Parturition, oxytocin, inflammation, myocyte damage and obesity; A study of myometrium and haematological parameters in human pregnancy and labour at term

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Abstract

The process of parturition resulting in the delivery of a newborn is a fundamental event ensuring survival of the species. In humans, the main clinical problems of parturition include activation of the process too early or too late resulting in the delivery of pre-term and post-term infants, both with their own implications for future health for the mother and baby. Additionally, where parturition systems are not activated correctly, dysfunctional labour with the resulting need for caesarean delivery (CS), in addition to atonic post-partum haemorrhage can also ensue. Overall, in the UK up to 40% of pregnancies are affected by one of these problems. However, the exact processes involved in the initiation and maintenance of parturition in the human are not fully understood. With such an important event, influences are most likely to be multi-factorial, with hormonal, mechanical, inflammatory, biochemical and maternal environmental factors playing a part.

The aims of this thesis were to investigate influences on parturition in human pregnancy. Firstly, the myometrial transcriptional effects of long term exposure to the uterotonic oxytocin (OT) were examined. Further investigation of the myometrial and maternal peripheral response to uterine contractions *in-vitro* and *in-vivo* was also made with particular reference to the role of inflammation and myocyte damage. Additionally, the influence of maternal factors, particularly obesity, on the myometrial *in-vitro* contractile function and response to OT was studied.

Initially, 150 gene arrays were produced using the Illumina platform. The samples were derived from myometrium taken at pre-labour CS which subsequently underwent functional contractility experiments in an organ bath. Five drug environments were studied, namely OT, acetic acid (OT vehicle), ML7 (a tocolytic acting via inhibition of myosin light chain kinase), ML7 & OT and finally DMSO (ML7 vehicle). Additionally, five time-points of 0, 1, 2, 4, and 6 hours after drug addition were used, resulting in 5 samples for each drug and time combination. The results indicated that despite a clear enhancement of myometrial contractile activity by OT, this functional response does not appear to be mediated by cellular transcription. However, there was a clear contraction and time dependent transcriptional wave, with overrepresentation

of genes associated with inflammation and cellular damage/apoptosis, and down-regulation of pathways concerning cellular metabolism.

These findings were confirmed by QPCR on further myometrial samples undergoing additional *in-vitro* functional studies. In addition to the temporal and contractile association with the inflammatory response, our data suggest inflammation occurs in response to myocyte cellular damage regardless of mode of damage e.g. contractile or chemically induced. This was demonstrated by inflammatory upregulation in myometrium exposed to the tocolytic agents nifedipine and ritodrine, which is not seen in response to ML7. Additionally, the myometrial inflammatory response was enhanced by the infective agent LPS. However, contrary to other proposals, the enhanced inflammatory response of the myometrium did not alter or promote the *in-vitro* contractile ability of the myometrium or its response to OT. This myometrial transcriptional data therefore suggests that the inflammatory response of labour is associated with contraction, chemical or infection induced myometrial cellular damage, but would not be considered necessary for a contractile response.

Our *in-vivo* study of peripheral changes in the maternal circulation again supported our *in-vitro* myometrial data. Data showed that the effect of pregnancy at term was limited to increased white cell count driven by a neutrophilia, with no suggestion of leukocyte priming prior to labour. Additionally, term pregnancy is associated with an increase in CRP, an increase in GCSF (corresponding with the neutrophilia) in addition to suppression of the chemokines CCL11 and CCL22. Subsequently, we found that repeated blood samples taken at 2 hourly intervals during term labour induced dramatic changes in inflammatory cells and inflammatory mediators in the maternal circulation. Importantly, these changes occur in a co-ordinated time and contraction dependent manner, with the degree of inflammation associated with the length of time in labour and the degree of myocyte damage as measured by circulating CK and Mb.

Our study of the influence of maternal factors on myometrial contractile ability and response to OT examined *in-vitro* myometrial contractility of 609 myometrial strips from 85 women. We demonstrated that maternal obesity does not impair spontaneous or OT induced myometrial contractions *in-vitro*.

Furthermore, maternal age, ethnicity, parity, previous caesarean delivery, gestation at delivery and birthweight do not influence *in-vitro* myometrial spontaneous or OT induced contractile activity. This therefore suggests that the observed implication of these maternal and infant factors on parturition *in-vivo* (high rates of induction of labour, high rates of intrapartum caesarean delivery and post partum haemorrhage) cannot be explained by an effect on myometrial contraction *per se*. This therefore merits further investigation as to alternative mechanisms to ultimately promote and effective, uncomplicated and safe labour and vaginal delivery for at risk mothers.

In summary, this thesis provides evidence that the myometrial contractions of human labour, whether spontaneous or OT induced are capable of inducing a temporal wave of transcriptional changes associated with the processes of inflammation, cellular damage/apoptosis with inhibition of cellular metabolic processes. In addition, maternal peripheral circulating factors mirror the myometrial transcriptional changes. These changes are highly comparable with those seen in response to exercising skeletal muscle, and in this model have been shown to play an important role in muscle repair and remodelling after exercise. Therefore, we would suggest that the inflammatory reaction typically associated with human labour occurs as a non-specific response to contraction induced cellular damage and may play a role in postpartum repair and remodelling of the uterus.

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Full Publications

Menzies FM, Higgins CA, Shepherd MC, Nibbs RJB, Nelson SM. Mast cells reside in myometrium and cervix, but are dispensable in mice for successful pregnancy and labor. *Immunology and Cell Biology* 2012;90(3):321-29

Higgins CA, Martin W, Anderson L, Blanks AM, Norman JE, McConnachie A, et al. Maternal Obesity and its Relationship With Spontaneous and Oxytocin-Induced Contractility of Human Myometrium In Vitro. *Reproductive Sciences* 2010;17(2):177-85.

Anderson L, Martin W, Higgins C, Nelson SM, Norman JE. The Effect of Progesterone on Myometrial Contractility, Potassium Channels and Tocolytic Efficacy. *Reproductive Sciences* 2009;16(11):1052-61.

Published Abstracts

Menzies FM, Oldham R, Higgins CA, Nibbs RJB, Nelson SM. Temporal Analysis of Chemokine Production and Immune Cell Influx into the Uterus in Mice and Humans. *Reproductive Sciences* 2011;18(3):243A-43A.

Menzies FM, Oldham R, Higgins CA, Nibbs RJ, Nelson SM. Temporal analysis of chemokine production and immune cell influx into the uterus in mice and humans. *Immunology* 2010;131:61-61.

Higgins CA, McConnachie A, Nibbs RJB, Nelson SM. Temporal Changes to Leukocyte Subpopulations, Chemokine Receptor Expression and Circulating Cytokines during Human Parturition. *Reproductive Sciences* 2010;17(3):179A-79A.

Higgins CA, Martin W, Blanks AM, Catalano R, Thornton S, Nelson SM. Myometrial Cytokines and Chemokines during Human Labour: A Co-Ordinated Time and Contraction Dependent Event. *Reproductive Sciences* 2010;17(3):179A-79A.

Higgins CA, Martin W, Blanks AM, Nelson SM. Lipopolysaccharide induces and intense inflammatory response to myometrium, but is not associated with a functional contractile response *in-vitro*. *Bjog-an International Journal of Obstetrics and Gynaecology* 2010;117(5):631-32.

Higgins CA, Martin W, Blanks A, Thornton S, Nelson SM. Investigation of Myometrial Gene Expression in Response to Sustained Spontaneous and Oxytocin Induced Contraction *In-vitro*. *Reproductive Sciences* 2009;16(3):292A-93A.

Higgins CA, Martin W, Blanks A, Thornton S, Norman JE, Nelson SM. Maternal obesity does not impair spontaneous or oxytocin-induced contractility of human myometrium in vitro. *Reproductive Sciences* 2008;15(2):113A-14A.

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I would therefore like to dedicate this thesis to my Dad, Dr John Higgins and my beautiful daughter Katie Mary Foley.

Author's Declaration

The contents of this thesis have not been submitted elsewhere for any other degree, diploma or professional qualification.

This thesis has been composed by me, and I have been responsible for patient recruitment, tissue collection and laboratory studies and analysis unless otherwise acknowledged.

Claire Angela Higgins, July 2012.

List of Abbreviations

AA	Acetic Acid
AP	action potential
APC	Allophycocyanin
ARM	Artificial rupture of membranes
BMI	Body Mass Index
Ca/Ca2+	Calcium
$CaCl_2$	Calcium Chloride
CK	Creatine Kinase
Cl-	Chloride
CO_2	Carbon Dioxide
COX	cyclooxygenase
CRP	C reactive protein
CS	Caesarean section/delivery
Ct	Cycle threshold
CV	Coefficient of variation
DAG	Diacylglycerol
DDW	Deionised water
DEPC	Diethylpyrocarbonate
DMSO	Dimethyl sulphoxide
EGF	Epidermal Growth Factor
FBS	Foetal Bovine Serum
FITC	Fluorescein isothiocyanate
GO	Gene Ontology
IOL	Induction of labour
IP3	inositol-1,4,5-triphosphate
IQR	Interquartile Range
K+	Potassium
KCl	Potassium Chloride
KEGG	Kyoto Encyclopaedia of Genes and Genomes
KH_2PO_4	Monopotassium Phosphate
LMP	Last menstrual period
LPS	Lipopolysaccharide
Mb	Myoglobin

MFI	Mean Fluoresence intensity
$MgSO_4$	Magnesium Sulphate
ML7	1-(5-Iodonaphthalene-1-sulfonyl)-1H-hexahydro-1,4-diazepine hydrochloride
MLC	Myosin Light Chain
MLCK	Myosin Light Chain Kinase
MLCP	Myosin Light Chain Phosphatase
Na+	Sodium
NaCl	Sodium Chloride
NaHCO3	Sodium Bicarbonate
NIL	Not in Labour
NP	Non-pregnant
O_2	Oxygen
OT	Oxytocin
OTR	Oxytocin Receptor
PBS	Phosphate Buffered Saline
PE	Phycoerythrin
PG	Prostaglandin
PIP_2	phosphatidylinositol 4,5-bisphosphate
PKC	protein kinase C
PMB	Polymixin B
PPH	postpartum haemorrhage
QPCR	quantitative real time polymerase chain reaction
TES	tris(hydroxymethyl)methyl-2-amino-ethanesulfonic acid
WBC	White Blood Cell

Chapter 1

General Introduction and themes of thesis

1 Introduction

1.1 Human parturition

The process of human parturition leading to delivery of a newborn infant is essential to secure the survival of mankind. Worldwide figures indicate that during the year 2012 there will be an estimated 134,475,000 live births adding to the population, with 774,000 in the UK[1]. Despite parturition being such a fundamental event, the exact processes involved in the initiation and maintenance of human parturition are not fully understood.

In humans, normal pregnancy is classically considered as spanning 40 weeks from the date of the last menstrual period (LMP), with term being considered at any time after 37 completed weeks. During pregnancy it is essential that the contractile apparatus of the uterus remains relaxed and quiescent to accommodate the growing foetus with the cervix remaining rigid and closed. Successful parturition is achieved at term when the cervix starts to soften (ripen) with breakdown of the collagen fibres. At the same time the uterus becomes more responsive and active with the resultant contractile activity typically required for labour and delivery of the newborn. After delivery the uterus involutes with eventual return of normal function and preparation for future pregnancy. The typical stages and timeline for the uterus in pregnancy are illustrated in Figure 1.

Figure 1 Phases of myometrial activation

Depiction of the typical stages and approximate timing of the activity of the contractile apparatus during pregnancy and parturition.

1.2 The clinical problems

The clinical implications of incorrect activation of myometrial associated parturition pathways can be considered as three categories; activation too early, activation too late or dysfunctional activation either during labour or in the post-partum period. These are summarised below in Figure 2.

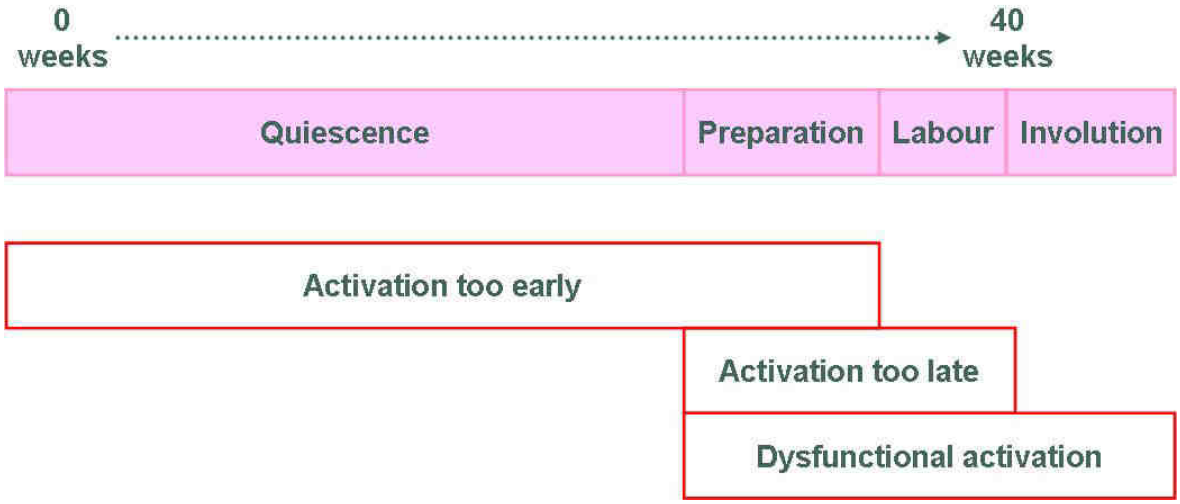

Figure 2 Phases of myometrial activation

Graphical depiction of timing of inappropriate activation of parturition pathways resulting in clinical problems with the mother or infant.

It is estimated that overall in the UK up to 40% of pregnancies may be affected by incorrect activation of parturition pathways[2-8], with each described in more detail in the following sections.

It remains unclear as to why aberrant activation of myometrial associated parturition pathways occurs, however, knowledge regarding these processes including modulating factors has the potential to help prevent abnormal activation and promote safe, effective and efficient parturition and delivery of the newborn infant.

1.2.1 Activation too early

Activation of the parturition processes prior to 37 completed weeks of pregnancy is considered too early and may result in the delivery of a pre-term infant. It is estimated that 1 in 10 of all babies born world wide are born prematurely, with rates ranging from 4% to 20%[2]. Scottish figures for 2010 indicate preterm

delivery affected 7.5% of all births and 6% of all singleton births[3]. English data show similar figures with the rate of preterm delivery standing at 7.3% of live born singletons 2010-2011[4]. Additionally, recent data reviews have suggested preterm birth rates appear to be increasing, both in developed and developing populations[5].

The implications of pre-term delivery can be severe with estimate of 1million children per year dying worldwide as a result of prematurity, with risks of severe physical and mental disabilities or difficulties inversely related to gestation in those who survive[2 6-11]. Additionally there are long term implications for both the mother and wider family following the birth of a pre-term infant[10 12-14]. In terms of the economic costs, infants born prematurely commonly require intensive neonatal care input along with lifelong supportive costs for any residual physical or learning difficulties [10 11 15-17].

It must be acknowledged that premature delivery does not necessarily occur as a result of pre-term labour, with approximately 30% of premature deliveries being iatrogenic[18]. In these situations, early delivery either by induction of labour or caesarean delivery, is expedited as a medical response when there is maternal or foetal risk if the pregnancy were to continue, many of which are linked to hypertensive or pre-eclamptic pathologies[18 19].

The remaining 70% of preterm deliveries occur secondary to either pre-term labour with intact membranes and premature activation of the uterine contractile machinery (40-45%), or following premature rupture of membranes (25-30%)[18 20]. In approximately 50% of those experiencing spontaneous preterm birth no identifiable cause is found. Therefore, the aetiology can be considered multifactorial with risk factors including multiple pregnancy, maternal body mass index (BMI), maternal age, previous or familial history of premature birth, cervical dysfunction, maternal ethnicity, maternal infection, antepartum haemorrhage, cigarette smoking, high alcohol consumption, use of recreational drugs, socioeconomic status and maternal stress[18 19 21-31].

It remains unclear as to whether the processes of term and preterm labours can be considered identical with the only difference being gestation. The occurrence of increased uterine contractility and cervical ripening and dilatation are certainly common to both situations. However, the concept of the ‘preterm

parturition syndrome' has been proposed where preterm activation of the contractile machinery is influenced by pathological signalling pathways influenced by intrauterine infection, uterine ischaemia, uterine overdistension, abnormal allogenic recognition, allergic-like reaction, cervical disease, and endocrine disorders [23]. Many of the factors suggested to be involved in this pathological activation are discussed later in this chapter.

1.2.2 Activation too late

As term approaches most women will labour spontaneously. In the United States, prolonged or 'post-dates' pregnancies are estimated to affect 5-10% of all pregnancies, and where no obstetric intervention is undertaken estimated rates of 18% of all singleton pregnancies last >41 weeks, 10% (range 3% to 14%) >42 weeks and 4% (range 2% to 7%) continue beyond 43 weeks[32]. English data for 2010-2011 indicate that deliveries after 42 weeks account for 3.6% of all live births and 2.3% of all births with spontaneous onset[4]. Additionally, at >42 weeks gestation, 33.9% of deliveries at this gestation will occur following spontaneous onset of labour compared with 79.0% at 40 weeks gestation[4]. Conversely, 34.3%, 19.6% and 11.9% of all inductions of labour occur beyond at 40, 41 and 42 week gestation respectively, with 56.5% of all labours at >42 weeks requiring induced onset[4].

Post dates pregnancies have implications for both the mother and the infant. Post dates pregnancy increases the rates of antepartum and intrapartum stillbirth[33-35], perinatal death and morbidity many of which are related to meconium aspiration[36-40]. Some have also shown increased risks of shoulder dystocia, intrapartum caesarean section for both foetal distress and failure to progress in labour, instrumental deliveries, severe perineal trauma and injury, and post partum haemorrhage (PPH)[38 40-44]. Additionally, new data suggest that post-term birth may also affect development of the child in later life, with increased risks of behavioural and emotional problems in childhood[45].

Interestingly, family history tends to increase the incidence of post dates pregnancy, with both maternal and paternal influences if they themselves were born postdates[46]. This suggests that the contractile mechanisms and responsiveness of the myometrium may be genetically determined.

It is recommended practice in the UK to offer induction of labour to women with uncomplicated pregnancies between 41+0 and 42+0 weeks gestation[44]. As discussed earlier, induction of labour is also undertaken at earlier gestations for other clinical reasons where the mother or infant may be compromised[18 19]. Therefore, overall, induction of labour accounted for 22.5% of all live births in Scotland 2010[3].

However, induction of labour (IOL) is not without risks itself. The process can last longer[47] and be perceived to be more painful than spontaneous labour, increasing the need for regional analgesia[48]. Additionally, there are risks of uterine hyperstimulation and rupture. Induction of labour may not always work, and some have suggested higher rates of intrapartum caesarean delivery or assisted vaginal delivery in those undergoing elective induction of labour compared with expectant management[48-54]. However, this may be influenced by parity, maternal age, cervical length or medical interpretation of length of labour rather than the induction process itself[47 52 55 56]. IOL also has both physical and emotional implications for the mother[57].

1.2.3 Dysfunctional activation

Dysfunctional activation of the myometrial contractile machinery can result in two main clinical problems. Firstly, dysfunctional labour may present as poor uterine contractility which may result in the need for an assisted vaginal delivery (forceps or ventouse) or delivery by intrapartum caesarean section. Secondly, failure of contraction mechanisms post delivery can result in atonic post partum haemorrhage (PPH) with excessive blood loss following delivery.

Overall caesarean section (CS) rates remain high at 26.6% of live births in Scotland with 11.3% of all deliveries classed as elective CS and 15.3% as emergency CS[3]. Failure to progress in labour has been identified as a major aetiological factor in the decision to perform an emergency CS, and is cited as a primary indication in up to 53% of intrapartum CS deliveries in singleton cephalic pregnancies[58-61]. This occurs despite augmentation of labour with the uterotonin Syntocinon® as up to 81% of these women have received oxytocin (OT) prior to CS[7 14].

There are specific maternal and pregnancy characteristics which appear to negatively influence and tend to suppress uterine contractility *in-vivo*. These include maternal BMI[62-67], maternal age[68-71], nulliparity[72 73], previous mode of delivery[74-78], gestation[79 80] and birthweight[79]. The mechanisms for this negative influence of patient characteristics upon myometrial contractile function remain uncertain.

PPH is one of the most common obstetric emergencies and haemorrhage at anytime in pregnancy represents the sixth leading cause of maternal mortality in the UK during 2006-2009[81]. PPH is defined as a loss of >500ml of blood from the genital tract which can either be within 24 hours of delivery (primary PPH) or between 24 hours and 12 weeks post delivery (secondary PPH). The rate of PPH in England is approximately 12%[4]. In addition major obstetric haemorrhage is considered to be the loss of ≥2500mls of blood or the requirement of a transfusion of ≥5units of blood. Rates of major obstetric haemorrhage have increased in Scotland, with an incidence of 3.7 per 1000 live births in 2003-2005 and 5.18 per 1000 live births in 2009. Notably, atony of the uterus secondary to dysfunction of the contractile mechanisms rendering the uterus unable to contract sufficiently to control bleeding is responsible for up to 50% of PPH cases[82 83].

Overall, it remains unclear as to why aberrant activation of myometrial associated parturition pathways occurs with the clinical outcomes described above. The next section will attempt to summarise current knowledge of the contractile apparatus involved in parturition and factors known to influence activation of contractile pathways.

1.3 The Uterus and parturition related contractile machinery

The uterus is a hollow fibromuscular organ which sits within the female pelvis, bounded anteriorly by the bladder and posteriorly by the rectum[84]. It is subject to multiple changes throughout a woman's life; cyclical changes related to menstruation, accommodation of the growing foetus during pregnancy, force generation during labour and subsequent post partum remodelling. In terms of size, in the nulliparous adult, the uterus is approximately 6-8cm long, 5cm wide, with the walls being 2.5 cm thick and can weigh up to 70 grams[85]. During pregnancy, hyperplasia and cellular hypertrophy of the uterus result in an enlargement of four to five times with a final weight of approximately 1000 grams and the ability to accommodate a total volume of up to 5 litres[85 86].

Anatomically, the uterus can be considered in two parts; the upper muscular uterine body (or corpus) and the lower fibrous uterine cervix[84 87] (Figure 3). Of note is the area described as the isthmus in the non-pregnant uterus; an area 0.5-1.0cm long which is still considered part of the corpus and is bounded superiorly by the anatomical internal os and inferiorly by the histological internal os[84 87-89](Figure 3). It is the isthmus which is thought to expand after 28-30 weeks gestation to approximately 2.5cm in length and is at this point referred to as the lower uterine segment, an anatomical area important in caesarean delivery. Of note, despite being thinner walled than the upper uterine corpus during pregnancy, the lower segment consists of approximately 70-80% uterine myocyte[90 91], and displays a similar *in-vitro* contractile ability to samples from the upper segment[92].

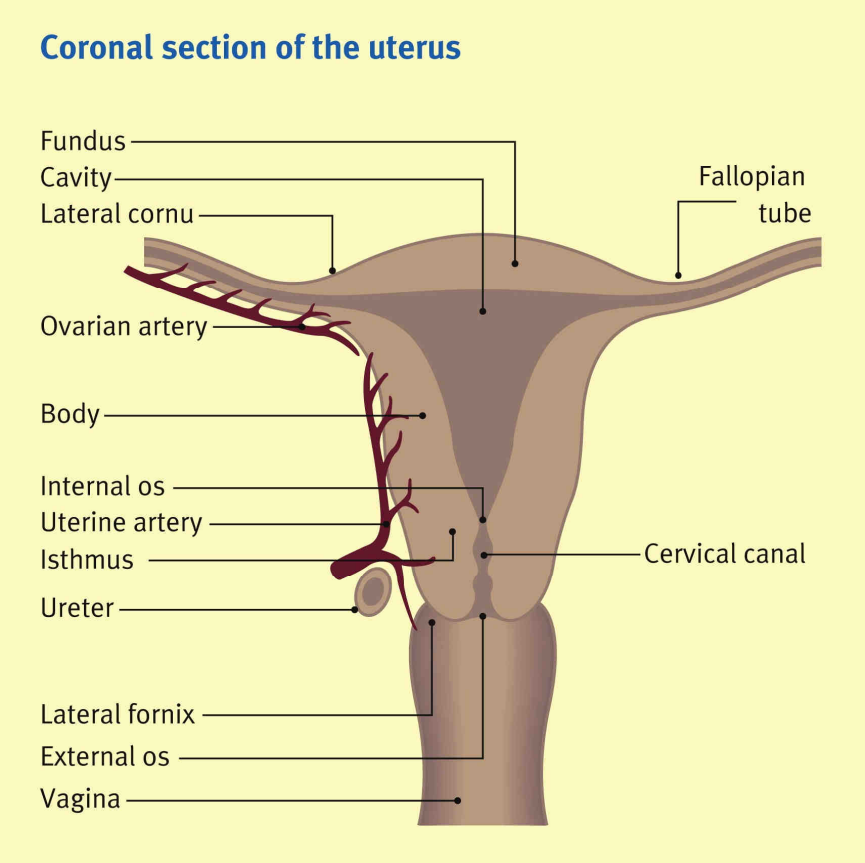

Figure 3 Gross anatomy of the human uterus
Reproduced from Anatomy of Uterus, Harold Ellis[87]

During pregnancy, the expulsive forces of the muscular corpus must be balanced against the resistance or competence of the cervix in order to facilitate in-utero development of the infant until term with subsequent changes in the balance of forces allowing for delivery. The current knowledge regarding influences on this change in balance between the cervix and the uterine body will be further discussed in the remainder of this chapter.

1.3.1 The uterine cervix

In many aspects, the cervix appears to be a continuation of the uterus (Figure 3). However it differs in its constitution with sparse contractile myocytes and a preponderance of fibrous connective tissue[89 93-95]. It is this connective tissue which contributes to the tensile structure of the cervix during pregnancy. At term the connective tissue degrades, softening the cervix, thus allowing 'ripening' and dilatation to facilitate passage and delivery of the neonate. This cervical remodelling occurs not only during the active phase of labour, but also towards term, with additional changes during postpartum repair[93 96 97].

The most abundant protein within the cervix is collagen which contributes to the firm tensile structure[93 94]. Cervical collagen is made up from Type I and Type III[93] with evidence of Type IV collagen in association with smooth muscle cells and blood vessels[94 95]. During ripening, there is a loss of tissue integrity mainly through changes to collagen fibril structure, disorganisation of collagen bundles, oedema and an increased volume of glycosaminoglycans[93 96]. An important constituent of the extracellur matrix are the glycosaminoglycans, particularly hyaluronan and proteoglycans (versican, decorin, biglycan, fibromodulin and asporin)[93 96]. These are thought to modulate the disruption and disorganisation of the collagen bundles, promote the accompanying increase in water content and oedema of the cervix typically seen during cervical remodelling[93 94 96].

Additionally, elastin fibres comprise 0.9-2.4% of cervical connective tissue in the pregnant and non-pregnant state and may also contribute to the recoil of the cervix during pregnancy and in the post partum remodelling period[96]. The contractile myocytes within the cervix account for between 8.9- 15.5% of the cervix from the distal to proximal end[95 98]. The cervical myocytes are capable of both spontaneous and drug induced contractile activity, however since they are sparse in number, it is probable their only function may be protection of surrounding blood vessels and closure of the cervix post delivery[89].

1.3.2 The uterine corpus

The uterine corpus is the upper largest part of the uterus which connects inferiorly with the isthmus and cervix and on each side with a fallopian tube (Figure 3). Anatomically, the fundus is considered the area of the corpus above the entrance of the fallopian tubes[87]. The uterine body is made up of 3 layers, the outer perimetrium (covers the outer peritoneal surface of the uterus), the inner endometrium (consisting of ciliated columnar epithelium) and the middle thicker layer of myometrium (the muscular portion of the uterus).

As this thesis is concerned with uterine contractility, the following section considers the contractile apparatus specific to the myometrium.

1.4 Myometrium – the uterine contractile machinery

1.4.1 Anatomical organisation

The myometrium is the largest layer of the uterus and provides the contractile element essential to the normal functioning of the female reproductive tract. It consists mainly of contractile muscle cells (myocytes) interspersed with connective tissue, blood vessels, lymphatic vessels and nerves[99]. In pregnancy the walls of the uterus are approximately 7mm thick and remain relatively stable across gestation from 26 weeks[100]. It is this myometrial layer of the uterine wall which undergoes cellular hyperplasia and hypertrophy during pregnancy.

Little definite evidence regarding the organisation of the myometrial cells in the human uterus in both the pregnant and non-pregnant state is known. In other animals, particularly those with two uterine horns, for example rodents and rabbits, there appears to be two distinct muscular layers with an outer longitudinal and an inner circular layer[99 101-103].

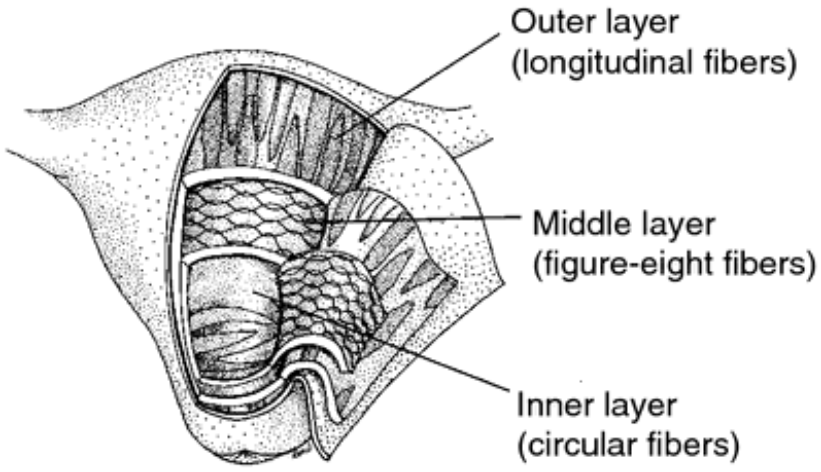

Figure 4 Layers of the myometrium

Proposed organisation of the layers of muscle fibres within the myometrial layer of the uterus body Layers of myometrium showing the three layers of smooth muscle fibre. Image downloaded from http://medical-dictionary.thefreedictionary.com/myometrium[104]

Some consider the muscular fibres of the myometrium in humans to be arranged in three layers; the thin outer layer (stratum supravasculare) consisting of longitudinal fibres, the thickest middle layer (stratum vasculare) of swirling intertwined fibres surrounding blood vessels and the thin inner layer (stratum

subvasculare) which appears to have circular fibres[85 99 105] (Figure 4). This arrangement is thought to determine the force and direction of contractions during labour[85]. Additionally the arrangement of the inner layer surrounding blood vessels is thought to play a protective role in stemming bleeding after delivery of the placenta[85].

However, others have not found the layers in the human uterus to be so distinct and alternative suggestions of a myometrial junctional layer in women of reproductive age have been made[106 107 108](Figure 5). This junctional layer has been studied mainly in the non-pregnant uterus where *in-vivo* imaging has shown peristaltic waves which vary in direction (cervico-fundal or fundo-cervical) dependent on the stage of the menstrual cycle[109 110]. The inner zone of myometrium has been found to have a higher smooth muscle density with a higher ratio of nucleus to cytoplasm within these cells suggesting both structural and cellular differences between the inner and outer zones of the myometrium[111 112]. The role of the junctional layer during pregnancy and parturition is uncertain, and MRI imaging of the myometrium during pregnancy has suggested that zones become indistinct from early gestation, with a return normal within 6 months post delivery[113]. Therefore, activity of the junctional layer may be limited to the non-pregnant state.

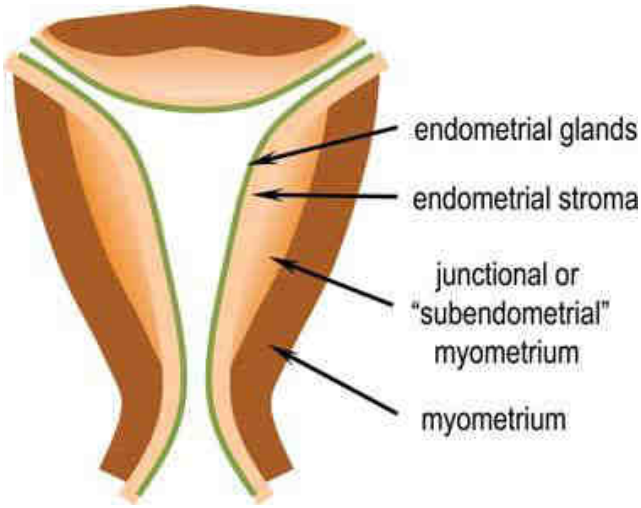

Figure 5 Layers of the myometrium

Illustration of the alternative layers of myometrium with the existence of the inner junctional or subendometrial myometrial zone and an outer myometrial zone. Image reproduced from Aguilar et al[106]

Further MRI studies in combination with computational modelling have attempted to describe the directionality of myometrial myocyte fibres[105] (Figure

6). This suggests that fibres run mainly in a circular direction implying a cylindrical form to the uterus with longitudinal fibres at the fundus[105]. Further computational modelling of the uterus using a combination of *in-vivo* imaging and pressure monitoring techniques with *in-vitro* physiology studies hope to determine the organisation of fibres and functional direction of contractile forces for both the pregnant and non-pregnant uterus[105 114 115].

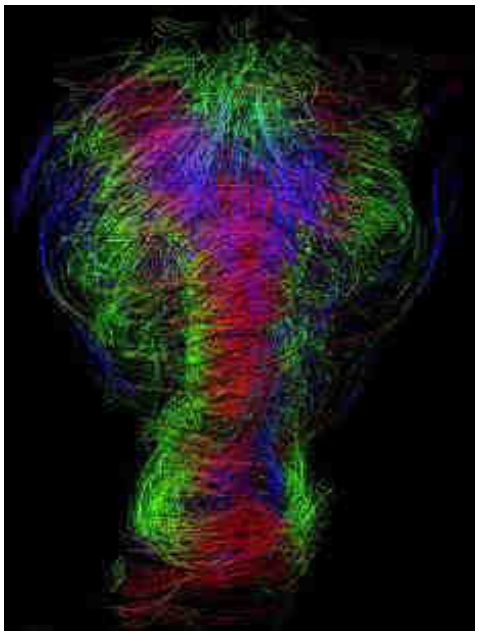

Figure 6 Global myometrial fibre structure of the uterus (dorsal view).

Circular direction of fibres at the cervix and surrounding the uterine cavity are illustrated with a suggestion of longitudinal fibres at the fundus. Red coloured fibres indicate transverse, left to right direction, green indicates through plane and blue top-down direction. Fibre direction is computed from MR images using the FACT algorithm (fibre assignment by continuous tracking) Further details and image from Weiss et al[105]

1.4.2 Myometrial myocytes and intracellular mechanics

The main cellular constituent of the myometrium is the contractile myocyte. As in other smooth muscle organs, these are spindle shaped cells which are connected to each other via gap junctions and form smooth muscle bundles with the exact arrangement and orientation of these bundles not completely certain as described above[116] (Section 1.4.1). Smooth muscle, including myometrium, differs from skeletal muscle as contractions are involuntary and the cells are adapted for long sustained contractile activity.

In the myometrial smooth muscle cell as in other myocytes, contractions are dependent on the final common pathway of an interaction between actin and

myosin causing shortening of the cell[116]. Unlike skeletal muscle where actin filaments are attached to Z-lines and therefore allow contraction in one longitudinal direction, the actin filaments of smooth muscle cells are anchored to dense bodies which are scattered through the cytoplasm of the cell[106 101 117](Figure 7A and Figure 7B). This is thought to facilitate the ability of the myocyte to stretch and accommodate contents of the hollow organ whilst maintaining contractile ability and strength. The dense bodies are attached via intermediate filaments to dense bands which are found around the circumference of the cell attached to the plasma membrane and therefore aid the shortening of the cell during a contraction[117] (Figure 7A & Figure 7B).

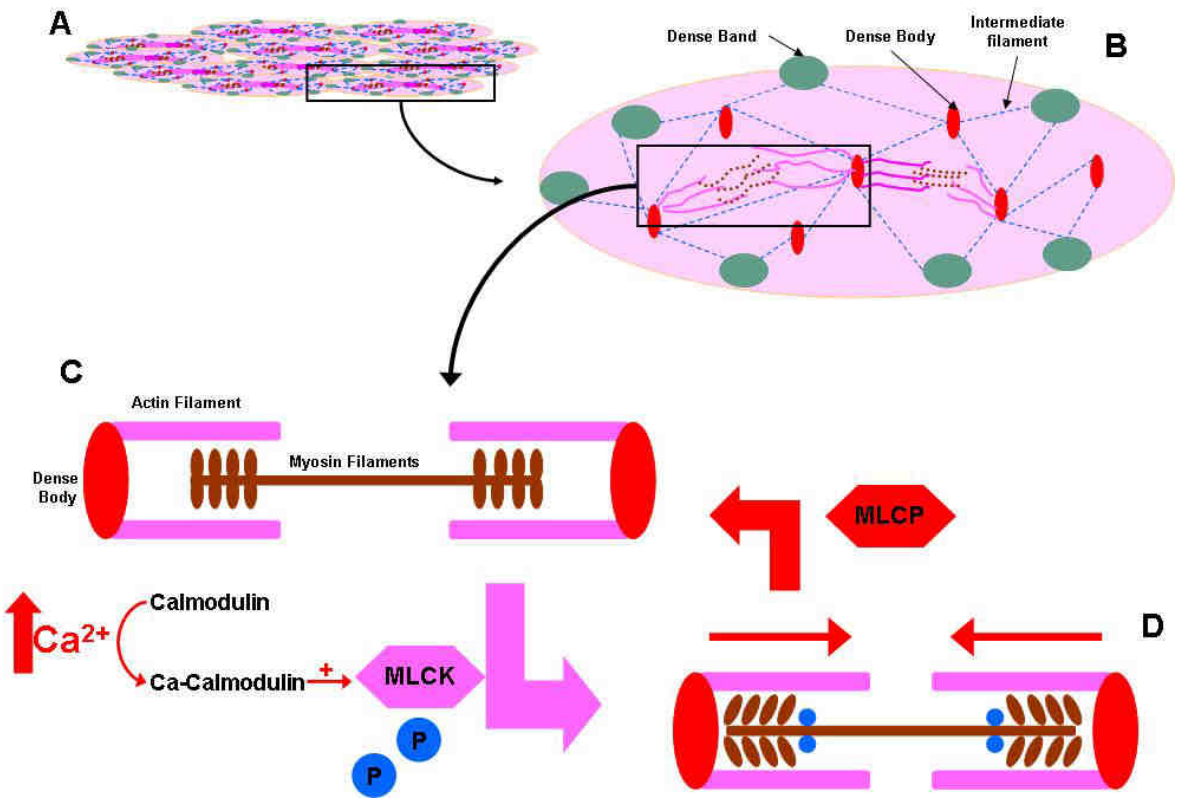

Figure 7 The myometrial smooth muscle cell contractile machinery

(A) myocytes packed into bundles connected by GAP junctions. (B) Single myometrial smooth muscle cell illustrating presence of actin and myosin filaments attached to dense bodies within the cytoplasm. Dense bodies are connected via intermediate filament (blue dotted line) to dense bands on cell membrane. (C) Single actin and myosin complex attached to a dense body in the relaxed state. In the presence of increased intracellular calcium, a calcium-calmodulin complex is formed which in turn activated the enzyme Myosin Light Chain Kinase (MLCK). Activated MLCK catalyses the phosphorylation of myosin causing cross bridge formation between actin and myosin filaments and change in the direction of the neck region of the myosin. This results in a contraction by shortening of the distance between the two anchor points (dense bodies) (D). Contractions are terminated when the phosphorylation is reversed, a process facilitated by Myosin Light Chain Phosphatase (MLCP)[106]

Common to all types of muscle cells, contractions are under the control of intracellular calcium ($[Ca^{2+}]_i$,) levels (Figure 7). With an increase in $[Ca^{2+}]_i$, a calcium-calmodulin complex is formed which binds to the calmodulin-binding site on myosin light chain kinase (MLCK), thus activating its enzymatic activity. This activation of MLCK promotes phosphorylation of the 20kDA regulatory myosin light chains in turn initiating actin-myosin ATPase on myosin heavy chains allowing cross bridge formation with actin thereby facilitating cellular contraction. It has been suggested that this is the common final pathway in myocyte contractions since *in-vitro*, MLCK inhibitors have rendered both rat and human myometrial samples unable to contract, even when exposed to the uterotonin oxytocin or in response to cellular depolarisation by Potassium Chloride (KCl) [118].

Additionally, it has been shown that in contrast to the non-pregnant state, myometrium during pregnancy has the ability to produce a higher level of tissue stress or contraction for any given level of myosin light chain phosphorylation[119], thus suggesting there is adaptation of the contractile mechanisms during pregnancy which favour a pro-contractile phenotype.

Phosphorylation of the myosin heads is reversed as the intracellular calcium concentrations decrease and by actions of the enzyme Myosin Light Chain Phosphatase (MLCP). Calcium is then extruded from the cell by sodium/calcium counter transporter (Na/Ca exchange) and Ca-ATPase transporter or replenishment of the intracellular calcium stores of the sarcoplasmic reticulum[106 120].

Additionally, the contractile response to the influx of a certain amount of calcium can be variable, this is especially noticeable after cellular stimulation with uterotonic agents, for example OT[106]. This is known as calcium sensitisation and the process is mediated by an impairment of MLCP activity, thereby facilitating phosphorylation of the MLC and cross-linking with actin. The process appears to be mediated via the receptor coupled activation of Rho-kinase (ROK) by RhoA-GTP and CPI-17 (17-kDa-protein-kinase C-potentiated inhibitor) by DAG-PKC [106 117 121-126]

1.4.3 Action potential generation

Myometrial smooth muscle, both pregnant and non-pregnant has the ability to produce spontaneous contractile activity without hormonal or neuronal stimulation, while maintaining the ability to respond to endogenous or exogenous agonists. Spontaneous contractile activity is preceded by depolarisation of the cell[116 127] (Figure 8). The resting membrane potential is maintained by trans-membrane ionic gradients of Calcium (Ca^{2+}), Sodium (Na^{+}), Chloride (Cl^{-}) and Potassium (K^{+}), created by energy dependent pumping of ions against their respective electrochemical gradients combined with ion-specific permeability of the plasma membrane. Concentrations of Ca^{2+}, Na^{+} and Cl^{-} are lower within the intracellular environment, and K^{+} higher in the intracellular space. It is movement of these ions across the cell membrane via specialised membrane ion channels which initiates cell depolarisation, AP generation, voltage-gated calcium entry and contractile activity.

Action potentials in the contracting myometrium have a specific shape which determines calcium entry with consistent increases in $[Ca^{2+}]_I$ preceding contractile force generation, with a return to baseline $[Ca^{2+}]_I$ prior to relaxation[127 128]. The levels of $[Ca^{2+}]_i$ determine the extent of the contractile force generated by the myometrium. In the presence of a depleted extracellular calcium environment, force is greatly reduced where increases in $[Ca^{2+}]_i$ are dependent on calcium release from the sarcoplasmic reticulum[128]. Additionally, changes in $[Ca^{2+}]_i$ tend to follow a pattern of an initial fast increase with cellular depolarisation followed by a plateau phase and fast repolarisation and reduction in $[Ca^{2+}]_i$, and this seems to determine the pattern of contractile force generated[127 128] (Figure 8). An example of the action potential generated by the myocyte, the corresponding intracellular Ca^{2+} levels and the contractile force produced by the cell is illustrated in Figure 8[115].

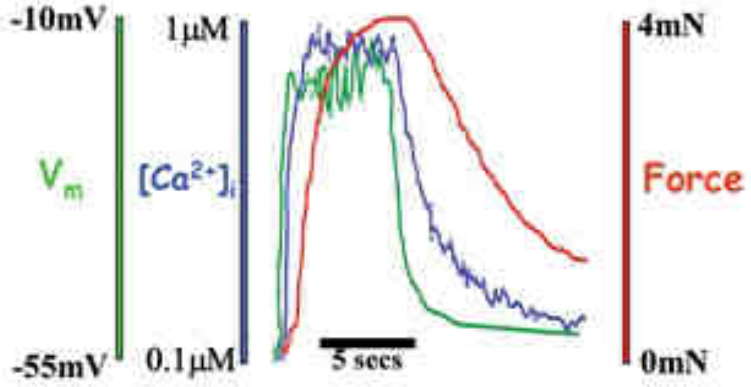

Figure 8 Smooth muscle action potential

The relationship between the action potential (green line), $[Ca^{2+}]_i$ (blue line) and force generation (red line) in smooth muscle. Reproduced from Taggart et al[115]

Additionally, it is of interest to note that the resting membrane potential in the myometrial myocyte alters with gestation[129]. During pregnancy the membrane potential becomes progressively more negative (i.e. cell membrane is hyperpolarised) peaking in mid-pregnancy at the reversal potential for potassium (-80 mV). As term approaches, resting membrane potential depolarises to near threshold at approximately -55 mV[129]. This implies that the myometrial cells are more difficult to depolarise at earlier gestations and therefore are intrinsically less excitable during the quiescent phase of pregnancy than towards term[129].

1.4.4 Ions and Ion Channels

Contractile activity in the myocyte is dependent on depolarisation of the cell, with ultimate increases in intracellular calcium levels which facilitate the MLCK mediated final pathway of actin and myosin cross-linking. The control of membrane potential via the influx and efflux of ions across the cell membrane requires the involvement of specialised ion channels.

In the human myometrial cell the inward depolarising current is carried mainly by the influx of calcium (Ca^{2+}), with potassium (K^+) efflux concerned with repolarisation to the resting state. Ca^{2+} influx is mediated mainly via opening of the voltage gated L-type calcium channels found in the cell membrane allowing rapid influx of calcium and an increased $[Ca^{2+}]_i$ with maximal opening at 0mV[116] [130-132]. There may be an additional contribution via T-type low voltage activated calcium channels[132 133] and also from the calcium stores in the sarcoplasmic reticulum, however, in experimental situations where the sarcoplasmic

reticulum was the main source of calcium, contractile activity fell to approximately 26% of that observed with Ca^{2+} influx from the extracellular environment[128]. Following the Ca^{2+} influx, the cell becomes repolarised primarily by outward movement of K^+ via Ca^{2+} sensitive K^+ channels (BK_{Ca} or maxi-K) and voltage gated K^+ channels (K_V)[116 130]. Initially, the membrane potential is maintained at around -20 mV for the duration of the contraction with subsequent full repolarisation to -55mV[106 116].

Other ions and ion channels, namely sodium and chloride, also play a part in the maintenance of resting membrane potential and cell excitablility[116 130]. In particular, chloride channels may be involved in the generation of the plateau phase of the action potential particularly in response to the agonist oxytocin[130 134]. However, sodium and chloride involvement in action potential development and contractile activity overall does not appear to be to the same degree as calcium and potassium.

1.4.5 Uterine pacemaker cells

It has been proposed that similar to cardiac smooth muscle, contractions of the myometrium are under the control of pacemaker type cells which are capable of spontaneous depolarisation with the subsequent propagation of an action potential through the uterine tissue. However, unlike in cardiac muscle pacemaker cells have never been defined within the uterus, either histologically or anatomically. It has therefore been proposed that the spontaneous electrical activity of the myometrium is an inherent property of these smooth muscle cells with cells able to depolarise at random and act as a pacemaker for that particular contraction. This would certainly be supported by *in-vivo* human data suggesting the 'hot spots' of activity can develop at any point within the myometrial tissue, thereby further negating the proposal of a fixed area of pacemaker type cells[135]. Additionally, virtual modelling of the gravid uterus has suggested that a heterogeneity of excitable cells throughout the uterus in addition to cellular communication via gap junctions is essential for the types and force of contraction required by the uterus for the process of parturition[136].

1.4.6 Gap junctions and action potential propagation

In the myometrium, it is essential that a co-ordinated contraction of many muscles cells produces sufficient contractile activity in the correct direction for progress in labour and delivery of the infant. Therefore the cells have developed a way to work in concert via GAP junctions (connexins). These are connecting channels which allow the flow of intracellular ions thus causing mass depolarisation and a synchronous tissue contraction. It has also been suggested that differential expression of connexins through the myometrial tissue may play a role in determining directionality of electrical activity and therefore contractility of the uterus[116]. Additionally, the speed of action potential propagation in the labouring uterus (0.25m/s) is faster than in the non-labouring uterus (0.1m/s), with the increase most probably facilitated by increased gap junction formation[114]. In the myometrium the main gap junction proteins are connexin-43, connexin-26, connexin-40 and connexin-45[116]. The numbers of connexin-43 gap junctions tends to increase at term[137-140] while the expression of connexin-26 tends to fall[138] and is thought to be influenced by both hormonal exposure[141 142] and stretch[142]. Certainly absence of connexin-43 is associated with delayed parturition in the mouse model[143] suggesting this may be considered the most important myometrial connexin protein with a role in facilitating sufficient myometrial contractile activity necessary for labour.

1.5 Modulators of contractile activity

Despite much research into the parturition associated contractile pathways, the exact mechanisms involved in the initiation and propagation of labour in the human remain uncertain. The following section will address the two main agonists, namely oxytocin and prostaglandins, which can directly promote contractile activity in the myometrium. Additionally, some of the known factors which are thought to modulate myometrial contractile ability and either positively of negatively influence the progression of human parturition will be discussed.

1.5.1 Oxytocin

Oxytocin (OT) is involved in many aspects of human reproduction with functions in the promotion of uterine contractility during labour and the postpartum period, in addition to involvement in the milk let down reflex allowing delivery of milk to the neonate during suckling. OT is a small nonapeptide hormone released by the posterior pituitary (Figure 9), with evidence for autocrine and paracrine production in gestational tissues towards term[91 144 145].

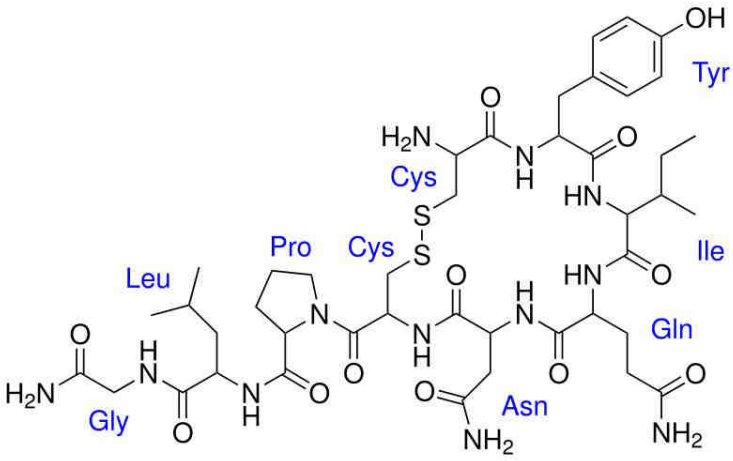

Figure 9 Chemical structure of Oxytocin

In terms of oxytocin in human parturition, 'extracts of the posterior pituitary' were first found to promote contractility of the pregnant animal uterus in 1906 by Dale[146 147]. In 1909, Blair Bell first began to use the 'extract' in clinical obstetric practice to control uterine bleeding during post partum haemorrhage

and bleeding secondary to placenta praevia and caesarean section[148]. In modern obstetrics, Syntocinon®, a synthetic oxytocin is widely used to induce and modulate uterine contractions to facilitate labour and delivery of the infant, in addition to its role in the prevention and treatment of post partum haemorrhage where it is routinely given to women in order to aid delivery of the placenta in the third stage of labour.

With regard to the role of OT in the modulation of myometrial contractile activity, its overall effect is to increase contraction frequency, duration and force. This enhancement of myometrial contractility is achieved by increasing the frequency of cellular action potential generation, extending the plateau phase of the action potential and promotion of calcium sensitisation (impairment of MLCP activity via activation of rho-associated kinase (ROK) by GTP-rhoA and CPI-17 by DAG-PKC [121])[106 116 125 126 149]. An illustrative example of the alteration to the contractile force produced by the myometrium in response to a single addition of OT is illustrated in Figure 10.

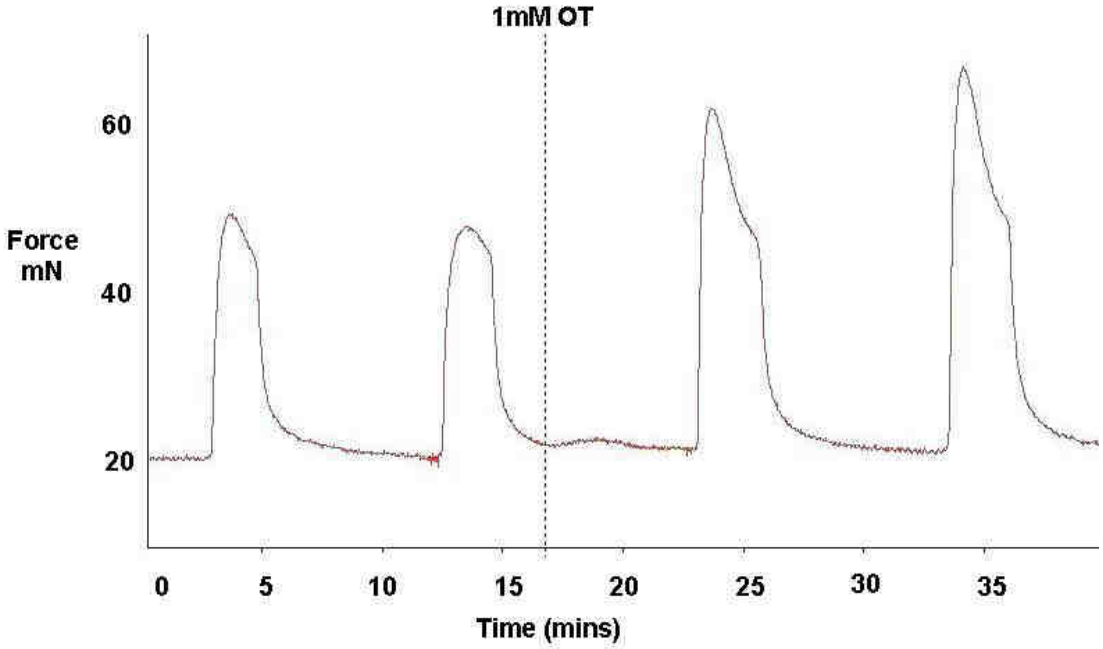

Figure 10 Trace of myometrial contractile activity

Illustration of myometrial contractile activity from organ bath experiment. The first 2 contractions indicate the contractile force of non-agonist induced spontaneous myometrial activity, while the subsequent two contraction curves indicate the changes which occur in response to stimulation with OT. OT increases the amplitude, duration and rate of contraction while decreasing the rate of relaxation. The latter observation is most probably due to OT-induced sensitisation of contractile machinery to Ca^{2+}

OT stimulation of myometrial cells facilitates two types of contractions which are typical of smooth muscle organs. Firstly, the phasic contraction with an intermittent contraction and relaxation phase, an example of this type of contractile activity is illustrated in Figure 10. Phasic contractions of the uterus are suitable for the process of labour and the passage of the neonate, with the intermittent relaxation phase necessary to restore blood flow both to the active myometrium in addition to the placenta and the neonate. In response to OT, the phasic contractions tend to increase in frequency, amplitude and overall force, with a prolonged contraction pattern. The second type of contraction seen in smooth muscle is the sustained tonic contraction. In the myometrium this is essential in the postpartum period to prevent haemorrhage particularly from vessels at the newly exposed myometrium at the site of placental bed detachment. In response to OT stimulation, the onset of this tonic contraction is hastened and the contraction is maintained[124 150].

To produce its effects on the myometrium, OT acts via the cell surface seven transmembrane G-protein coupled oxytocin receptor (OTR). Evidence suggests that with increasing gestation, the concentration of OTRs on the myometrial cells increases, particularly towards term[151-153]. As discussed earlier, contractility of the myometrial myocyte hinges on the interaction and cross linking of myosin with actin[154] which is ultimately dependent upon increased intracellular calcium. OT facilitates an increase in intracellular calcium as it binds the OTR resulting in activation of phospholipase C isoforms ß1, ß2 and ß3 via $G\alpha_q$. This catalyses the hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2) with a resultant increase in inositol-1,4,5-triphosphate (IP_3) and 1,2-diacylglycerol (DAG)[155]. IP_3 acts on the sarcoplasmic reticulum to release its calcium stores, thereby increasing the availability of cytoplasmic calcium[156]. The increased cytoplasmic calcium has a direct effect upon calcium-sensitive cationic channels which depolarise the cell and open voltage gated L-type calcium channels and calcium can influx from the extracellular environment[134]. The other second messenger DAG is thought to activate plasma membrane calcium channels in addition to mediating effects via protein kinase C (PKC)[155 157]. The subsequent influx of large quantities of calcium binds to the calcium binding protein calmodulin which acts to initiate activity of the enzyme MLCK thereby facilitating the final common contraction pathway of actin and myosin cross-linking via myosin light chain phosphorylation. Activation of the OTR and

the cascade of intracellular events culminating in an increase in intracellular calcium and cellular contraction are depicted in Figure 11.

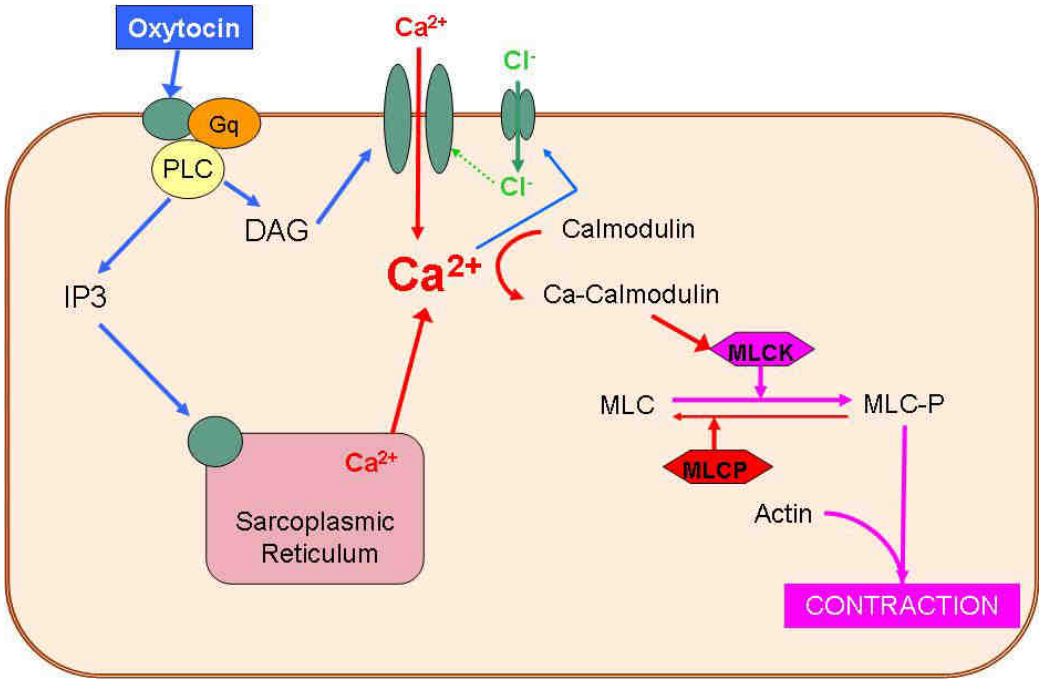

Figure 11 Intracellular events following OTR stimulation with OT (contraction pathway)
Diagram of the intracellular events which occur to produce a contractile response to OT activation of the oxytocin receptor (OTR) on a myometrial cell.

In addition to the above contraction pathway, OT is known to increase the sensitivity of the contractile machinery to calcium by altering the activity ratio of MLCK (pro-contraction) and MLCP (pro-relaxation) via activation of Rho-kinase (ROK) by RhoA-GTP, and also activation of CPI-17 (17-kDa-protein-kinase C-potentiated inhibitor) by DAG-PKC pathway [116 121 124 149 158]. This overall results in suppression of MLCP activity and allows the plateau phase typical of an agonist induced contraction to occur. The exact mechanisms by which OTR activation leads to ultimate activation of Rho-kinase and CPI-17 are not fully certain, but most likely ROK activation occurs via OTR associated G-protein activation which facilitates conversion of RhoA-GDP to RhoA-GTP enabling activation of Rho-kinase[106 124]. Concerning the activation of CPI-17, the 2nd messenger DAG is involved in the activation of protein kinase-C (PKC) which promotes CPI-17 activity, in addition to CPI-17 activation by Rho-kinase[106 125 126]. A diagram of the pathways involved in OT and agonist induced calcium sensitisation in uterine smooth muscle is illustrated in Figure 12.

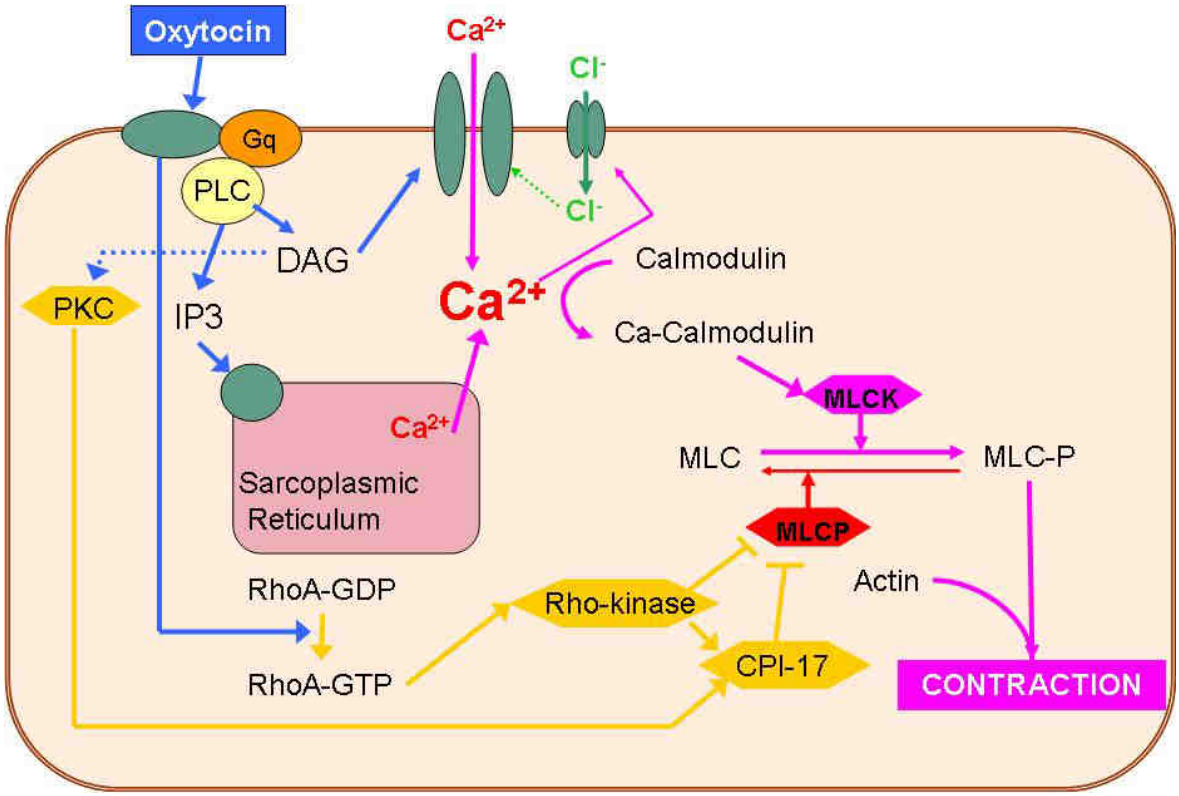

Figure 12 Intracellular events following OTR stimulation with OT

Diagram of intracellular events occurring in response to OT activation of the oxytocin receptor (OTR) on a myometrial cell culminating in cellular contraction. In addition, the mechanisms of calcium sensitisation in response to OT are illustrated with suppression of MLCP activity via 1.Rho-kinase and 2.DAG/PKC activation of CPI-17.

The responsiveness of myometrium to contractile agonists, particularly OT is increased with gestation[124]. In many animals, including humans, OTR number and expression of the OTR gene increases, peaking in the myometrium prior to the onset of labour, with a return to low levels post partum[153 159]. This subsequent decrease in OT binding is thought to be a result of OT exposure causing internalisation and downregulation of OTR mRNA transcription[160 161] and may represent a process of cellular contractile regulation. Additionally, OTR expression has been found to increase in human amnion tissue prior to labour, with the OT stimulation thought to be involved in prostaglandin production[162] suggesting a complimentary role for OT in uterine stimulation. The expression of both OT and OTR in myometrial tissue appears to be under regulation from the sex steroids oestrogen and progesterone, with oestrogen mediating the increase in both OT and OTR expression and progesterone increasing OT but decreasing OTR expression[160 161]. Uterine stretch is also thought to increase OTR expression both in cultured human myometrial cells and pregnant uterine horns of rats and sheep compared to the non-pregnant horn[161 163],and OT levels are

influenced by cervical stretch (the Ferguson reflex) particularly during delivery of the neonate[164-166].

During pregnancy, the uterus is exposed to endogenous oxytocin, which similar to other animals appears to be released in a pulsatile manner increasing towards term and particularly during labour[167]. During labour itself, circulating OT levels peak during delivery and expulsion of the foetus which may be part of the Ferguson reflex (distension of the cervix or vagina causing increased uterine contractility)[164-166]. Certainly in the animal model, mechanically mediated stretch of the cervix during delivery of the pups appears to stimulate afferent nerves to the hypothalamus with subsequent release of OT into the circulation[168-171]. In primates, the oxytocin pulses observed during pregnancy have a diurnal variation, peaking nocturnally during periods of darkness, which correlates with observed increases in periods of myometrial activity during these hours and can be inhibited by administration of an OT antagonist[172]. Gestational and diurnal variations in labour onset, and non-labour antenatal uterine activity (Braxton Hicks contractions) are also seen in human pregnancy[173-178] and may be explained by a diurnal variation in OT release[179]

It is also thought that prolonged antenatal and intrapartum exposure to OT may facilitate changes to the myometrial proteome and promote a pro-contractile profile. There is a suggestion of global protein changes after one hour of OT exposure in fresh term myometrial samples which were contracting in an organ bath environment[180]. This has shown up-regulation of the expression of 13 proteins associated with 5 functional classes, namely cytoskeletal function (septin-2, cytokeratin-19, desmin, transgelin) contractile/oxidative stress (annexin A3, heat shock protein 70, protein disulfide isomerise, thioredoxin), extracellular matrix (Osteoglycin, BIGH3), energy metabolism (EH domain protein 2, triose phosphate isomerise, pyruvate kinase) and protein synthesis (EF1γ)[180]. However, the protein changes identified by this approach are subject to method related selection bias and it is unclear whether the changes in expression are due to post-translational modifications or new gene expression. It is therefore important to study gene and protein expression independently, with gene arrays assessing a wider range of molecular changes. This is addressed within the work presented in Chapter 3 of this thesis.

Despite an important functional contractile role for OT, there does seem to a suggestion of redundancy, with OTR null mice still able to labour at the correct gestation, but with subsequent death of the pups due to absence of milk let-down during suckling[181]. Additionally, evidence has suggested that despite the increase in the concentration of OTRs on the myocytes at term, the ability of the myometrium to achieve phasic contractions is dependent on the working of the intracellular contractile machinery rather than exposure to oxytocin[182].

1.5.2 Prostaglandins

Prostaglandins (PGs) are biologically active lipid hormones which have both autocrine and paracrine actions. In terms of parturition, along with OT, prostaglandins are considered to be one of the main myometrial contractile agonists with an additional role in cervical ripening and softening to allow passage of the neonate. Prostaglandin (PG) $F_{2\alpha}$, PGE_2 and PGI_2 (prostacyclin) are made endogenously by the uterus. In particular, within the myometrium, there has been localisation of enzymes involved in the synthesis of PGs, namely cyclooxygenase (COX) and prostacyclin synthase to the myocytes with gestation dependent increases[183-185].

With regard to PG use in obstetric practice, Misoprotol (PGE_1 synthetic agonist) is used to facilitate cervical softening prior to surgical termination or for induction of contractions during medical terminations of pregnancy. Dinoprostone (PGE_2 synthetic agonist) is used for cervical ripening during the process of induction of labour, and Carboprost ($PGF_{2\alpha}$ synthetic agonist) is used in the treatment of postpartum haemorrhage to promote a tonic uterine contraction[186 187].

Endogenous PGs are derived from arachadonic acid (AA), which is a constituent of the cell membrane. After the initial step of AA release from the cell membrane via phospholipase-A2 (PLA2), the conversion of AA to PGs is mainly mediated by the COX enzymes COX-1 and COX-2, with specific synthase enzymes determining the final type of prostaglandin formation[188-192]. Of note, intracellular AA is suggested to play a direct part in the activation of Rho-kinase, which as described above can mediate calcium sensitisation and thus contractility of the myometrial cell[149].

PG receptors are coupled to G-proteins with a specific second messenger system[191]. The main function of $PGF_{2\alpha}$ appears to be in the stimulation of myometrial contractile activity. The exact mechanism of action is uncertain however, stimulation of the $PGF_{2\alpha}$ receptor (FP) mediate cell membrane depolarisation and a rise in intracellular calcium via influx from the extracellular environment, thus resulting in contractile activity[116 191].

The second main prostaglandin PGE_2 has varying actions dependent on receptor sub-type. Activation of the PGE_2 receptors E1 and E3 tend to stimulate contractility of the uterus (E1 via calcium mobilisation, E3 via inhibition of cAMP)[191], and E2 and E4 tend to relax the muscle via stimulation of cAMP[116 191 193 194]. The receptor sub-types have been traditionally thought to vary in expression throughout the uterus with E1 and E3 found to be fundal promoting contractile activity, and E2 and E4 in the lower segment and cervical tissue promoting relaxation[116 194 195]. However, contractile response to PG has more recently been found to be consistent between paired samples from the upper and lower uterine segments[196]. Prostacyclin (PGI_2) is thought to play a role in uterine relaxation, however this can be negated by $PGF_{2\alpha}$ or OT, and production of PGI_2 does not appear to alter with gestation[194].

Similar to OTR regulation, expression of receptors for $PGF_{2\alpha}$ and PGE_2 appear to change throughout pregnancy with decreases of up to 45% in the pro-contractile receptors FP and EP3 during pregnancy compared with non-pregnant myometrium[197]. In addition FP and EP receptor expression appear to be influenced by the sex steroids oestrogen and progesterone[190 198]. The production of prostaglandins within gestational tissues is influenced by gestation, labour and also inflammatory cytokines[188-191 199 200] and is discussed further later in this chapter.

1.5.3 Interchangeable role of the uterine contractile agonists oxytocin and prostaglandin

The two main agonists of myometrial contractile activity, namely OT and PG are described above. With such an important physiological process as parturition, there are advantages to having an element of redundancy between stimulating mechanisms. This is certainly demonstrated where the parturition process in OT

null mice has been examined. Surprisingly for such a powerful agonist, these mice laboured spontaneously with no evidence of prolonged active phase and were able to deliver viable pups at the normal expected gestation[181].

Additionally, the role of OT was studied in rats which had undergone disruption of the pelvic nerves thereby interrupting signalling via afferent pathways in response to cervical dilatation at delivery of the pups[168 201]. Repeated sampling and measurement of OT levels were certainly decreased in these rats, indicating the role of the Ferguson reflex, however blood OT was not completely abolished and administration of exogenous OT did not appear to reduce delivery time[168]. This suggests alternative pathways for both OT release and expulsion of pups in the absence of surge in circulating OT[168 171].

In terms of parturition in PG knock out mice, modulation of various PG specific pathways including $PGF_{2\alpha}$ receptor (FP) null mice and COX-1 null mice, labour is delayed since PGs are essential for luteolysis and in its absence, there is no decrease in circulating progesterone levels, an essential event for labour onset in the mouse[202-205]. However, where ovaries are removed to mimic the progesterone drop, labour and delivery of the pups occurs as normal[189 202-204 206]. Again, as with the OT null mouse, this suggests that myometrial stimulation by PGs is not essential for the process of parturition.

The effects of manipulating both OT and PG in the same mouse have been studied using the COX-1/OT null mouse. These mice demonstrate an ability to establish labour at the correct time (most probably due to the absence of the OT luteotropic effects allowing progesterone levels to fall), however, the process of parturition is prolonged[203 205]. Taken together with evidence from the single knock-out mice, this suggests that OT and PG can be considered interchangeable as myometrial agonists, however, in the absence of both, contractile and labour difficulties occur. The importance of both agonist systems was further supported by studies in sheep where simultaneous administration of an OT antagonist and a COX-2 antagonist has the ability to suppress the onset of induced labour and prevent progression where pre-term labour contractility had already been established[207 208].

1.5.4 Inflammation, infection and modulation of myometrial contractile activity

The concept of labour as an inflammatory event is well established[209-217], with 8189 citations found via Pubmed using the basic search criteria 'labour' and 'inflammation' (search performed July 2012). In 1981, Liggins first proposed that inflammatory cells were responsible for mediating changes in the cervix, leading to cervical remodelling and ripening[218]. Indeed, increases in numbers of immune cells (mainly neutrophils and macrophages) in response to labour are present within gestational tissues including the myometrium and cervix[219-221]. There is also a co-ordinated increase in cell adhesion molecules, cytokines and chemokines within these tissues suggesting the uterus can exert control over the inflammatory process in labour[220-223]. Certainly, from within our department, *in-vivo* gene array transcription data from the cervix and myometrium has shown labour is associated with a core inflammatory response, with particular increases in the chemokines CXCL3, CXCL5, CXCL8, CCL2 and CCL20[211].

In addition to a role in leukocyte chemoattraction, inflammatory cytokines also have the ability to stimulate production of prostaglandins (mainly via COX-2 stimulation) in the amnion, chorion, decidua and myometrium, which can occur both in the presence and absence of infection induced cytokine upregulation and can lead to uterine contractility[188 199 224 225]. It is of interest to note that COX-2 expression in myometrium is low during gestation and increased at the time of parturition (COX-2 is an inducible enzyme and upregulation is secondary to substances which can be associated with perceived cell damage e.g. cytokines, mechanical stretch, infection, hypoxia), where COX-1 (constitutively expressed in tissues) tends to remain stable throughout[204 226-229]. In terms of the relationship between inflammation and contractility, it follows that increased cytokines and PGs would positively enhance myometrial contractile activity, and this has been the basis of current theories regarding initiation of both term and in particular pre-term labour[210 216 217 230-236].

This obviously makes inflammation a possible target through which uterine contractions and human labour may be manipulated; suppressing the inflammatory process to prevent contractility in the case of pre-term labour or enhancing inflammation in an attempt to promote a contractile phenotype

where induction of labour is undertaken in the post-dates pregnancy[233 237 238]. However, dampening of the inflammatory response by systemic administration of COX inhibitors in pregnancy has proven to be inconsistent in preventing pre-term labour and delivery[239-241]. Furthermore, despite some evidence of prolonging gestation with COX inhibitors, they also demonstrate a poor neonatal side-effect profile, particularly concerning neonatal morbidity including necrotizing enterocolitis, premature closure of ductus arteriosis in utero, impairment of renal function and oligohydramnios[241].

It also remains unclear as to whether activation of inflammatory pathways precedes labour and can therefore be regarded as a stimulant of labour, or whether inflammation occurs as a response to labour. In humans this is difficult to determine, and *in-vivo* studies have examined tissues taken at caesarean delivery prior to the onset of labour and those taken intrapartum at both term and pre-term gestations[211 213 242-246]. However, there are inevitable problems with these comparisons in that there will be normal variation between subjects at the same gestation, and reasons for undergoing caesarean delivery at term and pre-term will differ and may contribute to variations. Even in mouse models, the mechanisms of cervical ripening differ between those mediated by progesterone withdrawal and infection, with an inflammatory cell mediated pro-inflammatory response and significant upregulation of COX-2 in response to infection[247]. This suggests an alternative mechanism for ripening in the non-infective, spontaneously labouring cervix at term[247 248]. Furthermore, evidence also suggests that smooth muscle contractility may potentially be suppressed by inflammatory mediators[249 250]. In intestinal smooth muscle studies, IL1ß and TNFα are associated with suppressing the activity of CPI-17, thereby preventing calcium sensitization with resultant bowel dysmotility[249 250].

The transcription factor, Nuclear Factor-κB (NF-κB) has been proposed to have an essential role in the activation of inflammatory pathways in non-gestational and gestational tissues, and hence may play a part in control of parturition[226 237 251-255]. Specifically, NF-κB acts to promote the transcription of cytokine, chemokines including TNFα, IL-1ß, CXCL8(IL-8) and inflammatory related enzymes including COX-2, and inhibition of NF-κB leads to decreased PG production[226 227 251 255 256]. Additionally, NF-κB has been found to be induced in response to not only cytokines, but also mechanical uterine stretch with a

suggestion of involvement in the promotion of OTR transcription[226 254]. It has also been proposed as a possible target for tocolytic therapies in the prevention of pre-term labour and potentially would display a better side effect profile in the manipulation of inflammation than the COX inhibitors[237 254], certainly evidence suggests progesterone may exhibit anti-inflammatory actions via suppression of NF-κB[257]. However, there is evidence of alternative transcription factors involved in the inflammatory response in some cell types[258 259], suggesting that blockage of NF-κB alone may not be sufficient to suppress the inflammatory and contractile response in uterine tissue.

In terms of infection associated inflammation, the clinical concern is of premature activation of uterine contractile activity resulting in pre-term labour which has been associated with infection (maternal systemic or local intrauterine infection) in up to 40% of cases[18 235 260-262]. Certainly, in the mouse model, exposure to the TLR-4 agonist lipopolysaccharide (LPS) is a recognised method whereby pre-term labour is successfully induced[263]. Infection is a classically recognised stimulus for mounting an inflammatory response[264 265]. Therefore, based on infection induced inflammation, the inflammatory response has been proposed as being pivotal in the initiation and propagation of human labour (term and pre-term) [210 216 217 230-236].

The theory regarding pathways involved in infection driven parturition hinges on an infective stimulus recruiting leukocytes to the affected tissue which in turn increases cytokine production and initiation of prostaglandin production[199 231]. It would therefore follow that using antibiotics to reduce bacterial load would potentially reduce inflammation and therefore inhibit the contractile response. However, despite the use of antibiotics (particularly in human pre-term labour and prevention of preterm labour with intact membranes) leading to reduced maternal intrauterine infections, there has been no concomitant reduction in preterm birth, delivery within 48 hours or perinatal mortality rates[261 266 267]. It has been suggested that these results may reflect firstly an inability of the antibiotic to penetrate and treat the infected tissues, or perhaps in these situations the inflammatory response is already established which cannot be altered by removal of the infective stimulus[261]. However, it has been proposed that treatment of culture proven infections in the antenatal period may have a

preventative effect on the development of infection associated preterm labour, particularly in high risk women[231 261 268-271].

1.5.5 Tocolytics and modulation of myometrial contractile activity

Where premature activation of myometrial contractile pathways occur, attempts can be made to suppress contractile activity via the administration of tocolytic agents. Discussed below are those specifically relevant to or examined within the main body of this thesis.

1.5.5.1 Myosin Light Chain Kinase inhibitors

There are a number of myosin light chain kinase (MLCK) inhibitors including wortmannin and ML9 available which have been used in vitro to demonstrate the importance of the contractile common final pathway via MLCK.

During the course of this thesis, we chose to use ML7 (1-(5-Iodonaphthalene-1-sulfonyl)-1H-hexahydro-1,4-diazepine hydrochloride, molecular weight 452.7, Sigma UK I2764), a selective MLCK inhibitor due to its specificity and its ability to inhibit contractile mechanisms of our myometrial samples (for details see Chapter 2, Section 2.3.3.2)[272-275]. Since MLCK is an essential component of the contractile pathway, inhibiting the activity of this enzyme will prevent phosphorylation of myosin light chain thereby preventing actin and myosin cross-linking and ultimately preventing cellular contraction (Figure 11).

ML7 is not used in clinical practice since MLCK inhibition would have profound effects on other contractile cells including cardiac, gastrointestinal and vascular smooth muscle myocytes and certainly blocking contractility of these cells *in-vivo* would most likely be fatal. However, it does provide an excellent substance to use in the study of contractile pathways *in-vitro* with the advantage of little interruption to upstream cellular events.

1.5.5.2 Calcium channel antagonists (Nifedipine)

Nifedipine is a calcium channel antagonist and acts to reduce the entry of calcium into the cell via blockage of the L-type voltage gated calcium channels calcium[186 187]. As discussed earlier, the main source of calcium entry is via the L-type calcium channel, are therefore blocking this channel with nifedipine leads to significant reduction in the cells ability to increase intracellular calcium levels. Since calcium is essential for the contractile machinery, reduced levels

of intra-cellular calcium therefore lead to suppression of myocyte contractions (Figure 13). Nifedipine is used in clinical practice as a tocolytic in the treatment of established pre-term labour (unlicensed in the UK) and has been recommended by a Cochrane Review as 'preferable to other tocolytic agents, mainly betamimetics'[239 276].

1.5.5.3 β_2 adrenergic receptor agonist (Ritodrine, Terbutaline)

Ritodrine acts as an agonist at the cell surface β_2 adrenergic receptor which promotes smooth muscle relaxation and is therefore considered as a uterine tocolytic. It exerts relaxatory effects on smooth muscle mainly by increasing levels of cyclic –AMP and its activation of cAMP-dependent protein kinase-A (PKA). PKA acts to alter intracellular events including opening of Ca^{2+} sensitive K^+ channels (BK_{Ca} or maxi-K) leading to cell membrane hyperpolarization thereby rendering depolarization and entry of calcium to the myocyte more difficult (Figure 13)[116]. In addition it inhibits MLCK activity and gap junction permeability, further promoting cellular relaxation[116 186 187]. Ritodrine is used in clinical practice licensed in the UK as a tocolytic in the treatment of established pre-term labour and has been shown to decrease the number of women in preterm labour delivering within 48hrs[239 277], however there is insufficient evidence for its use in prophylactic or maintenance therapies[278 279].

1.5.5.4 Potassium channel opener (Levcromakalim)

Levcromakalim is an ATP-dependent potassium channel ($K_)$ opener and works to hyperpolarize the cell membrane, therefore making it more difficult for the cell to depolarize with the net effect of promoting relaxation in smooth muscle (Figure 13)[280]. In the clinical situation it has been used as an antihypertensive medication, and has been proposed as a possible tocolytic[280 281].

1.5.5.5 Progesterone

Progesterone is a sex steroid hormone which has been considered to act as a suppressor of uterine contractility. It has also been suggested that pre-treatment of myometrium *in-vivo* with progesterone can render the myometrium less sensitive to the uterotonic effects of oxytocin and more sensitive to the

effects of tocolytics, including ß2-agonists[282 283], nifedipine and indomethacin[284]. However, this is not replicated by all studies, and importantly, *in-vivo* pre-treatment with progesterone does not seem to affect response to uterotonins or tocolysis[285 286]. Additionally evidence for its use *in-vivo* has been conflicting with no difference in rates of preterm delivery in twin pregnancy[287] and inconsistency of contractile inhibition in *in-vitro* and *in-vivo* singleton models[285 287-291]. The mechanisms by which progesterone can have a tocolytic effect is unknown, and had previously been thought to be via manipulation of potassium channels however we were unable to confirm this proposal[285]. The effects therefore, may be mediated by systemic inflammatory suppression or reduction in PG production via suppression of NF-κB[257 285].

1.5.5.6 Oxytocin receptor antagonist (Atosiban)

Atosiban is an OTR antagonist which has been shown to inhibit myometrial contractions in vitro[292] (Figure 13). Evidence of its success as a tocolytic in clinical practice has been variable[293], but has been shown to reduce the number of women giving birth within 7 days when treated for pre-term labour[239]. There is currently insufficient evidence to support its use in the prophylaxis of preterm labour[294].

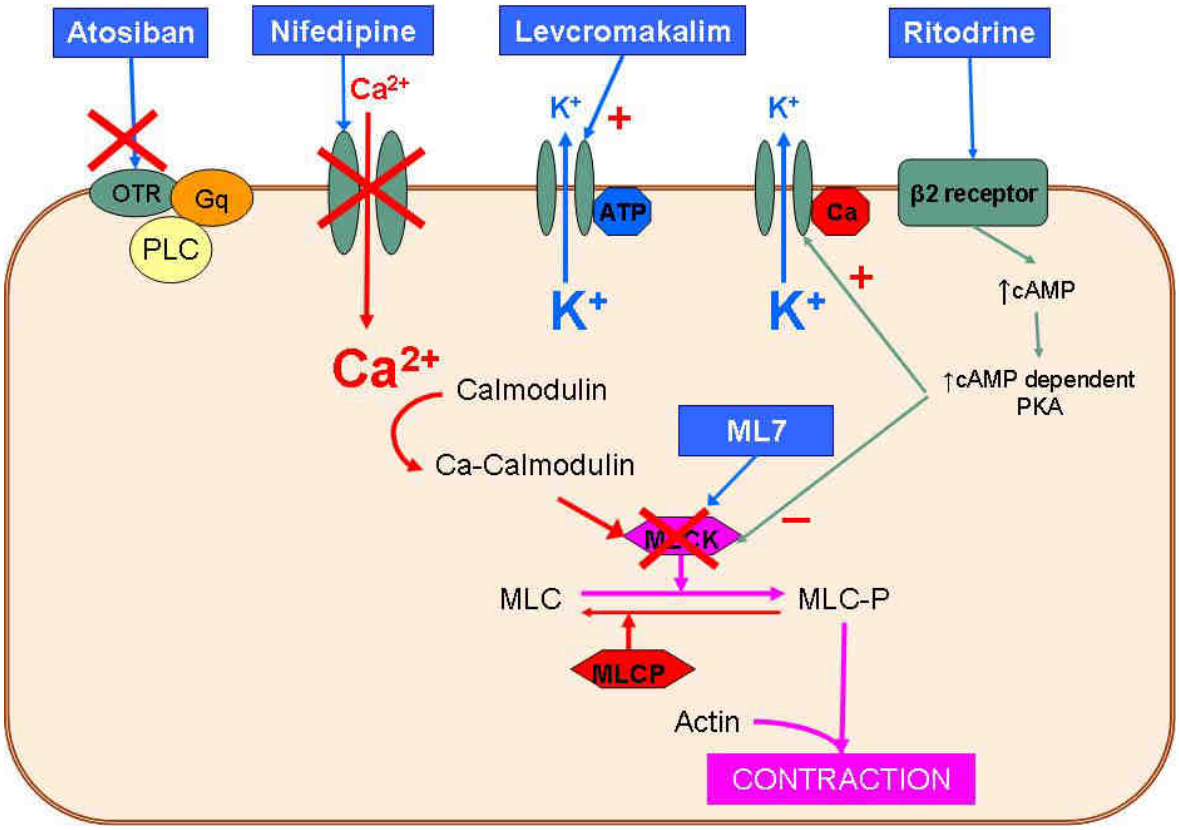

Figure 13 Mode of Action of Tocolytic agents

Graphical illustration of mode of action for selected methods of tocolysis namely Atosiban, Nifedipine, Levcromakalin, Ritodine and ML7. All apart for ML7 and levcromakalim are used in clinical practice in the UK for the management of preterm labour.

Overall, in terms of tocolytics, no single medication has been fully successful at halting the progress of labour, especially once labour has become established. This suggests redundancy in the system, which would certainly be plausible and desirable for such an important process as human parturition. Suggestions have been made to combine tocolytic medications for the desired effect, but as yet no combination has been adopted in clinical obstetrics, with further work still to be performed.

1.5.6 Maternal Characteristics

Despite the wealth of knowledge regarding the physiological processes underlying myometrial contractility, the maternal phenotype is capable of disrupting these mechanisms. Specifically there is considerable evidence for the implications of maternal BMI and other maternal and pregnancy factors affecting parturition *in-vivo*[62-80 295 296]. Many of these factors can be regarded as related to myometrial contractility function and or response to OT. These characteristic will be briefly discussed below.

1.5.6.1 Body mass index (BMI)

Maternal BMI can influence many aspects of pregnancy. In particular to parturition, a low BMI<17 is associated with a higher risk of preterm labour and delivery[19 297], while increased BMI is associated with higher rates of post date pregnancy[62 65 298], induction of labour[63-65], intrapartum emergency caesarean delivery for dysfunctional labour despite augmentation with oxytocin (OT) [62-64 66 67] and post partum haemorrhage independent of mode of delivery[63 65 66].

Collectively these observational studies suggest that obesity has an inhibitory effect on myometrial function and response to OT. Additionally, *in-vitro* contractility studies have suggested that organ bath environments with a high lipid or leptin content, representative of the circulatory maternal environment of those with a high BMI, can negatively affects contractile ability[299-301]. Also, it has been suggested from rat studies that high fat diets pre-pregnancy and during pregnancy are associated with a lower myometrial expression of contraction associated proteins including connexin-43[302].

1.5.6.2 Parity

Multiparity and previous vaginal delivery is associated with a shorter labour and a lower rate of caesarean delivery compared with nulliparous women[303 304]. Additionally, parity can influence the success of induction of labour, with women who are nulliparous undergoing elective induction of labour compared with spontaneous labour having an increased risk of requiring intrapartum caesarean delivery for dysfunctional labour. However, this does not seem to

hold true for parous women[49 52 305]. With increasing parity, the pressure generated by the uterus throughout labour which results in a normal vaginal delivery is less than in the nulliparous woman[72]. This suggests that firstly the myometrium of parous women may be damaged by a previous pregnancy, labour and remodelling. Alternatively, laxity of the pelvic floor after a previous delivery may facilitate passage of the neonate with a concomitant reduction in the required contractile force by the uterus.

It is therefore uncertain as to whether parity has a direct effect on myometrial contractile ability or if observed clinical effects are a result of other parity related factors.

1.5.6.3 Maternal age

Maternal age is implicated in many pregnancy outcomes[306]. In terms of contractility and labour, increasing maternal age is associated with increased rates of post-dates pregnancies, induction of labour and intrapartum caesarean delivery for dysfunctional labours[68-71 307 308]. This implies that increasing maternal age may play a role in the negative regulation of myometrial contractile function.

1.5.6.4 Previous anatomical damage or surgery

The most common surgical scarring of the uterus is found after previous caesarean delivery. Rates of caesarean delivery are increasing and currently account for approximately over 25% of live births in the UK 2010[3 4]. Therefore the numbers of women embarking on a subsequent pregnancy with a uterine scar is increasing.

The risks following previous caesarean delivery are mainly associated with scar dehiscence or rupture during labour, with RCOG patient literature quoting rates of 0.2-0.8%[74]. In terms of ability to deliver after previous CS, rates are high at approximately 72-76%, with further positive and negative influences from previous vaginal delivery, reasons for previous CS delivery, maternal BMI, age, induction of labour and foetal weight[74-78]. However, despite clinical evidence suggesting a negative effect on uterine contractility, it is of interest to note that

progress in the first stage of labour is comparable between women with a previous CS and women without[309].

1.5.6.5 Birth weight and uterine wall tension

In general, smooth muscle cells have the ability to stretch between 4-5 times in length whilst maintaining contractile ability. This is thought to be due to the attachment of actin to the randomly scattered dense bodies (Figure 7). In pregnancy, stretch and therefore uterine wall tension increases in the latter part of gestation as the uterus accommodates the growing foetus, placenta and amniotic fluid. In situations where there is over distension of the wall as in multiple births and polyhydramnios, there is an increased risk of preterm labour [3 18 23 310], however, it has been proposed that other factors not related to uterine wall stretch contribute to the risk of pre-term labour in these situations[311]. Additionally, increasing neonatal birthweight which may also be used as a surrogate marker for uterine stretch seems to be negatively associated with initiation of uterine contractility resulting in prolonged pregnancy[79].

In-vitro, stretch of myometrial primary culture cells (pregnant not in labour) resulted in an increased expression of OTR[163], however, no increase was observed in cells derived from non-pregnant and pregnant in labour samples[163]. Additionally cells which are stretched *in-vitro* upregulate their expression of inflammatory mediators[254 312 313], however, it is unclear if this is a reaction to cellular damage or if inflammation has the ability to stimulate contractility. *In-vivo*, where ultrasound imaging of the uterine wall has been utilised to measure uterine wall tension, there was no evidence of increased tension in those delivering preterm or those with twin gestations[100]. Taken together, this would suggest that there is ambiguity surrounding the role of uterine wall tension or stretch and its role in the onset of labour in humans.

1.6 Summary

Despite much evidence regarding the mechanisms of uterine contractility, the exact molecular pathways involved in the initiation and propagation of successful human labour at term is unclear. With such a fundamental process, there is likely to be multiple pathways allowing for an element of redundancy in the situation where one pathway fails. However *in-vitro* and *in-vivo* investigation of the functional and transcriptional response to prolonged contractions and the potential differential response to known clinical mediators of oxytocin, tocolysis, infection, inflammation, and maternal BMI will provide an invaluable insight into the workings of human pregnant myometrium.

1.7 Main themes of thesis

The work presented in this thesis aims to explore the roles of many of the factors described above in relation to human labour. The main themes of this thesis are summarised below.

- The transcriptional changes induced in myometrium in response to long term (hours) exposure to oxytocin

- The time and contraction dependent transcriptional changes induced in human myometrium in response to repeated contractile activity.

- The contractile and transcriptional response of myometrium to tocolysis

- The contractile and transcriptional response of human myometrium to a pro-inflammatory/infective LPS environment

- The *in-vivo* changes in the maternal circulation in response to pregnancy, delivery by pre-labour planned caesarean section and the temporal changes during labour with particular reference to leukocyte numbers, activation status, chemokine receptor expression, circulating cytokines, CRP and measures of myocyte damage CK and Mb

- The *in-vitro* contractile response (spontaneous and oxytocin induced) of myometrium and the influence of its *in-vivo* maternal environment including maternal BMI, birthweight, gestation and parity.

Chapter 2

Materials and methods

2 Methods

Multiple experimental designs and methodologies are used throughout this thesis and adapted in order to address the specific research question. Each specific experimental design is described in detail within the relevant chapters. In broad terms two types of biological samples were collected. Firstly myometrial biopsies were collected and used for the *in-vitro* organ bath contractility experiments, gene array and QPCR. Secondly, maternal blood samples were taken for analysis of various *in-vivo* circulating parameters in relation to pregnancy and delivery. The subjects and tissue handling for each biological sample is described below, with subsequent description of experimental procedures. Details of each individual experiment and the characteristics for the women contributing to each of these experiments are provided at the beginning of each results chapter.

2.1 Myometrial Biopsies - Subjects

Ethical approval for sample collection was obtained from the North Glasgow University Hospitals Research Ethics Committee Reference 06/S0704/105 and all patients gave written informed consent to participate.

Myometrial biopsies were obtained from non-laboring women with a singleton pregnancy, at term (≥ 37 weeks gestation) undergoing routine elective cesarean delivery under regional anesthesia at the Princess Royal Maternity Hospital, Glasgow, UK. Women were excluded if they had any clinical signs of impending labour (including uterine contractions or ruptured membranes), placenta praevia, uterine abnormalities, or pre-existing medical conditions, including pre-existing or gestational diabetes and epilepsy or use of opiate based medications (including methadone), which could potentially affect contractility. Information relating to the pregnancy was recorded for each woman including body mass index (BMI) at time of booking, maternal age, parity, gestation at the time of delivery, indication for planned caesarean delivery and birth weight.

2.2 Myometrial Biopsies - Tissue handling and storage

Following delivery of the baby and prior to delivery of the placenta, full thickness, myometrial biopsies were taken from the upper lip of the lower

uterine segment incision in the midline. All samples were taken prior to routine administration of an intravenous 5IU syntocinon bolus. Tissue samples were placed in a buffered Krebs solution (NaCl 133mmol/L, KCl 4.7 mmol/L, glucose 11.1mmol/L, $MgSO_4$ 1.2 mmol/L, KH_2PO_4 1.2 mmol/L, $CaCl_2$ 1.2mmol/L and N-tris(hydroxymethyl)methyl-2-amino-ethanesulfonic acid [TES] 10 mmol/L), stored at 4^{o}C and used within 12 hours of collection.

Samples taken from the lower uterine segment in this manner have demonstrated *in-vitro* functional contractile activity, comparable to samples from the fundus[92 196]. The differential transcriptional profile of the upper and lower uterine segments in the human is difficult to assess firstly due to difficulties in accessing upper segment samples, and secondly the *in-vivo* functional difference between the two areas during labour and its inevitable influence on transcriptional profiles[91 243 314]. However, we have not found any regional variation in myometrial transcription throughout the length of the uterine horn in the mouse model[315]. Additionally, the myometrial thickness of the lower segment in the third trimester prior to labour does not appear to be affected by previous caesarean section[316]. In particular for this study in which we examine myometrial gene transcription, samples taken in this manner from the lower uterine segment consist almost entirely of myocytes[91]. Thus down stream analysis is representative of the gene expression in myometrial cells.

Tissue was snap frozen at a variety of time points after dissection as described in each individual chapter. Samples which were set up in the organ bath as detailed below and were snap frozen once experiments were complete according to the pre-set time allocated to that specific contractility study. Samples taken from the organ bath were loosened from tension and removed from the bath making sure that they were not actively contracting at the time. The ends of the strips distal to the silk tie were removed to minimise any impact of tissue necrosis which may have occurred as a result of the ties. The sample was then split transversely into 2 equal portions and excess fluid was removed from the sample surface by placing on blotting paper prior to weighing. Samples were then immediately snap frozen in liquid nitrogen and stored at -80°C.

2.3 Myometrial Biopsies - *In-vitro* contractility organ bath studies

2.3.1 Tissue preparation

Myometrium was dissected along the plane of the muscle fibers into longitudinal strips 2x2x10mm, avoiding any scar or damaged tissue and blood vessels. Each tissue strip was tied at each end with silk and suspended in a separate 10ml organ bath filled with Krebs-Henseleit solution (NaCl 118 mmol/L, NaHCO3 25 mmol/L, KCl 4.7 mmol/L, glucose 11.1 mmol/L, MgSO4 1.2 mmol/L, KH2PO4 1.2 mmol/L, CaCl2 2.5 mmol/L (pH 7.4)). The Krebs solution was made fresh daily. Each bath was constantly perfused with 95% O_2/5% CO_2, and maintained at 37 ^oC. Myometrial strips were then placed under isometric conditions with a 20mN resting tension. Contractility data were recorded via a tension transducer (FT03, Grass Technologies, Slough, UK) and the signal amplified and stored in a commercial data acquisition system (Octal ML228 Bridge Amplifier; PowerLab ML870/P, Chart, version 3.6, all ADInstruments). Studies on myometrium performed in this manner have been shown to be suitable for examining spontaneous myometrial contractility and response to chemical manipulation[92 317].

Only strips that displayed spontaneous, regular rhythmic activity between 2 and 3 hours were used for experiments. Basal spontaneous activity was recorded for up to 3 contractions before the addition of any drugs or vehicle control to the organ bath.

2.3.2 Interpretation of contractility trace

Spontaneous activity was recorded between 2 and 3 hours in all strips with regular activity. Three measures of contractile activity were assessed, frequency, amplitude and activity integral and are illustrated in Figure 14. Frequency was determined as the time interval between the peaks of two contractions occurring at the time point of interest. Frequency was measured in Hertz (Hz) with subsequent conversion to contractions/hour where appropriate. Contractile force was determined as the difference between baseline tension and maximal contraction amplitude in milli-Newtons (mN). A summary measure of contractile activity was determined by the area under the tension curve for

one contraction and expressed as activity integral. For both amplitude and activity integral, spontaneous activity was determined as an average of three contractions, with drug response measured as one contraction occurring at the time point of interest.

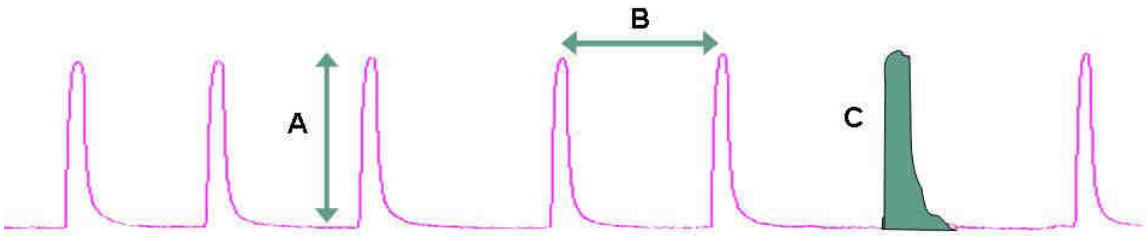

Figure 14 Illustration of the three measures of contractile force

(A) Amplitude measured from baseline to peak of contraction (mN). (B) Frequency measured by the time difference between the peak of 2 contractions (contractions/hour). (C) Activity integral determined by the area under the curve of a contraction (mN.s)

2.3.3 Drugs used in contractility studies

Preparation of drugs used in the contractility experiments and the methods of addition to the 10ml organ bath are described in the following section. All drug additions were undertaken where the tissue was in a rest period as indicated by a return to baseline tension, in other words, drugs were not added whilst the tissue was undergoing a contraction.

2.3.3.1 Oxytocin

Oxytocin (OT) is a major endogenous uterotonin involved in human labour. The hormone is a small nanopeptide produced by the posterior pituitary with evidence for autocrine and paracrine production in gestational tissues towards term[91 144 145]. The immediate cellular effects of oxytocin are mediated via the cell surface G-coupled oxytocin receptor, which has the net result of increasing intracellular calcium which in turn promotes cellular contraction. In clinical practice, the synthetic version Syntocinon® is widely used in the induction and augmentation of labour and in the promotion of contractility in the third stage of labour to assist delivery of the placenta and prevent atonic post-partum haemorrhage.

2.3.3.1.1 Drug preparation

Oxytocin was purchased as oxytocin acetate salt (molecular weight 10007.19, Sigma UK 75968) and the 5mg vial dissolved in 4.96ml 2% acetic acid making a 1mM OT stock solution, which was divided into aliquots, stored at -20°C, defrosted and diluted in fresh Krebs solution as needed on day of experiment.

2.3.3.1.2 Oxytocin concentration response

Samples were collected from 25 women, with a total of 54 strips examined. Myometrial strips all achieved spontaneous regular activity within 2 hours. Since OT acts via cell membrane receptor, it has an almost immediate effect on contractility. Therefore, a traditional logarithmic cumulative addition of oxytocin was undertaken in the range 10pM to 10μM (10^{-12} to 10^{-6} M), with an interval of 20 minutes between each addition. An example of the contractile pattern obtained in response to cumulative additions of OT is demonstrated in Figure 15.

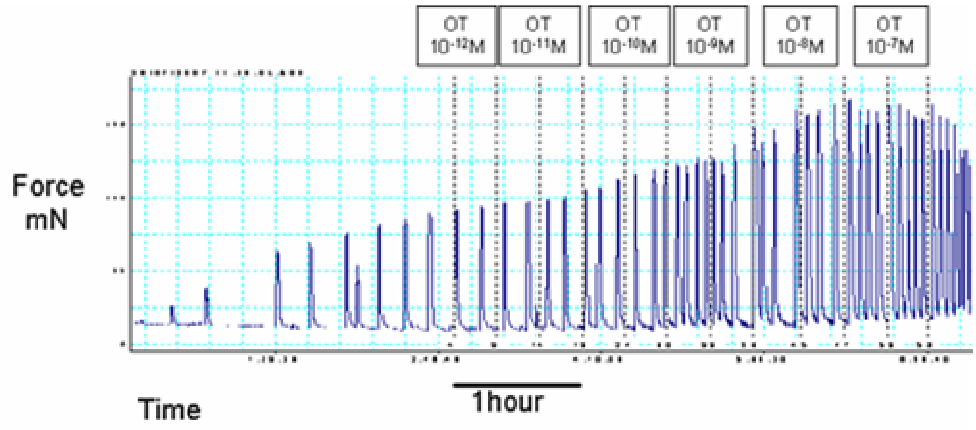

Figure 15 OT dose response contractile activity trace

An illustrative functional trace demonstrating the contractile response to cumulative additions of OT.

A Log EC_{50} [oxytocin] (the concentration of OT required for the tissue to demonstrate 50% the maximal response) was calculated based on the average % increase in amplitude from baseline at each bath concentration, with the overall Log EC_{50} [oxytocin] = -9.896M, (Figure 16).

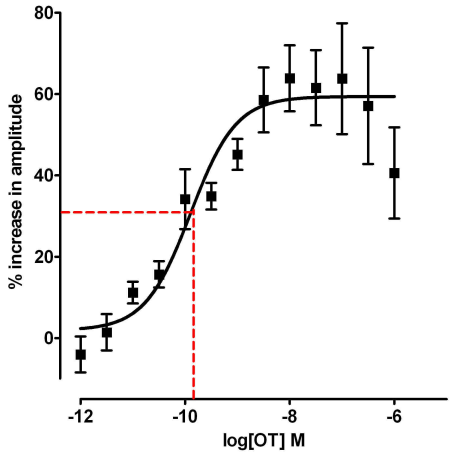

Figure 16 OT concentration response curve

Concentration response curve showing the effect of oxytocin (10^{-12} to 10^{-6}M) on percentage increase in contraction amplitude from baseline measures (n subjects=25, n strips = 54). Values shown are mean with SEM and shown with a sigmoidal dose-response nonlinear regression curve. The red dotted line indicates the LogEC50 (the concentration of oxytocin required to demonstrate 50% of the maximal response)

2.3.3.1.3 Oxytocin addition to study response to a single concentration

The concentration response experiments suggested that a final bath concentration of 1nM would be appropriate for promoting contractility in response to a single addition. However, it was imperative for transcriptional analysis that overstimulation resulting in a prolonged tonic phase was avoided. Therefore, a single addition of oxytocin was undertaken giving a final bath concentration of 1nM, 10nM or 100nM. An example of the effect of these concentrations is demonstrated in Figure 17. These data support the use of a bath concentration of 1nM OT since at this concentration there is promotion of myometrial contractility, avoiding the tonic phase induces by the higher concentration of 10nM and 100nM. Therefore, a final bath concentration of 1nM oxytocin was used in subsequent experiments where a single addition of the drug was undertaken.

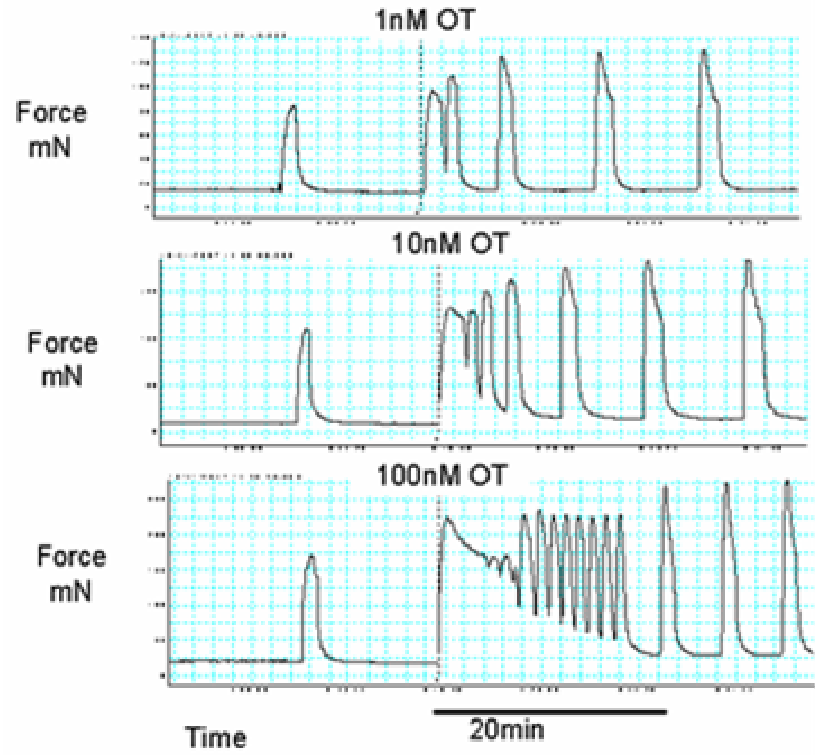

Figure 17 Myometrial contractile response to a single addition of OT

Example of the effect of a single concentration of 1nM, 10nM or 100nM OT on spontaneously contracting myometrial strips *in-vitro*.

2.3.3.1.4 Oxytocin addition to study response to a single concentration

The final bath concentration of 1nM oxytocin was achieved by a single addition of 10µl of 1mM oxytocin stock solution to the organ bath which contains a volume of 10ml of Krebs solution.

2.3.3.2 ML7

ML7 (1-(5-Iodonaphthalene-1-sulfonyl)-1H-hexahydro-1,4-diazepine hydrochloride, molecular weight 452.7, Sigma UK I2764) is a selective myosin light chain kinase inhibitor (MLCK) with the overall effect of inhibition of the contractile mechanisms[272-275]. MLCK is an essential component of the pathway leading to myocyte contraction, and therefore blocking the activity of this enzyme will prevent phosphorylation of myosin light chain thereby preventing actin and myosin cross-linking and ultimately, the initiation of a contraction is prevented.

2.3.3.2.1 Drug preparation

The 5mg vial of ML7 was dissolved in 110.5µl Dimethyl sulphoxide (DMSO) to give a 100mM ML7 stock. This was stored in aliquots at -20°C, defrosted and used as needed on day of experiment.

2.3.3.2.2 ML7 concentration response

49 myometrial strips from 9 women were assessed to determine the ideal concentration of ML7 required for complete inhibition of contractile activity.

The method of action of ML7 is intracellular, and thus the effect of exposure is not immediately apparent. Therefore, traditional concentration response experiments with cumulative additions of drugs (as described for oxytocin) could not be undertaken. Thus after spontaneous activity was established in a strip, it was exposed to a single concentration of ML7 in the range 12.5µM to 300µM. Measurements of contractile activity were taken prior to addition of ML7, and at 30, 60 and 120 minutes after addition. After two hours of exposure, a single concentration of OT 1nM was added to the bath to ensure complete blockage of contractions had been achieved. Measures of contraction amplitude and frequency were made, and percentage decrease from baseline calculated at 30, 60 and 120 minutes after addition.

Dose response for percentage reduction in contraction amplitude and frequency were compiled (Figure 18). The inhibition of contraction amplitude observed using the highest concentration of ML7 (300 µM) 99.2 ± 0.5% ($p<0.001$). Furthermore, an ML7 concentration of ≥200 µM was sufficient to maintain complete inhibition of contractile response to oxytocin (Figure 19).

