Chapter 3

Follistatin Isoforms in the Placenta

3.1 Introduction

Whilst this thesis has only addressed the dominant forms, there have been suggestions that follistatin has potentially six different isoforms (Sugino *et al.*, 1993).

Mature core polypeptides FS315 and FS288 both have two potential N-linked glycosylation sites (Asn-Xaa-Ser/Thr) at residues 95 and 259, with findings suggesting that the Asn-259 is more prone to incorporation of carbohydrate chains than the Asn-95 (Esch *et al.*, 1987; Shimasaki *et al.*, 1988a; Shimasaki *et al.*, 1988b). Also FS315 can be proteolytically cleaved at the C-terminal end to give the FS303 isoform which will form both glycosylated and non-glycosylated variants (Esch *et al.*, 1987; Inouye *et al.*, 1991). The biological differences between these isoforms have only very recently been investigated as outlined in Chapter 1 – Section 1.6.2.

As yet none of the investigating groups have fully determined the individual actions of each of the follistatin isoforms. In fact it is difficult to determine exactly which isoform current assays are measuring. Most research groups review their own results but spend little time considering the variability between the assay they have developed and results obtained by other groups. The assays used currently are shown in Table 3-1 along with the variability of results that are obtained in similar tissues. The differences in the values clearly indicate that these assays must be measuring different isoforms and/or having variable cross-reactivity with isoforms for follistatin or follistatin-like proteins. There have also been a number of assays developed that state they measure free follistatin 288 and 315 isoforms (Wang *et al.*, 1996; McConnell *et al.*, 1998; Schneyer *et al.*, 2004a). Whilst this is potentially correct, there has not been enough comparable sampling done to determine whether each of these assays is measuring the same isoform.

The high affinity of all follistatin isoforms for activin has the potential for activin to interfere with assays. Some assays have utilized denaturing of analytes (Knight *et al.*, 1996), others have employed dissociation agents and detergents (McFarlane *et al.*, 1996; Evans *et al.*, 1998; O'Connor *et al.*, 1999) to countermand this action. Whilst some assay designs allow for the cross reactivity from the activin/follistatin complex, for example by the use of dissociation steps, these methods do not take other considerations into account. As previously discussed (Section 1.7), follistatin can also bind other members of the TGFβ family such as BMP's and myostatin (Iemura *et al.*, 1998). As such, this affinity for TGFβ members also has the potential to cross-react in follistatin assays so that levels of follistatin may be over or under assessed.

Not only is there potential for interference from the activin/follistatin complex, there is also cross reactivity between the various isoforms of follistatin, along with the more recently discovered follistatin like proteins. Some authors make it clear they understand that other isoforms can interfere with the assay for example O'Connor et al. (1999), who assume that the FS315 cross reacts with the FS288 assay (O'Connor *et al.*, 1999). In fact, given there are potentially six isoforms of follistatin, there should also be specific assays for their measurement in order to determine the different actions of the individual isoforms of follistatin.

Most assays have been developed using porcine or bovine isoforms, whilst some have been developed using a mixture of the two. The variety of isoforms being used and the species involved has also not been investigated in enough detail to validate the specificity of isoforms between species. Homology between species is high enough to make it difficult to raise antibodies against the peptide itself, therefore polyclonal antibodies are most often used. Polyclonal antibodies have a high affinity

but can recognize a mixture of epitopes and are therefore not as specific. Monoclonal antibodies are expensive to produce and are raised against only one epitope however generally have low affinity. At this stage there are no standards available for purchase so that each research group has had to develop their own set of standards creating additional difficulties when comparing results obtained by different studies. Currently, there is no particular assay being used more frequently than any others. Each of the assays is individual laboratory-based and there has been no real comparison between scientific results.

Wakatsuki *et al.* (1996), and more recently Schneyer *et al.* (2004), have developed the only specific assays for FS315 (Wakatsuki *et al.*, 1996; Schneyer *et al.*, 2004b). Most other assays are for FS288 or to measure both of the predominant isoforms.

A paper by Schneyer *et al.* (1996) showed high levels of FS315 in serum whilst FS288 was higher in follicular fluid (Schneyer *et al.*, 1996). Gilfillan and Robertson (1994) are the only group to show a follistatin variation across the menstrual cycle, all other groups show no variability in this. Most groups agree that follistatin rises steadily with increasing gestation, (Khoury *et al.*, 1995; Wakatsuki *et al.*, 1996; Evans *et al.*, 1998) although Gilfillan and Robertson (1994) show only a narrow range and no variation across gestation (Gilfillan & Robertson, 1994).

There is some debate between groups regarding how follistatin levels differ between post menopausal women, normal cycling women and men. However all groups' results are equally valid, but are probably measuring a combination of different isoforms and this also supports the theory that each isoform has its own unique set of biological actions.

Table 3-1. Follistatin Assay result variability (n/a-not applicable)

Assay	Original Assay Author	Paper utilizing assay	Pregnancy	Women	Men	Other tissues
ELISA Total Follistatin	(Evans *et al.*, 1998)	(Evans *et al.*, 1998)	1st tri 0.847 ±0.26ng/ml 3rd tri 2.80 ±093ng/ml	Cycling 0.627 ±0.196ng/ml Post-menopausal 1.42ng/ml	0.450 ±0.16ng/ml	Follicular fluid 252ng/ml Placental homogenate 3.11 ±0.4 ng/g 16wk amniotic fluid 3.63 ±1.5ng/ml Term amniotic fluid 0.890 ±0.32ng/ml
ELISA Total Follistatin	(Evans *et al.*, 1998)	(Keelan *et al.*, 1999)	n/a	n/a	n/a	109-554pg/mg across all membranes
SPICA Total follistatin	(Evans *et al.*, 1997)	(Schneider-Kolsky *et al.*, 2000)	2-8ng/ml	n/a	n/a	n/a
RIA Total Follistatin	(O'Connor *et al.*, 1999)	(O'Connor *et al.*, 1999)	7.4-72.9ng/ml	n/a	n/a	n/a
RIA Total Follistatin	(Khoury *et al.*, 1995)	(Khoury *et al.*, 1995)	4-35ng/ml range	IVF,cycling 4-35 ng/ml	n/a	Follicular fluid 274.6 ±13.4ng/ml
RIA Total Follistatin	(Khoury *et al.*, 1995)	(Woodruff *et al.*, 1997)	0-500ng/ml	n/a	n/a	n/a
RIA Total Follistatin	(Khoury *et al.*, 1995)	(McConnell *et al.*, 1998)	n/a	Cycling 10.1±1.6ng/ml Post-menopausal 10.5 ±0.6ng/ml	14.1±1.6 ng/ml	n/a
RIA Total Follistatin	(Sugawara *et al.*, 1990)	(Kettel *et al.*, 1996)	n/a	Pre,mid and post pubertal girls 4-6.0 ng/ml Cycling and post menopausal women 6.3-6.9ng/ml No changes observed with menstrual cycle	4-6.0 ng/ml	n/a
RIA Free follistatin 288 & 315	(Wang *et al.*, 1996)	(Woodruff *et al.*, 1997)	0 – 30 ng/ml	n/a	n/a	n/a
2-site chemiluminiscent assay Free follistatin 288 & 315	(McConnell *et al.*, 1998)	(McConnell *et al.*, 1998)	n/a	Cycling <1ng/ml Post menopausal <1 ng/ml	<1ng/ml	n/a
2-site immunoassay Free FS315	(Schneyer *et al.*, 2004b)	(Schneyer *et al.*, 2004b)	n/a	2-60ng/ml	2-60ng/ml	Follicular fluid Undetected

There is growing evidence that the different isoforms of follistatin have a variety of actions and responses. The strongest evidence for this comes from the different distribution of isoforms FS315 and FS288 across the tissues, compartmentalization of the isoforms and the different interactions with cellular membranes. A number of papers have alluded to differences in isoform expression affecting functions of the target tissues, however there have been no studies that have investigated the difference in isoform mRNA expression.

The difficulty in understanding follistatin *in vivo* is due to the multiplicity of its isoforms. Whilst three main protein sizes (~31, 33, and 35 kDa) have been named as FS288, FS303, FS315 respectively, and are regularly referred to in the literature, a number of other molecular weight proteins have been noted on Western Blot analysis, however none have been characterised. Table 3.2 outlines molecular weight bands previously reported for follistatin (Range 18 kDa – 78 kDa). In particular larger molecular weight proteins have been reported across a number of species; human (65 kDa and 78 kDA) and bovine (65kDa) (Michel *et al.*, 1996; Glister *et al.*, 2006).

Michel *et al.* (1996) suggest that the higher molecular weight proteins present in human cerebrospinal fluid may be due to a brain-specific posttranslational modification or an unknown molecule with a high degree of homology to follistatin, whilst Glister *et al.* (2006) suggests that the 65 kDa molecule is due to a dimerisation of follistatin molecules. In this study they were unable to determine if the 65 kDa complex remained under reducing conditions due the abolition of antibody binding under reducing conditions.

Table 3-2 Western Blot bands for follistatin as previously reported

Authors	Antibody	Tissue source	Band Size
(Sidis *et al.*, 2006)	Anti- Myc (clone 4A6) Myc tagged proteins	Human FS 288 FS 315 FS 303 FSTL-3	38-44 (quadret) 42-47 (triplet) 45-50 (doublet) 36
(McPherson *et al.*, 1999)	Monoclonal 288 17/2 (Evans *et al.*, 1998) Monoclonal 315 H10 (Knight *et al.*, 1996)	Human prostate tumor cell line PC3	31 34 35 39 42
(Michel *et al.*, 1996)	Rabbit polyclonal against human FS residues 123-134 (Saito *et al.*, 1991)	Porcine standards Porcine Aortic Endothelial cells Human CSF Porcine follicular fluid	22 31 35 39 45 33 41 20 39 45 65 78 18 28 31
(Wang *et al.*, 1996)	Monoclonal to rhFS288	Human ovarian carcinoma cell lines (PA-1)	32 35 44
(Glister *et al.*, 2006)	Monoclonal to rhFS288 Clone 1/1 and 8/1	Bovine follicular fluid	31 33 35 37 41 65

Interestingly, in the work by Glister *et al.* (2006), the relative abundance of follistatin isoforms seen in follicular fluid showed distinct shifts associated with antral follicle growth, suggesting that individual isoforms have potentially different roles in folliculogenesis (Glister *et al.*, 2006). Recent work by Schneyer *et al.* (2004b), using the newly developed FS315 assay, showed that FS315 is the major isoform for follistatin in circulation as previously suggested, due to it inability to interact with cell surfaces (Schneyer *et al.*, 2004b). Whilst within follicular fluid FS315 remained

undetectable in both normal and PCOS women. This compartmentalization of isoforms is also indicative of distinct physiological roles.

Recent work by Sidis *et al.* (2006) on activin-driven proliferation in testicular tumour (TT) cells showed that follistatin isoforms and FSTL-3 modulated differently. FS288 suppressed the proliferation of the TT cells whilst FS315 enhanced proliferation, again suggesting different biological actions *in vivo* (Sidis *et al.* , 2006).

Work in our laboratory on sheep using two different antibodies for follistatin, both JMCK20 and #204 (outlined in Chapter 2 – Antibodies), has shown that assays for each antibody show remarkably different patterns of follistatin release leading up to and following parturition, as shown in Figure 3.1. Antibody JMCK20 shows a relatively constant concentration of follistatin prior to parturition however concentrations begin dropping almost immediately at the onset of parturition, followed by a rapid increase to peak at 40 hours post delivery. Antibody #204 shows follistatin concentrations plummet in the 20 hours prior to delivery to reach nadir at 3 hours prior to birth and then begin to increase following delivery to reach a peak at 24 hours postpartum (Rae *et al.*, 2003). Again this work suggests that the two antibodies are recognizing different follistatin isoforms or perhaps an as-yet unrecognized follistatin or follistatin family member. Given the widespread nature of follistatin distribution and actions on the body, this is an area that will require extensive future research to further characterize follistatin isoforms.

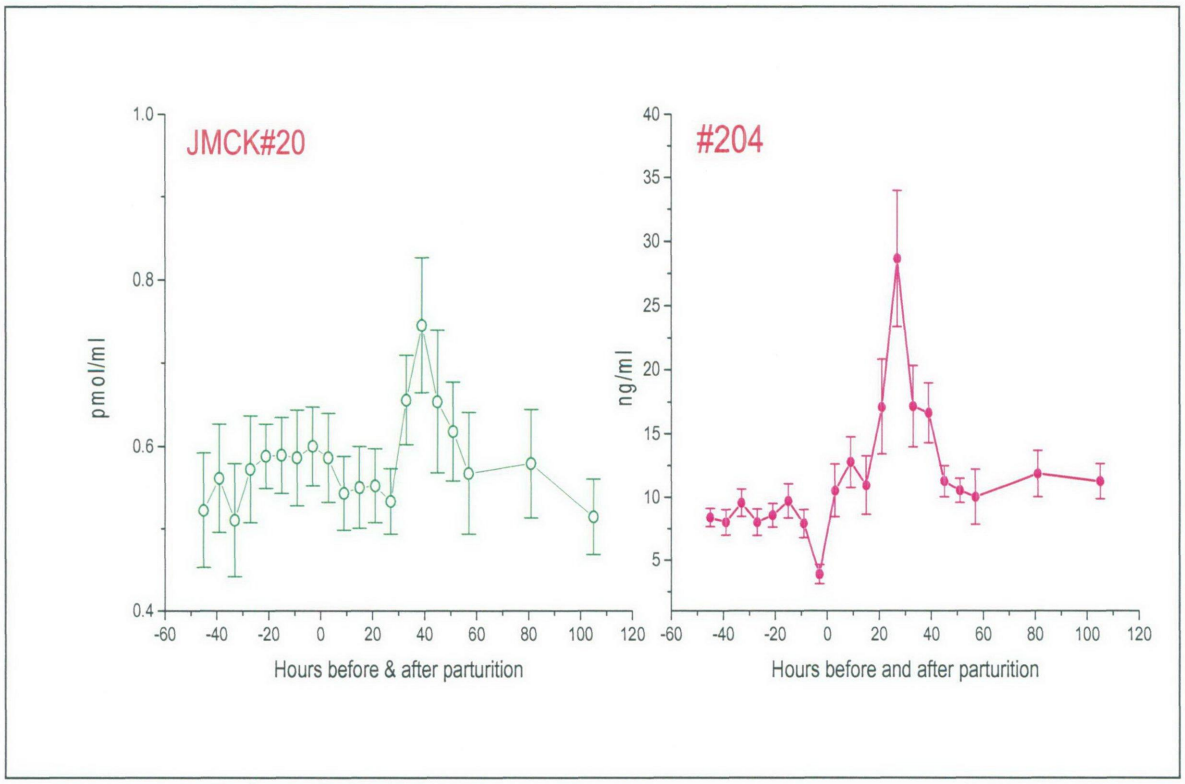


Figure 3-1 Profile of differential antibody recognition across parturition in ewes (Rae *et al.* , 2003)

With the different distribution of follistatin in tissues, this study was designed to determine if mRNA for the major follistatin isoforms (FS288 and FS315) showed differences in expression within the placenta that related to the onset of labour. Using both Con A chromatography and Heparin Affinity columns, the heparin binding and degree of glycosylated isoforms of follistatin were also assessed in these labour groups in the hope of elucidating further information of the different actions of follistatin isoforms.

3.2 Materials and Methods

3.2.1 General Methods

General sample collection and ethics approval have been described in Chapter 2. Briefly, post-delivery samples were divided according to patient groups as previously outlined (Spontaneous onset vaginal delivery, Induced onset vaginal delivery and Non-labouring LSCS delivery). For RT-PCR studies (Experiment 3.1), cotyledon tissue from the placenta was dissected into 100 mg portions immediately following delivery and placed into RNA*later* (Ambion). A total of 5 placentae were used in each group.

In Experiment 3.2 and 3.3 placentae was homogenized at a ratio of 1:4 with homogenizing buffer and stored in 1 ml aliquots at 4°C for further analysis. For chromatographic studies a pool of 10 placenta samples was used for each patient group (10x 1ml aliquots). Individual pools were made up for Con-A chromatography and Heparin Affinity Chromatography. Following chromatography studies, resulting pools were concentrated using a an Amicon 50ml stirred cell concentrator (Amicon Corporaton, Danvers, MA, USA) and samples were analysed using Western Blot procedures as outlined in Section 3.2.3. However all samples needed amplification of signal and were further concentrated using activated CNBr Sepharose beads outlined below in this section.

Complete laboratory procedures are given in Appendix 1 – Specific Labarotory Procedures, however brief methods for the individual procedures are outlined below.

SDS-PAGE and Western Blot

Equivalent placental protein concentrations were added to sample buffer (0.125M Tris-HCl, 0.07% SDS, 0.6% Glycerol, 10% β-mercaptoethanol, Bromophenol Blue) to a dilution of 1:2 (ie – 50% sample and 50% sample buffer). All samples were heated

to 95°C for 5 minutes and then fractionated on 4.06% stacking and 12.5% separating SDS-PAGE gels (Mighty Small Tall System, Hoefer, San Francisco, CA, USA) run at 15 mA/gel. Non-transferred gels were stained in Roeder's stain (0.25mg/ml Coomassie R-250, 25% Isopropanol, 10% Acetic Acid). Proteins were then electro-transferred onto nitrocellulose membrane for 1 hour at 40 V using 298 mA and transfer buffer (25mM Tris, 200mM glycine, 15% methanol, pH 8.2-8.4). Post-transfer gels were stained in Roeder's stain to check transfer efficiency. Transfer quality was also checked by Ponceau S (0.2% Ponceau, 3% Trichlooacetic acid, 3% sulfosalicyclic acid) staining of membranes. After blotting, membranes were blocked for 1 hour in 10% milk in PBS (0.05M Phosphate, 0.15M NaCl, pH 7.4). Membranes were then incubated overnight on a rocker in PBS containing 1% milk in PBS with JMCK20 antibody (1/6,250). Membranes were washed 5 times for 5 minutes each in Tris wash buffer (20mM Tris-base, 0.15M NaCl, 0.1%Triton-X 100, pH 8.2-8.4), and then incubated for 3 hours on rocker with anti-chicken biotin secondary antibody in ELISA buffer containing 1% milk (1/10,000). After washing again in Tris wash buffer membrane was incubated with strepavidin alkaline phosphatase in ELISA buffer (1/10,000) for 1 hour. Visualisation of bands on membrane occurred using the addition of BCIP/NBT (Pierce Chemical Co.).

For negative controls- SDS electrophoresis gels were run in duplicate. The control gel is transferred at the same time as positive gel. Both membranes were blocked for 1 hour in 10% milk in PBS (0.05M Phosphate, 0.15M NaCl, pH 7.4). Negative control membranes were then incubated overnight on a rocker in PBS containing 1% milk in PBS without JMCK20 antibody. Both membranes were washed 5 times for 5 minutes each in Tris wash buffer (20mM Tris-base, 0.15M NaCl, 0.1%Triton-X 100, pH 8.2-8.4), and then incubated for 3 hours on rocker with anti-chicken biotin

secondary antibody in ELISA buffer containing 1% milk (1/10,000). After washing again in Tris wash buffer membranes were incubated with strepavidin alkaline phosphatase in ELISA buffer (1/10,000) for 1 hour. Visualisation of bands on membrane occurred using the addition of BCIP/NBT (Pierce Chemical Co.) for the equivalent length of time for both positive and negative membranes

Sepharose Coupling

1g of CNBr Sepharose 4B was mixed and centrifuged at 3000rpm with 50 ml of 0.1M HCl solution four times with decanting in between washes. The resulting 3.5 ml of gel was washed in 5 ml of coupling buffer (0.1M $NaHCO_3$, 0.5M NaCl, pH 8.3) per gram of dry gel. Antibody/coupling buffer was added to the gel, in this case using JMCK20 antibody, at 1/330 diluted with coupling buffer. Gel was blocked in blocking buffer (1M ethanolamine or 0.2M glycine pH 8.0) for 2 hours whilst mixing. Antibody/coupling buffer and gel were mixed overnight at 4°C on a rocker. Gel was centrifuged with supernatant removed and then washed in alternating coupling buffer followed by acetate buffer (0.1M acetate, 0.5M NaCl, pH 4.0) a total of four times. Sample was incubated with resultant gel (1/20) and mixed overnight on a rocker. A further four washes with PBS with centrifuging and removal of supernatant in between prepared the gel.

3.2.2 Experiment 1 – Identification of Follistatin Isoforms using Reverse Transcription-Polymerase Chain Reaction

This study aims to determine expression for follistatin isoforms FS288 and FS315 within placental tissues using Reverse Transcription Polymerase Chain Reaction (RT-PCR). Primers have been designed so that mRNA expression for the two major follistatin isoforms can be compared. Placental tissues have been obtained from 5

women from each of the three differing labour groups (Spontaneous, Induced and LSCS) so that isoform differences associated with labour onset can be determined.

RNA Extraction

Total RNA's were isolated using Trizol reagents (Life Technologies, Inc., Gaithersburg, MD) according to the manufacturer's instructions. Homogenized samples were incubated for 5 minutes at room temperature and then 0.25 ml of Phenol:Chloroform:Isoamyl Alcohol (25:24:1) (Life Technologies, Inc., Gaithersburg, MD) was added. Samples were gently shaken and incubated at room temperature for 2-3 minutes prior to centrifuging 13,000rpm for 10 minutes at 4°C. The aqueous phase was collected and an equal volume of chloroform (Amresco, Solon, OH) was added. The mixture was vortexed and centrifuged again at 4°C with the aqueous phase again being collected. 0.5 ml isopropyl alcohol (BDH) was added and samples incubated for 20 minutes at room temperature. Samples were then centrifuged at 12,000 rpm for 30 minutes at 4°C and the resulting pellet was retained and rinsed in 0.5 ml 75% (v/v) ice cold ethanol. Samples were centrifuged again (12,000 for 15 minutes at 4°C) and air dried. The pellet was then re-suspended in 50 µL of DEPC treated water with 0.5 µL of RNA inhibitor (Fisher Biotech) added to suppress the actions of any RNase contaminants. Concentrations of RNA were determined using the Beckman DU 600 spectrophotometer (Beckman Instruments, Jan Ramon, CA). All RNA products were checked using RNA gel analysis to determine the product quality and to confirm concentration. Additionally, all RNA products were checked by the PCR products of amplification of the housekeeping gene β-actin primers.

