The role of the paraoxonases in pre-eclampsia and labour

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Abstract

Human parturition involves interaction of hormonal, neurological, mechanical stretch and inflammatory pathways and the placenta plays a crucial role. The paraoxonases (PONs 1-3) protect against oxidative damage and lipid peroxidation, modulation of endoplasmic reticulum stress and regulation of apoptosis. Nothing is known about the role of PON2 in the placenta and labour. Since PON2 plays a role in oxidative stress and inflammation, both features of labour, the hypothesis was that placental PON2 expression would alter during labour. PON2 was examined in placentas obtained from women who delivered by caesarean section and were not in labour and compared to the equivalent zone of placentas obtained from women who delivered vaginally following an uncomplicated labour. Samples were obtained from 12 sites within each placenta: 4 equally spaced apart pieces were sampled from the inner, middle and outer placental regions. PON2 expression was investigated by Western blotting and real time PCR. Two PON2 forms, one at 62 kDa and one at 43 kDa were found in all samples. No difference in protein expression of either isoform was found between the three sites in either the labour or non-labour group. At the middle site there was a highly significant decrease in PON2 expression in the labour group when compared to the non-labour group for both the 62 kDa form ($p=0.02$) and the 43 kDa form ($p=0.006$). No spatial differences were found within placentas at the mRNA level in either labour or non-labour. There was, paradoxically, an increase in PON2 mRNA in the labour group at the middle site only. This is the first report to describe changes in PON2 in the placenta in labour. The physiological and pathological significance of these remains to be elucidated but since PON2 is anti-inflammatory further studies are warranted to understand its role.

Pre-eclampsia (PE) is associated with maternal and placental oxidative stress. The second aim was to determine the expression of PON2 in the placenta in pre-eclampsia. PON2 was examined in placentas obtained from non-labouring women who delivered by caesarean section (normal pregnancy and pre-eclampsia) and compared to women who delivered vaginally (normal pregnancy and pre-eclampsia). Samples were obtained from 8 sites within each placenta: Four equally spaced apart pieces were sampled from the inner and 4 from the middle regions. PON2 expression was investigated by Western blotting and qRT-PCR. Two PON2 bands (62 kDa and 43 kDa were found in all samples. When PON2 expression in the non-labour control group was compared to the non-labour PE group at the inner placental site no differences were found. At the middle placental site a reduction

in both PON2 isoforms was observed in the non-labour PE group compared to the non-labour control group (p=0.02 for both isoforms). No difference was found between labour control and labour PE at the inner site. A reduction in PON2 was observed in the labour PE group compared to the labour control group (PON2 62 kDa p=0.008, PON2 43kDa p=0.001). No differences in mRNA were found. Conclusion: This is the first study to investigate the expression of PON2 in PE. Given the protective roles of paraoxonases future studies including measurement of PONs in maternal blood, PONs gene polymorphism and paraoxanase induction experiment on this family of proteins may reveal new insights into understanding PE.

The third aim was to perform the same experiments as above but for PON3. There was no difference in expression of PON3 between the three sites (inner, middle, outer) within individual placentas for both labour and non-labour. PON3 was significantly decreased in the labour group when compared to the non-labour group at the inner site. No other differences were found. There was a significant decrease in PON3 expression in the PE non-labour group compared to the control non-labour group in the middle site only. There was a significant decrease in the PE labour group compared to the control labour at the middle site only.

Finally preliminary experiments were performed to determine if different zones of the placenta were more susceptible to ischemic-reperfusion injury *in vitro*. The number of experiments was not sufficient to make definitive conclusions but the pilot data suggest this should be explored more in future studies include more placenta tissue exposed to ischemic-reperfusion injury to understand how placental stress might affect placental function.

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Author's Declaration

I declare that, except where explicit reference is made to the contribution of others, that this dissertation and the work described within it was carried out entirely by myself and has not submitted for any other degree at the University of Glasgow or any other institution.

Signature----------

Samy Alwarfaly

September 2014

Publications

1. Samy Alwarfaly, Akrem Abdulsid, Kevin Hanretty and Fiona Lyall. Paraoxonase2 Protein Is Spatially Expressed in the Human Placenta and Selectively Reduced in Labour.*PlosOne.*May-2014. http://www.plosone.org/article/info%3Adoi%2F10.1371%2Fjournal.pone.0096754

2. Samy Alwarfaly, Akrem Abdulsid, Kevin Hanretty and Fiona Lyall. Paraoxonase2 is spatially reduced in the human placenta in pre-eclampsia.

   Under review (*PlosOne*. Aug 2014)

Conferences

1. Samy Alwarfaly, Akrem Abdulsid, Kevin Hanretty and Fiona Lyall. PON 2 expression in labour and pre-eclampsia. The 61 annual meeting of the Society of Gynaecologic Investigation (SGI), Florence-Italy, 25-28 March 2014.

2. Samy Alwarfaly, Akrem Abdulsid, Kevin Hanretty and Fiona Lyall. Paraoxonase3 expression is spatially down-regulated in the human placenta in labour. International Federation of Placenta Associations IFPA-EPG meeting, Paris-France, 9-12 September 2014. (Abstract accepted for poster presentation).

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Dedication

I would like to dedicate this work to my first teachers, my father, my mother and my grandmothers, who always inspired me to seek education and knowledge.

To my dear wife for all her support, guidance and patience.

To my family and friends both in Scotland and Libya.

List of Abbreviations

ANOVA	Analysis Of Variance
ATF6	Activating transcription factor 6
ATF6	Activating transcription factor 6
ATP	Adenosine triphosphate
bp	Base pair
BAS	Bovine Serum Albumin
BMI	Body mass index
cDNA	Complementary Deoxy-Ribonucleic Acid
CO2	Carbon dioxide
CRH	Corticotrophin-releasing hormone
CS	Caesarean Section
CT value	Threshold cycle value
DAMPs	Damage-associated molecular patterns
DHEA-S	Dehydroepiandrosterone-sulfate
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
eIF2α	Eukaryotic Initiation Factor 2 (eIF2)
ELC/S	Elective caesarean section
EMC/S	Emergency caesarean section
ER	Endoplasmic reticulum
FGR	Fetal growth restriction
GRP78	78 kDa glucose-regulated protein
GRP94	Heat shock protein 90kDa beta member 1
HCG	Human chorionic gonadotropin
HCL	Hydrochloric acid
HDL	High Density Lipoprotein
hPGH	Human placenta growth hormone
HPL	Human placental lactogen
HRP	Horseradish peroxidase
HSP70	70 kilo Dalton heat shock proteins

IgG	Immunoglobulin
IQR	Interquartile range
IUGR	Intrauterine growth restriction
JNK	c-Jun N-terminal kinases
KDa	kilo Dalton
LDL	Low Density Lipoprotein
LG	Labour group
LG-PE	Labour group-pre-eclampsia
LH	Luteinizing hormone
MCP-1	Monocyte chemotactic protein 1
mg	Milligram
mg/L	Milligrams per liter
mL	Millilitre
mM	Mili-molar
mmHg	Millimeter of mercury
mRNA	Messenger ribonucleic acid
Na+/H+	Sodium–hydrogen
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
ng	Nanogram
NLG	Non-labour group
NLG-PE	Non-labour group Pre-eclampsia
P38	Mitogen-activated protein kinase
PBS	Phosphate buffer saline
PE	Pre-eclampsia
PGH	Placental growth hormone
PIH	Pregnancy induced hypertension
PlGF	Placental growth factor
PON	Paraoxonase
PON1	Paraoxonase 1
PON2	Paraoxonase 2
PON3	Paraoxonase 3
QRT-PCR	Quantitative real time polymerase chain reaction
RNA	Ribonucleic acid
ROS	Reactive oxygen species
rpm	Revolutions per minute

RQ	Relative quantification of gene expression.
RT-PCR	Reverse transcriptase polymerase chain
SDS-PAGE	Sodium deodecyl sulfate polyacrylamide gel electrophoresis
sFlt1	Soluble fms-like tyrosine kinase-1
STBMs	Syncytiotrophoblast micro particles
STD	Stock standard solution
SVD	Spontaneous vaginal delivery)
TEMED	N, N tetramethylethylenediamine
TGF	Transforming growth factor
TNF-α	Tumour necrosis factor-α
TRPV	Transient Receptor Potential Vanilloid
UPR	Unfolded protein response
VEGF	Vascular endothelial growth factor
VLDL	Very-low-density lipoprotein
Wks.	Weeks
XBP1	X-box binding protein 1
β-ME	beta-Mercapto-ethanol
μg	Microgram
μL	Microliter

1 Introduction

1.1 The placenta

1.1.1 Anatomy

The human placenta connects the growing fetus to the uterine wall and allows transfer of nutrients, prevents immune rejection, eliminates fetal waste and secretes hormones that support the pregnancy. The placenta is a disc shape organ, weighing on an average about 470 g (Berirschke *et al*., 2006). In early pregnancy the placenta is formed from the union of the chorion and the allantois. The pregnancy endometrium is named decidua and the attachment site of the placenta to the uterus is called the decidua basalis or basal plate (maternal surface) Figure 1.1 A and B.

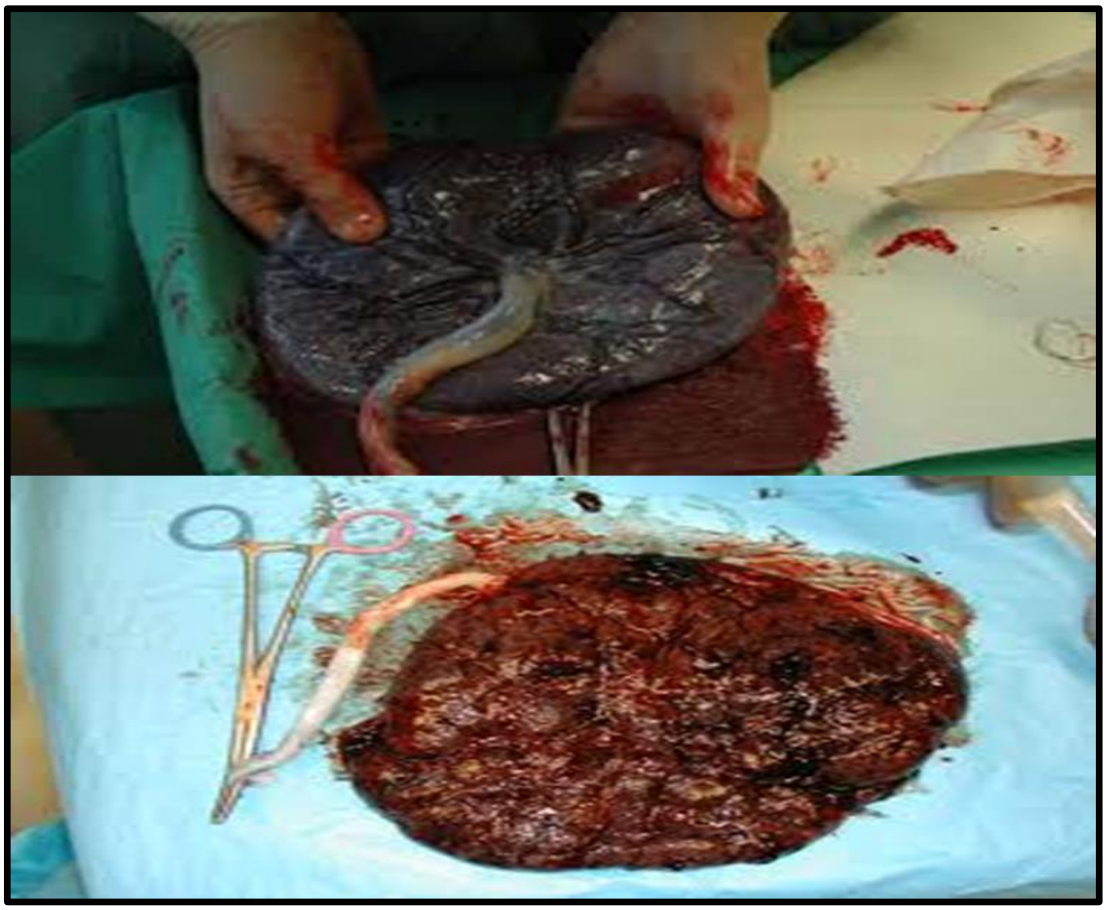

Figure 1-1: Illustration of the term human placenta. The upper shows the fetal side of the placenta. The umbilical cord inserts within the chorionic plate. The lower shows the maternal side which is contact with uterus and divided into cotyledons.

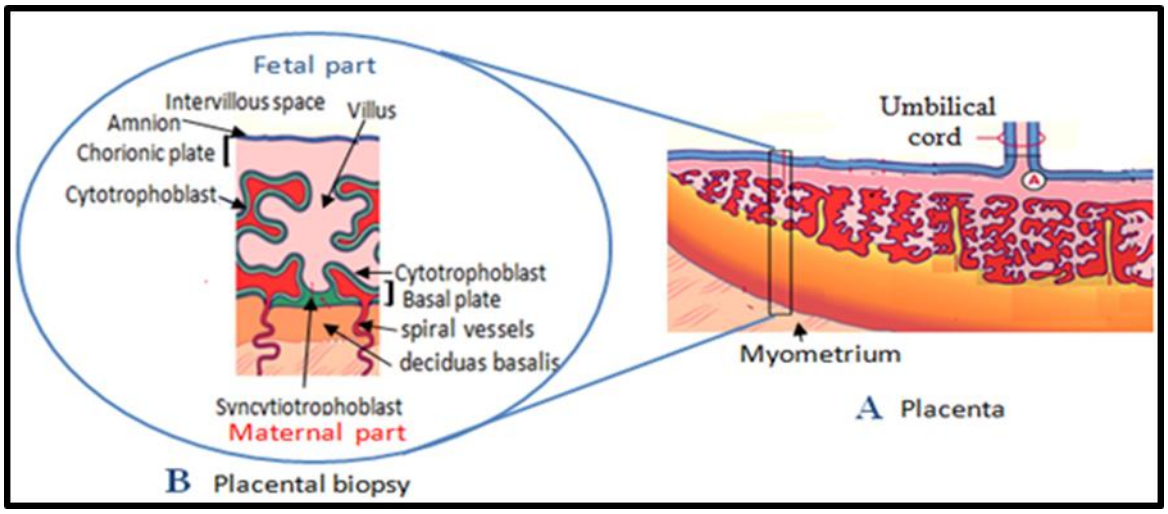

Figure 1-2: Structure of human placental: A- Diagram of human placenta shows the umbilical cord and longitudinal section of placenta. B- Magnified view of full thickness of human placenta showing the fetal and maternal sides. Figure adapted from http://www.embryology.ch/anglais/fplacenta/villosite01.html

The placenta is made of about 10-40 irregular shaped lobules named cotyledons. Major branches of the umbilical circulation supple each cotyledon. Cotyledon shapes are defined by placental setae. Cotyledons consist of villous trees structures that are bathed in maternal blood. The chorionic plate is the fetal surface where the umbilical cord inserts eccentrically in most placentas. The umbilical cord contains two arteries and one vein. The amnion faces the fetus and forms single layer of epithelium with basement membrane and avascular connective tissue base (Rampersad *et al*., 2011). The vessels that spread over amnion surface are chorionic vessels.

1.1.2 Early developmental stages of placenta

1.1.2.1 Stage of Pre-implantation:

Decidualization is endometrium changes in structure in anticipation of implantation. After fertilization the fertilized egg called zygote, which transported through the fallopian tube to the uterine cavity, a process of 3-4 days. In fallopian tube the zygote undergoes a series of mitotic division to form a ball of cells called a morula, which after entry into the uterine cavity developed to a blastocyst. The blastocyst is formed of a fluid filled cavity surrounding by differentiated trophoblast forming a single layer of mononucleotide trophoblasts called the trophectoderm that forms the extraembryonic membrane and placenta. At one end of blastocyst there is an asymmetric group of cells forms the inner

cell mass that develop into the fetus (Boyd and Hamilton, 1970; Barnea *et al*., 1992; Benirschke and Kaufmann, 2000).

1.1.2.2 Pre-lacunar stage:

For successful pregnancy, implantation of blastocyst to the uterine wall must occur. The pre-lacunar stage begins on day 7 post conception as microvilli emanating from trophectoderm; these microvilli make the initial contact with uterus epithelium. Attachment of the blastocyst follows soon through a complex interplay between both the decidua and trophoblast cells. Apposition of blastocyst is the first step in implantation followed by an adhesion step, in which blastocyst adhesion to decidua with inner cell mass (Schlafke and Enders, 1975). The blastocyst thereby undergoes interstitial implantation within connective tissue and close to maternal blood vessels that will provide the blood flow to the placenta. During invasion of the blastocyst the cytotrophoblast proliferate and fuse to form double layer, with outer cells forming of differentiated multinucleated syncytiotrophoblast that comes in direct contact with the maternal tissue. 6 to 7 days post-conception the embryo embedded in the uterus and epithelial cells grow over the site of implantation (Hertig *et al.,* 1956). The inner cell mass also differentiates into bilaminar disk of embryoblast and amnioblast (Rampersad *et al*., 2011).

1.1.2.3 Lacunar stage:

Following invasion, about eight days after conception, multiple vacuoles are formed inside the syncytiotrophoblast. These vacuoles coalesce to form lacunae. These spaces will eventually form the intervillous space. Erosion of maternal blood capillaries will fill the intervillous space with maternal blood. Placenta spiral arteries will undergo physiological changes include trophoblast invasion and wall destruction (Lyall, 2007). During the time of organogenesis the low oxygen environment protect the fetus from reactive oxygen species (Lyall, 2007). During the low oxygen period, nutrients necessary for embryo development are obtained from plasma filtrate and uterine glandular secretions in the intervillous space. At the end of first trimester feto-maternal exchange of oxygen and nutrients start after the spiral arterioles begin to perfuse the intervillous space (Hustin *et al*., 1988).

1.1.3 Villous tree developments

Villi are finger like outgrowths of trophoblast that form the tree like structure and proliferate into the intervillous space. These are primary villi contain an outer layer of multinucleated syncytiotrophoblasts and mono-nucleated cytotrophoblasts. At 5 weeks of gestation the primary villi are invaded by allantoic mesenchyme derived from the embryoblast, they are now called secondary villi, from which the feto-placental blood vessels will develop. The villous core of the villi is known as stroma and is composed of a loose network of fibroblast cells and hofbauer cells. By 5-6 weeks of gestation the first signs of "vasculogenesis" are evident. All subsequent generations of vascularized villi are subclasses of these tertiary villi. Mesenchymal villi give rise to mature intermediate villi, from which terminal villi will sprout. Intermediate villi eventually become stem villi. Terminal villi formed from non-branching angiogenesis are the last to form vessels because they are in contact with the trophoblast to allow nutrients and oxygen exchange. The villious tree is established as villi develop throughout the placenta forming a complex network which supports the growing fetus by the maternal circulation. Term placenta contains 60-70 main stem villi each of which is at a centre of a villous tree; these main villi subdivide into 2-5 branches (Page, 1993). Final stem villi have a single arteriole and a venue and contain up to 10 capillaries. By term the area of exchange of the villi is about 14 square meters (Martal and Cedard, 1993).

1.1.4 Functions of the placenta

The placenta play unique functions includes metabolism of many compounds and also part of the feto-maternal flux that supplies the fetal needs. The placenta performs maternal-fetal transport, hormone synthesis, is a barrier to vertical transmission of pathogens and an immunological interface (Illsely, 2011).

1.1.4.1 Placental transport

The placenta functions as a transport system where it transfers nutrients, ions, gases, water to the fetus and waste is also eliminated via placenta. Active transport systems deal with different plasma concentration of various molecules. As pregnancy continue the placenta grows too so as to increase blood flow into the placenta; this involves altered dimensions and surface area of the exchange barrier by formation of terminal villi and increased

number of microvilli on the syncytiotrophoblast surface and change in transport activity. Transport is inversely proportional to the thickness of the exchange barrier which decreases during gestation (Sibley *et al*., 1998),

Placenta transport systems

Maternal-fetal exchange across the placenta is reviewed in (Sibley and Boyd, 1988; Sibley *et al*., 1998; Illsely, 2011). Below are listed the different exchange methods in the placenta.

1. Passive diffusion: oxygen, gases, urea and free fatty acids transport via the placenta is by passive diffusion. Specifically, oxygen transport between maternal spiral arteries and intervillous space (O_2 concentration 90 mmHg). CO_2 is brought back from the uterine vein (O_2 concentration 30-45 mmHg) to the maternal circulation (O_2 concentration 30mmHg). The umbilical artery carries out the de-oxygenated blood back to the intervillous space (17mmHg).

2. Facilitated diffusion:
   The trans-membrane proteins embedded in the plasma membrane facilitate the rate of transport down a concentration gradient in an ATP independent fashion. For example, there is a family of 12 diffusion protein members facilitating glucose transport.

3. Active transport:
   Active transport is ATP dependent and there are many carrier systems for this type of transport. Amino acids are an example.

4. Receptor transport:
   This system is mediated by endocytosis. Protein transport take place this way, however, protein transport by this way is very restricted except for IgG. Receptor mediated endocytosis-receptors on microvilli bind IgG- coated pits are formed which are nipped off and move to the cytoplasm. Lipid transport also occurs by receptor mediated endocytosis; maternal plasma lipoproteins are taken up by receptors.

5. Ions- diffusion system:

Sodium-ion transport occurs via diffusion as well as sodium transporters (Na^+/H^+ exchanger) and (Na^+/K^+ exchanger). Similarly, potassium diffusion occurs through potassium channels. Calcium diffusion occurs through calcium channels for example transient receptor potential vanilloid (TRPV family).

1.1.4.2 Excretion function of placenta

Uric acid, urea and creatinine are waste products that are transferred to the maternal circulation by placenta through diffusion

1.1.4.3 Endocrine functions of the placenta (hormone synthesis)

The placenta is a remarkable organ with diverse functions. It plays an important role as a secretory organ. Different hormones that are important for pregnancy are produced by the placenta, specifically, from the syncytial layer of chorionic villi. The placenta produces many hormones and proteins such as hormones, human chorionic gonadotropin (hCG), oestrogens, progesterone, human placental lactogen (hPL), human placental growth hormone (hPGH) (Lacroix *et al*., 2002). The placenta also secretes others peptide hormones such as oxytocin, inhibin, Corticotrophin-releasing hormone (CRH) and prolactin. It also produces many growth factors that promote uterine blood flow and placental development such as vascular endothelial growth factor (VEGF), placental growth factor (PlGF), epidermal growth factor (EGF), and transforming growth factor (TGF).

- **Human chorionic gonadotrophin (HCG):**

HCG is a glycoprotein has a molecular weight of approximately 38,000 Dalton with 237 amino acids organized in two dissimilar non-covalently linked subunits designated alpha (α) and beta (β) (Wu et al., 1994). HCG is produced in the syncytium and acts on Luteinizing hormone (LH) receptors. A critical role of hCG is to maintain and stimulates the corpus luteum (in the ovary) to produce progesterone. Additionally, hCG suppresses the maternal immune response to prevent fetal rejection. In the case of a male fetus, it stimulates fetal leydig cells to produce testosterone in order to affect male sexual development (Kay et al., 2011).

- **Estrogen**

Placenta production of estrogen is dependent on maternal and fetal adrenal production of the precursor dehydroepiandrosterone-sulfate (DHEA-S). Estrogen increases uterine blood flow and increases expression of proteins needed for continued progesterone release as well as helps prepare the breasts for lactation (Kallen, 2004).

- **Progesterone**

After week 8, the placenta takes over production of progesterone from the ovary. Production is dependent on maternal cholesterol stores and progesterone is essential for uterine quiescence (Kallen, 2004). In addition, progesterone modulates the maternal immune response to the feto-placental allograft; this hormone also primes the breasts for lactation.

- **Human placental lactogen (hPL)**

HPL is structurally district from pituitary growth hormone, 95% sequence homology to growth hormone. It is made in syncytium. hPL early in pregnancy modifies metabolic state of mother to facilitate energy supply to fetus by stimulating maternal food intake and weight gain.

- **Human placental growth hormone (hPGH)**

hPGH is another example of a trophoblast hormone, which allows maternal metabolic adaptation to pregnancy. hPGH increases throughout pregnancy and gradually replaces maternal pituitary growth hormone. It is the major determinant of maternal insulin resistance in pregnancy. Function includes mobilization of maternal glucose for fetus. HPGH suggesting effect fetal growth after it has been identified in cord blood (Freemark, 2006).

1.2 Normal parturition

1.2.1 Definition

Labour is a process of giving birth to a baby, and is defined as a process of myometrium contraction followed by cervical ripening, effacement, dilatation and finally, fetus expulsion. It involves three phases: dilation of the reproductive tract by cervical remodelling, rupture of the membranes and then initiation of uterine contraction in a rhythmic way.

In humans, parturition includes interaction of immunity, mechanical stretch, hormonal, neurological and inflammatory pathways in which the placenta plays a crucial role (Challis *et al*., 2000; Keelan *et al*., 2003; Petraglia *et al*., 2010; MacIntyre *et al*., 2012). Placenta hormones such as corticotrophin releasing hormone and oxytocin are crucial neuroendocrine mediates involved in parturition (MacIntyre *et al*., 2012). The onset of labour is controlled by the interaction between endocrine and immune pathways.

Pregnancy is a unique in that the maternal immune response tolerates the fetal allograft. The mother's immune system could be activated by fetal amniotic fluid derived stretch-mediated damage-associated molecular patterns (DAMPs) that contribute to labour initiation (Petraglia, *et al*., 2010). Furthermore, Challis, *et al*. (2000) and Keelan, *et al*. (2003) proposed that there are two phases of the physiological parturition; the first phase is started with entry of amniotic components into uterine vessels that could promote mechanical stretching. The second phase is the uterine myometrium contraction.

Increasing evidence suggests that inflammatory markers play an important role in physiological parturition. However, many pro-inflammatory mediators such as cytokines and prostaglandins are directly implicated in labour (Challis *et al*., 2000; Keelan *et al* 2003; MacIntyre *et al.*, 2012). Previous studies showed that during labour inflammatory mediators such as, cytokines are produced by infiltration of activated leucocytes into the myometrium, cervix and placenta, and they are involved in initiation of labour. Placenta hormones such as CRH and oxytocin are crucial neuroendocrine mediators involved in parturition (MacIntyre *et al*., 2012). The onset of labour is controlled by the interaction between endocrine and immune pathways.

1.2.2 Parturition and link with oxidative stress

Oxidative stress occurs when there is an over production of reactive oxygen species compared to the intrinsic anti-oxidant defences. It may induce a range of cellular responses depending upon the severity of the insult and the compartment in which reactive oxidative species are generated (Roberts and Hubel, 2009; Burton and Janiaux, 2011). Labour is also associated with placental alterations in several pathways linked to oxidative stress (Cindrova-Davies *et al*., 2007). Uterine contractions during labour are also associated with an intermittent utero-placental perfusion, providing the basis for ischemia-reperfusion type injury to the placenta. In relation to this, Doppler ultrasound studies have demonstrated a linear inverse relationship between uterine artery resistance and the intensity of the uterine contractions during labour (Brar *et al*., 1988).

1.3 Pre-eclampsia

1.3.1 Definition

Pre-eclampsia affects about 2 to 3% of all pregnancies but this can be much higher in underdeveloped countries. Pre-eclampsia (PE) is an important cause of maternal morbidity and mortality worldwide and a leading cause of iatrogenic prematurity and fetal growth restriction (Redman and Sargent, 2005). PE has been defined by the International Society for the Study of Hypertension in Pregnancy and was defined as a blood pressure of >140/90 mm Hg on at least 2 occasions at least 6 hours apart occurring after 20 weeks' gestation and accompanied by proteinuria (>300 mg/L in a 24 hour urine collection) with no other underlying clinical problems.

During a healthy pregnancy maternal spiral arteries are remodelled. They become dilated and lose their responsiveness to vaso-constrictive stimuli. Blood then enters the intervillous space in a non-pulsatile manner and under low pressure (Lyall, 2006). In PE spiral artery remodeling is partial or incomplete (Lyall *et al*., 2013). The resulting high pressure flow results in hydrostatic damage to the placental villi. Furthermore perfusion by intermittent pulses of fully oxygenated arterial blood is thought to lead to fluctuations in oxygen delivery resulting in oxidative stress (Roberts and Hubel, 2009; Burton and Janiaux, 2011). The maternal syndrome is, at least in part, due to the maternal response to this damaged

placenta (Myatt, 2004). Placental oxidative stress is a feature of PE ((Burton and Janiaux, 2011, Myatt, 2004) and labor (Cindrova-Davies *et al*., 2007).

1.3.2 Pre-eclampsia placenta

The placenta plays a vital role in the pathophysiology of PE. Suboptimal placenta blood flow due to incomplete spiral artery remodeling might be the first cause.

1.3.2.1 Failed trophoblast invasion and placental abnormalities in pre-eclampsia

Many histological studies have shown that spiral arteries in placenta, decidua and myometrium undergo physiological changes in normal pregnancy (Lyall, 2007). Normally, these changes result in formation of high capacity low resistance vessels due to replacing the endothelium and smooth muscle of the vessels with fibrinoid matrix and invasive trophoblast cells. The dilated vessels deliver oxygen and nutrients to the placenta and the growing fetus. In PE the trophoblast invasion of spiral arteries in the placental bed is restricted and the endovascular trophoblast invasion is also limited to the decidua vessels in many cases, and about half of myometrium vessels lose these physiological changes, Figure 3. Nevertheless, myometrium vessels may be undergo partial transformation (Lyall, 2013) that will lead to intermittent contraction resulting in bursts of blood flow and ischemic-reperfusion injury to the placenta that is a feature of PE. Consequently, failure of spiral artery remodeling in PE may lead to altered blood flow to the placenta, ensuing oxidative stress and release of endothelial damaging factors into maternal circulation.

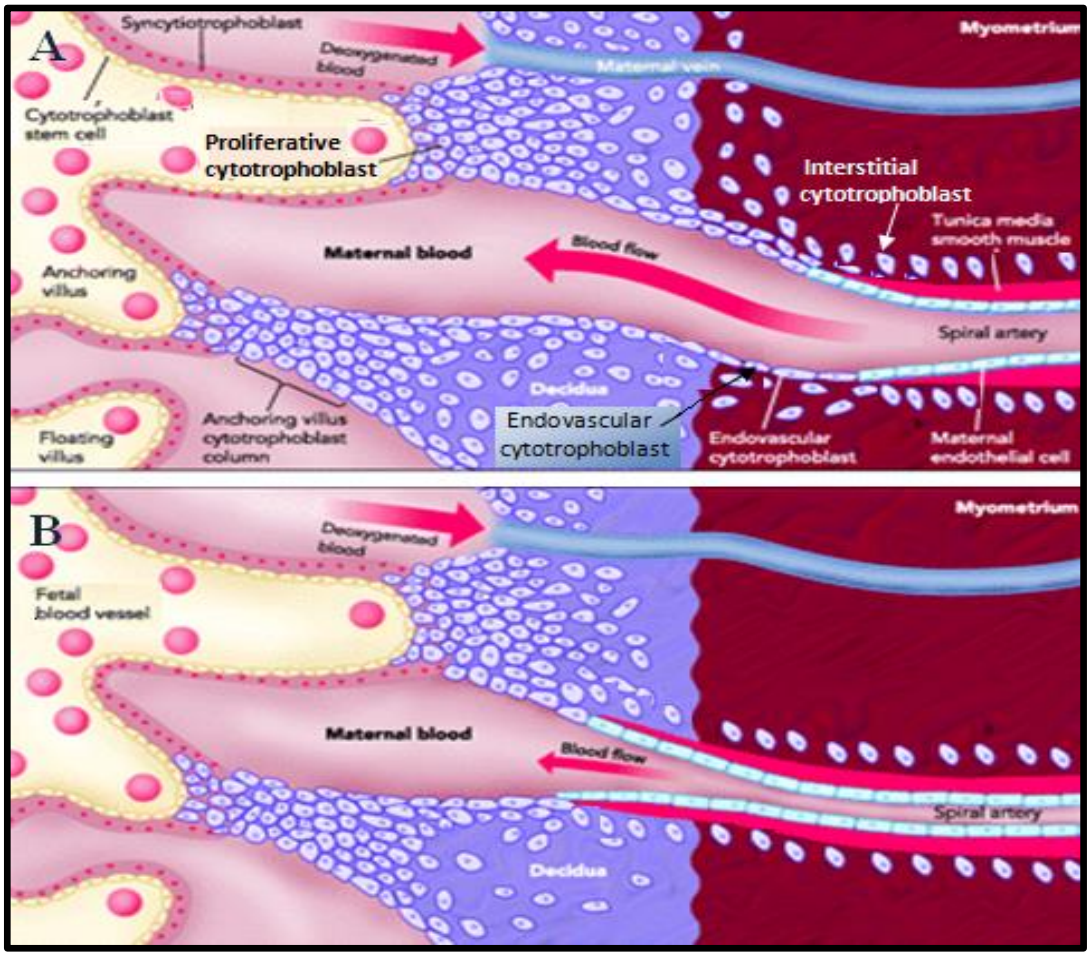

Figure 1-3: Cytotrophoblast cells Invasion of the uterine spiral arteries. Normal pregnancy (upper panel): The maternal spiral arteries are invaded by invasive cytotrophoblast cells replacing the vascular endothelial layer and the musculoelastic wall, which will form large wide, low resistant blood vessels that provide high blood flow to the fetus. In pre-eclampsia (lower panel): Spiral arteries undergo incomplete remodeling the consequence of which is low blood flow to the fetus due to the thick wall and narrow lumen of spiral arteries. Figure adapted from Wang *et al*. (2009).

1.3.2.2 Placenta pathology and apoptosis

Decidua arteriolopathy, infarcts, abruption placentas, impaired growth and Tenny-Parker changes (increased surface budding of placental syncytium) are obvious changes in the placenta in PE (Lyall and Belfort, 2007). These changes become much worse in pre-term PE. Tenny-parker changes include protrusions, containing normal nuclei and section of syncytial bridges, these changes may be a response to the alteration in oxygen content or blood flow to the intervillous space. However, as a part of syncytiotrophoblast turnover, there is another form of syncytial knot that removes the old syncytial nuclei.

Knot like apoptotic shedding may be a way of turnover that involves the apoptotic pathway. Moreover, necrotic shedding of the syncytium happens when the aponecrotic knots that resemble the apoptotic knots appear with plasma membrane defect, and

incomplete apoptotic substrate cleavage replace the apoptotic knots, and this will result in shedding membrane bound vesicles to the maternal blood, leading to induce maternal inflammatory response. Syncytiotrophoblast micro particles (STBMs) are a feature of PE (Southcombe *et al*., 2011).

