An investigation into stress, inflammatory and apoptotic pathways in the placenta in labour, pre-eclampsia and fetal growth restriction

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A THESIS SUBMITTED IN FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (PHD) TO THE UNIVERSITY OF GLASGOW

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May 2018

Abstract

The mechanisms that are involved in maintaining a human pregnancy to term, and the switches that lead to a normal labour and pregnancy outcome or indeed an adverse outcome such as miscarriage, pre-eclampsia (PE), fetal growth restriction (FGR) or preterm labour, are complex but the role of the placenta is crucial to them all. Heat shock proteins (HSPs) play an important role in regulating signal transduction, inhibition of apoptosis and cellular proliferation and differentiation. Apoptosis has been implicated in both PE and labour. In the apoptotic pathway, HSPs act at several stages to prevent cell death initiated by stress-induced damage. Toll-like receptors (TLR) are the principal signalling molecules through which mammals sense infection. Since these pathways play a role in oxidative stress and inflammation, both features of labour and PE, the hypothesis was that placental stress pathways (HSP70, HSP27, HSF1-2, small HSPs family, DNAJAB, HSP60, GRP78, HSP90AA, HSP90B1 and HSP105), inflammatory mediates (TLRs, MyD88 and NF-κB) and apoptotic markers (caspases (CASP) 3, 7 and 9) pathways expression would alter during labour and pregnancies complications such as PE and FGR and this would vary in different placental zones perhaps due to the variation in blood supply to each zone or link to ischemic-reperfusion (IR) injury and are some zones of placenta more susceptible to IR injury. HSPs, TLRs and caspases were examined in placentas obtained from women who delivered by caesarean section (non-labour) and were not in labour and compared to the equivalent zone of placentas obtained from women who delivered vaginally (labour) 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. The expression was investigated by Western blotting (HSP70, HSF1, HSP27 and P-HSP27) and real time PCR (HSP families, HSF1-2, TLRs, MyD88, NF-κB and caspases).

The first aim of this study was to examine the spatial expression of HSPs, caspases and TLR genes in placentas. Two HSF1 forms were observed in all samples which, according to the data sheet represent the phosphorylated (P-HSF1) and non-phosphorylated (NP-HSF1) forms. HSP70, HSF1 and HSP27 are expressed in a spatial manner in the placenta with the highest expression being in the middle zone in the labour group (HSF1, both forms) and in both the labour and the non-labour groups (HSP70 and HSP27). HSF1 mRNA expression was spatially distributed in the non-labour group with highest expression being in the outer zone. No spatial differences were noticed in both P-HSP27

protein expression and mRNA expression of other HSPs families, HSF2, TLRs, MyD88, NF-κB and caspases3, 7 and 9 genes.

Human parturition involves interaction of hormonal, neurological, mechanical stretch and inflammatory pathways and the placenta plays a crucial role. The second aim of this study was to determine these genes expression in the non-labour group compare to the labour group at the inner, middle and outer placental zones. When non-labour groups were compared with the labour group at the three zones the changes found can be summarized as follows:

HSP70 protein expression was increased in the labour group compared to the non-labour group and in contrast to that, HSP27 protein and mRNA expression were increased in the non-labour group both at the middle zone.

Phosphorylated-HSP27 (P-HSP27) expression was increased in the non-labour group compared to the labour group at the inner (all ser sites) and outer (ser 15 and 78) zones. No differences were found at the middle zone. HSF1 protein expressions (both forms) was increased in the non-labour, group compared to the non-labour group, paradoxically, HSF1 mRNA expression was increased in in the labour group at the inner zone.

HSPB6, HSP32 and DNAJB1 mRNA expression was increased in the non-labour group compared to the labour group at the middle zone. HSPB2, HSP90AA and HSP90B1 mRNA expression was increased in the labour group compared to the non-labour group at the outer zone. TLR5 and CASP7 mRNA expression was increased in labour group compared to the non-labour group at the middle zone. TLR9-10 mRNA expression was neglectable. No other changes were found for any other genes.

PE is associated with maternal and placental oxidative stress. The third aim was to determine the expression of genes of the same pathways described above in the placenta in pregnancies complicated with PE and FGR compare to the normal pregnancies. HSPs, TLRs and caspases were examined in placentas obtained from non-labouring women who delivered by caesarean section (normal pregnancy and PE) and compared to women who delivered vaginally (normal pregnancy and PE). Samples were obtained from 12 sites within each placenta: Four equally spaced apart pieces were sampled from the inner, middle and outer, however, due to time constraints only the inner and middle zones were investigated in PE group (except HSP27 and HSP70). Samples from FGR placentas were obtained from 8 sites within each placenta represent inner and outer zones. Western blotting and qRT-PCR was used for the analysis as required.

When PE (non-labour and labour) and FGR (labour only) groups were compared with the controls (non-labour and labour) at the two zones, the changes found can be summarized as

follows: HSP70 was increased in the non-labour-PE group compared to the non-labour control group at the inner zone. No other differences were found. Non- Phosphorylated-HSF1 (NP-HSF1) protein expression and HSF1 mRNA expression were increased in the PE group (non-labour and labour) compared to the control group (non-labour and labour) at the inner zone. HSP27 and P-HSP27 protein expression was increased in the PE (labour) compared to the control (labour) at the middle zone. No changes were found in the mRNA expression of HSP27. There was a significant increase in P-HSP27 protein expression in labour PE compared to the labour control at the inner (ser 78 and 82) and middle (ser 15) zones.

CASP7 and CASP9 mRNA expression was increased in the non-labour control compared to the non-labour PE at the inner (CASP7) and middle (CASP9) zones. CASP3 mRNA expression was increased in the labour PE compared to labour control at the middle zone.

There was decreased in mRNA expression of HSPB6, TLR5 and TLR1 in the PE group non-labour compared to the controlnon-labour at the inner (HSPB6) and middle (TLR1 and TLR5) zones.

Towards the end of the PhD time preliminary experiments were performed to determine if different zones of the placenta were more susceptible to heat shock and IR injury *in vitro.* The markers mentioned above were used and pieces of placenta from each zone were exposed to 2% oxygen then 8% oxygen for different time periods to create an IR model. The number of experiments were 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 IR injury and heat shock to understand how placental stress might affect placental function.

Obesity is characterized by a chronic, low-grade inflammation and an impaired intracellular stress defence system. Obesity increases the risk for PE. The last aim was to screen for different HSPs and CASPs families in placentas obtained from 4 BMI groups (non-labour). In general, HSPs expression was reduced whereas caspases expression was increased in the morbid obesity group (BMI>40) compared to the normal BMI group. This indicates reduced cellular protection and increased cellular damage respectively in placentas from obese women.

In summary this thesis has reiterated the importance of controlled sampling of the placenta for studying pregnancy disorders. The results show that spatial changes in inflammatory and stress markers occur in both labour and PE. Trying to make sense of the data is very difficult however the preliminary data from the *in vitro* studies suggests that further studies exposing different zones of the placenta to stress may help to understand the process that

take place during stress. Clearly the bigger aim would be, one day, to try and decide which of the pathways might be targeted for therapy for pre-term labour and PE.

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Acknowledgement

First of all, I owe it all to Almighty God for granting me the strength to undertake this research task and enabling its completion. Thanks God for all the blessings that make the impossible possible

The completion of this work was possible with the support of several people to whom I would like to express my sincere gratitude.

I would like to thank my co-supervisor Dr Kevin Hanretty for the support and help in the sample collection process. I would also thankful to all staff at the delivery suite at the Southern General Hospital for all their help with placenta sample collection, which was crucial for this project.

I would like to thank Libyan embassy for providing the funding for my project without which this research would not have been possible.

I would like to thank Mr David Young for the advice given on statistical analysis

I would also like to thank my colleague at the department, Mrs Anne Theriaul for her help and unlimited support.

I am very much indebted to my family and friends in Libya and here in Glasgow, who encouraged and helped me at every stage of my personal and academic life. They were the joy of my life and they will be forever.

I would to thank my husband for his love, support and constant patience that inspired me to complete this work as you made our future look brighter.

Dedication

A special feeling of gratitude to my loving parents, whose endless love, care, encouragement filled me with joy, strength and tenacity each day of my life.

For my loving mother and my deceased father, I would like to dedicate this work to you both.

Author’s Declaration

I declare that, except where explicit reference is made to the contribution of others, that the composition of this thesis 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.

Signed ……

Akrem Abdulsid

May 2018

Publication

1. Alwarfaly, S., Abdulsid, A., Hanretty, K., and Lyall, F. (2014)*Paraoxonase 2 protein is spatially expressed in the human placenta and selectively reduced in labour*. PLoS ONE, 9 (5). e96754. ISSN 1932-6203 (doi:10.1371/journal.pone.0096754)

Poster and oral presentations

Abdulsid A., Walker S., Hanretty K., Lyall F. (2011).Heat shock protein 70 is expressed in a temporal manner in the placenta and up-regulated during labour. Tensvo Research Day, Glasgow, UK.

Abdulsid A. and Lyall F (2012). Heat Shock Protein 70 Expression Is Spatially Distributed in Human Placenta and Selectively Up-regulated during Labour and Pre-eclampsia. Women Research day, Glasgow, UK.

Abdulsid A. and Lyall F (2013). Heat shock protein 27 expression is spatially distributed in human placenta and selectively regulated during pre-eclampsia. ISSHP, Tromso, Norway.

Abdulsid A.' Hanretty K., Lyall F. (2014). Stress, inflammatory and apoptotic pathways expression in human placenta of normotensive pregnancy and pregnancy complicated by FGR or pre-eclampsia. 3 minutes competition at University of Glasgow, Glasgow, UK.

Abdulsid A.' Hanretty K., Lyall F (2014). HSP 27 and 70 expressions in the placenta of labour FGR cases versus control labour cases. ISGE meeting, Florence, Italy.

Abdulsid A.' Hanretty K., Lyall F (2014). TLR Expression in the Placenta during Labour and Pre-eclampsia. ISGE meeting, Florence, Italy.

Abdulsid A.' Alwarfaly S., Hanretty K., Lyall F. (2014). PON 2 Expression in Labour and Pre-Eclampsia. SGI 61st Annual Scientific Meeting, Florence, Italy.

Abdulsid A.' Hanretty K., Lyall F (2014). HSP 27 and 70 expressions in the placenta of labour FGR cases versus control labour cases. ISGE meeting, Florence, Italy. Young research winner.

Abdulsid A.' Hanretty K., Lyall F (2014). Heat shock transcription factor 1 expression is spatially distributed and selectively down-regulated in the human placenta during labour. IFPA meeting, Paris, France.

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Definitions/Abbreviations

Ab/Am	Antibiotic antimycotic solution
AIF	Apoptosis-inducing factor
ANOVA	Analysis Of Variance
Apaf1	Apoptotic protease activation factor-1
APC	Antigen presentation cells
APS	Ammonium per sulphate
ASK1	Apoptosis signal-regulating kinase 1
ATF6	Activating transcription factor 6
ATP	Adenosine triphosphate
BAD	Bcl-2-associated death promoter
Bcl-2	B cell lymphocytic-leukaemia proto-oncogene released proteins
BiP	Binding immunoglobulin protein
BMI	Body mass index
Bp	Base pair
BSA	Bovine serum albumin
C pneumonia	Chlamydia pneumonia
cAMP	Cyclic adenosine momophosphate
CASP3	Caspase-3
CASP7	Caspase-7
CASP9	Caspase-9
CD14	Cluster of differentiation 14
cDNA	Complementary Deoxy-Ribonucleic Acid
CEMACH	Confidential Enquiries into Maternal Deaths
CHD	Coronary heart disease
CMV	Cytomegalovirus
CO	Carbon monoxide
COX2	Cyclooxygenase 2
CRH	Corticotrophin-releasing hormone
CRP	C-reactive protein
CS	Caesarean section
CT value	Threshold cycle value
C_T	Threshold cycle

CTB	Cytotrophoblasts
CVD	Cardiovascular diseases
CXCL	Chemokine CXC motif ligand
CXCL8	Chemokine CXC motif ligand 8
DAMPs	Damage-associated molecular patterns
DBP	Diastolic blood pressure
DD	N-terminal death domain
DIC	Disseminated intravascular coagulation
DISC	Death-inducing signalling complex
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
EFW	Estimated fetal weight
EGF	Epidermal growth factor
eIF2α	Eukaryotic Initiation Factor 2 (eIF2)
ELC/S	Elective caesarean section
ELISA	Enzyme-linked immunosorbent assay
EMC/S	Emergency caesarean section
EndoG	Endonuclease G
ER	Endoplasmic reticulum
ERAD	ER associated degradation
ETB	Endovascular trophoblasts
EVT	Extra villous trophoblast
FADD	Fas receptor with Fas associated-death-domain
FBS	Fetal bovine serum
FEEL-1	Fasciclin, EGF-like, laminin-type EGF-like, and link domain-containing scavenger receptor-1
FFA	Free fatty acid
FGR	Fetal growth restriction
GDM	Gestational diabetic mellitus
GRP78	Glucose regulated protein 78
GRP94	Heat shock protein 90kDa beta member 1
H	Hour
HCG	Human chorionic gonadotropin
HCL	Hydrochloric acid

HNE	4-hydroxy-2-nonenal adducts
HO-1	Hemoxygenase-1
hPGH	Human placental growth hormone
hPL	Human placental lactogen
HRP	Horseradish peroxidise
HSBP1	Heat shock protein binding factor 1
HSEs	Heat shock elements
HSF1	Heat shock transcription factor 1
HSP70	70 kilo Dalton heat shock proteins
HSP90AA	Heat shock protein 90kDa alpha (cytosolic), class A member 1
HSP90B1	Heat shock protein 90kDa alpha (cytosolic), class B member 1)
HSPA5	Heat shock 70 kDa protein 5
HSPH1	Heat shock 105kDa/110kDa protein 1
HSPs	Heat shock proteins
HSR	Heat shock response
IFN-B	Inducing interferon-B
IFN-γ	Interferon-γ
IgG	Immunoglobulin G
IHC	Immunohistochemistry
IL	Interleukin
IL1	Interleukin 1
iNOS	Inducible nitric oxide synthase
IQR	Interquartile range
IRAKs	Interleukin-1 receptor-associated kinases
Ire1	Inositol-requiring 1
IRF	Interferon regulation factor
IUGR	Intrauterine growth restriction
JNK	c-Jun N-terminal kinase
KDa	kilo Dalton
LDL	Low-density lipoprotein
LG	Labour group
LG-FGR	Labour-FGR
LG-PE	Labour-PE
LH	Luteinizing hormone
LIF	Leukemia inhibitory factor

LOX-1	Lectin-like oxidized LDL receptor-1
MAP	Mitogen-activated protein
MAPKAP	MAP kinase activated protein
MAPKAPKs	MAP kinase-activated protein kinase
MAPKs	Mitogen-activated protein kinases
Mcl-1	Myeloid leukemia cell differentiation protein
MCP-1	Monocyte chemotactic protein 1
MDPK	Myotonic dystrophy protein kinase
Mg	Milligram
mg/L	Milligrams per litre
MHC	Major histocompatibility complex
MKBP	Myotonic dystrophy protein kinase-binding protein
mL	Millilitre
mM	Mili-molar
mmHg	Millimeter of mercury
MMP	Matrix metalloproteinase
mRNA	Messenger ribonucleic acid
MyD88	Myeloid differentiation factor 88
NaOH	Sodium hydroxide solution
NBD	N-terminal ATPase domain
NCBI	National center for biotechnology information
NDRG1	N-myc downstream-regulated gene 1
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
Ng	Nanogram
NK	Natural killer
NLG	Non-labour group
NLG-PE	Non-labour-PE
NO	Nitric oxide
PAMPs	Pathogen-associated molecular patterns
Par-4	Prostate apoptosis response 4
PBS	Phosphate buffered saline
PCOS	Polycystic ovarian syndrome
PE	Pre-eclampsia
PECAM-1	Platelet endothelial cell adhesion molecule-1
PERK	PKR-like endoplasmic reticulum kinase

PG	Primigravida
PGH	Placental growth hormone
PIC	Protease Inhibitor Cocktail
PIH	Pregnancy induced hypertension
PlGF	Placental growth factor
PlGF	Placental growth factor
PRRs	Pattern-recognition receptors
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	Room temperature
RT-PCR	Reverse transcriptase polymerase chain
SARM	Sterile-alpha and armadillo motif containing protein
SBD	Substrate binding domain
SBP	Systolic blood pressure
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide
sEng	Soluble endoglin
sFlt-1	Soluble fms-like tyrosine kinase receptor-1
SGA	Small for gestational age
sHSP	Small HSPs
SREC-1	Scavenger receptor expressed by endothelial cell-I
ssRNA	Single stranded RNA
STAT1	Signal transducers and activators of transcription
STB	Syncytiotrophoblast
STD	Stock standard solution
SVD	Spontaneous vaginal delivery
TEMED	N,N,N,N tetramethylethylenediamine
TGF	Transforming growth factor
TGF-β	Transforming the growth factor beta
TIR	Toll/IL-1 receptor domain
TLR	Toll like receptors
TNF	Tumour necrosis factor

TNFα	Tumour necrosis factor α
TRAIL	TNF-related apoptosis-inducing ligand
TRAM	TRIF-related adapter molecule
TRIF	Toll/IL-1 receptor domain-containing adaptor inducing interferon-β
UPR	Unfolded protein response
USS	Ultrasound scan
UV	Ultraviolet
vCTB	villous cytotrophoblast
VEGF	Vascular endothelial growth factor
VEGF-A	Vascular endothelial growth factor-A
VTE	Venous thromboembolic
WHO	World Health Organization
Wks	Weeks
XD/XO	xanthine dehydrogenase to xanthine oxidase
β-ME	Beta-Mercaptoethanol
μg	Microgram
μL	Microliter
2D	Two-dimensional
8-isoPGF α	8-iso prostaglandin F2α (oxidative stress marker)

Chapter I: General introduction

1 Introduction

1.1 The placenta

The human placenta is a unique organ that connects the growing fetus to the uterine wall via the umbilical cord. According to the Oxford English dictionary a placenta is a flattened circular shape organ formed in the uterus of pregnant mammals, that provides nourishment, maintaining, waste elimination and gas exchange of the fetus through the umbilical cord (Dictionary, online) (Benirschke et al., 2012c). It also produces hormones that support pregnancy and fights against infection. In early pregnancy the placenta is formed from the union of the chorion and the allantois.

1.1.1 The term placenta

The full term human placenta is a disc like and round to oval organ in about 90 % of cases (Benirschke et al., 2012c). In approximately 10% of the cases, however, it has an abnormal shape, such as placenta duplex, placenta succenturiate, zonary placenta and placenta membranacea (Torpin, 1969). At term, the placenta is on average 22 cm in diameter, 2.5 cm central thickness and an average weight about 470 gram (Benirschke et al., 2012a). Those measurements show considerable individual variation depending on several factors such as mode of delivery, time of cord clamping and time between delivery and examination. As the placenta is a highly vascular organ, the time of cord clamping is important because fetal and maternal blood loss can have an impact on the measurement (Bouw et al., 1976).

The fetal surface named as the chrorionic or amniotic plate has a glossy appearance and facing the amniotic cavity, it is a vascular membrane that contains the chorionic vessels (Figure 1-1). Chorionic vessels branch out over the placenta and form a star like pattern network, where the arteries cross superficial to the veins. The chorionic vessel network is covered by a single epithelium layer. The chorionic plate has an opaque appearance because of a huge number of collagen fibers accompany the vessels. There are transparent areas in between, which are black and shine because of maternal blood in the intervillous

space (Benirschke et al., 2012c). The transparency of the chorionic plate decreases near the placental margin.

The maternal surface of the placenta is an opaque surface originating from the separation of the organ from the uterine wall (Figure 1-1). This process of separation subdivides the junctional zone between the placenta and uterine wall into the basal plate and placental bed. The basal plate is the attachment site of the placenta to the uterus and it is composed of decidual cells and trophoblastic embedded in an extracellular fibrinoid, debris and blood clots (Benirschke et al., 2012c). The placental bed remains in the uterus after delivery. The placenta is subdivided by grooves into about 10-40 irregular shaped lobules named cotyledons. Major branches of the umbilical circulation supply each cotyledon. Cotyledons consist of villous trees structure bathed in maternal blood.

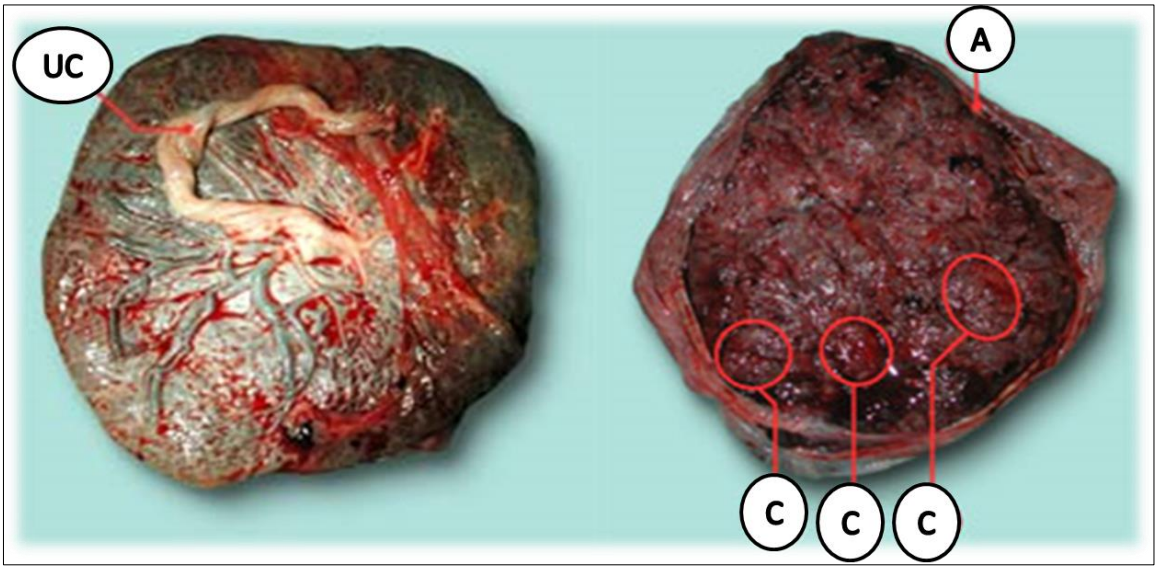


Figure 1-1: Pictures of the term human placenta. The left panel shows the fetal side of the placenta and umbilical cord inserts within the chorionic plate. The right shows the maternal side which is contact with uterus and divided into cotyledons. (C): cotyledons. (A): amnion. (UC): umbilical cord.

1.1.2 Placenta shape and cord insertion:

The umbilical cord contains two arteries and one vein, it is approximately 55-60 cm in length and inserts eccentrically in most placentas into the chrorionic plate (Rampersad et al., 2011). The cord insertion point and the placenta shape are important structurally parameters but not functionally and algorithms can be used to obtain the shape index into either round (Salafia et al., 2007) or oval shapes (Pathak et al., 2010). It has been suggested (Salafia et al., 2010) that increased variability in the pattern of placenta growth associated with different placenta function dimensions, as assessed by the ratio of birth and placenta weights that may indicate utero-placental pathology. The umbilical cord is usually inserted near the center of the placenta as a result of a blastocyst orientation during the implantation. It has been shown (Pathak et al., 2010) that eccentric is the common site of cord insertion. The ratio of fetal to placenta weight is lower in a off-center cord insertion, suggesting placenta function dimension (Yampolsky et al., 2009). High blood vessels resistance of the umbilical circulation is associated with eccentric insertion of the umbilical cord (Nordenvall et al., 1991).

1.1.3 Early development of the human placenta

1.1.3.1 The decidua of the endometrium

Preparation of the endometrium for implantation depends on hormonal stimulation as well as the interaction between the blastocyst and the endometrium (Loke et al., 1995, Gellersen et al., 2007, Ramathal et al., 2010). This interaction is mediated by several factors such as growth factors, cytokines and adhesion molecules, which are secreted by the endometrium and the blastocyst (Cross et al., 1994, Lindhard et al., 2002). Preparation of the endometrial stroma (decidualization) is the changes that occur in the endometrium structure in anticipation of pregnancy. The decidua is a specialized, modified endometrium of pregnancy and is a function of hemochorial placentation that has a common process of trophoblast invasion. The decidualization is started in the mid-secretory phase of each menstrual cycle, in pregnancy and due to high level of progesterone continues throughout the pregnancy. Decidualization is mainly dependent on oestrogen, progesterone and regulatory molecules that include cytokines, chemokines and proteases (Dimitriadis et al., 2010). Cytokines include leukaemia inhibitory factor (LIF) (Laird et al., 1997) and interleukin (IL) 11 (Dimitriadis et al., 2003) both are important factors in the implantation process. The decidua is composed of three parts, decidua

basalis that lies beneath the site of blastocyst implantation, decidua capsularis which overlies the blastocyst and decidua parietalis which line the rest of the uterus.

1.1.3.2 Early development of the blastocyst

Following fertilization, the fertilized egg called the zygote, moves down the fallopian tube to the uterine cavity. In the fallopian tube the zygote undergoes mitotic division to form a ball of cells (morula), which are surrounded by the zona pellucida that helps to hold them together. On day 4-5 post conception the morula subsequently develops into a blastocyst soon after entering the uterine cavity. The blastocyst is formed of a fluid filled cavity surrounded by differentiated trophoblast forming a single outer layer of multinucleated syncytiotrophoblast (STB) that later forms the extraembryonic membrane and placenta (Luckett, 1978). At one end of the blastocyst the inner cell mass is formed by an asymmetric group of cells that give rise to the embryo (Boyd and Hamilton, 1970, Selwood and Johnson, 2006, Benirschke et al., 2012a).

1.1.4 Implantation of the blastocyst

1.1.4.1 Pre-lacunar stage

Blastocyst implantation is the important key step for pregnancy to occur. Apposition of the blastocyst is the first step in implantation then followed by an adhesion step (Schlafke and Enders, 1975). The ability of the blastocyst to adhere to the epithelium (apposition) is dependent on the interaction between cell surface receptors at the site of implantation and receptor on the blastocyst (Lessey and Castelbaum, 2002, Lindhard et al., 2002). Epithelia receptive development depends mainly on estrogen and progesterone production by the corpus luteum after ovulation, if blastocyst approaches the endometrium after day 24 of the cycle the potential for adhesion is decreased as a result of the synthesis of anti-adhesive glycoproteins that prevent receptor interaction (Navot et al., 1991). The pre-lacunar stage begins on day 6-7 post conception, microvillus emanating from the trophectoderm make the initial contact with the uterus epithelium (Figure 1-2). Attachment of the blastocyst follows soon through a complex interplay between both decidua and trophoblast cells. The blastocyst begins interstitial implantation close to the maternal blood vessels which will provide the placenta with blood flow. The blastocyst adhesion involves modification in to the expression of cellular adhesion molecules such as integrin. The area of the uterus underlying the placenta (placenta bed), contains spiral arteries that play a role in supporting

placenta. By day 7 post conception the embryo is 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).

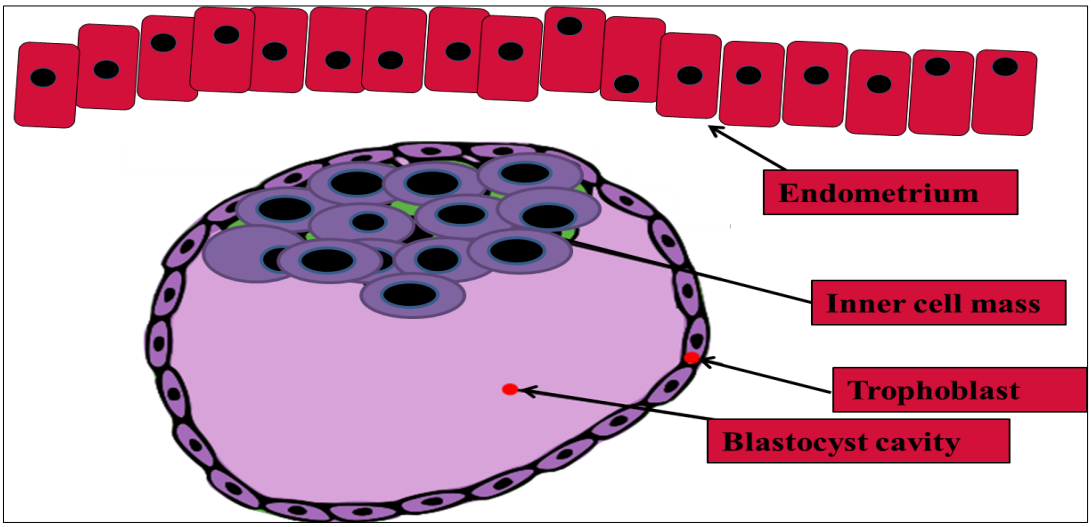


Figure 1-2: Illustration shows the blastocyst just before implantation (apposition). The blastocyst contains a single layer of trophoblast (trophectoderm) which is the outer layer of cells comprised of trophoblast cells seen in purple (arrow), inner cell mass and blastocyst cavity (arrows). The endometrium is seen in red (arrow).

1.1.4.2 Lacunar stage

Around 8 days post conception; multiple vacuoles filled with fluid begin to develop within the STB mass. These vacuoles coalesce and become confluent, forming the lacunae. The separating lamellae of STB are called the trabeculae and their appearances indicate the beginning of placenta trabecular stage. After formation of the lacunae the trophoblasts subdivides into three layers the first one is facing the blastocyst and is called the primary chorionic plate, the second layer is the lacunar together with the trabeculae system together and the third layer is facing the endometrium and called the cytotrophoblastic shell (Benirschke et al., 2012a). After implantation is completed the trophoblast undergo further differentiation into inner villous cytotrophoblast (vCTB) and extra villous trophoblast (EVT), each one has distinct functions when it makes contacts with the maternal tissue (Loke et al., 1995). The vCTB villous are the precursor cells that differentiate into STB. The EVT because of their location migrate into decidua and myometrium (attaching to the extracellular matrix, degrading and then migrating), and then penetrating the maternal vasculature this is the beginning of the process of trophoblast invasion (Meekins et al.,

1994, Pijnenborg et al., 2006, Pijnenborg et al., 1981). The EVT then follow two pathways either interstitially or follow an endovascular route. The interstitial trophoblasts invade the maternal decidua and myometrium in the placenta bed and surround the spiral arteries. The endovascular trophoblasts (ETB) enter the lumen of spiral arteries. This process is regulated by several factors including decidua, trophoblast cells and other diffusible factors (Lyall, 2006). The cells invading the uterus reach up to an inner third of the myometrium, where some undergo fusion forming giant cells (Al-Lamki et al., 1999). In the early stages of implantation, endometrium superficial venules undergo dilation to form sinusoids, which are subsequently eroded into by the STB (Hertig et al., 1956) forming the venous circulation and maternal arteries circulation will be established by the end of the first trimester (Burton et al., 1999). Subsequently, the early inter-villous space is formed to allow the intercommunication network in particular oxygen and nutrient transport between mother and fetus (Burton et al., 1999). In the first 10 weeks of pregnancy there is no maternal blood flow going to the intervillous space due to trophoblast plugs, this will result in low oxygen levels until losing the trophoblast plugs (Lyall and Belfort, 2007). During the organogenesis stage the low oxygen environment protects the fetus from reactive oxygen species (ROS) (Burton et al., 1999). Plasma filtrate and uterine glandular secretions in the intervillous space provide the nutrients necessary for embryo development. At the end of the first trimester the oxygen concentration rises three fold, and may precipitate in a pregnancy complication by inducing the oxidative stress (Burton et al., 1999). However, the placenta expresses anti-oxidants in normal pregnancy which help protect from the onset of oxidative stress (Burton et al., 1999). Fetal-maternal exchange of oxygen and nutrients starts at the end of the first trimester after the spiral arterioles begin to perfuse the intervillous space (Hustin et al., 1988).

1.1.5 Development of the placenta and utero-placenta circulation

1.1.5.1 Villous structure and development

The villi are complex tree-like projections of the chorionic plate into the inter-villous space. These projections represent primary villi, which are composed of core of mononucleated cytotrophoblasts (CTB) and are covered by an outer layer of multinucleated STB. Their presence marks the beginning of the villous stages of placentation. Trophoblasts form the villi cover which is bathed initially by the clear fluid

and later by the maternal blood. The secondary villi is formed from the primary villi around week five of gestation after invading the extraembryonic mesenchyme, which then initiates the development of primitive villous trees and the feto-placenta circulation. When primitive villous trees contact the trophoblastic shell, they are called anchoring villi. Mesenchymal villi give rise to the mature intermediate villi, from which terminal villi will sprout. Non-branching angiogenesis gives rise to terminal villi and because they are in contact with the trophoblast, they are the last to form vessels that allow the exchange of oxygen and nutrients. Fetal vessels are present inside the villi that connect to the fetal circulatory system via the umbilical cord. However, the fetal and placental circulations develop independently with a little communication up to the sixth week (Corner, 1929). With subsequent villi development, the villous tree is established throughout the feto-placenta circulation forming a complex network which supports the growing fetus by the maternal.

1.1.5.2 Trophoblast invasion

Trophoblasts are the first cell lineage to differentiate during development. Trophoblasts play a critical role at the fetal-maternal interface. Trophoblasts are derived from the trophoectoderm of a developing blastocyst, after eight days of fertilization and initial blastocyst implantation the trophoblast has differentiated into an outer multinucleated syncytium (primitive STB) and inner mononuclear CTB. After implantation is complete the trophoblast undergo further differentiation into villous and EVT. The villous gives rise to the chorionic villi of the placenta with functions in the transport of oxygen and nutrient between the mother and fetus, as well as placental hormone and growth factor production. The invading extra villous trophoblast cells follow two routes, endothial trophoblast (ETB) cells which penetrates the lumen of maternal spiral arteries, and the interstitial trophoblast cells which migrate into decidua and myometrium, then penetrate the maternal vasculature forming the placenta bed and surrounding the spiral arteries (Pijnenborg, 1994). This is a controlled process regulated by several factors including decidua, trophoblast cells and other diffusible factors (Lyall, 2006). The perivascular trophoblasts at eight weeks form around 20% of myometrium spiral arteries and 60% at 16-18 weeks (Pijnenborg et al., 1983).

After 11 weeks of gestation the number of CTB is reduced probably due to the formation of a multinuclear giant cells after the fusion of the CTB cells (Pijnenborg, 1996). The ETB invasion occurs in two different waves. Firstly, a decidual phase completed within 10

weeks and the second myometrium phase starting 4-5 weeks later (Pijnenborg et al., 1983). The morphological basis of this theory is still unclear (Lyall, 2005). However, (Robson et al., 2001) reported that endovascular migration into myometrium arteries is a continuous progressive process rather than two different waves. The interstitial trophoblast migrates and invades the uterine tissue and anchors the placenta to the uterus. Uterine spiral arteries undergo physiological modification as a result of the interaction of the invasive ETB and the arterial vessel wall. As a result the spiral arteries are converted into distended, inelastic flaccid vessels without maternal vasomotor control. This process is termed a conversion which forms a vessel of low resistance and high capacitance in order to meet fetal requirements**.** The remodelling can be classified into three phases; trophoblast invasion without vascular changes, vascular remodelling by perivascular interstitial trophoblast and trophoblast infiltration of arterial wall (Kaufmann et al., 2003).

During the invasion process the trophoblast becomes embedded in a wall of spiral arteries, replacing the endothelial cell lining, and destroying the elastic and muscular coat which is replaced by an amorphous fibrinoid material. The conversion of the spiral arteries occurs by end of the first trimester (Lyall and Belfort, 2007). This process involves mainly the spiral arteries in the centre of the placental bed, the density of invasion of spiral arteries decreases towards the placenta margin (Brosens, 1988).

The source of ETB cells and route of invasion is still controversial between the intravasation, and extravasation models. In the intravasation model the spiral arteries are infiltrated by interstitial and perivascular trophoblast. In the extravasation model the endovascular trophoblasts enters the spiral arteries from the intervillous space and migrate along the arterial lumen retrograde to blood flow to replace the endothelium and infiltrate the wall and centrifugally invade media and adventitia. One group (Kam et al., 1999) suggested a combination of two hypotheses, infiltration and replacement by interstitial trophoblast followed by replacement of endothelium by a different population of trophoblast.

1.1.6 Mechanism of control of the trophoblast invasion

Invasion of the decidua and myometrium by trophoblast occurs in a controlled manner. The villous trophoblast changes their differentiation into anchoring trophoblast due to decidua contact either through a paracrine pathway or by a direct contact with the extracellular matrix (Kam et al., 1999). Signals from the decidual extracellular matrix play

a role in this differentiation (Vićovac et al., 1995). However, (cytokines) paracrine factors effect the trophoblast express specific antigens such as the integrin cell-extracellular matrix antigens, metalloproteinases, adhesion kinase, transforming growth factor-β 1, insulin like-growth factor-2, vascular endothelial factor and its receptor (Myatt, 2002).

1.1.6.1 Role of integrin and cell adhesion molecules:

The extra villous trophoblast expresses extracellular molecules such as integrin, selectins and immunoglobulin on the trophoblast surface to enable their adhesion to the extracellular matrix in decidua which control their invasion include (Kreis and Vale, 1993). The integrin is acting as a receptor for the extracellular matrix which plays an important role in the trophoblast invasion. Proliferating and invasive trophoblast secrete different kinds of integrin (Damsky et al., 1994). In addition, proliferating cells secrete cell adhesion molecules for example E-cadherin which response for cell to cell contact (Kreis and Vale, 1993).

1.1.6.2 Role of PECAM-1

Platelet endothelial cell adhesion molecule-1 (PECAM-1) is a transmembrane glycoprotein present in a wide range of cell including endothelial. Few studies suggested that the CTB that invades spiral arteries and replace the endothelial cells express PECAM-1. However, it has been shown that that PECAM-1 does not play a role in trophoblast (Blankenship and Enders, 1997, Holthe et al., 2004, Lyall et al., 2001) .

1.1.6.3 Role of proteases

Trophoblast invade the decidua and myometrium after degrading the extracellular matrix by releasing enzymes known as proteases matrix metalloproteinase (MMPs) (Grahamm and Lala, 1992). Proteases matrix metalloproteinase include MMP-1 (interstitial collagenase) degrade fibrillar collagens, MMP-2 (72-kDa type VI collagenase, gelatinase A), MMPs 9 (92-kDa type IV collagenase, gelatinases B) degrade denatured collagens, MMP-3 (stromelysin), MMP-7 (matrilysin) have several substrate include fibronectin, laminin, elastin, and collagens III, V, and IX (Myatt, 2002). MMP-14, MMP-15, MMP-16, and MMP-17 present on the cell surfaces and activates progelatinase-A (Lyall, 2006). Trophoblast invasion also involves a balance between the release of MMPs and their tissue inhibitors (Lyall, 2006).

1.1.6.4 Oxygen tension and trophoblast invasion

Before twelve weeks of pregnancy the villous tissue is separated from the maternal circulation by a trophoblast layer indicating that during the early stages of gestation the placenta develops in a physiological low oxygen environment (Burton et al., 2001). It was reported that at 10-12 weeks of gestation maternal blood flow comes into the intervillous space with an increase in oxygen tension (Jauniaux et al., 2003a). This alteration in oxygen tension affects the trophoblast invasion as low tension prevents invasion by EVT.

Other factors include apoptosis, carbon monoxide (CO) and hemeoxygenase (HO), nitric oxide synthase and trophoblast invasion, oxygen tension, trophoblast invasion and human leukocyte antigen-G and natural killer cells.

1.1.7 Placenta functions

Placenta is a remarkable organ with diverse functions. These unique functions include materno-fetal transport, hormone synthesis and production, metabolism of several compounds, barrier to vertical transmission of pathogens and an immunological interface.

1.1.7.1 Hormone synthesis and secretion

Placenta plays an important role as a secretory organ by synthesizes of important hormones that support pregnancy. Most of the placenta hormones are produced and secreted from CTB, STB, or both. Most of these placenta hormones are proteins hormones; however progesterone and oestrogen are steroid hormones. Placenta hormones include estrogen, progesterone, human chorionic gonadotropin (hCG), human placental lactogen (hPL), human placental growth hormone (hPGH) (Lacroix et al., 2002). It also produces many growth factors that promote uterine blood flow and placental development such as placental growth factor (PlGF), epidermal growth factor (EGF), vascular endothelial growth factor (VEGF), and transforming growth factor (TGF). Furthermore it also secretes others peptide hormones such as oxytocin, inhibits, corticotrophin-releasing hormone (CRH) and prolactin (Kay et al., 2011).

1.1.7.2 Placental transport system

Nutrients, ions, gases and water are transported to the fetus and waste products are transferred via the placenta. Several materno-fetal exchange systems are involved include passive diffusion, active transport, facilitated diffusion, ion diffusion systems and receptor

transport; these are reviewed in (Sibley and Boyd, 1988, Kay et al., 2011). Excretion of waste products such as uric acid, urea and creatinine are transferred to the maternal circulation by the placenta through diffusion.

1.1.1 Placental sampling and mRNA Analyses

Studies of gene expression have been used to investigate the pathophysiology of several placental diseases. Special collection procedures are required in order to obtain accurate representative sample, these procedures firstly start with mode of delivery. A placenta delivered after vaginal delivery either spontaneous or induced is associated with metabolic, endocrine and cytokine changes, generation of oxidative and other stresses, activation of signalling pathways and gene transcription compared to the placenta delivered after caesarean section (Burton et al., 2014, Yung et al., 2014a). Many of the changes in normal placenta after labour are also observed in the placenta from a pre-eclampsia (PE) patient (Cindrova-Davies et al., 2007b). Therefore, the placenta delivered by a caesarean section (CS) should be subject to analysis to separate from the effects of labour. Second variations in the time interval between delivery and sample collection will be important. Inevitably, the placenta is exposed to a period of ischaemia following separation from the uterine wall and as mRNA degrades rapidly following delivery, freezing of tissue samples should be done rapidly, preferably within 10 min. several studies reported that after 10 min oxidative and metabolic changes are detectable (Serkova et al., 2003, Yung et al., 2014a). One group recommended that the placenta sample should be taken in a systemic uniform random sampling in which the first sample is chosen randomly and then a pre-determined pattern decides the sites of the other samples (Mayhew, 2008). In addition, others, recommended that samples should be taken from at least ten placentas because of the intra and inter placenta variability in gene expression (Avila et al., 2010). Our own publications (which make up part of this thesis) found a clear zonal variation in gene expression across individual placenta (Abdulsid et al., 2013a, Abdulsid et al., 2013b, Abdulsid and Lyall, 2014, Alwarfaly, 2015) and this varied from gene to gene. Placenta samples should be washed thoroughly in buffered saline to remove maternal contaminants, frozen rapidly in liquid nitrogen, and then stored in a -80°C freezer until processing. A housekeeping gene should be used to increase the accuracy of normalization (Benirschke et al., 2012b).

1.2 Normal Parturition

Pregnancy is characterized by a complex interplay of inflammatory events regulated by both the innate and acquired immune systems. In humans parturition involves interaction of hormonal, neurological, mechanical stretch and inflammatory pathways and the placenta plays a crucial role (Challis et al., 2000, Keelan et al., 2003, Petraglia et al., 2010, MacIntyre et al., 2012).

1.2.1 Definition

Labour (parturition) is a physiological process during which the fetus, membranes, umbilical cord, and placenta are expelled from the uterus. Labour is initiated by hormones which cause the muscular walls of the uterus to contract and the process is achieved with changes in the biochemical connective tissue and with gradual effacement and dilatation of the uterine cervix as a result of rhythmic uterine contractions of sufficient frequency, intensity, and duration (Norwitz, 2003). Obstetricians have divided labour into three stages which occurs in a continuous manner. The first stage begins with regular uterine contractions and ends when the cervix fully dilated at 10 cm. The second stage begins with full cervical dilation and ends with delivery of the fetus. The third stage is the period between delivery of the fetus and the delivery of the placenta.

1.2.2 Parturition as an inflammatory process

It is known that normal pregnancy is a pro-inflammatory state (Redman et al., 1999), which increases in normal pregnancy as the pregnancy advances (Duckitt and Harrington, 2005).

Inflammatory mediators such as cytokines and prostaglandins in the cervix, myometrium, placenta and fetal membranes play a crucial role in parturition. (Kelly, 1996, Thomson et al., 1999, Jabbour et al., 2009, MacIntyre et al., 2012, Osman et al., 2003, Gomez-Lopez et al., 2013). Leukocytes, predominantly neutrophils and macrophages, infiltrate the myometrium especially lower uterine segment during labour at term adaptive immune cells, and specifically T cells, play a role in the onset of spontaneous labour at term pregnancy (Thomson et al., 1999, Gomez-Lopez et al., 2013). Leukocyte infiltration has been demonstrated in all these tissue (Gomez-Lopez et al., 2011). In addition, neutrophil chemokines and other inflammatory mediators are over-expressed in the chorio-amniotic membranes, even in the absence of leukocyte infiltration (histologic chorioamnionitis)

(Haddad et al., 2006, Gomez-Lopez et al., 2013).

The inflammatory cell infiltration may be attracted by an increase in cell adhesion molecule and E-selectin expression on the vascular endothelium of myometrium, placenta, cervix, and fetal membranes at or immediately after the onset of labour (Winkler et al., 1998, Thomson et al., 1999, Ledingham et al., 2001, Gomez-Lopez et al., 2013). Coincident with infiltration of leucocytes is the production of pro-inflammatory cytokines (IL-1β, IL-6, IL-8) and tumour necrosis factorα (TNFα) (Osman et al., 2003, Gomez-Lopez et al., 2013) (Figure 1-3). In addition, it has been shown that two T-cell subsets of memory-like T cells; cluster of differentiation (CD) (CD3+ CD4+ CD45RO+ and CD3+ CD4CD8CD45RO+ cells) were identified in the chorio-decidua of women who had spontaneous labour (Gomez-Lopez et al., 2013). Leukocytes particularly neutrophils contribute to cervical remodelling by releasing proteolytic enzymes such as collagenase and elastase that effect on collagen fibre molecule, which provides the rigidity for cervix that will result in cervical ripening (Jabbour et al., 2009).

The initiation of this process could involve neutrophil chemotactic agents such as IL-8. Stimulation of IL-8 occur either in the cervical fibroblast or monocytes which are potent sources of IL-8, prostaglandin-E and nitric oxide (NO) (Kelly, 2002). It has been proposal that labour would result by release of two activating and effector molecules waves, which together create local responses leading to induction of myometrium contractions and extracellular degradation in cervical canal and promote spontaneous labour (Gomez-Lopez et al., 2013). Activating molecules include prof-inflammatory cytokine (IL1β, TNFα) and chemokines (chemokine CXC motif ligand 8 or IL8 (CXCL8), CXCL10) (Gomez-Lopez et al., 2011). Effector molecules include MMPs and prostaglandins (Maymon et al., 2000, Xu et al., 2002, Gomez-Lopez et al., 2013).

The biochemical events associated with labour involve increased IL-1β (Allport et al., 2001). IL-1β and TNFα increase the production of proteinases including MMP-1, MMP-3, MMP-9 and cathepsin S that can digest the collagen and elastin fibres in the extra-cellular matrix of the cervix (Ledingham et al., 2000a, Kelly, 2002). It has been reported that IL-1α and IL-1β have been shown to induce proMMP-9, cyclooxygenase 2 (COX2) and Prostaglandin-E2 production, all of which play essential roles in myometrium contraction and cervical ripening and dilution (Imada et al., 1997, Gomez-Lopez et al., 2013) (Figure 1-3).

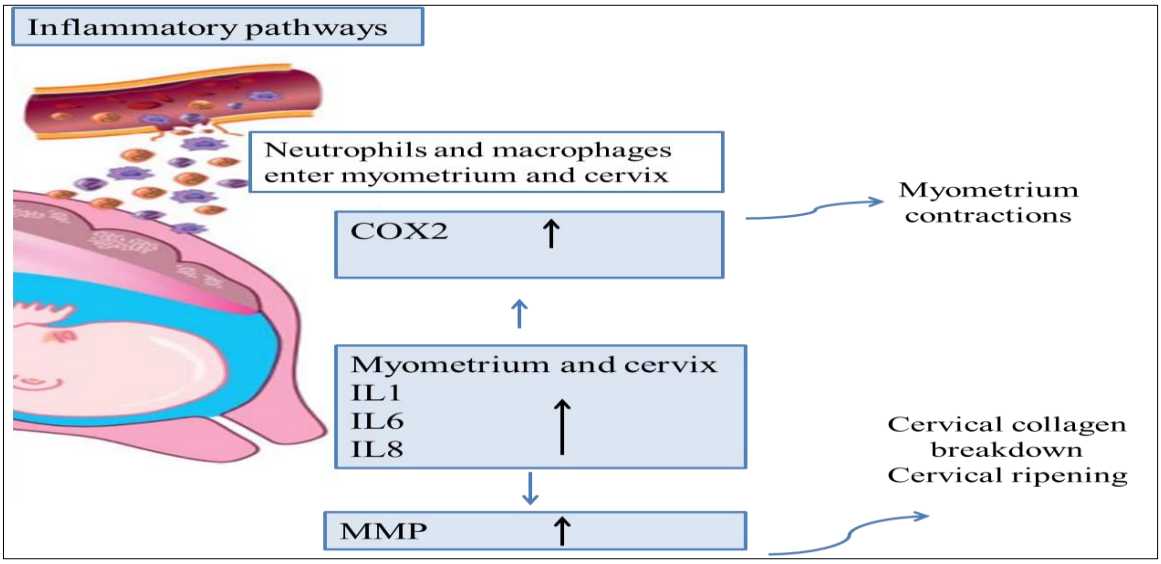


Figure 1-3: Inflammatory pathway in parturition is associated with the influx of leukocytes (mainly neutrophils and macrophages) into the myometrium and cervix during labour, result in release IL 1, IL6 and IL8. These cytokines up-regulate MMP expression and activity in the cervix, causing collagen breakdown and cervical ripening. In the myometrium, IL1 activity leading to more calcium entry to cells, also cytokines increase COX2 expression, causing increase of prostaglandin production both will stimulate myometrium contractions. Modified from (Jabbour et al., 2009).

NO is an inflammatory mediator that is increased at term and along with prostaglandin-E2 increases blood vessel permeability for leukocyte trafficking (Ledingham et al., 2000b). Cytokines release leads to up-regulation of several inducible enzymes including COX2 and inducible nitric oxide synthase (iNOS). COX2 is induced rapidly during labour and play a role in conversion of free arachidonic acid into prostaglandin precursors (Slater et al., 1999). *In vitro* studies also suggest that hypoxia-reoxygenation increases COX2 which may in turn play a role in augmentation or even initiation of labour (Burdon et al., 2007). Prostaglandin is a potential stimulator for uterine contractions. Clinically, iNOS has an important role in cervical ripening (Ledingham et al., 2000a). In addition, human placental trophoblast shows activation of the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), COX2 and iNOS during labour (Allport et al., 2001). Furthermore, corticotrophin releasing hormone and oxytocin secreted from placenta are crucial mediators involved in human parturition (MacIntyre et al., 2012). Increase in levels of cleaved caspase-3 (CASP3) and cleaved caspase-9 (CASP9) confirm evidence of placental apoptosis during labour (Cindrova-Davies et al., 2007b).

Several studies showed that inflammatory gene expression is profoundly altered during parturition (Bollopragada et al., 2009). The initiating signal that drives these inflammatory

events is still unknown; however, some studies showed that Toll like receptors (TLRs) that are responsible for receiving these signals increased toward end of pregnancy and during labour (Cindrova-Davies et al., 2007b). In addition, chemotactic ability of leukocytes increases in those taken from blood samples of labouring women compared to the non-labouring women (Yuan et al., 2009).

Prostaglandins are well established agent in having a primary action in cervical ripening and have considerable clinical use where labour is associated with poor progression of cervical ripening (Kelly, 1996). Prostaglandins formation is known to be stimulated by pro-inflammatory cytokines that are secreted locally by intrauterine tissues. Just before labour, the myometrium switch from prostacyclin synthesis (uterine contraction suppressor) to Prostaglandins-E1 and Prostaglandins-F2 synthesis (uterotonic). Prostaglandins arise by action of COX enzymes, primarily via transcriptional up-regulation of the inducible isoform COX2 (Kniss, 1999).

Studies showed that anti-inflammatory pathways are also involved in the mechanism of parturition, for example, during labour IL10 levels increase in amniotic fluid (Gotsch et al., 2008). In addition, it has been reported (Maldonado-Perez et al., 2009) that anti-inflammatory lipid mediators such as lipoxins increase in both synthetic capacity and receptor density of these molecules in the myometrium during labour. Moreover, other research group has been shown that mechanical stretch could promote a wave of entry of amniotic fluid components into the uterine blood vessel and this would be followed by the second big wave of subsequent myometrium contraction (Kobayashi, 2012).

1.2.3 Parturition and link with oxidative stress

The contractions that occur during labour are associated with intermittent utero-placental perfusion and could lead to an ischemia-reperfusion (IR) type injury to the placenta (Fleischer et al., 1987, Brar et al., 1988). This intermittent perfusion can be expected to provide the basis for IR type injury of the placenta. Indeed 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).

Labour is also associated with placental alterations in several pathways linked to oxidative stress (Many and Roberts, 1997, Cindrova-Davies et al., 2007b, Sitras et al., 2008, Lim et al., 2012, Díaz-Castro et al., 2015). Oxidative stress is related with conversion of xanthine

dehydrogenase to xanthine oxidase (XD/XO) a marker of IR injury, and the latter increases in labouring placentas compared to the non-labour controls (Many and Roberts, 1997). In addition, during labour there is a reduction in tissue concentrations of ATP, depletion of vitamin C in maternal blood and high glutathione levels in umbilical venous blood compared to the non-labour controls (Bloxam and Bobinski, 1984, Woods et al., 2002, Raijmakers et al., 2003).

During oxytocin-induced contractions, a 50% reduction in flow into the intervillous space was found compared to when no contractions occurred. This suggests that intermittent perfusion of the intervillous space would lead to an IR injury of the placenta. Reactive oxygen species (ROS) and the oxidant/antioxidant balance can be affected as a result (Mccord, 1993). Other studies looking at heat shock proteins (HSPs), Superoxide dismutase (SOD) is an enzyme that alternately catalyzes the dismutation (or partitioning) of thesuperoxide (O_2^-) radical into either ordinary molecular oxygen (O_2) or hydrogen peroxide (H_2O_2). Mn-SOD, Cu/Zn-SOD We conclude that oxidative stress is a potent inducer of placental synthesis and release of proinflammatory factors. Most of these effects are mediated through the p38 MAPK and nuclear factor-kappaB pathways and can be effectively blocked by vitamins C and E in vitro. Peroxidation of lipids and oxidative damage mediators also show an association between labour and placental oxidative stress (Cindrova-Davies et al., 2007b, Abdulsid et al., 2013b, Abdulsid et al., 2013a, Díaz-Castro et al., 2015).

1.3 Pre-eclampsia (PE)

PE is a disorder of widespread vascular endothelial dysfunction and vasoconstriction which occurs after the 20th weeks of pregnancy and can present as late as 4-6 weeks after delivery. PE remains to be one of the leading causes of maternal morbidity and mortality worldwide. PE affects about 2-3% of all pregnancy but this can be much higher in underdeveloped countries. About 50 thousand women die in developing countries each year from PE disorder (Duley, 1992). Maternal mortality is far less in developed nations due to proper perinatal observation; nevertheless morbidity still remains the leading cause of admissions to intensive unit care (Tang et al., 1997). PE is an important cause of fetal growth restriction and iatrogenic prematurity (Redman and Sargent, 2005). Of all preterm births 8% are due to delivery of women who develop severe PE early in gestation (Ananth and Vintzileos, 2006). PE is a common cause of still birth and early neonatal death (Ngoc et al., 2006). PE is also associated with several pregnancy complications such as placenta abruption and caesarean section decision. Women who develop PE are at an increased risk of cardiovascular disease later in life (Myatt et al., 2014b).

1.3.1 Definition of PE

A number of classifications for hypertensive disorders in pregnancy have been proposed (Davey and MacGillivray, 1988, Chung et al., 2001). According to the International Society for the Study of Hypertension in Pregnancy (ISSHP) (Myatt et al., 2014b), PE is currently defined as a blood pressure of >140/90 mm Hg on two occasions at least six hours apart occurring in women who was normotensive before 20th weeks gestation and accompanied by proteinuria (>300 mg/L in a 24 hour urine collection) but without evidence of end-organ damage. However, since this system does not take into account the multisystem nature of PE, patients with other characteristics such as thrombocytopenia, abnormal liver function or fetal growth restriction (FGR) may be overlooked (Chung et al., 2001). PE is also defined as a cardiovascular and renal dysfunction identified by blood pressure and proteinuria measurements. In women with pre-existing essential hypertension, PE is diagnosed if systolic blood pressure (SBP) has increased by 30 mm Hg or if diastolic blood pressure (DBP) has increased by 15 mm Hg. Sever PE is defined as the presence of PE with presence one of the following symptoms and signs being present (Table 1-1) (Paruk and Moodley, 2000). Eclampsia is defined as a pregnancy-specific seizure disorder that cannot be attributable to other causes in a woman with PE (Chesley,

1984). Eclampsia is estimated to occur in 1 of 200 cases of PE when magnesium prophylaxis is not administered (Livingston et al., 2003). Delivery of the fetus and placenta are the only way to relieve PE symptoms.

Table 1-1: Severe PE criteria.

1) SBP of 160 mm Hg or higher or DBP of 110 mm Hg or higher on 2 occasions at least 6 hours apart.
2) Proteinuria of more than 5 g in a 24-hour collection or more than 3+ on 2 random urine samples collected at least 4 hours apart.
3) Oliguria (< 400 mL in 24 hours).
4) Epigastric pain.
5) Impaired liver function.
6) Pulmonary oedema.
7) Thrombocytopenia.
8) HELLP syndrome.
9) Placental abruption

Neurological symptoms or signs:

- Persistent headaches.
- Visual blurring.
- Altered consciousness.

1.3.2 PE risk

The incidence of PE is higher in women with a history of PE, multiple gestations, and chronic hypertension or underlying renal disease. Gestational hypertension and proteinuria predict an increased risk for developing PE. However, there is an increased risk associated with hypertension when accompanied by other systemic involvement even without proteinuria (Myatt et al., 2014b). There are several risk factors for PE include maternal: old age> 40, nulliparity, black race, high BMI, diabetes mellitus, chronic renal disease and chronic hypertension (Knight et al., 2010, Practice, 2002, Ziadeh and Yahaya, 2001, Sohlberg et al., 2012, O'Brien et al., 2003, Staff et al., 2014). Fetal factors include multiple pregnancy and environmental factors (Long and Oats, 1987, Neela and Raman, 1993). There is also a correlation between PE and maternal genes (Roberts and Cooper, 2001).

1.3.3 Clinical presentation

Maternal organ systems that are susceptible to excessive inflammation and endothelial damage are the CNS, kidneys, lungs, liver, systemic vasculature, coagulation, and the heart. Patients frequently have epigastric or right upper quadrant pain, along with headache, visual changes, and nausea or vomiting, seem to be more predictive than are laboratory parameters for adverse maternal outcomes (Sibai, 2004, Cavkaytar et al., 2007). The HELLP syndrome is a serious complication in pregnancy characterized by haemolysis, elevated liver enzymes and low platelet count occurring in 0.5 to 0.9% of all pregnancies and in 10–20% of cases with severe PE. Complications of HELLP syndrome are abruption placentae, disseminated intravascular coagulation (DIC), pulmonary oedema, acute renal failure, eclampsia and sub-capsular liver haematoma (Haram et al., 2009).

1.3.4 PE classification

Several schemes have been proposed for PE classification, and the most common is to divide cases clinically into early and late-onset PE (term and pre-term PE), according to the gestational age at diagnosis or when delivery is necessitated. The majority of cases are late-onset, usually defined as PE that occurs after 34 weeks of the gestational age. Early-onset is often considered more severe, as it is associated with a higher rate of complications such as FGR and risk of maternal cardiovascular complications after delivery (Paruk and Moodley, 2000). It has been proposed that PE can be classified on the basis of pathophysiology into ‘placental’ and ‘maternal’ causation or mix of them (Redman and Sargent, 2005). In the placental class, it is postulated that malperfusion leads to placental stress and hence the release of cytokines and angiogenic regulators, which will then cause maternal endothelial cell activation. Recently (Redman et al., 2014) suggested that there may be two placental causes for PE, the first is an extrinsic placental mechanism is the dysfunctional remodelling of the uteroplacental spiral arteries that feeding blood into the intervillous space, the second is intrinsic placental mechanism which due to an increasing placental size leads to a restricted intervillous perfusion. In the maternal class, 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 maternal classification is based on normal pregnancy being a pro-inflammatory state, with an activation of circulating immune cells (Redman and Sargent, 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 an existing inflammation (Redman et al., 1999). Several studies showed 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 and Harrington, 2005).

1.3.5 Pathophysiology of PE

PE is a condition of imbalance between factors produced by the placenta and the maternal reaction to them. Although the cause of PE remains largely unknown, but a 3-stage model is suggested for the (placental type) of PE (Redman and Sargent, 2010). The 1st stage is dysregulated immunological factors; the 2nd stage is an abnormal placentation with reduced trophoblast invasion and failure to fully remodel maternal spiral arteries and the 3rd stage is the clinical signs of PE hypertension and proteinuria due to an excessive maternal inflammatory response and endothelial dysfunction as a result of placenta oxidative and endoplasmic reticulum (ER) stresses (Redman and Sargent, 2005). Abnormal placentation, immunologic intolerance, cardiovascular and inflammatory changes, genetic and environmental factors are important factors in PE development (Cunningham et al., 2014).

1.3.5.1 Immunological factors in PE

Immunological factors have long been considered to be a key factor in PE. One important component is a poorly understood dysregulation of maternal tolerance to paternally derived placental and fetal antigens. One important theory was based on a model of immune-regulation in pregnancy that is based on shifting of the maternal immune response away from Th1 (T-helper1 cell-mediated graft rejection responses) to Th2 (T-helper2 antibody-mediated responses) that result in protecting the conceptus from the maternal cell mediated immune attack (Wegmann et al., 1993). In PE, the pregnancy shift to Th2 either does not occur or is reversed early in the disorder. Thus, Th1 responses are not suppressed and production of cytokine interferon-γ (IFN-γ) is normal. There is a possibility that fetal antigen play a role in this regulation (Sargent et al., 2006). In addition, the endothelial cell dysfunction may be partially due to an extreme activation of leukocytes in the maternal blood circulation, as evidenced by an up-regulation of Th1. Immune responses may play an important role in PE by restricting trophoblast invasion through the interaction of trophoblast major histocompatibility complex (MHC) and decidual natural killer (NK) cells in early pregnancy and through the interaction of STB debris and circulating NK cells

to stimulate the intense systemic inflammatory response in late pregnancy (Sargent et al., 2006).

1.3.5.2 Abnormal placentation

The mechanisms that are involved in maintaining a human pregnancy to term, and the switches that lead to a normal labour and pregnancy outcome or indeed an adverse outcome such as miscarriage, PE, FGR or preterm labour, are complex but the role of the placenta is crucial to them all (Petraglia et al., 2010, Challis et al., 2000, Roberts and Escudero, 2012, Burton and Jauniaux, 2011). Abnormal placentation and trophoblastic invasion of uterine spiral arteries is a major cause of hypertension associated with PE syndrome (Redman and Sargent, 2005, Zhou et al., 1997). During a healthy pregnancy maternal spiral arteries are dramatically remodelled. They become widely dilated and lose their responsiveness to vasoconstrictive stimuli. Thus blood enters the inter-villous space in a non-pulsatile manner and under low pressure (Lyall, 2006). PE is characterized by defective in spiral arteries remodelling, high resistance of utero-placental circulation, and inadequate placenta perfusion. The frequent findings in the PE are low number of trophoblast invasion of the maternal spiral arteries and muscular media persists these changes involve myometrial arteries primarily (Pijnenborg et al., 1991). Incomplete trophoblastic invasion of the spiral arteries is directly correlated with the severity of subsequent maternal hypertension. In PE spiral artery remodelling is partial or incomplete (Lyall, 2006). The ensuing high pressure flow results in a hydrostatic damage to the placental villi. Acute atherosis is also a characteristic of decidual changes (Lyall, 2003, Staff et al., 2014). Furthermore perfusion by intermittent pulses of fully oxygenated arterial blood is thought to lead to fluctuations in oxygen delivery resulting in oxidative stress and release of systemic vasoactive compounds (Burton and Jauniaux, 2011, Roberts and Hubel, 2009). These compounds cause vasoconstriction, endothelial damage, inflammation hypercoagulability and platelet dysfunction, which all contribute to organ dysfunction and clinical feature of PE. The maternal syndrome is, at least in part, due to the maternal response to this damaged placenta. This is known as the two -stage model of PE (Roberts and Hubel, 2009).

Placental pathology is generally worse in an early-onset PE however there is no histological evidence that spiral artery remodelling is restricted to early-onset disease only (Lyall et al., 2013a). There appears to be a spectrum of changes both within all the spiral arteries of individual cases and across gestational ages (Lyall et al., 2013a).

1.3.5.3 Endothelial dysfunction

In PE, new hypertension and proteinuria arise secondarily to diffuse maternal endothelial and inflammatory dysfunction. Imbalance of proangiogenic and antiangiogenic factors produced by the placenta may play a major role in mediating endothelial dysfunction. Markers of endothelial cell injury can be demonstrated in women who develop PE before they became symptomatic. These markers include endothelin, cellular fibronectin, plasminogen activator inhibitor-1 and von Willebrand factor (Taylor et al., 1997, Friedman et al., 1995). A poorly perfused placenta resulting from insufficient trophoblast invasion and placental bed vascular remodelling, may cause the placenta to secrete placental factors into the maternal circulation and this will altered the endothelial cell activity. This activation will lead to change in the vascular sensitivity to activate coagulation, and reduce vascular integrity resulting in the pathophysiological changes of PE. Oxidative stress, inflammation and circulatory maladaptation also contribute to the endothelial dysfunction and PE pathogenesis (Staff et al., 2010).

1.3.5.4 Angiogenic factors in PE

Angiogenesis is important for successful placentation and the normal interaction between trophoblasts and uterus endothelium. The circulating proangiogenic factors secreted by the placenta include a vascular endothelial growth factor (VEGF), soluble vascular endothelial growth receptor-1 (sVEGFR-1), sVEGFR-1 also known as soluble fms-like tyrosine kinase receptor-1 (sFlt-1), placental growth factor (PlGF) and soluble endoglin (sEng). The receptor sFlt-1 is a soluble isoform of Flt-1, which is a transmembrane receptor for VEGF. Although sFlt-1 lacks the transmembrane domain, it contains the ligand-binding region and is capable of binding circulating VEGF and PlGF, hence preventing these growth factors from binding to the transmembrane receptors. Thus, sFlt-1 has an antiangiogenic effect. VEGF and PlGF are growth factors produced by the placenta and the serum level of PlGF rises much more during pregnancy. In women who later developed PE there is an increased level of sFlt1 (antagonist of VEGF and PIGF) (Maynard et al., 2003, Thadhani et al., 2004, Vatten et al., 2007) and decrease levels of both VEGF and PIGF (Taylor et al., 2003, Maynard et al., 2003, Levine et al., 2004, Thadhani et al., 2004). In early onset PE, there are elevated levels of sFlt-1, sEng with reduced PlGF (Maynard et al., 2003, Staff et al., 2013) and the serum level of sFlt-1 was higher in women who developed severe early PE (< 34 wk) than it was in women who developed mild PE at term (Levine et al., 2004) .

However, late onset PE is not characterized by low circulating levels of PlGF (biomarker for poor placental function) and elevated sFlt1 but the angiogenic imbalance is therefore not likely to explain all features of the heterogeneous PE syndrome (Staff et al., 2013).

The vascular endothelial growth factor A (VEGF-A) levels in PE are biologically active and one study suggested that the cause of an increased vascular permeability of PE is due to loss of the repression of VEGFR1 signalling by PlGF-1, and $VEGF_{165}b$ (Bills et al., 2011). The sEng is a soluble isoform of co-receptor for transforming the growth factor beta (TGF-β). As sEng contains the TGF-β binding domain, it can bind to circulating TGF-β and decrease circulating levels. TGF-β is a proangiogenic molecule, so the net effect of high levels of sEng is anti-angiogenic. sEng is found in the blood of a women with PE and its level in maternal circulation correlates with the syndrome severity (Levine et al., 2006, Venkatesha et al., 2006). sEng inhibits TGF-β in endothelial cells and also inhibits TGF-β -1 activation of NO mediated vasodilatation (Venkatesha et al., 2006). There is also evidence that sEng may act in concert with sFlt1 to induce severe PE (Venkatesha et al., 2006).

1.3.5.5 Genetics factors in PE

PE has been shown to involve multiple genes. Several genes (maternal and paternal) have been studied for their association with PE, including genes known to play a role in cardiovascular disease (Williams and Morgan, 2012). There is a family risk of PE between close relative. However, as the PE is a genetically and phenotypically complex disease, it is unlikely that any single gene will be shown to play a dominant role in its development (Liu et al., 2015).

1.3.6 Prediction of PE

In the past two decades, our understanding of PE has increased greatly. In vivo and in vitro studies have increased our knowledge of the mechanisms involved in failed conversion of the maternal spiral arteries (Lyall et al., 2013b) and this can lead to an IR injury of the placenta (Burton et al., 2009a). PE is a multisystem disorder with an inflammation role involve in their pathogenesis (Roberts et al., 1989). The organs involvements include liver, kidney, CNS, cardiovascular system and placenta. However, although this has helped to direct research efforts to better understand the PE etiology, it

has not had a significant effect on the clinical management for PE patients or indeed how the condition is predicted reliably.

There are several promising attempts for prediction and the most successful one was to measure factors for example placental derived factors, implicated in the pathophysiology of PE to try predicting the PE. None have as yet been adopted for use in a routine clinical practice due to problems such as adequate sensitivity and specificity (Conde-Agudelo et al., 2009, Redman et al., 2014). Measurement of urinary kallikrein was shown to have a high predictive value, but it was not reproducible (Kyle et al., 1996). Although work on sFlt-1, PlGF, and VEGF has been promising, their positive predictive value in predicting PE have yet to be evaluated in a prospective fashion. In addition, (Meads et al., 2008) reviewed 27 predict tests for PE, only a few reached specificities above 90%. Tests that reached specificities above 90% were body mass index greater than 34, alpha-fetoprotein and uterine artery Doppler (bilateral notching). Sensitivity of higher than 60% was achieved only by uterine artery Doppler resistance index and combinations of indices. Kallikreinuria (sensitivity >80% and specificity >90%), cellular and total fibronectin (specificity >90%) seem worthy of further investigation (Meads et al., 2008).

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., 2014a). Add to this the factors being measured are not present in all women with PE (Myatt et al., 2014a). All of this supports the idea that sub-groups of PE exist which all end up presented as high blood pressure and proteinuria.

1.3.7 PE and link with oxidative stress

Oxidative stress occurs when the production of ROS overwhelms 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 (Burton and Jauniaux, 2011, Roberts and Hubel, 2009). There is an irrefutable evidence of placental oxidative stress in PE, including increased concentrations of protein carbonyls, lipid peroxides, nitrotryosine residues, glutathione and DNA oxidation (Burton and Jauniaux, 2011, Myatt and Cui, 2004, Serdar et al., 2003, Orhan et al., 2003). In PE, there are increased levels of placental N-myc downstream-regulated gene 1 (NDRG1) (response for

hypxia) expression (Choi et al., 2007), hypoxia-activated genes (Vaiman et al., 2005), STB apoptosis (Leung et al., 2001, Allaire et al., 2000), maternal serum cell-free fetal DNA (Sekizawa et al., 2003), maternal serum TNF-α, IL-6, IL-8 (Johnson et al., 2002) and maternal serum leptin (Laivuori et al., 2006). PE, and the deleterious effect of ROS may be counteract by an antioxidant therapy, and there is the need to investigate the optimum dosing and timing of antioxidants administration, since an inappropriate antioxidant treatment in pregnant women may have deleterious consequences, reducing placental cells proliferation until to cell death (Fiore and Capasso, 2008). The superoxide dismutase gene SOD3 Ala40Thr single nucleotide polymorphism in a Caucasian population from Hungary also increased in blood with the oxidative stress in severe FGR complicated PE (Rosta et al., 2009). Due to the deficiency of the superoxide dismutase activity, the concentration of superoxide anion concentration is increased, which in conjunction with increased concentration of iron would result in a greater oxidative stress (Zhang et al., 2005). In addition, NO reacts with a superoxide during the deficiency of superoxide dismutase activity forming a strong oxidizing agent (peroxynitrite (ONOO)) that initiates lipid peroxidation.

1.4 Fetal growth restriction (FGR)

1.4.1 Definition

FGR is the failure of the fetus to achieve its genetically pre-determined potential size due to adverse genetic or environmental factors. FGR is a serious complication of pregnancy; it affects 3-10% of pregnancies, and is generally defined as an estimated fetal weight (EFW) at or below the 10th percentile for his or her gestation age in weeks by using the Standardized Scottish (or other) Birth Weight Table (Resnik, 2002, Alberry and Soothill, 2007). Perinatal morbidity and mortality increases markedly as birth weight falls from the 10th to the first percentile (Resnik, 2002). Severe FGR is defined when the fetal weight estimation is less than the 3th percentile and most consistently associated with adverse perinatal outcome (Unterscheider et al., 2013). This definition excludes fetuses that are small for gestational age (SGA), in which the estimated weight is less than a cut off weight for a given population, often because of constitutional factors. There are numerous standard curves for fetal growth that has been published and it is well recognized that growth may be influenced by factors such as race, gender and socioeconomic environment. Customized growth curves for ethnicity, parental height and weight and fetus gender are in development in order to improve the sensitivity and specificity of a diagnosis (Gardosi, 2006, Groom et al., 2007), in addition, using a customized rather than population based growth curve is more accurate (Figueras et al., 2007). Furthermore, it would thus seem good to use a single customized standard chart for all racial groups within a specific geographic area (Greene et al., 2008). Perinatal morbidity is more predicted with asymmetric fetal estimated weight less than 3th percentile. FGR can be classified along with the Doppler measurement of umbilical/uterine artery blood flow; abnormal measurement is associated with poor prognosis (Albaiges et al., 2000). It contributes to a third of antepartum death, 10 fold rises in perinatal mortality and also increases health complications later on in an adult life.

1.4.2 Pathophysiology of FGR

FGR is not a specific disease, but rather a manifestation of many possible fetal and maternal disorders. However, although the aetiology is multifactorial; placenta insufficiency is the most common cause and is usually due to poor utero-placental blood flow and placental infarcts (Alberry and Soothill, 2007). FGR with PE and abruption

placenta are pregnancy disorders associated with placental ischemia and share many similar pathological and pathophysiological features and are usually associated with failure to delivery oxygen and nutrients to the placenta (Roberts, 2014). Early onset PE and chronic hypertension with superimposed PE usually have the most profound effect on fetal growth (Ødegård et al., 2000). There is a strong association between FGR and chromosomal disorders such as trisomy 13, 18, and 21, those are frequently growth restricted (Snijders et al., 1993, Resnik, 2002). In addition, there are several factors that seems to be associated with FGR such as prothrombin gene mutation, antiphospholipid syndrome, maternal nutritional abnormalities, cytomegalovirus infection, or fetal alcohol syndrome (Kupferminc et al., 2000, Resnik, 2002). FGR fetuses placentas will frequently have abnormal size and function, FGR placentas are usually much smaller than normal placentas (Heinonen et al., 2001). Krebs et al. (Krebs et al., 1996) found severe abnormalities at the terminal villous, which explains vascular impedance observed clinically using Doppler velocimetry.

1.4.3 Causes of FGR

There are numerous causes leading to FGR without a direct link to the placenta, and these including genetic abnormalities such as imbalances of chromosome number, mosaicism and single gene mutations. Environmental causes like maternal infection (cytomegalovirus), drugs (opiates, cocaine, and some anti-hypertensive drugs) and severe alcohol consumption may influence the fetal growth. Also, smoking-mediated through nicotine that mediates secretion of vasoconstrictive catecholamine to which the placenta adapts by increasing angiogenesis and excessive apoptosis. FGR occurs when gas exchange and nutrient delivery to the fetus are not sufficient (Roberts, 2014); this process can occur because of a maternal disease such as chronic hypertension, diabetic, autoimmune disease, protein-calorie malnutrition and uterine malformations. Placental causes include multiple gestations, chronic abruption, cord and placental abnormalities and placenta previa (Severi et al., 1999).

1.4.4 Diagnosis and Surveillance

FGR screening is based on clinical examination and identification of risk factors such as previous history of pregnancy with FGR, PE and a low pre-pregnancy weight. Screening includes abdominal palpation, symphysis to fundal (S-F) height measurement, and Ultrasound scan (USS) examination. For most cases, an estimation fetal weight (EFW) at

or below the 10th percentile is used to identify fetuses at risk. However, it is important to understand that this is not a definitive cut off for utero-placental insufficiency. USS evaluation of the fetus is considered the standard for the diagnosis of FGR as it gives a reasonably precise estimates of fetal weight, as well as the ability to follow interval growth and the pattern of growth abnormality (symmetric or asymmetric). However, although a diagnosis of placenta insufficiency can be based on USS parameters, FGR is often a diagnosis of exclusion. When a small fetus is detected, diagnostic signs also include abnormal umbilical artery Doppler impedance indexes, reduced amniotic fluid volume, decreased fetal growth rate, and fetal karyotype abnormality.

1.4.5 Fetal and maternal health risks

The growth restricted fetus can experience several complications in the neonatal period due to growth insults as well as antepartum and intrapartum factors which include neonatal asphyxia, meconium aspiration and hypoglycaemia. The FGR infant is at an increased risk of mortality, necrotizing enterocolities and a need for respiratory support (Garite et al., 2004). The FGR infant also associated with spastic cerebral palsy (Blair and Stanley, 1990). Later on in the adult life of the FGR is a significant risk factor diabetes, chronic hypertension, ischemic heart disease and obstructive lung disease (Barker, 2001). The underlying placental dysfunction suggests that there would be a similar increase in future maternal morbidity and mortality among women whose pregnancies are complicated by FGR, especially when it is early-onset and severe, resulting in a preterm birth. Few studies found that there is an inverse relationship between infant birthweight and cardiovascular mortality in the mother (Smith et al., 2000a, Smith et al., 2005). This may be related to the adverse effects of an increased total cholesterol, higher triglycerides, increased BMI, increased insulin resistance, genetic pathway and socioeconomic status found among women with a history of FGR.

1.4.6 The placenta in FGR

At the same time, there are direct placenta dependent causes that result in FGR. A decrease in placental weight and a reduction in functional placental units are generally related to FGR (Heinonen et al., 2001, Pardi et al., 2002). Failure of remodeling of maternal spiral arteries is another cause of FGR, villus injury and thrombotic lesions (Parham, 2004, Burton et al., 2009a, Roberts, 2014). These lesions can be diagnosis by

sonographic examination which may reveal placenta lakes in severe cases of FGR. These lakes are described by an area of hypo-echogenicity and defined as ischemic thrombotic lesions. The placenta has a significant reserve capacity, and due to the accumulation of utero-placenta lesions and villus injury, this leads to the clinical apparent placental dysfunction.

Apoptotic pathways is associated with FGR placentas, there is increased level of placental NDRG1 expression (response for hypoxia) (Choi et al., 2007), there is no changes in the Hypoxia-activated genes in FGR (Vaiman et al., 2005), STB apoptosis (Leung et al., 2001, Allaire et al., 2000) and DNA damage (Takagi et al., 2004). There is also no change in maternal serum cell-free fetal DNA (Sekizawa et al., 2003), maternal serum TNF-α, IL-6, IL-8 (Johnson et al., 2002) and maternal serum leptin (Laivuori et al., 2006). ER stress also plays a role in the pathophysiology of unexplained FGR, with speculation that this arises secondary to mal-perfusion (Burton et al., 2009b).

1.5 Maternal obesity

Obesity is a condition characterised by excess of body fat, frequently resulting in a significant impairment of health and longevity (WHO, 2000). Body mass index (BMI) is used to classify obesity. Obesity is a chronic multifactorial disease caused by genetic, behavioural and environmental factors that leads to serious health consequences. Obese adults are at significantly higher risk for cancer, cardiovascular and metabolic morbidity and mental disease, as well as overall mortality (Calle and Kaaks, 2004, Kenchaiah et al., 2002, Friedman and Brownell, 1995, Allison et al., 1999). In addition, the health resources of a population are increase as the number of obese individuals in that population increases (Popkin et al., 2001, 2013).

1.5.1 Obesity classification

BMI is the body weight of an individual in kilograms divided by their height in metres squared [weight (kg)/height (m2)]. A BMI below 18.5 is categorised at underweight, a BMI of 18.5-24.9 is normal/healthy weight, a BMI of 25.0-29.9 is overweight and a BMI of 30 and above is obese (Table 1-2).

Table 1-2: Classification of weight status according to BMI (WHO, 2000).

BMI (kg/m2	Classification
<18.5	Underweight
18.5-24.9	Normal weight
25-29.9	Overweight
30-34.9	Obese I
35-39.9	Obese II
>40	Obese III

1.5.2 Obesity and pregnancy

Obesity in pregnancy is increasing worldwide and is associated with increased risk of adverse outcomes for both mother and child (Ng et al., 2014, Lutsiv et al., 2015). Obesity complicates around 20% of pregnancies in a typical antenatal clinic in the UK and leads to short- and long-term complications affecting the fetus, mother and the pregnancy (Huda et al., 2010, Jarvie and Ramsay, 2010, Kerrigan and Kingdon, 2010, McGuire et al., 2010). Pre-pregnant obesity is associated with increased risk of PE, gestational diabetic mellitus (GDM), venous thromboembolism (VTE) and CS (Jarvie and Ramsay, 2010, Flick et al., 2010, Faucher and Barger, 2015, Sween et al., 2015).

Obese pregnant women are more likely to give birth to an infant with congenital anomalies (Stothard et al., 2009) and obesity also lowers detection rates of fetal anomalies during prenatal USS (Dashe et al., 2009).

Multiple studies have been shown that maternal obesity and excessive weight gain during pregnancy are associated with large for gestational age infants (Faucher and Barger, 2015). Offspring of obese mothers have a higher perinatal morbidity and mortality, higher birth weight and long-term health problems such as coronary heart disease (Haeri et al., 2009, Ferraro et al., 2012). The odds ratios for these outcomes directly increase with pre-pregnant BMI (Schrauwers and Dekker, 2009, Flick et al., 2010).

1.5.3 The rising trends in maternal obesity

Obesity in the pregnant population has increased by more than 70% over a decade and 1 in 5 pregnant women in the United Kingdom is obese (Heslehurst et al., 2010). This lead to increased risk for several clinical and obstetric complications such preterm delivery, instrumental delivery, operative and anaesthetic issues and birth injury (Sebire et al., 2001, Torloni et al., 2009a, Torloni et al., 2009b, Lutsiv et al., 2015). Maternal obesity has also been linked to the development of obesity in offspring via a fetal over-supply of glucose and/or lipids in utero (Faucher and Barger, 2015).

1.5.4 Maternal complications associated with maternal obesity

1.5.4.1 Maternal mortality

Maternal obesity is overrepresented in maternal deaths in developed countries. The Confidential Enquiries into Maternal Deaths (CEMACH) report on maternal death in the 2003-2005 triennium showed that 28% of mothers who died overweight or obese (Lewis, 2007), although in the published report 2008-2011 maternal obesity prevalence rate was running at 16% and so it has been chosen as CEMACH's principal project with focus on maternal health (Lewis, 2011). This finding reflect the fact that obesity is a leading cause of mortality by either direct or indirect causes include PE, cardiovascular diseases (CVD) and thromboembolism.

1.5.4.2 Gestational diabetes mellitus (GDM)

Diabetes mellitus is a metabolic disorder characterized by persistent hyperglycemia, with disturbance of carbohydrate metabolism resulting from a defect in insulin secretion (Consultation, 1999). There is a strong correlation between obesity and GDM (Sebire et al., 2001, Torloni et al., 2009a). Torloni *et al* (Torloni et al., 2009a) reported that morbid obesity BMI >40 kg/m^2 is associated with five times the increased risk of GDM. Other risk factors related to the development of GDM are age, parity, family history and ethnicity (Group, 2008).

1.5.4.3 Thromboembolic complications

Venous thromboembolic (VTE) complications are a leading direct cause of maternal mortality in the UK and other developed countries (Bourjeily et al., 2010). There is two and half times more risk for obese women to develop VTE compared to lean pregnant women (Knight, 2008). The possible mechanisms for VTE may related to several factors include inflammatory damage to the venous endotheium, sedentary lifestyle and high maternal coagulation factors (Lippi and Franchini, 2008).

1.5.4.4 Effects of obesity on labour and delivery

Obese pregnant women have an increased incidence of labour induction, CS, infection and postpartum haemorrhage (Zhang et al., 2007, Sebire et al., 2001, Poobalan et al., 2009,

Heslehurst et al., 2008). In addition, obese women have prolonged labour and reduction in uterine contraction (Kominiarek et al., 2011).

1.5.4.5 Hypertensive disease in pregnancy

Several studies show that maternal obesity is associated with increased risk of gestational hypertension (O'Brien et al., 2003). Two large population-based studies also showed strong positive associations between pre-pregnancy BMI and the risk of PE (Sebire et al., 2001, O'Brien et al., 2003). In addition, (Bellamy et al., 2007) showed that women who develop PE are more likely to develop CVD later in life. The association between the PE prevalence and high BMI may be explained by inflammation, oxidative stress and insulin resistance (Oteng-Ntim and Doyle, 2012).

1.5.5 Obesity and PE

PE risk is linked with greater BMI; the mechanisms for this relationship remain unclear. Several of the postulated causal pathways are studied but most of them from observational studies and inevitably suffer from potential limitations (Sattar and Freeman, 2012). These pathways include obesity association with metabolic syndrome include hypertension and proteinuria, dyslipidaemia, insulin resistance, inflammation and altered vascular function.

There is much evidence that obesity is a major risk factor for essential hypertension and risk of proteinuria in non-pregnant subjects (Whaley-Connell et al., 2006). Obesity is associated with the mild form of PE rather than the severe form (Bodnar et al., 2007). Furthermore, there is a clear association between PE and subsequent CVD risk especially in early onset PE (Sattar and Freeman, 2012).

PE is characterized by dyslipidaemia, predominantly hypertriglyceridemia, linked to this, the plasma levels of free fatty acid (FFA) and low-density lipoprotein (LDL) molecules concentrations are also increased (Sattar and Greer, 2002). There are several mechanisms that show the associations between lipid profile and PE, these include LDL molecules, which are more prone to oxidation and the rise in FFA may facilitate lipid accumulation in some organs such as liver and kidney, placenta (Sattar et al., 1996). The relative endothelial dysfunction in obese pregnancy is in part explained by altered levels of plasma cytokines IL6 and IL10 with obesity (Stewart et al., 2007). In addition, high maternal BMI is associated with increased levels of circulating leptin secreted by placentas in PE patients (Chappell et al., 2002).

However, raise lipids levels in PE could also serve as a protective mechanism (improve transfer of placental nutrient), thereby counteracting deficiencies arising from compromised placental blood flow (Sattar and Freeman, 2012).

Several researchers suggested that PE arises from an inflammatory state. In mothers with PE there are high circulating levels of pro-inflammatory cytokine level (Lyall et al., 1997, Freeman et al., 2004), as well as changes of inflammatory profile in the placenta of obese women (Walsh, 2007). Obesity is linked to a low-grade inflammatory response in pregnancy and some postulated that obesity increase PE risk through inflammatory pathway, however, this relationship does not approved (Ramsay et al., 2002) (Walsh,2007). There are no studies of anti-inflammatory interventions that have addressed the prevention or treatment of PE, trials of low does aspirin have been approached but remains to approved, however aspirin has less effect on this pathways (Sattar and Freeman, 2012). In addition, it has been shown (Poston et al., 2006) that concomitant supplementation with vitamin C and vitamin E does not prevent PE in women at risk, but does increase the rate of low birthweight. Furthermore, it has been reported that no evidence support the use of antioxidants during pregnancy for the prevention of PE and other outcomes (Salles et al., 2012).

Lipid peroxides are increased in healthy pregnancy (Little and Gladen, 1999), however few findings are available on oxidative stress in obese pregnancy. In addition, obesity puts overload on the ER due to an accumulation of misfolded proteins, lipid over-supply and increased demand on the synthetic machinery (Özcan et al., 2004). Increased glucose metabolism can also cause an increase in ROS in the mitochondria. It has been shown (Houstis et al., 2006) by using gene expression analysis, a role for ROS in both TNFα and glucocorticoid models of insulin resistance. As both ER and oxidative stress are known to induce inflammatory signalling cascades (Özcan et al., 2004, Kamata et al., 2005), these may provide another explanation by which obesity disrupts insulin signalling.

PE is characterized by maternal vascular dysfunction. Maternal obesity is associated with an atherogenic LDL sub-fraction phenotype (Meyer et al., 2013). In addition, elevation in plasma levels of asymmetric dimethylarginine (ADMA) (NO synthesis inhibitor) has been shown to predict coronary heart disease (CHD) and PE (Roberts et al., 2011). ADMA levels are effected by many factors such as obesity and dietary factors, few studies postulated their role as link between obesity and PE (Roberts et al., 2011).

Insulin resistance could be a major link between PE and obesity as it is involved in several abnormal pathways such as dyslipidemia, vascular dysfunction, inflammation and PE. Reducing insulin resistance via either lifestyle changes or pharmacotherapy might therefore lead to decrease PE risk. In addition, it has been shown (De Leo et al., 2011) that continuing metformin therapy throughout pregnancy resulted in significant reduction in pregnancy complications for example PE in women with polycystic ovarian syndrome (PCOS) and hyperinsulinemia. However, conflicted results came from another study (Vanky et al., 2010).

Successful placentation is essential for healthy pregnancy outcome, and in PE partial failure of trophoblast invasion is likely to play a major role in the disease process (Lyall et al., 2013b). There is no clear causal link between obesity and PE but few speculatives have been carried out including how obesity can influence placentation and placental function and thereby contribute to the primary pathology in PE (Sattar and Freeman, 2012). Plasma adiponectin concentrations are elevated in women with PE, perhaps as a salvage mechanism (Ramsay et al., 2003).

Physical activity and lifestyle interventions seems to be associated with lower PE risk (Sorensen et al., 2003). Weight loss via bariatric surgery can considerably decrease PE risk (Bennett et al., 2010). In addition, the current evidence does not favour use of antioxidant supplements to prevent PE (Rumbold et al., 2008). Thus, while obesity appears causally related to risk of PE, there appears to be no single mechanism that is able to explain this link (Sattar and Freeman, 2012).

1.5.6 Obesity management during pregnancy

There are currently no universal guidelines for management of the obese woman during pregnancy and recent review of evidence relating to interventions targeting weight loss showed a lack in appropriate interventions (Birdsall et al., 2009). Diet and exercise have a role in the management of obesity in pregnancy and the ideal weight gain for various degrees of maternal obesity is still unknown. Ideally, women would try to achieve a lower BMI prior to pregnancy and attend for pre-conceptual counselling clinic according to the ACOG Committee opinion no. 549 (2013).

It is recommended that obese women should give birth in consultant-led units in view of the increased risk of induction of labour and emergency caesarean section. It is also recommended that the psychological, social and emotional needs of each obese pregnant

woman should be recognised, despite their care being largely consultant-led. The support of a named midwife should be available to all women throughout the childbearing experience.

1.6 Thesis aims

Uterine contractions during labour are associated with intermittent utero-placental perfusion providing the basis for ischemia-reperfusion type injury to the placenta. Doppler ultrasound studies have demonstrated a linear inverse relationship between uterine artery resistance and the intensity of the uterine contractions during labour. Labour is associated with placental alterations in several pathways linked to oxidative stress.

PE is an important cause of maternal death worldwide and a leading cause of iatrogenic prematurity and FGR. In PE spiral artery remodeling is partial or incomplete in many cases. The ensuing 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.

HSPs play an important role in regulating signal transduction, inhibition of apoptosis and cellular proliferation and differentiation. Apoptosis has been implicated in both PE and labour. In the apoptotic pathway, HSPs act at several stages to prevent cell death initiated by stress-induced damage. TLRs are the principal signalling molecules through which mammals sense infection. Since these pathways play a role in oxidative stress and inflammation, both features of labour and PE. The hypothesis was that placental stress pathways, inflammatory mediators and apoptotic markers would alter during labour and pregnancies complications such as PE and FGR. Furthermore changes in expression vary in different placental zones perhaps due to the variation in blood supply to each zone or due to the fact that some zones of the placenta are more susceptible to IR injury.

Clearly the bigger aim would be, one day, to try and decide which of the pathways might be targeted for therapy for pre-term labour and PE.

1.6.1 Placenta tissue experiments study

The first aim of this project was to investigate the spatial expression of both protein and mRNA of stress proteins HSP70, HSF1, HSP27 and P-HSP27and mRNA expression of

(i) Stress genes (HSF2, HSPB2-8, HSP32, HSP40, HSP60, GRP78, HSP90AA, HSP90B1 and HSP105).
(ii) Inflammatory mediators (TLRs).
(iii) Apoptotic pathway markers (CASP3, 7 and 9)

For the above, placentas were obtained from non-labour and labour groups and the spatial distribution was assessed by comparing the inner, middle and outer placenta zones within individual placentas.

The second aim of this project was to compare expression of both the protein and mRNA of stress proteins HSP70, HSF1, HSP27 and P-HSP27 and mRNA expression of

(i) Stress genes (HSF2, HSPB2-8, HSP32, HSP40, HSP60, GRP78, HSP90AA, HSP90B1 and HSP105)
(ii) Inflammatory mediators (TLRs)
(iii) Apoptotic pathway markers (CASP3, 7 and 9)

For the above, placentas were obtained from non-labour and labour groups and the difference in expression was assessed by comparing the inner, middle and outer placenta zones of the non-labour group with the respective zone of the labour group.

The third aim of this project was to compare both the protein and mRNA expression of stress proteins HSP70, HSF1, HSP27 and P-HSP27 and mRNA expression of

(i) Stress genes (HSF2, HSPB2-8, HSP32, HSP40, HSP60, GRP78, HSP90AA, HSP90B1 and HSP105)
(ii) Inflammatory mediators (TLRs)
(iii) Apoptotic pathway markers (CASP3, 7 and 9)

For the above, placentas were obtained from non-labour PE, labour PE, non-labour control and labour control groups. Due to time constraints only the inner and middle zones were examined for the mRNA work. Protein analysis of HSP70 and HSP27 was performed on all placenta zones. The aim was to determine if there was a difference, at

each zone, for labour PE versus control labour and non--labour PE versus non-labour control.

1.6.2 Tissue culture experiments study

The aim of the first study was to develop an *in vitro* model of the hypoxia-reperfusion that the placenta is exposed to during labour contractions and also in PE as a result of failed spiral artery physiological conversion. The ain was to measure expression of pre-defined proteins or mRNA at placental samples taken from different zones and then exposed to this stress. This might help explain why protein expression varied between zones in labour and PE in the initial experiments performed in this thesis. Specifically, at the protein level HSP70 and HSP27 were studied and at the mRNA level HSP70, HSP27, HSF1, HSP90AA and HSP90B1 were studied.

The aim of second study was to determine how heat shock (classical way of inducing stress proteins) affected the expression of pre-defined proteins or mRNA. The aim was also to determine if any zones of the placenta were more susceptible to heat shock which might help explain why protein expression varied between zones in labour and PE. Specifically at the protein level HSP70 and HSP27 were studied and at the mRNA level HSP70, HSP27, HSPB6, HSPB2, HSP32, DNAJAB1, HSP90AA, HSP90B1, HSPD1, HSF1, HSF2, CASP3, CASP7 and CASP9 were studied.

1.6.3 BMI study

Nothing is known about HSPs expression in the placenta of obese women. However since obesity is associated with oxidative stress and inflammation, the aim was to screen for different HSPs and CASPs families in the human placenta and then compare their expression in placentas from 4 different BMI groups at the inner, middle and outer placental zones. Specifically at the protein levels of HSP70 and HSP27 were studied and at the mRNA level HSP70, HSP27, HSPB6, HSPB2, HSP32, DNAJAB1, HSPD1, HSPA5, HSP90AA, HSP90B1, HSPH1, CASP3, CASP7 and CASP9 were studied.

Chapter II: Materials and methods

2 Materials and methods

2.1 Patient consenting process

2.1.1 Identification

Women who underwent elective caesarean section surgery for obstetric reasons were identified as a potential participant. The participants in the control groups of non-labour and obese group were identified by reviewing the surgical list of the obstetrics operation theatre. The participant control labour group were identified by review the antenatal ward list or labour room notice board showing those who were in in first stage of their labour. The participants in the PE and fetal growth restriction group were identified by reviewing the maternity department notes.

2.1.2 Patient recruitment

Women were approached when they were admitted to the maternity department. During this routine meeting with them, I would introduce my study to them and request invites them to consider participating in it; they would also be given an information sheet. The period between approach (i.e the first introduction of the project to prospective participants) and obtaining consent varied from a few hours to 2 days. Written consent was always obtained through a signed consent form, and the women were given time making their decision between their admission into a labour or a recovery room and a few hours prior to the delivery. This is the time period that the women will have in order to decide whether they want to take part in the study and to sign the consent form. It was made clear to the women that they would always have the right to withdraw from participating at any time, irrespective of the fact that they had signed a consent form. If a woman was interested in participating in the study once they were approached at the maternity department, I would discuss the study further and answer any questions she might have. All consent forms were filed along with the clinical notes pertaining to the patient. A copy was kept in the study file at the Medical Genetics Department.

2.1.3 Ethical approval

Human term placentae were collected from pregnant women at the Southern General Hospital, Glasgow. The ethical approval for this project was approved by the West of Scotland Research Ethics service and was in compliance to the terms in the Declaration of Helsinki (REC reference number 13/WS/0149, IRAS ID 130896), consent forms and information sheets. Signed patient consent was obtained prior to delivery. The information and consent sheets were also approved by the ethics committee. All signed consent sheets were stored in case of the need for an audit arose (appendix 1).

2.2 Subjects

The participants were pregnant women at the Southern General Hospital, Glasgow. The patient's details are shown in (Table 2-1) and (Table 2-2). Placentas were collected from:

2.2.1 Control groups:

Women who had uncomplicated pregnancies and either had spontaneous vaginal delivery at term (labour group (LG), n= 6) or deliveries by elective caesarean section (non-labour group (NLG), n= 6). All those in the labour group had spontaneous labour; this was a tight group in term of length of delivery (the minimum time taken for labour was 3 hours and the maximum was 8 hours). CS was performed for obstetric reasons such as breach presentation, previous caesarean section or because of maternal request. All chosen placentas were confirmed free of infection by pathology report. The control groups studied had no underlying maternal conditions such as hypertension, PE, diabetes or gestational diabetes or other medical disorders. There was no fetal pathology such as fetal anomalies or FGR.

2.2.2 Obese group:

Obesity is a condition characterized by excess of body fat and consequently resulting in a significant impairment of health. BMI (kg/m^2) is defined as persons's weight (kg) divide by the height (m^2). It is a simple index that is commonly used to classify overweight as BMI >25 and obesity BMI >30 in adults. Obese women who had uncomplicated pregnancies delivered by elective CS were classified into three groups according to their BMI (Table 2-2).

Obesity group 1: BMI between 30 and 35 (n=6), group 2: BMI between 35 and 40 (n=6), group 3: BMI equal or greater than 40 (n=6). The control placental samples were collected from women with normal BMI i.e BMI between 19 and 25(n=6).

2.2.3 PE group:

This group includes women who had pregnancies complicated by PE. Non-labour PE (n=5) and labour PE (n=7) were compared to non-labour and labour normotensive pregnancies. Late onset PE is defined as blood pressure of >140/90 mm Hg on at least 2 occasions at least 6 hours apart occurring in women were normotensive before pregnancy after 20 weeks' gestation and accompanied by proteinuria (>300 mg/L in a 24 hour urine collection) with no other underlying clinical problems or maternal disease such as renal impairment (Brown et al., 2001).

2.2.4 FGR group

This group includes women who had pregnancies complicated by FGR. FGR is a concept signifying that the fetus has not achieved its optimal growth and it generally defined as EFW at or below the 10^{th} weight percentile for his or her gestation age in weeks. Severe FGR when EFW is at or below 3^{th} weight percentile and is probably a more reliable cut off for associated perinatal morbidity (Resnik, 2002). However, some of the FGR definitions used in the published literature vary, or can be used inappropriate. About 50-70% of the babies in research publications are actually constitutionally small, with fetal growth appropriate for maternal size and ethnicity (Alberry and Soothill, 2007). The Royal College of Obstetricians and Gynaecologists in Green–top Guideline No. 31 have of defined FGR as "Growth restriction implies a pathological restriction of the genetic growth potential. As a result, growth restricted fetuses may manifest evidence of fetal compromise (abnormal Doppler studies, reduced liquor volume)". All the samples used in this study (n=8) were obtained from a sever group with EFW< 5^{th} weight percentile.

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); LG-FGR (labour-FGR); PG (primigravida); 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
LG-FGR	27	G4P2	NO	37	1880	310	SVD	29
LG-FGR	33	G2P1	NO	35+2	1850	370	EMC/S	34.5
LG-FGR	30	G2P1	YES	34+4	1056	350	EMC/S	18.1
LG-FGR	32	G8P0	NO	37+4	1990	265	EMC/S	39
LG-FGR	22	PG	NO	37+6	2368	470	EMC/S	21.3
LG-FGR	28	G3P2	NO	37+2	2075	324	EMC/S	20.1
LG-FGR	23	PG	NO	38+4	2880	427	SVD	22.4
LG-FGR	42	PG	YES	36.6	2180	442	EMC/S	18.1

Table 2-2: BMI patient details.

Sample	Age years	Parity	Smoker	Gestation (weeks/ days)	Birth weight (g)	Placenta weight (g)	Mode of delivery	BMI
BMI 1-1	26	G2P1	YES	39	2860	683	ELC/S	23
BMI 1-2	22	PG	YES	39+2	3050	465	ELC/S	21
BMI 1-3	32	G2P1	NO	39	4255	605	ELC/S	27
BMI 1-4	25	G2P1	NO	40	5250	980	ELC/S	24.1
BMI 1-5	32	G2P1	NO	39	3510	716	ELC/S	24.4
BMI 1-6	36	G2P1	NO	39+1	4220	630	ELC/S	23
BMI 2-1	34	G2P1	NO	41+3	3100	450	ELC/S	30.6
BMI 2-2	34	G3P2	NO	39+1	3555	685	ELC/S	32.4

BMI 2-3	33	G2P1	YES	38+6	3450	625	ELC/S	33.2
BMI 2-4	29	G2P1	NO	37+3	3210	675	ELC/S	32.2
BMI 2-5	43	G4P3	NO	37	3515	750	ELC/S	32.8
BMI 2-6	37	PG	NO	38+3	3460	595	ELC/S	33.1
BMI 3-1	25	G2P1	NO	39+5	3770	690	ELC/S	36.7
BMI 3-2	40	G3P2	NA	38+4	4850	940	ELC/S	36.6
BMI 3-3	32	PG	NA	38+5	2740	430	ELC/S	37.9
BMI 3-4	36	G2P1	NA	39+3	4035	790	ELC/S	35.9
BMI 3-5	33	G3P2	YES	40+1	3725	722	ELC/S	37.4

BMI 3-6	22	G3P2	NO	38+4	3840	680	ELC/S	37
BMI 4-1	29	G3P2	NO	39+3	4015	995	ELC/S	40.3
BMI 4-2	46	G3P1+1	NO	38	2845	590	ELC/S	42.3
BMI 4-3	42	G3P2	NO	39+3	3550	685	ELC/S	44.7
BMI 4-4	25	G3P1+1	NO	38+4	3575	645	ELC/S	49
BMI 4-5	38	G2P1	NO	39	3736	695	ELC/S	47.2
BMI 4-6	33	G2P1	NO	39+2	2940	494	ELC/S	52

2.3 Buffers

Water (dH_2O) used in buffer preparation was Milli-Q RG Ultrapure.

2.3.1 Phosphate buffered saline (PBS)

- PBS **(**Sigma-Aldrich, cat.no. P4417-50 TAB)

Phosphate buffered saline: A solution of one PBS tablet per 100 ml dH_2O

2.3.2 Tissue homogenization buffers (1 L)

- Tris (Sigma-Aldrich, cat.no. 93352)
- EDTA (Sigma-Aldrich, cat.no. E6758)
- Sucrose (Sigma-Aldrich, cat.no. S9378)
- Protease Inhibitor Cocktail (PIC) (Sigma Aldrich cat. no. P8340-5ml)

1L of dH_2O, 25mM Tris (3.02g), 1mM ethylenediaminetetraacetic acid (EDTA) (0.4g), 250mM sucrose (85.6g). The pH adjusted to 7.6. The buffer was stored at 4°C. On the experiment day 12.5µl/ml of PIC designed for use with mammalian cells (250µl in 20ml of the buffer) was added.

2.3.3 Bradford Assay buffers

- Bovine Serum Albumin (BSA) (Sigma-Aldrich, cat.no. A7906-50G)
- Bradford reagent (Sigma-Aldrich, cat.no. B6916-500ML)

Stock standard solution for Bradford assay contained 10mg of BSA in 10ml dH2O

2.3.4 Western blotting buffers

- 10% sodium dodecyl sulphate (SDS) (Sigma-Aldrich, cat.no. 35000)
- Bromophenol blue (solid) (Sigma-Aldrich, cat.no. B0126-25G)
- Glycerol (Sigma-Aldrich, cat.no.G2025)
- Tris (Sigma-Aldrich, cat.no. 93352)
- Acrylamide (Sigma-Aldrich, cat.no.A3699-100ML)

- Ammonium persulfate (Sigma-Aldrich, cat. No. A3678-100G)
- TEMED: N,N,N,N tetramethylethylenediamine (Sigma-Aldrich, cat.no.T9281-100ML).

2.3.4.1 Sample loading buffer (2x)

1.2ml of 1M Tris, PH 6.8, 2ml Glycerol, 4ml of 10% SDS, 0.8ml of dH_2O, 2ml of 1M dithiolthreitol (DTT) and a few bromophenol blue crystals.

2.3.4.2 Buffer A for the resolving gel (1 L, pH 8.8)

One L of dH_2O contained 1.5M Tris (181.65 g) and 0.4% SDS (40 ml of 10% SDS).

The pH was adjusted to 8.8.

2.3.4.3 Buffer B for the stacking gel

One L of dH_2O contained 1M of Tris (60.55g) and 0.4 % SDS (40 ml of 10% SDS). The pH was adjusted to 6.8.

2.3.4.4 Composition of resolving and stacking gels (Table 2-3)

Table 2-3: Resolving and stacking gels compositions.

Reagent	**Resolving gel (2 gels) (10%)**	**Stacking gel (2 gels) (4%)**
	Volume	Volume
30% acrylamide/bis-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 for 2 gels	20 ml for 2 gels

2.3.4.5 10% ammonium per sulphate (APS) solution

To 1ml of dH_2O, 0.1 g of APS was added and allowed to dissolve

2.3.4.6 Running buffer (5x)

Four L of dH_2O contained 25mM Tris (60.4 g), 190mM glycine (288 g), 0.4% SDS (200ml of 10% SDS) was added. The pH was adjusted to 8.3.

2.3.4.7 Running buffer diluted

Diluted 1:5 on the day of experiment: 0.5L running buffer (5x) added to 2 L of dH_2O.

2.3.5 Transfer of proteins to nitrocellulose: buffers required

- Tris (Sigma-Aldrich, cat.no. 93352)
- NaOH (Sigma-Aldrich, cat.no. 655104)
- Glycine (Sigma-Aldrich, cat.no. G8898-500G)

Transfer buffer in 1L: 800ml of dH_2O, 25mM of Tris (3 g), 190mM glycine (14.4 g), 20% methanol (200 ml).

Sodium hydroxide solution (NaOH): 0.1 g NaOH in 100ml dH2O.

2.3.6 Immuno-detection of proteins: buffer required

2.3.6.1 TBSTB (in 1 Litre, PH 7.5)

- Tris (Sigma-Aldrich, cat.no. 93352)
- NaCl (Sigma-Aldrich, cat.no.S7653)
- Tween-20 (Sigma-Aldrich, cat.no.P1379)
- Bovine serum albumin (BSA) (Sigma-Aldrich, cat.no. A7906)

700 ml of dH_2O contained 20 mM Tris (2.4g), 5M HCL (3ml, pH 7.5), 0.5M NaCL (29.2g), 0.4% Tween-20 (4 ml) and 0.25% BSA (2.5g) was mixed and final volume of 1 L was made and stored at 4°C.

2.3.7 TAE buffer for gel electrophoresis

- Tris (Sigma-Aldrich, cat.no. 93352)

- EDTA (Sigma-Aldrich, cat.no. E6758)
- Glacial acetic Acid (Sigma-Aldrich, cat.no. 1005706-3X1.5ML)

1L of dH_2O contained 242 g, 0.5M EDTA (100 ml, pH 8.3) and 57.1 ml of glacial acetic acid. This was stored at room temperature.

2.3.8 Supplemented media for tissue culture (hypoxia-reperfusion and heat shock experiments).

- Medium199 (M199) (with Earle's salts, L-glutamine and sodium bicarbonate. The liquid was supplied sterile-filtered, cell culture tested) (Sigma-Aldrich, cat. no. M4530-100ML).
- 5% fetal bovine serum (heat inactivated) (FBS) (Sigma-Aldrich, cat. no. F9665)
- 1% antibiotic antimycotic solution (Ab/Am) (Sigma-Aldrich, cat. no. A5955)

Every 20 ml aliquot of M199 media was supplemented with 5% of FBS (1ml) and 1% Ab/Am (0.2ml).

2.4 Placenta sampling methods

Placentas were obtained after delivery and washed with tap water to remove blood. The amnion was cut off and then the whole placenta was weighed. The placenta was divided into three zones (inner, middle and outer) by three imaginary concentric circles and was labelled with 12 numbers before a photograph was taken (Figure 2-1). Each circle was divided into quadrants. A full thickness piece of placenta, approximately ~1 cm^3 was obtained from each of the three zones by taking measurements from the umbilical cord insertion point: inner zone being the inner third closest to cord insertion point, the middle zone being the third located roughly in the middle of the distance between the umbilical cord insertion point and the periphery, and the outer zone being the third closes to the periphery of the placenta. This was all performed as previously described and is shown in (Abdulsid et al., 2013b, Abdulsid et al., 2013a, Abdulsid and Lyall, 2014, Alwarfaly, 2015). In the FGR placentas, as placenta were smaller, samples were obtained from two zones (inner and outer). Within each zone four separate samples were obtained representing the four quadrants. Placentas had a central cord insertion. Samples were immediately flash frozen in liquid nitrogen. The remaining part of the placenta was transported to the pathology department and sections were examined by a pathologist.

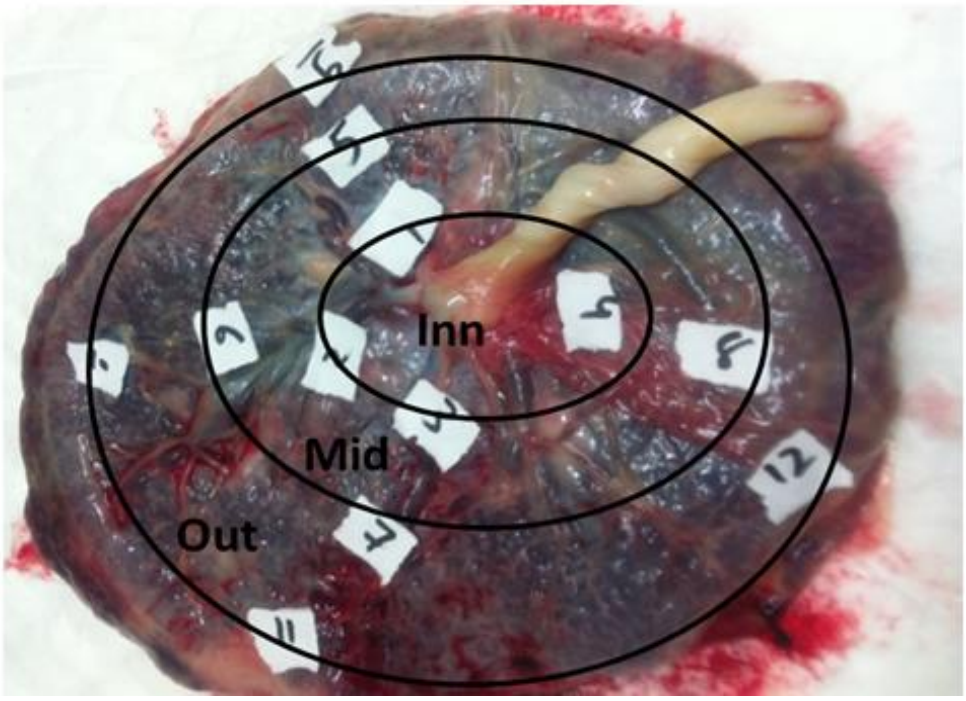


Figure 2-1: Photograph of placenta shown the 12 sites within the three placenta zones where samples were obtained in each individual placenta.

2.4.1 Collection and processing of placental tissue at the delivery suite

Tissue samples were collected according to systemic sampling methods. They were washed three times in PBS (phosphate buffered saline) until any remaining traces of blood was washed away then, flash frozen in liquid nitrogen. The liquid nitrogen has been collected in sterile containers and all equipment used for the tissue collection was sterile. Cryostat containers were cooled on dry ice and each was labelled and used to store a sample of tissue. Snap frozen tissue was then brought to the laboratory on dry ice and stored in -80 °C until required.

2.5 Protein expression analysis:

2.5.1 Placenta tissue homogenisation

An appropriate volume of tissue homogenising buffer (section 2.3.2) supplemented with protease inhibitor cocktail was aliquot into a universal container and kept on wet ice. Relevant tissue samples were removed from –80 °C storage and ground down to a fine powder in liquid nitrogen using a mortar and pestle. Homogenising buffer was added to the fine powder at a final ratio of 3:1 (v:w). The sample was then homogenised using 5 bursts of 10 second at a speed 20 with 1 minute (min) of cooling interval on ice between

bursts using a rotor-stator homogeniser (polytron® PT 1600E, Lucerne, Kinematica) was used. Placenta homogenates were then spun at 5000g for 10 min at 4°C to remove debris. The supernatant containing the total protein (both particular and cytosolic fractions) was extracted and divided into aliquots and stored at -70°C. The amount of protein in each sample was quantified by the method of Bradford.

2.5.2 Protein estimation using Bradford assay

The protein concentration was determined according to the Bradford dye-binding method (Bradford, 1976). Bradford assay: is a spectroscopic analytical procedure used to measure the concentration of protein in a solution. It is subjective, *i.e.*, dependent on the amino acid composition of the measured protein. The quantification is based on the colour change of Coomassie Blue G-250 in response to various concentrations of protein. is based on an absorbance shift of the dye Coomassie Brilliant Blue G-250 in which under acidic conditions the red form of the dye is converted into its bluer form to bind to the protein being assayed. The stock standard solution (STD) of bovine serum albumin (BSA) (1 mg per ml) (section 2.3.3) was used to create the standard curve. A series of standard solution (STD) using the dilutions below were prepared as shown in (Table 2-4).

Table 2-4: Standards stock dilution (µg/ml).

STD concentration (µg/ml)	**Vol. stock std (µl)**	**Vol. of d H2O (µl)**
	n/a	50
200	10	40
400	20	30
600	30	20
800	40	10
1000	50	n/a

Samples were diluted 1:40 (39 µl of dH_2O and 1 µl of protein homogenate). Measurement of protein samples was performed in duplicate and samples were read in a BioPhotometer® (Eppendorf). A standard curve was created of absorbance against different BSA concentrations and this was used to find the concentration of protein samples concentration (Figure 2-2). For a Western blot, 50 mg of protein loaded into each well. The calculation to convert µg to mg = reading value divided by 1000. The value obtained was then multiplied by 40 (dilution factor).

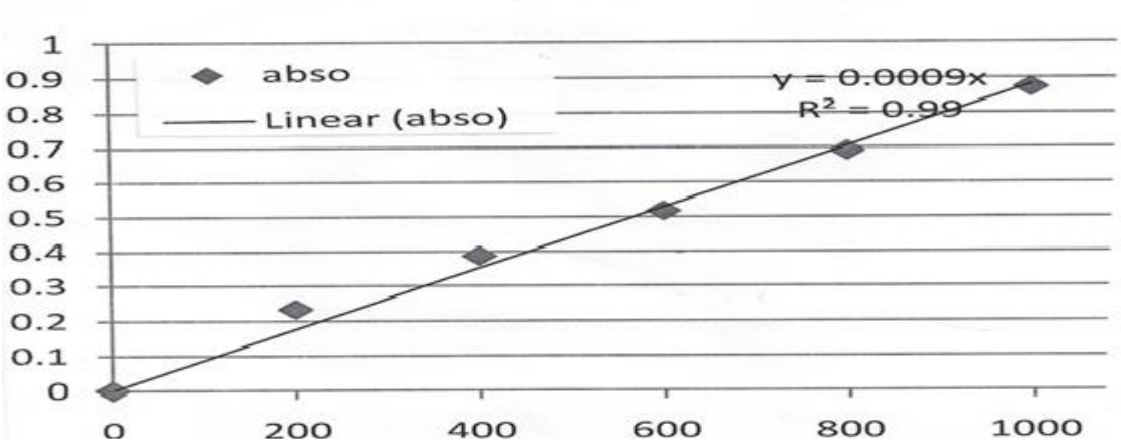


Figure 2-2: Example of standard curve.

2.5.3 Western blotting

Sodium dodecyl sulphate polyacrylamide (SDS-PAGE) gel electrophoresis separates proteins according to their molecular weight. Proteins migration is determined by the negative charge to mass ratio. Proteins separation is determined by the size of the proteins as well as the size of the pores of the gel. The protocol in this study used a 10% resolving gel and 4% stacking gel.

2.5.3.1 Samples preparation and gel electrophoresis

Equal concentration of protein samples (including control) were denatured and reduced in the presence of mercatoethanol and sodium deodecyl sulphate (SDS) (2.4.4.1) by boiling for 5 min in heat block at 95 °C before loading. After brief centrifugation**,** each well was loaded with 50 µl of protein samples (50 mg of protein). 20 µl of pre-stained SDS-PAGE protein molecular weight standards (Low range, BIO-RAD, cat.no. 161-0305) was loaded to the first well. 50 µl of positive control (same normal placenta used for all gels) was loaded to the second well and used as a reference sample for subsequent densitometry. Samples were resolved on polyacrylamide gels (4-10%) (section: 2.4.4.2 to 2.4.4.5). Samples were separated using the Protean II® xi Cell System (Bio-Rad Laboratories, Hemelhempstead, UK) immersed in running buffer (section 2.4.4.6) at 300 V, 27 mA for the first hour (h) then the current was turned up to 30 mA, for a total time period of about 5 hours (h) until the dye front was close to the bottom of the gel (Figure 2-3).

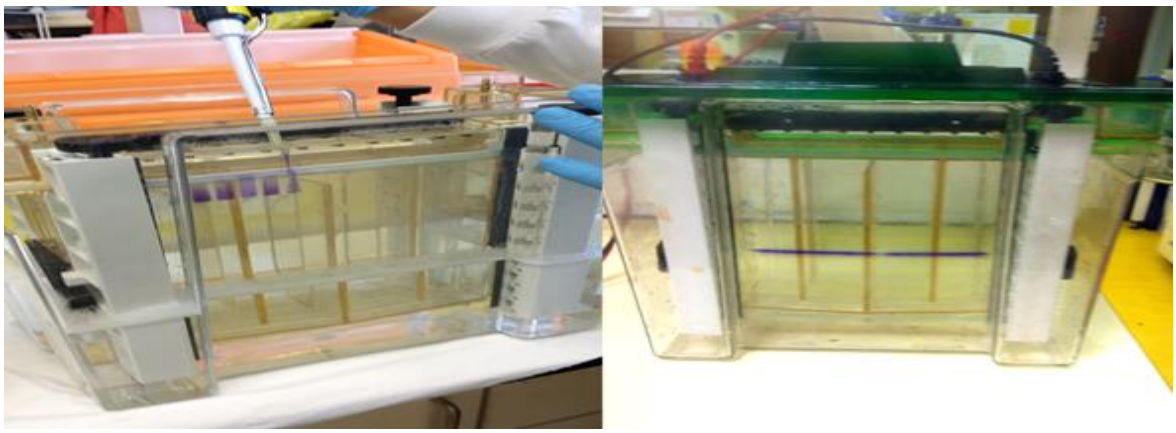


Figure 2-3: PROTEAN II xi systems by Bio-Rad Laboratories: photograph shows a sample being loaded (left) and a gel that is running (right).

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

Resolved proteins were electro-transferred onto Whatman Protran® nitrocellulose transfer membranes (Sigma-Aldrich, cat.no. Z61360). Two blotting papers and nitrocellulose paper were kept in transfer buffer (section 2.4.5) for 20 min. The transfer was performed using a Trans-Blot SD Semi-Dry® Electrophoretic Transfer Cell (Bio-Rad, cat.no. 170-3940) at 22 Volts (V) and a constant current (membrane area x 2.5) for 30 min. Successful transfer was indicated by full transfer of the prestained molecular weight markers onto the nitrocellulose membrane. Complete transfer of protein was demonstrated using Ponceau S solution staining and then de-staining of the nitrocellulose was carried out in 0.1% NaOH solution (Figure 2-4).

2.5.3.3 Immuno-detection of proteins (Immune-blotting)

Membranes were blocked in 5% normal donkey serum (Serotec, cat.no CO6SBZ) or 5% normal goat serum (Sigma-Aldrech, cat.no. cat.G9023) in TBSTB (section 2.3.6) for 1h at room temperature (RT) with gentle agitation. Primary antibodies were pre-absorbed for 30 min in 5% normal human serum (Sera Laboratories International, cat.no. S-123-H) in TBSTB at room temperature during the blocking stage. Membranes were then exposed to specific primary antibodies and incubated for 1h at RT (Table 2-5). Different positive control protein samples were run when appropriate (Table 2-6). The membranes were washed three times for 5 min each in TBSTB (section 2.3.6.1) before adding appropriate horseradish peroxidise (HRP) conjugated anti-immunoglobulin G (IgG) secondary antibody at the appropriate different dilution in TBSTB buffer for 1h at RT (Table 2-7). After secondary antibody incubation membranes were washed twice in TBSTB for 5 min each and then in water for 5 min.

Table 2-5: Primary antibodies

Antibody	Supplier	Catalogue No	Host	Dilution
HSP27	Cell Signalling Technology	2402	Mouse mono-clonal	1:1000
P-HSP27 (Ser15)	Cell Signalling Technology	2404S	Rabbit polyclonal	1:1000
P-HSP27 (Ser78)	Cell Signalling Technology	2405S	Rabbit polyclonal	1:1000
P-HSP27 (Ser82)	Cell Signalling Technology	2401S	Rabbit polyclonal	1:1000
HSF1	Enzo	ADI-SPA-901	Rabbit polyclonal	1:1000
HSP70	Enzo	ADI-SPA-812	Rabbit polyclonal	1:1000
Actin-β	Abcam	Ab8227	Rabbit polyclonal	1:2000
Caspase-3	Enzo	ADI-AAP-113	Rabbit polyclonal	1:1000
TLR4	Abcam	ab47839	Rabbit polyclonal	1:500
TLR4	Abcam	ab22048	Mouse monoclonal	1:1000
TLR2	Santa Cruz	Sc8690	Goat polyclonal	1:200
TLR4	Santa Cruz	Sc-8694(C18)	Goat polyclonal	1:200

Table 2-6: Cell lysate positive control

Cells	Supplier	Catalogue number	Cell type	Source	Function
HL-60	Santa Cruz	Sc-2209	Whole cell lysate	Ready made	Positive control
HEL92.1.7	Santa Cruz	Sc-2270	Cell lysate	Ready made	Positive control
Caco-2	Santa Cruz	Sc-2262	Cell lysate	Ready made	Positive control

Table 2-7: Secondary antibodies

Antibody	Supplier	Catalogue	Host	Dilution
Donkey anti-mouse IgG-HRP	Abcam	ab6820	Donkey polyclonal antibody to mouse	1:1000
Donkey anti-Rabbit IgG-HRP	Abcam	ab7083	Donkey polyclonal antibody to rabbit	1:3000
gout anti-Mouse IgG-HRP	Abcam	ab6789	Goat polyclonal antibody to Mouse	1:2000
Rabbit-anti-Mouse IgG-HRP	Abcam	ab6728	Rabbit polyclonal antibody to Mouse	1:2000
Donkey anti-Goat IgG-HRP	Santa Cruz	Sc-2033	Donkey polyclonal antibody to Goat	1:200
Rabbit anti-Goat IgG-HRP	Santa Cruz	Sc-2768	Rabbit polyclonal antibody to Goat	1:1000
goat anti-Rabbit IgG-HRP	Cell-Signalling Technology	7074	Goat polyclonal antibody to rabbit	1:1000

2.5.3.4 Detection

Immunologically reactive protein were visualised using the enhance Amersham ECL® Western Blotting Substrate (Amersham Pharmacia Biotech, GE healthcare, cat. No RPN2106). Chemiluminescent images of immune-detected bands were obtained on sensitive autoradiography X-ray films (Kodak film. Sigma- Aldrich, cat.no. Z373508-50EA) after exposed for different times periods (e.g. 1 min, 30 seconds then 10 seconds) until bands were clearly not saturated). The films were developed using an Ecomax x-ray film processor (Protec Medical Systems, cat.no. 1186-3-0000). Intensities of bands on exposed films were quantitatively analysed using a multi-Analysis® software Bio-Rad GS-700® imaging densitometer. The density of the placental reference sample (added to every gel) was used as internal control and other bands density were expressed relative to it.

2.5.3.5 Loading control

Ponceau S solution also was used to ensure the transfer and equality of sample loading (Figure 2-4).

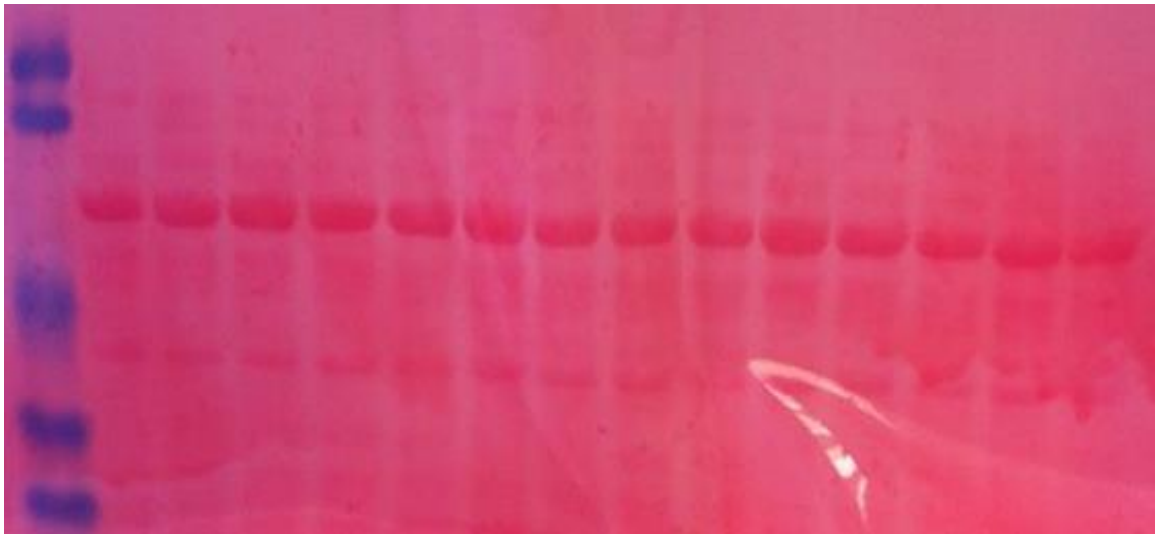


Figure 2-4: Example of nitrocellulose membrane illuminated with Ponceau S solution.

2.6 RNA extraction from placenta tissue

2.6.1 RNA extraction

Total RNA was extracted using the RNeasy Midi Kit (Qiagen .cat.no. 75142) from snap frozen placenta samples. RNA extraction after overnight incubation in RNALater® (Sigma-Aldrich, cat.no R0901) was also attempted at the beginning of the thesis but because of poor yields of RNA obtained compared to the freshly frozen samples all subsequent samples were snap frozen immediately after collection.

When required placenta samples were removed from -80° C storage and placed in a pre-weighed plate to determine the correct weight of starting material (maximum 250 mg of tissue was used at any one time). The sample was disrupted in 4 ml RLT buffer (provided with the kit) supplemented with Beta-Mercaptoethanol (β-ME) (10 µl β-ME per 1ml of RLT Buffer). Homogenisation of tissue was carried out using a Rotor-stator homogeniser (Polytron PT1600E, Lucerne, Kinematica) at full speed setting for 45 seconds. Homogenised lysate from each sample was then spun at 4000g, 25°C for 10 min. The supernatant was then removed carefully to a new 15ml Falcon tube (Sigma-Aldrich, cat.no.Z617849) and then 4 ml of ethanol (70%) was added to the supernatant and mixed by shaking vigorously until the solution was clear.

RNasey® Midi kit spin columns already contained within the 15 ml collection tubes were retrieved and 4 ml of mixed supernatant (ethanol and supernatant) was added to the column which was then spun at 4000g, 25°C for 5 min. The flow-through was discarded. Next the column was washed three times in 4.0 ml of RW1 buffer (provided with the kit) then 2.5 ml of RPE (twice). After each wash the column was spun at 4000g, 25°C, for 5, 5 and 2 min respectively. For RNA elution, the RNeasy column was transferred to a new 15 ml collection tube. 250 µl of RNase-free water was added into the RNeasy silica-gel membrane and allowed to stand for 1 min. After that the columns were spun at 4000g, 25°C for 3 min. This step was then repeated twice. Then RNA was aliquoted and stored at -80°C.

2.6.2 Quantification of RNA

Total RNA concentration (ng/µl) was calculated using a Nanodrop 1000™ spectrophotometer (Thermo Scientific). The ratio of absorbance at 260/280 nm indicates

the RNA purity; a value ratio close to 2 indicates optimal purity. The final measurements were in ng/µl (Figure 2-5) (Figure 2-6).

Name | Report Full Mode: Ignore

Sample ID	User ID	Date	Time	ng/ul	A260	A280	260/280	260/230
blank	Sandy	02/05/2012	21:47	0.16	0.004	0.006	0.65	0.25
2PE1	Sandy	02/05/2012	21:48	376.08	9.402	4.484	2.10	2.28
2PE6	Sandy	02/05/2012	21:49	305.19	7.630	3.596	2.12	2.26
6PE1	Sandy	02/05/2012	21:50	346.98	8.674	4.131	2.10	2.31
6PE6	Sandy	02/05/2012	21:51	256.52	6.413	3.013	2.13	2.33
7PE1	Sandy	02/05/2012	21:52	513.83	12.846	5.929	2.17	2.24
7PE6	Sandy	02/05/2012	21:53	357.28	8.932	4.238	2.11	2.27
8PE1	Sandy	02/05/2012	21:54	392.43	9.811	4.678	2.10	2.27
8PE6	Sandy	02/05/2012	21:55	154.39	3.860	1.802	2.14	2.19

Figure 2-5: Example of transcript from Nanodrop reading in ng/µl. The ratio of absorbance at 260 nm and 280 nm (260/280) is given an indication of RNA purity.

