respiration by 42% ( $\rho$ =0.0156) (Figure 7.7 C), ATP production by 44% ( $\rho$ =0.0156) (Figure 7.7 D), proton leak by 34% ( $\rho$ =0.0313) (Figure 7.7 E) and maximal respiration capacity by 41% ( $\rho$ =0.0313) (Figure 7.7 F), when compared to control cells cultured in serum-containing medium. The spare respiratory capacity ( $\rho$ =0.0156) (Figure 7.7 G) and the non-mitochondrial respiration ( $\rho$ =0.0156) (Figure 7.7 H) were also significantly decreased in the serum-free group compared to control. These results suggest that serum-starvation alters the mitochondrial respiration and bioenergetics in placental trophoblast cells. Inhibition of AOX1 by raloxifene attenuated the serum-starvation induced decrease in mitochondrial function in terms of basal respiration, ATP production, proton leak and maximal respiration capacity (Figure 7.7 C–F), in all the three concentrations (1, 10 and 100 nM) of the drug in a dose-dependent manner and the results are comparable to the controls. Similarly, GPER1 activation and AOX1 inhibition by G1 prevented the serum-starvation induced mitochondrial dysfunction in a dose-dependent way at 1, 10 and 100 nM concentrations in terms of basal respiration, ATP production, proton leak and

maximal respiration capacity, and the results are also comparable to the controls (Figure 7.7 C–F). While raloxifene treatment significantly increased the non-mitochondrial respiration ( $\rho$ =0.0469) (Figure 7.7 H) and spare respiratory capacity ( $\rho$ =0.0469) (Figure 7.7 G) from the serum-free group at only 1 nM and 100 nM, respectively. Likewise, the non-mitochondrial respiration ( $\rho$ =0.0156) (Figure 7.7 H) and spare respiratory capacity ( $\rho$ =0.0156 and 0.0313) (Figure 7.7 G) were significantly higher in the G1 treatment group than the serum-free group at 100 nM, and 1 and 100 nM concentrations, respectively. These data suggest that AOX1 activation and/or loss of GPER1 function may produce the mitochondrial dysfunction in the condition of growth factor deprivation.



Figure 7.7 Mitochondrial respiration in placental trophoblast cells. (A) Schematic of the mitochondrial stress test and respiration parameters. OCR was measured under basal conditions followed by sequential injections of oligomycin, FCCP, and rotenone/antimycin A. Oxygen consumption rate (OCR) was measured by sequential addition of compounds in pre-treated cells (B) and individual parameters, basal respiration (C), ATP production (ATP-linked respiration) (D), proton leak (E), maximal respiration (F), spare respiratory capacity (G), and nonmitochondrial respiration (H), were calculated. In B, data are presented as the mean ± standard error of the mean (S.E.M) of 7 independent experiments, with 3-10 replicate wells for each group (control and pretreatment groups) in each experiment. In C-H, data are presented as the percent (%) of the control group. Statistical significances (p values) were calculated using t-tests (paired, non-parametric) using GraphPad Prism software and a  $\rho$  value of less than 0.05 was considered statistically significant.  $*\rho < 0.05$ , serum-free vs control or pre-treatments as indicated; ns, non-significant.

#### 7.3.6.2 Glycolytic capacity in trophoblast culture

The extracellular acidification rate (ECAR) was measured in basal condition and after sequential injections of oligomycin, FCCP, and rotenone/antimycin A in pre-treated cells in serum-free medium and with the addition of raloxifene (1, 10 and 100 nM) and G1 (1, 10 and 100 nM) and the cellular glycolytic parameters were calculated. The ECAR and the glycolytic parameters are shown in Figure 7.8 A–F. As shown in Figure 7.8 B, ECAR

was increased after addition of oligomycin, an inhibitor of mitochondrial ATP synthase (Complex V) in the ETC, in all groups (control and pre-treated). The increase in ECAR after addition of oligomycin is due to reduction of ATP production by ATP synthase that decreases the ATP/ADP ratio, which induces glycolytic pathway, as the cells attempt to maintain their energy balance by generating ATP via glycolysis. The level of ECAR after addition of oligomycin revealed the cellular maximum glycolytic capacity that the cell can sustain in response to energy demand. A concomitant increase in ECAR was also observed after addition of FCCP and rotenone/antimycin A, as the cells shift to a more glycolytic state in order to maintain energy balance. However, there is no significant difference in glycolytic parameters in terms of baseline and maximal glycolytic rate, and glycolytic reserve capacity between control vs serum-free and serum-free plus raloxifene or G1 treatment (Figure 7.8 C-E). These results indicate that serum-starvation does not significantly alter the cellular glycolytic capacity, suggesting that growth factor signalling may not have an impact on cellular glucose utilization via glycolysis in placental trophoblast cells. In addition, cellular preference for mitochondrial oxidative phosphorylation versus glycolysis for energy generation can be predicted from the ratio of ECAR/OCR, where a high value of ECAR/OCR indicates that cells mostly rely on glycolysis to produce energy [558]. In this experiment, low ECAR/OCR values for the control and in pre-treated cells (raloxifene at all three concentrations and G1 at only 100 nM concentration) were observed indicating that their relatively higher preference on mitochondrial oxidative phosphorylation for energy than glycolysis compared with serum-free group (Figure 7.8 F). The increase in ECAR/OCR ratio in serum-free group is because, in serum-free group mitochondrial oxygen consumption (OCR) was decreased, which increased the relative ratio of ECAR/OCR.



Figure 7.8 Glycolytic capacity in placental trophoblast cells. (A) Schematic of mitochondrial stress test and parameters of glycolytic capacity. Extracellular acidification rate (ECAR) was measured by sequential addition of compounds in pre-treated cells (B) and individual parameters,

baseline glycolysis (C), maximal glycolysis (D), glycolytic reserve capacity (E), and ECAR/OCR ratio (F), were calculated. In B, data are presented as the mean  $\pm$  standard error of the mean (SEM) of 7 independent experiments, with 3–10 replicate wells for each group (control and pre-treatment groups) in each experiment. In C-E, data are presented as the percent (%) of the control group. Statistical significance ( $\rho$ ) values were calculated using t-test (paired, non-parametric) using GraphPad Prism software and a  $\rho$  value of less than 0.05 was considered statistically significant. \*  $\rho$ < 0.05, serum-free *vs* control or pre-treatments as indicated; ns, non-significant.

## 7.4 Discussion

A summary of the key findings of this study is presented in Table 7.1. Our data indicate that serum-starvation of the placental trophoblast HTR8/SVneo cell line induces oxidative damage related changes in biochemical parameters, in particular lipid peroxidation and DNA oxidation. Increased oxidative damage to DNA and lipid have also been reported in aging tissue such as placenta associated with post-dates and unexplained fetal death [229] and in the brain in Alzheimer's disease [397,431]. We also observed a similar change in the oxidation of lipid in placental explants cultured in growth factor deficient medium (Chapter 6 and [229]). These results indicate that the placental trophoblast may provide a useful model of aging in a human tissue that uniquely ages in a 9 months period of time. The cell line model is particularly beneficial to study aging as it is easier to genetically manipulate and allow testing of the target sites of a range of different therapeutic agents.

	(+) Serum	(–) Serum	(-) Serum	(–) Serum (+) Raloxifene
			(1) 01	(1) Ituromiene
P-p70S6K	$\uparrow$	$\downarrow$	$\uparrow$	<b>↑</b>
P-AKT	$\uparrow$	$\rightarrow$	1	$\uparrow$
Р-АМРК	$\downarrow$	1	$\downarrow$	1
4HNE	$\downarrow$	1	$\downarrow$	$\rightarrow$
8OHdG	$\downarrow$	$\uparrow$	1	$\rightarrow$
Mitochondrial OCR	$\uparrow$	$\rightarrow$	↑	<b>↑</b>
ATP production	1	$\rightarrow$	1	1
Proton leak	$\uparrow$	$\rightarrow$	1	←
4HNE, 4-hydroxynonenal; 8OHdG, 8-hydroxy-2-deoxy guanosine; AMPK, 5' adenosine				
mono-phosphate activated protein kinase; ATP, adenosine tri-phosphate; G1, G-protein				
coupled estrogen receptor 1 agonist; OCR, oxygen consumption rate; p70S6K, phosphoprotein				
70 ribosomal protein S6 kinase.				

**Table 7.1**A summary of the results.

To test our hypothesis that the aging of the placenta is mediated through AOX1 activation via GPER1, we have treated cells with an AOX1 inhibitor, raloxifene and a GPER1 agonist, G1 which is also thought to act via AOX1. Raloxifene, which is a potent inhibitor of human AOX1, has been shown to reduce 4HNE production by blocking AOX1 activation in renal tissue [226]. Raloxifene has also been shown to reduce oxidative damage by reducing the release of ROS in vascular endothelial cells in rats [225] and brain microvascular endothelial cells in human [505]. Activation of GPER1 by its agonist G1 has been shown to induce protective anti-inflammatory effects in multiple sclerosis [235] and cardioprotective effects against ischemia-reperfusion injury [236-239] in rodent models, and reduces ROS induced toxicity in human renal epithelium cells [506] via reduction of mitochondrial ROS generation [239]. Moreover, GPER1 activation by chronic G1 treatment has been shown to reduce lipid oxidation via reduction of oxidative

stress in renal tissue [226], and the reduction of oxidative damage is suggested to be mediated via reduction of NADPH-stimulated superoxide generation [240]. GPER1 is highly expressed in the placental syncytiotrophoblast apical membrane, suggesting that this receptor may play a role in modulating AOX1 and oxidative damage within the placenta [229]. We have demonstrated that the AOX1 inhibitor raloxifene is also able to block serum-starvation induced oxidative damage to the lipid and DNA in a placental cell line. This was confirmed using the GPER1 agonist G1 that has been shown to reduce 4HNE production (a measure of lipid peroxidation) in the placental trophoblast cells, possibly by blocking AOX1-mediated, mitochondrial or NADPH-induced ROS generation [240]. These results suggest that that low estrogen concentrations may lead to a reduction of the cell surface estrogen receptor (GPER1) mediated inhibition of AOX1 and consequently placental oxidative damage and impaired function.

We have also examined the effect of serum deprivation on the nutrient sensing mTORC1 pathway. mTORC1 is a regulator of cellular growth and metabolism, and the cellular energy sensing AMPK pathway, which controls cellular activities when energy is depleted. As discussed in an earlier section, extracellular amino acids are required for activating mTORC1, and its activity is essential to transport of amino acids across placental membrane. mTORC1 inactivation is associated with reduced fetal growth in IUGR pregnancies [144,152] and an activation of placental mTORC1 signalling in association with fetal overgrowth in human [149] and in mice [158]. Therefore, it is likely that inactivation of mTORC1 in response to growth factors depletion may reduce the amino acids flux across the placenta. Moreover, mTORC1 stimulates mitochondrial respiration and ATP production by promoting synthesis of nucleus-encoded mitochondrial proteins via inhibition of the eukaryotic translation initiation factor 4E

(eIF4E)-binding proteins (4E-BPs) [559]. Inhibition of mTORC1 causes a reduction of ATP production associated with impaired mitochondrial function and glycolysis [559]. There has also been a direct link between mTORC1 and AMPK. AMPK is normally inactive, however, during starvation or stress, cellular energy level depletion activates the AMPK pathway, which inhibits energy-consuming anabolic processes by preventing mTORC1 activation through activating and/or by inhibiting the events that regulate mTORC1, and induces catabolic processes such as autophagy to restore cellular energy levels [157].

As expected we observed a significant decrease in mTORC1 activity, measured in terms of phosphorylation p70S6K and AKT, after serum starvation. Our data are consistent with inhibition of mTORC1 signalling after growth factor (insulin) and amino acid deprivation [560]. The effect of which was blocked following treatment with both raloxifene and G1. These results suggest that inactivation of mTORC1 in response to growth factors deprivation is mediated by AOX1 activation as well as loss of GPER1 stimulation, which may reduce the amino acids flux across the placenta. Estrogen receptor activation at GPER1 by agonist G1 may trigger cellular uptake of essential amino acids by controlling amino acid transporters [561] and subsequent activation of mTORC1. While, inhibition of AOX1 by raloxifene increases mTORC1 activity, possibly by inducing autophagy via AMPK activation [562]. Autophagy maintains cellular amino acid levels under nutrient starvation [563]; increased autophagic function increases cellular amino acid regeneration and subsequent activation of mTORC1. Our result are compatible with a role of raloxifene in activation of mTOR and AKT in a breast cancer cell line [562]. Moreover, removal of growth factors significantly increased AMPK activation. Our data are consistent with an activation of AMPK by ROS mediated oxidative stress [564]. AMPK appears to control

the redox-state and mitochondrial function [565], and AMPK activation correlates with an increase in cellular AMP:ATP ratio [564]. AMPK also activates fatty acid β-oxidation in mitochondria [565]. GPER1 activation by its agonist G1 significantly attenuated growth factor depletion-induced increase in AMPK activity, while raloxifene not suppress the AMPK activation. These results suggest that AMPK activation during growth factors deprivation may be mediated by GPER1, and a reduction of AMPK activity after G1 treatment may be due an increase in ATP production [566,567], or reduction of mitochondrial [239] or NADPH-induced ROS generation [240]. GPER1 activation may promote cellular glucose and pyruvate uptake [568,569], leading to an increase in glucose/pyruvate metabolism by glycolysis or by mitochondrial oxidative phosphorylation, resulting in the generation of ATP. While, in consistent with our data, raloxifene has been shown to activate AMPK, leading to an increase in autophagy [562]. Together with these results, it is likely that in times of nutrient deficiency the mTORC1/AKT pathway is regulated via both AOX1 and GPER1, while, GPER1 controls the AMPK pathway (Figure 7.9). Our results confirm that serum-withdrawal, which contains growth factors, as well as other micronutrients and antioxidants, promotes oxidative damage to lipids and DNA, and changes in mTORC1 and AMPK pathways and that these changes are mediated by AOX1 activation and/or loss of GPER1 function.


Figure 7.9 Growth factor removal (serum-starvation) induces ROS mediated oxidative damage in lipids and DNA, alters mTORC1/AKT and APMK activity and induce mitochondria dysfunction via AOX1 and GPER1. Both raloxifene and G1 can block the ROS induced alteration of DNA and lipid oxidation, mTORC1/AKT and AMPK activation and mitochondrial dysfunction. 4E-BPs, eukaryotic translation initiation factor 4E-binding proteins; 4HNE, 4-hydroxynonenal; 8OHdG, 8-hydroxy-2deoxy guanosine; AMPK, 5' adenosine mono-phosphate activated protein kinase; ATP, adenosine tri-phosphate; GPER1, G-protein coupled estrogen receptor 1; mTORC1, mammalian target of rapamycin complex 1; NADPH, nicotinamide adenine dinucleotide phosphate; p70S6K, phosphoprotein 70 ribosomal protein S6 kinase; ROS, reactive oxygen species. Mitochondrial dysfunction and increased oxidative damage are often associated with ageassociated disorders including such as Alzheimer's disease, Parkinson's disease, and Huntington's disease [570]. Mitochondria are the primary source of ROS generation and oxidative stress during the aging process [571,572]. ROS is generated when electrons escape from the ETC complexes, in particular, Complex I and Complex III, during normal and abnormal respiration [573,574]. ROS causes damage to mitochondrial components (DNA, lipids, and proteins) resulting in leakage of electrons from the ETC, which further facilitate ROS generation [575]. Progressive ROS generation and oxidative stress during aging impair mitochondrial function by affecting the enzymatic function of mitochondrial ETC complexes (complexes I, II, IV, and V) that regulate oxidative phosphorylation and energy homeostasis [574,576]. There is an age-associated decline of activities of mitochondrial complexes I, III, III, IV and V in rats heart and brain tissue [577,578] and in mouse kidney [574]. Similarly, an age-related reduction of enzyme activities of ETC complexes for oxidative phosphorylation and ATP synthesis has been demonstrated in human skeletal muscle [579-581], heart [582], and brain [583]. These data provide strong evidence that mitochondrial dysfunction might be a driving force for accelerated aging. Therefore, evaluating mitochondrial function offers a great opportunity to study oxidative stress and aging.

The Extracellular Flux (XF) Cell Mito Stress Test is a method for measuring mitochondrial function in cells and in tissues [584]. Using inhibitors of respiratory chain complexes and uncoupling agents, this technique is able to quantify different components of mitochondrial function, including respiration in the basal condition, the rate of ATP generation, proton leak rate, maximum and spare respiratory capacity, and non-mitochondrial respiration rate in real time [585]. We have measured mitochondrial

respiratory function and bioenergetic parameters of the placental trophoblast HTR8/SVneo cells using Seahorse Bioscience XF96 Extracellular Flux Analyser with XF Cell Mito Stress Test Kit. To assess the effect of growth factor deprivation on mitochondrial function, the assay was performed after culturing in serum-free medium. Placental trophoblast under growth factor deficiency showed a significant reduction of mitochondrial function, in terms of basal respiration rate, ATP production, proton leak, maximal respiration rate and spare respiratory capacity, when compared to control. Cells store energy in the form of ATP and ATP production is an important function of mitochondria. A decrease in mitochondrial ATP production is demonstrated in human aging [575,581]. Reduction of enzymatic activity of mitochondrial ETC, in particular ATP synthase (Complex IV) activity, is correlated with a significant increase in mitochondrial ROS production [586,587], and both are associated aging. In addition, a decrease in proton leak is also directly linked to an increase in mitochondrial ROS production, and is associated with pathologic conditions, such as obesity, diabetes, aging and age-related diseases [543-545]. To investigate the pathways that control the alteration of mitochondrial function in response to growth factor deprivation, we used an AOX1 inhibitor, raloxifene and GPER1 agonist G1, as we observed that AOX1 activation and loss of GPER1 function lead to placental oxidative damage, and alteration of mTORC1 and AMPK pathways in growth factor deficiency. We observed an increase in mitochondrial respiration function, in particular, ATP production, after treatment with both raloxifene and G1. Our result is consistent with the role of GPER1 activation by estrogen treatment in activating the enzymes involved in mitochondrial ETC and ATP production [566,567]. GPER1 activation induces ATP generation via the mitochondrial oxidative phosphorylation and glycolysis by stimulating cellular glucose uptake and pyruvate transportation into mitochondria [568,569]. GPER1 activation with estrogen (βestradiol) has been shown to increase mitochondrial membrane potential (proton leak) and decrease mitochondrial ROS production in mice heart [239], and in human brain microvascular endothelial cells [505]. Also estrogen activation by  $\beta$ -estradiol increases proton leak through ATP synthase component in rat liver mitochondria [588]. Raloxifene, an AOX1 inhibitor (also act as estrogen receptor modulator) suppress mitochondrial ROS production in human brain cells [99]. These results suggest that AOX1 induced ROS may cause oxidative damage in mitochondrial components that further facilitates mitochondrial ROS production and impairs mitochondrial function. Raloxifene and/or G1 blocks AOX1 that may reduce the AOX1 induced or mitochondrial ROS production, resulting in the attenuation of mitochondrial dysfunction. Whereas, the profiles of cellular glycolytic flux that was estimated using the mitochondrial stress test assay suggest that growth factors removal may not affect the cellular glucose utilization via glycolysis in placental trophoblast cells. Both the mitochondrial respiration and glycolysis suggested that placental trophoblasts preferentially derive much more energy using the mitochondrial pathway than glycolysis, and that serum starvation reduces mitochondrial oxygen consumption but not glycolysis. The reduction of mitochondrial respiration by growth factor removal could also be due to the decrease in pyruvate (an intermediate product of glycolysis)-dependent oxygen uptake by mitochondria, as aging is shown to be associated with pyruvate-dependent reduction of mitochondrial oxygen consumption [547,549,550]. These results will provide a basis for further study of mitochondrial function and bioenergetics of the placental trophoblast cells.

## **CHAPTER 8**

## **Conclusion and Future Directions**

#### 8.1 Concluding remarks

The effect of placental pathology on pregnancy outcomes has been a subject of much scientific interest for many years. There is accumulating evidence that demonstrates an association between oxidative stress and placental aging that contribute to poor pregnancy outcomes. However, the pathways that regulate placental aging and the underlying mechanism remain unknown. The studies within this thesis provide insights into the molecular mechanisms of placental aging.

A primary aim of this study was to measure biochemical markers of oxidative damage and aging in placentas associated with unexplained stillbirth. To fulfil this aim, at first, we studied if late gestational tissues show evidence of oxidative damage and other biochemical signs of aging, including, oxidation of DNA/RNA, lipid peroxidation, altered autophagic potential, and changes in protein expression. We then investigated if placental tissues from pregnancies associated with unexplained stillbirth show evidence of these biochemical parameters of aging. Finally, we compared the results from both late-term and unexplained stillbirth pregnancies with the results from healthy term pregnancies.

A further aim was to develop an *in vitro* human placental explant and a placental cell line culture model to test the pathways that regulate oxidative damage and aging in the placenta in pathologic pregnancies. For this, we induced *in vitro* oxidative stress in both the placental explants and in placental trophoblast cells to generate an aging-like phenotype. Using both placental explants and placental trophoblast cell line we tested the pathways that control placental oxidative damage and aging. We also investigated if oxidative stress causes a change in placental protein expression patterns and perturbs mitochondrial homeostasis in the placental trophoblast cells. Finally, we tested if modulation of pathways that regulate oxidative damage in the placenta can attenuate the changes in protein expression and mitochondrial dysfunction.

A main and novel finding of this thesis is that both aldehyde oxidase 1 (AOX1) activation and loss of estrogen activation at G-protein coupled estrogen receptor 1 (GPER1) cause placental oxidative damage and aging via increases in reactive oxygen species (ROS) generation and oxidative stress in the placenta.