1.3.3 Pre-eclampsia and link with oxidative stress

Pregnancy is a state of oxidative stress. In part is due to the considerable metabolic activity of the placenta and generation of reactive oxygen species. There is irrefutable evidence of placental oxidative stress in PE, including increased concentrations of protein carbonyls, lipid peroxides, nitrotryosine residues and DNA oxidation (Myatt and Cui, 2004; Burton and Janiaux, 2011). It is enhanced in PE due to imbalance of pro and anti-oxidants in the placenta. There are also signs of endoplasmic reticulum stress in the syncytoptrophoblast that has adverse effects on protein synthesis, and oxidative stress in mitochondria alters energy metabolism.

1.4 PONS

The paraoxonases (PON) are multifaceted and pleiotropic enzymes encoded by three highly conserved genes (PON1, PON2, and PON3) located on chromosome 7q21.3–22.1 (Primo-Parma *et al*., 1996). The three members of PON family are 60% identical at the amino acid level and approximately 70% similarity at the nucleotide level.The paroxonases were named so because the substrate for PON1 is paraoxon which is the active metabolite of the organophosphorus insecticide parathion. PON2 and 3 lack this esterase activity despite the similar nomenclature. PON1, 2 and 3 are lactones and PON2 has the highest activity of the three PONs (Primo-Parma *et al*., 1996; Draganov and La Du, 2004; Stoltz *et al*., 2007; Teiber *et al*., 2008; Horke *et al*., 2010). PON1, 2 and 3 can hydrolyse acyl-homoserine lactones ((Draganov and La Du, 2004), the latter are molecules which mediate bacterial quorum-sensing signals important in the regulation of expression of virulence factors and inducing a host inflammatory response (Primo-Parma *et al*., 1996; Ng *et al*., 2001; Stoltz *et al*., 2007; Horke *et al*., 2010). They have multifunctional roles and are involved in various biochemical pathways. These include protection against oxidative damage and lipid peroxidation, modulation of endoplasmic reticulum stress, regulation of cell proliferation/apoptosis contribution to innate immunity and detoxification of reactive

molecules and bioactivation of drugs (Primo-Parma *et al*., 1996; Draganov and La Du, 2004). Phylogenetic analysis has shown that PON1 and PON3 arose from gene duplication of the ancestral PON2 gene (Primo-Parma *et al*., 1996; Draganov and La Du, 2004). PON1 and PON3 are circulating proteins associated with high-density lipoproteins (HDL) (Deakin *et al*., 2002). PON2 is expressed in many tissues and is cell associated (Ng *et al*., 2001). PON1 and PON3 proteins are present in plasma and reside in the high-density lipoprotein fraction (Ng *et al*., 2001) and protect against oxidative stress by hydrolyzing certain oxidized lipids in lipoproteins, macrophages, and atherosclerotic lesions (Sierksma *et al*., 2007; Jaouad *et al*., 2006). Paraoxonases are important detoxifying and anti-oxidative enzymes with roles being described in organophosphate poisoning, diabetes, obesity, cardiovascular diseases, and innate immunity (Camps *et al*., 2009; Shih and Lusis, 2009). Research in the PON family has increased greatly in the last few years, particularly in the cardiovascular field (Martinelli1 *et al*., 2012). Until the work of this thesis was started nothing was known about the role of PON2 and PON3 in the placenta or whether it plays a role in labour and PE. As stated above PONs play a role in oxidative stress and inflammation, both features of labour and PE.

1.4.1 PON1

PON1 protein consists of 354 amino acids residues with molecular mass 43-47 kDa (Hassett *et al*., 1991). PON1 is mainly synthesized by the liver and then secreted into plasma. The human PON1 mRNA was detected only in liver (Reddy *et al*., 2001). It associates with high-density lipoprotein (HDL) in the circulation (Deakin *et al*., 2002). Small amounts of PON1 were detected in very low-density lipoprotein (VLDL) and postprandial chylomicrons (Draganov and La Du, 2004). PON1 hydrolyses several substrates; these include organophosphate insecticides and nerve gases, lipid hydroperoxides, lactones and thiolactones (Mackness *et al*., 1991; Jakubowski *et al*., 2000; Draganov and La Du. 2004; Dragomir *et al*., 2005; Furlong *et al*., 2008). PON1 is a potent anti-atherosclerotic enzyme (Mackness *et al*., 1993; Watson *et al*., 1995; Aviram *et al* 2000; Jaouad *et al*., 2006; Sierksma *et al*., 2007). In human, Kerkeni *et al*. (2006) suggested that the protection role of PON1 against the coronary heart disease is due to PON1 ability to hydrolysis of homocysteine thiolactone. Furthermore, Wang *et al*. (2012) showed that low PON1 plasma levels were associated with increased risk of coronary arterial disease. However, the antioxidant mechanism is still unclear and it is thought to be due to an existence of an enzymatic mechanism rather than PON1 activity of chelating of

copper ion or lipid transfer to HDL from LDL (Mackness *et al*., 1993). In humans with atherosclerosis, PON1 immunostaining increases in smooth muscle and endothelial cells with disease progression and this is thought to be due to up-regulation by oxidative stress (Mackness *et al*., 1997). In mice, PON levels negatively correlated with atherosclerosis (Shih *et al*., 1998; Ng *et al*., 2006; Shih *et al*., 2007).

1.4.2 PON2

PON2 is an intracellular protein with molecular weight about 44 kDa (Primo-Parma *et al*., 1996; Reddy *et al*., 2001; Ng *et al*., 2001; Draganov and La Du, 2004). PON2 is more widely expressed and is found in a variety of tissues including the liver, lung, heart, intestine and stomach (Primo-Parma *et al*., 1996; Reddy *et al*., 2001; Ng *et al*., 2001; Draganov and La Du, 2004). Nothing is known about the function of PON2 in the placenta and the only previous report showing expression was from our own group (Alwarfaly *et al*., 2014). Furthermore, Ng *et al*. (2001) detected that PON2 is also expressed in the endothelial cells of arterial wall, smooth muscle and macrophages. PON2 enzyme activity is limited to cells however there is the possibility of rapid degradation of PON2 following cellular secretion. In contrast to PON1 and PON3, PON2 is undetectable in HDL or LDL (Ng *et al*., 2001). PON2 has n-terminal similar to PON1 and PON3 but it appears to remain intracellular associated with the plasma membrane of cells (Ng *et al*., 2001). PON2 has anti-oxidant properties and protects against LDL oxidation (Ng *et al*., 2001, Rosenblat *et al*., 2003).

PON2 expression and activity is up regulated in response to oxidative stress. Ng and co-workers (2001) demonstrated that PON2 is also able to prevent oxidation of mildly oxidized LDL. However how this cellular antioxidant properties of PON2 protects against oxidative stress is still unknown and need further investigation (Ng *et al*., 2004).

PON2 has several polymorphisms that are associated with number of pathological conditions, two common polymorphisms were identified a pair of amino acid substitution with alanine or glycine at positions 148 (A148G) and either cysteine or serine at position 311 (S311C). A148G polymorphism has been associated with variation of total and LDL cholesterol, fasting blood glucose levels and with birth weight (Hegele *et al*., 1997; Hegele *et al*., 1998; Hegele *et al*., 1999). S311C polymorphism has been related with coronary artery disease, ischemic stroke in patients with type 2 diabetes mellitus and Alzheimer's disease (Sanghera *et al*., 1998; Wang *et al*., 2002; Kao *et al*., 2002; Janka *et al*., 2002; Chen *et al*., 2003). Furthermore, despite the report of 2 isoforms (found on Western blots)

there is no literature reporting where the isoforms have different functions.

1.4.3 PON3

Among the three PON enzymes, PON3 was the last to be discovered. PON3 is primarily synthesised in the liver, and then secreted to the circulation, where it is associated with HDL but in much lower levels than PON1. PON3 mRNA is expressed primarily in the liver, a low level of PON3 expression can also be found in the kidney (Reddy *et al*., 2001). PON3 has a molecular mass of approximately 40 kDa and also has antioxidant properties. Two polymorphisms were identified in the PON3 gene at position 311 and 324. At position 311 is serine to threonine and at position 324 is glycine to aspartic acid (Campo *et al*., 2004). Unlike PON1 and PON2, PON3 has very limited or lack of paraoxonase and arylesterase activities, but both enzymes PON1 and PON3 hydrolyse aromatic and long-chain aliphatic lactones like dihydrocoumarin that can inhibit the oxidation of low density lipoprotein (LDL), a function that is believed to slow the initiation and progression of atherosclerosis (Reddy *et al*., 2001). In addition, PON3 hydrolyses some drugs like statin lactones (lovastatin and simvastatin) and a diuretic spironolactone (Draganov and La Du, 2004). Moreover, beside its function in cardiovascular disease PON3 is associated with obesity (Shih *et al*., 2007). Shih et al. (2007) showed that transgenic mice expressing high human PON3 had low body weight and low plasma leptin levels compared to their littermates. In contrast to PON1 and PON2, PON3 expression does not appear to change in response to oxidative stress (Reddy *et al*., 2001).

1.5 Ischemic-reperfusion injury model

Ischemic reperfusion injury is defined as tissue damage caused as a result of return of blood flow after a period of ischemia. During the ischemic period the lack of oxygen and nutrient creates an ischemic tissue condition and the length of time that any tissue can survive oxygen deprivation varies from organ to organ. Restoration of blood supply leads through inflammation and oxidative damage; this is mainly due to free radical activity and oxidative stress induction. Ischemic-reperfusion insult is characterized by oxidant production, leucocyte–endothelial cell adhesion, platelet–leucocyte aggregation, increased microvascular permeability and decreased endothelium-dependent relaxation. Ischemic reperfusion injury can lead to multi-organ damage and death (Schofield et al., 2013; Brown

et al., 2013; Karatzas et al., 2014). Paraoxonases may be involved in ischemic-reperfusion injury, due to their known functions, and therefore are of interest to study in the setting of pre-eclampsia.

1.6 Hypothesis

Nothing is known about the role of PONs in the placenta or whether they play a role in labour. However since PON2 and PON3 play a role in oxidative stress and inflammation, both features of labour and pre-eclampsia, we hypothesised that placental PON2 and PON3 expression would increase during labour and pre-eclampsia. We also hypothesised that expression would alter at different zones of the placenta.

- The null hypothesis was there is no different in PONs expression when comparing two groups of non-labour to labour and normotensive to pre-eclampsia
- The true hypothesis was there is a real difference when comparing two groups of non-labour to labour and normotensive to pre-eclampsia.

1.7 Aims

- The first aim of this study was to examine the spatial expression of PON2 and PON3 protein and mRNA expression in placentas obtained from women who delivered by caesarean section and were not in labour (non-labour group) and placentas obtained from women who delivered vaginally following an uncomplicated labour (labour group).
- The second aim was to compare the PON2 and PON3 protein and mRNA expression in placentas of non-labour and labour groups at the inner, middle and outer sites of the placenta.
- The third aim was examine the PON2 and PON3 mRNA expression in placenta of non-labour pre-eclampsia, labour- pre-eclampsia compared them to normotensive non-labour and labour groups.

2 Materials and methods

2.1 Ethical approval

Ethical approval was performed according to the Declaration of Helsinki. The project was approved by the West of Scotland Research Ethics Service- REC 4 (REC reference number 13/WS/0149, IRAS ID 130896). All information sheets, consent form and ethical approval for this study are shown in Appendix 1. Signed patient consent was obtained prior to delivery. All the consent was obtained by Dr A. Abdulsid. Placental samples were stored within the Institute of Medical Genetics.

2.2 Subjects

Human term placentas were collected from pregnant women at the Southern General Hospital, Glasgow. Placentas were collected from: (1) women who had uncomplicated pregnancies and delivered at term either spontaneous vaginally (labour group) or by elective caesarean section (non-labour group). The labour group was all spontaneous labour and a tight group (labour time minimum 3 hours and maximum 8 hours). Caesarean sections were performed for obstetric reasons such as breach presentation, previous caesarean section or maternal request. Patient consent was obtained prior to delivery. The groups studied had no underlying maternal conditions such as hypertension, PE, diabetes, gestational diabetes or any other medical disorders. There was no fetal pathology such as FGR. (2) Women who had pregnancies complicated by PE (non-labour group or labour group). (3) Women who pregnancies complicated by FGR. PE was defined as pregnancy-induced hypertension (blood pressure ≥140/90 mm Hg) and proteinuria (≥300 mg/24 h) in women who were normotensive before pregnancy and had no other underlying clinical problems, such as renal disease (Brown *et al*., 2001). The patients' details are shown in Table 2-1 below.

Table 2-1: Patient clinical details. NLG (non-labour group); LG (Labour group); NLG-PE (non-labour-PE); LG-PE (labour-PE); EMC/S (emergency caesarean section); ELC/S (elective caesarean section); SVD (spontaneous vaginal delivery); BMI (body mass index); PG (prim gravida); G refers to the number of pregnancies the woman has had and P refers to the number of live children from these pregnancies. PO+2=2 miscarriages

Sample	**Age years**	**Parity**	**Smoker**	**Gestation (weeks/ days)**	**Birth weight (g)**	**Placenta weight (g)**	**Mode of delivery**	**BMI**
NLG	22	G3P2	NO	38 +4	3840	680	ELC/S	37
NLG	34	G2P1	NO	41+3	3100	450	ELC/S	30.6
NLG	26	G2P1	YES	39	2860	683	ELC/S	23
NLG	22	PG	YES	39+2	3050	465	ELC/S	21
NLG	34	G3P2	NO	39+1	3555	685	ELC/S	32.4
NLG	32	G2P1	NO	39	4255	605	ELC/S	27
LG	27	PG	NO	40+2	4192	585	SVD	24
LG	28	PG	NO	40+4	3646	595	SVD	28
LG	22	PG	NO	41+3	3940	700	EMC/S	34
LG	25	PG	NO	41+3	3310	486	SVD	24
LG	28	G3P2	NO	37+6	3354	636	SVD	26
LG	25	G4P3	NO	41+1	3870	535	SV	33.9
NLG-PE	34	G3P2	NO	39	3490	592	ELC/S	27
NLG-PE	33	PG	NO	36+6	2800	450	ELC/S	39
NLG-PE	28	G2P0	YES	36+3	4525	1309	ELC/S	57.3
NLG-PE	38	G2P1	NO	39	3736	695	E/CLS	47.2
NLG-PE	29	G2P1	NO	37 +3	3210	675	ELC/S	32.2
LG-PE	42	G6P4	NO	36+6	2248	449	EMC/S	29
LG-PE	32	PG	NO	38+1	2812	521	SVD	23
LG-PE	30	PG	NO	28+5	1230	265	EMC/S	29.7
LG-PE	32	PG	NO	38+3	2990	420	EMC/S	21.9
LG-PE	28	PG	NO	40+6	3530	692	SVD	26.7
LG-PE	17	PG	NO	29+2	1260	317	EMC/S	23
LG-PE	35	PO+2	YES	35+6	2210	780	SVD	29.2

2.3 Patient clinical data analysis

The combined analysis for all the patients shown in Table 2-1 is shown in Table 2-2. There was no significant difference in maternal age, placental weight and birth weight between all groups except gestational age at delivery was reduced in the LG-PE compared to the LG-C. Smoking did not affect the results. Comparison of groups was performed with the one way ANOVA statistic test. Comparison of two groups was performed with the Mann-Whitney test.

Table 2-2: Shows the demographics of patients used for placenta collection

CATEGORY	NLG-C (n=6)	LG-C (n=6)	NLG-PE (n=5)	LG-PE (n=7)	p-value
Maternal age	28.33±5.7	26±2.28	32.4±4.03	30.57±6.88	p=0.2
Placenta weight	594.7±110.5	589.5±75.0	662.2±164.12	591.7±161.4	p=0.7
Birth weight	3443±537	3719±347	3552±645.54	2959±567	p=0.09
No. Prim gravida	1	4	1	6	
Gestation age at delivery	39.3±1.0	40.31±1.4	37.64±1.29	38.55±2.024	NLG v LG (p=0.2) NLG v NLG-PE (p=0.05) LG v LG-PE (p=0.02)
No. smokers	2	0	1	1	

2.4 Sampling methods

Placentas were obtained directly after delivery, washed in water and the amnion was removed. The placenta was divided into zones. For each control patient placental samples (~1 cm^3) were obtained from three zones by taking measurements from the cord insertion point: 0–2 cm (inner zone), 2–4 cm (middle zone) and 4–6 cm (outer zone) of the placenta (Figure 2-1). Within each zone four separate samples were obtained representing the four

quadrants as previously described (Abdulsid *et al*., 2013, Abdulsid *et al*., 2014, Abdulsid and Lyall, 2013). Placentas had a central cord insertion. Samples were rinsed and immediately flash frozen in liquid nitrogen.

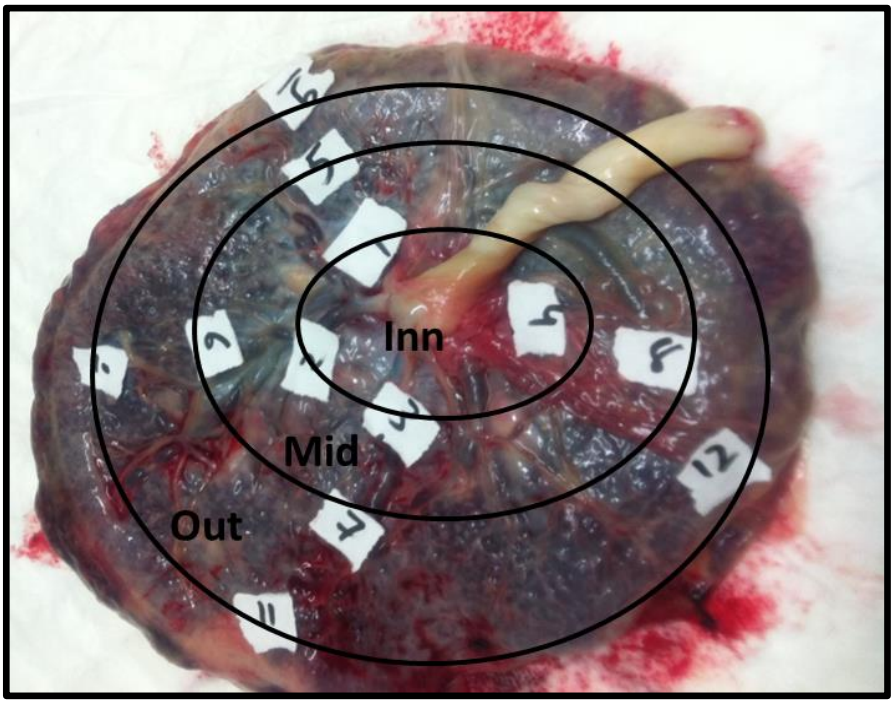

Figure 2-1: picture shows areas where samples were taken in each individual placenta.

2.5 Collection and processing of the placenta tissue

2.5.1 Tissue collected for protein and molecular analysis

All equipment used for the tissue collection was sterile and the appropriate number of cryostat containers was put in dry ice to ensure they were cold. Tissue samples were collected and washed three times in phosphate buffered saline (PBS) until the blood was washed away, then immediately flash frozen in liquid nitrogen. Snap frozen tissue was then brought to the lab on dry ice and kept at -80 C until processed.

2.6 RNA extraction from placenta tissue

2.6.1 RNA extraction

RNA was extracted from placenta samples by using the RNeasy Midi Kit (Qiagen cat. no. 75142) Figure 2-2. Three steps were followed as per the manufacturer's instructions.

- **Preparation of working solution:** A working solution was made prior to use according to the Qiagen kit recommendations: β-Mercaptoethanol (β-ME) was added to RLT buffer (10µl β-ME per 1ml of RLT buffer). Buffer RPE was supplied as a concentrate; 11ml was added to 44ml of ethanol to obtain 55ml of Buffer RPE.
- **Homogenization of tissue:** The frozen tissue sample was removed from -80° C storage and placed in a pre-weighted plate to determine the correct amount of starting material (maximum 250 mg of tissue). The suitable amount of placenta tissue was disrupted in 4 ml RLT buffer (buffer provided with kit). The tissue was then homogenised using a (Polytron PT1600E, Lucerne) Rotor-stator homogeniser (Kinematica) at maximal setting speed for 3x20 seconds until uniform homogeneity. After that, the homogenising lysate from each sample was spun in the centrifuge (4000g, 25°C for 10 minutes). The supernatant was then transferred carefully to a new 15ml Falcon tube (Sigma-Aldrich, cat.no.Z617849). Then 4.0ml of ethanol (70%) was added to the supernatant. The tube was immediately shaken vigorously until the solution was clear.
- **Extraction of total RNA:** an appropriate number of RNasey® Midi kit spin columns already contained within the 15 ml collection tubes (provided in kit) were retrieved and 4 ml of mixed supernatant (ethanol and supernatant) was added to the column which was then spun in centrifuge (4000g, 25°C for 5 minutes). The flow-through was discarded**.** This step was repeated twice until all supernatant had gone. Next the column was washed three times as follows: 4.0ml of RW1 buffer (provided with kit) was then added to the RNeasy column which was then spun in the centrifuge (4000g, 25°C, for 5 minutes); the flow-through was discarded again. RPE buffer (2.5 ml) (provided with the kit) was then added to each RNeasy column and spun down in the centrifuge (4000g, 25°C for 2 minutes). Another 2.5 ml of RPE buffer was added to each column and spun again in the centrifuge (4000g, 25°C for 5 minutes) in order to dry the RNeasy silica-gel membrane. For RNA elution, the RNeasy column was transferred to a new 15 ml collection tube

(provided with the kit). Of RNase-free water (250 µl) was added into the RNeasy silica-gel membrane and allowed to stand for 1 minute. After that the columns were spun down in the dcentrifuge (4000g, 25°C for 3 minutes). This step was then repeated with same volume of RNase-free water.

Figure 2-2: RNeasy Midi Kit (Qiagen .cat.no. 75142).

2.6.2 Quantification of RNA

The quantity of total RNA concentration (ng/µl) present in the sample was calculated by using a spectrophotometer NanoDrop 1000™ (Thermo Scientific, USA). The Nanodrop was first blanked with 1.5µl of RNAase-free water (same elution material) and the obtained value in the blank state served as a comparison value. After that the RNA samples were measured one by one. The reading at 260 nm gives an estimation of the amount of RNA. The ratio of absorbance at 260/280 reading indicates the RNA purity of the sample; a ratio close to 2 indicates optimal purity. The final measurements were in ng/µl. (Figure 2-3 and 2-4).

Sample ID	User ID	Date	Time	ng/ul	A260	A280	260/280	260/230	Constant	Cursor Pos.	Cursor abs.	340 raw
9PE1	Sandy	15/01/2013	20:03	425.39	10.635	5.087	2.09	2.19	40.00	230	4.856	0.016
	Sandy	15/01/2013	20:07	0.92	0.023	0.005	4.29	0.27	40.00	230	0.086	0.033
9PE1	Sandy	15/01/2013	20:08	419.27	10.482	4.981	2.10	2.17	40.00	230	4.839	0.042
9PE6	Sandy	15/01/2013	20:09	137.90	3.447	1.609	2.14	2.01	40.00	230	1.717	0.029
9PE11	Sandy	15/01/2013	20:09	318.94	7.974	3.731	2.14	2.20	40.00	230	3.626	0.040
10PE1	Sandy	15/01/2013	20:10	127.37	3.184	1.463	2.18	2.12	40.00	230	1.501	0.047
10PE6	Sandy	15/01/2013	20:13	417.34	10.434	4.966	2.10	2.05	40.00	230	5.084	0.039
11PE1	Sandy	15/01/2013	20:14	396.64	9.916	4.699	2.11	2.19	40.00	230	4.520	0.029
11PE6	Sandy	15/01/2013	20:15	202.41	5.060	2.357	2.15	2.11	40.00	230	2.403	0.037

Figure 2-3: Example of transcript from Nanodrop reading in ng/µl (arrow). The ratio of absorbance at 260 nm and 280 nm (260/280) (Arrow) is given an indication of RNA purity.

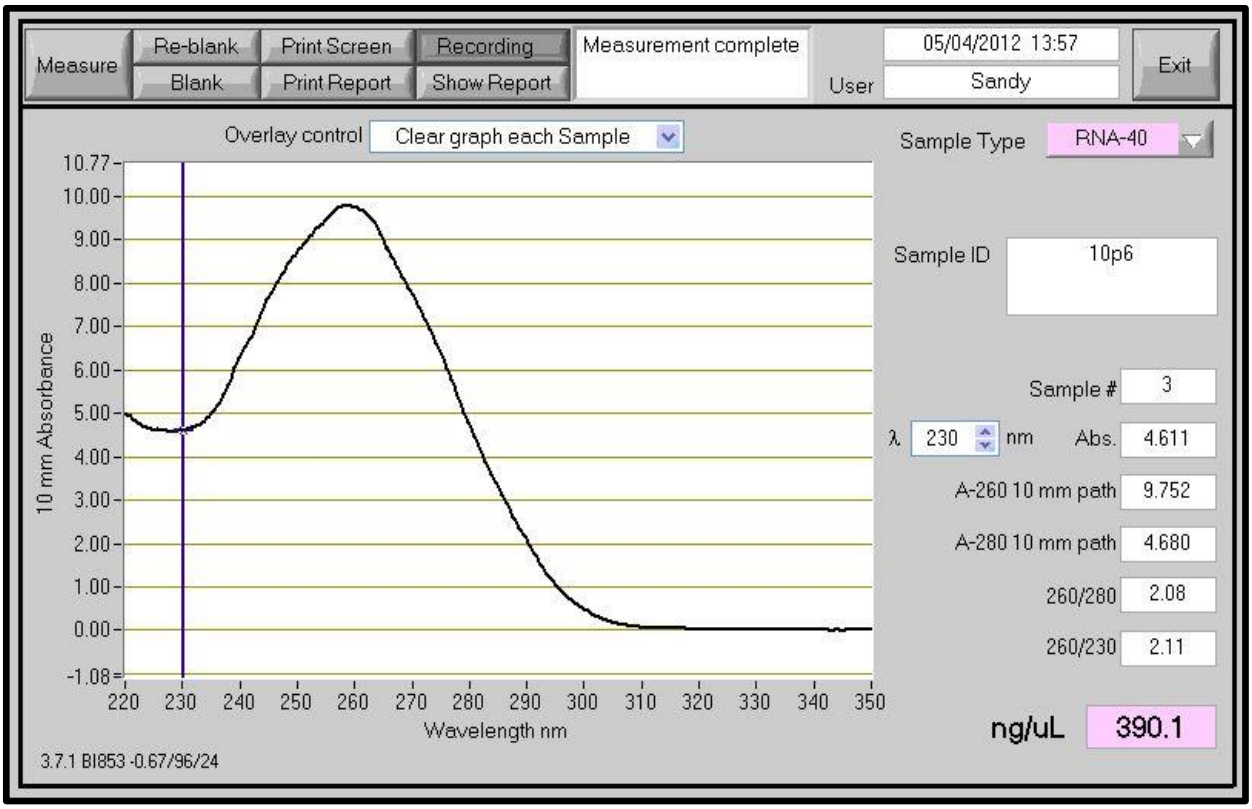

Figure 2-4: RNA concentration of placenta sample number 10p6 in ng/µl (390.1)

2.7 Converting mRNA into cDNA (Reverse transcription)

RNA cannot serve as temple for PCR so a reverse transcription step is required. The protocol for the reverse transcription was performed using the Quanti-Tech Kit (Qiagen,

cat. no 205310) Figure 2-5 and Go Script™ reverse transcriptase (Promega, cat. no. A5003). As a result of optimization process in our lab, the quality of cDNA obtained using GoScript™ reverse transcriptase reaction was better than reverse transcriptase of the Qiagen kit.

Figure 2-5: Quanti-Tech Kit (Qiagen, cat.no 205310)

Reverse transcription including two steps as following:

2.7.1 Genomic DNA elimination reaction:

mRNA and Quanti-Tect® kit were removed from -80 C and -20 C storages respectively and kept on wet ice at all times. Then amount of RNA required for 100 ng was calculated based on their original concentration.

$$\text{Required RNA (µl)} = \frac{100\text{ng} \times 1\text{µl}}{\text{Original concentration (ng/µl)}}$$

For each DNA elimination reaction in each PCR tubes the following was made:

- Wipe-out buffer- 2µl
- RNA (100ng)- X (volume depends on each sample conc) all, and RNase free water Y all (total 12 µl)
- Total reaction volume- 14µl

Once the reaction was set up, the tubes were put through the gOUT program, using a thermal cycler (DNA Engine®, Bio-Rad).

- 3 minute at 42°C

2.7.2 Reverse transcript (RT) reaction in each PCR tube:

The master mix was made up for the reverse transcription reaction as follows:

- GoScript Reverse transcriptase - 1µl
- RT Buffer -4µl
- RT primer Mix-1µl

Once the reaction was complete, the PCR tubes were removed and the master mix was made up. A volume of 6µl of master mix was then added to each tubes containing 14µl of RNA that had gone through the gnu wipe-out program.

- Total reaction volume 20 µl

PCR tubes were put again into the thermal cycler using the RT4 program,

- 30 minutes at 42 °C
- 5 minutes at 95 °C (enzyme deactivation)

Once the reaction was completed, cDNA was stored in -20 C.

2.8 Testing cDNA quality

The cDNA quality was checked using the human cDNAOK!® kit (Microzone, cat. no. 2HCDOK-150). The human cDNAOK mix and mega Mix-Gold are supplied from Microzone and it was not known for the customers. Interpretation of results as following: expected fragment size: 125 bp, 250, 375, 500 and 650 bp. If all 5 fragments are observed the cDNA is likely to be okay. The 500 bp (arrow) fragment is derived from an internal control and should always be present (even in negative control), if not, PCR has failed and needs repeating. However, if less than 5 fragments are observed then the cDNA is likely to not be okay.

Methods: In each tube was added

- Human cDNAOK!® Kit mix 7.5µl
- Master mix Gold 12.5µl
- H_2O 4µl

- cDNA 1µl
- Total volume of reaction 25µl

Once the reaction was complete the PCR tubes were put into the thermal cycler program (cDNAOK)

- 95°C for 30 seconds
- Then for 35 cycles:
    - 95°C for 30 seconds
    - 59°C for 20 seconds
    - 72°C for 45 seconds

Once completed, the PCR products were separated on a 2% agarose gel at 100 volts constant voltage Figure 2-6.

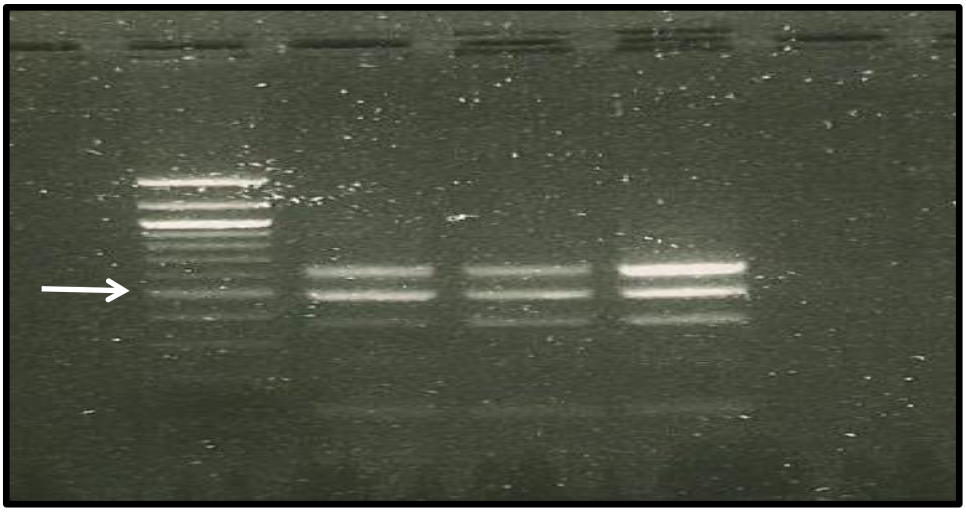

Figure 2-6: PCR multiplex using the Human cDNAOKı® Kit (Microzone, cat. no. 2HCDOK-150). cDNA was made using the Quantitect® kit and GoScript™ reverse transcriptase. Products were separated on 2% agarose gels using a 100 bp ladder (Sigma-Aldrich, cat. no.P1473).

2.9 Quantitative real time polymerase chain reaction (qRT-PCR)

All equipment and bench space were sterilized with 70% ethanol before starting the experiment. cDNA was removed from -20°C storage and allowed to thaw on wet ice. The TaqMan Master Mix (Applied Biosystem, cat. no 4369514) was used to investigate mRNA levels of different genes by using the StepOnePlus® qPCR machine (Applied Biosystems).

TaqMan gene assays were used according to manufacture guidelines. β-actin was used as an endogenous control. Placenta cDNA (primer design) (Figure 2-7) was used as a reference sample. RNA free water was used as negative control (Figure 2-7). Samples and reference sample were diluted 1/5 (1µl cDNA+ 4µl dH_2O). The master-mix was made for every primer used within a single experiment.

Typical master-mix and cDNA per would be as follows:

- Master mix --- 10µl
- dH_2O----- 4µl
- diluted cDNA- 5µl
- TaqMan Gene Expression Assay 1µl
- Total volume--- 20µl

PON2 TaqMan Gene Expression Assay (Applied Biosystems, cat.no: Hs00165563_m1), PON3 TaqMan Gene Expression Assay (Applied Biosystems, cat. no: Hs01023629_m1 and cat no: Hs01023630_m1).

The experiment was set up in microamp® fast optical 96 well plates (Applied Biosystems, cat. no. 4346906). A plastic cover was added after the plate was set up. The plate was mixed and then spun in a centrifuge (4860g for 90 seconds) in order to remove any bubbles after which the plate was put in a qPCR StepOnePlus machine and the run was performed according to following qPCR programme:

Holding stage:

- 50 C for 2 minutes
- 95°C for 10 minutes

Cycling stage X50:

- 95 C for 0.15 minutes
- 60 C for 1 minute

Once the programme was completed, the plate was kept in the fridge.

Different gene expressions were compared using the comparative C^t methods of Applied Biosystem software® v2.1 in order to obtain a relative quantification (RQ) of gene expression. Example of PON2 and PON3 amplification plot is shown in Figure 2-8. TaqMan® Assays are primer and probe sets based on 5’ nuclease chemistry using TaqMan® MGB (minor groove binder) probes, the most quantitative gene expression technology available by Applied Biosystems.

