The Relationship Between Pro-inflammatory Cytokines and Prostaglandin Production in the Human Amnion

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December 2017

This thesis is submitted for the degree of Doctor of Philosophy at Imperial College London

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This thesis is dedicated to my family

Abstract

Preterm labour (PTL) management may be improved through better understanding of term labour. Existing research suggests that myometrial inflammation stimulates the initiation of labour at term. However, this theory has recently been challenged by group data, derived from a collection of gestational tissues. In this thesis, amnion samples were selected and prolabour gene expression, inflammation and the steroid receptor pathways for the glucocorticoid and progesterone receptors (PRs) studied.

It was hypothesised that (i) amnion inflammation occurs in early labour (TEaL) and, consequently, may have a role in the onset of human parturition; (ii) prolabour gene expression is increased in amnion samples in TEaL and that (iii) both are related to a decline in progesterone (P4) signalling. It was also speculated whether (iv) P4 acts via PRs and not glucocorticoid receptors (GRs) and whether (v) P4 function in the amnion can be enhanced by the addition of cyclic AMP were investigated.

Analysis suggested that amnion inflammation occurs predominantly in TEsL but also in TEaL leaving it unclear whether amnion inflammation has a role in human labour onset. Increased prolabour gene expression was observed more significantly in TEsL and in PTL, was dependent on the underlying aetiology.

Human amnion epithelial cells were acquired from women undergoing term elective caesarean section. Cells were treated with P4 and a cAMP agonist, forskolin, independently and in combination, to investigate whether the effects of an *in vitro* IL-1β treatment may be reduced, in the presence and absence of mechanical stretch. Both P4 treatment alone, as well as P4 +F treatment reduced IL-1β-driven expression. However, stretch-induced changes in expression could not be reduced through P4 treatment, alone or in combination with forskolin. P4 maintained its capacity for reducing IL-1β driven COX-2 mRNA expression in the absence of GR, whilst overexpressing PR isoforms enhanced P4-driven action.

Declaration of Originality

All work presented in this thesis was performed by myself unless otherwise stated in the text.

Copyright Declaration

Acknowledgements

I would like to express my sincerest thanks to my supervisors: Professor Mark Johnson and Dr Suren Sooranna. I am so grateful for the opportunity to have experienced such academic expertise, ambition and drive. This experience has not only nurtured my academic but also my personal development; and for that I will be eternally grateful.

Kaiyu, thank you for patiently answering my never-ending list of questions throughout my time in the IRDB. Your expertise has inspired me both inside and outside of the lab, as a mentor and as a friend.

Thank you to all the members of the MJ group who consented and chased samples for me. Natasha, thank you for allowing us access to the tissue bank you so conscientiously acquired. Thanks also to Bronwen for your help with many-a-multiplex. A huge thank you to Pei: you went above and beyond ensuring I never lost a precious sample once it was consented. Your support and advice throughout my lab years have been influential, so thank you.

Thank you to my fellow lab mates, both in IRDB and CW. Particular thanks go to my predecessors: Angela, Hector, Julia and Laura; I have each one of you to thank for shaping my PhD experience. Together you all placed bets on how long my positivity would last, and perhaps in turn taught me to go beyond my own limits! Angela, your work ethic was truly inspiring, although 5 years on I can still hear you calling my name; once you left, your legacy of 'Anaaaaa', lived on. Laura, I am still in awe of your organisational skills and thank you for such thought-provoking chats. Julia, thank you for encouraging us with your enthusiasm and surprise post-it notes of positivity – I still have them! Hector, thank you for the use of your desktop during your clinical sabbatical – and *all* that that entailed!! Despite running at 110 mph, you always made time to advise me on stretch, FAK and beyond! Alice, you came into the group only recently but I am so grateful for your positive presence, especially during the last year of my PhD. You helped me appreciate the little things -mostly edible and from your very well stocked desk drawer! Pei, Sophia, Lydia, Nish, Johann, Matt, Ida, Alex, Katerina, Priya, Gav and AnnieBelle – thank you for making life in the MJ research

group so fun. Together, we made up a truly unique lab family where cake and hilarity were always in abundance, and where I came to feel at home.

Huge thanks go to Professor Bennett's group and the rest of the 3rd floor, IRDB. A special thanks goes to Kim, Roberta and Yooni. Kim, thank you for taking me under your wing, passing on your amnion wisdom and for always questioning the sun's presence in London's grey skyline! Roberta, thank you for sharing in my positivity: for always managing to spot those sunsets from the IRDB lab window (despite them always being part-obstructed by Wormwood Scrubs Prison!). Yooni, thank you for being so helpful and acting as a guardian figure to all of us on the 3rd floor. Together, you all made my experience on the 3rd floor such a joy and I am truly sad to leave this unique place of work.

To my MSc-PhD friends: Kacie and Diana. We did it! I will remain eternally grateful for the support I received from both of you as we progressed through our MSc and PhD years. Thank you for being there when it mattered. When 'life' happened, you were both there to share the highs, the lows, the tears and the laughs.

To my non-research friends: thank you for sticking with me! You always made me feel included and never missed an opportunity to make a placenta-related joke!

Doug, thank you for being such a source of encouragement during my PhD. You remained patient, positive and understanding throughout and endured my out-of-hours lab trips with grace.

To my family, thank you for your unconditional love, support and faith from the beginning. Whether it was in person, or in spirit, this PhD would not have been possible without you and is as much yours as it is mine.

Thank you to all the women who selflessly donated their placentas to research.
And finally, to the preterm babies I know personally: thank you for sparking my interest in reproductive biology and motivating me to pursue research in this area. I truly hope we find a way to prevent babies like you, being born too early in the future.

Publications and Conference Presentations

1. **Das A**, Sooranna SR, Johnson MR. Stretch and Inflammation induced changes in the Amnion: Can the Anti-inflammatory Effect of Progesterone Be Enhanced in Stretched IL-1β Stimulated Human Amnion Cells, Society for Gynecological Investigation, 64th Annual Meeting, 2017, Orlando, USA (Poster)
2. Waldorf, K.M.A., Singh, N., Mohan, A.R., Young, R.C., Ngo, L., **Das, A**., Tsai, J., Bansal, A., Paolella, L., Herbert, B.R. and Sooranna, S.R., 2015. Uterine overdistention induces preterm labor mediated by inflammation: observations in pregnant women and nonhuman primates. *American journal of obstetrics and gynecology*, *213*(6), pp.830-e1
3. Sivarajasingam SP, **Das A**, Herbert BR, Fais MF, Singh N, Imami N, Johnson MR Immunological Changes In the Choriodecidua, Placenta and Amnion in Preterm Deliveries Secondary to Chorioamnionitis, Society for Gynecologic Investigation, 64th Annual Meeting, 2017, Orlando, USA (Poster)
4. Sivarajasingam SP, **Das A**, Herbert BR, Singh N, Imami N, Johnson MR Inflammation in Choriodecidua and Amnion in Preterm Birth Subtypes 2nd European Spontaneous Preterm Birth Conference, Gothenburg, Sweden, 2016 (Poster Presentation)
5. **Das. A.,** Singh. N., Sooranna S., Johnson MR. Stretch-induced changes in the amnion, San Francisco Society for Gynecologic Investigation, 62nd Annual Meeting, San Francisco, USA, 2015 (Poster Presentation)
6. Yulia A, Georgiou EX, **Das A**, Lei K, Sooranna SR, Terzidou V, Johnson MR Enhancement of Anti-Inflammatory Effect by the Combination of Progesterone and cAMP on IL-1β-stimulated human myometrial cells. Society for Gynecologic Investigation, 61st Annual Meeting, Florence, Italy, 2014 (Poster Presentation)
7. Yulia A, Georgiou EX, **Das A**, Lei K, Sooranna SR, Terzidou V, Johnson MR Enhancement of Anti-Inflammatory Effect by the Combination of Progesterone and cAMP on IL-1β-stimulated human myometrial cells. Society for Gynecologic Investigation, 61st Annual Meeting, Florence, Italy, 2014 (Oral Presentation)

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Abbreviations

AA:	arachidonic acid
ANOVA:	analysis of variance
AP-1:	activator protein-1
AR:	androgen receptor
cAMP:	cyclic adenosine monophosphate
CAPs:	contraction-associated proteins
cDNA:	complementary DNA
C/EBP:	CAAT/enhancer binding protein
COX:	cyclo-oxygenase
CRH:	corticotrophin-releasing hormone
Dex:	dexamethasone
DMEM:	dulbecco's modified eagles' medium
DMSO:	dimethyl sulfoxide
DNA:	deoxyribonucleic acid
dNTP:	deoxyribonucleoside triphosphates
EDTA:	ethylenediaminetetraacetic acid
ERK:	extracellular-signal-regulated kinases
FBS:	fetal bovine serum
FKBP:	FK506 binding protein
GAPDH:	glyceraldehyde 3-phosphate dehydrogenase
GR:	glucocorticoid receptor
HSD11β1:	11β-hydroxysteroid dehydrogenase type 1
Hsp:	heat shock protein
Ig:	immunoglobulin
IκBα:	nuclear factor-kappa B inhibitor α
IKK:	inhibitory κB kinase
IL-1β:	interleukin-1 beta
IFN-γ:	Interferon-γ
IPTL	idiopathic preterm labour
MAPK:	mitogen-activated protein kinase
MMP:	matrix metalloproteinase
NF-κB:	nuclear factor-kappa B

OTR:	oxytocin receptor
P4:	progesterone
PBS:	phosphate buffered saline
PKA:	protein kinase A
PKC:	protein kinase C
PLC:	phospholipase C
PR:	progesterone receptor
PRE:	progesterone responsive element
PTLA	preterm labour, secondary to abruption
PTLC	preterm labour, secondary to chorioamnionitis
PTNL	preterm not in labour
PTLP	preterm labour, secondary to polyhydramnios
PTTWNL	preterm twin not in labour
PTTWL	preterm twin in labour
qPCR:	quantitative PCR
RNA:	ribonucleic acid
rpm:	revolutions per minute
RT-PCR:	reverse transcription polymerase chain reaction
SDS:	sodium dodecyl sulphate
siRNA:	small interfering RNA
SP-A:	surfactant protein-A
TEaL	term early labour
TEsL	term established labour
TNF-α:	tumor necrosis factor-α
TNL	term not in labour
USMCs:	uterine smooth muscle cells

1. Introduction

1.1 Human parturition

Human parturition is characterised by the expulsion of the fetus and the fetal membranes during the course of labour (1, 2). The process by which parturition occurs varies between species, however human parturition is distinct due to structural differences, the co-existence of a relatively large fetal head and a narrow pelvis (3). This process comprises of physiological events involving gestational tissues described below.

1.1.1 Gestational Tissues in pregnancy and labour

The gestational tissues comprise of both maternal and fetal tissues. The myometrium, cervix and decidua are of maternal origin, whilst the fetal membranes (amnion and chorion) and placenta are of fetal origin. Together these tissues and their communication are responsible for supporting pregnancy as well as triggering labour onset. (Figure 1.1).

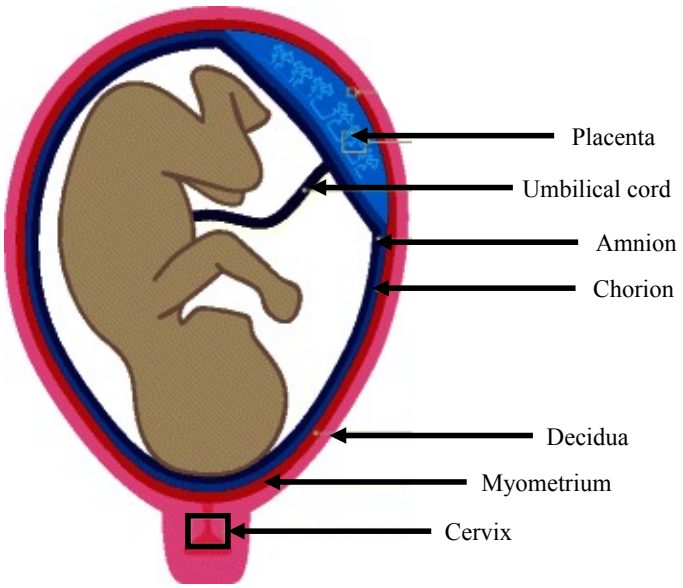

Figure. 1.1 The gestational tissues of the human uterus. [Modified from (4)]

Throughout pregnancy the uterus, a muscular organ, is responsible for holding the expanding fetus. The uterus consists of the myometrium, the cervix and the decidua. Though each play important roles throughout human pregnancy, this thesis focuses on research from the amnion; and thus the fetal membranes will be discussed in greater detail.

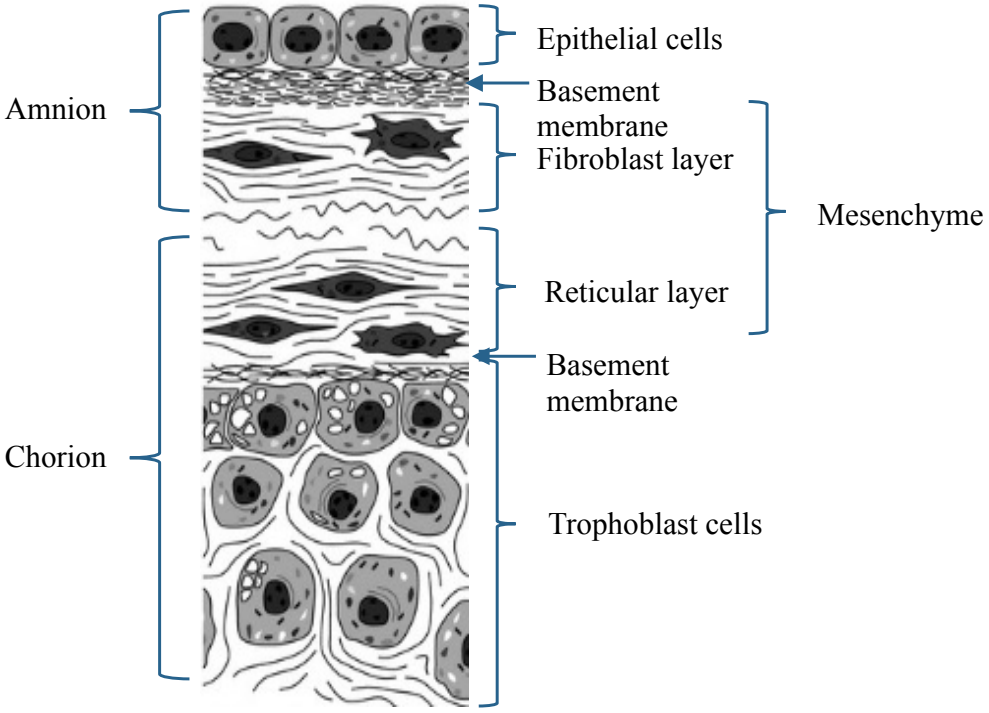

Figure 1.2 The human fetal membranes [Modified from (5)]

The fetal membranes consist of the amnion and the chorion (Figure 1.1 & Figure 1.2). During gestation, they expand with the growing fetus and amniotic fluid as well as acting as a barrier towards infection (19). On the inner layer of the fetal membranes lies the amnion, which interacts with the amniotic fluid. The amnion is made up of five different layers, epithelial cells and basement membrane. Together the fibroblast layer and surrounding reticulin make up the strongest component of the amnion, whilst the inner mesenchyme lies adjacent to the chorion. The amnion supports the fetal sac, offering it tensile strength. Though it lacks vascular tissue, it has been suggested that the amnion triggers labour onset through progressively increasing mechanical stretch, cytokine and prostaglandin (PG) production (19).

The chorion meanwhile consists of three layers: a trophoblast layer, chorionic basement membrane and reticular layer. The main constituent of the chorion is formed of the reticular layer, which remains attached to the chorionic basement membrane. The trophoblast layer acts as the connection between fetal membranes and decidua. Both chorion and amnion contribute to PG synthesis and hence are also associated with labour onset (22-24).

The placenta facilitates nutrient and gas exchange between mother and fetus (6, 7). It is made up of both lymphoid and non-lymphoid cells. Its neuroendocrinological properties enable it to manage hormone production including progesterone (P4), gonadotrophins and also corticosteroid-releasing hormone (CRH). In this way, the placenta is involved in both maintaining pregnancy but also triggering parturition (2, 8, 9).

1.1.2 Term labour and phases of parturition

Human gestation lasts around 40 weeks, although ‘term labour’ is defined as being between 37 and 42 weeks, when calculated from the last menstrual period (LMP). Variations in the timing of physiological labour taking place though may occur due to patient heterogeneity (10).

Human parturition can be divided into: quiescence, activation, stimulation and uterine involution (11). In early pregnancy, uterine quiescence is largely due to the actions of the steroid hormone, P4 (12-15). In many species, P4 levels remain high throughout pregnancy, only falling before the onset of labour. In humans however, circulating P4 levels do not decline before labour, but only after the delivery of the placenta (16).

CRH, human placental lactogen (HPL) and nitric oxide (NO) are all additional signalling components responsible for increasing levels of intracellular cyclic adenosine and guanosine monophosphate (cAMP and cGMP), which are important in later pregnancy. Elevated cAMP and cGMP concentrations reduce calcium signalling. As a result, cell contractility is decreased and this maintains quiescence (17, 18).

The activation phase involves a rise in contractile-associated protein (CAP) expression, including prostaglandin (PG) E and F receptors as well as oxytocin receptor (OTR). Receptor activation leads to the phosphorylation of myosin light-chain kinase. In addition, actin and myosin association occur (17-20) as well as myometrial contractility (21). Furthermore, increased connexin-43 (Cxn 43) expression between myometrial cells through the formation of gap junctions facilitates electrical signalling and coordinated uterine activity (17, 22-24). The stimulation phase is defined by the start of myometrial contractions and results in the expulsion of the fetus and placenta. The uterine involution phase that follows the stimulation phase returns the uterus to its pre-pregnancy state (17, 25).

1.1.3 Preterm labour

Preterm birth is the principal reason for neonatal death and in 2013, was responsible for almost 1 million deaths worldwide (26). Preterm labour (PTL) is the onset of labour before 37 weeks of gestation (27). The degree of prematurity may vary from severely preterm (<28 weeks), very preterm (28 to <32 weeks) to: moderate-to-late preterm (32 to <37 weeks). Moderate-to-late prematurity comprises 84.3% of all preterm births, whilst mortality rates depend on the level of prematurity (27-29). Not only are babies born preterm more susceptible to death as a result of infection, but their development is often impaired. Preterm birth is associated with blindness, deafness and compromised learning (30, 31).

Though evaluating the economic cost is largely dependent on population study and other variables, one review has estimated that in the first year of its life, an extremely premature baby will cost in excess of US $100,000 (32). Furthermore, preterm birth contributes to a substantial financial burden to health services (33, 34).

1.1.3.1 Causes of preterm birth

Preterm birth has various aetiologies and therefore it is more accurate to define it as a syndrome rather than a single disease (27). Its presentation can be sub-categorised into spontaneous (70%) or iatrogenic (30%) (Table 1.1) (27, 35). However, globally, the most significant cause of preterm birth is infection (35).

Spontaneous	**Iatrogenic**
Infection	Hypertension
Premature rupture of membranes	Diabetes
Idiopathic contractions	Intrauterine growth restriction
Multiple pregnancy	
Cervical dysfunction	
Antepartum Haemorrhage	
Stress	
Malnutrition	

Table 1.1 Causes of preterm birth [Adapted from (27)]

1.1.3.2 Management of PTL

The main strategy for PTL management is to delay parturition by 24-48 hours. Tocolytic drugs such as calcium channel blockers and OTR antagonists are administered to inhibit uterine contractions. With these drugs, the aim is to achieve a 24-48 hour delay to allow adequate time for corticosteroids (administered to the mother) to promote fetal lung maturity (36, 37). The main risk factors for premature birth are: previous PTL and a short cervix (38, 39). Thus, much of the research in this area has focused on prevention strategies. Two interventions previously disregarded as ineffective include: antibiotic prophylaxis (40) and bed rest (41). Though there is debate surrounding the efficacy of a cervical pessary (42), studies in this area are still on going.

Current PTL management approaches include: cervical cerclage (43) and the administration of P4 in singleton pregnancies. The OPPTIMUM trial however, recently found no significant decrease in the rate of PTL after vaginal P4 supplementation in high-risk singleton pregnancies (44) and progestins have consistently failed to prevent PTL in multiple pregnancies where excessive uterine distention is a factor (45). Therefore, it is even more important to establish the mode of P4's pro-quiescent action with the future aim of developing and refining ways to prevent and treat PTL.

1.2 Uterine Distention

1.2.1 The role of stretch in pregnancy

As pregnancy progresses, the uterus gradually expands to allow fetal and placental growth. This results in the maintenance of a constant, intra-amniotic pressure (46, 47). P4 and myometrial relaxants (NO and cAMP) (18, 48) support myometrial hyperplasia, hypertrophy and also inhibit CAP gene expression (49-51). As pregnancy comes to an end, uterine growth ceases. This disrupts the harmonised growth between the uterus and fetus, resulting in an increase in uterine wall tension. Stretch interacts with increased placental CRH and amniotic fluid surfactant proteins and lipids, all leading to the start of labour (23, 35, 52-54).

1.2.2 Excessive uterine distention and PTL

Excessive stretch, for example in the case of multiple pregnancy and polyhydramnios, is associated with PTL. It has been suggested that the increased incidence of PTL in higher order pregnancies could be due to the larger placental volume whereby this larger volume contributes to an elevated production of pro-contractile signals, e.g. CRH, known to trigger the onset of labour (55, 56). However, this fails to explain the cases of stretch-induced PTL if polyhydramnios is a cause, and therefore is considered unlikely. Furthermore, when CRH levels were compared between twin pregnancies born preterm and those born at term, no significant difference was observed in CRH levels (41).

Subjecting myometrial or amnion cells to *in vitro* mechanical stretch results in elevated production of pro-inflammatory mediators, such as IL-6 and IL-8. In addition, expression of CAPs, such as cyclo-oxygenase-2 (COX-2) and OTR also increased via nuclear factor κappa B (NF-κB) and activator protein-1 (AP-1) (57-62).

Stretch studies involving animal models have also demonstrated an increase in CAP expression in the myometrial gravid uterine horn compared to the myometrial non-gravid horn (50, 51). Uterine overdistention in pigtail macaques also displayed elevated levels of pro-inflammatory substances and resulted in PTL. These results mimic what was seen in women with polyhydramnios or a twin pregnancy (63). However, Ou, *et al* observed that in order for OTR levels to be significantly increased, stretch was not in itself adequate enough, highlighting the importance of endocrine signalling (50); in the timing of labour.

1.3 Inflammation in human pregnancy

Inflammation plays an important role in the innate immune response (64). It can be recognised by five classical signs: heat, pain, redness, swelling and loss of function. When the body experiences tissue injury or infection it responds accordingly in order to combat the insult and prevent further damage. This response leads to the recruitment and release of cytokines and chemokines. Immune cells, including neutrophils and macrophages are also recruited which then stimulate tissue regeneration (64).

Disruptions to the normal inflammatory regulation may lead to the development of disease (65). In the context of pregnancy, disruptions to the cytokine network are usually associated with spontaneous abortion, pre-eclampsia and PTL (66).

1.3.1 Cytokines and chemokines in human pregnancy

Cytokines are a family of proteins involved in the regulation of inflammation. They can be sub-categorised. Pro-inflammatory cytokines, IL-1α, IL-1β, IL-6, IL-8, TNFα and IFNγ, are regulated by T helper (Th) 1 cells. Anti-inflammatory cytokines, IL-4, IL-10, as well as adaptive immunity cytokines such as GM-CSF (67), are mediated by Th2 cells (67).

Chemokines are chemotactic proteins, which can be classified depending on the positioning of the N-terminal cysteine residues: CC family (CCL2 and CCL5); C family (XCL1); CXC family (CXCL1 and CXCL2) and CX3C family (CX3CL1) (67). These, chemotactic proteins stimulate cervical ripening and remodelling through their ability to recruit neutrophils into the fetal membranes and cervix (68). Local T cells produce Th2 cytokines, though they are mainly produced from lymphoid tissues: placenta and trophoblast. In a healthy human pregnancy, there is a shift in how Th1 and Th2 regulate inflammation. With increasing gestational age Th1, which is involved in cell-mediated immunity, becomes more dominant. Disruptions to this may occur in pathological examples of inflammation and cause an abnormal production of inflammatory cytokines; which may be involved in cases of pre-eclampsia, spontaneous abortion and preterm birth (69).

1.3.2 Inflammation in term labour

Term labour is commonly linked with inflammation of the cervix, myometrium and fetal membranes (70). This association is also indicated through the elevated levels of circulating pro-inflammatory cytokines and chemokines (71-73). In particular, increased levels of interleukin-1β (IL-1β), IL-6 and tumour necrosis factor α (TNFα) are observed in the amnion, myometrium, and choriodecidua during parturition (74-76). While myometrial inflammation has often been thought to trigger the onset of labour, recent evidence from our group suggests otherwise (76). Comparing non-labouring myometrial tissues with myometrial samples taken from various stages of labour, cytokine mRNA and protein levels were elevated only in established labour. Though neutrophils increased with labour onset they did not appear to infiltrate into the myometrium. This evidence suggests that perhaps inflammation is not a cause of labour onset but more a result of this change (76). While these recent findings have

been observed in human myometrial tissue, this thesis attempts to assess whether data obtained from human amnion tissue support this theory.

1.3.3 Infection and inflammation in PTL

Infection is a major cause of PTL and is also commonly associated with inflammation (77). The inflammatory response produced after pathogenic exposure from an infection generates a similar up-regulation of white blood cells as seen in term labour (54). An influx of neutrophils and macrophages can be observed as labour progresses and uterine contractions commence, resulting in parturition (78). Animal models have been used to study inflammation-induced PTL. Mice lacking macrophages did not go into PTL when exposed to bacterial lipopolysaccharide (LPS) (79), nor when exposed to a broad-spectrum inhibitor treatment (80). These studies demonstrate the important role played by inflammation in PTL as a result of infection.

1.3.4 Contraction associated proteins (CAPs)

1.3.4.1 Prostaglandins and their biosynthesis in human parturition

Prostaglandins (PGs) are biologically active eicosanoids. Stimulatory PGs such as PGE2 and PGF2α play a vital role in human parturition.

The precursor to PGs is arachidonic acid (AA). Cytoplasmic phospholipase $A2_2$ acting on cell membrane phospholipids results in AA production (Figure 1.3). In this pathway COX enzymes are fundamental to the production of PGs.

Whilst COX-1 is ubiquitously expressed in most tissues (81), COX-2 is an inducible enzyme. The COX promoter site contains binding sites for important pro-inflammatory transcription factors, such as, NF-κB (82, 83). COX-2 mediates the irreversible conversion of AA to PGH2, (84) and acts as the rate-limiting step in PG biosynthesis (84, 85).

Depending on cell type, PGH2, may be converted into PGs: TXA2, PGI2, PGD2, PGE2 or PGF2α (86) which may signal through specific receptors: TP, I2, D2, EP1-4 and FP respectively (86). The levels of receptor sub-types are dependent on the time of labour. For example, relaxatory receptors decrease at the time of labour onset (87, 88) whilst pro-contractile FP and EP3 increase (78) (Figure 1.3).

PGs contribute to the onset of labour by promoting:

1. Rupture of membranes, MMP activation and cervical dilatation (89); and
2. Increased intracellular calcium resulting in myometrial contractions (90-92).

In addition, studies have observed increased PG levels in amniotic fluid, intrauterine tissues and maternal blood (84, 93-97); thus supporting the involvement of PG biosynthesis in human parturition.

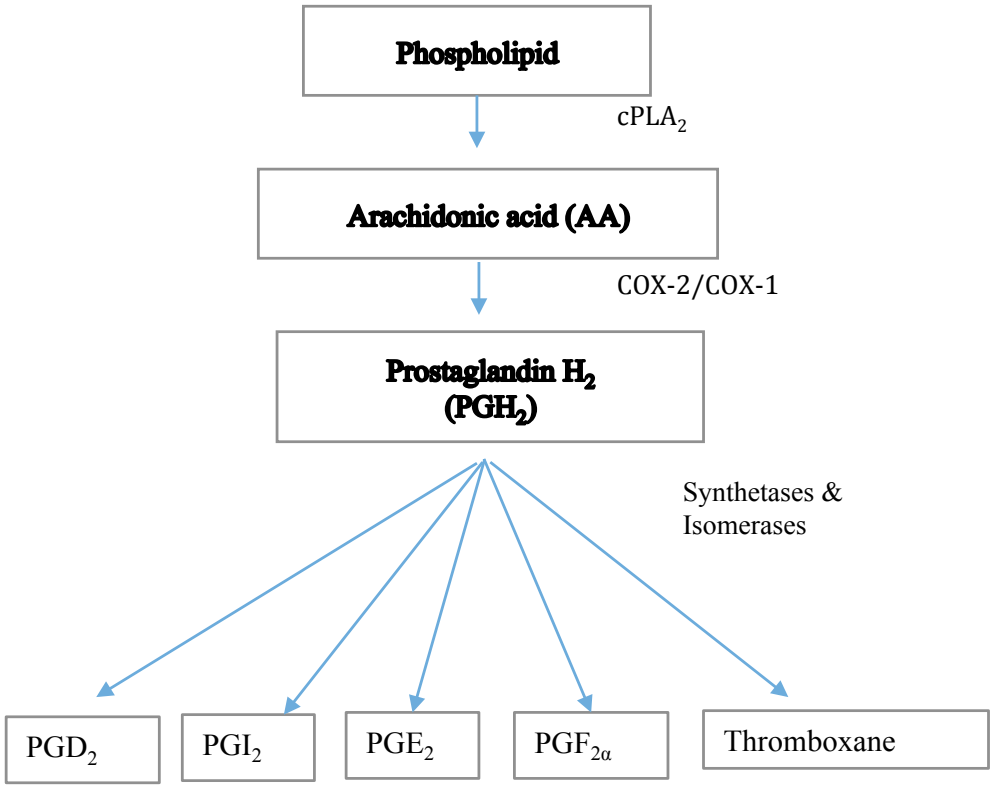

Figure 1.3 Prostaglandin biosynthetic pathways. [Modified from (98)]

The importance of COX-2 in parturition has been well established and prior to the onset of labour it appears to be up regulated in all gestational tissues (99-102). COX-2 mRNA and protein levels increased at term and during labour whilst COX-1 did not (99, 103-105).

Labour onset is typically associated with elevated levels of PGE2 seen in the human amnion and this has been linked with a partial induction of the COX-2 gene (106, 107). In addition, *in vitro* studies have shown inhibiting COX-2 synthesis decreased contractility (108) and lengthened parturition in animal models (109). With the available research, it is well accepted that COX-2 plays a crucial role in labour onset. As a result, it is understandable why regulatory mechanisms of COX-2 have been a dominant aspect of research. For these reasons, in this thesis it also is used as a major indictor for the onset of labour in the human amnion.

1.3.4.2 Oxytocin and oxytocin receptor in human parturition

Oxytocin (OT) is a nonapeptide hormone associated with human labour, which signals through oxytocin receptor (OTR). Its ability to cause the uterus to contract was first observed in 1906 (110) and it is still used today to induce the onset of labour and stimulate uterine contractions in pregnant women. Studies observing the impact of OT and OTR in the amnion have used PGE2 response as a predictor both in animal and *in vitro* models, as an indicator for labour onset (86, 111). In the *in vitro* setting, OT administration resulted in an increase in PGE2 response (76). Towards the end of pregnancy, rabbit amnion demonstrated increased OTR and PGE2 (112). These two models support the notion that OT and its receptor play a role in the initiation of labour.

1.3.4.3 Connexin-43 (Cxn 43)

Optimal delivery conditions include co-ordinated uterine contractions. These are achieved through the action of gap junctions, of which a key component is Cxn 43. Studies have shown an up-regulation of Cxn 43 with the onset of labour (23, 113). In addition, in 82% of animals, a conditional knockout for the protein extended the length of pregnancy (114).

1.4 Progesterone (P4) & its role in parturition

Progesterone (P4) (Figure 1.4) maintains pregnancy by working in conjunction with oestrogen to allow the fetus to be accommodated within the uterus (115). The absence of P4, for example, induced by a luteectomy, resulted in uterine contractions and pregnancy loss (116). Thus, the role of P4 in pregnancy has been demonstrated to maintain myometrial quiescence and repress inflammation (117). P4 action may also be mediated by the glucocorticoid receptor (GR) (118, 119); however whether this is the case in the human amnion will be assessed in this thesis.

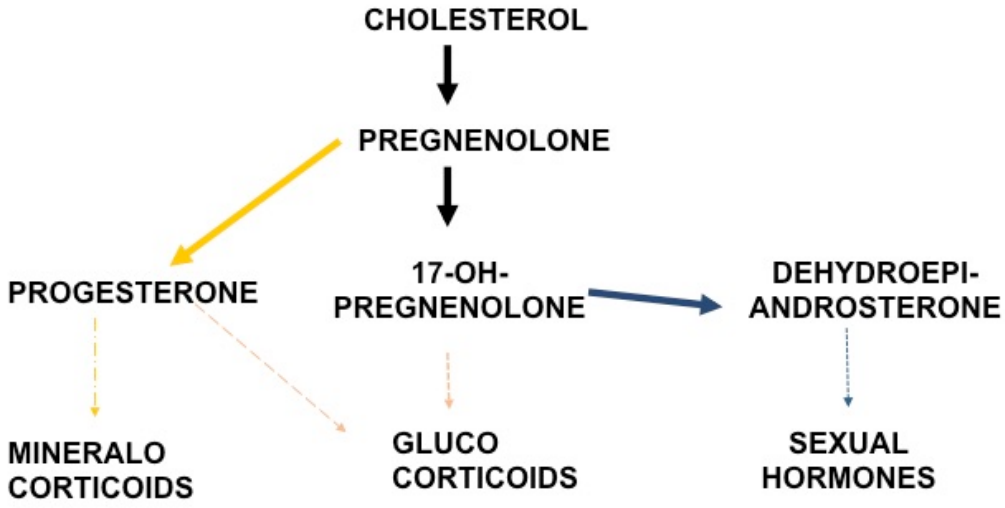

Figure 1.4 Biosynthesis of Steroid Hormones [Modified from (120)]

1.4.1 Nuclear progesterone receptors (nPR)

Genomic nuclear progesterone receptor (nPR) occurs as two main isoforms: PR-A (~94kDa) and PR-B (~116kDa) (121, 122). There was also speculation of another nuclear variant of the PR, PR-C, whose expression levels appeared to increase with the onset of labour. In the myometrial cell line, hTERT-HM, this presence seemed to repress the action of PR-B. It was speculated that this might act as a factor for P4 withdrawal (123). However, since then it has come to light that this more truncated PR-C form may not truly be present (124, 125) and evidence suggests that this was in fact an artefact (126-129).

In general, PR-B acts as a transcriptional activator of P4 responsive genes whilst PR-A acts as transcriptional repressor (130), though this classification has been debated recently (44). Studies within myometrial cells suggest that with advancing gestational age, PR-A's repressive influence on PR-B increases and thus activates a functional progesterone withdrawal and triggers parturition (131). Furthermore, it was found that IL-1β and LPS stimulation were able to enhance PR-A's ability to inhibit PR-B. These findings from Mesiano's group further reinforce the idea that labour onset is determined by this functional progesterone withdrawal (132). Whether this mechanism of action is prevalent in the human amnion remains to be seen.

1.4.2 Membrane progesterone receptors (mPRs)

Membrane progesterone receptors (mPRs) have been recently recognised in humans (133) and three GPCRs have been characterised: mPRα, β and γ (134). Another distinct family of PRs are PGRM1 and PGRMC2 (135, 136) though less is known about their action and roles.

1.4.3 PR co-regulators, chaperones, co-chaperones

Optimum PR function involves the presence of chaperones and co-chaperones: heat shock proteins (Hsp40, Hsp70, Hsp90, Hsc70/Hsp90) organising protein (Hop) and p23 (137-139). Unbound to a ligand, PR remains transcriptionally inactive. Furthermore, depending on the functional purpose, other co-chaperones may exist in other receptor complexes such as FK506 binding proteins: FKBP51, FKBP52 (140, 141). Knockout mice for FKBP52 exhibit resistance to P4 in the uterus despite normal PR expression and systemic P4 (140, 141). Mice void of FKBP51 display no known reproductive issues however FKBP51 has still been shown to have an important role: it has the ability to inhibit GR binding affinity (142, 143). This trait is relevant as it may help identify how P4 exerts its anti-inflammatory effect in the human amnion.

Co-regulators have the ability to boost or reduce transcriptional activity once the ligand-bound receptor has undergone dimerization and made its way to the nucleus (130). Steroid receptor co-activator-2 (SRC-2) displays dual functionality, switching its responsibilities depending on its intracellular conditions (130, 144-146). Thus, P4 action in myometrial cells and their sensitivity is heavily dependent on PR expression and its corresponding co-regulators. This relationship is yet to be fully determined in amnion cells.

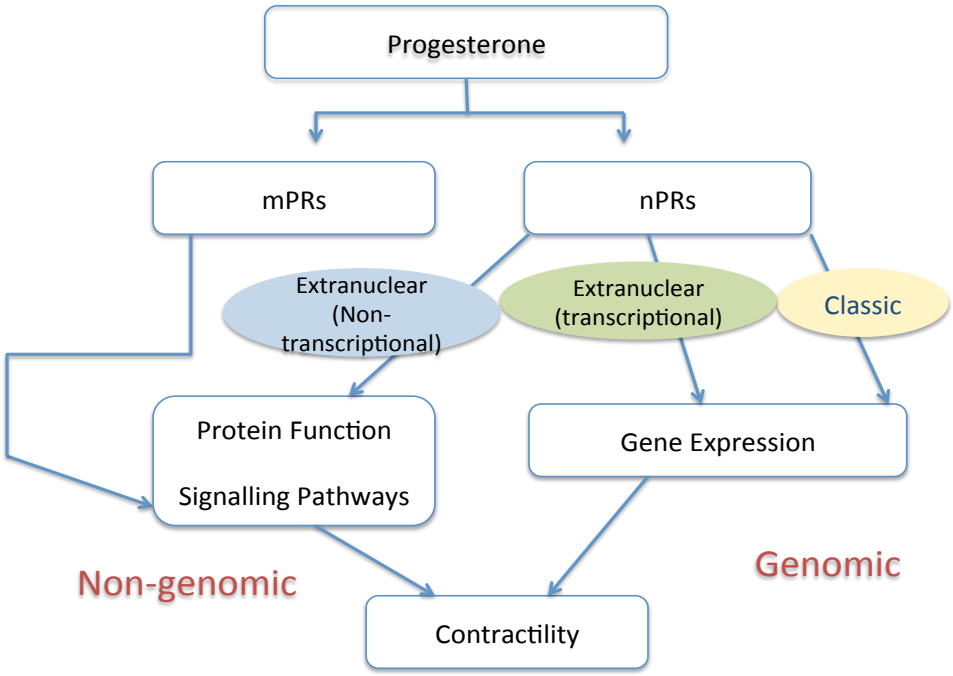

Figure 1.5 Genomic and non-genomic P4 signalling. [Adapted from (147)]

1.4.4 Regulation of P4 action

1.4.4.1 Genomic P4 action

Genomic P4 action has been proposed to be the primary way P4 acts in pregnancy and parturition (115) (Figure 1.5). Once P4 binding occurs in the cytoplasm, PR forms a dimer; which is able to move to the nucleus. Here the dimer binds to P4 response elements (PRE) and DNA sequences upstream from the start site of transcription (148-150).

The drug mifepristone (RU486), an antiprogestin displaying both agonistic and antagonistic properties, has the ability to stimulate parturition (151-153). It also has the ability to act on nPRs by encouraging both receptor dimerization as well as PRE binding (154, 155). Thus demonstrating the significance of genomic P4 action in reproduction.

1.4.4.2 Non-genomic P4 action

P4 has the ability to generate phenotypical change rapidly (156), a phenomenon inexplicable through genomic P4 signalling and one which may take hours to evoke the same result (157, 158). Non-genomic P4 action has been an intriguing area of research as it is able to create new proteins independent of DNA transcription or RNA translation. This signalling may involve: mPRs, nPR variants as well non-PRs including OTR and GABA receptor (159). P4, via this form of non-genomic signalling, has been shown to lessen levels of intracellular calcium (160, 161) a key component for generating effective contractility in myometrial cells.

1.4.4.3 P4 withdrawal

Unlike in most animals, P4 concentrations continue to rise throughout human pregnancy and are maintained even during labour (162-164). *Csapo* demonstrated that the drop in P4 or ‘P4 withdrawal’ was what gave rise to the start of labour in animals (165). However, in humans, P4 decreases only after the expulsion of the placenta (166-170). As mentioned previously mifepristone, the nPR antagonist, has the ability to bring about parturition at any stage of pregnancy (151-153) and also indicates the importance of nPRs in maintaining uterine quiescence (16, 171). Therefore, the term ‘functional P4 withdrawal’ has been used to describe the fact that whilst P4 levels remain elevated, P4 may lose its as labour approaches (16, 171).

1.4.4.3.1 Genomic mechanisms of functional P4 withdrawals

In order to maintain uterine quiescence during pregnancy, genomic P4 action works to suppress inflammation and contractility by inhibiting production of CAPs including: COX2 (119, 172, 173), OTR, and Cxn 43 (174-176).

It has also been observed that nPRs may interact with other transcription factors and therefore cause indirect genomic P4 signalling. One such example is the activation of the mitogen-activated protein kinase (MAPK) pathway through the interaction of Src-homology 3 (SH3) domain of Src tyrosine kinases. This however seems only to involve PR-B and not PR-A (177).

PR-B has been shown to enhance gene transcription through its relationship with co-activator proteins (130, 178). PR-A, however, seemed to have the opposite effect by inhibiting transcription with associated co-repressors (130, 178-180). More recently though, it is considered that the ratio of these two receptor isoforms determines a change in final phenotype (147).

1.4.4.3.2 Non genomic mechanisms of functional P4 withdrawal

The role of transmembrane receptors has also been implicated in functional P4 withdrawal, in particular PGRMC-1 and -2. Although a decrease in the mRNA expression levels of these two receptors was observed in both term and preterm myometrium (181, 182), there is limited evidence of P4's binding ability to these receptors (183, 184). Non-genomic mechanisms of functional P4 withdrawal remain unclear in light of contradictory evidence.

1.4.5 Cross-talk between NF-κB and steroid receptors

1.4.5.1 NF-κB

NK-κB plays an important role in inflammation (185) and makes up a family of transcription factors. The most ubiquitous NK-κB subtypes are p65 and p50 and can be found as heterodimers in the cytoplasm. These are bound to IκBα, their inhibitor and in this state are inactive (185). However, once activated, NK-κB separates from IκBα, and passes through to the nucleus where it acts as a major pro-inflammatory transcription factor (185).

1.4.5.2 Activator protein 1 (AP-1)

The family of AP-1 proteins is another major pro-inflammatory transcription factor system. The best-known members of this family are c-Fos and c-Jun, which form heterodimers. Similar to NK-κB, AP-1 is activated via phosphorylation (186) and following this, translocates to the nucleus. Here, it modulates transcription by binding to response elements or interacting with other transcription factors. The MAPK signalling pathway is an essential component in allowing signal transduction from the cell membrane, arriving at AP-1. Constituents of this cascade include extracellular signal-related kinases (ERK) 1/2, p38 and Jun N-terminal kinase (JNK), which are activated by phosphorylation (186). This cascade is contained and regulated through the action of MAPK phosphatase-1 (MKP-1), which dephosphorylates MAPK (187, 188).

1.4.5.3 The link between steroid receptors NF-κB

Liganded steroid receptors, such as PR and GR, have been shown to reduce NF-κB activity, and as mentioned previously parturition is closely linked with inflammation. Therefore, given the role NF-κB plays in mediating inflammatory gene expression, the association between it and steroid receptors suggests an important role in the maintenance of uterine quiescence during pregnancy and labour onset.

In addition, although P4 activates PR, it does also have the ability to bind to GR; meaning it is possible that some of its activity may be mediated by the latter (189).
Primary cultured trophoblast cells, taken at term, showed minimal PR expression suggesting the inhibitory P4 action was down to GR-mediated signalling (190). Cortisol, an important glucocorticoid, remains the specific ligand for GR (189).

Towards the latter part of pregnancy, higher cortisol levels may selectively compete with P4 to bind with GR, resulting in increased CRH levels with progressing gestation.

This serves as an example of the cross talk between steroid receptors and NF-κB in pregnancy. It also poses an interesting question as to which receptor is favoured, if any, in P4 signalling in the amnion which will be discussed later in this thesis.

1.5 Cyclic adenosine monophosphate (cAMP)

Cyclic adenosine monophosphate (cAMP) is a commonly expressed second messenger involved in physiological processes through its involvement in protein kinase activation, regulating calcium transport and gene expression (191). cAMP is generated as a result of a ligand-receptor binding interaction; usually a G-protein-coupled receptor (GPCR) to a specific ligand is able to incite the generation of intracellular cAMP via adenylyl cyclase. Activated adenylyl cyclase catalyses the conversion of ATP to cAMP (192, 193). Protein kinase A (PKA) is a very important and common cAMP-dependent target. The binding of cAMP molecules to PKA liberates 2 subunits that phosphorylate specifically intended proteins (194). These subunits, once activated, can in turn cause phosphorylation of other cytoplasmic molecules, translocate or affect transcription factor activity (192).