The work presented in this thesis has generated the following conclusions:

- Placentas from late-term pregnancies show biochemical signs of aging in the form of increased oxidised DNA (8-hydroxy-2-deoxyguanosine, 8OHdG) and increased oxidised lipid (4-hydroxynonenal, 4HNE). Similar aging related biomarkers have been demonstrated in association with unexplained stillbirth (Chapter 5).
- 2. The level of AOX1, which is an important redox regulated pathway of cellular ROS formation [207], is increased in placental tissues obtained from both late-gestation and stillbirth pregnancies (Chapter 5). This finding suggests that the AOX1 may function as an important regulator in inducing placental oxidative damage and aging.
- 3. The demonstration of cell surface estrogen receptor GPER1 localisation on the apical surface of the normal placental syncytiotrophoblast (Chapter 5) indicates the plausibility of estrogen inhibition of AOX1 activity in the placenta under physiological conditions. This finding supports the possibility that low estrogen

concentrations may lead to loss of the cell surface estrogen receptor (GPER1) mediated inhibition of AOX1, resulting an induction of AOX1 activity and ROS generation, consequent placental oxidative damage and impaired function.

- 4. In vitro culture of placental explants and trophoblast cells in growth factor deficient medium induces oxidative stress, measured in terms of lipid peroxidation (4HNE production) and DNA oxidation (8OHdG) in placental trophoblast cells. Blocking of AOX1 by raloxifene and inducing GPER1 receptor activity by its agonist G1 attenuate growth factor starvation induced oxidative stress in the placental explants and trophoblast cells (Chapter 5, 6 and 7), suggesting AOX1 and GPER1 mediated regulation of placental oxidative damage.
- 5. The protein expressions of sirtuins (SIRT1, SIRT2 and SIRT6), which are known to be implicated in life span regulation in experimental organisms [482-485,493,495,496], are downregulated in placental explants under oxidative stress (Chapter 6). Modulation of oxidative stress inducing pathways, AOX1 or GPER1 attenuates the oxidative stress induced downregulation of sirtuins in placental explants (Chapter 6).
- 6. Induction of *in vitro* oxidative stress in placental trophoblast cells inhibited the activity of mammalian target of rapamycin complex 1 (mTORC1), a sensor of cellular nutrients [143], and increases 5' adenosine monophosphate stimulated protein kinase (AMPK) activity, a key sensor for cellular energy [533]. Inhibition of AOX1 or GPER1 activation attenuates the oxidative stress induced changes in mTORC1 and AMPK activity in placental trophoblast cells (Chapter 7).

7. Induction of *in vitro* oxidative stress in placental trophoblast cells causes mitochondrial dysfunction and changes in the mitochondrial bioenergetic parameters including, a reduction of mitochondrial respiration function, ATP production and proton leak (Chapter 7). A decrease in mitochondrial ATP production is demonstrated in human aging [575,581] and is correlated with a significant increase in mitochondrial ROS production [586,587]. Reduction of mitochondrial proton leak is directly linked to an increase in mitochondrial ROS production and is associated with aging and age-related diseases [543-545]. Inhibition of AOX1 activity or induction of receptor activity at GPER1 attenuates the oxidative stress induced mitochondrial dysfunction possibly via reducing ROS production (Chapter 7). This finding further supports the importance of the AOX1 and GPER1 pathways in mediating placental oxidative damage.

Altogether, the results presented in this thesis support the hypothesis that placental oxidation is regulated by estrogen activation at the GPER1 and inhibition of AOX1 leading to the inhibition of ROS generation. Oxidative stress in the placenta may cause a loss of GPER1 activation, lead to an increase in AOX1 activation, resulting in increased ROS generation, which increases DNA oxidation, lipid peroxidation, alters lysosome-autophagosome function, and causes changes in protein expression and mitochondrial function, and all these changes may accelerate the aging process in the placenta.

Our study identifies potential biomarkers of oxidative damage and aging in stillbirth placentas that raise the possibility that these biomarkers of placental oxidative damage aging may be released into maternal blood where they may have diagnostic value in predicting the fetus at risk for stillbirth. This study also identifies potential therapeutic targets such as AOX1 that may arrest the oxidative damage and premature aging to placentas in pregnancies identified at high risk of stillbirth. This is particularly valuable when extreme prematurity precludes delivery and therapeutic intervention is the only possible solution.

The successful utilisation of the *in vitro* aging model in this study indicates that the placenta may provide a tractable model to study aging in a human tissue that uniquely ages in a 9 months period of time. The collective findings within this thesis will provide significant contributions to knowledge of the mechanisms of aging in other tissue such as brain, skin, and kidney. Moreover, the role of AOX1 and GPER1 in inducing oxidative damage in placenta warrants further investigation in this area and provides an interesting topic for future research into human aging. Treatments targeting AOX1 and GPER1 to prevent oxidative damage in placenta could also be translated to prevent premature aging in other organs in humans.

Although the thesis has reached its aims, there were some unavoidable limitations. One of the limitations of our study was that there is still a lack of knowledge on the specific role of AOX1 and GPER1 in placental function due to the unavailability of genetically engineered organisms or animal models.

#### 8.2 Future directions

Future work will be aimed at addressing some of the limitations of the current study and extending the ideas explored here to study several other relevant pregnancy pathologies.

The identification of potential biomarkers of oxidative damage and aging in stillbirth placenta which warrants further investigation in this area. There is a possibility that these biomarkers could be found in maternal blood as placenta continuously releases materials into maternal system throughout the pregnancy. Thus, one of the more important future extensions of the work will involve the identification of placenta derived biomarkers of aging in maternal blood that may lead to the development of novel diagnostic tools for predicting the fetus at risk for stillbirth and other placental aging associated complications.

As mentioned, it would be important to explore the importance of AOX1 and GPER1 in pregnancy and their specific role on placental function. For this, further study using a genetically modified population of placental trophoblast cells or using experimental animal models need to be conducted. Particularly, manipulation of the genes encoding AOX1 and/or GPER1 could be performed using retroviral-mediated gene transduction or CRISPR technologies. Studies are now underway in our laboratory to produce AOX1 and GPER1 knockout populations of placental trophoblast cells. There remain many other genes that have been identified to be associated with aging process in placenta but were unable to be followed up due to time constraints, which could form the basis of many more future projects.

# **CHAPTER 9**

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# **APPENDIX** A

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# **REVIEW ARTICLE**

#### WILEY AJRI American Journal of Reproductive Im

# Oxidative stress, placental ageing-related pathologies and adverse pregnancy outcomes

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Roger Smith, Mothers and Babies Research Centre Hunter, Hunter Medical Research Institute, Newcastle, NSW, Australia. Email: Roger.Smith@newcastle.edu.au Oxidative stress (OS), an imbalance between free radical generation and antioxidant defence, is recognized as a key factor in the pathogenesis of adverse pregnancy outcomes. Although OS is a common future of normal pregnancy, persistent, overwhelming OS leads to consumption and decline of antioxidants, affecting placental antioxidant capacity and reducing systems. The accumulation of OS causes damage to lipids, proteins and DNA in the placental tissue that induces a form of accelerated ageing. Premature ageing of the placenta is associated with placental insufficiency that prevents the organ meeting the needs of the foetus, and as a consequence, the viability of the foetus is compromised. This review summarizes the literature regarding the role of OS and premature placental ageing in the pathophysiology of pregnancy complications.

## KEYWORDS

intrauterine growth restriction, oxidative stress, placental ageing, pre-eclampsia, preterm birth, senescence, stillbirth

# 1 | INTRODUCTION

All living organisms have limited life cycles, and ageing is part of that life cycle. Each organ within an organism also exhibits ageing-related changes; the placenta is no exception. The placenta, a specialized organ formed during pregnancy, grows throughout gestation, performs multiple functions, including endocrine regulation and nourishment of the foetus,<sup>1</sup> but also ages and is discarded at the end of pregnancy, while the foetus may live for another hundred years. So placental ageing is a normal physiologic phenomenon.<sup>2</sup> However, there are likely to be some placentas which show signs of ageing earlier than others, in the same way as some individuals age more quickly than others. Premature ageing and degenerative changes in the placenta may reduce the functional capacity of the placenta and lead to abnormal pregnancy outcomes. The placenta is the primary organ for transferring nutrients from the mother to the foetus, so growth and function of the placenta are precisely regulated and coordinated to ensure the optimal growth and development of the foetus. The placenta exchanges nutrients, for example oxygen, amino acids, carbohydrates,

minerals and waste products, for example carbon dioxide between the maternal and foetal circulatory systems.<sup>3</sup> It releases hormones into both the maternal and foetal circulations to affect uterine function, maternal metabolism, foetal growth and development. Moreover, it metabolizes some substances and can release metabolic products into both foetal and maternal circulations. The placenta can help to protect the foetus against certain xenobiotic molecules, infections and maternal diseases. Therefore, the adequate function of this organ is crucial for a normal physiologic gestational process and a healthy baby as a final outcome.

In this review, we focus on the role of OS in the pathophysiology of pregnancy complications, beginning with a brief overview of placental development at different stages of gestation. We then discuss the biochemical markers of ageing and OS-induced placental ageing. Finally, we discuss the studies indicating that OS and placental ageing play a role in the pathophysiology of abnormal pregnancies, with a particular emphasis on pregnancy complicated by spontaneous preterm birth, intrauterine growth restriction, pre-eclampsia, pregnancy loss and stillbirth.

# 2 | HUMAN PLACENTAL DEVELOPMENT

In human embryonic development, the blastocyst is formed by 5-6 days after fertilization and is composed of the outer trophectoderm layer and the inner cell mass.<sup>4</sup> The blastocyst makes contact with the endometrium and invades into the decidua of the endometrium at approximately 6-7 days after fertilization.<sup>5</sup> Immediately after attachment to the endometrium, the trophectoderm layer proliferates rapidly and differentiates into an inner layer of mononuclear cytotrophoblasts and a multinucleated outer epithelial layer known as the syncytiotrophoblast.<sup>3</sup> The syncytiotrophoblast is a terminally differentiated cell layer which is formed by the fusion of multiple cytotrophoblasts, a process called syncytialization. The combination of inner cytotrophoblasts and outer syncytiotrophoblast forms finger-like structures called primary chorionic villi.<sup>3</sup> At the initial phase of differentiation, these villi are distributed symmetrically over the chorion. As gestation progresses, the chorionic villi grow like branches of a tree (arborization) and accumulate asymmetrically towards the uterine wall where the embryo is attached.<sup>3</sup> After the invasion of mesenchymal cells into the centre of the primary villi forming secondary villi, foeto-placental blood vessels arise inside the villi at the 5th week of gestation to form tertiary villi.<sup>6</sup> The placental vasculature system is essential for transferring nutrients, gases and hormones to the growing foetus. The proper branching of placental blood vessels (angiogenesis) is part of a successful pregnancy. Inadequate placental development, trophoblast invasion and vascular remodelling, as well as abnormal placental angiogenesis, have been reported in pathological pregnancies such as intrauterine growth restriction and pre-eclampsia.<sup>5,7,8</sup>

In the first trimester, the chorionic villi of the placenta are large, and the blood vessels in the villi are not prominent. In addition to villous trophoblast, an additional set of mononuclear trophoblasts, termed the extravillous trophoblast, grows outside the villi and extends into the decidualized endometrium.<sup>3,9</sup> During the first trimester of differentiation (up to 11-12 weeks), these extravillous trophoblasts erode into and plug the uterine spiral arteries and restrict the ability of the oxygenated maternal blood to access the placenta.<sup>10</sup> Consequently, the early stages of human embryonic development occur in an environment of low oxygen tension.<sup>11</sup> The hypoxic environment is thought to be necessary for the initial differentiation of the trophoblasts: in fact, miscarriage has been reported in cases of the early arrival of oxygenated blood in the intervillous space.<sup>12</sup> As the placenta matures and increases in size in the second trimester, the villi become smaller and more vascular. The syncytiotrophoblast cell layer draws up into "syncytial knots" which are small clusters of nuclei, leaving a single cytotrophoblast layer. Later the extravillous trophoblasts replace the endothelial layer covering the smooth muscle of the spiral arteries and render them flaccid and non-contractile.<sup>13</sup> The trophoblast plugs are gradually dislodged from the spiral arteries after 11-12 weeks of gestation, and maternal blood invades from the maternal spiral arteries into the intervillous spaces.<sup>11,14</sup> This process is associated with a sharp rise in oxygen tension, increased free radical generation and a burst of OS within the placental tissues; however, this OS returns to baseline upon a surge of antioxidant activity, as placental cells gradually acclimate to the new oxidative surroundings.<sup>15</sup> The nutrients, gases and growth factors carried by maternal blood are readily taken up by the large surface of the syncytiotrophoblast allowing the foetus to grow in an oxygen- and nutrient-rich environment. A mature placenta in the third trimester has small and highly vascularized chorionic villi to support the blood gas and nutrient exchange of the maternal-foetal circulation required by the growing foetus approaching term gestation. Syncytial knots are prominent in the third-trimester chorionic villi. Figure 1 illustrates the development of human placental chorionic villi at different stages of gestation.

# 3 | APOPTOSIS AND ITS ROLE IN THE TROPHOBLAST FUNCTION

Apoptosis, or programmed cell death, is crucial to the development and homoeostasis of all multicellular organisms and for many organs including the placenta. Apoptosis is known to occur in a number of biological processes, both physiologic and pathologic. Trophoblast apoptosis is a physiologic event in normal pregnancy, increases with advancing gestational age and is higher in post-term pregnancies and therefore is considered as a normal process in the development and ageing of the placenta.<sup>16,17</sup> Apoptosis is proposed to occur as a normal event during the formation of the villous trophoblast bilayer and syncytiotrophoblast formation from cytotrophoblasts (trophoblast differentiation).<sup>18</sup> However, it is likely that placental insults can alter the regulation of apoptosis in the trophoblasts, possibly by modulating trophoblast cell turnover.<sup>18</sup> Cultured trophoblasts exposed to hypoxia show marked upregulation of activity of tumour suppressor protein p53, enhanced expression of the pro-apoptotic Mtd-1 and decreased expression of the anti-apoptotic Bcl-2, all of which promote apoptosis,<sup>19-21</sup> and the apoptosis is more marked in hypoxia/ re-oxygenation.<sup>22</sup> Additionally, an upregulated p53 and decreased Bcl-2-mediated increased apoptosis in placental syncytiotrophoblast are associated with some pregnancy pathologies, including intrauterine growth restriction (IUGR) and pre-eclampsia.<sup>23,24</sup> Syncytial knots, a characteristic feature of syncytiotrophoblast apoptosis, increase in placentas associated with pre-eclampsia and IUGR.<sup>24,25</sup> In contrast, apoptosis decreases in extravillous trophoblasts in pregnancies complicated by pre-eclampsia and is associated with reduced trophoblast invasion.<sup>26</sup> Thus, apoptosis is differently regulated in villous and extravillous trophoblasts in normal placental development.

# 4 | AGEING, OS AND PLACENTAL AGEING

# 4.1 | Cellular senescence and ageing

Ageing can be defined as an age-dependent decline and deterioration of functional properties at the cellular, tissue and organ level, leading to a decreased adaptability to internal and external stress and an increased vulnerability to disease and mortality.<sup>27</sup> Age-related diseases and premature ageing syndromes are often characterized by



**FIGURE 1** Development of human placental chorionic villi. Cross sections of (A) primary villi arborization, (B) firsttrimester, (C) second-trimester and (D) third-trimester villi. The chorionic villi in the first trimester (B) are large, covered by two layers of cells, cytotrophoblasts and syncytiotrophoblasts, and the blood vessels in the villi are not prominent. Second-trimester placental villi (C) are more vascular with a single cytotrophoblast layer. The third trimester (D) has highly vascularized chorionic villi, and syncytial knots are prominent

short telomeres and reduced or complete loss of telomerase activity.<sup>28</sup> Telomeres are nucleoprotein structures comprised of doublestranded DNA region of TTAGGG repeats which is typically 10-15 kb long in humans, located at the termini of the chromosomes and are essential for chromosomal stability and cell survival.<sup>29</sup> Telomeres protect DNA ends from breaks, end-to-end fusion and degradation by forming a protective cap with a 150- to 200-nucleotide-long G-rich single-stranded telomere overhang and telomere-binding protein complexes.<sup>28</sup> Telomeres are progressively shortened with each cell division, and shortening is accelerated as a consequence of environmental stressors and insults, such as hyperglycaemia, hypoxia and OS.<sup>30-33</sup> Once a critical shortening of telomeres is attained, cells enter a state of irreversible metabolic arrest known as senescence, which leads to a process of cellular or tissue ageing.<sup>29,34</sup> Cell senescence is distinct from apoptotic cell death. Senescence is a biological ageing process in which cells change morphologically, in gene and protein

expression, and in the activation of key signalling constituents (such as p38 and p53) that determine the fate of a tissue.<sup>35</sup> Cellular senescence has been associated with a gradual deterioration of functional characteristic of the cell, although there is no evidence that senescent cells undergo a cell death pathway. Senescent cells are resistant to apoptosis or programmed cell death through the overexpression of Bcl-2 protein, leading to the accumulation of these cells within tissues.<sup>36</sup> The accumulation of senescent cells within tissues contributes to the ageing process and generates age-related phenotypes by altering metabolic function, degrading structural components, reducing tissue renewal and repair, changing the behaviour of neighbouring cells or the extracellular environment, and reducing the pool of growthcompetent mitotic cells.<sup>37</sup> Premature senescence can also occur, independent of telomere size, as a consequence of progressive DNA damage, telomere uncapping and telomere dysfunction caused by extrinsic or intrinsic stressors including OS, resulting in end-to-end

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fusion and aggregation of telomeric DNA.<sup>1,38,39</sup> Telomere length is regulated by the enzyme telomerase, a specific reverse transcriptase, capable of adding telomeric repeats to the ends of the chromosome.<sup>40</sup> Telomerase consists of a catalytic protein component, telomerase reverse transcriptase (TERT) and an RNA template component, telomerase RNA component (TERC). TERC is widely expressed, but TERT expression is tightly regulated and is considered to be the ratelimiting factor in telomerase activity.<sup>41</sup> The absence of functional telomerase or loss of telomerase activity leads to progressive telomere shortening during cell division.<sup>34,40</sup> Telomere shortening may also be associated with a lack of adequate damage repair mechanisms that protect against DNA damage.<sup>39</sup> Due to their high oxidation potential, the guanine-rich residues in telomeres are extremely susceptible to free radical attack.<sup>42</sup> There is a clear relationship between OS and telomere length and telomerase activity, the indicators of cellular senescence and ageing.<sup>43</sup> Therefore, measurement of telomere length and telomerase activity can be used as biological markers for tissues suffering OS and age.43-45

# 4.2 | OS and placental ageing

OS is an important contributing factor in the pathophysiology of complicated pregnancies. OS is described as an imbalance in the generation of reactive oxygen species (ROS) and the ability of antioxidant defences to scavenge them. OS can arise from increased ROS production and/or defects in antioxidant defence mechanisms.46 These ROS are oxygen free radicals that contain one or more unpaired electrons, produced from the reduction in molecular oxygen and generated as by-products of aerobic respiration and metabolism. These molecules have diverse chemical properties and are capable of activating and modulating various signalling pathways, including those involved in cell growth, differentiation and metabolism.<sup>47</sup> They can also induce cellular oxidative damage by interacting with DNA and intracellular macromolecules such as proteins and membrane lipids, leading to cellular malfunction that may initiate pathological processes. The free radical theory of ageing<sup>48</sup> postulates that ageing and degenerative diseases associated with ageing are due to the oxidative damage by ROS on cellular components. Moreover, the mitochondrial free radical theory of ageing<sup>49</sup> proposes that ROS damage mitochondrial DNA (mtDNA), proteins and other macromolecules that lead to respiratory chain dysfunction. Mutant mtDNA induces an increased production of ROS, further facilitating mtDNA damage and creates a self-amplifying deterioration.<sup>49</sup> The increased generation of ROS can cause lipid peroxidation, protein damage and several types of DNA lesions in cells, which may result in altered or complete loss of cellular function, compromised tissue and organ function, and ageing. Mechanistically, OS induces activation of processes, including repair pathways, inhibition of cell proliferation (transient cell-cycle arrest or senescence) or apoptosis.<sup>50</sup> OS activates a specific p53 transcriptional response, mediated by p44/p53 and p66, which regulates the cellular response to DNA damage, leading to a halt in proliferation via senescence or apoptosis and contributes to ageing.<sup>51</sup> To counterbalance the ROS, cells have endogenous antioxidant systems, including non-enzymes, for example vitamin C and E, and glutathione (GSH), enzymes, for example superoxide dismutase (SOD), glutathione peroxidases (GSH-Ps), glutathione S-transferase (GSH-T) and catalase (CAT), and trace elements, for example copper, zinc, manganese and selenium.<sup>52</sup>

Pregnancy itself is a state of OS, arising from the increased metabolic activity in placental mitochondria and an increased ROS production due to the higher metabolic demand of the growing foetus.53,54 Superoxide anions produced by placental mitochondria appear to be a major source of ROS and lipid peroxidation that contribute to the OS in the placenta.<sup>55</sup> Although a physiologic balance between ROS and antioxidant activity is maintained in normal pregnancies,<sup>56</sup> an imbalance may increase OS. The placenta experiences a heightened level of OS in certain pathologic pregnancies, especially those that are complicated by maternal smoking, gestational diabetes, foetal growth restriction, pre-eclampsia and miscarriage.<sup>42,57,58</sup> Often antioxidant activity is upregulated in response to OS. However, persistent, overwhelming OS leads to consumption and decline of antioxidants, and affects placental antioxidant capacity and reducing systems.<sup>54</sup> In the post-mature placenta, the accumulation of OS damage to lipids, proteins and DNA in the placental tissue may induce a form of advanced ageing.<sup>42</sup> Premature ageing can occur when the intrauterine environment is affected by conditions that increase OS, causing irreversible changes in placental tissue.<sup>8,59</sup> It has been hypothesized that ageing of the placenta is usually associated with placental insufficiency, preventing this organ from meeting the needs of the foetus, and as a consequence, the viability of the foetus is compromised.<sup>42</sup> Figure 2 summarizes the effect of oxidative stress on placental function and pathological events at different stages during pregnancy.