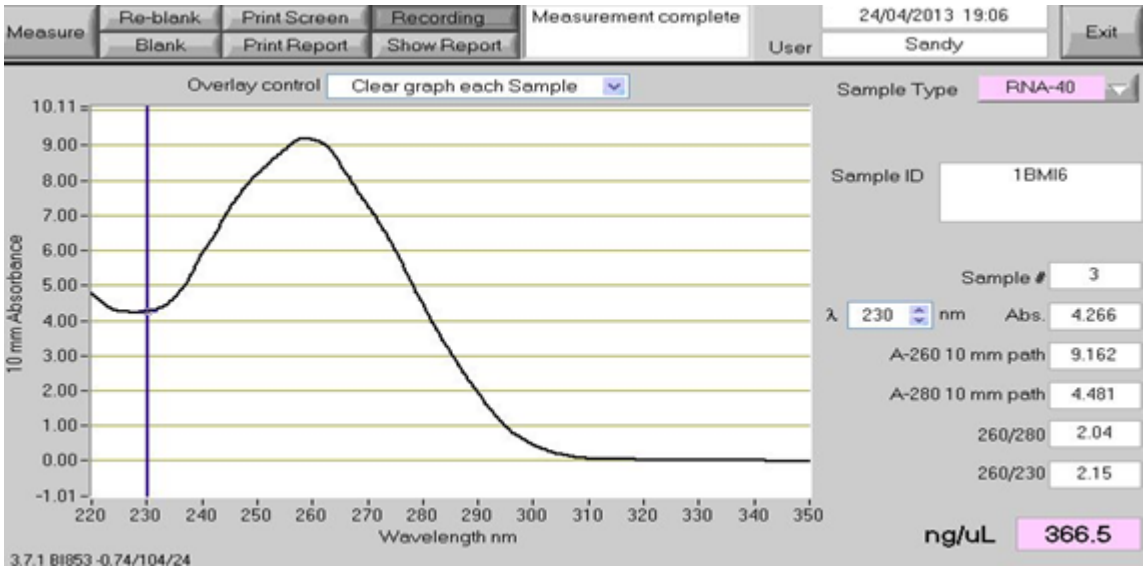


Figure 2-6: RNA concentration of representative placenta sample: number 1BMI6 in ng/µl (366.5).

2.6.3 Reverse transcription (converting mRNA into cDNA)

RNA was reverse-transcribed to first strand complementary DNA (cDNA) using an adapted protocol that used the QuantiTech Kit (Qiagen, cat.no 205310) and GoScript™ (Promega, cat.no. A5003). As a result of an initial optimization process, the quality of cDNA obtained using GoScript™ reverse transcriptase was better quality than reverse transcriptase of the Qiagen kit.

Reverse transcription reaction included two steps as follows:

- Genomic DNA elimination reaction:

Based on their original concentration the amount of RNA required for 100 ng was calculated according to following equation:

$$\textbf{Required RNA (µl)} = 100ng\ X\ 1\mu l/orginal\ concentration\ (^{ng}/_{\mu l})$$

For each DNA elimination reaction (in each PCR tube) the mixture was: 12 µl of RNA (100 ng) from the previous calculation, 2 µl of wipe-out buffer (Qiagen kit) was added to mixture and tubes were put through gOUT program, using a thermal cycler (DNA Engine®, Bio-Rad) for 3 min at 42 °C.

- Reverse transcript (RT) reaction

Once the gDNA wipeout reaction was completed, to each tube 1 µl of GoScript Reverse transcriptase ((Promega), 1 µl RT primer Mix (Qiagen kit) and 4 µl RT Buffer (Qiagen kit) were added and PCR tubes were again placed in the thermal cycler and the RT4 program was used as follows; 30 min at 42°C and 5 min at 95°C. Once the reaction was completed, the cDNA was stored at -20 °C.

2.6.4 Testing cDNA quality

The cDNA quality was tested using the human cDNAOK!® kit (Microzone, cat.no. 2HCDOK-150). The human cDNAOK mix and megaMix-Gold are supplied from Microzone and the primers sequence was held by company. Interpretation of the results as following: expected fragment size: 125 bp, 250, 375, 500 and 650 bp. If all 5 fragments are observed the cDNA intergrate is 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. The PCR reaction was as following; 7.5 µl of human cDNAOK!® Kit mix, 12.5µ master mix Gold, 4µl RNase-free water and 1µl cDNA (RNase-free water was added to the negative control). PCR tubes were put into the thermal cycler program (cDNAOK)

- 95°C for 30 seconds
- 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 a constant voltage of 50 V (Figure 2-7).

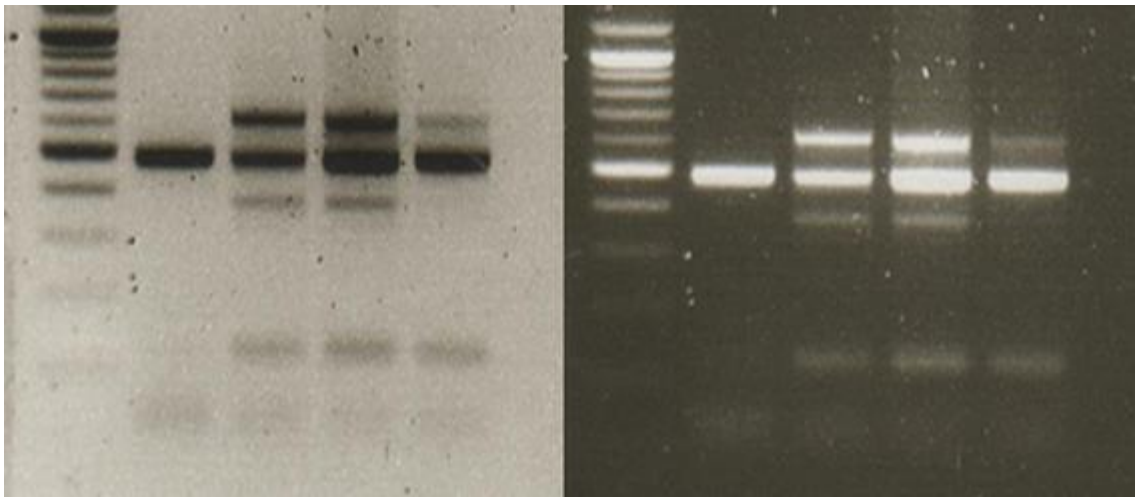


Figure 2-7: Gel showing products of the PCR multiplex reaction using the Human cDNAOK!® Kit (Microzone, cat.no. 2HCDOK-150). cDNA was made using Quantitect® kit and GoScript™ reverse transcriptase. Products were separated on 2% agarose gel using 100 bp moelcualr ladders. The negative control was run in first lane. The other lanes show different samples that showed good quality cDNA .

2.6.5 Gel electrophresis using 2% agarose gels

1g of agarose (Sigma-Aldrich, cat.no. 05066-50G) was added to 50 ml of TAE (section 2.4.7) buffer then dissolved in a microwave for 30, 20 and 10 seconds with a short interval between each time. 7µL of Sybr safe DNA gel stain (Invitrogen, cat.no. S33102) was added. Gel electrophoresis was performed using RunOne™ Electrophoresis cell (EmbTec, cat.no. E15659). The loading was as following; DNA ladder (Sigma-Aldrich, cat.no.P1473) was loaded in the first well, dH2O as negative control in the second well,

then samples were then added in order and finally the positive control. The run was at constant 50 V. the gel was then viewed on a UV transilluminator and a photograph was taken.

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

Gene-specific real-time PCR TaqMan gene expression assays were predesigned by Applied Biosystems. All gene expression assays have FAM™ reporter dye at the 5' end of the probe, TaqMan® MGB probe and a non-fluorescent quencher at the 3' end of the probe. Both the TaqMan® MGB probes and primers have been premixed to a concentration of 18 uM for each primer and 5 uM for the probe. All gene expression assays amplicon length were less than 150 bp (endogenous control amplicon Length was 171 bp) and all were linked to FAM-MGB dye. These assays were readymade and supplied by Applied Biosystems (cat.no 4331182). If possible, the primers were selected span exons in order to exclude the amplification of genomic DNA.

Real-time PCR was performed from reverse transcribed cDNA samples using the Quantitative PCR StepOnePlus® machine (applied Biosystems), following the manufacturer's instructions. Briefly, 100 ng of cDNA (including the reference sample) were diluted 1:5 in RNase-free water and then added to a 96-well MicroAmp® Fast Optical Reaction Plate (Applied Biosystems, cat.no. 4346906) with 10 µl of TaqMan Master mix (Applied Biosystems, cat.no. 4369514), 4 µl RNase-free water and 1 µl TaqMan Gene Expression Assay (either gene-specific inventoried assays or endogenous reference assays). β-actin (Applied Biosystems, cat.no. Hs99999903_ml) was used as an internal control for normalising relative expression levels in the different RNA samples. The choice of endogenous control is important and for this work the endogenous control was β-actin, which has previously been demonstrated as a suitable endogenous control in this laboratory. Placenta cDNA (primer design) was used as a positive reference sample. Thermal cycling and fluorescent monitoring were performed using the StepOnePlus® machine (Applied Biosystems).

PCR amplification was performed using the following conditions: initial holding stage at 50 °C for 2 min and then 95 °C for 10 min, followed by a total of 50 cycles (15 seconds at 95 °C for denaturation and 1 min at 60 °C for anneal/extend). During the extension step of each cycle the fluorescence data were collected. Negative controls using RNase-free water as template were included to test for the presence of any genomic DNA contamination.

TaqMan gene expression assays of different genes which were used in my studies are shown in (Table 2.8), (Table 2-9) and (Table 2-10). All assays were inventoried assays (prepared by Applied Biosystems) and have a unique catalogue number (4331182). This means that primer sequences and details are withheld by the company.

Table 2-8: TaqMan gene expression assays of stress pathways. Hs means Homo sapiens, m means an assay whose probe spans an exon junction, s an assay whose primers and probes are designed within a single exon. Such assays, by definition, detect genomic DNA. g an assay that may detect genomic DNA. The assay primers and probe may also be within a single exon.

Human assay ID	Gene name/Gene aliases	Gene Symbol	Assay Design	Amplicon length
Hs00328933_m1	heat shock protein, alpha-crystallin-related, B6/ (HSP20)	HSPB6	probe spans exons	96
Hs00166138_m1	crystallin, alpha A / (HSPB4, HSP20)	CRYAA	probe spans exons	123
Hs00157107_m1	crystallin, alpha B / (HSPB5, HSP20)	CRYAB	probe spans exons	66
Hs00205056_m1	heat shock 22kDa protein 8 /HSP22	HSPB8	probe spans exons	66
Hs03044127_g1	heat shock 27kDa protein 1	HSPB1	probe spans exons	145
Hs00155436_m1	heat shock 27kDa protein 2 /(HSPB2-C110, MKBP)	HSPB2	probe spans exons	116
Hs00272204_s1	heat shock 27kDa protein 3	HSPB3	both primers and probe map within a single exon	77
Hs00205296_m1	heat shock 27kDa protein family, member 7 (cardiovascular)/ (ccV HSP)	HSPB7	probe spans exons	79
Hs01110250_m1	heme oxygenase (decycling) 1/ HSP32	HMOX1	probe spans exons	82
Hs00428680_m1	DnaJ (Hsp40) homolog, subfamily B, member 1/(HSP40)	DNAJB1	probe spans exons	130
Hs01036753_g1	heat shock 60kDa protein 1 (chaperonin)	HSPD1	probe spans exons	81
Hs00359163_s1	heat shock 70kDa protein 1A	HSPA1A	both primers and probe map within a single exon	124

Hs00607129_gH	heat shock 70kDa protein 5 (glucose-regulated protein, 78kDa)	HSPA5	probe spans exons	146
Hs00743767_sH	Heat shock protein 90α	HSP90AA1	both primers and probe map within a single exon	133
Hs00427665_g1	heat shock protein 90kDa beta (Grp94), member 1	HSP90B1	probe spans exons	135
Hs00971475_m1	heat shock 105kDa/110kDa protein 1	HSPH1	probe spans exons	114
Hs00232134_m1	heat shock transcription factor 1	HSF1	probe spans exons	102
Hs00988308_g1	heat shock transcription factor 2	HSF2	probe spans exons	68

Table 2-9: TaqMan gene expression assays of inflammatory pathways: TLRs, MYD88 and NFKB1. SA: single exons

Human assay ID	Gene name/Gene aliases	Gene	Assay Design	Amplicon length
Hs00413978_m1	toll-like receptor 1	TLR1	probe spans exons	72
Hs00610101_m1	toll-like receptor 2	TLR2	probe spans exons	80
Hs01551078_m1	toll-like receptor 3	TLR3	probe spans exons	132
Hs00152939_m1	toll-like receptor 4	TLR4	probe spans exons	89
Hs00152825_m1	toll-like receptor 5	TLR5	probe spans exons	78
Hs00271977_s1	toll-like receptor 6	TLR6	both primers and probe map within SA	122
Hs00152971_m1	toll-like receptor 7	TLR7	probe spans exons	125
Hs00152972_m1	toll-like receptor 8	TLR8	probe spans exons	89
Hs00152973_m1	toll-like receptor 9	TLR9	probe spans exons	139
Hs01675179_m1	toll-like receptor 10	TLR10	probe spans exons	121

Hs00182082_m1	myeloid differentiation primary response 88	MYD88	Probe spans exons	104
Hs00765730_m1	nuclear factor of kappa light polypeptide gene enhancer in B-cells	NFKB1	probe spans exons	66

Table 2-10: TaqMan gene expression assays of apoptotic pathways: CASP3, CASP7, CASP9 and ACTB. B-Actin TaqMan® gene expression assay ID beginning with "Hs999999…" and ending in "_m1" identifies that amplifies a region spanning an exon junction.

Human assay ID	Gene name/Gene aliases	Gene Symbol	Assay Design	Amplicon length
Hs00234387_m1	caspase 3, apoptosis-related cysteine peptidase	CASP3	probe spans exons	100
Hs00154261-m1	caspase 9, apoptosis-related cysteine peptidase	CASP9	probe spans exons	65
Hs00169152-m1	caspase 7, apoptosis-related cysteine peptidase	CASP7	probe spans exons	76
Hs99999903-m1	B-actin	ACTB	amplicon spans exons and probe does not span exons	171

2.6.7 qRT-PCR data analysis

Gene expression in different samples was quantified using the comparative C_T method ($\Delta\Delta C_T$) as described by Livak and Schmittgen (Livak and Schmittgen, 2001) and according to Applied Biosystem guides (part number. 4376785). There are two different methods of analysing data from real-time, quantitative PCR experiments: absolute quantification and relative quantification. The absolute quantification determines the transcript copy number usually comparing to a standard curve while the relative quantification related the PCR signal (or fold change in gene expression level) of a target gene in a one group to the positive control after normalising to an endogenous reference gene. We followed the relative quantification method based on C_T values. Briefly, threshold cycle (C_T) is the fractional cycle at which the amount of PCR amplified gene reaches a fixed threshold in exponential phase, C_T is determined from a log-linear curve where PCR signal is plotted against the cycle number, and was obtained from the PCR machine. The C_T values of each sample were then imported into Microsoft Excel at end of PCR reaction.

The amount of transcript gene detected at a certain cycle number is directly related to the initial amount of target in the sample. In order to determine the amount of specific transcripts present in cDNA, firstly, C_T values for each sample should be normalised to obtain ΔC_T. For each group; subtracting the mean C_T value of endogenous reference gene β-actin from the corresponding mean C_T value of specific gene (SG) (ΔC_T = C_T SG - C_T β-actin). Then concentration of a specific gene in the samples relative to the positive control was normalised by subtraction to obtain $\Delta\Delta C_T$ values ($\Delta\Delta C_T$ = ΔC_T sample - ΔC_T of positive control). Finally, the relative expression which is often named as RQ value was determined by raising 2 to the power of the negative value of $\Delta\Delta C_T$ ($2^{-\Delta\Delta C_T}$) for each sample (Amount of target = $^{-\Delta\Delta}C_T^2$).

2.6.8 Evaluation of TaqMan gene expression assay product by performing gel electrophoresis

This step was done in order to ensure that TaqMan gene expression assays were working properly. Amplified PCR products were then run on a 2% agarose gel to detect the right Amplicon length for the specific gene. For example, HSP70 gene expression Amplicon length was 124 base pairs; the band was exactly at 125 base pair confirming that it was the specific gene (Figure 2-8).

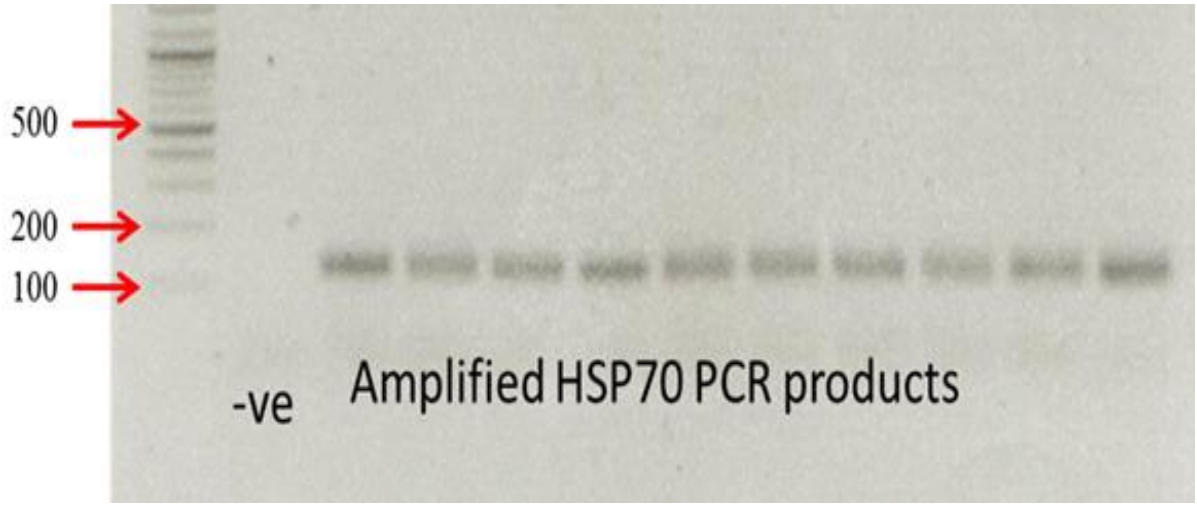


Figure 2-8: 2% gel electrophoresis of PCR products of HSP70 TaqMan gene expression assay, band corresponded to amplicon length of the TaqMan 124 base pairs. Negative control was loaded in first lane showed no expression.

2.7 Mammalian tissue culture

All cell culture procedures were carried out in in a Biological Safety Unit (BSU) Class II hoods using standard aseptic techniques and sterile instruments. The tissue culture flasks, dishes and pipettes were obtained from Corning (UK). Culture reagents were purchased from Sigma-Aldrich.

2.7.1 Hypoxia-normoxia (model of IR injury) tissue culture

This experiment was undertaken to investigate the effect of exposing placenta tissue to 2% O_2 (hypoxia) for one hour followed by exposing the same placental samples to 8% O_2 (normoxia) (normal O_2 concentration in placenta) for four time points (2, 6, 12 and 22 h). Control samples were exposed to 8% O_2 for the entire duration. This tissue experiment was done to recreate the type of ischemic reperfusion of placenta that occurs in labour and PE and to examine this effect on expression of selected genes. During labour, due to uterine muscle contractions the blood vessels contract and relax giving an IR kind of injury. In PE, uterine spiral artery remodelling is incomplete and blood flow may occur in pulsatile manner which is also an IR kind of injury. The aim was to determine whether pilot data from this group of experiments revealed any data suggesting that more experiments were needed. It was known at the start of this group of experiments that there was only enough time to perform pilot experiments.

The experiment was carried out as follows:

2.7.1.1 Experiment preparation

One day before experiment (in the lab): Two NuAir® incubators were set to 2% (hypoxia) and 8% (normoxia) O_2 concentration. Three T5 culture flasks (labelled inner, middle and outer zones) with 10 ml of supplemented media (section 2.3.8) were placed in the 8% O_2 incubator (normal placenta oxygen concentration) for 24 h before tissue collection. These sets of culture flask were transported to the maternity unit on the day of tissue collection with the lids sealed.

Twelve culture Plates (24 well culture plates) containing two ml of supplemented media (section 2.3.8) were placed in the 8% O_2 incubator (normal placenta oxygen concentration) for 24 h prior to tissue collection. These sets of twelve plates remained in the laboratory.

2.7.1.2 In the hospital collection room

Step 1: Three pieces of placenta tissue representing the inner, middle and outer zones ($2cm^3$) were taken and each was cut into four parts. The first piece was used as baseline reference hence it was immediately snap frozen in liquid nitrogen. The other three pieces were collected and then transported to the laboratory in the T5 culture flasks (Figure 2-9 A).

2.7.1.3 On the day of experiment (in the lab):

Step 2: The placental tissue was transported to the Yorkhill Hospital laboratory in three 8% O_2 T5 culture flasks; each flask contained inner, middle or outer zones placenta tissue. Each flask contained three pieces; the first piece was used as a second reference point after sample transportation to the lab in order to investigate transport time effect on stress genes expression. The other two pieces were cut down into four equal sized pieces. Eight culture plates were removed from the 8% O_2 incubator (four plates for the experiment group and four plates for the control group; each plate was used for each time point i.e 2, 6, 12 and 22 h. Four pieces (in the first row) were added to the experimental cultural plates and four pieces (in the first row) were added to the control cultural plates. In each well one piece from each zone was added. The four experimental plates were put in the 2% O_2 incubator and the four control plates were put back into 8% O_2 incubator (Figure 2-9 B).

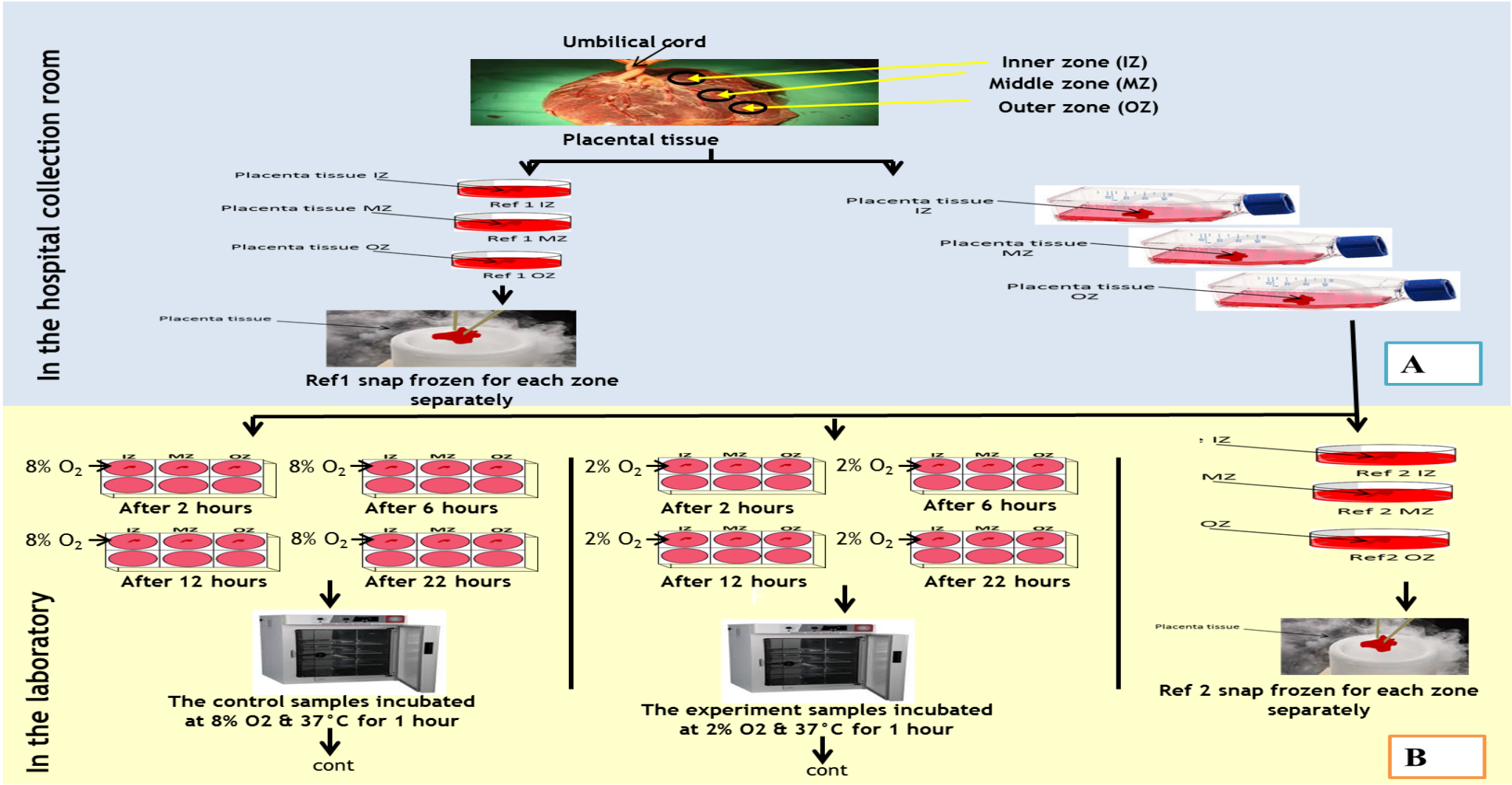
2.7.1.4 The experiment (in the lab):

Step 3: The hypoxia exposure time for all experimental placental pieces (inner, middle and outer) was 1 h at 2% O_2. After that the tissue plates were removed from the incubator, samples were transferred to pre-equilibrated 8% O_2 medium (the other four plates) and transferred back to the 8% O_2 incubator. The recovery time in 8% O_2 for the experiment plates were 2, 6, 12 and 22 h respectively. The control samples were incubated for 1h in 8% O_2 incubator rather than 2% O_2. They then underwent the same second step and were placed back in the 8% O_2 incubator (Figure 2-9 C). Thus the control samples did not get exposed to the low oxygen environment.

2.7.1.5 Experiment recovery time (in the lab):

Step 4: At the end of each time point (2, 6, 12, 22 h) the relevant tissue plate was taken from the 8% O_2 incubator and the three pieces of placenta tissue of the inner, middle and outer zones from both experiment (2% O_2 -8% O_2) and control wells (8%O_2 -8% O_2) were collected, snap frozen in liquid nitrogen and stored at -80 °C. One ml of tissue culture media was taken in each point and stored for cytokine analysis should time allow for this in the future (Figure 2-9 D).

When required tissues were taken from -80 °C storage, RNA and protein was then extracted from the frozen tissue and processed according to the protein or molecular analysis protocol.


In the hospital collection room
Umbilical cord
Inner zone (IZ)
Middle zone (MZ)
Outer zone (OZ)
Placental tissue
Placenta tissue IZ
Placenta tissue MZ
Placenta tissue OZ
Ref 1 IZ
Ref 1 MZ
Ref 1 OZ
Placenta tissue
Ref1 snap frozen for each zone separately
A
8% O2
After 2 hours
After 6 hours
After 12 hours
After 22 hours
The control samples incubated at 8% O2 & 37°C for 1 hour
cont
2% O2
After 2 hours
After 6 hours
After 12 hours
After 22 hours
The experiment samples incubated at 2% O2 & 37°C for 1 hour
cont
Ref 2 IZ
Ref 2 MZ
Ref2 OZ
Placenta tissue
Ref 2 snap frozen for each zone separately
B
In the laboratory

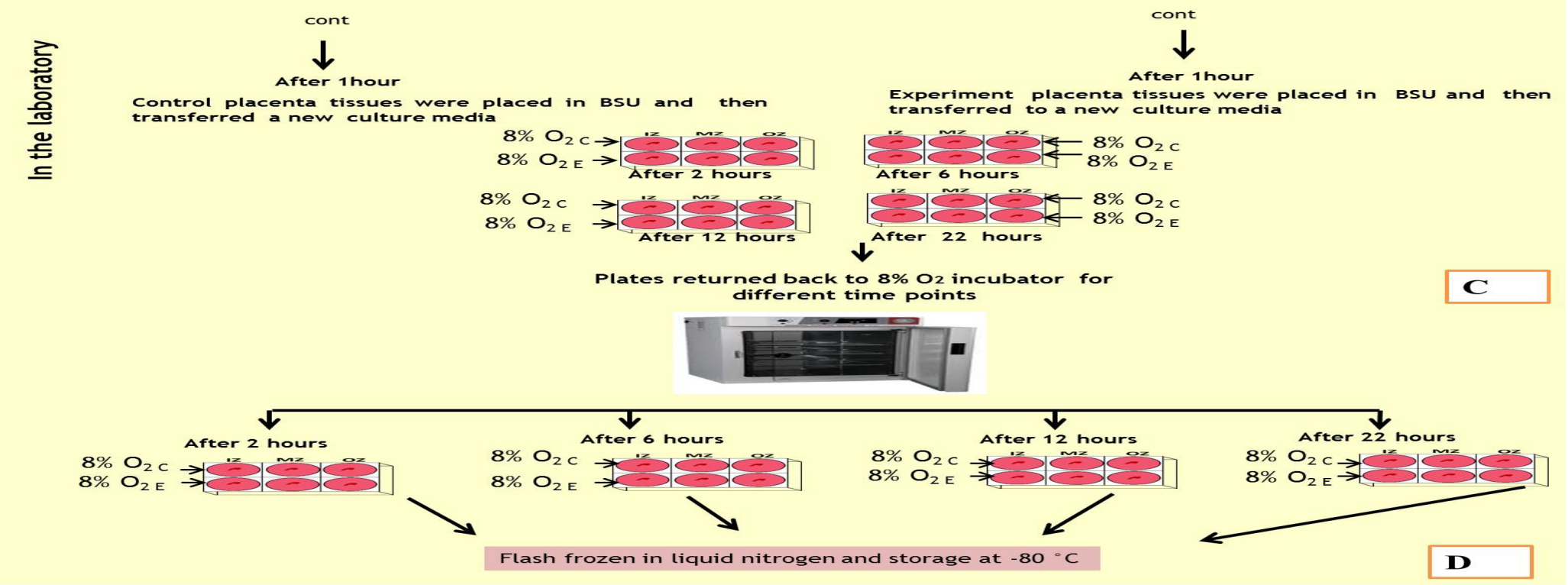


Figure 2-9: Figure showing the time course of hypoxia-reperfusion experiment. (A) Collection of first reference samples from (inner (IZ), middle (MZ) and outer (OZ) placental zones). The rest of placenta tissue samples were collected in three flasks (8% O_2) for each of the three zones. (B) in the lab: the second reference samples were collected from the three zones, the remaining placenta tissue samples were cut up and distributed into control (8% O_2) and experiment (2% O_2), then each were put into relevant incubator. (C) After one hour all plates were taken out the incubators and were placed in the biological safety unit (BSU) and then transferred into a new culture media (8% O_2). All were returned back to the (8% O_2) incubator for different time points. (D) After each time point (2, 6, 12 and 22 h) one plate containing control and experiment tissue for inner, middle and outer zones was taken out of the incubator, the tissue were snap frozen in liquid nitrogen, then stored at -80 °C.

2.7.2 HSP induction experiment

The heat shock protein induction experiment was undertaken to investigate the effect of exposing placenta tissue from three different placental zones to heat shock (43 °C) for 30 min followed by a recovery period compared to the control group which did not receive heat shock and remained at 37 °C throughout. This experiment was undertaken to investigate whether a classical "shock" would differentially induce heat shock proteins in different placental zones. The aim was to determine whether pilot data from this group of experiments revealed any data suggesting that more experiments were warranted in the future. Heat shock is the classical way to induce heat shock proteins therefore a decision was made to try and determine the effects of heat shock exposure on the different placental zones. If there was a response the next step would be determine if different placental zones were differentially sensitive to heat shock and this might give some clues to help understand why the protein expression of HSPs proteins can vary at different zones of the placenta.

The experiment was carried out as follows:

2.7.2.1 Experiment preparation (in the lab):

All media preparation was done on the day of the experiment. An oven incubator (Philip Harris ltd, Shenstone) was adjusted to 43 °C and NuAir® incubators were set to 20% O_2, 90% humidity and 37 °C.

Three T5 culture flasks (labelled inner, middle and outer zones) containing 10 ml of supplemented media (section 2.3.8), ten culture plates (24 well culture plates) containing two ml of supplemented media in each well and 15 bijous containing four ml of supplemented media were placed in the 20% O_2 at 37 °C incubator for four hours before tissue collection. These culture flasks were transported to the maternity unit on the same day for tissue collection with the lids sealed while the plates and bijous remained in the laboratory.

2.7.2.2 In the hospital collection room:

Step 1: Tissue collection: The T5 culture flasks were retrieved from the incubator and transported to the theatre. One piece of tissue was collected for each placenta zone IZ, MZ and OZ. Each piece was cut into three and one section was used as a baseline reference

hence was immediately snap frozen in liquid nitrogen. The two remaining pieces of tissue collected (2X3= 6) were put three T5 culture flasks (Figure 2-10 A).

2.7.2.3 On experiment day (in the lab):

Step 2: The tissue was transported to Yorkhill Hospital. The placenta tissue was removed quickly from the cultural flasks. Each piece from each zone (2) was cut up into five equal sized pieces, five pieces were used as controls and five were experimental (Figure 2-10 B).

2.7.2.4 The experiment (in the lab)

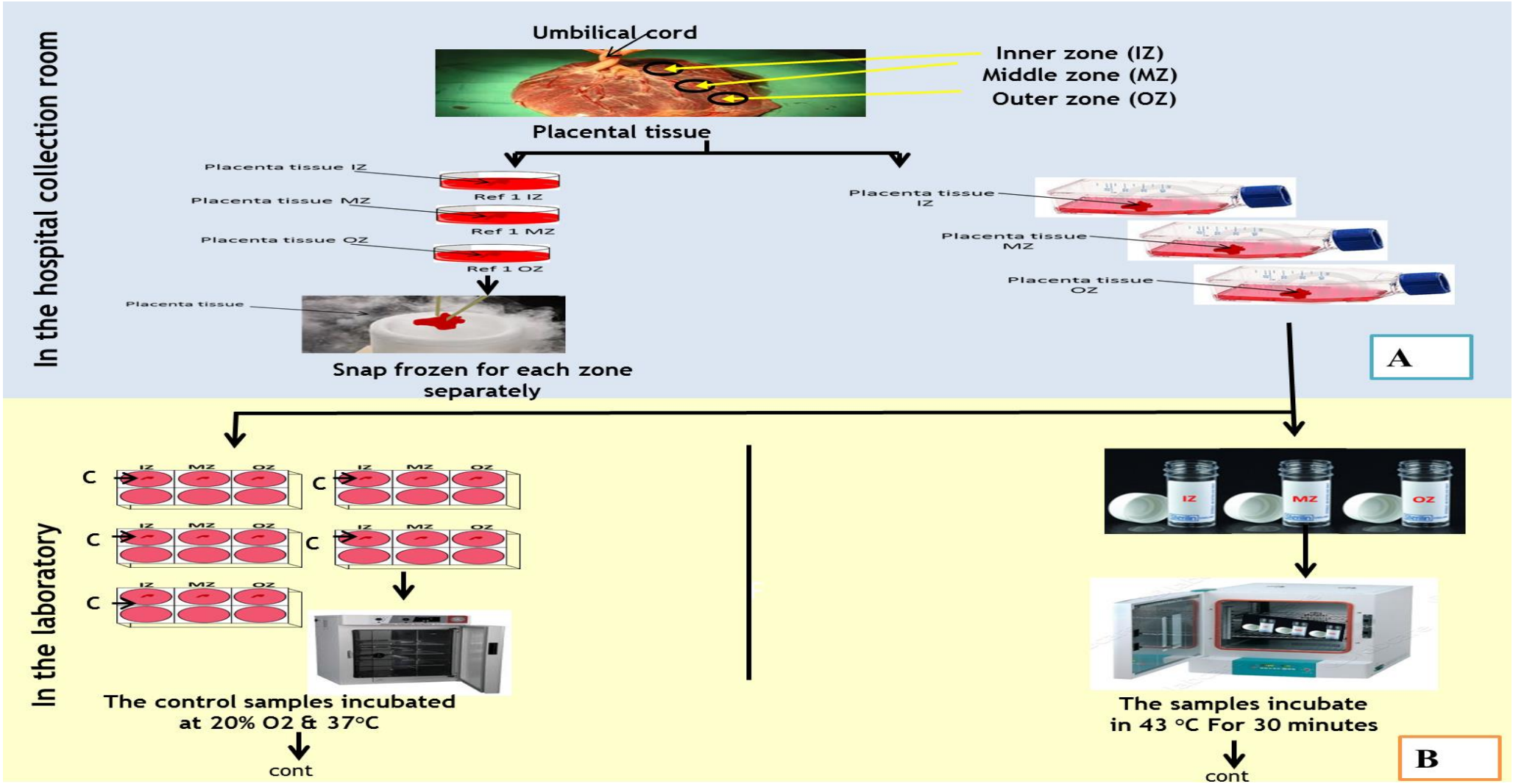
Step 3: Experimental placenta pieces were placed into pre incubated bijous to then be placed in an oven adjusted at 43 °C for 30 min. Control samples were placed into pre incubated plates and were incubated for 30 min at 37 °C.

After heat shock in the oven experimental tissue was placed into pre incubated plates in order to be incubated at 37 °C. Tissues were allowed to recover from that point on for periods of 1, 3, 6, 12 or 24 h (Figure 2-10 C).

2.7.2.5 Experiment recovery period (in the lab)

Step 4: At each time point (1, 3, 6, 12, 24 h) the relevant tissue plate containing pieces of inner, middle and outer zones of both heat shocked and control samples were collected and snap frozen in liquid nitrogen. 1 ml of tissue culture media was taken in each point and stored for cytokine analysis should time allows in the future (Figure 2-10 D).

RNA and protein were then extracted from the frozen tissue and process according to protein or molecular analysis protocol.


In the hospital collection room
Umbilical cord
Inner zone (IZ)
Middle zone (MZ)
Outer zone (OZ)
Placental tissue
Placenta tissue IZ
Ref 1 IZ
Placenta tissue MZ
Ref 1 MZ
Placenta tissue OZ
Ref 1 OZ
Placenta tissue
Snap frozen for each zone separately
Placenta tissue IZ
Placenta tissue MZ
Placenta tissue OZ
A
In the laboratory
C
IZ
MZ
OZ
The control samples incubated at 20% O2 & 37°C
cont
The samples incubate in 43 °C For 30 minutes
cont
B

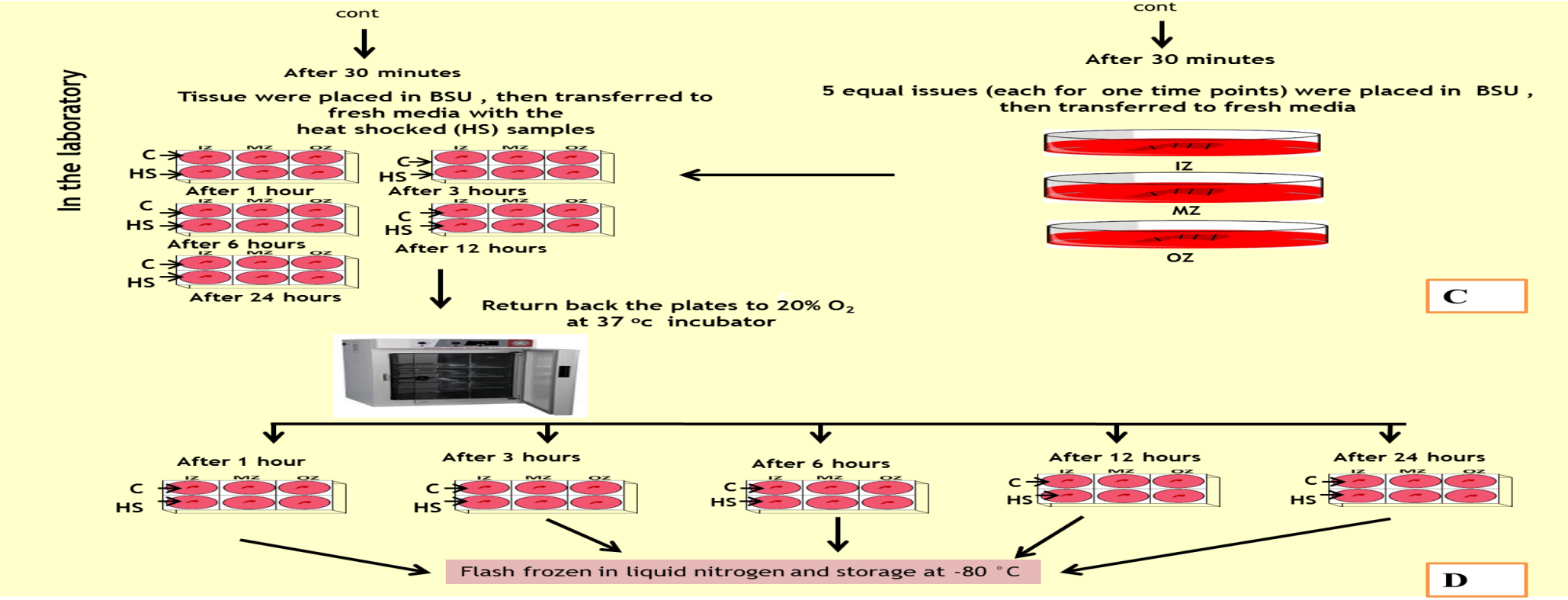


Figure 2-10: Figure showing the time course of heat shock induction experiment. (A) Collection of first reference samples from (inner (IZ), middle (MZ) and outer (OZ) placental zones) and collection of placenta samples in three flasks represent the three zones. (B) Placenta samples were cut down and were distributed into controls or experiment (heat shock). Controls were placed in 37°C incubator and experiments were placed in 43°C oven for 30 min. (C) After 30 min plates were taken out of the incubator and oven, were put in the BSU and then were transferred into a new culture media (37° C). All were placed back to the (37 °C) incubator for different time points. (D) after each time point (1, 3, 6, 12 and 24 h) one plate contain control and experiment tissue for IZ, MZ and OZ was taken out of the incubator and snap frozen in liquid nitrogen, then stored at –80 °C.

2.8 Statistical analysis:

Statistical analysis was performed using GraphPad prism 5. Results are expressed as median unless otherwise stated. Repeated measures one way ANOVA (Friedman test) followed by Dunn's post hoc test were used to compare the difference in the three groups of parametric data for the samples from same placenta. A Kruskal-Wallis test was used to compare difference within three or four groups of non-parametric data for the samples from different placenta. Comparison between the two groups was performed using Mann Whitney test (two tailed) and using whiskers: min to max. Statistical significance was considered with p values of <0.05. Graphs show box and whiskers plots. The statistical analysis of hypoxia-reperfusion and heat shock experiments was performed using Minitab17 software. General linear model two-way ANOVA was using with Turkey pair-wise comparison post hoc test. Two factors (hypoxia and time) or (heat shock and time) were measured. Graphs show interaction plots were demonstrated.

2.9 Troubleshooting

2.9.1 Optimization of Santa Cruz and Abcam TLR2 and TLR4 antibodies

I started looking at TLR2 and TLR4 protein expression using commercially available antibodies used in published papers. However, it soon become clear that these antibodies either do not work or produce many non-specific bands, I have detailed the procedure followed in troubleshooting. In the end we were refunded by the companies for the cost of the antibodies. Summary of several experiments that were carried out is shown below

2.9.2 Different protocols

Different western blotting protocols were attempted but the correct molecular weight bands were not detected. These protcols include using different reagents (TSBA, PBS and TSHB), using milk as blocker and overnight incubation of primary antibody. Primary antibodies that had been used are shown in (Table 2-11).

2.9.3 Western blotting of membrane protein

Membrane proteins were separated and run according to the Western blot protocol but also multiple non-specific bands were detected.

Table 2-11: Different primary and secondary antibodies were run for TLR2 and TLR4

Antibody	Company/ catalogue number	Secondary antibody	Observation	Action
TLR4	Santa Cruz (C-18): Sc-8694. 1:200	donkey anti-goat IgG-HRP: Sc-2033, 1:100	multiple bands	secondary was diluted and different blocker protocols were tried
TLR4	Abcam mouse monoclonal ab22048. 1:200	gout anti-mouse IgG-HRP ab6789 1:2000	no band	positive control run
TLR2	Santa Cruz (C-19): Sc-8690. 1:200	donkey anti-goat IgG-HRP: Sc-2033 1:100	no bands	different primary antibody concentrations were tried
TLR4	Abcam Rabbit polyclonal, ab47839. 1:500	donkey anti-Rabbit IgG-HRP ab7083. 1:3000	no bands	positive control was run

2.9.4 Running the positive control using Santa Cruz TLR4 antibody

Positive controls were purchased from Santa Cruz; the company literature stated that they are detected by the TLR4 antibody. It became clear that these positive controls do not

detect the antibody. These are two positive controls that I tried from Santa Cruz. 1) Positive control caco-2 cell lysate: Sc-2262**.** 2) Positive control Hl-60 whole cell lysate: Sc-2209. Next a different control supplied by a collaborator was used. No bands were detected by both protocols.

2.9.5 Blotting using only secondary antibodies

Several Western blots were run in standard way but without primary antibodies in order to detect if there is any nonspecific bands. Multiple non-specific bands were detected.

Chapter III: HSP70, HSF1 and HSF2 expression in normal pregnancies and pregnancies complicated with PE and FGR (HSP70 only).

3 Introduction

3.1 Heat shock proteins

Heat-shock proteins (HSPs) are a family of protein that are expressed by all cells and organisms. They have many important physiological functions as well as helping cells to cope with stressful situations. Some HSPs are expressed constitutively while others are induced by a range of damaging insults including heat shock, ischemia, hypoxia, oxidative stress and physical injury (Lanneau et al., 2010). HSPs perform chaperone function by stabilizing new protein to ensure correct folding. HSPs are required for proper folding and activity of protein in the cell (Saibil, 2013). HSPs are classified according to their molecular weight into two groups: the small HSPs family and the high molecular weight HSPs. The small HSPs are ATP independent and their functions controlled by their phosphorylation status. The high molecular weights HSPs include HSP60, HSP70, HSP90 and HSP110 families. They are ATP dependent chaperones. HSPs have become an area of intensive research due to their remarkable properties, their role in disease and the potential to modulate their expression for therapeutic benefit.

3.1.1 Discovery

In 1962, it was observed that exposing salivary gland of the fruit fly (Drosophila busckii) to heat induced a characteristic pattern of puffing on the polytene chromosomes (Ritossa, 1962). This indicated heat shock induced gene expression and rapidly induced the synthesis of a new set of proteins, which eventually became known as HSPs (Tissiéres et al., 1974). HSPs are highly conserved and are found in virtually all living organisms, from prokaryotes to eukaryotes (Feder and Hofmann, 1999). HSPs are expressed in distinct compartments of the cell including cytoplasm, nucleus, mitochondria and ER. They are associated with various structural proteins such as actin and collagens (de Jong et al., 2009).

3.1.2 Functions

3.1.2.1 Intra-cellular HSPs

HSPs are chaperones being important in proper folding of nascent proteins, refolding, assembly and degradation of proteins, in fact, not all, but most of the HSP function is as chaperones (Horváth et al., 2008). Their expressions allow cells to survive sub-lethal conditions by binding to partially denatured proteins, dissociating protein aggregates and regulating correct folding (Jolly and Morimoto, 2000, Young et al., 2004, Sarto et al., 2000, de Jong et al., 2009, Horváth et al., 2008). HSPs have been shown to be involved in cell differentiation, tissue development, apoptosis and hormonal stimulation (Sarto et al., 2000). The ER is a major site for production of proteins and controls this process: if it overburdened by misfolded proteins this will triggers cell death by apoptosis (Young et al., 2004). All HSP families contain of members expressed either constitutively or regulated inductively and are targeted to several cellular compartments (Schmitt et al., 2007). For example, in contrast to the HSP90, which is expressed constitutively, HSP27 and HSP70 are highly inducible proteins (Schmitt et al., 2007). HSPs also have an important role under normal physiological conditions with the constitutively expressed protein making up to 5-10% of total cellular protein. Under stressful conditions the concentration can be increased two to three fold (Pockley, 2003). HSPs function under non-stress conditions by carrying denatured proteins to the proteasome and helping newly synthesised protein fold properly. When cells exposed to stress, the expression of HSPs are increased and became upmost important to the cells to ensure protein confirmation in the correct form. In addition to their role as molecular chaperones they also influence transport, aggregation, and folding of other proteins. HSPs play a critical role in modulating the execution of the apoptotic signalling pathways to maintain cell survival and to ensure that stress induced damage does not trigger cell death inappropriately (Schmitt et al., 2007, Latchman, 2001). They are two apoptotic pathways, intrinsic and extrinsic pathways, HSPs play an important role in regulation of these pathways. The intrinsic pathways (mitochondria) can be induced by HSP10 and HSP60 (Samali et al., 1999) and inhibited by HSP70, HSP27 and HSP90 (Bruey et al., 2000a, Beere et al., 2000, Pandey et al., 2000). The extrinsic pathways (receptor mediated) are inhibited by HSP27, HSP90 and HSP70 (Charette et al., 2000, Sato et al., 2000, Garrido et al., 2003). (Table 3-1) shows major HSPs families and their intra-cellular location and roles.

Table 3-1: functions and intra-cellular location of major HSPs Adapted from (Pockley, 2003, Calderwood et al., 2007a, Noble et al., 2008, Grubišic, 2005)

HSP families names	Intracellular location	Functions
Small HSPs α,β crystalline	Cytoplasm	Cytoskeletal stabilisation
HSP25/HSP27	Cytoplasm and nucleus	Molecular chaperone and anti-apoptotic
HSP32	Cytoplasm	Anti-oxidant (heam catabolism)
HSP40	Cytoplasm and nucleus	Co-factor of HSP70
HSP47	ER	Processing of pro-collagen
HSP60 (chaperonins)	Cytoplasm and mitochondria	Assist correct folding, assembly of multimeric complexes and anti-inflammatory
HSP70		
Inducible:HSP70/HSP72	Cytoplasm and nucleus	Molecular chaperone
Constitutive: HSC70/HSP75	Cytoplasm and nucleus	
GRP78	mitochondria	Mitochondria chaperone
HSP90	Cytoplasm and nucleus	ATPase activity, HSP70 down regulates HSF1 activity Molecular chaperone In non-stress cellular condition HSP90 maintenance of the HSF1 monomeric state
HSP110	Cytoplasm and nucleus	Molecular chaperone Thermal tolerance

3.1.2.2 Extra-cellular HSPS

HSPs were initially believed to be solely intracellular, where they have their cytoprotective functions (molecular chaperones). However, it is now well established that HSPs can be also be present extracellularly where they display a different function (Mambula et al., 2007, De Maio and Vazquez, 2013, Binder, 2014). HSPs outside the cell act as cellular communicators in response to stress by alerting other cells especially immune system cells to avoid the propagation of the insult (De Maio, 2014, De Maio, 2011, Binder, 2014, De Maio and Vazquez, 2013). It has been reported (Gastpar et al., 2004) that HSP70 was expressed in human tumour plasma membranes. HSP70 expression has also been shown in several tissues such as vascular smooth muscle, blood mononuclear cells and neuronal cells (Robinson et al., 2005, Liao et al., 2000, Hunter-Lavin et al., 2004). Recombinant HSP70 can modulate the immune system by activation of macrophages; this activation can measured by increases in calcium levels and cytokine release (Asea et al., 2000). HSP70 bound with high affinity to the plasma membrane, elicited a rapid intracellular calcium flux, activated nuclear factor (NF)-kappaB and upregulated the expression of pro-inflammatory cytokines tumor necrosis factor (TNF)-alpha, interleukin (IL)-1beta and IL-6 in human monocytes. In addition, several studies have demonstrated that HSP70 is capable of activating cells of the immune system (Vega et al., 2008, Henderson and Pockley, 2010). HSP 60 and HSP70 have been detected in blood serum samples (Pockley et al., 2008). HSP60 was detected in the plasma membrane of mitochondria and cardiac myocytes (Gupta and Knowlton, 2002, Lin et al., 2007). Furthermore, HSP60 has been found in plasma in some disease conditions (Pockley et al., 2000). GRP78 (HSPA5), which is ER resident, has also been detected in the extracellular compartment human peripheral circulation (Delpino and Castelli, 2002). In addition, a homologous form of cytosolic HSP90 (GRP94) was also detected outside the baby hamster kidney cells secreted into a serum-free medium (Evdokimovskaya et al., 2012). The small heat shock protein families (sHSPs) are also secreted by cells and modulate the immune system (van Noort et al., 2012). HSP27 has been detected in blood during pathological diseases such as chronic pancreatitis and pancreatic carcinoma (Liao et al., 2009).

The mechanism of export of HSPs to the extracellular compartment has been also subject of controversy among researchers (De Maio, 2014, Henderson and Pockley, 2012, De Maio, 2011). Different mechanisms have been proposed including translocation across the plasma membrane, endolysosomal pathways and the passive release after cell death by

necrosis (De Maio, 2014, Mambula and Calderwood, 2006b, Vega et al., 2008, Mambula and Calderwood, 2006a).

HSP70 secretion does not follow a leader classical signal pathways (Calderwood et al., 2007a). It was suggested (Nickel and Seedorf, 2008) that HSP70 may be exported via an alternative mechanism, which has been named the non-classical or unconventional secretory pathway but the mechanism for this pathways has not yet emerged. Another way of HSP extra-cellular secretion is via secretory vesicles in a similar to interlukin-1β (leaderless protein) secretion from macrophage (MacKenzie et al., 2001, De Maio, 2011). HSP27, HSC70, HSP70 and HSP90 have been shown to be released extra-celluarly from heat shocked B-cells via exosomes (Clayton et al., 2005). HSP70 is also released via lysosomal endosomes especially from tumour cells that transfer intracellular proteins to the plasma membrane then release them to the extra-cellular space (Mambula and Calderwood, 2006b). In addition, HSP90 has been proposed as being exported via exosomes or by direct translocation across the plasma membrane (McCready et al., 2010, Li et al., 2012b)

3.1.3 Synthesis of HSPs

HSP production is a key part of the heat shock response. HSPs expression can be induced by several insults other than heat shock; these insults include steroid hormones, hyperthermia, ROS, hypoxia, ischemia, bacterial infection and inflammation (Patel et al., 1995, Horváth et al., 2008, Kukreja et al., 1994, Norton and Latchman, 1989, Richard et al., 1996, SLAKEY et al., 1993, Zheng et al., 2004). Heat shock transcription factor 1 (HSF1) is the stress responsive transcriptional activator responsible for the inducible transcription of genes encoding HSPs. It binds to regulatory heat shock elements present in the promoter region of all heat shock genes (Yao et al., 2006). In non-stress conditions HSF1 is present as a monomer in the cytosol. After stress, damaged protein induced phosphorylation of HSF1 and then HSF1 is translocated into the nuclear compartment. In the nucleus, HSF1 forms homotrimers and bind to HSP gene promoter regions, leading to induction of HSP gene transcription. Upon cell recovery, HSF is dephosphorylated, however, little is known about the fate of HSF1 after its dissociation from DNA and many controversies in this area. It has been suggested (Yang et al., 2008) that HSF1 may be a substrate for chaperone mediated autophagy. In addition, the fate of the disulphide linked HSF1 monomers is also not known, if they reduced and allowing the HSF1 to retain to an inactive state in the cytoplasm or just degraded after its dissociation from DNA (Neef et al., 2011).

3.1.4 HSPs and the immune system

The extracellular HSPs functions have been investigated intensively for example their interaction with the immune system (Calderwood et al., 2007b, Srivastava, 2000, Calderwood et al., 2005, Binder, 2014). HSPs plays important roles include pro-inflammation and lead to cytokine release (Asea et al., 2002), anti-inflammation (Van Eden et al., 2005, Hauet-Broere et al., 2006) and antigen presentation (Noessner et al., 2002). Extra-cellular HSPs which function as anti-inflammatory agent include HSP20 (Wang et al., 2009), HSP27 (Calderwood et al., 2007a) and HSP32 (Yeh et al., 2009). Other immune functions of extra-cellular HSPs include HSP60 as pro-inflammation (Lin et al., 2007) and HSP70 as pro-inflammation and antigen presentation (Lin et al., 2007, Gastpar et al., 2004, Liao et al., 2000, Noessner et al., 2002). HSP90 functions as pro-immune (Calderwood et al., 2007a). Secreted HSPs, including HSP70, can take part in immune surveillance. They can capture antigens and interact with receptors on antigen presenting cells. HSP70 complexes are able to deliver antigens to major histocompatibility complex (MHC) class I and II molecules on the antigen presenting cell (APC) cell surface and lead to the presentation of tumor antigens to T lymphocytes. HSP70 also has anti-inflammatory properties. For example it can bind to, and activate, human monocytes, inhibiting the secretion of inflammatory cytokines, such as TNF-α, IL-1β, and IL-6 and increasing secretion of the anti-inflammatory IL-10 (Asea et al., 2000).

3.1.5 Families of heat shock protein

HSPs are classified into six major families: HSP110, HSP90, HSP70, HSP60, HSP40, HSP32 (heme oxygenase-1), small HSPs (sHSPs) where the monomer size typically ranges between 12- to 30-kDa (e.g HSP20, HSP27 and αβ-crystalline) and ubiquitin (Latchman, 2001, Pockley, 2003, Kappe et al., 2003). The sHSP subfamily consists of 10 members (HSPB1-HSPB10). It was proposed by one group (Taylor and Benjamin, 2005) to classify the sHSPs into major categories (class I and II) according to their subcellular location, gene expression patterns and transcriptional regulation. Different HSPs families will be discussed in more detail in the following chapters. HSP70 will be discussed in this chapter.

3.2 Heat shock protein 70 (HSP70)

HSP70 is the most abundant chaperone and exist in different cellular compartments. The HSP70 family comprises at least 13 members, which are different from each other by amino acid sequence, expression level and cellular location, this family also contains 50 J proteins (DNAJ or HSP40 proteins) (Kampinga et al., 2009, Tavaria et al., 1996). The diversity of the HSP40 co-chaperones allow recruitment of different members of the HSP70 family to multiple cellular location and activities other than chaperone roles (Kampinga and Craig, 2010). HSP70 is the most conserved protein in evolution (Lindquist and Craig, 1988, Daugaard et al., 2007). Immunoglobulin heavy-chain binding protein (BiP) is the ER orthologue of the HSP70 family. BIP is intricately involved in most functions of this organelle through its interactions with a variety of substrates and regulatory proteins (Behnke et al., 2015).

3.2.1 HSP70 structure

The HSP70 family function depends on their ability to bind and release target proteins coupled with conformational changes in HSP70, driven by hydrolysis and release of ATP. HSP70 has two major functional domains. The N-terminal ATPase domain (NBD) binds ATP and hydrolyses it to ADP lead to conformational changes in other domains (Mayer and Bukau, 2005). The second domain is the substrate binding domain (SBD) that can be further divided into substrate binding subdomain and C-terminal subdomain. The substrate binding subdomain contains a groove that recognizes exposed hydrophobic segments of target protein. The C-terminal subdomain is rich in alpha helical structure and acts as a 'lid' for the substrate binding groove (Lund, 2001). These domains are connected by an inter-domain linker (Figure 3-1). SBD and NBD interact when HSP70 is ADP bound (Swain et al., 2007). When HSP70 is ATP bound, the lid is open with low affinity for peptide and rapid kinetics of binding and release. When HSP70 is ADP the lid is closed and affinity for peptides is increased while kinetics of binding and release is reduced. When peptides bind the SBD it stimulates the ATPase activity helping to promote target protein loading of HSP70. The HSP70 intrinsic ATPase activity is low and for his chaperone activity HSP70 needs a co-chaperone HSP40 (Henderson and Pockley, 2012). HSP70 activity depends on the interaction between these domains and their interaction with the co-chaperone for example HSP40 and nucleotide exchange factors (Zuiderweg et al., 2013). The binding of an extended segment of a polypeptide chain to HSP70 can prevent mis-folding, aggregation and help to maintain the unfolded state for translocation

to another cellular compartment. HSP40 has a diverse function and targets specific sites of HSP70. HSP40 is thought to act as a primary substrate for HSP70 that stimulates the HSP70 ATPase (Kampinga and Craig, 2010, Saibil, 2013).

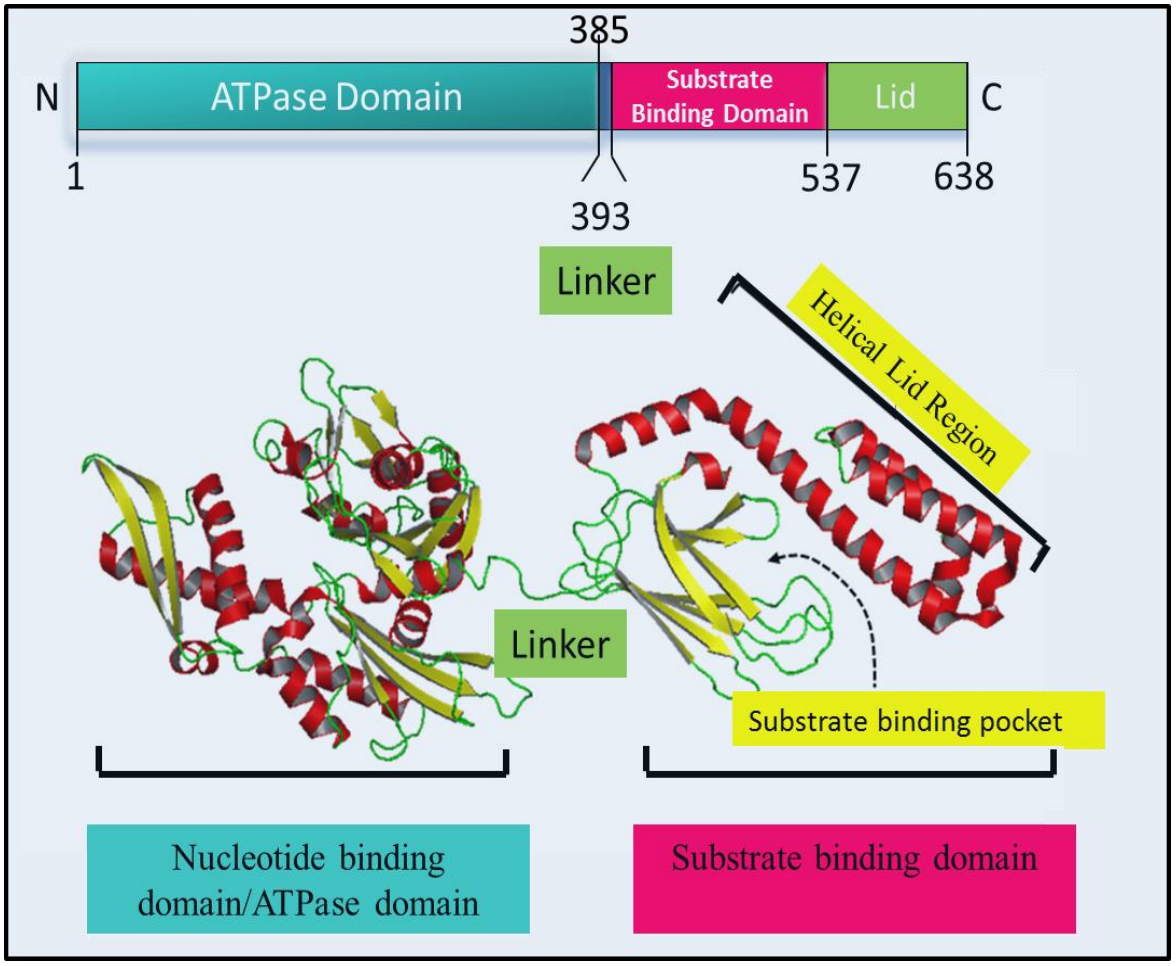


Figure 3-1: HSP70 gene structure and domains: HSP70 contain two domains; nucleotide binding domain/ATPase domain and substrate binding domain modified from (Grubišic, 2005).

3.2.2 HSP70 family members and chaperone function

The HSP70 family contain several slightly different HSP70 proteins, they all share a common domain structure but with different function and cellular locations. HSC70 (HSPA8/HSP73) is a constitutively expressed chaperone protein. HSP70 (HSPA1A) is a stress inducible one. HSP70 is one of the best studied HSPs (Nollen and Morimoto, 2002, Clerico et al., 2015). Both forms are localised in the cytoplasm and the nucleus (Brown et al., 1993, Noble et al., 2008). The HSP70 family also include HSP78 (ER) and HSP75 (mitochondria), both function as chaperones (Noble et al., 2008). HSP70 has been localised in the extra-cellular space (Schmitt et al., 2007).

3.2.3 HSP70 chaperone function

In non-stress conditions HSP70 and HSC70 act as molecular chaperones and their functions include assisting protein folding and refolding, degradation of unstable protein and misfolded proteins and transport of proteins between different cellular compartments. HSP70 is induced under stress conditions to maintain cellular stability and protect cells against damage (Schmitt et al., 2007, Clerico et al., 2015). HSP70 also binds to all newly forming polypeptides on ribosomes in cells to keep them from folding inappropriately while they are being synthesized. HSP70 protein with ATP, HSP40 and HSP90 binds the misfolded proteins. However, If refolding does not occur, then Bag-1 and E3-ubiquitin ligase CHIP binds to HSP70, then this complex targets the misfolded protein to the proteasome where it is ubiquinated and degraded (Lanneau et al., 2010).

3.2.4 HSP70 and anti-apoptotic function

HSP70 can block apoptosis regulator BAX translocation and prevent mitochondrial outer membrane permeability. HSP70 can bind the apoptotic protease activating factor 1 (Apaf-1) and prevent formation of the apoptosome (Apaf-1, cytochrome C, CASP 9) and activation of CASP3 (Joly et al., 2009). HSP70 can also stabilize the induced myeloid leukemia cell differentiation protein (Mcl-1) which prevents BAX activation and mitochondrial mediated CASP3 mediated apoptosis (Sharp et al., 2013). Co-chaperone HSP40 with HSP70 can inhibit several apoptotic signalling molecules include tumour necrosis factor (TNF) induced apoptosis, assembly of the death inducing signalling complex (DISC), inhibiting transmembrane protein-Fas and TNF-related apoptosis-inducing ligand (TRAIL) (Joly et al., 2009, Sharp et al., 2013). HSP70 can block c-Jun N-terminal kinases (JNK) induced release of Bcl-2-associated death promoter (BAD) from mitochondria leading to block of CASP8 activation, and binding apoptosis signal-regulating kinase 1 (ASK1) and block mitogen-activated protein (MAP) kinase induced apoptosis (Joly et al., 2009).

3.2.5 HSP70 and inflammatory pathways

HSP70 has a profound effect on immune pathways and generally shown to have anti-inflammatory effects by reducing the activation of the pro-inflammatory gene transcription include NF-κB in myocyte cells (Shimizu et al., 2002). HSP70 form a complex with NF-κB that may be cell and stimulus specific. HSP70/ NF-κB interaction can decrease levels of TNF, IL-1, MMP9 and other pro-inflammatory mediators that are regulated by NF-κB

(Sharp et al., 2013). HSP70 decrease MMP processing from inactive to active forms (Sharp et al., 2013). In addition, HSP70 decreases MMP9 activity through NF-κB. HSP70 can also down regulate MMP2 possibly through interactions of HSP70 with signal transducers and activators of transcription (STAT1) (Kim et al., 2012).

HSP70 can affect both the innate and adaptive immune systems, and generally appear to be pro-inflammatory. HSP70 can stimulate Toll like receptor 2 (TLR2), TLR4 and cluster of differentiation 14 (CD14) pathways on monocytes-macrophages and dendritic cells and activate intracellular NF-κB, interferon regulatory factor (IRF) and STAT3 signalling (Joly et al., 2009). HSP70-peptide complexes can activate CD8+ and CD4+ T cell responses. (Joly et al., 2009, Sharp et al., 2013). HSP70 protein appears to be released into blood after acute disorders like myocardial infarction (Giffard et al., 2008).

3.2.6 HSP70 and cardio protection

HSP70 is expressed in myocardial tissue under assault and provides cardiac protection against myocardial injuries and stress, including IR injury (Latchman, 2001, Lepore et al., 2001, Liu et al., 2012). A study (Liu et al., 2007) was performed using liposome-mediated gene transfer of HSP70 into rat myometrium showed that overexpression of HSP70 provided protection against IR injury. In addition, up-regulation of HSP70 plays a role in myocardial adaptation to acute ischemia stress (Kingma, 1999). Heat shock induced expression of HSC70 was shown to restrict the degree of myocardial damage caused by ischemia in rat heart myogenic cells (Mestril et al., 1994).

HSP70 is important in regulating the myocardial innate immune response and cardiac function after IR injury (Liu et al., 2012). HSP70 promotes cardiomyocyte survival by the conservation of antioxidants and inhibition of pro-apoptotic pathways including caspase and Fas mediated death cascades (Suzuki et al., 2002, Zhao et al., 2007, de Jong et al., 2009). HSP70 induces pro-inflammatory cytokines via activation of TLR4 in macrophages and myocardium (Liu et al., 2012). In addition, overexpression of HSP70 in myocardium tissue improved post-ischemic cardiac contractility, which inversely correlated with infarct size (Plumier et al., 1995, de Jong et al., 2009). In a rabbit model of IR injury intra-myocardial injection of recombinant adenovirus–human–HSP70 constructs resulted in a twofold reduction in infarct size (Okubo et al., 2001). Increased levels of HSP70 expression was associated with improvement of ventricular function after cardiopulmonary bypass-associated myocardial IR injury (Yan et al., 2013).

Besides its function as a molecular chaperone in cardiac tissues, HSP70 is also involved in inflammatory responses after cardiac surgery (de Jong et al., 2009). High levels of pre-operative myocardial HSP70 showed reduced levels of biochemical markers of myocardial injury postoperatively, whereas patients with low levels of myocardial HSP70 pre-operative showed an almost twofold increase of biomarkers of myocardial injury after surgery (Demidov et al., 1999, Giannessi et al., 2003).

HSP70 induces cardiac activity tolerance to endotoxin by reducing TNF-α response to endotoxin in macrophages and also inhibiting NF-κB activity, TNF- α and ICAM-1 levels in cardiac tissues (Ao et al., 2009, Su et al., 2010b, Liu et al., 2012)

The level of HSP70 is significantly decreased in diabetic myocardium because of insulin deficiency (Liu et al., 2012). HSP70 is commonly found in atherosclerotic plaques in atherogenesis and it appears to be a protective factor against cellular stress (Dupont et al., 2008). HSP70 expression was decreased significantly in the hypertrophied myocardium from peripheral vascular disease (Kawada and Ishii, 2009).

3.2.7 HSP70 in cancer

HSP70 is expressed in malignant tumours of different origins (Mosser and Morimoto, 2004) and its expression correlates with lymph node metastases, increased cell proliferation and therapeutic drug resistance in breast cancer (Ciocca et al., 1993, Vargas-Roig et al., 1998). HSP70 and HSP90 are very interesting HSPs from drug discovery perspective as both have been shown to be highly expressed in cancer (Santarosa et al., 1997, Nanbu et al., 1997, Trieb et al., 1998, Ricaniadis et al., 2001).

3.2.8 HSP70 gene regulation

3.2.8.1 Heat shock elements (HSEs)

Genomic studies on the HSP70 promoter showed that heat shock induce a rapid binding of HSFs to a region of the 5 nGGAn sequence named HSEs. Regulation of HSPs genes is mediated by the interaction of HSF with HSEs in the heat shock promoter regions (Voellmy, 1993).

3.2.8.2 Heat shock transcription factors (HSFs)

Heat shock factors are a family of 4 transcription factors (HSF1-4) (Åkerfelt et al., 2010, Nakai et al., 1997). HSF1 is the stress responsive transcriptional activator responsible for the inducible transcription of genes encoding HSPs. It binds to regulatory heat shock elements present in the promoter region of all heat shock genes (Yao et al., 2006, Sarge et al., 1993). HSF2 has the characteristics of a temperature sensitive protein and it is inactivated when exposed to heat. HSF2 is more selective in function and also induced during differentiation and early development (Pirkkala et al., 2001). It has been shown that HSF2 associated with the expression of chaperone protein-encoding genes via heteromultimerization with HSF1 (Sandqvist et al., 2009). In addition, HSF2 in concert with HSF1, promotes the expression of a αB-crystallin (Shinkawa et al., 2011).

HSF1 also regulates the expression of genes involved in other cell survival aspects such as protein degradation, signal transduction, energy generation, carbohydrate metabolism and cytoskeleton formation (Trinklein et al., 2004, Gonsalves et al., 2011). HSF1 promotes survival and proliferation of highly malignant cells (Mendillo et al., 2012, Santagata et al., 2013, Vihervaara and Sistonen, 2014).

HSF1 is usually present in the cytoplasm as a latent monomer that has no ability for DNA binding. In non-stress conditions HSF1 is phosphorylated on ser303 and ser307 residues and its activity is repressed through the interaction of the chaperone proteins HSP70, HSP90 and HSP40 (Shi et al., 1998, Zou et al., 1998, Guo et al., 2001). Upon stress, HSF1 is phosphorylated from a monomer (inactive) to a trimer (activated) translocate from the cytosol to the nucleus in order to bind DNA (Sarge et al., 1993, Pockley, 2003, Neef et al., 2011) (Figure 3-2). Disulphide bonds stabilize trimer formation, HSP trimers bind to HSP gene promoter regions, leading to induction of HSP gene transcription. This results in increased transcription of HSP70 and eventually increased translation of more HSP70 protein. HSP70 is needed for the activation of heat shock factor1 in mammalian cells (Ahn et al., 2005). HSF1 is hyper phosphorylated during HSF1-dependent transactivation; this process is repressed through a negative feedback inhibition. Induction of HSPs has to be tightly controlled as inappropriate growth control may lead to cell death. One mechanism that regulates HSP expression is the binding of HSP70 to the HSF1 activation domain (Shi et al., 1998, Neef et al., 2011). The second mechanism is the interaction between heat shock protein binding factor 1 (HSBP1) and HSP70, which lead to inhibition of the capacity of HSF1 to bind to DNA (Satyal et al., 1998). For example, HSP70 gene

transcription is down-regulated by interaction of HSP70 or HSBP1 with the HSF trimers (Pockley, 2003). Upon cell recovery, HSF1 fate is still unclear and two mechanisms have been suggested either HSF1 is dephosphorylated and retained in the cytoplasm or HSF1 is degraded (Neef et al., 2011). HSFs also play other important functions spanning from heat shock response to metabolism, development, lifespan and disease in particular cancer and neurodegenerative disorders.

HSF1 is involved in balancing cellular processes during stress and then enables their rapid re-establishment once suitable conditions for proliferation have been restored. HSF1 also controls target genes in cell stress, development and cancer progression (Mendillo et al., 2012, Le Masson et al., 2011, Vihervaara et al., 2013).

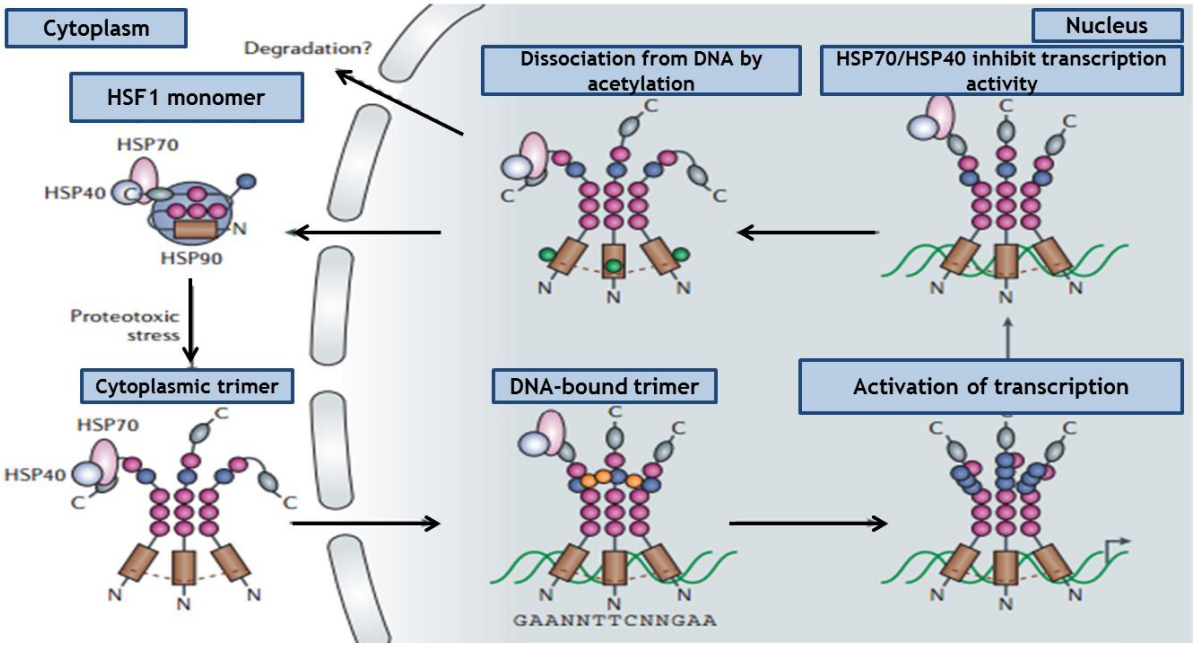


Figure 3-2: HSF1 activation and degradation cycle. In non-stress conditions, HSF1 exists as an inactive monomer in the cytoplasm. Upon stress HSF1 form homotrimers linked together by disulphide bond and become localized to the nucleus to bind to HSE in the promoters of stress-responsive genes. HSF1 becomes dissociated from DNA after HSP70 and HSP40 re-associate with the HSF1 transactivation domain. Adapted with permission from (Neef et al., 2011).

HSF2 is not activated in response to classical stress stimuli, but under developmentally related conditions. Animal studies showed that HSF2 is not able to functionally substitute for HSF1 or to rescue the heat shock response in HSF1 knockout mice (McMillan et al., 1998, Xiao et al., 1999). Human HSF2 and HSF4 are capable of complementing the viability defect and conferring thermotolerance in S. cerevisiae cells carrying a lethal HSF deletion (Liu et al., 1997, Tanabe et al., 1999). However, different members of the HSF

family could also cooperate in order to regulate expression of their target gene, for example, HSF3 has a dominant role in the regulation of the heat shock response and directly influences HSF1 activity (Tanabe et al., 1998).

HSF2 is a non-stress-responsive member of the HSF family. However, HSF2 plays an important role in controlling development and differentiation (Åkerfelt et al., 2010), for example, several studies showed that HSP70 transcription induced by hemin in K562 cells is mediated by HSF2 (Morimoto, 1998, Theodorakis et al., 1989, Schuetz et al., 1991). Furthermore, in K562 cells undergoing hemin-mediated erythroid differentiation, the high level of HSF2 protein levels is preceded by transcriptional induction of the HSF2 gene, accompanied by increased HSF2 mRNA. In contrast HSF2 is down-regulation during TPA-mediated megakaryocytic differentiation (PIRKKALA et al., 1999).

HSF2 binds to the promoters of HSPs and modulates the activity of HSF1 through formation of HSF1-HSF2 heterotrimers (Sandqvist et al., 2009). HSF2 deficiency has been shown to reduce the temperature at which HSF1 is activated (Shinkawa et al., 2011). HSF2 is a short-lived protein with a tissue and developmental stage specific expression pattern (Björk et al., 2010). HSF2 activity is regulated by its levels within the cells (Sandqvist et al., 2009, Björk and Sistonen, 2010). HSF2 expression decrease during mitosis in human cell lines which corresponds to the HSP70 induction and protection against stress induced mitotic abnormalities and apoptosis (Elsing et al., 2014).

3.3 Results

3.3.1 Patient clinical data analysis

3.3.1.1 Normotensive, PE and FGR groups:

NLG normotensive compared to LG normotensive groups. NLG and LG normotensive groups then compared to the NLG and LG PE groups. LG normotensive groups compared to the FGR group (Table 3-2). The comparisons between groups carried out by using one way ANOVA. Significant p values were sub-analysed individually (Table 3-3).

Table 3-2: Patient's clinical details; the descriptive analysis showed the mean and standard deviation. Analyses between the five groups were performed using one way ANOVA, P value < 0.05 was considered statistically significant. Non-labour controls (NLG-C), labour controls (LG-C), non-labour PE (NLG-PE), labour PE (LG-PE) and labour FGR (LG-FGR).

Category	NLG-C (n=6)	LG-C (n=6)	NLG-PE (n=5)	LG-PE (n=7)	LG-FGR (n=8)	p-value
Maternal age (years)	28.33±5.7	26±2.28	32.4±4.03	30.57±6.88	28.88±37	p=0.9
Placenta weight (g)	594.7±110.5	589.5±75	662.2±164.12	591.7±161.4	369.8±71.2	p=0.001
Birth weight (g)	3443±537	3719±347	3552±645.54	2959±567	2035±516.3	p=0.0007
No prier gravida	1	4	1	6	3	
Gestation age at delivery (weeks)	39.3±1.0	40.31±1.4	37.64±1.29	38.55±2.024	36.7±1.3	P=0.001
No. Smokers	2	0	1	1	2	
BMI	28.50±6.01	28.33±4.6	38.56±11.35	25.46±2.93	25.3±7.9	P=0.02

Table 3-3: Patients clinical details; statistical analysis of the groups that were statistically significant in Table 2-2 above. The analysis between two groups was performed using the Mann Whitney test, p<0.05 was statistically significant.

Category	p values group comparison
p value analysis for placenta weight	NLG-C v LG-C (p=0.9) NLG-C v NLG-PE (p=0.7) LG-C v LG-PE (p=0.7) LG-C v LG-FGR (p=0.0007)
p value analysis for birth weight	NLG-C v LG-C (p=0.2) NLG-C v NLG-PE (p=0.9) LG-C v LG-PE (p=0.03) LG-C v LG-FGR (p=0.0007)
p value analysis for gestation age	NLG-C v LG-C (p=0.2) NLG-C v NLG-PE (p=0.05) LG-C v LG-PE (p=0.1) LG-C v LG-FGR (p=0.004)
p value analysis for BMI	NLG-C v LG-C (p=0.9) NLG-C v NLG-PE (p=0.09) LG-C v LG-PE (p=0.3) LG-C v LG-FGR (p=0.3)

3.3.2 Analysis of protein and mRNA HSP70 expression in normal pregnancy

Experiment 1: This experiment was designed to test if there was a spatial difference in protein expressions of HSP70 within individual placentas obtained from women who were not in labour (non-labour group) and from women who were in labour (labour group). First the non-labour group was compared. Examples of Western blots showing HSP70 protein expressions for the non-labour are shown in (Figure 3-3). The graphs and statistical analysis are shown below the blots. Comparison between zones was performed using Friedman test analysis. There was a significant difference in protein expression of HSP70 between the three zones of the non-labour group and the labour group (p=0.04 for both). The sub-analysis showed that there was a significant decrease in HSP70 expression

in outer zones compared to the middle zones for both non-labour and labour groups (p=0.03, p=0.02 respectively).

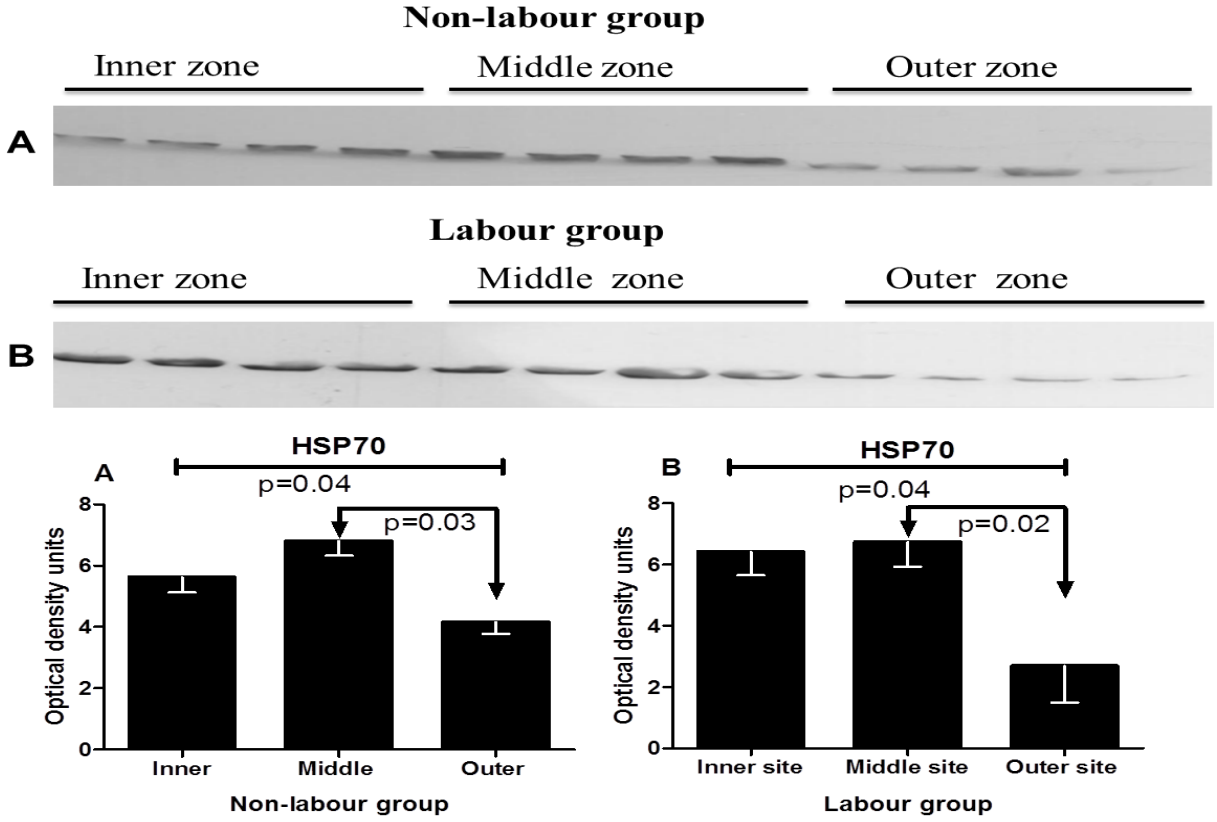


Figure 3-3: Representative images of Western blots showing HSP70 protein expressions in inner, middle and outer zones of individual placentas of non-labour group (A) and labour group (B). Four quadrants were sampled in each zone. Graphs show the median for HSP70 protein expressions. Comparison between zones was performed using Friedman analysis.

Experiment 2: This experiment was designed to test if there was any difference in HSP70 protein expression between labour and non-labour groups at the inner, middle or outer zones of placentas. Western blots showing placental protein expression of HSP70 in the non-labour and the labour at the inner, middle and outer zones are shown in (Figure 3-4). The graphs and statistical analysis are shown below the blots. There was a significant decrease in HSP70 expression in the non-labour group when compared to the labour group at the middle zone (p=0.008). There was no significant difference in HSP70 expression in the non-labour group when compared to the labour group at the inner and outer zones (p=0.2, p=0.06 respectively).

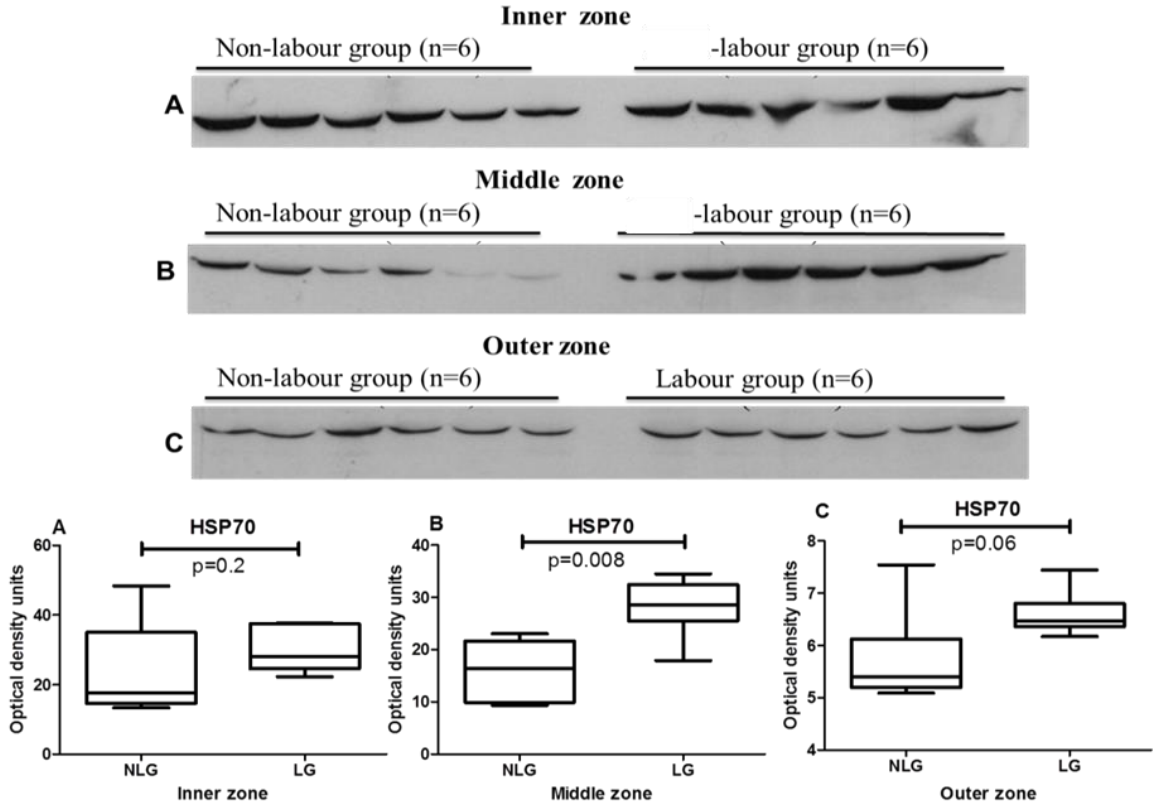


Figure 3-4: Representative images of Western blots showing HSP70 expressions in the inner, middle and outer placental zones in non-labour and labour (n=6 in each group). The graphs show the box and whiskers analysis of the blots. Comparison between zones was performed using Mann-Whitney analysis. NLG non-labour group, LG labour group.

Experiment 3: This experiment was designed to test if there were any differences in mRNA expression of HSP70 within individual placentas at different zones (inner, middle and outer) in either labour or non-labour. No spatial differences were found within placentas at the mRNA level in non-labour group and labour group (p=0.95, p=0.74). (Figure 3-5) shows the HSP70 RQ values in the inner, middle and outer zones for non-labour (A) and labour (B).

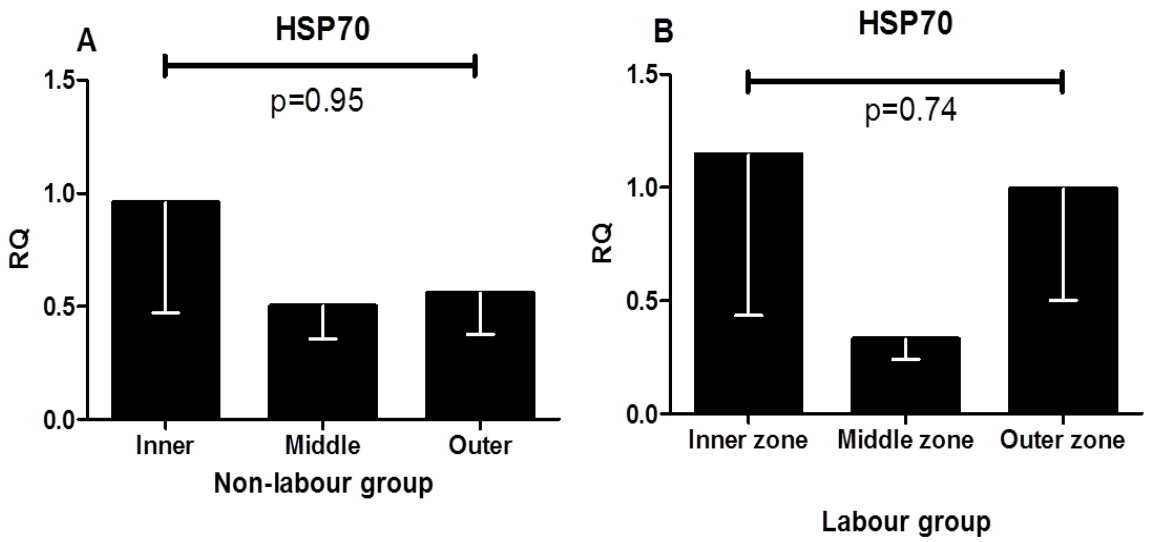


Figure 3-5: RQ values for HSP70 mRNA measurements in inner (n=4), middle (n=4) and outer zones (n=4) of individual placentas (non-labour and labour groups). Four quadrants were sampled in each zone. Graphs show the median for HSP70 mRNA expressions. Comparison between zones was performed using Friedman analysis.

Experiment 4: This experiment was designed to test if there were any differences in mRNA expression of HSP70 in the non-labour compared to the labour group at the inner, middle and outer zones. Figure 4 shows the HSP70 RQ values in the non-labour compared to the labour group at the inner (A), middle (B) and outer zones (C). There was no significant difference in mRNA expression of HSP70 in the non-labour group compared to the labour group at the inner, middle and outer zones (p=0.69, p=0.39, p=0.93 respectively) (Figure 3-6).

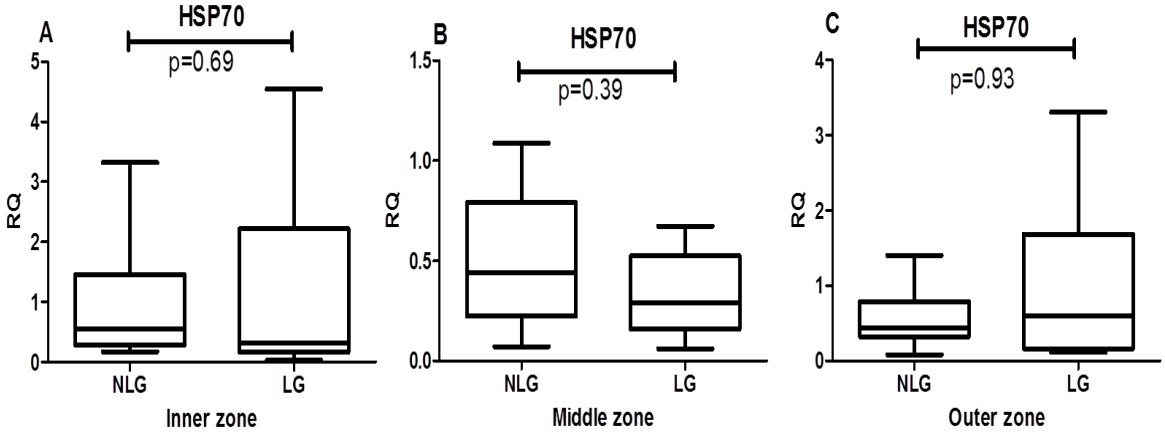


Figure 3-6: RQ values for HSP70 mRNA measurements in non-labour (n=6) compared to the labour group (n=6) at the inner, middle and outer placental zones. Comparison between zones was performed using Mann-Whitney analysis.

3.3.3 Analysis of protein and mRNA HSP70 expression in pregnancies complicated with PE

Experiment 1: This experiment was designed to test if there was any difference in protein expression of HSP70 between non-labour control and non-labour PE groups at the inner, middle and outer zones of placentas. Western blots showing placental HSP70 protein expression in the non-labour control and the non-labour PE at the inner, middle and outer zones are shown in (Figure 3-7). The graphs and statistical analysis are shown below the blots. There was a significant decrease in HSP70 expression in the non-labour control group when compared to the non-labour PE group at the inner zone (p=0.02). There was no significant difference in HSP70 expression in the non-labour control group when compared to the non-labour PE group at the middle and outer zones (p=0.16, p=0.71 respectively).

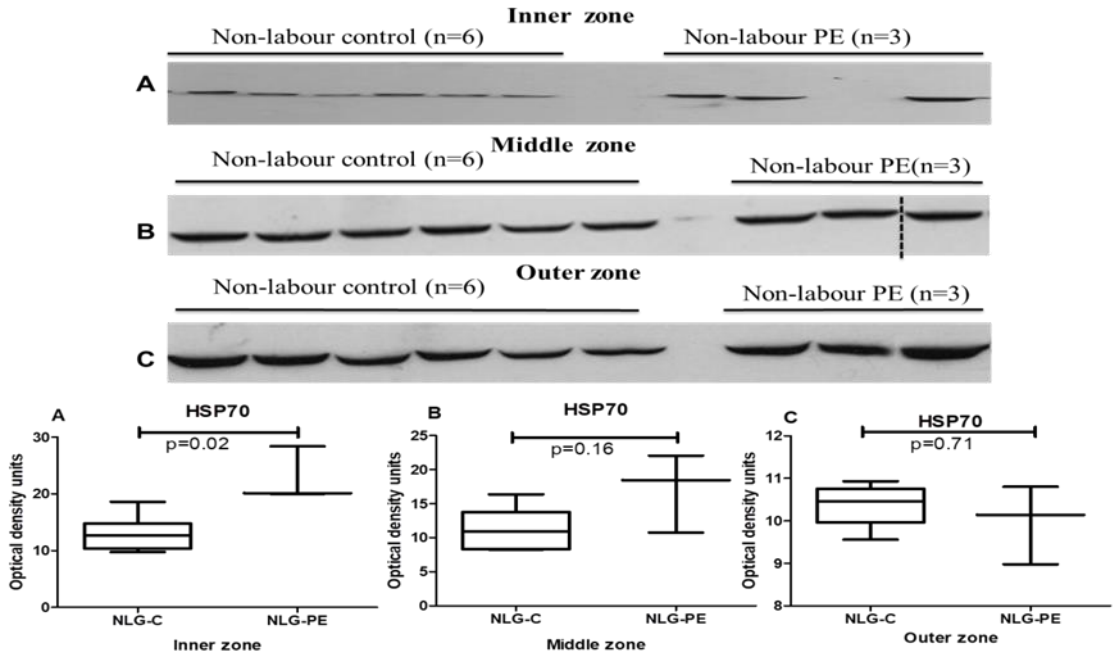


Figure 3-7: Representative images of Western blots showing HSP70 expressions in the inner, and outer placental zones in non-labour control (n=6) and non-labour PE (n=3). The graphs show the box and whiskers analysis of the blots of three placenta zones. Middle Western blot image empty lane was removed (dotted lines). Comparison between zones was performed using Mann-Whitney analysis. 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 HSP70 expression between labour control and labour PE groups at the inner, middle and outer zones of placentas. Western blots showing placental HSP70 protein expression in the labour control and the labour PE at the inner, middle and outer zones are shown in (Figure 3-9). The graphs and statistical analysis are shown below the blots. There was no

significant difference in HSP70 expression in the labour control group compared to the non-labour PE group at the inner, middle and outer zones (p=0.6, p=0.9, p=0.1 respectively).

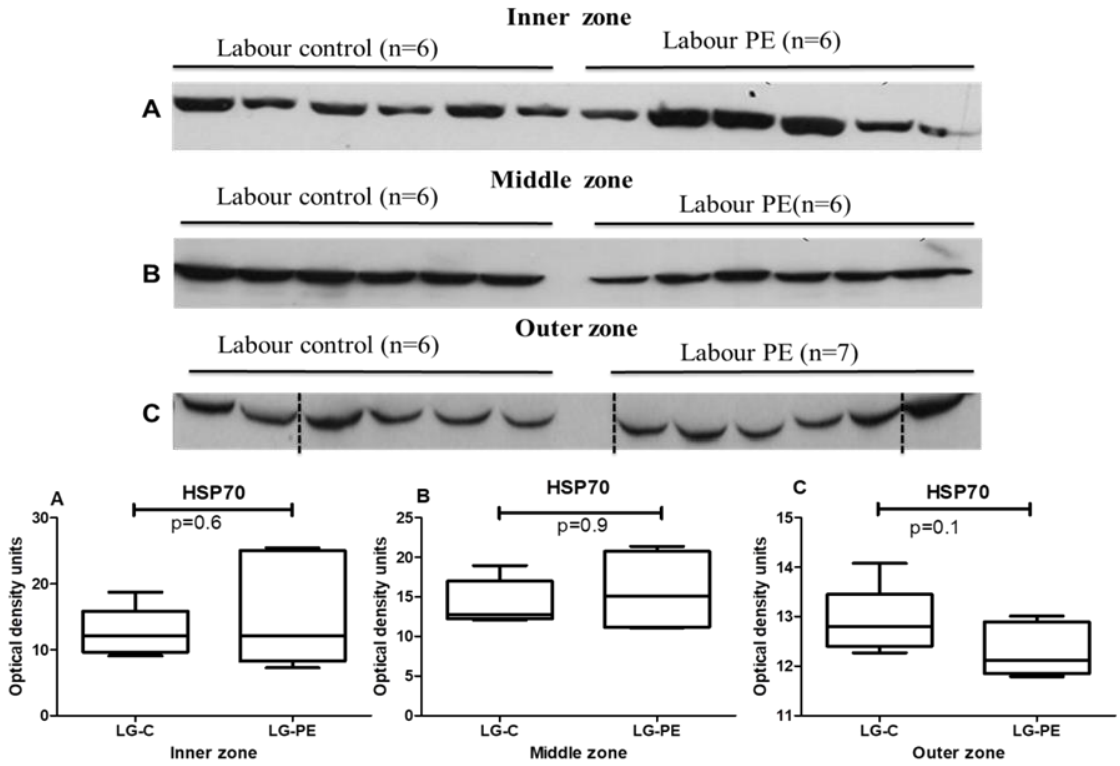


Figure 3-8: Representative images of Western blots showing HSP70 expressions in the inner and middle placental zones in labour control and labour PE groups (n=6 for each group). Western blot image empty lanes were removed (dotted lines). The graphs show the box and whiskers analysis of the blots. Comparison between zones was performed using Mann-Whitney analysis. LG-C labour control group, LG-PE labour PE group.

Experiment 3: This experiment was designed to test if there were any differences in mRNA expression of HSP70 in the non-labour control compared to the non-labour PE group at the inner, middle and outer placental zones. (Figure 3-10) shows the HSP70 RQ values in the non-labour control compared to the non-labour PE group at the inner (A), middle (B) and outer (C) zones. There was no significant difference in HSP70 mRNA in the non-labour control group compared to the non-labour PE at the inner, middle and outer zones (p=0.9, p=0.09, p=0.71 respectively).

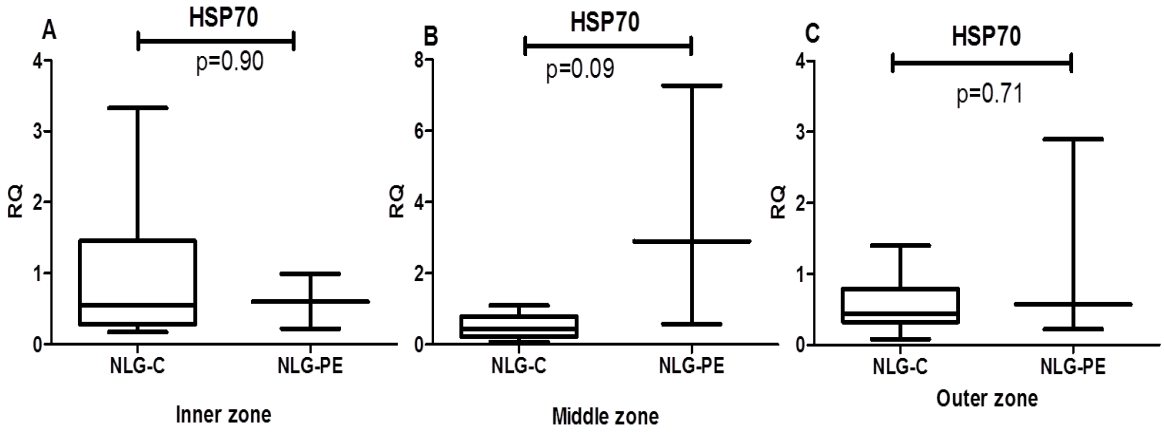


Figure 3-9: RQ values for HSP70 mRNA measurements in inner, middle and outer placental zones for the non-labour control group (n=6) compared with non-labour PE groups (n=3). Comparison between zones was performed using Mann-Whitney analysis.

Experiment 4: This experiment was designed to test if there was any difference in mRNA expression of HSP70 between labour control and labour PE groups at the inner, middle and outer zones of placentas. Figure 8 shows the HSP70 RQ values in the labour control compared to the labour PE group at the inner (A), middle (B) and outer (C) zones. Comparison between zones was performed using Mann Whitney test analysis. There was no significant difference in mRNA expression of HSP70 in the labour control group compared to the labour PE group in the inner, middle and outer placental zones (p=0.48, p=0.06,p=0.48 respectively) (Figure 3-11).

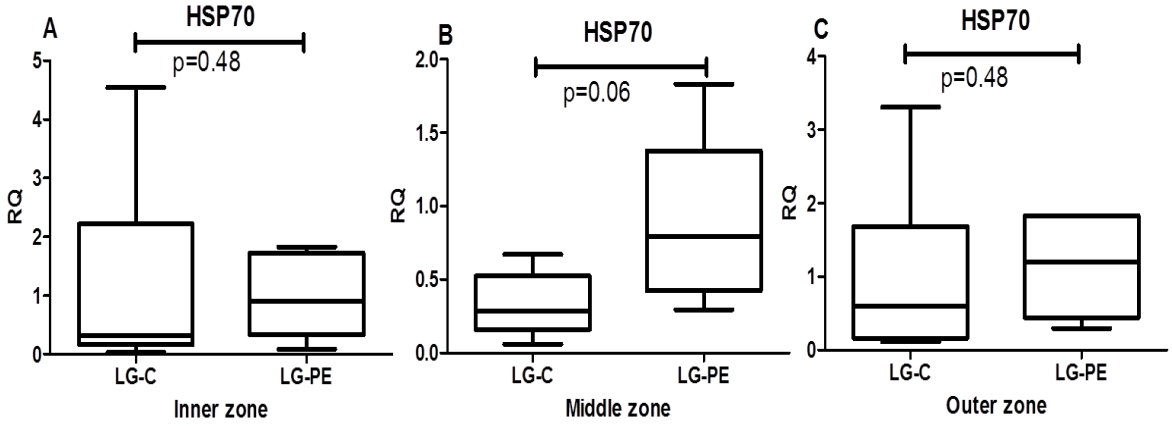


Figure 3-10: RQ values for HSP70 mRNA measurements in inner, middle and outer placental zones for labour control group (n=6) compared with labour PE groups (n=6). Comparison between zones was performed using Mann-Whitney analysis.

Experiment 5: This experiment was designed to test if there was any difference in mRNA expressions of HSP70 between normotensive pregnancies and pregnancies complicated with PE (both labour and non-labour cases were combined) at the inner, middle and outer zones of placentas. (Figure 3-12) shows the HSP70 RQ values in the normotensive pregnancies group compared to the PE pregnancies group. Comparison between zones was performed using Mann Whitney test analysis. There was a highly significant decrease in mRNA expression of HSP70 in the normotensive pregnancies group compared to the pregnancies complicated with PE group in the middle (p=0.009). No difference was found in the inner and outer placental zones (p=0.54, p=0.12).

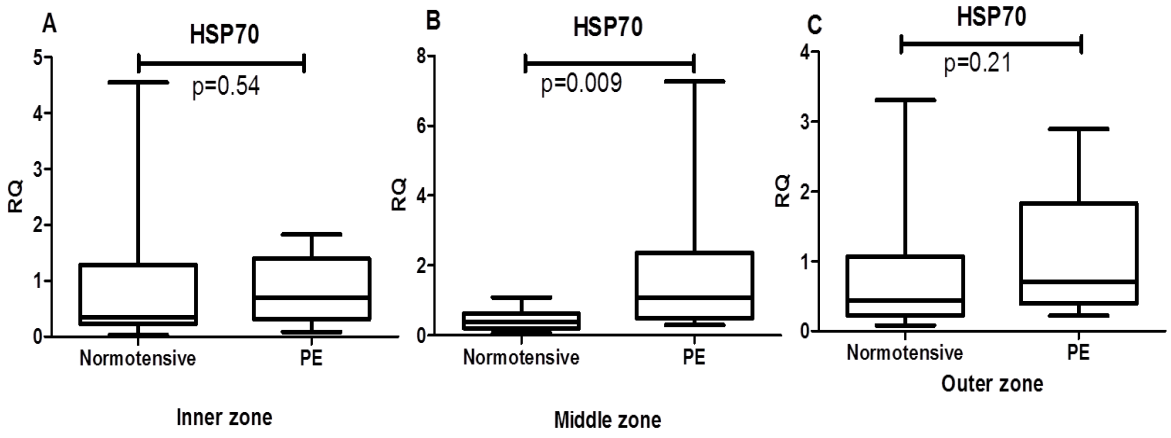


Figure 3-11: RQ values for HSP70 mRNA measurements in normotensive pregnancies group (non-labour and labour were combined) (n=12) compared to the pregnancies complicated with PE group (non-labour and labour were combined) (n=12) at the inner, middle and outer placental zones. Comparison between zones was performed using Mann-Whitney analysis.

3.3.4 Analysis of protein and mRNA expression of HSP70 in pregnancies complicated with FGR.

Experiment 1: This experiment was designed to test if there was any difference in HSP70 protein and mRNA expressions between labour control and labour FGR groups at the inner or outer zones of placentas. Due to time constraints only one set of experiments was performed for labour groups and in the inner and outer zones only. The aim was to determine whether pilot data from this group of experiments revealed any data suggesting that more experiments were warranted. Western blots showing placental HSP70 expression at the inner zone (upper panel) and placental HSP70 expression at the outer zone (lower panel) of the labour control group and the labour-FGR group are shown in (Figure 3-13). The graphs and statistical analysis are shown below the blots. There was a

significant decrease in HSP70 expression in the labour-FGR group compared to the control labour group in the outer zone (p=0.01). There was no significant difference in the HSP70 expression between the labour control and labour FGR groups at the inner zone (p=0.17).

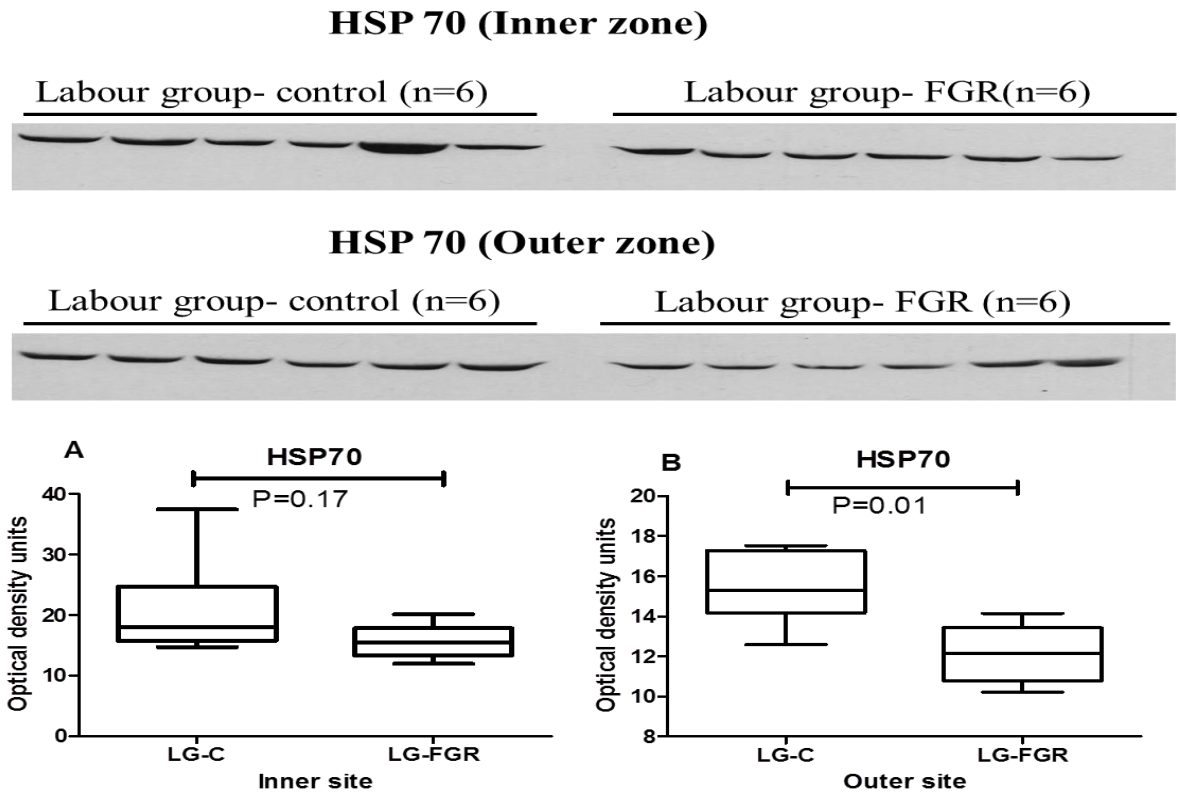


Figure 3-12: Representative images of Western blots showing HSP70 expression at the inner zone (upper panel) and the outer zone (lower panel) in labour control (n=6) and labour FGR group (n=6). The graphs show the box and whiskers analysis of the blots. Comparison between zones was performed using Mann-Whitney analysis. LG-C labour group control, LG-FGR labour group FGR.

Experiment 2: tested whether there was any difference between labour control and labour FGR at the inner or outer zones at the HSP70 mRNA level. The results are shown in (Figure 3-14). No differences were found in the inner or outer zones in the HSP70 mRNA expression.

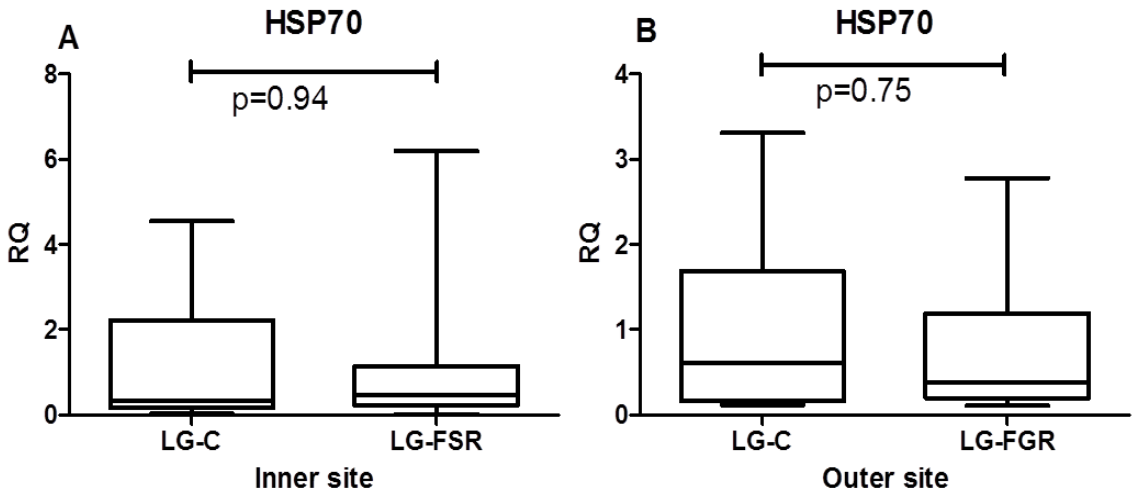


Figure 3-13: RQ values for HSP70 mRNA measurements in inner and outer placental zones for labour control group (n=6) compared with labour FGR-groups (n=6). Comparison between zones was performed using Mann-Whitney analysis.

3.3.5 Analysis of protein and mRNA expression of HSF1 in normal pregnancy

Experiment 1: This experiment was designed to test if there was a spatial difference in protein expression of HSF1 within individual placentas obtained from women who were not in labour (non-labour group) and from women who were in labour (labour group). First the non-labour groups were compared. Two main bands were detected in all placenta samples, which, according to the data sheet represent the phosphorylated 80 kDa and non-phosphorylated 65 kDa forms. Examples of Western blots showing HSF1 (phosphorylated 80 kDa) and (non-phosphorylated 65 kDa) expressions for non-labour are shown in (Figure 3-15). The graphs and statistical analysis are shown below the blots. Comparison between zones was performed using Friedman test analysis. There was no significant difference in protein expression of either form between the three zones of the non-labour group (p=0.65, p=0.12 respectively).

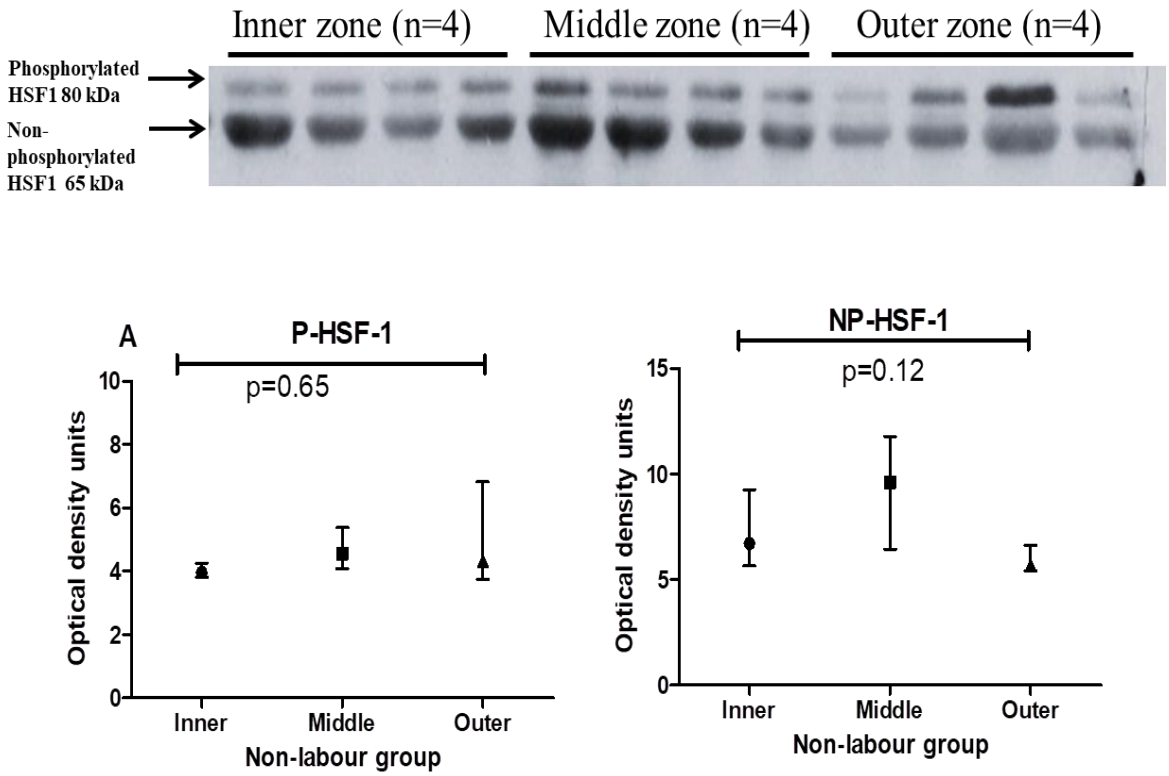


Figure 3-14: Representative image of Western blot showing expression of HSF1 phosphorylated and non- phosphorylated forms expressions in inner, middle and outer zones of individual placentas of non-labour group. Four quadrants were sampled in each zone. Graphs show median and interquartile range for HSF1 phosphorylated and non-phosphorylated forms expressions. Comparison between zones was performed using Friedman analysis. P-HSF-1= phosphorylated HSF1, NP-HSF-1= non-phosphorylated HSF1.

Experiment 2: This experiment was designed to test if there was a spatial difference in expression of HSF1 within individual placentas obtained from women who were in labour. Two main bands were detected in all placenta samples, which, according to the data sheet represent the phosphorylated 80 kDa and non-phosphorylated 65 kDa forms. Examples of Western blots showing HSF1 (phosphorylated 80 kDa) and (non-phosphorylated 65 kDa) expressions for labour group are shown in (Figure 3-16). There was a significant decrease in both phosphorylated and non-phosphorylated forms at the outer zones compared to the inner and middle zones of the labour group (p=0.04 for both). The sub-analysis showed that there was a significant decrease in both phosphorylated and non-phosphorylated forms of HSF1 expression in outer zones compared to the inner zone for labour group (p=0.02 for both).

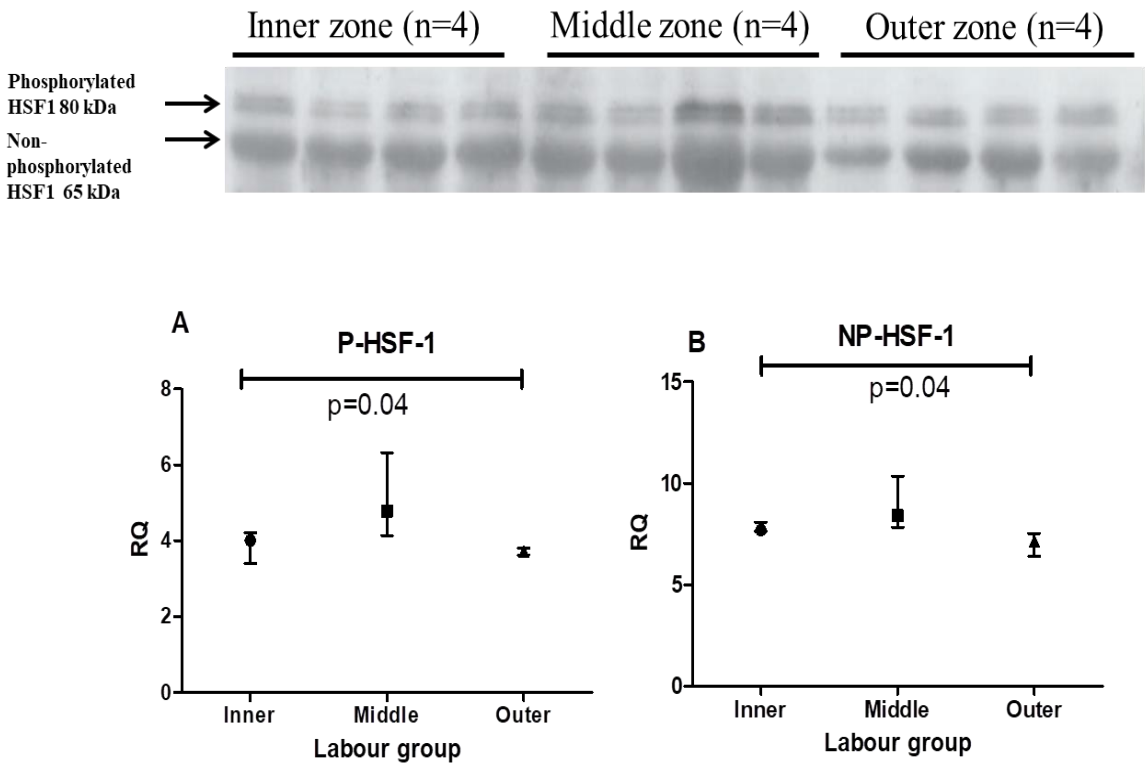


Figure 3-15: Representative image of Western blot showing HSF1 phosphorylated and non-phosphorylated forms expressions in inner, middle and outer zones of individual placentas of labour group. Four quadrants were sampled in each zone. Graphs show median and interquartile range for HSF1 phosphorylated and non-phosphorylated forms expressions. Comparison between zones was performed using Friedman analysis. P-HSF-1= phosphorylated HSF1, NP-HSF-1= non-phosphorylated HSF1.

Experiment 3: This experiment was designed to test if there was any difference in HSF1 phosphorylated and and non-phosphorylated forms expression between labour and non-labour groups at the inner, middle or outer zones of placentas. Western blots showing placental protein expression of HSF1 in the non-labour and the labour at the inner zone are shown in (Figure 3-17) phosphorylated 80 kDa (upper panel) and non-phosphorylated 65 kDa (lower panel). The graphs and statistical analysis are shown below the blots. There was a significant decrease in HSF1 expression in the labour group when compared to the non-labour group for both the phosphorylated form and the non-phosphorylated forms at the inner zone (p=0.02, p=0.04).

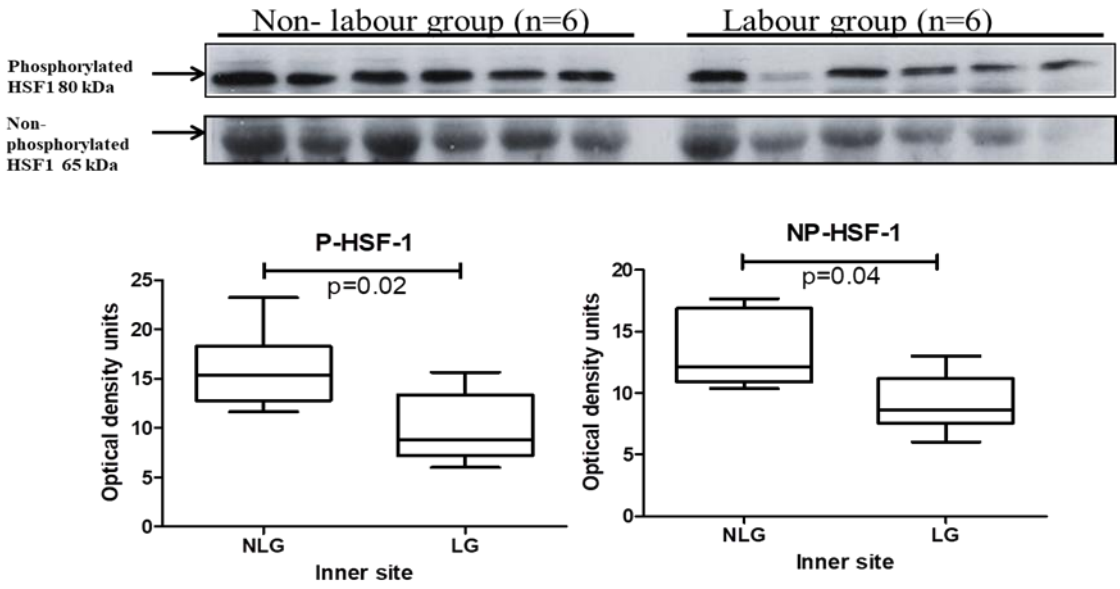


Figure 3-16: Representative images of Western blot showing HSF1 phosphorylated form (upper panel) and HSF1 non-phosphorylated form (lower panel) expressions in the inner placental zone in non-labour and labour (n=6 in each group). Both bands of Western blot image showed separately. The graphs show the box and whiskers analysis of the blots. Comparison between zones was performed using Mann-Whitney analysis. NLG non-labour group, LG labour group.

Western blots showing placental HSF1 phosphorylated form (upper panel) and HSF1 non-phosphorylated form (lower panel) expressions in non-labour and labour at the middle zone are shown in (Figure 3-18). The graphs and statistical analysis are shown below the blots. There was a highly significant decrease in HSF1 expression in the labour group when compared to the non-labour group for both the phosphorylated and non-phosphorylated forms at the middle zone (p=0.01, p=0.008 respectively).

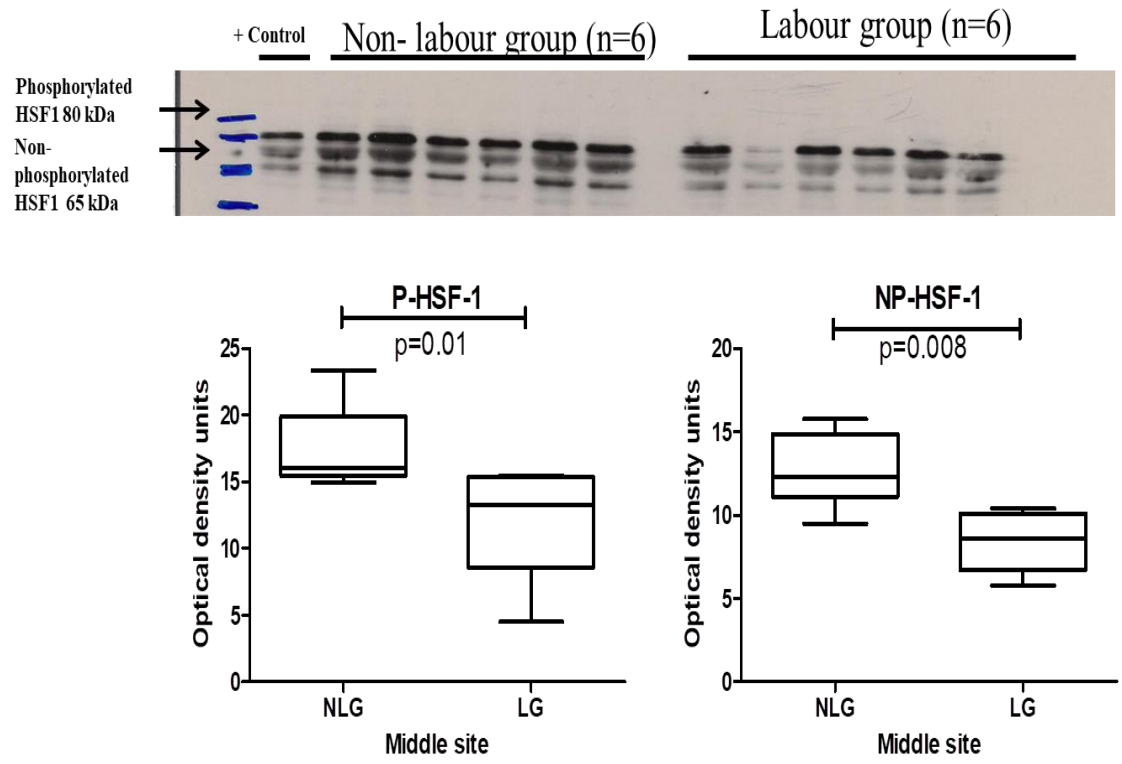


Figure 3-17: Representative image of Western blot showing HSF1 phosphorylated form (upper panel) and HSF1 non-phosphorylated form (lower panel) expressions in the middle placental zone in non-labour and labour (n=6 in each group). First lane is the protein marker and second lane is the positive control sample. The graphs show the box and whiskers analysis of the blots. Comparison between zones was performed using Mann-Whitney analysis. NLG non-labour group, LG labour group.

Western blots showing placental HSF1 phosphorylated form (upper panel) and HSF1 non-phosphorylated form (lower panel) expressions in the non-labour and the labour at the outer zone are shown in (Figure 3-19). The graphs and statistical analysis are shown below the blots. There was no significant difference in HSF1 expression at the outer zone for both forms (p=0.30, p=0.48 respectively).

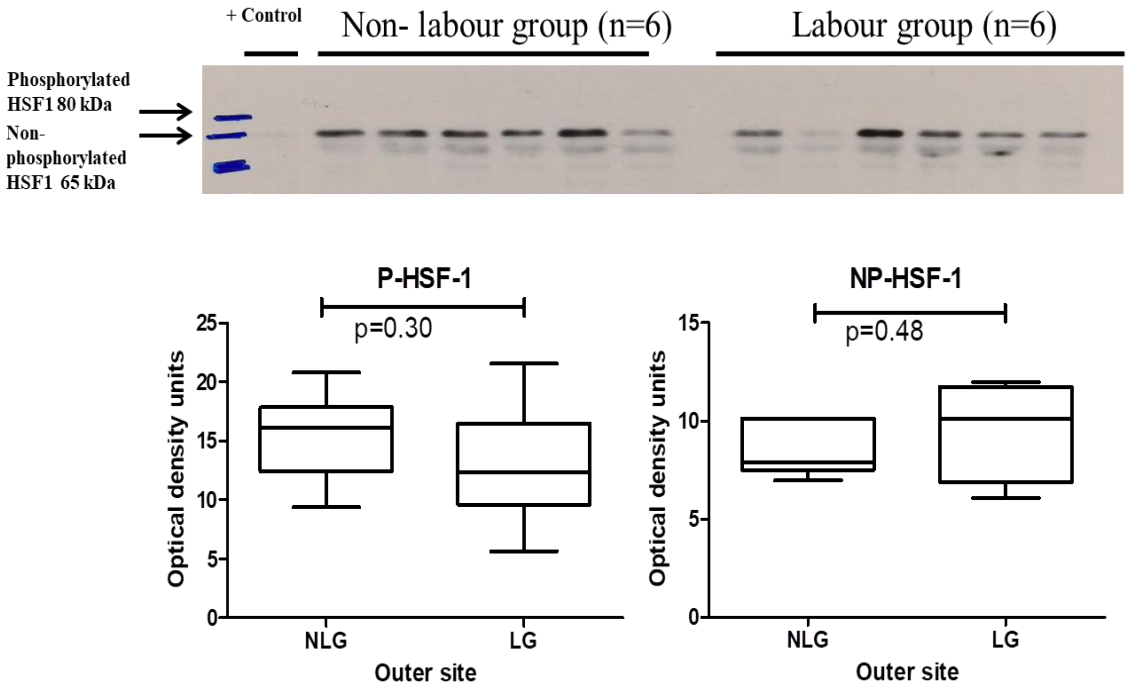


Figure 3-18: Representative image of Western blot showing HSF1 phosphorylated form (upper panel) and HSF1 non-phosphorylated form (lower panel) expressions in the outer placental zone in non-labour and labour (n=6 in each group). First lane is the protein marker and second lane is the positive control sample. The graphs show the box and whiskers analysis of the blots. Comparison between zones was performed using Mann-Whitney analysis. NLG non-labour group, LG labour group.