1.5.1 The role of cAMP in the human uterus

cAMP is involved in reproductive processes including ovarian cell proliferation, oocyte maturation and pregnancy (195, 196). In pregnancy, cAMP is thought to maintain uterine quiescence (191, 193), but may also have the opposite effect in certain cases.

Prostacyclin, known for activating cAMP/PKA signalling was thought to only contribute towards myometrial quiescence. Yet, when term myometrial tissue was exposed to long-term prostacyclin, myometrial contractility was stimulated via the elevated expression of CAPs, e.g. Cxn43, calponin and alpha-smooth muscle actin (197). Recent evidence has shown that in the myometrium, COX-2 expression was also increased due to cAMP (198). Thus the ultimate function of cAMP may be down to the fine balance of its differing results (197). Findings from our research group have indicated that a drop in cAMP/PKA activity may result in labour onset (199). Links have also been made between this functional decline of cAMP/PKA in mediating OTR expression as well as generating myometrial activation and the start of parturition (199). Thus, there is evidence for a dual functionality for cAMP in the myometrium whilst its role in the human amnion remains to be determined.

1.5.2 cAMP and P4 interactions

Currently, P4 administration is used to decrease the risk of PTL in high-risk women by around 40% (200). However, as previously mentioned, P4 is ineffective in stretch-induced PTL and this has been backed up by data from group findings (59). Current strategies therefore aim to understand the cause for this as well as investigate whether P4's anti-inflammatory effect can be enhanced.

In recent years, cAMP's anti-inflammatory role has been observed (201-204) to counteract the action of pro-inflammatory NFkB. In addition, in endometrial stromal cells, using cAMP and P4 together, promoted decidualisation more effectively than either agent alone (205). Our own recent findings suggest that forskolin, a cAMP agonist, enhances P4's anti-inflammatory effect resulting in a lessening of IL-1ß-induced COX-2 expression, nuclear transfer of p65 as well as DNA binding (206). These data go some way to suggest a mechanism that might enhance P4 action. Its role and whether this summative effect is present in the amnion is yet to be determined.

1.6 Hypothesis and Aims

1.6.1 Hypotheses

In human amnion tissue, it was hypothesised that:

1. Amnion inflammation occurs in early labour and, consequently, may have a role in the onset of human labour;
2. Prolabour gene expressions is increased in amnion samples in early labour and that both are related to a decline in progesterone signalling.

In primary amnion cells in culture, it was hypothesised that:

1. Mechanical stretch and inflammation drive pro-labour gene expression;
2. cAMP alone represses stretch and inflammation-induced gene expression;
3. P4 function in the amnion can be enhanced by the addition of cAMP: The combination of cAMP and P4 is more potent than either agent alone in the repression of stretch and inflammation-induced gene expression.
4. P4 acts via progesterone receptors and not glucocorticoid receptors to inhibit stretch and inflammation-induced gene expression.

1.6.2 Aims and Objectives

1. Evaluate the molecular changes observed in vivo with the onset of labour using a human amnion tissue bank;
2. Define whether P4 and/or cAMP represses the stretch and inflammation-induced changes in gene expression and through which pathways; and
3. Identify the mechanisms by which P4 may exert its anti-inflammatory effect in primary human amnion cells.

2. Materials and Methods

2.1 Materials

2.1.1 Chemicals, Reagents and Solvents

Absolute Ethanol	Fisher Scientific
Ampicillin	Sigma-Aldrich
Bovine Serum Albumin (BSA)	Sigma-Aldrich
Deoxynucleotide triphosphate (dNTP)	Invitrogen
Dimethyl Sulfoxide (DMSO)	Sigma-Aldrich
Dithiothreitol (DTT)	Bio-Rad
Ethylenediaminetetraacetic Acid (EDTA)	Sigma-Aldrich
Isopropanol	Fisher Scientific
Methanol	Fisher Scientific
Magnesium Chloride ($MgCl_2$)	Applied Biosystems
Non-fat dried milk powder	Applichem
PCR 10x buffer	Applied Biosystems
Oligo-dT random primers	Applied Biosystems
RNaseZap	Ambion
Potassium Chloride (KCl)	Sigma-Aldrich
Sodium Chloride (NaCl)	Sigma-Aldrich
SYBR Green	Applied Biosystems
NaOH	Sigma-Aldrich
Oligonucleotides and primers	Invitrogen

Antibodies

2.1.1.1 Primary antibodies

AP-1 phospho-c-Fos (Ser32)	Thermo Scientific, #MS-113-R7
AP-1 phospho-c-Jun (Ser63)	Santa Cruz Biotechnology, sc-822
α-tubulin	Santa Cruz Biochemicals, SC-8035
β-actin	Abcam, Ab6276
Connexin-43	Cell Signalling, 3512
c-Fos	Cell Signalling, 4384
c-Jun	Cell Signalling, 9165S
COX-2	Santa Cruz Biochemicals, SC-1745
ERK1/2	Cell Signalling, 9102
FKBP51	Santa Cruz Biotechnology, sc-11514
GAPDH	Millipore, MAB 374
GR	Santa Cruz Biochemicals, SC-1003
HSD11β1	Abcam, ab83522
HSP70	Abcam, ab2787
HSP90	Abcam, ab1429
IκBα	Santa Cruz Biotechnology, sc-371
JNK	Cell Signalling, 9252
MKP-1	Santa Cruz Biotechnology, sc-370
NCoR	Cell Signalling, 5948
OTR	Santa Cruz Biotechnology, sc-8102
p65	Santa Cruz Biotechnology, sc-8008
Phospho-ERK1/2 (Thr202/Thr204)	Cell Signalling, 9101

Phospho-ERK1/2 (Thr202/Thr204)	Cell Signalling, 9101
Phospho-NF-κB p65 (Ser536)	Cell Signalling, 3031
Phospho-NF-kB p65 (Ser536)	Cell Signalling, 3031
PR	Santa Cruz, sc-7208
SMRT	Santa Cruz Biotechnology, sc-13554
	& sc-1610; Abcam, ab2780
SRC1	Cell Signalling, 2191
SRC2	Bioss, 13157R
SRC3	Cell Signalling, 2126

2.1.2.2 Secondary antibodies

Anti-goat IgG, HRP-linked antibody	Dako,P0449
Anti-mouse IgG, HRP-linked antibody	Cell Signalling, 7076
Anti-rabbit IgG, HRP-linked antibody	Cell Signalling, 7074

2.1.3 Buffers, Solutions and Gels

Cell Lysis Buffer	1mL 10x cell lysis buffer
	1 tablet protease inhibitor
	100µL phosphatase inhibitor
	8900µL dH_2O

Phosphate Buffered Saline (PBS)	140mM NaCl
	2.5mM KCl
	1.5mM KH_2PO_4, pH 7.2
	10mM Na_2HPO_4, pH7.2
Sodium Dodecyl Sulphate (SDS)	25mM Tris-base
	250mM glycine
	0.1% (w/v) SDS
	pH 8.3
Tris Buffered Saline-Tween 20 (TBS-T)	130mM NaCl
	20mM Tris-HCl, pH 7.6
	Adjusted to pH 7.4 with HCl
	0.1% Tween 20
Western Blocking Buffer	5% (w/v) non-fat milk in TBS-T
Western Antibody Incubation Buffers	5% (w/v) non-fat milk in TBS-T 5% (w/v) BSA in TBS-T
	1% (w/v) non-fat milk in TBS-T

2.1.4. Cell Culture Materials and Media

Cell strainer (70μM)	VWR International
Dulbecco's Modified Eagles' Medium (DMEM)	Sigma-Aldrich
Fetal Bovine Serum	Sigma-Aldrich
L-glutamine	Sigma-Aldrich
Penicillin/Streptomycin	Sigma-Aldrich
Tissue Culture Plastic-ware	Sigma-Aldrich
Trypsin/EDTA	Sigma-Aldrich
6 well flexible-bottom plates	Flexcell International Corp.

2.1.5. Enzymes

DNase I	Life Technologies
MuLV reverse transcriptase	Applied Biosystems
RNase inhibitor	Applied Biosystems

2.1.6. Inhibitors and Treatments

Complete Protease Inhibitors	Roche Diagnostics
Forskolin	Sigma-Aldrich
Halt Phosphatase Inhibitor Single-Use Cocktail	Thermo Scientific
Interleukin 1β (IL-1β)	Sigma-Aldrich
Mifepristone (RU486)	Sigma-Aldrich
Progesterone	Sigma-Aldrich

2.1.7. Kits

Amaxa Nucleofector Kit:	Lonza
cDNA synthesis and SYBR Green for qPCR	Applied Biosystems
Homogenising CK28-R Precellys tubes	Stretton Scientific
RNeasy mini Kit:	Qiagen

2.1.8. SDS-PAGE electrophoresis and Western blotting materials

Amersham Hyperfilm ECL	GE Healthcare
Clarity Western ECL substrate	BioRad
Cell Lysis buffer	Cell Signalling
Nu-PAGE® LDS Sample Buffer	LifeTechnologies
Precision Plus Protein^{TM} Dual Color Standards	Biorad
SDS PAGE precast gels	Biorad

2.1.9. siRNA

ON-TARGET plus Non-Targeting siRNA:	Dharmacon, D-001810-01-05
ON-TARGETplus SMARTpool for GR:	Dharmacon, L-003424-00-0005
ON-TARGETplus SMARTpool for PR:	Dharmacon, L-003433-00-0005

2.2 Methods

2.2.1 *In vitro Studies*

2.2.1.1.Tissue Specimens

Institutional ethics committee approval was granted for the study. Healthy patients with no known factors such as were invited to partake in this study. Patients experiencing induction of labour, gestational diabetes mellitus (GDM), pre-eclampsia or obstetric cholestasis were not considered. Once patients had provided written consent, term or preterm human fetal membranes were obtained and stored depending on experimental protocol. Human amnion samples were either chilled at 4°C in phosphate-buffered saline (PBS) ready for processing within maximum 1 hour or snap-frozen at -80°C.

2.2.1.2 Primary Cell Culture

For cell culture, fetal membranes were attained from women undergoing an elective caesarean section at term (between 38-40 weeks) due to: breech presentation, prior caesarean section or maternal request. Human amnion was separated chorion-decidua and placed in PBS. Following 3 washes in PBS, the one membrane was then divided into smaller strips and incubated in in 0.5 mmol/litre EDTA at room temperature for 15 minutes. Following this, these strips were washed again in PBS and then transferred into 2.5g/litre dispase at 37°C. After a 45 minute incubation, the strips were transferred to 'full' culture medium: DMEM, (10% fetal calf serum and 100 mU/mL penicillin and 100 μg/mL streptomycin) and shaken vigorously for 4 minutes to separate amnion epithelial cells. The liquid, cell suspension was centrifuged at 3000 rpm for 10 minutes. Full DMEM culture medium, was used to resuspend the cell pellets. The cells were then cultured into 6-well flexible bottomed culture plates in 2.5ml DMEM full culture medium. On days 3-4, when cells were 95% confluent, medium as aspirated off and replaced with 2.5ml serum-starving (serum free) medium.

2.2.2. The stretch model

Cells were subjected to 11% static mechanical stretch for up to 24 hours and in accordance to manufacturer's protocol and guidance: http://www.flexcellint.com/; Flexcell International Corp., McKeesport, PA. The controls used were cells unstretched, yet grown and treated in similar conditions. Though the stretch model cannot offer a direct comparison between excessive stretch-related cases in pregnancy,

it does offer a means to study the impact of *in vitro* stretch on cell signalling and gene expression.

2.2.3. Transfection

Amnion cells were cultured in PS- and LG-free, 10% FCS DMEM medium until ~60% confluent. Cells were transfected using DharmaFECT 2 (Dharmacon) for siRNA according to the manufacturer's protocol. Expression vectors were transfected at concentrations of 300 ng per well. pSG5 was used as an empty expression vector. For each 6 well plate, the transfection reagent contained 4µl of Dharmafect transfection reagent with 196 µl of DMEM (phenol-red; serum-, PS- and LG-free) and incubated at RTP for 5 minutes. The siRNA (10µl of 20µM siRNA) was then added to the mixture in combination with 190µl of DMEM (again, phenol-red; serum, PS- and LG-free). This solution was then incubated at RTP for 20 minutes. The mixture that the cells were grown thus contained: 400µl of the reaction mixture and 1600µl of 10% FC DMEM for cells to be ready to be treated. Following treatment, supernatant was removed and cells harvested ready for mRNA and protein extraction.

2.2.4. Long PCR product Amplification

DNA polymerase was used in order to make completing the PCR process possible. Each 50 µl reaction used: 200 µM of each dNTP, 50nM of each sense and antisense primer, 20 ng template DNA, 1 x PCR buffer and 0.5 µl of Promega *pfu* Taq.
Heating to 95˚C for 2 minutes denatured templates of DNA. This was then followed by 35 thermo-cycles of 95˚C for 30 seconds. Primer annealing occurred between 60˚C for 30 sec, and primer extension at 72˚C for 5 min. The final extension was carried out at 72˚C for 10 min. Gel electrophoresis was used to analyse the final products.
Agarose was dissolved in fresh 1 x TBE and heated until boiling, in order to prepare 2% agarose gels. Once the solution had cooled to 50˚C, SYBR safe was added and it was poured into a mould. Upon setting, the gel was immersed into a gel tank of 1x TBE buffer. The wells were loaded with DNA samples (containing 1 x DNA loading buffer).
Time needed for electrophoresis was dependent on DNA size, but the process was carried out at 120V. Comparing the relative mobility of DNA fragments with restriction fragments of known sized markers enabled the DNA fragment size to be estimated.

Gels, from DNA that would undergo purification, were imaged on a dark reader. The band was then cut.

2.2.5. Purifying DNA from agarose gel

The GenElute™ Gel Extraction Kit was used in order to purify DNA fragments from agarose gels. The gel band was weighed and a solubilisation solution was added based on the kit protocol.

Incubation of the mixture was carried out at 50-65˚C for 10 min. For every 100mg of gel, 100 µl of isopropanol was added to the mixture. Following various washing protocols, the column was put into a new micro centrifuge tube. 50µl ddH_2O was added to this new column and incubated at RTP for 3 minutes, then centrifuged at 17000×g RTP for 1 minute.

A Nano Drop Nd-1000 spectrophotometer was used to calculate the DNA concentration.

2.2.6. Digestion of restriction enzymes digestion and ligation

The digestion process required the addition of 20 µl of DNA, 5 µl of 10×restriction enzyme buffer, 1 µl of restriction enzyme and nuclease-free water. This was then incubated for 1-4 hours at 37˚C.

Following digestion, positive cloned constructs were examined through the running of agarose gels or purified using the PCR purification kits for subsequent ligation.

For ligation, purified DNA fragments (30-50ng) was used in addition to a 5:1 molar ratio of another parental vector. The final mixture consisted of 10 µl with: 1×T4 DNA ligase buffer and 1 µl T4 DNA ligase. These reactions were incubated for 2 hours at RTP.

2.2.7. Colony PCR

Colony PCR was used to evaluate the positive clone after ligation and transformation. A colony was picked from an agar plate and resuspended with 20 µl of water. From this, a small volume was kept to inoculate LB for the growth of minicultures. 15 µl was heated at 95˚C, for 5-10 minutes, in order to lyse the cells.
Centrifugation was carried out at 16,000×g, for 2 minutes in order to remove any debris. From the supernatant, 1µl was used to act as a template in the PCR reaction.

2.2.8. DNA sequencing

The plasmid was sent for sequencing following positive clone selection. This was provided by Imperial College London and the MRC clinical sciences service. Results were analysed using specialist DNAMAN software.

2.2.9. RNA extraction and qPCR

RNA extraction and purification of total RNA from amnion cells were carried out using an RNeasy mini kit as per the manufacturer's protocol. RNA extraction for human amnion tissue involved by placing tissue in pre-cooled Precellys tubes containing RLT buffer (Qiagen). These tissues were homogenised using a Precellys homogeniser at 5000rpm in 2 x 20 second cycles. Following this, total RNA was extracted using the same RNeasy mini kit and the manufacturer's protocol was followed.

A NanoDrop Nd-1000 spectrophotometer was used to determine the concentration and purity of RNA using the 260/280 ratio (a ratio of ~2.0 was deemed satisfactory) and RNA was stored at -80°C.

1.5µg of RNA was heated to 70 degrees Celsius for 5 minutes and then reverse transcribed to cDNA: using 1µl 50µM oligo dT random primers, 4µL 10x PCR buffer, 8µL 25mM $MgCl_2$, 8µL 10mM dNTPs, 1µL 50µM oligo dT random primers, 1µL MuLV reverse transcriptase and 1µL RNase inhibitor.

Primer 3 software was used to design sets of primers. The Max Poly-X' setting was set to 3.

In order to check the resulting primer sequences matched the gene of interest, they were input into Nucleotide Blast:
(https://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE_TYPE=BlastSearch) .

Quantitative real-time PCR required the use of SYBR Green. The Rotor Gene R-G 3000 was used as it measures fluorescence, which is a result of the dye-binding to double-stranded DNA. The protocol consisted of: a pre-PCR cycle of 10 minutes at 95˚C, up to 45 cycles of 95˚C for 20 seconds, 58-60˚C for 20 seconds and 72˚C for 20 seconds followed by an extension at for 15 seconds.

The final step involves a melt over the temperature range of 72-99°C rising by 1 degree. On the first step, the wait is 15 seconds whilst from then on, the wait becomes 5 seconds for each subsequent step. The cycle threshold, in which fluorescence reaches a predetermined threshold, was used for quantitative analyses.

A ten-fold dilution series was used to determine a standard curve. This was done for each set of primers from the concentrated DNA template. All mRNA data were expressed and normalised to the amount of constitutively expressed GAPDH.

Table 2.1: Primer Pair Sequences with Gene Accession Numbers

Group	Name	Forward (F) and Reverse (R) primer sequence (5'-3')	Genebank/EMBL Accession no.	Product size (bp)
Housekeeping Gene	**GAPDH**	F:tgatgacatcaagaaggtggtgg R: tccttggaggccatgtaggccat	BC014085	240
Progesterone responsive genes	**FKBP51**	F: tccctcgaatgcaactctct R: gccacatctctgcagtcaaa	NM_001145775	194
	HSD11B1	F:accttcgcagagcaatttgt R:gccagagaggagacgacaac	NM_005525	226
Contraction associated proteins	**PTGS2**	F:tgtgcaacacttgagtggct R:actttctgtactgcgggtgg	AY151286	297
	OXTR	F:agaagcactcgcgcctctt R: aggtgatgtcccacagcaact	NM_000916	102
	GJA1	F:aattcagacaaggcccacag R: catggcttgattccctgact	NM_000165	214
Nuclear receptors	**PGR**	F: agcccacaatacagcttcgag R: tttcgacctccaaggaccat	NM_000926	293
	NR3C1	F:cttccagaaccatggtagcc R: tacgaaactccacccaaagg	NM_002425	166
Chemokines & Cytokines	**IL1A**	F:aatgacgccctcaatcaaag R:tgggtatctcaggcatctcc	NM_000575.4	226
	IL1B	F: gctgaggaagatgctggttc R:tccatatcctgtccctggag	NM_000576	240
	IL6	F: agtgaggaacaagccagagc R: gaggtgcccatgctacattt	NM_000600	246
	IL8	F: gccttcctgattttgcagc R: cgcagtgtggtccactctca	NM_000584	150
	IL10	F: tgccttcagcagagtgaaga R: ggtcttggttctcagcttgg	NM_000572	170
	CXCL1	F: gaaagcttgcctcaatcctg R: gcctctgcagctgtgtctct	NM_001511	174
	CXCL2	F: ctgctcctgctcctggtg G: gctttctgcccattcttgag	NM_002089	191

CCL2	F: tctgtgcctgctgctcatag R: agatctccttggccacacaatg	NM_002982	203
CCL5	F: ccatattcctcggacaccac R: tgtactcccgaacccatttc	NM_002985	263
TNFα	F: gagaagggtgaccgactcag R: ggttgagggtgtctgaagga	NM_000594	174

2.2.10. Whole-cell protein extraction

The extraction buffer used for whole-cell protein extraction consisted of cell lysis buffer, 1 protease tablet and 100µl phosphatase inhibitor per 10 ml. This cocktail was applied onto 6-well plates. In order to separate and remove all cell debris, the lysate was centrifuged at 13,000xg for 20 minutes at 4˚C.

Pre-cooled Precellys tubes were used to weigh human amnion tissue pieces. Depending on each the weight of each piece, 10µl cell lysis buffer was added per 1mg. Amnion tissue underwent the 2 x 20 second cycles at 5000rpm homogenisation process using the Precellys homogeniser. Following this, centrifugation of the lysate was completed as above at 13,000xg for 20 minutes at 4˚C.

Samples were then aliquoted and stored at -80˚C.

2.2.11. Protein Assay

The DC protein assay (Biorad) was used as per manufacturer's protocol. Reference standards were made up using bovine serum albumin (BSA). The Optimax microplate reader (Molecular Devices) was used as the plate reader and the plate was read at 650nm.

2.2.12. Western blot analysis

NuPAGE LDS sample buffer was used to denature proteins. Following the addition of NuPAGE, the mixture was then heated at 75˚C for 10 minutes. Western blotting was performed through electrophoresis via a PVDF membrane and Trans-Blot Turbo transfer system (Biorad). Membranes were immersed into blocking buffer (5% milk

and 1 x TBST) for 1 hour at RTP. Membranes were then incubated overnight with primary antibody at 4˚C. TBST was used to wash the membranes again prior to secondary antibody incubation at RTP for 2 hours. Immunodetection was carried out using Clarity Western ECL substrate (Biorad).

2.2.13. Chemokine/Cytokine Assay

BioRad Cell Lysis buffer was supplemented with 2mM PMSF and Biorad QG solution to lyse human amnion tissue. Tissue pieces were homogenised using the Precellys homogeniser (4 x 20 second cycles at 5000rpm).

Tissue media was separated from cell debris through centrifugation at 13, 000 x g (20 minutes at 4˚C). Human Bio-Plex© ProTM assays were used to measure the concentrations of chemokine and cytokines including: IL- 1α, IL-1β, IL-6, IL-8, CCL2, CCL5, CCL11, CCL20. These were performed according to the manufacturer's instructions and were read using a Bio-Plex© 200 reader and Bio-Plex Manager© v7.1 software. Tissue weight was used to normalise the concentrations of chemokine and cytokine results.

2.2.14. Statistical analysis

The Kolmogorov-Smirnoff test was used to first test all data for normality. Following this, a Student *t* test (for two groups) was used for normally distributed data or an an ANOVA followed by a Dunnett *post hoc* test (three or more groups). For match paired data not normally distributed (two groups), a Wilcoxon matched pairs test was used.
Nor non-paired, not normally distributed data, the Mann–Whitney *U* test was used. A Friedman's Test, with a Dunn's Multiple Comparisons *post hoc* test was used for comparing three or more groups. Statistical significance was considered $p<0.5$.

3. A functional assessment of prolabour gene expression, cytokine profiling and nuclear receptors in human amnion tissue

3.1 Prolabour gene expression

A marked increase in prolabour gene expression particularly COX-2, OTR and Cxn 43 in the myometrium is widely associated with term labour and myometrial activation (207). In addition, activated macrophages release inflammatory mediators including cytokines, which stimulate PG synthesis in gestational tissues (208), for which the amnion is largely responsible (86, 111). COX-2 is the rate-limiting step in PG biosynthesis; it is an inducible enzyme, which is up regulated in all gestational tissues with the onset of labour (100-102).

The heightened leukocyte infiltration into the myometrium during parturition supports the pre-existing theory that there is a link between labour and inflammation (71, 73, 209). In addition, Norman and colleagues demonstrated the association between inflammatory cytokines stimulated myometrial contractions (210).
However, a recent publication from Singh *et al* suggested that the presence of inflammation in term myometrial tissue might not cause term labour but simply be a consequence of labour (76). Similarly, the role of amnion-inflammation is uncertain in different causes of PTL.

Intrauterine infection, for example, has been implicated in the onset of PTL. Where infectious agents activate macrophages present in gestational tissues, including the fetal membranes, stimulating the release of inflammatory cytokines (71, 73, 209, 211).

In Section A, this chapter aims to:

1. Assess the presence of inflammatory cytokines and prolabour gene expression in amnion samples to determine whether inflammation occurs in early labour and may therefore have a role in its onset or only occurs in established labour and may therefore only be a consequence.
2. Assess inflammation in amnion samples from different causes of PTL to define whether each type of PTL is associated with its own unique cytokine-chemokine profile.
3. To relate changes in inflammation to prolabour gene expression in human amnion samples.

3.2 Assessment of GR and PR expression

Primary cell data carried out by the group identified a potential role for GR in P4 signalling (59). As a result, GR mRNA and protein were investigated in this chapter. The two major isoforms of PR work in conjunction; PR-B mediates P4 action whilst PR-A has long been held to oppose this effect, although recent data suggest PR-A has its own transcriptional effects (173) (131). In human labour, it has been suggested that a switch from PR-B dominance to PR-A dominance occurs, which is the basis of one potential explanation for the functional P4 withdrawal. (131, 212, 213)

In Section B, this chapter aims to:

- Characterise the changes in pattern expression of GR, the PR-A/PR-B ratio, as well as PR co-activators and chaperone expression in human amnion with the onset of human labour.

3.2.1 PR chaperones, co-chaperones and co-regulators

PR's function is a direct result of its relation with other associated proteins, chaperones and co-regulators. During the onset of labour it has been observed that PR function and binding to its ligand decreases. This finding is another potential explanation for functional P4 withdrawal (214).

3.2.2 Chaperones and co-chaperones

Heat shock protein 70 (HSP70) and heat shock protein 90 (HSP90) are chaperone proteins that facilitate the nuclear receptor binding (215, 216). Co-chaperones, such as FKBP506-binding protein 51 (FKBP51) and FKBP52, interact with HSP 90 (217). Findings have demonstrated FKBP51 as P4 responsive and it has also been shown to inhibit nuclear receptor activity (218, 219). As a result, FKBP51 may be involved in signalling via negative feedback mechanisms (142).

3.2.3 Co-regulators

A wide number of non-DNA binding proteins named co-regulators exist displaying tissue- and gene-specific actions (220, 221). Their role in parturition and functional P4 withdrawal has been speculated (213, 222) and they can also be sub-categorised into co-activators and co-repressors. In general, the former enhances gene transcription by interacting with liganded nuclear receptors (223), whilst the latter binds to unbound nuclear receptors and inhibits transcription (223). However, the reverse is commonly also true, with co-repressors enhancing transcription and co-activators inhibiting transcription (224). The p-160 co-activator family consists of steroid receptor co-activators (SRC) 1,2 and 3 (225), which together with other co-regulators, aid chromatin remodelling at the PR complex (225, 226). It is thought that PR-B's ability to bind for SRC-1 and SRC-2 is greater than that of PR-A (130).

Within parturition, the most prominent active co-repressors are nuclear receptor co-repressor (NCoR) and silencing mediator for retinoid or thyroid hormone (SMRT). Interestingly, in cell lines, SMRT has been observed to bind with PR-A more favourably than PR-B (130), although others have suggested that neither are involved in PR action (220). In general, although they have been detected at the mRNA level in the myometrium their presence has not been well characterised in the amnion (227).

The roles of steroid receptor chaperones and co-regulators are not known in human amnion. Therefore, this chapter aims to define changes in GR and PR expression in human amnion with the onset of human labour.

3.3 Chapter Aims Summary:

To define the changes in inflammation, prolabour gene and steroid receptors in human amnion:

1. With advancing gestation
2. With the onset of term labour
3. With the onset of PTL of defined phenotype

3.4 Demographic data

3.4.1 Amnion tissue bank

Samples were taken from a large collection of amnion, obtained from women at different stages of pregnancy and labour (term and preterm). In labour, the human amnion is largely responsible for PG synthesis, which is essential for the onset of labour. Vital components of the PG synthetic pathway are COX-2 and arachidonic acid, the PG precursor, both of which are present at high concentrations in the amnion.

The following four groups of samples were compared:

- Preterm not in labour (PTNL): 24-36 weeks of gestation
- Term not in labour (TNL): >37 weeks of gestation
- Term early labour (TEaL): 3-4 contractions in 10 minutes and <3cm dilated
- Term established labour (TEsL): 3-4 contractions in 10 minutes and >3cm dilated

The demographic data for these groups are shown in Table: 3.1

In order to define the changes in expression by PTL phenotype, PTNL samples were compared with

- **Idiopathic preterm labour, (IPTL)**

  - 24-26 weeks of gestation, women who were delivered preterm with no evidence of infection or previous surgery or other pathology
- **PTL secondary to chorioamnionitis (PTLC)**

  -Infection-induced PTL at 24-36 weeks
- **PTL secondary to polyhydramnios (PTLP)**

  - Excessive amniotic fluid compared to an amniotic fluid index >24 cm. Dilation of cervix did not surpass 3cm in all samples.
- **PTL secondary to abruption (PTLA)**

  -24-36 weeks gestation, delivered due to a variety of causes including shock, compromised fetus or PV bleeding.

This study also consisted of

- **Preterm twins both not in labour (PTTWNL)**
- **Preterm twin gestation in labour (PTTWL)**

  The demographic data for these groups are shown in Table 3.2.

	PTNL	TNL	TEaL	TEsL
No. of Samples	18	17	18	18
Maternal Age	35 ± 1.7	32.9 ± 1.2	34.6 ± 0.7	34.5 ± 0.9
Gestational Age	30.8 ± 0.7	39.0 ± 0.2	38.6 ± 0.3	39.3 ±0.3

Table 3.1 Demographic data for term amnion samples. Comparison of preterm not in labour (PTNL), term not in labour (TNL) term early labour (TEaL) and term established labour (TEsL) patients. Data are expressed as mean +/- SEM.

	PTNL	IPTL	PTLC	PTLA	PTLP	PTTWNL	PTTWL
No. of Samples	17	17	12	4	3	10	12
Maternal age	34.5 ±1.9	33.8 ± 1.5	33.4 ± 1.7	30.8± 2.1	29.3± 4.4	34.9 ± 1.3	35.2 ± 2.5
Gestational age	30.4 ± 0.6	35.0 ± 0.6	29.6 ± 0.8	32.9 ±1.9	32.9 ±0.6	35.6 ± 0.4	34.1 ± 0.4

Table 3.2 Demographic data for preterm amnion samples. Comparison of preterm not in labour (PTNL), idiopathic preterm labour (IPTL), preterm labour secondary to chorioamnionitis (PTLC), preterm labour secondary to polyhydramnios (PTLP), preterm labour secondary to abruption (PTLA), preterm twin gestations not in labour (PTTWNL) and preterm twin gestation in labour (PTTWL). Data are expressed as mean ± SEM.

3.5 Section A: Results

3.5.1 What inflammatory changes are observed in term and preterm labour in the human amnion?

3.5.1.1 Does inflammation occur in term early labour (TEaL) in the human amnion?

Human amnion tissue was used to assess whether inflammation could be implicated in the onset of term labour. Table 3.3 shows a summary of the inflammatory cytokines investigated to observe these changes in term amnion tissue.

	PTNL vs TNL	TNL vs TEaL	TNL vs TEsL	TEaL vs TEsL
CXCL1	-	-	** ↑	-
CXCL2	-	-	-	-
CCL25	-	-	-	-
IL-8	-	** ↑	*** ↑	-
IL10	-	-	-	-
IL-16	** ↓	-	-	-
CCL2	-	-	* ↑	-
CCL7	-	-	-	-
CCL20	-	-	** ↑	-
TNFα	-	-	-	-
CXCL6	* ↑	-	-	-
CCL1	-	-	-	-
IL6	-	* ↑	** ↑	-
IL-1β	-	-	* ↑	-

Table 3.3. Does inflammatory cytokine-chemokine profile in term labour. Protein was extracted using bead homogenisation, quantified and protein lysate was used to run human Bio-Plex© Pro™ chemokine/cytokine assays. Normality was determined via a Kolmogorov-Smirnov test. PTNL vs TNL; TNL vs TEaL; TNL vs TEsL and TEaL vs TEsL were compared using an unpaired t test for normally distributed data and a Mann Whitney testing for non-normally distributed data. * $p<0.05$; ** $p<0.01$; *** $p<0.001$ PTNL: Preterm not in Labour [n=18]; TNL: term not in labour [n=17] ; TEaL: term early labour [n=18]; TEsL: term established labour [n=18].

There were significant changes observed across preterm and term amnion tissues. These were minimal changes with advancing gestation, with lower levels of IL-16 and higher CXCL6 levels in TNL samples compared with PTNL samples (Figure 3.1 A-B).

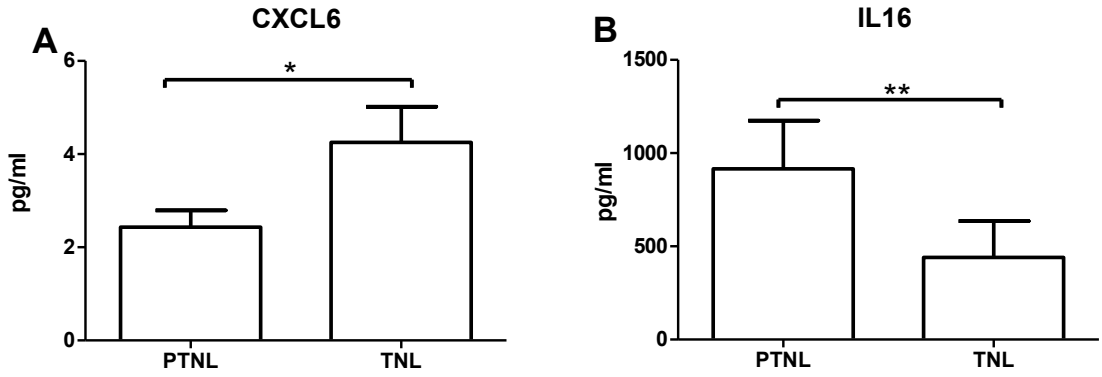

Figure 3.1. The cytokine-chemokine profile with advancing gestation in term amnion. Protein was extracted using bead homogenisation, quantified and protein lysate was run in was used to run human Bio-Plex© Pro™ chemokine/cytokine assays. Normality was determined via a Kolmogorov-Smirnov test followed by an unpaired t test for normally distributed data and Mann Whitney testing for non-normally distributed data. * $p<0.05$; ** $p<0.01$; PTNL: Preterm not in Labour [n=18]; TNL: term not in labour [n=17].

With the onset of labour, both IL-8 and IL-6 were significantly elevated in TEaL samples compared to TNL samples (Figure 3.2). As labour progressed, CXCL1, IL-8, CCL2, CCL20, IL-6 and IL-1β were all significantly raised in TEsL compared with TNL samples (Figure 3.3). However, no change was observed in the cytokine-chemokine profile between samples that were in TEaL and samples in TEsL.

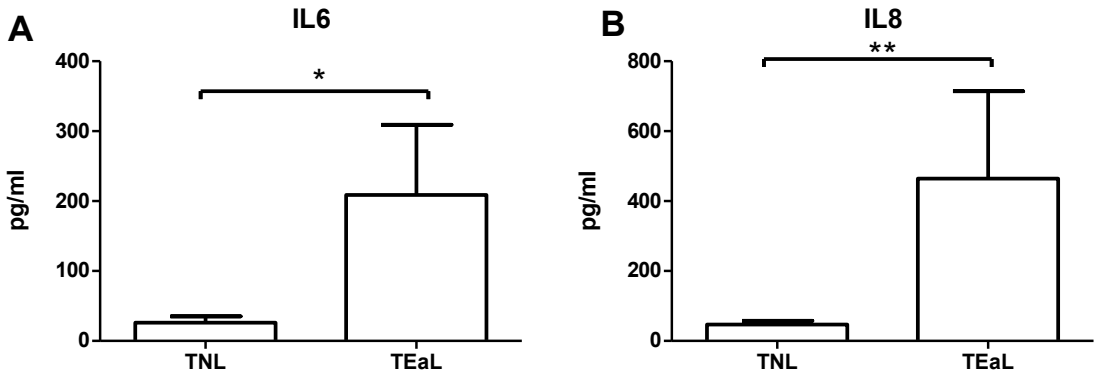

Figure 3.2. The cytokine-chemokine profile with the onset of term labour in the amnion. Protein was extracted using bead homogenisation, quantified and protein lysate was run in was used to run human Bio-Plex© Pro™ chemokine/cytokine assays. Normality was determined via a Kolmogorov-Smirnov test followed by an unpaired t test for normally distributed data and Mann Whitney testing for non-normally distributed data. * $p<0.05$; ** $p<0.01$; TNL: term not in labour [n=17]; TEal: term early labour [n=18];

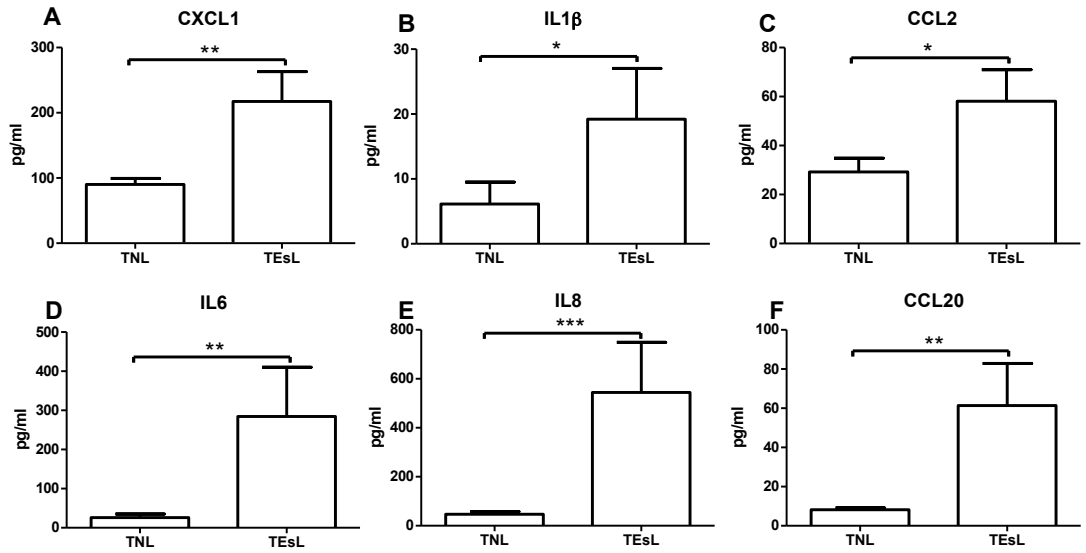

Figure 3.3. The cytokine-chemokine profile in term labour in the amnion. Protein was extracted using bead homogenisation, quantified and protein lysate was run in was used to run human Bio-Plex© Pro™ chemokine/cytokine assays. Normality was determined via a Kolmogorov-Smirnov test followed by an unpaired t test for normally distributed data and Mann Whitney testing for non-normally distributed data. * $p<0.05$; ** $p<0.01$; *** $p<0.001$; TNL: term not in labour [n=17]; TEsL: term established labour [n=18].

3.5.1.2 Is PTL of differing aetiologies associated with its own unique cytokine-chemokine profile?

Human amnion tissue lysate from PTL of differing subtypes was used to assess whether each type of PTL is associated with its own unique cytokine-chemokine response. Table 3.4 shows a summary of the inflammatory cytokines investigated to observe these changes in preterm amnion tissue.

	PTNL vs IPTL	PTNL vs PTLC	PTNL vs PTLA	PTNL vs PTLP	PTTWNL vs PTTWL
CXCL1	*↑	*** ↑	* ↑	-	* ↑
CXCL2	-	*** ↑	-	* ↑	* ↑
CCL25	-	* **↑	-	-	-
IL-8	*** ↑	*** ↑	-	-	-
IL10		*** ↑	-	-	** ↑
IL-16	-	-	** ↓	-	* ↑
CCL2	** ↑	*** ↑	-	-	-
CCL7	* ↑	*** ↑	-	* ↑	-
CCL20	* ↑	*** ↑	-	-	-
TNFα	-	*** ↑	-	-	** ↑
CXCL6	** ↑	** ↑	-	-	-
CCL1	-	*** ↑	-	-	** ↑
IL6	*** ↑	*** ↑	-	-	-
IL-1β	* ↑	*** ↑	-	-	-

Table 3.4 Does cytokine-chemokine profile depend on the underlying cause of PTL? Protein was extracted using bead homogenisation, quantified and protein lysate was used to run human Bio-Plex© Pro™ chemokine/cytokine assays. Normality was determined via a Kolmogorov-Smirnov test. All singleton preterm subtypes were compared to PTNL using an unpaired t test for normally distributed data and Mann Whitney testing for non-normally distributed data, PTTWNL was compared to PTTWL, * $p<0.05$; ** $p<0.01$; *** $p<0.001$ Preterm not in labour (PTNL)[n=17], idiopathic preterm labour (IPTL) [n=17], preterm labour secondary to chorioamnionitis (PTLC) [n=12], preterm labour secondary to polyhydramnios (PTLP) [n=3] , preterm labour secondary to abruption (PTLA) [n=4] , preterm twin not in labour (PTTWNL) [n=10] , preterm twin in labour (PTTWL) [n= 12].

IPTL samples showed increased levels of CXCL1, IL-8, CCL2, CCL7, CCL20, CXCL6, IL-6 and IL- 1β. PTLC showed elevated chemokine-cytokine profile across all mediators except IL-16. PTLA samples showed lower CXCL1 and IL-16 levels compared to PTNL samples whilst samples that were PTLP showed elevated levels of CXCL2 and CCL7 (Figure 3.4). PTTWL showed elevated levels of CXCL1, CXCL2, CCL1, IL-10, IL-16 and TNF α compared to PTTWNL samples (Figure 3.5).

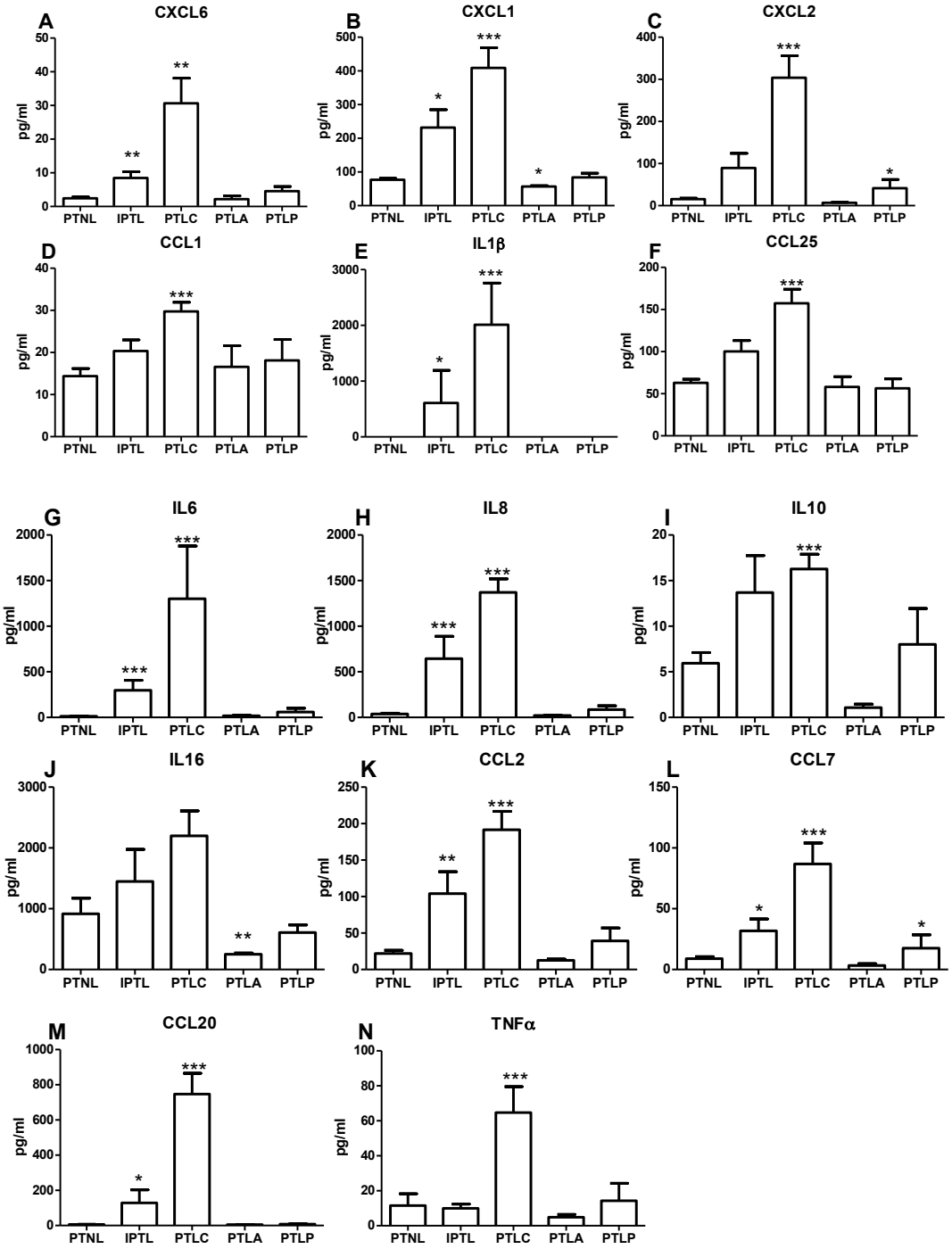

Figure 3.4. Does cytokine-chemokine profile depend on the underlying cause of PTL? Protein was extracted using bead homogenisation, quantified and protein lysate was run in was used to run human Bio-Plex© Pro™ chemokine/cytokine assays. Normality was determined via a Kolmogorov-Smirnov test followed by an unpaired t test for normally distributed data and Mann Whitney testing for non-normally distributed data. PTNL vs. PTL subtype: * $p<0.05$; ** $p<0.01$; *** $p<0.001$; Preterm not in labour (PTNL)[n=17], idiopathic preterm labour (IPTL) [n=17], preterm labour secondary to chorioamnionitis (PTLC) [n=12], preterm labour secondary to polyhydramnios (PTLP) [n=3] , preterm labour secondary to abruption (PTLA) [n=4].

