Modelling physiological reproductive inflammatory networks *in vivo.*

Sarah Louise Field

Submitted in accordance with the requirements
for the degree of Doctor of Philosophy

The University of Leeds
School of Medicine

September 2014

I confirm that the work submitted is my own work, except where work has formed part of jointly authored publications has been included. The contribution of myself and other authors to this work has been explicitly indicated below. I confirm that appropriate credit has been given within the thesis where reference has been made to the work of others.

Abstracts

Field SL, Cummings M, Burns PA, Simpson NAB, Orsi NM. “Immune-related pathways are activated in murine endometrial epithelial and stromal cells in response to mating”. Society for Gynaecological Investigation Conference 2014 (Florence). Contributions to this publication were: SL Field – sample collection, laboratory analyses, data analyses and presentation, writing of the abstract and preparation of the poster. M Cummings – laboratory analyses and student support. NAB Simpson and PA Burns – student supervision. NM Orsi – student supervision and comments on abstract/poster. This abstract was based on part of Chapter 2 – Results.

Orsi NM, Dasgupta T, McSara H, Savage R, Adebayo J, Cummings M, Gunawardena J and Field SL. “Bayesian Modelling of Murine Lactational Networks In Vivo”. Society for Gynaecological Investigation Conference 2014 (Florence). Contributions to this publication were: SL Field – laboratory analyses, data analyses and presentation, network interpretation, writing of the abstract and preparation of the poster. T Dasgupta and J Adebayo – preparation and interpretation of the network data. H McSara – sample collection. R Savage – statistical analyses. M Cummings – network interpretation and comments on first draft of poster. J Gunawardena – review of network analyses. NM Orsi – student supervision, network interpretation and comments on abstract/poster. This abstract was based on part of Chapter 3 – Results.

Field SL, Sharma V, Ekbote UV, Gopichandran N, Baskind NE, Walker JJ, Simpson NAB, Burns PA & Orsi NM. “Cytokines in follicular fluid: a potential tool to define oocyte maturity in intracytoplasmic sperm injection?” Society for Reproduction and Fertility Conference 2010 (Sutton Bonnington). Contributions to this publication were: SL Field – sample collection and laboratory analyses, patient selection and application of inclusion/exclusion criteria, data analysis and presentation, writing of the abstract and preparation of the poster. V Sharma and JJ Walker – Directors of clinical studies. UV Ekbote – sample collection and laboratory analyses. N Gopichandran and NE Baskind – collection of clinical and demographic data. NAB Simpson and PA Burns – student

supervision. NM Orsi – student supervision and comments on abstract/poster. This abstract was based on part of Chapter 4 – Results.

Publications

Field SL, Dasgupta T, Cummings M, & Orsi NM (2013). "Cytokines in ovarian folliculogenesis, oocyte maturation and luteinisation." Molecular Reproduction and Development 81(4):284-314. Contributions to this publication were: SL Field – literature review, inclusion/exclusion of studies, manuscript preparation and submission. T Dasgupta – preparation of Bayesian network figure, review of Bayesian network section. M Cummings – comments on first and final draft of manuscript. NM Orsi – supervision of SL Field, comments on first and final draft of manuscript. This paper forms part of Chapter 4 – Introduction.

Acknowledgements

This thesis would not have been possible without the support of countless others. It has been a testament of dedication and determination.

Chapter 2: Inflammatory pathways in the murine uterine response to seminal plasma.

This research has been carried out by a team which has included myself, Dr Michele Cummings and Dr Nicolas Orsi. Michele Cummings provided invaluable help with the LCM and microarray work, assisting in the laboratory in all aspects. Nicolas Orsi provided supervision and comments on the design of the study and the results.

Chapter 3: Bayesian modelling of cytokine interactions *in vivo*.

This research has been carried out by a team which has included myself, Dr Tathagata Dasgupta, Hema McSara, Dr Richard Savage, Julius Adebayo, Dr Jeremy Gunarwardena and Dr Nicolas Orsi. Dr Tathagata Dasgupta and Professor Jeremy Gunawardena (with support from Julius Adebayo) provided the expertise and equipment to generate the Bayesian networks, and aided with interpretation of the results. Hema McSara completed the animal work. Dr Richard Savage assisted with the preparation of the hierarchical cluster analysis and heatmap. Dr Nicolas Orsi provided supervision and comments on the design of the study and the results.

Chapter 4: Modelling follicular fluid cytokines in relation to oocyte maturation.

This research has been carried out by a team which has included myself, Dr Tathagata Dasgupta, Julius Adebayo, Professor Jeremy Gunawardena and Dr Nicolas Orsi. In addition, samples and demographic data were provided by the Leeds Reproductive Unit, under the supervision of Dr Vinay Sharma. Dr Tathagata Dasgupta and Dr Jeremy Gunarwardena (assisted by Julius Adebayo) provided the expertise and equipment to generate the Bayesian networks, and aided with interpretation of the results. Dr Nicolas Orsi provided supervision and comments on the design of the study and the results.

I would like to thank my supervisors. Firstly, Dr Nicolas Orsi, to whom I am indebted for support to undertake this course of study on a part-time basis. This thesis would not have been completed without your academic experience, support and guidance, given despite completing a medical degree and foundation years as a junior doctor. I hope I have lived up to your drive for perfection, and I thank you wholeheartedly for everything you have done. I thank Mr Nigel Simpson and Dr Philip Burns for your guidance and pastoral care, essential aspects of any supervision team. Again, without your guidance and encouragement this thesis would not have been possible.

Dr Tathagata Dasgupta has provided invaluable discussion on the design, structure and meaning of Bayesian network analysis, and contributed to the methodology within this thesis. His unique approach to network discovery (assisted by Julius Adebayo) has enabled the advances in biological knowledge within this thesis, and I hope to continue our good work in the future. Professor Jeremy Gunarwardena welcomed me into his laboratory on my visit to the Systems Biology department at Harvard Medical School for which I am grateful.

The team at the LIBAC and LICAP laboratory have been inspirational and without whom I would have been lost. I especially would like to thank Uma Ekbote, who was a constant colleague in the lab and furnished some great discussions as well as helping with the Luminex marathon, for which I am greatly indebted. Also, Dr Michele Cummings, who provided both practical and emotional support. I hope to continue working with the team at Leeds and look forward to a fruitful scientific future.

The clinical sample collection and data harvesting could not have been done without the dedication and accuracy of the team at the Leeds IVF unit. I particularly thank Mrs Vinay Sharma and her team for providing the clinical samples, and Drs Ellissa Baskind and Nadia Gopichandran for collecting all the information for the massive follicular fluid database. Nadia in particular has become a very close friend, and I have enjoyed putting the world to rights with you!

There have been several experiments which (for the sake of my examiners and for my own sanity!) have not made it into this thesis. Firstly, I would like to thank Dr Julie Fisher and Dr Cassey McCrae for welcoming me into their lab to conduct NMR analysis on follicular fluid. It was a shame that this technique was not sensitive enough to detect differences, but I learnt a lot and spent many a happy hour in your company! Secondly,

I would like to thank Dr Anna Nicolaou and Dr Karen Massey for teaching me mass spectrometry.

This thesis is dedicated to the memory of my dad, Kenny Ebbrell, who passed away while I was writing. I hope I have done you proud.

Finally, but by no means least, my family! Thank you to my mum Bev and her partner Cath, as well as my in laws Liz and Garry. In particular, thank you to Liz for many hours of childcare! I thank my husband Chris and my children Max and Aimee for supporting me through this PhD journey. Although it has taken nearly 6 years to complete, it has been a great experience and very much added to my skills as a scientist. I can safely promise, though, never to do it again! I look forward to returning to ‘normal’ and enjoying our family time.

Abstract

The immune and reproductive systems have long been known to be inextricably linked, with components of immune pathways, particularly cytokines, mediating processes such as ovarian/menstrual cyclicity, endometrial remodelling, mating-induced immunomodulation, implantation, pregnancy, parturition and lactation. The nature of this involvement has often been investigated at the level of single mediators, with little consideration of the fact that cytokines are increasingly understood to function as complex networks. This study aimed to characterise inflammatory networks using both traditional and novel machine-learning Bayesian network-based methods in the context of keystone aspects of reproduction, *viz*., in the endometrial response to seminal plasma, cytokine:hormone interactions during lactation, and oocyte maturation following controlled ovarian hyperstimulation.

'Traditional' pathway analyses used to examine the murine endometrial response to seminal plasma revealed previously unidentified mediators and showed compartmentalised epithelium/stroma-specific responses. However, they proved ineffective in describing novel cytokine interactions. This led to the development a highly effective novel Bayesian network-based approach to explore cytokine:hormone networks during murine lactation. This revealed that prolactin, a putative potent immunomodulator, was far less influential than expected *in vivo*. The method also identified previously unknown cytokine interactions and described features such as synergy and antagonism. Further refinement of these network analyses as modified variational Bayesian state space models enabled the display of core, conserved subnetworks (communities) of human follicular fluid cytokines whose interactions varied with oocyte maturity. Moreover, these cytokine signatures also allowed the prediction of an oocytes' fertilisability potential, with potential attendant benefits to assisted conception.

This thesis represents the first endeavour to model inflammatory networks *in vivo* in any setting to date. It has revealed their central role, functional conservation and key features of cytokine interactions across a spectrum of reproductive processes. Further development of this methodology appears set to offer invaluable new insights into the complex immune signalling that underpins reproductive biology.

Contents

List of Figures

List of Tables

Abbreviations

ACU	assisted conception unit
AMH	anti-Müllerian hormone
APC	antigen presenting cell
AREG	amphiregullin
ART	assisted reproductive technology
bFGF	basic-fibroblast growth factor
BHC	Bayesian hierarchical cluster
BMP	bone morphogenetic protein
BTC	betacellulin
cAMP	cyclic adenosine 3', 5'-monophosphatase
CCR	correct classification rate
CD	cycle day
CGH	comparative genomic hybridisation
CL	corpus luteum
COC	cumulus oophorus complex
COH	controlled ovarian hyperstimulation
COX	cyclooxygenase
cRNA	complementary ribonucleic acid
CRP	C-reactive protein
CSF	colony stimulating factor
CTACK	cutaneous T-cell attracting chemokine
CNTF	ciliary neurotrophic factor
Cy3	cyanine
DAG	directed acyclic graph
DC	dendritic cell
DFA	discriminant factor analysis
E_2	17β-oestradiol
ECM	extracellular matrix
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor

ELISA	enzyme-linked immunosorbent assay
EREG	epiregullin
FISH	fluorescence *in situ* hybridisation
FF	follicular fluid
FGF	fibroblast growth factor
FSH	follicle stimulating hormone
GC	granulosa cell
G-CSF	granulocyte colony stimulating factor
GDF	growth differentiation factor
GM-CSF	granulocyte macrophage colony stimulating factor
Gn	gonadotrophin
GnRH	gonadotrophin releasing hormone
GRO-α	growth-regulated oncogene-α
GV	germinal vesicle
Has	hyaluronidase synthase
hCG	human chorionic gonadotrophin
HFEA	Human Fertilization and Embryology Authority
HGF	hepatocyte growth factor
HMG	human menopausal gonadotrophin
ICAM	intercellular adhesion molecule
ICSI	intra-cytoplasmic sperm injection
IFN	interferon
IGF	insulin like growth factor
IL	interleukin
INHB	inhibin
IP-10	interferon gamma-induced protein 10
IVF	*in vitro* fertilization
IVM	*in vitro* maturation
KC	keratinocyte chemokine
KL	kit ligand
LCM	laser capture microdissection
LH	luteinising hormone
LIF	leukaemia inhibitory factor

MAPK	mitogen activated protein kinase
MCP	monocyte chemotactic protein
M-CSF	macrophage colony stimulating factor
MHC	major histcompatability complex
MI	metaphase I
MIF	macrophage migration inhibitory factor
MIG	monokine induced by gamma interferon
MII	metaphase II
MII-F	metaphase II (fertilised)
MII-NF	metaphase II (not fertilised)
MIP	macrophage inflammatory protein
MMP	matrix metalloproteinase
mRNA	messenger ribonucleic acid
MPD	menupur daily
NGF	nerve growth factor
NHS	National Health Service
NK	natural killer
NO	nitric oxide
NOS	nitric oxide synthase
O	oocyte
OCT	optimal cutting compound
OHSS	ovarian hyperstimulation syndrome
OSM	oncostatin M
P_4	progesterone
PC	principal component
PCA	principal components analysis
PCOS	polycystic ovary syndrome
PDGF	platelet-derived growth factor
PG	prostaglandin
PID	pelvic inflammatory disease
POF	premature ovarian failure
PRL	prolactin
PTGER	prostaglandin receptor
PTGS	prostaglandin synthase

ra	receptor antagonist
RANTES	regulated on activation, normal T cell expressed and secreted
ROR	reduced ovarian reserve
SCF	stem cell factor
SDF	stromal derived factor
SEM	standard error of mean
SET	single embryo transfer
STAT	signal transducer and activator of transcription
TC	theca cell
TGF	transforming growth factor
T_h1	type 1 T helper cell
T_h2	type 2 T helper cell
T_h17	T helper 17 cell
THBS	thrombospondin
TIMP	tissue inhibitor of matrix metalloproteinases
TNF	tumour necrosis factor
TNFR	tumour necrosis factor receptor
TRAIL	TNF-related apoptosis-inducing ligand
VBSSM	variational Bayesian state space model
VCAM	vascular cell adhesion molecule
VEGF	vascular endothelial growth factor
vWF	von Willebrand Factor
ZP	zona pellucida

1. Introduction

1.1 The role of immune networks in reproductive physiology

The immune system underpins many physiological processes, and reproduction is no exception. In all animals, reproductive organs play host to a large population of leukocytes, which change dynamically in composition in response to the hormonal environment [1-5]. Tissue responses to reproductive events such as ovulation, implantation and parturition are akin to an inflammatory response, with both leukocytes and reproductive tissues themselves producing, and exhibiting functional responses to, bioactive proteins such as cytokines [6]. This results in a complex network of interactions between host tissue cells (including resident macrophages), fibroblasts and immune effector cells via the intermediary of these agents. These mediators also have neuroendocrine effects, thereby implicating the immune system in all aspects of reproductive function [7, 8]. Indeed, both the innate and adaptive systems (Figure 1-1) influence reproductive outcome from conception to adulthood in women and other mammalian dams (Table 1-1).