Experiment 4 this experiment was designed to test if there were any differences in mRNA expression of HSF1 within individual placentas at different zones (inner, middle and outer) in either labour or non-labour. In the non-labour group there was a significant increased at the outer zone (p=0.0001). No spatial differences were found within placentas at the mRNA level in labour group (p=0.4). (Figure 3-20) shows the HSF1 RQ values in the inner, middle and outer zones for non-labour (A) and labour (B).

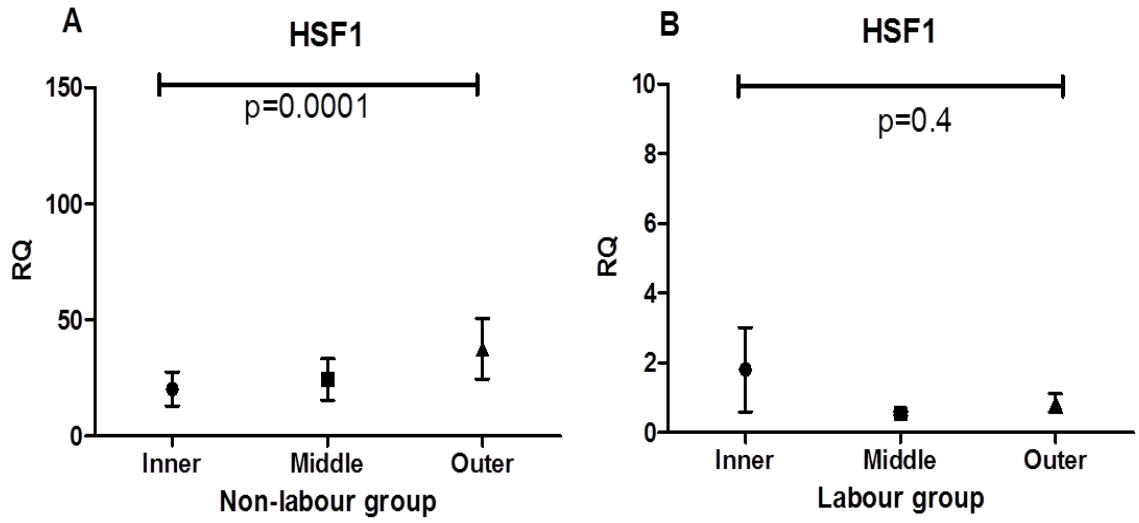


Figure 3-19: RQ values for HSF1 mRNA measurements in in inner (n=4), middle (n=4) and outer zones (n=4) of individual placentas (non-labour and labour groups). Four quadrants were sampled in each zone. Graphs show the median for HSF1 mRNA expressions. Comparison between zones was performed using Friedman analysis.

Experiment 5 this experiment was designed to test if there were any differences in mRNA expression of HSF1 in the non-labour compared to the labour group at the inner, middle and outer zones. (Figure 3-21) shows the HSF1 RQ values in the non-labour compared to the labour group at the inner (A), middle (B) and outer zones (C). There was, paradoxically, an increase in HSF1 mRNA in the labour group at the inner zone (p=0.01). No differences were found in HSF1 mRNA expression at the middle and outer zones (p=1, p=0.8).

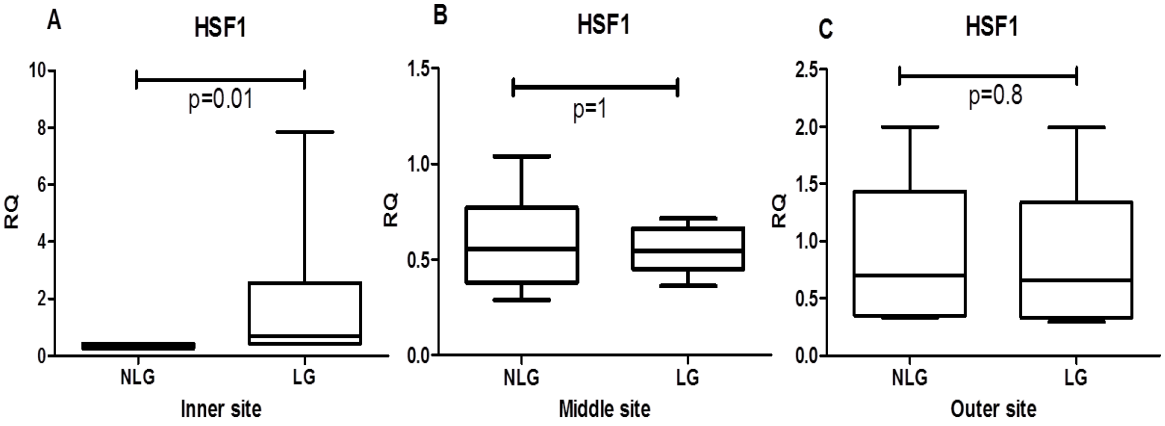


Figure 3-20: RQ values for HSF1 mRNA measurements in non-labour (n=6) compared to the labour group (n=6) at the inner, middle and outer placental zones. Comparison between zones was performed using Mann-Whitney analysis.

3.3.6 Analysis of protein and mRNA expression of HSF1 in pregnancies complicated with PE

Experiment 1: This experiment was designed to test if there was any difference in HSF1 phosphorylated and non-phosphorylated forms expression between non-labour control and non-labour PE groups at the inner and the middle zones of placentas. Due to time constraints only two placental zones experiments were analysed. Western blots showing placental HSF1 protein expression in the non-labour control and the non-labour PE at the inner zone are shown in (Figure 3-22) phosphorylated 80 kDa (upper panel) and non-phosphorylated 65 kDa (lower panel). The graphs and statistical analysis are shown below the blots. There was a significant decrease in HSF1 expression in the non-labour control group when compared to the non-labour PE group for the non-phosphorylated forms (p=0.04). There was no significant difference in the phosphorylated form at the inner zone (p=0.24).

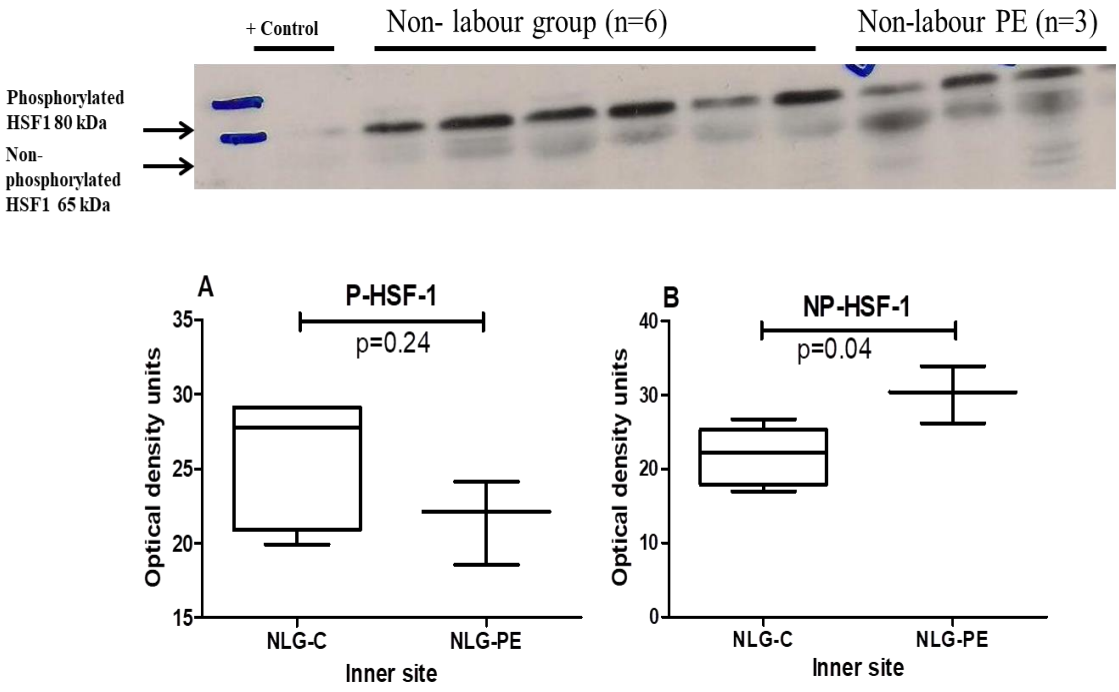


Figure 3-21: Representative image of Western blot showing HSF1 phosphorylated form (upper panel) and HSF1 non-phosphorylated form (lower panel) expressions in the inner placental zone in non-labour control (n=6) and non-labour PE (n=3). First lane is the protein marker and second lane is the positive control sample. The graphs show the box and whiskers analysis of the blots. Comparison between zones was performed using Mann-Whitney analysis. NLG-C non-labour control group, NLG-PE non-labour PE group.

Western blots showing placental HSF1 phosphorylated form (upper panel) and HSF1 non-phosphorylated form (lower panel) expressions in the non-labour control and the non-labour PE at the middle zone are shown in (Figure 3-23). The graphs and statistical analysis are shown below the blots. There was no significant difference in HSF1 expression in the non-labour group when compared to the non-labour PE group for both the phosphorylated and non-phosphorylated forms at the middle zone (p=0.54, p=0.89 respectively).

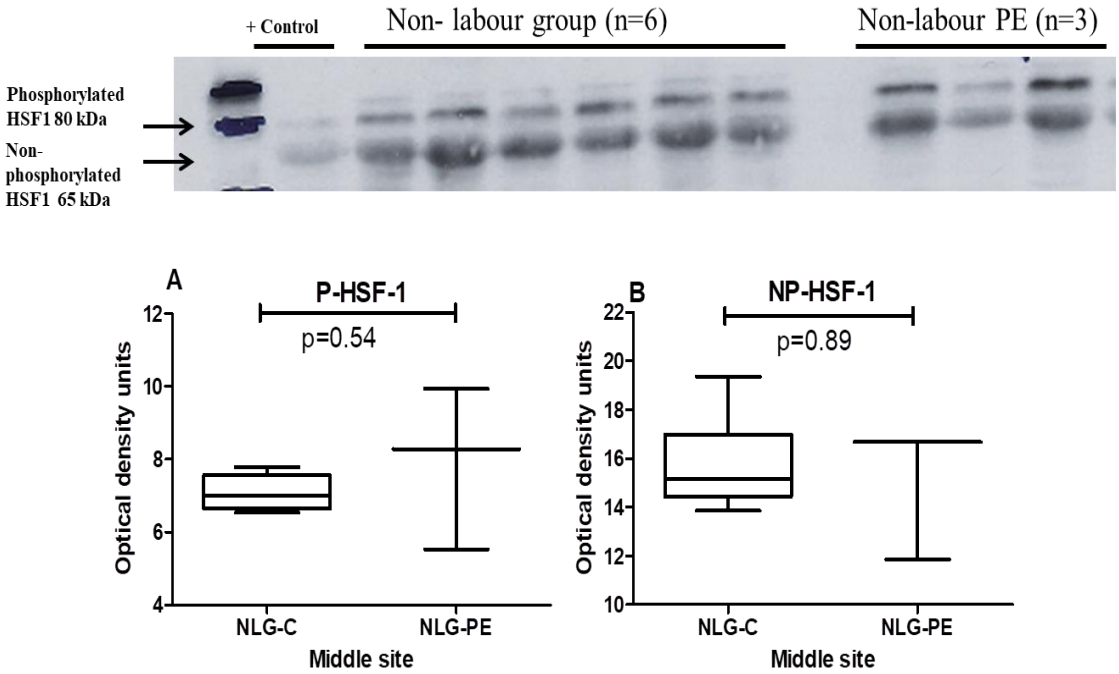


Figure 3-22: Representative image of Western blot showing HSF1 phosphorylated form (upper panel) and HSF1 non-phosphorylated form (lower panel) expressions in the middle placental zone in non-labour control (n=6) and non-labour PE (n=3). First lane is the protein marker and second lane is the positive control sample. The graphs show the box and whiskers analysis of the blots. Comparison between zones was performed using Mann-Whitney analysis. 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 HSF1 phosphorylated and non-phosphorylated forms expression between the labour control and the labour PE groups at the inner and the middle zones of placentas. Western blots showing placental HSF1 expression in labour control and PE at the inner zone is shown in (Figure 3-24) phosphorylated 80 kDa (upper panel) and non-phosphorylated 65 kDa (lower panel). The graphs and statistical analysis are shown below the blots. There was a highly

significant decrease in HSF1 expression in the labour control group when compared to the labour PE group for the non-phosphorylated form (p=0.002). There was no significant difference in the phosphorylated form at the inner zone (p=0.39).

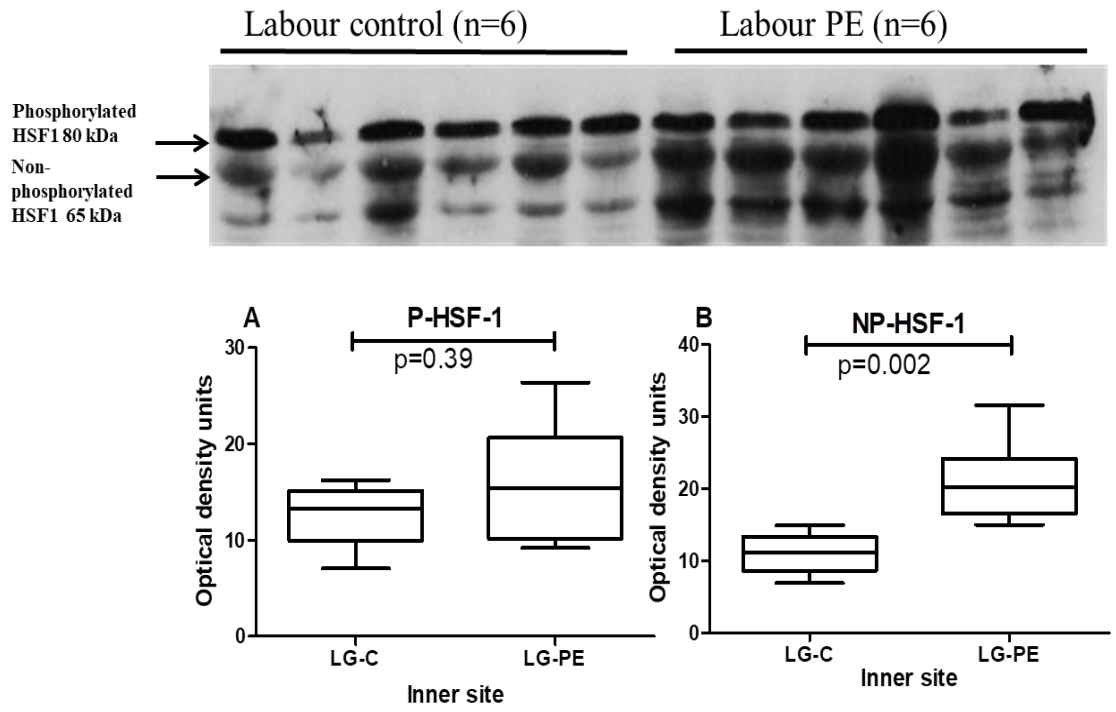


Figure 3-23: Representative image of Western blot showing HSF1 phosphorylated form (upper panel) and HSF1 non-phosphorylated form (lower panel) expressions in the inner placental zone in labour control and PE (n=6 for both groups). The graphs show the box and whiskers analysis of the blots. Comparison between zones was performed using Mann-Whitney analysis. LG-C labour control group, LG-PE labour PE group.

Western blots showing placental HSF1 phosphorylated form (upper panel) and HSF1 non-phosphorylated form (lower panel) expressions in the labour control and the labour PE at the middle zone are shown in (Figure 3-25). The graphs and statistical analysis are shown below the blots. There was a significant decrease in HSF1 expression in the labour PE group when compared to the labour control group for the non-phosphorylated form at the middle zone (p=0.004). There was no significant difference in phosphorylated form at the middle zone (p=0.17).

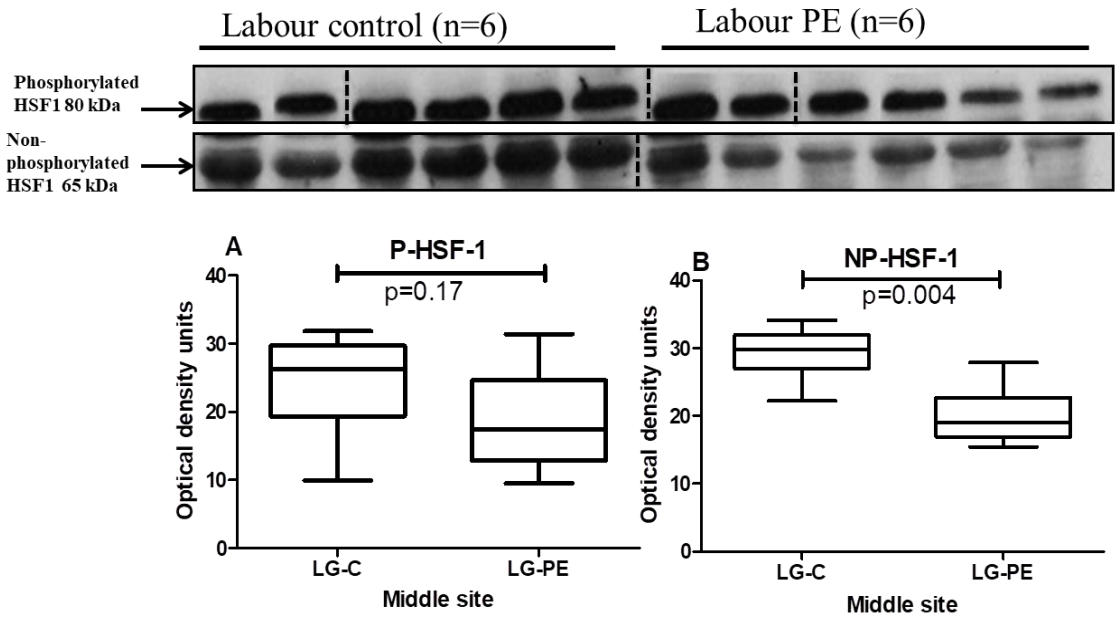


Figure 3-24: The graphs presentations show the box and whiskers analyisis of HSF1 phosphorylated form and HSF1 non-phosphorylated form expressions in the middle placental zone in labour control and PE (n=6 for both groups). Both bands of Western blot image showed separately. As curved gel, areas of splicing of gel image where indicated (dotted lines). Comparison between zones was performed using Mann-Whitney analysis. LG-C labour control group, LG-PE labour PE group.

Experiment 3: This experiment was designed to test if there were any differences in mRNA expression of HSF1 in the non-labour control compared to the non-labour PE group at the inner and middle placental zones. (Figure 3-26) shows the HSF1 RQ values in the non-labour control compared to the non-labour PE group at the inner (A) and middle (B) zones. There was a significant decrease in mRNA expression of HSF1 in the non-labour control group compared to the non-labour PE at the inner zone (p=0.02). No differences were found in HSF1 mRNA expression at the middle zone (p=0.1).

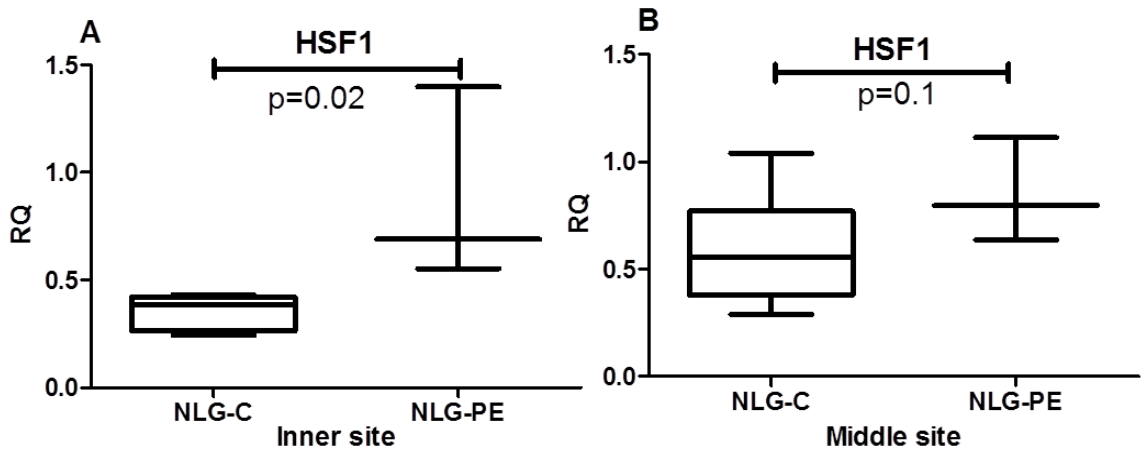


Figure 3-25: RQ values for HSF1 mRNA measurements in inner and middle placental zones for non-labour control group (n=6) compared with non-labour PE groups (n=3). Comparison between zones was performed using Mann-Whitney analysis.

Experiment 4: This experiment was designed to test if there was any difference in mRNA expressions of HSF1 between the labour control and the labour PE groups at the inner or middle zones of placentas. (Figure 3-27) shows the HSF1 RQ values in the labour control compared to the labour PE group at the inner (A) and middle (B) zones. Comparison between zones was performed using Mann Whitney test analysis. There was no significant difference in mRNA expression of HSF1 in the labour control group compared to the labour PE group in the inner or middle placental zones (p=0.1, p=0.06 respectively).

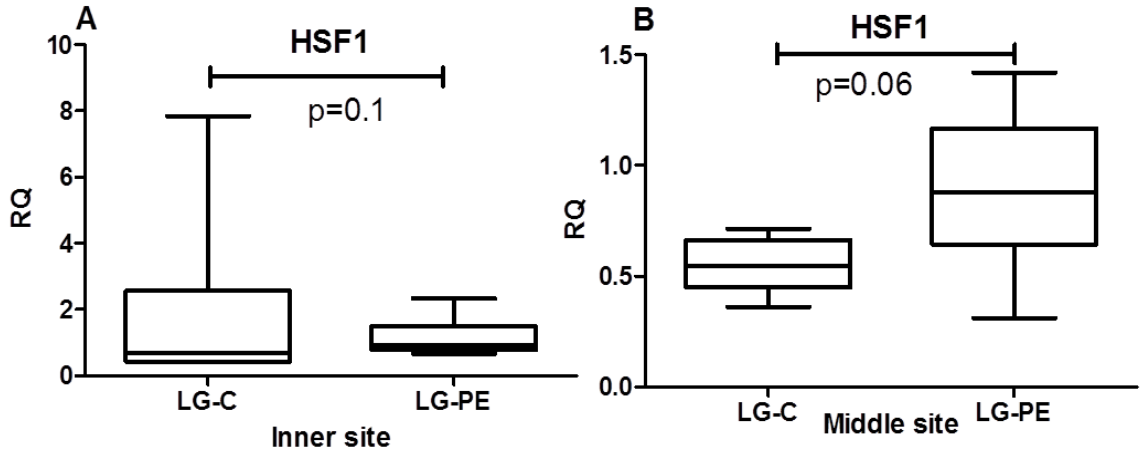


Figure 3-26: RQ values for HSF1 mRNA measurements in inner and middle placental zones for labour control group (n=6) compared with labour PE groups (n=6). Comparison between zones was performed using Mann-Whitney analysis.

Experiment 5: This experiment was designed to test if there was any difference in mRNA expressions of HSF1 between normotensive pregnancies and pregnancies complicated with PE regardless the labour factor at the inner or middle zones of placentas. (Figure 3-28) shows the HSF1 RQ values in the labour control compared to the labour PE group at the inner (A) and middle (B) zones. Comparison between zones was performed using Mann Whitney test analysis. There was a highly significant decrease in mRNA expression of HSF1 in the normotensive pregnancies group compared to the pregnancies complicated with PE group in the inner and middle placental zones (p=0.007, p=0.01).

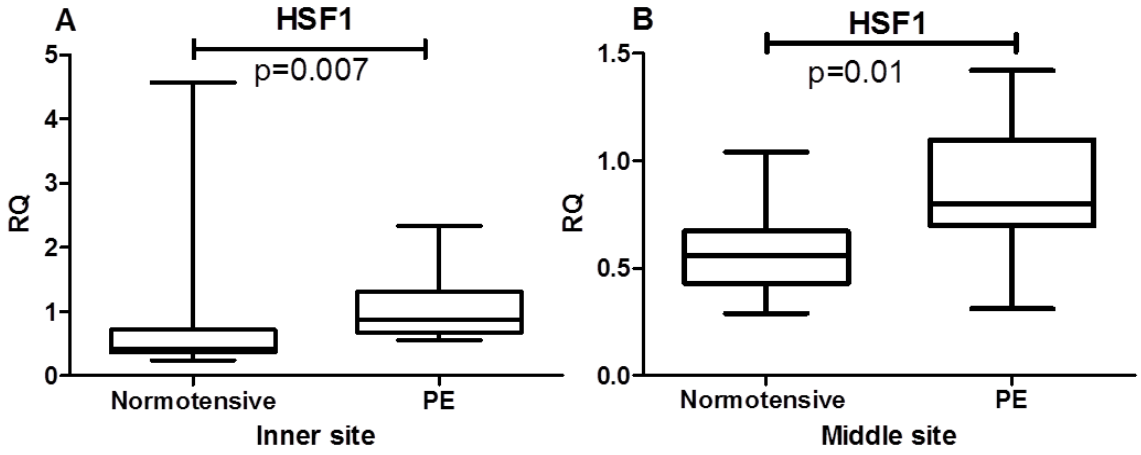


Figure 3-27: RQ values for HSF1 mRNA measurements in normotensive pregnancies group (n=12) compared to the pregnancies complicated with PE group (n=12) at the inner and middle placental zones. Comparison between zones was performed using Mann-Whitney analysis.

3.3.7 Analysis of mRNA expression of HSF2 in normal pregnancies and pregnancies complicated with PE at the inner and middle zones.

Experiment 1: This experiment was designed to test if there was a spatial difference in mRNA expression of HSF2 within individual placentas obtained from women who were not in labour (non-labour group) and from women who were in labour (labour group). (Figure 3-29) shows the HSF2 RQ values within individual placenta in the non-labour and labour group at the inner (A) and middle (B) zones. Comparison between zones was

performed using Friedman test analysis. There was no significant difference in mRNA expression of HSF2 between the three zones of the non-labour and labour groups (p=0.9 for both).

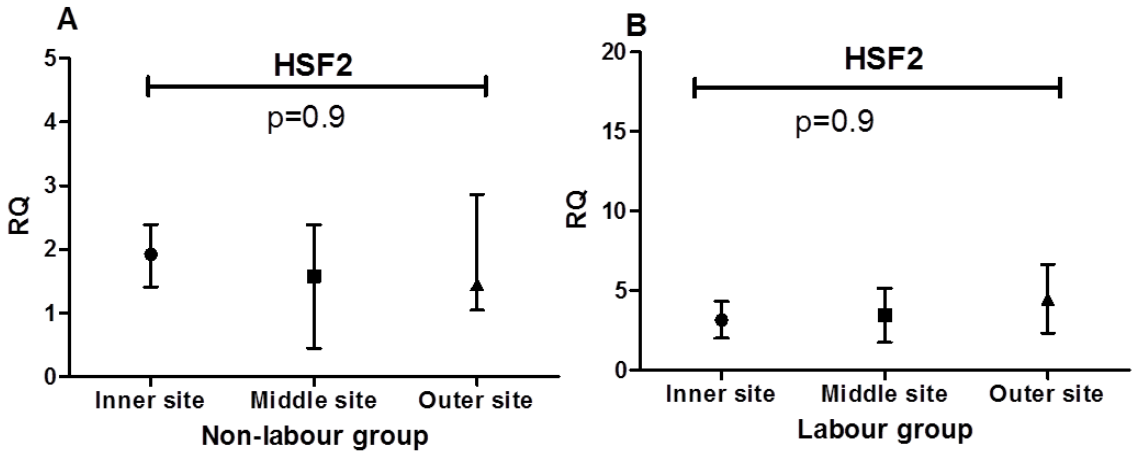


Figure 3-28: RQ values for HSF2 mRNA measurements in in inner (n=4), middle (n=4) and outer (n=4) zones of individual placentas (non-labour and labour groups). Four quadrants were sampled in each zone. Graphs show the median for HSF2 mRNA expressions. Comparison between zones was performed using Friedman analysis.

Experiment 2: This experiment was designed to test if there was any difference in mRNA expression of HSF2 between the labour and the non-labour groups at the inner, middle or outer zones of placentas. (Figure 3-30) shows the HSF1 RQ values in the non-labour group compared to the labour group at the inner (A), middle (B) and outer (C) placental zones. Comparison between zones was performed using Mann Whitney test analysis. There was no significant difference in mRNA expression of HSF2 in the non-labour group when compared to the labour group at the inner, middle and outer zones (p=0.69, p=0.58, p=0.24 respectively).

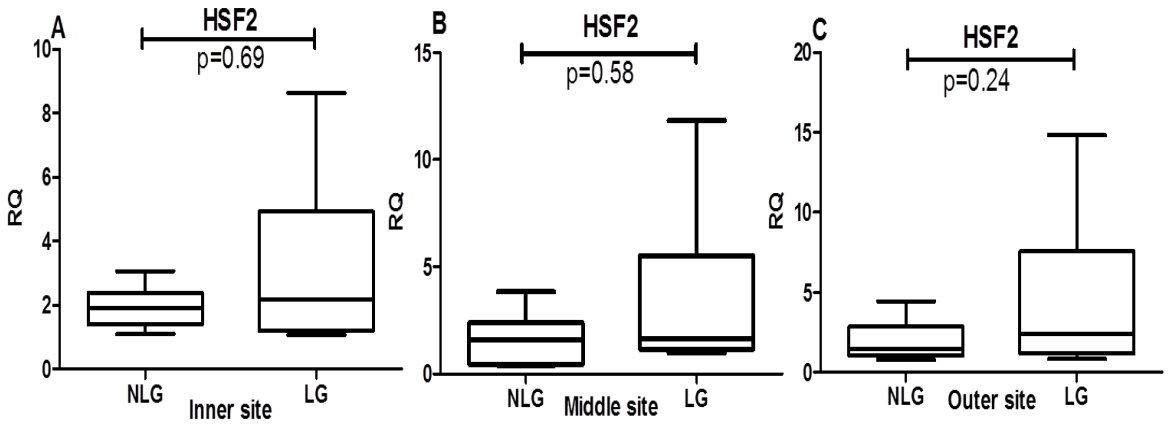


Figure 3-29: RQ values for HSF2 mRNA measurements in non-labour group (n=6) compared to the labour group (n=6) at the inner, middle and outer placental zones. Comparison between zones was performed using Mann-Whitney analysis.

Experiment 3: This experiment was designed to test if there was any difference in mRNA expressions of HSF2 between the non-labour control and the non-labour PE groups at the inner or middle placental zones. (Figure 3-31) shows the HSF1 RQ values in the non-labour control group compared to the non-labour PE group at the inner (A) and middle (B) placental zones. Comparison between zones was performed using Mann Whitney. There was no significant difference in the mRNA expression of HSF2 was found at the inner and middle zones (p=0.90 for both).

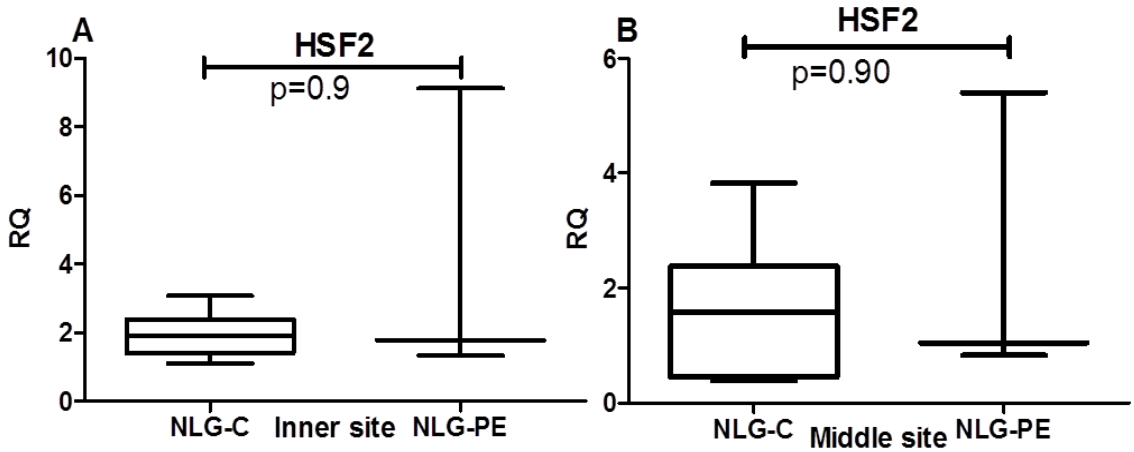


Figure 3-30: RQ values for HSF2 mRNA measurements in non-labour control group (n=6) compared to the non-labour PE group (n=3) at the inner and middle placental zones. Comparison between zones was performed using Mann-Whitney analysis.

Experiment 4: This experiment was designed to test if there was any difference in mRNA expressions of HSF2 between labour control and labour PE groups at the inner or middle placental zones. (Figure 3-32) shows the HSF1 RQ values in the labour control group compared to the labour PE group at the inner (A) and middle (B) placental zones. Comparison between zones was performed using Mann Whitney. There was no significant difference in the HSF2 mRNA was found at the inner and middle zones (p=0.32, p=0.95).

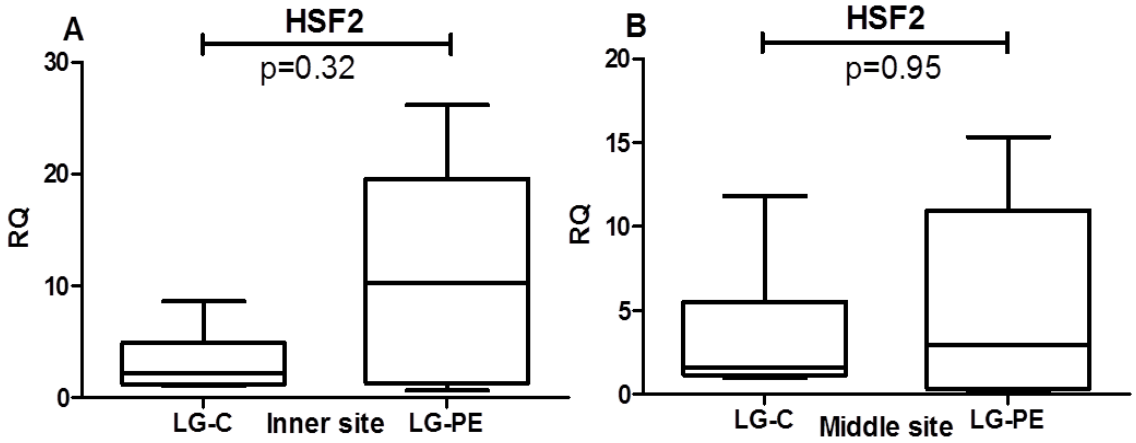


Figure 3-31: RQ values for HSF2 mRNA measurements in labour control group (n=6) compared to the labour PE group (n=6) at the inner and middle placental zones. Comparison between zones was performed using Mann-Whitney analysis.

Experiment 5: This experiment was designed to test if there was any difference in mRNA expressions of HSF2 between normotensive pregnancies and pregnancies complicated with PE regardless the labour factor at the inner or middle zones of placentas. (Figure 3-33) shows the HSF2 RQ values in the labour control compared to the labour PE group at the inner (A) and middle (B) zones. Comparison between zones was performed using Mann Whitney test analysis. There was no significant decrease in mRNA expression of HSF2 in the normotensive pregnancies group compared to the pregnancies complicated with PE group in the inner and middle placental zones (p=0.14, p=0.93).

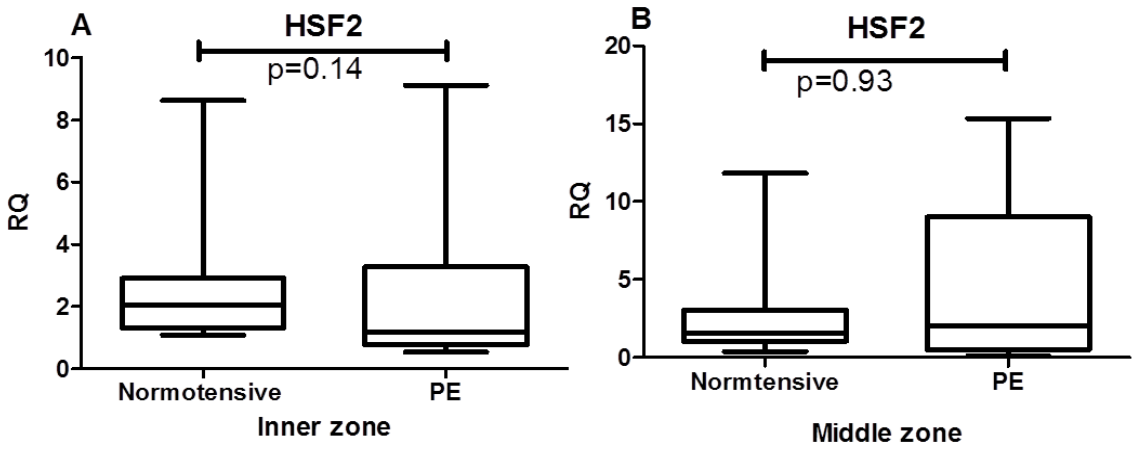


Figure 3-32: RQ values for HSF2 mRNA measurements in normotensive pregnancies group (n=12) compared to the pregnancies complicated with PE group (n=12) at the inner and middle placental zones. Comparison between zones was performed using Mann-Whitney analysis.

3.3.8 Results summary

3.3.8.1 Summary results for HSP70 expression in normal pregnancy:

Experiment 1: Within individual placentas there was a significant difference in protein expression of HSP70 between the three zones of the non-labour group and the labour group (p=0.04 for both). The sub-analysis showed that there was a significant decrease in HSP70 expression in outer zones compared to the middle zones for both non-labour and labour groups (p=0.03, p=0.02 respectively

Experiment 2: There was a significant decrease in HSP70 expression in the non-labour group when compared to the labour group at the middle zone (p=0.008). No other differences were found when comparing labour with non-labour.

Experiment 3: Within individual placentas no differences in mRNA expression for HSP70 were found either in the labour or non-labour groups.

Experiment 4: There was no significant difference in mRNA expression for HSP70 in the non-labour group when compared to the labour group at the inner, middle and outer zones

3.3.8.2 Summary results for HSP70 expression in pregnancies complicated with PE

Experiment 1: When the PE groups (non-labour) were compared with the control groups (non-labour) for protein expression of HSP70 at the inner, middle and outer zones the changes found can be summarized as follows: There was a significant decrease in protein expression of HSP70 in the non-labour control group compared to the non-labour PE group at the inner zone ($p=0.02$). No other differences were found when comparing non-labour control with non-labour PE.

Experiment 2: When the PE groups (labour) were compared with the control groups (labour) for protein expression of HSP70 at the inner, middle and outer zones the changes found can be summarized as follows: There was no significant difference in HSP70 expression in the labour control group compared to the non-labour PE group at the inner, middle and outer zones.

Experiment 3: There was no significant difference in HSP70 mRNA in the non-labour control group when compared to the non-labour PE at the inner, middle and outer zones.

Experiment 4: There was no significant difference in HSP70 mRNA in the labour control group when compared to the labour PE at the inner, middle and outer zones.

Experiment 5: When non labour and labour was combined, there was a highly significant decrease in mRNA expression of HSP70 in the normotensive pregnancies group compared to the pregnancies complicated with PE group in the middle ($p=0.009$). No difference was found in the inner and outer placental zones.

3.3.8.3 Summary results for HSP70 expression in pregnancy complicated with FGR

Experiment 1: When the FGR groups (labour) were compared with the control groups (labour) for protein expression of HSP70 at the inner and outer zones the changes found can be summarized as follows: there was a significant decrease in protein expression of HSP70 in the FGR labour group compared to the labour control group at the outer zone ($p=0.01$). No other differences were found when comparing FGR labour with labour control at the inner zone.

Experiment 2: There was no significant differences were found when labour control and labour FGR groups were compared at the inner or outer zones.

3.3.8.4 Summary results for HSF1 expression in normal pregnancy:

Experiment 1: Within individual placentas there was no significant spatial difference in protein expression of either form of HSF1 between inner, middle and outer placenta zones of the non-labour group

Experiment 2: Within individual placentas there was a significant decrease in both phosphorylated and non-phosphorylated forms in outer zone compared to the inner and middle zones of the labour group ($p=0.04$ for both). The sub-analysis showed that there was a significant decrease in HSF1 expression in outer zones compared to the inner zone for both forms ($p=0.02$ for both).

Experiment 3: There was a significant decrease in HSF1 expression in the labour group when compared to the non-labour group for both forms at the inner zone ($p=0.02$, $p=0.04$ respectively) and at the middle zone ($p=0.01$, $p=0.008$ respectively). No other changes were found at the outer zone.

Experiment 4: Within individual placentas there was a significant spatial difference in the mRNA HSF1 in the non-labour group with high expression at the outer zone ($p=0.0001$). No spatial differences were found within placentas of the labour group.

Experiment 5: There was, paradoxically, an increase in HSF1 mRNA in the labour group at the inner zone ($p=0.01$). No differences were found in HSF1 mRNA expression at the middle and outer zones.

3.3.8.5 Summary results for HSF1 expression in pregnancies complicated with PE

Experiment 1: When the PE groups (non-labour) were compared with the control groups (non-labour) for protein expression of HSF1 at the inner, middle and outer zones the changes found can be summarized as follows: there was a significant decrease in protein expression of HSF1 in the non-labour control group compared to the non-labour PE group for the non-phosphorylated forms ($p=0.04$) at the inner zone. There was no significant difference in the phosphorylated form at the inner zone. There was no significant difference in HSF1 expression was found at the middle zone.

Experiment 2: When the PE groups (labour) were compared with the control groups (labour) for protein expression of HSF1 for both forms at the inner and middle zones the changes found can be summarized as follows: There was a significant decrease in HSF1

expression in the labour control group when compared to the labour PE group for the non-phosphorylated form at the inner zone (p=0.002). There was a significant decrease in HSF1 expression in the labour PE group when compared to the labour control group for the non-phosphorylated form at the middle zone (p=0.004). There was no significant difference in the phosphorylated form at the inner and middle zone (p=0.39).

Experiment 3: There was a significant decrease in mRNA expression of HSF1 in the non-labour control group compared to the non-labour PE at the inner zone (p=0.02). No differences were found in HSF1 mRNA expression at the middle zone.

Experiment 4: There was no significant difference in mRNA expression of HSF1 in the labour control group compared to the labour PE group in the inner or middle placental zones.

3.3.8.6 Summary results for mRNA expression of HSF2

Experiment 1: No spatial differences were found in mRNA expression of HSF2 within placentas in labour or non-labour groups.

Experiment 2: No differences were found in mRNA expression of HSF2 between labour and non-labour groups at the inner, middle and outer zones.

Experiment 3: No difference in mRNA expression of HSF2 was found between non-labour control and non-labour PE at the inner and middle zones

Experiment 4: No difference in mRNA expression of HSF2 was found between labour control and labour PE at the inner and middle zones

Experiment 5: When non labour and labour was combined, there was no significant difference in mRNA expression of HSF1 in the normotensive pregnancies group compared to the pregnancies complicated with PE group in the inner and middle placental zones.

3.4 Discussion

3.4.1 HSP70 and present findings

This study shows for the first time that HSP70 is expressed in a spatial manner in the placenta with the highest expression being in the middle zone in both labour and non-labour groups. The study also shows the importance of using a systematic method to sample the placenta. Previous reports of HSP70 expression, and indeed most studies of expression of other proteins, do not take this into account. Therefore by taking a single sample or averaging protein expression of several samples may well mask possible changes in expression. The increase in HSP70 in labour, PE and FGR at precise zones suggests that there is a controlled spatial change in HSP70 expression. The physiological and pathological significance of these remains to be elucidated but oxidative stress is the common link.

3.4.2 Key findings HSP70

The main findings of this study were that HSP70 is expressed in a spatial manner in the placenta with the highest expression being in the middle zone in both labour and non-labour groups ($p=0.04$). There was a significant increase in protein expression of HSP70 in labour group compared to the non-labour group at the middle zone ($p=0.008$). There was no significant difference in mRNA expression of HSP70 between the labour and the non-labour groups. There was a significant increase in protein expression of HSP70 in non-labour PE compared to non-labour control group ($p=0.02$) at the inner zone. There was no change in protein expression of HSP70 in labour PE compared to the labour control group. No changes were found on mRNA level. However, when non-labour and labour groups combined there was a significant increase in mRNA expression of HSP70 in PE compared to the normotensive group at the middle zone ($p=0.009$). In the FGR study there was a significant increase in protein expression of HSP70 in labour control compared to the labour FGR group at the outer zone ($p=0.04$).

3.4.3 HSF1 and present findings

Heat shock proteins (HSPs) are induced in cells as a protective mechanism to cope with cellular stress. The activation of the heat shock response is mediated by heat shock transcription factor 1 (HSF1). HSFs (HSF1-4) bind to the promoter regions of target genes.

HSF1 exists in a complex with HSP40, HSP70 and HSP90 as inactive monomers; after stress HSF1 is and trimerizes. Two HSF1 bands were observed which; according to the data sheet represent the phosphorylated 80 kDa (active form) and non-phosphorylated 65 kDa (inactive form).

3.4.4 Key findings HSF1

This study shows for the first time that protein expression of HSF1 is spatial distributed in the placenta with the highest expression being in the middle zone in the labour group ($p=0.04$). There was no significant difference in non-labour group. There was a significant increase in non-labour group compared to the labour group for both forms phosphorylated and non-phosphorylated at the inner zone ($p=0.02$, $p=0.04$ respectively) and middle zone ($p=0.01$, $p=0.008$ respectively). No change was found at the outer zone. There was a spatial distribution in mRNA expression of HSF1 in the non-labour group ($p=0.001$). No change was found in the labour group. There was a paradoxical increase in mRNA expression of HSF1 in the labour group compared to the non-labour at the inner zone ($p=0.01$). No difference was found at the middle zone. There was a significant increase in the non-phosphorylated form of labour PE compared to the labour control in the inner and middle zones ($p=0.04$, $p=0.004$ respectively). There was a significant increase in mRNA expression of HSF1 in non-labour PE compared to the non-labour control at the inner zone ($p=0.02$). No other changes were found. When all labour and non-labour groups were combined, there was a significant increase in mRNA expression of HSF1 in PE compared to the normotensive group at the inner and middle zones ($p=0.007$, $p=0.01$ respectively). (Table 3-4) showed comparison in protein and mRNA expression of both HSP70 and HSF1.

Table 3-4: Summary of different comparison in protein and mRNA expression of HSP70 (blue arrow) and HSF1 (red arrow) in non-labour group (NLG), labour group (LG), non-labour-PE (NLG-PE) and labour group-PE at the inner, middle and outer zones of placentas. Both HSF1 forms; phosphorylated (P) and non-phosphorylated (NP) were included. Spatial distribution also was included. Comparison was pointed by arrows to either increase, decrease or no change.

Protein expression	**Inner**	**Middle**	**Outer**
Spatial distribution	Yes for HSP70 both NLG and LG groups, highest in the middle zone Yes for HSF1 (both forms) only LG, highest in the middle zone.		
NLG versus LG	↔ ↑ (both forms)	↓ ↑ (both forms)	↔ ↔
NLG-PE versus NLG-C	↑ ↑ (NP)	↔ ↔	
LG-PE versus LG-control	↔ ↑ (NP)	↔ ↑ (NP)	
mRNA			
Spatial distribution	No for HSP70 Yes for HSF1 only NLG, highest in outer zone.		
NLG versus LG	↔ ↓	↔ ↔	↔ ↔
NLG-PE versus NLG-C	↔ ↑	↔ ↔	
LG-PE versus LG-C	↔ ↔	↔ ↔	

3.4.5 Key findings HSF2

There was no spatial difference in mRNA expression of HSF2. There was no difference when non-labour and labour groups compared at the inner, middle and outer placental zones. There was no difference comparing both non-labour and labour PE group with both

labour and non-labour control. When labour and non-labour groups were combined there was no difference in mRNA expression of HSF2.

3.4.6 HSP70 expression in labour, PE and FGR

The main components of the HSP70 family are HSP72 (HSP 70i) (induced during cell stress) and HSP73 (HSC70) which is constitutively expressed in all cells. Both have very similar amino acid sequences. Both are involved in translocation of proteins from the cytosol into the ER and mitochondria and in protein folding during and after synthesis. Other family members include GRP75 and GRP78. HSP70 can rescue cells from death by inhibiting activation effector caspases (Jäättelä, 1999). There has been some confusion over the use of names for HSPs including HSP70 (Kampinga et al., 2009). The HSP70 used in this study is part of the HSPA family. HSPA1A (also called HSP70-1; HSP72; HSPA1) and HSPA1B (also called HSP70-2) differ by only two amino acids and are believed to be fully interchangeable proteins. Together with HPA6 (Heat shock 70kD protein 6 (HSP70B)) they are the most inducible HSPs. HSPA8 was designated previously as (HSC70; HSC71; HSP71; HSP73) and is an essential "house-keeping" HSPA member and is involved in co-translational folding and protein translocation across intracellular membranes (Kampinga et al., 2009).

Under non-stressful conditions constitutively expressed members of each HSP family are found in almost all organelles including the nucleus, cytoplasm, ER and mitochondria. By interacting with proteins and peptides they play an important role in cell and organ survival. HSPs are induced in response to cell stresses including heat shock, oxidative stress, ultraviolet radiation, IR injury, viral infections, nutrient deprivation, hypoxia, physical damage, ischemia and chemicals. The perils of subsequent protein misfolding are controlled by two mechanisms: (i) the molecular chaperones (including HSPs) that facilitate assembly, folding and translocation of proteins as well as the refolding of denatured proteins and (ii) the ubiquitin-proteasome system which regulates the degradation of misfolded proteins which cannot be renatured (Borges et al., 2012).

Although originally thought to bind directly to the signalling receptors TLR2, TLR4, cluster of differentiation (CD) 40, or CD91 it is now known that HSP70 binds to scavenging receptors including lectin-like oxidized LDL receptor-1 (LOX-1), scavenger receptor expressed by endothelial cell-I (SREC-1), and fasciclin, EGF-like, laminin-type

EGF-like, and link domain-containing scavenger receptor-1 (FEEL-1). On binding to the receptor it is thought that HSP70 then signals to the TLR2 receptor which in turn signals MyD88 activation leading to the phosphorylation of ERK which can trigger the activation of an undetermined transcription factor that will bind the IL-10 gene promoter leading to IL-10 production (Borges et al., 2012). Interestingly IL-10 can be pro-inflammatory at the end of labour and it has been proposed that this inflammatory action of a usually anti-inflammatory cytokine might be an evolutionary adaptation to accelerate parturition and delivery and allow the fetus rapidly to exit a hostile environment (Gibb WL, 2006). Labour is a normal physiological process but PE is a disorder. It is possible in PE that IL-10 production could be a protective response and future *in vitro* studies could determine if this is mediated by HSP70.

Apoptosis has been implicated in both PE and labour. In the apoptotic pathway, HSPs act at several stages to prevent cell death initiated by stress-induced damage. For example HSP70 inhibits CASP 3 and 9. Thus it is possible HSP70 acts to keep the rate of apoptosis in check (Borges et al., 2012).

HSP70 also has anti-inflammatory properties. For example it can bind to, and activate, human monocytes, inhibiting the secretion of inflammatory cytokines, such as TNF-α, IL-1β, and IL-6 (Asea et al., 2000) and increasing secretion of the anti-inflammatory IL-10. Since labour and PE are pro-inflammatory states, HSP70 could again be a protective mechanism to counter-balance this.

There are some publications in which HSP70 expression in the placenta and changes during adverse pregnancy have been reported. The major point to highlight is that controlled sampling and the confounding effects of labour have not been considered. These studies can be summarized as follows.

Divers *et al* (Divers et al., 1995) also performed an immunohistochemical (IHC) study of HSP70 expression in pre-term labour, term labour, term non-labour and pre-term cesarean section for PE or FGR. No changes in HSP70 were reported however different tissues were studied namely amniochorion and basal plate. The placenta itself was not examined and controlled sampling was not performed.

Li *et al* reported increased expression of HSP70 in placenta of what they termed “placental vascular disease” (PE, IUGR and PE plus IUGR all combined in one group) compared

with term non-diseased placentae (Li et al., 1996). All were delivered by caesarean section. No systematic sampling was performed and labour was not studied.

Shah *et al* used IHC to assess HSP70 expression in paraffin sections of placentae from normal term pregnancies and reported immunostaining on cell types, both in cytoplasm and nucleus (Shah et al., 1998). Site of sampling or labour was not assessed.

Ziegert *et al* (Ziegert et al., 1999) reported that HSP70 was expressed in placenta and reported no difference between labour and non-labour however no data or p values were shown to support this statement and no systematic sampling was performed. Similarly (Li et al., 1996) found no difference between labour and non-labour but similar issues applied. Several years ago we examined HSP70 expression in placentas from normal and PE with or without IUGR. At that time no clear difference in expression was found but we too did not control for labour or sampling area (Hnat et al., 2005).

Barut *et al* preformed immunofluorescence on paraffin sections and examined HSP70 expression. HSP70 expression was reported to be increased on STB, CTB and extravillous trophoblast in PE and on endothelial cells in intrauterine growth restriction (Barut et al., 2010). This study did not control for placental site or labour.

Kim *et al* reported that the presence of a uterine artery notch in a mixed group of normal pregnant, PE and PE plus IUGR was associated with increased eNOS and HSP70 in basal plate samples taken from patients who underwent caesarean section (Kim et al., 2010). Placental villous tissue was not studied.

Padmini and Lavanya using western blotting and immunofluorescence found that protein expression of HSP70 was increased in PE endothelial cells when compared to normotensive subjects (Padmini and Lavanya, 2011a).

In addition, the same research group (Padmini and Lavanya, 2011a, Padmini et al., 2012a) using ELISA found that expression of HSP70 was increased by 30.2% in PE endothelial cells when compared to normotensive subjects.

Some studies have examined HSP70 expression in early pregnancy. (Jauniaux et al., 2000) demonstrated a sharp increase in HSP70 during 8-9 weeks of gestation when blood flow to the placenta is initiated. They proposed that the placenta up regulates protective proteins to protect it at this time as the initiation of blood flow represents an oxidative stress insult. (Hempstock et al., 2003) found increased syncytial HSP70 immunostaining in early

pregnancy miscarriage placentae compared with placentae match for gestation from pregnancies terminated for psychosocial reasons. Onset of the maternal circulation is associated with a three-fold rise in the oxygen concentration within the placenta resulting in increased generation of reactive oxygen species. (Jauniaux et al., 2003b) examined HSP70 and nitrotyrosine expression in placentae obtained from surgically terminated pregnancies between 8-13 weeks of gestation. They sampled the inner and outer third. Immunoreactivity for HSP70 and nitrotyrosine residues was greater in samples from peripheral than from central regions of normal placentas and from missed miscarriages compared to controls. They proposed that oxidative damage to the trophoblast, induced by premature onset of the maternal placental circulation is a key factor in early pregnancy loss.

Johnstone *et al* measured HSP70 in purified CTB cells obtained from normal and PE pregnancies (Johnstone et al., 2011). HSP70 was reported to be reduced in the cells from PE cases however labour and site of sampling was not studied. Changes due to the shock of enzyme digestion and cell purification are also confounding factors.

A mechanism which was proposed for increased HSP70 and the link to labour was as follows. Intracellular HSP70 binds to the progesterone receptor, thus functions as a co-repressor of this receptor and suppresses progesterone binding to the nuclear response element (Chaiworapongsa et al., 2008).

Hung *et al* exposed term placenta explants from non-labour cases to IR *in vitro* and showed this led to increased HSP72 expression and this could be reduced in the presence of reactive oxygen species scavengers (Hung et al., 2001).

Malyshev *et al* showed that oxidative stress increases NF-κB which in turn activates nitric oxide synthase, nitric oxide release and subsequently HSP70 induction in several organs (Malyshev et al., 1995). Blocking nitric oxide synthase activity inhibited HSP70 induction. Our lab group have previously shown that villous endothelial eNOS (Myatt et al., 1997), nitrotyrosine (a marker of peroxynitrite produced by superoxide and nitric oxide interaction (Myatt et al., 1996) and 4-hydroxy-2-nonenal adducts (HNE) a marker for lipid peroxidation (Hnat et al., 2005) are both increased in PE as is serum placental nitric oxide accumulation (Lyall et al., 1995). Hence future studies that will investigate the role of nitric oxide synthase in increasing HSP70 in the placenta would be recommended. A study

by (Kakui et al., 2003) reported that protein expression of nitric oxide synthase isoforms were not altered in labour however nitric oxide synthase activity was not investigated.

HSPs can be detected in the circulation. The few reported studies of HSP70 serum concentrations in PE and labour are conflicting (Fukushima et al., 2005, Chaiworapongsa et al., 2008).

3.4.7 HSF1 in labour and PE

HSF1 is a prime coordinator of transcription in stressed cells and involved in a multitude of physiological processes (Vihervaara and Sistonen, 2014). Upon stress HSF1 controls the composition of the entire chaperone machinery and adjusts the expression of ubiquitin through the proteasomal pathway (Vihervaara et al., 2013). HSF1 is the stress responsive transcriptional activator responsible for the inducible transcription of genes encoding HSPs. It binds to regulatory heat shock elements present in the promoter region of all heat shock genes (Yao et al., 2006). It has been shown that active HSF is stably bound to DNA and any turnover of transcription activator is not required for rounds of HSP70 transcription (Yao et al., 2006).

The expression of HSF1 was investigated. Similar to HSP70 there was a spatial distribution in protein expression of HSF1 in the labour group for both isoforms with the highest being in the middle zone. There was no change in HSF1 protein expression in the non-labour placentas. However, at the mRNA level there was spatial differences only in non-labour group. The complexity of these changes makes it difficult to make clear conclusions however the middle site appears to be the site that, for reasons to be explained, shows more changes. Also differences in labour and non-labour may also relate to the fact that labour is a large stress compared to non-labour. Further difficulties in explaining the findings relate to what causes the initial labour contractions to start versus the consequences of labour. The fact that mRNA did not always parallel the protein changes may also be linked to the same explanation as well as possible differences in mRNA/protein stability/turnover in different zones and in labour versus non-labour. Overall it can be seen that changes in mRNA and protein are complex and the most important conclusion of all is the need for researcher to be consistent in sampling methods within their own studies and if comparisons are to be made with other studies then it is important that everyone agrees on a sampling methods. To date despite much discussion on this, this has not been achieved.

There are several post translational modifications sites on HSF1 including phosphorylation, acetylation and sumoylation sites on different serine and threonine sites that can be activated by stress active kinases resulting in either activation or inhibition (Xu et al., 2012). HSF1 is strongly induced during stress condition and the phosphorylation level of HSF1 correlates well with its transcriptional activity (Seo et al., 2006). Our findings show that both isoforms of HSF1 were increased in the non-labour groups compared to the labour at the inner and middle zones, this may be explain that in labour it is possible that HSF1has increased and then retained to normal level some time before placenta was delivered. This is correlated with our results that have been shown increased in HSP70, which induced by HSF1 in labour group compared to the non-labour (Abdulsid et al., 2013b).

The non-phosphorylated form of HSF1 was increased in the non-labour and labour PE groups compared to the non-labour and labour control groups. Diminished or poor placental function in PE increases the chances of apoptosis (Allaire et al., 2000). HSP70 is an anti-apoptotic protein that can influence the apoptotic cascade at multiple sites. Over expression of HSF1 and HSP70 are suggesting their protective role in relation to the oxidative stress generated in PE.

Disruption of HSF1 results in reduced constitutive expression of several HSPs, which is intimately associated with impaired redox homeostasis and mitochondrial damage (Yan et al., 2002). There are few studies on HSF1 expression in placentas and PE most of them are from one research group (Padmini and Lavanya, 2011a, Padmini and Lavanya, 2011b, Padmini et al., 2012a, Padmini et al., 2012b).

Padmini *et al* (Padmini and Lavanya, 2011b) showed that non-phosphorylate HSF1 (NP-HSF1) in normotensive women was detected mainly in cytoplasm, whereas phosphorylate HSF1 (P-HSF1) in PE women was detected at high level in the nuclear fraction. Systematic sampling and labour were not included.

Padmini *et al* (Padmini and Lavanya, 2011a, Padmini and Lavanya, 2011b) reported increased nuclear fraction of phosphorylated HSF1 protein expression in placentae from PE cases compare with uncomplicated pregnancies. As shown from this study not controlling for labour or sampling zone might alter the findings.

Oddly the same group (Ramayya et al., 1997) reported that there was no mRNA expression of HSF1 in placenta. However, in our study mRNA expression was found.

3.4.8 HSF2 in labour and PE

HSF2 is not activated in response to classical stress stimuli, but under developmentally related conditions. There was no spatial distribution on protein or mRNA expression of HSF2 in non-labour and labour groups, also there was no significant differences when comparing different groups at different placental zones. As HSF2 appears to have a role in development and differentiation but not stress it would be of interest to follow the expression of HSF2 in placental zones throughout gestation. In addition, it clear that HSF1 and HSF2 function in response to stress are not linked to each other.

Chapter IV: HSP27 and P-HSP27 expression in normal pregnancies and pregnancies complicated with PE and FGR (HSP27 only).

4 Introduction

Heat-shock proteins (HSPs) are a family of proteins expressed by all cells. HSPs are chaperones important in the folding, assembly and degradation of proteins. Their expressions allow cells to survive to sub-lethal conditions by binding to partially denatured proteins, dissociating protein aggregates and regulating correct folding (Sarto et al., 2000). Heat shock proteins are expressed in response to a wide variety of physiological and environmental insult such as heat, inflammation, reactive oxygen species, ischemia and some anticancer drugs (Sarto et al., 2000, Macario and Conway, 2006, Lanneau et al., 2010). HSPs also play an important role in regulating signal transduction, inhibition of apoptosis and cellular proliferation and differentiation. HSP27 is a molecular chaperone and several different mechanisms involve in their cytoprotective activity.

4.1 Heat shock protein 27 (HSP27)

HSP27 (also known HSPB1) belongs to the family of small heat shock proteins (sHSPs) (15-30 kDa) which include ubiquitin; α-crystallin and HSP20 (Schmitt et al., 2007). HSP27 is ATP-independent and protects against stress-induced aggregation of proteins. HSP27 present in many cell types especially all types of muscle cells. It is over expressed during different stages of cell differentiation and development and becomes phosphorylated in response to various stimuli through interaction with different kinases (Sarto et al., 2000). In response to stress, changes in expression of HSP27 occurs and, like many proteins, HSP27 function can also be regulated by at the post-translational level (Ghayour-Mobarhan et al., 2012).

4.1.1 HSP27 gene structure

One of the best studied small HSPs is HSP27 (Kampinga et al., 2009). HSP27 contains two terminals (Figure 4-1). The first one is less conserved and referred to as the N-terminus and contains a WDPF motif necessary for multimer formation and protein

oligomerization. Multimers are reduced to dimers by phosphorylation of consensus MK2 phosphorylation sites at serine residues (ser) 15, 78 and 82 (Ghayour-Mobarhan et al., 2012). The second terminal is the C-terminus which includes α-crystallin motif that is highly conserved among species. The crystallin domain is necessary for dimer formation, while the flexible region has been suggested to participate in the interaction with target proteins, to be involved in oligomerization, and to be important for solubility (Salinthone et al., 2008, Gusev et al., 2002). The sHSPs are often found in oligomeric complexes involving one or more family members.(Lelj-Garolla and Mauk, 2005) HSP27 activation is stimulus dependent.

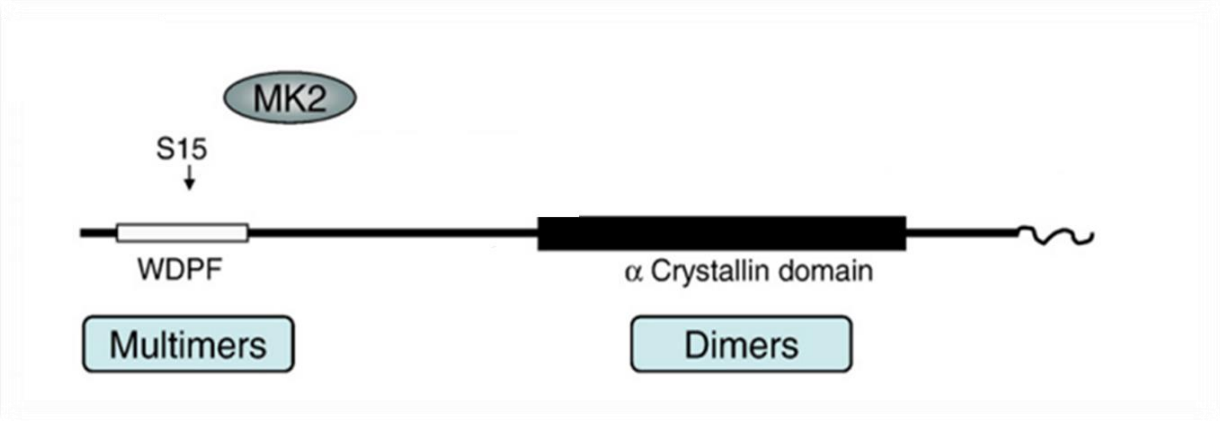


Figure 4-1: HSP27 gene structure. The N-terminus of HSP27 contains a hydrophobic WDPF motif, which is adjacent to a mitogen-activated protein kinases associated protein (MAPKAP) kinases (MK2) phosphorylation site at Ser15. The C-terminal 18–20 amino acids include an α-crystallin motif that is highly conserved among species and a flexible C-terminal tail modified from (Salinthone et al., 2008).

4.1.2 Post-translational modifications

HSP27 undergoes different types of post-translation modification. HSP27 is the main methylglyoxal modified protein in different cancer cells that are characterized by high level of glycolysis (Sakamoto et al., 2002). Under conditions of oxidative stress and during ischemia and reperfusion, HSP27 undergoes S-thiolation (Eaton et al., 2002). HSP27 undergoes multisite phosphorylation at three serine sites (ser 15, 78 and 82), which is named as phosphorylated-HSP27 (P-HSP27). HSP27 can oligomerize into large aggregates up to 800 kDa, but it can also form hetero-oligomers with other sHSP family members (e.g. HSP20 and αA-crystallin). This phosphorylation causes a decrease in

HSP27 oligomers size (Rogalla et al., 1999). Large oligomers are the structural organization of HSP27 required for its anti-apoptotic activity, chaperone activity and protection against oxidative stress (Rogalla et al., 1999, Bruey et al., 2000b, Mehlen et al., 1997, Kato et al., 1994b). Regulation of these modification control by cellular signalling pathways of HSP27 and involve archetypal phosphorylation cascades, such as MAPKAP and PRAK. (Ghayour-Mobarhan et al., 2012) that can be induced by a variety of stress including differentiating agents, mitogens, inflammatory cytokines such as TNF (Döppler et al., 2005, Garrido, 2002). Phosphorylation of HSP27 has been shown to result in complex dissociation and the subsequent loss of chaperoning activity (Welsh and Gaestel, 1998). Activation of phosphatases and inhibition of upstream signalling pathways are involoving in inhibition of HSP27 phosphorylation (Cairns et al., 1994). Phosphorylation of ser-15 seems to induce only a small effect on oligomerization (Kostenko and Moens, 2009). The residues corresponding to ser-15 and ser-82 in human HSP27 are conserved throughout the animal kingdom. It was reported (Stokoe et al., 1992, Paul et al., 2010) that ser-82 is phosphorylated much faster than either ser-15 or ser-78. Oligomerization of HSP27 is affected by the phosphorylation pattern but how phosphorylation governs the oligomerization process is not known (Henderson and Pockley, 2012).

It was reported, (Bruey et al., 2000b, Garrido, 2002) that in tumour cells growing *in vivo* or grown at confluence *in vitro*, cell-cell contacts induce the formation of large oligomers, whatever the phosphorylation status of the protein. These post-translational modifications are observed within minutes after cellular stress. This rapidity of modification implicates HSP27 in the early response even before HSP27 gene transcriptional activation (Jozefowicz-Okonkwo et al., 2009). One study (Henderson and Pockley, 2012) revealed that stimulus-induced HSP27 phosphorylation is often transient, so that HSP27 phosphatases are also implicated in controlling the phosphorylation status of HSP27.

4.1.3 Functions of HSP27

There are several intracellular function of HSP27 such as chaperone activity, control of apoptotic signalling, regulation of cell glutathione levels, inhibition of actin polymerization, stabilization of actin filament arrays as well as protection against heat shock, oxidative stress and mechanical stress (Brown et al., 2007, Ghayour-Mobarhan et al., 2012). HSP27 is expressed during both skeletal and cardiac muscle development and has been shown to be involved in a diverse array of cellular processes (Brown et al., 2007). HSP27 can bind to proteins and affect their function, and there is evidence that HSP27

inhibits enzymes in the apoptotic pathway (Ghayour-Mobarhan et al., 2012). HSP27 also plays a role in atherosclerosis (Martin-Ventura et al., 2004), in regulation of cytokine production from monocytes as well as expression of toll-like receptors (De et al., 2000), and may play a role in protecting the immature human myocardium in response to stress during open heart surgery (Walker et al., 2013).

4.1.3.1 HSP27 and chaperone function

HSP27 act as a protein chaperone by binding to unfolded proteins and preventing their aggregation (Knauf et al., 1994). Un-phosphorylated HSP27 forms large multimers, which are responsible for protection against cellular stress, while phosphorylation results in conformational changes and formation of smaller di- and tetrameric units (Kato et al., 1994b) and in cooperation of other chaperones allow the target proteins to regain their native structure (Ehrnsperger et al., 1997).

4.1.3.2 HSP27 and actin filament functions

In addition to its chaperone function, HSP27 also play an important role as a regulator of structural integrity and membrane stability, cytoskeleton formation, cell cycle progression, pro-inflammatory gene expression, muscle contraction, signal transduction pathways and apoptosis (Welsh and Gaestel, 1998, Charette et al., 2000, Gerthoffer, 2005, Garrido et al., 2006, Stetler et al., 2008). Under stress, phosphorylation of HSP27 has been shown to improve cytoskeleton stability in cells that are known to express constitutively wild type HSP27 (Lavoie et al., 1995). One of important functions of P-HSP27 is the regulatory role of P-HSP27 in actin filament functions and their associated processes such as muscle contraction, cell division, differentiation and migration (Kostenko et al., 2009). In its un-phosphorylated form, HSP27 inhibits actin polymerization while in P-HSP27 allow small oligomers to bind to actin and stabilizing them (Dominguez and Holmes, 2011). HSP27 also protect cytoskeleton of neonatal cardiac cells (van de Klundert et al., 1998a).

4.1.3.3 HSP27 and anti-apoptotic function

Un-phosphorylated and phosphorylated HSP27 exhibit anti-apoptotic functions via different mechanisms. P-HSP27 binds to Daxx and inhibits TNFα and Fas-mediated apoptosis or stimulates the pro-survival Akt pathway (Benn et al., 2002, Arrigo, 2000, Charette et al., 2000, Garrido et al., 2006), activates protein kinase Akt, phosphorylating Bax which prevents pore formation in the outer mitochondria membrane and thus prevents

liberation of cytochrome c from mitochondria (Havasi et al., 2008). HSP27 also seems to inhibit the Ask1-JNK pathway which through Bax leads to cytochrome c leakage (Stetler et al., 2009). Un-phosphorylated HSP27 can block apoptosis pathways as it inhibit the activation of pro-caspase-9 by its interaction with cytochrome c upon its release from the mitochondria, thus preventing the correct formation and function of the apoptosome complex (Bruey et al., 2000a, Garrido et al., 2006, Paul et al., 2010, Lanneau et al., 2007, Concannon et al., 2003). In the extrinsic apoptotic pathway, HSP27 prevents translocation of activated Bid to the mitochondrial membrane and thus inhibits apoptosis (Arya et al., 2007). In addition, HSP27 activation of transcriptional activity of NF-κB can be another pathway providing for anti-apoptotic activity of HSP27 (Mymrikov et al., 2011). However, other studies have also shown increased HSP27 protection via mechanisms other than interaction with the apoptotic processes (Paul et al., 2002).

4.1.4 HSP27 and oxidative stress

Oxidative stress is accompanied by increased levels of toxic ROS, such as peroxides and free radicals. HSP27 has important anti-oxidant function by maintaining glutathione in its reduced form, to decrease ROS (Arrigo, 2007). Un-phosphorylated HSP27 reduced ROS production, enhanced glutathione levels, and provided cellular protection against oxidative stress (Mehlen et al., 1997). HSP27 and αB-crystallin decrease the intracellular level of iron, and as a results they interfere with formation of hydroxyl radicals via the Fenton reaction (Arrigo, 2007). Cell culture of hippocampal HiB5 cells expressing high level of HSP-2D (where ser-15 is not substituted by asp) displayed better survival rates and actin stability against heat shock than cells over-expressing HSP27-2A (Geum et al., 2002). This showed that phosphorylation of HSP27 is important for maintenance of the actin fibres and thus enhances thermos-resistance (Geum et al., 2002). Furthermore, demonstrated that HSP27 protected cells against oxidative stress (Rogalla et al., 1999).

HSP27 can also protect against oxidative stress by the nonspecific chaperone-like activity that prevents aggregation of partially denatured proteins by their renaturation or proteasome-dependent degradation.

4.1.5 HSP27 and ER stress

HSP27 was reported to be induced as a result of ER stress (Ito et al., 2005). There are several conditions that lead to disturbance in ER homeostasis for example, protein misfolding, changes in calcium intracellular levels and oxidative stress, evolve an ER

stress response in order to cope with the protein load in the organelle (Zhang and Kaufman, 2004). The ER has evolved highly specific signalling pathways that causes a general attenuation of translation, a transcriptional activation of chaperones, and the ER associated degradation (ERAD) of misfolded proteins by the proteasome; this response is named unfolded protein response (UPR) (Kaufman, 2002). HSP27 is involved in proteasome-apoptotic pathway *in vitro* and *in vivo* by binding to polyubiquitin chains and to the 26S proteasome (Parcellier et al., 2003). The ubiquitin-proteasome pathway is involved in the activation of transcription factor (NF-κB) by degrading its inhibitor (I-κBα). Overexpression of HSP27 increases NF-κB nuclear rearrangement, the degradation of phosphorylated I-κBα was dependent on the interaction of HSP27 with the 26S proteasome (Parcellier et al., 2003). It was shown (Kato et al., 2002) that P-HSP27 and αB-crystallin are recruited to protein aggresomes in muscle atrophy. This finding is suggested to be the ability of HSP27 to bind to ubiquitin (Parcellier et al., 2005).

4.1.6 HSP27 and Disease

HSP27 has a role in human pathogenesis (Figure 4-2) for example; HSP27 expression levels are increased in many cancer cells and their expression contributes to the malignant properties of cancer cells such as increased tumorigenicity and treatment resistance, and inhibition of apoptosis (Calderwood et al., 2006, Wyttenbach et al., 2002, Kostenko and Moens, 2009). HSP27 expression associated with pathogenesis of neurodegenerative disorders, inherited peripheral neuropathy and cardiovascular disease (Ferns et al., 2006, Welsh and Gaestel, 1998, Houlden et al., 2008). HSP27 also has been localized to the extra-cellular compartment (Calderwood et al., 2007a). In animal studies enhanced HSP27 phosphorylation is associated with nephrotic syndrome and diabetic nephropathy (Smoyer et al., 1996, Park et al., 2008). Animal studies showed that HSP27 also play an important role in prevention of pulmonary oedema and viral infection (Liu et al., 2009, Singh et al., 2007).

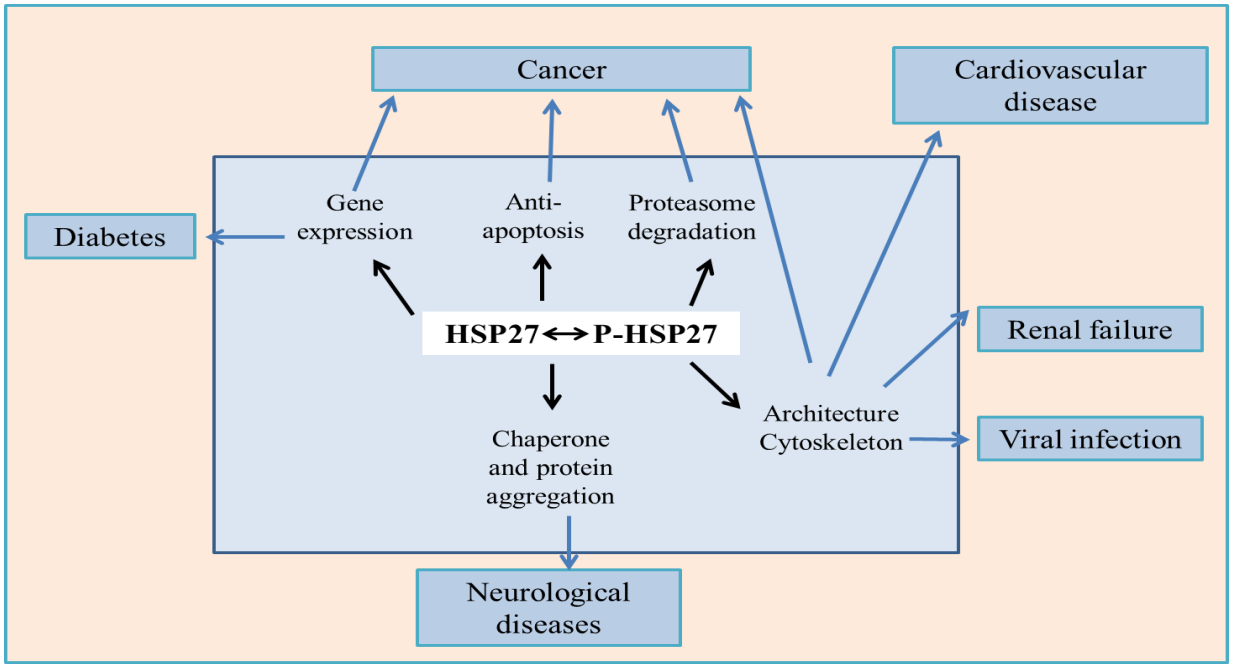


Figure 4-2: P-HSP27 functions and its possible implication in diseases. HSP27 may be present in un-phosphorylated and phosphorylated forms depending on the cellular stress conditions. The central part is referred to the normal function of HSP27 and P-HSP27 (pink). The outer part is referred to the pathogenic conditions (blue) adapted from (Henderson and Pockley, 2012).

4.1.7 HSP27 as a therapeutic agent

HSP27 regulates multiple processes in cells and therefore, it may be a potentially important therapeutic target. For example, atovastin which modulates HSP27 phosphorylation can be potentially useful in the case of ischemic heart disease (Brundel et al., 2008). Electro-injection of HSP27 in retinal ganglion cells protects them from apoptosis and can be a treatment agent of some neurodegenerative disorders (O'Reilly et al., 2010). HSP27 probably can protect hepatocytes against liver IR injury (Ye et al., 2011). HSP27 affects many different processes and uncontrolled increase in HSP27 expression correlates with increased resistance to anticancer drugs.

4.2 Results

4.2.1 Patient clinical data analysis

Patient data analysis is the same as section (3.4.1.1), results analysis is shown in (Table 3-2) (Table 3-3).

4.2.2 Analysis of protein and mRNA HSP27 expression in normal pregnancy

Experiment 1: This experiment was designed to test if there was a spatial difference in protein expressions of HSP27 within individual placentas obtained from women who were not in labour (non-labour group) and from women who were in labour (labour group). First the non-labour group was compared. Examples of Western blots showing HSP27 protein expressions for the non-labour are shown in (Figure 4-3). The graphs and statistical analysis are shown below the blots. Comparison between zones was performed using Friedman test analysis. There was a significant difference in protein expression of HSP27 between the three zones of the non-labour group and the labour group (p=0.04 for both). The sub-analysis showed that there was a significant decrease in HSP27 expression in inner zone compared to the middle zone for non-labour group (p=0.02).

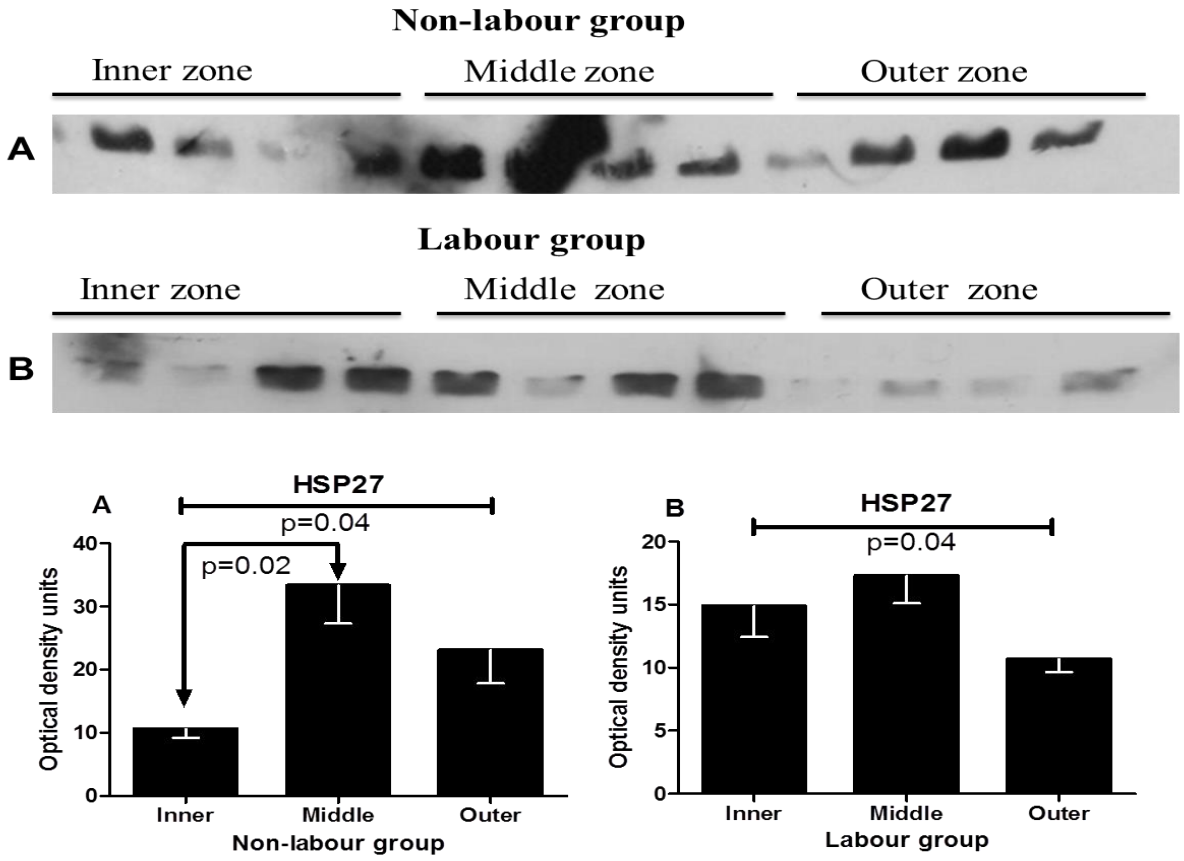


Figure 4-3: Representative images of Western blots showing HSP27 protein expressions in inner, middle and outer zones of individual placentas of non-labour group (A) and labour group (B). Four quadrants were sampled in each zone. Graphs show the median for HSP27 protein expressions. Comparison between zones was performed using Friedman analysis.

Experiment 2: This experiment was designed to test if there was any difference in protein expression of HSP27 between labour and non-labour groups at the inner, middle or outer zones of placentas. Western blots showing placental protein expression of HSP27 in the non-labour and the labour at the inner, middle and outer zones are shown in (Figure 4-4). The graphs and statistical analysis are shown below the blots. There was a significant decrease in HSP27 expression in the labour group when compared to the non-labour group at the middle zone (p=0.002). There was no significant difference in HSP27 expression in the non-labour group when compared to the labour group at the inner and outer zones (p=0.93, p=0.69 respectively).

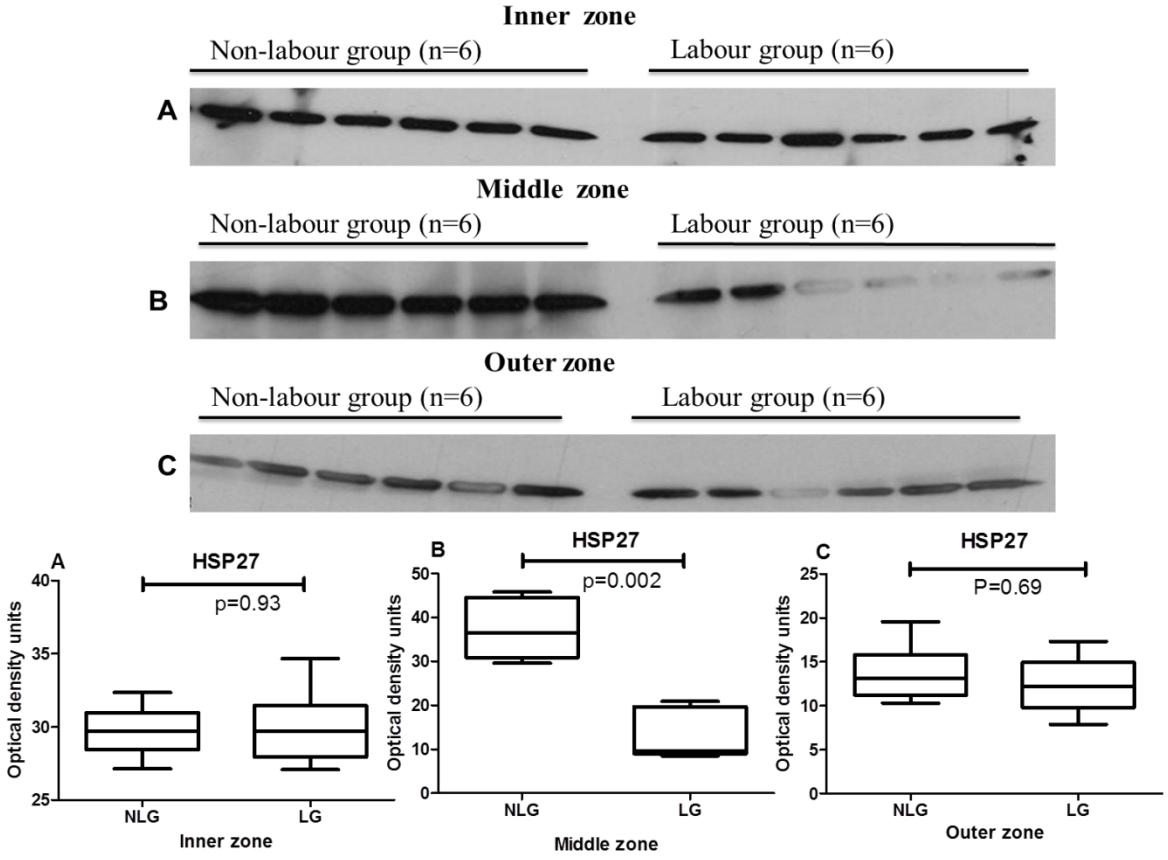


Figure 4-4: Representative images of Western blots showing HSP27 expressions in the inner, middle and outer placental zones in non-labour and labour (n=6 in each group). The graphs show the box and whiskers analysis of the blots. Comparison between zones was performed using Mann-Whitney analysis. NLG non-labour group, LG labour group.

Experiment 3: This experiment was designed to test if there were any differences in mRNA expression of HSP27 within individual placentas at different zones (inner, middle and outer) in either labour or non-labour. No spatial differences were found within placentas at the mRNA level in non-labour group and labour group (p=0.74, p=0.95). (Figure 4-5)

shows the HSP27 RQ values in the inner, middle and outer zones for non-labour (A) and labour (B).

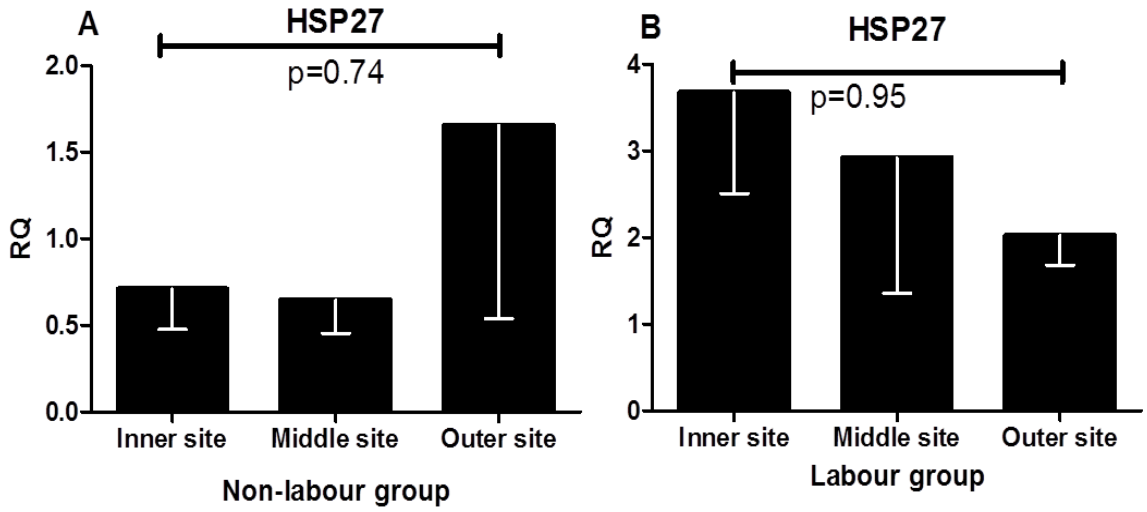


Figure 4-5: RQ values for HSP27 mRNA measurements in in inner (n=4), middle (n=4) and outer zones (n=4) of individual placentas (non-labour and labour groups). Four quadrants were sampled in each zone. Graphs show the median for HSP70 mRNA expressions. Comparison between zones was performed using Friedman analysis.

Experiment 4: This experiment was designed to test if there were any differences in mRNA expression of HSP27 in the non-labour compared to the labour group at the inner, middle and outer zones. (Figure 4-6) shows the HSP27 RQ values in the non-labour compared to the labour group at the inner (A), middle (B) and outer zones (C). There was a significant decrease in mRNA expression of HSP27 in the labour group compared to the non-labour group at the inner and middle zones (p=0.02, p=0.008 respectively). No difference was found at the outer zone (p=0.1).

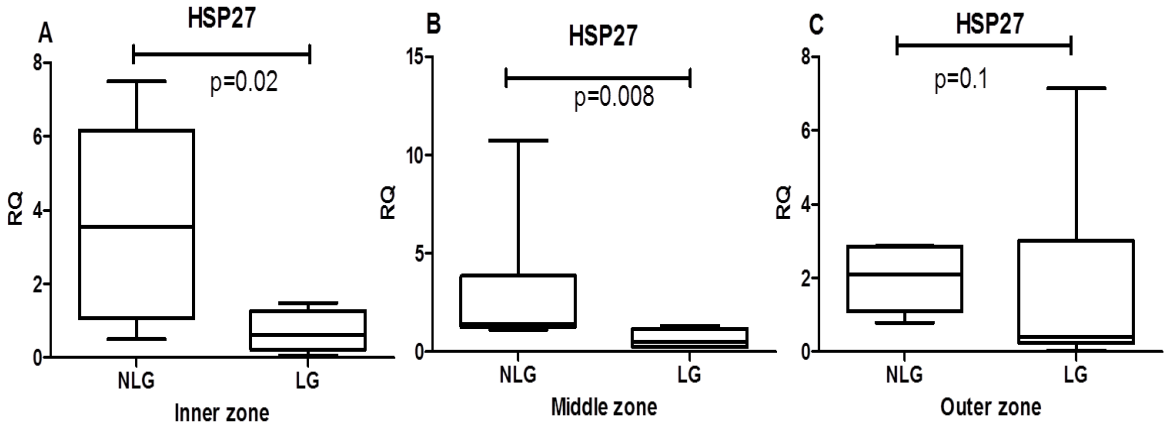


Figure 4-6: RQ values for HSP27 mRNA measurements in non-labour (n=6) compared to the labour group (n=6) at the inner, middle and outer placental zones. Comparison between zones was performed using Mann-Whitney analysis.

4.2.3 Analysis of protein and mRNA expression of HSP27 in pregnancy complicated with PE.

Experiment 1: This experiment was designed to test if there was any difference in protein expression of HSP27 between non-labour control and non-labour PE groups at the inner, middle and outer zones of placentas. Western blots showing placental HSP27 protein expression in the non-labour control and the non-labour PE at the inner, middle and outer zones are shown in (Figure 4-7). The graphs and statistical analysis are shown below the blots. There was no significant difference in HSP27 expression in the non-labour control group when compared to the non-labour PE group at the inner, middle and outer zones (p=0.16, p=0.60, p=0.43 respectively).

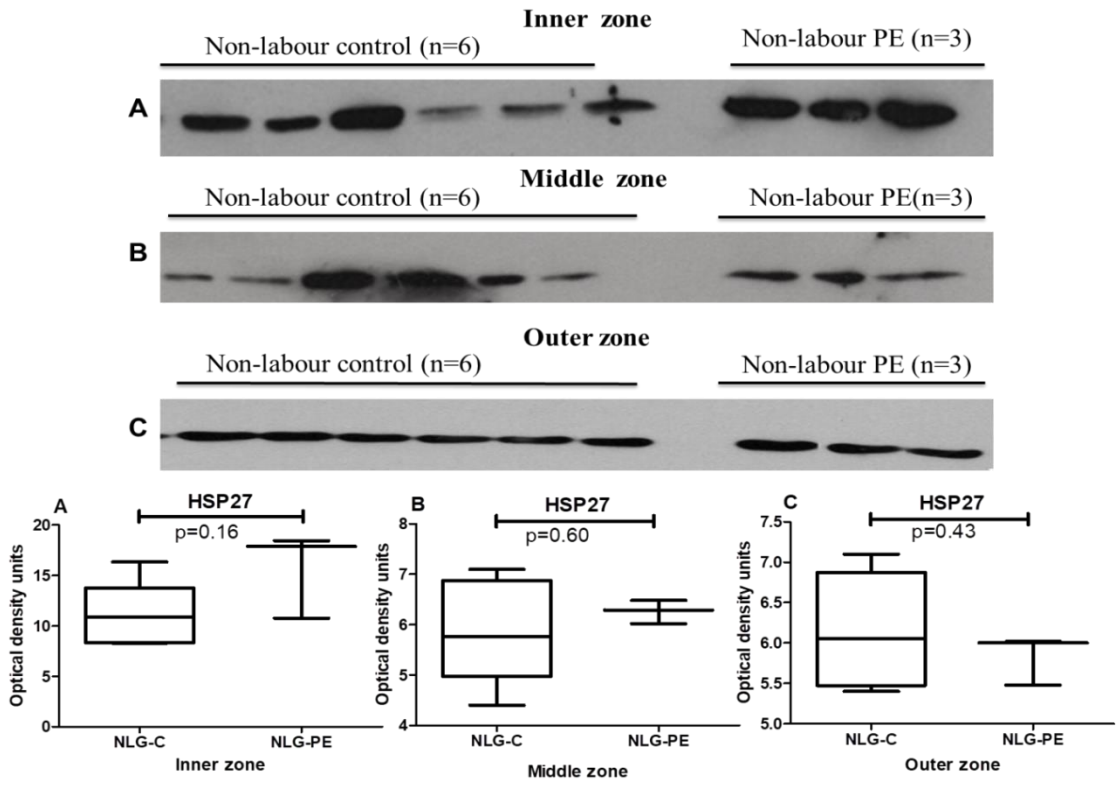


Figure 4-7: Representative images of Western blots showing HSP27 expressions in the inner, middle and outer placental zones in non-labour control (n=6) and non-labour PE (n=3). The graphs show the box and whiskers analysis of the blots. Comparison between zones was performed using Mann-Whitney analysis. 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 HSP27 expression between labour control and labour PE groups at the inner, middle and outer zones of placentas. Western blots showing placental HSP27 protein expression in the labour control and the labour PE at the inner, middle and outer zones are shown in (Figure 4-8). The graphs and statistical analysis are shown below the blots. There was a significant decrease in HSP27 expression in labour control compared to the labour PE at the middle and outer zones (p=0.01, p=0.04 respectively). No difference was found in HSP27 expression at the inner zone (p=0.9).

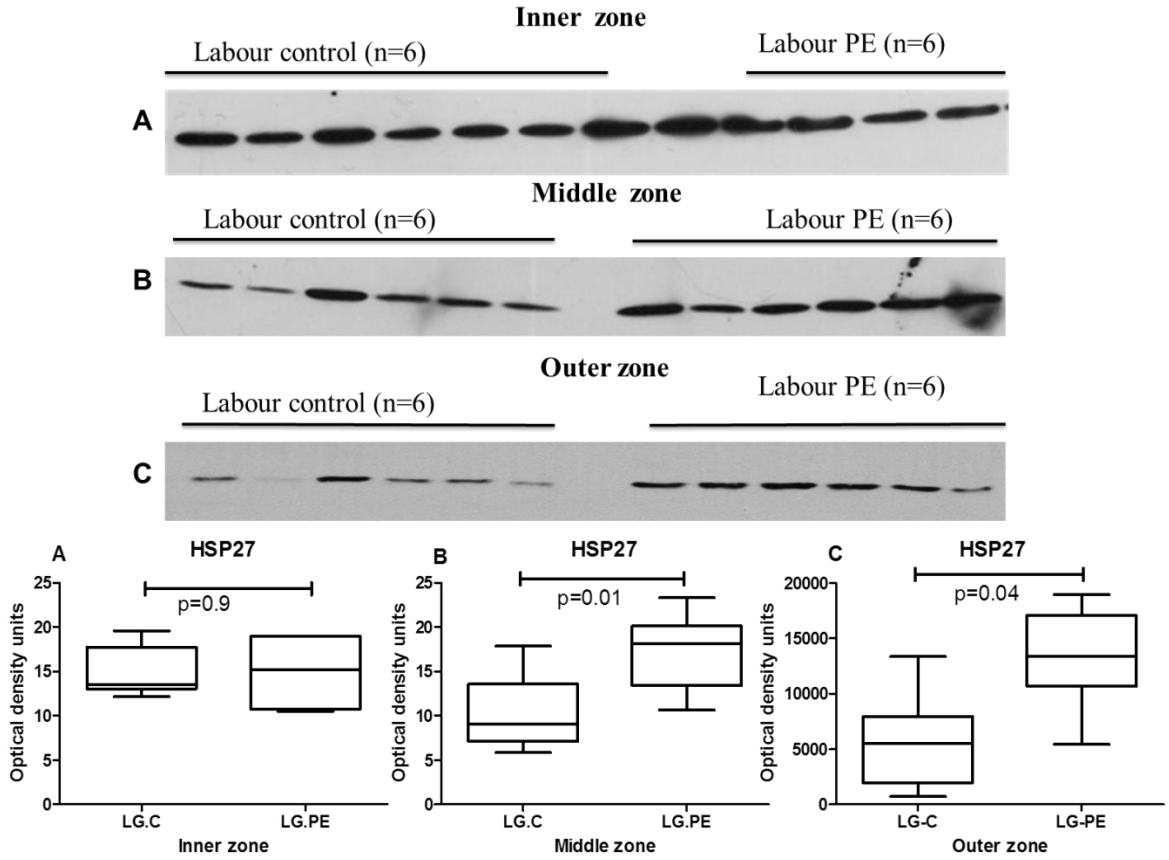


Figure 4-8: Representative images of Western blots showing HSP27 expressions in the inner, middle and outer placental zones in labour control and labour PE groups (n=6 for each group). The graphs show the box and whiskers analysis of the blots. Comparison between zones was performed using Mann-Whitney analysis. LG-C labour control group, LG-PE labour PE group.

Experiment 3: This experiment was designed to test if there were any differences in mRNA expression of HSP27 in the non-labour control compared to the non-labour PE group at the inner, middle and outer placental zones. (Figure 4-10) shows the HSP27 RQ values in the

non-labour control compared to the non-labour PE group at the inner (A), middle (B) and outer (C) zones. There was no significant difference in HSP27 mRNA in the non-labour control group compared to the non-labour PE at the inner, middle and outer zones (p=0.9, p=0.4, p=0.71 respectively).

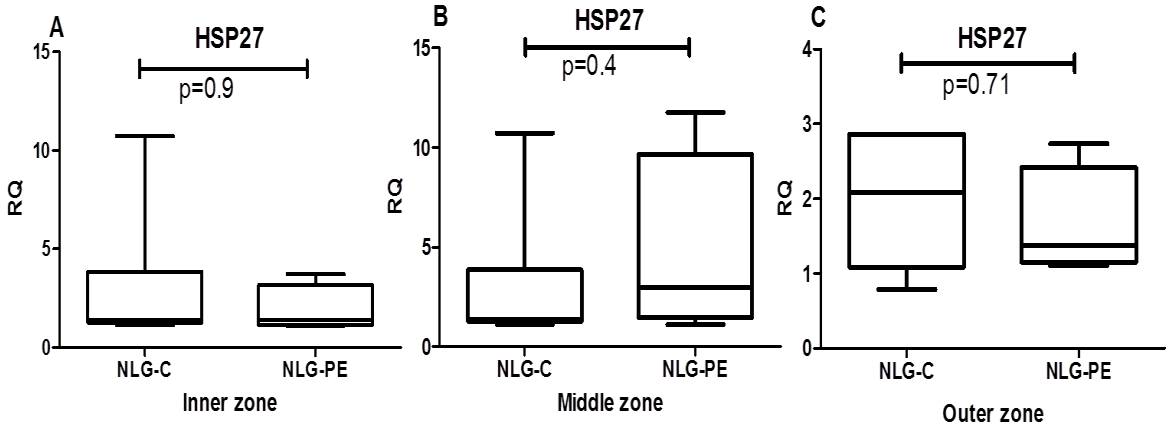


Figure 4-9: RQ values for HSP27 mRNA measurements in inner, middle and outer placental zones for the non-labour control group (n=6) compared with non-labour PE groups (n=4). Comparison between zones was performed using Mann-Whitney analysis.

Experiment 4: This experiment was designed to test if there was any difference in mRNA expressions of HSP27 between labour control and labour PE groups at the inner, middle and outer zones of placentas. (Figure 4-11) shows the HSP27 RQ values in the labour control compared to the labour PE group at the inner (A), middle (B) and outer (C) zones. Comparison between zones was performed using Mann Whitney test analysis. There was no significant difference in mRNA expression of HSP27 in the labour control group compared to the labour PE group in the inner, middle and outer placental zones (p=0.5, p=0.3,p=0.30 respectively).

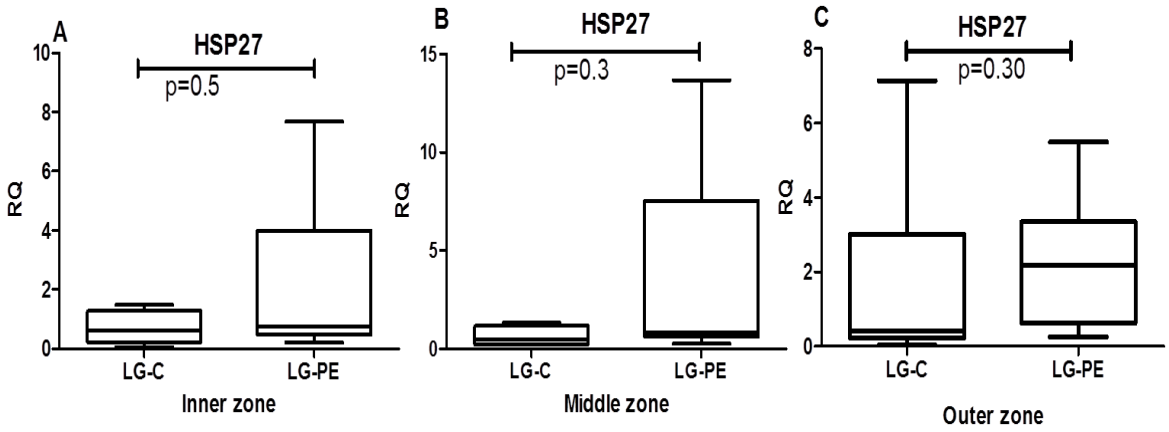


Figure 4-10: RQ values for HSP27 mRNA measurements in inner, middle and outer placental zones for labour control group (n=6) compared with labour PE groups (n=6). Comparison between zones was performed using Mann-Whitney analysis.

Experiment 5: This experiment was designed to test if there was any difference in mRNA expressions of HSP27 between normotensive pregnancies and pregnancies complicated with PE regardless the labour factor at the inner, middle and outer zones of placentas. (Figure 4-12) shows the HSP27 RQ values in the labour control compared to the labour PE group at the inner (A), middle (B) and outer (C) zones. Comparison between zones was performed using Mann Whitney test analysis. There was no significant difference in mRNA expression of HSP27 when the normotensive pregnancies group compared to the pregnancies complicated with PE group in the inner, middle and outer (p=0.9, p=0.72, p=0.72 respectively).

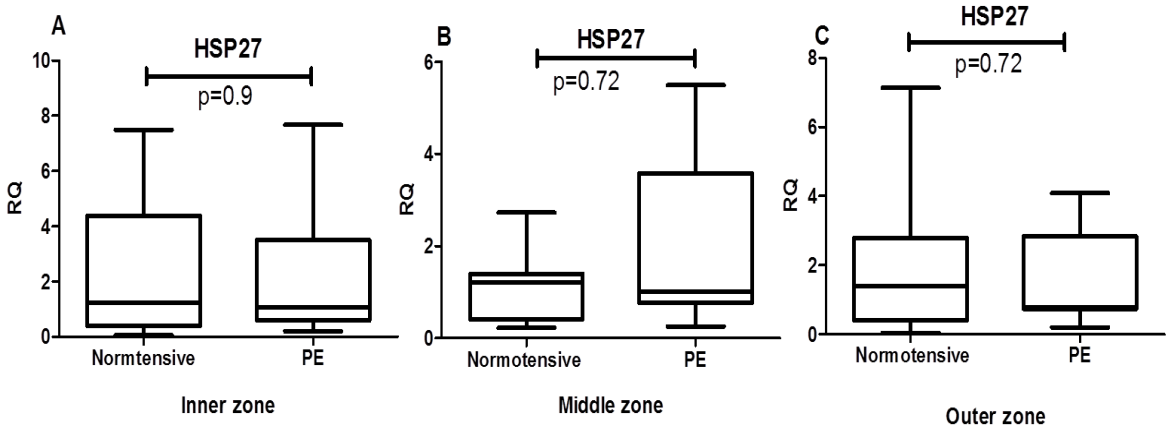


Figure 4-11: RQ values for HSP27 mRNA measurements in normotensive pregnancies group (n=12) compared to the pregnancies complicated with PE group (n=9) at the inner, middle and outer placental zones. Comparison between zones was performed using Mann-Whitney analysis.

4.2.4 Analysis of protein and mRNA expression of HSP27 in pregnancies complicated with FGR.

Experiment 1: This experiment was designed to test if there was any difference in HSP27 expressions between labour control and labour FGR groups at the inner or outer zones of placentas. Due to time constraints only one set of experiments was performed for labour groups and in the inner and outer zones only. The aim was to determine whether pilot data from this group of experiments revealed any data suggesting that more experiments were warranted. Western blots showing placental HSP27 expression at the inner zone (upper panel) and placental HSP27 expression at the outer zone (lower panel) in labour control and labour FGR are shown in (Figure 4-13). The graphs and statistical analysis are shown below the blots. There was no significant difference in the HSP27 protein expression between the labour control and labour FGR groups at the inner or outer zones (p=0.09, p=0.81 respectively).

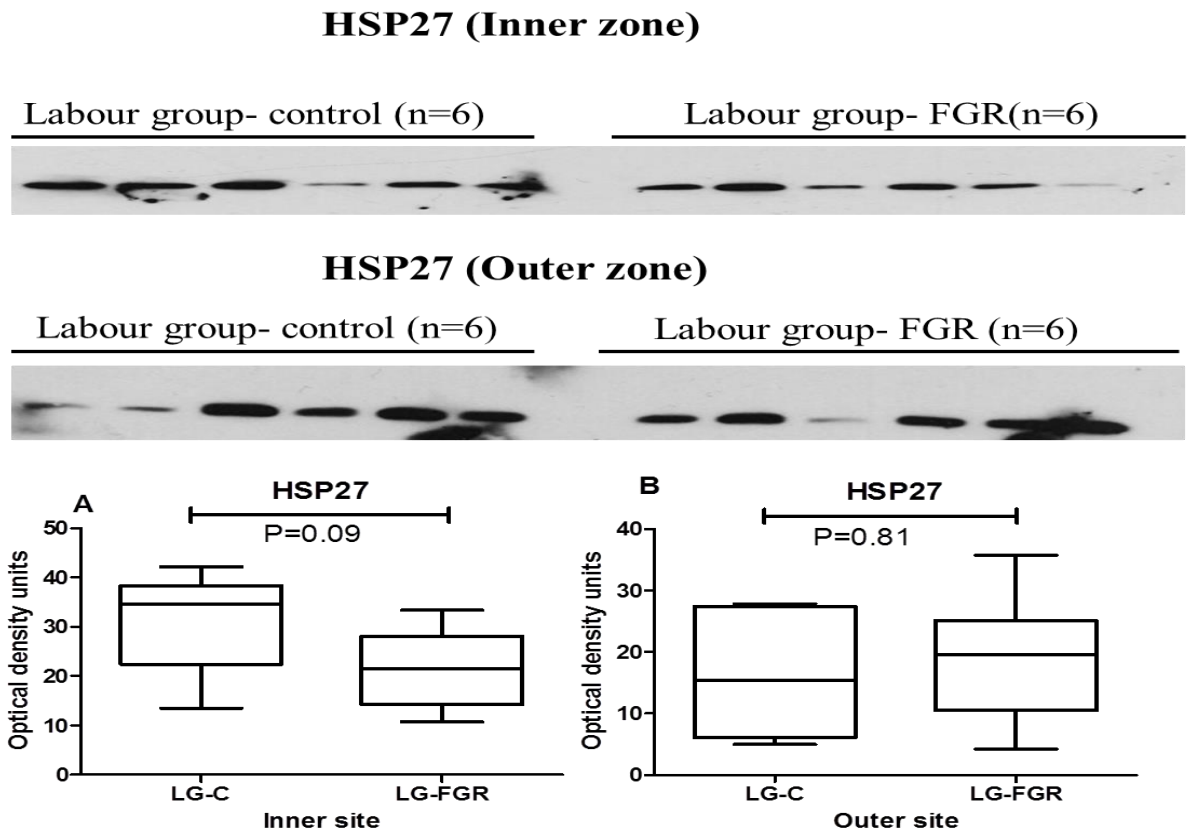


Figure 4-12: Representative images of Western blots showing HSP27 expression at the inner zone (upper panel) and the outer zone (lower panel) in labour control (n=6) and labour FGR group (n=6). The graphs show the box and whiskers analysis of the blots. Comparison between zones was performed using Mann-Whitney analysis. LG-C labour group control, LG-FGR labour group fetal growth restriction.

Experiment 2: This experiment was tested whether there was any difference between labour control and labour FGR of mRNA expression of HSP27 at the inner or outer zones. The results are shown in (Figure 4-14). There was a significant decrease in HSP27 mRNA expression in the labour control compared to the labour FGR groups at the inner and the outer zones (p=0.008, p=0.02 respectively).

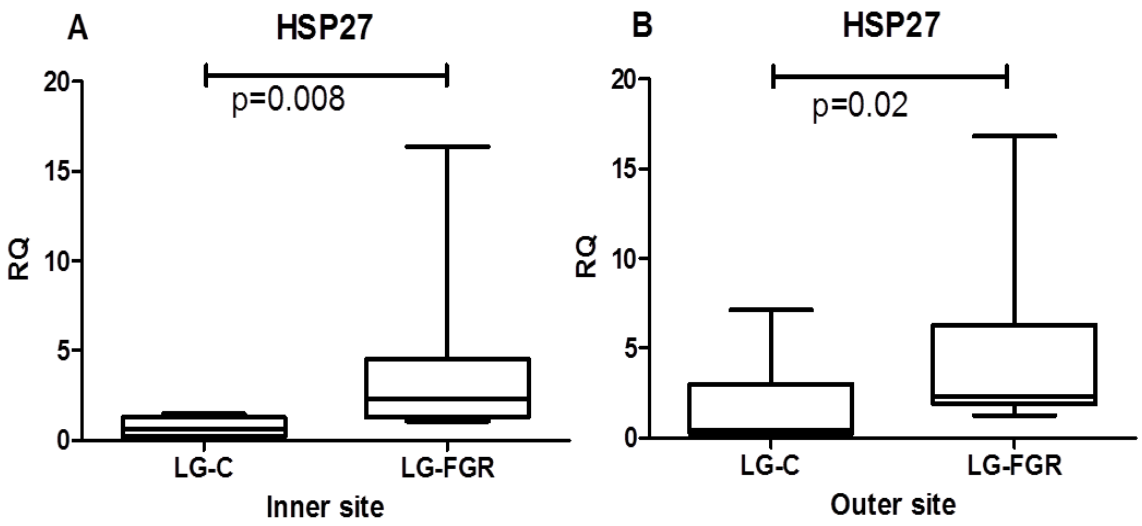


Figure 4-13: RQ values for HSP27 mRNA measurements in inner and outer placental zones for labour control compared with labour FGR groups. Comparison between zones was performed using Mann-Whitney analysis.

4.2.5 Analysis of protein expression of P-HSP27 at ser 15, 78 and 82 in normal pregnancies and pregnancies complicated with PE.

Experiment 1: This experiment was designed to test if there was a spatial difference in expression of P-HSP27 at ser 15, 78 and 82 within individual placentas obtained from women who were not in labour. Examples of Western blots showing P-HSP27 at ser (15, 87 and 82) expression for 2 different placentas (all non-labour) are shown in (Figures 4-15) (Figures 4-16) (Figures 4-17). The graphs and statistical analysis are shown below the blots. Friedman test analysis showed there was no significant difference in expression of P-HSP27 at ser 15 (p=0.4, p=0.06), ser 78 (p=0.65, p=0.27) and ser 82 (p=0.65, p=0.27) between the three zones (inner, middle, outer) within individual placentas for the non-labour group.

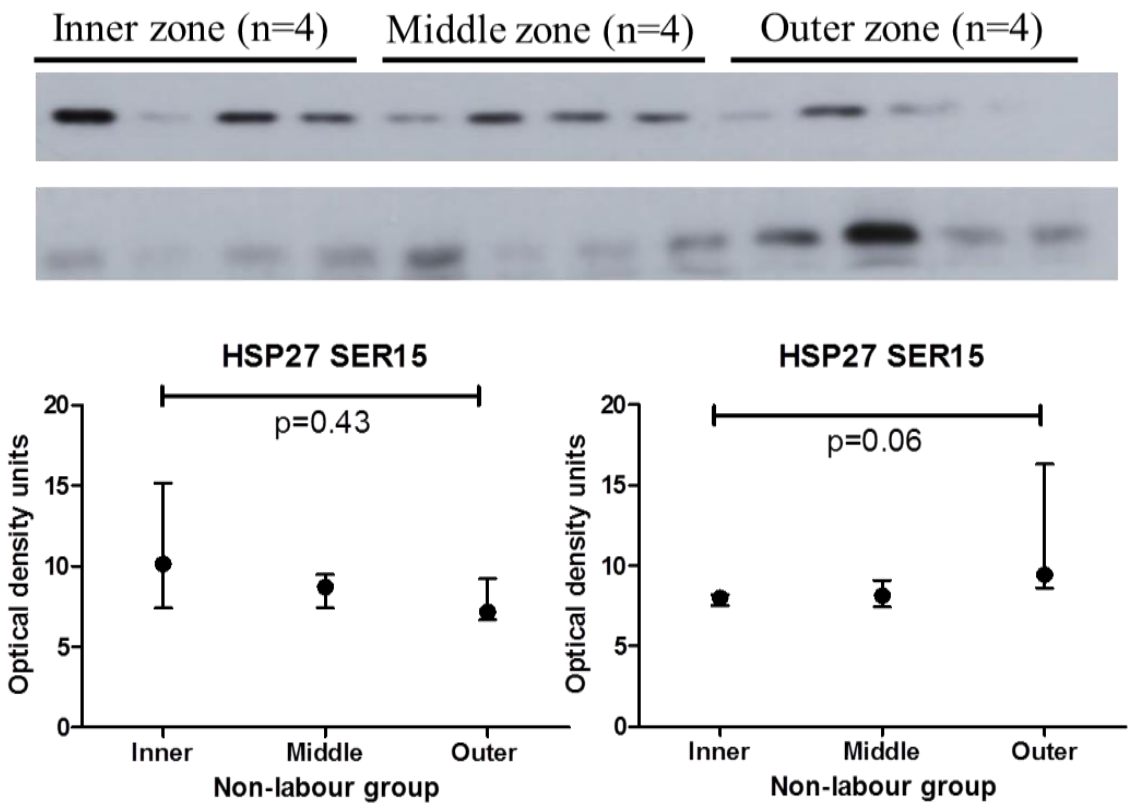


Figure 4-14: Representative images of Western blots showing P-HSP27 ser 15 expression in inner, middle and outer zones of two individual placentas (non-labour group). Four quadrants were sampled in each zone. Graphs show median and interquartile range for P-HSP27 ser 15 expressions. Comparison between zones was performed using Friedman analysis.

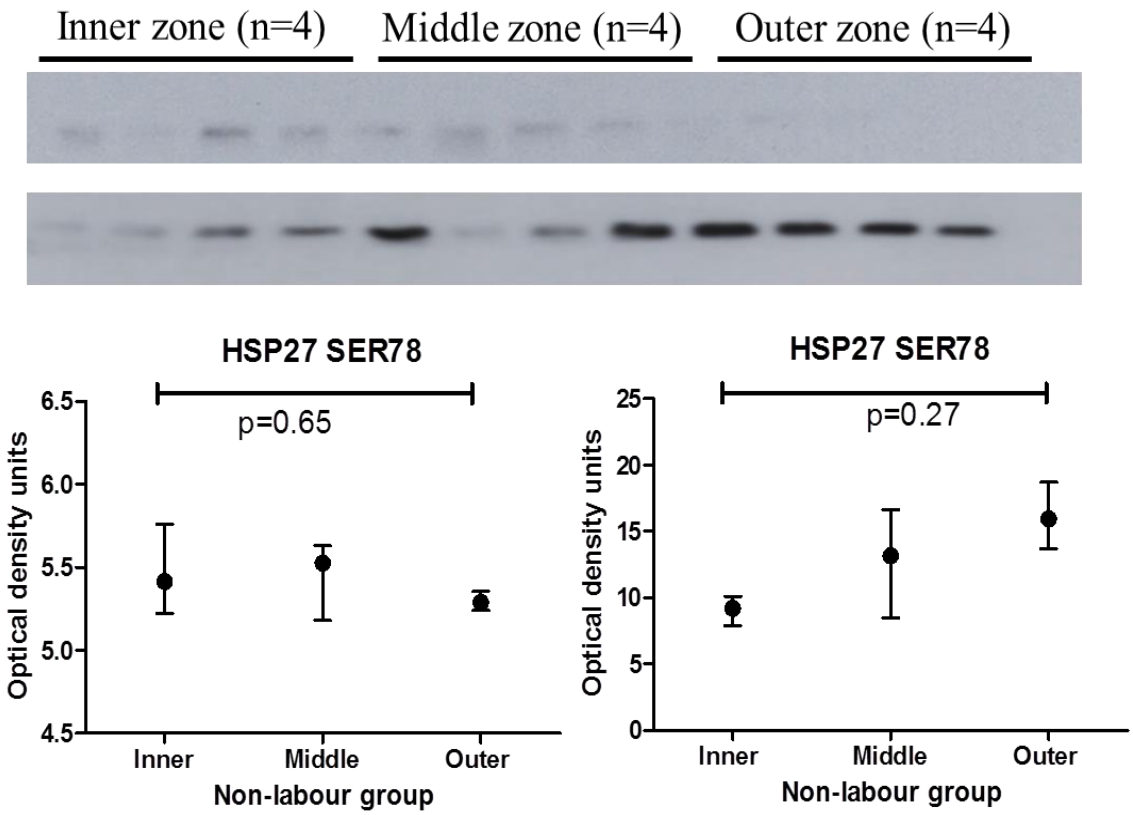


Figure 4-15: Representative images of Western blots showing P-HSP27 ser 78 expression in inner, middle and outer zones of two individual placentas (non-labour group). Four quadrants were sampled in each zone. Graphs show median and interquartile range for P-HSP27 ser 78 expressions. Comparison between zones was performed using Friedman analysis.

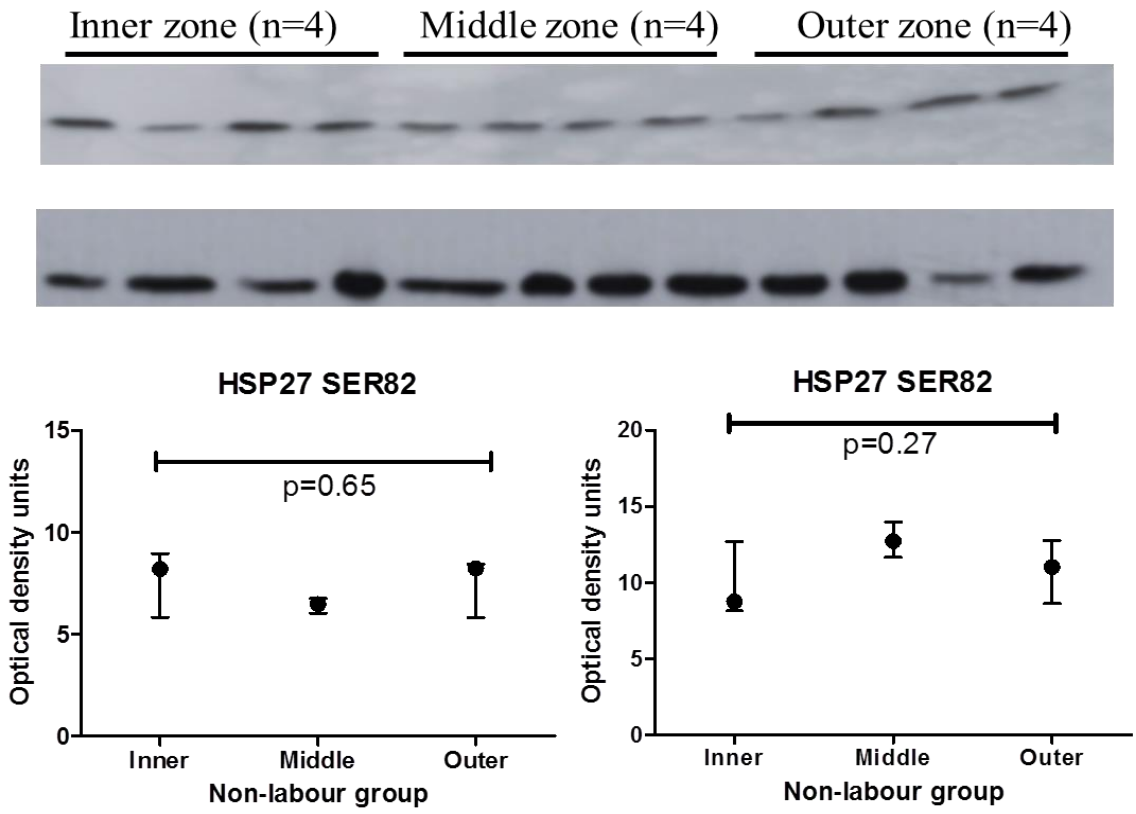


Figure 4-16: Representative images of Western blots showing P-HSP27 ser 82 expression in inner, middle and outer zones of two individual placentas (non-labour group). Four quadrants were sampled in each zone. Graphs show median and interquartile range for P-HSP27 ser 82 expression. Comparison between zones was performed using Friedman analysis.

Experiment 2: This experiment was designed to test if there was a spatial difference in expression of P-HSP27 at ser 15, ser 78 and ser 82 within individual placentas obtained from women who were in labour. Examples of Western blots showing P-HSP27 at ser (15, 87 and 82) expression for 2 different placentas (all labour) are shown in (Figure 4-18) (Figure 4-19) (Figure 4-20). The graphs and statistical analysis are shown below the blots. Friedman test analysis showed there was no significant difference in expression of P-HSP27 at ser 15 (p=0.43, p=0.39), ser 78 (p=0.27) and ser 82 (p=0.65) between the three zones (inner, middle, outer) within individual placentas for the labour group.

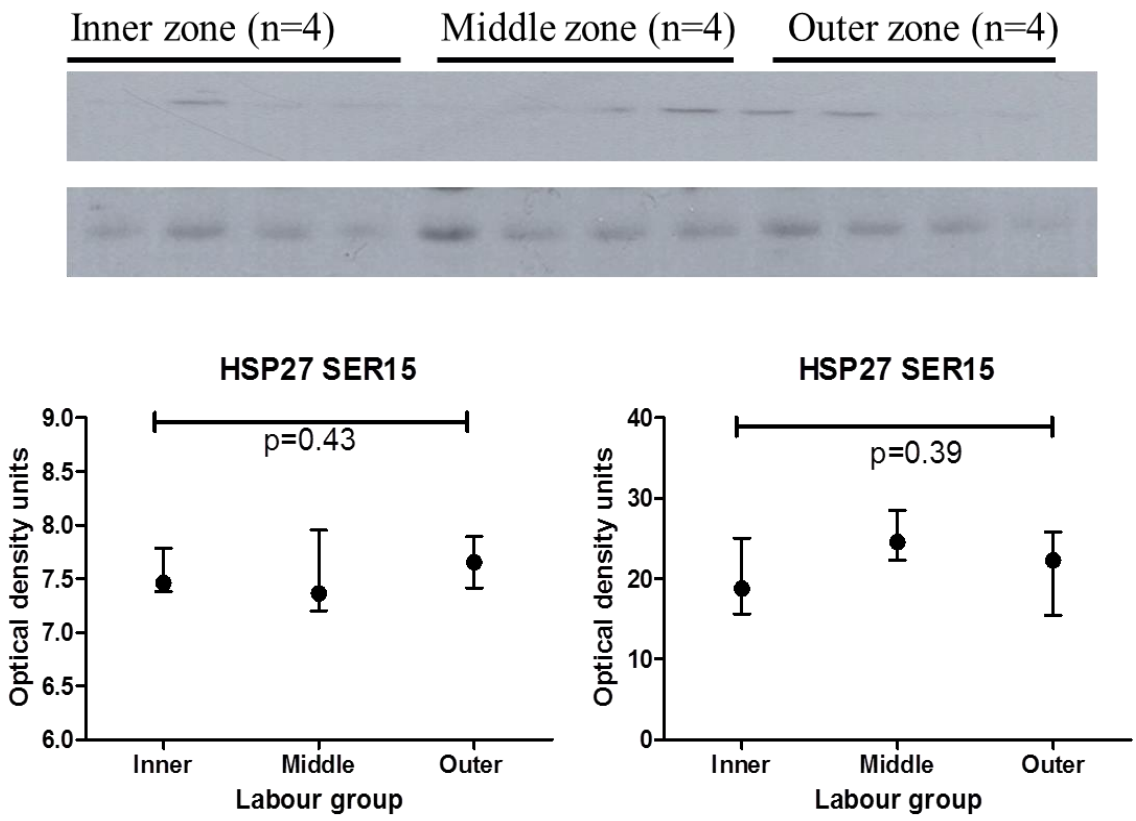


Figure 4-17: Representative images of Western blots showing P-HSP27 ser 15 expression in inner, middle and outer zones of two individual placentas (labour group). Four quadrants were sampled in each zone. Graphs show median and interquartile range for P-HSP27 ser 15 expression. Comparison between zones was performed using Friedman analysis.

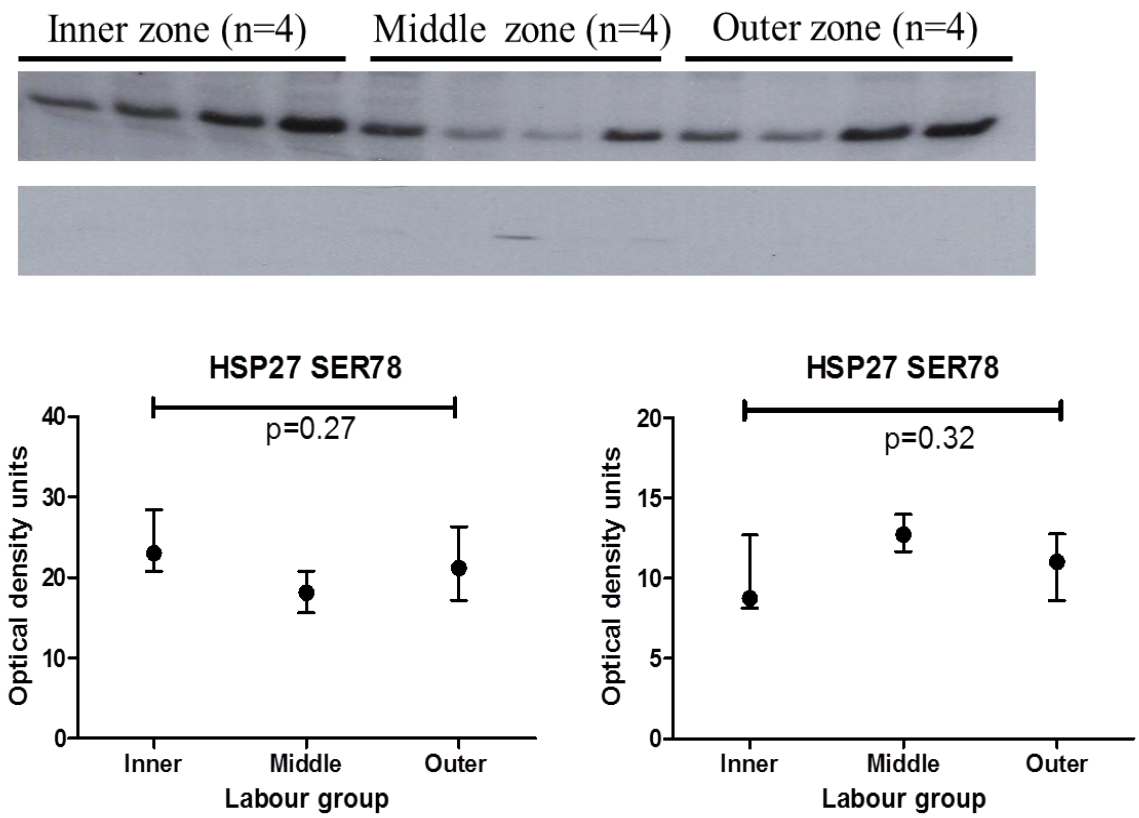


Figure 4-18: Representative images of Western blots showing P-HSP27 ser 78 expression in inner, middle and outer zones of two individual placentas (labour group). Four quadrants were sampled in each zone. Graphs show median and interquartile range for P-HSP27 ser 78 expression. Comparison between zones was performed using Friedman analysis.