DNase Digestion of RNA

In order to eliminate any genomic DNA from the newly isolated RNA, 15 µg of RNA was incubated at 37°C with 5 µL of DNase I (RQ-DNase, Promega Corp., Madison, WI) and appropriate concentrations of buffer for 40 minutes, then at 70°C for 10 minutes to inactivate DNase I and then transferred to a freezer for later use.

Reverse Transcription

Extracted RNA was placed into RNA free tubes and reverse transcribed by incubating at 42°C for 45 minutes. This was then prepared using a final cDNA volume of 40 µL using 5 µg RNA of total RNA and 50 pmoles of oligo-dT (Promega), denatured in a solution of reverse transcriptase buffer (Fisher Biotech), 25 mM of each deoxyribonucleotide trisphosphate, 40 units of RNase Inhibitor (Promega), and 1 µL of reverse transcriptase (Fisher Biotech). The reaction was terminated by heating to 70°C for 10 minutes followed by cooling to 4°C. The quality of the cDNA was determined by the amplification of the PCR products for the housekeeper gene β-actin primers.

Primer Design

Primers were designed around the two DNA sequences for follistatin. The first HUMFOLLI1 (Accession No. M19480) gives the primary structure for follistatin gene exon 1-5 as outlined by Shimasaki *et al.* (Shimasaki *et al.*, 1988a; Shimasaki *et al.*, 1988b). The second sequence HUMFOLLI2 (Accession No. M19481) gives the sequence for human follistatin exon 6 (Shimasaki *et al.*, 1988a). The genetic sequence is shown in Figure 3.3 with blue shaded areas being the introns and the red typed sequence being specific to FS288.

Primers were designed to pick up each of the isoforms 288 and 315 as shown in Figure 3.2. Primers are also shown as the underlined regions on the genetic sequence in Figure 3.3. All primers were designed using Primer 3 and ANGIS software to ensure primers were appropriate and minimize false positive results. In order to minimize replication of genomic DNA primer pairs were designed to span a large intron of the gene. Primers were designed to have melting points of >60°C to promote highly specific annealing of primers to RNA. Primer sequences, melting points and expected product sizes are shown in Table 3.3.

Table 3-3 Primer sequences used for RT-PCR

Kym's Reference	Sequence 5'-3'	Diagram reference in Figure 3-2		Primer length	Product length	Melt point
β- actin	ctggaacggtgaaggtgaca		Forward	20	150	66.21
β- actin	aagggacttcctgtaacaatgca		Reverse	23	150	65.48
FS315 (1)	gggatttcaaggttgggaga	a	Forward	20	198	64.94
FS315 (1)	ttcctcctcttcctcggtgt	b	Reverse	20	198	65.01
FS288 (1)	gagtgtgccatgaaggaagc	c	Forward	20	300	64.78
FS288 1c	tggctagatgggggaataca	d	Reverse	20	300	64.05

PCR

PCR cycle conditions and concentrations of reagents are listed in Tables 3.4 and 3.5. All primers and patient samples were run under equivalent conditions. Each patient sample was run on a minimum of three occasions, using β-actin as a positive control and replacement of primers with water in negative controls.

Table 3-4 Reagent conditions for RT-PCR

Reagent	**Volume added**
cDNA	5 µl
DNA Polymerase Buffer	5 µl
dNTP	2 µl
MgCl2	2 µl
Forward primer	1 µl
Reverse primer	1 µl
DNA polymerase	1 µl
DEPC treated RNA free H2O	33 µl
Final reaction volume	**50 µl**

Table 3-5 RT-PCR Cycle conditions

Stage	**Temperature**	**Length**	**Cycle number**
Denature	94°C	60 seconds	1 cycle
Denature	94°C	40 seconds	40 cycles
Anneal	60°C	45	
Elongate	72°C	40 seconds	
Denature	94°C	40 seconds	1 cycle
Final Elongate	72°C	10 minutes	1 cycle

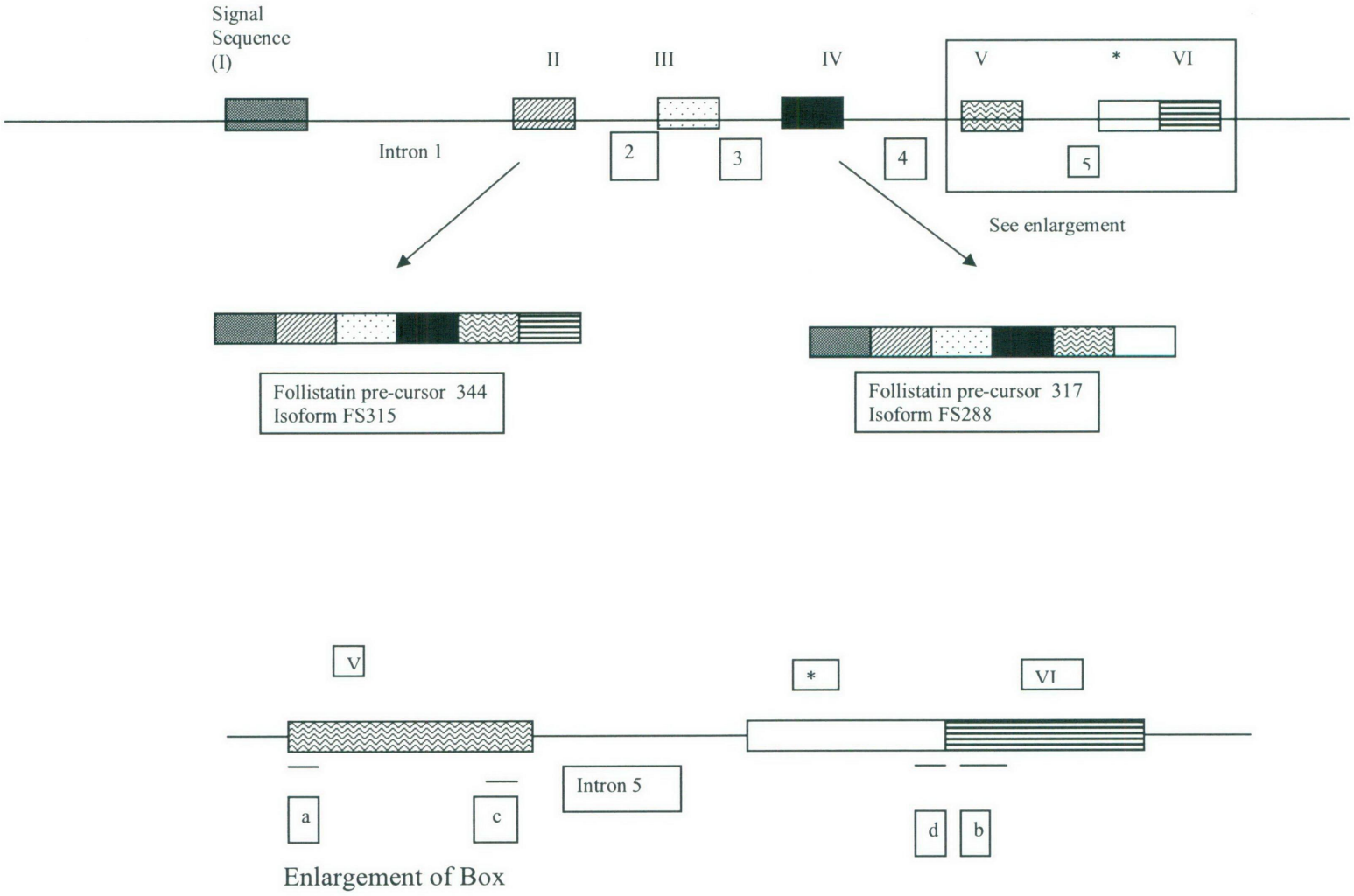


Figure 3-2 – Arrangements of introns, exons and primers for RT- PCR

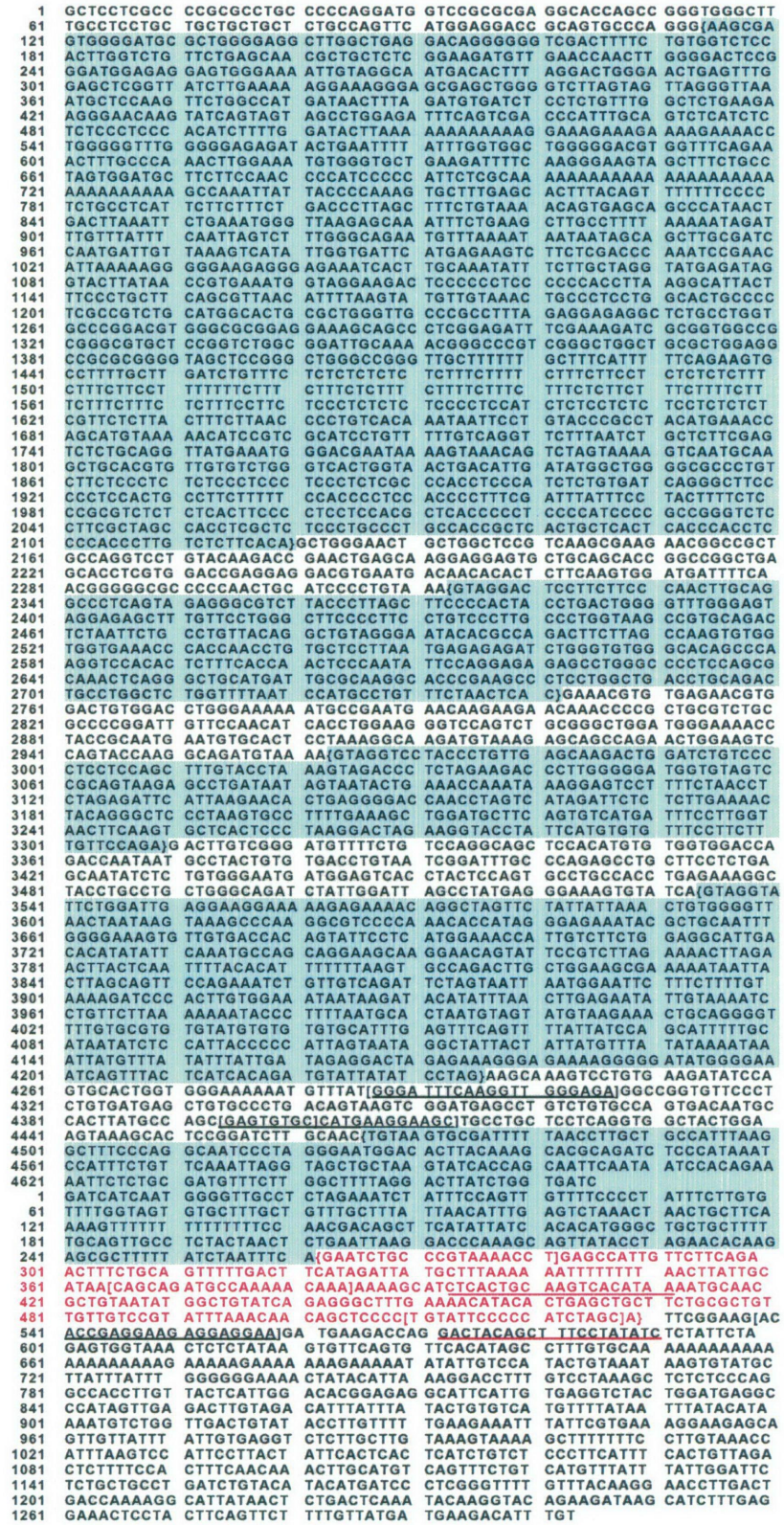


Figure 3.3 Gene Sequence for Follistatin (Accession Numbers M19480 and M19481)

Identification of PCR products

The amplified products were subjected to electrophoresis on 3% agarose (Gibco BRL) gels stained with 0.5 µg/ml ethidium bromide. 10 µL of each PCR product with 2 µl bromophenol blue loading buffer was analysed in parallel with 100 bp DNA ladder (Promega) as a standard. Electrophoresis was run at 120V for ~ 1.5 hours. After gel electrophoresis was completed, gels were visualized under ultraviolet light and photographed with a computer linked Polaroid Camera.

3.2.3 Experiment 2 – Characterisation of Heparin Binding Follistatin Isoform Differences between Labouring Groups

Heparin Affinity Chromatography

This study was designed to assess the heparin binding of the follistatin isoforms and determine differences, if any, between the three patient groups. Briefly, the heparin affinity column was washed with 20 ml of 0.1% Triton-X solution followed by 50 ml starting buffer. 10 ml of pooled placenta was run through a heparin column which was then rinsed with starting buffer until no further protein was detected by Bradford's reagent in the fractions. These fractions were then pooled and known as the Heparin Unbound. The column was then rinsed with elution buffer until the resulting fractions were negative for protein using Bradford's reagent. These fractions were then combined and known as the Heparin Bound pool. The column was rinsed with ~10 ml of starting buffer and the procedure was repeated three times with each patient pool to ensure all possible binding proteins were separated.

Following collection individual pools were concentrated and analysed originally using Western Blotting, however signals were too weak to elucidate differences between

patient groups. Then using CNBr Sepharose beads activated with JMCK20 antibody, pools were coupled to the beads and the resulting immunoprecipitated sample was analysed via Western Blotting.

3.2.4 Experiment 3 - Characterisation of Glycosylation of Follistatin Isoform Differences between Labouring Groups

ConA Chromatography

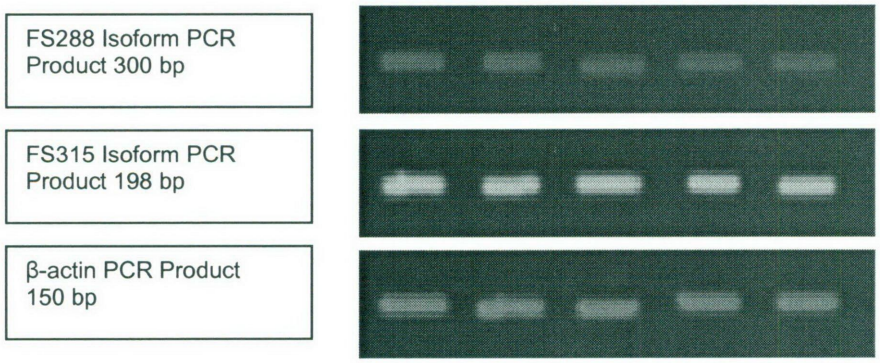
This study was designed to determine differences, if any, between the glycosylated forms of follistatin isoforms and how they relate to the onset of labour. Briefly, Con A column was washed with 5 ml 1.0M glucose solution followed by 15 ml of starting buffer. 10 ml of pooled placenta was run through a Con A chromatography column which was then rinsed with starting buffer until no further protein was detected by Bradford's reagent in the fractions. These fractions were then pooled and called the Con A Unbound. Increasing concentrations of glucose solutions (0.2M – 1.0M) were used to elute the Con A bound protein and again the column was rinsed with the solution until Bradford's reagent test for protein was negative. These fractions were then pooled and called the Con A Bound. The column was rinsed with ~10 ml starting buffer and procedure was repeated three times with each patient pool to ensure all possible binding proteins were separated.

Following collection, individual pools were concentrated and analysed originally using Western Blotting, however signals were too weak to elucidate differences between patient groups. Then using CNBr Sepharose beads activated with JMCK20 antibody, pools were coupled to the beads and the resulting immunoprecipitated sample was analysed with Western Blotting.

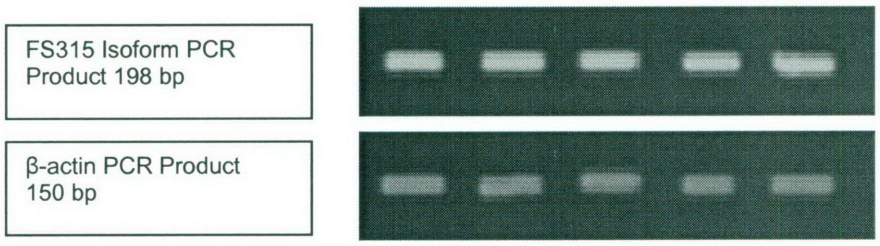
3.3 Results

3.3.1 Experiment 1 – Identification of Follistatin Isoforms using RT-PCR

SPONTANEOUS



INDUCED



LSCS

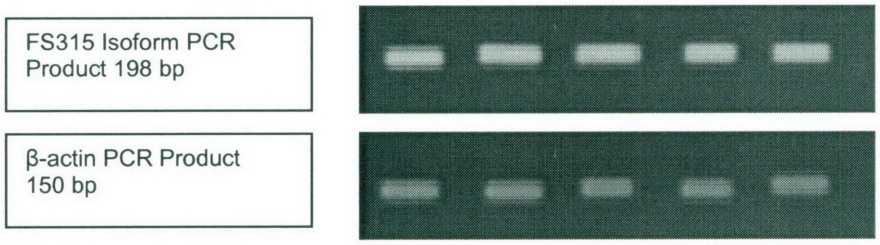


Figure 3-4 RT-PCR Products for β-actin, FS288 and FS315 isoform.

As shown in Figure 3.4 all patient groups show PCR products for β-actin and FS315, however only the spontaneous onset patients show the PCR product for FS288. Interestingly, it appears that more FS315 mRNA is present when compared to FS288 as bands are much more intense for the FS315 PCR product.

3.3.2 Experiment 2 – Characterisation of Heparin Binding Follistatin Isoform Differences between Labouring Groups

Initial studies, using a reduced transfer time in the Western blotting, confirmed the presence of the well recognized 31kDa band for follistatin (Figure 3.5) that is widely referred to in the literature and outlined in Table 3.2. The 29kDa band present only in the spontaneous patient group is possibly a degradation product of FS288. However Roeder's staining of post-transferred electrophoresis gels revealed many larger molecular weight proteins still un-transferred, so that for all further studies transfer time was extended to ensure the full range of protein was available to analyse, however lower molecular weight bands are extremely faint and very difficult to see on the following images, although clear to the naked eye. The lower molecular bands seen are reported in the subsequent tables rather than on the images.

Proteins that have been separated using heparan affinity chromatography and recognized by the JMCK20 antibody have been visualised using Western Blot procedures. Figure 3.6 shows the original placental pools for patient groups, Spontaneous Onset (SPON), Induced Onset (IND) and patients that have had no onset of labour but have had a Lower Segment Caesarean Section (LSCS). Figure 3.7 and Figure 3.8 show protein bands that have been bound and remained unbound to the heparin affinity column. Both the similarities and the differences between protein bands in each group have been identified and tabulated in Table 3.6.

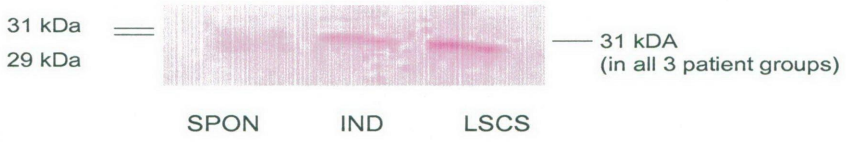


Figure 3-5 Original placental pools with reduced transfer time using JMCK20 antibody

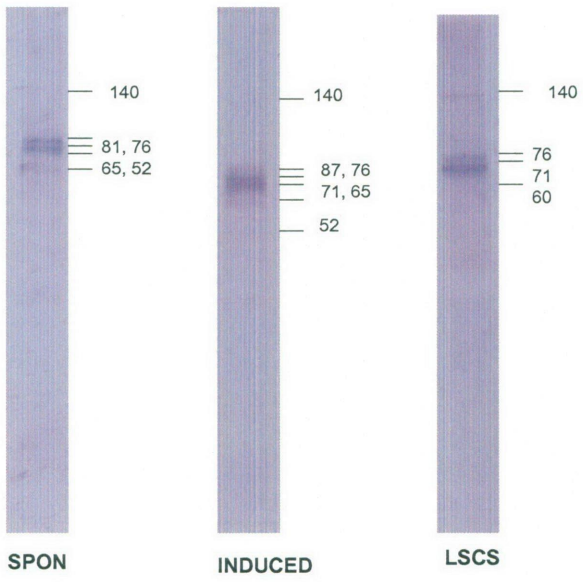


Figure 3-6 Original Starting pools bound to JMCK20 sepharose and analysed via Western Blot using JMCK20 antibody

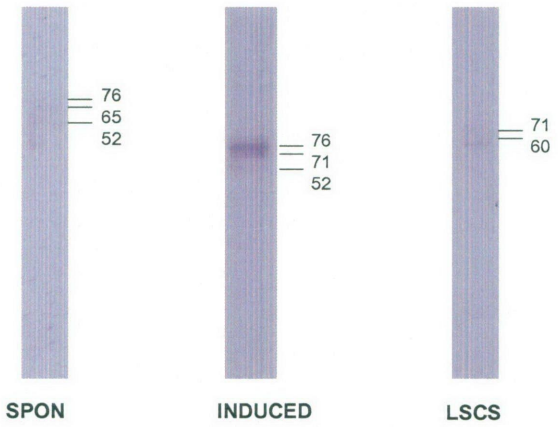


Figure 3-7 Heparin unbound sample bound to JMCK20 sepharose and analysed via Western Blot using JMCK20 antibody

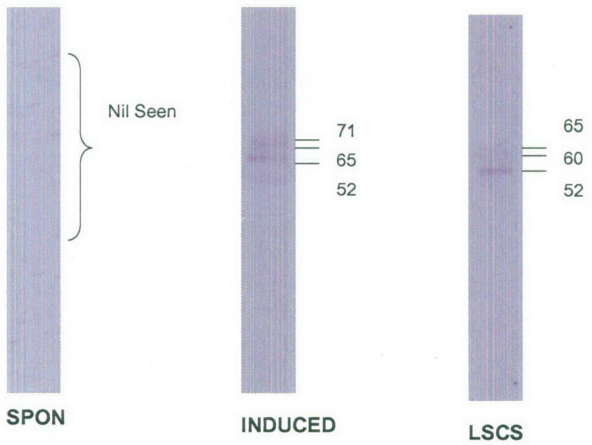


Figure 3-8 Heparin Bound sample bound to JMCK20 sepharose and analysed via Western Blot using JMCK20 antibody

Table 3-6 Similarities and differences of original pool, heparin unbound and heparin bound between patient groups

SAMPLE	SPONTANEOUS ONSET	INDUCED ONSET	NIL ONSET (LSCS)
Starting Pool CK20 Bound	140 81 76 65 52 31	140 87 76 71 65 52 31	140 76 71 60 31
Heparin Unbound CK20 Bound	76 65 52	76 71 52	 71 60
Heparin Bound CK20 Bound	Nil Seen	71 65 52	 65 60 52

3.3.3 Experiment 3 - Characterisation of Glycosylation of Follistatin Isoform Differences between Labouring Groups

Proteins that have been separated using Con A chromatography and recognized by the JMCK20 antibody have been visualised using Western Blot procedures. Figure 3-9 and Figure 3-10 show protein bands that have been bound and remained unbound to the Con A column. Both the similarities and the differences between protein bands in each group have been identified and tabulated in Table 3-7.