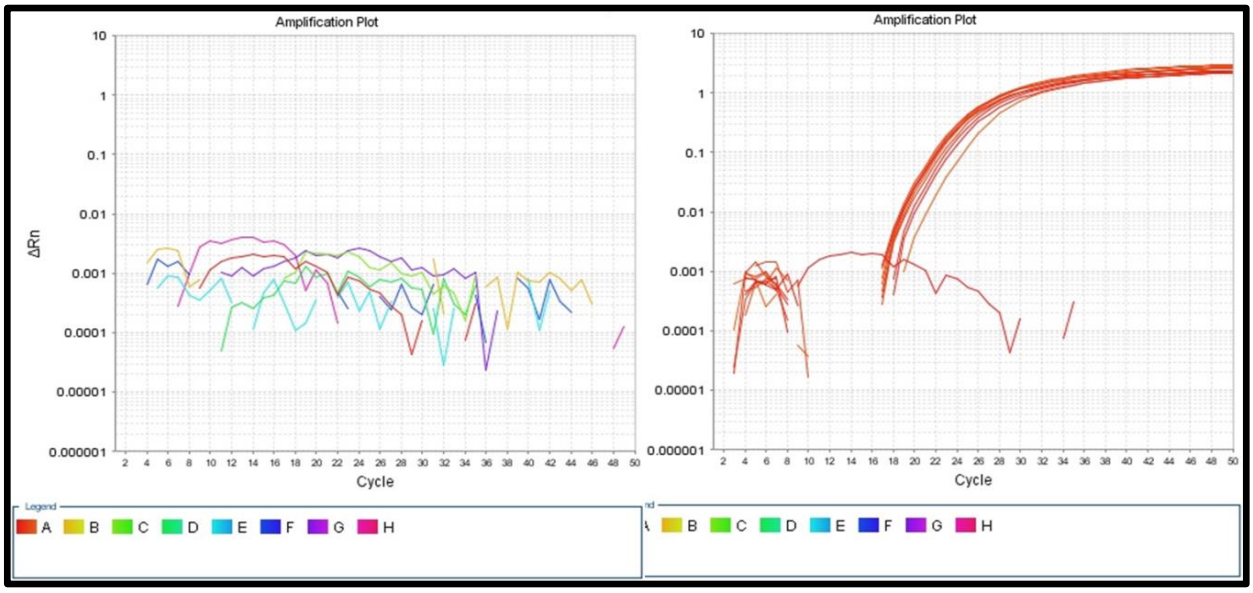

Figure 2-7: Left panel: The negative control (RNA free water) showed no expression, Right panel: Placenta cDNA (positive control) showed clear expression from cycle number 16.

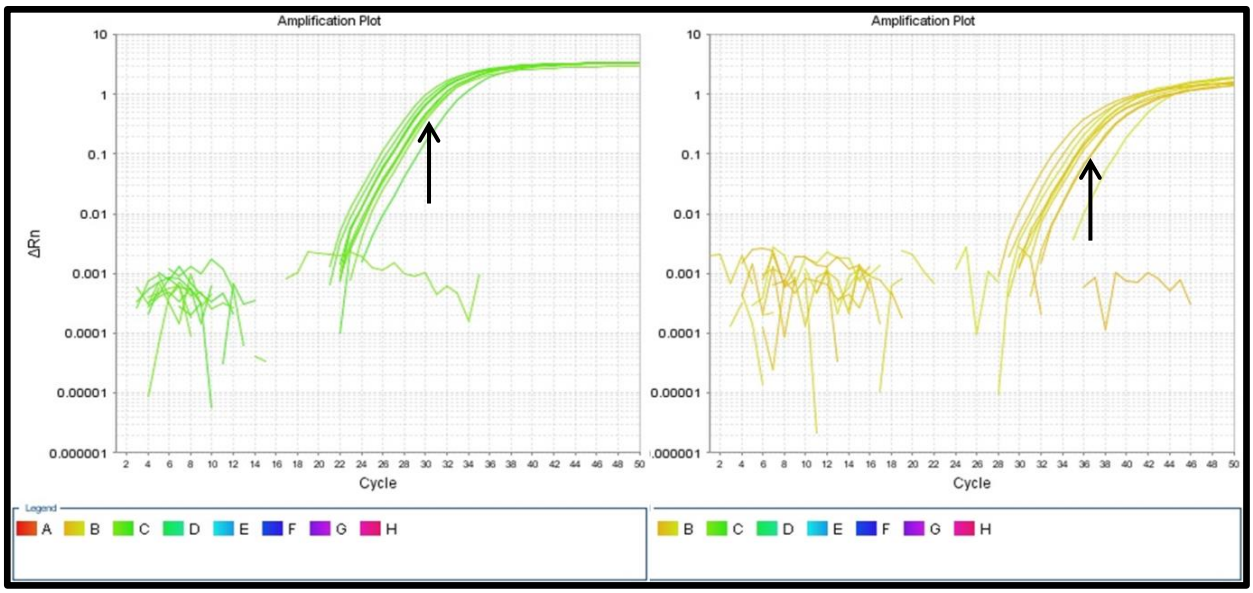

Figure 2-8: Left panel: PON2 gene expression the average Ct. Value (arrow) around cycle. No. 30. Right panel: PON3 gene expression the amplification started after 35 cycles the average Ct. Value (arrow) around cycle. No. 38.

2.10 Placental tissue preparation for protein expression analysis

2.10.1 Homogenisation of placenta tissue

Homogenising buffer contained: 25 mM Tris buffer (25 mM Tris 75 mM NaCl with the pH adjusted to 7.6), 1mM Ethylenediaminetetraacetic acid (EDTA), and 250 mM sucrose. The buffer was stored at 4C. 12.5µl/ml mammalian cell protease inhibitor cocktail (Sigma Aldrich cat. no. P8340-5ml) was added immediately prior to use on the day of the experiment. Relevant tissue samples were retrieved from –80 C storage. Tissue samples were ground down to a fine powder in liquid nitrogen using a pestle and mortar. An appropriate volume of homogenising buffer supplemented with protease inhibitor cocktail was added to the fine tissue powder. Samples were homogenized using a rotor-stator homogeniser (polytron® PT 1600E, Lucerne, Kinematica) at speed 20 for each sample, with 5x10 second intervals and 1 minute cool down on wet ice in between. The tissue homogenate was spun in a centrifuge (4860g for 10 minutes at 4 C) to remove debris. The supernatant, containing the total protein (both particular and cytosolic fraction), was extracted, allocated and stored at -80 C.

For each tissue sample

- The sample was ground down and kept cold. Bijous was labelled with the relevant sample name were weighed in grams without the lid on, and then balanced to zero. The relevant sample was retrieved to be homogenised and put into its relevant bijou determine the weight of the tissue. Then 3 times the amount of buffer (ml) for the weight of tissue sample (grams) was added.
- Tissue homogeniser: Homogenisation was switched on by using speed 20 for each sample x5, for 10 second each with 1 minute cooling interval. The tissue sample was left in the bijou on ice at 4 C.
- Centrifugation: 15 ml labelled conical tube containing lysate at 4860g for 10 min at 4 C. The supernatant is systolic protein and the pellet is “junk” protein. New 1.5 ml Eppendorf’s was retrieved and the supernatant (total/cytosolic protein) was pipetted off into labelled Eppendorfs. The pellet (junk protein) was discarded.
- Tissue homogeniser: Homogenisation was switched on by using speed 20 for each sample x5, for 10 second each with 1 minute cooling interval. The tissue sample was left in the bijou on ice at 4 °C.

- Centrifugation: 15 ml labelled conical tube containing lysate was spun using 4860g for 10 min at 4 °C. Supernatant will be systolic protein and the pellet will be junk protein. New 1.5 ml Eppendorfs was retrieved and the supernatant (total/cytosolic protein) was pipetted, ensuring that the Eppendorfs were labelled, and that the pellet (junk protein) was discarded into the bin.

2.10.2 Protein estimation using Bradford assay

The method described by (Bradford, 1976) was used to quantify protein concentration. Sample protein concentration can be measured by mixing protein solutions with the acidic brown comassie-dye reagent (Bradford reagent), which alters it to a bluish colour. Changing of the colour alters the absorbance maximum of the protein from 465 nm to 595 nm. The colour response of the sample protein is compared to that of standard samples which is usually bovine serum albumin (BSA). The stock standard solution (STD) of bovine serum albumin (BSA) (1 mg per ml) was used to create the standard curve as shown in Table 2-3:

Table 2-3: Concentration of STD

STD concentration (µg/ml)	**Vol. stock STD (µl)**	**Vol. of d H_2O (µl)**
0	N/A	50
200	10	40
400	20	30
600	30	20
800	40	10
1000	50	N/A

Procedure

Samples were diluted by 1/40 (39 µl of dH_2O and 1 µl of homogenate). Measurements of different concentrations were prepared using a BioPhotometer® (Eppendorf). Standards were used to create the standard curve against which the sample's concentration was measured. Example of standards curve is shown in Figure 2-9. For a Western blot 50 µg

was required for loading. The calculation (the reading value /1000 (convert µg to mg (mg/ml) x40 (dilution factor) 1000 to convert µg to mg (mg/ml), divided by the dilation factor of 40 the dilution factor. For 50 µg the appropriate volume could then be calculated for loading.

Protocol summary:

The stock standard solution: 1 mg per ml of BSA, 20 mg in 20 ml H_2O. 6x 0.5 ml Eppendorf tubes were labelled for making up the different concentration of standard solutions as shown in Table 3.

Sample dilution: Relevant samples were retrieved from freezer then Eppendorfs tubes were labelled. Samples were diluted by 1/40 (39 µl of dH_2O and 1 µl of homogenate). For standards assay preparation: New 1.5 ml Eppendorf tubes were labelled for each STD solution and 10 µl of each STD solution was added to their relevant tubes. For sample assay preparation: 2 new 1.5ml Eppendorfs tubes were labelled for each sample, 10 µl of the diluted sample was added into each tube. Bradford's reagent was retrieved from fridge and 200 µl was added to each tube which was containing 10 µl of standards or sample. All of them was vortex and was allowed to stand for 30 minutes. 180 µl of each sample/standard solution mixed with the Bradford's reagent was added to their relevant curvette, and then analyse using a biophotometer was carried out. Standard curve was created using standards and against this curve the sample concentrations was measured as shown in Figure 3. For a Western blot 50 µg was required for sample loading, calculation was carried out as following the reading value /1000x40 (1000 to convert µg to mg) mg/ml. (40 the dilution factor).

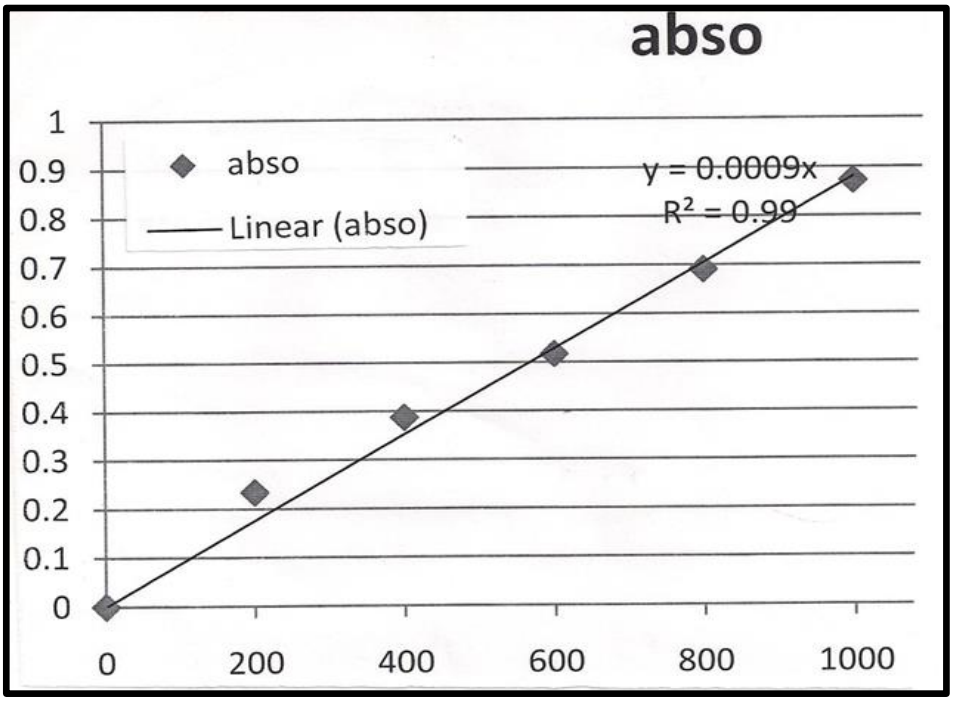

Figure 2-9: Picture represents one example of standard curve reading

2.10.3 Western blot

Sodium deodecyl sulphate (SDS) is used to denature and reduce the protein resulting in loss of the three dimensional structure of the protein. Migration of proteins by SDS polyacrylamide gel electrophoresis (SDS-PAGE) is determined by negative charge of the SDS. Separation of proteins is determined by the size of the pores of the gel thus a 10% resolving gel and 4% stacking gel was used.

2.10.3.1 Samples preparation and gel electrophoresis

Samples (including control) were mixed 1:1 with loading buffer and boiled on a heat block for 5 minutes at 95 C before loading. Each well was loaded with 50µl of protein. 20µl of pre-stained SDS-PAGE Standards (Low range, BIO-RAD, cat. no. 161-0305) was loaded to allow the size of proteins to be identified. 50 µl of positive control (placenta homogenate) was loaded into the gel and used as a reference sample. Samples were separated using the Protean II® xi Cell System (Bio-Rad Laboratories, Hemelhempstead, UK) at 300 V, 27 Amp for the first hour then turned up to 30 Amp, total time period of about 5 hours Figure 2-10. Summary of western blot protocol is as follows:

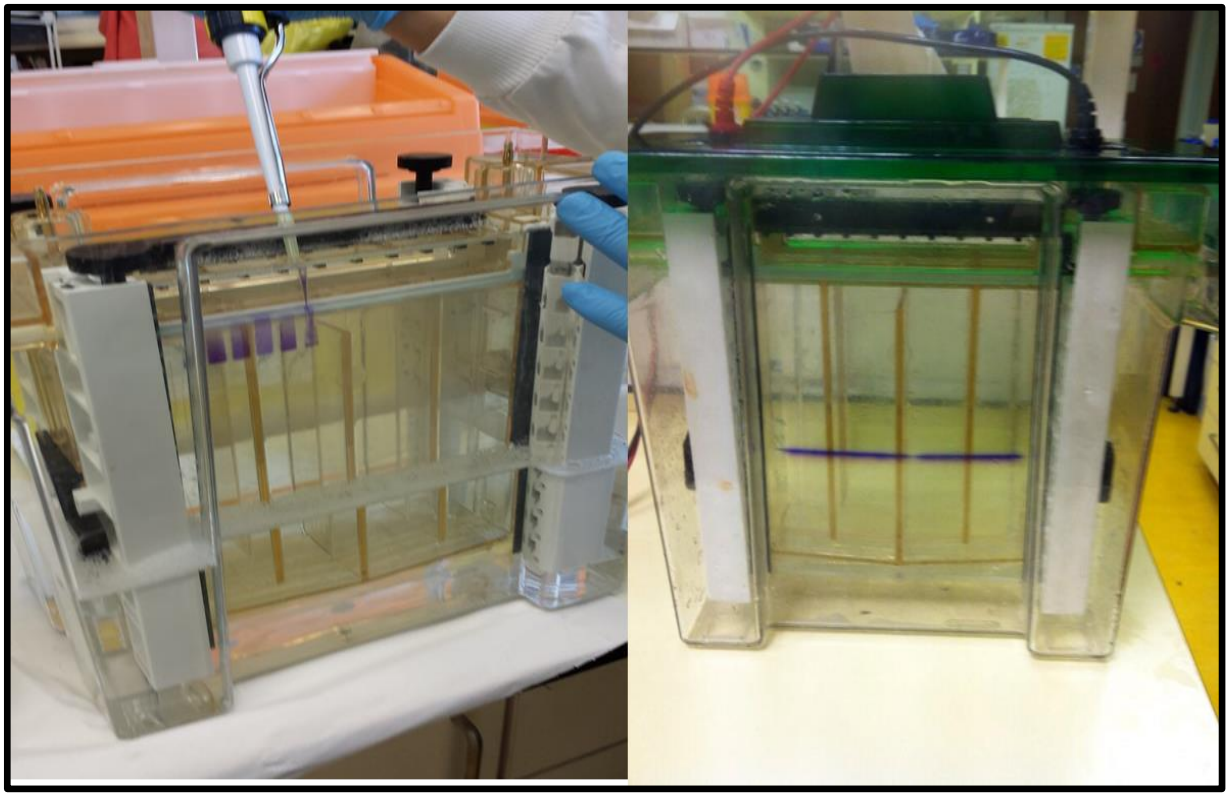

Figure 2-10: PROTEAN II xi systems by BIO-Rad Laboratories

2.10.3.2 Reagents for gel preparation:

Buffer A' for resolving gel:

- 1 litre of dH_2O, 181.65 g of Tris, and 40 ml of 10% SDS was added. Allowed to dissolve. pH to 8.8

Buffer B' for stacking gel:

1 litre of dH_2O, 60.55g of Tris, and 40 ml of 10% SDS solution was added. Allowed to dissolve. pH 6.8

Running buffer

3.8 L of dH_2O, 60.4 g of Tris, 288 glycine, 200 ml of 10% SDS solution. pH to 8.3

Running buffer diluted:

- 0.5 L running buffer (5x), 2 L of dH_2O.

Resolving and stacking gels: see Table 2-4.

Table 2-4: Resolving (left column) and stacking gels (right column) composition

Reagent	**Resolving gel (2 gels) (10%)**	**Stacking gel (2 gels) (4%)**
	Volume	Volume
30% acrylamide	24 ml	2.7 ml
50% glycerol	4.8 ml	N/A
Buffer A	18 ml	N/A
Buffer B	N/A	5 ml
Distilled water	24.8 ml	12.2 ml
10% AMPS	360 µl	100 µl
TEMED	36 µl	20 µl
Total	36x2 ml	20 ml

10% AMPS solution (AMPS-ammonium per sulphate)

- 1 ml of dH_2O, 0.1 g of ammonium persulfate (Sigma-Aldrich, cat. No. A3678-100G).
- TEMED: N,N,N,N tetramethylethylenediamine (Sigma-Aldrich, cat.no.T9281-100ML).

Sample preparation

- Samples (and control) were put on heat block for 5 minute at temp 95C.
- The central reservoir of a cooling tank was filled with running buffer to check for leaks (PROTEAN II xi system by Bio-Rad Laboratories, UK). See Figure 13.
- 20 µl of molecular ladder (BIO-RAD, 161-0305) was loaded onto one well of the gel, and once samples were heated, 50 µl of each was added to their relevant wells in the gel.
- Gels were run at 300 V, 27 Amp for first hour then up to 30 Amp for a total time period of about 5 hours until the dye front had reached the bottom.

2.10.3.3 Transfer of proteins to Nitrocellulose (semi-dry transfer):

Proteins were transferred to Whatman Protran® nitrocellulose transfer membranes (Sigma-Aldrich, cat. no. Z61360). Two blotting papers and the nitrocellulose paper were kept in transfer buffer for 20 minutes. The transfer was undertaken using the Trans-Blot SD Semi-Dry® Electrophoretic Transfer Cell (Bio-Rad, cat. no. 170-3940) with paper for blotting in the following order: 1) first blotting paper, 2) then the nitrocellulose paper, 3) then the gel on top of the nitrocellulose, 4) then the second piece of blotting paper. The transfer was performed at 22 Volts for 30 minutes, the current (mAmp) used depends on the dimensions of the gel (current= gel area x2.5) Figure 2-11. Once transfer was completed the nitrocellulose was stained with Ponceau S solution to determine that protein had been transferred. Once done, the nitrocellulose was destained in 0.1% NaOH solution Figure 2-11.

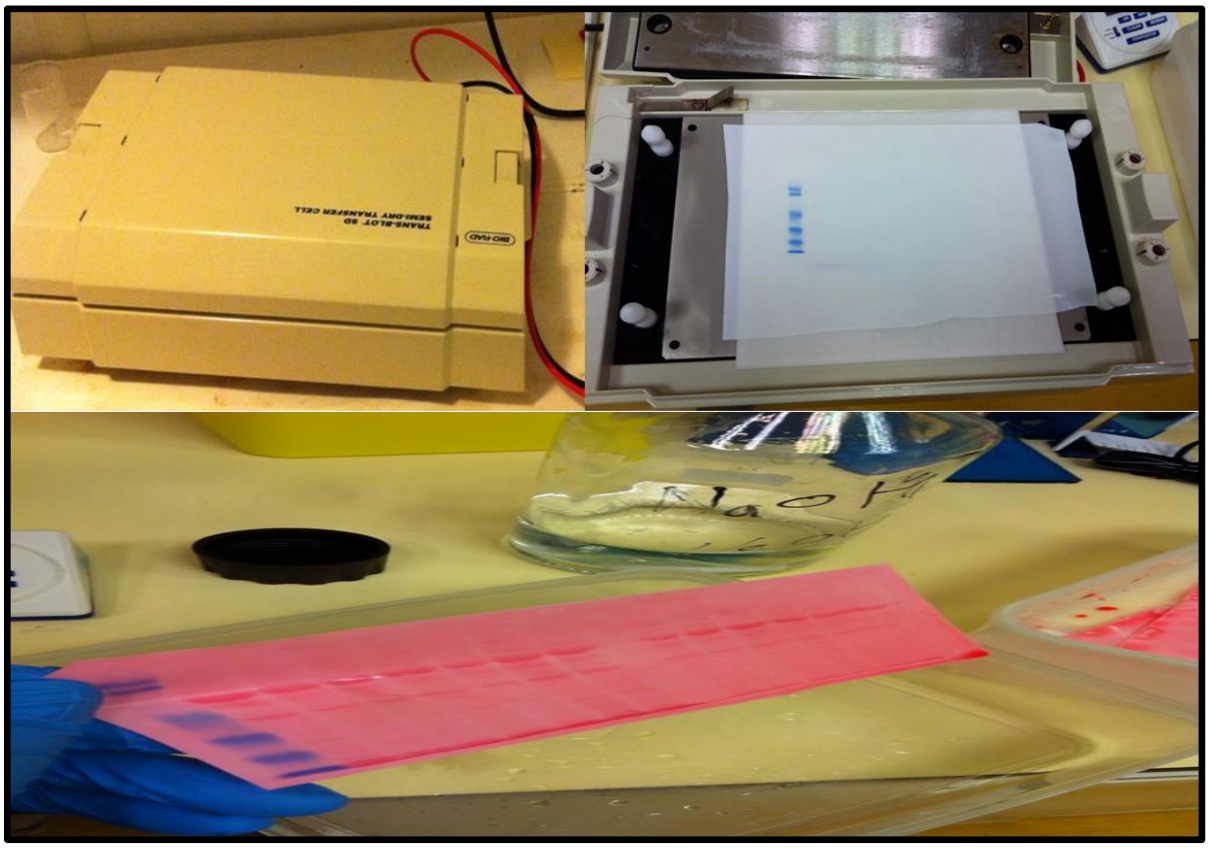

Figure 2-11: Upper panel: Semi-dry transfer cell and lower panel: Illumination of membrane by Ponceau S solution to ensure transfer.

Protocol summary

Reagents: Transfer buffer: 800 ml H2O, 3 g Tris, 14.4 g glycine and 200 ml methanol. Once the gels have run the stacker gel and bottom of the resolving gel were removed and both were discard. Remaining gel was measured and put into a box containing the 2 pieces of blotting paper. Nitrocellulose paper was cut (20 cm x 15 cm) and was put into the box containing transfer buffer for 20 minutes. Semi dry transfer cell (BIO RAD, 170-3940) was retrieve and then 20 minutes later one piece of blotting paper was placed and then the nitrocellulose, gel was placed on top of the nitrocellouse, and then the second piece of blotting paper was placed on the top. Transfer at 22 V for 30 minutes was done, the current (mA) used depends on the dimensions of the gel (current= gel area x2.5).Visualise the nitrocellulose was carried out using Ponceau S solution (Figure 14) in order to check protein presence. Once done, de-stain nitrocellulose in 0.1% NaOH solution was carried out.

2.10.3.4 Immuno-detection of proteins (Immune-blotting)

Reagent: TBSTB (1 Litre): 1L of dH_2O, 2.4g Tris, 3ml of concentrated HCL (pH 7.5), 29.2g NaCL, 4ml Tween 20 and 2.5g BSA. Summary of protocols for immune detection of protein is in Tables 5, 6, and 7).

Membranes were blocked in 5% normal donkey serum (Serotec, cat. no CO6SBZ) for PON3 or 5% normal goat serum (Sigma-Aldrich, cat.G9023) for PON2 Table 2-5.

Table 2-5: Blocking protocol for PON2 and PON3

PON2 blocker	PON3 blocker
5% normal goat serum in TBSTB for 1 hour at room temperature.	5% normal donkey serum in TBSTB for 1 hour at room temperature.

Primary antibodies were pre-absorbed for 30 minutes in 5% normal human serum (Sera Laboratories International, cat.no. S-123-H) in TBSTB at room temperature during the blocking stage. Membranes were incubated with appropriate primary antibodies solution, for example: PON2 (Santa Cruz cat.no. sc-373981), PON3 (Santa Cruz, cat.no. sc-21152) Table 2-6.

Table 2-6: Primary antibodies protocol for PON2 and PON3

PON2 primary antibody	PON3 primary antibody
5% Normal Human Serum (NHS) in TBSTB with Mouse monoclonal anti human PON2 primary antibody (Santa Cruz cat.no. sc-373981) (1:200) for 1 hour at room temperature	5% Normal Human Serum (NHS) in TBSTB with Goat polyclonal anti human PON3 primary antibody (Santa Cruz, cat.no. sc-21152) (1:200) for 1 hour at room temperature.

Membranes were rinsed and washed in TBSTB (3X5 minutes), and then incubated with appropriate horseradish peroxidase conjugated secondary antibodies for PON2 (Goat anti-mouse IgG-HRP, Santa Cruz, cat.no. sc-2005), for PON3 (Donkey anti-goat IgG-HRP, Santa Cruz, cat.no. sc-2020) for 1 hour at room temperature Table 2-7.

Table 2-7: Secondary antibodies protocol for PON2 and PON3

PON2 secondary antibody	PON3 secondary antibody
Goat anti-mouse secondary antibody 1:2000 For 1 hour at room temperature	Donkey anti-goat secondary antibody 1:2000 For 1 hour at room temperature

The membranes were washed in TBSTB for 2x5 minutes and in water for 1x5 minutes after secondary antibody incubation.

2.10.3.5 Detection

Immunologically reactive proteins were visualised using the Amersham ECL® Western Blot detection system (Amersham Pharmacia Biotech, GE healthcare, cat. No RPN2106 Figure 2-12. Membranes were placed in a film cassette and films were exposed in a dark room for an appropriate period of time (e.g. 1 min, 30 sec then 10 sec) to obtain the correctly exposed bands suitable for scanning. Exposures were selected that were not saturated. Bands on films were scanned using a Bio-Rad GS-700® imaging densitometer. The reading for placental control sample (same one used in all experiments) was used as internal control and the density of the other bands was expressed relative to it.

Figure 2-12: Detecting using ECL solution for 1minute

2.10.4 Loading controls

2.10.4.1 β Actin

Actin β was used as loading control to ensure the equal loading among samples Figures 3-12 and 3-15. β Actin protocol according to the following Table 2-8:

Table 2-8: Actin β protocol

Blocker	Primary antibody	Secondary antibody
5% normal donkey serum in TBSTB for one hour at room temperature.	Anti-actin Rabbit polyclonal antibody (ab8227) in TBSTB and pre-absorbed in 5% normal human serum 1:2000 for one hour at room temperature.	HRP-donkey anti rabbit secondary antibody (ab7083) in TBSTB 1:3000 for one hour at room temperature.

2.10.4.2 Ponceau S Solution

Ponceau S solution was used to ensure the protein transfer and to show equal loading among samples Figure 2-13.

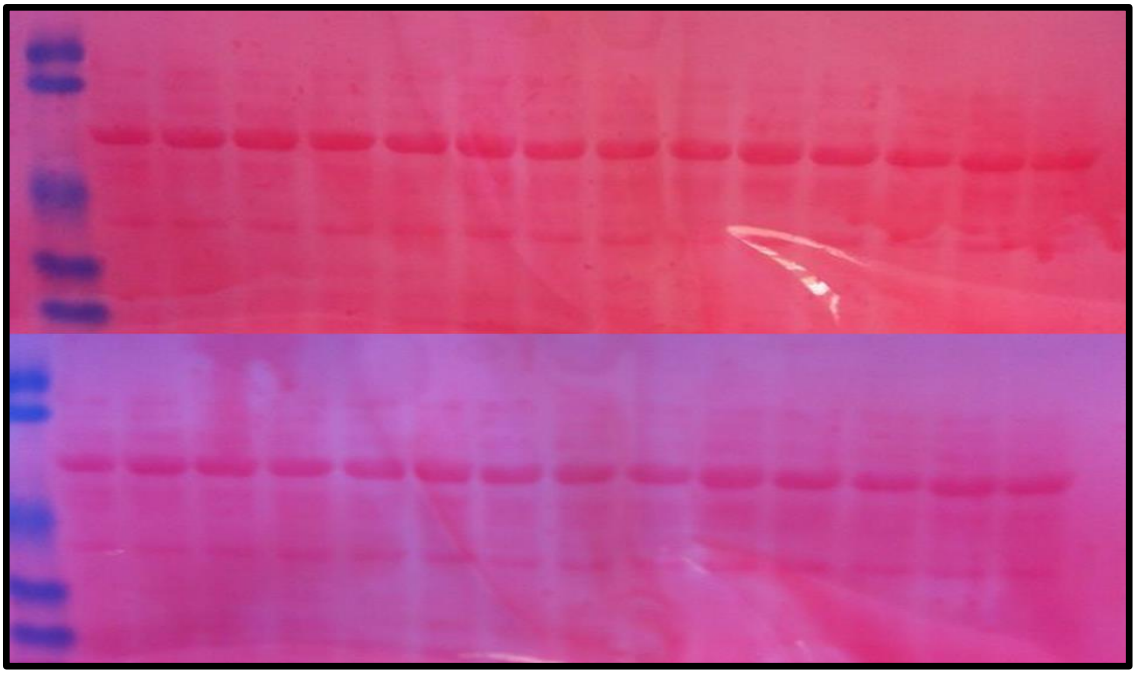

Figure 2-13: Example of two membranes illuminated with Ponceau S solution, which used as a loading control.

2.11 Hypoxia-normoxia (model of ischemic-reperfusion injury) tissue culture

The reperfusion experiments was undertaken to investigate the effect of exposing placenta tissue to 2% (hypoxia) for one hour followed by exposure to 8% (normoxia) O_2 for 4 time points (2, 6, 12 and 22 hours). The oxygen concentration in placenta tissue is 8%. This tissue experiment was done to recreate the type of reperfusion of placenta that occurs in labour and PE when vessels contract and relax and to examine this effect on expression of selected genes. The experiments carry out as follows Table 2-9:

Table 2-9: Preparation steps for reperfusion experiment

Preparation (day before experiment)
• The internal surface of incubators and cell culture chamber were cleaned using 70% ethanol the day before experiment. • The incubators were set up 24 hours before tissue collection: NuAir incubator 1 at 2% O_2 (hypoxia)

NuAir incubator 2 at 8% O_2 (normoxia)

- Six 24 well culture plates and three T5 culture flasks were retrieved.
- Every 20 ml of M199 media was supplemented with 1ml of FBS (fetal bovine serum) and 0.2ml Ab/Am (antibiotics/ antimycotics).

Medium199 (with Earle's salts, L-glutamine and sodium bicarbonate, liquid, sterile-filtered, cell culture tested) (Sigma, cat. no. M4530-100ML).

5% fetal bovine serum (heat inactivated) (Sigma-Aldrich, cat. no. F9665)

1% antibiotic antimycotic solution (Sigma-Aldrich, cat. no. A5955)

- 10 ml of supplemented media was added to the labelled culture flasks and 1 ml of supplemented media was added to 6 wells of six 24 well culture plates.
- Plates (24 well culture plates) were put into each incubator that was set to the desired oxygen concentration for 24 hour prior to tissue collection so that that desired oxygen concentration was reached.
- Three T5 culture flasks with 10 ml of supplemented media were put in 8% O2 incubator that closes to placenta oxygen concentration for 24 hours before tissue collection.

The experiment:

- Step one: Normal placenta tissue was collected. The T5 culture flasks were removed from the incubator. For base line control three piece of placenta tissue (inner, middle and outer areas) were removed and immediately flash snap in liquid nitrogen. The rest of each piece of tissue collected was cut into three parts and put into 8% O_2.

In culture lab:

- Step two: The tissue was taken to the laboratory at Yorkhill Hospital and the second reference point of placenta tissue was taken.
- Step three: The placenta tissue was removed quickly and cut up into equal sized pieces. The 24 wells culture plates were removed from both incubators and placenta tissues were distributed into an equal number of wells in each plate, after which the tissue culture plates were retained back to their relevant incubators.
- Step four: The hypoxia exposure time for all placental pieces (inner, middle and outer) was 1 hour at 2% O_2, after which the tissues plates were removed, placed in

pre-equilibrated 8% O_2 medium and transferred to the 8% O_2 incubator. The experimental times were after 2, 6, and 12 and 22 hours after the 2% O_2 plates were placed in 8% O_2 incubator. Control samples underwent the same process except the first hour was in 8% O_2 medium rather than 2% O_2.

- Step five: at each time point (2, 6, 12, 22 hours) the relevant tissue plate were taken from the incubator and tissue at inner, middle and outer areas were snap frozen in liquid nitrogen. 1ml of tissue culture media were taken in each point and stored for cytokine analysis should time allow in the future.

RNA were then extracted from the frozen tissue and RNA was later reverse transcribed into cDNA and used for subsequent investigation of PON2 and PON3 mRNA analysis as described earlier.

Tissue protein was obtained using the homogenisation process described earlier and PON2 and PON3 protein expression were investigated using the Western blot method.

2.12 Statistical analysis:

Statistical analysis was performed using Graph Pad prism 5 using analysis of variance for non-parametric data (Friedman test) for 3 groups. Comparison of two groups was performed by the Mann Whitney test. Graphs show box and whiskers plots. The hypoxia-normoxia experiment statistical analysis was performed using Minitab 17, using two ways ANOVA to measure the response compared to two factors (hypoxia and time). Graphs show interaction plots.