# 5 | OS, PLACENTAL AGEING AND ADVERSE PREGNANCY OUTCOMES

## 5.1 | OS and spontaneous preterm birth

Preterm birth is defined as birth before 37 weeks of gestation, affects 5%-18% of pregnancies and is a leading cause of infant morbidity and mortality. Most of the preterm births occur after the spontaneous onset of labour (with or without preterm premature rupture of the membrane, pPROM), but the precise mechanisms of onset of preterm labour remain unclear.<sup>60</sup> Labour induces changes of gene expression in chorioamniotic membranes that are consistent with the localized acute inflammatory response, despite the absence of histologically detectable inflammation.<sup>61</sup> It has been hypothesized that cellular apoptosis transmits an inflammatory signal that stimulates parturition.<sup>62</sup> Although they are resistant to apoptosis, senescent cells may transmit both inflammatory and senescence-promoting signals to induce labour.<sup>35</sup> It has also been suggested that labour is associated with senescence-associated changes in the placental membranes mediated by the p38 MAPK pathway, including telomere shortening, p38 MAPK activation and increased expression of p21 and SA-β-galactosidase.<sup>63</sup> OS at term induces DNA damage and telomere shortening, which



ROS inducers (Hypoxia,

High metabolic activity)

FIGURE 2 Effect of oxidative stress on placental function and pathological events in pregnancy

accelerates telomere-dependent senescence of the foetal membranes, resulting in senescence-associated inflammatory activation that may contribute to parturition.<sup>64</sup> It has long been thought both term and preterm labour have similar processes that occur through a "common pathway". The activation of this common pathway through physiologic signals results in term labour, while preterm labour is a "syndrome," which occurs from spontaneous activation of one or more of the components of the common pathway induced by multiple pathologic processes or risk factors.<sup>60</sup> Spontaneous preterm labour or pPROM is likely to be triggered by premature placental ageing caused by OS-induced damage and premature senescence of the intrauterine tissues, especially the foetal membranes of the placenta, <sup>65-67</sup> and vascular, endocrine or immune system dysfunction.<sup>68</sup> ROS activates NF-kappa B, which stimulates COX-2 expression and systemic inflammation. Infection, inflammation or exogenous factors (eg lead) upregulate ROS, resulting in OS-induced tissue injury, and the consequent decrease in antioxidant defences is likely to increase the risk of preterm birth.<sup>69</sup> Preterm birth is also associated with ROS-mediated redox imbalance (balance between pro- and antioxidants). In preterm birth, increased placental and maternal serum levels of oxidized metabolites (malondialdehyde) with reduced levels of antioxidant (GSH, selenium, GSH-T) are observed compared to term labour.<sup>70-73</sup> However, the expression of Mn-SOD in foetal membranes of women in preterm labour is increased, likely to constrain the inflammatory processes and OS.<sup>74</sup>

# 5.2 | OS and IUGR

IUGR, also known as foetal growth retardation, is a failure of a foetus to reach its genetic growth potential. IUGR is a leading cause of foetal, neonatal and perinatal morbidity and mortality.<sup>75</sup> IUGR is defined as an estimated foetal weight of less than the 10<sup>th</sup> percentile for gestational age.<sup>18</sup> Most intrauterine deaths, in particular, those that are classified as unexplained, are associated with IUGR. Around 76% of intrauterine deaths are associated with IUGR.<sup>76,77</sup> IUGR also strongly affects the long-term health of survivors.<sup>78</sup> Some potential risk factors for IUGR include maternal smoking, infection, obesity, malnutrition and chromosomal abnormalities, but the majority of cases remain unexplained.<sup>79</sup> The most common aetiology for IUGR is uteroplacental dysfunction, due to diminished maternal uteroplacental blood flow.<sup>80</sup> The placenta is the central organ for transporting nutrients and oxygen from the mother to the foetus. Inadequate function of this organ limits the supply of critical substrates to support the normal aerobic growth of the foetus.<sup>81</sup> Recently, it has been hypothesized that placental insufficiency originates in the early stage of gestation when the trophoblast invades spiral arteries in the placental bed.<sup>81</sup> This process requires high energy availability for cell growth, proliferation and metabolic activity that generates ROS and OS. Inadequate trophoblastic invasion to the spiral arteries may occur when the chorioallantoic villi encounter an injury caused by stimuli or mediators.<sup>82</sup> Among the diverse number of stimuli or mediators, OS has the leading role.<sup>22</sup>

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Consequently, incompletely developed spiral arteries cause ischaemia (hypoxia)-reperfusion that exacerbates the OS and contributes to damage of the placental tissue.<sup>18</sup>

Damage resulting from OS predominantly occurs to membrane lipids, proteins and nuclear and mitochondrial DNA. Plasma and tissue levels of malondialdehvde (MDA), an end product of fatty acid oxidation, are frequently measured as indicators of lipid peroxidation and OS. The levels of MDA and xanthine oxidase (XO, an enzyme that generates ROS) are higher in maternal plasma, umbilical cord plasma and placental tissues of the patients with IUGR pregnancy compared to healthy pregnancies.<sup>83</sup> which suggest that OS has a role in IUGR. In nuclear and mitochondrial DNA, 8-hydroxy-2'-deoxyguanosine (8-OHdG, an oxidized derivative of deoxyguanosine) is one of the predominant forms of free radical-induced oxidative lesions, and has therefore been widely used as a biomarker for oxidative DNA damage, as well as OS. The levels of 8-OGdH and redox factor-1 (ref-1) are significantly higher in placentas from IUGR compared to normal pregnancies.<sup>84-86</sup> Ref-1 is a redox regulator that repairs oxidative DNA damage, and its concentration increases in response to oxidative damage. Placental antioxidant levels and antioxidant activity are also altered in pregnancies complicated by IUGR. In IUGR, the SOD and GSH-Px activities in maternal plasma, cord blood and placental tissues are increased, while CAT activity is decreased.<sup>83</sup> The mRNA levels of the reducing systems, glutaredoxin and thioredoxin, are also depleted in placentas with IUGR.<sup>87</sup> Moreover, the IUGR placenta shows signs of ageing markers, including shortening of telomere length and reduced telomerase activity. A significantly shorter telomere and/or an absent or reduced telomerase activity are observed in the placentas from IUGR  $\operatorname{pregnancies}^{41,88-90}$  with a reduced expression of hTERT, which is the rate-limiting factor in the telomerase activity.<sup>41</sup> Also, the expression of telomere-induced senescence markers p21 and p16 is elevated, and anti-apoptotic protein Bcl-2 is decreased in IUGR placentas.88 Together with increased OS markers and reduced antioxidants capacity, the evidence of ageing markers supports the concept of the role of OS in placental ageing and IUGR.

# 5.3 | OS and pre-eclampsia

Pre-eclampsia is a hypertensive disorder of human pregnancy, and it frequently occurs in association with IUGR. Pre-eclampsia affects 5%-7% of all pregnancies worldwide and remains a leading cause of foetal growth retardation, premature delivery and maternal death.<sup>54,91,92</sup> The main features of pre-eclampsia are new-onset maternal hypertension (blood pressure ≥140/90 mmHg), reduced uteroplacental blood flow, proteinuria (≥300 mg/24 h), oedema and occurrence primarily in nulliparous women in their third trimester.<sup>54</sup> Among the two distinct subtypes, early-onset pre-eclampsia (occurs before 34 weeks) confers a higher risk of life-threatening maternal complications and foetal and perinatal death, than the late-onset (occurs at 34 weeks or later), and early delivery is the only treatment.<sup>93</sup> This disorder develops during pregnancy, and the rapid and complete recovery after childbirth indicates that the placenta has a pivotal role in the pathogenesis of this disease.<sup>94</sup> Although the aetiology of pre-eclampsia is still subject to debate, the basic pathologic event in pre-eclampsia is an injury to the vascular endothelium<sup>95</sup> that is mediated by OS from increased placental ROS or decreased antioxidant activity.<sup>96</sup> Consequently, trophoblastic invasion to the spiral arteries is inhibited that limits the spiral artery remodelling to the decidual portions and the myometrial segments of the arteries remain narrow and contractile.<sup>97</sup> Therefore, in pre-eclampsia, increased vascular resistance in the placenta leads to reduced uteroplacental perfusion.<sup>97,98</sup> The resultant hypoxia or ischaemia, together with intermittent perfusion, is associated with the conversion of xanthine dehydrogenase to XO and the increased XO activity provokes ROS synthesis in the placenta.<sup>99,100</sup> Both pre-eclampsia and IUGR share similar pathophysiology that is associated with defective placentation, but pre-eclampsia (with or without IUGR) is distinguished from IUGR (without pre-eclampsia) by extension of disturbances into the maternal vasculature.<sup>97,101</sup>

In pre-eclampsia, both the circulating and placental tissue levels of markers of OS are elevated and antioxidant capacities are compromised.<sup>100,102</sup> Polyunsaturated fatty acids, which are found in abundance in the cell membrane and in circulating lipoproteins, are highly susceptible to oxidation by free radicals to form lipid peroxides, and the process is called lipid peroxidation.<sup>103</sup> When lipid peroxidation is initiated, it becomes self-propagating and continues until it is interrupted by an antioxidant. Normal pregnancy is associated with increased free radical production, lipid peroxidation and OS; however, antioxidant activity is also upregulated<sup>56</sup> that counterbalances free radical generation and oxidative damage. In contrast, pre-eclampsia is associated with increased lipid peroxidation in the maternal circulation and the placenta and decreased antioxidant activity.103-105 Superoxide anions produced by the enzyme XO in the placental mitochondria appear to be a major source of OS and contribute to an overall increase in maternal blood and placental lipid peroxidation in pre-eclamptic women.<sup>55,104</sup> Two major end-products of lipid peroxidation, MDA and 4-hydroxynonenal (4-HNE), are frequently measured as indicators of lipid peroxidation and OS. Increased placental and serum levels of MDA and 4-HNE and placental XO expression of pre-eclamptic women are observed compared with normotensive subjects, 55,99,103,106,107 whereas maternal circulating and placental levels of antioxidants, for example, CAT, GPX and SOD, are decreased in pre-eclampsia compared to healthy pregnancy.<sup>103,105,106,108</sup> Also, the expression of 8-OHdG is increased in both maternal blood and the placental trophoblast in pregnancy complicated by pre-eclampsia with or without IUGR.<sup>84-86</sup> The level of ref-1 that repairs oxidative DNA damage is also higher in the pre-eclamptic placenta.<sup>84-86</sup> Serum levels of derivatives of reactive oxygen metabolites (d-ROMs), for example, organic hyper oxides, are also increased in pre-eclamptic women,<sup>85,86</sup> indicating increased ROS in maternal circulation from which they are produced. The increased ROS in the maternal circulation may originate from the placenta, as the d-ROMs decrease following delivery.<sup>85</sup> Additionally, in pre-eclamptic placentas with or without IUGR, telomeres are shorter, and telomerase activity is reduced compared to healthy placentas.<sup>38,41</sup>

# 5.4 | OS and early pregnancy loss

OS has been implicated in early pregnancy loss. There is a sharp increase in oxygen tension when the maternal blood enters into the placenta, and this associated with a burst of OS.<sup>15</sup> It is not until about 11-12 weeks of gestation that the maternal blood invades into the intervillous space. The arrival of oxygenated blood before 10-11 weeks leads to deterioration of the syncytiotrophoblast caused by OS, resulting in loss of pregnancy, including spontaneous miscarriage and recurrent pregnancy loss.<sup>12,69</sup> The high levels of OS markers, such as nitrotyrosine residues, 4-HNE adducts and heat shock protein 70 in the placentas from early pregnancy loss,<sup>15</sup> suggest that increased ROS generation is due to premature establishment of maternal-placental perfusion, resulting in oxidative damage to the trophoblasts with subsequent termination of the pregnancy.<sup>46</sup> The expression of these markers is induced in vitro by exposing early placental villi to 21% oxygen and is associated with increased ROS production.<sup>109</sup> This OS in early stage of pregnancy can impair a number of cell functions, including matrix remodelling, angiogenesis, cytotrophoblast proliferation, migration and fusion, and endocrine function,<sup>110</sup> resulting in pregnancy loss.

# 5.5 | OS, placental ageing and stillbirth

Stillbirth, which is intrauterine foetal death at or after 20 weeks of gestation, is a major obstetric complication. Although a number of risk factors for stillbirth have been identified including advanced maternal age, obesity, smoking, late gestational age and IUGR,<sup>111,112</sup> most cases remain unexplained. Recently studies on stillbirth have postulated an association between stillbirth and placental pathology, including infarction, vessel wall thickening and calcification and dysfunction.<sup>42,113-115</sup> A 2016 study shows a significant reduction in telomere length in placentas associated with unexplained stillbirth indicating a telomere-dependent senescence in the placenta, suggesting that this may cause premature placental ageing and placental dysfunction leading to foetal death.<sup>113</sup> We have hypothesized that OS causes changes in proteins, lipids and DNA in the placenta, which may induce a form of advanced ageing, leading to placental insufficiency and an inability to meet the demands of the growing foetus that ultimately causes foetal demise.42

# 6 | SUMMARY

There is accumulating evidence that demonstrates an association between OS and placental ageing that contribute to poor pregnancy outcomes. Altered cellular metabolism is observed in several pathological situations, and these metabolic shifts that elevate ROS generation can increase telomere shortening or induce telomerase dysfunction leading to premature senescence and ageing. Conversely, dysfunctional telomerase may itself induce altered metabolic and mitochondrial functions that may, in turn, cause further OS deregulation. OS also activates processes or mediators that cause inhibition AJRI American Journal of

of cellular proliferation or increased apoptosis. Premature placental ageing is the consequence of OS-induced damage to lipids, proteins and DNA in the placental tissue that may cause cellular senescence or cell death in the placenta, leading to placental dysfunction and insufficiency. OS-induced endothelial dysfunction contributes to the pathogenesis of pregnancy complications, including pre-eclampsia, IUGR, preterm birth and recurrent pregnancy loss. Alteration of antioxidant capacity or changes apoptosis regulation in the placenta are also significant factors that contribute to the pathophysiology of abnormal pregnancies.

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# Is there a role for placental senescence in the genesis of obstetric complications and fetal growth restriction?

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#### Cellular senescence and aging

A key feature of aging is a progressive loss of function at the cellular, tissue, and organ level, resulting in a reduced adaptability to stress and an increased vulnerability to disease and mortality.<sup>1</sup> In mitotic tissues, the progressive accumulation of senescent cells is thought be one of the causal factors for aging.<sup>2</sup> Thus, the biomarkers of cellular senescence can be used as markers of tissue aging. Such biomarkers of cellular senescence have been summarized in a later section (see *Biomarkers of senescence*).

Senescent cells within tissues contribute to the aging process and disease development by altering normal cellular function, changing the behavior of neighboring cells, degrading structural components such as the extracellular matrix, and accelerating the loss of tissue regeneration capacity by reducing stem and progenitor cells.<sup>2</sup> Elimination

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0002-9378/\$36.00 © 2017 Elsevier Inc. All rights reserved. https://doi.org/10.1016/j.ajog.2017.11.567 The placenta ages as pregnancy advances, yet its continued function is required for a successful pregnancy outcome. Placental aging is a physiological phenomenon; however, there are some placentas that show signs of aging earlier than others. Premature placental senescence and aging are implicated in a number of adverse pregnancy outcomes, including fetal growth restriction, preeclampsia, spontaneous preterm birth, and intrauterine fetal death. Here we discuss cellular senescence, a state of terminal proliferation arrest, and how senescence is regulated. We also explore the role of physiological placental senescence and how aberrant placental senescence alters placental function, contributing to the pathophysiology of fetal growth restriction, pre-eclampsia, spontaneous preterm labor/birth, and unexplained fetal death.

**Key words:** aging, cellular senescence, cyclin-dependent kinase, DNA damage, fetal death, fetal growth restriction, mammalian target of rapamycin complex, membrane rupture, mitogen-activated protein kinase, oxidative stress, phosphoinositide 3-kinase, placental aging, preeclampsia, preterm birth, preterm labor, reactive oxygen species, senescence-associated heterochromatin foci, small for gestational age, stillbirth, telomere, tumor suppressor protein p53, p16, senescence-associated beta-galactosidase, senescence-associated secretory phenotype

of senescent cells can delay aging-associated disorders in mice.<sup>3</sup>

Cellular senescence is a state of irreversible, terminal arrest of cell proliferation (growth), triggered by a plethora of intrinsic and extrinsic stimuli or stressors. These stimuli or stressors include short or dysfunctional telomeres, DNA damage (telomeric or genomic DNA), and DNA damageresponse mediators, strong mitogenic signals (eg, overexpression of oncogenic renin-angiotensin system [RAS], a mutant RAS-p21 protein, reninangiotensin system involves transmitting signals and activating signaling cascades, including mitogen-activated protein kinase [MAPK] and phosphoinositide 3-kinase/mammalian target of rapamycin complex pathways), epigenomic disruption (chromatin disruption), overexpression of certain oncogenes, deteriorating mitochondrial function, and oxidative stress created by reactive oxygen species (ROS) (reviewed in references 4-6) (Figure 1).

The stressors that trigger senescence act by 2 major pathways controlled through stabilization of the tumor suppressor protein p53 and transcriptional inactivation of the cyclin-dependent kinases (CDKs). The suppression of CDKs is produced by transcriptional activation of the CDK inhibitor p21 (also termed p21<sup>Cip1</sup>) in concert with the CDK inhibitor, p16 (also known as p16<sup>INK4a</sup>) and retinoblastoma tumor suppressor protein (pRB) (reviewed in references 7 and 8) (Figure 1).

When activated, p53 inhibits cell proliferation via activation of its transcriptional target p21.<sup>8</sup> Both p21 and p16 maintain the protein pRB in its hypophosphorylated and active state.<sup>8,9</sup> Active pRb suppresses the E2F1 (a member of E2F family of transcription factors, which induce gene transcriptions that are essential for cell proliferation)-dependent expression of genes that regulate progression of the G1/S phase of the cell cycle, and thereby irreversibly blocks cell cycle entry<sup>10</sup>

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Telomere-dependent replicative senescence and stress-induced premature senescence act through the modulation of proteins p53 and Rb. Senescence stimuli, such as DNA damage, strong mitogenic signals, overexpression of oncogenes, epigenomic disruption, telomere dysfunction, and ROS engage in cell signaling cascades that cause activation of 1 or both of the pathways that regulate cell senescence, the p53-p21 and p16-pRB pathways. Activation of p53 induces the expression of a CDK inhibitor, p21. Senescence stimuli, which involve the p16-pRB pathway upregulate the expression of another CDK inhibitor, p16. Both p21 and p16 suppress the phosphorylation and inactivation of pRB, and hereby maintain its hypophosphorylated and active state. Active pRB halts cell cycle progression by inhibiting gene transcription via downregulating transcription factor E2F. Senescent cells remain metabolically active, despite their terminal growth arrest, and secrete proinflammatory cytokines, chemokines, growth factors, and proteases, collectively termed the senescence-associated secretory phenotype.

CDK, cyclin-dependent kinase; pRB, retinoblastoma tumor suppressor protein; Rb, retinoblastoma; ROS, reactive oxygen species. Sultana. Placenta senescence and complications of pregnancy. Am J Obstet Gynecol 2017.

(see Panel 1 for cell cycle). Silencing of E2F target genes is mediated by pRB-dependent reorganization of chromatin into distinct heterochromatin structures that accumulate in the nucleus of senescent cells termed senescence-associated heterochromatin foci (SAHF).<sup>9</sup> Interestingly, a senescent cell can reenter the cell cycle following inhibition of p53 if the cell senescence occurred because of activation of the p53-p21 pathway; however, cells that senesce solely via the p16-pRB pathway are unable to resume proliferation, even after the inhibition of p53, pRB, or p16.<sup>11</sup> **Causes of cellular senescence.** A critically short telomere is thought to be one factor initiating cellular senescence. Telomeres are highly conserved repetitive DNA regions, consist of tandem arrays of the hexanucleotide sequence TTAGGG in the human, which is typically 10–15 kb long,<sup>12</sup> located at the end of linear chromosomes, and are essential for chromosomal stability and cell survival.<sup>13,14</sup>

Telomeres protect DNA ends from double-strand breaks, end-to-end fusion, and degradation by forming a protective cap with a guanine-rich single-stranded telomere overhang and telomere-binding protein complexes.<sup>15,16</sup> Because of an inability to replicate telomeric DNA at the ends of chromosomes (known as the endreplication problem of eukaryote DNA), telomeres are progressively shortened every time a cell divides.<sup>2</sup> When telomeres reach a critical minimum length, their protective structure is distorted, resulting in the exposure of DNA ends and a DNA damage response, which lead to the activation of the cellular senescence pathway.<sup>5,14,17,18</sup> This phenomenon is commonly known as replicative senescence. Telomere shortening is also accelerated as a consequence of environmental stressors and insults, such as hyperglycemia, hypoxia, and oxidative stress,<sup>19-22</sup> which lead to the oxidation of the guanosine residues. Telomere length is regulated by the enzyme telomerase, which is a specific reverse transcriptase capable of

#### PANEL 1 CELL CYCLE

The cell cycle or cell-proliferation cycle is a series of events that take place in a mitotic cell to produce 2 daughter cells. In eukaryotic cells, the stages of the cell cycle are divided into 2 major phases: interphase and the M phase.

- **Interphase:** During the interphase the cell grows in size and makes a copy of the cell's DNA (called DNA replication) to prepare for the cell division. The interphase is comprised of 3 stages: G1, S, and G2.
- G<sub>1</sub>. In the first gap phase, the cell increases in size, copies organelles, and makes the molecular building blocks it will need in later steps. The G<sub>1</sub> checkpoint control mechanism ensures that everything is ready for DNA synthesis.
- S phase. DNA synthesis occurs during this phase. It also duplicates a microtubule-organizing structure called the centrosome. The centrosomes help separate DNA during M phase.
- G<sub>2</sub>. The cell continues to grow in the second gap phase and synthesizes proteins and organelles. During this phase microtubules begin to reorganize to form a spindle. The G<sub>2</sub> checkpoint control mechanism ensures that everything is ready to enter the M phase and divide.