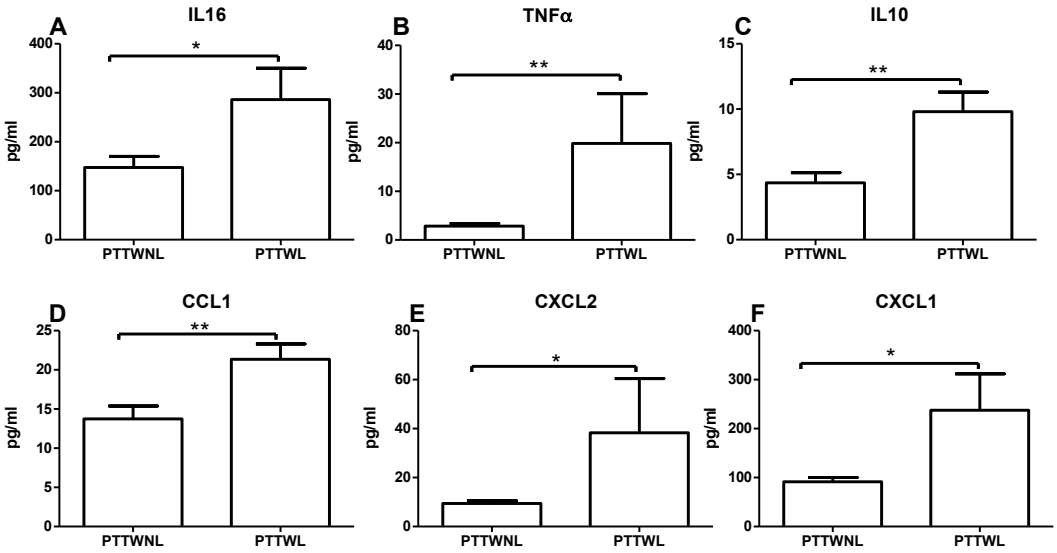

Figure 3.5. Cytokine and Chemokine levels in preterm twin gestations in the amnion. Protein was extracted using bead homogenisation, quantified and protein lysate was run in was used to run human Bio-Plex© Pro™ chemokine/cytokine assays. Normality was determined via a Kolmogorov-Smirnov test followed by an unpaired t test for normally distributed data and Mann Whitney testing for non-normally distributed data. * $p<0.05$; ** $p<0.01$; Preterm twins not in labour (PTTWNL) [n=10] were compared with samples that were preterm twins in labour (PTTWL) [n=12].

3.5.2 What changes are observed in prolabour gene expression in term and preterm labour?

3.5.2.1 Does prolabour gene expression increase with the onset of labour?

mRNA and protein obtained from human amnion tissue was used to determine prolabour gene expression with the onset of term labour in the amnion. Table 3.5 shows a summary of prolabour genes investigated.

	PTNL vs TNL	TNL vs TEaL	TNL vs TEsL	TEaL vs TEsL
COX-2 mRNA	-	-	-	-
COX-2 protein	-	-	* ↑	-
OTR mRNA	** ↓	-	-	-
OTR protein	-	-	-	*** ↑
Cxn 43 mRNA	-	-	-	-
Cxn43 protein	-	-	-	* ↑

Table 3.5. How does prolabour gene expression change in term amnion? Protein and qPCR were carried out for COX-2, OTR and Cxn 43. Normality was determined via a Kolmogorov-Smirnov test. PTNL vs TNL; TNL vs TEaL; TNL vs TEsL and TEaL vs TEsL were compared using an unpaired t test for normally distributed data and a Mann Whitney testing for non-normally distributed data. * $p<0.05$; ** $p<0.01$; *** $p<0.001$ PTNL: Preterm not in Labour [n=18]; TNL: term not in labour [n=17] ; TEaL: term early labour [n=18]; TEsL: term established labour [n=18].

3.5.2.1.1 CAP mRNA in term amnion

No significant difference was observed in COX-2 or Cxn 43 mRNA between preterm not in labour samples and term not in labour samples, or between varying stages of term labour investigated in this study. However, there was a trend towards an increase in COX-2 mRNA as labour progressed from TNL through TEaL and into TEsL. OTR mRNA from TNL samples was significantly lower than PTNL samples and also showed a trend towards a decrease as labour progressed into TEsL. Although there was no significant difference between Cxn 43 mRNA levels across amnion samples at term, there was a trend towards an increase in TEaL compared with TNL as well as TEsL samples (Figure 3.6).

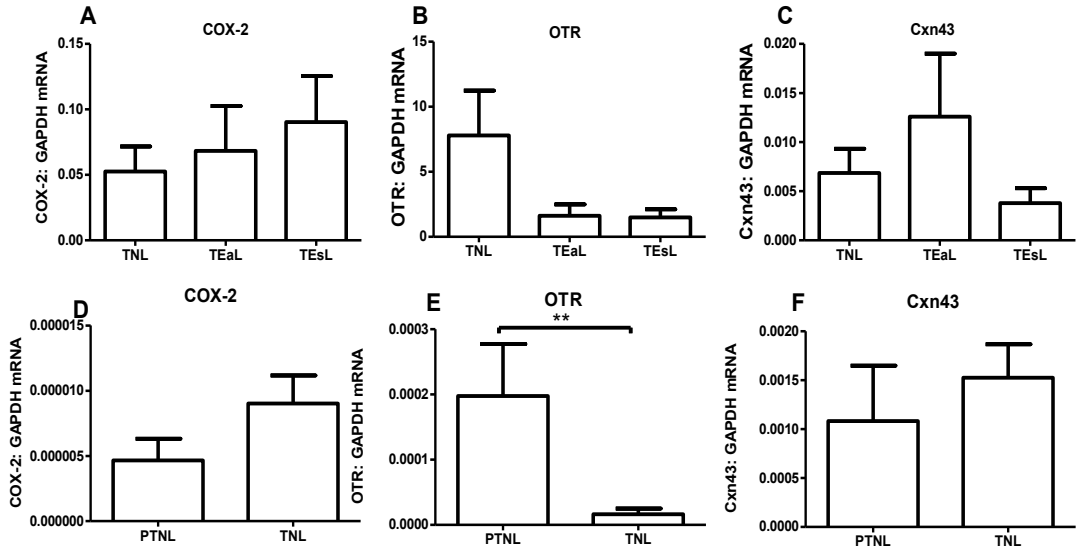

Figure 3.6. Does prolabour expression increase during term labour in the amnion? Bead homogenisation was used to carry out RNA extraction. RNA was then converted into cDNA and quantative PCR was carried out. A & D: COX-2 ; B & E: OTR; C & F: Cxn-43; Data are expressed as mean +/- SEM. Normality was determined using Kolmogorov-Smirnov test. Between TNL, TEal and TEsL samples, an ANOVA was used for normally distributed data whilst a Kruskal-Wallis test was used non-normally distributed. For analysis between PTNL and TNL an unpaired t test was used for normally distributed data, whilst Mann Whitney testing was used to non-normally distributed data. ** $p<0.01$; PTNL: Preterm not in Labour [n=18]; TNL: term not in labour [n=17] ; TEaL: term early labour [n=18]; TEsL: term established labour [n=18].

3.5.2.1.2 COX-2 protein in term amnion

Assessing protein in amnion tissue was carried out using western blotting. Though 40 µg of each protein sample was loaded, patient heterogeneity between amnion samples accounted for the differences in loading control. No significant changes were observed in COX-2 protein levels between PTNL and TNL, between labour onset or as labour progressed from TEaL to TEsL. However, there was a significant increase in COX-2 protein in samples from TEsL compared with samples taken from TNL (Figure 3.7).

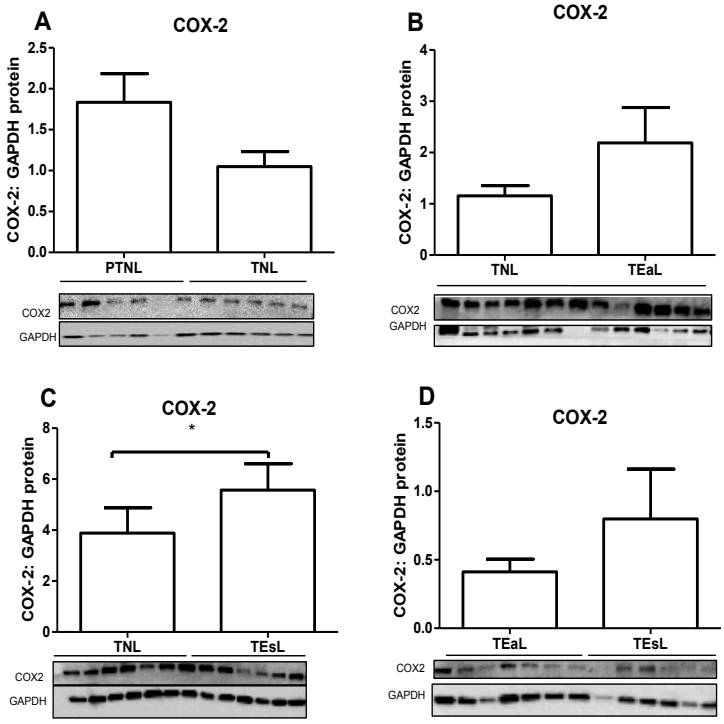

Figure 3.7. COX-2 protein levels in term labour in the amnion. Bead homogenisation was used to carry out protein extraction. Protein was quantified through western blotting for COX-2 to compare A. preterm not in labour (PTNL) to term not in labour (TNL), B. term not in labour (TNL) to term early labour (TEaL), C. term not in labour (TNL) to term established labour (TEsL). D. Term early labour (TEaL) to term established labour (TEsL). A representative western blot is shown in conjunction with densitometric analysis. Data are expressed, as mean +/- SEM. Normality was determined using Kolmogorov-Smirnov test. An unpaired t test was used for normally distributed data, whilst Mann Whitney testing was used to non-normally distributed data * $p<0.05$ PTNL: Preterm not in Labour [n=18]; TNL: term not in labour [n=17] ; TEaL: term early labour [n=18]; TEsL: term established labour [n=18].

3.5.2.1.3 OTR protein levels in term amnion

No change was observed in OTR protein between PTNL and TNL samples or with labour onset. There was a significant increase in OTR protein levels in TEsL compared with TEaL samples (Figure 3.8).

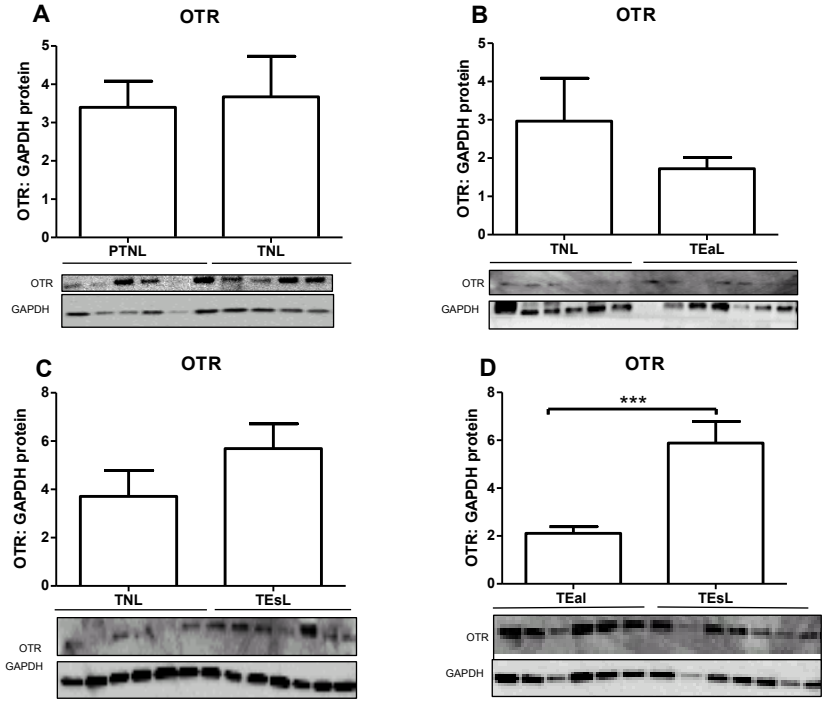

Figure 3.8. OTR protein levels in term labour in the amnion. Bead homogenisation was used to carry out protein extraction. Protein was quantified through western blotting for OTR to compare A. preterm not in labour (PTNL) to term not in labour (TNL), B. term not in labour (TNL) to term early labour (TEaL), C. term not in labour (TNL) to term established labour (TEsL). D. Term early labour (TEaL) to term established labour (TEsL). A representative western blot is shown in conjunction with densitometric analysis. Data are expressed, as mean +/- SEM. Normality was determined using Kolmogorov-Smirnov test. An unpaired t test was used for normally distributed data, whilst Mann Whitney testing was used to non-normally distributed data *** $p<0.001$ PTNL: Preterm not in Labour [n=18]; TNL: term not in labour [n=17] ; TEaL: term early labour [n=18]; TEsL: term established labour [n=18].

3.5.2.1.4 Cxn 43 protein levels in term amnion

A trend towards an increase in Cxn 43 was seen in TNL compared with samples that were PTNL, however no statistically significant differences were observed. TEsL samples showed a significant increase in Cxn 43 when compared with TEaL samples (Figure 3.9).

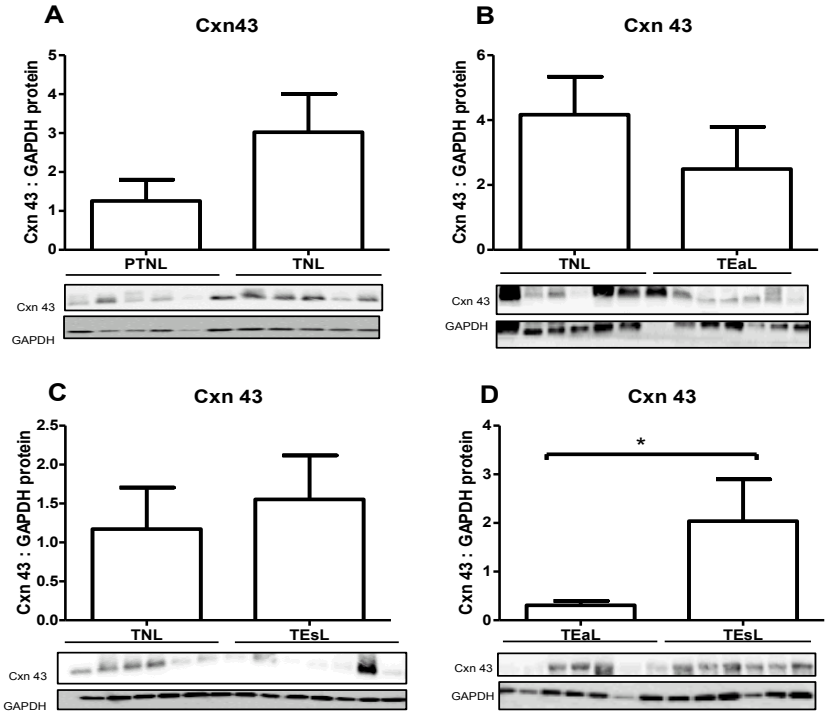

Figure 3.9 Cxn43 protein levels in term labour in the amnion. Bead homogenisation was used to carry out protein extraction. Protein was quantified through western blotting for Cxn 43 to compare A. preterm not in labour (PTNL) to term not in labour (TNL), B. term not in labour (TNL) to term early labour (TEaL), C. term not in labour (TNL) to term established labour (TEsL). D. Term early labour (TEaL) to term established labour (TEsL). A representative western blot is shown in conjunction with densitometric analysis. Data are expressed, as mean +/- SEM. Normality was determined using Kolmogorov-Smirnov test. An unpaired t test was used for normally distributed data, whilst Mann Whitney testing was used to non-normally distributed data. * $p<0.05$ PTNL: Preterm not in Labour [n=18]; TNL: term not in labour [n=17] ; TEaL: term early labour [n=18]; TEsL: term established labour [n=18].

3.5.2.2 Is prolabour gene expression altered with different type of PTL?

mRNA and protein obtained from human amnion tissue was used to determine prolabour gene expression in PTL of different subtypes. Table 3.6 shows a summary of prolabour genes investigated.

	PTNL vs IPTL	PTNL vs PTLC	PTNL vs PTLA	PTNL vs PTLP	PTTWNL vs PTTWL
COX-2 mRNA	*** ↑	-	** ↑	-	-
COX-2 protein	-	-	* ↓	-	-
OTR mRNA	* ↓	*** ↓	-	* ↓	-
OTR protein	-	* ↑	-	-	-
Cxn 43 mRNA	*** ↓	* ↓	* ↓	-	-
Cxn43 protein	-	-	-	-	** ↑

Table 3.6. How does prolabour gene expression differ in preterm amnion? Protein and qPCR were carried out for COX-2, OTR and Cxn 43. Normality was determined via a Kolmogorov-Smirnov test. All singleton preterm subtypes were compared to PTNL using an unpaired t test for normally distributed data and Mann Whitney testing for non-normally distributed data, PTTWNL was compared to PTTWL, * $p<0.05$; ** $p<0.01$; *** $p<0.001$ Preterm not in labour (PTNL)[n=17], idiopathic preterm labour (IPTL) [n=17], preterm labour secondary to chorioamnionitis (PTLC) [n=12], preterm labour secondary to polyhydramnios (PTLP) [n=3] , preterm labour secondary to abruption (PTLA) [n=4] , preterm twin not in labour (PTTWNL) [n=10] , preterm twin in labour (PTTWL) [n= 12].

3.5.2.2.1 CAP mRNA in preterm amnion

There were significant increases in COX-2 mRNA in IPTL and as well as PTLA. There were significant decreases in OTR mRNA in IPTL, PTLC, and PTLP, both secondary to PTL. PTLA samples displayed significantly decreased levels of Cxn 43. There were no significant differences in CAP mRNA between preterm twin gestations, though there was a decreased trend in OTR mRNA levels in PTTWL compared with PTTWNL There was a trend towards increased COX-2 mRNA and Cxn 43 in PTTWL compared with PTTWNL (Figure 3.10).

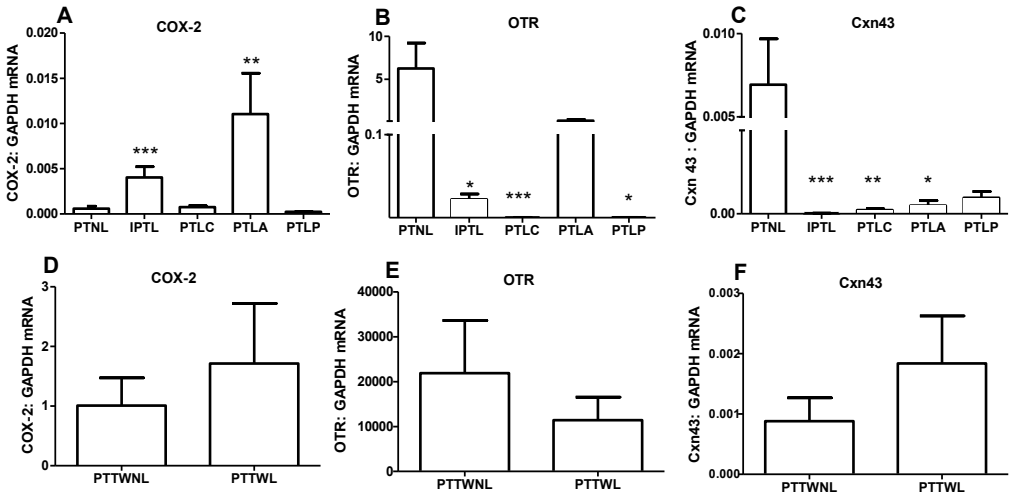

Figure 3.10 How does prolabour mRNA change with differing causes of PTL in the amnion? Bead homogenisation was used to carry out RNA extraction. RNA was then converted into cDNA and quantative PCR was carried out. A & D: COX-2; B & E: OTR; C & F: Cxn-43; Normality was determined via a Kolmogorov-Smirnov test followed by an unpaired t test for normally distributed data and Mann Whitney testing for non-normally distributed data. All PTL subtypes were compared to PTNL; PTTWNL vs. PTTWL; * $p<0.05$; ** $p<0.01$; *** $p<0.001$; Preterm not in labour (PTNL)[n=17], idiopathic preterm labour (IPTL) [n=17], preterm labour secondary to chorioamnionitis (PTLC) [n=12], preterm labour secondary to polyhydramnios (PTLP) [n=3] , preterm labour secondary to abruption (PTLA) [n=4] , preterm twin not in labour (PTTWNL) [n=10] , preterm twin in labour (PTTWL) [n= 12].

3.5.2.2.2 COX-2 protein levels in preterm amnion

PTLA showed significantly decreased levels of COX-2 protein compared with PTNL. No significant differences were seen in COX-2 protein levels across all other preterm singleton samples when compared with PTNL samples. Interestingly however, samples from PTTWL showed a trend towards an increase compared with PTTWNL samples (Figure 3.11).

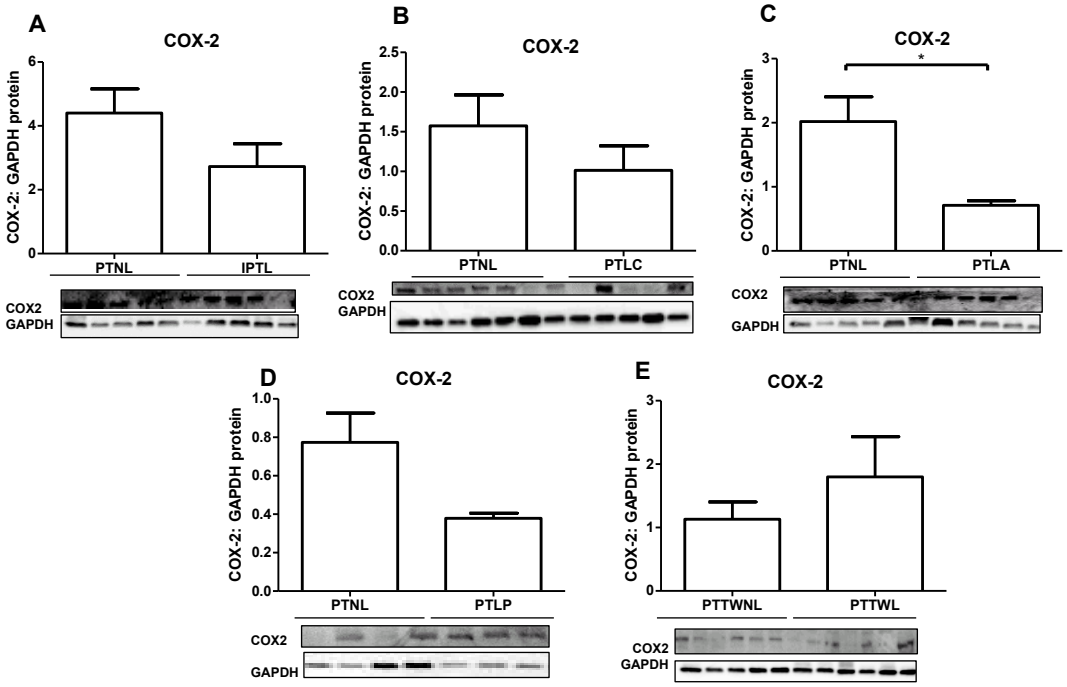

Figure 3.11. COX-2 protein levels in different types of PTL in the amnion. Bead homogenisation was used to carry out protein extraction. Protein was quantified through western blotting for COX-2 to compare **A.** preterm not in labour (PTNL) to idiopathic labour (IPTL), **B.** preterm not in labour (PTNL) to preterm labour due to chorioamnionitis (PTLC), **C.** preterm not in labour (PTNL) to preterm labour abruption (PTLA) **D.** preterm not in labour (PTNL) to preterm labour polyhydramnios (PTLP) **E.** preterm twin not in labour (PTTWNL) to preterm twins in labour (PTTWL). A representative western blot is shown in conjunction with densitometric analysis. Data are expressed, as mean +/- SEM. Normality was determined using Kolmogorov-Smirnov test. An unpaired t test was used for normally distributed data, whilst Mann Whitney testing was used to non-normally distributed data * $p<0.05$. Preterm not in labour (PTNL)[n=17], idiopathic preterm labour (IPTL) [n=17], preterm labour secondary to chorioamnionitis (PTLC) [n=12], preterm labour secondary to polyhydramnios (PTLP) [n=3] , preterm labour secondary to abruption (PTLA) [n=4] , preterm twin not in labour (PTTWNL) [n=10] , preterm twin in labour (PTTWL) [n= 12].

3.5.2.2.3 OTR protein levels in preterm amnion

Compared to PTNL samples, only PTLC and PTLP samples showed significantly elevated OTR protein levels. No other significant changed was noted in OTR protein levels in singleton preterm samples or in twin samples (Figure 3.12).

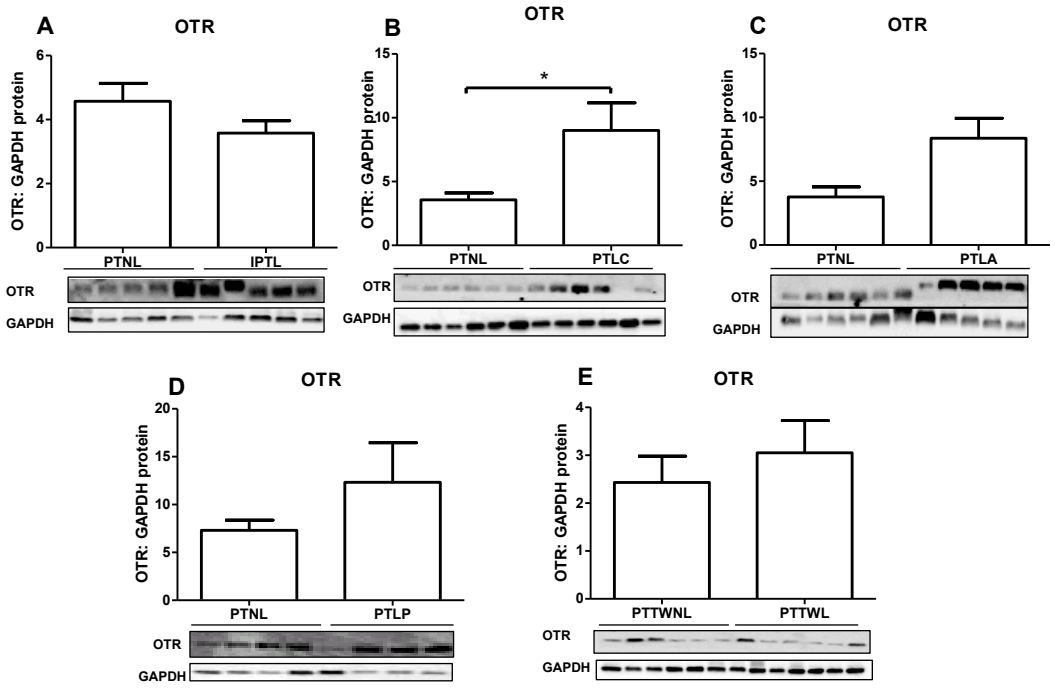

Figure 3.12. OTR protein levels in different types of PTL in the amnion. Bead homogenisation was used to carry out protein extraction. Protein was quantified through western blotting for OTR to compare **A.** preterm not in labour (PTNL) to idiopathic labour (IPTL), **B.** preterm not in labour (PTNL) to preterm labour due to chorioamnionitis (PTLC), **C.** preterm not in labour (PTNL) to preterm labour abruption (PTLA) **D.** preterm not in labour (PTNL) to preterm labour polyhydramnios (PTLP) **E.** preterm twin not in labour (PTTWNL) to preterm twins in labour (PTTWL). A representative western blot is shown in conjunction with densitometric analysis. Data are expressed, as mean +/- SEM. Normality was determined using Kolmogorov-Smirnov test. An unpaired t test was used for normally distributed data, whilst Mann Whitney testing was used to non-normally distributed data. * $p<0.05$. Preterm not in labour (PTNL)[n=17], idiopathic preterm labour (IPTL) [n=17], preterm labour secondary to chorioamnionitis (PTLC) [n=12], preterm labour secondary to polyhydramnios (PTLP) [n=3] , preterm labour secondary to abruption (PTLA) [n=4] , preterm twin not in labour (PTTWNL) [n=10] , preterm twin in labour (PTTWL) [n= 12].

3.5.2.2.4 Cxn 43 protein levels in preterm amnion

Significantly increased Cxn 43 protein levels were observed in PTTWL when compared with PTTNL. There was a trend toward a decrease in Cxn 43 protein levels in PTLC, PTLA and PTLP samples. Interestingly, there was a trend towards an increase in idiopathic PTL compared with PTNL samples (Figure 3.13).

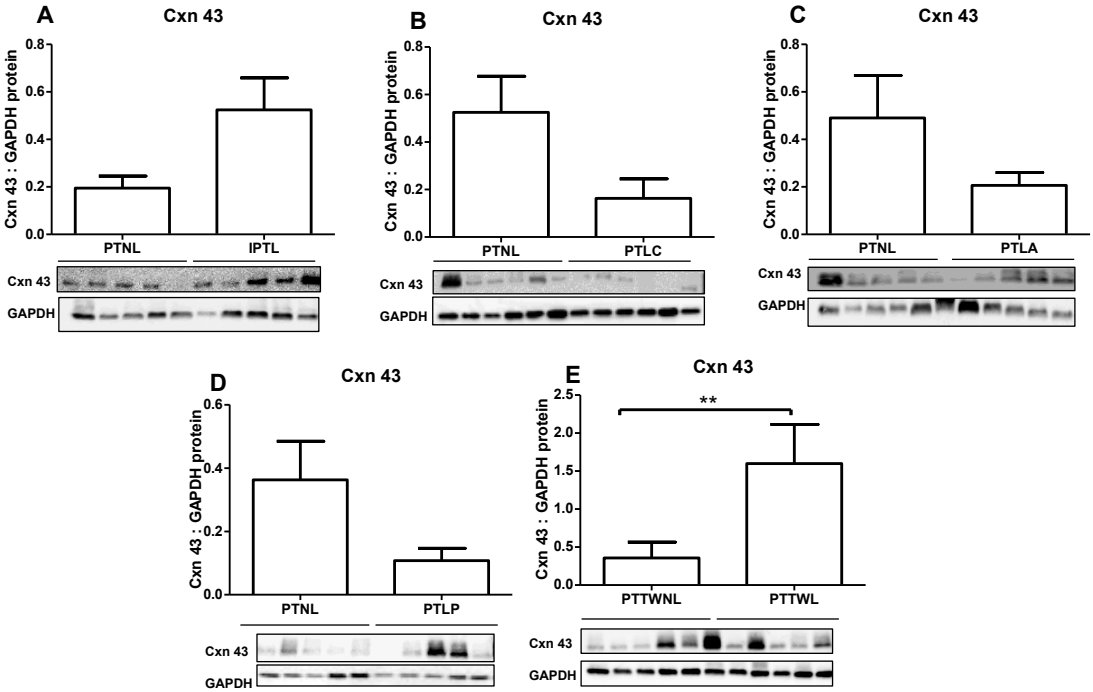

Figure 3.13. Cxn 43 protein levels in different types of PTL in the amnion. Bead homogenisation was used to carry out protein extraction. Protein was quantified through western blotting for Cxn 43 to compare **A.** preterm not in labour (PTNL) to idiopathic labour (IPTL), **B.** preterm not in labour (PTNL) to preterm labour due to chorioamnionitis (PTLC), **C.** preterm not in labour (PTNL) to preterm labour abruption (PTLA) **D.** preterm not in labour (PTNL) to preterm labour polyhydramnios (PTLP) **E.** preterm twin not in labour (PTTWNL) to preterm twins in labour (PTTWL). A representative western blot is shown in conjunction with densitometric analysis. Data are expressed, as mean +/- SEM. Normality was determined using Kolmogorov-Smirnov test. An unpaired t test was used for normally distributed data, whilst Mann Whitney testing was used to non-normally distributed data. **$p<0.01$. Preterm not in labour (PTNL)[n=17], idiopathic preterm labour (IPTL) [n=17], preterm labour secondary to chorioamnionitis (PTLC) [n=12], preterm labour secondary to polyhydramnios (PTLP) [n=3] , preterm labour secondary to abruption (PTLA) [n=4] , preterm twin not in labour (PTTWNL) [n=10] , preterm twin in labour (PTTWL) [n= 12].

3.6 Results - Section B

3.6.1 Are there consistent patterns in steroid activity in term and preterm labour?

3.6.2 Are there patterns in nuclear receptor expression in term and preterm amnion?

Nuclear receptor expression in term and preterm amnion was assessed by evaluating mRNA and protein expression through qPCR western blotting. Tables 3.7 and 3.8 show a summary of the data.

	PTNL vs TNL	TNL vs TEaL	TNL vs TEsL	TEaL vs TEsL
PR-B mRNA	* ↓	-	-	-
PR-Total mRNA	-	-	-	-
PR-B : PR-Total ratio mRNA	-	-	-	-
PR-A protein	** ↑	-	* ↑	-
PR-B protein	-	-	-	-
PR-A:PRB ratio protein	* ↑	-	-	-
GR mRNA	-	-	-	-
GR protein	-	-	-	-

Table 3.7 Are there consistent patterns in nuclear receptor expression in term amnion? Protein and qPCR were carried out for PR and GR. Normality was determined via a Kolmogorov-Smirnov test. PTNL vs TNL; TNL vs TEaL; TNL vs TEsL and TEaL vs TEsL were compared using an unpaired t test for normally distributed data and a Mann Whitney testing for non-normally distributed data. * $p<0.05$; ** $p<0.01$; *** $p<0.001$ PTNL: Preterm not in Labour [n=18], TNL: term not in labour [n=17] ; TEaL: term early labour [n=18] TEsL [n=18] : term established labour [n= 18].

	PTNL vs IPTL	PTNL vs PTLC	PTNL vs PTLA	PTNL vs PTLP	PTTWNL vs PTTWL
PR-B mRNA	-	-	-	-	-
PR-Total mRNA	-	-	-	-	-
PRB:PR-Total ramRNA	-	-	-	-	-
PR-A protein	-	-	-	-	-
PR-B protein	-	-	-	-	-
PR-B protein	-	-	-	-	-
PR-A:PRB ratio protein	* ↑	-	-	-	-
GR mRNA	-	-	-	-	-
GR protein	-	-	-	* ↑	-

Table 3.8 Are there consistent patterns in nuclear receptor expression in preterm amnion? Protein and qPCR were carried out for PR and GR. Normality was determined via a Kolmogorov-Smirnov test. All singleton preterm subtypes were compared to PTNL using an unpaired t test for normally distributed data and Mann Whitney testing for non-normally distributed data, PTTWNL was compared to PTTWL, * $p<0.05$; Preterm not in labour (PTNL)[n=17], idiopathic preterm labour (IPTL) [n=17], preterm labour secondary to chorioamnionitis (PTLC) [n=12], preterm labour secondary to polyhydramnios (PTLP) [n=3] , preterm labour secondary to abruption (PTLA) [n=4] , preterm twin not in labour (PTTWNL) [n=10] , preterm twin in labour (PTTWL) [n= 12].

3.6.2.1 PR-B and PR-Total mRNA levels in term amnion

There was a significant decrease in PR-B mRNA levels in TNL samples compared with PTNL samples. No change was observed in PR-B or PR-Total mRNA levels across the gestations at term, though there did appear to be a trend towards a decrease in the PR-B: PR-Total ratio as labour progressed, between TEaL and TEsL samples (Figure 3.14).

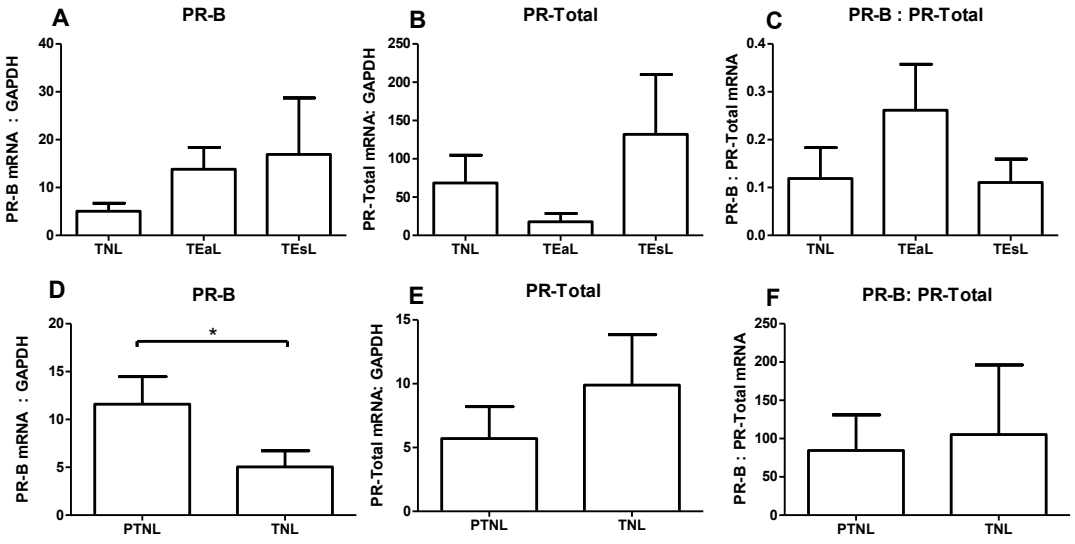

Figure 3.14. PR-B and PR-Total mRNA levels in term labour amnion. Bead homogenisation was used to carry out RNA extraction. RNA was then converted into cDNA and quantative PCR was carried out. A & D: PR-B; B & E: PR-Total; C & F: PR-B: PR-Total; Key: preterm not in labour (PTNL); term not in labour (TNL) term early labour (TEaL), term established labour (TEsL). Data are expressed, as mean +/- SEM. Normality was determined using Kolmogorov-Smirnov test. Between TNL, Teal and TEsL samples, a 1-way ANOVA was used for normally distributed data whilst a Kruskal-Wallis test was used non-normally distributed. For analysis between PTNL and TNL an unpaired t testing was used for normally distributed data, whilst Mann Whitney testing was used to non-normally distributed data * $p<0.05$; PTNL: Preterm not in Labour [n=18], TNL: term not in labour [n=17] ; TEaL: term early labour [n=18] TEsL [n=18] : term established labour [n= 18].

3.6.2.2 PR-B and PR-Total mRNA levels in preterm amnion

There was a trend towards an increase in PR-total mRNA levels in PTLP when compared with PTNL samples. In addition, there seemed to be a trend towards an increase in PR-B: PR-Total mRNA levels in IPTL compared with PTNL. However, these trends were not statistically significant. (Figure 3.15) No significant differences were observed between preterm twin gestations (Figure 3.16).

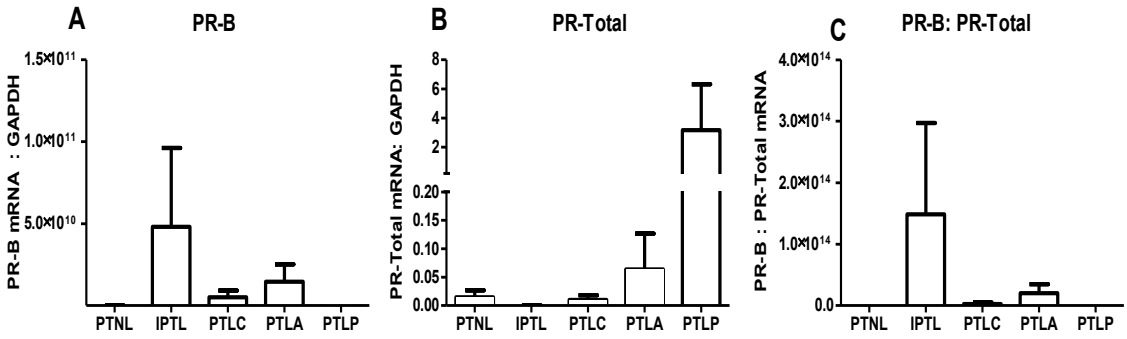

Figure 3.15. PR-B and PR-Total mRNA levels in preterm labour amnion. Bead homogenisation was used to carry out RNA extraction. RNA was then converted into cDNA and quantative PCR was carried out. A: PR-B; B: PR-Total; C: PR-B: PR-PR-Total; Key: preterm not in labour (PTNL), idiopathic preterm labour (IPTL), preterm labour secondary to chorioamnionitis (PTLC), preterm labour secondary to polyhydramnios (PTLP), preterm labour secondary to abruption (PTLA). Data are expressed, as mean +/- SEM. Normality was determined using Kolmogorov-Smirnov test. All preterm singleton samples were compared with PTNL. Preterm not in labour (PTNL)[n=17], idiopathic preterm labour (IPTL) [n=17], preterm labour secondary to chorioamnionitis (PTLC) [n=12], preterm labour secondary to polyhydramnios (PTLP) [n=3] , preterm labour secondary to abruption (PTLA) [n=4].

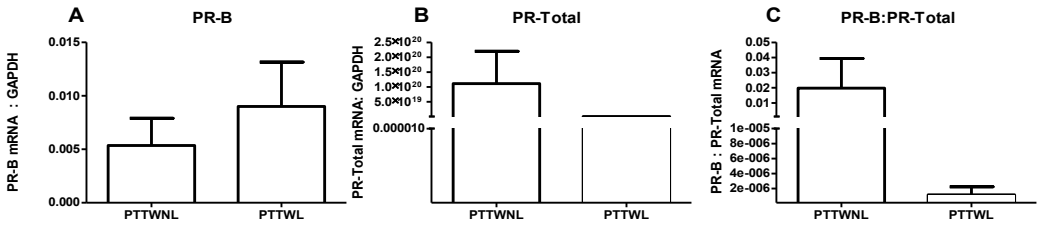

Figure 3.16. PR-B and PR-Total mRNA levels in preterm amnion. Bead homogenisation was used to carry out RNA extraction. RNA was then converted into cDNA and quantative PCR was carried out. A: PR-B; B: PR-Total; C: PR-B: PR-PR-Total; preterm twin gestations not in labour (PTTWNL) and preterm twin gestation in labour (PTTWL). Data are expressed, as mean +/- SEM. Normality was determined using Kolmogorov-Smirnov test. PTTWNL [n=10] were compared with PTTWL [n=12] using either an unpaired t test for normally distributed data or a Mann Whitney test was used to non-normally distributed data.

3.6.2.3 PR-A and PR-B Protein Levels in the amnion at term

There were significant increases in PR-A protein levels in TNL compared with PTNL. In addition, this also occurred in TEsL samples compared with TNL. No other statistically different changes were observed across the differing gestations between PR-A and PR-B levels (Figure 3.17).

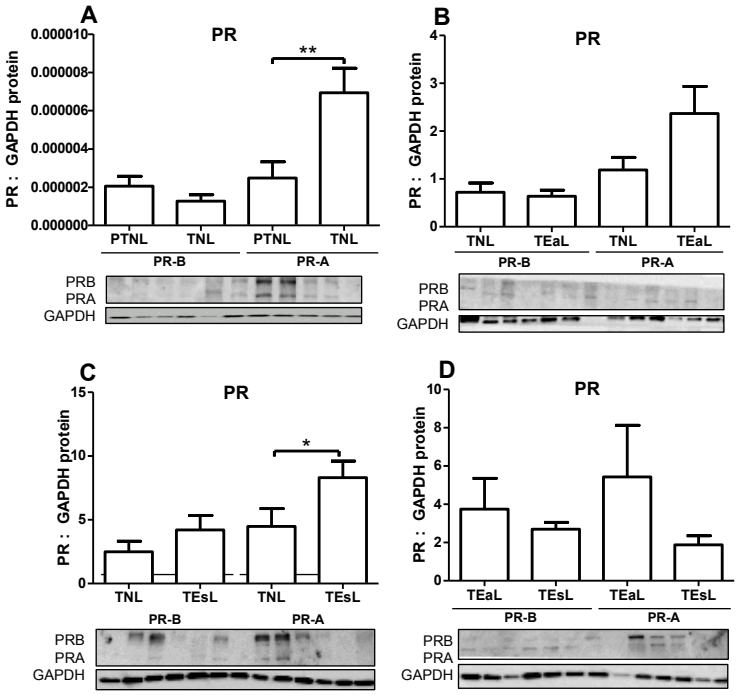

Figure 3.17. PR-A/B protein levels in term labour amnion. Bead homogenisation was used to carry out protein extraction. Protein was quantified through western blotting for PR to compare A. preterm not in labour (PTNL) to term not in labour (TNL), B. term not in labour (TNL) to term early labour (TEaL), C. term not in labour (TNL) to term established labour (TEsL). D. Term early labour (TEaL) to term established labour (TEsL). A representative western blot is shown in conjunction with densitometric analysis. Data are expressed, as mean +/- SEM. Normality was determined using Kolmogorov-Smirnov test. An unpaired t test was used for normally distributed data, whilst Mann Whitney testing was used to non-normally distributed data * $p<0.05$; ** $p<0.01$; PTNL: Preterm not in Labour [n=18], TNL: term not in labour [n=17] ; TEaL: term early labour [n=18] TEsL [n=18] : term established labour [n= 18].