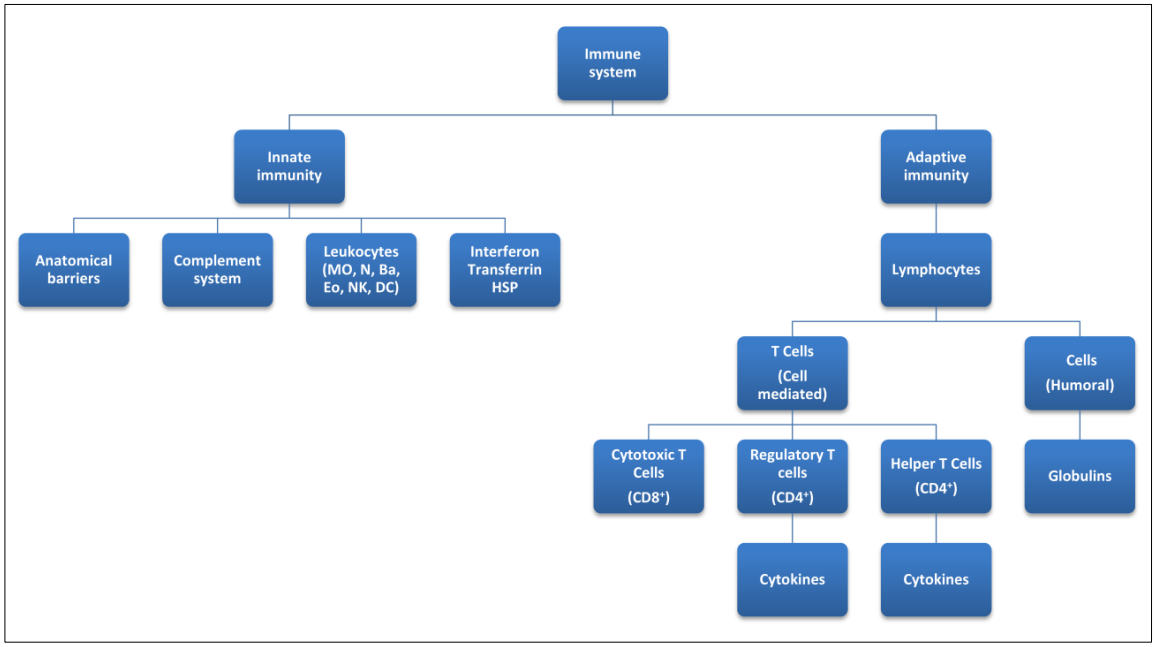


Figure 1-1: Schematic showing the components of the immune system. MΦ – macrophage, N – neutrophil, Ba – basophil, Eo – eosinophil, NK – natural killer cell, DC – dendritic cell, HSP – heat shock protein. Adapted from Muehlebein *et al* 2010 [9].

Reproductive Event		Roles of the immune System	Mechanism
Puberty		Unknown	Unknown
Ovulation		Oocyte release	Leukocytes and cytokines
		Priming of the endometrium	T cells and NK cells
		Ovarian repair	Neutrophil
Pregnancy	*Implantation*	Mediate tolerance	Uterine NK cells, leukocytes
		Facilitate implantation	
	Pregnancy maintenance	Mediate tolerance	T regs, endometrial NK cells, IgG
		Placentation	
		Immunoeducation of the newborn	
	Birth	Induction of labour	Increase in inflammatory cells and processes
Lactation		Direct transfer of immune factors	Production and secretion of IgA lactoferrin and lysosyme in breast milk
		Immunoeducation of the newborn	
Menopause		Unknown	Unknown

Table 1-1: Immune system involvement in major reproductive events.

IgG – Immunoglobulin G, NK – natural killer cells, T regs – regulatory T cells. Adapted from Abrams and Miller (2011) [6].

1.1.1 The ovarian cycle

The ovarian and menstrual cycles provide a prime example of the multiple roles of the immune system in reproductive physiology. Folliculogenesis, the concurrent production of mature oocytes capable of fertilization, ovulation and formation of the corpus luteum (CL) are all mediated by a coordinated network of hormones, cytokines, gonadotrophins and an array of other factors (described in more detail in Chapter 4) [2, 10-14]. A wide array of immune effector cells are permanently present in the ovary. They surround follicles from their primordial stage of development through to the formation of the Graafian follicle and subsequent ovulation, providing regulatory cytokine support, thereby actively mediating both ongoing follicle growth and parallel attrition through atresia [2, 15, 16]. Ovulation itself has often been noted to be an inflammatory event, mediated by cells of the immune system and the cytokines, prostaglandins and matrix metalloproteinases that they produce in order to break down the follicular wall and release the oocyte to the oviduct [13, 17-21].

Once ovulation has occurred, the formation of the corpus luteum (CL - whose role is to produce high levels of progesterone to support the subsequent pregnancy until this role is taken over by the foetoplacental unit) is mediated by leukocytes such as macrophages and T cells [22-25]. Cytokines such as vascular endothelial growth factor (VEGF) [26], interferon (IFN)-γ [27-29] and tumour necrosis factor (TNF)-α [30, 31] are critical to ensuring the adequate vascularization and structural integrity of the CL, such that failures in these signaling mechanisms can lead to luteal phase defects and infertility in women [32-34]. Subsequent regression of the corpus luteum into the corpus albicans is also mediated by leukocytes and the cytokines they produce [35, 36].

Immune cells and their effector cytokines are intimately linked with reproductive hormones, particularly estradiol (E_2) and progesterone (P_4), varying alongside these throughout the menstrual or oestrus cycle [6]. This leads to an apparent conflict between the reproductive and immune systems, as it has been shown that the low E_2 levels seen throughout the follicular phase lead to a higher risk of autoimmune disease, while the higher levels at ovulation and during pregnancy provide an immunosuppressive environment which increases the risk of infection [37, 38].

1.1.2 Endometrial remodelling and response to mating

Alongside folliculogenesis, the endometrium undergoes significant remodelling in response to hormonal signals in order to prepare for an impending pregnancy. This involves growth, remodelling and breakdown governed by a complex interplay of hormones, cytokines, eicosanoids and matrix metalloproteinases [5, 7, 39-42]. This process is evident in the majority of mammalian species to a greater or lesser extent and is described in more detail in Chapter 2. If mating and subsequent implantation of a blastocyst do not occur, regression of the corpus luteum in the absence of embryo-derived human chorionic gonadotrophin (hCG) or species-sepcific equivalent (platelet activating factor in mice, interferon-tau in bovids and ovids) leads to a fall in P_4, which triggers menstruation in women, and endometrial remodelling in other species which do not shed their endometrium. These processes are driven by an influx of leukocytes, drawn by chemotactic cytokines [43].

Following mating, as well as providing protection from invading pathogens in the female genital tract, the endometrium must adapt to tolerate the non-self antigens of the sperm and conceptus allograft. Seminal plasma proteins induce a coordinated maternal inflammatory response which recruits leukocytes to the endometrium [5, 15,

44-46], subsequently culminating in a selective systemic immunomodulation geared at tolerating paternal antigens and enabling successful fertilization and implantation [47-49].

1.1.3 Pregnancy

Despite the fact that maternal and foetal cells differ in their genetic constitution by 50%, the maternal immune system does not reject the foetal allograft during pregnancy. Trowsdale and Betz (2006) coined the phrase 'temporary self' to describe this situation where the maternal immune system demonstrates tolerance to the presence of foreign antigens and treats the foetus as 'self' for the period of pregnancy [50]. This modification of the maternal immune system is made even more remarkable by the fact that foetal cells cross into the maternal bloodstream and can persist for many years/lifelong, a phenomenon known as microchimaerism [51-53]. This process is facilitated by the fact that the conceptus trophoblast is uniquely designed to minimise maternal immune activation due to a lack of constitutive and inducible major histocompatibility complex (MHC) molecules on the presenting villous surface [50, 54], while alterations in the maternal T cell pool reduce the likelihood of an immune response at the materno-foetal interface [54]. In addition to the local changes in the endometrium, the maternal immune system dampens cell-mediated immunity systemically whilst maintaining antibody-mediated immunity, a change which protects the developing foetus but leaves the mother vulnerable to infection [55]. For example, if a woman becomes infected with *Toxoplasma gondii* during pregnancy, she is more likely to pass the infection to her foetus during late gestation when E_2 levels are at their highest [56].

1.1.4 Parturition

The initiation of labour was traditionally considered to be an endocrine-mediated event, although evidence increasingly suggests that the trigger may actually be inflammatory in nature [57, 58]. The foetus is thought to provide many of these signals, for example through the production of phospholipids and pulmonary-derived surfactant proteins which mediate lung maturation. In turn, this triggers foetal macrophages to migrate to the uterine wall where they participate in increasing local levels of prostaglandins and cytokines. [58, 59]. On the other hand, phospholipids increase corticotrophin releasing hormone, further contributing to membrane rupture, cervical effacement and increased myometrial contractility [60-62]. Cervical and myometrial changes are mediated by a rapid influx of leukocytes, with concurrent cytokine, prostaglandin and hormone

production driving cervical ripening and myometrial activity as a functional syncytium [63-65]. The role of the inflammatory/immune system in this process is perhaps best exemplified in women by the fact that if a mother contracts an infectious disease during late pregnancy, the resulting inflammatory reaction can trigger labour and result in a pre-term birth, with its attendant *sequelae* in both the mother and foetus [66].

1.1.5 Lactation

Nourishment of the newborn is costly to the mother, in humans amounting to an additional daily energy expenditure of approximately 500kcal [67, 68]. However, a large body of evidence suggests that this expenditure has immunological benefits for both the mother and the newborn. For example, provision of maternal milk has been shown to protect the newborn from infectious disease, and also confers protection against allergy and asthma in later life [69-74]. In the foetus, immunologic defence is present, although this is immature. During pregnancy, IgG antibodies cross the placenta to provide the foetus with a passive immunity which is depleted in the first 6-12 months of life. Furthermore, during birth, the foetal gut is colonised by maternal vaginal and faecal bacteria, establishing a competent mucosal surface which offers some degree of microbiomial protection against enteric pathogens. This colonisation is continued in the breastfed infant by transfer of live bacteria through breast milk, such that the infant gut microbiome resembles that of adults by the age of 2 [75, 76]. Breast milk provides additional maternal immune resources in the form of secretory IgA, leukocytes and non-specific anti-microbial factors which supplement pregnancy-acquired immunity [73, 74, 77-81]. These factors combine to influence subsequent immune development in the newborn, improving thymic production of T cells and infant responses to vaccination [77, 82].

In terms of maternal benefits, lactation confers a change on the cyclicity of the endometrium creating lactational amenorrhoea in women, where the menstrual cycle is suspended, providing a natural break in fertility. Suckling delays the resumption of normal ovarian cycles by disrupting the pattern of pulsatile hypothalamic GnRH release and pituitary LH production [83]. Although the mechanisms behind this are not fully understood, the change in ovarian cyclicity is believed to involve a modulation of the functional immune cells and cytokines which mediate follicular growth.

1.1.6 Menopause

Involvement of the immune system in the onset of menopause is subject to speculation. Although the onset of menopause may not be directly immune-mediated, severe or repeated infection during the life of a woman can result in late-onset menses and early menopause, leading to a reduced reproductive lifespan. Certain autoimmune diseases, such as Type I diabetes and rheumatoid arthritis, as well as sporadic infection with mumps virus exhibit an increased risk of early menopause [84-88]. The menopause itself is associated with a reduction in E_2 and P_4 with a concomitant general trend towards immune senescence [89]. Figure 1-2 represents a schematic demonstrating this global interaction of the immune system with the reproductive cycle of the female.

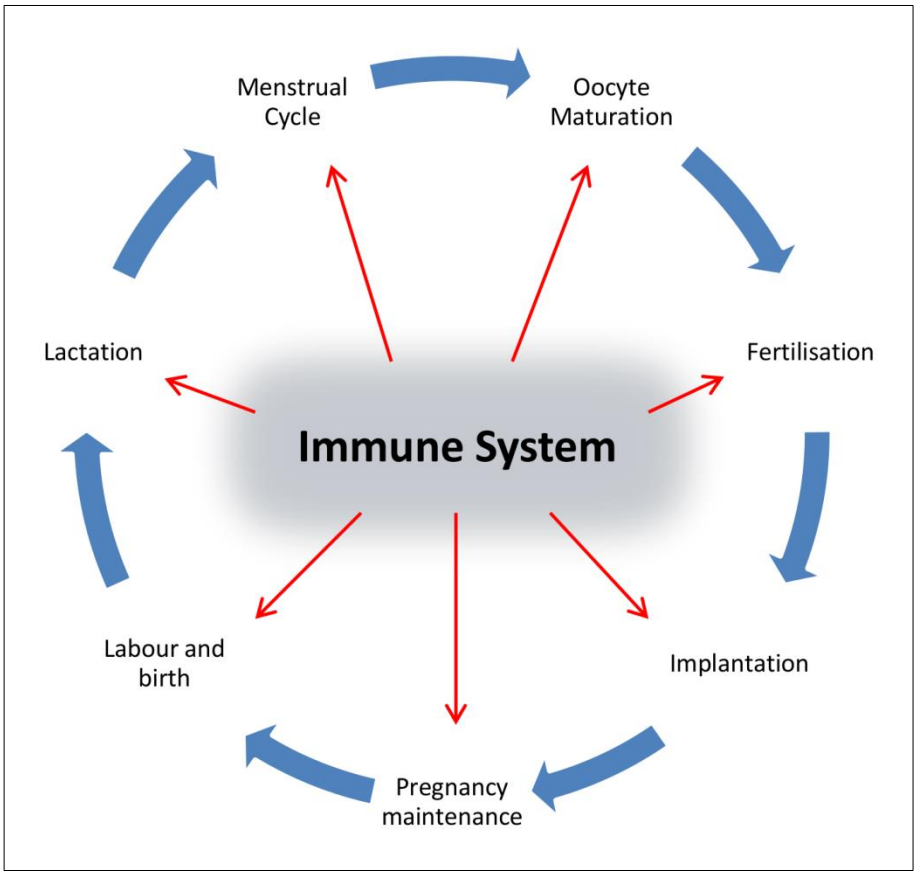


Figure 1-2: Schematic diagram illustrating the multiple reproductive processes influenced by the immune system in women.