**M phase:** During the M phase, cell growth stops and cellular energy is focused on the orderly division into 2 daughter cells. At this stage the cell separates its DNA into 2 sets and divides its cytoplasm, forming 2 new cells.

 $G_1$ , gap 1;  $G_2$ , gap 2; M, mitotic; S, synthesis phase.

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adding telomeric repeats to the ends of the chromosome.<sup>23</sup>

Telomerase consists of a catalytic protein component, telomerase reverse transcriptase, and an RNA template component, telomerase RNA component. Telomerase reverse transcriptase is considered to be the rate-limiting factor in the telomerase activity.<sup>24</sup> The absence of a functional telomerase or loss of telomerase activity leads to a progressive telomere shortening during cell division, resulting in telomere-dependent replicative senescence and an inability to further divide when a critically short telomere length is reached.<sup>18,23,25</sup>

Senescence can also be induced independently of telomere length by a process termed premature senescence. Premature senescence leads to premature aging and is linked to several disease processes.<sup>26</sup> Premature senescence occurs as a consequence of progressive DNA damage and the DNA damage response, telomere uncapping, and telomere dysfunction caused by extrinsic or intrinsic stressors including oxidative stress by ROS, resulting in end-to-end fusion and aggregation of telomeric DNA.<sup>27-29</sup> ROS stimulates senescence by inducing DNA damage and by engaging p53-p21 and p16-pRB signal transduction cascades, either directly or indirectly.5 Genomic damage or epigenomic perturbation, including dysfunctional telomeres and DNA double-strand breaks, activates the DNA damage response. The resulting signal transduction pathways then lead to arrest of the cell cycle.<sup>30</sup>

Features of cellular senescence. Senescent cells are distinct from their proliferation-competent counterparts; the former display altered characteristics, morphologically, in gene and protein expression and in the activation of key signaling constituents.<sup>2</sup> Morphologically, senescence cells are enlarged, multinucleated, often double in volume, and adopt a flattened or more spindle-shaped morphology, depending on the type of senescence inducer.<sup>5</sup> Senescent cells are resistant to apoptosis or programmed cell death through the overexpression of the antiapoptotic Bcl-2

protein, leading to the accumulation of these cells within tissues.<sup>31</sup>

Senescent cells display significant changes in their secretory phenotype. Senescent cells remain metabolically active, despite their terminal growth arrest, and secrete proinflammatory cytokines, chemokines, growth factors, and proteases, collectively termed the senescence-associated secretory phenotype.<sup>32</sup> The expression of interleukin (IL)-1 $\alpha$ , IL-1 $\beta$ , IL-6, IL-8, and tumor necrosis factor- $\alpha$  have been shown to increase in senescent cells.<sup>6,33</sup> Increased expression of matrix metalloproteinases (enzymes that degrade extracellular matrix proteins such as collagen and elastin) is also common.<sup>34</sup>

The senescence-associated secretory phenotype in senescent cells can induce senescence in neighboring cells,<sup>35</sup> alter the behavior of surrounding cells and tissue homeostasis by activating various cell-surface receptors and their signal transduction pathways, and induce tumorigenesis and malignant progression of nearby premalignant cells.<sup>6,8,30,36</sup> Senescence-associated  $\beta$ -galactosidase (SA- $\beta$ -gal) activity is increased in senescent cells and has been widely used as a biomarker for cellular senescence.<sup>37</sup> The SA- $\beta$ -gal most likely derives from increased lysosomal beta-galactosidase, associated with the increased lysosomal biogenesis that occurs in senescent cells.<sup>38</sup> Despite its apparent specificity for senescent cells, SA- $\beta$ -gal is not required for senescence.<sup>38</sup>

Perturbation of mitochondrial homeostasis is also an important characteristic feature of cellular senescence. Aging is generally linked to a progressive mitochondrial dysfunction.<sup>39</sup> Mitochondrial dysfunction is characterized by increased ROS generation; impaired mitochondrial dynamics (imbalance in fission and fusion; typically more fusion, resulting in the formation of abnormally enlarged mitochondria); depolarization of the inner membrane, which stalls the mitochondrial electron transport chain; reduced 5' adenosine triphosphate generation and increased 5' monophosphate-activated adenosine protein kinase activation; reduced adenine dinucleotide nicotinamide

oxidase/nicotinamide adenine dinucleotide hydroxide ratio and altered metabolism; and mitochondrial Ca<sup>2+</sup> accumulation.<sup>40</sup> These changes in mitochondrial function can induce the activation of p53-p21 and/or p16-pRB signaling pathways that eventually lead to cellular senescence<sup>40</sup> (Figure 2).

Increased mammalian target of rapamycin complex 1 (mTORC1) kinase activity is a common feature of senescent cells. mTORC1 is a conserved serine/ threonine kinase, belonging to the phosphoinositide 3-kinase family that induces anabolism by regulating protein translation and nucleotide and lipid biogenesis, and inhibits the catabolic process by blocking autophagy (a process that involves fusion of acid and proteolytic enzyme containing lysosomes with autophagosomes that contain damaged organelles and misfolded proteins that is central to the cell recycling system).<sup>41</sup>

Persistent mTORC1 signaling in senescent cells may result from defects in the sensing of amino acids and growth factor starvation.<sup>42</sup> In senescent cells increased mTORC1 activity promotes synthesis while inhibiting protein cellular proliferation.<sup>36,43</sup> mTORC1 activation activates intracellular signaling cascades that regulate mitochondrial function and apoptosis<sup>44</sup> while concurrently inhibiting autophagy,<sup>45</sup> which leads to the accumulation of damaged cellular contents including misfolded proteins as well as lipid droplets that can be seen by light microscopy as granular cytoplasmic inclusions surrounding the nucleus of senescent cells.46,47

Interestingly, mTORC1 inhibition by rapamycin not only delays the progression of cellular senescence but also prevents the permanent loss of proliferative capacity and allows the arrested cells to re-enter the cell cycle.<sup>36,48-50</sup> Rapamycin can also prolong the life span of various species including yeast, flies, and mice<sup>44,51,52</sup> by blocking the effects of mTORC1.<sup>36,44</sup> In addition, metformin, a popular hypoglycaemic agent, has recently been shown to extend longevity in worms<sup>53</sup> and mice,<sup>54</sup> possibly by modulating several age-related



Changes in mitochondrial function trigger cellular senescence via activation of p53-p21 and/or p16-pRB signal transduction cascades. Figure adapted from Ziegler et al.<sup>40</sup>

pRB, retinoblastoma tumor suppressor protein.

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pathways, including mitochondrial function and 5' adenosine monophosphate-activated protein kinase activity and the nutrient-sensing mTORC1 pathway (reviewed in reference 51).

**Biomarkers of senescence.** The importance of senescence in aging and several age-related pathological conditions has led to the identification of several senescence biomarkers (Table). The current methods to assess biomarkers of cell and tissue senescence have been reviewed by Bernardes et al.<sup>55</sup> Expression of  $\beta$ -galactosidase (SA- $\beta$ -gal) is known to be one of the well-characterized and simplified methods to detect senescence in vitro culture cells as well as for aged tissues in vivo. The assay that measures

SA- $\beta$ -gal activity expressed by senescent cells can be detectable at pH 6.0 by immunohistochemistry.<sup>37</sup> SA- $\beta$ -gal is expressed in senescent cells but not in other cell types and is shown to increase in an age-dependent manner in human skin samples<sup>37</sup> and therefore is a widely used and reliable marker for the detection of senescent cells in a variety of species and pathological conditions.<sup>56-61</sup>

Another important biomarker of senescence is SAHF, both in cultured cells and in vivo. In the senescent cell nucleus, the chromatin undergoes dramatic remodeling through the formation of domains of facultative heterochromatin foci, called SAHF,<sup>62</sup> which can be visualized under 4,6-diamidino-2microscopy as phenylindole-stained punctate areas. SAHF irreversibly silences and represses several E2F-target genes (eg, cyclin A)<sup>62</sup> and are triggered by p16 or p53 pathway activation.<sup>63</sup> Transcription starting sites are absent in SAHF regions which are enriched in transcriptionsilencing histone, for example, HP1, macroH2A, H3Lys9me3 (trimethylation of lysine 9 in histone 3).<sup>63</sup> Other protein complexes that have shown to be accumulated at SAHF include chromatin regulators HIRA, Asf1, and HMGA, which are considered as valuable biomarkers of senescence.55

The senescence-associated secretory phenotype, which is characterized by the secretion of inflammatory signals that resembles a local immune response, is a hallmark of senescent cells. The expression of inflammatory cytokines (IL-6) or chemokines (IL-8) has been extensively used as biomarkers for measuring senescence in cells and in tissue.<sup>64</sup> p16-pRB and p53-p2 are 2 major cellular pathways that are involved in induction of cellular senescence as described in the previous section. Increased levels and/or activity of p16, p53, and p21 have been shown to be associated with cell senescence and are considered as important biomarkers of cell senescence and tissue aging.<sup>59,65-</sup> Other cellular senescence markers include telomere shortening and dvsfunction<sup>69,70</sup> and an activated and persistent DNA-damage response.<sup>55</sup>

# Cellular senescence and placental aging in pathological pregnancies

**Physiological and pathological placental senescence and aging.** The placental syncytiotrophoblast is a multinucleated, single layer of terminally differentiated cells covering the chorionic villi. The layer is replenished by the fusion of cytotrophoblasts with the overlying layer of syncytiotrophoblast, resulting in a huge syncytium with multiple nuclei.

Mature (term) placental syncytiotrophoblast displays molecular markers of cellular senescence, for example, SA- $\beta$ -gal and an increased expression of the CDK inhibitors p16 and p21 and tumor suppressor p53.<sup>75</sup> Heterochromatin foci can be seen within the nuclei resulting from reorganisation of chromatin structures.<sup>47</sup> Evidence of oxidative damage and aging in the syncytiotrophoblast increases as gestation advances<sup>76</sup> and is associated with mammalian target of rapamycin complex activation and telomere shortening.

Fusion of cytotrophoblasts with the syncytiotrophoblast is a physiological process by which differentiated cytotrophoblast cells are incorporated into the syncytiotrophoblast that starts at approximately 12 weeks and continues until term.<sup>77</sup> This process is essential to achieve the rapid and extensive expansion of the placental villi, contributing to the overall growth of the placenta and constant damage repair of the chorionic villi, which is accomplished through further fusion with underlying cytotrophoblasts.

This process requires an endogenous human defective retroviral element encoding *ERVWE1*, also known as syncytin-1 (syncytin-A in mice), which is expressed in all trophoblast cell lineages. Expression of *ERVWE1* causes cell fusion a process that induces cellular senescence in normal and cancer cells.<sup>75</sup> In syncytin-A knockout mouse embryos, failure of cytotrophoblast cell fusion results in intrauterine growth restriction (IUGR) and fetal demise in midgestation.<sup>78</sup> In humans, a reduced expression of syncytin-1 has been observed in placentas associated with IUGR and preeclampsia.<sup>79</sup>

Trophoblast senescence is a physiological phenomenon and is expected

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Biomarkers of senescence			
Biomarkers	Trend	References	
SA- $\beta$ -gal	+	37, 56–61	
SAHF			
H3Lyn9me3, H1, macroH2A	+	71-73	
HMGA, HP1	+	72, 74	
HIRA, Asf1	+	71	
SASP			
IL-6, IL-8	+	64	
Senescence inducers			
p16	+	59, 65, 66	
p53/p21	+	65, 67, 68	
Telomere length and DDR	_	55, 69	
DDR. DNA damage response: //, interleukin: S	A- $\beta$ - $aal$ , senescence-associated $\beta$ -galactic	osidase: SAHF. senescence-	

DDR, DNA damage response; IL, interleukin; SA- $\beta$ -gal, senescence-associated  $\beta$ -galactosidase; SAHF, senescence-heterochromatin foci; SASP, senescence-associated secretory phenotype.

Sultana. Placenta senescence and complications of pregnancy. Am J Obstet Gynecol 2017.

to progress gradually as pregnancy advances to term, that is with placental aging. However, premature or accelerated senescence and aging can occur as a result of placental stress that can lead to placental and clinical pathology. Premature or accelerated senescence happens when the placenta encounters stressors including, oxidative, mitochondrial, or endoplasmic reticulum stress, which therefore contribute to the pathophysiology of pregnancy complications, such as preeclampsia and fetal growth restriction (FGR).

Low levels of stress can induce adaptive responses, including upregulation of antioxidant capacities and cell turnover by autophagy, moderate levels may interfere with stem cell behavior and reduce cell proliferation, while elevated levels of stress can cause the release of proinflammatory cytokines and antiangiogenic factors, and may contribute to the pathophysiology of preeclampsia, while chronic stress may accelerate senescence of the trophoblast.<sup>80</sup> The consequences of accelerated senescence in the cytotrophoblast/syncytiotrophoblast are potentially compromised placental

nutrient transport that can cause compromised fetal growth, with or without preeclampsia.

Likewise, maternal decidual cells and fetoplacental membranes display features of senescence as pregnancy approaches term.<sup>47,81</sup> A progressive natural physiological senescence and aging of decidual cells and placental membranes may be important for modulating the cell signaling pathways that are required for the onset of labor at term. Increased expression of cellular senescence signals, including p53, p21, senescence-associated secretory phenotype (IL-6 and IL-8), and SA- $\beta$ -gal from both the maternal decidua and fetal membranes has been found to be associated with labor at term,<sup>81,82</sup> which may contribute to human parturition.

Early secretion of the senescenceassociated inflammatory signals (such as IL-1 $\beta$ , IL-6, and IL-8) caused by senescence of the chorioamniotic membranes triggered by pathological processes may promote premature membrane rupture and spontaneous preterm labor.<sup>47,83</sup> It is likely that placental aging determines pregnancy duration and parturition,<sup>84</sup> and premature aging may lead to early onset of labour.

Placental senescence in small for gestational-age fetuses and neonates. FGR, also called small for gestational age is defined as an estimated fetal weight below the 10th percentile for gestational age<sup>85</sup> and affects more than 15% of worldwide.86 pregnancies Poor placentation and placental dysfunction are known to predispose to FGR. Placental dysfunction caused by the failure of trophoblast invasion and maternal spiral artery transformation, caused by ROS-mediated oxidative stress<sup>87-89</sup> has been reported in FGR.<sup>90</sup> ROS-induced oxidative damage affects membrane lipids, proteins, and nucleic acids (both DNA and RNA).<sup>89</sup>

In genomic and mitochondrial DNA, 8-hydroxy-2'-deoxyguanosine (8-OHdG; an oxidized derivative of deoxyguanosine) is one of the predominant forms of ROS-induced oxidative DNA lesions and has therefore been widely used as a biomarker for oxidative DNA damage. The level of 8-OHdG is reported to be significantly higher in placentas associated with FGR.<sup>91-93</sup>

Increased trophoblast senescence has been observed in FGR. FGR placentas display senescence markers, including short telomeres, telomere aggregation or dysfunction, and a reduction of telomerase activity.<sup>24,28,94-99</sup> Specifically a strong association between reduction of placental trophoblast telomere length and FGR pregnancies has been reported.<sup>24,94,98-100</sup> An absent or a decrease in telomerase activity is also observed in the placentas from FGR pregnancies.<sup>24,94-96</sup> FGR placentas display upregulation of the senescence markers p21 and p16, tumor suppressor protein p53, IL-6, and a reduced expression of antiapoptotic protein Bcl-2.<sup>27,94</sup>

There is also an elevated level of SAHF in the FGR placenta.<sup>100</sup> The presence of oxidized DNA as 8-OHdG is increased in placental trophoblast complicated by FGR.<sup>27</sup> Overall, there is a strong association between reduction of telomere length in placental trophoblast and DNA damage and FGR, suggesting that senescence in trophoblast cells may contribute to the etiology of FGR.

**Preeclampsia and placental senescence.** Preeclampsia is a hypertensive disorder of pregnant women and often occurs in association with FGR. Preeclampsia is the leading cause of maternal and neonatal death and preterm birth, affecting 5–7% pregnancies worldwide.<sup>101-103</sup>

Preeclampsia is characterized by newonset maternal hypertension (blood pressure  $\geq$ 140/90 mm Hg), diminished uteroplacental blood flow, proteinuria ( $\geq$ 300 mg per 24 hours), and edema.<sup>103</sup> An injury to the vascular endothelium is the basic pathological event in preeclampsia,<sup>89,104</sup> caused by placental oxidative and endoplasmic reticulum stress,<sup>80,105</sup> which are known to trigger cellular senescence and may therefore contribute to the clinical features of this pregnancy complication.

Increased placental or trophoblast senescence has been demonstrated in preeclampsia in terms of senescence biomarkers, including short telomeres, telomere aggregation and dysfunction and reduced telomerase activity, increased senescence-associated secretory phenotype, and increased expression of tumor suppressor p53 and CDK inhibitors p16 and p21.

In preeclamptic placentas the formation of telomere (or nuclear) aggregate (SAHF) is increased compared with placentas from normotensive women<sup>24,28,106</sup> The expression of senescence inducers p53, p21, and p16 are higher in pregnancy complicated by preeclampsia.<sup>27,107-109</sup> Moreover, a high level of proinflammatory cytokine (IL- $1\beta$  and IL-6) profile can be demonstrated in preeclampsia.27,110 DNA oxidation as measured by the expression of 8-OHdG in preeclamptic placenta is higher than in the healthy placentas.<sup>27</sup>

**Placental senescence in spontaneous preterm labor/birth.** Preterm birth is the leading cause of neonatal death and the second leading cause of infant mortality.<sup>111</sup> Spontaneous preterm birth may occur after the spontaneous onset of labor with or without preterm premature rupture of the membrane

(pPROM). Both term and preterm labor occur through activation of a common pathway characterized by increased myometrial contractility, cervical ripening (dilatation) and decidua/chorioamniotic membrane activation, and chorioamniotic membranes rupture<sup>112,113</sup> and is likened to an inflammatory activation, particularly of cytokines and chemokines, in the gestational membranes.<sup>114</sup>

In term delivery, physiological signals activate the pathway to labor, while in preterm labor several pathological processes or conditions induce labor by activating 1 or more of the components of this pathway.<sup>112</sup> Labor promotes alterations of gene expression in placental membranes, which are compatible with the localized acute inflammatory response, without evidence of histologically observable inflammation.<sup>115</sup>

Labor is also associated with the expression of senescence-associated signals in the placental chorioamniotic membranes, for example, telomere length reduction, and increased expression of p53, p21, senescence-associated secretory phenotype (IL-6 and IL-8), and SA- $\beta$ -gal, mediated through the activation of the p38 MAPK pathway.<sup>82,116</sup>

Senescent cells may transmit inflammatory (cytokines and chemokines, the senescence-associated secretory phenotype) and senescence-promoting signals, which may cause changes in gene expression patterns in chorioamniotic membranes (overexpression of IL-8, IL-6, toll-like receptor 2 and superoxide dismutase) and in amniotic fluid (IL-1 $\alpha$ , IL-1 $\beta$ , IL-6, IL-8) that stimulate labor.<sup>115</sup> Increased levels of antiinflammatory cytokines and chemokines, for example, tumor necrosis factor- $\alpha$ , IL-1 $\beta$ , IL-6, and IL-8, have been found in cervicovaginal secretions in women who deliver preterm, that are associated with early-onset labor,<sup>83</sup> and early initiation of these inflammatory signals is likely to promote premature labor.

Chorioamniotic membranes from spontaneous preterm labor without acute histological chorioamnionitis (inflammation of the fetal membranes) show signs of cellular senescence, for examples, increased levels of *CDKN1A* (the gene that encodes p21) and SA- $\beta$ -gal, and downregulated CDK and cyclins (CCNA2, CCNB1, and CCNE1) compared with preterm not-in-labor membranes.<sup>117</sup>

Telomeric DNA fragments released from senescent fetal cells into the amniotic fluid may induce amniotic cell senescence via the p38 MAPK activation and stimulate sterile inflammatory signals that promote parturition.<sup>118</sup> Although there is a strong association between inflammatory activation and labor (both term and preterm), whether theses inflammatory signals result in the induction of labor remains unclear.

Premature senescence of the intrauterine tissues, especially the fetal membranes, triggered by senescence stimuli such as oxidative DNA damage by ROS, may contribute to spontaneous or pPROM,<sup>119-121</sup> preterm labor possibly via inflammatory signals (the senescence-associated secretory phenotype). Increased expression of the biomarkers of the senescence phenotype, for example, p53, p21 and p38 MAPK were observed in the fetal membranes in preterm births with pPROM compared with spontaneous preterm and term deliveries.121

The senescence phenotype could be induced in vitro in term fetal membranes by exposure to cigarette smoke extract. Because smoke causes oxidative stress, these data suggest that ROS-mediated damage to the fetal membranes may result in premature senescence in fetal membranes in pPROM.<sup>121</sup>

Significantly shorter telomeres are also found in fetal membranes in pPROM compared with spontaneous preterm births with intact membranes, indicating that premature senescence and aging of the placental membranes may lead to pPROM.<sup>122</sup> Studies using a mouse model suggest that in normal mouse pregnancy, progressive uterine decidual and fetal membrane senescence occur as term approaches,<sup>81</sup> while uterine p53-deficient transgenic mice show premature and accelerated decidual senescence, with increased levels of p21, IL-8, and other cytokines, and this is associated with spontaneous preterm birth.  $^{123\text{-}125}$ 

Interestingly, an additional deletion of the p21 gene can prevent spontaneous preterm birth, indicating that p21-dependent senescence in the decidua causes preterm birth in the mouse.<sup>124</sup>

Placental senescence and aging in late gestation and fetal death. There is evidence of oxidative damage and aging in late gestational tissues.<sup>76</sup> It has been hypothesized that in late pregnancy, fetal needs for nutrients and oxygen rises, if the demands exceed the placenta's ability to transfer, the placenta experiences stress that stimulates ROS generation and oxidative stress, and the resulting oxidative damage leads to aging in the placental tissue.<sup>126,127</sup> The risk of fetal death increases exponentially late in pregnancy, especially after 41 weeks of gestation,<sup>128,129</sup> suggesting that placental aging plays a key role in the clinical features of this complication.