3.6.2.4 PR-A/PR-B protein ratio in amnion at term

There was a significant increase in the ratio of PR-A/PR-B protein levels in TNL compared with PTNL, however no other change was observed across the differing gestations at term (Figure 3.18).

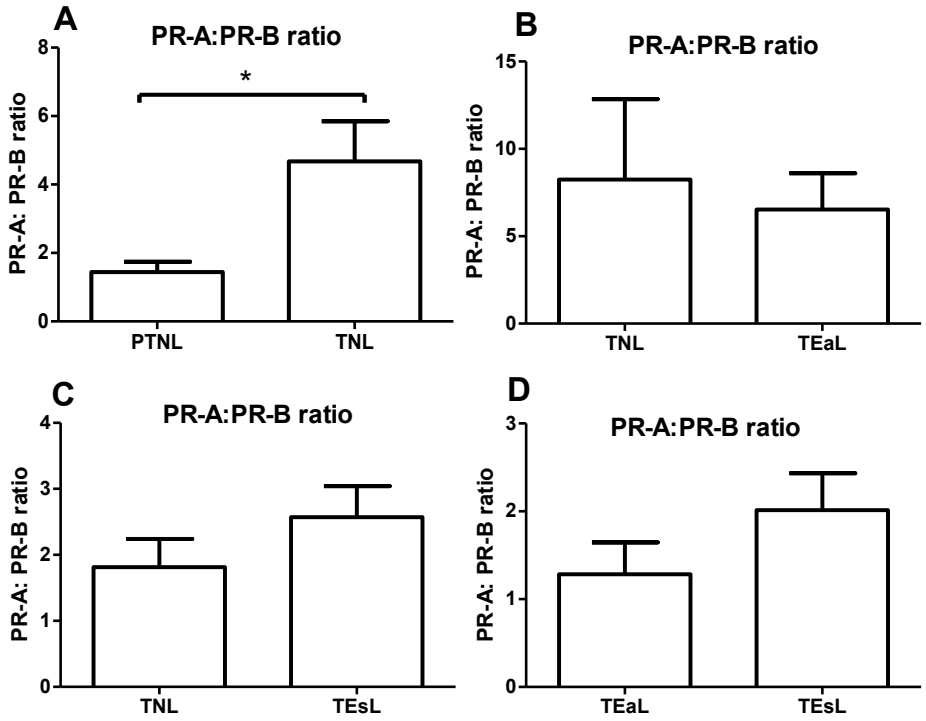

Figure 3.18. PR-A/B protein levels in term labour. Bead homogenisation was used to carry out protein extraction. Protein was quantified through western blotting for PR to compare A. preterm not in labour (PTNL) to term not in labour (TNL), B. term not in labour (TNL) to term early labour (TEaL), C. term not in labour (TNL) to term established labour (TEsL). D. Term early labour (TEaL) to term established labour (TEsL). A representative western blot is shown in conjunction with densitometric analysis. Data are expressed, as mean +/- SEM. Normality was determined using Kolmogorov-Smirnov test. An unpaired t test was used for normally distributed data, whilst Mann Whitney testing was used to non-normally distributed data. * $p<0.05$; PTNL: Preterm not in Labour [n=18], TNL: term not in labour [n=17] ; TEaL: term early labour [n=18] TEsL [n=18] : term established labour [n= 18].

3.6.2.5 PR-A/PR protein ratio in preterm amnion

No change was observed in PR-A/PR-B protein levels in all preterm amnion tissues (Figure 3.19) when compared with PTNL samples. In addition, no change was observed between PTTWNL and PTTWL samples.

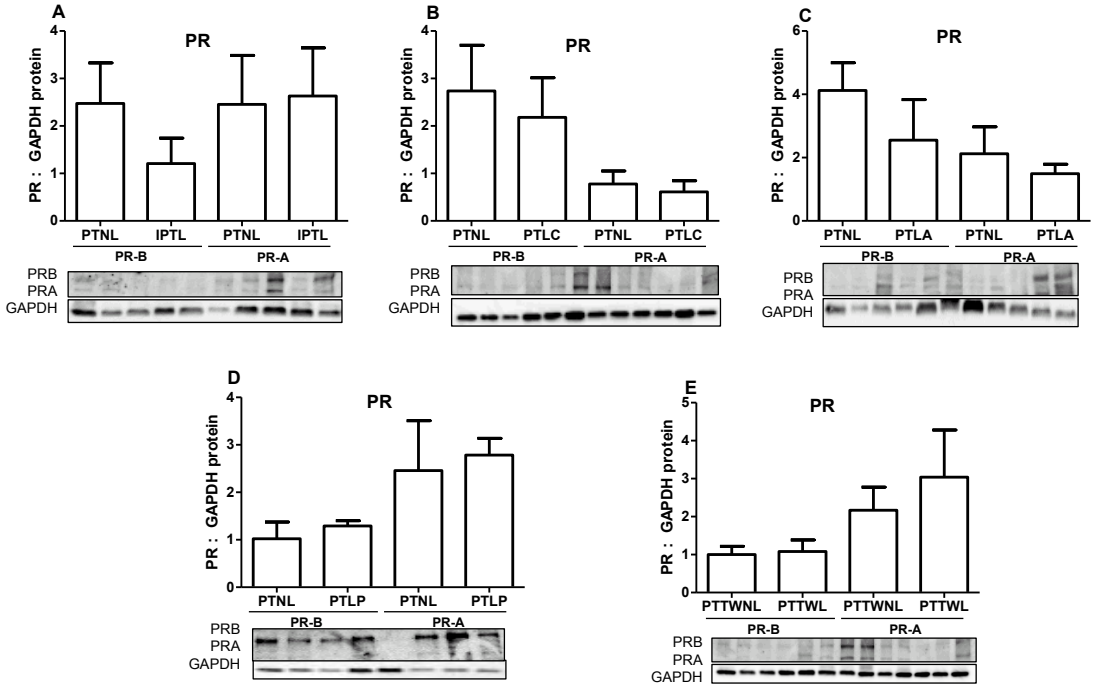

Figure 3.19. PR-A/B protein levels in preterm labour. Bead homogenisation was used to carry out protein extraction. Protein was quantified through western blotting for PR to compare **A.** preterm not in labour (PTNL) to idiopathic labour (IPTL), **B.** preterm not in labour (PTNL) to preterm labour due to chorioamnionitis (PTLC), **C.** preterm not in labour (PTNL) to preterm labour polyhydramnios (PTLP) **D.** preterm not in labour (PTNL) to preterm labour abruption (PTLA) **E.** preterm twin not in labour (PTTWNL) to preterm twins in labour (PTTWL). A representative western blot is shown in conjunction with densitometric analysis. Data are expressed, as mean +/- SEM. Normality was determined using Kolmogorov-Smirnov test. An unpaired t test was used for normally distributed data, whilst Mann Whitney testing was used to non-normally distributed data. Preterm not in labour (PTNL)[n=17], idiopathic preterm labour (IPTL) [n=17], preterm labour secondary to chorioamnionitis (PTLC) [n=12], preterm labour secondary to polyhydramnios (PTLP) [n=3] , preterm labour secondary to abruption (PTLA) [n=4] , preterm twin not in labour (PTTWNL) [n=10] , preterm twin in labour (PTTWL) [n= 12].

3.6.2.6 PR-A PR-A/PR-B Ratio in preterm amnion

There was no significant change in PR-A/PR-B ratio in PTLC, PTLA, and PTLP when compared with PTNL samples. In addition there were no significant changes between twin samples. However, interestingly, there the ratio was significantly increased in IPTL compared with PTNL (Figure 3.20).

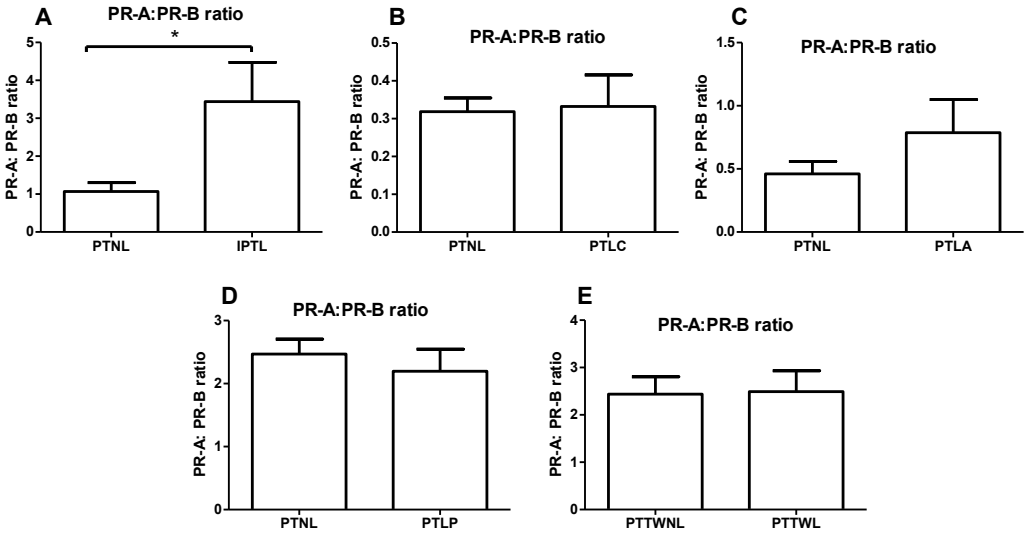

Figure 3.20. PR-A/PR-B protein levels in preterm labour. Bead homogenisation was used to carry out protein extraction. Protein was quantified through western blotting for PR to compare **A.** preterm not in labour (PTNL) to idiopathic labour (IPTL), **B.** preterm not in labour (PTNL) to preterm labour due to chorioamnionitis (PTLC), **C.** preterm not in labour (PTNL) to preterm labour polyhydramnios (PTLP) **D.** preterm not in labour (PTNL) to preterm labour abruption (PTLA) E**.** preterm twin not in labour (PTTWNL) to preterm twins in labour (PTTWL). A representative western blot is shown in conjunction with densitometric analysis. Data are expressed, as mean +/- SEM. Normality was determined using Kolmogorov-Smirnov test. An unpaired t test was used for normally distributed data, whilst Mann Whitney testing was used to non-normally distributed data. * $p<0.05$. Preterm not in labour (PTNL)[n=17], idiopathic preterm labour (IPTL) [n=17], preterm labour secondary to chorioamnionitis (PTLC) [n=12], preterm labour secondary to polyhydramnios (PTLP) [n=3] , preterm labour secondary to abruption (PTLA) [n=4] , preterm twin not in labour (PTTWNL) [n=10] , preterm twin in labour (PTTWL) [n= 12].

3.6.2.7 GR mRNA levels in term and preterm amnion

As previously mentioned, studies from primary myometrial cells have suggested that GR may be involved in P4 signalling (119). As a result, GR mRNA and, later in the chapter, protein levels were assessed. No statistically significant changes were observed across term gestations. There was no difference noted between GR mRNA levels between PTNL and TNL. There was a trend towards an increase in GR mRNA levels in TEaL compared with TNL and TEsL samples. Similarly, no difference was observed between in GR mRNA samples from preterm twin gestations. There was a trend towards an increase in GR mRNA levels in samples that were PTLC as well as PTLP samples, though these changes were not statistically significant (Figure 3.21).

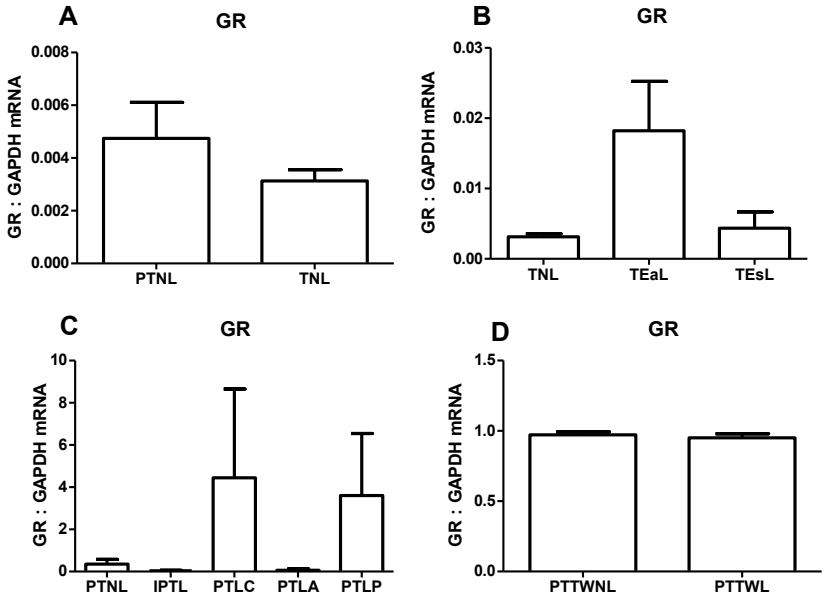

Figure 3.21. GR mRNA levels in term and preterm labour. Bead homogenisation was used to carry out RNA extraction. RNA was then converted into cDNA and quantative PCR was carried out. Data are expressed as mean +/- SEM. Normality was determined using Kolmogorov-Smirnov test. A: Between Term Not in Labour (TNL),Term Early Labour (TEal) and Term Established Labour (TEsL) samples, a 1-way ANOVA was used for normally distributed data and a Kruskal-Wallis test for non-normally distributed data. B: For analysis between Preterm Not in Labour (PTNL) and TNL an unpaired t test was used for normally distributed data and Mann Whitney testing for non-normally distributed data. C: All preterm singleton samples [idiopathic preterm labour (IPTL), preterm labour secondary to chorioamnionitis (PTLC), preterm labour secondary to polyhydramnios (PTLP), preterm labour secondary to abruption (PTLA)] were compared with PTNL. D: Preterm twin gestations not in labour (PTTWNL) were compared to preterm twin gestation in labour (PTTWL) using either unpaired t testing for normally distributed data or Mann Whitney testing was used to non-normally distributed data. Preterm not in labour (PTNL)[n=17], TNL: term not in labour [n=17] ; TEaL: term early labour [n=18] TEsL [n=18] : term established labour [n= 18], idiopathic preterm labour (IPTL) [n=17], preterm labour secondary to chorioamnionitis (PTLC) [n=12], preterm labour secondary to polyhydramnios (PTLP) [n=3] , preterm labour secondary to abruption (PTLA) [n=4] , preterm twin not in labour (PTTWNL) [n=10] , preterm twin in labour (PTTWL) [n= 12].

3.6.2.8 GR protein levels in term amnion

No significant changes were observed in GR protein expression between PTNL and TNL In addition, no other significant differences were observed term labouring samples (Figure 3.22).

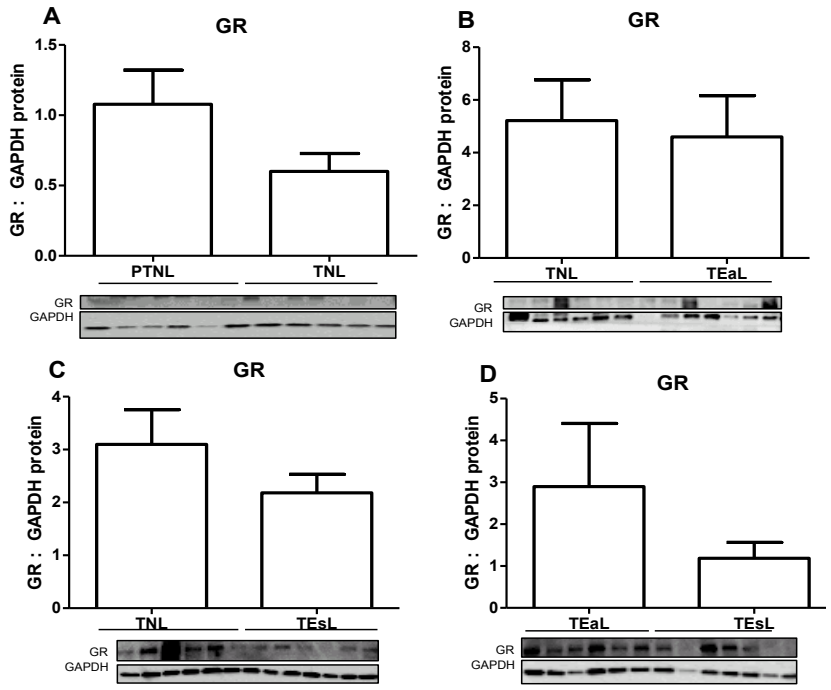

Figure 3.22. GR protein levels during the onset of labour. Bead homogenisation was used to carry out protein extraction. Protein was quantified through western blotting for GR to compare A. preterm not in labour (PTNL) to term not in labour (TNL), B. term not in labour (TNL) to term early labour (TEaL), C. term not in labour (TNL) to term established labour (TEsL).D. term early labour (TEaL) to term established labour (TEsL). A representative western blot is shown in conjunction with densitometric analysis. Data are expressed, as mean +/- SEM. Normality was determined using Kolmogorov-Smirnov test. An unpaired t test was used for normally distributed data, whilst Mann Whitney testing was used to non-normally distributed data. Preterm not in labour (PTNL)[n=17], TNL: term not in labour [n=17] ; TEaL: term early labour [n=18] TEsL [n=18] : term established labour [n= 18],

3.6.2.9 GR protein levels in preterm amnion

No significant differences were observed between GR protein expression levels in IPTL, PTLC or PTLA compared with PTNL samples. No change was observed between PTTWL compared with PTTWNL. PTLP showed significantly increased GR protein expression compared with PTNL samples (Figure 3.23).

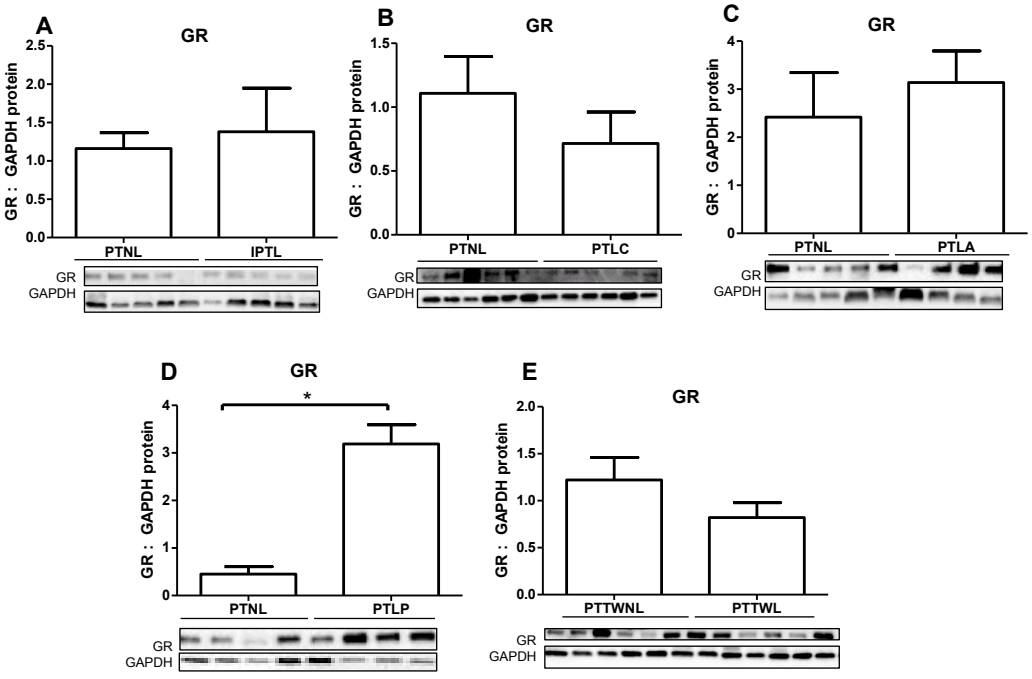

Figure 3.23. GR protein levels in preterm labour. Bead homogenisation was used to carry out protein extraction. Protein was quantified through western blotting for GR to compare **A.** preterm not in labour (PTNL) to idiopathic labour (IPTL), **B.** preterm not in labour (PTNL) to preterm labour due to chorioamnionitis (PTLC), **C.** preterm not in labour (PTNL) to preterm labour polyhydramnios (PTLP) **D.** preterm not in labour (PTNL) to preterm labour abruption (PTLA) **E.** preterm twin not in labour (PTTWNL) to preterm twins in labour (PTTWL). A representative western blot is shown in conjunction with densitometric analysis. Data are expressed, as mean +/- SEM. Normality was determined using Kolmogorov-Smirnov test. An unpaired t test was used for normally distributed data, whilst Mann Whitney testing was used to non-normally distributed data. * $p<0.05$ Preterm not in labour (PTNL)[n=17], idiopathic preterm labour (IPTL) [n=17], preterm labour secondary to chorioamnionitis (PTLC) [n=12], preterm labour secondary to polyhydramnios (PTLP) [n=3] , preterm labour secondary to abruption (PTLA) [n=4] , preterm twin not in labour (PTTWNL) [n=10] , preterm twin in labour (PTTWL) [n= 12].

3.6.3 Are there patterns in co-regulator expression in term and preterm amnion?

The expression of co-regulators was assessed by evaluating mRNA and protein expression through qPCR western blotting. Tables 3.9 and 3.10 show a summary of the data.

	PTNL vs TNL	TNL vs TEaL	TNL vs TEsL	TEaL vs TEsL
FKBP51 mRNA	-	-	-	-
HSP 70 mRNA	-	-	-	-
HSP 70 protein	-	-	-	-
HSP 90 mRNA	-	-	-	-
HSP 90 protein	-	-	-	-
SRC-1 mRNA	-	-	-	-
SRC-1 protein	** ↑	* ↑	-	-
SRC-2 mRNA	-	-	* ↓	-
NCoR mRNA	-	-	-	-

Table 3.9 Are there consistent patterns in steroid co-regulators in term amnion? Protein and qPCR were carried out for FKBP51, HSP70, HSP90, SRC-1, SRC-2 and NCoR. Normality was determined via a Kolmogorov-Smirnov test. PTNL vs TNL; TNL vs TEaL; TNL vs TEsL and TEaL vs TEsL were compared using an unpaired t test for normally distributed data and a Mann Whitney testing for non-normally distributed data. * $p<0.05$; ** $p<0.01$; *** $p<0.001$ PTNL: Preterm not in Labour, TNL: term not in labour; TEaL: term early labour; TEsL: term established labour Preterm not in labour (PTNL)[n=17], TNL: term not in labour [n=17] ; TEaL: term early labour [n=18] TEsL [n=18] : term established labour [n= 18].

	PTNL vs IPTL	PTNL vs PTLC	PTNL vs PTLA	PTNL vs PTLP	PTTWNL vs PTTWL
FKBP51 mRNA	-	-	* ↑	-	-
HSP 70 mRNA	-	-	-	-	-
HSP 70 protein	-	-	** ↓	* ↓	-
HSP 90 mRNA	-	-	-	-	-
HSP 90 protein	-	*** ↓	-	-	-
SRC-1 mRNA	-	-	-	-	** ↑
SRC-1 protein		*** ↓	-	* ↑	-
SRC-2 mRNA	-	-	-	-	-
NCoR mRNA	-	-	-		-

Table 3.10 Are there consistent patterns in steroid co-regulators in preterm amnion? Protein and qPCR were carried out for FKBP51, HSP70, HSP90, SRC-1, SRC-2 and NCoR. Normality was determined via a Kolmogorov-Smirnov test. All singleton preterm subtypes were compared to PTNL using an unpaired t test for normally distributed data and Mann Whitney testing for non-normally distributed data, PTTWNL was compared to PTTWL, * $p<0.05$; Preterm not in labour (PTNL)[n=17], idiopathic preterm labour (IPTL) [n=17], preterm labour secondary to chorioamnionitis (PTLC) [n=12], preterm labour secondary to polyhydramnios (PTLP) [n=3] , preterm labour secondary to abruption (PTLA) [n=4] , preterm twin not in labour (PTTWNL) [n=10] , preterm twin in labour (PTTWL) [n= 12].

3.6.3.1 FKBP51 mRNA levels in term and preterm amnion

There was a decreased trend in FKBP51 mRNA between PTNL and TNL but no significant difference. An increased trend was also observed between TNL and TEsL. There was also a trend towards a decrease in mRNA levels in PTTWL compared with PTTNL. There was a significant increase in FKBP51 mRNA levels in PTLA compared with PTNL samples (Figure 3.24).

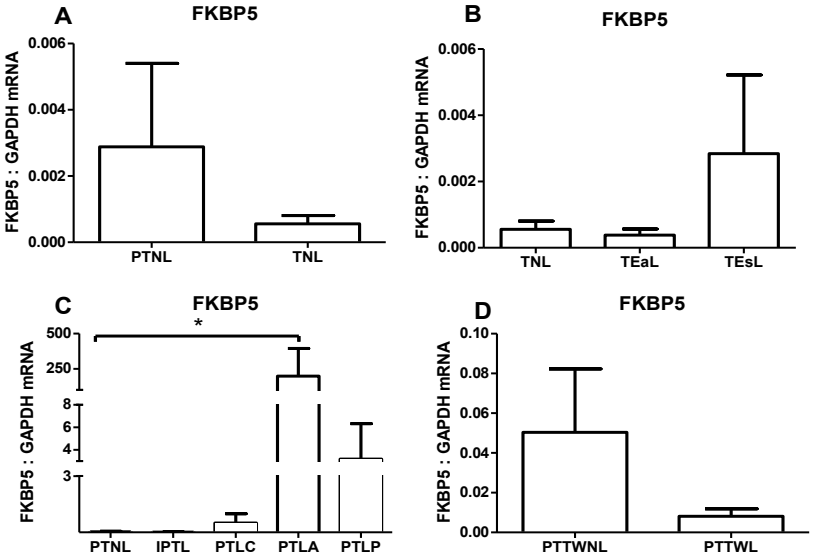

Figure 3.24. FKBP51 mRNA levels in term and preterm labour. Bead homogenisation was used to carry out RNA extraction. RNA was then converted into cDNA and quantative PCR was carried out. Data are expressed as mean +/- SEM. Normality was determined using Kolmogorov-Smirnov test. A: Between Term Not in Labour (TNL),Term Early Labour (TEal) and Term Established Labour (TEsL) samples, a 1-way ANOVA was used for normally distributed data and a Kruskal-Wallis test for non-normally distributed data. B: For analysis between Preterm Not in Labour (PTNL) and TNL an unpaired t test was used for normally distributed data and Mann Whitney testing for non-normally distributed data. C: All preterm singleton samples [idiopathic preterm labour (IPTL), preterm labour secondary to chorioamnionitis (PTLC), preterm labour secondary to polyhydramnios (PTLP), preterm labour secondary to abruption (PTLA)] were compared with PTNL. D: Preterm twin gestations not in labour (PTTWNL) were compared to preterm twin gestation in labour (PTTWL) using either unpaired t testing for normally distributed data or Mann Whitney testing was used to non-normally distributed data. * $p<0.05$; Preterm not in labour (PTNL)[n=17], TNL: term not in labour [n=17] ; TEaL: term early labour [n=18] TEsL [n=18] : term established labour [n= 18], idiopathic preterm labour (IPTL) [n=17], preterm labour secondary to chorioamnionitis (PTLC) [n=12], preterm labour secondary to polyhydramnios (PTLP) [n=3] , preterm labour secondary to abruption (PTLA) [n=4] , preterm twin not in labour (PTTWNL) [n=10] , preterm twin in labour (PTTWL) [n= 12].

3.6.3.2 HSP70 mRNA levels in term and preterm amnion

There was a trend towards a decrease in HSP70 mRNA in TNL compared with PTNL samples. However, there was no significant change in HSP70 mRNA levels across term gestations. There was an increased trend in HSP 70 mRNA in PTLC and PTLP samples (Figure 3.25).

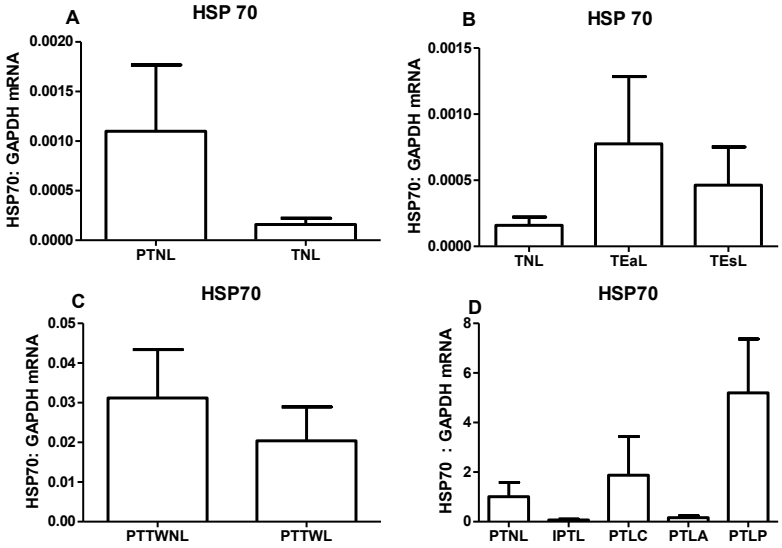

Figure 3.25. HSP70 mRNA levels in term and preterm labour. Bead homogenisation was used to carry out RNA extraction. RNA was then converted into cDNA and quantative PCR was carried out. Data are expressed as mean +/- SEM. Normality was determined using Kolmogorov-Smirnov test. **A:** Between Term Not in Labour (TNL),Term Early Labour (TEal) and Term Established Labour (TEsL) samples, a 1-way ANOVA was used for normally distributed data and a Kruskal-Wallis test for non-normally distributed data. B: For analysis between Preterm Not in Labour (PTNL) and TNL an unpaired t test was used for normally distributed data and Mann Whitney testing for non-normally distributed data. C: All preterm singleton samples [idiopathic preterm labour (IPTL), preterm labour secondary to chorioamnionitis (PTLC), preterm labour secondary to polyhydramnios (PTLP), preterm labour secondary to abruption (PTLA)] were compared with PTNL. D: Preterm twin gestations not in labour (PTTWNL) were compared to preterm twin gestation in labour (PTTWL) using either unpaired t testing for normally distributed data or Mann Whitney testing was used to non-normally distributed data Preterm not in labour (PTNL)[n=17], TNL: term not in labour [n=17] ; TEaL: term early labour [n=18] TEsL [n=18] : term established labour [n= 18], idiopathic preterm labour (IPTL) [n=17], preterm labour secondary to chorioamnionitis (PTLC) [n=12], preterm labour secondary to polyhydramnios (PTLP) [n=3] , preterm labour secondary to abruption (PTLA) [n=4] , preterm twin not in labour (PTTWNL) [n=10] , preterm twin in labour (PTTWL) [n= 12].

3.6.3.3 HSP70 protein levels in term amnion

Though there was an increased trend in HSP70 protein between PTNL and TNL samples, no significant differences were observed in HSP70 protein levels across term deliveries (Figure 3.26).

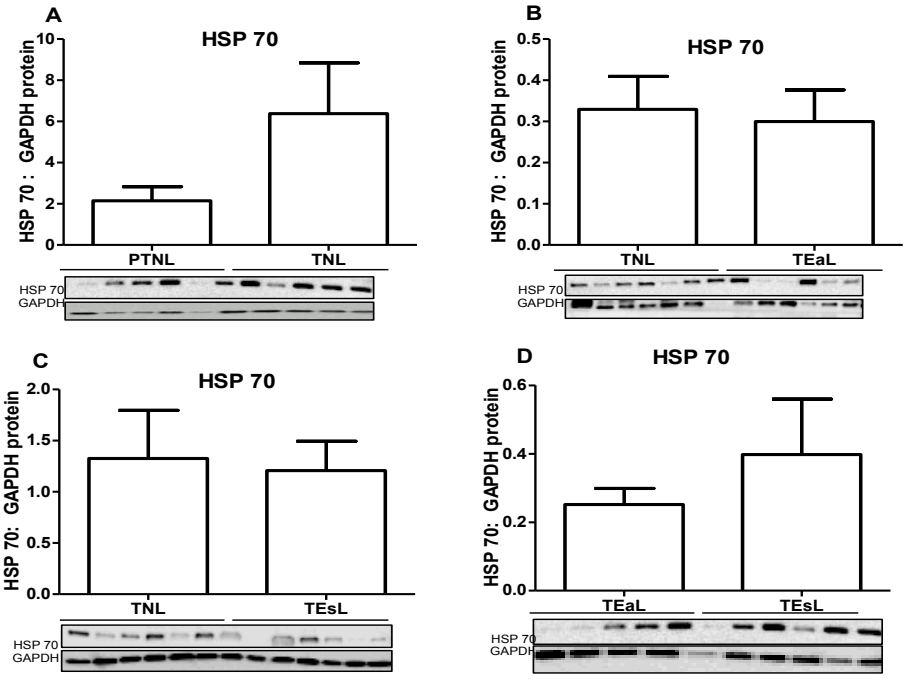

Figure 3.26. HSP70 protein levels in term labour. Bead homogenisation was used to carry out protein extraction. Protein was quantified through western blotting for HSP70 to compare A. preterm not in labour (PTNL) to term not in labour (TNL), B. term not in labour (TNL) to term early labour (TEaL), C. term not in labour (TNL) to term established labour (TEsL). D. Term early labour (TEaL) to term established labour (TEsL). A representative western blot is shown in conjunction with densitometric analysis. Data are expressed, as mean +/- SEM. Normality was determined using Kolmogorov-Smirnov test. An unpaired t test was used for normally distributed data, whilst Mann Whitney testing was used to non-normally distributed data. Preterm not in labour (PTNL)[n=17], TNL: term not in labour [n=17] ; TEaL: term early labour [n=18] TEsL [n=18] : term established labour [n= 18].

3.6.3.4 HSP70 protein levels in preterm amnion

No change in HSP70 protein levels were observed in IPTL or PTLC compared with PTNL. There were significant decreases in HSP70 protein levels in PTLP and PTLA samples compared with PTNL. No change was observed between twin gestations (Figure 3.27).

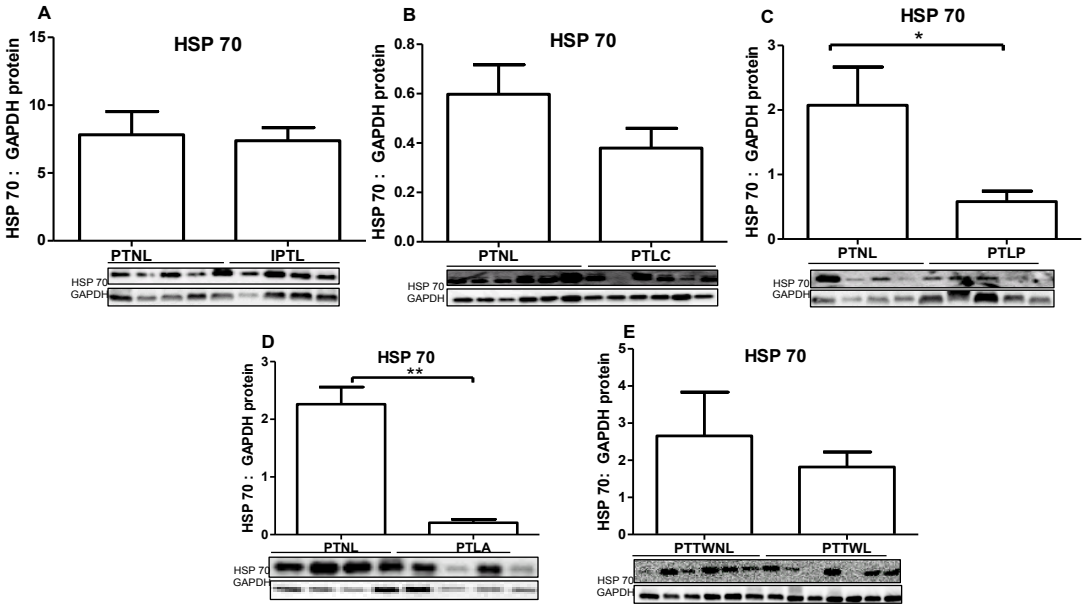

Figure 3.27. HSP70 protein levels in preterm labour. Bead homogenisation was used to carry out protein extraction. Protein was quantified through western blotting for HSP70 to compare **A.** preterm not in labour (PTNL) to idiopathic labour (IPTL), **B.** preterm not in labour (PTNL) to preterm labour due to chorioamnionitis (PTLC), **C.** preterm not in labour (PTNL) to preterm labour polyhydramnios (PTLP) **D.** preterm not in labour (PTNL) to preterm labour abruption (PTLA) E**.** preterm twin not in labour (PTTWNL) to preterm twins in labour (PTTWL). A representative western blot is shown in conjunction with densitometric analysis. Data are expressed, as mean +/- SEM. Normality was determined using Kolmogorov-Smirnov test. An unpaired t test was used for normally distributed data, whilst Mann Whitney testing was used to non-normally distributed data. * $p<0.05$ Preterm not in labour (PTNL)[n=17], idiopathic preterm labour (IPTL) [n=17], preterm labour secondary to chorioamnionitis (PTLC) [n=12], preterm labour secondary to polyhydramnios (PTLP) [n=3] , preterm labour secondary to abruption (PTLA) [n=4] , preterm twin not in labour (PTTWNL) [n=10] , preterm twin in labour (PTTWL) [n= 12].

3.6.3.5 HSP 90 mRNA levels in term and preterm amnion

No significant changes were observed between PTNL and TNL samples at mRNA level (Figure 3.28) or protein level (Figure 3.29). In addition, no other differences were observed in HSP90 mRNA levels between term samples (Figure 3.29). There was an increased trend in HSP 90 mRNA in PTLC and PTLP samples however no significant changes were observed in PTL subtypes (Figure 3.28).

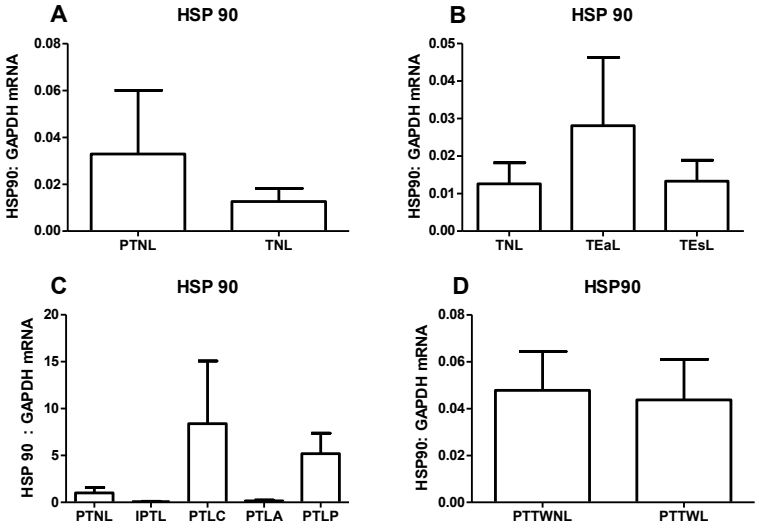

Figure 3.28. HSP90 mRNA in term and preterm labour. Bead homogenisation was used to carry out RNA extraction. RNA was then converted into cDNA and quantative PCR was carried out. Data are expressed as mean +/- SEM. Normality was determined using Kolmogorov-Smirnov test. A: Between Term Not in Labour (TNL),Term Early Labour (TEal) and Term Established Labour (TEsL) samples, a 1-way ANOVA was used for normally distributed data and a Kruskal-Wallis test for non-normally distributed data. B: For analysis between Preterm Not in Labour (PTNL) and TNL an unpaired t test was used for normally distributed data and Mann Whitney testing for non-normally distributed data. C: All preterm singleton samples [idiopathic preterm labour (IPTL), preterm labour secondary to chorioamnionitis (PTLC), preterm labour secondary to polyhydramnios (PTLP), preterm labour secondary to abruption (PTLA)] were compared with PTNL. D: Preterm twin gestations not in labour (PTTWNL) were compared to preterm twin gestation in labour (PTTWL) using either unpaired t testing for normally distributed data or Mann Whitney testing was used to non-normally distributed data. Preterm not in labour (PTNL)[n=17], TNL: term not in labour [n=17] ; TEaL: term early labour [n=18] TEsL [n=18] : term established labour [n= 18], idiopathic preterm labour (IPTL) [n=17], preterm labour secondary to chorioamnionitis (PTLC) [n=12], preterm labour secondary to polyhydramnios (PTLP) [n=3] , preterm labour secondary to abruption (PTLA) [n=4] , preterm twin not in labour (PTTWNL) [n=10] , preterm twin in labour (PTTWL) [n= 12]. idiopathic preterm labour (IPTL) [n=17], preterm labour secondary to chorioamnionitis (PTLC) [n=12], preterm labour secondary to polyhydramnios (PTLP) [n=3] , preterm labour secondary to abruption (PTLA) [n=4] , preterm twin not in labour (PTTWNL) [n=10] , preterm twin in labour (PTTWL) [n= 12].

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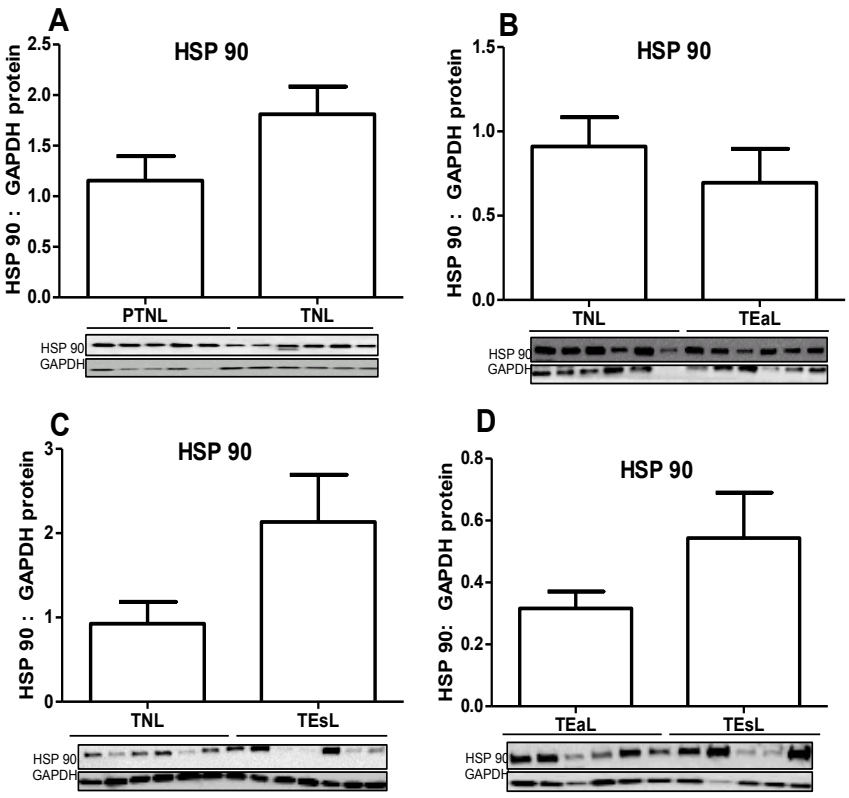

Figure 3.29. HSP90 protein levels in term labour. Bead homogenisation was used to carry out protein extraction. Protein was quantified through western blotting for HSP90 to compare A. preterm not in labour (PTNL) to term not in labour (TNL), B. term not in labour (TNL) to term early labour (TEaL), C. term not in labour (TNL) to term established labour (TEsL).D. term early labour (TEaL) to term established labour (TEsL). A representative western blot is shown in conjunction with densitometric analysis. Data are expressed, as mean +/- SEM. Normality was determined using Kolmogorov-Smirnov test. An unpaired t test was used for normally distributed data, whilst Mann Whitney testing was used to non-normally distributed data [n=17-18].

3.6.3.6 HSP 90 protein levels in preterm amnion

No significant changes in HSP90 levels were observed in IPTL, PTLA or PTLP samples, when compared with PTNL samples. There was a significant decrease in HSP90 in chorioamnionitis samples when compared with PTNL. No change was observed between preterm twin gestations (Figure 3.30).