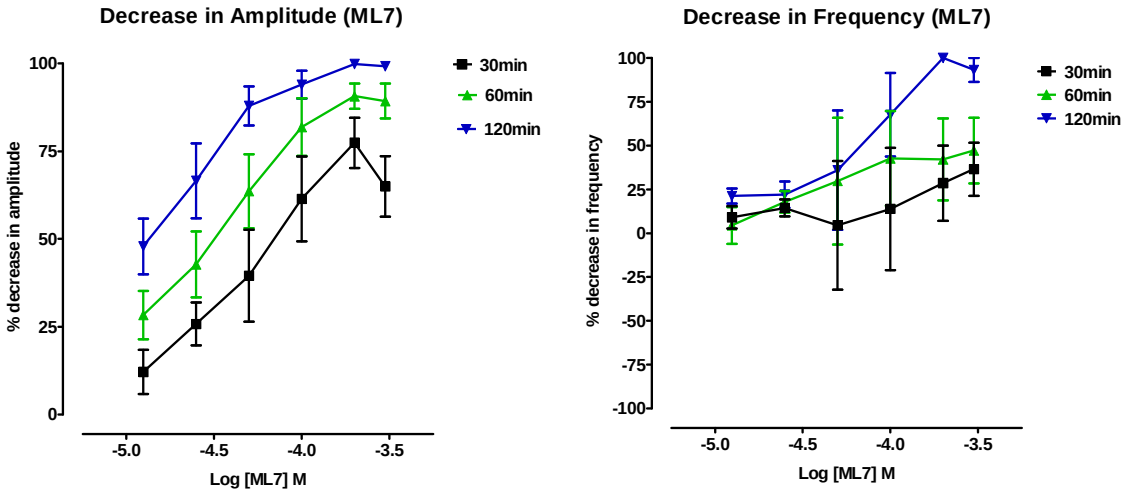

Figure 18 ML7 concentration response curves

Concentration response curves showing the effects of ML7 on the magnitude and frequency of spontaneous contractions of strips of myometrium from pregnant women. Values are means ± sem. n subjects = 9, n strips = 49

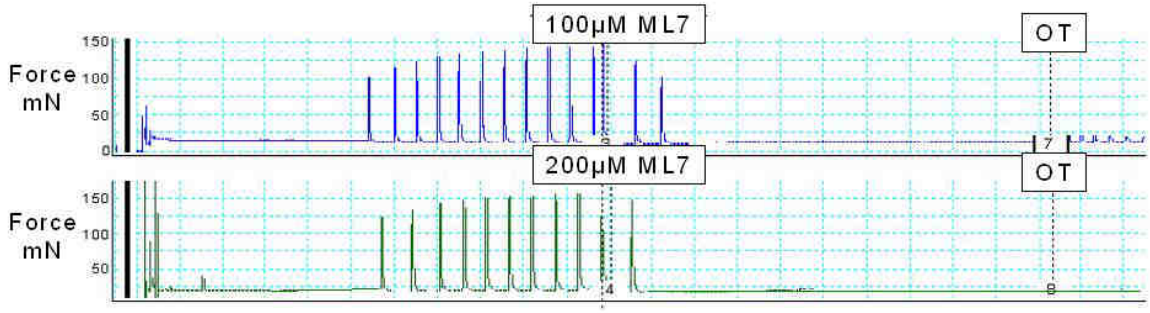

Figure 19 Myometrial contractile response to OT after pre-incubation with ML7

An example of functional contractility trace indicating the ability of a concentration of 200µM of ML7 to completely inhibit contractility even in the presence of OT whereas at the lower concentration of 100µM contractions return, albeit lower amplitude when OT is added.

2.3.3.2.3 ML7 addition to study response to a single concentration

A single addition of 20µl or 100mM stock ML7 was undertaken to give a final bath concentration of 200µM.

2.3.3.3 Peptide 18

Peptide 18 (molecular weight 1324.6) is a selective myosin light chain kinase inhibitor[318]. Similar to ML7, Peptide 18 requires entry to the cell to exhibit its inhibitory effect on the enzyme myosin light chain kinase

2.3.3.3.1 Drug preparation

The Peptide 18 (Calbiochem UK 475981) 5mg vial was dissolved in 750µl DMSO to give a 5mM Peptide 18 stock solution. Aliquots were stored at -20°C, defrosted, and used as needed on day of experiment.

2.3.3.3.2 Peptide 18 concentration response

Concentration response curve experiments for Peptide 18 were conducted in the same way as for ML7 using 26 strips from 3 women. The documented IC_{50} for peptide 18 is 50nM[318], therefore a drug concentration range of 0.5nM to 50µM was used, with contractile activity observed for 2 hours after addition when a single addition of 1nM OT was undertaken. Again, measures of contraction amplitude and frequency were made, and percentage decrease from baseline calculated at 30, 60 and 120 minutes after addition.

The inhibition of contraction amplitude observed using the highest concentrations of Peptide 18 (50 µM) was 8.0±1.2%, and overall Peptide 18 at the concentrations used appeared to ineffective at blocking contractile activity in myometrial biopsies. Additionally, despite addition of Peptide 18, subsequent exposure to OT caused an immediate increase in contractility in all strips. Peptide 18 was therefore not selected as the drug of choice for inhibition of myometrial contractility for the future experiments.

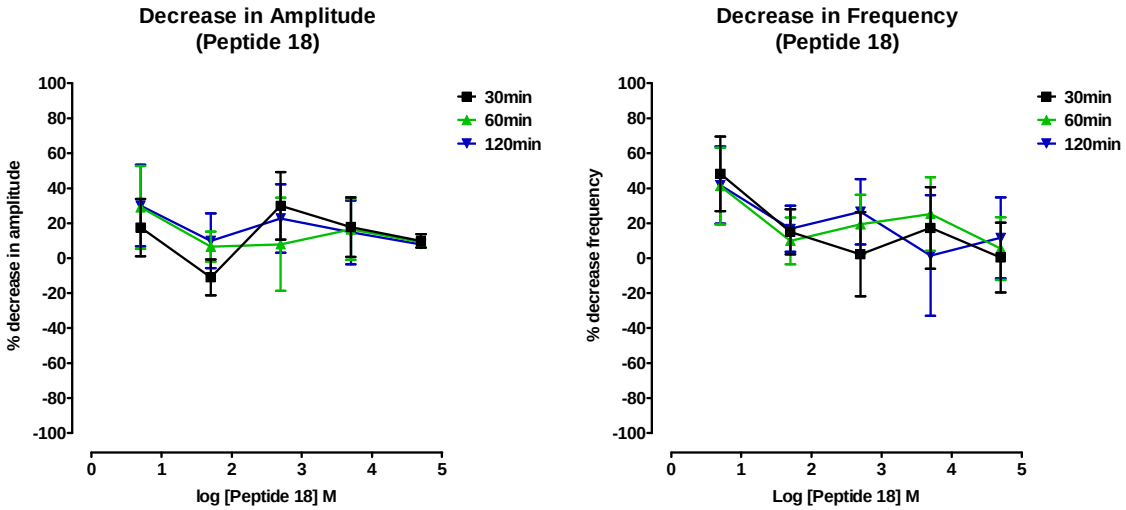

Figure 20 Peptide 18 concentration response curves

Concentration response curves showing the effects of Peptide 18 on the magnitude and frequency of spontaneous contractions of strips of myometrium from pregnant women. Values are means ± sem. n subjects=3, n strips=26.

2.3.3.4 Acetic Acid vehicle

2% acetic acid is used as the vehicle for dissolving oxytocin. It is stored at room temperature, and diluted in Krebs on the day of the experiment in the same way as the 1mM OT stock solution with 10µl of 2% acetic acid added to the 10ml organ bath of Krebs solution.

2.3.3.5 Dimethy Sulphoxide (DMSO) vehicle

Dimethyl sulphoxide (DMSO) is used as the vehicle for dissolving both ML7 and Peptide 18. 100% DMSO is stored at room temperature, and added to the 10 ml of Krebs in the organ bath, undiluted at a volume of 20µl.

2.3.3.6 Ritodrine

Ritodrine acts as an agonist at the cell surface $ß_2$ adrenergic receptor which promotes smooth muscle relaxation and is therefore considered as a uterine tocolytic. Ritodrine is used in clinical practice licensed in the UK as a tocolytic in the treatment of established pre-term labour[239].

2.3.3.6.1 Drug preparation

Ritodrine (Sigma, UK) was dissolved in Normal Saline (0.9% NaCl) to give a stock solution of 1M and stored in aliquots at -20°C.

2.3.3.6.2 Ritodrine concentration response

These were previously carried out in the department where it was demonstrated that a final bath concentration of 10mM was required for maximal inhibition of contractile activity[285]. This concentration was achieved by the addition of 100µl of 1M stock solution to the 10ml organ bath.

2.3.3.7 Nifedipine

Nifedipine is a calcium channel antagonist and acts to reduce the entry of calcium to the cell via the L-type voltage gated calcium channels. Since calcium is essential for the contractile machinery, reduced levels of intra-cellular calcium therefore lead to blockage of contractions. Nifedipine is used in clinical

practice as a tocolytic in the treatment of established pre-term labour (unlicensed in the UK)[239].

2.3.3.7.1 Drug preparation

Nifedipine (Sigma, UK) was dissolved in ethanol to give a concentration of 0.1M. This was further diluted with DDW to give a working stock solution of 10mM to be used when single additions of Nifedipine were made. Aliquots of stock solution were stored at -20°C.

2.3.3.7.2 Nifedipine concentration response

These were previously carried out within the department and demonstrated the concentration of nifedipine required for maximal inhibition of contractile activity was 10μM [285]. This final bath concentration was achieved by the addition of 10μl of 10mM stock solution to the 10ml bath.

2.3.3.8 Progesterone

Progesterone is a sex steroid hormone and is produced mainly by the placenta in term human pregnancies. In non-human pregnancy, withdrawal of progesterone is considered the initiator of labour. Some consider progesterone to act as a suppressor of uterine contractility. Traditionally progesterone compounds required to be reconstituted in high concentrations of ethanol. However, these high concentrations of ethanol itself have a tocolytic effect on myometrium, and therefore the alternative water soluble progesterone was used in these experiments.

2.3.3.8.1 Drug preparation

Water-soluble progesterone (Sigma, UK) was dissolved in normal saline (0.9% NaCl) to give a stock solution of 10mM, with aliquots stored at -20°C

2.3.3.8.2 Progesterone concentration response

These were previously performed in the laboratory and demonstrated the concentration of water soluble progesterone required for maximal inhibition of contractility was 1mM which was achieved by the addition of 1ml of 10mM stock to the 10ml organ bath[285].

2.3.3.9 Levcromakalim

Levcromakalim is an ATP-dependent potassium channel opener and works to hyperpolarize the cell membrane, therefore making it more difficult for the cell to depolarize with the net effect of promoting relaxation in smooth muscle.

2.3.3.9.1 Drug preparation

Levcromakalim (Sigma, UK) was dissolved in DMSO to give a stock solution of 100mM, which was further diluted with DDW to give a working stock solution of 10mM. Aliquots were stored at -20°C.

2.3.3.9.2 Levcromakalim concentration response

These were previously carried out in our laboratory and demonstrated that a final bath concentration of 10μM was required for maximal inhibition of contractile activity[285]. This concentration was achieved by the addition of 10μl of 10mM stock to the 10ml organ bath.

2.3.3.10 Lipopolysaccharide (LPS)

Lipopolysaccharides (LPS) are a group of molecules made up from a lipid and a polysaccharide and are present in the cell walls of Gram-negative bacteria. They act via the TLR4 receptors to induce an intense pro-inflammatory response with release of cytokines. They can contribute to contamination of solutions used for *in-vitro* experiments.

2.3.3.10.1 Drug preparation

5mg of LPS (*Salmonella typhosa*) (Sigma-Aldrich) was dissolved in 50ml of DDW to give a stock solution of 0.1mg/ml. This was aliquoted and stored at -20◦C with 10μl of stock added to each 10ml bath when required to give a final bath concentration of 0.1μg/ml. This final bath concentration was based on previous work where LPS at this level was able to induce a significant inflammatory response when cultured with rat aortas[319].

2.3.3.11 Polymixin B (PMB)

Polymixin B (PMB) is a decapeptide obtained from *Bacillus polymyxa* which is recognized as a potent antibiotic with the ability to bind and neutralize bacterial endotoxin (LPS) [320]. It is therefore widely used in the solutions for *in-vitro* experiments to remove any contaminating LPS.

2.3.3.11.1 Drug preparation

90mg of PMB (Sigma-Aldrich) was dissolved in 3ml of DDW to give a stock of 30mg/ml. Aliquots were stored at -20◦C. Where the addition of PMB was required, 10µl of stock was added to each 10ml organ bath to give a final bath concentration of 30µg/ml. This concentration of PMB was based on previous work in the laboratory of Professor Martin which showed this concentration of PMB was sufficient to suppress the inflammatory reaction of rat aorta while in culture[319].

2.4 Myometrial Biopsies - RNA extraction, quantification and quality assessment

The frozen myometrial samples were processed in a random order, one at a time. The total RNA extraction protocol has been adapted from that used by collaborators in Warwick, where they have experience of extracting high quality RNA from small myometrial samples.

In summary, immediately on removal from the -80°C freezer, the sample was homogenized in 1ml of Trizol with a hand held homogenizer with disposable tips. The samples then underwent an initial centrifugation at 8000xg at 4°C allowing removal of any excess fibrous tissue from the homogenate. The 'clean' homogenate was then incubated with 0.2ml of 1-Bromo-3-Chloro-Propane (Sigma UK, B9673) for 3 minutes at room temperature, followed by separation into the organic and aqueous phases while samples were centrifuged for 15 minutes at 8000 X g at 4°C. Of note, 1-Bromo-3-Chloro-Propane was substituted for chloroform after problems with contamination of our stock of chloroform led to RNA degradation.

The aqueous phase was then carefully obtained and 0.5 x aqueous phase volume of 100% ethanol added. The sample was then processed according to the

manufacturers protocol for RNeasy MiniKit (QIAGEN) from step 10, RNeasy Fibrous Tissue Mini Handbook 01/2003. The optional DNase treatment steps were not performed and RNA was eluted into 30µl of RNase-free water with the elution step repeated using the first elute in order to increase the final concentration of RNA.

Total RNA was quantified on the Nanodrop ND-1000 spectrophotometer, which is a cuvette free method of measuring concentrations of RNA, DNA or proteins on microsamples (1µl). RNA quality and integrity was assessed using the BioAnalyzer 2100 (Agilent RNA6000 Nano LabChips®), and performed as a service at the Sir Henry Welcome Functional Genomics Facility, University of Glasgow. Samples were considered to be of good quality if they showed clear peaks at 18S and 28S and produced a RNA integrity number (RIN) >7. The RIN is a measure of RNA integrity developed by Agilent technologies, and aims to provide a standardized measure of RNA quality thereby removing individual interpretations. The algorithm developed by Agilent Technologies classifies total RNA on a scale from 1 to 10 with 1 being the most degraded and 10 being most intact.

2.5 Myometrial Biopsies - RNA amplification and Biotin labeling

Once total RNA was extracted from the myometrial sample, and quality confirmed, the RNA underwent amplification and biotinylation prior to hybridization onto the Illumina gene array chips. This was done using approximately 500ηg of total RNA, following the recommended method using the Illumina Total Prep RNA Amplification Kit (Ambion) according to manufacturers instructions. This labeled cRNA was quantified on the Nanodrop ND-1000 spectrophotometer and quality assessed on the BioAnalyser 2100.

2.6 Myometrial Biopsies - Illumina Human Ref 6 hybridisation and gene array

This was outsourced and performed at The Wellcome Trust, Clinical Research Facility, University of Edinburgh, however, I did attend to observe the methods used. The biotin labelled cRNA prepared above was diluted to give 1.5µg of

cRNA in a final volume of 10µl which was then used for hybridisation to the gene array chips.

The principle of the technique is that each Illumina chip can run 6 individual samples in parallel. For each sample there are >46000 oligonucleotide probes which corresponds to 25440 annotated genes with the remaining probes coding for genes with provisional annotations, non-coding transcripts or experimentally confirmed mRNA sequences. Illumina uses BeadArray™ technology where the chip is covered in beads held in microwells on the surface of the chip. Each individual bead is covered by hundreds of thousands of identical oligonucleotide probes and attached to the bead via a 29 base address sequence (Figure 21) with each bead being present up to 30 times per array. The address sequence is used to map the array prior to any hybridisation. Each probe has been selected from databases including NCBI and RefSeq, and has been selected using a multistep algorithm to account for factors including lack of similarity to other genes, sequence complexity, absence of highly repeated sequence in genome and distance from 3prime end of the transcript, with each probe being 50 bases in length.

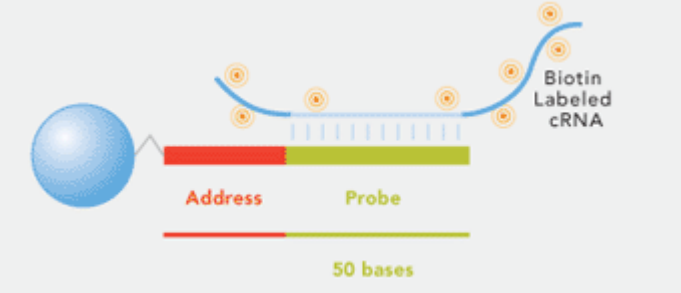

Figure 21 Illustration of Illumina beads and attached probes

An illustration of a 50 base probe linked to the bead via a short 29 base address sequence. Each bead is covered in hundred of thousands of identical gene specific probes and each bead is repeated up to 30 times per array. The probe is hybridised to the biotin labelled cRNA from the study sample. (Illustration reproduced from www.illumina.com)

Once hybridisation of the study sample to the chip has taken place, the BeadChips are washed and stained. They are then read using the BeadArray Reader in conjunction with the pre-determined map for that specific chip. The intensity of staining is indicative of the quantity of gene present in the sample.

2.7 Myometrial Biopsies - Quantitative reverse transcription polymerase chain reaction (QPCR)

The principle of QPCR is that it allows monitoring of the PCR reaction as it occurs in real time using the starter molecule complementary DNA (cDNA) which is made using the messenger RNA (mRNA) templates isolated from the sample of interest. TaqMan technology uses a fluorescent probe which accumulates as the gene of interest accumulates during the PCR reaction. Each oligonucleotide probe has a reporter fluorescent dye of the 5 prime end, with a quencher dye on the 3 prime end and while the probe is intact the quencher limits the fluorescent energy release. During the PCR reaction, if the probe's target sequence is present, it anneals downstream from the primer site and as the primer is extended the probe is cleaved at the 5 prime end by the action of the enzyme Taq DNA polymerase. This cleavage of the reporter dye from the quencher dye allows the expression of the florescent energy which is then recorded. As the PCR reaction progresses, the more target gene sequence is present and therefore the fluorescence emitted increases.

2.7.1 Preparation of cDNA and quantification process

Quantification of gene expression was carried out using sample of mRNA extracted above. Batches of 40µl of cDNA was synthesised from approximately 1.8µg of total RNA from each sample using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems) according to manufacturers protocol. Each time a batch of cDNA was made, a simultaneous control experiment was performed on a sample of 'spare' total RNA. The master mix for the control sample excluded the multiscribe reverse transcriptase, and therefore acted as our negative control run for each 96-well TaqMan plate.

All primer probes used for gene expression quantification were purchased as pre-designed, inventoried Homo-Sapien TaqMan Gene Expression Assays from Applied Biosystems with details in Table 1. In addition all probes used spanned exon junction and was therefore unlikely to and is therefore unlikely to detect genomic DNA contamination. For normalisation of quantitative results, ß-actin was used as an endogenous control/reference gene.

Gene Symbol	Gene Name	Applied Biosystems Assay ID
B-Actin	Human ACTB (B-Actin) endogenous control	4310881E
CCL2	Chemokine (C-C motif) ligand 2	Hs00234140_m1
CCL3	Chemokine (C-C motif) ligand 3	Hs00234142_m1
CCL8	Chemokine (C-C motif) ligand 8	Hs00271615_m1
CCL20	Chemokine (C-C motif) ligand 20	Hs00171125_m1
CXCL1	Chemokine (C-X-C motif) ligand 1	Hs00605382_m1
CXCL2	Chemokine (C-X-C motif) ligand 2	Hs00601975_m1
CXCL5	Chemokine (C-X-C motif) ligand 5	Hs00171085_m1
CXCL8	Interleukin 8	Hs00174103_m1
IL1B	Interleukin 1 Beta	Hs00174097_m1
IL6	Interleukin 6	Hs00985641_m1
IL10	Interleukin 10	Hs00174086_m1
TNFα	Tumor necrosis factor (TNF superfamily, member 2)	Hs00174128_m1
CHURC1	Churchill domain containing 1	Hs00540886_m1
IFIT2	Interferon induced protein with tetratricopeptide repeats 2	Hs00533665_m1

Table 1 Target assay mixes and endogenous control probes used in QPCR
All probes were inventoried and obtained from Applied Biosystems, Cheshire, UK.

To determine the appropriate concentration of sample to be used for each probe, serial dilutions were carried out with appropriate dilution determined by a Ct value around 28-30. 1µl of undiluted sample was used for CCL2, CCL3, CCL8, CXCL5, IL10 and TNFα with 1µl of a 1 in 10 dilution of sample required for ß-actin, CXCL1, CXCL2, CXCL8, IL6, CCL20, IL1ß, CHURC1 and IFIT2.

Quantification was performed on an Applied Biosystems 7900 using a 96 well plate with a different probe and sample combination in each well. Each well contained 12.5µl of TaqMan mastermix, 10.25µl of DEPC (Diethylpyrocarbonate) treated water, 1.25µl of target assay mix (probe) and 1µl of sample (either undiluted of diluted 1 in 10). The thermal cycler conditions were 50°C for 2 minutes, 95°C for 10 minutes followed by 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. For each probe, samples were run in duplicate and control samples were run for every second plate using DDW and the no-RT control

2.7.2 Choice of endogenous control gene

Our results were normalised to an endogenous control (reference gene), and therefore results are an expression of the relative quantification of the gene of interest in relation to the endogenous control. A selection of potential endogenous control genes were chosen based on previous work investigating myometrial gene expression in both our department and others[140]. We examined the expression of the endogenous control genes 18S, B-actin and GAPDH on a random selection of 10 myometrial RNA samples at a 1/10 dilution, with each probe run in triplicate for each sample. As suggested by the application note from Applied Biosystems, the endogenous control should be the gene which shows the most stable expression in our tissue of interest (myometrium). We therefore based our choice on the sample mean Ct value with the narrowest SD. The trial of the above probes gave the following results; 18S (mean ct=13.84, SD=1.15, ß-actin (mean ct=22.11, SD=0.79, GAPDH (mean ct=24.65, SD=2.13) and therefore ß-actin was chosen as our endogenous control.

2.7.3 Analysis and determination of target gene expression relative to β-actin

PCR data were captured and analysed using the automated photometric detector and detection software (ABI Prism 7900, Sequence Detection System v2.3). Threshold values for each probe were determined and used throughout for all PCR cycles using that probe (Table 2). The threshold value indicates background level of fluorescence for that probe.

Gene symbol	Threshold value	Gene symbol	Threshold value
B-Actin	0.258	CXCL8	0.259
CCL2	0.200	IL1B	0.381
CCL3	0.249	IL6	0.315
CCL8	0.193	IL10	0.200
CCL20	0.190	TNFα	0.148
CXCL1	0.130	CHURC1	0.063
CXCL2	0.233	IFIT2	0.254
CXCL5	0.288		

Table 2 Threshold values used for each of the probes used in QPCR
Threshold values used for each of the probes for the determination of Ct values

The Ct (cycle threshold) value indicates the cycle number at which the fluorescence passes the set threshold value (background value) and is therefore a way to determine the concentration of the gene of interest. Ct values were then determined for each well and since samples were run in duplicate, the mean Ct value was used and where Ct values differed by more that 1.2 Ct values, they were excluded and samples repeated. The expression was then determined relative to the B-Actin value for the sample which provides a ΔCt (Ct (target gene) – Ct (endogenous control) = ΔCt). In general the lower the Ct values, the greater the amount of gene present in the sample.

2.8 Myometrial Biopsies - Determination of time points for temporal contractility studies

Within the context of our studies, we were interested in performing temporal analyses of gene transcription. We therefore aimed to establish a time-line description of 1) the length of time which the myometrial tissue strips would maintain the ability to contract following a single addition of OT1nM in the *in-vitro* organ bath environment and 2) the times at which changes in gene expression in response to contractions are observed.

2.8.1 Functional testing of time-points

To assess the temporal contractile ability of the myometrial strips in our *in-vitro* organ bath, four functional experiments were carried out (n patients=4, n strips

from each patient=6). Myometrium was used immediately after collection with each strips set up in a separate organ bath as previously described. Each strip was given 2 hours to establish spontaneous activity, after which time OT was added to each bath to give a final bath concentration of 1nM. One lane at a time was stopped at a time point of 0, 1, 2, 4 or 6 hours after addition of OT, and one strip left to contract overnight.

When each lane was terminated, the myometrial strip was halved, weighed, snap frozen in liquid nitrogen, and then stored at -80°C.

In all experiments, contractions were established in response to OT, with the time at which contractions ceased approximately 8hours after addition of OT in each experiment. It was also noted that approximately 30-60 minutes prior to cessation of contractile activity, the amplitude of contractions decreased. An example of the functional output from these experiments is demonstrated in Figure 22.

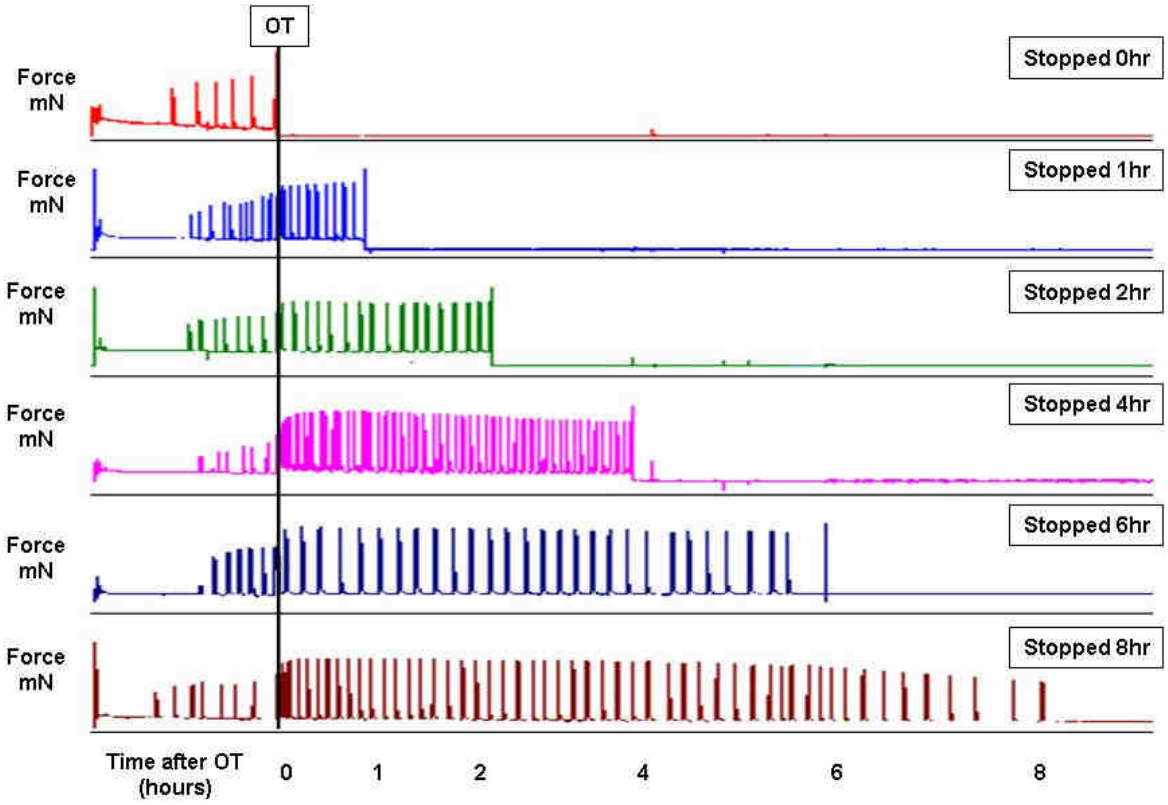

Figure 22 Illustration of the functional contractile data from an OT timeline experiment

This shows establishment of spontaneous contractile activity, response to OT and the maximum time for which contractility is sustained being approximately 8 hours following OT stimulation.

2.8.2 Transcriptional testing of the time-line

Samples from three of the above functional experiments were processed according to the total RNA extraction protocol. They were quantified, and quality assessed, and good quality total RNA had been extracted. The RNA was then prepared according to the Ambion total prep kit protocol and Illumina Human Ref 6 Beadchip arrays were performed as described above.

Results from the transcriptional study indicated that there was a temporal element to gene changes with 12, 4, 37, 1036 and 655 genes differentially expressed compared with 0hr at the 1,2,4,6, and overnight timepoints respectively.

2.8.3 Timeline determination

Data from both the functional and transcriptional experiments suggest that the appropriate choice of maximal timepoint was 6 hours after drug addition. Additionally, the transcriptional data suggest the largest changes to occur between 4 and 6 hours and that earlier time points of less than one hour are not necessary since minimal gene changes are occurring by 1 hour.

2.9 Myometrial Biopsies – analysis of protein products released by contracting samples

To assess the inflammatory protein products which were synthesised and released by contracting myometrium *in-vitro*, analysis of the Krebs solution from the organ bath was undertaken

Strips of myometrium were set up to contract spontaneously as described above. 7 hours after initial tension was applied, experiments were terminated and 5ml of Krebs solution from the organ bath was frozen and stored at -80°C. For comparison, a separate sample of Krebs which had never been exposed to tissue but had been in an organ bath, bubbled with carbogen at 37°C for 7 hours was also frozen and stored at -80°C.

Samples were used neat then prepared and analysed using a Biorad Bioplex Pro Human Cytokine 8-Plex Panel 1 M50-000007 and analysed on the Biorad Bioplex

System- Luminex 100 according to manufacturers instructions. The principles of multiplex analysis are described in detail below in section2.14. The specific proteins assessed by this assay are IL2, IL4, IL6, IL10, CXCL8, GM-CSF, IFNγ and TNFα. Inter-assay variation CV is <30% and Intra-assay CV<20%.

2.10 Maternal blood samples - Subjects

Ethical approval for sample collection was obtained from the North Glasgow University Hospitals Research Ethics Committee Reference 08/S0704/54 and all patients gave written informed consent to participate.

Women were recruited into three comparison groups as follows:

1. Non-pregnant (NP group) women volunteers of reproductive age during day 2-5 of their menstrual cycle (or day 3-6 of their pill free week if taking combined oral contraceptive). Women were excluded if they were taking a progesterone only contraceptive preparation or did not have a cycle. A single sample was obtained from these women.

2. Pregnant women at term attending for a planned caesarean delivery (CS group) who did not demonstrate any signs of labour including uterine contractions or spontaneous rupture of membranes. A sample was obtained from these women in the morning of admission prior to their operation, with a subsequent sample obtained 1 hour following delivery of the placenta. All caesarean deliveries occurred prior to 1pm and were uncomplicated.

3. Pregnant women at term attending for induction of labour (IOL group) by artificial rupture of membranes (ARM) $\pm$ syntocinon who did not demonstrate any current signs of labour including uterine contractions or spontaneous rupture of membranes. Women were excluded if they required priming of the cervix with prostaglandins. A baseline sample was obtained from these women immediately prior to ARM, with subsequent samples taken at 2 hourly intervals post ARM with the final sample obtained 1 hour after delivery of the placenta.

Women in all groups were excluded if they were currently taking anti-histamines, antibiotics (including benzylpenicillin prophylaxis during labour), steroids, or had received steroid injections during the current pregnancy to promote foetal lung maturity. They were also excluded if they were currently suffering from a minor cough or cold or if they developed symptoms of sepsis

during CS or labour including a core temperature measurement of ≥38°C during labour.

2.11 Maternal blood samples - Tissue handling and storage

Blood was obtained directly into 2 standard blood sampling tubes, with approximately 3mls into K_2EDTA tube (purple top) and 4-5mls in a lithium heparin tube (green top). Blood collected in the purple tube was processed in the NHS accredited haematology laboratory at Glasgow Royal Infirmary which provided standard haematological measures including a differential white blood cell count. Blood from the green tubes was used immediately for flow cytometry (approximately 1ml) and any remaining blood in this tube was centrifuged at 1200 X g for 15 minutes at 4°C and the plasma stored at -80°C for cytokine, chemokine, C-reactive protein (CRP), creatine kinase (CK) and myoglobin (Mb) assays.

2.12 Maternal blood samples - processing for differential white cell counts

The blood sample taken in the K_2EDTA (purple topped) tube was sent directly to the on-site NHS haematology laboratory at Glasgow Royal Infirmary. Samples were processed within 1 hour and counts were derived using Laser Flow Cytometry on whole blood samples adequately mixed with K_2EDTA, analysed using the standard automatic mode (SYSMEX XE2100 Analyser, Sysmex America, Inc). Counts were provided for total white blood cells (t-WBC), neutrophils, lymphocytes, monocytes, eosinophils and basophils.

2.13 Maternal blood samples - Flow Cytometry analysis of white cell surface chemokine receptor expression

The principle of flow cytometry is the ability to count cells which are suspended in fluid and differentiate between cell sub-types using characteristics including cell size and intracellular content. In addition, cells can be stained with fluorescent antibodies and further categorised according to expression of cell surface markers.

Laser is used to detect forward and side scatter of light as it passes perpendicular to the line of flow of cells, with forward scatter representative of cell size and side scatter intracellular content. Where cell surface markers are examined, when the laser hits the fluorochrome, it transfers to the excited state and emits fluorescence which is picked up by the automated sensors. A diagram of this process is illustrated in Figure 23.

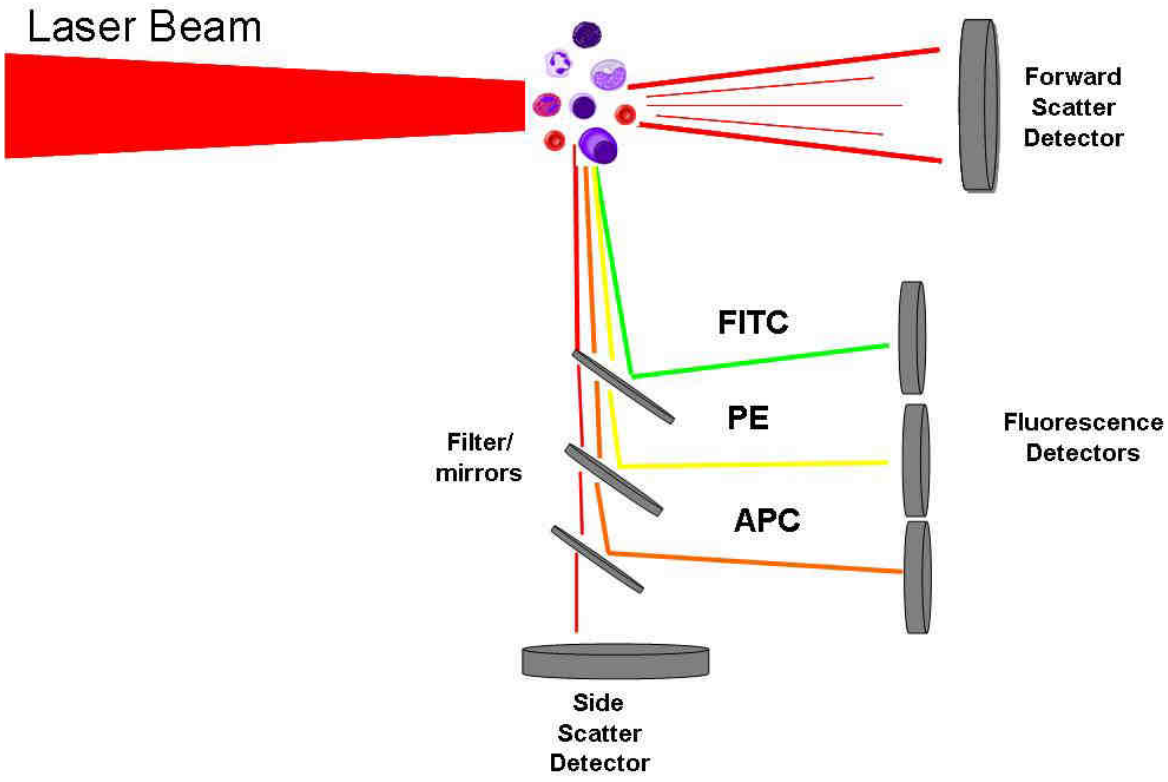

Figure 23 Illustration of flow cytometry.

Cells suspended in solution are hydrodynamically focussed to form a single cell line. A laser beam passes perpendicular to the flow of the cells and light is scattered forward to the forward scatter detector and also sideways to the side scatter detector. In addition where cell surface markers are examined, fluorescent light is released in the case of the experiments in this thesis, the associated fluorescences are (Fluorescein isothiocyanate (FITC), Phycoerythrin (PE) and Allophycocyanin (APC)).

2.13.1 Blood processing for flow cytometry white cell expression of chemokine receptors

Thirteen individual tubes were prepared with the combinations of antibodies and their associated fluorescence (Fluorescein isothiocyanate (FITC), Phycoerythrin (PE) or Allophycocyanin (APC)) for each tube listed in Table 3. 5µl of each antibody (undiluted) was used for each tube. Details of each antibody used are listed in Table 4.

Tube	FITC antibody	PE antibody	APC antibody
1	nil	Nil	nil
2	CD3	CXCR1	CD4
3	CD3	CXCR2	CD4
4	CD3	CXCR1	CD8
5	CD3	CXCR2	CD8
6	CD3	CCR2	CD4
7	CD3	CCR6	CD4
8	CD3	CCR2	CD8
9	CD3	CCR6	CD8
10	CD66b	CXCR1	CD11b
11	CD66b	CXCR2	CD11b
12	CD14	CCR2	CD11b
13	Isotype FITC	Isotype PE	Isotype APC

Table 3 Antibody combinations for flow cytometry experiments

Combinations of FITC, PE and APC antibodies used for flow cytometry analysis of whole blood.

Within 20 minutes of the blood sample being taken into the lithium heparin (green topped) tube, 50µl of whole blood was transferred by pipette into each tube and mixed with the antibodies by vortex. The tubes were then incubated in the dark at room temperature for 15 minutes. Red cell lysing buffer (1 in 10 dilution of 10x BD Pharm Lyse™ Lysing solution (BD Bioscience) with DDW) was brought to room temperature and 1ml was then added to each tube, with a further incubation in the dark at room temperature for 20 minutes. Samples were then centrifuged at 1200 X g for 5 minutes at 4ºC, the supernatant was discarded and 2ml of wash buffer (1 in 100 dilution of FBS with PBS) was added to each tube, mixed by vortex, and centrifuged for 5mins at 1200 X g at 4ºC. This wash was repeated one further time. The sample was prepared for analysis by flow cytometry by adding 0.5ml of wash buffer. Samples were then stored on ice and analysed in order, with 50,000 events recorded for each tube. Analysis was performed using BD FACs Calibur machine running the acquisition software BD CellQuest™ Pro, BD Biosciences, V1.c.6fcd.

Antibody	Fluorescence & species	Concentration	Antibody type Clone name	Supplier Cat no
CD3	FITC Mouse anti-Human CD3	Pre diluted*	Monoclonal HIT3a	BD Biosciences 555339
CD66b	FITC Mouse anti-Human CD66b	Pre diluted*	Monoclonal G10F5	BD Biosciences 555724
CD14	FITC Mouse anti-Human CD14	Pre diluted*	Monoclonal MSE2	BD Biosciences 555397
CXCR1	PE mouse anti-human CD181	0.2mg/ml	Monoclonal 5A12	BD Biosciences 555940
CXCR2	PE Mouse Anti-Human CD182	0.2 mg/ml	Monoclonal 6C6	BD Biosciences 555933
CCR2	PE Mouse Anti-Human CCR2	25 µg/ml	Monoclonal #48607	R&D systems FAB151P
CCR6	PE Mouse anti-Human CD196	Pre diluted*	Monoclonal 11A9	BD Biosciences 551773
CD4	APC Mouse anti-Human CD4	Pre diluted*	Monoclonal RPA-T4	BD Biosciences 555349
CD8	APC Mouse anti-Human CD8	Pre diluted*	Monoclonal RPA-T8	BD Biosciences 555369
CD11b	APC Mouse anti-Human CD11b/Mac-1	Pre diluted*	Monoclonal ICRF44	BD Biosciences 550019
Isotype FITC	FITC Mouse IgG2a K	Pre diluted*	Monoclonal G155-178	BD Biosciences 555573
Isotype PE	PE Mouse IgG1 K	Pre diluted*	Monoclonal MOPC-21	BD Biosciences 555749
Isotype APC	APC Mouse IgG1 K	Pre diluted*	Monoclonal MOPC-21	BD Biosciences 555751

Table 4 Antibody types and details for flow cytometry experiments

Details for each antibody used including clone name, species of generation and supplier. 5µl of each antibody are used in each experimental tube with no further dilutions. *Antibody reagent has been pre-diluted for use at the recommended volume per test where company has typically used 1 X 10e6 cells in a 100-µl experimental sample (a test).

2.13.2 Interpretation of flow cytometry data

Each sample was analysed individually using the analysis software on BD CellQuest™ Pro, BD Biosciences, V1.c.6fcd. Cell types were gated according to forward scatter (the intensity of light signal attributed to cell size) and side scatter (intensity light signal attributable to intracellular components e.g. granules and cell inclusions) into granulocytes, monocytes and lymphocytes as shown in Figure 24.

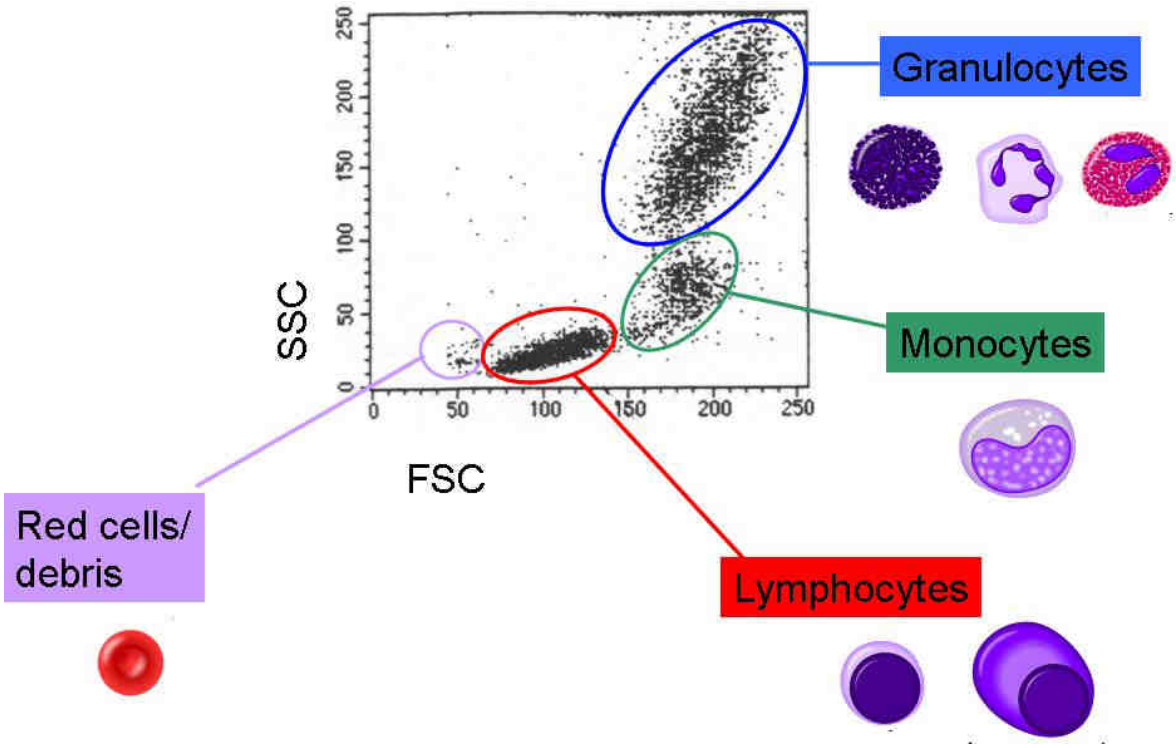

Figure 24 Flow cytometry data

An example of flow cytometry data output illustrating gating of white blood cell types into granulocytes, monocytes and lymphocytes based of side scatter (SSC) and forward scatter (FSC) of light.

Granulocytes were further gated using the markers CD11b and CD66b, with neutrophils defined as CD66b+ granulocytes, and activated neutrophils defined as CD66b+/CD11b+ granulocytes. Cell surface expression of the chemokine receptors CXCR1 and CXCR2 were then analysed firstly for the percentage of neutrophils (CD66b+ granulocytes) or activated neutrophils (CD66b+/CD11b+ granulocytes) expressing either receptor, and secondly for the cell surface density of that receptor as measured by the mean fluorescent intensity (MFI).

Monocytes were defined from the population of large cells with little granularity which were CD14+ with activated monocytes defined as CD14+/CD11b+ monocytes, The cells were then assessed for the % expressing the receptor CCR2 and the density of cell surface expression (MFI).

Lymphocytes were defined as smaller cells with low granularity and were further gated using the cell surface marker CD3 indicating T-lymphocyte cells. Additional gating was undertaken and defined T-helper lymphocyte cells as CD3+/CD4+ and T-cytotoxic lymphocyte cells and CD3+/CD8+. Since neither express CCR2, this was used as a negative control to further define these

lymphocyte subtypes. Analysis of the % of cells expressing the chemokine receptors CXCR1, CXCR2 and CCR6, and the density of expression was then undertaken.

Numbers of cells of interest which express the receptor being examined are expressed as a % of the cells gated. MFI (mean fluorescent intensity) expresses the geometric mean of light signal intensity and is an indication of the density of cell surface expression of the receptor of interest.

Samples were processed and analysed immediately after collection in order to minimise dead cells and debris within the samples, with further exclusion on the basis of gating by forward and side scatter parameters.

2.14 Maternal blood samples - Measurement of circulating cytokines and chemokines using multiplex technology

Traditional methods for protein quantification, such as ELISA or Western blotting, were used to quantify one single protein at a time. More recent mutiplex technologies allow the simultaneous measurement of a number of proteins in one single sample. We undertook multiplex analysis of protein in the plasma samples using pre-designed kits from Millipore.

An illustrative summary of the Milliplex™ MAP technology is shown in Figure 25 and employs immunoasays on the surface of fluorescent-coded beads (microspheres). Each microsphere is dyed to create a distinct colour, then coated with hundreds of one specific capture antibody therefore, one colour of bead is specific for one single capture antibody. Any analyte present in the study sample will combine with the capture antibody and this is turn can combine with a biotinylated detection antibody. Following a further incubation with a streptavidin- phycoerythrin conjugate the sample is ready for processing. Flow cytometry technology is used to detect the microspheres, with one laser able to detect with microsphere dye colour and the other the florescent dye on the detector molecule. A standard curve is run on each plate for each analyte and is used to determine sample concentrations.

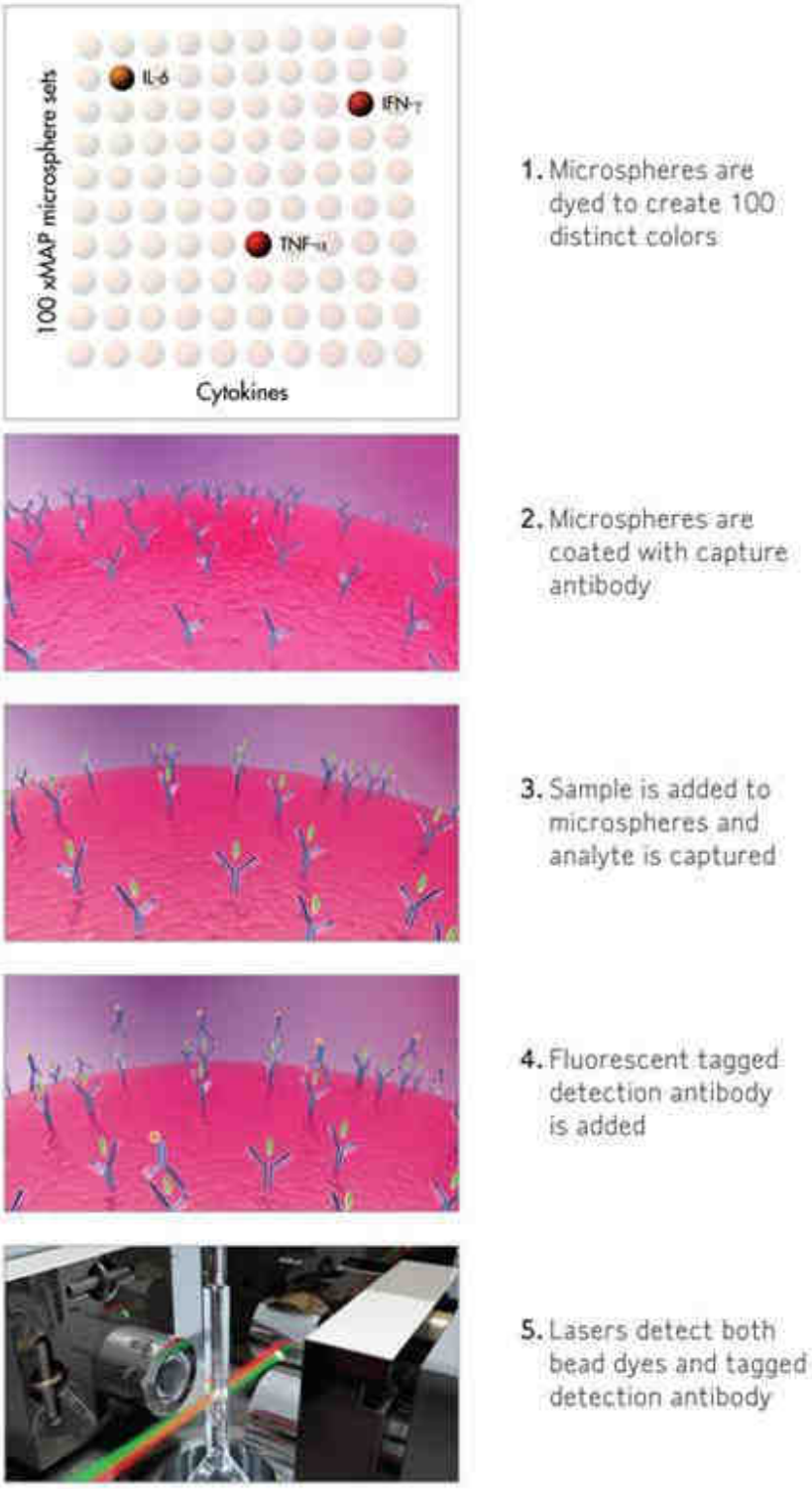

Figure 25 Illustration of principles behind multplex microsphere technology
Illustration reproduced from www.millipore.com

2.14.1 Processing of plasma samples for cytokine measurement

Circulating cytokines were measured in each individual sample using a 39-plex mixed human cytokine/chemokine plate and a 1-plex plate for CCL5 (RANTES) (Milliplex Human Cytokine/Chemokine Panel, MPXHCYTO-60K) which assessed a total of 40 circulating cytokines in the maternal plasma samples. The intra-assay coefficient of variation (CV) for the assays was 2-13%, and the inter assay CV 5-17%. The specific cytokines and their corresponding minimum detection sensitivity using these kits with an overnight incubation are listed in Table 5. Of note, the antibody used to detect GRO (growth regulated oncogene) measures all 3 isoforms of GRO, with the final measurement representing the sum of GROα(CXCL1) + GROß(CXCL2) + GROγ(CXCL3) (personal communications from technicalservice@merckgroup.com, Merck Millipore).

Cytokine	Expected minimum detection (pg/ml)
EGF (Epidermal Growth Factor)	2.7
CCL11 (Eotaxin)	1.2
FGF-2 (Basic Fibroblast Growth Factor)	1.8
Flt-3 (CD135, fms-like tyrosine kinase receptor-3)	2.6
CX3CL1 (Fractalkine)	6.0
GCSF (granulocyte colony stimulating factor)	0.5
GMCSF (granulocyte macrophage colony stimulating factor)	9.5
CXCL1 + CXCL2 + CXCL3 (GRO (growth regulated oncogene))	10.1
Interferon- α2 (IFNα2)	24.5
Interferon-γ (IFNγ)	0.1
Interleukin-1α (IL1α)	3.5
IL1ß	0.4
IL1ra	2.9
IL2	0.3
IL3	2.1
Il4	0.6
IL5	0.1
IL6	0.3
IL7	1.8
CXCL8 (IL8)	0.2
IL9	0.7

IL10	0.3
IL12 (p40)	10.5
IL12 (p70)	0.4
IL13	0.4
IL15	0.4
IL17	0.2
CXCL10 (Interferon-γ Inducible Protein (IP10))	1.2
CCL2 (Monocyte Chemotactic protein-1 (MCP1))	0.9
CCL7 (Monocyte Chemotactic protein-3 (MCP3))	2.0
CCL22 (Macrophage derived chemokine (MDC))	3.7
CCL3 (Macrophage inflammatory protein 1α (MIP1α))	3.5
CCL4 (Macrophage inflammatory protein 1ß (MIP1ß))	4.5
sCD40L (soluble CD40 ligand)	1.9
sIL2Rα	4.4
TGFα	0.4
TNFα	0.1
TNFß	1.9
VEGF (vascular endothelial growth factor)	5.8
CCL5 (RANTES)	1.0

Table 5 List of circulating cytokines/chemokines examined.

Circulating cytokines measured using 39-plex human cytokine/chemokine panel from milipore. CCL5 (RANTES) analysed on separated 1-plex (dilution of sample 1 in 100)

All plasma samples, with the exception of those used to measure CCL5, were used undiluted. A 1 in 100 dilution of sample was recommended when using the kit to assess CCL5 with washing buffer prior to use. All samples were run in duplicate with the mean taken as the concentration value of that cytokine for the individual sample.

2.15 Maternal blood samples - Measurement of circulating c-reactive protein (CRP)

CRP was quantified on the stored plasma samples by ELISA (Randox hsCRP immunoturbidimetric assay, cat No. CP3885) as directed by the manufacturers instructions. The minimum detection for this assay is 0.9mg/l, with and intra-assay CV of <4%. The reference range for CRP measurement in a normal population using this kit is 0.9-5 mg/l.

2.16 Maternal blood samples - Measurement of circulating creatine kinase (CK)

In this study, circulating CK measurements were performed on the stored plasma samples within the accredited NHS Biochemistry laboratory at Glasgow Royal Infirmary. Samples were processed using the ABBOTT ARCHITECT analyser and provided a measure of total circulating CK. The limit of detection for this assay is 5.1U/L, with an intra-assay CV <2%, and inter-assay CV of <4%.

2.17 Maternal blood samples - Measurement of circulating myoglobin (Mb)

Circulating Mb concentrations were determined using commercially available ELISA kit (Calbiotech, Cat No. MG017C, 96 tests). The stored plasma samples were diluted to 1/10 prior to analysis. Concentrations were determined using Multiskan® EX using Ascent software for iEMS (Thermo Electon Corporation, Finland). The lower limit of detection for this assay is 5.0ng/ml.

2.18 Statistical Analysis

Statistical analysis was adapted for each specific experiment and is discussed in full within each individual chapter.

Chapter 3

Effect of oxytocin and time on myometrial transcriptional profile

3 Effect of oxytocin and time on myometrial transcriptional profile

3.1 Introduction

The hormone oxytocin (OT) plays a major role in the stimulation of uterine contractility. OT is secreted by the posterior pituitary, with evidence also suggesting paracrine and autocrine production within the pregnant uterus especially towards term[91 144 145].

'Extracts of the posterior pituitary' were first found to promote contractility of the pregnant animal uterus in 1906 by Dale[146 147]. In 1909, Blair Bell first began to use the 'extract' in clinical obstetric practice to control uterine bleeding during post partum haemorrhage and bleeding secondary to placenta praevia and caesarean section[148].

OT was first isolated and synthesized by Vincent du Vigneaud 1953[321-324]. His work allowed the synthesis of Syntocinon® (Alliance Pharmaceutical), a synthetic oxytocin, which is widely used in modern obstetric practice to induce and modulate uterine contractions to facilitate labour and delivery of the infant. Syntocinon® also plays an important role in the prevention and treatment of post partum haemorrhage, and is given to women to aid delivery of the placenta in the third stage of labour. Conversely blockage of oxytocin receptor (OTR) activity can reduce uterine contractions. Atosiban is an OTR antagonist which has been shown to inhibit myometrial contractions in vitro[292]. Evidence of its success as a tocolytic in clinical practice has been variable[293], but has been shown to reduce the number of women giving birth within 7 days when treated for pre-term labour[239].

Despite the extensive clinical use of OT and OTR antagonists the molecular and cellular mechanisms underlying OT-induced modulation of uterine contractility are not completely understood. Elucidation of OT signalling has major clinical implications for patient care in obstetrics as OT has been implicated in the aetiology and pathogenesis of preterm delivery (7.5% of all births and 6% of all singleton births in Scotland 2010[3]), induction of labour (22.5% of live births in Scotland 2010[3]) and major obstetric haemorrhage (≥2500mls or requiring

transfusion of ≥5units of blood affects 5.18 per 1000 live births with >50% attributable to uterine atony[83]).

In addition to these significant clinical problems caesarean section (CS) rates remain high at 26.6% of live births in Scotland in 2010, with 11.3% of all deliveries occurring as elective CS and 15.3% as emergency CS[3]. Failure to progress in labour has been identified as a major aetiological factor in the decision to perform an emergency CS, and is cited as a primary indication in up to 53% of intrapartum CS deliveries in singleton cephalic pregnancies[58-61]. This occurs despite augmentation of labour with Syntocinon, as up to 81% of these women have received OT in the form of Syntocinon® prior to CS[19 20].

Current knowledge concerning the contractile effect of OT on myometrium is limited to the short term and immediate effects, with a well described pathway as illustrated in Figure 26.

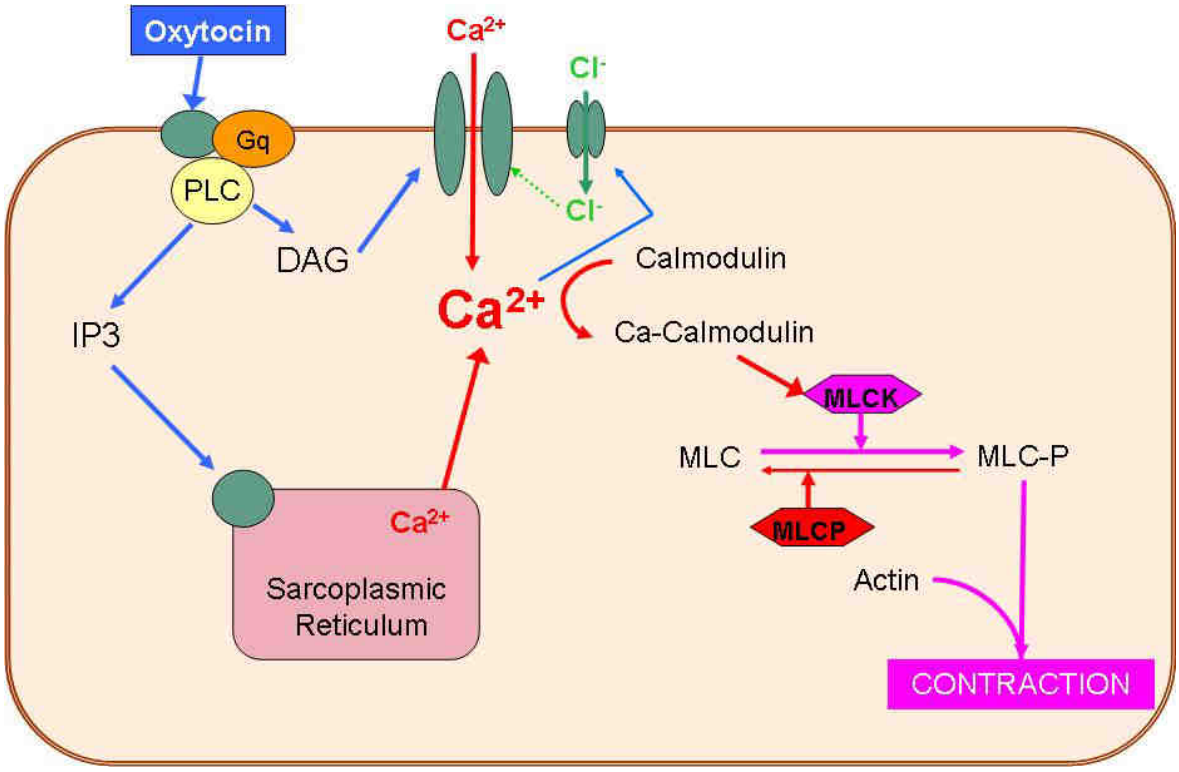

Figure 26 Intracellular events following OTR stimulation with OT

Summary diagram of intracellular events occurring in response to OT activation of the oxytocin receptor (OTR) on a myometrial cell culminating in cellular contraction.

Contractions of the myometrial myocyte have much in common with other contractile tissues in that contractility hinges on the interaction and cross

linking of myosin with actin[154]. This interaction requires phosphorylation of myosin light chain (MLC) which is mediated by the enzyme myosin light chain kinase (MLCK). MLCK becomes activated in the presence of the calcium-calmodulin complex, which is formed in the presence of intracellular calcium. Thereby, increased intracellular calcium is required for this final pathway. OT facilitates an increase in intracellular calcium as it binds the OTR (a seven transmembrane $G\alpha_q$ protein coupled receptor) resulting in activation of phospholipase C isoforms ß1, ß2 and ß3. This catalyses the hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2) with a resultant increase in inositol-1,4,5-triphosphate (IP_3) and 1,2-diacylglycerol (DAG)[155]. IP_3 acts on the sarcoplasmic reticulum to release its calcium stores, thereby increasing the availability of cytoplasmic calcium[156]. The increased cytoplasmic calcium has a direct effect upon calcium-sensitive cationic channels which depolarise the cell, opening voltage gated L-type calcium channels, leading to a full action potential[134]. The other second messenger DAG is thought to activate plasma membrane calcium channels in addition to mediating effects via protein kinase C[155 157]. The subsequent influx of large quantities of calcium binds to the calcium binding protein calmodulin thereby facilitating activation of MLCK and initiation of the final common contraction pathway of actin and myosin cross-linking. In addition to the above contraction pathway, OT is known to increase the sensitivity of the contractile machinery to calcium by altering the activity ratio of MLCK (pro-contraction) and MLCP (pro-relaxation) as detailed in Chapter 1, Section 1.5.1[116 158].

This knowledge of the immediate effects of OT on myometrial contractility has been used extensively in physiological and pharmacological studies. However, there is difficulty in applying this to human labour, a process which last hours rather than seconds. Additionally, the uterus is exposed to endogenous oxytocin throughout pregnancy as it is released in a pulsatile manner increasing towards term and particularly during labour[167]. In primates, these oxytocin pulses have a diurnal variation, peaking nocturnally during periods of darkness, which correlates with observed increases in periods of myometrial activity during these hours and can be inhibited by administration of an OT antagonist[172]. Gestational and diurnal variations in labour onset, and non-labour antenatal uterine activity (Braxton Hicks contractions) are also seen in human pregnancy[173-178 30-35] and may be explained by a diurnal variation in OT release[179]. The purpose of Braxton

Hicks contractions have not been fully elucidated, however they are popularly regarded as 'practice contractions' required by the uterus to preparing for the process of labour including the process of cervical ripening[325 326]. To this end it may be postulated that long term exposure to OT during pregnancy may result in transcriptional changes to 'prime' the myometrial cells thereby enhancing the development of a pro-contractile phenotype which facilitates an effective and efficient labour.

The mechanisms which underly these OT effects on uterine contractility are unclear. At a cellular level, cultured myometrial cells which were exposed to OT for 1 hour showed an increase in Regulator of G Protein Signalling 2 (RGS2) mRNA[327]. Additionally, there is evidence of an effect on the myocyte proteome of long term (1 hour) exposure to OT while contracting in an *in-vitro* organ bath environment. This has shown up-regulation of the expression of 13 proteins associated with 5 functional classes, namely cytoskeletal function (septin-2, cytokeratin-19, desmin, transgelin) contractile/oxidative stress (annexin A3, heat shock protein 70, protein disulfide isomerise, thioredoxin), extracellular matrix (Osteoglycin, BIGH3), energy metabolism (EH domain protein 2, triose phosphate isomerise, pyruvate kinase) and protein synthesis (EF1γ)[180].

These data suggest that long-term OT exposure may promote transcriptionally regulated phenotypic changes in the myometrium, which are required for the sustained uterine activity necessary for normal labour. An increased understanding of the mechanisms involved in OTR stimulation and its resultant cellular effects has the potential to guide the development of possible pharmacologic agents for the prevention, reversal or stimulation of uterine contractions. This knowledge may then be used to facilitate the development of novel and effective strategies to reduce the incidence of preterm birth, optimise the safe induction of labour leading to successful vaginal birth and avoidance of intrapatrum caesarean deliveries, in addition to minimising post-partum haemorrhage.

3.2 Hypothesis

The overall hypothesis to be examined in this chapter regarding the role of long term oxytocin exposure and the effect on myometrial contractile function and gene transcription is summarised in Figure 27.

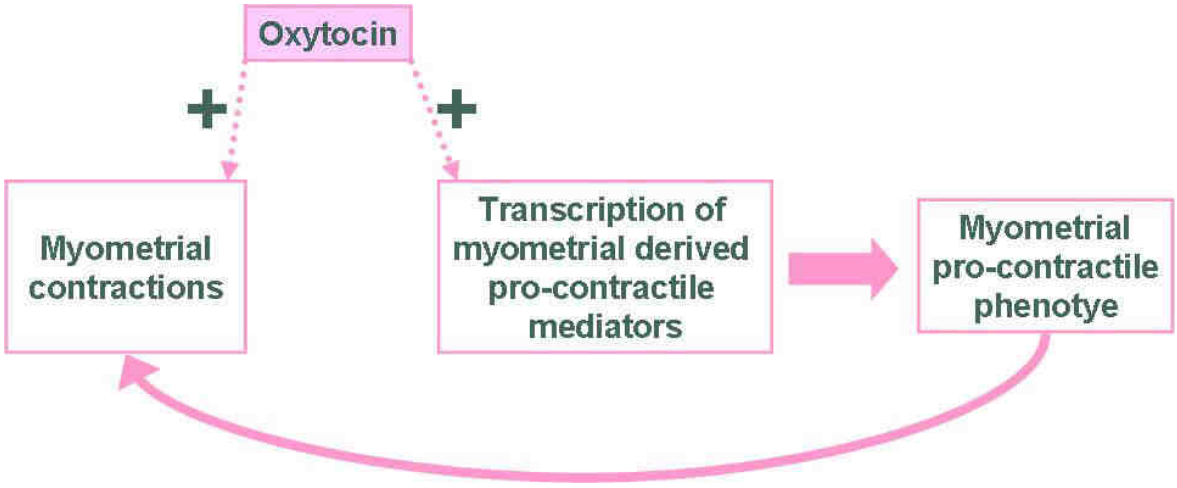

Figure 27 Chapter 3 Overall Hypothesis

Summary of the overall hypothesis concerning the functional and transcriptional effects of oxytocin exposure on *in-vitro* contracting human myometrium.

Overall, we propose that in addition to the well described acute physiological contractile effect of OT upon the myometrial myocyte, that longer term (hours) exposure to OT induces a transcription led contractile phenotype in human myometrium. This contractile phenotype is then able to facilitate sustained uterine activity and ultimately an effective and efficient labour.

To clarify this, examination of the time-dependent transcriptional response of myometrium to spontaneous and OT-induced contractions *in-vitro* was undertaken with additional comparisons made where contractions were blocked downstream by a myosin light chain kinase (MLCK) inhibitor, ML7 (see Figure 26).

The specific hypotheses to be tested are

1. Oxytocin exposure will induce time dependent transcriptional changes over and above the transcriptional profile of spontaneously contracting myometrium.

2. Oxytocin exposure will induce time dependent transcriptional changes in myometrial samples even where contractions are inhibited by blocking MLCK function prior to oxytocin exposure.

3. Temporal transcriptional changes occur within myometrium in response to contractile activity.

3.3 Methods

Functional *in-vitro* organ bath experiments were performed with subsequent temporal analysis of myometrial gene expression. A summary of the experimental design used in this chapter is shown in Figure 28.

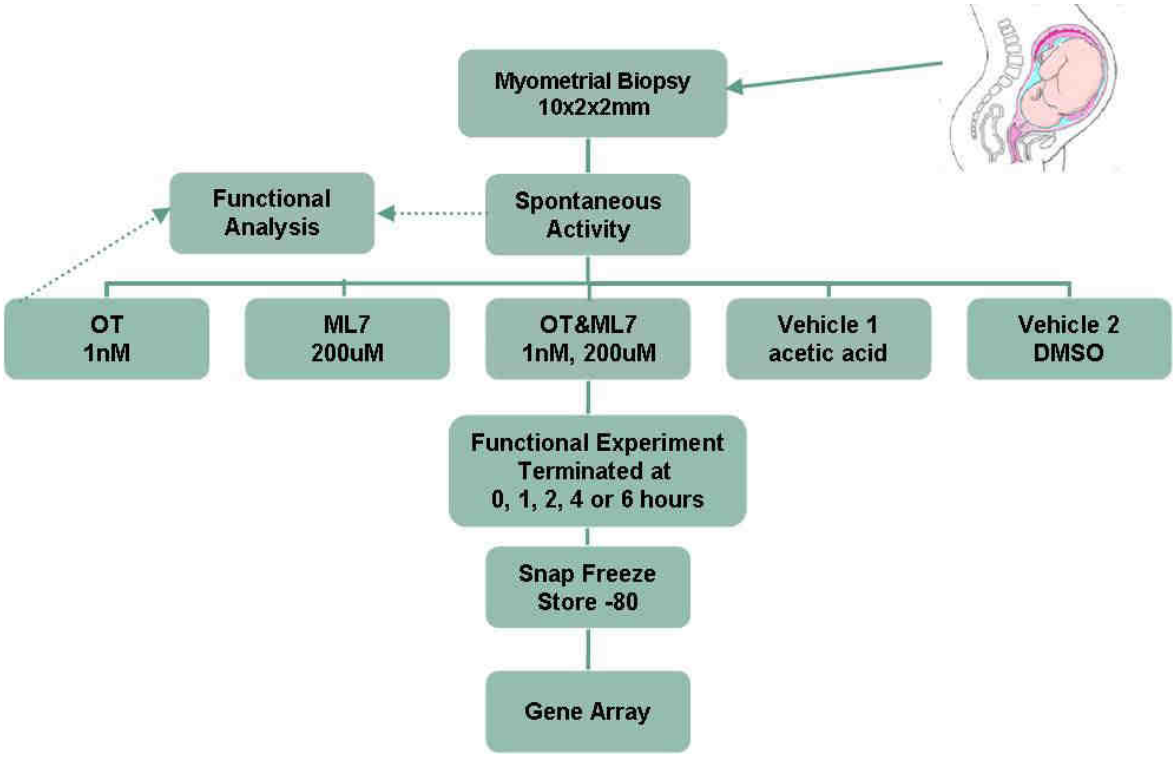

Figure 28 Chapter 3 Experimental Design

Illustration of experimental design used to examine the functional and transcriptional effects of long term (hours) exposure of myometrium to oxytocin *in-vitro*. Study groups are defined by drug environment (OT, ML7, OT&ML7, acetic acid (AA) and DMSO) and length of exposure (0, 1, 2, 4 & 6 hours) myometrial strips in each group n = 5.

3.3.1 Patient Selection

Myometrial strips were obtained from term pre-labour planned caesarean deliveries (n patients=25) as described previously in Chapter 2, Section 2.1.

3.3.2 In vitro contractility studies

In-vitro contractility studies were performed as previously described in Chapter 2, Section 2.3. Myometrial strips (n strips from each patient=5) were dissected and suspended in separate organ baths under physiological conditions with a 20mN tension applied. After establishment of spontaneous contractions (2 hours), a single addition of ML7 (final bath concentration 200μM) was undertaken

into two of the baths, and a single addition of the vehicle DMSO (20µl) into one other bath. Once contractile activity had been suppressed in the ML7 treated samples (approximately 1 hour and 30minutes after addition), OT (final bath concentration 1nM) was added to one of the remaining baths and one of the baths pre-treated with ML7. The OT vehicle acetic acid (10µl of 2% AA) was added to the one remaining bath. All drug preparations, additions and justification for use and of final bath concentrations are previously described in detail in Chapter 2.

The experiments were terminated at either 0, 1, 2, 4 or 6 hours after the time at which the addition of OT was undertaken. The myometrial strips were prepared for storage, weighed, snap frozen in liquid nitrogen and stored at -80°C.

3.3.3 Contractile Analysis

Spontaneous contractile activity was recorded where stable, regular rhythmic activity occurred at 2 hours after initial application of tension, prior to any drug additions and at variable time intervals during the course of the experiment while the tissue remained in the organ bath. Contractile activity (amplitude, frequency and activity integral) are measured as previously described in Chapter 2, Section 2.3.2.

Contractility data are described as medians (IQR) and analysed using a Kruskal-Wallis test or Mann-Whitney U-test comparing measures of contractility between study groups (defined as organ bath drug environment and time at which experiment was terminated) at each time point over the length of the experiment.

3.3.4 RNA extraction and Illumina gene array

RNA was extracted from each frozen sample in random order (n=125), quantified and RNA integrity checked as describe in Chapter 2. The samples then underwent amplification and biotin labelling. Individual Illumina (Human Ref 6) gene arrays were performed on each individual sample using the protocol described in Chapter 2. Illumina Arrays were outsourced and performed by The Wellcome Trust Clinical Research Facility, University of Edinburgh.

3.3.5 QPCR validation

Gene array validation was performed on the stored RNA samples above. Genes used for validation were CXCL1, CXCL2, CXCL5, CXCL8, CCL2, CCL3, CCL8, CCL20, IL1B, IL6, IL10, TNFα, IFIT2, and CHURC1. QPCR was carried out using the housekeeping gene B-actin, and performed according to the protocol described in Chapter 2, Section 2.7.

3.3.6 Statistical analysis of gene array output

Data analysis of the gene array output was performed by FIOS Genomics, University of Edinburgh using a combination of R programming with the Lumi and Limma packages from Bioconductor[41-43].

Briefly, the raw data (BeadStudio scan outputs (.txt files)) from the Illumina arrays was passed through a quality control step, transformed using variance stabilisation followed by robust spline normalisation[328]. Pearson correlation analysis between arrays was then performed with subsequent exclusion of any arrays which did not pass this quality control step.

These transformed data from arrays were then reduced to a single set of relevant values for each drug/time exposure (e.g. OT at 0hrs, OT at 1hr etc) using linear model fitting of the normalised data. These data sets were then analysed using Empirical Bayes statistics t-test coupled with p value adjustment for multiple testing using the Benjamini & Hochberg method to test differential expression by drug exposure and across time as appropriate [329 330]. Fold changes for differential expression by drug and time were also calculated.

3.3.7 Statistical analysis of Q-PCR data and array validation

Validation of the array findings was performed using Q-PCR data for the probes CXCL1, CXCL2, CXCL5, CXCL8, CCL2, CCL3, CCL8, CCL20, IL1B, IL6, IL10, TNFα, IFIT2, and CHURC1. These were chosen as they represented genes which were over expressed in contracting samples (those associated with inflammation) with the addition of IFIT2 which demonstrated a >2 fold down regulation in most samples in the array and CHURC1 which demonstrated up to 2 fold down regulation in most samples in the array.

Lists of fold changes were then compiled from the gene array data for each gene probe for each drug exposure in comparison to the baseline time 0 hours (e.g. fold change for CXCL1 for samples exposed to OT, 1hr vs 0hrs, 2hr vs 0hrs, 4hrs vs 0hrs and 6hrs vs 0hrs). Gene array fold change was expressed as logFC (log base 2 fold change of the gene with a positive values indicating up regulation).

Q-PCR data were calculated in the same manner to give the same output for comparison purposes. Fold changes and ranges were calculated using the $2^{-\Delta\Delta Ct}$ method for relative gene expression with ΔΔCt +SD and ΔΔCt –SD where SD is standard deviation for the ΔΔCt value[46 47]. The fold change value was subsequently log(2) transformed.

Correlation analysis was then performed to assess the similarity between the array and Q-PCR data using Pearsons correlation testing since data had been normalised with log transformation with a $p<0.05$ considered significant.

Other analyses of Q-PCR are undertaken within this chapter. These are performed on the raw untransformed data using the Mann-Whitney U-test where comparisons are made between two groups. No adjustments are made for multiple testing for these data as with our relatively small dataset adjustment may reduce the chance of making a type I error, but may lead to and increase in the chance of making a type II error and a need to increase our sample size[331-334]. A $p<0.05$ was considered significant.

3.3.8 KEGG and GO enrichment analysis

This analysis was performed on the transformed and normalised data generated from the Illumina Arrays and allows exploration of the underlying biological changes occurring within the dataset.

Gene ontology (GO) is a database describing gene products in a consistent fashion[335]. It categorises gene products in terms of their associated biological processes (BP), cellular components (CC) or molecular functions (MF). Genes which had a raw significance values of $p<0.001$ for the differential contrast of interest were used regardless of the extent of fold change. The GO categories relating to these differentially expressed genes were compiled as a list of

upregulated and downregulated categories with enrichment significance values supplied for each category.

The Kyoto Encyclopaedia of Genes and Genomes (KEGG) is a similar database to the GO database, however some categories differ and it is useful to consider both databases to gain insight into the transcription driven cellular changes[336]. The stringency of matching to KEGG is higher than GO, therefore a raw significance value of $p<0.01$ for the differential expression of genes in the contrast of interest was used. Again a list of upregulated and downregulated categories was compiled with enrichment significance values supplied for each category.

3.4 Results

3.4.1 Patient demographics

Samples were taken from 25 patients, with five myometrial strips dissected from each sample. Data are available from 5 strips for each environment at each time point. Demographic details for these patients are listed in Table 6.

N Women		25
N Strips		125
Maternal BMI (kg/m^2)	Median (IQR) [Range]	24 (21.9, 26.5) [18.5,34.6]
Age (years)	Median (IQR) [Range]	31 (27,35) [21,41]
Gestation (days)	Median (IQR) [Range]	273 (273, 275) [263,284]
Baby birth weight (kg)	Median (IQR) [Range]	3.47 (3.28, 3.70) [2.30, 4.19]
Indication for CS	N (%) Previous LUSCS N (%) breech N (%) maternal request N (%) prev traumatic delivery	20 (80%) 1 (4%) 2 (8%) 2 (8%)
Parity	N (%) =0 N (%) =1 N (%) ≥2	2 (8%) 16 (64%) 7 (28%)

Table 6 Patient demographic details

Demographic details of patients examining the myometrial functional and transcriptional effects of long term exposure to oxytocin.

3.4.2 Summary baseline contractility data

An example of the contractile trace for a 6 hour experiment, which illustrates the timing and functional effect of each drug addition is shown in Figure 29.

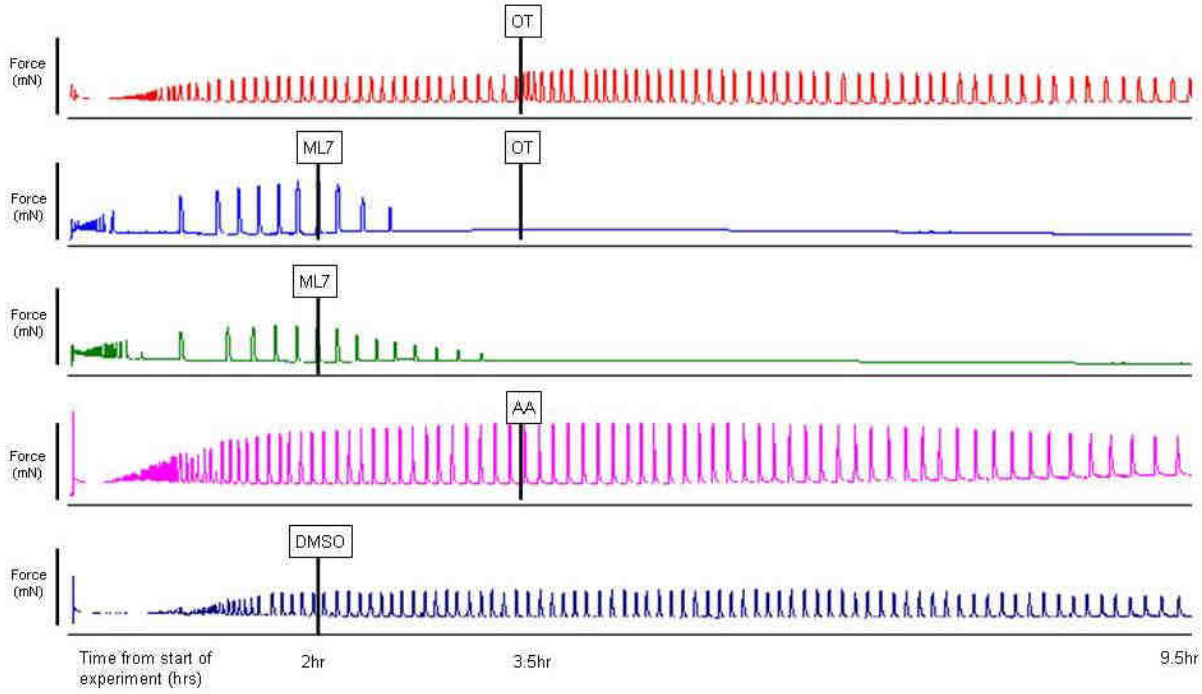

Figure 29 Example of contractility trace from experiments in Chapter 3

Contraction trace from an experiment terminated 6hours following time at which addition of OT was undertaken. This illustrates the timing and functional effects of each drug addition. OT (oxytocin), ML7 (myosin light chain kinase inhibitor), AA (acetic acid, the OT vehicle), DMSO (the ML7 vehicle)

A summary of the baseline contractility (amplitude, frequency and activity integral) of myometrial samples at 2 hours after initial application of tension and prior to drug additions is shown in Figure 30 and Figure 31. Data demonstrate that there was no difference in baseline contractility regardless of which drug was destined to be added to the organ bath, or how long the experiment was destined to last (amplitude, $p \geq 0.2$, frequency, $p \geq 0.1$ and activity integral, $p \geq 0.3$ for all comparisons).

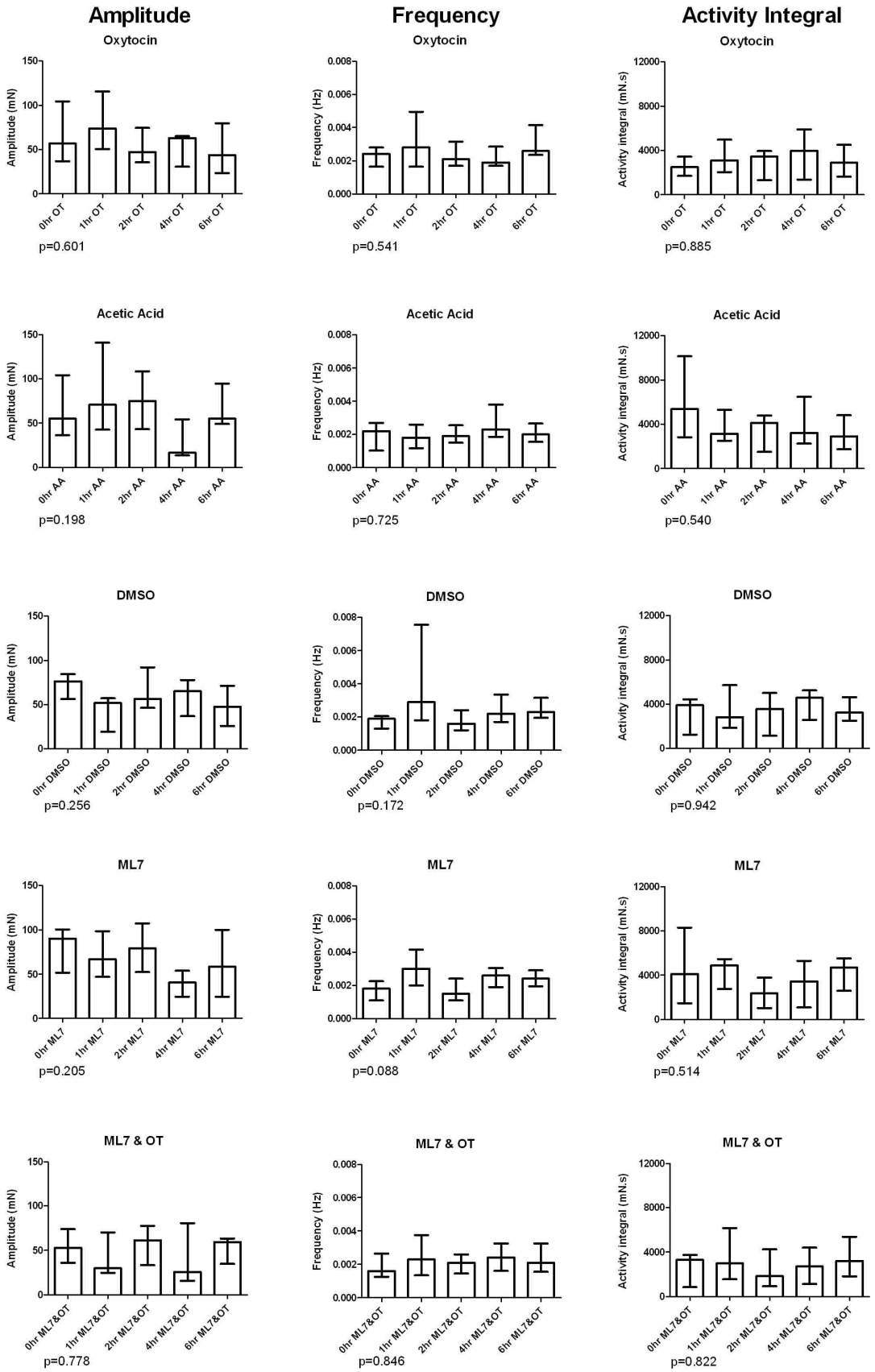

Figure 30 Baseline measures of contractility analysed by eventual length of experiment

Illustration of baseline measures of contractility (amplitude, frequency and activity integral) in myometrial strips assessed 2 hours after initial application of tension before any drug additions were undertaken. Data are shown as bars indicating median and IQR, with Kruskal Wallis p values indicated under each graph and show no difference regardless of the destined length of experiment.

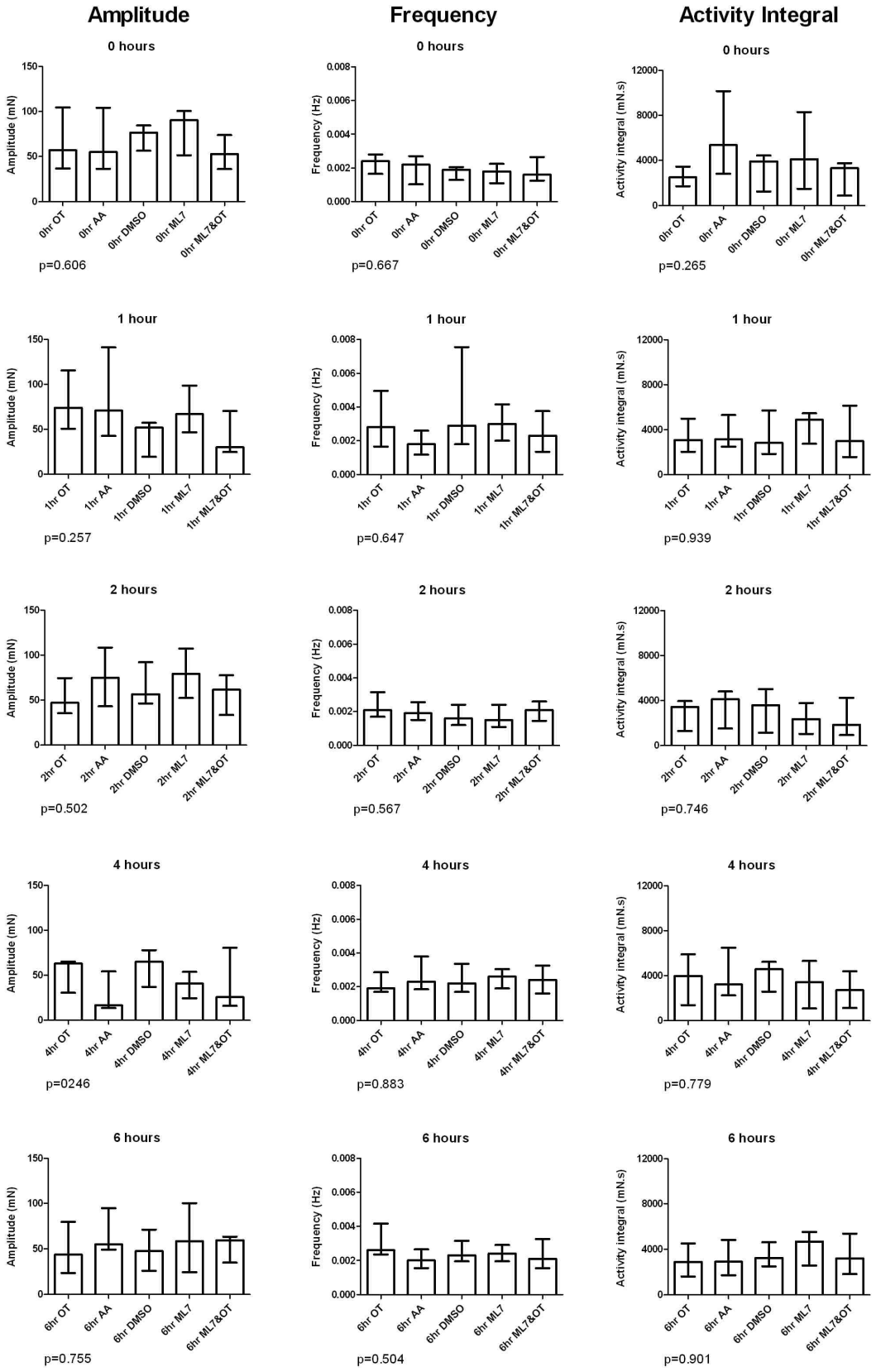

Figure 31 Baseline measure of contractility analysed by eventual type of drug exposure

Illustration of baseline measures of contractility (amplitude, frequency and activity integral) in myometrial strips assessed 2 hours after initial application of tension before any drug additions were undertaken. Data are shown as bars indicating median and IQR, with Kruskal Wallis p values indicated under each graph and show no difference regardless of the destined drug addition.

3.4.3 Array QC and normalisation

Following transformation and normalisation of the array data, correlation plots indicated that although inter-array correlations were high, four arrays exhibited lower correlations and were subsequently removed from the data set. Transformation and normalisation were repeated on the remaining 121 samples that were subsequently used for the array analysis.

The samples which were removed were all from different groups resulting in the following groups only having data from 4 myometrial strips; ML7 at 6 hours, OT and 4 hours, ML7 and 4 hours, and AA at 4 hours.

3.4.4 Array validation

Selected transcripts were identified from the array lists for verification using QPCR. The gene targets chosen represented different expression profiles within the array in comparison to expression at time 0hrs, and included CCL2, CCL3, CCL8, CCL20, CXCL1, CXCL2, CXCL5, CXCL8, IL1B, IL6, IL10, TNFα, IFIT2, and CHURC1. The Log2 fold change was calculated for the PCR data using the technique above, and Pearson correlation analysis was performed. All gene targets were in good agreement with the array data ($r>0.686$, $p<0.001$) with the exception of CCL2 ($r=0.326$, $p=0.161$) and CHURC1 ($r=0.155$, $p=0.515$). The correlation plots are demonstrated in Figure 32. Therefore it can be assumed that the gene expression changes seen in the array data reflect reliable data.

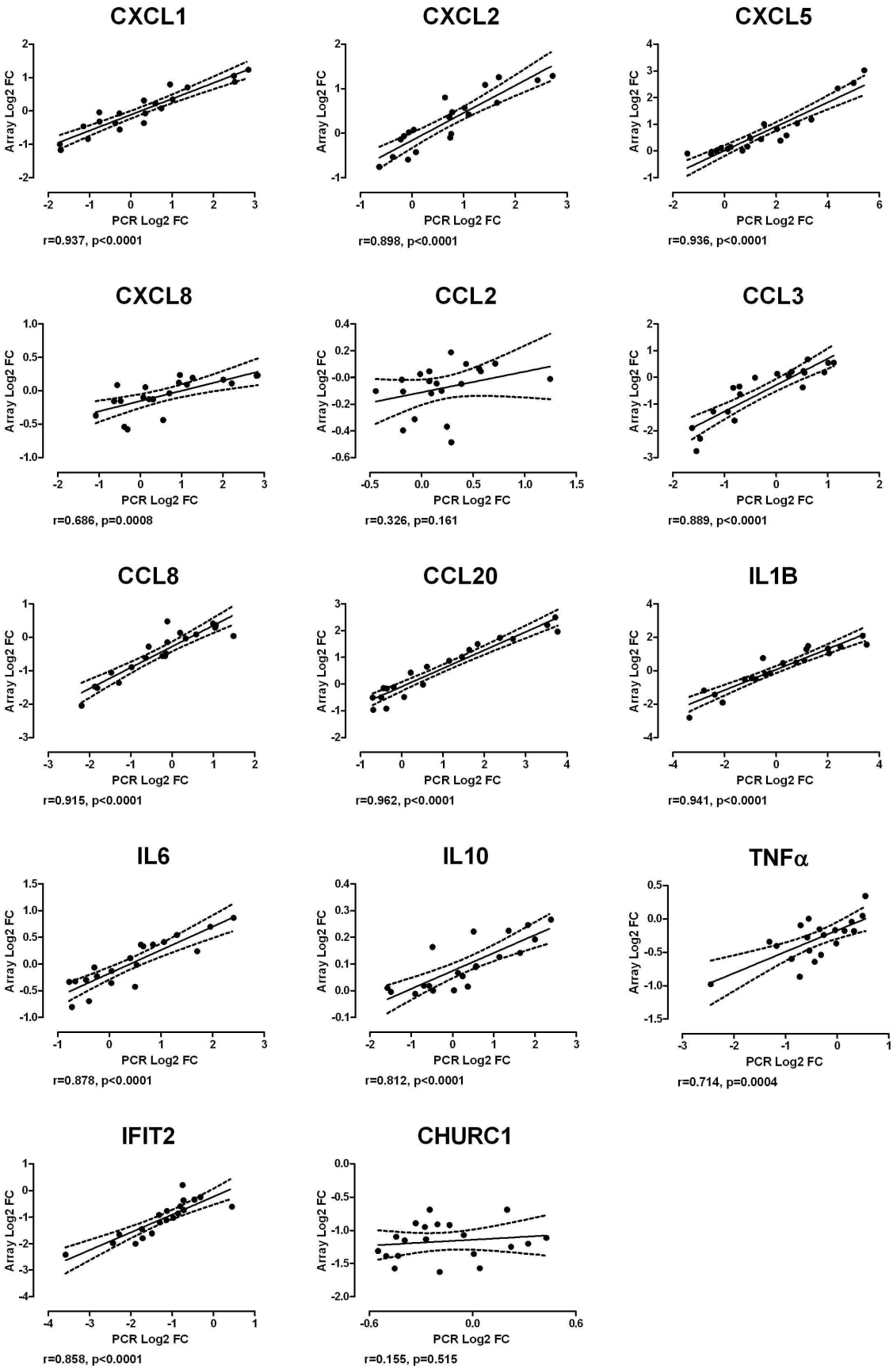

Figure 32 Correlation analysis of array data with QPCR data.

Fold changes were calculated for each drug environment relative to baseline time 0 hours. Data are shown as scatter plots with regression line and 95% confidence interval. Pearson correlation analysis was performed for each gene probe, with p values demonstrated under each graph.

3.4.5 Hypothesis 1 – OT exposure will induce time dependent transcriptional changes over and above the transcriptional profile of spontaneously contracting myometrium (OT vs AA)

3.4.5.1 Contractility Data

The spontaneous activity of the myometrial strips was characterized by regular contractions 2 hours after initial tension applied (baseline) and also immediately prior to addition of either OT or AA (pre) and is illustrated in Figure 33. There were no significant differences in the spontaneous activity in strips which received OT or acetic acid at either baseline or immediately prior to drug addition (AA vs OT baseline (amplitude $p=0.51$, frequency $p=0.06$, activity integral $p=0.09$) AA vs OT pre (amplitude $p=0.82$, frequency $p=0.23$, activity integral $p=0.43$).

Addition of 1nM OT had an immediate stimulatory effect on contractile activity compared with vehicle (median % change (IQR) amplitude 12.5 (6.1,50.7) vs 1.4 (0.3,6.5), $p<0.001$, frequency 526.7 (282.7,807.4) vs 0.0 (-12.8,10.7) $p<0.001$, activity integral 151.8 (89.3,311.0) vs -1.7 (-9.2,8.0), $p<0.001$). A stimulatory effect of OT compared with acetic acid persisted throughout the course of each experiment, but differences became less significant with time (Figure 33).

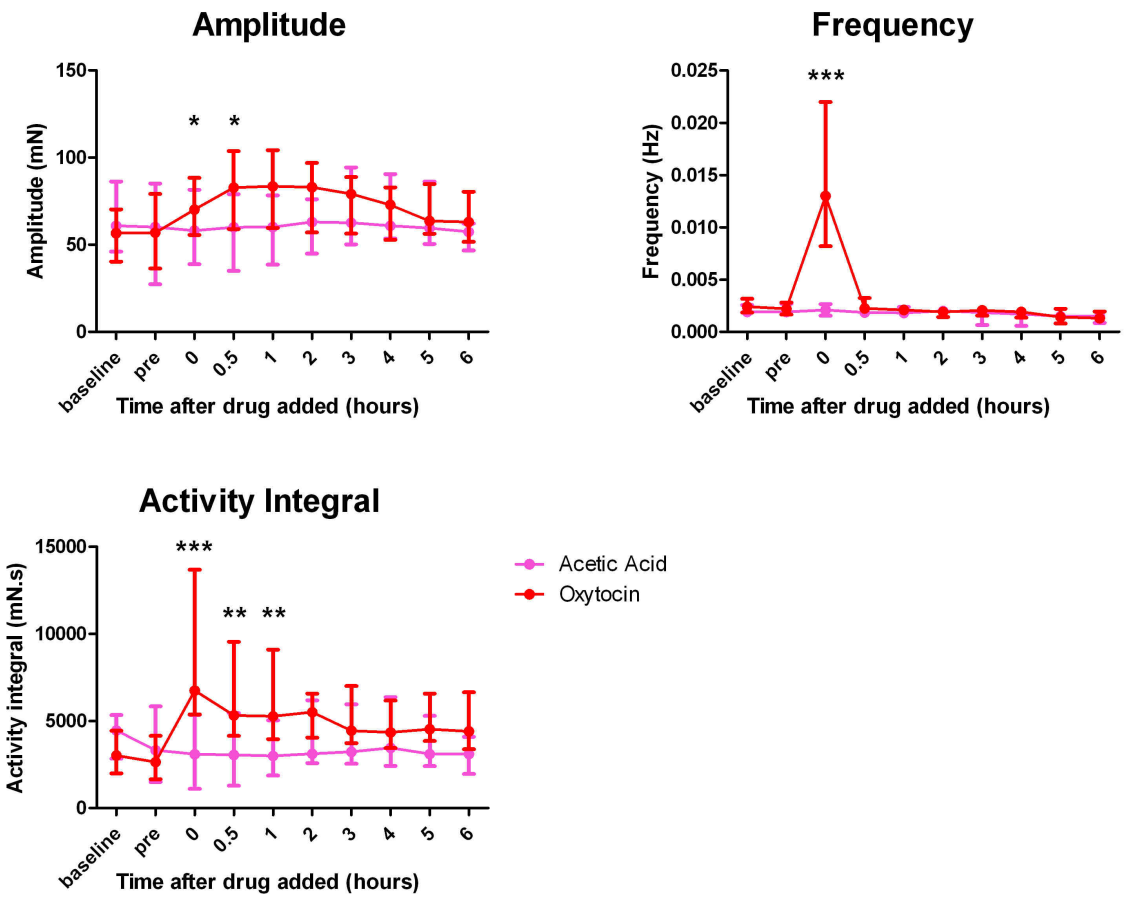

Figure 33 The effect of OT relative to its AA vehicle on *in-vitro* myometrial contractility

Illustration of the effect of OT relative to its AA vehicle on *in-vitro* myometrial contractility as measured by frequency, amplitude and activity integral throughout the time course of the experiment. All measures of contractility are expressed as median and IQR with n = 25 at baseline, pre-drug and 0mins, n = 20 at 0.5 and 1 hour, n = 15 at 2 hours, n = 10 at 3 and 4 hours and n = 5 at 5 and 6 hours. Mann-Whitney U-tests were performed to assess the difference in contractility between OT and AA treated strips at each timepoint. *p<0.05, **p<0.01, ***p<0.001

3.4.5.2 Myometrial transcription data from array

Using an adjusted significance of p<0.05, both OT and AA vehicle treated myometrium demonstrated a time-dependent transcriptional wave with differential expression of 183 genes at 4hours and 1759 genes at 6hours in the OT treated group, and 804 genes at 4hours and 2262 genes at 6 hours in the AA vehicle treated group (Table 7). However, using an adjusted significance of p<0.05, no genes were significantly altered by OT when compared to AA vehicle at any of the 5 time points (Table 8), and no significant difference was found between time dependent differentially expressed genes in the OT or vehicle group (Table 9).

Comparison	n genes differentially expressed adjusted p<0.05	Comparison	n genes differentially expressed adjusted p<0.05
OT (1hr-0hr)	0	AA (1hr-0hr)	0
OT (2hr-0hr)	0	AA (2hr-0hr)	0
OT (4hr-0hr)	183	AA (4hr-0hr)	804
OT (6hr-0hr)	1759	AA (6hr-0hr)	2262

Table 7 Differential gene expression in OT and AA relative to 0 hours

The number of genes differentially expressed in myometrium relative to time 0 hours in response to treatment with OT or AA vehicle using an adjusted significance value of p<0.05.

Comparison	n genes differentially expressed adjusted p<0.05
OT vs AA 0hr	0
OT vs AA 1hr	0
OT vs AA 2hr	0
OT vs AA 4hr	0
OT vs AA 6hr	0

Table 8 Differential gene expression OT vs AA

The number of genes differentially expressed in myometrium between exposure to OT and AA vehicle at each time point using an adjusted significance value of p<0.05

Comparison	n genes differentially expressed adjusted p<0.05
OT(1hr-0hr) vs AA (1hr-0hr)	0
OT (2hr-0hr)vs AA (2hr-0hr)	0
OT (4hr-0hr) vs AA (4hr-0hr)	0
OT (6hr-0hr) vs AA (6hr-0hr)	0

Table 9 Differential gene expression OT vs AA relative to 0 hours

The difference in number of genes expressed in myometrium showing no significant changes in expression relative to 0 hours when groups are compared using an adjusted significance value of p<0.05

3.4.5.3 Myometrial Q-PCR confirmation of array data

QPCR examination of the probes CXCL1, CXCL2, CXCL5, CXCL8, CCL2, CCL3, CCL8, CCL20, IL1B, IL6, IL10, TNFα, IFIT2, and CHURC1 relative to ß-actin is illustrated in Figure 34. This confirmed the gene array data with no significant differences in gene expression between samples exposed to OT or AA vehicle, except at the 2 hour timepoint for CXCL8 where expression was higher in response to OT ($p=0.03$).

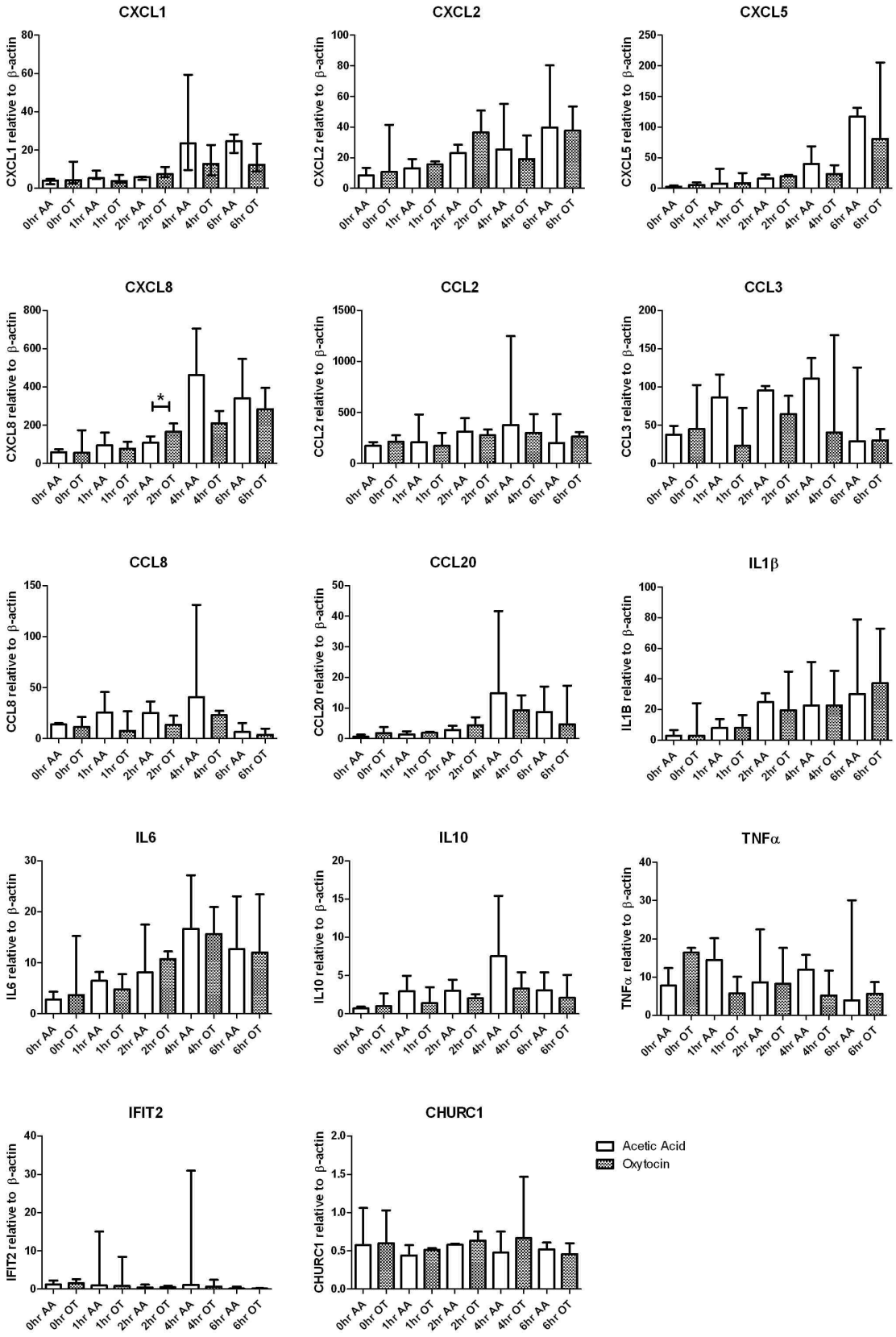

Figure 34 QPCR gene expression OT vs AA

QPCR confirmation of no significant difference in myometrial gene transcription between samples exposed to OT or AA vehicle at each timepoint after first exposure. Data are shown as bars indicating median and IQR with clear bars indicating AA data and shaded bars indicating OT data. *p<0.5

3.4.6 Hypothesis 2 - OT exposure will induce time dependent transcriptional changes in myometrial samples even where contractions are inhibited by blocking MLCK function prior to oxytocin exposure (ML7 vs ML7 & OT)

3.4.6.1 Contractility Data

Addition of ML7 resulted in a diminution in contractile activity with complete cessation of activity within a median time of 49 minutes (IQR 34 - 61mins) for those exposed to ML7 only, and 40 minutes (IQR 31.5 -54mins) for those treated with ML7 with subsequent addition of OT. There was no significant difference in the response between the two groups (p=0.4). Additionally where OT was added to a strip pre-treated with ML7, there was no subsequent activity in that strip, with contractions remaining absent.

3.4.6.2 Myometrial transcription data from array

Using an adjusted significance of p<0.05 and p<0.01 both ML7 and ML7 & OT treated groups demonstrated a time-dependent transcriptional wave (Table 10). However, using an adjusted significance of p<0.05, no genes were significantly altered by the addition of OT in myometrial strips pre-treated with ML7 at any of the 5 time points (Table 11).

Comparison	n genes differentially expressed		Comparison	n genes differentially expressed	
	adjusted p<0.05	adjusted p<0.01		adjusted p<0.05	adjusted p<0.01
ML7 (1hr-0hr)	0	0	ML7 & OT (1hr-0hr)	0	0
ML7 (2hr-0hr)	4	0	ML7 & OT (2hr-0hr)	2	0
ML7 (4hr-0hr)	67	27	ML7 & OT (4hr-0hr)	279	71
ML7 (6hr-0hr)	764	222	ML7 & OT (6hr-0hr)	2237	802

Table 10 Differential gene expression in ML7 and ML7 & OT relative to 0 hours

The number of genes differentially expressed in myometrium relative to time 0 hours in response to treatment with ML7 or ML7 & OT using an adjusted significance value of p<0.05.

Comparison	n genes differentially expressed adjusted p<0.05
ML7 vs ML7 & OT 0hr	0
ML7 vs ML7 & OT 1hr	0
ML7 vs ML7 & OT 2hr	0
ML7 vs ML7 & OT 4hr	0
ML7 vs ML7 & OT 6hr	0

Table 11 Differential gene expression ML7 vs ML7 & OT

The number of genes differentially expressed between myometrium treated with ML7 only or ML7 & OT at each time point using an adjusted significance value of p<0.05

3.4.6.3 Myometrial Q-PCR confirmation of array data

QPCR examination of the probes CXCL1, CXCL2, CXCL5, CXCL8, CCL2, CCL3, CCL8, CCL20, IL1B, IL6, IL10, TNFα, IFIT2, and CHURC1 relative to ß-actin is illustrated in Figure 35. This confirmed the gene array data with no significant difference in gene expression between samples exposed to ML7 or ML7 & OT, except at the 6 hour timepoint for IFIT2 and 2 hour timepoint for CHURC1 where expression was higher in the ML7 only group ($p=0.032$ and $p=0.016$ respectively).