1.2 Molecular mediators

As outlined above, immune processes are key to all facets of the reproductive cycle. These multiple effects are mediated by signalling molecules, principally cytokines, matrix metalloproteinases and prostanoids under the influence of both peptide and steroid hormones. The following sections will introduce these mediators which take centre stage throughout the various chapters of this thesis.

1.2.1 Cytokines

Cytokines are small soluble signalling glycoproteins probably best known for their immunoregulatory properties [90]. However, in recent years, cytokines have increasingly been recognised as growth factors governing cell proliferation, differentiation, function and fate [91, 92]. Cytokines are produced by more than one cell type and, in the same vein, commonly have more than one target tissue, although they can also operate in an autocrine or paracrine manner. Cytokines are also characterised by a specific array of singular functional adaptations (*viz*., synergy, antagonism, functional redundancy) whose characterisation will form a core part of this thesis. There is a plethora of cytokines which ranges from the better known interleukins (ILs), colony stimulating factors (CSFs), tumour necrosis factors (TNFs) and transforming growth factors (TGFs) through to perhaps less familiar growth factors and peptide hormones such as prolactin (PRL). As integral signalling proteins to the orchestration of an array of physiological processes, deregulations in cytokine production and imbalances in their relative interactions underlie pathophysiological processes as diverse as cancer, autoimmunity and cardiovascular disease [93-102]. Analogously, their central role in reproductive success in creating an immunopermissive and embryotrophic environment supporting gametogenesis, fertilisation, pre-implantation embryo development, blastocyst implantation, placentation, foetal development and lactation highlights the fact that inflammatory dysfunctions underpin a broad spectrum of reproductive disorders [92, 103-107].

1.2.2 Prostanoids

Prostanoids are a subgroup of the eicosanoid family of lipid mediators, comprising prostaglandins, thromboxanes and prostacyclins. This group of mediators is formed by the *de novo* conversion of free fatty acid substrates (e.g. arachidonic acid) by cyclooxygenase (COX) in response to exogenous stimuli. Prostaglandins mediate many physiological processes, but are intrinsic participants in inflammatory reactions. In the reproductive setting, prostaglandins are best known for their involvement in the onset of labour, particularly in terms of promoting cervical ripening and myometrial

contractility [108]. Moreover, prostanoids (and in particular prostaglandins) are induced by cytokines and *vice versa*, thereby highlighting the breadth of mediators actively involved in physiological reproductive inflammatory networks [109-111].

1.2.3 Matrix metalloproteinases

Although their name implies a role in the turnover and degradation of the extracellular matrix (ECM), MMPs serve a much wider function as immune mediators [112]. These zinc-independent endopeptidases have the ability to directly modulate cytokine activity, particularly in terms of the repression of chemokine activity by proteolysis [113-115]. MMP activity can be induced by prostaglandins, a function typically exhibited in the decidua where leukocyte migration and cytokine release is increased as a result of their activity [116].

These key mediators act in a coordinated network to promote an environment conducive to oocyte maturation, fertilisation, implantation, pregnancy maintenance and parturition. However, much of the research conducted to date in the reproductive arena has broadly focussed on these molecules in isolation such that comparatively little is known about the networks/pathways implicated when these are investigated in the context of their interactions with other inflammatory mediators.

1.3 Clinical relevance

1.3.1 Female infertility

Subfertility affects one in seven couples in the UK according to the Human Fertilisation and Embryology Authority (HFEA, 2011/12). Infertility is caused by a complex interaction of factors, including genetics, lifestyle, environment, physiology and pathology, although in many cases the condition remains idiopathic. There are significant medical, psychosocial, ethicolegal, religious and economic implications associated with infertility and its treatment. High multiple birth rates can result from the latter, which have considerable health risks for the mother and baby, resulting in significant financial burden to the healthcare system [117-120]. Female factor infertility accounts for a third of all infertility cases and results from a variety of pathologies including ovulatory dysfunction, (hypothalamic pituitary dysfunction or hypothalamic pituitary failure), endometriosis, polycystic ovary syndrome (PCOS), pelvic inflammatory disease (PID), and premature ovarian failure (POF)/reduced ovarian reserve (ROR). Table 1-2 demonstrates the aetiology of infertility according the HFEA.

Aetiology		% of all subfertility cases	References
Female Factor infertility		29	[121, 122]
Male Factor Infertility (MFI)		30	[121, 123, 124]
Unexplained Infertility		25	[121, 123, 125, 126]
Multiple Infertility Causes		9-15	[121, 123, 127]
Ovarian Dysfunction	Polycystic ovarian syndrome (PCOS)	7	[121, 128]
	Premature ovarian Failure (POF)	1	[121]
Pelvic inflammatory disease (PID)		12-16	[121, 129]
Endometriosis		3.5	[121, 130, 131]
Uterine factors		0.4	[121, 123]

Table 1-2: Aetiology of infertility.

Distribution of various aetiologies involved in infertility, data from HFEA (2011/12) relates to women undergoing assisted conception.

1.3.2 Treatment for female infertility

Treatment options for female infertility include surgery (e.g. ablation of endometriosis), hormone therapy, ovulation induction (using anti-oestrogens or gonadotrophins) and assisted conception techniques such as *in vitro* fertilisation (IVF) and intracytoplasmic sperm injection (ICSI). Since the success of the first IVF live birth (Louise Brown in 1978), assisted conception now accounts for approximately 1-2% of all live births within the UK [132]. Assisted conception often includes several processes, such as ovulation induction, oocyte collection and fertilisation, and embryo transfer. Success rates for these treatments are variable, with IVF live birth rates in the UK remaining at 25% per cycle (HFEA, 2011/12).

Much research is focused on improving assisted conception success rates, particularly in terms of understanding the complex mechanisms which provide competent oocytes for fertilisation and embryo development. The reduction in the number of embryos transferred is a prime target for the lowering of the number of multiple pregnancies, with some success. In the USA, when clinics were discouraged from transferring 3 or more embryos following IVF in 1998, the triplet or higher order multiple rate attributable to IVF dropped from 48% to 34%, although the number of twin births continues to rise [133]. Recent guidelines suggest moving towards elective single embryo transfer (eSET), a choice which more parents are selecting due to the risks of multiple pregnancy [133]. The success rates of eSET vary from clinic to clinic, although in the UK around 16% of women who choose this treatment option attain a live birth (HFEA,

2011/12). With the drive towards eSET, there is an additional focus on the choice of the best oocyte to fertilise, and the best embryo to transfer in order to maximise the chance of achieving a viable pregnancy and live birth.

Recently, in order to improve the quality of oocytes collected, there has been a rise in the popularity of so called natural cycle (NC)-IVF. This approach aims to reduce the risk of side effects of controlled ovarian hyperstimulation (COH) such as ovarian hyperstimulation syndrome (OHSS), and to provide the highest quality oocyte for subsequent fertilisation [134]. The International Society for Mild Approaches in Assisted Reproduction (ISMAAR) defines NC-IVF as oocytes collected within a spontaneous menstrual cycle without the assistance of medication [135]. However, this approach has its failings; spontaneous cycles often only yield only one oocyte, inherently reducing the chances of successful fertilisation and implantation. Moreover, intervention is often required in terms of the administration of exogenous human chorionic gonadotrophin (hCG) to promote oocyte maturation in a timely manner for collection. Oocyte collection fails in as many as 43% of all cycles started, contributing to the low NC-IVF success rates [136, 137]. Notwithstanding, the oocytes retrieved are typically of a higher quality than those obtained following COH[138, 139]. The implications of this will be discussed further in Chapter 4.

An alternative approach is modified natural cycle IVF (MNC-IVF), where the risk of premature luteinisation is mitigated by the administration of a GnRH antagonist for a short period of time (usually 2-4 days) and/or hCG to initiate final oocyte maturation prior to oocyte collection [135, 140, 141]. Comparison of success rates with COH is difficult, as NC-IVF is often considered in terms of cumulative pregnancy rates rather than pregnancy rates per started cycle. Nonetheless, a 43% pregnancy rate after 3 started cycles remains low [137] such that the drive to improve success rates within COH cycles remains. In line with this, a requirement to understand the changes in the microenvironment of the follicle and oocyte quality following COH in order to optimise the stimulation protocols is much needed.

1.3.3 Embryo transfer and implantation

Another area presenting an opportunity for the improvement of assisted conception success rates is the preparation of the endometrium for implantation. Implantation of an embryo requires the synchronised development of both the embryo and endometrium. Once an oocyte has been fertilised and has developed into a blastocyst, it must attach to the luminal endometrial epithelium and invade the underlying decidualising stroma

over the course of a very small window of opportunity [41]. Studies which have assessed implantation rates following embryo transfer estimate that around 26-35% of all transfers result in a clinical pregnancy, with a pre-clinical pregnancy loss of approximately 19% [142-144]. This compares to an implantation rate in NC/MNC-IVF of up to 50% [136, 137]. This suggests an asynchrony between folliculogenesis and endometrial remodelling in stimulated cycles, which subsequently reduces the chance of achieving a pregnancy/live birth.

Coordinated preparation of the endometrium for implantation is driven by oestrogen (E_2) and progesterone (P_4), and these hormones can be used at supraphysiological levels to prepare the endometrium for embryo transfer either with or without prior gonadotrophin stimulation [145, 146]. However, there are a number of processes which occur prior to the embryo reaching the uterus in natural cycles. It is thought that semen (its seminal plasma fraction in particular) provides a signal to the female reproductive tract to prepare for implantation, and to induce maternal immunotolerance of the sperm surface paternal antigens [147]. To date, the mechanisms governing this induction of tolerance, endometrial priming and subsequent receptivity remain very poorly understood, and will be the focus of Chapter 2 [148].

1.3.4 Role of the immune system in infertility

The immune system has been implicated in various pathologies and dysregulations which contribute to infertility. These are often mediated by communication failures between the immune and endocrine systems which result in difficulties in conception, implantation and the maintenance of pregnancy. From the quality of oocytes produced through folliculogenesis to implantation and subsequent pregnancy, disruption to the immune system can result in failures which affect fertility to a lesser or greater degree, as discussed below.

1.3.4.1 Oocyte quality

Immune/inflammatory mediators (cytokines, MMPs, prostaglandins) have been shown to influence oocyte quality. Studies involving controlled ovarian hyperstimulation have sought to determine whether intrafollicular cytokine profiles in particular can be used as predictive markers to determine the developmental competence of an oocyte and, in certain cases, even predict the outcome of assisted conception cycles [149-151]. However, most studies have been limited by focussing on single or small groups of mediators, disregarding the fact that cytokines are increasingly believed to operate as

part of complex network systems [91, 92, 152-154]. This approach will form part of the remit of Chapter 4, where follicular fluid (FF) cytokine signatures will be related to the fertilisation potential of oocytes collected during ICSI-based assisted conception cycles.

1.3.4.2 Fertilisation

Post coitum, immunological and neuroendocrine systems are activated to prevent the maternal immune system attacking the foreign antigens present in sperm. In some situations, this development of sperm-specific immunotolerance fails to occur and sperm are destroyed in the maternal tract before they reach the oocyte, causing infertility. In this respect, infertile women have been shown to have a higher prevalence of anti-sperm antibodies and/or seminal plasma hypersensitivity compared to their fertile counterparts. However, when these women are treated with assisted conception techniques which allow bypass of the maternal tract mucosal defences, their live birth rates are comparable to those of controls [155-158].

1.3.4.3 Implantation

The preparation of the endometrium for implantation and immune tolerance is mediated by a range of immune effector cells, such as natural killer (NK) cells. A failure at any stage of this process has been suggested to be one of the contributory mechanisms underlying recurrent miscarriage. Recent research has shown that couples who share human leukocyte antigen (HLA) alleles are at high risk of recurrent implantation failure, indicating that the similarity between male and female antigens does not activate the initial inflammatory response in the female tract responsible for conferring immunotolerance [159, 160]. Indeed, there appears to be an evolutionary mechanism which favours the selection of mates who are immunologically different, in that both mice and women prefer the pheromones in the body odour of prospective sexual partners with dissimilar MHC alleles [161-165]. Thus, although an immunological basis for implantation failure is an accepted proposition, there remains a paucity of studies which explore the underlying physiological response to insemination and implantation [166].

1.3.4.4 Maintenance of pregnancy

Once implantation has occurred, the maternal immune system is geared to maintain the allograft tolerance developed following exposure to seminal plasma. However, this represents walking an immunological tightrope such that any mild immune/inflammatory deviations during pregnancy alone can result in miscarriage and

premature labour. For example, the complement system (which forms part of the innate immune system) must be tightly regulated to maintain a successful pregnancy; a lack of decay accelerating factor (encoded by the CD55 gene) results in recurrent early miscarriage in women, likely due to an overactive complement system [167, 168]. Similarly, the premature activation of the primed uterus in late pregnancy by inflammation/immune system stimulation can lead to an untimely rupture of the foetal membranes and uterine contractions, resulting in premature labour and birth. Oft-quoted examples in this context include bacterial vaginosis, sexually transmitted diseases and group B streptococci, which have all been linked to premature birth in women via immune system activation [169-171].

1.4 Rationale, hypothesis and aims

The immune system is involved at all stages of the reproductive cycle, from the ovarian cycle and menarche through to pregnancy and birth, lactation and menopause. However, the physiological basis of both the identity and the interactions of its multiple mediators remains remarkably poorly understood. The central unifying theme in this thesis is thus to explore the mechanisms providing the basis of immune mediator involvement at select, landmark points in female reproductive life.

1.4.1 Research hypothesis

Throughout the theme of this thesis, the following research hypothesis was tested:

"cytokine-based immune networks underpin the physiological processes governing the endometrial response to seminal plasma, lactation and oocyte maturation following ovarian stimulation. The structure of these networks can be revealed, displayed and explored using both traditional and novel machine-learning data handling methodologies".

This hypothesis was tested using a number of reproductive systems and methodologies. The aims of each Chapter is presented below.

1.4.2 Aims of studies

1.4.2.1 Inflammatory pathways in the murine endometrial response to seminal plasma (Chapter 2)

The aim of the studies contained within this first experimental chapter was to discover the immune pathways involved in the endometrial response to seminal plasma (including its selectively epithelial and stromal components) and the establishment of foetal allograft immunotolerance using a murine model system.