3 Results

3.1 PON2 expression in the labour group

Experiment 1: This experiment was designed to test if there was a spatial difference in expression of PON2 within individual placentas obtained from women who were not in labour. Two main bands were detected in all placenta samples, one at 62 kDa and one at 43 kDa. These are named as isoform 1 62kDa and isoform 43 kDa throughout. Examples of Western blots showing PON2 (isoform 1, 62 kDa) expression for 3 different placentas (all non-labour) are shown in Figure 3-1 (blots A-C). Examples of Western blots showing PON2 (isoform 2, 43 kDa) expression for the same three placentas are also shown in Figure 17 (blots D-F). Friedman test analysis showed there was no difference in expression of either isoform between the three sites (inner, middle, outer) within individual placentas. The graphs for blots A-C (isoform 1, 62 kDa) are shown in Figure 18 (A-C) and the graphs for blots D-F (isoform 2, 43 kDa) are shown in Figure 3-2 (D-F).

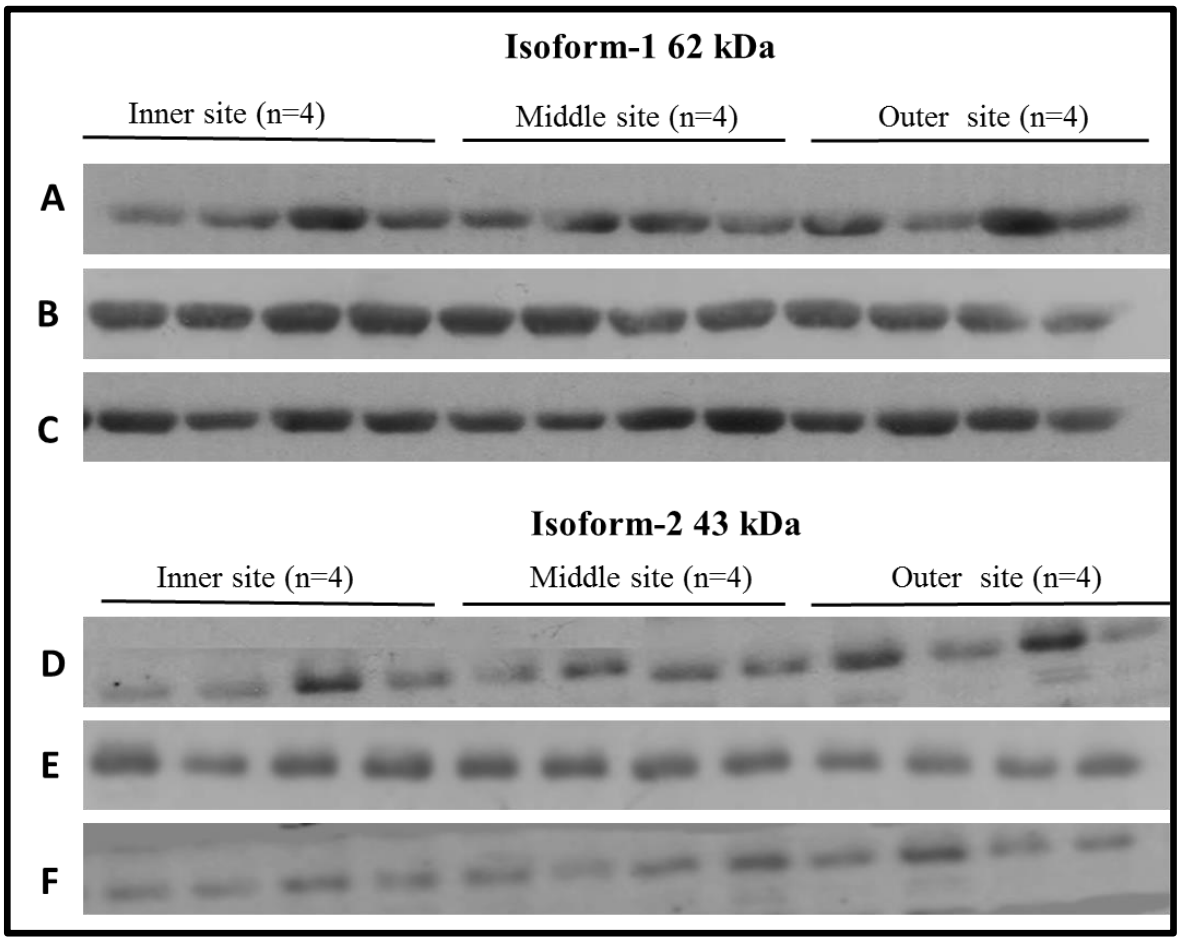

Figure 3-1: Western blots showing PON2 expression in inner, middle and outer sites of three individual placentas (non-labour group). Four quadrants were sampled in each zone.

A, B and C show the 62 kDa isoform 1 for the three placentas shown in Figure 17. D, E and F show the 43 kDa isoform 2 for the same three placentas also shown in Figure 17.

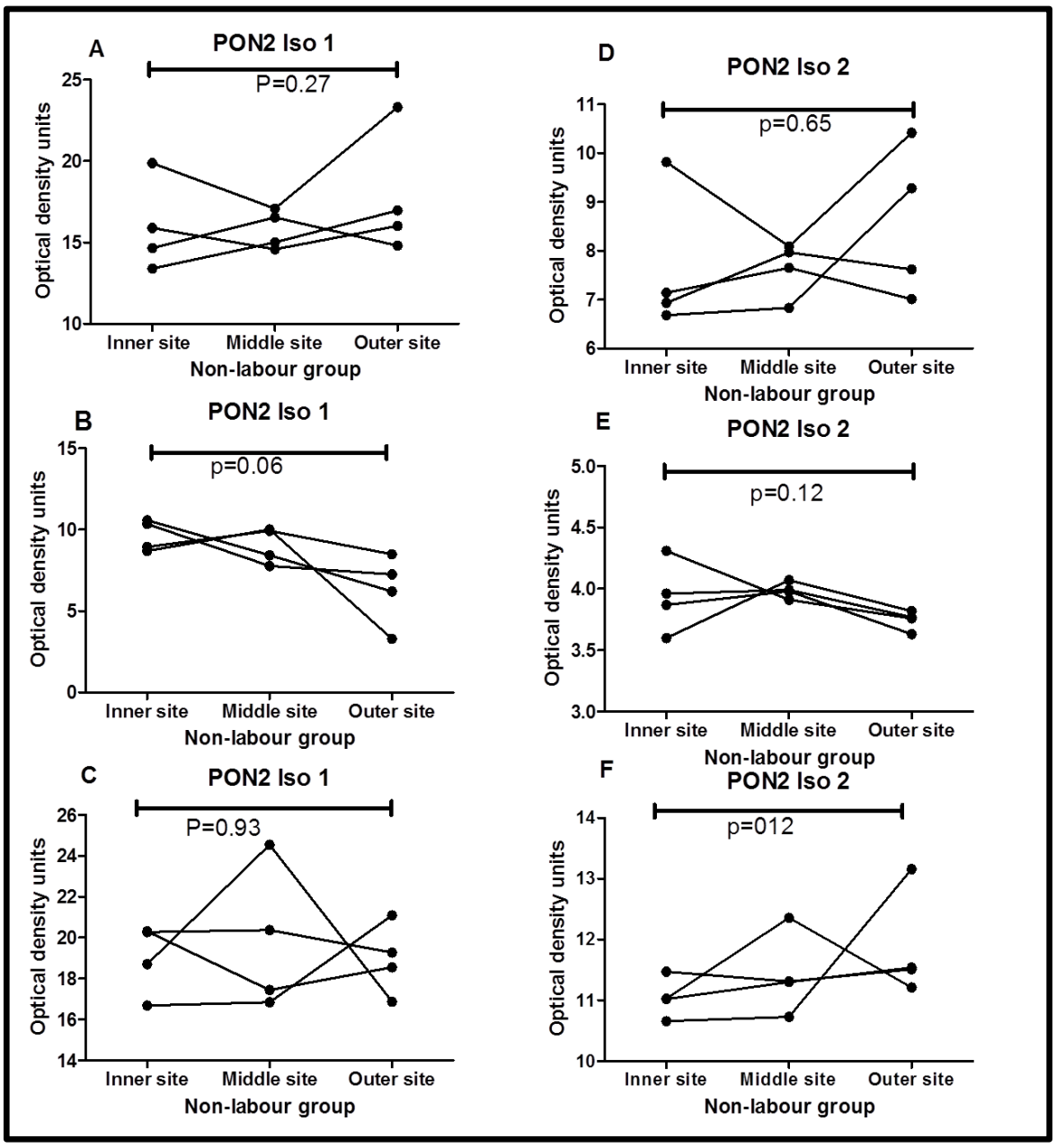

Figure 3-2: Graphs show median and interquartile range for PON2 isoforms 1 and 2 for the combined 4 quadrants sampled in the inner, middle and outer zones of three different placentas (A-C, D-F non-labour group) shown in Figure 17. Comparison between zones was performed using Friedman analysis**.**

Experiment 2: This experiment was designed to test if there was a spatial difference in expression of PON2 within individual placentas obtained from women who were in labour. Two isoforms (62 kDa and 43 kDa) were also identified in all placenta samples. Examples of Western blots showing PON2 (isoform 1, 62 kDa) expression for 3 different placentas

(all labour) are shown in Figure 3-3 (blots A-C). Examples of Western blots showing PON2 (isoform 2, 43 kDa) expression for the same three placentas are also shown in Figure 19 (blots D-F). Friedman test analysis showed there was also no difference in expression of either isoform between the three sites (inner, middle, outer). The graphs for blots A-C (isoform 1, 62 kDa) are shown in Figure 20 (A-C) and the graphs for blots (isoform 2, 43 kDa) are shown in Figure 3-4 (D-F).

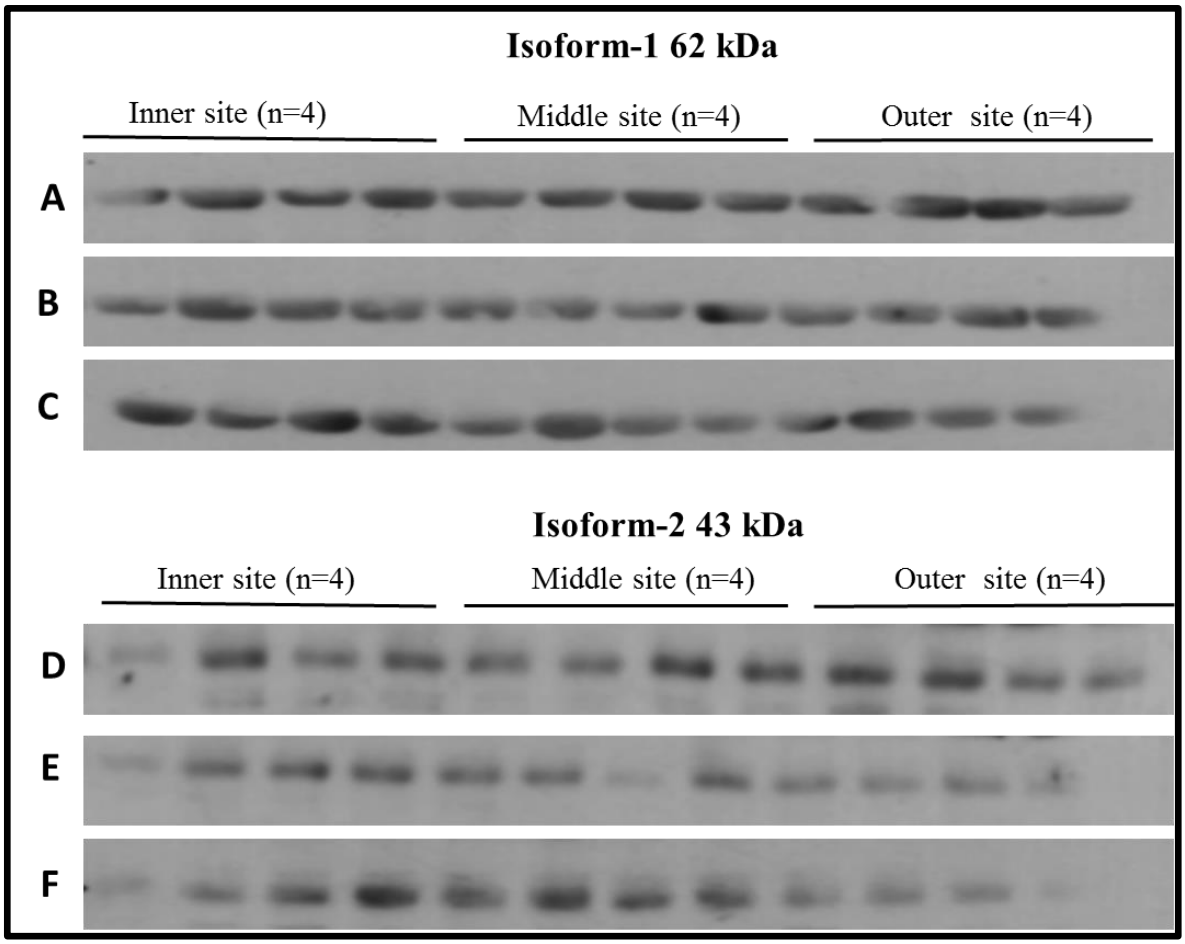

Figure 3-3: Western blots showing PON2 expression in inner, middle and outer sites of three individual placentas (labour group). Four quadrants were sampled in each zone. A, B and C show the 62 kDa isoform 1. D, E and F show the 43 kDa isoform 2.

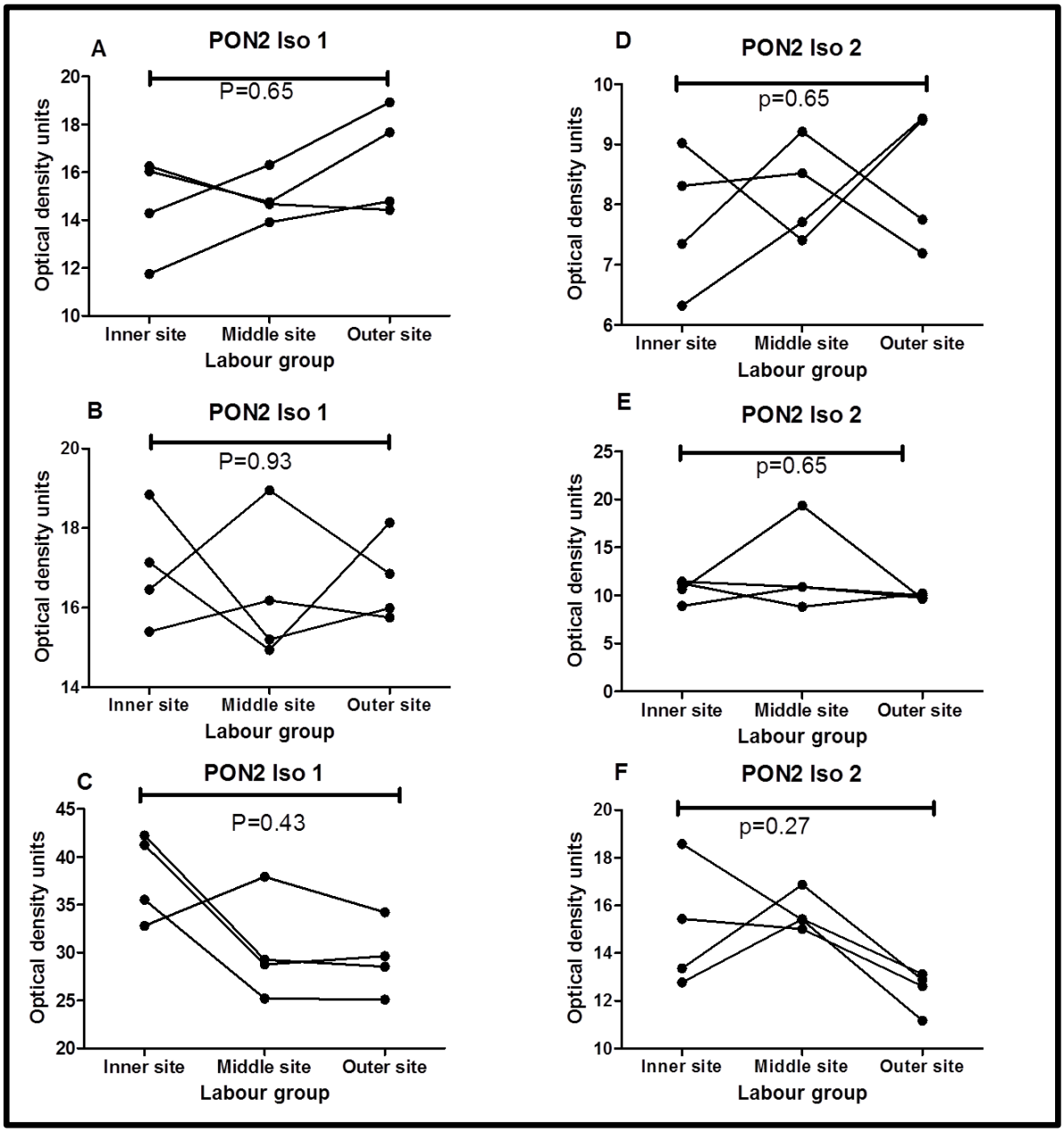

Figure 3-4: Graphs show median and interquartile range for the 4 quadrants sampled in the inner, middle and outer zones of each of the three placentas (labour group) shown in Figure 19. Comparison between zones was performed using Friedman analysis.

In summary for experiment 1 and 2, there was no spatial difference in expression of either PON2 isoform within individual placentas both in non-labour and in labour groups.

Experiment 3: This experiment was designed to test if there was any difference in PON2 expression between labour and non-labour groups at the inner, middle or outer areas of placentas. Western blots showing placental PON2 expression in non-labour and labour at the inner site are shown in Figure 3-5 (upper panel 62 kDa isoform 1; lower panel 43 kDa isoform 2). The graphs and statistical analysis are shown below the blots. No differences were found.

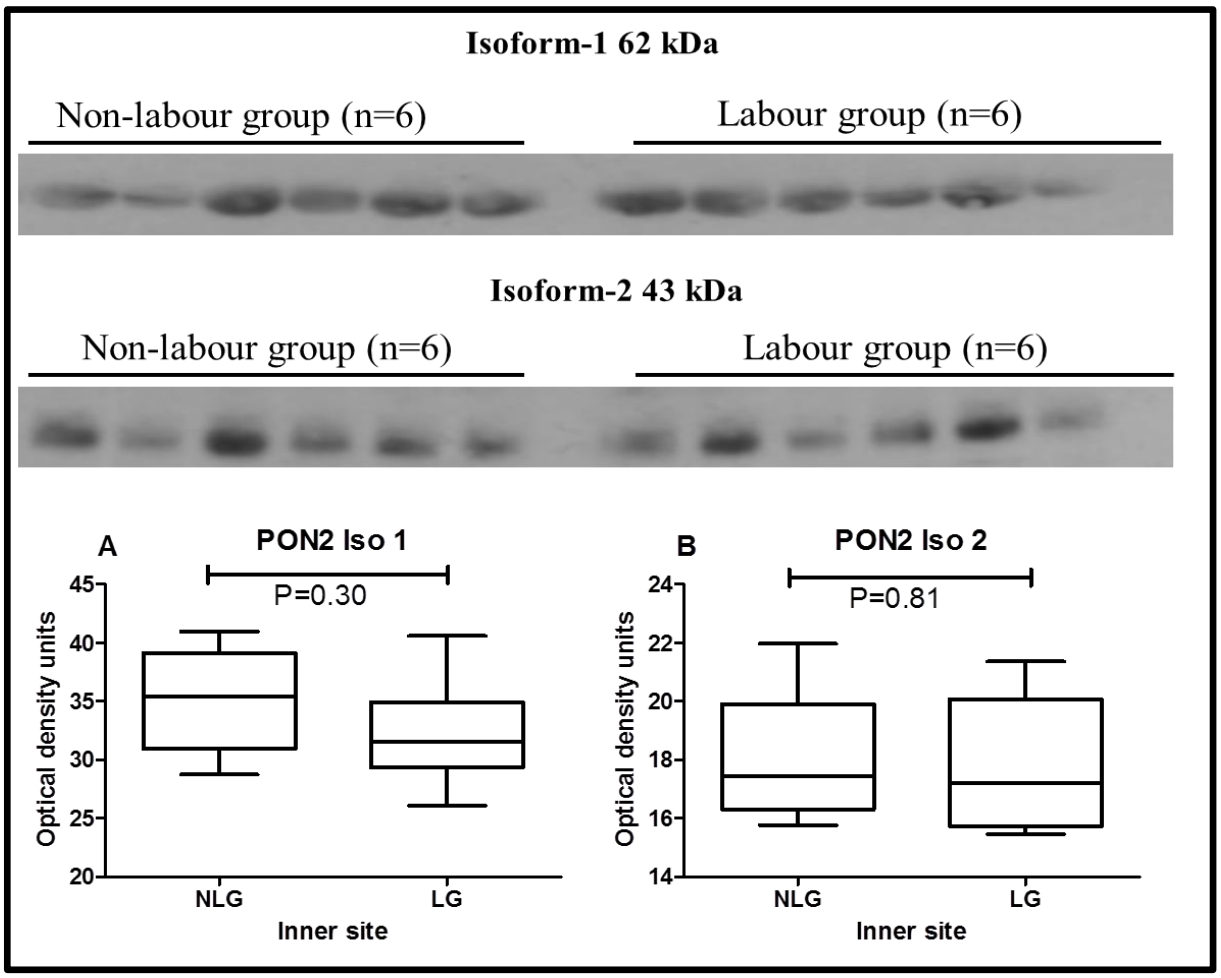

Figure 3-5: Western blots showing PON2 62 kDa isoform 1 (upper panel) and PON2 43 kDa isoform 2 (lower panel) expressions in the inner placental site in non-labour and labour (n=6 in each group). The graphs show the box and whiskers analysis of the blots. Comparison between zones was performed using Mann-Whitney analysis. Graph A, 62 kDa isoform 1; Graph B, 43 kDa isoform 2. NLG non-labour group, LG labour group.

Western blots showing placental PON2 expression in non-labour and labour at the middle site are shown in Figure 3-6 (upper panel 62 kDa isoform 1; lower panel 43 kDa isoform 2). The graphs and statistical analysis are shown below the blots. There was a highly significant decrease in PON2 expression in the labour group when compared to the non-

labour group for both the 62 kDa isoform 1, (p=0.02) and the 43 kDa isoform 2, (p=0.006).

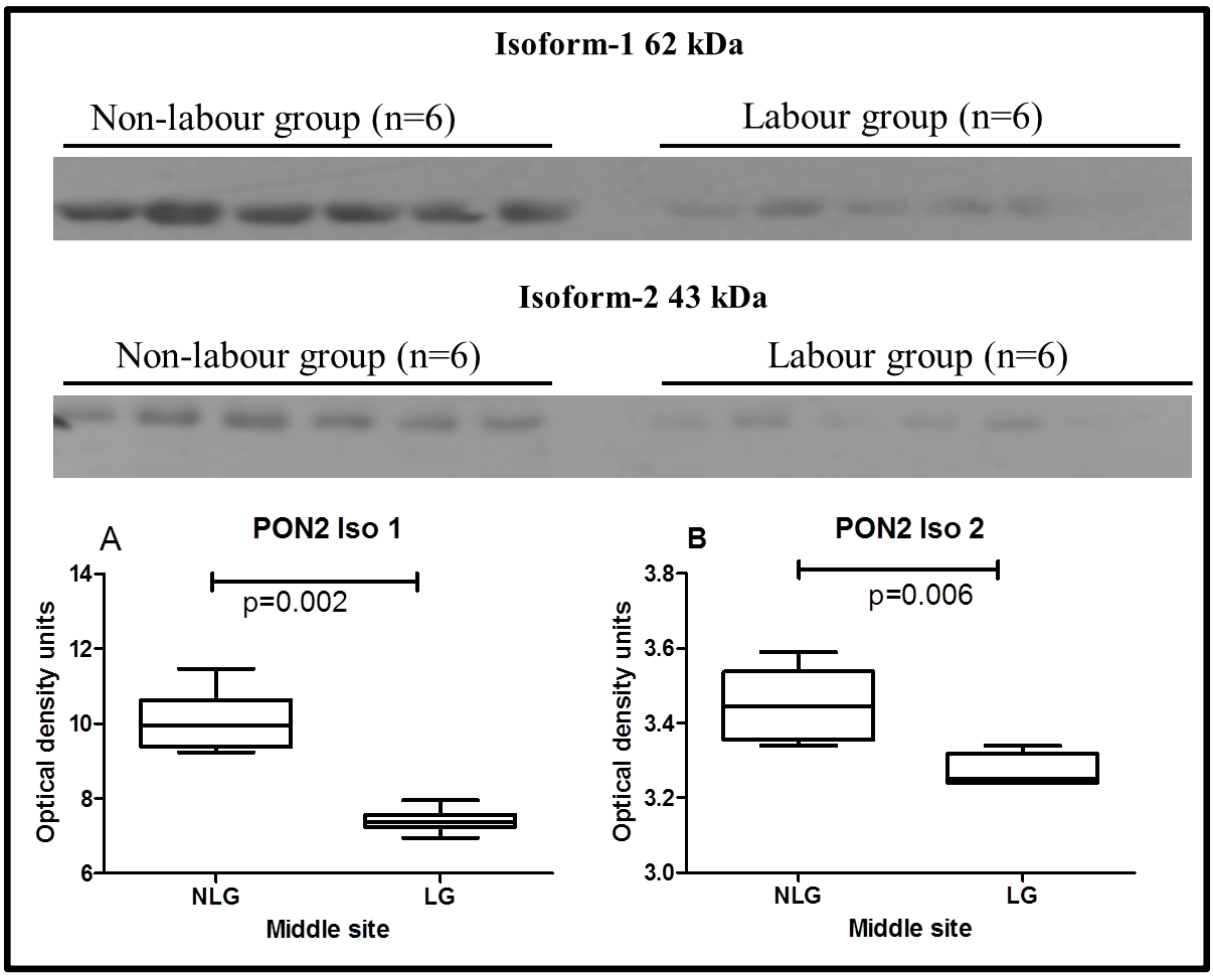

Figure 3-6: Western blots showing PON2 62 kDa isoform 1 (upper panel) and PON2 43 kDa isoform 2 (lower panel) expressions in the middle placental site in non-labour and labour (n=6 in each group). The graphs show the box and whiskers analysis of the blots. Comparison between zones was performed using Mann-Whitney analysis. Graph A, 62 kDa isoform 1; Graph B, 43 kDa isoform 2. NLG non-labour group, LG labour group.

Western blots showing placental PON2 expression in non-labour and labour at the outer placental site are shown in Figure 3-7 (upper panel 62 kDa isoform 1; lower panel 43 kDa isoform 2). The graphs and statistical analysis are shown below the blots. No differences were found.

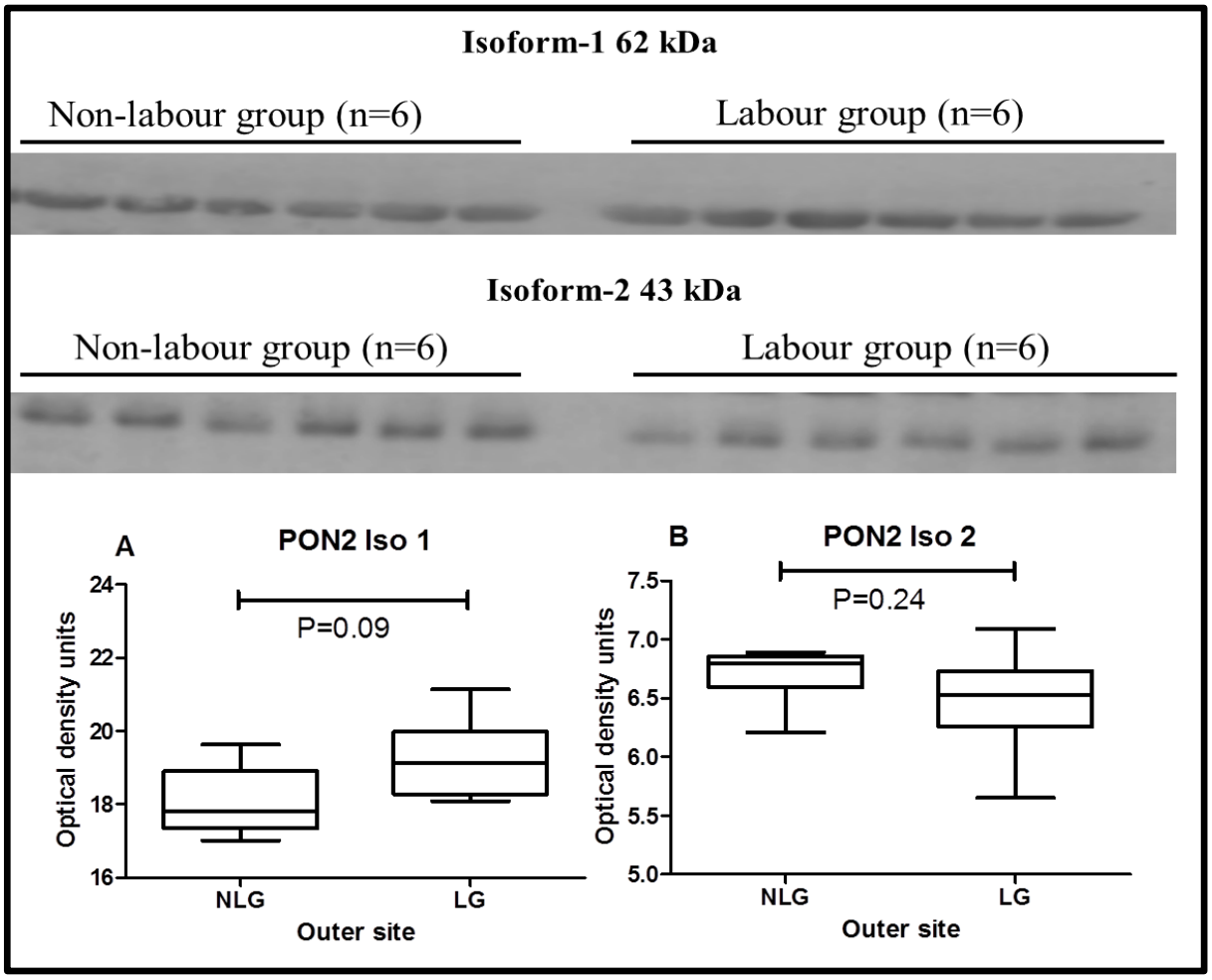

Figure 3-7: Western blots showing PON2 62 kDa isoform 1 (upper panel) and PON2 43 kDa isoform 2 (lower panel) expressions in the outer site in non-labour and labour (n=6 in each group). The graphs show the box and whiskers analysis of the blots. Comparison between zones was performed using Mann-Whitney analysis. Graph A, 62 kDa isoform 1; Graph B, 43 kDa isoform 2. NLG non-labour group, LG labour group.

Experiment 4: This experiment was designed to test if there were any differences in PON2 mRNA expression within individual placentas at different zones in either labour or non-labour. Figure 3-8 shows the PON2 RQ values in the inner, middle and outer zones for non labour (A) and labour (B). Just as for both PON2 protein isoforms, no spatial differences were found at the mRNA level in either labour or non-labour.

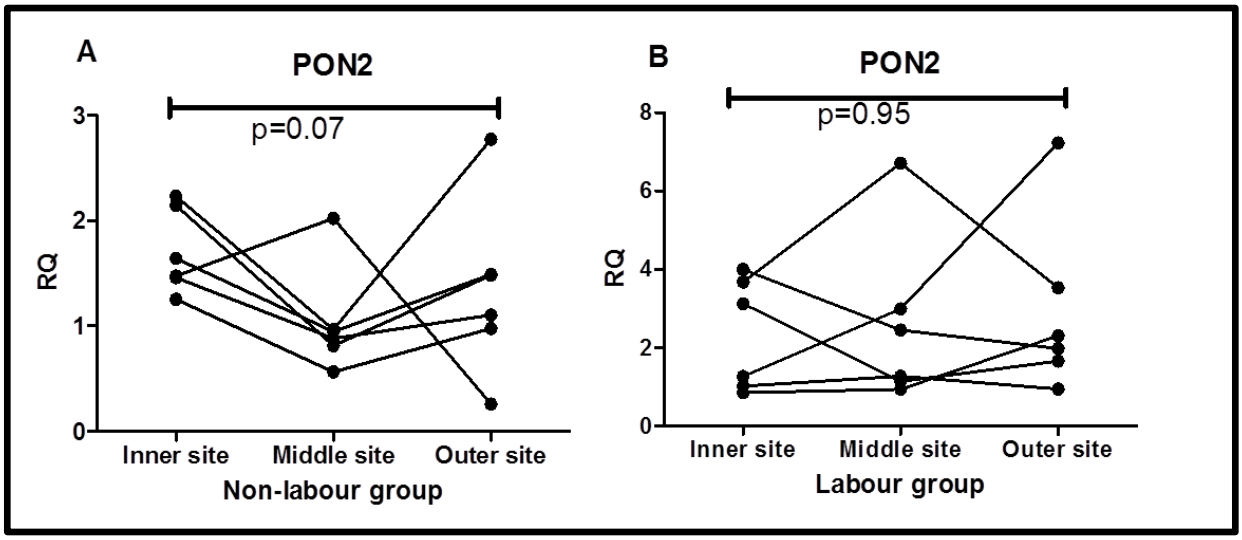

Figure 3-8: RQ values for mRNA measurements in inner, middle and outer placental sites within individual placentas. A non-labour and B labour. Comparison between zones was performed using Friedman analysis.

Experiment 5 was designed to test whether there was any difference between labour and non-labour at the inner, middle our outer sites of PON2 at the mRNA level. The results are shown in Figure 3-9. As for PON2 protein no differences were found between labour and non-labour at the inner or outer placental sites. There was, paradoxically, an increase in PON2 mRNA in the labour group at the middle site.