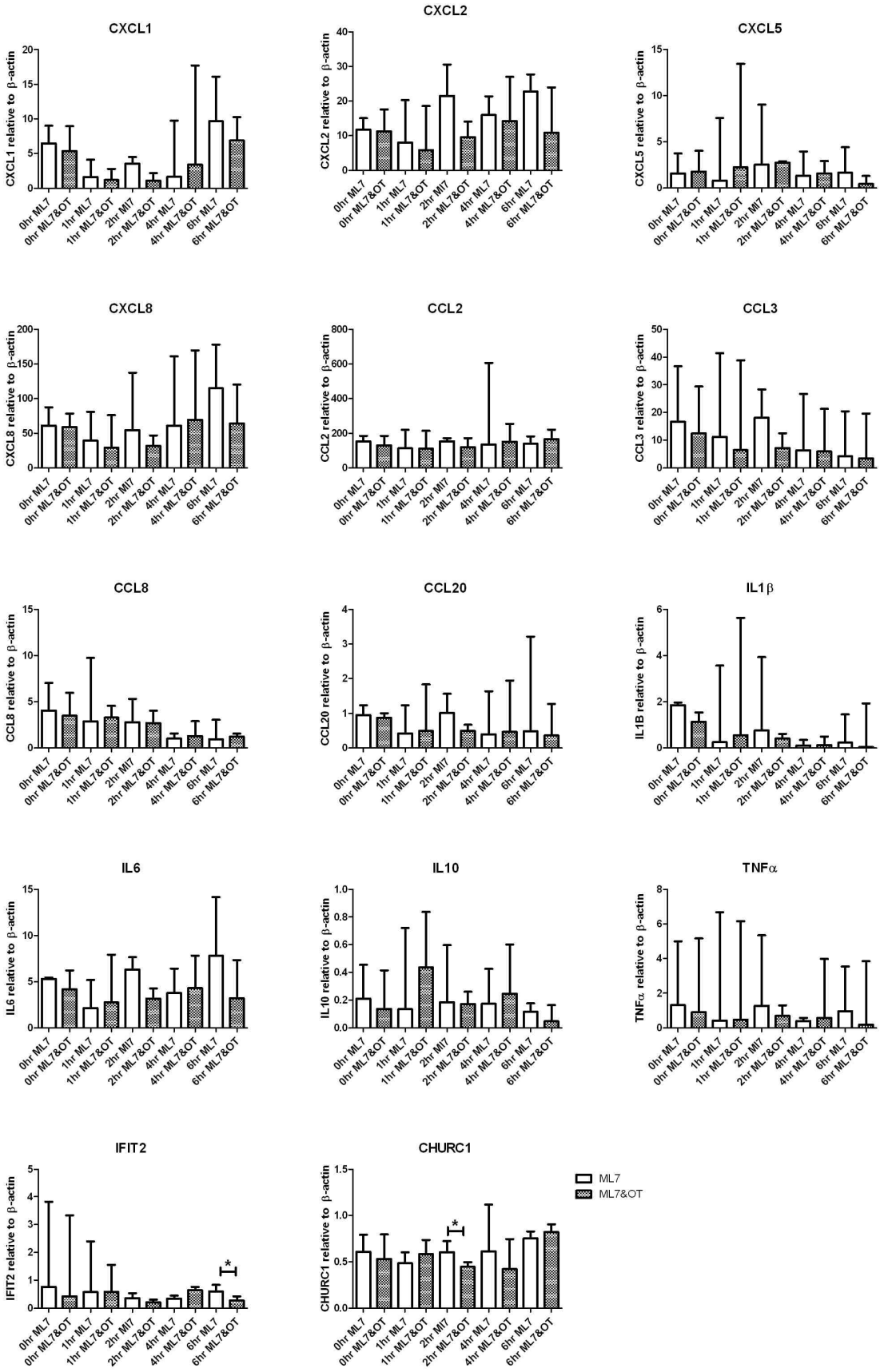

Figure 35 QPCR gene expression ML7 vs ML7&OT

QPCR confirmation of no significant difference in myometrial gene transcription between samples exposed to ML7 or ML7 & OT at each timepoint after first exposure. Data are shown as bars indicating median and IQR with clear bars indicating ML7 data and shaded bars indicating ML7 & OT data. *p<0.05

3.4.7 Hypothesis 3 - Temporal transcriptional changes occur within myometrium in response to contractile activity (DMSO vs ML7) and relative to time 0 hours (AA & DMSO & OT over time)

3.4.7.1 Contractility Data

The contractile activity of the myometrial strips used in this section is illustrated in Figure 36. Addition of AA (OT vehicle) or DMSO (ML7 vehicle) to the organ bath did not have an effect on any aspect of contractile activity over time as measured using a Kruskal Wallis test, (amplitude $p=0.9$, $p=0.8$, frequency $p=0.1$, $p=0.2$, activity integral $p=0.8$, $p=0.7$ for AA and DMSO respectively). Additionally there was no significant difference between contractile activity of strips exposed to AA or DMSO at any timepoint (amplitude, $p>0.5$, frequency, $p>0.4$, activity integral $p>0.2$ at each timepoint). OT had a stimulatory effect on contractile activity as discussed in Hypothesis 1 (Section 3.4.5) and ML7 had an inhibitory effect on all aspects of contractile activity as discussed in Hypothesis 2 (Section 3.4.6).

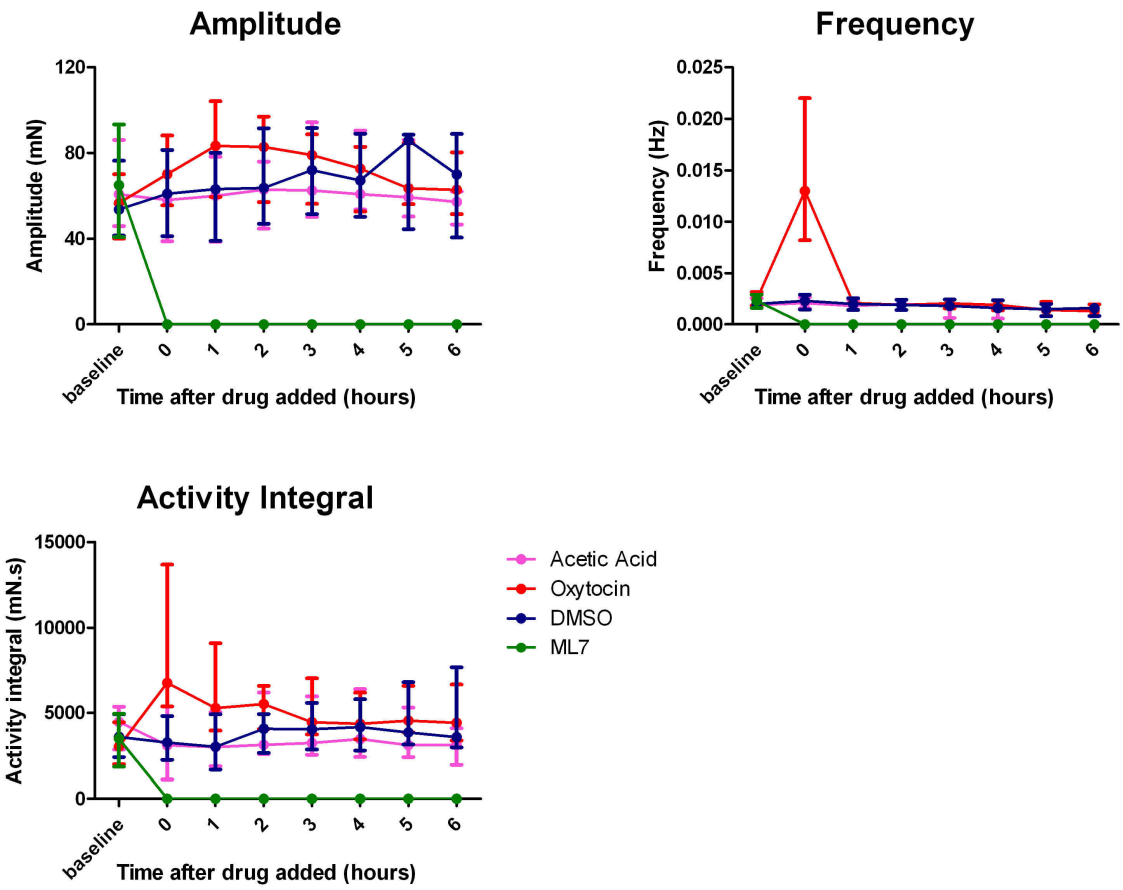

Figure 36 The effect of OT, AA vehicle, ML7 or DMSO vehicle on *in-vitro* myometrial contractility

Illustration of the effect of OT, AA vehicle, ML7 and DMSO vehicle on *in-vitro* myometrial contractility as measured by frequency, amplitude and activity integral throughout the time course of the experiment. All measures of contractility are expressed as median and IQR with n = 25 at baseline, pre-drug and 0mins, n = 20 at 0.5 and 1 hour, n = 15 at 2 hours, n = 10 at 3 and 4 hours and n = 5 at 5 and 6 hours.

3.4.7.2 Myometrial transcription data from array

3.4.7.2.1 Pattern of contraction associated transcriptional changes

To ensure pooling of contracting samples was appropriate, comparisons were made between gene expression at each timepoint in the AA group with both OT (as illustrated earlier) and DMSO (Table 12). Using an adjusted significance of $p<0.05$, no differences were found, therefore data from samples which contracted *in-vitro* in response to OT, AA and DMSO were pooled as one group.

Comparison	n genes differentially expressed adjusted p<0.05	Comparison	n genes differentially expressed adjusted p<0.05
OT vs AA 0hr	0	DMSO vs AA 0hr	0
OT vs AA 1hr	0	DMSO vs AA 1hr	0
OT vs AA 2hr	0	DMSO vs AA 2hr	0
OT vs AA 4hr	0	DMSO vs AA 4hr	0
OT vs AA 6hr	0	DMSO vs AA 6hr	0

Table 12 Differential gene expression OT vs AA and DMSO vs AA

The number of genes differentially expressed between myometrium treated with OT or AA, and DMSO or AA at each time point using an adjusted significance value of p<0.05

Analysis of the contracting group (pooled OT, AA and DMSO) revealed a time-dependent transcriptional wave with 13, 114, 2006, 4760 genes differentially expressed using an adjusted significance value of p<0.01 at 1, 2, 4 and 6 hours respectively (Table 13).

Comparison	n genes differentially expressed adjusted p<0.05	adjusted p<0.01
Pooled contracting samples (1hr-0hr)	101	13
Pooled contracting samples (2hr-0hr)	387	114
Pooled contracting samples (4hr-0hr)	3973	2006
Pooled contracting samples (6hr-0hr)	7034	4760

Table 13 Differential gene expression in pooled contracting samples relative to 0 hours

The number of genes differentially expressed relative to time 0 hours in contracting samples (pooled data from samples exposed to OT, AA, DMSO) using an adjusted significance value of p<0.05.

The transcriptional wave for the pooled contracting samples is further illustrated by heatmaps in Figure 37 (all fold changes) and Figure 38 (fold changes > 2). On the heatmap, the blue colour indicates down regulation and red upregulation, with the deeper the colour indicating a larger fold change. A greater number of red and blue lines and an increase in the intensity of colour is seen at the longer timepoints of 4 and 6 hours consistent with a time dependent transcriptional wave.

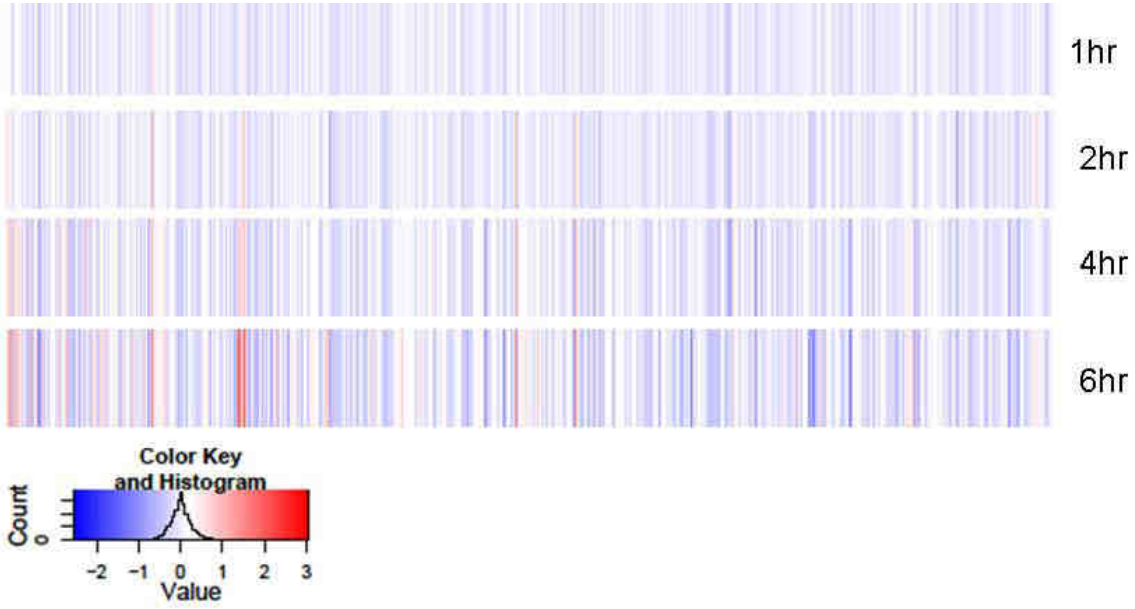

Figure 37 Heatmap of transcriptional changes relative to O hours for pooled contracting samples

Heatmap illustrating the pattern of transcriptional changes relative to time 0hr in pooled contracting samples (AA, DMSO and OT). Data are shown for all fold changes where adjusted p<0.01. Blue colour indicated down regulation and red upregulation, with the deeper the colour indicated a higher fold change. With increasing time contracting, samples display an increased number and extent of gene fold changes.

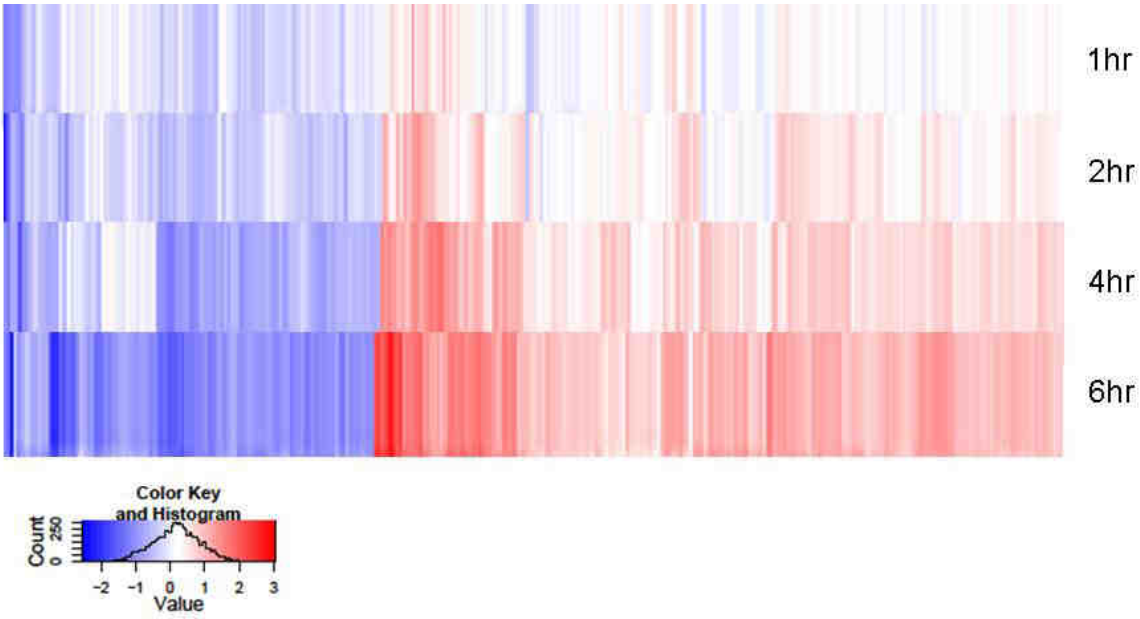

Figure 38 Heatmap of transcriptional changes relative to 0 hours for poked contracting samples (fold change>2)

Heatmap illustrating the pattern of transcriptional changes relative to time 0hr in pooled contracting samples (AA, DMSO and OT). Data are shown for fold changes>2, where adjusted p<0.01. Blue colour indicated down regulation and red upregulation, with the deeper the colour indicated a higher fold change. With increasing time contracting, samples display an increased number and extent of gene fold changes.

A further time-dependent transcriptional wave was found when samples which were rendered unable to contract by addition of ML7 were compared with their vehicle treated contracting samples (DMSO) (Table 14).

Comparison	n genes differentially expressed	
	adjusted p<0.05	adjusted p<0.01
ML7 vs DMSO 0hr	2	0
ML7 vs DMSO 1hr	4	2
ML7 vs DMSO 2hr	25	6
ML7 vs DMSO 4hr	487	175
ML7 vs DMSO 6hr	3320	1548

Table 14 Differential gene expression in ML7 vs DMSO

The number of genes differentially expressed between ML7 and DMSO vehicle at each time point using an adjusted significance value of p<0.05

3.4.7.2.2 Contraction associated overexpressed genes

The top 20 genes which are upregulated at the 6 hour timepoint in contracting samples compared with the baseline at time 0 hours are shown in Table 15. There is over representation of cytokine mediated processes with 7 of the 20 genes transcribing a cytokine, chemokine or receptor (IL11, CXCL5, IL1A, CCL20, IL23A, IL1B, IL13RA2). Additionally, the genes PTGS2 and FST are also involved in the inflammatory response.

Gene Symbol	Gene Name	Log 2 Fold Change	Adj p value
IL11	interleukin 11	2.777602	4.03E-15
CXCL5	chemokine (C-X-C motif) ligand 5	2.637802	9.47E-23
IL1A	interleukin 1, alpha	2.436311	1.42E-09
MYEOV	myeloma overexpressed gene (in a subset of t(11;14) positive multiple myelomas)	2.326521	1.52E-11
NA	probeset not currently assigned to a gene symbol	2.261165	3.45E-14
CCL20	chemokine (C-C motif) ligand 20	2.14653	4.11E-07
MMP1	matrix metallopeptidase 1 (interstitial collagenase)	2.089201	3.12E-06
SERPINB2	serpin peptidase inhibitor, clade B (ovalbumin), member 2	1.951359	3.58E-07
FLJ27255	hypothetical LOC401281	1.930037	1.70E-14
IL23A	interleukin 23, alpha subunit p19	1.821352	3.99E-17
KRT34	keratin 34	1.799177	4.51E-06
GJB2	gap junction protein, beta 2, 26kDa	1.797835	5.59E-11
AKR1B1	aldo-keto reductase family 1, member B1 (aldose reductase)	1.771979	3.05E-25
RCC1	regulator of chromosome condensation 1	1.751471	1.49E-10
PTGS2	prostaglandin-endoperoxide synthase 2 (prostaglandin G/H synthase and cyclooxygenase)	1.726473	6.53E-07
IL1B	interleukin 1, beta	1.698531	0.000277
HIST2H2AA3	histone cluster 2, H2aa3	1.684515	1.45E-07
FST	follistatin	1.676597	1.57E-07
EVI1	ecotropic viral integration site 1	1.668135	7.32E-11
IL13RA2	interleukin 13 receptor, alpha 2	1.658026	2.27E-13

Table 15 Top 20 genes upregulated in contracting samples (6 hours relative to 0 hours)

Pooled contracting samples top 20 genes with the largest fold change which are differentially upregulated at 6 hours compared with 0 hours.

Furthermore, many of the same overexpressed genes are common to the data in Table 16 where there is again overexpression of genes involved in the inflammatory process in contracting myometrial samples compared with those which are not contracting after treatment with ML7 (DMSO vs ML7). There is an overlap of 25% of the genes expressed in both comparisons, with 5 genes common to both lists, namely IL1B, CXCL5, IL11, IL1A and CCL20. Additionally, the inflammatory associated genes FCER1G, CCL3, CD14, CCL3L3 (chemokine (C-

C motif) ligand 3-like 3[337]), CCL4 L1 (chemokine (C-C motif) ligand 4-like 1[338]), FCGR2A, SLAMF1, CD163 and IL1RN also up-regulated in response to contractions when compared with non-contracting samples.

Gene Symbol	Gene Name	Log 2 Fold Change	Adj p value
IL1B	interleukin 1, beta	3.47154	0.054566
CXCL5	chemokine (C-X-C motif) ligand 5	3.02576	6.66E-06
IL11	interleukin 11	2.94093	0.010186
IL1A	interleukin 1, alpha	2.91841	0.051357
FCER1G	Fc fragment of IgE, high affinity I, receptor for; gamma polypeptide	2.89977	0.015421
CCL3	chemokine (C-C motif) ligand 3	2.84458	0.071062
KYNU	kynureninase (L-kynurenine hydrolase)	2.76708	0.016709
CCL20	chemokine (C-C motif) ligand 20	2.69377	0.106052
CD14	CD14 molecule	2.67815	0.002216
CCL3L3	chemokine (C-C motif) ligand 3-like 3 (functionally different isoform of CCL3 with differing receptor interactions. CCL3L3 interacts with CCR5, role inhibiting HIV cell entry)	2.55156	0.118392
MYEOV	myeloma overexpressed gene (in a subset of t(11;14) positive multiple myelomas)	2.52618	0.043751
CCL4L1	chemokine (C-C motif) ligand 4-like 1 (functionally different isoform of CCL4 with differing receptor interactions. CCL4L1 interacts with CCR5, role inhibiting HIV cell entry)	2.52287	0.110971
FCGR2A	Fc fragment of IgG, low affinity IIa, receptor (CD32)	2.51975	0.032364
SLAMF1	signaling lymphocytic activation molecule family member 1	2.48996	0.057718
TPSAB1	tryptase alpha/beta 1	2.44531	0.069854
CD163	CD163 molecule	2.42854	0.042647
KYNU	kynureninase (L-kynurenine hydrolase)	2.38369	0.007839
HCK	hemopoietic cell kinase	2.35412	0.000129
IL1RN	interleukin 1 receptor antagonist	2.34052	0.115504
SERPINB2	serpin peptidase inhibitor, clade B (ovalbumin), member 2	2.30318	0.132766

Table 16 Top 20 genes upregulated in DMSO samples relative to ML7 samples

Top 20 overexpressed genes as assessed by fold change in contracting sample compared with non-contracting samples (ML7) at 6 hour timepoint

3.4.7.2.3 KEGG& GO Enrichment analysis of contraction associated genes

Examination of the genes expressed by myometrium in relation to contraction using KEGG and GO enrichment analysis showed an overall up-regulation of inflammatory processes and processes associated with cellular damage or death, with down regulation of processes concerned with cellular metabolism.

In the pooled contracting samples (OT, AA and DMSO) compared to time 0hours, KEGG pathways which were upregulated in at least three of the timepoint comparisons were pathways 5220 'Chronic myeloid leukemia', 5213 'Endometrial cancer', 4012 'ErbB signalling pathway', 4640 'Hematopoietic cell lineage', 4010 'MAPK signalling pathway', 4810 'Regulation of actin cytoskeleton', and 5211 'Renal cell carcinoma'. Down regulation was seen for pathway 650 'Butanoate metabolism', 251 'Glutamate metabolism', 563 'Glycosylphosphatidylinositol (GPI)-anchor biosynthesis', 640 'Propanoate metabolism', and 380 'Tryptophan metabolism'. A full table of KEGG pathway analysis for contracting myometrium over time is provided in Appendix I.

Additionally, contracting samples relative to time 0hrs were associated with upregulation in 3 or more comparison with GO associated biological processes (BP) of chromatin assembly (GO:0031497), chromatin assembly or disassembly (GO:0006333), chromosome organization and biogenesis (GO:0051276), DNA packaging (GO:0006323), establishment and/or maintenance of chromatin architecture (GO:0006325), macromolecule metabolic process (GO:0043170) , nucleosome assembly (GO:0006334), positive regulation of cellular process (GO:0048522), protein-DNA complex assembly (GO:0065004), regulation of apoptosis (GO:0042981) and regulation of programmed cell death (GO:0043067). Upregulation in 3 or more comparisons were also seen for the molecular functions of binding (GO:0005488) and protein binding (GO:0005515) in addition to the cellular component terms of chromatin (GO:0000785) and chromosome (GO:0005694). The cellular component terms of intracellular (GO:0005622), intracellular membrane-bound organelle (GO:0043231), intracellular organelle (GO:0043229), intracellular part (GO:0044424), membrane-bound organelle (GO:0043227), organelle (GO:0043226) are also seen to be upregulated at the three timepoints of 2, 4 and 6 hours, however, they also appear to be

downregulated at the later timepoints of 4 and 6 hours compared with the baseline at 0 hours. A full table of GO pathway analysis for contracting myometrium over time is provided in Appendix II.

Comparison between contracting and non-contracting myometrial samples (DMSO vs ML7) demonstrated that contracting myometrium was associated with an upregulation of the KEGG pathways 4060 'Cytokine-cytokine receptor interaction', 4210 'Apoptosis', 4640 'Hematopoietic cell lineage', 4010 'MAPK signaling pathway', and 4620 'Toll-like receptor signaling pathway' at all timepoints from 0- 6 hours. A full table of KEGG pathway analysis for contracting myometrium compared with non-contracting myometrium (ML7 Vs DMSO) is provided in Appendix III.

Additionally contracting samples were associated with enrichment of 22 gene ontology (GO) pathways in contracting samples for 3 or more of the timepoints examined, with over representation of processes associated with inflammation. These are represented by the contraction associated upregulation of the biological processes of 'acute inflammatory response' (GO:0002526), 'behaviour' (GO:0007610), 'chemokine activity' (GO:0008009), 'chemokine receptor binding' (GO:0042379), 'chemotaxis' (GO:0006935), 'defense response' (GO:0006952), 'fever' (GO:0001660), 'immune response' (GO:0006955), 'immune system process' (GO:0002376), 'inflammatory response' (GO:0006954), 'locomotory behaviour' (GO:0007626), 'negative regulation of cell proliferation' (GO:0008285), 'response to chemical stimulus' (GO:0042221), 'response to external stimulus' (GO:0009605), 'response to stimulus' (GO:0050896), 'response to wounding' (GO:0009611), 'taxis' (GO:0042330), and the molecular functions of 'cytokine activity' (GO:0005125) and 'G-protein-coupled receptor binding' (GO:0001664). The biological processes of 'response to protein stimulus' (GO:0051789) and 'response to unfolded protein' (GO:0006986) are down regulated in contracting myometrium while 'response to stress' (GO:0006950) is both up and down regulated in response to contractions. A full table of GO pathway analysis for contracting myometrium compared with non-contracting myometrium (ML7 Vs DMSO) is provided in Appendix IV.

Overall this enrichment analysis using both KEGG and GO suggests that contractions are associated with myometrial transcriptional upregulation of

inflammatory processes and processes concerning cellular damage, stress or cell death. In addition, the transcription of genes which are associated with cellular metabolic processes tend to be downregulated.

3.4.7.3 Myometrial Q-PCR confirmation of array data

QPCR examination of the probes CXCL1, CXCL2, CXCL5, CXCL8, CCL2, CCL3, CCL8, CCL20, IL1B, IL6, IL10, TNFα, IFIT2, and CHURC1 relative to ß-actin was performed on the pooled contracting samples (AA, OT and DMSO) and is shown in Figure 39. The data from this time series shows a time dependent trend for increased expression of these inflammatory mediators, with many showing a significantly higher expressed at the later timepoints of 4 and 6 hours.

Additionally, analysis of QPCR data examining the contracting samples (DMSO) compared with the non-contracting ML7 treated samples is shown in Figure 40. This again confirmed the gene array findings suggesting a time and contraction dependent upregulation of inflammatory processes with significant overexpression of all inflammatory mediators with the exception of CCL2 at the 6 hour timepoint in the contracting samples (Figure 40).

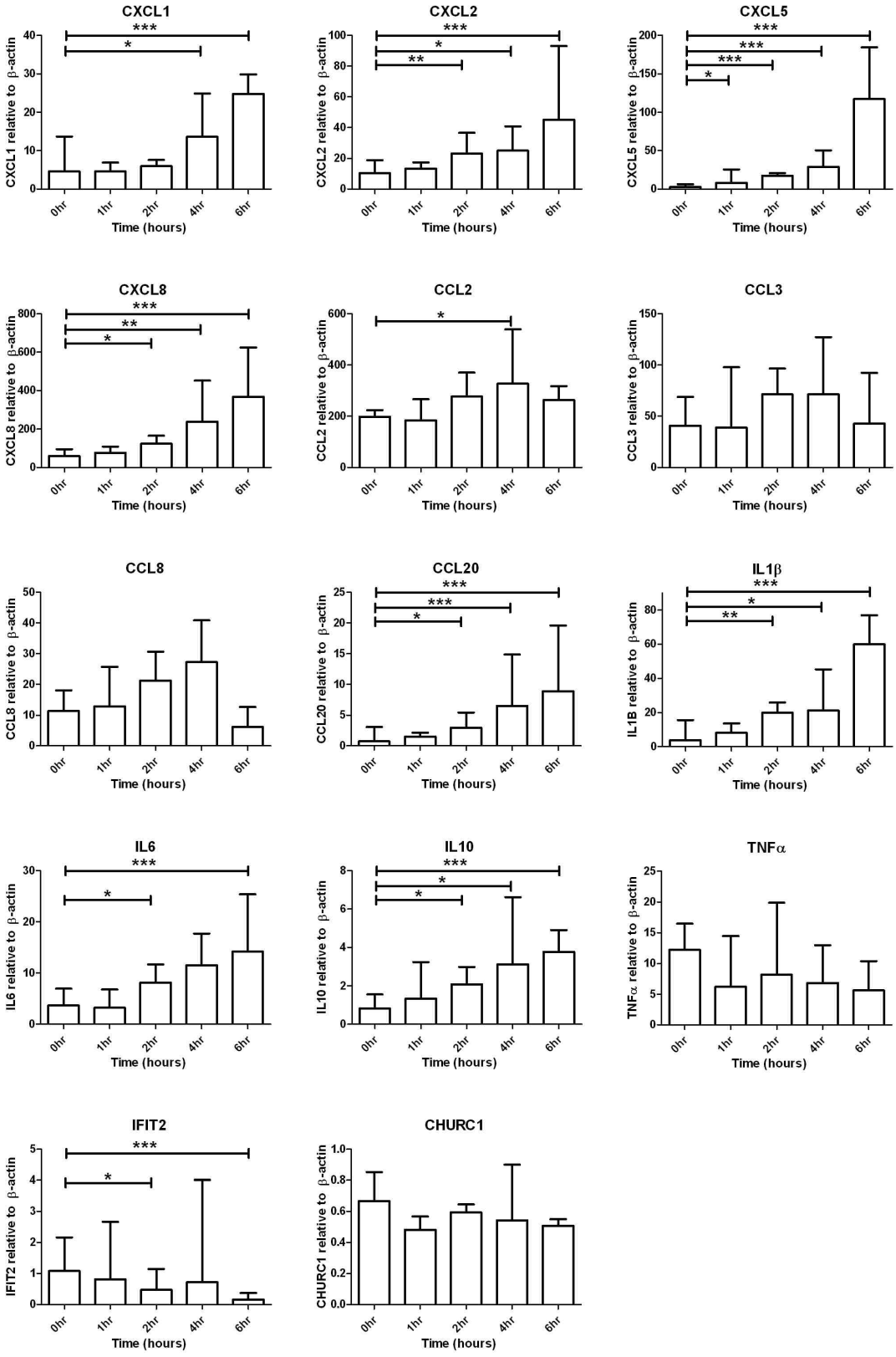

Figure 39 QPCR gene expression pooled contracting samples over time

QPCR confirmation of array data with time dependent increase in expression of inflammatory related genes in contracting myometrial samples compared with a baseline of 0 hours. Differential expression was more likely to be significant at the longer timepoints of 4 and 6 hours. Data are shown as bars indicating median and IQR. Differential analysis was performed for each timepoint compared with 0hrs using Mann-Whitney U-test with lines demonstrating significance values between 2 samples under star and end of each line. *p<0.5, **p<0.01, ***p<0.001

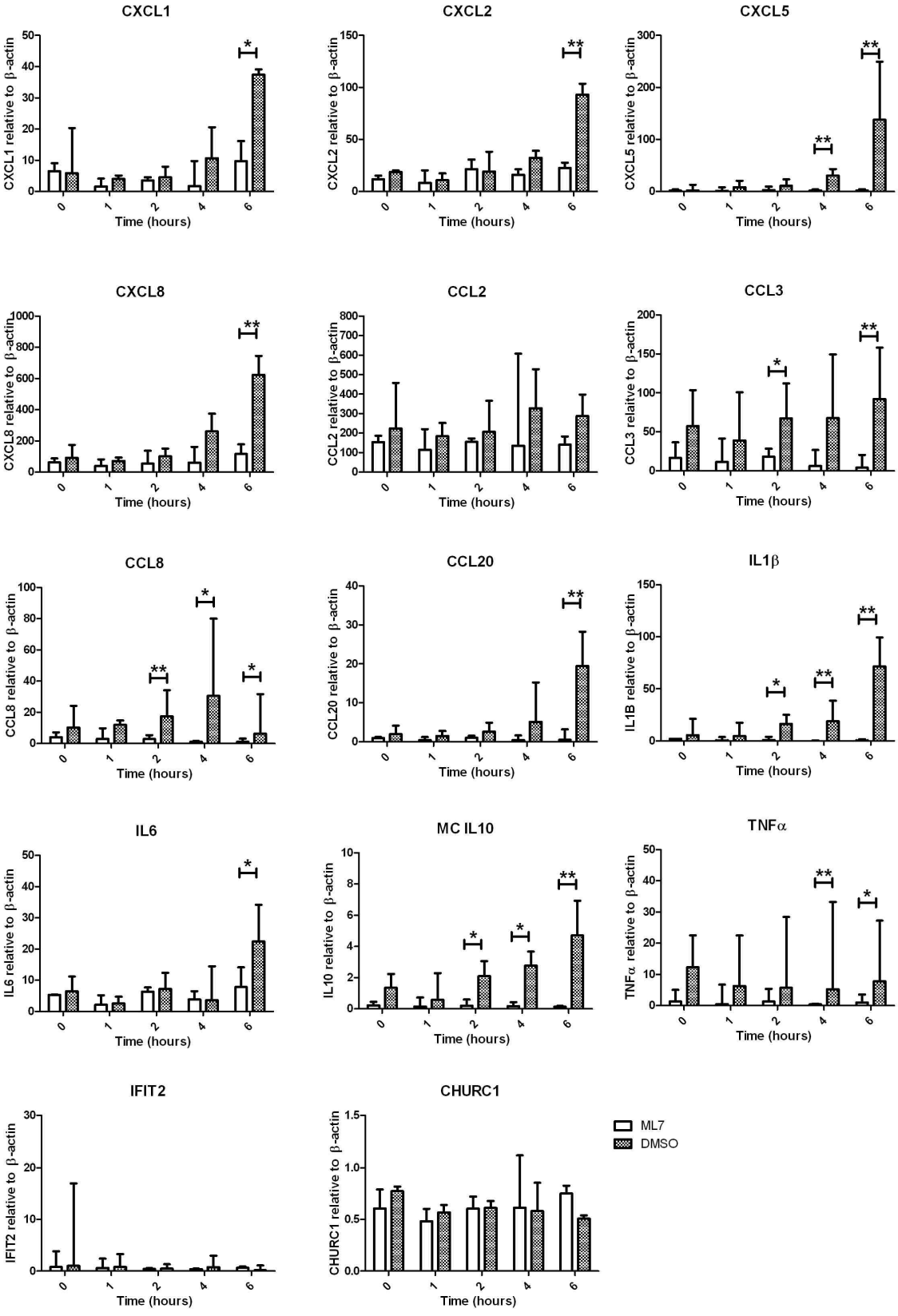

Figure 40 QPCR gene expression ML7 vs DMSO

QPCR confirmation of array data with significantly higher expression of inflammatory related genes in contracting myometrial samples (DMSO) compared with non-contracting samples (ML7) which is particularly apparent at the longer timepoints of 4 and 6 hours. Data are shown as bars indicating median and IQR with clear bars indicating ML7 data and shaded bars indicating DMSO data. Analysis was performed between different drug exposure at each timepoint using Mann-Whitney U-test with lines demonstrating significance values between 2 samples under star and end of each line. *p<0.5, **p<0.01

3.5 Summary of Results

3.5.1 Myometrial functional and transcriptional response to long term exposure to OT

- Myometrium has a recognised increase in overall contractile activity as measured by amplitude, frequency and activity integral in response to exposure to oxytocin

- OT exposure does not significantly alter gene transcription in contracting myometrial samples.

3.5.2 Myometrial transcriptional response to long term exposure to OT where contractions have been blocked by inhibition of MLCK.

- OT exposure does not significantly alter gene transcription in ML7 treated non-contracting myometrial samples.

3.5.3 Temporal transcriptional changes in contracting myometrium

- Contracting myometrium (spontaneous and OT induced) exhibits a time dependent alteration in gene transcription.

- Contracting myometrium exhibits a transcriptional wave over and above that seen in non-contracting ML7 treated samples

- KEGG & GO enrichment analysis demonstrates a time and contraction dependent up-regulation of inflammatory processes and processes associated with cellular damage or death, with down regulation of processes concerned with cell metabolism in myometrial tissue.

3.6 Discussion

The data presented in this chapter demonstrate that *in-vitro* myometrial contractile activity is positively affected by exposure to oxytocin. However, long term exposure to OT and its stimulatory effect does not appear to be modulated via transcriptional changes. Rather our data would suggest that OT acts to facilitate pre-existing myometrial proteins and contractility pathways.

Additionally, myometrial contractions *in-vitro*, whether OT induced or spontaneous, demonstrates a 'temporal wave' of transcriptional changes over and above non-contracting samples, with time related up-regulated genes demonstrating a preponderance of pathways concerned with inflammation and cellular damage or apoptosis, and down-regulation of pathways concerning cellular metabolism.

The concept of human labour as an inflammatory event is well recognised, however the cause, timing and role is unknown. Our data suggest uterine contractions during labour are associated with a myometrial derived inflammatory response in addition to myocyte cellular damage, death and reduced cellular metabolic function.

3.6.1 Myometrial functional and transcriptional response to long term exposure to OT

Our functional results concerning the stimulatory effect of OT on myometrial contractility are in agreement with the widely established role of OT as a uterotonic agent and most certainly support the physiology behind Blair Bell's initial use of oxytocin in the primary prevention/treatment of atonic post partum haemorrhage[148].

Despite contractile stimulation by OT, our hypotheses that long term (hours) exposure to OT would induce transcriptional changes in addition to those seen in spontaneously contracting myometrium or in non-contracting myometrium (where MLCK function had been blocked), was not supported by our data.

Previous evidence has suggested there may be a transcriptional role for long term exposure to OT which may 'prime' the myometrial cells and lead to an

efficient and effective labour [167 179 180 327]. However, despite contractile stimulation by OT, we were unable to demonstrate any transcriptional changes in addition to transcriptional changes seen in spontaneously contracting myometrium. This suggests that long term OT stimulation of myometrial cells promotes contractile behaviour via facilitation of pre-existing proteins and contractility pathways.

Despite our evidence, it must be acknowledged that since this study considered only a single addition of OT rather than continuous infusion therefore, firm conclusions about transcriptional effects of OT on myometrium may be hampered. An infusion would have been more comparable to the *in-vivo* situation for both spontaneous and OT induced labours. Additionally, with a single dose of OT, there may be a natural decrease in the bioavailability within the organ bath over time via the actions of an oxytocinase present in the myometrial samples. The half life of OT *in-vivo* is estimated at lying anywhere between 3-15 minutes with evidence of pregnancy, gestation and labour specific variations[50-54]. However, the half life of OT in our *in-vitro* model of labour in the absence of maternal serum is unknown, nevertheless, we presume it longer since no other routes of metabolism or excretion are present apart from the myometrial derived oxytocinase. A slight drop in the OT bioavailability in the organ bath may explain the drop in the contractile stimulatory effect of OT in our samples after one hour of exposure. However, the contractility measures of amplitude and activity integral did remain higher in the OT treated group than the control group despite a loss of significance.

These factors were certainly considered at the study design stage of this study; however, we opted to have a 'controlled' stimulation of contractile activity by OT. Thereby, we were able to avoid myometrial overstimulation or development of a tonic contraction as this would not be representative of normal labour, and a tonic contraction itself may have caused transcriptional changes which would hamper investigation of the OT pathway. An additional factor to be considered was the presence of endogenous oxytocin within the myometrial samples[91]. However, we assume that the comparisons back to a baseline measures would be sufficient to control for any stimulatory effect which the endogenous OT may have upon the myometrial samples.

Since OT itself does not have transcriptional effect on the myometrium, this suggests that despite OT being the agonist for Braxton Hicks contractions, the role of Braxton Hicks in priming or preparing the uterus for labour is through the action of repeated contractions rather than exposure to OT. In this situation comparisons can be made with exercise training in the skeletal muscle system where the stimulus for contractions is neuronal rather than agonist induced. It is well known that exercise training improves performance and prevents injury. Evidence demonstrates that even after one episode of exercise induced damage, there is less cellular damage following repetition of the exercise, less muscle soreness and recovery of muscle strength is quicker, and is referred to as the 'repeat bout effect'[55-60]. Additionally, assessment of gene expression in trained and untrained muscles performing the same exercise have shown suppression of inflammatory pathways, blunting of the stress response, down regulation of glucose metabolism, mitochondrial structure, and oxidative phosphorylation, with enhancement of gene expression in processes involving the extracellular matrix and cytoskeleton development and organization[61].

3.6.2 Myometrial transcriptional response to contractions and time

We have clearly demonstrated that time and contraction dependent increases in myometrial transcription occur *in-vitro*. In particular these are associated with upregulation of genes associated with inflammatory processes and cellular damage or apoptosis, with down regulation of genes associated with cellular metabolism.

This is in agreement with the widely held concept of labour as an inflammatory event and also the global transcriptional changes seen by others who have compared labouring and non-labouring myometrial samples *in-vivo*[62-67]. However, we are the first to demonstrate that myometrial derived inflammatory changes occurs in relation to contractions and time. To assess these temporal changes in a controlled manner it has been necessary to use *in-vitro* data as *in-vivo* contractility is difficult to assess and control. However, the overlap between our *in-vitro* and *in-vivo* data are further assessed in Chapter 4 and suggests significant similarities between human labour and our *in-vitro* model. Using our *in-vitro* model of labour also allowed comparison between multiple

treatment and time groups, especially where we can examine the stimulatory effects of OT and the inhibitory effects of ML7.

Interestingly, our data suggest that the transcriptional effects of myometrial contractions may be reversed by suppression of contractile activity via blockage of MLCK. This may simply reflect the tocolytic effect of ML7; however, it may also reflect a direct transcriptional effect or modulation of upstream cellular event by ML7 in the myometrial myocyte[339-341]. It may have therefore been beneficial to have an additional non-treated, non-contracting baseline control sample. However, these concerns are partially addressed in Chapter 4, Hypothesis 4, which suggests that gene expression is comparable between non contracting samples and those where contractions are suppressed secondary to exposure to ML7.

Additionally, contracting skeletal muscle has demonstrated similar pathway changes at the mRNA level following a single bout of endurance exercise in healthy, young sedentary males. Compared to baseline measures pre-exercise, array analysis revealed an upregulation of inflammatory processes (in particular inflammatory cell recruitment) and those associated with cell stress, regulation of proteolysis, apoptosis, and cell growth and differentiation[342]. However, contrary to our findings, there was a transient increase in transcription of genes associated with metabolism[342]. Others have also shown exercise associated gene array changes in skeletal muscle with upregulation of processes associated with cell growth regulation, DNA damage response, stress response, energy metabolism, inflammation, extracellular matrix structure and function[343]. This then suggests that these pathways are altered in myocytes in a non-specific manner in response to contractions.

Throughout this chapter, QPCR confirmation of array data has been performed. We acknowledge that the samples used for validation are the same as those used for gene array and that validation should ideally be performed on a separate set of samples. However, although not formally tested, separate *in-vitro* contracting samples are analysed in Chapter 4 which support the array findings of an association between contractions and inflammatory upregulation. Additionally, in the process of array validation there was a bias towards the use of QPCR probes associated with inflammation. However this was deemed to be

appropriate and allowed further exploration of contraction associated inflammation since the pathway analysis demonstrated enhancement of inflammatory processes. Despite this, further examination of transcriptional markers of cellular damage, stress and apoptosis would be of merit in the future.

3.7 Conclusion

In-vitro myometrial contractions induce a transcriptional wave which is not significantly different to that produced under the influence of OT despite significant differences in functional activity. This suggests that in addition to contractions *per se* having a significant time dependent effect on the myometrial transcriptome that the specific actions of oxytocin are limited to the modification of pre-existing myometrial proteins and contractility pathways.

Additionally, the time and contraction dependent myometrial transcriptional wave is associated with upregulation of pathways concerning inflammation and cellular damage or apoptosis, with down regulation of cellular metabolic pathways.

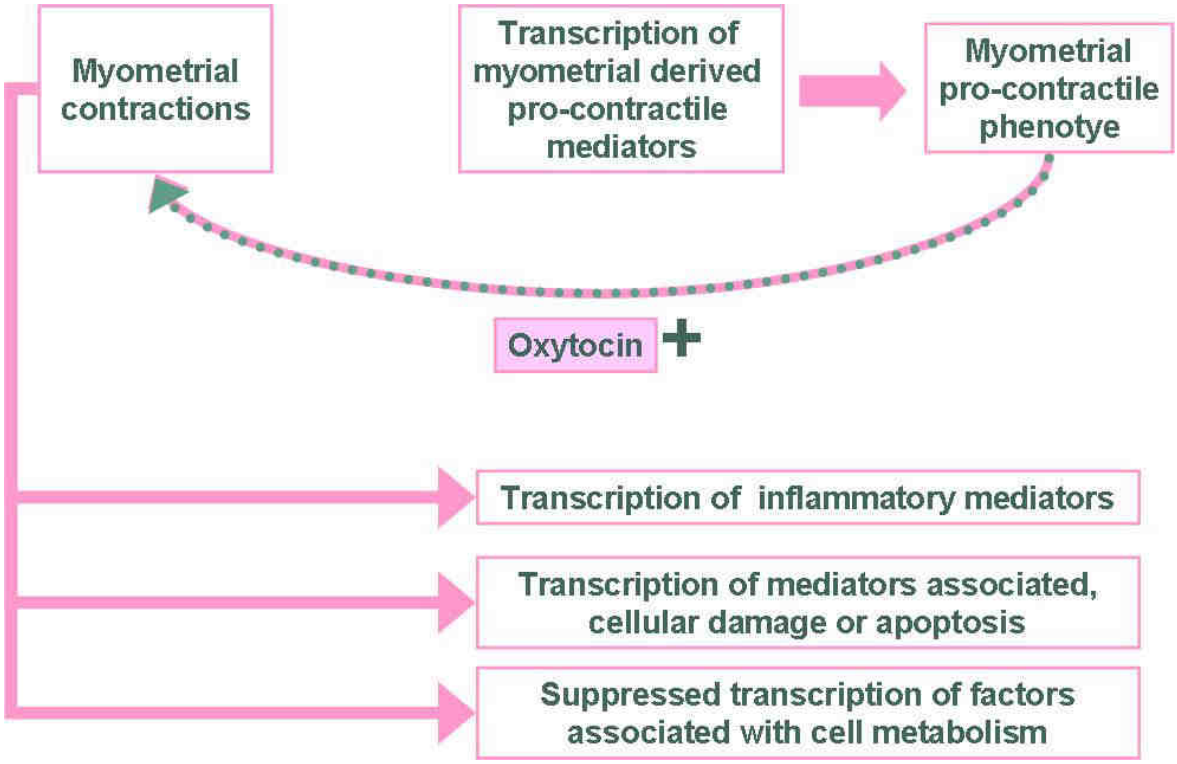

Figure 41 Chapter 3 Amended Hypothesis

Summary of the amended hypothesis regarding the effect of oxytocin on myometrial contractility and gene transcription. Oxytocin has a well recognised stimulatory effect upon myometrial contractility, however this does not appear to be modulated via transcriptional changes, rather if facilitates pre-existing myometrial proteins and contractility pathways. In addition, myometrial contractions (spontaneous or OT induced) appear to induce a transcriptional temporal wave dominated by inflammatory mediators.

Chapter 4

Inflammatory changes in myometrium in response to time, contractions, tocolysis and infection

4 Inflammatory changes in myometrium in response to time, contractions, tocolysis and infection

4.1 Introduction

In the previous chapter, our *in-vitro* model of labour has shown evidence that oxytocin (OT) *per se* does not appear to alter the transcriptional profile of myometrium. However, the array does suggest that time and contraction dependent gene changes occur, with a high preponderance of inflammatory mediators including cytokines and chemokines.

The concept of labour as an inflammatory event is well established[209-217], with 8189 citations found via Pubmed using the basic search criteria 'labour' and 'inflammation' (search performed July 2012). Increases in numbers of immune cells (mainly neutrophils and macrophages) are present within gestational tissues including the myometrium and cervix[219-221]. There is also a co-ordinated increase in cell adhesion molecules and cytokines within these tissues suggesting that the uterus can exert control over the inflammatory process in labour[220-223]. Certainly, from within our department, *in-vivo* gene array transcription analysis from the cervix and myometrium has shown labour is associated with a core inflammatory response, with particular increases in the chemokines CXCL3, CXCL5, CXCL8, CCL2 and CCL20[211]

Premature activation of uterine contractile activity resulting in pre-term labour is associated with infection in up to 40% of cases[18 235 260]. Certainly, in the mouse model, exposure to the TLR-4 agonist lipopolysaccharide (LPS) is a recognised method whereby pre-term labour is successfully induced[263]. Infection is a classically recognised stimulus for mounting an inflammatory response[264 265]. Therefore, a pivotal role for inflammation in the initiation and propagation of human labour (term and pre-term) has been proposed [210 216 217 230-236].

This obviously makes inflammation a possible target through which uterine contractions and human labour may be manipulated, suppressing the inflammatory process to prevent contractility in the case of pre-term labour or enhancing inflammation in an attempt to promote a contractile phenotype

where induction of labour is undertaken in the post-dates pregnancy[233 237 238]. However, despite this theory, dampening of the inflammatory response by systemic administration of inhibitors of the cyclooygenase enzyme (COX inhibitors) or progesterone in pregnancy has proven to be unreliable in preventing pre-term labour in the high risk pregnancy [239-241 287 289 290].

Overall, labour is unquestionably an inflammatory event. However, the cause, timing and role of the inflammatory changes associated with human labour are unknown. We therefore sought to provide a temporal description of the myometrial derived inflammatory cytokines and chemokines associated with spontaneous myometrial contractions *in-vitro* and examine the overlap with those produced *in-vivo* during term human labour. We also sought to describe inflammatory and functional changes to myometrium which occur in response to a range or tocolytic agents and subsequently in response to a pro-inflammatory, infective environment. Additionally, a preliminary examination of contraction induced myometrial inflammatory protein products is made.

4.2 Hypothesis

The hypotheses in this chapter regarding myometrial contraction and inflammation are summarised in Figure 42.

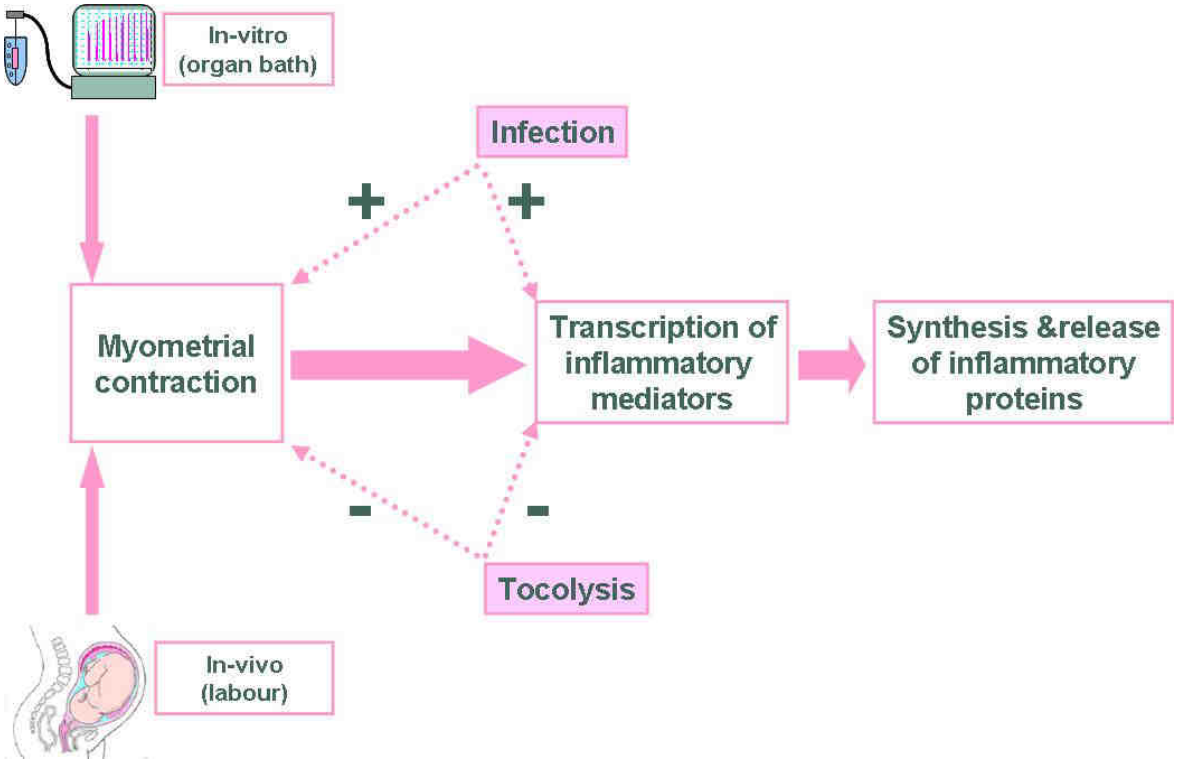

Figure 42 Chapter 4 Overall Hypothesis

Summary of the hypotheses to be examined regarding the association between 1) myometrial contractions and myometrial derived inflammatory mediators, 2) the overlap between myometrial response to *in-vitro* contractions and spontaneous human labour, 3) the influence of tocolysis on myometrial contractile function and transcription of inflammatory mediators, and 4) the influence of an infective/inflammatory environment on myometrial contractile function and transcription of inflammatory mediators.

We propose that our *in-vitro* model of labour will reveal a contraction dependent increase in the myometrial gene transcription of inflammatory mediators, and that these changes are influenced by time. We also propose that there will be an overlap of our *in-vitro* data with transcriptional changes *in-vivo*.

Additionally, we want to examine the functional and transcriptional effects of various chemical methods of tocolysis, and propose that tocolytic agents suppress myometrial contractions and thereby lead to suppression of inflammatory mediator transcription. In particular, we will examine the transcriptional effects of ML7 in comparison to non-contracting but metabolically active myometrial samples to confirm the appropriateness of ML7 as a non-contracting comparison sample in our initial gene array experiment.

The proposal that infection driven inflammation will enhance both contractile function and inflammatory transcription within the myometrium will be examined by assessing the *in-vitro* response to an infective, pro-inflammatory environment with specific comparisons with an endotoxin free environment.

Lastly, we conduct a preliminary test to examine if selected inflammatory genes which are upregulated in contracting myometrium result in protein synthesis and release into their surrounding environment.

The specific hypotheses to be tested are

1. Myometrial transcription of inflammatory mediators is associated with contractions *in-vitro*.

2. Myometrial transcription of inflammatory mediators is associated with contractions *in-vitro* in a time dependent manner.

3. Myometrial transcription of inflammatory mediators *in-vitro* overlaps with those seen *in-vivo*.

4. Myometrial contractility and transcription of inflammatory mediators *in-vitro* is suppressed by chemical tocolysis.

5. Myometrial contractility and transcription of inflammatory mediators *in-vitro* is enhanced in response to an infective and inflammatory stimulus (LPS).

6. Myometrium *in-vitro* can synthesise and release inflammatory protein product.

4.3 Methods

4.3.1 Hypothesis 1 - Myometrial transcription of inflammatory mediators is associated with contractions *in-vitro*.

A summary of the experiment design for this hypothesis is illustrated in Figure 43.

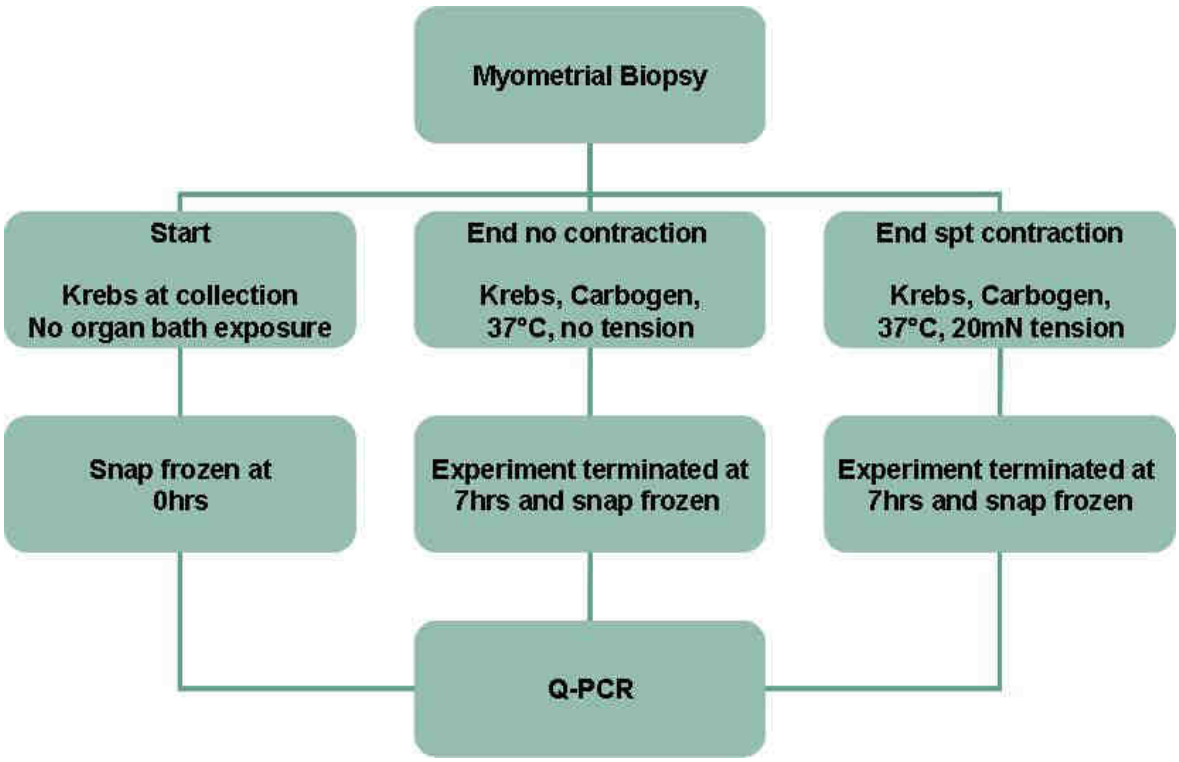

Figure 43 Chapter 4, Hypothesis 1 Experimental Design

Illustration of experimental design to examine the effect of *in-vitro* contractions on inflammatory gene expression as measured using Q-PCR. Three study group environments and timing of sample freezing is shown.

Samples were obtained from patients undergoing non-labouring caesarean delivery at term, and within 2 hours of collection the sample was cut into strips as previously described. One strip was weighed and snap frozen in liquid nitrogen at this start point (start). A second strip was placed without ties or stretch in an organ bath containing Krebs solution, bubbled with carbogen and maintained at 37°C (end no contraction). A third strip was set up to spontaneously contract as previously described (end spt contraction). After 7 hours in the organ baths, experiments were terminated and samples weighed, snap frozen and stored at -80°C.

RNA extraction and Q-PCR were performed on each individual sample examining the gene expression of inflammatory mediators (CXCL1, CXCL2, CXCL5, CXCL8, CCL2, CCL3, CCL8, CCL20, IL1ß, IL6, IL10 and TNFα). The expression of CCL20 in myometrial samples was low, and if both duplicates were undetected by the plate reader, expression of this gene was calculated as 0%. Data are expressed relative to the housekeeping gene ß-actin.

Contractility data are described as medians (IQR) and analysed using a Kruskal-Wallis test examining either amplitude, frequency of activity integral over the length of the experiment. Analysis of Q-PCR results was performed on the raw untransformed data using the Mann-Whitney U-test. No adjustments are made for multiple testing for these data as with our relatively small dataset adjustment may reduce the chance of making a type I error, but may lead to and increase in the chance of making a type II error and a need to increase our sample size[331-334]. A $p<0.05$ was considered significant.

4.3.2 Hypothesis 2 - Myometrial transcription of inflammatory mediators is associated with contractions *in-vitro* in a time dependent manner

A summary of the study design for this hypothesis is illustrated in Figure 44. Q-PCR was performed on the myometrial samples which were used for the gene array experiment in the previous chapter. Gene expression of inflammatory mediators (CXCL1, CXCL2, CXCL5, CXCL8, CCL2, CCL3, CCL8, CCL20, IL1ß, IL6, IL10 and TNFα) relative to ß-actin was performed on each sample individually. Since the array suggested no significant difference in gene expression in contracting myometrium regardless of environment (OT, acetic acid or DMSO) results were pooled and analysed as one 'contracting group' with 15 individual samples at each of the time-points.

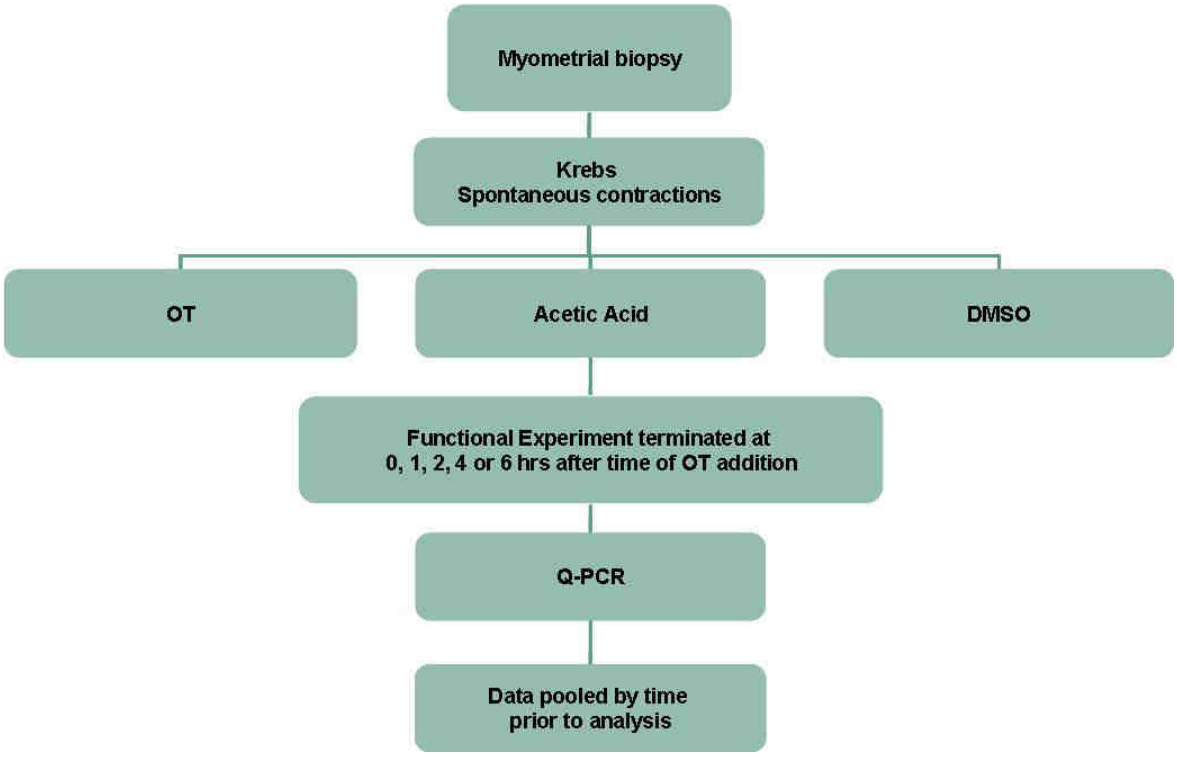

Figure 44 Chapter 4, Hypothesis 2 Experimental Design

Illustration of experimental design to examine the effect of contractions and time on myometrial inflammatory gene expression measured using Q-PCR. Three study group environments and timing of sample freezing is shown. Of note despite different environmental exposures, previous analysis has shown no environmental effect on gene transcription, therefore samples are pooled for analysis with n=15 samples for each time point.

Contractility data are described as medians (IQR) and analysed using a Kruskal-Wallis test or Mann-Whitney U-test comparing measures of contractility between study groups (defined as time at which experiment was terminated) at each time point over the length of the experiment. The raw untransformed Q-PCR data was analysed using Pearson's correlations and was performed for each gene probe to investigate its relationship with time. A $p<0.05$ was considered significant.

4.3.3 Hypothesis 3 - Myometrial transcription of inflammatory mediators *in-vitro* overlaps with those seen *in-vivo*

The myometrial gene expression in samples from our *in-vitro* model of labour was compared with genes differentially expressed in contracting myometrium from women in term labour. To do this we used our original array data from contracting samples (those exposed to oxytocin, acetic acid or DMSO) and ran an overlap analysis with a previously performed gene array on *in-vivo* myometrial labouring samples. A summary of the experimental design is illustrated in Figure 45.

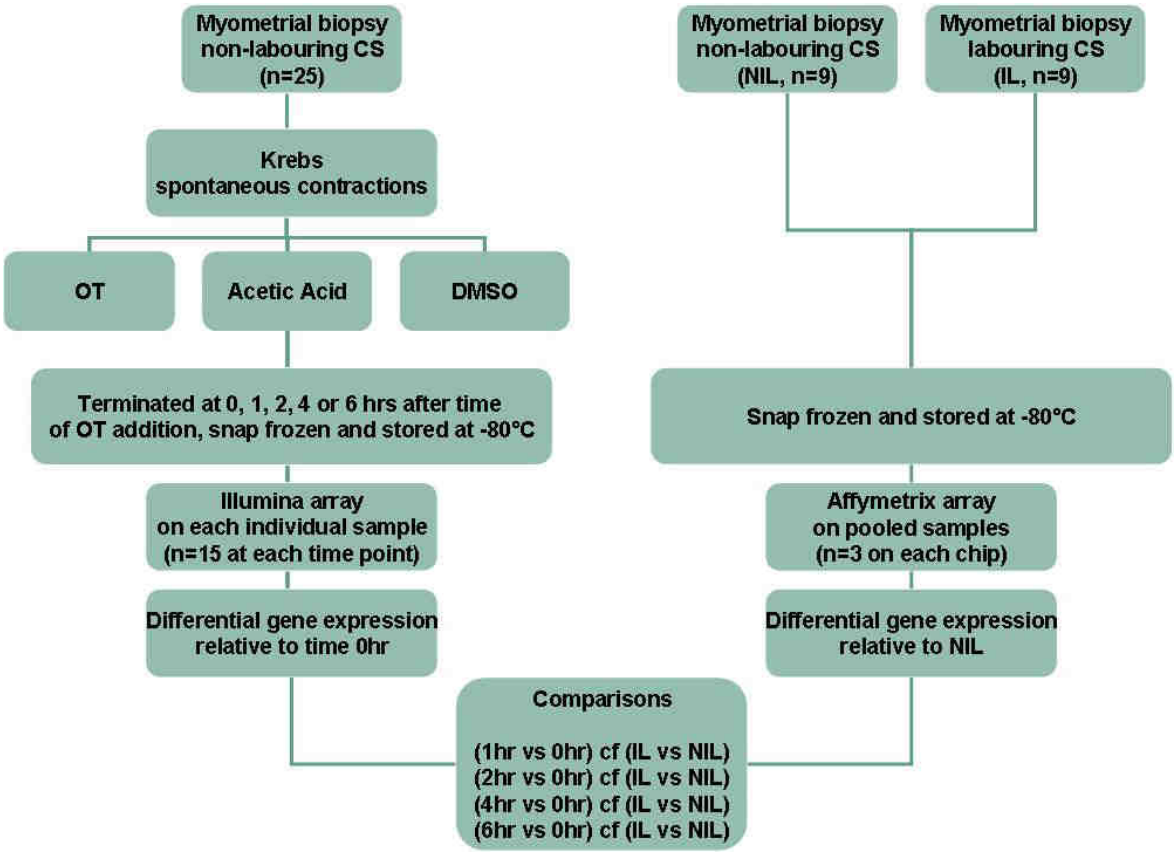

Figure 45 Chapter 4, Hypothesis 3 Experimental Design

Illustration of experimental design to examine the overlap between myometrial inflammatory gene expression in contracting samples *in-vitro* (1, 2, 4 and 6hrs relative to time 0hrs) and *in-vivo* (NIL vs IL). Of note despite different environmental exposures, *in-vitro* samples are pooled for analysis with n=15 samples for each time point.

The *in-vivo* data had been collected and analysed previously[211]. It is derived from samples of myometrium which were taken during caesarean delivery in the same manner as samples collected for the *in-vitro* experiments. Samples were taken from women at term (>37 weeks gestation) either before the onset of

labour (NIL, n=9) or from women during spontaneous labour with a cervical dilatation between 4 and 10cm who were undergoing emergency caesarean section for presumed foetal distress (IL, n=9). RNA was isolated from each sample individually. Subsequently, 3 samples were pooled and analysed on one chip using the Affymetrix v2 U133 plus 2 gene arrays using the One-Cycle Eukaryotic Target Labelling Assay protocol (Affymetrix, Santa Clara, CA).

The *in-vitro* data are derived from myometrial samples obtained at term pre-labour caesarean deliveries (n patients = 25, n strips from each patient = 3) where samples then subsequently contracted in an organ bath. The 3 samples from each patient were exposed to either OT, acetic acid or DMSO after establishment of spontaneous contractile activity. Experiments were terminated at 0, 1, 2, 4 & 6hrs after drug addition. Illumina gene arrays were compiled for each individual sample (n=75) and were analysed together, n=15 at each time point (previous work shows OT alters contractions but not transcription in myocytes).

Since the arrays were performed on different platforms, they were not directly comparable. Therefore the Affymetrix data were reanalysed and the probesets that were significantly different between IL and NIL samples using an adjusted $p<0.01$ were selected (t-tests with significance correction using the Benjamini & Hochberg method for multiple testing). Of these genes that were differentially expressed on the Affymetrix array, only those which had a gene symbol common to the Illumina array were included for further analysis (n=814). The differential gene expression for the *in-vivo* data (IL vs NIL) were then compared with differential gene expression from the *in-vitro* data relative to time 0 hours (1hr vs 0hr, 2hr vs 0hr, 4hr vs 0hr and 6hr vs 0hr). A list of genes common to both *in-vivo* and *in-vitro* work was compiled for each comparison as outlined in Figure 45. The cellular pathways associated with the genes on this list were determined using the Kyoto Encyclopaedia of Genes and Genomes (KEGG). All analysis of array data was performed by Fios Genomics Ltd, Edinburgh.

Additional analysis of previously performed Q-PCR data for the chemokines CCL2, CCL20 and CXCL8 relative to ß-actin was also undertaken. Supplementary comparison with data from Hypothesis 1 in this chapter (start, end no contraction, end spt contraction) was also performed as baselines (NIL and start)

were felt to be more comparable to each other than the 0 hour timepoint where contractions had already been established. As the Q-PCR on the NIL and IL samples had been performed using a different concentration of sample than for the Q-PCR performed during the other experiments in this thesis, fold changes and ranges were considered and calculated using the $2^{-\Delta\Delta Ct}$ method for relative gene expression with ΔΔCt +SD and ΔΔCt –SD where SD is standard deviation for the ΔΔCt value[344 345].

4.3.4 Hypothesis 4 - Myometrial contractility and transcription of inflammatory mediators *in-vitro* is suppressed by chemical tocolysis

A summary of the methods used to investigate the response of myometrium to tocolysis is illustrated in Figure 46.

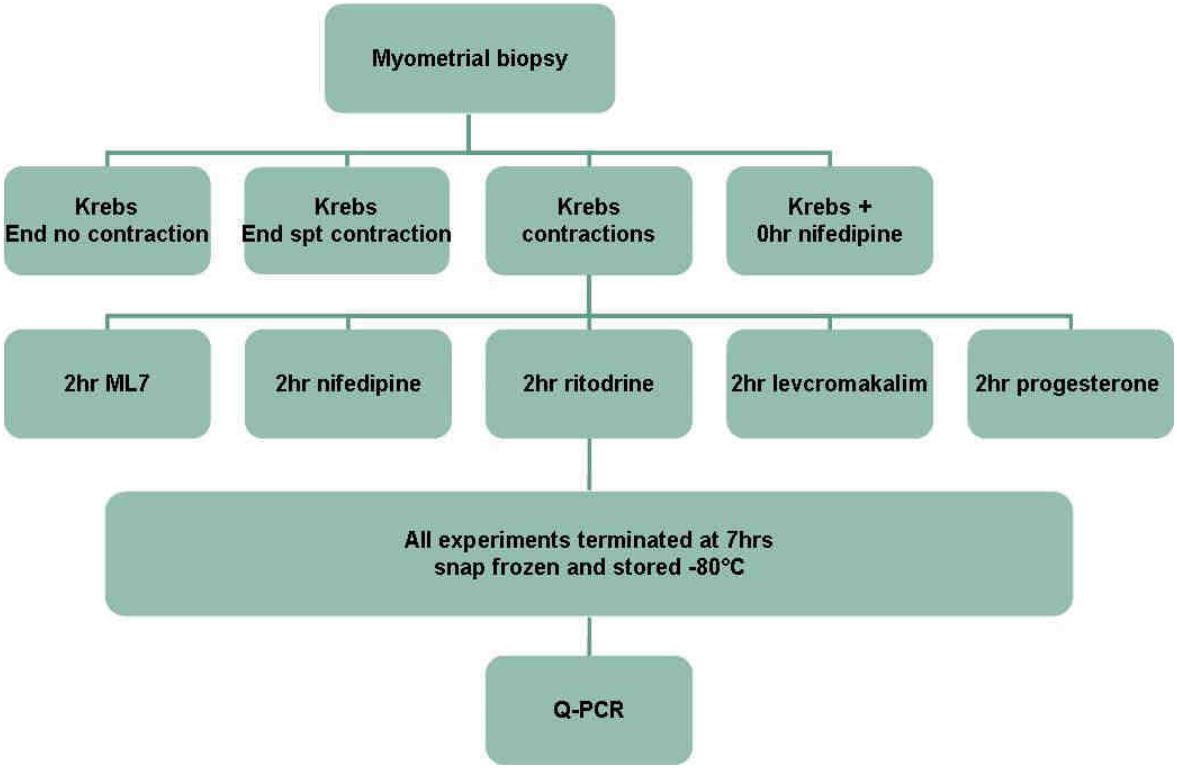

Figure 46 Chapter 4, Hypothesis 4 Experimental Design

Illustration of experimental design to examine the functional and transcriptional effect of chemical tocolysis on *in-vitro* myometrial contractions and inflammatory gene expression as measured using Q-PCR. Organ bath environments and timing of sample freezing is shown.

Samples of myometrium were dissected into strips and set up in individual organ baths of Krebs solution at 37°C, with 20mN tension applied. Once regular

contractile activity was established (approximately 2 hours after initial tension was applied), various tocolytic agents namely ML7, nifedipine, ritodrine, levcromakalim and water soluble progesterone were added to the organ bath. Final bath concentrations were ML7 (200μM), nifedipine (10 μM), ritodrine (10mM), levcromakalim (10 μM) and progesterone (1mM) determined as outlined in Chapter 2. Additionally, data are available for strips exposed to nifedipine (10 μM) throughout the experiment from time of initial tension application (0hr nifedipine). Data for additional comparison is provided from 1) fresh strips frozen at the start of the experiment, 2) strips placed without ties or stretch in an organ bath containing Krebs solution, bubbled with carbogen and maintained at 37°C (end no contraction) and 3) a control strip which spontaneously contracted in normal Krebs solution (end spt contraction) (Chapter 4, Hypothesis 1). Contractile activity was observed for a total of 7 hours from initial tension being applied after which time the experiments were terminated and samples stored at -80°C. Subsequent Q-PCR was performed on each individual sample examining the gene expression of inflammatory mediators (CXCL1, CXCL2, CXCL5, CXCL8, CCL2, CCL3, CCL8, CCL20, IL1ß, IL6, IL10 and TNFα) relative to ß-actin. The expression of CCL20 in myometrial samples was low, and if both duplicates were undetected by the plate reader, expression of this gene was calculated as 0%.

Contractility data are described as medians with IQR. The raw untransformed Q-PCR data were analysed using the non-parametric Kruskall-Wallis and Mann-Whitney U-tests as appropriate. No adjustments are made for multiple testing for these data as with our relatively small dataset adjustment may reduce the chance of making a type I error, but may lead to and increase in the chance of making a type II error and a need to increase our sample size[331-334]. A $p<0.05$ was considered significant.

4.3.5 Hypothesis 5 - Myometrial contractility and transcription of inflammatory mediators *in-vitro* is enhanced in response to an infective and inflammatory stimulus (LPS)

A summary of the methods used to investigate the effects of a pro inflammatory environment of myometrium is illustrated in Figure 47.

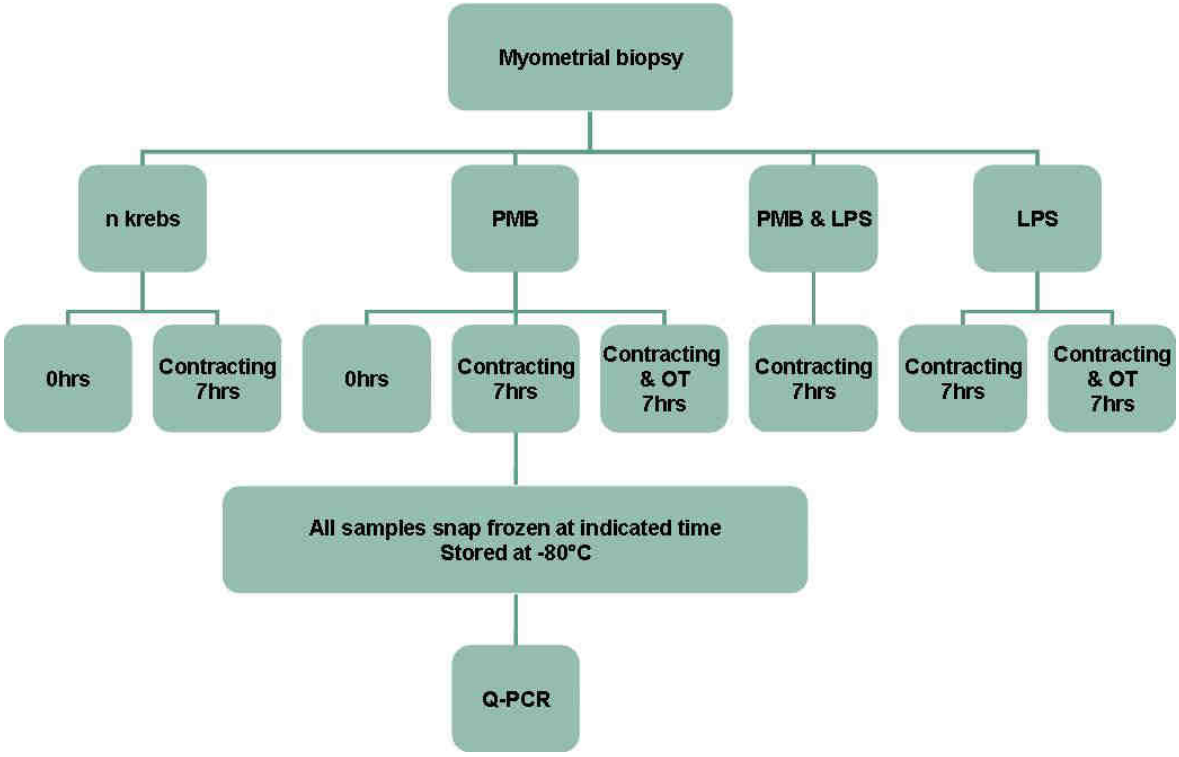

Figure 47 Chapter 4, Hypothesis 5 Experimental Design

Illustration of experimental design to examine the functional and transcriptional effect of an infective, pro-inflammatory environment (Lipopolysaccharide (LPS)) on *in-vitro* myometrial spontaneous and OT induced contractions and inflammatory gene expression as measured using Q-PCR. Comparisons are made with an endotoxin free environment (Polymixin-B (PMB)) and untreated Krebs solution. Organ bath environments and timing of sample freezing is shown.

Myometrial samples were immediately split into two part following collection at caesarean delivery, with one sample placed in normal Krebs solution, and the other in Krebs solution treated with Polymixin-B (PMB). Myometrial strips were dissected and one strip from each environment was immediately snap frozen ('n Krebs start' and 'PMB start'). Further strips were suspended in separate organ baths of carbogen gassed Krebs solution under isometric conditions with a resting tension of 20mN. Two baths underwent addition of PMB (30µg/ml) to inactivate endotoxin ('PMB'), and the remaining two baths had an addition of Lipopolysaccharide ('LPS') (100µg/ml). A further bath had a combination of PMB

and LPS ('PMB &LPS') and there was a sixth control bath of normal Krebs solution with no drug additions ('n Krebs'). After establishment of spontaneous contractions, OT (1nM) was added to one PMB bath and one LPS bath ('PMB & OT' and 'LPS & OT'). Contractions were then observed for a further 5 hours with amplitude, frequency and activity integral recorded at 0, 0.5, 1, 2, 3, 4 and 5 hours. At this time tissues were snap frozen with subsequent gene expression of inflammatory mediators (CXCL1, CXCL2, CXCL5, CXCL8, CCL2, CCL3, CCL8, CCL20, IL1β, IL6, IL10 and TNFα) relative to ß-actin performed using Q-PCR.

Contractility data are described as medians (IQR) and analysed using a Kruskal-Wallis test or Mann-Whitney U-test as appropriate. The raw untransformed Q-PCR data were analysed using the non-parametric Kruskall-Wallis and Mann-Whitney U-tests as appropriate. No adjustments are made for multiple testing for these data as with our relatively small dataset adjustment may reduce the chance of making a type I error, but may lead to and increase in the chance of making a type II error and a need to increase our sample size[331-334]. A $p<0.05$ was considered significant.

4.3.6 Hypothesis 6 – Myometrium *in-vitro* can synthesise and release inflammatory protein product

A summary of the methods used for out preliminary examination of the synthesis and release of inflammatory proteins by spontaneously contracting myometrium *in-vitro* is illustrated in Figure 48

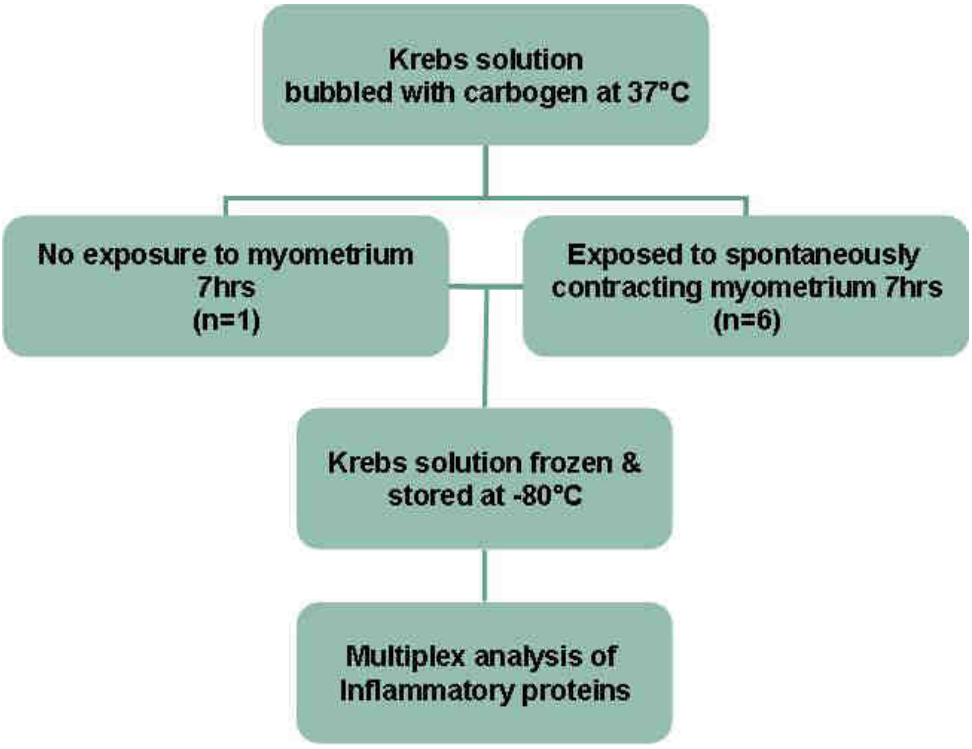

Figure 48 Chapter 4, Hypothesis 6 Experimental Design

Illustration of experiment design used to provide a preliminary examination of the synthesis and release of inflammatory proteins by myometrium which spontaneously contracts in an organ bath of carbogen bubbled Krebs solution at 37°C.

Strips of myometrium from 6 different patients were set up to contract spontaneously as previously described. 7 hours after initial tension was applied, experiments were terminated and 5ml of Krebs solution from the organ bath was frozen and stored at -80°C (n=6). A separate sample of Krebs which had never been exposed to tissue but had been bubbled with carbogen at 37°C for 7 hours was also frozen and stored at -80°C (n=1).

Samples were prepared and analysed using a Biorad Bio-Plex Pro Human Cytokine 8-Plex Panel 1 M50-000007 and analysed on the Biorad Bioplex System- Luminex 100 (Chapter 2, Section 2.5 and 2.6). Data are presented as raw fluorescence readings and % change from baseline, where baseline is considered expression in Krebs solution which has not been exposed to tissue and was calculated for each sample individually.

4.4 Results

4.4.1 Hypothesis 1 - Myometrial transcription of inflammatory mediators and the association with *in-vitro* contractions

4.4.1.1Patient Demographics

Myometrial samples were collected for 13 women. Data were excluded if the sample had not been handled correctly (1 sample from 'start group' was not frozen within 2 hours, 7 samples from end not contracting group were not in an environment of 37°C, 1 sample from end spontaneous contraction group did not contract). The characteristics of the patients from which the samples used for final analysis are shown in Table 17.

N Women		13
N Strips	Start End no contraction End spt contraction	12 6 12
Maternal BMI (kg/m^2)	Median (IQR) [Range]	29.6 (25.0,31.2) [17.24,48.2]
Age (years)	Median (IQR) [Range]	32.0 (30.0, 35.0) [25.0,41.0]
Gestation (days)	Median (IQR) [Range]	274 (273,276) [273,280]
Baby birth weight (kg)	Median (IQR) [Range]	3.78 (3.45, 4.02) [3.29,4.20]
Indication for CS	N (%) Previous LUSCS N (%) Other	9 (69.2%) 4 (30.8%)
Parity	N (%) =0 N (%) =1 N (%) ≥2	2 (15.4%) 9 (69.2%) 2 (15.4%)

Table 17 Hypothesis 1 - Patient demographic details

Demographic details of patients included in experiment to examine the effect of *in-vitro* contractions on inflammatory gene expression as measured using Q-PCR (Chapter 4 Hypothesis 1).

4.4.1.2Contractility data

A total of 12 strips were used to provide contractile data (end spt contraction) which is summarized below in Figure 49. Overall, Kruskal-Wallis testing showed no significant changes in amplitude (p=0.93), frequency (p=0.39) and activity integral (p=0.79) throughout the length of the experiment in the contracting strips.

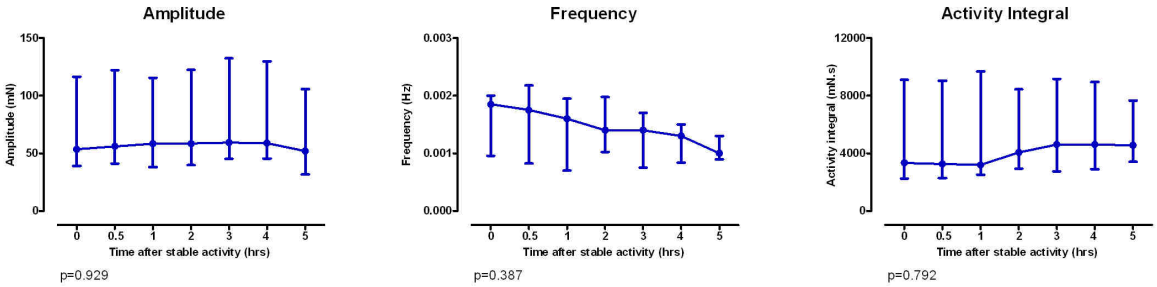

Figure 49 Spontaneous contractile activity of myometrial strips

Illustration of spontaneous contractile activity (amplitude, frequency and activity integral) in myometrial strips used in this experiment (n=12). Data are shown as median (IQR) with p-values indicated for Kruskal Wallis test of contractile activity over time showing no significant change.

4.4.1.3Myometrial transcription of inflammatory mediators

Compared with baseline measures, all inflammatory probes tested significantly increased in the samples which were not contracting but still metabolically active in the Krebs solution at 37°C (start vs end no contraction). In the myometrial samples which contracted (end spt contraction), a further increase was seen in all twelve probes, with increases significantly greater than the non-contracting counterpart for all except TNFα (Figure 50).

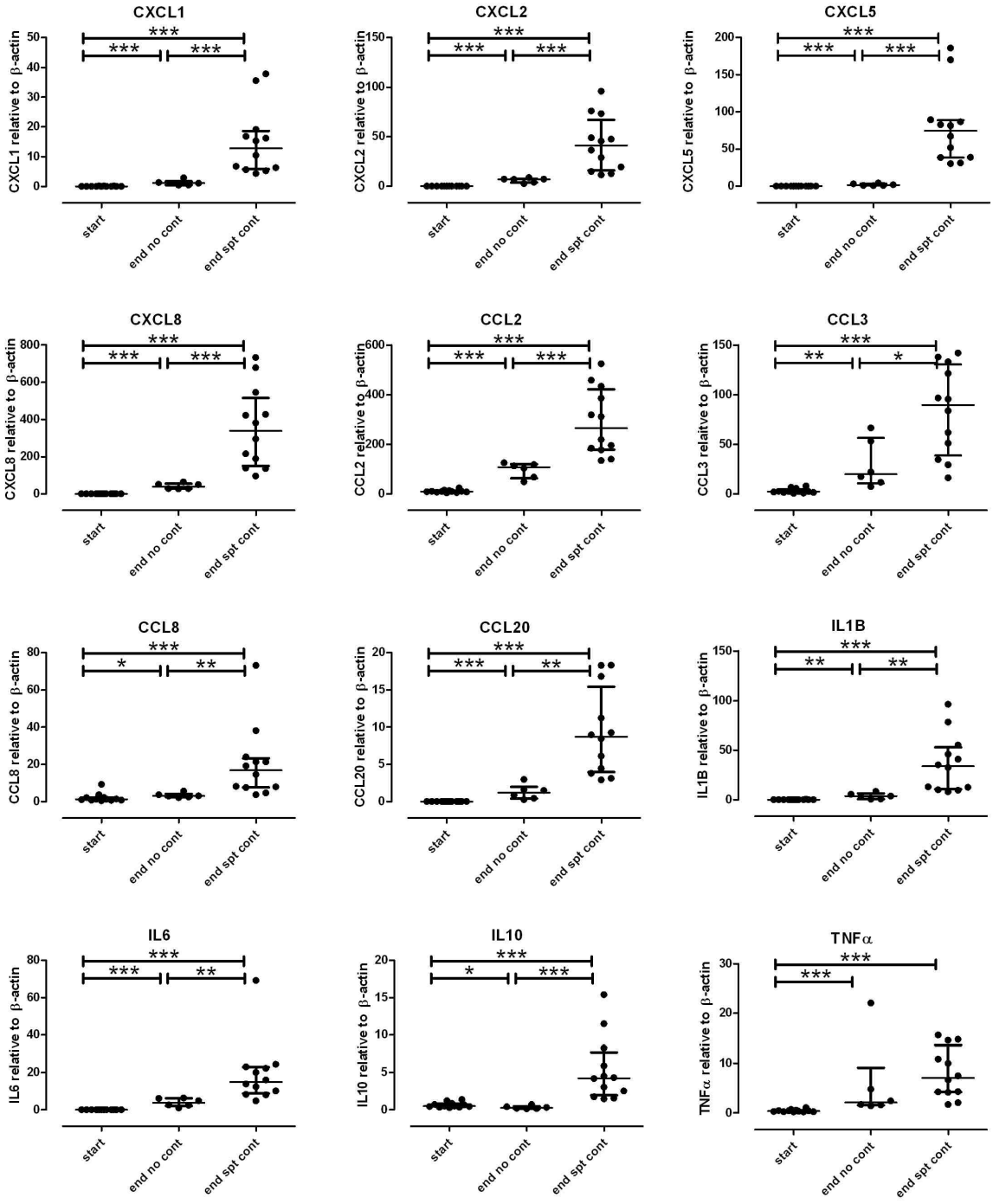

Figure 50 QPCR data contracting vs non-contracting myometrium

Expression of inflammatory markers in myometrium at start of experiment (start) and after 7hrs in an organ bath of normal Krebs solution at 37°C not contracting (end no cont) or in an organ bath at 37°C spontaneously contracting (end spt cont). Raw data are shown with median and IQR. Analysis performed using Mann-Whitney U test with lines demonstrating significance value between 2 samples under start and end of each line. *p<0.05, ** p <0.01, ***p<0.001

4.4.2 Hypothesis 2 - Myometrial transcription of inflammatory mediators and the temporal association with contractions *in-vitro*

4.4.2.1Patient Demographics

Samples were taken from 25 patients, with three myometrial strips from each sample, with data from 15 strips at each time point. Demographic details for these patients are listed in Table 6.

N Women		25
N Strips		75
Maternal BMI (kg/m^2)	Median (IQR) [Range]	24 (21.9, 26.5) [18.5,34.6]
Age (years)	Median (IQR) [Range]	31 (27,35) [21,41]
Gestation (days)	Median (IQR) [Range]	273 (273, 275) [263,284]
Baby birth weight (kg)	Median (IQR) [Range]	3.47 (3.28, 3.70) [2.30, 4.19]
Indication for CS	N (%) Previous LUSCS N (%) Other	20 (80%) 5 (20%)
Parity	N (%) =0 N (%) =1 N (%) ≥2	2 (8%) 16 (64%) 7 (28%)

Table 18 Hypothesis 2 - Patient demographic details

Demographic details of patients included in experiment for Chapter 4 Hypothesis 2 examining myometrial transcription of inflammatory mediators and the temporal association with *in-vitro* contractions.

4.4.2.2Contractility Data

Functional contractile data are presented in Figure 51.

No significant difference for any measure of contractile activity at any time point was found between groups (defined as the time at which the sample was frozen) when analysed by time at which the strip was frozen (Kruskal-Wallis or Mann-Whitney test at each time point; amplitude, $p>0.35$; frequency, $p>0.36$; activity integral, $p>0.37$).

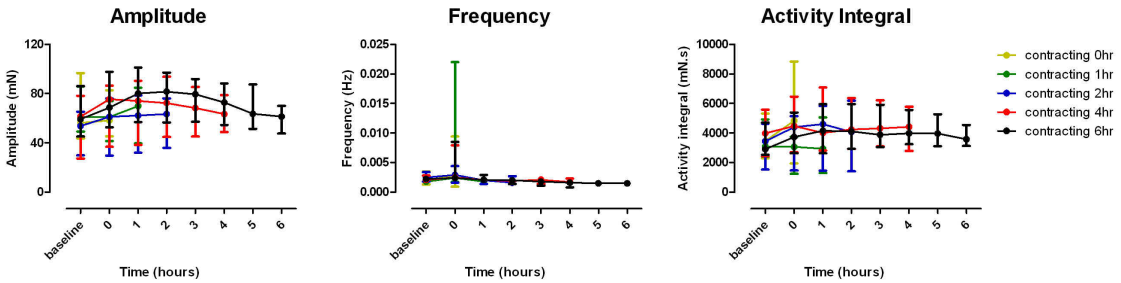

Figure 51 Contractile activity of myometrium over time

Illustration of contractile activity (amplitude, frequency and activity integral) in myometrial strips, yellow = experiment terminated at 0hr, green = experiment terminated at 1hr, blue = experiment terminated at 2hrs, red = experiment terminated at 4hrs and black = experiment terminated at 6hrs (n=15 in each group). There was no significant difference in contractile pattern between groups at any of the time points. Data are shown as median and IQR.

4.4.2.3Myometrial transcription of inflammatory mediators

Q- PCR data for contracting myometrial strips are illustrated in Figure 52. This demonstrated a clear positive correlation between duration of contraction and transcription of the genes CXCL1, CXCL2, CXCL5, CXCL8, CCL20, IL1β, IL6 and IL10 ($r>0.47$, $p<0.001$ for all). Despite up-regulation of CCL2, CCL3, CCL8 and TNFα in response to contractions (Figure 50), the changes in these cytokines did not appear to be associated with time in these samples.

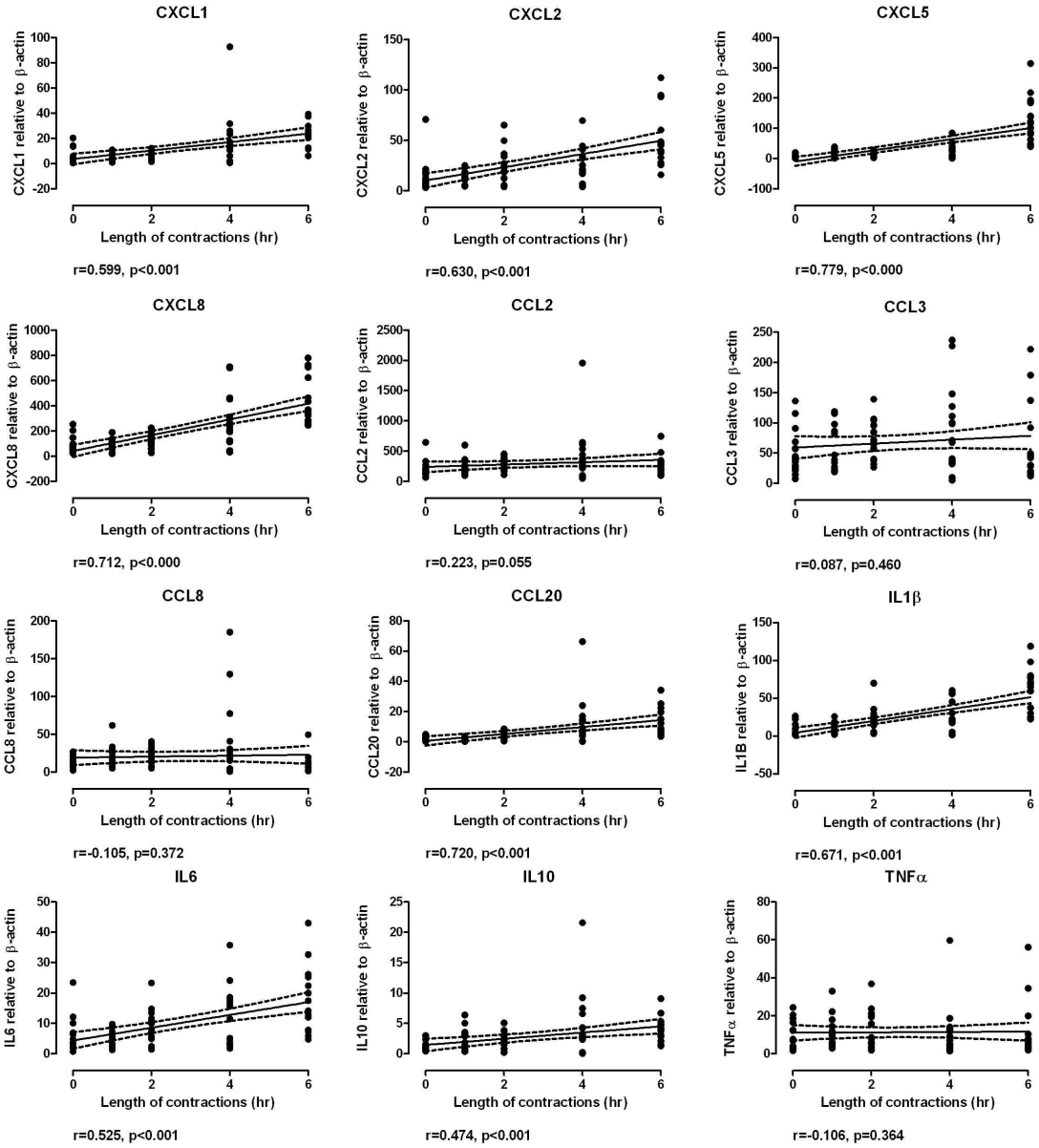

Figure 52 QPCR correlation of inflammatory marker with time

Time dependent changes in myometrial transcriptional expression of selected cytokines with significant time and contraction dependent increases in CXCL1, CXCL2, CXCL5,CXCL 8, CCL20, IL1B, IL6 and IL10 (n strips at each time point = 15). Raw data are shown with regression line and 95% confidence interval. Analysis was performed using Spearmans correlation, r and p values are shown for each gene.

4.4.3 Hypothesis 3 - Myometrial transcription of inflammatory markers *in-vitro* overlaps with those seen *in-vivo*

4.4.3.1Patient Demographics

Demographic details for the patients in each of the study groups are listed below in Table 19.

Study group		*In-vitro* contractility study	*In-vivo* NIL	*In-vivo* IL
N Women		25	9	9
N Strips/samples		75	9	9
Maternal BMI (kg/m^2)	Median (IQR) [Range]	24 (21.9, 26.5) [18.5,34.6]	Not recorded	Not recorded
Age (years)	Median (IQR) [Range]	31 (27,35) [21,41]	26 (21.5,32.0)	24 (22.5,31.0)
Gestation (days)	Median (IQR) [Range]	273 (273, 275) [263,284]	273 (272, 277)	283 (274, 288)
Baby birth weight (kg)	Median (IQR) [Range]	3.47 (3.28, 3.70) [2.30, 4.19]	3.26 (2.89,3.37)	3.50 (3.33, 4.26)
Indication for CS	N (%) Previous CS N (%) Other	20 (80%) 5 (20%)	5 (55.5%) 4 (44.5%)	0 9 (100%)
Parity	N (%) =0 N (%) =1 N (%) ≥2	2 (8%) 16 (64%) 7 (28%)	/ 5 (55.5%) /	/ 1 (11.1%) /
Dilatation at CS (cm)	Median (IQR) [Range]	/	/	6.0 (5.0,10.0)
Length of labour (hr)	Median (IQR) [Range]	/	/	11.6 (5.3,13.5)
Interval from membrane rupture to delivery (hr)	Median (IQR) [Range]	/	/	10.7 (4.5,11.7)

Table 19 Hypothesis 3 - Patient demographic details

Demographic details of patients included in experiment for Chapter 4 Hypothesis 3 examining the overlap in myometrial transcription between the *in-vitro* model of labour and the *in-vivo* term human labour.

4.4.3.2Contractility data

In-vitro contractility data are described previously in section 4.4.2.2 and Figure 51. Data describing cervical dilatation, length of labour and time from membrane rupture to delivery in the *in-vivo* labouring group are described above in Table 19.

4.4.3.3 Myometrial differential gene expression and overlap analysis of gene array data

Analysis of the Affymetrix data demonstrated that using an adjusted p<0.01, 1050 probesets were significantly different between NIL and IL samples. Of these, 954 had a gene symbol with 814 having a gene symbol common to the Illumina arrays.

Analysis of the overlap of these differentially expressed genes from *in-vivo* contractions with the *in-vitro* contractile profile at adjusted p<0.05 and adjusted p<0.01 is shown Table 20. Of note a larger number of differentially expressed genes from the *in-vivo* data overlap with differentially expressed genes from the longer 4 and 6 hour timepoints *in-vitro*.

Comparison	n genes overlapping	
	Adjusted p<0.05 for *in-vitro* comparison	Adjusted p<0.01 for *in-vitro* comparison
(1hr vs 0hr) cf (IL vs NIL)	8	0
(2hr vs 0hr) cf (IL vs NIL)	18	4
(4hr vs 0hr) cf (IL vs NIL)	178	91
(6hr vs 0hr) cf (IL vs NIL)	292	192

Table 20 Number of genes overlapping between *in-vitro* and *in-vivo* myometrial samples

Overlap summary analysis of myometrial differential gene expression (adjusted p<0.01) in samples which have contracted *in-vivo* relative to samples taken prior to onset of labour (IL vs NIL) and samples which have contracted *in-vitro* for varying lengths of time relative to time 0hr using adjusted p<0.05 or p<0.01 (1hr vs 0hr, 2hr vs 0hr, 4hr vs 0hr and 6hr vs 0hr).

A summary of the cellular processes represented by the overlapping genes and determined using KEGG is provided in Table 21. Notably inflammatory processes dominated in each of the comparisons, in particular those involved with cytokine to cytokine receptor interactions. The full table of KEGG determined cellular processes for this *in-vivo* and *in-vitro* overlap analysis can be found in Appendix V.

Comparison at adjusted p<0.01 for both *in-vivo* and *in-vitro* data

(2hr v 0hr) cf (IL v NIL)		(4hr v 0hr) cf (IL v NIL)		(6hr v 0hr) cf (IL v NIL)	
Pathway	n genes	Pathway	n genes	Pathway	n genes
Not assigned	3	Not assigned	54	Not assigned	125
Cytokine-cytokine receptor interaction	1	Cytokine-cytokine receptor interaction	4	Cytokine-cytokine receptor interaction	8
		Jak-STAT signaling pathway	4	Complement and coagulation cascades	6
		MAPK signaling pathway	4	Jak-STAT signaling pathway	5
		Calcium signaling pathway	4	MAPK signaling pathway	5
		GnRH signaling pathway	4	Neuroactive ligand-receptor interaction	5
		Long-term depression	3	Regulation of actin cytoskeleton	5
		Long-term potentiation	3	Calcium signaling pathway	4
		Melanogenesis	3	Hematopoietic cell lineage	4
		Complement and coagulation cascades	2	GnRH signaling pathway	3
		Hematopoietic cell lineage	2	Insulin signaling pathway	3

Table 21 Top 10 cellular processes expressed in both *in-vitro* and *in-vivo* myometrial samples

Top 10 cellular processes as determined by KEGG represented by overlapping differentially expressed genes from *in-vivo* (NIL vs IL) and *in-vitro* (1, 2, 4 and 6hours relative to 0hours) myometrial samples. Significance level for differential expression was set at an adjusted p<0.01.

4.4.3.4Myometrial gene expression and comparison of fold changes between *in-vivo* and *in-vitro* samples

Supplementary analysis was performed on the available QPCR data for CCL2, CCL20 and CXCL8 relative to ß-actin and Fold change analysis is shown in Table

22. Additionally, Q-PCR data from Hypothesis 1 earlier in this chapter (sample groups: start, end no contraction and end spt contraction) were also analysed as baseline measures (NIL and start) were felt to be more comparable to each other than the 0hr timepoint where contractions had already been established. Fold change analysis showed upregulation of all three chemokines assessed, with fold changes smallest for the *in-vitro* time series, which may reflect the baseline 'time 0hr' measure as previously discussed. Using a comparable baseline measure (NIL and start) *in-vitro* differential chemokine expression was higher than that observed in the *in-vivo* data set for all three genes.

Comparison	CCL2	CCL20	CXCL8
	Fold Change (range)	Fold Change (range)	Fold Change (range)
IL relative to NIL	11.5 (4.6,28.8)	113.0 (13.2,966.8)	105.2 (13.5,819.3)
1hr relative to 0hr	1.1 (0.5,2.3)	1.3 (0.6,2.9)	1.1 (0.5,2.5)
2hr relative to 0hr	1.3 (0.7,2.5)	2.6 (1.2,5.7)	1.6 (0.8,3.4)
4hr relative to 0hr	1.8 (0.4,8.8)	6.3 (0.9,43.2)	2.8 (0.4,21.4)
6h relative to 0hr	1.3 (0.6,2.7)	9.3 (3.9,21.7)	5.9 (3.3,10.5)
End no contraction relative to Start	9.7 (4.5,21.2)	1570.5 (788.3,3129.2)	1027.8 (612.9,1723.2)
End contraction relative to Start	27.9 (8.3,94.1)	12648.5 (3465.8,46160.2)	7600.5 (2271.8,25427.5)

Table 22 QPCR determined fold changes for *in-vitro* and *in-vivo* myometrial samples

Fold changes in CCL2, CCL20 and CXCL8 measured using Q-PCR relative to B-actin and baseline. The fold change and range given for each chemokine is determined using $2^{-\Delta\Delta Ct}$ method with ΔΔCt +SD and ΔΔCt –SD where SD is standard deviation for the ΔΔCt value.

4.4.4 Hypothesis 4 - Myometrial contractility and transcription of inflammatory markers *in-vitro* in response to chemical tocolysis

4.4.5 Patient Demographics

Myometrial samples were collected for 13 women. Data were excluded if the sample had not been handled correctly (7 samples from end not contracting group were not in an environment of 37°C), or if samples did not spontaneously contract (1 from end spontaneous contraction group, 2 from levcromakalim group) or organ baths/drugs were unavailable on the day of experiment (8 samples 0hr nifedipine, 7 samples ML7, 6 samples progesterone). The characteristics of the patients from which the samples used for final analysis are shown previously in Table 17, with number of strips for each environment illustrated in Table 23.

N women		13
N strips	End no contraction	6
	End spont contraction	12
	2hr ML7	6
	0hr nifedipine	5
	2hr nifedipine	13
	2hr ritodrine	13
	2hr levcromakalim	11
	2hr progesterone	7

Table 23 Number of myometrial strips exposed to each environment

Table illustrating number of myometrial strips analysed for each organ bath condition. Further patient demographic details are shown in Table 17.

4.4.5.1Contractility data

Functional contractility analysis of the effect of tocolytic addition to the organ baths at 2 hours after regular spontaneous activity was achieved is illustrated in Figure 53. This demonstrates that at the concentrations used in this experiment, nifedipine and ritodrine inhibited contractions in a manner comparable with the effect of ML7. Additionally, where nifedipine was present from the start of the experiment (0hr nifedipine) the myometrial strip was rendered unable to

contract at all. Levcromakalim and water soluble progesterone did not fully inhibit contractile activity with contractions continuing at reduced amplitude following progesterone addition, and contractions returning 2 hours following addition of levcromakalim.