1.4.2.2 Bayesian modelling of cytokine interactions in vivo (Chapter 3)

The aim of the studies contained within chapter 3 was to develop novel methods of discovering, displaying and exploring mediator (specifically cytokine) interactions with peptide and steroid hormones *in vivo* by using murine lactation as a model system. This required moving away from conventional, basic analysis to develop Bayesian machine learning-based mathematical models.

1.4.2.3 Modelling follicular fluid cytokines in relation to oocyte maturation (Chapter 4)

The aims of the studies contained within the final experimental chapter were:

- to use the mathematical methodologies developed in chapter 3 as a basis for improved modelling strategies;
- to discover, display and explore cytokine networks within human follicular fluid surrounding oocytes exposed to ovarian stimulation, thereby highlighting their likely involvement in maturation, and
- to classify maturation and the developmental viability of those oocytes based on their follicular fluid cytokine profile.

2. Inflammatory pathways in the murine uterine response to seminal fluid

2.1 Introduction

The endometrium is a dynamic tissue which undergoes growth, remodelling and breakdown (to a lesser or greater degree in different species) on a cyclical basis, in preparation for blastocyst implantation. Although implantation is a rate limiting step in the improvement of assisted conception success rates, the molecular mechanisms underlying the endometrial changes in readiness for implantation remain a poorly understood aspect of reproductive physiology. Various factors influence the rate of implantation in assisted conception, including the quality of the embryo transferred [172] and the synchronous endometrial receptivity [148, 173]. In rodents and various ungulates, constituents of seminal plasma are thought to prepare the endometrium for implantation in a concept dubbed 'seminal priming', a process which modifies components of the immune system and can influence subsequent events in pregnancy [174]. Although exposure to semen affects the endometrium directly in species such as rodents and swine, this priming phenomenon is likely to operate indirectly through a more widespread alteration of reproductive mucosal response in species such as humans, where ejaculate volumes are more modest and where seminal plasma remains largely confined to the vaginal vault and cervix. As the exploration of the response of the endometrium to seminal plasma in humans presents ethical and logistic issues, the mouse presents a convenient physiological system within which these responses can be explored. This section of the thesis will explore the global changes in gene expression of murine endometrial epithelial and stromal cells in response to coitus and exposure to seminal plasma, with a particular focus on the changes elicited in inflammatory and immune pathways in preparation for implantation.

2.1.1 Murine reproductive physiology

2.1.1.1 Uterine structure

In the mouse, the uterus is a bicornuate organ with two uterine horns joined to a single corpus. This organ comprises a muscle layer (myometrium) covered by a mucosal endometrial layer, featuring simple columnar epithelial cells (both ciliated and secretory) which involute into the underlying stroma and exhibit changing morphology according to cycle stage (Figure 2-1). The cells of endometrial layer undergo

proliferation, differentiation and regression/death on a cyclical basis (i.e. the menstrual cycle in humans and the oestrus cycle in rodents and other species), a process mainly regulated by changes in ovarian steroid hormones.

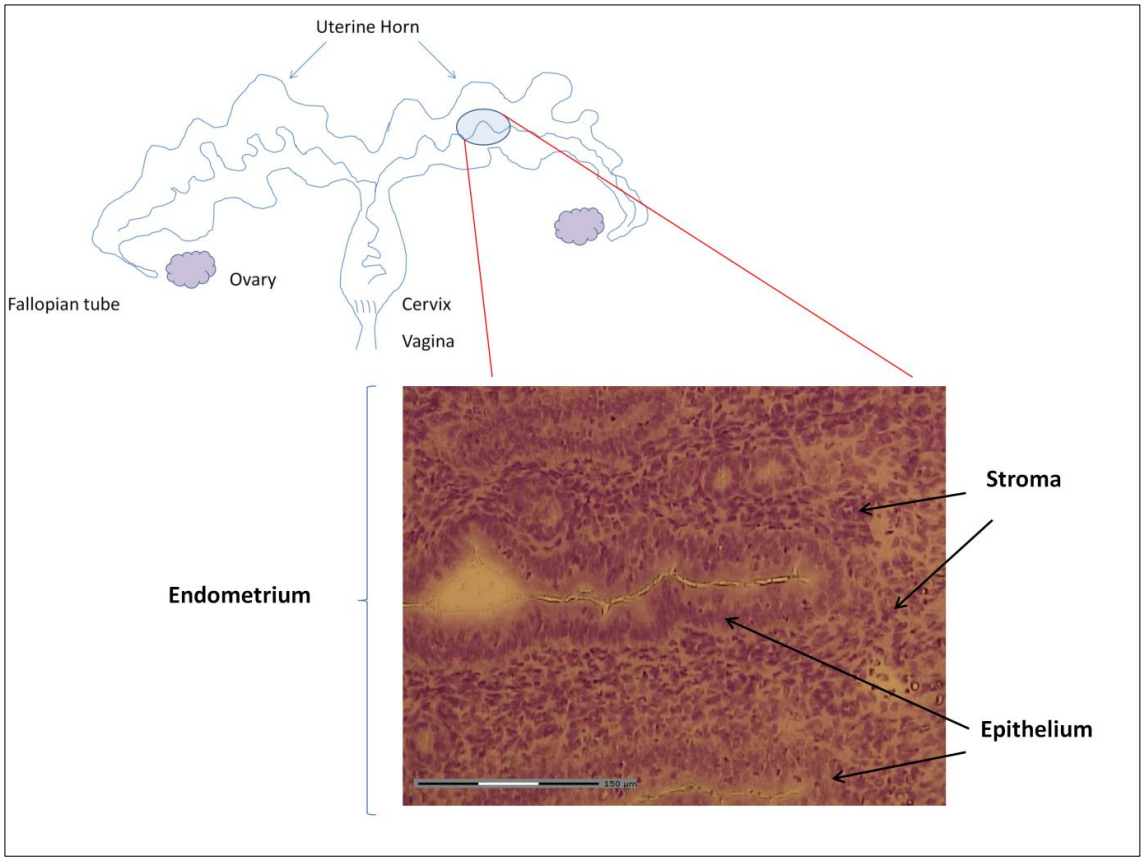


Figure 2-1: Gross anatomy of the murine uterus. The expanded panel displays the microscopic structure of the endometrium. Frozen sections were stained with cresyl violet and imaged at 20x magnification (image captured by the author). The scale bar indicates 150µm.

2.1.1.2 Murine oestrus cycle

The murine oestrus cycle generally lasts 4-5 days [175], and consists of four distinct stages, comprising dioestrus, proestrus, oestrus and metoestrus [176], each displaying distinct vaginal cytological characteristics [177] (Table 2-1). In response to dynamic changes in P_4 and E_2, the endometrium undergoes extensive remodelling (Figure 2-2), involving proliferation, differentiation and apoptosis alongside ECM remodelling, angiogenesis and immune cell infiltration [178, 179]. This immune cell influx is mediated by E_2, which draws macrophages, neutrophils and eosinophils into the uterine stroma [180-183]. It is noteworthy that the murine cycle does not involve

endometrial shedding/menstruation as featured in women. At oestrus, the female mouse becomes sexually receptive in response to E_2, although this phenomenon may also occur during late proestrus or early metoestrus [184].

Stage	Vaginal characteristics	Endometrial characteristics	Hormone profile
Dioestrus	Quiescent tissue, predominantly leukocytes	Low epithelial and stromal proliferation, neutrophils and macrophages present in stroma only, high numbers of mitotic bodies	E_2 low but rising, P_4 high
Proestrus	Predominance of nucleated epithelial cells, appearing in clusters or individually; some cornified cells	High glandular epithelium proliferation, low stromal proliferation, high apoptosis of glandular epithelium, neutrophils and macrophages present in stroma only, increased luminal dilation	E_2 rising, P_4 falling, FSH/LH surge at the end of this stage
Oestrus	Clusters of cornified squamous epithelial cells, with no visible nucleus and granular cytoplasm	Endometrium at its thickest, luminal epithelium proliferation at its highest, glandular epithelium proliferation low, elevated stromal proliferation, reduction in epithelial cell collagen type-IV, increased extracellular fluid, neutrophils and macrophages present in stroma only, evidence of apoptotic cells in the endometrium, increase luminal dilation	E_2 high then falling to basal levels, P_4 low
Metoestrus	Mix of cell types with a predominance of leucocytes and a few nucleated epithelial and/or cornified squamous epithelial cells	Glandular epithelium proliferation high, low stromal proliferation, highest apoptosis of luminal/glandular epithelium and stroma, neutrophils present in luminal epithelium and stroma, macrophages present in stroma and adjacent to luminal epithelial cells	E_2 falling, P_4 rising

Table 2-1: Characteristics of the vagina and endometrium at each stage of the murine oestrous cycle. E_2 - 17β-estradiol, P_4 – progesterone, LH – luteinising hormone, FSH – follicle stimulating hormone [185-187].

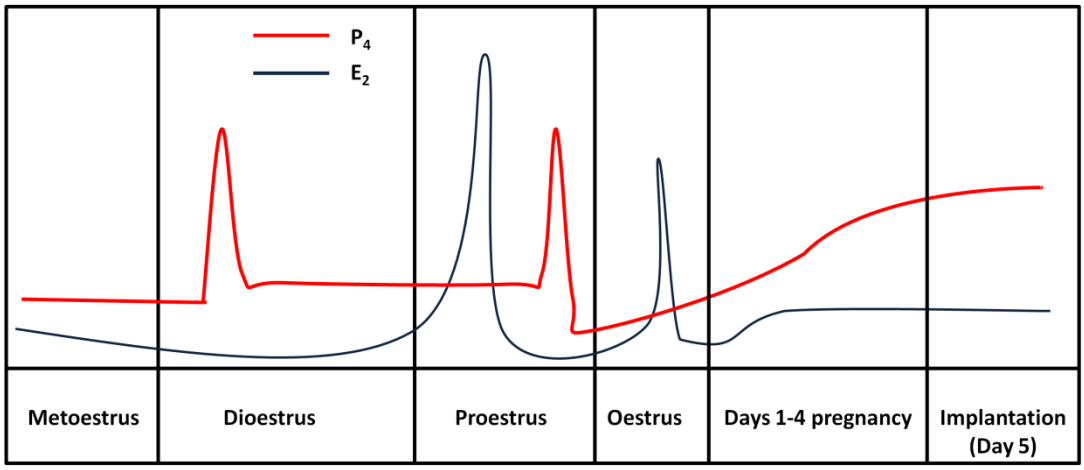


Figure 2-2: Schematic diagram representing hormone (E_2 and P_4) profiles throughout the murine oestrus cycle and early pregnancy. Adapted from Emanuele 2002 [45].

2.1.1.3 Murine mating

Mating behaviour consists of the male sniffing, following, mounting, mounting-with-intromission, and post-copulatory grooming of the female [188, 189]. Ejaculation may take place after one to more than 100 intromissions, marked by the male rolling over on his side, and usually occurs only once per day. The male accessory glands produce a secretion which hardens to form the vaginal plug, which may be used to confirm ejaculation. This persists for 16-24 hours and presents an obstacle to the female mating with other males [190].

2.1.1.4 Ejaculate constituents

The ejaculate comprises several constituents which are essential for the establishment of pregnancy. These components vary across species, but in general comprise sperm and seminal plasma arising from various accessory glands (Table 2-2). The main functions of seminal plasma are to support spermatozoa survival whilst inducing a concurrent alteration of the female reproductive tract microenvironment [191]. Interestingly, the ejaculate is not uniform *in vivo*, consisting of a pre-ejaculate from the urethral glands, followed by release of sperm from the epididymis/secretions from the prostate with subsequent ejaculation proper in a series of spurts, meaning that the female tract receives signals in a temporal and spatial manner which is difficult to replicate *in vitro* [192].

Source	Percentage	Component	Function
Testes	2-5%	Spermatozoa: 2-16x10^6 sperm per ejaculate in mice [193]	Fertilisation of the oocyte
Seminal vesicles	65-75%	Amino acids, citrate, enzymes, flavins, fructose, prostaglandins, proteins (including semenoclotin and TGF-β), transglutaminase, vitamin C [194-197]	Spermatozoa support and nutrition; suppression of the female inflammatory response; vaginal plug formation
Prostate	25-30%	Acid phosphatase, citric acid, fibrinolysin, prostate specific antigen, proteolytic enzymes, zinc [198]	Alkaline fluid neutralises the female reproductive tract; stabilisation of spermatozoa chromatin
Coagulating gland	<1%	Galactose, mucus, pre-ejaculate, sialic acid [199]	Increases sperm motility; vaginal plug formation

Table 2-2: Murine accessory glands and their contribution to the ejaculate.

2.1.1.5 Seminal plasma

Seminal plasma constitutes 95-98% of the ejaculate volume; this small variation being accounted for by the presence and size of accessory glands in different species [200]. Proteins comprise a large fraction of seminal plasma which are largely attributable to one of three families – spermadhesins, proteins containing fibronectin type II modules, or cysteine-rich secretory proteins [201, 202]. In addition, blood plasma proteins are also present in seminal plasma, including albumin, globulins, transferrin, immunoglobulins, complement factors and immune mediators such as cytokines and chemokines. These immunological factors include monocyte chemotactic protein (MCP)-1, macrophage inhibitory protein (MIP)-1α/β, regulated upon activation normal T cell expressed and secreted (RANTES), prostaglandins and TGF-β, all of which are believed to modulate endometrial function [197].

2.1.2 Importance of seminal plasma in the uterine response

Although traditionally viewed as a survival and transport medium for sperm, seminal plasma is now considered to have an equally significant role in preparing the uterus for pregnancy. The concept of seminal priming of the uterus has been prevalent in the literature for a number of years now, where evidence that the uterine response to mating originates from seminal plasma has been shown in experiments in hamsters, mice and rats [191]. In mice, laboratory protocols utilise the uterine response to seminal fluid to induce pseudopregnancy by mating females to vasectomised males.

This improves implantation rates at embryo transfer into surrogate dams, reduces miscarriage rates and prevents abnormalities which might otherwise occur in females not exposed to male reproductive fluids [203].