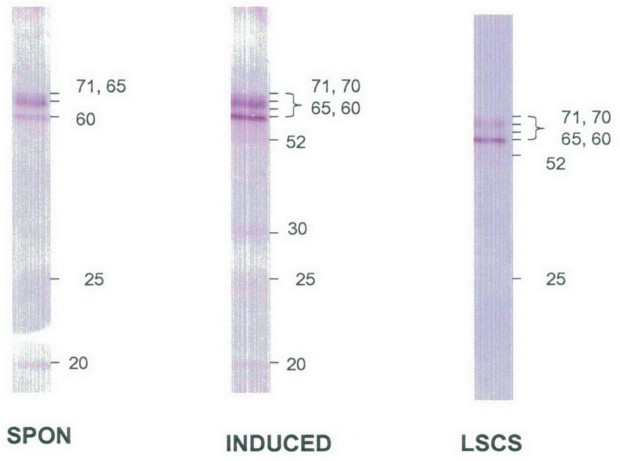


Figure 3-9 Con A bound follistatin isoforms as recognized using JMCK20 sepharose and analysed via Western lot using JMCK20 antibody

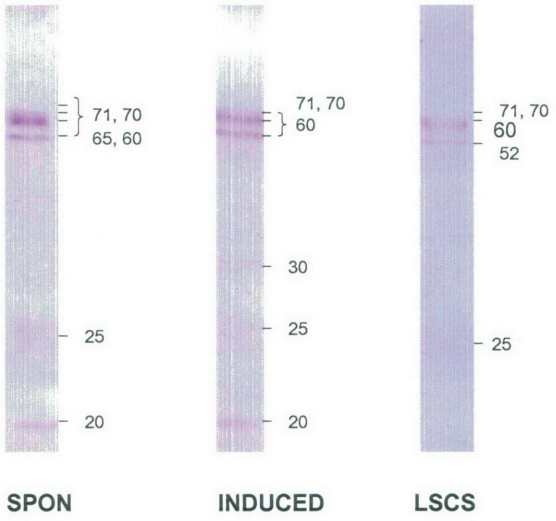


Figure 3-10 Con A Unbound follistatin isoforms as recognized using JMCK20 sepharose and analysed via Western Blot using JMCK20 antibody

Table 3-7 Similarities and differences of ConA Unbound and Con A Bound between patient groups

Sample	SPONTANEOUS ONSET	INDUCED ONSET	NIL ONSET (LSCS)
Original Starting Pool JMCK20 Bound	140 81 76 65 52 31	140 87 76 71 65 52 31	140 76 71 60 31
Con A Bound JMCK20 Bound	71 65 60 25 20	71 70 65 60 52 30 25 20	71 70 65 60 52 25
Con A Unbound JMCK20 Bound	71 70 65 60 41 25 20	71 70 60 30 25 20	71 70 60 52 25

3.4 Discussion

This study has aimed to investigate the role that isoforms, both heparin binding and glycosylation variants, and potentially novel follistatin proteins have in the placenta of spontaneous onset of labour, and determine if there is a different pattern of expression in patients undergoing inductions and those who have elective caesarean deliveries.

mRNA studies indicate that mRNA for both FS288 and FS315 was present in the placenta of those patients presenting with a natural and spontaneous onset of labour, whilst patients undergoing an induction and those having a planned caesarean delivery seem to only have mRNA for FS315. Interestingly, all patient groups seemed to show increased amounts of FS315 mRNA when a visual comparison of FS315 to the FS288 is done. Many previous studies have suggested that FS315 is the predominant form of follistatin in circulation and it appears to be true for the mRNA of the placenta as well. However with only small patient numbers in the mRNA studies these results if viewed alone must be taken with some caution.

As discussed in Section 1.6.2, all evidence to date suggests that FS315 is the activin binding protein in circulation, whilst FS288 acts to sequester activin to the cell surface and induce degradation via the early endosomes before activin initiates Smad pathways of signal transduction. This suggests that the natural labouring process requires removal of free activin via binding to FS288 from the placenta maternal/fetal interfaces. All patients have increased amounts of FS315 mRNA compared to FS288 suggesting that FS315 is also increased in circulation however its role still remains unclear.

Using Western Blotting JMCK20 antibody detected bands as outlined in Tables 3-6 and 3-7 along with Figures 3.5-3.10. In initial studies using a transfer time of 40

minutes (Figure 3-5) JMCK20 detected bands at 29 kDa and 31 kDA for spontaneous patients and at 31 kDa only for induced and LSCS patients. The band present at molecular weight 29 kDa is probably the FS288 isoform whilst 31 kDa is the FS315 isoform as reported previously (Michel *et al.*, 1996; Wang *et al.*, 1996; McPherson *et al.*, 1999; Glister *et al.*, 2006). The Western Blotting results confirm those results obtained using RT-PCR where mRNA for both isoforms is seen in spontaneous patients, but FS315 alone in induced and LSCS patients.

It seems clear that the antibody used in this study (JMCK20) recognized molecular weight proteins similar to those reported by other groups (Table 3-2) with a range of weights from 20 kDa through to 87 kDa. Interestingly, it appears that the higher molecular weight proteins recognized by the antibody are the predominant forms recognized by this follistatin antibody in the placenta. However as outlined in the introduction to this chapter, none of these higher molecular weight proteins have been characterised with speculation that these may be a novel follistatin-like molecule.

As seen in Figure 3-6, original starting pools of placenta, using an extended transfer time (60 minutes) the three patient groups showed some differences in the bands detected by the JMCK20 antibody prior to having been separated by heparin affinity and immunoprecipitated with JMCK20. Interestingly, the bands seen are at much higher molecular weights than anticipated. The band present at 140 kDa in all patient groups is the antibody complex. Whilst bands of 65 kDa and 78 kDa have been previously reported, it was surprising that the intensity of staining indicated that these bands were the predominant forms of follistatin or follistatin-like proteins. The 76 kDa band seen in all three patient groups is probably the 78 kDa band reported by Michel *et al.* (1996), in human cerebrospinal fluid, whilst the 81 kDa in spontaneous,

the 87 kDa in induced and the 71 kDa bands seen in induced and LSCS patients are probably varying glycosylated and un-glycosylated forms of this band. Spontaneous and induced patients also show a 65 kDa band that corresponds to the band reported by Michel *et al.* (1996) in humans and Glister *et al.* (2006) in bovine follicular fluid. Interestingly, both spontaneous and induced onset patients show a 52 kDa band, that are not seen in caesarean patients although they contain a 60 kDa band that is possibly a glycosylated version of this.

Although these larger molecular weight proteins have not been characterised, the binding of the JMCK20 antibody does give us some insights into their character. JMCK20 antibody has been designed around sequence 121-133 of the first follistatin domain. The study by Michel *et al.* (1996) that also recognises larger molecular weight proteins, utilized a different antibody from the one used here. However their rabbit polyclonal antibody was designed around a similar peptide sequence, amino acids 123-134, which suggests that both antibodies are recognizing the same proteins. This region of follistatin domain one forms part of site 1 for activin binding and contains a number of hydrophobic residues that form a concave shape allowing the convex of the activin molecule to bind within this area (Thompson *et al.*, 2005). As these larger molecular weight proteins bind within this region, it would appear that these new proteins also contain a similar region or similar follistatin domain, thus suggesting firstly that they have a follistatin domain and secondly that they too may have the ability to bind activin.

When we review heparin binding characteristics it seems clear that spontaneous labour patients show little in the way of larger molecular weight proteins that bind to heparan whilst both induced and caesarean section patients contain large levels of both heparin binding and non-heparin binding proteins. Both the 71 kDa and the 76

kDa have little interaction with heparin, with either the 3-dimensional arrangement of the molecule interfering with the heparin binding sequence or this heparin binding sequence is missing altogether similar to FSTL-3 (Sidis *et al.*, 2006). Despite containing follistatin domain 1, FSTL-3 does not have the HBS in this domain and therefore cannot interact with cell surface (Sidis *et al.*, 2006).

Onset of labour appears to effect the types of heparin binding proteins present with clear similarities and differences noted between patients as shown in Table 3-6. However all comparisons are between the larger molecular weight proteins of follistatin that are yet to be completely characterised. Spontaneous and induced patients both exhibit 52 kDa and 76 kDa bands that are not binding to heparin whilst induced and caesarean section patients both contain a non-heparin binding protein of 71 kDa as seen in the original starting pool. Interestingly, although caesarean patients have the 76 kDa protein present in their starting profile it is not present in either the heparin bound or non-heparin bound samples and we can only assume it has been lost in the chromatography process. Other bands of interest are the 60 kDa seen in caesarean patients. This band is not present in either of the other two patient groups and we theorise that this follistatin-like protein is somehow degraded, possibly through endosomal endocytosis in the labouring process.

When heparin bound proteins are reviewed, we see no heparin binding proteins present in the spontaneous labour patients, whilst induced and LSCS patients both contain a few bands ranging from 52 kDa and 71 kDa. The induced patients contain the 65 kDa protein whilst the LSCS patients contain the 60 kDa bands. In the induced patient group we see both the 52 kDa and the 71 kDa bands present in both heparin binding and non-binding fractions. We presume this is due to the binding capacity of the heparin affinity column being exceeded, the possibility that these

proteins can only weakly interact with heparin and due to the intensity of bands would surmise that 52 kDa is heparin unbound and 71kDa is heparin bound.

It is extremely difficult to formulate reasons why we see the majority of these differences, as the data obtained clearly relates to a much larger follistatin protein than has been studied in the past, and all of this protein's actions are unknown to us at this stage. It is also important to keep in mind the pitfalls of this experiment, namely that while every effort has been made to keep protein concentrations loaded into the gel equivalent, there are some minor discrepancies. Possibly these differences are why some bands are not obvious when we compare groups, particularly in the lower molecular weight proteins, however this is not a quantitative study and our object was merely to record the presence or absence of bands rather than quantify them.

As described, follistatin has two potential N-linked glycosylation sites (Asn-Xaa-Ser/Thr) at residues 95 and 259 in the mature core follistatins (Esch *et al.*, 1987; Shimasaki *et al.*, 1988a; Shimasaki *et al.*, 1988b). N-linked glycosylation is a co-translational process occurring in the endoplasmic reticulum. It involves the transfer of a pre-cursor oligosaccharide $GlcNAc_2Man_9Glc_3$ to the asparagine residues in the protein chain and this is known to influence protein folding. A recent study on the role of glycosylated proteins and their relationship to function suggested that N-linked glycosylated proteins are almost exclusively involved in transport and binding functions (Gupta & Brunak, 2002). All information to date regarding follistatin suggest that FS315 is the isoform involved in transport, probably within the circulation whilst FS288 is involved in binding to the cell surface and acting there.

This study aimed to elucidate if glycosylation variants of follistatin have roles that can be differentiated due to the onset of labour. Table 3.7 shows the similarities and

differences between the three labouring groups. As seen in the heparan affinity studies, the non-heparan binding 70 kDa /71 kDa band is again present, however it is present in all three patient groups and appears on both glycosylated and non-glycosylated fractions, with no real differences between patient groups. We would presume that the binding capacity of the column has been exceeded however the differences between fractions are so minor it is difficult to determine the predominant band.

The 65 kDa band observed in the starting pools (Figure 3.6) is again only seen in spontaneous labour and induced labour groups and shows strong binding to Con A (Figure 3.9) suggesting it is a glycosylated protein. However we see this band appear in the Con A bound fractions of LSCS patients. All three patient groups exhibit a 60 kDa protein that is shown in both Con A bound and unbound fractions, however it appears slightly more intensely stained in the Con A bound suggesting it is a glycosylated protein and that the column binding capacity has been saturated with either these follistatin-like proteins or other glycosylated proteins.

Interestingly, the only patient group that shows the traditional lower molecular weight follistatin 31 kDa is the induced labour group. What is most surprising is that this is most likely to be the FS288 isoform and it seems more unusual as the induced patient group does not show the mRNA for FS288 suggesting that there is a source other than the placenta for this band in this patient group. The presence of this band is seen even after extended transfer times, suggesting that concentrations of this protein are very high as extended transfer time results in the protein passing through the membranes. It is also interesting that it shows in both glycosylated and non-glycosylated fractions suggesting it has exceeded column capacity.

All three patient groups show a band at 25 kDa, both glycosylated and non-glycosylated variants and both spontaneous and induced labour groups also show a lower molecular weight band of 20 kDa. Whilst proteins similar to this lower molecular weight have been previously reported by Michel *et al.*, (1996) with 20 kDa seen in human CSF, 22 kDa in porcine standards and 18 kDa in porcine follicular fluid (Michel *et al.*, 1996), they are uncharacterized and may be potentially new follistatin-like proteins, follistatin family members or the degradation products of some of the larger follistatin proteins.

Chapter 4

Follistatin in Pregnancy and Parturition

4.1 Follistatin and other proteins in Pregnancy

In pregnancy, shortly after conception, the maternal system alters its natural basal endocrine concentrations across many systems to facilitate the growth and development of the fetus. These adaptations are both extreme and remarkable, Figure 4.1 shows the process of establishment of pregnancy.

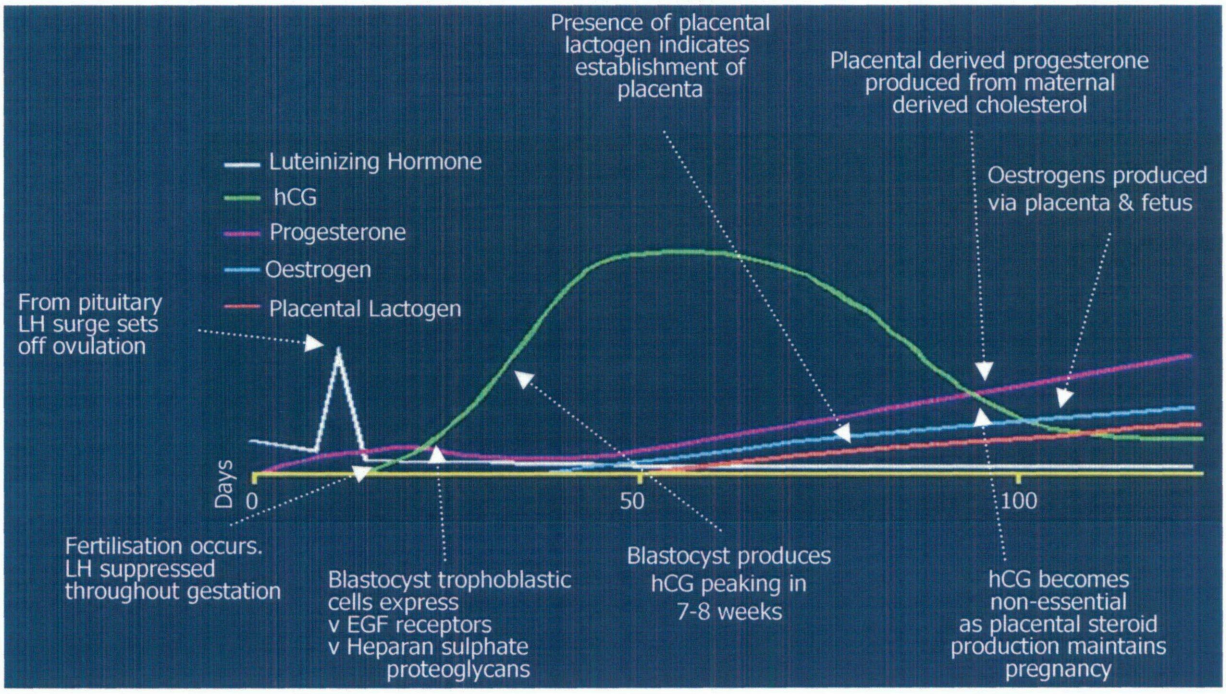


Figure 4-1 Establishment of pregnancy

Following fertilisation, the formation of the placenta that will support the developing fetus is reliant on the blastocyst attaching and implanting to the uterine wall. The first attachment phase involves adherence of the trophoblast cells of the blastocyst to the luminal epithelial cells of the endometrium, known as the decidualisation reaction. The stromal epithelial cells on the uterine wall immediately below the blastocyst begin

expressing heparin binding EGF-like growth factor (HB-EGF) (Isaacs & Murphy, 2002; Chobotova *et al.*, 2005). The trophoblast cells on the outer surface of the blastocyst in contact with the endometrium express both EGF and heparan sulfate proteoglycans (Carson *et al.*, 1998; Paria *et al.*, 1999). The presence of these receptors provides double binding for the heparin binding EGF-like growth factor which then allows for invasive development of the placenta. Members of the transforming growth factor β superfamily have been implicated in the implantation process. Angiogenesis, cell differentiation and remodelling are important parts of the implantation process and are roles in which activin has been implicated. Activin has been shown to promote decidualisation with stromal cells producing both activin receptor and activin βA subunits in high concentrations with its onset (Jones *et al.*, 2002a).

One of the markers for decidualisation is the production of prolactin (PRL). Prolactin is generally released via the pituitary, however it has been established by a number of groups that PRL is also synthesized and released by decidualised endometrial stromal cells (Golander *et al.*, 1988; Thrailkill *et al.*, 1989; Gellersen *et al.*, 1994; Frank *et al.*, 1995). The stimulus for this release is still unclear with suggestions including interleukin 1β (Frank *et al.*, 1995), insulin (Thrailkill *et al.*, 1989), lymphocytes (Gellersen *et al.*, 1994) and decidual prolactin releasing factor (Golander *et al.*, 1988; Thrailkill *et al.*, 1989; Frank *et al.*, 1995). It has also been postulated that progesterone receptors regulate the expression and synthesis of prolactin in the decidua (Wang *et al.*, 1994; Brosens *et al.*, 1999). Progesterone receptor A is predominant in endometrial stromal cells and its activation by progesterone stimulates cAMP and leads to the synthesis of decidual prolactin (Brosens *et al.*, 1999). However this then acts in a feedback system, as the increases in prolactin lead to decreases in the progesterone receptor A

expression suggesting a reduced uptake of progesterone by the myometrium (Brosens *et al.*, 1999).

Jones and colleagues (2002a) established that decidualisation of stromal cells had occurred through the increased levels of PRL. The production of PRL ceased on administration of the activin binding protein follistatin. Follistatin's activin regulatory role is well established and it now appears as if the pair are also implicated in placental development and potentially regulate placental PRL.

During pregnancy levels of inhibin A, activin A and follistatin are greatly increased (Schneider-Kolsky *et al.*, 2000) which is most apparent in the third trimester. The levels of activin and follistatin increase in the mother in the third trimester of pregnancy with activin A being predominant in all pregnant women (Draper *et al.*, 1997; Petraglia, 1997; Schneider-Kolsky *et al.*, 2000; Wang *et al.*, 2002).

The placenta has both an autocrine and paracrine role in pregnancy, with the increased levels of hormones in maternal circulation probably due to placental production (Keelan *et al.*, 1999b). Studies by a variety of groups have shown that the placenta produces mRNA's that encode inhibins, activins and follistatin. de Kretser *et al.* (1994), suggested that inhibin A and B, activin A, AB, and B and three forms of follistatin were present in placental homogenate, whilst Yokoyama *et al.* (1995) showed only follistatins and activin A (de Kretser *et al.*, 1994; Yokoyama *et al.*, 1995). If correlation between the activities of activins, inhibins and follistatin isoforms is to be established, further research is clearly required to clarify which forms of these peptides are produced within the placenta.

It has been suggested, based on the *in vitro* data, that the feto-placental unit is responsible for production and regulation of these peptide hormones. Unfortunately the regulation process *in vivo* is still very poorly understood. What is known, however, is that fetal and trophoblast membranes produce activin type II and type IIB receptors mRNA as well as producing follistatins, inhibins and activins (Petraglia *et al.*, 1994b). The

presence of the receptors alongside the peptides suggests an autocrine/paracine role in the feto-placental unit.

4.2 Follistatin and other proteins in Parturition

Despite the vast quantity of information available on follistatin, activin and inhibin it is still unclear whether these hormones have a role in the onset of parturition. The majority of scientific investigation into labour and its initiation have focused on two major areas. These are, (1) inflammatory responses due to the cytokine pathways and (2) responses due to endocrine/paracrine stimulation. Both of these changes create a complex series of cascade events that all lead to the increase in myometrial contractility, which then establishes labour.