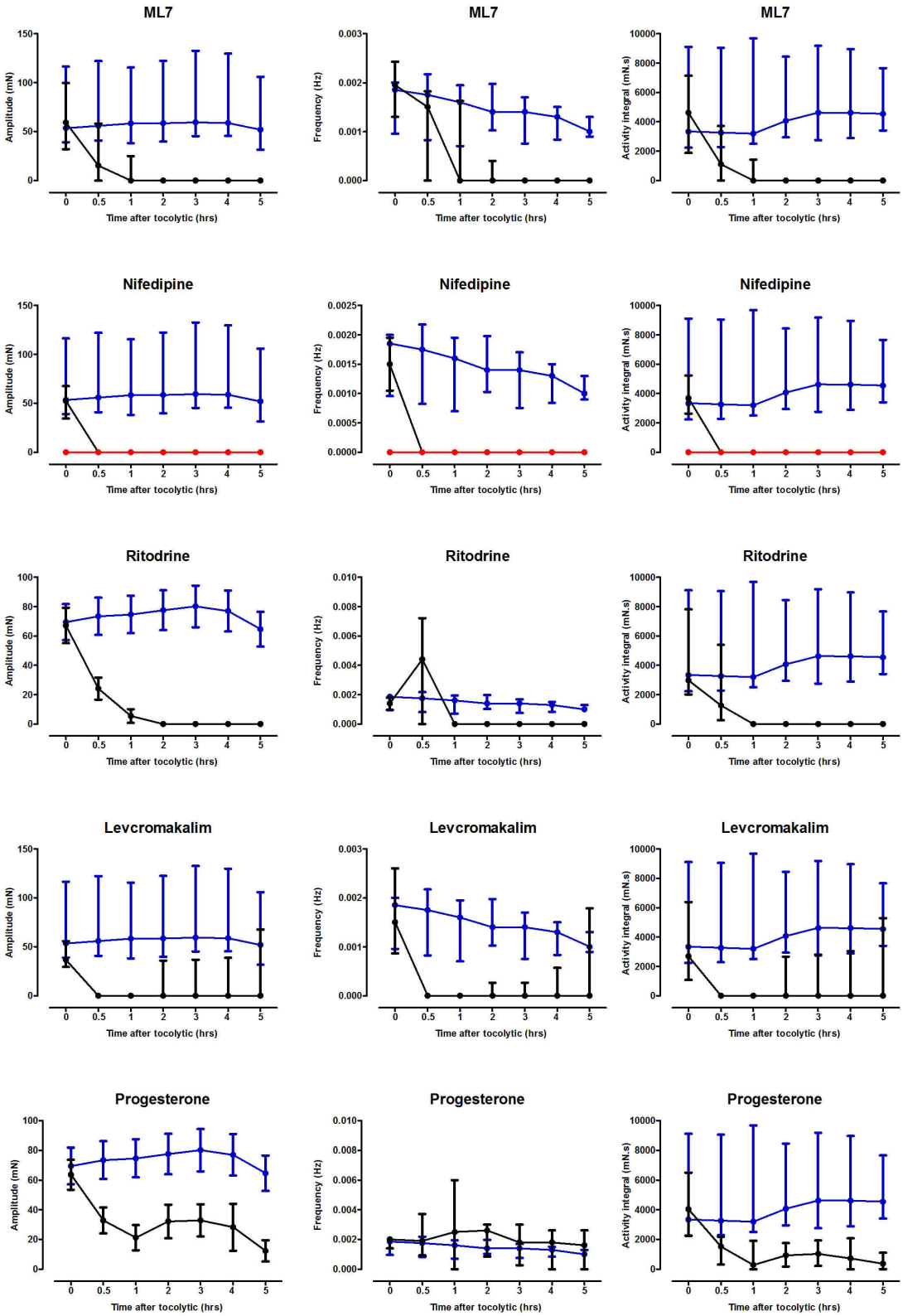

Figure 53 *In-vitro* contractile activity response to tocolytics

The effect of tocolysis on *in-vitro* myometrial contractility in response to the addition of either ML7 (n=6), Nifedipine (n=13), Ritodrine (n=13), Levcromakalim (n=11) or Progesterone (n=7). Comparison data from spontaneously contracting strips are shown in blue on each graph (n=12). Data from exposure to nifedipine from start of the experiment is illustrated in red. Three measures of contractility are used amplitude, frequency and activity integral (graphs from left to right). Data are shown as median and IQR. Complete inhibition of contractile activity is achieved by ML7, nifedipine and ritodrine whereas, levcromakalim and progesterone are unable to fully inhibit contractions.

4.4.5.2Myometrial transcription of inflammatory mediators

Q-PCR analysis of cytokine targets was performed on all samples and comparisons made firstly between those exposed to effective tocolysis (ML7, nifedipine and ritodrine) and corresponding spontaneously contracting samples (Figure 54). As expected from our initial experiments, ML7 exposure reduced inflammatory marker expression compared with spontaneously contracting samples, with the exception of CXCL2, $p=0.055$ (CXCL1, $p=0.022$; CXCL5, $p<0.001$; CXCL8 $p=0.013$; CCL2, $p=0.006$; CCL3, $p=0.001$; CCL8, $p<0.001$; CCL20, $p<0.001$; IL1β, $p=0.002$; IL6, p0.013; IL10, $p<0.001$; TNFα, $p=0.003$).

However, despite abolition of contractions with nifedipine or ritodrine (Figure 53), the expression of all inflammatory markers was comparable with expression in the spontaneously contracting myometrial strips with the exception of CXCL1 which had a higher expression in the 0hr nifedipine group ($p=0.040$) and CCL8 which was lower in the ritodrine group ($p=0.024$). Additionally, expression levels of the measured inflammatory markers were significantly higher after exposure to nifedipine or ritodrine compared with ML7 with the exception of CXCL2 and IL6 which tended to be higher, but differences were not significant (Figure 54). Furthermore, time of nifedipine addition to the organ bath (0 hours or 2 hours) did not lead to any significant difference in expression of the selected inflammatory markers between these two groups (Figure 54).

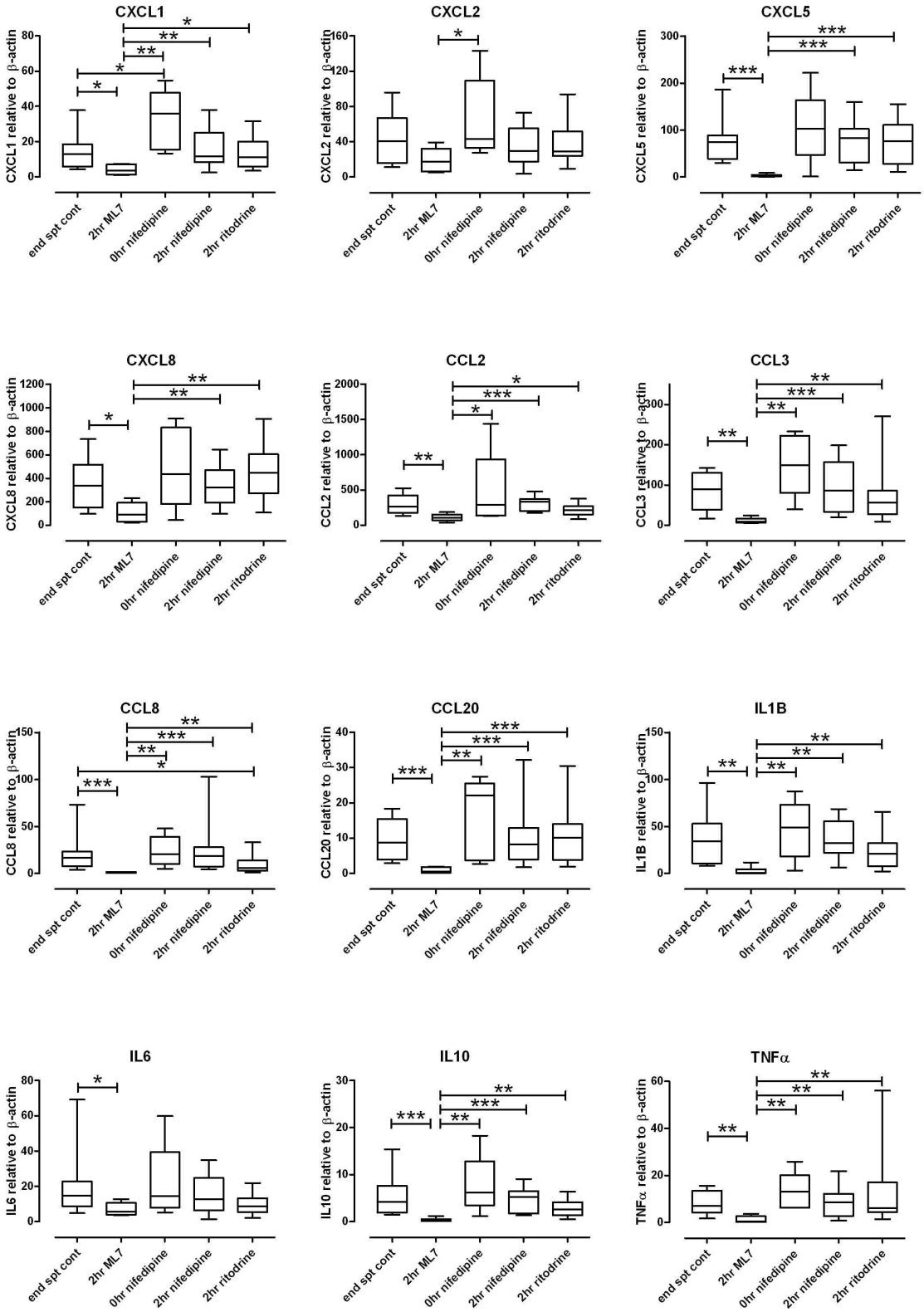

Figure 54 QPCR analysis of myometrial strips exposed to successful tocolysis

Transcriptional expression of inflammatory mediators CXCL1,2,5,8, CCL2,3,8,20, IL6, IL10, IL1B and TNFα relative to β-actin in myometrial samples spontaneously contracting in normal Krebs solution at 37°C (n=12) compared with samples where contractions were abolished by ML7 (n=6), nifedipine (addition at 0hrs (n=5) and 2hrs (n=13)) and ritodrine (n=13). Raw data are shown as box and whisker plots with median, IQR and range shown. Analysis performed using Mann-Whitney U test with lines demonstrating significance value between 2 samples under start and end of each line. *p<0.05, ** p <0.01, ***p<0.001

To investigate if chemically blocking the contraction with the tocolytics ML7, nifedipine or ritodrine resulted in a 'non-contracting' pattern of gene expression, samples were compared with a metabolically active but not contracting sample (end no contraction) (Figure 55). This demonstrated that expression of inflammatory markers in ML7 treated strips was comparable with the 'non-contracting' pattern (end no contraction), with the exception of CCL8 where expression is lower in the ML7 treated strips ($p=0.002$). However, expression of inflammatory markers in the nifedipine and ritodrine tocolytic groups is overall higher than the non-contracting samples. Results are significant for all comparisons with the exception of CXCL5 and IL1β for nifedipine at 0hr ($p=0.052$ for both), CCL3 and CCL8 for ritodrine treated strips ($p=0.087$, $p=0.105$ respectively) and TNFα for all (0hr nifedipine, $p=0.052$; 2hr nifedipine, $p=0.236$, ritodrine, $p=0.087$) (Figure 55).

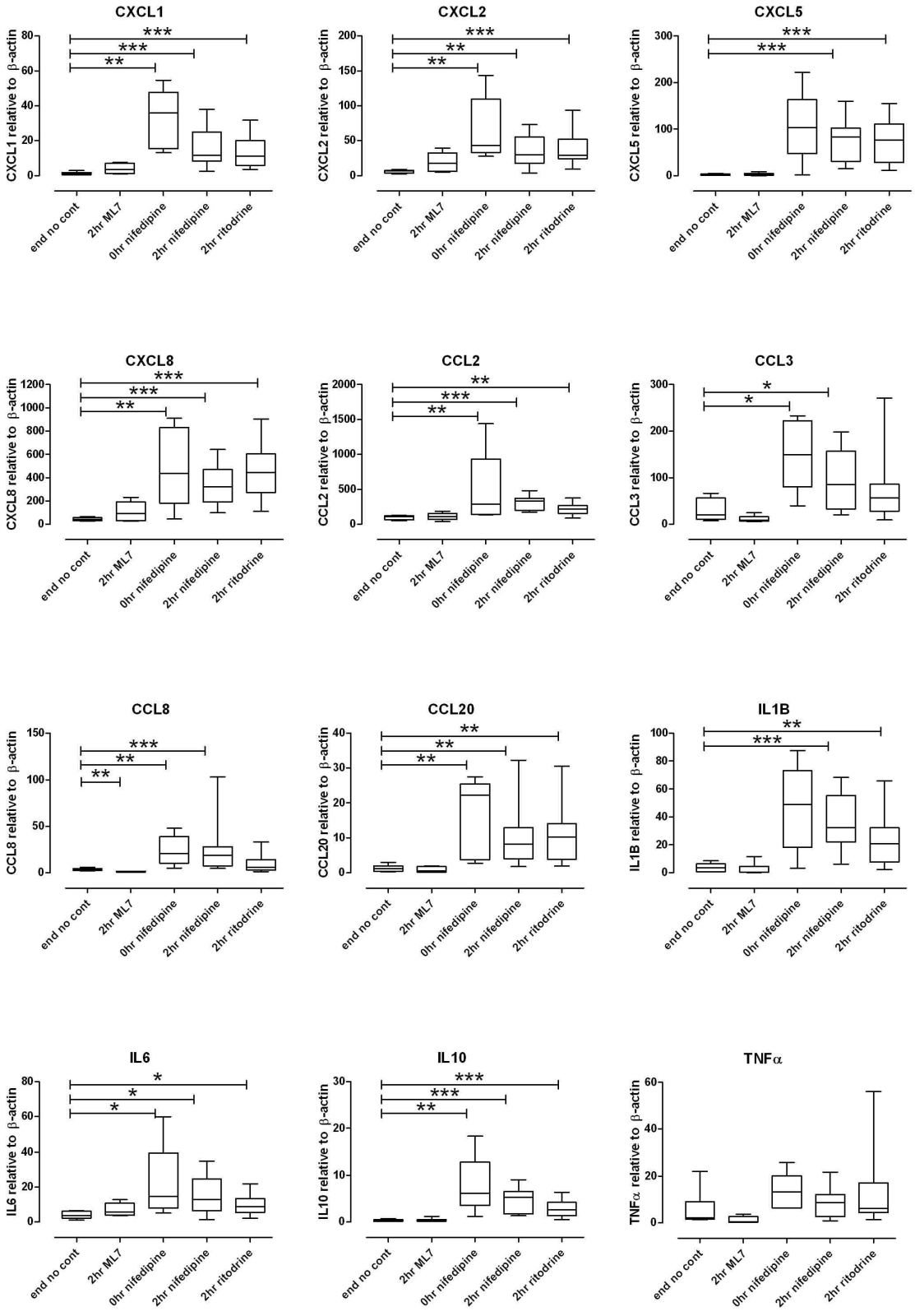

Figure 55 QPCR analysis of myometrial strips exposed to successful tocolysis

Transcriptional expression of inflammatory mediators CXCL1,2,5,8, CCL2,3,8,20, IL6, IL10, IL1B and TNFα relative to β-actin in myometrial samples physiologically active but not contracting in normal Krebs solution at 37°C (n=12) compared with samples where contractions were abolished by ML7 (n=6), nifedipine (addition at 0hrs (n=5) and 2hrs (n=13)) and ritodrine (n=13). Raw data are shown as box and whisker plots with median, IQR and range shown. Analysis performed using Mann-Whitney U test with lines demonstrating significance value between 2 samples under start and end of each line. *p<0.05, ** p <0.01, ***p<0.001

To investigate if partial inhibition of contractility by levcromakalim and progesterone affected inflammatory gene transcription, Q-PCR data were compared using a Kruskal-Wallis test with data from spontaneously contracting strips (end spt contraction vs levcromakalim vs progesterone) (Figure 56). No significant differences were found for any of the inflammatory markers measured. Additionally, when compared with the tocolytics nifedipine and ritodrine added at 2 hours, (levcromakalim vs progesterone vs nifedipine vs ritodrine) no difference was found using a Kruskal-Wallis test, with the exception of CCL8 ($p=0.005$) (Figure 56).

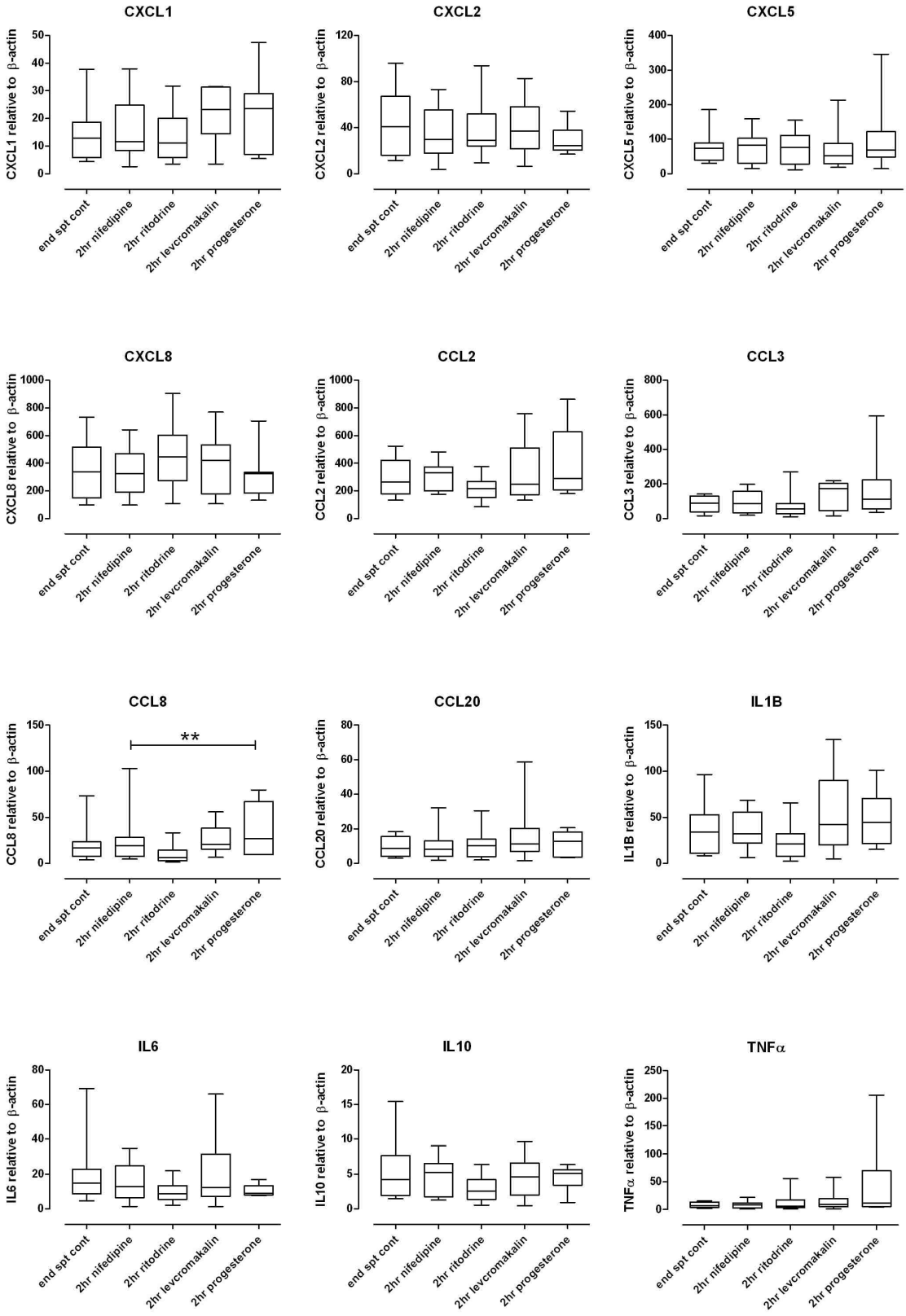

Figure 56 QPCR analysis of myometrial strips exposed to all tocolysis

Transcriptional expression of inflammatory mediators CXCL1,2,5,8, CCL2,3,8,20, IL6, IL10, IL1B and TNFα relative to β-actin in myometrial samples spontaneously contracting in normal Krebs solution compared with samples where contractions were abolished (nifedipine and ritodrine) and partially abolished (levcromakalim and progesterone). Raw data are shown as box and whisker plots with median, IQR and range shown. Analysis performed using Kruskal-Wallis test with line demonstrating significance value between the 4 samples under the line. ** p <0.01

4.4.6 Hypothesis 5 - Myometrial contractility and transcription of inflammatory markers *in-vitro* in response to an infective and inflammatory stimulus (LPS)

4.4.6.1Patient Demographics

Myometrial samples were collected for 6 women. Data were excluded if the sample did not achieve regular spontaneously contractile activity (1 from n Krebs group, 2 from PMB group). The characteristics of the patients with number of strips for each environment are illustrated in Table 24.

N Women		6
N Strips	n Krebs start PMB start n Krebs PMB PMB & LPS LPS PMB & OT LPS & OT	6 6 5 4 5 6 6 6
Maternal BMI (kg/m^2)	Median (IQR) [Range]	30.2 (28.0,32.1) [23.0,34.5]
Age (years)	Median (IQR) [Range]	35.0 (32.8,35.0) [32.0,35.0]
Gestation (days)	Median (IQR) [Range]	276.0 (273.0,277.8) [273.0,280.0]
Baby birth weight (kg)	Median (IQR) [Range]	3.9 (3.5,4.1) [3.3,4.2]
Indication for CS	N (%) Previous CS N (%) Other	4 (66.7%) 2 (33.3%)
Parity	N (%) =0 N (%) =1	1 (16.7%) 5 (83.3%)

Table 24 Hypothesis 5 - Patient demographic details

Demographic details of patients included in experiment for Chapter 4 Hypothesis 5 examining the functional and transcriptional effect of an infective, pro-inflammatory environment (Lipopolysaccharide (LPS)) on myometrium *in-vitro*.

4.4.6.2Contractility data

Functional contractile data for each environment in the absence of OT and in the presence of OT are presented in Figure 57. No significant difference for any measure of contractile activity was found to be influenced by organ bath environment at any time point (Kruskal-Wallis test at each time point; amplitude, $p>0.390$; frequency, $p>0.116$; activity integral, $p>0.304$). Notably,

LPS did not enhance contractile activity of response to OT (Mann-Whitney U-test at each time point; amplitude, p>0.093; frequency, p>0.240; activity integral, p>0.240).

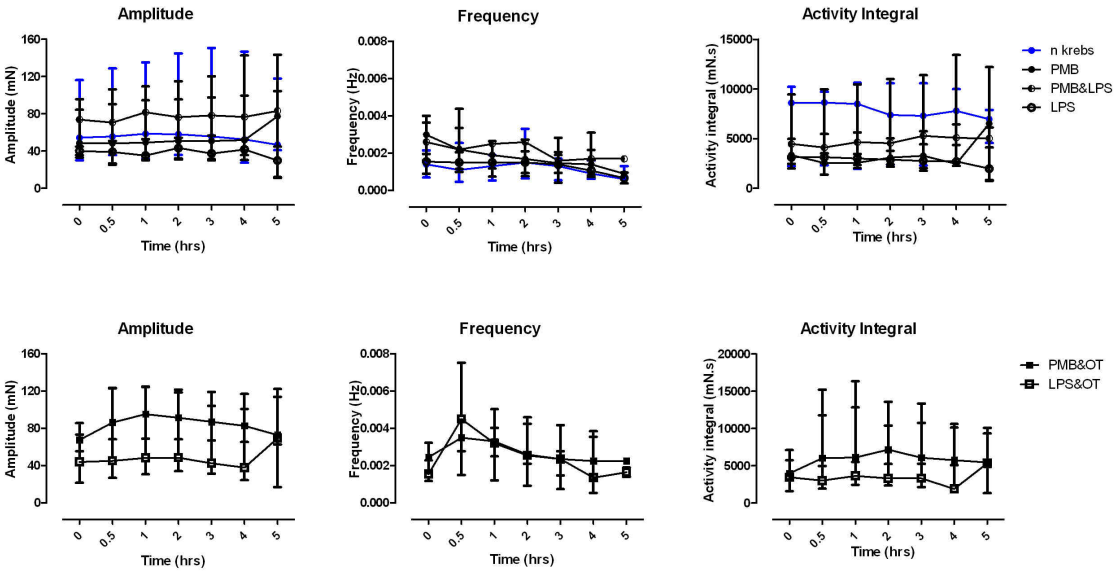

Figure 57 Contractile activity of strips exposed to LPS of PMB

Illustration of contractility data (amplitude, frequency and activity integral (AUC)) for samples which contracted in an environment of LPS or PMB. Contractions were either spontaneous or enhanced with a single addition of OT. Data are shown as median and IQR for each time point. Spontaneous contractility and response to OT was not enhanced by an LPS induced pro-inflammatory environment.

4.4.6.3Myometrial transcription of inflammatory mediators

Q-PCR data demonstrated that contractions induced an upregulation of inflammatory mediator transcription in myometrium exposed to normal untreated Krebs solution (n Krebs start v n Krebs) and PMB treated, endotoxin free solution (PMB start vs PMB) (Figure 58). This suggests an inflammatory reaction driven by contractions rather than infection. Strips which were treated with both LPS & PMB expressed inflammatory mediators at a comparable level with PMB alone, suggesting that PMB is sufficient to eradicate endotoxins from the organ bath environment (Figure 58). Additionally, strips which contracted in a PMB treated environment had a tendency to express lower levels of inflammatory mediators than strips in normal Krebs, but a significant difference was seen for only CCL8 (p=0.016), CCL20 (p=0.032) and IL6 (0.032) (Figure 58).

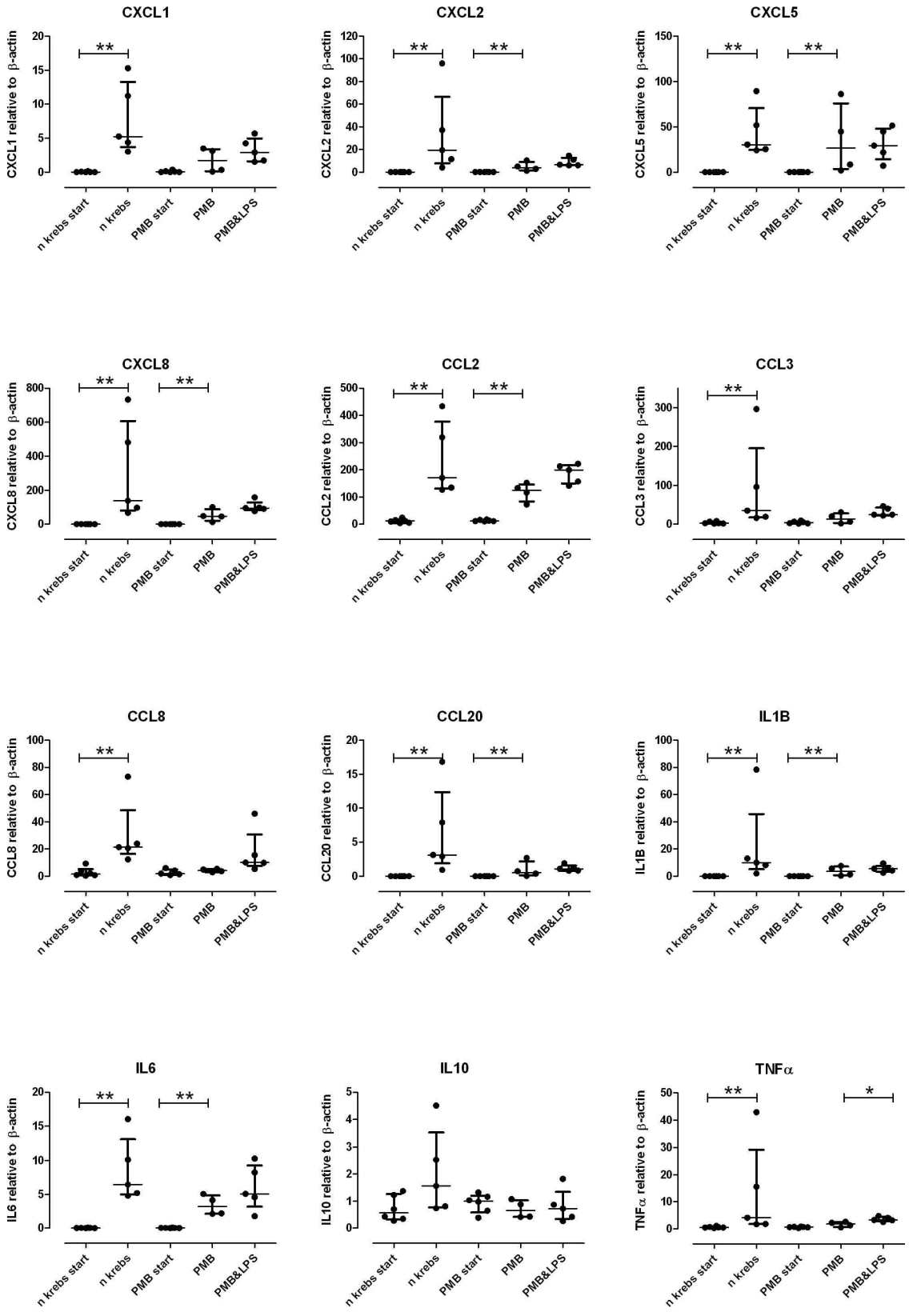

Figure 58 QPCR analysis of myometrial strips exposed to LPS or PMB

Transcriptional expression of inflammatory mediators CXCL1,2,5,8, CCL2,3,8,20, IL6, IL10, IL1B and TNFα relative to β-actin in myometrial samples in normal Krebs or Krebs treated with PMB or PMB & LPS. Comparisons are made between transcription at start of experiment and strips which have spontaneously contracted. Contractions induce transcription of inflammatory mediators even when environmental endotoxins are removed. Data shown as scatter plots with median and IQR indicated. Analysis performed using Mann-Whitney U test with lines demonstrating significance value between 2 samples under start and end of each line. *p<0.05, ** p <0.01

The presence of LPS in the organ bath leads to the induction of inflammatory cytokine transcription, with a significant rise for all ($p<0.05$) with the exception of CXCL5 ($p=0.352$) and CCL8 ($p=0.067$) when compared with strips in a PMB treated environment (Figure 59). Additionally, the data suggest that the addition of LPS to the organ bath environment tends to increase the expression of inflammatory mediators compared with untreated Krebs, however this was not significant.

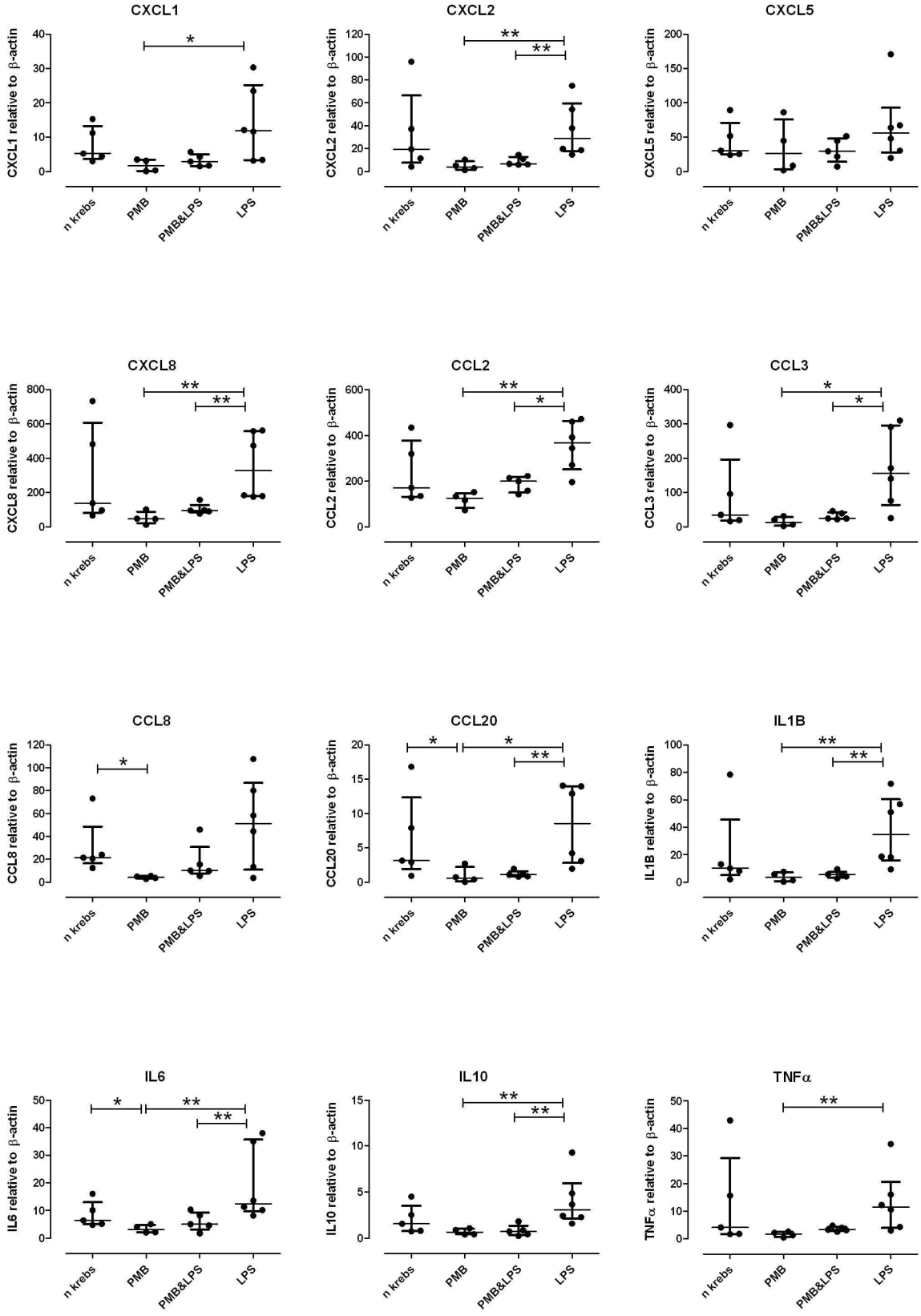

Figure 59 QPCR analysis of myometrial strips exposed to LPS, PMB or LPS&PMB

Transcriptional expression of inflammatory mediators CXCL1, 2, 5, 8, CCL2, 3, 8, 20, IL6, IL10, IL1B and TNFα relative to β-actin in myometrial samples spontaneously contracting in 1) normal Krebs solution, 2) Krebs treated with PMB for endotoxin eradication), 3) Krebs treated with PMB and LPS or 4) Krebs with LPS added to promote a pro-inflammatory environment. LPS induces transcription of inflammatory mediators compared with PMB treated samples. Data shown as scatter plots with median and IQR indicated. Analysis performed using Mann-Whitney U test with lines demonstrating significance value between 2 samples under start and end of each line. *p<0.05, ** p <0.01

There was also a significant increase in the inflammatory mediators observed in the presence of LPS where contractions were induced by OT when compared with OT induced contractions in the presence of PMB ($p<0.05$ for all) with the exception of CXCL5 ($p=0.3939$) (Figure 60).

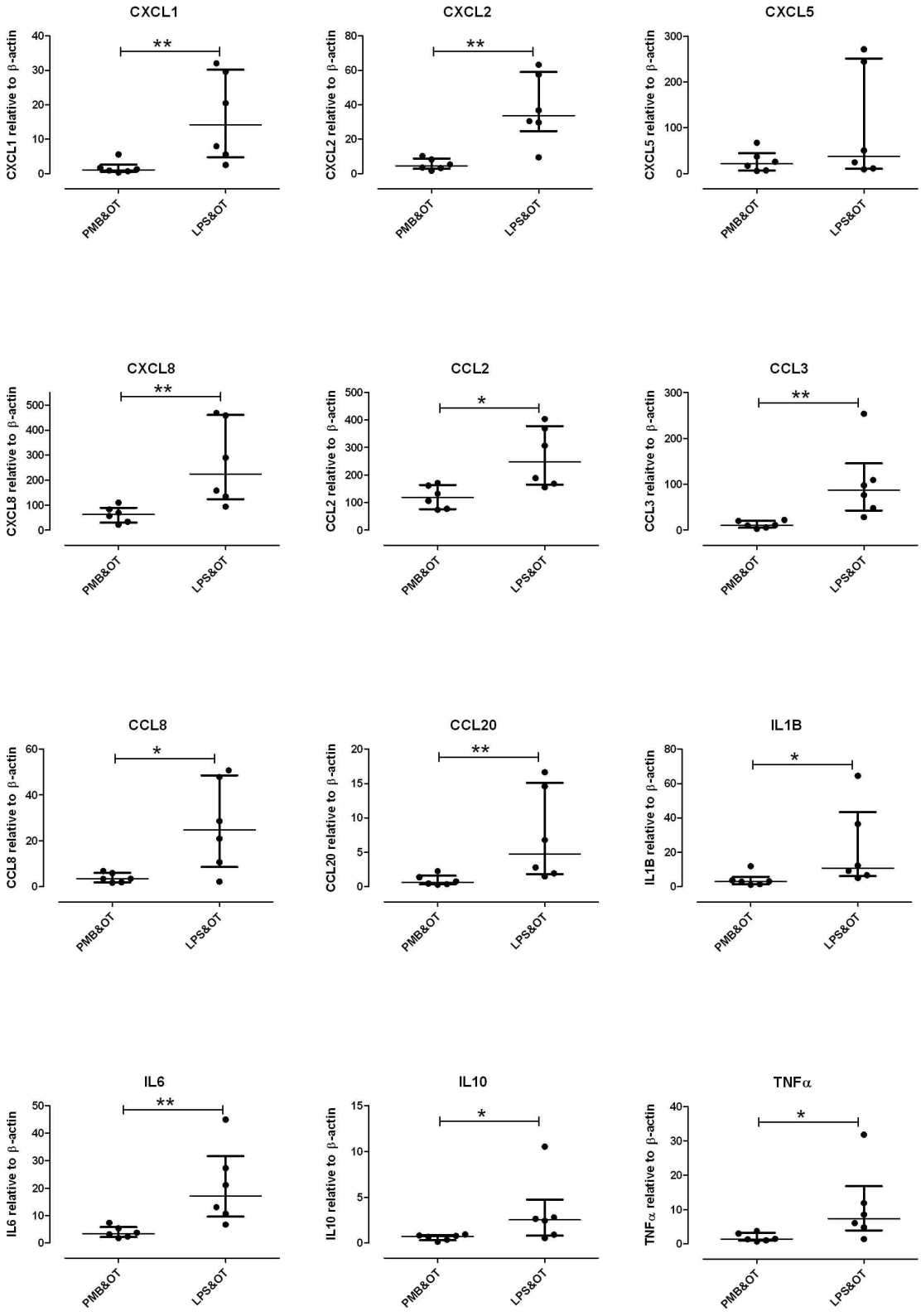

Figure 60 QPCR analysis of myometrial strips exposed to LPS&OT or PMB&OT

Transcriptional expression of inflammatory mediators CXCL1,2,5,8, CCL2,3,8,20, IL6, IL10, IL1B and TNFα relative to β-actin in myometrial samples in Krebs treated with PMB or Krebs with LPS with contractions induced by a single addition of OT. Environmental LPS promotes transcription of inflammatory mediators. Data shown as scatter plots with median and IQR indicated. Analysis performed using Mann-Whitney U test with lines demonstrating significance value between 2 samples under start and end of each line. *p<0.05, ** p <0.01

4.4.7 Hypothesis 6 – Synthesis and release of inflammatory protein products from contracting myometrium *in-vitro*

Expression of the inflammatory proteins Il2, IL4, IL6, Il10, CSCL8, GM-CSF, IFNγ and TNFα in Krebs solution is illustrated in Figure 61. This showed an overall trend to increased detection of all measured proteins with the exception of IL2 where there was a slight decrease (median decrease -13.54% (IQR -44.79, 10.42)). Notable increases in the median level of protein expression occurred for Il6 (1881% (IQR 570.3, 18200.0%)) and CXCL8 (5013% (425.8,16819%)), with moderate increases for TNFα (84.6% (30.8, 948.1%)) and IL10 (15.8% (2.6, 72.1%)).

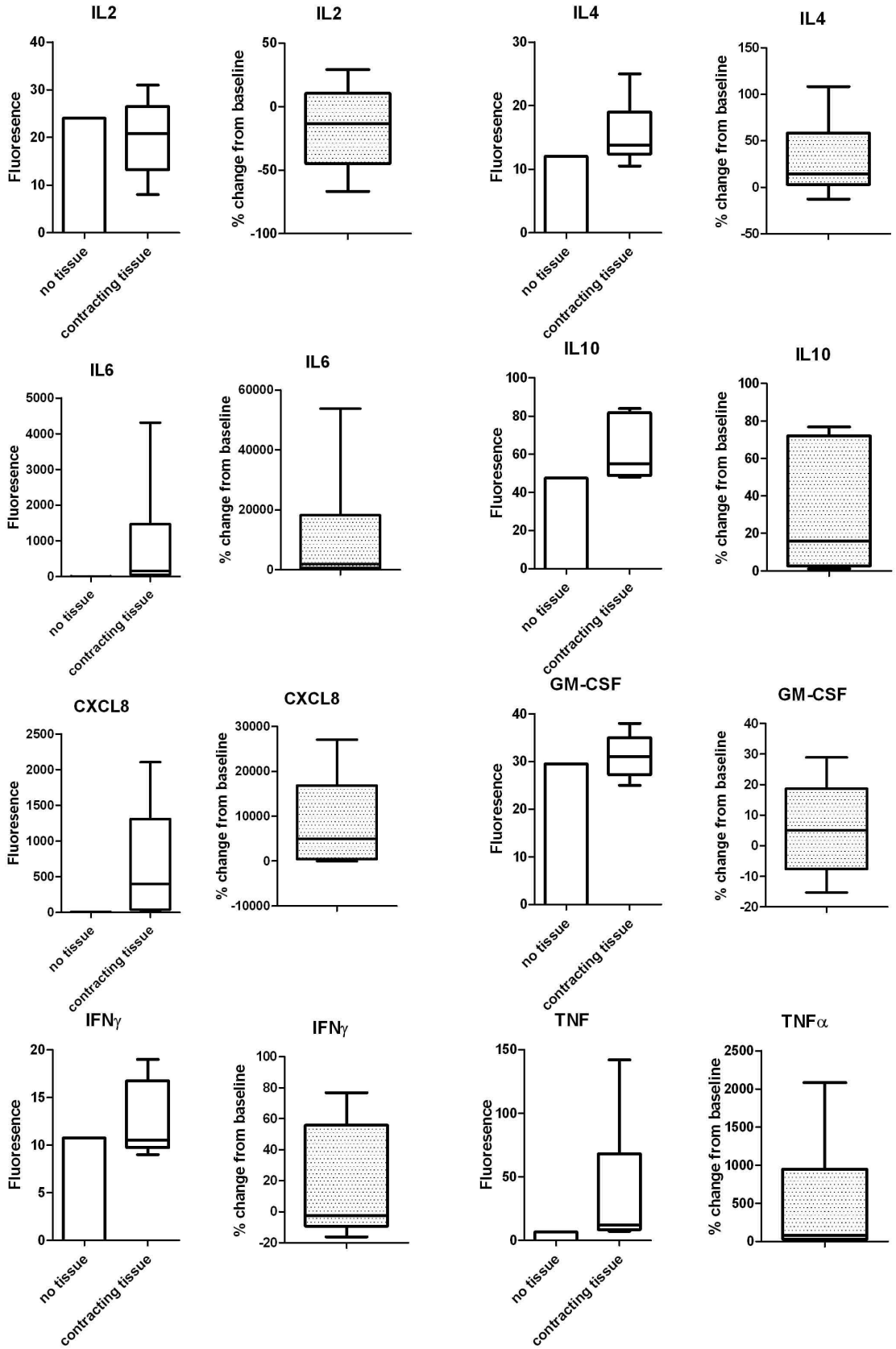

Figure 61 Bioplex analysis of inflammatory proteins in organ bath solution

Expression of the inflammatory proteins IL2, IL4, IL6, IL10, CXCL8, GM-CSF, IFNγ and TNFα in Krebs solution which has nor been exposed to tissue (n=1) and Krebs solution which has been exposed to contracting myometrium for 7 hours (n=6). Measures of expressed fluorescence in each environment and % change from baseline are shown. Data are shown as box and whisker plots with median, IQR and range.

4.5 Summary of Results

4.5.1 Myometrial inflammation and *in-vitro* contractions

- Contractions induce transcription of inflammatory mediators in myometrial samples over and above the transcription seen in non contracting ex-vivo samples kept in physiological conditions.

4.5.2 Temporal association between myometrial inflammation and *in-vitro* contractions

- Transcription of inflammatory mediators in contracting myometrium occurs in a time dependent manner.

4.5.3 Overlap of *in-vitro* and *in-vivo* myometrial transcriptional profile in response to contractions

- There is an overlap of contraction associated myometrial genes between those derived in response to *in-vivo* contractions (IL vs NIL) and *in-vitro* contractions with a preponderance of genes associated with the inflammatory response.

4.5.4 Myometrial contractile and inflammatory response to *in-vitro* tocolysis

- Effective tocolysis of myometrial strips is comparable between ML7, nifedipine and ritodrine.

- Levcromakalim and water soluble progesterone only partially inhibit contractile activity of myometrial strips.

- Transcription of inflammatory mediators is suppressed in ML7 treated samples compared with spontaneously contracting samples, and is comparable with non-contracting myometrial samples kept in physiological conditions.

- Despite abolition of contractile activity, transcription of inflammatory mediators in myometrium is not suppressed by exposure to nifedipine or ritodrine compared with spontaneously contracting samples.

- Transcription of inflammatory mediators in myometrium is comparable between those which have spontaneously contracted and where contractions are chemically blocked or partially suppressed by nifedipine, ritodrine, levcromakalim or progesterone.

4.5.5 Myometrial contractile and inflammatory response to an *in-vitro* pro-inflammatory, infective environment

- Spontaneous and OT induced myometrial contractile activity *in-vitro* is not enhanced by a pro-inflammatory, infective environment nor is it suppressed by an endotoxin free environment.

- Contractions induce transcriptional up-regulation of inflammatory mediators in myometrial strips incubated in normal untreated Krebs solution and PMB (endotoxin free) Krebs solution.

- In myometrial samples undergoing spontaneous and OT induced contractions, LPS induces an up-regulation of inflammatory mediator transcription when compared with strips contracting in a PMB treated (endotoxin free) environment.

4.5.6 Synthesis and release of myometrial inflammatory proteins *in-vitro*

- There is a non-significant trend for inflammatory protein products to be present in Krebs solution exposed to myometrium in an organ bath environment compared with Krebs solution which has not been exposed to myometrium.

4.6 Discussion

The data presented in this chapter demonstrates that *in-vitro* myometrial contractile activity is associated with a transcriptional upregulation of inflammatory mediators in a time dependent manner. Additionally, there is overlap with the genes upregulated in response to human labour suggesting contractions *per se* are associated with the recognised inflammatory response of the myometrium during human labour.

We also found that chemical manipulation of contractility has an affect on transcriptional expression of inflammatory mediators, with a suggestion that method of tocolysis, regardless of efficacy of suppressing contractile activity, influences the inflammatory response. Additionally, although a pro-inflammatory/infective environment did promote a myometrial inflammatory response, this did not have the expected effect of enhancing contractility, suggesting myometrial derived inflammatory mediators may not directly drive myometrial contractility.

4.6.1 Myometrial inflammatory response and contractility

We have provided evidence which demonstrates inflammatory up-regulation within non-contracting ex-vivo myometrial samples in a physiological environment. This inflammatory response is significantly augmented when myometrial strips exhibit spontaneous contractile activity. This suggests that the inflammatory up-regulation seen in our *in-vitro* model of labour occurs as a response to tissue contractions rather than ex-vivo tissue degradation.

Further support for association between myometrial contractions and the inflammatory response is provided by our temporal analysis of the gene expression of inflammatory mediators. This clearly demonstrates a time dependent relationship whereby the longer the myometrial sample contracted in the organ bath, the greater the inflammatory transcriptional response.

We have demonstrated overlap between the transcription of inflammatory mediators by contracting myometrium *in-vitro* and *in-vivo*. The degree of overlap between the array data are perhaps not as striking as we thought it may be. However, this may simply reflect the fact the baseline measures (NIL and

0hr time point) were not truly comparable as strips at the 0hr time point had contracted in the organ bath and a degree of inflammatory response would have been already be initiated. Therefore, it follows that fold changes at the subsequent time points would not be as dramatic as between the nil and labour samples.

Despite this, when the QPCR data for the chemokines CCL2, CCL20 and CXCL8 are compared between the *in-vivo* samples (IL vs NIL) and the *in-vitro* samples (end spt contractions vs start) the trend for upregulation of these chemokines in both situations is reproduced, with the extent of fold change higher for the *in-vitro* samples. This may represent the difference in an increased amount of cellular damage within the *in-vitro* myometrium sample. Additionally it may suggest that labouring myometrium *in-vivo* does not require to wield such an extreme inflammatory response since there will be a leukocyte response with trafficking, whereas this obviously cannot occur in an organ bath. This merits further investigation and further confirmation of the overlap between the reaction of the myometrium during labour and in the organ bath environment with larger numbers of inflammatory mediators and sample sizes.

Overall, our data suggest that the inflammatory changes associated with human labour occur in response to uterine contractions. Additionally, the overlap reassuringly suggests that our *in-vitro* organ bath experimental set up is a representative model suitable for the study of myometrial responses in human labour.

4.6.2 Tocolysis and myometrial contractile and inflammatory response

Our hypothesis that the chemical tocolytic agents would lead to suppression of both contractile activity and the myometrial inflammatory response was not fully supported by our data. However, data do suggest that myometrial cells initiate an inflammatory reaction in response to chemically induced, non-contractile cellular stress.

Considering the functional contractile response of myometrium to tocolysis, we found variable responses which are in agreement with data from other *in-vitro*

contractility studies[118 285 346]. In our hands, ML7, nifedipine and ritodrine were able to fully inhibit contractility while levcromakalim and progesterone were not as reliable with only partial inhibition. This is also reflected in clinical practice where the efficacy of tocolytic agents can be variable, with only ritodrine and the oxytocin receptor antagonist Atosiban licensed for use in prevention of pre-term labour in the UK, and nifedipine widely used 'off licence' for this purpose[186 239]. Additionally, the inconsistency of progesterone to inhibit contractility in our *in-vitro* model has also been seen previously *in-vitro* and in the clinical situation[285 287-291].

In terms of the myometrial inflammatory response to tocolysis, results did not reflect our initial hypothesis, and despite complete abolition of contractions by nifedipine and ritodrine, transcription of inflammatory mediators was comparable to that of tissue which had spontaneously contracted. Transcription of inflammatory mediators in response to nifedipine and ritodrine is also higher than in the non contracting tissue under physiological conditions. Furthermore, myometrium exposed to nifedipine from the outset and therefore never contracted has a comparable inflammatory profile to tissue where nifedipine was added after contractions were established. This suggests an alternative stimulus whereby the myometrium requires to initiate an inflammatory response, and that this may be a reflection of the method of tocolysis.

The mechanisms whereby nifedipine and ritodrine exert their tocolytic effects are through manipulation of upstream cellular events; nifedipine blocks calcium entry into cells via L-type voltage gated calcium channels [186 187], and ritodrine acts via the cell surface β_2 adrenergic receptor via cAMP-dependent protein kinase A to inactivate myosin light chain kinase (MLCK), reduce intracellular calcium and increase permeability to potassium [186 187]. These mechanisms therefore have the potential not only to affect the contractile function of the cell, but also other essential cellular functions. This could be interpreted by the cell as 'damage', thereby the cell responds by mounting an inflammatory stress response.

This suggested proposal is also partially supported by the response of the myometrial strips to ML7 whereby complete abolition of contractions is achieved and the inflammatory transcriptional profile is suppressed to levels comparable

with myometrium which is physiologically active but non-contracting. The mechanism of tocolysis employed by ML7 is to specifically inhibit smooth muscle MLCK activity, which in terms of cellular function is a downstream event mainly concerned with cell contractility [272-275]. Blocking this downstream event would therefore not necessarily be interpreted by the cell as a threat or 'damage', with no need to initiate an inflammatory response. However, there is some evidence of alternative actions of ML7 which have the potential to also influence some upstream transcriptional and cellular events[339-341].

The transcription of inflammatory mediators in ML7 treated samples is comparable with non-contracting, physiologically active samples. Again this suggests that ML7 is a relatively appropriate way to block contraction without affecting other cellular functions. Additionally it has been previously demonstrated that MLCK phosphorylation of myosin is the common final pathway essential in the generation of myometrial contractile force[118]. It can therefore be concluded that ML7 as a method of inhibiting myometrial contractions *in-vitro* provides a suitable contrast whereby the effects of spontaneous and oxytocin induced contractions can be studied, with the added advantage of knowing the sample has the ability to contract unlike using non-contracting samples only.

Our results regarding the effect of progesterone on myometrial inflammatory response are not in agreement with previous suggestions where progesterone and progesterone related hormones have been regarded as an 'anti-inflammatory' [347-349]. This may be due to differences in the timing of hormone exposure; pre-treating prior to any contractions compared with exposure once spontaneous contractile activity is already established.

4.6.3 Infection and myometrial contractile and inflammatory response

Our Q-PCR data have confirmed that an LPS rich environment induces an exaggerated inflammatory response in contracting myometrium compared with a non-inflammatory PMB environment. However, interestingly, this inflammatory upregulation does not alter or enhance *in-vitro* myometrial spontaneous contractile activity or its response to OT.

Again, this is contrary to our initial hypothesis, and also the proposals of others regarding the mechanisms involved in the initiation and propagation of human labour, especially those associated with inflammation with or without the presence of infection[18 199 210 216 217 230-236 260-262]. This suggests that the mechanism which promotes labour in the presence of infection may not be directly mediated by myometrial produced inflammatory mediators. This may explain why studies examining the role of antibiotic therapy in the treatment or prevention of pre-term labour with intact membranes have been unsuccessful[261 266 267 350]. However, this is an *in-vitro* model, and during human labour, other factors, for example infiltration of leukocytes into the myometrium may account for premature activation of contractility.

However, our data would suggest that inflammation *per se* is not necessary for contractions to occur as is demonstrated in the PMB samples. Additionally, despite the absence of an infective stimulus in the PMB treated myometrium, an inflammatory response still occurs as a result of myometrial contractility. This suggests that *in-vivo*, COX-inhibitors have not been particularly effective at preventing pre-term birth in humans since suppression of inflammatory response does not necessarily suppress myometrial contractile activity.

4.6.4 Synthesis and release of myometrial inflammatory proteins *in-vitro*

Our data concerning levels of inflammatory proteins in the organ bath which has been exposed to contracting myometrium in-vitro shows a tendency towards a release of these proteins into the surrounding environment. However, we must interpret these data with caution as this experiment was only designed to assess if these proteins could be measured in the Krebs solution. However, we did not have the appropriate control group of Krebs which has been exposed to non-contracting myometrial tissue, and we are therefore unable to conclude that myometrial contractions are responsible for the release of inflammatory proteins.

Conclusion

We have presented novel data examining the *in-vitro* time and contraction dependent myometrial inflammatory changes and the overlap with changes occurring within contracting myometrium *in-vivo*.

We have demonstrated that myometrial transcription of inflammatory cytokine and chemokines at term occurs in a co-ordinated time and contraction dependent manner. Thereby, this suggests that the inflammatory response typical of human labour occurs as an acute reaction to myometrial contractile damage. Additionally, our data suggest that the degree of inflammatory response mounted by the myometrium does not influence the functional contractility of the tissue. Rather, the level of inflammation may be more appropriately interpreted as an indication of myometrial cellular stress, which is non-specific and can be induced not only by contractions but also exposure to chemical manipulation of cell function or a pro-inflammatory infective environment.

Overall, the evidence tends to support much of the hypotheses proposed at the start of this chapter, with modifications summarised below in Figure 62.

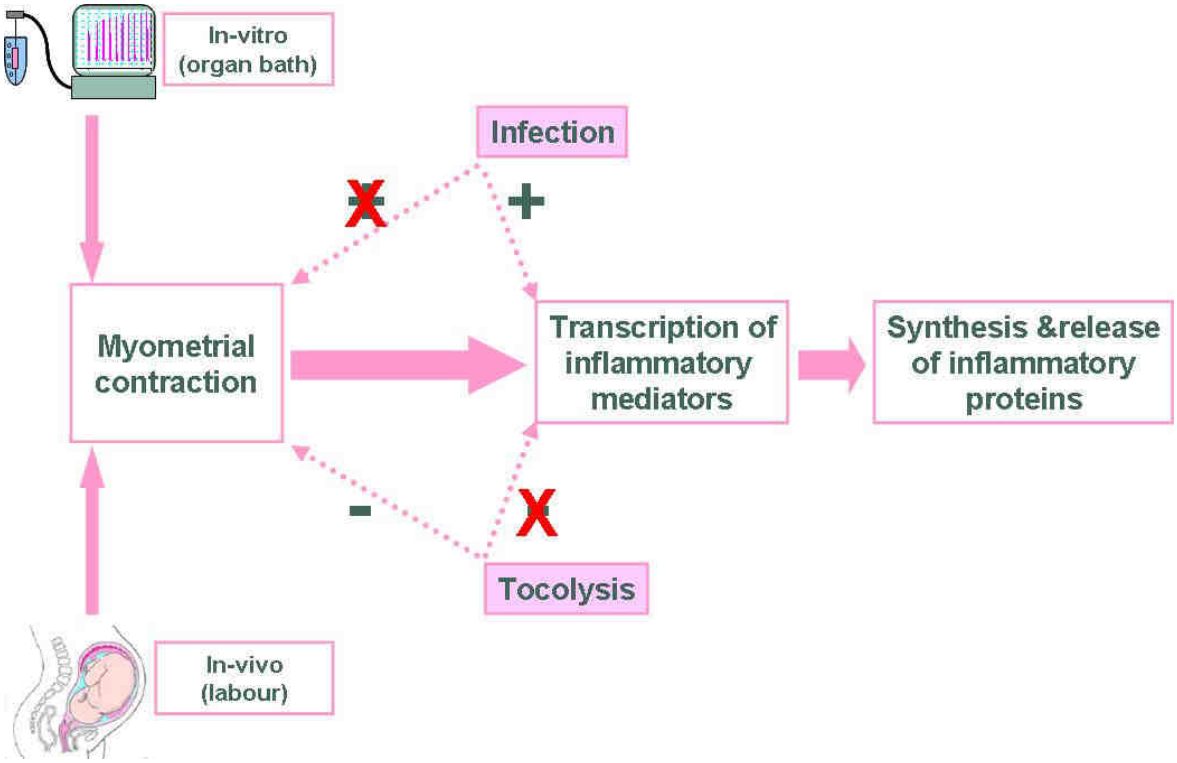

Figure 62 Chapter 4 Amended Hypothesis

Summary of the hypotheses examined regarding myometrial contractions and myometrial derived inflammatory mediators, the overlap between *in-vitro* contraction and those seen in human labour and the influence of tocolysis or an infective/inflammatory environment. Red crosses indicate areas which were not supported by our experiments.

Chapter 5

Circulating white blood cells, inflammatory cytokines and markers of myocyte damage in response to human labour

5 Circulating white blood cells, inflammatory cytokines and markers of myocyte damage in response to human labour

5.1 Introduction

Trafficking of leukocytes involved in the inflammatory process to areas of tissue damage is a basic physiological response forming part of the acute inflammatory reaction which should be self limiting and protective for the host[264 265 351-353]. The acute inflammatory process can occur for a number of reasons including defence against infection, defence and clearing of non-infective tissue damage and stimulation of tissue repair and healing. Inflammatory changes are associated with the process of human labour both at term and pre-term, with a striking increase in leukocyte numbers, predominantly neutrophils and macrophages, throughout the uterus into the myometrium and cervix[219 220 354]. Of note, the acute inflammatory reaction of human labour occurs in a non-infective environment in normal pregnancy, involves inflammatory cells derived from the maternal circulation[354], is self limiting and results in uterine involution and return to normal function.

Characterisation of changes to circulating leukocyte numbers during pregnancy have been described, with a gestation dependent increase in overall white cell count driven by a neutrophilia[355-357]. Within the context of pregnancy, alterations in the levels of other white cell sub-sets are conflicting with some suggesting a reduction in lymphocytes and an increase in monocytes at term compared with non-pregnant controls, while others have observed no changes[355-361]. It is also well recognised that labour induces further changes in maternal circulating white cells, with increases in total white cell count and neutrophils, a decrease in lymphocytes, eosinophils and monocytes all of which occur in the absence of infective stimuli[359 362-364]. These changes are consistent with those seen in acute inflammatory reactions in response to other stimuli including infection and surgical trauma[264 365-371].

Additionally, it has been proposed that maternal leukocytes may be activated or ‘primed’ in the peripheral blood prior to labour (pre-term and term) and

therefore have increased responsiveness to circulating agonists and an ability to migrate to gestational tissues prior to the commencement of labour[360 372]. However, others have suggested no change to cellular immune function during pregnancy, except during delivery itself where a suppression of mitogenic function in circulating lymphocytes has been observed[356].

In conjunction with the changes in white cells during pregnancy, there are also alterations to circulating inflammatory mediators and other markers of inflammation. Certainly, C-reactive protein (CRP) levels increase from an early gestation[373 374]. Concentrations of circulating cytokines can change throughout pregnancy, with intrapartum increases in IL6 and IL10, however results have been inconsistent and many without comparison with non-pregnant individuals[375-378].

The actual timing and role of the changes in circulating leukocytes, cytokines and the leukocyte infiltration into the uterus are yet to be determined. Current proposals suggest an overall inflammatory environment in gestational tissue with an increase in numbers and reactivity of cells within the maternal circulation are required for the initiation and propagation of cervical ripening, myometrial contractility and therefore labour[210 211 216 217 219 379-382]. However, evidence from other contractile muscle systems, in particular exercising skeletal muscle, observe similar inflammatory changes in the circulation and the contracting tissue and suggests that inflammatory changes occur as a reaction to myocyte damage and play a role in clearing cellular debris with subsequent tissue repair and remodelling[383-389]. Of note, the changes seen in both circumstances, especially the predominance of infiltrating neutrophils and monocytes is consistent with the classical changes of acute inflammation[264].

This reactionary model of acute inflammation as a response to myocyte damage may therefore also apply to the uterus in human labour. There is inevitable damage to the myometrial tissue during labour, confirmed by myocyte cell structure disruption,[354] and increases in circulating myocyte derived proteins including creatine kinase and myoglobin,[390-392] in the absence of increased cardiac specific troponin I[390]. Additionally, the gene array and transcription data shown in the preceding chapters has suggested that up-regulation of

inflammatory mediators in human myometrium is a contraction and time dependent event.

Despite evidence of both myocyte damage and inflammatory changes, the acute time and labour dependent changes to circulatory inflammatory mediators and markers of muscle damage have not previously been described in a longitudinal manner. Additionally, comparison with changes seen in response to delivery after a non-labouring caesarean section and with non-pregnant controls will provide a better understanding of inflammation during human labour. Description of these time and contraction induced inflammatory changes will aid our understanding of the physiology of myometrial contractility, and in addition could advocate the use of human labour as a model of an inflammatory process which is self-limiting with subsequent repair, remodelling and regeneration after the initial insult.

5.2 Hypotheses

Based on our *in-vitro* transcriptional data and evidence from exercising skeletal muscle, the working paradigm regarding the role of inflammation can be summarised in Figure 63.

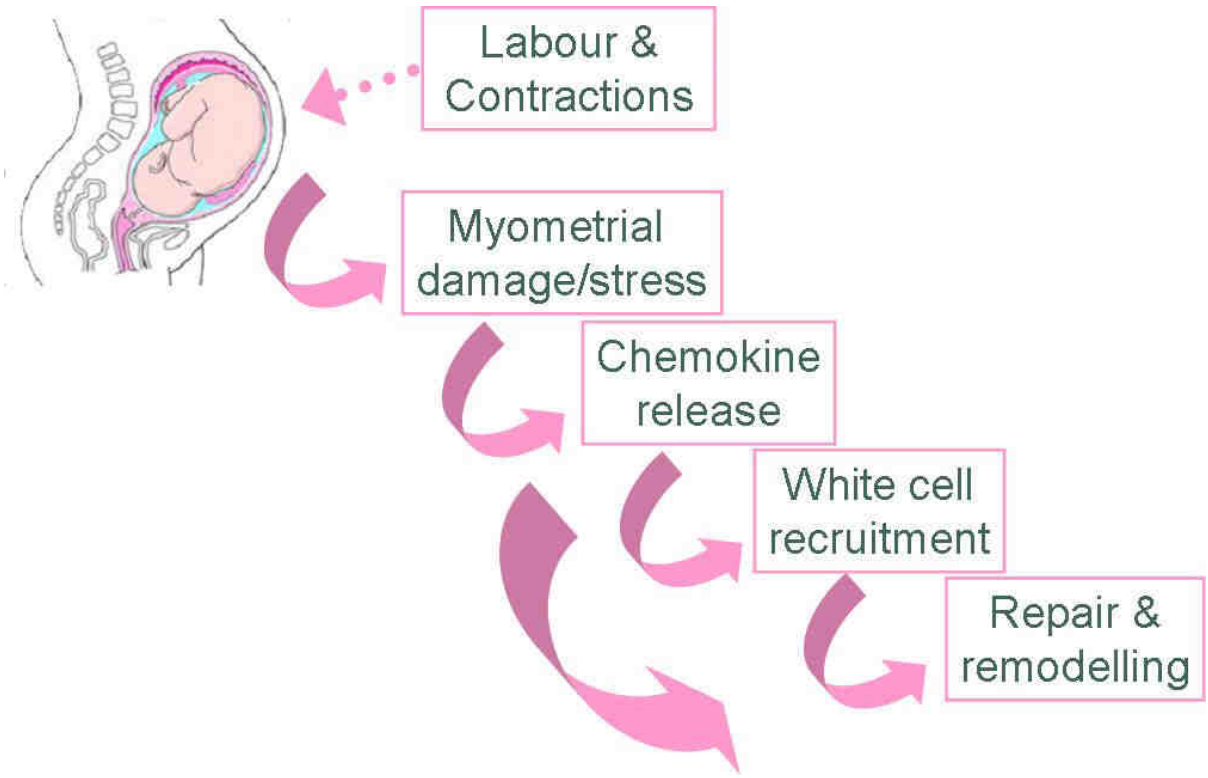

Figure 63 Chapter 5 Overall Hypothesis
Working paradigm concerning the role of inflammation in term human labour.

Overall, we propose that myometrium responds to contractile damage of labour by releasing inflammatory signals, an event which occurs after the initiation of contractions. In turn, we propose that these inflammatory mediators may play a pivotal role in the chemo-attraction and subsequent influx of leukocytes into the myometrium.

The four main hypotheses which will be tested are

1. Pregnancy at term induces changes to circulating inflammatory parameters when compared with non-pregnant controls.

2. Term delivery by either planned, non-labour caesarean section (CS) or induction of labour (IOL) with syntocinon will result in a shift towards a

pro-inflammatory profile in the maternal circulation, with an enhanced response in mothers who labour.

3. In term pregnancies, changes observed as a result of IOL occur in a time and contraction dependent manner in response to labour.

4. Promotion of a pro-inflammatory phenotype in the maternal circulation in response to labour is associated with myometrial myocyte damage.

The inflammatory parameters will be assessed for a time series during term induction of labour (IOL) with syntocinon. IOL was used as a model of labour with a clear pre-labour baseline. Additionally, we have seen in our array data that stimulation of contraction with exogenous oxytocin does not influence myometrial gene transcription. Measures will be compared with data from two control groups, firstly non-pregnant females during the early follicular phase of the menstrual cycle, and secondly those undergoing delivery by planned non-labouring caesarean section (CS) at term. This second comparison group was included as post-partum remodelling of the myometrium would still be required, but likely to be less since contractile activity and therefore myometrial damage should be lower.

The parameters measured within the maternal peripheral circulation will consider both systemic inflammation and indirect measures of myocyte damage and include the following;

1) circulating leukocyte differential counts

2) expression of leukocyte cell surface markers and chemokine receptors

3) circulating cytokine and chemokine levels

4) circulating c-reactive protein (CRP) levels

5) circulating creatine kinase (CK) and myoglobin (Mb).

A brief overview of each of the parameters analysed and the underlying rationale is provided below.

5.2.1 Differential leukocyte count

White blood cells (WBC) or leukocytes are the immunological cells which play a pivotal role in the body's defensive mechanisms. The numbers and types of circulating white cells can change with recognised patterns being considered a typical response to a defined stimulus. For example, an acute inflammatory response to a non-specific stimulus or injury is characterised by an initial sharp increase in circulating neutrophils driven by release of 'new' neutrophils from the bone marrow, with an accompanying later decrease in circulating monocytes and eosinophils[264 365].

The leukocyte parameters considered in clinical practice and within the context of these experiments are defined in the Table 25.

Leukocyte parameter	Cell description
Total white cell count	A summative measure of the white blood cell population in the circulation
Neutrophil	A type of granulocyte containing a multilobed nucleus and neutrophilic granules with a major role in phagocytosis involved in the initial acute inflammatory/innate immune response. Neutrophils represent the largest population of leukocytes in the circulation.
Lymphocyte	A small dense leukocyte with 3 major types 1) B cells (10-15% of lymphocytes) which when activated become plasma cells and secrete antibodies 2) T cells (CD3+ lymphocytes, 75% of lymphocytes) further subdivided into - CD4+ T-cell (T-helper/Th cells) activate other cells e.g. B-cells and macrophages. 4 further sub-classes of T-helper cells are recognised Th1 (pro-inflammatory), Th2 (anti-inflammatory), Th17 and Treg (suppressor). Memory Th cell can develop after activation. - CD8+ T-cell (T-cytotoxic/Tc cells) role in direct destruction of damaged or infected cells. Memory Tc cells can develop after activation. 3) Natural killer cells (NK cells) have the ability to kill 'non-self' cells and are considered part of the innate immune response
Monocyte	A circulating leukocyte which matures and differentiates into a macrophage within tissue where its primary role is phagocytosis
Eosinophil	A type of granulocyte involved in defence against parasite infection, and allergy, also role in acute inflammatory/innate immune response[393]
Basophil	A type of granulocyte representing <1% of total white cell count with possible role in Th2 response (anti-inflammatory) and B cell activation[394]

Table 25 White blood cell (leukocyte) parameters

Summary table of white blood cell (leukocyte) parameters measured within the context of this experiment.

5.2.2 Leukocyte cell surface markers and chemokine receptors

The specific trafficking of leukocytes to areas where they are required can be driven by local release of chemokines, which via the appropriate chemokine receptor on the cell surface will mediate the influx of the particular subset of cells required. Chemokine receptors exist as mainly transmembrane, G-protein coupled receptors, with activation stimulating downstream cellular functional responses which include adhesion molecule expression, chemokine/cytokine release and of course chemotaxis and migration of the leukocyte to the area of

chemokine release[395 396]. The leukocytes which typically increase in number within uterus (myometrium and cervix) in response to labour are predominantly neutrophils and macrophages, with T-lymphocytes increasing in the myometrium of the lower segment only[219 354]. These cell types were therefore studied in the maternal circulation.

As described in Chapter 2, Section 2.13, flow cytometry allows cell types and properties to be analysed. Dependent on the characteristics assessed, cells were defined as neutrophils (CD66b+ granulocytes), activated neutrophils (CD66b+/CD11b+ granulocytes), monocytes (CD14+ monocytes), activated monocytes (CD14+/CD11b+ monocytes), T-helper lymphocytes (CD3+/CD4+ lymphocytes) or T-cytotoxic lymphocytes (CD3+/ CD8+ lymphocytes). A study was then made of the chemokine receptors expressed by each white cell subset, with the particular chemokine receptors examined being CXCR1, CXCR2, CCR2 and CCR6.

CD11b (Mac-1, $\alpha M\beta_2$) is a cell surface molecule which is constitutively expressed in low levels on the surface of neutrophils and monocytes, and is also found in a preformed state within cytoplasmic granules. Activation of these leukocytes by CXCL8 via CXCR1 and CXCR2 causes rapid mobilisation (minutes) of cytoplasmic CD11b with an enhanced cell surface expression[397 398], and is a typical innate immunity response to sepsis. CD11b plays a crucial role in leukocyte chemotaxis, adhesion and sequestration from circulation to the area of tissue damage by increasing the cells adhesive capacity to the endothelium via facilitating binding to the cell adhesion molecule ICAM-1. A high density of cell surface expression of CD11b is therefore consistent with neutrophils and monocytes being in an 'activated state'.

CXCR1 and CXCR2 are the main chemokine receptors present on the cell surface of granulocytes, with evidence suggesting expression on T-lymphocytes, but to a much lesser extent[399]. The main ligand for both receptors is CXCL8 which is bound with a high affinity[400 401]. In addition, CXCR2 is a more promiscuous receptor and additional ligands include CXCL1, 2, 3 and CXCL5[402]. Activation of the receptors plays a significant role in activation of the neutrophils and promotes chemoattraction and migration to the site of chemokine release.

CCR2 (CD192) is primarily expressed by monocytes and subsequently macrophages once migration into the tissue has occurred[402]. CCR2 is the predominant receptor for the ligand CCL2 which is involved in the chemotaxsis of monocytes to areas of inflammation[402 403]. CCR2 can also interact with the CC-ligands CCL7[404], CCL8[405], CCL13[402 406].

CCR6 is expressed by lymphocytes (CD4+ve T-helper cells, CD8+ve T-cytotoxic cells, and B-cells) but not by granulocyte, NK-cells or monocytes. The corresponding chemotactic ligand for CCR6 is CCL20[402 407].

5.2.3 Circulating cytokines and chemokines

Cytokines are small signalling molecules which are released by cells (mostly activated leukocytes, fibroblasts and endothelial cells) and have a key role in the mediation of inflammation and immunity in response to tissue damage. They can act in an autocrine, paracrine or endocrine manner. Additionally, chemokines (chemotactic cytokines) are a type of cytokine which act via leukocyte chemokine cell surface receptors and stimulate chemotaxis and trafficking of white blood cells in both health and disease.

The cytokines and chemokines assessed in the circulation of women in this experiment and a description of their main functional roles is outlined below in Table 26.

Cytokine	Function
EGF (Epidermal Growth Factor)	Stimulates cell proliferation , differentiation and survival
CCL11 (Eotaxin)	Chemotactic for eosinophils via CCR3
FGF-2 (Basic Fibroblast Growth Factor)	Growth factor involved in angiogenesis during wound healing and tumour development
Flt-3 (CD135, fms-like tyrosine kinase receptor-3)	Growth factor that increases number of immune cells by activating haematopoietic progenitors
CX3CL1 (Fractalkine)	Chemotactic for monocytes and Tcells via CX3CR1
GCSF (granulocyte colony stimulating factor)	Growth factor which stimulates bone marrow

	production, maturation and release of granulocytes
GMCSF (granulocyte macrophage colony stimulating factor)	Growth factor stimulating bone marrow production and release of granulocytes and monocytes
CXCL1, 2, and 3 (GRO (growth regulated oncogene))	Primarily involved in the chemotaxsis of neutrophils via CXCR2. The assay used measures GRO as a whole and does not separate into isoforms.
Interferon- α2 (IFNα2)	Involved in response to viral infection
Interferon-γ (IFNγ)	Main cytokine involved in the Th1 pro-inflammatory response
Interleukin-1α (IL1α)	Produced by macrophages and epithelial cells, promotes leukocyte adhesion, T-cell and macrophage activation, stimulates release of acute phase proteins from liver, considered an endogenous pyrogen
Interleukin-1β (IL1β)	See IL1α
IL1ra	IL1 receptor antagonist
IL2 (T-cell growth factor)	Produced by T cells, promotes T cell proliferation, activates B cells and NK cell
IL3 (multicolony CSF)	Produced by T cells, plays a role in haematopoiesis
IL4	Produced by Tcells and mast cells, IgE synthesis and Th2 response
IL5	Eosinophil growth and differentiation, B cell activation
IL6	Role in final differentiation of B cell and generation of Th17 cells. Induces acute phase response proteins (including CRP) from liver, endogenous pyrogen, mainly released by monocytes/macrophages and also contracting muscle (a 'myokine'). Involved in resolution of inflammation via shift from innate to adaptive response.
IL7	Released by bone marrow, stimulates growth and differentiation of T cells and B cells
CXCL8 (IL8)	Chemotaxis of neutrophils, and weak attraction

	of basophils and T cells via CXCR1 and CXCR2
IL9	Enhances mast cell activity
IL10	Produced by T cells and macrophages, 'anti-inflammatory cytokine', potent suppressor of macrophage function, can block NF-κβ, inhibits synthesis of pro-inflammatory cytokines including IFNγ, IL2, IL3, TNFα, GMCSF
IL12 (p40) (IL12β subunit)	Produced by macrophages, involved in the stimulation of T-cells and NK cells and production of IFNγ and TNFα
IL12 (p70) (IL12)	Produced by macrophages, involved in the stimulation of T-cells and NK cells and production of IFNγ and TNFα
IL13	Produced by Th2, involved in allergen response
IL15	Stimulates growth of T cells and NK cells
IL17	Produced by CD4+ T cells, stimulates IL6, IL8, GCSF and PGE2 release from epithelial, endothelial and fibroblastic cells
CXCL10 (Interferon-γ Inducible Protein (IP10))	Chemoattractant for monocytes and T cells via CXCR3. produced in response to IFNγ, antiangiogenic.
CCL2 (Monocyte Chemotactic protein-1 (MCP1))	Chemoattractant for monocytes and basophils via CCR2
CCL7 (Monocyte Chemotactic protein-3 (MCP3))	Chemoattractant for macrophages via CCR1, CCR2, CCR3
CCL22 (Macrophage derived chemokine (MDC))	Chemoattractant for NK cells, activated Tcells, monocytes and dendritic cells via CCR4
CCL3 (Macrophage inflammatory protein 1α (MIP1α))	Chemoattractant for lymphocytes via CCR1 and CCR5, inhibits proliferation of haematopoietic stem cells
CCL4 (Macrophage inflammatory protein 1β (MIP1β))	Chemoattractant for lymphocytes, especially T-helper cells via CCR1 and CCR5
sCD40L (soluble CD40 ligand)	Induces B cell activation via Th cells
sIL2Rα (sCD25)	Produced by activated macrophage, involved in regulation of IL2 activity
TGFα (transforming growth factor α)	Produced by macrophages, involved in epithelial development, related to EGF

TNFα (tumour necrosis factor α)	Produced by macrophages and T cells endogenous pyrogen, activates neutrophils, endothelial cells and acute phase reactants, role in wound healing
TNFß (tumour necrosis factor ß)	Produced by activated T lymphocytes similar activity as TNFα but less potent
VEGF (vascular endothelial growth factor)	Growth factor which promotes angiogenesis, also chemoattractant for monocytes
CCL5 (RANTES)	Chemoattractant for monocytes, Th cells and eosinophils via CCR3, CCR5 and CCR1. Involved in histamine release from basophils and activation of eosinophils.

Table 26 Circulating cytokine/chemokine parameters

Summary of the circulating cytokines/chemokines measured within the context of this experiment and a brief summary of their functions.

5.2.4 C-reactive protein (CRP)

C-reactive protein (CRP) is an acute phase response protein which is manufactured by hepatocytes as part of the systemic response to acute inflammation, infection or tissue damage. Its production is predominantly under the control of circulating IL6, and its appearance in the circulation occurs approximately 6 hours following stimulation, peaking at approximately 48hours. The half life of CRP in the circulation is 19hours, and importantly remains constant regardless of the health of the individual. It is therefore widely used in clinical practice as a marker of systemic response to treatment as return to baseline measures is taken to be indicative of resoloution[408].

In response to pathogen induced inflammation, CRP mimics the actions of antibodies and can opsonise the pathogen and activate the complement cascade. CRP does not display specificity and therefore recognises a broad range of pathogens. However, the production of CRP is not specific to bacterial infections and levels rise in response to any trigger which increases circulating IL6.

5.2.5 Creatine kinase (CK)

Creatine kinase (CK) is an enzyme responsible for the catalysing the reaction of creatine to creatine phosphate (a high energy compound found in high concentrations within muscle cells and provides the energy required during myocyte contractions). It is found as 2 iso-enzymes CK-MM or CK-BB, and the majority of the total CK in the body is present within muscle cells. During muscle damage or degradation, CK is released into the circulation and is therefore used as a biomarker of muscle damage. Circulating CK was traditionally used in the diagnosis of myocardial infarction, however, CK is non-specific and rises are also seen following surgical procedures, including abdominal surgery, reaching a peak on the second post-operative day[409]. In pregnancy the concentration of CK within uterine cells has been shown to increase with gestation[410] and thus can be considered a suitable marker of uterine myocyte damage. Its half life in the circulation is approximately 15.5hrs.

5.2.6 Myoglobin (Mb)

Myoglobin (Mb) is the primary oxygen binding protein found in myocytes. It contains iron and therefore gives muscle its red colour; haemoglobin is the equivalent molecule found in red blood cells. The protein is a single chain apoprotein of 153 or 154 amino acids surrounding a central haem molecule. Previously believed to be absent from smooth muscle, more recent data have demonstrated its presence at both gene and protein levels within the myometrium[411] and it is certainly found to be present at a transcription level within our gene array data. Mb is an intracellular protein and enters the circulation at times of muscle damage, and in a similar manner to CK rises are associated with myocardial infarction and surgery involving muscle disruption[409] [412] In addition, release of myoglobin from the myocyte appears to occur up to 24 hours prior to the peak in circulating CK[409], its half life in the circulation is approximately 10 minutes and is cleared by the renal system.

5.3 Materials and methods

An illustration of the overall study design for this chapter is shown in Figure 64. Patients were recruited into three study groups (non-pregnant (NP), pregnant undergoing a planned, non-labour caesarean section (CS) and pregnant undergoing induction of labour (IOL)) as described in the methods chapter. Blood samples were taken from women as previously described at the time-points ascribed to their particular study group, with approximately 3mls into K_2EDTA tube (purple top) and 4-5mls in a lithium heparin tube (green top).

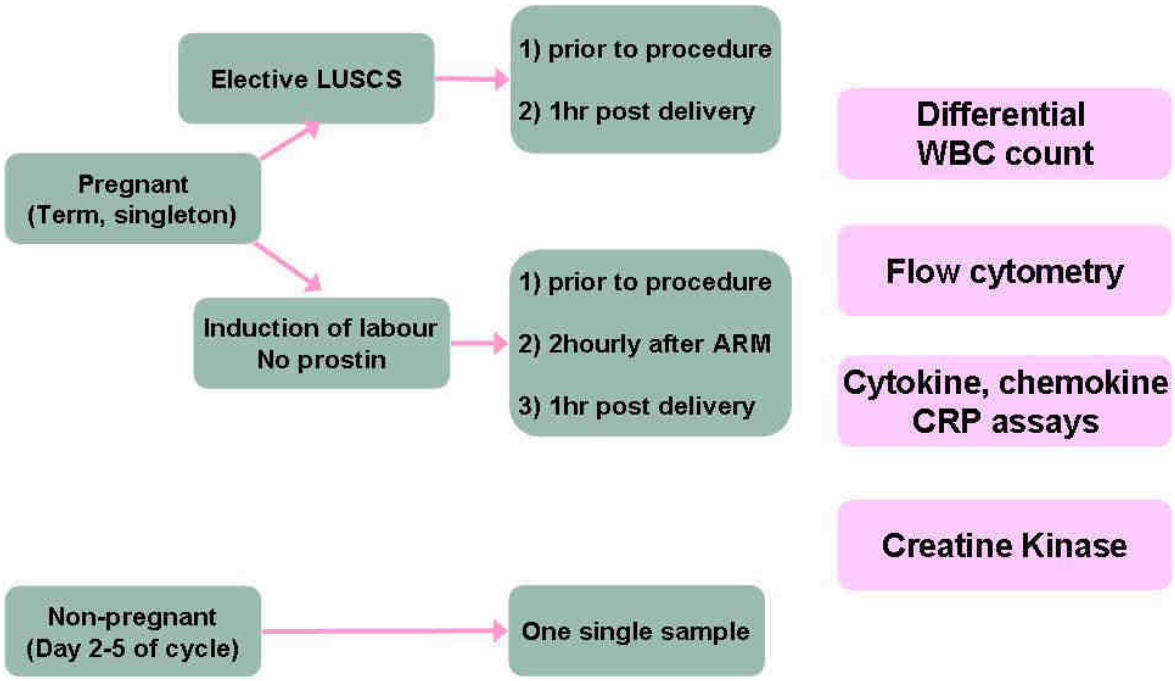

Figure 64 Chapter 5 Experimental Design

Illustration of overall study design to examine circulating inflammatory parameters associated with pregnancy and labour showing 3 study groups, timing of samples and the 4 main parameters measured.

Blood samples were then processed as described in the Methods section with measures being made for differential white cell counts, flow cytometry for leukocyte cell surface markers and chemokine receptors CD11b, CCR2, CCR6, CXCR1 and CXCR2. Plasma from the blood samples was stored at -80◦C and subsequently used to measure circulating cytokines, chemokines, C-reactive protein (CRP), creatine kinase (CK) and myoglobin (Mb) as described in the methods section.

5.3.1 Data analysis

Demographic data are presented as medians, IQR, range with non-parametric Kruskal-Wallis, Mann-Whitney and Chi-squared test as appropriate. Demographic data were analysed using Minitab® 15.1.30.0. For biochemical data, a one way ANOVA was used to compare baseline data between the three groups, with unpaired and paired t-tests to compare between 2 groups as appropriate. Where comparison between non-pregnant (NP) and pregnant at term (PT) groups are made the PT group is the pooled pre-CS and pre-IOL data (n=16). To improve analysis in relation to the assumption of normal distribution, data for white cell differential counts, cytokines, CRP, CK and Mb were analysed on a log-transformed scale. Where results for cytokine quantification were outwith the pre-determined sensitivity of the multiplex kit, a minimum of 50% of expected readings was considered the minimum required for analysis (i.e. 4 readings in each comparison group, 8 readings for pooled pregnant at term data or 21 readings where correlation analysis was performed). No adjustments are made for multiple testing for these data as with our relatively small dataset adjustment may reduce the chance of making a type I error, but may lead to and increase in the chance of making a type II error and a need to increase our sample size[331-334]. A $p<0.05$ was considered significant.

Where temporal changes as a result of labour were examined, data were analysed by calculating the % time of total duration of labour (time of ARM=0%, and 1 hour post delivery of placenta = 100%) at which the sample was obtained and performing a time correlation analysis using a Pearson correlation. Statistical analysis of biochemical data were performed using GraphPad Prism® V5.00.

5.4 Results

5.4.1 Patient Demographics

The characteristics of each patient group are outlined in Table 27. No significant differences between all three groups for age or BMI were detected. Parity differed between the groups, with only one woman in the non-pregnant group and 3 women in the IOL group having had a previous pregnancy compared with those in the CS group where all women had at least one previous full term pregnancy ($p=0.002$). Previous mode of delivery also differed between groups with only women in the CS group having had a previous caesarean delivery ($p<0.001$), however whether or not women had a previous SVD was not significantly different between groups ($p=0.446$). No significant differences were found between the 2 pregnant groups in terms of maternal BMI, gestation at time of starting the study or baby's birthweight. As expected, the estimated blood loss during caesarean section was higher compared with that during induction of labour ($p=0.018$).

Indications for caesarean delivery in the index pregnancy in the CS group were, breech presentation (n=1), previous traumatic vaginal delivery (n=1) and previous caesarean delivery (previous indications were 5 intrapartum caesareans for presumed foetal distress and 1 planned delivery for malposition). Indications for IOL were post dates (n=4), reduced maternal reporting of foetal movement (n=3) and elevated blood pressure in the absence of other symptoms of pre-eclampsia (n=1).

In the NP group 4 women were using a combined oral contraceptive preparation with 2 women on their 4th pill free day and 2 women on their 6^{th} pill free day. One woman was using the Mirena intrauterine system and blood was taken on day 5 of her cycle. The remaining 3 women were not using any hormonal contraceptive preparations with 2 women on day 2 of their cycle and 1 on day 3.

		Study Group			3 group comparison	2 group comparison
		NP	CS	IOL	p-value	p-value
	N women	8	8	8	/	/
Age (years)	Median	29.0	30.5	27.0		
	(IQR)	(27.0,30.8)	(25.5,36.0)	(21.0,31.5)	P=0.295	/
	[range]	[23.0,32.0]	[22.0,38.0]	[21.0,37.0]		
Parity	N (%) =0	7 (87.5%)	0.(0.0%)	5 (62.5%)		
	N (%) =1	1 (12.5%)	7 (87.5%)	1 (12.5%)	0.002	/
	N (%) ≥2	0 (0.0%)	1 (12.5%)	2 (25.0%)		
Previous SVD†	N (%) =0	7 (87.5%)	5 (62.5%)	5 (62.5%)		
	N (%) =1	1 (12.5%)	3 (37.5%)	1 (12.5%)	0.446	/
	N (%) ≥2	0 (0.0%)	0(0.0%)	2 (25.0%)		
Previous LUSCS	N (%) =0	8 (100%)	2 (25.0%)	8 (100%)		
	N (%) =1	0 (0%)	6 (75.0%)	0 (0%)	<0.001	/
	N (%) ≥2	0(0%)	0 (0.0%)	0(0%)		
Gestation at first sample (days)	Median		274.5	286.5		
	(IQR)	n/a	(273.3,275.0)	(271.5,292.8)	n/a	0.345
	[range]		[272.0,281.0]	[269.0,293.0]		
Baby wt (kg)	Median		3.49	3.60		
	(IQR)	n/a	(3.31,3.74)	(3.34,3.84)	n/a	P=0.462
	[range]		[3.26,3.94]	[3.03,4.00]		
Maternal BMI	Median	23.4	24.5	24.5		
	(IQR)	(21.5,24.9)	(23.2,28.5)	(23.6,32.8)	P=0.411	/
	[range]	[20.1,26.0]	[20.0,32.0]	[18.5,36.0]		
EBL (mls)	Median		400	300		
	(IQR)	n/a	(400,500)	(263,375)	/	P=0.018
	[range]		[300,700]	[150,400]		
Labour length(mins)	Median			433		
	(IQR)	n/a	n/a	(308,533)	/	/
	[range]			[255,775]		

Table 27 Patient demographic details

Patient characteristics for each study group, non-pregnant (NP), those attending for non-labouring planned caesarean delivery (CS) and those attending at term for induction of labour by artificial rupture of membrane and syntocinon (IOL). †Chi-squared test performed on pooled SVD data ie previous SVD 'yes' or previous SVD 'no'.

5.4.2 Hypothesis 1 – Pregnancy at term induces a difference in circulating inflammatory parameters.

5.4.2.1White Blood Cells

Baseline measure of differential white blood cell counts for the three study groups (NP, pre-CS and pre-IOL) are illustrated in Figure 65 with direct comparisons between non-pregnant (NP) and pregnant at term (PT (pre-CS + pre-IOL)) groups annotated below each graph.

There were significant differences between the NP and PT groups for baseline measures of overall WBC (NP vs PT, $p<0.001$) with differences observed between all 3 comparison groups (NP vs CS-pre vs IOL-pre, $p<0.001$, NP vs CS-pre, $p=0.027$, NP vs IOL-pre, $p=0.001$, CS-pre vs IOL pre, $p=0.011$). This increase in overall white cell count appears to be driven by a neutrophilia (NP vs PT, $p<0.001$) and again varies between the three groups (NP vs CS-pre vs IOL-pre, $p<0.001$, NP vs CS-pre, $p=0.001$, NP vs IOL-pre, $p=0.01$, CS-pre vs IOL-pre, $p<0.001$). There was an overall lower lymphocyte count in pregnancy (NP vs PT, $p=0.09$), however this was only significant between NP and pre-CS groups (NP vs CS-pre ($p=0.008$)). Eosinophils also differed in response to pregnancy (NP vs PT, $p=0.008$), which with further testing was significant between the non-pregnant group and women prior to induction of labour (NP vs IOL-pre ($p=0.005$)). Basophils counts were lower in pregnancy (NP vs PT, $p=0.034$), however analysis between all 3 groups did not show any significant differences (NP vs CS-pre vs IOL-pre, $p=0.109$, NP vs CS-pre, $p=0.065$, NP vs IOL-pre, $p=0.125$, CS-pre vs IOL-pre, $p=0.766$). Pregnancy did not appear to induce any significant changes to the circulating number of monocytes (NP vs PT, $p=0.162$).

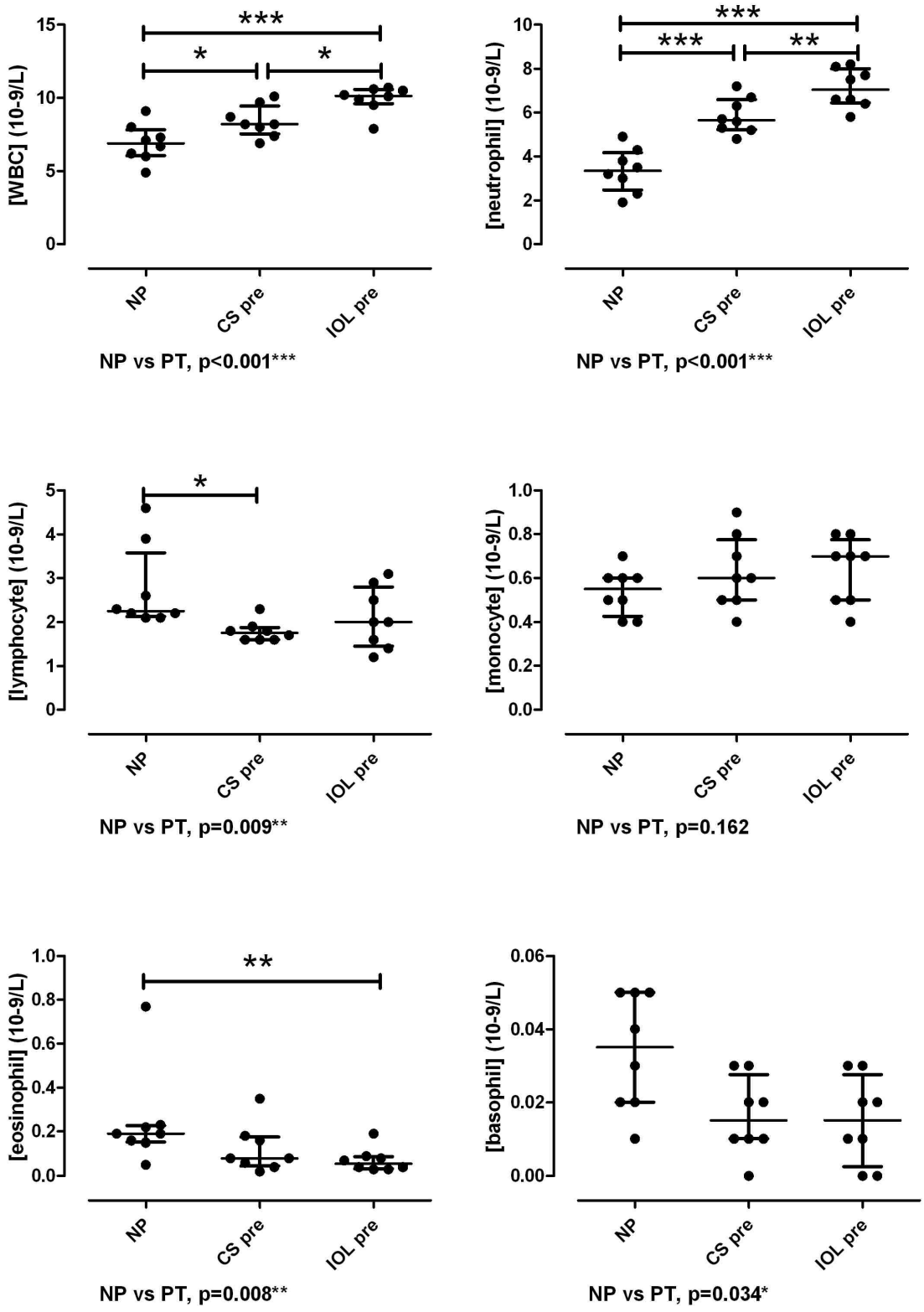

Figure 65 Baseline measure of WBC subtypes

Baseline concentrations of circulating white blood cells for each study group with p values for the comparison between non-pregnant and pooled pregnant at term groups (NP vs PT). Raw data are shown as scatter plots with median and IQR. Data were log transformed prior to analysis. Unpaired t-tests were performed with lines demonstrating significance value between 2 samples under start and end of each line *p<0.05, **p≤0.01, ***p≤0.001

5.4.2.2Leukocyte cell surface markers and chemokine receptors

5.4.2.2.1 Granulocytes

An example of the flow cytometry profile used to assess the granulocyte parameters is shown in Figure 66. Data were initially gated using forward and side scatter parameters with granulocytes defined as large cells with high granularity (Region 3 (R3) on diagram). These selected cells were then gated based on there positive expression of CD66b and CD11b and defined as activated granulocytes (R5 on diagram). Cells defined by R5 were then further gated based on their cell surface expression of the chemokine receptor CXCR1 or CXCR2.

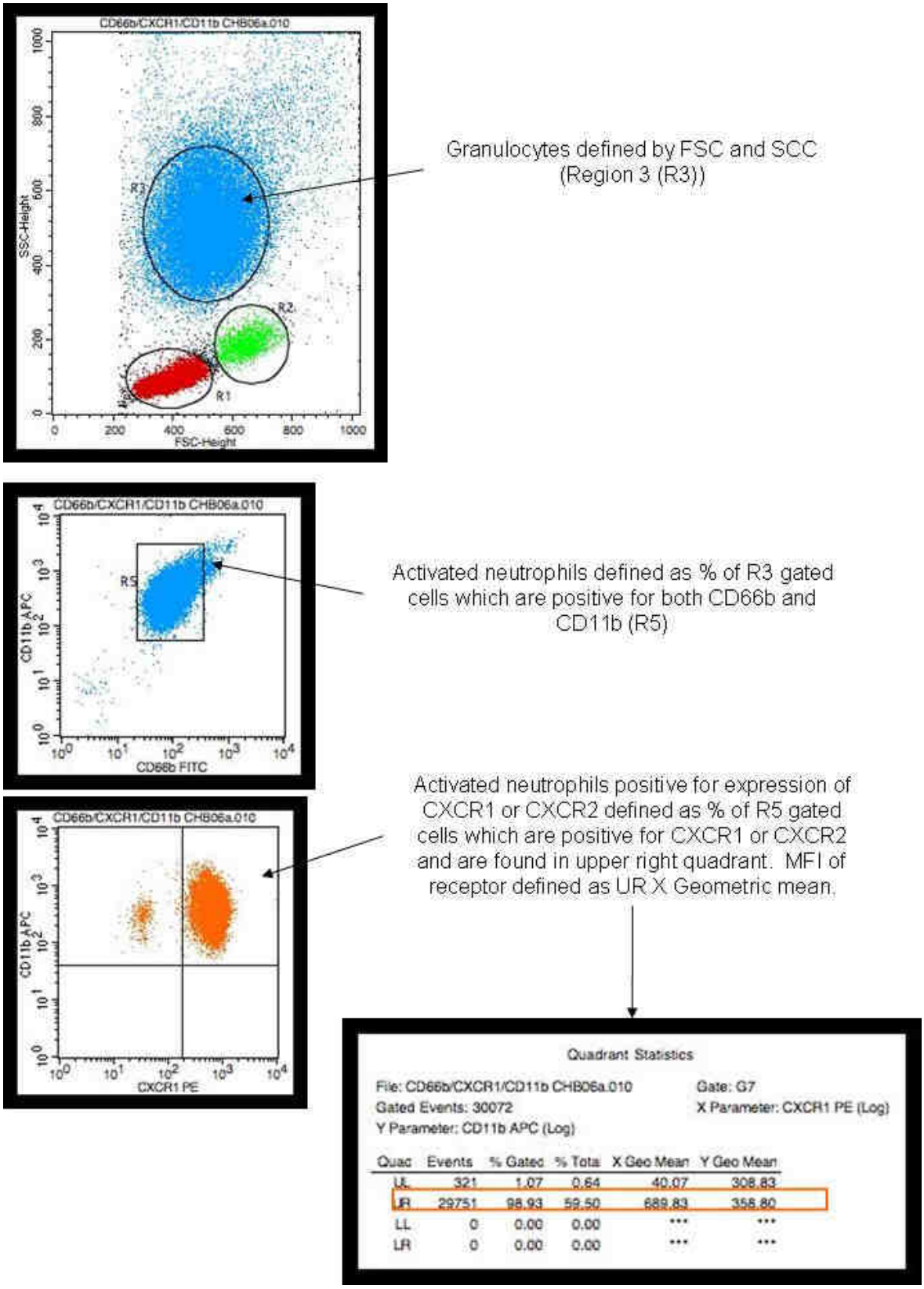

Figure 66 Representative flow cytometry profile used for granulocyte analysis

Initial gating based on forward and side scatter (R3). Subsequent gating was based on expression of the cell surface marker CD66b and CD11b, with further analysis for cell surface expression of the chemokine receptors CXCR1 and CXCR2.

Granulocyte cell surface marker data are illustrated in Figure 67. The percentage of CD66b+ granulocytes expressing CD11b did not differ significantly with pregnancy (NP vs PT, p=0.62) or between the 3 groups (p=0.34). The density of CD11b did not differ with pregnancy (NP vs PT, p=0.60) however, it was higher in the CS-pre group than the IOL-pre group (CS-pre vs IOL-pre, p=0.009), with no significant difference found between non-pregnant women and those prior to CS (NP vs CS-pre, p=0.11) or between non-pregnant and women and those prior to IOL (NP vs IOL-pre, p=0.36).

The percentage of CD11b+/CD66b+ granulocytes (activated neutrophils) expressing CXCR1 and the density of cell surface expression was not significantly different with pregnancy (NP vs PT, p=0.64, p=0.5271 respectively) or between the 3 groups (p=0.81, p=0.81 respectively). The percentage of activated neutrophils expressing CXCR2 was higher in pregnancy (NP vs PT, p=0.009) and in women pre-IOL compared with the non-pregnant group (NP vs IOL-pre, p=0.022), however this difference was not found between non-pregnant women and those prior to CS (NP vs CS-pre, p=0.069) or between the 2 pregnant groups (CS-pre vs IOL-pre, p=0.69). Conversely, the density of CXCR2 cell surface expression was lower in the IOL group compared with the non-pregnant group (NP vs IOL-pre, p=0.008), and again no significant difference was found between the non-pregnant group and those prior to CS (NP vs CS-pre, p=0.34) or between the 2 pregnant groups (CS-pre vs IOL-pre, p=0.40) and additionally no significant difference was found with respect to pregnancy (NP vs PT, p=0.08) (Figure 67).

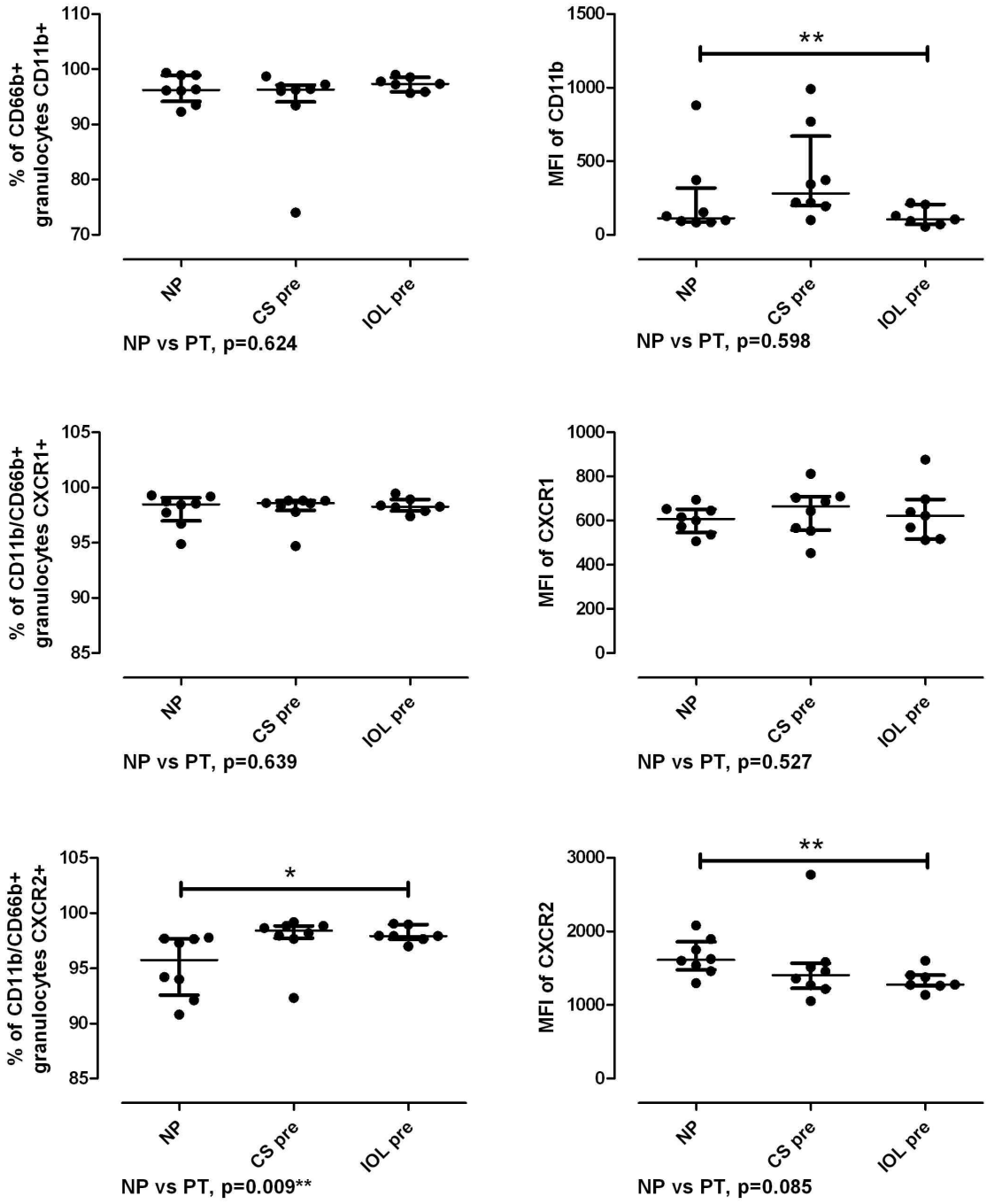

Figure 67 Baseline measure of granulocytes

Baseline measurements for granulocytes for each study group with p values for the comparison between non-pregnant and pooled pregnant at term groups (NP vs PT). Parameters examined are 1) the % of CD66b+ granulocytes expressing CD11b and the cell surface density of CD11b, 2) the % of CD11b+/CD66b+ granulocytes expressing CXCR1 and the cell surface density of CXCR1 and 3) the % of CD11b/CD66b+ granulocytes expressing CXCR2 and the cell surface density of CXCR2. The cell surface density is indicated by the mean fluorescence intensity (MFI). Raw data are shown with median and IQR. Data were log transformed prior to analysis, Unpaired t-tests were performed with lines demonstrating significance value between 2 samples under start and end of each line *p<0.05,p≤0.01**

5.4.2.2.2 Monocytes

An example of the flow cytometry profile used to assess the monocyte parameters is shown in Figure 68. Data were initially gated using forward and side scatter parameters with monocytes defined as large cells with lower granularity than granulocytes (Region 2 (R2) on diagram). These selected cells were then gated based on there positive expression of CD14 and CD11b and defined as activated monocytes (R5 on diagram). Cells defined by R5 were then further gated based on their cell surface expression of the chemokine receptor CCR2.

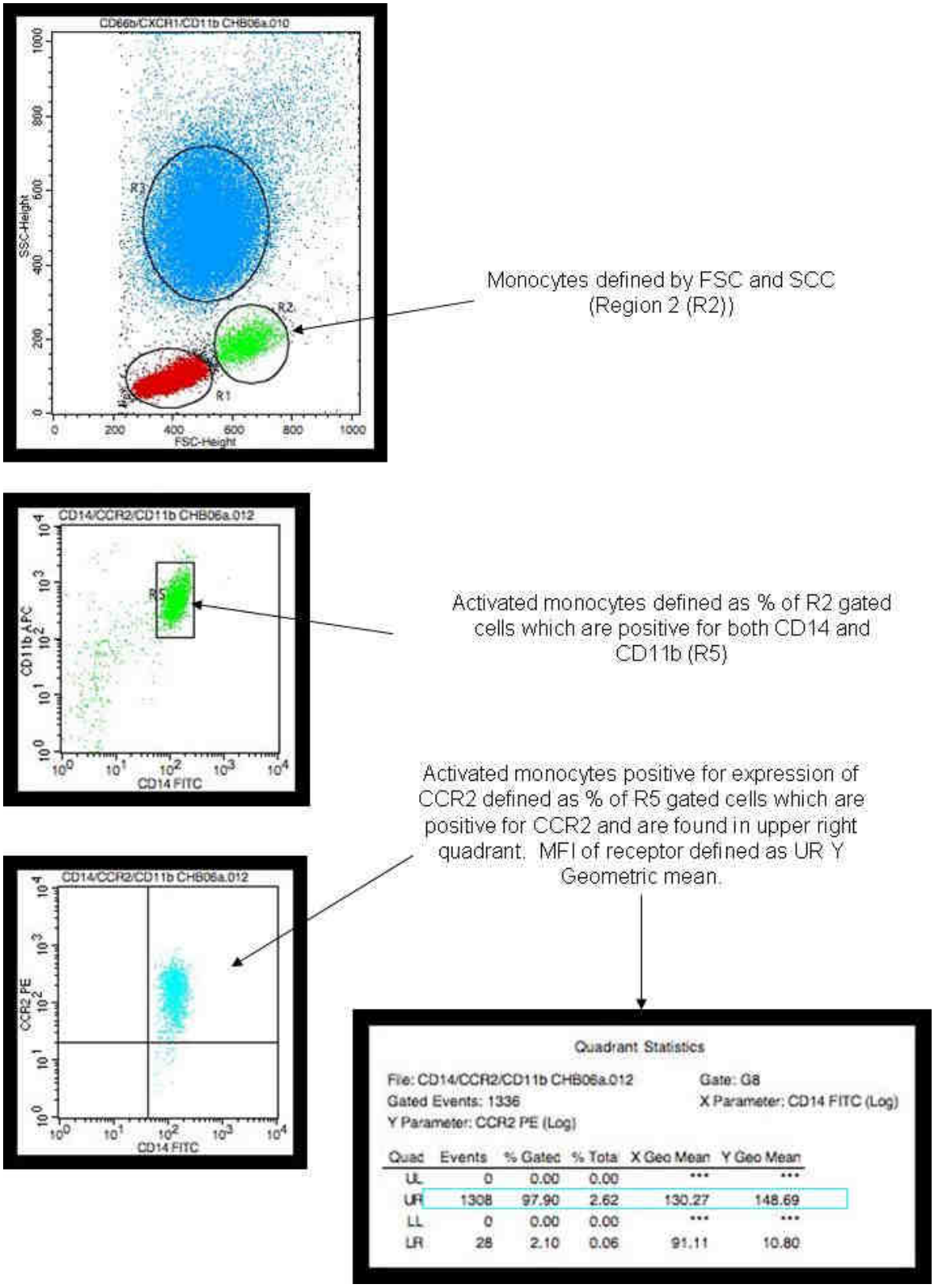

Figure 68 Representative flow cytometry profile used for monocyte analysis

Initial gating based on forward and side scatter to define monocyte population (R2). Subsequent gating was based on expression of the cell surface marker CD14 and CD11b, with further analysis for cell surface expression of the chemokine receptor CCR2.