Each accessory gland appears to influence fertility to a greater or lesser extent. In female mice mated with seminal vesicle-deficient males, embryos implant at normal rates but foetoplacental growth is retarded and live birth rates are reduced [204-206]. Where the coagulating glands, prostate and/or seminal vesicles are surgically removed, successful pregnancy is significantly reduced such that the impact on fertility is attributed to reduced sperm motility and survival resulting in poor fertilisation rates [199, 206-208]. Comparable surgical interventions in hamsters results in slower embryo cleavage and increased miscarriage rates post-implantation [209]. Interestingly, female mice establishing a successful pregnancy without exposure to the fraction of seminal plasma derived from the seminal vesicles exhibit a longer gestation without a reduction in litter size [206]. The prostate gland component of seminal plasma influences pregnancy to a lesser extent in rodents where its removal results in slightly lower pregnancy rates, a feature not noted with the removal of coagulating glands [199]. Thus, these studies indicate that seminal plasma performs a greater function than that of a simple, passive carrier fluid for spermatozoa. Indeed, uterine physiological changes can be induced by semen and/or seminal plasma, indicating that the physical stimulation of the mating act is not required [210].

2.1.3 Maternal immune response to seminal plasma

Post coitum, the first response of the uterus to seminal fluid exposure is that of endometrial cytokine synthesis, a process followed by an influx of leukocytes (Figure 2-3) [211]. Seminal plasma proteins interact with E_2-primed uterine epithelial cells, resulting in the synthesis of a large array of cytokines and other immune factors, including granulocyte macrophage colony stimulating factor (GM-CSF), IL-6, IL-8, monocyte chemoattactant protein (MCP-1), MMPs and prostaglandins (PGs) amongst others [212, 213]. Within hours of this peak in cytokine production, inflammatory cells, particularly leukocytes, invade the cervix as the primary site of semen deposition in humans, pigs, rabbits and rodents [147, 214-219]. Studies involving the artificial transfer of seminal plasma into gilts have demonstrated that this maternal response is evident without the physical act of mating [210]. Beyond the immediate uterine environment, seminal plasma can influence other maternal structures, including newly formed corpora lutea where macrophage populations are indirectly modulated to mediate tissue remodelling [45].

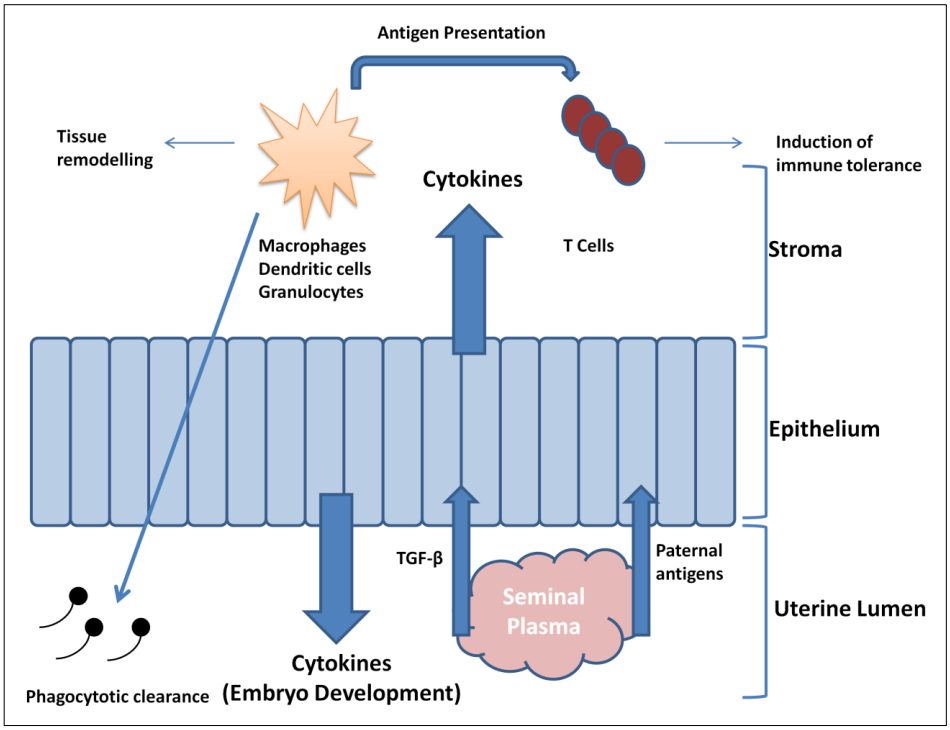


Figure 2-3: Schematic diagram representing the murine uterine response to seminal plasma. Adapted from Robertson (2007) [220]

2.1.3.1 Myeloid cells

As has been shown in mice and swine, myeloid lineage cells such as macrophages, dendritic cells and granulocytes of circulatory origin accumulate in the uterine stromal tissue, while macrophages, neutrophils and granulocytes traverse the epithelium and accumulate in the uterine cavity post coitum (Figure 2-4) [216, 221, 222]. This initial neutrophil response resolves prior to implantation, with a concurrent drop in cytokine profiles and rise in P_4 [213]. However, leukocytes residing within the endometrium remain for several days, proliferating throughout the peri-implantation period [210]. Maternal macrophages and neutrophils within the uterine lumen following semen deposition phagocytose slow-moving or damaged sperm, thereby increasing the likelihood of fertilisation by competent sperm [147, 191]. There is also evidence to suggest that the female response to seminal plasma is male partner-specific in mice, providing a potential mechanism for an increased chance of successful pregnancy from the fittest male [223]. The immune tolerance conferred by seminal plasma in mice is in part MHC class II+ specific, meaning that subsequent sperm depositions by a different

male may be subject to immune attack [211, 224, 225]. Figure 2-4 depicts the relative distribution of myeloid cells through the post mating period.

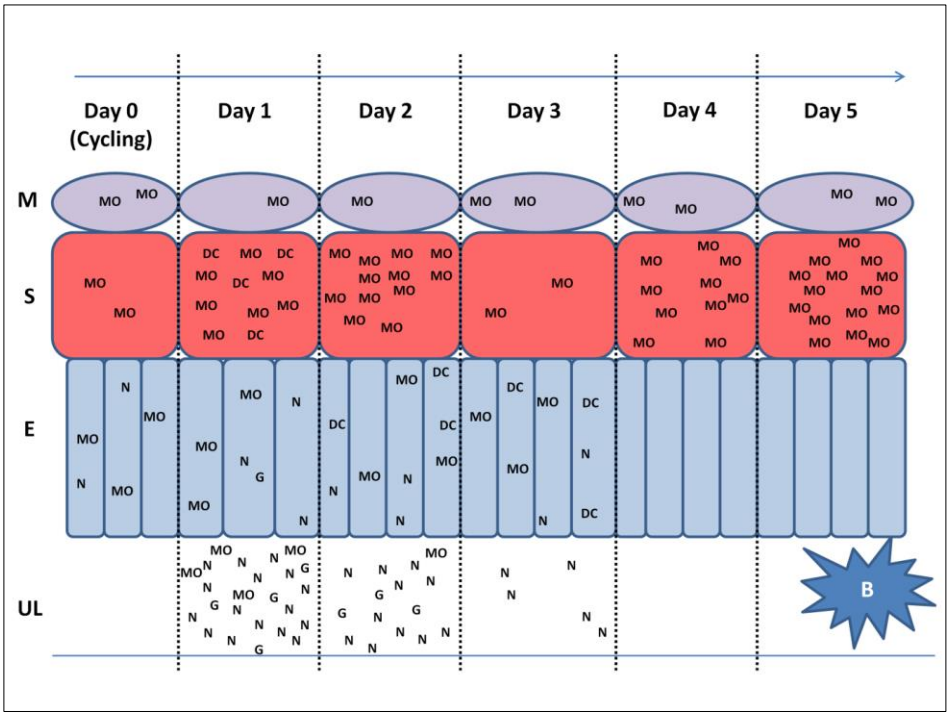


Figure 2-4: Schematic diagram showing the relative distribution of immune cells across the first five days of murine pregnancy. M – myometrium, S – stroma, E – epithelium, UL – uterine lumen, DC – dendritic cell, G – granulocyte, MO – macrophage, N – neutrophil, B – blastocyst.

2.1.3.2 Role of T cells in the uterine response to seminal plasma

In mice, the main function of this influx of immune cells is to prepare the maternal immune system for paternal antigen tolerance upon implantation of the conceptus. Modulation of the T cell response in the uterus is essential in this process. Upon initial semen deposition in the female reproductive tract, paternal antigens are presented to maternal $CD4^+$ and $CD8^+$ T cells by antigen presenting cells (APCs) within the lymph nodes which drain the uterus [46, 224]. These cells then become functionally anergic/hyporesponsive, enabling tolerance of the conceptus [226]. A concurrent expansion of the $CD4^+CD25^+Foxp3^+$ regulatory T cell (Treg) pool facilitates embryo implantation [54, 227]. The strength of this maternal response depends on the composition of seminal plasma, particularly the paternal antigen content and concentrations of TGF-β [225]. Seminal plasma TGF-β in humans and pigs is largely of

the TGF-β1 isoform (although TGF-β2 and 3 are also present) which is secreted in an inactive form which is activated in the acidic environment of the vagina [228]. In the presence of activated TGF-β, naïve $CD4^+CD25^-$ T cells differentiate into $Foxp3^+$ suppressor/anergic T cells [229]. There is the potential for the prostaglandin content of seminal plasma to synergise with TGF-β in this regard, as PGE_2 enhances the inhibitory effect and induces a regulatory phenotype in human $CD4^+CD25^-$ T cells *in vitro* [230].

2.1.4 Cytokine and immune mediator response to seminal plasma

As outlined above, seminal plasma induces the expression of endometrial cytokines and chemokines. Chemokines are a group of small cytokines responsible for leukocyte chemotaxis. However for the sake of simplicity from here on in, the term cytokine will be used to refer to both cytokines and chemokines given that this traditional segregation according to their functional properties is increasingly being brought into question. Several cytokines have been implicated in the maternal response to seminal plasma, as discussed below.

2.1.4.1 Colony stimulating factors

GM-CSF, also known as colony stimulating factor (CSF)-2, was initially thought to drive the development of macrophages and dendritic cells from their precursors [231], although other research has shown it to be a key mediator of the inflammatory response [232-234]. Several groups have proposed that GM-CSF serves as a messenger between local tissue and haematopoetic cells, particularly during an inflammatory response [235-237]. Within the uterus, the endometrial epithelium has been shown to be responsible for the vast majority of GM-CSF produced in naturally cycling mice and women [238, 239]. This production fluctuates according to hormonal profile:- E_2 induces GM-CSF while P_4 inhibits its production [240]. This cytokine is thought to be a major mediator of the endometrial response to seminal plasma. Within 12 hours of mating, seminal plasma induces a 20-fold increase in lumenal GM-CSF released by endometrial epithelial cells in mice [240, 241], an effect only observed when the seminal vesicles of the donor male remain intact [213]. GM-CSF recruits dendritic cells (DCs) and macrophages to the endometrium post coitum, with both cell types expressing the GM-CSF receptor (GM-CSFR) [242]. GM-CSF has been shown to have a number of effects including the regulation of glucose uptake and inhibition of apoptosis in the pre-implantation embryo [243]. This transient rise in GM-CSF abrogates before implantation in response to rising P_4 levels during early pregnancy

[240]. There is mounting evidence that GM-CSF controls the ability of macrophages and DCs to elicit a T cell response during early pregnancy in mice, increasing their APC capacity in terms of antigen uptake, processing and presentation [48, 244-247]. In this respect, insufficient GM-CSF may lead to impaired T-cell activation and therefore reduced tolerance and altered APC profiles, which are recognised contributory factors in both infertility and miscarriage [48, 248, 249].

Macrophage colony stimulating factor (M-CSF, also known as CSF-1) production by the endometrial epithelium correlates with macrophage distribution [250]. In the mouse, macrophages are evenly distributed at dioestrus, while at proestrus and oestrus they are increasingly associated with the epithelium, driven by a peak of E_2-induced M-CSF at proestrus [251]. However, as M-CSF is a relatively weak chemotactic agent for macrophages, together with the fact that M-CSF mRNA is not significantly increased post coitum compared to naturally cycling mice, it is unlikely to act in isolation in recruiting macrophages to the endometrium in response to mating [252].

2.1.4.2 IL-6 family of cytokines

The IL-6 family (including cardiotrophin-1, IL-6, IL-11, IL-27, IL-31, LIF and neurotrophic factor) comprises a diverse group of mediators involved in the regulation of the acute phase and immune responses [253-255]. IL-6 is expressed in uterine epithelial cells of the naturally cycling mouse in response to E_2 and P_4 profiles, with levels highest at proestrus [212, 238]. This expression is, however, not uniform – expression at the luminal surface of cultured murine epithelial cells is 2.5-5 fold higher than at the basal surface [256], suggesting that IL-6 is primed to respond to incoming paternal antigens in these polarised cells. Endometrial stromal cells also produce IL-6 in response to IL-1α, although this response occurs to a lesser extent than that seen in epithelium [256].

IL-6 is a known mediator of the acute inflammatory response, and switches the immune response from a neutrophil-mediated to a monocyte/macrophage mediated event via induction of intracellular adhesion molecule (ICAM)-1 and MCP-1 [257]. Alongside this switch, IL-6 also directs the differentiation of naïve $CD4^+$ T Cells into the Th17 lineage, with a concurrent inhibition of Treg cell differentiation, promoting their conversion to a Th17 phenotype cells via the downregulation of FoxP3 [258-260]. IL-6 mRNA is induced by seminal plasma proteins, including TGF-β, as seen in human cervical epithelial cells [211, 223, 261] and mid-cycle endometrial epithelial cells [262] *in vitro.* However, it remains to be seen if these relationships hold true *in vivo*.

Another member of the IL-6 family, LIF, has been shown to be essential for implantation in mice, since LIF null mice exhibit a lack of implantation and minimal decidualisation [263, 264]. As LIF null embryos implant normally when transferred into wild type recipients, this implantation defect is thought to be maternally regulated [265]. LIF mRNA has been reported to rise in murine glandular epithelium at day 4 post mating, with a concurrent spike in protein expression in response to an increase in E_2 production [266, 267]. However, the control of LIF expression varies between species: in the rabbit, P_4 appears to be its main regulator instead [268]. Seminal plasma also induces LIF mRNA expression in human endometrial epithelial cells *in vitro* [262]. However, the transition through the peri-implantation period from initial semen deposition to implantation remains poorly defined.