A recent study by Mait et al<sup>76</sup> reported that placentas from unexplained intrauterine fetal death display evidence of oxidative damage and aging. Increased expression of 8-OHdG (a marker of DNA oxidation) and 4-hydroxynonenal (a marker of lipid peroxidation) have been observed in fetal death—associated placentas,<sup>76</sup> compared with term placentas; expression of both these markers has also been described to increase in aging tissues,<sup>130</sup> such as the brain in Alzheimer's disease.<sup>131,132</sup>

Also, a dysregulated lysosomal distribution and an increased autophagosome size with failure of autophagosome-lysosome fusion have also been noted in placentas associated with fetal death, suggesting an overall inhibition of autophagy. Placentas from late-term pregnancies show similar changes in oxidation of DNA and lipid, lysosomal distribution, and larger autophagosomes compared with placentas from women delivered at term.<sup>76</sup>

Increased expression of aldehyde oxidase 1 (an enzyme that is known to be involved in ROS generation<sup>133</sup>) is observed in placentas from both fetal death and late-term pregnancies. In vitro placental explants deprived of growth factors show similar changes in oxidation of lipid, lysosomal distribution, and autophagosome size, which can be blocked by inhibitors of aldehyde oxidase 1, suggesting that this enzymes plays a key role in placental aging.<sup>76</sup>

Ferrari et al<sup>134</sup> demonstrated that unexplained fetal death—associated placentas exhibit shortened telomeres. The authors observed an overall 2-fold reduction of telomere length in placentas from fetal death (both early and late term) with or without growth restriction compared with term live-birth placentas. They also reported that the telomere length in fetal death placentas is comparable with those of pPROM, while telomeres are shorter in fetal death compared with spontaneous preterm birth.<sup>134</sup>

Taken together, reduced telomere length, increased DNA and lipid oxidation, and inhibition of autophagy, changes that are consistent with cellular senescence and aging, indicate that placental senescence and aging is an etiological factor in fetal death.

#### **Concluding remarks**

Senescence has both beneficial and detrimental effects on gestational tissue, depending on the cell type and timing of onset. While physiological senescence in placental trophoblasts appears to be necessary for the formation of the syncytium, and growth and function of the placenta, it is likely that placental cell senescence plays a key role in pathogenesis of a number of adverse pregnancy outcomes, including FGR, preeclampsia, spontaneous preterm birth, and intrauterine fetal death. The senescence-associated secretory phenotype, especially matrix metalloproteinase that is released by the syncytiotrophoblast in early gestational tissue, may be necessary for trophoblast penetration during the lacunar stage of very early placentation.47

There is also a link between placental senescence and the onset of labor. Spontaneous preterm labor and pPROM may be promoted by premature and

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accelerated senescence of placental membranes and decidua that can be induced by several endogenous and exogenous factors, such as ROS. The physiological programming of senescence may be essential in determining the timing of labor onset.

In FGR the increased expression of biomarkers of DNA damage, reduction of telomere length and telomerase activity, upregulation of senescence inducing p53 and p16, and elevated levels of senescence-associated secretory phenotype and SAHF support the concept that placental senescence and aging contribute to FGR.

There is also evidence of placental oxidative DNA damage, and premature senescence in late gestational tissues. Therefore, it would appear that aging is a key factor that may affect function in the short but important life span of the placenta.

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#### Glossary of terms

**Telomeres:** highly conserved repetitive DNA regions and consist of tandem arrays of the hexanucleotide sequence, TTAGGG, in the human. Telomeres protect DNA ends from breaks, end-to-end fusion, and degradation by forming a protective cap with a guanine-rich single-stranded telomere overhang.

**Telomerase:** a reverse transcriptase enzyme, which regulates telomere length by adding telomeric repeats to the ends of chromosomes.

**Replicative senescence:** dependent on telomere length and occurs as a result of progressive telomere shortening during mitotic cell division. DNA polymerases are unable to replicate DNA at the ends of chromosomes (known as the end-replication problem of eukaryote DNA) leaving  $\sim$  50–200 bp of unreplicated telomeric DNA in each round of DNA replication. When telomeres reach a critical minimum length, their protective structure is distorted (leads to dysfunctional telomeres), resulting in the exposure of DNA ends and a DNA damage response, which leads to the activation of the cellular senescence pathway.

**Premature senescence:** is independent of telomere length and occurs as a consequence of DNA damage and the DNA damage response caused by stress such as elevated reactive oxygen species, activation of oncogenes, telomere dysfunction, and cell-cell fusion.

**RAS:** renin-angiotensin system, a mutant renin-angiotensin system-p21 protein, renin-angiotensin system involves transmitting signals and activating signaling cascades, including mitogen-activated protein kinase and phosphoinositide 3-kinase/mammalian target of rapamycin complex pathways

**Chromatin:** Chromatin is a mass of genetic material composed of DNA and proteins, primarily histones, which condense to form chromosomes during eukaryotic cell division. Chromatin compresses the DNA into a compact unit that will be less voluminous and can fit within the nucleus. Histones help to organize DNA into structures called nucleosomes by providing a base on which the DNA can be wrapped around. Posttranslational modification to histone proteins, which includes methylation, phosphorylation, and acetylation, can cause disruption in chromatin structure.

**Heterochromatin:** a chromatin variety in which DNA, which codes inactive genes (turned off), is more condensed and associated with structural proteins. Heterochromatin protects chromosome integrity and gene regulation, while DNA, which codes genes that are actively transcribed (turned on), is more loosely packaged and associated with RNA polymerases, referred to as euchromatin.

**Cyclin-dependent kinase:** a family of multifunctional enzymes that can phosphorylate various protein substrates involved in cell cycle progression.

Cyclin-dependent kinase inhibitors, p16 and p21: proteins that inhibit cyclin-dependent kinase and are involved in cell cycle arrest at the G1 phase.

**p53:** a tumor suppressor gene.

**pRB:** retinoblastoma protein is a tumor suppressor, which plays a pivotal role in the negative control of the cell cycle and in tumor progression. The retinoblastoma protein represses gene transcription by directly binding to the transactivation domain of *E2F* genes and by binding to the promoter of these genes as a complex with *E2F*.

*E2F*: a group of genes that code transcription factors, such as *E2F1* and *E2F2*, in higher eukaryotes. The *E2F* family plays a crucial role in the control of cell cycle and action of tumor suppressor proteins. E2F proteins can mediate both cell proliferation and p53-dependent/independent apoptosis. The retinoblastoma protein binds to the E2 transcription factor 1 that preventing it from interacting with the cell's transcription machinery.

Antiapoptotic Bcl-2: a regulator protein that regulates cell death via apoptosis by inhibiting apoptosis (antiapoptotic).

*ERVWE1*: *ERVW-1 gene* (endogenous retrovirus group W envelope member 1) is a human defective retroviral fusogen found in humans and other primates that encodes the protein syncytin-1. Syncytin-1 is a cell-cell fusion protein, highly expressed in normal placental tissue whose function is most well characterized in placental development.

**p38 MAPK:** a member of mitogen-activated protein kinase, which mediates a wide variety of cellular behaviors in response to extracellular stimuli.

**mTORC1:** a conserved serine/threonine kinase that induces anabolism by regulating protein translation, nucleotide, and lipid biogenesis and inhibits the catabolic process by blocking autophagy.

**AMPK:** 5' adenosine monophosphate-activated protein kinase plays a key role as a master regulator of cellular energy homeostasis. The kinase is activated in response to stresses that deplete cellular 5' adenosine triphosphate supplies such as low glucose, hypoxia. Cellular stresses that inhibit 5' adenosine triphosphate production or increase its consumption change the 5' adenosine monophosphate/5' adenosine triphosphate ratio and activate the pathway. 5' Adenosine monophosphate-activated protein kinase activation positively regulates signaling pathways that replenish cellular 5' adenosine triphosphate supplies, including fatty acid oxidation and autophagy.

# **Expert Review**

**Reactive oxygen species:** oxygen-free radicals that contain 1 or more unpaired electrons, produced as byproducts of mitochondrial respiration and metabolism, and are capable of activating and modulating various signaling pathways, including those involved in cell growth, differentiation, and metabolism. Examples include peroxides, superoxide, hydroxyl radical, and singlet oxygen.

**SA-\beta-gal:** senescence-associated beta-galactosidase is a hydrolase enzyme that catalyzes the hydrolysis of  $\beta$ -galactosides into mono-saccharides only in senescent cells. Therefore, expression of SA- $\beta$ -gal is considered to be a biomarker of cellular senescence.

**8-OHdG:** 8-hydroxy-2'-deoxyguanosine is an oxidized derivative of deoxyguanosine. In genomic and mitochondrial DNA, 8-hydroxy-2'-deoxyguanosine is one of the major product of free radical-induced oxidative lesions and has therefore been widely used as a biomarker for DNA damage and oxidative stress.

**Mitochondrial fusion and fission:** Mitochondria are dynamic organelles that constantly fuse (fusion) and divide (fission) and are termed mitochondrial dynamics. Mitochondria fusion and fission are important for mitochondrial inheritance and for the maintenance of mitochondrial functions. Fusion helps mitigate stress by mixing the contents of partially damaged mitochondria as a form of complementation. Fission is needed to create new mitochondria, but it also contributes to quality control by enabling the removal of damaged mitochondria and can facilitate apoptosis during high levels of cellular stress.

#### OBSTETRICS

# **Evidence that fetal death is associated with placental aging**

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**BACKGROUND:** The risk of unexplained fetal death or stillbirth increases late in pregnancy, suggesting that placental aging is an etiological factor. Aging is associated with oxidative damage to DNA, RNA, and lipids. We hypothesized that placentas at >41 completed weeks of gestation (late-term) would show changes consistent with aging that would also be present in placentas associated with stillbirths.

**OBJECTIVE:** We sought to determine whether placentas from late-term pregnancies and unexplained stillbirth show oxidative damage and other biochemical signs of aging. We also aimed to develop an in vitro term placental explant culture model to test the aging pathways.

**STUDY DESIGN:** We collected placentas from women at 37-39 weeks' gestation (early-term and term), late-term, and with unexplained stillbirth. We used immunohistochemistry to compare the 3 groups for: DNA/RNA oxidation (8-hydroxy-deoxyguanosine), lysosomal distribution (lysosome-associated membrane protein 2), lipid oxidation (4-hydroxynonenal), and autophagosome size (microtubule-associated proteins 1A/1B light chain 3B, LC3B). The expression of aldehyde oxidase 1 was measured by real-time polymerase chain reaction. Using a placental explant culture model, we tested the hypothesis that aldehyde oxidase 1 mediates oxidative damage to lipids in the placenta.

**RESULTS:** Placentas from late-term pregnancies show increased aldehyde oxidase 1 expression, oxidation of DNA/RNA and lipid, perinuclear location of lysosomes, and larger autophagosomes compared to placentas from women delivered at 37-39 weeks. Stillbirth-associated placentas showed similar changes in oxidation of DNA/RNA and lipid, lysosomal location, and autophagosome size to placentas from late-term. Placental explants from term deliveries cultured in serum-free medium also showed evidence of oxidation of lipid, perinuclear lysosomes, and larger autophagosomes, changes that were blocked by the G-protein-coupled estrogen receptor 1 agonist G1, while the oxidation of lipid was blocked by the aldehyde oxidase 1 inhibitor raloxifene.

**CONCLUSION:** Our data are consistent with a role for aldehyde oxidase 1 and G-protein-coupled estrogen receptor 1 in mediating aging of the placenta that may contribute to stillbirth. The placenta is a tractable model of aging in human tissue.

**Key words:** aging, aldehyde oxidase 1, autophagosome, DNA/RNA oxidation, fetal death, G-protein-coupled estrogen receptor 1, lipid oxidation, placenta, placental explant culture, raloxifene, stillbirth

#### Introduction

Unexplained fetal death is a common complication of pregnancy occurring in approximately 1 in 200 pregnancies in developed countries<sup>1</sup> and more frequently in the developing world. While no cause has been established, the rate of fetal death rises rapidly as gestation progresses >38 weeks.<sup>2</sup> Johnson et al<sup>3</sup> proposed the operational definition of aging as an increase in risk of mortality with time, which is consistent with a role for aging in the etiology of stillbirth (Figure 1).<sup>4</sup> Supporting this view, a histopathological study of placentas associated with cases of unexplained intrauterine death at

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#### **EDITORS' CHOICE**

term revealed that 91% showed thickening of the maternal spiral artery walls, 54% contained placental infarcts, 10% had calcified areas, and 13% demonstrated vascular occlusion<sup>5</sup>; another study reported increased atherosclerosis,<sup>6</sup> changes associated with aging in other organs. Supporting a link between placental aging and stillbirth, Ferrari et al<sup>7</sup> recently reported that telomere length is reduced in placentas associated with stillbirth. Fetal growth restriction is also associated with both stillbirth and telomere shortening.<sup>8</sup> We therefore sought to determine whether placentas from women who delivered >41 completed weeks (late-term) or had stillbirth had biochemical evidence of aging. As markers of aging we chose to measure 8-hydroxydeoxyguanosine (8OHdG) (a marker of DNA oxidation) and 4-hydroxynonenal (4HNE) (a marker of lipid oxidation) as both have been described to increase in the brain with aging, and the enzyme aldehyde oxidase (AOX), which is known to generate oxidative damage in the kidney. Aging is also known to affect the effectiveness of the intracellular recycling process that involves fusion of acidic hydrolase containing lysosomes with autophagosomes; we therefore sought changes in these intracellular organelles in the late-term placentas and those associated with stillbirth.

#### Materials and Methods Ethics, collection, and processing of tissues

This study was approved by the human research ethics committee of the Hunter New England Health Services and the University of Newcastle, Australia. Human placentas were collected after written informed consent was obtained from the patients by midwives. Placentas were collected from women at 37-39 weeks' gestation undergoing cesarean delivery for previous cesarean delivery or normal vaginal delivery, women at  $\geq$ 41 weeks'



Kaplan-Meier plot of number of continuing pregnancies as function of gestational age and plot of unexplained stillbirth per 1000 continuing pregnancies; data from Omigbodun and Adewuyi<sup>10</sup> and Sutan et al.<sup>2</sup> Plot shows increase in risk of stillbirth with time consistent with operational definition of aging proposed by Johnson et al<sup>3</sup> and relatively small number of pregnancies at risk of stillbirth by 41 weeks because of prior delivery. Reproduced with permission.<sup>4</sup>

gestation undergoing cesarean delivery or normal vaginal delivery, and women who had stillborn infants undergoing vaginal delivery. Placentas were collected immediately after delivery and processed without further delay. Villous tissues were sampled from multiple sites and prepared for histology and RNA extraction. For each placenta, tissues were obtained from at least 5 different regions of the placenta and 4-5 mm beneath the chorionic plate. Samples from each individual placenta were immediately frozen under liquid nitrogen and stored at  $-80^{\circ}$ C until subsequent experiments. For histology experiments, tissues were fixed in 2% formaldehyde for 24 hours, stored in 50% ethanol at room temperature, and embedded in paraffin. To create a placental roll a 2-cm strip of chorioamniotic membrane was cut from the periphery of the placenta keeping a small amount of placenta attached

to the membrane. The strip was rolled around forceps leaving residual placenta at the center of the cylindrical roll. The cylindrical roll was then cut perpendicular to the cylindrical axis to obtain 4-mm thick sections and fixed in formalin. Placentas from patients with infection, diabetes, preeclampsia, placenta previa, intrauterine growth restriction, or abruption were excluded.

#### **Reagents and antibodies**

Antibodies against lysosomeassociated membrane protein 2 (LAMP2) and AOX1 were obtained BD Biosciences from (Sydney, Australia) and Proteintech (Rosemont, IL), respectively. Antibody against LC3B and G-protein-coupled estrogen receptor 1 (GPER1) were obtained from Novus Biologicals (Littleton, CO). Antibodies against 8OHdG and 4HNE were purchased from Abcam (Melbourne, Australia). Dulbecco modified Eagle medium antibiotic-antimycotic, (DMEM), NuPAGE (Thermo Fisher Scientific, Melbourne, Australia) precast 12-well protein gel, and prolong gold antifade mounting media with 4',6diamidino-2-phenylindole (DAPI), Alexa conjugated secondary antibodies were obtained from Thermo Fisher Scientific Australia Pty. The horseradish peroxide conjugated secondary antibodies were purchased from Cell Signaling Technology (Danvers, MA). Fetal bovine serum (FBS) was obtained from Bovogen Biologicals Pty Ltd (Melbourne, Australia). Protease inhibitor and phosphatase inhibitor were supplied by Roche (Sydney, Australia). Raloxifene was purchased from Sigma-Aldrich (Sydney, Australia) and G1 was supplied by Tocris-Bioscience (Bristol, United Kingdom). The BCA protein assay kit was obtained from Thermo Fisher Scientific Australia Pty. All other chemicals were purchased from either Ajax Finechem Ptv Ltd (Sydney, Australia) or Sigma-Aldrich.

#### **Placental explant culture**

For in vitro experiments, human term placentas (all at 39 weeks of gestation) were obtained from women with normal singleton pregnancies without any symptoms of labor after an elective (a scheduled repeat) cesarean delivery. Placentas were collected immediately after delivery and prepared for explant culture. Villous tissues of placentas were randomly sampled from different regions of the placenta 4-5 mm beneath the chorionic plate. Tissues were washed several times with Dulbecco phosphate-buffered saline under sterile conditions to remove excess blood. Villous explants of  $\sim 2 \text{ mm}^3$ were dissected and placed into 100mm culture dishes (30 pieces/dish) containing 25 mL of DMEM supplemented with 2 mmol/L L-glutamine, 1% sodium-pyruvate, and 1% penicillin/streptomycin  $(100 \times)$  solution with the addition of 10% (vol/vol) FBS and cultured in a cell culture chamber at 37°C temperature under 95% air (20% oxygen) and 5% carbon dioxide



Confocal microscopy showed increased 8-hydroxy-deoxyguanosine (80hdG) staining (red) in nuclei from **B**, late-term and **C**, stillbirth placentas compared to **A**, 37- to 39-week placentas. 4',6-Diamidino-2-phenylindole (DAPI) (blue) staining identifies nuclei. **D**, Late-term and stillbirth placentas have increased intensity of nuclear 80HdG staining (P < .0001 for late-term placentas and P = .0005 for stillbirth placentas, Mann-Whitney test) compared to 37- to 39-week placentas. **D**, Open and filled circles represent 37- to 39-week cesarean nonlaboring (n = 10) and vaginal delivery laboring (n = 8) placentas, respectively; open and filled squares represent late-term laboring cesarean (n = 5) and laboring vaginal delivery (n = 13) placentas, respectively; and filled triangles represent third-trimester laboring vaginal delivery unexplained stillbirth placentas (n = 4). Each point in graph represents average intensity of 80HdG of 60 nuclei in 6 images per placenta photographed at ×100 magnification and 1.4 optical resolution. Scale bar = 20  $\mu$ m. Microscopy also indicates increased staining in cytosol of late-term and stillbirth placentas representing oxidized RNA (8-hydroxyguanosine) that is also detected by antibody.

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for 24 hours. At day 2, villous explants were transferred to fresh 30-mL growth medium and incubated in a cell culture chamber for 90 minutes and washed in DMEM without FBS (referred to as "serum-free medium" or "growth factor deficient medium"). Next 6-7 pieces of villous tissue weighing approximately 400 mg were transferred to a culture dish (60 mm) containing 6 mL of serum-free medium with or without the addition of pharmacological agents, for example, raloxifene (1 nmol/L) or the

GPER1 agonist G1 (1 nmol/L), for subsequent incubation for 24 hours. At the end of 24 hours some tissues were fixed in 2% formaldehyde, subjected to routine histological processing, and embedded in paraffin wax, and some tissues were immediately frozen in liquid nitrogen and stored at  $-80^{\circ}$ C until subsequent experiments. For each placental explant culture, samples were also collected at time 0 hours, ie, before incubation in serum-free medium, and were formalin fixed and stored frozen at  $-80^{\circ}$ C until further experiments.

#### Western blotting

Western blotting was performed as previously described.9 Samples of placenta (1 g) were crushed under liquid nitrogen. Aliquots of 100 mg of placental tissues were homogenized in 1 mL of lysis buffer (phosphatebuffered saline, 1% Triton-X-100, 0.1% Brij-35, 1 X protease inhibitor, 1 X phosphatase inhibitor, pH 7.4). The protein concentration of each placental extract was measured using a BCA protein assay kit (Thermo Fisher Scientific) and 40  $\mu$ g of placental extract was separated by electrophoresis in NuPAGE bis-tris precast 12-well gels for 50 minutes at a constant 200 V. Separated proteins were then transferred to nitrocellulose membrane using a Novex (Thermo Fisher Scientific) transfer system for 70 minutes and blocked overnight at 4°C with 1% bovine serum albumin (BSA) in trisbuffered saline with 0.1% tween-20 (TBST). The membranes were then incubated with primary antibody in 1% BSA in TBST for 2 hours at room temperature, then washed 3 times with TBST, followed by incubating with horseradish peroxide conjugated secondary antibodies in 1% BSA in TBST for an hour. After 3 further washes with TBST, the immunoreactive bands were developed in Luminata reagent (Merck Millipore, Billerica, MA) and detected using an Intelligent Dark Box LAS-3000 Imager (Fuji Photo Film, Tokyo, Japan).