Monocyte data are illustrated in Figure 69. No difference was seen with pregnancy in the % of CD14+ monocytes expressing CD11b or the density of CD11b expression (np vs PT, p=0.61, p=0.24 respectively). On further analysis the density of CD11b was higher in those prior to CS (NP vs CS-pre, p=0.034, CS-pre vs IOL-pre, p=0.013), with no difference between non-pregnant and those prior to IOL (NP vs IOL-pre, p=0.77).

The % of CD14+/CD11b+ monocytes expressing CCR2 reduced slightly with pregnancy which was significant (NP vs PT, p=0.022), however was not found to differ significantly between the 3 groups (NP vs CS-pre vs IOL-pre, p=0.070). The density of CCR2 expression did not differ over all with pregnancy (NP vs PT, p=0.079) however density appeared to be lower in the pre-IOL group compared with the non-pregnant group (NP vs IOL-pre, p<0.005), with no significant differences between non-pregnant women and those prior to CS (NP vs CS-pre, p=0.498) or between the 2 pregnant groups (CS-pre vs IOL-pre, p=0.077).

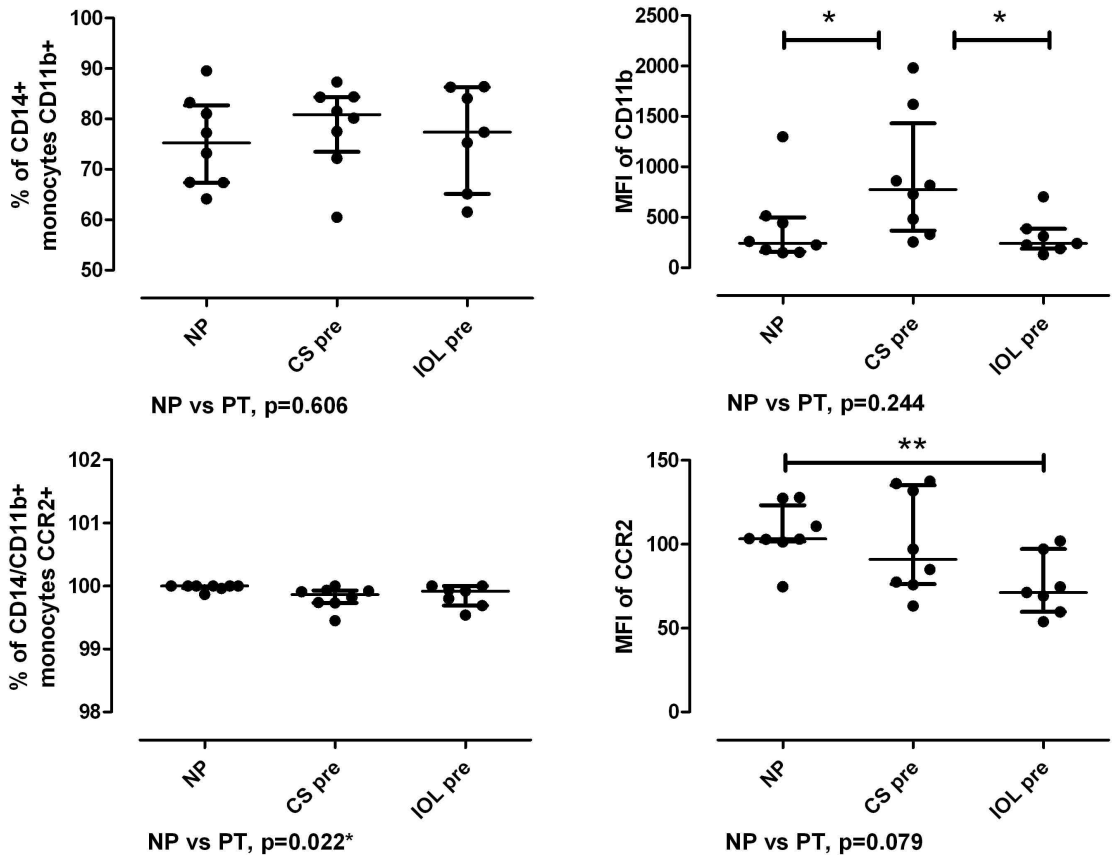

Figure 69 Baseline measure of monocytes

Baseline measurements for monocytes for each study group with p values for the comparison between non-pregnant and pooled pregnant at term groups (NP vs PT). Parameters examined are 1) the % of CD14+ monocytes expressing CD11b and the cell surface density of expression of CD11b and 2) the % of CD14+/CD11b+ monocytes expressing CCR2 and the cell surface density of expression of CCR2. The cell surface density is indicated by the mean fluorescence intensity (MFI). Raw data are shown with median and IQR. Data were log transformed prior to analysis, Unpaired t-tests were performed with lines demonstrating significance value between 2 samples under start and end of each line *p<0.05,p≤0.01**

5.4.2.2.3 Lymphocytes

An example of the flow cytometry profile used to assess the lymphocyte parameters is shown in Figure 70. Data were initially gated using forward and side scatter parameters with lymphocytes defined as small cells with low granularity (Region 1 (R1) on diagram). These selected cells were then gated based on there positive expression of CD3 and defined as T-lymphocytes (R4). APC fluorescence of R4 gated indicated expression of CD4 (or CD8 dependent on tube combination) and R5 gated cells defined T-cell population as either CD4+ (Th cells) or CD8+ (Tc cells). Cells defined by R5 were then further gated based on their cell surface expression of the chemokine receptors CXCR1, CXCR2 or CCR6.

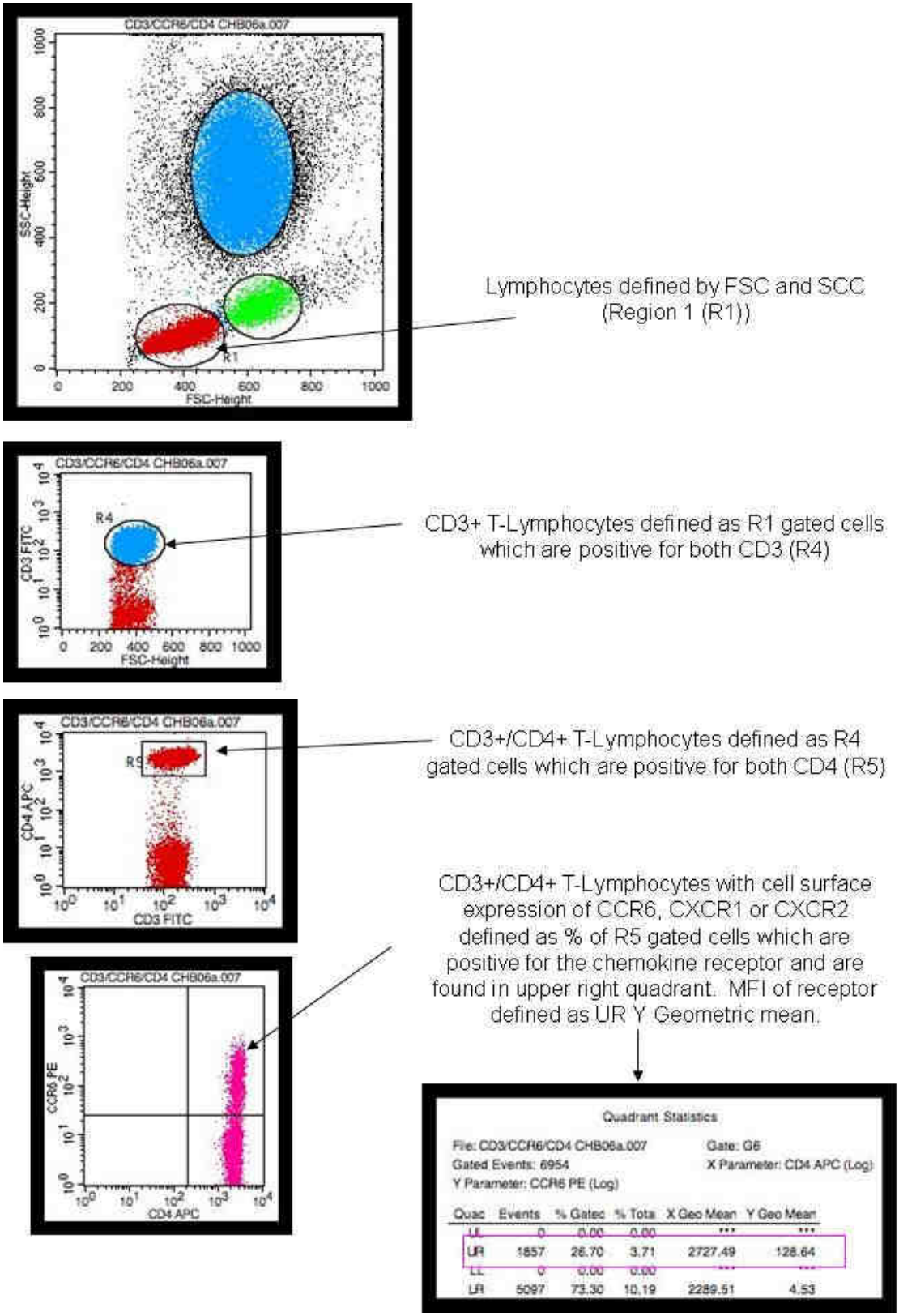

Figure 70 Representative flow cytometry profile used for lymphocyte analysis

Initial gating based on forward and side scatter to define lymphocyte population (R1). Subsequent gating was based on expression of the cell surface marker CD3 to define T-lymphocytes. Analysis was then undertaken to examine the proportion of T-lymphocytes which were CD4+ or CD8+ (R5), with further analysis for cell surface expression of the chemokine receptor CXCR1, CXCR2 and CCR6.

The percentage of CD3+ lymphocytes (T cells) expressing CD4 (T helper cell (Th-cell)) or CD8 (T cytotoxic (Tc-cell)) did not differ with pregnancy (NP vs PT, p=0.21, p=0.51 respectively) or between the 3 study groups (p=0.41, p=0.69 respectively) (Figure 71).

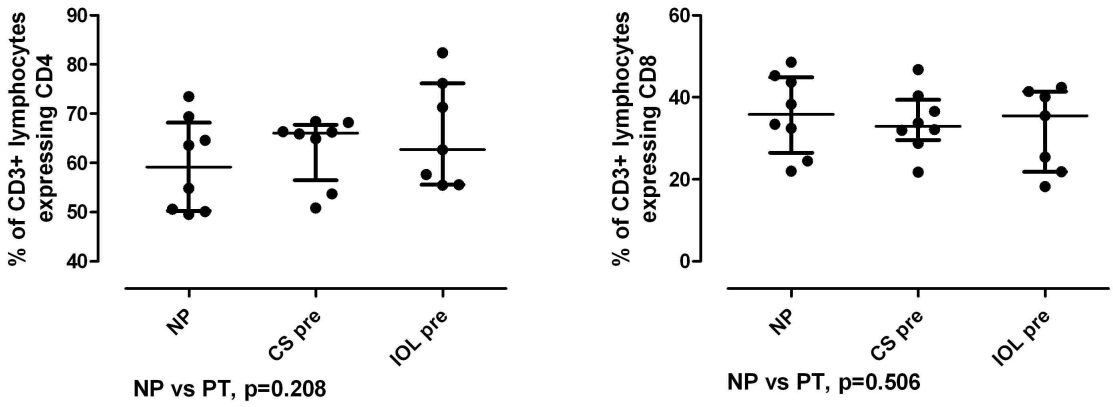

Figure 71 Baseline measures for CD3+/CD4+ and CD3+/CD8+ lymphocytes

Baseline measurements of the % of circulating CD3+/CD4+ (T helper lymphocytes) and the % of CD3+/CD8+ (T cytotoxic lymphocytes) for each study group with p values for the comparison between non-pregnant and pooled pregnant at term groups (NP vs PT). Raw data are shown with median and IQR. Data were log transformed prior to analysis.

Data for Th-cells (CD3/CD4+ lymphocytes) are presented in Figure 72. The percentage of Th-cells expressing CXCR1, CXCR2 or CCR6 did not differ with pregnancy (NP vs PT, p=0.96, p=0.87, p=0.613 respectively) or between groups (p=0.98, p=0.95, p=0.86 respectively). The density of cell surface expression of CXCR1 and CXCR2 on Th-cells was lower in pregnancy (NP vs PT, p=0.011, p=0.002 respectively). CXCR1 MFI was higher in the NP group compared to those prior to CS (NP vs CS-pre, p=0.030), however no difference in CXCR1 density were found for the remaining comparisons (NP vs IOL-pre, p=0.06, CS-pre vs IOL-pre, p=0.91). The density of CXCR2 on Th-cells was higher in the NP group compared with both pregnant groups (NP vs CS-pre, p=0.021, NP vs IOL-pre, p=0.026), no difference existed between the pregnant groups (CS-pre vs IOL-pre, p=0.92). Density of CCR6 expression on Th-cells did not significantly change with pregnancy (NP vs PT, p=0.15), however further analysis suggested expression was lower in those prior to IOL (NP vs IOL-pre, p=0.038, CS-pre vs IOL-pre, p=0.046), with no significant difference between NP and CS-pre groups (NP vs CS-pre, p=0.66).

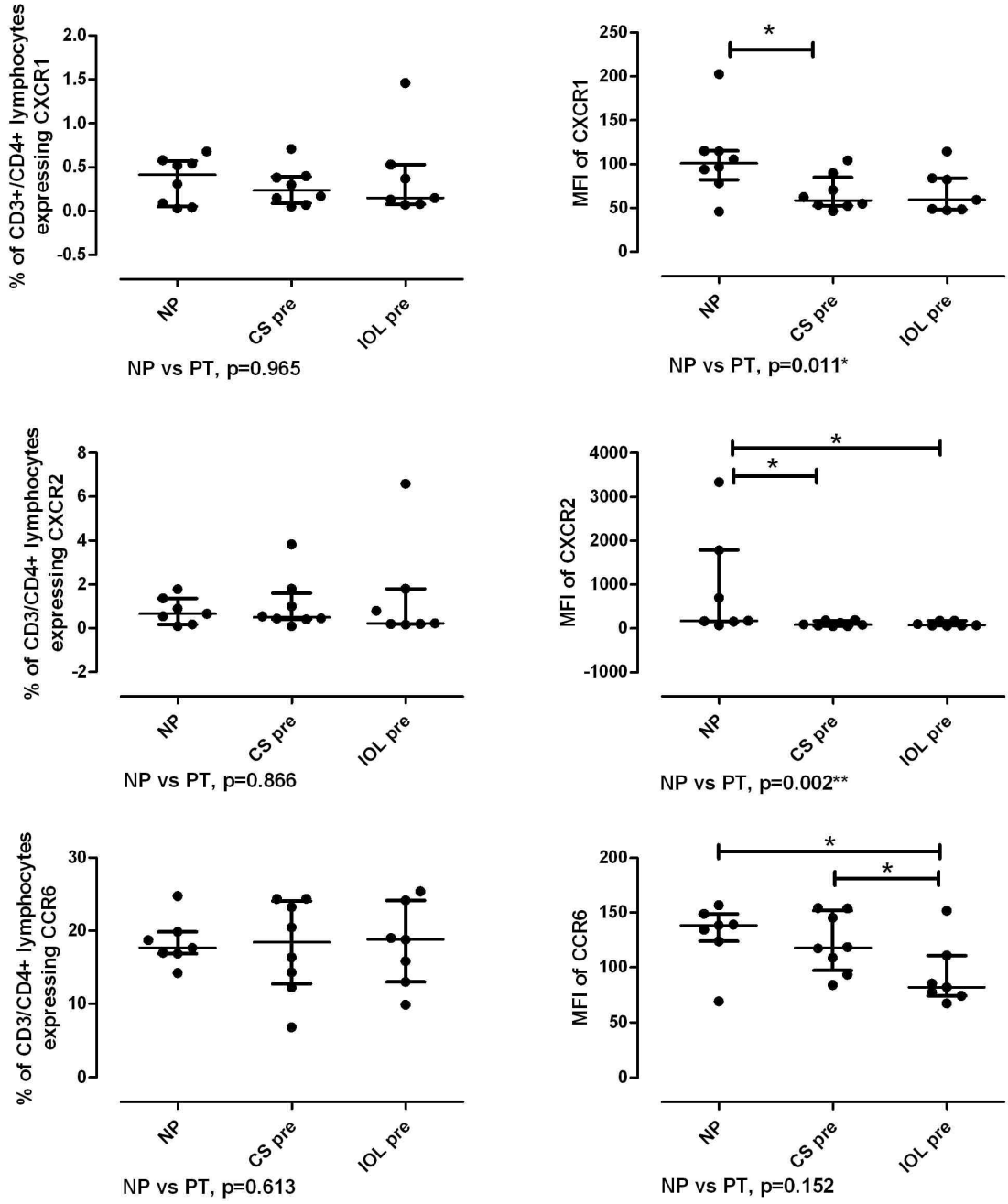

Figure 72 Baseline measures of CD3+/CD4+ lymphocytes

Baseline measurements for CD3+/CD4+ lymphocytes (T-helper cells) for each study group with p values for the comparison between non-pregnant and pooled pregnant at term groups (NP vs PT). Parameters examined are 1) the % of T-helper lymphocytes expressing CXCR1 and cell surface density of CXCR1, 2) the % or T-helper lymphocytes expressing CXCR2 and the cell surface density of CXCR2, and 3) the % of T-helper lymphocytes expressing CCR6 and the cell surface density of CCR6. The cell surface density is indicated by the mean fluorescence intensity (MFI). Raw data are shown with median and IQR. Data were log transformed prior to analysis, Unpaired t-tests were performed with lines demonstrating significance value between 2 samples under start and end of each line *p<0.05, **p<0.01.

Data for Tc-cells (C3/CD8+ lymphocytes) is illustrated in Figure 73. Similarly, the % of Tc-cells expressing CXCR1, CXCR2 or CCR6 did not differ with pregnancy (NP vs PT, p=0.79, p=0.427, p=0.43 respectively) or between groups (p=0.79, p=0.59, p=0.74 respectively). Similarly, the density of CXCR1 and CXCR2 expression fell with pregnancy (NP vs PT p<0.001, p=0.006 respectively). Further analysis confirmed CXCR1 MFI was lower with pregnancy (NP vs CS-pre, p=0.006, NP vs IOL-pre, p=0.003), but did not differ between pregnant groups (CS-pre vs IOL-pre, p=0.73). The density of CXCR2 expression was higher in the NP groups compared with CS-pre (NP vs CS-pre, p=0.04), however no significant difference was found for CXCR2 between the other groups (NP vs IOL-pre, p=0.055, CS-pre vs IOL-pre, p=0.62). CCR6 density of expression did not significantly differ with pregnancy (NP vs PT, p=0.38) or between the three groups (p=0.16).

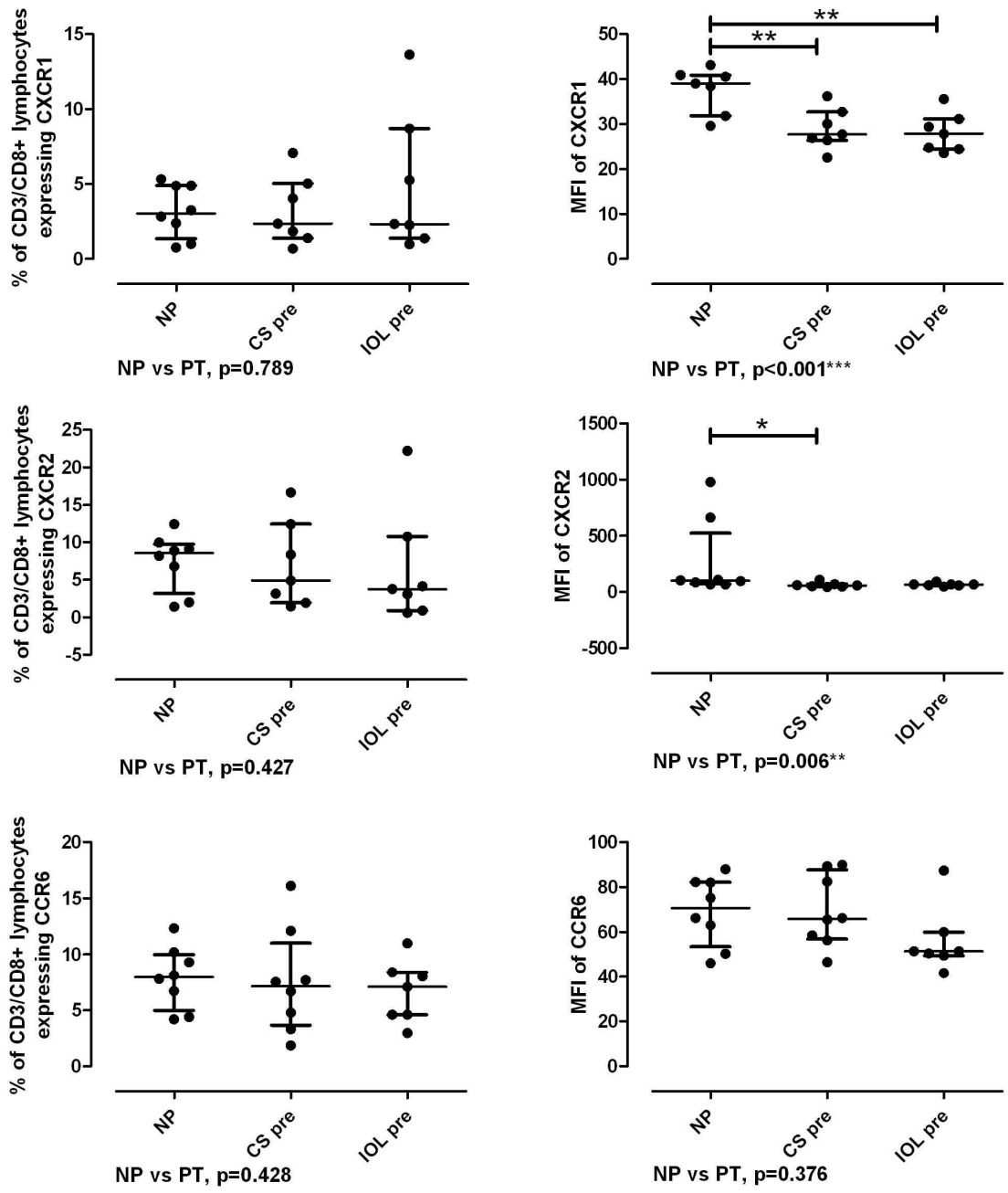

Figure 73 Baseline measures of CD3+/CD8+ lymphocytes

Baseline measurements for CD3+/CD8 lymphocytes (T-cytotoxic) for each study group with p values for the comparison between non-pregnant and pooled pregnant at term groups (NP vs PT). Parameters examined are 1) the % of T-cytotoxic lymphocytes expressing CXCR1 and cell surface density of CXCR1, 2) the % or T-cytotoxic lymphocytes expressing CXCR2 and the cell surface density of CXCR2, and 3) the % of T-cytotoxic lymphocytes expressing CCR6 and the cell surface density of CCR6. The cell surface density is indicated by the mean fluorescence intensity (MFI). Raw data are shown with median and IQR. Data were log transformed prior to analysis, Unpaired t-tests were performed with lines demonstrating significance value between 2 samples under start and end of each line *p<0.05, **p<0.01.

5.4.2.3Circulating cytokines and chemokines

Due to limitations of the range of sensitivities for cytokines in human plasma, a full complement of results is available for CCL11 (eotaxin), FGF2, GCSF, GMCSF, GRO (CXCL1, 2, 3), IL6, CXCL8 (IL8), IL10, CXCL10 (IP10), CCL2 (MCP-2), CCL22 (MDC), CCL4 (MIP1b) and TNFα. In cases where more than 50% of values for an individual analyte were missing in each group (n<4), they were excluded from analysis. Missing values for these analytes were below the threshold of sensitivity. Data for each of the analytes is shown in Table 28.

Significant differences between the three groups were noted for 3 of the cytokines tested, namely FGF2 (p=0.003), GCSF (p=0.008), and CXCL1, 2, 3 (GRO)(p=0.002). Measures of FGF2 were lower in those pre-IOL compared with both their non-pregnant and pre-CS counterparts (NP vs CS-pre (p=0.1379), NP vs IOL-pre (p=0.033), CS-pre vs IOL-pre (p=0.002)), however, when pregnancy data were pooled, no significant difference was found between NP and PT individuals (NP vs PT (p=0.552)). Measures of CXCL1,2,3 (GRO) were higher in the pre-CS group with no significant difference between non-pregnant and pre-IOL groups (NP vs CS-pre (p=0.035), NP vs IOL-pre (p=0.65), CS-pre vs IOL-pre (p=0.002)), however, again no significant difference was found between NP and PT individuals when pregnancy data were pooled (NP vs PT (p=0.955)). In terms of GCSF a higher circulating concentration was seen in both pregnant groups compared with non-pregnant values (NP vs PT (p=0.003)), however, the only significant difference was between the non-pregnant and pre-IOL group (NP vs CS-pre (p=0.06), NP vs IOL-pre (p=0.006)) but did not differ between the 2 pregnant groups (CS-pre vs IOL-pre (p=0.18)).

Cytokine/ Chemokine (pg/ml)		Study Group			p value unpaired t-test			
		NP	CS-pre	IOL-pre	NP v PT	NP v CS	NP v IOL	CS v IOL
EGF	Median (IQR) [range] N	28.93 (22.82,152.2) [12.18,488.4] 6	31.42 (3.97,60.42) [6.12,64.06] 6	10.84 / [7.840,13.83] 2	0.199	0.370	/	/
CCL11	Median (IQR) [range] N	294.6 (171.4,432.4) [63.9,647.8] 8	103.3 (92.74,231.8) [75.69,429.2] 8	187.2 (119.9,221.2) [66.66,263.0] 8	0.044*	0.081	0.142	0.569

Cytokine/ Chemokine (pg/ml)		Study Group			p value unpaired t-test			
		NP	CS-pre	IOL-pre	NP v PT	NP v CS	NP v IOL	CS v IOL
FGF-2	Median (IQR) [range] N	75.1 (63.3,90.1) [46.1,112.4] 8	93.33 (87.92,103.3) [55.85,119.4] 8	56.75 (40.79,68.85) [21.92,71.68] 8	0.552	0.138	0.033*	0.002**
Flt-3	Median (IQR) [range] N	5.62 (2.195,35.11) [1.30,44.69] 4	38.25 / / 1	5.335 / [1.3,9.37] 2	/	/	/	/
CX3CL1	Median (IQR) [range] N	27.04 (9.83,73.21) [4.25,173.0] 7	21.27 (8.520,27.04) [4.830,66.83] 7	60.07 (36.93,734.6) [27.04,1370] 5	0.592	0.530	0.117	0.031*
GCSF	Median (IQR) [range] N	19.24 (10.69,36.17) [2.61,102.3] 8	43.22 (23.97,78.88) [17.02,129.6] 8	74.74 (43.28,99.61) [33.22,154.3] 8	0.003**	0.061	0.006**	0.179
GMCSF	Median (IQR) [range] N	56.80 (34.43,140.9) [5.63,1815.0] 8	76.97 (31.43,190.9) [8.450,306.3] 8	46.89 (24.38,110.6) [10.29,139.7] 8	0.704	0.964	0.571	0.504
CXCL1,2,3,	Median (IQR) [range] N	1169 (801.7,1493) [604.1,1589] 8	1687 (1207,2140) [940.6,3038] 8	804.0 (481.2,992.2) [238.1,1218] 8	0.955	0.035*	0.065	0.002**
IFNα2	Median (IQR) [range] N	13.70 (4.935,65.61) [0.79,79.87] 5	115.4 / [42.18,188.6] 2	15.24 (12.92,58.17) [12.15,72.48] 4	/	/	0.645	/
IFNγ	Median (IQR) [range] N	24.37 (5.508,82.55) [1.89,140.7] 8	8.800 (2.140,14.23) [2.070,91.04] 7	6.510 (2.688, 55.49) [2.340,67.31] 8	0.225	0.256	0.385	0.757
IL1α	Median (IQR) [range] N	128.8 (90.24,375.6) [86.50,1555] 7	166.8 (78.90, 375.7) [59.58, 938.0] 8	192.2 (111.8, 271.5) [67.67, 274.3] 6	0.714	0.818	0.701	0.878
IL1ß	Median (IQR) [range] N	7.15 / [1.91,12.39] 2	1.34 / / 1	1.34 / / 1	/	/	/	/

Cytokine/ Chemokine (pg/ml)		Study Group			p value unpaired t-test			
		NP	CS-pre	IOL-pre	NP v PT	NP v CS	NP v IOL	CS v IOL
IL1ra	Median	2.64	6.96	2.64	/	/	/	/
	(IQR)	/	/	/				
	[range]	/	/	/				
	N	1	1	1				
IL2	Median	5.30	2.640	1.160	/	/	/	0.912
	(IQR)	/	(0.8550, 3.390)	(0.995,3.850)				
	[range]	[1.50,154.1]	[0.7800, 4.000]	[0.930,4.550]				
	N	3	5	5				
IL3	Median	9.49	5.24	29.27	/	/	/	/
	(IQR)	/	/	/				
	[range]	[0.75,66.12]	/	/				
	N	3	1	1				
Il4	Median	/	/	75.88	/	/	/	/
	(IQR)	/	/	/				
	[range]	/	/	/				
	N	0	0	1				
IL5	Median	1.51	0.38	/	/	/	/	/
	(IQR)	/	/	/				
	[range]	[0.62,2.4]	[0.23,0.53]	/				
	N	2	2	0				
IL6	Median	63.93	8.820	11.79	0.054	0.051	0.167	0.857
	(IQR)	(31.49,272.1)	(4.335, 63.76)	(3.025, 283.6)				
	[range]	[15.06,669.7]	[3.360, 185.2]	[2.690, 549.3]				
	N	6	6	5				
IL7	Median	49.21	5.660	7.405	/	0.219	/	/
	(IQR)	(12.88,103.9)	(3.665, 38.72)	(3.600, 11.21)				
	[range]	[4.630,118.2]	[2.000, 41.44]	[3.600, 11.21]				
	N	4	5	2				
CXCL8	Median	27.35	17.57	16.03	0.112	0.111	0.204	0.767
	(IQR)	(19.62,47.23)	(6.930, 30.19)	(6.675, 42.35)				
	[range]	[12.38,71.94]	[3.010, 63.20]	[4.740, 65.12]				
	N	8	8	8				
IL9	Median	19.98	2.89	4.940	/	/	0.324	/
	(IQR)	(9.563,25.31)	/	(1.985, 44.09)				
	[range]	[6.46,26.72]	/	[1.640, 78.71]				
	N	4	1	5				
IL10	Median	2.575	2.200	1.815	0.734	0.891	0.374	0.279
	(IQR)	(1.76,4.99)	(1.605, 5.960)	(1.535, 3.040)				
	[range]	[0.82,23.90]	[1.260, 21.52]	[1.490, 3.130]				
	N	8	8	6				

Cytokine/ Chemokine (pg/ml)		Study Group			p value unpaired t-test			
		NP	CS-pre	IOL-pre	NP v PT	NP v CS	NP v IOL	CS v IOL
IL12 (p40)	Median	62.75	207.4	179.8	/	/	/	/
	(IQR)	(8.120,1.87.1)	/	(170.6,192.5)				
	[range]	[8.120,187.1]	/	[170.6,192.5]				
	N	3	1	3				
IL12 (p70)	Median	8.36	2.940	2.340	0.573	/	0.511	/
	(IQR)	(1.95,197.6)	(1.360, 372.5)	(1.855, 202.8)				
	[range]	[1.36,704.2]	[1.360, 372.5]	[1.490, 402.1]				
	N	7	3	5				
IL13	Median	37.82	1.150	/	/	/	/	/
	(IQR)	(5.64,331.6)	/	/				
	[range]	[2.94,382.8]	/	/				
	N	5	1	0				
IL15	Median	0.81	0.92	1.330	0.374	/	0.383	/
	(IQR)	(0.525,1.815)	(0.68,3.30)	(0.765, 3.31)				
	[range]	[0.48,1.92]	[0.68, 3.30]	[0.480, 4.57]				
	N	5	3	5				
IL17	Median	47.46	18.97	12.29	0.125	0.294	0.117	0.589
	(IQR)	(13.41,81.63)	(5.990, 66.67)	(3.140, 57.51)				
	[range]	[9.8,149.8]	[3.980, 140.6]	[1.720, 116.2]				
	N	8	6	8				
CXCL10	Median	512.2	823.8	565.5	0.126	0.050	0.424	0.136
	(IQR)	(318.7,569.2)	(537.7, 1464)	(425.5, 718.7)				
	[range]	[298.7,642.9]	[272.0, 4959]	[225.0, 903.2]				
	N	8	8	8				
CCL2	Median	327.1	235.4	229.9	0.111	0.262	0.112	0.489
	(IQR)	(219.7,423.0)	(210.3, 305.0)	(172.2, 282.4)				
	[range]	[194.3,536.3]	[157.6, 503.3]	[95.36, 469.1]				
	N	8	8	8				
CCL7	Median	7.075	7.700	6.290	0.465	0.806	0.339	0.431
	(IQR)	(3.045,36.66)	(2.313, 26.20)	(1.830, 10.28)				
	[range]	[2.11,143.9]	[2.110, 51.04]	[1.550, 12.18]				
	N	8	6	5				
CCL22	Median	1192	547.2	566.1	0.031*	0.022*	0.106	0.703
	(IQR)	(878.0,1314)	(406.4, 1035)	(349.8, 1369)				
	[range]	[498.9,1614]	[259.6, 1291]	[321.4, 1978]				
	N	8	8	8				
CCL3	Median	47.82	41.21	38.60	0.7001	0.529	0.863	0.772
	(IQR)	(39.07,82.01)	(28.08, 97.76)	(22.71, 177.5)				
	[range]	[37.17,162.0]	[25.20, 124.2]	[21.12, 178.3]				
	N	7	5	7				

Cytokine/ Chemokine (pg/ml)		Study Group			p value unpaired t-test			
		NP	CS-pre	IOL-pre	NP v PT	NP v CS	NP v IOL	CS v IOL
CCL4	Median	80.85	57.55	42.98	0.375	0.506	0.401	0.787
	(IQR)	(36.75,176.5)	(24.02, 95.48)	(18.55, 176.9)				
	[range]	[16.38,403.5]	[20.47, 323.2]	[6.170, 217.6]				
	N	8	8	8				
sCD40L	Median	3849	4130	3447	0.928	/	0.880	/
	(IQR)	(2056,4946)	/	(1551, 7070)				
	[range]	[1726,5044]	/	[738.3, 8610]				
	N	4	1	8				
sIL2Rα	Median	25.72	8.570	1.180	/	/	/	/
	(IQR)	(23.25,28.19)	(5.770, 51.51)	/				
	[range]	[23.25,28.19]	[4.560, 65.05]	/				
	N	2	5	1				
TGFα	Median	6.280	8.010	2.750	0.266	0.816	0.113	0.103
	(IQR)	(3.67,10.14)	(3.490, 8.440)	(0.483, 9.143)				
	[range]	[3.46,49.27]	[3.220, 12.99]	[0.340, 21.06]				
	N	7	7	6				
TNFα	Median	2.27	4.830	3.865	0.580	0.246	0.886	0.205
	(IQR)	(1.698,7.125)	(3.198, 9.235)	(1.183, 7.113)				
	[range]	[1.320,15.89]	[1.740, 15.44]	[0.810, 8.380]				
	N	8	8	8				
TNFβ	Median	0.78	1.0	1.12	/	/	/	/
	(IQR)	(0.74,1.60)	(0.78,1.88)	/				
	[range]	[0.74,1.60]	[0.78,1.88]	/				
	N	3	3	1				
VEGF	Median	108.2	146.5	113.5	0.404	0.626	0.331	0.483
	(IQR)	(51.83,525.9)	(70.67, 161.9)	(14.46, 305.9)				
	[range]	[34.78,669.0]	[16.60, 352.8]	[4.000, 347.5]				
	N	8	7	4				
CCL5	Median	35173	12464	1466	0.303	0.575	/	/
	(IQR)	(5116,56938)	(6870, 19638)	(422.3, 12007)				
	[range]	166.0,130414	[362.4, 23230]	[422.3, 12007]				
	N	7	8	3				

Table 28 Baseline measures of circulating cytokines/chemokines

Table showing baseline levels of circulating cytokines for each study group. Raw data are presented with analysis performed on normalised log transformed data. Where boxes are marked with / this indicates that less than 50% of expected values were present (n expected in each group n=8 or N=16 in pooled pregnant at term group (PT)), therefore analysis not performed. *p<0.05, **p≤0.01, ***p≤0.001

5.4.2.4Circulating CRP

Baseline measures of CRP were higher with pregnancy (NP vs PT, P=0.002) which was confirmed by further analysis showing no significant difference between the two pregnant groups (CS-pre vs IOL-pre, p=0.25), with levels in pregnancy significantly higher (NP vs CS-pre (p=0.039), NP vs IOL-pre (p=0.002)) (Figure 74).

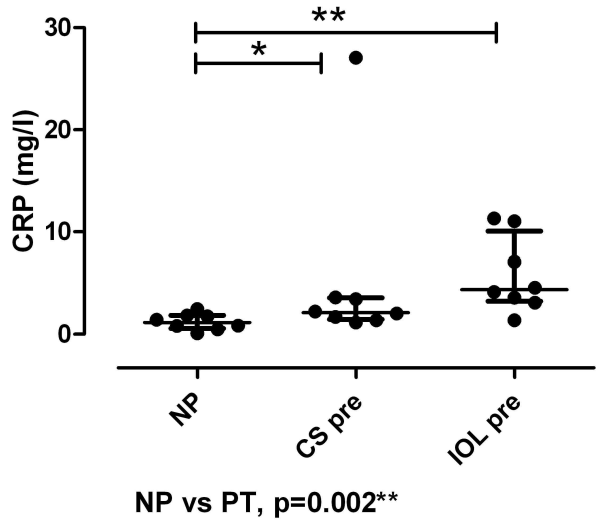

Figure 74 Baseline measures of circulating CRP

Baseline measure of circulating CRP for each study group with p values for the comparison between non-pregnant and pooled pregnant at term groups (NP vs PT). Raw data are shown as scatter plots with median and IQR. Data were log transformed prior to analysis. Unpaired t-tests were performed with lines demonstrating significance value between 2 samples under start and end of each line*p<0.05,p≤0.01,**

5.4.3 Hypothesis 2 – Term delivery by either planned, non-labour CS or IOL with syntocinon will result in a shift towards a pro-inflammatory profile in the maternal circulation, with and enhanced response in mothers who labour

5.4.3.1White Blood Cells

Pre and post delivery values for each white cell subset were firstly compared within groups (Figure 75). Changes induced as a result of delivery by either CS or IOL showed significant increases in circulating t-WBC (CS-pre vs CS-post ($p=0.03$), IOL-pre vs IOL-post ($p<0.001$)) and neutrophils (CS-pre vs CS-post ($p=0.005$), IOL-pre vs IOL-post ($p<0.001$)), with decreases in lymphocytes (CS-pre vs CS-post $p=0.02$, IOL-pre vs IOL-post ($p=0.001$)) and eosinophils (CS-pre vs CS-post ($p=0.015$), IOL-pre vs IOL-post ($p=0.015$)). Additionally, circulating monocytes increase pre to post IOL ($p=0.006$).

The extent of changes in white cell concentrations in response to delivery was significantly larger in response to IOL than to CS for t-WBC ($p<0.001$), neutrophils ($p<0.001$), lymphocytes ($p=0.006$), monocytes ($p=0.002$) and eosinophils ($p=0.02$). These differences are illustrated in Figure 76.

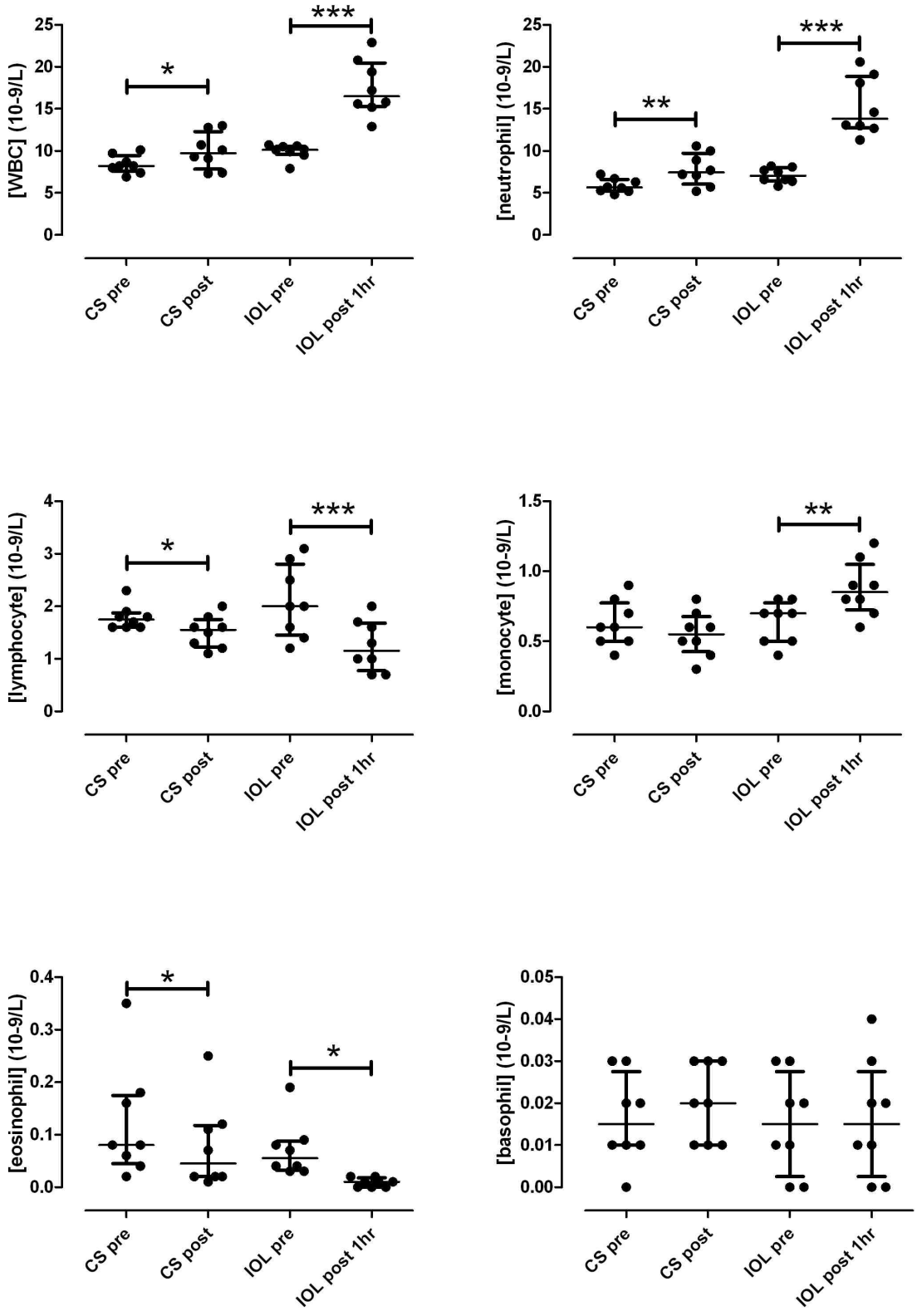

Figure 75 WBC subtypes pre and post delivery

Concentration of circulating white blood cell sub-sets pre and post delivery by planned caesarean delivery (CS) or vaginal delivery after induction of labour with syntocinon only (IOL). Raw data are shown as scatter plots with median and interquartile ranges indicated. Data were log transformed prior to analysis. Paired t-tests were performed with lines demonstrating significance value between 2 samples under start and end of each line *p<0.05, **p≤0.01, ***p≤0.001

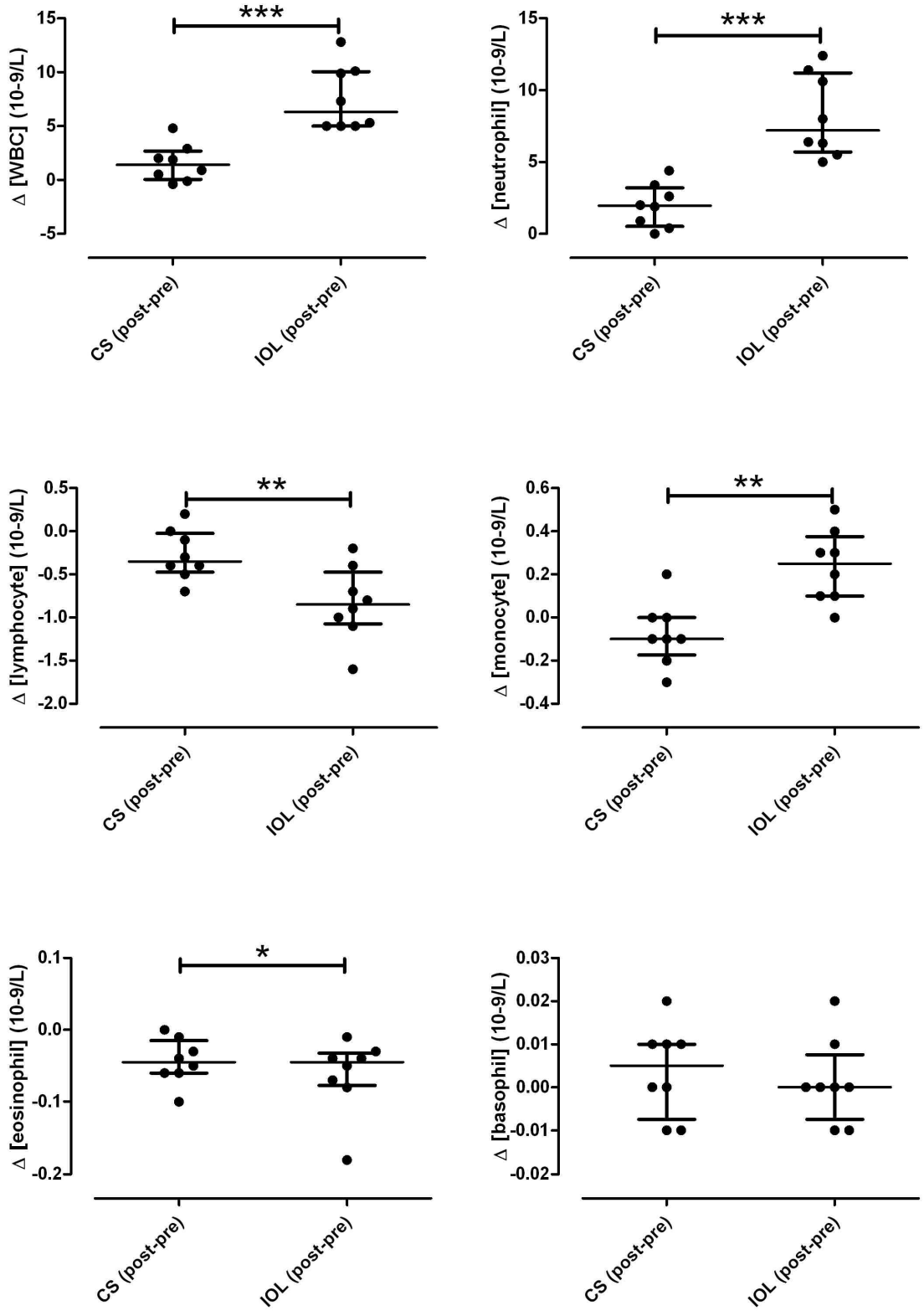

Figure 76 ΔWBC subtypes (post-pre) delivery

Change in circulating white blood cells post to pre delivery for CS and IOL. Raw data are shown with scatter plots with median and interquartile range indicated. Analysis was performed on log transformed data where log Δ CS = (log CS post)- (log CS pre) and log Δ IOL = (log IOL post) – (log IOL pre). Unpaired t-tests were performed with lines demonstrating significance value between 2 samples under start and end of each line. *p<0.05, **p≤0.01, ***p≤0.001

5.4.3.2Leukocyte cell surface markers and chemokine receptors

5.4.3.2.1 Granulocytes

The percentage of CD66+ granulocytes expressing CD11b did not change in response to delivery by either CS or IOL (p=0.21, p=0.59 respectively). There was a trend for a fall in the density of expression of CD11b in response to delivery, with the fall being significant in the CS group (CS-pre vs CS-post p=0.047, IOL-pre vs IOL-post p=0.69) (Figure 77).

The % of CD11b/CD66b+ granulocytes expressing CXCR1 increased with IOL but not CS (CS-pre vs CS-post (p=0.14), IOL-pre vs IOL-post, p=0.02), with the same pattern for CXCR2 (CS-pre vs CS-post (p=0.39), IOL-pre vs IOL-post (p=0.005)). Additionally the density of expression of CXCR1 fell in response to both CS and IOL (CS-pre vs CS-post (p=0.007), IOL-pre vs IOL-post (p=0.001)). CXCR2 MFI also fell in response to delivery (CS-pre vs CS-post (p=0.043), IOL-pre vs IOL-post, (p<0.0001)) (Figure 77).

The extent of changes to any of the measured granulocyte parameters did not differ significantly by mode of delivery (Figure 78).

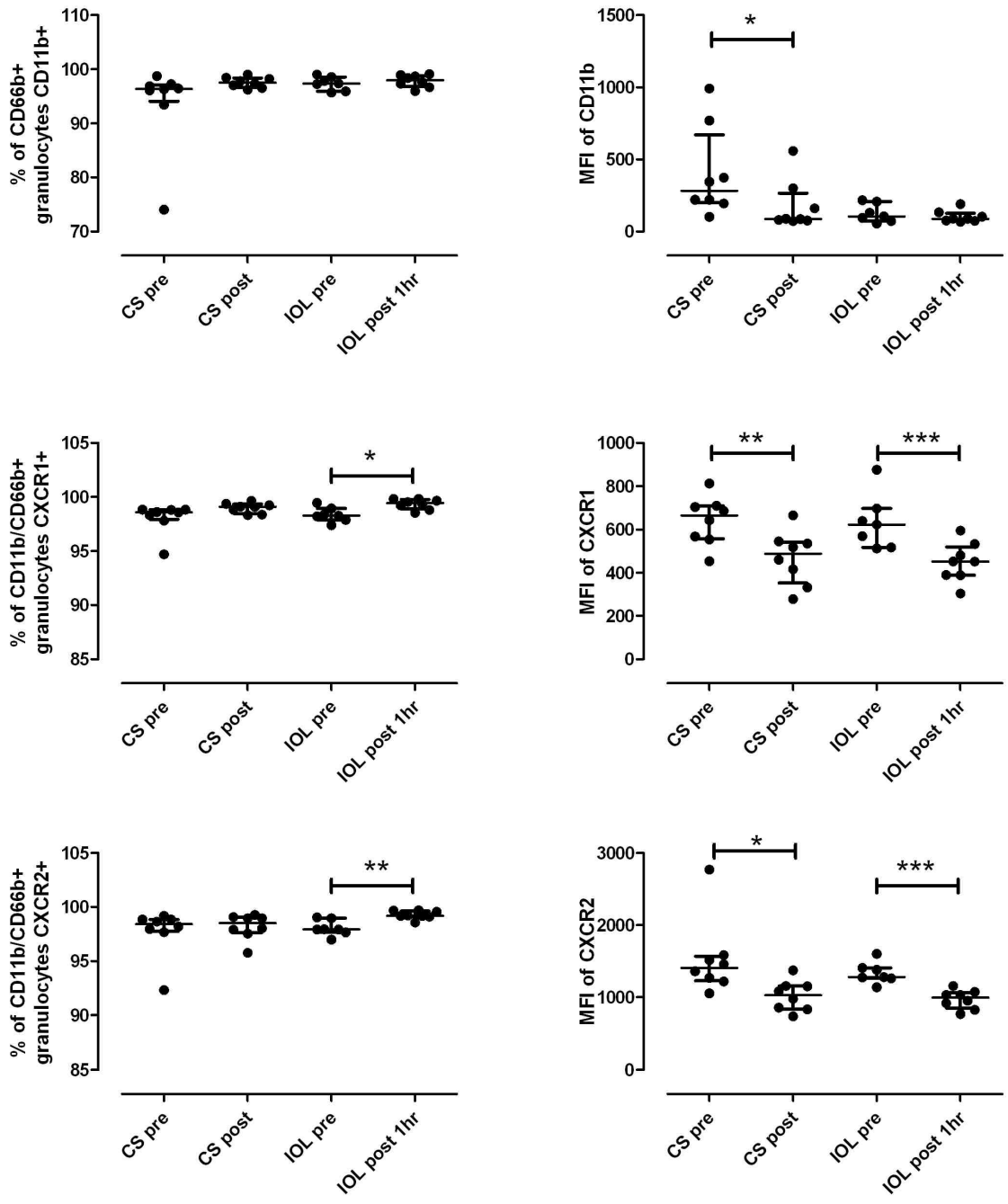

Figure 77 Granulocytes pre and post delivery

Assessment of granulocytes in the maternal circulation in response to delivery by either planned caesarean delivery (CS) or vaginal delivery after induction of labour (IOL). Parameters examined are 1) the % of CD66b+ granulocytes expressing CD11b and the cell surface density of CD11b, 2) the % of CD11b+/CD66b+ granulocytes expressing CXCR1 and the cell surface density of CXCR1 and 3) the % of CD11b/CD66b+ granulocytes expressing CXCR2 and the cell surface density of CXCR2. The cell surface density is indicated by the mean fluorescence intensity (MFI). Raw data are shown with median and IQR. Data were log transformed prior to analysis, Paired t-tests were performed with lines demonstrating significance value between 2 samples under start and end of each line. *p<0.05,p≤0.01, ***p≤0.001.**

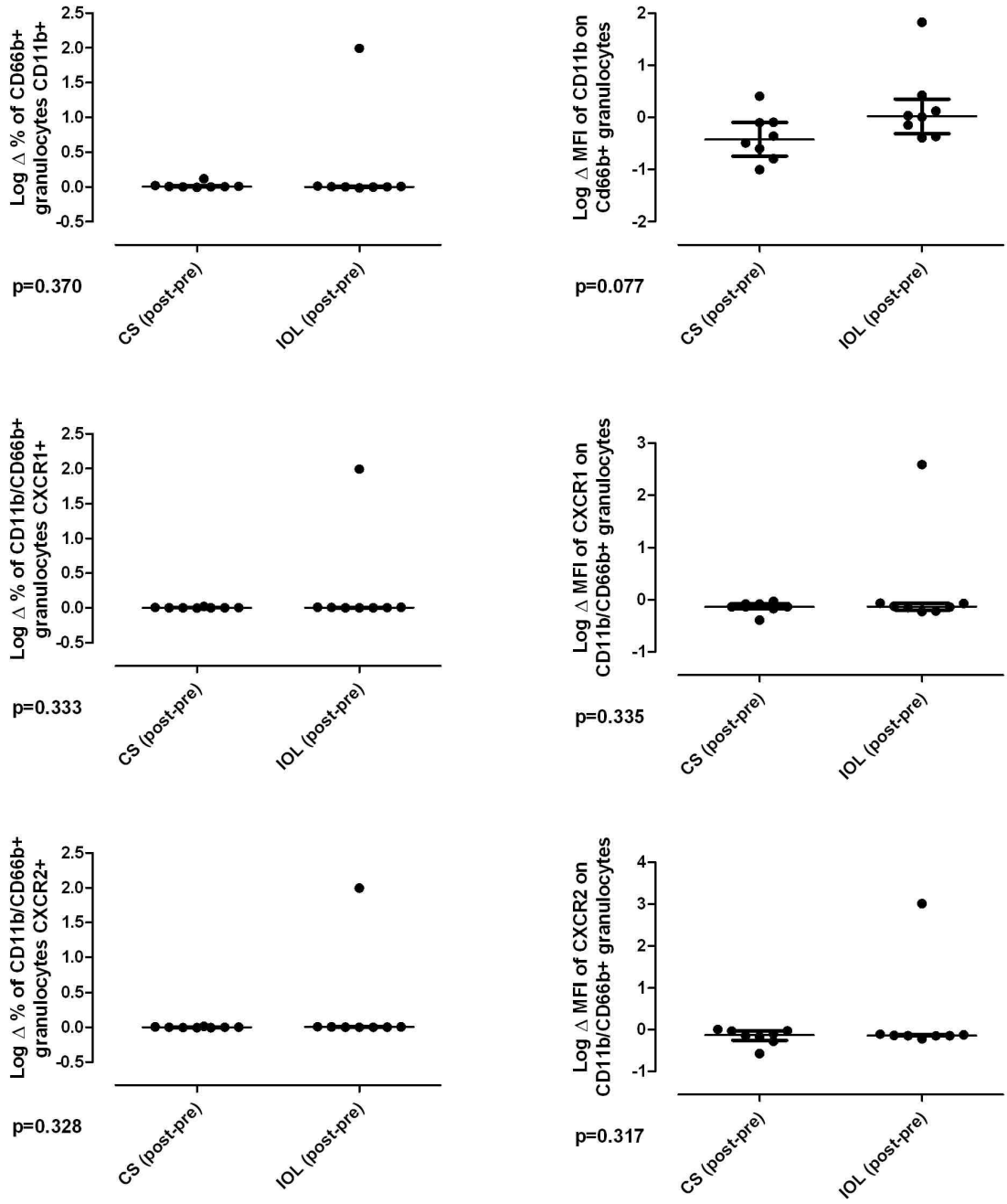

Figure 78 Δgranulocytes (post-pre) delivery

Change in the % of circulating granulocytes expressing CD11b, CXCR1 and CXCR2 and the density of expression (MFI) post to pre delivery for CS and IOL. Transformed data are shown with scatter plots with median and interquartile range indicated. Analysis was also performed on log transformed data where log Δ CS = (log CS post)- (log CS pre) and log Δ IOL = (log IOL post) – (log IOL pre) and p values are shown for each graph.

5.4.3.2.2 Monocytes

Monocyte data in response to delivery are illustrated in Figure 79. The percentage of monocytes expressing CD11b did not change significantly in response to CS (p=0.89), however the density of expression fell (p=0.04). In response to IOL, the % of monocytes expressing CD11b increased (p=0.014), but density of expression did not alter significantly (p=0.09).

CCR2 MFI decreased in response to CS (CS-pre vs CS-post (p=0.028)), however no other measured monocyte parameters changed significantly in response to delivery (Figure 79).

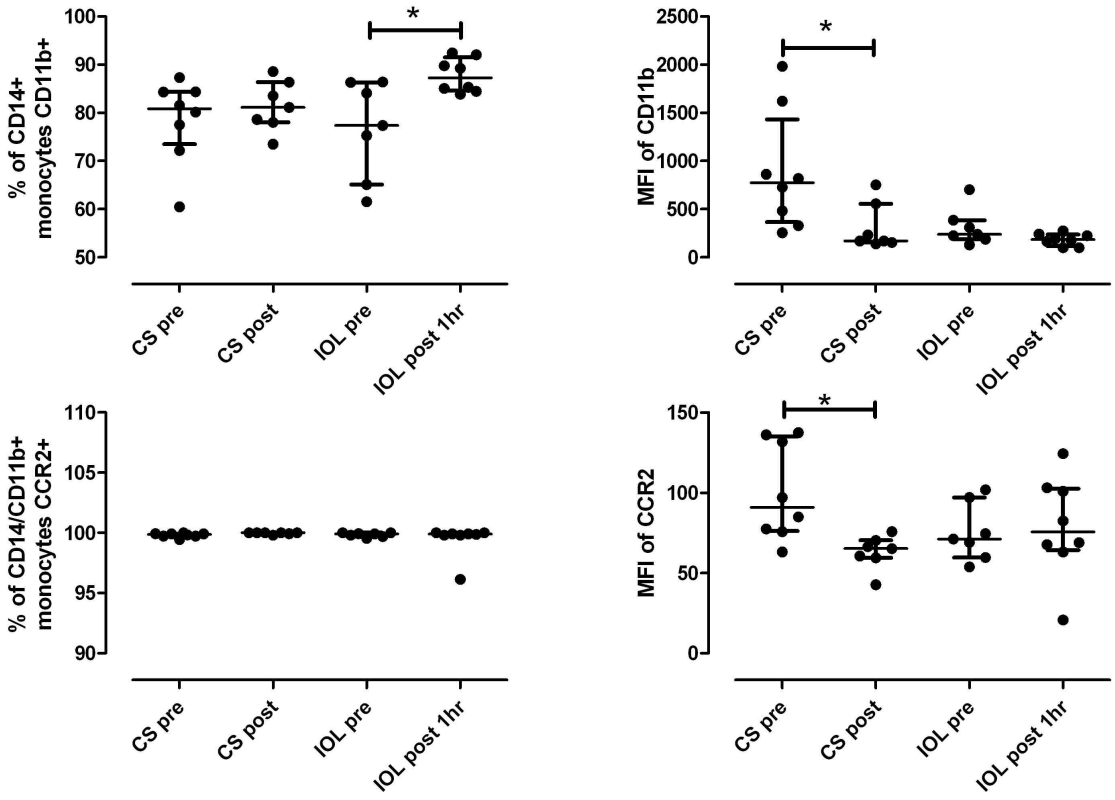

Figure 79 Monocytes pre and post delivery

Assessment of monocytes in the maternal circulation in response to delivery by either planned caesarean delivery (CS) or vaginal delivery after induction of labour (IOL). Parameters examined are 1) the % of CD14+ monocytes expressing CD11b and the cell surface density of CD11b, and 2) the % of CD14/CD11b+ monocytes expressing CCR2 and the cell surface density of CCR. The cell surface density is indicated by the mean fluorescence intensity (MFI). Raw data are shown with median and IQR. Data were log transformed prior to analysis, Paired t-tests were performed with lines demonstrating significance value between 2 samples under start and end of each line. *p<0.05,p≤0.01, ***p≤0.001.**

The extent of changes to any of the measured monocyte parameters did not differ significantly by mode of delivery (Figure 80).

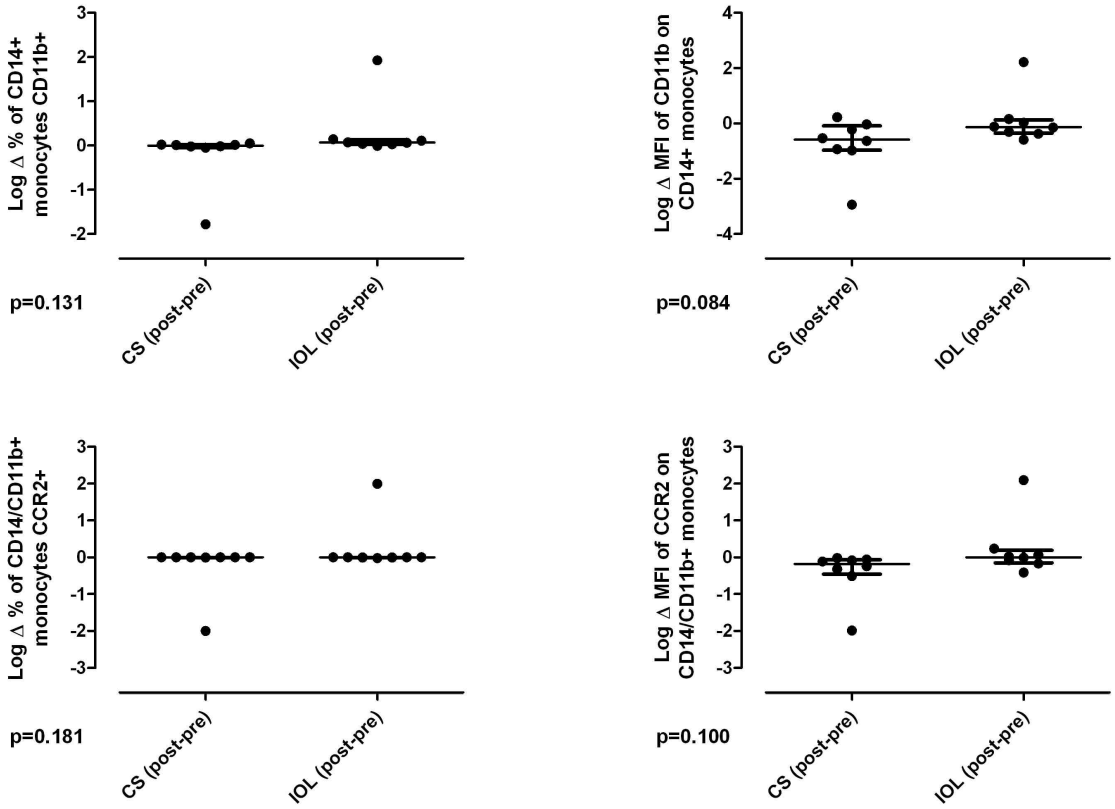

Figure 80 Δmonocytes (post-pre) delivery

Change in the % of circulating monocytes expressing CD11b and CCR2 and the density of expression (MFI) post to pre delivery for CS and IOL. Transformed data are shown with scatter plots with median and interquartile range indicated. Analysis was also performed on log transformed data where log Δ CS = (log CS post)- (log CS pre) and log Δ IOL = (log IOL post) – (log IOL pre) and p values are shown for each graph.

5.4.3.2.3 Lymphocytes

The percentage of T-cells (CD3+ lymphocytes) expressing either CD4 (T-h cells) or CD8 (T-c cells) did not change significantly in response to delivery by either CS (CD3+/CD4+ CS-pre vs CS-post (p=0.22), CD3+/CD8+ CS-pre vs CS-post (p=0.12)) or IOL(CD3+/CD4+ IOL-pre vs IOL-post (p=0.70), CD3+/CD8+ IOL-pre vs IOL-post (p=0.69)) (Figure 81). No significant difference according to mode of delivery was confirmed by further analysis illustrated in Figure 82.

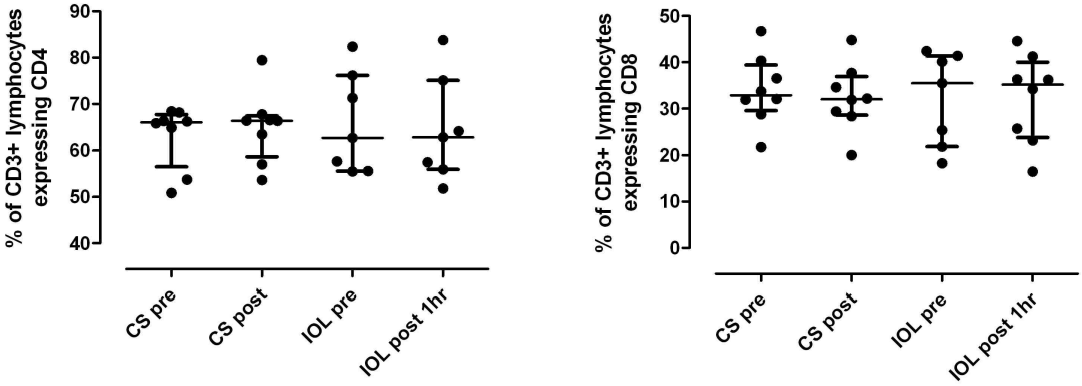

Figure 81 CD3+/CD4+ and CD3+/CD8+ lymphocytes pre and post delivery

Measurements of the % of circulating CD3+/CD4+ (T helper lymphocytes) and the % of CD3+/CD8+ (T cytotoxic lymphocytes) in the maternal circulation in response to delivery by either planned caesarean delivery (CS) or vaginal delivery after induction of labour (IOL). Raw data are shown with median and IQR. Data were log transformed prior to analysis.

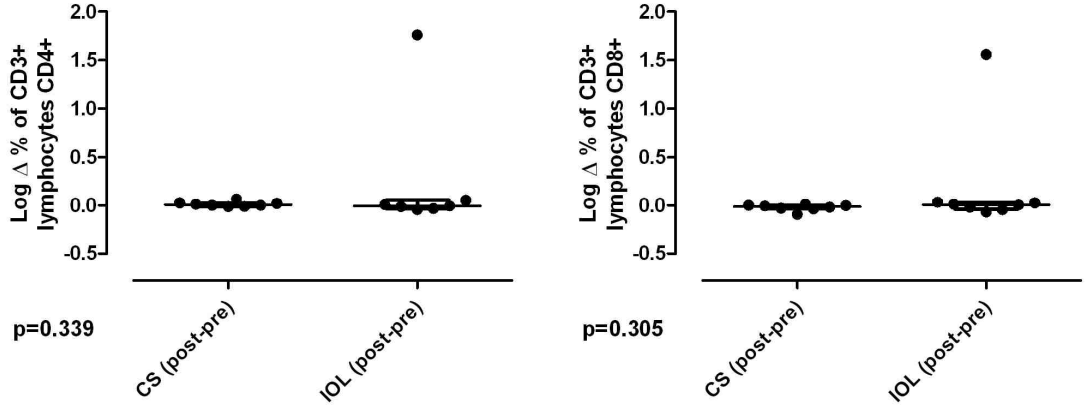

Figure 82 Δ CD3+/CD4+ and CD3+/CD8+ lymphocytes (post-pre) delivery

Change in the % of circulating CD3+ lymphocytes expressing either CD4 (T-helper cells) or CD8 (T-cytotoxic cells) post to pre delivery for CS and IOL. Transformed data are shown with scatter plots with median and interquartile range indicated. Analysis was also performed on log transformed data where log Δ CS = (log CS post)- (log CS pre) and log Δ IOL = (log IOL post) – (log IOL pre) and p values and shown for each graph.

All measures of chemokine receptor expression on T-h cells (CD3/CD4+ lymphocytes) were unchanged by delivery with the exception of the density of CXCR2 and CCR6 which decreased in response to CS (CS-pre vs CS-post p=0.037, p=0.027 respectively), however this was not observed in response to IOL (IOL-pre vs IOL-post p=0.17, p=0.50 respectively) (Figure 83). The extent of changes to any of the measured T-h lymphocyte parameters did not differ significantly by mode of delivery (Figure 84).

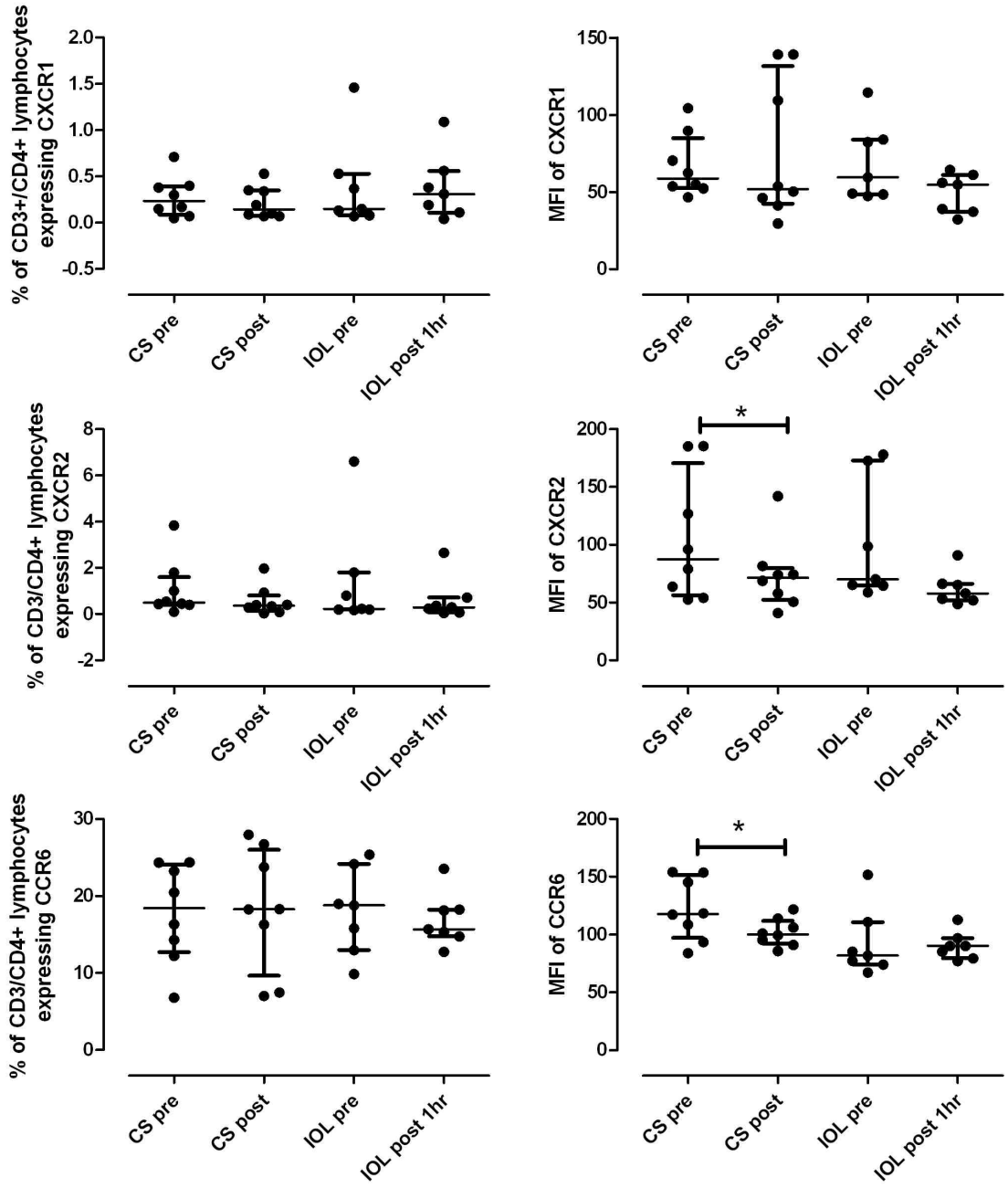

Figure 83 CD3+/CD4+ lymphocytes pre and post delivery

Assessment of CD3+/CD4+ lymphocytes (T-helper cells) in the maternal circulation in response to delivery by either planned caesarean delivery (CS) or vaginal delivery after induction of labour (IOL). Parameters examined are 1) the % of T-helper lymphocytes expressing CXCR1 and cell surface density of CXCR1, 2) the % or T-helper lymphocytes expressing CXCR2 and the cell surface density of CXCR2, and 3) the % of T-helper lymphocytes expressing CCR6 and the cell surface density of CCR6. The cell surface density is indicated by the mean fluorescence intensity (MFI). Raw data are shown with median and IQR. Data were log transformed prior to analysis, Paired t-tests were performed with lines demonstrating significance value between 2 samples under start and end of each line. *p<0.05, **p<0.01.

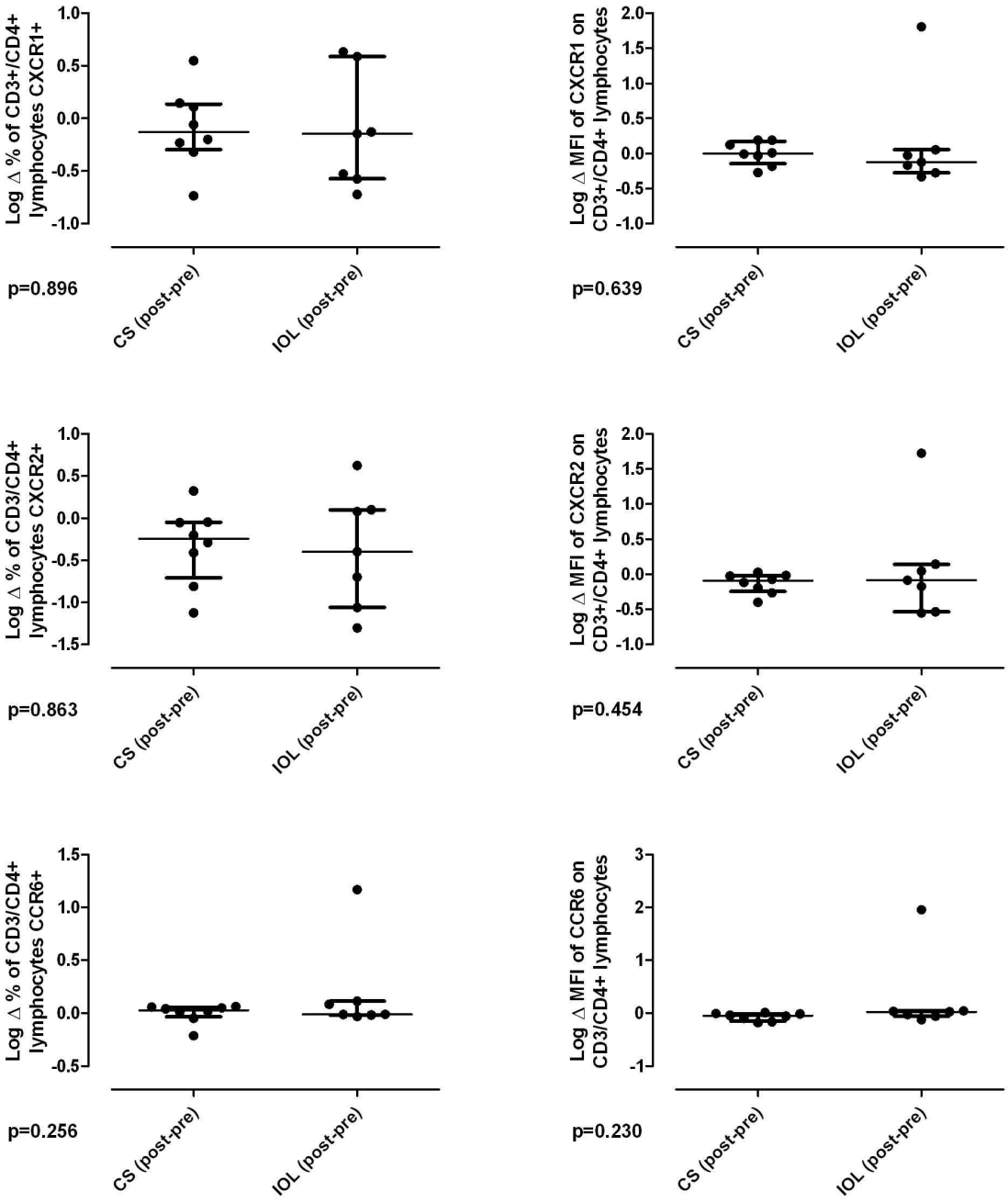

Figure 84 Δ CD3+/CD4+ lymphocytes (post-pre) delivery

Change in % of circulating CD3/CD4+ lymphocytes (T-helper cells) expressing CXCR1, CXCR2 and CCR6 post to pre delivery for CS and IOL. Transformed data are shown with scatter plots with median and interquartile range indicated. Analysis was also performed on log transformed data where log Δ CS = (log CS post)- (log CS pre) and log Δ IOL = (log IOL post) – (log IOL pre) and p values are shown for each graph.

Analysis of T-c cell chemokine receptor expression of CXCR1 and CXCR2 showed no significant response to either type of delivery. In response to IOL, the % of T-cytotoxic cells expressing CCR6 decreased (p=0.048), however density of expression remained stable (p=0.98). In response to CS, the % of T-cytotoxic cells expressing CCR6 did not alter significantly (p=0.048), while the density of expression fell (p=0.045) (Figure 85). The extent of changes to any of the measured T-c lymphocyte parameters did not differ significantly by mode of delivery (Figure 86).

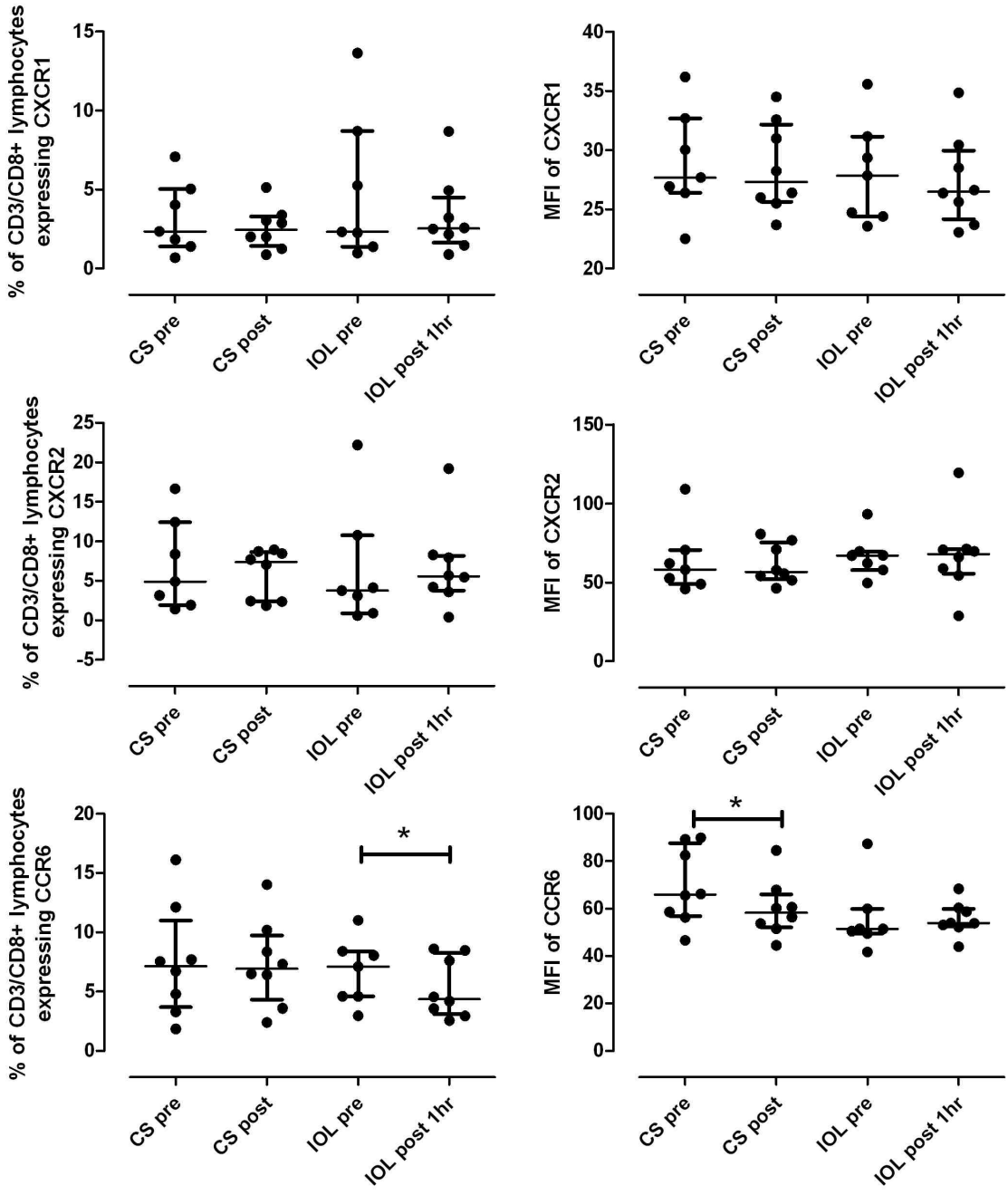

Figure 85 CD3+/CD8+ lymphocytes pre and post delivery

Assessment of CD3+/CD8 lymphocytes (T-cytotoxic) in the maternal circulation in response to delivery by either planned caesarean delivery (CS) or vaginal delivery after induction of labour (IOL). Parameters examined are 1) the % of T-cytotoxic lymphocytes expressing CXCR1 and cell surface density of CXCR1, 2) the % or T-cytotoxic lymphocytes expressing CXCR2 and the cell surface density of CXCR2, and 3) the % of T-cytotoxic lymphocytes expressing CCR6 and the cell surface density of CCR6. The cell surface density is indicated by the mean fluorescence intensity (MFI). Raw data are shown with median and IQR. Data were log transformed prior to analysis, Paired t-tests were performed with lines demonstrating significance value between 2 samples under start and end of each line. *p<0.05, **p<0.01.

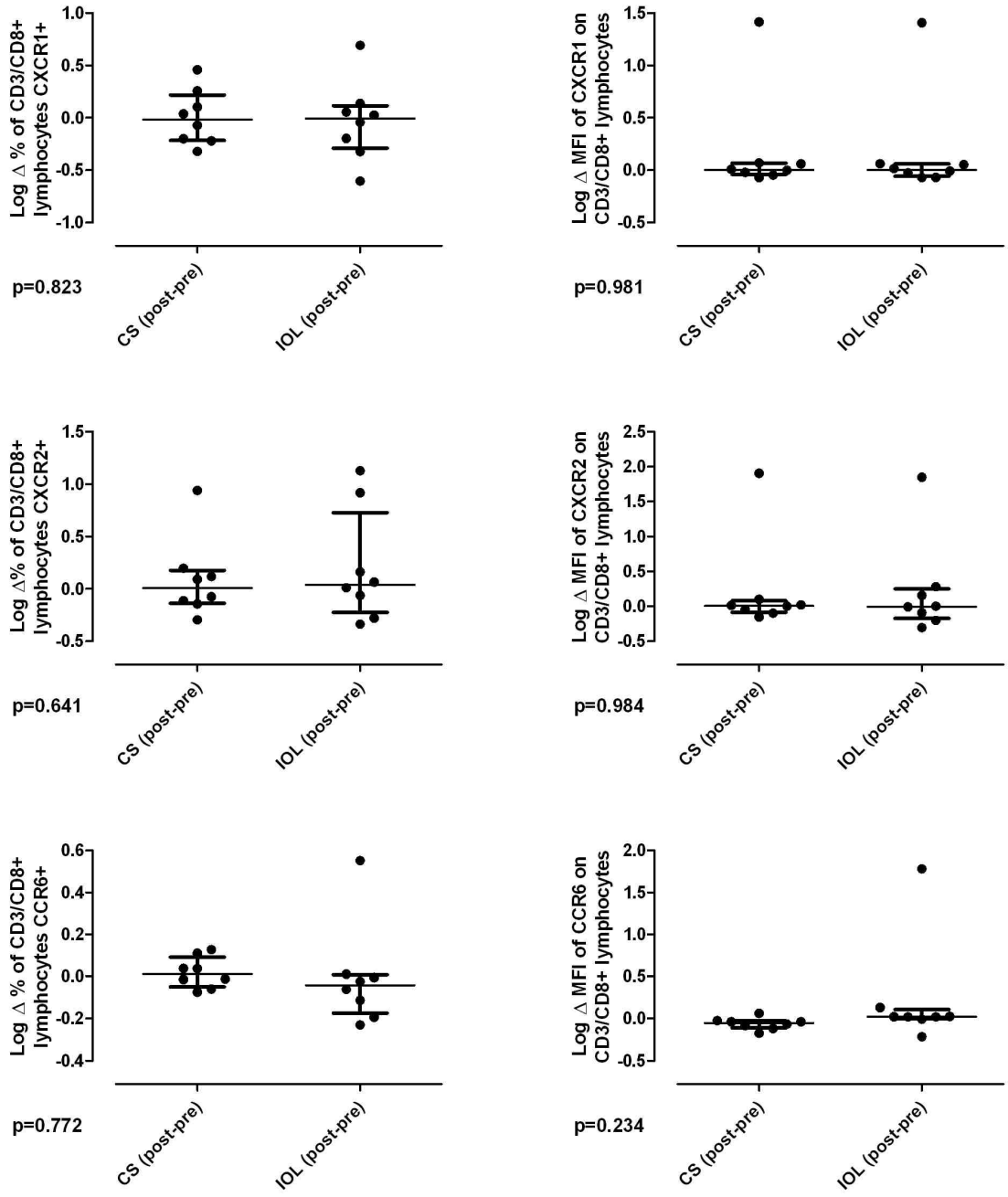

Figure 86 ΔCD3+/CD8+ lymphocytes (post-pre) delivery

Change in % of circulating CD3/CD8+ lymphocytes (T-cytotoxic cells) expressing CXCR1, CXCR2 and CCR6 post to pre delivery for CS and IOL. Transformed data are shown with scatter plots with median and interquartile range indicated. Analysis was also performed on log transformed data where log Δ CS = (log CS post)- (log CS pre) and log Δ IOL = (log IOL post) – (log IOL pre) and p values are shown for each graph.

5.4.3.3Circulating cytokines and chemokines

Cytokine data are described in Table 29. Data for the cytokines FLT3, IL1β, IL1RA, IL3, IL4, IL5, IL12p40, IL13 and TNF β was not analysed for any comparison since more than four values were missing as measures for these analytes were below the threshold of sensitivity.

Four cytokines were found to change in response to CS but not IOL; FGF2 and TGFα decreased (CS-pre vs CS-post p= 0.004, and p=0.006 respectively), while IL2 and IL6 increased, (CS-pre vs CS-post p=0.003 and p=0.04 respectively).

There were changes to a further 8 cytokines in response to IOL but changes were not significant in response to CS. CCL11, IFNγ and CXCL10 decreased (IOL-pre vs IOL-post, p=0.007, p=0.034, p=<0.001 respectively). There were significant increases in GCSF, CXCL1,2,3, IL10, CCL2 and CCL7 (IOL-pre vs IOL-post, p=0.021, p=0.008, p=0.002. p=0.001, p=0.048 respectively).

Cytokine/ Chemokine (pg/ml)		Study Group		Paired t-test	Study Group		Paired t-test	log Δ CS vs log Δ IOl
		CS-pre	CS-post	p-value n pairs	IOL-pre	IOL-post	p-value n pairs	p-value
EGF	Median (IQR) [range] N	31.42 (3.97,60.42) [6.12,64.06] 6	6.205 (3.190,62.58) [3.918,81.33] 4	0.087 4	10.84 [7.840,13.83] 2	13.94 (6.115,19.31) [3.970,22.35] 6	/	/
CCL11	Median (IQR) [range] N	103.3 (92.74,231.8) [75.69,429.2] 8	98.56 (87.26,209.9) [78.23,293.1] 8	0.056 8	187.2 (119.9,221.2) [66.66,263.0] 8	137.8 (104.3,173.3) [54.46,200.3] 8	0.007** 8	0.142
FGF-2	Median (IQR) [range] N	93.33 (87.92,103.3) [55.85,119.4] 8	56.04 (40.66,75.31) [34.20,88.27] 8	0.004** 8	56.75 (40.79,68.85) [21.92,71.68] 8	55.47 (39.41,70.02) [16.68,73.67] 8	0.751 8	0.022*
Flt-3	Median [range] N	38.25 1	34.79 1	/	5.34 [1.30,9.37] 2	12.30 [4.65,19.94] 2	/	/

Cytokine/ Chemokine (pg/ml)		Study Group		Paired t-test	Study Group		Paired t-test	log Δ CS vs log Δ IOl
		CS-pre	CS-post	p-value n pairs	IOL-pre	IOL-post	p-value n pairs	p-value
CX3CL1	Median (IQR) [range] N	21.27 (8.520,27.04) [4.830,66.83] 7	33.58 (7.455,56.05) [4.250,66.83] 5	0.0513 5	60.07 (36.93,734.6) [27.04,1370] 5	50.14 (31.21,418.9) [4.250,1379] 6	0.726 5	0.778
GCSF	Median (IQR) [range] N	43.22 (23.97,78.88) [17.02,129.6] 8	76.38 (34.85,96.95) [18.26,158.5] 8	0.198 8	74.74 (43.28,99.61) [33.22,154.3] 8	141.5 (86.13,413.1) [68.91,496.4] 8	0.021* 8	0.133
GMCSF	Median (IQR) [range] N	76.97 (31.43,190.9) [8.450,306.3] 8	41.95 (22.06,160.6) [15.24,311.0] 8	0.131 8	46.89 (24.38,110.6) [10.29,139.7] 8	70.69 (23.06,145.3) [12.22,190.1] 8	0.221 8	0.047*
CXCL1,2,3,	Median (IQR) [range] N	1687 (1207,2140) [940.6,3038] 8	1239 (917.1,1590) [883.3,3025] 8	0.070 8	804.0 (481.2,992.2) [238.1,1218] 8	1160 (792.1,1621) [641.6,3273] 8	0.008** 8	0.001***
IFNα2	Median (IQR) [range] N	115.4 [42.18,188.6] 2	103.0 [42.90,163.1] 2	/	15.24 (12.92,58.17) [12.15,72.48] 4	15.24 (4.940,58.52) [0.7900,95.64] 5	0.712 4	/
IFNγ	Median (IQR) [range] N	8.800 (2.140,14.23) [2.070,91.04] 7	10.08 (5.135,50.74) [3.330,89.72] 5	0.813 5	6.510 (2.688, 55.49) [2.340,67.31] 8	4.140 (2.850,28.26) [2.040,57.72] 8	0.034* 8	0.175
IL1α	Median (IQR) [range] N	166.8 (78.90, 375.7) [59.58, 938.0] 8	139.7 (85.96, 298.4) [54.86, 844.1] 7	0.178 7	192.2 (111.8, 271.5) [67.67, 274.3] 6	185.1 (101.7, 198.1) [89.78, 204.2] 5	0.225 5	0.760
IL1ß	Median N	1.34 1	1.64 1	/	1.34 1	1.34 1	/	/
IL1ra	Median [range] N	6.46 1	5.38 1	/	2.64 1	4.21 [3.93,5.68] 3	/	/
IL2	Median (IQR) [range] N	2.640 (0.8550, 3.390) [0.7800, 4.000] 5	3.295 (1.505, 4.673) [0.9300,5.110] 4	0.003** 4	1.160 (0.995,3.850) [0.930,4.550] 5	1.410 (0.830, 11.10) [0.810, 18.16] 5	0.274 4	0.504

Cytokine/ Chemokine (pg/ml)		Study Group		Paired t-test	Study Group		Paired t-test	log Δ CS vs log Δ IOl
		CS-pre	CS-post	p-value n pairs	IOL-pre	IOL-post	p-value n pairs	p-value
IL3	Median	5.24	4.62	/	29.57	32.13	/	/
	N	1	1		1	1		
Il4	Median			/	75.88		/	/
	N	0	0		1	0		
IL5	Median [range]	0.38 [0.23,0.53]	0.74	/	0	0	/	/
	N	2	1					
IL6	Median (IQR) [range]	8.820 (4.335, 63.76) [3.360, 185.2]	23.80 (16.34, 50.40) [12.11, 133.8]	0.040* 6	11.79 (3.025, 283.6) [2.690, 549.3]	63.68 (34.01, 158.8) [19.97, 248.5]	0.093 5	0.350
	N	6	7		5	8		
IL7	Median (IQR) [range]	5.660 (3.665, 38.72) [2.000, 41.44]	18.51 (2.873 , 32.36) [2.210, 32.42]	0.182 4	7.405 [3.600, 11.21]	4.825 [2.90, 6.75]	/	/
	N	5	4		2	2		
CXCL8	Median (IQR) [range]	17.57 (6.930, 30.19) [3.010, 63.20]	7.76 (4.593, 17.95) [2.720, 64.48]	0.060 8	16.03 (6.675, 42.35) [4.740, 65.12]	24.74 (12.81, 43.79) [9.130, 64.91]	0.330 8	0.057
	N	8	8		8	8		
IL9	Median (IQR) [range]	2.89	2.84	/	4.940 (1.985, 44.09) [1.640, 78.71]	4.990 (3.110, 17.91) [2.080, 29.43]	0.762 5	/
	N	1	1		5	5		
IL10	Median (IQR) [range]	2.200 (1.605, 5.960) [1.260, 21.52]	3.150 (2.210, 5.890) [1.610, 19.67]	0.191 8	1.815 (1.535, 3.040) [1.490, 3.130]	16.29 (6.478, 29.59) [2.030, 101.6]	0.002** 6	<0.001***
	N	8	8		6	8		
IL12 (p40)	Median [range]	207.4	174.1	/	179.8 [170.6,192.5]	209.6 [193.5,220.5]	/	/
	N	1	1		3	3		
IL12 (p70)	Median (IQR) [range]	2.940 [1.360, 372.5]	226.8 [3.090, 450.6]	/	2.340 (1.855, 202.8) [1.490, 402.1]	6.945 (2.473, 101.0) [2.270, 131.1]	0.888	/
	N	3	2		5	4		
IL13	Median [range]	1.15	0	/	0	2.245 [0.87,3.72]	/	/
	N	1				2		

Cytokine/ Chemokine (pg/ml)		Study Group		Paired t-test	Study Group		Paired t-test	log Δ CS vs log Δ IOl
		CS-pre	CS-post	p-value n pairs	IOL-pre	IOL-post	p-value n pairs	p-value
IL15	Median (IQR) [range] N	0.92 [0.68, 3.30] 3	1.965 [0.840, 3.090] 2	/	1.330 (0.765, 3.31) [0.480, 4.57] 5	1.905 (1.440, 2.958) [0.780, 4.990] 6	0.712	/
IL17	Median (IQR) [range] N	18.97 (5.990, 66.67) [3.980, 140.6] 6	9.995 (4.365, 65.85) [3.720, 143.8] 6	/	12.29 (3.140, 57.51) [1.720, 116.2] 8	4.925 (3.763, 24.30) [2.120, 82.66] 8	0.095	/
CXCL10	Median (IQR) [range] N	823.8 (537.7, 1464) [272.0, 4959] 8	660.1 (474.7, 1357) [288.4, 3676] 8	0.061 8	565.5 (425.5, 718.7) [225.0, 903.2] 8	399.8 (313.1, 429.5) [158.5, 460.2] 8	<0.001*** 8	0.013*
CCL2	Median (IQR) [range] N	235.4 (210.3, 305.0) [157.6, 503.3] 8	282.6 (232.7, 449.2) [146.3, 553.5] 8	0.211 8	229.9 (172.2, 282.4) [95.36, 469.1] 8	397.4 (241.4, 592.0) [146.4, 1093] 8	0.001** 8	0.027*
CCL7	Median (IQR) [range] N	7.700 (2.313, 26.20) [2.110, 51.04] 6	4.260 (2.650, 14.49) [2.110, 42.43] 7	0.138 6	6.290 (1.830, 10.28) [1.550, 12.18] 5	8.290 (5.440, 12.67) [4.190, 17.14] 7	0.048* 4	0.003**
CCL22	Median (IQR) [range] N	547.2 (406.4, 1035) [259.6, 1291] 8	539.2 (373.2, 879.6) [227.0, 1228] 8	0.270 8	566.1 (349.8, 1369) [321.4, 1978] 8	484.2 (312.1, 1201) [230.4, 1514] 8	0.154 8	0.492
CCL3	Median (IQR) [range] N	41.21 (28.08, 97.76) [25.20, 124.2] 5	34.34 (19.08, 103.1) [9.660, 148.2] 5	0.357 5	38.60 (22.71, 177.5) [21.12, 178.3] 7	30.35 (24.52, 114.4) [23.40, 152.2] 7	0.216 7	0.708
CCL4	Median (IQR) [range] N	57.55 (24.02, 95.48) [20.47, 323.2] 8	36.55 (25.53, 70.74) [24.16, 310.9] 8	0.1977 8	42.98 (18.55, 176.9) [6.170, 217.6] 8	30.77 (15.68, 117.5) [5.580, 141.5] 8	0.259 8	0.545
sCD40L	Median (IQR) [range] N	4130 1	2649 (1783, 5586) [1563, 10393] 7	/	3447 (1551, 7070) [738.3, 8610] 8	3861 (1507, 4600) [727.8, 5633] 8	0.777 8	/

Cytokine/ Chemokine (pg/ml)		Study Group		Paired t-test	Study Group		Paired t-test	log Δ CS vs log Δ IOl
		CS-pre	CS-post	p-value n pairs	IOL-pre	IOL-post	p-value n pairs	p-value
sIL2Rα	Median	8.570	8.300		1.180	9.835		
	(IQR)	(5.770, 51.51)	(4.525, 41.88)	0.2357			/	/
	[range]	[4.560, 65.05]	[1.810, 56.22]	4		[3.200, 16.47]		
	N	5	5		1	2		
TGFα	Median	8.010	2.200		2.750	4.860		
	(IQR)	(3.490, 8.440)	(1.250, 5.940)	0.006**	(0.483, 9.143)	(3.590, 8.245)	0.213	0.011*
	[range]	[3.220, 12.99]	[1.150, 12.51]	7	[0.340, 21.06]	[3.340, 9.140]	5	
	N	7	7		6	5		
TNFα	Median	4.830	4.185		3.865	3.245		
	(IQR)	(3.198, 9.235)	(1.893, 6.803)	0.054	(1.183, 7.113)	(1.433, 7.108)	0.454	0.359
	[range]	[1.740, 15.44]	[1.320, 14.29]	8	[0.810, 8.380]	[0.580, 8.120]	8	
	N	8	8		8	8		
TNFß	Median	1.0	0.85		1.12	1.0		
	[range]	[0.78,1.88]	[0.74,2.18]	/			/	/
	N	3	3		1	1		
VEGF	Median	146.5	103.7		113.5	26.79		
	(IQR)	(70.67, 161.9)	(29.10, 137.4)	0.0801	(14.46, 305.9)	(1.600, 168.4)	0.101	0.474
	[range]	[16.60, 352.8]	[24.98, 245.3]	7	[4.000, 347.5]	[1.600, 201.6]	4	
	N	7	7		4	5		
CCL5	Median	12464	12098		1466	6211		
	(IQR)	(6870, 19638)		/			/	/
	[range]	[362.4, 23230]	[1907, 22939]		[422.3, 12007]	[3493, 8929]		
	N	8	3		3	2		

Table 29 Circulating cytokine/chemokine levels pre and post delivery

Cytokine levels pre and post delivery. Raw data are presented with analysis performed on normalised log transformed data. Where changes in levels are examined, log Δ CS = (log CS post)- (log CS pre) and log Δ IOL = (log IOL post) – (log IOL pre). Where boxes are marked with / this indicates that less than 4 values were present (n expected in each group n=8), therefore analysis was not performed.

5.4.3.4Circulating CRP

CRP did not change significantly in response to delivery by either CS or IOL, (CS-pre vs CS-post p=0.05, IOL-pre vs IOL-post, p=0.18). However the extent of change in CRP levels was significantly more in response to delivery following IOL compared with CS (log Δ CS vs log Δ IOL, p=0.024). Data are shown in Figure 87.

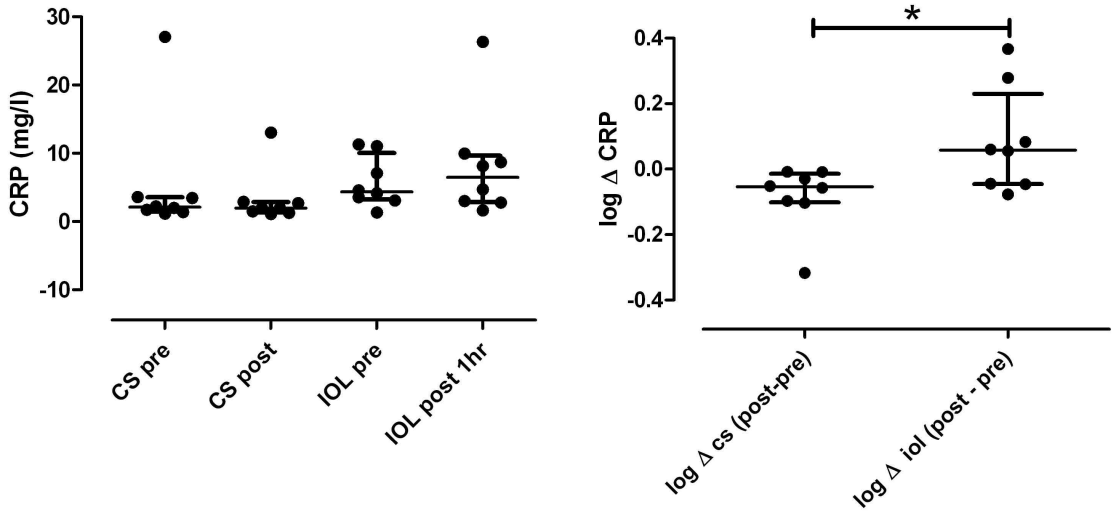

Figure 87 Circulating CRP pre and post delivery and ΔCRP (post-pre) delivery

Maternal circulating CRP in response to delivery by CS or IOL at term. No significant changes were induced, however the extent of change in CRP levels was significantly higher in response to IOL. Raw data and log transformed data are shown. Analysis was performed on log transformed data where log Δ CS = (log CS post)- (log CS pre) and log Δ IOL = (log IOL post) – (log IOL pre). Paired and unpaired t-tests were performed as appropriate with lines demonstrating significance value between 2 samples under start and end of each line. *p<0.05

5.4.4 Hypothesis 3 – In term pregnancies, circulatory changes observed pre and post IOL occur in a time dependent manner.

5.4.4.1Differential white blood cell count

Correlation plots and regression lines are shown for the normalised, log transformed values for each white cell sub-set in Figure 88. Levels of circulating white cells were correlated with the time sample was taken during labour, with time 0% equivalent to IOL –pre sample and time 100% equivalent to IOL-post sample. Changes in circulating leukocyte numbers occurred in a time dependent manner for WBC ($r=0.68$, $p<0.0001$), neutrophils ($r=0.68$, $p<0.0001$), lymphocytes ($r=-0.35$, $p=0.022$), monocytes ($r=0.45$, $p=0.002$) and eosinophils ($r=-0.60$, $p<0.0001$).

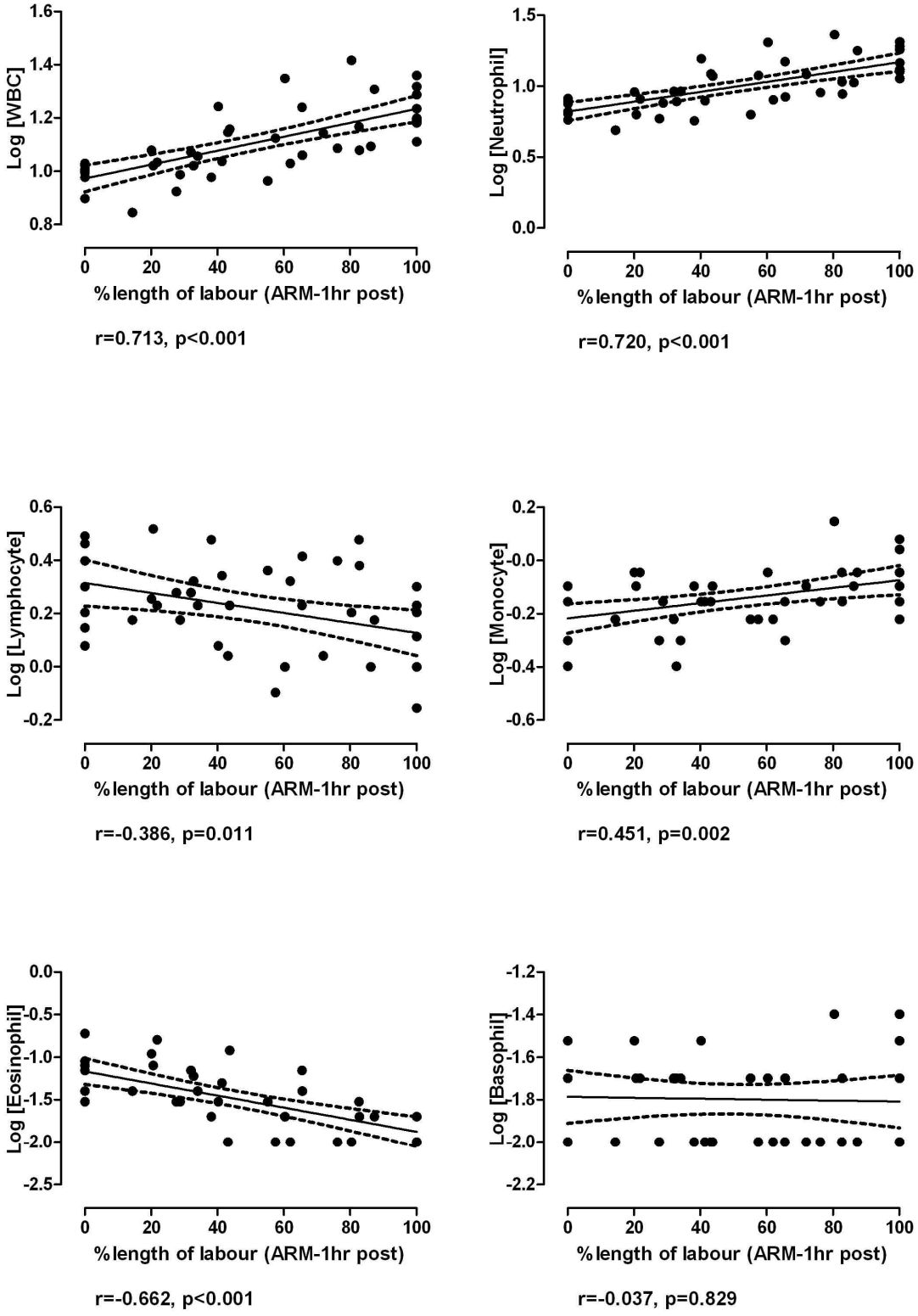

Figure 88 Correlation of WBC subtype with time in labour

Correlation plots and regression lines for each white blood cell sub-type and % length of labour (ARM-1hr post delivery of placenta). Log transformed data are shown. Pearson correlation r values and p values are shown for each graph.

5.4.4.2Leukocyte cell surface markers and chemokine receptors

5.4.4.2.1 Granulocytes

The association between granulocyte cell surface markers and receptors is illustrated in Figure 89, with significant changes occurring for CXCR1 and CXCR2 expression on neutrophils The percentage of neutrophils expressing CXCR1 and CXCR2 both were positively associated with the time in labour (CXCR1 r=0.44, p=0.003, CXCR2 r=0.48, p=0.001), with the density of cell surface expression of both of these receptors falling with time in labour (CXCR1 r=-0.56, p<0.001, CXCR2 r=-0.48, p=0.001).