2.1.4.3 Interleukin-8

Present in human endometrial epithelium and, to a lesser extent, stromal cells, IL-8 is thought to coordinate the recruitment of neutrophils and granulocytes to the endometrium [269] and drive the proliferation of endometrial stromal cells prior to decidualisation [270]. The ability of IL-8 to maintain stromal cell growth in a paracrine manner is thought to be a major factor in the pathogenesis of endometriosis [271, 272]. Seminal plasma (particularly the TGF-β fraction) induces the release of IL-8 from human non-pregnant cervical explants and stabilises IL-8 mRNA transcripts in endometrial stromal cells [223, 261, 273, 274]. In the mouse, the gene encoding IL-8 has been deleted, along with its receptor CXCR1 [275]. Functional murine homologues to IL-8 include keratinocyte derived chemokine (KC/CXCL1) lipopolysaccharide-inducible CXC chemokine (LIX/CXCL5) and macrophage inflammatory protein (MIP-2/CXCL2), all of which share the extracellular loop reactive+ motif (Glu-Leu-Arg) which confers the chemoattractant properties of IL-8 [276].

2.1.4.4 Other identified chemokines

MCP-1 is another chemokine thought to mediate the maternal response to seminal plasma. In women, MCP-1 is secreted by the endometrial epithelium into the uterine lumen and stroma compartments, and its levels correlate with successful implantation [143, 277]. A potent chemoattractant for monocytes/macrophages [278], MCP-1 mRNA is very highly expressed in murine endometrial epithelial cells post mating, and if this translates into protein levels may account for the large influx of macrophages to the endometrium [251, 252].

Expressed on days 1 and 5 post mating in murine endometrial epithelium, RANTES is chemotactic for T cells, eosinophils and basophils [252, 279]. However, there is evidence to suggest that RANTES also recruits and activates macrophages, as uterine lumenal exposure to this chemokine results in a four-fold increase in macrophage numbers [252]. RANTES expression in the endometrium is stimulated by IFN-γ, IL-1β and TNF-α, with IL-4 antagonising TNF-α-induced RANTES production *in vitro* [280, 281].

2.1.4.5 Prostaglandins

Prostaglandins (PGs) are lipid mediators which are intimately linked with the immune system, both inducing and being induced by various cytokines. Although seminal PGs and prostacyclins are a major contributors to the induction of the maternal endometrial response, recent evidence shows that their production is also stimulated in the endometrium by seminal plasma, and that they are involved in the decidualisation process. In particular, PGE_2 and PGI_2 increase at the time of implantation in a localised manner [282-284], while PGI_2 appears to be essential for blastocyst implantation and decidualisation in the mouse [285]. PGs are produced from arachidonic acid by the enzymatic action of proteins encoded by prostaglandin endoperoxide synthase genes (PTGS;, also known as cyclooxygenases, COX), in conjunction with specific synthases [286]. Although COX-2 is generally considered to be inducible while COX-1 is constitutively expressed, both are inducible and developmentally controlled within the uterus. COX-2 is essential for successful implantation and pregnancy in mice [287] as highlighted by the fact that it is expressed at implantation sites within the endometrium and that COX-2 deficient mice exhibit multiple reproductive defects surrounding implantation and decidualisation [288-290]. Both COX-1 and COX-2 are transiently expressed post-mating in pigs [210] and mice, with COX-1 peaking at day 4 and COX-2 peaking at day 1 post coitum in murine whole uterine preparations [288]. COX-1 expression is governed by the prevailing hormonal milieu, being induced to its highest levels at day 4 by rising P_4 levels originating from developing corpora lutea (CLs) [288]. By contrast, COX-2 production appears to be independent of hormonal control, as demonstrated in ovariectomised mice [288]. Subsequent studies have localised COX-2 expression to epithelial cells in human endometrium, with expression peaking during the proliferative phase of the menstrual cycle *in vitro* [291-293].

Little is known about the function of PGs in the endometrial response to seminal plasma. Several studies speculate that PGs, particularly $PGF_{2\alpha}$, promote epithelial cell

proliferation and survival, with reduced rates of apoptosis [294, 295]. PGE_2 is thought to influence angiogenesis, particularly in conjunction with endometrial stromal cell activity, via the induction of VEGF in the mink and pig [296, 297], which may in turn induce $PGF_{2\alpha}$ production [298]. However, translating these findings from mustelids to other species must be approached with caution, as this family has developed a unique reproductive strategy of delayed implantation which can last up to 10 months and resumes upon environmental stimuli [299-301]. More recently, studies have shown that PG synthesis is induced by seminal plasma in the porcine uterus, with concurrent angiogenesis extending throughout the preimplantation period [302, 303]. Interestingly, the modulation of the CL by seminal plasma may be mediated by endometrial-derived PGE_2, which antagonises the luteolytic effect of $PGF_{2\alpha}$ in gilts [304]. Further speculative roles in PG function include the regulation of angiogenesis/vascular function, mitogenesis, tissue invasion, ECM remodelling and immunosuppression, although these remain to be demonstrated conclusively within the endometrium [305-309].

2.1.4.6 Matrix metalloproteinases (MMPs)

MMPs are zinc-dependent endopeptidases capable of degrading a large range of ECM proteins, with involvement in cell-surface receptor cleavage, cytokine activation/inactivation and release of apoptotic ligands (e.g. FAS-ligand) [310, 311]. They are traditionally grouped according to substrate specificity and cellular localisation, although with the emergence of new MMPs which do not fit into the current divisions, this categorisation may become redundant (Table 2-3). MMPs are regulated by circulating P_4 via the transcription factor lefty [40, 312], either by inhibition of mRNA synthesis or modulation of MMP-inducing cytokines [40]. In addition to their role in ECM remodelling, MMPs are intricately linked with cytokine biology. For example, MMP-1, -2, -3 and -9 cleave IL-1β into biologically inactive fragments, thereby reducing inflammation [313]. Conversely, MMP-2, -3, and -9 can also cleave IL-1β precursors into an active form, providing a mechanism for both up and downregulation of protein activity [116]. Epidermal growth factor (EGF) and platelet derived growth factor (PGDF) induce the transcription of MMP-1 mRNA in human endometrial stromal cells [314]. This interaction with cytokines also occurs at the cell surface, where MMP-7 can cleave and release TNF-α from the cell membrane [315].

Tissue remodelling, particularly in the endometrial stroma, occurs throughout the peri-implantation period, both prior to and during decidualisation [312, 316]. Several MMPs have been shown to mediate this process, with macrophages being the main source of these proteases under the influence of cytokines and prostaglandins [317]. However,

the function of MMPs varies across species. In humans, for example, these enzymes are the main mediators responsible for triggering endometrial shedding; this is not seen in rodents, where endometrial remodelling merely causes regression [42, 312]. MMP-7 protein expression in rat whole uterus increases sharply on day 1 post mating [318]. In mice, MMP-2 mRNA is expressed in the subepithelial stroma on days 3-5 post mating, while MMP-9 mRNA is expressed locally at the site of implantation at day 5 [319]. Tissue inhibitors of MMPs (TIMPs) are also induced within 12 hours of mating in the endometrial stroma [319].

Category	MMP
Collagenases	MMP-1, MMP-8, MMP-13
Gelatinases	MMP-2, MMP-9
Stromelysins	MMP-3, MMP-10, MMP-11
Matrilysins	MMP-7, MMP-26
Enamelysin	MMP-20
Metalloelastase	MMP-12
Membrane bound MMPs	MMP-14, MMP-15, MMP-16, MMP-17, MMP-24, MMP-25
Other	MMP-19, MMP-21, MMP-23A and B, MMP-27, MMP-28

Table 2-3: Classification of matrix metalloproteinases

2.1.4.7 Transforming growth factor (TGF)

TGF-β is present in large quantities in seminal plasma, but is also induced within the maternal endometrium post mating. In humans, the three isoforms of TGF-β are expressed within all endometrial cells, increasing in response to rising P_4 profiles [320-323]. However, there appear to be species-specific differences in the temporal regulation of endometrial TGF-β expression. In rodents, TGF-β expression is high during late dioestrus and proestrus, possibly indicating a role in endometrial restructuring from one cycle to the next [324]. This is in contrast to the high expression in human endometrium during the late proliferative/early secretory stages which correspond to low levels of proliferation and high levels of differentiation [322].

Within the murine endometrium, TGF-β1 and -2 have been identified at the foeto-maternal interface [325]. Neutralising antibodies to these cytokines reduce implantation rates, suggesting a pivotal role in decidualisation and the attachment of the blastocyst to the endometrial wall [326]. Concurrent with ECM remodelling, the signalling molecules (SMAD proteins) involved in the TGF-β signalling cascade are expressed

during the peri-implantation period concentrated at the lumenal epithelium both post mating and at implantation [327, 328]. The TGF-β modulation of ECM remodelling is thought to be achieved via the regulation of MMPs. Human endometrial stromal cell-derived TGF-β2 has been shown to suppress epithelial cell MMP-7 production *in vitro*, thereby providing a control for erroneous ECM breakdown [329]. However, alongside the caveat of species differences in endometrial TGF-β function, endometrial cells display different expression patterns depending on the *in vitro* system used, rendering these data difficult to transfer both across species and to the *in vivo* setting [330].

Little remains known about the TGF-β endometrial response to seminal plasma, with most research focussed on the effects of exogenous TGF-β within seminal plasma itself, and its role in decidualisation. One study showed that TGF-β2 is expressed in murine endometrial epithelial cells in the pre-implantation period [325]. In human endometrial stromal cells TGF-β1 inhibits proliferation and induces COX-2 [331]. However, further evidence is required in order to elucidate the roles of TGF-β isoforms in the endometrial response to seminal plasma.

Although individual immune mediators of the endometrial response to seminal plasma have been identified as discussed above, few studies have extended analyses to measuring large numbers of mediators in single samples to identify the global immune response at the level of the transcriptome. Many studies have focussed on profiling gene expression in the window of implantation, particularly in women following ovarian stimulation, in order to identify genes involved in endometrial receptivity in preparation for embryo transfer and in relation to implantation difficulties in endometriosis [332-336]. Only one, however, has explored global gene expression in the cervix following stimulation with seminal plasma. Sharkey *et al* (2012) performed expression microarray analysis on biopsies of human cervix pre- and post- coitus, and revealed that seminal fluid activated pathways including the inflammatory response, immune response, immune cell trafficking, cellular movement, and antigen presentation [211]. Within these pathways, GM-CSF, IL-6 and IL-8 mRNA were prominently upregulated post coitum, as were COX-2 and various MMPs, suggesting active leukocyte recruitment and ECM remodelling [211]. However, the reporting of these findings was scant, and only related to a single time point, which limits the interpretation of the results. As such, to date, there are no gene expression studies examining the murine endometrial response to seminal plasma *in vivo* throughout the pre-implantation period.

2.2 Aims

The aims of this chapter were:

- To identify changes in gene expression in response to seminal plasma relative to oestrus and dioestrus at four time points (days 1, 2, 3 and 4) throughout the pre-implantation period
- To identify pathways involved in the endometrial response to seminal plasma
- To identify differences between epithelial and stromal response in this process
- To evaluate whether the pathway analysis approach is sufficient to describe immune pathway involvement in the murine endometrial response to seminal plasma.

2.3 Materials and methods

An overview of methodology is presented in Figure 2-5. Detailed methods for each stage will be presented below.

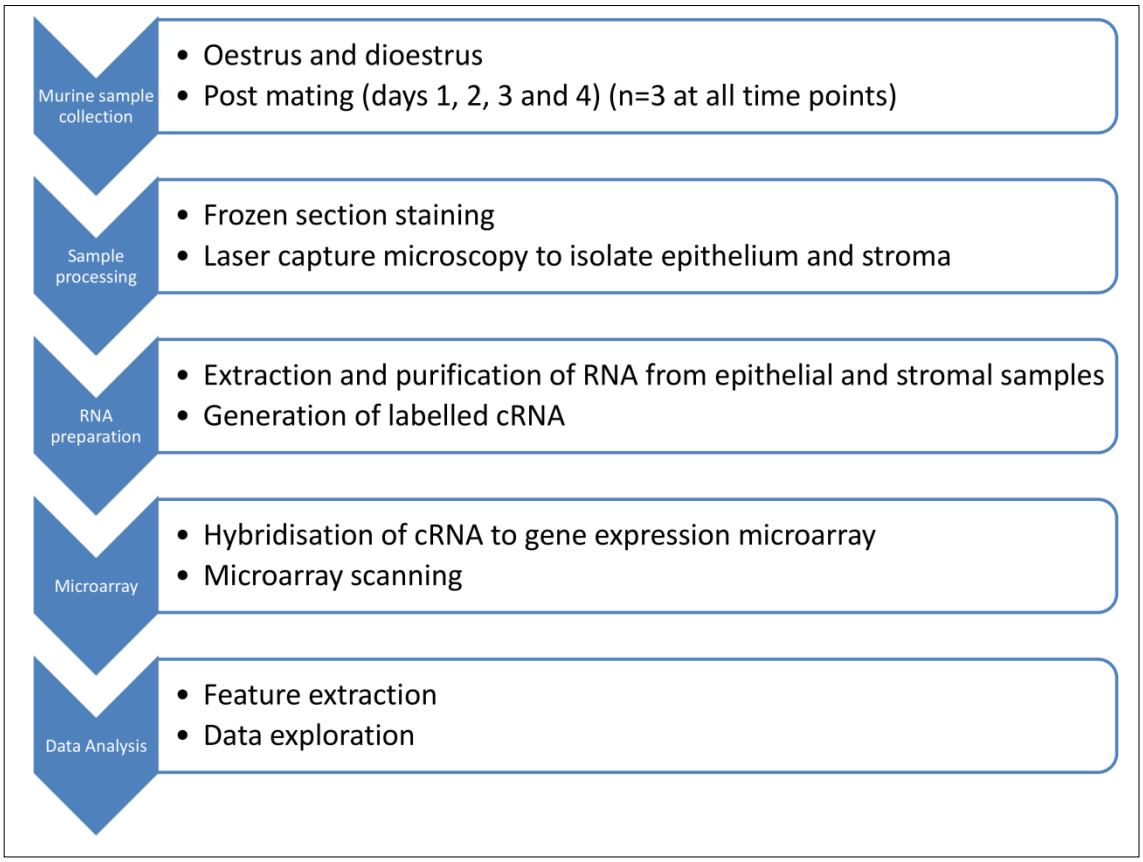


Figure 2-5: Methodology employed to explore immune networks within the murine endometrium

2.3.1 Murine husbandry

Male CD1 mice aged 10-12 weeks were individually housed while females (aged 6 weeks) of the same strain were group housed (10 per cage) and allowed to acclimatise to the unit environment for 1 week. Mice had *ad libitum* access to water and Standard Expanded Beekay diet (B&K, Grimston, Aldborough, UK). The lighting cycle was 14h:10h light:dark, (05:30 on; 19:30 off) and humidity and temperature were maintained at 55-65% and 21.5±1°C.