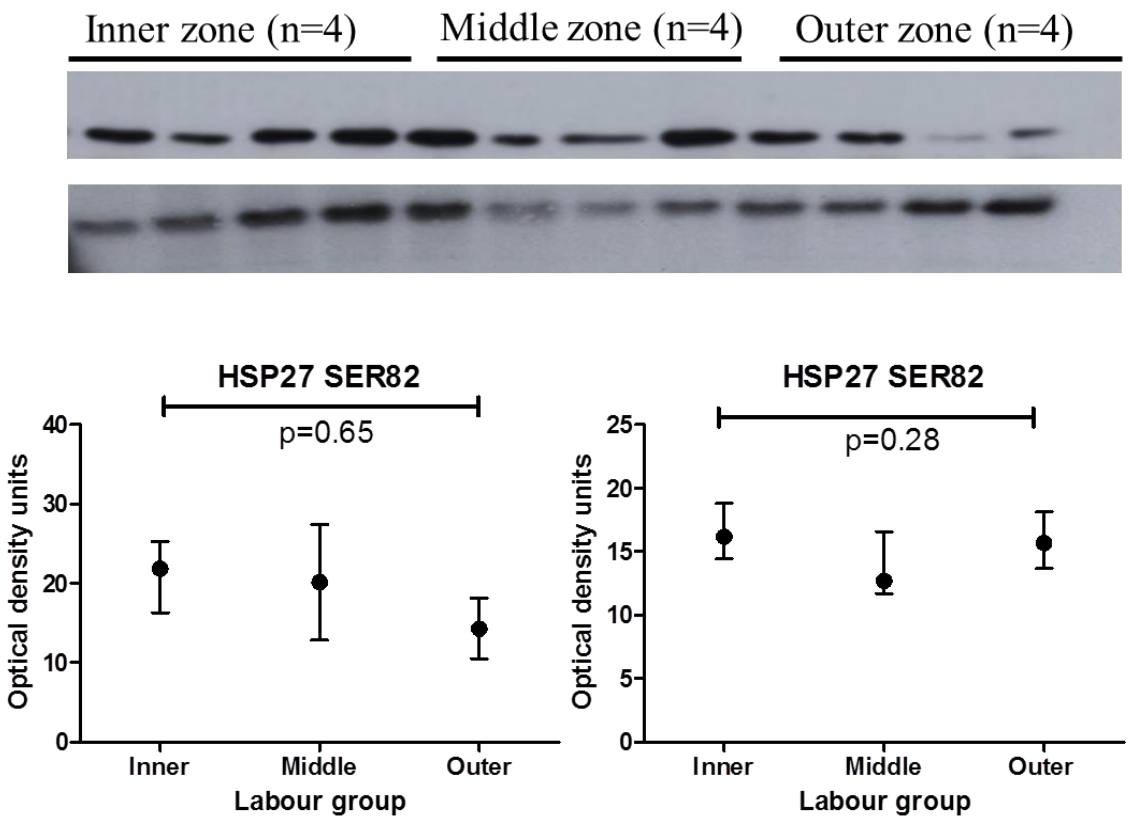


Figure 4-19: Representative images of Western blots showing P-HSP27 ser 82 expression in inner, middle and outer zones of two individual placentas (labour group). Four quadrants were sampled in each zone. Graphs show median and interquartile range for P-HSP27 ser 82 expression. Comparison between zones was performed using Friedman analysis.

Experiment 3: This experiment was designed to test if there was any difference in P-HSP27 at ser 15, ser 78 and ser 82 expressions between non-labour and labour groups at the inner, middle and outer zones of placentas. Western blots showing placental P-HSP27 at ser 15 (upper panel), ser 78 (middle panel) and ser 82 (lower panel) expression in non-labour and labour at the inner site are shown in (Figure 4-21). The graphs and statistical analysis are shown below the blots. There was a significant decrease in P-HSP27 expression in the labour group when compared to the non-labour group at the inner zone for all P-HSP27 at ser 15, ser 78 and ser 82 ($p=0.008$, $p=0.01$, $p=0.05$ respectively).

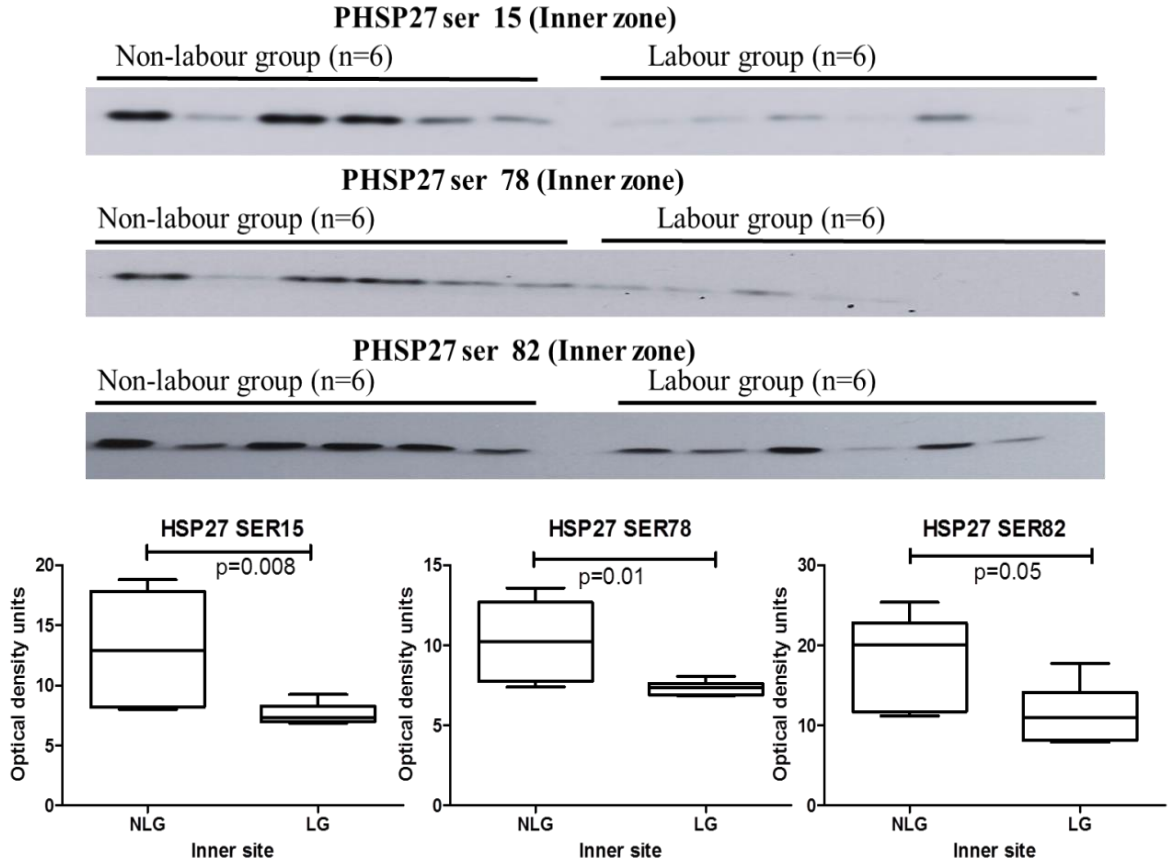


Figure 4-20: Representative images of Western blots showing PHSP27 at ser 15 (upper panel), ser 78 (middle panel) and ser 82 (lower panel) expression in the inner placental zone in non-labour and labour (n=6 in each group). The graphs show the box and whiskers analysis of the blots. Comparison between zones was performed using Mann-Whitney analysis. NLG non-labour group, LG labour group.

Western blots showing placental P-HSP27 at ser 15 (upper panel), ser 78 (middle panel) and ser 82 (lower panel) expression in non-labour and labour at the middle zone are shown in (Figure 4-22). The graphs and statistical analysis are shown below the blots. There was no significant difference in P-HSP27 expression at the middle zone for all P-HSP27 at ser 15, 78 and 82 (p=0.13, p=0.30, p=0.69 respectively).

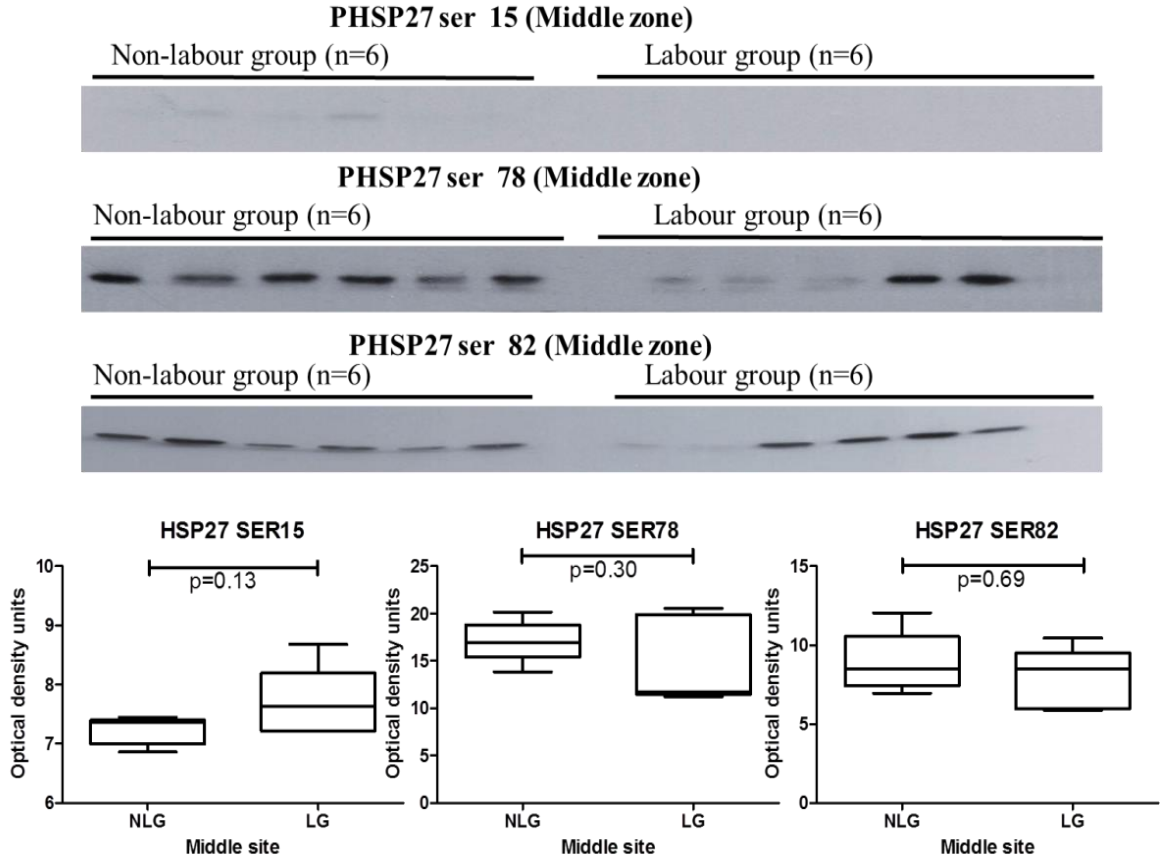


Figure 4-21: Representative images of Western blots showing P-HSP27 at ser 15 (upper panel), ser 78 (middle panel) and ser 82 (lower panel) expression in the middle placental zone in non-labour and labour (n=6 in each group). The graphs show the box and whiskers analysis of the blots. Comparison between zones was performed using Mann-Whitney analysis. NLG non-labour group, LG labour group.

Western blots showing placental P-HSP27 at ser 15 (upper panel), ser 78 (middle panel) and ser 82 (lower panel) expression in non-labour and labour at the outer zone are shown in (Figure 4-23). The graphs and statistical analysis are shown below the blots. There was a highly significant decrease in P-HSP27 expression in the labour group when compared to the non-labour group at the outer zone for P-HSP27 at ser 15 and ser 78 (p=0.004, p=0.002respectively). There was no significant difference in P-HSP27 at ser 82 at the outer zone (p=0.09).

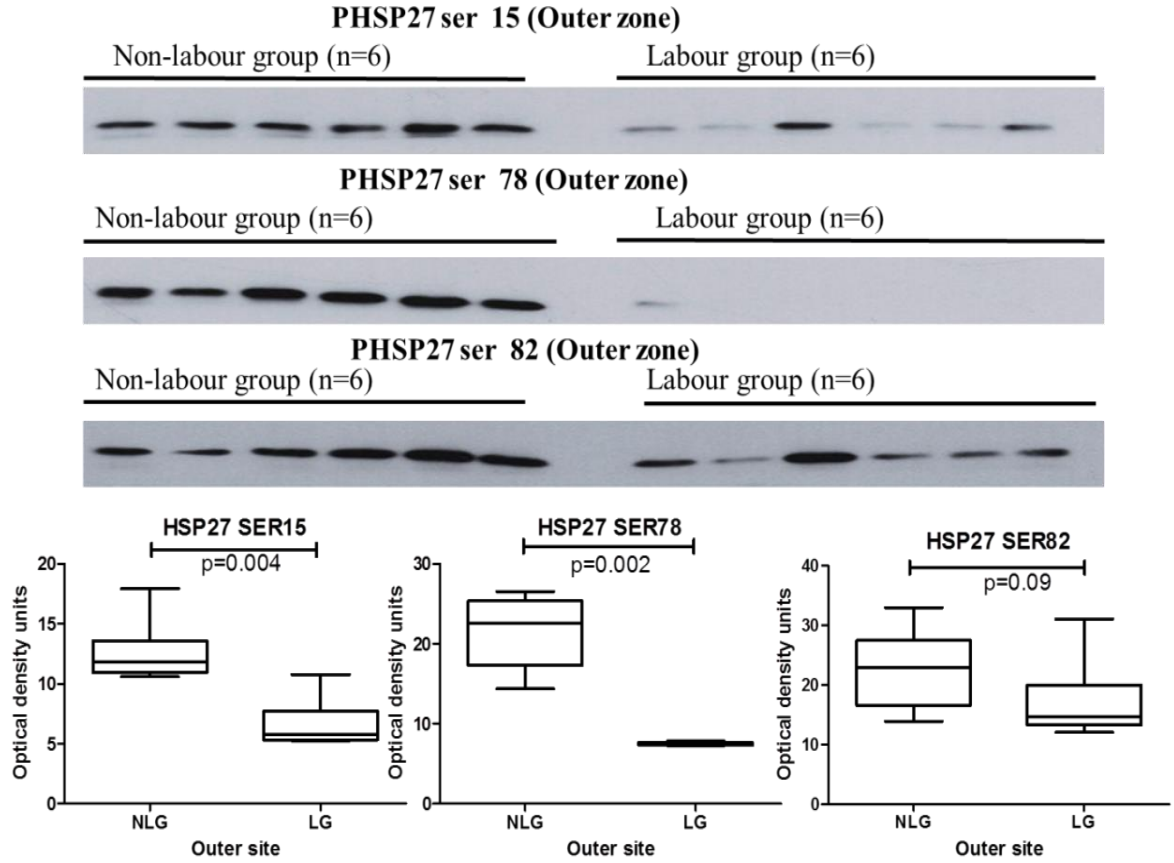


Figure 4-22: Representative images of Western blots showing PHSP27 at ser 15 (upper panel), ser 78 (middle panel) and ser 82 (lower panel) expression in the outer placental zone in non-labour and labour (n=6 in each group). The graphs show the box and whiskers analysis of the blots. Comparison between zones was performed using Mann-Whitney analysis. NLG non-labour group, LG labour group.

Experiment 4: This experiment was designed to test if there was any difference in P-HSP27 at ser 15, 78 and 82 expressions between labour control and labour PE groups at the inner or middle zones of placentas. Due to time constraint only labour group was compared. Western blots showing placental P-HSP27 at ser 15 (upper panel), ser 78 (middle panel) and ser 82 (lower panel) expression in the labour control and the labour PE at the inner zone are shown in (Figure 4-24). The graphs and statistical analysis are shown below the blots. There was a highly significant decrease in P-HSP 27 at ser 78 and ser 82 expression in the labour control group compared to the labour PE group at the inner zone (p=0.03, p=0.002 respectively). There was no significant difference in the P-HSP27 at ser 15 expression between the labour control group and the labour PE group at the inner zone (p=0.13).

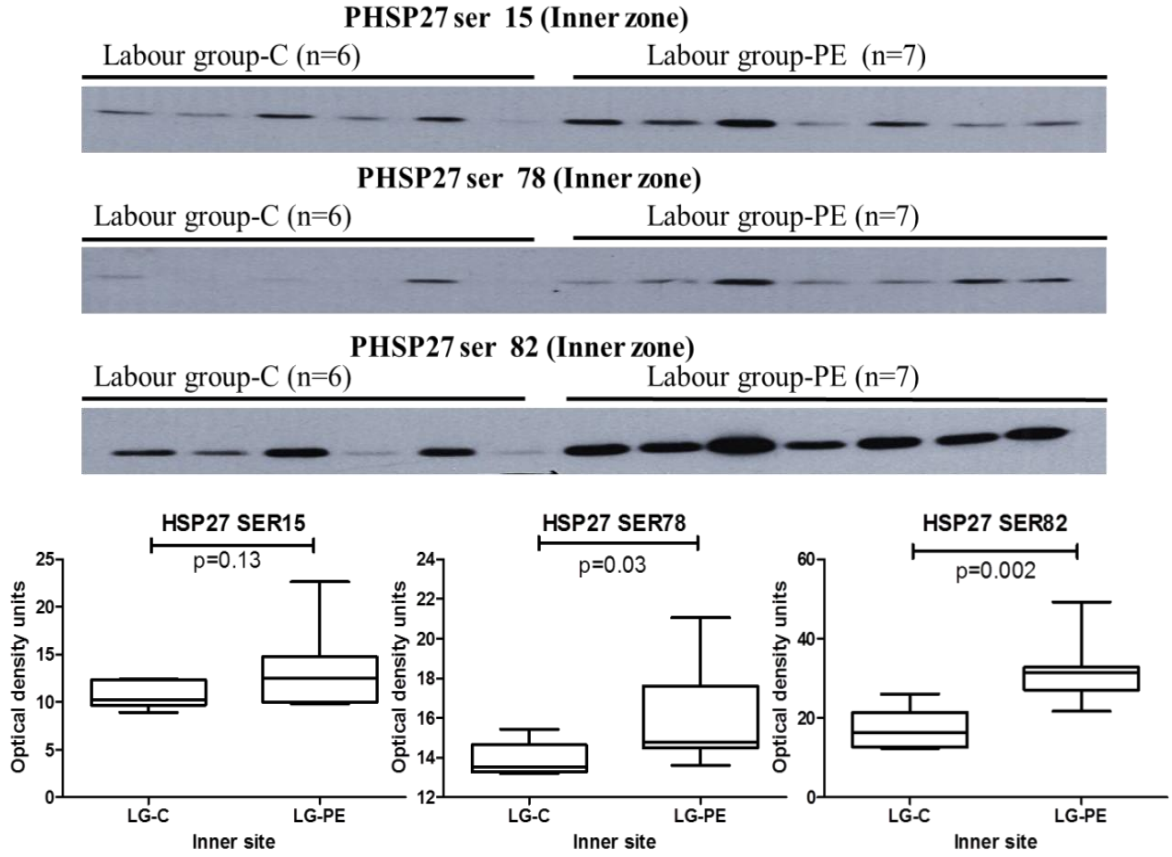


Figure 4-23: Representative images of Western blots showing PHSP27 at ser 15 (upper panel), ser 78 (middle panel) and ser 82 (lower panel) expression in the inner placental zone in labour control (n=6) and labour PE group (n=7). The graphs show the box and whiskers analysis of the blots. Comparison between zones was performed using Mann-Whitney analysis. LG-C labour group control, LG-PE labour group pre-eclampsia.

Western blots showing placental P-HSP27 at ser 15 (upper panel), ser 78 (middle panel) and ser 82 (lower panel) expression in the labour control and the labour PE at the middle zone are shown in (Figure 4-25). The graphs and statistical analysis are shown below the blots. There was a significant decrease in P-HSP 27 at ser 15 in the labour control group compared to the labour PE group at the middle zone (p=0.04). There was no significant difference in the P-HSP27 at ser 78 and ser 82 expression between the labour control and the labour PE groups at the middle zone (p=0.83, p=0.13 respectively).

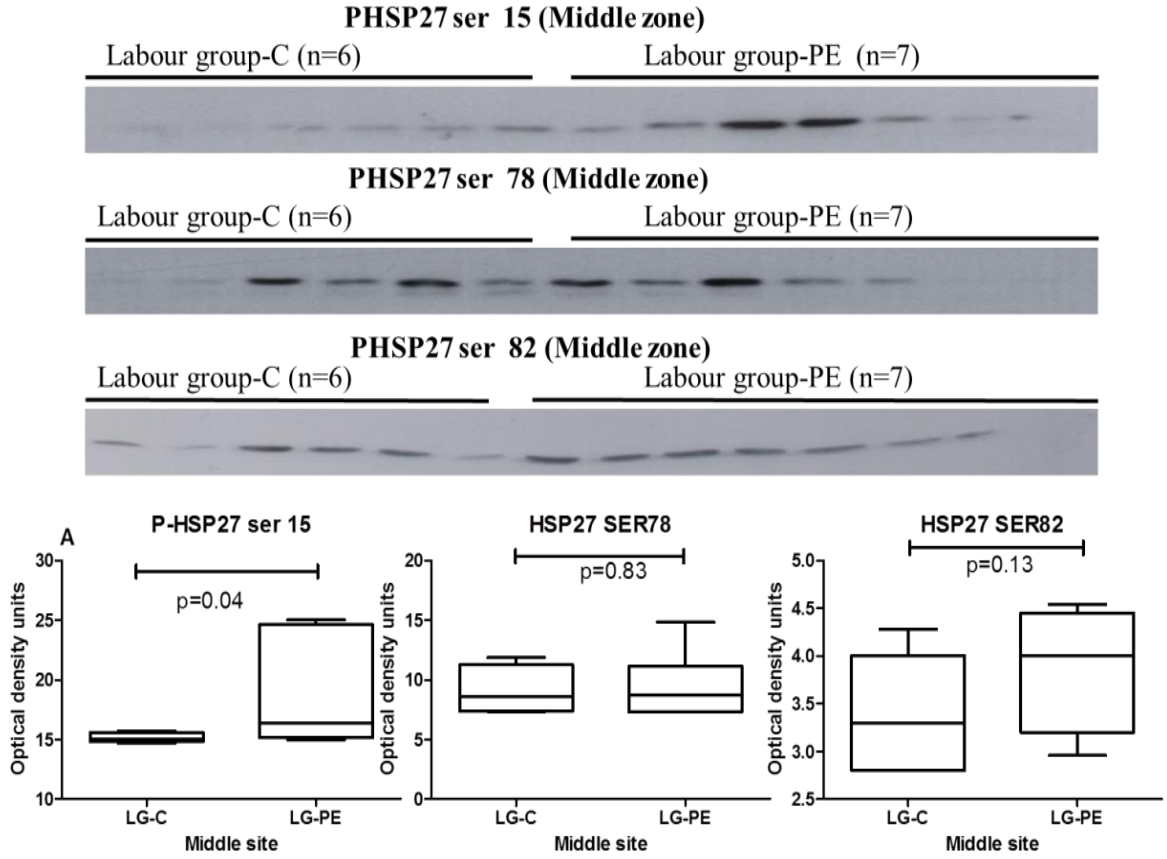


Figure 4-24: Representative images of Western blots showing PHSP27 at ser 15 (upper panel), ser 78 (middle panel) and ser 82 (lower panel) expression in the middle placental zone in labour control (n=6) and labour PE group (n=7). The graphs show the box and whiskers analysis of the blots. Comparison between zones was performed using Mann-Whitney analysis. LG-C labour group control, LG-PE labour group pre-eclampsia.

4.2.6 Results summary

4.2.6.1 Summary results for HSP27 expression in normal pregnancy:

Experiment 1: Within individual placentas there was a significant difference in protein expression of HSP27 between the three zones of the non-labour group and the labour group (p=0.04 for both). The sub-analysis showed that there was a significant decrease in HSP27 expression in inner zones compared to the middle zone for the non-labour (p=0.02).

Experiment 2: There was a significant decrease in HSP27 expression in the labour group when compared to the non-labour group at the middle zone (p=0.002). No other differences were found when comparing labour with non-labour groups at the inner and outer zones.

Experiment 3: Within individual placentas no differences in mRNA expression for HSP27 were found either in the labour or non-labour groups.

Experiment 4: There was a significant decrease in mRNA expression of HSP27 in the labour group compared to the non-labour group at the inner and middle zones (p=0.02, p=0.008 respectively). No difference was found at the outer zone.

4.2.6.2 Summary results for HSP27 expression in pregnancy complicated with PE

Experiment 1: When the PE groups (non-labour) were compared with the control groups (non-labour) for protein expression of HSP27 at the inner, middle and outer zones the changes found can be summarized as follows: There was no significant difference in HSP27 expression in the non-labour control group when compared to the non-labour PE group at the inner, middle and outer zones.

Experiment 2: When the PE groups (labour) were compared with the control groups (labour) for protein expression of HSP27 at the inner, middle and outer zones the changes found can be summarized as follows: There was a significant decrease in HSP27 expression in labour control compared to the labour PE at the middle and outer zones (p=0.01, p=0.04 respectively). No difference was found in HSP27 expression at the inner zone.

Experiment 3: There was no significant difference in HSP27 mRNA in the non-labour control group compared to the non-labour PE at the inner, middle and outer zones.

Experiment 4: There was no significant difference in mRNA expression of HSP27 in the labour control group compared to the labour PE group in the inner, middle and outer placental zones.

Experiment 5: When non-labour and labour was combined, there was no significant difference in mRNA expression of HSP27 when the normotensive pregnancies group compared to the pregnancies complicated with PE group in the inner, middle and outer zones.

4.2.6.3 Summary of HSP27 expression in FGR group

Experiment 1: There was no significant difference in the HSP27 protein expression between the labour control and labour FGR groups at the inner or outer zones.

Experiment 2: There was a significant decrease in mRNA expression of HSP27 in the labour control compared to the labour FGR groups at the inner and the outer zones (p=0.008, p=0.02 respectively).

4.2.6.4 Summary of results for P-HSP27 at ser 15, ser 87 and ser 82

Experiment 1: There was no significant difference in expression of P-HSP27 at ser 15, ser 78 and ser 82 between the three zones within individual placentas in the non-labour group.

Experiment 2: There was no significant difference in expression of P-HSP27 at ser 15, ser 78 and ser 82 between the three zones within individual placentas in the labour group.

Experiment 3: There was a significant decrease in P-HSP27 expression in the labour group when compared to the non-labour group at the inner zone for all P-HSP27 at ser 15, 78 and 82 (p=0.008, p=0.01, p=0.05 respectively). There was no significant difference in P-HSP27 expression at the middle zone. There was a highly significant decrease in P-HSP27 expression in the labour group when compared to the non-labour group at the outer zone for P-HSP27 at serine 15 and ser 78 (p=0.004, p=0.002 respectively). There was no significant difference in P-HSP27 at serine 82 at the outer zone (p=0.09).

Experiment 4: There was a highly significant decrease in P-HSP 27 at ser 78 and ser 82 expression in the labour control group compared to the labour PE group at the inner zone (p=0.03, p=0.002 respectively). There was no significant difference in the P-HSP27 at ser 15 expression between the labour control group and the labour PE group at the inner zone (p=0.13). There was a significant decrease in P-HSP 27 at ser 15 in the labour control group compared to the labour PE group at the middle zone (p=0.04). There was no significant difference in the P-HSP27 at ser 78 and ser 82 expressions between the labour control and the labour PE groups at the middle zone.

4.3 Discussion

4.3.1 HSP27 and present findings

This study shows for the first time that HSP27 is expressed in a spatial manner in the placenta with the highest expression being in the middle zone in both labour and non-labour groups. It therefore shows the importance of using a systematic method to sample the placenta. Most previous reports of placental protein expression do not take this into account. Taking a single or a few samples or averaging protein expression of several samples may well mask possible changes in expression. The study also shows that HSP27 protein and mRNA are reduced in the labour group compared to non-labour group at defined zones (inner for mRNA and middle for protein expression). In PE patients there was a significant decrease in HSP27 expression in labour control compared to the labour PE at the middle and outer zones. In addition, there was a significant decrease in mRNA expression of HSP27 in the labour control compared to the labour FGR groups at the inner and the outer zones. Apart from the reported changes and their link to placental pathology the results have important implications for how results in placental disease (and perhaps other organs) can be influenced by sampling methods.

4.3.2 P-HSP27 and present finding

HSP27 can be phosphorylated at ser 15, 78 and 82 by MAPKAPK-2 and 3 (Garrido et al., 2012). Phosphorylation favours small oligomers to form whereas de-phosphorylation favours formation of large oligomer (Garrido et al., 2012). Small and large forms may have different functions for example, larger forms are important in chaperone and anti-oxidant roles whereas smaller forms are important in actin regulation (Garrido et al., 2012). The HSP 27 gene contains two functional HSE binding sites, a cAMP response element as well as HSF-1 and 2 binding sites (Garrido et al., 2012). This study aims to understand whether any changes in of P-HSP27 occurs at defined zones during labour or PE.

4.3.2.1 HSP27 expression in labour

The key finding of this study was the fall in both HSP27 mRNA and protein at the inner and middle zones of the placenta during labour and which was particularly striking in the middle zone. Many HSPs are increased to protect against stress in disease states (Garrido et al., 2012) however in the present study HSP27 was reduced. One reason for this may be that a fall in HSP27 may be necessary to facilitate the inflammatory steps of labour which

is, after all, a normal physiological process, not a disease. HSP27 protects against apoptosis, decreases oxidative stress, reduces the pro-inflammatory cytokine balance, stabilizes actin, and inhibits NFκB activation (Garrido et al., 2012) but during labour the opposite effect of these events needs to occur.

Previous publications of HSP27 expression in the placenta are few. The major point we wish to highlight is that controlled sampling and the confounding effects of labour have not been considered (Abdulsid et al., 2013a). These studies can be summarized as follows.

One study examined the expression of HSP27 in placenta in labour and non-labour (Cindrova-Davies et al., 2007b). No significant differences were found however a different approach was taken: since different regions of the placenta were not compared, it is impossible to directly compare the present study with that one.

In another study HSP27 was reported to be unaltered in the placenta in samples from labour and non-labour. However only one biopsy was taken from each placenta and no quantification analysis was performed or presented (Li et al., 1996).

Shah *et al* showed that HSP27 was detected in intermediate trophoblast and syncytotrophoblast cells of the first and the second trimester (Shah et al., 1998). This result also in contrast to our finding that showed high protein expression of HSP27 in the term placenta.

Wataba *et al* showed that HSP27 and 70 were increased in syncytial knots, avascular villi and the presence of thrombus whereas both were reduced in the presence of infarction suggesting different stresses evoke different responses in HSPs in the placenta and the response may vary depending on the area of the placenta exposed to the stress (Wataba et al., 2004).

Mineva *et al* reported that HSP27 was detected exclusively in the STB layer (Mineva et al., 2008). No systemic sampling was performed and labour was not studied.

Matalon *et al* examined HSP27 distribution in the first trimester by using a human trophoblast explant model (Matalon et al., 2008). This study showed that during the culture period trophoblast cells express HSP27 which varied according to their stage of differentiation: migratory cells express HSP27 in the cell cytoplasm; non-proliferating villous-trophoblast cells express HSP27 in the nucleus and cytoplasm and proliferating villous cell did not express HSP27. This study suggested that HSP27 expression in

trophoblast is modulated in concordance with migration dependent parameters. Furthermore, 6-mercaptopurine (inhibitor of EVT migration) decreased HSP27 nuclear expression and that was associated with decreased MMP2 activity, NO production and decreased inducible (iNOS) expression (Matalon et al., 2008).

Our group has previously examined the expression of HSP70 in the placenta (Abdulsid et al., 2013b). In non-labour HSP70 was reduced in the outer zone of the placenta compared with the middle area. In contrast in the present study HSP27 was reduced in the inner zone compared with the middle zone. With regard to labour our previous study showed that HSP70 was increased in the inner and middle areas, the same findings for HSP27. At this stage it is only possible to speculate why such zonal differences exist but may relate to the functions of HSP27 and 70, some of which differ and some overlap (Wataba et al., 2004, Concannon et al., 2003, Borges et al., 2012). Placental separation is an important part of labour. Herman et al (Herman et al., 2002) showed that the process of placental separation from the uterine wall can be divided into three distinct phases i.e. latent, contraction/detachment and expulsion. They showed that placental separation is accomplished by means of an orderly multiphasic process with a definite direction and sequence. They found in most cases the placenta separated from the uterine wall in a "down-up" separation i.e. initiating from the lower pole. Interestingly cases with a previous Cesarean section had a higher rate of up-down separation. In contrast in the case of "fundal placentae" separation started at the placental poles (bipolar separation) and the central area of the placenta was the last to separate. It would be therefore of interest in a future study to investigate whether there was a link between the zonal distribution of HSP27 or HSP70 and the method of placental separation.

Placentas collected at term by caesarean section are not subjected to the stress of labour however one possibility is that zonal differences in HSPs might reflect the fact that labour is not far off and that the molecular steps to allow labour to precede have started. Thus it would be interesting to compare placentas from the second trimester where labour is not close to determine if such zonal differences still exist. In contrast at labour zonal differences in HSPs may be linked to the response to the stress of labour, extent of exposure to hypoxia or may contribute to the process that allows the placenta to separate at delivery.

It has been shown that HSP27 regulates apoptosis through key components of the apoptotic signaling pathway, in particular, those involved in caspase activation and apoptosis (Concannon et al., 2003). HSP70 can also inhibit CASP3 and 9 (Borges et al., 2012). HSP70, via the TLR-2 receptor, can increase IL-10 production; IL-10 can be pro-inflammatory at labour which may accelerate parturition (Gibb WL, 2006). This may also explain why HSP70 increases at labour when HSP27 decreases. Of interest is the observation that reduced MMP2 activity has been shown to be linked to reduced HSP27 (Matalon et al., 2008). Whether this is linked to the zonal distribution requires further investigation. The expression of HSP27 was also reported to be reduced in placentae from SGA neonates although zonal distribution was not investigated (Cañete et al., 2011).

In summary HSP27 is expressed in a spatial manner in the human placenta and changes in expression occur during labour suggest that HSP27 may be part of the signaling process of labour and thus warrants further investigation particularly with regard to a role in pre-term labour.

4.3.3 P-HSP27 (ser 15, 78 and 82) in labour and PE

HSP27 is a stress induced chaperone molecule known to regulate cytoskeletal stability, protein folding, cellular stress, and prevention of apoptosis in different cells (Bolhuis and Richter-Landsberg, 2010). Cellular functions of HSP27 are controlled by phosphorylation of HSP27 at three ser sites (Ghayour-Mobarhan et al., 2012).

When P-HSP27 was assessed in this study the findings showed that there was no spatial distribution for P-HSP27 of either ser sites (ser 15, 78 and 82) in both the non-labour and the labour groups despite of the clear spatial distribution in protein expression of HSP27, which increased especially in the middle zone within individual placentas. Whether the observation that phosphorylation changes were not noted reflects something about the function of HSP27 in the placenta remains to be elucidated.

Similar to the HSP27 protein expression there was increased expression of P-HSP27 at the three ser sites (15, 78 and 82) in the non-labour group compared to the labour but in contrast to HSP27 findings where the highest expression was in the middle zone, in P-HSP27 the expression was much higher in the inner and the outer placenta zones. The explanation for less P-HSP27 in labour group but may perhaps allow labour to progress and the placenta to detach as repair mechanisms are now reduced. Again these findings

further emphasize that without careful sampling methods differences between study samples can be missed.

For the PE group and due to time constrain, only the labour group was investigated. The P-HSP27 was a significant increased in the labour PE group compared to the labour control at the inner for (ser 78 and 82) and the middle for (ser15) zones. As PE is associated with a high level of apoptosis, these results may be explained as increased level of P-HSP27 is a defence mechanism to protect cells from oxidative stress and apoptosis that over-rules the effects observed in labour alone. Overall protein expression of HSP27 was increased in the labour PE group compared to normotensive women but at the middle and outer zones. Again a zonal variation is an important factor and highlighted the urgent need for optimization of placental sampling methods.

Shin *et al* showed that P-HSP27 was increase in PE placentas compared to the control (Shin et al., 2009). They obtained 5 tissue pieces from the feto-maternal interface but there was no clear systemic sampling was performed and labour was not controlled for.

We found an increase in phosphorylation at the ser 15 site that matched the increase in protein expression in the PE labour group compared with the control labour group at the middle zone. Since phosphorylation favours small oligomers, meaning there are fewer larger oligomers that play anti-oxidant roles, and since PE is associated with fewer anti-oxidants, the reduction in large oligomers may contribute to a more pro-inflammatory state in the PE placenta.

Small HSPs have been studied in myometrium during labour. The myometrium undergoes substantial remodeling at the time of labour including rearrangement of the cellular contractile machinery. Since HSP27 can modulate actin polymerisation one study investigated changes in small HSPs in the myometrium at labour (MacIntyre et al., 2008). A 69% decrease in the small HSP αB-crystalline was found in the myometrium at labour plus multiple isoforms of HSP27. In addition, immunoblotting using phosphor-specific HSP27 antibodies (P-HSP27 at ser15, 78, and 82) detected marked changes in HSP27 phosphorylation at labour. P-HSP27 ser15 was 3.0-fold higher in labouring myometrium. In contrast, levels of HSP27 ser 82 were 85% less in labouring myometrium. There was no significant change in HSP27 ser 78. It was proposed that decreased expression of αB-crystallin at the time of labour liberates HSP27 enabling it to participate in other cellular events such as cytoskeletal remodeling. Clearly the functions and structure of the

myometrium and placenta are different, however since both play a role in labour we carried out experiments investigate the expression of αB-crystallin within the placenta during labour (chapter V). Also that particular study highlights how different changes in HSP27 can occur depending on the cellular event to be targeted.

These finding showed a little conflict but although the spatial manner of HSP27 was not the same, the high expression was in similar way especially increased expression in non-labour compared to the labour and also decreased expression in labour control compared to the PE. Future studies will be undertaken to determine whether any other know post-translational changes occurs in these samples. Placenta zones distribution also showed a little difference this is not clear and further studies include immunohistochemistry will help to understand this variation.

To our knowledge this is the first study to show a decrease in p-HSP27 associated with an increase in placental apoptosis during labour. This study suggests a role for P-HSP27 in placental trophoblast apoptosis during labour and PE pregnancies. However, we chose to study p-HSP27 for two reasons. First, because P-HSP27 is involved in the control of apoptosis and is expressed in trophoblast cells, second as labour and PE are pro-inflammatory conditions and is characterized by high level of apoptosis.

In view of the findings of this study future studies will be directed to understand whether any changes in phosphorylation of HSP27 or MAPKAPK-2 or 3 occurs at defined zones during labour.

4.3.4 HSP27 expression in PE and FGR

This study shows that HSP27 protein expression is increased in placentae from women with PE who were in labour when compared to women who were in labour but did not have pregnancies complicated by PE. Furthermore this increase in HSP27 protein was only seen at the middle and outer zones (Abdulsid and Lyall, 2014). It also shows the importance of using a systematic method to sample the placenta. The pathological significance of this finding remains to be elucidated but oxidative stress is the common link between labour and PE.

The present study was designed to compare pregnancies complicated by PE with uncomplicated pregnancies, but to control for labour. Controlling for labour is important because we have shown that placental HSP27 expression is altered during labour (Abdulsid

et al., 2013a). We previously reported, for non-labour, significantly less HSP27 protein in the inner placenta region compared with both the middle region within an individual placenta. For labour there was also less HSP27 protein in the inner region compared with the middle region for an individual placenta. When non-labour was compared with labour there was significantly less HSP27 in the labour group at both the middle zone compared with the non-labour group. Similar to HSP27 protein, there was less HSP27 mRNA in the labour group in both the middle and the outer zones compared with the non-labour group. This study suggested that placental HSP27 might play a role in labour and is spatially controlled. Thus, we were careful to control for this in the present study

There were no differences noted at the mRNA level however, it is possible that mRNA levels had increased then returned to normal some time before the placentae were delivered. The PE patients used in this study had mostly later-onset disease. Some, but not all, changes in PE can be different depending on whether the disorder is early- or late-onset (Staff et al., 2013). However, late-onset disease can also be severe PE. It would be of interest in future studies to find if this is the case for HSP27, but this study did not have sufficient numbers to allow statistical analysis if PE cases were split into two groups according to gestation.

Apoptosis is a component of normal development and differentiation in most tissues, however, in the FGR placenta at term increased apoptosis is a common feature (Gorman et al., 2005). HSP27 has been shown to be involved in the control of apoptotic pathways in several cells (Aiko et al., 2002, Paul et al., 2002, Gorman et al., 2005).

There was no significant difference in the protein expression of HSP27; however, there was a significant increase in mRNA expression of FGR group compared to the control group at both the inner and outer zone. The reason for this finding is still unknown. Whether the increase in mRNA reflects new protein synthesis about to begin can only be speculated.

Geisler *et al* performed IHC and showed that HSP27 protein expression was twice that in placentas from patients with severe PE and FGR compared to the placentas from normotensive women (Geisler et al., 2004). In this study, no systematic sampling was performed and labour was not studied.

Wataba *et al* performed IHC and reported that HSP27 protein expression was increase in the CTB and STB layer of the FGR placentas at second and third trimester (Wataba et al.,

2004). HSP27 was decreased in CTB and STB around infraction regions. They suggested that although the villous cells around the infarction histologically appeared viable, they may have received lethal damage, and as a result the expression of HSPs was decreased. Systemic sampling method was not performed.

Webster *et al* performed Western blots and showed that HSP27 protein expression was up-regulated in preterm PE placentas compared to term normotensive placentas (Webster et al., 2007). They concluded that HSP27 is a critical stress-induced protein and it may well have been up-regulated due to the stressors specific to PE. However, the drawback in this study was labour and systematic sampling method also does not taken in account.
Jin *et al* performed Western blotting and reported that HSP27 protein expression was up-regulated in trophoblast of PE placenta compared to control (Jin et al., 2008). They concluded that HSP27 might take part in the regulation of the phased development of PE and pathogenesis of PE. This study was similar to the present findings of protein expression but they did not perform RT-PCR. However, one central placental sample was taken and labour was not studied.

Shin *et al* performed Western blotting and IHC to investigate HSP27 and P-HSP27 protein expression (Shin et al., 2009). It was shown that HSP27 protein expression was increased in PE placentas compared to the controls. Immunoprecipitation analysis showed that the interaction between HSP27 and MAPK (p38 and ERK) was significantly increased in PE placentas. They concluded that phosphorylation HSP27 might be induced by p38 and ERK in PE placentas. No clear systemic sampling was performed.

Bahr *et al* performed Western blotting and IHC on human and animal placentas (early, mid and late gestation) (Bahr et al., 2014). In this study they reported that human P-HSP27 expression was reduced in FGR placentas compared to control placentas. Again the site of sampling or labour was not assessed. In the animal study it has been reported that HSP27 expression increased in early gestation in the FGR group and this expression was significantly increased by near-term as compared to controls (Bahr et al., 2014). P-HSP27 was significantly increased at early gestation but paradoxically decreased near term in the FGR group compared to the control. IHC showed that P-HSP27 protein is localized to the trophoblast cells of the placenta throughout gestation (Bahr et al., 2014). They concluded that decreased P-HSP27 at term is present when placental apoptosis is increased during FGR. This could be a factor leading to the decreased placental weight observed during FGR.

Chapter V: Analysis of mRNA expression of small HSP family in human placenta of normal pregnancies and pregnancies complicated with PE

5 Small HSP family (sHSP)

5.1 Introduction

5.1.1 Definition

Small HSPs (sHSPs) are a family of widespread stress proteins, which are found widely in nature. The sHSP vary in size from 12 to 30 kDa (Narberhaus, 2002, Franck et al., 2004, Haslbeck et al., 2005), and are present in the cytosol of most cells and tissues even in the absence of stress factors. Most of the sHSP have the α-crystallin domain which is characterized by the presence of a conserved sequence of about 80–100 residues (Kriehuber et al., 2010). Some members (HSP27, α-crystallin) form large oligomeric assemblies ranging from 150 to 800 kDa (Narberhaus, 2002, Haslbeck et al., 2005), whereas other sHSP, which include HSPB2, HSPB6 form small oligomeric assemblies. HSPB8 exists as monomer or dimer (Chowdary et al., 2004, Bukach et al., 2009, Prabhu et al., 2012). sHSP family structure include the α-crystallin domain consists of a β-sandwich comprising two antiparallel β-sheets, followed by a short C-terminal extension and a long loop extending from the β-sandwich (van Montfort et al., 2001). However, other sequence characteristics are the less conserved including the N-terminal domain, the variable central region, and the variable region of C-terminal tails (Hu et al., 2008).

The sHSP family form large oligomeric complexes that are in a dynamic equilibrium with smaller structures (Orejuela et al., 2007). These oligomers can either be homo or hetero oligomers involving different members of the sHSP family. For example, HSPB1 can interact with HSPB8 in a similar manner as the αA-αB-crystallin pair (HSPB4, HSPB5) (Sun et al., 2004). Differential serine phosphorylation plays an important role in the function of mammalian sHSPs (Kato et al., 2002). Chaperone activity of the sHSPs family is modulated by the dynamic structural organization of sHSPs into oligomers by post-translational modification and by other factors like heat shock. The sHSPs are responsible for the binding of misfolded proteins and their further transfer to the ATP-dependent

chaperones or to the protein degradation molecules such as proteasomes or autophagosomes (Haslbeck et al., 2005). Unlike other chaperons sHSPs lack ATPase domains (Marini et al., 2005). However, despite the fact that there are high structural similarities of the conserved α-crystallin domain of sHSP, the various members of the family do not necessarily possess similar functions *in vivo*. For example, HSP27 (HSPB1) has an anti-apoptotic action (both upstream and downstream) of the apoptosome while HSPB8 shows a pro-apoptotic activity in a cell-specific manner (Gober et al., 2003).

In humans, 10 α-crystallin–related sHSPs have now been named HSPB1–HSPB10 in accordance with the guidelines of the National Centre for Biotechnology Information (NCBI) (Kappe et al., 2003) (Table 5-1). The best-known representatives are HSP27 (HSPB1) (discussed in Chapter 4), αB-crystallin (HSPB5), and HSP20 (HSPB6). In addition, there are other low molecular weight HSPs, like HSP32 which sometimes also included as sHSPs but because they lack the α-crystallin domain, they do not belong to this family.

Table 5-1: α-Crystallin-related small heat-shock proteins (HSPB1–HSPB10) adapted from (Hu et al., 2008, Mymrikov et al., 2011).

HSPB	Molecular weight (kDa)	Alternative name	Heat induction	Tissue location
HSPB1	27	HSP27	Yes	Uterus, skin, platelets, myocardium, breast tumours, ovarian and endometrium carcinoma
HSPB2	22	MKBP (myotonic dystrophy protein kinase-binding protein)	No	Cardiac and skeletal muscle
HSPB3	17/27		No	Cardiac and skeletal muscle
HSPB4	20	αA-crystalline	No	Lens
HSPB5	20	αB-crystalline	Yes	Myocardium, vascular wall, lens, lung, kidney and head
HSPB6	20	HSP20	No	Myocardium, liver, lung, kidney, brain, neck tumor and platelets
HSPB7	23/25	CvHSP (cardiovascular heat shock protein)	?	Heart and skeletal muscle
HSPB8	22-25	HSP22	Cell type dependent	Heart, liver, lung, kidney, brain and skeletal
HSPB9	17.5		?	Testis
HSPB10	27/85	ODF1 (outer dense fiber protein)	?	Testis

5.1.2 HSPB2 (heat shock 27kDa protein 2)

HSPB2 is a member of the sHSP family and is highly expressed in heart and skeletal muscle (Suzuki et al., 1998, Nakagawa et al., 2001, de Thonel et al., 2012). HSPB2

appears to be essential for skeletal muscle development and the maintenance of its structure and function (Nakagawa et al., 2001, de Thonel et al., 2012). HSPB2 activates the myotonic dystrophic kinase (DMPK) and protects its kinase activity after a heat inducible stress, thereby acting as a molecular chaperone of DMPK (Suzuki et al., 1998). HSPB2 activity like other HSPs is regulated by post-translated phosphorylation of its serine site by MAP kinase-activated protein kinase (MAPKAPKs), which are activated by phosphorylation of p38 MAP kinase (Rouse et al., 1994).

HSPB2 is present in the cytosol as granules in association with the outer membrane components of the mitochondria and protect the cells from stresses such as oxidative stress, heat shock, radiation and anti-cancer drugs (Nakagawa et al., 2001, Yoshida et al., 2004, Chang et al., 2011). In a rat model it has been shown (Shama et al., 1999) that HSPB2 protein expression increased sharply in heart and skeletal muscle following exposure to hypoxia-reoxygenation. In addition, it has been shown (Pinz et al., 2008) that following IR injury, HSPB2 protect cardiac energetic properties while HSPB5 protects the diastolic contractile performance. HSPB2 is involved in tumourigenesis which may be related to the ability to protect cells against apoptosis (Laskowska, 2006, Calderwood et al., 2006). HSPB2 is associated with pathological conditions such as ectopic endometrium of women with endometriosis and in patient with myotonic dystrophy (Ota et al., 1997, Sugiyama et al., 2000).

5.1.3 HSPB3 (HSP17/27)

HSPB3 is expressed in adult smooth muscle, heart, brain and fetal tissues (Boelens et al., 1998, Asthana et al., 2012). HSPB3 exhibits a target protein dependent chaperone like activity (Asthana et al., 2012). HSPB3 induced production of IL8, soluble ICAM-1 and monocyte chemo-attractant protein 1 by brain astrocytes and leptomeningeal smooth muscle cells (Bruinsma et al., 2011). Mutation R7S located in the N-terminal part of HSPB3 is associated with development of axonal motor neuropathy (Kolb et al., 2010).

5.1.4 HSPB4 (CRYAA, αA-crystallin)

αA-crystallin can induced by heat shock and belongs to the sHSP family (Horwitz, 2000). αA-crystallin, a tissue specific structural protein of the ocular lens maintains the transparency and refractive index of the lens (Derham and Harding, 1999, Horwitz, 2000). It is synthesised in lens differentiating fibres and it extremely stable proteins so it maintain

the lens transparently (Srinivasan et al., 1992). αA-crystallin has chaperone like activity (Banerjee et al., 2014). The two forms of α-crystallin (αA-crystallin and αB-crystallin) share 57% sequence homology and have been reported to form multimeric complexes with each other at a ratio of 3:1 (Christopher et al., 2014). αA-crystallin can act as a molecular chaperones preventing the aggregation of other lens crystallins and proteins that have become unfolded by trapping the protein in a high molecular weight complex (Derham and Harding, 1999). αA and αB gene products are differentially expressed; αA is preferentially restricted to the lens and αB is expressed widely in multiple tissues including the retina, heart, skeletal muscle, kidney and lung (Derham and Harding, 1999, Horwitz, 2000, Christopher et al., 2014). Defects in this gene cause autosomal dominant congenital cataract (Litt et al., 1998). In addition, it was shown (Xu et al., 2015) that knockout of αA-crystallin inhibited pathologic neovascularization through the VEGF and VEGFR2 signalling pathways. αAcrystallin mutations are usually accompanied by development of different forms of cataract (Moreau and King, 2012). Furthermore, it was reported that αA-crystallins displayed significant protection to oxidative stress induced cell death (Christopher et al., 2014).

5.1.5 HSPB5 (CRYAB, αB-crystallin)

αB-crystallin (HSPB5) and HSP27 are members of the sHSP family and are characterized by a considerable degree of sequence and functional similarity (Mineva et al., 2008, Reddy and Reddy, 2015). HSPB5 is a heat inducible protein (Dubin et al., 1990), and is produced in the presence of several types of cellular stress (Bluhm et al., 1998, Muchowski et al., 1999, Mineva et al., 2008). HSPB5 can be phosphorylated at ser19, ser45, and ser59, which reduces the complex size (Peschek et al., 2013). HSPB5 phosphorylation influences chaperone activity (Ecroyd et al., 2007, Reddy and Reddy, 2015). MAPKAPK2 kinases are responsible for ser59 phosphorylation, p42/p44 MAPK are responsible for ser45 phosphorylation and the signalling pathway responsible for phosphorylation of ser19 is currently unknown (Reddy and Reddy, 2015).

5.1.5.1 HSPB5 functions

HSPB5 exert several cellular functions including chaperone activity (Derham and Harding, 1999, Arrigo et al., 2007, Srinivas et al., 2008), protects cells from stress and inhibits apoptosis (Webster, 2003, Kamradt et al., 2005), inhibits inflammation (Arrigo et al., 2007, Arac et al., 2011), stabilizes actin filaments and decreases actin de-polymerization (Wang and Spector, 1996, Ghosh et al., 2007). It has also been suggested to have a role in the

ubiquitin/proteasome pathway (den Engelsman et al., 2003). In addition, HSPB5 is able to protect cytoskeletal elements from ischemic injury and aggregation (Mineva et al., 2008). Transgenic mice overexpressing HSPB5showed a protective ability against IR injury in the heart (Ray et al., 2001).

HSPB5 is highly expressed in eye lens and protects lens epithelial cells from apoptosis triggered by oxidative stress (Kronschläger et al., 2013). HSPB5 plays an important role in preventing cell death. Increased expression of HSPB5 enhances resistance to thermal stress, UV light and other stress conditions (Boelens, 2014).

HSPB5 is the most abundant of the sHSP family in cardiac cells, with expression levels varying between 0.1% and 2% of the soluble protein content (Christians et al., 2012). Cardiac tissue overexpressing HSPB5 exhibited less tissue damage and preserved contractile function when subjected *ex vivo* to IR injury (Ray et al., 2001). In addition, HSPB5 can suppress pressure overload cardiac hypertrophy (Kumarapeli et al., 2010)

In humans, HSPB5 is playing critical roles in angiogenesis and autoimmune diseases, and expressed highly in breast cancer and Alzheimer disease (Ousman et al., 2007, Link et al., 2003). In addition, HSPB5 mutation can trigger many diseases such as cataract and cardiomyopathy (Sacconi et al., 2012).

5.1.6 HSPB6 (heat shock protein, alpha-crystallin-related B6, HSP20)

HSPB6 (HSP20) was first isolated from rat and skeletal muscle (Kato et al., 1994a). HSPB6 belongs to the group of ubiquitously expressed sHSP and its expression does not seem to be heat-inducible (Taylor and Benjamin, 2005). HSPB6 undergoes post-translational modifications (phosphorylation), and these modifications are might provide little change in HSPB6 activity in the cell (Mymrikov et al., 2011). HSPB6 can be phosphorylated at ser16 by a cAMP-dependent protein kinase A (Dreiza et al., 2010) and cGMP dependent protein kinase G (Beall et al., 1997, Woodrum et al., 1999). All member of sHSP family contain the α-crystallin domain and they interact with each other (Mchaourab et al., 2009). HSPB6 interact with β-crystallin, HSP27 and HSPB8 forming hetero-oligomeric complexes (Sugiyama et al., 2000, Bukach et al., 2009, Fontaine et al., 2005). HSPB2/MKBP and HSP20 were shown to bind partially to actin-associated proteins (Golenhofen et al., 2004). In addition, HSPB2/MKBP co-localization with mitochondria suggests that it may function as a molecular chaperone, protecting from apoptosis by

inhibiting cytochrome c efflux inhibiting the mitochondrial-dependent death pathways (Nakagawa et al., 2001).

5.1.6.1 HSPB6 functions

HSPB6 participates in regulation of smooth muscle contraction. This function is controlled by phosphorylation of HSPB6 at ser16, which is facilitated by activation of nucleotide-dependent protein kinases (cAMP). Phosphorylated HSPB6 correlated with relaxation of carotid artery smooth muscle (Beall et al., 1999, Rembold et al., 2000). Phosphorylated HSPB6 is also involve in vascular smooth muscle relaxation, which could be beneficial if cellular stress in the heart causes vasoconstriction (Flynn et al., 2003). Relaxation induced by HSPB6 phosphorylation was not accompanied by de-phosphorylation of myosin light chains (Rembold et al., 2000). The exact mechanism of HSPB6 action on smooth muscle relaxation and cell motility remains elusive (Mymrikov et al., 2011).

5.1.6.2 HSPB6 chaperone function

The α-crystallin domain is shared by all members of the sHSP family and is thought to be important for their molecular chaperone activity. It was believed that HSPB6 is a poor chaperone than β-crystallin in preventing insulin aggregation (van de Klundert et al., 1998b). However, it was shown that HSPB6 chaperone activity measured with different model protein substrates *in vitro,* was comparable to that of β-crystallin or HSPB8 (Mymrikov et al., 2010).

5.1.6.3 HSPB6 and cardio protective role

HSPB6 play an important role in cardio protection. Phosphorylation of HSPB6 in cardiomyocites is induced by isoproterenol (Chu et al., 2004). Prolonged isoproterenol treatment resulting in apoptosis and overexpression of HSPB6 prevented apoptosis induced by the isoproterenol (Fan et al., 2004). It has been shown (Fan et al., 2005b) that HSPB6 was effective in protection of cardiomyocytes against IR injury. This kind of injury leads to translocation of HSPB6 to actin filaments resulting in its stabilization (Fan et al., 2004). Overexpression of HSPB6 leads to a strong anti-apoptotic effect (Fan et al., 2005a) which may be due to an increased protein ratio of Bcl-2/Bax and reduced CASP3 activity (Fan et al., 2005b). In addition, HSPB6 activates Akt and thus reduces doxorubicintriggered oxidative stress and cardiotoxicity (Fan et al., 2008). Phosphorylated HSPB6 might be

also be involved in regulation of autophagy thus protecting the cell from apoptosis (Qian et al., 2009). HSPB6 also interacts with and inhibits NF-κβ which is responsible for cytokine synthesis during sepsis (Wang et al., 2009). The cardio protective effect of HSPB6 can be at least partly due to its chaperone-like activity (Mymrikov et al., 2011).

5.1.7 HSPB7 (heat shock 27kDa protein family, member 7 (cardiovascular, cvHSP)

The expression of most members of the sHSP family is developmentally regulated and many can be induced by various forms of stress (Davidson et al., 2002). The sHSPs are involved in a variety of cellular processes, mostly relating to cytoskeletal rearrangements and apoptosis (Quinlan, 2002, Arrigo, 2000, Kappé et al., 2003). HSPB7 was first identified and designated as a cardiovascular HSP due to its high expression in the heart (Krief et al., 1999). HSPB7 is characterized by its small molecular mass, approximately 20kDa, and a highly conserved α-crystallin domain (Augusteyn, 2004, Chiu et al., 2012). HSPB7 is localized within the cytosol or is associated with myofibrils in cardiac or skeletal muscle cells (Doran et al., 2007). HSPB7 was linked to sporadic heart failure on genome-wide association studies (Cappola et al., 2010, Villard et al., 2011). HSPB7 is a potential early biomarker after myocardial infraction and serves as an independent risk factor of acute coronary syndrome (Chiu et al., 2012). In addition, HSPB7 prevented aggregation of disease-associated proteins with an expanded polyQ stretch (Vos et al., 2010).

5.1.8 HSPB8 (heat shock 22kDa protein 8)

HSPB8 belongs to the sHSP family and is closely related to other members of the family especially to HSP27 (Sun et al., 2004). HSPB8 is heat-inducible (Chowdary et al., 2004). HSPB8 interact with HSPB1mimicking the phosphorylation by MAPKAP2 kinase (Rual et al., 2005). HSPB8 can be phosphorylated at ser24 and ser/thr87 (Villén et al., 2007). HSPB8 interacts with other sHSP family members such as HSPB6 and αB-crystallin (Fontaine et al., 2005).

5.1.8.1 HSPB8 chaperone function

Similar to other sHSP family, HSPB8 has chaperone-like activity, more important HSPB8 is also able to prevent the accumulation of protein aggregates in the cell (Chowdary et al., 2004, Mymrikov et al., 2011). Chaperone activity of HSPB8 also prevents formation of the aggresomes formed by the R120G mutant of B-crystallin, which correlates with

desmin-related cardiomyopathy (Zobel et al., 2003, Sanbe et al., 2007). Overexpression of HSPB8 was accompanied by reduction in amyloid aggregates leading to improved cardiac function and survival (Sanbe et al., 2009). In addition, it was shown that HSPB8 was more effective than HSPB1 in preventing in vivo aggregation of polyglutamine containing proteins (Carra et al., 2005). Furthermore, HSPB8 interacts with amyloid -peptides and inhibits the death of cardiovascular cells (Wilhelmus et al., 2006).

5.1.8.2 HSPB8 and inflammation

Several HSPs families are involved in stimulation of APC, chaperoning of antigen proteins and induces synthesis of certain interleukins, TNF, and NO (Srivastava, 2002). HSPB8 and HSPB4 are involved in the activation of APC through a TLR4 dependent mechanism (Roelofs et al., 2006). In addition, it has been shown (Roelofs et al., 2006) that HSPB8 plays a role in inflammatory process of autoimmune diseases, for example, HSPB8 was abundantly expressed in synovial tissues of rheumatoid arthritis patients.

5.1.8.3 HSPB8 and cardiac protection

HSPB8 is involved in cell survival as transit ischemia resulted in increased expression of HSPB8 (Depre et al., 2001). In chronic myocardium dysfunction (hibernating myocardium) due to coronary artery disease, HSPB8 was upregulated (Depre et al., 2004). The molecular mechanisms of HSPB8 action in cardiomyocytes is still not clear; it is believed that HSPB8 produces its anti-apoptotic by activation of protein kinase Ak (Danan et al., 2007). In addition, it was shown that HSPB8 also possesses metabolic and survival properties that seem to be due to activation of AMP-dependent protein kinase (Danan et al., 2007). It was postulated (Danan et al., 2007) that HSPB8 is able to produce pre-conditioning and survival effects by activating the ε-isoform of protein kinase C and the inducible NO synthase. Furthermore, it has been reported that these features are efficient in protecting myocardium against irreversible damage and may be explained by the direct interaction of HSPB8 with AMP-dependent protein kinase and its translocation to perinuclear space (Danan et al., 2007).

5.2 Results

5.2.1 Patient clinical data analysis

Patient data analysis is the same as section (3.4.1.1), results analysis is shown in (Table 3-2) (Table 3-3).

5.2.2 Undetermined sHSPs

Presence of HSPB3 (heat shock 27kDa protein 3) and HSPB4 (CRYAA, crystallin, alpha A) are undetermined in the human placenta.

5.2.3 HSPB2 expression in normal pregnancies and PE

Experiment 1: This experiment was designed to test if there was a spatial difference in mRNA expression of HSPB2 within individual placentas obtained from women who were not in labour (non-labour group) and from women who were in labour (labour group). Comparison between zones was performed using Friedman test analysis. There was no significant difference in HSPB2 mRNA expression between the three zones of the non-labour and labour groups (p=0.57 for both) (Figure 5-1).

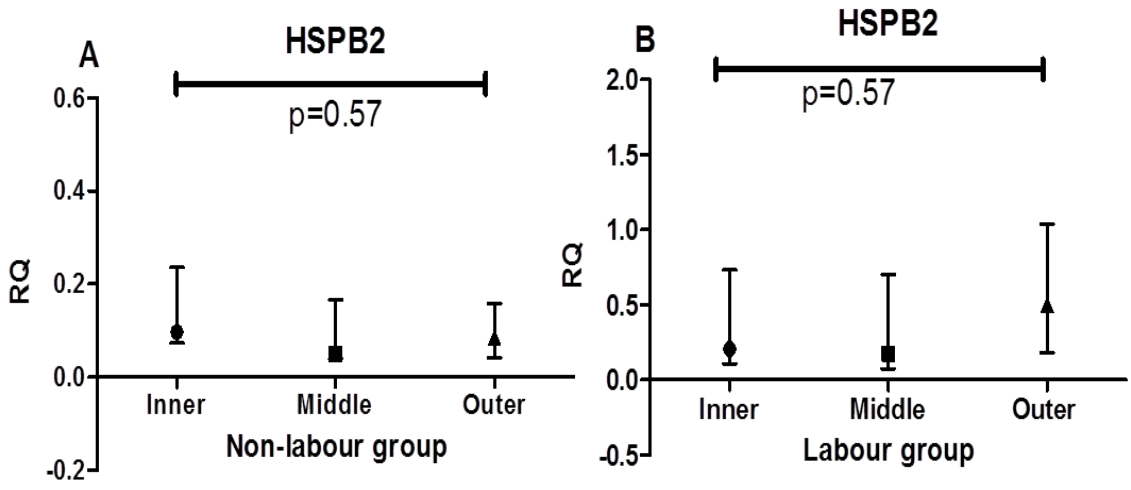


Figure 5-1: RQ values for HSPB2 mRNA measurements in in inner (n=4), middle (n=4) and outer zones (n=4) of individual placentas (non-labour and labour groups, 6 placentas in each group). Four quadrants were sampled in each zone. Graphs show the median for HSPB2 mRNA expressions. Comparison between zones was performed using Friedman analysis.

Experiment 2: This experiment was designed to test if there was any difference in mRNA expression of HSPB2 between labour and non-labour groups at the inner, middle or outer zones of placentas. Comparison between zones was performed using Mann Whitney test analysis. There was a significant decrease in HSPB2 mRNA expression in the non-labour group when compared to the labour group at the outer zone (p=0.02). No difference was found at the inner and middle zones (p=0.13, p=0.06) (Figure 5-2).

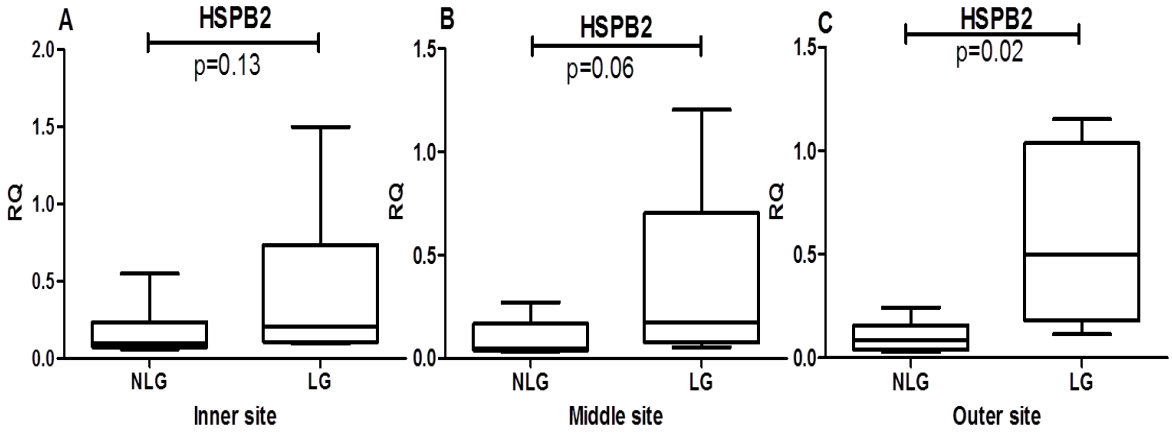


Figure 5-2: RQ values for HSPB2 mRNA measurements in non-labour (n=6) compared to the labour group (n=6) at the inner, middle and outer placental zones. Comparison between zones was performed using Mann-Whitney analysis.

Experiment 3: This experiment was designed to test if there was any difference in mRNA expressions of HSPB2 between non-labour control and non-labour PE groups at the inner or middle zones of placentas. Comparison between zones was performed using the Mann Whitney test. There was no significant difference in HSPB2 mRNA at the inner and middle zones (p=0.16, p=0.26) (Figure 5-3).

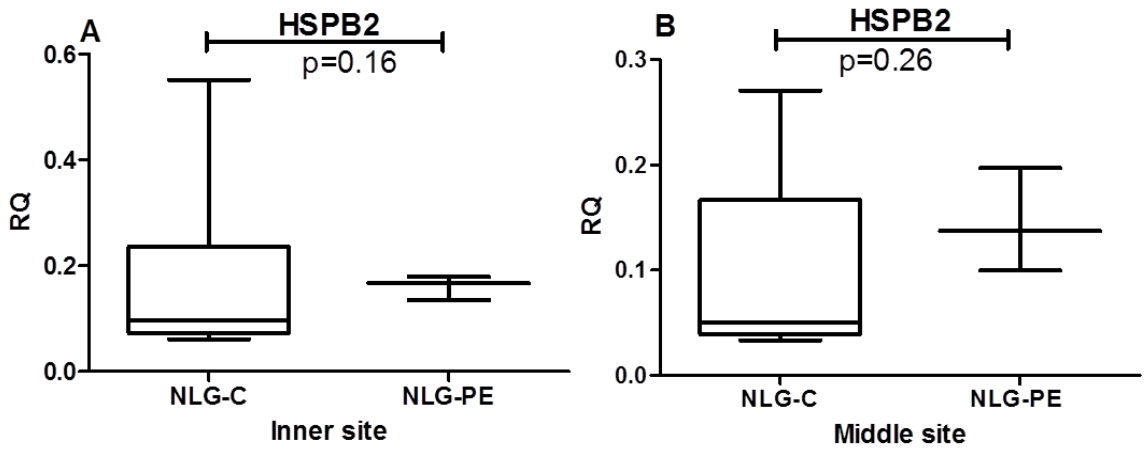


Figure 5-3: RQ values for HSPB2 mRNA measurements in non-labour control (n=6) compared to the non-labour PE groups (n=3) at the inner and middle placental zones. Comparison between zones was performed using Mann-Whitney analysis.

Experiment 4: This experiment was designed to test if there was any difference in mRNA expressions of HSPB2 between labour control and labour PE groups at the inner or middle zones of placentas. Comparison between zones was performed using Mann Whitney test analysis There was no significant difference in HSPB2 mRNA in the labour control group compared to the labour PE group in the inner or middle placental zones (p=0.6 for both) (Figure 5-4).

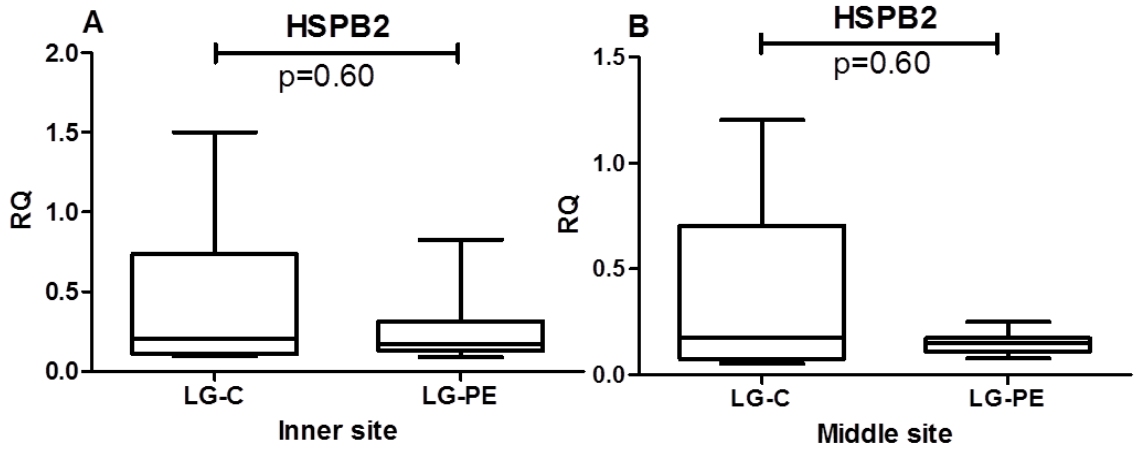


Figure 5-4: RQ values for HSPB2 mRNA measurements in labour control (n=6) compared to the labour PE groups (n=6) at the inner and middle placental zones. Comparison between zones was performed using Mann-Whitney analysis.

5.2.4 HSPB5 expression in normal pregnancies and PE

Experiment 1: This experiment was designed to test if there was a spatial difference in mRNA expression of HSPB5 (CRYAB) within individual placentas obtained from women who were not in labour (non-labour group) and from women who were in labour (labour group). Comparison between zones was performed using Friedman test analysis. There was no significant difference in mRNA expression of CRYAB between the three zones of the non-labour and labour groups (p=0.74 for both) (Figure 5-5).

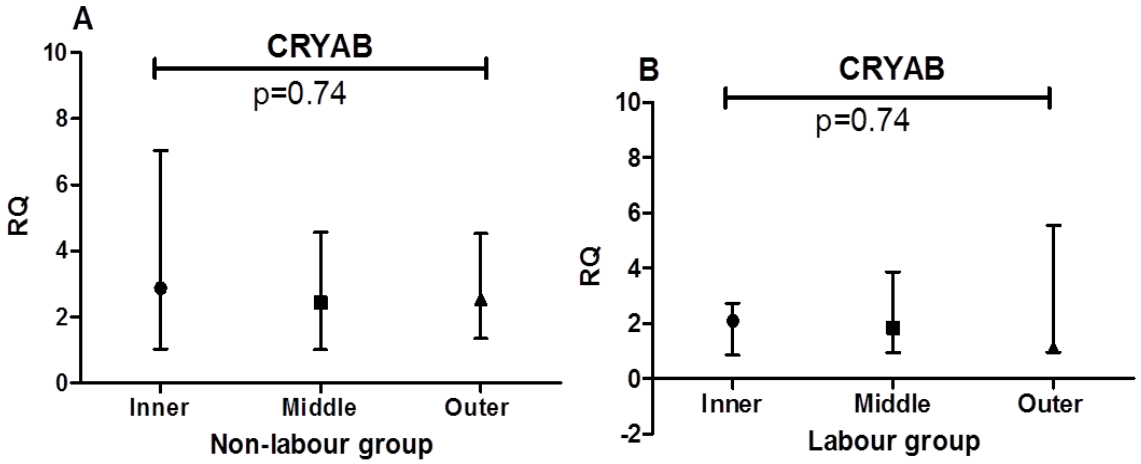


Figure 5-5: RQ values for CRYAB mRNA measurements in in inner (n=4), middle (n=4) and outer (n=4) zones of individual placentas (non-labour and labour groups, 6 placentas in each group). Four quadrants were sampled in each zone. Graphs show median and interquartile range for CRYAB mRNA expressions. Comparison between zones was performed using Friedman analysis.

Experiment 2: This experiment was designed to test if there was any difference in mRNA expression of CRYAB between labour and non-labour groups at the inner, middle or outer zones of placentas. Comparison between zones was performed using the Mann Whitney test analysis. There was no significant difference in CRYAB mRNA expression in the labour group when compared to the non-labour group at the inner, middle and outer zones (p=0.48, p=0.81, p=0.30) (Figure 5-6).

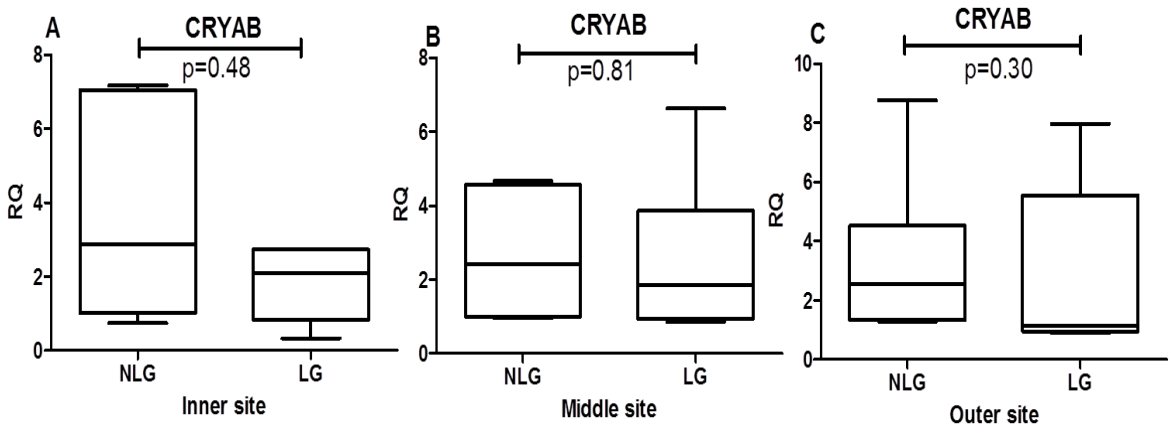


Figure 5-6: RQ values for CRYAB mRNA measurements in non-labour (n=6) compared to the labour (n=6) group at the inner, middle and outer placental zones. Comparison between zones was performed using Mann-Whitney analysis.

Experiment 3: This experiment was designed to test if there was any difference in mRNA expressions of CRYAB between non-labour control and non-labour PE groups at the inner or middle zones of placentas. Comparison between zones was performed using the Mann Whitney test analysis. There was no significant difference in the CRYAB mRNA at the inner and middle placental zones (p=0.54, p=0.38) (Figure 5-7).

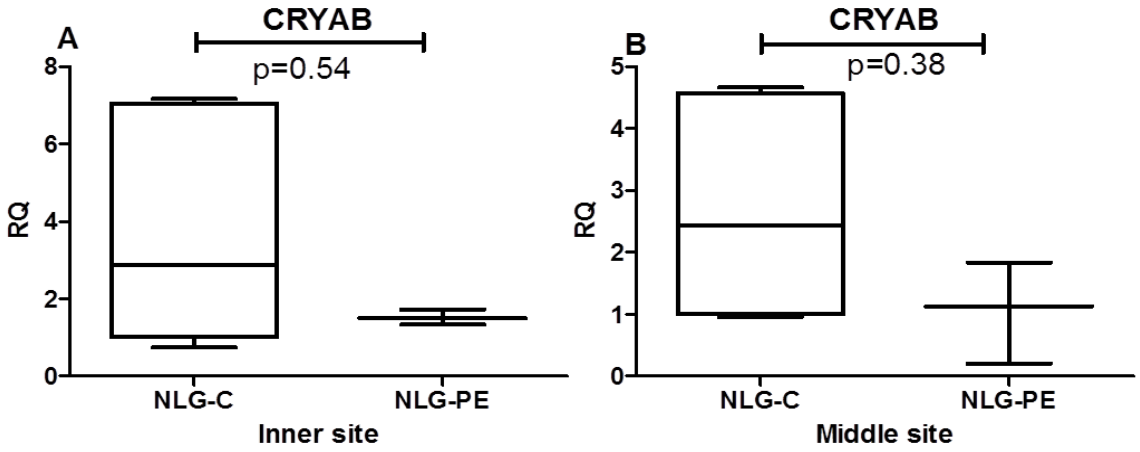


Figure 5-7: RQ values for CRYAB mRNA measurements in non-labour control (n=6) compared to the non-labour PE (n=3) groups at the inner and middle placental zones. Comparison between zones was performed using Mann-Whitney analysis.

Experiment 4: This experiment was designed to test if there was any difference in mRNA expressions of CRYAB between labour control and labour PE groups at the inner or middle zones of placentas. Comparison between zones was performed using the Mann

Whitney test analysis. There was no significant difference in CRYAB mRNA in the labour control group compared to the labour PE group in the inner or middle placental zones (p=0.95, p=0.32) (Figure 5-8).

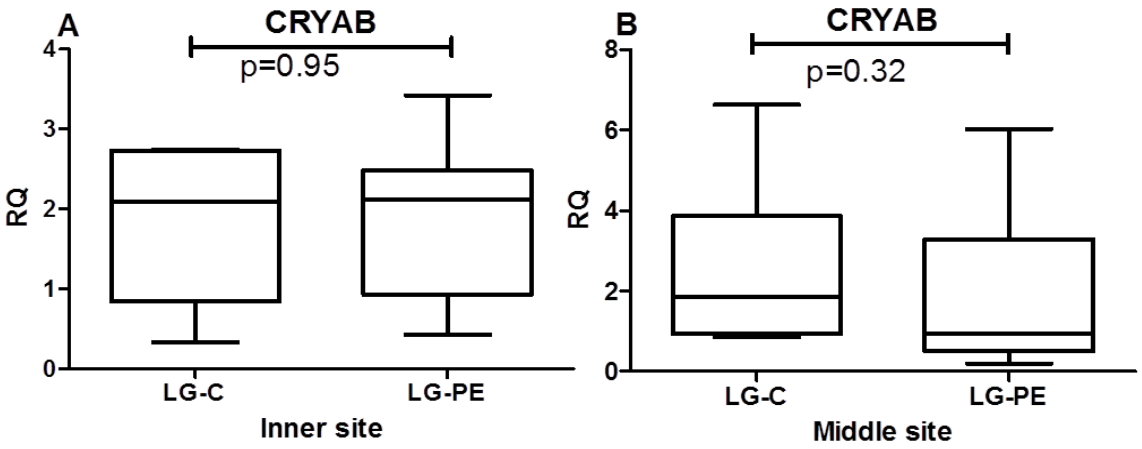


Figure 5-8: RQ values for CRYAB mRNA measurements in labour control (n=6) compared to the labour PE (n=6) groups at the inner and middle placental zones. Comparison between zones was performed using Mann-Whitney analysis.

5.2.5 HSPB6 expression in normal pregnancies and PE

Experiment 1: This experiment was designed to test if there was a spatial difference in mRNA expression of HSPB6 within individual placentas obtained from women who were not in labour (non-labour group) and from women who were in labour (labour group). Comparison between zones was performed using Friedman test analysis. There was no significant difference in mRNA expression between the three zones of the non-labour and labour groups (p=0.74, p=0.95 respectively) (Figure 5-9).

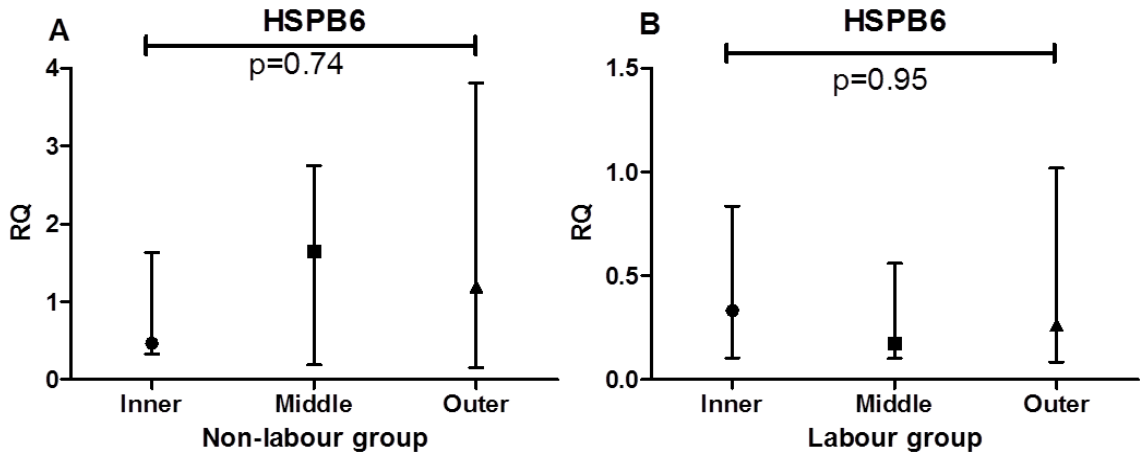


Figure 5-9: RQ values for HSPB6 mRNA measurements in in inner (n=4), middle (n=4) and outer (n=4) zones of individual placentas (non-labour and labour groups, 6 placentas in each group). Four quadrants were sampled in each zone. Graphs show median and interquartile range for HSPB6 mRNA expressions. Comparison between zones was performed using Friedman analysis.

Experiment 2: This experiment was designed to test if there was any difference mRNA expression of HSPB6 between labour and non-labour groups at the inner, middle or outer zones of placentas. Comparison between zones was performed using Mann Whitney test analysis. There was a significant decrease in mRNA expression of HSPB6 in the labour group when compared to the non-labour group at the middle zone (p=0.01). No difference was found at the inner and outer zones (p=0.3 for both) (Figure 5-10).

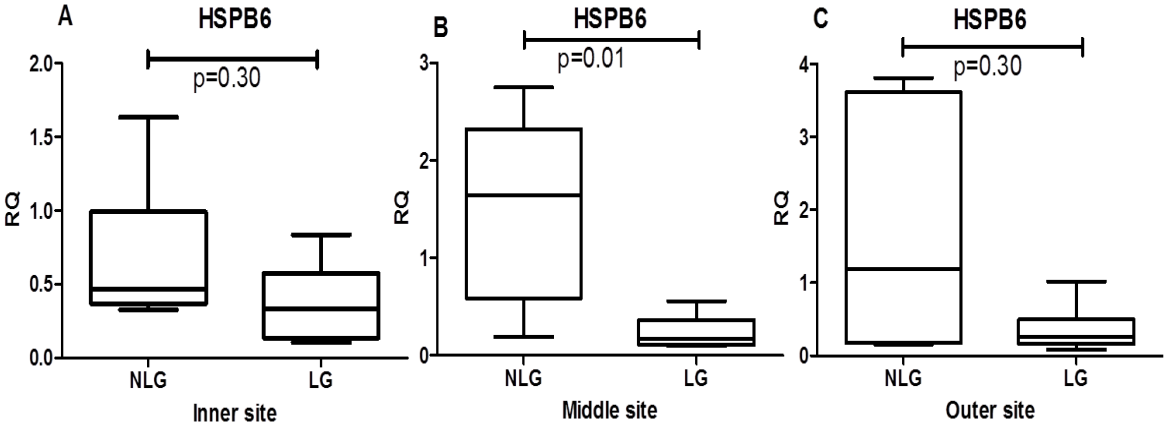


Figure 5-10: RQ values for HSPB6 mRNA measurements in non-labour (n=6) compared to the labour group (n=6) at the inner, middle and outer placental zones. Comparison between zones was performed using Mann-Whitney analysis.

Experiment 3: This experiment was designed to test if there was any difference in mRNA expressions of HSPB6 between non-labour control and non-labour PE groups at the inner or middle zones of placentas. Comparison between zones was performed using Mann Whitney test analysis. There was a significant decrease in HSPB6 mRNA in the non-labour PE group compared to the non-labour control group in the inner zone (p=0.02). There was no significant difference in the HSPB6 mRNA was found at the middle zone (p=0.09) (Figure 5-11).

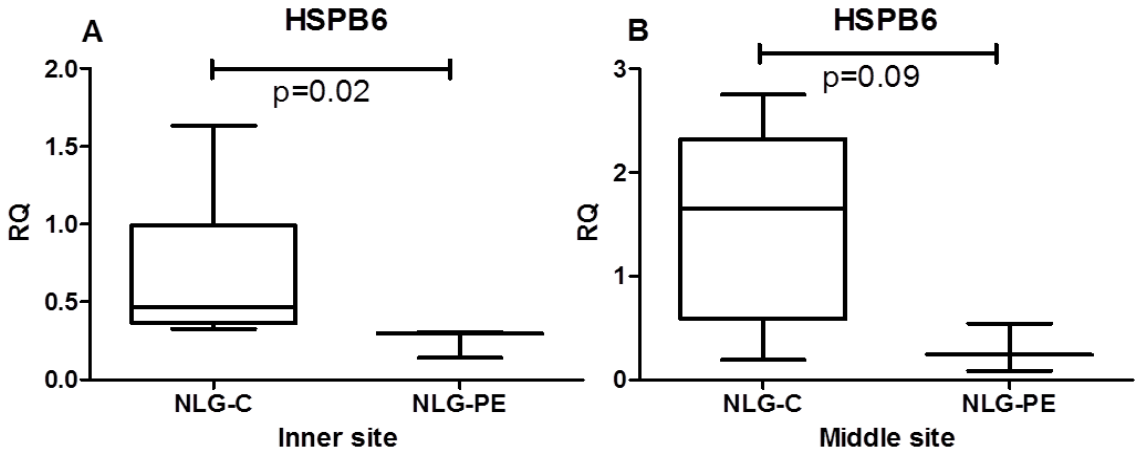


Figure 5-11: RQ values for HSPB6 mRNA measurements in non-labour control (n=6) compared to the non-labour PE (n=3) groups at the inner and middle placental zones. Comparison between zones was performed using Mann-Whitney analysis.

Experiment 4: This experiment was designed to test if there was any difference in mRNA expressions of HSPB6 between labour control and labour PE groups at the inner or middle zones of placentas. Comparison between zones was performed using Mann Whitney test analysis. There was no significant difference in HSPB6 mRNA in the labour control group compared to the labour PE group in the inner or middle placental zones (p=0.52, p=1) (Figure 5-12).

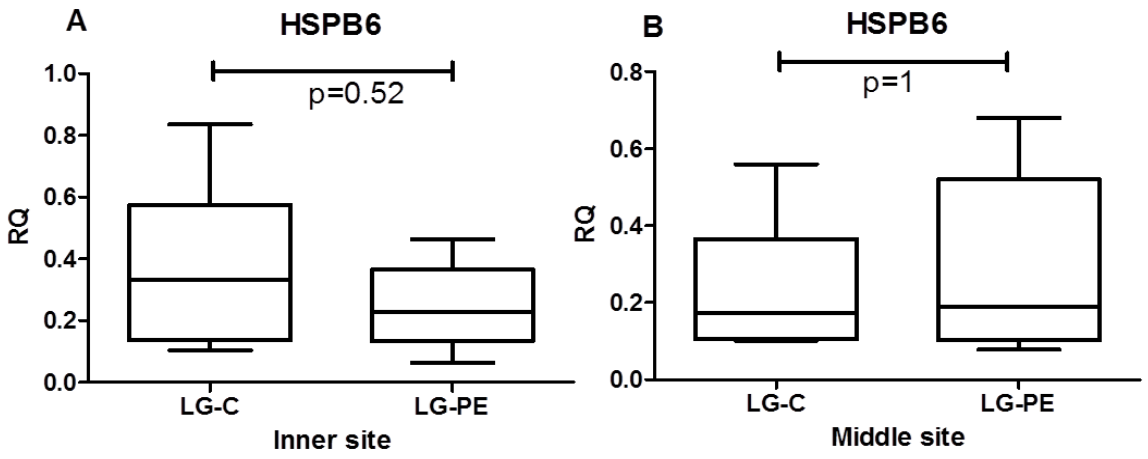


Figure 5-12: RQ values for HSPB6 mRNA measurements in labour control (n=6) compared to the labour PE (n=6) groups at the inner and middle placental zones. Comparison between zones was performed using Mann-Whitney analysis.

5.2.6 HSPB7 expression in normal pregnancies and PE

Experiment 1: This experiment was designed to test if there was a spatial difference in mRNA expression of HSPB7 within individual placentas obtained from women who were not in labour (non-labour group) and from women who were in labour (labour group). Comparison between zones was performed using Mann Whitney test analysis. There was no significant difference in mRNA expression of HSPB7 between the three zones of the non-labour and labour groups (p=1, p=0.57) (Figure 5-13).

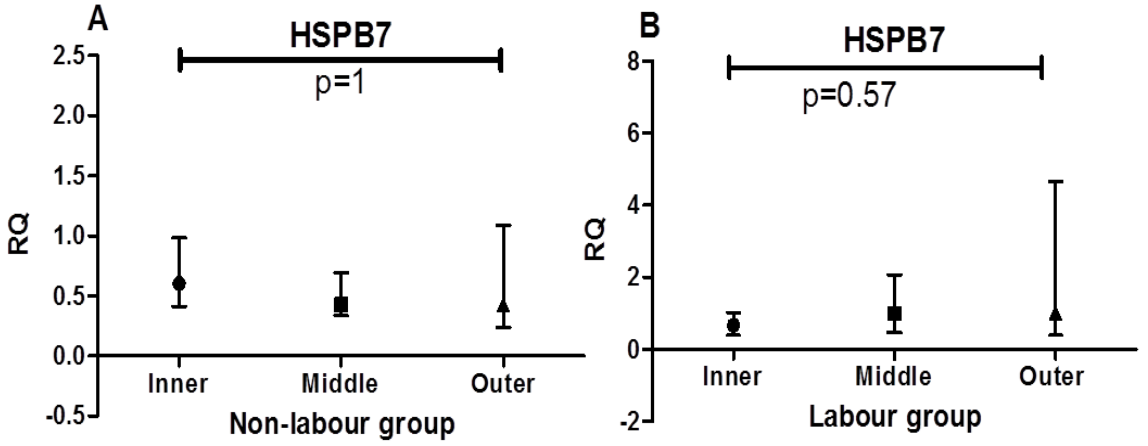


Figure 5-13: RQ values for HSPB7 mRNA measurements in in inner (n=4), middle (n=4) and outer (n=4) zones of individual placentas (non-labour and labour groups, 6 placentas in each group). Four quadrants were sampled in each zone. Graphs show median and interquartile range for mRNA expressions of HSPB7. Comparison between zones was performed using Friedman analysis.

Experiment 2: This experiment was designed to test if there was any difference in mRNA expression of HSPB7 between labour and non-labour groups at the inner, middle or outer zones of placentas. Comparison between zones was performed using the Mann Whitney test analysis. There was no significant difference in mRNA expression of HSPB7 in the labour group when compared to the non-labour group at the inner, middle and outer zones (p=1, p=0.17, p=0.24) (Figure 5-14).

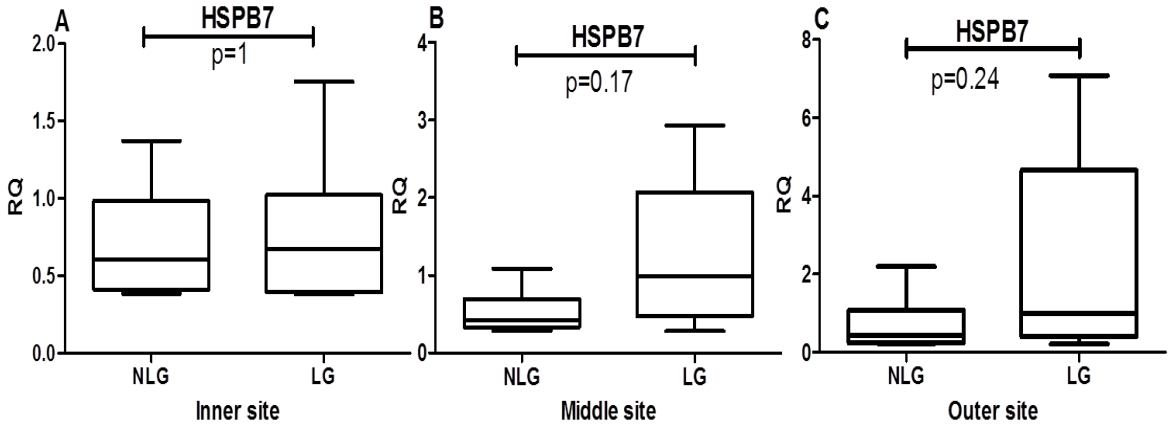


Figure 5-14: RQ values for HSPB7 mRNA measurements in non-labour (n=6) compared to the labour (n=6) group at the inner, middle and outer placental zones. Comparison between zones was performed using Mann-Whitney analysis.

Experiment 3: This experiment was designed to test if there was any difference in mRNA expressions of HSPB7 between non-labour control and non-labour PE groups at the inner or middle zones of placentas. Comparison between zones was performed using Mann Whitney test analysis. There was no significant difference in the HSPB7 mRNA at the inner and middle placental zones (p=0.54, p=0.90) (Figure 5-15).

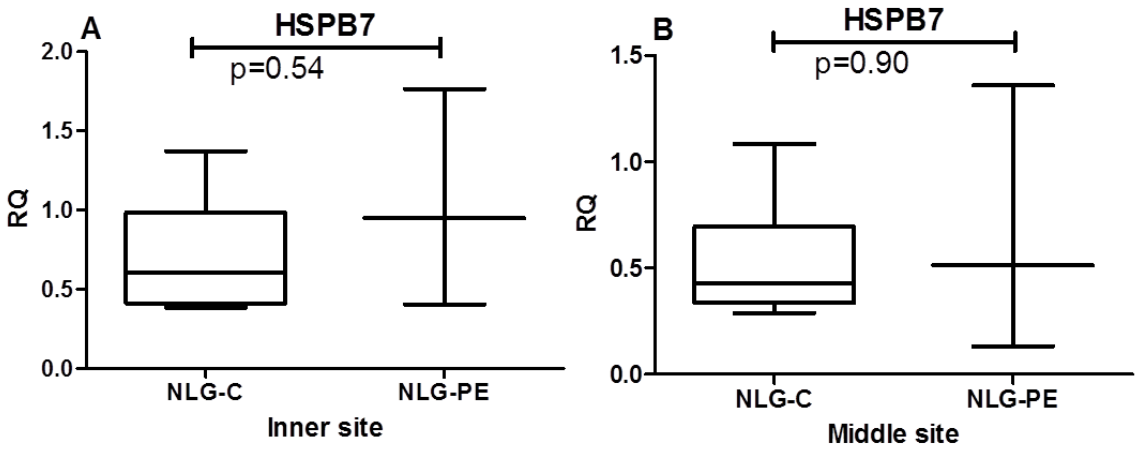


Figure 5-15: RQ values for HSPB7 mRNA measurements in non-labour control (n=6) compared to the non-labour PE (n=3) groups at the inner and middle placental zones. Comparison between zones was performed using Mann-Whitney analysis.

Experiment 4: This experiment was designed to test if there was any difference in mRNA expressions of HSPB7 between labour control and labour PE groups at the inner or middle zones of placentas. Comparison between zones was performed using the Mann Whitney test analysis There was no significant difference in HSPB7 mRNA in the labour control group compared to the labour PE group in the inner or middle placental zones (p=0.32, p=0.22) (Figure 5-16).

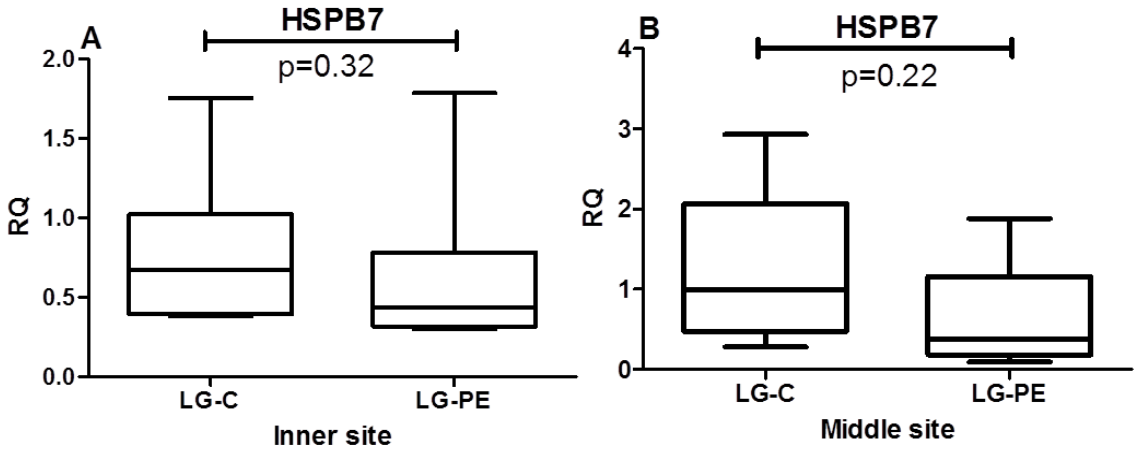


Figure 5-16: RQ values for HSPB7 mRNA measurements in labour control (n=6) compared to the labour PE (n=6) groups at the inner and middle placental zones. Comparison between zones was performed using Mann-Whitney analysis.

5.2.7 HSPB8 expression in normal pregnancies and PE

Experiment 1: This experiment was designed to test if there was a spatial difference in mRNA expression of HSPB8 within individual placentas obtained from women who were not in labour (non-labour group) and from women who were in labour (labour group). Comparison between zones was performed using Friedman test analysis. There was no significant difference in mRNA expression of HSPB8 between the three zones of the non-labour and labour groups (p=0.57, p=0.14 respectively) (Figure 5-17).

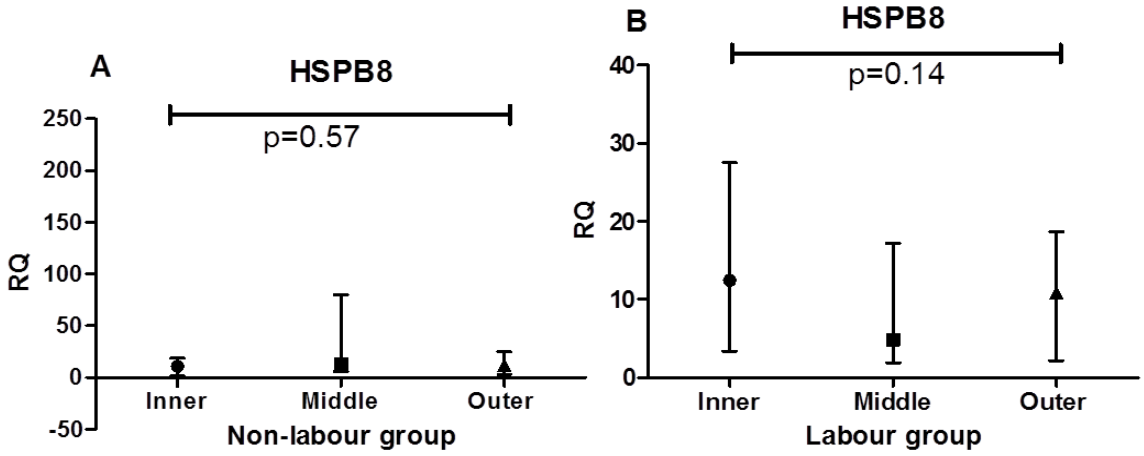


Figure 5-17: RQ values for HSPB8 mRNA measurements in in inner (n=4), middle (n=4) and outer (n=4) zones of individual placentas (non-labour and labour groups, 6 placentas in each group). Four quadrants were sampled in each zone. Graphs show the median for mRNA expressions HSPB8. Comparison between zones was performed using Friedman analysis.

Experiment 2: This experiment was designed to test if there was any difference in mRNA expression of HSPB8 between labour and non-labour groups at the inner, middle or outer zones of placentas. Comparison between zones was performed using Mann Whitney test analysis. There was no significant difference in mRNA expression of HSPB8 in the labour group when compared to the non-labour group at the inner, middle and outer zones (p=0.39, p=0.24, p=0.81) (Figure 5-18).

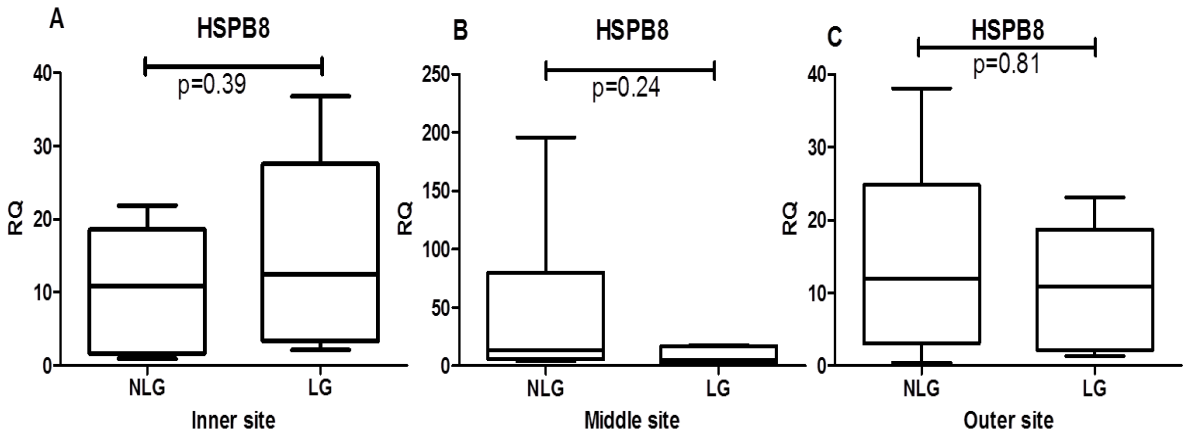


Figure 5-18: RQ values for HSPB8 mRNA measurements in non-labour (n=6) compared to the labour (n=6) groups at the inner, middle and outer placental zones. Comparison between zones was performed using Mann-Whitney analysis.

Experiment 3: This experiment was designed to test if there was any difference in mRNA expressions of HSPB8 between non-labour control and non-labour PE groups at the inner or middle zones of placentas. Comparison between zones was performed using Mann Whitney test analysis. There was no significant difference in HSPB6 mRNA at the inner and middle placental zones (p=0.9, p=09) (Figure 5-19).

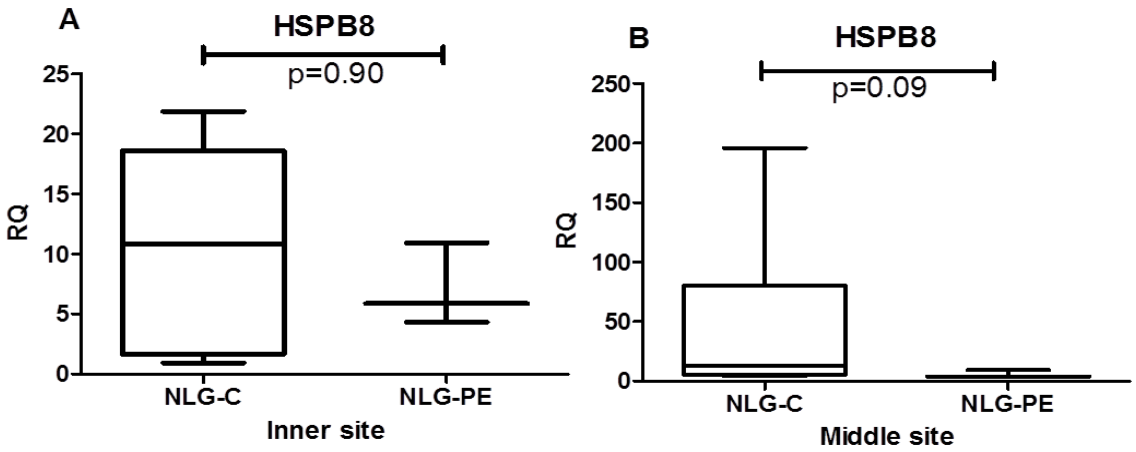


Figure 5-19: RQ values for HSPB8 mRNA measurements in non-labour control (n=6) compared to the non-labour PE (n=3) groups at the inner and middle placental zones. Comparison between zones was performed using Mann-Whitney analysis.

Experiment 4: This experiment was designed to test if there was any difference in mRNA expressions of HSPB8 between labour control and labour PE groups at the inner or middle zones of placentas. Comparison between zones was performed using Mann Whitney test

analysis There was no significant difference in HSPB8 mRNA in the labour control group compared to the labour PE group in the inner or middle placental zones (p=0.45, p=0.6) (Figure 5-20).

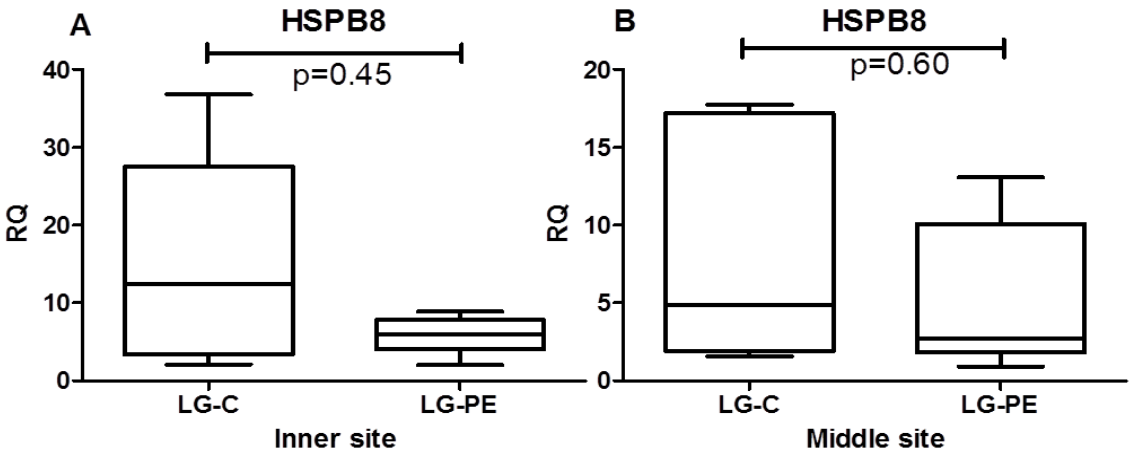


Figure 5-20: RQ values for HSPB8 mRNA measurements in labour control (n=6) compared to the labour PE (n=6) groups at the inner and middle placental zones. Comparison between zones was performed using Mann-Whitney analysis.

5.2.8 Results summary

5.2.8.1 HSPB2

Experiment 1: There was no significant spatial difference in mRNA expression of HSPB2 expression between the three zones of the non-labour and labour groups.

Experiment 2: There was a significant decrease in mRNA expression of HSPB2 in the non-labour group when compared to the labour group at the outer zone (p=0.02). No difference was found at the inner and middle zones.

Experiment 3: There was no significant difference in mRNA expression of HSPB2 in the non-labour control group compared to the non-labour PE group in the inner or middle placental zones.

Experiment 4: There was no significant difference in mRNA expression of HSPB2 in the labour control group compared to the labour PE group in the inner or middle placental zones.

5.2.8.2 CRYAB

Experiment 1: There was no significant spatial difference in mRNA expression of CRYAB between the three zones of the non-labour and labour groups.

Experiment 2: There was no significant difference in CRYAB mRNA expression in the non-labour group when compared to the labour group at the inner, middle and outer zones

Experiment 3: There was no significant difference in the mRNA expression of CRYAB in the non-labour PE group compared to the non-labour control group at the inner and middle zones.

Experiment 4: There was no significant difference in mRNA expression of CRYAB in the labour control group compared to the labour PE group at the inner and middle placental zones.

5.2.8.3 HSPB6

Experiment 1: There was no significant spatial difference in mRNA expression of HSPB6 expression between the three zones of the non-labour and labour groups.

Experiment 2: There was a significant decrease in mRNA expression of HSPB6 in the labour group when compared to the non-labour group at the middle zone ($p=0.01$). No difference was found at the inner and outer zones.

Experiment 3: There was a significant decrease in HSPB6 mRNA in the non-labour PE group compared to the non-labour control group in the inner zone ($p=0.02$). There was no significant difference in the HSPB6 mRNA was found at the middle zone.

Experiment 4: There was no significant difference in mRNA expression of HSPB6 in the labour control group compared to the labour PE group in the inner or middle placental zones.

5.2.8.4 HSPB7

Experiment 1: There was no significant spatial difference in mRNA expression of HSPB7 between the three zones of the non-labour and labour groups.

Experiment 2: There was no significant difference in mRNA expression of HSPB7 in the non-labour group when compared to the labour group at the inner, middle and outer zones

Experiment 3: There was no significant difference in the mRNA expression of HSPB7 in the non-labour PE group compared to the non-labour control group at the inner and middle zones.

Experiment 4: There was no significant difference in mRNA expression of HSPB7 in the labour control group compared to the labour PE group at the inner and middle placental zones.

5.2.8.5 HSPB8

Experiment 1: Overall there was no significant spatial difference between the three zones of the placenta in HSPB8 expression for the non-labour and labour groups.

Experiment 2: There was no significant different in HSPB8 expression in the labour group compared to the non-labour group at the inner, middle and outer zones of placentas.

Experiment 3: There was no significant increase in HSPB8 expression in the non-labour PE group compared to the non-labour control group in the inner and middle zones.

Experiment 4: There was no significant difference between the labour control and labour PE groups at the inner and middle zones.

5.3 Discussion

The mRNA expression of sHSPs of members of the family was investigated using qRT-PCR. The inner, middle and outer zones for non-labour and labour groups were analysed but due to time constraints only the inner and middle zones for the PE group were studied. The aim was to determine whether pilot data from this group of experiments revealed any data suggesting that more experiments were warranted and as screen study for mRNA expressions of sHSPs in the human placenta.

HSPB3 and HSPB4 were not detected in the human placenta. Only HSPBs 1-8 were studied as 9 and 10 are testicular HSPs. There were only a few significant differences in mRNA expression of sHSPs between non-labour versus labour groups and normotensive versus pregnancies complicated with PE (Table 5-2).

Table 5-2: Summary of different comparison in mRNA expression of the sHSP family in the non-labour group (NLG), labour group (LG), non-labour-PE (NLG-PE) and labour group-PE (LG-PE) at the inner (I), middle (M) and outer (O) zones. Comparison was pointed by arrows to either increase, decrease or (No) for no change.

mRNA	HSPB2	HSPB5	HSPB6	HSPB7	HSPB8
Spatial distribution	No	No	No	No	No
NLG versus LG	↓(O)	No	↑(M)	No	No
NLG-PE versus NLG-C	No	No	↓(I)	No	No
LG-PE versus LG-C	No	No	No	No	No

5.3.1 HSPB2 (HSP22) and present findings

HSB2 expression was significantly decreased in the non-labour compared to the labour group at the outer zone. Importantly the present work shows that expression varies on the zone of the placenta the piece of tissue is taken from. Nothing is known about the role of HSPB2 in the placenta or whether it plays a role in labour or PE.

5.3.2 HSPB2 in labour and PE

HSPs are induced in response to cell stresses including heat shock, oxidative stress, ultraviolet radiation, ischemia-reperfusion injury, viral infections, nutrient deprivation, hypoxia and ischemia. This study shows for this first time that HSPB2 mRNA is expressed in the placenta and this expression increased in labour group at outer zone. The physiological and pathological significance of this remains to be elucidated. Labour is associated with inflammation, apoptosis and oxidative stress and HSB2 is an anti-oxidant chaperone that protect cells against these conditions.

5.3.3 HSPB5 and present findings

HSB5 was shown to be expressed in the placenta in this study. However, there were no significant changes in mRNA expression of HSB5 both in non-labouring, labouring and PE placentas.

5.3.4 HSPB5 and labour and PE

Normal human labour involves the interaction of hormonal, neurological, mechanical stretch and inflammatory pathways and the placenta plays a crucial role. Labour has been shown to induce placental oxidative stress, which in turn activates ER stress *in vitro* (Yung et al., 2007, Cindrova-Davies et al., 2007b). Heat shock proteins are found in several tissues involved in human reproduction. Members of all HSP families are found in the endometrium of healthy women (Tabibzadeh and Broome, 1999). It has been shown (Tian et al., 2013) that mRNA expression of HSPB5 is detected at the implantation sites in mouse uteri. In addition, mRNA expression of HSPB5 was increased in mouse uterus once delayed implantation is activated by estrogen (Su et al., 2010a). Recently, it has been shown that HSPB5 is expressed in mouse decidua and also protects against oxidative and inflammatory stress conditions during decidualization (Zuo et al., 2014). Furthermore, it has been shown (Zuo et al., 2014) that HSB5 was significantly induced and phosphorylated in stromal cells of mouse decidua when exposed to oxidative stress. In humans, one study showed that HSPB5 is expressed in endometrium during the implantation window (Gruidl et al., 1997).

There is one publication showing HSPB5 expression in the placenta (Mineva et al., 2008). This study showed using immunofluorescence that HSPB5 was expressed in the stroma of placental villi and there was an inter-individual HSPB5 expression variation. Protein and

mRNA expression of HSPB5 also detected in low amounts in term placenta compared to HSP27 (Mineva et al., 2008). However, systematic sampling method was not performed and labour was not investigated so it is not possible to compare this finding to our study.

5.3.5 HSPB6 and present finding

HSB6 mRNA expression was significantly decreased in the labour group compared to the non-labour group at the middle zone. However, HSPB6 also more less in the non-labour PE group compared to non-labour C especially at the inner zone. Importantly the present work shows again that expression varies on the zone of the placenta the piece of tissue is taken from. Nothing is known about the role of HSPB6 in the placenta or whether it plays a role in labour or PE.

5.3.6 HSPB6 expression in labour and PE

The key finding was the fall in mRNA expression of HSPB6 at the middle zone of the placenta during labour. Many HSPs are increased to protect against oxidative damage and lipid peroxidation and regulation of apoptosis (Garrido et al., 2012) however in the present finding HSPB6 was reduced. One reason for this may be that a fall in HSPB6 may be necessary to facilitate the inflammatory steps of labour which is, after all, a normal physiological process, not a disease. A reduction in HSPB6 may help to initiate or promote labour. Protein expression of HSPB6 is therefore worth investigating in further studies. There was a significant decrease in mRNA expression of HSPB6 in the non-labour PE compared to the non-labour control at the inner zone. The reason for that is not clear, but there may be two parallel pathways; one associated with labour and one with PE and they may not necessarily work in the same direction.

5.3.7 HSPB7 and HSPB8

No changes in mRNA expression of HSPB7 or HSPB8 between non-labour and labour groups or normotensive compared to PE pregnancies were found. There was no zonal distribution. Nothing is known about the role of HSPB7 or HSPB8 in the placenta or whether it plays a role in labour or PE.

5.3.8 Linking sHSPs expression to oxidative stress and cell death

Oxidative stress occurs when the production of ROS overwhelms the intrinsic anti-oxidant defences. Contraction of the uterus leads to IR injury that can alter placental sHSP expression (Cindrova-Davies et al., 2007b).

Members of the sHSP family exhibit several properties include chaperone activities, potent anti-apoptotic activity, pro-angiogenic properties and anti-inflammatory property involving interactions with several clients. Mitochondria play an important role in cell death via production of excess ROS (Ott et al., 2007). HSPB5 has anti-apoptotic functions that protect cells against stress. This is essential for the protective function of HSPB5 that involves P38 and ERK activity (Li and Reiser, 2011). In addition, it has been shown that HSPB5 prevents apoptosis induced upon oxidative stress and upon treating with drugs such as doxorubicin and staurosporin (Mehlen et al., 1996, Kamradt et al., 2001, Bakthisaran et al., 2014). HSPB5 has been shown to prevent TNF-α-induced cell death and over-expression leads to increased levels of glutathione and reduce levels of TNF-α- induced increase in ROS (Mehlen et al., 1996). Under stress conditions, the sHSP family has the ability to interact with cytoskeletal elements and protect them against apoptosis and cell death. There seems to be an apoptotic signalling pathway linking cytoskeletal damages to mitochondria (Bakthisaran et al., 2014).

HSPB5 and HSPB6 interact with the pro-apoptotic molecule, Bax and inhibit its translocation into mitochondria and the subsequent release of cytochrome c (Mao et al., 2004, Fan et al., 2005b). In addition, HSPB5 interacts with cytochrome c and prevents interaction of Apaf-1 with pro-caspase 9 to form apoptosome (McGreal et al., 2012). HSPB5 also prevents pro-caspase-3 conversion to active CASP3 (Kamradt et al., 2002, Jeong et al., 2012). Furthermore, HSPB5 interacts with the pro-apoptotic molecules include Bax and Bcl-Xs and prevents apoptosis by inhibiting their translocation into mitochondria (Mao et al., 2004).

In the heart HSPB6 protects cardiac cells from IR injury induced apoptosis, improves recovery of cardiac function (Fan et al., 2005b). HSPB6 interacts with the Bax (pro-apoptotic protein) and preventing its translocation to mitochondria, leading to decreased CASP3 activation (Fan et al., 2005b).

It has been shown that HSPB2 is a negative regulator of the caspase dependent extrinsic apoptotic pathway mediated by the Fas and FADD adaptors (Oshita et al., 2010). HSPB2 prevents activation of CASP8 and 10 (Oshita et al., 2010).

In compare to anti-apoptotic properties of the sHSPs, HSPB8 appears to have dual roles (Bakthisaran et al., 2014). It is possible that is a result of anti-apoptotic functions of other HSPs or HSPB8 pro-apoptotic property is cell type specific (Bakthisaran et al., 2014). HSPB8 protect the heart from IR injury and exhibits an anti-apoptotic property by activation of the Akt pathway (Danan et al., 2007, Acunzo et al., 2012).

Chapter VI: Analysis of mRNA expression of HO-1 (HSP32), DNAJ (HSP40) and HSPD1 (HSP60) families in human placenta of normal pregnancies and pregnancies complicated with PE

6 HO-1, DNAJ and HSPD1

6.1 Introduction

6.1.1 Heme oxygenase (decycling)-1 (HO-1, HSP32)

Hemoxygenase-1 (HO-1) was discovered by Tenhunen et al (Tenhunen et al., 1968) and is an enzyme that facilitates the degradation of heme. This reaction uses NADPH as cofactor and yields the by-products carbon monoxide (CO), ferrous iron and biliverdin, which is then subsequently converted into bilirubin by biliverdin reductase (Ponka, 1999, Idriss et al., 2008, Barbagallo et al., 2013). All three products instead display potent cytoprotective properties. CO has been demonstrated to display several of the same physiological functions as NO (Bainbridge and Smith, 2005). CO act as a neurotransmitter inhibits platelet aggregation and is a vascular smooth muscle relaxant (Lyall et al., 2000). In addition, the second and third catalytic compounds of heme degradation (biliverdin and ferrous iron) contribute to HO's antioxidant effect (Bainbridge and Smith, 2005). Biliverdin and its subsequent breakdown product bilirubin demonstrate potent antioxidant characteristics (McGeary et al., 2003).

HO exists in three isoforms; the first isoform is HO-1, a 32 kDa protein induced by several stimuli includes heavy metals, ROS, NF-κβ and hypoxia inducible factor (Idriss et al., 2008, Syapin, 2008, Wagner et al., 2003). The second isoform HO-2 is a constitutively synthesized 36-kDa protein and is generally non-inducible. Its specific function is thought to be maintaining the basic homeostatic balance of heme within body (Bainbridge and Smith, 2005). The third isoform is HO-3, which was originally found in the rat. The function of HO-3 is unknown but the enzyme is believed to be inactive (Hayashi et al., 2004, Idriss et al., 2008, Levytska et al., 2013). HO-1, which is known as HSP32 is the one of greatest interest in relation to this project.

6.1.1.1 HO-1 functions

It is now recognized that HO-1 is involved in the control of vascular tone, anti-inflammatory, anti-apoptotic, angiogenic, anti-oxidant and cytoprotective functions (Christova et al., 1999, Sass et al., 2003, Levytska et al., 2013). However, although physiologically moderate quantities of heme are cytoprotective as they induce the rapid up-regulation of HO-1; large heme amounts can be toxic by mediating oxidative stress and inflammation (Wagener et al., 2003, Idriss et al., 2008, Bainbridge and Smith, 2005). In heart, HO-1 provides cardiac cells protection by several routes include sequestration of iron by ferritin, antioxidant activity of bilirubin and vasodilation effect of CO (Balla et al., 2007, Kawamura et al., 2005, Wagener et al., 2003, Idriss et al., 2008).

6.1.1.2 HSP32 and anti-apoptotic and anti-inflammatory functions

HO-1 exerts anti-apoptotic functions in several ways including CO production, which has been shown to have a cytoprotective effects (Vulapalli et al., 2002), bilirubin has been shown to protect cells against oxidative damage. HO-1 decrease TNF, hyperglycemia and iron (Petrache et al., 2000, Idriss et al., 2008). In addition, various models of tissue culture have been shown that HO-1 inhibit apoptosis by suppressing cytotoxic, inflammatory, and signalling cytokines (Zhen-Wei et al., 2007, Rushworth and MacEwan, 2008).

It has been shown that HO-1 exerts cytoprotective and anti-inflammatory functions in the innate immunity dominated liver IR injury (Tsuchihashi et al., 2007). The antioxidant activity of bilirubin may feed through to anti-atherogenic properties, possibly by protecting LDL from oxidation (Mayer, 2000). In addition to regulating oxidative stress, increased HO-1 activity is anti-inflammatory, attenuating angitenstion II-induced prostaglandin-E_2 and F2α synthesis (Volti et al., 2003). HO-1 plays an important role in endothelial homeostasis and resistance to injury through protein kinase c-ε activity (Mylroie et al., 2015).

6.1.1.3 Linking of HSP32 expression to oxidative stress and IR injury

HO-1 is involved in the oxidative stress response (Petrache et al., 2000, Onda et al., 2015). Biliverdin and bilirubin catalytic by-products of heme possess antioxidant properties (Petrache et al., 2000). In addition, iron can stimulate ferritin synthesis which has been suggested to decrease the cellular oxidant potential (Choi and Alam, 1996). Furthermore, low CO levels provide protection against oxidant stress (Otterbein et al., 1999).

The HO-1– bilirubin pathway can protects cells from IR injury, and restricts vascular smooth muscle cell growth by increasing the release of CO (Clark et al., 2000, Onda et al., 2015). In a rat model, mono-therapy with either (CO or biliverdin) did not alter the survival of heart grafts, however both treatments increased survival with a significant decrease of myocardial injury and improved cardiac function (Nakao et al., 2005).

6.1.1.4 HO-1 expression in the placenta

There are several studies on HO-1 expression in human pregnancy, mouse and rat placentas (Lyall et al., 2000, Zhao et al., 2008, Kreiser et al., 2003, McLean et al., 2000, Bilban et al., 2009, Ehsanipoor et al., 2013, Onda et al., 2015). There are mixed reports on the expression of HO-1 and the association with several pregnancy complications such as spontaneous abortion, recurrent miscarriage and PE (Bainbridge and Smith, 2005, Kweider et al., 2014, Wong et al., 2012). HO-1 enzyme was identified in STB and CTB cells as well as the vascular endothelium (Lyall et al., 2000). One study showed mRNA expression of HO-1 increases with advancing gestation (Bainbridge and Smith, 2005). HO-1 mRNA and protein expression decreased under hypoxic conditions while increased in glucose deficient media (Bainbridge and Smith, 2005).

HO and its catalytic products may play a significant role in the progression of a healthy pregnancy to term. It has been shown that HO-1 antibody had no effect on trophoblast invasion whereas the presence of the HO-2 antibodies significantly inhibition trophoblast invasion, also presence of HO inhibitor zinc protoporhyrin-9 led to decrease of trophoblast invasion, thus suggest that HO-2 may be important in controlling trophoblast invasion (McCaig and Lyall, 2009).

In normal pregnancy, the physiological placental expression of HO-1 prevents systemic vasculopathy by lowering the release of antiangiogenic proteins including sFLT-1 and soluble endoglin as well as to promote PlGF activity and inducing the production of CO, a potent vasodilator (Levytska et al., 2013, Ahmed and Ramma, 2014, Onda et al., 2015). In addition, it has been suggested (Levytska et al., 2013, Onda et al., 2015) that HO-1 manipulation may restore the physiology of the trophoblast layer through CO production and decrease sFLT-1 levels seen in PE. HO-1 may play an important role as modulator of innate immune responses in pregnancy (Kahlo et al., 2013).

Several researchers have attempted to profile HO expression, localization, and activity in the PE placenta with conflicting results. It has been reported (Ahmed et al., 2000) that

expression HO-1 was decreased in PE, this was in contrast to a positive association between placental HO-1 expression and feto-placental vascularization in normal pregnancy. However, other studies (Barber et al., 2001) showed that only endothelial HO-2 was decreased in PE. End-tidal breath CO levels, which indicates a decrease in the activity of HO were lower in PE patient in compare to the healthy pregnant women, supporting the role of CO as a physiologic vasodilator implicated in gestational hemodynamic changes (Kreiser et al., 2004, Zhao et al., 2015).

Recently, it has been shown (Linzke et al., 2014) that CO acts as a key molecule in successful pregnancy by modulating the uterine NK cells, which result in the promotion of the remodelling of maternal spiral arteries. Since invasive CTB express HO-2 the CTB may a source of CO (Lyall et al., 2000). Furthermore, it has been shown that sofalcone, a gastric antiulcer agent in clinical use, potently increased HO-1 mRNA and protein in both primary trophoblasts and human umbilical vein endothelial cells (Onda et al., 2015). Sofalcone also decreased the secretion of sFlt-1from primary trophoblasts (Onda et al., 2015).

6.1.2 DNAJ (HSP40) heat shock protein

DNAJ/HSP40s have been found in the nucleus, the cytosol, endosomes, mitochondria, ribosomes and ER. The DNAJ family of co-chaperone proteins play a role in cell stress protection, folding of nascent polypeptides, refolding of denatured or aggregated proteins and protein degradation (Cyr and Ramos, 2015). HSP70 binds to unfolded hydrophobic regions of proteins, and its activity is controlled by ATP binding, hydrolysis, and nucleotide exchange cycle (Qiu et al., 2006, Cyr and Ramos, 2015). In the ATP bound form, HSP70 has a low affinity for substrate proteins. Upon hydrolysis of the ATP to ADP, HSP70 undergoes a conformational change that increases its affinity for substrate proteins (Cyr and Ramos, 2015). The HSP70 proteins are assisted and regulated by several different co-chaperones and these include the DNAJ family. The family can use their various domain structures to bind HSP70, help load the substrates on HSP70 and stimulate the ATPase activity of HSP70 (Cyr and Ramos, 2015). In addition, some of the members of the family DNAJ also regulate the activity of other chaperones including HSP90 (Brychzy et al., 2003). Furthermore, DNAJ can act as chaperones by themselves through binding to certain unfolded proteins (Qiu et al., 2006).

In mammals, more than 44 DNAJ homologs with diverse activities have been reported, however the exact numbers remain unknown (Qiu et al., 2006, Kim et al., 2013, Cyr and Ramos, 2015). They are subdivided into types I, II and III based on the presence of conserved regions (Li et al., 2009). All DNAJ family proteins contain the J domain (through which they bind to their partner HSP70), which consist of 70 amino acid residues that form four -helices and encompass the conserved tripeptide of histidine, proline and aspartic acid (Kostenko et al., 2014). DNAJ proteins in addition to the J domain contain other conserved regions, which are important to their functions (Cajo et al., 2006). DNAJ can be phosphorylated similar to other HSPs (Dephoure et al., 2008, Kostenko et al., 2014).. DNAJB1 is mediates repression of transcriptional activity of the HSF1(Kostenko et al., 2014).

DNAJB1 is the homolog; subfamily B member and its mRNA expression was investigated in non-labour, labour and PE placentas in this study.

6.1.3 HSPD1 (HSP60)

The HSPD family (HSP60, also known as chaperonins) are ubiquitous protein folding machines which prevent protein aggregation by binding non-native proteins, and facilitate folding and unfolding of proteins (Kampinga et al., 2009). The HSPD1 family of chaperones is one of the most abundant classes of molecular chaperones present in the mitochondria (Horváth et al., 2008). HSPD1 has both the anti and pro-apoptotic roles. Cytosolic HSPD1 functions as an anti-apoptotic protein by preventing translocation of Bax into mitochondria and promotes cell survival, however, HSPD1 also promotes pro-caspase 3 maturation, which lead to caspase mediated cell death (Arya et al., 2007).

HSPD1 is regulated by its co-chaperoning HSP10 when performing its function (Shan et al., 2003). HSP10 (known as HSPE) is a 10 kDa highly conserved, mitochondria resident protein, which co-chaperones with the mitochondrial HSPD1. HSP10 alone is widely involved in protecting cells from stresses caused by infection and inflammation (Jia et al., 2011).

6.1.3.1 HSPD1 functions

HSPD1 responsible for the transportation and refolding of proteins from the cytoplasm to the mitochondria, and is also involved in prevention of denaturation of protein during stress (Boshoff, 2015). HSPD1 is important for the correct folding of many proteins in the

cell; under both normal and stress conditions, the folding function of HSPD1 requires a cooperation of HSP70 (Boshoff, 2015). Mitochondrial HSPD1 is heat inducible and also plays a role in protein assembly and trafficking (Seo et al., 2010, Xu et al., 2011). Furthermore, HSPD1 is involved in the replication and transmission of mitochondrial DNA.

HSPD1 plays a role in the production of pro-inflammatory cytokines and also plays both pro-apoptotic and anti-apoptotic roles, depending on their localisation (Knowlton and Gupta, 2003, Boshoff, 2015). The cytoplasmic HSPD1 forms a complex with proteins responsible for apoptosis and prevent apoptosis (Itoh et al., 2002). The cytoplasmic HSPD1 is also involved in immune response and cancer (Itoh et al., 2002). Under stress conditions cytoplasmic HSPD1 is imported into the mitochondria by HSP70 (Itoh et al., 2002).

6.1.3.2 HSPD1 immunological roles

It has been suggested (Hansen et al., 2003) that HSPD1 may play a role in the immune response and autoimmune disease. Different HSPD1 can bind to several cell surface receptors such as TLRs, suggesting there are several subfamilies of HSPD1 (Ranford et al., 2000). HSPD1 induces cytokine secretion as well as activation of immune system cells such as monocytes, macrophages and dendritic cells (Hansen et al., 2003). There is a twist in the immunological function of HSPD1 because there are two different types (bacteria and mammalian) of HSPD1 proteins. It has been shown (Ranford et al., 2000) that bacterial HSPD1 causes the immune system to produce anti-chaperonin antibodies, which then recognize and attack human HSPD1 resulting in autoimmune disease (Ranford et al., 2000). HSPD1 has been associated with stress response, diabetes, cancer and certain genetic diseases (Imatoh et al., 2009, Olvera-Sanchez et al., 2011). In addition, the association between antibodies against HSPD1 and high serum cholesterol has been shown at the beginning of the atherosclerosis in humans (Foteinos and Xu, 2009). It has been suggested (Olvera-Sanchez et al., 2011) that there is a role for HSPD1-like protein in cholesterol distribution, as in placental steroidogenesis. Moreover , HSPD1 are likely to suppress the maternal anti-foetal immune response via TGFβ production, thus contributing to pregnancy maintenance (Giacomelli et al., 2004).