#### Immunohistochemistry

Fluorescent immunohistochemistry (IHC) was performed according to previously published methods.<sup>9</sup> We deparaffinized and hydrated  $6-\mu$ m paraffin placental sections, then heated them with tris-EDTA buffer (pH 9) in a microwave oven for antigen retrieval. The sections were blocked with 1% BSA in TBST for an hour at room temperature. The sections were incubated with primary antibodies overnight and washed 3 times with TBST, before incubation with Alexaconjugated secondary antibodies for

#### FIGURE 3

Changes in lysosomal distribution in late-term and stillbirth placentas



Immunohistochemistry of lysosome-associated membrane protein 2 (LAMP2) (red); lysosomal marker showed that lysosomes predominantly localize to apical surface of **A**, 37- to 39-week placentas, whereas lysosome distribution extends to perinuclear and basal surface of syncytiotrophoblast in **B**, late-term and **C**, stillbirth placentas. Intensity calculation across syncytiotrophoblast showed that distribution of LAMP2 in **F**, late-term (n = 5) and **G**, unexplained stillbirth (n = 4) placentas shifts to perinuclear and basal surface whereas lysosome distribution in **D**, 37- to 39-week cesarean (n = 5) and **E**, vaginal delivery (n = 5) placentas remained in apical region of syncytiotrophoblast. 4',6-Diamidino-2-phenylindole (DAPI) (blue) staining identifies nuclei. **D** to **G**, Each colored line represents results on individual placenta, and shows mean intensity of LAMP2 across syncytiotrophoblast at 5 random sites per image (**A** to **C**, example represented by light green line) for 6 separate images per placenta. Images were photographed at ×100 magnification; scale bar = 20  $\mu$ m.

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90 minutes. The sections were mounted with prolong gold antifade mounting media with DAPI. The fluorescent photographs for Figures 2 to 7 and Supplementary Figures 1 to 3 were taken on a Nikon eclipse 90i confocal microscope (Nikon Instruments Inc, Melville, NY). The fluorescent photographs for Figure 8 were taken on Nikon eclipse Ti fluorescence microscope (Nikon Instruments Inc).

# RNA isolation and real-time polymerase chain reaction

Placental tissues were crushed under liquid nitrogen. Approximately 100 mg of

crushed placental tissues were homogenized in 2 mL of Trizol reagent (Thermo Fisher Scientific) with an Ultra Turrax (IKA Works, Staufen im Breisgau, Germany) homogenizer. Total RNA was extracted from the Trizol-extract by Direct-zol RNA MiniPrep (Zymo Research, Irvine, CA). The RNA was treated with DNAse and purified with a RNA Clean and Concentrator-5 kit (Zymo Research). The RNA quality was checked by running the DNAse-treated sample in agarose gel with ethidium bromide in 1X Tris-acetate-EDTA buffer. The purified RNA was used to make complementary DNA using a SuperScript III first-strand

synthesis system kit (Thermo Fisher Scientific), The complementary DNA was used to run real-time polymerase chain reaction (PCR) by TaqMan primers for (assay ID: Hs00154079\_m1; AOX1 Thermo Fisher Scientific) and TaqMan gene expression master mix (Thermo Fisher Scientific) with an internal control of 18s ribosomal RNA Thermo Fisher quantify Scientific to messenger RNA (mRNA) for AOX1. We used a SYBR (Thermo Fisher Scientific) green master mix to quantify mRNA for GPER1 (forward primer 5'-CGTCCTGTG CACCTTCATGT-3' backward primer 5'-AGCTCATCCAGGTGAGGAAGAA-3')



4-Hydroxynonenal (4HNE) (red) immunostaining in **A**, 37- to 39-week, **B**, late-term, and **C**, stillbirth placentas. 4',6-Diamidino-2-phenylindole (DAPI) (blue) staining identifies nuclei. **D**, Intensity of 4HNE is significantly increased in late-term (P < .0001, Mann-Whitney test) and stillbirth (P = .0014, Mann-Whitney test) placentas. **D**, Open and filled circles represent 37-to 39-week cesarean nonlaboring (n = 20) and vaginal delivery laboring (n = 14) placentas, respectively; open and filled squares represent late-term laboring cesarean (n = 10) and vaginal delivery (n = 18) placentas, respectively; and filled triangles represent third-trimester laboring vaginal delivery unexplained stillbirth placentas (n = 4). **D**, Each point represents mean intensity per unit area for 6 images taken for each individual placenta. Images were photographed at ×100 magnification; scale bar = 20  $\mu$ m.

(Invitrogen, Waltham, MA) with respect to beta-actin as an internal control using a 7500 PCR system (Applied Biosystems, Foster City, CA).

#### **Statistical analysis**

Sample numbers are shown in the legends to individual figures. The data in Figures 2, 4 to 6, and 8 were analyzed using the Mann-Whitney test (2-way) and results are presented as scatter plots showing the median. The data in Figure 7 and Supplementary Figures 2 and 3 were analyzed using the Wilcoxon matched-pairs signed rank test and results are presented as mean showing SEM. All the *P* values were calculated using GraphPad Prism

software (Version 7; Graph Pad Software Inc, San Diego, CA). A P value of  $\leq .05$  was considered statistically significant.

## Results

#### Subject characteristics

Demographic and clinical characteristics of the study participants are reported in the Table.

# Relationship between stillbirth risk and length of gestation

To illustrate the relationship between stillbirth risk and length of gestation we created a Kaplan-Meier plot of the data on human gestational length in a population with relatively low levels of medical intervention from Omigbodun and Adewuyi<sup>10</sup> and combined it with the data on risk of stillbirth per 1000 continuing pregnancies from Sutan et  $al^2$  (Figure 1). The data illustrate that stillbirth is consistent with an aging etiology as defined by Johnson et al.<sup>3</sup>

#### **DNA/RNA oxidation**

We sought evidence of placental DNA/ RNA oxidation as measured by 8OHdG, as a marker of DNA/RNA oxidation that was previously observed in aging tissues<sup>11</sup> such as the brain in Alzheimer disease.<sup>12</sup> IHC was performed for 8OHdG and the average intensity of 8OHdG staining in nuclei/frame demonstrated a significant increase in DNA/RNA oxidation in late-term and stillbirthassociated placentas (Figure 2).

#### Movement and clustering of lysosomes in late-term and stillbirth placentas

Misfolded proteins and damaged mitochondria are normally recycled in autophagosomes in a process that involves autophagosome fusion with proteolytic enzyme-containing lvsosomes. Accumulation of abnormal protein is thought to play a role in aging, particularly in the brain, for instance the accumulation of tau and amyloid protein in Alzheimer disease<sup>13,14</sup> and mutant huntingtin in Huntington disease.<sup>15</sup> In Huntington disease, the distribution of the lysosomes within neurones is altered with increased perinuclear accumulation of lysosomes.<sup>16</sup> We used a lysosomal marker, LAMP2, to analyze the distribution of lysosomes in the placenta by IHC. IHC showed lysosomes positioned on the apical surface of early-term placental syncytiotrophoblast (Figure 3, A, D, and E), whereas lysosomes relocated to the perinuclear and the basal surface in lateterm and stillbirth placentas (Figure 3, B, C, F, and G).

#### Lipid oxidation in placental tissue

The increase in DNA oxidation that we demonstrated suggested free radical damage that might also lead to lipid peroxidation. Lipid peroxidation



Immunofluorescence staining of LC3B (green) in **A**, 37- to 39-week, **B**, late-term, and **C**, unexplained stillbirth placentas. 4',6-Diamidino-2-phenylindole (DAPI) (blue) staining indicates nuclei. Autophagosome size was quantified using NIS element (Nikon) software and diameter was measured at arbitrary intensity range of 1000-3000, diameter range 0.2-1  $\mu$ m, and circularity range 0.5-1. **D**, Analysis showed that late-term and stillbirth placentas have significantly larger (P = .012 and P = .0019, respectively, Mann-Whitney test) autophagosomes than 37- to 39-week placentas. **D**, Open and filled circles represent 37- to 39-week cesarean nonlaboring (n = 11) and vaginal delivery laboring (n = 10) placentas, respectively; open and filled squares represent late-term laboring cesarean (n = 8) and laboring vaginal delivery (n = 15) placentas, respectively; and filled triangles represent unexplained stillbirth placentas (n = 4). Each point in graph represents average diameter of LC3B particles in 6 images taken for each placenta. Original magnification, ×100; scale bar = 20  $\mu$ m. Arrowheads indicate autophagosomes (LC3B positive particles).

has been observed to increase in Alzheimer disease as measured by the formation of 4HNE.<sup>17</sup> We therefore performed IHC for 4HNE in lateterm, stillbirth, and 37- to 39-week placental tissue. This revealed a marked, statistically significant increase in 4HNE staining in late-term syncytiotrophoblast that we also observed in placentas associated with stillbirth shown in Figure 4.

#### Larger autophagosomes containing 4HNE occur in late-term and stillbirth-associated placentas

Inhibition of autophagosome function with failure of fusion with lysosomes leads to an increase in autophagosome size.<sup>18,19</sup> This process leads to inhibition of overall autophagic function that is seen in Alzheimer disease,<sup>18</sup> disease,19 Danon and neurodegeneration.<sup>20</sup> We detected autophagosomes using IHC with an antibody against LC3B. We observed a significant increase in the size of autophagosomes (Figure 5, D) in both late-term (Figure 5, B) and stillbirth-associated (Figure 5, C) placentas compared to 37- to 39week placentas (Figure 5, A). Duallabeled fluorescence immunostaining showed that the larger autophagosomes of late-term and stillbirth placentas contained 4HNE, a product of

# Role of AOX1 in placental oxidative damage

AOX1 is a molybdoflavoenzyme, which oxidizes a range of aldehydes including 4HNE into corresponding acids and peroxides.<sup>21</sup> We provide evidence that AOX1 is involved in the generation of the increased 4HNE observed in late-term and stillbirthassociated placentas using colocalization. Dual-labeled fluorescence IHC showed that AOX1 colocalizes to 4HNE-positive particles in late-term (Figure 6, A to C) and stillbirth (Figure 6, D to F) placentas. Additionally, real-time quantitative PCR showed that late-term and stillbirth placentas expressed significantly higher mRNA for AOX1 than 37- to 39-week placentas (Figure 6, G). These data support the concept that AOX1 plays a role in the oxidative damage that occurs in the late-term and stillbirth-associated placentas.

# Pharmacological inhibition of AOX1 using placental explant culture

Our data provide clear evidence for increased lipid oxidation, disordered lysosome-autophagosome interactions, and increased AOX1 expression in the late-term and stillbirth placental syncytiotrophoblast. To determine if these events were causally linked we developed a placental explant culture system using term placental tissue cultured in serum-free (growth factor-deficient) medium. IHC showed that serum deprivation significantly increased production of 4HNE at 24 hours after incubation (Figure 7, A to C, F, and G). We also found a significant increase in the size of autophagosomes (Supplementary Figure 2) and a change in lysosomal distribution to a perinuclear location >24-hour incubation in serum-free medium (Supplementary Figure 3). We sought to determine cause-and-effect relationships between the development of lipid oxidation observed when placental explants were cultured in the



Representative dual-labeled fluorescence immunostaining in **A** to **C**, late-term and **D** to **F**, stillbirth placentas showed that AOX1-positive particles (green) are colocalized with 4HNE (red). **C** and **F**, Orange dots (arrowheads) indicate colocalization. Nuclei are stained with 4',6-diamidino-2-phenylindole (blue). **G**, Real-time polymerase chain reaction showed that expression of AOX1 mRNA is increased in late-term (P = .0097) and stillbirth (P = .012) placentas compared to early-term placentas. Original magnification ×100; scale bar = 20  $\mu$ m.

absence of serum, and AOX1. To achieve this we used a potent AOX1 inhibitor, raloxifene<sup>22</sup> and a GPER1 agonist, G1. We used the GPER1 agonist G1 as we had detected GPER1 expression on the apical surface of syncytiotrophoblast (Figure 8, A and B) and the GPER1 agonist has been shown to inhibit production of 4HNE in the kidney.<sup>23</sup> Both raloxifene and G1 inhibited the production of 4HNE in the serum-starved placental explants >24 hours of treatment (Figure 7, D to G). G1 also prevented the changes in lysosomal distribution within the syncytiotrophoblast (Supplementary Figure 3).

#### Presence of the cell surface estrogen receptor GPER1 on the apical surface of the syncytiotrophoblast

As the GPER1 agonist had evident effects in placental explant cultures we undertook characterization of GPER1 expression in placental tissue. The expression of GPER1 in a section of placenta roll (described in the "Materials and Methods" section) detected by fluorescent IHC showed that GPER1 is expressed in placental villi (Figure 8, A), which, at higher magnification  $(\times 100)$ , was localized to the apical surface of placental villi (Figure 8, B). Real-time PCR for GPER1 showed that placental villi have significantly higher expression of GPER1 than amnion, chorion, or decidua (Figure 8, C). Western blot for GPER1 also confirmed higher protein levels of GPER1 in placental villous tissue than amnion, chorion, or decidua (Figure 8, D). The demonstration of GPER1 localization on the apical surface of the syncytiotrophoblast indicates the plausibility of estrogen inhibition of AOX1 activity in the placenta.

#### Comment

Our data indicate that between 37-39 and 41 weeks of gestation dramatic changes occur in the biochemistry and physiology of the placenta. In particular there is increased oxidative damage to DNA/RNA and lipid, a change in position of lysosomes that accumulate at the perinuclear and basal surface of the syncytiotrophoblast, the formation of larger autophagosomes associated with oxidized lipid, and increased expression of the enzyme AOX1. The same changes are observed in placentas associated with stillbirth. Some of our results are semiquantitative as this is the nature of Western analysis; nevertheless, the robustness of our results is supported by the use of multiple end points for aging, and the biological plausibility of the reported links. Further supporting our hypothesis, similar changes in oxidation of lipid, localization of lysosomes, and size of autophagosomes occurred in placental explants deprived of growth factors, and these changes were blocked by inhibition of AOX1.

Stillbirth occurs in approximately 1 in 200 pregnancies in developed countries.<sup>1</sup> The *Lancet*<sup>1</sup> and the *BMJ*<sup>24</sup>



Fluorescence immunostaining with antibody against 4HNE (red) in **A**, serum-starved placental explant at time 0 (just before starvation); **B**, 24 hours after culturing in medium containing fetal bovine serum (FBS) (control treatment); **C**, 24 hours after starvation (culturing in medium without FBS); **D**, 24 hours after treatment with aldehyde oxidase 1 inhibitor, raloxifene (RLX); and **E**, 24 hours after treatment with membrane estrogen receptor G-protein-coupled estrogen receptor 1 agonist, G1. Intensity calculation showed that production of 4HNE (induced by serum starvation) is significantly reduced after treating placental explants with **F**, RLX and **G**, G1. Data are mean  $\pm$  SEM, \**P* < .05 (N = 6). Original magnification, ×20; scale bar = 100  $\mu$ m. 4',6-Diamidino-2-phenylindole (DAPI) (blue) staining indicates nuclei.

recently highlighted gaps in our knowledge of this condition. Stillbirth frequently occurs in the setting of fetal growth restriction and in this setting telomere shortening and oxidative damage have been observed in associated placentas.<sup>25</sup> The risk of stillbirth per 1000 continuing pregnancies rises dramatically >38 weeks of gestation. We have suggested<sup>4</sup> that stillbirths in late gestation are a consequence of placental aging. More than 90% of pregnancies have delivered by the end of the 40th week of gestation,<sup>10</sup> consequently changes that occur in the placenta in pregnancies that have gone past the usual term have little effect on population-level infant survival, since most have already delivered. Such late-gestation changes may exist in a kind of Medawar<sup>26</sup> shadow that allows deleterious

genes to persist in the population if their damaging effects occur after reproduction, especially if the same genes exert positive actions earlier in pregnancy. This Medawar<sup>26</sup> shadow effect has been proposed to explain the high prevalence of Huntington disease associated with increased fertility in early life but disastrous neurological deterioration after reproduction has occurred.<sup>27</sup> Our immunofluorescence data show high levels of 8OHdG and 4HNE in lateterm and stillbirth placentas supporting this postulated pathway to placental aging. Increases in oxidative damages to DNA and lipid have also been reported in Alzheimer disease.17,28

We have also seen marked accumulation of particles positive for the lysosomal marker LAMP2 in the perinuclear and basal side of the syncytiotrophoblast of late-term placentas and placentas associated with stillbirth. This phenomenon closely resembles lysosomal positioning that occurs in cells under nutritional stress.<sup>29</sup> Autophagy is an important cellular recycling process that involves fusion of acidic lysosomes with the autophagosomes. Our data show that stillbirth and late-term placentas contain larger autophagosomes than 37- to 39-week placentas indicating inhibition of the autophagic process in these placentas. Our data further indicate that the autophagosomes are coated with oxidized lipid in the form of 4HNE, which may play a role in the failure of lysosomalautophagosome fusion. Such disturbances in the function of



Fluorescence IHC showed that GPER1 (green) is localized predominantly in placenta in section of term placental roll photographed at **A**,  $\times 10$  magnification. **B**, GPER1 (green) was shown to localize in apical layer of syncytiotrophoblast of placental villi when photographed at  $\times 100$  magnification. **A** and **B**, Scale bar = 100 and 20  $\mu$ m, respectively. **C**, Real-time quantitative PCR data showed that messenger RNA (mRNA) for GPER1 is expressed in higher amounts in term placenta, whereas amnion, chorion, and decidua show very low expression of GPER1. Expression of mRNA for GPER1 follows order: decidua<chorion<amnion<placenta. **D**, Western blot of protein extract from breast cancer cell line Michigan Cancer Foundation-7 (MCF-7), term placenta, myometrium, amnion, chorion, and decidua. **D**, Placenta, myometrium, and MCF-7 cell lines expressed higher amounts of GPER1 than amnion, chorion, or decidua. Western-blotting data showed that all tissues expressed glycosylated GPER1 (\*\* or \*\*\*) and nonglycosylated nascent GPER1 (\*). **E**, SYPRO Ruby stain (Thermo Fisher Scientific) of same polyvinylidene fluoride membrane is used as internal loading control.

DAPI, 4',6-diamidino-2-phenylindole; MW, molecular weight.

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autophagosomes may lead to the accumulation of abnormal protein and deterioration in the function of the syncytiotrophoblast.

Stillbirth is not restricted to the late-term setting and is known to be associated with cigarette smoking and growth restriction. It seems likely that smoking accelerates aging-related pathways. Evidence for this is the finding that telomere length is reduced in the fetuses of women who actively smoke during pregnancy,<sup>30</sup> and similar changes are to be expected in the placentas of smokers. Down syndrome is associated with advanced aging or progeria<sup>31,32</sup> and with increased rates of stillbirth,<sup>33,34</sup>

raising the possibility that accelerated placental aging may play a part in stillbirth related to Down and some other congenital anomalies. Similarly placental abruption is associated with growth restriction, maternal smoking, and stillbirth, and placental aging may play a part in this condition.<sup>35,36</sup>

We used cultured term placental explants to interrogate the pathways leading to the lipid oxidation and disturbed autophagosome function. We measured production of 4HNE and the diameter of autophagosomes following serum depletion. We observed a significant increase in 4HNE and a significant increase in autophagosome size suggesting inhibition of autophagy by oxidative damage as we previously observed in the stillbirth and late-term placentas. Raloxifene, a potent inhibitor of AOX1, has been shown to reduce oxidative damage in endothelial cells.<sup>37</sup> We demonstrated that the AOX1 inhibitor raloxifene is also able to block oxidative damage to the lipid in placental explants. The role of AOX1 was confirmed using the GPER1 agonist G1 that has been shown to block AOX1 activation and reduce 4HNE in renal tissue.<sup>23</sup> G1 also blocked changes in lysosomal positioning within the explants. We report the novel finding of the presence of the cell surface estrogen receptor GPER1 on the syncytiotrophoblast apical membrane, suggesting that this receptor may play a role in modulating oxidative damage within the placenta. It has been shown that urine from pregnant women carrying a fetus with postmaturity syndrome has lower estrogen:creatinine ratios than that from women carrying normal fetuses.<sup>38</sup> These data support the possibility that low estrogen concentrations may lead to loss of the cell surface estrogen receptor (GPER1) mediated inhibition of AOX1 and consequently placental oxidative damage and impaired function.

The changes in the late-term placenta occur as the fetus continues to grow and to require additional supplies of nutrients. Postmaturity

CC Characteristic	37-39 wk	Late-term	Stillbirth	ı		
Cases, n	34	28	4			
Gestational ages, wk	$\textbf{38.57} \pm \textbf{0.15}$	$41.46\pm0.06$	32	32.57	39	40.14
Fetal growth restriction, n	0	0	No	Yes	No	Yes
Maternal age, y	$31.03 \pm 0.88$	$\textbf{28.81} \pm \textbf{1.15}$	30.21	± 2.68		
Vaginal birth	41.20%	64.30%	100.00%	6		
BMI, kg/m <sup>2</sup> , at second trimester or at birth	$\textbf{29.10} \pm \textbf{1.50}$	$\textbf{28.52} \pm \textbf{1.10}$	27.40	± 2.40		
Ethnicity						
Caucasian	82.35%	96.42%	75.00%	6		
Smoker	17.64%	17.85%	0.00%	6		

syndrome is a condition seen in postdates infants who show evidence of late gestation failure of nutrition.<sup>39</sup> Normal human infants born at term have 12-14% body fat whereas postmaturity syndrome is associated with the birth of a baby with severe wrinkling of the skin due to loss of subcutaneous fat. Postmaturity syndrome is rarely seen in modern obstetric practice where delivery is usually effected <42 weeks of gestation using induction of labor or cesarean delivery if labor has not occurred spontaneously. While none of the infants born to mothers in our study exhibited postmaturity syndrome, our data suggest that the physiological function of the placenta >41 completed weeks is showing evidence of decline that has many features in common with aging in other tissues. The known exponential increase in unexplained intrauterine death that occurs >38 weeks of gestation may therefore be a consequence of aging of the placenta and decreasing ability to adequately supply the increasing needs of the growing fetus. This knowledge may impact on obstetric practice to ensure infants are born before the placenta ages to the point of critical failure.<sup>40</sup> However, it is notable that not all placentas in our late-term cohort

exhibited evidence of aging and it is known that infants born later in gestation have lower rates of special school needs, with those born at 41 weeks having the lowest rates.<sup>41</sup> The conflicting pressures of late gestation increases in stillbirth and falling rates of intellectual disability make obstetric care at this time very challenging; diagnostics that can predict pregnancies at increased risk of stillbirth would be useful and some progress in their development has been made.<sup>42</sup> Our data also indicate that the placenta may provide a tractable model of aging in a human tissue that uniquely ages in a 9-month period of time. The results suggest that the rate of aging of the placenta varies in different pregnancies and raises the possibility that the rate of aging of the placenta may parallel the rate of aging of the associated fetus carrying the same genome. Our work identifies potential therapeutic targets such as AOX1 that may arrest the oxidative damage to placentas in pregnancies identified at high risk of stillbirth when extreme prematurity precludes delivery. Finally, our data raise the possibility that markers of placental oxidative damage and AOX1 mRNA may be released into maternal blood where they may have diagnostic value in predicting the fetus at risk for stillbirth.