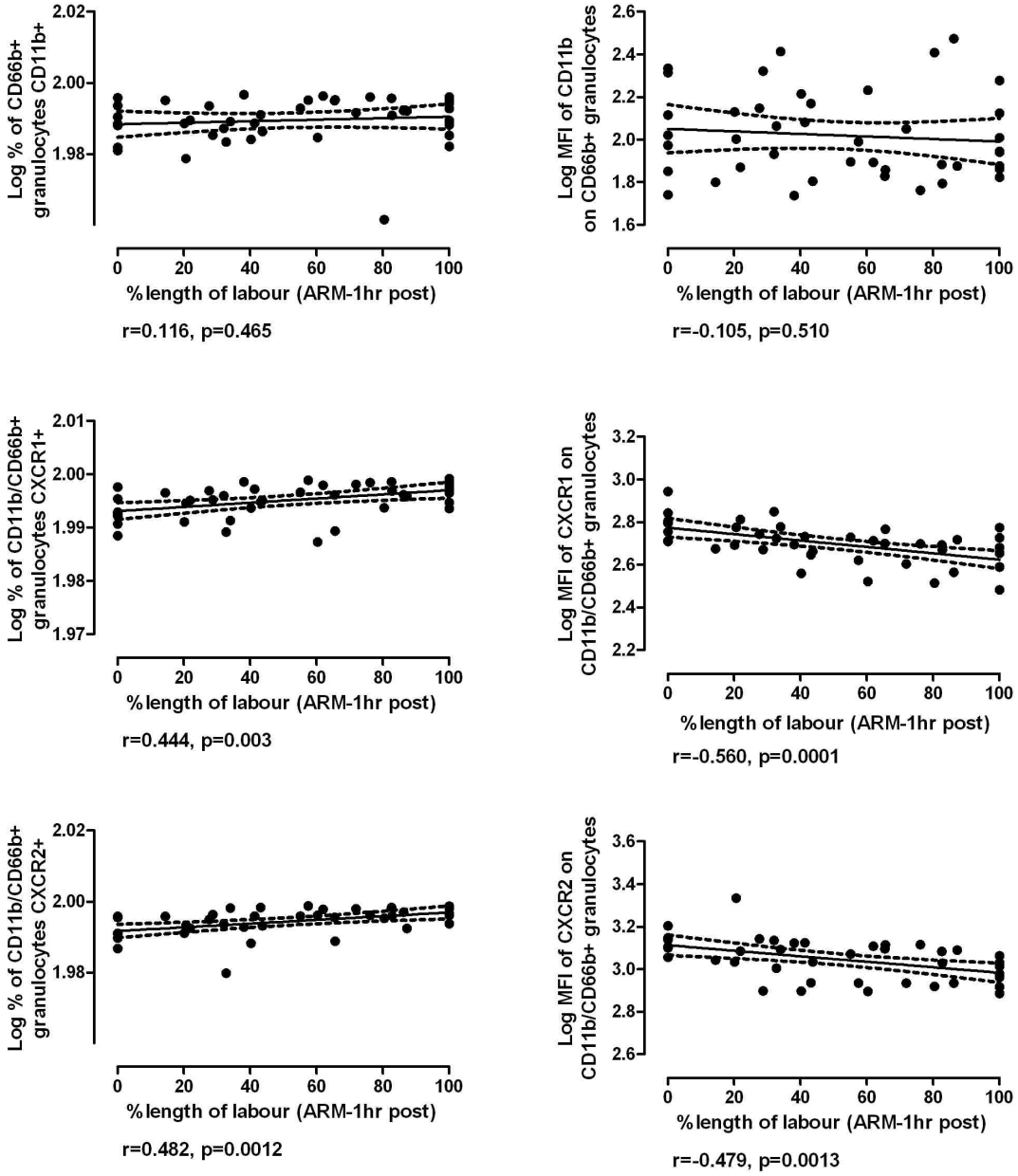

Figure 89 Correlation of granulocytes with time in labour

Correlation plots and regression lines with 95% confidence intervals for % of circulating granulocytes expressing CD11b, CXCR1 and CXCR2, and the cell surface density of expression (MFI) plotted against the length of time in labour (ARM-1hr post delivery of placenta). Data shown and used for analysis was log transformed, Pearson correlation r values and significance p values and shown for each graph.

5.4.4.2.2 Monocytes

No changes in monocytes receptor expression occurred in association with length of time in labour (Figure 90).

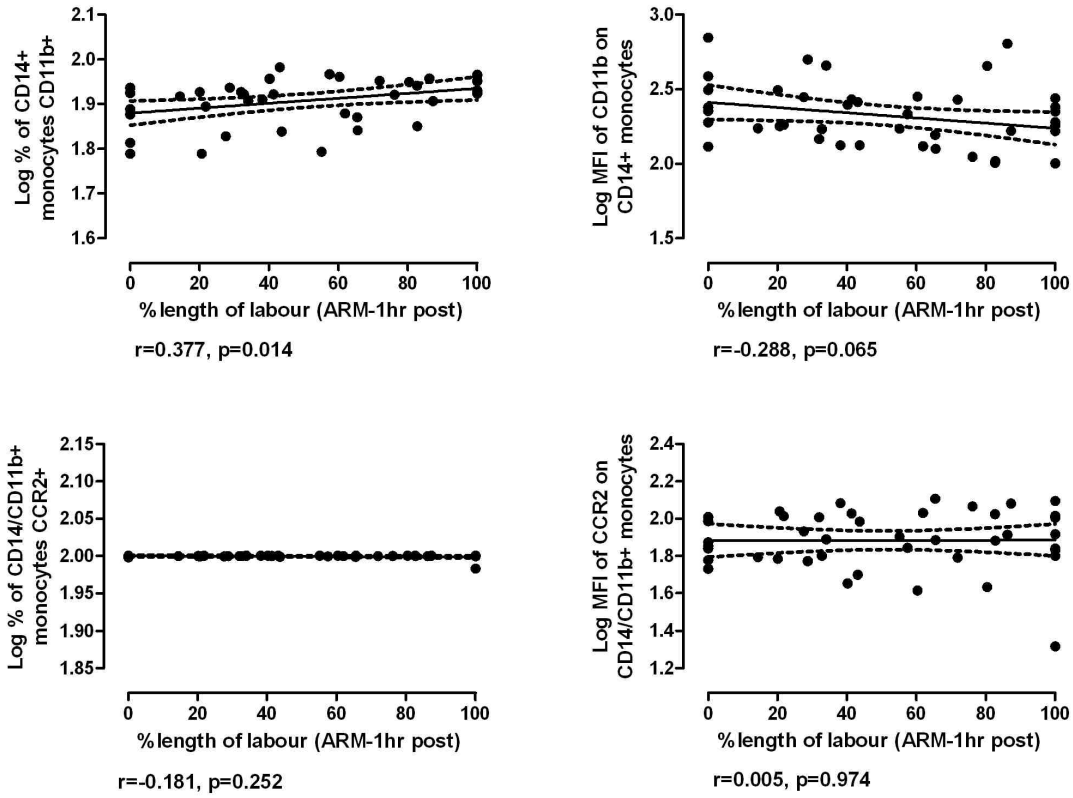

Figure 90 Correlation of monocytes with time in labour

Correlation plots and regression lines with 95% confidence intervals for % of circulating monocytes expressing CD11b, CCR2, and the cell surface density of expression (MFI) plotted against the length of time in labour (ARM-1hr post delivery of placenta). Data shown and used for analysis was log transformed, Pearson correlation r values and significance p values and shown for each graph.

5.4.4.2.3 Lymphocytes

Changes in lymphocytes are illustrated in Figure 91, Figure 92 and Figure 93. Despite no significant difference between pre and post IOL sample, a negative correlation was found between time in labour and the MFI of CXCR1 on T-helper cells (r=-0.324, p=0.042).

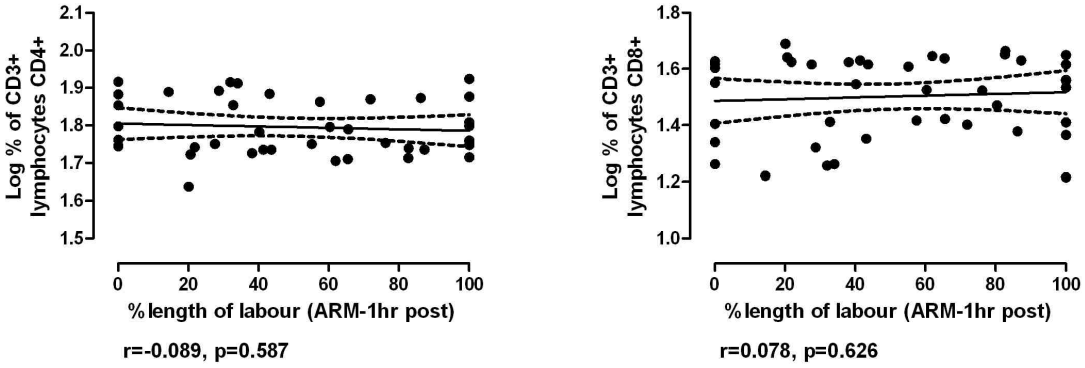

Figure 91 Correlation of CD3+/CD4+ and CD3+/CD8+ lymphocytes with time in labour

Correlation plots and regression lines with 95% confidence intervals for % of circulating CD3+ lymphocytes expressing CD4 (T-helper cells) of CD8 (T-cytotoxic cells) plotted against the length of time in labour (ARM-1hr post delivery of placenta). Data shown and used for analysis was log transformed, Pearson correlation r values and significance p values and shown for each graph.

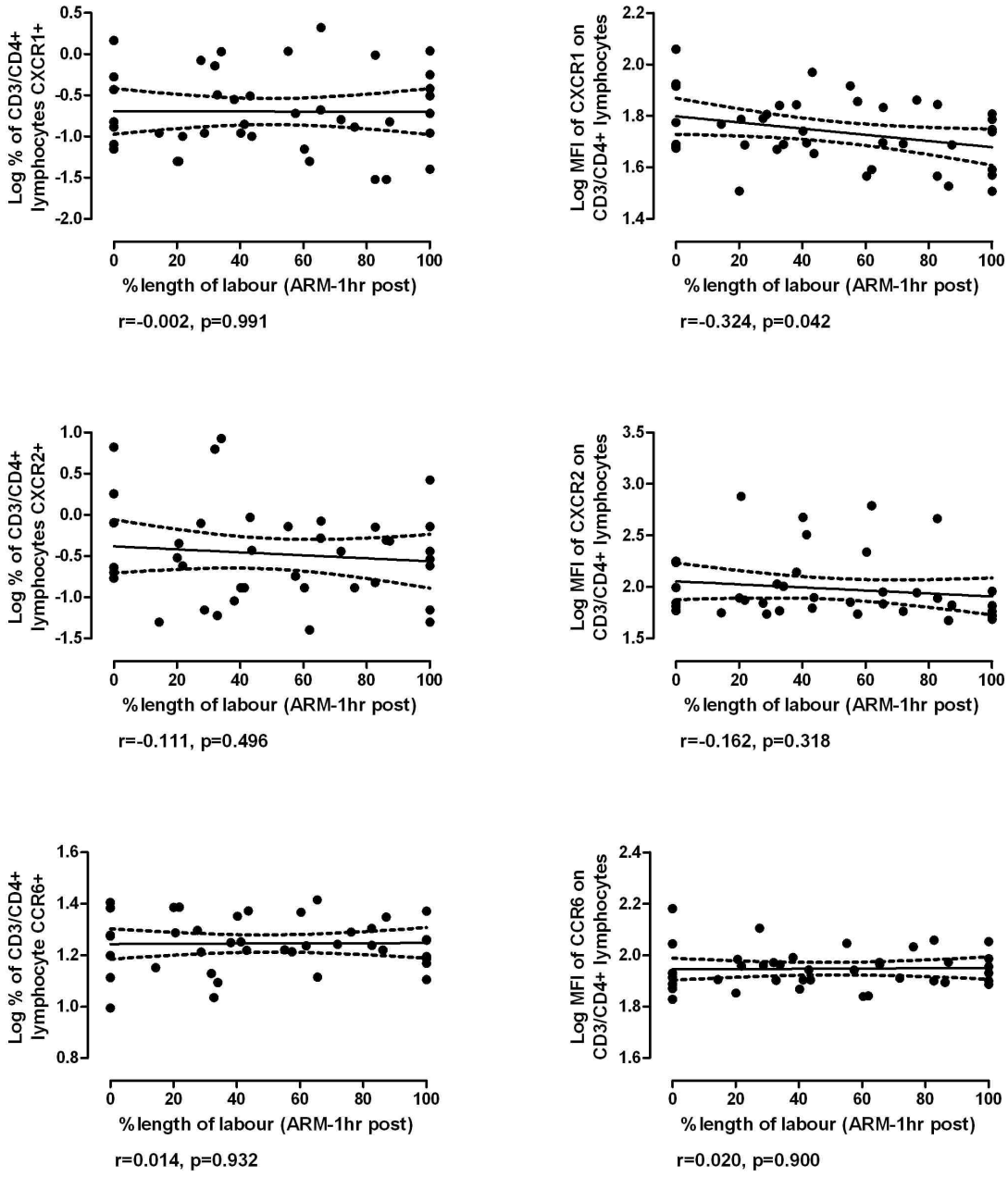

Figure 92 Correlation of CD3+/CD4+ lymphocytes with time in labour

Correlation plots and regression lines with 95% confidence intervals for % of circulating CD3/CD4+ lymphocytes (T-helper cells) expressing CXCR1, CXCR2 and CCR6 and the cell surface density of expression (MFI) plotted against the length of time in labour (ARM-1hr post delivery of placenta). Data shown and used for analysis were log transformed, Pearson correlation r values and significance p values and shown for each graph.

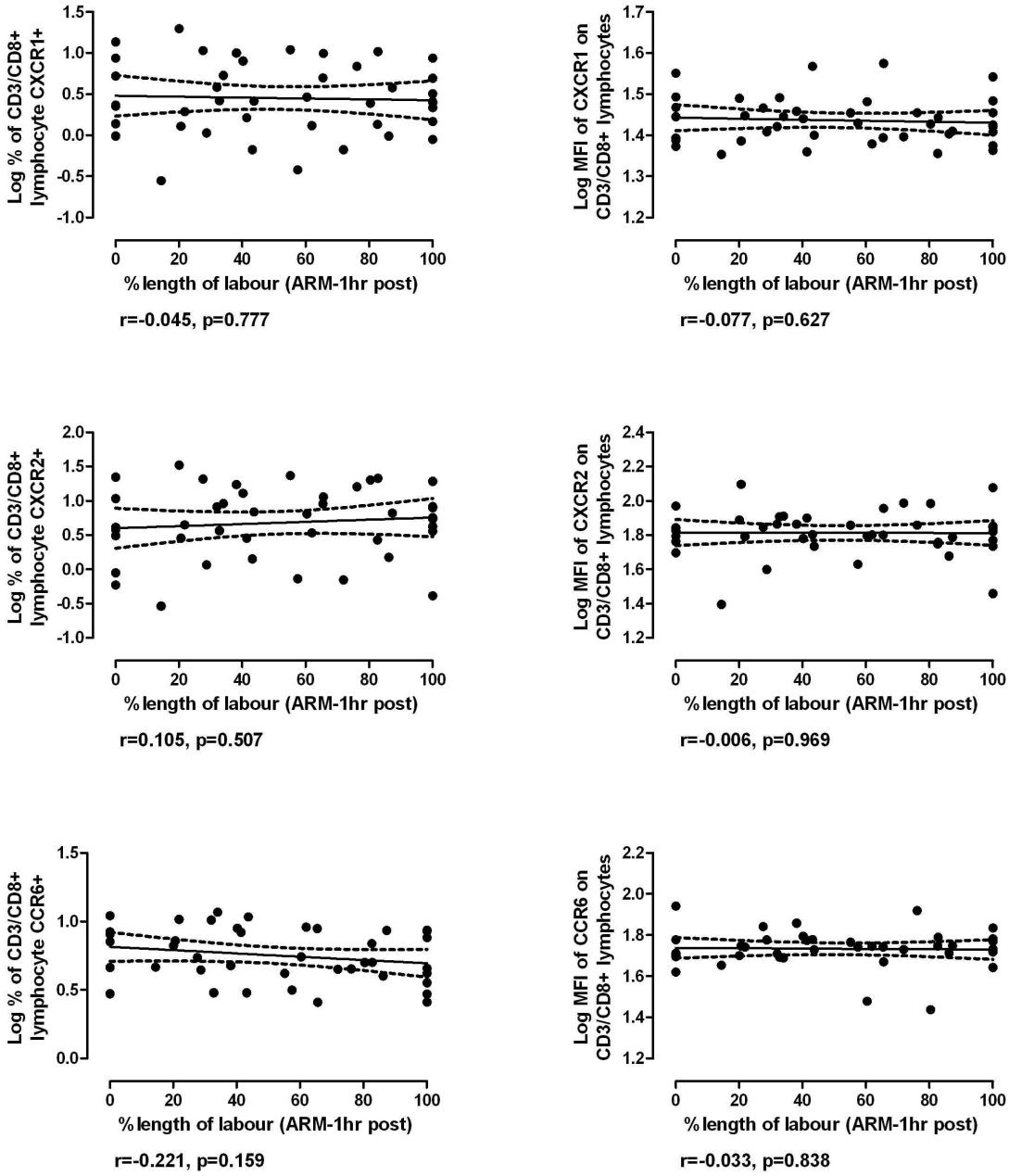

Figure 93 Correlation of CD3+/CD8+ lymphocytes with time in labour

Correlation plots and regression lines with 95% confidence intervals for % of circulating CD3/CD8+ lymphocytes (T-cytotoxic cells) expressing CXCR1, CXCR2 and CCR6 and the cell surface density of expression (MFI) plotted against the length of time in labour (ARM-1hr post delivery of placenta). Data shown and used for analysis were log transformed, Pearson correlation r values and significance p values and shown for each graph.

5.4.4.3Circulating cytokines and chemokines

All circulating cytokines were examined for the relationship between expression levels at the time in labour at which the sample was taken (Table 30). The cytokines FLT3, IL1ß, ILRA, IL3, IL4, IL5, IL7, IL12p40, IL13, sIL2Rα, TNFß and CCL5 were excluded as there were <50% of expected readings.

Cytokines which showed a significant positive correlation with time of labour were GCSF ($r=0.50$, $p<0.001$), CXCL1, 2, 3 ($r=0.30$, $p=0.047$), IL6 ($r=0.48$, $p=0.002$), CXCL8 ($r=0.35$, $p=0.023$), IL10 ($r=0.58$, $p<0.001$), CCL2 ($r=0.34$, $p=0.025$), CCL7 ($r=0.35$, $p=0.042$). CXCL10 showed a negative association with time in labour ($r=-0.32$, $p=0.036$).

Cytokine/Chemokine	Correlation with % time		Cytokine/Chemokine	Correlation with % time	
	n pairs	r-value	p-value	n pairs	r-value	p-value
EGF	25	0.643	0.760	IL9	24	-0.036	0.869
CCL11	43	-0.161	0.303	IL10	38	0.577	<0.001***
FGF-2	43	-0.055	0.725	IL12 (p40)	19	/	/
Flt-3	11	/	/	IL12 (p70)	22	0.108	0.631
CX3CL1	35	0.031	0.859	IL13	3	/	/
GCSF	43	0.504	<0.001***	IL15	30	0.198	0.294
GMCSF	43	0.135	0.388	IL17	41	0.026	0.869
CXCL1,2,3	43	0.305	0.047*	CXCL10	43	-0.320	0.036*
IFNα2	26	-0.219	0.282	CCL2	43	0.342	0.025*
IFNγ	42	0.015	0.925	CCL7	34	0.351	0.042*
IL1α	31	-0.142	0.446	CCL22	43	0.007	0.963
IL1β	3	/	/	CCL3	38	0.050	0.767
IL1ra	11	/	/	CCL4	43	-0.028	0.858
IL2	26	0.118	0.567	sCD40L	40	-0.107	0.513
IL3	5	/	/	sIL2Rα	8	/	/
Il4	4	/	/	TGFα	32	0.331	0.065
IL5	0	/	/	TNFα	43	0.013	0.936
IL6	40	0.481	0.002**	TNFβ	3	/	/
IL7	10	/	/	VEGF	24	-0.146	0.497
CXCL8	43	0.346	0.023*	CCL5	16	/	/

Table 30 Correlation of circulating cytokines/chemokines with time in labour

Table illustrating correlation of measured cytokines with % time during IOL (ARM-1hr post delivery of placenta). Analysis was performed using Pearson's correlation using log transformed data. Where <50% of expected cytokine readings were obtained (n<22) the cytokine was excluded from analysis. *p<0.05, **p≤0.01, ***p≤0.001

5.4.4.4Circulating CRP

Circulating maternal CRP was not correlated with % length of labour (r=0.10, p=0.51) (Figure 94 A). However, as described above, IL6 levels increase in response to labour (Table 30), and since IL6 stimulation of hepatocyte CRP production can take 6 hours, a further analysis was undertaken to examine changes in CRP which occur over time as measured in hours. Additionally, since each patient had a different labour length, changes were explored for each patient individually. This analysis suggests that circulating CRP levels initially remains static and increases or there is a tendency for CRP to increase acutely in those where labour is longer and thus IL6 has had time to increase and exert an effect on CRP production by the liver (Figure 94 B)

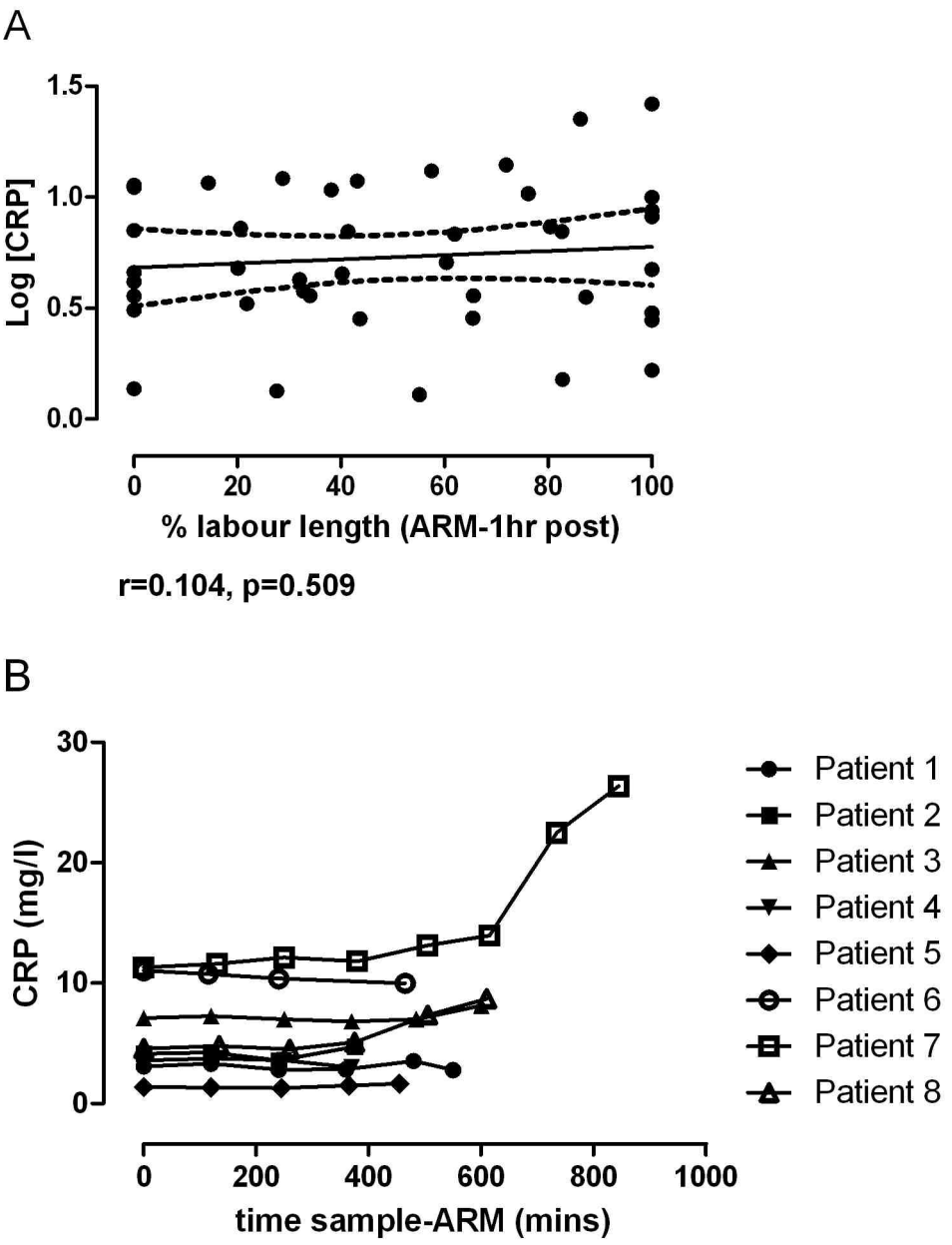

Figure 94 Correlation of maternal circulating CRP with time in labour

(A) Correlation plot and regression line with 95% confidence interval for CRP according to % length of labour (ARM-1hr post delivery of placenta). Pearson correlation r and p values are shown. (B) Point and line plots for raw CRP levels demonstrating values for each individual patient across time in minutes.

5.4.5 Hypothesis 4 - Promotion of a pro-inflammatory phenotype in the maternal circulation is associated with myometrial myocyte damage resulting from repeated contractions during labour.

5.4.5.1CK and Mb in response to pregnancy and delivery

Baseline measures of for creatine kinase (CK) and myoglobin (Mb) are illustrated in Figure 95. Circulating CK did not differ significantly between the 3 groups (NP vs CS-pre vs IOL-pre, p=0.19) or between pregnant and non-pregnant women (NP vs PT p=0.07). Baseline measures of Mb were found to differ between the 3 groups (NP vs CS-pre vs IOL-pre, p=0.037) with lower levels found in women pre-IOL (np vs CS-pre p=0.56, np vs IOL-pre p=0.020, CS-pre vs IOL-pre p=0.047), however, no difference was found between non=pregnant and the pooled pregnancy values (NP vs PT p=0.08).

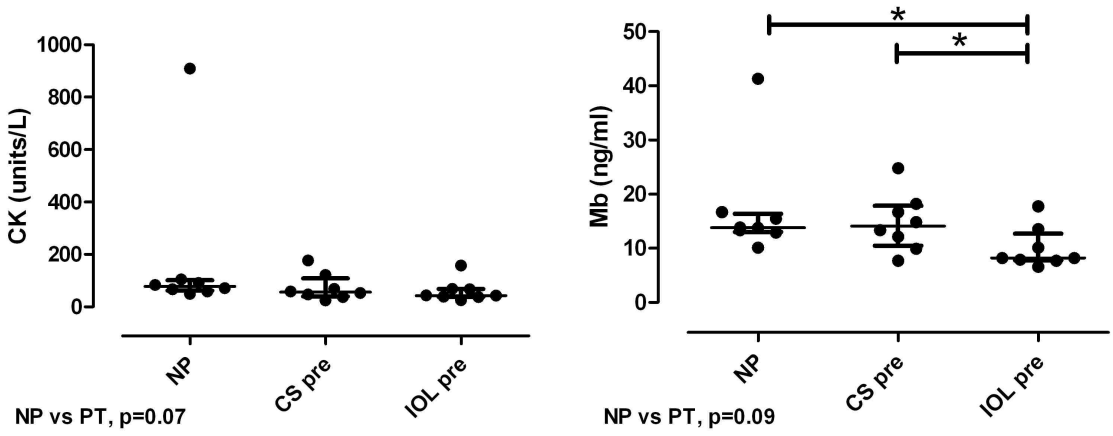

Figure 95 Baseline measures of CK and Mb

Baseline measures of circulating CK and Mb in non-pregnant, pregnant at term pre-CS and pregnant and term pre-IOL. Raw data are shown as scatter plots with median and interquartile ranges indicated. Data were log transformed prior to analysis. Unpaired t-tests were performed with lines demonstrating significance value between 2 samples under start and end of each line *p<0.05

Delivery by both CS and IOL induced a significant increase in both circulating CK and Mb (CK: CS-pre vs CS-post, p=0.003, IOL-pre vs IOL post, p=0.005, Mb: CS-pre vs CS-post, p<0.001, IOL-pre vs IOL post, p<0.001) (Figure 96).

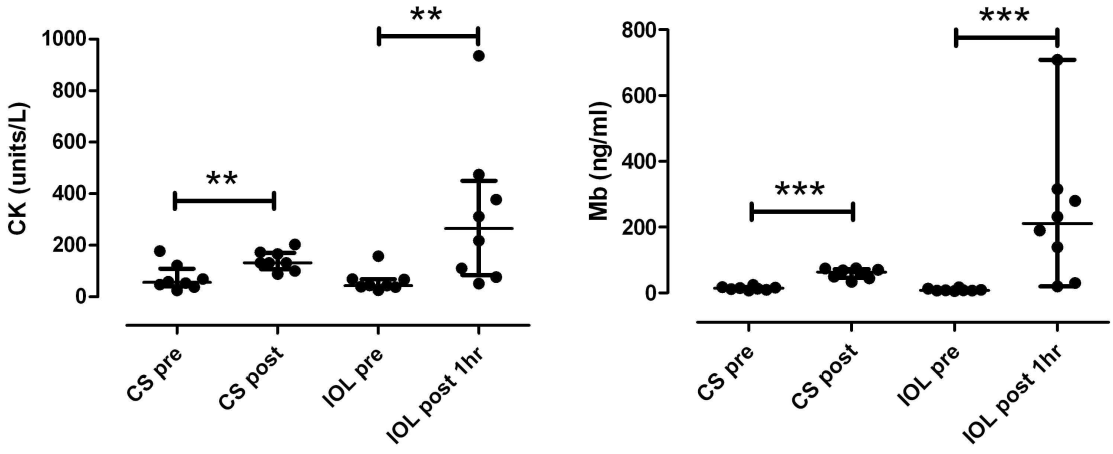

Figure 96 Circulating CK and Mb pre and post delivery

Concentration of circulating CK and Mb pre and post delivery by planned caesarean delivery (CS) or vaginal delivery after induction of labour with syntocinon only (IOL). Raw data are shown as scatter plots with median and interquartile ranges indicated. Data were log transformed prior to analysis. Paired t-tests were performed with lines demonstrating significance value between 2 samples under start and end of each line. *p<0.05, **p≤0.01, ***p≤0.001

The difference in both CK and Mb was greater in response to IOL, however the difference was only significant for the change in circulation Mb (CK: log Δ CS vs log Δ IOL, p=0.13, Mb: log Δ CS vs log Δ IOL, p=0.012) (Figure 97).

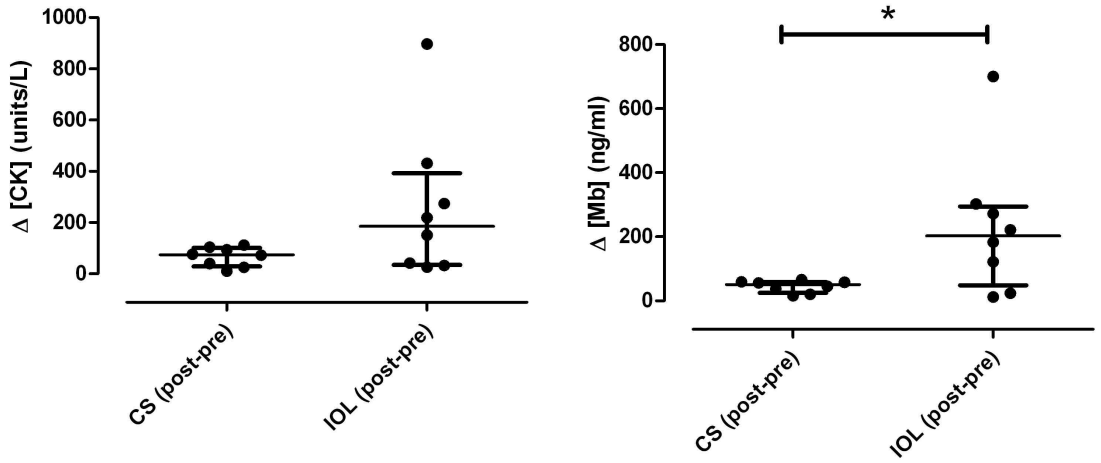

Figure 97 ΔCK and ΔMb (post-pre) delivery

Change in circulating levels of CK and Mb post to pre delivery for CS and IOL. Raw data are shown as scatter plots with median and interquartile range indicated. Analysis was performed on log transformed data where log Δ CS = (log CS post)- (log CS pre) and log Δ IOL = (log IOL post) – (log IOL pre). Unpaired t-tests were performed with lines demonstrating significance value between 2 samples under start and end of each line. *p<0.05

Additionally the change in circulating CK and Mb levels occurred in a time dependent manner during labour (CK r=0.48, p=0.001, Mb r=0.60, p<0.001) (Figure 98).

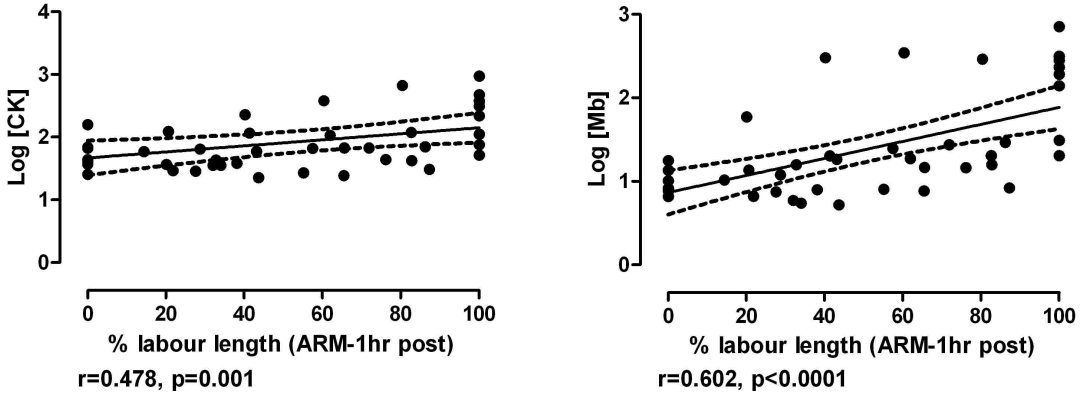

Figure 98 Correlation of CK and Mb with time in labour

Correlation plot and regression lines with 95% confidence intervals for circulating CK and Mb levels during labour (ARM-1hr post delivery of placenta). Log transformed data are shown and Pearson correlation r and p values are shown.

5.4.5.2CK and Mb and correlation with markers of inflammation

To evaluate the association between inflammation in labour and contraction induced myometrial cell damage, the correlation between CK levels, Mb, white blood cell subsets and circulating inflammatory markers was examined.

All white blood cell subsets demonstrated a significant correlation with both circulating CK and Mb levels; WBCs (CK; $r=0.56$, $p=0.0001$, Mb; $r=0.67$, $p<0.0001$), neutrophils (CK; $r=0.53$, $p=0.0003$, Mb; $r=0.68$, $p<0.0001$), lymphocytes (CK; $r=-0.32$, $p=0.036$, Mb; $r=-0.51$, $p=0.0005$), monocytes (CK; $r=0.50$, $p=0.0006$, Mb; $r=0.50$, $p=0.0007$), eosinophils (CK; $r=-0.48$, $p=0.002$, Mb; $r=-0.51$, $p=0.001$) and basophils (CK; $r=0.33$, $p=0.044$, Mb; $r=0.89$, $p=0.014$) (Figure 99 and Figure 100).

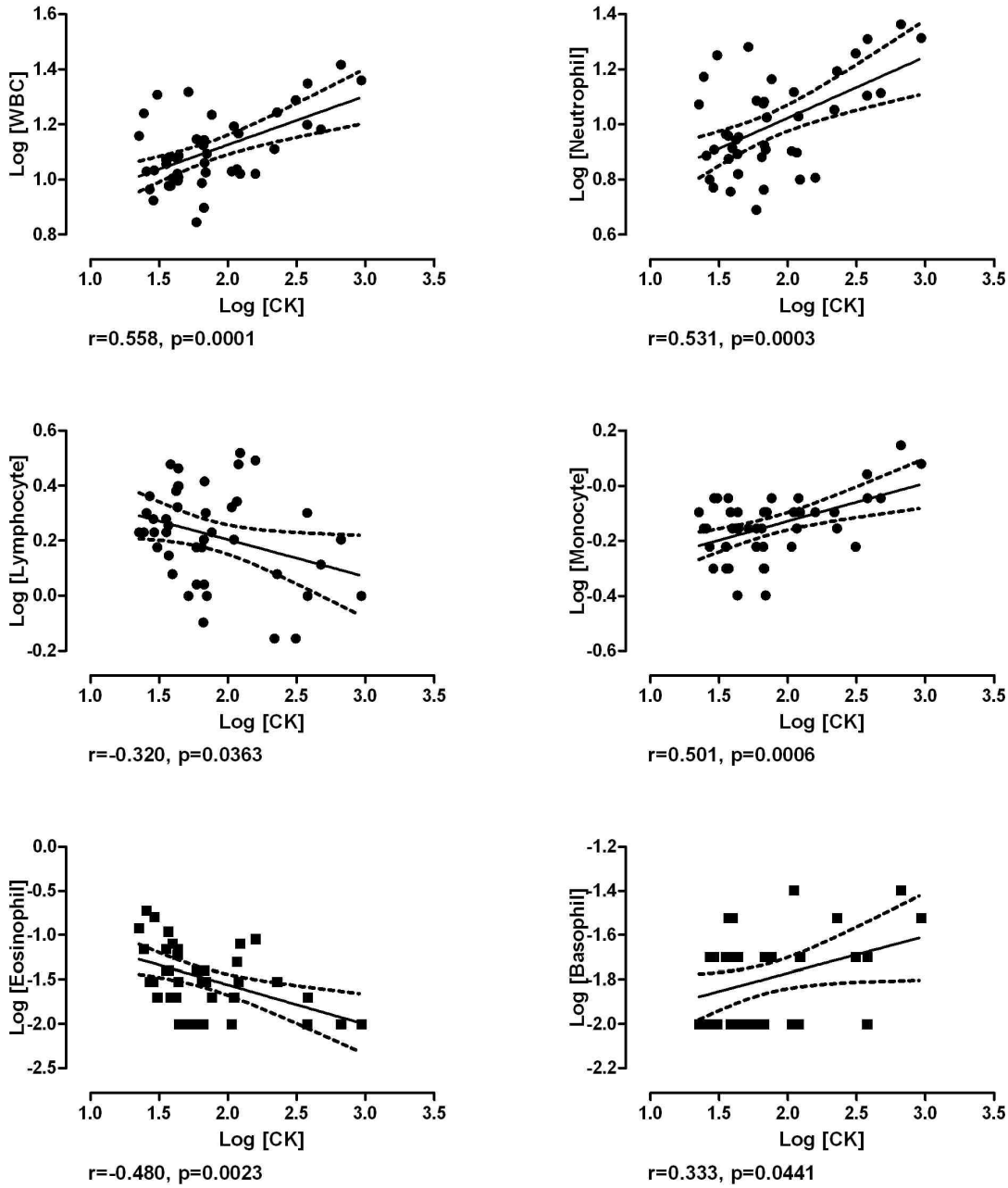

Figure 99 Correlation of WBC subtype with CK

Correlation plots, regression lines with 95% confidence intervals for circulating CK levels and white cell sub-sets. Log transformed data are shown with Pearson correlation r and p-values shown.

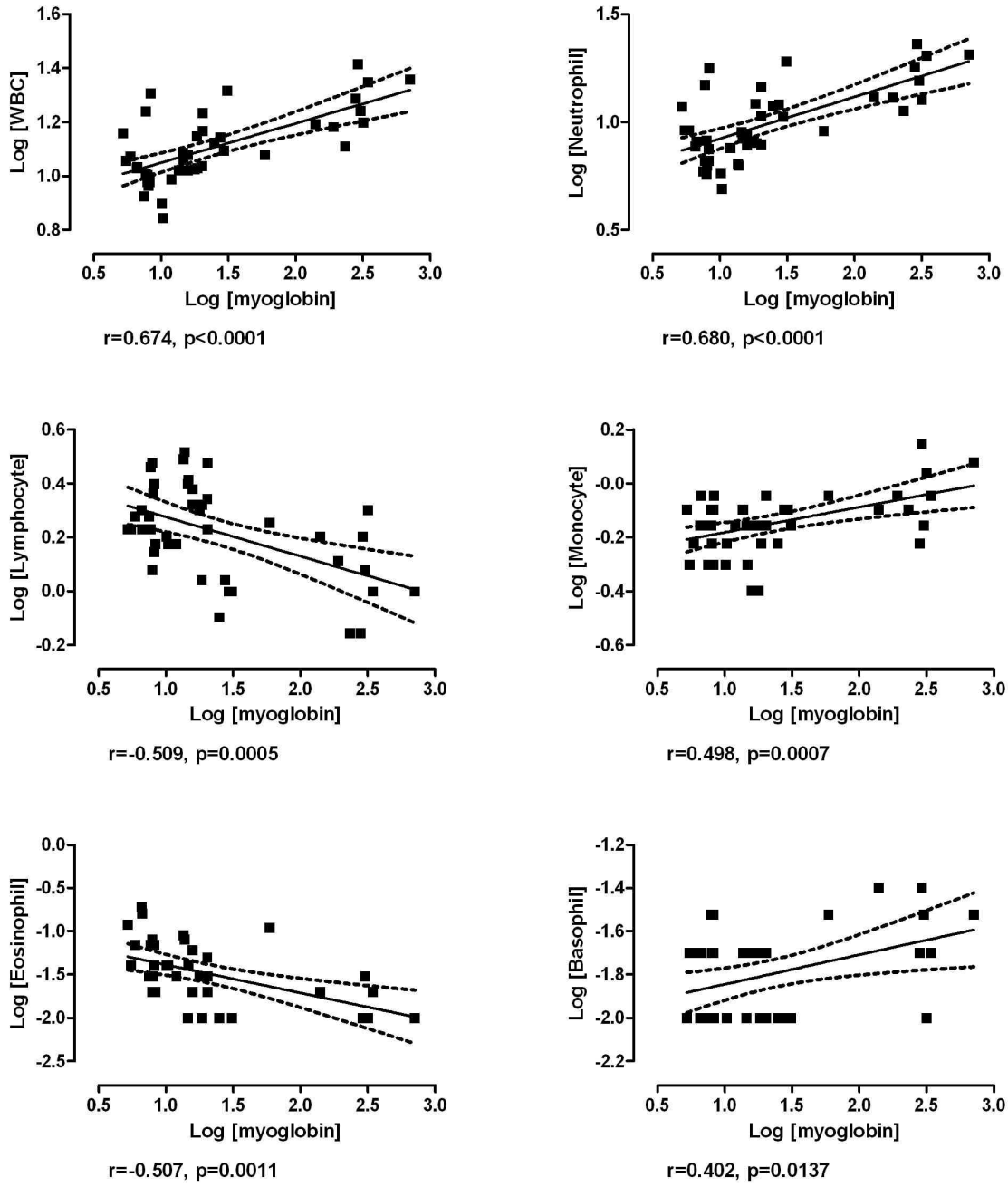

Figure 100 Correlation of WBC subtype with Mb

Correlation plots, regression lines with 95% confidence intervals for circulating Mb levels and white cell sub-sets. Log transformed data are shown with Pearson correlation r and p-values shown.

Additionally, circulating CK and Mb levels have a significant positive correlation with levels of GCSF, IL10 and CCL2, and a negative correlation with IFNγ, IL17, CCL3, CCL4 and VEGF. IL2 has a negative correlation with CK and IL6 is positively correlated with Mb (Table 31).

Cytokine/Chemokine		Correlation with CK		Correlation with MB	
	n pairs	r-value	p-value	r-value	p-value
EGF	25	-0.045	0.831	-0.077	0.714
CCL11	43	0.225	0.146	0.092	0.555
FGF-2	43	0.043	0.787	-0.054	0.731
Flt-3	11	/	/	/	/
CX3CL1	35	-0.302	0.078	-0.215	0.216
GCSF	43	0.511	0.0005***	0.588	<0.0001***
GMCSF	43	-0.212	0.172	-0.085	0.588
CXCL1,2,3,	43	0.160	0.307	0.202	0.193
IFNα2	26	0.205	0.316	0.084	0.684
IFNγ	42	-0.492	0.0009***	-0.413	0.007**
IL1α	31	-0.248	0.179	-0.194	0.295
IL1ß	0	/	/	/	/
IL1ra	11	/	/	/	/
IL2	26	-0.414	0.036*	-0.363	0.069
IL3	5	/	/	/	/
Il4	4	/	/	/	/
IL5	0	/	/	/	/
IL6	40	0.276	0.085	0.364	0.021*
IL7	10	/	/	/	/
CXCL8	43	-0.037	0.814	0.104	0.509
IL9	24	0.176	0.411	0.148	0.491
IL10	38	0.472	0.003**	0.659	<0.0001***
IL12 (p40)	19	/	/	/	/
IL12 (p70)	22	0.075	0.740	0.017	0.941

Cytokine/Chemokine		Correlation with CK		Correlation with MB	
	n pairs	r-value	p-value	r-value	p-value
IL13	0	/	/	/	/
IL15	30	0.055	0.774	0.134	0.479
IL17	41	-0.473	0.002**	-0.390	0.012*
CXCL10	43	0.071	0.651	0.041	0.795
CCL2	43	0.325	0.034*	0.397	0.008**
CCL7	34	-0.013	0.943	0.022	0.901
CCL22	43	-0.294	0.056	-0.163	0.296
CCL3	38	-0.485	0.002**	-0.402	0.012*
CCL4	43	-0.524	0.0003***	-0.487	0.0009***
sCD40L	40	0.031	0.851	0.006	0.970
sIL2Rα	8	/	/	/	/
TGFα	32	-0.163	0.373	-0.072	0.695
TNFα	43	0.219	0.158	0.205	0.188
TNFß	0	/	/	/	/
VEGF	24	-0.478	0.018*	-0.512	0.011*
CCL5	16	/	/	/	/

Table 31 Correlation of circulating cytokines/chemokines with CK and Mb

Table illustrating correlation of measured cytokines with circulating CK and Mb during IOL (ARM-1hr post delivery of placenta). Analysis was performed using Pearson's correlation using log transformed data. Where <50% of expected cytokine readings were obtained (n<22) the cytokine was excluded from analysis. *p<0.05, **p≤0.01, ***p≤0.001

5.5 Summary of Results

5.5.1 Pregnancy and maternal peripheral inflammation

- Pregnancy at term is associated with a state of low grade inflammation in the maternal circulation at term with a tendency for a higher CRP and a neutrophil driven leucocytosis as compared with non-pregnant women.

- Our flow cytometry data do not show evidence of priming of maternal circulating leukocytes at term as compared with non-pregnant women.

- Pregnancy at term is associated with minimal alteration in circulating cytokine concentrations with only GCSF increasing and CCL11 and CCL22 decreasing.

5.5.2 Labour and maternal peripheral inflammation

- Labour induces a time dependent increase in total leukocytes, neutrophils and monocytes with a decrease in lymphocytes and eosinophils. The extent of change is greater in response to labour than to delivery by non-labouring CS.

- Labour induces a time dependent increase in the % of granulocytes expressing CXCR1 and CXCR2 with a fall in the cell surface density of the receptor.

- Labour induces a time dependent increase in circulating GCSF, GRO (CXCL1,2,3), IL6, IL10, CXCL8, CCL2 and CCL3.

- Labour induces a time dependent decrease in circulating CXCL10.

- Labour tends to increase the level of circulating CRP in a time dependent manner.

5.5.3 Muscle damage, labour and maternal peripheral inflammation

- Circulating markers of muscle damage (CK and Mb) at term are not altered from non-pregnant levels.

- Labour induces a time dependent increase in muscle damage (as measured by circulating CK and Mb) which is greater than in response to delivery by non-labouring CS.

- Peripheral total white cell counts, neutrophils, monocytes and basophils are positively associated with the extent of muscle damage during labour.

- Peripheral lymphocytes and eosinophils are negatively associated with extent of muscle damage during labour.

- Muscle damage during labour as measured by CK is positively correlated with GCSF, IL10 and CCL2, and negatively correlated with IFN γ, IL2, IL17, CCL3 and CCL4.

- Muscle damage during labour as measured by Mb is positively correlated with GCSF, IL6, IL10 and CCL2, and negatively correlated with IFN γ, IL17, CCL3 and CCL4.

5.6 Discussion

The data presented in this chapter demonstrate that peripheral inflammatory changes in the maternal circulation seen in response to pregnancy at term appear to be limited to increased total white cell counts driven by a neutrophilia, with no suggestion of leukocyte priming prior to labour. Additionally, term pregnancy is associated with an increase in CRP, an increase in GCSF corresponding with the neutrophilia and a suppression of the cytokines CCL11 and CCL22.

Subsequently, we found that term labour induces dramatic changes in inflammatory cells and mediators in the maternal circulation. Importantly, these changes occur in a co-ordinated time and contraction dependent manner, with the degree of inflammation associated with the time in labour and the degree of myocyte damage as measured by circulating CK and Mb.

5.6.1 Pregnancy and maternal peripheral inflammation

Our data confirm the well known changes to circulating leukocyte numbers in pregnancy with an overall increase in total white cell count, driven by increased neutrophills[355 356 358]. We also found a decrease in circulating lymphocytes, eosinophils and basophil counts, with no significant change in monocytes induced by pregnancy at term, which may reflect a dilutional effect of pregnancy.

Specific analysis of leukocyte cell surface markers and chemokine receptors do not suggest priming of circulating maternal leukocytes in term pregnancy. In particular, there is no change in the expression of CD11b on the neutrophil or monocyte cell population, suggesting activation status of these cells is comparable with the non-pregnant controls. In addition the percentage of CD3+ lymphocytes (T-cells) that were either CD4+ or CD8+ did not alter with pregnancy, however we were unable to comment on the CD4+ cell sub-types (Th1, Th2, Th17 or Treg) in this study which may have given further information as to the type of Th cell response to be expected in pregnancy. Concerning chemokine receptor expression, again results do not suggest that term pregnancy promotes priming of leukocytes since only the percentage neutrophils expressing CXCR2 increases in pregnancy with a non significant trend to a

reduction in the density of CXCR2 expression. Contrary to leukocyte priming, our data suggest that pregnancy actually decreases chemokine receptor expression on monocytes, Th and Tc cells with a reduced number on monocytes expressing CCR2, and a decreased density of CXCR1 and CXCR2 expression on Th and Tc cells.

Compared with non-pregnant women, pregnancy was only associated with alterations in the circulating cytokines GCSF, CCL11 and CCL22. The increase in GCSF is consistent with previous results[378] and also its proposed production by gestational tissues[413]. This increase would also explain the typical neutrophilia of pregnancy since it promotes release of neutrophils from the bone marrow, and has been suggested to represent a compensatory increase in innate immunity, while there is some suppression of the adaptive immune response in pregnancy[378]. The cause for the decrease in circulating CCL11 in pregnancy is unclear, however it has been reported by others[378] and certainly fits with the reduced numbers of circulating eosinophils. The role of decreased CCL22 is similarly uncertain, however, a reduction in pregnant cervico-vaginal fluid has been reported and it was postulated that it may have a suppressive effect upon the maternal innate immune response[414]. Additionally, comparisons across the three groups suggested FGF-2 levels were higher pre-CS compared with pre-IOL, and lower pre-IOL compared with NP levels with a similar pattern for CXCL1,2&3 levels. However, when pregnant data were pooled giving n=16, no significant differences were found when compared with the NP controls, and therefore differences in data may simply reflect patient variability, but would necessitate further investigation with larger numbers.

Our observation of a significantly higher plasma CRP in pregnant women is in agreement with previous descriptions of an early gestational and persistent rise in CRP[373 374]. Certainly, it has been suggested that a CRP>10mg/L (upper range of normal in non-pregnant individuals[408]) may be normal for pregnancy[373], with a suggested upper limit of 15mg/L[374]. Reassuringly, markers of muscle damage, namely CK and Mb did not differ with pregnancy in the groups examined implying no effect of pregnancy upon muscular damage or remodelling.

Overall, our data suggest that pregnancy does induce a shift to a state of low grade inflammation as indicated by the recognised changes of neutrophilia,

raised CRP and GCSF, but there is no suggestion of leukocyte priming or acute inflammatory changes in preparation for labour at term.

5.6.2 Labour and maternal peripheral inflammation

The overall response to delivery by either CS or IOL produces a pattern of acute inflammatory changes. Given that the extent of change was more pronounced in response to IOL this suggests that the observed inflammatory changes may be contraction dependent. Furthermore many of the inflammatory changes seen with delivery by IOL occur in a time dependent manner, as suggested by the correlations with time in labour.

5.6.2.1Leukocytes

In terms of circulating leukocyte numbers, there is agreement with previous studies in labour with an increase total white cell count, increased neutrophils, and decreases in lymphocyte and eosinophil numbers [359 362-364], with a greater change in response to labour when compared with a non-labouring caesarean delivery[360]. Specifically, we observed the total white cell count to reach a maximum level of $22.09x10^9$/L, with a neutrophil count of $20.6x10^9$/L in the absence of infective stimuli. The accompanying reduction in the density of cell surface expression of CXCR1 and CXCR2 on circulating activated neutrophils post delivery suggests that the majority of circulating neutrophils tend to be immature. This tendency toward immature neutrophils, typical of an acute inflammatory response[264], is most probably driven by two factors; trafficking of mature activated neutrophils into the myometrium and other inflammatory and immune tissues[219] in conjunction with release of neutrophils stores from the bone marrow under the influence of increased circulating GCSF.

The reduction in circulating lymphocytes and eosinophils is consistent with previous findings in labour[362 363], and suggests that there may be a degree of cellular recruitment from the circulation to the contracting uterus. In response to delivery, there is a decrease in the density of expression of CCR6 on circulating T-lymphocytes with no change in the % of Th or Tc cells expressing this receptor. This suggests that the type of T-cell sequestered into the inflammatory tissue is likely to express CCR6. Of note, CCR6 is the only

chemokine receptor which is attracted via CCL20, which although not included on the serum cytokine panel used in this experiment, transcription of CCL20 was significantly upregulated in contracting myometrium (Chapter 3 and Chapter 4).

Our monocyte data suggest there is an increase in circulating monocytes in response to labour, with no significant change found pre to post delivery by CS. Previous studies examining monocyte changes with labour have described either numbers increasing, falling and remaining static[362 363]. However, our study has the advantages of using repeated measures rather than one single measure during an arbitrary timepoint in labour in addition to our comparison back to baseline measures taken directly before delivery.

5.6.2.2Cytokines

In response to delivery, we found that a limited number of circulating cytokine concentrations changed. In particular, significant changes were seen in response to IOL rather than CS with increases in GCSF, GRO (CXCL1,2,3), IL10, CCL2 and CCL7. Of note all these cytokines were also positively associated with time in labour suggesting that an increased inflammatory response occurs in advancing labour. Additionally, despite no significant change pre to post delivery by IOL, there were positive associations found between time in labour and the cytokines IL6 and CXCL8. Three cytokines were found to fall in response to IOL, namely CCL11, IFN-γ and CXCL10 with CXCL10 being negatively associated with time in labour.

The changes in circulating inflammatory proteins suggests an association with and therefore perhaps an effect of labour, especially since we have controlled for delivery with the CS comparison group. Certainly, the specific cytokine changes which we have observed are consistent with recognised labour associated inflammatory changes to circulating white cells[359 362-364] and leukocyte infiltration to gestational tissues[219 220 354]. Specifically, GCSF is consistent with peripheral neutrophilia, GRO and CXCL8 with neutrophil chemotaxis, CCL2 and CCL7 with monocyte chemotaxsis, and IL6 with muscle damage and an overall upregulation of the acute inflammatory response. The cytokines IL10, CXCL10 and IFNγ may be associated with a suppression of the initial inflammatory response with a tendency toward repair and resolution. These changes may

occur via suppression of macrophage function and NFkB activity by IL10 and reduced chemotaxis of CD4+ T-lymphocytes by a reduced level of CXCL10 and suppression of an overall pro-inflammatory environment with a reduction in IFNγ levels.

Additionally, the levels of circulating chemokines may also be regulated by interaction with atypical chemokine receptors (ACRs)[415-418]. These are structurally similar to other chemokine receptors, however they do not have the ability to stimulate intracellular signalling pathways, and thus interaction of chemokines with ACRs does not stimulate migration and sequestration of peripheral leukocytes as is seen with 'traditional' chemokine receptor interactions. Recognised ACRs or 'decoy receptors' include D6 and the Duffy antigen receptor for chemokines (DARC)[415-419]. They are thought to play a role in 'scavenging' chemokines by interacting with high affinity and internalising a number of chemokines without activation cellular activity[419 420]. There has also been proposal that ARCs may facilitate leukocytes acting as a 'sink' and 'resevoir' for circulating chemokines and thus exerting a further element of control over the inflammatory response and leukocyte trafficking, releasing chemokines when required[419 421]. Concerning muscle damage, evidence from the myocardium post infarction has also suggested that D6 may play a role in preventing excessive inflammation and muscle remodelling[422]. This then raises the possibility of a further comparable role for D6 and possibly other ACRs in the labouring myometrium, especially considering the high density of D6 expression within the placenta and gestational tissues during pregnancy[420 423].

5.6.2.3CRP

Despite increases in other inflammatory indices in response to delivery, there was only a non significant trend for CRP to increase, however the extent of increase was significantly greater in response to labour rather than CS. This may reflect the timing of the serum sampling in that CRP rises in the circulation may not be as acute as other mediators since the time delay between IL6 mediated stimulation of hepatic CRP production and its appearance in the circulation is approximately 6hours. Despite this, in the patients with later serum sampling there is evidence of an acute rise in CRP to levels surpassing the clinically

accepted maximum of 10mg/L. Again this suggests inflammation occurs as a reaction to labour rather than being necessary for it progression.

5.6.3 Muscle damage, labour and maternal peripheral Inflammation

5.6.3.1Myometrial contractile damage

Overall, the rise in circulating CK and Mb is consistent with the finding of others for human labour[391 392], response to OT induced tonic contraction of the pregnant and non-pregnant uterus[424] and also surgical trauma in the non obstetric patient[409]. When the type of delivery is taken into account IOL enhances the extent of change in the level of circulating Mb, however, the change in circulating CK is comparable between IOL and CS. This is in keeping with the findings of peak rises in Mb on the first post-operative day and maximal CK on the second post-operative day[409].

Although CK and Mb are not specific to myometrial myocytes[411], the contracting uterus is the most likely source of these proteins since no subject displayed evidence of alternative muscle damage; no complaints of chest pain consistent with myocardial infarction and no skeletal muscle injury or exercise since all were confined to a labour room bed for the duration of the labour. This is in accordance with previous data where no rise in troponin I is observed[390] suggesting a non-cardiac cause for muscle damage.

The causative link between the repeated contractions typical of human labour and increased myometrial cellular damage is suggested by the temporal association with both CK and Mb in the IOL group. This link with repeated contractions is further supported by the higher levels of CK and Mb in response to IOL compared with CS. This occurs despite the skeletal muscle disruption and sustained tonic contraction following the standard 5iu bolus and infusion of syntocinon which occurs during caesarean section.

5.6.3.2Contractile damage and leukocytes

As with time in labour, contraction induced damage as measured by CK and Mb are both correlated with the changes in peripheral circulating leukocytes. This suggests that changes in circulating maternal leukocytes in labour can be considered a time and contraction dependent event, and the pattern of changes may reflect the reactive inflammatory process with bone marrow release of the neutrophils required and reduction in other leukocyte types most probably reflecting trafficking into damaged myometrium and gestational tissues in addition to sequestration into other inflammatory related tissues.

5.6.3.3Contractile damage and cytokines

In addition to temporal changes to inflammatory cytokines during labour, there were also correlations with the extent of muscle damage as measured by circulating CK and Mb. In particular, GCSF, IL6, IL10, and CCL2 were all positively associated with these markers of muscle damage. There were additional negative associations between CK and Mb with IFNγ, IL17, CCL3 and CCL4, and CK with IL2. The combination of these correlations would suggest that a high level of uterine muscle damage can stimulate and also suppresses the release of certain cytokines, thereby exerting control over the acute inflammatory reaction in addition to possible modulation of circulating chemokines by atypical chemokine receptors (ARCs). This may in turn limit the cellular damage by infiltrating leukocytes and perhaps promote a repair and remodelling phenotype.

5.6.4 Exercising skeletal muscle and inflammation

The observed contraction dependent inflammation of human labour has marked similarities to that reported for exercising skeletal muscle. In this parallel situation, it is suggested that contractions of skeletal muscle cause myocyte damage, which in turn drives myocyte release of inflammatory mediators and promotion of the ensuing circulating inflammatory changes and leukocyte influx into the damaged muscle [383 386 389 425-429].

In response to contractions of exercising skeletal muscle, damage has been measured by both direct changes (MRI and disruption to cell ultrastructure) and indirect measures (increases in circulating CK, Mb and slow twitch skeletal myosin heavy chain fragments)[386 387]. These changes occur in the absence of increased cardiac troponin T[387 390] thereby suggesting release from the exercising muscle rather than cardiac muscle.

The subsequent myocyte release of inflammatory cytokines shows that some are common to our labour data, in particular increases in IL6, IL10, CXCL8 and CCL4[384 385 428]. The inflammatory reaction which ensues in the skeletal muscle shows a cellular influx of predominantly neutrophils and macrophages[383 386 429] which is comparable to the pattern seen in the uterus during labour[219]. Of note, there is also a similar peripheral leukocyte response[383 426 430] with increased neutrophils and decreased lymphocytes. Additionally, there is a similarity in the systemic inflammatory response to surgery, trauma, and sepsis[366-370 409] with a similar CK and Mb response[409] suggesting this is a non-specific acute reaction to muscle damage.

Clinically, the inflammation associated with skeletal muscle exercise presents as muscle soreness in the following hours and days, a phenomenon which is well recognised and referred to as delayed onset muscle soreness (DOMS), with the degree of soreness dependent on the type of exercise and amount of damage induced[386]. The proposed timing and sequence of events involved in DOMS was proposed by Smith over 20 years ago and illustrated in Figure 101[389].

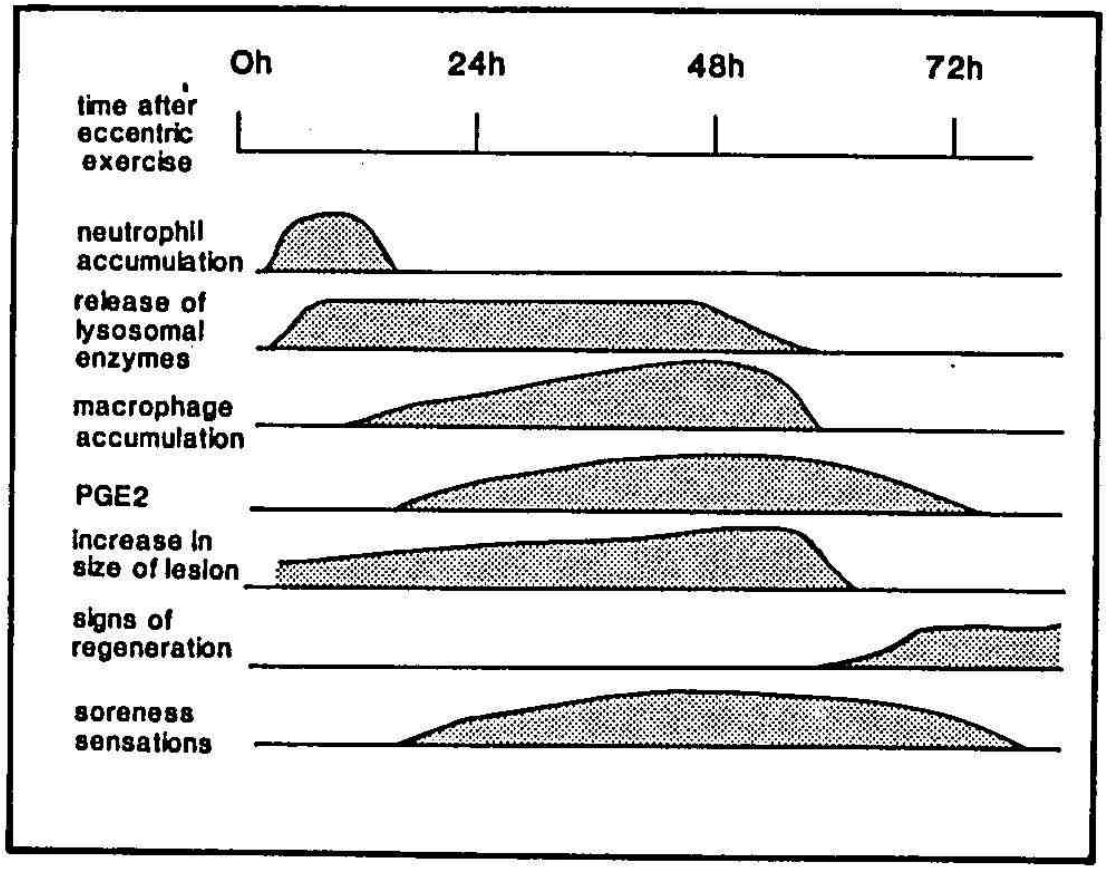

Figure 101 Inflammation and cellular damage in exercising skeletal muscle

The sequence of inflammatory events in response to contraction induced myocyte damage in exercising skeletal muscle. Taken from *Acute inflammation: the underlying mechanism in delayed onset muscle soreness?, L Smith,1991*[389]

In this model, leukocytes (mainly neutrophils and macrophages) are thought to play a pivotal role in clearing damaged tissue with subsequent muscle repair and remodelling. Certainly in CCR2 null mice the repair of exercise damaged muscle is impaired and is thought to be an effect of reduced monocyte/macrophage recruitment despite a sparing of the neutrophil response[431]. In addition, some CC chemokines (CCL2, CCL3 and CCL4) produced at the site of injury are thought to have a direct effect upon healing and repair of the damaged myocytes[432 433].

5.6.5 The role of inflammation in labour

Overall, our data would suggest an association between contraction induced myocyte damage and the recognised inflammatory changes typical of term labour in humans. This does not provide direct evidence of the initiating event, however, the concept of labour associated inflammation as a reaction to muscle

damage is somewhat supported by the inflammatory time course in the skeletal muscle model.

The role of the inflammatory response in human labour is not addressed in this work. However, there is a suggestion that rather than being involved in the initiation and propagation of labour, inflammation is required for tissue repair and remodelling. Certainly, an inflammatory response appears to aid uterine involution in humans which is delayed in women who underwent a non-labouring caesarean section compared with normal labour, regardless of breast-feeding[434] [435]. Additionally, monocyte infiltration to the uterus is not necessary for normal labour to occur in the CCR2 knock out mouse[315], but appears to be required for skeletal muscle repair[431]. There is also preliminary observational evidence from our lab which suggests that CCR2 deficient mice are more prone than their wild-type counterparts to birthing difficulties in their second and third pregnancies.

5.7 Conclusion

This is the first study to examine the maternal cellular and humoral inflammatory response to term pregnancy and labour and relate this to the degree of myocyte damage.

Collectively our data suggest that the previously described inflammatory changes observed in human labour at term occur in response to contractile myometrial damage and may play a principal role in tissue repair and remodelling rather than the initiation of labour. Overall, the evidence tends to support the hypothesis proposed at the start of this chapter and is summarised in Figure 102.

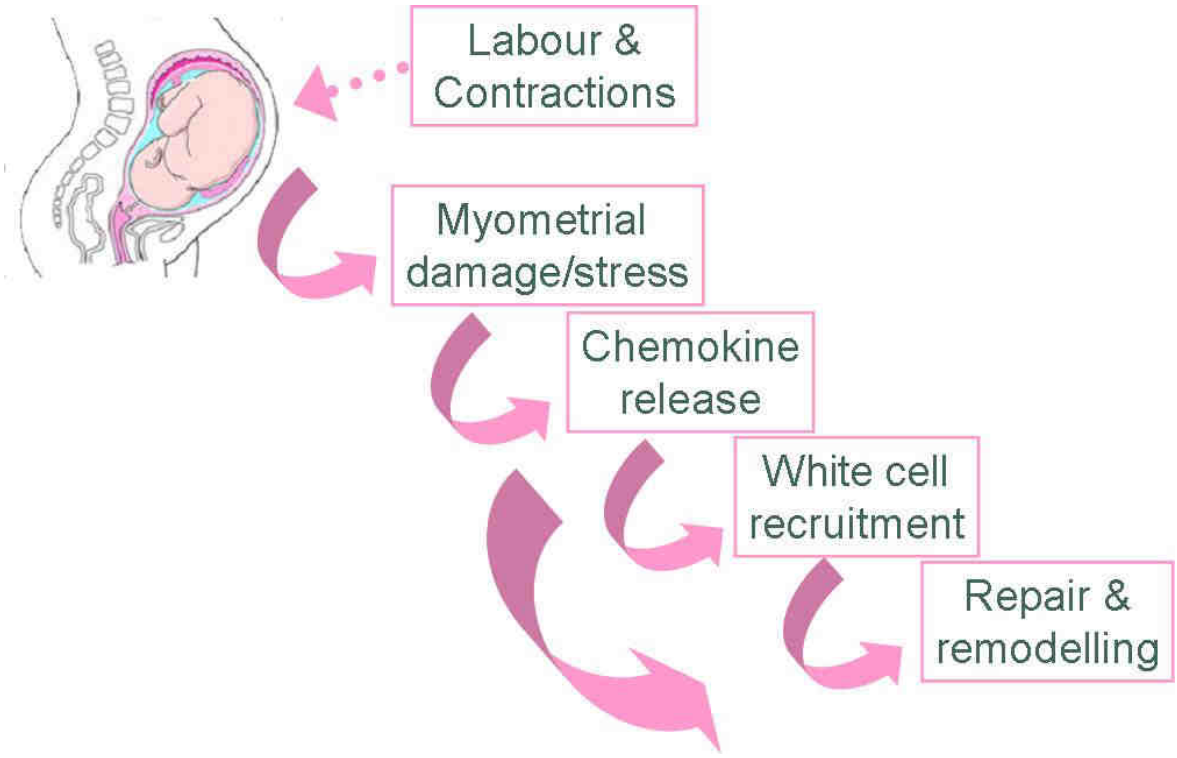

Figure 102 Overall Hypothesis Chapter 5

Proposed hypothesis summarising the inflammatory events of human labour.

Chapter 6

Maternal and pregnancy characteristics and myometrial contractility *in-vitro*

6 Maternal and pregnancy characteristics and myometrial contractility *in-vitro*

6.1 Introduction

Obesity is a major health problem across the world. In the UK, obesity affects one-fifth of the female population, with 18.8% of all females in the reproductive age group (16-44 years) classed as obese[436-438]. Similar rises have been observed in the pregnant population in the UK and Ireland with up to one in five women booking for antenatal care being clinically obese with a BMI ≥30kg/m^2 [2 295 439 440]. To date prospective cohort studies have demonstrated a wide variety of perinatal complications associated with maternal obesity which have been suggested to be independent of glucose tolerance status[63 295 296 441], including a reduction in spontaneous term labour[62], with a concomitant increase in necessity for post-dates induction of labour[63-65], and an increased risk of emergency caesarean delivery (CS) for dysfunctional labour[63 64 66 67], despite induction and augmentation of labour by oxytocin (OT) analogues[62 66]. Furthermore the incidence of post-partum haemorrhage is increased with maternal obesity which is independent of mode of delivery and use of OT[63 65 66]. Collectively these observational studies suggest that obesity has an inhibitory effect on myometrial function and response to OT.

Traditionally the ineffectual progress in labour associated with maternal obesity has been attributed to either foetal macrosomia, narrowing of the pelvic outlet by adipose tissue deposition or inadequate active second stage of labour. However, caesarean delivery is required in a significant proportion of women even in the absence of macrosomia[442], furthermore magnetic resonance imaging (MRI) studies of pelvis do not support a role for adiposity obstructing labour[443], and lastly intrauterine pressures exerted during active second stage are comparable in obese and lean women[444].

Given that myometrial function may be the common link between intrapartum and postpartum complications, myocyte specific explanations have been proposed, with the increases in myocyte cell membrane lipid content in obese women thought to be responsible[299 445]. Certainly pregnancy in obese women is

associated with exaggeration of the normal metabolic response and development of a dyslipidaemic environment, characterized by hypertriglyceridaemia and low high density lipoproteins[446], which could potentially facilitate alterations to the membrane lipid composition. Acute lipid loading of myocyte cellular membranes *in-vitro* with cholesterol or low-density lipoproteins has also been associated with a reduction in both spontaneous and OT induced contractility, with improvement in myocyte activity with lipid reduction[299 300]. Alternatively, or in addition, obese pregnant women are hyperleptinaemic[446], with leptin being an adipocyte derived hormone whose circulating levels are directly proportional to the amount of adipose tissue in the body. In terms of uterine function, leptin has been shown to have a direct inhibitory effect on myometrial contractile activity *in-vitro*[301]. Studies in rats have also suggested that high fat diets pre-pregnancy and during pregnancy are associated with a lower myometrial expression of contraction associated proteins including connexin-43 which may influence contractile ability[302].

Additionally, other maternal characteristics including maternal age[68-71], parity[72 73], previous mode of delivery[74-78], gestation[79 80] and birthweight[79] have shown associations with clinical outcomes which can be regarded as related to myometrial contractility function and or response to OT.

6.2 Hypothesis

The overall hypothesis to be examined in this chapter is summarised below in Figure 103.

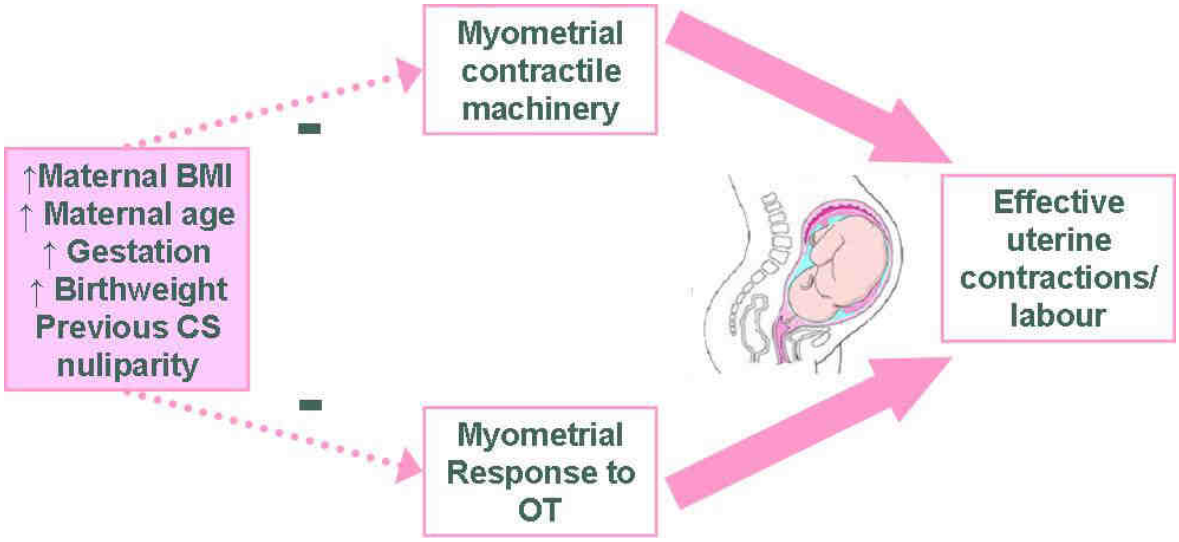

Figure 103 Overall Hypothesis Chapter 6

Summary of the overall hypothesis concerning the effect of maternal characteristics on myometrial spontaneous contractility and myometrial contractile response to oxytocin (OT).

Overall we hypothesised that where myometrium has a chronic exposure during pregnancy to a hyperlipidaemic and hyperleptinaemic environment (implied by a high early pregnancy body mass index (BMI)) that both spontaneous and OT induced contractility would be impaired and that this would underlie the clinical association with impaired myometrial function and apparent resistance to intervention with OT. Additionally, high maternal age, increasing gestation, high birthweight, previous delivery by CS and low parity would be associated with reduced myometrial contractility and response to OT as suggested by the increased need for induction of labour and intrapartum caesarean section.

The specific hypotheses to be tested are

1. Spontaneous *in-vitro* myometrial contractility is impaired by maternal obesity in a body mass index (BMI) dependent manner.

2. Myometrial contractile response *in-vitro* to OT is impaired by maternal obesity in a body mass index (BMI) dependent manner.

3. Spontaneous *in-vitro* myometrial contractility is influenced by other maternal characteristics including age, parity, gestation and birthweight.

4. Myometrial contractile response *in-vitro* to OT is influenced by other maternal characteristics including age, parity, gestation and birthweight.

6.3 Methods

6.3.1 Patient Selection

Myometrial biopsies were obtained as described previously in Chapter 2. Samples were collected on consecutive days; one sample per day was obtained from the first patient of the day providing inclusion criteria were met. Indications for planned caesarean delivery were, previous caesarean delivery (n = 65), breech presentation (n = 8), maternal request (n = 6), previous traumatic vaginal delivery (n = 5) and foetal reasons (n = 1).

Early pregnancy demographic data were recorded for each woman including body mass index, maternal age and parity. At the time of delivery the gestation, indication for elective caesarean delivery and birth weight were also recorded.

6.3.2 *In-vitro* contractility and response to oxytocin (OT)

Myometrial strips were dissected and placed under a 20mN tension in separate organ baths and left to develop spontaneous contractility as previously described in Chapter 2. Where OT response was studied, a single addition of the drug was undertaken after the establishment of regular contractile activity, resulting in a bath concentration of 1nM with the contractile response observed for 60 minutes. OT concentration response curves were performed on separate sample strips by using cumulative additions of OT in the range 10^{-12} to 10^{-6} M, with an interval of 20 minutes between each addition.

6.3.3 Contractile analysis

Spontaneous activity was recorded where stable, regular, rhythmic activity was established within 4 hours after application of the 20mN resting tension. A record of samples achieving spontaneous activity within 2 hours and between 2-4 hours was made. Three measures of contractile activity were assessed: frequency, amplitude and activity integral as described in Chapter 2. Frequency was determined from the time interval between the peaks of two contractions occurring at the time point of interest, and was expressed as contractions/hour.

Contractile force was determined as the difference between baseline tension and maximal contraction amplitude in milli-Newtons (mN). A summary measure of contractile activity was determined by the area under the tension curve and expressed as activity integral in mN.s. For both amplitude and activity integral, spontaneous activity was determined by the average of three contractions when activity was regular, with response to a single concentration of OT measured as one contraction occurring at the time point of interest, either 0, 30 or 60 minutes after addition of OT to the bath. Contractile measurements of amplitude were recorded for OT concentration-response curves, with values recorded immediately prior to the next drug addition. Concentration-response curves were constructed for each patient using percentage increase in amplitude from baseline activity. A log EC_{50} [OT] was calculated for each strip.

6.3.4 Statistical analysis

For all strips achieving spontaneous contractions within 4 hours, contractile activity was analyzed using linear mixed effects regression models[447] with random patient effects for each outcome, to account for the natural correlation in each measurement between strips taken from the same woman. To improve the fit of each model in relation to the assumption of normally distributed residuals, contractile frequency was analyzed on a log transformed scale, whilst amplitude and activity integral were analyzed on a square root scale. The patient level estimates of the average contractile activity measures were extracted from each model and correlated amongst themselves and with other patient characteristics. Since the patient level average activity measures were estimated quantities, robust confidence intervals and p-values were derived by applying a bootstrap method[448] estimating the required correlations from 10,000 replicated datasets.

For each woman, the number of strips that developed spontaneous contractions within 2 hours was analyzed using logistic regression, in relation to pregnancy characteristics, to assess factors associated with the rate with which spontaneous contractions were achieved. The effect of each characteristic was assessed individually, and multivariate models were used to assess which characteristics were independently associated with the rate of achieving spontaneous contractions.

Data from the single concentration OT experiments were analyzed with similar linear mixed effects models; contractile activity measures at all four time points (pre-OT and 0, 30 or 60 minutes post-OT exposure) were included, with hierarchical random effects for women and strips within women, allowing for a general symmetric correlation structure between measurements at the four time points. The time response of each measure was modeled using fixed effects for time. Patient characteristics were then added to each regression model as fixed effects to assess whether they were associated with response to OT, either at all time points or at specific time points, through the inclusion of covariate-by-time interaction terms.

Finally, linear mixed effects regression models were used to model log EC_{50} as estimated in the OT concentration experiment. Patient level predicted values were extracted and correlated with patient characteristics, with robust confidence intervals and p-values derived by bootstrapping with 10,000 replicated datasets. Estimated correlations and model effects are reported as estimate [95% confidence interval], p-value. No adjustments are made for multiple statistical testing.

Statistical analysis of data in this chapter was outsourced and performed by The Robertson Centre for Biostatistics, University of Glasgow.

6.3.5 Study Power

Since the primary aim of the study was to estimate the association between contractile measurements and BMI, the sample size of 85 women had 80% power to detect a correlation of 0.300 between maternal BMI and the model predicted patient level average for each measurement. With an average of 7.2 strips analyzed for each patient, this is equivalent to a correlation of 0.112 between maternal BMI and contractile measurements from a single strip.

6.4 Results

6.4.1 Demographic data

Myometrial biopsies were obtained from 85 women with maternal BMI ranging from 18.0 – 42.2kg/m^2. World Health Organisation (WHO) definitions of BMI revealed that in our study group 2 women (2.3%) were underweight (BMI < 18.5), 38 women (44.7%) were in the normal range (BMI = 18.5-24.99), 23 (27.1%) were overweight (BMI = 25.0-29.99), 13 (15.3%) were obese class I (BMI = 30.0-34.99), 6 (7.1%) were obese class II (BMI = 35.0-39.99) and 3 (3.5%) obese class III (BMI ≥ 40). Strips were analyzed where spontaneous contractions were observed within 4 hours (609 strips) and in two experimental subsets, namely a single concentration OT response group (56 strips from 39 women) and an OT concentration-response group (40 strips from 21 women, of whom 15 were included in the analysis of the response to a single concentration of OT). Patient characteristics are shown for each group in Table 32; no significant differences were observed between those women included or excluded from each subgroup.

		Spontaneous Contractions	Single Oxytocin 1nM Response			Oxytocin Concentration Response		
			Included	Excluded	p-value	Included	Excluded	p-value
N Women		85	39	46		21	64	
N Strips		609	56			40		
BMI (kg/m^2)	Median (IQR) [Range]	25.0 (22.9, 30.0) [18.0, 42.2]	24.3 (22.3, 28.0) [18.5, 42.2]	26.1 (23.0, 31.8) [18.0, 40.0]	p=0.202	27.3 (23.6, 34.2) [18.0, 42.2]	24.9 (22.4, 28.6) [18.0, 40.0]	p=0.141
Age (years)	Median (IQR) [Range]	31.0 (27.0, 35.0) [19.0, 42.0]	31.0 (27.0, 34.5) [21.0, 41.0]	32.0 (27.0, 35.0) [19.0, 42.0]	p=0.418	32.0 (28.0, 35.0) [19.0, 42.0]	31.0 (27.0, 34.2) [21.0, 41.0]	p=0.556
Gestation (days)	Median (IQR) [Range]	273 (273, 275) [259, 284]	273 (273, 274) [259, 284]	273 (273, 275) [263, 282]	p=0.874	274 (273, 275) [269, 276]	273 (273, 275) [259, 284]	p=0.378
Birth Weight (kg)	Median (IQR) [Range]	3.55 (3.18, 3.78) [2.18, 4.72]	3.50 (3.20, 3.73) [2.18, 4.72]	3.62 (3.21, 3.83) [2.48, 4.57]	p=0.449	3.66 (3.31, 3.97) [2.30, 4.72]	3.54 (3.17, 3.70) [2.18, 4.57]	p=0.418
Ethnicity	N (%) Caucasian N (%) Non-Caucasian	79 (92.9%) 6 (7.1%)	35 (89.7%) 4 (10.3%)	44 (95.7%) 2 (4.3%)	p=0.406	20 (95.2%) 1 (4.8%)	59 (92.2%) 5 (7.8%)	p=1.000
Indication for Caesarean	N (%) Previous Caesarean N (%) Other	63 (74.1%) 22 (25.9%)	30 (76.9%) 9 (23.1%)	33 (71.7%) 13 (28.3%)	p=0.627	16 (76.2%) 5 (23.8%)	47 (73.4%) 17 (26.6%)	p=1.000
Parity	N (%) =0 N (%) =1 N (%) ≥2	11 (12.9%) 53 (62.4%) 21 (24.7%)	5 (12.8%) 23 (59.0%) 11 (28.2%)	6 (13.0%) 30 (65.2%) 10 (21.7%)	p=0.551	2 (9.5%) 12 (57.1%) 7 (33.3%)	9 (14.1%) 41 (64.1%) 14 (21.9%)	p=0.282

Table 32 Patient demographic details

Characteristics of women included in the main study group ('Spontaneous Contractions'), and each substudy group ('Single Oxytocin 1nM Response' and 'Oxytocin Concentration Response').

Spontaneous contractile activity

Variations in spontaneous activity of myometrial strips between women and between strips were accounted for with linear mixed effects models, giving an average frequency across the 85 women of 7.22 (range 4.39, 11.89) contractions/hour, with an average amplitude of 55.57 (range 24.74, 98.72) mN and activity integral of 3383 (range 1335, 6365) mN.s. No correlation was found between maternal BMI and any indices of spontaneous myometrial contractile activity: frequency (r=-0.03 [-0.26, 0.30], p=0.780), amplitude (r=0.12 [-0.07, 0.30], p=0.228), activity integral (r=-0.13, [-0.32, 0.08], p=0.231). As expected, there was a correlation between increasing maternal BMI and increasing infant birth weight (r=0.54, [0.38, 0.68], p<0.001). However, in terms of myometrial activity, birth weight, maternal age, parity, previous caesarean delivery and gestational age were unrelated to spontaneous contractility (Table 33).

		Frequency	Amplitude	Activity Integral	BMI
BMI	Correlation (95% CI) p-value	-0.03 (-0.26, 0.30) p=0.780	0.12 (-0.07, 0.30) p=0.228	-0.13 (-0.32, 0.08) p=0.231	
Age	Correlation (95% CI) p-value	-0.13 (-0.33, 0.08) p=0.205	-0.02 (-0.23, 0.26) p=0.815	-0.02 (-0.23, 0.19) p=0.874	0.15 (-0.07, 0.34) p=0.171
Gestational age	Correlation (95% CI) p-value	-0.13 (-0.34, 0.09) p=0.274	0.00 (-0.23, 0.24) p=0.950	0.05 (-0.15, 0.22) p=0.580	-0.03 (-0.21, 0.13) p=0.684
Birth weight	Correlation (95% CI) p-value	0.01 (-0.19, 0.20) p=0.927	0.10 (-0.12, 0.35) p=0.436	-0.12 (-0.31, 0.04) p=0.195	0.54 (0.38, 0.68) p<0.001
Previous caesarean delivery	Correlation (95% CI) p-value	0.16 (-0.07, 0.35) p=0.166	-0.13 (-0.31, 0.07) p=0.196	0.07 (-0.14, 0.28) p=0.497	0.20 (-0.04, 0.44) p=0.092
Parity	Correlation (95% CI) p-value	0.10 (-0.12, 0.31) p=0.382	-0.10 (-0.37, 0.13) p=0.451	0.07 (-0.11, 0.25) p=0.397	0.21 (-0.02, 0.40) p=0.061

Table 33 Correlation between maternal characteristics and contractile activity

Correlations between patient average frequency, amplitude and activity integral of spontaneous contractions, based on linear mixed effects models of transformed variables, and maternal BMI, age, gestational age, birth weight, number of previous caesarean deliveries and parity.

The association between maternal characteristics and the proportion of strips contracting spontaneously within 2 hours is shown in Table 34. Univariate analysis demonstrated that maternal BMI, ethnicity and gestational age at birth

did not appear to affect time to development of spontaneous activity. However, strips were more likely to develop regular spontaneous activity within 2 hours in older mothers, those with increased parity relative to nulliparous women or if they had a previous caesarean delivery. Greater infant birth weight appeared to be associated with a reduced probability of spontaneous contraction within 2 hours. Subsequent multivariate logistic regression of the proportion of strips spontaneously contracting within 2 hours found that the previously observed association with parity was explained by the association with maternal age. Nevertheless, independent associations were found for maternal age, birth weight and previous caesarean delivery, with the latter independent of the indication for previous caesarean section. Therefore, despite contractile strength being unaffected by maternal characteristics, strips taken from mothers over 35 years, with a previous caesarean delivery or with smaller babies tended to spontaneously contract sooner than those from other women.

Factor	Level	N strips/Total (%)	Univariate Association		Multivariate Association	
			Odds Ratio (95% CI)	p-value	Odds Ratio (95% CI)	p-value
BMI (kg/m^2)	<25	272/299 (91.0%)	1.00	0.856	-	
	25-30	131/143 (91.6%)	1.08 (0.53, 2.20)			
	>30	150/167 (89.8%)	0.88 (0.46, 1.65)			
Age (years)	< 30	220/249 (88.4%)	1.00	<0.001	1.00	p=0.002
	30-34	174/198 (87.9%)	0.96 (0.54, 1.70)		0.86 (0.44, 1.68)	
	35+	159/162 (98.1%)	6.98 (2.12, 22.99)		5.21 (1.50, 18.10)	
Gestation (days)	≤ 39 wks	304/333 (91.3%)	1.00	0.648	-	
	> 39 wks	249/276 (90.2%)	0.88 (0.51, 1.52)			
Birth Weight (kg)	< 3.5 kg	250/262 (95.4%)	1.00	0.001	1.00	p<0.001
	≥ 3.5 kg	274/313 (87.5%)	0.34 (0.17, 0.66)		0.31 (0.15, 0.61)	
Ethnicity	Caucasian	511/560 (91.2%)	1.00	0.227	-	
	Non-Caucasian	42/49 (85.7%)	0.58 (0.25, 1.35)			
Previous caesarean delivery	No	127/149 (85.2%)	1.00	0.012	1.00	p=0.013
	Yes	416/450 (92.4%)	2.12 (1.20, 3.75)		2.36 (1.21, 4.58)	
Parity	0	75/87 (86.2%)	1.00	0.021	-	
	1	339/377 (89.9%)	1.43 (0.71, 2.86)			
	≥ 2	139/145 (95.9%)	3.71 (1.34, 10.26)			

Table 34 Association between maternal characteristics and time to developing contractile activity

Associations between patient characteristics and probability of spontaneous contraction within 2 hours, from univariate logistic regression models for each characteristic, and from multiple logistic regression model found by forward stepwise regression approach.

6.4.2 Contractile response to a single addition of oxytocin (OT)

Exposure to a concentration of 1nM OT produced an immediate increase in contractile activity in the strips, with model predictions of the average response for each time point for frequency, amplitude and activity integral shown in Figure 104. Maternal BMI was unrelated to all measures of contractile response to 1nM OT. The estimated changes in each contractile measure for every 5kg/m^2 increase in BMI were: for log frequency, +0.03 [-0.04, 0.09], p=0.454; for the square root of amplitude, -0.29 [-0.89, 0.31], p=0.352; for the square root of activity integral, -4.9 [-10.6, 0.8], p=0.098. Additionally, there was no evidence that contractile response to OT was affected by any other patient characteristic including maternal age, gestation, birth weight, ethnicity, parity or previous caesarean delivery (Table 35).

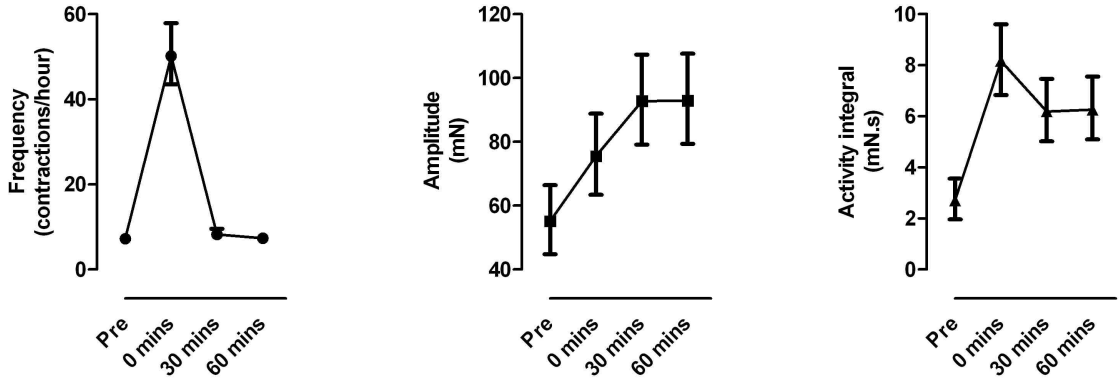

Figure 104 Contractile response to OT

Model predictions of average myometrial contractile activity (with 95% confidence interval) as measured by frequency, amplitude and activity integral following exposure to a single concentration (1nM) oxytocin.

Measure	Factor	Estimate	95% CI	p-value
Frequency	BMI	0.03	(-0.04, 0.09)	0.454
	Maternal Age	-0.08	(-0.29, 0.13)	0.469
	Gestational Age	0.09	(-0.04, 0.22)	0.175
	Birth weight	0.21	(-0.08, 0.51)	0.162
	Ethnicity	0.34	(0.00, 0.68)	0.058
	Indication for LUSCS	-0.07	(-0.27, 0.13)	0.489
	Parity	0.02	(-0.09, 0.14)	0.664
Amplitude	BMI	-0.29	(-0.89, 0.31)	0.352
	Maternal Age	-0.63	(-2.06, 0.81)	0.398
	Gestational Age	-0.65	(-1.51, 0.21)	0.145
	Birth weight	0.06	(-2.66, 2.77)	0.966
	Ethnicity	1.36	(-0.95, 3.67)	0.256
	Indication for LUSCS	-0.20	(-1.81, 1.40)	0.805
	Parity	0.44	(-0.41, 1.29)	0.317
Activity Integral	BMI	-4.9	(-10.6, 0.8)	0.098
	Maternal Age	3.5	(-10.7, 17.7)	0.628
	Gestational Age	-7.5	(-15.8, 0.8)	0.084
	Birth weight	4.4	(-23.0, 31.8)	0.756
	Ethnicity	12.8	(-10.0, 35.6)	0.277
	Indication for LUSCS	1.7	(-14.1, 17.4)	0.838
	Parity	7.9	(-0.2, 15.9)	0.063

Table 35 Effects of maternal characteristics on contractile response to OT

Estimated effects of BMI (per 5 kg/m^2 increase), maternal age (per 10 year increase), gestational age (per 5 day increase), birth weight (per 500 g increase), ethnicity (Caucasian vs. Other), indication for caesarean delivery (LUSCS) (Previous LUSCS vs. Other) and parity (per additional pregnancy) on the contractile response to a single concentration of 1nM OT

6.4.3 Oxytocin (OT) concentration response curves

Cumulative additions of OT in the range 10^{-12} to 10^{-6} M resulted in a gradual increase in amplitude from baseline median 53.9 mN (35.0 - 81.5) to maximal amplitude at 10^{-7}M of 101.7 mN (81.6-117.2) with a slight fall in amplitude after maximal activity. Based on the model, the expected mean log EC_{50} [OT] was -9.7 (5-95% range -10.7, -8.7), and was unrelated to maternal BMI (r=0.03 [-0.36, 0.35], p=0.977) (Figure 105). Parity, maternal age, gestational age, birth weight and previous caesarean delivery were similarly unrelated to log EC_{50} [OT] (Table 36).

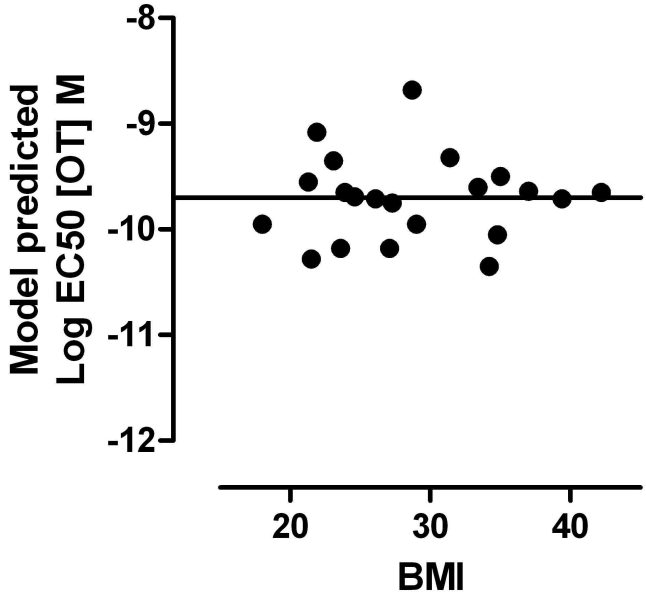

Figure 105 Correlation of maternal BMI and log EC_{50} [OT]

Scatter plot of model predicted log EC_{50} [OT] relative to maternal BMI, with line showing average log EC_{50} [OT]

		log EC_{50}
BMI	Correlation (95% CI) p-value	0.00 (-0.36, 0.35) p=0.977
Age	Correlation (95% CI) p-value	0.02 (-0.49, 0.38) p=0.846
Gestational Age	Correlation (95% CI) p-value	-0.06 (-0.44, 0.26) p=0.713
Birth Weight	Correlation (95% CI) p-value	0.15 (-0.30, 0.55) p=0.513
Previous LUSCS	Correlation (95% CI) p-value	0.12 (-0.30, 0.49) p=0.509
Parity	Correlation (95% CI) p-value	0.15 (-0.32, 0.57) p=0.438

Table 36 Correlation between maternal characteristics and log EC_{50} [OT]

Correlations between patient average log EC_{50} for the response of amplitude to OT, based on a linear mixed effects model, and maternal BMI, age, gestational age, birth weight, number of previous LUSCS and parity.

6.5 Summary of Results

6.5.1 Maternal BMI and spontaneous contractile activity

- Maternal BMI does not influence myometrial spontaneous contractile activity *in-vitro*

6.5.2 Maternal BMI and contractile response to oxytocin (OT)

- Maternal BMI does not influence myometrial contractile response to OT *in-vitro*

6.5.3 Maternal and pregnancy characteristics and spontaneous contractile activity

- Maternal and pregnancy characteristics of age, ethnicity, parity, previous caesarean delivery, gestation and birth weight do not influence myometrial spontaneous contractile activity (amplitude, frequency and activity integral) *in-vitro*.

- There is a tendency for the maternal characteristics of older age (>35 years), previous caesarean delivery or smaller baby to shorten the time required for myometrium *in-vitro* to establish spontaneous activity despite no influence on contractile strength.

6.5.4 Maternal and pregnancy characteristics and contractile response to oxytocin (OT)

- Maternal and pregnancy characteristics of age, ethnicity, parity, previous caesarean delivery, gestation and birth weight do not influence myometrial contractile response to OT *in-vitro*.

6.6 Discussion

6.6.1 Myometrial contractility and maternal BMI

In this study we clearly demonstrate that maternal obesity is not associated with an alteration in *in-vitro* myometrial function. Specifically maternal obesity does not impair the ability of the myometrium to generate spontaneous contractile activity as measured by contraction amplitude, frequency of contractions and activity integral. Furthermore, we demonstrate that increasing maternal BMI does not alter the myometrial response to either a single concentration of OT or cumulative additions of OT (concentration response). These findings are applicable to the obstetric population since the maternal BMI distribution in our sample set is comparable with that in both the general and obstetric female population[436 439].

The dyslipidaemic environment of obese mothers[446 449] has the potential to modulate cellular membrane lipid content[445 450 451]. In particular the areas with a high cholesterol content may be affected, namely lipid rafts or caveolae, which are associated with and can alter efficacy of calcium channels[451] and OT receptors[450] – key systems in regulating myometrial contractile responses. The increasing hyperlipidaemic state of pregnancy across gestation[452] may therefore facilitate an increase in cellular membrane activity in preparation for labour. In support of this, OT receptors[450] and caveolae[453] are noted to increase in number with increasing gestation. Furthermore cholesterol deposition within the cell membrane allows stabilization of the OT receptor with enhanced agonist affinity[450]. These lipid dependent effects on OT receptors may ensure an adequate response to OT in obese women.

The findings related to maternal BMI and contractility are in agreement with other data which found no relationship between BMI and *in-vitro* spontaneous contractile activity in addition to no relationship with response to the tocolytics nifedipine and indomethacin[454]. However, there is a contrast with other previous smaller studies examining *in-vitro* myometrial spontaneous contractile activity. This potentially reflects technical differences with respect to the organ bath work in terms of organ bath solutions, temperature and the degree of tension applied to the strip. It may also reflect the number of women in the

study or the previous lack of accounting for inter-strip variability, with the current study including at least 3, and an average of 7.2 strips per patient, ultimately increasing the robustness of our findings[66 300 301]. Additionally, in previous studies examining the alteration of the membrane composition, differences in maternal BMI were not considered[300 301]. These studies may therefore only reflect the effect of acute changes in membrane composition in comparison to this study where samples were exposed to the hyperlipidaemic and hyperleptinaemic environment for the prolonged period of pregnancy where myometrium may adapt to maintain a pro-contractile state.

The observed response to a single concentration of OT is of interest; initial high frequency contractions with subsequent return to baseline frequency, and maintenance of an increased amplitude and activity integral over 1 hour. This reflects the ideal pattern of contractions required for the safe delivery of an infant, with a balance between frequency and force providing sufficient energy to encourage passage through the pelvic outlet, but still allowing time for recovery of uterine and placental blood flow between contractions.

6.6.2 Contractility and other maternal characteristics

Similar to the relationship with maternal BMI, increased maternal age is known to be associated with increased rates of dysfunctional labour, augmentation of labour with OT, and an increase in the need for intrapartum caesarean delivery[68 69]. Data presented show no relationship between maternal age and the ability of the myometrium to produce a contractile force *in-vitro*, and in fact myometrium from older mothers achieves stable contractile activity quicker than younger mothers. This suggests that myometrium *per se* is not affected by age, lending support to the alternative hypothesis that contractility is modulated by age related changes in the uterine blood supply (see section 6.6.3.1 for further discussion)[69].

Our data also confirm other large observational studies where increasing maternal BMI is correlated with increased birth weight[63 296], and although birthweight may be surrogate for uterine stretch our data do not suggest a relationship with myometrial contractile activity, either spontaneous or OT induced.

This study similarly does not demonstrate a relationship between previous caesarean delivery and myometrial activity. This is consistent with our sampling techniques avoiding scar tissue, the high incidence of successful vaginal birth[77] and no difference in progress in the first stage of labour after a previous caesarean delivery[309]. It would also suggest that the increased risk of scar rupture during induction of labour is not related to a problem with the contractile mechanisms *per se*, but reflects the weakness of the uterine scar.

6.6.3 Alternative mechanisms of poor contractility *in-vivo*

Given the lack of an impact of maternal obesity on the relationship of myometrial function and OT, we would suggest that alternative mechanisms are responsible for the observed high rates of dysfunctional labour in obese mothers.

6.6.3.1 Alteration in microvascular function and accumulation of lactic acid

Microvascular function is impaired in conjunction with maternal obesity at all stages of pregnancy[455 456] and this impairment in circulation may result in the local accumulation of metabolic waste products including lactic acidic – a known inhibitor of myocyte contractile function[457]. Notably we have also observed a time dependent decrease in myometrial function *in-vitro* consistent with a detrimental effect of metabolite accumulation on myocyte function. Consistent with this blood from myometrial capillaries of women experiencing dysfunctional labour has a significantly lower pH, higher lactate and lower oxygen saturation than samples from women not in labour, and those labouring normally with or without syntocinon[458]. Increases in maternal age also affect microvascular function[459] and in addition, evidence also suggests age related changes to the walls of the larger uterine arteries[69], which may explain the increased rate of dysfunctional labour in this group of women[68 69]. Interestingly, women with pre-eclampsia have also been shown to have microvascular dysfunction[460], and although little evidence exists to the length of labour in this group of women, available data suggest either no difference[461] or a prolonged induction to delivery time compared to case matched unaffected women[462].

6.6.3.2Asynchrony of uterine contractions and cervical ripening

The rate of cervical dilatation in obese women is also significantly slower than normal weight women[62] and despite OT augmentation and significant improvement in myometrial contractility, caesarean delivery may still be required for failure to progress[463]. Changes in cervical structure occur across gestation including remodelling of cervical smooth muscle[97] with longer cervical length in the second trimester predictive of caesarean at term and cervical length positively associated with maternal obesity[464]. These gradual changes in cervical structure occur in conjunction with gestation related changes in the myometrium and indeed mathematical modelling of the acute changes in association with labour suggest that asynchrony of uterine and cervical structural changes result in slow progress, which is difficult to overcome despite normal or high contractile pressures[465]. The increased risk of caesarean delivery may therefore be in part iatrogenic as if in the obese mother, the cervix does lag behind the uterus in its ability to prepare for labour, cervical priming may be more difficult, and syntocinon commenced prior to sufficient change in composition or effacement of the cervix. This may lead to a situation where smooth muscle cells are still within the tubular structure of the cervix, and when stimulated with exogenous OT they contract, thereby providing resistance to the myometrial force and ultimately the cervix in unable to dilate[466]. This is in part supported by the finding that over 50% of women who in their first pregnancy had a caesarean delivery performed for dysfunctional labour, managed to have a vaginal delivery in the subsequent pregnancy[77], and suggests that consideration for alternative cervical priming regimes should be given to women with higher BMI.

These changes in cervical dilatation do not, however, explain the observed increase in postpartum haemorrhage in conjunction with vaginal deliveries. Instead this association may simply reflect the increased birth weight of offspring of obese mothers[63 296] with a concomitant increase in placental size and thereby placental bed size the main source of atonic bleeding.

6.7 Conclusion

The data presented within this chapter demonstrate through the use of *in-vitro* contractility studies that maternal obesity does not impair spontaneous or OT induced myometrial contractions *in-vitro*, suggesting that the observed implication of obesity on parturition in vivo (high rates of induction of labour, high rates of intrapartum caesarean delivery and post partum haemorrhage) cannot be explained by an effect on myometrial contraction *per se*. Suggestions for alternative mechanisms which may be responsible for the observed increased risk in myometrial related complications have been proposed, which may include impaired microvascular function or asynchrony in preparation of the cervix for labour. Both mechanisms merit further investigation as targets to ultimately promote labour, decrease rates of intrapartum caesarean delivery for dysfunctional labour and reduce post partum haemorrhage in obese mothers.

Chapter 7

Discussion and Future Research

7 Discussion and Future Research

The process of parturition resulting in the delivery of a newborn is a fundamental event ensuring survival of the species. In humans, the main clinical problems of parturition include activation of the process too early or too late resulting in the delivery of pre-term and post-term infants, both with their own implications for future health of the mother and baby[8 14 37 44 45 467-470]. Additionally, where parturition systems are not activated correctly, dysfunctional labour with the resulting need for caesarean delivery may occur[58-61 471], with further complications still feasible after the delivery namely atonic post-partum haemorrhage[4 81-83]. Overall, in the UK approximately 40% of pregnancies are affected by one of these problems [3 4 58-61 83]. However, the exact processes involved in the initiation and maintenance of parturition in the human are not fully understood. With such an important event, influences are most likely to be multi-factorial, with hormonal, mechanical, inflammatory, biochemical and maternal environmental factors playing a part.

In this thesis I have explored a range of factors associated with human parturition. The advantages of using a temporal analysis approach in combination with *in-vitro* and *in-vivo* data are revealed, all of which I believe to add strength to our findings and conclusions.

7.1 Oxytocin

The hormone oxytocin (OT) plays a major role in the stimulation of uterine contractility and can help facilitate human parturition, both when labour onset is spontaneous or induced. In this section we focussed on the long term (hours) cellular effects of OT on the contracting myometrium as it has been suggested that OT may prepare the myocytes for efficient and effective labour by transcriptional changes influencing a pro-contractile phenotype[180 327].

We confirmed Blair Bell's century old observation of the uterotonic effects of OT on our *in-vitro* samples with immediate increases in contractile activity following addition to the organ bath[148]. However, despite evidence suggesting 'priming of the uterus' by long term exposure to OT, our *in-vitro* time and contraction series analysis did not reveal any transcriptional changes over and

above those seen in spontaneously contracting samples. Possible reasons for our observation are discussed within the chapter, and overall our results may suggest that the uterotonic effects of OT occur by actions facilitating or modulating pre-existing intracellular pro-contractile proteins rather that de-novo transcriptional changes.

7.2 The myometrial temporal transcriptional wave and inflammation

To our knowledge, we are the first to examine the time dependent transcriptional changes which take place within human myometrium in response to repeated contractile activity. The most striking finding was the temporal transcriptional wave which was dominated by genes associated with the cellular processes of inflammation, cellular damage and apoptosis with a concomitant down regulation of genes associated with cellular metabolism. Previous data from our department suggest similar changes to myometrial transcription are seen *in-vivo* in response to labour[211]. Despite limitations of *in-vivo* data with only 2 timepoints ('not in labour' or 'in labour'), we found an overlap of contraction associated genes with a preponderance of genes associated with the inflammatory response.

Confirmation that the inflammatory response was contraction induced was suggested by comparison with metabolically active but non-contracting samples. Here we found that despite evidence of an inflammatory response in the non-contracting, metabolically active samples, the response was greatly enhanced over and above these levels where the myometrium contracted. These data suggest that it is the repeated physical contractile activity of the myometrium which is the driving force behind the pro-inflammatory shift associated with human labour.

We also examined the paradigm of infection causing inflammation which in turn activates contractile activity. However, this proposal was not confirmed by our data. In contracting myometrium exposed to an infective LPS environment, the transcription of genes associated with inflammation was certainly greatly upregulated compared to contracting myometrium in a non-infective/non-inflammatory environment. However, despite enhancement of these pro-

inflammatory changes, we observed no impact on any measure of contractile activity. This is contrary to other proposals which have suggested inflammation is necessary to promote contractility in the myometrium [210 216 217 230-236]. This discordance may underlie why trials of anti-inflammatory medication and antibiotics in the prevention and treatment of preterm labour have demonstrated non-consistent results[239-241 261 271 287 289 290 350 472].

Additionally, the link between cellular damage and a non-specific inflammatory response was suggested by the use of various tocolytic agents. We found the results to be initially surprising, with myometrium treated with the tocolytic agents nifedipine, ritodrine, levcromakalim and progesterone mounting an inflammatory response comparable with that seen in contracting samples. However, the tocolytic ML7 showed suppression of inflammation. This suggests that inflammatory upregulation is influenced by the method of tocolysis. In this case, the difference may be explained by the level of the contractile pathway which is altered. Where 'down-stream' processes are blocked (e.g. MLCK blockage by ML7) the inflammatory response is inhibited, whereas when 'up-stream' events are modulated (e.g. calcium entry inhibition via nifedipine) this effects many other cellular activities which are not contraction related. The abnormal regulation of these cellular processes may be perceived by the cell as cellular damage, thereby leading to the tissue upregulating inflammatory processes. However, our data do not prove this theory and further investigation of myometrial reactions to chemical tocolytics including prostaglandin inhibitors, would be of interest.

Overall, data suggest that the myometrium is able to mount a non-specific inflammatory response to cellular damage whether this is induced by contractions, infection or chemical means. Additionally, with an extended exposure to cellular damage, for example contraction time, the more intense the degree of inflammatory response mounted by the myometrium.