As the gestational period gets to the end of its forty weeks, many processes are altering for both the mother and the child. Prolactin, which began to increase at decidualisation, is now at levels ten times greater than prior to pregnancy (Buckman & Peake, 1976; Cregan *et al.*, 2002). As full term approaches, a rise in the estrogen/progesterone ratio, first discussed by Csapo (1956) and validated by modern methods since, brings about many complex endocrine changes to initiate parturition, although the primary cause for the alteration in the ratio is yet to be established (Csapo, 1956; Albrecht & Townsley, 1978; Lahav *et al.*, 1980; Wilson, 1983; Keresztes *et al.*, 1988; Romero *et al.*, 1988; Maltier *et al.*, 1989; Wu, 1991; Mitchell & Wong, 1993; Leslie *et al.*, 1994).

The uterine smooth musculature acts in two distinctly different functional units. The upper area, the corpus (~70% smooth muscle), remains quiescent throughout pregnancy, and undergoes several fold increases in size across pregnancy (Schwalm & Dubrauszky, 1966). The lower segment exhibits unchanged muscle tone and undergoes only minor changes in size. The composition of each of these areas is closely related to the function required of it. Due to the nature of the contractions of the corpus,

communication between cells is essential to facilitate the propagation of the action potentials required for myometrial contraction. Large amounts of extracellular matrix aid the communication between corpus smooth muscle cells through the gap junctions (Garfield & Hayashi, 1981; Hendrix *et al.*, 1992; Ciray *et al.*, 1995). The major function of the cervix is to remain non-compliant until late pregnancy when dilation is required for delivery.

The endometrium is the major production site for prostaglandins throughout pregnancy. As full term approaches, the endometrium is beginning to incorporate large amounts of free arachidonic acid into stores in the decidua and membranes (Hansen *et al.*, 1999). Arachidonic acid is one of the precursors required for the production of prostaglandins (PG). Alteration of the estrogen/progesterone ratio increases the concentration of available calcium ions which then liberate phospholipase A_2. This in turn increases the arachidonic acid and PG production (Hansen *et al.*, 1999).

The uterus is incredibly sensitive to PG throughout the gestational period. The PGs most often linked with labour are PGE_2 and $PGF_2\alpha$. PG has been linked to cervical softening which is one of the first physical signs of imminent parturition. For some time prostaglandins have been used by obstetricians to induce the cervix to ripen for treatment of delayed labour onset. The increasing estrogen/progesterone ratio acts simultaneously to increase PG availability and increases the number of oxytocin receptors in the endometrium. Oxytocin stimulates further release of PG by the endometrium and aids the onset of parturition by lowering the excitation threshold of myometrial cells to a level at which an action potential will occur (Liggins, 1973; Blanks & Thornton, 2003).

Activin A levels are higher in labouring women compared to gestational-age matched non-labouring women (Florio *et al.*, 1996; Draper *et al.*, 1997; Petraglia, 1997; Schneider-Kolsky *et al.*, 2000; Wang *et al.*, 2002). Petraglia and colleagues (1993) have

shown treatment with activin A will stimulate *in vitro* cultures of amnion cells to produce prostaglandins and placental cells to produce oxytocin (Petraglia *et al.*, 1993) Recent work by Pleyvak *et al.* (2003), again confirms that activin A increases across gestation and shows both pre-term and term labour patients have higher circulating activin A in labour when compared to non-labouring gestational aged matched controls (Plevyak *et al.*, 2003). Whilst this links activin A and also follistatin, its regulator, closely with onset of labour, neither receptors ActIA, IIA and IIB nor the activin βA subunit are localised to the myometrial cells of these women (Schneider-Kolsky *et al.*, 2001). Schneider-Kolsky and colleagues (2001) established with immunohistochemistry that these three receptors and the βA subunit are localised within the vascular structure of the myometrial smooth muscle, suggestiong activin and follistatin have an indirect role in initiation of labour.

Although other studies on the uterine myometrium have shown that, whilst other myometrial proteoglycans decrease, heparan sulfate proteoglycans in the myometrium increase by 46% in active labour (Hjelm *et al.*, 2002). As discussed in Section 1.6 (Follistatin and its binding), follistatin contains a heparin binding site within the follistatin domain 1. It is therefore possible that follistatin or the follistatin/activin complex may have a direct role in labour and its initiation.

The ripening of the cervix prior to dilation has great similarity to an inflammatory reaction. A number of inflammatory cytokines can be detected in cervico-vaginal secretions at term; interleukin 1α, and 1β (IL-1α and IL-1β), tumour necrosing factor α (TNFα), interleukin 6 (IL-6) and interleukin 8 (IL-8) (Bowen *et al.*, 2002). The initiation of the inflammatory pathway involves the release of inflammatory cytokines particularly interleukin-1β, 6, and 8 as well as tumour necrosing factor α (TNFα) (Fortunato *et al.*, 2001).

Activin A and follistatin have been closely linked to the acute phase responses in inflammation and trauma. The onset of labour, particularly premature labour, has been studied with this in mind. A recent study of inflammatory responses suggests that activin A increases to stimulate the release of the inflammatory cytokines with follistatin peaking approximately four hours later (Jones *et al.*, 2000). It is thought that the role of follistatin in the inflammatory response is in a short-loop feedback to modulate the actions of activin A. Although there is some evidence that follistatin may respond directly or have additional facilitative roles in certain inflammatory conditions such as severe food poisoning, sepsis and meningitis, follistatin is elevated whereas activin remained unchanged (Jones *et al.*, 2000; Michel *et al.*, 2000; Phillips *et al.*, 2001). This evidence suggests that both activin and follistatin are linked. However, it seems possible that increases in follistatin may be stimulated by factors other than activin. In the inflammatory responses necessary for wound repair FLRG/FSTL-3 has been linked to follistatin, although further research to determine whether FLRG/FSTL-3 becomes part of the inflammatory pathway with follistatin and activin is necessary (Wankell *et al.*, 2001a).

It has been shown granulocyte numbers increase rapidly in cervical tissue as dilation progresses (Winkler *et al.*, 1998). Granulocytes increase in tissues that have been associated with the release of matrix degrading enzymes such as matrix metalloproteinase's (MMP) from their granules. These enzymes appear to mediate the tissue remodeling required in cervical tissues in order to facilitate the progress of dilation. IL-8 has been shown to attract granulocytes as well as activate and degrade leukocytes. Prostaglandin E_2 can stimulate the production of IL-8 from cervical tissues (Bowen *et al.*, 2002).

IL-6 has been previously identified as a marker for amniotic infection and according to some authors it is rarely identified prior to onset of labour (Bowen *et al.*, 2002). In

infection IL-6 is produced by the amnion, chorion and decidua (Keelan *et al.*, 1999a), however, one group has identified it in low levels through the pre-labour stage in cervical sections (Kemp *et al.*, 2002) with levels increasing as labour becomes further established (Winkler *et al.*, 1998). IL-6 stimulates the production of prostaglandins by the amnion and decidua (Kemp *et al.*, 2002). As labour becomes established in the dilation stage, the levels of IL-1α and IL-1β, IL-6, and IL-8 all increase to reach peak values once the cervix is fully dilated (Steinborn *et al.*, 1996).

In the clinical setting, there are very few assays developed that can be utilized in the routine biochemical screening of patients for inflammation. Whilst our understanding of interleukins is growing and it is clear they are involved in most inflammatory states, there is yet to be an assay developed for them that has the reliability and speed required by a hospital biochemistry laboratory. However in order to determine inflammatory states in patients another inflammatory marker, C-reactive protein (CRP), is often used. CRP is produced rapidly by the hepatocytes in response to both viral and bacterial infection and also shows alteration in levels with chronic inflammatory conditions such as rheumatoid arthritis and Crohn's disease (Edwards *et al.*, 1982; Gewurz *et al.*, 1982). CRP rises rapidly in inflammatory conditions. Whilst the mechanism for its release has not been adequately studied, IL- 1 and -6 are clearly implicated (Dinarello, 1984; Nishimoto *et al.*, 1989).

The CRP assay is robust and unaffected by patient drug treatments, abnormal serum proteins and any other patient blood variables such as polycythemia and anaemia (Gewurz *et al.*, 1982). CRP has been considered as a marker for pre-term labour (Farb *et al.*, 1983; Watts *et al.*, 1993), however the body of literature on this subject itself suggests its use is as a marker for infection in patients with premature rupture of membranes necessitating and/or causing early delivery rather than premature labour onset (Foulon *et al.*, 1995).

In order for dilation of cervix to occur, the myometrial tissues undergo a series of contractions that facilitates this dilation and eventual expulsive phases of labour. Throughout pregnancy the growth of the myometrium ensures that the dominant force whilst labouring will be an expulsive vector from fundus to cervix (Buhimschi *et al.*, 2003). Similar to cardiac muscle, it has been established that the thinner the myometrial wall the greater the generated uterine wall stress (Buhimschi *et al.*, 2003). Whilst in the dilation stage of labour, the fundal and anterior myometrium thin symmetrically and it appears as though contractions occur isometrically (Buhimschi *et al.*, 2003).

The main function of the cervix is to remain non-compliant until late pregnancy when it undergoes the softening necessary for cervical dilation essential for parturition. This softening is generated through a decrease in collagen concentration and proteoglycans. Proteoglycan concentrations decrease by approximately 50% in the ripened cervix (Norman *et al.*, 1991). The function of proteoglycans in the uterus is thought to be essential for the uterine remodeling that is necessary at parturition onset and for postpartum healing (Hjelm *et al.*, 2001). The most dominant of the proteoglycans present in the corpus of the uterus are heparan sulfate proteoglycans, biglycan and in much smaller amounts, versican and decorin (Hjelm *et al.*, 2001). However, in the cervix only decorin and biglycan are found (Hjelm *et al.*, 2001). Gap junctions are needed by the corpus and increase in both size and quantity prior to labour, becoming important for syncytial contraction of the smooth muscle (Ciray *et al.*, 1995). Some groups suggest that that the function of the heparan sulfate proteoglycans in the corpus is to influence the functionality of these developing gap junctions (Hjelm Cluff *et al.*, 2005).

In all placental mammals, oxytocin has been implicated in the parturition process, however its measurement is surrounded by difficulties particularly as its release from the posterior hypothalamus is pulsatile (Blanks & Thornton, 2003). As the levels of oxytocin increase this further stimulates the release of prostaglandins and further establishes

labour. Oxytocin is often administered to induce labour when onset is delayed however its role in the initiation phase of labour is still to be established. At this stage it is known to establish uterine smooth muscle contractions however, induction of labour with oxytocin is well known in obstetric circles to have an increased risk of emergency caesarean sections as a percentage of patients have a failure to progress to effective contractions.

As full term approaches there is a dramatic increase in the number of oxytocin receptors in the uterus (Blanks & Thornton, 2003). Oxytocin though its receptors in the myometrium act both directly to stimulate further myometrial contractions and indirectly to further increase the production of PGE_2 and $PGF_2\alpha$ (Blanks *et al.*, 2003). This then additionally increases the levels of calcium ions in the myometrium and aids myometrial action potentials. As labour continues, there is continued positive feedback to increase production of prostaglandins and promote the myometrial contractility so that the fetus is expelled from the uterus.

Asymmetrical thickening of the myometrium occurs immediately post-partum, with the thinnest area occurring over the point of placental insertion (Buhimschi *et al.*, 2003). The thinning of the myometrium increases the wall tension of the uterus in this region and is critical for placental separation (Buhimschi *et al.*, 2003). The intensity of the myometrial contractions are greater during this stage than during any prior stage. It is thought that the presence of the fetus and amniotic sac prevent the myometrial contractions from gaining this intensity, and therefore protect against early placental separation (Deyer *et al.*, 2000).

During the recovery stage of delivery, the puerperium (6-8 week period following delivery), the uterus is undergoing repairs and returning to pre-pregnancy size. In this time the area of placental insertion remains the thinnest segment of the uterus (Buhimschi *et al.*, 2003). The active stages of labour have been investigated with aims

to prevent many of the complications associated with pre-term delivery, however in the post-partum stage very little of the biochemical processes has been characterised. The difficulties with tissue sampling have made this almost impossible to study in women. It has been long established that the high levels of progesterone and estrogen essential for maintenance of pregnancy will return to pre-pregnancy levels within a number of hours, whilst the changes in prolactin levels are determined by the nursing status of the mother.

Uterine involution involves substantial tissue destruction, repair and remodeling by changes to the extracellular matrix, cellular proliferation and apoptosis. Using ultrasonographic studies, it appears that the endometrium remains very thin in the first three days following delivery (Salamonsen, 2003). The involution of the uterus is related to the extensive loss of collagen. Matrix metalloproteinase's (MMP) are proteolytic enzymes that have been implicated in the areas of matrix remodeling (Ravanti & Kahari, 2000). In rat studies MMP-7 is found to peak on days 1 to 2 in postpartum uterine tissues. Between days 7 to 17 it appears that the endometrium undergoes normal endometrial healing as seen in the re-epithelialisation stage of the menstrual cycle (Salamonsen, 2003). From days 26 to 56 the endometrium is most likely to resemble the menstrual cycle proliferative phase tissue where stromal restoration is occurring (Salamonsen, 2003).

In the healing of any wound inflammation is an essential part of the process, with the huge amount of tissue remodeling that occurs following parturition it is clear that many of the inflammatory pathways will be involved. Follistatin is implicated in a range of inflammatory responses (Keelan *et al.*, 1999a; Jones *et al.*, 2000; Phillips *et al.*, 2001) but whether it has a role in the postpartum inflammation is uncertain.

4.3 Other Factors effecting Follistatin across Parturition

4.3.1 Gender

There are significant gender differences in the mortality and morbidity of the fetus with the male fetus having greatly reduced outcomes when compared to the female fetus, in association with pre-eclampsia, placental insufficiency, infection, preterm delivery, and intra-uterine growth restriction (Vatten & Skjaerven, 2004). The male gender dominates in numbers of preterm deliveries and perinatal mortality (Stevenson *et al.*, 2000). Some researchers have suggested that the gestational period is influenced by gender however, results have suggested both a reduced gestational period (Cooperstock & Campbell, 1996) and an extended gestational period (Divon *et al.*, 2002) so that the data related to gestational period is far from clear despite both studies being from extremely large patient populations (>1,000,000 and >600,000 births assessed respectively by the authors).

The vast majority of women are screened in the second trimester using multiple biochemical screening to create a risk estimate of certain fetal abnormalities, most particularly Down Syndrome and neural tube defects. Generally, the screening test consists of maternal serum alpha fetoprotein (MSAFP), hCG, estradiol, and more recently inhibin. However, a number of authors have suggested that some of these maternal serum results are dependant on the gender of the fetus, most particularly MSAFP which is decreased whilst both hCG and inhibin are elevated in the presence of a female fetus (Sowers *et al.*, 1983; Leporrier *et al.*, 1992; Lockwood *et al.*, 1993; Yung Hang Lam, 2001). Surprisingly, very limited research has been carried out to determine the impact of fetal gender on both the maternal inflammatory and endocrinology systems which both have strikingly important roles throughout pregnancy and parturition, and potentially effects on the mothers longer term health.

It has been suggested that survival of the immunohisto-incompatible fetus is dependant on a shift of the uterine environment to a Th-2 allergenic immune response rather than the Th-1 phagocytotic response that would result in rejection of the fetus by the maternal system (Wegmann *et al.*, 1993). Studies of allergic conditions such as asthma have shown that 33% of asthmatic women have a worsening of their condition through pregnancy. Recent work suggests that it is in those women carrying female fetuses (Murphy *et al.*, 2003). In the presence of a female fetus these asthmatic mothers have an increased requirement for inhaled glucocorticoid treatment. This result suggests that fetal sex has an influence on maternal immune function during pregnancy.

The actions of glucocorticoids are well known as mediators of the inflammatory responses of the immune system. Under normal conditions the placenta controls the exposure of the fetus to the actions of glucocorticoids by the presence of 11β hydroxysteroid dehydrogenase 2 (11βHSD-2) (Benediktsson *et al.*, 1997). However, in normal non-asthmatic mothers placental 11βHSD-2 activity is significantly higher with female than male fetuses, suggesting that within the placenta the intracellular cortisol concentrations are lower in the female. Cortisol actions are mediated predominantly by glucocorticoid receptors with mineralcorticoid receptors having a reduced ability to regulate cortisol's effects both centrally and peripherally. Interestingly both glucocorticoid and mineralcorticoid receptor mRNA expression is reduced in the male fetus. Placental immune function is regulated by glucocorticoids via cytokines such as IL-1β, IL-10 and TNFα. The mRNA of all three cytokines has been quantified and found to be significantly up-regulated in the placenta of females compared to males, again suggesting that the intracellular cortisol concentrations are lower intracellularly in placenta of females (Clifton & Murphy, 2004).

Follistatin is widely recognized for its role in the inhibition and modulation of members of the transforming growth factor β family, namely activin and BMP's, with it preferentially binding to activin, (Moore *et al.*, 1994; Schneyer *et al.*, 2003). All three peptides increase in maternal circulation across gestation, with activin concentrations being predominant, followed by decreases in all three post –delivery (Florio *et al.*, 1999; Schneider-Kolsky *et al.*, 2000). Current evidence suggests follistatin is implicated in both pregnancy and parturition (Schneider-Kolsky *et al.*, 2000; de Kretser *et al.*, 2002; Schneider-Kolsky *et al.*, 2002). Of the three peptides, maternal inhibin concentrations have been shown to increase when the fetus is female, particularly in the second trimester (Wallace *et al.*, 1999), however neither activin nor follistatin has had this phenomenon investigated, however in view of its relationship with both activin and inhibin, and the reduced inhibin across gestation in maternal serum when carrying male fetuses the data obtained in this study has been reviewed in light of fetal gender.

Inhibin, activin and follistatin are all produced in a large number of locations throughout pregnancy. Each is produced by the placenta in large concentrations that may feed into the maternal circulation (Keelan *et al.*, 1999b), however the maternal system also has the ability to produce each of these peptides independent of the placenta. Other sources of follistatin are outlined in Chapter 1, Section 1.4 in Table 1.1. From this information it is obvious that there are multiple possible sources for the follistatin rises seen in maternal serum and therefore it is difficult to determine the origins, and the stimulating factor for the increased levels of each of these peptides in the maternal circulation.

4.3.2 Labour length

Literature studies reviewing the effects of labour length on reproductive hormones were surprisingly limited in this area. The vast body of literature on labour length deals mainly

with the associated clinical complications that evolve when labour is prolonged. Parturition in women is divided into three stages. The first stage is divided into the latent and the active stage, with the latent stage occurring whilst cervical dilation to 4cm occurs. The active stage is the dilation from 4 cm through to full dilation of the cervix. The second stage of labour is from a fully dilated cervix through to the delivery of the fetus, and the third stage is from fetal delivery through to placental delivery.

It appears that the length of labour is somewhat related to ethnicity, with black and American Indian women having shorter second stage labour, and Asian women having longer second stage labour when compared to Anglo-Saxon women (Albers *et al.*, 1996; Albers, 1999; Greenberg *et al.*, 2006). Parity also has a great effect on the length of labour with nullliparous women having significantly longer labour in all stages (Albers, 1999). The position of the fetus also can have significant and often detrimental effects on the length of labour (Senecal *et al.*, 2005).

From the limited studies available, it appears that maternal plasma cortisol and estradiol increase in labour whilst prolactin decreases, however only a limited number of patients have been used in this particular study, and the differences due to the length of labour are yet to be established (Kubota *et al.*, 1987).

4.3.3 Parity

It has been shown that the outcome of the first pregnancy is a powerful predictor of the outcome of subsequent pregnancies, that is if the first pregnancy ends in a miscarriage there is a 20% likelihood that the 2nd will also (Anokute, 1987; Regan *et al.*, 1989). However, if the first is successful then there is only a 5% chance of miscarriage in future pregnancies. Increasing parity has increased risks of malpresentation of fetus for delivery, maternal obesity, maternal anemia and preterm delivery. However, these women have a significantly reduced rate of operative vaginal deliveries, emergency

caesarean section, and prolongation of labour (Mocanu *et al.*, 2000). Multiparous women also have a reduced rate of induction (Chan & Lao, 1999). However, anecdotally obstetricians have stated that if the first pregnancy needs an induction of labour, then in subsequent pregnancies it will become increasingly difficult to induce that patient into labour.

Whilst all of the above data has been known and patient advice accordingly given by clinicians, little in the way of research into the primary cause of the effect of parity on maternal physiology has been investigated.

Parturition is clearly associated with inflammatory pathways, however the role of follistatin in this process is unknown. This project aimed to investigate the role of follistatin through the labouring and post-partum stages of pregnancy. Follistatin was compared to estrogen, progesterone, prolactin, cortisol, activin A, TNFα and the clinical indicator of inflammation, C-reactive protein.

This study into follistatin across parturition, has prompted investigations into whether concentrations of follistatin are influenced by fetal gender, maternal parity and labour length, in a preliminary attempt to elucidate the effect on both the maternal and fetal systems.

4.4 Methods:

4.4.1 General Methods

Details regarding recruitment of patients, ethics approval and sample collections have been previously described in Chapter 2. However, for studies investigating fetal gender, labour length and parity the criteria for group allocations has been modified as shown below.

4.4.2 Effect of Fetus and Fetal Gender

Maternal serum samples were taken using a patient cohort of 50 women who underwent vaginal delivery, subjects were retrospectively divided according to fetal sex (Males n= 21, Females n= 29). A smaller cohort of patients from LSCS deliveries (n=11) were also used as shown in Table 4.1 The mean weights of males were 3342.95g compared to 3243.96g in the female. These differences in weight were not significant so further sorting using weight, as a variable was not necessary.

Table 4-1 Numbers of male and female delivered in vaginal and LSCS onset groups

Fetal Gender Samples	Vaginal Delivery	LSCS Delivery
Maternal Serum – Male	21	6
Maternal Serum - Female	29	5
Fetal cord - Male	15	3
Fetal cord – Female	22	4
Total samples used	50	11

4.4.3 Effect of Labour Length

Using the same cohort of patients, subjects were retrospectively divided into those having no labour (LSCS), and those vaginal delivery patients having short, medium or long labour as shown below in Table 4.2. The labour length is defined as the time taken for the patient to complete all stages (contraction, dilation, fetal delivery, and placental expulsion) of labour. Short labour is defined as less that 3 hours, Medium labour is between 3 and 6 hours, whilst Long Labour is greater than 6 hours. A small number of patients were excluded from the original cohort due to insufficient detail on labour length in medial records.