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K.M. and R.S. hold patents through the University of Newcastle on aldehyde oxidase 1 as a therapeutic target and the use of placental aging-related markers as diagnostics to predict stillbirth.

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#### **SUPPLEMENTARY FIGURE S1** Oxidized lipids within autophagosomes of late-term placentas



Representative dual-labeled fluorescence immunostaining showed that **A**, LC3B, autophagosome marker (green), is colocalized with **B**, 4-hydroxynonenal (4HNE), marker of lipid peroxidation (red). **C**, Orange dots (arrowheads) indicate colocalization. 4',6-Diamidino-2-phenylindole (blue) staining indicates nuclei. Original magnifications  $\times 100$ ; scale bar = 20  $\mu$ m.

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Serum Starvation time (h)

Fluorescence immunostaining with antibody against LC3B (green) in **A**, serum-starved placental explant at time 0 (just before starvation) and **B**, 24 hours after starvation. 4',6-Diamidino-2-phenylindole (DAPI) (blue) staining indicates nuclei. **C**, Immunohistochemical analysis showed that size of autophagosomes (LC3B-positive particles) increased 24 hours after serum starvation compared to 0 hours. Data presented as mean  $\pm$  SEM, \*\*\**P* = .0002 (N = 13). Scale bar = 20  $\mu$ m. *Maiti et al. Fetal death and placental aging. Am J Obstet Gynecol 2017.* 



Fluorescence immunostaining with antibody against lysosome-associated membrane protein 2 (LAMP2) (red) in **A**, serum-starved placental explant at time 0 (just before starvation); **B**, 24 hours after culturing in medium containing fetal bovine serum (FBS); **C**, 24 hours after starvation (culturing in medium without) FBS; and **D**, 24 hours after treatment with GPER agonist, G1. 4',6-Diamidino-2-phenylindole (DAPI) (blue) staining indicates nuclei. **E**, Intensity calculation across syncytiotrophoblast showed that distribution of LAMP2 at 24 hours after starvation shifts to perinuclear and basal surface compared to control treatment (N = 7). **E**, Each colored line represents mean intensity of LAMP2 across syncytiotrophoblast at 5 random sites per image for 6 separate images per experiment. **E**, Each colored bar indicates mean of area under curve (AUC) of corresponding colored line and statistical differences were calculated. Original magnifications, ×40; scale bar = 20  $\mu$ m; error bar, SEM; \**P* < .05 (N = 7).

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antibody (Labvision), which then examined by 2 pathologists. The expression of VEGF is given score; negative (0), weak (1), positive (2) and strong (3).

**Result**: A total of 191 subjects were recruited, in which 99% (n = 189) had spontaneous vaginal deliveries. The common histological findings are increased syncytial knots (19.4%) and reactive changes (3.33%). The mean cotinine level is significantly higher in SHS group ( $16.35 \pm 12.84$ ). The VEGF expression scores are lower in this group ( $1.06 \pm 0.52$ ). There is significant negative correlation of the cotinine level with the VEGF expression scores; An increament of every 1 ng/ml cotinine level significant; ly reduce VEGF score 0.77 points (p < 001).

**Conclusion**: Cotinine as a byproduct of nicotine are significantly correlated with lower placenta VEGF expression in pregnant SHS.

#### P2.37. THE MANAGEMENT OF PLACENTAL ABRUPTION

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**Background**: In Japan, placental abruption is the most common causes of neonatal cerebral palsy and maternal critical state such as Disseminated intravascular coagulation (DIC). The aim of this study is to explore the prognostic factors of placental abruption in Japan.

**Study design**: The approval of ethical committee of Nagoya University Hospital was obtained. We send questionnaire to all obstetric institutions including clinic, tertiary hospital in Aichi prefecture in Japan and collected clinical information about cases with placental abruption to deliver a baby in 2012 (n = 65356). The numbers of annual births in Aichi accounts for about 7 % in Japan. The questionnaire recovery rate was 98.6%. The neonates were divided into two groups; good or poor prognosis group. A poor prognosis group was defined as cases having any of the following characteristics: stillbirth, < 3 at 5 min of Apgar score, < 7.0 of umbilical artery pH, and  $\geq -12$ mmol/L of umbilical artery BE. We conducted univariate analysis to compare good and poor groups. Multivariate analysis was performed to find risk factors of neonatal poor prognosis.

**Results**: 132 cases of placental abruption was enrolled. The incidence was 0.21% (132/63219). The peak of onset was 36 weeks of gestation. Independent risk of neonatal poor prognosis with placental abruption was lower abdominal pain (OR 3.1, 95%CI 1.2-7.8), decrease of fetal movement (OR 5.8, 95%CI 1.5-22.2), retroplacental hematoma (OR 5.9, 95%CI 1.3-25.9), and maternal transfusion (OR 7.8, 95%CI 3.1-20.1).

**Conclusions:** The maternal symptoms of lower abdominal pain and decrease of fetal movement might predict poor prognosis of neonates. In addition, poor prognosis of neonates were often associated with maternal risk of blood transfusion. Those information would be important for the management of placental abruption.

#### P2.38.

#### THE HUMAN PLACENTA AT 41 WEEKS OF GESTATION SHOWS EVIDENCE OF AGING WITH SHORTENED TELOMERES, DNA OXIDATION AND CHANGES IN IGFR2, AUTOPHAGY AND MTOR

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Risks of unexplained intrauterine death rise exponentially late in gestation. Previous histopathological studies of placentas from patients with intrauterine death have demonstrated vessel thickening, calcification and infarction; changes associated with aging in other tissues. We hypothesised that placentas from pregnancies beyond 39 weeks of gestation would show biochemical changes consistent with aging. In this study, we have compared markers of aging in term and post-dated placentas.

We collected placentas from women at 38 weeks of gestation (term) and placentas collected at 41 weeks of gestation (post-dated). We compared the two groups for: telomere length measured by real-time PCR, oxidative DNA (8-hydroxy guanosine), LIPD damage (4-HNE), autophagosome

formation (LC3) assessed by immunohistochemistry and mTOR and autophagic pathways using immunohistochemistry and western blots. Telomeres from placentas at 41 weeks were shorter (P = 0.05) and immunohistochemistry revealed significantly increased 8-hydroxy guanosine (0.008) and 4-HNE (0.0025) compared to those at 38 weeks of gestation. Phos-AKT (S473) was reduced in post-dated placentas (p = 0.04) and correlated with phos-mTOR s2481 an mTORC2 marker ( $R^2 = 0.4212$ , 0.002); phos-p70S6 kinase (T389), a substrate for mTORC1 rose (p=0.0008), and phos-p70S6K and AKT were strongly negatively correlated  $(R^2 = -0.4018, p = 0.003)$ . Western analysis showed that LAMP2, a lysosomal marker, rose in post-dated placentas (p=0.005) and immunohistochemistry demonstrated a change in the distribution of LAMP2 to the basal side of the syncytiotrophoblast in post-dated placentas. Additionally IGF2R, a major component of the lysosomal pathway, increased (p=0.02) in post-dated placentas. Expression of IGF2R was negatively correlated with both phos-AKT ( $R^2 = -0.32$ , p<0.01) and phos-mTOR (s2481) ( $R^2 = -0.38$ , p < 0.01). Immunohistochemistry for LC3 demonstrated a significantly higher number of autophagosomes in post-dated placentas (p < 0.02). Fluorescent double staining with LC3 and 4-HNE showed oxidation of lipids in autophagosomes. Our data are consistent with the hypothesis that placental ageing is associated with the late gestation increase in fetal death.

P2.39.

# USE OF AN *IN VITRO* HUMAN PLACENTAL EXPLANT CULTURE MODEL TO INTERROGATE THE EFFECTS OF STARVATION ON PLACENTAL FUNCTION.

Zakia Sultana, Kaushik Maiti, Roger Smith. Mother and Babies Research Centre, HMRI, University of Newcastle, NSW, Australia

The placenta transports essential nutrients, hormones, small molecules and gases from maternal blood to the fetus. Throughout the pregnancy syncytiotrophoblasts experience varying exposure to nutrients in maternal plasma and growth factors and are known to express the nutrient sensor mTOR. mTOR is known to respond to growth factors. mTOR is also known to regulate pathways that affect aging and oxidative stress. We performed in vitro placental villi culture to investigate changes in mTOR activation, autophagosome formation and lipid oxidation in response to nutrient and growth factor deprivation.

Placenta villi from normal pregnancies were cultured in serum free medium with or without the addition of rapamycin for different incubation time periods. Protein extract and tissue samples were analysed by western blotting and immunohistochemistry.

Phosphorylation of the mTORC1 substrate p70S6 kinase was inhibited significantly 2 h after starvation (p = 0.0002), but restored by 4 h (P = 0.0082) and thereafter. We also observed that during starvation the lysosomes move from the apical side to the basal side of the syncytio-trophoblast membrane. Moreover, there was a significant increase in the number of autophagosomes and an increased expression of 4HNE, a lipid oxidation inducer, in the autophagosome vacuoles during starvation. However, addition of rapamycin at 2 h after incubation in serum free medium successfully blocked mTORC1 reactivation and inhibited lipid oxidation within autophagosomes.

Our data shows that serum starvation caused changes in mTORC1 activation, increases in lipid peroxidation and autophagosome formation. Serum starvation also causes changes in lysosomal distribution within the syncytiotrophoblast. The pharmacological inhibition of lipid oxidation by rapamycin indicates a link between mTORC1 and free radical generation during starvation and removal of growth factors.

#### P2.40.

#### RESTRICTION OF PLACENTAL GROWTH FROM CONCEPTION IN THE SHEEP RESULTS IN CHANGES IN PLACENTAL STRUCTURE AND GROWTH FACTOR EXPRESSION THAT ARE INDEPENDENT OF WHETHER THE FETUS BECOMES HYPOXAEMIC IN LATE GESTATION

Song Zhang<sup>1</sup>, Paige Barker<sup>1</sup>, Kimberley Botting<sup>1</sup>, Claire Roberts<sup>2</sup>, Christine McMillan<sup>1</sup>, Caroline McMillen<sup>1</sup>, Janna Morrison<sup>1</sup>. <sup>1</sup> University of South Australia, Adelaide, SA, Australia; <sup>2</sup> University of Adelaide, Adelaide, SA, Australia

#### Scientific Abstracts

#### F-143

Sex-Divergent Expression Patterns of Fatty Acid Regulatory Genes in Rat Placenta. Amy Loverin, Haimei Wang, Christian Wiscombe, Lisa A Joss-Moore\*. University of Utah, Salt Lake City, UT, United States.

**INTRODUCTION:** Appropriate fetal development depends upon maternal-fetal transfer of long chain polyunsaturated fatty acids (LCPUFA). The placenta provides the interface for regulated maternalfetal transfer of LCPUFA as well as local metabolism of LCPUFA. Placental LCPUFA transport and metabolism is regulated by suites of fatty acid transport proteins (FATP) and fatty acid binding proteins (FABP). Expression of FATPs and FABPs is governed by the fatty acid responsive peroxisome proliferator activated receptor gamma (PPARy). Emerging evidence suggests that placental LCPUFA transport and metabolism differs between placenta supporting male fetuses and placenta supporting female fetuses. However the effect of placental sex on expression of PPAR $\gamma$ , FATPs, and FABPs is unknown. We hypothesize that, in the rat, mRNA transcript levels of PPARy, FATPs, and FABPs will differ between placenta supporting male fetuses and placenta supporting female fetuses. METHODS: Placenta from pregnant Sprague Dawley rats were surgically collected at embryonic day 21 (term embryonic day 23). Placental lipid levels were qualitatively examined using Oil-red-O staining of frozen sections. Whole placental homogenate was used to measure mRNA transcript levels of PPARy, FATPs (FATP1, FATP2, FATP4, CD36, Lipase), and FABPs (FABP1, FABP3, FABP4) using real-time reverse transcriptase PCR. Placenta supporting male fetuses (male placenta) and placenta supporting female fetuses (female placenta) were treated as separate groups. Sample size was 6-8 per group, collected from 6-8 different litters. Statistical significant difference between means was defined as p≤0.05 by Mann Whitney U test.

**RESULTS:** Data are expressed as female as % male  $\pm$  SD, \*p<0.05. Female placenta appeared to have greater lipid storage in the basal zone than male placenta. Homogenate of female placenta had greater mRNA transcript levels of PPAR $\gamma$  (191 $\pm$ 15%\*), FATP4 (119 $\pm$ 13%\*), Lipase (172 $\pm$ 44%\*), and FABP1 (219 $\pm$ 93%\*), compared to male placenta. mRNA transcript levels of FATP1, FATP2, CD36, FABP3 and FABP4 were not significantly different between female and male placenta.

**CONCLUSION:** We conclude that, in the rat, mRNA transcript levels of PPAR $\gamma$  as well as select FATPs and FABPs differ between placenta supporting female fetuses and placenta supporting male fetuses. Given the apparent higher lipid content in female placenta, we speculate that PPAR $\gamma$  is directly regulating the increased mRNA levels. Ongoing studies are evaluating the sex-divergence of protein levels and location of FATPs and FABPs in the rat placenta.

#### F-144

Tissue Factor Pathway Inhibitor Regulation of Endovascular Trophoblast Cell Development and Uterin Spiral Artery Remodeling. <u>Masanaga Muto†</u>,<sup>2</sup> Damayanti Chakraborty†,<sup>2</sup> Regan L Scott†,<sup>2</sup> Michael J Soares.<sup>1,2</sup> <sup>1</sup>Fetal Health Research, Children's Research Institute, Children's Mercy, Kansas City, MO, United States; <sup>2</sup>Institute for Reproductive Health and Regenerative Medicine, Departments of Pathology and Pediatrics, University of Kansas Medical Center, Kansas City, KS, United States.

**INTRODUCTION:** Hemochorial placentation is characterized by the development of trophoblast cells specialized to interact with uterine and fetal vascular beds. These specialized trophoblast cells arise from a trophoblast stem (TS) cell population possessing the capacity to differentiate into multiple trophoblast cell lineages. Among the differentiated trophoblast lineages are cells that acquire an endothelial cell-like phenotype, termed "endovascular trophoblast cells". In this investigation, we examine the physiological significance of tissue factor pathway inhibitor (TFPI), an endothelial regulator of hemostasis in endovascular trophoblast development and invasion.

**METHODS:** RNA sequencing of TS cells was used to identify candidate regulatory mechanisms controlling the development of endovascular trophoblast cell lineage. Placentation site specific expression patterns of TFPI, a differentiation-dependent anti-coagulation regulatory factor, was

examined by immunocytochemical analyses. Functional evaluation of the role of TFPI at the maternal-fetal interface was determined utilizing trophoblast-specific lentiviral delivery of *Tfpi* shRNAs.

**RESULTS:** Trophoblast cell differentiation was characterized by transcript signatures consistent with acquisition of an endothelial celllike phenotype. Among the upregulated transcripts were components of the coagulation pathway, including tissue factor pathway inhibitor (Tfpi). In vitro TS cell TFPI knockdown significantly altered the TS cell phenotype. TFPI was expressed in endovascular trophoblast cells within the rat placentation site. In vivo trophoblast-specific TFPI knockdown significantly decreased placental weights at gestation day 15.5. Placentation sites of TFPI disrupted embryos exhibited a significant decrease in intrauterine trophoblast cell invasion and an increase in fibrinogen deposition associated with uterine spiral arterioles.

**CONCLUSION:** In summary, these results implicate TFPI as a regulator of endovascular trophoblast cell invasion, uterine spiral artery remodeling, and hemostasis at the maternal-fetal interface. (Supported by a Lalor Foundation Postdoctoral Fellowship; NIH HD020676, HD079363; Sosland Foundation)

#### F-145

Pyroptotic Cell Death and NLRP3 Inflammasome Function in Hofbauer Cells. <u>Seth Guller\*</u>, Zhonghua Tang, Jessica Tan, Gil Mor, Vikki M Abrahams. *Yale University SOM, New Haven, CT, United States.* INTRODUCTION: Recent studies have indicated that alterations in the number and gene expression of Hofbauer cells (HBCs) may play a role in microbial-driven/cytokine-mediated inflammation in placenta. Pyroptosis is a form of rapid cell death mediated by NLRP3 inflammasome, a multiprotein complex which drives the secretion of IL-1 $\beta$ . It has been shown that pyroptotic cell death can be triggered by bacterial lipopolysaccharide (LPS) and adenosine triphosphate (ATP). However, the potential role of pyroptosis in HBC pathophysiology remains unclear. The aim of this study was to explore the regulation of pyroptotic cell death and inflammasome function in HBCs.

**METHODS:** HBCs (n=4) were isolated from human term placentas with high yield and purity. Cells were treated with 1 ng/ml LPS for 4 h and then with 5 mM ATP for up to 2 h. Pyroptosis was examined by microscopy. Lactate dehydrogenase (LDH) assay and Capases-1 Glo assays were carried out to measure cell death, and caspase-1 enzyme activity, respectively. The synthesis and secretion of cytokine IL-1 $\beta$  was examined by Western blot and ELISA.

**RESULTS:** Pyroptotic changes including an increase in cell size, nuclear condensation, and the formation of perinuclear 'balloon-shaped' vesicles was induced approximately 3-fold in response to combined treatment with LPS and ATP, LPS and ATP treatment increased supernatant levels of LDH activity five-fold compared to HBCs treated with LPS alone. Similarly, caspase-1 activity increased ten-fold compared in cells treated with LPS and ATP compared to controls. ATP treatment alone did not enhance IL-1 $\beta$  secretion but enhanced LPS-mediated IL-1 $\beta$  scretion from 1000 pg/ml to 20,000 pg/ml. Preincubation of HBCs with NLRP3 silencing RNA (siRNA), but not scrambled RNA, suppressed the ATP-enhanced increase in IL-1 $\beta$  scretion in LPS-treated cells approximately 85%.

**CONCLUSION:** Collectively, our data indicate that LPS and ATP treatment stimulated NLRP3 inflammasome activation and pyroptosis in HBCs. Since HBCs have been demonstrated to play an important role in microbial-associated placental/fetal inflammation, this suggests that future studies targeting the HBC inflammasome may provide a unique approach to reduce inflammation at the placental-fetal interface.

#### F-146

Growth Factor Depletion in Placental Cells Increases Lipid Peroxidation and Reduces Mitochondrial Function and mTOR Activity via Aldehyde Oxidase Mediated Pathways. Zakia Sultana<sup>†</sup>, Kaushik Maiti, Roger Smith<sup>\*</sup>, Philip M Hansbro, Prema Mono Nair. University of Newcastle, Newcastle NSW, Australia.

**INTRODUCTION:** Biochemical evidence of placental aging in the form of increased oxidised lipid (4 hydroxynonenal (4HNE)) has been demonstrated in association with fetal death (Am J Obstet Gynecol

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2017;217:441). The increased production of 4HNE was replicated in serum deprived placental explants and shown to depend on aldehyde oxidase (AOX1) activity. It was unclear how serum deprivation impacted upon nutrient sensing pathways and mitochondrial function and the relationship to AOX1 activity. **Objective:** We have used the HTR8/SVneo placental cell line to test the hypothesis that serum deprivation induced changes in nutrient sensing pathways and mitochondrial function were downstream from AOX1.

**METHODS:** To induce oxidative damage we removed growth factors from HTR8/SVneo cells by culturing them in serum free medium for 24 hours. To inhibit AOX1 activity the cells were treated with raloxifene, a potent AOX1 inhibitor, or G1, a GPER1 agonist, that is also known to inhibit AOX1 activity. Lipid peroxidation was quantified using an ELISA for 4HNE. Western blotting was used to measure phosphorylation of S6K that is regulated by mTORC1 and AKT, an mTOR regulator. A Seahorse extracellular flux (XF) analyser was used to evaluate mitochondrial function including cAMP production and oxygen consumption rate.

**RESULTS:** Serum-starved HTR8/SVneo cells showed evidence of increased lipid oxidation as 4HNE (p=0.015, N=4). A significant decrease in phosphorylation of both S6K (p=0.007, N=4) and AKT (p=0.007, N=4) were observed after serum deprivation. The XF assay (N=3) showed a 2-fold decrease of both mitochondrial basal respiration (oxygen consumption rate) and ATP generation after serum-starvation. The increases in 4HNE and the decreases in S6K, AKT, mitochondrial cAMP and oxygen consumption were all blocked by inhibition of AOX1 using either raloxifene or G1.