7.3 Maternal peripheral inflammatory changes in pregnancy and during labour

Compared to non-pregnant controls, we found only minor changes to circulating markers of inflammation, with an overall picture of low grade inflammation

associated with term pregnancy. These changes included the well described neutrophil driven leukocytosis[355-357], concomitant increased GCSF and lower CCL11 and CCL22, with CRP levels increased despite remaining within the accepted 'normal range'[373 374]. In terms of leukocyte cell surface marker expression, we found no difference between groups and no evidence of leukocyte priming in the circulation of women at term. This is contrary to suggestions of others[372], however, in contrast to other studies, our data can be considered more representative of both term pregnancy induced and parturition induced changes since we used repeated measures on the same patients with a non-labouring baseline measurement in addition to having data from two comparison groups; a non-pregnant population and those pre and post a non-labouring caesarean delivery. Ideally, it would also be advantageous to examine gestation dependent changes to leukocyte cell surface chemokine receptor expression, and data collection for this is currently being undertaken within our department.

In terms of a temporal wave of inflammatory changes in response to labour, we have been able to demonstrate that this also occurs within the maternal circulation. Additionally by using the comparison group of women undergoing non-labouring caesarean delivery (CS), we were able to control for delivery of an infant. Therefore, given that the extent of change was more pronounced in response to labour this suggests that the observed inflammatory changes may be contraction dependent. Overall, the inflammatory changes within the maternal circulation during labour tend to represent those observed during an acute inflammatory response which is commonly associated with other stimuli including infection and surgical trauma[264 365-371]. We also found that alterations to circulating leukocyte numbers and cytokine concentrations were consistent with the chemotaxis of leukocytes into labouring myometrium which has been noted by other studies[219 220]. Additionally we also found an overlap with the *in-vitro* myometrial transcription data with similar responses for CXCL1, CXCL2, IL10, IL6, CXCL8 and CCL2 which suggest the circulating cytokines may originate from the contracting myometrium.

CRP levels showed a tendency to increase the longer the labour progressed. However, differential levels for CRP pre-labour to post-labour were not

significant, though this is most probably a reflection of the delay between initial inflammatory response and hepatic manufacture of CRP.

Overall, we suggest that pregnancy *per se* results in a recognised low grade inflammatory response. However the labour associated inflammatory changes seem to be restricted to a non-specific acute inflammatory response during the period where the uterus is undergoing repeated contractions. Additionally we found no evidence to suggest circulating leukocytes undergo priming or preparation for the event of parturition.

7.4 Myometrial and systemic inflammatory responses and the association with cellular damage

The association between the inflammatory response of labour, and contraction induced myometrial cell damage is proposed by the combination of data using both in our *in-vitro* and *in-vivo* models of labour.

Using the *in-vitro* model, the observed myometrial transcriptional changes were time and contraction dependent, and were dominated by the concomitant upregulation of genes involved in the cellular processes of inflammation and cellular damage/apoptosis. Additionally, as discussed earlier, a similar non-specific inflammatory transcriptional response can also occur as a result of non-contractile myocyte damage i.e. infection or chemical mediated damage.

This relationship between myocyte damage and inflammation was further confirmed by the *in-vivo* data showing a strong association between maternal circulatory inflammatory response and the release of the muscle damage specific proteins creatine kinase (CK) and myoglobin (Mb).

Overall, these data supports our earlier proposal of inflammation in labour as a non-specific response to cellular damage, including contraction induced damage, with additional support suggested by the similarities with exercising skeletal muscle models[383 386 389 425-429].

7.5 The uterus as an exercising muscle

Overall the evidence supplied within the context of this thesis tends to support the concept of the labouring uterus as an exercising muscle, with significant similarities between labour and the cellular and humoral responses by skeletal muscle to exercise. Research on exercising skeletal muscle suggests that contractions cause myocyte damage, which in turn drives myocyte release of inflammatory mediators and promotion of the ensuing circulating inflammatory changes including leukocyte influx into the damaged muscle [383 386 389 425-429]

The inflammatory response seen in the exercising skeletal muscle model has been suggested to be involved with the repair and remodelling of damaged muscle tissue with leukocytes (mainly neutrophils and macrophages) playing a pivotal role in clearing damaged tissue with subsequent muscle repair, remodelling and restoration of function[389 429]. Certainly in CCR2 null mice the repair of exercise damaged muscle is impaired and is thought to be an effect of reduced monocyte/macrophage recruitment despite a sparing of the neutrophil response[431]. In addition, some CC chemokines (CCL2, CCL3 and CCL4) and IL6 produced at the site of injury are thought to have a direct effect upon healing and repair of the damaged myocytes[432 433 473].

The role of the inflammatory response in human labour is not addressed in this work. However, there is a suggestion that rather than being involved in the initiation and propagation of labour, inflammation is required for tissue repair and remodelling. Certainly, an inflammatory response appears to aid uterine involution in humans which is delayed in women who underwent a non-labouring caesarean section compared with normal labour, regardless of breast-feeding[434 435]. Additionally, monocyte infiltration to the uterus is not necessary for normal labour to occur in the CCR2 knock out mouse[315], but appears to be required for skeletal muscle repair[431]. There is also preliminary observational evidence from our lab which suggests that CCR2 deficient mice are more prone than their wild-type counterparts to birthing difficulties in their second and third pregnancies.

If our proposal regarding the role of inflammation in labour is correct, it would suggest that the inflammatory process may be necessary for sufficient involution of the postpartum uterus, and may also be required for adequate repair of

damaged tissues in order that they may be used again in the future for pregnancy and parturition. Furthermore, our data suggest that inflammation may not be a suitable target in the manipulation of labour, either in the pre-term situation or where labour induction is undertaken since its pivotal role may be in limiting damage, repairing tissue and ultimately restoring tissue function.

7.6 The contracting uterus and the influence of maternal and pregnancy factors

Despite the robustness of the physiological processes underlying human parturition, the maternal phenotype is capable of disrupting these mechanisms. Specifically there is considerable evidence for the implications of maternal BMI and other maternal and pregnancy factors affecting parturition *in-vivo*[62-80 295 296]. In terms of parturition and maternal BMI, complications associated with myometrial contractility include high rates of post dates induction of labour[63-65], high numbers of intrapartum caesarean deliveries for dysfunctional labour[63 64 66 67] and high rates of post partum haemorrhage independent of mode of delivery and use of OT[63 65 66].

Despite these clinical population based observations, our *in-vitro* model of labour showed no association between myometrial contractile ability including response to OT and maternal characteristics, in particular maternal BMI. These data therefore suggest an alternative mechanism for the observed clinical outcomes associated with maternal factors, particularly BMI, and myometrial contractile function. Suggestions for an alternative include altered microvascular function with accumulation of lactic acid, or asynchrony of uterine contractions and cervical ripening.

7.7 Future research

From the work presented in this thesis, many novel concepts have been suggested. Inevitably, confirmation and further exploration of our findings will be required; therefore areas and suggestions for possible future works are outlined below.

In Chapter 3 we have attempted to address the transcriptional role of long term (hours) exposure of myometrium to OT. Our data would suggest no additional transcriptional role for OT with effects mainly concerned with facilitation and modulation of pre-existing proteins. However, we were unable to address the possible confounding factor of inherent oxytocinase activity within the samples themselves. Therefore, to strengthen our findings, it would be important to repeat the organ bath experiment and perhaps use a continuous infusion of OT rather than the one single addition. This would also have the added advantage of being more comparable to the clinical situation of induction of labour. However, it may also bring difficulties as differential myometrial contractile responses may be obtained and a decision regarding consistency between experiments would need to be determined, e.g. rate of infusion or a measure of contractility.

In Chapter 4 we have suggested a temporal aspect to the inflammatory response seen in human labour. In addition, a possible mechanism of contraction induced cellular damage has been proposed as the causative factor with a great deal of overlap with data from contracting skeletal muscle. In view of this proposed causative link, it would be pertinent to examine further the gene array data with complementary QPCR, with particular reference to correlations between inflammatory upregulation and transcription of genes related to stress proteins. There has been particular interest in the role of myometrial Heat Shock Proteins in human labour[474-477], and certainly in view of our data, a relationship with inflammation would seem logical.

The difficulties concerning the overlap analysis of *in-vitro* and *in-vivo* gene array data was discussed within Chapter 4. These issues were somewhat addressed by the QPCR analysis with never contracted and contracting samples from our studies. However, the probes used were limited and it is pertinent that these

QPCR inflammatory data are completed for additional comparison and confirmation that the *in-vitro* model emulates the *in-vivo* occurrences during human parturition. Since completion of this thesis, samples are currently being processed and it is anticipated data will be available later in the year.

In Chapter 5, our *in-vivo* data concerning measurable factors in the maternal circulation of women during pregnancy at term and in response to delivery have shown results comparable with our transcriptional data. However, we must acknowledge that numbers are small, although it does represent essential initial pilot data. Therefore, to confirm our results, these experiments would require to be repeated on larger numbers. This would have the additional benefit of providing essential intrapartum clinical 'normal' values for the measured parameters. This has the potential to affect clinical management during labour including selective use of intrapartum antibiotic therapy, administration of antibiotics to the newborn and choice of analgesia, especially regional epidural anaesthesia. Additionally, these parameters may be able to guide the obstetric clinician to decision making for determination of those who are progressing in labour and have a good chance of achieving a vaginal delivery and those who are not. Markers of muscle cell damage may also have the potential to be used as a marker of pre-term labour and importantly determining those who are at risk of pre-term birth therefore helping guide clinical decision making for tocolytic therapy, antenatal steroid administration and in-utero hospital transfer.

Our *in-vivo* maternal circulation data included those undergoing non-labouring caesarean delivery and those undergoing induction of labour. Since labour was being induced, we have inevitably been unable to study circulatory changes induced prior to and during spontaneous labour. These may be identical to those seen in our study, but perhaps occur over a longer time period. However, repeated measures data from women as they approach term and the relationship with delivery outcome does require to be studied in order to address fully the relationship between uterine contractility, cellular damage and inflammation, in particular leukocyte priming prior to labour onset.

Additionally, any changes in maternal circulating inflammatory measures throughout pregnancy would also be of interest. Since the completion of this thesis we have been involved in ongoing sampling of women throughout

pregnancy, with samples taken in each gestation. These have been processed for FACS with data analysis to take place this summer. These stored samples will also be analysed for a number of inflammatory cytokines and delivery outcome data for these women are also available. It is hoped this would add further evidence for the pattern and role of inflammation throughout the length of pregnancy.

A potentially wide reaching aspect of the findings within this thesis is the use of human labour in the study of systemic inflammatory conditions where it is essential to promote tissue repair and restoration of function to the affected organs. Human labour could provide an excellent model since we propose that contraction induced muscle damage leads to a local and systemic inflammatory response with subsequent repair and remodelling of the uterus with restoration of function. Therefore, further exploration of the mechanisms and role of inflammation in human parturition is certainly merited by those working both within the field of obstetrics and in other clinical specialities. Within our department further *in-vivo* studies examining the role of inflammation in parturition are being undertaken. Specifically, local and humoral inflammatory changes over the course of pregnancy and also within the post-partum period are being assessed in the mouse model[478]. In addition, a comparison between wild-type and CCR2 knock-out mice is being undertaken which we hope will provide data regarding post-partum uterine remodelling and the importance of the myometrial inflammatory infiltrate in facilitating repair and return to function after delivery. This is of particular interest as we have preliminary observational evidence from our lab which suggests that CCR2 deficient mice are more prone than their wild-type counterparts to birthing difficulties in their second and third pregnancies.

Lastly, in Chapter 6, we have provided strong evidence of a lack of association between maternal BMI and the inherent ability of the myometrium to contract and respond appropriately to OT stimulation. Alternative mechanisms for the observed contractile functional problems were discussed. These would merit investigation as reducing rates of induction of labour, caesarean delivery and post partum haemorrhage among this group of women would obviously be advantageous. One option may be to attempt to reduce the build up of lactic acid within the contracting uterine system. There is a potential that lactic acid

build up and therefore contractile efficiency could be lessened by promoting aerobic metabolism of the uterine muscle during labour. In the skeletal and cardiac muscle system this is achieved via improving cardio-respiratory fitness through physical activity and aerobic exercise, which also hold true within pregnancy[479-482]. The effects of maternal fitness on the resultant parturition related outcomes could be assessed using ongoing cohort studies, for example the UK Pregnancies Better Eating and Activity Trial (UPBEAT)[483], which may then provide more evidence for the advantages of promoting physical exercise during pregnancy.

7.8 Summary and Final Conclusions

In this thesis I have explored a selection of factors associated with human parturition. Overall I have provided further evidence that human labour is an inflammatory process and that contrary to the current paradigm of inflammation driving contractions, our data suggest labour associated inflammation is a non-specific response to contraction induced cellular damage. Additionally, based on comparison with complimentary data from the exercising skeletal muscle model, we have suggested that the peripheral and local inflammatory response of labour may play a role in tissue repair and remodelling post delivery.

The functional and cellular influences of the uterotonic OT, various tocolytics, an inflammatory environment and maternal pregnancy characteristics were investigated with details of findings relating to inflammation in parturition illustrated in Figure 106 and an overall summary of the main findings of this thesis illustrated below in Figure 107.

Overall, understanding of these pathways aids the understanding of myometrial and inflammatory physiology involved in human pregnancy and labour. Ultimately increasing our knowledge surrounding these aspects will aid the safe and effective delivery of maternity care and delivery of the newborn infant in the future.

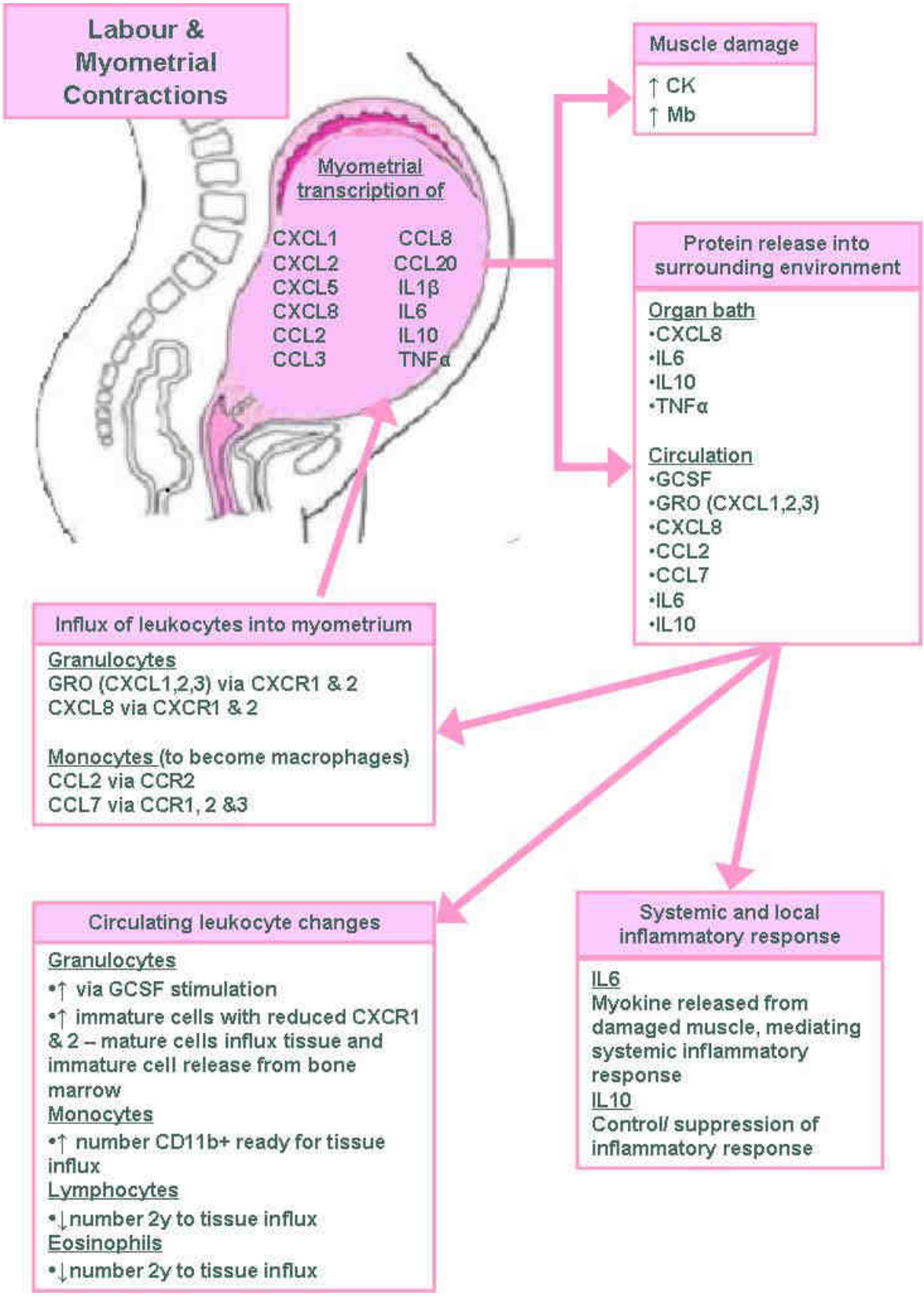

Figure 106 Main experimental findings

Illustration summarising the main findings relating specifically to inflammation and parturition from the research presented in this thesis.

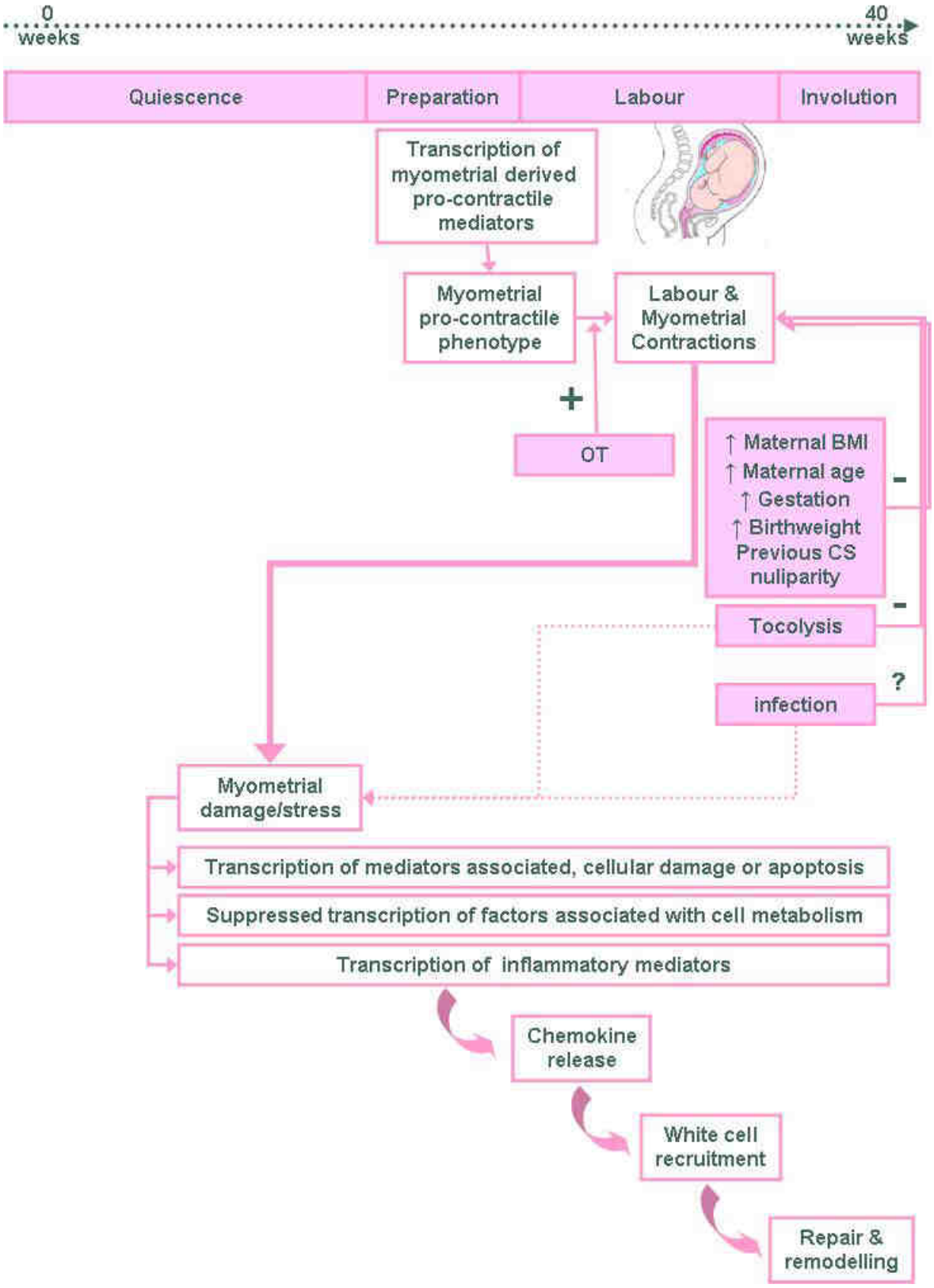

Figure 107 Summary overall hypothesis

Illustration summarising the main findings from the research presented in this thesis, and proposal of how the findings relate to one another. These are shown against the time course of the normal process of human pregnancy and parturition.

Appendices

Appendix I

Tabular description of cellular pathways represented by enrichment pathway analysis using the Kyoto Encyclopedia of Genes and Genomes (KEGG) performed on genes differentially expressed between contracting and non-contracting myometrium (DMSO vs ML7)

Timepoint of comparison	KEGG path ID	Direction of change	Pathway description	P value
1	530	Up	Aminosugars metabolism	0.014436
	4320	Up	Dorso-ventral axis formation	0.014436
	51	Up	Fructose and mannose metabolism	0.042081
	31	Up	Inositol metabolism	0.036786
	5130	Up	Pathogenic Escherichia coli infection - EHEC	0.002134
	5131	Up	Pathogenic Escherichia coli infection - EPEC	0.002134
	3320	Up	PPAR signaling pathway	0.038261
	521	Up	Streptomycin biosynthesis	0.013899
	410	Down	beta-Alanine metabolism	0.01657
	650	Down	Butanoate metabolism	0.000386
	4020	Down	Calcium signaling pathway	0.035786
	5213	Down	Endometrial cancer	0.026338
	5214	Down	Glioma	0.011754
	251	Down	Glutamate metabolism	0.027044
	4730	Down	Long-term depression	0.024759
	4070	Down	Phosphatidylinositol signaling system	0.026033
	640	Down	Propanoate metabolism	0.006078
	5216	Down	Thyroid cancer	0.024724
	380	Down	Tryptophan metabolism	0.009613
	220	Down	Urea cycle and metabolism of amino groups	0.027044
	280	Down	Valine, leucine and isoleucine degradation	3.84E-05
2	5220	Up	Chronic myeloid leukemia	0.021641
	5213	Up	Endometrial cancer	0.044284
	4012	Up	ErbB signaling pathway	0.004599
	4640	Up	Hematopoietic cell lineage	0.015721
	4010	Up	MAPK signaling pathway	0.032488
	1510	Up	Neurodegenerative Diseases	0.014454
	5215	Up	Prostate cancer	0.016757
	4810	Up	Regulation of actin cytoskeleton	0.028046
	5211	Up	Renal cell carcinoma	0.003974

Timepoint of comparison	KEGG path ID	Direction of change	Pathway description	P value
	5216	Up	Thyroid cancer	0.021965
	4940	Up	Type I diabetes mellitus	0.023474
	4310	Up	Wnt signaling pathway	0.02244
	1430	Down	Cell Communication	0.047093
	4110	Down	Cell cycle	0.042796
	4512	Down	ECM-receptor interaction	0.002597
	4510	Down	Focal adhesion	0.018488
	563	Down	Glycosylphosphatidylinositol(GPI)-anchor biosynthesis	0.035083
	626	Down	Naphthalene and anthracene degradation	0.026887
	860	Down	Porphyrin and chlorophyll metabolism	0.011957
4	4520	Up	Adherens junction	0.002895
	4662	Up	B cell receptor signaling pathway	0.029954
	4110	Up	Cell cycle	0.028058
	5110	Up	Cholera - Infection	0.030657
	5220	Up	Chronic myeloid leukemia	0.017961
	4710	Up	Circadian rhythm	0.025869
	5213	Up	Endometrial cancer	0.045391
	5120	Up	Epithelial cell signaling in Helicobacter pylori infection	0.01974
	4012	Up	ErbB signaling pathway	0.001778
	4640	Up	Hematopoietic cell lineage	0.026284
	4630	Up	Jak-STAT signaling pathway	0.011826
	4010	Up	MAPK signaling pathway	0.002139
	4330	Up	Notch signaling pathway	0.006577
	5020	Up	Parkinson's disease	0.007434
	4810	Up	Regulation of actin cytoskeleton	0.019129
	5211	Up	Renal cell carcinoma	0.000115
	5222	Up	Small cell lung cancer	0.012028
	4120	Up	Ubiquitin mediated proteolysis	0.046959
	252	Down	Alanine and aspartate metabolism	0.005555
	970	Down	Aminoacyl-tRNA biosynthesis	0.000185
	330	Down	Arginine and proline metabolism	0.008413
	120	Down	Bile acid biosynthesis	0.040353
	650	Down	Butanoate metabolism	0.001764
	710	Down	Carbon fixation	0.007408
	4110	Down	Cell cycle	0.012775
	62	Down	Fatty acid elongation in mitochondria	0.002018

Timepoint of comparison	KEGG path ID	Direction of change	Pathway description	P value
	251	Down	Glutamate metabolism	0.002742
	561	Down	Glycerolipid metabolism	0.012818
	563	Down	Glycosylphosphatidylinositol(GPI)-anchor biosynthesis	0.00423
	340	Down	Histidine metabolism	0.003174
	785	Down	Lipoic acid metabolism	0.030769
	310	Down	Lysine degradation	0.000558
	401	Down	Novobiocin biosynthesis	0.030769
	670	Down	One carbon pool by folate	0.020531
	30	Down	Pentose phosphate pathway	0.04851
	640	Down	Propanoate metabolism	0.0019
	230	Down	Purine metabolism	0.008051
	240	Down	Pyrimidine metabolism	0.038987
	620	Down	Pyruvate metabolism	0.009903
	72	Down	Synthesis and degradation of ketone bodies	0.009862
	380	Down	Tryptophan metabolism	0.001015
	220	Down	Urea cycle and metabolism of amino groups	0.032333
	290	Down	Valine, leucine and isoleucine biosynthesis	6.69E-05
	280	Down	Valine, leucine and isoleucine degradation	2.05E-08
6	5221	Up	Acute myeloid leukemia	0.000523
	4520	Up	Adherens junction	0.003332
	4210	Up	Apoptosis	0.02304
	4662	Up	B cell receptor signaling pathway	0.002492
	5219	Up	Bladder cancer	0.007922
	4110	Up	Cell cycle	0.018868
	5110	Up	Cholera - Infection	0.016786
	5220	Up	Chronic myeloid leukemia	2.36E-05
	4060	Up	Cytokine-cytokine receptor interaction	0.01071
	5050	Up	Dentatorubropallidoluysian atrophy (DRPLA)	0.018439
	4320	Up	Dorso-ventral axis formation	0.001782
	5213	Up	Endometrial cancer	0.009171
	5120	Up	Epithelial cell signaling in Helicobacter pylori infection	0.005253
	4012	Up	ErbB signaling pathway	1.48E-05
	4664	Up	Fc epsilon RI signaling pathway	0.015789
	5214	Up	Glioma	0.024981

Timepoint of comparison	KEGG path ID	Direction of change	Pathway description	P value
	4640	Up	Hematopoietic cell lineage	0.004815
	5040	Up	Huntington's disease	0.004539
	4910	Up	Insulin signaling pathway	0.006982
	4630	Up	Jak-STAT signaling pathway	0.004352
	4010	Up	MAPK signaling pathway	0.000217
	1510	Up	Neurodegenerative Diseases	0.011112
	5223	Up	Non-small cell lung cancer	0.013185
	4330	Up	Notch signaling pathway	0.00064
	4115	Up	p53 signaling pathway	0.048119
	5215	Up	Prostate cancer	0.001128
	3050	Up	Proteasome	0.012732
	4810	Up	Regulation of actin cytoskeleton	0.00013
	5211	Up	Renal cell carcinoma	1.12E-06
	3010	Up	Ribosome	0.047831
	5222	Up	Small cell lung cancer	0.004815
	4660	Up	T cell receptor signaling pathway	0.005601
	252	Down	Alanine and aspartate metabolism	0.016712
	970	Down	Aminoacyl-tRNA biosynthesis	7.55E-05
	440	Down	Aminophosphonate metabolism	0.028894
	530	Down	Aminosugars metabolism	0.015349
	330	Down	Arginine and proline metabolism	0.025237
	120	Down	Bile acid biosynthesis	0.006358
	780	Down	Biotin metabolism	0.011277
	363	Down	Bisphenol A degradation	0.043088
	650	Down	Butanoate metabolism	0.010411
	62	Down	Fatty acid elongation in mitochondria	0.008981
	71	Down	Fatty acid metabolism	0.00212
	790	Down	Folate biosynthesis	0.030531
	51	Down	Fructose and mannose metabolism	0.014888
	52	Down	Galactose metabolism	0.013354
	5214	Down	Glioma	0.03614
	251	Down	Glutamate metabolism	0.024264
	563	Down	Glycosylphosphatidylinositol(GPI)-anchor biosynthesis	0.025707
	340	Down	Histidine metabolism	0.001863
	310	Down	Lysine degradation	0.001086
	626	Down	Naphthalene and anthracene degradation	0.003519
	520	Down	Nucleotide sugars metabolism	0.005428

Timepoint of comparison	KEGG path ID	Direction of change	Pathway description	P value
	860	Down	Porphyrin and chlorophyll metabolism	0.009929
	640	Down	Propanoate metabolism	0.000622
	230	Down	Purine metabolism	0.017488
	240	Down	Pyrimidine metabolism	0.003291
	620	Down	Pyruvate metabolism	0.005539
	4130	Down	SNARE interactions in vesicular transport	0.006358
	380	Down	Tryptophan metabolism	0.000211
	130	Down	Ubiquinone biosynthesis	0.019822
	290	Down	Valine, leucine and isoleucine biosynthesis	0.004133
	280	Down	Valine, leucine and isoleucine degradation	4.40E-06

Appendix II

Tabular description of cellular pathways represented by enrichment pathway analysis using the Gene Ontology (GO) performed on genes differentially expressed in contracting myometrial samples (AA, DMSO, OT) at timepoints 1hr, 2hr, 4hr and 6 hours relative to baseline 0 hours.

Timepoint of comparison	Direction of change	Ontology group	GO path ID	Pathway description	Enrichment P value
1	Up	MF	GO:0003706	ligand-regulated transcription factor activity	0.000622173
	Up	BP	GO:0048522	positive regulation of cellular process	0.000691733
	Up	MF	GO:0005515	protein binding	0.00023372
	Up	MF	GO:0003704	specific RNA polymerase II transcription factor activity	0.000788204
	Down	MF	GO:0005487	nucleocytoplasmic transporter activity	0.000848817
2	Up	MF	GO:0005488	binding	2.57E-05
	Up	CC	GO:0000785	chromatin	9.67E-06
	Up	BP	GO:0031497	chromatin assembly	3.72E-06
	Up	BP	GO:0006333	chromatin assembly or disassembly	1.15E-05
	Up	CC	GO:0044427	chromosomal part	0.000417698
	Up	CC	GO:0005694	chromosome	0.000166287
	Up	BP	GO:0051276	chromosome organization and biogenesis	0.000392357
	Up	MF	GO:0003677	DNA binding	0.000506751
	Up	BP	GO:0006323	DNA packaging	5.38E-07
	Up	BP	GO:0006325	establishment and/or maintenance of chromatin architecture	0.000151035
	Up	MF	GO:0008201	heparin binding	0.000340187
	Up	CC	GO:0005622	intracellular	0.000130194
	Up	CC	GO:0043231	intracellular membrane-bound organelle	0.000203262
	Up	CC	GO:0043229	intracellular organelle	0.000157989
	Up	CC	GO:0044424	intracellular part	0.000141089
	Up	BP	GO:0043170	macromolecule metabolic process	0.000105493

Timepoint of comparison	Direction of change	Ontology group	GO path ID	Pathway description	Enrichment P value
	Up	CC	GO:0043227	membrane-bound organelle	0.000206202
	Up	MF	GO:0003676	nucleic acid binding	0.000680035
	Up	CC	GO:0000786	nucleosome	1.51E-06
	Up	BP	GO:0006334	nucleosome assembly	1.27E-06
	Up	CC	GO:0005634	nucleus	2.03E-05
	Up	CC	GO:0043226	organelle	0.000161434
	Up	BP	GO:0006996	organelle organization and biogenesis	0.000625805
	Up	MF	GO:0005515	protein binding	3.66E-05
	Up	BP	GO:0006611	protein export from nucleus	0.000948706
	Up	BP	GO:0065004	protein-DNA complex assembly	9.71E-05
	Up	BP	GO:0042981	regulation of apoptosis	0.000404679
	Up	BP	GO:0043067	regulation of programmed cell death	0.000473937
	Down	MF	GO:0003689	DNA clamp loader activity	0.000987566
	Down	MF	GO:0033170	DNA-protein loading ATPase activity	0.000987566
4	Up	MF	GO:0050681	androgen receptor binding	0.000545202
	Up	BP	GO:0030521	androgen receptor signaling pathway	0.000967225
	Up	BP	GO:0006915	apoptosis	5.16E-05
	Up	MF	GO:0005488	binding	2.01E-05
	Up	BP	GO:0065007	biological regulation	1.01E-05
	Up	BP	GO:0043283	biopolymer metabolic process	1.63E-06
	Up	BP	GO:0043412	biopolymer modification	0.000217054
	Up	BP	GO:0007049	cell cycle	0.000585277
	Up	BP	GO:0008219	cell death	9.40E-05
	Up	BP	GO:0048468	cell development	0.000335693
	Up	BP	GO:0008283	cell proliferation	8.65E-05
	Up	BP	GO:0044237	cellular metabolic process	0.000484337
	Up	CC	GO:0000785	chromatin	3.64E-06
	Up	BP	GO:0031497	chromatin assembly	2.86E-05
	Up	BP	GO:0006333	chromatin assembly or disassembly	1.30E-06

Timepoint of comparison	Direction of change	Ontology group	GO path ID	Pathway description	Enrichment P value
	Up	CC	GO:0044427	chromosomal part	0.000359826
	Up	CC	GO:0005694	chromosome	0.000127255
	Up	BP	GO:0051276	chromosome organization and biogenesis	1.32E-05
	Up	BP	GO:0016265	death	9.40E-05
	Up	BP	GO:0006323	DNA packaging	1.36E-05
	Up	BP	GO:0006325	establishment and/or maintenance of chromatin architecture	1.06E-05
	Up	BP	GO:0000082	G1/S transition of mitotic cell cycle	0.000158293
	Up	CC	GO:0005622	intracellular	2.06E-05
	Up	CC	GO:0043231	intracellular membrane-bound organelle	2.98E-05
	Up	CC	GO:0043229	intracellular organelle	0.000208213
	Up	CC	GO:0044424	intracellular part	1.01E-05
	Up	BP	GO:0043170	macromolecule metabolic process	1.00E-06
	Up	CC	GO:0043227	membrane-bound organelle	3.08E-05
	Up	BP	GO:0008152	metabolic process	4.58E-05
	Up	BP	GO:0043066	negative regulation of apoptosis	0.000385525
	Up	BP	GO:0048519	negative regulation of biological process	3.54E-05
	Up	BP	GO:0048523	negative regulation of cellular process	5.56E-05
	Up	BP	GO:0043069	negative regulation of programmed cell death	0.000467171
	Up	CC	GO:0000786	nucleosome	7.66E-06
	Up	BP	GO:0006334	nucleosome assembly	5.79E-06
	Up	CC	GO:0005634	nucleus	5.89E-08
	Up	CC	GO:0043226	organelle	0.000217066
	Up	MF	GO:0016773	phosphotransferase activity, alcohol group as acceptor	0.000510285
	Up	BP	GO:0048518	positive regulation of biological process	4.73E-06
	Up	BP	GO:0048522	positive regulation of cellular process	4.83E-06
	Up	BP	GO:0045935	positive regulation of nucleobase, nucleoside, nucleotide and nucleic acid metabolic process	0.000203091
	Up	BP	GO:0045941	positive regulation of transcription	0.000234308
	Up	BP	GO:0043687	post-translational protein modification	3.20E-05
	Up	BP	GO:0044238	primary metabolic process	3.53E-05
	Up	BP	GO:0012501	programmed cell death	6.99E-05
	Up	MF	GO:0005515	protein binding	1.23E-07

Timepoint of comparison	Direction of change	Ontology group	GO path ID	Pathway description	Enrichment P value
	Up	BP	GO:0006464	protein modification process	9.60E-05
	Up	BP	GO:0065004	protein-DNA complex assembly	2.41E-06
	Up	BP	GO:0042981	regulation of apoptosis	7.95E-06
	Up	BP	GO:0050789	regulation of biological process	2.48E-06
	Up	BP	GO:0051726	regulation of cell cycle	0.0004283 9
	Up	BP	GO:0050794	regulation of cellular process	4.97E-06
	Up	BP	GO:0000079	regulation of cyclin-dependent protein kinase activity	0.0006238 79
	Up	BP	GO:0050793	regulation of developmental process	0.0003641 92
	Up	BP	GO:0045682	regulation of epidermis development	0.0008666 89
	Up	BP	GO:0019222	regulation of metabolic process	0.0003048 49
	Up	BP	GO:0043067	regulation of programmed cell death	1.15E-05
	Up	BP	GO:0006950	response to stress	0.0003689 38
	Up	MF	GO:0015081	sodium ion transmembrane transporter activity	0.0009724 21
	Down	MF	GO:0030554	adenyl nucleotide binding	2.17E-05
	Down	MF	GO:0032559	adenyl ribonucleotide binding	0.0001146 52
	Down	BP	GO:0009308	amine metabolic process	9.02E-05
	Down	BP	GO:0006519	amino acid and derivative metabolic process	0.0002632 05
	Down	BP	GO:0006520	amino acid metabolic process	5.70E-05
	Down	MF	GO:0005524	ATP binding	6.52E-05
	Down	BP	GO:0009058	biosynthetic process	2.40E-05
	Down	BP	GO:0019752	carboxylic acid metabolic process	4.60E-07
	Down	MF	GO:0003824	catalytic activity	1.50E-11
	Down	CC	GO:0005623	cell	3.06E-05
	Down	BP	GO:0051301	cell division	0.0003040 09
	Down	CC	GO:0044464	cell part	2.98E-05
	Down	BP	GO:0044249	cellular biosynthetic process	1.27E-05
	Down	BP	GO:0044237	cellular metabolic process	7.83E-05
	Down	MF	GO:0050897	cobalt ion binding	0.0005699 87
	Down	BP	GO:0051188	cofactor biosynthetic process	4.69E-05
	Down	BP	GO:0051186	cofactor metabolic process	2.12E-05
	Down	CC	GO:0000777	condensed chromosome kinetochore	0.0003810 24

Timepoint of comparison	Direction of change	Ontology group	GO path ID	Pathway description	Enrichment P value
	Down	CC	GO:0000779	condensed chromosome, pericentric region	0.000658513
	Down	CC	GO:0005737	cytoplasm	1.94E-11
	Down	CC	GO:0044444	cytoplasmic part	5.78E-12
	Down	BP	GO:0006259	DNA metabolic process	6.31E-05
	Down	BP	GO:0006281	DNA repair	0.000372685
	Down	BP	GO:0006260	DNA replication	0.000112245
	Down	BP	GO:0051656	establishment of organelle localization	0.000985606
	Down	BP	GO:0046483	heterocycle metabolic process	6.43E-05
	Down	CC	GO:0005622	intracellular	7.24E-15
	Down	CC	GO:0043231	intracellular membrane-bound organelle	7.88E-14
	Down	CC	GO:0043229	intracellular organelle	1.08E-10
	Down	CC	GO:0044446	intracellular organelle part	3.46E-09
	Down	CC	GO:0044424	intracellular part	4.84E-14
	Down	CC	GO:0000776	kinetochore	0.000112545
	Down	MF	GO:0016874	ligase activity	0.000197695
	Down	CC	GO:0043227	membrane-bound organelle	8.35E-14
	Down	CC	GO:0031974	membrane-enclosed lumen	5.24E-07
	Down	BP	GO:0008152	metabolic process	9.37E-06
	Down	BP	GO:0051310	metaphase plate congression	0.000562598
	Down	MF	GO:0008168	methyltransferase activity	0.000653603
	Down	CC	GO:0042579	microbody	0.000121067
	Down	CC	GO:0031980	mitochondrial lumen	4.52E-05
	Down	CC	GO:0005759	mitochondrial matrix	4.52E-05
	Down	CC	GO:0044429	mitochondrial part	2.52E-06
	Down	CC	GO:0005739	mitochondrion	5.52E-13
	Down	BP	GO:0032787	monocarboxylic acid metabolic process	0.000552538
	Down	BP	GO:0006807	nitrogen compound metabolic process	0.000461512
	Down	MF	GO:0004518	nuclease activity	0.000355999
	Down	MF	GO:0000166	nucleotide binding	0.0005553 4
	Down	CC	GO:0043226	organelle	1.16E-10
	Down	CC	GO:0043233	organelle lumen	5.24E-07

Timepoint of comparison	Direction of change	Ontology group	GO path ID	Pathway description	Enrichment P value
	Down	CC	GO:0031090	organelle membrane	7.45E-05
	Down	CC	GO:0044422	organelle part	4.59E-09
	Down	BP	GO:0006082	organic acid metabolic process	5.60E-07
	Down	CC	GO:0000940	outer kinetochore of condensed chromosome	0.000127627
	Down	MF	GO:0016491	oxidoreductase activity	0.000184099
	Down	CC	GO:0005777	peroxisome	0.000121067
	Down	BP	GO:0043507	positive regulation of JNK activity	0.000985606
	Down	BP	GO:0044238	primary metabolic process	0.000262287
	Down	BP	GO:0045426	quinone cofactor biosynthetic process	4.26E-05
	Down	BP	GO:0042375	quinone cofactor metabolic process	4.26E-05
	Down	BP	GO:0006974	response to DNA damage stimulus	0.00019872
	Down	MF	GO:0004540	ribonuclease activity	0.000288313
	Down	MF	GO:0008641	small protein activating enzyme activity	2.09E-05
	Down	CC	GO:0005819	spindle	0.000900018
	Down	MF	GO:0016740	transferase activity	0.000448862
	Down	MF	GO:0016765	transferase activity, transferring alkyl or aryl (other than methyl) groups	2.76E-05
	Down	MF	GO:0016741	transferase activity, transferring one-carbon groups	0.000105435
	Down	BP	GO:0006399	tRNA metabolic process	0.000909314
	Down	BP	GO:0006744	ubiquinone biosynthetic process	4.26E-05
	Down	BP	GO:0006743	ubiquinone metabolic process	4.26E-05
6	Up	MF	GO:0030554	adenyl nucleotide binding	0.000569117
	Up	MF	GO:0032559	adenyl ribonucleotide binding	0.000118964
	Up	BP	GO:0006916	anti-apoptosis	0.000142591
	Up	BP	GO:0006915	apoptosis	9.33E-09
	Up	MF	GO:0005524	ATP binding	8.08E-05
	Up	MF	GO:0005488	binding	7.77E-10
	Up	BP	GO:0065007	biological regulation	4.19E-11
	Up	BP	GO:0043285	biopolymer catabolic process	0.000658938
	Up	BP	GO:0043283	biopolymer metabolic process	1.40E-13

Timepoint of comparison	Direction of change	Ontology group	GO path ID	Pathway description	Enrichment P value
	Up	BP	GO:0043412	biopolymer modification	2.34E-14
	Up	BP	GO:0007596	blood coagulation	0.000257161
	Up	BP	GO:0007049	cell cycle	8.74E-05
	Up	BP	GO:0008219	cell death	3.18E-08
	Up	BP	GO:0048468	cell development	2.74E-06
	Up	BP	GO:0030154	cell differentiation	5.12E-05
	Up	BP	GO:0016049	cell growth	0.000445274
	Up	BP	GO:0008283	cell proliferation	1.00E-06
	Up	BP	GO:0048869	cellular developmental process	5.12E-05
	Up	BP	GO:0044265	cellular macromolecule catabolic process	0.000386038
	Up	BP	GO:0044260	cellular macromolecule metabolic process	2.77E-11
	Up	BP	GO:0044237	cellular metabolic process	1.76E-09
	Up	BP	GO:0009987	cellular process	6.09E-05
	Up	BP	GO:0044257	cellular protein catabolic process	0.000279914
	Up	BP	GO:0044267	cellular protein metabolic process	1.23E-11
	Up	CC	GO:0000785	chromatin	2.21E-05
	Up	BP	GO:0031497	chromatin assembly	0.000121759
	Up	BP	GO:0006333	chromatin assembly or disassembly	2.09E-07
	Up	BP	GO:0016568	chromatin modification	7.09E-06
	Up	CC	GO:0016585	chromatin remodeling complex	0.000923263
	Up	CC	GO:0005694	chromosome	0.000252343
	Up	BP	GO:0051276	chromosome organization and biogenesis	2.04E-08
	Up	BP	GO:0050817	coagulation	0.000300563
	Up	MF	GO:0005125	cytokine activity	0.000599409
	Up	CC	GO:0005737	cytoplasm	0.000192793
	Up	BP	GO:0016265	death	3.18E-08
	Up	BP	GO:0006323	DNA packaging	5.22E-06
	Up	BP	GO:0006325	establishment and/or maintenance of chromatin architecture	1.87E-08
	Up	BP	GO:0000082	G1/S transition of mitotic cell cycle	4.05E-05
	Up	BP	GO:0010467	gene expression	1.68E-05

Timepoint of comparison	Direction of change	Ontology group	GO path ID	Pathway description	Enrichment P value
	Up	MF	GO:0008083	growth factor activity	0.000464648
	Up	BP	GO:0007599	hemostasis	0.000544573
	Up	BP	GO:0051329	interphase of mitotic cell cycle	0.000700009
	Up	CC	GO:0005622	intracellular	8.81E-09
	Up	CC	GO:0043231	intracellular membrane-bound organelle	1.73E-06
	Up	CC	GO:0043229	intracellular organelle	6.66E-06
	Up	CC	GO:0044424	intracellular part	3.07E-08
	Up	BP	GO:0007242	intracellular signaling cascade	1.72E-06
	Up	MF	GO:0016301	kinase activity	1.90E-08
	Up	BP	GO:0043170	macromolecule metabolic process	6.88E-14
	Up	BP	GO:0000165	MAPKKK cascade	0.000771931
	Up	CC	GO:0043227	membrane-bound organelle	1.82E-06
	Up	BP	GO:0008152	metabolic process	3.47E-09
	Up	BP	GO:0043632	modification-dependent macromolecule catabolic process	0.000201007
	Up	BP	GO:0019941	modification-dependent protein catabolic process	0.000201007
	Up	BP	GO:0043066	negative regulation of apoptosis	5.37E-05
	Up	BP	GO:0048519	negative regulation of biological process	1.98E-06
	Up	BP	GO:0048523	negative regulation of cellular process	7.59E-06
	Up	BP	GO:0043069	negative regulation of programmed cell death	7.32E-05
	Up	BP	GO:0007219	Notch signaling pathway	0.000977857
	Up	CC	GO:0031981	nuclear lumen	2.84E-05
	Up	CC	GO:0044428	nuclear part	3.41E-05
	Up	CC	GO:0005654	nucleoplasm	1.62E-05
	Up	CC	GO:0044451	nucleoplasm part	1.51E-05
	Up	BP	GO:0006334	nucleosome assembly	0.000172674
	Up	MF	GO:0000166	nucleotide binding	0.000119412
	Up	CC	GO:0005634	nucleus	5.12E-11
	Up	CC	GO:0043226	organelle	5.51E-06
	Up	BP	GO:0006996	organelle organization and biogenesis	7.74E-06
	Up	BP	GO:0006796	phosphate metabolic process	7.33E-09
	Up	MF	GO:0004721	phosphoprotein phosphatase activity	0.000520766
	Up	BP	GO:0006793	phosphorus metabolic process	7.33E-09

Timepoint of comparison	Direction of change	Ontology group	GO path ID	Pathway description	Enrichment P value
	Up	BP	GO:0016310	phosphorylation	2.15E-07
	Up	MF	GO:0016773	phosphotransferase activity, alcohol group as acceptor	5.88E-09
	Up	BP	GO:0043065	positive regulation of apoptosis	0.0009126 17
	Up	BP	GO:0048518	positive regulation of biological process	3.92E-10
	Up	BP	GO:0031325	positive regulation of cellular metabolic process	0.0004088 31
	Up	BP	GO:0048522	positive regulation of cellular process	8.14E-10
	Up	BP	GO:0051094	positive regulation of developmental process	0.0007156 91
	Up	BP	GO:0009893	positive regulation of metabolic process	0.0002977 95
	Up	BP	GO:0045933	positive regulation of muscle contraction	0.0003816 14
	Up	BP	GO:0045935	positive regulation of nucleobase, nucleoside, nucleotide and nucleic acid metabolic process	2.19E-05
	Up	BP	GO:0051254	positive regulation of RNA metabolic process	1.57E-05
	Up	BP	GO:0045987	positive regulation of smooth muscle contraction	8.26E-05
	Up	BP	GO:0045941	positive regulation of transcription	3.04E-05
	Up	BP	GO:0045893	positive regulation of transcription, DNA-dependent	1.26E-05
	Up	BP	GO:0043687	post-translational protein modification	1.61E-15
	Up	BP	GO:0044238	primary metabolic process	1.08E-10
	Up	BP	GO:0012501	programmed cell death	1.74E-08
	Up	BP	GO:0006470	protein amino acid dephosphorylation	0.0002734 65
	Up	BP	GO:0006468	protein amino acid phosphorylation	2.17E-08
	Up	MF	GO:0005515	protein binding	7.03E-26
	Up	BP	GO:0030163	protein catabolic process	0.0005975 74
	Up	MF	GO:0004672	protein kinase activity	2.46E-07
	Up	BP	GO:0007243	protein kinase cascade	1.05E-05
	Up	BP	GO:0019538	protein metabolic process	1.15E-10
	Up	BP	GO:0006464	protein modification process	6.55E-15
	Up	MF	GO:0004674	protein serine/threonine kinase activity	1.18E-05
	Up	BP	GO:0065004	protein-DNA complex assembly	0.0005518 18
	Up	MF	GO:0004713	protein-tyrosine kinase activity	5.99E-05
	Up	BP	GO:0051603	proteolysis involved in cellular protein catabolic process	0.0002247 64

Timepoint of comparison	Direction of change	Ontology group	GO path ID	Pathway description	Enrichment P value
	Up	MF	GO:0032555	purine ribonucleotide binding	0.0003846 87
	Up	MF	GO:0005102	receptor binding	0.0005222 73
	Up	BP	GO:0042981	regulation of apoptosis	1.90E-07
	Up	BP	GO:0050789	regulation of biological process	1.73E-11
	Up	BP	GO:0065008	regulation of biological quality	0.0005588 59
	Up	BP	GO:0051726	regulation of cell cycle	0.0006379 63
	Up	BP	GO:0001558	regulation of cell growth	0.0003599 88
	Up	BP	GO:0008361	regulation of cell size	0.0001557 78
	Up	BP	GO:0031323	regulation of cellular metabolic process	0.0001058 78
	Up	BP	GO:0050794	regulation of cellular process	2.50E-10
	Up	BP	GO:0050793	regulation of developmental process	1.32E-05
	Up	BP	GO:0010468	regulation of gene expression	9.29E-05
	Up	BP	GO:0040008	regulation of growth	0.0001733 36
	Up	BP	GO:0043549	regulation of kinase activity	0.0007124 91
	Up	BP	GO:0019222	regulation of metabolic process	8.48E-06
	Up	BP	GO:0019219	regulation of nucleobase, nucleoside, nucleotide and nucleic acid metabolic process	0.0001375 82
	Up	BP	GO:0043067	regulation of programmed cell death	3.49E-07
	Up	BP	GO:0045859	regulation of protein kinase activity	0.0004639 2
	Up	BP	GO:0051252	regulation of RNA metabolic process	0.0001421 24
	Up	BP	GO:0045449	regulation of transcription	0.0002447 09
	Up	BP	GO:0006357	regulation of transcription from RNA polymerase II promoter	1.31E-08
	Up	BP	GO:0006355	regulation of transcription, DNA-dependent	0.0001620 74
	Up	BP	GO:0006950	response to stress	5.48E-05
	Up	BP	GO:0009611	response to wounding	0.0001473 18
	Up	MF	GO:0032553	ribonucleotide binding	0.0003846 87
	Up	BP	GO:0032774	RNA biosynthetic process	9.26E-05
	Up	BP	GO:0016070	RNA metabolic process	0.0003267 24

Timepoint of comparison	Direction of change	Ontology group	GO path ID	Pathway description	Enrichment P value
	Up	MF	GO:0003702	RNA polymerase II transcription factor activity	0.000989057
	Up	BP	GO:0006350	transcription	9.96E-05
	Up	MF	GO:0003713	transcription coactivator activity	0.000870098
	Up	MF	GO:0003712	transcription cofactor activity	1.94E-05
	Up	MF	GO:0008134	transcription factor binding	5.86E-05
	Up	BP	GO:0006366	transcription from RNA polymerase II promoter	1.71E-07
	Up	MF	GO:0030528	transcription regulator activity	6.33E-05
	Up	MF	GO:0016564	transcription repressor activity	8.28E-05
	Up	BP	GO:0006351	transcription, DNA-dependent	8.16E-05
	Up	MF	GO:0016772	transferase activity, transferring phosphorus-containing groups	7.79E-07
	Up	BP	GO:0006512	ubiquitin cycle	2.59E-06
	Up	BP	GO:0006511	ubiquitin-dependent protein catabolic process	0.000179517
	Up	BP	GO:0042060	wound healing	0.000315407
	Down	BP	GO:0009308	amine metabolic process	0.00086876
	Down	BP	GO:0009058	biosynthetic process	0.000178283
	Down	MF	GO:0016884	carbon-nitrogen ligase activity, with glutamine as amido-N-donor	0.000773059
	Down	BP	GO:0019752	carboxylic acid metabolic process	5.26E-05
	Down	MF	GO:0003824	catalytic activity	3.37E-10
	Down	CC	GO:0005623	cell	9.59E-07
	Down	CC	GO:0044464	cell part	9.18E-07
	Down	BP	GO:0044249	cellular biosynthetic process	0.000596365
	Down	BP	GO:0044237	cellular metabolic process	1.85E-07
	Down	BP	GO:0051188	cofactor biosynthetic process	5.47E-05
	Down	BP	GO:0051186	cofactor metabolic process	5.33E-06
	Down	CC	GO:0005737	cytoplasm	2.84E-11
	Down	CC	GO:0044444	cytoplasmic part	1.67E-12
	Down	BP	GO:0006259	DNA metabolic process	4.31E-05
	Down	BP	GO:0006281	DNA repair	4.31E-05
	Down	MF	GO:0004519	endonuclease activity	0.000997769
	Down	MF	GO:0004521	endoribonuclease activity	0.000111669
	Down	MF	GO:0004527	exonuclease activity	0.000911533

Timepoint of comparison	Direction of change	Ontology group	GO path ID	Pathway description	Enrichment P value
	Down	MF	GO:0016796	exonuclease activity, active with either ribo- or deoxyribonucleic acids and producing 5'-phosphomonoesters	0.000335386
	Down	MF	GO:0004532	exoribonuclease activity	0.000780098
	Down	MF	GO:0016896	exoribonuclease activity, producing 5'-phosphomonoesters	0.000780098
	Down	BP	GO:0009296	flagellum biogenesis	0.0008966 3
	Down	BP	GO:0043064	flagellum organization and biogenesis	0.0008966 3
	Down	BP	GO:0006783	heme biosynthetic process	0.000753702
	Down	BP	GO:0042168	heme metabolic process	0.000624421
	Down	MF	GO:0016788	hydrolase activity, acting on ester bonds	0.000456605
	Down	CC	GO:0005622	intracellular	1.50E-16
	Down	CC	GO:0043231	intracellular membrane-bound organelle	1.10E-21
	Down	CC	GO:0043229	intracellular organelle	1.84E-13
	Down	CC	GO:0044446	intracellular organelle part	3.64E-05
	Down	CC	GO:0044424	intracellular part	2.29E-16
	Down	CC	GO:0005764	lysosome	0.000114044
	Down	CC	GO:0000323	lytic vacuole	0.000114044
	Down	CC	GO:0043227	membrane-bound organelle	1.22E-21
	Down	CC	GO:0031974	membrane-enclosed lumen	6.19E-08
	Down	BP	GO:0008152	metabolic process	6.54E-10
	Down	MF	GO:0008168	methyltransferase activity	7.35E-07
	Down	CC	GO:0005740	mitochondrial envelope	0.000994704
	Down	CC	GO:0031980	mitochondrial lumen	5.12E-09
	Down	CC	GO:0005759	mitochondrial matrix	5.12E-09
	Down	CC	GO:0044429	mitochondrial part	1.57E-09
	Down	CC	GO:0005761	mitochondrial ribosome	1.79E-08
	Down	CC	GO:0005763	mitochondrial small ribosomal subunit	0.000862723
	Down	CC	GO:0005739	mitochondrion	1.16E-21
	Down	BP	GO:0006379	mRNA cleavage	0.000750444
	Down	BP	GO:0016071	mRNA metabolic process	0.000501094
	Down	BP	GO:0006397	mRNA processing	0.000329943

Timepoint of comparison	Direction of change	Ontology group	GO path ID	Pathway description	Enrichment P value
	Down	MF	GO:0004518	nuclease activity	5.77E-06
	Down	BP	GO:0006139	nucleobase, nucleoside, nucleotide and nucleic acid metabolic process	6.65E-05
	Down	CC	GO:0005730	nucleolus	0.000774181
	Down	CC	GO:0000313	organellar ribosome	1.79E-08
	Down	CC	GO:0000314	organellar small ribosomal subunit	0.000862723
	Down	CC	GO:0043226	organelle	2.07E-13
	Down	CC	GO:0043233	organelle lumen	6.19E-08
	Down	CC	GO:0031090	organelle membrane	0.000884751
	Down	CC	GO:0044422	organelle part	4.80E-05
	Down	BP	GO:0006082	organic acid metabolic process	6.49E-05
	Down	MF	GO:0016491	oxidoreductase activity	0.000913126
	Down	BP	GO:0006733	oxidoreduction coenzyme metabolic process	0.000599605
	Down	BP	GO:0044238	primary metabolic process	3.05E-07
	Down	BP	GO:0008104	protein localization	0.000952195
	Down	BP	GO:0015031	protein transport	0.000658299
	Down	BP	GO:0045426	quinone cofactor biosynthetic process	5.19E-05
	Down	BP	GO:0042375	quinone cofactor metabolic process	5.19E-05
	Down	MF	GO:0016854	racemase and epimerase activity	4.09E-05
	Down	MF	GO:0016857	racemase and epimerase activity, acting on carbohydrates and derivatives	0.000152277
	Down	BP	GO:0006974	response to DNA damage stimulus	6.06E-06
	Down	BP	GO:0009719	response to endogenous stimulus	5.85E-05
	Down	MF	GO:0004540	ribonuclease activity	9.04E-08
	Down	MF	GO:0003723	RNA binding	6.20E-07
	Down	BP	GO:0006396	RNA processing	2.23E-08
	Down	MF	GO:0016741	transferase activity, transferring one-carbon groups	1.32E-07
	Down	BP	GO:0006399	tRNA metabolic process	0.000239044
	Down	BP	GO:0006744	ubiquinone biosynthetic process	5.19E-05
	Down	BP	GO:0006743	ubiquinone metabolic process	5.19E-05
	Down	CC	GO:0005773	vacuole	0.000170295

Appendix III

Tabular description of cellular pathways represented by enrichment pathway analysis using the Kyoto Encyclopedia of Genes and Genomes (KEGG) performed on genes differentially expressed between contracting and non-contracting myometrium (DMSO vs ML7)

Timepoint of comparison	KEGG path ID	Direction of change	Pathway description	P value
0hr	5010	Up	Alzheimer's disease	0.000904
	4210	Up	Apoptosis	0.000255
	4060	Up	Cytokine-cytokine receptor interaction	0.006752
	4640	Up	Hematopoietic cell lineage	7.35E-08
	4010	Up	MAPK signaling pathway	0.006978
	5060	Up	Prion disease	0.021749
	4620	Up	Toll-like receptor signaling pathway	0.011167
	4940	Up	Type I diabetes mellitus	3.79E-05
	592	Down	alpha-Linolenic acid metabolism	0.010812
	590	Down	Arachidonic acid metabolism	0.038558
	565	Down	Ether lipid metabolism	0.022259
	564	Down	Glycerophospholipid metabolism	0.04839
	591	Down	Linoleic acid metabolism	0.022259
	4010	Down	MAPK signaling pathway	0.011172
	4370	Down	VEGF signaling pathway	0.049789
1hr	5010	Up	Alzheimer's disease	0.003804
	4210	Up	Apoptosis	0.002398
	4060	Up	Cytokine-cytokine receptor interaction	0.001087
	4640	Up	Hematopoietic cell lineage	0.002745
	4010	Up	MAPK signaling pathway	0.009029
	5060	Up	Prion disease	0.04306
	4350	Up	TGF-beta signaling pathway	0.034319
	4620	Up	Toll-like receptor signaling pathway	0.004079
	4940	Up	Type I diabetes mellitus	0.000374
	440	Down	Aminophosphonate metabolism	0.006429
	650	Down	Butanoate metabolism	0.041306
	71	Down	Fatty acid metabolism	0.044705
	281	Down	Geraniol degradation	0.03566
	563	Down	Glycosylphosphatidylinositol(GPI)-anchor biosynthesis	0.009751
	340	Down	Histidine metabolism	0.036406
	310	Down	Lysine degradation	0.00017

Timepoint of comparison	KEGG path ID	Direction of change	Pathway description	P value
	626	Down	Naphthalene and anthracene degradation	0.008012
	190	Down	Oxidative phosphorylation	0.008805
	620	Down	Pyruvate metabolism	0.036406
	450	Down	Selenoamino acid metabolism	0.019402
	380	Down	Tryptophan metabolism	0.000819
	280	Down	Valine, leucine and isoleucine degradation	0.039646
2hr	4920	Up	Adipocytokine signaling pathway	0.027409
	5010	Up	Alzheimer's disease	0.001984
	4612	Up	Antigen processing and presentation	0.046953
	4210	Up	Apoptosis	0.000849
	4060	Up	Cytokine-cytokine receptor interaction	0.007604
	4664	Up	Fc epsilon RI signaling pathway	0.030458
	4640	Up	Hematopoietic cell lineage	0.000115
	4010	Up	MAPK signaling pathway	0.008094
	4620	Up	Toll-like receptor signaling pathway	0.001966
	4940	Up	Type I diabetes mellitus	0.007253
	624	Down	1- and 2-Methylnaphthalene degradation	0.006828
	632	Down	Benzoate degradation via CoA ligation	0.008525
	5120	Down	Epithelial cell signaling in Helicobacter pylori infection	0.045513
	564	Down	Glycerophospholipid metabolism	0.045513
	903	Down	Limonene and pinene degradation	0.009129
	4010	Down	MAPK signaling pathway	0.008121
	400	Down	Phenylalanine, tyrosine and tryptophan biosynthesis	0.039824
4hr	5010	Up	Alzheimer's disease	0.002605
	4612	Up	Antigen processing and presentation	0.039482
	4210	Up	Apoptosis	0.003343
	4662	Up	B cell receptor signaling pathway	0.046014
	4514	Up	Cell adhesion molecules (CAMs)	0.039431
	4610	Up	Complement and coagulation cascades	0.008568
	4060	Up	Cytokine-cytokine receptor interaction	1.85E-07
	5120	Up	Epithelial cell signaling in Helicobacter pylori infection	0.007791

Timepoint of comparison	KEGG path ID	Direction of change	Pathway description	P value
	4664	Up	Fc epsilon RI signaling pathway	0.004858
	4640	Up	Hematopoietic cell lineage	8.85E-09
	533	Up	Keratan sulfate biosynthesis	0.046649
	4010	Up	MAPK signaling pathway	0.025465
	4650	Up	Natural killer cell mediated cytotoxicity	0.000357
	4620	Up	Toll-like receptor signaling pathway	1.07E-05
	4940	Up	Type I diabetes mellitus	0.006772
	252	Down	Alanine and aspartate metabolism	0.017707
	650	Down	Butanoate metabolism	0.002328
	710	Down	Carbon fixation	0.032387
	20	Down	Citrate cycle (TCA cycle)	0.009972
	790	Down	Folate biosynthesis	0.001422
	51	Down	Fructose and mannose metabolism	0.001617
	480	Down	Glutathione metabolism	0.006418
	630	Down	Glyoxylate and dicarboxylate metabolism	0.006544
	190	Down	Oxidative phosphorylation	0.008509
	400	Down	Phenylalanine, tyrosine and tryptophan biosynthesis	0.02342
	640	Down	Propanoate metabolism	0.000507
	620	Down	Pyruvate metabolism	0.000248
	720	Down	Reductive carboxylate cycle (CO2 fixation)	0.00395
	500	Down	Starch and sucrose metabolism	0.041838
	72	Down	Synthesis and degradation of ketone bodies	0.02951
	280	Down	Valine, leucine and isoleucine degradation	4.60E-05
6hr	5221	Up	Acute myeloid leukemia	0.003155
	4520	Up	Adherens junction	0.00736
	4210	Up	Apoptosis	0.038984
	4360	Up	Axon guidance	0.0404
	4110	Up	Cell cycle	0.012101
	5220	Up	Chronic myeloid leukemia	0.001144
	4710	Up	Circadian rhythm	0.048279
	5210	Up	Colorectal cancer	0.010021
	4060	Up	Cytokine-cytokine receptor interaction	7.37E-06
	5120	Up	Epithelial cell signaling in Helicobacter pylori infection	0.035117
	4664	Up	Fc epsilon RI signaling pathway	0.00736

Timepoint of comparison	KEGG path ID	Direction of change	Pathway description	P value
	4640	Up	Hematopoietic cell lineage	4.96E-05
	5040	Up	Huntington's disease	0.017556
	4630	Up	Jak-STAT signaling pathway	0.001254
	533	Up	Keratan sulfate biosynthesis	0.026056
	4010	Up	MAPK signaling pathway	0.000714
	4650	Up	Natural killer cell mediated cytotoxicity	0.000998
	4330	Up	Notch signaling pathway	0.008837
	5212	Up	Pancreatic cancer	0.013166
	770	Up	Pantothenate and CoA biosynthesis	0.026056
	5130	Up	Pathogenic Escherichia coli infection - EHEC	0.019503
	5131	Up	Pathogenic Escherichia coli infection - EPEC	0.019503
	5215	Up	Prostate cancer	0.032029
	4810	Up	Regulation of actin cytoskeleton	0.00051
	5211	Up	Renal cell carcinoma	3.69E-05
	4660	Up	T cell receptor signaling pathway	0.006004
	4620	Up	Toll-like receptor signaling pathway	0.013185
	4120	Up	Ubiquitin mediated proteolysis	0.046777
	624	Down	1- and 2-Methylnaphthalene degradation	0.00334
	252	Down	Alanine and aspartate metabolism	0.000226
	960	Down	Alkaloid biosynthesis II	0.025849
	970	Down	Aminoacyl-tRNA biosynthesis	0.000101
	330	Down	Arginine and proline metabolism	0.022743
	632	Down	Benzoate degradation via CoA ligation	4.68E-05
	100	Down	Biosynthesis of steroids	0.01176
	363	Down	Bisphenol A degradation	0.024077
	650	Down	Butanoate metabolism	0.000209
	930	Down	Caprolactam degradation	0.005768
	710	Down	Carbon fixation	0.037292
	272	Down	Cysteine metabolism	0.046992
	62	Down	Fatty acid elongation in mitochondria	0.006592
	71	Down	Fatty acid metabolism	0.004423
	790	Down	Folate biosynthesis	0.017674
	51	Down	Fructose and mannose metabolism	0.000455
	251	Down	Glutamate metabolism	0.034037
	903	Down	Limonene and pinene degradation	6.62E-05

Timepoint of comparison	KEGG path ID	Direction of change	Pathway description	P value
	310	Down	Lysine degradation	0.015297
	626	Down	Naphthalene and anthracene degradation	0.016942
	401	Down	Novobiocin biosynthesis	0.019556
	190	Down	Oxidative phosphorylation	6.57E-09
	360	Down	Phenylalanine metabolism	0.034037
	400	Down	Phenylalanine, tyrosine and tryptophan biosynthesis	0.000176
	860	Down	Porphyrin and chlorophyll metabolism	1.10E-05
	640	Down	Propanoate metabolism	0.005595
	230	Down	Purine metabolism	0.003756
	240	Down	Pyrimidine metabolism	0.000249
	620	Down	Pyruvate metabolism	0.020305
	3020	Down	RNA polymerase	0.037292
	643	Down	Styrene degradation	0.019556
	380	Down	Tryptophan metabolism	0.009394
	350	Down	Tyrosine metabolism	0.009394
	4120	Down	Ubiquitin mediated proteolysis	0.019251
	220	Down	Urea cycle and metabolism of amino groups	0.034037
	280	Down	Valine, leucine and isoleucine degradation	0.000701

Appendix IV

Tabular description of cellular pathways represented by enrichment pathway analysis using the Gene Ontology (GO) performed on genes differentially expressed between contracting and non-contracting myometrium (DMSO vs ML7)

Timepoint of comparison	Direction of change	Ontology group	GO path ID	Pathway description	Enrichment P value
0hr	Up	BP	GO:0032288	myelin formation	0.000302
	Up	BP	GO:0030913	paranodal junction assembly	0.000302
	Down	BP	GO:0051353	positive regulation of oxidoreductase activity	0.000906
	Down	CC	GO:0005662	DNA replication factor A complex	0.000818
	Down	MF	GO:0035014	phosphoinositide 3-kinase regulator activity	0.000833
1hr	Up	BP	GO:0001525	angiogenesis	0.000924
	Up	BP	GO:0001660	fever	0.000755
	Up	BP	GO:0032755	positive regulation of interleukin-6 production	0.000755
	Down	BP	GO:0042158	lipoprotein biosynthetic process	0.000666
	Down	BP	GO:0006497	protein amino acid lipidation	0.000547
2hr	Up	BP	GO:0002526	acute inflammatory response	0.000381
	Up	BP	GO:0007610	behavior	1.63E-05
	Up	BP	GO:0051641	cellular localization	0.000757
	Up	BP	GO:0006935	chemotaxis	2.53E-07
	Up	BP	GO:0006952	defense response	6.16E-06
	Up	BP	GO:0051649	establishment of cellular localization	0.000638
	Up	BP	GO:0006887	exocytosis	2.22E-05
	Up	BP	GO:0001660	fever	3.71E-06
	Up	BP	GO:0031649	heat generation	2.22E-05
	Up	BP	GO:0006955	immune response	0.00011
	Up	BP	GO:0002376	immune system process	9.93E-06
	Up	BP	GO:0006954	inflammatory response	5.32E-08
	Up	BP	GO:0007626	locomotory behavior	9.65E-07
	Up	BP	GO:0008285	negative regulation of cell proliferation	0.000889
	Up	BP	GO:0017157	regulation of exocytosis	0.000332
	Up	BP	GO:0042221	response to chemical stimulus	6.92E-05
	Up	BP	GO:0009605	response to external stimulus	1.32E-06
	Up	BP	GO:0050896	response to stimulus	5.88E-05
	Up	BP	GO:0006950	response to stress	9.22E-06
	Up	BP	GO:0009611	response to wounding	7.27E-07

Timepoint of comparison	Direction of change	Ontology group	GO path ID	Pathway description	Enrichment P value
	Up	BP	GO:0046903	secretion	0.000387
	Up	BP	GO:0032940	secretion by cell	8.51E-05
	Up	BP	GO:0045045	secretory pathway	0.0003
	Up	BP	GO:0042330	taxis	2.53E-07
	Up	BP	GO:0001659	thermoregulation	7.74E-05
	Up	BP	GO:0016192	vesicle-mediated transport	0.000243
	Up	CC	GO:0044421	extracellular region part	0.000275
	Up	CC	GO:0005615	extracellular space	2.00E-05
	Up	MF	GO:0008009	chemokine activity	1.80E-06
	Up	MF	GO:0042379	chemokine receptor binding	1.96E-06
	Up	MF	GO:0005125	cytokine activity	3.66E-06
	Up	MF	GO:0001664	G-protein-coupled receptor binding	1.03E-05
	Up	MF	GO:0005149	interleukin-1 receptor binding	0.000261
	Up	MF	GO:0001871	pattern binding	5.12E-05
	Up	MF	GO:0042834	peptidoglycan binding	0.000121
	Up	MF	GO:0005102	receptor binding	0.000278
	Down	BP	GO:0008633	activation of pro-apoptotic gene products	0.000831
	Down	BP	GO:0051789	response to protein stimulus	1.35E-05
	Down	BP	GO:0006950	response to stress	0.000732
	Down	BP	GO:0006986	response to unfolded protein	1.35E-05
4hr	Up	BP	GO:0002253	activation of immune response	0.000583
	Up	BP	GO:0002526	acute inflammatory response	2.90E-05
	Up	BP	GO:0002250	adaptive immune response	4.78E-06
	Up	BP	GO:0002460	adaptive immune response based on somatic recombination of immune receptors built from immunoglobulin superfamily domains	4.78E-06
	Up	BP	GO:0006915	apoptosis	6.83E-10
	Up	BP	GO:0019724	B cell mediated immunity	0.000222
	Up	BP	GO:0007610	behavior	4.02E-07
	Up	BP	GO:0007154	cell communication	0.000144
	Up	BP	GO:0008219	cell death	1.16E-09
	Up	BP	GO:0048468	cell development	1.01E-05
	Up	BP	GO:0030154	cell differentiation	6.69E-06
	Up	BP	GO:0008283	cell proliferation	0.000519
	Up	BP	GO:0048869	cellular developmental process	6.69E-06
	Up	BP	GO:0006935	chemotaxis	1.99E-10
	Up	BP	GO:0051181	cofactor transport	0.000914
	Up	BP	GO:0001816	cytokine production	2.73E-06

Timepoint of comparison	Direction of change	Ontology group	GO path ID	Pathway description	Enrichment P value
	Up	BP	GO:0050663	cytokine secretion	0.000627
	Up	BP	GO:0016265	death	1.16E-09
	Up	BP	GO:0006952	defense response	2.62E-11
	Up	BP	GO:0032502	developmental process	0.000367
	Up	BP	GO:0001660	fever	0.00028
	Up	BP	GO:0030097	hemopoiesis	2.90E-06
	Up	BP	GO:0048534	hemopoietic or lymphoid organ development	6.45E-06
	Up	BP	GO:0048872	homeostasis of number of cells	9.78E-05
	Up	BP	GO:0006959	humoral immune response	0.000955
	Up	BP	GO:0002252	immune effector process	0.000552
	Up	BP	GO:0006955	immune response	3.98E-17
	Up	BP	GO:0002520	immune system development	2.45E-06
	Up	BP	GO:0002376	immune system process	7.81E-23
	Up	BP	GO:0016064	immunoglobulin mediated immune response	0.000199
	Up	BP	GO:0048305	immunoglobulin secretion	0.00028
	Up	BP	GO:0006917	induction of apoptosis	6.92E-06
	Up	BP	GO:0008624	induction of apoptosis by extracellular signals	0.000237
	Up	BP	GO:0012502	induction of programmed cell death	7.33E-06
	Up	BP	GO:0006954	inflammatory response	7.92E-15
	Up	BP	GO:0032635	interleukin-6 production	0.000154
	Up	BP	GO:0007242	intracellular signaling cascade	0.000327
	Up	BP	GO:0030595	leukocyte chemotaxis	2.64E-05
	Up	BP	GO:0002521	leukocyte differentiation	0.000727
	Up	BP	GO:0050900	leukocyte migration	8.79E-06
	Up	BP	GO:0007626	locomotory behavior	9.67E-10
	Up	BP	GO:0030099	myeloid cell differentiation	5.94E-06
	Up	BP	GO:0043066	negative regulation of apoptosis	9.07E-05
	Up	BP	GO:0048519	negative regulation of biological process	7.94E-05
	Up	BP	GO:0008285	negative regulation of cell proliferation	0.000104
	Up	BP	GO:0048523	negative regulation of cellular process	0.000147
	Up	BP	GO:0051093	negative regulation of developmental process	0.000329
	Up	BP	GO:0043069	negative regulation of programmed cell death	0.000104
	Up	BP	GO:0051048	negative regulation of secretion	0.000627
	Up	BP	GO:0030593	neutrophil chemotaxis	0.000914

Timepoint of comparison	Direction of change	Ontology group	GO path ID	Pathway description	Enrichment P value
	Up	BP	GO:0018212	peptidyl-tyrosine modification	0.000125
	Up	BP	GO:0018108	peptidyl-tyrosine phosphorylation	9.78E-05
	Up	BP	GO:0016310	phosphorylation	0.000894
	Up	BP	GO:0002821	positive regulation of adaptive immune response	0.000511
	Up	BP	GO:0002824	positive regulation of adaptive immune response based on somatic recombination of immune receptors built from immunoglobulin superfamily domains	0.000511
	Up	BP	GO:0043065	positive regulation of apoptosis	1.05E-05
	Up	BP	GO:0048518	positive regulation of biological process	1.22E-07
	Up	BP	GO:0050867	positive regulation of cell activation	0.000334
	Up	BP	GO:0048522	positive regulation of cellular process	2.38E-06
	Up	BP	GO:0032270	positive regulation of cellular protein metabolic process	0.000459
	Up	BP	GO:0001819	positive regulation of cytokine production	5.11E-05
	Up	BP	GO:0051094	positive regulation of developmental process	1.74E-07
	Up	BP	GO:0050778	positive regulation of immune response	4.70E-05
	Up	BP	GO:0002684	positive regulation of immune system process	8.58E-07
	Up	BP	GO:0032755	positive regulation of interleukin-6 production	0.00028
	Up	BP	GO:0002696	positive regulation of leukocyte activation	0.000334
	Up	BP	GO:0043410	positive regulation of MAPKKK cascade	0.000627
	Up	BP	GO:0051240	positive regulation of multicellular organismal process	1.71E-07
	Up	BP	GO:0050731	positive regulation of peptidyl-tyrosine phosphorylation	1.60E-05
	Up	BP	GO:0045937	positive regulation of phosphate metabolic process	4.68E-06
	Up	BP	GO:0042327	positive regulation of phosphorylation	3.30E-06
	Up	BP	GO:0043068	positive regulation of programmed cell death	1.22E-05
	Up	BP	GO:0001934	positive regulation of protein amino acid phosphorylation	9.97E-07
	Up	BP	GO:0051247	positive regulation of protein metabolic process	0.000586
	Up	BP	GO:0031401	positive regulation of protein modification process	6.53E-06

Timepoint of comparison	Direction of change	Ontology group	GO path ID	Pathway description	Enrichment P value
	Up	BP	GO:0048584	positive regulation of response to stimulus	0.000333
	Up	BP	GO:0009967	positive regulation of signal transduction	0.000422
	Up	BP	GO:0032760	positive regulation of tumor necrosis factor production	0.00083
	Up	BP	GO:0012501	programmed cell death	9.22E-10
	Up	BP	GO:0006468	protein amino acid phosphorylation	0.00043
	Up	BP	GO:0007243	protein kinase cascade	0.000191
	Up	BP	GO:0009306	protein secretion	0.000141
	Up	BP	GO:0042981	regulation of apoptosis	5.56E-09
	Up	BP	GO:0050865	regulation of cell activation	0.000592
	Up	BP	GO:0045595	regulation of cell differentiation	7.54E-05
	Up	BP	GO:0042127	regulation of cell proliferation	9.79E-05
	Up	BP	GO:0001817	regulation of cytokine production	0.000649
	Up	BP	GO:0050793	regulation of developmental process	8.24E-10
	Up	BP	GO:0050776	regulation of immune response	0.000165
	Up	BP	GO:0002682	regulation of immune system process	1.23E-05
	Up	BP	GO:0032675	regulation of interleukin-6 production	8.95E-05
	Up	BP	GO:0002694	regulation of leukocyte activation	0.000592
	Up	BP	GO:0051239	regulation of multicellular organismal process	1.80E-05
	Up	BP	GO:0045637	regulation of myeloid cell differentiation	0.000273
	Up	BP	GO:0050730	regulation of peptidyl-tyrosine phosphorylation	1.08E-05
	Up	BP	GO:0019220	regulation of phosphate metabolic process	7.34E-06
	Up	BP	GO:0051174	regulation of phosphorus metabolic process	7.34E-06
	Up	BP	GO:0042325	regulation of phosphorylation	4.27E-06
	Up	BP	GO:0043067	regulation of programmed cell death	7.59E-09
	Up	BP	GO:0001932	regulation of protein amino acid phosphorylation	1.88E-06
	Up	BP	GO:0031399	regulation of protein modification process	1.53E-05
	Up	BP	GO:0042221	response to chemical stimulus	1.64E-07
	Up	BP	GO:0009605	response to external stimulus	2.74E-15
	Up	BP	GO:0050896	response to stimulus	7.76E-11
	Up	BP	GO:0006950	response to stress	1.36E-11
	Up	BP	GO:0009611	response to wounding	1.19E-15
	Up	BP	GO:0033363	secretory granule organization and biogenesis	0.00028

Timepoint of comparison	Direction of change	Ontology group	GO path ID	Pathway description	Enrichment P value
	Up	BP	GO:0007165	signal transduction	0.000401
	Up	BP	GO:0042330	taxis	1.99E-10
	Up	CC	GO:0005602	complement component C1 complex	0.000271
	Up	CC	GO:0005576	extracellular region	3.48E-05
	Up	CC	GO:0044421	extracellular region part	6.12E-05
	Up	CC	GO:0005615	extracellular space	2.62E-07
	Up	CC	GO:0005887	integral to plasma membrane	0.000145
	Up	CC	GO:0031226	intrinsic to plasma membrane	0.000189
	Up	CC	GO:0016020	membrane	4.38E-05
	Up	CC	GO:0005886	plasma membrane	4.64E-05
	Up	CC	GO:0044459	plasma membrane part	0.000697
	Up	MF	GO:0015665	alcohol transmembrane transporter activity	0.000722
	Up	MF	GO:0008009	chemokine activity	8.86E-06
	Up	MF	GO:0042379	chemokine receptor binding	1.02E-05
	Up	MF	GO:0005125	cytokine activity	2.46E-08
	Up	MF	GO:0001664	G-protein-coupled receptor binding	0.000153
	Up	MF	GO:0005149	interleukin-1 receptor binding	3.72E-05
	Up	MF	GO:0060089	molecular transducer activity	6.27E-09
	Up	MF	GO:0042834	peptidoglycan binding	0.000295
	Up	MF	GO:0015166	polyol transmembrane transporter activity	0.000722
	Up	MF	GO:0005515	protein binding	0.000154
	Up	MF	GO:0004872	receptor activity	1.42E-05
	Up	MF	GO:0005102	receptor binding	7.72E-06
	Up	MF	GO:0005057	receptor signaling protein activity	0.000392
	Up	MF	GO:0004871	signal transducer activity	6.27E-09
	Down	BP	GO:0044260	cellular macromolecule metabolic process	0.000393
	Down	BP	GO:0044267	cellular protein metabolic process	0.000595
	Down	BP	GO:0051789	response to protein stimulus	9.02E-06
	Down	BP	GO:0006950	response to stress	0.00017
	Down	BP	GO:0006986	response to unfolded protein	9.02E-06
	Down	CC	GO:0005737	cytoplasm	3.29E-05
	Down	CC	GO:0005622	intracellular	5.07E-07
	Down	CC	GO:0043231	intracellular membrane-bound organelle	0.000115
	Down	CC	GO:0043229	intracellular organelle	0.000536
	Down	CC	GO:0044424	intracellular part	6.87E-07
	Down	CC	GO:0043227	membrane-bound organelle	0.000116

Timepoint of comparison	Direction of change	Ontology group	GO path ID	Pathway description	Enrichment P value
	Down	CC	GO:0043226	organelle	0.000545
	Down	MF	GO:0030554	adenyl nucleotide binding	0.000397
	Down	MF	GO:0032559	adenyl ribonucleotide binding	0.000178
	Down	MF	GO:0005524	ATP binding	0.000142
6hr	Up	BP	GO:0002526	acute inflammatory response	0.000427
	Up	BP	GO:0001525	angiogenesis	0.000756
	Up	BP	GO:0006915	apoptosis	5.31E-11
	Up	BP	GO:0007610	behavior	1.03E-06
	Up	BP	GO:0065007	biological regulation	1.14E-06
	Up	BP	GO:0043412	biopolymer modification	1.49E-05
	Up	BP	GO:0001568	blood vessel development	0.000475
	Up	BP	GO:0055080	cation homeostasis	0.000547
	Up	BP	GO:0007154	cell communication	2.58E-05
	Up	BP	GO:0008219	cell death	4.18E-10
	Up	BP	GO:0048468	cell development	3.46E-08
	Up	BP	GO:0030154	cell differentiation	8.87E-09
	Up	BP	GO:0016477	cell migration	0.000531
	Up	BP	GO:0006928	cell motility	0.000179
	Up	BP	GO:0008283	cell proliferation	1.14E-07
	Up	BP	GO:0048869	cellular developmental process	8.87E-09
	Up	BP	GO:0044260	cellular macromolecule metabolic process	0.000388
	Up	BP	GO:0044267	cellular protein metabolic process	0.000252
	Up	BP	GO:0006935	chemotaxis	8.61E-10
	Up	BP	GO:0019221	cytokine and chemokine mediated signaling pathway	1.72E-05
	Up	BP	GO:0001816	cytokine production	0.000161
	Up	BP	GO:0016265	death	4.18E-10
	Up	BP	GO:0006952	defense response	4.53E-08
	Up	BP	GO:0032502	developmental process	5.19E-05
	Up	BP	GO:0030218	erythrocyte differentiation	0.000588
	Up	BP	GO:0034101	erythrocyte homeostasis	0.000588
	Up	BP	GO:0030097	hemopoiesis	3.34E-06
	Up	BP	GO:0048534	hemopoietic or lymphoid organ development	3.26E-06
	Up	BP	GO:0048872	homeostasis of number of cells	0.000845
	Up	BP	GO:0006955	immune response	6.51E-08
	Up	BP	GO:0002520	immune system development	8.20E-07
	Up	BP	GO:0002376	immune system process	3.05E-12
	Up	BP	GO:0006954	inflammatory response	8.31E-11

Timepoint of comparison	Direction of change	Ontology group	GO path ID	Pathway description	Enrichment P value
	Up	BP	GO:0032635	interleukin-6 production	5.05E-06
	Up	BP	GO:0007242	intracellular signaling cascade	8.77E-06
	Up	BP	GO:0030595	leukocyte chemotaxis	3.16E-06
	Up	BP	GO:0050900	leukocyte migration	4.73E-06
	Up	BP	GO:0051674	localization of cell	0.000179
	Up	BP	GO:0007626	locomotory behavior	2.93E-08
	Up	BP	GO:0033002	muscle cell proliferation	0.000344
	Up	BP	GO:0030099	myeloid cell differentiation	9.57E-06
	Up	BP	GO:0043066	negative regulation of apoptosis	0.000184
	Up	BP	GO:0048519	negative regulation of biological process	2.77E-09
	Up	BP	GO:0008285	negative regulation of cell proliferation	8.51E-05
	Up	BP	GO:0048523	negative regulation of cellular process	2.20E-09
	Up	BP	GO:0051093	negative regulation of developmental process	2.41E-05
	Up	BP	GO:0042347	negative regulation of NF-kappaB import into nucleus	0.000872
	Up	BP	GO:0045934	negative regulation of nucleobase, nucleoside, nucleotide and nucleic acid metabolic process	0.000363
	Up	BP	GO:0043069	negative regulation of programmed cell death	0.000224
	Up	BP	GO:0051253	negative regulation of RNA metabolic process	0.000695
	Up	BP	GO:0051048	negative regulation of secretion	0.000574
	Up	BP	GO:0016481	negative regulation of transcription	0.000168
	Up	BP	GO:0048513	organ development	0.000654
	Up	BP	GO:0006796	phosphate metabolic process	0.000167
	Up	BP	GO:0006793	phosphorus metabolic process	0.000167
	Up	BP	GO:0016310	phosphorylation	0.000109
	Up	BP	GO:0043065	positive regulation of apoptosis	0.000191
	Up	BP	GO:0048518	positive regulation of biological process	3.88E-09
	Up	BP	GO:0048522	positive regulation of cellular process	8.21E-09
	Up	BP	GO:0001819	positive regulation of cytokine production	0.000726
	Up	BP	GO:0051094	positive regulation of developmental process	0.000253
	Up	BP	GO:0043068	positive regulation of programmed cell death	0.000231
	Up	BP	GO:0051254	positive regulation of RNA metabolic process	0.000903

Timepoint of comparison	Direction of change	Ontology group	GO path ID	Pathway description	Enrichment P value
	Up	BP	GO:0009967	positive regulation of signal transduction	0.000441
	Up	BP	GO:0045893	positive regulation of transcription, DNA-dependent	0.000805
	Up	BP	GO:0043687	post-translational protein modification	3.57E-06
	Up	BP	GO:0012501	programmed cell death	8.79E-11
	Up	BP	GO:0006468	protein amino acid phosphorylation	6.49E-06
	Up	BP	GO:0007243	protein kinase cascade	1.54E-07
	Up	BP	GO:0019538	protein metabolic process	0.000237
	Up	BP	GO:0006464	protein modification process	1.65E-05
	Up	BP	GO:0042981	regulation of apoptosis	1.40E-07
	Up	BP	GO:0050789	regulation of biological process	6.03E-07
	Up	BP	GO:0065008	regulation of biological quality	8.63E-06
	Up	BP	GO:0045595	regulation of cell differentiation	0.00011
	Up	BP	GO:0042127	regulation of cell proliferation	1.06E-06
	Up	BP	GO:0050794	regulation of cellular process	3.68E-06
	Up	BP	GO:0001817	regulation of cytokine production	9.04E-05
	Up	BP	GO:0050793	regulation of developmental process	1.50E-08
	Up	BP	GO:0045682	regulation of epidermis development	3.98E-05
	Up	BP	GO:0032675	regulation of interleukin-6 production	5.89E-05
	Up	BP	GO:0045637	regulation of myeloid cell differentiation	0.000856
	Up	BP	GO:0043067	regulation of programmed cell death	2.14E-07
	Up	BP	GO:0006357	regulation of transcription from RNA polymerase II promoter	0.000132
	Up	BP	GO:0042221	response to chemical stimulus	4.52E-07
	Up	BP	GO:0009605	response to external stimulus	3.74E-12
	Up	BP	GO:0050896	response to stimulus	0.000541
	Up	BP	GO:0006950	response to stress	1.31E-07
	Up	BP	GO:0009611	response to wounding	3.77E-12
	Up	BP	GO:0007165	signal transduction	3.66E-05
	Up	BP	GO:0048731	system development	0.000598
	Up	BP	GO:0042330	taxis	8.61E-10
	Up	BP	GO:0001944	vasculature development	0.000591
	Up	CC	GO:0005887	integral to plasma membrane	1.49E-05
	Up	CC	GO:0031226	intrinsic to plasma membrane	2.42E-05
	Up	CC	GO:0044459	plasma membrane part	8.53E-05
	Up	MF	GO:0005488	binding	4.48E-05
	Up	MF	GO:0008009	chemokine activity	3.52E-05

Timepoint of comparison	Direction of change	Ontology group	GO path ID	Pathway description	Enrichment P value
	Up	MF	GO:0042379	chemokine receptor binding	4.25E-05
	Up	MF	GO:0005125	cytokine activity	3.61E-06
	Up	MF	GO:0001664	G-protein-coupled receptor binding	0.000355
	Up	MF	GO:0060089	molecular transducer activity	0.000952
	Up	MF	GO:0015205	nucleobase transmembrane transporter activity	0.000784
	Up	MF	GO:0005515	protein binding	3.64E-13
	Up	MF	GO:0004672	protein kinase activity	0.000536
	Up	MF	GO:0005057	receptor signaling protein activity	0.000159
	Up	MF	GO:0004871	signal transducer activity	0.000952
	Down	BP	GO:0042773	ATP synthesis coupled electron transport	2.44E-07
	Down	BP	GO:0009058	biosynthetic process	2.86E-09
	Down	BP	GO:0044249	cellular biosynthetic process	6.71E-11
	Down	BP	GO:0044237	cellular metabolic process	7.11E-14
	Down	BP	GO:0042769	DNA damage response, detection of DNA damage	0.000517
	Down	BP	GO:0006118	electron transport	4.14E-07
	Down	BP	GO:0010467	gene expression	3.87E-07
	Down	BP	GO:0006091	generation of precursor metabolites and energy	8.29E-07
	Down	BP	GO:0042168	heme metabolic process	0.000331
	Down	BP	GO:0006886	intracellular protein transport	0.000532
	Down	BP	GO:0065002	intracellular protein transport across a membrane	0.000233
	Down	BP	GO:0046907	intracellular transport	0.000777
	Down	BP	GO:0009059	macromolecule biosynthetic process	8.29E-08
	Down	BP	GO:0043170	macromolecule metabolic process	2.37E-07
	Down	BP	GO:0008152	metabolic process	1.92E-15
	Down	BP	GO:0006120	mitochondrial electron transport, NADH to ubiquinone	4.54E-07
	Down	BP	GO:0006139	nucleobase, nucleoside, nucleotide and nucleic acid metabolic process	4.04E-07
	Down	BP	GO:0042775	organelle ATP synthesis coupled electron transport	2.44E-07
	Down	BP	GO:0006119	oxidative phosphorylation	2.30E-08
	Down	BP	GO:0043043	peptide biosynthetic process	0.000517
	Down	BP	GO:0006778	porphyrin metabolic process	0.000959
	Down	BP	GO:0044238	primary metabolic process	6.85E-11
	Down	BP	GO:0006457	protein folding	1.17E-05
	Down	BP	GO:0015031	protein transport	0.000396

Timepoint of comparison	Direction of change	Ontology group	GO path ID	Pathway description	Enrichment P value
	Down	BP	GO:0051789	response to protein stimulus	0.000359
	Down	BP	GO:0006986	response to unfolded protein	0.000359
	Down	BP	GO:0006396	RNA processing	1.65E-06
	Down	BP	GO:0016072	rRNA metabolic process	0.000233
	Down	BP	GO:0006364	rRNA processing	0.000747
	Down	BP	GO:0019748	secondary metabolic process	0.000353
	Down	BP	GO:0033013	tetrapyrrole metabolic process	0.000959
	Down	BP	GO:0006412	translation	1.44E-09
	Down	BP	GO:0006399	tRNA metabolic process	6.81E-06
	Down	BP	GO:0008033	tRNA processing	7.92E-05
	Down	CC	GO:0005623	cell	0.000754
	Down	CC	GO:0044464	cell part	0.00074
	Down	CC	GO:0005737	cytoplasm	2.24E-10
	Down	CC	GO:0044444	cytoplasmic part	2.05E-12
	Down	CC	GO:0031975	envelope	6.13E-08
	Down	CC	GO:0005622	intracellular	3.15E-21
	Down	CC	GO:0043231	intracellular membrane-bound organelle	1.46E-21
	Down	CC	GO:0043232	intracellular non-membrane-bound organelle	0.000957
	Down	CC	GO:0043229	intracellular organelle	9.34E-18
	Down	CC	GO:0044446	intracellular organelle part	3.20E-10
	Down	CC	GO:0044424	intracellular part	1.20E-20
	Down	CC	GO:0015934	large ribosomal subunit	0.000386
	Down	CC	GO:0032991	macromolecular complex	1.61E-09
	Down	CC	GO:0043227	membrane-bound organelle	1.56E-21
	Down	CC	GO:0031974	membrane-enclosed lumen	7.82E-14
	Down	CC	GO:0005740	mitochondrial envelope	4.24E-10
	Down	CC	GO:0005743	mitochondrial inner membrane	9.76E-12
	Down	CC	GO:0005762	mitochondrial large ribosomal subunit	3.22E-07
	Down	CC	GO:0031980	mitochondrial lumen	5.95E-14
	Down	CC	GO:0005759	mitochondrial matrix	5.95E-14
	Down	CC	GO:0031966	mitochondrial membrane	1.56E-09
	Down	CC	GO:0044455	mitochondrial membrane part	1.27E-10
	Down	CC	GO:0044429	mitochondrial part	1.22E-19
	Down	CC	GO:0005746	mitochondrial respiratory chain	4.16E-07
	Down	CC	GO:0005747	mitochondrial respiratory chain complex I	6.10E-08
	Down	CC	GO:0005761	mitochondrial ribosome	1.30E-16
	Down	CC	GO:0005763	mitochondrial small ribosomal subunit	2.61E-06

Timepoint of comparison	Direction of change	Ontology group	GO path ID	Pathway description	Enrichment P value
	Down	CC	GO:0005739	mitochondrion	2.91E-25
	Down	CC	GO:0030964	NADH dehydrogenase complex	6.10E-08
	Down	CC	GO:0043228	non-membrane-bound organelle	0.000957
	Down	CC	GO:0031981	nuclear lumen	0.000296
	Down	CC	GO:0044428	nuclear part	0.000314
	Down	CC	GO:0044452	nucleolar part	0.000734
	Down	CC	GO:0005730	nucleolus	0.000193
	Down	CC	GO:0005634	nucleus	9.53E-06
	Down	CC	GO:0000315	organellar large ribosomal subunit	3.22E-07
	Down	CC	GO:0000313	organellar ribosome	1.30E-16
	Down	CC	GO:0000314	organellar small ribosomal subunit	2.61E-06
	Down	CC	GO:0043226	organelle	1.02E-17
	Down	CC	GO:0031967	organelle envelope	6.13E-08
	Down	CC	GO:0019866	organelle inner membrane	8.09E-11
	Down	CC	GO:0043233	organelle lumen	7.82E-14
	Down	CC	GO:0031090	organelle membrane	0.000275
	Down	CC	GO:0044422	organelle part	4.16E-10
	Down	CC	GO:0043234	protein complex	2.54E-05
	Down	CC	GO:0045271	respiratory chain complex I	6.10E-08
	Down	CC	GO:0030529	ribonucleoprotein complex	7.35E-09
	Down	CC	GO:0033279	ribosomal subunit	2.85E-07
	Down	CC	GO:0005840	ribosome	5.16E-08
	Down	CC	GO:0015935	small ribosomal subunit	0.000258
	Down	MF	GO:0003824	catalytic activity	1.67E-06
	Down	MF	GO:0050662	coenzyme binding	0.000352
	Down	MF	GO:0003688	DNA replication origin binding	0.000573
	Down	MF	GO:0009055	electron carrier activity	4.66E-08
	Down	MF	GO:0008168	methyltransferase activity	0.000175
	Down	MF	GO:0050136	NADH dehydrogenase (quinone) activity	1.10E-06
	Down	MF	GO:0008137	NADH dehydrogenase (ubiquinone) activity	1.10E-06
	Down	MF	GO:0003954	NADH dehydrogenase activity	1.10E-06
	Down	MF	GO:0003676	nucleic acid binding	2.87E-05
	Down	MF	GO:0016491	oxidoreductase activity	2.28E-06
	Down	MF	GO:0016651	oxidoreductase activity, acting on NADH or NADPH	3.69E-06
	Down	MF	GO:0016655	oxidoreductase activity, acting on NADH or NADPH, quinone or similar compound as acceptor	3.08E-06

Timepoint of comparison	Direction of change	Ontology group	GO path ID	Pathway description	Enrichment P value
	Down	MF	GO:0003723	RNA binding	7.88E-05
	Down	MF	GO:0003735	structural constituent of ribosome	3.90E-07
	Down	MF	GO:0016741	transferase activity, transferring one-carbon groups	0.000223
	Down	MF	GO:0004549	tRNA-specific ribonuclease activity	0.000915

Appendix V

Tabular description of cellular pathways represented by enrichment pathway analysis using the Kyoto Encyclopedia of Genes and Genomes (KEGG) performed on overlapping differentially expressed genes from in-vivo (NIL vs IL) and in-vitro (1, 2, 4 and 6hours relative to 0hours) myometrial samples. Significance level for differential expression was set at an adjusted p<0.01.

	N gene overlapping for their differential expression between in-vivo and in-vitro data adjusted p<0.01		
Process	(2hr vs 0hr) cf (IL vs NIL)	(4hr vs 0hr) cf (IL vs NIL)	(6hr vs 0hr) cf (IL vs NIL)
Not assigned	3	54	124
Cytokine-cytokine receptor interaction	1	4	8
Complement and coagulation cascades		2	6
Jak-STAT signaling pathway		4	5
MAPK signaling pathway		4	5
Neuroactive ligand-receptor interaction		1	5
Regulation of actin cytoskeleton		1	5
Calcium signaling pathway		4	4
Hematopoietic cell lineage		2	4
GnRH signaling pathway		4	3
Insulin signaling pathway		2	3
Glycolysis / Gluconeogenesis		1	3
Lysine degradation		0	3
Valine, leucine and isoleucine degradation		0	3
Long-term depression		3	2
Long-term potentiation		3	2
Melanogenesis		3	2
Focal adhesion		2	2
Glycan structures - biosynthesis 2		2	2
Small cell lung cancer		2	2
Apoptosis		1	2
Arachidonic acid metabolism		1	2
Axon guidance		1	2
Cell adhesion molecules (CAMs)		1	2

Fructose and mannose metabolism	1	2
mTOR signaling pathway	1	2
Natural killer cell mediated cytotoxicity	1	2
Purine metabolism	1	2
Systemic lupus erythematosus	1	2
Vibrio cholerae infection	1	2
p53 signaling pathway	0	2
Propanoate metabolism	0	2
Toll-like receptor signaling pathway	0	2
Ubiquitin mediated proteolysis	0	2
Cell junctions	2	1
ErbB signaling pathway	2	1
Glycosphingolipid biosynthesis - ganglio series	2	1
Glycosphingolipid biosynthesis - globo series	2	1
Wnt signaling pathway	2	1
ABC transporters - General	1	1
Acute myeloid leukemia	1	1
Allograft rejection	1	1
Aminosugars metabolism	1	1
Arginine and proline metabolism	1	1
Asthma	1	1
Autoimmune thyroid disease	1	1
Biosynthesis of steroids	1	1
Cell cycle	1	1
Chronic myeloid leukemia	1	1
Drug metabolism - other enzymes	1	1
ECM-receptor interaction	1	1
Endometrial cancer	1	1
Epithelial cell signaling in Helicobacter pylori infection	1	1
Galactose metabolism	1	1
Gap junction	1	1
Glycan structures - biosynthesis 1	1	1
Keratan sulfate biosynthesis	1	1
O-Glycan biosynthesis	1	1
Oxidative phosphorylation	1	1
PPAR signaling pathway	1	1
Pyrimidine metabolism	1	1

Renin-angiotensin system	1	1
Starch and sucrose metabolism	1	1
Streptomycin biosynthesis	1	1
T cell receptor signaling pathway	1	1
Thyroid cancer	1	1
VEGF signaling pathway	1	1
3-Chloroacrylic acid degradation	0	1
Ascorbate and aldarate metabolism	0	1
beta-Alanine metabolism	0	1
Bile acid biosynthesis	0	1
Butanoate metabolism	0	1
Carbon fixation in photosynthetic organisms	0	1
Fatty acid metabolism	0	1
Folate biosynthesis	0	1
Glycerolipid metabolism	0	1
Glycosylphosphatidylinositol(GPI)-anchor biosynthesis	0	1
Graft-versus-host disease	0	1
Histidine metabolism	0	1
Inositol metabolism	0	1
Limonene and pinene degradation	0	1
Nicotinate and nicotinamide metabolism	0	1
Pancreatic cancer	0	1
Prion disease	0	1
Pyruvate metabolism	0	1
Renal cell carcinoma	0	1
SNARE interactions in vesicular transport	0	1
Tight junction	0	1
Tryptophan metabolism	0	1
Urea cycle and metabolism of amino groups	0	1

Appendix VI

Full Publications containing work undertaken in this thesis

Menzies FM, Higgins CA, Shepherd MC, Nibbs RJB, Nelson SM. Mast cells reside in myometrium and cervix, but are dispensable in mice for successful pregnancy and labor. *Immunology and Cell Biology* 2012;90(3):321-29

Higgins CA, Martin W, Anderson L, Blanks AM, Norman JE, McConnachie A, et al. Maternal Obesity and its Relationship With Spontaneous and Oxytocin-Induced Contractility of Human Myometrium In Vitro. *Reproductive Sciences* 2010;17(2):177-85.

Anderson L, Martin W, Higgins C, Nelson SM, Norman JE. The Effect of Progesterone on Myometrial Contractility, Potassium Channels and Tocolytic Efficacy. *Reproductive Sciences* 2009;16(11):1052-61.

Appendix VII

Published abstracts containing work undertaken in this thesis

Menzies FM, Oldham R, Higgins CA, Nibbs RJB, Nelson SM. Temporal Analysis of Chemokine Production and Immune Cell Influx into the Uterus in Mice and Humans. *Reproductive Sciences* 2011;18(3):243A-43A.

Menzies FM, Oldham R, Higgins CA, Nibbs RJ, Nelson SM. Temporal analysis of chemokine production and immune cell influx into the uterus in mice and humans. *Immunology* 2010;131:61-61.

Labour is a co-ordinated process and mechanisms required for its initiation in humans are not fully understood. Studies suggest that immune cells play an important role in stimulating myometrial contractions through creation of an inflammatory environment. Inflammation is a necessary component of implantation and pregnancy, however excessive inflammation can also be detrimental and has been associated with miscarriage. To further understand the role of immune cells and their influx into the uterus during labour, we sought to characterise the chemokine profile of the pregnant, labouring and post partum uterus within a mouse model. In order to achieve this, we obtained uterine horn tissue from C57BL/6 female mice at term pregnancy (day 18), during labour or during the post partum period (n=5-6 mice per group). Quantitative real-time PCR (qRT-PCR) analysis revealed that term pregnancy in mice is associated with an increase in expression of chemokines involved in monocyte, macrophage and neutrophil chemotaxis, some of which were further upregulated during labour and the post partum period. Macrophages, neutrophils and T cells within these tissues were also enumerated by qRT-PCR and immunohistochemistry. Furthermore, analysis of circulating chemokine levels in humans throughout labour demonstrated that this process is associated with a temporal increase in chemokines involved in monocyte and neutrophil trafficking. These studies show that an inflammatory type response is evident within the uterus throughout labour, however immune cells and the chemokine which attract them, continue to increase beyond labour initiation in order to aid in the post partum remodelling of the uterus.

Higgins CA, McConnachie A, Nibbs RJB, Nelson SM. Temporal Changes to Leukocyte Subpopulations, Chemokine Receptor Expression and Circulating Cytokines during Human Parturition. *Reproductive Sciences* 2010;17(3):179A-79A.

Background: Human labour is associated with significant induction of chemokine mediated leukocyte trafficking into myometrium and cervix. We sought to characterise the temporal changes to the peripheral inflammatory response in term pregnancy during induction of labour(IOL) & non-labour planned caesarean delivery(CS).

Methods: Blood samples were obtained from non-pregnant females (day 2-5 of cycle)(NP=8), pre CS (n=8), post CS (n=8), and pre, post&2hrly during induction of labour (n=8). Analyses were made for 1)counts of peripheral blood leukocytes (PBL), 2)PBL chemokine receptor (PBLCR) profile (CXCR1, CXCR2, CCR2, CCR6) characterised by flow cytometry and 3)plasma cytokine concentrations measured using 39 multiplex ELISA. Data was analysed by ANOVA, paired and 2-sample t-tests.

Results: Total white cell count (tWCC) was increased during pregnancy ($p<0.001$) reflecting increased neutrophils ($p<0.001$). CS & IOL both induced acute increases in tWCC, neutrophils & monocytes ($p<0.001$ for all), while lymphocytes and esoinophils decreased ($p<0.05$ for both). The extent of change was greater with IOL for tWCC, neutrophils, lymphocytes and monocytes ($p<0.001$ for all). The changes in tWCC and leukocyte subpopulations strongly correlated with % length of labour ($r>0.39$, $p<0.01$ for all). PBL chemokine expression did not differ with pregnancy, however with IOL the number of CXCR1&2 neutrophils increased, with a corresponding decrease in density of expression ($p<0.05$ for both). No differences were observed for CCR2+ or CCR6+ leukocytes. Only GCSF increased in pregnancy ($p=0.01$), with further increases in response to IOL, in addition GRO, IL6, IL10, CCL2 and CCL7 increased with labour ($P<0.01$ for all). No differences in circulating cytokines in response to CS were observed.

Conclusions: Induction of labour is associated with a systemic acute inflammatory response characterised by changes in the circulating leukocyte profile and inflammatory cytokines and chemokine production. Notably this is associated with the % length of labour, and neutrophils characteristics typical of those newly recruited from bone marrow. Changes during pregnancy appear to be limited to increased neutrophil count and corresponding GCSF with no suggestion of leukocyte priming prior to labour.

Higgins CA, Martin W, Blanks AM, Catalano R, Thornton S, Nelson SM. Myometrial Cytokines and Chemokines during Human Labour: A Co-Ordinated Time and Contraction Dependent Event. *Reproductive Sciences* 2010;17(3):179A-79A.

Introduction: The cause of the inflammatory changes associated with human labour are unknown. We sought to provide a temporal and functional description of the myometrial derived inflammatory cytokines and chemokines associated with myometrial contractions in-vitro and the overlap with human labour.

Methods: Myometrial strips from term pre-labour caesarean deliveries (patients=25, strips from each patient=3) were suspended in an organ bath. After establishment of spontaneous contractions, oxytocin (OT) was added to 1 strip/patient, the remaining strips continued spontaneous contractions, and were terminated at 0, 1, 2, 4 & 6hrs after drug addition. Illumina gene arrays were compiled for each individual sample (n=75) and were analysed together, n=15 at each time point (previous work shows OT alters contractions but not transcription in myoctes). Differential gene expression analysis was undertaken with subsequent validation by qPCR and functional enrichment analysis by GO&KEGG. Overlap comparison of significant genes was made with previously undertaken affymetrix gene arrays from myometrium at term not-in-labour (NIL=9) and in labour (IL=9).

Results: In-vitro studies revealed a time-dependent transcriptional wave with 13, 114, 2006 & 4760 genes differentially expressed at 1, 2, 4 & 6hrs respectively (adjusted $p<0.01$). GO & KEGG enrichment analysis showed up-regulation of inflammatory processes, with predominance of cytokines and chemokines. qPCR validation confirmed increased CXCL1, 2,5,8, CCL20, IL6,IL8 & IL1B expression, with all positively associated with the duration of contractions ($r>0.36$, $p<0.001$ for all). Analysis of the overlap of this in-vitro contractile profile with genes differentially expressed in myometrium of spontaneous labouring women ($p<0.01$) demonstrated an overlap with 126,163, 323, 417 genes at 1, 2, 4 & 6hrs. Notably again inflammatory processes dominated.

Conclusions: Inflammatory cytokine and chemokine release from myometrium at term occurs in a co-ordinated time and contraction dependent manner, implying inflammation in labour may occur as an acute response to myometrial contractions.