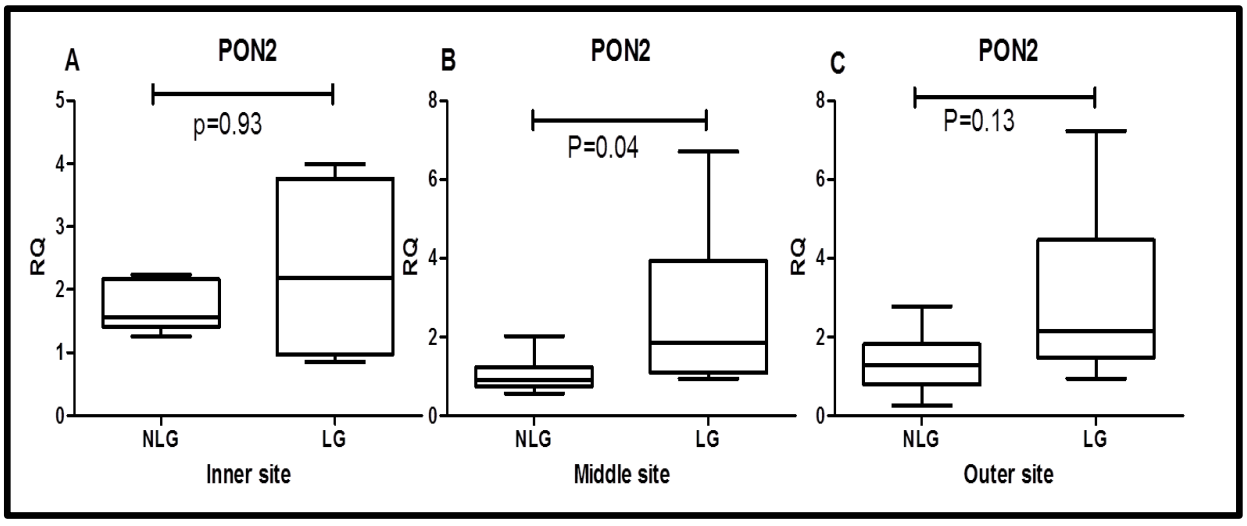

Figure 3-9: RQ values for mRNA measurements in inner (A), middle (B) and outer (C) placental sites for labour compared with non-labour. Comparison between zones was performed using Mann-Whitney analysis.

3.2 PON2 expression in pre-eclampsia placentas

Experiment 1: This experiment was designed to test if there was any difference in PON2 expression between non-labour control and non-labour PE groups at the inner, middle or outer sites of placentas. Western blots showing placental PON2 expression in non-labour control and non-labour PE at the inner site are shown in Figure 3-10 (upper panel 62 kDa isoform 1; lower panel 43 kDa isoform 2). The graphs and statistical analysis are shown below the blots. No differences were found.

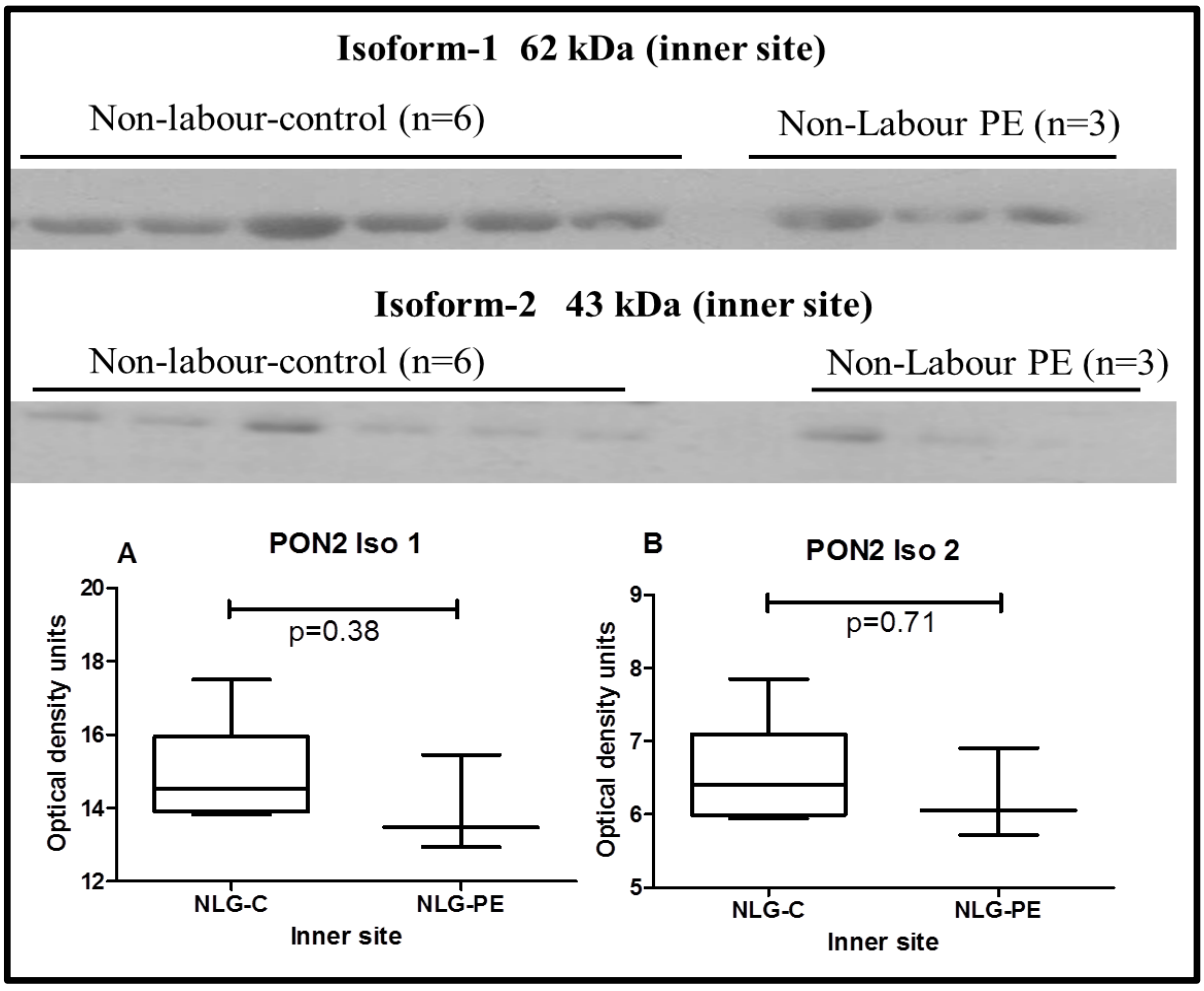

Figure 3-10: Western blots showing PON2 62 kDa isoform 1 (upper panel) and PON2 43 kDa isoform 2 (lower panel) expressions in the inner placental site in non-labour control (n=6) and non-labour PE (n=3). The graphs show the box and whiskers analysis of the blots. Comparison between zones was performed using Mann Whitney analysis. Graph A, 62 kDa isoform 1; Graph B, 43 kDa isoform 2. NLG-C (non-labour control group), NLG-PE (non-labour pre-eclampsia group).

Western blots showing placental PON2 expression in non-labour control and non-labour PE at the middle site are shown in Figure 3-11 (upper panel 62 kDa isoform 1; lower panel 43 kDa isoform 2). The graphs and statistical analysis are shown below the blots. There

was a highly significant decrease in PON2 expression in the non-labour PE group when compared to the non-labour control group for both the 62 kDa isoform 1, (p=0.02) and the 43 kDa isoform 2, (p=0.02). Figure 3-12 shows an example of β-actin analysis. Shown are the same samples used in Figure 3-11. There was no difference between the non-labour control and non-labour PE groups despite the reduction in the PON2 in the non-labour PE group.

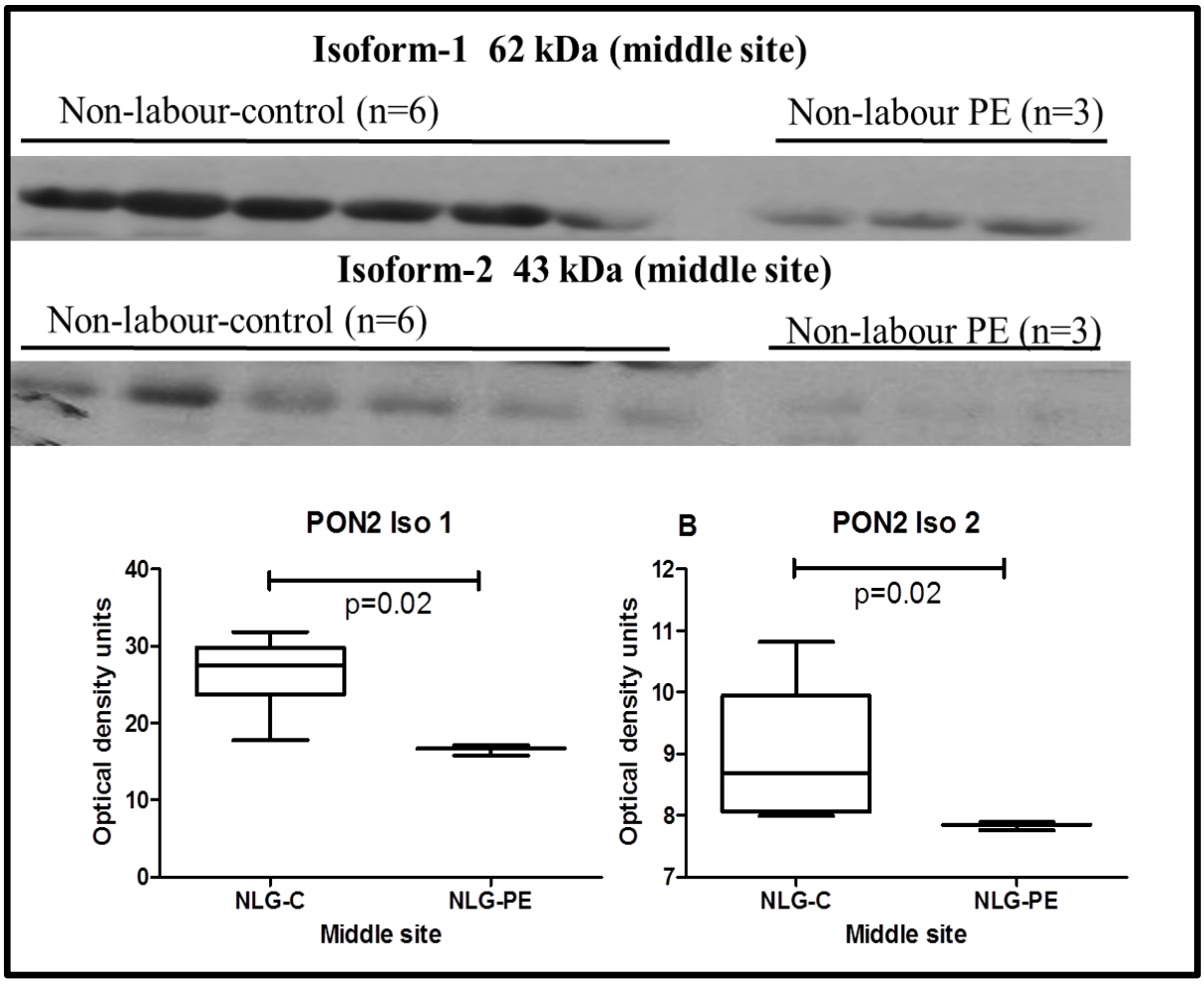

Figure 3-11: Western blots showing PON2 62 kDa isoform 1 (upper panel) and PON2 43 kDa isoform 2 (lower panel) expressions in the middle placental site in non-labour control (n=6) and non-labour PE (n=3). The graphs show the box and whiskers analysis of the blots. Comparison between zones was performed using Mann Whitney analysis. Graph A, 62 kDa isoform 1; Graph B, 43 kDa isoform 2. NLG-C (non-labour control group), NLG-PE (non-labour PE group).

B-actin is expressed in cells and is commonly used as a reference for loading. It is important to adjust the sample reading (western blot protocol) to for any observed changes in β actin protein levels. The equality of loading was tested by using actin-β as a loading control. Western blot showing actin-β expression in the non-labour control and non-labour

PE at the middle site are shown in figure 3-12. No differences were found between β actin in the groups compared in this thesis.

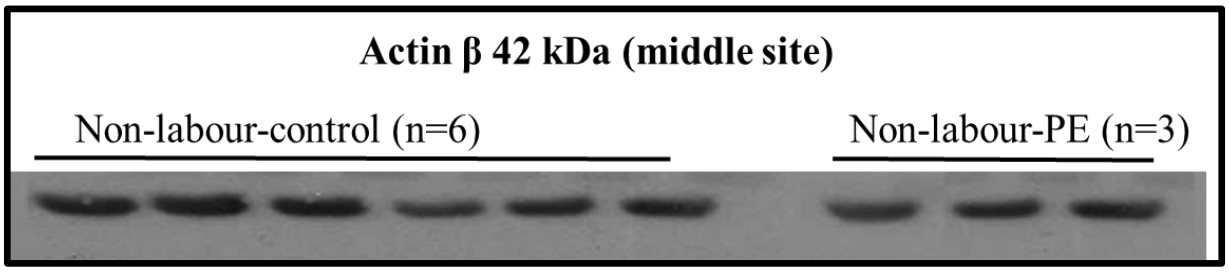

Figure 3-12: Western blots showing actin-β antibody as loading control to compare the same placenta samples in Figure 27. Non-labour control group compared to the non-labour PE group at the middle site.

Western blots showing placental PON2 expression in non-labour control and non-labour PE at the outer site are shown in Figure 3-13 (upper panel 62 kDa isoform 1; lower panel 43 kDa isoform 2). The graphs and statistical analysis are shown below the blots. No differences were found.

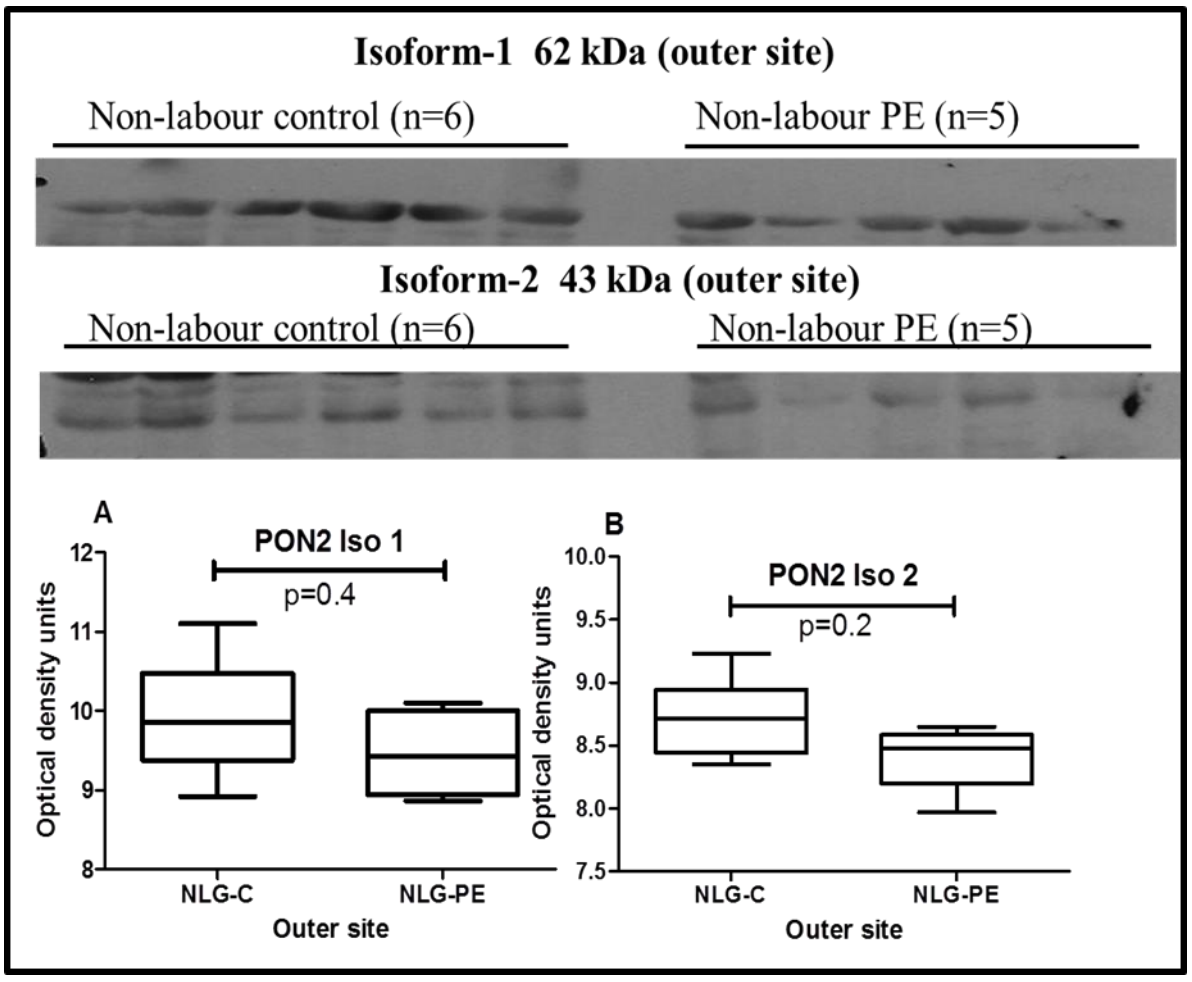

Figure 3-13: Western blots showing PON2 62 kDa isoform 1 (upper panel) and PON2 43 kDa isoform 2 (lower panel) expressions in the outer placental site in non-labour control (n=6) and non-labour PE (n=5). The graphs show the box and whiskers analysis of the blots. Comparison between zones was performed using Mann Whitney analysis. Graph A, 62 kDa isoform 1; Graph B, 43 kDa isoform 2. NLG-C (non-labour control group), NLG-PE (non-labour PE group).

Experiment 2: This experiment was designed to test if there was any difference in PON2 expression between labour control and labour PE groups at the inner, middle or outer sites of placentas. Western blots showing placental PON2 expression in labour control and labour PE at the inner site are shown in Figure 3-14 (upper panel 62 kDa isoform 1; lower panel 43 kDa isoform 2). The graphs and statistical analysis are shown below the blots. No differences were found.

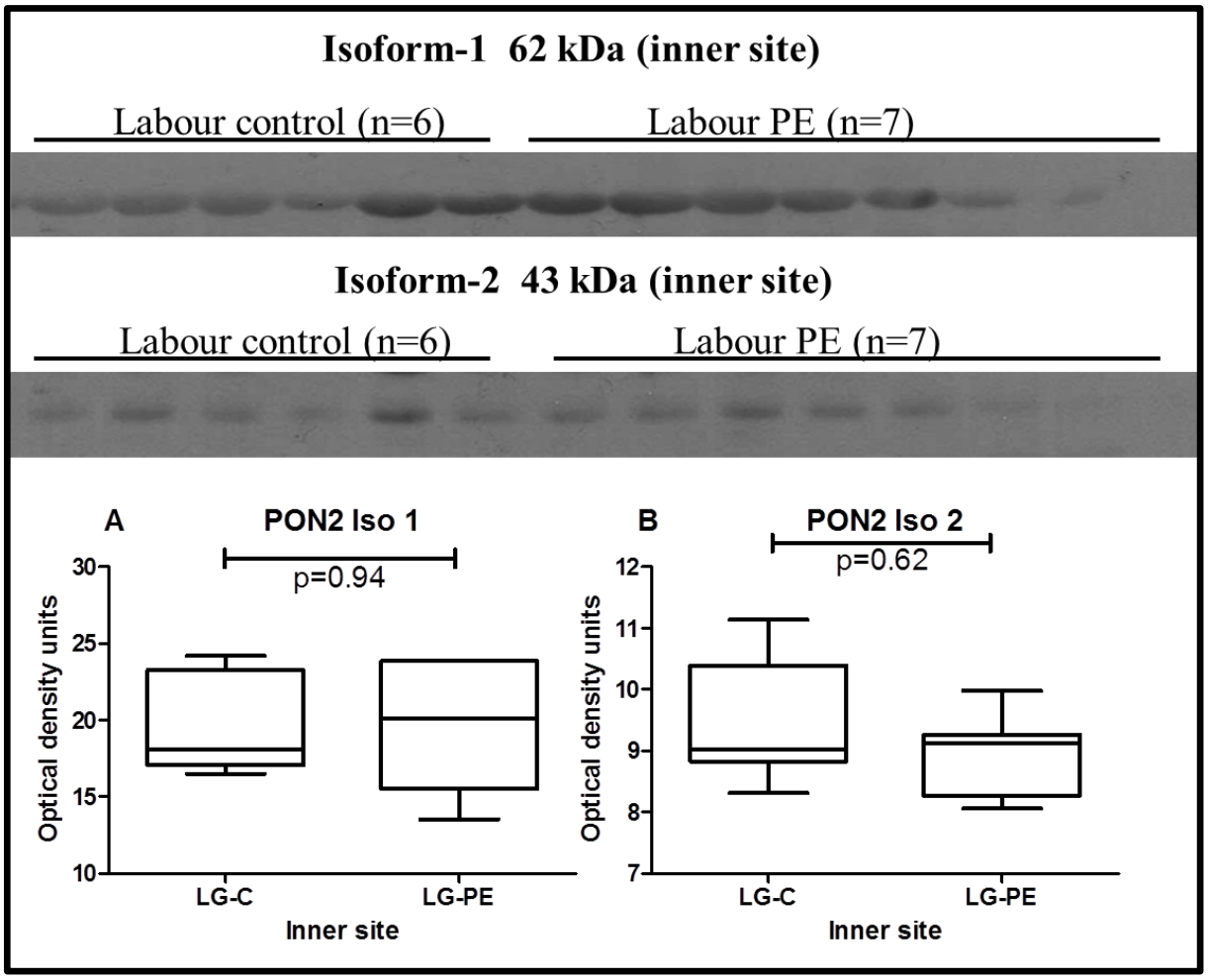

Figure 3-14: Western blots showing PON2 62 kDa isoform 1 (upper panel) and PON2 43 kDa isoform 2 (lower panel) expressions in the inner placental site in labour control (n=6) and labour PE (n=7). The graphs show the box and whiskers analysis of the blots. Comparison between zones was performed using Mann Whitney analysis. Graph A, 62 kDa isoform 1; Graph B, 43 kDa isoform 2. LG-C labour control group, LG-PE labour pre-eclampsia group.

Western blots showing placental PON2 expression in labour control and labour PE at the middle site are shown in Figure 3-15 (upper panel 62 kDa isoform 1; lower panel 43 kDa isoform 2). The graphs and statistical analysis are shown below the blots. There was a highly significant decrease in PON2 expression in the labour PE group when compared to the non-labour control group for both the 62 kDa isoform 1, (p=0.008) and the 43 kDa isoform 2, (p=0.001). Figure 3-15 shows an example of β-actin analysis. Shown are the same samples used in Figure 29. There was no difference between the labour control and labour PE groups despite the reduction in the PON2 in the labour PE group.

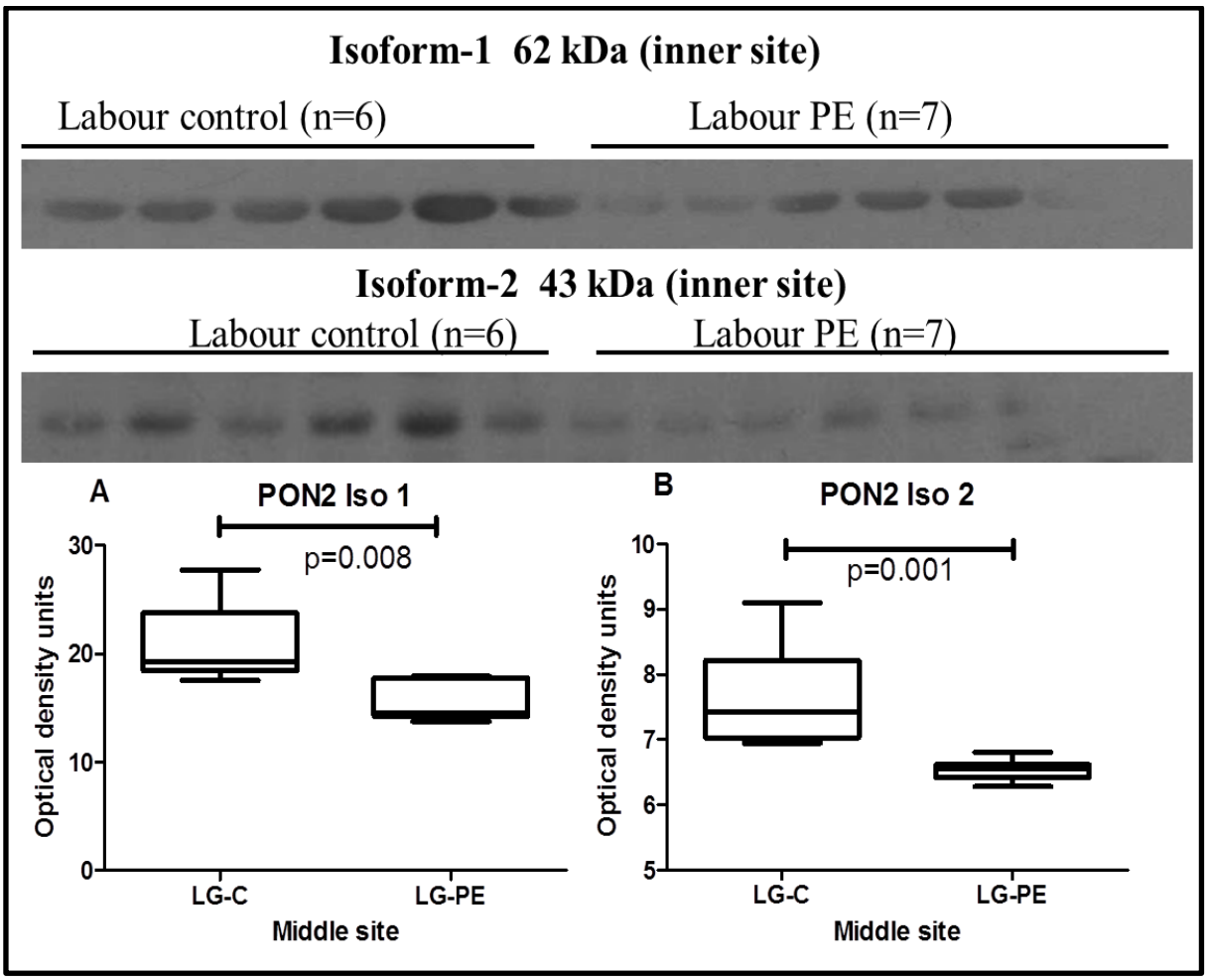

Figure 3-15: Western blots showing PON2 62 kDa isoform 1 (upper panel) and PON2 43 kDa isoform 2 (lower panel) expressions in the middle placental site in labour control (n=6) and labour PE (n=7). The graphs show the box and whiskers analysis of the blots. Comparison between zones was performed using Mann Whitney analysis. Graph A, 62 kDa isoform 1; Graph B, 43 kDa isoform 2. LG-C labour control group, LG-PE labour PE group.

The equality of loading was tested by using actin-β as a loading control. Western blot showing actin-β expression in the labour control and labour PE at the middle site are shown in figure 3-16.

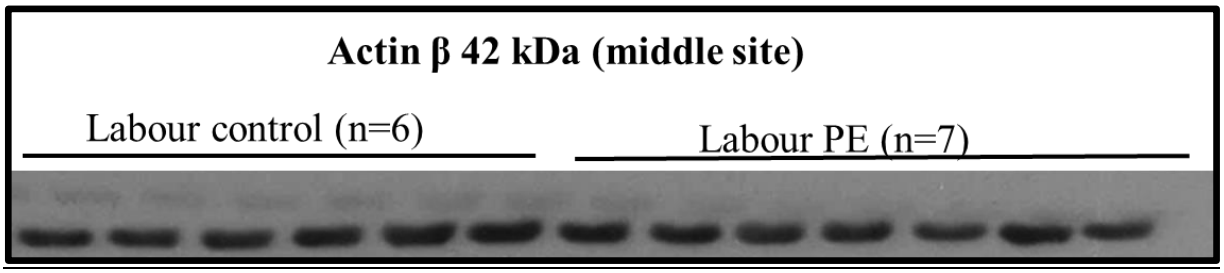

Figure 3-16: Western blots showing actin-β antibody as loading control to compare the same placenta samples in Figure 29. Labour control group compared to the labour PE group at the middle site.

Western blots showing placental PON2 expression in labour control and labour PE at the outer site are shown in Figure 3-15 (upper panel 62 kDa isoform 1; lower panel 43 kDa isoform 2). The graphs and statistical analysis are shown below the blots. No differences were found.

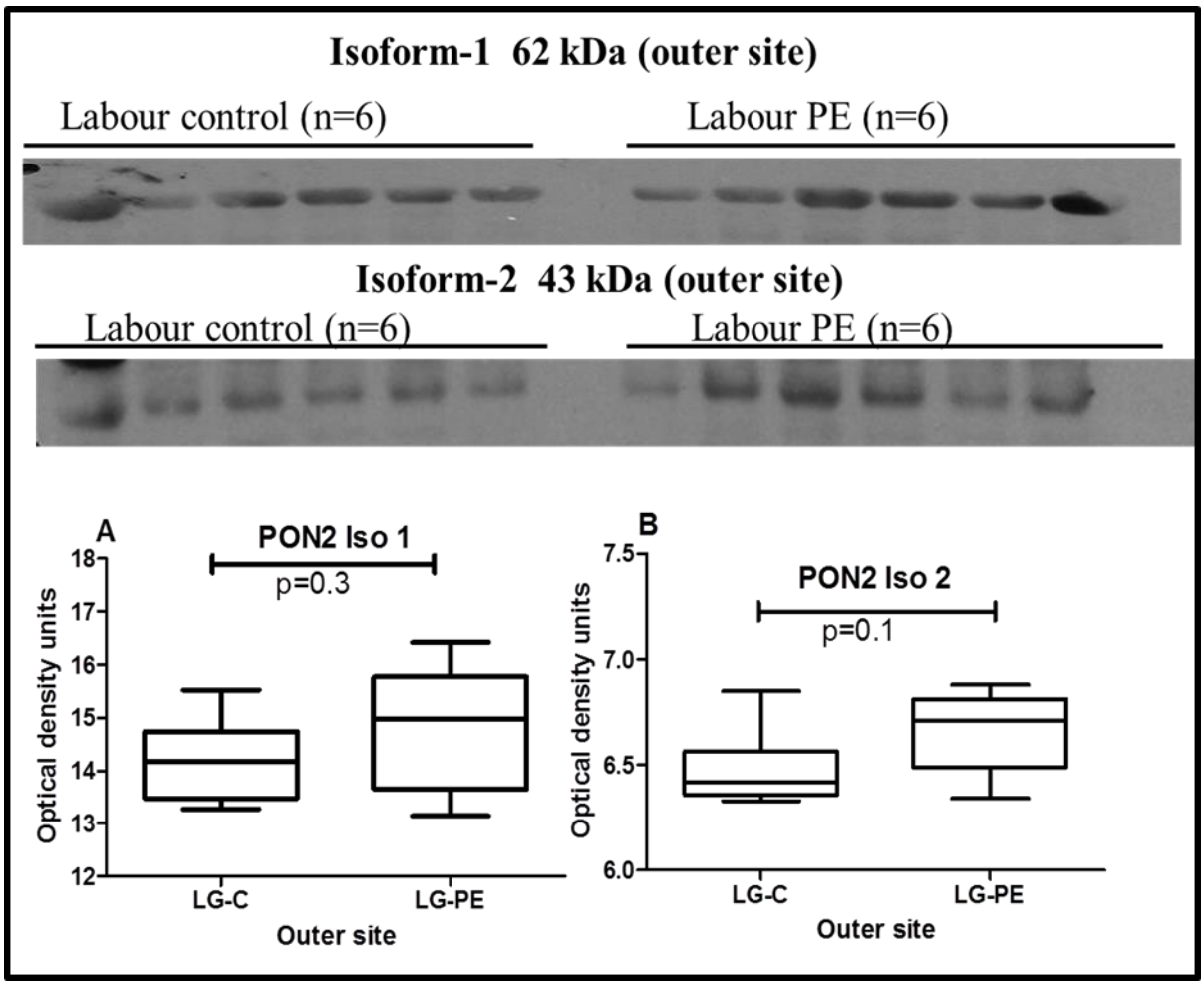

Figure 3-17: Western blots showing PON2 62 kDa isoform 1 (upper panel) and PON2 43 kDa isoform 2 (lower panel) expressions in the outer placental site in labour control (n=6) and labour PE (n=6). The graphs show the box and whiskers analysis of the blots. Comparison between zones was performed using Mann Whitney analysis. Graph A, 62 kDa isoform 1; Graph B, 43 kDa isoform 2. LG-C labour control group, LG-PE labour PE group.

Experiment 3: This experiment was designed to test whether there was any difference between non-labour control and non-labour PE at the inner and middle sites at the PON2 mRNA level. The results are shown in Figure 3-16. No differences were found at both inner and middle sites.

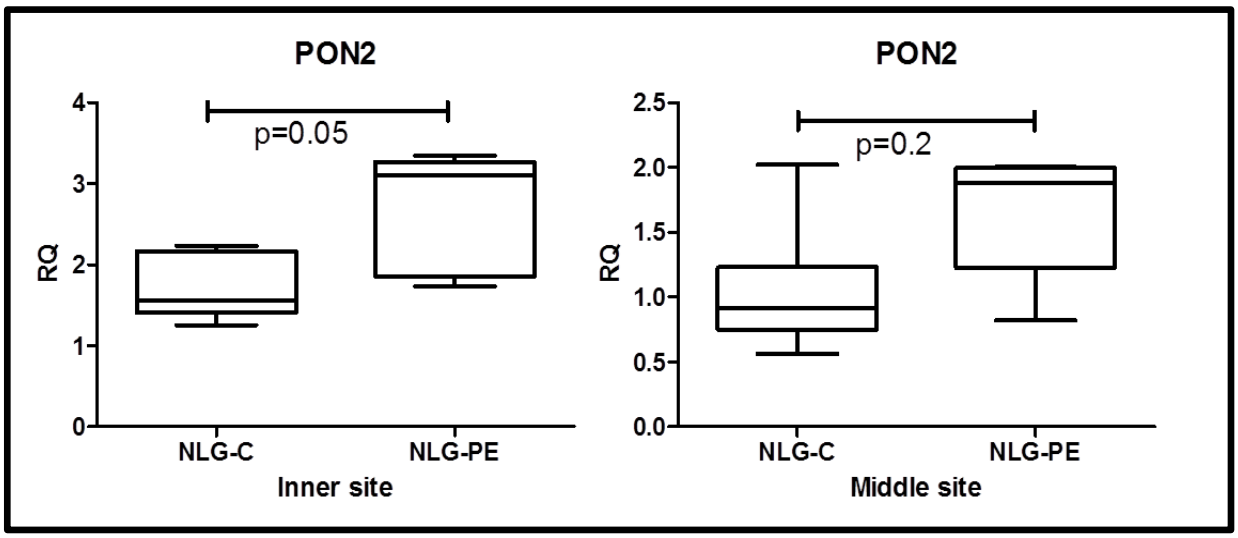

Figure 3-18: RQ values for PON2 mRNA measurements in inner and middle placental sites for non-labour control compared with non-labour PE. Comparison between zones was performed using Mann-Whitney analysis.