**CONCLUSION:** Serum-depletion that removes growth factors increases lipid peroxidation while decreasing mTORC1 activity and mitochondrial energy generation, all these changes are downstream from the enzyme AOX1. AOX1 plays a central role in regulating placental cell responses to growth factor deprivation, AOX1 may play a similar role in the aging placenta phenotype that is associated with stillbirth. *\*Figure(s) will be available online.* 

#### F-147

Chronic Placental Inflammation and Sex-Specific Assocations at Term. <u>Hannah Leah Bromberg</u><sup>+</sup>,<sup>2</sup> Joan Krickellas<sup>+</sup>,<sup>2</sup> Sylvia Dygulski<sup>+</sup>,<sup>2</sup> Serena Chen<sup>+</sup>,<sup>2</sup> Michael Joyce<sup>+</sup>,<sup>2</sup> Julian Nepola<sup>+</sup>,<sup>2</sup> Beata Dygulska,<sup>1</sup> Pramod Narula,<sup>1</sup> Sanford Lederman,<sup>1</sup> Carolyn M Salafia<sup>\*</sup>.<sup>2</sup> <sup>1</sup>New York Presbyterian Brooklyn Methodist Hospital, Brooklyn, NY, United States; <sup>2</sup>Placental Analytics, LLC, New Rochelle, NY, United States.

**INTRODUCTION:** Placental pathology studies have been hampered by the fact that most placentas are discarded from births considered to be of "well" newborns with uncomplicated pregnancies. Case-control studies have suggested associations of placental pathology with pregnancy compromise, but cohort studies do not exist. From 2013-2016, a mandate for uniform placental examination yielded a unique opportunity to study placental associations with newborn pathology at a population level. We present the first 25+% of a 1000 case birth cohort, related to the questions of "what is the rate of chronic villitis in a delivery population at term?" and "does chronic villitis have sex-specific newborn and placental correlates?" METHODS: From 2013-2016, all placentas were referred for gross and histopathologic examination, the latter performed by a single observer (CMS). Gross measurements and diagnoses were performed with clinical information of gestational age only. We have selected 20 cases from each month from 2013 to August 2016, and are extracting maternal, placental and newborn data. We had previously reported that chronic plac ental inflammation was more common in male fetuses born extremely preterm (<32 weeks). Here we report our results to date on the distribution of chronic inflammatory placental lesions, their associations with infant gender and their sex-specific correlates with other placental measures and newborn status. We elected conservative non-parametric testing throughout, considering p<0.05 significant.

**RESULTS:** Chronic amnionitis in extraplacental membranes was significantly more common in male (5%) v female (0.03%) fetuses (p=0.03). Chronic inflammation in villi, chorionic plate or basal decidual plasma cells did not differ by sex. Any chronic placental inflammation was associated with longer GA in females (p=0.05), and shorter (but not

significant) in males, a significantly smaller major axis of the placenta in males (p=0.004) with a similar trend in females (p=0.095), and a significantly smaller head circumference in males only (p=0.049 v p=0.79 in females). Chronic placental villitis was significantly more common in women with parity  $\leq 1$  with female newborns (p=0.02) but not withmale newborns (p=0.50). There was no difference by gender in NICU admissions or Apgar <1 at 5 minutes.

**CONCLUSION:** In this preliminary report of a larger epidemiologic cohort, sex-specific associations are identified in relation to chronic placental villitis, a lesion with differential diagnosis of congenital viral infection or maternal placental "immune" interaction. Effects on head circumference in males may be specially germane to fetal programming of neurodevelopmental disability.

#### F-148

**Defining the Secretome of Mouse Placental Endocrine Cells.** <u>Tina Napsot</u>, Amanda N Sferruzzi-Perri\*. *University of Cambridge, Cambridge, United Kingdom.* 

**INTRODUCTION:** During pregnancy, many physiological changes occur in the mother that enable the support of fetal growth. These include changes in the maternal cardiovascular, pulmonary, immune and metabolic systems, and are signalled, in part, by changes in placental hormone production. Failures in maternal adaptation may result in pregnancy complications, including abnormal birthweight and gestational diabetes. However, a complete identification of the proteins secreted by the placenta that mediate the changes in maternal physiology is lacking. This study aimed to unbiasedly characterise the secretory output of cultured placental endocrine cells.

**METHODS:** We used the mouse placenta as the endocrine and transport functions are performed by discrete zones that can be physically separated. Whole placenta (WP) and isolated endocrine junctional zones (Jz) were obtained on day 16 of pregnancy (0.75 of term, maximal Jz size). WP and Jz trophoblast were then isolated by density-gradient centrifugation, seeded at 10<sup>4</sup>/ml cell density and cultured in 10% serum for up to 120h (n=6). Cells were cultured in serum-free medium 24h prior to any downstream experiment. Mass spectrometry was used to identify the proteins secreted from Jz and WP cultures.

**RESULTS:** In both WP and Jz cultures, ~20% of the cells were lost within the first 24h of culture, and a further 20% were lost by 48h. Cell proliferation was detected at 72h and 96h and gene expression analyses revealed this was due to fibroblast contamination and expansion. By 120h, the majority of WP and Jz cells were not viable. Protein concentration of the conditioned media increased by 66% and 44% in Jz and by 225% and 25% in WP at 24h and 48h, respectively (n=3). Mass spectrometry on the conditioned media identified there were 825 proteins released by both the Jz and WP cultures at the 48h timepoint. Pathway analysis identified these to be involved in activities such as binding and catalytic activity and included proteins like IGF2, PRLs and PRPs.

**CONCLUSION:** We were able to prepare primary cultures of mouse placental trophoblast cells. These cell cultures secreted proteins that have been previously implicated in the endocrine regulation of maternal physiology. The number of endocrine versus transport trophoblast cells in the WP and Jz cultures is currently being investigated. Experiments will be repeated on cultures prepared from placentas at other days of pregnancy to assess the ontogenic changes in the placental secretome.

#### F-149

The Interaction between LIN28B and AR in the Placenta. Erin S <u>McWhorter</u>; Rachel C West, Quinton A Winger\*, Gerrit J Bouma\*. *Colorado State University, Fort Collins, CO, United States.* 

**INTRODUCTION:** Preeclampsia (PE) and intrauterine growth restriction (IUGR) are significant causes of infant and maternal disorders, which can lead to long-term health effects in the child. In humans, impaired trophoblast differentiation and invasion into the maternal spiral arteries is thought to be an underlying cause associated with these placental disorders as studies evaluating placentas in women with preeclampsia consistently show maternal and fetal vascular abnormalities. LIN28 is an RNA-binding protein necessary for maintaining pluripotency in stems

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#### F-150

Novel Localization of Hepcidin at the Human Maternal-Fetal Interface. Elizabeth Taglauer<sup>†</sup>,<sup>1</sup> Danielle Wuebbolt,<sup>2</sup> Elizabeth Tully,<sup>3</sup> Fionnuala Breathnach,<sup>3</sup> Amir Khan,<sup>4</sup> Sarbattama Sen<sup>\*, 5</sup> <sup>1</sup>Children's Hospital Boston, Boston, MA, USA; <sup>2</sup>Royal College of Surgeons Ireland, Dublin, Ireland; <sup>3</sup>Royal College of Surgeons Ireland, Dublin, Ireland; <sup>4</sup>Trinity College Dublin, Dublin, Ireland; <sup>5</sup>Brigham and Women's Hospital, Boston, MA, USA.

**INTRODUCTION:** Hepcidin is a well-characterized peptide hormone involved in iron homeostasis. Recently, hepcidin has been implicated in alterations of iron transfer to the fetus during pregnancy. We have found that maternal serum hepcidin is upregulated by pro-inflammatory conditions such as maternal obesity, which may contribute to impaired maternal-fetal iron transport. Hepcidin also has significant antimicrobial properties, which may play a role in protection against transplacental infections. Hepcidin mRNA and protein in first trimester placentas. However, a comprehensive localization analysis of this protein within the term placenta has not been performed to date. We hypothesized that hepcidin is expressed within key functional areas the term placenta.

**METHODS:** Using immunofluourence with confocal microscopy, we visualized hepcidin expression in healthy, term placental tissues (N=6). Our analysis included villous tissue, basal plate, decidua and fetal membranes.

**RESULTS:** We identified hepcidin in the outer syncitiotrophobst layer ubiquitously within the placental villous tissue. Additionally, we found consistent hepcidin expression in the endothelium of both large and small fetal blood vessels throughout placenta from all patients in our study.

**CONCLUSIONS:** Hepcidin is consistently expressed in the term fetal placenta, specifically within areas that are in direct contact with maternal blood. Given hepcidin's key role in iron homeostasis and its significant antimicrobial properties, our findings raise the possibility that hepcidin may be actively produced by the fetal placenta for a variety of functions during pregnancy. Further analysis of placental hepcidin could identify novel targets to improve fetal nutrient transfer and prevent transplacental infections.

#### F-151

Unexplained Antepartum Stillbirth Is Associated with Biochemical Evidence of Placental Aging. <u>Kaushik Maiti</u>,<sup>1</sup> Zakia Sultana,<sup>1</sup> John Aitken,<sup>2</sup> Roger Smith<sup>\*</sup>.<sup>1</sup> <sup>1</sup> University of Newcastle, Newcastle, NSW, Australia; <sup>2</sup>University of Newcastle, Newcastle, NSW, Australia.

**INTRODUCTION:** Risks of unexplained stillbirth rise late in gestation consistent with aging. We hypothesized that placentas after 41 completed weeks of gestation would show biochemical changes consistent with ageing that would also occur in placentas from stillbirths.

**METHODS:** We collected placentas from women at 37-39 weeks gestation (term, n=35), at 41<sup>+</sup> weeks (post-dates, n=29), and associated with unexplained still-birth (n=4). We compared the first two groups for: qPCR for telomere length and expression of aldehyde oxidase (AOX1), oxidized DNA (8-hydroxy-guanosine), oxidized lipid (4-hydroxynonenal, 4HNE), autophagosome formation (LC3B), lysosomal distribution (LAMP2) and mTOR pathways using immunohistochemistry (IHC) and western blot. We compared stillbirth placentas with term placentas for oxidative damage and distribution of autophagosomes and lysosomes. We tested whether aldehyde oxidase mediated the oxidative damage to lipids and the formation of autophagosomes using placental explant cultures.

**RESULTS:** Placentas at 41 weeks had shorter telomeres(p=0.0339), higher oxidized DNA (p<0.0001) and 4HNE (p<0.0001), and larger autophagosomes (p=0.012). mRNA for AOX1, which generates 4HNE was increased (p=0.0097) andIHC demonstrated a change in the distribution of LAMP2, a lysosomal marker, to the basal side of the syncytiotrophoblast in post-dates placentas and Western blot showed increased mTORC1 activity (phos-P70S6 kinase, p=0.0033). All stillbirth placentas showed increased oxidative damage, increased AOX1, altered LAMP2 distribution and larger autophagosomes as seen in post-dates samples. Placental explant culture without serum significantly increased

AOX1, 4HNE, and autophagosomes size at 24 hours. The production of 4HNE was inhibited by the rapamycin (mTOR inhibitor), raloxifene (AOX1 inhibitor), and G1, an agonist for the G-protein estrogen receptor 1. **CONCLUSIONS:** Oxidative damage to DNA and lipids, accumulation of larger autophagosomes and altered lysosomal distribution are seen in placentas from both post-dates and unexplained antepartum stillbirth. 4HNE production in explant culture was inhibited by mTOR inhibition, AOX1 inhibition and estrogenic stimulation. These data suggest that placental aging involving oxidative damage to lipids and disruption of autophagy is mediated by AOX1, mTOR and inhibited by cell surface estrogen signaling, these are potential targets for therapeutics to reduce the risk of stillbirth.

#### F-152

High Maternal Omega-3 Fatty Acid Levels in Hawaiian Women Impair Placental Lipid Storage. <u>Fernanda L Alvarado†</u>,<sup>1</sup> Virtu Calabuig-Navarro†,<sup>1</sup> Pai-Jong S Tsai,<sup>2</sup> Perrie O'Tierney-Ginn.<sup>1</sup> <sup>1</sup> Case Western Reserve University, Cleveland, OH, USA; <sup>2</sup>University at Buffalo, Buffalo, NY, USA.

**INTRODUCTION:** Placentas of obese women have higher lipid content compared to lean women. We have previously shown that supplementation of overweight and obese women with omega-3 fatty acids, decreases placental esterification pathways and total lipid content, in a mid-western population (Ohio). We hypothesized that placental lipid esterification pathways and storage would be similar between lean and obese women living in a region of high omega-3 intake, such as Hawaii.

**METHODS:** 84 healthy, normal glucose tolerant women (pregravid BMI 16-53 kg/m<sup>2</sup>) from Honolulu Hawaii, were recruited at scheduled term cesarean delivery. Maternal plasma DHA levels were analyzed by mass spectrophotometry. Expression of key genes involved in fatty acid esterification (*PPARg, DGAT1, FAS, and ACCa*) were measured in placental tissue using qPCR. Total lipids were extracted from placental tissue via the Folch method. One-way ANOVA was used to assess differences between groups. P value <0.05 was considered statistically significant.

**RESULTS:** As expected, maternal DHA levels were, on average, higher in this cohort (383  $\mu$ mol/L) as compared to pregnant women with similar characteristics in Ohio (156  $\mu$ mol/L, n=8). Furthermore, DHA levels were higher in lean Hawaiian women compared to overweight and obese women (*P*=0.01). Placental lipid content and expression of DGAT1, FAS, PPARg and ACCa were similar (*P*>0.05) between lean, overweight and obese women.

	Lean	Overweight	Obese	P value
n	29	24	31	
Pregravid BMI (kg/m <sup>2</sup> )	21.1±2.2	27.6±1.3	37.2±7	< 0.0001
Maternal DHA (µmol/L)	453±187	360±116	336±123	0.01
Gestational Age (wks)	38±0.6	38±0.4	39±0.3	0.207
Birth Weight (g)	3295±409	3459±538	3591±448	0.05
Placental weight (g)	472±88	523±99	562±98	0.002
Total lipid content (mg/g tissue)	18±6	15±7	17±4	0.28

**CONCLUSIONS:** Though overweight and obese Hawaiian women have lower DHA levels compared to their lean counterparts, these levels remain over twice as high as Ohio women. These relatively high plasma omega 3 levels in overweight and obese Hawaiian women may suppress the ability of the placenta to esterify and store lipids to the same levels of lean women. This curbed ability to store lipids may increase fetal growth. properties and prevents hypoxia/reoxygenation induced oxidative stress in cytotrophoblasts. We hypothesized that addition of melatonin would improve mitochondrial respiration in trophoblasts isolated from placentas of obese women

**METHODS:** Villous cytotrophoblasts were isolated from placental tissue collected at term by cesarean section either from obese (prepregnancy BMI 37.1 ± 6.0, n=9) and lean (BMI 21.6 ± 2.4, n=5) women. Cytotrophoblast synctyialized over 72 hr culture with melatonin 0.1 - 100  $\mu$ M for the last 24hr. Syncytiotrophoblast mitochondrial respiration was measured by Seahorse XF24 analyzer. Oxygen consumption rates were normalized to total cellular protein. Basal respiration, ATP coupled, maximum respiration, spare capacity and non-mitochondrial respiration were measured. Expression of mitochondrial respiratory chain complexes I-V in the cultured cells, normalized to βactin, was measured by western blot. The effects of melatonin on trophoblast respiration and expression of mitochondrial complexes were analyzed with one-way ANOVA and paired t-test as appropriate

**RESULTS:** Maternal clinical characteristics were similar between the patients except for BMI and placental weight. All fetuses were appropriate for gestational age with no adverse clinical outcomes. No significant improvement in mitochondrial respiration occurred with addition of melatonin to trophoblasts of lean women. However in trophoblasts from obese women, melatonin (10  $\mu$ M and 100  $\mu$ M) significantly increased maximal respiration (p = 0.02 and p = 0.003 respectively) and spare capacity (p =0.01 and p= 0.009 respectively) compared to the untreated control. There were no differences detected in the expression of the mitochondrial respiratory chain complexes I-V in trophoblasts treated with melatonin

**CONCLUSIONS:** Mitochondrial respiration of trophoblasts from obese women was significantly improved with the addition of melatonin. Improved spare respiratory capacity, the cellular reserve that can respond to stress, could impart a protective effect to the placenta and fetus in response to additional stressors. The effect of melatonin may be via reduction of oxidative stress or increased respiratory chain activity rather than increased expression of the mitochondrial respiratory chain complexes.

#### **O-075**

Evidence That Stillbirth Is Linked to Placental Aging. Kaushik Maiti, Zakia Sultana, John Aitken, Roger Smith. University of Newcastle, Newcastle, Australia.

**INTRODUCTION:** Stillbirths rise late in gestation. We hypothesised that placentas from pregnancies beyond 40 completed weeks of gestation would show biochemical changes of aging that would also occur in placentas associated with stillbirth.

**METHODS:** We collected placentas from women at 38 weeks of gestation(term), at 41 weeks of gestation (post-dated) and associated with stillbirth. We compared the first two groups for: telomere length measured by real-time PCR, oxidised DNA (8-hydroxyguanosine), lipid oxidation (4-hydroxynonenal (4HNE)), autophagosome formation and mTOR pathways using immunohistochemistry and western blots. We mimicked the post-term changes using placental explant cultures in serum free media in the presence and absence of the mTOR inhibitor rapamycin. Changes in oxidative damage and mTOR pathways were analysed by western blotting and immunohistochemistry. Results were compared with placentas from 3 cases of stillbirth.

**RESULTS:** Telomeres from placentas at 41 weeks were shorter (P=0.033), DNA showed increased oxidation (8-hydroxyguanosine (p=0.008)), and there was increased lipid oxidation(4-HNE (p<0.0001) which co-localised within autophagosomes which were increased in size and number(p=0.02) compared to those at 38 weeks of gestation. Immuno-histochemistry demonstrated a change in the distribution of LAMP2, a lysosomal marker, to the basal side of the syncytiotrophoblast in post-dated placentas. All three placentas from stillbirths showed increased 4-HNE and altered LAMP2 distribution.

\**Figure(s)* will be available online.

Western blot data showed increased mTORC1 activity as assessed with phos-p70S6 kinase(p=0.003) in post-dated placentas. In placental explant culture without serum the same changes in lipid oxidation, autophagosomes and lysosomes as seen in post-dates placentas were seen and the effects on 4-HNE were blocked by rapamycin.

**CONCLUSIONS:** Our data support the hypothesis that ageing occurs in post-dates placentas and involves telomere shortening, DNA and lipid oxidation, and alterations in lysosome and autophagosome structure. The alterations in lipid oxidation also occur in association with stillbirth and are down stream of mTORC1 activation and can be blocked by the mTORC1 inhibitor rapamycin.

#### **O-076**

Sex Differences in Gene Expression Are Restricted to Extraembryonic Tissue in Early Post-Implantation Embryos. Kathleen E O'Neill,<sup>1</sup> Monica Mainigi,<sup>1</sup> Tami Stuart,<sup>2</sup> David Condon,<sup>3</sup> Rebecca A Simmons.<sup>2</sup> <sup>1</sup>University of Pennsylvania, Philadelphia, PA, USA; <sup>2</sup>University of Pennsylvania, Philadelphia, PA, USA; <sup>3</sup>University of Pennsylvania, Philadelphia, PA, USA.

**INTRODUCTION:** Although male and female embryos carry the same autosomal DNA, sex-specific transcription/translation occurs and results in phenotypic differences prior to the time of gonadal differentiation including rate of embryo development, survival after vitrification, cell number at the blastocyst stage and glucose metabolism. Previous studies showed sexual dimorphism in gene expression in whole embryos. We hypothesize that there will be varying degrees of sexual dimorphism in gene expression in embryonic vs. extraembryonic tissue.

**METHODS:** Mouse embryos were obtained at 6.5 dpc from naturally mated C57BL/6 mice. Epiblasts were dissected from the ectoplacental cones (EPC) and were genotyped for sex. RNA was isolated and amplified from epiblasts and EPCs from male (n=3) and female embryos (n=3) and RNA-Seq libraries were prepared. We obtained  $\geq$ 50 million reads per biologic replicate.

**RESULTS:** 782 of the 24,029 unique transcripts (identified using EdgeR) present in the EPC were differentially expressed between male and female embryos using a FDR <0.01. In contrast, we identified only seven transcripts differentially expressed between male and female epiblasts using a FDR<0.01, all of which were sex-linked. Expression of 469 of the 782 autosomal DE transcripts was higher in female vs. male EPC. Pathway analysis, using DAVID, revealed that >50 of the transcripts upregulated in female ectoplacental cones were involved in VEGF, EGF, cytokine and p53 signaling, cell adhesion, and/or angiogenesis. 248 autosomal transcripts were increased in males vs. females, including genes regulating chromatin remodeling and DNA methylation.

**CONCLUSIONS:** Our data demonstrate that early post-implantation embryo transcriptomic sexual dimorphism is restricted to extraembryonic tissue. Pathways that were enriched in female EPC favor robust placental development and may be one explanation for the preferential ability of female fetuses to adapt to certain adverse intrauterine exposures. Genes modulating epigenetic processes were upregulated in male EPC. These changes may explain the differences in DNA methylation that have been observed in male vs. female embryos.

#### **O-077**

**Is Lactate a Potential Therapeutic Target for Endometriosis?** Syed F Ahmad, <sup>1</sup> Erin Greaves, <sup>1</sup> Philippa TK Saunders, <sup>2</sup> Andrew W Horne. <sup>1</sup> Queen's Medical Research Institute, The University of Edinburgh, Edinburgh, United Kingdom; <sup>2</sup>Queen's Medical Research Institute, The University of Edinburgh, Edinburgh, United Kingdom.

**INTRODUCTION:** Endometriosis is a chronic, hormone-dependent disorder defined by the growth of endometrial tissue outside uterus, commonly on the peritoneum. We have shown that peritoneal mesothelial cells of women with endometriosis have an altered metabolism with increased energy production (glycolysis) resulting in increased biosynthesis of lactate. We hypothesize that ectopic endometrial tissue may use the excess lactate produced by mesothelial cells as an energy source enhancing their establishment and growth.

**METHODS:** Endometrium (eutopic and ectopic), peritoneum (from distal and adjacent sites to endometriosis lesions) and peritoneal mesothelial cells (HPMC) were collected from women with or without endometriosis.

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