6.2 Results

6.2.1 Patient clinical data analysis

Patient data analysis is the same as section (3.4.1.1), results analysis is shown in (Table 3-2) (Table 3-3).

6.2.2 HO-1 expression in normal pregnancies and PE

Experiment 1: This experiment was designed to test if there was a spatial difference in mRNA expression of HSP32 within individual placentas obtained from women who were not in labour (non-labour group) and from women who were in labour (labour group). Comparison between zones was performed using Friedman test analysis. There was no significant difference in mRNA expression of HSP32 between the three zones of the non-labour and labour groups (p=0.74, p=0.95 respectively) (Figure 6-1).

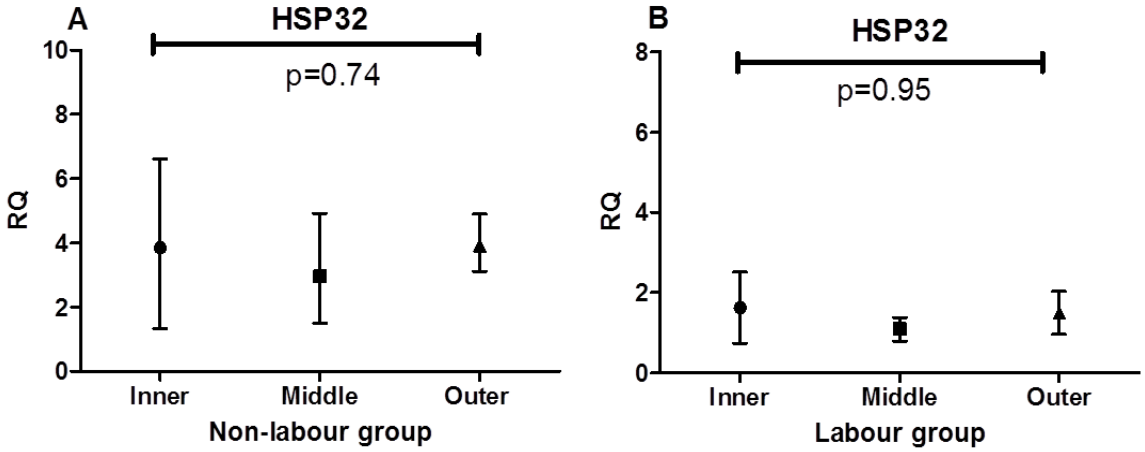


Figure 6-1: RQ values for HSP32 mRNA measurements in in inner (n=4), middle (n=4) and outer (n=4) zones of individual placentas (non-labour and labour groups). Four quadrants were sampled in each zone 6 placentas were studied in each group. Graphs show the median for mRNA expressions of HSP32. Comparison between zones was performed using Friedman analysis.

Experiment 2: This experiment was designed to test if there was any difference mRNA expression of HSP32 between labour and non-labour groups at the inner, middle or outer zones of placentas. Comparison between zones was performed using Mann Whitney test analysis. There was a significant decrease in mRNA expression of HSP32 in the labour group when compared to the non-labour group at the middle and outer zone (p=0.04, p=0.008 respectively). No difference was found at the inner zone (p=0.17) (Figure 6-2).

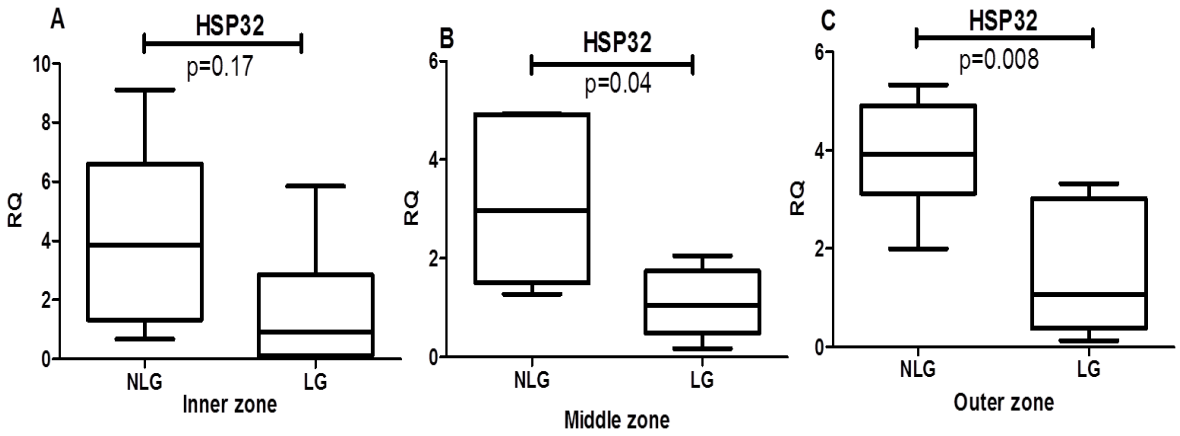


Figure 6-2: RQ values for HSP32 mRNA measurements in non-labour (n=6) compared to the labour group (n=6) at the inner, middle and outer placental zones. Comparison between zones was performed using Mann-Whitney analysis.

Experiment 3: This experiment was designed to test if there was any difference in mRNA expressions of HSP32 between non-labour control and non-labour PE groups at the inner or middle zones of placentas. Comparison between zones was performed using the Mann Whitney test. There was no significant difference in the HSP32 mRNA was found at the inner and middle zones (p=0.71, p=0.54) (Figure 6-3).

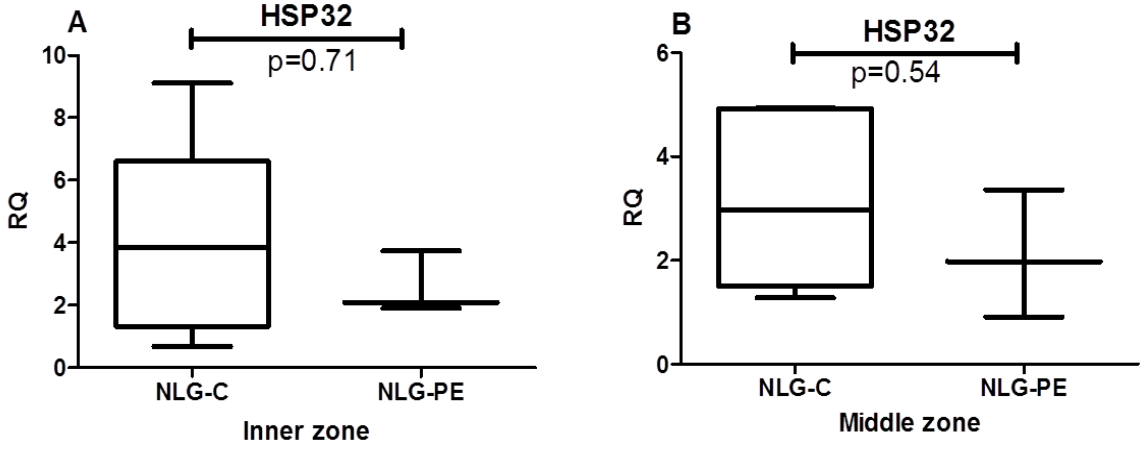


Figure 6-3: RQ values for HSP32 mRNA measurements in non-labour control (n=6) compared to the non-labour PE (n=3) groups at the inner and middle placental zones. Comparison between zones was performed using Mann-Whitney analysis.

Experiment 4: This experiment was designed to test if there was any difference in mRNA expressions of HSP32 between labour control and labour PE groups at the inner or middle zones of placentas. Comparison between zones was performed using Mann Whitney test analysis. There was no significant difference in HSP32 mRNA in the labour control group

compared to the labour PE group in the inner or middle placental zones (p=0.17, p=0.58) (Figure 6-4).

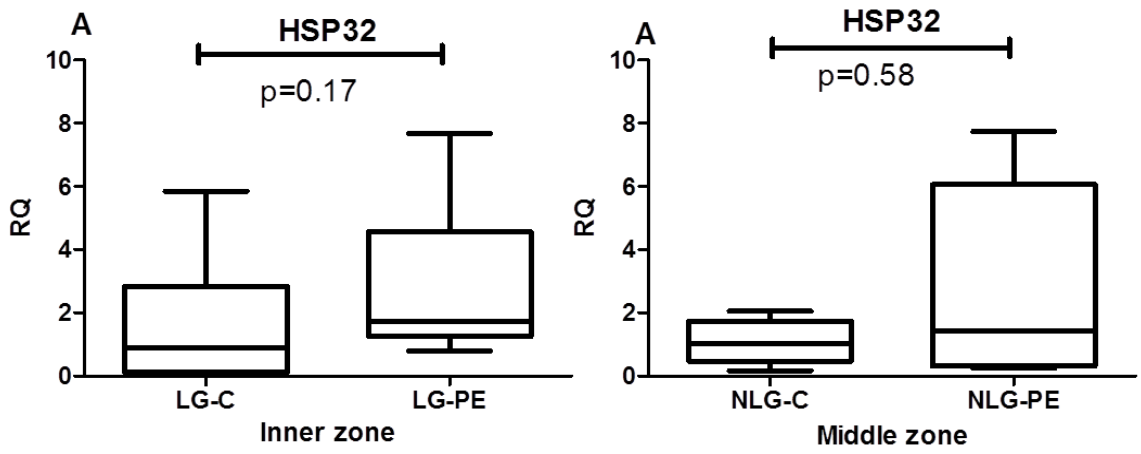


Figure 6-4: RQ values for HSP32 mRNA measurements in labour control (n=6) compared to the labour PE groups (n=6) at the inner and middle placental zones. Comparison between zones was performed using Mann-Whitney analysis.

6.2.3 DNAJAB1 expression in normal pregnancies and PE

Experiment 1: This experiment was designed to test if there was a spatial difference in mRNA expression of DNAJB1 within individual placentas obtained from women who were not in labour (non-labour group) and from women who were in labour (labour group). Comparison between zones was performed using Friedman test analysis. There was no significant difference in mRNA expression of DNAJB1 between the three zones of the non-labour and labour groups (p=0.18, p=0.14 respectively) (Figure 6-5).

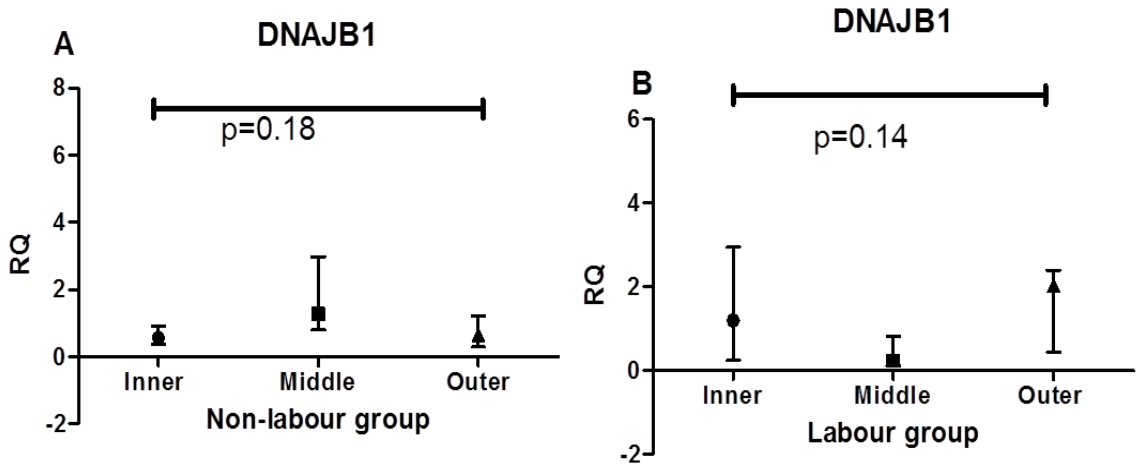


Figure 6-5: RQ values for DNAJB1 mRNA measurements in in inner (n=4), middle (n=4) and outer (n=4) zones of individual placentas (non-labour and labour groups, 6 placentas in each group). Four quadrants were sampled in each zone. Graphs show the median for mRNA expressions of DNAJB1. Comparison between zones was performed using Friedman analysis.

Experiment 2: This experiment was designed to test if there was any difference in mRNA expression of DNAJB1 between labour and non-labour groups at the inner, middle or outer zones of placentas. Comparison between zones was performed using Mann Whitney test analysis. There was a significant decrease in mRNA expression of DNAJB1 in the labour group when compared to the non-labour group at the middle zone (p=0.04). No difference was found at the inner and outer zones (p=0.58, p=0.24) (Figure 6-6).

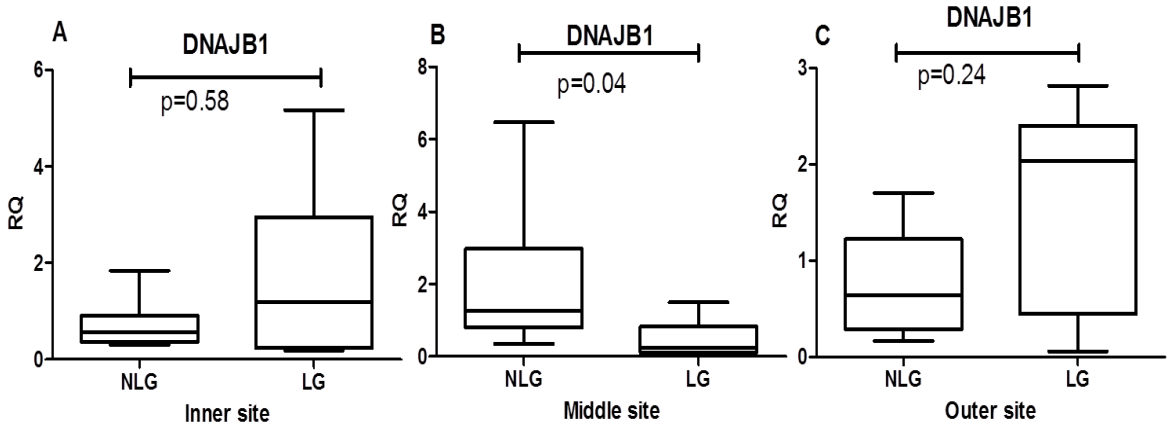


Figure 6-6: RQ values for DNAJB1 mRNA measurements in non-labour (n=6) compared to the labour (n=6) group at the inner, middle and outer placental zones. Comparison between zones was performed using Mann-Whitney analysis.

Experiment 3: This experiment was designed to test if there was any difference in mRNA expressions of DNAJB1 between non-labour control and non-labour PE groups at the inner

or middle zones of placentas. Comparison between zones was performed using the Mann Whitney test analysis. There was no significant difference in the DNAJB1 mRNA at the inner and middle placental zones (p=0.54 for both) (Figure 6-7).

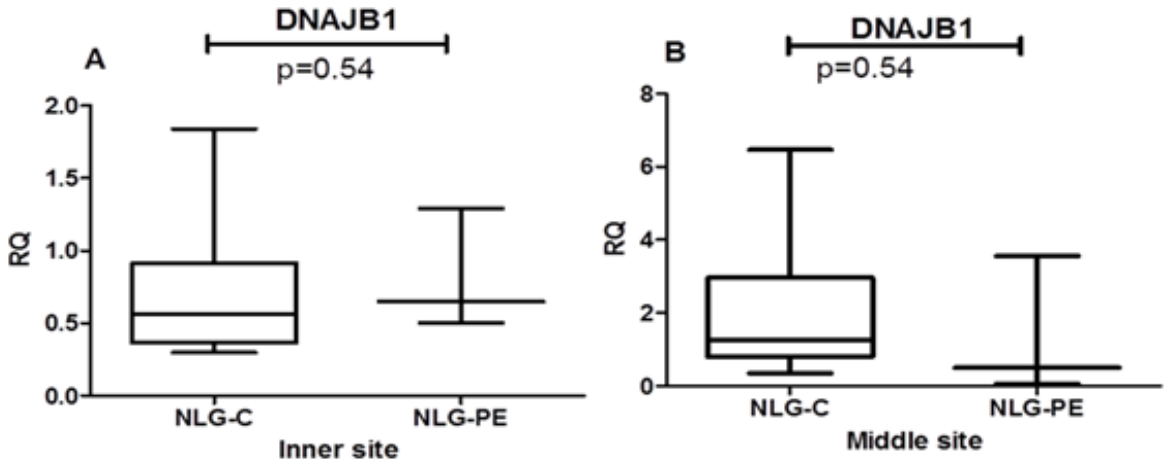


Figure 6-7: RQ values for DNAJB1 mRNA measurements in non-labour control (n=6) compared to the non-labour PE (n=3) groups at the inner and middle placental zones. Comparison between zones was performed using Mann-Whitney analysis.

Experiment 4: This experiment was designed to test if there was any difference in mRNA expressions of DNAJB1 between labour control and labour PE groups at the inner or middle zones of placentas. Comparison between zones was performed using the Mann Whitney test analysis. There was no significant difference in DNAJB1 mRNA in the labour control group compared to the labour PE group in the inner or middle placental zones (p=0.68, p=0.32) (Figure 6-8).

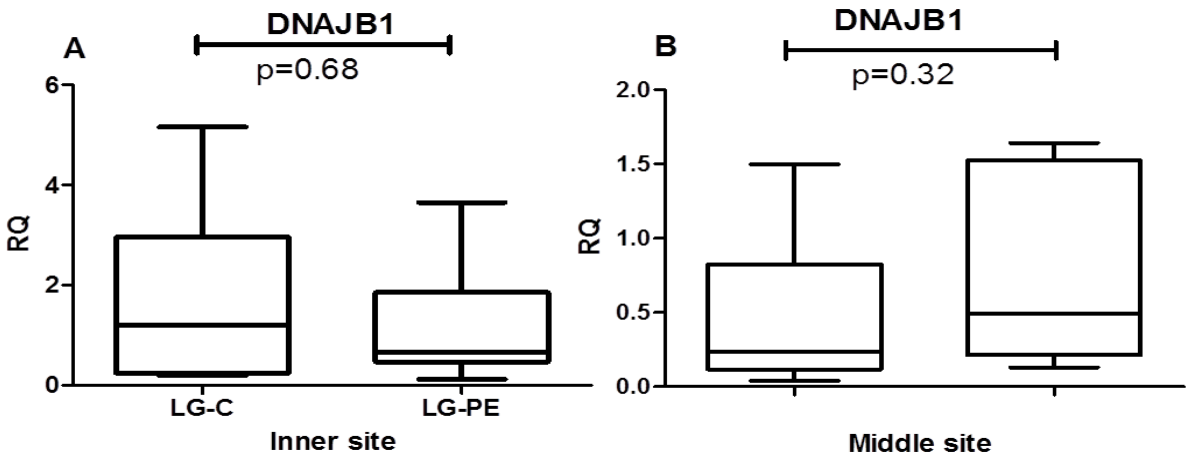


Figure 6-8: RQ values for DNAJB1 mRNA measurements in labour control (n=6) compared to the labour PE (n=6) groups at the inner and middle placental zones. Comparison between zones was performed using Mann-Whitney analysis.

6.2.4 HSPD1 expression in normal pregnancies and PE

Experiment 1: This experiment was designed to test if there was a spatial difference in mRNA expression of HSPD1 within individual placentas obtained from women who were not in labour (non-labour group) and from women who were in labour (labour group). Comparison between zones was performed using Friedman test analysis. There was no significant difference in mRNA expression of HSPD1 between the three zones of the non-labour and labour groups (p=0.25, p=0.05 respectively). The sub-analysis for the labour group showed that there was a significant decrease in RNA expression of HSPD1 at the middle zone compared to the inner zone (p=0.02) (Figure 6-9).

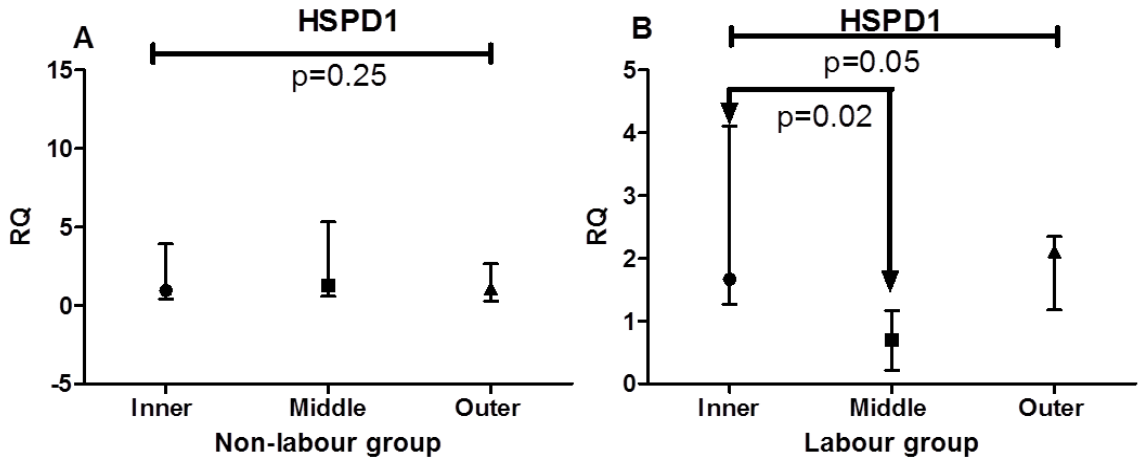


Figure 6-9: RQ values for HSPD1 mRNA measurements in in inner (n=4), middle (n=4) and outer (n=4) zones of individual placentas (non-labour and labour groups, 6 placentas in each group). Four quadrants were sampled in each zone. Graphs show the median for mRNA expressions of HSPD1. Comparison between zones was performed using Friedman analysis.

Experiment 2: This experiment was designed to test if there was any difference in mRNA expression of HSPD1 between labour and non-labour groups at the inner, middle or outer zones of placentas. Comparison between zones was performed using Mann Whitney test analysis. There was no significant difference in mRNA expression of HSPD1 in the labour group when compared to the non-labour group at the inner, middle and outer zones (p=0.3, p=0.24, p=0.3) (Figure 6-10).

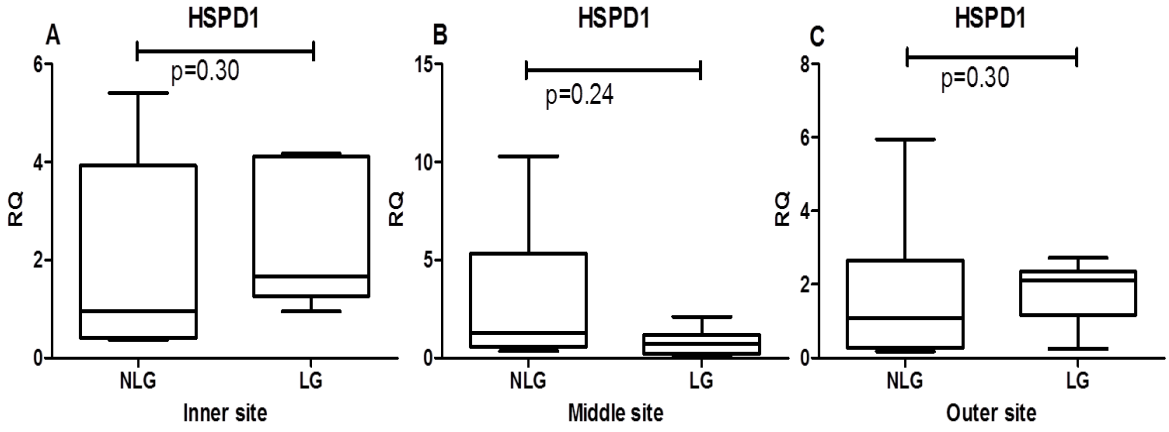


Figure 6-10: RQ values for HSPD1 mRNA measurements in non-labour (n=6) compared to the labour (n=6) group at the inner, middle and outer placental zones. Comparison between zones was performed using Mann-Whitney analysis.

Experiment 3: This experiment was designed to test if there was any difference in mRNA expressions of HSPD1 between non-labour control and non-labour PE groups at the inner or middle zones of placentas. Comparison between zones was performed using Mann Whitney test analysis. There was no significant difference in the HSPD1 mRNA at the inner and middle placental zones (p=1, p=0.54) (Figure 6-11).

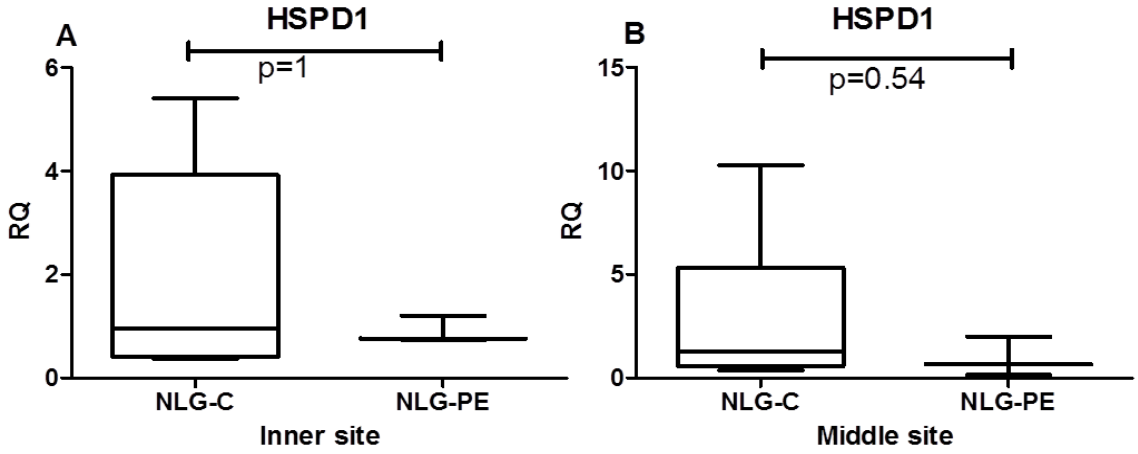


Figure 6-11: RQ values for HSPD1 mRNA measurements in non-labour control (n=6) compared to the non-labour PE (n=3) groups at the inner and middle placental zones. Comparison between zones was performed using Mann-Whitney analysis.

Experiment 4: This experiment was designed to test if there was any difference in mRNA expressions of HSPD1 between labour control and labour PE groups at the inner or middle zones of placentas. Comparison between zones was performed using the Mann Whitney test analysis. There was no significant difference in HSPD1 mRNA in the labour control group compared to the labour PE group in the inner or middle placental zones (p=0.68, p=0.32) (Figure 6-12).

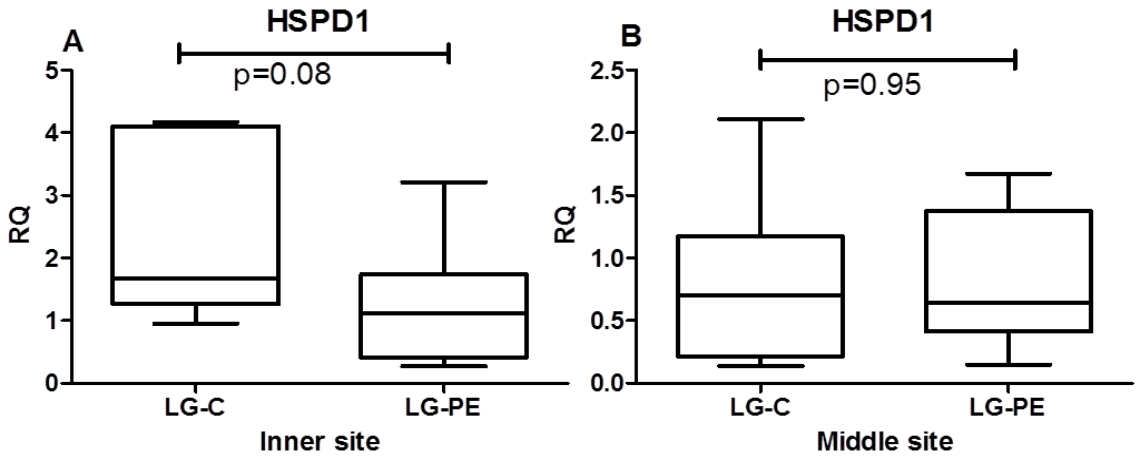


Figure 6-12: RQ values for HSPD1 mRNA measurements in labour control (n=6) compared to the labour PE (n=6) groups at the inner and middle placental zones. Comparison between zones was performed using Mann-Whitney analysis.

6.2.5 Results summary

6.2.5.1 HSP32

Experiment 1: There was no significant spatial difference in mRNA expression of HSP32 expression between the three zones of the non-labour and labour groups.

Experiment 2: There was a significant decrease in mRNA expression of HSP32 in the labour group when compared to the non-labour group at the middle and outer zone (p=0.04, p=0.008 respectively). No difference was found at the inner zone.

Experiment 3: There was no significant difference in the mRNA expression of HSP32 in the non-labour control group compared to the non-labour PE group in the inner or middle placental zones.

Experiment 4: There was no significant difference in mRNA expression of HSP32 in the labour control group compared to the labour PE group in the inner or middle placental zones.

6.2.5.2 DNAJB1

Experiment 1: There was no significant spatial difference in mRNA expression of DNAJB1 between the three zones of the non-labour and labour groups.

Experiment 2: There was a significant decrease in mRNA expression of DNAJB1 in the labour group when compared to the non-labour group at the middle zone (p=0.04). No difference was found at the inner and outer zones.

Experiment 3: There was no significant difference in the mRNA expression of DNAJB1 in the non-labour PE group compared to the non-labour control group at the inner and middle zones.

Experiment 4: There was no significant difference in DNAJB1 mRNA in the labour control group compared to the labour PE group at the inner and middle placental zones.

6.2.5.3 HSPD1

Experiment 1: There was no significant spatial difference in mRNA expression of HSPD1 between the three zones of the non-labour and labour groups. The sub-analysis for the labour group showed that there was a significant decrease in RNA expression of HSPD1 at the middle zone compared to the inner zone (p=0.02)

Experiment 2: There was no significant difference in mRNA expression of HSPD1 in the labour group when compared to the non-labour group at the inner, middle and outer zones.

Experiment 3: There was no significant difference in the mRNA expression of HSPD1 in the non-labour PE group compared to the non-labour control group at the inner and middle zones.

Experiment 4: There was no significant difference in mRNA expression of HSPD1 in the labour control group compared to the labour PE group at the inner and middle placental zones.

6.3 Discussion

6.3.1 HO-1 and present findings

The main finding in this study was that HO-1 mRNA expression was significant decreased in labour compared to the non-labour group at the middle and outer placental zones. One possible explanation is as labour is an inflammatory process the fall in HO-1 would allow the inflammatory pathways to go further and continue as natural process. However, importantly the present work shows that expression varies on the zone of the placenta the piece of tissue is taken from. There was no spatial distribution or significant difference in HO-1 expression between non-labour PE and labour PE groups compare to the non-labour and labour control groups.

HO-1 expression has been studied in both human and mouse placentas. In human placental tissue, the results have been inconsistent, it might due to the intra- and inter-placental tissue heterogeneity and different gestational ages studied (Zhao et al., 2015). Most studies were performed using either term or preterm placentas. For most studies, the tissue collected was only from the placenta without the placental bed, and investigated mostly for IHC studies. In our study we followed a systematic sampling methods by dividing placenta into three zones according to distance from cord insertion point (inner, middle and outer zones) (Abdulsid et al., 2013b, Abdulsid et al., 2013a, Abdulsid and Lyall, 2014). Because of time constraints it was only possible to perform qRT-PCR experiments and future plans would be to investigate HO-1 protein expression and IHC protein localization according to the systemic sampling protocol.

6.3.2 HO-1 in labour and PE

The mechanism of CO mediated vasodilatation has been investigated, and it is has been suggested that the CO/HO system may be involved in the control of placental vascular function and may protect the STB and endothelium against oxidative stress injury (Lyall et al., 2000). It has been shown (Appleton et al., 2002) that HO activity was directly dependent on O_2 concentration. Thus, although HO protein and mRNA expression increased under hypoxic conditions, there is a progressive decrease in HO activity with low O_2 concentration. Placentas of PE cases have large and numerous infractions areas compared to the normal pregnancies (Benirschke et al., 1998).

HO enzyme expression and activity may vary across the placenta. Consequently, it is critical that the sampling site be identified and considered when comparing various studies.

There are some publications in which HO-1 expression in the placenta and changes during adverse pregnancy have been reported. Again the main point to highlight is that systemic sampling and the confounding effects of labour have not been considered. These studies can be summarized as follows.

Lyall *et al* (Lyall et al., 2000) presented the most systematic study that investigated the HO-1 and HO-2 using ICH and western blotting, by collecting placentas and placental bed samples from first and second trimesters as well as term placentas. This study showed that HO-1 protein expression was very low compared to HO-2 and not gestational age dependent. Within the placental bed, HO-1 was expressed in EVT but absent from villous CTB (Lyall et al., 2000).

One group performed RT-PCR, Western blotting and IHC to investigate HO-1 and HO-2 in human term placenta (McLean et al., 2000). This study showed that HO-1 was detected in placenta tissue at the protein and molecular level. IHC showed wide distribution of HO-1 including STB, endothelium and smooth muscle of placenta blood vessels however doubt has been cast as to how much staining is non-specific with the antibodies used. However, although this group collected the placental samples from non-labouring and labouring placentas there was no any comparisons carried out or showed within the study for each. No systemic sampling was performed.

Other research group performed RT-PCR, Western blotting and IHC to investigate HO-1 and HO-2 in first trimester as well as term human placenta (Yoshiki et al., 2000). Similar to the above study (McLean et al., 2000) HO-1 was detected in placenta tissue at the protein and molecular level. IHC showed that distribution of the two HO isoforms had distinct topographic patterns: HO-1 was observed in STB layer with traces in the underlying CTB. In addition, the study also showed that HO-1 expression did not change throughout gestation. It is not appropriate to compare this study to our findings because there was no controlled sampling and labour was not investigated.

Ahmed *et al* (Ahmed et al., 2000) performed RT-PCR, IHC and western blotting to investigate HO-1 in first, second trimesters and term normal pregnancy, term PE placental tissue was also investigated. The level of HO-1 mRNA expression increased with increasing gestation in normotensive samples but not PE. HO-1 protein expression was

increased 2.6 fold in term compared to first trimester placentas. There was a significant decrease in HO-1 mRNA expression in PE placentas compared to normotensive placenta.

Barber *et al* (Barber et al., 2001) performed IHC and Western blotting to investigate HO-1 and HO-2 in term placenta and placenta bed of normal, PE and FGR pregnancies. HO-1 protein was undetectable in the placental homogenates collected from both the PE and the normotensive placentas. This follow up study investigated mRNA expression of HO-1 not protein, further studies according to controlled sampling would be recommend.

McLaughlin *et al* (McLaughlin et al., 2003) performed Western blotting to investigate HO-1 and HO-2 from four placental regions (chorionic villi, chorionic plate, basal plate and fetal membranes) of term human placenta of normotensive and PE pregnancies. This study showed that HO-1 expression was increased in chorionic villi and fetal membranes from PE compared with normotensive pregnancy. However, although samples were taken from four placenta regions but there were no controlled sampling methods within placenta. Labour was not studied.

Lash *et al* (Lash et al., 2003) performed IHC and Western blotting to investigate HO-1 and HO-2 in three areas of term non-labour placenta of normotensive and PE pregnancies (morphologically normal chorionic villi, peri-infarct chorionic villi, and infarct chorionic villi). This study showed that there were no significant differences in HO-1 protein levels in healthy and infarcted tissue for both normotensive and PE placentas. IHC analysis showed decreases in both HO-1 and HO-2 protein expression in all damaged tissue. In addition, they compared HO enzymatic activity in microsomes isolated from normal and infarcted chorionic villi and found decreased HO activity in the damaged tissue under optimized conditions (Lash et al., 2003). No systemic sampling method was performed and labour was not studied.

Newby *et al* (Newby et al., 2005) performed Western blotting to investigate HO-1 and HO-2 in trophoblast cell cultures. This study showed that HO-1 expression in STB was significantly lower than in CTB in standard conditions. In addition, there was no difference in HO-1 expression in CTB transferred to 2% O2 for different times. However, exposure of STB cultures to hypoxia for 12 h resulted in a significant decrease in HO-1 protein expression.

Bilban et al (Bilban et al., 2009) collected first trimester human placental tissues and compared the mRNA expression profiles of EVT compared to the less invasive CTB. This

study showed that HO-1 was down-regulated in EVT. In addition, a high expression of HO-1 in the proliferative, Ki67-positive cell column was detected, in contrast to the low levels observed in non-cycling, Ki67-negative, invasive EVT (Bilban et al., 2009).

Yamamoto *et al* (Yamamoto et al., 2014) performed RT-PCR on placenta and serum samples from PE and normal pregnancy patients. Then JEG-3 cells were cultured with PE and normal pregnant sera. This study showed that HO-1 mRNA expression in placenta was decreased significantly in PE patients compared to normal pregnancy patients. In addition, it has been shown that there was no correlation between PlGF and sFlt-1 mRNA expression and HO-1mRNA expression.

Recently, it was shown (Kaitu'u-Lino et al., 2015) that mRNA and protein expression of HO-1 is not reduced in PE and does not regulate sFlt-1 or soluble endoglin production in human placentas. In the present study there was no significant difference in mRNA expression of HO-1 between PE and normotensive pregnancies. Although, there was similarity in the findings controlled sampling and labour effect were not investigated.

These contradicting results from different studies add confusion to the potential role of HO-1 in the pathogenesis of PE. However, the disease state, subjects and experimental variability may explain some of these differences.

6.3.3 DNAJB1 and the present findings

The main finding was that DNAJB1mRNA expression was significantly decreased in the labour group when compared to the non-labour group at the middle placental zone. Recently, it has been shown that DNAJB1 inhibited p53-mediated apoptosis (Cui et al., 2015). The reason for the fall in DNAJB1mRNA expression is unclear but possible explanation is this would allow the apoptotic pathways in labour to progress.

However, importantly the present work shows that expression varies depending on the zone of the placenta the piece of tissue is taken from. There was no spatial distribution or any significant difference in DNAJB1 expression between non-labour and labour PE compare to the non-labour and labour control.

There are few publications in which DNAJ expression in the placenta. These studies can be summarized as follows.

One study showed that DNAJ is expressed in the placenta (Ohtsuka, 1993). DNAJ has also been showed that detected in umbilical vein endothelial cells (Chellaiah et al., 1993).

One group used cDNA oligonucleotide microarray analysis to investigate DNAJAB1 expression in uncomplicated term placenta of labour and non-labour (Lee et al., 2010). They reported that mRNA expression of DNAJAB1was increased (3.83 fold) in the labour group compared to the non-labour. No systemic sampling method was performed. In contrast on the present study DNAJAB1 was decreased in labour at the middle zone.

6.3.4 HSPD1 and present findings

This study showed that HSPD1 mRNA is expressed in human placenta. The key finding that mRNA expression of HSPD1 is expressed in spatial manner in the placenta with highest expression being in the middle zone in labour group. However, there were no significant changes in mRNA expression of HSPD1 in non-labouring, labouring and PE placentas.

6.3.5 HSPD1 expression in labour and PE

HSPs are essential for cellular survival under stressful conditions. The heat shock response is a mechanism that that protects cell from damage from heat stress by up-regulating the expression of genes that code for HSPs including HSPD1 (Vargas-Parada et al., 2001). HSPD1, similar to other HSPs, is up-regulated in response to different stresses for example it serve as defence mechanisms against neurotoxicity produced by ROS in neurodegenerative diseases (Calabrese et al., 2007, Rossi et al., 2002).

There are some publications in describing HSPD1 expression in the placenta and changes during adverse pregnancy. The major point to highlight is that controlled sampling and the confounding effects of labour have not been considered. These studies can be summarized as follows.

An IHC study of amniochorion and basal plate HSPD1 expression in pre-term labour, term labour, term non-labour and pre-term cesarean section for PE or FGR was reported (Divers et al., 1995). This study reported that HSPD1 expression was relatively constant throughout the last trimester and was unchanged by labour. The placenta itself was not examined and controlled sampling was not performed.

Immunofluorescence was used to investigate HSPD1 and HSPD1 antibody complex expression in term, pre-term and FGR placentas (Ziegert et al., 1999). This study showed that HSPD1 protein expressed in placenta, no significant difference between all groups. No data or p values were shown to support this statement and no systematic sampling was performed.

Placental HSPD1 protein expression, detected using IHC, was decreased in the CTB and STB around infracted regions (Wataba et al., 2004). The author concluded that although the villous cells around the infarction appeared histologically viable, they may have received lethal damage, and as a result, the expression of HSPs was decreased. Systemic sampling method was not performed.

Proteomic and Western blotting has been used to investigate HSPD1 expression in highly purified placental CTB obtained from normal and PE pregnancies, both non-labour and labour placentas were collected (Johnstone et al., 2011). This study showed that HSPD1 was significant decreased in the cells from PE cases compared to the normotensive subjects. Although placentas were collected from non-labour and labour group the effect of labour was not taken in account. A systemic sampling method was not performed.

Western blotting, IHC and immunoprecipitation was used by another group to investigate HSPD1 protein expression in STB mitochondria of term placentas (Olvera-Sanchez et al., 2011). This study concluded that HSPD1 is related to placental steroidogenesis and cholesterol transport across mitochondria. Systemic sampling methods were not performed and labour was not studied.

Chapter VII: Analysis of mRNA expression of HSPA5 (GRP78), HSP90 (HSP90AA and HSP90B1) and HSPH1 (HSP105) families in human placenta of normal pregnancies and pregnancies complicated with PE

7 HSPA5, HSP90 and HSPH1 expression

7.1 Introduction

7.1.1 Glucose regulated protein (GRP78, HSPA5)

In human cells the ER forms a vast and dynamic membrane network (English and Voeltz, 2013). The ER plays an important function in intracellular calcium homeostasis. Protein translocation into the ER is the first step in the biogenesis of many proteins of mammalian cells (Melnyk et al., 2015). ER stress can be induced by conditions that causing an alteration in the intraluminal oxidative status such as Ca^{++} signalling disturbance and misfolded protein accumulation, which could lead to induction and progression of apoptosis (Grubišic, 2005).

GRP78 is also known as BiP and recently as heat shock 70 kDa protein 5 (HSPA5) belongs to HSP70 family. GRP78 is located in the ER lumen and can be induced by ER stress (particularly hypoxia and hypoglycaemia) (Shields et al., 2011, Gonzalez-Gronow et al., 2012). Newly synthesized proteins are translocated to the ER where GRP78 binds to them to maintain their correct folding and oligomerization. GRP78 also act as an apoptotic regulator by protecting the cell against stress induced death or induced cell death by activation CASP3, 7 and 8 of the extrinsic pathways (Grubišic, 2005, Gonzalez-Gronow et al., 2007, Gonzalez-Gronow et al., 2012). Furthermore, GRP78 plays a critical role in the quality control of proteins processed in the ER and the regulation of ER signalling in response to the UPR or ER stress (Ma and Hendershot, 2004b, Zhang and Zhang, 2010).

7.1.1.1 GRP78 immunological roles

HSPs have three immunological regulatory functions: inside the cell, on the cell membrane as signalling receptors, and in the extracellular environment as stress cytokines. GRP78 has potent immunological activity when released from the internal cell environment into the extracellular compartment (Panayi et al., 2004). GRP78 has anti-inflammation roles such as down regulating the immune system by several mechanisms include cytokine production IL-10, IL-1 and TNF) and down regulating HLA-DR and CD86 (Panayi et al., 2004, Corrigall et al., 2004).

7.1.2 Heat shock protein 90 (HSP90)

HSP90 is most abundant intracellular protein in mammalian cells; it prevents aggregation of non-native proteins in an ATP dependent manner (Picard, 2002). HSP90 is also essential for a wide range of protein assembly, trafficking, folding, and degradation processes (Clare and Saibil, 2013). HSP90 plays a role in stabilization of protein at later stages of folding (Clare and Saibil, 2013). HSP90 activation is regulated by ATP-induced large conformational changes, co-chaperones and post-translational modifications such as phosphorylation, acetylation and nitrosylation (Li et al., 2012a). HSP90 is dependent on ATP hydrolysis and interacts with more than 20 co-chaperones, for chaperone activity (Taipale et al., 2010, Prodromou, 2012).

HSP90 also play a role in kinases signalling and regulation of transcription machinery such as steroid receptors and P53 (Khurana and Bhattacharyya, 2015). It also influences steroid receptor function (Pratt et al., 2004, Grad and Picard, 2007, Sanchez, 2012). HSP90 is a major regulator of cancer cell growth and proliferation by assisting oncogenes such as several protein kinases and transcription factors (Khurana and Bhattacharyya, 2015). HSP90 inhibition results in the simultaneous blockade of multiple signalling pathways and can sensitize tumour cells to anticancer drugs, hence HSP90 inhibitors play a major role in cancer treatment (Kumalo et al., 2015, Proia and Kaufmann, 2015). Another function of HSP90 is to act as buffer for genetic variation by rescuing mutated proteins with altered properties (Taipale et al., 2010).

7.1.2.1 Intracellular location

HSP90 is a large dimeric protein found in almost every compartment of eukaryotic cells. Cytosolic HSP90 constitutes 1-2% of total protein. Under stress condition HSP90

translocates to other cellular compartments including the nucleus and mitochondria (Taipale et al., 2010). Furthermore, HSP90 exists in the extracellular space where it is thought to interact with immune system (Schmitt et al., 2007).

7.1.2.2 HSP90 isoforms and transcriptional regulation

HSP90 is generally expressed in the cytoplasm, ER, mitochondria and the nucleus (Csermely et al., 1998, Krishna and Gloor, 2001, Chen et al., 2005). Although HSP90 is mainly a cytoplasmic chaperone, a small fraction of HSP90 (about 3%) is present in the nucleus (Khurana and Bhattacharyya, 2015). There are two major cytosolic isoforms of HSP90, which interact with a number of co-chaperones that regulate its ATPase cycle and determine its substrate proteins (Chiosis et al., 2013). Cytosolic HSP90, which is presented in two isoforms: α-HSPC2 (HSP90AA, inducible form) and β-HSPC3 (HSP90AB1, constitutive form) differ in its expression pattern, HSP90AB being abundant during normal physiological conditions and HSP90AA expressed during stress (De Maio and Vazquez, 2013). These two isoforms are the result of gene duplication (Krone and Sass, 1994). Other types of molecular chaperones include the ER resident homologue GRP94, and a mitochondrial variant TRAP1(Kumalo et al., 2015).

Despite its high basal abundance HSP90 is transcriptionally induced in response to different stress conditions such as heat shock, hypoxia and IR injury (Csermely et al., 1998). Mammalian genomes contain genes for both constitutively and inducible HSP90 isoforms. Inducible HSP90 is controlled by HSF1 (Whitesell and Lindquist, 2009). Under normal conditions HSP90 prevents the activation of HSP gene expression by its interaction with HSF1(Zou et al., 1998). There are other transcription factors which induce HSP90 expression such as NF-κB (Ammirante et al., 2008) and STAT3 (following IL-6 stimulation) or by STAT1 following interferon-γ stimulation (Stephanou et al., 1997, Ripley et al., 1999). The interface with HSF1 occurs in two ways, STAT1 interacts with HSF1 synergistically, and STAT3 antagonizes HSF1 activity (Sekimoto et al., 2010). In addition, HSP90 initiates a potent inflammatory response via transcriptional modulation of NF-κB and STAT3 (Bohonowych et al., 2014).

In this study HSP90AA and HSP90AB1 was investigated in normotensive and PE pregnancies. HSP90 protein expression was run for optimization but due to time constrain we only mRNA experiments were performed.

7.1.3 HSPH1 (HSP105/HSP110 family)

The HSP105/HSP110 family consists of HSP105α and HSP105β, HSP105α is expressed constitutively and induced by different forms of stress, while HSP105β, an alternatively spliced form of Hsp105α, is expressed specifically during mild heat shock (Hatayama et al., 1994, Ishihara et al., 1999). Both isoforms of HSP105 are composed of an N-terminal ATP binding site, a β-sheet, a loop and a C-terminal α-helix domain similar to the HSP70-family proteins (Ishihara et al., 2003). HSP105α is present in the cytoplasm but not the nucleoli (Ishihara et al., 1999) whereas HSP105β localized in the nucleus of mammalian cells (Saito et al., 2007). However, it has been shown that HSP105α accumulated in the nucleus of cells (Yamagishi et al., 2010). HSP105α exists as both non-phosphorylated and phosphorylated forms and is phosphorylated by protein kinase 2 at ser 509 (Ishihara et al., 2003).

HSP105 exist as a complex with HSP70 and HSC70 the constitutive member of the HSP70 family (Hatayama et al., 1998, Wang et al., 2000, Bracher and Verghese, 2015) and suppresses the chaperone activity of HSC70 in cells (Ishihara et al., 2003, Yamagishi et al., 2004). HSP105α and HSP105β suppress the aggregation of denatured proteins that occurs in cell under stress conditions, in which cellular ATP levels decrease markedly (Yamagishi et al., 2003, Bracher and Verghese, 2015) and also supresses apoptosis through the induction of HSP70 (Yamagishi et al., 2010). In addition, although both isoforms of HSP105 do not shown ATPase activity, their addition to HSP70/HSP40 enhanced ATP hydrolysis greater than that of the HSP40-stimulated HSP70 ATPase activity (Yamagishi et al., 2000). HSP105β but not HSP105α up-regulates HSP70 expression through signal transducer STAT-3 in cells (Yamagishi et al., 2003, Yamagishi et al., 2009).

7.2 Results

7.2.1 Patient clinical data analysis

Patient data analysis is the same as section (3.4.1.1), results analysis is shown in (Table 3-2) (Table 3-3).

7.2.2 HSPA5 expression in normal pregnancies and PE

Experiment 1: This experiment was designed to test if there was a spatial difference in mRNA expression of HSPA5 within individual placentas obtained from women who were not in labour (non-labour group) and from women who were in labour (labour group). Comparison between zones was performed using Friedman test analysis. There was no significant difference in mRNA expression of HSPA5 between the three zones of the non-labour and labour groups (p=0.95, p=0.25 respectively) (Figure 7-1).

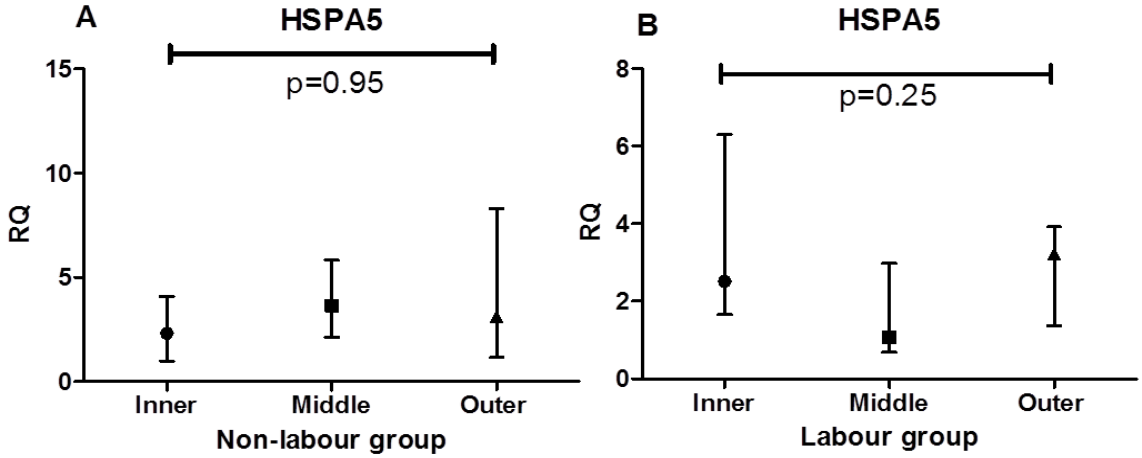


Figure 7-1: RQ values for HSPA5 mRNA measurements in in inner (n=4), middle (n=4) and outer (n=4) zones of individual placentas (non-labour and labour groups, 6 placentas in each group). Four quadrants were sampled in each zone. Graphs show the median and for mRNA expressions HSPA5. Comparison between zones was performed using Friedman analysis.

Experiment 2: This experiment was designed to test if there was any difference in mRNA expression of HSPA5 between labour and non-labour groups at the inner, middle or outer zones of placentas. Comparison between zones was performed using Mann Whitney test analysis. There was no significant difference in mRNA expression of HSPA5 in the labour group when compared to the non-labour group at the inner, middle and outer zones (p=0.58, p=0.09, p=0.69) (Figure 7-2).

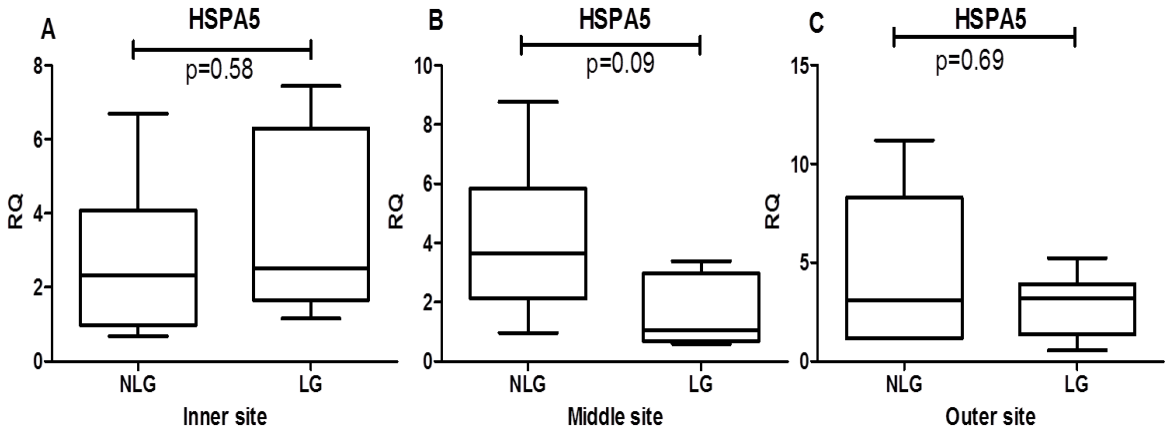


Figure 7-2: RQ values for HSPA5 mRNA measurements in non-labour (n=6) compared to the labour (n=6) groups at the inner, middle and outer placental zones. Comparison between zones was performed using Mann-Whitney analysis.

Experiment 3: This experiment was designed to test if there was any difference in mRNA expressions of HSPA5 between non-labour control and non-labour PE groups at the inner or middle zones of placentas. Comparison between zones was performed using Mann Whitney test analysis. There was no significant difference in the mRNA expression of HSPA5 at the inner and middle placental zones (p=0.9, p=0.26) (Figure 7-3).

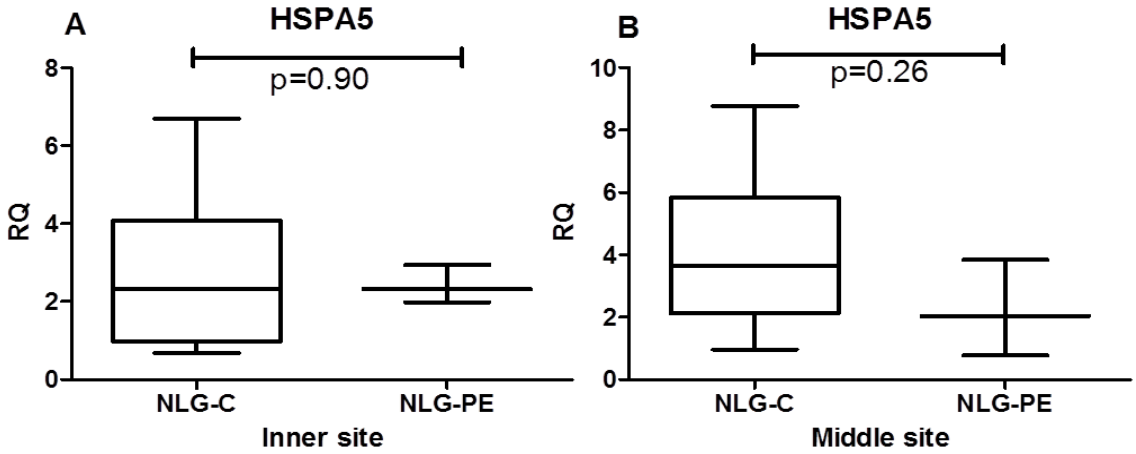


Figure 7-3: RQ values for HSPA5 mRNA measurements in non-labour control (n=6) compared to the non-labour PE (n=3) groups at the inner and middle placental zones. Comparison between zones was performed using Mann-Whitney analysis.

Experiment 4: This experiment was designed to test if there was any difference in mRNA expressions of HSPA5 between labour control and labour PE groups at the inner or middle

zones of placentas. Comparison between zones was performed using Mann Whitney test analysis. There was no significant difference in mRNA expression of HSPA5 in the labour control group compared to the labour PE group in the inner or middle placental zones (p=0.77, p=0.11) (Figure 7-4).

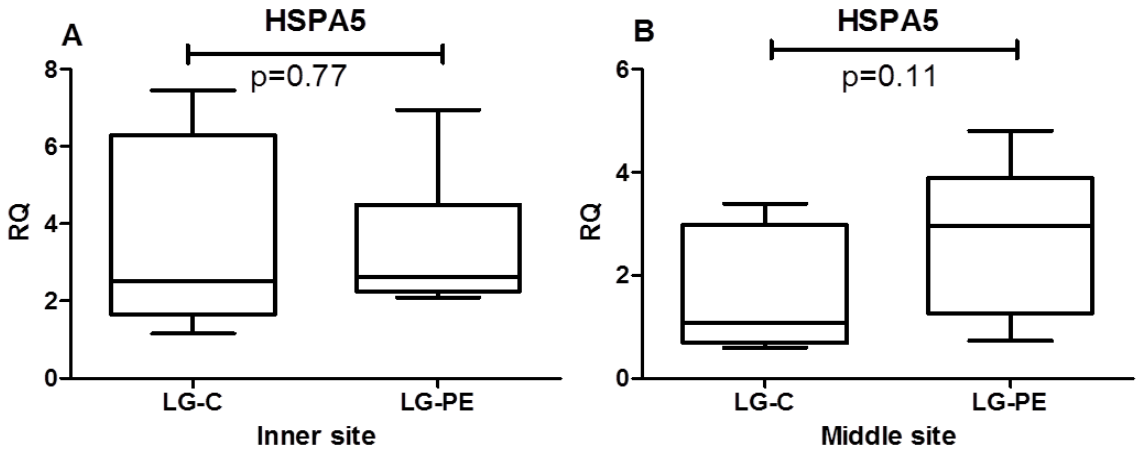


Figure 7-4: RQ values for HSPA5 mRNA measurements in labour control (n=6) compared to the labour PE (n=6) groups at the inner and middle placental zones. Comparison between zones was performed using Mann-Whitney analysis.

7.2.3 HSP90AA expression in normal pregnancies and PE

Experiment 1: This experiment was designed to test if there was a spatial difference in mRNA expression of HSP90AA within individual placentas obtained from women who were not in labour (non-labour group) and from women who were in labour (labour group). Comparison between zones was performed using Mann Whitney test analysis. There was no significant difference in mRNA expression of HSP90AA between the three zones of the non-labour and labour groups (p=25, p=0.57) (Figure 7-5)

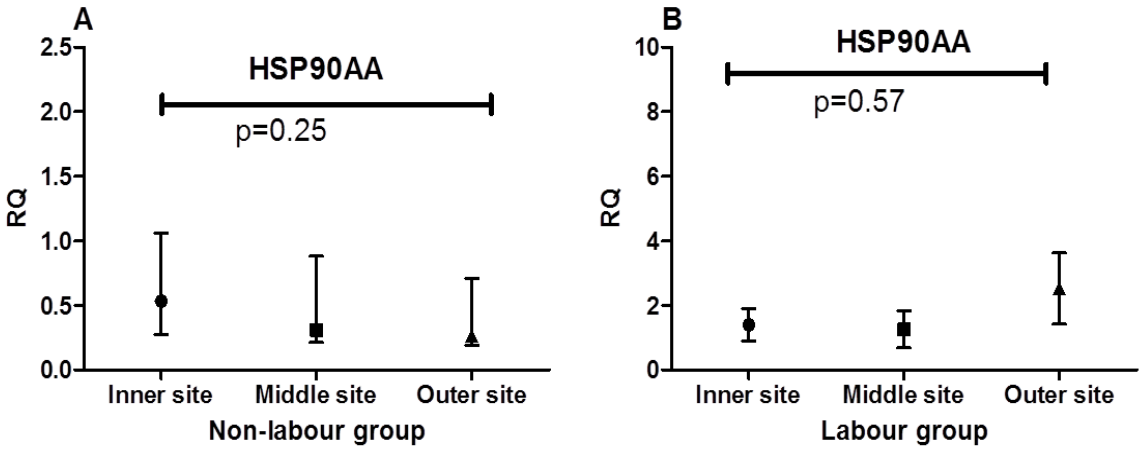


Figure 7-5: RQ values for HSP90 AA mRNA measurements in in inner (n=4), middle (n=4) and outer (n=4) zones of individual placentas (non-labour and labour groups, 6 placentas in each group). Four quadrants were sampled in each zone. Graphs show the median for mRNA expressions of HSP90AA. Comparison between zones was performed using Friedman analysis.

Experiment 2: This experiment was designed to test if there was any difference in mRNA expression of HSP90AA between labour and non-labour groups at the inner, middle or outer zones of placentas. Comparison between zones was performed using Mann Whitney test analysis. There was a highly significant decrease in mRNA expression of HSP90AA in the non-labour group compared to the labour group at the outer zone ($p=0.008$). There was no significant difference in mRNA expression of HSP90AA in the non-labour group when compared to the labour group at the inner or middle zones ($p=0.17$, $p=0.30$) (Figure 7-6).

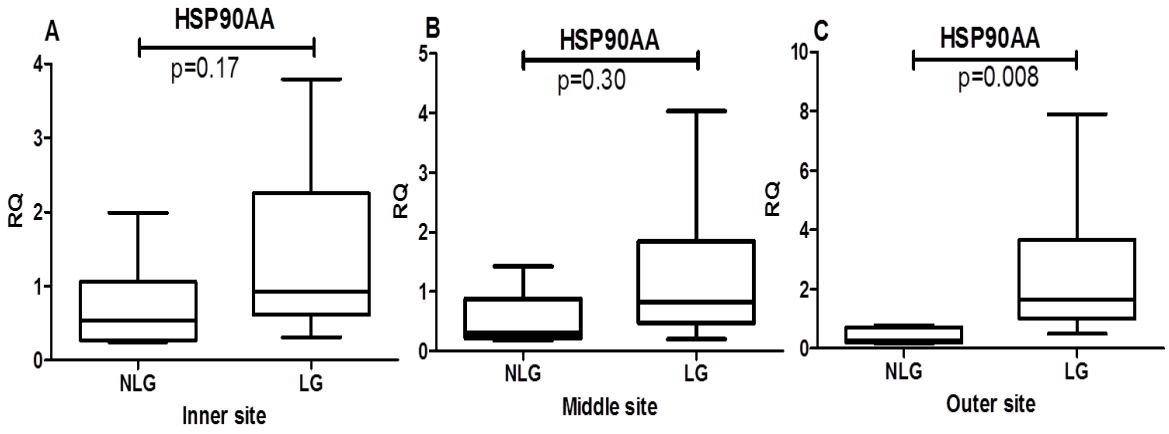


Figure 7-6: RQ values for HSP90AA mRNA measurements in non-labour (n=6) compared to the labour (n=6) group at the inner, middle and outer placental zones. Comparison between zones was performed using Mann-Whitney analysis.

Experiment 3: This experiment was designed to test if there was any difference in mRNA expressions of HSP90AA between non-labour control and non-labour PE groups at the inner or middle zones of placentas. Comparison between zones was performed using the Mann Whitney test analysis. There was no significant difference in HSP90AA mRNA at the inner and middle placental zones (p=1, p=0.38) (Figure 7-7).

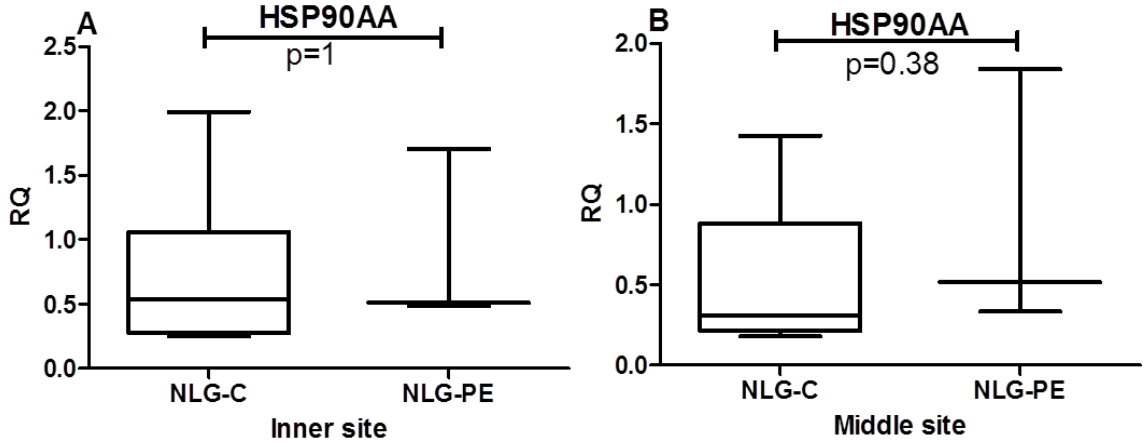


Figure 7-7: RQ values for HSP90AA mRNA measurements in non-labour control (n=6) compared to the non-labour PE (n=3) groups at the inner and middle placental zones. Comparison between zones was performed using Mann-Whitney analysis.

Experiment 4: This experiment was designed to test if there was any difference in mRNA expressions of HSP90AA between labour control and labour PE groups at the inner or

middle zones of placentas. Comparison between zones was performed using Mann Whitney test analysis. There was no significant difference in HSP90AA mRNA in the labour control group compared to the labour PE group in the inner or middle placental zones (p=0.86 for both) (Figure 7-8).

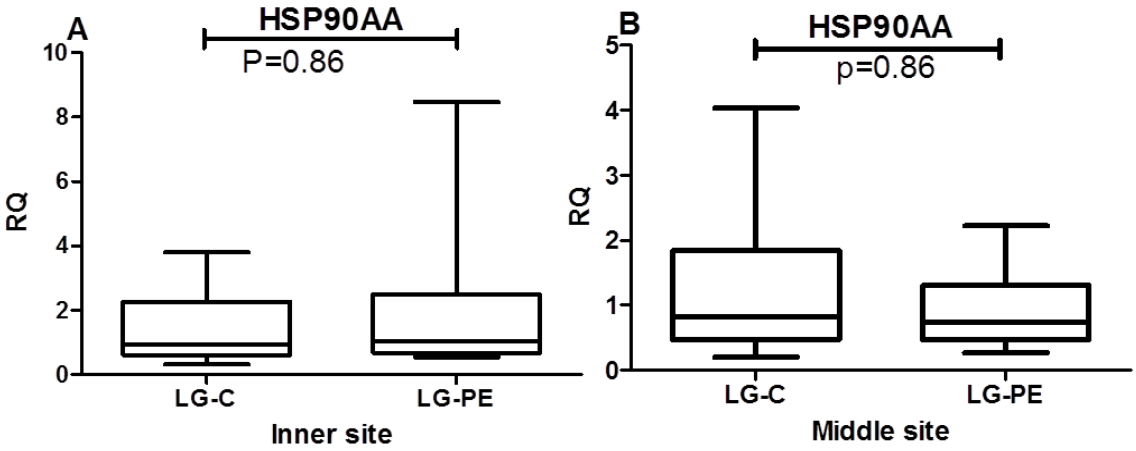


Figure 7-8: RQ values for HSP90AA mRNA measurements in labour control (n=6) compared to the labour PE (n=6) groups at the inner and middle placental zones. Comparison between zones was performed using Mann-Whitney analysis.

7.2.4 HSP90B1 expression in normal pregnancies and PE

Experiment 1: This experiment was designed to test if there was a spatial difference in mRNA expression of HSP90B1 within individual placentas obtained from women who were not in labour (non-labour group) and from women who were in labour (labour group). Comparison between zones was performed using Mann Whitney test analysis. There was no significant difference in mRNA expression of HSP90AA between the three zones of the non-labour and labour groups (p=0.33, p=0.74) (Figure 7-9).

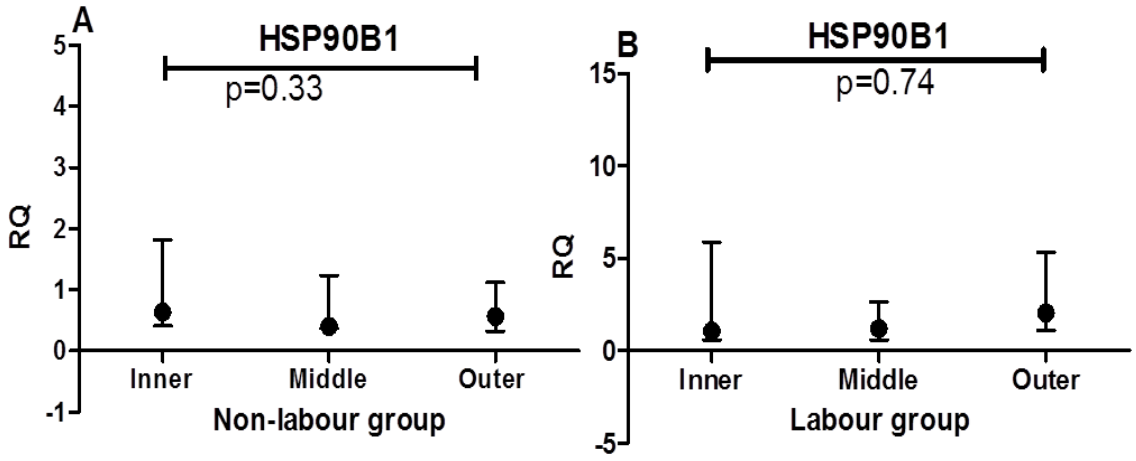


Figure 7-9: RQ values for HSP90B1 mRNA measurements in in inner (n=4), middle (n=4) and outer (n=4) zones of individual placentas (non-labour and labour groups, 6 placentas in each group). Four quadrants were sampled in each zone. Graphs show the median for mRNA expressions of HSP90B1. Comparison between zones was performed using Friedman analysis.

Experiment 2: This experiment was designed to test if there was any difference in mRNA expression of HSP90B1 between labour and non-labour groups at the inner, middle or outer zones of placentas. Comparison between zones was performed using Mann Whitney test analysis. There was a highly significant decrease in HSP90B1 mRNA expression in the non-labour group compared to the labour group at the outer zone (p=0.01). There was no significant difference in HSP90B1 mRNA expression in the non-labour group when compared to the labour group at the inner and middle zones (p=0.24, p=0.06) (Figure 7-10).

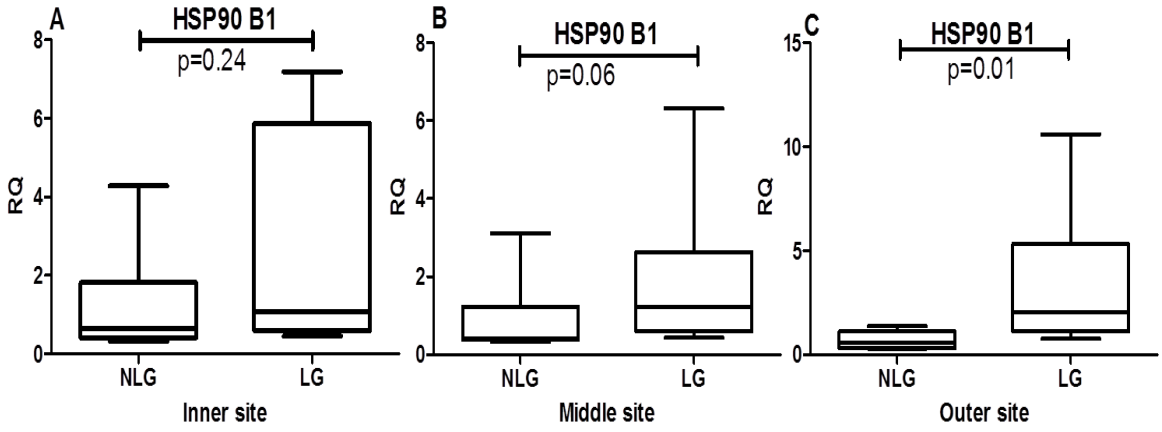


Figure 7-10: RQ values for HSP90B1 mRNA measurements in non-labour (n=6) compared to the labour (n=6) group at the inner, middle and outer placental zones. Comparison between zones was performed using Mann-Whitney analysis.

Experiment 3: This experiment was designed to test if there was any difference in mRNA expressions of HSP90B1 between non-labour control and non-labour PE groups at the inner or middle zones of placentas. Comparison between zones was performed using Mann Whitney test analysis. There was no significant difference in HSP90B1 mRNA at the inner and middle placental zones (p=0.16 for both) (Figure 7-11).

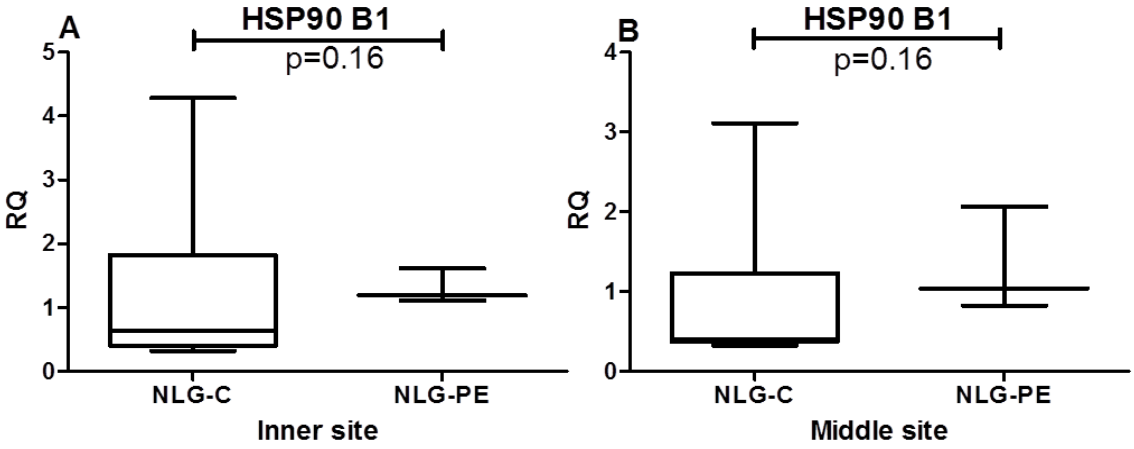


Figure 7-11: RQ values for HSP90B1 mRNA measurements in non-labour control (n=6) compared to the non-labour PE (n=3) groups at the inner and middle placental zones. Comparison between zones was performed using Mann-Whitney analysis.

Experiment 4: This experiment was designed to test if there was any difference in mRNA expressions of HSP90B1 between labour control and labour PE groups at the inner or middle zones of placentas. Comparison between zones was performed using the Mann Whitney test analysis. There was no significant difference in HSP90B1 mRNA in the labour control group compared to the labour PE group in the inner or middle placental zones (p=0.95, p=0.68) (Figure 7-12).

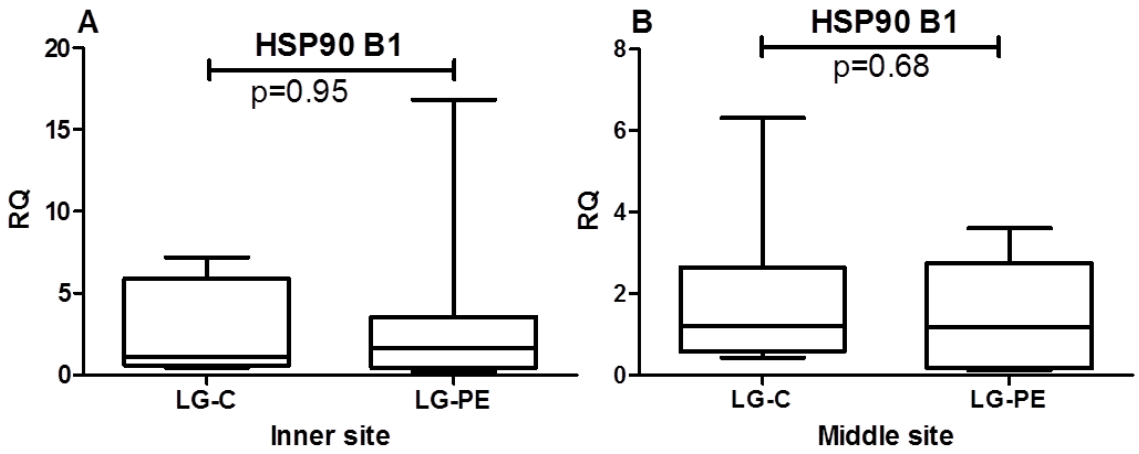


Figure 7-12: RQ values for HSP90B1 mRNA measurements in labour control (n=6) compared to the labour PE (n=6) groups at the inner and middle placental zones. Comparison between zones was performed using Mann-Whitney analysis.

7.2.5 HSPH1 expression in normal pregnancies and PE

Experiment 1: This experiment was designed to test if there was a spatial difference in mRNA expression of HSPH1 within individual placentas obtained from women who were not in labour (non-labour group) and from women who were in labour (labour group). Comparison between zones was performed using Friedman test analysis. There was no significant difference in mRNA expression of HSPH1 between the three zones of the non-labour and the labour groups (p=0.95, p=0.42 respectively) (Figure 7-13).

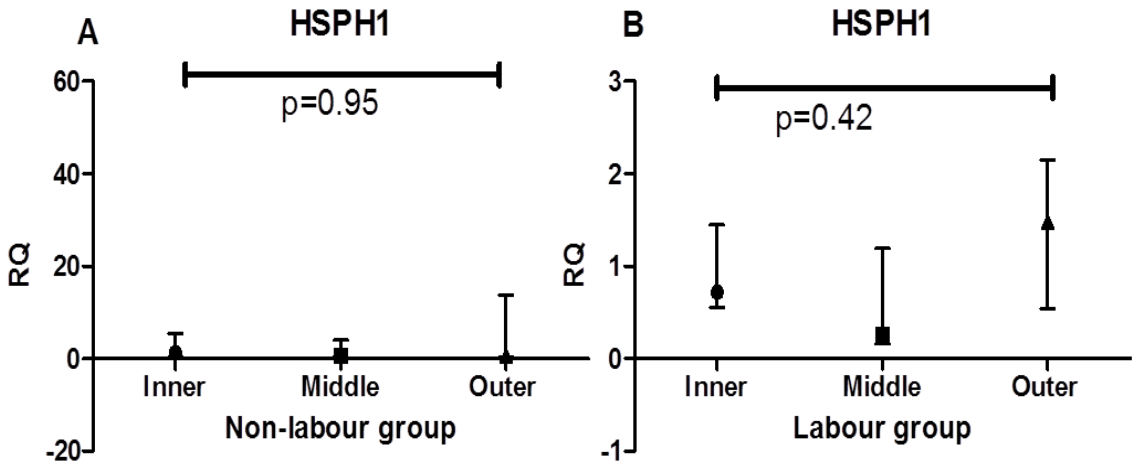


Figure 7-13: RQ values for HSPH1 mRNA measurements in in inner (n=4), middle (n=4) and outer (n=4) zones of individual placentas (non-labour and labour groups, 6 placentas in each group). Four quadrants were sampled in each zone. Graphs show the median for mRNA expressions of HSPH1. Comparison between zones was performed using Friedman analysis.

Experiment 2: This experiment was designed to test if there was any difference mRNA expression of HSPH1 between labour and non-labour groups at the inner, middle or outer zones of placentas. Comparison between zones was performed using Mann Whitney test analysis. There was no significant difference in mRNA expression of HSPH1 in the labour group when compared to the non-labour group at the inner, middle and outer zones (p=0.81, p=0.13, p=0.81) (Figure 7-14).

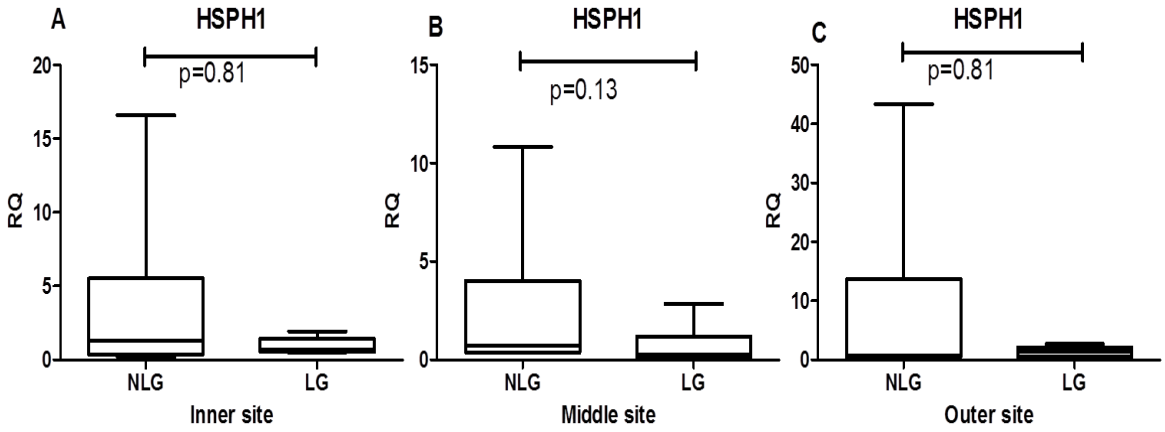


Figure 7-14: RQ values for HSPH1 mRNA measurements in non-labour (n=6) compared to the labour group (n=6) at the inner, middle and outer placental zones. Comparison between zones was performed using Mann-Whitney analysis.

Experiment 3: This experiment was designed to test if there was any difference in mRNA expressions of HSPH1 between non-labour control and non-labour PE groups at the inner or middle zones of placentas. Comparison between zones was performed using Mann Whitney test analysis. There was no significant difference in the HSPH1 mRNA was found at the inner and middle placental zones (p=0.26, p=0.38) (Figure 7-15).

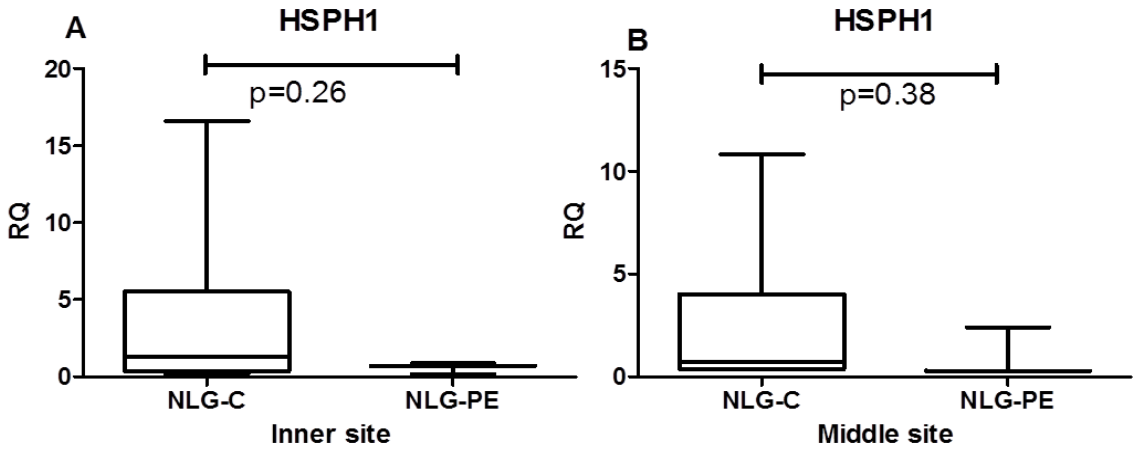


Figure 7-15: RQ values for HSPH1 mRNA measurements in non-labour control (n=6) compared to the non-labour PE (n=3) groups at the inner and middle placental zones. Comparison between zones was performed using Mann-Whitney analysis.

Experiment 4: This experiment was designed to test if there was any difference in mRNA expressions of HSPH1 between labour control and labour PE groups at the inner or middle zones of placentas. Comparison between zones was performed using Mann Whitney test analysis. There was no significant difference in HSPH1 mRNA in the labour control group compared to the labour PE group in the inner or middle placental zones (p=0.68, p=0.32) (Figure 7-16).

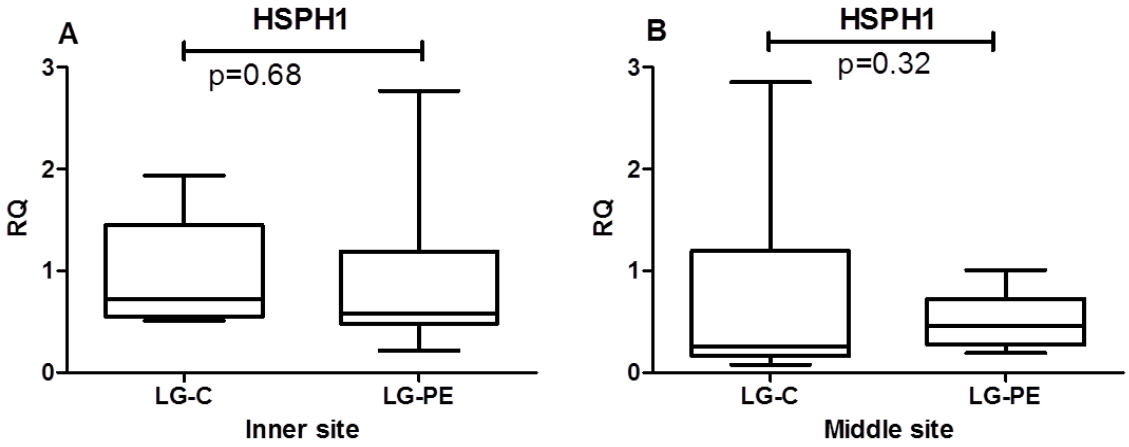


Figure 7-16: RQ values for HSPH1 mRNA measurements in labour control (n=6) compared to the labour PE (n=6) groups at the inner and middle placental zones. Comparison between zones was performed using Mann-Whitney analysis.

7.2.6 Results summary

7.2.6.1 HSPA5

Experiment 1: Overall there was no significant spatial difference in mRNA expression of HSPA5 between the three zones of the placenta for both the non-labour and the labour groups.

Experiment 2: There was no significant difference in mRNA expression of HSPA5 in the labour group when compared to the non-labour group at the inner, middle and outer zones.

Experiment 3: There was no significant increase in mRNA expression of HSPA5 in the non-labour PE group compared to the non-labour control group in the inner and middle zones.

Experiment 4: There was no significant difference in mRNA expression of HSPA5 in the labour control group compared to the labour PE group in the inner or middle placental zones.

7.2.6.2 HSP90AA

Experiment 1: There was no significant spatial difference in mRNA expression of HSP90AA between the three zones of the non-labour and labour groups.

Experiment 2: There was a significant decrease in mRNA expression of HSP90AA in the non-labour group compared to the labour group at the outer zone ($p=0.008$). There was no significant difference at the inner and middle zones.

Experiment 3: There was no significant difference in the mRNA expression of HSP90AA in the non-labour PE group compared to the non-labour control group at the inner and middle zones.

Experiment 4: There was no significant difference in mRNA expression of HSP90AA in the labour control group compared to the labour PE group at the inner and middle placental zones.

7.2.6.3 HSP90B1

Experiment 1: There was no significant spatial difference in mRNA expression of HSP90B1 between the three zones of the non-labour and labour groups.

Experiment 2: There was a highly significant decrease in HSP90B1 mRNA expression in the non-labour group compared to the labour group at the outer zone (p=0.01). There was no significant difference in HSP90B1 mRNA expression in the non-labour group when compared to the labour group at the inner and middle zones.

Experiment 3: There was no significant difference in the mRNA expression of HSP90B1 in the non-labour PE group compared to the non-labour control group at the inner and middle zones.

Experiment 4: There was no significant difference in mRNA expression of HSP90B1 in the labour control group compared to the labour PE group at the inner and middle placental zones.

7.2.6.4 HSPH1

Experiment 1: Overall there was no significant spatial difference in mRNA expression of HSPH1 between the three zones of the non-labour and the labour groups.

Experiment 2: There was no significant difference in mRNA expression of HSPH1 in the labour group when compared to the non-labour group at the inner, middle and outer zones.

Experiment 3: There was no significant difference in the mRNA expression of HSPH1 in the non-labour PE group compared to the non-labour control group at the inner and middle zones.

Experiment 4: There was no significant difference in mRNA expression of HSPH1 in the labour control group compared to the labour PE group at the inner and middle placental zones.

7.3 Discussion

7.3.1 GRP78 expression in the labour and PE

No changes in mRNA expression of GRP78 were found between non-labour and labour groups or between normotensive and PE pregnancies. There was no zonal distribution.

There are few publications in which GRP78 expression in the placenta, and changes during adverse pregnancy have been reported. These studies can be summarized as follows.

Mass spectrometry and two-dimensional (2D) electrophoresis was used to investigate expression of several trophoblast genes in non-labour at gestation 28-40 weeks and was compared to cases of PE (Jin et al., 2008). They reported that GRP78 was up-regulated in human trophoblast of PE placenta compared to the control. However, controlled sampling was not performed and labour was not studied.

Western blotting and electronic microscopy was used to investigate different stress pathways including GRP78 in 2nd trimester and term of placentas obtained from non-labouring PE patient (Yung et al., 2014b). Controls included 2nd trimester, pre-term and normal term, both labour and non-labour placentas. This study showed that GRP78 was increased in early-onset PE compared with both late-onset PE and controls. They found similar findings to our study, that there was no difference in GRP78 expression in late onset PE compared with controls however, they investigated protein levels and the present study measured mRNA. In addition, systematic sampling methods and labour were not studied by Yung *et al* (Yung et al., 2014b).

Some studies have examined GRP78 expression in early pregnancy and its roles in trophoblast invasion. These studies can be summarized as follows.

ICH and Western blot analysis was used to detect GRP78 in trophoblastic cells of first trimester placenta (Arnaudeau et al., 2009). It was concluded that GRP78 is involved in regulation of P53 and trophoblast invasion.

Western blotting and 2D electrophoresis analyses was used to investigate the effects of the cytokine IL11 (regulator of endometrium cell adhesion and trophoblast invasion) on GRP78 expression in villous trophoblast cells and EVT of first trimester placentas (Sonderegger et al., 2011). They found that IL11 stimulated secretion of GRP78 in EVT and could play a role in trophoblast invasion.

In addition, some studies have examined GRP78 expression in early pregnancy and its roles in early pregnancy loss. These studies can be summarized as follows:

Western blotting, IHC and proteomic analysis was used to investigate GRP78 in decidua from early pregnancy loss cases compared to controls (termination of pregnancy for psychological cause) (Liu et al., 2010). GRP78 was significant decreased in early pregnancy loss group.

Immunofluorescence, IHC and Western blotting was used to investigate GRP78 expression in decidua from early pregnancy loss compared to controls (Gao et al., 2012). This study showed that GRP78 was up-regulated in decidua cells treated with H_2O_2 in a dose dependent manner. They concluded that high GRP78 associated with oxidative stress induced ER stress and might be involved in development of early pregnancy loss.

A few studies reported that GRP78 could be uses as predictive marker of PE. These studies can be summarized as follows.

ELISA analysis was used to investigate GRP78 (C-term fragment, N-term fragment and full-length GRP78) and GRP78 autoantibodies in serum of PE women at term, women at the 1st trimester who subsequently developed PE and control sera both at 1st trimester and term pregnancy (Laverriere et al., 2009). There was no significant difference in autoantibodies-GRP78 complexes or total GRP78 at both first trimester and at term. However, the ratio of C-terminal GRP78 over full length GRP78 was significantly increased in plasma of PE patients compared with controls both during the first trimester and at term. They concluded that C-terminal GRP78 reflects the invasive properties of cells and could be used as PE predictive marker in early pregnancy.

ELISA and Western blotting was used to investigate GRP78 expression in term placentas and anti-GRP78 antibodies were measured in serum of women with PE (mild and severe PE) compared with controls (Rezanezhad et al., 2013). There was no significant difference between PE and healthy pregnancies however sub-analysis showed that anti-GRP78 antibodies were increased in severe PE cases when compared to controls.

A few studies have used cell culture to induce ER stress and then investigate GRP78 expression as ER stress marker. These studies can be summarized as follows

Western blotting and RT-PCR was used to investigate the role of GRP78 in trophoblast fusion using the BeWo choriocarcinoma cell line as a model of CTB fusion (Fradet et al.,

2012) as well as purified CTB from 1st trimester and term placenta of PE and control subjects (Fradet et al., 2012). This study showed that chemical inhibitors of GRP78 led to a decrease in forskolin-induced fusion capacity of BeWo cells (this highlighted role of membrane GRP78 in trophoblast fusion). In PE placentas they showed that although GRP78 mRNA was increased in CTB, the membrane GRP78 was significantly decreased compared to control CTB. They suggested that relocation of GRP78 from the ER to the trophoblast cell surface membrane is probably altered in PE.

In PE there is impaired GRP78 relocation from ER to cell surface. Prostate apoptosis response 4 (Par-4) is a protein identified as a partner of GRP78 relocation to the cell surface in prostate cancer cells. Cohen *et al* performed Western blotting, IHC and qRT-PCR to investigate GRP78 expression in trophoblast cell surface and whether (Par-4) is present in trophoblast and its role in translocation of GRP78 in term control CTB compared to the term PE CTB (Cohen et al., 2013). This study showed that that overexpression of Par-4 led to an increase of cell surface expression of GRP78 and they suggested that regulates the invasive property of evCTB (Cohen et al., 2013).

Western blotting had also been used to investigate GRP78 expression in the BeWo choriocarcinoma cell line as a model of placenta trophoblast (Repo et al., 2014). This study found that GRP78 protein expression was increased after exposure to ROS inducer agents (ethanol and nicotine).

7.3.2 HSP90AA and HSP90AB1 and the present findings

This study shows that there was no spatial distribution in mRNA expression of HSP90AA or HSP90B1 between the three zones of the non-labour and labour groups. The key finding was the fall in mRNA expression of HSP90AA and HSP90AB1 in the non-labour compared to the labour group at the outer zone. There was no significant difference in mRNA expression of HSP90AA or HSP90AB1 in the normotensive group compared to the PE pregnancies. The study again shows the importance of using a systematic method to sample the placenta.

7.3.3 HSP90AA and HSP90AB1 expression in the labour and PE

HSP90 is a highly conserved molecular chaperone, assisting the folding and conformational stabilization of several proteins (client) that play a crucial role in growth, cell survival, and developmental processes (Zuehlke and Johnson, 2010). HSP90 substrates include steroid hormone receptors, receptor tyrosine kinases, signalling kinases and NF-κβ (Zuehlke and Johnson, 2010). There are two isoforms of HSP90, HSP90α (inducible) and HSP90β (constitutive). HSP90 has been identified as essential for placenta formation in mice. HSP90 knockout fetuses grow until embryonic day 9.5 and then succumb due the non-expansion of the allantoic vasculature to form placental labyrinths (Voss et al., 2000).

There are a few publications in which HSP90 expression in the human placenta and changes during adverse pregnancy have been reported. These studies can be summarized as follows.

One research group performed an IHC study of HSP90 expression in pre-term labour, term labour, term non-labour and pre-term cesarean section for PE or FGR (Divers et al., 1995). No changes in HSP90 were reported however different tissues were studied namely amniochorion and basal plate. The placenta itself was not examined and controlled sampling was not performed.

On other research group used IHC to assess HSP90 expression in paraffin sections of placentae from normal term pregnancies and reported immunostaining on cell types, primarily in the nucleus (Shah et al., 1998). This study also showed that HSP90 staining decreased with advanced gestation. Site of sampling or labour was not assessed.

Ziegert *et al* performed immunofluorescence to investigate HSP90 and HSP90 antibody complex expression in term, pre-term and FGR placentas (Ziegert et al., 1999). This study showed that HSP90 protein was expressed in placenta and there was no significant difference between all groups. No data or p values were shown to support this statement and no systematic sampling was performed.

Wataba *et al* performed IHC to investigate HP90 in second and third trimester of FGR placentas (Wataba et al., 2004). This study showed that HSP90 protein expression was increased in the CTB and STB layer. In addition, HSP90 were increased in syncytial knots

and avascular villi whereas it was reduced in the presence of infarction. Systemic sampling methods were not performed.

Sotiriou *et al* performed IHC to investigate HSP90 in first trimester of missed miscarriages and term placentas (Sotiriou et al., 2004). This study showed that HSP90 was increased in chorionic villi of 1st trimester placentas in the STB, CTB, vessel and stroma cells compared to term placentas. In addition, there was a statistically significant increase of HSP90A expression in chorionic villi of 1st trimester placentas only in the CTB cells compared to full-term placentas (Sotiriou et al., 2004).

Cindrova-Davies *et al* performed Western blotting to compare oxidative stress markers in the non-labour, short labour and long labour. (Cindrova-Davies et al., 2007b) This study reported that there was significant increase in HSP90 protein expression in long labour group compared to the short labour group. Since systemic sampling was not performed, it is impossible to directly compare the present study with that one.

Zhang *et al* performed Western blotting to investigate HSP90 expression in term placentas of PE and controls (Zhang et al., 2011). This study showed that HSP90 protein expression was decreased in PE placentas. No systemic sampling was performed and labour was not studied.

Padmini and Lavanya performed Western blotting and immunofluorescence to investigate HSP90 (Padmini and Lavanya, 2011a). This study found that protein expression of HSP90 was increased in endothelial cells of PE placenta when compared to normotensive subjects. Systemic sampling was not performed and labour was not studied.

The same research group (Padmini and Lavanya, 2011a, Padmini et al., 2012a) using ELISA and Western blotting found that expression of HSP90 was increased in PE endothelial cells when compared to normotensive subjects.

Ermini et al performed Western blotting, double affinity chromatography and mass spectrometry to investigate HSP90 in trophoblast cells line exposed to different oxygen concentration and placenta tissue of 1st and 3rd trimester placentas (Ermini et al., 2013). This study reported that HSP90 expression is not affected by varying oxygen tension. However, the O-glycosylated isoform of HSP90 is expressed in an O_2 dependent manner. They concluded that oxygen tension is crucial in modulating glycosylation of proteins during placental development.

Yung *et al* performed Western blotting to investigate HSP90 over different time periods (10, 20, 30 and 45 min) after delivery of non-labour normotensive placentas (Yung et al., 2014a). This study showed that HSP90 did not change over time period. Systemic sampling was not performed.

7.3.4 HSPH1 (HSP105/HSP110) and present finding

The study showed that HSPH1 mRNA is expressed in human placenta. No changes in mRNA expression of HSPH1 between non-labour and labour groups or normotensive compared to PE pregnancies were found. There was no zonal distribution.

7.3.5 HSPH1 expression in the placenta

One study investigated HSP105 expression in human chorionic villus tissue at 10-17 weeks of a normal pregnancy after exposure to heat shock or sodium arsenite or cadmium chloride (Honda et al., 1991). This study showed that HSP105 protein expression was increased after exposure to heat shock, sodium arsenite and cadmium chloride through 10-17 weeks of pregnancy (Honda et al., 1991).

Chapter VIII: Analysis of mRNA expression of Caspases (CASP3, 7 and 9) apoptotic markers in human placenta of normal pregnancies and pregnancies complicated with PE

8 Caspases (CASP3, 7 and 9) expression

8.1 Introduction

8.1.1 Apoptosis

Apoptosis is a form of programmed cell death, which is responsible for the removal of unwanted cells during development and adult homeostasis. Apoptosis is an active form of cell death dependent on the internal machinery of the cell; this process is distinguished from necrosis which is an unplanned and accidental death caused by external factors (Balvan et al., 2015) During apoptosis cells shrink, detach and end up forming small pieces (apoptotic bodies), which are cleared immediately by phagocytes (Muñoz-Pinedo, 2012). During apoptosis, cells maintain integrity of their plasma membrane which helps to avoid inflammation. Cell death by necrosis, which occurs due to uncontrolled tissue damage, is a passive form of cell death that triggers inflammation pathways. Apoptosis is known to occur in a number of biologic processes, both physiological for example involution and atrophy of post-lactational breast and pathological including all neoplastic growths, cytotoxic cells and tissues exposed to low dosage of toxic stimuli (Smith et al., 1997). Abnormal regulation of cell execution of apoptosis is accomplished by activation of caspases, which are regulated by Bcl-2 family members and which are the final effectors in apoptosis (Steller, 1995, Cohen, 1997). Apoptosis in different tissues has been implicated in the onset and progression of several diseases include cancer, autoimmune disease and neurodegenerative disorders (Leung et al., 2001, Shalini et al., 2014)

Caspases (CASPs) are a family of cysteine proteases that play critical roles in apoptosis, necrosis and inflammation (Muñoz-Pinedo, 2012, Shalini et al., 2014). Caspases exist as inactive proenzymes that are proteolytically activated at conserved aspartic residues to produce two subunits, large and small, that dimerize to form the active enzyme (Alnemri et al., 1996). The caspase family consist of 10 members 1-12 (CASP1-10). Caspases are regulated by post translational modification to ensure that they can be rapidly activated

leads to an irreversible proteolytic cascade that ends up killing the cell. CASPs are classified into two groups based on their sequence homology and their role in cascade: initiator (apical) and executioner (effector) caspase. Initiator (CASPs 2, 8, 9 and 10) have a long domain in the N-terminus that are responsible for interactions between initiator caspases and the molecules which activate them (Mattson and Chan, 2002, Muñoz-Pinedo, 2012). The effector caspases (CASPs 3, 4, 5, 6, 7, 11, 12 and 13) are inactive until they are cleaved by initiator caspases (Mattson and Chan, 2002, Muñoz-Pinedo, 2012).

Apoptosis has been reported to be a common feature observed in trophoblast cells in the PE and FGR placenta at term (Leung et al., 2001, Bahr et al., 2014). Abnormal regulation of apoptosis has been implicated in the onset and progression of a broad range of diseases (Hetts, 1998, Muñoz-Pinedo, 2012, Shalini et al., 2014).

Apoptosis is controlled by two mechanisms: the mitochondrial (intrinsic) pathway and death receptor (extrinsic) pathway.

8.1.1.1 Intrinsic pathways

The outer mitochondrial membrane becomes permeabilized, resulting in release of molecules to the cytosol by a reaction controlled by Bcl-2 and Bcl-2 protein (B cell lymphocytic-leukaemia proto-oncogene released proteins), which contain pro-apoptotic members such as Bax (Zong et al., 2001, Lanneau et al., 2008). Bax (alone or in combination with sessile proteins) may play a role in pore formation in outer mitochondrial membrane (Zamzami and Kroemer, 2001). The intrinsic pathway is based on the cytochrome *c* dependent activation of CASPs, special proteases which are involved in cleavage and processing of more than 300 different cellular proteins (Fischer et al., 2003). Cytochrome c is an intermembrane protein; different signals include oxidative stress irradiation and other stress lead to release it from mitochondria, which once in the cytosol, it interacts with Apaf1. Oligomerization of Apaf-1 then binds to cytosolic pro-caspase-9 and leading to formation of apoptosome (Li et al., 1997), which leads to activation of pro-caspase-9, which in turn cleaves pro-caspase-3, the main caspase effector during apoptosis (Mignotte and Vayssiere, 1998, Mymrikov et al., 2011) (Figure 8-1).

8.1.1.2 Extrinsic pathway

The extrinsic pathway apoptosis is dependent on special membrane receptors know as plasma membrane death receptors that are members of the TNF receptor gene superfamily,

their activation leads to the direct activation of the receptor-proximal CASP8 or CASP10 in the death-inducing signalling complex (DISC) (Elmore, 2007).

The Fas receptor plays an important role in induction of apoptosis activating two different signal pathways. First by binding of a special ligand (FasL) to the Fas receptor promotes interaction of the Fas receptor with Fas associated-death-domain (FADD) protein which activates pro-caspase-8 and downstream CASPs (CASP3, 6, and 7) (Charette et al., 2000) (Figure 8-1). Secondly, binding of Fas-ligand promotes interaction of the Fas receptor with Daxx, and induction of apoptosis relies on translocation of Daxx from the nucleus to the cytosol, and its interaction with Fas receptor is probably followed by activation of Ask1.

In addition, apoptosis can be activated through ER/Ca^{2+}-mediated CASP12 pathways. Mitochondria can also release AIF and endonuclease G (EndoG), which lead to apoptosis independent of caspase activation (Kwak, 2013).

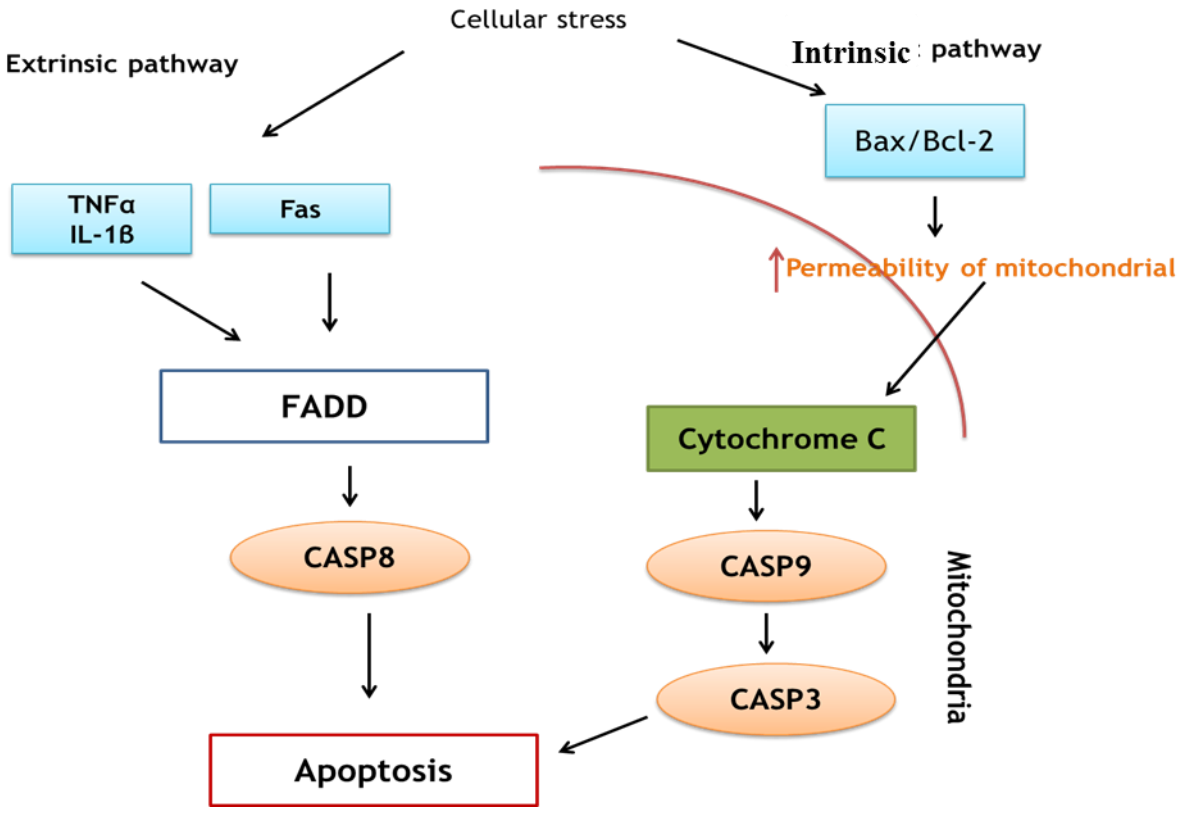


Figure 8-1: Apoptotic signaling pathways with overview of two major caspases-dependent apoptotic including (1) cytokines/Fas-mediated CASP8 pathways, (2) mitochondrial-mediated CASP9 pathways. Modified from (Kwak, 2013).

8.1.2 CASPs in inflammation and immunity

CASPs play an important role in inflammation pathways, for example CASP1 is involved in processing and maturation of inflammatory mediators such as IL-1β and IL-18 (Ghayur et al., 1997). There are several caspases, which have been identified as important mediators of the innate immune response including CASP1, 4, 5 and 12. Pyroptosis is triggered by CASP1 after its activation by various inflammasomes and results in lysis of the affected cell (Miao et al., 2011). Both pyroptosis and apoptosis are programmed cell death mechanisms but are dependent on different caspases and results in cellular laysis and release of the cytosolic contents to the extracellular space (Miao et al., 2011). CASP12 can inhibit the inflammatory response by inhibiting CASP1.

8.1.3 Apoptosis and HSPs

HSPs are a family of proteins that respond to different kinds of environmental and physiological stress (Garrido et al., 2006). HSPs function as molecular chaperones in regulating cellular homeostasis and promoting cell survival by blocking caspase-dependent apoptosis. HSPs have been shown to block apoptosis by interfering with caspase activation.

HSP27 functions include acting as a protein chaperone, control of apoptosis, regulation of cell glutathione levels, inhibition of actin polymerisation as well as protection against heat shock, oxidative stress and mechanical stress (Lanneau et al., 2010). HSP27 is known to be involved in the inhibition of apoptosis in several tissues (Paul et al., 2002). HSP27 inhibits CASP3 and 9 (GARRIDO et al., 1999), HSP27 increases cell survival by affecting both upstream signalling and downstream effector events in the apoptotic pathway (Tezel and Wax, 2000, Lanneau et al., 2008). In the placenta, HSP27 is localized in the trophoblast cells and upregulated in the placenta of PE (Shin et al., 2010, Abdulsid and Lyall, 2014). During labour HSP27 is down-regulated suggesting a role for this molecule during pregnancy (Abdulsid et al., 2013a). HSP27 prevent activation of pro-caspase-9 and 3 like Bcl-2 (GARRIDO et al., 1999). HSP27 does not prevent cytochrome *C* and AIF release from the mitochondria or translocation of AIF to the nucleus (inhibitory effect is specific for cytochrome *C*, caspase dependent apoptotic pathway). HSP27 interacts with cytosol cytochrome *C* and prevents apoptosome formation (Parcellier et al., 2006). P-HSP27 is associated with the Daxx pathway (caspase-independent pathway) (Charette et

al., 2000). The non-phosphorylated oligomer is the active form of HSP27 in caspase-dependent pathway (Garrido et al., 2006).

HSP70 inhibits apoptosis downstream of the release of cytochrom *c* and upstream of the activation of CASP3 by modulation of the apoptosome (Schmitt et al., 2007). HSP70 has anti-apoptotic activity which includes: binding to the Apaf-1 and prevent recruitment of pro-caspase-9 to the apoptosome (Saleh et al., 2000, Li et al., 2000). HSP70 also binds to AIF and inhibits chromatin condensation (Ravagnan et al., 2001), HSP70 rescues cells from late apoptosis (TNF-induced apoptosis), prevents downstream morphological changes, acts at an early stage by preventing JNK activation (Meriin et al., 1999, Lanneau et al., 2008). Furthermore, HSP70/Bag-1 regulates Raf-1/ERK kinase and cell growth in response to stress (Song et al., 2001).

HSP90 can inhibit apoptosis by preventing caspase activation as a result of a negative effect on Apaf-1 function (Pandey et al., 2000). In addition, HSP90 anti-apoptotic properties is also reflected by its capacity to interact with phosphorylated serine kinase Akt, phosphorylated Akt can phosphorylate the Bcl-2 family protein Bad and CASP9 (Schmitt et al., 2007) leading to their inactivation and to cell survival. Furthermore, it has been shown that p53 represses HSP90 gene expression in Ultraviolet (UV)-irradiated cells (Zhang et al., 2004). Moreover, the HSP90 family protein GRP94 was shown to inhibit the action of the calcium-dependent proteases calpains and protect against IR injury induced apoptosis involving calpains (Bando et al., 2003).

Cytosolic HSPD1 forms a complex with the pro-apoptotic protein Bax (Gupta and Knowlton, 2002). Under stress conditions HSPD1 and Bax dissociate and Bax translocates to the mitochondria and induces apoptosis and the interaction with HSPD1 prevents the apoptosis.

8.2 Results

8.2.1 Patient clinical data analysis

Patient data analysis is the same as section (3.4.1.1), results analysis is shown in (Table 3-2) (Table 3-3).

8.2.2 CASP3 mRNA expression in the labour and PE

Experiment 1: This experiment was designed to test if there was a spatial difference in mRNA expression of CASP3 within individual placentas obtained from women who were not in labour (non-labour group) and from women who were in labour (labour group). Comparison between zones was performed using Friedman test analysis. There was no significant difference in mRNA expression of CASP3 between the three zones of the non-labour and labour groups (p=0.42, p-0.74) (Figure 8-2).

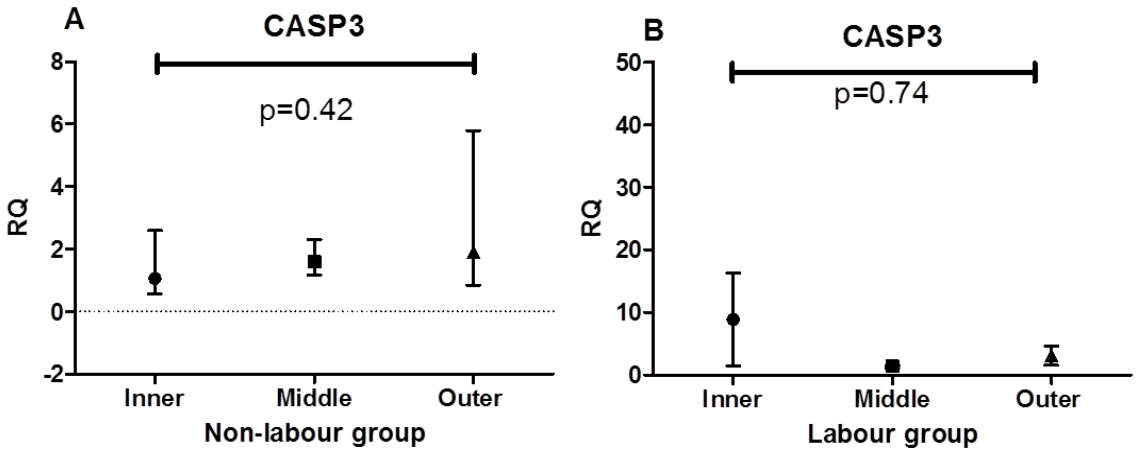


Figure 8-2: RQ values for CASP3 mRNA measurements in in inner (n=4), middle (n=4) and outer (n=4) zones of individual placentas (non-labour and labour groups, 6 placentas in each group). Four quadrants were sampled in each zone. Graphs show the median for mRNA expressions of CASP3. Comparison between zones was performed using Friedman analysis.

Experiment 2: This experiment was designed to test if there was any difference in mRNA expression of CASP3 between labour and non-labour groups at the inner, middle or outer zones of placentas. Comparison between zones was performed using Mann Whitney test analysis. There was no significant difference in mRNA expression of CASP3 in the non-

labour group when compared to the labour group at the inner, middle and outer zones (p=0.48, p=0.58, p=0.93 respectively) (Figure 8-3).

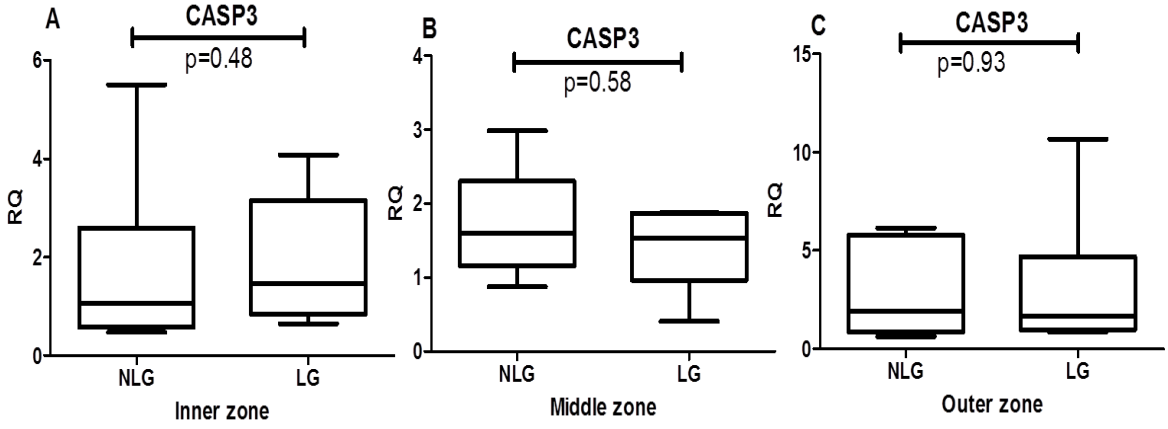


Figure 8-3: RQ values for CASP3 mRNA measurements in non-labour (n=6) compared to the labour (n=6) group at the inner, middle and outer placental zones. Comparison between zones was performed using Mann-Whitney analysis.

Experiment 3: This experiment was designed to test if there was any difference in mRNA expressions of CASP3 between non-labour control and non-labour PE groups at the inner or middle zones of placentas. Comparison between zones was performed using the Mann Whitney test. There was no significant difference in CASP3 mRNA at the inner and middle zones (p=0.26, p=1 respectively) (Figure 8-4).

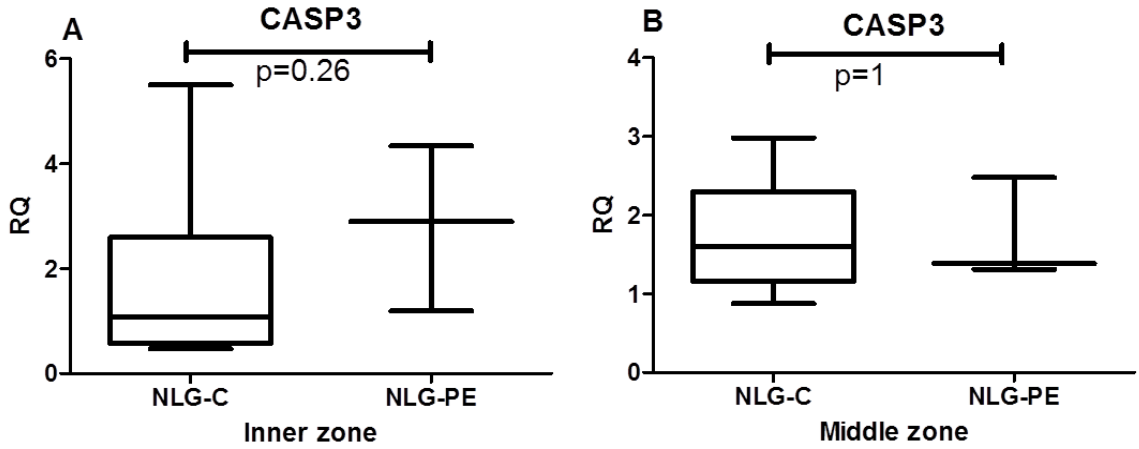


Figure 8-4: RQ values for CASP3 mRNA measurements in non-labour control (n=6) compared to the non-labour PE (n=3) groups at the inner and middle placental zones. Comparison between zones was performed using Mann-Whitney analysis.

Experiment 4: This experiment was designed to test if there was any difference in mRNA expressions of CASP3 between labour control and labour PE groups at the inner or middle zones of placentas. Comparison between zones was performed using the Mann Whitney test analysis. There was a significant decrease in mRNA expression of CASP3 in the labour PE group compared to the labour control group at the middle placental zone (p=0.04). No difference was found at the inner zone (p=013) (Figure 8-5).

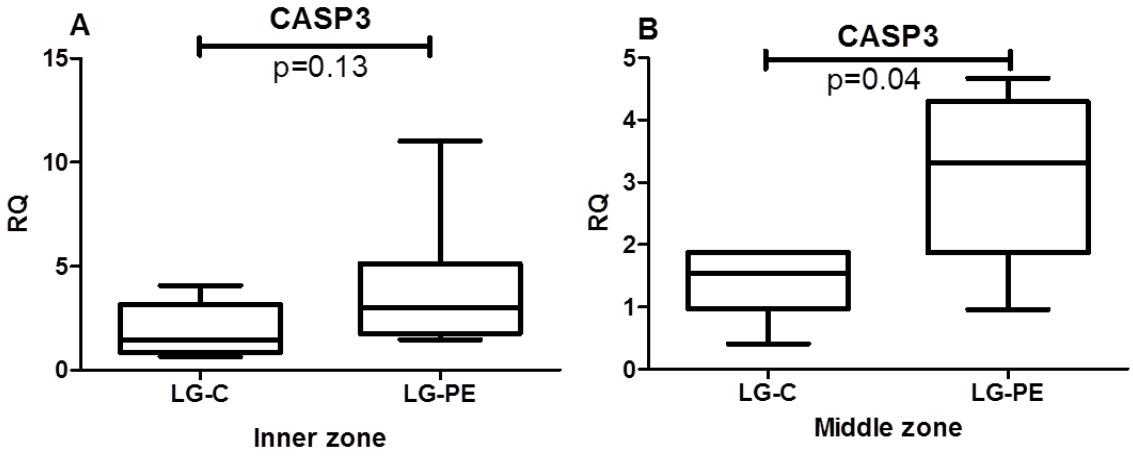


Figure 8-5: RQ values for CASP3 mRNA measurements in labour control (n=6) compared to the labour PE (n=6) groups at the inner and middle placental zones. Comparison between zones was performed using Mann-Whitney analysis.

8.2.3 CASP7 mRNA expression in the labour and PE

Experiment 1: This experiment was designed to test if there was a spatial difference in mRNA expression of CASP7 within individual placentas obtained from women who were not in labour (non-labour group) and from women who were in labour (labour group). Comparison between zones was performed using Friedman test analysis. There was no significant difference in mRNA expression between the three zones of the non-labour and labour groups (p=0.14, p-0.95) (Figure 8.6).

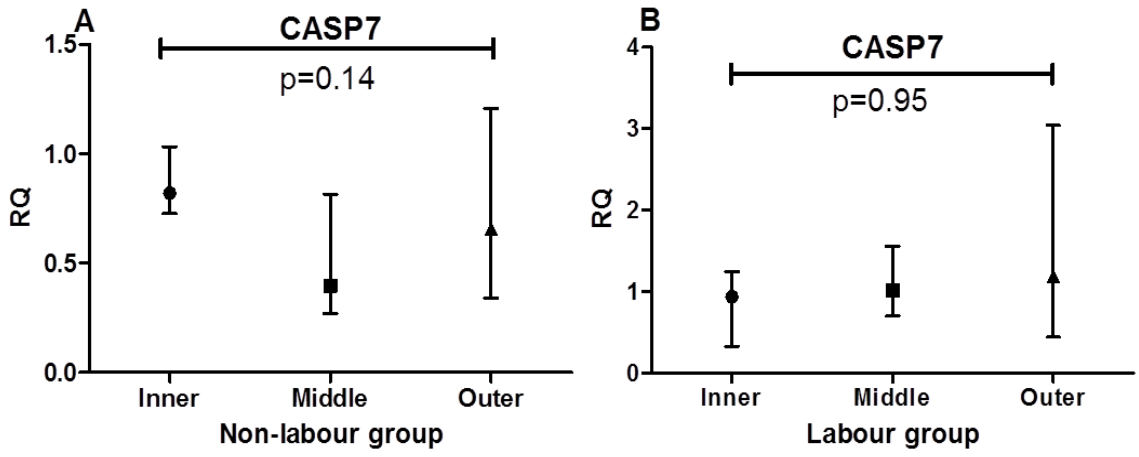


Figure 8-6: RQ values for CASP7 mRNA measurements in in inner (n=4), middle (n=4) and outer (n=4) zones of individual placentas (non-labour and labour groups, 6 placentas in each group). Four quadrants were sampled in each zone. Graphs show the median for mRNA expressions of CASP7. Comparison between zones was performed using Friedman analysis.

Experiment 2: This experiment was designed to test if there was any difference in mRNA expression of CASP7 between labour and non-labour groups at the inner, middle or outer zones of placentas. Comparison between zones was performed using the Mann Whitney test analysis. There was a significant decrease in mRNA expression of CASP7 in the non-labour group when compared to the labour group at the middle zone (p=0.04). No difference was found at the inner and outer zones (p=0.81, p=0.3) (Figure 8-7).

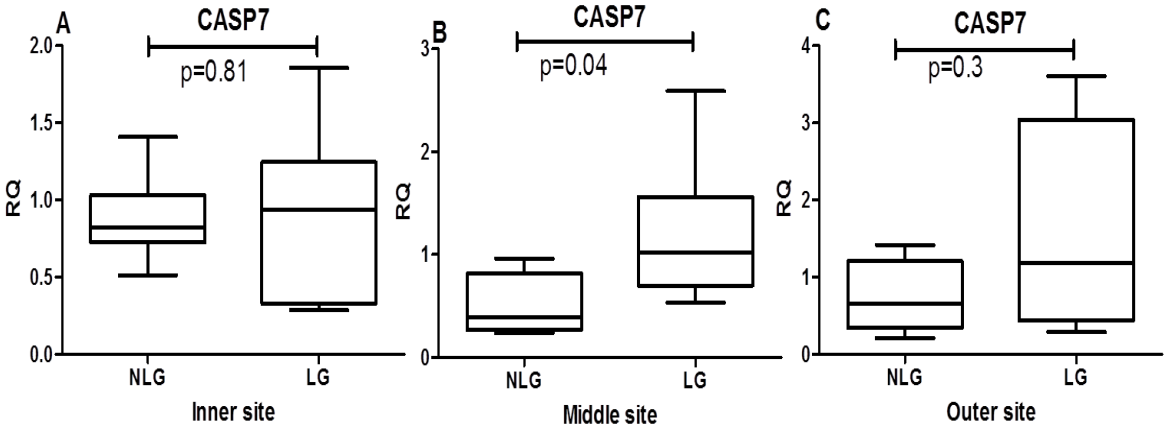


Figure 8-7: RQ values for CASP7 mRNA measurements in non-labour (n=6) compared to the labour (n=6) group at the inner, middle and outer placental zones. Comparison between zones was performed using Mann-Whitney analysis.

Experiment 3: This experiment was designed to test if there was any difference in mRNA expressions of CASP7 between non-labour control and non-labour PE groups at the inner or middle zones of placentas. Comparison between zones was performed using the Mann Whitney test. There was a significant decrease in the CASP7 mRNA in the non-labour-PE group compared to the non-labour control group at the inner zone (p=0.04). There was no significant difference in the CASP7 mRNA was found at the middle zone (p=0.16) (Figure 8.8).

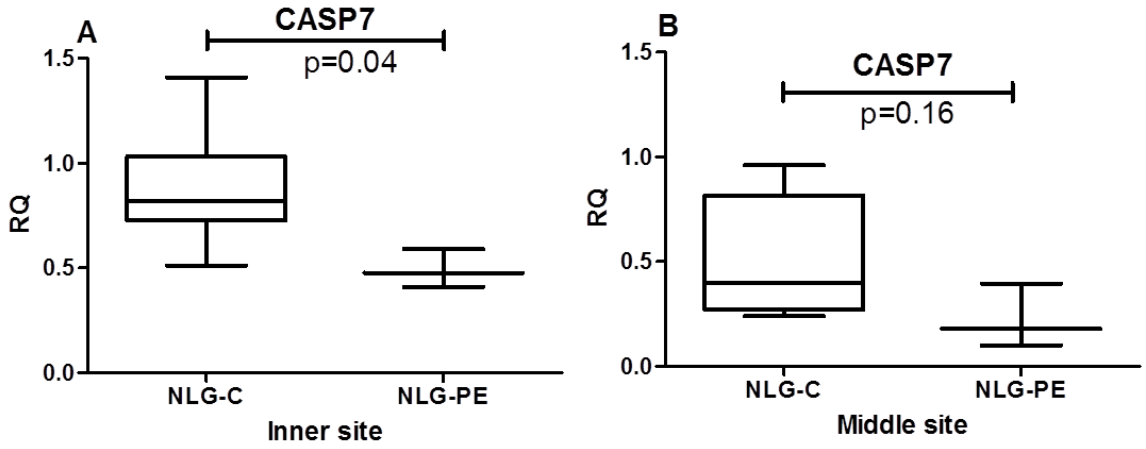


Figure 8-8: RQ values for CASP7 mRNA measurements in non-labour control (n=6) compared to the non-labour PE (n=3) groups at the inner and middle placental zones. Comparison between zones was performed using Mann-Whitney analysis.

Experiment 4: This experiment was designed to test if there was any difference in mRNA expressions of CASP7 between labour control and labour PE groups at the inner or middle zones of placentas. Comparison between zones was performed using Mann Whitney test analysis. There was no significant difference in mRNA expression of CASP7 in the labour control group compared to the labour PE group in the inner or middle placental zones (p=0.95, p=0.08) (Figure 8-9).

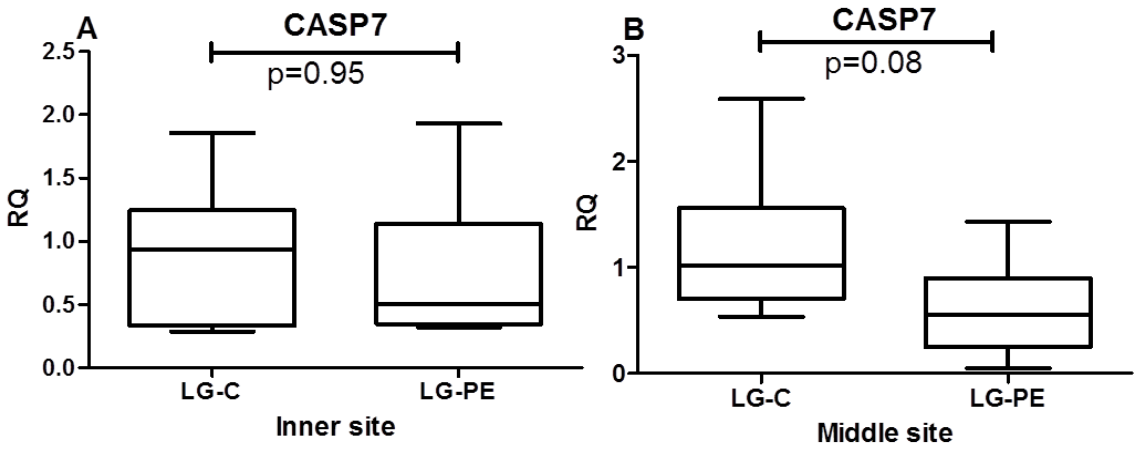


Figure 8-9: RQ values for HSPB2 mRNA measurements in labour control (n=6) compared to the labour PE (n=6) groups at the inner and middle placental zones. Comparison between zones was performed using Mann-Whitney analysis.

8.2.4 CASP9 mRNA expression in the labour and PE

Experiment 1: This experiment was designed to test if there was a spatial difference in mRNA expression of CASP9 within individual placentas obtained from women who were not in labour (non-labour group) and from women who were in labour (labour group). Comparison between zones was performed using Friedman test analysis. There was no significant difference in mRNA expression between the three zones of the non-labour and labour groups (p=0.42, p=0.95) (Figure 8-10).