Table 4-2 Numbers of patients used in labour groups

Group	Patient numbers	Fetal samples
No Labour (LSCS)	5	7
Short Vaginal Labour (< 3 hours)	16	17
Medium Vaginal Labour (> 3 hours)	19	15
Long Vaginal Labour (> 6 hours)	15	9
Total samples	55	48

4.4.4 Assays

Samples outside the standard curve of the assays were diluted with charcoal stripped male serum. A pool of male serum was collected from blood donations made to the Red Cross Blood Bank and charcoal stripped (50 mg/ml) to remove the high levels of steroid hormones. The male serum pool was assayed after charcoal stripping and concentrations of estradiol, cortisol and progesterone were undetectable, however

prolactin could not be removed from the pool. The prolactin concentration in the serum pool was very low compared to the samples from females and was accounted for in the calculation of concentrations in samples.

Every sample was then diluted with charcoal stripped male serum to either a 1:5 or 1:20 dilution. The male stock was used as a control for each analysis of samples. None of the patient serum analysed for C-reactive protein required dilution using the pooled serum.

Progesterone, estradiol, cortisol and prolactin were analysed by the automated analyzer, Access from Beckman-Coulter Inc. (Fullerton, CA, 92834, USA) at Tamworth Pathology Laboratory. Beckman Coulter and New England Area Health Service Pathology kindly provided the reagents for these analyses at no cost to the university. The Access analyser was calibrated on a daily basis using the calibrators provided with each assay. Quality controls were run at the time of analyses.

Progesterone

The Access Immunoassay system uses a competitive binding immunoezymatic assay utilizing rabbit antibodies to progesterone, a progesterone-alkaline phosphatase conjugate and goat anti-rabbit antibodies. This gives an assay range of 8.00 – 400.0 nmol/L, with sensitivity to 8.00 nmol/L.

Estradiol

The Access Immunoassay analyzer uses a competitive binding immunoenzymatic assay. The methodology is similar to the progesterone assay with samples being added to the reaction vessel containing paramagnetic particles coated with goat anti-rabbit, rabbit anti-estradiol and a TRIS buffered protein solution. The assay has a range of 0.20 – 360 nmol/L.

Cortisol

The Acces Immunoassay analyser uses a competitive binding immunoenzymatic assay which is similar to that of both Estradiol and Progesterone. The assay uses goat anti-rabbit capture antibodies, rabbit anti-cortisol and a cortisol-alkaline phosphatase conjugate solution. This assay has a range of 11-1655 nmol/L.

Prolactin

Prolactin on the Access Immunoassay system uses a simultaneous one-step immunoenzymatic (sandwich) assay which uses polyclonal goat anti-prolactin alkaline phosphatase conjugate and mouse monoclonal anti-prolactin antibody. This assay gives a range of results from 2500 – 20,000 uIU/ml.

Follistatin

Total follistatin was determined in plasma using a radioimmunoassay as previously described (O'Connor *et al.*, 1999). The assay uses a rabbit antiserum (#204) raised against purified 35 kDa bovine follistatin. A human recombinant follistatin (FS288) was used as both standard and tracer. The dissociation reagents were modified to 10% triton X-100, 3% sodium deoxycholate and 0.5% SDS. Cross reactivity with inhibin and activin was less than 0.5%. The detection limit was 0.25 ng/ml and the intra-assay and inter-assay CVs were 6% and 12%.

C Reactive Protein

This is an automated assay run on the Synchron analyzer by Beckman Coulter Inc. (Fullerton, CA, 92834, USA). The assay is an immunoturbinometric assay utilizing goat polyclonal anti-C reactive protein antibody. The assay has a sensitivity of 0.2 mg/L and a range of 0.2 – 280 mg/L.

Activin

The Activin A assay measuring both free and follistatin bound activin has been previously described (Knight *et al.*, 1996). This ELISA uses human recombinant activin A as standard (NHPP). The mean sensitivity was 0.01 ng/ml and the mean intra- and inter-assay coefficient of variation values were 6.2 and 6.9% respectively.

TNFα

This ELISA, available from Beckman Coulter Inc. (Fullerton, CA, 92834, USA), is an immunological one-step sandwich assay using a primary monoclonal antibody anti-TNFα in the presence of a second monoclonal antibody linked to alkaline phosphatase. Following incubation and washing the bound enzymatic activity is detected using chromogenic substrate with the intensity of colouration proportional to TNFα concentration. The assay has a sensitivity of 5 pg/ml, intra-assay precision between 1.6 – 10% and interassay precision between 5.4 - 12%.

4.4.5 Data Analysis

The raw hormone concentration data were log transformed to normalize the data and equalize the variances. The data were then analysed by two way analysis of variance (ANOVA), utilizing the Student-Newman-Keuls (SNK) post test to compare individual means. All the calculations were carried out using the GLM procedure and repeated statement of the SAS computer package (SAS Institute Inc., Cary, NC). The values are presented as mean ± SEM.

4.5 Results

To differentiate the changes occurring in the labouring patient, vaginal delivery patients were subdivided into patients undergoing spontaneous onset of labour and those medically induced into labour via operative rupture of membranes and the introduction of intravenous syntocin (an oxytocin derivative used to promote labouring).

Estradiol and Progesterone

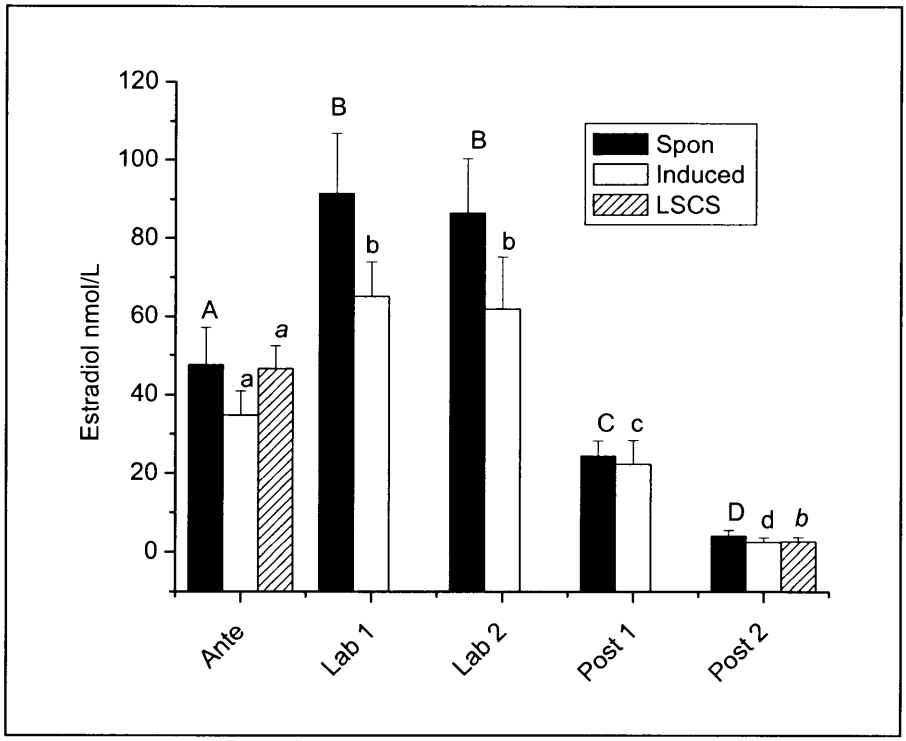


Figure 4-2 The effect of parturition on maternal serum estradiol concentrations. Significant differences within the spontaneous onset group are indicated with upper case letters. Significant differences within the induced onset group are indicated with lower case letters. Significant differences within the LSCS delivery group are indicated with italicized lower case letters.

Results from the traditional gestational hormones, estradiol and progesterone, show the expected pattern of concentration changes across the sample period (Figure 4.2 and 4.3). That is, peak concentrations in early labour compared to the antenatal period, that drop dramatically by the early post partum period when the placenta is removed.

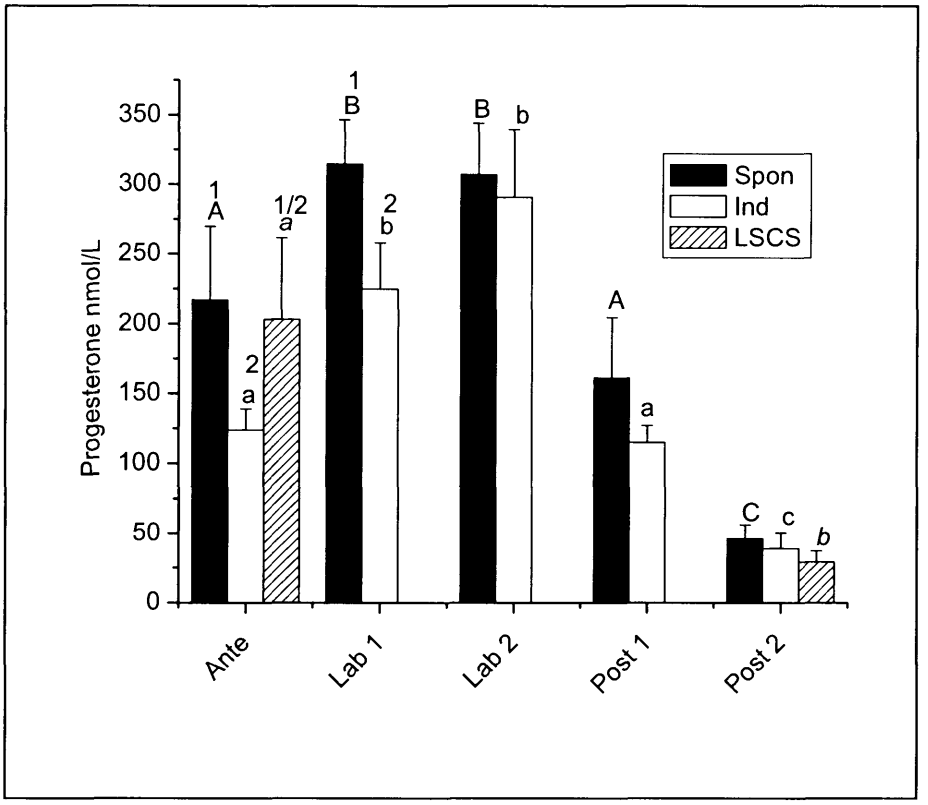


Figure 4-3 The effect of parturition on maternal serum progesterone concentrations. Significant differences within the spontaneous onset group are indicated with upper case letters. Significant differences within the induced onset group are indicated with lower case letters. Significant differences within the LSCS delivery group are indicated with italicized lower case letters. Significant differences between groups are indicated with numbers.

Estradiol and progesterone show that in early labour, spontaneous onset patients have higher concentrations of both of these hormones when compared to the induced patient group (Estradiol 91.4 nmol/L ± 15.5 to 65.1 nmol/L ± 8.8 respectively and Progesterone 314.5 nmol/L ± 32.0 to 224.8 nmol/L ± 32.9). Whilst there was a trend to maintaining this increased concentration of both hormones in the spontaneous labour patients, it was not deemed significant.

Table 4-3 % Concentration for Maternal Estradiol, Progesterone and Follistatin

	Labour (>3cm dilation)	(<3 hours from delivery)	(>3 hours from delivery)
Estradiol Spon nmol/L (%)	86.6 (100%)	23.3 (29.9%)	4.2 (4.8%)
Estradiol Induced nmol/L (%)	61.9 (100%)	32.2 (32.2%)	2.4 (4%)
Progesterone Spon nmol/L (%)	306.8 (100%)	160.7 (52.4%)	46.1 (15%)
Progesterone Induced nmol/L (%)	290.4 (100%)	114.8 (39.5%)	39.2 (13.5%)
Follistatin Spon ng/ml (%)	57.9 (100%)	57.6 (100%)	26 (44%)
Follistatin Induced ng/ml (%)	20.5 (100%)	26.9 (130%)	24 (117.1%)

In order to assess if follistatin reduced in a similar manner, Table 4.3 shows % concentration remaining, calculated as a percentage of the hormone concentration at late labour when cervical dilation is greater than 3 cm. Estradiol and progesterone clearances were compared with follistatin concentrations (taken from Figure 4.11) for correlations (Table 4.1) however none were observed. In both vaginal delivery groups estradiol and progesterone fell dramatically following delivery (Spontaneous - Estradiol Post partum < 3hrs from delivery – 29.9% Post partum > 3hrs from delivery – 4.8%; Progesterone Post partum < 3hrs from delivery – 52.3% Post partum > 3hrs from delivery – 15%) confirming the placenta as the major source of circulating maternal concentrations of these hormones.

However follistatin concentrations in these two patient groups showed some variations. The induced group showed follistatin concentrations continue to rise in early post partum and decline slightly by the post partum stage (Post partum < 3hrs from delivery - 130% Post partum > 3hrs from delivery – 117.1%), whilst in the spontaneous labour group it

remains elevated from labour (Post partum < 3hrs from delivery – 100%) to eventually decline in the later time period following birth (Post partum > 3hrs from delivery – 44%).

Cortisol

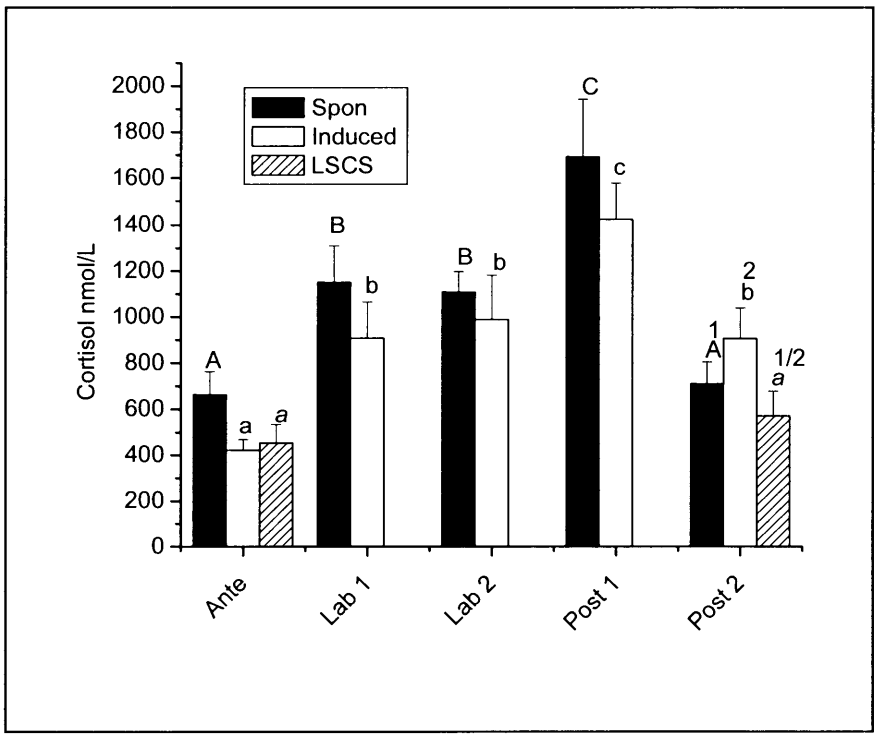


Figure 4-4 The effect of parturition on maternal serum cortisol concentrations. Significant differences within the spontaneous onset group are indicated with upper case letters. Significant differences within the induced onset group are indicated with lower case letters. Significant differences within the LSCS delivery group are indicated with italicized lower case letters. Significant differences between groups are indicated with numbers.

In both vaginal delivery groups cortisol (Figure 4.4) rises from the antenatal period across labour to peak at the early post partum sample (Spontaneous Ante- 663.9 nmol/L ± 52.6, Lab2- 1108.1 nmol/L ± 89.9, Post1- 1691.6 nmol/L ± 251.2; Induced Ante- 421.1 nmol/L ± 43.6, Lab2- 987.9 nmol/L ± 194.3, Post1 – 1424.2 nmol/L ± 154.2).

In the spontaneous labour patients these results showed increasing significant concentrations whilst for induced patients antenatal samples were significantly less than all subsequent samples. In the late post-partum sample period a decrease in the spontaneous group is seen. Interestingly, the differences in late postpartum between

each group are significant with induced patients having the highest concentration (906.7 nmol/L ± 132.4) compared with spontaneous labour and LSCS deliveries (708.5 nmol/L ± 96.1 and 571.7 nmol/L ± 106.4) respectively.

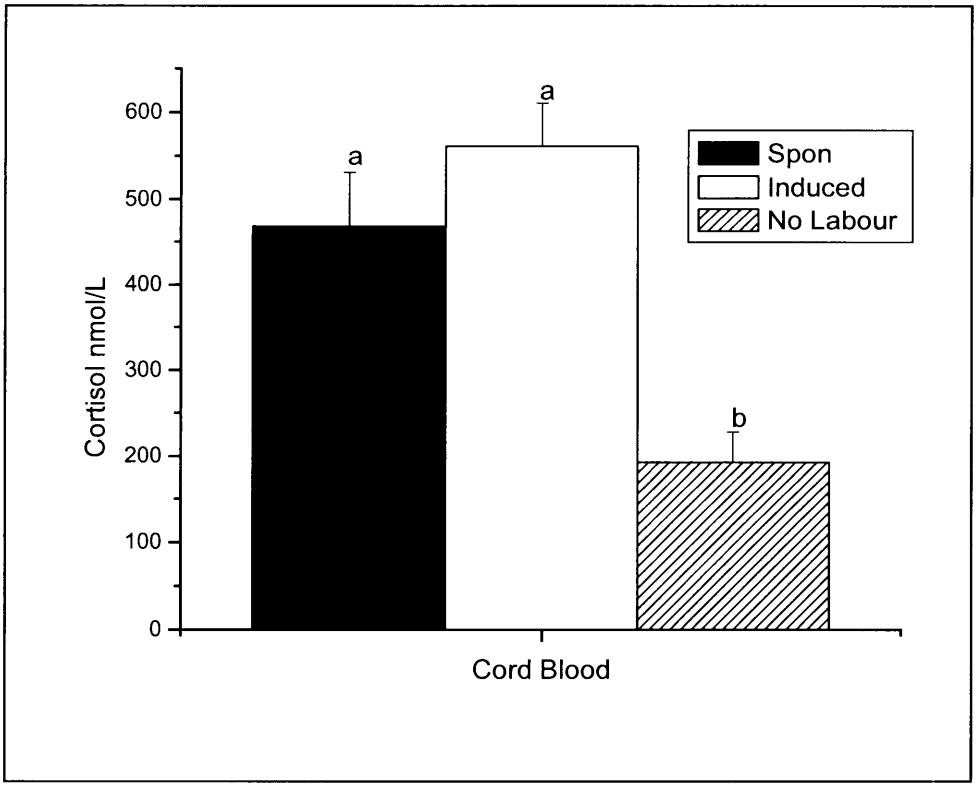


Figure 4-5 The effect of type of delivery on cord blood cortisol concentrations. Significant differences between groups are shown using lower case letters.

Fetal serum cortisol is elevated in spontaneous and induced patients (467.9 nmol/L ± 63.5 and 561.6 nmol/L ± 49.8 respectively) when compared to the LSCS delivery patient group (192.5 nmol/L ± 35.8).

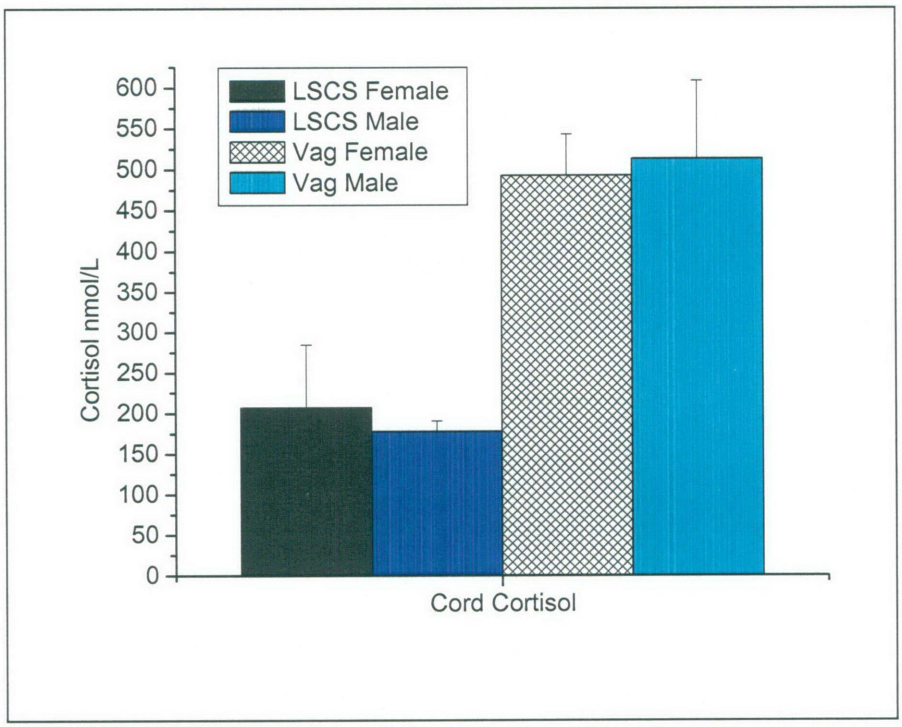


Figure 4-6 The effect of fetal gender on cord blood cortisol concentrations in vaginal and LSCS deliveries.

Fetal cord cortisol as shown in Figure 4.5 and shows vaginal delivery babies with significantly higher cortisol than their LSCS counterparts, however there is no difference in concentrations between the fetal gender of these two groups as seen in Figure 4.6 (Vag Female- 492.3 nmol/L ± 50.4, Vag Male- 512.9 nmol/L ± 95.4 versus LSCS Female- 207.3 nmol/L ± 77.5, LSCS Male- 177.7 nmol/L ± 12.9).