Experiment 4: This experiment was designed to test whether there was any difference between labour controls and labour PE at the inner and middle sites at the PON2 mRNA level. The results are shown in Figure 3-17. No differences were found at both inner and middle sites.

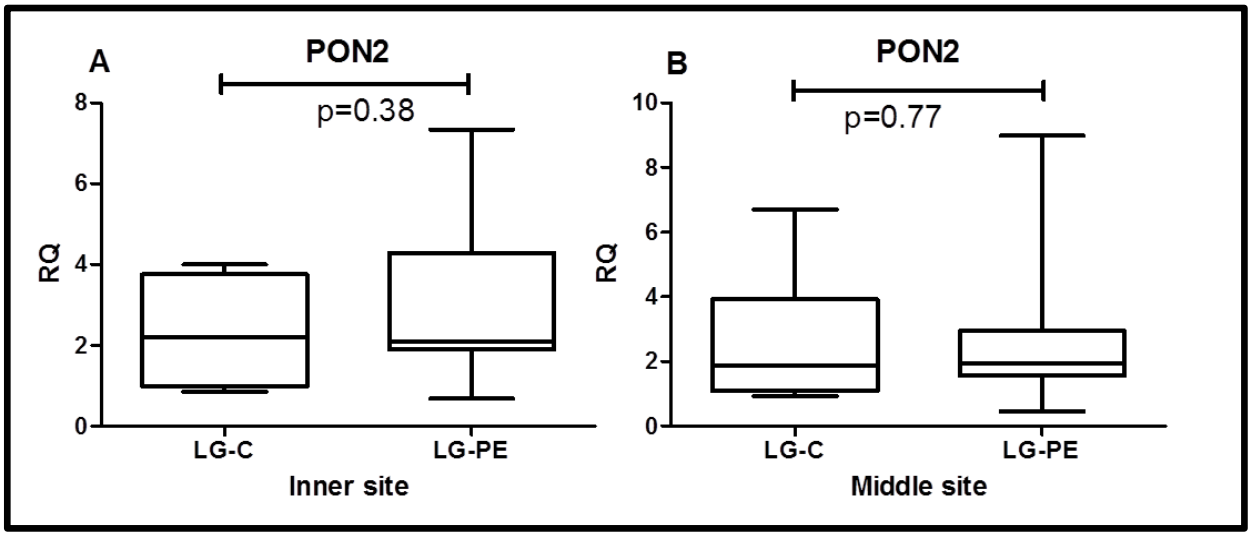

Figure 3-19: RQ values for PON2 mRNA measurements in inner and middle placental sites for labour control compared with labour PE. Comparison between zones was performed using Mann-Whitney analysis.

Experiment 5: This experiment was designed to test whether there was any difference between normotensive pregnancies and pregnancy complicated with pre-eclampsia at the inner and middle sites at the PON2 mRNA level. The results are shown in Figure3-18. There was no significant difference at inner and middle sites.

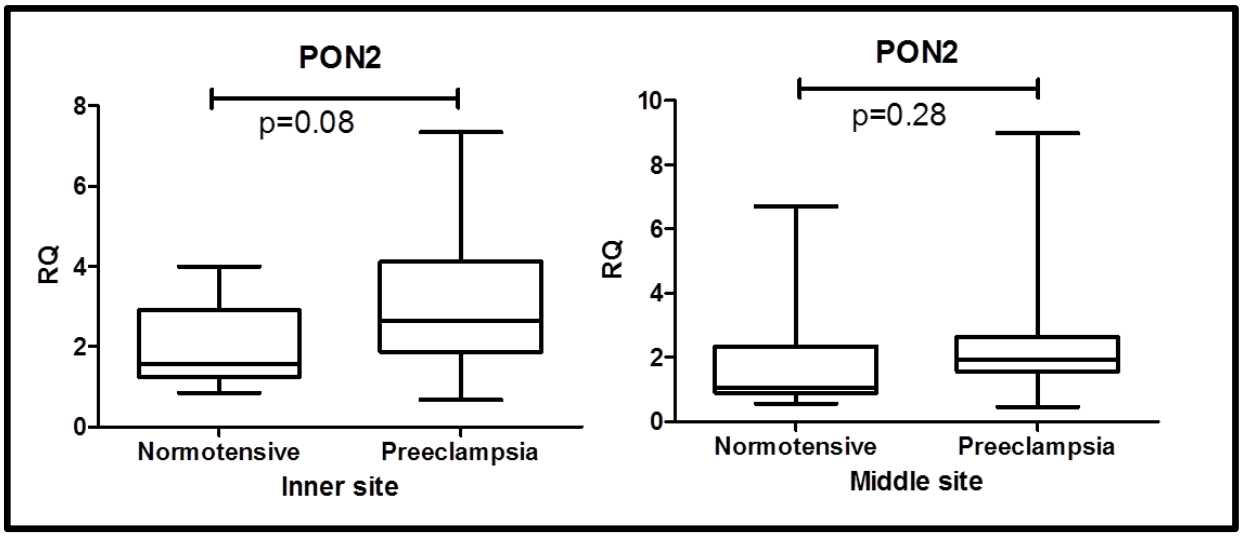

Figure 3-20: RQ values for PON2 mRNA measurements in inner and middle placental sites for normotensive pregnancy compared with pregnancy complicated with pre-eclampsia. Comparison between zones was performed using Mann-Whitney analysis.

3.3 PON2 expression in ischemic-reperfusion tissue cultural experiment

Due to time constraints only one set of experiments was performed for all of the below. The aim was to determine whether pilot data from this group of experiments revealed any data suggesting that more experiments were warranted.

Experiment 1: This experiment involved exposing placenta samples to 2% O_2 (hypoxia) for one hour and then 8% O_2 (normoxia). The control samples were exposed to 8% O_2 (normoxia). Four lengths of time points (3, 6, 12 and 22 hours) were used for re-oxygenation. PON2 protein expression was investigated using Western blot analysis. PON2 mRNA was investigated using RT-qPCR. Statistical analysis was performed using the Minitab 17 package and the two way ANOVA test was used. Interaction plot were plotted out to demonstrate the findings. Two PON2 isoforms (62 kDa and 43 kDa) were identified. Examples of Western blots showing PON2 (isoform 1, 62 kDa and isoform 2, 43 kDa) expression for the set of experiments performed on placental tissue obtained from the inner site are shown in Figure 3-19. Two ways ANOVA analysis showed there was no significant difference in expression of either isoform 1 and 2 due to hypoxia reperfusion (p=0.6, p=0.8 respectively for both isoforms) at the inner site Figure 3-20. It was interesting that expression of PON2 was reduced in both groups, perhaps due to protein turnover.

Figure 3-21: Western blots showing PON2 62 kDa isoform 1 (upper panel) and PON2 43 kDa isoform 2 (lower panel) expressions in the inner placental site. Ref 1 represents the sample collected in the labour suite; Ref 2 represents the sample after transfer to the laboratory. Different time points (3, 6, 12 and 22 hours) for the tissues following exposure to hypoxia then normoxia (E) are shown. Control tissue exposed to normoxia only for the same times are indicated as with C.

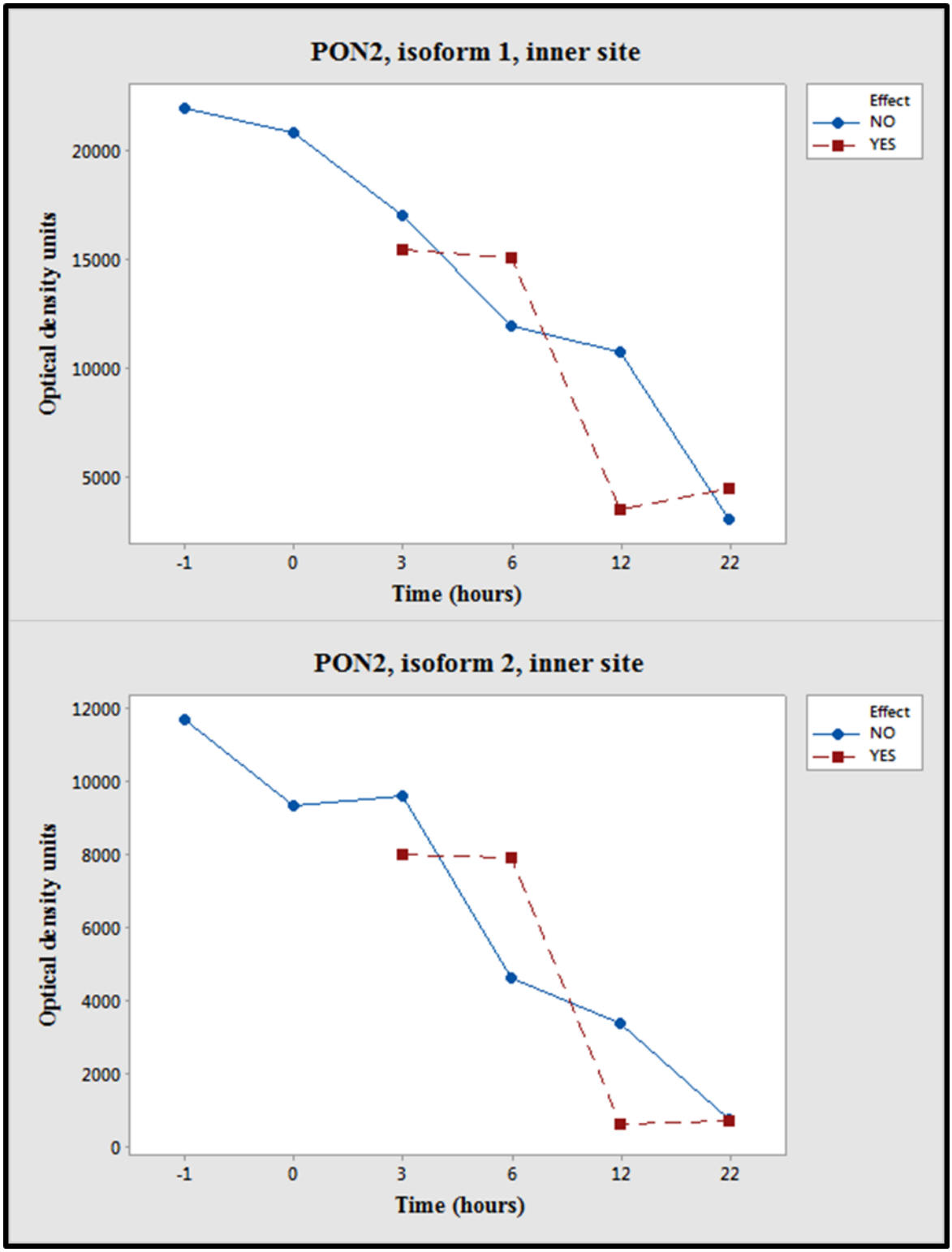

Figure 3-22: The graphs show the interaction plots analysis for PON2 protein expression at the inner site. The graph shows data of the samples exposing to hypoxia reperfusion (Red dotted line, YES) compared to the control samples (Blue continuous line, NO) at different time points. Upper panel isoform 1, lower panel isoform 2. Hypoxia-Rep ((hypoxia-Reperfusion). O2 (oxygen).

Experiment 2: This experiment was the same as the above experiment 1 but this time the middle area of the placenta was exposed to hypoxia-reperfusion. Two PON2 isoforms (62 kDa and 43 kDa) were identified again in all samples. Examples of Western blots showing PON2 (isoform 1, 62 kDa and isoform 2, 43 kDa) expression for middle site are shown in Figure 3-21. For hypoxia-reperfusion for comparison of paired points there was also no difference for both isoforms (isoform1 p=0.1, isoform 2 p=0.9 respectively). The interaction plot is shown in Figure 3-22. Again expression of PON2 was reduced in both groups with increasing time, perhaps due to protein turnover.

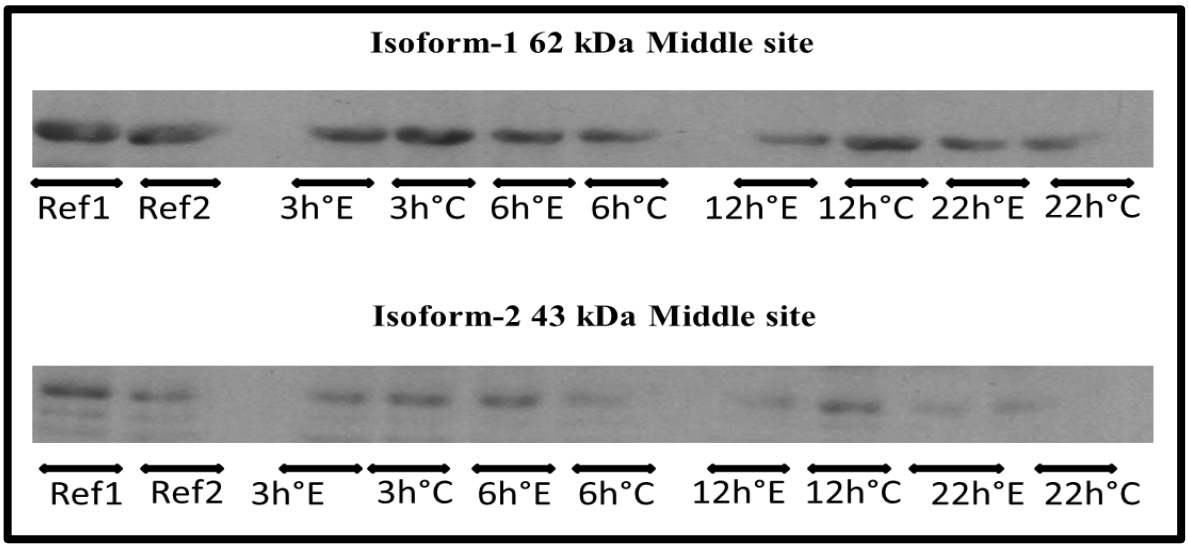

Figure 3-23: Western blots showing PON2 62 kDa isoform 1 (upper panel) and PON2 43 kDa isoform 2 (lower panel) expressions in the middle placental site. Ref 1 is the sample collected in the labour suite. Ref 2 is the sample once transferred to the laboratory. Different time points (3, 6, 12 and 22 hours) for the tissues following exposure to hypoxia then normoxia (E) are shown. Control tissue exposed to normoxia only for the same times are indicated as with C.

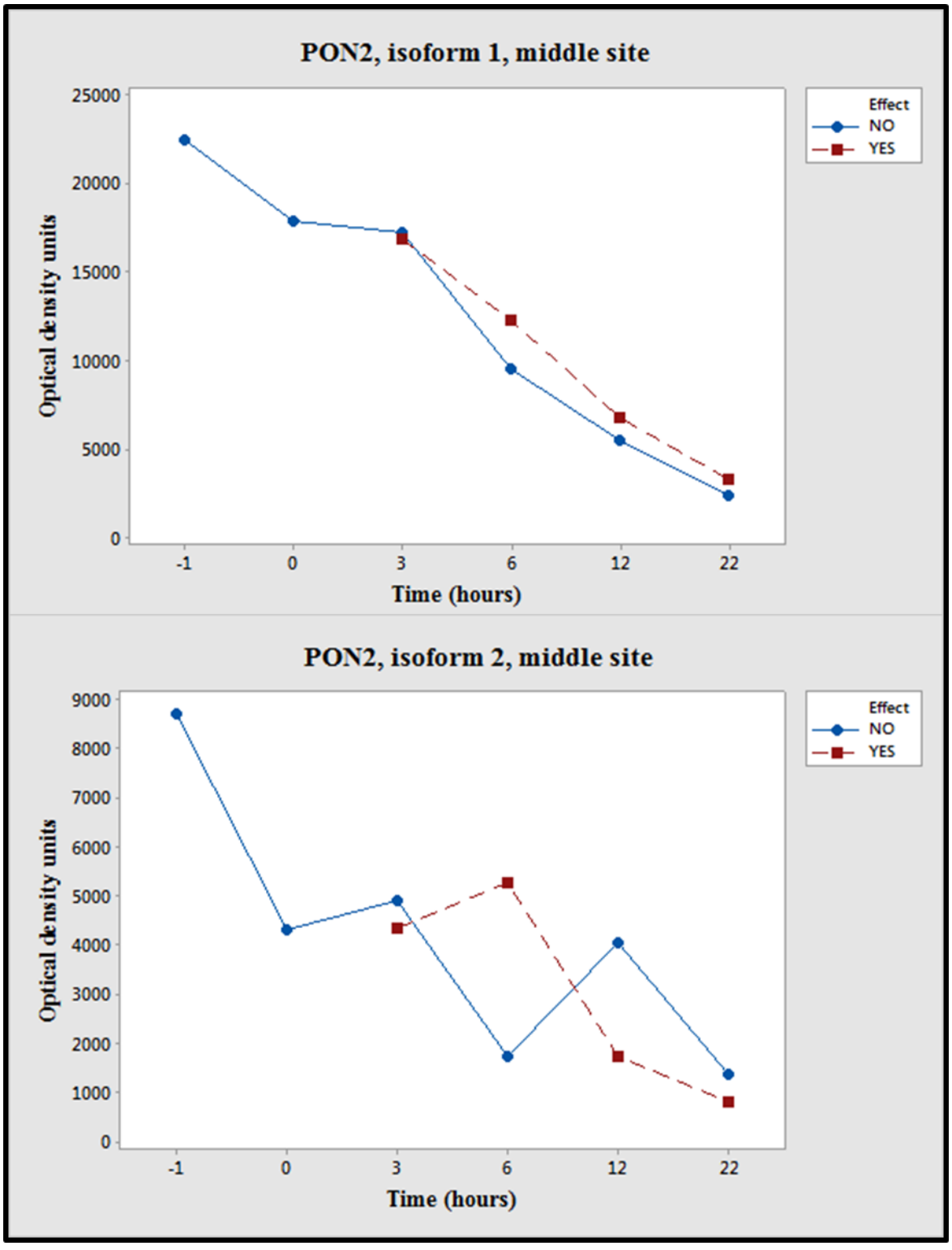

Figure 3-24: The graphs show the interaction plots analysis for PON2 protein expression at the middle site. The graph shows data of the samples exposing to hypoxia reperfusion (Red dotted line, YES) compared to the control samples (Blue continuous line, NO) at different time points. Upper panel isoform 1, lower panel isoform 2. Hypoxia-Rep ((hypoxia-Reperfusion). O2 (oxygen).

Experiment 3: This experiment was the same as the above experiment 1 but this time the outer site of the placenta was exposed to hypoxia-reperfusion. Two PON2 isoforms (62 kDa and 43 kDa) were identified again in all samples Figure 3-23. Two way ANOVA analysis showed there was no significant difference in expression of either isoform 1 and 2 due to hypoxia then reperfusion (p=0.9 isoform 1, p=0.3 isoform 2) at the outer site. The interaction blot is shown in Figure 3-24. Again expression of PON2 was reduced in both groups, perhaps due to protein turnover.

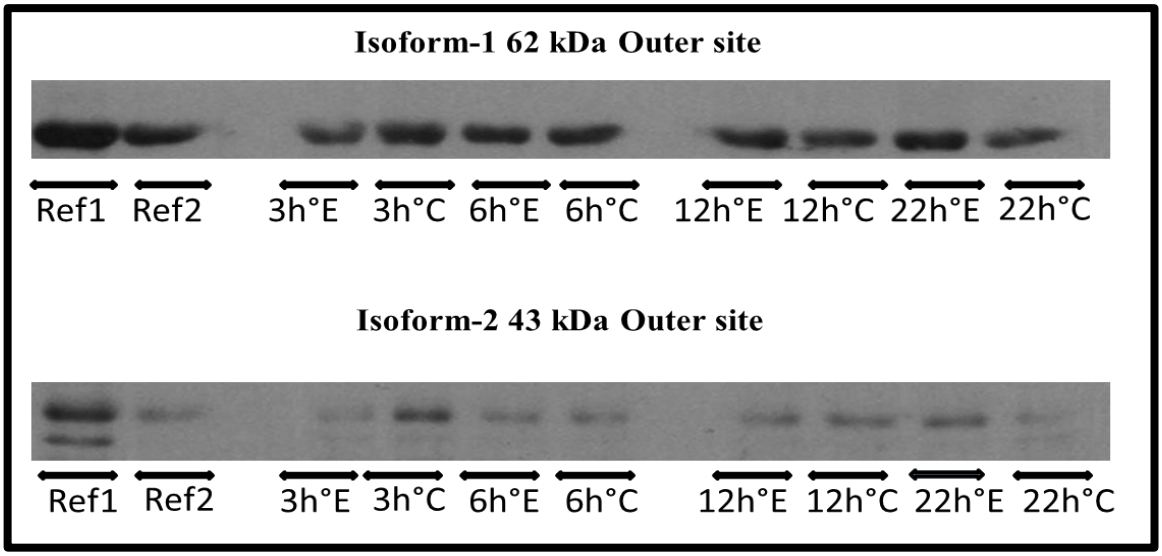

Figure 3-25: Western blots showing PON2 62 kDa isoform 1 (upper panel) and PON2 43 kDa isoform 2 (lower panel) expressions in the outer placental site. Ref 1 is the sample collected in the labour suite. Ref 2 is the sample collected once transferred to the laboratory. Different time points (3, 6, 12 and 22 hours) for the tissues following exposure to hypoxia then normoxia (E) are shown. Control tissue exposed to normoxia only for the same times are indicated as with C.

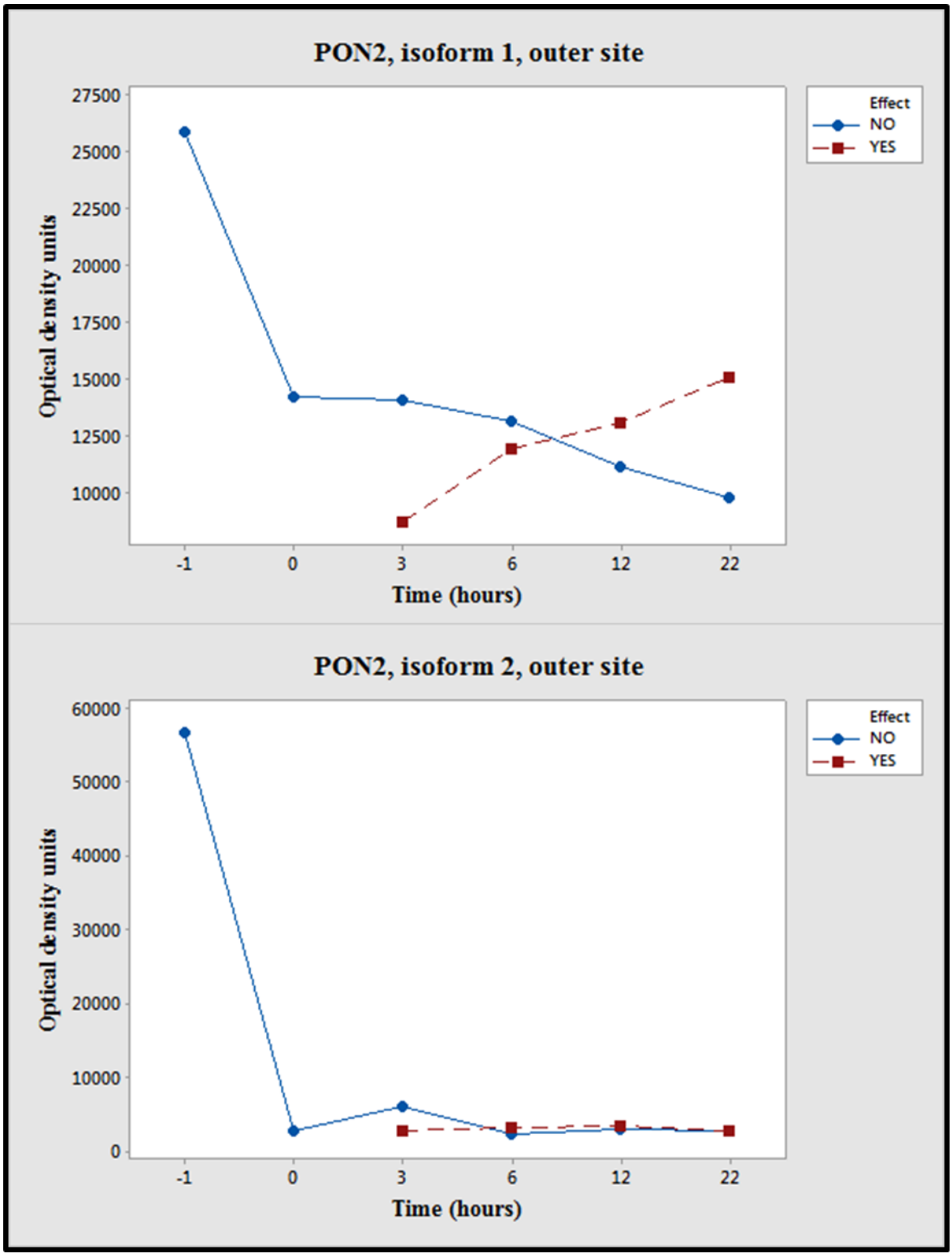

Figure 3-26: The graph shows the interaction plot analysis of the data for PON2 protein expression at the outer site. The graph shows data of the samples exposing to hypoxia reperfusion (Red dotted line, YES) compared to the control samples (Blue continuous line, NO) at different time points. Upper panel isoform 1, lower panel isoform 2. Hypoxia-Rep ((hypoxia-Reperfusion). O2 (oxygen).

The following set of data is the same experiment as above but this time mRNA was analyzed by RT-PCR.

Experiment 4: This experiment was designed to test if there was any difference in PON2 mRNA expression between tissues were exposing to hypoxia reperfusion and control at the inner site of placentas according to four different time points. There was no significant difference in expression of PON2 due to hypoxia reperfusion effect (p=0.4). The interaction plot is shown in Figure 3-25.

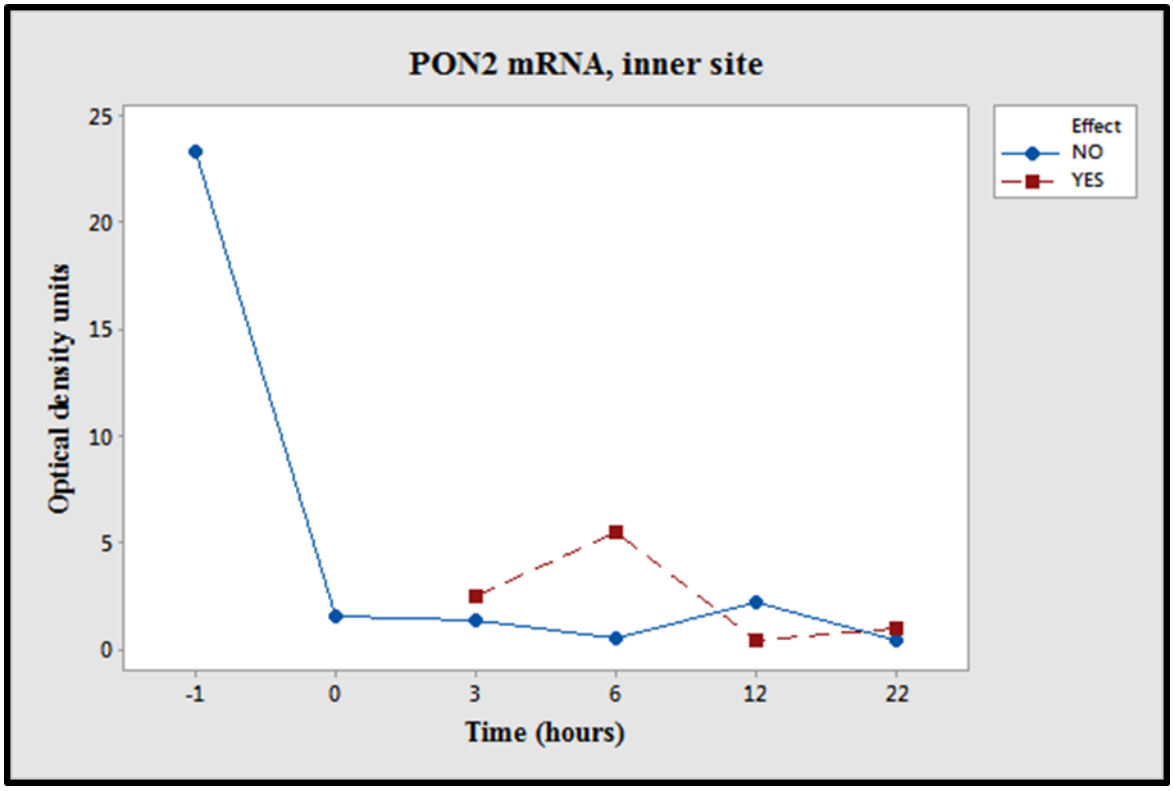

Figure 3-27: The graphs show the interaction plots analysis of the data for PON2 mRNA at the inner site. The graph shows data of the samples exposing to hypoxia reperfusion (Red dotted line, YES) compared to the control samples (Blue continuous line, NO) at different time points.

Experiment 5: This experiment was designed to test if there was any difference in PON2 mRNA expression between tissues were exposing to hypoxia reperfusion at the middle site of placentas according to four different time points. There was no significant difference in expression of PON2 due to hypoxia reperfusion (p=0.3). However at 6 hours the experimental group showed a clear increase. This suggests that further experiments to increase the number of experiments would then show whether this was a real statistical changes. The interaction blot is shown in Figure 3-26.

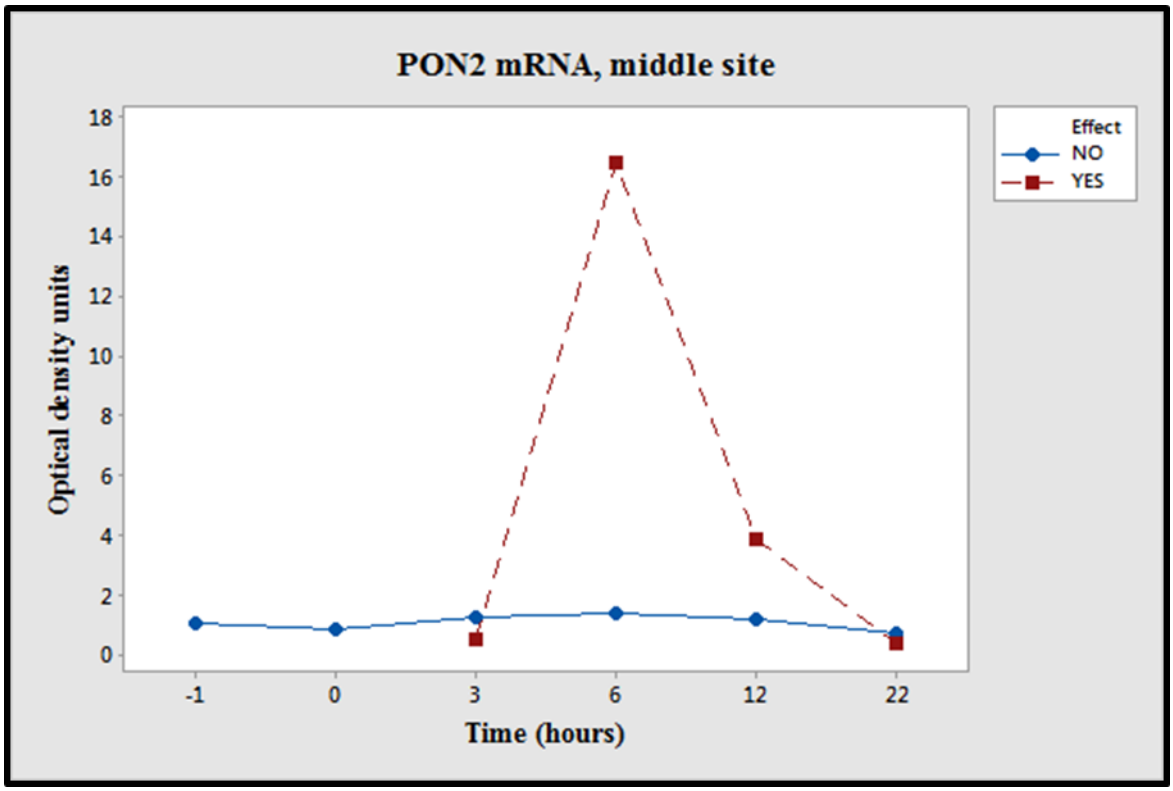

Figure 3-28: The graphs show the interaction plots analysis of the data for PON2 mRNA at the middle site. Graph samples exposing to hypoxia reperfusion (Red dotted line, YES) compared to the control samples (Blue continuous line, NO) at different time points.

Experiment 6: This experiment was designed to test if there was any difference in PON2 mRNA expression between tissues were exposing to hypoxia reperfusion and control at the outer site of placentas according to four different time points. There was no significant difference in expression of PON2 due to hypoxia reperfusion (p=0.3). However at 6 hours the experimental group again showed a clear increase. This suggests that further experiments to increase the number of experiments would then show whether this was a real statistical changes. The interaction blot is shown in Figure 3-27.