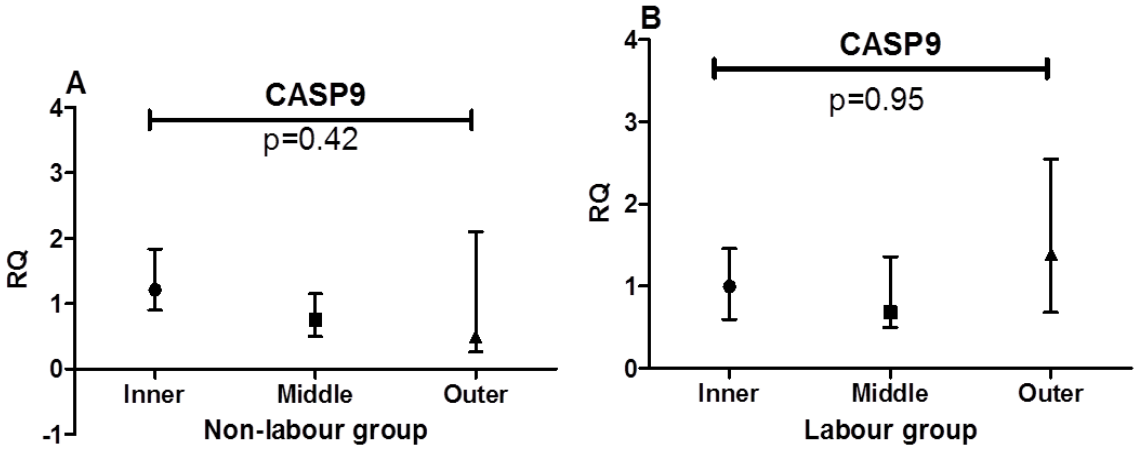


Figure 8-10: RQ values for CASP9 mRNA measurements in in inner (n=4), middle (n=4) and outer (n=4) zones of individual placentas (non-labour and labour groups, 6 placentas in each group). Four quadrants were sampled in each zone. Graphs show the median for mRNA expressions of CASP9. Comparison between zones was performed using Friedman analysis.

Experiment 2: This experiment was designed to test if there was any difference in mRNA expression of CASP9 between labour and non-labour groups at the inner, middle or outer zones of placentas. Comparison between zones was performed using the Mann Whitney test analysis. There was no significant difference in mRNA expression of CASP9 in the non-labour group when compared to the labour group at the inner, middle and outer zone (p=0.39, p=0.93, p=0.3) (Figure 8-11).

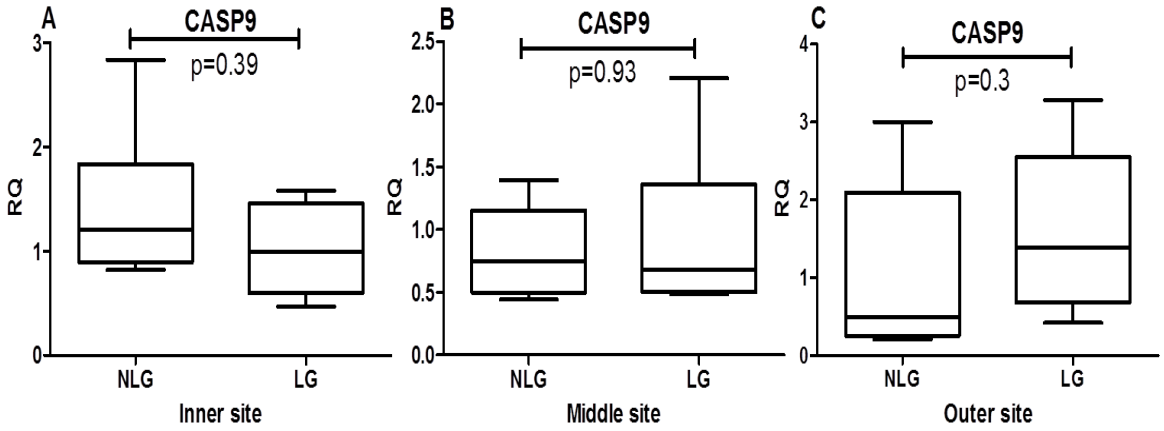


Figure 8-11: RQ values for CASP9 mRNA measurements in non-labour (n=6) compared to the labour (n=6) group at the inner, middle and outer placental zones. Comparison between zones was performed using Mann-Whitney analysis.

Experiment 3: This experiment was designed to test if there was any difference in mRNA expressions of CASP9 between non-labour control and non-labour PE groups at the inner or middle zones of placentas. Comparison between zones was performed using the Mann Whitney test analysis. There was a significant decrease in the mRNA expression of CASP9 in the non-labour PE group compared to the non-labour control at middle zone (p=0.04). No difference was found at the inner zone (p=0.26) (Figure 8-12).

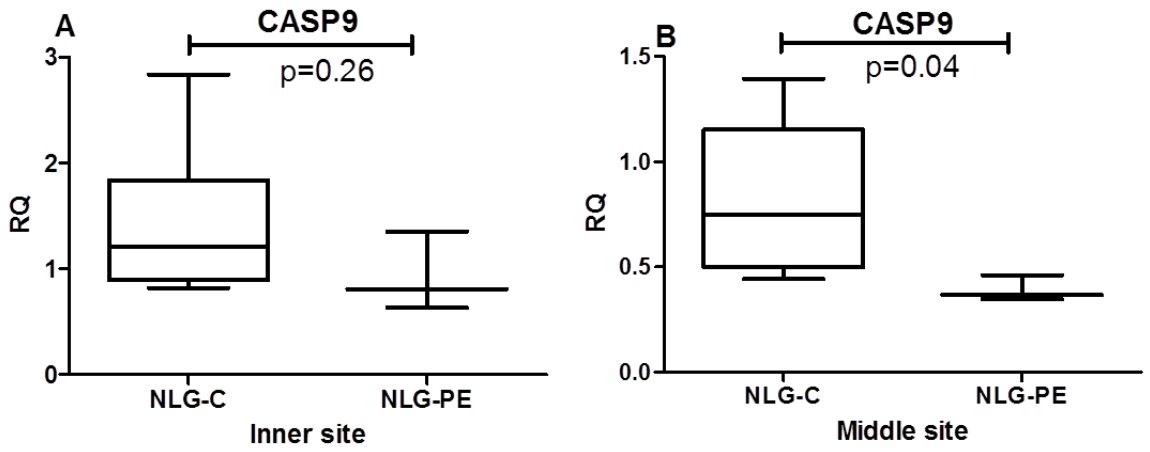


Figure 8-12: RQ values for CASP9 mRNA measurements in non-labour control (n=6) compared to the non-labour PE (n=3) groups at the inner and middle placental zones. Comparison between zones was performed using Mann-Whitney analysis.

Experiment 4: This experiment was designed to test if there was any difference in mRNA expressions of CASP9 between labour control and labour PE groups at the inner or middle zones of placentas. Comparison between zones was performed using the Mann Whitney test analysis. There was no significant difference in CASP9 mRNA in the labour control group compared to the labour PE group in the inner or middle placental zones (p=0.86, p=0.6) (Figure 8-13).

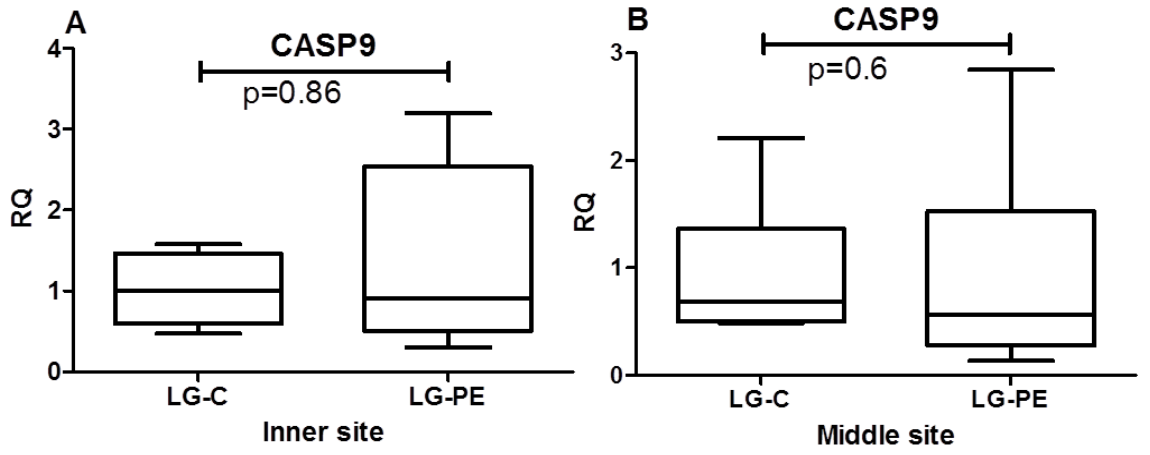


Figure 8-13: RQ values for CASP9 mRNA measurements in labour control (n=6) compared to the labour PE (n=6) groups at the inner and middle placental zones. Comparison between zones was performed using Mann-Whitney analysis.

8.2.5 Results summary

8.2.5.1 CASP3

Experiment 1: There was no significant spatial difference in mRNA expression of CASP3 expression between the three zones of the non-labour and labour groups.

Experiment 2: There was no significant difference in mRNA expression of CASP3 in the non-labour group when compared to the labour group at the inner, middle and outer zones

Experiment 3: There was no significant difference in the mRNA expression of CASP3 in the non-labour control group compared to the non-labour PE group at the inner zone and middle zones.

Experiment 4: There was a significant increase in mRNA expression of CASP3 in the labour PE group compared to the labour control group at the middle placental zone ($p=0.04$). No difference was found at the inner zone.

8.2.5.2 CASP7

Experiment 1: There was no significant spatial difference in mRNA expression of CASP7 expression between the three zones of the non-labour and labour groups.

Experiment 2: There was a significant decrease in mRNA expression of CASP7 in the non-labour group when compared to the labour group at the middle zone ($p=0.04$). No difference was found at the inner and outer zones.

Experiment 3: There was a significant decrease in the mRNA expression of CASP7 in the non-labour-PE group compared to the non-labour control group at the inner zone ($p=0.04$). There was no significant difference in the CASP7 mRNA was found at the middle zone.

Experiment 4: There was no significant difference in mRNA expression of CASP7 in the labour control group compared to the labour PE group in the inner or middle placental zones.

8.2.5.3 CASP9

Experiment 1: There was no significant spatial difference in mRNA expression of CASP9 expression between the three zones of the non-labour and labour groups.

Experiment 2: There was no significant difference in mRNA expression of CASP9 in the non-labour group when compared to the labour group at the inner, middle and outer zones.

Experiment 3: There was a significant decrease in the mRNA expression of CASP9 in the non-labour PE group compared to the non-labour control at middle zone ($p=0.04$). No difference was found at the inner zone.

Experiment 4: There was no significant difference in mRNA expression of CASP9 in the labour control group compared to the labour PE group in the inner or middle placental zones.

8.3 Discussion

8.3.1 CASPs mRNA expression and key findings

There was no spatial distribution in mRNA expression of CASP3, 7 and 9 across individual placentas. When non-labour was compared to the labour group, there was a significant decrease in mRNA expression of CASP7 in the non-labour group at the middle zone. There was no significant difference in mRNA expression of CASP3 and CASP9. When the non-labour control was compared to the non-labour PE there was a significant decrease in the mRNA expression of CASP7 (inner) and CASP9 (middle) in the non-labour-PE group. When the labour control was compared to the labour PE group, there was a significant increase in mRNA expression of CASP3 in the labour PE group at the middle zone. No other differences were found. Although the reason for this differential expression is unclear, again it highlights the importance for systemic sampling methods.

8.3.2 CASPs mRNA expression in the human placenta during labour and PE

Apoptosis is a feature of villous trophoblast throughout pregnancy and is an essential feature of placental invasion and STB function as well as playing a role in maternal immune tolerance. The amount of apoptosis in placental villi changes throughout normal pregnancy. The incidence of apoptosis in pregnancy increases from the first to the third trimester (Smith et al., 2000b), and markedly accelerates beyond 40 wks of gestation (Athapathu et al., 2003, Sharp et al., 2010). Apoptosis within the villus is predominantly localized to the STB but not CTB at term and a very low incidence in CTB of 1st trimester placenta (Burton et al., 2003). In addition, STB shows feature consistent with apoptosis including CASP8 and 9 activation, cytokeratin-18 cleavage, and DNAse activity (Kadyrov et al., 2001, De Falco et al., 2004, Sharp et al., 2010).

Trophoblast apoptosis is regulated by the interplay of pro and anti-apoptotic events (Heazell and Crocker, 2008). Trophoblast express TNF receptors, Fas and Fas ligand, and TNF-related apoptosis-inducing ligand (TRAIL), which may play a role in both apoptosis and immune regulation (Abrahams et al., 2004c, Bai et al., 2009). In addition, other factors control trophoblast apoptosis including the transcription factor p53 and members of the Bcl-2 family (Sharp et al., 2010).

Apoptosis that occurs in the placenta from following vaginal delivery may be of little physiological consequence because by this time of delivery the placenta will have completed its function. However, it has been shown (Cindrova-Davies et al., 2007b) that intermittent perfusion of the placenta stimulates apoptotic changes, which may explain increased apoptosis in PE placentas (Leung et al., 2001). Apoptotic debris transported into the maternal circulation in PE can lead to activation of maternal endothelium.

One study examined the expression of cleaved CASP3, 9 in placentas of non-labour, short and long labour (Cindrova-Davies et al., 2007b). This study showed that there was no difference in CASP3 and 9 expression between labored groups, however, IHC examination showed an increased in trophoblast apoptosis as evidenced by the cleavage of cytokeratin filaments in the labour group. In our study there was a significant increase in CASP7 in the labour group but not CASP3 and 9, however, it is not possible to compare the two studies due to differences in sampling methods.

Di Santo *et al* performed experiments using human term placentas using both villous explant culture and dual perfusion (Di Santo et al., 2003). They compared trophoblast viability during perfusion and in explants cultured under various conditions by monitoring several factors include apoptotic cell death. They performed TUNEL assays and IHC to analysis apoptotic cell death by detecting CASP3. This study showed that prominent cell death after 7 h in explant culture compared to dual perfusion experiment in which the expression was similar as in native tissue.

Increased levels of villous trophoblast apoptosis have been identified in placental pathologies including PE (Heazell et al., 2008). In PE increased levels of syncytial derived cytokeratin M30 neoepitope can be detected in maternal serum (Hamad et al., 2009).

Apoptosis has been implicated in both labour and PE. In the apoptotic pathway, many key proteins and cytokines are involved. For example, HSPs act at several stages to prevent cell death initiated by stress-induced damage (Jäättelä, 1999, Kampinga et al., 2009, Borges et al., 2012).

In pregnancy complications there is exaggerated apoptosis due to IR injury and oxidative stress (Sharp et al., 2010). In addition, villous trophoblast from placentas of pregnancies complicated by PE show increased susceptibility to apoptosis, these changes rendering these cells more vulnerable to oxidative damage (Leung et al., 2001, Ishihara et al., 2002).

In PE apoptosis has been associated with a reduction in STB with excessive syncytial loss and alterations in a variety of pro- and anti-apoptotic proteins. There are some publications which show that caspase expression in the placenta changes during adverse pregnancy. These studies can be summarized as follows.

One research group performed electronic microscopy to investigate apoptosis markers (apoptotic nuclei) in villous trophoblast of (>34 wk of gestation) placentas of PE compared to control pregnancies (Leung et al., 2001). This study showed that there was increased apoptosis in PE placentas.

Another research group performed IHC and electronic microscopy to investigate apoptosis markers in villous trophoblast in term placentas (Ishihara et al., 2002). This study showed that there was an increased TUNEL-positive nuclei in PE in both STB and CTB. BcL-2 was reduced in severe PE but there was no difference in Fas.

Hung *et al* performed Immunofluorescence to investigate CASP3 in villus tissue of term non-labour placenta exposed to hypoxia-reoxygenation (Hung et al., 2002). This study reported that there was increased active CASP3 staining in the STB and fetal endothelial cells (similar to us).

Bose *et al* performed villous trophoblast culture (mum blood) to investigate CASP7 expression after magnesium induced placental apoptosis with or without aspirin (Bose et al., 2005). This study showed that cleaved and activated form of CASP7 was absent in the presence of aspirin.

Chen *et al* performed cell based ELISA and electronic microscopy to investigate how anti-phospholipid antibodies affect trophoblast shedding from the placenta (Chen et al., 2009). This study showed decreased CASP3 and 7 in shed trophoblasts. They concluded that anti-phospholipid antibodies are transported into trophoblasts where they affect the regulation of the cell cycle leading to excess death and trophoblast shedding (Chen et al., 2009).

Shomer *et al* performed trophoblast culture experiments to examine the effect of microvesicles on early-stage or term trophoblast and endothelial cells. They used flow cytometry and fluorescence microscopy to investigate CASP3 and 7 activities. This study investigated the effect of microvesicles (membrane vesicles, found in blood circulation of pregnant women) of normal and PE pregnancies (Shomer et al., 2013). The study showed

that in endothelial cells, microvesicles of healthy pregnant women reduced CASP3 and 7 activities. Microvesicles of women with PE increased term trophoblast apoptosis and inhibited early-stage trophoblasts migration compared with cells exposed to microvesicles of healthy pregnant women. They concluded that microvesicle effects on trophoblast cells vary according to the physiological/pathological state of a pregnant woman (Shomer et al., 2013).

Toro *et al* used human placenta choriocarcinoma BeWo, first trimester Swan-71 cell lines and human placental explants to investigate CASP3 expression in response to addition of leptin. (Toro et al., 2014). This study showed that CASP3 was decreased after addition of leptin in does dependent manner. They suggested that leptin has an anti-apoptotic effect in the placenta is in part mediated by the p53 pathway.

Co *et al* (Co et al., 2015) performed Western blotting and IHC to investigate inflammatory and apoptotic markers in term placenta of PE compared to control subjects. This study showed that CASP9 was up-regulated in PE placenta compared to controls.

Chapter IX: Analysis of mRNA expression of TLRs, MyD88 and NF-κB families in human placenta of normal pregnancies and pregnancies complicated with PE

9 TLR expression in the placenta during labour and PE

9.1 Introduction

9.1.1 Toll-like receptors (TLRs) and innate immunity

TLRs are a type of pattern recognition receptors (PRRs) that has been shown to play a crucial role in the innate immune response and inflammation process. TLRs are membrane spanning receptors that bind to and are activated by, several products include exogenous micro-organisms products ligands, such as bacterial, viral, fungi and parasitic and endogenous ligands including heat shock protein 60 and 70, fibronectin and surfactant protein A (Beutler, 2009).

9.1.2 Human immune system

The immune system is a system of biological structures and processes that allow the host defence mechanism against pathogens. The immune system detects a wide range of pathogens and distinguishes them from the human's own tissue. The immune system is divided into two categories which are adaptive (specific) immunity and innate (non-specific) immunity (Grasso, 2003).

9.1.2.1 Adaptive immunity

The adaptive immune response is a subsystem of the immune system that allows the human immune system to attack pathogens. The adaptive immune system is characterized by specificity and memory and the responses work through humoral immunity and cell-mediated immunity. The cells of the acquired immune system are T and B lymphocytes. Major functions of adaptive immunity are recognition of (non-self) antigens by antigen presentation, generation of the immune responses and subsequently developing the

immunological memory for cells to eliminate pathogen in future infection. Adaptive immunity responses provide long lasting protection and counter effect of stronger attacks by the same pathogen on subsequent exposure.

9.1.2.2 Innate immune system

The innate immune provides immediate defence against infection through opsonisation, phagocytosis and activation of the complement and inflammatory cascades. Immune responses are initiated by PRRs, which recognize microbial components that are essential for structures and survival of the microorganism (Beutler, 2009). Different PRRs react with specific ligands and lead to distinct anti-pathogen responses. PRRs can be divided into few categories based on their subcellular localization and function (Table 9-1) (Hargreaves and Medzhitov, 2005).

TLRs form the major class of PRRs in drosophila, and mammals including humans (Kumar et al., 2009). TLRs play a major role in pathogen recognition and initiation of inflammatory and immune responses. Recognition of pathogen-associated molecular patterns (PAMPs) through TLRs, either alone or in hetrodimerization with other TLR or non- TLR receptors, triggers signals responsible for activation of innate immune response (Kumar et al., 2009, Beutler, 2009). Stimulation of TLRs by pathogen products will result in induction of anti-pathogen genes and inflammatory cytokines through activation of signalling pathways. TLRs also trigger dendritic cell maturation that lead to increase in the antigen-presenting capacity. In addition, TLRs help to initiate the adaptive immune system through the activation of professional antigen-presenting.

Table 9-1: Pattern Recognition Receptors (PRRs), location and functions.

Location	PRRs Types	Function
Soluble PRR	c-reactive protein (CRP), acute phase proteins and mannan-binding lectin	Opsonins and pathogen elimination by activation of phagocytic and complement systems (Gazi and Martinez-Pomares, 2009).
Intra-membrane PRR	TLRs (1,2,4,5 and 11) scavenger receptors (Taylor et al., 2005)	Enhance the antigen uptake and medicate the signalling pathways (Geijtenbeek et al., 2004).
Intracellular PRR	TLRs(3,7,8 and 9)	Medicate the signalling pathways by activation of gene expression and production of pro-inflammatory cytokines (Iwasaki and Medzhitov, 2004).

9.1.3 TLRs

TLR family members are expressed differentially in a variety of cells and tissues, including innate immune system cells such as T and B lymphocytes and non-immune cells. TLRs are localized both on the cellular membrane and within the cellular compartment. TLRs are highly conserved family of type I trans-membrane glycoprotein receptors with molecular weights between 90 and 115 kDa and containing 16-28 extracellular leucine-rich repeat domains (Akira, 2004a). In 1996, insect Toll initially identified as a receptor essential for dorso-ventral axis in early development of Drosophila has been shown to be essential for immune response against fungal infection (Lemaitre et al., 1996). These findings led to identification of TLRs in mammals; of twelve (TLR1–12) have been identified to date (Takeda and Akira, 2001, Akira, 2004b, Beutler, 2009). TLR11 function has not been documented in humans (Yarovinsky et al., 2005). TLRs are located either on the cell membrane (TLR1, TLR2, TLR4, TLR5 and TLR6) or intracellularly (TLR3, TLR7, TLR8 and TLR9). TLRs are characterized by a cytoplasmic signalling domain and extracellular domains. The cytoplasmic domain of TLRs is similar to IL-1 receptor family and it is called Toll/IL-1 receptor (TIR) (O'Neill and Dinarello, 2000, Liew et al., 2005). However, despite this similarity the extracellular domains are structurally unrelated. TIR also exists as intracellular proteins that are named adaptors as they bind through this domain to ligand receptors in order to transmit signals inside the cells (Bauer, 2008). Myeloid differentiation factor 88 (MyD 88) is one of these adaptors that binds to TLRs and

IL-1RI. TLRs and IL-1RI share similar pathways that activate the NF-κB, MAPKs, p38 and c-Jun N-terminal kinase (JNK). Activation of these pathways results in set of genes being expression that lead to production of essential molecules for the activation and regulation of immune system including chemokines, cytokines and co-stimulatory factors (Bauer, 2008). However, TLRs bind to different adapters which specifically mediate certain pathways such as MyD 88-adapter, TRIF-related adapter molecule (TRAM) and TIR domain-containing adapter-inducing interferon-B (IFN-B).

9.1.4 TLRs and their ligands

TLRs recognise their own unique ligands known as PAMPs. TLR ligands are varied including bacterial cell wall or DNA components, viral, fungal and parasites. TLRs also binds with autologous molecules include heat shock proteins, intracellular products and genomic DNA (Ohashi et al., 2000a, Kumar et al., 2009).

9.1.4.1 TLR1, TLR2 and TLR6

The TLR1 gene is ubiquitously expressed, and at higher levels than other TLR genes. TLR2 recognizes components from a variety of microorganisms including gram negative and positive bacteria, spirochetes and mycoplasma (Takeda et al., 2003). TLR2 is a membrane protein which recognises of bacterial lipo-peptides and is up-regulated during various disorders. TLR2 ligand recognition involves cooperation with other TLR family members, in particular TLR6 and TLR1 to mediate intracellular signalling (Farhat et al., 2008). TLR2 with TLR1 or TLR6 heterodimerization developed to expand the ligand spectrum to enable the innate immune system to recognize several different structures of lipo-peptides present in various pathogens (Farhat et al., 2008).

TLR6 functionally cooperates with TLR2 to recognize microbial lipo-peptides (Takeuchi et al., 2001). A TLR6 polymorphism may be associated with an increased risk of several diseases for example legionnaire's disease (Misch et al., 2013). Furthermore, TLR1, TLR2, and TLR6 polymorphisms are associated with increased susceptibility to skin structure infections (Stappers et al., 2014).

9.1.4.2 TLR3

TLR3 mediates immune responses toward viral dsRNA. TLR3 play a role in response to herpes viruses (Gregory and Damania, 2009). TLR3 is also activated by influenza A and Kaposi's sarcoma-associated herpes viruses (Guillot et al., 2005). In addition, TLR3 has

unique function to recognition of dsRNA as it expressed in mature dendritic cells (Muzio et al., 2000). TLR3 has unique structural features among TLRs, it lacks the proline residue. The activation of TLR3 by viral ligand and TLR4 by bacteria LPS result in increased trophoblast secretion of chemokines which lead to monocyte and neutrophil chemotaxis.

9.1.4.3 TLR4

TLR4 detects lipopolysaccharide from gram negative bacteria and is important in the activation of innate immune system (Takeda et al., 2003). In addition, TLR4 recognizes some endogenous ligands such as HSPs (Table 9-2). HSP60 is the best studied HSP for activation of the immune system, this activation is mediated by TLR4 (Vabulas et al., 2001). In addition, TLR4 seems to be responsible for the inflammatory responses elicited by HSPs and both TLR2 and TLR4 are required for recognition of HSP70 (Asea et al., 2002).

9.1.4.4 TLR5

TLR5 recognizes bacterial flagellin, which is a principal component of bacteria flagella of gram negative bacteria and contributes to its virulence (Hayashi et al., 2001a, Takeda et al., 2003). TLR5 activation stimulates TNF production through NF-κB pathways (Sharma et al., 2013).

9.1.4.5 TLR7, TLR8 and TLR9

TLR7, TLR8 and TLR9 are highly homologous to each other. TLR7 and TLR8 recognise single stranded RNA (ssRNA) viruses including Influenza and Coxsackie B viruses (Arpaia and Barton, 2011). In addition, TLR7 has been shown to play a significant role in the pathogenesis of autoimmune disorders. Antiviral response by TLR8 is through activation of NF-κB (Arpaia and Barton, 2011). TLR9 is involved in the recognition of unmethylated CpG sequences of bacterial DNA. TLR9 is expressed intra-cellularly, within the endosomal compartments and functions to alert the immune system of viral and bacterial infections by binding to DNA CpG motifs (Arpaia and Barton, 2011). Activation of TLR9 leads to the initiation of pro-inflammatory reactions and secretion of cytokines.

Table 9-2: TLRs ligands redrawn from (Koga and Mor, 2010, Chang, 2010)

TLRs	Ligands		References
	Exogenous	Endogenous	
TLR1	Bacterial tri-acylated lipopeptides with TLR2		(Jin et al., 2007).
TLR2	Gram-positive bacteria peptidoglycan, Lipopeptides, lipoteichoic acids, zymosan	HSP60, HSP70 and HMGB1	(Takeuchi et al., 1999, Gantner et al., 2003, Campos et al., 2001, Asea et al., 2002)
TLR3	Viral double-strand-RNA	Host dsRNA	(Alexopoulou et al., 2001)
TLR4	Lipopolysaccharides LPS, Fibronectin, fibrinogen	HSP22, HSP60,HSP70 HSP96,	(Poltorak et al., 1998, Monick et al., 2003, Asea et al., 2002, Ohashi et al., 2000b)
TLR5	Flagellin		(Hayashi et al., 2001b).
TLR6	Diacyle lipo-peptides with TLR2, Zymosan from fungal cell wall		(Kang et al., 2009)
TLR7	Single strand viral RNA	Self ssRNA	(Heil et al., 2004, Lund et al., 2003)
TLR8	Single strand RNA	Self ssRNA	(Heil et al., 2004)
TLR9	Non-methylated CpGDNA, Herpes-virus, Chromatine-IgG	self DNA	(Lund et al., 2003, Hemmi et al., 2000a)
TLR10	Unknown, Interact with TLR2		

9.1.5 Myeloid differentiation primary response gene (88)

MyD88 was discovered in mice (Lord et al., 1990). MyD88 is a pivotal signalling protein in the innate immune system which participating in TLR and IL-1 signalling pathways during host response to infection (Kawai and Akira, 2011). These pathways regulate the activation of numerous pro-inflammatory genes. MyD88 is composed of an N-terminal death domain (DD) (Pettersen et al., 2004) and a highly conserved C-terminal TIR (Medzhitov et al., 1998a). DD is responsible for downstream signalling through kinases of the interleukin-1 receptor-associated kinases (IRAKs) (Avbelj et al., 2011). The MyD88 TIR domain integrates signals from upstream TLR and IL-1 receptors (Avbelj et al., 2014). TLRs trigger the formation of TIR-domain dimers, which results in downstream signalling and expression of genes involved in the immune system (Avbelj et al., 2014).

9.1.6 Nuclear factor kappa light chain enhancer of activated B cells (NF-κβ)

NF-κB transcription factors can both induce and repress gene expression by binding to discrete κB elements of DNA sequences, in promoters and enhancers (Hayden and Ghosh, 2008). NF-κB also plays an important role in influencing gene expression events that impact on cell survival, differentiation and proliferation (Hayden and Ghosh, 2008). NF-κB is activated through various posttranslational modifications and translocate to the nucleus where it binds to specific DNA sequences and promotes transcription of target genes (Hayden and Ghosh, 2008). NF-κB is a key transcription factor in the TLR-mediated signalling pathway, and leads to in the regulation of pro-inflammatory cytokine and PG production (Allport et al., 2001, Lawrence, 2009, Drexler and Foxwell, 2010). TLRs are activated by PAMPs of gram-negative and gram-positive bacteria, this is followed by a cascade of cytoplasmic molecular events by activation of the inactive form of the NF-κB by phosphorylation of its serine residues and allowing its translocation to a nucleus (Jesic et al., 2014). Activation of the NF-κB pathway is associated with activation of apoptosis in placentas from women who underwent labour (Lee et al., 2010).

9.1.7 TLRs signalling pathways

Several studies have shown that TLRs are activated by a wide range of ligands. However, the first step in the initiation of signalling by TLRs is their homo or hetero-dimerization. This step of pairing provides a special structural form in the cytoplasm where the adapter

molecules bind and transmit signalling inside the cell (Kumar et al., 2009). The differences in the TLRs pathways are due to the presence of many adapters that activate different biochemical pathways. This activation leads to expression of pro-inflammatory cytokines, chemokines and other immune genes. However, the adapters not only control which pathways that TLRs are going to activate but also these adapter play an essential role in regulation of the TLR cascades (Kawai and Akira, 2010). In addition to these adapters there are a several endogenous proteins that interact with TLR signalling at adapter level in order to inhibit any over activation that may damage cells (Bauer, 2008, Kawai and Akira, 2010).

9.1.7.1 MyD88 dependent pathway

MyD 88 is a crucial member for signalling induced by TLRs and IL-1 receptors (Muzio et al., 1998, Bonnert et al., 1997, Medzhitov et al., 1998b, Kawai and Akira, 2010). All TLRs that activate MyD88 are similar to the IL-1 RI pathway in its activation of NF-κB and MAPKs. MyD88 binding to ligand TLRs results in the association of the IRAKs to the receptor signalling complexes. All TLRs, except TLR3, share common signalling pathways that require MyD88 and MyD88 adaptor like Mal. Activation of this pathway leads to TRAK6/IKK complex activation and results in activation of NF-κB and MAPK (Tanimura et al., 2008, O'Neill et al., 2009).

9.1.7.2 The TRIF dependent pathway

TLR3 and TLR4 can signal through a MyD88-independent manner. This signalling occurs through adaptor Toll/IL-1 receptor domain-containing adaptor inducing interferon-β (TRIF). TRIF can activate the NF-κB pathway and LPS-mediated phosophorylation of transcription interferon regulation factors (IRF) resulting in interferon3 production of type I interferons and inducible genes (Hemmi et al., 2000b) that are responsible for antiviral activity and acquired immunity (Tanimura et al., 2008). Sterile-alpha and armadillo motif containing protein (SARM) is negative protein adaptor for TLRs that blocks TRIF-dependent signalling (Jiang et al., 2006).

9.2 Results:

9.2.1 Patient clinical data analysis

Patient data analysis is the same as section (3.4.1.1), results analysis is shown in (Table 3-2) (Table 3-3).

9.2.2 Analysis of mRNA TLRs 1-8 expression in normal pregnancy

Experiment 1: This experiment was designed to test if there was a spatial difference in expression of mRNA of TLRs within individual placentas obtained from women who were not in labour (non-labour group) and from women who were in labour (labour group). Comparison between zones was performed using Friedman test analysis. There was very low expression of TLR9 and TLR10 in the human placenta (ct value more than 35 cycles). There was no significant difference in mRNA expression between the three zones of the non-labour and labour groups for TLR1 ($p=0.95$, $p=0.25$ respectively), TLR2 ($p=0.14$, $p=0.42$ respectively), TLR3 ($p=0.95$, $p=0.57$ respectively) and TLR4 ($p=0.42$ for both) (Figure 9-1)

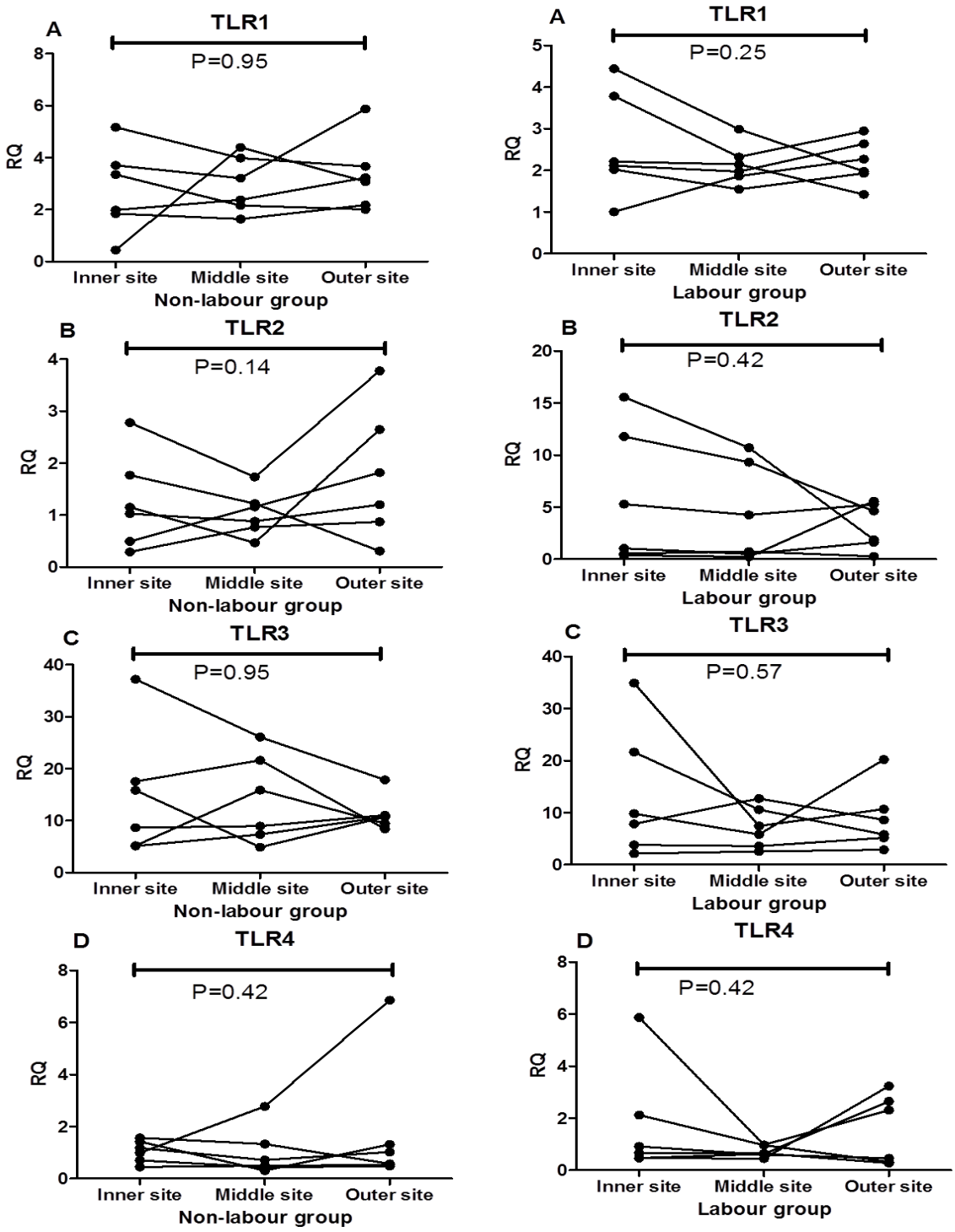


Figure 9-1: RQ values for TLR1-4 mRNA measurements in in inner, middle and outer zones of individual placentas of non-labour (n=6) and labour groups (n=6). Four quadrants were sampled in each zone. Graphs show the median for TLR1-4 mRNA expressions. Comparison between zones was performed using Friedman analysis.

There was also no significant difference in mRNA expression between the three zones of the non-labour and labour groups for TLR5 (p=0.25, p=0.57 respectively), TLR6 (p=0.42, p=0.57 respectively), TLR7 (p=0.95, p=0.7 4respectively) and TLR8 (p=0.74 for both) (Figure 9-2).

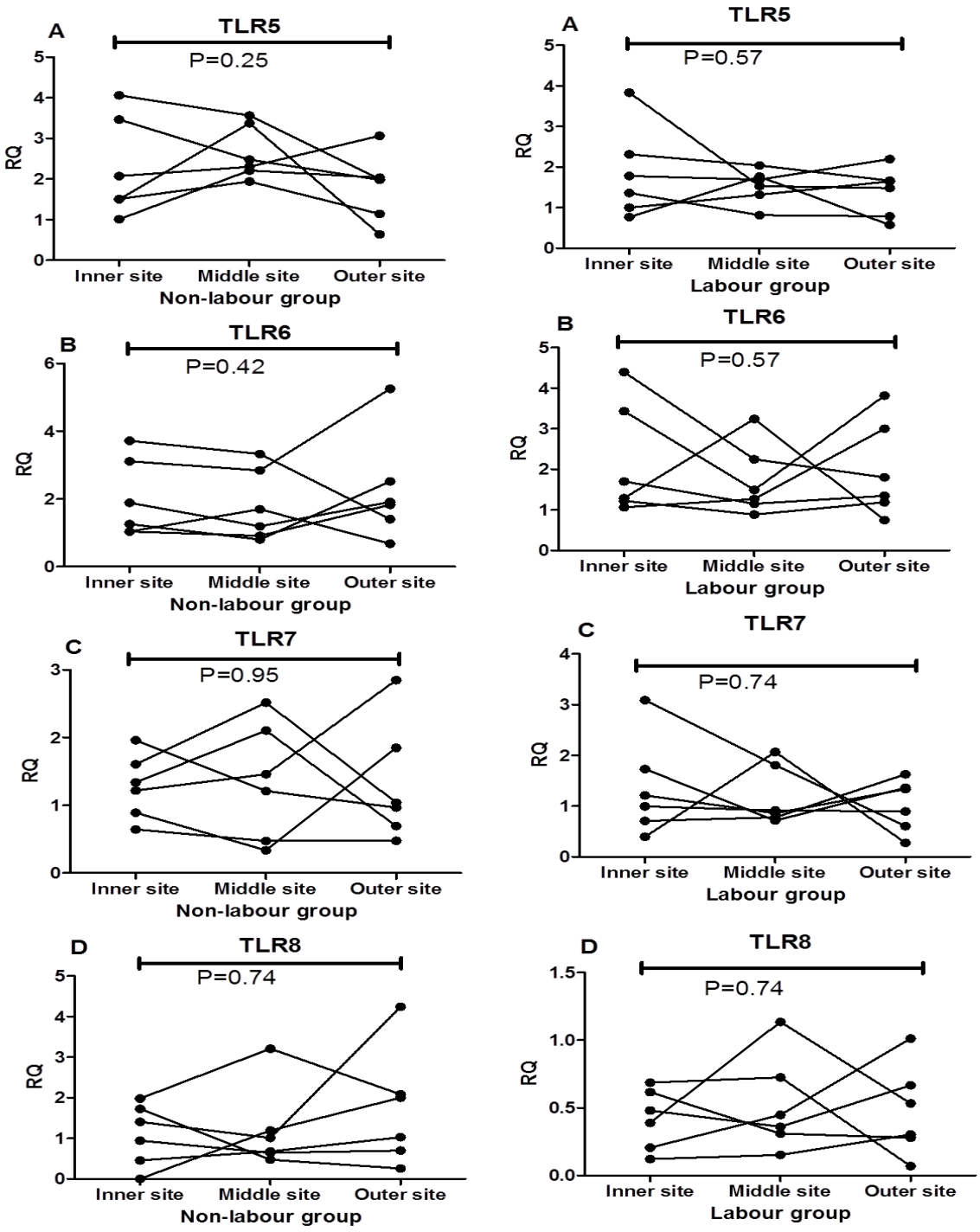


Figure 9-2: RQ values for TLR5-8 mRNA measurements in in inner, middle and outer zones of individual placentas of non-labour (n=6) and labour groups (n=6). Four quadrants were sampled in each zone. Graphs show the median for TLR5-8 mRNA expressions. Comparison between zones was performed using Friedman analysis.

Experiment 2: This experiment was designed to test if there was any difference TLR1-8 mRNA expression between labour and non-labour groups at the inner, middle or outer zones of placentas. Comparison between zones was performed using Mann Whitney test analysis. There was no significant difference in TLR1 and TLR2 mRNA expression in the non-labour group when compared to the labour group at the inner zone (p=0.81, p=0.39 respectively), middle zone (p=0.13, p=0.81 respectively) and the outer zone (p=0.06, p=0.30 respectively) (Figure 9-3).

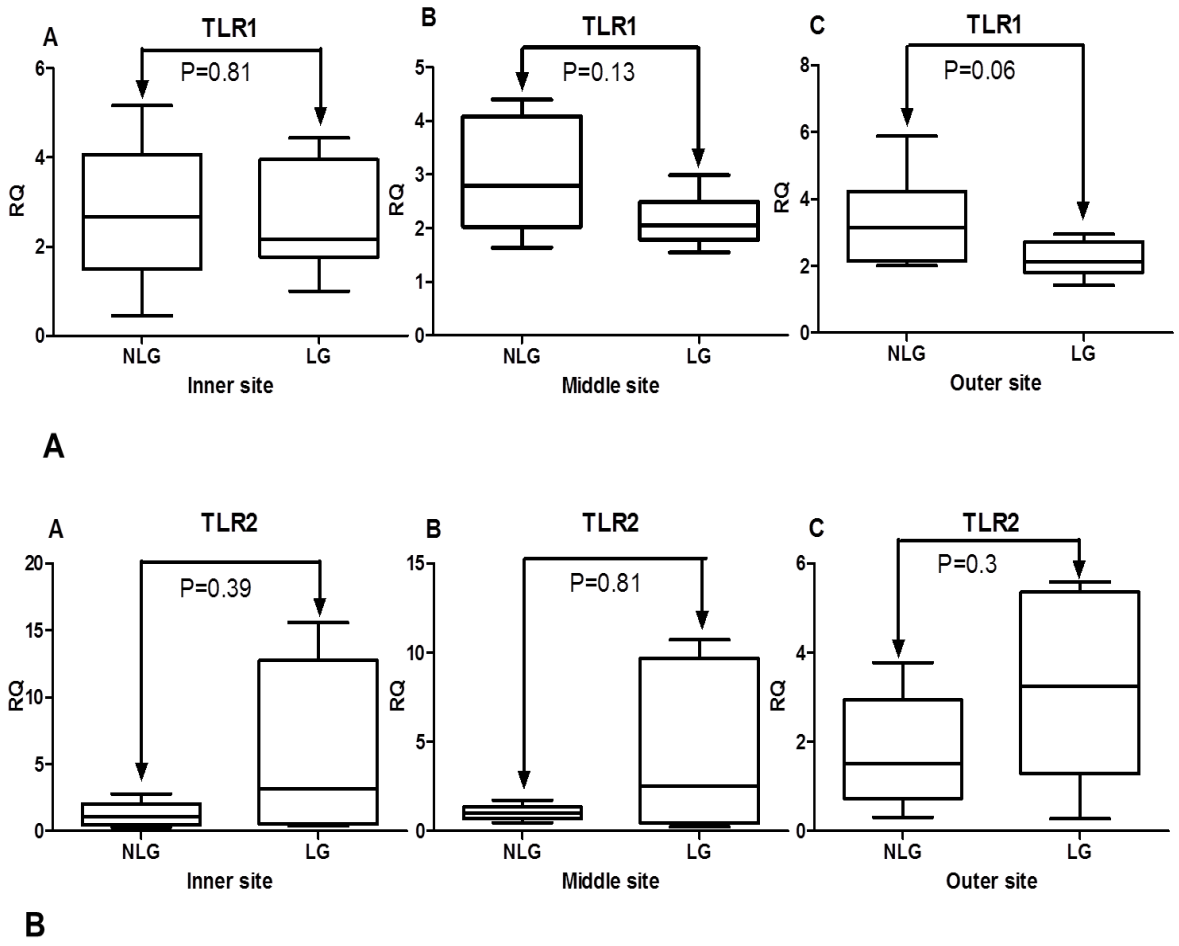


Figure 9-3: RQ values for TLR1 (A) and TLR2 (B) mRNA measurements in non-labour (n=6) compared to the labour group (n=6) at the inner, middle and outer placental zones. Comparison between zones was performed using Mann-Whitney analysis.

There was no significant difference in TLR3 and TLR4 mRNA expression in the non-labour group when compared to the labour group at the inner zone (p=0.6, p=0.9 respectively), middle zone (p=0.1, p=0.9 respectively) and the outer zone (p=0.1, p=0.6 respectively) (Figure 9-4).

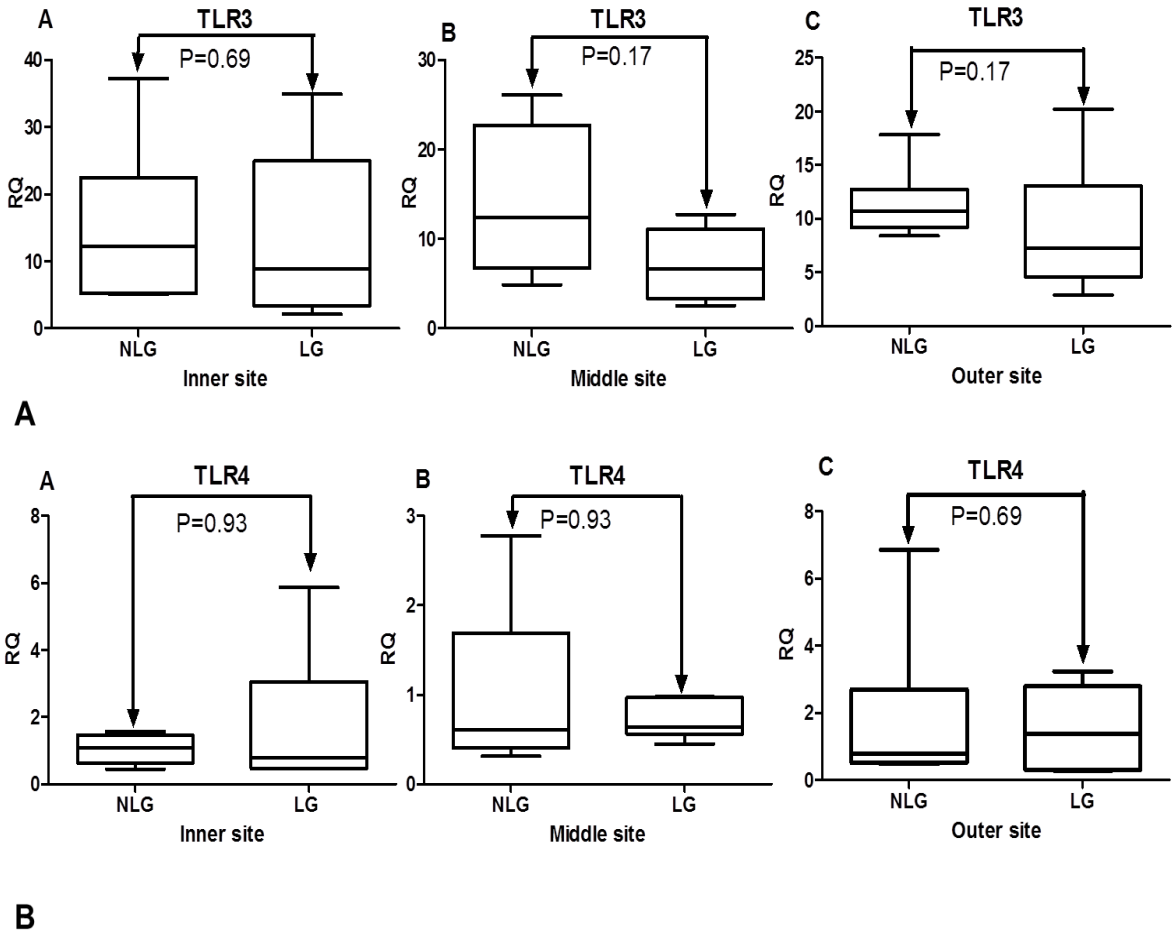


Figure 9-4: RQ values for TLR3 (A) and TLR4 (B) mRNA measurements in non-labour compared to the labour group at the inner, middle and outer placental zones. Comparison between zones was performed using Mann-Whitney analysis.

There was a significant decrease in TLR5 mRNA expression in the labour group when compared to the non-labour group at the middle zone (p=0.004). There was no significant difference in TLR6 mRNA expression in the non-labour group when compared to the labour group at the middle zone (p=1). There was no significant difference in TLR5 and TLR6 mRNA expression in the non-labour group when compared to the labour group at the inner zone (p=0.4, p=0.6 respectively) and the outer zone (p=0.3, p=0.6 respectively) (Figure 9-5).

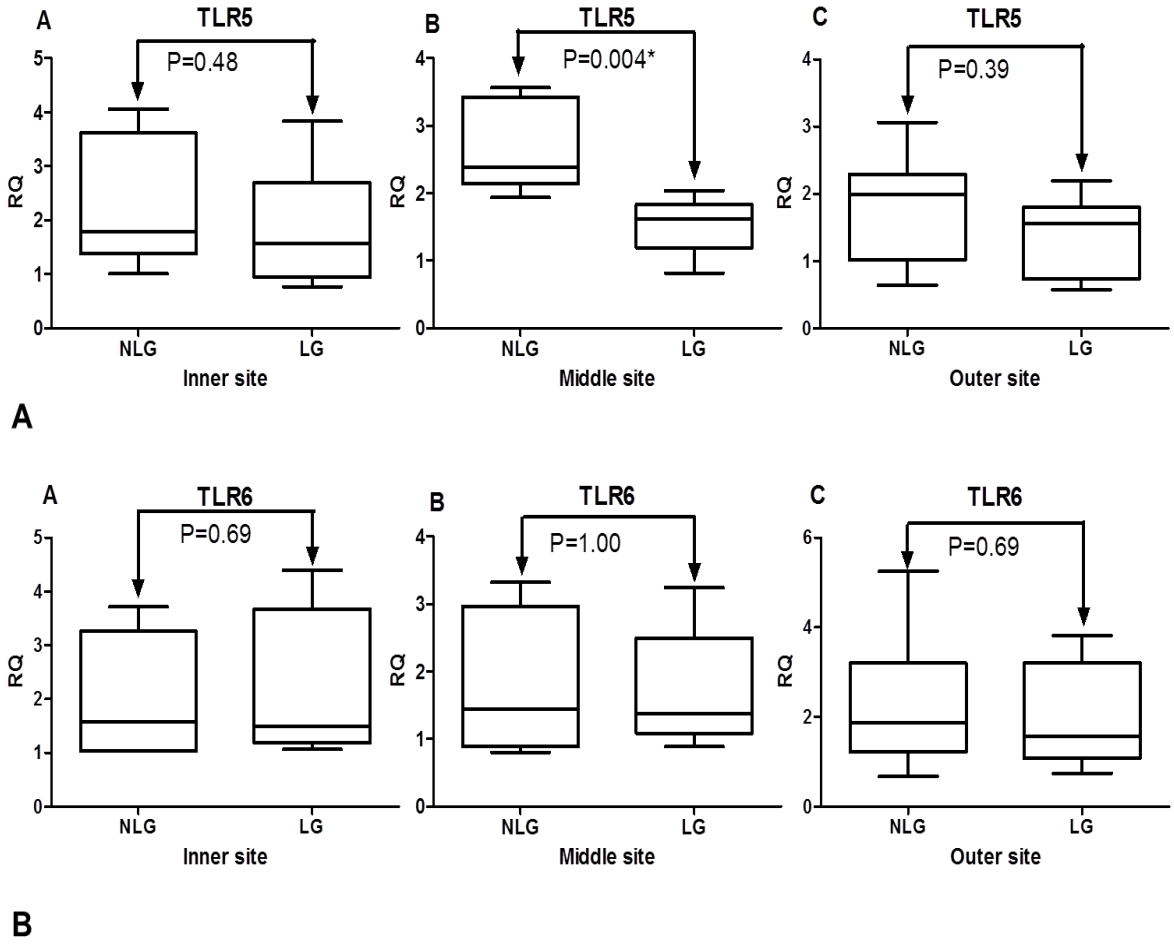


Figure 9-5: RQ values for TLR5 (A) and TLR6 (B) mRNA measurements in non-labour compared to the labour group at the inner, middle and outer placental zones. Comparison between zones was performed using Mann-Whitney analysis.

There was no significant difference in TLR7 and TLR8 mRNA expression in the non-labour group when compared to the labour group at the inner zone (p=0.8, p=0.1 respectively), middle zone (p=0.8, p=0.09 respectively) and the outer zone (p=0.6, p=0.06 respectively) (Figure 9-6).

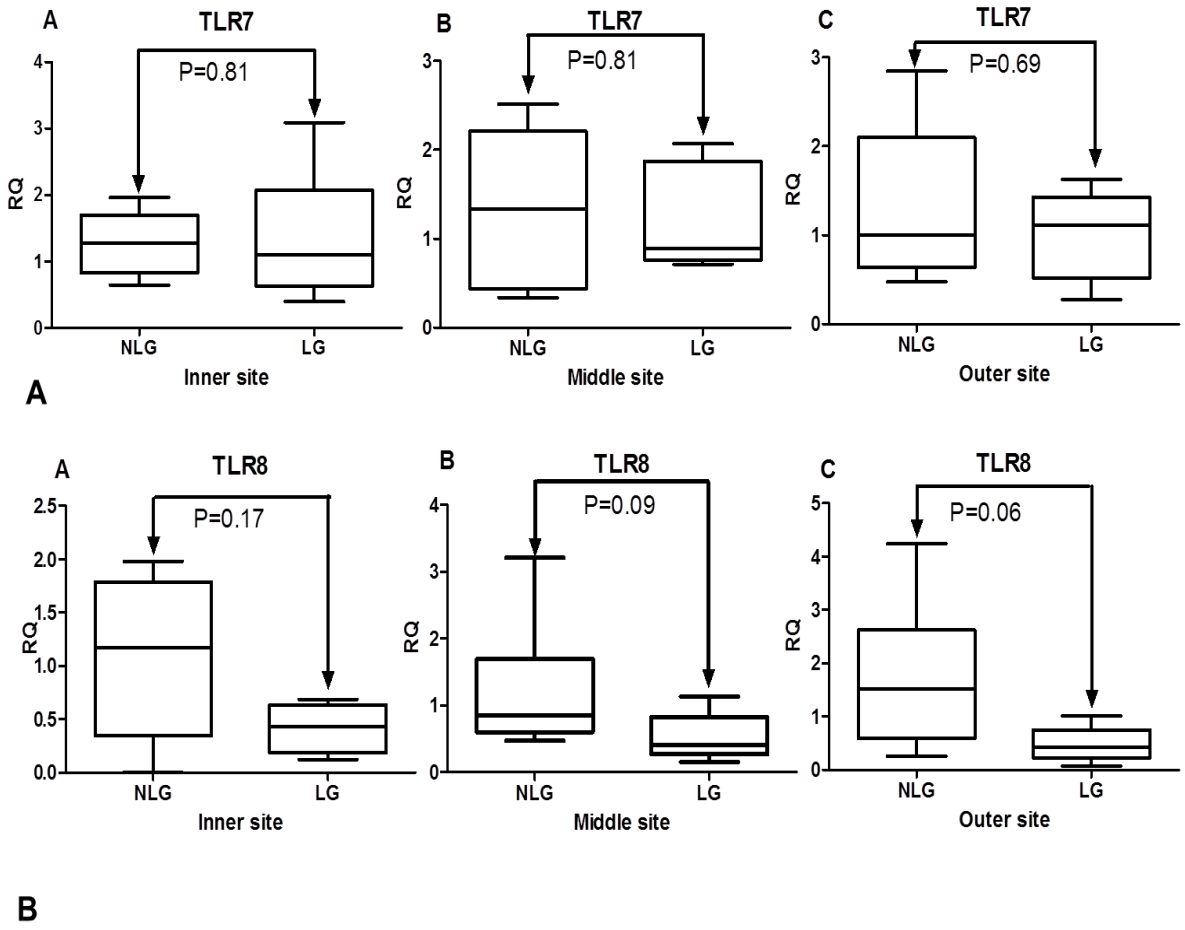


Figure 9-6: RQ values for TLR7 (A) and TLR8 (B) mRNA measurements in non-labour compared to the labour group at the inner, middle and outer placental zones. Comparison between zones was performed using Mann-Whitney analysis

9.2.3 Analysis of mRNA TLRs 1-8 expression in pregnancies complicated with PE

Experiment 1: This experiment was designed to test if there was any difference in TLR 1-8 mRNA expressions between non-labour control and non-labour PE groups at the inner or middle zones of placentas. Comparison between zones was performed using Mann Whitney. First the non-labour groups were compared. There was a significant decrease in TLR1 mRNA expression in the non-labour PE group compared to the non-labour control group at the middle zones (p=0.02). There was no significant difference in TLR1 mRNA expression in the non-labour PE group compared to the non-labour control group at the inner zones (p=1) (Figure 9-7).

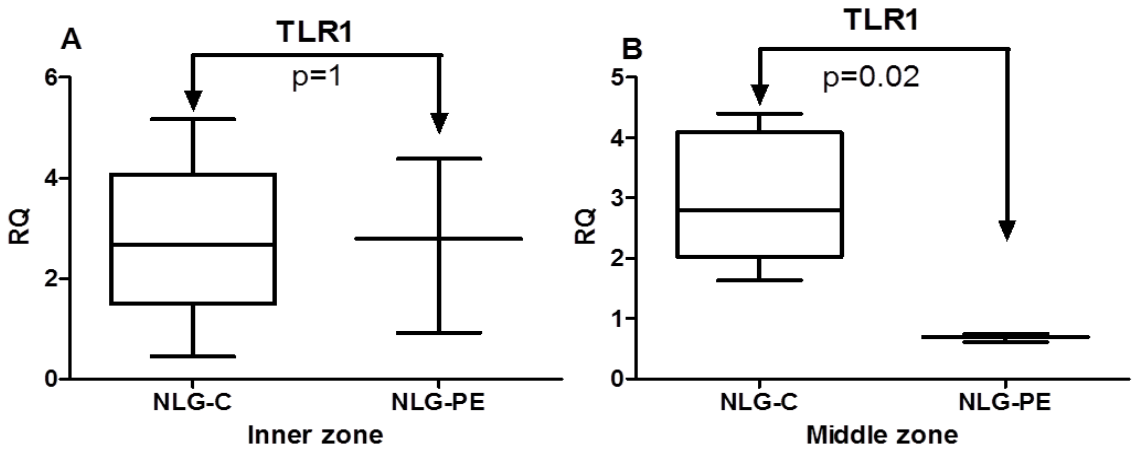


Figure 9-7: RQ values for TLR1 mRNA measurements in the non-labour PE group compared to the non-labour control group at the inner and middle placental zones. Comparison between zones was performed using Mann-Whitney analysis.

There was no significant difference in TLR2 mRNA expression in the non-labour PE group compared to the non-labour control group at the inner and middle zones (p=1, p=0.1 respectively) (Figure 9-8).

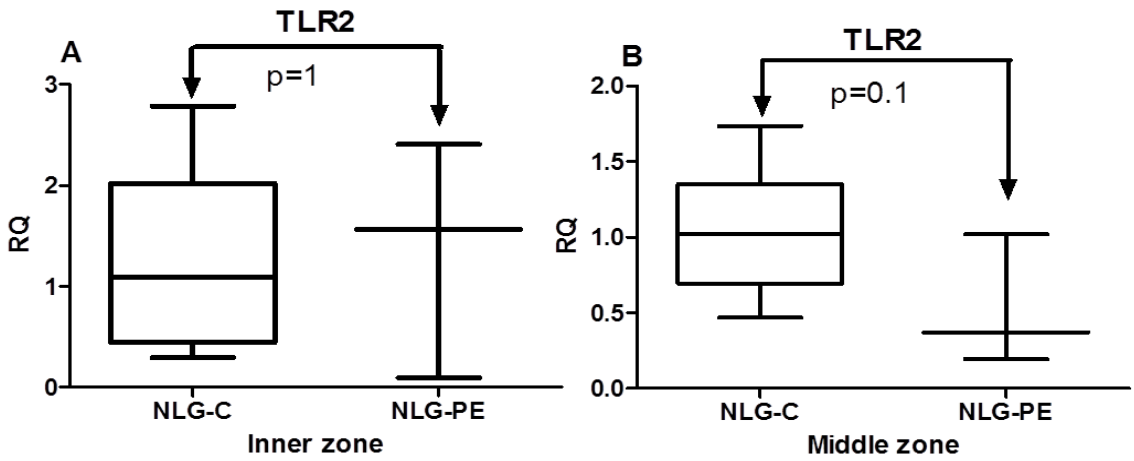


Figure 9-8: RQ values for TLR2 mRNA measurements in the non-labour PE group compared to the non-labour control group at the inner and middle placental zones. Comparison between zones was performed using Mann-Whitney analysis.

There was no significant difference in TLR3 and TLR4 mRNA expression in the non-labour PE group compared to the non-labour control group at the inner (p=0.7, p=0.1 respectively) and middle zones (p=0.7 for both) (Figure 9-9).

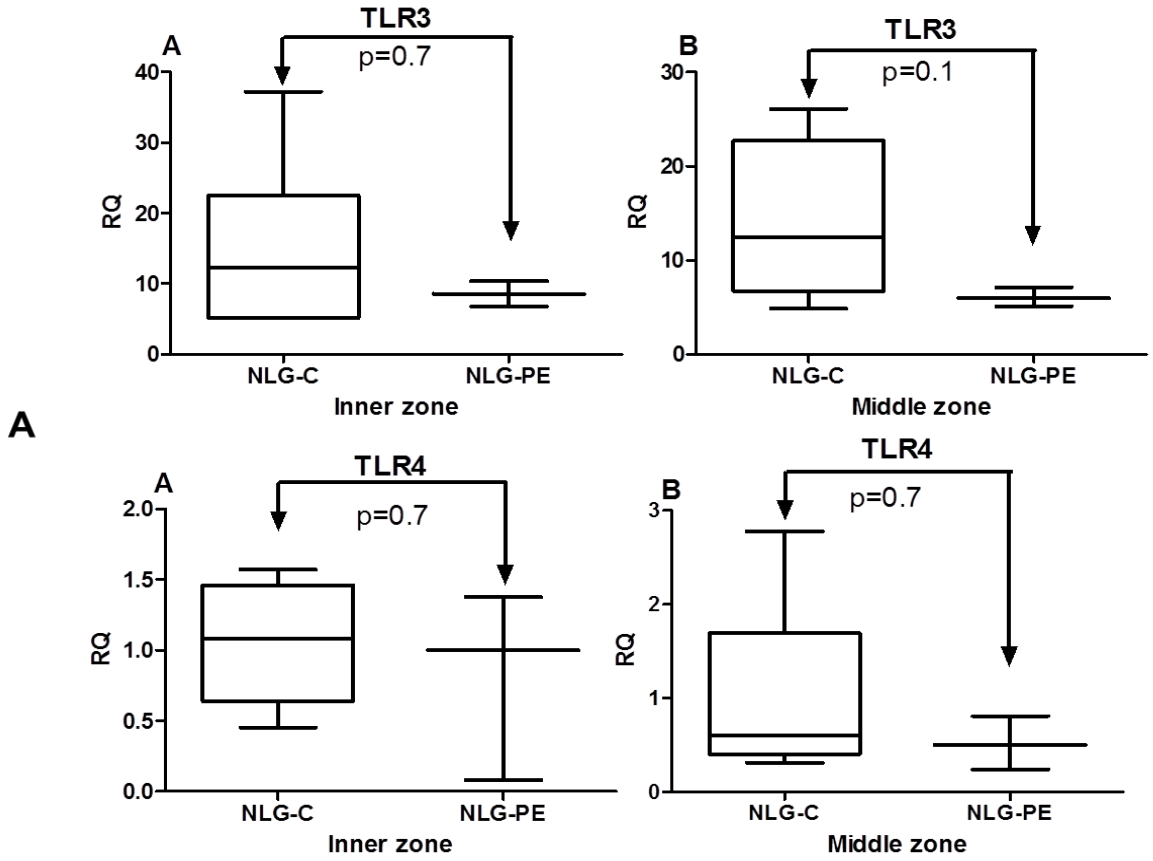


Figure 9-9: RQ values for TLR3 (A) and TLR4 (B) mRNA measurements in the non-labour PE group compared to the non-labour control group at the inner and middle placental zones. Comparison between zones was performed using Mann-Whitney analysis.

There was a significant decrease in TLR5 mRNA expression in the non-labour PE group compared to the non-labour control group at the middle zones (p=0.02). There was no significant difference in TLR5 mRNA expression in the non-labour PE group compared to the non-labour control group at the inner zones (p=0.09) (Figure 9-10).

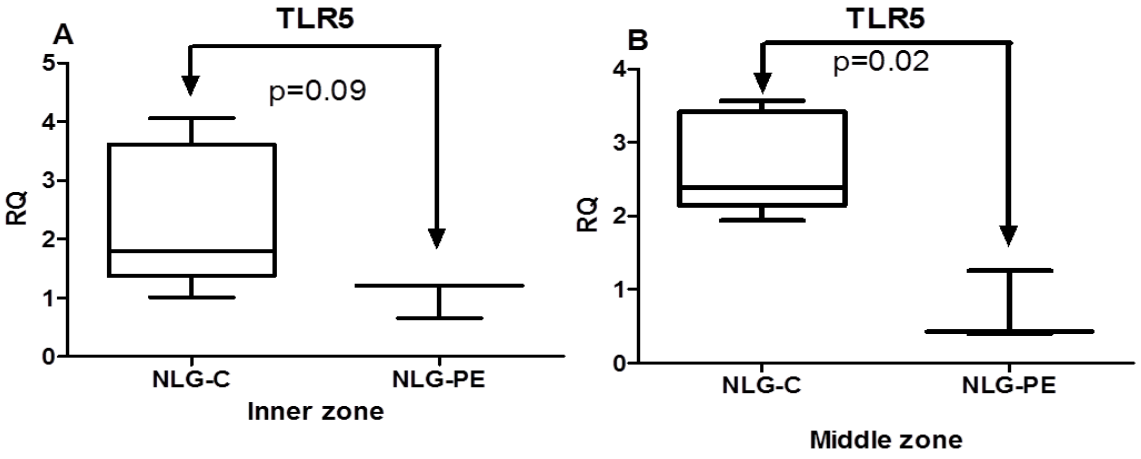


Figure 9-10: RQ values for TLR5 mRNA measurements in the non-labour PE group compared to the non-labour control group at the inner and middle placental zones. Comparison between zones was performed using Mann-Whitney analysis.

There was no significant difference in TLR6 mRNA expression in the non-labour PE group compared to the non-labour control group at the inner and middle zones (p=0.5, p=0.1 respectively) (Figure 9-11).

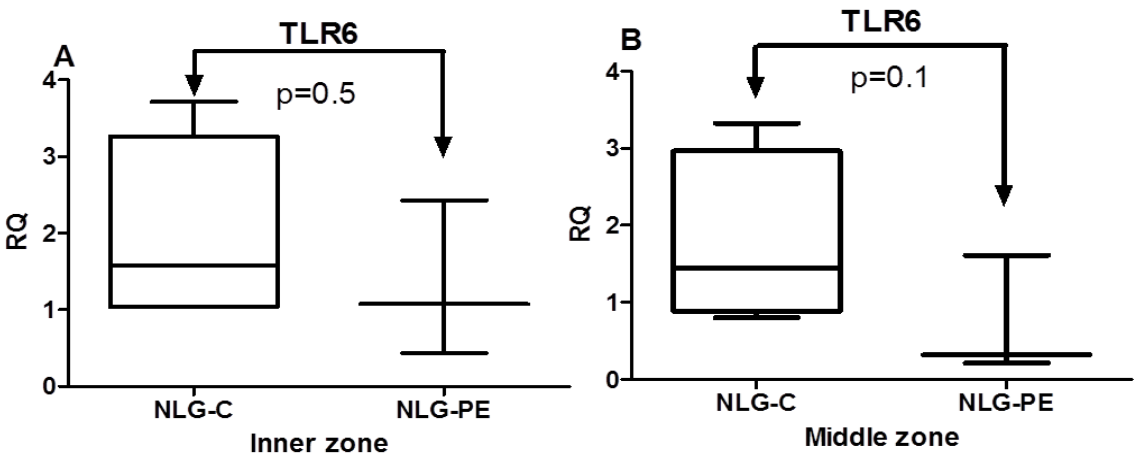


Figure 9-11: RQ values for TLR6 mRNA measurements in the non-labour PE group compared to the non-labour control group at the inner and middle placental zones. Comparison between zones was performed using Mann-Whitney analysis.

There was no significant difference in TLR7 and TLR8 mRNA expression in the non-labour PE group compared to the non-labour control group at the inner (p=0.5, p=0.7 respectively) and middle zones (p=0.09 for both) (Figure 9-12).

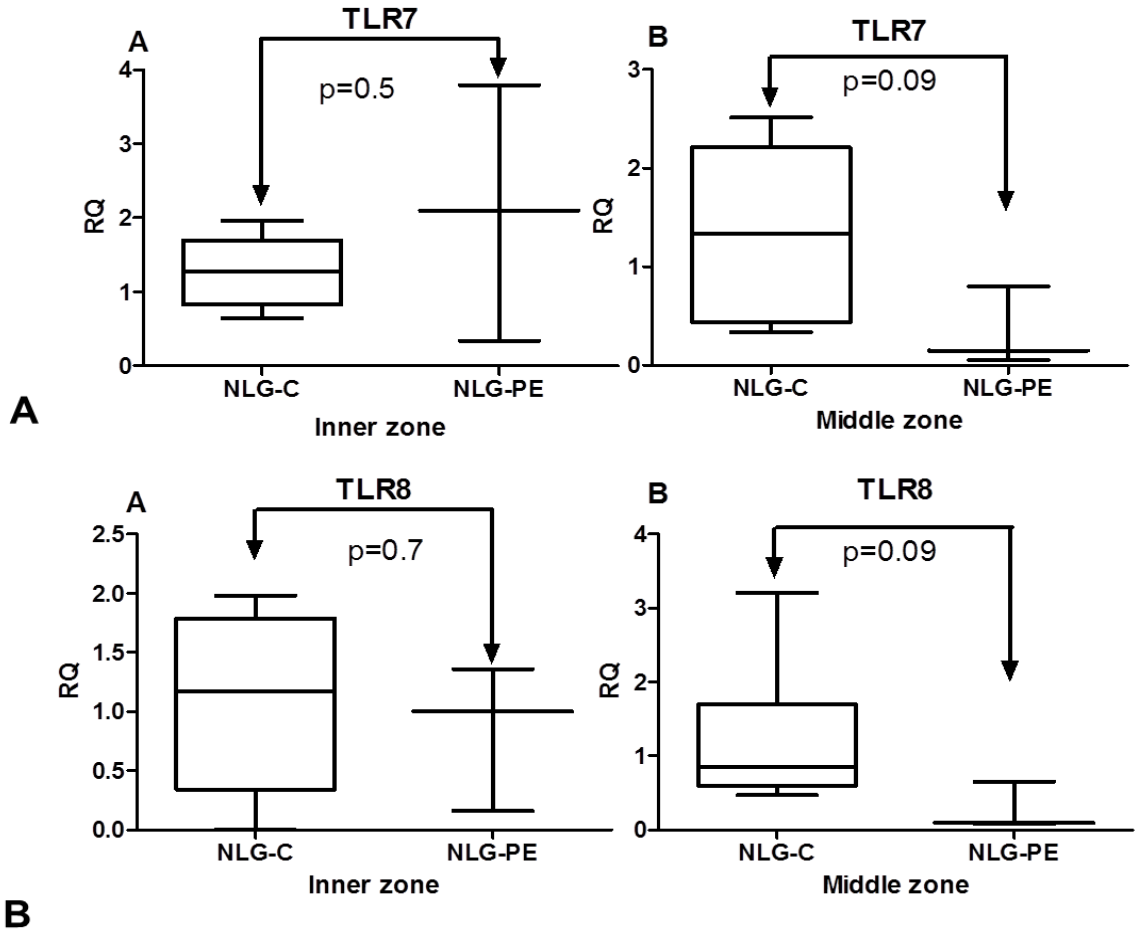


Figure 9-12: RQ values for TLR7 (A) and TLR8 (B) mRNA measurements in the non-labour PE group compared to the non-labour control group at the inner and middle placental zones. Comparison between zones was performed using Mann-Whitney analysis.

Experiment 2: This experiment was designed to test if there was any difference in TLRs 1-8 mRNA expressions between labour control and labour PE groups at the inner or middle zones of placentas. Comparison between zones was performed using the Mann Whitney analysis test. There was a significant decrease in mRNA expression of TLR1 in the labour PE group compared to the labour control group at the inner and middle zones (p=0.04, p=0.002 respectively) (Figure 9-13).

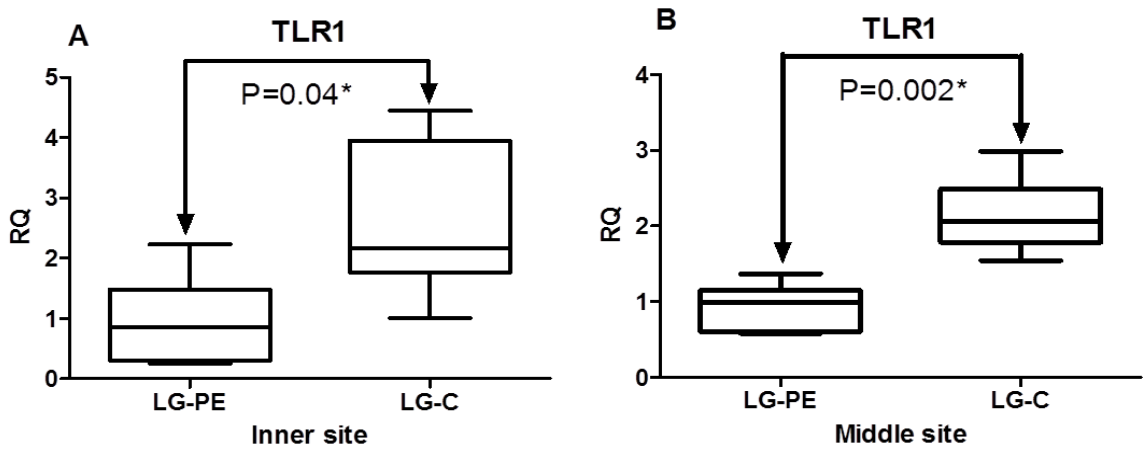


Figure 9-13: RQ values for TLR1 mRNA measurements in the labour PE group compared to the labour control group at the inner and middle placental zones. Comparison between zones was performed using Mann-Whitney analysis.

There was no significant difference in TLR2 mRNA expression in the labour PE group compared to the labour control group at the inner and middle zones (p=0.09, p=0.4 respectively) (Figure 9-14).

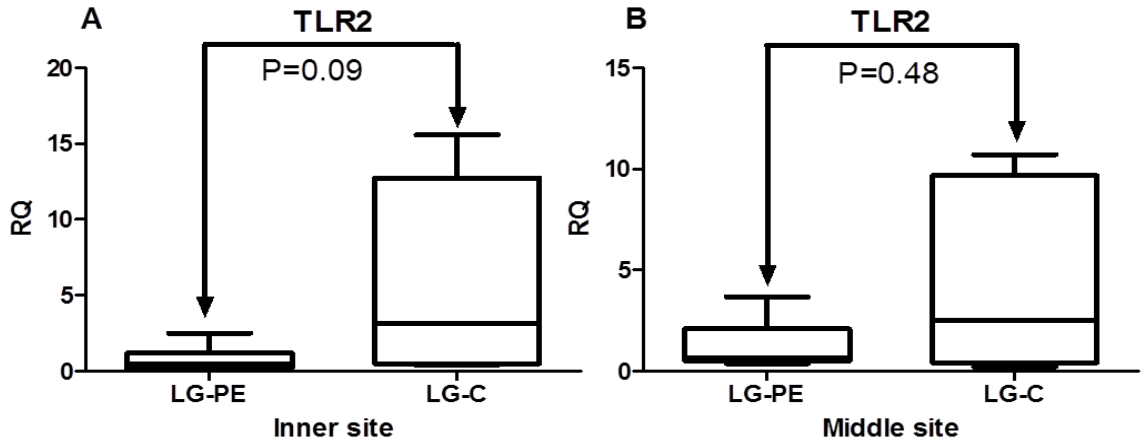


Figure 9-14: RQ values for TLR2 mRNA measurements in the labour PE group compared to the labour control group at the inner and middle placental zones. Comparison between zones was performed using Mann-Whitney analysis

There was no significant difference in TLR3 and TLR4 mRNA expression in the labour PE group compared to the labour control groups at the inner zone (p=0.6, p=0.4 respectively) and the middle zones (p=0.3, p=0.06 respectively) (Figure 9-15).

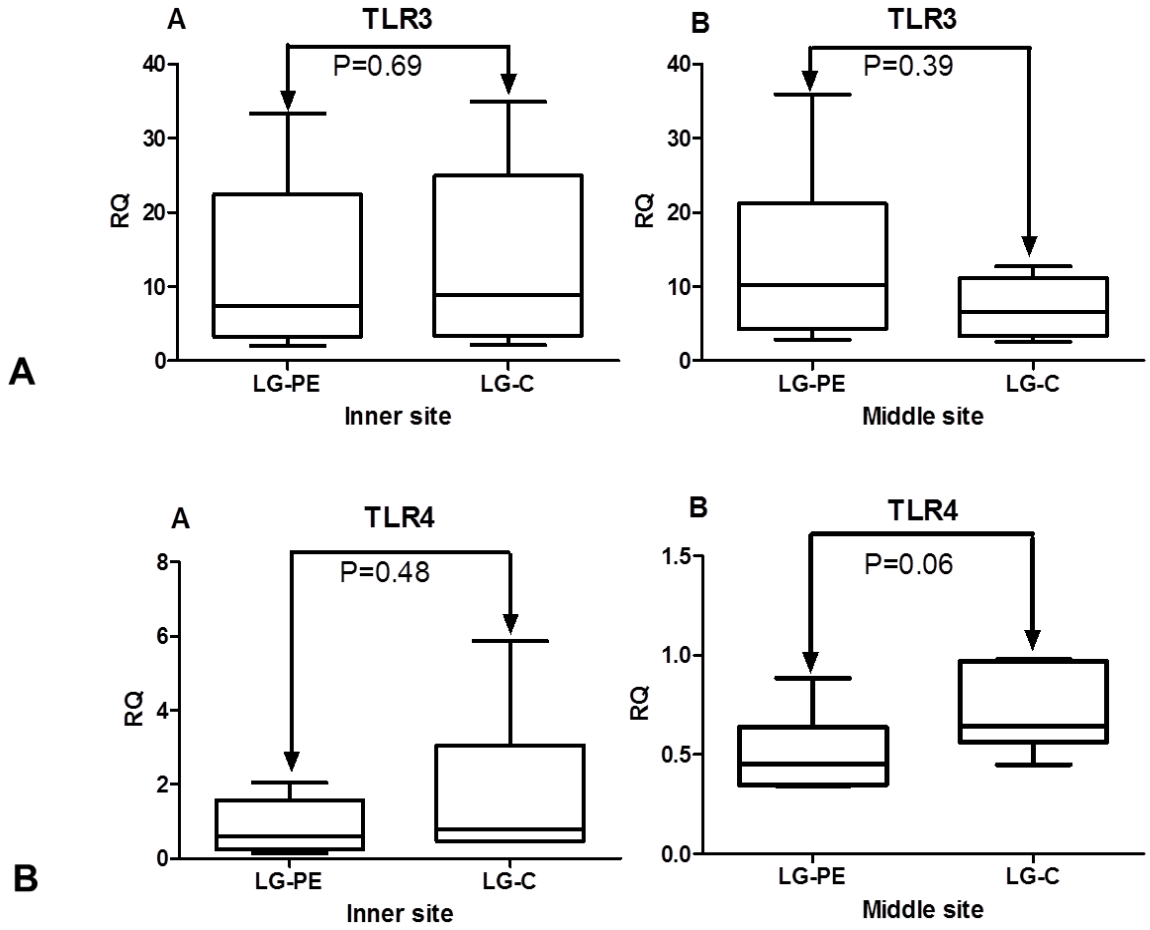


Figure 9-15: RQ values for TLR3 (A) and TLR4 (B) mRNA measurements in the labour PE group compared to the labour control group at the inner and middle placental zones. Comparison between zones was performed using Mann-Whitney analysis.

There was no significant difference in TLR5 and TLR6 mRNA expression in the labour PE group compared to the labour control groups at the inner zone (p=0.9, p=0.1 respectively) and the middle zones (p=0.5, p=0.1 respectively) (Figure 9-16).

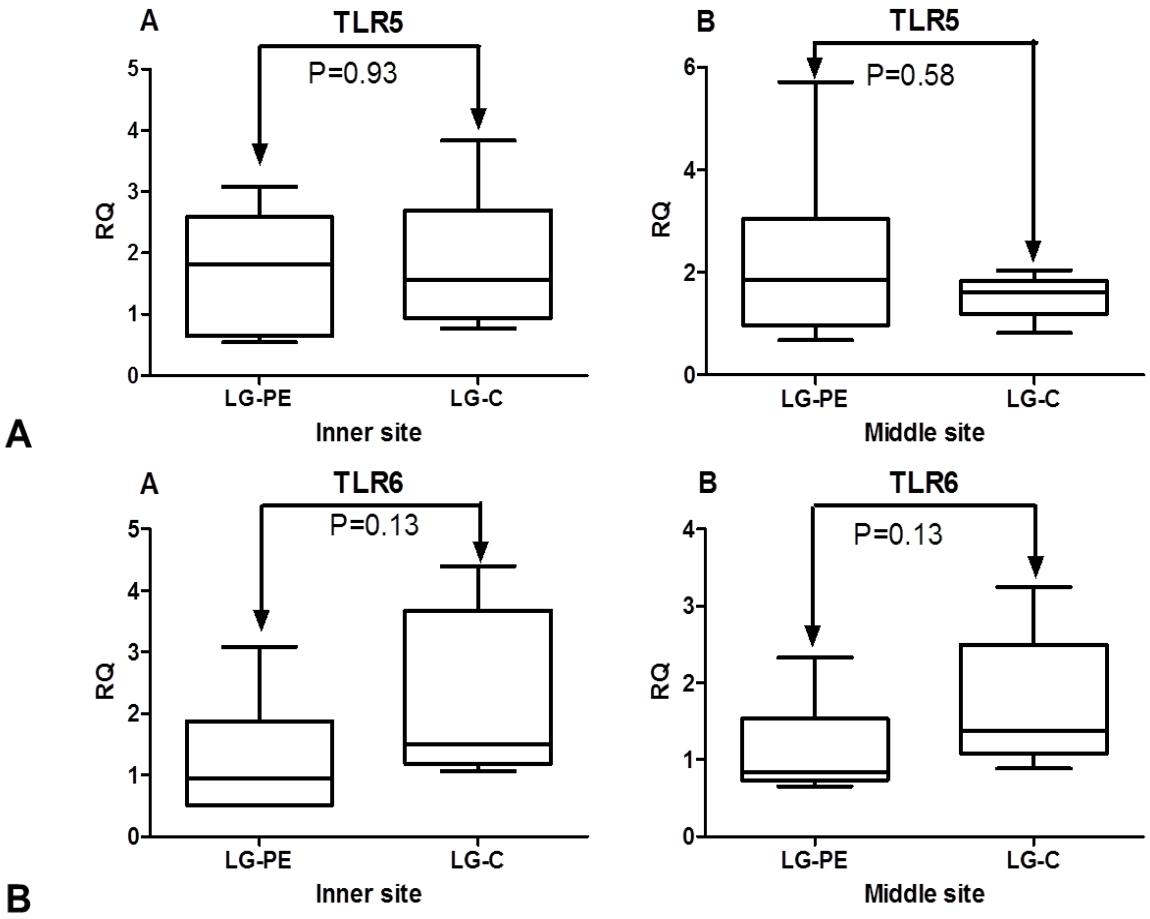


Figure 9-16: RQ values for TLR5 (A) and TLR6 (B) mRNA measurements in the labour PE group compared to the labour control group at the inner and middle placental zones. Comparison between zones was performed using Mann-Whitney analysis

There was no significant difference in TLR7 and TLR8 mRNA expression in the labour PE group compared to the labour control groups at the inner zone (p=0.09, p=0.2 respectively) and the middle zones (p=0.5, p=0.3 respectively) (Figure 9-17).

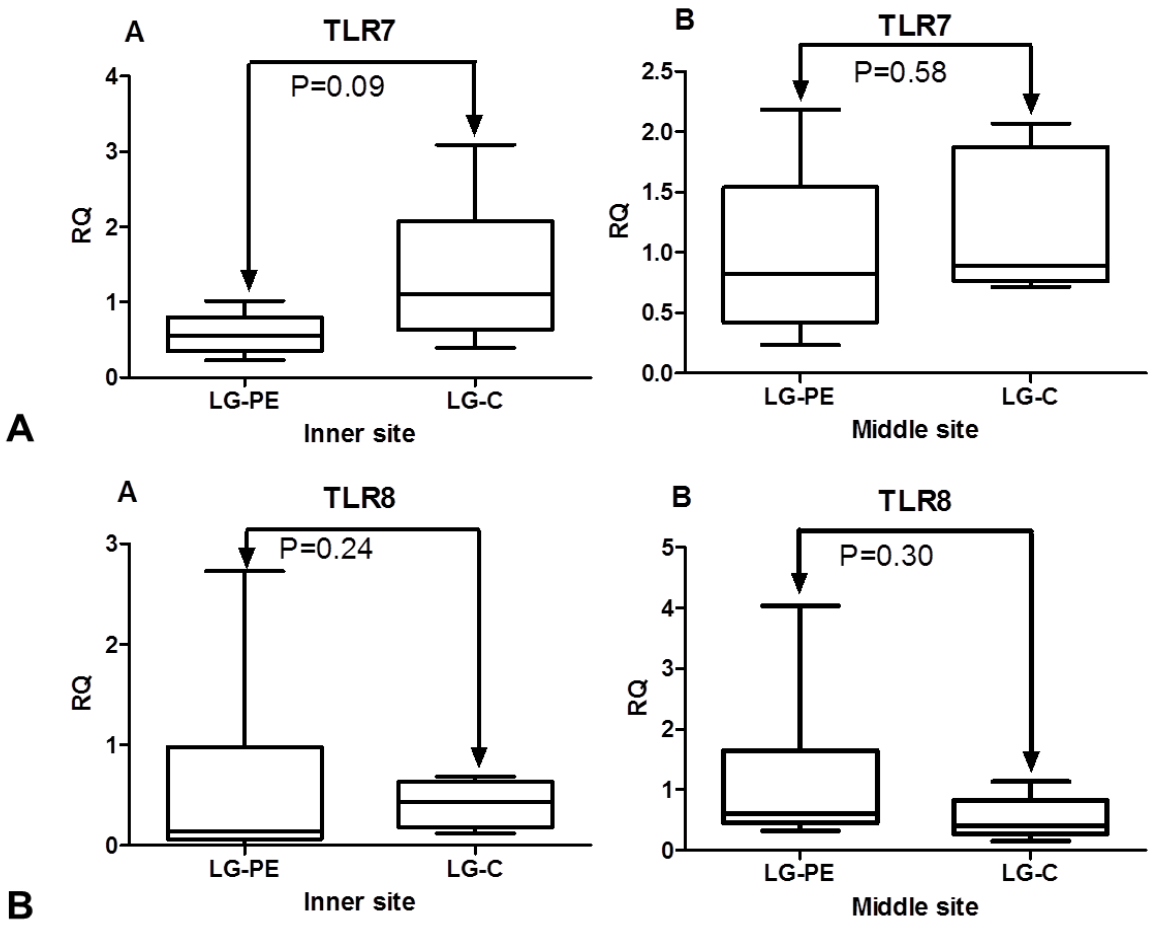


Figure 9-17: RQ values for TLR7 (A) and TLR8 (B) mRNA measurements in the labour PE group compared to the labour control group at the inner and middle placental zones. Comparison between zones was performed using Mann-Whitney analysis.

Experiment 5: This experiment was designed to test if there was any difference in TLR 1-8 mRNA expressions between normotensive pregnancies and pregnancies complicated with PE and included non-labour and labour groups combined for both at the inner and middle zones of placentas. Comparison between zones was performed using Mann Whitney test analysis. There was a significant decrease in TLR1 mRNA expression in the pregnancies complicated with PE group compared to the normotensive pregnancies group in the inner and middle placental zones (p=0.04, p=0.0002 respectively) (Figure 9-18).

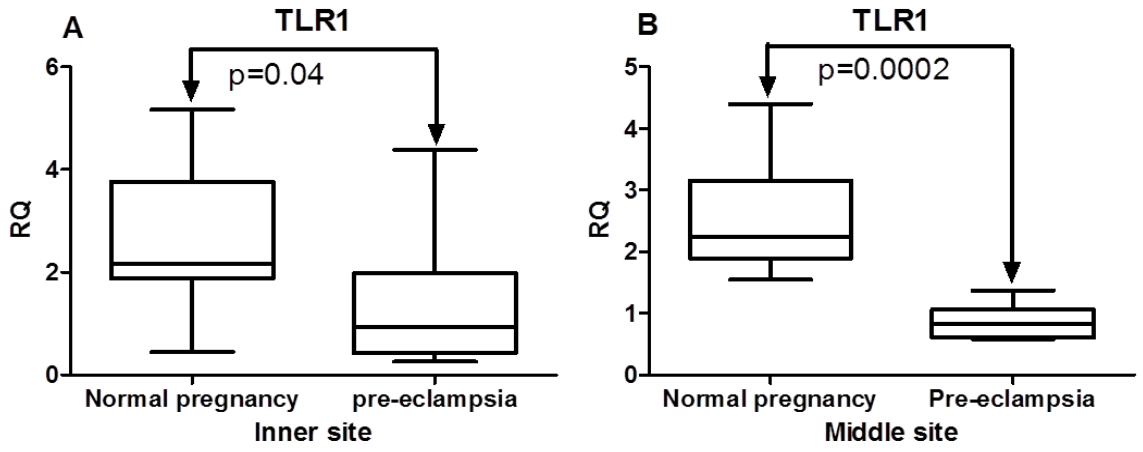


Figure 9-18: RQ values for TLR1 mRNA expression in the PE pregnancy group compared to the normotensive pregnancy group at the inner and middle placental zones. Comparison between zones was performed using Mann-Whitney analysis.

There was no significant difference in TLR2 mRNA expression in the pregnancies complicated with PE group compared to the normotensive pregnancies group in the inner and middle placental zones (p=0.06, p=0.2 respectively) (Figure 9-19).

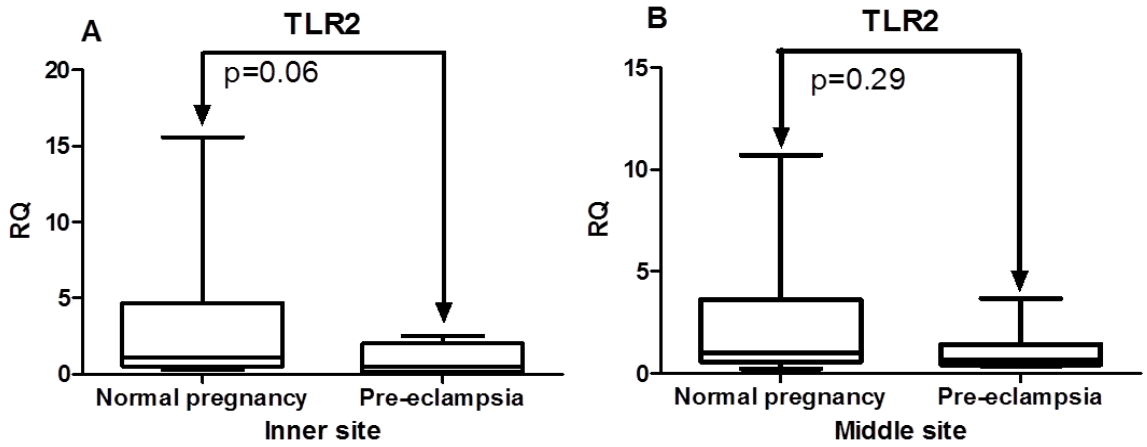


Figure 9-19: RQ values for TLR2 mRNA expression in the PE pregnancy group compared to the normotensive pregnancy group at the inner and middle placental zones. Comparison between zones was performed using Mann-Whitney analysis.

There was no significant difference in TLR3 and TLR4 mRNA expression in the pregnancies complicated with PE group compared to the normotensive pregnancies group in the inner zone (p=0.6, p=0.2 respectively) and middle placental zones (p=0.9, p=0.2 respectively) (Figure 9-20).

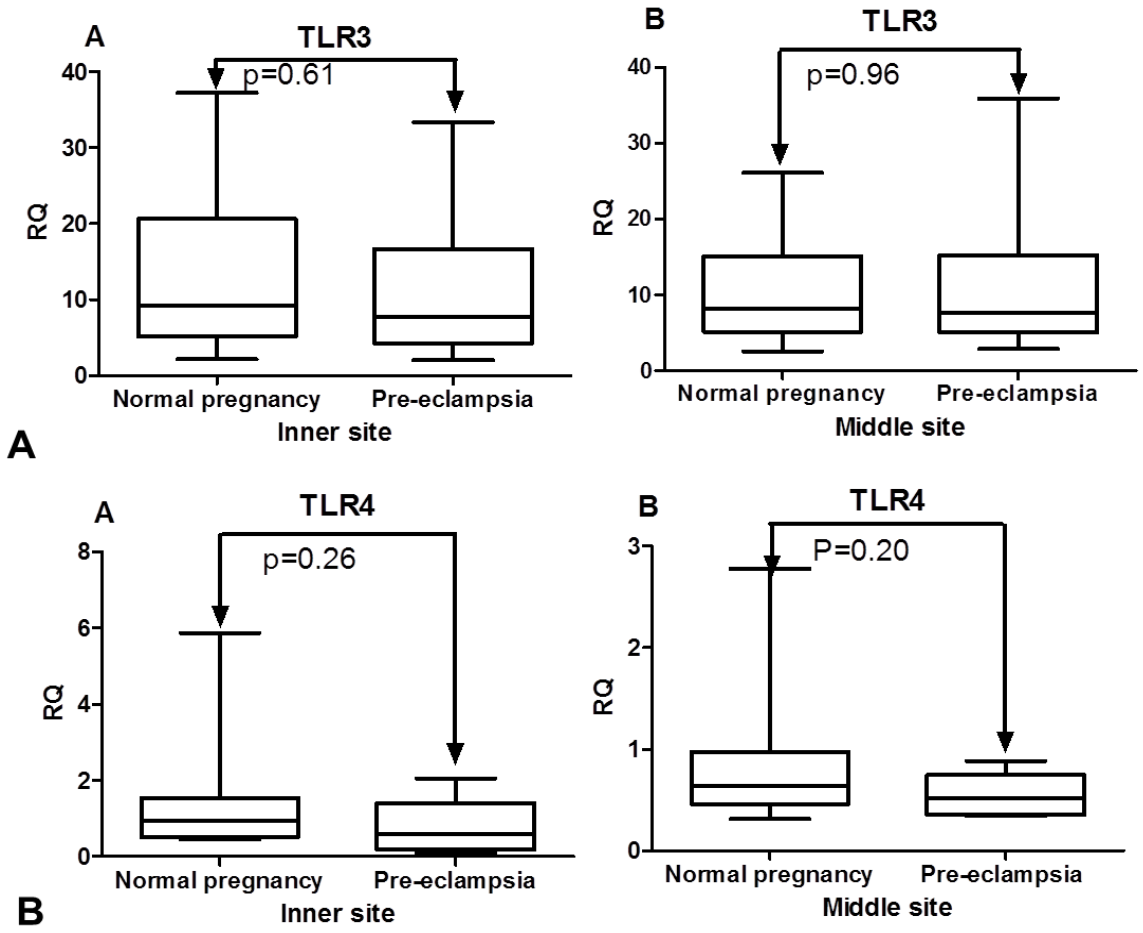


Figure 9-20: RQ values for TLR3 (A) and TLR4 (B) mRNA expression in the PE pregnancy group compared to the normotensive pregnancy group at the inner and middle placental zones. Comparison between zones was performed using Mann-Whitney analysis.

There was no significant difference in TLR5 and TLR6 mRNA expression in the pregnancies complicated with PE group compared to the normotensive pregnancies group in the inner zone (p=0.3, p=0.08 respectively) and middle placental zones (p=0.2, p=0.05 respectively) (Figure 9-21).

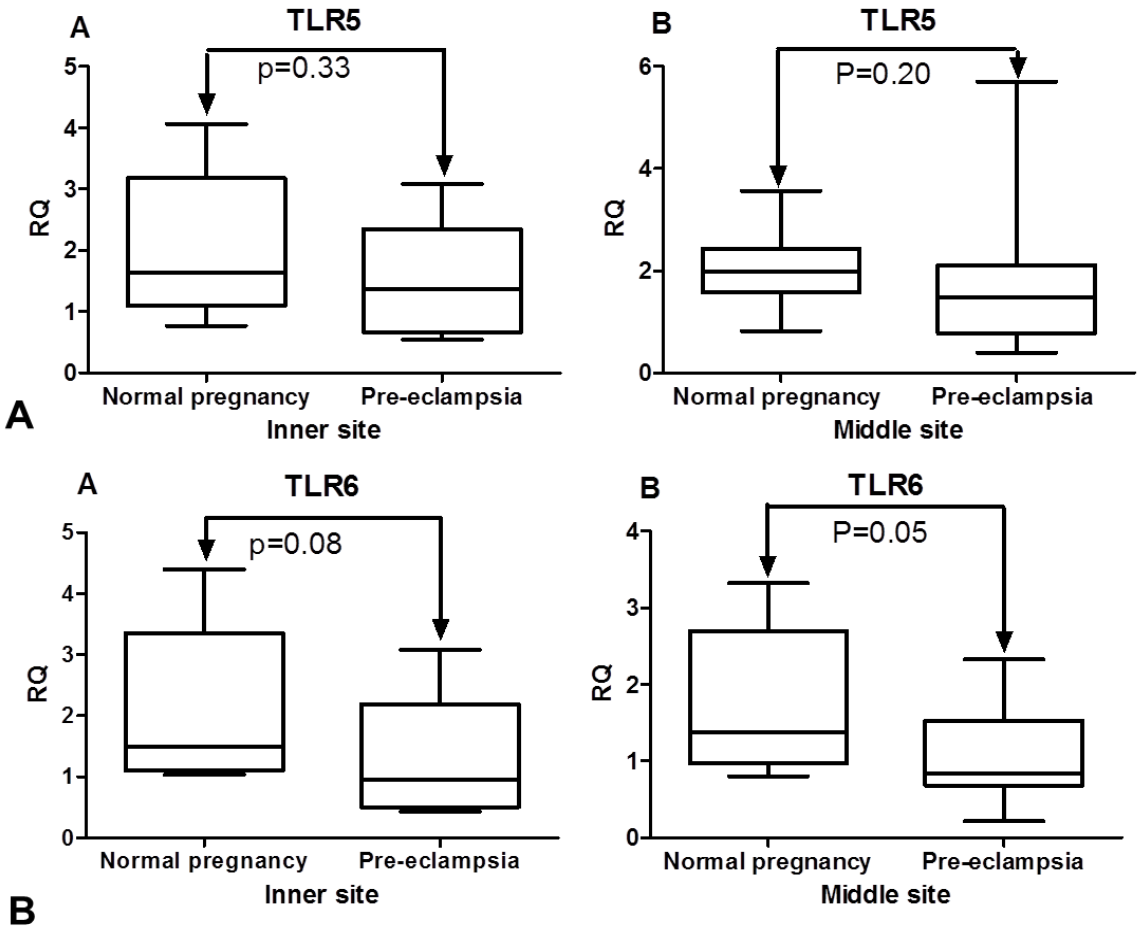


Figure 9-21: RQ values for TLR5 (A) and TLR6 (B) mRNA expression in the PE pregnancy group compared to the normotensive pregnancy group at the inner and middle placental zones. Comparison between zones was performed using Mann-Whitney analysis.

There was no significant difference in TLR7 and TLR8 mRNA expression in the pregnancies complicated with PE group compared to the normotensive pregnancies group in the inner zone (p=0.05, p=0.2 respectively) and middle placental zones (p=0.2, p=0.8 respectively) (Figure 9-22).

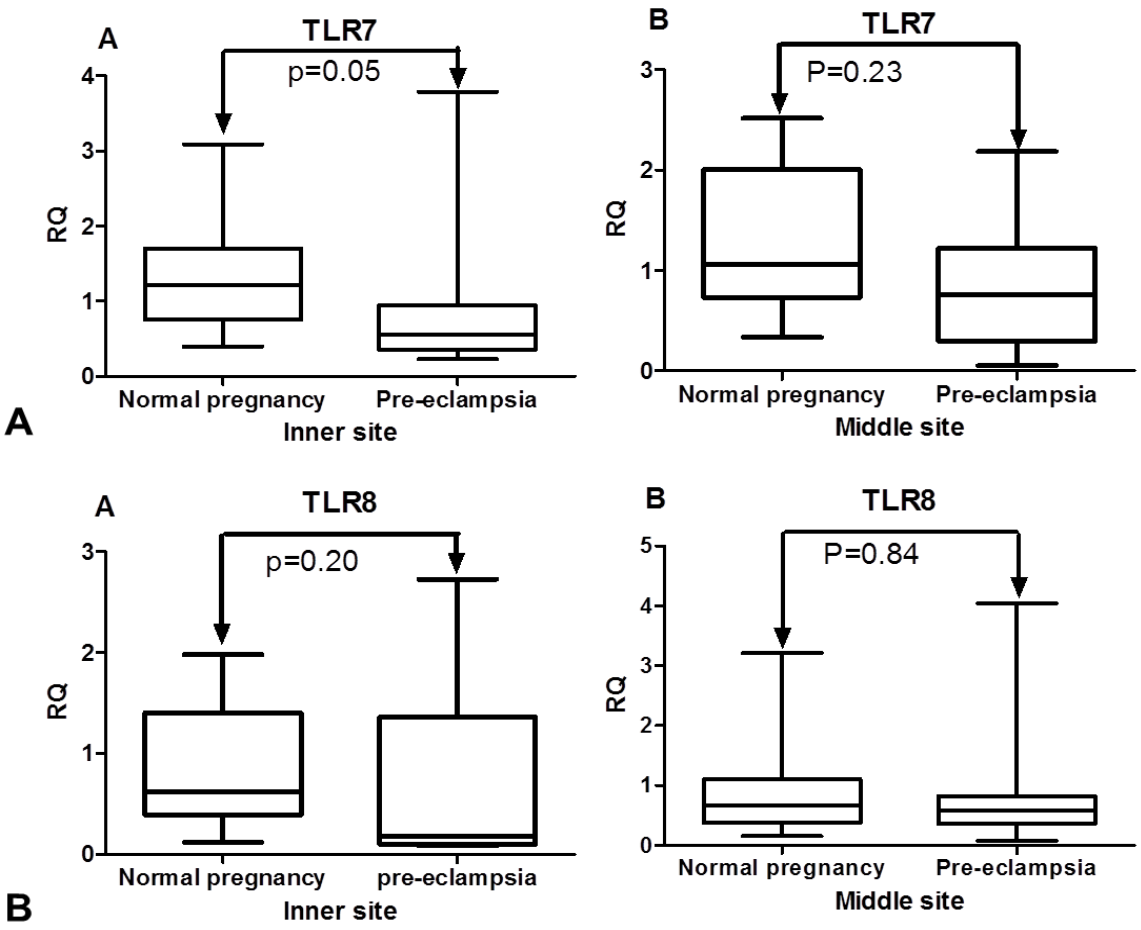


Figure 9-22: RQ values for TLR7 (A) and TLR8 (B) mRNA expression in the PE pregnancy group compared to the normotensive pregnancy group at the inner and middle placental zones. Comparison between zones was performed using Mann-Whitney analysis.

9.2.4 Analysis of mRNA of MYD88 expression in normal pregnancy

Experiment 1: This experiment was designed to test if there was a spatial difference in expression of MYD88 mRNA within individual placentas obtained from women who were not in labour (non-labour group) and from women who were in labour (labour group). Comparison between zones was performed using Friedman test analysis. There was no significant difference in MYD88 mRNA expression between the three zones of the non-labour and labour groups (p=0.5, p=0.7 respectively) (Figure 9-23).

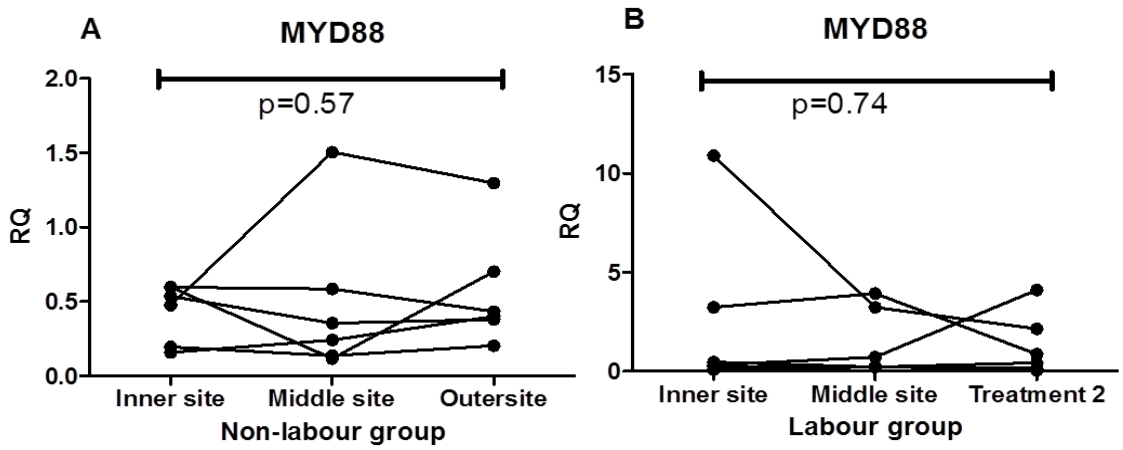


Figure 9-23: RQ values for MYD88 mRNA measurements in in inner, middle and outer zones of individual placentas (non-labour and labour groups). Four quadrants were sampled in each zone. Graphs show the median for MYD88 mRNA expressions. Comparison between zones was performed using Friedman analysis.

Experiment 2: This experiment was designed to test if there was any difference MYD88 mRNA expression between labour and non-labour groups at the inner, middle or outer zones of placentas. Comparison between zones was performed using Mann Whitney test analysis. There was no significant difference in MYD88 mRNA expression in the non-labour group when compared to the labour group at the inner, middle and outer zones (p=1, p=0.3, p=0.8 respectively) (Figure 9-24).

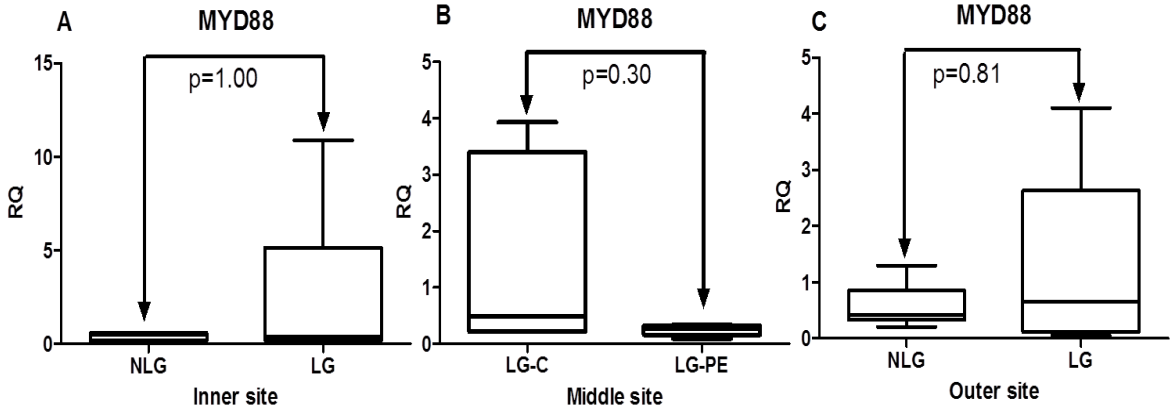


Figure 9-24: RQ values for MYD88 mRNA measurements in non-labour compared to the labour group at the inner, middle and outer placental zones. Comparison between zones was performed using Mann-Whitney analysis.

9.2.5 Analysis of mRNA MyD88 expression in pregnancies complicated with PE

Experiment 1: This experiment was designed to test if there was any difference in MYD88 mRNA expressions between non-labour control and non-labour PE groups at the inner or middle zones of placentas. Comparison between zones was performed using Mann Whitney. There was a significant decreased in the MYD88 mRNA expression in the non-labour PE group compared to the non-labour control group at the inner zone (p=0.02). There was no significant difference in the MYD88 mRNA expression in the non-labour control group compared to the non-labour PE group at the middle zone (p=0.9) (Figure 9-25).

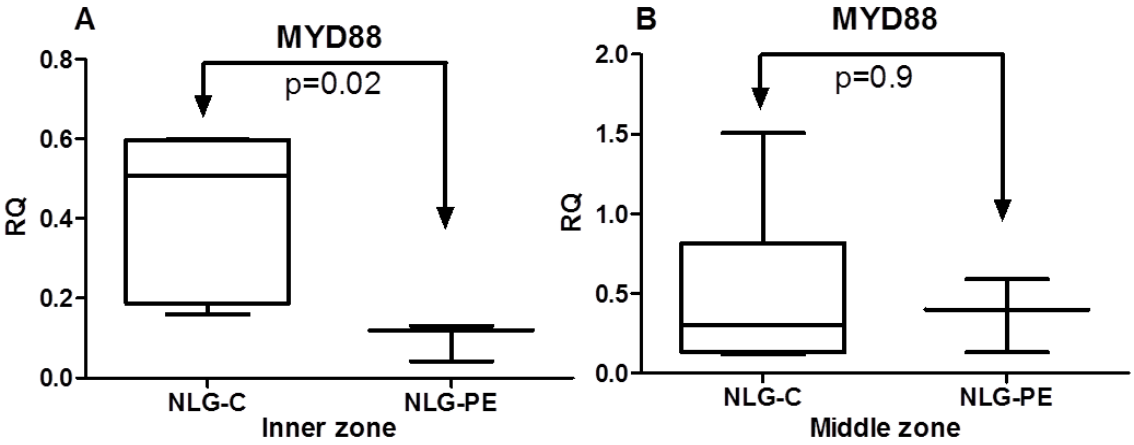


Figure 9-25: RQ values for MYD88 mRNA measurements in non-labour control compared to the non-labour PE groups at the inner and middle placental zones. Comparison between zones was performed using Mann-Whitney analysis.

Experiment 2: This experiment was designed to test if there was any difference in MYD88 mRNA expressions between labour control and labour PE groups at the inner or middle zones of placentas. Comparison between zones was performed using Mann Whitney test analysis. There was no significant difference in MYD88 mRNA in the labour control group compared to the labour PE group in the inner or middle placental zones (p=0.5, p=0.3 respectively) (Figure 9-26).

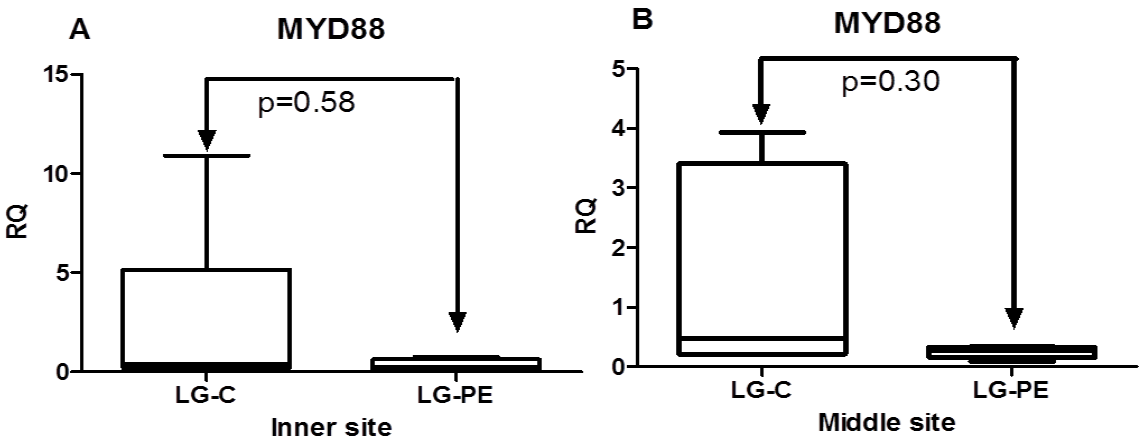


Figure 9-26: RQ values for MYD88 mRNA measurements in labour control compared to the labour PE groups at the inner and middle placental zones. Comparison between zones was performed using Mann-Whitney analysis.

Experiment 3: This experiment was designed to test if there was any difference in MYD88 mRNA expressions between normotensive pregnancies and pregnancies complicated with PE regardless the labour factor at the inner or middle zones of placentas. Comparison between zones was performed using Mann Whitney test analysis. There was no significant difference in MYD88 mRNA expression in the pregnancies complicated with PE group compared to the normotensive pregnancies groups in the inner and middle placental zones (p=0.1, p=0.2 respectively) (Figure 9-27).

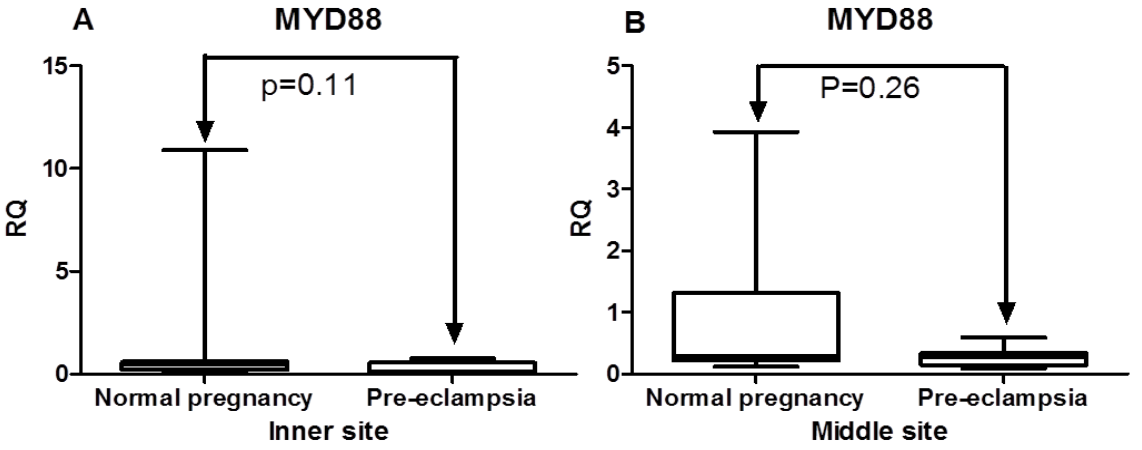


Figure 9-27: RQ values for MYD88 mRNA measurements in normotensive pregnancies group compared to the pregnancies complicated with PE group at the inner and middle placental zones. Comparison between zones was performed using Mann-Whitney analysis.

9.2.6 Analysis of mRNA NF-κB expression in normal pregnancy

Experiment 1: This experiment was designed to test if there was a spatial difference in expression of NF-κB mRNA within individual placentas obtained from women who were not in labour (non-labour group) and from women who were in labour (labour group). Comparison between zones was performed using Friedman test analysis. There was no significant difference in NF-κB mRNA expression between the three zones of the non-labour and labour groups (p=0.7, p=0.5 respectively) (Figure 9-28).

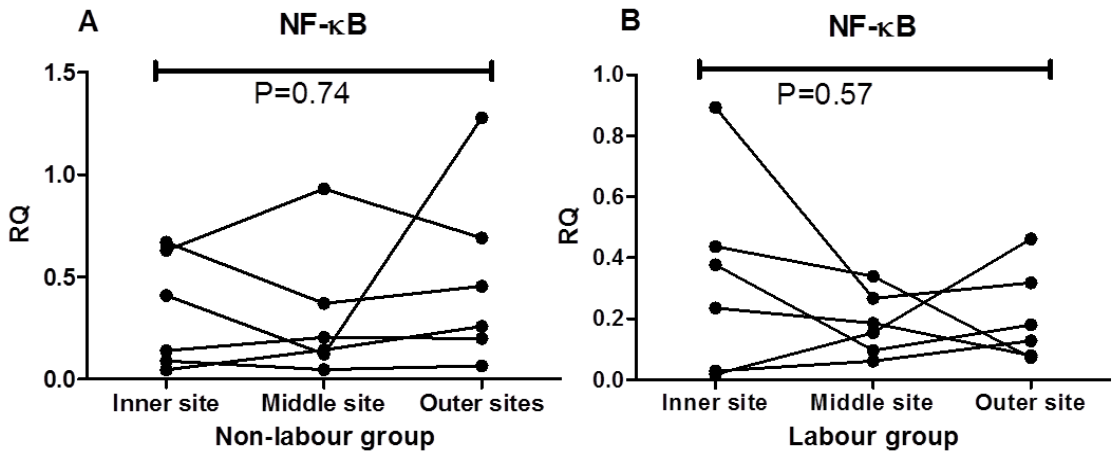


Figure 9-28: RQ values for NF-κB mRNA measurements in in inner, middle and outer zones of individual placentas (non-labour and labour groups). Four quadrants were sampled in each zone. Graphs show the median for NF-κB mRNA expressions. Comparison between zones was performed using Friedman analysis.

Experiment 2: This experiment was designed to test if there was any difference NF-κB mRNA expression between labour and non-labour groups at the inner, middle or outer zones of placentas. Comparison between zones was performed using Mann Whitney test analysis. There was no significant difference in NF-κB mRNA expression in the non-labour group when compared to the labour group at the inner, middle and outer zones (p=8, p=0.8, p=0.3 respectively) (Figure 9-29).

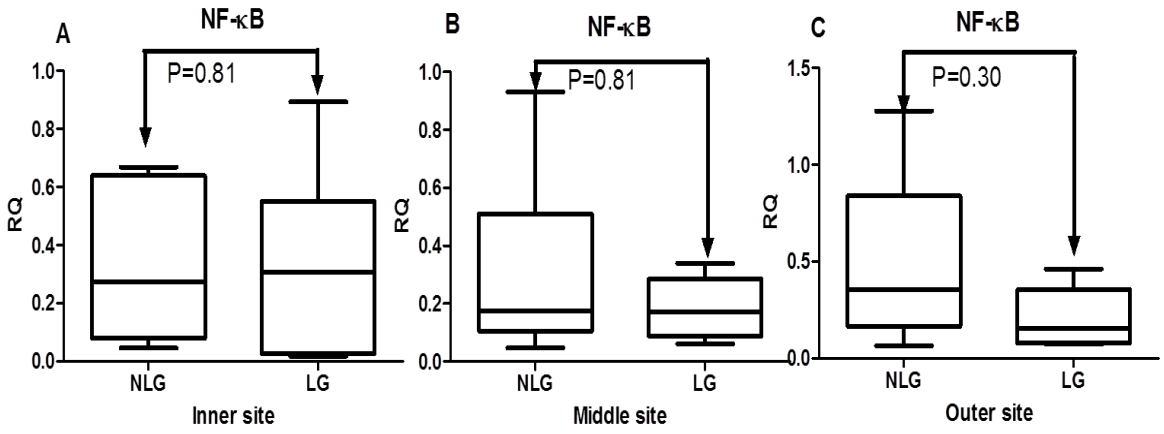


Figure 9-29: RQ values for NF-κB mRNA measurements in non-labour compared to the labour group at the inner, middle and outer placental zones. Comparison between zones was performed using Mann-Whitney analysis.

9.2.7 Analysis of mRNA NF-κB expression in pregnancies complicated with PE

Experiment 1: This experiment was designed to test if there was any difference in NF-κB mRNA expressions between non-labour control and non-labour PE groups at the inner or middle zones of placentas. Comparison between zones was performed using Mann Whitney. There was no significant difference in the NF-κB mRNA was found at the inner and middle zones (p=0.3, p=0.7) (Figure 9-30).

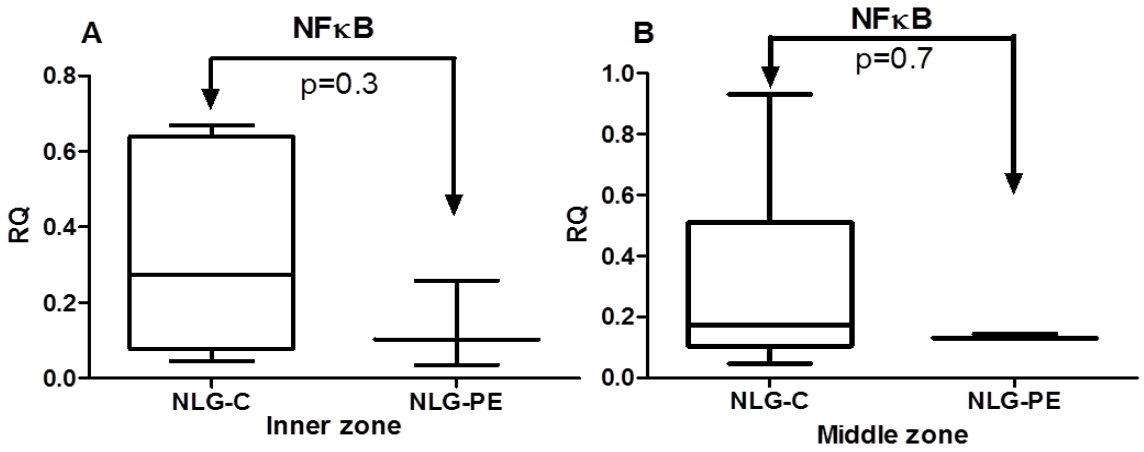


Figure 9-30: RQ values for NF-κB mRNA measurements in labour control compared to the labour PE groups at the inner and middle placental zones. Comparison between zones was performed using Mann-Whitney analysis.

Experiment 2: This experiment was designed to test if there was any difference in NF-κB mRNA expressions between labour control and labour PE groups at the inner or middle zones of placentas. Comparison between zones was performed using Mann Whitney test analysis. There was no significant difference in NF-κB mRNA in the labour control group compared to the labour PE group in the inner or middle placental zones (p=0.93, p= 1 respectively) (Figure 9-31).

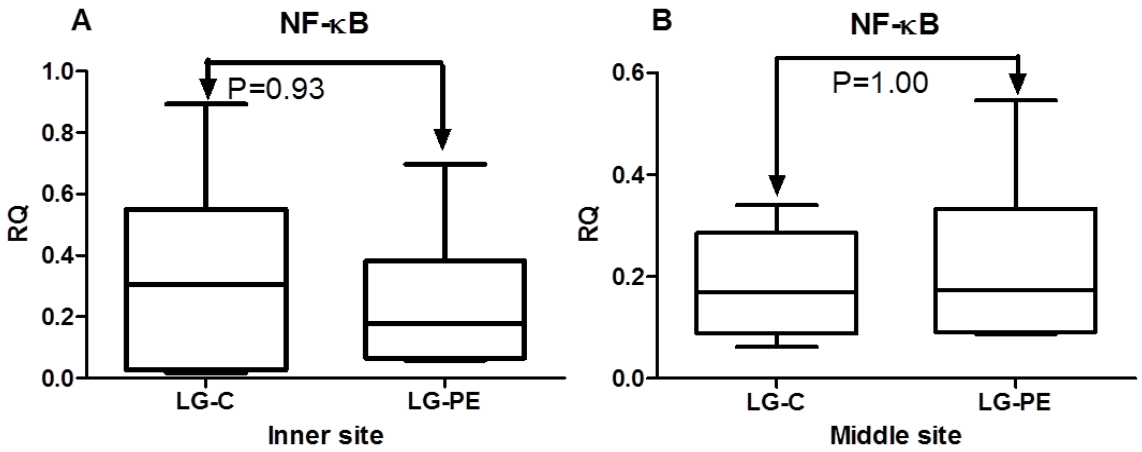


Figure 9-31: RQ values for NF-κB mRNA measurements in labour control compared to the labour PE groups at the inner and middle placental zones. Comparison between zones was performed using Mann-Whitney analysis.

Experiment 3: This experiment was designed to test if there was any difference in NF-κB mRNA expressions between normotensive pregnancies and pregnancies complicated with PE regardless the labour factor at the inner or middle zones of placentas. Comparison between zones was performed using Mann Whitney test analysis. There was no significant difference in NF-κB mRNA expression in the pregnancies complicated with PE group compared to the normotensive pregnancies groups in the inner and middle placental zones (p=0.6, p=0.7 respectively) (Figure 9-32).

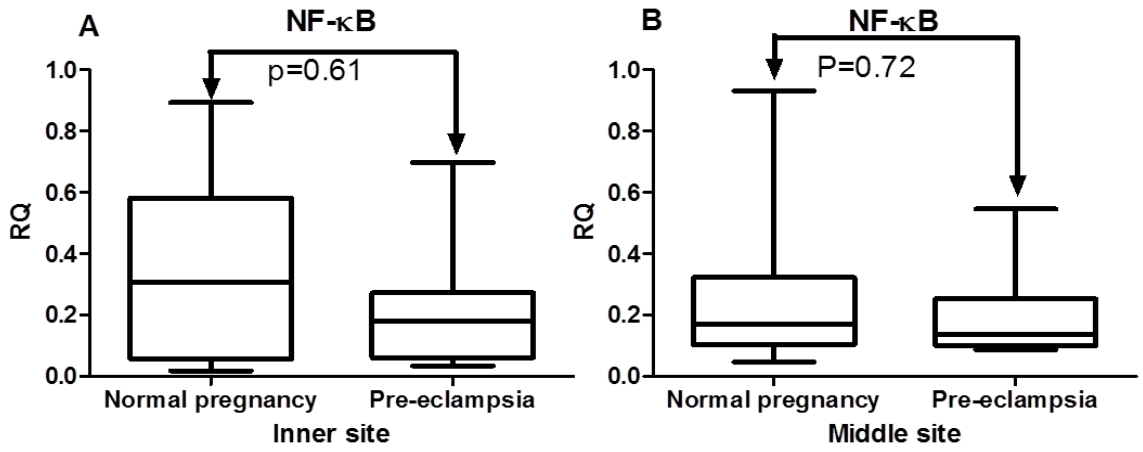


Figure 9-32: RQ values for NF-κB mRNA measurements in normotensive pregnancies group compared to the pregnancies complicated with PE group at the inner and middle placental zones. Comparison between zones was performed using Mann-Whitney analysis.

9.2.8 Results summary

9.2.8.1 TLRs

Experiment 1: Within individual placentas no spatial differences in mRNA for TLRs 1-8 were found either in the labour or non-labour groups.

Experiment 2: There was a significant decrease in TLR5 mRNA expression in the labour group when compared to the non-labour group at the middle zone (p=0.004). No other differences were found when comparing labour with non-labour.

Experiment 1: When the PE groups (non-labour) were compared with the control groups (non-labour) at the middle and inner zones the changes found can be summarized as follows: there was a significant decrease in TLR1 mRNA expression in the non-labour PE group compared to the non-labour control group at the middle zones (p=0.02). There was a significant decrease in TLR5 mRNA expression in the non-labour PE group compared to the non-labour control group at the middle zones (p=0.02).

Experiment 2: When the PE groups (labour) were compared with the control groups (labour) at the middle and inner zones the changes found can be summarized as follows: There was a significant decrease in TLR1 mRNA expression in the labour PE group compared to the labour control group at the inner and middle zones (p=0.04, p=0.002 respectively).

Experiment 3: When all non-labour and labour was combined. There was a significant decrease in TLR1 mRNA expression in the pregnancies complicated with PE group compared to the normotensive pregnancies group in the inner and middle placental zones.

9.2.8.2 MyD88

Experiment 1: No spatial differences were found in mRNA expression of MYD88 within placentas in labour or non-labour groups.

Experiment 2: No differences were found in mRNA expression of MYD88 between labour and non-labour groups within each zone.

Experiment 1: There was a significant decreased in the mRNA expression of MYD88 in the non-labour PE group compared to the non-labour control group at the inner zone.

Experiment 2: No difference was found in mRNA expression of MYD88 between labour control and labour PE groups at the inner or middle zones of placentas.

Experiment 3: When all non-labour and labour was combined. There was no significant difference in mRNA expression of MYD88 was found between normotensive pregnancies and pregnancies complicated with PE group at the inner and middle placental zones.

9.2.8.3 NF-κβ

Experiment 1: No spatial differences within placentas were found for labour or non-labour.

Experiment 2: No differences were found between labour and non-labour at each site.

Experiment 1: No difference was found between non-labour control and non-labour PE when either inner or middle zones were compared.

Experiment 2: No difference was found between labour control and labour PE when either inner or middle zones were compared.

Experiment 3: When all non-labour and labour was combined. There was no significant difference in mRNA expression of NF-κB was found between normotensive pregnancies and pregnancies complicated with PE group at the inner and middle placental zones.

9.3 Discussion

9.3.1 TLRs and present findings

This study shows that TLR9 and TLR10 mRNA were expressed in very low amounts in the human placenta. There was no spatial distribution of mRNA expression of TLRs 1-8 within individual placentas either in the labour or non-labour groups. When the labour group was compared to the non-labour group, only TLR5 mRNA expression showed a significant difference with a fall in the labour group at the middle zone. The reason for that is unclear.

Again due to time constrain only inner and middle zones of the PE group placentas were compared to the normotensive controls. When the non-labour PE group was compared to the non-labour control group, there was a significant decreased in TLR1 and TLR5 mRNA expression in the non-labour PE compared to the non-labour control at the middle zone. When the labour PE group was compared to the labour control group, there was a significant decrease in TLR1 mRNA expression in the labour PE group compared to the labour control group at the inner and middle zones. Furthermore, when all non-labour and labour was combined, there was a significant decrease in TLR1 mRNA expression in the pregnancies complicated with PE compared to the normotensive pregnancy group; this occurred at the inner and middle placental zones. The key finding in this study was that significant changes seem to be involved only TLR1 and TLR5. The study also shows the importance of using a systematic method to sample the placenta. Previous reports of TLRs expression do not take this into account. Therefore by taking a single sample or averaging protein expression of several samples may well mask possible changes in expression.

9.3.2 MyD88 and NF-κB and the present findings

There was no spatial distribution in both MyD88 and NF-κB mRNA expression within placentas. There was a significant decreased in the mRNA expression of MyD88 in the non-labour PE group compared to the non-labour control group at the inner zone. No other difference was found.

9.3.3 TLRs expression at the maternal-fetal interface

Innate immune responsive against microorganism at the maternal-fetal interface may have a significant impact on the pregnancy outcome. The maternal-fetal interface is a unique immunological site that tolerates the allogeneic fetus and provides the host immunity against pathogens. In addition, TLRs could provide a mechanism of cytokine production at the maternal-fetal interface and might have a role in the initiation and maintenance of labour (Abrahams et al., 2004a).