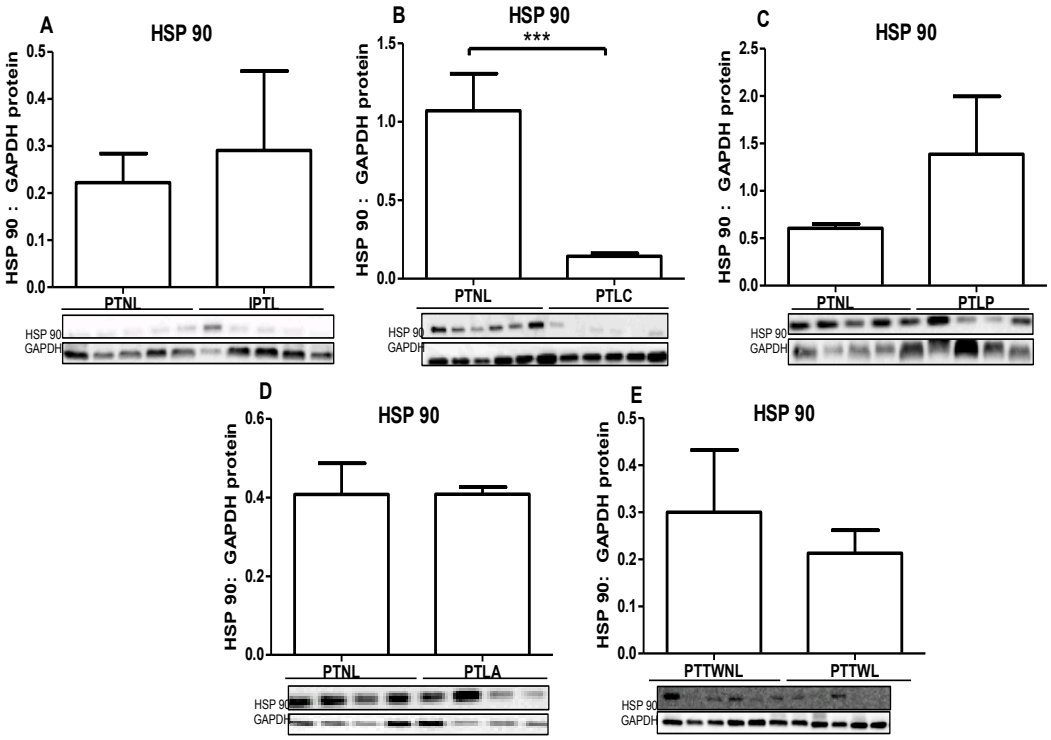

Figure 3.30. HSP90 protein levels in preterm labour. Bead homogenisation was used to carry out protein extraction. Protein was quantified through western blotting for HSP90 to compare **A.** preterm not in labour (PTNL) to idiopathic labour (IPTL), **B.** preterm not in labour (PTNL) to preterm labour due to chorioamnionitis (PTLC), **C.** preterm not in labour (PTNL) to preterm labour polyhydramnios (PTLP) **D.** preterm not in labour (PTNL) to preterm labour abruption (PTLA) E**.** preterm twin not in labour (PTTWNL) to preterm twins in labour (PTTWL). A representative western blot is shown in conjunction with densitometric analysis. Data are expressed, as mean +/- SEM. Normality was determined using Kolmogorov-Smirnov test. An unpaired t test was used for normally distributed data, whilst Mann Whitney testing was used to non-normally distributed data. *** $p<0.001$ Preterm not in labour (PTNL)[n=17], idiopathic preterm labour (IPTL) [n=17], preterm labour secondary to chorioamnionitis (PTLC) [n=12], preterm labour secondary to polyhydramnios (PTLP) [n=3] , preterm labour secondary to abruption (PTLA) [n=4] , preterm twin not in labour (PTTWNL) [n=10] , preterm twin in labour (PTTWL) [n= 12].

3.6.3.7 SRC-1 mRNA levels in term and preterm amnion

SRC-1 mRNA levels tended to decrease in TNL compared to PTNL samples but no significant change was observed in SRC-1 mRNA levels across all term gestation. Within preterm amnion samples there was a trend towards an increase in SRC-1 mRNA levels compared with PTNL, but this was not significant. SRC-1 mRNA levels significantly increased in PTTWL compared with PTTWNL samples (Figure 3.31).

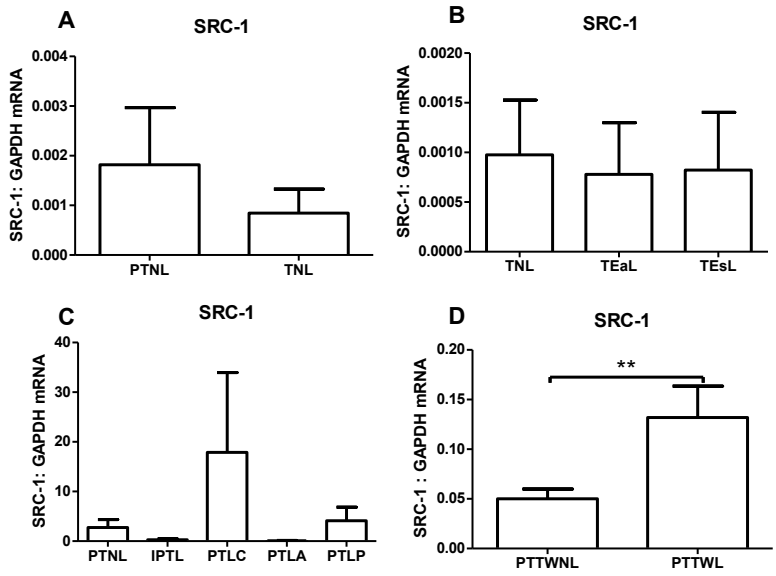

Figure 3.31. SRC-1 mRNA levels in term and preterm labour. Bead homogenisation was used to carry out RNA extraction. RNA was then converted into cDNA and quantative PCR was carried out. Data are expressed as mean +/- SEM. Normality was determined using Kolmogorov-Smirnov test. A: Between Term Not in Labour (TNL),Term Early Labour (TEal) and Term Established Labour (TEsL) samples, a 1-way ANOVA was used for normally distributed data and a Kruskal-Wallis test for non-normally distributed data. B: For analysis between Preterm Not in Labour (PTNL) and TNL an unpaired t test was used for normally distributed data and Mann Whitney testing for non-normally distributed data. C: All preterm singleton samples [idiopathic preterm labour (IPTL), preterm labour secondary to chorioamnionitis (PTLC), preterm labour secondary to polyhydramnios (PTLP), preterm labour secondary to abruption (PTLA)] were compared with PTNL. D: Preterm twin gestations not in labour (PTTWNL) were compared to preterm twin gestation in labour (PTTWL) using either unpaired t testing for normally distributed data or Mann Whitney testing was used to non-normally distributed data ** $p<0.01$; Preterm not in labour (PTNL)[n=17], TNL: term not in labour [n=17] ; TEaL: term early labour [n=18] TEsL [n=18] : term established labour [n= 18], idiopathic preterm labour (IPTL) [n=17], preterm labour secondary to chorioamnionitis (PTLC) [n=12], preterm labour secondary to polyhydramnios (PTLP) [n=3] , preterm labour secondary to abruption (PTLA) [n=4] , preterm twin not in labour (PTTWNL) [n=10] , preterm twin in labour (PTTWL) [n= 12]. idiopathic preterm labour (IPTL) [n=17], preterm labour secondary to chorioamnionitis (PTLC) [n=12], preterm labour secondary to polyhydramnios (PTLP) [n=3] , preterm labour secondary to abruption (PTLA) [n=4] , preterm twin not in labour (PTTWNL) [n=10] , preterm twin in labour (PTTWL) [n= 12].

3.6.3.8 SRC-1 protein levels in term amnion

SRC-1 protein levels were significantly higher in TNL compared with PTNL samples. There was a trend towards an increase in SRC-1 protein levels in TEaL compared with TNL samples. SRC-1 also significantly increased in TEsL compared with TNL. Furthermore a trend towards an increase in TEsL compared with TEaL was observed (Figure 3.32).

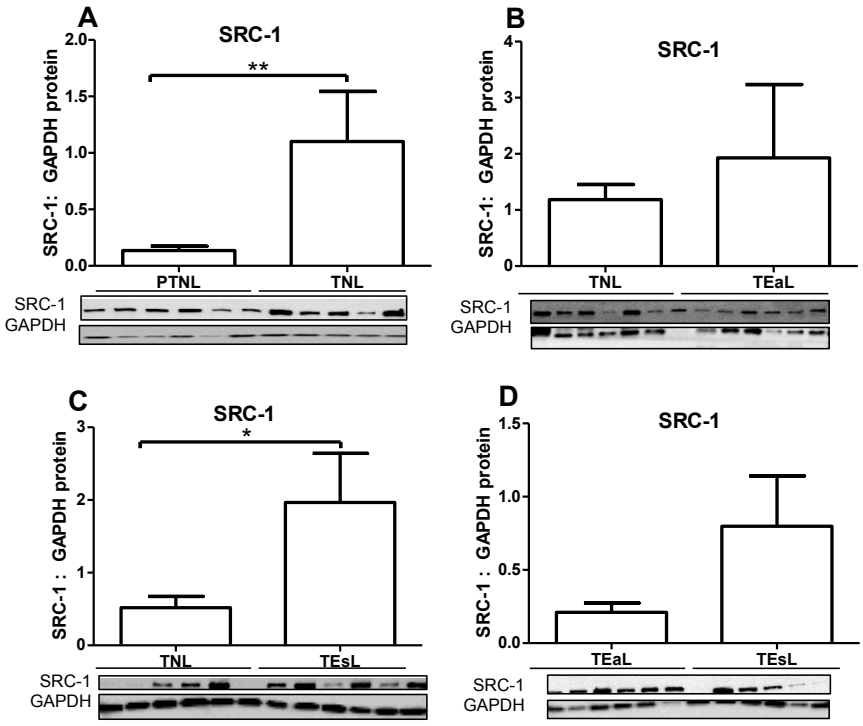

Figure 3.32. SRC-1 protein levels in term labour. Bead homogenisation was used to carry out protein extraction. Protein was quantified through western blotting for SRC-1 to compare A. preterm not in labour (PTNL) to term not in labour (TNL), B. term not in labour (TNL) to term early labour (TEaL), C. term not in labour (TNL) to term established labour (TEsL).D. term early labour (TEaL) to term established labour (TEsL). A representative western blot is shown in conjunction with densitometric analysis. Data are expressed, as mean +/- SEM. Normality was determined using Kolmogorov-Smirnov test. An unpaired t test was used for normally distributed data, whilst Mann Whitney testing was used to non-normally distributed data * $p<0.05$; ** $p<0.01$; [n=17-18].

3.6.3.9 SRC-1 protein levels in preterm amnion

A trend towards a reduction in SRC-1 protein levels was observed IPTL and PTLA, when compared with PTNL samples. A significant reduction was observed in PTLC when compared with PTNL samples. Interestingly the reverse, a significant increase was observed in PTLP when compared with PTNL. No change was observed in SRC-1 levels when comparing preterm twin gestations (Figure 3.33).

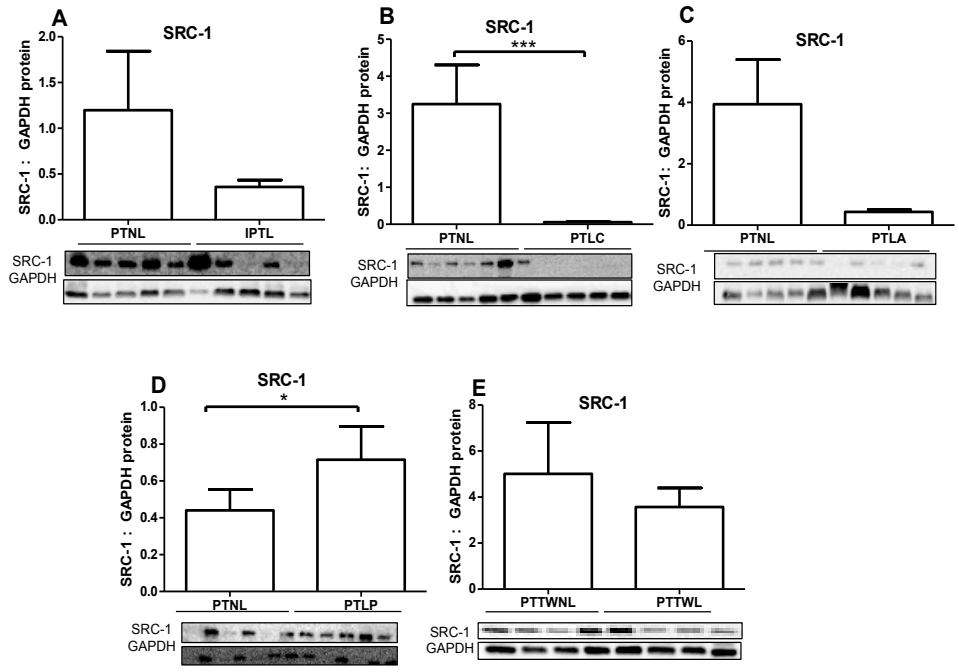

Figure 3.33. SRC-1 protein levels in preterm labour. Bead homogenisation was used to carry out protein extraction. Protein was quantified through western blotting for SRC-1 to compare **A.** preterm not in labour (PTNL) to idiopathic labour (IPTL), **B.** preterm not in labour (PTNL) to preterm labour due to chorioamnionitis (PTLC), **C.** preterm not in labour (PTNL) to preterm labour polyhydramnios (PTLP) **D.** preterm not in labour (PTNL) to preterm labour abruption (PTLA) **E.** preterm twin not in labour (PTTWNL) to preterm twins in labour (PTTWL). A representative western blot is shown in conjunction with densitometric analysis. Data are expressed, as mean +/- SEM. Normality was determined using Kolmogorov-Smirnov test. An unpaired t test was used for normally distributed data, whilst Mann Whitney testing was used to non-normally distributed data * $p<0.05$ Preterm not in labour (PTNL)[n=17], idiopathic preterm labour (IPTL) [n=17], preterm labour secondary to chorioamnionitis (PTLC) [n=12], preterm labour secondary to polyhydramnios (PTLP) [n=3] , preterm labour secondary to abruption (PTLA) [n=4] , preterm twin not in labour (PTTWNL) [n=10] , preterm twin in labour (PTTWL) [n= 12].

3.6.3.10 SRC-2 mRNA levels in term and preterm amnion

There was a decreased trend in SRC-2 mRNA between PTNL and TNL. SRC-2 mRNA levels were significantly decreased in TEsL compared with TNL and TEaL samples. There was a trend towards an increase in preterm samples: PTLC, PTLA and PTLP compared with PTNL samples. In addition, there was an increased trend in SRC-2 mRNA levels in PTTWL compared with PTTWNL samples (Figure 3.34).

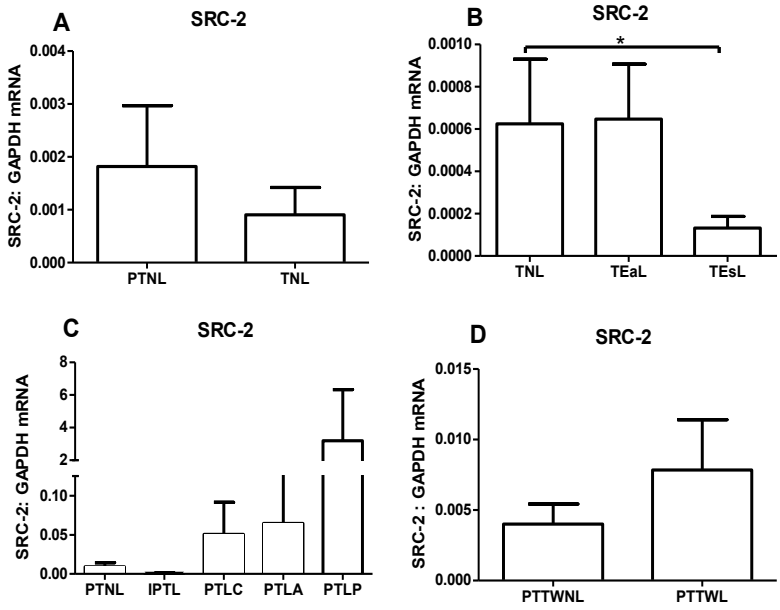

Figure 3.34. SRC-2 mRNA levels in term and in term and preterm labour. Bead homogenisation was used to carry out RNA extraction. RNA was then converted into cDNA and quantative PCR was carried out. Data are expressed, as mean +/- SEM. Normality was determined using Kolmogorov-Smirnov test. A: Between Term Not in Labour (TNL),Term Early Labour (TEal) and Term Established Labour (TEsL) samples, a 1-way ANOVA was used for normally distributed data and a Kruskal-Wallis test for non-normally distributed data. B: For analysis between Preterm Not in Labour (PTNL) and TNL an unpaired t test was used for normally distributed data and Mann Whitney testing for non-normally distributed data. C: All preterm singleton samples [idiopathic preterm labour (IPTL), preterm labour secondary to chorioamnionitis (PTLC), preterm labour secondary to polyhydramnios (PTLP), preterm labour secondary to abruption (PTLA)] were compared with PTNL. D: Preterm twin gestations not in labour (PTTWNL) were compared to preterm twin gestation in labour (PTTWL) using either unpaired t testing for normally distributed data or Mann Whitney testing was used to non-normally distributed data ** $p<0.01$; Preterm not in labour (PTNL)[n=17], TNL: term not in labour [n=17] ; TEaL: term early labour [n=18] TEsL [n=18] : term established labour [n= 18], idiopathic preterm labour (IPTL) [n=17], preterm labour secondary to chorioamnionitis (PTLC) [n=12], preterm labour secondary to polyhydramnios (PTLP) [n=3] , preterm labour secondary to abruption (PTLA) [n=4] , preterm twin not in labour (PTTWNL) [n=10] , preterm twin in labour (PTTWL) [n= 12]. idiopathic preterm labour (IPTL) [n=17], preterm labour secondary to chorioamnionitis (PTLC) [n=12], preterm labour secondary to polyhydramnios (PTLP) [n=3] , preterm labour secondary to abruption (PTLA) [n=4] , preterm twin not in labour (PTTWNL) [n=10] , preterm twin in labour (PTTWL) [n= 12].

3.6.3.11 SRC-2 & 3 protein in term and PTL

Very faint bands were sporadically detectable for SRC-2 and SRC-3 across term and preterm gestations. However, they were not strong enough to carry out accurate densitometric analysis and therefore could not be accurately quantified.

3.6.3.12 NCoR mRNA levels in term and preterm amnion

There were no significant differences observed in NCoR mRNA levels between PTNL and TNL samples. No other significant differences were observed across term and preterm amnion samples. There was a trend towards lower NCoR mRNA levels in TEaL compared with TNL or TEsL samples. There was also a trend towards an increase in NCoR mRNA levels in PTLC compared with PTNL samples but these trends were not significant (Figure 3.35).

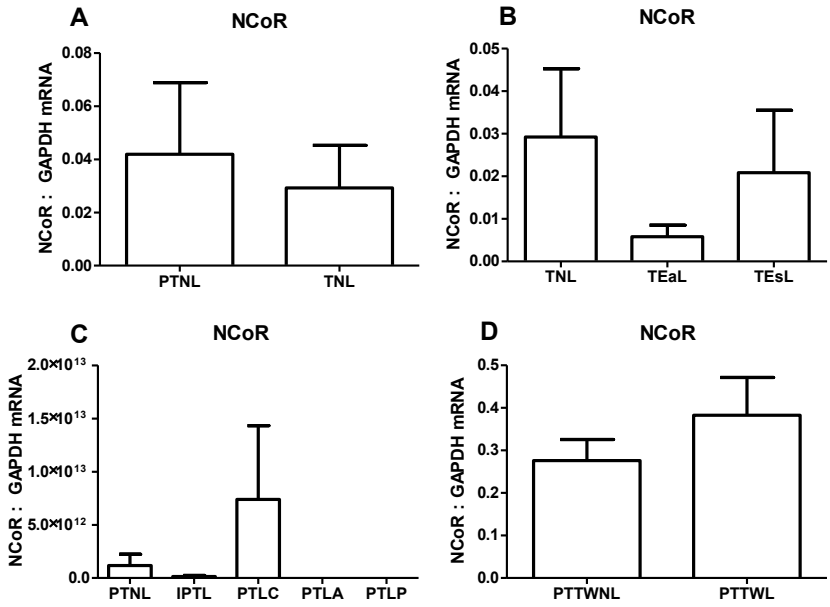

Figure 3.35. NCoR mRNA levels in term and preterm labour. Bead homogenisation was used to carry out RNA extraction. RNA was then converted into cDNA and quantative PCR was carried out. Data are expressed, as mean +/- SEM. Normality was determined using Kolmogorov-Smirnov test. A: Between Term Not in Labour (TNL), Term Early Labour (TEal) and Term Established Labour (TEsL) samples, a 1-way ANOVA was used for normally distributed data and a Kruskal-Wallis test for non-normally distributed data. B: For analysis between Preterm Not in Labour (PTNL) and TNL an unpaired t test was used for normally distributed data and Mann Whitney testing for non-normally distributed data. C: All preterm singleton samples [idiopathic preterm labour (IPTL), preterm labour secondary to chorioamnionitis (PTLC), preterm labour secondary to polyhydramnios (PTLP), preterm labour secondary to abruption (PTLA)] were compared with PTNL. D: Preterm twin gestations not in labour (PTTWNL) were compared to preterm twin gestation in labour (PTTWL) using either unpaired t testing for normally distributed data or Mann Whitney testing was used to non-normally distributed data. Preterm not in labour (PTNL)[n=17], TNL: term not in labour [n=17] ; TEaL: term early labour [n=18] TEsL [n=18] : term established labour [n= 18], idiopathic preterm labour (IPTL) [n=17], preterm labour secondary to chorioamnionitis (PTLC) [n=12], preterm labour secondary to polyhydramnios (PTLP) [n=3] , preterm labour secondary to abruption (PTLA) [n=4] , preterm twin not in labour (PTTWNL) [n=10] , preterm twin in labour (PTTWL) [n= 12]. idiopathic preterm labour (IPTL) [n=17], preterm labour secondary to chorioamnionitis (PTLC) [n=12], preterm labour secondary to polyhydramnios (PTLP) [n=3] , preterm labour secondary to abruption (PTLA) [n=4] , preterm twin not in labour (PTTWNL) [n=10] , preterm twin in labour (PTTWL) [n= 12].

3.6.23 NCoR & Silencing mediator for Retinoid or Thyroid Hormone (SMRT) levels

NCoR and silencing mediator for retinoid or thyroid hormone (SMRT) levels were not detectable in the amnion tissues from our research bank. To ensure there were no errors in experimental technique, a positive control was used (Figure 3.36 & 3.37).

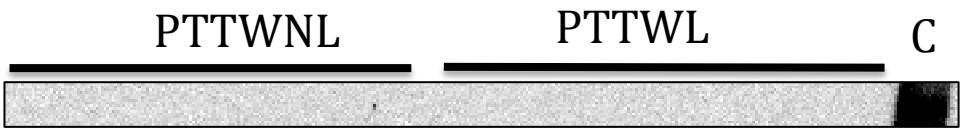

Figure 3.36. Nuclear receptor co-repressor (NCoR) Bead homogenisation was used to carry out protein extraction. Protein was quantified through western blotting for NCoR. Compared preterm twins no labour (PTTWNL) to preterm twins in labour (PTTWL). 20 μg HeLa nuclear extract [C] was used as a positive control.

Figure 3.37. Silencing mediator for retinoid or thyroid hormone (SMRT) Bead homogenisation was used to carry out protein extraction. Protein was quantified through western blotting for SMRT. Compared preterm twins no labour (PTTWNL) to preterm twins in labour (PTTWL); 20 μg HeLa nuclear extract [C] was used as a positive control.

3.7 Discussion – Section A

3.7.1 An assessment of the cytokine profile and prolabour gene expression in term and preterm amnion tissue

This study highlights key patterns in the cytokine-chemokine profile as labour ensues in the human amnion. It also identifies changes in prolabour gene expression, both in term and PTL, using human amnion tissue. Prolabour gene expression at the protein level increased as labour progressed at term as well as in the context of inflammation and stretch-induced PTL.

3.7.2 Does inflammation occur in TEaL?

Identifying cytokine-chemokine patterns expressed during labour may allow better development of clinical biomarkers to predict abnormalities in pregnancy.

The cytokine-chemokine data presented here add to the concept that human labour at term is characterised by increased pro-inflammatory cytokines: (IL-1β, IL-6, TNFα) and chemotactic IL-8 and CCL-2, important for neutrophil recruitment and activation (69, 75, 211, 228-233). The data presented here show increased levels of IL-8, IL-6 in TEaL (Figure 3.2) and TEsL samples (Figure 3.3) when compared with TNL samples. Furthermore, increased levels of CCL2, CXCL1 and IL-1β in TEsL samples (Figure 3.3) were also observed.

The data presented shares similarities with recent work by Singh *et al* who demonstrated that myometrial inflammation might actually be a result of labour as opposed to being a cause of term labour (76). However, Singh *et al* found no significant difference in IL-6, IL-8 or TNFα between PTNL samples and TNL samples. Instead, they found significantly increased IL-6, IL-8 and CXCL1 levels in TEsL myometrial tissue samples compared with TNL samples (76). Singh *et al* suggests inflammation observed in the myometrium at term parturition, may not be directly responsible for the initiation of labour but rather a consequence. This may not entirely be the case in the amnion where the data presented here showed increased IL-6 and IL-8 in TEaL and TEsL samples, when compared with TNL samples.

IL-16 concentrations decreased whilst CXCL6 concentration increased, when comparing PTNL to TNL amnion samples (Figure 3.1) These data correlate with studies which used elevated IL-16 present in amniotic fluid as a biomarker to predict preterm birth (234-236). CXCL6 is known for its pro-inflammatory properties and is involved in neutrophil recruitment and activation (43). In amniotic fluid, it has been shown to increase with gestational age (237) as well as in labouring term myometrium when compared with non-labouring controls (238).

3.7.3 Does the inflammatory cytokine-chemokine profiling of PTL depend on its underlying cause?

The preterm data presented here correlates with the existing literature surrounding cytokine expression, and goes some way to examine the differences between PTL in the context of differing pathologies.

In general, PTL has been associated with elevated pro-inflammatory cytokines (239-241). These findings complement data presented here, showing increased levels (CXCL1, CXCL1, IL-8, CCL2, CCL7, CCL20, CXCL6, IL-6 and IL- β) in IPTL samples (Figure 3.4) as well as PTLC samples. A study looking at fetal plasma concentration linked with PTLC showed elevated IL-6 (242), supporting our observations (Figure 3.4). Intriguingly, IL-7 was only increased in PTLP and PTLC samples, when compared with PTNL samples. A study found maternal serum cytokine levels in women who delivered preterm (following a threatened preterm labour (TPTL) diagnosis) to have elevated IL-7 compared with 'term' women and 'control' women, without TPTL (243).

Elevated IL-16 levels in amniotic fluid have been used as a biomarker for PTL (234-236)and in the current series; amnion IL-16 levels were increased in PTLA samples only. The levels were otherwise similar to PTNL controls (Figure 3.4).

3.7.4 Cytokine-chemokine profile in uterine overdistention

Our group has shown *in vitro* stretch leads to cytokine and chemokine release in amnion and myometrial cells (60, 244). In addition, the data presented here (Figure 3.5) showing increased TNFα, correlates with the increased cytokine expression (TNFα and IL-6 mRNA) observed in women with polyhydramnios, seen in both the myometrium and the amnion (63).

3.7.5 How does prolabour gene expression differ in term and preterm amnion?

3.7.5.1 COX-2 expression in term and PTL

COX-2 as previously mentioned, plays a vital role in PG production (245). At the mRNA level, there was a trend towards an increase as term labour progressed (Figure 3.6). At the protein level, COX-2 increased in TEsL compared with TNL (Figure 3.7). This was similar to other studies that found increased COX-2 mRNA during term labour onset as well as studies that created labour-like environments *in vitro* (73, 99, 103, 104, 246-251). Findings presented here were also consistent with studies observing increased COX-2 protein after labour onset (246, 247, 252).

Although elevated COX-2 has been observed in labour (245) its precise expression in different underlying causes of preterm birth has been investigated in less detail. Data presented here showed increased COX-2 mRNA in idiopathic PTL as well as PTL abruption samples. However, the COX-2 protein levels were lower in the abruption samples. COX-2 mRNA has been shown to be regulated transcriptionally and display constitutive stability in term amnion (253), while COX-2 is a so-called suicide protein, such if it is active it is targeted for destruction (254). These factors may explain the differences in COX-2 mRNA and protein levels.

One study, focusing on the epigenetics of preterm birth in the human myometrium, investigated differential methylation across tissue groups: preterm without labour, idiopathic PTL and preterm twin gestations in labour. (255) Although there was a correlation between hypomethylation and increased mRNA of PGES-1, no difference in methylation status was noted to explain differing COX-2 expression across tissue groups. Although this study does not explain differential COX-2 gene expression, it does highlight the need for further investigation into the regulatory mechanisms

involved in contraction-associated genes in PTL (255). There were also trends towards increased COX-2 mRNA (Figure 3.10) and protein (Figure 3.11) in polyhydramnios and PTTWL. PTL in the context of polyhydramnios and preterm twin gestations may represent examples of stretch-induced preterm birth. It has previously been shown that subjecting primary human amnion and myometrial cells to stretch *in vitro* results in increased COX-2 expression (60, 244).

3.7.5.2 OTR expression in term and PTL

The data presented here support the existing literature that gives OTR an important role in the progression of labour. No significant change was observed at the mRNA level across term labour however OTR protein increased in established labour, when compared with early labouring samples (Figure 3.8). Wathes *et al*, used *in situ* hybridization to investigate OTR expression in term and preterm human gestational tissues following labour onset, and their work supports this finding (256). Though they were unable to quantify their amnion data, they did find increased OTR at the mRNA level in the myometrium from women late into their pregnancy (256). OTR at the mRNA level was significantly decreased in idiopathic PTL as well as samples with chorioamnionitis and polyhydramnios (Figure 3.10). Interestingly though at the protein level chorioamnionitis samples showed increased levels of OTR (Figure 3.12). It has been suggested that increased OTR may enhance inflammatory cytokine production in the context of PTL (257, 258). Myometrial cells subjected to *in vitro* inflammatory conditions increased OTR levels (257). This study also proposed that in extremely inflammatory environments, such as in chorioamnionitis, an OTR decline could also indicate a compromised ability to regulate labour or an inflammatory response. (257). However, the disparity between mRNA and protein could suggest differential pathways for OTR mRNA, its protein degradation and regeneration, similar to that of COX-2 (254).

3.7.5.3 Cxn 43 expression in term and PTL

Gap junctions link different myometrial cells facilitating the spread of action potentials between cells. In general, much less is known about its role and function in the amnion. However elevated Cxn 43 levels were observed in damaged human fetal membranes following fetoscopic surgery (259). More recently, *in vitro* damage to fetal membranes was shown to also over stimulate Cxn 43 levels (260). These findings suggest that elevated Cxn 43 may prevent healthy integrin signals and catalyse collagen degradation, which may result in membrane rupture.

The data presented suggest that Cxn 43 protein levels increased in TEsL compared with TEaL (Figure 3.9) however there was a large amount of variability between tissue samples. These findings are supported by formative work that demonstrated similar results in the myometrium with labour progression (23). Cxn 43 mRNA however, decreased in idiopathic PTL as well as PTL secondary to chorioamnionitis and abruption. This however, contrasts with the increased Cxn 43 observed following insult to fetal membranes (259). The difference between these results could be explained through differing methodologies used to collect fetal membranes; the amnion tissue obtained from women in this study were snap frozen following emergency caesarean section, whereas human fetal membranes in Barrett *et al*'s research were taken following fetoscopic surgery.

PTTWL samples displayed higher Cxn 43 protein levels when compared with PTTWNL (Figure 3.13). Twin gestations are often associated with stretch-induced preterm birth and animal stretch models have shown stretch-induced increases of myometrial Cxn 43 during pregnancy (49). These findings correlate with data presented here in the amnion and could offer an explanation for the increased Cxn 43 observed in human preterm twin labouring amnion samples.

Furthermore, Ou *et al* found that in rats, stretch-induced Cxn 43 expression could be inhibited through the action of P4 (49) (261-263). Although this study proposed a potential therapeutic technique aimed at inhibiting myometrial Cxn 43, it also leaves the question: could a stretch (or otherwise)-induced up-regulation of prolabour genes be reduced through the action of P4 in other tissue types? This question, in the context of amnion will be investigated in a later chapter.

3.7.5.4 Summary of Section A

To summarise the findings from this chapter, it is important to refer back to the initial questions:

- Does inflammation occur in term early labour?
- How does prolabour gene expression change with advancing gestation and as labour progresses?
- Does each subtype of PTL display a unique cytokine-chemokine profile?

Although there were increases in IL-6 and IL-8 in TEaL compared with TNL samples, a marked elevation in cytokine-chemokine profile was observed in TEsL, compared with TNL samples. In addition, elevated COX-2 protein levels were observed when compared with TNL samples, whilst OTR and Cxn43 levels were higher in TEsL samples compared with TNL. Together these data may potentially add to the theory that inflammation observed in gestational tissues is a consequence of labour.

Furthermore, assessing the expression of inflammatory cytokines from preterm amnion tissue suggest that each subtype of PTL may exhibit a different cytokine-chemokine profile.

However, in the case of western blotting data, it is important to note a large amount of variability between tissues. In order to come to a more robust conclusion, it would be useful to use multiple housekeeping proteins as an alternative to GAPDH, to fully assess the differences observed.

3.8 Discussion - Section B:

3.8.1 Are there consistent patterns in steroid activity in term and preterm amnion?

Human fetal membranes are steroid responsive and in this study, the expression of nuclear receptor expression and coactivators were examined from term and preterm human amnion tissue.

3.8.2 Progesterone Receptor (PR) in term and PTL

Though minimal levels of PR have previously been reported in the human amnion (178, 264) other evidence has demonstrated an increase in receptor isoform A during term parturition in amnion cells (178).

The data presented here support a change in dominance between PR isoforms and suggest that the theory of a functional P4 withdrawal may also be relevant in the amnion as well as the myometrium. The mRNA data (Figure 3.14) showed a significant decrease in PR-B levels from PTNL to TNL samples, whilst at the protein level, (Figure 3.17 & 3.18) there was a significant increase in the PR-A: PR-B ratio when comparing these samples together. The shift from PR-B dominance to PR-A dominance as has been reported in the myometrium with the onset of labour at term (117, 212, 265). Although significant differences were not observed at the mRNA or protein levels, in the amnion in the current study, there were trends towards this pattern of change. Idiopathic PTL samples displayed a significant shift in PR-A: PR-B ratio at the protein level (Figure 3.20). However, no other significant changes were observed in PR expression at the mRNA (Fig 3.15) or protein level (Figure 3.19 & 3.20) when comparing PTNL samples with preterm sub-types. In addition, no significant change in PR expression was observed between preterm twin gestations (Figure 3.16, 3.19 & 3.20).

As mentioned above, given that low PR levels have previously been reported characterised in the human amnion (178, 266), the non-significant changes may be due to the low levels of PR present in amnion tissue, raising the question of how important a role PR plays in the amnion. Some evidence has been presented for the

existence of PR-C (123), but later evidence suggests that this isoform did not exist (125).

Mesiano's group have demonstrated opposing actions of PR-A and PR-B *in vitro*; PR-B was able to stimulate genes that were anti-inflammatory whilst PR-A worked in the reverse, up-regulating pro-inflammatory genes in human myometrial cells (173). In addition, PR-A's effect on inhibiting PR-B activity has also been documented in the human amnion *in vitro* (178). This highlights that the presence and function of PR may be dependent on tissue type as well as aetiology of labour.

3.8.3 GR in term and PTL

No significant changes were observed at the mRNA or protein levels of GR between PTNL and TNL, at the onset of labour or as labour progressed through to TEsL. In addition, no significant changes were observed between each type of PTL (Figure 3.21-23). Limited studies exist demonstrating GR levels in human amnion tissue at similar stages of labour. However, in the nuclei of amnion cells, GR protein was highly expressed at both term and preterm (267). Findings from placental tissues showed no difference in GR levels at term or preterm (268). This differed from *in vitro* work in myometrial cells (119), which proposes a role for GR in facilitating P4 signalling. Given there were also trends towards increases in GR protein levels in polyhydramnios samples and chorioamnionitis samples (Figure 3.29) it suggests that the role of GR in the context of labour in the human amnion requires further investigation.

3.8.4 Chaperone and co-chaperone proteins in term and PTL

Limited data exists for the presence of chaperone and co-chaperone proteins in term and PTL amnion tissue. The data presented here demonstrate non-significant trends towards an increase in HSP 70 mRNA (Figure 3.25) in labouring samples at term compared with TNL. At the protein level, HSP 70 did not display any significant changes but non-significant increases were observed with gestation and again with the onset of labour (Figure 3.26). In term and PTL serum and placental tissue samples, HSP 70 levels increased, particularly in chorioamnionitis samples (269) whilst data from amniotic fluid show similar results (270). Thus, the above findings from the current study agree with the existing literature and suggest an increase as labour progresses. With PTL, there were significant decreases of HSP70 in polyhydramnios and abruption samples at the protein level (Figure 3.27). These findings oppose what

was found in serum and placental tissue samples, which showed elevated HSP70 (82). HSP 70 has been characterised both intracellularly (anti-inflammatory) and extracellularly, displaying contrasting properties (271, 272). For this reason, perhaps the role of HSP70 is dependent on tissue as well as underlying cause for PTL.

HSP 90 not only plays a role as a chaperone to PR but also has been implicated in the context of inflammation and has been linked with NF- κB (273). In human myometrial tissue from the group's tissue bank, HSP 90 protein levels were significantly increased in PTLC samples (Georgiou et al., unpublished observations). This observed difference correlated with increased HSP 90 mRNA levels observed in a ewe model of PTL (274) and taken together suggested that perhaps the increase in myometrial tissue in chorioamniotis samples, was a result of infection-associated inflammation. These results differ to the data presented here where protein levels were significantly lower in chorioamnionitis (Figure 3.30). The fact that tissue taken from myometrial and amnion tissue were from the same patients from the group's tissue bank add to the theory that differing aetiologies of PTL present themselves differently depending on tissue type.

Data presented here showed FKBP51 mRNA significantly increased (Figure 3.24) in samples with abruption and showed increased trends in polyhydramnios samples when compared with PTNL samples. These correlate with findings from corresponding myometrial tissue of the same patient (Georgiou *et al.,* unpublished observations), which found similar increased FKBP5 protein levels in PTL subtypes including chorioamnionitis. Further work is required to determine the role of FKBP5 in the context of PTL of varying aetiology. However, FKBP51 has been shown to boost NF-κB action in various models of cancer (275-277) and this fact has been used postulate a link between infection-associated inflammation, often present in cases of PTL. Group findings obtained using snap-frozen myometrial tissue could not be reproduced in vitro using *ex vivo* myometrial explants (Georgiou *et al*., unpublished observations). This highlights the complexity and importance of defining the role of such chaperones to aid in our understanding of PTL.

Detailed characterisations of these chaperones have not been carried out in human amnion tissue of varying aetiology until now. These findings provide a basis for further investigation into the roles each play in the human amnion.

3.8.5 Co-regulators in term and PTL

Other studies have noted no significant differences in SRC-1 levels in term labour in myometrial samples (278) nor any differences in the amnion (267). In this study, only SRC-1 and SRC-2 could be detected at the mRNA level where no difference was seen as labour progressed (Figure 3.31). At the protein level (Figure 3.32) SRC-1 levels were higher in TNL samples compared with PTNL samples. In addition, TEsL showed a significant increase in SRC-1 compared with TNL. These differences suggest some involvement during labour in the amnion, although further investigation is required to determine its precise role. Interestingly though, at the protein level in the context of chorioamnionitis, SRC-1 dropped significantly (Figure 3.33). There are no other reports of such a finding in the literature in the context of chorioamnionitis, a similar decline has been observed in the myometrium (unpublished data Georgiou *et al*).. No significant changes were observed in SRC-1 between preterm twin gestations. These findings suggest that the expression changes with not only the onset of labour but also due in part, to the aetiology of the PTL.

SRC-2 at the mRNA level significantly decreased in TEsL compared to TNL (Figure 3.34). But no significant differences were observed between PTL subtypes and PTNL samples. Though bands were faintly present for SRC-2 at the protein level, they were below the level of detection for densitometric analysis, thus only SRC-1 was quantified at the protein level. Other studies however, noted no change in amnion or choriodecidua in SRC-2 or 3 (267). In contrast to this however, SRC-2 and SRC-3 nuclear localisation decreased as labour approached, in the myometrium (278). In addition, mice deficient of both SRC-1 and 2 demonstrated delayed onset of parturition (279) and therefore suggests a role for both in the onset of labour, which remains to be determined in the amnion.

Neither SMRT nor NCoR were detectable at the protein level (Figure 3.36 & Figure 3.37). In a myometrial study using the group's tissue bank, SMRT could also not be detected in western analysis (unpublished data Georgiou *et al*). The lack of both presenting at the protein level could also be due to tissue degradation through processing and handling in the laboratory. In addition, it may suggest that neither have a prominent role in the amnion during parturition.

3.8.6 Summary of Section B

Addressing the initial aims for this study:

- Can consistent patterns be observed in steroid activity?
- How does expression of PR (A/B), co-regulators and chaperones change in term and PTL?

The ratio of PR-A and PR-B is often taken as an indicator to mark the adjustment from uterine quiescence to activation. These data suggest that though this may be the case, further work is required to fully characterise PR's role in the human amnion. In addition, the data presented shows the expression pattern of nuclear receptor mediators in the human amnion. The observed changes in expression differed depending on the type of term and PTL. In addition, insignificant changes in PR levels may indicate that these nuclear receptor coactivators may be working independently of PR. These data elude to the complex role that nuclear receptors and coregulators may play in the human amnion during human parturition.

4. The impact of Progesterone on the inflammatory and stretch-induced response.

4.1 Introduction

4.2 The role of progesterone

P4 is an important steroid hormone, which plays a key role in reproductive function (280, 281). Pivotal research by Csapo demonstrated that as circulating P4 levels decreased, uterine sensitivity to prolabour mediators increased, thus highlighting the importance of P4 in early human pregnancy (116, 282, 283). Furthermore, administration of mifepristone (RU486), the P4 and glucocorticoid antagonist, induced labour (151), emphasises the importance of P4 in the maintenance of early and established pregnancy.

4.3 P4 as an anti-inflammatory agent

Spontaneous PTL is most commonly associated with inflammation (65). P4 remains the only drug thought to reduce the risk of spontaneous PTL in high-risk singleton pregnancies by up to 40% (200, 284); however, its ability to reduce the risk of PTL has recently been questioned (44). In higher order pregnancies however, P4 administration does not reduce the risk of preterm labour (285-288) possibly suggesting that the onset of labour may be different in singleton and in multiple pregnancies.

P4 is thought to exert its anti-inflammatory effect by repressing NFκB through its interaction with PR-B (172, 266, 289). Further, P4 has also been shown to impede myometrial sensitivity to oxytocin and PGs (116, 285, 290). PR-B is thought to be responsible for facilitating most of P4's action, however studies have shown that PR-A may also be involved (117) indicating that P4 responsiveness may be dependent on receptor subtype. A shift between PR sub-units occurs with labour onset; there is a shift towards PR-A dominance as PR-A expression increases in the myometrium. (178, 212). In the amnion, it has been shown that PR-A has the ability to also inhibit PR-B activity (21). The suppression of genomic P4 action may be due to the change in sub-type ratio and may result in a withdrawal of P4 activity (265) as suggested by research in primates and human (208, 212, 265, 291).

Alternatively, myometrial relaxation could also be accounted for by non-genomic P4 actions(160). Uterine stretch increases with advancing gestation and aside from its role in maintaining uterine quiescence; P4 has also been shown to stimulate hyperplasia and hypertrophy in the myometrium (49-51, 292). Excessive uterine distension observed in multiple pregnancies and in women with polyhydramnios has been linked with a heightened risk of PTL (293-295). Experiments simulating excessive uterine stretch have shown that doing so may result in premature initiation of labour (296, 297).

In addition, it has been suggested that the human uterus changes in response to uterine stretch meaning that its receptivity to P4 action may also be affected. It has been speculated that this trait may be a contributing factor to functional P4 withdrawal before labour onset. However, work in myometrial cells have also suggested that uterine stretch may also be unaffected by P4 action (59)
Previously, our group has found that *in vitro* mechanical stretch elevates COX-2 expression in both myometrial and amnion cells and induces OTR expression independently of MAPK (57, 58, 60, 61, 298, 299). It has been shown that P4 does not have the ability to inhibit stretch-induced COX-2 and IL-8 expression in myometrial cells (59). However this has not been investigated in human amnion cells and therefore will be explored in this chapter.

4.4 Enhancing P4's anti-inflammatory effect

Given the limitations of P4 for the prevention of PTL, alternatives are also being explored, including enhancing P4 action. The second messenger, cAMP has the potential to enhance P4 action as well as having anti-inflammatory properties and being able to promote myometrial relaxation, acting via protein kinase A (PKA) (191, 193). Cyclic AMP enhances agonist bound-PR activity and can convert RU486 from being a PR antagonist into a partial agonist (300, 301).

However, mechanisms of cAMP activity remain poorly understood. Though research has highlighted cAMP's ability to enhance P4 activity (205, 302, 303) the reverse has also been observed (205, 304) suggesting that cAMP may have varying effects

depending on the prevailing environment. The transcriptional activity of nuclear receptors, such as GR, is susceptible to cAMP (305-307). As of yet, cAMP administration in the context of P4 has been investigated in the myometrium and endometrium (205), but whether the combination has enhanced anti-inflammatory characteristics in the human amnion remains to be determined.