2.3.2 Murine mating protocol

Oestrus synchronisation was achieved via the Whitten effect by introducing male urine soaked bedding to the female cage 72 hours prior to mating. Oestrus was confirmed by vaginal smear and receptivity to the male. Females were naturally mated to males (1 female:1 male). Five successful copulations were observed prior to leaving the male and female together overnight. Females were then group housed according to time of mating, before being sacrificed under Schedule 1 of the Animals (Scientific Procedures) Act, 1986. Animals were sacrificed at 24, 48, 72 and 96 hours post initial mating ± 1 hour (*n*=3 at each time point). Controls were provided by naturally cycling females at the oestrus and dioestrus stages (*n*=3 for both), as determined by vaginal smear.

2.3.3 Uterine tissue fixation

Within five minutes of culling, mice were dissected and the ovaries, uterine horns and vagina were retrieved. Immediately after harvest, the tissue was placed on a cork block and coated in optimal cutting temperature (OCT) compound. The block was then placed in isopentane slush pre-cooled with liquid nitrogen until all the OCT became opaque. Once fully frozen, the blocks were stored on dry ice before transfer to -80°C freezer for storage.

2.3.4 Uterine tissue sectioning

Blocks were removed from -80°C storage and transported on dry ice for frozen sectioning using a Leica CM3050S Cryostat (Leica Biosystems, UK). The cryostat chamber temperature was set to -16°C, and blocks were allowed to equilibrate to this temperature for 30 minutes. Blocks were trimmed at 30µm section depth until sufficient tissue depth was achieved, with subsequent sections cut at 12µm. Sections were mounted onto Arcturus PEN membrane glass slides (Applied Biosystems, CA, USA), and kept on dry ice prior to staining.

2.3.5 Uterine tissue staining

All plasticware and staining jars were treated with RNAse-ZAP, rinsed three times in Milli-Q H_2O, and dried thoroughly (overnight) before use. Ethanol solutions for the staining protocol were prepared using RNAse-free water and molecular grade absolute ethanol. 100% anhydrous ethanol was prepared by adding 15g molecular sieve beads (3 Angstrom, 1-2 mm) to 500 ml ethanol.

Slides were stained using buffered cresyl violet staining solution - 40µl 0.5 M Tris-HCl pH 8.0 was added to 1 ml cresyl violet staining solution (1g cresyl violet solubilised in 75% ethanol and filtered) immediately prior to use. Staining with cresyl violet was chosen as this compound does not affect RNA quality.

Slides were stained using the following protocol, adapted from Cummings *et al* 2011 [337]:

- 95% ethanol, 30 seconds
- 75% ethanol, 30 seconds
- 50% ethanol, 30 seconds
- Lay slide flat, gently wipe edges with tissue and mark with barrier pen. Add 300 µl of buffered cresyl violet stain; 40 seconds
- 50% ethanol, seconds
- 75% ethanol, 30 seconds
- 95% ethanol, 30 seconds
- 100% ethanol, 30 seconds
- 100% ethanol, 30 seconds
- 100% ethanol, 5 minutes

Ethanol was used in order to preserve RNA quality, with a minimum concentration of 50%. All slides were blotted between each solution to reduce carry over. Solutions were changed after every two slides to prevent OCT saturation. Slides were stored at -80°C.

2.3.6 Laser capture microscopy

A Zeiss Palm® Microbeam UV laser capture microscope, equipped with PALM@Robo software version 3.2 (Carl Zeiss, Herts, UK) was used to perform laser capture microscopy (LCM). Samples were captured with cut energy set at 51-60 and laser speed of 20. Endometrial epithelial and stromal samples were identified by morphology under light microscopy (20x magnification), highlighted by the in-built drawing tool, excised by laser and collected into adhesive caps (Carl Zeiss, Herts, UK) individually.

2.3.7 RNA preparation

2.3.7.1 RNA extraction

RNA extraction was performed using a Qiagen RNA extraction kit (Qiagen, UK). Each captured sample was suspended in 350µl of RLT buffer with 3.5µl β-mercaptoethanol. Each sample was vortexed and stored at -80°C prior to RNA extraction. The extraction protocol was performed as per manufacturer's instructions with minor modifications; one extra RPE and 80% ethanol wash step was incorporated in order to reduce the carry-over of impurities and improve RNA quality. Samples were eluted from the spin column by immediate addition of 14µl RNAse-free H_2O, with 0.5µl RNASecure (Ambion) to the eluate. Samples were concentrated using a Savant SpeedVac® concentrator (Eppendorf, Hamburg, Germany) for 30 minutes or until dry, then resuspended in 4µl RNAse-free water and stored at -80°C until use.

2.3.7.2 Assessment of RNA quality

RNA quality was assessed using the Agilent RNA 6000 Nano kit (Agilent, UK) on a 2100 Bioanalyser (Agilent, UK) equipped with 2100 Expert software following the manufacturer's instructions. The chip was processed using the RNA Eukaryote Total RNA Nano function.

2.3.7.3 Fluorescent cRNA generation

Fluorescent cRNA was prepared using the Agilent One Colour Low Input Quick AMP labelling kit. 10ng of RNA was added to the labelling reaction. Purification of RNA was performed using the RNEasy Mini kit (Quiagen UK) following the manufacturer's instructions.

2.3.7.4 Flourescent cRNA quality assessment

Fluorescent cRNA quality was assessed using the NanoDrop 1000 (Thermo-Scientific UK). Cyanine (Cy3) concentration (pmol/µl), RNA 260/280 ratio and cRNA concentration (ng/µl) were recorded. The yield and specific activity were calculated for each sample as follows:

Yield (µg cRNA) = (Concentration of cRNA x elution volume)/1000

Specific Activity (pmol Cy3/µg cRNA) = (Concentration of Cy3/concentration cRNA) x 1000

2.3.8 RNA hybridisation and microarray scanning

Samples were hybridised to Agilent SurePrint G3 Mouse GE 8x60k Microarrays following the manufacturer's instructions. Hybridisation was performed in an Agilent hybridisation oven (Agilent, UK), hybridisation time was set to 17 hours at 65°C with a rotation speed of 20rpm. Subsequent washes were completed within 1 hour. Slides were scanned immediately to reduce potential variations in signal intensities due to environmental contaminants. Microarrays were scanned using an Agilent 'C' scanner (Agilent, UK), with a scan resolution of 3µm.

2.3.9 Data presentation and statistical analysis

2.3.9.1 Feature extraction

Feature extraction was performed using Feature Extraction version 11.0.1.1 (Agilent Technologies, UK). Sample quality was assessed utilising the inbuilt quality control metrics. 11 metrics were applied to the microarray, covering aspects such as alignment, signal intensity and reproducibility, providing a range within which a microarray is considered 'good' (Table 2-4). Microarrays falling outside of these 'good' criteria were evaluated for inclusion/exclusion from the analysis.

Metric name	Aspect assessed	'Good' criteria
Isgoodgrid	Alignment of the microarray correctly detected by the software	>1
AnyColorPrcntFeatNonUn	Percentage of non-uniform outliers	<1
gNegCtrlAveNetSig	Negative control statistics, average net signal (mean signal minus scanner offset, indicates dynamic range)	<40
gNegCtrlAveBGSubSig	Average background subtracted signal	-10 to 5
gNegCtrlSDevBGSubSig	Standard deviation of the BG sub-signal	<10
gSpatialDetrendRMSFilter	Low signal background – the higher the number, the more background detected and removed	<15
gNonCntrlMedCVProcSign	Reproducibility in repeated non-control probes (median %CV)	0 to 8
gE1aMedCVProcSignal	Spike-in reproducibility plot (%CV)	0 to 8
absGE1E1aSlope	Slope of spike-in dose response curve	0.9 to 1.2
DetectionLimit	Limit of detection of spike-in	0.01 to 0.2
gDDN	Direction dependent noise (background) from green channel	-15 to 15

Table 2-4: Quality control metrics applied to Agilent 1-colour gene expression microarrays. 'Good' criteria show the range of scores indicating that a particular microarray is of high enough quality to be included in analysis.

2.3.9.2 Sample size calculation

Sample size and desired fold difference were calculated using the Microarray Sample Size Computation tool available at http://bioinformatics.mdanderson.org/microarraysamplesize/. The calculation was based on 55821 entities, with an acceptable false positive rate of 5%, power (percentage of differentially expressed entities likely to be detected by the experiment) of 0.8, and a standard deviation of 0.7.

2.3.9.3 Data exploration

Data were visualised and analysed using GeneSpring version 12.5 (Agilent Technologies, UK). Data were normalised by dividing the signal intensity of each entity by 75% of the average signal intensity of all entities. Samples were examined by the inbuilt principal components analysis (PCA) to identify major outliers. Due to the sample size, a 5-fold change cut off was applied to the data. Pathway analysis was performed using the condition averages in order to identify immune pathways active in

the murine endometrium. P values were corrected for multiple comparisons using Benjamini and Hochberg's false discovery rate (FDR). The results from the pathway analysis were used in subsequent cluster analyses.

2.3.9.4 Cluster analysis

Entities identified through pathway analysis were subjected to cluster analysis in IBM SPSS version 19. The analysis was performed for epithelium and stroma individually, based on a two-step cluster analysis using Ward linkage. At each iteration, the ∆coefficient was calculated, with the largest ∆coefficient defining the number of clusters. Identified entities in each cluster were visualised and graphed in GraphPad Prism version 6.

2.4 Results

2.4.1 Microscopic changes in the murine endometrium

Epithelial and stromal cells differed in microscopic appearance at different points during the oestrus cycle and post coitum. At dioestrus, epithelial cells appeared reduced in height compared to oestrus, while stromal cells were round/oval (Figure 2-6). Mitotic bodies were evident within the epithelium.

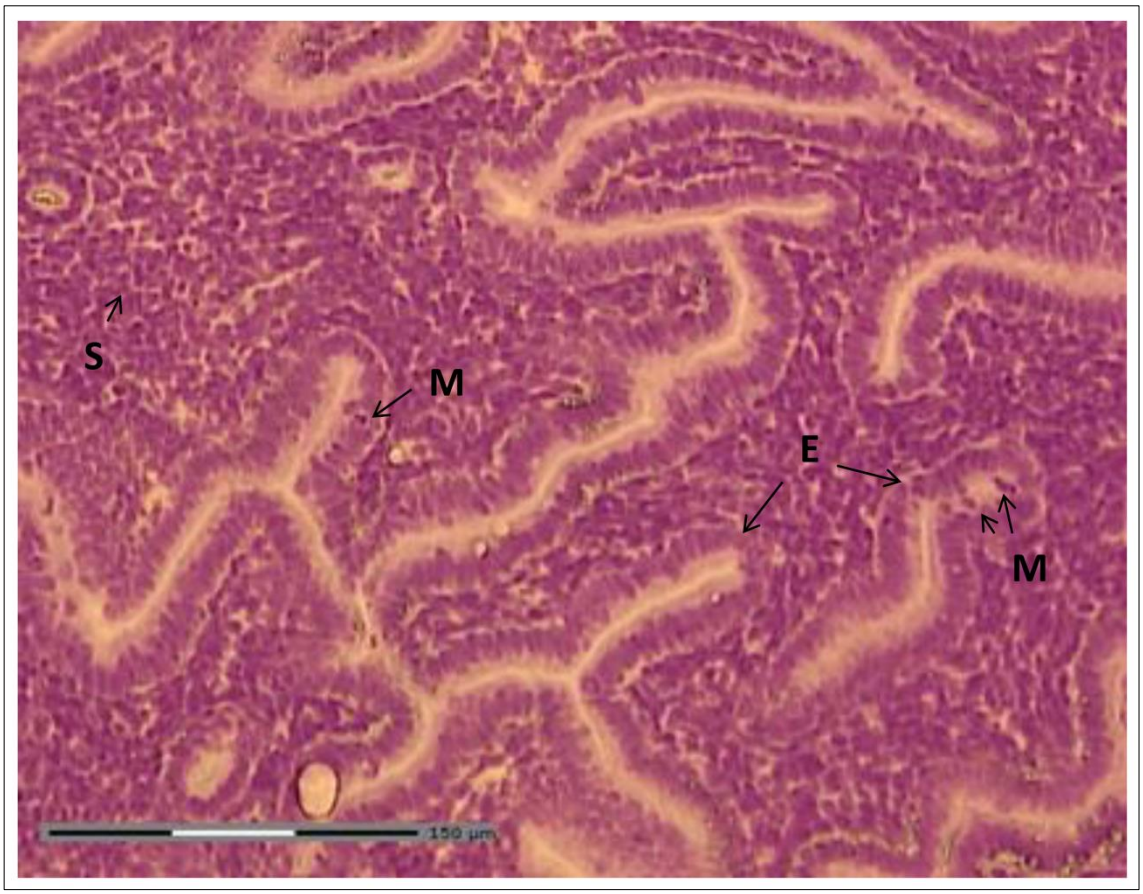


Figure 2-6: Endometrial epithelial and stromal cells at dioestrus. Frozen sections were stained with cresyl violet and visualised at 20x magnification. E – epithelium, M – mitotic bodies, S – rounded/oval stromal cells.

Within the oestrus endometrium, the epithelial cells became very large, tall columnar cells (Figure 2-7). There was evidence of vacuolar degeneration and apoptotic cells within the epithelium, and the stromal compartment maintained a fibroblast-like appearance. The uterine lumen also showed evidence of dilation.