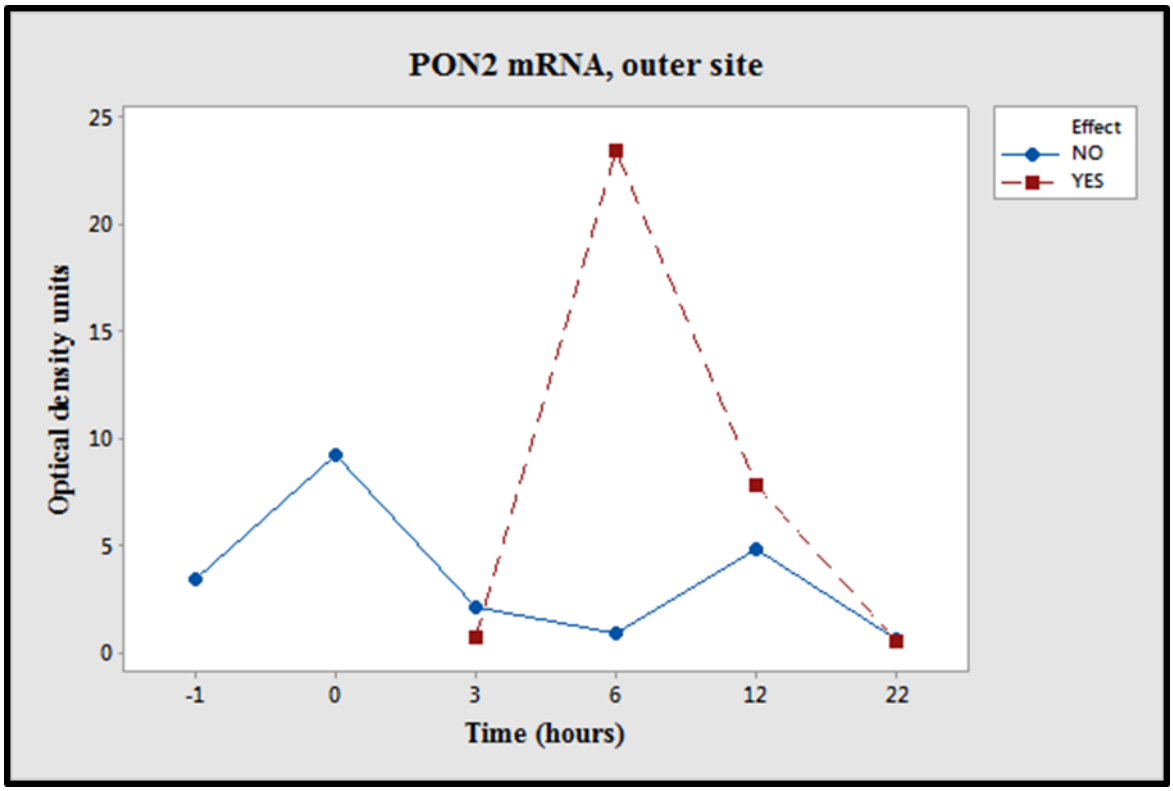

Figure 3-29: The graphs show the interaction plots analysis of the data for PON2 mRNA at the outer site. Graph show samples exposed to hypoxia (Red dotted line, YES) compared to the control samples (Blue continuous line, NO) at different time points.

In summary although these data are very preliminary there was an obvious increase in PON2 mRNA in inner, middle and outer placental areas when exposed to hypoxia re-oxygenation at the 6 hours point. Future experiments should follow this up to determine if this is a real statistical effect.

3.4 PON3 expression in Labour

Experiment 1: This experiment was designed to test if there was a spatial difference in expression of PON3 within individual placentas obtained from women who were not in labour. All samples expressed PON3. Examples of Western blots showing PON3 expression for 3 different placentas (all non-labour) are shown in Figure 3-28. Friedman test analysis showed there was no difference in expression of PON3 between the three sites (inner, middle, outer) within individual placentas.

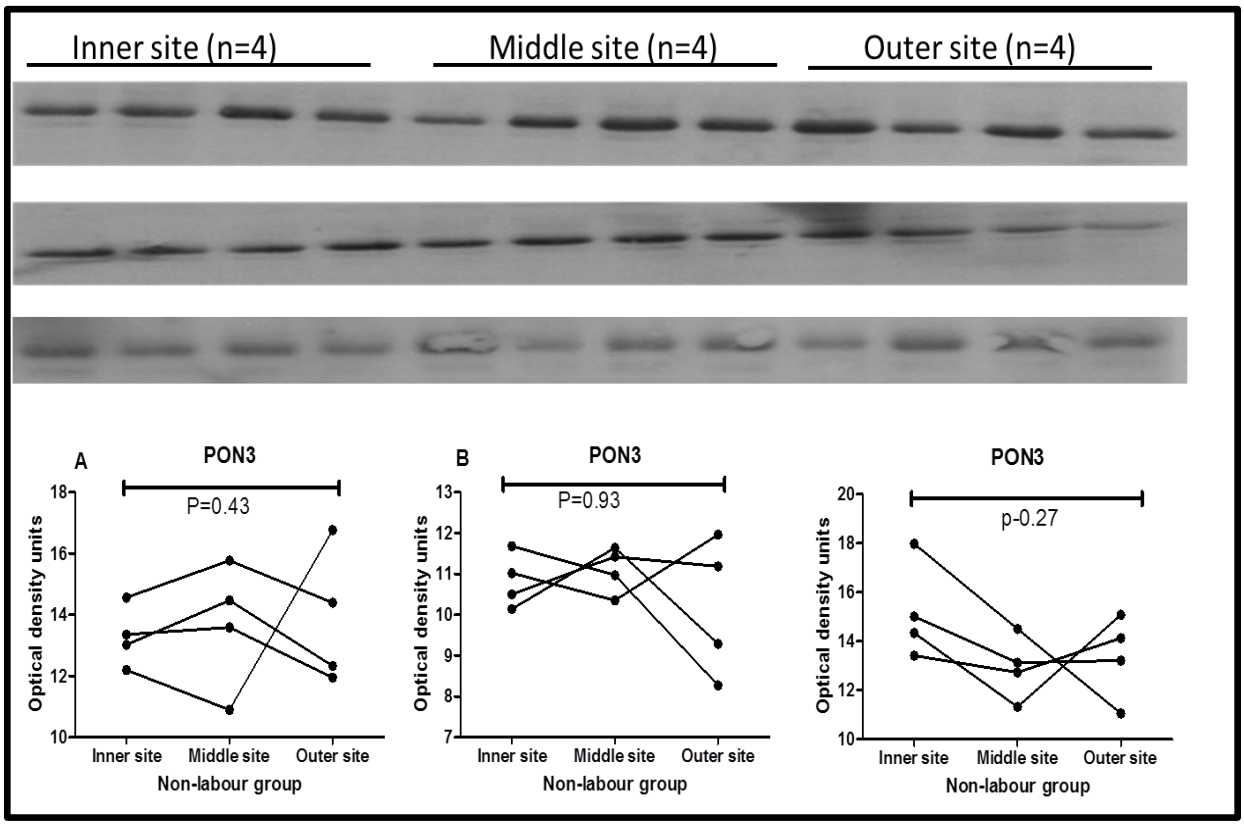

Figure 3-30: Western blots showing PON3 expression in inner, middle and outer sites of three individual placentas (non-labour group). Four quadrants were sampled in each zone. Graphs show the median for PON3.

Experiment 2: This experiment was designed to test if there was a spatial difference in expression of PON3 within individual placentas obtained from women who were in labour. Examples of Western blots showing PON3 expression for 3 different placentas (labour) are shown in Figure 3-29. Friedman test analysis showed there was also no difference in expression between the three sites (inner, middle, outer).

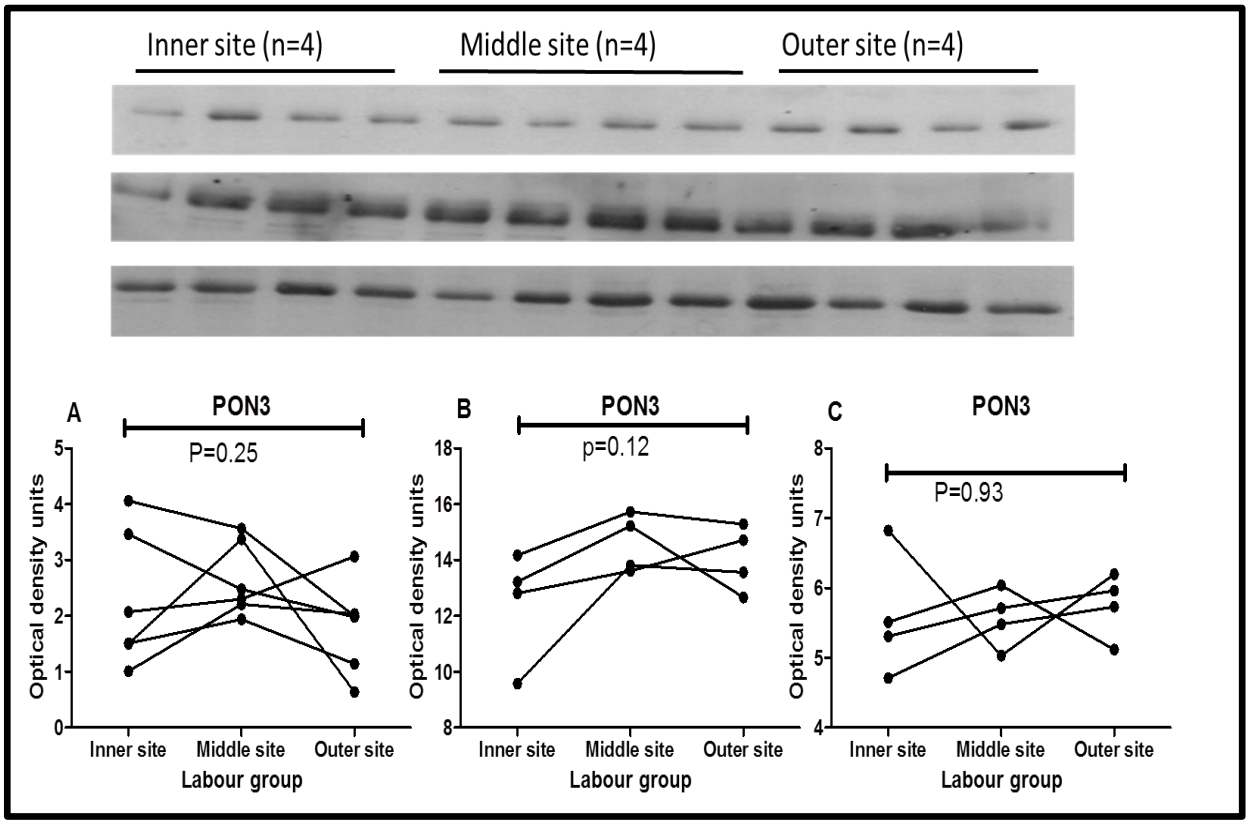

Figure 3-31: Western blots showing PON3 expression in inner, middle and outer zones of three individual placentas (labour group). Four quadrants were sampled in each zone. Graphs show the median for PON3

Experiment 3: This experiment was designed to test if there was any difference in PON3 expression between labour and non-labour groups at the inner, middle or outer areas of placentas. Western blots showing placental PON3 expression in non-labour and labour at the inner, middle and outer zones are shown in Figure 3-30. The graphs and statistical analysis are shown below the blots. PON3 was significantly decreased in the labour group when compared to the non-labour group at the inner site (p=0.02). No other differences were found when comparing the middle and outer sites of non-labour to the labour groups (p=0.24, p=0.09).

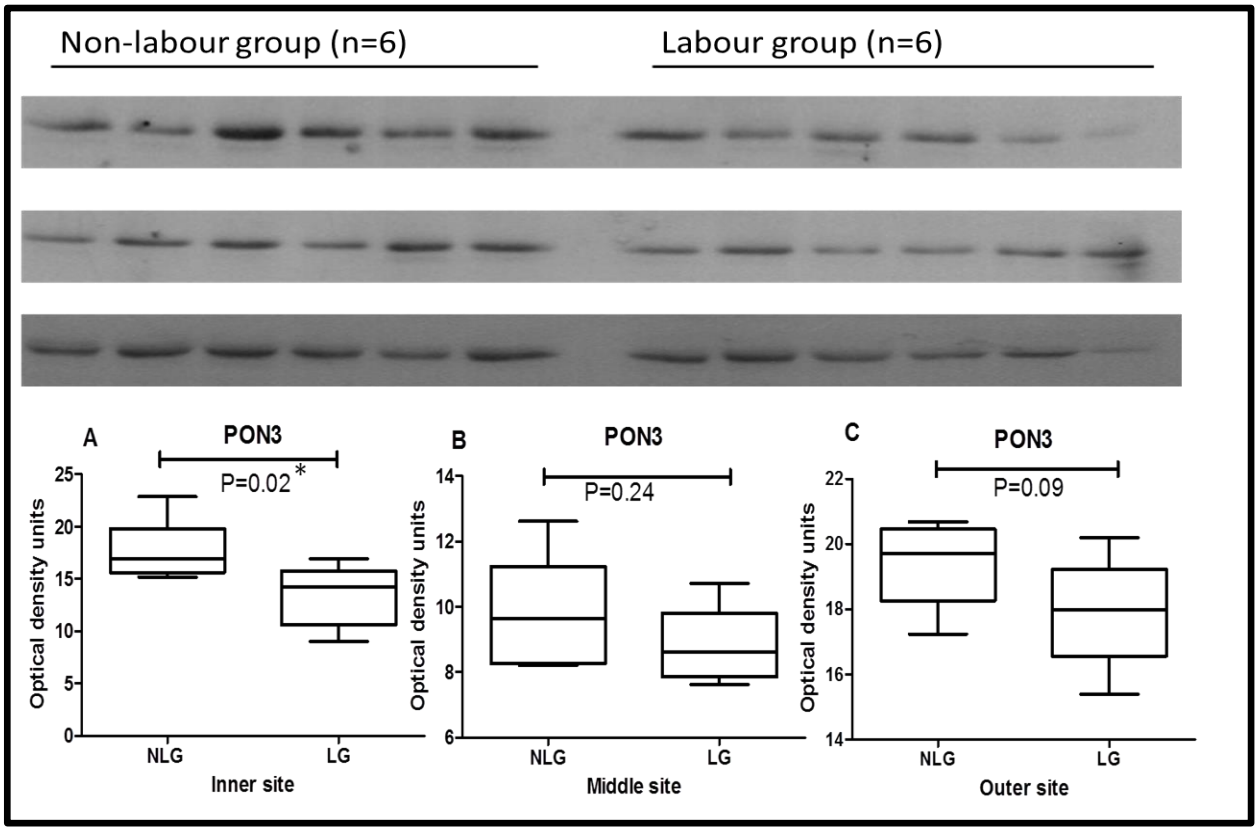

Figure 3-32: Western blots showing PON3 expression in non-labour group compared to the labour group at the inner, middle and outer zones. Graphs show the median for PON3.

3.5 PON3 expression in Pre-eclampsia

Experiment 1: This experiment was designed to test if there was any difference in PON3 expression between non-labour control and non-labour PE groups at the inner or middle areas of placentas. Western blots showing placental PON3 expression in non-labour control and non-labour PE at the inner site are shown in Figure 3-31. The graphs and statistical analysis are shown below the blots. There was a significant decreased in PON3 expression in the PE non-labour group compared to the control non-labour group in the middle sites (p=0.02). However, no difference was found in the inner site (p=0.16).

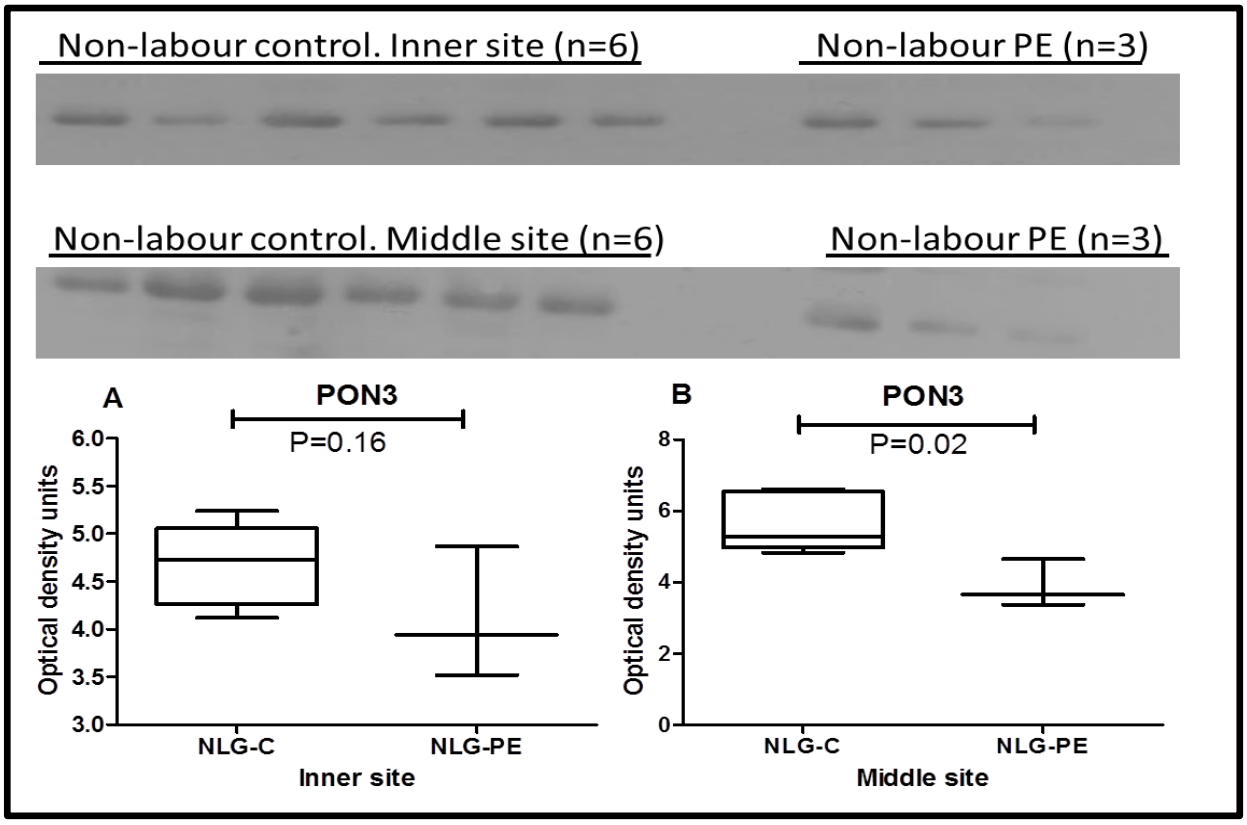

Figure 3-33: Western blots showing PON3 expression in non-labour control compared to non-labour PE at the inner and middle zones. Data analysis shows the median for PON3 expression.

Experiment 2: This experiment was designed to test if there was any difference in PON3 expression between labour control and labour PE groups at the inner or middle areas of placentas. Western blots showing placental PON3 expression in labour control and labour PE at the inner site are shown in Figure 3-32. The graphs and statistical analysis are shown below the blots. There was a significant decrease in the PE labour group compared to the control labour at the middle sites (p=0.04). At the inner there was a trend but not significant (p=0.06).

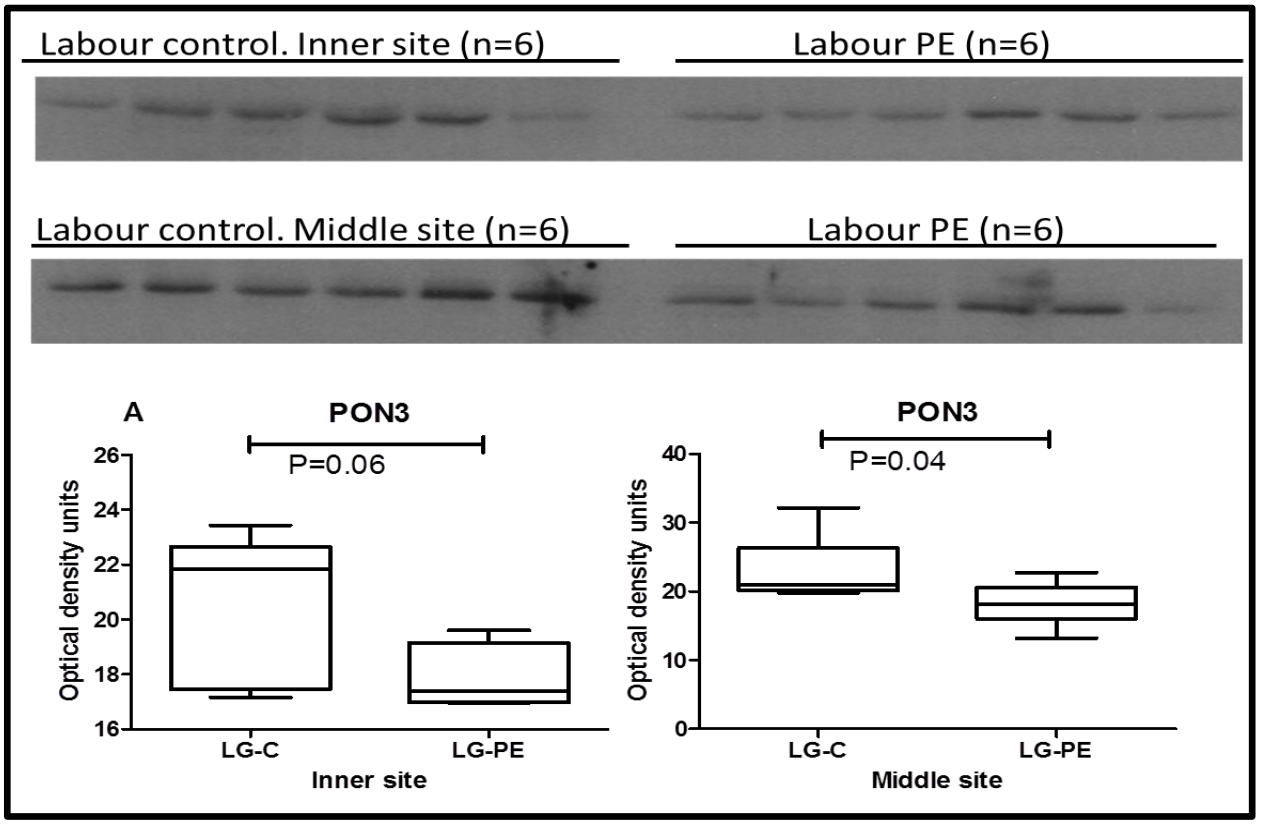

Figure 3-34: Western blots showing PON3 expression in labour control compared to labour PE at the inner and middle zones. Data analysis shows the median for PON3 expression.

3.6 PON3 expression in hypoxia-reperfusion tissue cultural experiment

Experiment 1: This experiment undertaken involved exposing placenta samples to 2% O_2 (hypoxia) for one hour and then 8% O_2 (normoxia) compared to the control samples which were exposed to only 8% O_2 (normoxia) at four time points 3, 6, 12 and 22 hours. PON3 protein expression was investigated using Western blot analysis. Statistical analysis was performed using the Minitab 17 package and the two way ANOVA test was used. Interaction plot were plotted out to demonstrate the findings. Western blots showing PON3 expression for the inner site of placenta sample is shown in Figure 3-33. Two ways ANOVA analysis showed there was no significant difference in expression of PON3 due to hypoxia reperfusion at the inner site (p=0.8). The interaction plot is shown in Figure 3-33. As for the PON2 protein there was a gradual fall with time.

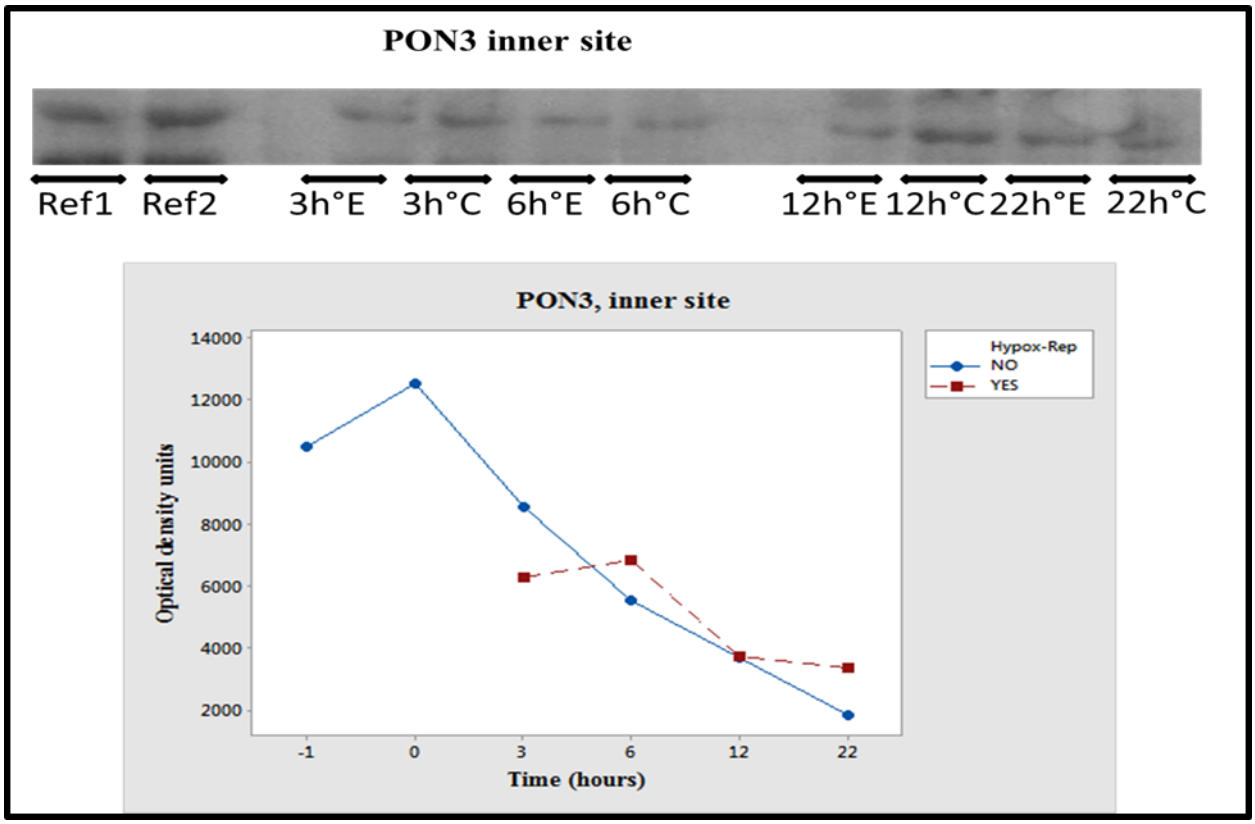

Figure 3-35: Western blots showing PON3 expression (upper panel) in the inner placental zone. Ref 1 is the sample collected in the labour suite. Ref 2 is the sample collected after arriving in the laboratory. Different time point (3, 6, 12 and 22 hours) of reperfusion was performed. Graphs show the interaction plots analysis of the data for PON3 protein expression at the inner site. Samples exposed to hypoxia reperfusion are shown as a (red dotted line, YES) and compared to the control samples (Blue continuous line, NO) at different time points.

Experiment 2: This experiment was the same as the above but now the middle site of the placenta was assessed in its response to hypoxia reperfusion. Example of Western blots showing PON3 expression for the inner site of the placenta sample is shown in Figure 3-34 Two way ANOVA test analysis showed no significant differences (p=0.7). The interaction plot is shown in Figure 3-34 below the blots.

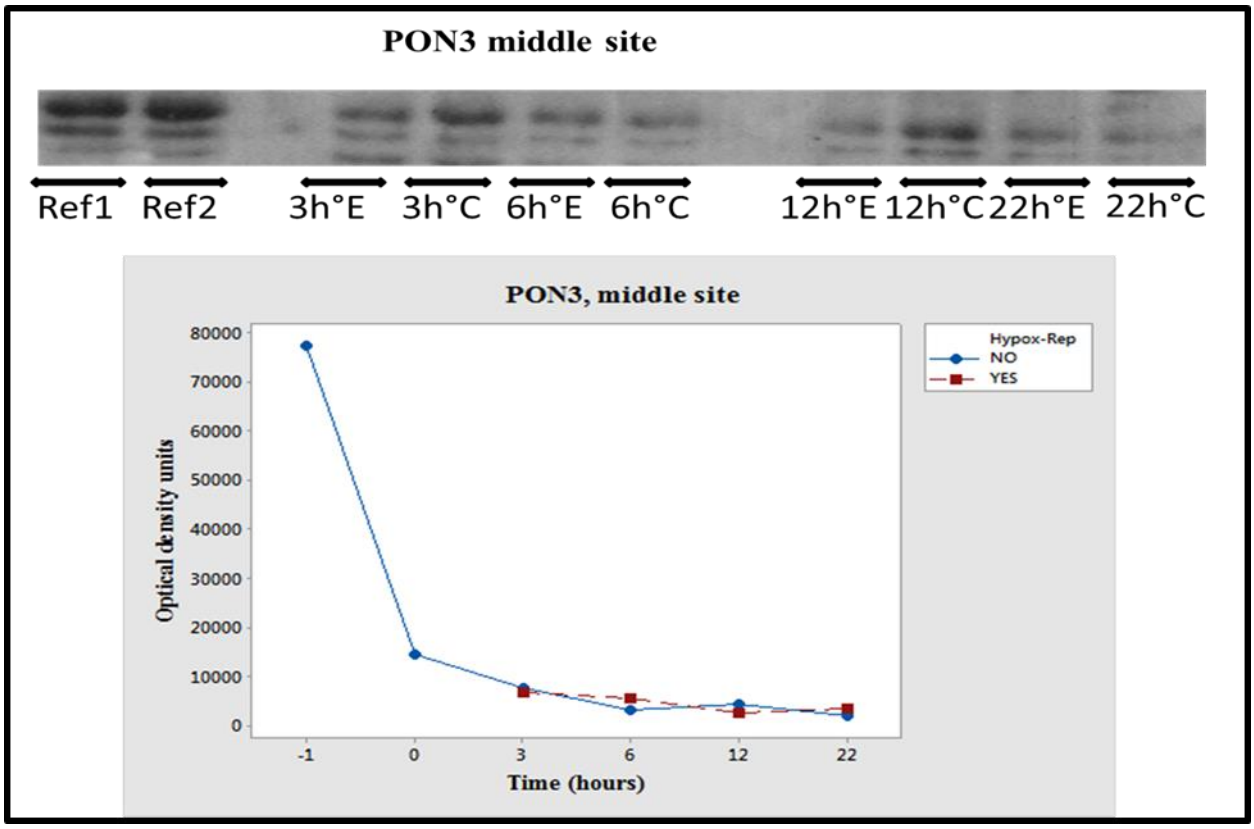

Figure 3-36: Western blots showing PON3 expression (upper panel) in the middle placental site. Ref 1 is the sample collected in the labour suite. Ref 2 is the sample collected after arriving in the laboratory. Different time point (3, 6, 12 and 22 hours) of reperfusion was performed. Graphs show the interaction plots analysis of the data for PON3 protein expression at the middle site. Samples exposed to hypoxia reperfusion are shown as a (red dotted line, YES) and compared to the control samples (Blue continuous line, NO) at different time points.

Experiment 3: This experiment was designed to test if there was any difference in PON3 protein expression between tissues following exposing placental samples from the outer site to hypoxia reperfusion. Examples of Western blots showing PON3 expression are shown in Figure 3-35 Again no difference was found between the two groups (p=0.1 for both). The interaction plot is also shown in Figure 3-35.

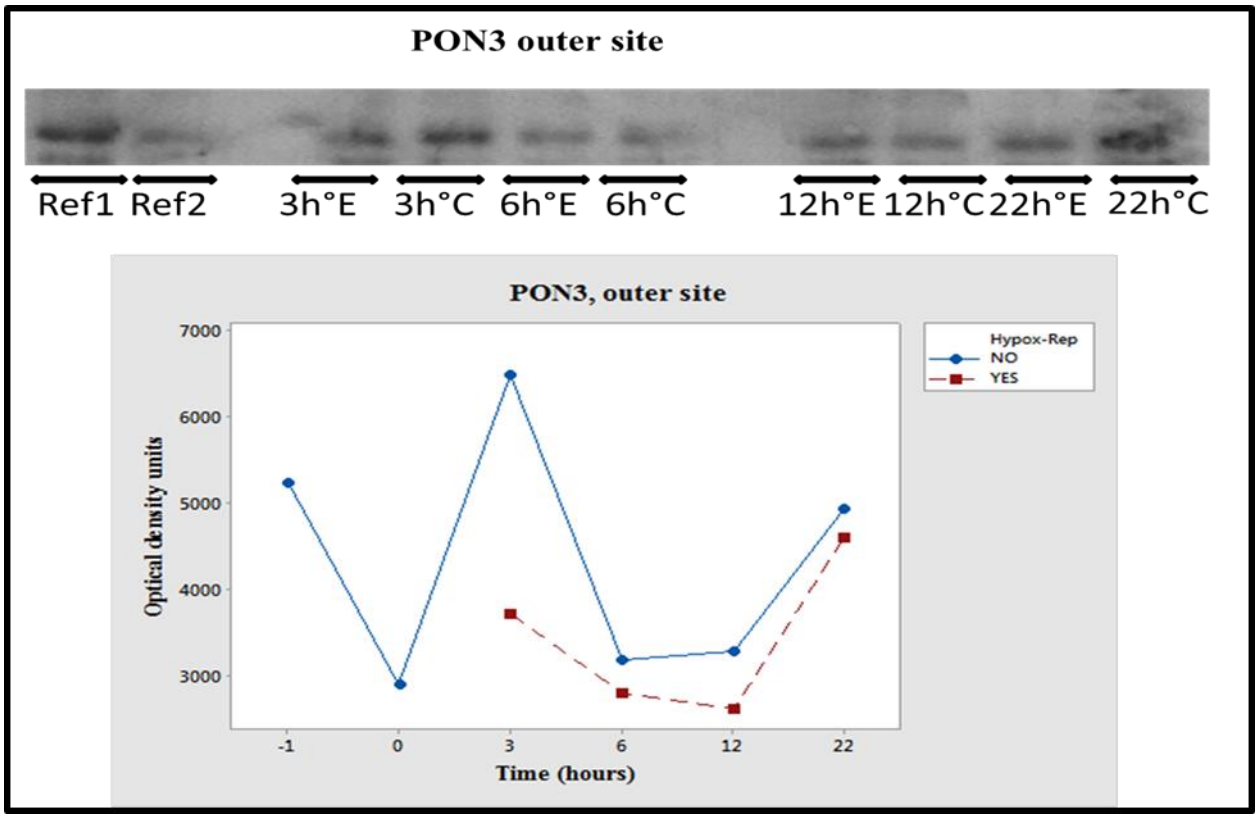

Figure 3-37: Western blots showing PON3 expression (upper panel) in samples from the outer placental site. Ref 1 is the sample collected in the labour suite. Ref 2 is the sample collected after transport to the laboratory. Different time point (3, 6, 12 and 22 hours) of reperfusion was performed. Graphs show the interaction plots analysis of the data for PON3 protein expression at the outer site. Samples exposed to hypoxia reperfusion are shown as a (red dotted line, YES) and compared to the control samples (Blue continuous line, NO) at different time points.