4.5 Chapter Aims

This chapter investigated the effect of P4 administration in amnion cells.

1. Whether P4 administration was effective in reducing stretch-induced gene expression.
2. Whether P4 has the ability to repress IL-1β induced prolabour expression in human amnion cells in the presence and absence of *in vitro* mechanical stretch.
3. Whether P4's efficacy may be enhanced through the addition of a cAMP agonist (forskolin).

4.6 Results

4.7 The impact of stretch on COX-2 expression in human amnion cells

Human amnion cells were exposed to 11% mechanical stretch for 15, 30, 60, 120 240, 360, 720 and 1440 minutes. COX-2 mRNA expression increased significantly at 60-1440 minutes (Figure 4.1A) and increased significantly at all time points at the protein level (Figure 4.1B). IL-6 mRNA also significantly increased from 240 minutes through to 1440 minutes (Figure 4.2C). Though there was a trend towards increased TNFα and IL-8 expressions of both the mRNA (Figure 4.2) and protein (Figure 4.3) level, no significant changes were seen.

In addition to evaluating the levels of these cytokines, the chemokines: CXCL1, CXCL2 were also observed. As found in Chapter 3, tissue lysate from labouring preterm twin gestations showed increased TNFα, CXCL1, CXCL2 when compared to non-labouring samples of the same gestational age. Given that cases of twins are often linked with stretch-induced preterm labour, it was hypothesised that human amnion cells subjected to *in vitro* stretch may also show similar increased cytokine-chemokine profiling. Unfortunately though, when cell supernatant was quantified using a multiplex assay for CXCL1 and CXCL2, both were too dilute and beyond detection meaning no accurate comparisons could be made.

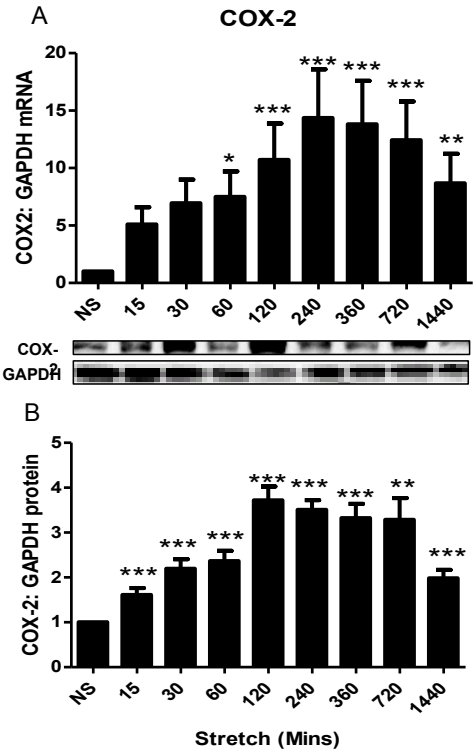

Figure 4.1 COX-2 mRNA and protein expression levels in human amnion cells in response to *in vitro* stretch. Human amnion cells were isolated as described in *Materials and Methods* and subjected to 11% *in vitro* stretch for up to 1440 minutes. **A:** RNA was extracted, cDNA synthesised and qPCR ran for COX-2 and normalised to GAPDH**. B:** In a separate experiment, human amnion cells were isolated and stretched as before but analysed for protein analysis. Protein was extracted and quantified and western blotting for COX-2 was performed. A representative western blot is shown. Results were corrected to unstretched controls. Normality was tested using a Kolmogorov-Smirnov test followed by an ANOVA and Bonferroni's multiple comparison *post-hoc* test was used. * $p<0.05$ versus NS control ; ** $p<0.01$ versus NS control; *** $p<0.001$ versus NS control. [n= 8].

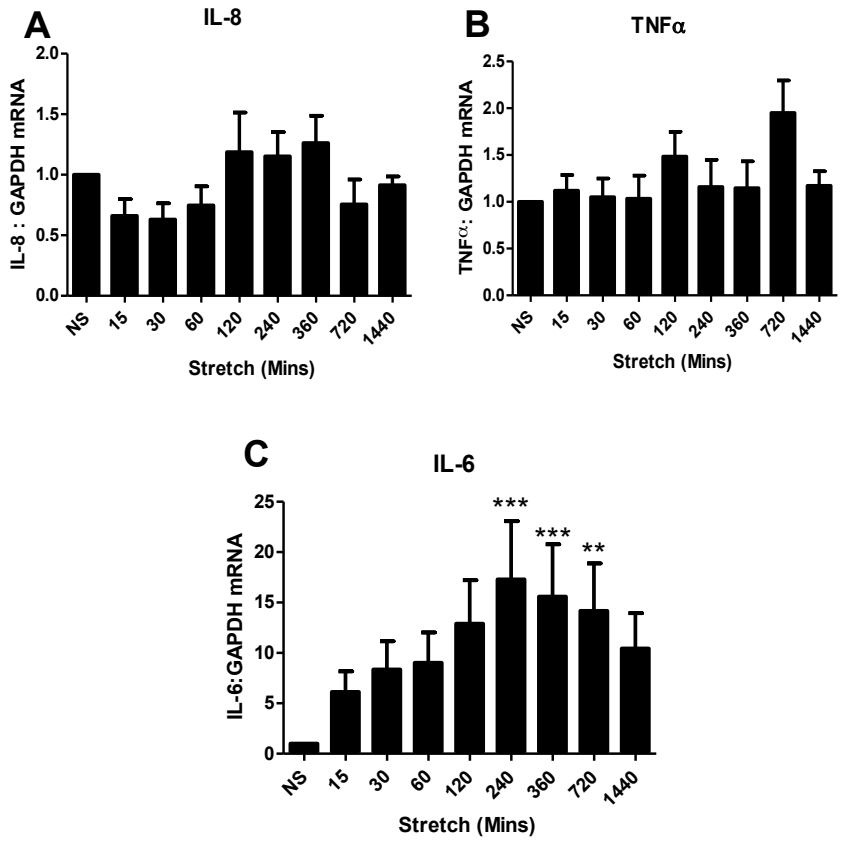

Figure 4.2 IL-8, TNF α and IL-6 mRNA expression levels in human amnion cells in response to *in vitro* stretch. Human amnion cells were isolated as described in *Materials and Methods* and subjected to 11% *in vitro* stretch for up to 1440 minutes. RNA was extracted, cDNA synthesised and qPCR ran for **A:** IL-8 and **B:** TNF α **C: IL-6** Results were corrected to upstretched controls. Normality was tested using a Kolmogorov-Smirnov test followed by an ANOVA and Bonferroni's multiple comparison *post-hoc* tests. ** $p<0.01$ versus NS control ***; $p<0.001$ versus NS control [n= 8].

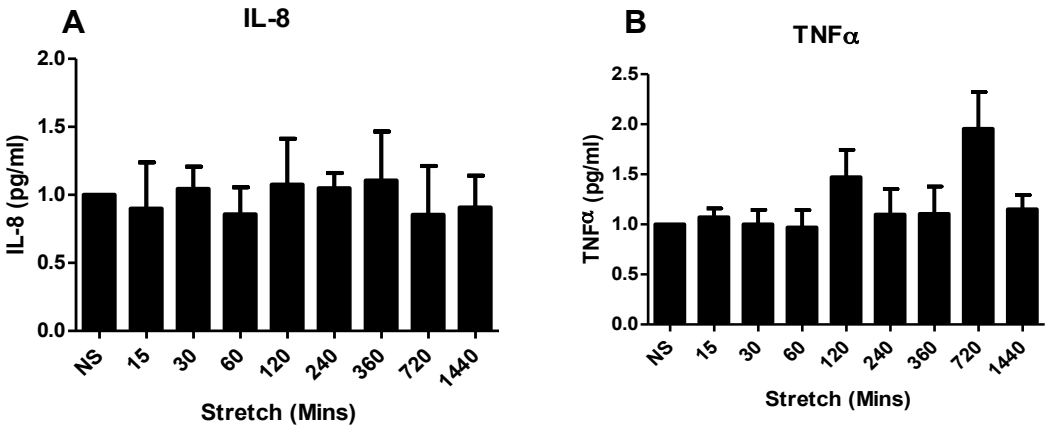

Figure 4.3 IL-8 and TNF α mRNA levels in human amnion cells in response to *in vitro* stretch. Human amnion cells were isolated as described in *Materials and Methods* and subjected to 11% *in vitro* stretch for up to 1440 minutes. Cell supernatant was used to run human Bio-Plex © Pro™ cytokine-chemokine assays for **A:** IL-8 and **B:** TNF α as per the manufacturer's protocol. Normality was tested using a Kolmogorov-Smirnov test followed by an ANOVA and Bonferroni's multiple comparison *post-hoc* test was used [n= 8].

4.8 Does P4 affect stretch-induced gene expression? Can P4 action be enhanced by the addition of the cAMP agonist, forskolin?

As mentioned previously, P4 administration is used clinically to prevent the onset of PTL. Human amnion cells were treated with doses of 1 and 10 μM of P4 and 10 μM Forskolin for 6 hours, prior to stretch treatment (2 hours). The stretch time course demonstrated that COX-2 mRNA and protein both increased (Figure 4.1). Since expression increased after 2 hours at both the mRNA level and protein level, the 2 hour (120 minute) time point was used for all other stretch related experiments in this chapter (Figure 4.1B).

P4 treatment both alone and in combination with cAMP agonist, forskolin, was used to determine whether stretch-induced changes in gene expression could be reduced *in vitro*. Neither 1 μM nor 10uM alone P4, or in combination with 10-μM forskolin was able to repress stretch-induced COX-2 expression. The combined treatment showed no difference in efficacy in preventing stretch-induced changes at neither mRNA nor protein level (Figure 4.4). In fact the combination of 1 μM and 10 μM with forskolin in stretched cells resulted in significantly higher COX-2 mRNA and protein levels.

Similarly, neither P4 alone (1μM or 10μM), nor in combination with forskolin had any effect in reducing stretch-induced IL-6 (Figure 4.5 A & B) TNFα mRNA (Figure 4.5E & F) or protein (Figure 4.5A & B).

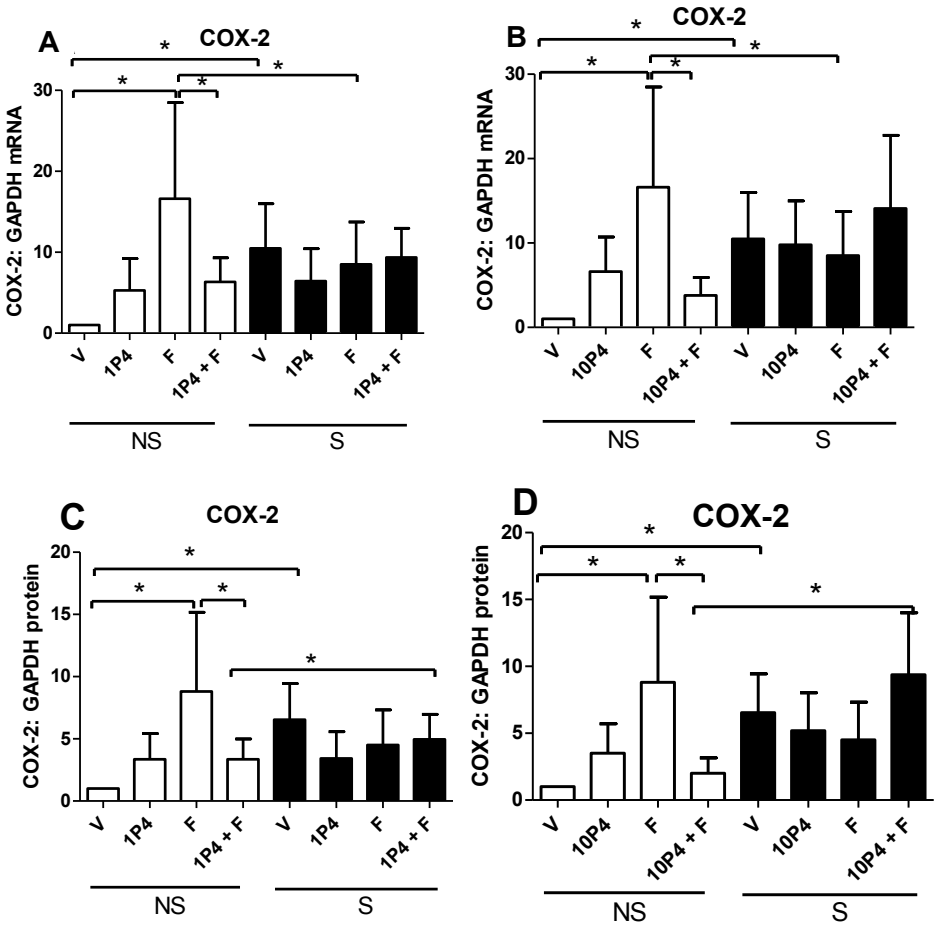

Figure 4.4 Stretch–induced COX-2 mRNA and protein expression levels in human amnion cells were unchanged following P4 and cAMP treatment. Human amnion cells were isolated as described in *Materials and Methods*. They were treated for 6 hours with ethanol vehicle, P4 (1μM or 10μM) and F (10μM) and subjected to 11% *in vitro* stretch for 2 hours. **A & B:** RNA was extracted, cDNA synthesised and qPCR ran for COX-2 and normalised to GAPDH. **C & D:** The experiment was repeated for protein analysis. Protein was extracted and quantified and western blotting for COX-2 was performed and normalised to GAPDH. Results were corrected to unstretched controls. Normality was tested using a Kolmogorov-Smirnov test. For normally distributed data an ANOVA followed by Bonferroni's multiple comparison *post-hoc* test was used; data were compared using Friedman's Test, with a Dunn's Multiple Comparisons post hoc test for data that were not normally distributed. Wilcoxon matched pairs test for data that were not normally distributed and paired *t* test for data that were normally distributed when comparing data individual stretched samples vs. unstretched samples of the same treatment e.g. NS V vs. S V. *$p<0.05$ versus NS control [n= 8].

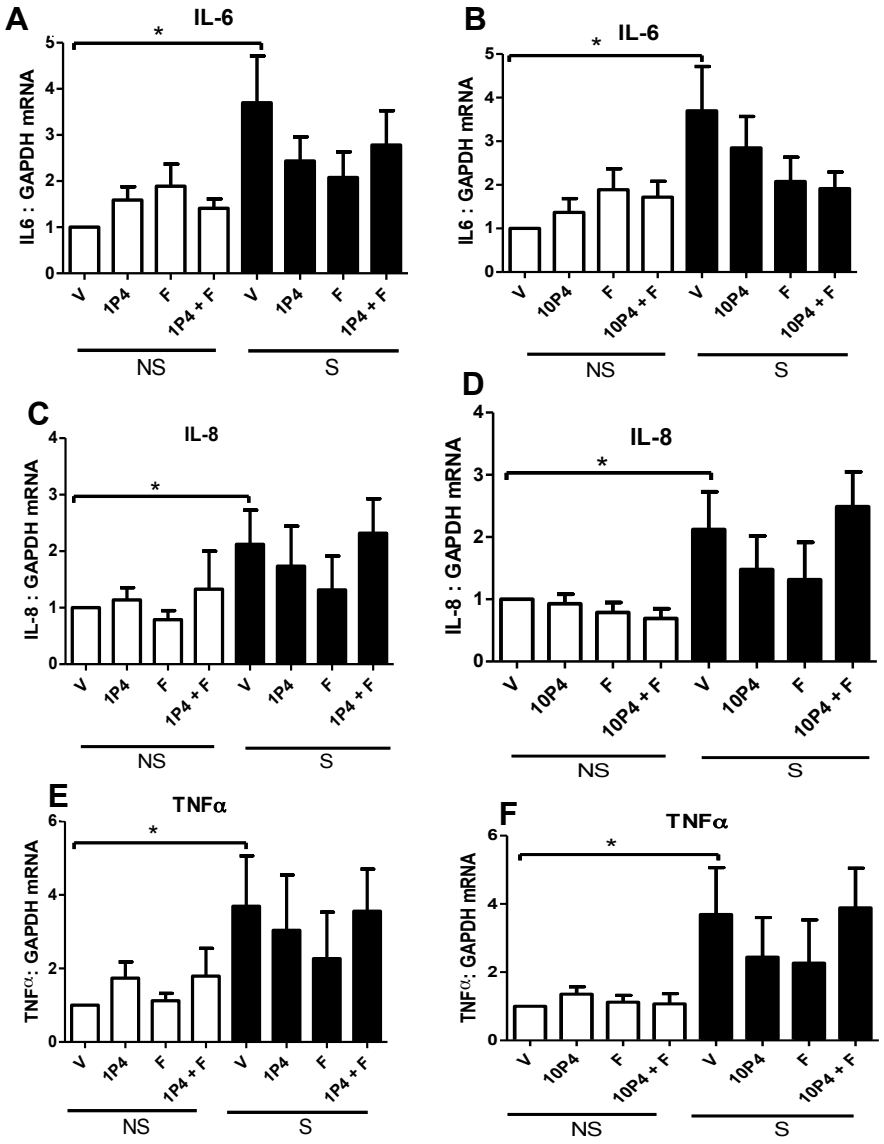

Figure 4.5 Stretch–induced mRNA expression levels in human amnion cells were unchanged following P4 and cAMP treatment. Human amnion cells were isolated as described in *Materials and Methods*. They were treated for 6 hours with ethanol vehicle, P4 (1μM or 10μM) and F (10μM) and subjected to 11% *in vitro* stretch for 2 hours. RNA was extracted, cDNA synthesised and qPCR ran for A & B:IL-6 C &D: IL-8 and E & F: TNF α and normalised to GAPDH. Results were corrected to unstretched controls. Normality was tested using a Kolmogorov-Smirnov test. For normally distributed data an ANOVA followed by Bonferroni's multiple comparison *post-hoc* test was used; data were compared using Friedman's Test, with a Dunn's Multiple Comparisons post hoc test for data that were not normally distributed. Data were compared using Wilcoxon matched pairs test for data that were not normally distributed and paired *t* test for data that were normally distributed when comparing data individual stretched samples vs. unstretched samples of the same treatment e.g. NS V vs. S V. * $p<0.05$ versus NS control [n= 8].

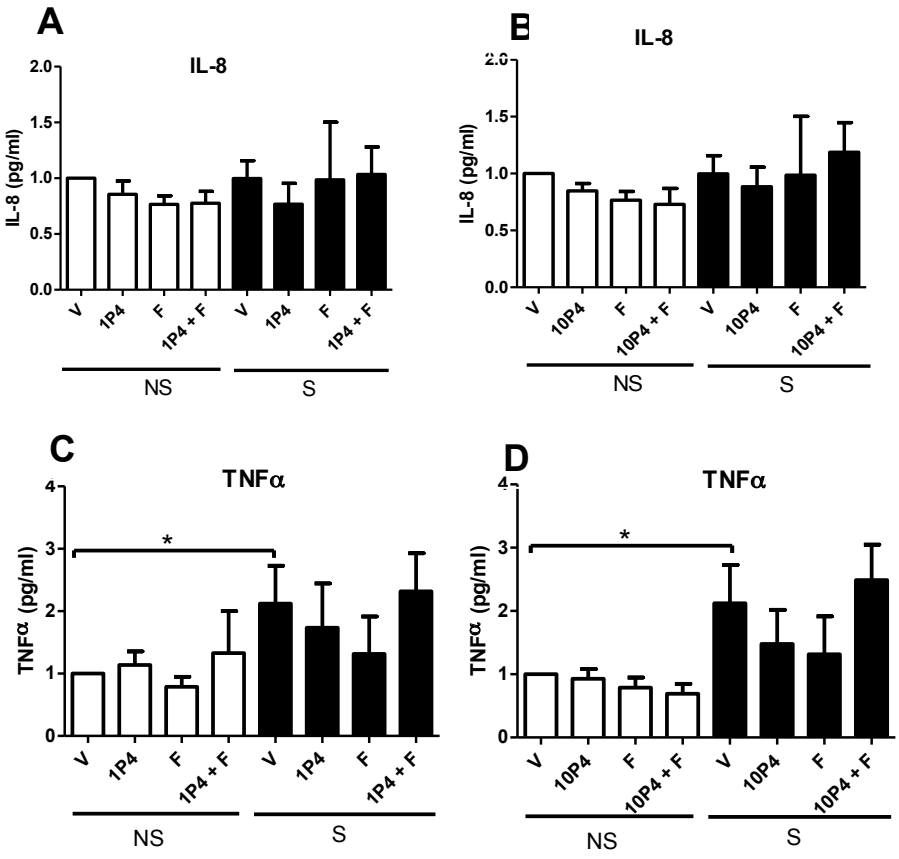

Figure 4.6 Stretch–induced changes to IL-8 and TNFα expression levels in human amnion cells were unchanged following P4 and cAMP treatment. Human amnion cells were isolated as described in *Materials and Methods*. They were treated for 6 hours with ethanol vehicle, P4 (1μM or 10μM) and F (10μM) and subjected to 11% *in vitro* stretch for 2 hours. Cell supernatant was used to run human Bio-Plex © Pro™ cytokine-chemokine assays for **A. & B:** IL-8 and **C&D:** TNFα. And were run as per the manufacturer's protocol. Normality was tested using a Kolmogorov-Smirnov test. For normally distributed data an ANOVA followed by Bonferroni's multiple comparison *post-hoc* test was used; data were compared using Friedman's Test, with a Dunn's Multiple Comparisons post hoc test for data that were not normally distributed. Wilcoxon matched pairs test for data that were not normally distributed and paired *t* test for data that were normally distributed when comparing data individual stretched samples vs. unstretched samples of the same treatment e.g. NS V vs. S V. * $p<0.05$ versus NS control [n= 8].

4.9 Does *in vitro* mechanical stretch alter P4 responsive gene expression activity?

Several genes have been identified as being P4 responsive. From microarray analysis and validation (see appendix for Gene Accession) both FKBP5 (Figure 4.8) and HSD11β1 (Figure 4.7) were up regulated by P4. The effect of *in vitro* stretch on P4 responsive genes was investigated in order to ascertain whether P4 action in the presence of mechanical stretch utilises a different mechanistic pathway. Human amnion cells were treated for 6 hours with P4 (1 μM and 10 μM) both alone and then in combination with forskolin. Stretch did not alter P4 induced increase in 11ßHSD mRNA or FKBP5 mRNA involving either dose of P4. There was also no difference observed between 11ßHSD or FKBP5 mRNA levels involving: 1 μM P4 alone and 1 μM P4 + forskolin. However, when human amnion cells were treated with the combination of 10μM P4 + F, in the presence of stretch, 11ßHSD and FKBP5 mRNA levels increased significantly when compared with unstretched cells treated in the same way. A significant increase in FKBP5 and 11ßHSD mRNA was also observed between stretched samples: 10P4 μM and 10P4 μM + F. The addition of forskolin alone significantly increased 11ßHSD mRNA (Figure 4.8) or FKBP5 mRNA in stretched samples compared with unstretched controls with the same treatment (Figures 4.7).

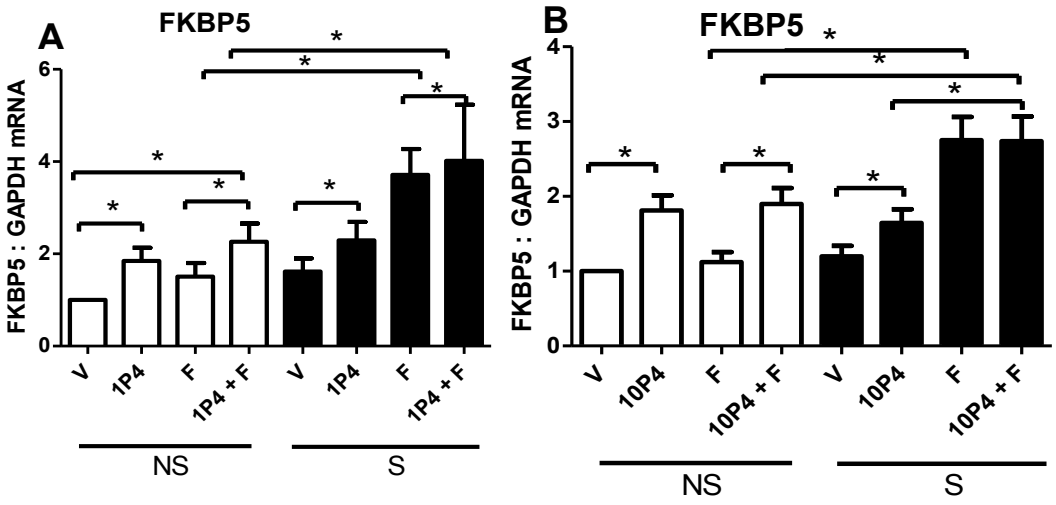

Figure 4.7 The effect of *in vitro* stretch on P4 and cAMP action in human amnion cells Human amnion cells were isolated as described in *Materials and Methods*. They were treated for 6 hours with ethanol vehicle, P4 (1μM or 10μM) and F (10μM) and subjected to 11% *in vitro* stretch for 2 hours. RNA was extracted, cDNA synthesised and qPCR ran for **A & B:** FKBP5 and normalised to GAPDH. Results were corrected to unstretched controls. Normality was tested using a Kolmogorov-Smirnov test. For normally distributed data an ANOVA followed by Bonferroni's multiple comparison post hoc test was used; data were compared using Friedman's Test, with a Dunn's Multiple Comparisons post hoc test for data that were not normally distributed. Wilcoxon matched pairs test for data that were not normally distributed and paired *t* test for data that were normally distributed when comparing data individual stretched samples vs. unstretched samples of the same treatment e.g. NS V vs. S V. * $p<0.05$ versus NS control [n= 8].

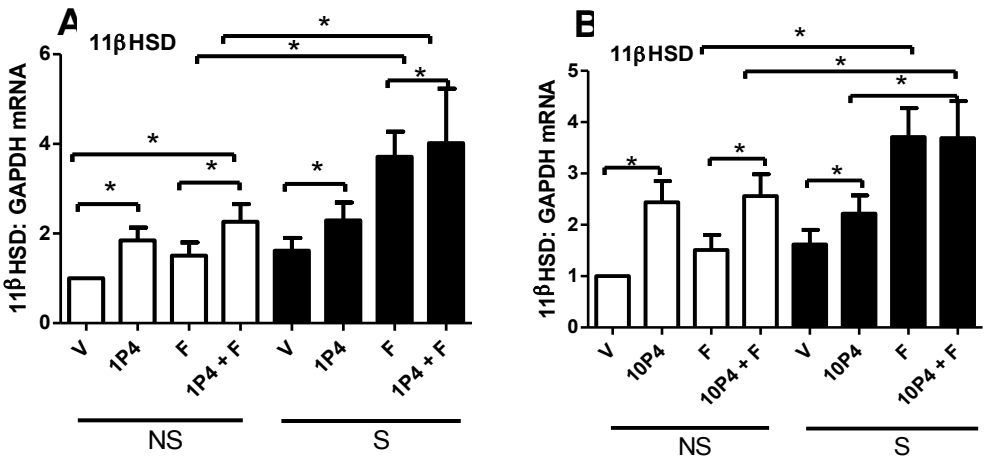

Figure 4.8 The effect of *in vitro* stretch on P4 and cAMP action in human amnion cells
Human amnion cells were isolated as described in *Materials and Methods*. They were treated for 6 hours with ethanol vehicle, P4 (1μM or 10μM) and F (10μM) and subjected to 11% *in vitro* stretch for 2 hours. RNA was extracted, cDNA synthesised and qPCR ran for **A & B:** 11ßHSD and normalised to GAPDH. Results were corrected to unstretched controls. Results were corrected to unstretched controls. Normality was tested using a Kolmogorov-Smirnov test. For normally distributed data an ANOVA followed by Bonferroni's multiple comparison post hoc test was used; data were compared using Friedman's Test, with a Dunn's Multiple Comparisons post hoc test for data that were not normally distributed. Wilcoxon matched pairs test for data that were not normally distributed and paired *t* test for data that were normally distributed when comparing data individual stretched samples vs. unstretched samples of the same treatment e.g. NS V vs. S V. * $p<0.05$ versus NS control [n= 8].

4.10 Can P4's anti-inflammatory action be enhanced in order to repress IL-1β-induced pro-labour expression in human amnion cells in the presence and absence of in vitro stretch?

4.10.1 IL-1β-induced pro-labour expression

Given that spontaneous PTL is associated with infection and inflammation (308) it was important to create an *in vitro* environment, which mimicked such inflammatory conditions. For this reason, IL- β treatment (1ng/ml, following a colleague's dose response work using human amnion cells) was used to construct a time course experiment (Figure 4.9). This confirmed that IL-1β treatments up-regulated COX-2 mRNA and protein expression at 2, 4, 6 hours and mRNA expression at 24 hours. As a result of these findings, subsequent IL-1β treatments were carried out for 6 hours.

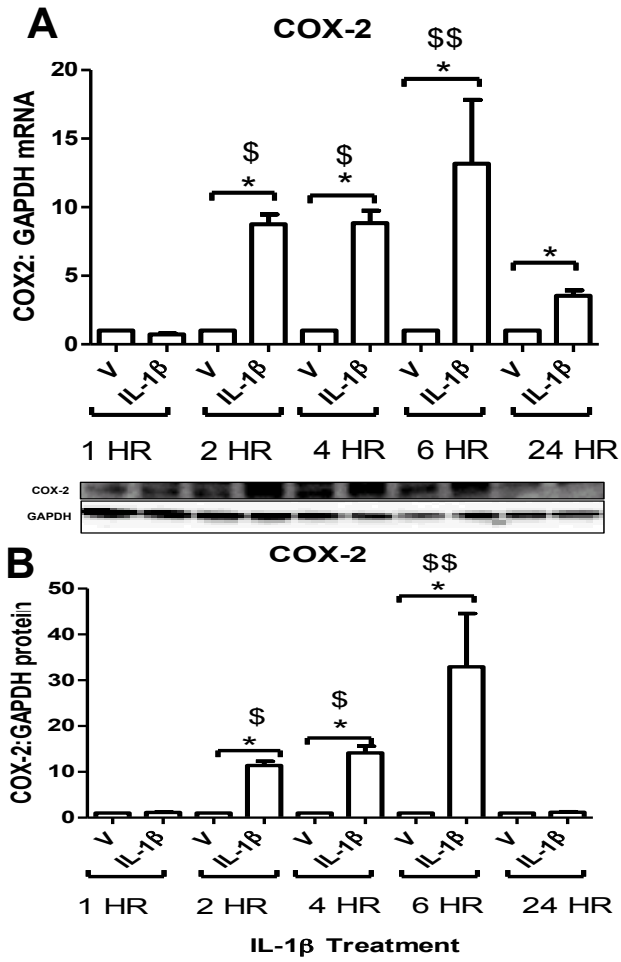

Figure 4.9 COX-2 mRNA and protein expression levels in human amnion cells in response to IL-1 β treatment. Human amnion cells were isolated as described in *Materials and Methods* and treated with IL-1 β (1ng/ml) for up to 24 hours. **A:** RNA was extracted, cDNA synthesised and qPCR ran for COX-2 and normalised to GAPDH**. B:** In a separate experiment, human amnion cells were isolated and stretched as before but analyzed for protein analysis. Protein was extracted and quantified and western blotting for COX-2 was performed (Results were corrected to unstretched controls. Normality was tested using a Kolmogorov-Smirnov test followed by an ANOVA and Bonferroni's multiple comparison *post-hoc* tests was used. * $p<0.05$ versus NS control; \$ $p<0.05$ versus *(IL-1 β after 1 hour*; \$\$ $p<0.01$ versus *(IL-1b after 1 hour)*; [N= 8].

4.10.2 Can P4 reduce IL-1β-induced pro-labour expression in human amnion cells in the presence and absence of *in vitro* stretch?

The ability of P4 (1μM and 10μM) in repressing IL-1β-induced prolabour expression of COX-2, OTR, IL-6, IL-8, and TNFα was investigated. IL-1β stimulation as previously demonstrated in (Figure 4.9) resulted in increased COX-2 mRNA and protein levels (Figure 4.10) which could be reduced by both 1μM and 10μM P4, in the absence of stretch at both the mRNA and protein levels. In the presence of stretch, COX-2 mRNA and protein increased compared to unstretched controls. IL-1β treatment also resulted in increased COX-2 mRNA and protein expression, compared to untreated stretched vehicle. However, there was no significant difference in COX-2 expression between unstretched and stretched IL-1β treated samples. The addition of 1 μM and 10 μM P4 administration did not alter any stretch-induced and IL-1β-stimulated increases in COX-2 mRNA or protein levels.

Similar patterns of expression were seen for OTR mRNA and protein (Figure 4.11) as well as IL-6 mRNA (Figure 4.12 A &B); IL-1β treatment in both stretched and unstretched samples increased the gene of interest and in the absence of stretch, both doses of P4 (1 μM and 10 μM) were able to repress this change. They had no effect on stretched samples in reducing either stretch/inflammation-induced changes however. Although TNFα mRNA (Figure 4.12 E & F) and protein expression (Figure 4.13 C & D) followed a similar pattern at the protein level in the presence and absence of stretch, at the mRNA level, only 10 μM was able to repress IL-1β induced TNFα expression.

IL-1β treatment in the presence of stretch also resulted in increased IL-8 mRNA (Figure 4.12 C&D) and protein (Figure 4.13 A &B). In the absence of stretch, both doses of P4 (1 μM and 10 μM) were able to repress this change at the mRNA level. Interestingly, the protein levels showed no stretch-induced change (Figure 4.13) and also showed that P4 at both doses had no significant effect, despite a trend towards a repression.

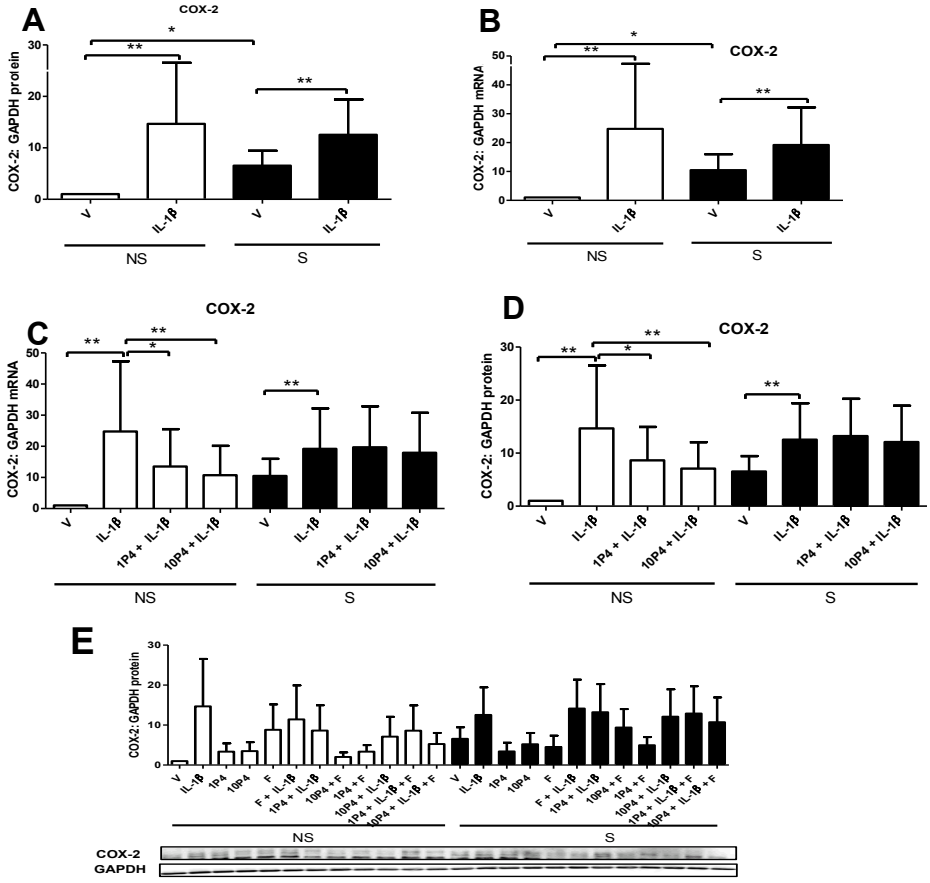

Figure 4.10 P4 treatment is able to reduce IL-1 β induced COX-2 mRNA and protein expression levels in human amnion cells in the absence of *in vitro* stretch. Human amnion cells were isolated as described in *Materials and Methods*. They were subjected to 11% *in vitro* stretch for 2 hours and treated for 6 hours with ethanol vehicle, P4 (1μM or 10μM) and IL-1 β (1ng/ml). **A & C.**
RNA was extracted, cDNA synthesised and qPCR ran for COX-2 and normalised to GAPDH. **B, D, E.** Protein was extracted, quantified and western blotting for COX-2 was performed and normalised to GAPDH. **E.** A representative blot is shown with all data points. Normality was tested using a Kolmogorov-Smirnov test. **A & B:** Wilcoxon matched pairs test for data that were not normally distributed and paired *t* test for data that were normally distributed. They were also used for **C & D** when comparing data individual stretched samples vs. unstretched samples of the same treatment e.g. NS V vs. S V. **C & D:** For normally distributed data an ANOVA followed by Bonferroni's multiple comparison post hoc test was used; data were compared using Friedman's Test, with a Dunn's Multiple Comparisons post hoc test for data that were not normally distributed. * $p<0.05$; ** $p<0.01$ [N= 8].

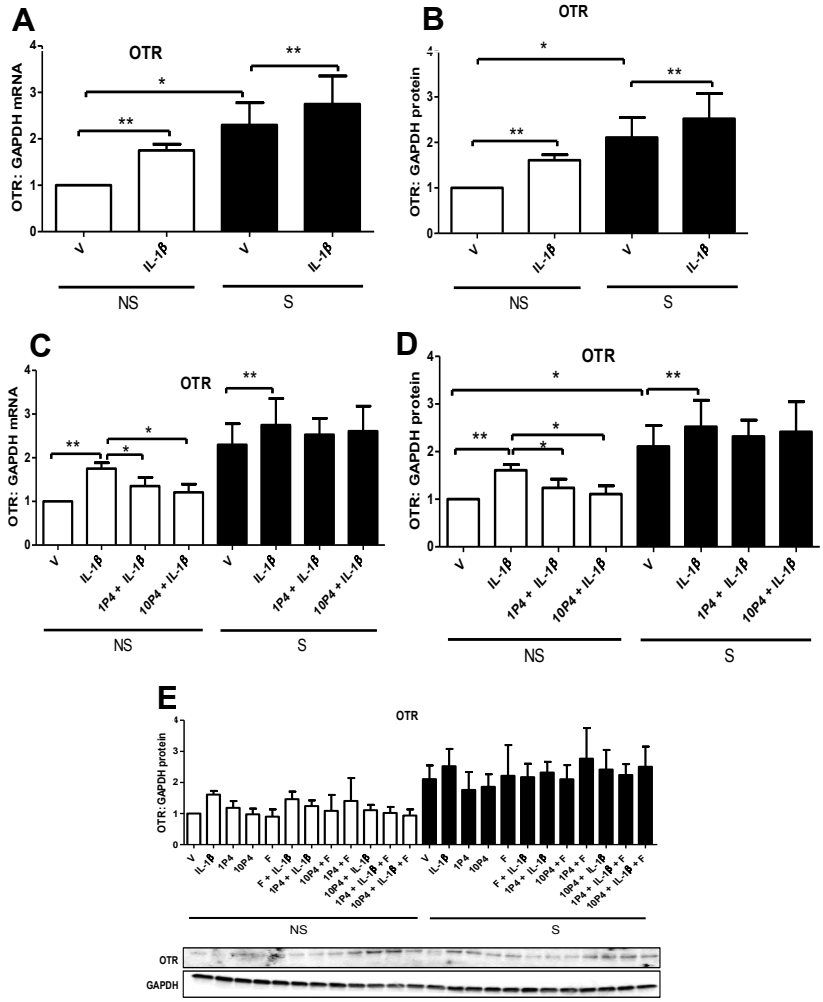

Figure 4.11 P4 treatment is able to reduce IL-1β -induced OTR mRNA and protein expression levels in human amnion cells in the absence of *in vitro* stretch. Human amnion cells were isolated as described in *Materials and Methods*. They were subjected to 11% *in vitro* stretch for 2 hours and treated for 6 hours with ethanol vehicle, P4 (1μM or 10μM) and IL-1β (1ng/ml). **A &C.**

RNA was extracted, cDNA synthesised and qPCR ran for OTR and normalised to GAPDH. **B, D, E.** Protein was extracted, quantified and western blotting for OTR was performed and normalised to GAPDH. **E.** A representative blot is shown with all data. Normality was tested using a Kolmogorov-Smirnov test. **A & B:** Wilcoxon matched pairs test for data that were not normally distributed and paired *t* test for data that were normally distributed. They were also used for **C & D** when comparing data individual stretched samples vs. unstretched samples of the same treatment e.g. NS V vs. S V. **C&D:** For normally distributed data an ANOVA followed by Bonferroni's multiple comparison post hoc test was used; data were compared using Friedman's Test, with a Dunn's Multiple Comparisons post hoc test for data that were not normally distributed. * $p<0.05$; ** $p<0.01$ [N= 8].

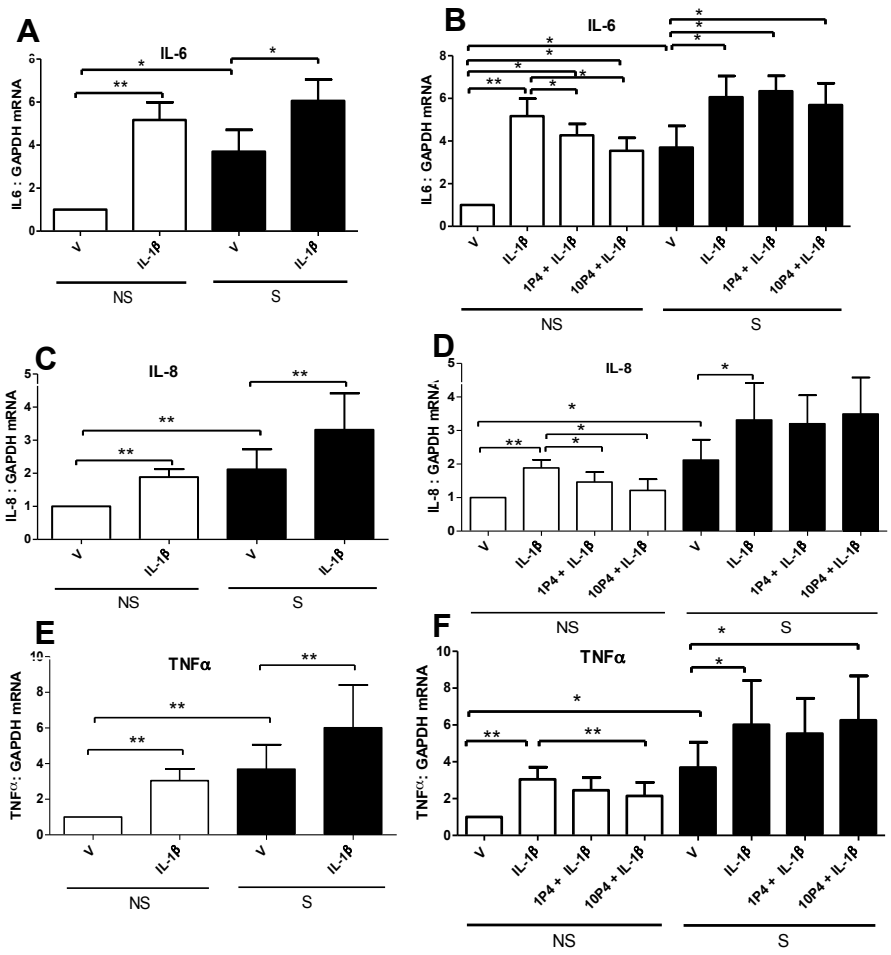

Figure 4.12 P4 treatment is able to reduce IL-1β -induced IL-6, IL-8, and TNFα mRNA and protein expression levels in human amnion cells in the absence of *in vitro* stretch. Human amnion cells were isolated as described in *Materials and Methods*. They were subjected to 11% *in vitro* stretch for 2 hours and treated for 6 hours with ethanol vehicle, P4 (1μM or 10μM), and IL-1β (1ng/ml). RNA was extracted, cDNA synthesised and qPCR ran for **A & B: IL-6; C&D: IL-8; E&F:** TNFα and normalised to GAPDH. Results were corrected to unstretched controls. Normality was tested using a Kolmogorov-Smirnov test. **A, C, E:** Wilcoxon matched pairs test for data that were not normally distributed and paired *t* test for data that were normally distributed. They were also used for **B, D, E** when comparing data individual stretched samples vs. unstretched samples of the same treatment e.g. NS V vs. S V; **B, D, E:** For normally distributed data an ANOVA followed by Bonferroni's multiple comparison post hoc test was used; data were compared using Friedman's Test, with a Dunn's Multiple Comparisons post hoc test for data that were not normally distributed. * $p<0.05$; ** $p<0.01$ [N= 8].