9.3.4 TLR signalling and function at the maternal-fetal interface

Differential responses to TLR4 and TLR2 ligation in trophoblast cells: Zymosan and LPS stimulation of TLR2 and TLR4 of term placental i*n vitro* cultural experiment induced high expression of IL-6 and IL-8 cytokine without effect mRNA or protein expression (Holmlund et al., 2002). These finding suggested that trophoblast have ability to recognize pathogen and initiate immune response through activation of immune cells (Koga et al., 2014). However, the response pattern for TLR ligation depends on the type of stimuli. HSP60 induces apoptosis in first-trimester trophoblast through TLR4 whereas LPS did not. This different effect of two different TLR4 ligands may be explained by different use of adaptor molecules (Koga and Mor, 2010, Koga et al., 2014). First trimester trophoblast cells are able to recognize and specifically respond to viral products in a highly regulated fashion (Abrahams et al., 2006). This different between first and third trimester trophoblast may be explained by presence TLR6 in third trimester. Koga et al showed that TLR4 ligation by LPS inhibit trophoblast migration (Koga et al., 2014).

9.3.5 TLRs expression in female reproductive tract

Little is known about TLR expression and distribution in the female reproductive tract in humans. It has been shown that TLR1, 2, 3, 5 and 6 are present in the epithelia of different regions of the female reproductive tract (Fazeli et al., 2005). One study showed that TLR4 is only present in the endocervix, endometrium and uterine tubes and absent in vagina and ectocervix (Fazeli et al., 2005). Moreover, mRNA expression of TLRs was up-regulated in uterine and cervical tissue of pregnant compared to the non-pregnant mice (Gonzalez et al., 2007). This study highlighted that there was different expression of TLRs mRNA between uterus, cervix and placenta, placenta TLR4 was down-regulated (Gonzalez et al., 2007).

9.3.6 TLRs expression in decidua and amnion

There are few studies regarding TLRs expression in decidua and amnion. The presence of all 10 TLR mRNA expression have been shown in the first trimester and the term decidua (Krikun et al., 2007a). In amnion, TLR4 is expressed by amniotic epithelium cells indicating that TLR4 is poised to monitor amniotic fluid for the presence of pathogens (Ma et al., 2006). In addition, it has been reported that TLR4 is highly expressed in the amnion of preterm infected placentas compared to term placenta (Hayati et al., 2010). Furthermore, TLR2 is also highly expressed in amniotic cell and decidua cells of infected placentas compared to non-infected placentas in the preterm situation (Hayati et al., 2010). These findings suggested TLR2 play an important role in protection of the preterm placenta against invading pathogens.

9.3.7 TLRs expression in placental tissue

The placenta constitutes a physical barrier against invading pathogens. Several studies have shown that all 10 TLRs are expressed by the placenta as well as co-receptors such as CD14 (Zarember and Godowski, 2002, Nishimura and Naito, 2005b, Klaffenbach et al., 2005a, Mitsunari et al., 2006a, Patni et al., 2009). Cultured cells from term placenta showed both STB and CTB cells express TLR2, 3, 4, 5, 6 and 9 (Holmlund et al., 2002, Mitsunari et al., 2006a). TLR2 and TLR4 are highly expressed on intermediate trophoblasts in free cell islands, cell column, decidua and trophoblasts covering the peripheral chorionic villi, but not the villous core (Holmlund et al., 2002, Kumazaki et al., 2004b, Beijar et al., 2006).

It has been reported that some TLRs are expressed in temporal manner, for example TLR6 (Koga and Mor, 2010). Abrahams et al has been shown that the first trimester trophoblasts express TLR1, 2, 3 and 4 but not TLR6 (Abrahams et al., 2004b), while TLR6 is expressed by third trimester trophoblast (Mitsunari et al., 2006a). In addition, Beijar et al compared TLR4 expression in the placenta from the first and the third trimester and showed that TLR4 is highly expressed in the term placenta (Beijar et al., 2006). These findings may suggest that the placenta in first trimester is less response to invading pathogens when compared to the term placenta.

TLRs are expressed by other placenta cells such as Hofbauer, macrophage and endothelial cells. IHC studies showed that TLR4 is expressed by these cells in the term placenta (Kumazaki et al., 2004b). In addition, Ma et al showed that TLR2 is highly expressed in

endothelial cells and macrophage with weaker expression in STB and fibroblast, while TLR4 showed stronger expression in STB and fibroblasts (Ma et al., 2006). These data suggested that not only immune system cells response to invading pathogen but also trophoblast and other cells within the placenta may be involved in physiological protection of the placenta (Koga and Mor, 2010, Koga et al., 2014).

There are several publications of TLRs, MyD88 and NF-κB expression in the placenta. Most of these studies used IHC analysis to detect TLR localization. Few studies compared the labour and non-labour in normal pregnancies. These studies can be summarized as follows.

Kumazaki *et al* performed IHC study to investigate TLR4 protein expression in 2^{nd} and 3^{rd} trimester placentas of normal and complicated pregnancies (Kumazaki et al., 2004a). This study showed that TLR4 was localized to the extra-villous trophoblasts, intermediate trophoblasts/X cells in the degenerative villi, and villous Hofbauer cells of all preterm and term placentas and also to the inflammatory cells in placentas with chorioamnionitis (Kumazaki et al., 2004a). In addition, TLR4 staining was increased in the villous Hofbauer cells of preterm chorioamnionitis placentas. They suggested that TLR4 plays a role in activation of the innate immune system in response to infectious pathogens in preterm placentas (Kumazaki et al., 2004a).

It has been shown, using RT-PCR that in the term placenta choriocarcinoma cell lines expressed the TLR1-10 and co-receptors CD14,MyD88,TIRP and TRIF mRNA (Klaffenbach et al., 2005a).

Nishimura and Naito performed RT-PCR to investigate TLR1-10 in several tissues include placenta (Nishimura and Naito, 2005a). All TLR1-10 were detected in placenta.

Klaffenbach *et al* performed RT-PCR to investigate TLR1-10, fluorescence-activated cell sorter analysis to investigate TLR1, 2, 4 and 9 and activation of NF-κB was determined in a reporter assay system in the term human placenta and trophoblast cell lines JAR and BeWo (Klaffenbach et al., 2005b). This study showed that all TLR1-10 were detected in term placenta. LPS induced mRNA and protein expression of TLR2 and also activated NF-κB in the JAR cells.

Mitsunari *et al* performed multiplex PCR to investigate mRNA expression for TLR1-6 and TLR9 in term non-labour human placental explants (Mitsunari et al., 2006b). TLR2, 3, 4,

5 and 6 was expressed in STB and CTB, while TLR1 and 9 were not detected. IHC analysis showed that TLR2, 4 and 6 protein expression was detected in the STB (Mitsunari et al., 2006b). A difference to the present study was that in the present study mRNA expression of TLR1-8 were detected but not TLR9-10; however, labour was not studied.

Krikun *et al* performed Western blotting, IHC and RT-PCR to investigate TLRs in decidual tissue of 1st trimester and term non-labour pregnancies (Krikun et al., 2007b). This study showed that decidua of 1st trimester as well as term trimester expresses mRNA for all TLR1-10. All TLRs apart from TLR2, 7 and 9 were expressed at lower levels in term than in 1st trimester decidual cells.

Ma *et al* performed IHC, Western blotting and qRT-PCR to localize TLR2 and TLR4 in the terminal and stem villi of term placenta (Ma et al., 2007). TLR2 was localized to endothelial cells and macrophages, and to a lesser extent to STB and fibroblasts. TLR4 was more prominent in STB and endothelial cells. In addition, Western blot and mRNA results showed that TLR2 and TLR4 were expressed mostly in STB whereas myofibroblasts expressed only TLR4. They concluded that there exists cell type-specific patterns of TLR function in placenta which likely regulate innate immune response at the maternal-fetal interface (Ma et al., 2007)

Patni *et al* performed qRT-PCR to investigate TLR1-10 expression in human term placentas of non-labour and labour groups (Patni et al., 2009). This study showed that all TLR1-10 were expressed in human placentas. In addition, TLR2 and TLR5 were significantly increased in labour group. In our study TLR5 was decreased in the labour group and no significant changes were found in TLR2 expression. A systematic sampling method was not performed in the other study.

Lee *et al* compared non-labour and labour placentas and reported that mRNA expression of TLR8 and NF-κB were increased in the labour group (Lee et al., 2010). However, no systemic sampling method was performed.

A recent study performed IHC, Western blotting and qRT-PCR to investigate TLR3 and TLR4 expression in 1st and 3rd trimester placental explants after exposure to either bacteria or viral antigens (Lye et al., 2015). TLR3 and TLR4 mRNA expression increased from the 1st to 3rd trimester, and the receptors localized to CTB in the 1st trimester and to STB in the 3rd trimester (Lye et al., 2015). In addition, TLR3 and TLR4 expression decreased in the 3rd trimester after exposure to viral antigen. They concluded that bacterial infections likely

alter exposure of the fetus to toxins during early pregnancy whereas viral exposure may disrupt fetal protection in later stages of pregnancy (Lye et al., 2015).

9.3.8 TLRs in complicated pregnancies

Recent studies have linked TLRs to many pregnancy disorders such as PE, preterm labour and early pregnancy loss.

PE effect at least 2-3 % of all pregnancies and it is a major cause of maternal morbidity and mortality. Despite progress in understanding the cause of PE, the aetiology remains unclear. The placenta has been shown to play an important role in the development of this serious syndrome (Roberts and Escudero, 2012). Recent investigations have suggested that immune response and inflammation activated by infectious or non-infectious pathogen or host derived molecules may be important factor in pathogenesis of PE (Koga et al., 2014). The activation of host derived molecules by TLRs may be a link between PE and activation of the maternal immune system (Holmlund et al., 2007). TLR4 expression was reported to be increased in trophoblast in women with preterm delivery PE compared to the term PE (Kim et al., 2005). In addition, Rahman et al (Rahman et al., 2006) described the relationship between high levels of TLR4 expression in endothelial cells and placenta vascular disease.

It has been shown that injection of poly (I:C) (TLR3 agonist) into wild type mice induces a strong pro-inflammation response by trophoblast cells resulting in a change in the distribution and activation of immune cells at the feto-maternal interface (Koga et al., 2009). It was concluded that poly (I:C) induces preterm delivery via a TLR3-dependent manner (Koga et al., 2009).

A few studies have examined TLRs expression in complicated pregnancies such as pre-term and PE. These studies can be summarized as follows.

Kim *et al* performed double IHC to investigate TLR2 and TLR4 expression in trophoblasts at the placental bed of labour and non-labour normal term pregnancies and labour and non-labour of pre-term and term pregnancies complicated with PE or chorioamnionitis (Kim et al., 2005). This study showed that there was no significant difference in TLR4 protein expression in the interstitial trophoblast between the labour and the non-labour normotensive groups. TLR4 was increased in the interstitial trophoblasts of PE (both

combined term and pre-term) compared to the pre-term labour with or without chorioamnionitis. There were no significant changes of TLR2 between all groups.

Rindsjo *et al* performed IHC to investigate the expression of TLR2 in 2nd and 3rd trimester placentas with chorioamnionitis compared to normal term controls (Rindsjo et al., 2007). TLR2 was localized to the CTB and STB cell layer and decidual stromal cells. TLR2 expression was decreased in placentas with chorioamnionitis compared to normal controls. In addition, TLR2 expression was high in 2nd compared to 3rd trimester placentas. There was a significantly higher TLR2 expression in placentas from live-born babies than still-birth babies (Rindsjo et al., 2007). It was concluded that TLR2 expression in the trophoblast could be involved in the response to infectious pathogens in the placenta.

Hayati *et al* performed IHC to detect TLR2 and TLR4 in amniotic cells and decidual cells in infected placentas and normal controls (Hayati et al., 2010). TLR2 expression was increased in the amniotic cells and decidua cells in infected pre-term placentas. They concluded that TLR2 plays a role in the innate immunity in bacterial and viral infection in the placenta.

Pineda *et al* performed immunofluorescence and confocal microscopy to detect TLR2, 3, 4 and 9 in term human placentas of PE and normal subjects (Pineda et al., 2011). All 4 TLRs were expressed in the trophoblast, villous stroma and vascular endothelium. TLR2, 3, 4 and 9 expression were increased in PE placentas (Pineda et al., 2011).

Bernardi *et al* performed Western blotting to investigate TLR4, MyD88 and NF-κB expression in term normotensive and PE placentas (Bernardi et al., 2012). This study showed that TLR4 and NF-κB was increased in PE placentas compared to the controls. They concluded that the TLR4- NF-κB pathway is upregulated in PE, and this probably generated local inflammatory response that is followed by local oxidative damage.

Chaparro *et al* performed RT-PCR and ELISA to investigate TLR2 and TLR4 mRNA expression in hypertensive disorders and PE placentas compared to the placentas of normotensive subjects (Chaparro et al., 2013). This study showed that TLR2 was significantly increased in hypertensive disorders placentas.

9.3.9 Immune mechanisms and PE

9.3.9.1 Infectious microorganisms in PE

Several studies have shown that maternal infection and PE are connected, and this link may be through TLRs activation (Abrahams et al., 2005, Kim et al., 2005). It has been suggested that infectious pathogens such as cytomegalovirus (CMV) and chlamydia pneumonia (C. pneumonia) may contribute to cardiovascular disease and atherosclerosis (Martin-Ventura et al., 2004). Since PE has many pathological feature similar to atherosclerosis this link may exist in PE. Several investigators have examined CMV and C. pneumonia in PE (von Dadelszen et al., 2003, Teran et al., 2003). It has been reported, using ELISA, that there was an increase in C. pneumonia antibodies in PE (Teran et al., 2003). Other researchers detected elevated anti-CMV antibodies levels in women with early onset PE compared to late onset PE (von Dadelszen et al., 2003). LPS activates an inflammatory response and even a low dose of LPS injected into pregnant rats produced pathological changes similar to PE (Faas et al., 1994).

9.3.9.2 TLR polymorphisms and susceptibility to PE

TLR polymorphisms can affect the susceptibility to PE. It has been reported that maternal TLR4 polymorphisms alter the susceptibility to early onset PE and HELLP syndrome (van Rijn et al., 2008). In addition, the presence of two TLR4 SNPs (Asp299Gly and Thr399Ile) and TLR2 Arg753Gln was associated with a normal pregnancy outcome (Hirschfeld et al., 2007).

Chapter X: Investigation of several HSPs and CASPs (tissue culture experiment)

10 Hypoxia-reperfusion tissue culture experiment

10.1 Introduction

The placenta performs key transport, metabolic and secretory functions to support fetal development. The human placenta is made up of a tree of villi; some villi are free-floating while anchoring villi are attached to the basal plate. The floating villi are covered by the STB, which is bathed in maternal blood and regulates maternal-fetal exchange. In early pregnancy trophoblast differentiation is controlled by the oxygen concentration (Genbacev et al., 1997). At change to the end of first trimester the blood flow starts in the inter-villous space and oxygen tension increases from 2% to 8% (Burton et al., 1999). Changes in oxygen tension are thought to be linked to trophoblast invasion of the placental spiral arteries. PE and FGR are linked with exposing the placenta villi to hypoxia (Kingdom and Kaufmann, 1997, Newby et al., 2005).

10.1.1 Response of ischemic placenta to reperfusion during peri-partum period

Different events are associated with changes in function of the placenta during the peri-partum period. The placenta is subject to several changes in homeostasis of metabolism due to change in blood supply. During uterine contractions of labour, there are cycles of normal blood flow alternating with episodes of ischemia followed by reperfusion. Placenta detachment from the uterine wall during third stage of delivery leads to severe impairment of blood flow and will result in complete ischemia in the placenta. Uterine contractions develop in late pregnancy long before start of proper labour, these early contractions may have a pre-conditioning effect providing increased tolerance of severe hypoxia in the fetus and placenta in preparation for labour (Schneider, 2009). However, this mechanism of pre-conditioning may not be sufficient to protect against oxidative stress generated during vaginal delivery as is clear when comparing placentas of non-labour patients to patients who underwent labour (Cindrova-Davies et al., 2007b).

10.1.2 Free radicals and oxidative stress in normal pregnancy

Free radicals are reactive molecules that have one or more unpaired electrons. ROS like hydrogen peroxide, super oxide and hydroxyl radicals form as a product of oxygen metabolism and have important roles in cell signalling (Valko et al., 2006); however in high quantities they are associated with cell damage. Polyunsaturated fatty acids in the plasma membrane as well as lipoproteins are susceptible to the oxidation (lipid peroxidation process). ROS are generated from different sources for example mitochondria via cytochrome P450 and the electron transport chain (Bobba et al., 2008). Oxygen radicals are generated by oxidation of catecholamine and metabolism of arachidonic acid by both the lipoxygenase and cyclooxygenase pathways (Halliwell and Gutteridge, 1990). Lipid and hydrogen peroxides initiate the lipid peroxidation reaction that continues until it is interrupted by antioxidants (Sies, 1997). Antioxidants are either endogenous such as catalase, superoxide dismutase and glutathione peroxidase or are obtained from the diet such as vitamin A, C and E (Gutteridge, 1995).

PE is a syndrome characterized by a generalized systemic maternal inflammatory response, associated with diffuse endothelial cell dysfunction. The placenta is the organ which is thought to where the disorder originates. Many theories have been suggested including that deportation of placental debris into the maternal circulation is a key element in development of the syndrome (Redman and Sargent, 2000). One potential source of the debris is turnover of the placental epithelial STB layer through apoptosis which is increased in PE as a result of oxidative stress (Hung et al., 2002).

10.1.3 Endoplasmic reticulum (ER) stress

ER stress has recently been identified as a major regulator of cell homeostasis through its involvement in post-translational protein modifications and folding, and its capacity to activate the UPR.

The ER is where synthesis of growth factors, polypeptide hormones and plasma membrane proteins and their post-translational protein modification takes place, and its capacity to activate the UPR. ER stress is a pathophysiological phenomenon closely linked with oxidative stress (Wang and Kaufman, 2012). Because of the ER role in protein folding and transport, the ER is also rich in Ca^{2+} dependent molecular chaperones for example GRP78, GRP94 and BiP which stabilize protein folding (Schröder and Kaufman, 2005, Orrenius et al., 2003, Ma and Hendershot, 2004a). The ER lumen contains a high concentration of

Ca^{2+} ions and it is also present in an oxidative environment which is critical for the formation of disulphide bonds. Any disturbances of these conditions cause accumulation of unfolded proteins in the ER, triggering the evolutionarily conserved UPR. The UPR functions to restore the homeostatic balance within the ER, but if this cannot be achieved it activates the apoptotic pathways (Burton et al., 2009b). However, as ER, the UPR require high energy, this would be disturbed by minor metabolic changes. The initial role for the UPR is to re-establish normal ER function by a mechanism involving transcriptional programs that induce an expression of genes that enhance the protein folding capacity and promote misfolded proteins degradation. Accumulation of unfolded and misfolded proteins activates the ER stress-response pathways and the UPR pathway. The UPR comprises three signalling pathways: the protein kinase (PKR)-like endoplasmic reticulum kinase (PERK)–eIF2α pathway, which attenuates non-essential protein synthesis; and the ATF6 and IRE1–XBP1 pathways, which increase the folding capacity by up-regulation of the ER chaperones GRP78 and GRP94 and phospholipid biosynthesis. However, if this mechanism fails, the ER-associated protein degradation (ERAD) pathway facilitates protein degradation (Schröder and Kaufman, 2005). Excessive and prolonged ER stress triggers cellular apoptosis (Xu et al., 2005). IR induces oxidative stress and activates the ER stress (Yung et al., 2007).

10.1.4 IR experiments

For many years, there has been interest in understanding the role that placental oxidative stress plays in the pathophysiology of complications of human pregnancy (Hubel, 1999, Myatt and Cui, 2004, Burton and Jauniaux, 2004, Lyall et al., 2013b). The cause of the oxidative stress is not certain, but several pregnancy complications such as PE and FGR have been associated with abnormal uterine spiral arteries conversion, suggesting that impaired perfusion of the placenta is the initiating insult (Brosens et al., 1972, Brosens, 1977, Kim et al., 2003). During a healthy pregnancy maternal spiral arteries are dramatically remodelled. They become widely dilated and lose their responsiveness to vasoconstrictive stimuli. Thus blood enters the intervillous space in a non-pulsatile manner and under low pressure (Lyall, 2006). Abnormal conversion of spiral arteries is associated with retention of smooth muscle cells in the spiral artery leading to poor or intermittent perfusion of the placenta (Hung et al., 2001). Hypoxia-reoxygenation experiments have demonstrated that placental oxidative stress can be induced rapidly by an

IR type insult that can alter placental gene and protein expression (Cindrova-Davies et al., 2007b).

Tissue damage caused by IR injury represents a serious event, which leads to deterioration of organ function. IR injury is associated with transient oxygen deprivation due to vascular occlusion and subsequent reperfusion following blood flow restoration. Tissue damage during an ischemic period is aggravated more through ROS generation and inflammation responses. IR injury occurs during organ transplantation, surgical intervention, and diseases such as circulatory shock, myocardial infraction and toxic injuries (Lorenzen et al., 2013, Karatzas et al., 2014).

10.2 Results

As time was limited one set of experiments was performed for all the data shown below, with a view to zooming in on particular time points should any obvious trends be spotted. As will be evident there appeared to a trend for differences to be apparent around 6 h and this would require future experiments.

10.2.1 Protein and mRNA expression of HSP70 analysis using hypoxia-reperfusion tissue culture experiment.

Experiment 1A: This experiment undertaken involved exposing placenta samples to 2% O2 (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 h. Ref 1 is the sample collected in the labour suite and -1 time point which reflected time before experiment carried out in laboratory. Ref 2 is the sample collected after arriving in the laboratory and 0 time point reflected time when the experiment started. These two time point (-1 and 0) were advised by department statistician. HSP70 protein expression was investigated using Western blot analysis. HSP70 mRNA was investigated using RT-qPCR. The interaction plot was used to demonstrate the findings. Examples of Western blots showing HSP70 expression for the set of experiments performed on placental tissue obtained from the inner zone are shown in (Figure 10-1). The interaction plot showed that expression of HSP70 in the treated group increased slightly from 3 h then gradual fall after 6 h. The control group showed that HSP70 expression increased from Ref1 to Ref2 points after that the expression increased sharply until 3 h when it decreased dramatically.

Overall both groups show that there was an increase in HSP70 expression, the control group from Ref2 point and treated group from 6 h before both showed a gradual fell with time.

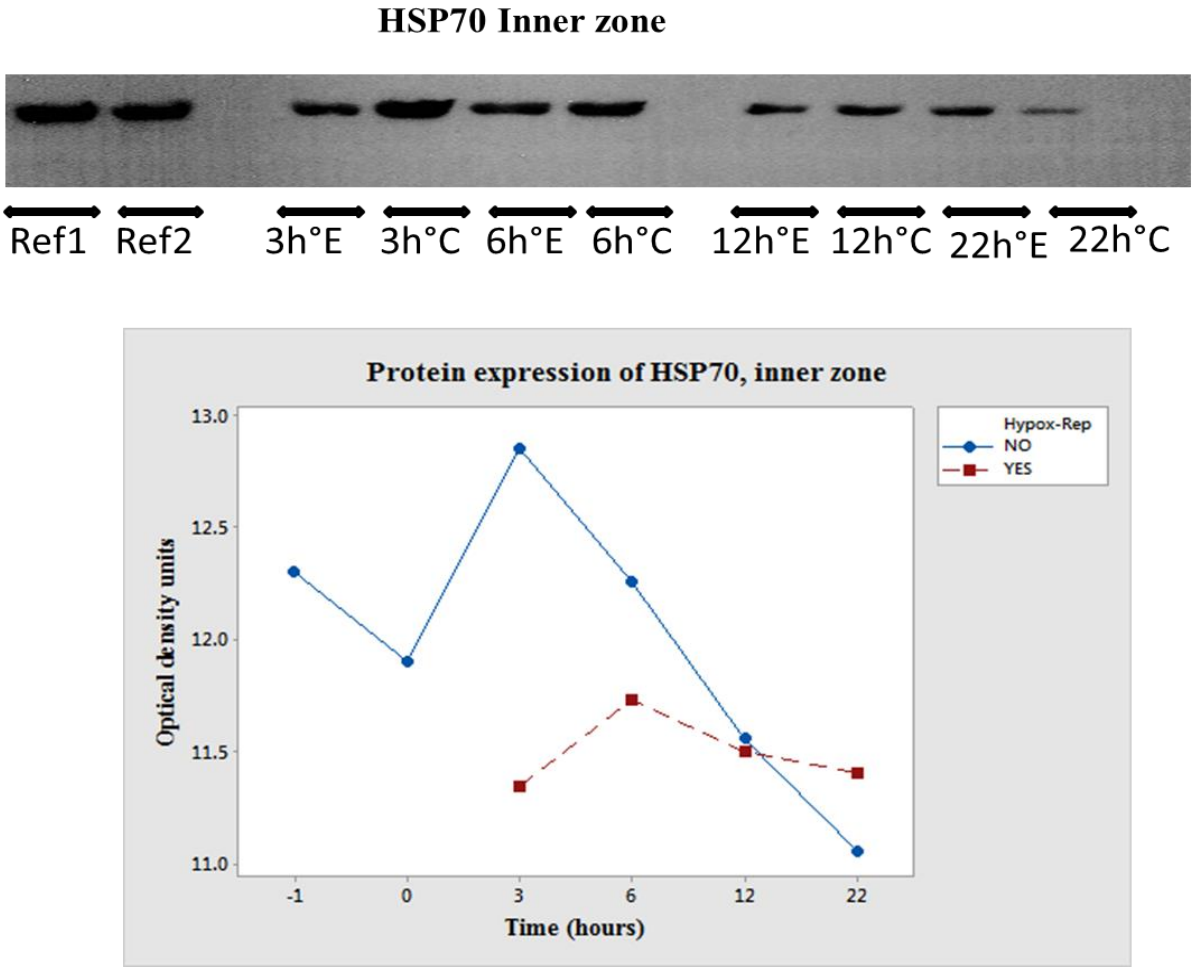
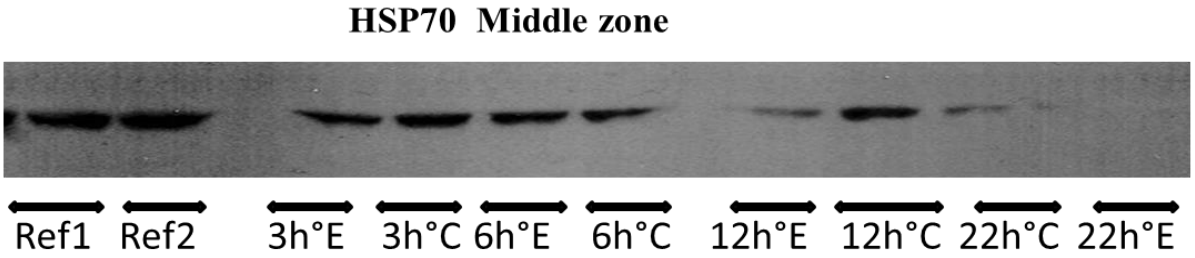


Figure 10-1: Representative image of Western blot showing HSP70 expression (upper panel) in the inner placental zone. Ref1 is the sample collected in the labour suite. Ref2 is the sample collected after arriving in the laboratory. Different time points (3, 6, 12 and 22 h) of reperfusion were performed. The graph shows the interaction plot analysis of the data for HSP70 protein expression at the inner zone. 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 1B: This experiment was the same as the above but now the middle zone of the placenta was assessed in its response to hypoxia-reperfusion. Example of Western blots showing HSP70 expression for the middle zone of the placenta sample is shown in (Figure 10-2). The interaction plot showed that expression of HSP70 in the treated group fell gradually over time. The control group showed that HSP70 expression increased slightly until 3 h when the expression decreased gradually. Overall both groups show that HSP70 expression was reduced over time in both groups, perhaps due to protein turnover.



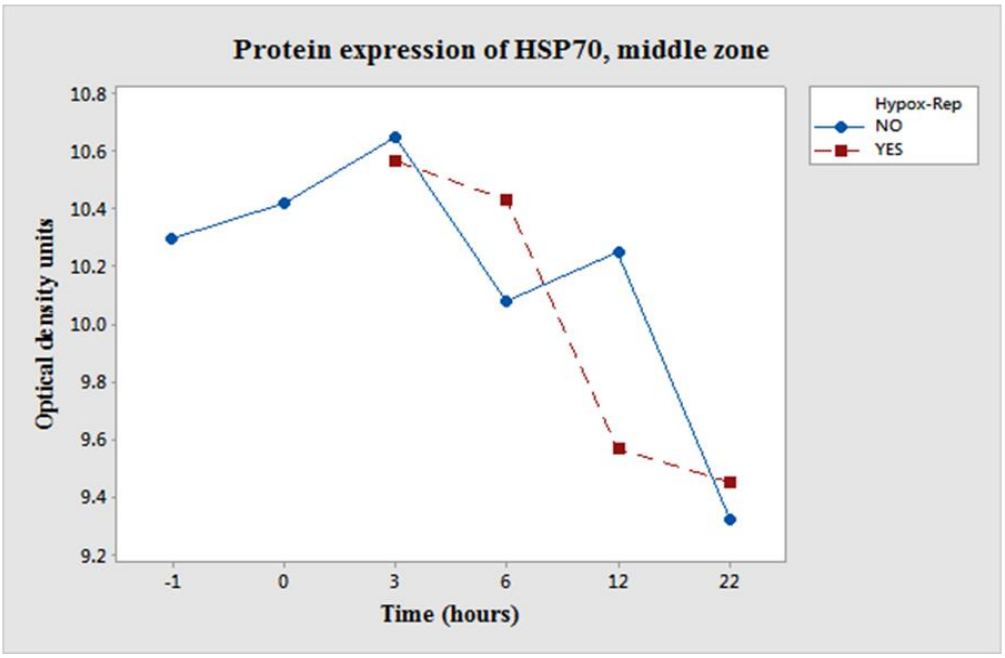


Figure 10-2: Representative image of Western blot showing HSP70 expression (upper panel) in the middle placental site. Ref1 is the sample collected in the labour suite. Ref2 is the sample collected after arriving in the laboratory. Different time points (3, 6, 12 and 22 h) of reperfusion were performed. The graph shows the interaction plot analysis of the data for HSP70 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 1C: This experiment was the same as the above but now the outer zone of the placenta was assessed in its response to hypoxia-reperfusion. Examples of Western blots showing HSP70 expression are shown in (Figure 10-3). The interaction plot showed that expression of HSP70 in treated group fell sharply over time with a slight increase after 6 h until 12 h when expression fell gradually. The control group showed that HSP70 expression decreased slightly until Ref2 point when the expression increased sharply, after 3 h the expression fall with slight increase after 12 h. Overall both groups show that HSP70 expression was reduced over time, the HSP70 expression in control group showed a peak at 3 h.

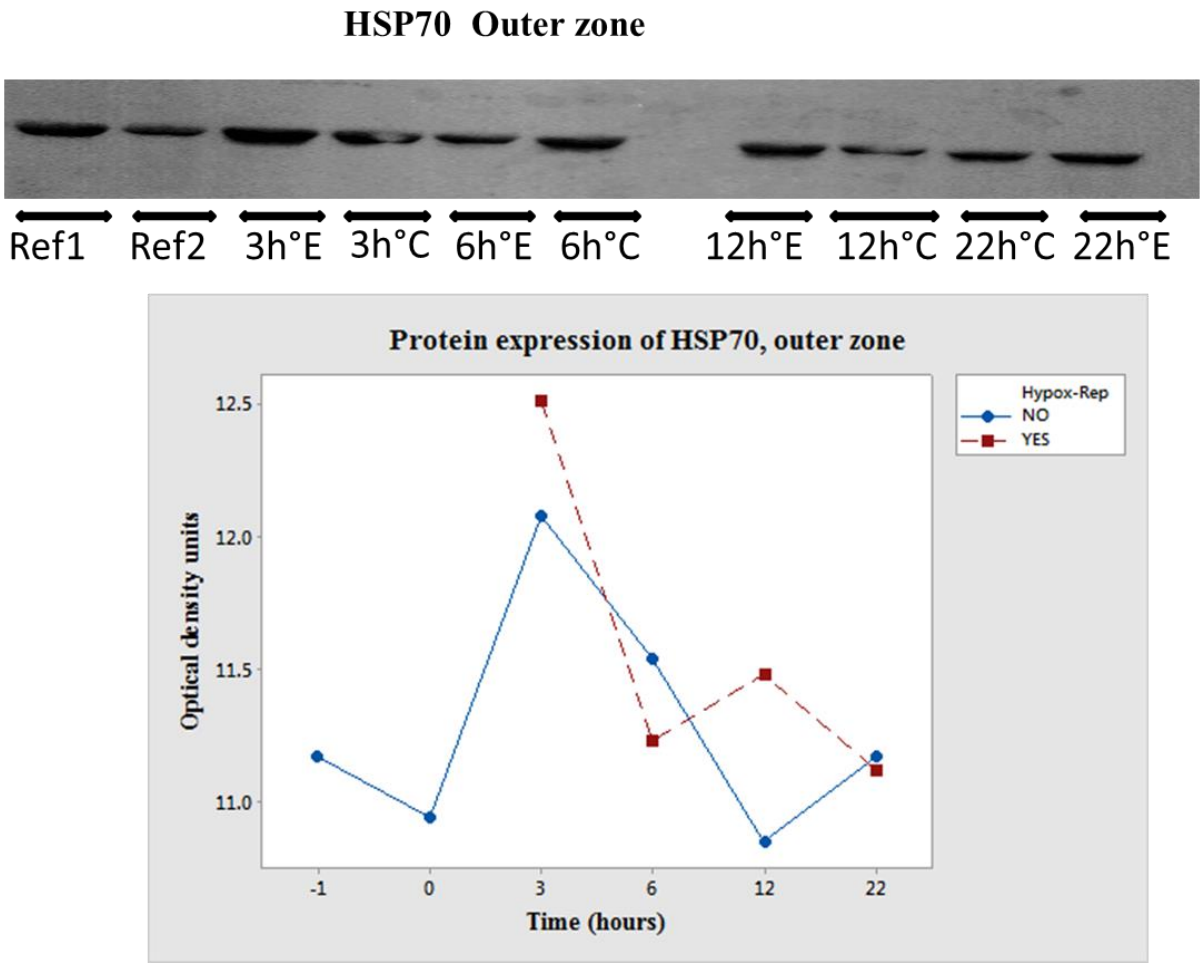


Figure 10-3: Representative image of Western blot showing HSP70 expression (upper panel) in the outer placental zone. Ref1 is the sample collected in the labour suite. Ref2 is the sample collected after arriving in the laboratory. Different time points (3, 6, 12 and 22 h) of reperfusion were performed. The graph shows the interaction plot analysis of the data for HSP70 protein expression at the outer zone. 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 2A: This experiment was designed to test if there was any difference in mRNA expression of HSP70 between tissues were exposing to hypoxia-reperfusion and control at the inner zone of placentas at four different time points (3, 6, 12 and 22 h). HSP70 mRNA was investigated using RT-qPCR. The interaction plot was used to demonstrate the findings (Figure 10-4). The interaction plot showed that mRNA expression of HSP70 in the treated group increased sharply from 3-6 h; the peak was at the 6 h and then decreased after that until 12 h when it increased again. The control group showed that mRNA expression of HSP70 continued without changes over the time until 6h when it increased slightly. Overall the treated group showed that there was a sharp peak of HSP70 expression at 6 h compared to the control group that showed small peak at 6-12 h.

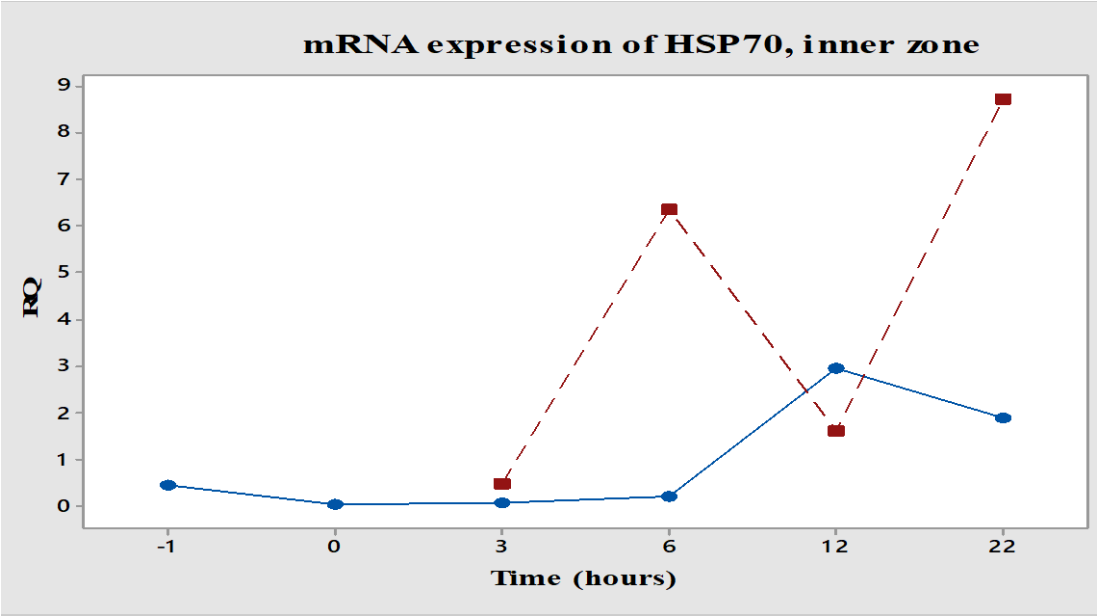


Figure 10-4: The graph shows the interaction plot analysis of the data for HSP70 mRNA at the inner zone. The graph shows data of the samples exposing to hypoxia-reperfusion (Red dotted line, YES) compared to the control samples (Blue continuous line, NO) at different time points.

Experiment 2B: This experiment was the same as the above but now the middle zone of the placenta was assessed in its response to hypoxia-reperfusion. The interaction plot showed that mRNA expression of HSP70 in the treated group increased sharply after 3 h. The control group showed that there was a slight increase in HSP70 expression after 6 h then before decreased after 12 h (Figure 10-5). However at 6 hours the experimental group showed a clear increase.

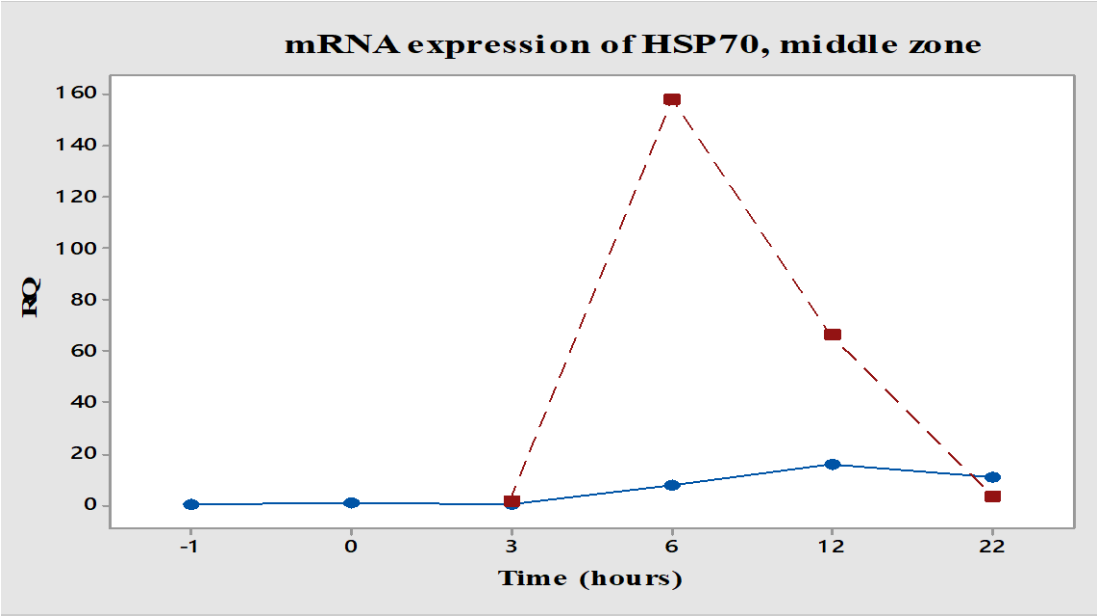


Figure 10-5: The graph shows the interaction plot analysis of the data for HSP70 mRNA at the middle zone. The graph shows data of the samples exposing to hypoxia-reperfusion (Red dotted line, YES) compared to the control samples (Blue continuous line, NO) at different time points.

Experiment 2C: This experiment was the same as the above but now the outer zone of the placenta was assessed in its response to hypoxia-reperfusion. The interaction plot showed that mRNA expression of HSP70 in the treated group increased sharply after 3 h until 12 h before it falling. The control group showed a slight increase in HSP70 expression after 6 h then decreased after 12 h. However at 6 hours the experimental group showed a clear increase (Figure 10-6).

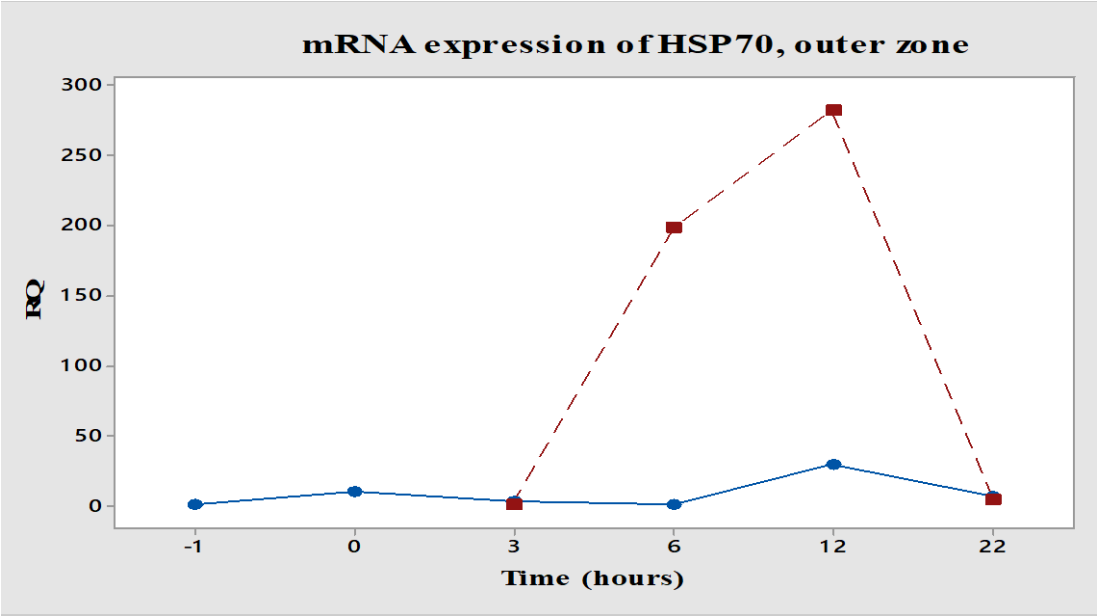


Figure 10-6: The graph shows the interaction plot analysis of the data for HSP70 mRNA at the outer zone. The graph shows data of the samples exposing to hypoxia reperfusion (Red dotted line, YES) compared to the control samples (Blue continuous line, NO) at different time points.

10.2.2 Analysis of protein and mRNA expression of HSP27 in the hypoxia-reperfusion tissue culture experiment.

The experiments below are the same as those above for HSP70 and therefore less text detail are included.

Experiment 1A: Examples of Western blots showing HSP27 expression for the set of experiments performed on placental tissue obtained from the inner zone are shown in (Figure 10-7). The interaction plot showed that expression of HSP27 in the treated group decreased gradually over time. The control group showed that HSP27 expression also decreased slowly over time (Figure 10-7). Overall both groups showed that HSP27 expression was reduced over time, perhaps due to protein turnover.

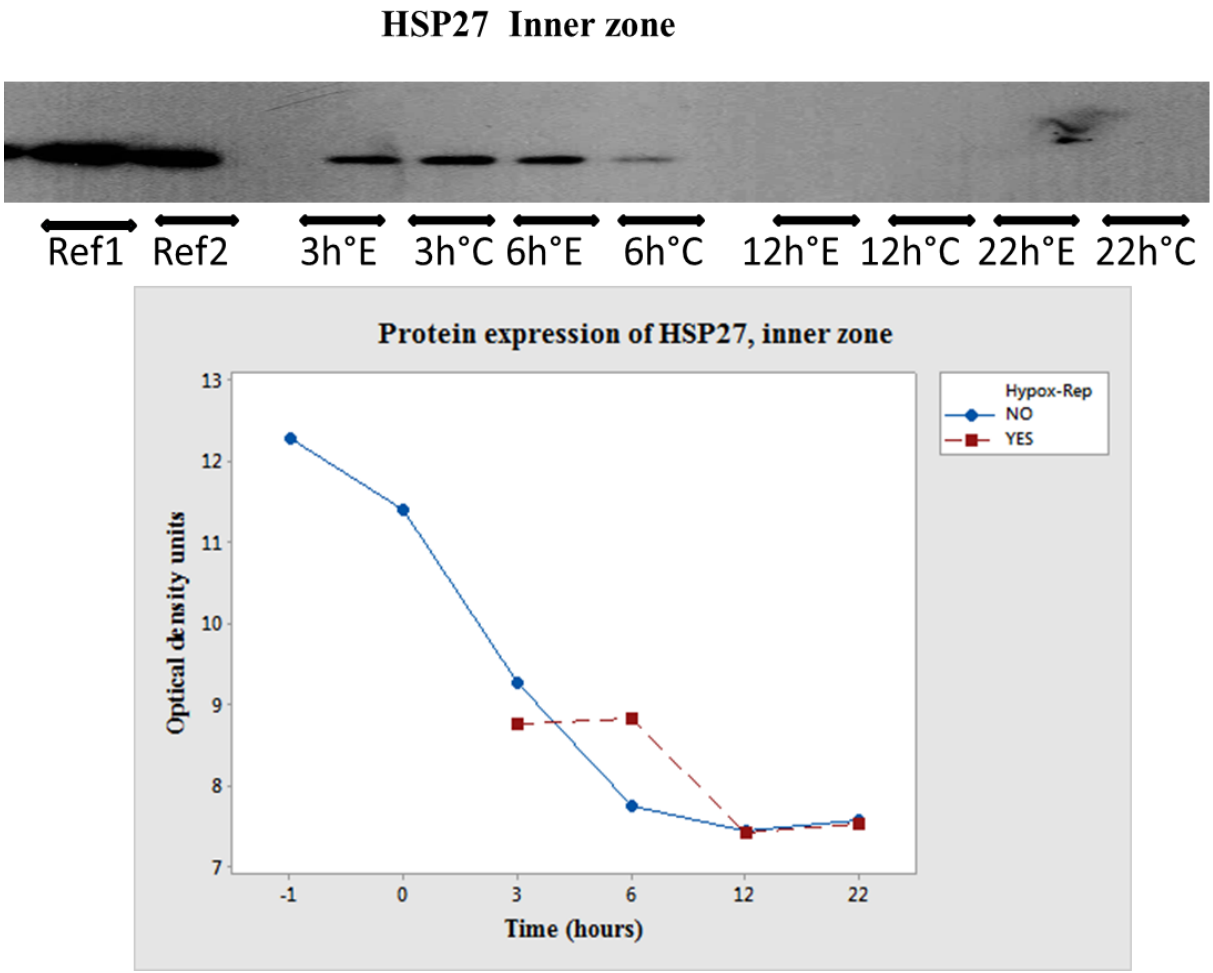


Figure 10-7: Representative image of Western blot showing HSP27 expression (upper panel) in the inner placental zone. Ref1 is the sample collected in the labour suite. Ref2 is the sample collected after arriving in the laboratory. Different time points (3, 6, 12 and 22 h) of reperfusion were performed. The graph shows the interaction plot analysis of the data for HSP27 protein expression at the inner zone. 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 1B: This experiment was the same as the above but now the middle zone of the placenta was assessed in its response to hypoxia-reperfusion. Examples of Western blots showing HSP27 expression for the middle zone of the placenta sample are shown in (Figure 10-8). The interaction plot showed that expression of HSP27 in the treated group decreased gradually over time. The control group showed that HSP27 expression increased slightly from Ref1 to Ref2 point before it decreased gradually over time (Figure 10-8). Overall both groups showed that HSP27 expression was reduced over time in, perhaps due to protein turnover.

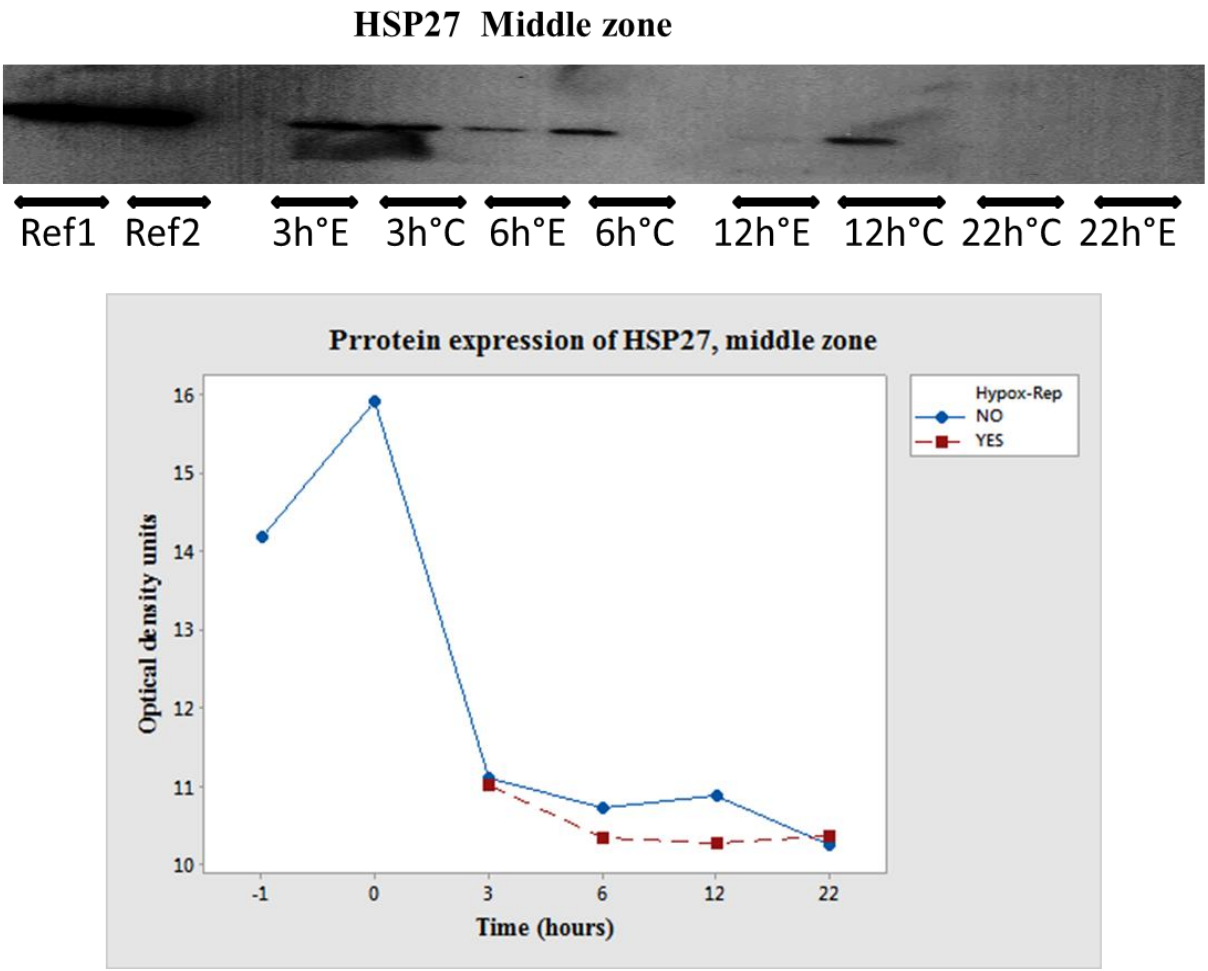


Figure 10-8: Representative image of Western blot showing HSP27 expression (upper panel) in the middle placental zone. Ref 1 is the sample collected in the labour suite. Ref 2 is the sample collected after arriving in the laboratory. Different time points (3, 6, 12 and 22 h) of reperfusion were performed. The graph shows the interaction plot analysis of the data for HSP27 protein expression at the middle zone. 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 1C: This experiment was designed to test if there was any difference in HSP27 protein expression between tissues following exposing placental samples from the outer zone to hypoxia-reperfusion. Examples of Western blots showing HSP27 expression are shown in (Figure 10-9). The interaction plot showed that expression of HSP27 in the treated group decreased gradually over time. The control group showed that HSP27 expression also decreased over time (Figure 10-9). Overall both groups showed that HSP27 expression was reduced over time, perhaps due to protein turnover.

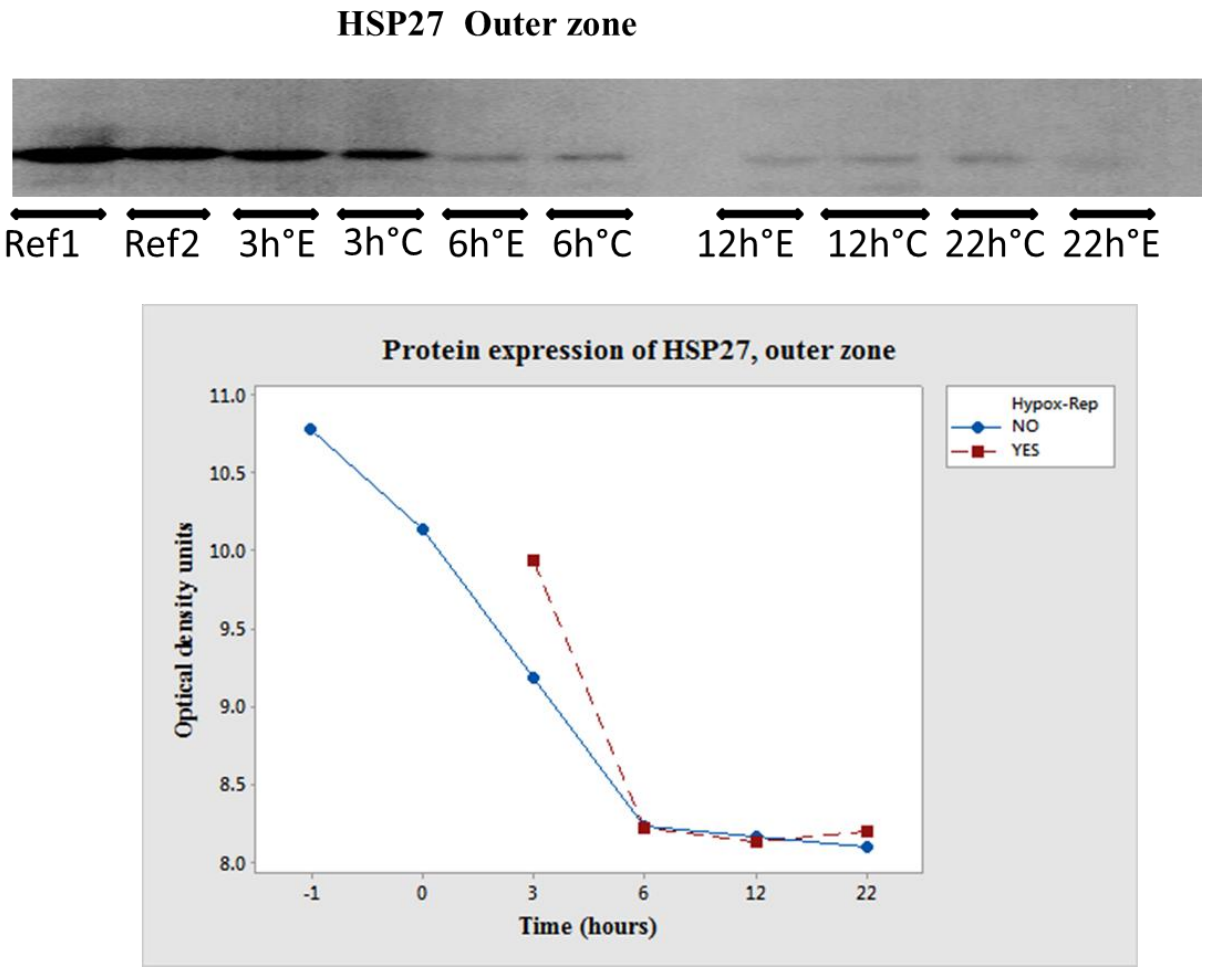


Figure 10-9: Representative image of Western blot showing HSP27 expression (upper panel) in the outer placental zone. Ref 1 is the sample collected in the labour suite. Ref 2 is the sample collected after arriving in the laboratory. Different time points (3, 6, 12 and 22 h) of reperfusion were performed. The graph shows the interaction plot analysis of the data for HSP27 protein expression at the outer zone. 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 2A: This experiment was designed to test if there was any difference in HSP27 mRNA expression between tissues were exposing to hypoxia reperfusion at the inner zone of placentas according to four different time points. The interaction plot showed that expression of HSP27 in the treated group did not change with time. The control group showed that HSP27 expression decreased sharply from Ref1 to Ref2 point then did not change over time (Figure 10-10).

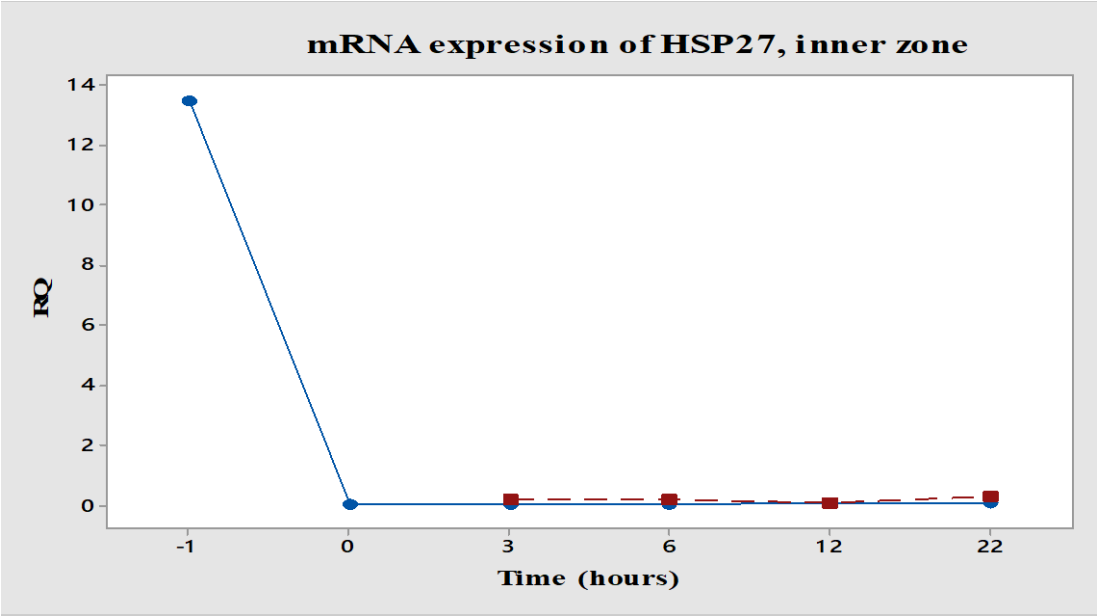


Figure 10-10: The graph shows the interaction plot analysis of the data for HSP27 mRNA at the inner zone. The graph shows data of the samples exposing to hypoxia-reperfusion (Red dotted line, YES) compared to the control samples (Blue continuous line, NO) at different time points.

Experiment 2B: This experiment was designed to test if there was any difference in HSP27 mRNA expression between tissues were exposing to hypoxia-reperfusion at the middle zone of placentas at four different time points. The interaction plot showed that mRNA expression of HSP27 in the treated group increased sharply after 3 h until 6 h then fell. The control group showed that there was a slight increase in HSP27 expression between 3-12 h then decreased after 12 h. However at 6 hours the experimental group showed a clear increase (Figure 10-11).

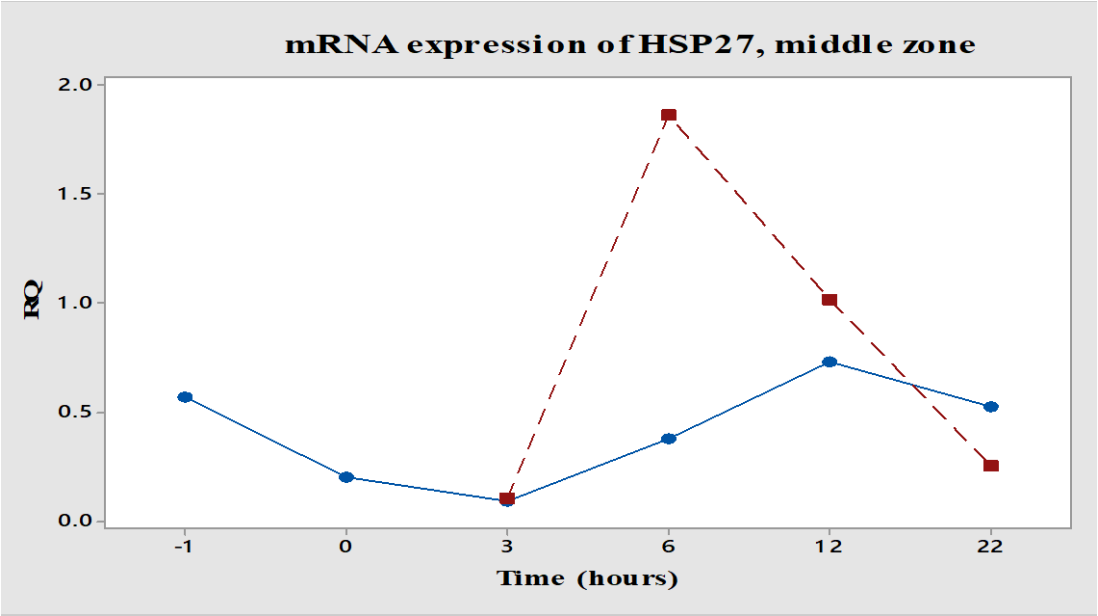


Figure 10-11: The graph shows the interaction plot analysis of the data for HSP27 mRNA at the middle zone. The graph shows data of the samples exposing to hypoxia-reperfusion (Red dotted line, YES) compared to the control samples (Blue continuous line, NO) at different time points.

Experiment 2C: This experiment was designed to test if there was any difference in mRNA expression of HSP27 between tissues exposed to hypoxia-reperfusion at the outer zone of placentas at four different time points. The interaction plot showed that mRNA expression of HSP27 in the treated group increased sharply after 3 h until 12 h then fell. The control group showed that there was a slight increase in HSP27 expression between –Ref1 and Ref2 points and 6-12 then decreased until 12 h where there was a slight increased. However at 3 hours the experimental group showed again a clear increase (Figure 10-12).

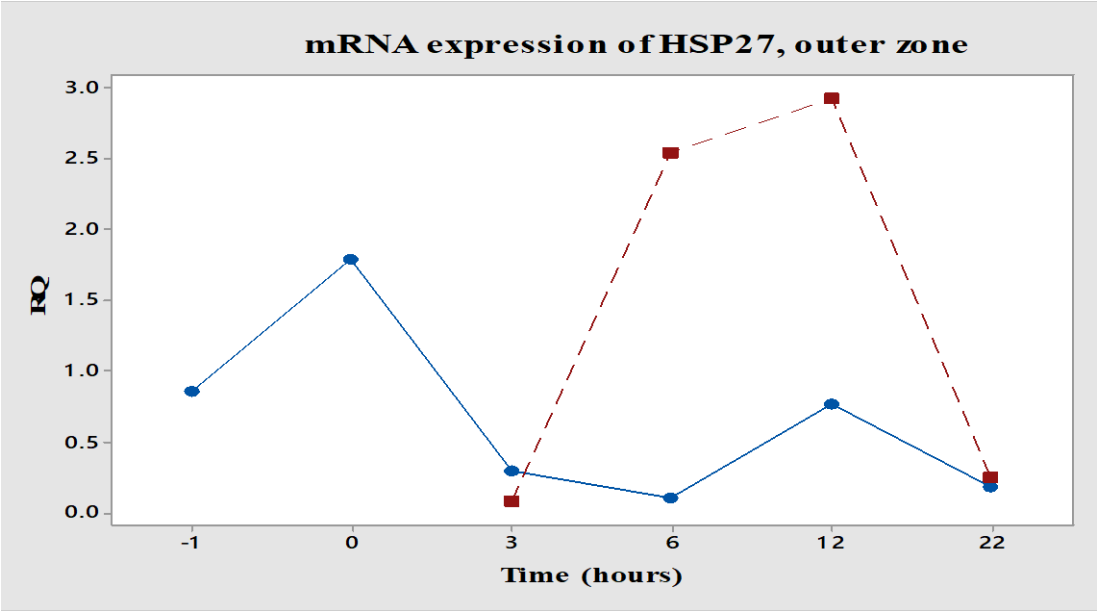


Figure 10-12: The graph shows the interaction plot analysis of the data for HSP27 mRNA at the outer zone. The graph shows data of the samples exposing to hypoxia-reperfusion (Red dotted line, YES) compared to the control samples (Blue continuous line, NO) at different time points.

10.2.3 Analysis of mRNA expression of HSF1 in the hypoxia-reperfusion tissue culture experiment.

Experiment 1A: This experiment was designed to test if there was any difference in HSF1 mRNA expression between tissues exposed to hypoxia-reperfusion at the inner zone of placentas at four different time points. The interaction plot showed that mRNA expression of HSF1 in the treated group increased gradually after 3 h until 12 h then increased further. The control group showed that there was a sharp decrease in HSF1 expression between Ref1 and Ref2 points then there was a slight increase after 6 h before falling after 12 h. (Figure 10-13).

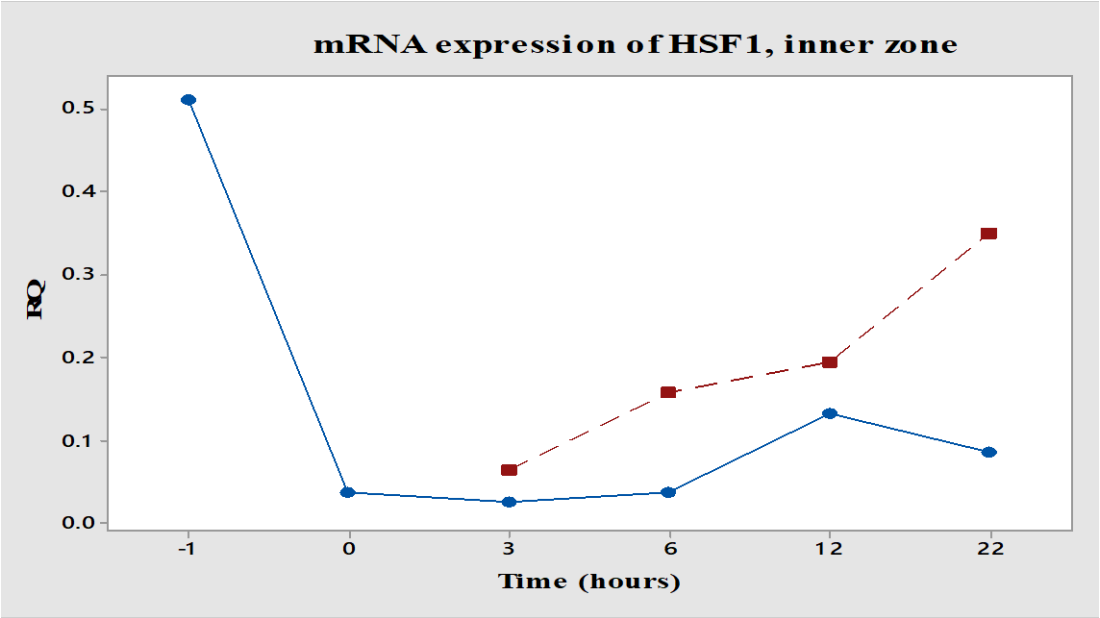


Figure 10-13: The graph shows the interaction plot analysis of the data for HSF1 mRNA at the inner zone. The graph shows data of the samples exposing to hypoxia-reperfusion (Red dotted line, YES) compared to the control samples (Blue continuous line, NO) at different time points.

Experiment 1B: This experiment was designed to test if there was any difference in HSF1 mRNA expression between tissues exposed to hypoxia-reperfusion at the middle zone of placentas at four different time points. The interaction plot showed that mRNA expression of HSF1 in the treated group increased gradually after 3 h until 12 h then declined. The control group showed that there was a gradual increase in HSF1 expression from 3-12 h then decreased after 12 h (Figure 10-14).

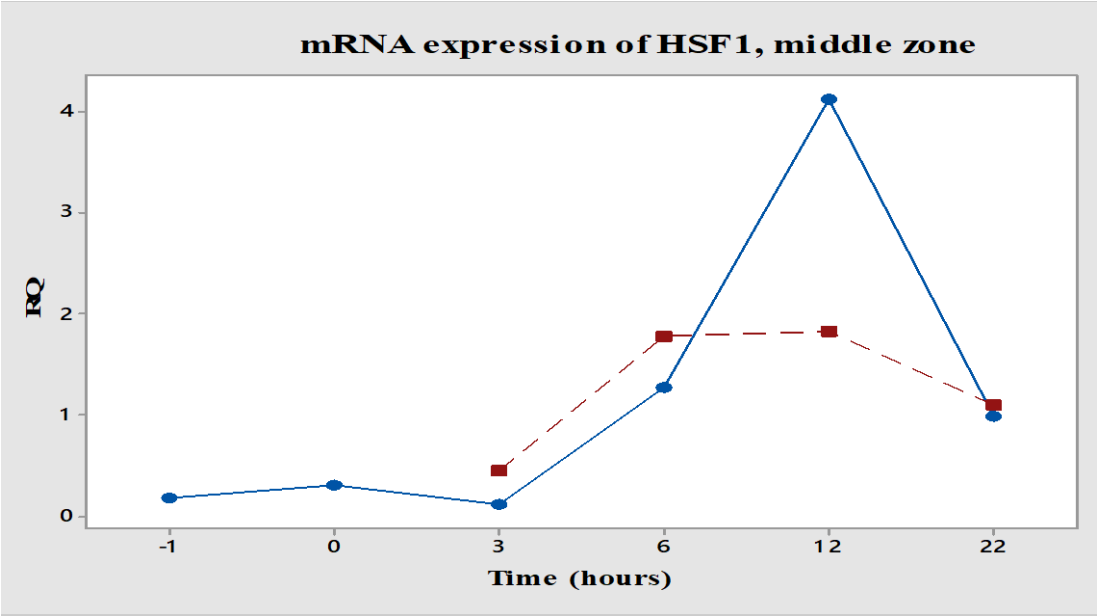


Figure 10-14: The graph shows the interaction plot analysis of the data for HSF1 mRNA at the middle zone. The graph shows data of the samples exposing to hypoxia-reperfusion (Red dotted line, YES) compared to the control samples (Blue continuous line, NO) at different time points.

Experiment 1C: This experiment was designed to test if there was any difference in mRNA expression of HSF1 between tissues exposed to hypoxia-reperfusion at the outer zone of placentas at four different time points. The interaction plot showed that mRNA expression of HSF1 in the treated group increased sharply after 3 h then fell after 6 h. The control group showed that there was a slight increase in HSF1 expression from Ref1 to Ref2 time point and 6-12 h then declined again (Figure 10-15).

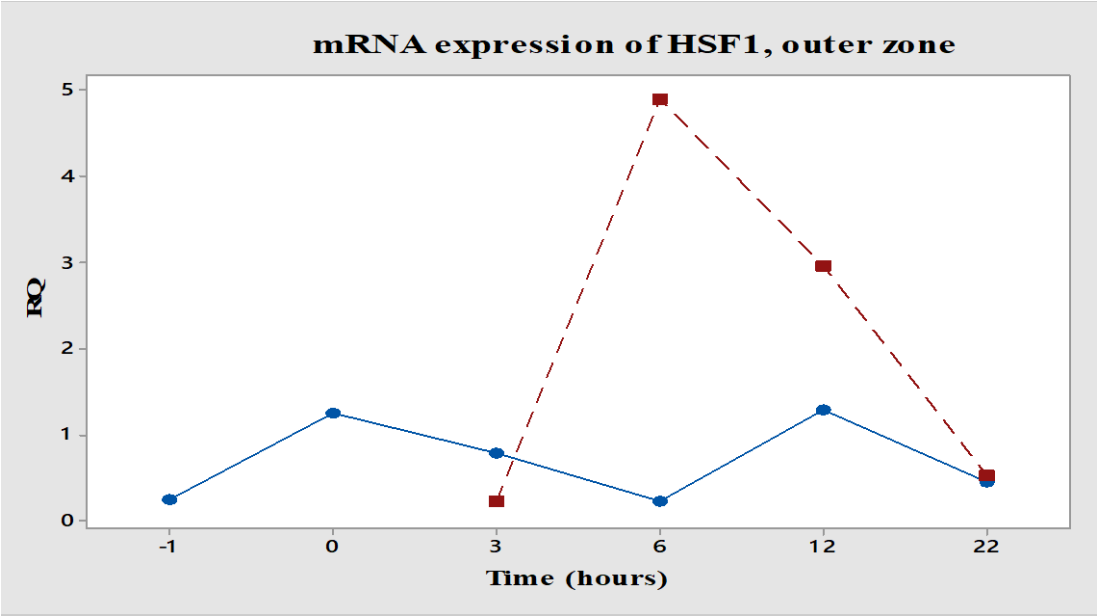


Figure 10-15: The graphs show the interaction plot analysis of the data for HSF1 mRNA at the outer zone. The graph shows data of the samples exposing to hypoxia-reperfusion (Red dotted line, YES) compared to the control samples (Blue continuous line, NO) at different time points.

10.2.4 Analysis of mRNA expression of HSP90AA in the hypoxia-reperfusion tissue culture experiment.

Experiment 1A: This experiment was designed to test if there was any difference in HSP90AA mRNA expression between tissues exposed to hypoxia-reperfusion at the inner zone of placentas at four different time points. The interaction plot showed that expression of HSP90AA in the treated group remained unaltered. The control group showed that HSP90AA expression decreased sharply from Ref1 to Ref2 time point then remained unchanged over time (Figure 10-16).

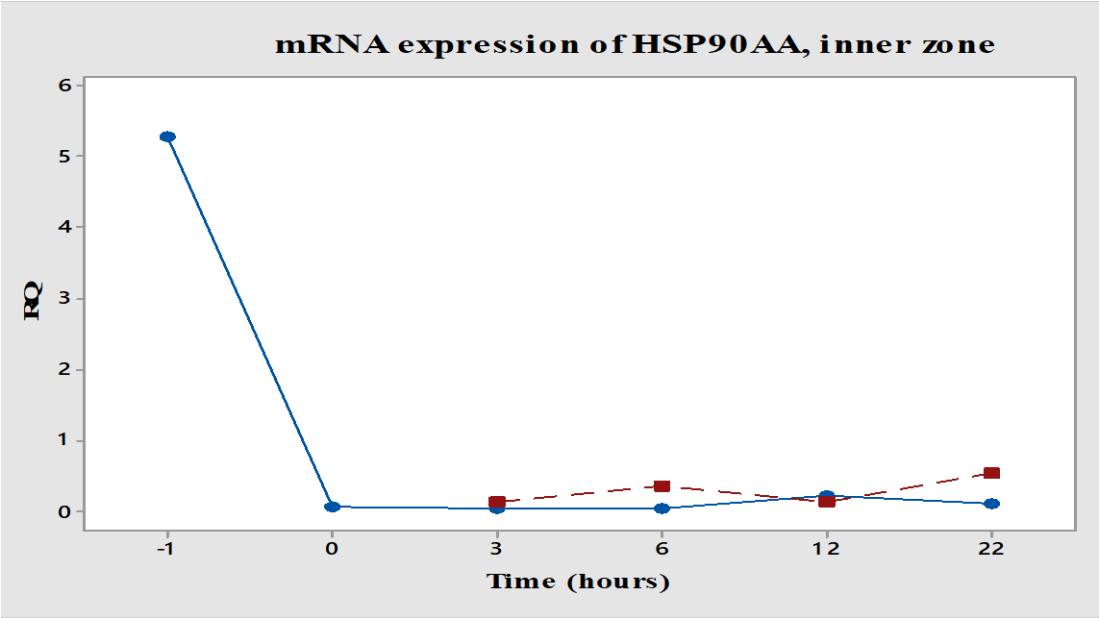


Figure 10-16: The graph shows the interaction plot analysis of the data for HSP90AA mRNA at the inner zone. The graph shows data of the samples exposing to hypoxia-reperfusion (Red dotted line, YES) compared to the control samples (Blue continuous line, NO) at different time points.

Experiment 1B: This experiment was designed to test if there was any difference in HSP90AA mRNA expression between tissues exposed to hypoxia-reperfusion at the middle zone of placentas at four different time points. The interaction plot showed that mRNA expression of HSP90AA in the treated group increased sharply after 3 h until 6 h before falling. The control group showed that there was a slight increase in HSP90AA expression between 6-12 h before falling (Figure 10-17).

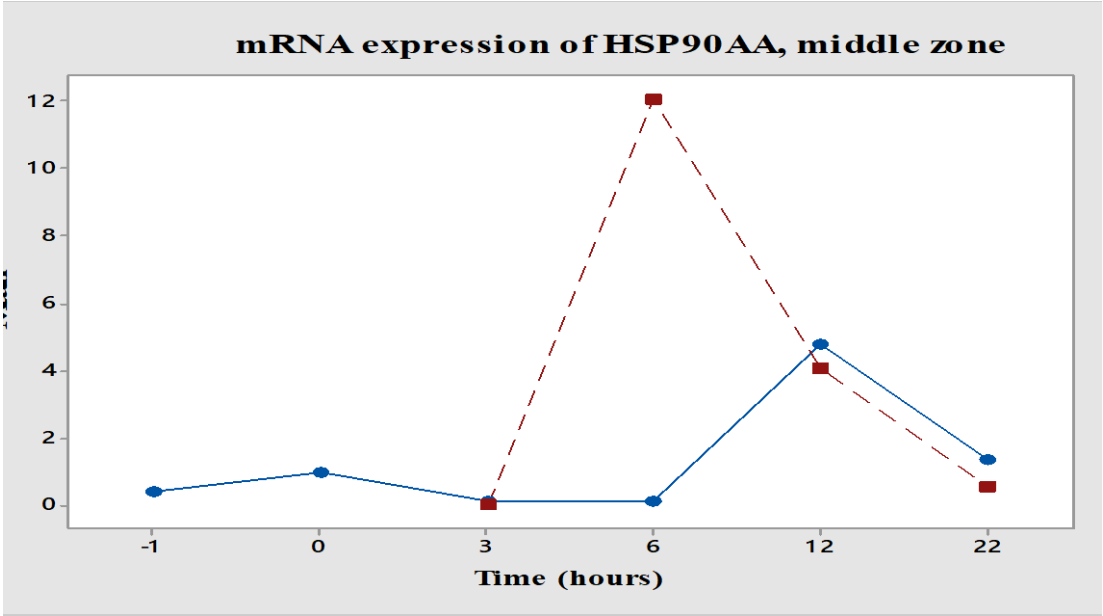


Figure 10-17: The graph shows the interaction plot analysis of the data for HSP90AA mRNA at the middle zone. The graph shows data of the samples exposing to hypoxia-reperfusion (Red dotted line, YES) compared to the control samples (Blue continuous line, NO) at different time points.

Experiment 1C: This experiment was designed to test if there was any difference in mRNA expression of HSP90AA between tissues exposed to hypoxia-reperfusion at the outer zone of placentas at four different time points. The interaction plot showed that mRNA expression of HSP90AA in the treated group was increased sharply after 3 h until 12 h before falling. The control group showed that there was a slight increase in HSP90AA expression between Ref1 and Ref2 time point and 6-12 h then it decreased (Figure 10-18).

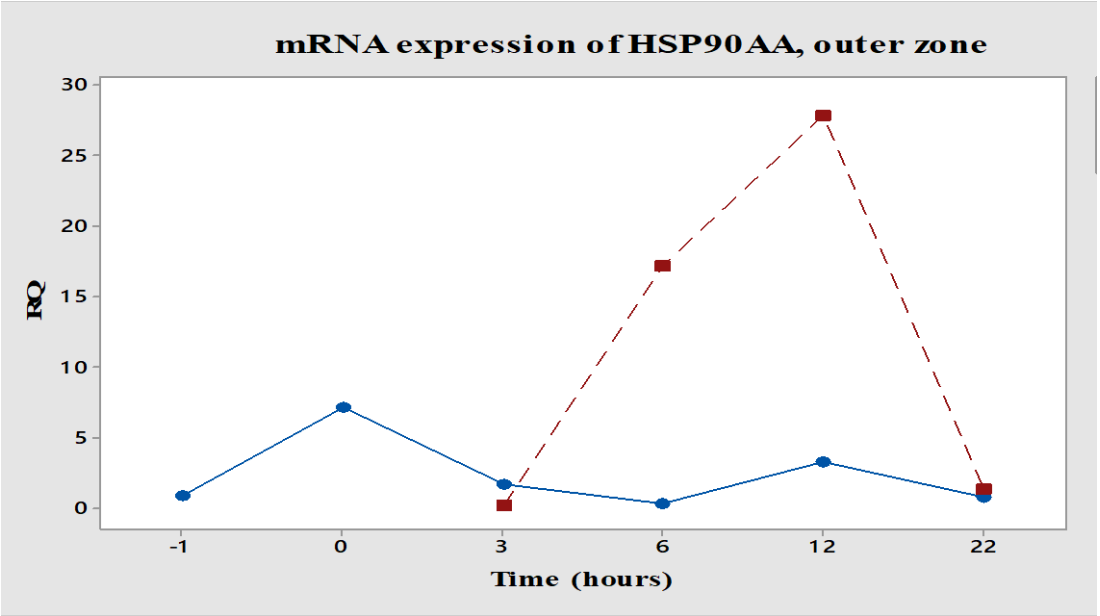


Figure 10-18: The graph shows the interaction plot analysis of the data for HSP90AA mRNA at the outer zone. The graph shows data of the samples exposing to hypoxia-reperfusion (Red dotted line, YES) compared to the control samples (Blue continuous line, NO) at different time points.

10.2.5 Analysis of mRNA expression of HSP90B1 in the hypoxia-reperfusion tissue culture experiment.

Experiment 1A: This experiment was designed to test if there was any difference in HSP90B1 mRNA expression between tissues exposed to hypoxia-reperfusion at the inner zone of placentas at four different time points. The interaction plot showed that expression of HSP90B1 in the treated group remained the same over time. The control group showed that HSP90B1 expression decreased sharply from Ref1 to Ref2 time point then remained the same (Figure 10-19).

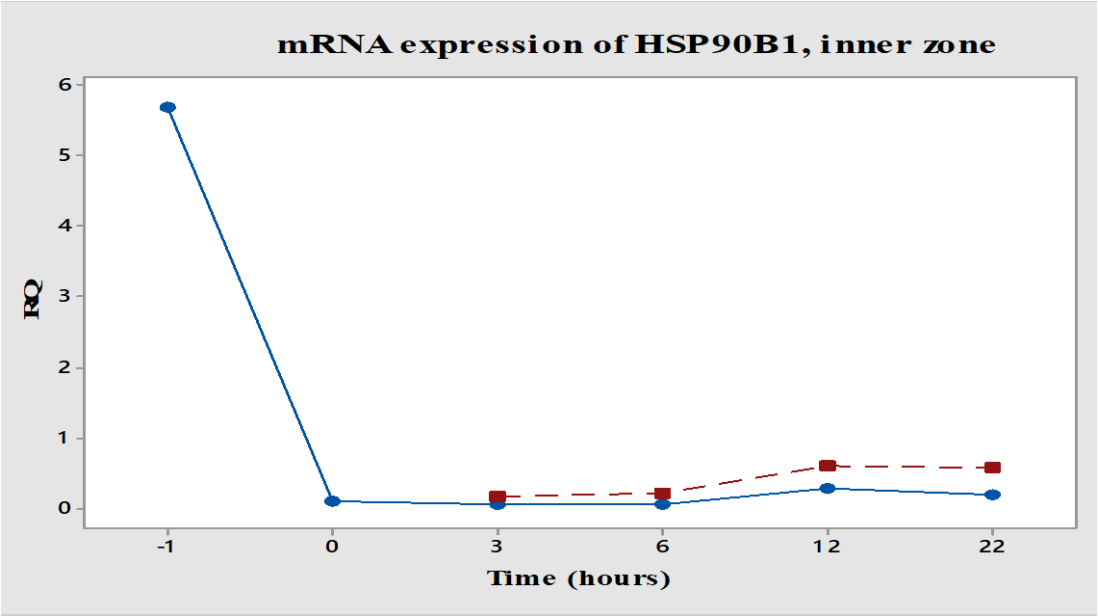


Figure 10-19: The graph shows the interaction plot analysis of the data for HSP90B1 mRNA at the inner zone. The graph shows data of the samples exposing to hypoxia-reperfusion (Red dotted line, YES) compared to the control samples (Blue continuous line, NO) at different time points.

Experiment 2B: This experiment was designed to test if there was any difference in HSP90B1 mRNA expression between tissues exposed to hypoxia reperfusion at the middle zone of placentas at four different time points. The interaction plot showed that mRNA expression of HSP90B1 in the treated group increased slightly after 3 h until 6 h before it fell. The control group showed that there was a slight increase in HSP90B1 expression from Ref1 to Ref2 time point and then there was a sharp increase from 6-12 h before falling (Figure 10-20).

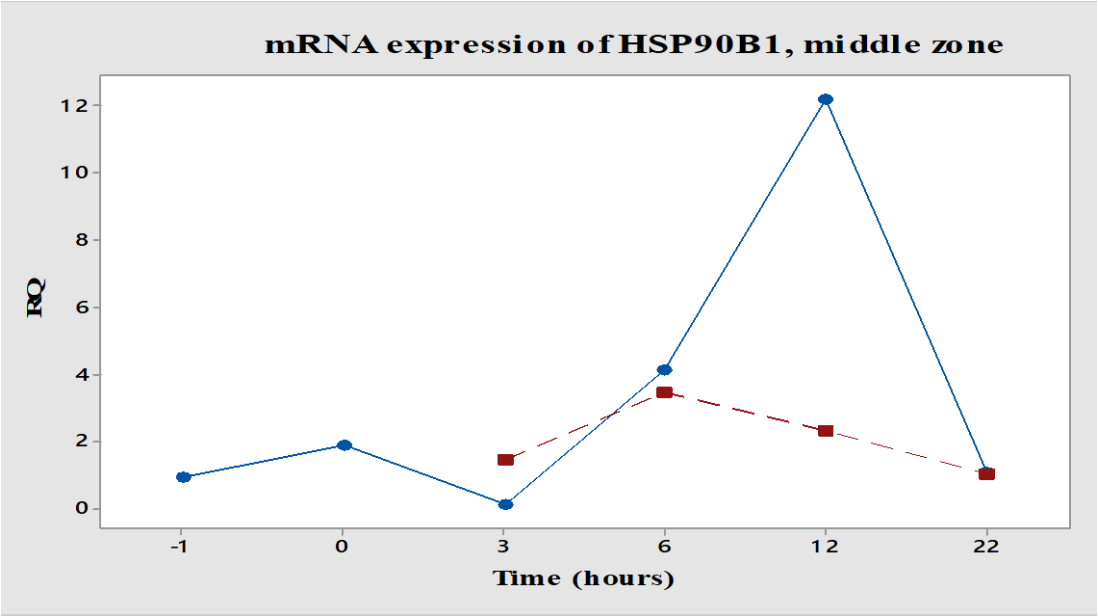


Figure 10-20: The graph shows the interaction plot analysis of the data for HSP90B1 mRNA at the middle zone. The graph shows data of the samples exposing to hypoxia-reperfusion (Red dotted line, YES) compared to the control samples (Blue continuous line, NO) at different time points.

Experiment 1C: This experiment was designed to test if there was any difference in mRNA expression of HSP90B1 between tissues exposed to hypoxia-reperfusion at the outer zone of placentas at four different time points. The interaction plot showed that mRNA expression of HSP90B1 in the treated group showed a sharp increase after 3 h until 6 h before falling. The control group showed that there was a slight increase in HSP90B1 expression from Ref1 to Ref2 time point and 6-12 h then decreased (Figure 10-21).

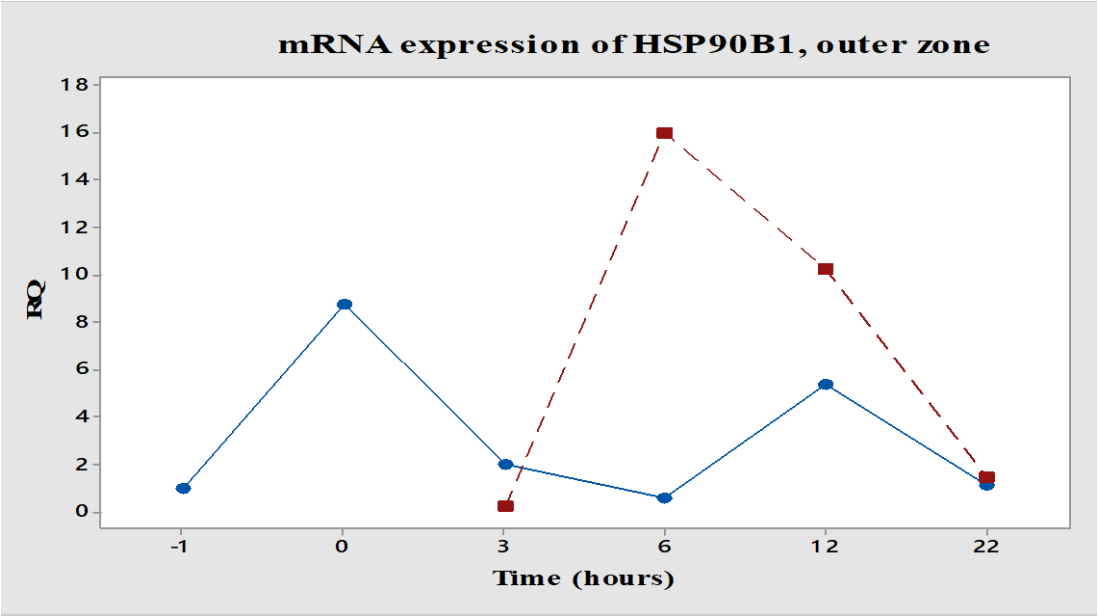


Figure 10-21: The graph shows the interaction plot analysis of the data for HSP90B1 mRNA at the outer zone. The graph shows data of the samples exposing to hypoxia-reperfusion (Red dotted line, YES) compared to the control samples (Blue continuous line, NO) at different time points.

10.3 Discussion

10.3.1 IR experiment and present findings

HSP70: There was no significant difference in mRNA and protein expression of HSP70 in the IR experiment over the time periods studied. HSF1: There was also no significant difference in mRNA expression of HSF1.

HSP27: There was a significant decrease in HSP27 protein expression (all zones) and mRNA (inner zone) over time, the letter perhaps due to protein turnover. There was no significant difference in protein expression of HSP27 due to hypoxia-reperfusion.

HSP90AA: There was a significant decrease in mRNA expression of HSP90AA over time only in the inner zone. There was no significant difference in mRNA expression of HSP90AA due to hypoxia-reperfusion. HSP90B1: there was a significant increase in mRNA expression of HSP90B1 due to hypoxia-reperfusion over time in the inner zone.

10.3.2 IR experiment and key findings

IR injury is a transient ischemia which when followed by reperfusion induces tissue damage. Several HSPs protect cells during IR injury and allow greater capacity for increased work during acute inotropic challenge; for example in the heart, HSP20 protects cardiac cells (Pinz et al., 2008) whereas HSPB5 protects diastolic contractile performance during IR (Hu et al., 2008).

There are several studies which have examined the effect of hypoxia on cultured trophoblasts (Hung et al., 2002, Hardy and Yang, 2002, Newby et al., 2005). Trophoblast cells remain viable for up to 72h in hypoxic conditions and CTB differentiation into syncytium is inhibited by exposure to hypoxia (Nelson et al., 1999, Newby et al., 2005).

In the present study, there were no significant changes in both protein and mRNA expression of (HSP27 and HSP70) and mRNA expression of (HSP90AA and HSF1) expression due to hypoxia-reperfusion. HSP90B1 expression showed significant increased due to the IR insult. However, there was significant decrease in HSP27 and HSP90AA expression over time but at different placenta zones.

Although these data are very preliminary there was an obvious increase in HSP70, HSP27 and HSP90AA mRNA expression when exposed to IR at the 6 h point. Future experiments should follow this up to determine if this is a real statistical effect.

The important point to highlight here is placental zonal variation in responding to IR insult. Our previous study (Abdulsid et al., 2013b) was to investigate protein and mRNA expression HSP70 in the labour and PE groups. In the labour HSP70 protein expression was higher in the labour group compared to the non-labour at the middle zone and there were no significant changes in HSP70 mRNA expression. In the PE group HSP70 protein was increased in non-labour PE group compared to the control group at the inner zone and no changes were noted at the mRNA level. In the IR experiment, protein expression of HSP70 a little higher in the treated group compared to the control at the 6 h time at the middle zone. There was a high mRNA expression of HSP70 in the treated group at all zones at time 6 h. These findings highlighted that IR insult had an effect on HSP70 expression at the 6 h time but in different placental zones.

Another study from our group (Abdulsid et al., 2013a, Abdulsid and Lyall, 2014) involved investigating protein and mRNA expression HSP27 in the labour and PE groups. In the

non-labour group HSP27 protein and mRNA expression was increased compared to the labour group at the inner (only mRNA) and middle zone (both). In the PE group, HSP27 protein expression was increased in the labour PE compared to the control at the middle and outer zones. No changes were found at the mRNA level. In the IR experiment protein expression of HSP27 was a slightly increased in the treated group compared to the control group on the 6 h time at the inner zone but there was a high mRNA expression of HSP27 in the treated group at the middle and outer zones at time 6 h. Again, these finding showed that IR had an effect on HSP27 expression especially at 6 h at middle and outer placental zones.

Other study from our group (chapter VII) involved investigating mRNA expression HSP90AA and HSP90B1 in the labour and PE groups. In the labour both HSP90AA and HSP90B1 mRNA expression was increased in the labour group compared to the non-labour group at the outer zone. In the PE group, there was no difference in HSP90AA and HSP90B1 mRNA expression. In the IR experiment, there was an increased in HSP90B1 mRNA expression due to IR effect at the inner zone. HSP90AA mRNA expression significantly decreased over time. This study showed a clear link between IR and increased HSP90B1 expression at the inner zone. It also showed a decreased HSP90AA expression over time again at the inner zone.

Another study (chapter III) involved investigating protein and mRNA expression of HSF1 in the labour and PE groups. At the molecular level, in the labour group, there was a significant increase in mRNA expression of HSF1 compared to the non-labour at the inner zone. In the PE group, there was high HSF1 mRNA expression in the non-labour PE group compared to the controls. In the IR experiment there was no changes over period of time due to the IR insult in any placental zones.

10.3.3 Placental oxidative stress in labour and PE

Oxidative stress is associated with activation of several pathways such as p38, MAPK, and the NF-κB signalling pathways. Oxidative stress also increase production of TNF-α, IL-1β and apoptotic changes localized principally in the STB (Hung et al., 2002, Hung et al., 2004, Bainbridge et al., 2006, Cindrova-Davies et al., 2007a). Antioxidant can block these changes and this suggesting role for ROS (Cindrova-Davies et al., 2007a)

Pregnancy is associated with an increase rate of lipid peroxidation and oxidative stress compared with non-pregnant women (Kaur et al., 2008). The placenta initially developed

in a hypoxic environment but toward end of first trimester, its vascularization develops and the circulation starts, with this comes an increase in the oxygen concentration. When mitochondria are exposed to high maternal oxygen levels the result will be increased production of ROS and the start of the oxidative stress process (Siddiqui et al., 2010).

There are many evidences for placental oxidative stress in labour and PE. Uterine contractions during labour are associated with intermittent utero-placental blood perfusion providing the basis for IR type injury to the placenta. 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). In addition, labour is also associated with placental alterations in several pathways linked to oxidative stress (Cindrova-Davies et al., 2007b). In PE spiral artery remodelling is partial or incomplete (Lyall, 2006) and perfusion by intermittent pulses of fully oxygenated arterial blood is thought to lead to fluctuations in oxygen delivery resulting in oxidative stress (Burton and Jauniaux, 2011). In PE, there are several evidences for oxidative stress including increased concentrations of protein carbonyls, lipid peroxides, nitrotryosine residues and DNA oxidation. Our group has shown that HSP27 and HSP70 are selectively up-regulated in labour and PE suggesting a link to placental oxidative stress (Abdulsid et al., 2013b, Abdulsid and Lyall, 2014).

10.3.4 Placenta ER and the inflammatory response

The ER is where synthesis of polypeptide hormones, growth factors and plasma membrane proteins and their post-translational modification takes place. IR and hypoxia induces oxidative stress and activates ER stress (Yung et al., 2007), through three ways: the first one by disturbance of calcium homeostasis as well as increased generation of ROS through mitochondrial pathways or by proteolytic cleavage of xanthine dehydrogenase to the xanthine oxidase form, which is higher in PE (Halliwell, 2005, Burton et al., 2009b). Secondly, the UPR can activate same intracellular inflammatory signalling pathways (Xu et al., 2005, Zhang and Kaufman, 2008). Thirdly, protein folding is an oxidative event that also generates ROS (Tu and Weissman, 2004). Ire1 activates the NF-kB pathway through phosphorylation of IKK, and the p38 MAPK pathway through ASK1 (Burton et al., 2009b).

In vitro, exposing placenta explants to IR generates oxidative stress and increased cytokine release such as TNFα, sFLT-1 and IL-1β (Hung et al., 2001, Hung et al., 2004, Cindrova-

Davies et al., 2007a). These changes can blocked by inhibition of the p38 and NF-κB pathways or addition of anti-oxidant vitamins C and E (Cindrova-Davies et al., 2007a, Cindrova-Davies, 2009).

Apoptosis and necrosis are common outcomes of IR injury. Apoptosis increased in villous trophoblast on exposure to oxygen concentrations less than 2% (Kilani et al., 2003). During the reperfusion phase, ROS are generated within mitochondria and also through the actions of enzymes such as xanthine oxidase (Collard and Gelman, 2001). Imbalances of ROS lead to loss of Ca^{+2} homeostasis, and promote opening of the mitochondrial pore lead to release of cytochrome *c* and activation of caspase pathways (Kowaltowski et al., 2001, Hung et al., 2002).

Chapter XI: Heat shock tissue culture experiment and investigation of HSPs and caspases at the inner, middle and outer placental zones.

11 Heat shock tissue culture experiment

11.1 Introduction

Heat shock is effect of exposing a tissue to a higher temperature than the physiological temperature. Hyperthermia induces complex cellular responses including protein aggregation, misfolding or unfolding due to the interactions of the exposed hydrophobic groups (Roti Roti, 2008). In addition, hyperthermia effects also include organelle fragmentation, changes in protein associations, and ROS generation (Roti Roti, 2008, Bruskov et al., 2002). Furthermore, hyperthermia may cause stalling of the DNA replication fork and double strand breaks, micronucleus formation, chromosome aberrations and inhibition of DNA repair processes (Roti Roti, 2007, Hintzsche et al., 2012, Eppink et al., 2012).

Heat shock induces the expression of the highly conserved HSPs, which belong to a superfamily sharing a protection function against cellular stress. In cells, there are basal levels of HSPs (constitutive HSPs). HSPs can also be induced by several pathological or stressful situations (inducible HSPs) to protect cells from stress (Lanneau et al., 2010). HSPs assist in protein refolding and salvage, as well as disaggregation of inappropriate protein collectives, and will promote the degradation of unrecoverable proteins (Saibil, 2013). Sub-lethal hyperthermia increases expression of several HSPs, which are consistent with the cellular need for protein repair and stabilization (Dong and Dong, 2008).

Hyperthermia is used in combination with anticancer drugs to increase their cytotoxicity (Sottile et al., 2015). One of the important results of hyperthermia is the producing of HSPs, which are able to associate with DNA repair pathways (Sottile et al., 2015). When tumour cells were exposed to sub-lethal heat shock prior to the chemotherapy, the latter was less effective (Kiang et al., 1998).

11.2 Results

As time was limited one set of experiments was performed for all the data shown below, with a view to zooming in on particular time points should any obvious trends be spotted. As will be evident there appeared to a trend for differences to be apparent around 6 h and this would require future experiments.

11.2.1 Analysis of protein and mRNA expression of HSP70 in the heat shock tissue culture experiment.

Experiment 1A, protein analysis: This experiment involved exposing placenta samples to 43° C for 30 min and then 37° C for different time periods. The control samples were exposed to 37° C only. Five time points (1, 3, 6, 12 and 24 h) were used for recovery. HSP70 protein expression was investigated using Western blot analysis. HSP70 mRNA was investigated using RT-qPCR. Interaction plots were prepared to demonstrate the findings. Examples of Western blots showing HSP70 expression for the set of experiments performed on placental tissue obtained from the inner zone are shown in (Figure 11-1). The interaction plot showed that expression of HSP70 in the heat shocked group decreased from 1-3 h then there was a sharp increase until 6 h when it falls down and then increased slightly. The control group showed that HSP70 expression increased slowly from 0-1 h after that it decreased gradually until 6 h when there was a slow increase from 6-12 h before it falls down after 12 h (Figure 11-1). Overall because this is based on one placenta it appears that the only clear change appeared to be the increase in HSP70 at the 6 h in the treatment group.

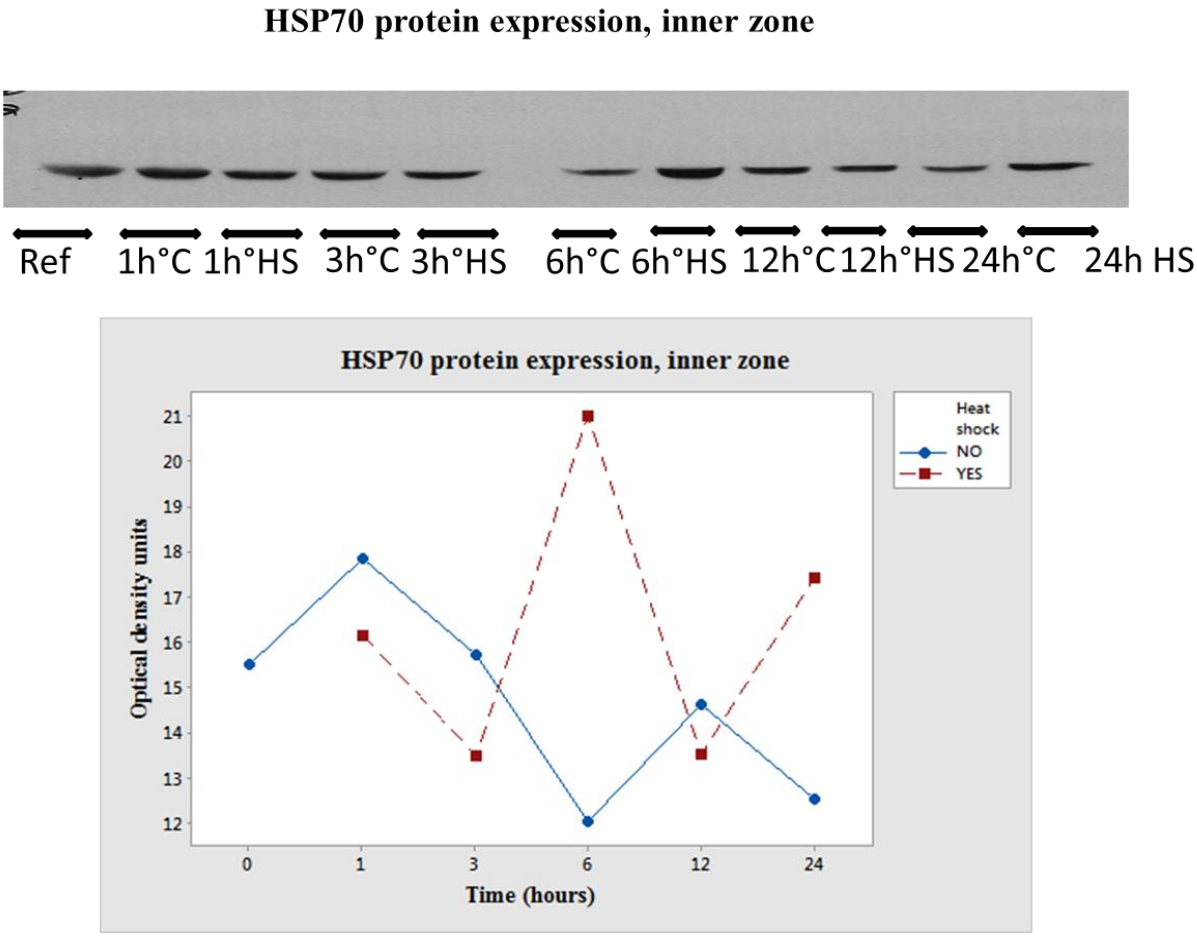


Figure 11-1: Representative image of Western blot showing HSP70 expression (upper panel) in the inner placental zone. Ref is the sample collected in the labour suite. Different time points (1, 3, 6, 12 and 24 h) of recovery at 37° C were studied. Graphs show the interaction plots analysis of the data for HSP70 protein expression at the inner zone. Samples exposed to heat shock are shown as a (red dotted line, YES) and compared to the control samples (Blue continuous line, NO) at different time points.

Experiment 1 B: This experiment involved exposing placenta samples to 43°C for 30 min and then recovery at 37° C for different time periods. The control samples were exposed to 37° C only. Examples of Western blots showing HSP70 expression for the set of experiments performed on placental tissue obtained from the middle zone are shown in (Figure 11-2). The interaction plot showed that protein expression of HSP70 in heat shocked group was decreased until 3 h then was increased sharply, after 6 h and there was a steady decrease until 24 h. The control group showed that HSP70 expression was increased until 1 h then decreased gradually until 6 h where there was a slight increase before it falls down again. Both group showed sudden decrease after 1h with sharp increase after 3 h for the heat shocked group and slight increase after 6 h for the control group before both groups showed fall down in HSP70 expression after 12 h (Figure 11-2).

Overall because this is based on one placenta and again it appears that the only clear change appeared to be the increase in HSP70 at 6 h in the treatment group.

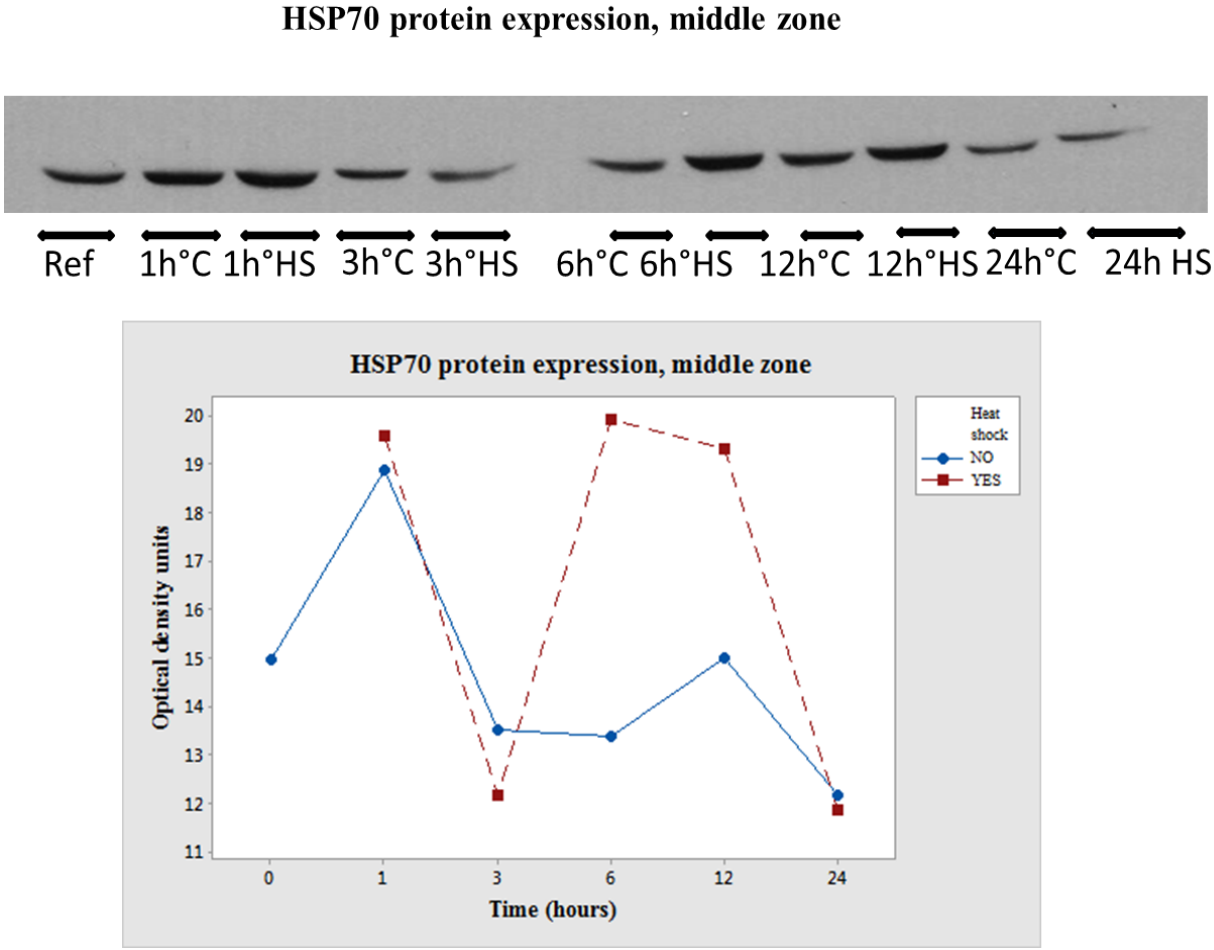


Figure 11-2: Representative image of Western blot showing HSP70 expression (upper panel) in the middle placental zone. Ref is the sample collected in the labour suite. Different time points (1, 3, 6, 12 and 24 h) of recovery at 37° C were performed. Graphs show the interaction plots analysis of the data for HSP70 protein expression at the middle zone. Samples exposed to heat shock are shown as a (red dotted line, YES) and compared to the control samples (Blue continuous line, NO) at different time points.

Experiment 1 C: This experiment involved exposing placenta samples to 43° C for 30 min and then 37° C for different time periods. The control samples were exposed to 37° C only. Examples of Western blots showing HSP70 expression for the set of experiments performed on placental tissue obtained from the outer zone are shown in (Figure 11-3). Interaction plot showed that expression of HSP70 in the heat shocked group slightly increased from 1-6 h then decreased gradually over time. In the control group HSP70 expression was decreased suddenly from 0-1 h then increased slightly between 1-3 h and 6-

12 h before it decreased again (Figure 11-3). Overall both groups show that expression of HSP70 was reduced, perhaps due to protein turnover.

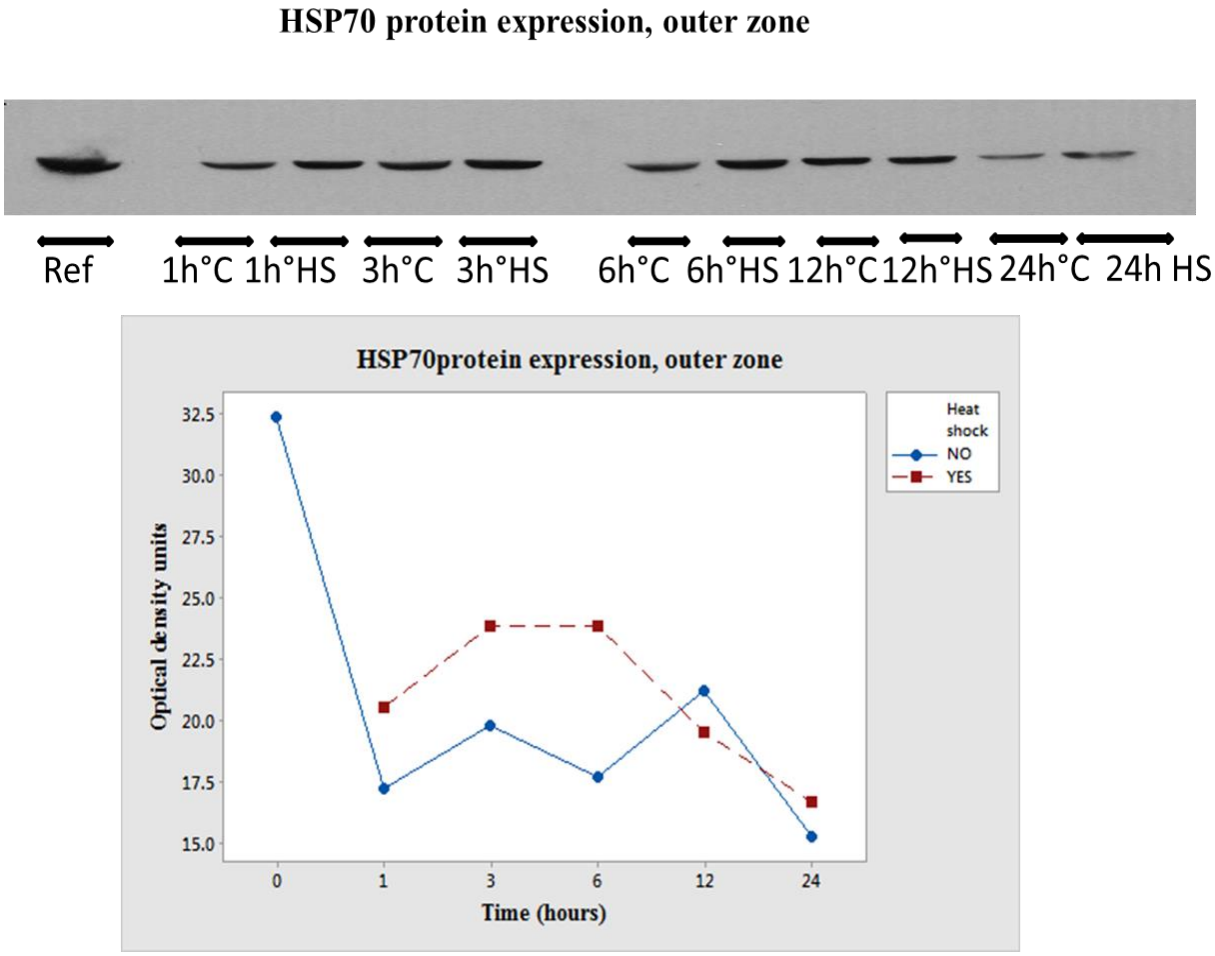


Figure 11-3: Representative image of Western blot showing HSP70 expression (upper panel) in the outer placental zone. Ref is the sample collected in the labour suite. Different time points (1, 3, 6, 12 and 24 h) of recovery at 37° C were studied. Graphs show the interaction plots analysis of the data for HSP70 protein expression at the outer zone. Samples exposed to heat shock are shown as a (red dotted line, YES) and compared to the control samples (Blue continuous line, NO) at different time points.

Experiment 2 A mRNA analysis: This experiment involved exposing placenta samples to 43° C for 30 min and then 37° C for different time periods. The control samples were exposed to 37° C only. Five time points (1, 3, 6, 12 and 24 h) were used for recovery. HSP70 mRNA was investigated using RT-qPCR. Interaction plots were used to demonstrate the findings. (Figure 11-4) shows mRNA expression of HSP70 for the set of experiments performed on placental tissue obtained from the inner zone. The interaction plot showed that mRNA expression of HSP70 in heat the shocked group increased sharply from 3-6 h then decreased gradually over time. The control group showed that mRNA

expression of HSP70 continued without changes over the time (Figure 11-4). Overall because this is based on one placenta and again it appears that the only clear change appeared to be the increase in HSP70 at 6 h in the treatment group.

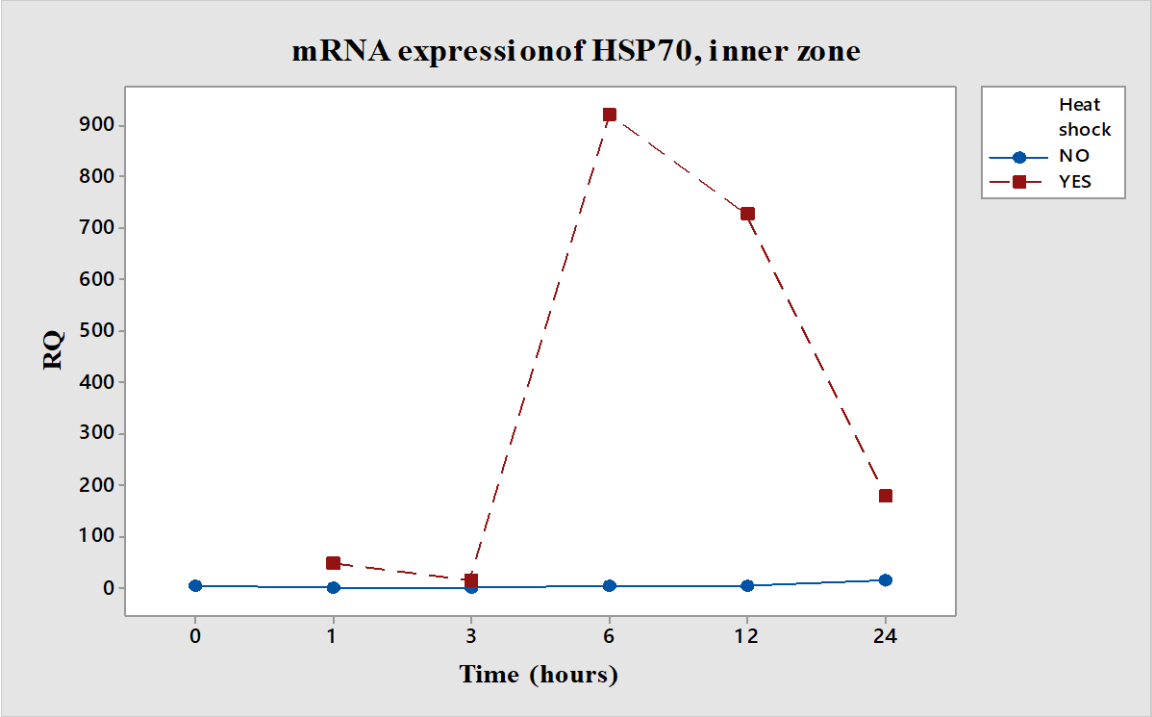


Figure 11-4: The graphs show the interaction plots analysis of the data for HSP70 mRNA at the inner zone. The graph shows data of the samples exposing to heat shock (Red dotted line, YES) compared to the control samples (Blue continuous line, NO) at different time points.

Experiment 2 B: This experiment involved exposing placenta samples to 43° C for 30 min and then 37° C for different time periods. The control samples were exposed to 37 ° C only. (Figure 11-5) shows HSP70 mRNA expression for the set of experiments performed on placental tissue obtained from the middle zone. The interaction plot showed that mRNA expression of HSP70 in heat shock group was increased sharply after 3 h then it decreased sharply after 6 h. The control group showed that there was a slight increase in HSP70 expression after 3 h then before decreased after 6 h (Figure 11-5). Overall both groups show that there was increase of HSP70 expression after 3 h until 6 h sharply for heat shocked group and slightly for the control group. Overall because this is based on one placenta and again it appears that the only clear change appeared to be the increase in HSP70 at 6 h in the treatment group.

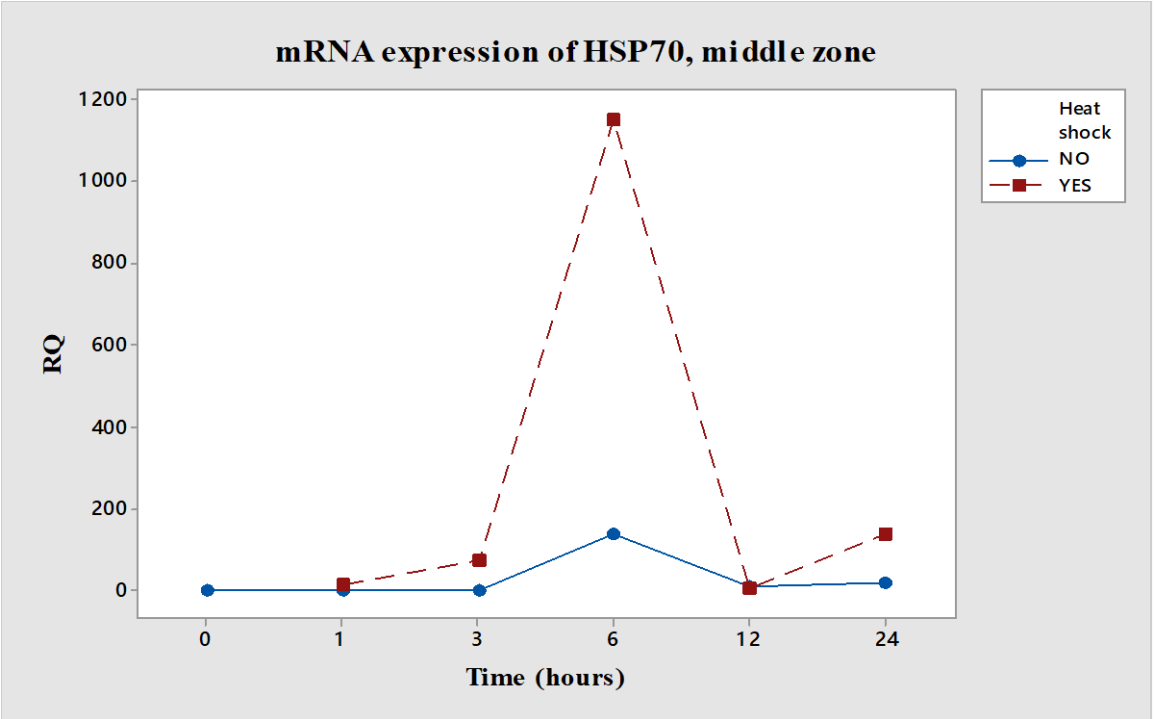


Figure 11-5: The graphs show the interaction plots analysis of the data for HSP70 mRNA at the outer zone. The graph shows data of the samples exposing to heat shock (Red dotted line, YES) compared to the control samples (Blue continuous line, NO) at different time points.

Experiment 2 C: This experiment involved exposing placenta samples to 43° C for 30 min and then 37° C for different time periods. The control samples were exposed to 37° C only. (Figure 11-6) shows HSP70 mRNA expression for the set of experiments performed on placental tissue obtained from the outer zone. The interaction plot showed that mRNA expression of HSP70 in heat shocked group decreased at 1 h then increased slightly at the 3 h before it decreased again at 6 h, at the 12 h mRNA expression of HSP70 showed that there was a sharp increase. The control group showed slight changes in mRNA expression of HSP70 with slight increase at the 3 h before it decreased again at the 6 h (Figure 11-6). Overall both groups showed that there was a small increase of HSP70 expression between 3-6 h then at the 12 h there was a large increase in the heat shocked group compared to the control group that showed a small increase.

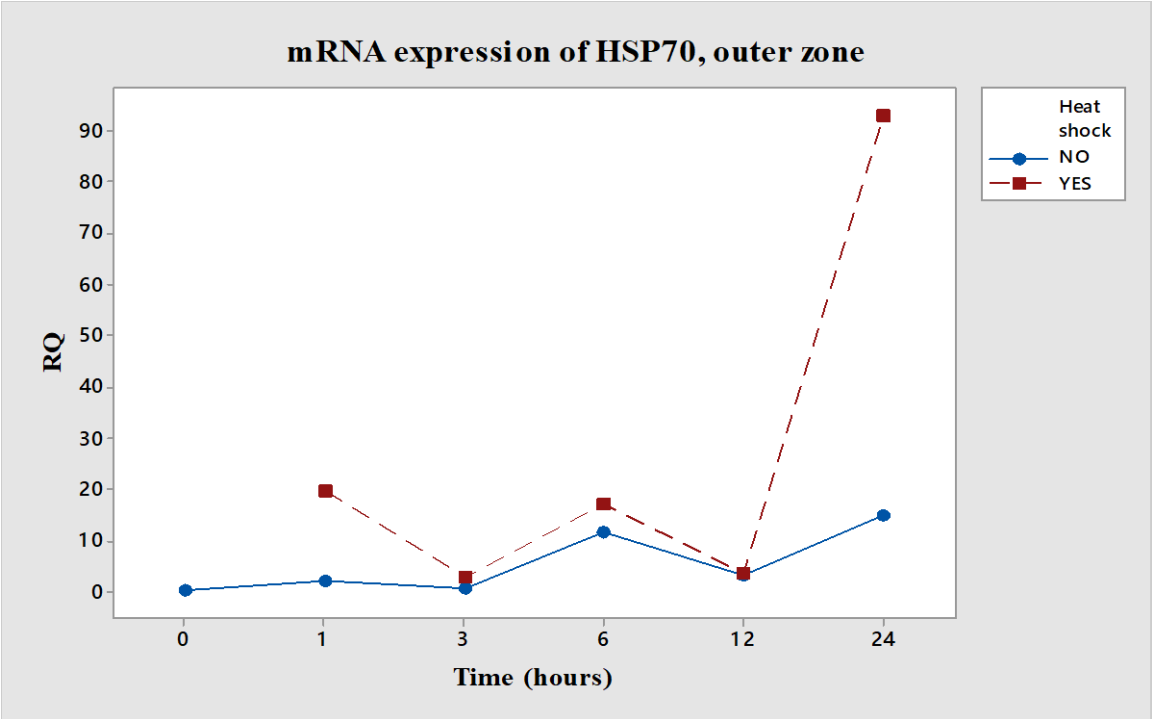


Figure 11-6: The graphs show the interaction plots analysis of the data for HSP70 mRNA at the outer zone. The graph shows data of the samples exposing to heat shock (Red dotted line, YES) compared to the control samples (Blue continuous line, NO) at different time points.

11.2.2 Analysis of protein and mRNA expression of HSP27 in the heat shock tissue culture experiment.

The experiments below are the same as those above for HSP70 and therefore less text detail are included.

Experiment 1 A, protein analysis: Examples of Western blots showing HSP27 expression for the set of experiments performed on placental tissue obtained from the inner zone are shown in (Figure 11-7). The interaction plot showed that expression of HSP27 in the heat shocked group was reduced from 1-3 h then increased after 3 h; the peak was at the 6 h and then decreased over time. The control group showed that HSP27 expression was decreased gradually from time period 0-6 h then slight increased after 6 h. Overall both groups show that there was a decrease in HSP27 expression over the time, perhaps due to protein turnover. There was a sharp increase in HSP27 expression in heat shocked group at the 6 h time point (Figure 11-7).

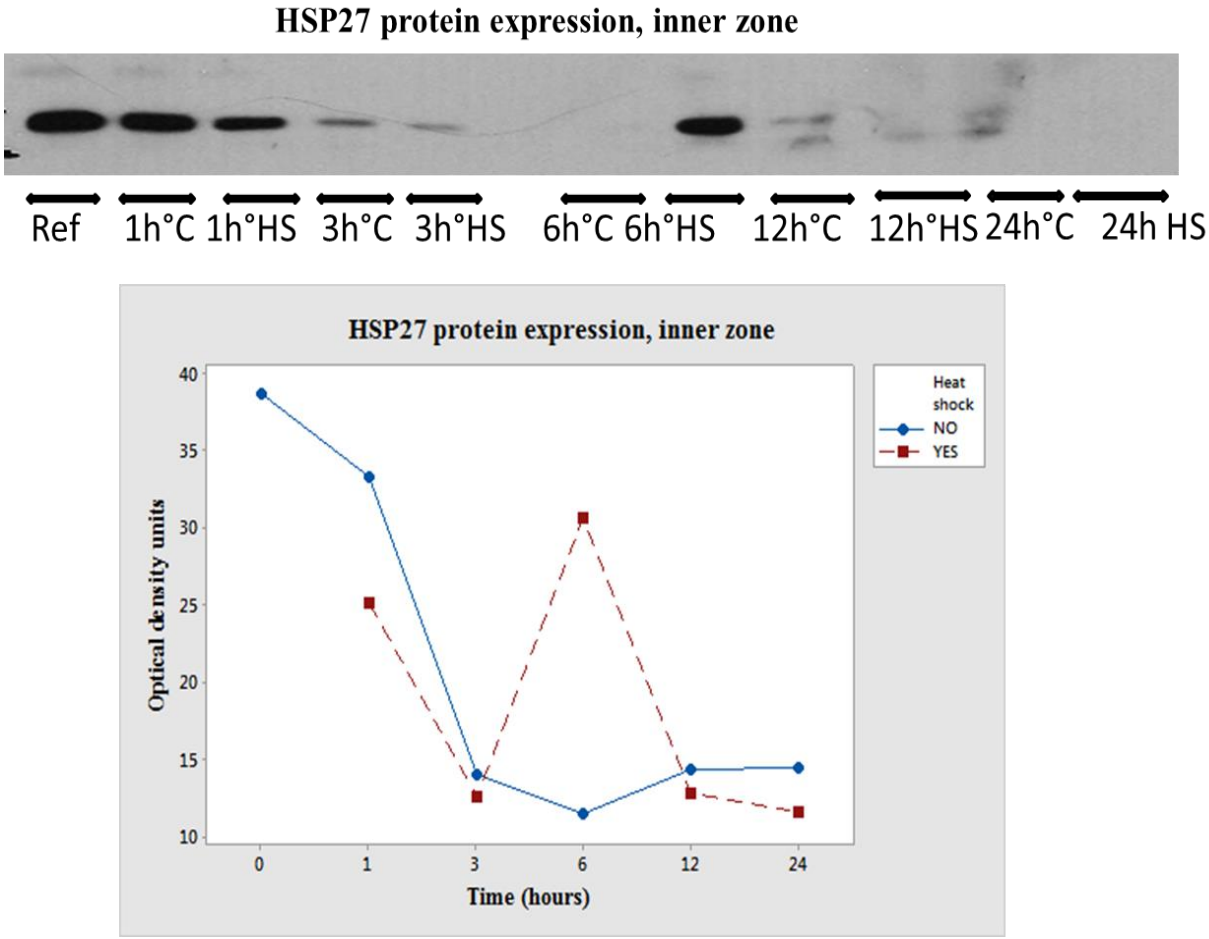


Figure 11-7: Representative image of Western blot showing HSP27 expression (upper panel) in the inner placental zone. Ref is the sample collected in the labour suite. Different time point (1, 3, 6, 12 and 24 h) of recovery at 37 °C was performed. Graphs show the interaction plots analysis of the data for HSP27 protein expression at the inner zone. Samples exposed to heat shock are shown as a (red dotted line, YES) and compared to the control samples (Blue continuous line, NO) at different time points.

Experiment 2 B: The interaction plot showed that expression of HSP27 in the heat shocked group was increased from 1-3 h then reduced sharply until 6 h after that there was a gradual decrease. The control group showed that HSP27 expression was decreased sharply from time period 0-3 h then gradually over time. Overall both groups show that there was a decrease in HSP27 expression over the time perhaps due to protein turnover, with slight increase in HSP27 expression of heat shocked group between 1-3 h of recovery period (Figure 11-8).