Higgins CA, Martin W, Blanks AM, Nelson SM. Lipopolysaccharide induces and intense inflammatory response to myometrium, but is not associated with a functional contractile response *in-vitro*. *Bjog-an International Journal of Obstetrics and Gynaecology* 2010;117(5):631-32.

Background: The TLR-4 agonist Lipopolysaccharide(LPS) has been implicated in the initiation of pre-term and term labour. We have examined whether LPS induces an inflammatory response in myometrium and whether this was associated with stimulation of contractile activity *in-vitro*.

Methods: Myometrial strips were exposed to LPS(100μg/ml) or Polymixin-B (30μg/ml) to inactivate endotoxin + exposure to a single concentration of oxytocin(OT,1nM). Contractions were observed for 5hours after stable activity or addition of OT and tissue frozen for Q-PCR.

Results: LPS induced a significantly higher expression of the inflammatory mediators; CXCL2,CXCL8,CCL2,CCL3,CCL8,CCL20,IL6 and IL1β($p<0.05$ for all). However, contractile amplitude, frequency or activity integral was not affected. Additionally, the contractile stimulatory effect of OT was not enhanced by LPS, despite further significant enhancement of the inflammatory response.

Conclusions: Activation of TLR-4 by LPS stimulates a significant up-regulation of myometrial chemokine production. However, this does not alter inherent *in-vitro* myometrial contractility, or response to OT.

Higgins CA, Martin W, Blanks A, Thornton S, Nelson SM. Investigation of Myometrial Gene Expression in Response to Sustained Spontaneous and Oxytocin Induced Contraction *In-vitro*. *Reproductive Sciences* 2009;16(3):292A-93A.

Introduction: Oxytocin (OT) is a major endogenous uterotonin which induces myometrial contractions by stimulating intracellular calcium release and voltage gated calcium entry. At present it is unclear whether oxytocin receptor signalling may have longer term actions on gene expression in addition to an acute physiological effect. To clarify this, we examined the time-dependent transcriptional response of myometrium to spontaneous and OT-induced contractions *in-vitro*.

Methods: Myometrial strips from term pre-labor planned caesarean deliveries (n patients=25, n strips from each patient=2) were suspended in an organ bath under physiological conditions. After establishment of spontaneous contractions, a single addition of either OT (1nM) or acetic acid vehicle (2μM) was undertaken and frequency, amplitude and activity integral of contractile activity was recorded. Contractions were terminated at either 0,1,2,4 or 6 hours after drug addition and individual Illumina gene arrays were performed on each sample (n=50), with array validation using qRT-PCR. Data from arrays were analysed using t-test coupled with p value adjustment for multiple testing using the Benjamini & Hochberg method.

Results: Addition of 1nM OT had an immediate stimulatory effect on contractile activity compared with vehicle (median % change (IQR) frequency 526.7 (524.8) vs 0.0 (23.5) $p<0.001$, amplitude 12.5 (44.6) vs 1.4 (6.1), $p<0.001$, activity integral 151.8 (222.0) vs -1.7 (17.2), $p<0.001$). OT and vehicle treated myometrium demonstrated a time-dependent transcriptional wave with differential expression of 804 genes at 4h and 2262 genes at 6 h. Using an adjusted significance of $p<0.05$, no genes were significantly altered by OT when compared to vehicle alone at any of the 5 time points.

Conclusion: *In-vitro* myometrial contractions induce a transcriptional wave identical to that produced under the influence of OT despite significant differences in functional activity. This suggests that contractions *per se* have a significant time dependent effect on the myometrial transcriptome and the specific actions of oxytocin are limited to the modification of pre-existing myometrial proteins.

Higgins CA, Martin W, Blanks A, Thornton S, Norman JE, Nelson SM. Maternal obesity does not impair spontaneous or oxytocin-induced contractility of human myometrium in vitro. *Reproductive Sciences* 2008;15(2):113A-14A.

Introduction: Maternal obesity is associated with an increased need for operative delivery due to ineffectual progress in labour despite oxytocin (OT) augmentation. It is unclear whether this reflects impaired myometrial contractility, spontaneous or OT-induced, or alternative mechanisms. To clarify this we have examined myometrial activity in-vitro and related this to maternal body mass index.

Methods: Lower uterine segment myometrial samples were obtained at term pre-labour elective caesarean section (n=43). Maternal BMI was calculated using early pregnancy weight (12-14 weeks). Myometrial strips were placed in an organ bath of Carbogen gassed Krebs solution and suspended under isometric conditions with a resting tension of 20mN. After establishment of spontaneous contractions, strips were exposed to a single concentration of OT (1nM), with contractions observed for 60 min. OT concentration-response curves, 10-12 M-10-7 M, were also undertaken (n=21). Amplitude and frequency of contractile activity was recorded using a PowerLab with Chart v3.6 software. Data were analysed using one-way ANOVA, paired t-test and Pearson correlation.

Results: Mean BMI was 26.25±5.19 kg/m2. Increasing BMI was not associated with a difference in spontaneous myometrial activity; mean amplitude=61.84mN, r=-0.10, p=0.51, mean frequency=7.68 contractions/hr, r=0.25, p=0.10. Exposure to a concentration of 1nM OT produced an immediate increase in activity in all samples (amplitude mean increase=19.74mN, p=<0.01, frequency mean increase=50.56 contractions/hr p<0.01). % increase in both amplitude and frequency respectively were not related to BMI at immediate (108.7% r=-0.08, p=0.59; 880.8% r=-0.03 p= 0.85), 30min (171.3% r=-0.06, p=0.72; 59.28% r=0.12 p=0.48) or 60min (173.8% r=-0.06, p=0.73; 47.58% r=0.10 p=0.56) timepoints. Analysis of OT concentration-response curves did not show a BMI dependent effect (mean BMI 28.44±6.05 kg/m2, mean Log EC50=-9.73M, r=0.25, p=0.27).

Conclusion: The association between maternal obesity and operative delivery is not due to impaired spontaneous myometrial activity or responsiveness to OT, and may reflect pelvic adiposity, or a decrease in bioavailabilty of OT with increasing BMI.

List of References

1. United States Census Bureau, International Data Base, International Programs. *http://www.census.gov/population/international/data/idb/informationGateway.php*, May 2012.
2. *March of Dimes, PMNCH, Save the Children, WHO. Born Too soon: The Global Action Report on Preterm Birth*. Geneva: World Health Organization, 2012.
3. Births in Scottish Hospitals, year ending 31 March 2010. Edinburgh: Information Services Division (ISD), NHS National Services, Scotland, 2011.
4. NHS Maternity Data, England: 2010-11, http://www.hesonline.nhs.uk/Ease/servlet/ContentServer?siteID=1937&categoryID=1815: Hospital Episode Statistics, HES online, 2012.
5. Blencowe H, Cousens S, Oestergaard MZ, Chou D, Moller A-B, Narwal R, et al. National, regional, and worldwide estimates of preterm birth rates in the year 2010 with time trends since 1990 for selected countries: a systematic analysis and implications. *The Lancet* 2012;379(9832):2162-72.
6. Nosarti C, Reichenberg A, Murray RM, Cnattingius S, Lambe MP, Yin L, et al. Preterm Birth and Psychiatric Disorders in Young Adult LifePreterm Birth and Psychiatric Disorders. *Archives of General Psychiatry* 2012;69(6):610-17.
7. Moster D, Lie RT, Markestad T. Long-term medical and social consequences of preterm birth. *New England Journal of Medicine* 2008;359(3):262-73.
8. Saigal S, Doyle LW. An overview of mortality and sequelae of preterm birth from infancy to adulthood. *The Lancet* 2008;371(9608):261-69.
9. Mwaniki MK, Atieno M, Lawn JE, Newton CRJC. Long-term neurodevelopmental outcomes after intrauterine and neonatal insults: a systematic review. *The Lancet* 2012;379:445-52.
10. Gilbert WM. The cost of preterm birth: the low cost versus high value of tocolysis. *Bjog* 2006;3:4-9.
11. Gilbert WM, Nesbitt TS, Danielsen B. The cost of prematurity: quantification by gestational age and birth weight. *Obstet Gynecol* 2003;102(3):488-92.
12. Saigal S, Pinelli J, Streiner DL, Boyle M, Stoskopf B. Impact of extreme prematurity on family functioning and maternal health 20 years later. *Pediatrics* 2010;126(1):7.
13. Singer LT, Fulton S, Kirchner HL, Eisengart S, Lewis B, Short E, et al. Parenting very low birth weight children at school age: maternal stress and coping. *J Pediatr* 2007;151(5):463-9.
14. Saigal S, Burrows E, Stoskopf BL, Rosenbaum PL, Streiner D. Impact of extreme prematurity on families of adolescent children. *J Pediatr* 2000;137(5):701-6.
15. Keller M, Felderhoff-Mueser U, Lagercrantz H, Dammann O, Marlow N, Huppi P, et al. Policy benchmarking report on neonatal health and social policies in 13 European countries. *Acta Paediatr*. 2010;99(11):1624-29.
16. Phibbs CS, Schmitt SK. Estimates of the cost and length of stay changes that can be attributed to one-week increases in gestational age for premature infants. *Early Hum. Dev*. 2006;82(2):85-95.
17. Petrou S. The economic consequences of preterm birth duringthe first 10 years of life. *BJOG: An International Journal of Obstetrics & Gynaecology* 2005;112:10-15.

18. Goldenberg RL, Culhane JF, Iams JD, Romero R. Epidemiology and causes of preterm birth. *The Lancet* 2008;371(9606):75-84.
19. Steer P. The epidemiology of preterm labour. *BJOG: An International Journal of Obstetrics & Gynaecology* 2005;112:1-3.
20. Gracie S, Lyon A, Kehler H, Pennell C, Dolan S, McNeil D, et al. All Our Babies Cohort Study: recruitment of a cohort to predict women at risk of preterm birth through the examination of gene expression profiles and the environment. *BMC Pregnancy and Childbirth* 2010;10(1):87.
21. Copper RL, Goldenberg RL, Das A, Elder N, Swain M, Norman G, et al. The preterm prediction study: maternal stress is associated with spontaneous preterm birth at less than thirty-five weeks' gestation. National Institute of Child Health and Human Development Maternal-Fetal Medicine Units Network. *Am J Obstet Gynecol* 1996;175(5):1286-92.
22. Muglia LJ, Katz M. CURRENT CONCEPTS The Enigma of Spontaneous Preterm Birth. *New England Journal of Medicine* 2010;362(6):529-35.
23. Romero R, Espinoza J, Kusanovic JP, Gotsch F, Hassan S, Erez O, et al. The preterm parturition syndrome. *Bjog* 2006;3:17-42.
24. Kyrgiou M, Koliopoulos G, Martin-Hirsch P, Arbyn M, Prendiville W, Paraskevaidis E. Obstetric outcomes after conservative treatment for intraepithelial or early invasive cervical lesions: systematic review and meta-analysis. *Lancet* 2006;367(9509):489-98.
25. Refuerzo JS. Impact of multiple births on late and moderate prematurity. *Seminars in Fetal and Neonatal Medicine* 2012;17(3):143-45.
26. Albertsen K, Andersen AMN, Olsen J, Gronbaek M. Alcohol consumption during pregnancy and the risk of preterm delivery. *Am. J. Epidemiol.* 2004;159(2):155-61.
27. Bada HS, Das A, Bauer CR, Shankaran S, Lester BM, Gard CC, et al. Low birth weight and preterm births: etiologic fraction attributable to prenatal drug exposure. *Journal of perinatology : official journal of the California Perinatal Association* 2005;25(10):631-7.
28. Henderson JJ, McWilliam OA, Newnham JP, Pennell CE. Preterm birth aetiology 2004-2008. Maternal factors associated with three phenotypes: spontaneous preterm labour, preterm pre-labour rupture of membranes and medically indicated preterm birth. *J. Matern.-Fetal Neonatal Med.* 2012;25(6):642-47.
29. Bhattacharya S, Raja EA, Mirazo ER, Campbell DM, Lee AJ, Norman JE. Inherited Predisposition to Spontaneous Preterm Delivery. *Obstetrics and Gynecology* 2010;115(6):1125-33.
30. Kesmodel U, Olsen SF, Secher NJ. Does alcohol increase the risk of preterm delivery? *Epidemiology* 2000;11(5):512-18.
31. Yuan W, Duffner A, Chen L, Hunt L, Sellers S, Bernal A. Analysis of preterm deliveries below 35 weeks' gestation in a tertiary referral hospital in the UK. A case-control survey. *BMC Research Notes* 2010;3(1):119.
32. Hussain AA, Yakoob MY, Imdad A, Bhutta Z. Elective induction for pregnancies at or beyond 41 weeks of gestation and its impact on stillbirths: a systematic review with meta-analysis. *BMC Public Health* 2011;11(Suppl 3):S5.
33. Smith GCS. Life-table analysis of the risk of perinatal death at term and post term in singleton pregnancies. *American Journal of Obstetrics and Gynecology* 2001;184(3):489-96.
34. Cotzias CS, Paterson-Brown S, Fisk NM. Prospective risk of unexplained stillbirth in singleton pregnancies at term: population based analysis. *Br. Med. J.* 1999;319(7205):287-88.

35. Hilder L, Costeloe K, Thilaganathan B. Prolonged pregnancy: evaluating gestation-specific risks of fetal and infant mortality. *Br. J. Obstet. Gynaecol.* 1998;105(2):169-73.
36. Hussain AA, Yakoob MY, Imdad A, Bhutta ZA. Elective induction for pregnancies at or beyond 41 weeks of gestation and its impact on stillbirths: a systematic review with meta-analysis. *BMC Public Health* 2011;11:12.
37. Gulmezoglu AM, Crowther CA, Middleton P. Induction of labour for improving birth outcomes for women at or beyond term. *Cochrane Database Syst Rev* 2006;18(4).
38. Gulmezoglu AM, Crowther CA, Middleton P, Heatley E. Induction of labour for improving birth outcomes for women at or beyond term. *Cochrane Database Syst Rev* 2012;2012(13).
39. Nakling J, Backe B. Pregnancy risk increases from 41 weeks of gestation. *Acta Obstetricia et Gynecologica Scandinavica* 2006;85(6):663-68.
40. Chantry AA, Lopez E. Fetal and neonatal complications related to prolonged pregnancy. *J. Gynecol. Obstet. Biol. Reprod.* 2011;40(8):717-25.
41. Alexander JM, McIntire DD, Leveno KJ. Forty weeks and beyond: Pregnancy outcomes by week of gestation. *Obstetrics and Gynecology* 2000;96(2):291-94.
42. Caughey AB, Bishop JT. Maternal complications of pregnancy increase beyond 40 weeks of gestation in low-risk women. *J. Perinatol.* 2006;26(9):540-45.
43. Greve T, Lundbye-Christensen S, Nickelsen CN, Secher NJ. Maternal and perinatal complications by day of gestation after spontaneous labor at 40-42 weeks of gestation. *Acta Obstetricia et Gynecologica Scandinavica* 2011;90(8):852-56.
44. NICE. Induction of Labour. 2nd ed. London: RCOG Press 2008.
45. El Marroun H, Zeegers M, Steegers EA, van der Ende J, Schenk JJ, Hofman A, et al. Post-term birth and the risk of behavioural and emotional problems in early childhood. *International Journal of Epidemiology* 2012.
46. Morken NH, Melve KK, Skjaerven R. Recurrence of prolonged and post-term gestational age across generations: maternal and paternal contribution. *BJOG: An International Journal of Obstetrics & Gynaecology* 2011;118(13):1630-35.
47. Harper LM, Caughey AB, Odibo AO, Roehl KA, Zhao Q, Cahill AG. Normal Progress of Induced Labor. *Obstetrics & Gynecology* 2012;119(6):1113-18 10.097/AOG.0b013e318253d7aa.
48. *NHS Maternity Statistics, England:2004-2005*. Leeds: The Information Centre CHS, 2006.
49. Yeast JD, Jones A, Poskin M. Induction of labor and the relationship to cesarean delivery: A review of 7001 consecutive inductions. *Am J Obstet Gynecol* 1999;180(3 Pt 1):628-33.
50. Seyb ST, Berka RJ, Socol ML, Dooley SL. Risk of cesarean delivery with elective induction of labor at term in nulliparous women. *Obstet Gynecol* 1999;94(4):600-7.
51. Cammu H, Martens G, Ruyssinck G, Amy JJ. Outcome after elective labor induction in nulliparous women: a matched cohort study. *Am J Obstet Gynecol* 2002;186(2):240-4.
52. Dublin S, Lydon-Rochelle M, Kaplan RC, Watts DH, Critchlow CW. Maternal and neonatal outcomes after induction of labor without an identified indication. *Am J Obstet Gynecol* 2000;183(4):986-94.

53. Maslow AS, Sweeny AL. Elective induction of labor as a risk factor for cesarean delivery among low-risk women at term. *Obstet Gynecol* 2000;95(6 Pt 1):917-22.
54. Grivell RM, Reilly AJ, Oakey H, Chan A, Dodd JM. Maternal and neonatal outcomes following induction of labor: a cohort study. *Acta Obstetricia et Gynecologica Scandinavica* 2011;91(2):198-203.
55. Prysak M, Castronova FC. Elective induction versus spontaneous labor: a case-control analysis of safety and efficacy. *Obstet Gynecol* 1998;92(1):47-52.
56. Rane SM, Guirgis RR, Higgins B, Nicolaides KH. Models for the prediction of successful induction of labor based on pre-induction sonographic measurement of cervical length. *J Matern Fetal Neonatal Med* 2005;17(5):315-22.
57. Shetty A, Burt R, Rice P, Templeton A. Women's perceptions, expectations and satisfaction with induced labour--a questionnaire-based study. *Eur J Obstet Gynecol Reprod Biol* 2005;123(1):56-61.
58. Leitch CR, Walker JJ. The rise in caesarean section rate: the same indications but a lower threshold. *BJOG: An International Journal of Obstetrics & Gynaecology* 1998;105(6):621-26.
59. McIlwaine G, Boulton-Jones C, Cole S, Wilkinson C. Caesarean Section in Scotland 1994/5: a National Audit. Edinburgh: Scottish Programme for Clinical Effectiveness in Reproductive Health, 1998.
60. EAGoCS. Report and Recommendations of the Expert Advisory Group on Caesarean Section in Scotland. Edinburgh: Scottish Programme for Clinical Effectiveness in Reproductive Health, 2001.
61. Thomas J, Paranjothy S. The national sentinel caesarean section audit report. London: Royal College of Obstetricians and Gynaecologists Clinical Effectiveness Support Unit, 2001.
62. Vahratian A, Zhang J, Troendle JF, Savitz DA, Siega-Riz AM. Maternal Prepregnancy Overweight and Obesity and the Pattern of Labor Progression in Term Nulliparous Women. *Obstet Gynecol* 2004;104(5):943-51.
63. N J Sebire, M Jolly, J P Harris, J Wadsworth, M Joffe, R W Beard, et al. Maternal obesity and pregnancy outcome: a study of 287 213 pregnancies in London. *Int J Obes Relat Metab Disords* 2001;25(8):1175-82.
64. Graves BW, DeJoy SA, Heath A, Pekow P. Maternal Body Mass Index, Delivery Route, and Induction of Labor in a Midwifery Caseload. *Journal of Midwifery & Women's Health* 2006;51(4):254.
65. Usha Kiran TS, Hemmadi S, Bethel J, Evans J. Outcome of pregnancy in a woman with an increased body mass index. *BJOG: An International Journal of Obstetrics & Gynaecology* 2005;112(6):768-72.
66. Zhang J, Bricker L, Wray S, Quenby S. Poor uterine contractility in obese women. *BJOG: An International Journal of Obstetrics & Gynaecology* 2007;114(3):343-8.
67. Barau G, Robillard PY, Hulsey TC, Dedecker F, Laffite A, Gerardin P, et al. Linear association between maternal pre-pregnancy body mass index and risk of caesarean section in term deliveries. *BJOG: An International Journal of Obstetrics & Gynaecology* 2006;113(10):1173-7.
68. Main DM, Main EK, Moore DH, 2nd. The relationship between maternal age and uterine dysfunction: a continuous effect throughout reproductive life.[see comment]. *American Journal of Obstetrics & Gynecology* 2000;182(6):1312-20.
69. Rosenthal AN, Paterson-Brown S. Is there an incremental rise in the risk of obstetric intervention with increasing maternal age? *BJOG: An*

International Journal of Obstetrics and Gynaecology 1998;105(10):1064-69.
70. Ecker JL, Chen KT, Cohen AP, Riley LE, Lieberman ES. Increased risk of cesarean delivery with advancing maternal age: Indications and associated factors in nulliparous women. *American Journal of Obstetrics and Gynecology* 2001;185(4):883-87.
71. Lialios G, Kaponis A, Adonakis G. Maternal age as an independent risk factor for cesarean delivery. *International Journal of Gynecology & Obstetrics* 1999;67(3):187-88.
72. Arulkumaran S, Gibb DMF, Lun KC, Heng SH, Ratnam SS. The effect of parity on uterine activity in labour. *BJOG: An International Journal of Obstetrics and Gynaecology* 1984;91(9):843-48.
73. Rane SM, Guirgis RR, Higgins B, Nicolaides KH. The value of ultrasound in the prediction of successful induction of labor. *Ultrasound in Obstetrics and Gynecology* 2004;24(5):538-49.
74. RCOG. Birth after Previous Caesarean Birth (Green-top Guideline No. 45). London: Royal College of Obstetricians and Gynaecologists, 2007.
75. Guise J-M, McDonagh MS, Osterweil P, Nygren P, Chan BKS, Helfand M. Systematic review of the incidence and consequences of uterine rupture in women with previous caesarean section. *BMJ* 2004;329(7456):19.
76. Kennare R, Tucker G, Heard A, Chan A. Risks of Adverse Outcomes in the Next Birth After a First Cesarean Delivery. *Obstetrics & Gynecology* 2007;109(2, Part 1):270-76 10.1097/01.AOG.0000250469.23047.73.
77. Hopkins F, Raine-Fenning N, Gee H. Prediction of vaginal delivery following caesarean section for failure to progress based on the initial aberrant labour pattern. *European Journal of Obstetrics & Gynecology and Reproductive Biology* 2002;101(2):121.
78. Tahseen S, Griffiths M. Vaginal birth after two caesarean sections (VBAC-2)—a systematic review with meta-analysis of success rate and adverse outcomes of VBAC-2 versus VBAC-1 and repeat (third) caesarean sections. *BJOG: An International Journal of Obstetrics & Gynaecology* 200;117(1):5-19.
79. Tita ATN, Lai Y, Bloom SL, Spong CY, Varner MW, Ramin SM, et al. Timing of delivery and pregnancy outcomes among laboring nulliparous women. *American Journal of Obstetrics and Gynecology* 2012;206(3):239.e1-39.e8.
80. Bailit JL, Gregory KD, Reddy UM, Gonzalez-Quintero VH, Hibbard JU, Ramirez MM, et al. Maternal and neonatal outcomes by labor onset type and gestational age. *American Journal of Obstetrics and Gynecology* 2010;202(3):245.e1-45.e12.
81. Saving Mothers' Lives: Reviewing maternal deaths to make motherhood safer: 2006–2008. *BJOG: An International Journal of Obstetrics & Gynaecology* 2011;118:1-203.
82. Brace V, Kernaghan D, Penney G. Learning from adverse clinical outcomes: major obstetric haemorrhage in Scotland, 2003-05. *Bjog-an International Journal of Obstetrics and Gynaecology* 2007;114(11):1388-96.
83. Lennox C M, L. Scottish Confidential Audit of Sever Maternal Morbidity, 7th Annual Report (Data from 2009). Edinburgh: Reproductive Health Programme, Healthcare Improvement Scotland, 2011.
84. Moore KL, Agur AMR. Pelvis and Perineum. *Essential clinical anatomy*. 3rd edition ed. Baltimore: Williams & Wilkins, 1995.
85. Ramsey EM. Anatomy of the uterus. In: Chard T, Grudzinskas JG, editors. *The Uterus*. Cambridge: Cambridge University Press, 1994:18-40.
86. Young RC. Myocytes, Myometrium, and Uterine Contractions. *Annals of the New York Academy of Sciences* 2007;1101(1):72-84.

87. Ellis H. Anatomy of the uterus. *Anaesthesia & Intensive Care Medicine* 2011;12(3):99-101.
88. Gray H. *Anatomy of the human body*. 20th ed. Philadelphia: Bartleby, 2000.
89. Calder AA. The cervix during pregnancy. In: Chard T, Grudzinskas JG, editors. *The Uterus*. Cambridge: Cambridge University Press, 1994:288-307.
90. Arrowsmith S, Quenby S, Weeks A, Burdyga T, Wray S. Poor spontaneous and oxytocin-stimulated contractility in human myometrium from postdates pregnancies. *PLoS ONE* 2012;7(5):10.
91. Blanks AM, Vatish M, Allen MJ, Ladds G, de Wit NC, Slater DM, et al. Paracrine oxytocin and estradiol demonstrate a spatial increase in human intrauterine tissues with labor. *J. Clin. Endocrinol. Metab.* 2003;88(7):3392-400.
92. Luckas MJ, Wray S. A comparison of the contractile properties of human myometrium obtained from the upper and lower uterine segments. *BJOG: An International Journal of Obstetrics & Gynaecology* 2000;107(10):1309-11.
93. Ludmir J, Sehdev HM. Anatomy and physiology of the uterine cervix. *Clin Obstet Gynecol* 2000;43(3):433-9.
94. Bauer M, Mazza E, Nava A, Zeck W, Eder M, Bajka M, et al. In vivo characterization of the mechanics of human uterine cervices. *Ann N Y Acad Sci* 2007:15.
95. Oxlund B, Ortoft G, Bruel A, Danielsen C, Bor P, Oxlund H, et al. Collagen concentration and biomechanical properties of samples from the lower uterine cervix in relation to age and parity in non-pregnant women. *Reproductive Biology and Endocrinology* 2010;8(1):82.
96. Timmons B, Akins M, Mahendroo M. Cervical remodeling during pregnancy and parturition. *Trends Endocrinol. Metab.* 2010;21(6):353-61.
97. Nelson SM, Khan AH, Norman JE. Cervical and myometrial physiology and preterm labor. *Expert Review of Obstetrics & Gynecology* 2007;2(2):179-92.
98. Rorie DK, Newton M. Histologic and chemical studies of the smooth muscle in the human cervix and uterus. *Am J Obstet Gynecol* 1967;99(4):466-9.
99. Garfield RE, Yallampalli C. Structure and function of uterine muscle. In: Chard T, Grudzinskas JG, editors. *The Uterus*. Cambridge: Cambridge University Press, 1994.
100. Sokolowski P, Saison F, Giles W, McGrath S, Smith D, Smith J, et al. Human Uterine Wall Tension Trajectories and the Onset of Parturition. *PLoS ONE* 2010;5(6):e11037.
101. Garfield RE, Somlyo AP. Structure of Smooth Muscle. In: Grover AK, Daniel EE, editors. *Calcium and Contractility: Smooth Muscle*: Humana Press, 1985.
102. Favaro RR, Salgado RM, Raspantini PR, Fortes ZB, Zorn TM. Effects of long-term diabetes on the structure and cell proliferation of the myometrium in the early pregnancy of mice. *Int J Exp Pathol* 2010;91(5):426-35.
103. Lambert FL, Pelletier G, Dufour M, Fortier MA. Specific properties of smooth muscle cells from different layers of rabbit myometrium. *Am J Physiol* 1990;258(5 Pt 1):C794-802.
104. Miller BF, O'Toole MT. *Miller-Keane encyclopedia and dictionary of medicine, nursing, and allied health*: Saunders, 2005.
105. Weiss S, Jaermann T, Schmid P, Staempfli P, Boesiger P, Niederer P, et al. Three-dimensional fiber architecture of the nonpregnant human uterus determined ex vivo using magnetic resonance diffusion tensor imaging.

The Anatomical Record Part A: Discoveries in Molecular, Cellular, and Evolutionary Biology 2006;288A(1):84-90.
106. Aguilar HN, Mitchell BF. Physiological pathways and molecular mechanisms regulating uterine contractility. *Human Reproduction Update* 2010;16(6):725-44.
107. Brosens J, Barker F, deSouza N. Myometrial zonal differentiation and uterine junctional zone hyperplasia in the non-pregnant uterus. *Human Reproduction Update* 1998;4(5):496-502.
108. Brosens JJ, de Souza NM, Barker FG. Uterine junctional zone: function and disease. *Lancet* 1995;346(8974):558-60.
109. Nakai A, Togashi K, Yamaoka T, Fujiwara T, Ueda H, Koyama T, et al. Uterine peristalsis shown on cine MR imaging using ultrafast sequence. *J Magn Reson Imaging* 2003;18(6):726-33.
110. Fujiwara T, Togashi K, Yamaoka T, Nakai A, Kido A, Nishio S, et al. Kinematics of the uterus: cine mode MR imaging. *Radiographics* 2004;24(1):3.
111. Scoutt LM, Flynn SD, Luthringer DJ, McCauley TR, McCarthy SM. Junctional zone of the uterus: correlation of MR imaging and histologic examination of hysterectomy specimens. *Radiology* 1991;179(2):403-7.
112. Brown HK, Stoll BS, Nicosia SV, Fiorica JV, Hambley PS, Clarke LP, et al. Uterine junctional zone: correlation between histologic findings and MR imaging. *Radiology* 1991;179(2):409-13.
113. Willms AB, Brown ED, Kettritz UI, Kuller JA, Semelka RC. Anatomic changes in the pelvis after uncomplicated vaginal delivery: evaluation with serial MR imaging. *Radiology* 1995;195(1):91-4.
114. Aslanidi O, Atia J, Benson AP, van den Berg HA, Blanks AM, Choi C, et al. Towards a computational reconstruction of the electrodynamics of premature and full term human labour. *Progress in Biophysics & Molecular Biology* 2011;107(1):183-92.
115. Taggart M, Blanks A, Kharche S, Holden A, Wang B, Zhang H. Towards understanding the myometrial physiome: approaches for the construction of a virtual physiological uterus. *BMC Pregnancy and Childbirth* 2007;7(Suppl 1):S3.
116. Blanks AM, Shmygol A, Thornton S. Myometrial function in prematurity. *Best Practice & Research Clinical Obstetrics & Gynaecology* 2007;21(5):807-19.
117. Taggart MJ, Morgan KG. Regulation of the uterine contractile apparatus and cytoskeleton. *Semin. Cell Dev. Biol.* 2007;18(3):296.
118. Longbottom ER, Luckas MJM, Kupittayanant S, Badrick E, Shmigol T, Wray S. The effects of inhibiting myosin light chain kinase on contraction and calcium signalling in human and rat myometrium. *Pflugers Archiv European Journal of Physiology* 2000;440(2):315-21.
119. Word RA, Stull JT, Casey ML, Kamm KE. Contractile elements and myosin light chain phosphorylation in myometrial tissue from nonpregnant and pregnant women. *J Clin Invest* 1993;92(1):29-37.
120. Arrowsmith S, Kendrick A, Wray S. Drugs acting on the pregnant uterus. *Obstetrics, Gynaecology & Reproductive Medicine* 2010;20(8):241-47.
121. Somlyo AP, Somlyo AV. Signal transduction through the RhoA/Rho-kinase pathway in smooth muscle. *Journal Of Muscle Research And Cell Motility* 2004;25(8):613-15.
122. Moran CJ, Friel AM, Smith TJ, Cairns M, Morrison JJ. Expression and modulation of Rho kinase in human pregnant myometrium. *Molecular Human Reproduction* 2002;8(2):196-200.

123. Woodcock NA, Taylor CW, Thornton S. Effect of an oxytocin receptor antagonist and rho kinase inhibitor on the [Ca++](i) sensitivity of human myometrium. *American Journal of Obstetrics and Gynecology* 2004;190(1):222-28.
124. Taggart MJ, Arthur P, Zielnik B, Mitchell BF. Molecular pathways regulating contractility in rat uterus through late gestation and parturition. *American Journal of Obstetrics and Gynecology* 2012;207(1):76.e15-76.e24.
125. Yasuda K, Nakamoto T, Yasuhara M, Okada H, Nakajima T, Kanzaki H, et al. Role of protein kinase Cß in rhythmic contractions of human pregnant myometrium. *Reproduction* 2007;133(4):797-806.
126. Webb BLJ, Hirst SJ, Giembycz MA. Protein kinase C isoenzymes: a review of their structure, regulation and role in regulating airways smooth muscle tone and mitogenesis. *British Journal of Pharmacology* 2000;130(7):1433-52.
127. Burdyga T, Wray S. Simultaneous measurements of electrical activity, intracellular [Ca2+] and force in intact smooth muscle. *Pflugers Arch* 1997;435(1):182-4.
128. Luckas MJM, Taggart MJ, Wray S. Intracellular calcium stores and agonist-induced contractions in isolated human myometrium. *American Journal of Obstetrics and Gynecology* 1999;181(2):468-76.
129. Parkington HC, Tonta MA, Brennecke SP, Coleman HA. Contractile activity, membrane potential, and cytoplasmic calcium in human uterine smooth muscle in the third trimester of pregnancy and during labor. *American Journal of Obstetrics and Gynecology* 1999;181(6):1445.
130. Sanborn BM. Relationship of Ion Channel Activity to Control of Myometrial Calcium. *J. Soc. Gynecol. Invest.* 2000;7(1):4-11.
131. Wray S, Jones K, Kupittayanant S, Li Y, Matthew A, Monir-Bishty E, et al. Calcium Signalling and Uterine Contractility. *J Soc Gynecol Investig* 2003;10(5):252-64.
132. Sanborn BM. Hormonal signaling and signal pathway crosstalk in the control of myometrial calcium dynamics. *Semin. Cell Dev. Biol.* 2007;18(3):305.
133. Blanks AM, Zhao Z-H, Shmygol A, Bru-Mercier G, Astle S, Thornton S. Characterization of the molecular and electrophysiological properties of the T-type calcium channel in human myometrium. *J Physiol* 2007;581(3):915-26.
134. Arnaudeau S, Lepretre N, Mironneau J. Chloride and monovalent ion-selective cation currents activated by oxytocin in pregnant rat myometrial cells. *American Journal of Obstetrics and Gynecology* 1994;171(2):491-501.
135. Eswaran H, Preissl H, Wilson JD, Murphy P, Robinson SE, Lowery CL. First magnetomyographic recordings of uterine activity with spatial-temporal information with a 151-channel sensor array. *American Journal of Obstetrics and Gynecology* 2002;187(1):145-51.
136. Benson AP, Clayton RH, Holden AV, Kharche S, Tong WC. Endogenous driving and synchronization in cardiac and uterine virtual tissues: bifurcations and local coupling. *Philos Transact A Math Phys Eng Sci* 2006;364(1842):1313-27.
137. Garfield RE, Blennerhassett MG, Miller SM. Control of myometrial contractility: role and regulation of gap junctions. *Oxf Rev Reprod Biol* 1988;10:436-90.
138. Orsino A, Taylor CV, Lye SJ. Connexin-26 and connexin-43 are differentially expressed and regulated in the rat myometrium throughout late

pregnancy and with the onset of labor. *Endocrinology* 1996;137(5):1545-53.
139. Chow L, Lye SJ. Expression of the gap junction proein connexin-43 is increased in the human myometrium toward term and with the onset of labor. *American Journal of Obstetrics and Gynecology* 1994;170(3):788-95.
140. Tattersall M, Engineer N, Khanjani S, Sooranna SR, Roberts VH, Grigsby PL, et al. Pro-labour myometrial gene expression: are preterm labour and term labour the same? *Reproduction* 2008;135(4):569-79.
141. Lye SJ, Nicholson BJ, Mascarenhas M, Mackenzie L, Petrocelli T. Increased expression of connexin-43 in the rat myometrium during labor is associated with an increase in the plasma estrogen progesterone ratio. *Endocrinology* 1993;132(6):2380-86.
142. Ou CW, Orsino A, Lye SJ. Expression of connexin-43 and connexin-26 in the rat myometrium during pregnancy and labor is differentially regulated by mechanical and hormonal signals. *Endocrinology* 1997;138(12):5398-407.
143. Doring B, Shynlova O, Tsui P, Eckardt D, Janssen-Bienhold U, Hofmann F, et al. Ablation of connexin43 in uterine smooth muscle cells of the mouse causes delayed parturition. *Journal of Cell Science* 2006;119(9):1715-22.
144. Chibbar R, Miller FD, Mitchell BF. Synthesis of oxytocin in amnion, chorion, and decidua may influence the timing of human parturition. *The Journal of Clinical Investigation* 1993;91(1):185-92.
145. Chibbar R, Wong S, Miller FD, Mitchell BF. Estrogen stimulates oxytocin gene expression in human chorio-decidua. *J. Clin. Endocrinol. Metab.* 1995;80(2):567-72.
146. Dale HH. On some physiological actions of ergot. *J. Physiol.-London* 1906;34(3):163-206.
147. Dale HH. The action of extracts of the pituitary body. *Biochemical Journal* 1909;4:427-47.
148. Bell WB. The pituitary body and the therapeutic value of the infundibular extract in shock, uterine atony, and interstinal paresis. *BMJ* 1909;2(2553):1609-13.
149. Shmygol A, Gullam J, Blanks A, Thornton S. Multiple mechanisms involved in oxytocin-induced modulation of myometrial contractility. *Acta Pharmacologica Sinica* 2006;27(7):827-32.
150. Thornton S, Smith SK. The physiological basis for administration of oxytocin antagonists in preterm labour. *Journal of the Royal Society of Medicine* 1995;88(3):166P-70P.
151. Blanks AM, Shmygol A, Thornton S. Regulation of oxytocin receptors and oxytocin receptor signaling. *Seminars in Reproductive Medicine* 2007;25(1):52-9.
152. Fuchs AR, Fuchs F, Husslein P, Soloff MS. Oxytocin receptors in the human-uterus during pregnancy and parturition. *American Journal of Obstetrics and Gynecology* 1984;150(6):734-41.
153. Soloff MS, Alexandrova M, Fernstrom MJ. Oxytocin receptors: triggers for parturition and lactation? *Science* 1979;204(4399):1313-5.
154. Word RA, Stull JT, Casey ML, Kamm KE. Contractile elements and myosin light chain phosphorylation in myometrial tissue from nonpregnant and pregnant women. *The Journal of Clinical Investigation* 1993;92(1):29-37.
155. Berridge MJ, Irvine RF. Inositol trisphosphate, a novel second messenger in cellular signal transduction. *Nature* 1984;312(5992):315-21.
156. Berridge MJ. Inositol trisphosphate and calcium signalling. *Nature* 1993;361(6410):315-25.

157. Shlykov SG, Sanborn BM. Stimulation of intracellular Ca2+ oscillations by diacylglycerol in human myometrial cells. *Cell Calcium* 2004;36(2):157-64.
158. Somlyo AP, Wu X, Walker LA, Somlyo AV. Pharmacomechanical coupling: the role of calcium, G-proteins, kinases and phosphatases. *Reviews of physiology, biochemistry and pharmacology* 1999;134:201-34.
159. Kimura T, Tanizawa O, Mori K, Brownstein MJ, Okayama H. Structure and expression of a human oxytocin receptor. *Nature* 1992;356(6369):526-29.
160. Gimpl G, Fahrenholz F. The Oxytocin Receptor System: Structure, Function, and Regulation. *Physiol. Rev.* 2001;81(2):629-83.
161. Mitchell BF, Schmid B. Oxytocin and its Receptor in the Process of Parturition. *J. Soc. Gynecol. Invest.* 2001;8(3):122-33.
162. Terzidou V, Blanks AM, Kim SH, Thornton S, Bennett PR. Labor and Inflammation Increase the Expression of Oxytocin Receptor in Human Amnion. *Biology of Reproduction* 2011;84(3):546-52.
163. Terzidou V, Sooranna SR, Kim LU, Thornton S, Bennett PR, Johnson MR. Mechanical stretch up-regulates the human oxytocin receptor in primary human uterine myocytes. *J. Clin. Endocrinol. Metab.* 2005;90(1):237-46.
164. Thornton S, Davison JM, Baylis PH. Plasma oxytocin during third stage of labour: comparison of natural and active management. *Br. Med. J.* 1988;297(6642):167-69.
165. Thornton S, Davison JM, Baylis PH. Plasma oxytocin during the first and second stages of spontaneous human labor. *Acta Endocrinologica* 1992;126(5):425-29.
166. Ferguson JKW. A study of the motility of the intact uterus at term. *Surg Gynecol and Obstet* 1941;73((3)):359-66.
167. Fuchs AR, Romero R, Keefe D, Parra M, Oyarzun E, Behnke E. Oxytocin secretion and human parturition - pulse frequency and duration increase during spontaneous labor in women. *American Journal of Obstetrics and Gynecology* 1991;165(5):1515-23.
168. Higuchi T, Uchide K, Honda K, Negoro H. Oxytocin release during parturition in the pelvic-neurectomized rat. *J. Endocrinol.* 1986;109(2):149-54.
169. O'Byrne KT, Ring JPG, Summerlee AJS. Plasma oxytocin and oxytocin neuron activity during delivery in rabbits. *Journal of Physiology (Cambridge)* 1986;370:501-14.
170. Summerlee AJ. Extracellular recordings from oxytocin neurones during the expulsive phase of birth in unanaesthetized rats. *J Physiol* 1981;321:1-9.
171. Higuchi T, Okere CO. Role of the supraoptic nucleus in regulation of parturition and milk ejection revisited. *Microsc Res Tech* 2002;56(2):113-21.
172. Honnebier M, Mecenas CA, Jenkins SL, Nathanielsz PW. Comparison of the myometrial response to oxytocin during daylight with the response obtained during the early hours of darkness in the fetectomized rhesus-Monkey at 160-172 days gestational-age. *Biology of Reproduction* 1993;48(4):779-85.
173. Vatish M, Steer PJ, Blanks AM, Hon M, Thornton S. Diurnal variation is lost in preterm deliveries before 28 weeks of gestation. *Bjog-an International Journal of Obstetrics and Gynaecology* 2010;117(6):765-67.
174. Dickinson JE, Godfrey M, Evans SF. Antenatal patterns of uterine activity in low-risk women: A longitudinal study. *Aust. N. Z. J. Obstet. Gynaecol.* 1997;37(2):149-52.
175. Nageotte MP, Dorchester W, Porto M, Keegan KA, Freeman RK. Quantitation of uterine activity preceding preterm, term, and postterm labor. *American Journal of Obstetrics and Gynecology* 1988;158(6):1254-59.

176. Main DM, Grisso JA, Wold T, Snyder ES, Holmes J, Chiu G. Extended longitudinal-study of uterine activity among low-risk women. *American Journal of Obstetrics and Gynecology* 1991;165(5):1317-22.
177. Moore TR, Iams JD, Creasy RK, Burau KD, Davidson AL. Diurnal and gestationl patterns of uterine activity in normal human-pregnancy. *Obstetrics and Gynecology* 1994;83(4):517-23.
178. Germain AM, Valenzuela GJ, Ivankovic M, Ducsay CA, Gabella C, Seronferre M. Relationship of circadian-rhythms of uterine activity with terma dn preterm delivery. *American Journal of Obstetrics and Gynecology* 1993;168(4):1271-77.
179. Fuchs AR, Behrens O, Liu HC. Correlation of nocturnal increase in plasma oxytocin with a decrease in plasma estradiol progesterone ratio in late pregnancy. *American Journal of Obstetrics and Gynecology* 1992;167(6):1559-63.
180. de Wit NCJ, Heck AJR, Thornton S. The effect of oxytocin and an oxytocin antagonist on the human myometrial proteome. *Reproductive Sciences* 2010;17(1):40-6.
181. Young WS, Shepard E, Amico J, Hennighausen L, Wagner KU, LaMarca ME, et al. Deficiency in mouse oxytocin prevents milk ejection, but not fertility or parturition. *J. Neuroendocrinol.* 1996;8(11):847-53.
182. Kawamata M, Tonomura Y, Kimura T, Sugimoto Y, Yanagisawa T, Nishimori K. Oxytocin-induced phasic and tonic contractions are modulated by the contractile machinery rather than the quantity of oxytocin receptor. *American Journal of Physiology - Endocrinology And Metabolism* 2007;292(4):E992-E99.
183. Moonen P, Klok G, Keirse M. Immunohistochemical localization of prostaglandin endoperoxide synthase and prostacyclin synthase in pregnant human myometrium. *Eur. J. Obstet. Gynecol. Reprod. Biol.* 1985;19(3):151-58.
184. Moonen P, Klok G, Keirse M. Distribution of prostaglandin endoperoside synthase and prostacyclin synthase in the late pregnant uterus. *Br. J. Obstet. Gynaecol.* 1986;93(3):255-59.
185. Moonen P, Klok G, Keirse M. Increase in concentrations of prostaglandin endoperoxide synthase and prostacyclin syntase in human myometrium in late pregnancy. *Prostaglandins* 1984;28(3):309-21.
186. British National Formulary, http://bnf.org.
187. McKay GR, JL. Walters MR. *Lecture Notes: Clinical Pharmacology and Therapeutics*. 8th ed: Wiley-Blackwell, 2010.
188. Terzidou V. Biochemical and endocrinological preparation for parturition. *Best Practice & Research Clinical Obstetrics & Gynaecology* 2007;21(5):729.
189. Olson DM, Ammann C. Role of the prostaglandins in labour and prostaglandin receptor inhibitors in the prevention of preterm labour. *Front. Biosci.* 2007;12:1329-43.
190. Olson DM. The role of prostaglandins in the initiation of parturition. *Best Practice & Research in Clinical Obstetrics & Gynaecology* 2003;17(5):717-30.
191. Kimura T, Ogita K, Kusui C, Ohashi K, Azuma C, Murata Y. What knockout mice can tell us about parturition. *Reviews of Reproduction* 1999;4(2):73-80.
192. Gross G, Imamura T, Muglia LJ. Gene knockout mice in the study of parturition. *J. Soc. Gynecol. Invest.* 2000;7(2):88-95.
193. Grigsby PL, Sooranna SR, Adu-Amankwa B, Pitzer B, Brockman DE, Johnson MR, et al. Regional expression of prostaglandin E2 and F2 alpha receptors

in human myometrium, amnion, and choriodecidua with advancing gestation and labor. *Biology of Reproduction* 2006;75(2):297-305.
194. Myatt L, Lye SJ. Expression, localization and function of prostaglandin receptors in myometrium. *Prostaglandins Leukot. Essent. Fatty Acids* 2004;70(2):137-48.
195. Astle S, Thornton S, Slater DM. Identification and localization of prostaglandin E-2 receptors in upper and lower segment human myometrium during pregnancy. *Molecular Human Reproduction* 2005;11(4):279-87.
196. Arulkumaran S, Kandola MK, Hoffman B, Hanyaloglu AC, Johnson MR, Bennett PR. The Roles of Prostaglandin EP 1 and 3 Receptors in the Control of Human Myometrial Contractility. *J. Clin. Endocrinol. Metab.* 2012;97(2):489-98.
197. Olson DM, Zaragoza DB, Shallow MC, Cook JL, Mitchell BF, Grigsby P, et al. Myometrial activation and preterm labour: evidence supporting a role for the prostaglandin F receptor--a review. *Placenta* 2003;24 Suppl A:S47-54.
198. Blesson CS, Buttner E, Masironi B, Sahlin L. Prostaglandin receptors EP and FP are regulated by estradiol and progesterone in the uterus of ovariectomized rats. *Reproductive Biology and Endocrinology* 2012;10:10.
199. Keelan JA, Blumenstein M, Helliwell RJ, Sato TA, Marvin KW, Mitchell MD. Cytokines, prostaglandins and parturition--a review. *Placenta* 2003;24(46):S33-46.
200. Bennett PR, Slater D, Sullivan M, Elder MG, Moore GE. Changes in amniotic arachidonic-acid metabolism associated with increased cyclo-oxygenase gene-expression. *Br. J. Obstet. Gynaecol.* 1993;100(11):1037-42.
201. Higuchi T, Uchide K, Honda K, Negoro H. Pelvic neurectomy abolishes the fetus-expulsion reflex and induces dystocia in the rat. *Exp. Neurol.* 1987;96(2):443-55.
202. Sugimoto Y, Yamasaki A, Segi E, Tsuboi K, Aze Y, Nishimura T, et al. Failure of parturition in mice lacking the prostaglandin F receptor. *Science* 1997;277(5326):681-83.
203. Blanks AM, Thornton S. Gene redundancy in parturition: lessonsfor tocolysis? *Preterm Birth*, 2007:56-59.
204. Tsuboi K, Sugimoto Y, Iwane A, Yamamoto K, Yamamoto S, Ichikawa A. Uterine expression of prostaglandin H-2 synthase in late pregnancy and during parturition in prostaglandin F receptor-deficient mice. *Endocrinology* 2000;141(1):315-24.
205. Gross GA, Imamura T, Luedke C, Vogt SK, Olson LM, Nelson DM, et al. Opposing actions of prostaglandins and oxytocin determine the onset of murine labor. *Proceedings of the National Academy of Sciences of the United States of America* 1998;95(20):11875-79.
206. Blanks AM, Thornton S. The role of oxytocin in parturition. *BJOG: An International Journal of Obstetrics & Gynaecology* 2003;110 Suppl 20:46-51.
207. Grigsby PL, Poore KR, Hirst JJ, Jenkin G. Inhibition of premature labor in sheep by a combined treatment of nimesulide, a prostaglandin synthase type 2 inhibitor, and atosiban, an oxytocin receptor antagonist. *American Journal of Obstetrics and Gynecology* 2000;183(3):649-57.
208. Scott JE, Grigsby PL, Hirst JJ, Jenkin G. Inhibition of prostaglandin synthesis and its effect on uterine activity during established premature labor in sheep. *J. Soc. Gynecol. Invest.* 2001;8(5):266-76.
209. Blank V, Hirsch E, Challis JRG, Romero R, Lye SJ. Cytokine signaling, inflammation, innate immunity and preterm labour - A workshop report. *Placenta* 2008;29:S102-S04.

210. Challis JR, Lockwood CJ, Myatt L, Norman JE, Strauss JF, Petraglia F. Inflammation and Pregnancy. *Reproductive Sciences* 2009;16(2):206-15.
211. Bollopragada S, Youssef R, Jordan F, Greer I, Norman J, Nelson S. Term labor is associated with a core inflammatory response in human fetal membranes, myometrium, and cervix. *American Journal of Obstetrics and Gynecology* 2009;200(1):104.e1-04.e11.
212. Haddad R, Tromp G, Kuivaniemi H, Chaiworapongsa T, Kim YM, Romero R. Spontaneous labor at term is characterized by a genomic signature of acute inflammation in the chorioamniotic membranes but not in the systemic circulation. *American Journal Of Obstetrics And Gynecology* 2004;191(6):S138-S38.
213. Mittal P, Romero R, Tarca AL, Gonzalez J, Draghici S, Xu Y, et al. Characterization of the myometrial transcriptome and biological pathways of spontaneous human labor at term. *J. Perinat. Med.* 2010;38(6):617-43.
214. Catalano RD, Lannagan TRM, Gorowiec M, Denison FC, Norman JE, Jabbour HN. Prokineticins: novel mediators of inflammatory and contractile pathways at parturition? *Molecular Human Reproduction* 2010;16(5):311-19.
215. Liggins G. Cervical ripening as an inflammatory reaction. In: EllwoodDA A, editor. *The Cervix in Pregnancy and Labor: Clinical and Biochemical Investigations*. Edinburgh: Churchill-Livingstone, 1981:1-9.
216. Bowen JM, Chamley L, Keelan JA, Mitchell MD. Cytokines of the Placenta and Extra-placental Membranes: Roles and Regulation During Human Pregnancy and Parturition. *Placenta* 2002;23(4):257-73.
217. Yellon SM, Mackler AM, Kirby MA. The role of leukocyte traffic and activation in parturition. *J. Soc. Gynecol. Invest.* 2003;10(6):323-38.
218. Liggins G. Cervical ripening as an inflammatory reaction. In: Ellwood DA, Anderson ABM, editors. *The Cervix in pregnancy and labour: clinical and biochemical investigations*. Edinburgh: Churchill Livingstone, 1981.
219. Thomson AJ, Telfer JF, Young A, Campbell S, Stewart CJR, Cameron IT, et al. Leukocytes infiltrate the myometrium during human parturition: further evidence that labour is an inflammatory process. *Hum. Reprod.* 1999;14(1):229-36.
220. Osman I, Young A, Ledingham MA, Thomson AJ, Jordan F, Greer IA, et al. Leukocyte density and pro-inflammatory cytokine expression in human fetal membranes, decidua, cervix and myometrium before and during labour at term. *Mol. Hum. Reprod.* 2003;9(1):41-45.
221. Winkler M. Role of cytokines and other inflammatory mediators. *BJOG: An International Journal of Obstetrics & Gynaecology* 2003;110:118-23.
222. LEDINGHAM MA, THOMSON AJ, JORDAN F, YOUNG A, CRAWFORD M, NORMAN JE. Cell Adhesion Molecule Expression in the Cervix and Myometrium During Pregnancy and Parturition. *Obstetrics & Gynecology* 2001;97(2):235-42.
223. Young A, Thomson AJ, Ledingham M, Jordan F, Greer IA, Norman JE. Immunolocalization of Proinflammatory Cytokines in Myometrium, Cervix, and Fetal Membranes During Human Parturition at Term. *Biology of Reproduction* 2002;66(2):445-49.
224. Hansen WR, Keelan JA, Skinner SJM, Mitchell MD. Key enzymes of prostaglandin biosynthesis and metabolism. Coordinate regulation of expression by cytokines in gestational tissues: a review. *Prostaglandins & Other Lipid Mediators* 1999;57(4):243-57.
225. Romero R, Mazor M, Tartakovsky B. Systemic administration of interleukin-1 induces preterm parturition in mice. *American Journal of Obstetrics and Gynecology* 1991;165(4):969-71.

226. Lindstrom TM, Bennett PR. The role of nuclear factor kappa B in human labour. *Reproduction* 2005;130(5):569-81.
227. Newton R, Kuitert LME, Bergmann M, Adcock IM, Barnes PJ. Evidence for Involvement of NF-Î°B in the Transcriptional Control of COX-2 Gene Expression by IL-1Î². *Biochemical and Biophysical Research Communications* 1997;237(1):28-32.
228. Yang X, Sheares KK, Davie N, Upton PD, Taylor GW, Horsley J, et al. Hypoxic induction of cox-2 regulates proliferation of human pulmonary artery smooth muscle cells. *American journal of respiratory cell and molecular biology* 2002;27(6):688-96.
229. Shimizu N, Ozawa Y, Yamaguchi M, Goseki T, Ohzeki K, Abiko Y. Induction of COX-2 expression by mechanical tension force in human periodontal ligament cells. *Journal of periodontology* 1998;69(6):670-77.
230. Goldenberg RL, Hauth JC, Andrews WW. Mechanisms of disease - Intrauterine infection and preterm delivery. *New England Journal of Medicine* 2000;342(20):1500-07.
231. Keelan JA. Pharmacological inhibition of inflammatory pathways for the prevention of preterm birth. *Journal of Reproductive Immunology* 2011;88(2):176-84.
232. Golightly E, Jabbour HN, Norman JE. Endocrine immune interactions in human parturition. *Molecular and Cellular Endocrinology* 2011;335(1):52-59.
233. Sykes L MD, Teoh TG, Bennett PR. Targeting Immune Activation in the Prevention of Pre-term Labour. *European Obstetrics & Gynaecology* 2011;6(2):100-06.
234. Romero R, Gotsch F, Pineles B, Kusanovic JP. Inflammation in pregnancy: Its roles in reproductive physiology, obstetrical complications, and fetal injury. *Nutrition Reviews* 2007;65(12):S194-S202.
235. Romero R, Espinoza J, Goncalves LF, Kusanovic JP, Friel L, Hassan S. The role of inflammation and infection in preterm birth. *Seminars in Reproductive Medicine* 2007;25(1):21-39.
236. Romero R, Espinoza J, GonÃ§alves LsF, Kusanovic JP, Friel LA, Nien JK. Inflammation in preterm and term labour and delivery. *Seminars in Fetal and Neonatal Medicine* 2006;11(5):317-26.
237. MacIntyre DA, Sykes L, Teoh TG, Bennett PR. Prevention of pre-term labour via the modulation of inflammatory pathways. *Journal of Maternal-Fetal and Neonatal Medicine* 2012;0(ja):1-16.
238. Elovitz MA. Anti-inflammatory interventions in pregnancy: Now and the future. *Seminars in Fetal and Neonatal Medicine* 2006;11(5):327-32.
239. RCOG. Tocolysis for Women in Preterm Labour, Green-top Guideline No. 1b. London: Royal College of Obstetricians and Gynaecologists, 2011.
240. Groom KM, Shennan AH, Jones BA, Seed P, Bennett PR. TOCOX—A randomised, double-blind, placebo-controlled trial of rofecoxib (a COX-2-specific prostaglandin inhibitor) for the prevention of preterm delivery in women at high risk. *BJOG: An International Journal of Obstetrics & Gynaecology* 2005;112(6):725-30.
241. King J, Flenady V, Cole S, Thornton S. Cyclo-oxygenase (COX) inhibitors for treating preterm labour. *Cochrane database of systematic reviews (Online)* 2005(2):CD001992.
242. Haddad R, Tromp G, Kuivaniemi H, Chaiworapongsa T, Kim YM, Mazor M, et al. Human spontaneous labor without histologic chorioamnionitis is characterized by an acute inflammation gene expression signature. *American Journal Of Obstetrics And Gynecology* 2006;195(2):394-405.

243. Bukowski R, Hankins GD, Saade GR, Anderson GD, Thornton S. Labor-associated gene expression in the human uterine fundus, lower segment, and cervix.[see comment]. *PLoS Medicine / Public Library of Science* 2006;3(6):e169.
244. Bethin KE, Nagai Y, Sladek R, Asada M, Sadovsky Y, Hudson TJ, et al. Microarray Analysis of Uterine Gene Expression in Mouse and Human Pregnancy. *Molecular Endocrinology* 2003;17(8):1454-69.
245. Aguan K, Carvajal JA, Thompson LP, Weiner CP. Application of a functional genomics approach to identify differentially expressed genes in human myometrium during pregnancy and labour. *Molecular Human Reproduction* 2000;6(12):1141-45.
246. Weiner CP, Mason CW, Dong Y, Buhimschi IA, Swaan PW, Buhimschi CS. Human effector/initiator gene sets that regulate myometrial contractility during term and preterm labor. *American Journal of Obstetrics and Gynecology* 2010;202(5):474.e1-20.
247. Holt R, Timmons BC, Akgul Y, Akins ML, Mahendroo M. The Molecular Mechanisms of Cervical Ripening Differ between Term and Preterm Birth. *Endocrinology* 2011;152(3):1036-46.
248. Mahendroo M. Cervical remodeling in term and preterm birth: insights from an animal model. *Reproduction* 2012;143(4):429-38.
249. Ohama T, Hori M, Momotani E, Iwakura Y, Guo F, Kishi H, et al. Intestinal inflammation downregulates smooth muscle CPI-17 through induction of TNF-alpha and causes motility disorders. *American Journal of Physiology-Gastrointestinal and Liver Physiology* 2007;292(5):G1429-G38.
250. Ohama T, Hori M, Sato K, Ozaki H, Karaki H. Chronic treatment with interleukin-1 beta attenuates contractions by decreasing the activities of CPI-17 and MYPT-1 in intestinal smooth muscle. *Journal of Biological Chemistry* 2003;278(49):48794-804.
251. Uwe S. Anti-inflammatory interventions of NF-kappaB signaling: potential applications and risks. *Biochem Pharmacol* 2008;75(8):1567-79.
252. Lim S, MacIntyre DA, Lee YS, Khanjani S, Terzidou V, Teoh TG, et al. Nuclear Factor Kappa B Activation Occurs in the Amnion Prior to Labour Onset and Modulates the Expression of Numerous Labour Associated Genes. *PLoS ONE* 2012;7(4):12.
253. Khanjani S, Kandola MK, Lindstrom TM, Sooranna SR, Melchionda M, Lee YS, et al. NF-kappaB regulates a cassette of immune/inflammatory genes in human pregnant myometrium at term. *J Cell Mol Med* 2011;15(4):809-24.
254. Hua R, Pease JE, Sooranna SR, Viney JM, Nelson SM, Myatt L, et al. Stretch and Inflammatory Cytokines Drive Myometrial Chemokine Expression Via NF-kappa B Activation. *Endocrinology* 2012;153(1):481-91.
255. Cookson VJ, Chapman NR. NF-kappaB function in the human myometrium during pregnancy and parturition. *Histol Histopathol* 2010;25(7):945-56.
256. Belt AR, Baldassare JJ, Molnar M, Romero R, Hertelendy F. The nuclear transcription factor NF-kappa B mediates interleukin-1 beta-induced expression of cyclooxygenase-2 in human myometrial cells. *American Journal of Obstetrics and Gynecology* 1999;181(2):359-66.
257. Hardy DB, Janowski BA, Corey DR, Mendelson CR. Progesterone Receptor Plays a Major Antiinflammatory Role in Human Myometrial Cells by Antagonism of Nuclear Factor-Î°B Activation of Cyclooxygenase 2 Expression. *Molecular Endocrinology* 2006;20(11):2724-33.
258. Eliopoulos AG, Dumitru CD, Wang C-C, Cho J, Tsichlis PN. Induction of COX-2 by LPS in macrophages is regulated by Tpl2-dependent CREB activation signals. *EMBO J* 2002;21(18):4831-40.

259. Wadleigh DJ, Reddy ST, Kopp E, Ghosh S, Herschman HR. Transcriptional Activation of the Cyclooxygenase-2 Gene in Endotoxin-treated RAW 264.7 Macrophages. *Journal of Biological Chemistry* 2000;275(9):6259-66.
260. Lamont RF. Infection in the prediction and antibiotics in the prevention of spontaneous preterm labour and preterm birth. *Bjog-an International Journal of Obstetrics and Gynaecology* 2003;110:71-75.
261. Klein LL, Gibbs RS. Use of microbial cultures and antibiotics in the prevention of infection-associated preterm birth. *American Journal of Obstetrics and Gynecology* 2004;190(6):1493-502.
262. Morken NH, Gunnes N, Magnus P, Jacobsson B. Risk of spontaneous preterm delivery in a low-risk population: the impact of maternal febrile episodes, urinary tract infection, pneumonia and ear-nose-throat infections. *European Journal of Obstetrics & Gynecology and Reproductive Biology* 2011;159(2):310-14.
263. Kaga N, Katsuki Y, Obata M, Shibutani Y. Repeated administration of low-dose lipopolysaccharide induces preterm delivery in mice: A model for human preterm parturition and for assessment of the therapeutic ability of drugs against preterm delivery. *American Journal of Obstetrics and Gynecology* 1996;174(2):754-59.
264. Ryan GB, Majno G. Acute inflammation. *American Journal of Pathology* 1977;86(1):185-276.
265. Playfair JHL. *Immunology at a Glance*. Sixth Edition ed. Oxford: Blackwell Science, 1996.
266. Kenyon SL, Taylor DJ, Tarnow-Mordi W, Grp OC. Broad-spectrum antibiotics for spontaneous preterm labour: the ORACLE II randomised trial. *Lancet* 2001;357(9261):989-94.
267. King J, Flenady V. Prophylactic antibiotics for inhibiting preterm labour with intact membranes. *Cochrane database of systematic reviews (Online)* 2002(4):CD000246.
268. Lamont RF. Infection in the prediction and antibiotics in the prevention of spontaneous preterm labour and preterm birth. *BJOG: An International Journal of Obstetrics and Gynaecology* 2003;110, Supplement 20(0):71-75.
269. Romero R, Oyarzun E, Mazor M, Sirtori M, Hobbins JC, Bracken M. Meta-analysis of the relationship between asymptomatic bacteriuria and preterm delivery low birth-weight. *Obstetrics and Gynecology* 1989;73(4):576-82.
270. McDonald HM, Brocklehurst P, Gordon A. Antibiotics for treating bacterial vaginosis in pregnancy. *Cochrane Database of Systematic Reviews* 2007(1):66.
271. Simcox R, Sin W-TA, Seed PT, Briley A, Shennan AH. Prophylactic antibiotics for the prevention of preterm birth in women at risk: A meta-analysis. *Australian and New Zealand Journal of Obstetrics and Gynaecology* 2007;47(5):368-77.
272. Saitoh M, Ishikawa T, Matsushima S, Naka M, Hidaka H. Selective inhibition of catalytic activity of smooth muscle myosin light chain kinase. *Journal of Biological Chemistry* 1987;262(16):7796-801.
273. Sigma-Aldrich. ML-7 Product Details, http://www.sigmaaldrich.com/catalog/ProductDetail.do?D7=0&N5=SEARCH_CONCAT_PNO|BRAND_KEY&N4=I2764|SIGMA&N25=0&QS=ON&F=SPEC.
274. Cohen P. The effective use of protein kinase inhibitors. *Sigma-Aldrich Application Notes* 2003:20-23.
275. Bain J, McLauchlan H, Elliott M, Cohen P. The specificities of protein kinase inhibitors: an update. *Biochemical Journal* 2003;371:199-204.

276. King JF, Flenady VJ, Papatsonis DN, Dekker GA, Carbonne B. Calcium channel blockers for inhibiting preterm labour. *Cochrane Database Syst Rev* 2003;1.
277. Anotayanonth S, Subhedar NV, Garner P, Neilson JP, Harigopal S. Betamimetics for inhibiting preterm labour. *Cochrane Database Syst Rev* 2004;18(4).
278. Whitworth M, Quenby S. Prophylactic oral betamimetics for preventing preterm labour in singleton pregnancies. *Cochrane Database Syst Rev* 2008;23(1).
279. Dodd JM, Crowther CA, Dare MR, Middleton P. Oral betamimetics for maintenance therapy after threatened preterm labour. *Cochrane Database Syst Rev* 2006;25(1).
280. Morrison JJ, Ashford MLJ, Khan RN, Smith SK. The effects of potassium channel openers on isolated pregnant human myometrium before and after the onset of labor - potential for tocolysis. *American Journal of Obstetrics and Gynecology* 1993;169(5):1277-85.
281. Suzuki S, Yano K, Kusano S, Hashimoto T. Antihypertensive effect of levcromakalim in patients with essential hypertension. Study by 24-h ambulatory blood pressure monitoring. *Arzneimittelforschung* 1995;45(8):859-64.
282. Galik M, Gaspar R, Kolarovszki-Sipiczki Z, Falkay G. Gestagen treatment enhances the tocolytic effect of salmeterol in hormone-induced preterm labor in the rat in vivo. *Am J Obstet Gynecol* 2008;198(3):e1-5.
283. Chanrachakul B, Pipkin FB, Warren AY, Arulkumaran S, Khan RN. Progesterone enhances the tocolytic effect of ritodrine in isolated pregnant human myometrium. *American Journal of Obstetrics and Gynecology* 2005;192(2):458-63.
284. Baumbach J, Shi SQ, Shi L, Balducci J, Coonrod DV, Garfield RE. Inhibition of uterine contractility with various tocolytics with and without progesterone: in vitro studies. *Am J Obstet Gynecol* 2012;206(3):16.
285. Anderson L, Martin W, Higgins C, Nelson SM, Norman JE. The Effect of Progesterone on Myometrial Contractility, Potassium Channels, and Tocolytic Efficacy. *Reproductive Sciences* 2009;16(11):1052-61.
286. Anderson L. The Myometrial Effects of Progesterone. University of Glasgow, 2009.
287. Norman JE, Mackenzie F, Owen P, Mactier H, Hanretty K, Cooper S, et al. Progesterone for the prevention of preterm birth in twin pregnancy (STOPPIT): a randomised, double-blind, placebo-controlled study and meta-analysis. *Lancet* 2009;373(9680):2034-40.
288. Rozenberg P, Chauveaud A, Deruelle P, Capelle M, Winer N, DesbriÃ¨re R, et al. Prevention of preterm delivery after successful tocolysis in preterm labor by 17 alpha-hydroxyprogesterone caproate: A randomized controlled trial. *American Journal of Obstetrics and Gynecology* 2012;206(3):206.e1-06.e9.
289. Mackenzie R, Walker M, Armson A, Hannah ME. Progesterone for the prevention of preterm birth among women at increased risk: A systematic review and meta-analysis of randomized controlled trials. *American Journal of Obstetrics and Gynecology* 2006;194(5):1234-42.
290. Dodd JM, Flenady VJ, Cincotta R, Crowther CA. Progesterone for the prevention of preterm birth - A systematic review. *Obstetrics and Gynecology* 2008;112(1):127-34.
291. Thornton S, Terzidou V, Clark A, Blanks A. Progesterone metabolite and spontaneous myometrial contractions in vitro. *Lancet* 1999;353(9161):1327-9.

292. Wilson RJ, Allen MJ, Nandi M, Giles H, Thornton S. Spontaneous contractions of myometrium from humans, non-human primate and rodents are sensitive to selective oxytocin receptor antagonism in vitro. *BJOG: An International Journal of Obstetrics & Gynaecology* 2001;108(9):960-6.
293. Papatsonis D, Flenady V, Cole S, Liley H. Oxytocin receptor antagonists for inhibiting preterm labour.[see comment]. *Cochrane Database of Systematic Reviews* 2005(3):CD004452.
294. Papatsonis D, Flenady V, Liley H. Maintenance therapy with oxytocin antagonists for inhibiting preterm birth after threatened preterm labour. *Cochrane Database of Systematic Reviews* 2009(1).
295. Dennedy MC, Avalos G, O'Reilly MW, O'Sullivan EP, Gaffney G, Dunne F. ATLANTIC-DIP: Raised Maternal Body Mass Index (BMI) Adversely Affects Maternal and Fetal Outcomes in Glucose-Tolerant Women According to International Association of Diabetes and Pregnancy Study Groups (IADPSG) Criteria. *J. Clin. Endocrinol. Metab.* 2012;97(4):E608-E12.
296. Cedergren MI. Maternal Morbid Obesity and the Risk of Adverse Pregnancy Outcome. *Obstet Gynecol* 2004;103(2):219-24.
297. Dietz PM, Callaghan WM, Cogswell ME, Morrow B, Ferre C, Schieve LA. Combined effects of prepregnancy body mass index and weight gain during pregnancy on the risk of preterm delivery. *Epidemiology* 2006;17(2):170-7.
298. Stotland NE, Washington AE, Caughey AB. Prepregnancy body mass index and the length of gestation at term. *American Journal of Obstetrics and Gynecology* 2007;197(4):378.e1-78.e5.
299. Smith RD, Babiychuk EB, Noble K, Draeger A, Wray S. Increased cholesterol decreases uterine activity: functional effects of cholesterol alteration in pregnant rat myometrium. *Am J Physiol Cell Physiol* 2005;288(5):C982-88.
300. Zhang J, Kendrick A, Quenby S, Wray S. Contractility and Calcium Signalling of Human Myometrium Are Profoundly Affected by Cholesterol Manipulation: Implications for Labor? *Reproductive Sciences* 2007;14(5):456-66.
301. Moynihan AT, Hehir MP, Glavey SV, Smith TJ, Morrison JJ. Inhibitory effect of leptin on human uterine contractility in vitro. *American Journal of Obstetrics and Gynecology* 2006;195(2):504.
302. Elmes MJ, Tan DSY, Cheng Z, Wathes DC, McMullen S. The effects of a high-fat, high-cholesterol diet on markers of uterine contractility during parturition in the rat. *Reproduction* 2011;141(2):283-90.
303. Vahratian A, Hoffman MK, Troendle JF, Zhang J. The impact of parity on course of labor in a contemporary population. *Birth* 2006;33(1):12-7.
304. Martel M, Wacholder S, Lippman A, Brohan J, Hamilton E. Maternal age and primary cesarean section rates: a multivariate analysis. *Am J Obstet Gynecol* 1987;156(2):305-8.
305. Heinberg EM, Wood RA, Chambers RB. Elective induction of labor in multiparous women. Does it increase the risk of cesarean section? *J Reprod Med* 2002;47(5):399-403.
306. Fraser AM, Brockert JE, Ward RH. Association of Young Maternal Age with Adverse Reproductive Outcomes. *New England Journal of Medicine* 1995;332(17):1113-18.
307. Black C, Kaye JA, Jick H. Cesarean delivery in the United Kingdom - Time trends in the general practice research database. *Obstetrics and Gynecology* 2005;106(1):151-55.

308. Marwick JC, Lynn R. High caesarean section rates among,women over 30 - Effect of age is continuous and international. *Br. Med. J.* 2001;323(7307):284-84.
309. Graseck AS, Odibo AO, Tuuli M, Roehl KA, Macones GA, Cahill AG. Normal first stage of labor in women undergoing trial of labor after cesarean delivery. *Obstet Gynecol* 2012;119(4):732-6.
310. Stock S, Norman J. Preterm and term labour in multiple pregnancies. *Semin Fetal Neonatal Med* 2012;15(6):336-41.
311. Many A, Hill LM, Lazebnik N, Martin JG. The association between polyhydramnios and preterm delivery. *Obstet Gynecol* 1995;86(3):389-91.
312. Loudon JA, Sooranna SR, Bennett PR, Johnson MR. Mechanical stretch of human uterine smooth muscle cells increases IL-8 mRNA expression and peptide synthesis. *Molecular Human Reproduction* 2004;10(12):895-9.
313. Shynlova O, Tsui P, Dorogin A, Lye SJ. Monocyte chemoattractant protein-1 (CCL-2) integrates mechanical and endocrine signals that mediate term and preterm Labor. *J. Immunol.* 2008;181(2):1470-79.
314. Sparey C, Robson SC, Bailey J, Lyall F, Nicholas Europe-Finner G. The Differential Expression of Myometrial Connexin-43, Cyclooxygenase-1 and -2, and GsÎ± Proteins in the Upper and Lower Segments of the Human Uterus during Pregnancy and Labor. *J. Clin. Endocrinol. Metab.* 1999;84(5):1705-10.
315. Menzies FM, Khan AH, Higgins CA, Nelson SM, Nibbs RJB. The Chemokine Receptor CCR2 Is Not Required for Successful Initiation of Labor in Mice. *Biology of Reproduction* 2012.
316. Durnwald CP, Mercer BM. Myometrial thickness according to uterine site, gestational age and prior cesarean delivery. *Journal of Maternal Fetal & Neonatal Medicine* 2008;21(4):247-50.
317. Crankshaw DJ, Morrison JJ. Methodology and pharmacological analysis of effects of uterotonic compounds in human myometrium in vitro. *American Journal of Obstetrics and Gynecology* 2011;205(2):6.
318. Product 475981 Myosin Light Chain Kinase Inhibitor Peptide 18 http://www.merck-chemicals.com/is-bin/INTERSHOP.enfinity/WFS/Merck-GB-Site/en_US/-/GBP/ViewPDF-Print.pdf?RenderPageType=ProductDetail&CatalogCategoryID=Dcib.s1OezAAAAEjtRl9.zLX&ProductUUID=0eqb.s1OqV8AAAEaiMssKi67&PortalCatalogUUID=ywGb.s1LAyMAAAEWzdUfVhTl. Nottingham: Merck Chemicals Limited, 2010.
319. McKendrick JD, Paisley K, Eason S, Mian KB, Martin W. Induction of nitric oxide synthase by endotoxin in rat isolated aorta but not in rat aortic smooth muscle cells grown in culture from explant. *Archives Internationales de Pharmacodynamie et de Therapie* 1995;330(2):206-24.
320. Ferrari D, Pizzirani C, Adinolfi E, Forchap S, Sitta B, Turchet L, et al. The Antibiotic Polymyxin B Modulates P2X7 Receptor Function. *J Immunol* 2004;173(7):4652-60.
321. Pierce JG, Gordon S, Duvigneaud V. Further distribution studies on the oxytocic hormone of the posterior lobe of the pituitary glasnd and the preparation of an active crystalline flavianate. *Journal of Biological Chemistry* 1952;199(2):929-40.
322. Vigneaud VD, Ressler C, Swan JM, Roberts CW, Katsoyannis PG, Gordon S. The synthesis of an octapeptide amide with the hormonal activity of oxytocin. *Journal of the American Chemical Society* 1953;75(19):4879-80.
323. Vigneaud VD, Ressler C, Trippett S. The sequence of amino acids in oxytocin, with a proposal for the structure of oxytocin. *Journal of Biological Chemistry* 1953;205(2):949-57.

324. Vigneaud VD. A Trail of Sulfa Research: From Insulin to Oxytocin. *Nobel Lectures, Chemistry 1942-1962*. Amsterdam: Elsevier Publishing Company, 1964.
325. Hicks J. On the contractions of the uterus throughout pregnancy: their physiological effects and their value in the diagnosis of pregnancy. *Transactions of the Obstetrical Society of London* 1871;13:216-31.
326. Dunn PM. John Braxton Hicks (1823â€“97) and painless uterine contractions. *Archives of Disease in Childhood - Fetal and Neonatal Edition* 1999;81(2):F157-F58.
327. Park ES, Echetebu CO, Soloff S, Soloff MS. Oxytocin stimulation of RGS2 mRNA expression in cultured human myometrial cells. *American Journal of Physiology - Endocrinology & Metabolism* 2002;282(3):E580-4.
328. Lin SM, Du P, Huber W, Kibbe WA. Model-based variance-stabilizing transformation for Illumina microarray data. *Nucleic Acids Research* 2008;36(2):e11.
329. Smyth GK. Limma: linear models for microarray data. In: Gentleman V, Carey S, Dudoit R, Irizarry WH, editors. *Bioinformatics and Computational Biology Solutions using R and Bioconductor, R*. New York: Springer, 2005:397-420.
330. Benjamini Y, Hochberg Y. Controlling the False Discovery Rate: A Practical and Powerful Approach to Multiple Testing. *Journal of the Royal Statistical Society. Series B (Methodological)* 1995;57(1):289-300.
331. Feise R. Do multiple outcome measures require p-value adjustment? *BMC Medical Research Methodology* 2002;2(1):8.
332. Rothman KJ. No adjustments are needed for multiple comparisons. *Epidemiology (Cambridge, Mass.)* 1990;1(1):43-6.
333. Bacchetti P. Peer review of statistics in medical research: the other problem. *BMJ* 2002;324(7348):1271-73.
334. Goodman SN. Multiple Comparisons, Explained. *Am. J. Epidemiol.* 1998;147(9):807-12.
335. The Gene Ontology, http://www.geneontology.org/, 2012.
336. KEGG: Kyoto Encyclopedia of Genes and Genomes, http://www.genome.jp/kegg/, 2012.
337. NCBI. CCL3L3 chemokine (C-C motif) ligand 3-like 3 [Homo sapiens] Gene ID: 414062 http://www.ncbi.nlm.nih.gov/gene/414062, 2012.
338. NCBI. CCL4L1 chemokine (C-C motif) ligand 4-like 1 [Homo sapiens] Gene ID: 9560 http://www.ncbi.nlm.nih.gov/gene/9560, 2012.
339. Dhawan J, Helfman DM. Modulation of acto-myosin contractility in skeletal muscle myoblasts uncouples growth arrest from differentiation. *Journal of Cell Science* 2004;117(17):3735-48.
340. Duran JM, Valderrama F, Castel S, Magdalena J, TomÃ¡s Mn, Hosoya H, et al. Myosin Motors and Not Actin Comets Are Mediators of the Actin-based Golgi-to-Endoplasmic Reticulum Protein Transport. *Molecular Biology of the Cell* 2003;14(2):445-59.
341. Helfman DM, Pawlak G. Myosin light chain kinase and acto-myosin contractility modulate activation of the ERK cascade downstream of oncogenic Ras. *Journal of Cellular Biochemistry* 2005;95(5):1069-80.
342. Mahoney DJ, Parise G, Melov S, Safdar A, Tarnopolsky MA. Analysis of global mRNA expression in human skeletal muscle during recovery from endurance exercise. *The FASEB Journal* 2005;19(11):1498-500.
343. Chen Y-W, Hubal MJ, Hoffman EP, Thompson PD, Clarkson PM. Molecular responses of human muscle to eccentric exercise. *Journal of Applied Physiology* 2003;95(6):2485-94.

344. Livak KJ, Schmittgen TD. Analysis of Relative Gene Expression Data Using Real-Time Quantitative PCR and the $2^{-\Delta\Delta Ct}$ Method. *Methods* 2001;25(4):402-08.
345. User Bulletin #2 ABI Prism 7700 Sequence Detection System, Relative Quantification of Gene Expression: Applied Biosystems, 2001.
346. Baumbach J, Shi S-Q, Shi L, Balducci J, Coonrod DV, Garfield RE. Inhibition of uterine contractility with various tocolytics with and without progesterone: in vitro studies. *American Journal of Obstetrics and Gynecology* 2011;206(3):254.e1-54.e5.
347. Norman JE, Yuan MF, Anderson L, Howie F, Harold G, Young A, et al. Effect of Prolonged In Vivo Administration of Progesterone in Pregnancy on Myometrial Gene Expression, Peripheral Blood Leukocyte Activation, and Circulating Steroid Hormone Levels. *Reproductive Sciences* 2011;18(5):435-46.
348. Critchley HOD, Jones RL, Lea RG, Drudy TA, Kelly RW, Williams ARW, et al. Role of inflammatory mediators in human endometrium during progesterone withdrawal and early pregnancy. *J. Clin. Endocrinol. Metab.* 1999;84(1):240-48.
349. Elovitz M, Wang Z. Medroxyprogesterone acetate, but not progesterone, protects against inflammation-induced parturition and intrauterine fetal demise. *American Journal of Obstetrics and Gynecology* 2004;190(3):693-701.
350. Hutzal CE, Boyle EM, Kenyon SL, Nash JV, Winsor S, Taylor DJ, et al. Use of antibiotics for the treatment of preterm parturition and prevention of neonatal morbidity: a metaanalysis. *American Journal of Obstetrics and Gynecology* 2008;199(6):620.e1-20.e8.
351. Serhan CN. Novel Lipid Mediators and Resolution Mechanisms in Acute Inflammation: To Resolve or Not? *The American Journal of Pathology* 2010;177(4):1576-91.
352. Janeway C. *Immunobiology: the immune system in health and disease*: Current Biology Publications, 1999.
353. Bochner BS. Road signs guiding leukocytes along the inflammation superhighway. *The Journal of allergy and clinical immunology* 2000;106(5):817-28.
354. Leong AS, Norman JE, Smith R. Vascular and myometrial changes in the human uterus at term. *Reproductive Sciences* 2008;15(1):59-65.
355. BALLOCH AJ, CAUCHI MN. Reference ranges for haematology parameters in pregnancy derived from patient populations. *Clinical & Laboratory Haematology* 1993;15(1):7-14.
356. Griffin JFT, Beck I. A longitudinal study of leucocyte numbers and mitogenesis during the last ten weeks of human pregnancy. *Journal of Reproductive Immunology* 1983;5(4):239-47.
357. Lurie S, Rahamim E, Piper I, Golan A, Sadan O. Total and differential leukocyte counts percentiles in normal pregnancy. *European Journal of Obstetrics & Gynecology and Reproductive Biology* 2008;136(1):16-19.
358. Valdimarsson H, Mulholland C, Fridriksdottir V, Coleman DV. A longitudinal study of leucocyte blood counts and lymphocyte responses in pregnancy: a marked early increase of monocyte-lymphocyte ratio. *Clinical & Experimental Immunology* 1983;53(2):437-43.
359. Dawson DW. Eosinophils and pregnancy. *Journal of Obstetrics & Gynaecology of the British Empire* 1953;60(5):727-31.

360. Luppi P, Haluszczak C, Betters D, Richard CAH, Trucco M, DeLoia JA. Monocytes are progressively activated in the circulation of pregnant women. *Journal of Leukocyte Biology* 2002;72(5):874-84.
361. Kraus T, Engel S, Sperling R, Kellerman L, Lo Y, Wallenstein S, et al. Characterizing the Pregnancy Immune Phenotype: Results of the Viral Immunity and Pregnancy (VIP) Study. *Journal of Clinical Immunology*:1-12.
362. Delgado I, Neubert R, Dudenhausen JW. Changes in white blood cells during parturition in mothers and newborn. *Gynecologic & Obstetric Investigation* 1994;38(4):227-35.
363. Rodriguez-Dennen F, Martinez-Ocana J, Kawa-Karasik S, Villanueva-Egan L, Reyes-Paredes N, Flisser A, et al. Comparison of hemodynamic, biochemical and hematological parameters of healthy pregnant women in the third trimester of pregnancy and the active labor phase. *BMC Pregnancy and Childbirth* 2011;11(1):33.
364. Griffin JFT, Beck I. CHANGES IN MATERNAL PERIPHERAL LEUCOCYTES AROUND DELIVERY. *BJOG: An International Journal of Obstetrics & Gynaecology* 1980;87(5):402-07.
365. Bass DA. Behavior of eosinophil leukocytes in acute inflammation. II. Eosinophil dynamics during acute inflammation. *The Journal of Clinical Investigation* 1975;56(4):870-79.
366. van Ginhoven TM, Dik WA, Mitchell JR, Nijenhuis MAS-t, van Holten-Neelen C, Hooijkaas H, et al. Dietary Restriction Modifies Certain Aspects of the Postoperative Acute Phase Response. *Journal of Surgical Research*;171(2):582-89.
367. Desborough JP. The stress response to trauma and surgery. *British Journal of Anaesthesia* 2000;85(1):109-17.
368. Bartal I, Melamed R, Greenfeld K, Atzil S, Glasner A, Domankevich V, et al. Immune perturbations in patients along the perioperative period: Alterations in cell surface markers and leukocyte subtypes before and after surgery. *Brain Behav. Immun.* 2010;24(3):376-86.
369. Bone RC, Balk RA, Cerra FB, Dellinger RP, Fein AM, Knaus WA, et al. Definitions for sepsis and organ failure and guidelines for the use of innovative therapies in sepsis. The ACCP/SCCM Consensus Conference Committee. American College of Chest Physicians/Society of Critical Care Medicine. *Chest* 1992;101(6):1644-55.
370. Thorley JD, Smith JW, Luby JP, Sanford JP. Peripheral blood lymphocyte response to acute infections in humans. *Infection and Immunity* 1977;16(1):110-14.
371. Levy MMMDF, Fink MPMDF, Marshall JCMD, Abraham EMD, Angus DMDMPHF, Cook DMDF, et al. 2001 SCCM/ESICM/ACCP/ATS/SIS International Sepsis Definitions Conference. *Critical Care Medicine* 2003;31(4):1250-56.
372. Yuan M, Jordan F, McInnes IB, Harnett MM, Norman JE. Leukocytes are primed in peripheral blood for activation during term and preterm labour. *Molecular Human Reproduction* 2009;15(11):713-24.
373. Picklesimer AH, Jared HL, Moss K, Offenbacher S, Beck JD, Boggess KA. Racial differences in C-reactive protein levels during normal pregnancy. *American Journal of Obstetrics and Gynecology* 2008;199(5):523.e1-23.e6.
374. Watts DHM, Krohn MAP, Wener MHM, Eschenbach DAM. C-Reactive Protein in Normal Pregnancy. *Obstetrics & Gynecology* 1991;77(2):176-80.
375. Makhseed M, Raghupathy R, Azizieh F, Farhat R, Hassan N, Bandar A. Circulating cytokines and CD30 in normal human pregnancy and recurrent spontaneous abortions. *Human Reproduction* 2000;15(9):2011-17.

376. Curry AE, Vogel I, Skogstrand K, Drews C, Schendel DE, Flanders WD, et al. Maternal plasma cytokines in early- and mid-gestation of normal human pregnancy and their association with maternal factors. *Journal of Reproductive Immunology* 2008;77(2):152-60.
377. Kronborg CS, Gjedsted J, Vittinghus E, Hansen TK, Allen JIM, Knudsen UB. Longitudinal measurement of cytokines in pre-eclamptic and normotensive pregnancies. *Acta Obstetricia et Gynecologica Scandinavica* 2011;90(7):791-96.
378. Kraus TA, Sperling RS, Engel SM, Lo Y, Kellerman L, Singh T, et al. Peripheral Blood Cytokine Profiling During Pregnancy and Post-partum Periods. *American Journal of Reproductive Immunology* 2010;64(6):411-26.
379. Hamilton S, Oomomian Y, Stephen G, Shynlova O, Tower CL, Garrod A, et al. Macrophages Infiltrate the Human and Rat Decidua During Term and Preterm Labor: Evidence That Decidual Inflammation Precedes Labor. *Biology of Reproduction*.
380. Gomez-Lopez N, Guilbert LJ, Olson DM. Invasion of the leukocytes into the fetal-maternal interface during pregnancy. *Journal of Leukocyte Biology*;88(4):625-33.
381. Bisits AM, Smith R, Mesiano S, Yeo G, Kwek K, MacIntyre D, et al. Inflammatory Aetiology of Human Myometrial Activation Tested Using Directed Graphs. *PLoS Comput Biol* 2005;1(2):e19.
382. Gorowiec MR, Catalano RD, Norman JE, Denison FC, Jabbour HN. Prokineticin 1 Induces Inflammatory Response in Human Myometrium A Potential Role in Initiating Term and Preterm Parturition. *American Journal of Pathology*;179(6):2709-19.
383. Walsh NP, Gleeson M, Shephard RJ, Gleeson M, Woods JA, Bishop NC, et al. Position Statement Part one: Immune function and exercise. *Exercise Immunology Review* 2011;17:6-63.
384. Pedersen BK. Exercise and cytokines. *Immunol Cell Biol* 2000;78(5):532-35.
385. Pedersen BK, Toft AD. Effects of exercise on lymphocytes and cytokines. *British Journal of Sports Medicine* 2000;34(4):246-51.
386. Clarkson PM, Hubal MJ. Exercise-induced muscle damage in humans. *American Journal of Physical Medicine & Rehabilitation* 2002;81(11 Suppl):S52-69.
387. Mair J, Koller A, Artner-Dworzak E, Haid C, Wicke K, Judmaier W, et al. Effects of exercise on plasma myosin heavy chain fragments and MRI of skeletal muscle. *Journal of Applied Physiology* 1992;72(2):656-63.
388. Peake J, Wilson G, Hordern M, Suzuki K, Yamaya K, Nosaka K, et al. Changes in neutrophil surface receptor expression, degranulation, and respiratory burst activity after moderate- and high-intensity exercise. *J Appl Physiol* 2004;97(2):612-18.
389. Smith LL. Acute inflammation: the underlying mechanism in delayed onset muscle soreness? *Medicine & Science in Sports & Exercise* 1991;23(5):542-51.
390. Shivvers SA, Wians FH, Jr., Keffer JH, Ramin SM. Maternal cardiac troponin I levels during normal labor and delivery. *American Journal of Obstetrics & Gynecology* 1999;180(1 Pt 1):122.
391. Kosowicz J, Gryczyska M, Drews K. ELEVATION OF MATERNAL SERUM MYOGLOBIN CONCENTRATIONS DURING DELIVERY. *BJOG: An International Journal of Obstetrics & Gynaecology* 1981;88(6):628-31.
392. Abramov Y, Abramov D, Abrahamov A, Durst R, Schenker J. Elevation of serum creatine phosphokinase and its MB isoenzyme during normal labor

and early puerperium. *Acta Obstetricia et Gynecologica Scandinavica* 1996;75(3):255-60.
393. Rothenberg ME, Hogan SP. THE EOSINOPHIL. *Annual Review of Immunology* 2006;24(1):147-74.
394. Karasuyama H, Mukai K, Obata K, Tsujimura Y, Wada T. Nonredundant Roles of Basophils in Immunity. *Annual Review of Immunology* 2011;29(1):45-69.
395. Rottman JB. Key Role of Chemokines and Chemokine Receptors in Inflammation, Immunity, Neoplasia, and Infectious Disease. *Veterinary Pathology Online* 1999;36(5):357-67.
396. Baggiolini M. Chemokines in pathology and medicine. *Journal of Internal Medicine* 2001;250(2):91-104.
397. Roberts PJ, Pizzey AR, Khwaja A, Carver JE, Mire-Sluis AR, Linch DC. The effects of interleukin-8 on neutrophil fMetLeuPhe receptors, CD11b expression and metabolic activity, in comparison and combination with other cytokines. *British Journal of Haematology* 1993;84(4):586-94.
398. White JR, Lee JM, Young PR, Hertzberg RP, Jurewicz AJ, Chaikin MA, et al. Identification of a Potent, Selective Non-peptide CXCR2 Antagonist That Inhibits Interleukin-8-induced Neutrophil Migration. *Journal of Biological Chemistry* 1998;273(17):10095-98.
399. Lippert U, Zachmann K, Henz BM, Neumann C. Human T lymphocytes and mast cells differentially express and regulate extra- and intracellular CXCR1 and CXCR2. *Experimental Dermatology* 2004;13(8):520-25.
400. NCBI. CXCR1 chemokine (C-X-C motif) receptor 1 [Homo sapiens] Gene ID: 3577 http://www.ncbi.nlm.nih.gov/gene/3577, 2012.
401. NCBI. CXCR2 chemokine (C-X-C motif) receptor 2 [Homo sapiens] Gene ID: 3579 http://www.ncbi.nlm.nih.gov/gene/3579, 2012.
402. Baggiolini M. Chemokines and leukocyte traffic. *Nature* 1998;392(6676):565-68.
403. NCBI. CCR2 chemokine (C-C motif) receptor 2 [Homo sapiens] Gene ID: 729230 http://www.ncbi.nlm.nih.gov/gene/729230, 2012.
404. Combadiere C, Ahuja SK, Van Damme J, Tiffany HL, Gao J-L, Murphy PM. Monocyte Chemoattractant Protein-3 Is a Functional Ligand for CC Chemokine Receptors 1 and 2B. *Journal of Biological Chemistry* 1995;270(50):29671-75.
405. Gong X, Gong W, Kuhns DB, Ben-Baruch A, Howard OMZ, Wang JM. Monocyte Chemotactic Protein-2 (MCP-2) Uses CCR1 AND CCR2B as Its Functional Receptors. *Journal of Biological Chemistry* 1997;272(18):11682-85.
406. Godiska R, Chantry D, Raport CJ, Schweickart VL, Trong HL, Gray PW. Monocyte chemotactic protein-4: tissue-specific expression and signaling through CC chemokine receptor-2. *Journal of Leukocyte Biology* 1997;61(3):353-60.
407. NCBI. CCR6 chemokine (C-C motif) receptor 6 [Homo sapiens] Gene ID: 1235 http://www.ncbi.nlm.nih.gov/gene/1235, 2012.
408. Pepys MB, Hirschfield GM. C-reactive protein: a critical update. *The Journal of Clinical Investigation* 2003;111(12):1805-12.
409. Laurence AS. Serum myoglobin and creatine kinase following surgery. *British Journal of Anaesthesia* 2000;84(6):763-6.
410. Payne RM, Friedman DL, Grant JW, Perryman MB, Strauss AW. Creatine kinase isoenzymes are highly regulated during pregnancy in rat uterus and placenta. *Am J Physiol Endocrinol Metab* 1993;265(4):E624-35.
411. Qiu Y, Sutton L, Riggs AF. Identification of myoglobin in human smooth muscle. *Journal of Biological Chemistry* 1998;273(36):23426-32.

412. Stone MJ, Waterman MR, Harimoto D, Murray G, Willson N, Platt MR, et al. Serum myoglobin level as diagnostic test in patients with acute myocardial infarction. *British Heart Journal* 1977;39(4):375-80.
413. McCracken S, Layton JE, Shorter SC, Starkey PM, Barlow DH, Mardon HJ. Expression of granulocyte-colony stimulating factor and its receptor is regulated during the development of the human placenta. *J. Endocrinol.* 1996;149(2):249-58.
414. Walter J, Fraga L, Orin M, Decker W, Gipps T, Stek A, et al. Immunomodulatory factors in cervicovaginal secretions from pregnant and non-pregnant women: A cross-sectional study. *BMC Infectious Diseases* 2011;11(1):263.
415. Nibbs R, Graham G, Rot A. Chemokines on the move: control by the chemokine â€œinterceptorsâ€ Duffy blood group antigen and D6. *Seminars in Immunology* 2003;15(5):287-94.
416. Mantovani A, Bonecchi R, Locati M. Tuning inflammation and immunity by chemokine sequestration: decoys and more. *Nat Rev Immunol* 2006;6(12):907-18.
417. Hansell C, Simpson C, Nibbs R. Chemokine sequestration by atypical chemokine receptors. *Biochem Soc Trans.* 2006;34(Pt 6):1009-13.
418. Graham GJ. D6 and the atypical chemokine receptor family: Novel regulators of immune and inflammatory processes. *European Journal of Immunology* 2009;39(2):342-51.
419. Hansell C, Hurson C, Nibbs R. DARC and D6: silent partners in chemokine regulation? *Immunol* 2010;89(2):197-206. Epub 2010 Dec 14.
420. Nibbs R, Wylie S, Yang J, Landau N, Graham G. Cloning and characterization of a novel promiscuous human beta-chemokine receptor D6. *J Biol Chem.* 1997;272(51):32078-83.
421. Novitzky-Basso I, Rot A. Duffy antigen receptor for chemokines and its involvement in patterning and control of inflammatory chemokines. *Front* 2012;3:266. Epub 2012 Aug 17.
422. Cochain C, Auvynet C, Poupel L, Vilar J, Dumeau E, Richart A, et al. The chemokine decoy receptor D6 prevents excessive inflammation and adverse ventricular remodeling after myocardial infarction. *Arterioscler Thromb Vasc Biol* 2012;32(9):2206-13. doi: 10.1161/ATVBAHA.112.254409. Epub 2012 Jul 12.
423. Borroni E, Bonecchi R, Buracchi C, Savino B, Mantovani A, Locati M. Chemokine decoy receptors: new players in reproductive immunology. *Immunol Invest* 2008;37(5):483-97.
424. Lanza A. Creatine kinase in serum after uterus contraction produced by oxytocin. *Cellular and Molecular Life Sciences* 1968;24(4):337-37.
425. Pizza FX, Koh TJ, McGregor SJ, Brooks SV, Pizza FX, Koh TJ, et al. Muscle inflammatory cells after passive stretches, isometric contractions, and lengthening contractions. *Journal of Applied Physiology* 2002;92(5):1873-8.
426. Pizza FX, Mitchell JB, Davis BH, Starling RD, Holtz RW, Bigelow N. Exercise-induced muscle damage: effect on circulating leukocyte and lymphocyte subsets. *Medicine & Science in Sports & Exercise* 1995;27(3):363-70.
427. Smith LL, McKune AJ, Semple SJ, Sibanda E, Steel H, Anderson R, et al. Changes in serum cytokines after repeated bouts of downhill running. *Applied Physiology, Nutrition, & Metabolism = Physiologie Appliquee, Nutrition et Metabolisme* 2007;32(2):233-40.
428. Smith LL, Anwar A, Fragen M, Rananto C, Johnson R, Holbert D. Cytokines and cell adhesion molecules associated with high-intensity eccentric exercise. *European Journal of Applied Physiology* 2000;82(1-2):61-7.

429. Tidball JG. Inflammatory processes in muscle injury and repair. *American Journal of Physiology - Regulatory, Integrative and Comparative Physiology* 2005;288(2):R345-R53.
430. Smith L, McCammon M, Smith S, Chamness M, Israel R, O'Brien K. White blood cell response to uphill walking and downhill jogging at similar metabolic loads. *European Journal of Applied Physiology and Occupational Physiology* 1989;58(8):833-37.
431. Lu H, Huang D, Saederup N, Charo IF, Ransohoff RM, Zhou L. Macrophages recruited via CCR2 produce insulin-like growth factor-1 to repair acute skeletal muscle injury. *Faseb J.* 2011;25(1):358-69.
432. Yahiaoui L, Gvozdic D, Danialou G, Mack M, Petrof BJ. CC family chemokines directly regulate myoblast responses to skeletal muscle injury. *J Physiol* 2008;586(16):3991-4004.
433. Lu H, Huang D, Ransohoff RM, Zhou L. Acute skeletal muscle injury: CCL2 expression by both monocytes and injured muscle is required for repair. *Faseb J* 2011;25(10):3344-55.
434. Negishi H, Kishida T, Yamada H, Hirayama E, Mikuni M, Fujimoto S. Changes in uterine size after vaginal delivery and cesarean section determined by vaginal sonography in the puerperium. *Archives of Gynecology & Obstetrics* 1999;263(1-2):13-6.
435. Mulic-Lutvica A, Axelsson O. Postpartum ultrasound in women with postpartum endometritis, after cesarean section and after manual evacuation of the placenta. *Acta Obstetricia et Gynecologica Scandinavica* 2007;86(2):210-17.
436. Hirani V. Chapter 5: Anthropometric Measures, Overweight, and Obesity. In: Bromley C, Sproston K, Shelton N, editors. *The Scottish Health Survey 2003 - Volume 2*. Edinburgh: Scottish Executive, 2005:168-206.
437. Hirani V. Chapter 6: Anthropometric measures, overweight, and obesity In: Sprotston K, Primatesta P, editors. *Health Survey for England 2003. Volume 2: Risk factors for cardiovascular disease*. London: Department of Health, 2004:143-80.
438. Statistics on Obesity, Physical Activity and Diet: England, February 2009 http://www.ic.nhs.uk/statistics-and-data-collections/health-and-lifestyles/obesity/statistics-on-obesity-physical-activity-and-diet-england-february-2009.
439. Kanagalingam MG, Forouhi NG, Greer IA, Sattar N. Changes in booking body mass index over a decade: retrospective analysis from a Glasgow Maternity Hospital. *BJOG: An International Journal of Obstetrics and Gynaecology* 2005;112(10):1431-33.
440. Heslehurst N, Ells LJ, Simpson H, Batterham A, Wilkinson J, Summerbell CD. Trends in maternal obesity incidence rates, demographic predictors, and health inequalities in 36 821 women over a 15-year period. *BJOG: An International Journal of Obstetrics & Gynaecology* 2007;114(2):187-94.
441. Cnattingius S, Bergstrom R, Lipworth L, Kramer MS. Prepregnancy Weight and the Risk of Adverse Pregnancy Outcomes. *N Engl J Med* 1998;338(3):147-52.
442. Smith GC. A population study of birthweight and the risk of caesarean section: Scotland 1980-1996. *BJOG: An International Journal of Obstetrics & Gynaecology* 2000;107(6):740-4.
443. Wischnik A, Lehmann K, Ziegler M, Georgi M, Melchert F. Does the 'fatty pelvis' exist? Quantitative computer tomography studies. *Z Geburtshilfe Perinatol* 1992;196(6):247-52.

444. Buhimschi CS, Buhimschi IA, Malinow AM, Weiner CP. Intrauterine Pressure During the Second Stage of Labor in Obese Women. *Obstet Gynecol* 2004;103(2):225-30.
445. Noble K, Zhang J, Wray S. Lipid rafts, the sarcoplasmic reticulum and uterine calcium signalling: an integrated approach. *Journal of Physiology* 2006;570(Pt 1):29-35.
446. Ramsay JE, Ferrell WR, Crawford L, Wallace AM, Greer IA, Sattar N. Maternal Obesity Is Associated with Dysregulation of Metabolic, Vascular, and Inflammatory Pathways. *J Clin Endocrinol Metab* 2002;87(9):4231-37.
447. Pinheiro J, Bates, Douglas *Mixed-Effects Models in S and S-PLUS*. 1st ed ed. New York ; London: Springer, 2000.
448. Efron BT, Robert J. *An introduction to the bootstrap*. 1 edition ed. London: Chapman & Hall/CRC, 1993.
449. Mazurkiewicz JC, Watts GF, Warburton FG, Slavin BM, Lowy C, Koukkou E. Serum lipids, lipoproteins and apolipoproteins in pregnant non-diabetic patients. *J Clin Pathol* 1994;47(8):728-31.
450. Gimpl G, Fahrenholz F. Cholesterol as stabilizer of the oxytocin receptor. *Biochimica et Biophysica Acta (BBA) - Biomembranes* 2002;1564(2):384.
451. Bastiaanse EML, Hold KM, Van der Laarse A. The effect of membrane cholesterol content on ion transport processes in plasma membranes. *Cardiovascular Research* 1997;33(2):272.
452. Sattar N, Greer IA, Louden J, Lindsay G, McConnell M, Shepherd J, et al. Lipoprotein Subfraction Changes in Normal Pregnancy: Threshold Effect of Plasma Triglyceride on Appearance of Small, Dense Low Density Lipoprotein. *J Clin Endocrinol Metab* 1997;82(8):2483-91.
453. Turi A, Kiss AL, Mullner N. ESTROGEN DOWNREGULATES THE NUMBER OF CAVEOLAE AND THE LEVEL OF CAVEOLIN IN UTERINE SMOOTH MUSCLE. *Cell Biology International* 2001;25(8):785.
454. Chiossi G, Costantine MM, Betancourt A, Hankins GDV, Longo M, Saade GR, et al. Effect of maternal body mass index on in vitro response to tocolytics in term myometrium. *American Journal of Obstetrics and Gynecology* 2010;203(3):5.
455. Stewart FM, Freeman DJ, Ramsay JE, Greer IA, Caslake M, Ferrell WR. Longitudinal Assessment of Maternal Endothelial Function and Markers of Inflammation and Placental Function throughout Pregnancy in Lean and Obese Mothers. *J Clin Endocrinol Metab* 2007;92(3):969-75.
456. Myers J, Hall C, Wareing M, Gillham J, Baker P. The effect of maternal characteristics on endothelial-dependent relaxation of myometrial arteries. *European Journal of Obstetrics & Gynecology and Reproductive Biology* 2006;124(2):158.
457. Larcombe-McDouall J, Buttell N, Harrison N, Wray S. In vivo pH and metabolite changes during a single contraction in rat uterine smooth muscle. *Journal of Physiology* 1999;518(Pt 3):783-90.
458. Quenby S, Pierce SJ, Brigham S, Wray S. Dysfunctional labor and myometrial lactic acidosis.[erratum appears in Obstet Gynecol. 2004 Jun;103(6):1344]. *Obstetrics & Gynecology* 2004;103(4):718-23.
459. Muller-Delp JM. Aging-induced adaptations of microvascular reactivity. *Microcirculation* 2006;13(4):301-14.
460. Anim-Nyame N, Gamble J, Sooranna SR, Johnson MR, Sullivan MH, Steer PJ. Evidence of impaired microvascular function in pre-eclampsia: a non-invasive study. *Clin. Sci.* 2003;104(4):405-12.
461. Edwards C, Witter FR. Preeclampsia, labor duration and mode of delivery. *International Journal of Gynecology & Obstetrics* 1997;57(1):39.

462. Griffiths AN, Hikary N, Sizer AR. Induction to delivery time interval in patients with and without preeclampsia: a retrospective analysis. *Acta Obstetricia et Gynecologica Scandinavica* 2002;81(9):867-9.
463. Blanch G, Lavender T, Walkinshaw S, Alfirevic Z. Dysfunctional labour: a randomised trial. *British Journal of Obstetrics & Gynaecology* 1998;105(1):117-20.
464. Smith GCS, Celik E, To M, Khouri O, Nicolaides KH. Cervical Length at Mid-Pregnancy and the Risk of Primary Cesarean Delivery. *The New England Journal of Medicine* 2008;358:In press.
465. Gee H, Taylor EW, Hancox R. A model for the generation of intra-uterine pressure in the human parturient uterus which demonstrates the critical role of the cervix. *Journal of Theoretical Biology* 1988;133(3):281.
466. Olah KSJ, Neilson JP. Failure to progress in the management of labour. *BJOG: An International Journal of Obstetrics and Gynaecology* 1994;101(1):1-3.
467. Moser K, Macfarlane A, Chow Y, Hilder L, Dattani N. Introducing new data on gestation-specific infant mortality among babies born in 2005 in England and Wales. *Health Stat Q.* 2007(35):13-27.
468. Kramer M, Demissie K, Yang H, Platt R, Sauve R, Liston R. The contribution of mild and moderate preterm birth to infant mortality. Fetal and Infant Health Study Group of the Canadian Perinatal Surveillance System. *JAMA.* 2000;284(7):843-9.
469. Singer L, Salvator A, Guo S, Collin M, Lilien L, Baley J. Maternal psychological distress and parenting stress after the birth of a very low-birth-weight infant. *JAMA.* 1999;281(9):799-805.
470. Taylor HG, Klein N, Minich NM, Hack M. Long-term family outcomes for children with very low birth weights. *Arch Pediatr Adolesc Med* 2001;155(2):155-61.
471. Brennan DJ, McGee SF, Rexhepaj E, O'Connor DP, Robson M, O'Herlihy C. Identification of a myometrial molecular profile for dystocic labor. *BMC Pregnancy and Childbirth* 2011;11:10.
472. King J, Flenady V. Prophylactic antibiotics for inhibiting preterm labour with intact membranes. *Cochrane Database Syst Rev* 2002;4.
473. Nielsen S, Pedersen BK. Skeletal muscle as an immunogenic organ. *Curr Opin Pharmacol* 2008;8(3):346-51.
474. White BG, Williams SJ, Highmore K, Macphee DJ. Small heat shock protein 27 (Hsp27) expression is highly induced in rat myometrium during late pregnancy and labour. *Reproduction* 2005;129(1):115-26.
475. MacIntyre DA, Tyson EK, Read M, Smith R, Yeo G, Kwek K, et al. Contraction in human myometrium is associated with changes in small heat shock proteins. *Endocrinology* 2008;149(1):245-52.
476. White BG, MacPhee DJ. Distension of the uterus induces HspB1 expression in rat uterine smooth muscle. *Am J Physiol Regul Integr Comp Physiol* 2011;301(5):7.
477. MacIntyre DA, Tyson EK, Read M, Smith R, Yeo G, Kwek K, et al. Contraction in human myometrium is associated with changes in small heat shock proteins. *Endocrinology* 2008;149(1):245-52.
478. Oldham RS, Menzies FM, Nibbs RJB, Nelson SM. Identification of a dominant T cell subpopulation in the uterine horn during postpartum remodelling. *Immunology* 2011;135:120-20.
479. Thompson PD, Buchner D, PiÃ±a IL, Balady GJ, Williams MA, Marcus BH, et al. Exercise and Physical Activity in the Prevention and Treatment of Atherosclerotic Cardiovascular Disease. *Arteriosclerosis, Thrombosis, and Vascular Biology* 2003;23(8):e42-e49.

480. Mandic S, Tymchak W, Kim D, Daub B, Quinney HA, Taylor D, et al. Effects of aerobic or aerobic and resistance training on cardiorespiratory and skeletal muscle function in heart failure: a randomized controlled pilot trial. *Clinical Rehabilitation* 2009;23(3):207-16.
481. Melzer K, Schutz Y, Boulvain M, Kayser B. Physical Activity and Pregnancy Cardiovascular Adaptations, Recommendations and Pregnancy Outcomes. *Sports Med.* 2010;40(6):493-507.
482. Jones AM, Carter H. The effect of endurance training on parameters of aerobic fitness. *Sports Med.* 2000;29(6):373-86.
483. UPBEAT. UK Pregnancies Better Eating and Activity Trial (UPBEAT), http://www.medscinet.net/upbeat/default.aspx. London, 2012.