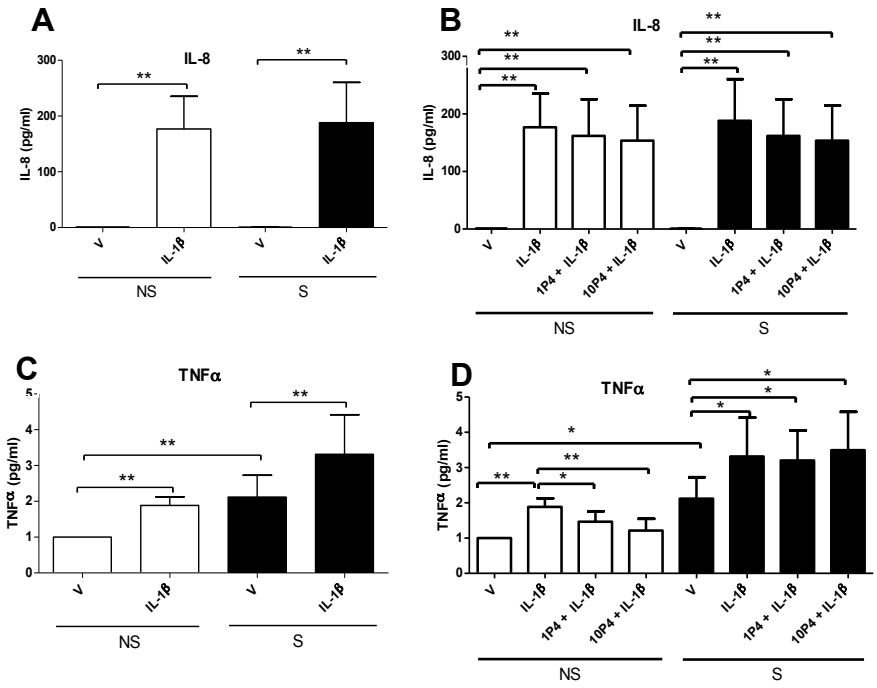

Figure 4.13 P4 treatment is able to reduce IL-1β -induced IL-8 and TNFa protein expression levels in human amnion cells in the absence of *in vitro* stretch. Human amnion cells were isolated as described in *Materials and Methods*. They were subjected to 11% *in vitro* stretch for 2 hours and treated for 6 hours with ethanol vehicle, P4 (1μM or 10μM) and IL-1β (1ng/ml). Cell supernatant was used to run human Bio-Plex © Pro™ cytokine-chemokine assays for **A. & B.** IL-8 and **C&D.** TNFα. And were run as per the manufacturer's protocol. Results were corrected to unstretched controls. Normality was tested using a Kolmogorov-Smirnov test. **A & C:** Wilcoxon matched pairs test for data that were not normally distributed and paired *t* test for data that were normally distributed. They were also used for **B & D:** when comparing data individual stretched samples vs. unstretched samples of the same treatment e.g. NS V vs. S V; **B & D:** For normally distributed data an ANOVA followed by Bonferroni's multiple comparison post hoc test was used; data were compared using Friedman's Test, with a Dunn's Multiple Comparisons post hoc test for data that were not normally distributed. * $p<0.05$; ** $p<0.01$ [N= 8].

4.10.3 Can P4's anti-inflammatory effect be enhanced when combined with a cAMP agonist?

One of the aims of this investigation was to determine whether the addition of cAMP agonist, forskolin could enhance P4's anti-inflammatory action in human amnion cells. Initial experiments found that a treatment of 10μM forskolin with either 1 μM or 10 μM was able to repress IL-1β-induced COX-2 mRNA expression. Therefore, this ability to enhance P4's ability to reduce IL-1β-induced pro-inflammatory expression was investigated further in the presence and absence of stretch
Forskolin treatment alone significantly increased COX-2 mRNA and protein in the absence of stretch (Figure 4.14 & 4.15) though no effect was seen in COX-2 expression in stretched samples. Forskolin was not able to significantly reduce COX-2 mRNA (Figure 4.14) or protein (Figure 4.15) despite a trend towards a decrease in unstretched samples. No significant change was observed in forskolin alone treatment in repressing either stretch-induced or IL-1β-stimulated COX-2 expression in stretched samples. Furthermore, the treatment of P4 (at either dose) + forskolin was unable to repress the IL-1β effect in stretched samples.

In unstretched samples P4 + forskolin was able to significantly reduce IL-1β stimulated COX-2 mRNA and protein expression. At the mRNA level, the efficacy of 1μM P4 treatment alone compared with 1μM P4 + forskolin in repressing IL-1β-induced COX-2 expression, showed no significant difference. Similarly, no such difference was seen between 10μM P4 treatment alone compared with 10 μM P4 + forskolin at the mRNA level (Figure 4.14). At the protein level (Figure 4.15), there was a significant difference in the repression of IL-1β driven COX-2 expression; treatment of 10μM + forskolin significantly decreased COX-2 protein levels further than 10 μM P4 treatment alone. However there was no additive effect of P4 + F for non-stretched cells.

OTR mRNA (Figure 4.16) and protein (Figure 4.17) expression followed a similar pattern to COX-2 in human amnion cells. The addition of forskolin to unstretched amnion cells resulted in significantly increased OTR mRNA and protein expression. No significant change was observed between vehicle and forskolin alone unstretched samples at either mRNA or protein level. Similarly, the addition of forskolin alone or

in combination with P4 had no effect in reducing either IL-1β induced or stretch-induced OTR increase.

Human amnion cells that were left unstretched however, showed a repression of IL-1β induced OTR expression at the mRNA level (Figure 4.16) when treated with the combination of 1 μM P4 + forskolin as well as 10 μM P4 + forskolin. There was no significant difference between the efficacy either dose of P4 treatment alone compared with the combined treatment of P4 + forskolin at the mRNA or protein level (Figure 4.17).

No significant increase was seen in IL-1β driven IL-6 expression when human amnion cells were treated with forskolin alone. However, there was a trend towards a decrease in IL-6 expression in stretched samples (Figure 4.18) Interestingly, forskolin alone was able to repress IL-1β-stimulated IL-6 expression in both unstretched and stretched cells. Treatment consisting of 1 μM and 10 μM of P4 was able to significantly reduce IL-1β stimulated IL-6 mRNA expression in unstretched cells. The repression observed from 10 μM P4 and forskolin together was significantly lower than 10 μM P4 treatment alone in unstretched cells. The addition of P4 + forskolin however was not sufficient to reduce IL-1 β driven IL-6 mRNA expression.

The addition of forskolin alone did not affect IL-8 mRNA (Figure 4.19) or protein (Figure 4.21) expression. However, forskolin was able to reduce the IL-1β-induced increase in IL-8 mRNA and protein expression in both unstretched and stretched samples. Similarly for IL-6, treatment of forskolin + either dose of P4 significantly decreased the IL-1β-induced increase in IL-8 in unstretched samples. Together, 10 μM P4 + forskolin resulted in a significantly lower IL-8 mRNA level than 10 μM P4 treatment alone, in unstretched amnion cells. IL-8 supernatant levels (Figure 4.21) also showed that treatment of either dose of P4 + forskolin was able to significantly reduce IL-8 expression more than P4 treatment alone. Human amnion cells that had been subjected to *in vitro* stretch showed a reduction only with forskolin only treatment but not with P4 alone nor a treatment of P4 + forskolin together.

Forskolin treatment alone did not affect TNFα. mRNA (Figure 4.20) or protein (Figure 4.22) levels. P4 alone (was not sufficient to repress IL-1 β induced TNFα mRNA expression in either the absence or presence of stretch at the mRNA level. A treatment of 10 μM P4 + forskolin was able to reduce the IL-1β-induced TNFα mRNA significantly in unstretched cells. Although forskolin alone was able to reduce IL-1β-induced TNFα up-regulation in stretched cells, neither P4 alone or with forskolin was able to repress mRNA expression or protein levels in the presence of in vitro stretch. These data suggest that forskolin does have anti-inflammatory effects, which are exerted in human amnion cells in the presence and absence of *in vitro* stretch.

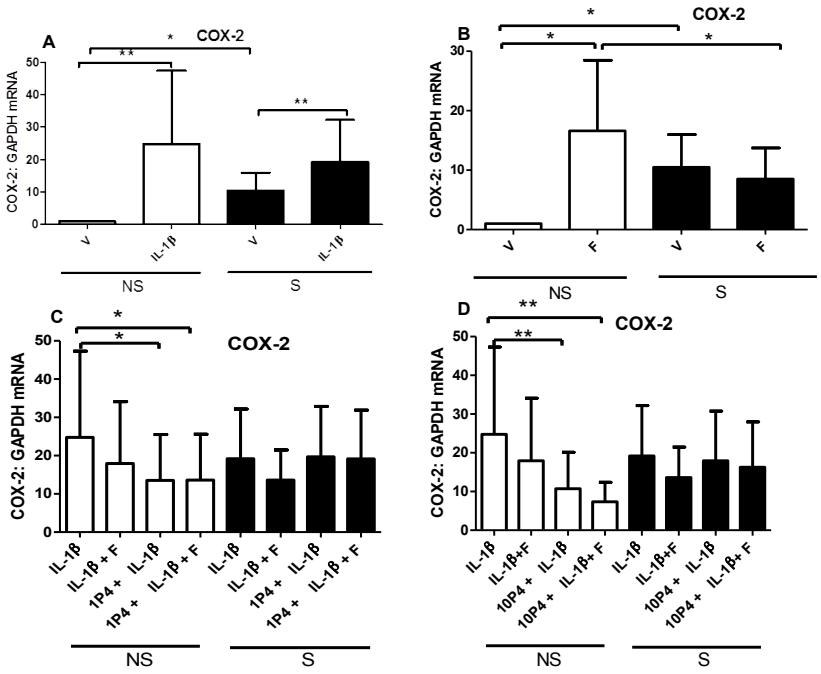

Figure 4.14 Can P4 treatment in combination with forskolin (F) reduce IL-1 β -induced COX-2 mRNA expression levels in human amnion cells in the absence and presence of *in vitro* stretch?

Human amnion cells were isolated as described in *Materials and Methods*. They were subjected to 11% *in vitro* stretch for 2 hours and treated for 6 hours with ethanol vehicle, P4 (1µM or 10µM), forskolin (10µM) and IL-1 β (1ng/ml). RNA was extracted, cDNA synthesised and qPCR ran for COX-2 and normalised to GAPDH. Results were corrected to unstretched controls. Normality was tested using a Kolmogorov-Smirnov test. **A & B:** Wilcoxon matched pairs test for data that were not normally distributed and paired *t* test for data that were normally distributed. They were also used for **C & D:** when comparing data individual stretched samples vs. unstretched samples of the same treatment e.g. NS IL-1 β vs. S IL-1 β ; **C & D:** For normally distributed data an ANOVA followed by Bonferroni's multiple comparison post hoc test was used; data were compared using Friedman's Test, with a Dunn's Multiple Comparisons post hoc test for data that were not normally distributed. * $p<0.05$ versus NS control; ** $p<0.01$ versus V control; versus NS control [N= 8].

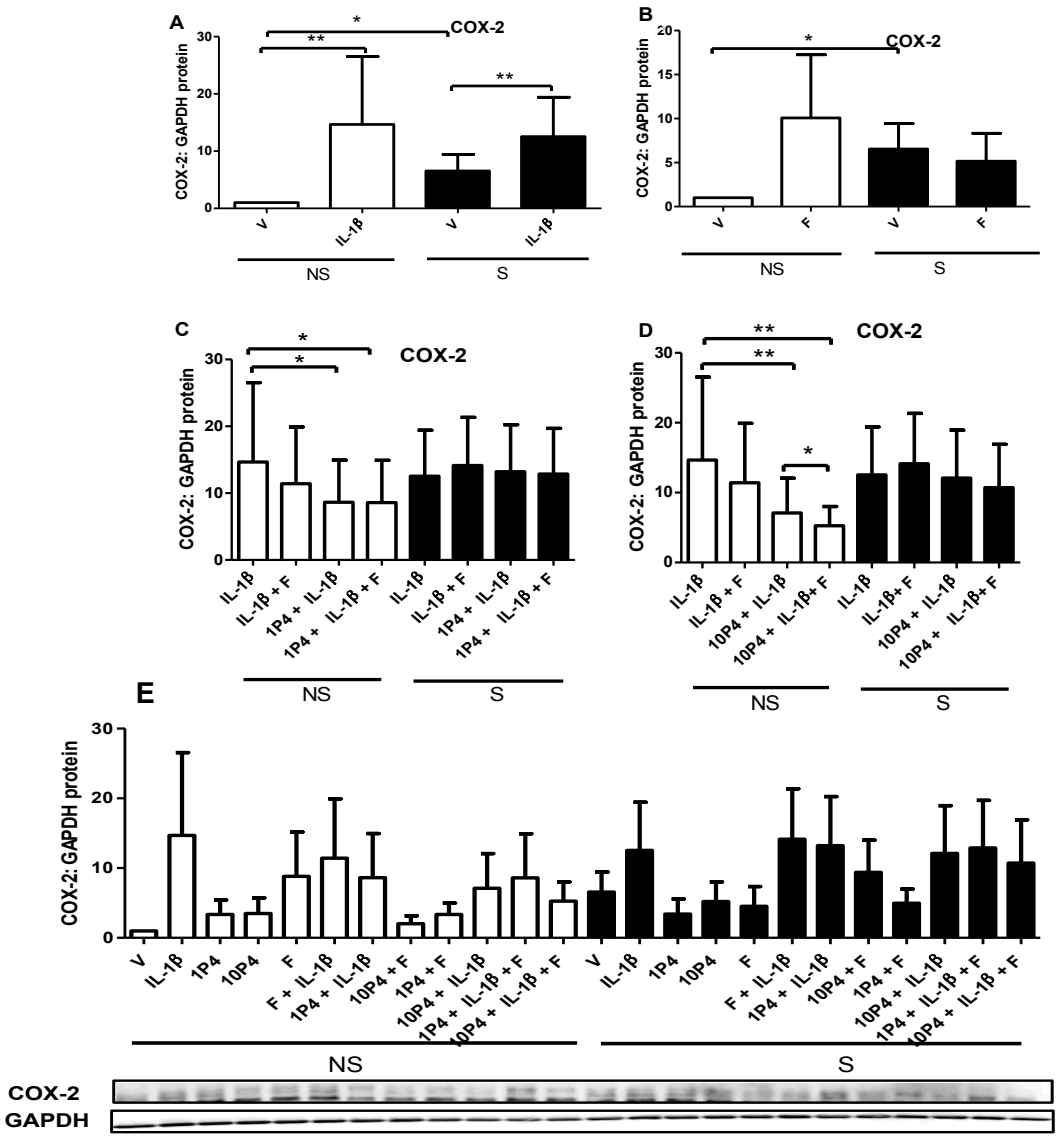

Figure 4.15 Can P4 treatment in combination with forskolin (F) reduce IL-1 β -induced COX-2 protein expression levels in human amnion cells in the absence and presence of *in vitro* stretch? Human amnion cells were isolated as described in *Materials and Methods*. They were subjected to 11% *in vitro* stretch for 2 hours and treated for 6 hours with ethanol vehicle, P4 (1μM or 10μM), forskolin (10μM) and IL-1 β (1ng/ml). **A-E:** Protein was extracted and quantified and western blotting for COX-2 was performed. Results were corrected to unstretched controls. A representative blot is shown. Normality was tested using a Kolmogorov-Smirnov test. **A & B:** Wilcoxon matched pairs test for data that were not normally distributed and paired *t* test for data that were normally distributed. They were also used for **C & D:** when comparing data individual stretched samples vs. unstretched samples of the same treatment e.g. NS IL-1 β vs. S IL-1 β ; **C & D:** For normally distributed data an ANOVA followed by Bonferroni's multiple comparison post hoc test was used; data were compared using Friedman's Test, with a Dunn's Multiple Comparisons post hoc test for data that were not normally distributed. * $p<0.05$; ** $p<0.01$ versus V control [N= 8].

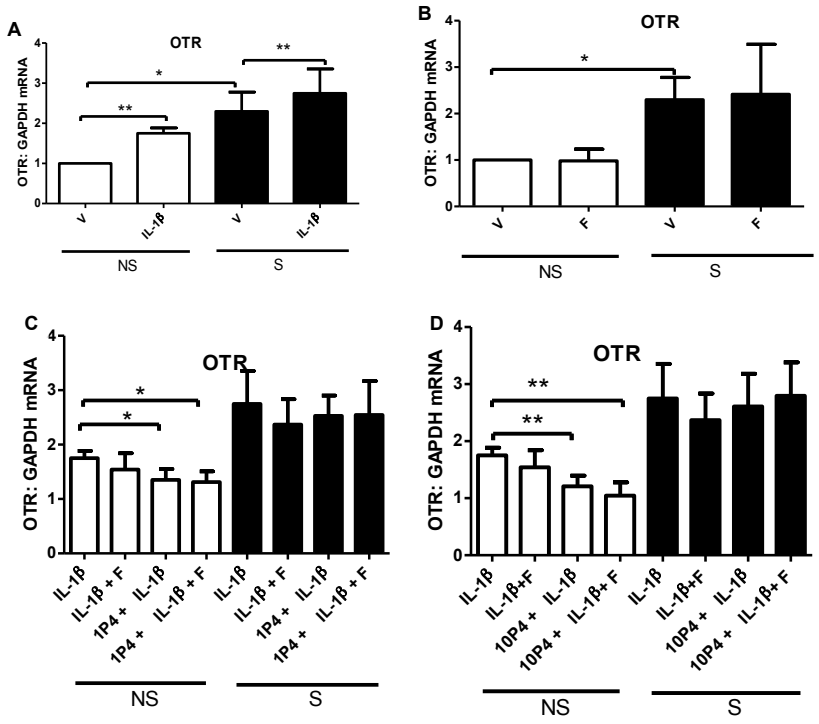

Figure 4.16 Can P4 treatment in combination with forskolin (F) reduce IL-1 β -induced OTR mRNA expression levels in human amnion cells in the absence and presence of *in vitro* stretch? Human amnion cells were isolated as described in *Materials and Methods*. They were subjected to 11% *in vitro* stretch for 2 hours and treated for 6 hours with ethanol vehicle, P4 (1µM or 10µM), forskolin (10µM) and IL-1 β (1ng/ml). RNA was extracted, cDNA synthesised and qPCR ran for OTR and normalised to GAPDH. Results were corrected to unstretched controls. Normality was tested using a Kolmogorov-Smirnov test. **A & B:** Wilcoxon matched pairs test for data that were not normally distributed and paired *t* test for data that were normally distributed. They were also used for **C & D:** when comparing data individual stretched samples vs. unstretched samples of the same treatment e.g. NS IL-1 β vs. S IL-1 β ; **C & D:** For normally distributed data an ANOVA followed by Bonferroni's multiple comparison post hoc test was used; data were compared using Friedman's Test, with a Dunn's Multiple Comparisons post hoc test for data that were not normally distributed. * $p<0.05$, ** $p<0.01$ v [N= 8].

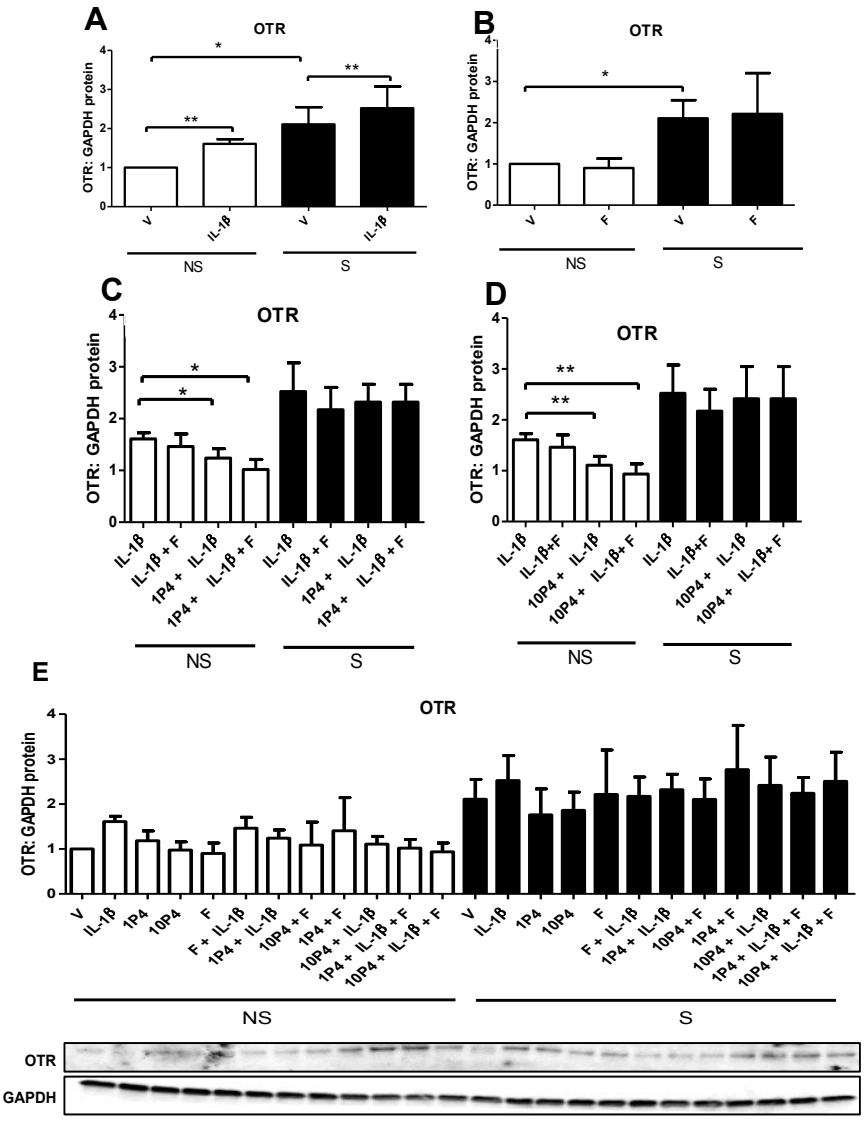

Figure 4.17 Can P4 treatment in combination with forskolin (F) reduce IL-1 β - induced OTR expression levels in human amnion cells in the absence and presence of *in vitro* stretch? Human amnion cells were isolated as described in *Materials and Methods*. They were subjected to 11% *in vitro* stretch for 2 hours and treated for 6 hours with ethanol vehicle, P4 (1μM or 10μM), forskolin (10μM) and IL-1 β (1ng/ml). **A-E:** Protein was extracted and quantified and western blotting for OTR was performed. Results were corrected to unstretched controls. A representative blot is shown. Normality was tested using a Kolmogorov-Smirnov test. **A & B:** Wilcoxon matched pairs test for data that were not normally distributed and paired *t* test for data that were normally distributed. They were also used for **C & D:** when comparing data individual stretched samples vs. unstretched samples of the same treatment e.g. NS IL-1 β vs. S IL-1 β ; **C & D:** For normally distributed data an ANOVA followed by Bonferroni's multiple comparison post hoc test was used; data were compared using Friedman's Test, with a Dunn's Multiple Comparisons post hoc test for data that were not normally distributed. * $p<0.05$; ** $p<0.01$ [N= 8].

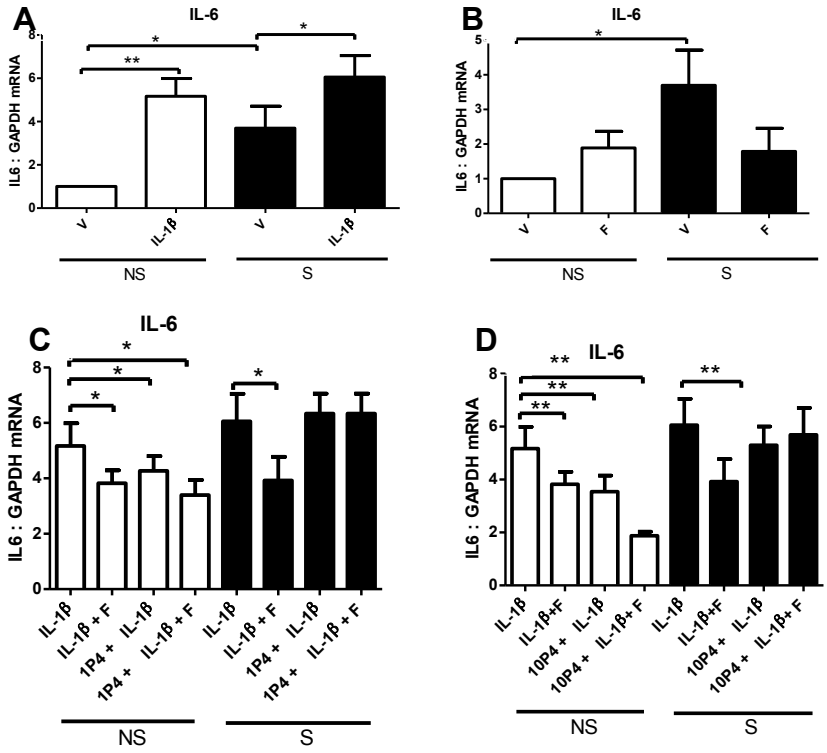

Figure 4.18 Can P4 treatment in combination with forskolin (F) reduce IL-1 β induced IL-6 mRNA expression levels in human amnion cells in the absence and presence of *in vitro* stretch? Human amnion cells were isolated as described in *Materials and Methods*. They were subjected to 11% *in vitro* stretch for 2 hours and treated for 6 hours with ethanol vehicle, P4 (1μM or 10μM), forskolin (10μM) and IL-1 β (1ng/ml). RNA was extracted, cDNA synthesised and qPCR ran for IL-6 and normalised to GAPDH. Results were corrected to unstretched controls. Normality was tested used a Kolmogorov-Smirnov test. test. **A & B:** Wilcoxon matched pairs test for data that were not normally distributed and paired *t* test for data that were normally distributed. They were also used for **C & D:** when comparing data individual stretched samples vs. unstretched samples of the same treatment e.g. IL-1 β vs. S IL-1 β ; **C & D:** For normally distributed data an ANOVA followed by Bonferroni's multiple comparison post hoc test was used; data were compared using Friedman's Test, with a Dunn's Multiple Comparisons post hoc test for data that were not normally distributed. * $p<0.05$, ** $p<0.01$ [N= 8].

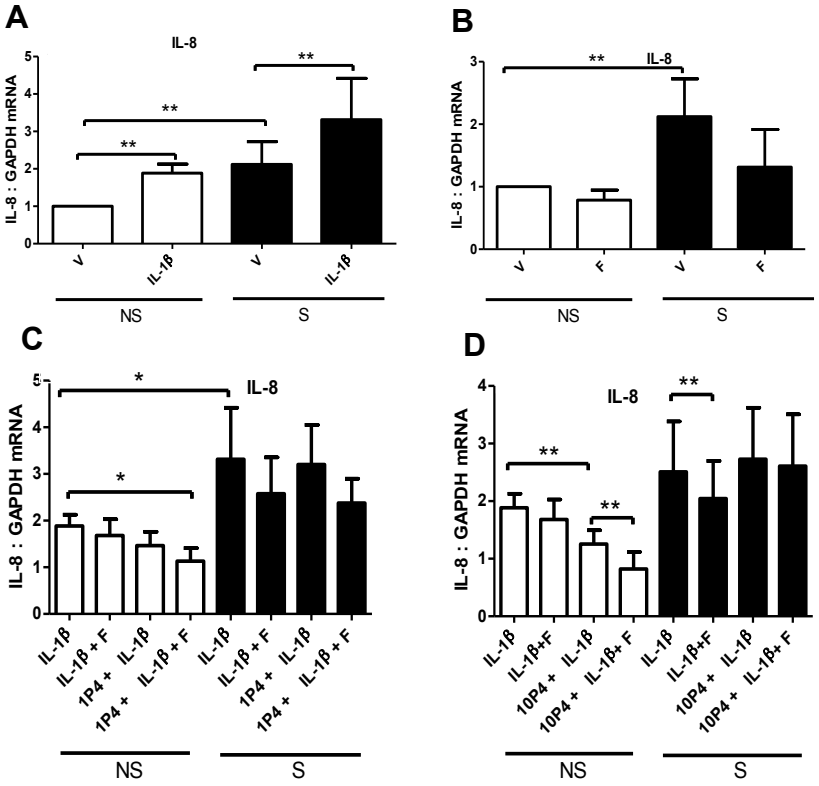

Figure 4.19 Can P4 treatment in combination with forskolin (F) reduce IL-1 β -induced IL-8 mRNA expression levels in human amnion cells in the absence and presence of *in vitro* stretch? Human amnion cells were isolated as described in *Materials and Methods*. They were subjected to 11% *in vitro* stretch for 2 hours and treated for 6 hours with ethanol vehicle, P4 (1μM or 10μM), forskolin (10μM) and IL-1 β (1ng/ml). RNA was extracted, cDNA synthesised and qPCR ran for IL-8 and normalised to GAPDH. Results were corrected to unstretched controls. Normality was tested used a Kolmogorov-Smirnov test. test. **A & B:** Wilcoxon matched pairs test for data that were not normally distributed and paired *t* test for data that were normally distributed. They were also used for **C & D:** when comparing data individual stretched samples vs. unstretched samples of the same treatment e.g. NS IL-1 β vs. S IL-1 β ; **C & D:** For normally distributed data an ANOVA followed by Bonferroni's multiple comparison post hoc test was used; data were compared using Friedman's Test, with a Dunn's Multiple Comparisons post hoc test for data that were not normally distributed. * $p<0.05$, ** $p<0.01$ [N= 8].

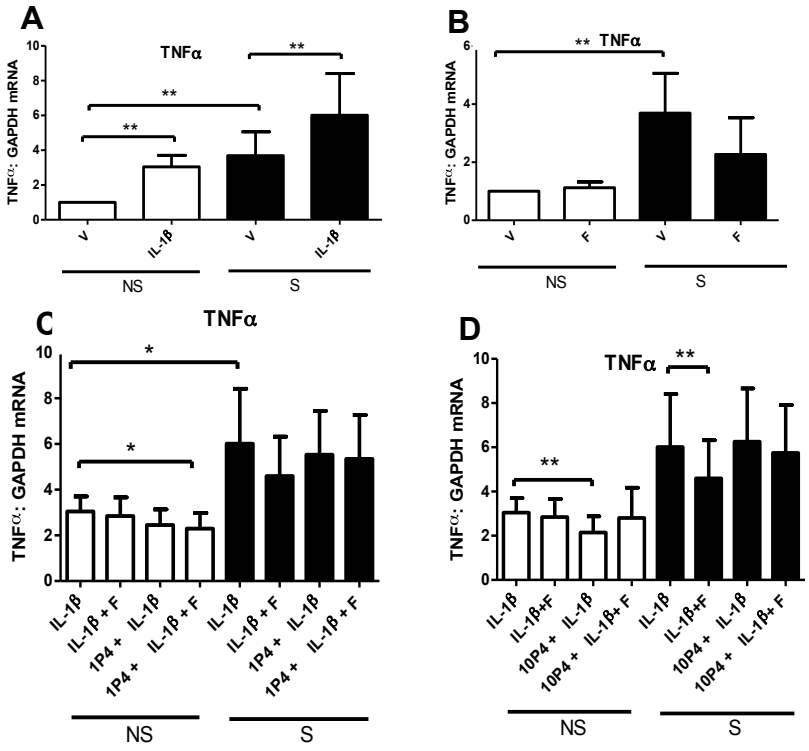

Figure 4.20 Can P4 treatment in combination with forskolin (F) reduce IL-1 β - induced TNF α **mRNA expression levels in human amnion cells in the absence and presence of *in vitro* stretch?** Human amnion cells were isolated as described in *Materials and Methods*. They were subjected to 11% *in vitro* stretch for 2 hours and treated for 6 hours with ethanol vehicle, P4 (1μM or 10μM), forskolin (10μM) and IL-1 β (1ng/ml). RNA was extracted, cDNA synthesised and qPCR ran for TNF α and normalised to GAPDH. Results were corrected to unstretched controls. Normality was tested used a Kolmogorov-Smirnov test. test. **A & B:** Wilcoxon matched pairs test for data that were not normally distributed and paired *t* test for data that were normally distributed. They were also used for **C & D:** when comparing data individual stretched samples vs. unstretched samples of the same treatment e.g. NS IL-1 β vs. S IL-1 β; **C & D:** For normally distributed data an ANOVA followed by Bonferroni's multiple comparison post hoc test was used; data were compared using Friedman's Test, with a Dunn's Multiple Comparisons post hoc test for data that were not normally distributed. * $p<0.05$, ** $p<0.01$ [N= 8].

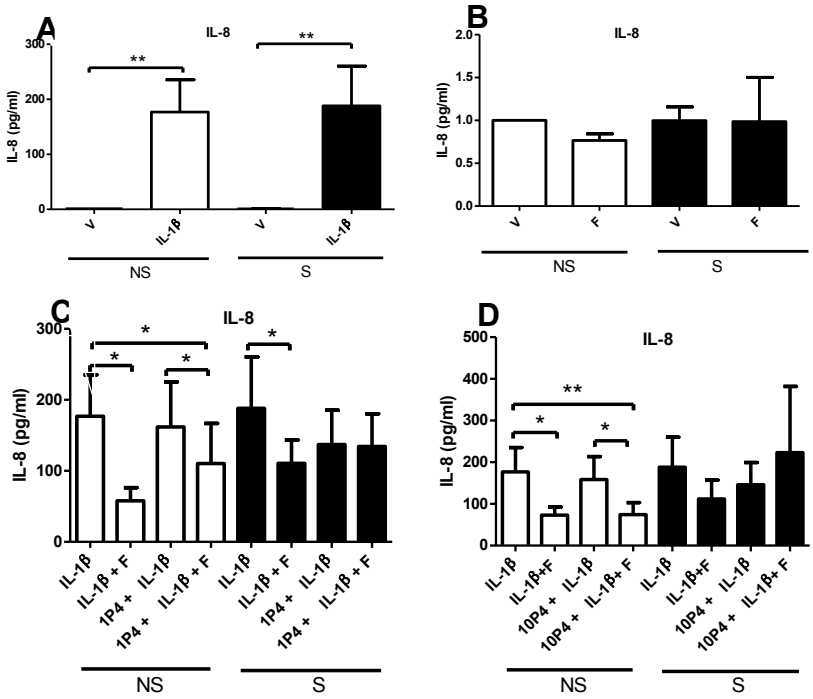

Figure 4.21 Can P4 treatment in combination with forskolin (F) reduce IL-1 β induced IL-8 expression levels in human amnion cells in the absence and presence of *in vitro* stretch? Human amnion cells were isolated as described in *Materials and Methods*. They were subjected to 11% *in vitro* stretch for 2 hours and treated for 6 hours with ethanol vehicle, P4 (1μM or 10μM), forskolin (10μM) and IL-1 β (1ng/ml). Cell supernatant was used to run human Bio-Plex © Pro ™ cytokine-chemokine assays for **IL-8** And were run as per the manufacturer's protocol. Results were corrected to unstretched controls. Normality was tested used a Kolmogorov-Smirnov test. test. **A & B:** Wilcoxon matched pairs test for data that were not normally distributed and paired *t* test for data that were normally distributed. They were also used for **C & D:** when comparing data individual stretched samples vs. unstretched samples of the same treatment e.g. NS IL-1 β vs. S IL-1 β; **C & D:** For normally distributed data an ANOVA followed by Bonferroni's multiple comparison post hoc test was used; data were compared using Friedman's Test, with a Dunn's Multiple Comparisons post hoc test for data that were not normally distributed. * $p<0.05$, ** $p<0.01$ N= 8].

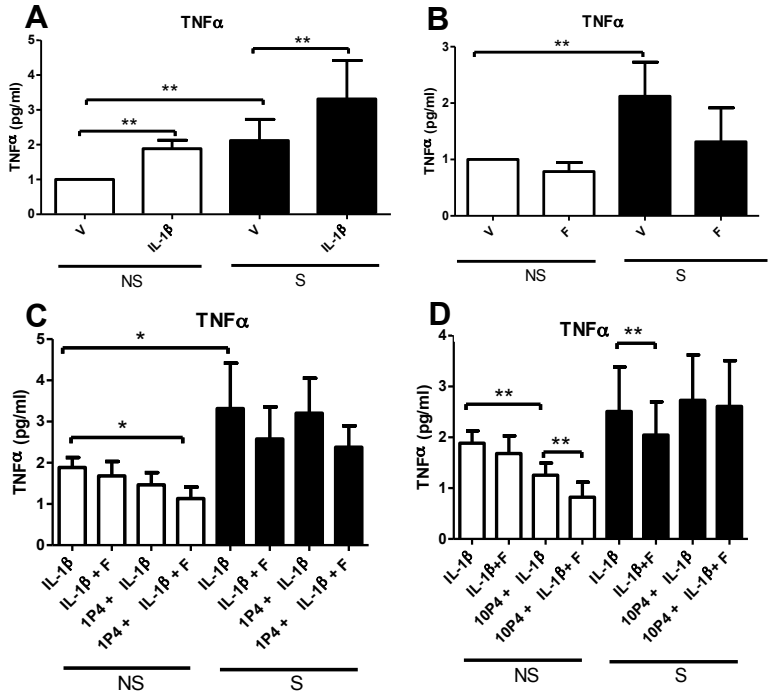

Figure 4.22 Can P4 treatment in combination with forskolin (F) reduce IL-1 β **induced** TNF α **mRNA expression levels in human amnion cells in the absence and presence of *in vitro* stretch?** Human amnion cells were isolated as described in *Materials and Methods*. They were subjected to 11% *in vitro* stretch for 2 hours and treated for 6 hours with ethanol vehicle, P4 (1μM or 10μM), forskolin (10μM) and IL-1 β (1ng/ml). Cell supernatant was used to run human Bio-Plex © Pro ™ cytokine-chemokine assays for TNF α. And were run as per the manufacturer's protocol. Results were corrected to unstretched controls. Normality was tested used a Kolmogorov-Smirnov test. **A & B:** Wilcoxon matched pairs test for data that were not normally distributed and paired *t* test for data that were normally distributed. They were also used for **C & D:** when comparing data individual stretched samples vs. unstretched samples of the same treatment e.g. NS IL-1 β vs. S IL-1 β; **C & D:** For normally distributed data an ANOVA followed by Bonferroni's multiple comparison post hoc test was used; data were compared using Friedman's Test, with a Dunn's Multiple Comparisons post hoc test for data that were not normally distributed. * $p<0.05$, ** $p<0.0$; [N= 8].

4.11 Discussion

The aim of this chapter was to answer 3 questions:

1. Does P4 administration repress stretch-induced gene expression in human amnion cells?
2. Can P4 repress IL-1β-induced pro-labour gene expression in amnion cells – in the presence and absence of stretch?
3. Can the effects of P4 be enhanced by the administration of the cAMP agonist, forskolin?

4.11.1 P4 does not affect stretch-induced prolabour expression

Initial *in vitro* stretch experiments (Figure 4.1-4.3) demonstrated that stretch elevated COX-2 mRNA and protein levels as well as the pro-inflammatory cytokines: IL-6, IL-8 and TNF α (60). P4 administration is used to reduce the risk of PTL in high-risk singleton pregnancies, however it has been deemed ineffective in cases of multiple pregnancy (286-288). The data presented here support these observations as the administration of 1μM or 10μM P4 administration failed to reduce stretch-induced prolabour expression of COX-2, IL-6, IL-8 or TNFα (Figure 4.4-4.6), at the protein or mRNA level.

In high-risk singleton pregnancies, P4 is thought to inhibit IL-1β-induced COX-2 expression and therefore reduce the risk of PTL. This result has been demonstrated in the hTERT myometrial cell line (172) where this involved inhibiting NF-κB binding to the COX-2 promoter. Further to this, data obtained from uterine smooth muscle cells showed that by targeting IKK, stretch-induced NF-κB activation could be inhibited and this also led to a reduction in stretch-induced COX-2 expression (309). These results suggested P4 could reduce stretch-induced changes however subsequent work done by Lei, *et al*. showed that this was not the case. P4 treatment was not able to repress stretch-induced changes in COX-2, IL-8 or OTR expression in human myometrial cells (59).

The findings from this study in the human amnion correlate with the group's myometrial work. It supports the theory that P4 does not have the ability to impair

stretch-induced expression and also alludes to differential mechanistic behaviour in singleton and multiple pregnancies.

4.11.2 Forskolin does not affect stretch-induced prolabour expression

The addition of the cAMP agonist, forskolin, had little significant effect in terms of reducing stretch-induced prolabour gene expression: COX-2, IL-6, IL-8 or TNFα (Figure 4.4-4.6), at the mRNA or protein level.

In unstretched amnion cells, COX-2 mRNA and protein levels significantly increased when forskolin was added alone (Figure 4.4). In comparison, unstretched amnion cells treated with (either dose of) P4 and forskolin together showed a trend towards a decrease in COX-2 levels. This suggests that the addition of forskolin alone may induce COX-2 levels in human amnion cells in Flexercell stretch plates. In stretched amnion cells however, forskolin treatment alone showed a decreased trend in COX-2 compared with unstretched cells with the same treatment (Figure 4.4) suggesting a potential inhibitory effect in the presence of mechanical stretch. Interestingly though, in stretched amnion cells, the combination of P4 doses with forskolin resulted in significantly higher COX-2 expression compared with forskolin alone in unstretched cells (Figure 4.4). It could be speculated that this may be an example of cAMP's altered function depending on its context and cell type.

The expression of other prolabour genes was unaltered by the combination of both treatments. Since this is a novel approach, there is limited evidence in the literature exploring whether the combined action of P4 and a cAMP agonist might reduce stretch-induced prolabour expression. However, since PTL, in particular cases of excessive uterine overdistention where P4 administration is known to be ineffective, this area remains an area for further investigation.

4.11.3 Stretch does not affect P4-dependent gene expression

The impact of *in vitro* mechanical stretch on P4 action was explored. Our research group has previously identified P4-regulated genes in uterine cells using a microarray study. In this microarray study and further validation experiments, it was found that MPA and P4 increased the expression of HSD11β1 and FKBP5 (59). These two genes acted as markers to assess whether mechanical stretch altered P4's ability to enhance gene expression. Results from this study showed that amnion cells subjected to mechanical stretch did not impair the ability of P4 to stimulate 11βHSD (Figure 4.7 A & B) or FKBP5 (Figure 4.8 A & B) gene expression. These data correspond to our group findings in myometrial cells (59). Suggesting that the ability of P4 to increase gene expression is unchanged stretch *in vitro*. However, it has been shown that P4 withdrawal may maintain uterine quiescence. As a result, it is still yet to be determined whether stretch may negatively impact P4 action using a non-genomic mechanism. However, the addition of forskolin in the presence of stretch did enhance the P4-driven expression significantly and supports data from Chen *et al.* (206) in myometrial cells.

4.11.4 Does P4 have the ability to repress IL- β induced COX-2 expression observed in amnion cells – in the absence and presence of stretch?

Following an initial time course experiment, which confirmed IL-1β treatment induced COX-2 expression (Figure 4.9), a subsequent experiment was performed to investigate the ability of P4 to reduce IL-1β stimulated prolabour gene expression in the presence and absence of stretch.

Stretch increased COX-2 mRNA and protein expression compared to unstretched amnion cells (Figure 4.10 A - E) as seen previously (Figure 4.1& 4.4). However, no further enhancement in COX-2 expression was observed following subsequent IL-1β treatment. Though there was a significant increase following IL-1β administration in stretched amnion cells, this elevation in COX-2 expression was not significantly different when compared with unstretched amnion cells with the same treatment. OTR (Figure 4.11 A-E) and the proinflammatory cytokines IL-6, IL-8 and TNFα (Figure

4.12 and 4.13) demonstrated similar results. It could be hypothesized that exerting two stimuli such as mechanical stretch and inflammation may produce an equivalent, but not summative effect on prolabour gene expression. Alternatively, perhaps IL-1β-driven mRNA synthesis differs from protein generation; COX-2 protein synthesis in synovial fibroblasts has recently been characterised via a p38-dependent mechanism (310) and alludes to the possibility of a more efficient post-transcriptional translation of COX-2 mRNA.

4.11.5 Does P4 have the ability to repress IL-1β stimulated prolabour gene expression in human amnion cells? Can its efficacy be enhanced through the administration of a cAMP agonist (forskolin)?

The data presented here showed that P4 treatment alone, at both doses: 1μM or 10μM, was able to repress COX-2 expression (Figure 4.10) OTR (Figure 4.11) at both the mRNA and protein levels in unstretched cells. P4 treatment alone was also able to reduce IL- β-stimulated pro-inflammatory cytokines of IL-6 (Figure 4.12 A & B) in unstretched amnion cells. Only 10μM P4 was able to repress IL- β-stimulated pro-inflammatory TNFα at the mRNA (Figure 4.12 E & F) level and protein level of TNFα at the protein level (Figure 4.13 C &D) in unstretched cells. P4 however, was unable to significantly reduce the IL-1β driven prolabour expressions in the presence of stretch. These findings support other studies involving P4 administration *in vitro* in myometrial cells where P4's anti-inflammatory ability was ineffective in the presence of mechanical stretch (59).

In addition, it was found that although IL-8 at the mRNA level could be reduced significantly by both doses of P4, (Figure 4.12 C &D) IL-8 levels observed through multiplex assay could not be significantly reduced (Figure 4.13 A &B). Similar results have been observed in human myometrial cells (Unpublished work by Angela Yulia), suggesting that perhaps IL-1β-stimulated IL-8 mRNA and protein expression could be mediated differently.