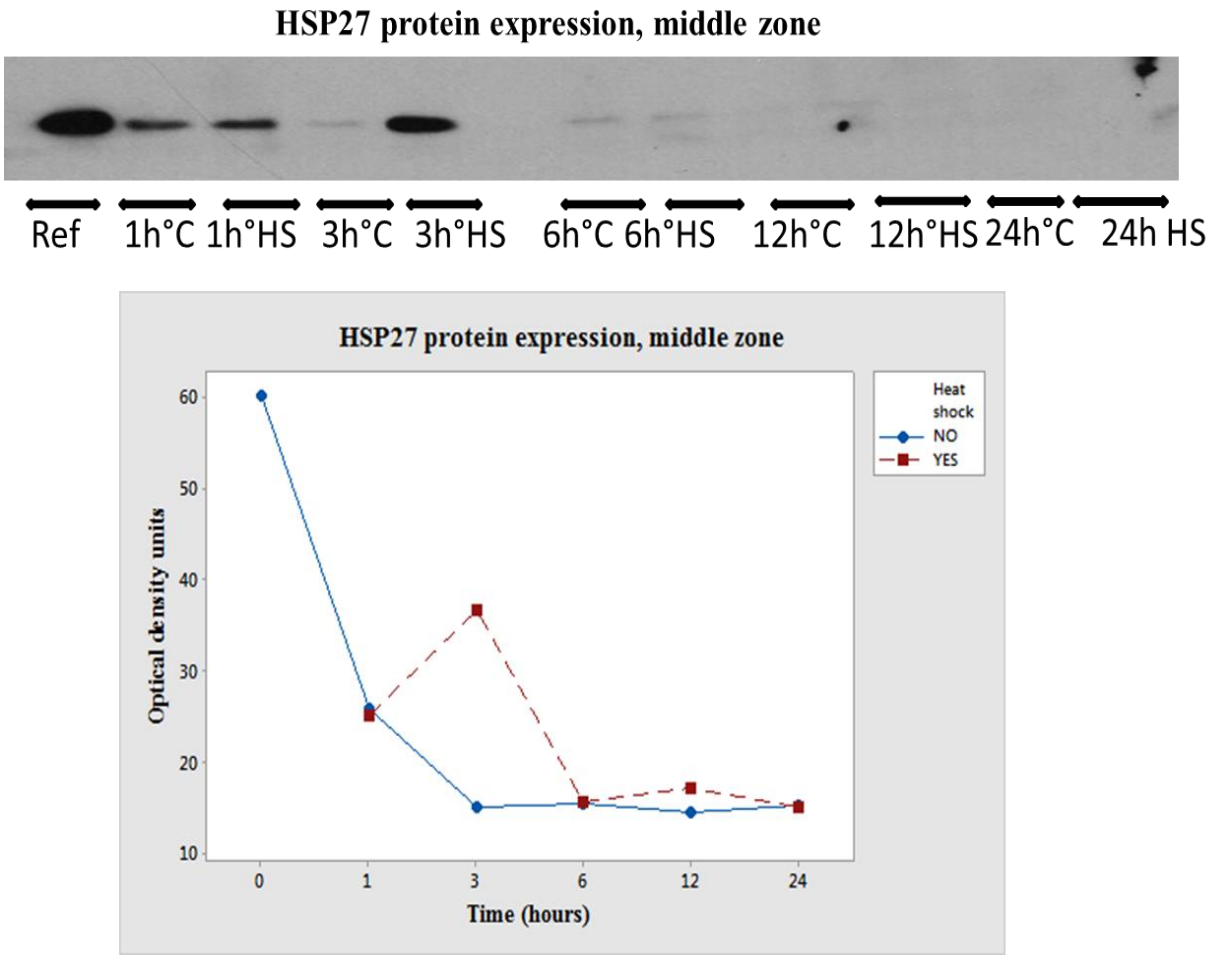


Figure 11-8: Representative image of Western blot showing HSP27 expression (upper panel) in the middle placental zone. Ref is the sample collected in the labour suite. Different time points (1, 3, 6, 12 and 24 h) of recovery at 37 ° C were performed. Graphs show the interaction plots analysis of the data for HSP27 protein expression at the middle zone. Samples exposed to heat shock are shown as a (red dotted line, YES) and compared to the control samples (Blue continuous line, NO) at different time points.

Experiment 2 C: Interaction plot showed that expression of HSP27 in heat shocked group was reduced over the recovery period with a slight increase from 3-6 h. In the control group HSP27 expression was decreased from 0-1 h then slight reduced over the time (Figure 11-9). Overall no major findings were observed with heat shock.

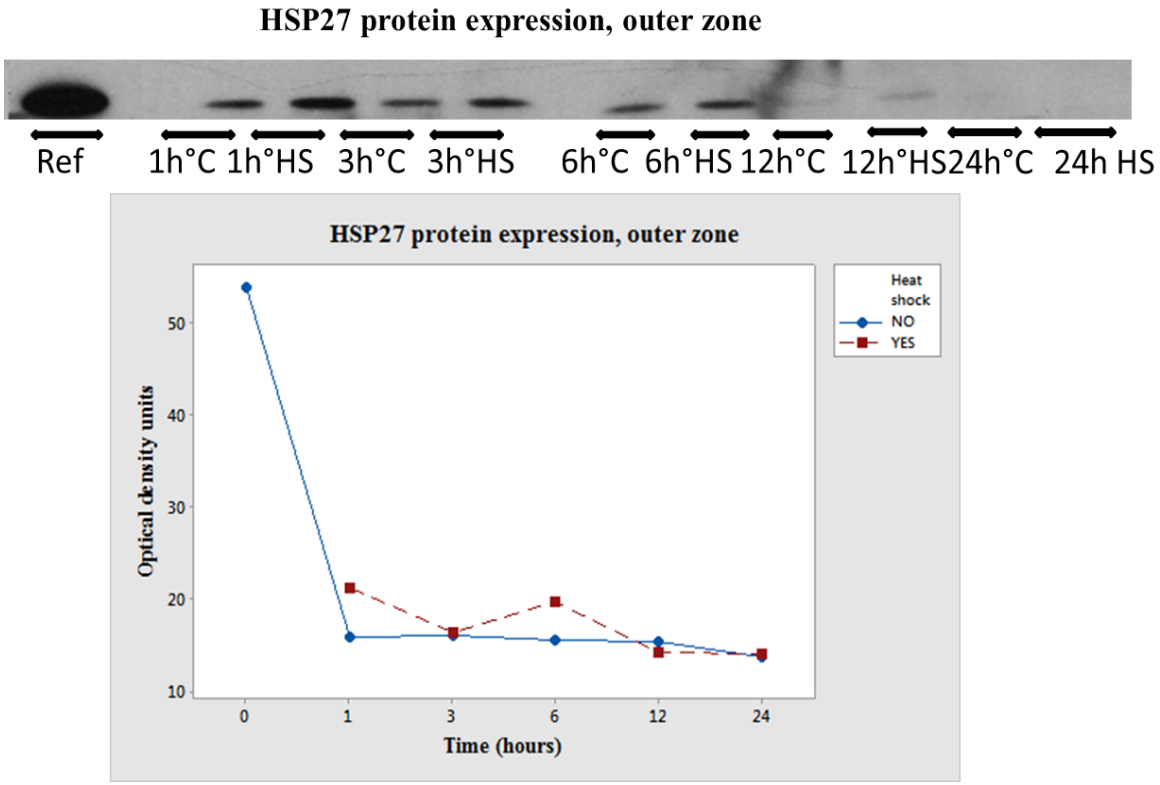


Figure 11-9: Representative image of Western blot showing HSP27 expression (upper panel) in the outer placental zone. Ref is the sample collected in the labour suite. Different time points (1, 3, 6, 12 and 22 hours) of recovery at 37 °C were performed. Graphs show the interaction plots of the data for HSP27 protein expression at the outer zone. Samples exposed to heat shock are shown as a (red dotted line, YES) and compared to the control samples (Blue continuous line, NO) at different time points.

Experiment 2 A: (Figure 11-10) shows mRNA expression of HSP27 for the set of experiments performed on placental tissue obtained from the inner zone. Interaction plot showed that mRNA expression of HSP27 in the heat shocked group was very high at 6 h then there was a sharp reduction. The control group showed that mRNA expression of HSP27 was reduced sharply from 0-1 h.

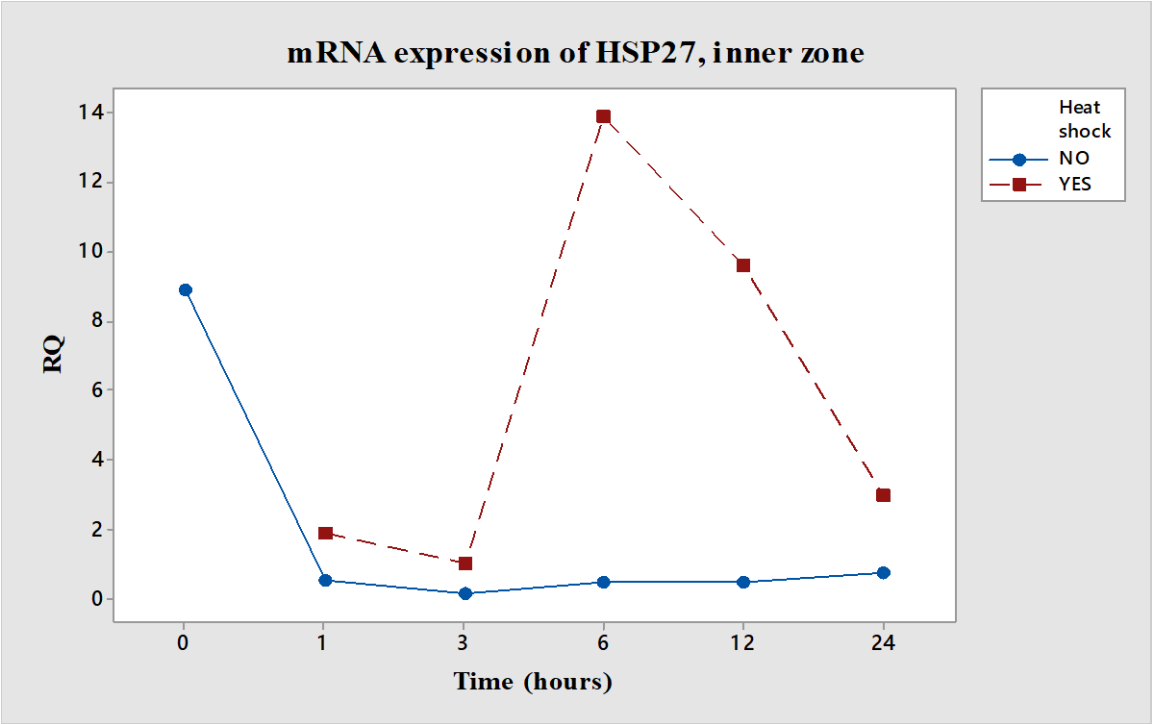


Figure 11-10: The graphs show the interaction plots analysis of the data for HSP27 mRNA at the inner zone. The graph shows data of the samples exposing to heat shock (Red dotted line, YES) compared to the control samples (Blue continuous line, NO) at different time points.

Experiment 2 B: The interaction plot showed that mRNA expression of HSP27 in the heat shocked group increased markedly at 6 h then there was a sharp reduction. The control group showed that mRNA expression of HSP27 was reduced from 0-3 h then increased slightly until 6 h before dropping down again (Figure 11-11).

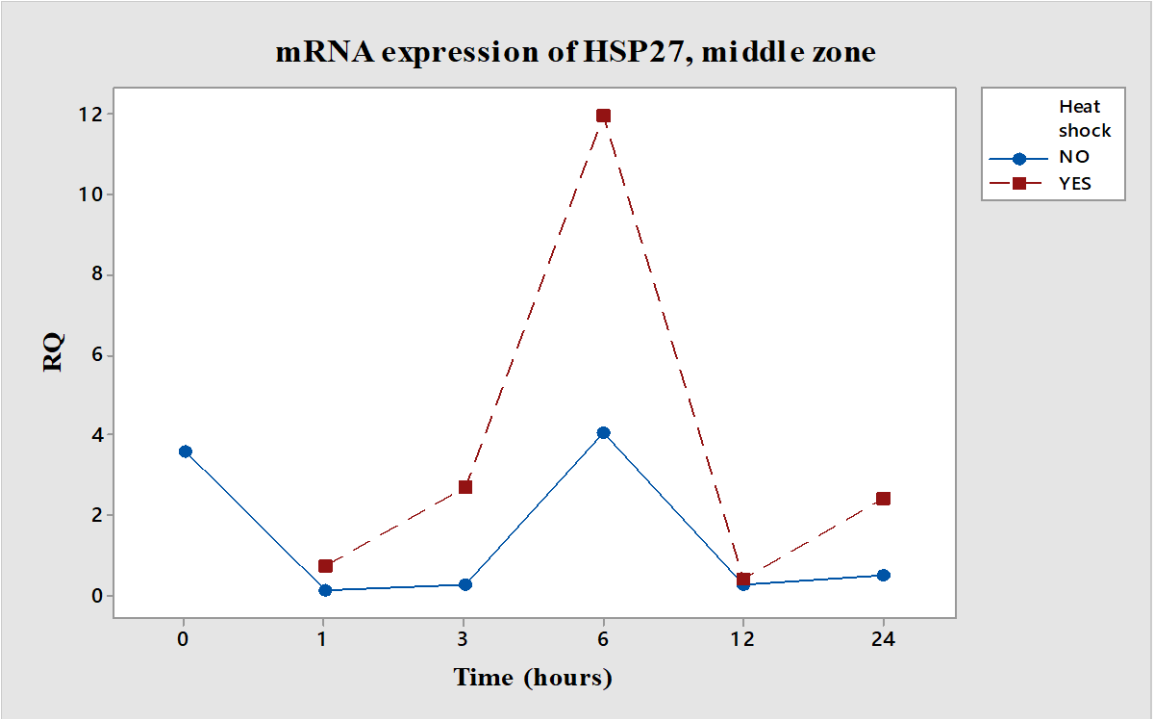


Figure 11-11: The graphs show the interaction plots analysis of the data for HSP27 mRNA at the middle zone. The graph shows data of the samples exposing to heat shock (Red dotted line, YES) compared to the control samples (Blue continuous line, NO) at different time points.

Experiment 2 C: The interaction plot showed that mRNA expression of HSP27 in the heat shocked group decreased sharply from 1-3 h then slightly increased until 6 h then decreased at 12 h there was a sharp increased. The control group showed that mRNA expression of HSP27 was reduced from 0-3 h then increased slightly until 6 h before drop down again.at the 12 h there was a slight increase (Figure 11-12).

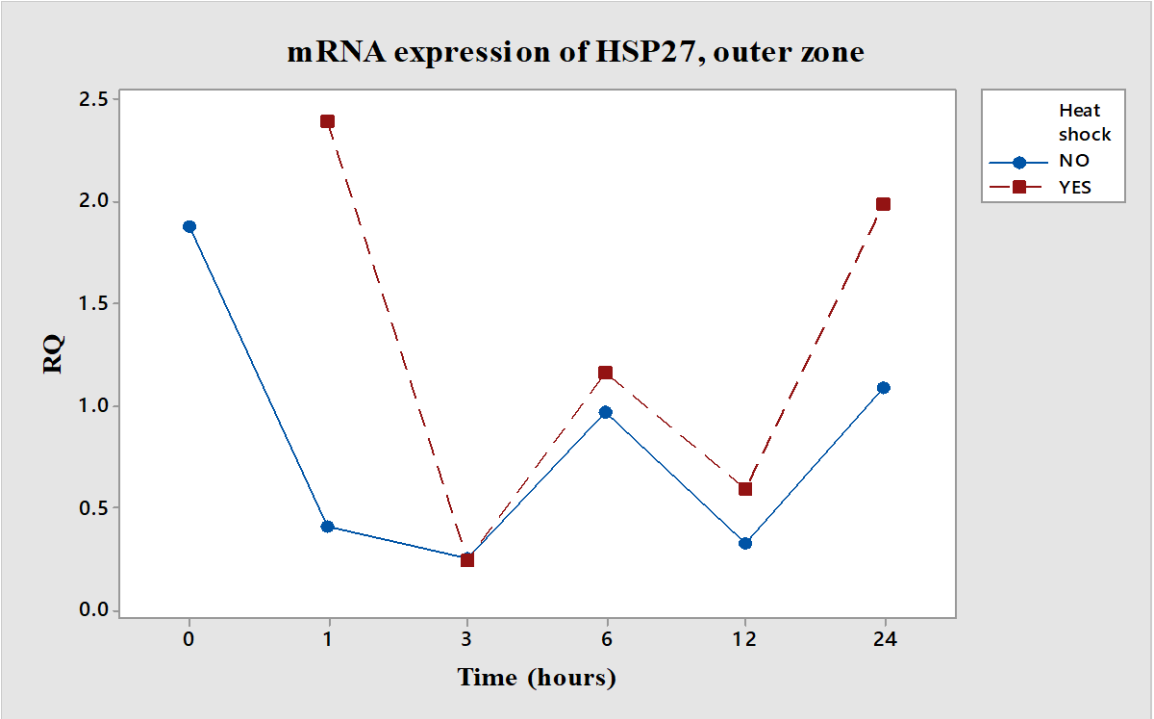


Figure 11-12: The graphs show the interaction plots analysis of the data for HSP27 mRNA at the outer zone. The graph shows data of the samples exposing to heat shock (Red dotted line, YES) compared to the control samples (Blue continuous line, NO) at different time points.

11.2.3 Several HSPs and caspases mRNA expression after exposure to the heat shock

Because there was a large volume of data, main finding will summarized in next section

11.2.3.1 HSP32:

It appears that the only clear change appeared to be the increase in HSP32 at 6 hour (h) in the treatment group.

11.2.3.2 HSP90AA:

There was an obvious increase in HSP90AA mRNA in inner, middle and outer placental zones when exposed to heat shock at the 6 h point.

11.2.3.3 HSP90B1:

It was clear that there was an increase in 3-6 h in the treated group at the inner and outer zones, however, at the middle zone the expression in the control group was slightly increased.

11.2.3.4 HSF1:

It appears that the only clear change appeared to be the increase in HSF1 at 6 h in the treatment group.

11.2.3.5 HSF2:

It appears that the only clear change appeared to be the decrease in HSF2 over time at the inner zone.

11.2.3.6 DNAJAB1:

It appears that the only clear change appeared to be the increase in DNAJAB1 mRNA in inner, middle and outer placental zones when exposed to heat shock at the 6 h point.

11.2.3.7 HSPB2:

It appears that the only clear change appeared to be the increase in HSB2 due to heat shock effect at the inner zone when exposed to heat shock at the 6h point.

11.2.3.8 HSPB6:

It appears that the only clear change appeared to be the increase in HSB6 at 6 h in the treatment group.

11.2.3.9 HSPD1:

Although these data are very preliminary there was an obvious increase in HSPD1 mRNA in inner, middle and outer placental zones when exposed to heat at the 6 h point.

11.2.3.10 CASP3:

It appears that there was an increase in mRNA expression of CASP3 due to heat shock effect at the inner zone and decrease in mRNA expression of CASP3 over time at the inner and middle zones, no changes at the outer zone.

11.2.3.11 CASP7:

There was a decrease in mRNA expression of CASP7 over time at the middle zone.

11.2.3.12 CASP9:

There were no changes in mRNA expression of CASP9 due to heat shock effect over time.

11.3 Discussion

11.3.1 HSP70 and HSP27 protein and mRNA expression and present findings

There was no significant difference in protein or mRNA expression of HSP27 and HSP70 due to heat shock. There was a significant decrease in HSP70 and HSP27 protein expression over time but at different zones.

11.3.2 Different HSPs mRNA expression and present findings

There was no significant difference in mRNA expression of HSP32, HSP90AA, HSP90B1, HSF1, DNAJAB1, HSPB6 and HSPD1 due to heat shock or time. There was a fall in HSF2 expression over time and there was a significant increase in HSPB2 due to heat shock, both at the inner zone.

11.3.3 CASPs mRNA expression and present findings

There was a fall in CASP3 and CASP7 mRNA expression over time at the middle zone. There was a significant increase in CASP3 due to heat shock at the inner zone. No changes in CASP9 were found.

There was a clear trend for increases in most HSPs and caspases mRNA expression at the 6 h time and this seems to be the key finding. As this set of work was performed close to the end of the thesis future experiments should follow this up to determine if this is a real statistical effect however, the preliminary results suggest there are.

11.3.4 Heat shock experiment

There is nothing in the literature to compare to this study; however sampling from different placental zones is a key point. Overall there was only significant increase in HSPB2 and CASP3 expression after exposure to heat shock, both at the inner zone. There was a significant decrease in HSF2, CASP3 and CASP7 over time, also at the inner zone. It seems that the inner zone is the most sensitive area to hyperthermia. Another important point was the time (6 h) for the highest expression of majority of genes that were studied.

Several studies have been carried out to investigate the effect of heat shock on HSP expression most of them on cell culture. Examples include:

Wiegant *et al* performed heat shock experiments on hepatoma cells of Reuber H35 rat (Wiegant et al., 1994). This study examined the effect of different stressors including heat shock, arsenite, cadmium, dinitrophenol and ethanol on cell survival and induction of HSPs. Heat shock was applied between 30 min to 60 min at 41° C, 41.5 ° C, 42 ° C, 42.5 ° C or 43 ° C by immersing the culture flasks in a water bath. They found that there was increased inhibition of protein synthesis as the temperature of the heat shock increased, with 10% fall at 43 ° C for 60 min. They concluded that the pattern of induced HSPs might be used as biomarker of chemical or stress exposure, however; they added that HSPs cannot be used as a general risk-assessment tool (Wiegant et al., 1994).

Ovelgonne *et al* performed heat shock experiment on Reuber H35 hepatoma cells to investigate HSF and HSP 60, HSP68 and HSP84 expression and to compare different stressful factors (Ovelgonne et al., 1995). This study showed that the rate of HSP synthesis correlated well with HSP mRNA levels and the stress response is regulated mainly at the transcriptional level (Ovelgonne et al., 1995). HSF was activated and they concluded that more processes than just HSF activations are involved in the induction of HSPs.

Kiang *et al* performed a heat shock experiment on T47-D cells, a human breast cancer cell line that expresses estrogen receptors, to investigate HSP expression by Western blotting analysis (Kiang et al., 1998). Heat shock was applied at 44° C. This study showed that there was an increased in HSP70, GRP78 and GRP94 expression in a time dependent manner and the maximal expression rate was between 2 and 4 h after heat shock. In addition, cells over expressing HSP70, GRP78 and GRP94 displayed resistance against a lethal temperature (47° C for 50 min) (Kiang et al., 1998).

Dong and Dong performed a heat shock experiment on sea cucumbers using sub-lethal and lethal temperatures between 30-40 ° C (Dong and Dong, 2008). This study found that there was an increased in survival rates of the sea cucumbers when they were exposed to 34° C, HSP70 expression level also increased and induced a thermotolerance (Dong and Dong, 2008). They concluded that there a close relationship existed between the induction of thermotolerance and the levels of HSP70.

Tazawa *et al* used heat shock to investigate HSP70 expression in microfluidic tissue culture and its relation to endothelial damage (Tazawa et al., 2015). This study reported that live cell imaging showed only minor changes in the appearance of heat-treated cells; however, increased HSP70 mRNA expression showed that the endothelial cells responded well to heat treatment (Tazawa et al., 2015). They concluded that heat-induced endothelial changes under stress and this introduces a potent tool for analysing endothelial secretions in tissue culture.

Chapter XII: Analysis of stress and apoptic pathways in placentas from four different BMI groups

12 BMI study

12.1 Introduction

The worldwide prevalence of obesity has increased markedly over the past few decades, with the World Health Organization (WHO) describing this trend as a 'global epidemic' posing a serious threat to public health (WHO, 2000). Obesity is asscoiated with hyperinsulinemia, dyslipidemia, higher blood pressure, impaired endothelial function and inflammatory up-regulation. The increasing prevalence of obesity among women during childbearing age is concern as obesity carries additional risk for both mother and fetus (Heslehurst et al., 2007).

12.1.1 Obesity and inflammation

Obesity is characterized by a chronic, low-grade inflammation and an impaired intracellular stress defence system (Fernández-Sánchez et al., 2011). Obesity-induced inflammation depends on a set of classical inflammatory mediators. In obese individuals the stress response is more complex and includes an impairment of the heat shock response (HSR), ER stress and down-regulation of the cellular antioxidant system (Bruce et al., 2003, Hotamisligil, 2010, Sies, 2007). In addition, there are high levels of inflammatory mediators, adipocytes and C-reactive protein (CRP) in the circulation of obese women (Bodnar et al., 2005a). Adipose tissues produce inflammatory mediators including TNF-α which leads to vasodilation, vascular permeability, endothelial damage, insulin resistance, and oxidative stress, and its levels related to adiposity degree and insulin resistance (Tzanavari et al., 2009, Fernández-Sánchez et al., 2011). Obesity is involved in development of type2 diabetes mellitus (Fernández-Sánchez et al., 2011). Inflammation is a result of increased oxidative stress in obese people and is related to endothelial dysfunction and insulin resistance, these changes lead to metabolic and vascular alterations (Fonseca-Alaniz et al., 2007). Adipose tissue produces 25% of systemic IL-6 which contributes to systemic inflammation related to obesity (Fernández-Sánchez et al., 2011). In addition, adipose tissue in obese people activates CD8 (+) T-cells, which activate

macrophages and the start of adipose inflammation (Nishimura et al., 2009). Furthermore, hypoxia is a result of adipose tissue overgrowth that leads to production of acute phase proteins and adipokines (Fernández-Sánchez et al., 2011).

12.1.2 Obesity and endothelial dysfunction

Endothelial dysfunction is characterized by a pro inflammtory, pre-coagulant and proliferative state that all lead to atherogenesis (Hadi et al., 2005). Obesity is associated with higher levels of superoxide radicals and nitrotyrosine in endothelial cells. In obesity there is increased oxidative stress and endothelial dysfunction associated with high levels of leptin (Galili et al., 2007). Inflammation, LDL oxidation, cytokines and ROS can stimulate endothelial dysfunction by secretion of adhesion molecules, which results in monocyte infiltration into the sub-endothelial space (Couillard et al., 2005). Obesity is associated with oxidative stress, which increases vascular endothelial permeability and promotes leukocyte adhesion (Hadi et al., 2005). Weight loss improves endothelial activation markers and vasodilation and also decreases cytokines (Hadi et al., 2005).

12.1.3 Obesity and insulin resistance

Metabolic syndrome features include obesity, insulin resistance, impaired glucose tolerance, hypertension and dyslipidemia. Insulin resistance is estimated to be present in two thirds of individuals with excess weight, this in turn strongly, predisposes to type2 diabetes and cardiovascular disease (Murdolo et al., 2013, Jeyabalan, 2013). Dyslipidemia and the increase in free fatty acid production from adipocytes has been suggested to contribute to oxidative stress and insulin resistance, which are obesity features (Jeyabalan, 2013). Oxidative stress markers are increased in obese individuals. For example, 8-iso prostaglandin F2α is an oxidative stress marker (8-isoPGF α), and the urine concentration is positively correlated with obesity and insulin resistance (Keaney et al., 2003). Oxidative stress is associated with the pathogenesis of insulin resistance via the inhibition of insulin signals and the dysregulation of adipokines (Houstis et al., 2006, Matsuda and Shimomura, 2013). In pregnancies complicated by PE with obesity, insulin resistance is common and can persist for several years, thus also increasing cardiovascular diseases risk (Laivuori et al., 2000).

12.1.4 Obesity and oxidative stress

Oxidative stress and production of ROS occur under several physiological and pathological conditions, which lead to tissue damage (Fernández-Sánchez et al., 2011, Matsuda and Shimomura, 2013). Oxidative stress is involved in many diseases such as obesity, cardiovascular disease, diabetes and cancer (Fernández-Sánchez et al., 2011, Matsuda and Shimomura, 2013). Obesity could induce systemic oxidative stress and in turn oxidative stress is associated with abnormal adipokine production (Esposito et al., 2006). C-reactive protein (CRP) is a marker of oxidative stress and is higher in obese people, in addition, antioxidant markers are lower in obese individuals (Chrysohoou et al., 2007). Furthermore, a diet high in fat and carbohydrates diet increase oxidative stress and inflammation in obese individuals (Patel et al., 2007). Oxidative stress and endothelial dysfunction may predispose obese women to develop PE (Jeyabalan, 2013).

12.1.5 The association of obesity with PE

Obesity has increased globally over last few decades. This increasing trend has substantial implications for pregnancy, as obesity is associated with infertility, spontaneous miscarriage, thromboembolic complications, gestational diabetes, stillbirth, caesarean section, large babies and hypertensive complications (Yogev and Catalano, 2009). Obesity is associated with a two to three fold increased risk of PE (Bodnar et al., 2005b). Obesity is associated with a risk for late onset PE as well as early onset PE, which is associated with perinatal morbidity and mortality (Catov et al., 2007). The association between PE risk and obesity has been reported in several countries and this supporting the evidence that weight loss reduces this risk (Hauger et al., 2008, Magdaleno Jr et al., 2012). Obesity is a potentially modifiable risk factor for PE and weight loss prior to pregnancy is encouraged in obese women (Yogev and Catalano, 2009).

12.2 Results

12.2.1 Patient clinical data analysis

12.2.1.1 BMI groups:

The patient details for the four BMI groups (Table 2-2) were compared by using one way ANOVA and are shown in (Table 12-1).

Table 12-1: Patient's clinical details; the descriptive analysis showed the mean and standard deviation. Analyses between the four groups were performed using one way ANOVA, P value < 0.05 was considered statistically significant.

Category	BMI<30 (n=6)	BMI 30-35 (n=6)	BMI 35-40 (n=6)	BMI>40 (n=6)	p-value
Maternal age	28.83±5.3	35.00±4.69	31.33±6.74	35.50±7.97	p=0.2
Placenta weight	679.8±170.7	630.0±103.1	708.7±166.8	684.0±169.3	P=0.8
Birth weight	3858±894	3381±183	3827±676	3444±459	p=0.4
No. PG	1	1	1	0	
Gestation age at delivery	39.21±0.39	38.60±1.54	39.03±0.70	38.86±0.54	P=0.6
No. smokers	2	1	1/3 NA	0	
BMI	24.58±2.7	32.38±0.95	36.91±0.69	45.92±4.34	

12.2.2 Analysis of protein and mRNA expression of HSP70 between 4 BMI groups.

Group 1 (BMI <30), group 2 (BMI 30-35), group 3 (BMI 35-40) and group 4 (BMI>40)
Experiment 1: This experiment was designed to test if there was any difference in protein expression of HSP70 between 4 BMI groups. Group 1 (BMI <30), group 2 (BMI 30-35),

group 3 (BMI 35-40) and group 4 (BMI>40) at the inner, middle and outer zones of placentas. Western blots showing placental protein expression of HSP70 in the 4 BMI groups at the inner zone are shown in (Figure 12-1). The graphs and statistical analysis are shown below the blots. There was no significant difference in HSP70 expression when 4 BMI groups compared (p=0.82) at the inner placental zone (Figure 12-1). Dunn's Multiple Comparison Test showed that there was no significant changes between different BMI groups.

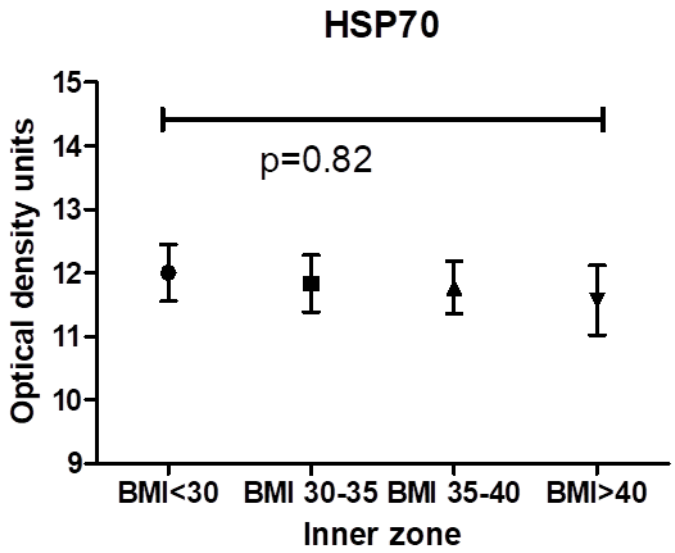


Figure 12-1: Graph shows the median HSP70 expressions in the inner placental zone in 4 BMI groups (n=6 in each group). Comparison between zones was performed using Kruskal Wallis analysis. Western blots images were not shown because of missing one set of the original gel image.

Western blots showing placental protein expression of HSP70 in the 4 BMI groups at the middle zone are shown in Figure 2. The graphs and statistical analysis are shown below the blots. There was no significant difference in HSP70 expression when the 4 BMI groups were compared (p=0.7) at the middle placental zone (Figure 12-2). Dunn's Multiple Comparison Test showed that there was no significant changes between different BMI groups.

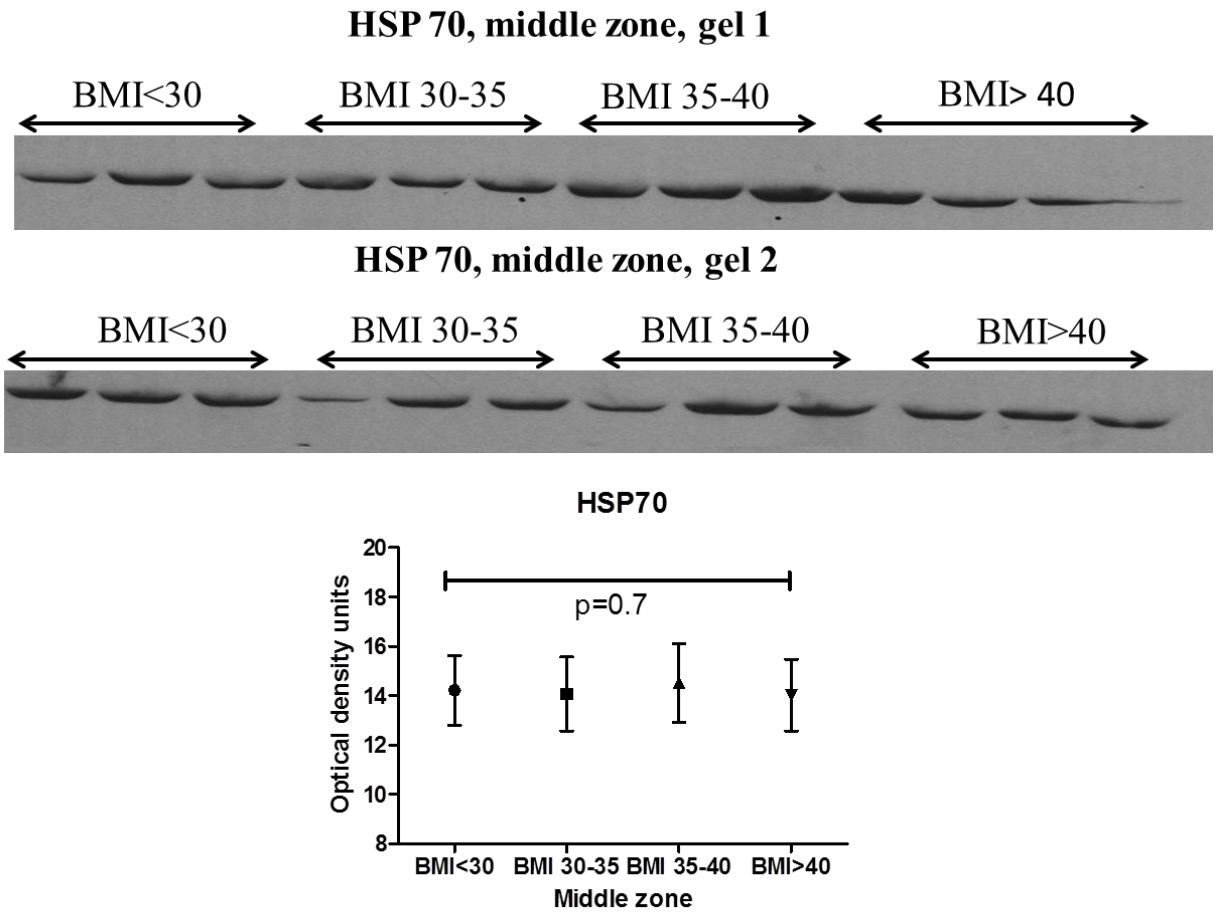


Figure 12-2: Representative images of Western blots showing HSP70 expressions in the middle placental zone in 4 BMI groups (n=6 in each group). Graphs show the median for HSP27 protein expressions. Comparison between zones was performed using Kruskal-Wallis analysis.

Western blots showing placental protein expression of HSP70 in the 4 BMI groups at the outer zone are shown in Figure 3. The graphs and statistical analysis are shown below the blots. There was no significant difference in HSP70 expression when 4 BMI groups compared (p=0.15) at the outer placental zone (Figure 12-3). Dunn's Multiple Comparison Test showed that there was no significant changes between different BMI groups.

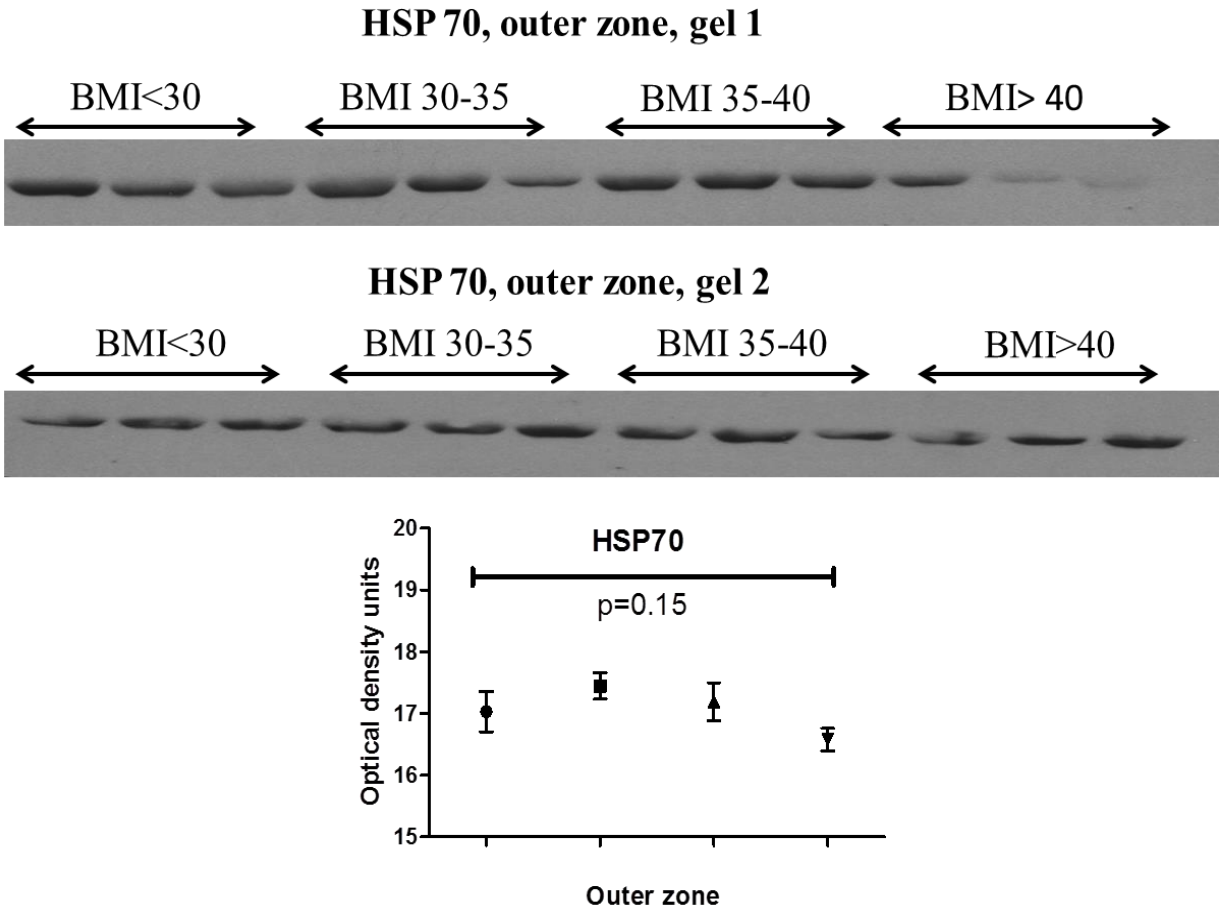


Figure 12-3: Representative images of Western blots showing HSP70 expressions in the outer placental zone in 4 BMI groups (n=6 in each group). Graphs show the median for HSP27 protein expressions. Comparison between zones was performed using Kruskal-Wallis analysis.

Experiment 2: This experiment was designed to test if there were any differences in mRNA expression of HSP70 between the 4 BMI groups at the inner, middle and outer zones. (Figure 12-4) shows the HSP70 RQ values when the 4 BMI groups were compared at the inner (A), middle (B) and outer zones (C). There was no significant difference in HSP70 expression when 4 BMI groups were compared at the inner, middle and outer placental zones (p=0.95, p=0.4, p=0.89 respectively) (Figure 12-4). Dunn's Multiple Comparison Test showed that there was no significant changes between different BMI groups. No changes were found in subanalysis.

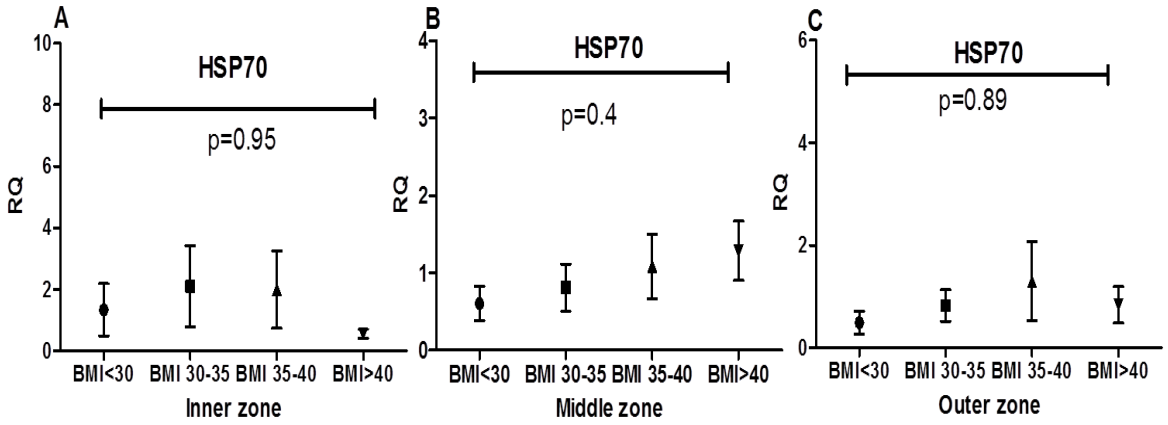


Figure 12-4: Graph showing mRNA expressions of HSP70 in the in 4 BMI groups (n=6 in each group) at the inner, middle and outer placental zones. Graphs show the median for mRNA expressions of HSP27. Comparison between zones was performed using Kruskal-Wallis analysis.

12.2.3 Analysis of protein and mRNA expression of HSP27 between 4 BMI groups.

Experiment 1: This experiment was designed to test if there was any difference in protein expression of HSP27 between 4 BMI groups. Group 1 (BMI <30), group 2 (BMI 30-35), group 3 (BMI 35-40) and group 4 (BMI>40) at the inner, middle and outer zones of placentas. Western blots showing placental protein expression of HSP27 in the 4 BMI groups at the inner zone are shown in (Figure 12-5). The graphs and statistical analysis are shown below the blots. There was a significant decrease in protein expression of HSP27 between the 4 BMI groups (p=0.006) at the inner placental zones. Dunn's Multiple Comparison Test showed that there was a significant diffences between different <30, 30-35 and 35-40 groups compared to the >40 BMI group. The subanalysis of all groups was as follows: HSP27 was signifiacnt decreased in >40 BMI group compared to the <30 BMI group (p=0.002), 30-35 BMI group (p=0.01) and 35-40 BMI group (p=0.002) (Figure 12-6).

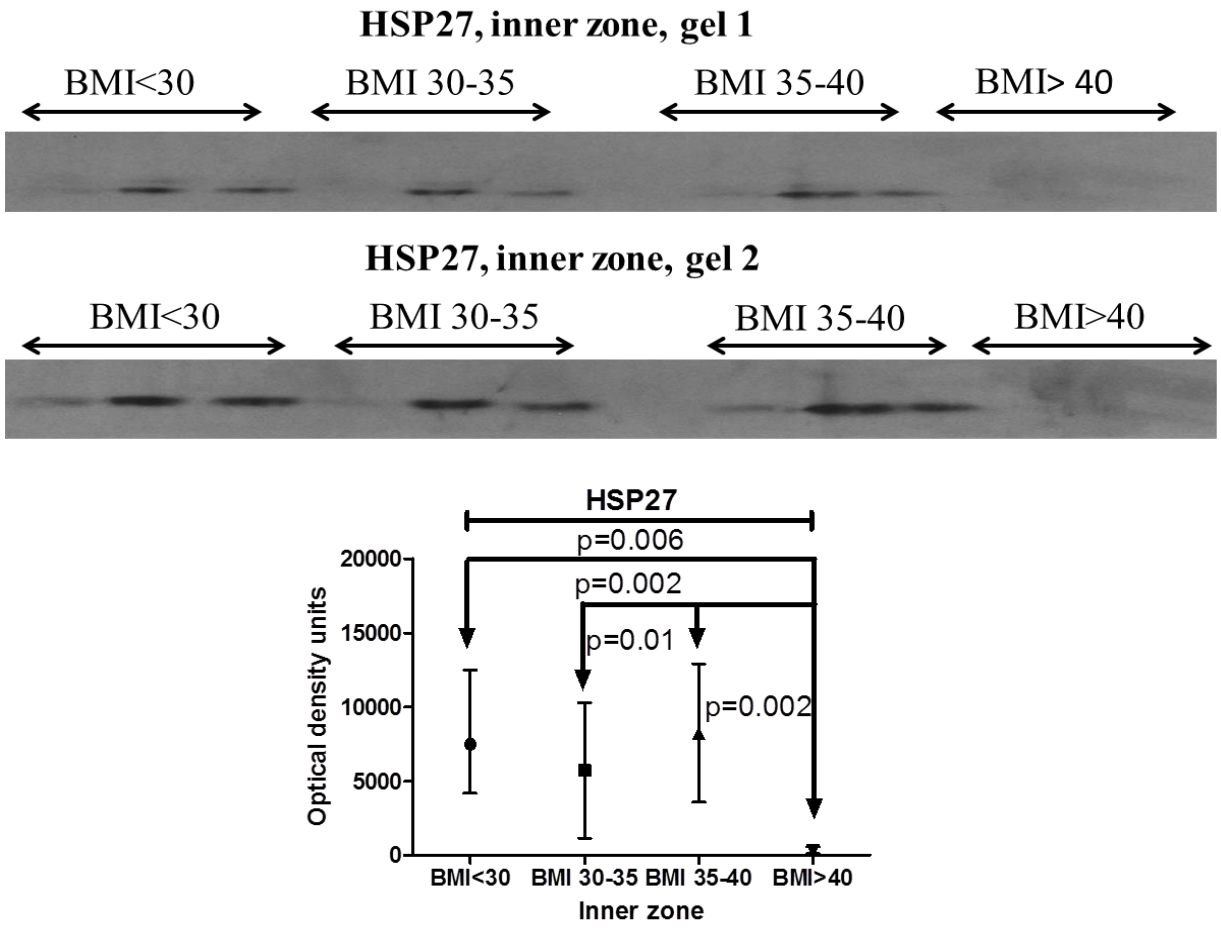


Figure 12-5: Representative images of Western blots showing HSP27 expressions in the inner placental zone in 4 BMI groups (n=6 in each group). Graphs show the median for HSP27 protein expressions. Comparison between zones was performed using Kruskal-Wallis analysis.

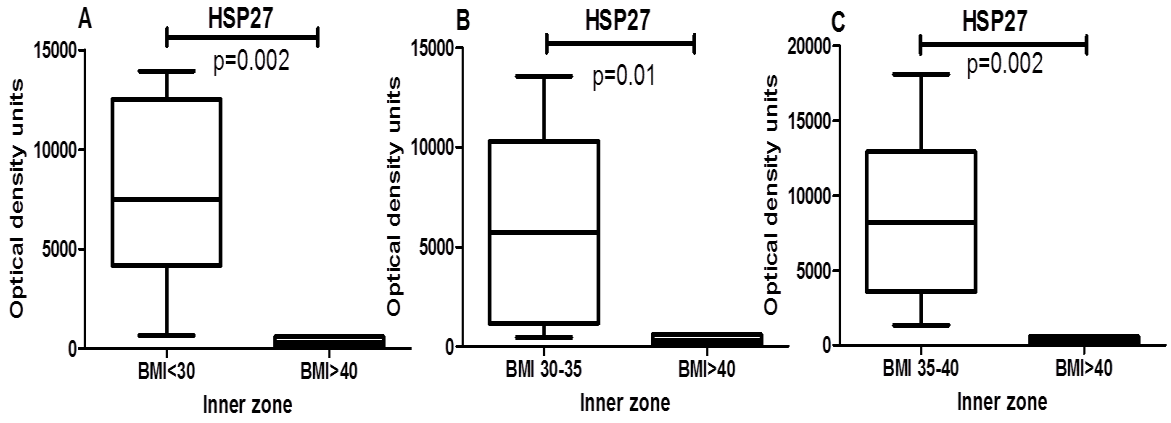


Figure 12-6: Graph showing the sub-analysis of 4 BMI groups HSP27 expression at the inner zone. Groups <30, 30-35 and 35-40 groups compared to the >40 BMI group (n=6 in each group). The graphs show the box and whiskers analysis of the blots. Comparison between zones was performed using Mann-Whitney analysis.

Western blots showing placental protein expression of HSP27 in the 4 BMI groups at the middle zone are shown in (Figure 12-7). The graphs and statistical analysis are shown below the blots. There was no significant difference in HSP27 expression when all 4 BMI groups were compared (p=0.06) at the middle placental zones (Figure 12-7). Dunn's Multiple Comparison Test showed that there was no diffences between the 4 groups. The subanalysis of all groups was as follows: HSP27 was significantly decreased in >40 BMI group compared to the <30 BMI group (p=0.02) and 35-40 BMI group (p=0.01). No other difference were found (Figure 12-8).

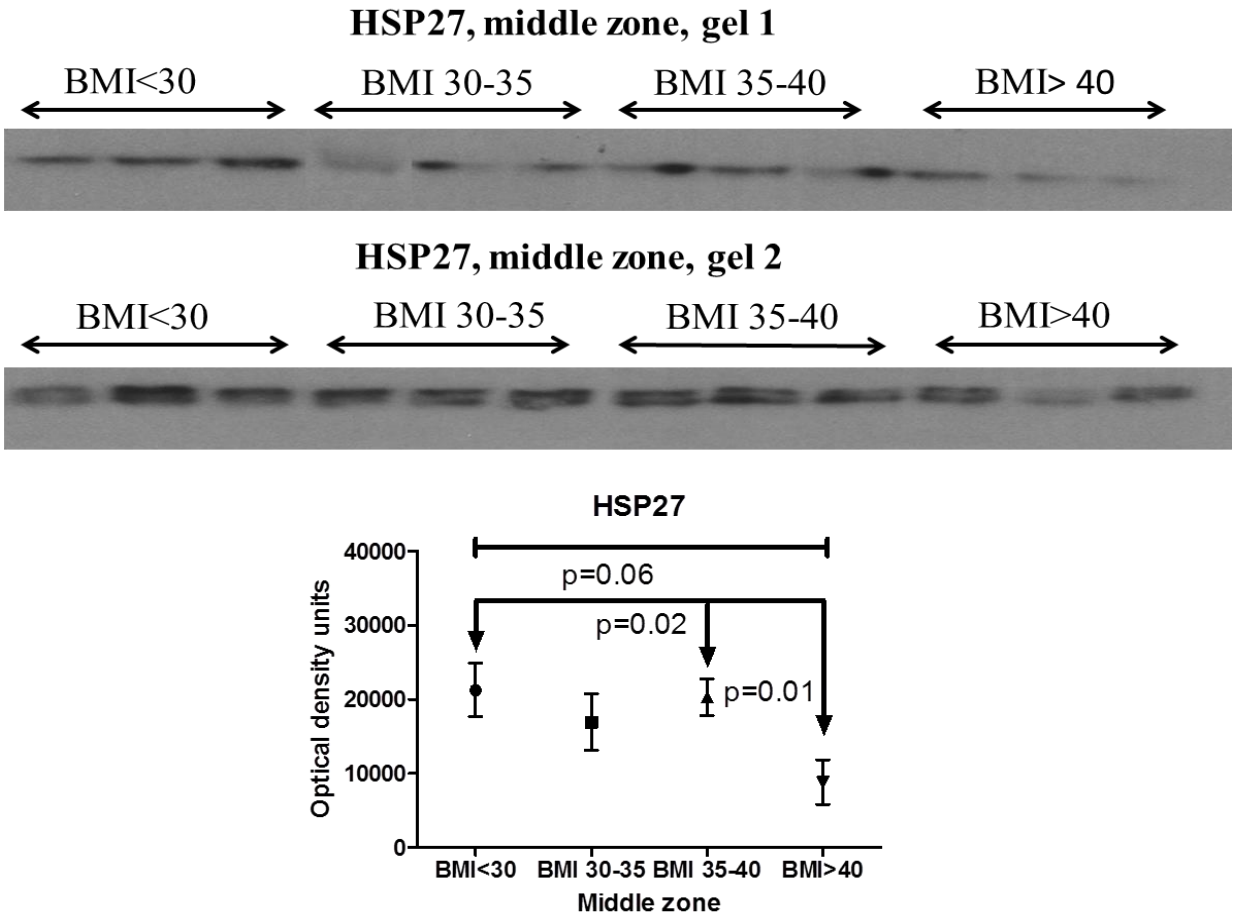


Figure 12-7: Representative images of Western blots showing HSP27 expressions in the middle placental zone in 4 BMI groups (n=6 in each group). Graphs show the median for HSP27 protein expressions. Comparison between zones was performed using Kruskal-Wallis analysis.

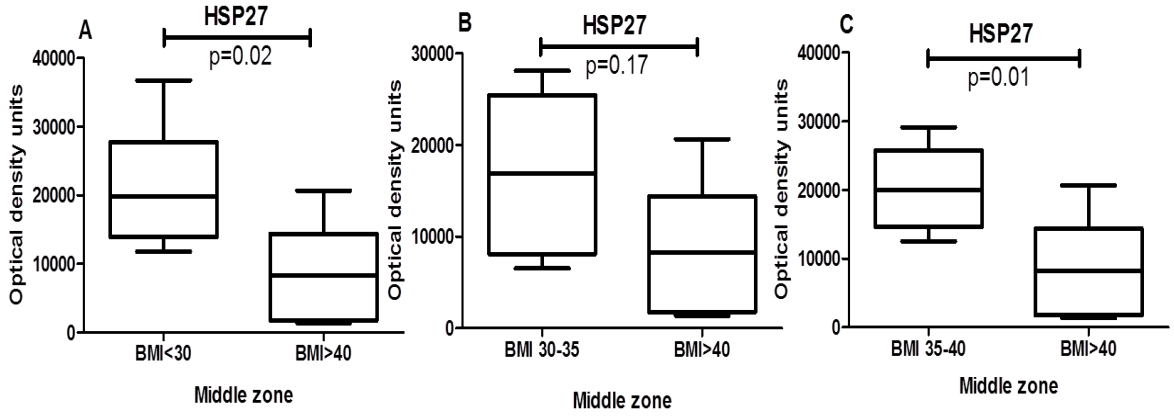


Figure 12-8: Graph showing the sub-analysis of 4 BMI groups for HSP27 expression at the middle zone. Groups <30, 30-35 and 35-40 groups compared to the >40 BMI group (n=6 in each group). The graphs show the box and whiskers analysis of the blots. Comparison between zones was performed using Mann-Whitney analysis.

Protein expression of HSP27 in the 4 BMI groups at the outer zone is shown in (Figure 12-9). The graphs and statistical analysis are shown below the blots. There was no significant difference in HSP27 expression when all 4 BMI groups compared (p=0.05) at the outer placental zones (Figure 12-9). Dunn's Multiple Comparison Test showed that there was a significant decrease in > 40 BMI group compared to the 30-35 BMI group. The sub-analysis of all groups was as follows: HSP27 was decreased significantly in >40 BMI group compared to the 35-40 BMI group (p=0.01) (Figure 12-10). No other significant differences were found.

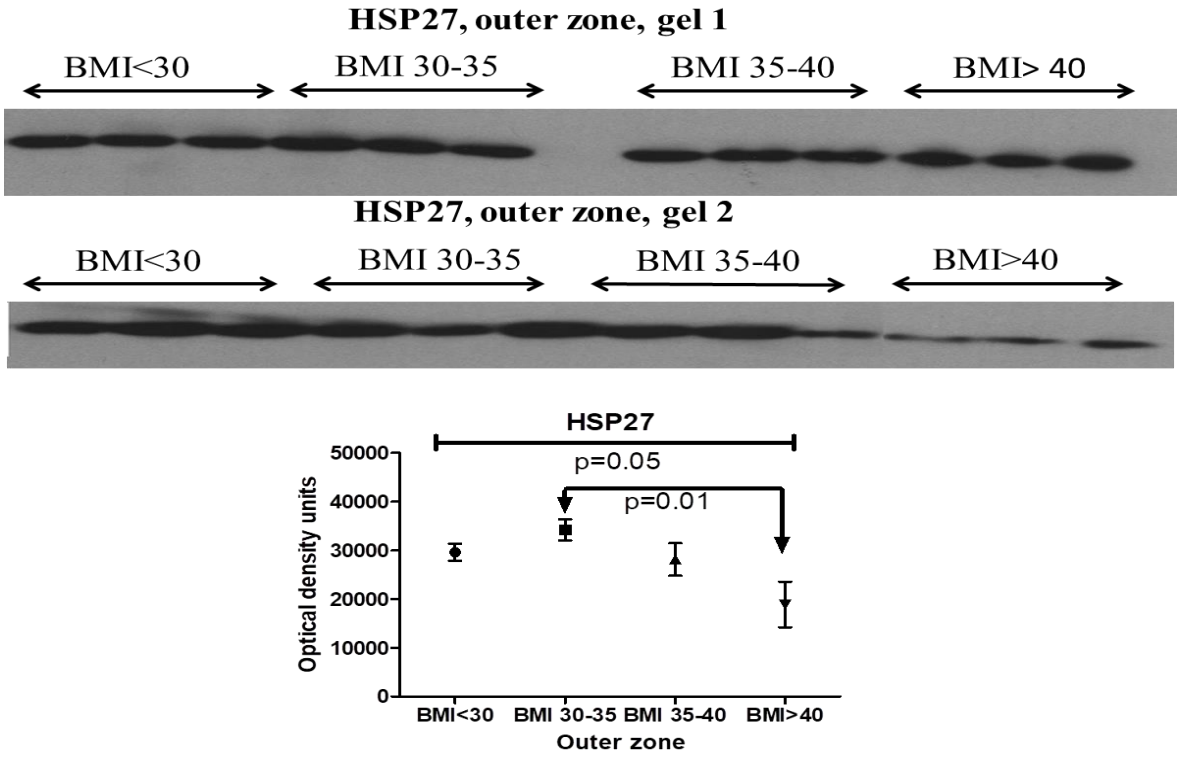


Figure 12-9: Representative images of Western blots showing HSP27 expressions in the middle placental zone in 4 BMI groups (n=6 in each group). Graphs show the median for HSP27 protein expressions. Comparison between zones was performed using Kruskal-Wallis analysis.

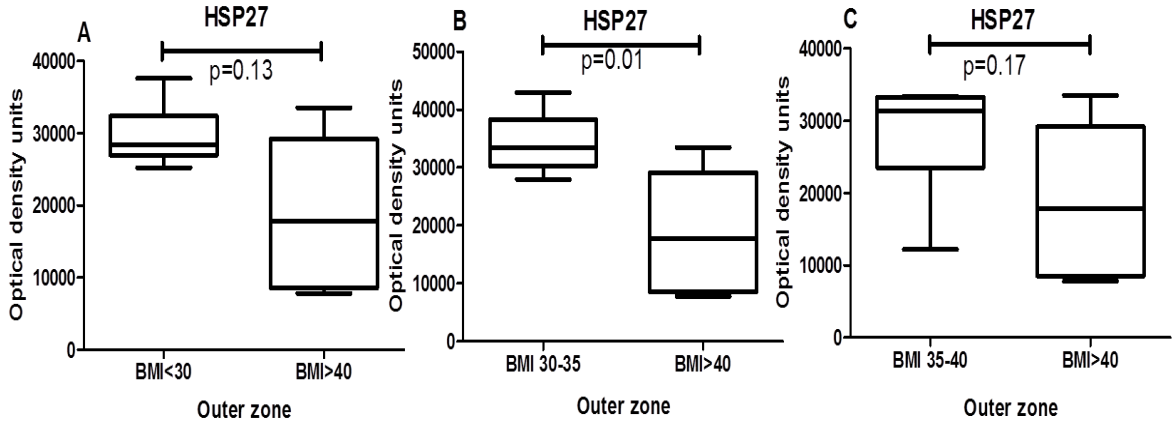


Figure 12-10: Graph showing the sub-analysis of 4 BMI groups HSP27 expression at the middle zone. Groups <30, 30-35 and 35-40 groups compared to the >40 BMI group (n=6 in each group). The graphs show the box and whiskers analysis of the blots. Comparison between zones was performed using Mann-Whitney analysis.

Experiment 2: This experiment was designed to test if there were any differences in mRNA expression of HSP27 between the 4 BMI groups at the inner, middle and outer zones. (Figure 12-11) shows the HSP27 RQ values when the 4 BMI groups were compared at the inner (A), middle (B) and outer zones (C). There was no significant difference in mRNA expression of HSP27 when 4 BMI groups were compared at the inner, middle and outer placental zones (p=0.09, p=0.6, p=0.6 respectively) (Figure 12-11). Dunn's Multiple Comparison Test showed that there was no significant changes between different BMI groups. Sub-analysis showed that there was a significant decrease in HSP27 expression in the >40 BMI compared to the <30 BMI group at the inner zone (p=0.03).

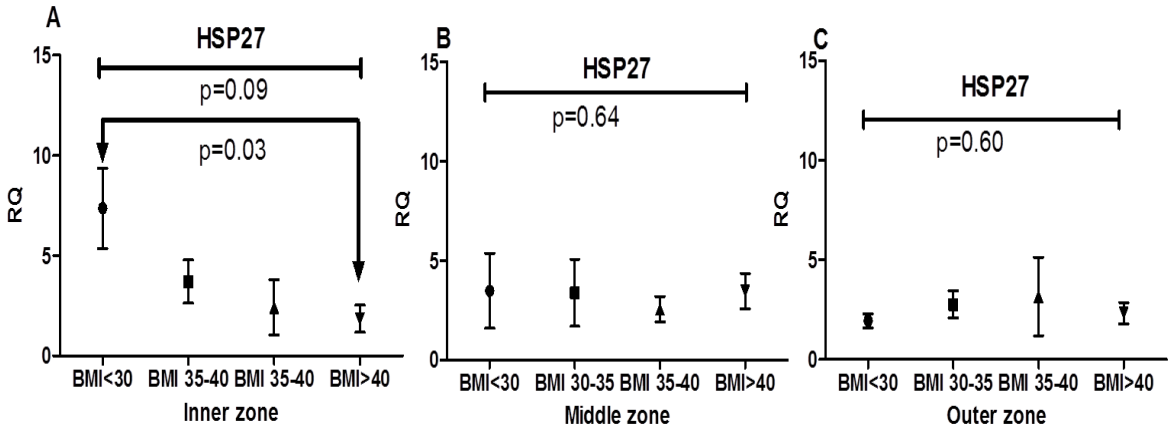


Figure 12-11: Graph showing mRNA expressions of HSP27 in the in 4 BMI groups (n=6 in each group) at the inner, middle and outer placental zones. Graphs show the median for mRNA expressions of HSP27. Comparison between zones was performed using Kruskal-Wallis analysis.

12.2.4 Analysis of mRNA expression of other HSPs families between 4 BMI groups.

Experiment 1: This experiment was designed to test if there were any differences in mRNA expression of HSPB6 (heat shock protein, alpha-crystallin-related, B6) between the 4 BMI groups at the inner, middle and outer zones. (Figure 12-12) shows the HSPB6 RQ values when the 4 BMI groups were compared at the inner (A), middle (B) and outer zones (C). There was no significant difference in HSPB6 expression when all 4 BMI groups were compared at the inner, middle and outer placental zones (p=0.13, p=0.34, p=0.23 respectively) . Dunn's Multiple Comparison Test showed that there was no significant changes between different BMI groups. No changes were found in sub-analysis (p=0.08 for all) (Figure 12-13).

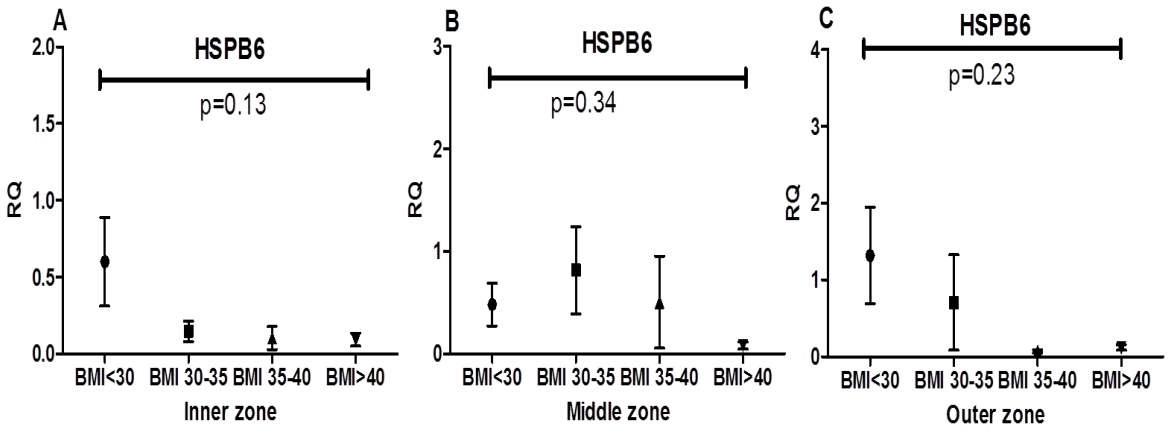


Figure 12-12: Graph showing mRNA expressions of HSPB6 in the in 4 BMI groups (n=6 in each group) at the inner, middle and outer placental zones. Graphs show the median for mRNA expressions of HSPB6. Comparison between zones was performed using Kruskal-Wallis analysis.

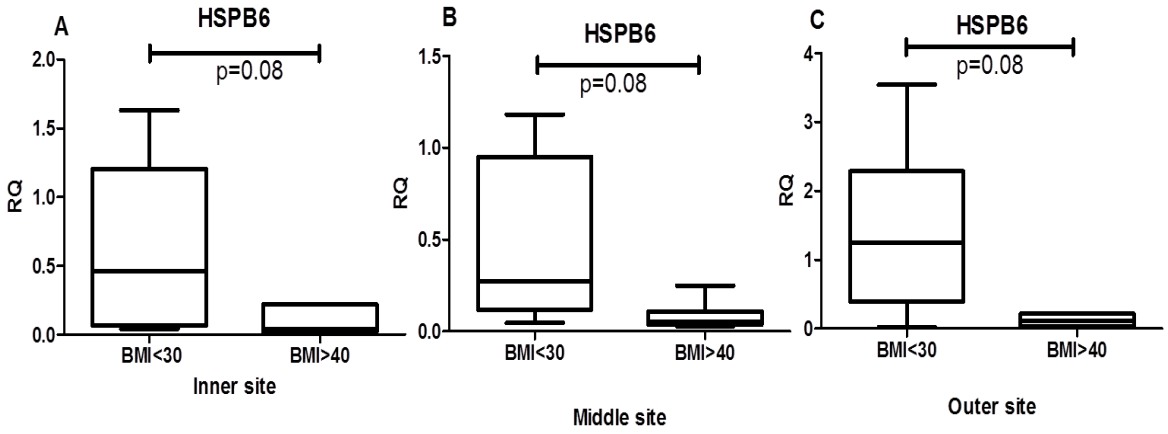


Figure 12-13: Graph showing the sub-analysis of 4 BMI groups HSPB6 expression at the middle zone. Groups <30, 30-35 and 35-40 groups compared to the >40 BMI group (n=6 in each group).The graphs show the box and whiskers analysis of the blots. Comparison between zones was performed using Mann-Whitney analysis.

Experiment 2: This experiment was designed to test if there were any differences in mRNA expression of HSPB2 (HSP27, protein 2) between the 4 BMI groups at the inner, middle and outer zones. (Figure 12-14) shows the HSPB2 RQ values when the 4 BMI groups were compared at the inner (A), middle (B) and outer zones (C). There was a significant difference in mRNA expression of HSPB2 when 4 BMI were compared at the inner zone (p=0.04). There was no significant difference in HSPB2 expression when all 4 BMI groups were compared at the middle and outer placental zones (p=0.65, p=0.18 respectively). Dunn's Multiple Comparison Test showed that there was no significant changes between different BMI groups. Sun-analysis of significant results showed that

there was a significant decrese in HSPB2 in the BMI>40 group compared to the BMI<30 group at the inner zone (p=0.01). No other changes were found in sub-analysis.

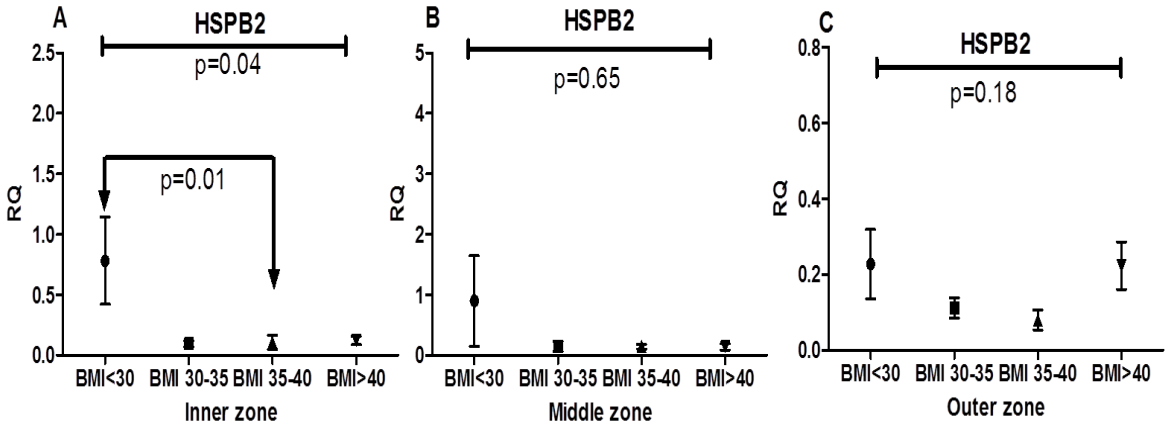


Figure 12-14: Graph showing mRNA expressions of HSPB2 in the in 4 BMI groups (n=6 in each group) at the inner, middle and outer placental zones. Graphs show the median for mRNA expressions of HSPB2. Comparison between zones was performed using Kruskal-Wallis analysis.

Experiment 3: This experiment was designed to test if there were any differences in mRNA expression of HMOX1 (HSP32) between the 4 BMI groups at the inner, middle and outer zones. (Figure 12-15) shows the HSP32 RQ values when the 4 BMI groups were compared at the inner (A), middle (B) and outer zones (C). There was a significant difference in mRNA expression of HSP32 when all 4 BMI groups were compared at the outer placental zone (p=0.04). Dunn's Multiple Comparison Test showed that there was no significant changes between different BMI groups. Sub-analysis of significant results showed that there was a significant decrease in HSP32 in BMI>40 group compared to BMI<30 group at inner, middle and outer zones (p=0.01,p=0.03, p=0.004 respectively).

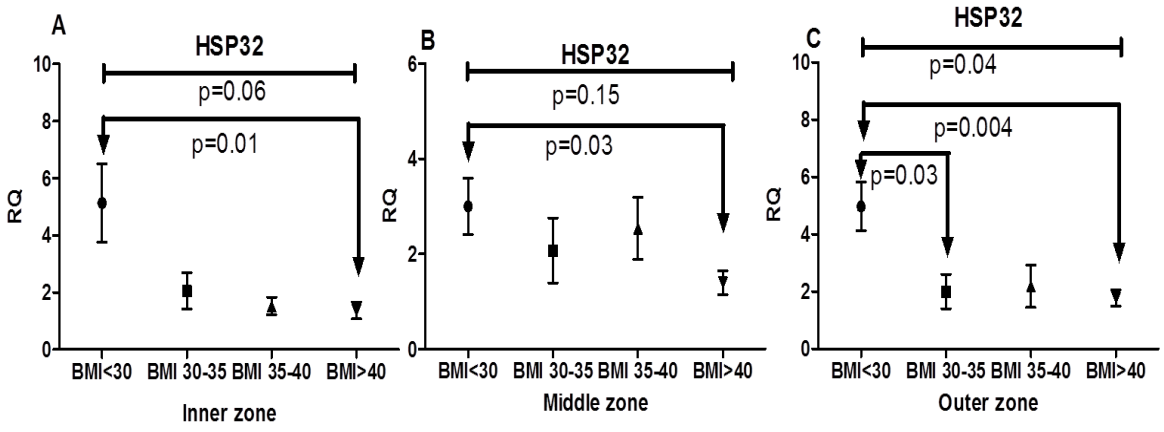


Figure 12-15: Graph showing mRNA expressions of HSP32 in the in 4 BMI groups (n=6 in each group) at the inner, middle and outer placental zones. Graphs show the median for mRNA expressions of HSP32. Comparison between zones was performed using Kruskal-Wallis analysis.

Experiment 4: This experiment was designed to test if there were any differences in mRNA expression of DNAJB1 between the 4 BMI groups at the inner, middle and outer zones. (Figure 12-16) shows the DNAJB1 RQ values when the 4 BMI groups were compared at the inner (A), middle (B) and outer zones (C). There was no significant difference in mRNA expression of DNAJB1 when all 4 BMI groups were compared at the inner, middle and outer placental zones (p=0.8 for all). Dunn's Multiple Comparison Test showed that there was no significant difference in mRNA expression of DNAJB1 in BMI>40 compared to the any other BMI group. Sub-analysis showed that there was no significant difference in DNAJB1 when all 4 BMI groups were compared. Sub-analysis showed no difference was found between all 4 BMI groups.

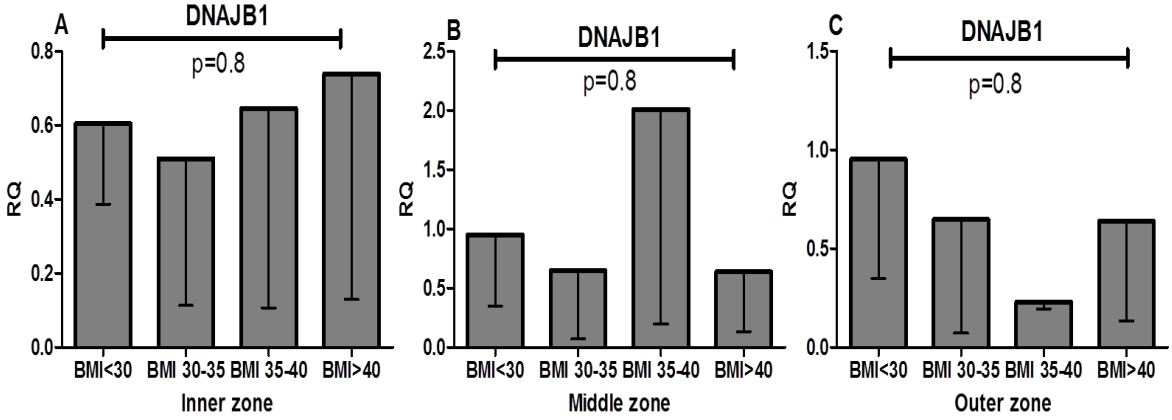


Figure 12-16: Graph showing mRNA expressions of DNAJB1 in the in 4 BMI groups (n=6 in each group) at the inner, middle and outer placental zones. Graphs show the median for mRNA expressions of DNAJB1. Comparison between zones was performed using Kruskal-Wallis analysis.

Experiment 5: This experiment was designed to test if there were any differences in mRNA expression of HSPD1 (HSP60) between the 4 BMI groups at the inner, middle and outer zones. (Figure 12-17) shows the HSPD1 RQ values when the 4 BMI groups were compared at the inner (A), middle (B) and outer zones (C). There was no significant difference in mRNA expression of HSPD1 when all 4 BMI groups were compared at the inner, middle and outer placental zones (p=0.6, p=0.3, p-0.6 respectivelyl). Dunn's Multiple Comparison Test showed that there was no significant difference in mRNA expression of HSPD1 in BMI>40 compared to the any other BMI group. Sub-analysis showed no difference in HSPD1 was found between all 4 BMI groups.

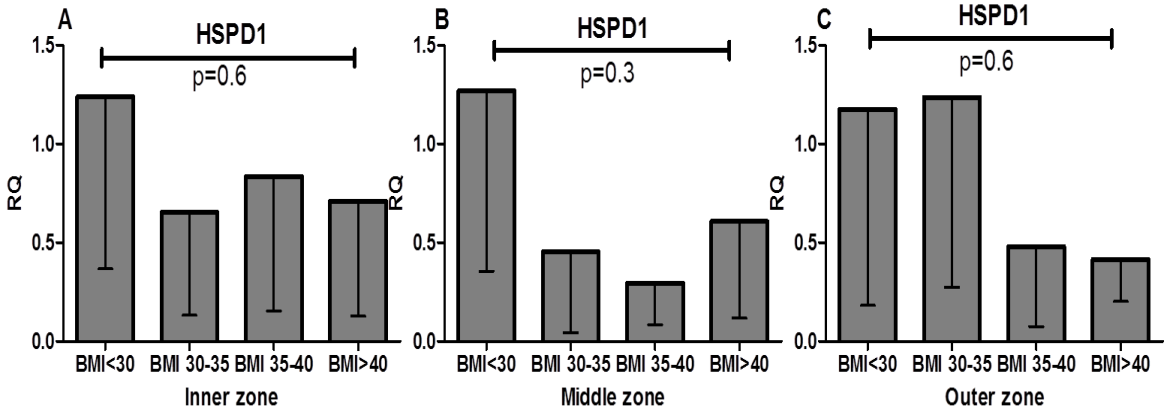


Figure 12-17: Graph showing the sub-analysis of 4 BMI groups mRNA expression of HSPD1 at the middle zone. Groups <30, 30-35 and 35-40 groups compared to the >40 BMI group (n=6 in each group). The graphs show the box and whiskers analysis of the blots. Comparison between zones was performed using Mann-Whitney analysis.

Experiment 6: This experiment was designed to test if there were any differences in mRNA expression of HSPA5 between the 4 BMI groups at the inner, middle and outer zones. (Figure 12-18) shows the HSPA5 RQ values when the 4 BMI groups were compared at the inner (A), middle (B) and outer zones (C). There was no significant difference in mRNA expression of HSPA5 when all 4 BMI groups were compared at the inner, middle and outer placental zones (p=0.3, p=0.6, p-0.1 respectivelyl). Dunn's Multiple Comparison Test showed that there was no significant difference in mRNA expression of HSPA5 in BMI>40 compared to the any other BMI group. Sub-analysis showed a significant decrease in HSPA5 in BMI>40 group compared to BMI 30-35 group (p=0.04). No other differences were found.

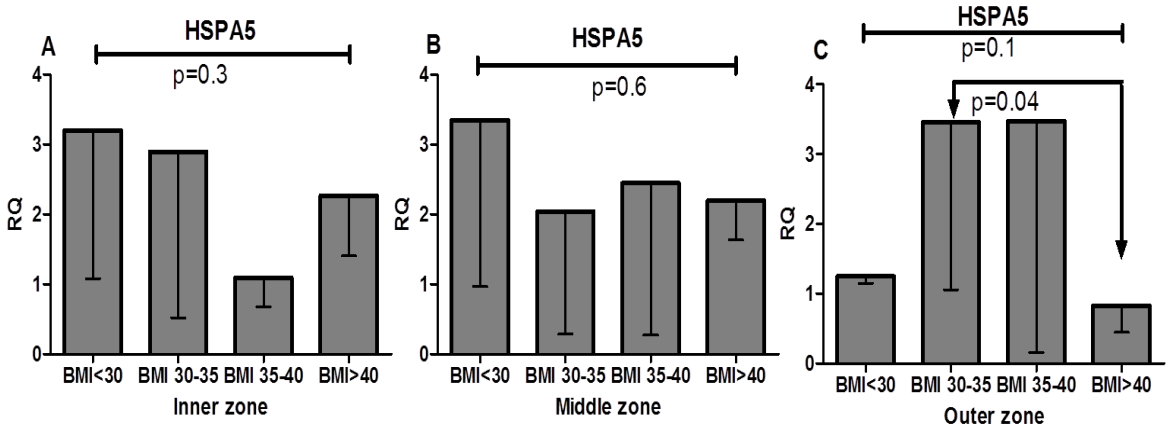


Figure 12-18: Graph showing the sub-analysis of 4 BMI groups mRNA expression of HSPA5 at the middle zone. Groups <30, 30-35 and 35-40 groups compared to the >40 BMI group (n=6 in each group).The graphs show the box and whiskers analysis of the blots. Comparison between zones was performed using Mann-Whitney analysis.

Experiment 7: This experiment was designed to test if there were any differences in mRNA expression of HSP90AA between the 4 BMI groups at the inner, middle and outer zones. (Figure 12-19) shows the HSP90AA RQ values when the 4 BMI groups were compared at the inner (A), middle (B) and outer zones (C). There was no significant difference in mRNA expression of HSP90AA when all 4 BMI groups were compared at the inner, middle and outer placental zones (p=0.79, p=0.31, p=0.28 respectively) . Dunn's Multiple Comparison Test showed that there was no significant changes between different BMI groups. No changes were found in sub-analysis.

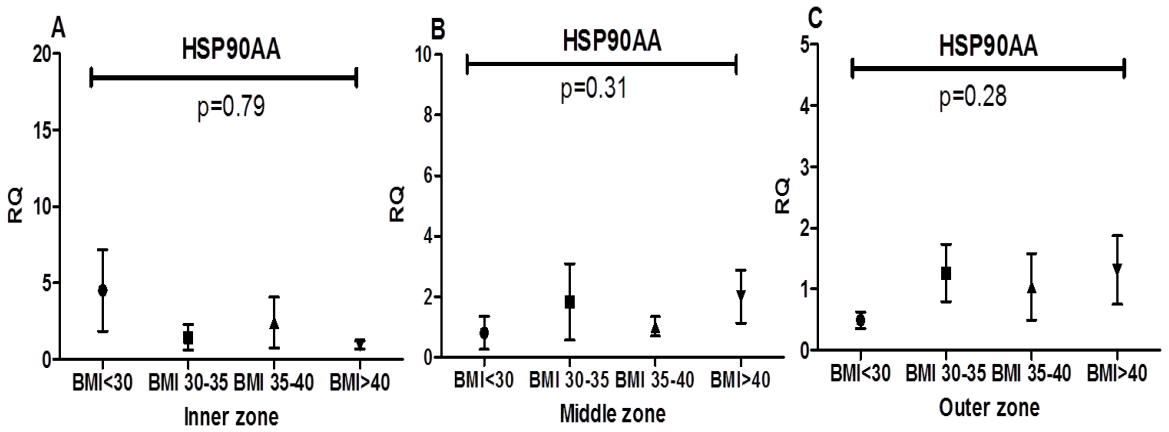


Figure 12-19: Graph showing mRNA expressions of HSP90AA in the in 4 BMI groups (n=6 in each group) at the inner, middle and outer placental zones. Graphs show the median for mRNA expressions of HSP90AA. Comparison between zones was performed using Kruskal-Wallis analysis.

Experiment 8: This experiment was designed to test if there were any differences in mRNA expression of HSP90B1 between the 4 BMI groups at the inner, middle and outer zones. (Figure 12-20) shows the HSP90B1 RQ values when the 4 BMI groups were compared at the inner (A), middle (B) and outer zones (C). There was no significant difference in HSP90B1 expression when all 4 BMI groups were compared at the inner, middle and outer placental zones (p=0.7, p=0.2, p=0.5 respectively) . Dunn's Multiple Comparison Test showed that there was no significant changes between different BMI groups. No changes were found in sub-analysis.

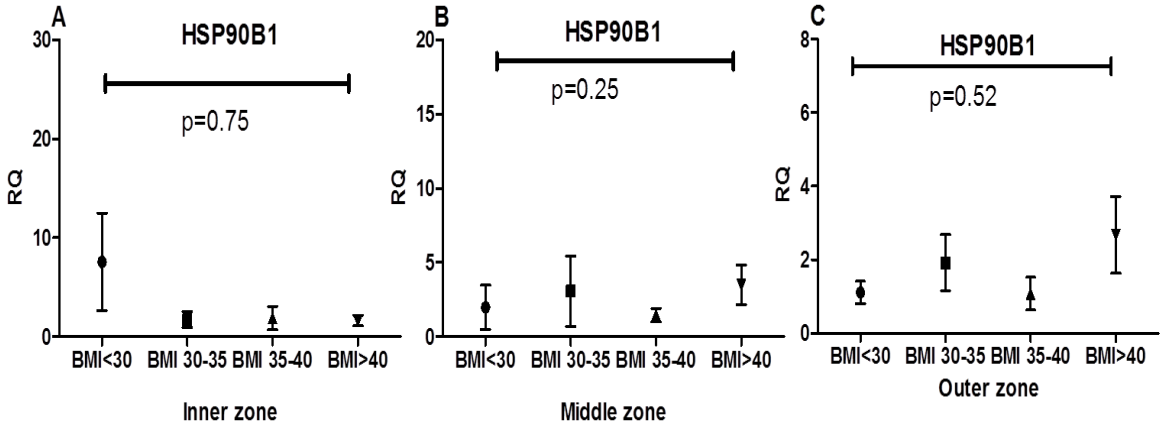


Figure 12-20: Graph showing mRNA expressions of HSP90B1 in the in 4 BMI groups (n=6 in each group) at the inner, middle and outer placental zones. Graphs show the median for mRNA expressions of HSP90B1. Comparison between zones was performed using Kruskal-Wallis analysis.

Experiment 9: This experiment was designed to test if there were any differences in mRNA expression of HSPH1 (heat shock 105kDa/110kDa protein 1) between the 4 BMI groups at the inner, middle and outer zones. (Figure 12-21) shows the HSPH1 RQ values when the 4 BMI groups were compared at the inner (A), middle (B) and outer zones (C). There was no significant difference in mRNA expression of HSPH1 when all 4 BMI groups were compared at the inner, middle and outer placental zones (p=0.2, p=0.2, p=0.6 respectivelyl). Dunn's Multiple Comparison Test showed that there was no significant difference in mRNA expression of HSPH1 in BMI>40 compared to the any other BMI group. Sub-analysis showed no difference in HSPH1 was found between all 4 BMI groups.

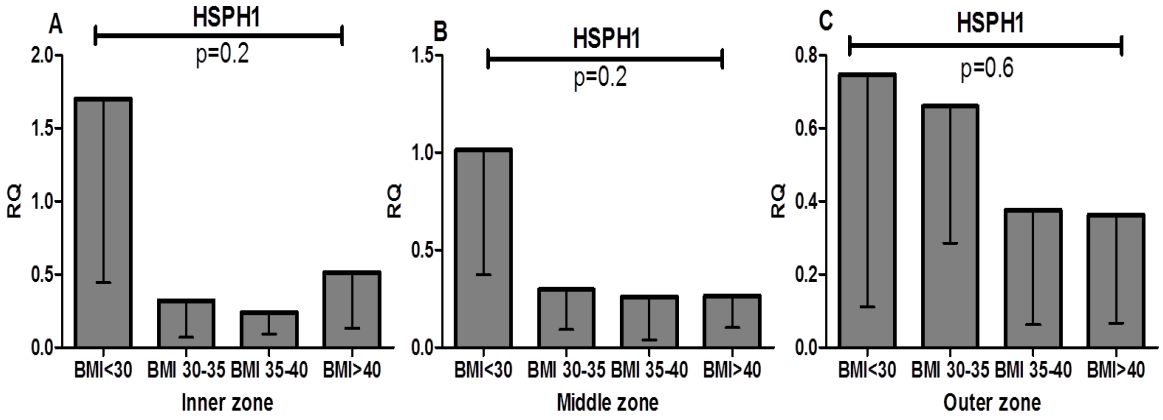


Figure 12-21: Graph showing the sub-analysis of 4 BMI groups mRNA expression of HSPH1 at the middle zone. Groups <30, 30-35 and 35-40 groups compared to the >40 BMI group (n=6 in each group). The graphs show the box and whiskers analysis of the blots. Comparison between zones was performed using Mann-Whitney analysis.

12.2.5 Analysis of mRNA expression of CASP3, 7 and 9 between 4 BMI groups.

Experiment 1: This experiment was designed to test if there were any differences in mRNA expression of CASP3 between the 4 BMI groups at the inner, middle and outer zones. (Figure 12-22) shows the CASP3 RQ values when the 4 BMI groups were compared at the inner (A), middle (B) and outer zones (C). There was a significant difference in mRNA expression of CASP3 when all 4 BMI groups were compared at the inner and middle placental zones (p=0.04, p=0.03 respectively). There was no siginifacnt difference at the outer zone (p=0.5). Dunn's Multiple Comparison Test showed that there was a significant decrese in mRNA expression of CASP3 in BMI>40 compared to the BMI 30-35 group at the inner zone. Sub-analysis of significant difference at the inner showed that there was a significant increase in CASP3 in BMI >40 group compared to the BMI<30 and BMI 30-35 groups (p=0.03, p=0.01 respectively). No difference was found between BMI 35-40 and BMI>40 groups at the inner zone (Figure 12-23).

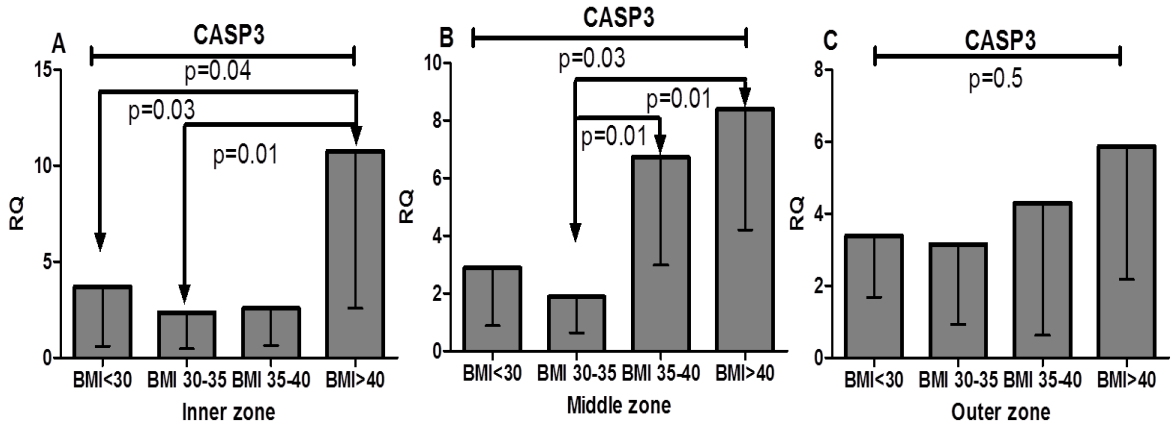


Figure 12-22: Graph showing mRNA expressions of CASP3 in the in 4 BMI groups (n=6 in each group) at the inner, middle and outer placental zones. Graphs show the median for mRNA expressions of CASP3. Comparison between zones was performed using Kruskal-Wallis analysis.

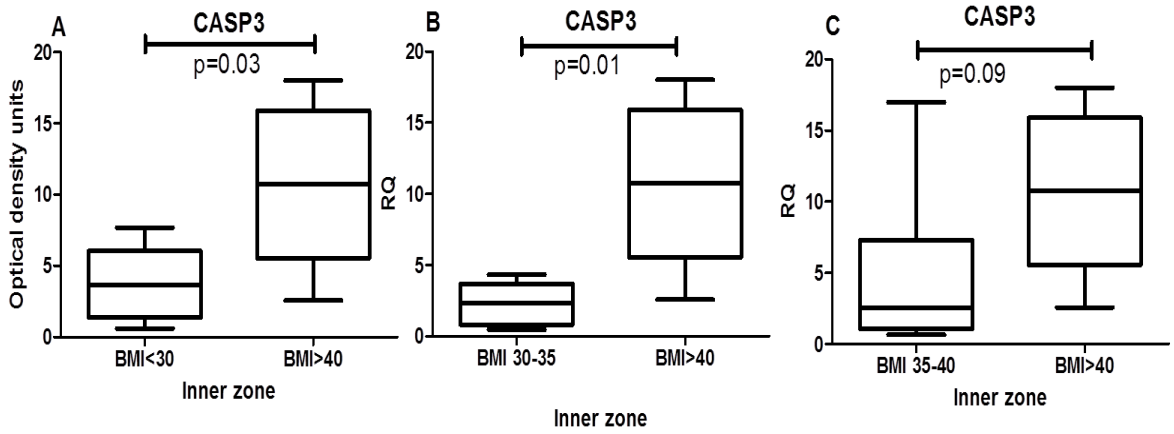


Figure 12-23: Graph showing the sub-analysis of 4 BMI groups mRNA expression of CASP3 at the inner zone. Groups <30, 30-35 and 35-40 groups compared to the >40 BMI group (n=6 in each group). The graphs show the box and whiskers analysis of the blots. Comparison between zones was performed using Mann-Whitney analysis.

Sub-analysis of significant difference at the middle zone showed that there was a significant increase in CASP3 in BMI >40 group compared to the BMI 30-35 groups at the middle zone (p=0.01). No difference was found between BMI<30 and BMI 35-40 compared to the BMI>40 groups at the middle zone (Figure 12-24). No sub-analysis was performed for the outer zone as there was no significant change.

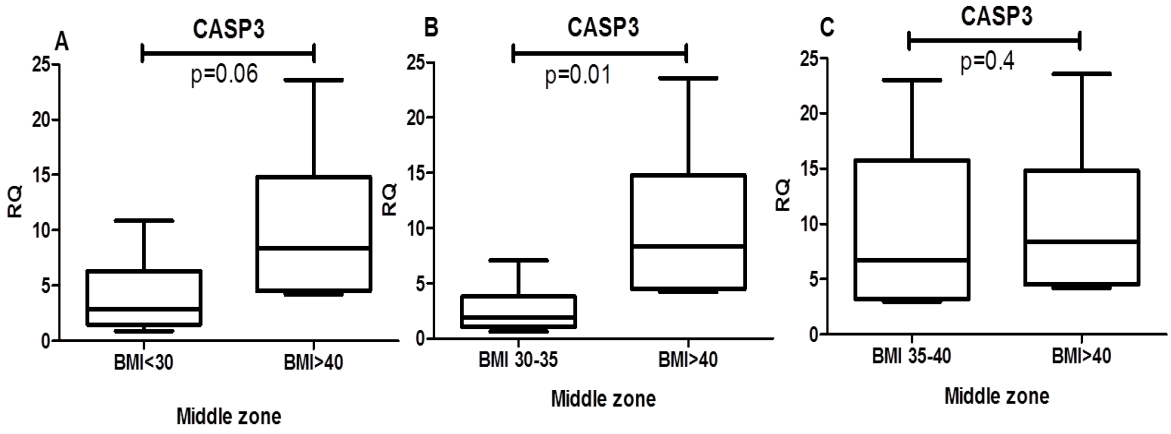


Figure 12-24: Graph showing the sub-analysis of 4 BMI groups mRNA expression of CASP3 at the middle zone. Groups <30, 30-35 and 35-40 groups compared to the >40 BMI group (n=6 in each group). The graphs show the box and whiskers analysis of the blots. Comparison between zones was performed using Mann-Whitney analysis.

Experiment 2: This experiment was designed to test if there were any differences in mRNA expression of CASP7 between the 4 BMI groups at the inner, middle and outer zones. (Figure 12-25) shows the CASP7 RQ values when the 4 BMI groups were compared at the inner (A), middle (B) and outer zones (C). There was no significant difference in mRNA expression of CASP7 when all 4 BMI groups were compared at the inner, middle and outer placental zones (p=0.9, p=0.1, p=0.6 respectively). Dunn's Multiple Comparison Test showed that there was no significant difference in mRNA expression of CASP7 in BMI>40 compared to other BMI groups. Sub-analysis showed that there was no significant difference in CASP7 when all 4 BMI groups were compared. Sub-analysis showed no difference was found between all 4 BMI groups.

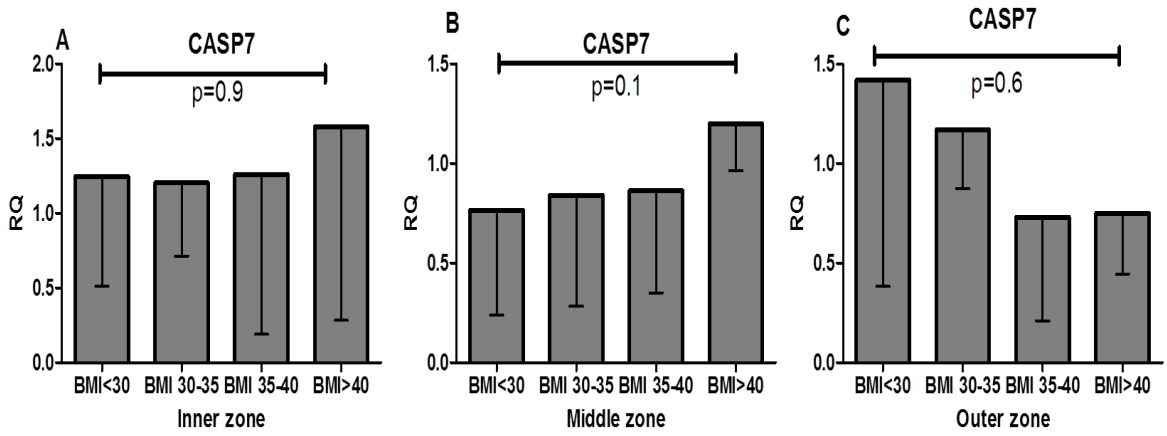


Figure 12-25: Graph showing mRNA expressions of CASP7 in the in 4 BMI groups (n=6 in each group) at the inner, middle and outer placental zones. Graphs show the median for mRNA expressions of CASP7. Comparison between zones was performed using Kruskal-Wallis analysis.

Experiment 3: This experiment was designed to test if there were any differences in mRNA expression of CASP9 between the 4 BMI groups at the inner, middle and outer zones. (Figure 12-26) shows the CASP9 RQ values when the 4 BMI groups were compared at the inner (A), middle (B) and outer zones (C). There was a significant difference in mRNA expression of CASP9 between the 4 BMI groups at the middle zone (p=0.01). There was no significant difference was found at the inner and outer placental zones (p=0.7, p=0.8 respectively). Dunn's Multiple Comparison Test showed that there was a significant increase in mRNA expression of CASP9 in BMI >40 group compared to the BMI 30-35 and 35-40 groups at the middle zone. Sub-analysis for significant results showed that there was a significant increase in mRNA expression of CASP9 in BMI >40 group compared to the BMI 30-35 and 35-40 groups at the middle zone (p=0.008, p=0.004 respectively). No difference was found between BMI<30 and BMI>40 groups (p=0.32) (Figure 12-27).

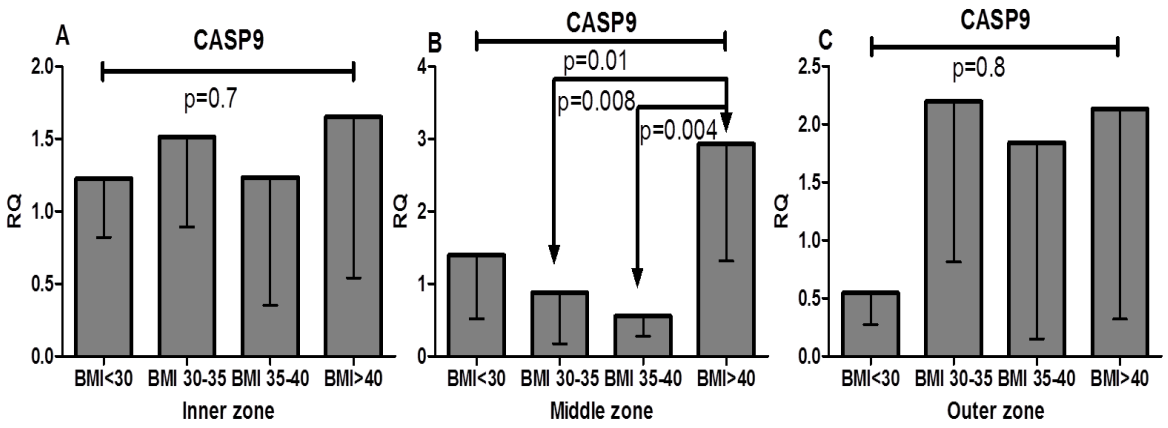


Figure 12-26: Graph showing mRNA expressions of CASP9 in the in 4 BMI groups (n=6 in each group) at the inner, middle and outer placental zones. Graphs show the median for mRNA expressions of CASP9. Comparison between zones was performed using Kruskal-Wallis analysis.

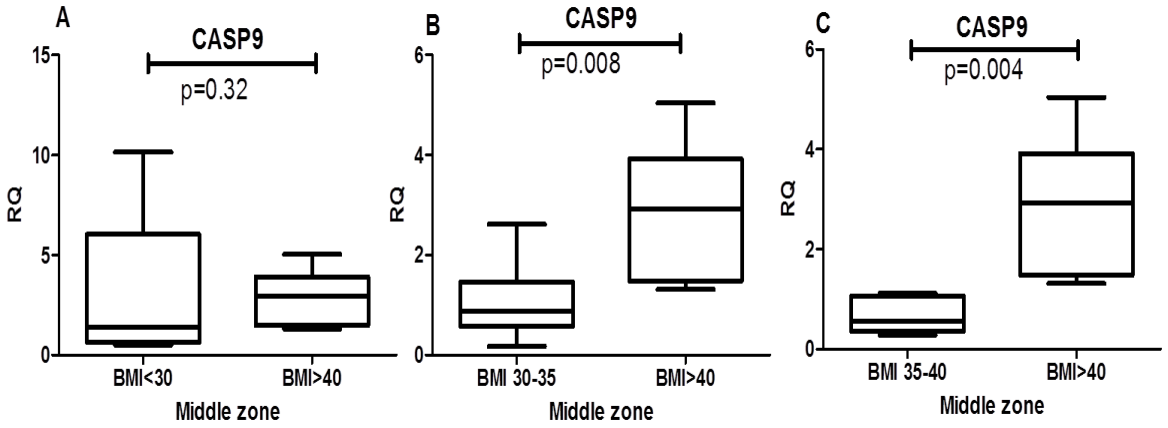


Figure 12-27: Graph showing the sub-analysis of 4 BMI groups mRNA expression of CASP3 at the middle zone. Groups <30, 30-35 and 35-40 groups compared to the >40 BMI group (n=6 in each group). The graphs show the box and whiskers analysis of the blots. Comparison between zones was performed using Mann-Whitney analysis.

12.2.6 Results summary

12.2.6.1 HSP70 expression in the 4 BMI groups

Experiment 1: There was no significant difference in protein expression of HSP70 when the 4 BMI groups were compared at the inner, middle and outer placental zones.

Experiment 2: There was no significant difference in mRNA expression of HSP70 when the 4 BMI groups compared at the inner, middle and outer placental zones.

12.2.6.2 HSP27 expression in the 4 BMI groups

Experiment 1: There was a significant decrease in protein expression of HSP27 between the 4 BMI groups (p=0.006) at the inner placental zones. The sub-analysis of all groups was as follows: HSP27 was significantly decreased in >40 BMI group compared to the <30 BMI group (p=0.002), 30-35 BMI group (p=0.01) and 35-40 BMI group (p=0.002). There was no significant difference in HSP27 expression when all 4 BMI groups were compared (p=0.06) at the middle placental zones. The sub-analysis of all groups was as follows: HSP27 was significantly decreased in >40 BMI group compared to the <30 BMI group (p=0.02) and 35-40 BMI group (p=0.01). There was no significant difference in HSP27 expression when all 4 BMI groups compared (p=0.05) at the outer placental zones. The sub-analysis of all groups was as follows: HSP27 was decreased significantly in >40 BMI group compared to the 35-40 BMI group (p=0.01).

Experiment 2: There was no significant difference in mRNA expression of HSP27 when 4 BMI groups were compared at the inner, middle and outer placental zones. However, sub-analysis showed that there was a significant decrease in HSP27 expression in the >40 BMI compared to the <30 BMI group at the inner zone (p=0.03).

12.2.6.3 Summary results for mRNA expression of other HSPs families between 4 BMI groups.

Experiment 1: There was no significant difference in HSPB6 expression when all 4 BMI groups were compared at the inner, middle and outer placental zones

Experiment 2: There was a significant difference in mRNA expression of HSPB2 when 4 BMI groups were compared at the inner zone (p=0.04). There was no significant difference in HSPB2 expression when all 4 BMI groups were compared at the middle and outer placental zones. Sub-analysis showed that there was a significant decrese in HSPB2 in the BMI>40 group compared to the BMI<30 group at the inner zone (p=0.01).

Experiment 3: There was a significant difference in mRNA expression of HSP32 when all 4 BMI groups were compared at the outer placental zone (p=0.04). Sub-analysis showed that there was a significant decrease in HSP32 in BMI>40 group compared to BMI<30 group at inner, middle and outer zones (p=0.01,p=0.03, p=0.004 respectively).

Experiment 4: There was no significant difference in mRNA expression of DNAJB1 when all 4 BMI groups were compared at the inner, middle and outer placental zones.

Experiment 5: There was no significant difference in mRNA expression of HSPD1 when all 4 BMI groups were compared at the inner, middle and outer placental zones.

Experiment 6: There was no significant difference in mRNA expression of HSPA5 when all 4 BMI groups were compared at the inner, middle and outer placental zones. However, sub-analysis showed a significant decrease in HSPA5 in BMI>40 group compared to BMI 30-35 group (p=0.04).

Experiment 7: There was no significant difference in mRNA expression of HSP90AA when all 4 BMI groups were compared at the inner, middle and outer placental zones.

Experiment 8: There was no significant difference in HSP90B1 expression when all 4 BMI groups were compared at the inner, middle and outer placental zones

Experiment 9: There was no significant difference in mRNA expression of HSPH1 when all 4 BMI groups were compared at the inner, middle and outer placental zones.

12.2.6.4 CASP3, 7 and 9 expression in the 4 BMI groups

Experiment 1: There was a significant difference in mRNA expression of CASP3 when all 4 BMI groups were compared at the inner and middle placental zones (p=0.04, p=0.03 respectively). There was no significant difference at the outer zone. Sub-analysis of significant difference at the inner zone showed that there was a significant increase in CASP3 in BMI >40 group compared to the BMI<30 and BMI 30-35 groups (p=0.03, p=0.01 respectively). Sub-analysis showed that there was a significant increase in CASP3 in BMI >40 group compared to the BMI 30-35 groups at the middle zone (p=0.01).

Experiment 2: There was no significant difference in mRNA expression of CASP7 when all 4 BMI groups were compared at the inner, middle and outer placental zones.

Experiment 3: There was a significant difference in mRNA expression of CASP9 between the 4 BMI groups at the middle zone (p=0.01). There was no significant difference was found at the inner and outer placental zones. Sub-analysis showed that there was a significant increase in mRNA expression of CASP9 in the BMI >40 group compared to the BMI 30-35 and BMI 35-40 groups at the middle zone (p=0.008, p=0.004 respectively).

12.3 Discussion

12.3.1 HSPs family expression in obese pregnant women and the present findings

This study describes, for the first time, expression of HSPs and CASPs families in different BMI groups. The most interesting results were the reduction in expresstion of some HSPs in the morbid obesity group (BMI>40) compared to the normal BMI group and obese grade 1 or 2. In contrast, CASP 3 and CASP9 was increased in the same group (BMI>40) compared to the normal BMI group and obese grade1 or 2. Reduced HSPs and increased CASPs indicate reduced cellular protection and increased cellular damage respectively.

12.3.2 Key findings of HSP70 and HSP27 protein and mRNA expression

HSP70: There was no significant difference in protein and mRNA expression of HSP70 when the 4 BMI groups were compared at the inner, middle and outer placental zones.

HSP27: There was a significant decrease in protein expression of HSP27 in the BMI >40 group at the inner, middle and outer placental zones. There was a significant decrease in mRNA expression of HSP27 in the BMI >40 group compared to the BMI<30 group at the inner zone, no other differences were found.

12.3.3 Key findings of other HSPs families mRNA expression

HSPB2: There was a significant decrese in HSPB2 in the BMI>40 group compared to the BMI<30 group at the inner zone. No other differences were found. HSP32: There was a significant decrease in HSP32 in the BMI>40 group compared to BMI<30 group at the inner, middle and outer zones. HSPA5: There was a significant decrease in HSPA5 in the BMI>40 group compared to the BMI 30-35 group. There was no significant difference in mRNA expression of other HSPs families when all 4 BMI groups were compared at the inner, middle and outer placental zones.

12.3.4 Key findings in mRNA expression of CASPs

CASP3: There was a significant increase in CASP3 in the BMI >40 group compared to the BMI<30 and BMI 30-35 groups at the inner and middle zones. CASP9: There was a significant increase in mRNA expression of CASP9 in the BMI >40 group compared to

both BMI 30-35 and BMI 35-40 groups at the middle zone. No difference was found in CASP7 expression.

12.3.5 Obesity and HSPs

HSPs act as molecular chaperones and demonstrate important protective functions in stressed cells. In the obese state, adipose tissue is characterized by an impaired intracellular stress defense system and unbalanced HSR. Several members of the HSP family have been identified as adipokines released upon cellular stress (Habich and Sell, 2015). The inducible isoform of HSP70, HSP72 is attracting most attention (Henstridge et al., 2014b). Several studies showed that HSP72 protects against disturbed metabolic homeostasis via multiple modes of action including improving skeletal muscle oxidation and decreasing inflammation (Chung et al., 2008, Gupte et al., 2009, Henstridge et al., 2014a). In addition, HSP60 has also been implicated in the cause of mitochondrial homeostasis and maintenance of insulin sensitivity, for instance HSP60 was decreased in insulin resistance and mitochondrial dysfunction (Kleinridders et al., 2013). HSP60 reduction in obese, diabetic mice was due to a disruption of leptin signalling and was restored by leptin treatment (Henstridge et al., 2014b). Furthermore, HSP40 was decreased in obese patients and positively correlated with maximum oxygen consumption. Of interest was that exercise restored the expression of HSP40 (Abubaker et al., 2013). HSP27 may also play a role in the aetiology of metabolic disease and expression is reduced in the skeletal muscle of insulin resistance rats (Gupte et al., 2008).

There are several studies on different interventions that stimulate the HSR against obesity, and showing a concomitant improvement of clinical outcomes. These interventions include heat therapy (Gupte et al., 2009), electrical therapy, physical exercise (Morino et al., 2008) and HSR pharmacological stimulation (Literati-Nagy et al., 2009).

However, while our knowledge of the mechanisms and the contribution of HSP are well documented for other conditions, little is known about their dysregulation during the course of obesity.

Apoptosis is a form of programmed cell death and has been observed in the placenta of normal pregnancies (Smith et al., 1997). Apoptosis was the subject of study in relation to the pathophysiology of PE (Levy et al., 2000). Placentas from PE and FGR patients showed enhanced apoptosis as compared to placentas of normal prenancy (Ishihara et al., 2002). However, the molecular mechansims leading to apopotosis are complex, and

several pathways are involved including bcl-2, CASP3 and NF-κB (Adams and Cory, 1998).

One research group performed spectrophotometer and enzymatic measurements to investigate oxidative stress markers and lipid profiles in placentas as well as maternal and fetal blood of obese women compared to control (Malti et al., 2014). In this study they used same systematic sampling methods to our study. This study showed that maternal, fetal and placenta triglyceride levels were increased in the obese group. In addition, oxidative stress markers were increased in the obese group (Malti et al., 2014). Furthermore, there was a variation in redox balance indicating high oxidative stress in the placenta and in the new born of the obese group. They concluded that maternal obesity is associated with metabolic alterations and dysregulation of redox balance in the mother and fetal-placenta unit (Malti et al., 2014).

Another study performed Western blotting, IHC and RNA sequencing on term placenta of obese and non-obese pregnant women (Saben et al., 2014). RNA-seq revealed 288 genes to be significantly different in placentas from obese women by1.4 fold (Saben et al., 2014). They showed that genes involved in angiogenesis and lipid metabolism were significantly decreased in placentas of obese women. They also showed that pro-inflammatory ILs (IL-6, IL-23A, IL21R and IL-12RB2) and certain chemokines were increased in obese women. In contrast, a significant decrease in pro-inflammatory IL1R1 and receptor protein was reported in placenta of obese women. In addition, there were a significant decrease in adiponectin receptors and number of hormones (CRH, Lep and CCK) in placentas of obese women compared to normal weight women. Furthermore, indicative of a lipotoxic environment, increased placental lipid was associated with decreased AMPK and increased activation of NF-κB in placenta from obese women. There was a 25% decrease in total antioxidant capacity. They concluded that maternal obesity leads to a lipotoxic placental environment that is associated with decreased regulators of angiogenesis and increased markers of inflammation and oxidative stress (Saben et al., 2014).

Chapter XIII: Difficulties and limitation

13 Difficulties

I was presented with some difficulties with placenta sample collection. When the project was started the medical genetics lab was located at Yorkhill hospital as was next the Queens mothers maternity hospital and so placenta collection was relatively straightforward. However during the project the maternity hospital and the vast majority of medical genetics staff relocated some distance away to the Southern General Hospital site. It was a monumental task to follow these women, identify suitable women and get the placentas fresh as I had to travel continually between the two sites by taxi.

The second limitation was when the medical genetics department was evacuated there was a long delay in my work as cell culture stopped for 18 months. The gas supply set up used for running the incubators in our laboratory was cancelled as this was on an NHS contract. The reason for this was at the start of my project it was a joint NHS University department. Two things happened; the University underwent restructure with removal of departments and no longer did the joint NHS University department exist. This situation has prevented me from performing the HSP induction and tissue culture experiments which were planned as an important component of my work. By the time the culture system was reinstated my time in the laboratory had finished.

One of main limitation in the tissue culture experiment was the sample size in both heat shock and hypoxic-reperfusion experiments as only one experiment in each group was performed. The second limitation was how to create *in vitro* condition similar to the real *in vivo* condition. For example as going from 2% to 8% in real life ischemia it is probably not as black and white as that. The third limitation was the transport time between two hospitals and it is effect on tissue culture experiments.

Chapter XIV: Summary and conclusions

14 Summary

Human parturition involves interaction of hormonal, neurological, mechanical stretch and inflammatory pathways and the placenta plays a crucial role. HSPs are induced in cells as a protective mechanism to cope with cellular stress. HSPs also play an important role in regulating signal transduction, inhibition of apoptosis and cellular proliferation and differentiation. The activation of HSPs is mediated by HSF1, which binds to the promoter regions of HSPs. Apoptosis has been implicated in both PE and labour. In the apoptotic pathway, HSPs act at several stages to prevent cell death initiated by stress-induced damage. For example HSP70 inhibits CASP 3 and 9. Thus it is possible HSP70 acts to keep the rate of apoptosis in check. TLR family members are expressed differentially in a variety of cells and tissues. TLR are the principal signalling molecules through which mammal's sense infection, so called innate immunity. This project investigated the stress, apoptotic and inflammatory pathways.

First aim: This study showed for the first time that (within placentas) HSP70 and HSP27 protein is expressed in a spatial manner in the labour and the non-labour groups with the highest expression being in the middle zone for both. HSF1 protein expression (both forms) was spatially distributed in the labour group placentas while HSF1 mRNA expression was spatially distributed in the non-labour group. No other changes were found.

At this stage it is only possible to speculate why such zonal differences exist but may relate to the functions of HSP70, HSP27 and HSF1, some of which differ and some overlap. Placental separation is an important part of labour and one study showed that the process of placental separation from the uterine wall can be divided into three distinct phases i.e. latent, contraction/detachment and expulsion. They showed that placental separation is accomplished by means of an orderly multiphasic process with a definite direction and sequence. They found in most cases the placenta separated from the uterine wall in a "down-up" separation i.e. initiating from the lower pole. Interestingly cases with a previous Cesarean section had a higher rate of up-down separation. In contrast in the case of "fundal placentae" separation started at the placental poles (bipolar separation) and the central area of the placenta was the last to separate. It would be therefore of interest in a future study to investigate whether there was a link between the zonal distribution of HSP27 or HSP70 and the method of placental separation. In addition, different genes

expression would vary in different placental zones perhaps due to the variation in blood supply to each zone or link to IR injury and are some zones of placenta more susceptible to IR injury. It may also be within zones there are subtle differences in signal pathways that mean each zone behaves differently.

Second aim: This study also showed for the first time that there was a zonal difference in expression of several proteins when comparing the non-labour group to the labour group. HSP70 protein expression was increased in the labour group compared to the non-labour group at the middle zone. In contrast, HSP27 protein expression was increased in the non-labour group when compared to the labour group. HSP27 mRNA expression was significantly increased in the non-labour group compared to the labour group at the inner and middle zones. No difference was found in HSP70 mRNA expression.

This study also shows that P-HSP27 expression was significantly increased in the non-labour group when compared to the labour group at the inner zone for all P-HSP27 (ser 15, 78 and 82). There was a significant increase in P-HSP27 expression in the non-labour group when compared to the labour group at the outer zone for P-HSP27 (ser 15 and ser 78). No difference was found at the middle zone.

HSF1 protein expression was increased in the non-labour group when compared to the labour group for both isoforms at the inner and middle zones. There was, paradoxically, an increase in HSF1 mRNA expression in the labour group at the inner zone.

In this study the mRNA expression of several stress genes was compared between labour and non-labour groups at the all placental zones. HSPB6, HSP32 and DNAJB1 were increased in non-labour group compared to the labour group at the middle zone. HSPB2, HSP90AA and HSP90B1 were increased in the labour group compared to the non-labour at the outer zone.

This study compared caspases (3, 7 and 9) mRNA expression in the non-labour group and labour group. CASP7 was increased in labour group at the middle zone.

This study also shows that mRNA expression of TLR9-10 was negligible in the placenta and the only difference of the inflammatory mediators (TLRs, MyD88 and NF-κB) was in mRNA expression of TLR5 when comparing the labour group to the non-labour group which was increased in the labour group and again at the middle zone.

Third aim: There was a significant decrease in protein expression of HSP70 in the non-labour control group compared to the non-labour PE group at the inner zone. No other difference in HSP70 was found when comparing the labour control with the labour PE. No difference in mRNA HSP70 expression was found between groups.

NP-HSF1 protein expression was increased in the non-labour and labour PE compared to the non-labour and labour control at the inner and middle zones. No changes in P-HSF1 expression were found. At the mRNA level there was increased in HSF1 expression in the non-labour PE compared to the non-labour control at the inner zone.

This study showed that protein expression of HSP27 was increased in the labour PE group compared to the labour controls at the middle and outer zones. There was no difference in non-labour PE compared to the non-labour controls. No changes at the mRNA level were found.

Due to time constraints only the labour group was compared for P-HSP27. P-HSP27 (ser 15, ser 78 and ser 82) was significantly increased in the labour PE group compared to labour control group at the inner zone (ser 78 and ser 82) and middle (ser 15) zones.

This study showed that mRNA expression of CASP3 was reduced in the labour PE group compared to the labour control group at the middle zone. There was a significant decrease in the non-labour PE group compared to the non-labour control group in mRNA expression of CASP7 (inner) and CASP9 (middle) zones.

TLR1, TLR5 and HSPB6 mRNA expression was significantly decreased in the non-labour PE group compared to the non-labour control group at the middle zone. TLR1 mRNA expression was also decreased in the labour PE group compared to the labour control group again at the inner and middle zone.

This project also included a small study to investigate protein and mRNA expression of HSP70 and HSP27 in the labour FGR compared to the labour control group at the inner and outer zones. HSP70 protein expression was significantly decreased in FGR group compared the control group at the outer zone, no changes in mRNA expression were found. HSP27 mRNA expression was increased in FGR group compared to the control group in both the inner and outer zone. No changes in protein expression were found.

The increase or decrease in expression of several proteins and genes in labour, PE and FGR at precise zones suggests that there is a controlled spatial change in their expression or a susceptibility to changes in expression. The physiological and pathological significance of these remains to be elucidated but oxidative stress is the common link. Oxidative stress occurs when the production of ROS (such as the superoxide anion) overwhelms the intrinsic anti-oxidant defenses (such as superoxide dismutase, glutathione peroxidase, ascorbate (vitamin C) and α-tocopherol (vitamin E). Excessive generation of superoxide can also lead to interactions with nitric oxide to form peroxynitrite ($ONOO^-$). Peroxynitrite is a powerful pro-oxidant. Under conditions of stress activation of redox-sensitive transcription factors, such as AP-1, p53 and NF-κB occurs to increase expression of pro-inflammatory and other cytokines, cell differentiation and apoptosis.

During a healthy pregnancy maternal spiral arteries are dramatically remodeled. They become widely dilated and lose their responsiveness to vasoconstrictive stimuli. Thus blood enters the intervillous space in a non-pulsatile manner and under low pressure

Uterine contractions during labour are associated with intermittent utero-placental perfusion. Doppler ultrasound studies have demonstrated a linear inverse relationship between uterine artery resistance and the intensity of the uterine contractions during labour

In PE spiral artery remodeling is partial or incomplete. The resulting high-pressure flow results in hydrostatic damage to the placental villi. In addition perfusion by intermittent pulses of fully oxygenated arterial blood is thought to lead to fluctuations in oxygen delivery resulting in oxidative stress.

The intermittent perfusion might explain the placental zonal variation in both labour and PE by different response to IR injury in each placental zone.

Placentas collected at term by caesarean section are not subjected to the stress of labour however one possibility is that zonal differences might reflect the fact that labour is not far off and that the molecular steps to allow labour to precede have started. Thus it would be interesting to compare placentas from the second trimester where labour is not close to determine if such zonal differences still exist. In contrast at labour zonal differences in some gene expression may be linked to the response to the stress of labour, extent of exposure to hypoxia or may contribute to the process that allows the placenta to separate at delivery.

in vitro studies: In this procedure placenta tissue was exposed to low oxygen levels, then exposed to normal oxygen levels (8%). These samples were then compared to the control samples that were only exposed to the normal oxygen level. This particular experiment focused on the expression of stress proteins.

With regards to reperfusion injury of ischemic placenta exposure to 8% oxygen following exposure to low oxygen for different periods of time significantly induced mRNA expression of HSP90B1 at the inner zone. There was a decrease in HSP27 protein expression (all zones) and of HSP27 mRNA in inner zone. HSP90AA was also decreased at the inner zone over time periods with less expression after 22 h recovery period, the letter perhaps due to protein turnover. No changes were found in HSP70 and HSF1 expression following exposure to I-R injury.

This *in vitro* data supports the idea the cellular stress markers are altered due to reperfusion of placenta tissue during labour and/or PE. In addition, although these data are very preliminary there was an obvious increase in HSP70, HSP27 and HSP90AA mRNA expression when exposed to IR at the 6 h point. Future experiments should follow this up to determine if this is a real statistical effect.

This project also included *in vitro* experiments in order to investigate expression of several stress genes and caspases in placenta tissues after exposure to heat shock and also to examine the effect on zonal distribution. In this study placenta tissue from all zones were exposed to heat shock for 30 min, then a recovery period for different time periods in compare to control which were kept all time at 37° C. A limitation of such studies is that by cutting the placenta into its 3 zones the structure of the placenta is no longer maintained in the way it would be exposed to blood flow *in vivo*.

In this study there was a decrease in HSP70 and HSP27 protein expression over time. No changes were noted at the mRNA level. There was a fall in HSF2 expression over time and there was an increase in HSPB2 due to heat shock, both at the inner zone. No other changes were found in stress genes.

There was a fall in CASP3 and CASP7 mRNA expression over time at the middle zone. There was an increase in CASP3 due to heat shock at the inner zone. No changes in CASP9 were found.

Again there was a clear trend for increases in mRNAs expression of most HSPs and caspases at the 6 h time and this seems to be the key finding. As this set of work was performed close to the end of the thesis future experiments should follow this up to determine if this is a real statistical effect however, the preliminary results suggest there are.

For the first time we showed that there was a clear zonal variation in response in both hypoxia-reperfusion and heat shock experiments.

There was a significant increase in HSP70 in the labour group compared to the non-labour but at the middle zone. There was a significant increase in HSP70 in the non-labour PE group compared to the non-labour control at the inner zone. In the heat shock experiment HSP70 protein expression was decreased over time at the inner and middle zones. No change in HSP70 expression was found in the hypoxia-reperfusion experiment. The *in vitro* heat shock experiment totally reflects the changes in HSP70 expression in labour and PE groups at the inner and middle zones.

There was a significant increase in HSP27 in the non-labour group compared to the labour at the inner and middle zones. HSP27 expression was increased in labour PE group compared to the labour control group at the middle zone. In the heat shock experiment HSP27 protein expression was decreased over time at the inner and middle zones. HSP27 expression was decreased over time at the inner zone in the hypoxia-reperfusion experiment. The *in vitro* heat shock and hypoxia-reperfusion experiments reflecst the changes in HSP27 expression in labour and PE groups the inner and middle zones.

There was a significant increase in HSP90AA, HSP90B1 and HSPB2 in the labour group compared to the non-labour at the outer zone. HSPB2 expression was increased in the non-labour control compared to the non-labour PE group at the middle zone. In the heat shock experiment HSP90AA, HSP90B1 and HSPB2 expression was increased at the 6h of recovery time at the inner zone. HSP90AA expression was decreased and HSP90B1 expression was increased over time at the inner zone in the hypoxia-reperfusion experiment. The *in vitro* heat shock and hypoxia-reperfusion experiments do not at all reflect the changes in HSP90AA, HSP90B1 and HSPB2 expression in labour and PE groups at the middle and outer zones.

There was a significant increase in CASP7 in the labour group compared to the non-labour at the middle zone. CASP7 and CASP9 expression was increased in the non-labour and labour control compared to the non-labour and labour PE at the inner (CASP9) and middle (CASP7) zones. In the heat shock experiment CASP7 expression was decreased over time

at the middle zone. CASP3 expression was increased due to heat shock at the inner zone. The *in vitro* heat shock and hypoxia-reperfusion experiments reflect the changes in CASPs expression in labour and PE groups at at the inner and middle zones.
It was a clear that *in vitro* heat shock and hypoxia-reperfusion experiments reflect, for the majority of changes, the genes in labour and PE groups in their placental zones.

Final aim: BMI study

This study describes, for the first time, placental expression of HSPs and CASPs families in different BMI groups. The most interesting observations were that some HSPs (HSPB2, HSP27, HSP32 and GRP78) were reduced in the morbid obesity group (BMI>40) compared to the normal BMI group and obese grade 1 or 2. In contrast, CASP 3 and CASP9 was increased in the same group (BMI>40) compared to the normal BMI group and obese grade1 or 2. Reduced HSPs and increased CASPs indicate reduced cellular protection and increased cellular damage respectively.

The experiments, within this thesis examined placenta gene and protein expression only. Whether factors within the systemic circulation are altered requires further investigation.

This project is novel because it looked at the expression of stress, apoptotic and inflammatory pathways within the human placenta of labour, PE and FGR groups using precise sampling methods and how these genes were influenced by zonal difference, BMI classification, ischemic-reperfusion and heat shock insults. It also examined how such expression was affected by pregnancy complication such as PE and FGR. This project for the first time shows the important of systemic sampling methods. This project allowed a better understanding about how these different genes are expressed in the placenta alone and how labour, PE and FGR can affect them. It has also highlighted the best ways in which such investigations could be extended. This project has contributed important data to the area of placenta research and could potentially help lead to an improvement in placenta protection during labour and PE. However it is clearly a complex process as the findings that zonal expression can differ so differently between different molecules and proteins makes interpretation of the studies very difficult. If researchers all use similar sampling methods it would make it much easier to compare studies and to move this field of research forward.

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16 Appendices

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Patient information sheets for collecting placentas and blood samples

Dr Akrem Abdulsid (Researcher)
Dr Kevin Hanretty (Consultant Obstetrician)
Prof Fiona Lyall (Professor)

Title: Understanding the cause of small birth weight babies and high blood pressure in pregnancy.

(Title of the project: An investigation of the regulation and functions of the stress pathways in pregnant women and in the human placenta.

Full title: An investigation of the regulation and functions of the inflammation, stress and apoptotic pathways in pregnant women and in the human placenta focused on the impact of pre-eclampsia and fetal growth restriction on these pathways.)

Invitation: you are being invited to take part in a research study. Before you decide it is important for you to understand why the research is being done and what it will involve. Please take time to read the following information carefully and discuss it with others if you wish. Ask us if there is anything that is not clear or if you would like more information. Take time to decide whether or not you wish to take part.

What is the purpose of the study? Some mums have high blood pressure when pregnant. In some cases the mums can be quite sick and the babies can be small for dates. The reasons for that are not known. We are studying the blood and afterbirth (placenta) as it may give us important clues to understanding the above conditions. We are interested in inflammatory and oxidative stress proteins which are involved in immunity response against microorganisms at maternal-fetal interface. We are trying to understand why this protein does not always work properly in pregnancies where the baby was small for dates or when the mum had very high blood pressure. Our study will last for 5 years to give us enough time to perform all the experiments and collect sufficient numbers of samples.

Why have I been chosen? To perform our research we therefore need to collect blood and placentas after the baby has been born. We will collect placentas from mums with high blood pressure, mums whose babies were small for dates and mums who had uncomplicated pregnancies.

Do I have to take part? It is up to you to decide whether or not to take part. If you do decide to take part you will be given this information sheet to keep and be asked to sign a consent form. If you decide to take part you are still free to withdraw at any time and without giving a reason. A decision to withdraw at any time, or a decision not to take part, will not affect the standard of care you receive.

What will happen if I take part? If you do take part we will collect blood samples, <1 teaspoon before and after delivery from a line that is already in place. Few placenta samples will be taken and transport to the laboratory. Then we will study different proteins to try and find out how they differ between the different pregnancies.

What are the possible benefits of taking part? This study will not be of any use to you if you had a small baby or high blood pressure. However by studying the placenta and blood markers we may understand these conditions better with a hope of one day finding a treatment.

If you wish to complain or have any concerns about any aspect of the way you have been approached or treated during the course of this study, the normal National Health Service complaints mechanism may be available to you.

Normally placentas are incinerated. We will take a few samples from the placenta for research and these samples will be stored until the end of the study and then will be discarded.

Incidental finding: as the implications of these results are as yet clinically uncertain, no action can be justified in direct response.

Will taking part in this study be kept confidential? All information which is collected about you (blood pressure, baby's weight, and your age, etc) will be kept strictly confidential. Any information about you which leaves the hospital will have your name and address removed so that you cannot be recognised from it i.e. it is will be strictly anonymous.

What will happen to the results of the study?

When our studies are complete we will publish them in scientific journals. If you are interested then a copy of the results can be obtained from us and our contact details are given.

Who is funding the research? Fund for this project covers by a PhD scholarship from Libyan Ministry of Higher Education and Scientific Research

The patient will be given a copy of the information sheet and a signed consent form to keep.

Thank you again for your time.

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CONSENT FORM

Title of the project: Understanding the cause of small birth weight babies and high blood pressure in pregnancy.

(F*ull title: An investigation of the regulation and functions of the inflammation, stress and apoptotic pathways in pregnant women and in the human placenta focused on the impact of pre-eclampsia and fetal growth restriction on these pathways.)*

Researcher's names: Dr. K. Hanretty (CI), Dr. A. Abdulsid (PI) and Prof. F. Lyall (AS)

Please initial Box

I confirm that I have read and understood the information sheet dated 16/05/2013 of the above study and I have had the opportunity to consider the information, ask questions and have had these answered satisfactorily. ☐

I understand that my participation is voluntary and that I am free to Withdraw at any time without giving any reason, without my medical care or legal rights being affected. ☐

I understand that sections of any of my medical notes may be looked at by responsible individuals from regulatory authorities where it is relevant to my taking part in research. I give permission for these individuals to have access to my records. ☐

I agree to blood samples being taken and to placental examination to be Undertaken. ☐

I agree to these samples being stored for the study time duration. ☐

I agree to take part in the above study ☐

Name of patient	Date	Signature
Name of person taking consent (If different from researcher)	Date	Signature
Doctor	Date	Signature

1 copy for the patient; 1 for researcher site file; 1 (original) to be kept in medical notes