Fetal cortisol concentrations were lowest in those delivered via caesarean section and concentrations subsequently increased significantly with increased labour length (LSCS- 192.5 nmol/L ± 35.8, Vag Short- 435.1 nmol/L ± 39.4, Vag Medium- 415.3 nmol/L ± 58.3, Vag Long-737.1 nmol ± 154.4) as shown in Figure 4.7.

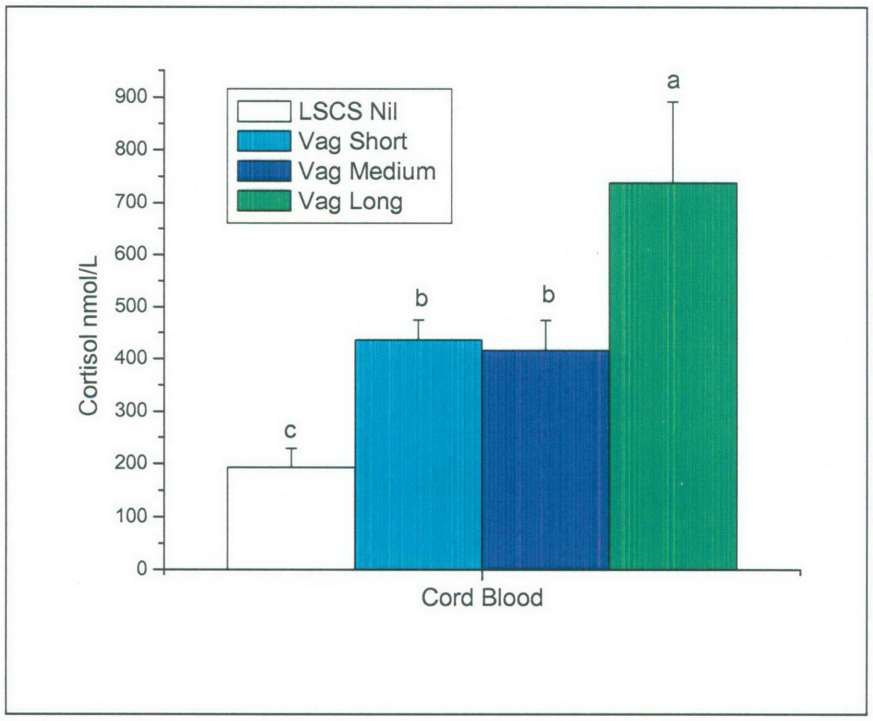


Figure 4-7 The effect of labour length on cord blood cortisol concentrations. Significant differences between groups are shown in lower case letters.

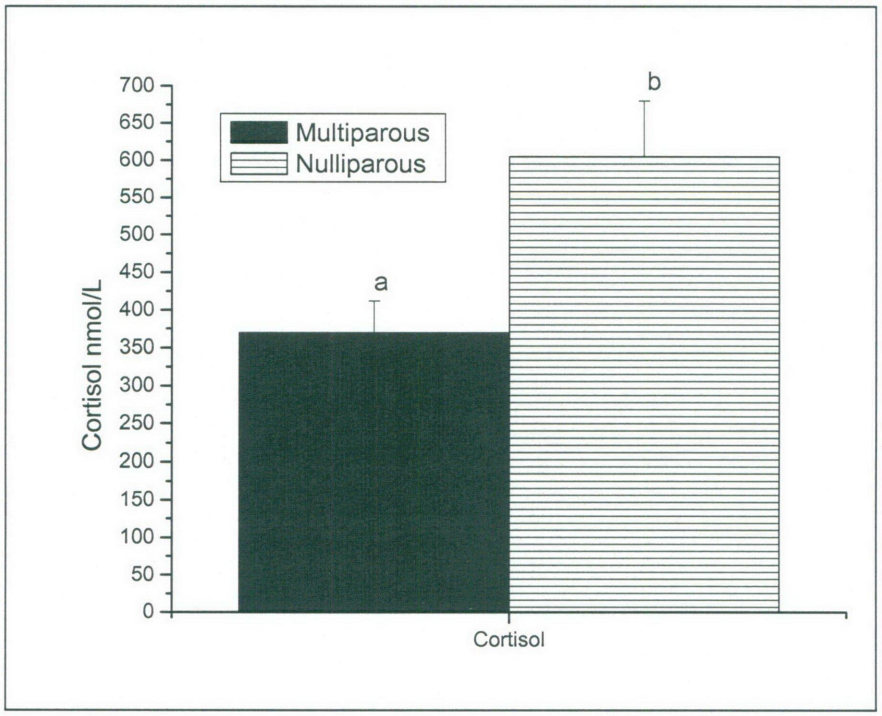


Figure 4-8 The effect of parity on cord blood cortisol concentrations. Significant differences between groups are shown in lower case letters.

Interestingly, cord blood cortisol concentrations appear to be affected by the parity of the mother (Figure 4.8), with significantly lower cortisol concentrations seen in the cord blood of multiparous women (369.9 nmol/L + 42.2) when compared to nulliparous women (605.0nmol/L + 74.9).

Prolactin

As shown in Figure 4.9, in the spontaneous labour patients there is a trend to increasing prolactin between the antenatal period and the final post partum sample (2773.6 ± 242.3 uIU/ml to 4823.9 ± 581.2 uIU/ml), however it is without significance. Induced patients show prolactin reaching its significant peak at late labour (5449.40 ± 890.71uIU/ml) and then dropping significantly to reach to early labour levels in the first post-partum period (3906.89 ± 806.32 uIU/ml).

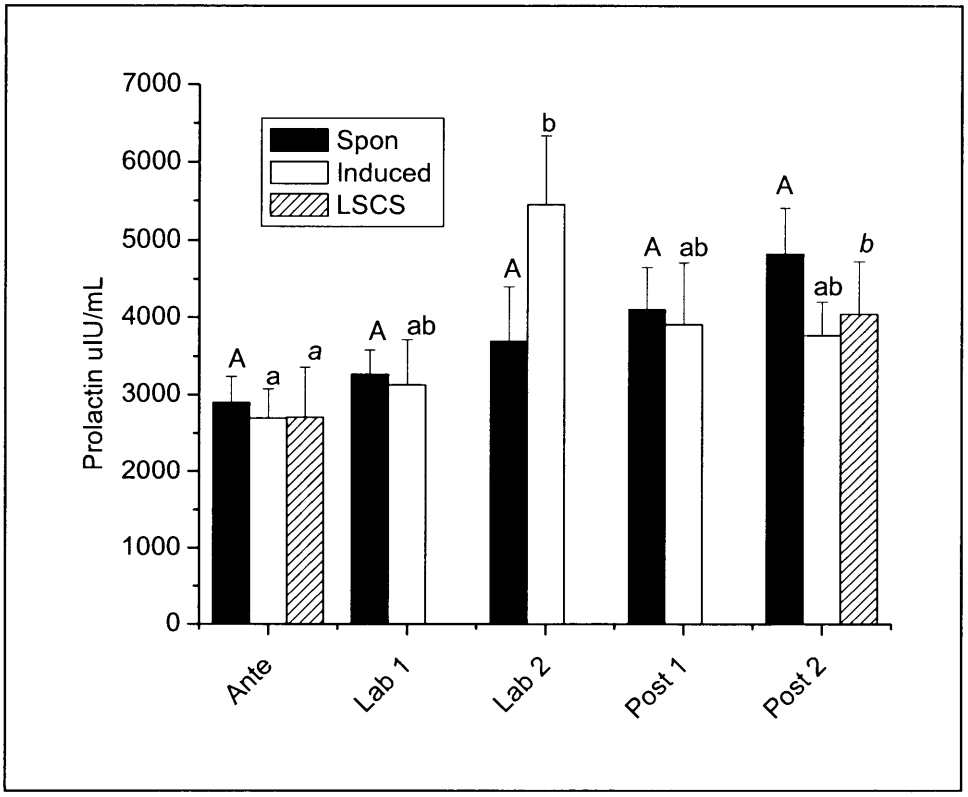


Figure 4-9 The effect of parturition on maternal serum prolactin concentrations. Significant differences within the spontaneous onset group are indicated with upper case letters. Significant differences within the induced onset group are indicated with lower case letters. Significant differences within the LSCS delivery group are indicated with italicized lower case letters.

Fetal cord serum was not analysed for prolactin in this study. Maternal serum prolactin show a trend indicating that prolactin concentrations are higher in patients who have a shorter labour when compared with patients who have medium and longer labour in the late labour (Lab2) sample and subsequent postpartum sampling (Lab2 Vag Short- 5337.48 uIU/ml ± 1712.9 compared to Vag Medium- 3576.2 uIU/ml ± 593.4 and Vag Long- 4213.7 uIU/ml ± 1059.6) as seen in Figure 4.10.

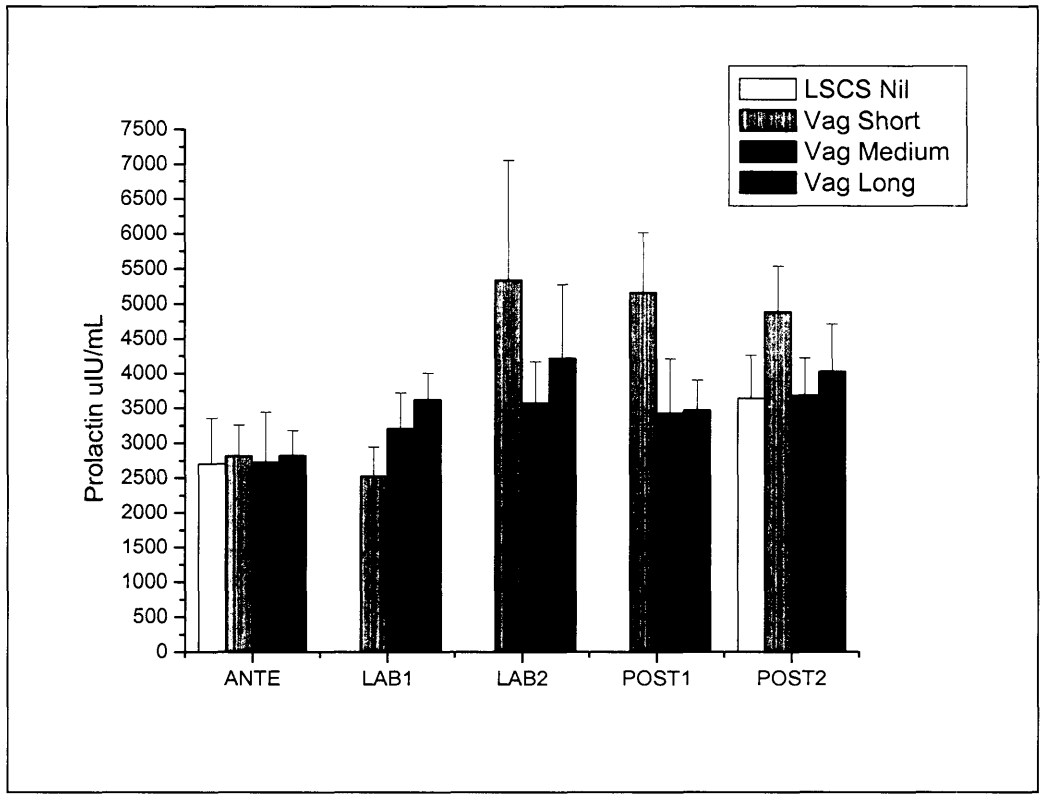


Figure 4-10 The effect of labour length on maternal serum prolactin concentrations

Follistatin

Follistatin concentration changes in maternal serum are profiled in Figure 4.11. In the spontaneous labour patients follistatin showed significant differences between antenatal, early labour, late labour and early post partum samples, whilst the induced patients

showed significant differences between labouring samples and the early post-partum sample.

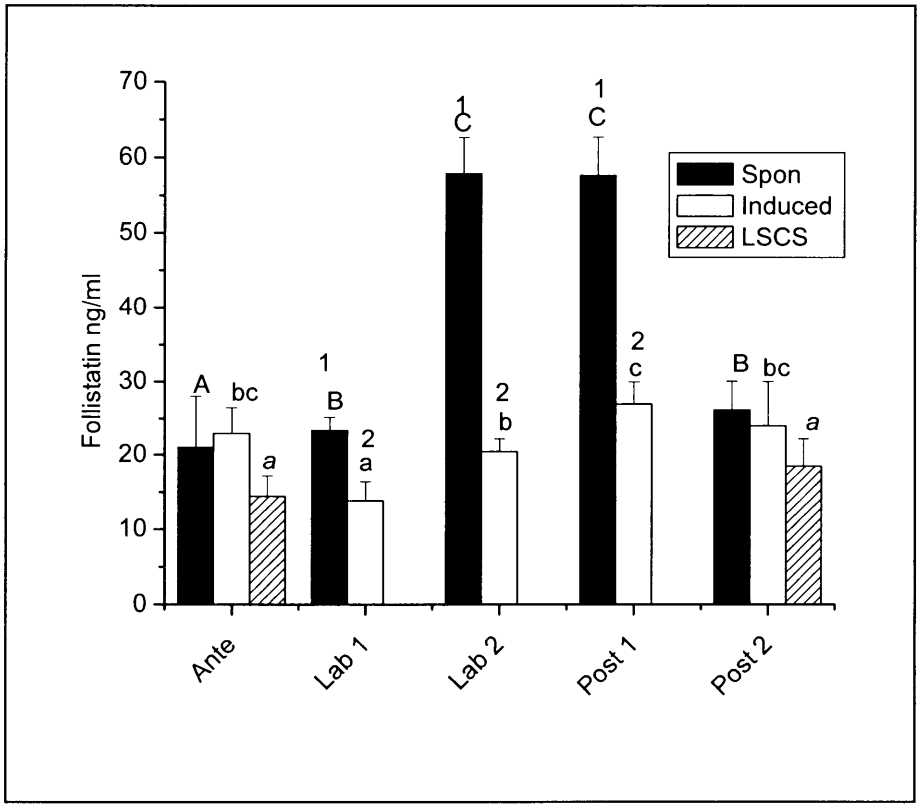


Figure 4-11 The effect of parturition on maternal serum follistatin concentrations. Significant differences within the spontaneous onset group are indicated with upper case letters. Significant differences within the induced onset group are indicated with lower case letters. Significant differences within the LSCS delivery group are indicated with italicized lower case letters. Significant differences between groups are indicated with numbers.

Of great interest are the differences seen between these two patient groups within both labouring samples and early post partum samples. In early labour, spontaneous patients have significantly higher concentrations of follistatin than induced patients (23.4 ng/ml ± 1.84 to 13.9 ng/ml ± 2.54 respectively) whilst in late labour there is a 2-fold difference which peaks in the spontaneous group. When the two groups are compared at late labour follistatin concentrations are 57.9 ng/ml ± 5.48 and 20.5 ng/ml ± 1.73 respectively. Follistatin remains significantly elevated in spontaneous labour patients in the early post-partum (57.6 ng/ml + 5.1) when compared to induced patients (26.9 ng/ml

+ 3.0). The induced patients show follistatin continues to rise from labour, into the early post-partum period and reaches its peak concentration here (26.9 ng/ml ± 3.0 to 20.5 ng/ml ± 1.73 in late labour). In the later post- partum period we see the follistatin concentration of both patient groups declining.

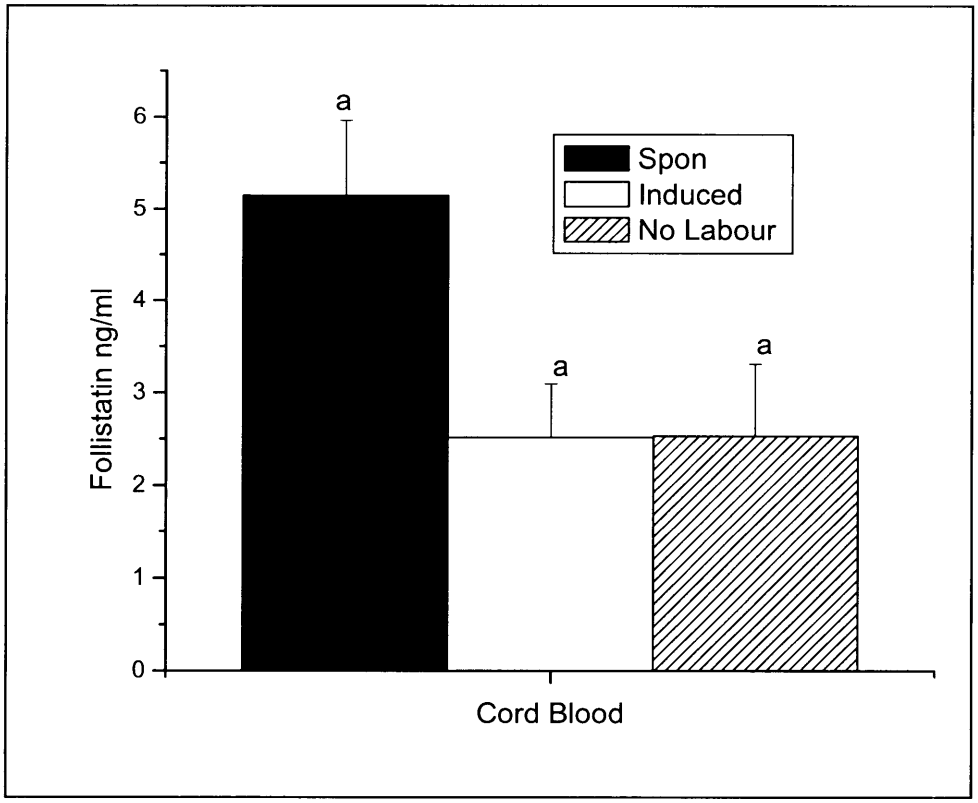


Figure 4-12 The effect of delivery type on cord blood follistatin concentrations. Significant differences are shown in lower case letters.

Figure 4.12 shows the effect of delivery type on fetal fetal cord follistatin. Whilst the spontaneous onset of parturition creates increased follistatin concentrations in the fetus (5.32 ng/ml ± 0.94) when compared to both induced (2.52 ng/ml ± 0.58) and LSCS delivery (2.53 ng/ml ± 0.78), due to the high variability between spontaneous patients it was not deemed significant.

Follistatin shows an obvious fetal gender effect regardless of delivery type however due to small sample sizes it is not deemed significant, with males showing higher fetal follistatin than females (Vag Male- 5.36 ng/ml ± 1.3, LSCS Male- 3.30 ng/ml ± 2.1 versus Vag female- 3.67 ng/ml ± 0.71, LSCS Female- 2.15 ng/ml ± 0.8) as shown in Figure 4.13.

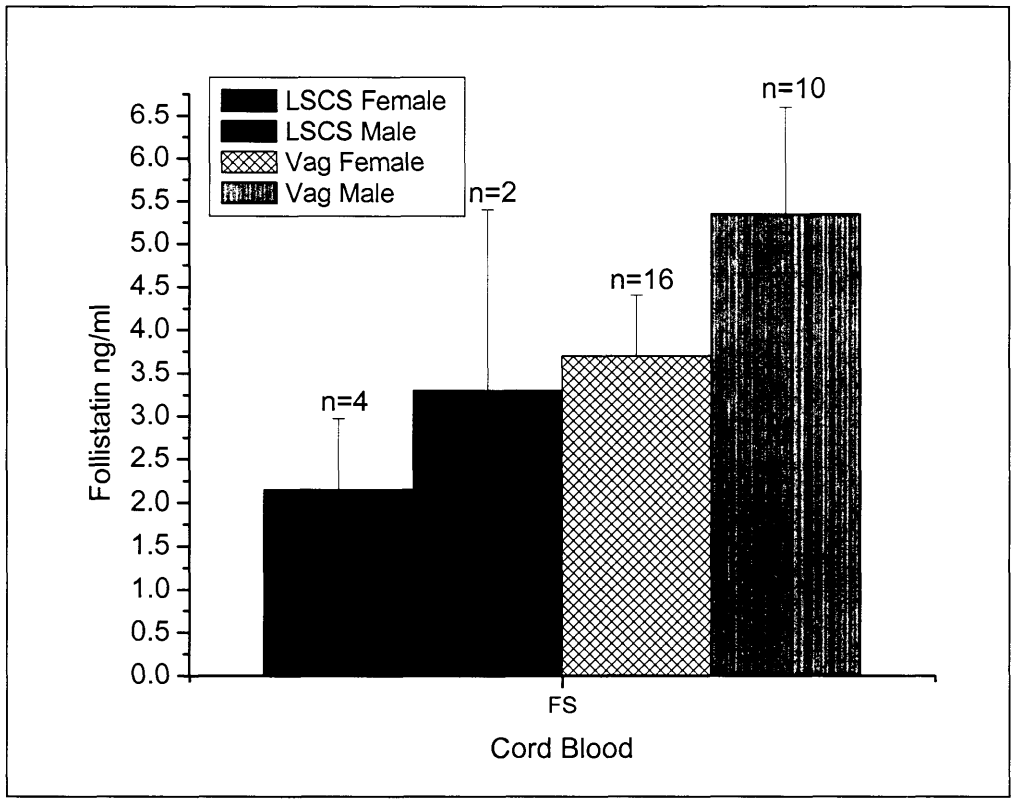


Figure 4-13 The effect of fetal gender on serum cord follistatin concentrations in vaginal and LSCS deliveries

Maternal serum follistatin was the analyte showing differences due to fetal gender and is shown in Figure 4.14, with mothers carrying male fetuses showing significantly higher serum follistatin Lab1 and Lab2 when compared to those carrying females (Lab1 Males- 30.9 ng/ml ± 0.3, Lab2 Males- 54.0 ng/ml ± 7.63, versus Lab1 Females-18.2 ng/ml ± 1.7, Lab2 Females -39.9 ng/ml ± 6.4). The effect of fetal gender appears to diminish following delivery.