Previous work from our group suggested that elevated cAMP levels through forskolin administration enhanced P4 repression of IL-1β-induced COX-2 expression (206).

Data presented here showed that in the absence of additional mechanical stretch, this effect was also seen in human amnion cells. In unstretched amnion cells, IL-1β-induced COX-2 gene expression (Figure 4.14 & 4.15), OTR (Figure 4.16 & 4.17), IL-6 (Figure 4.18), IL-8 (Figure 4.19 & Figure 4.21) TNFα. (Figure 4.20 & Figure 4.22) could all be significantly reduced through a treatment of P4 + forskolin. For COX-2 protein (Figure 4.15), IL-8 mRNA (Figure 4.19) and protein (Figure 4.21) and TNFα protein (Figure 4.22). This combined treatment was significantly more effective in reducing IL-1β-induced expression, compared with P4 treatment alone. Taken together, these studies highlight cAMP's anti-inflammatory properties when taken with P4 and support previous work in myometrial cells (206) showing similar results. However, it is also important to note is that for OTR mRNA (Figure 4.16) and IL-6 mRNA (Figure 4.18) there was no additive effect of forskolin and P4 on non stretch cells. In addition, there was a suggested forskolin inhibition on stretched cells in these sets of data.

Interestingly, forskolin alone was able to significantly reduce the IL-1β-induced increase in IL-8 protein levels (Figure 4.21) and IL-6 mRNA in both unstretched and stretched amnion cells (Figure 4.18). This suggests that cAMP's own complex anti-inflammatory properties may persist in the context of mechanical stretch. Though this is a promising outcome, it also encourages further work in this area to solidify the proposed theory.

4.1.1 Summary

These data confirms that a stretch-induced increase in prolabour gene expression in human amnion cells. P4 alone or joint with forskolin, were able to inhibit the stretch-induced increase in prolabour gene expression. However, since there was suggested decreased trend with forskolin treatment, the data suggest that further research should be done to investigate the role of cAMP alone has in reducing mechanical stretch-induced changes in human amnion cells.

In addition, this chapter found that P4 administration could be used to significantly reduce *in vitro* IL-1β-induced prolabour gene expression in the absence of mechanical stretch. It was found that the addition of P4 treatment (1μM or 10μM) with forskolin could also significantly repress IL-1β-induced prolabour expression in unstretched amnion cells. To further support these findings, in future work, the IL-1β-induced expression and activity of nuclear phospho-p65 could be investigated. In human myometrial cells, P4 repression of IL-1β-induced COX-2 expression has been linked with less NF-κB binding to COX-2 promoter, whilst the cocktail of P4+forskolin has shown to augment of this effect (206). Determining whether this is true for amnion cells should be explored. It is important to note P4's repression of IL-1β-induced expression was not apparent in stretched cells and adds to the hypothesis that tackling stretch-induced PTL may require an alternative approach.

Chapter 5. Does P4 exert its anti-inflammatory effect through PR or GR?

5.1 Introduction

5.1.1 P4 inflammatory action

Pivotal research by Csapo *et al*., demonstrated P4's key role in early pregnancy (116). It is now well established that P4 is fundamental for maintaining pregnancy in animals as well as humans. Prior to the onset of labour, a withdrawal can be observed in systemic P4 levels in most animals. In contrast, the circulating maternal, fetal and amniotic P4 levels in humans remain high. This observation in humans and primates, suggests the existence of a functional P4 withdrawal, whereby P4 activity is reduced as reproductive tissues become resistant to P4 action (1). Frydman *et. al.,* showed that the P4 and glucocorticoid antagonist, mifepristone (RU486) is able to bring about labour (151). In addition, P4 is administered to high-risk women to delay the onset of preterm birth (284). Taken together, these studies suggest that P4 is important for the maintenance of pregnancy in humans and raises the possibility that a withdrawal of P4 action is involved in the onset of parturition.

P4 is thought to exert its pro-gestational effects via different pathways in order to delay labour onset. Animal studies, investigating uterine distention in pregnancy (or otherwise) demonstrated elevated prolabour gene expression (49, 50), which could be targeted and reduced through P4 administration. In humans, however, P4 administration was not able to delay labour onset in multiple pregnancy (286, 288) or reduce prolabour gene expression in in vitro studies (59).

Despite these facts, the mechanisms through which P4 may exert its effect remain unclear. Previous work by the group has demonstrated that in myometrial cells P4 may work through both the GR and progesterone receptors (PRs) whilst, P4 has the ability to reduce inflammation-induced COX-2 production through GR (119). It is not known whether this is also true in primary human amnion cells.

5.1.2 Chapter Aims

Thus, this chapter aimed to investigate:

1. Through which receptor P4 was able to exert its anti-inflammatory effect: PR subtypes or GR
2. Whether P4's anti-inflammatory effect was enhanced through the over-expression of receptors PR-A, PR-B or GR
3. What impact modulating nuclear receptor expression has on P4 responsive genes

5.2 Results

5.3 Is P4 repression of IL-1β-driven COX-2 expression altered in the presence of mifepristone, RU486?

The main aim was to define through which receptor P4 mediates its anti-inflammatory action. However, it was first important to investigate IL-1β-driven COX-2 expression, in the absence of both receptors. As previously mentioned, RU486 mifepristone has the ability to induce labour at any point in gestation (151). RU486 treatment prior to IL-1β-treatment was able to able to reverse the P4-anti-inflammatory effect (Figure 5.1).

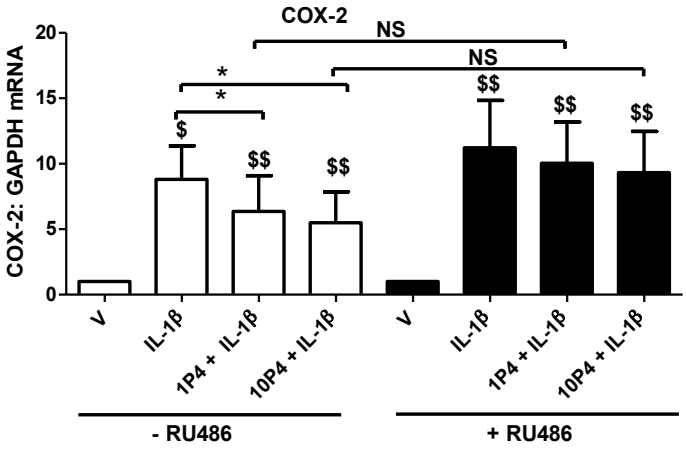

Figure 5.1. Is P4 repression of IL-1β-driven COX-2 expression altered in the presence of mifepristone, RU486? Human amnion cells were isolated as described in *Materials and Methods* and treated with IL-1β (1ng/ml), P4 (1 μM and 10μM) for 6 hours. RNA was extracted, cDNA synthesised and qPCR ran for COX-2 and normalised to GAPDH**.** Results were corrected to untreated vehicle controls. Normality was tested using a Kolmogorov-Smirnov test. For normally distributed data an ANOVA followed by Bonferroni's multiple comparison post hoc test was used; data were compared using Friedman's Test, with a Dunn's Multiple Comparisons post hoc test for data that were not normally distributed. Wilcoxon matched pairs test for data that were not normally distributed and paired *t* test for data that were normally distributed was used when comparing

1. RU486 treated samples of the same treatment; 2. 1 μM P4+ IL-1β vs 10 μM P4+ IL-1β; $ $p<0.05$ versus untreated vehicle control; $$ $p<0.01$ versus untreated vehicle control; * $p<0.05$; IL-1β versus IL-1β+P4; NS: not significant between RU486 un/treated samples [N= 8].

5.4 Is P4 repression of IL-1β-driven prolabour gene expression affected following changes to nuclear receptor expression?

5.4.1 Is P4 repression of IL-1β-driven COX-2 & OTR expression affected in the absence of GR?

Human amnion cells were transfected with siRNAs against GR (siGR) and also non-targeting siRNA, used as a control. The reduction in GR did not alter P4's ability to inhibit IL-1β-driven COX-2 (Figure 5.2A) or OTR (Figure 5.2 B) mRNA expression. No significant differences were observed between knocked down samples and samples treated with non-targeting controls.

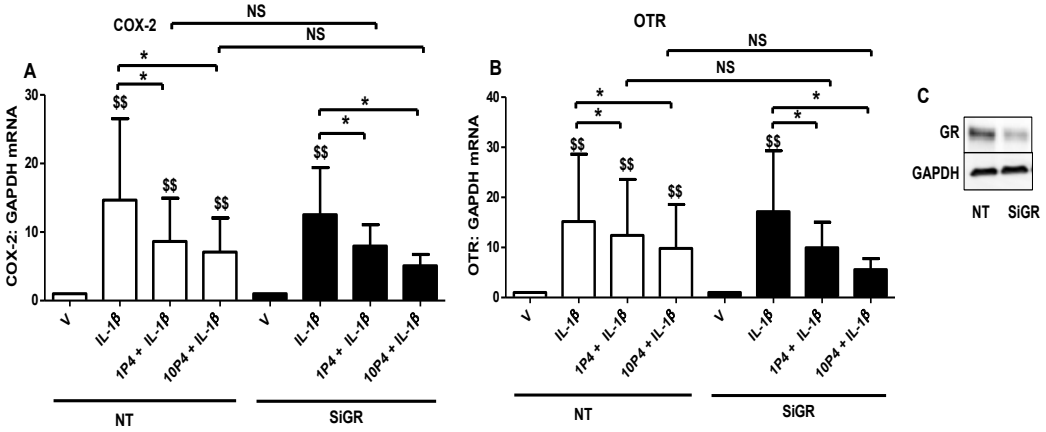

Figure 5.2. Is P4 repression of IL-1β-driven COX-2 & OTR expression altered in the absence of GR? Human amnion cells were isolated as described in *Materials and Methods* and then transfected with siRNA against GR (siGR) as well as non-targeting control siRNA (NT). Cells were incubated for 48 hours following transfection and were treated with IL-1β (1ng/ml), P4 (1 μM and 10μM) for 6 hours. RNA was extracted, cDNA synthesised and qPCR ran for **A:** COX-2 **B:** OTR and normalised to GAPDH**.** A representative blot of the transfection is shown. Results were corrected to untreated vehicle controls. Normality was tested using a Kolmogorov-Smirnov test. For normally distributed data an ANOVA followed by Bonferroni's multiple comparison post hoc test was used; data were compared using Friedman's Test, with a Dunn's Multiple Comparisons post hoc test for data that were not normally distributed. Wilcoxon matched pairs test for data that were not normally distributed and paired *t* test for data that were normally distributed was used when comparing 1. siGR treated samples to NT samples of the same treatment; 2. 1 μM P4+ IL-1β vs 10 μM P4+ IL-1β; $ $p<0.05$ versus untreated vehicle control; $$ $p<0.01$ versus untreated vehicle control; * $p<0.05$; IL-1β versus IL-1β+P4; NS: not significant between siGR and NT samples [N= 8].

5.4.2 Is P4 repression of IL-1β-driven COX-2 expression affected in the presence of overexpressed PR-A, PR-B or GR

Despite several attempts to knockdown PR, its basal level was only sporadically detectable in human amnion cells at a very low protein expression level making it challenging to knockdown. As previously mentioned, in the literature it has been suggested that endogenous PR levels are very low in the amnion (178, 264). As a result, both sets of receptors were over-expressed (Figure 5.3-5.5). In all cases, P4's ability to repress IL-1β-driven COX-2 and OTR expression was maintained. Overexpressing PR-A (Figure 5.3) was able to significantly enhance the effect of 1 μM P4 in repressing IL-1β-driven COX-2 mRNA expression and showed a trend towards a significant enhancement in this decrease with 10 μM P4 treatment. Significant enhancement in this repression was also observed following PR-B overexpression in human amnion cells with both doses of P4 (Figure 5.4 A&B). Repression of IL-1β-driven COX-2 and OTR mRNA expression was not altered by overexpressing GR (Figure 5.5 A &B).

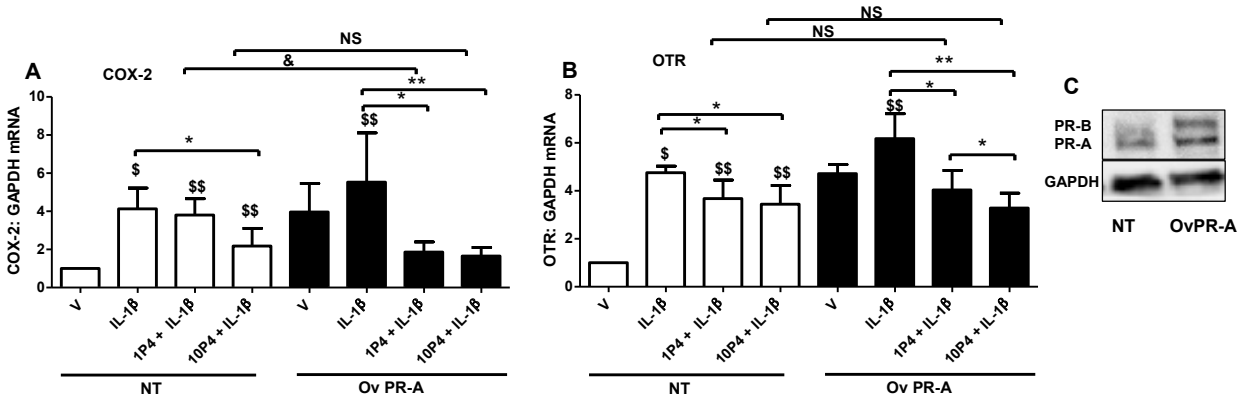

Figure 5.3. Is P4 repression of IL-1β-driven COX-2 & OTR expression altered when PR-A is overexpressed? Human amnion cells were isolated as described in *Materials and Methods* and then transfected with PR-A or SG5, used as a control. Cells were incubated for 48 hours following transfection and were treated with IL-1β (1ng/ml), P4 (1 μM and 10μM) for 6 hours. RNA was extracted, cDNA synthesised and qPCR ran for **A:** COX-2 **B: OTR** and normalised to GAPDH**.** A representative blot of the transfection is shown. Results were corrected to untreated vehicle controls. Normality was tested using a Kolmogorov-Smirnov test. For normally distributed data an ANOVA followed by Bonferroni's multiple comparison post hoc test was used; data were compared using Friedman's Test, with a Dunn's Multiple Comparisons post hoc test for data that were not normally distributed. Wilcoxon matched pairs test for data that were not normally distributed and paired *t* test for data that were normally distributed was used when comparing 1. Ov PR-A treated samples to NT samples of the same treatment; 2. 1 μM P4+ IL-1β vs 10 μM P4+ IL-1β; $ $p<0.05$ versus untreated vehicle control; $$ $p<0.01$ versus untreated vehicle control;
* $p<0.05$; IL-1β versus IL-1β+P4; ** $p<0.05$; IL-1β versus IL-1β+P4; & $p<0.05$; Ov-PR-A and NT samples NS: not significant between Ov PR-A and NT samples [N= 8].

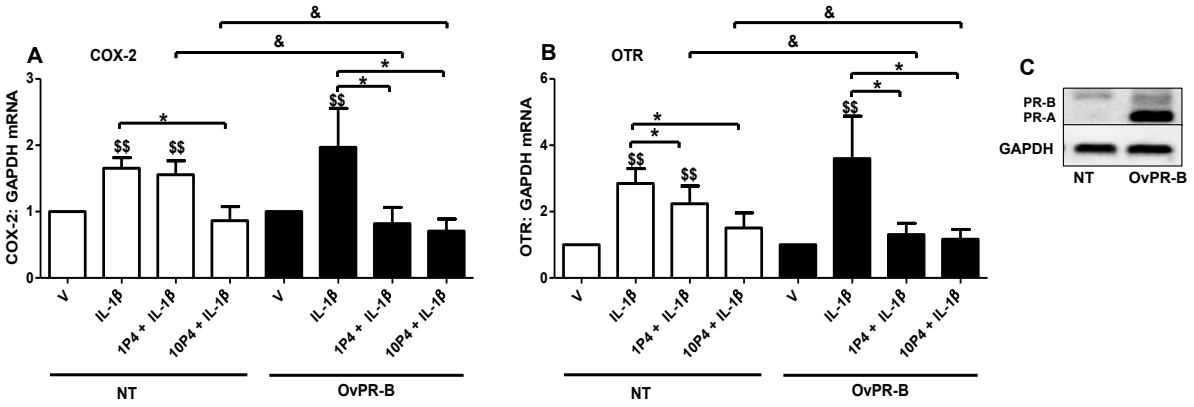

Figure 5.4. Is P4 repression of IL-1β-driven COX-2 & OTR expression altered when PR-B is overexpressed? Human amnion cells were isolated as described in *Materials and Methods* and then transfected with PR-B or SG5, used as a control. Cells were incubated for 48 hours following transfection and were treated with IL-1β (1ng/ml), P4 (1 μM and 10μM) for 6 hours. RNA was extracted, cDNA synthesised and qPCR ran for **A:** COX-2 **B: OTR** and normalised to GAPDH**.** A representative blot of the transfection is shown. Results were corrected to untreated vehicle controls. Normality was tested using a Kolmogorov-Smirnov test. For normally distributed data an ANOVA followed by Bonferroni's multiple comparison post hoc test was used; data were compared using Friedman's Test, with a Dunn's Multiple Comparisons post hoc test for data that were not normally distributed. Wilcoxon matched pairs test for data that were not normally distributed and paired *t* test for data that were normally distributed was used when comparing 1. Ov-PR-B treated samples to NT samples of the same treatment; 2. 1 μM P4+ IL-1β vs 10 μM P4+ IL-1β; $ $p<0.05$ versus untreated vehicle control; $$ $p<0.01$ versus untreated vehicle control; * $p<0.05$; IL-1β versus IL-1β+P4; & $p<0.05$; Ov-PR-B and NT samples; NS: not significant between Ov PR-B and NT samples [N= 8].

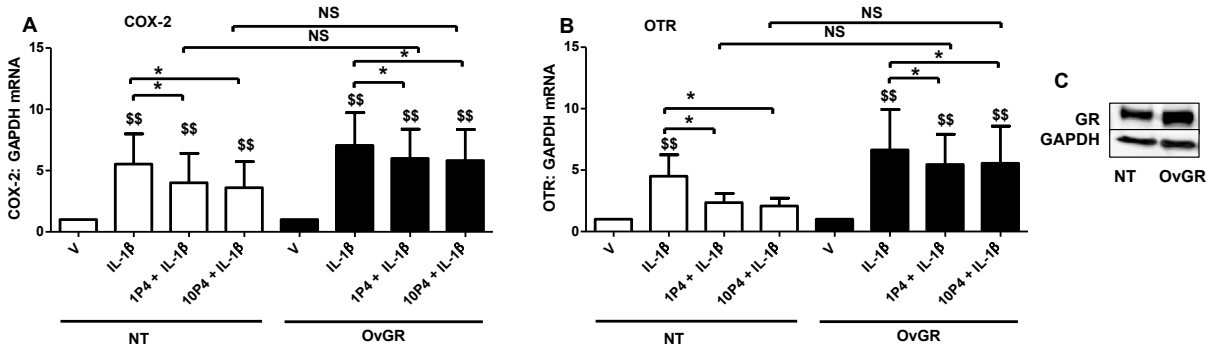

Figure 5.5. Is P4 repression of IL-1β-driven COX-2 & OTR expression altered when GR is overexpressed? Human amnion cells were isolated as described in *Materials and Methods* and then transfected with GR or SG5, used as a control. Cells were incubated for 48 hours following transfection and were treated with IL-1β (1ng/ml), P4 (1 μM and 10μM) for 6 hours. RNA was extracted, cDNA synthesised and qPCR ran for **A:** COX-2 **B: OTR** and normalised to GAPDH**.** A representative blot of the transfection is shown. Results were corrected to untreated vehicle controls. Normality was tested using a Kolmogorov-Smirnov test. For normally distributed data an ANOVA followed by Bonferroni's multiple comparison post hoc test was used; data were compared using Friedman's Test, with a Dunn's Multiple Comparisons post hoc test for data that were not normally distributed. Wilcoxon matched pairs test for data that were not normally distributed and paired *t* test for data that were normally distributed was used when comparing. 1. Ov-GR treated samples to NT samples of the same treatment 2. 1 μM P4+ IL-1β vs 10 μM P4+ IL-1β; $ $p<0.05$ versus untreated vehicle control; $$ $p<0.01$ versus untreated vehicle control; * $p<0.05$; IL-1β versus IL-1β+P4; NS: not significant between Ov GR and NT samples [N= 8].

5.5 Is P4 ability to modulate gene expression affected when nuclear receptor expression is altered?

5.5.1 Is P4's ability to modulate gene expression altered in the absence of GR?

As previously mentioned, 2 genes have been found to be modulated by P4 following a microarray study (NCBI GEO Accession GSE68171). FKBP5 and 11βHSD were identified and were used as markers for P4 action. The mRNA expression of both FKBP5 and 11βHSD were quantified following transfection with siGR. In the absence of GR, P4 maintained its ability to induce both FKBP5 (Figure 5.6 A) and 11βHSD (Figure 5.6B) Knocking down GR enhanced P4 induced 11βHSD expression following 1 and 10 μM P4 treatment. In addition, there was a trend towards a similar enhancement in FKBP5 mRNA expression following 10 μM P4 treatment (Figure 5.6A).

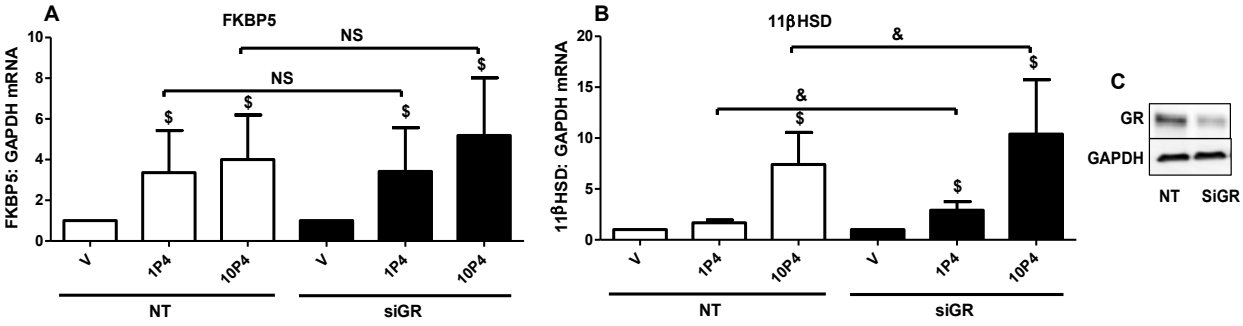

Figure 5.6. Is P4's ability to modulate gene expression altered in the absence of GR? Human amnion cells were isolated as described in *Materials and Methods* and then transfected with siRNA against GR (siGR) as well as non-targeting control siRNA (NT). Cells were incubated for 48 hours following transfection and were treated with P4 (1 μM and 10μM) for 6 hours. RNA was extracted, cDNA synthesised and qPCR ran for A: FKBP5 B: 11βHSD and normalised to GAPDH. Results were corrected to untreated vehicle controls. Normality was tested using a Kolmogorov-Smirnov test. Wilcoxon matched pairs test for data that were not normally distributed and paired *t* test for data that were normally distributed was used when comparing 1. siGR treated samples to NT samples of the same treatment. $ $p<0.05$ versus untreated vehicle control; & $p<0.05$; siGR and NT samples; NS: not significant between siGR and NT samples [N= 8].

5.5.2 Is P4's ability to modulate gene expression altered in the presence of overexpressed PR-A, PR-B and GR?

Overexpression of PR-A and PR-B significantly enhanced P4-induced FKBP5 and 11βHSD mRNA levels (Figure 5.7 and 5.8). GR overexpression did not affect P4-induced 11βHSD mRNA levels (Figure 5.7 and 5.8) however did significantly increase FKBP5 mRNA levels, following 10μM P4 treatment (Figure 5.7).

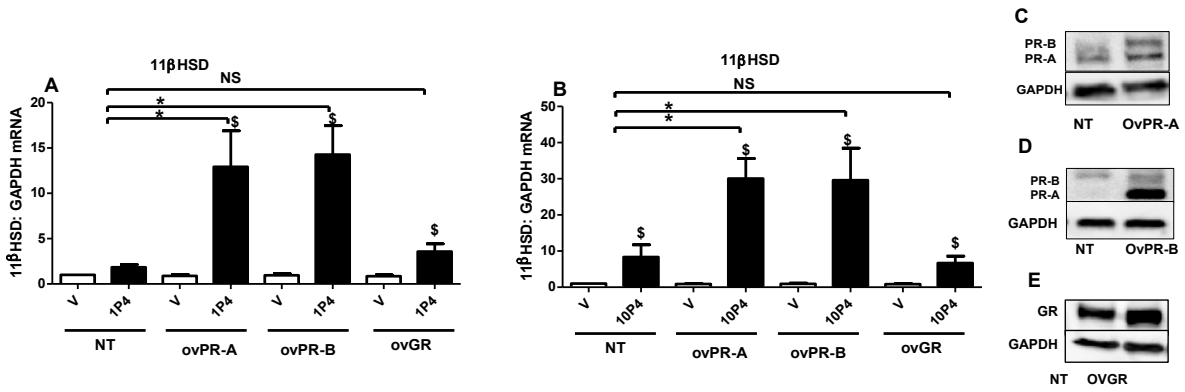

Figure 5.7. Is P4's ability to modulate gene expression altered in the presence of overexpressed PR-A, PR-B and GR? Human amnion cells were isolated as described in *Materials and Methods* and then transfected with PR-A, PR-B, GR or SG5, used as a control (NT). Cells were incubated for 48 hours following transfection and were treated with P4 [1 μM (A) and 10μM (B)] for 6 hours. RNA was extracted, cDNA synthesised and qPCR ran for FKBP5 and normalised to GAPDH. Results were corrected to untreated vehicle controls. Normality was tested using a Kolmogorov-Smirnov test. Wilcoxon matched pairs test for data that were not normally distributed and paired *t* test for data that were normally distributed was used when comparing 1. P4 treated samples to their corresponding vehicle samples. 2. P4 samples of transfected PR-A, PR-B, GR to NT control samples; * $p<0.05$; transfected P4 compared to NT P4 samples; NS: not significant between Ov GR and NT samples [N= 8].

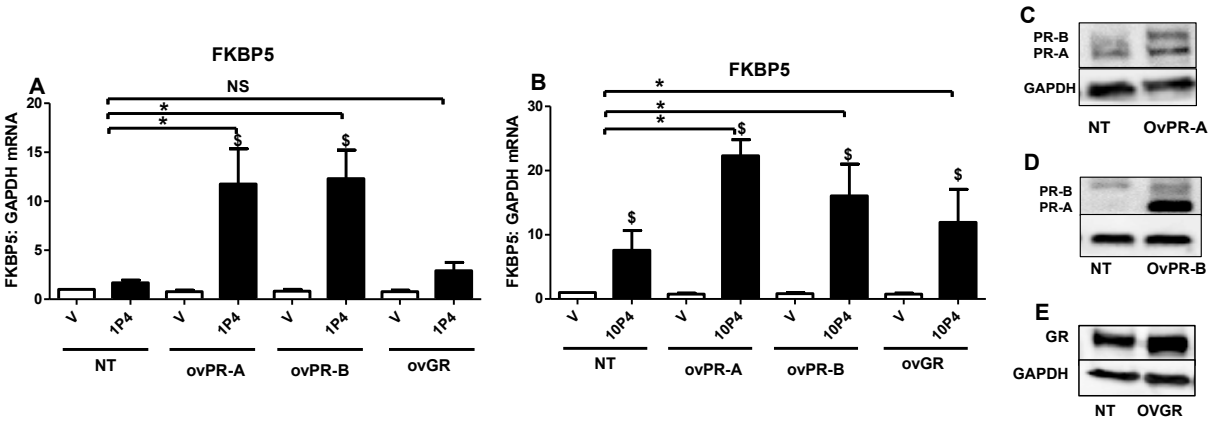

Figure 5.8. Is P4's ability to modulate gene expression altered in the presence of overexpressed PR-A, PR-B and GR? Human amnion cells were isolated as described in *Materials and Methods* and then transfected with PR-A, PR-B, GR or SG5, used as a control (NT). Cells were incubated for 48 hours following transfection and were treated with P4 [1 μM (A) and 10μM (B)] for 6 hours. RNA was extracted, cDNA synthesised and qPCR ran for 11βHSD and normalised to GAPDH. Results were corrected to untreated vehicle controls. Normality was tested using a Kolmogorov-Smirnov test. Wilcoxon matched pairs test for data that were not normally distributed and paired *t* test for data that were normally distributed was used when comparing 1. P4 treated samples to their corresponding vehicle samples. 2. P4 samples of transfected PR-A, PR-B, and GR to NT control samples; * $p<0.05$; transfected P4 compared to NT P4 samples; NS: not significant between Ov GR and NT samples [N= 8].

5.6 Discussion

The data presented in this chapter suggest that modulating the expression of PR-A, PR-B and GR does impact P4 activity as well as its inhibition of IL-1β-driven prolabour gene expression. Despite these results though, more work is required to determine which receptor plays the more dominant role in bringing about this effect in human amnion cells.

Previous group findings have demonstrated that in myometrial cells, P4 acts via GR to exert its anti-inflammatory effect and reduce IL-1β-driven COX-2 mRNA expression (119). In contrast, in the absence of GR, P4 maintained its ability to inhibit IL-1β-driven prolabour expression (Figure 5.2) in human amnion cells suggesting that this interaction was independent of GR.

In amnion cells were treated with the PR/GR antagonist, RU486, P4 lost its ability to significantly reduce IL-1β-driven COX-2 mRNA expression (Figure 5.1), but a trend in IL-1β-driven COX-2 mRNA was still apparent. This suggests either that RU486 inhibition was partial or that P4 acts via an alternative pathway to inhibit IL-1β-induced COX-2. Similarly, in myometrial cells, RU486 treatment reduced P4's inhibitory action; this effect was mediated via GR-induced MKP-1 activation (187). In the rat corpus luteum (311), P4 inhibited 20 alpha-hydroxysteroid dehydrogenase via GR, inducing a decline in TLR4-mediated macrophage activation (118). It is important to note that PR levels in the rat CL are also undetectable PR; similar to the situation in human amnion cells, where PR levels are so low that knock down is challenging. The previous study also found RU486 administration impeded P4 effects (311), whilst a specific PR agonist was unable to decrease TLR4 actions (118) suggesting a GR-mediated action.

The hypothesis that P4 acts via PR to inhibit NFκB activation is largely based on research by Kalkhoven *et al*, which highlighted that *in* vitro, a direct inhibition was present between PR and p65. Furthermore, they found a link between PR activity and TNFα; TNFα stimulation inhibited PR activity whilst, PR was able to reduce TNFα-stimulated activation of NFκB (289). Similar results were found in primary human amnion cells (266) and were also replicated in human myometrial cells (119). It was hypothesised that if P4 was acting through P4 receptor sub-types A and B, then

modulating their individual expressions may provide some evidence for this proposed theory. Low endogenous PR levels (178, 264) posed a challenge in identifying whether P4's anti-inflammatory actions were in fact working via PR-A or PR-B instead of GR. However, overexpression of receptor subtypes resulted in significantly enhanced P4 repression of IL-1β driven COX-2 mRNA expression (Figure 5.3 & 5.4).

However, it is important to note that during the overexpression process of PR receptor sub-types, the other receptor isoform ended up also being overexpressed. This may be due to PR-B being truncated and therefore resulting in a truncated version of PR-A, which may have been additionally over-expressed however this is unclear. Furthermore, it is not known whether there is a compensatory mechanism that may produce more PR-B when PR-A is overexpressed or whether is it endogenous PR-B being witnessed. However, unfortunately since endogenous PR is very low in the amnion, this remains unclear.

In contrast, no statistically significant enhancement was noted in P4's repression when GR was over-expressed (Figure 5.5). Overall, these data suggest that P4 acts via PR to repress IL-1β driven COX-2 mRNA.

In terms of P4-transactivation, it seems clear that P4 acts via PR to increase both FKBP5 and 11β HSD mRNA levels. P4 driven FKBP5 expression was unaltered in the absence of GR whilst, P4 driven 11β HSD mRNA expression increased significantly (Figure 5.6). These data suggest that GR might actually act as a decoy, binding P4 and preventing its ability to drive 11β HSD gene expression. Overexpression of all receptors resulted in significantly enhanced P4 driven FKBP5 mRNA expression following treatment with 10 μM P4, although the effect of GR was relatively less (Figure 5.7). P4 driven 11β HSD mRNA expression was only significantly enhanced when PR-A and PR-B were overexpressed and remained unaltered by the overexpression of GR. Together, these data suggest that although GR may have the potential to mediate P4 action, P4 predominantly exerts its effect on gene expression via PR-A and PR-B. In contrast, in myometrial cells, P4 operated through both PR and GR in order to mediate FKBP5 expression and through PR alone to stimulate mRNA expression of 11βHSD1 (59, 119).

These data suggest an important role for PR subtypes in mediating P4's anti-inflammatory activity within amnion cells. However, the results from this chapter suggest that P4 also has an affinity for other pathways too and therefore leaves it an area for further research.

Summary

This chapter has made an initial attempt to answer the question of which nuclear receptor P4 may be exerting its anti-inflammatory action through in primary human amnion cells. Whilst P4 is able to act via GR to inhibit IL-1β-driven COX-2 mRNA in human myometrial cells, the same cannot be concluded in amnion cells and appears to act through PR receptors. Though P4-induced FKBP5 and 11βHSD mRNA expression may be enhanced through the overexpression of P4 receptor subtypes, the data from RU486 treatment suggests that alternative mechanisms may also be involved. Additional assessment is required to further understand the mechanisms of P4 anti-inflammatory action.

Chapter 6: Final Discussion

Preterm birth is a major global health issue, which in the majority of countries over the last 50 years has increased in incidence. It is imperative to understand the nature of human pregnancy and labour better, before being able to design effective preventative strategies. Indeed, reinforcing this statement, recent data question the efficacy of the only medical approach to the prevention of PTL, P4 (44). This work seeks to understand how the amnion behaves in terms of inflammation, prolabour gene expression and the steroid (glucocorticoid and P4) pathways during pregnancy and the onset of term and PTL. Further it explores how P4 works in the amnion and whether its efficacy can be enhanced by the addition of a cAMP agonist. Understanding these elements of amnion physiology and pathology will help us define whether the amnion should be considered a target for therapies aiming to reduce the risk of PTL. In addition, the results of these studies may suggest alternative potential therapeutic approaches in high-risk women.

6.1 Main Findings & Future Work

The hypotheses tested whether

1. Amnion inflammation occurs in early labour (TEaL) and, consequently, may have a role in the onset of human labour;
2. Prolabour gene expression is increased in amnion samples in TEaL and that
3. Both are related to a decline in progesterone (P4) signalling.

In later chapters of this thesis, hypotheses tested that

4. P4 acts via PRs and not glucocorticoid receptors (GRs) and
5. P4 function in the amnion can be enhanced by the addition of cyclic AMP

were tested.

6.1.1 Amnion inflammation occurs in both early and established labour at term

Analysis proposed that amnion inflammation occurs most prominently in TEsL but is still present at TEaL. The data presented here showed a significant increase in IL6 and IL8 levels between term non-labouring and early labour amnion samples, a significant elevation in CXCL1, IL6, IL8, CCL2, IL-1β was observed between TNL and TEsL amnion samples.

6.1.2 Prolabour gene expression is increased in established labour at term

Prolabour gene expression was also observed more prominently in TEsL. Established labour samples also displayed: (i) higher COX-2 protein levels compared with non-labouring samples, and (ii) higher OTR protein levels and Cxn 43 levels compared with TEaL samples. These data suggest that the principal elevation in prolabour expression in human amnion occurs after the initial onset of labour and is therefore considered a consequence of labour, rather than a direct cause. These findings correlate with recent findings from Singh *et al,* who showed heightened cytokine profiling in established myometrial tissue samples at term (76).

Findings from amnion tissue also demonstrated that prolabour gene expression differs depending on the underlying aetiology of PTL. Some marked changes included an increase in OTR protein seen in samples from women with chorioamnionitis, which could be accounted for by OTR's role in stimulating proinflammatory cytokine production in PTL (257, 258). In addition, Cxn43 protein was elevated in PTTWL amnion samples. Twin gestations are often associated with stretch-induced PTL and the rise in Cxn43 expression has also been observed in animal studies of *in vitro* stretch in pregnant rats (50).

6.1.3 Steroid receptor activity and P4 signalling

An assessment of nuclear receptor expression in term and preterm amnion tissue suggested a shift in PR isoform ratios from PR-B to PR-A dominance with term labour onset. Though expression of P4 co-regulators did not show marked results, the inability to characterise certain co-factors such as NCoR and SMRT at the protein level supports the theory that they may not be involved in PR physiology in the amnion. However, alternative studies where NCoR has been characterised highlight how further research is often needed to confirm a cofactor's key role.

In conclusion, the assessment of gene expression in term and preterm labour is critical to our understanding and ability to treat PTL. This comprises: (i) the ability to observe a shift in PR-A: PR-B ratio as an indicator for the transition between uterine quiescence to activation, and (ii) the use of proinflammatory cytokine expression as a predictor for preterm birth. Each aspect plays a significant role in the management of PTL.

The ratio of PR-A and PR-B is often taken as an indicator to mark the adjustment from uterine quiescence to activation. These data suggest that though this may be the case, further work is required to fully characterise PR's role in the human amnion. In addition, the data presented shows the expression pattern of nuclear receptor mediators in the human amnion. The observed changes in expression differed depending on the type of term and PTL. In addition, insignificant changes in PR levels may indicate that these nuclear receptor coactivators may be working independently of PR. These data allude to the complex role and interaction nuclear receptors and coregulators may have in the human amnion during human parturition.

6.1.4 P4 function in the amnion can be enhanced through the addition of cAMP

Data from Chapter 4 highlighted that *in vitro* administration of P4 was able to significantly reduce IL-1β-induced prolabour gene expression in the absence of mechanical stretch. However, it was noted that the stretch-induced changes seen in human amnion cells subjected to mechanical *in vitro* stretch could not be inhibited through P4 treatment, alone or with forskolin. This suggests that further research is required in attempting to prevent stretch-induced PTL.

To enhance our understanding in this area, it will be important to investigate the expression and activity of nuclear phospho-p65 following IL-1β stimulation. Also, it will be important to evaluate whether the reduction of IL-1β-induced COX-2 expression, following P4 treatment correlates with less NF- κB binding to the COX-2 promoter and what impact additional forskolin would have.

6.1.5 P4 acts via PRs and not glucocorticoid receptors (GRs)

Determining the exact mechanism through which P4 is able to exert its anti-inflammatory effect requires further investigation. However, the data presented in Chapter 5 proposes that PR subtypes play an important role in mediating P4 activity in human amnion cells.

Knocking down GR did not impact P4's ability to reduce IL-1β driven COX-2 mRNA expression, nor did it affect P4 driven FKBP5 expression. Overexpression of PR subtypes however, was able to significantly enhance P4 driven activity. Taken together, these findings suggest that GR is not solely responsible for P4's inflammatory mechanism as was observed in the myometrium. Instead it is considered that though the dominant pathway involves P4 receptors, alternative mechanisms may also be involved in low or compromised PR environments in the amnion.

In order to examine the relationship between PR and NF-κB in human amnion cells, it will be important to incorporate TF: TF arrays and reporter constructs. Immunoprecipitation would evaluate the ability of both GR and PRs to bind in human amnion cells. It would also be important to confirm whether P4 treatment leads to a decrease in IL-1β-stimulated p65 phosphorylation and also whether nuclear

translocation occurs. Furthermore, evaluating P4- p65 binding to NF-κB binding elements of the COX-2 promoter will also help evaluate how these anti-inflammatory mechanisms operate.

Future work involving cAMP will also open up opportunities to explore the action of cAMP during altered nuclear receptor expression. Through knockdown and overexpression studies of PR and GR, it will be important to observe the role cAMP adopts and whether it is still able to enhance the action of P4 as well as evaluate mRNA expression of P4 responsive genes.

6.2 Conclusion

Data presented in this thesis supports the existing literature as well recent evidence from the group. In addition, it forms a foundation for further research into the possible roles of these prolabour factors in the amnion and the onset of term and preterm labour. It supports the theory that inflammation observed in gestational tissues is a consequence of labour and furthermore, may also aid in refining the development of current clinical biomarkers for the prediction of PTL. The ability to reduce IL-1β-driven gene expression in human amnion cells through both P4 and in combination with cAMP in the absence of *in vitro* stretch is an encouraging finding for future PTL management. Though the inability to reduce stretch-induced gene expression highlights the need for further investigation in this area. Defining the mechanisms through which P4 is able to target IL-1β-driven gene expression in human amnion cells will help our consolidate our understanding in this and hopefully help in the development of future PTL prevention strategies.

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Appendix

Appendix 1 Top 50 up-regulated genes by P4.

gene_assignment	Gene Symbol	RefSeq	[siNT down vs. siNT(P4)]
ATP-binding cassette, sub-family A (ABC1), member 1	ABCA1	NM_005502	-2.75844
FK506 binding protein 5	FKBP5	NM_001145775	-2.72812
LOC100131826	LOC100131826	AY358789	-2.64534
small nucleolar RNA, C/D box 26	SNORD26	NR_002564	-2.50998
hippocampus abundant transcript-like 1	HIATL1	NM_032558	-2.23635
vaccinia related kinase 2	VRK2	ENST0000042802 1	-2.10109
ERBB receptor feedback inhibitor 1	ERRFI1	NM_018948	-2.09597
small nucleolar RNA, H/ACA box 70G	SNORA70G	NR_033335	-2.06689
hypothetical protein DKFZp686O24166	DKFZp686O2416 6	NR_026750	-2.06126
mitochondrial ribosomal protein L45	MRPL45	NM_032351	-2.05865
transmembrane protein 199	TMEM199	NM_152464	-2.04879
postmeiotic segregation increased 2-like 1 pseudogene	PMS2L1	NR_003613	-2.03471
transmembrane protein 111	TMEM111	NM_018447	-2.00364
microRNA 622	MIR622	NR_030754	-1.99393
fatty acid binding protein 3, muscle and heart	FABP3	NM_004102	-1.99212
cirrhosis, autosomal recessive 1A	CIRH1A	NM_032830	-1.98269
small nuclear ribonucleoprotein polypeptide A	SNRPA1	NM_003090	-1.97423
adaptor-related protein complex 4, beta 1 subunit	AP4B1	NM_006594	-1.97143
solute carrier family 31 (copper transporters), member 1	SLC31A1	NM_001859	-1.94897
eukaryotic translation initiation factor 3, subunit L	EIF3L	NM_016091	-1.94246
solute carrier family 37	SLC37A3	NM_207113	-1.9315
polymerase (RNA) II (DNA directed) polypeptide G	POLR2G	NM_002696	-1.92569
cytochrome b5 reductase 1	CYB5R1	NM_016243	-1.91244
retinol dehydrogenase 11	RDH11	NM_016026	-1.91228
Niemann-Pick disease, type C1	NPC1	NM_000271	-1.912
amyloid beta (A4) precursor protein-binding, family B	APBB2	NM_004307	-1.90965
acetyl-CoA carboxylase alpha	ACACA	NM_198839	-1.90307
membrane protein, palmitoylated 1	MPP1	NM_002436	-1.90278
NHP2 non-histone chromosome protein 2-like 1	NHP2L1	NM_005008	-1.8907
presenilin associated, rhomboid-like	PARL	NM_018622	-1.87751
glutamic-oxaloacetic transaminase 1	GOT1	NM_002079	-1.86537

zinc finger protein 90	ZNF90	AK298173	-1.85633
Der1-like domain family, member 1	DERL1	NM_024295	-1.85021
eukaryotic translation initiation factor 3, subunit I	EIF3I	NM_003757	-1.84026
elongation factor Tu GTP binding domain containing 1	EFTUD1	NM_024580	-1.83168
3-hydroxy-3-methylglutaryl-CoA reductase	HMGCR	NM_000859	-1.82625
bifunctional apoptosis regulator	BFAR	NM_016561	-1.82429
SMG1 homolog, phosphatidylinositol 3-kinase-related kinase	SMG1	NM_015092	-1.82221
LAG1 homolog, ceramide synthase 5	LASS5	NM_147190	-1.81841
transforming growth factor, beta receptor II	TGFBR2	NM_001024847	-1.815
adaptor-related protein complex 3, mu 2 subunit	AP3M2	NM_001134296	-1.81218
excision repair cross-complementing rodent repair deficiency	ERCC3	NM_000122	-1.80516
peptidase domain containing associated with muscle regeneration	PAMR1	NM_015430	-1.79952
sushi, von Willebrand factor type A	SVEP1	NM_153366	-1.78903
keratin 18	KRT18	NM_000224	-1.78758
Williams Beuren syndrome chromosome region 22	WBSCR22	NM_017528	-1.78287
trafficking protein particle complex 4	TRAPPC4	NM_016146	-1.78025
tubby like protein 3	TULP3	NM_003324	-1.7782
X-prolyl aminopeptidase (aminopeptidase P) 3	XPNPEP3	NM_022098	-1.77432
karyopherin alpha 2	KPNA2	NM_002266	-1.7725