3.7 PON3 mRNA expression:

Although PON3 protein expression was very high at the protein level within placenta tissue, the PON3 at mRNA level using TaqMan assay was very low (Ct. Value was more than 35 cycle figure 2-8) or no amplification. According to TaqMan gene expression assay quality control if Ct value more than 35 cycles this mean that cDNA in the sample less than 5 copies. There was no record of PON3 gene being expression in placenta from Gene Card web site (Figure 3-36). Several attempts of optimization for TaqMan gene expression assay were undertaken include:

- Concentrated placenta cDNA (Primer Design) was run.
- Two different PON3 TaqMan gene expression assays (Applied Biosystem, cat no: Hs01023629_m1 and cat no: Hs01023630_m1) were used.

- Heart and myometrium tissue were investigated for PON3 mRNA gene expression
- Agarose gel 2% was run on PCR product showed double bands in different amplicon length indicated that expression was not PON3 Figure 3-37.

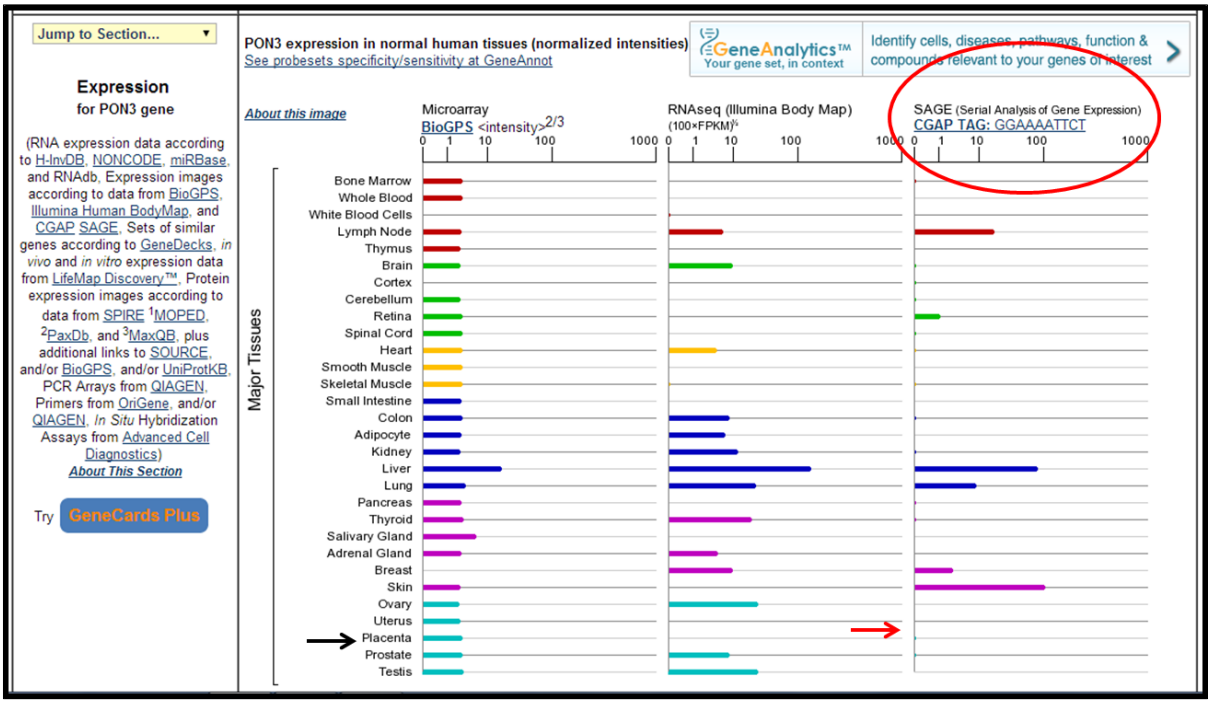

Figure 3-38: Print screen of Gene Card website showed that PON3 gene expression (red cycle) in placenta (black arrows) is so little (red arrow).

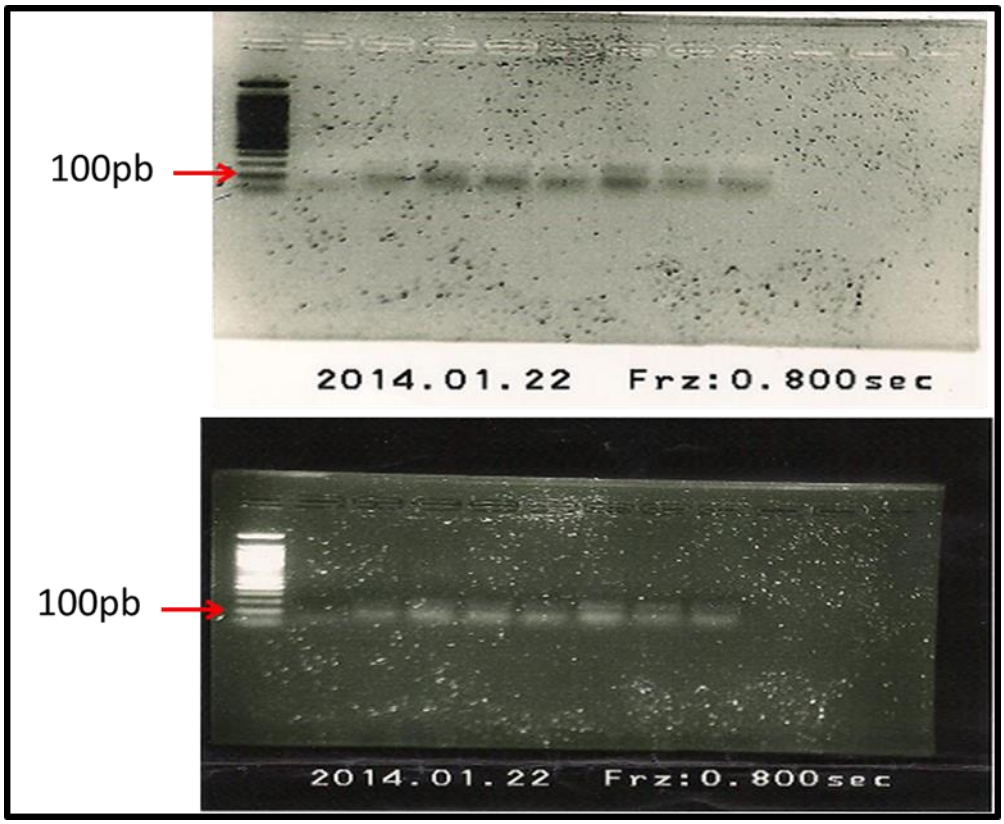

Figure 3-39: Graph showed qPCR product of PON3 TaqMan gene assay. Double bands were identified by run agarose gel at 100-120 bp and the amplicon length for the PON3 TaqMan gene assay is at the 69 bp.

This placenta, heart and myometrium did not express PON3 mRNA. There is no evidence in the literature that PON3 is expressed in heart or myometrium and our study was unable to find PON3 in this tissue. One possibility is that PON3 protein could be produced elsewhere within the body and then transported to the placenta where it deposits. This could account for the very high level detected with Western blots as there is a very high blood flow to the placenta. Another possibility is that the protein is extremely stable, while the mRNA is very unstable. This would mean that we would need little detectable mRNA to build up large deposits of the protein. Also it is important to remember that the mRNA level is not always directly comparable to the amount of detectable proteins, as some protein may degrade very quickly, while other proteins can be extremely stable. This is also true for the mRNA species as well, as some mRNA will be degraded or silenced (miRNA) very quickly and some mRNAs will be extremely stable within the cell. In this situation, it is more than likely that the results we have seen with TaqMan are the true negative results, that there is no detectable level of PON3 mRNA within placenta.

At the end of the results section a summarised table regards the main finding of the study was done and is shown in table 3-1.

Table 3-1: Results summary table

Gene	Protein	Molecular	Hypoxia-reperfusion injury
PON2	No spatial difference across individual placenta. There was a significant decrease in labour group compared to the non-labour group at the middle site only. There was a significant decrease in PE group compared to the control group at the middle site only.	No spatial difference across individual placenta. There was a significant decrease in labour group compared to the non-labour group at the middle site only. No changes were found.	There was a clear decrease over the time. There was no significant difference after exposed to hypoxia-reperfusion insult.
PON3	No spatial difference across individual placenta. There was a significant decrease in labour group compared to the non-labour group at the inner site only. There was a significant decrease in PE group compared to the control group at the middle site only.	There was no PON3 mRNA expression in placenta tissue.	There was a clear decrease over the time. There was no significant difference after exposed to hypoxia-reperfusion insult.

4 Discussion

4.1 PONs and present findings

PON2 and PON3 expression was significant decreased in labour and further less in pre-eclampsia group especially at the middle site. As labour is an inflammatory process the reduction in PONs would allow the inflammatory pathways to go further and continue as natural process. Importantly the present work shows that expression varies on the zone of the placenta the piece of tissue is taken from. This has important implications for placental research as the overwhelming majority of published papers do not control for sampling area. This may, at least in part, explain why there is such a discrepancy in published findings for similar studies applied to the placenta. In addition, it seemed odd that for so many studies on the placenta, and for any given protein, there seemed to be such a variety of conflicting papers. Part of this thesis therefore attempted to try and understand if this could, at least in part, be explained by sampling approaches. The present study has confirmed that protein expression varied at different areas of the placenta. With regard to labour controls or labour pre-eclampsia it is impossible to know what changes in protein expression contribute to labour or pre-eclampsia and what protein changes are a result of labour or pre-eclampsia. The fact that changes in protein expression are noted across zones suggested there is either something different about the inherent physiology of different zones of the placenta or it may be that there is something different about the blood supply to the different zones. It is beyond the work of this thesis to be able to answer that.

4.1.1 Key findings PON2

The main findings of this study were that there were no spatial differences within individual placentas for both labour and non-labour. At the middle site there was a significant decrease in PON2 expression in the labour group when compared to the non-labour group for both the 62 kDa form ($p=0.02$) and the 43 kDa form ($p=0.006$).

In PE, the main findings were here was no difference between PON2 in the non-labour control group compared to the non-labour PE group at the inner placental site or between the respective labour groups. A significant reduction in both PON2 isoforms was observed

in the non-labour PE group when compared to the non-labour control group and for the respective labour groups at the middle site.

The hypoxia-reperfusion experiments are preliminary. But there was an obvious increase in PON2 mRNA in inner, middle and outer placental areas when exposed to hypoxia re-oxygenation at the 6 hours' time point. Future experiments should follow this up to determine if this is a real statistical effect.

4.1.2 Key findings PON3

There was no difference in expression of PON3 between the three sites (inner, middle, outer) within individual placentas for both labour and non-labour. PON3 was significantly decreased in the labour group when compared to the non-labour group at the inner site. No other differences were found.

There was a significant decrease in PON3 expression in the PE non-labour group compared to the control non-labour group in the middle site only. There was a significant decrease in the PE labour group compared to the control labour at the middle site only. Again the hypoxia-reperfusion experiments are preliminary but worth pursuing.

4.2 Labour and PON

Normal human labour involves interaction of hormonal, neurological, mechanical stretch and inflammatory pathways and the placenta plays a crucial role. Labour has been show to induce placental oxidative stress, which in turn activates ER stress *in vitro* (Yung *et al.*, 2007; Cindrova-Davies *et al.*, 2007). The paraoxonases are involved in protecting against oxidative damage and lipid peroxidation, modulation of endoplasmic reticulum (ER) stress and regulation of apoptosis (Martinelli *et al.*, 2012; Li *et al*., 2014). Until recently nothing was known about the role of PONs in the placenta and how expression was affected by the stress of labour. This study examined PON2 expression in the placenta during labour and has now been published (Alwarfaly *et al*., 2014). No difference in protein expression of either PON2 isoforms was found between the three sites (inner, middle, outer) within either the labour or non-labour group. At the middle site there was a highly significant decrease in PON2 expression in the labour group when compared to the non-labour group

for both isoforms. A reduction in PON2 protein may help to initiate or promote labour.

PONs is glycosylated with high-mannose-type sugars, which are important for protein stability but are not essential for their enzymatic activities (Martinelli *et al.*, 2012). Whether alterations in glycosylation of PON2 could affect PON2 expression in the placenta would require future research. In contrast PON3 was significantly decreased in the labour group when compared to the non-labour group at the inner site. Whether the site of where the reduction of the protein is relevant remains to be discovered nonetheless a reduction was also found for PON3 too. There was a significant decrease in PON3 expression in the PE non-labour group compared to the control non-labour group in the middle site only. There was a significant decrease in the PE labour group compared to the control labour at the middle site only. Thus PON3 may just as important as PON2.

4.3 PE general

PE remains one of the leading causes of maternal morbidity and mortality worldwide for both mothers and babies. About 50,000 women die in developing countries per year from the disorder (Duley, 1992). The gold standard for management of PE has been the same for 100 years that is careful maternal observation for the signs of PE and delivery of women with symptoms that are severe. Maternal mortality is far less in developed countries due to proper perinatal observation; nevertheless morbidity still remains the leading cause of admissions to intensive care for pregnant women. (Tang *et al*., 1997) Delivery of women who develop severe PE early in gestation accounts for 8% of all preterm births (Ananth *et al*., 2006). With that come all the problems associated with prematurity.

During the past 20 years, our knowledge of PE has increased greatly. It is known that normal pregnancy is a pro-inflammatory state (Redman *et al.*, 1990) which increases in normal pregnancy as the pregnancy advances (Duckitt *et al*., 2005). Monocytes/macrophages play an important role (Faas *et al*., 2014). Redman (1990) has proposed therefore that PE is an excessive response to this stress. Women who already have an existing inflammatory disorder such as autoimmune disease, renal disease or metabolic syndromes are more likely to develop PE which supports the notion that it the entire inflammatory system has been pushed beyond which the mother cannot cope

(Duckitt *et al*., 2005).

4.3.1 Definition of PE

PE is currently defined as cardiovascular and renal dysfunction identified by blood pressure and proteinuria measurements. This is a historical definition coming from the fact these were the first observed symptoms in women which were then followed by eclampsia, which was then thought to be a pregnancy-specific seizure disorder (Chesley, 1984).

4.3.2 Risk

Hypertension and proteinuria predict increased risk for mother and baby however, even without proteinuria, there is an increased risk associated with gestational hypertension when accompanied by other systemic involvement. It has been stated that a key feature in studying PE is recognizing the fact that it is a syndrome which does not require proteinuria (Myatt *et al*., 2014).

4.3.3 Causes and prediction of PE

During the past 20 years, our knowledge of PE has increased greatly. I*n vivo* and *in vitro* (through venous circulation) studies have aided our understanding of the mechanisms involved in failed conversion of maternal spiral arteries (Lyall *et al*., 2013) and that this can lead to ischemia-reperfusion injury of the placenta (Burton *et al*., 2009). Known better is the role of inflammation, including endothelial dysfunction and recognizing that it is a multisystem disorder (Roberts *et al*., 1989). This has meant that less importance is put on blood pressure as the important contributing factor. The primary organ involvement may be hepatic, renal, cardiovascular, or placental. Although this has helped to direct research efforts to better understand the aetiology, it has not had a significant effect on how patients are managed or indeed how the condition is predicted reliably. One of the most promising attempts for prediction was to measure factors (such as placental derived factors) implicated in the pathophysiology of PE to try and predict the disorder. None have as yet been adopted for use in routine clinical practice due to problems such as adequate

sensitivity and specificity (Redman *et al.*, 2014).

Prediction and treatment of PE has been more successful in small studies than larger powered multi-centre studies. This has been put down to smaller studies having a more homogenous population and conversely larger multi-centre trials having a more heterogenous population (Myatt *et al*., 2014). Another problem of predictive studies is that factors being measured are not present in all women with PE (Myatt et al., 2014). All of this supports the idea that sub-groups of PE exist which all end up presenting as high blood pressure and proteinuria. Indeed this is supported by clinical and epidemiological data.

Placental pathology is generally worse in early onset PE however there is no histological evidence that spiral artery remodelling is restricted to early onset disease only (lyall *et al*., 2013). There appears to be a spectrum of changes both within all the spiral arteries of individual cases and across gestational ages (lyall *et al*., 2013). Where remodelling is defective it has been predicted to result in high-velocity, intermittent, maternal blood flow within the inter-villous space (Burton *et al*., 2009) which can induce placental oxidative stress through high shear rates and an ischemia/hypoxia–reperfusion type of injury (Burton *et al*., 2004, Redman *et al*., 2009). In vitro, placental oxidative stress causes release of anti-angiogenic factors which may reach the maternal circulation through the venous circulation (Cindrova-Davies *et al*., 2007). The presence of anti-angiogenic factors in the maternal circulation during the second trimester of pregnancy, for example sFlt1, is strongly positively the onset of PE (Vatten *et al*., 2007). However the maternal response to the deficient remodelling may also determine how well the pregnancy progresses. As well as inducing oxidative stress ischemia/hypoxia–reperfusion activates ER stress and apoptosis (Vatten *et al*., 2007).

PONs has been shown, as outlined below, to be involved in ER stress and apoptosis. Thus, in theory, a reduction in PON expression such observed in this work, would favour ER stress. Hormones, growth factors and plasma membrane proteins as well as protein post-translational modification occur in the ER. Knockout mice studies have shown that ER stress causes placental insufficiency leading to restriction of fetal development and growth (Iwawaki T *et al.*, 2009). Accumulation of unfolded and misfolded proteins activates ER stress-response pathways, also referred to as the unfolded protein response (UPR). The UPR is made up of three conserved signalling pathways. The first is the PERK–eIF2α pathway which attenuates non-essential protein synthesis. The ATF6 and IRE1–XBP1

pathways increase folding capacity by up-regulation of the ER chaperones GRP78 and GRP94. Furthermore the ER-associated protein degradation pathway facilitates protein degradation (Schroder *et al.,* 2005). Activation of ER stress pathways has been demonstrated n in PE and fetal growth restriction (Yung *et al*., 2014). Activation of placental UPR and stress-response pathways, including P-IRE1*α*, ATF6, XBP-1, GRP78 and GRP94, P-p38/p38 and HSP70, was higher in early-onset PE than in late-onset PE and normotensive controls with 34 weeks of gestation being where a clear difference between gestations was observed; a different placental sampling method was used to the one in this thesis. Cellular stress and protein unfolding also initiates other protective pathways such as the heat shock proteins which aid in folding and degradation of misfolded proteins and previous work from out laboratory has shown that heat shock protein 70 and 27 expression is spatially distributed in human placenta and selectively upregulated during labour and PE (Abdulsid *et al*., 2013 and 2014).

The majority of PE cases present in the last month of pregnancy. The cases used in our study were all late-onset. Women can, however, present at any gestational age with PE that can rapidly progresses to life-threatening disease over hours to days yet on other women the symptoms can remain the same and not get any worse. The 10% cases that present early on are markedly different than those at term (Myatt *et al.,* 2014). PE presenting before 37 weeks of gestation is when most of the small for gestational age babies are born and before 37 weeks is when the disorder tends to be more serious (Xiong *et al*., 2002). In our study birth weight was not different between the PE and control groups. Studies in our laboratory are now being performed on placentas from severely growth restricted babies to determine if PON2 expression is different. Long–term health consequences for women who have had early on-set PE are different too; epidemiological studies have shown the risk of cardiovascular disease in later life with earlier onset PE is 8 to 10 fold (Irgens *et al*., 2001; Mongraw-Chaffin *et al*., 2010) compared to 2 fold when PE presents close to term (Bellamy*et al*., 2007).

Numerous studies have been performed on measuring circulating factors in PE as well as assessing expression of a plethora placental factor such as those linked to inflammatory, anti-angiogenic, oxidative stress, or ER stress-mediated or other pathways. Whether studies such as these, including the present one, might help to categorise PE sub-types or helps to understand the disease process remains to be seen. Caution should be used in how factors are used to categorise PE because different factors can often be linked to a similar

pathway (Myatt *et al*., 2014). Given that circulating paraoxonases are emerging as unique protectors against cardiovascular diseases (Martinelli1 *et al*., 2012; Li *et al*., 2014). Future studies using ELISA to measure PONs in maternal circulation as predictive markers to measure PONs as predictive markers of PE will be of interest. There is one important human study; term human infants had more than 6-fold higher umbilical cord serum levels of PON3 than preterm (24-28 weeks of gestation) infants (Belteki *et al*., 2010).

The aetiology of the PE syndrome remains elusive and is pregnancy specific. Delivery of the placenta is the only cure and so, for this reason, it is generally thought that the root of the disorder lies in the placenta. One of the difficulties in studying PE is that it is a heterogenous disorder with a range in the way symptoms present clinically as well as heterogeneity in the pathological findings reported within the placenta. (Ohkuchi *et al.*, 2007; Wikstrom *et al*., 2006). In recent times more emphasis has been placed on how placental samples should be taken to ensure systematic sampling however the majority of published studies do not follow such procedures. At present, there are no agreed standards for placental sampling or storage of samples or for what minimal accompanying clinical information is required for interpretation of the results. However attempts are being made to put do this (Burton *et al*., 2014). Many factors can affect subsequent analysis; these include direct clinical and non-clinical factors (Myatt *et al*., 2014; Burton *et al*., 2014). Ethnicity can have effects on the placenta through genetic variations, diet or other factors. Both birth weight and placental weight were reported to be lower in Asian women compared to European and Afro-Caribbean ethnic groups, although the fetal/placental ratio was not different (perry *et al.,* 1995). Cigarette smoke also has been reported to alter placenta structure (Bush *et al*., 2000) as has alcohol (Burd *et al*., 2007) which has been linked to a reduction in placenta size, reduced blood flow and impaired nutrient transport. Body mass index is becoming a more and more serious global health issue and no less so in pregnant women. High maternal body mass index is negatively correlated with the fetal: placental weight ratio (perry *et al.,* 1995). Maternal obesity is also associated with increased oxidative stress in the placenta (Roberts *et al*., 2009) and with a global reduction in DNA methylation (Nomura *et al*., 2013). Placenta weight increases with maternal (Haavaldsen *et al* 2011) and paternal age.

Study groups have met to try and agree how to classify PE into groups (von Dadelszen *et al.*, 2003; Myatt *et al*., 2014). Several schemes have been proposed, and the most common is to divide cases clinically into early- and late-onset, according to the gestational age at

diagnosis or when delivery is necessitated. The majority (90%) of cases are late-onset, usually defined as the occurrence after 34 weeks gestational age. Of the two forms, early-onset is often considered the more severe, as it is associated with a higher rate of intrauterine growth restriction (IUGR) and risk of maternal cardiovascular complications after delivery (Paruk and Moodley, 2000; Dahlstrom *et al.*, 2008). Moreover, there is a greater prevalence of placental lesions indicative of maternal malperfusion (Moldenhauer *et al*., 2003; van der Merwe *et al.,* 2010; Nelson *et al*., 2014) as confirmed by a recent magnetic resonance imaging study (Sohlberg et al., 2014). In view of these differences, it has been proposed that PE can be classified on the basis of the pathophysiology into 'placental' and 'maternal' causation (Redman *et al*., 2000). In the former, it is postulated that malperfusion leads to placental stress and the release of cytokines and angiogenic regulators that cause maternal endothelial cell activation. In the latter, it is believed that the same final stage is reached due to a predisposing exaggerated maternal endothelial sensitivity to factors emanating from a relatively normal or excessively large placenta. The latter classification is based on normal pregnancy being a pro-inflammatory state, with activation of circulating immune cells (Redman *et al*., 2003) and elevated systemic oxidative stress as gestation advances (Belo *et al*., 2004). Therefore, 'maternal' PE has been suggested to be an excessive maternal response towards existing inflammation (Redman *et al.*, 1999). This is backed up by a growing body of evidence showing that women with chronic systemic inflammation, due to conditions such as renal disease, autoimmune diseases or metabolic syndromes, have an increased risk of developing PE near to term (Duckitt *et al*., 2005).

In placental PE there is deficient remodelling of the maternal spiral arteries (Papageorghiou *et al*., 2001; Lyall *et al*., 2013) shown by abnormal uterine artery Doppler waveforms and immunohistochemical analysis of placental bed spiral arteries This is thought to result in high-velocity, intermittent, maternal blood flow within the inter-villous space (Burton *et al*., 2009) which can induce placental oxidative stress as a result of high shear rates leading to an ischaemia/hypoxia–reperfusion type of injury (Burton *et al*., 2004; Redman , Sargent, 2009). Placental oxidative stress causes release of anti-angiogenic factors into the maternal circulation (Cindrova-Davies *et al*., 2007) in the maternal circulation during the second trimester of pregnancy levels of anti-angiogenic factors, such as sFlt1, positively correlate with onset of the syndrome (Vatten *et al.,* 2007).

The ER is where synthesis of polypeptide hormones, growth factors and plasma membrane

proteins and their post-translational modification takes place. Ischaemia/hypoxia–reperfusion induces oxidative stress and activates ER stress (Yung *et al.*, 2007; Yung *et al.* 2008). It has been shown using genetic manipulation studies that ER stress causes placental insufficiency, which in turn reduces fetal growth and development (Iwawaki *et al*., 2009). Accumulation of unfolded and misfolded proteins activates ER stress-response pathways and the unfolded protein response pathway (UPR). The UPR comprises three signalling pathways: the PERK–eIF2α pathway, which attenuates non-essential protein synthesis; and the ATF6 and IRE1–XBP1 pathways, which increase folding capacity by up-regulation of the ER chaperones GRP78 and GRP94 and phospholipid biosynthesis. In addition, the ER-associated protein degradation (ERAD) pathway facilitates protein degradation (Schroder and Kaufman, 2005). ER stress pathways in placentae from cases of fetal growth restriction show greater activation of ER stress compared to pregnancies complicated with PE and fetal growth restriction (Yung *et al*., 2008). More recently researchers have discussed separating findings based on gestational age at diagnosis and/or delivery. There is no agreement what this should be.

PE is commonly divided into early-onset (<34 weeks) and late-onset (≥34 weeks). As stated above researchers are now discussing the distinction between placental and maternal PE. It has been suggested placental cases are more likely to be early-onset with fetal growth restriction. Activation of placental UPR and stress-response pathways were shown to be higher in early-onset PE than in both late-onset PE and normotensive controls (NTCs) with 34 weeks of gestation being where a clear distinction was apparent. In that study there was no difference in placentas from ≥ 34 weeks PE and controls were indistinguishable. *In vitro* hypoxia-reoxygenation induced activation of UPR pathways in BeWo cells. The authors suggested that placental stress they had studied may be unlikely to be involved in late-onset PE however (Yung *et al.*, 2014). However there are many papers in the literature showing stress pathways altering in late-onset PE as discussed herein.

4.4 Linking PON2 expression to oxidative stress and ischemia-reperfusion injury

Oxidative stress occurs when the production of reactive oxygen species overwhelms the intrinsic anti-oxidant defences. Whether reductions in PON2 protein expression in the

placenta contribute to labour and PE or are a result of contractions or placental stress cannot be answered since human patients have been used. Contraction of the uterus leads to ischemic-reperfusion injury that can alter placental protein expression (CindrovaDavies *at al*., 2007). Furthermore Doppler ultrasound studies have been used to demonstrate an inverse relationship between uterine artery resistance and the intensity of uterine contractions during labour. In pregnant rhesus monkeys placental blood flow is almost completely stopped during sustained myometrium contractions as a result of compression of the arcuate and spiral arteries. The closest human model to this was performed on patients prior to termination of pregnancy at 17–20 weeks of gestation.

During oxytocin-induced contractions, a 50% reduction in flow into the intervillous space, as well as a fall in entry sites and volume, was found compared to when no contractions occurred (Borell et al 1965; Ramsey *et al*., 1980). This suggests that intermittent perfusion of the intervillous space would lead to an ischemic-reperfusion injury of the placenta which could affect reactive oxygen species and the oxidant/antioxidant balance (McCord, 1993). In keeping with this labour has been reported to be associated with placental alterations in several pathways linked to oxidative stress and this includes late-onset PE (Many *et al*., 1997; Elliott *et al*., 1998; Allport *et al*., 2001; Sitras *et al*., 2008; Lee *et al*., 2010; Peng *et al*., 2011; Lim *et al*., 2012). Other studies from our laboratory have shown selective up regulation of heat shock proteins HSP70 and 27 in labour and PE suggesting a link to placental oxidative stress (Abdulsid *et al.,* 2013 and 2014). Failure to modify spiral arteries is a feature of many cases of PE (Lyall *et al*., 2013). Some are partially remodelled, some fully remodelled and some not remodelled at all. Hence another parameter that could potentially affect PON expression might be linked to the physiological remodelled state of the spiral artery that supplies a particular area of the placenta.

4.5 PONS

The paraoxonases were named so because the substrate for PON1 is paraoxon which is the active metabolite of the organophosphorus insecticide parathion. PON2 and 3 lack this esterase activity despite the similar nomenclature. PON1 and PON3 proteins are present in plasma and reside in the HDL fraction and protect against oxidative stress by hydrolysing certain oxidized lipids in lipoproteins, macrophages, and atherosclerotic

lesions (Mackness *et al*., 1997; Reddy *et al*., 2001; Jaouad *et al*., 2006; Sierksma *et al*., 2007). Paraoxonases are important detoxifying and anti-oxidative enzymes with roles being described in organophosphate poisoning, diabetes, obesity, cardiovascular diseases, and innate immunity and with atherosclerosis (Shih *et al*., 1998; Ng *et al*., 2006; Shih *et al*., 2007; Camps J *et al*., 2009; Shih *et al*., 2009).

4.6 PON2 and cell death

ER stress activates the UPR pathway and pro-apoptotic CHOP protein in the presence of overwhelming ER stress (Oyadomari and Mori, 2004; Zhang *et al*., 2006; Song *et al*., 2008). Mitochondria also play a key role in cell death via production of excess reactive oxygen species. (Ott *et al*., 2007). It has been shown that human PON2 diminished not only R reactive oxygen species but also ER stress-induced apoptosis in vascular cells (Horke *et al*., 2007). PON2 is expressed in several tissues with antioxidant properties. It is capable of preventing cell-mediated oxidative modification of low density lipoprotein and ER stress-induced apoptosis. (Ramsey *et al*., 1980; Cindrova-Davies *et al*., 2007; Roberts and Hubel, 2009). PON2 is not present in serum lipoprotein fractions but exists as an intracellular protein found in almost every tissue, particularly at the perinuclear region, ER and mitochondria (Ng *et al*., 2001; Horke *et al*., 2007; Rothem *et al*., 2007). Natural substrates remain unknown albeit PON2, as part of the innate immune system, appears involved in the defence against infections by the human pathogen *Pseudomonas aeruginosa* (Rothem *et al*., 2007). Several studies demonstrated that PON2 protected macrophages, vascular and other cells against oxidative stress, whereas its down regulation reversed this effect (Ng *et al*., 2001; Aviram *et al*., 2004; Horke *et al*., 2007).

PON2 has been shown to be overexpressed in several cancers and it has been suggested that this may be due to the fact that PON2 confers resistance to apoptosis as well as oxidative stress (Witte *et al*., 2011). It has been shown that during ER stress, high levels of PON2 lowered redox-triggered induction of pro-apoptotic CHOP particularly via the JNK pathway, which prevented mitochondrial cell death signalling (Witte *et al*., 2011). Apart from CHOP, PON2 also diminished intrinsic apoptosis as it prevented mitochondrial superoxide formation, cardiolipin peroxidation, cytochrome *c* release, and caspase activation. Oxidized lipids can also induce pro-inflammatory genes, such as TNF-α and MCP-1, via NF-κB activation (Witte *et al*., 2011). Therefore one possibility

is that less placental PON2 would result in more oxidized lipids and more NF-κB activation which would promote inflammatory responses. Interestingly macrophages harvested from PON2-/- mice are more susceptible to cellular oxidative stress than wild-type macrophages (Fuhrman *et al*., 2008).

The mechanism by which PON2 modulates ROS production is still unclear (Meilin *et al*., 2010; Altenhöfer *et al*., 2010; Devarajan *et al*., 2011). Lactones have been suggested to be the natural substrates of PON2 and PON2 lactonase activity has been shown to correlate with this enzyme's biological antioxidant properties. Studies have shown increased risk of coronary artery disease, carotid atherosclerosis and stroke in patients with low paraoxonase activity (Chen *et al*., 2004). Since PE is a cardiovascular disorder future studies in PE include measurement of PONs enzyme activities are warranted. Specific gene polymorphisms for PON1 and PON2 have been reported in children born pre-term. (Chen *et al*., 2004).

In conclusion this is the first study to investigate the expression of PON2 in PE and there are the summary of the results:

1. PON2

There were expression of two isoform of PON2; isoform one (62 kDa) and isoform two (43 kDa) in all placenta samples. There was no significant difference in spatial expression of PON2 protein and mRNA of both isoforms across individual placenta of both labour and non-labour groups. There was no significant difference in PON2 protein and mRNA expressions of both isoforms between labour and non-labour groups at the inner and outer placenta sites. There was a significant decrease in PON2 protein and mRNA expressions of both isoform in labour group compared to the non-labour group at the middle site. There was no significant difference in PON2 protein expression of both isoforms between non-labour and labour controls when compared to non-labour and labour PE at the inner placenta site. There was a significant decrease in PON2 protein expressions of both isoform in non-labour and labour PE groups when compared to the non-labour and labour controls group at the middle site. No changes were found in the mRNA level when non-labour and labour control compared to the non-labour and labour PE. There was no significant difference when all normotensive groups compared to the PE groups.

2. PON2 expression after hypoxia-reperfusion insult

There was clear decrease in PON2 expression over the time. There was no significant difference in PON2 protein expression of both isoforms at inner, middle and outer placental sites after exposed to hypoxia reperfusion injury. There was a clear increase of PON2 mRNA in exposed group between 3-12 hours at the middle and outer sites but as it was a pilot study so there was no comparison.

3. PON3 expression

There was no PON3 mRNA expression in placenta tissue. There was no significant difference in spatial expression of PON3 protein across individual placenta of both labour and non-labour groups. There was a significant decrease in PON3 protein expressions in labour group compared to the non-labour group only at the inner site. There was a significant decrease in PON3 protein expressions in non-labour and labour PE group compared to the non-labour and labour control group at the middle site.

4. PON3 expression after hypoxia-reperfusion insult

There was clear decrease in PON2 expression over the time. There was no significant difference in PON3 protein expression at inner, middle and outer placental sites after exposed to hypoxia reperfusion injury.

The findings suggest, given the protective roles of paraoxonases, that future studies include clinical trials of therapeutic approaches that emerge to increase PONs activity may provide a novel of preventing PE and reveal new insights into our understanding of PE. Our findings also emphasise the heterogeneity of the placenta.

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6 Appendixes