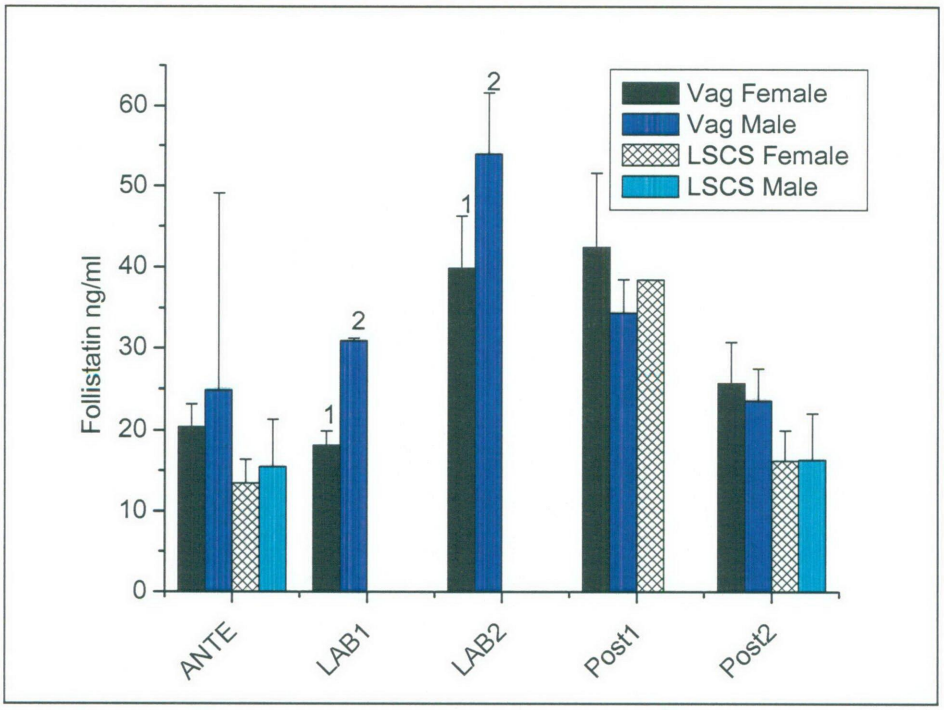


Figure 4-14 The effect of fetal gender on maternal serum follistatin concentrations. Significant differences between groups are shown with numbers.

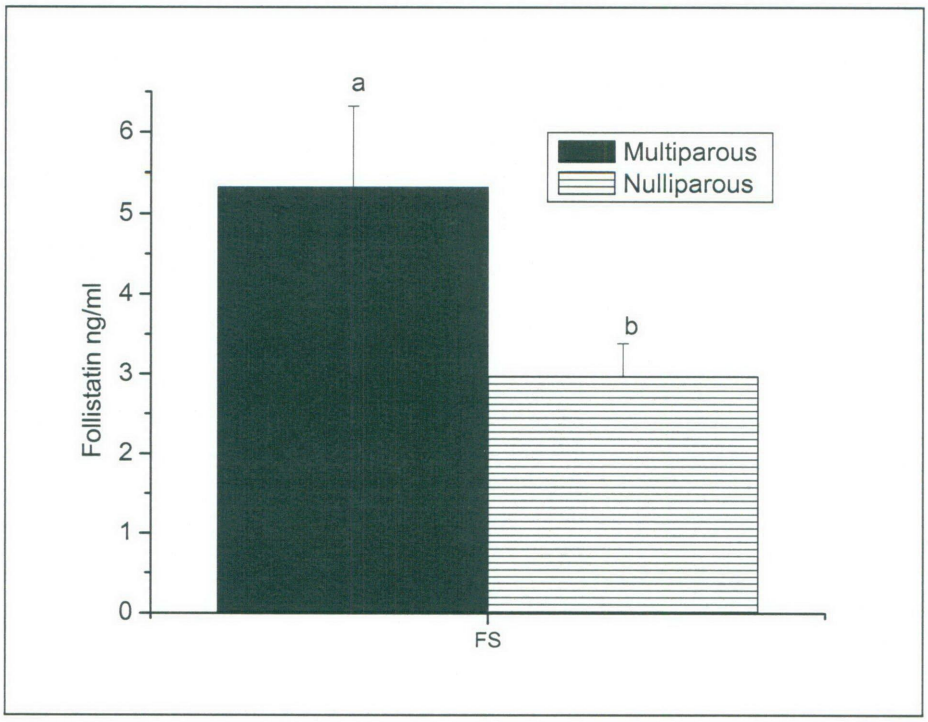


Figure 4-15 The effect of parity on cord blood follistatin concentrations. Significant differences shown with lower case letters.

Parity also effects the concentrations of follistatin in the cord blood with multiparous patients showing significantly increased follistatin in fetal cord blood (5.32 ng/ml + 1.0) when compared to nulliparous patients (2.97 ng/ml + 0.42) as shown in Figure 4-15.

C-Reactive Protein

As seen in Figure 4.16, C-reactive protein concentrations in all patient groups remain relatively steady from antenatal period and across labour into the early post partum period. However, in the late post-partum period, we see a 4-5 fold increase in C-reactive protein in all patients. Spontaneous patients increase from early postpartum 13.4 mg/L ± 3.96 to 43.7 mg/L ± 12.0 in the later post partum sample. Induced and LSCS patients increase from 11.0 mg/L ± 2.61 and 4.92 mg/L to 44.1 mg/L ± 9.69 and 55.2 mg/L ± 12.2 respectively.

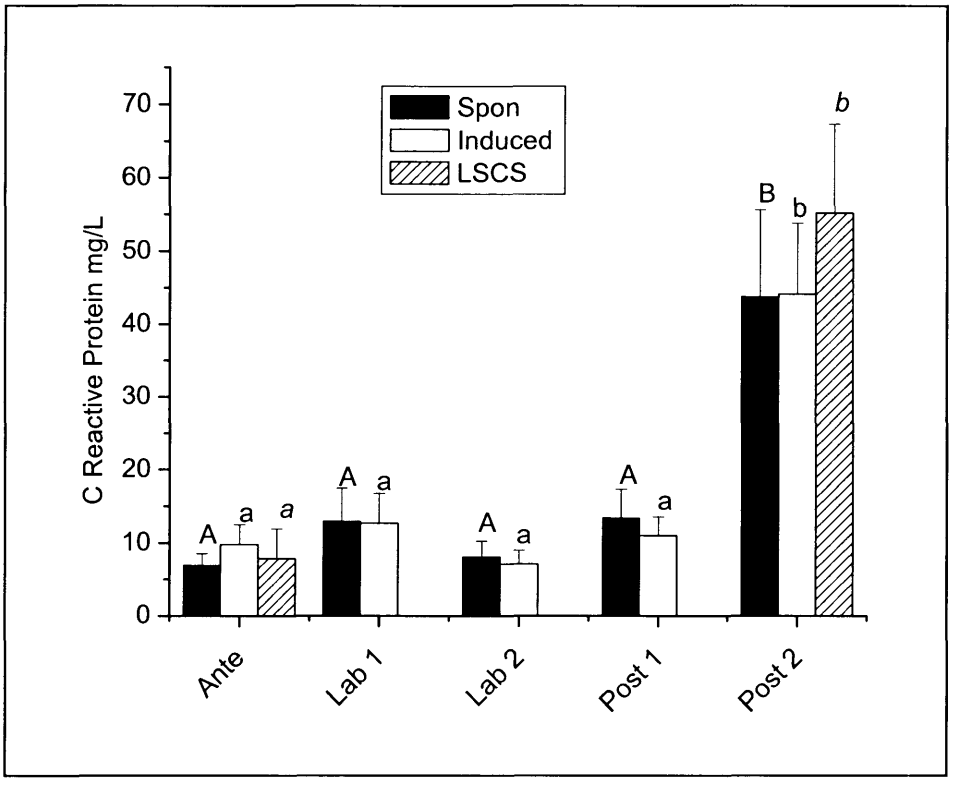


Figure 4-16 The effect of parturition on maternal serum C Reactive protein concentrations. Significant differences within the spontaneous onset group are indicated with upper case letters. Significant differences within the induced onset group are indicated with lower case letters. Significant differences within the LSCS delivery group are indicated with italicized lower case letters.

Fetal C reactive protein and the effect of delivery type is shown in Figure 4.17. Both Induced and LSCS delivery types showed similar fetal C reactive protein concentrations and minimal variation within their groups (0.21 mg/L ± 0.01 and 0.2 mg/L ± 0.01 respectively) whilst spontaneous deliveries show increased concentrations and great variability within the patient group (0.75 mg/L ± 0.55) and therefore no significant differences were noted between groups.

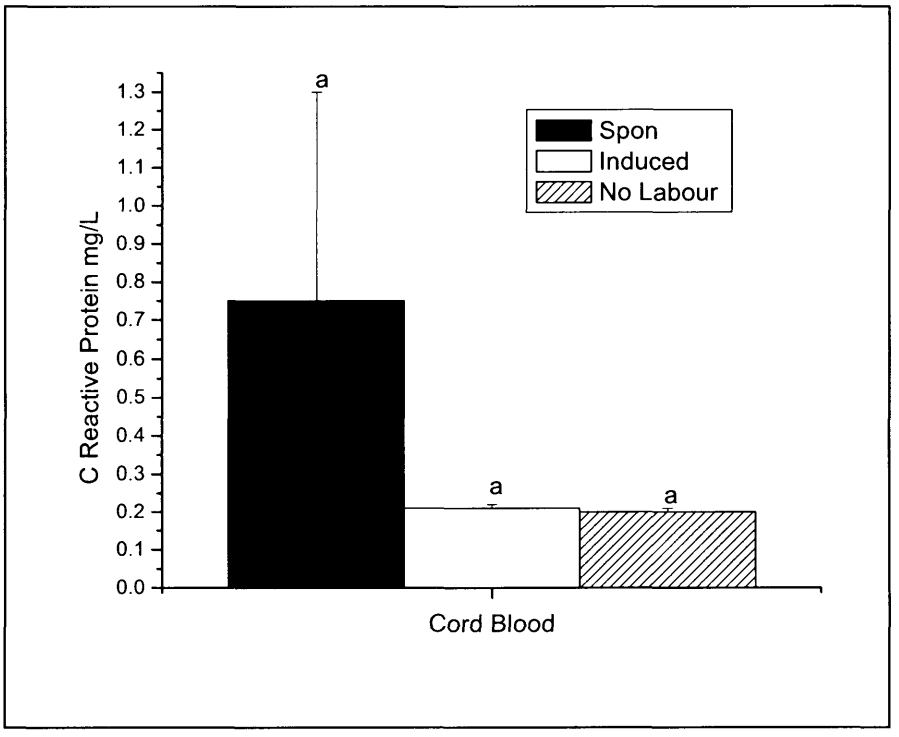


Figure 4-17 Fetal cord C Reactive protein and the effect of delivery type.

Figure 4.18 shows the changes in C Reactive protein and the effect of labour length on maternal serum. As shown in Figure 4.16 CRP shows no real changes until the late postpartum sampling period (Post2), and here it shows that the greatest increases seen in CRP due to length of labour are LSCS delivery and a long vaginal delivery (Vag Short- 31.7 mg/L ± 9.34, Vag Medium- 43.5 mg/L ± 11.2, LSCS-62.4 mg/L ± 11.4, Vag Long- 75.2 mg/L ± 21.7).

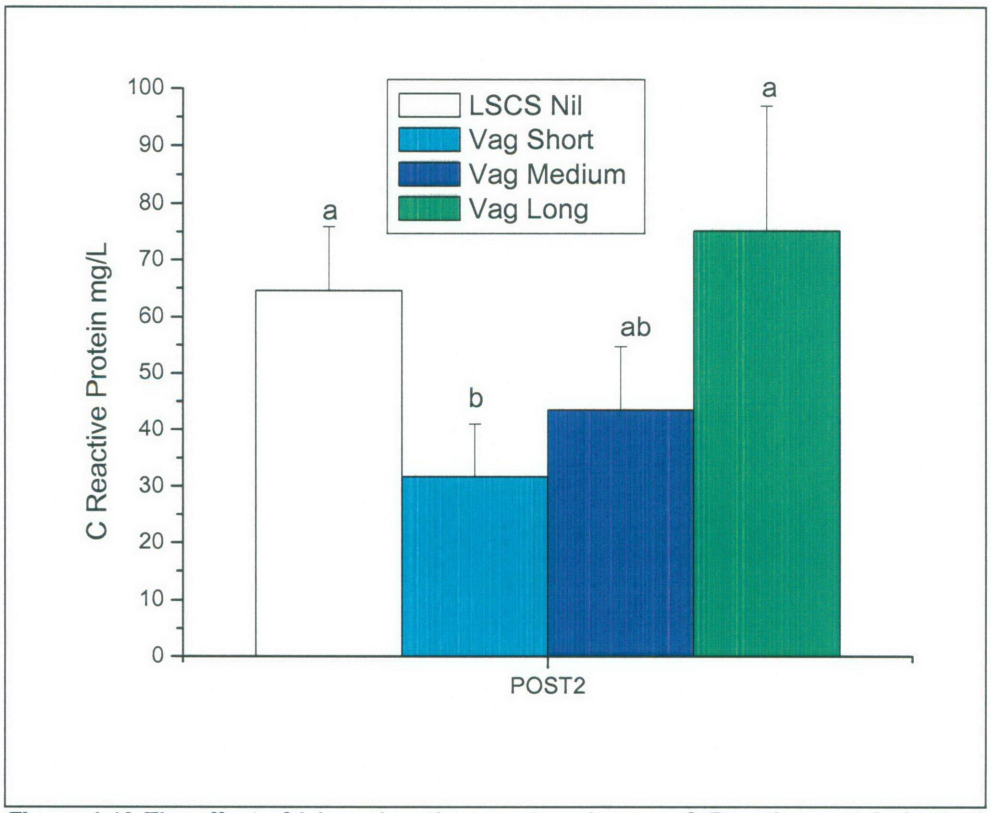


Figure 4-18 The effect of labour length on maternal serum C Reactive protein in the late post partum period. Significant differences are shown with lower case letters.

Activin

Activin is shown in Figure 4.19 and in the spontaneous labour patients we see activin significantly increasing from the antenatal sample (2.88 ng/ml ± 0.86) to reach its maximum (7.16 ng/ml ± 0.79) in late labour when the cervix is dilated to >3cm. The minimum activin concentration is reached in the late post-partum sample period (2.00 ng/ml ± 0.37). However in the induced patients we see the peak concentration reached in early labour (8.05 ng/ml ± 1.24) and then steadily decrease to the late post partum period (1.87 ng/ml ± 0.31). When comparing activin between groups we see significant differences between the two groups in the early labour period with induced labour

patients showing higher activin concentrations (8.05 ng/ml ± 1.24) than spontaneous labour patients (6.08 ng/ml ± 0.69).

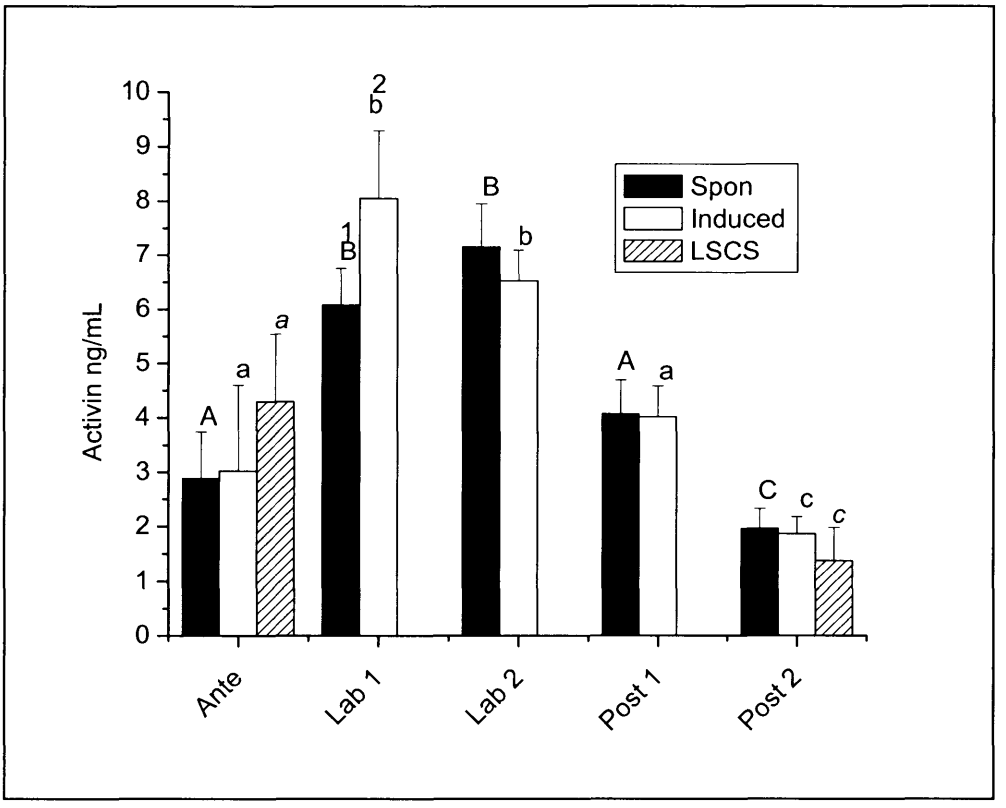


Figure 4-19 Activin across parturition. Significant differences within the spontaneous onset group are indicated with upper case letters. Significant differences within the induced onset group are indicated with lower case letters. Significant differences within the LSCS delivery group are indicated with italicized lower case letters. Significant differences between groups are indicated with numbers.

TNFα

Results for TNFα are surprising in the lack of obvious pattern for any onset group (Figure 4.20). Whilst there are no significant differences observed across any sampling period for any labour group or between labour groups, we do see a trend to increasing TNFα in early labour from the antenatal sample (Spontaneous labour patients Ante – 8.17 pg/ml ± 0.93 to Lab 1 – 18.2 pg/ml ± 3.8). In the spontaneous labour patient TNFα then remains relatively constant throughout all samples. Induced labour patients also show similar pattern (Ante – 8.92 pg/ml + 1.44 and Lab1- 15.72 pg/ml + 1.66) followed by

relatively constant TNFα results. Fetal TNFα assessed however it showed no differences in the effect of fetal gender, labour length or parity between groups (data not shown).

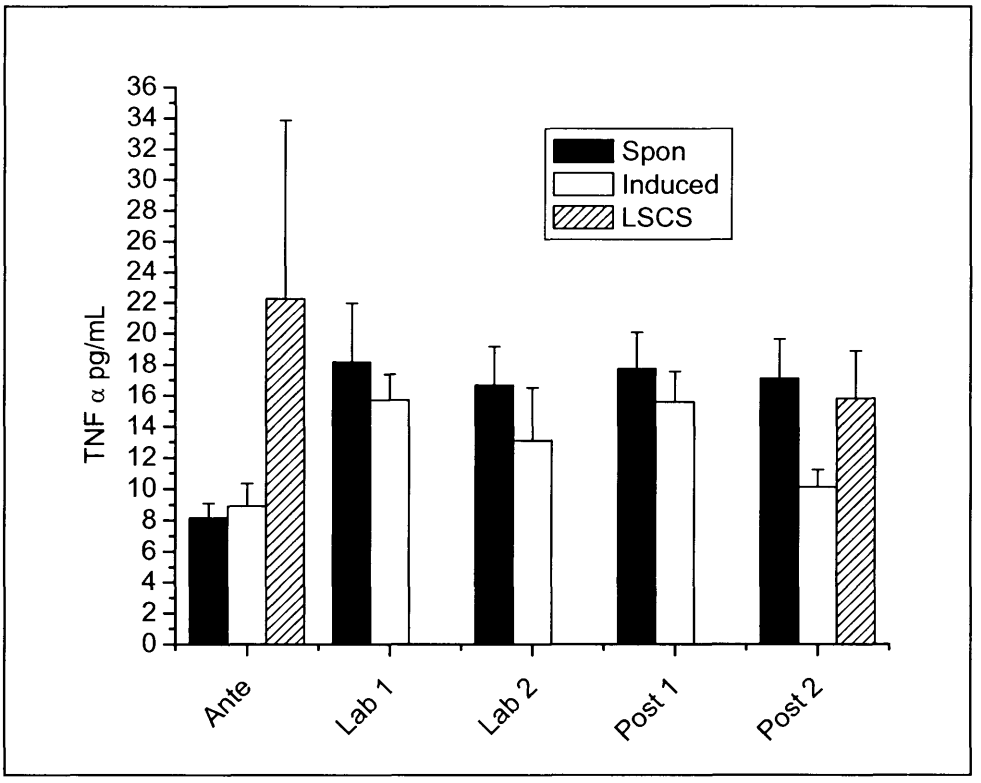


Figure 4-20 The effect of parturition on maternal serum TNFα concentrations. No significant differences noted.

4.6 Discussion

This study, which investigated the role of follistatin along with other reproductive hormones (progesterone, estradiol, prolactin), inflammatory markers (cortisol and C-reactive protein) and cytokines (activin and TNFα) has shown that there are clear indications of the involvement of follistatin in the parturition process. For some of the reproductive hormones considered there have been enlightening discoveries on potentially new areas for research.

Changes in estradiol and progesterone (Figure 4.2 and 4.3) respectively for the spontaneous and induced onset of labour as well as LSCS groups, progesterone and estradiol show continued increases from the antenatal to labouring samples. When the vaginal delivery patients are analysed using the type of onset, further differences were noted. It was not unexpected to see a trend for higher levels of progesterone and estradiol in the spontaneously labouring patient group. These patients have reached the final gestational stages and progesterone and estradiol levels have reached their peak values whilst in the induced patients, despite being at the end of the gestational period, the progesterone and estradiol levels are lower. The induced patients, for unknown reasons, do not go into natural labour and therefore the normal pattern of hormones in the final stages of pregnancy is altered.

The clearance of estradiol and progesterone from maternal serum is extremely rapid following delivery of the placenta as shown by the % concentration of hormones in Table 4-3. With the study by Kogure *et al.* (1996) indicating that the half-life for follistatin is approximately 4 minutes (Kogure *et al.*, 1996), if the placenta was the source of the follistatin in the maternal serum we would have expected to see concentrations for follistatin returning to antenatal concentrations or below within the first post partum period (< 3 hours from delivery). Whilst studies in Chapter 3 on mRNA show that isoform

FS315 is present in all patients while FS288 is seen only in spontaneous labour patients. As FS288 remains bound to cell surface it is unlikely that the high levels of follistatin seen in circulation are from the placenta. It also seems clear that follistatin remains elevated in circulation following placental delivery, whilst estradiol and progesterone fall to below antenatal concentrations rapidly. These results suggest that the follistatin increases seen in labour are from a different source, either in addition to placental follistatin or individually from another source such as systemic vascular endothelial cells, macrophages, the liver or the many other places capable of follistatin production.

There is mounting evidence to suggest that high levels of progesterone suppress the binding of oxytocin to the oxytocin receptor, which prevents activation of the uterine myometrium and production of prostaglandins (Dunlap & Stormshak, 2003; Friebe-Hoffmann, 2003). Therefore the reducing concentrations of progesterone seen in spontaneous patients once labour establishes, would thereby allow oxytocin greater binding ability to its receptor thus activating the actin and myosin fibres of the myometrium (Dunlap & Stormshak, 2003). However, in the medically induced group of patients, pharmacological doses of syntocin (the oxytocin derivative) used to induce labour create such high levels of oxytocin in the maternal system that the oxytocin receptor becomes saturated, activated and this increases contractility of the myometrium and stimulates prostaglandin production thus bypassing the natural onset process. Following delivery of child and placenta, the latter being the major site for estradiol and progesterone production in pregnancy, both spontaneous onset and medically induced patients have comparable levels of estradiol and progesterone for the remainder of the sampling period.

Prolactin is well known for its role in mammary development plus establishment and maintenance of lactation in the new mother (Buckman & Peake, 1976). Prolactin is produced by the maternal and fetal pituitary system throughout pregnancy as well as by

the decidua and membranes (Wang *et al.*, 1994). Both prolactin and oxytocin have long been interconnected for their joint roles in the suckling induced reflex arcs necessary for breast-feeding, however prolactin has not been previously profiled across labour. Recent studies have shown prolactin to be involved in the decidualisation process with its expression and synthesis in the decidua potentially regulated by activin A and follistatin (Jones *et al.*, 2002a).

In the induced labouring patient group (Figure 4.9), prolactin shows an increase in labour until > 3cm dilation when the mean value drops significantly for the rest of the sampling period. Further studies on prolactin in labour are necessary, particularly as the profile for the spontaneous onset patient shows a different pattern to the profile of the induced patient. With the increased levels of progesterone and estradiol in this patient group as well as the proposed increases in oxytocin availability, higher prolactin levels in this group were expected. These women are physically ready to give birth earlier than the induced patient group and therefore it was expected that prolactin would also be rising potentially in preparation for breast milk production, however we observed a trend of gradually rising prolactin in spontaneous women.

On detailed examination of the vaginal delivery group (Figure 4.9), it is observed that the induced patients have higher levels of prolactin in the later stages of labour than the spontaneous onset patients. Due to the large levels of introduced oxytocin used in the induction, it was expected that there would be additional increases in the observed levels of prolactin in the labouring samples of this group. In the postpartum samples it appears that that the cessation of the intravenous oxytocin administration decreases the subsequent levels of prolactin in the induced patient group to match those of the spontaneously labouring patient group.

As previously discussed prolactin has been linked to the stress response, and whilst no significant differences were noted (Figure 4.10), we noted a trend to increased prolactin in all patients from late labour to the end of the sampling period, with patients undergoing a short labour (<3 hours) having the highest prolactin. The significance of this observation is unclear, but anecdotal discussion with obstetricians and midwives have suggested that women undergoing a rapid delivery often go into shock and need subsequent treatment following delivery. Interestingly, cortisol data (data not shown) does not support the stress response in this patient group, suggesting that a different stimulus than stress has increased prolactin or prolactin is elevated for other undetermined reason in the short labour length group.

Whilst it has been reported that prolactin concentrations *in vitro* can be reduced by follistatin this study has failed to show those connections (Jones *et al.*, 2002a). Further, the previously documented increases across pregnancy (Buckman & Peake, 1976) were unable to be assessed here as insufficient sampling occurred in the very early antenatal period to establish a baseline value. However, given that prolactin is influenced by activin, stress, cortisol, feeding status etc, it is clear that complete understanding of prolactin's roles at parturition is multifactorial and therefore a much larger patient cohort and rigorous sampling protocol's are necessary to tease out its influence, if any, on parturition.

Although its role in the onset of parturition in women has not been clearly defined, fetal increases in cortisol levels by the adrenal cortex in other species such as sheep, initiates the timing of parturition. Cortisol is widely known as a stress hormone and shows increases with both physical and psychological stress. Cortisol is also known for its ability to stimulate gluconeogenesis in the liver when fasting, thereby increasing blood glucose levels. It has been identified as an anti-inflammatory agent that increases during

the acute phase response in order to suppress tissue response to injury (Riad *et al.*, 2002; Vogeser *et al.*, 2002).

In the antenatal period there are no differences in cortisol concentration between the groups (Figure 4.4). However, despite the small sample size at individual sample periods, it appears that patients are "less stressed" by caesarean section delivery as they show significantly lower cortisol concentrations in the postpartum stages than the patients undergoing vaginal delivery (Figure 4.4). Given the intense physical demands and the length of time required to sustain the physical strains of a vaginal delivery, this result is not surprising, although a larger LSCS patient cohort is necessary to determine the accuracy of this observation.

When considering the vaginal delivery patients results (Figure 4.4), these indicate that the spontaneous onset patients have slightly more cortisol postpartum when compared to the patients undergoing induction. Between the spontaneous and induced onset patient groups it was noted (Table 2.2) that the induced patients had reduced time of labouring when compared to the mean labouring time for the spontaneous onset patients (4.5 hours to 6.5 hours respectively). The increased levels of cortisol in the early postpartum stages for the spontaneous onset patients could therefore be attributed to the extended physical stress necessary for the mothers of this group. By 1-6 hours postpartum both groups show significantly different results show similar results, suggesting the stimulus for the maternal release of cortisol is stopped almost immediately following birth. By late postpartum results between groups are significantly different however in reverse order with induced patients showing higher cortisol concentrations that the other groups.

Cortisol is greatly increased in the fetus' that have undergone a vaginal delivery along with the maternal serum (Figure 4.4, Figure 4.5, Figure 4.6 and Figure 4.7) and has

been previously reported in other studies (Kubota *et al.*, 1987). It also appears that a longer vaginal labour increases the cortisol secretion of the fetus (Figure 4.7), with the fetal cortisol in long labour (737.1 nmol/L ±154.4) almost double that seen in either short (435.1 nmol/L ± 39.4) or medium labour (415.3 nmol/L ± 58.3). Fetal blood flow is estimated at 104.2 ml/min/kg which equates to approximately 343 ml/min in the average 3.2kg fetus (Ferrazzi *et al.*, 2001). Maternal blood volume is approximately 6-7 litres assuming a 30% increase in blood volume through pregnancy. At volumes such as these it is possible that the rise in cortisol seen in maternal serum through labour could potentially be fetal in origin. The increases noted from the antenatal samples to labouring samples (Figure 4.4) are approximately equal (Figure 4.7 ~400-500 nmol/L) to the concentrations seen in fetal cord samples. When data were reviewed in light of fetal gender, no alterations of cortisol due to fetal gender were observed.

Interestingly, it appears that the number of previous pregnancies impact on the concentrations of fetal cortisol, with children from multiparous patients showing decreased cortisol concentrations. The significance of this finding is unclear, however we speculate that with an increased number of previous deliveries that labour length tends to reduce and therefore a reduced fetal cortisol is not surprising. .

C-reactive protein (CRP) results (Figure 4.16) confirmed the work of previous studies that have shown that CRP is not a useful marker for onset of labour (Young *et al.*, 1991). In all labouring patients CRP had no significant change in maternal serum until the postpartum period (Figure 4.16). These results also showed no apparent correlation with follistatin in labour, despite both proteins being associated with the acute phase response.

C-Reactive protein (Figure 4.18) shows no changes in any patient group until the late postpartum sample (>3hours following delivery). This is the first sign of the acute phase

response occurring and the data clearly shows that labour length affects the concentrations of acute phase proteins being released with longer labour patients having significantly higher CRP (75.18 mg/L ± 21.73) compared to medium (43.51 mg/L ± 11.2) and short (31.67 mg/L ± 9.34). In fact the acute phase response generated in longer labour patients is similar to that generated by those patients undergoing a surgical delivery (62.4 mg/L ± 11.4) and suggests that the inflammatory responses generated in these two patient groups is similar, which has implications in the postpartum healing and potential treatment options for these women.

In the acute phase response, CRP appears to be released from the hepatocytes of the liver within 6-12 hours of the stimulus (Gewurz *et al.*, 1982) indicating the acute phase response becomes activated in all postpartum women as part of the uterine wound inflammation 6 hours following delivery regardless of type of onset or delivery. This is consistent with other types of stimulus to the acute phase response (Gewurz *et al.*, 1982). Additional studies over an extended time period would be interesting to determine how long CRP remains elevated in the postpartum female. With the activation of the acute phase response in all patients groups it is surprising that serum cortisol postpartum (Figure 4.4) in the LSCS group have remained lower despite the increased C-reactive protein as cortisol concentrations also rise in the acute phase response (Vogeser *et al.*, 2002).

In the fetus, C-reactive protein concentrations remain low in both induced and LSCS delivery groups, whilst in the spontaneous onset group results are elevated and highly variable. This suggests that a proportion of spontaneous onset deliveries have had the acute phase pathway stimulated prior to delivery in the fetus however, the significance of this is unknown.

This study confirmed reports by other groups that activin A is higher in labouring women when compared to the antenatal samples (Figure 4-19). Fetal cord and maternal serum

showed no patterns in activin concentrations when analysed in light of either fetal gender or labour length (data not shown).

Interestingly our data shows that activin is higher in early labour than in the antenatal period in both the spontaneous onset and induced patients whilst Schneider-Kolsky *et al.* (2000) showed the reverse (Schneider-Kolsky *et al.*, 2000). This study used the same assay and antibody as our study and were analysed by the same laboratory group. They reported concentrations of 16.6 ng/ml at 40 weeks gestation which then fell to 10 ng/ml in early labour. The average gestation for our antenatal samples was 35 weeks gestation so that we may have underestimated activin concentrations. As labour becomes further established the observed concentrations peak at approximately 14.6 ng/ml, however these concentration changes were not deemed significant. When comparing the two studies, Schneider-Kolsky *et al.* (2000) had also separated patients into induced and spontaneous onset of labour and showed no differences between these two patient groups. The differences seen between the published data of Schneider-Kolsky and the data presented in this chapter is perhaps a reflection of the medical complications of the patients chosen by the Schneider-Kolsky study. In the work of this thesis patients with any medical complications were excluded from the study

Interestingly our study has shown that activin increases significantly from the antenatal period to peak in early labour in both groups. It also has shown that activin A is increased in the induced patients when compared to spontaneous onset of labour patients. Work by Sawchenko *et al.* (1988) and more recently Florio *et al.* (1996), showed that oxytocin and activin showed the ability to effect the concentrations of each the other, so that perhaps the increased concentrations we noted in the induced patients is due to an up-regulation of activin due to the syntocin (oxytocin derivative) used in induction (Sawchenko *et al.*, 1988; Florio *et al.*, 1996). Postpartum concentrations of activin fall below the antenatal concentrations in all patient groups and are indicative that

the placenta is the source of the elevated maternal activin seen in pregnancy and parturition. Despite studies showing links between activin and prolactin in decidualisation this present study showed no correlations between activin and prolactin across labour or postpartum.

As discussed in Section 1.6.1, follistatin binds to activin in a 2:1 ratio (Shimonaka *et al.*, 1991; de Winter *et al.*, 1996; Thompson *et al.*, 2005). When we compared molar quantities of follistatin (using average molar weight of 32.5kDa) to activin (using average molar weight of 24kDa) we see that follistatin molar concentrations are still well above those needed to suppress activin activity. Activin molar concentrations are at ~0.25 moles/ml and therefore needs ~0.50moles/ml follistatin to suppress bioactivity. Instead follistatin molar concentrations are approximately 9-fold that of activin (Follistatin 1.8 moles/ml; Activin 0.2 moles/ml). At such increased concentrations of follistatin compared to activin, we would anticipate that these increases are not due to solely to suppression of activin bioavailability but to fulfill a role distinct to follistatin. This study shows very different follistatin concentrations in labour and post-partum for spontaneous patients when compared to induced patients, whilst activin was essentially the same for both patient groups. We therefore suggest that activin is not the target for follistatin.

Whilst it is well established that cytokines such as TNFα are involved with the initiation and mediation of inflammation, and that TNFα has been found in cervicovaginal secretions at term (Bowen *et al.*, 2002), this study showed no particular pattern suggesting any involvement with the onset of parturition or any particular association with type of labour onset. Possibly with a larger cohort of patients greater detail of TNFα and its roles in inflammatory events surrounding labour would emerge, however results obtained here suggest otherwise.

The aim of this study was to determine if follistatin is involved in the labouring process so that its potential use as a marker or clinical treatment target for preterm labour could be

further evaluated. Previous studies have been contradictory for follistatin's involvement in parturition. Some studies have shown follistatin increases in maternal serum with gestation however none of these groups has examined follistatin concentrations across the labouring process (Wakatsuki *et al.*, 1996; Woodruff *et al.*, 1997; Evans *et al.*, 1998; O'Connor *et al.*, 1999).

Studies have also shown high levels of follistatin in the placenta, amnion and choriodecidua (Petraglia, 1997; Keelan *et al.*, 1999b). Studies of the uterine myometrium have shown that whilst other myometrial proteoglycans decrease, heparan sulfate proteoglycans increase by 46% in active labour, (Hjelm *et al.*, 2002) which can potentially increase the interactions with follistatin. One of the roles of heparan sulfate proteoglycans is to influence the size and function of gap junctions (Hjelm *et al.*, 2001). Throughout labour studies also have shown the size and number of gap junctions to increase and it is thought that this is to promote the actions of the uterine muscular synctium necessary to ensure progressive contractions (Hjelm Cluff *et al.*, 2005; Cluff *et al.*, 2006).

The greatest heparan sulfate proteoglycan increase, as measured by Hjelm *et al.* (2002), coincided with the highest concentrations for follistatin measured in this study. This study has shown total follistatin to change positively through parturition in the spontaneous group (Figure 4.11) with follistatin concentrations increasing with vaginal dilation through the labouring process peaking at >3 cm dilation, when active labour is fully established. It also shows follistatin is elevated in women who are spontaneously labouring when compared to those undergoing an induction. Of particular interest is that in spontaneously labouring women who have only minimal vaginal dilation (0-3 cm) still show significant differences from the antenatal period, indicating that follistatin is rising whilst the pre-labour stage of cervical ripening occurs. Although activin A is also

increasing the quantity of follistatin in maternal circulation greatly exceeds that needed for the suppression of activin bioavailability.

Although research by Schneider-Kolsky *et al.* (2001), showed neither activin β_A subunits nor any of the three activin receptors were immunolocalised to the myometrial smooth muscle cells in active labour, perhaps the elevations noted in this study are due to the actions of the follistatin/activin complex or those of follistatin interacting with other members of the TGFβ family. The presence of FS288 mRNA in the spontaneous patient seen in Chapter 2 indicates the possibility of an interaction between FS288 and heparan sulfate proteoglycans, perhaps at the maternal decidual junction of the placenta, however these preliminary results are many steps away from a complete picture. *In situ* hybridization studies would be useful to determine the exact location of each of the follistatin isoforms in both the placenta and myometrium.

Schneider-Kolsky *et al.* (2000) performed a similar study on parturition using 36 women in spontaneous labour and plasma was assessed for both follistatin and activin. Results were correlated with cervical dilation, however no changes to follistatin concentrations were shown across labour. In the same study a group of 9 women undergoing inductions had serum collected at 60 minute intervals from the time of the amniotomy to delivery. However it should be noted that a number of these women had pre-existing medical conditions such as gestational diabetes and non-proteinuric hypertension. This group of women also showed no significant changes in either follistatin or activin. The Schneider- Kolsky *et al.* (2000) study utilised a different follistatin assay/antibody to the one used in this present study and therefore the results obtained are potentially measuring different isoforms from the assay utilized here. When considering all follistatin results, including these most recent, one must consider the multiplicity of follistatin isoforms and the potential diversity of actions of each of these isoforms.

Despite some evidence in other studies to the contrary, it is clear from our results that follistatin is likely to have a role to play in both the onset and parturition process. The data from Schneider-Kolsky *et al.* (2001) in labour, showing no changes to follistatin, along with this studie's finding suggest the potential for the isoforms for follistatin being differentially regulated across parturition of women (Schneider-Kolsky *et al.*, 2001). Recent *in vitro* work by Sidis *et al.* (2006), suggested that *in vivo* isoforms for follistatin have distinctly different roles, dependant on both the cell surface binding ability and on the localisation of both the protein and biosynthesis site. This work suggested that the heparin binding sequence along with the cell surface interactions of the isoform was critical to the role of follistatin, so that different isoforms will modify the actions of activin and other members of the TGFβ family differently (Sidis *et al.*, 2006).

There appears to be an effect due to fetal gender, with males showing higher fetal follistatin regardless of delivery type and mothers carrying males (Figure 4.13 and Figure 4.14) also showing a trend to higher serum follistatin throughout the antenatal and labour sampling period, however, this effect is abolished once delivery has taken place. Despite these results indicating that the male fetus is responsible for the high follistatin seen in maternal circulation when carrying males, the concentrations follistatin in the male fetus (4.96 ng/ml $\pm$ 1.2) are markedly below those observed in maternal circulation in any of the sampling periods (eg Lab1- 30.9 ng/ml $\pm$ 0.3), suggesting that a response to the male fetus is occurring within the maternal system.

Follistatin in the fetus at term appears to be affected by the number of previous pregnancies of the mother with the cord blood of fetus' from multiparous pregnancies higher that nulliparous pregnancies (Figure 4.15).

Studies on stress have linked prolactin and cortisol release to the physiological stress response, whilst clear links in our data have not been shown, perhaps using the length

of labour would find those correlations. When reviewing follistatin's results along with the stress marker, cortisol and the acute phase protein, C-reactive protein (Figures 4.4, 4.11 and 4.16), we see that C-reactive protein shows no correlations with the increases seen in follistatin in the spontaneous patient. However, in the spontaneous patient follistatin rises slightly in early labour and markedly at late labour whereas cortisol rises at early labour remains constant at late labour and is highest at early postpartum. The cortisol concentration increases show no correlations with follistatin. This suggests that follistatin increases due to stimuli other than that of stress or the acute phase response. While follistatin has been implicated in the acute phase response (de Kretser *et al.*, 1999) this clearly shows that the acute phase response we see triggered post partum is unrelated to follistatin, suggesting that follistatin is not driving the acute phase proteins post partum. Perhaps the increases in follistatin that we have documented in late labour and early postpartum are the trigger for the adrenals to release glucocortiods (Vanttinen *et al.*, 2002) that then initiate or propagate the release of the acute phase response proteins from the liver to assist in the post-partum 'mop up' and uterine re-modelling . We do note that follistatin concentrations remain elevated above antenatal concentrations in the post-partum period in all labour patient groups suggesting that the placenta is not the major source of the follistatin seen in maternal serum.

Whilst previous studies of inflammatory states, such as septicaemia, meningitis, and the post-operative state (Michel *et al.*, 1998; Michel *et al.*, 2000; Michel *et al.*, 2003) show that follistatin parallels the timing of the inflammatory marker CRP, this is not the case in labouring women and postpartum inflammation. As shown in Figure 4.11 and Figure 4.16, follistatin peaks at >3 cm dilation whilst C reactive protein peaks 6-12 hours postpartum. This would indicate that the inflammatory responses postpartum are distinct from that in the cervical ripening stages of parturition. Follistatin has been implicated previously in wound repair (Hubner *et al.*, 1996; Munz *et al.*, 1999b; Wankell

et al., 2001a; Wankell *et al.*, 2001b) and although decreases in follistatin values postpartum are evident in Figure 4.11, further studies with a range of inflammatory cytokines or an extended sampling period may help to differentiate follistatin's role in the postpartum healing process.

Although we have shown that follistatin is involved with the natural labouring process, there are still many aspects that need to be considered for further research. Using willing volunteers, daily serum measurements from 38 weeks would help establish our initial findings that follistatin is involved in the cervical ripening stage of parturition prior to active labour, as well as a longer period of sampling postpartum. We have previously reported that follistatin in sheep rises on the day of parturition and remains elevated in the 24-48 hours following parturition, therefore a longer sampling period would be valuable to assess this in women (McFarlane *et al.*, 2002). However, the placenta in sheep is of a different nature to that seen in women and it remains attached to the uterine wall for up to 48 hours.

Overall, this study has shown follistatin is strongly implicated in the spontaneous labouring woman, however further research is required to determine the exact role it plays in the initiation of parturition. This study looking at other effects on follistatin has clearly shown that follistatin is influenced by fetal gender in both the fetus and the mother, and that the maternal system responds to the gender of the fetus. Interestingly, activin showed no such effect, suggesting that follistatins roles in response to fetal gender are not due to the modulation of the actions of activin but are of follistatin alone.

It is interesting to speculate the sources for the greatly increased concentrations of follistatin in maternal circulation during labour. Clearly, the fetus is not the source, and it is unlikely that the placenta is as follistatin remains elevated in the postpartum periods long after the placenta has been removed. With such widespread follistatin mRNA and

protein distribution (Table 1.1) it will remain an extremely difficult task to determine the source. As shown in Chapter 3, follistatin isoforms show a differential pattern of mRNA expression in the placenta and it remains to be determined which isoforms are responsible for the increases noted in maternal serum and the role these play in maternal responses to fetal gender and labour length.