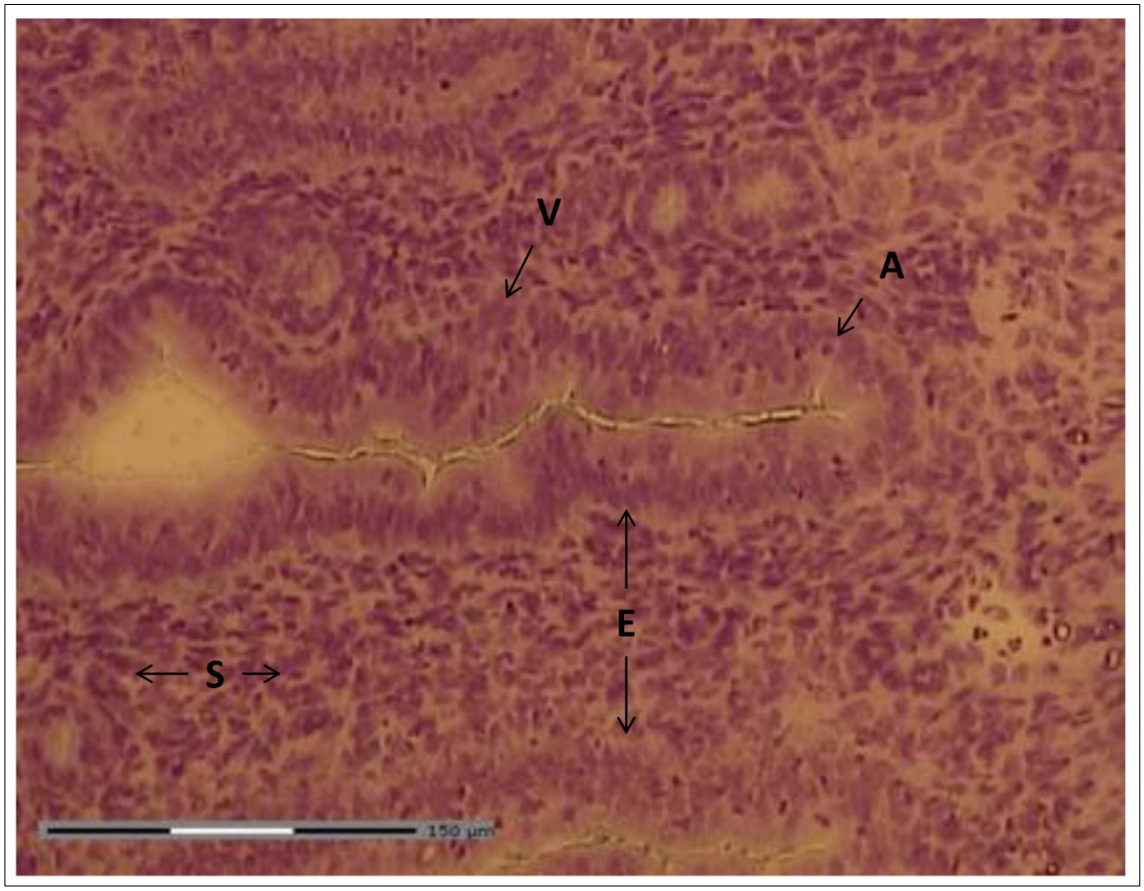


Figure 2-7: Endometrial epithelial and stromal cells at oestrus. Frozen sections were stained with cresyl violet and visualised at 20x magnification. A – apoptotic cells, E – epithelium, S – stromal cells, V – vacuolar degeneration.

Post coitum, the epithelium maintained a taller appearance at days 1 and 2, subsequently returning to a shortened state (similar to that seen at dioestrus) at days 3 and 4 (Figure 2-8). Stromal cells became progressively more rounded towards day 4 post coitum.

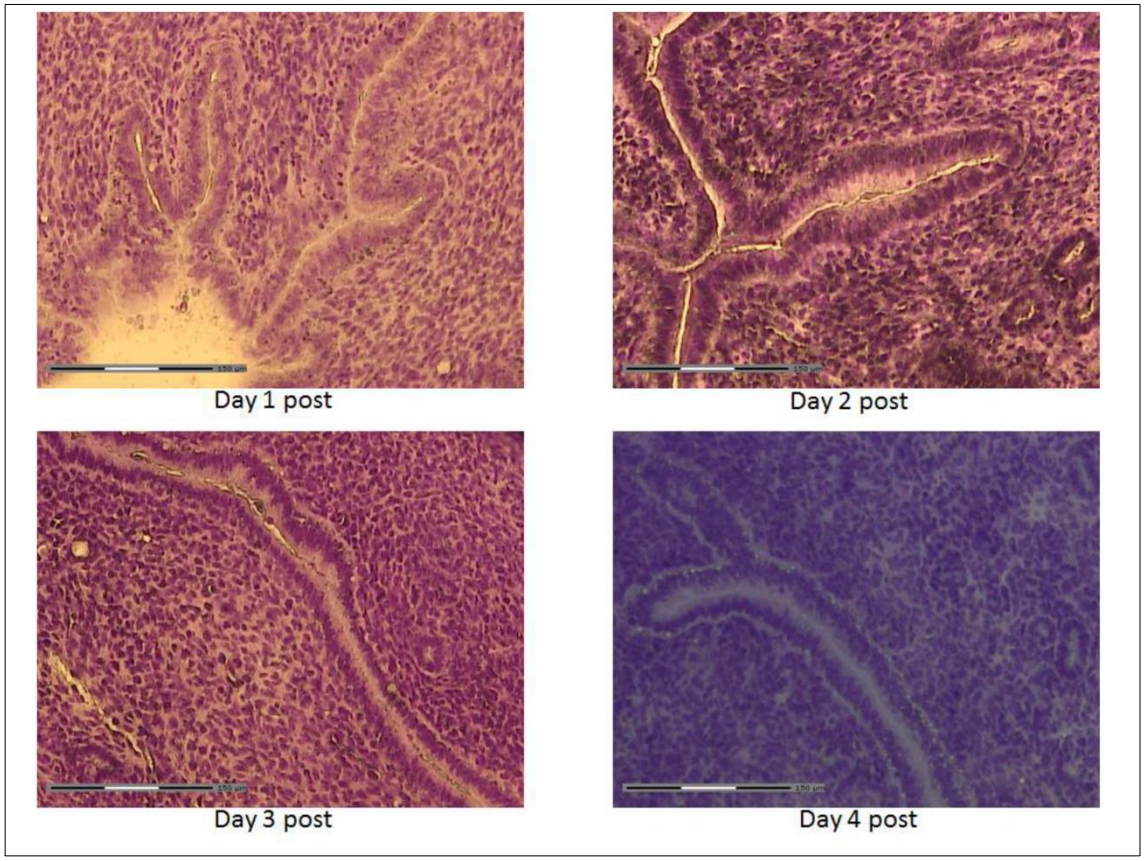


Figure 2-8: Endometrial epithelial and stromal cells on days 1-4 post coitum. Frozen sections were stained with cresyl violet and visualised at 20x magnification.

2.4.2 Sample quality control and filtering

2.4.2.1 Epithelial samples

Samples were assessed for hybridisation quality. Table 2-5 shows the number of quality control metrics passed for each epithelial sample.

Sample	Number of metrics passed
Dioestrus 1	2
Dioestrus 2	11
Dioestrus 3	7
Oestrus 1	11
Oestrus 2	7
Oestrus 3	11
Day 1-1	11
Day 1-2	3
Day 1-3	2
Day 2-1	7
Day 2-2	11
Day 2-3	11
Day 3-1	11
Day 3-2	10
Day 3-3	11
Day 4-1	10
Day 4-2	10
Day 4-3	11

Table 2-5: Quality control metrics for epithelial samples.

Samples passing less than 7 quality control metrics were excluded from subsequent analysis. Profile plots were examined for those samples passing 7 or more metrics (Figure 2-9).

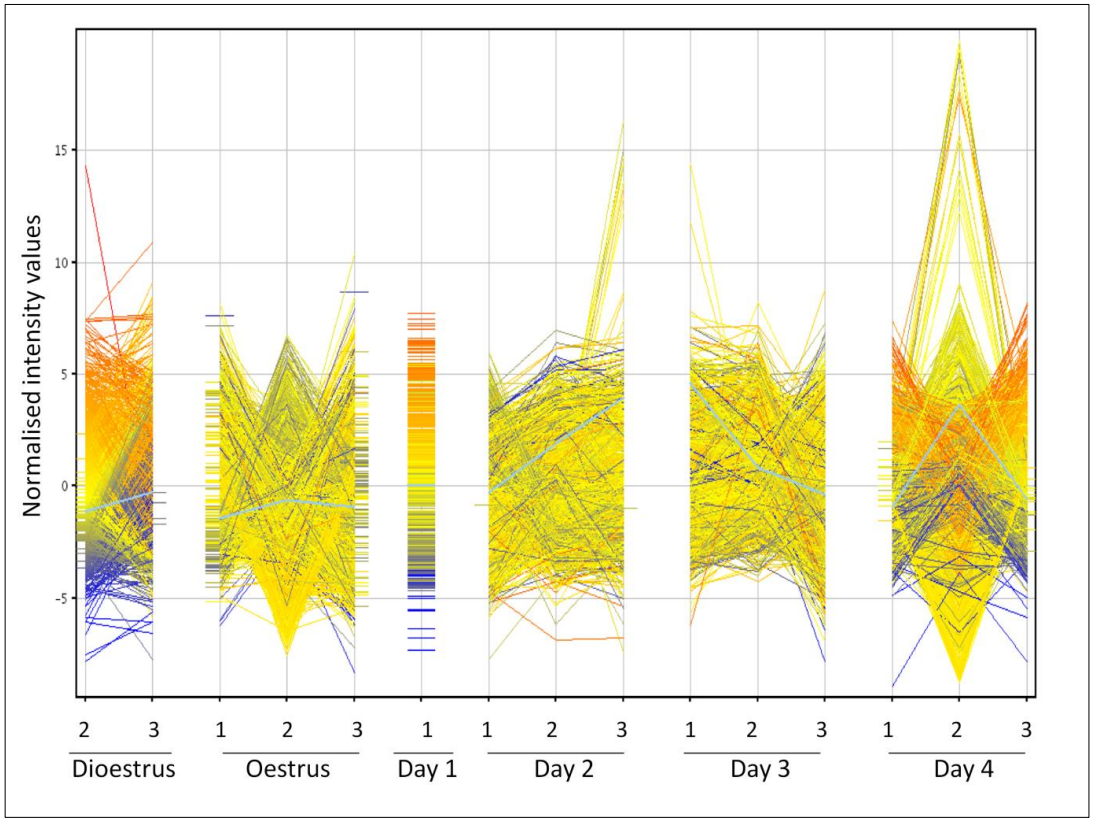


Figure 2-9: Endometrial epithelial cell sample profile plot. Normalised intensity values for each entity are displayed according to sample grouping.

Samples Oestrus 2 and Day 4-2 exhibited ‘mirroring’ or ‘inversion’ of their normalised intensity values compared to their replicates (Figure 2-9). Extended discussions with the gene expression microarray supplier (Agilent, UK) suggested that these samples may have been contaminated, however this was not conclusive. Initial PCA of the data (using the in-built PCA in Genespring) identified Oestrus 2 and Day 4-2 as outliers. Due to the low level of confidence in the results and the lack of correlation with their biological replicates, these samples were excluded from subsequent analysis. The remaining samples were averaged, and the resultant profile plots are displayed below (Figure 2-10). Individual entities (mRNA) appeared to exhibit differential expression over time.

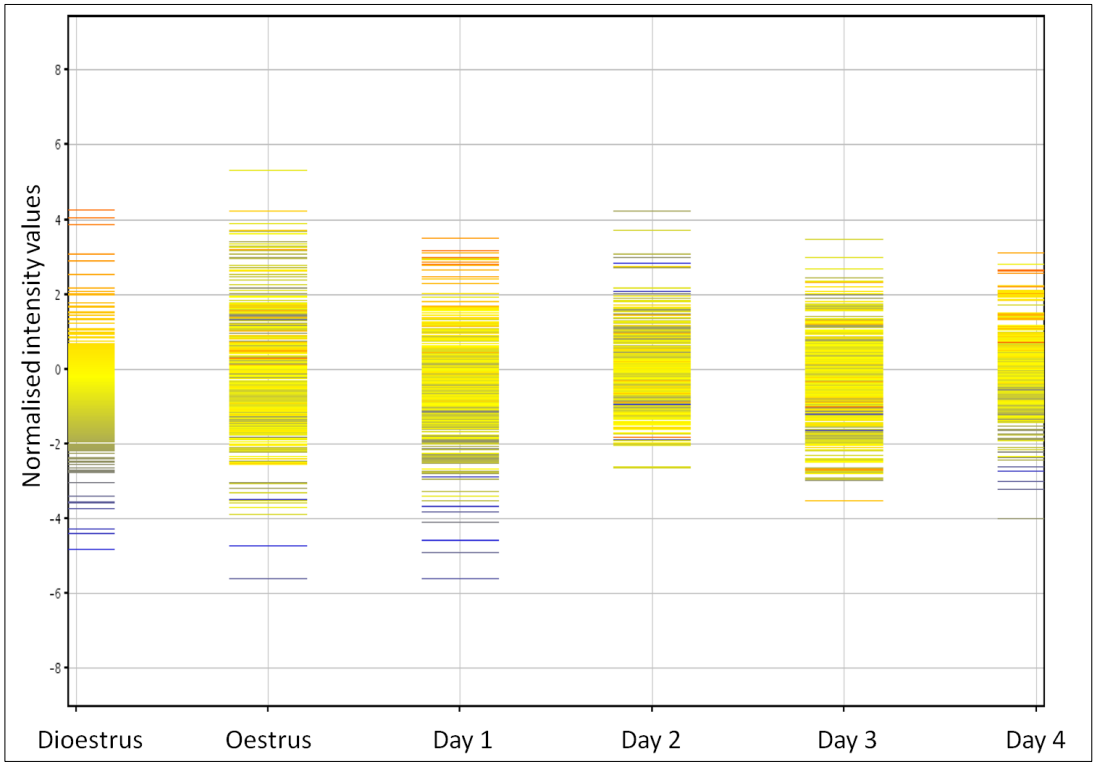


Figure 2-10: Averaged profile plots for epithelial samples. Normalised intensity values are displayed according to sample type. Individual entities (mRNA transcripts) are colour coded and exhibit differential expression over time.

2.4.2.2 Stromal samples

Samples were assessed for hybridisation quality. Table 2-6 shows the number of quality control metrics passed for each epithelial sample. As with the epithelium, stromal samples passing less than 7 quality control metrics were excluded from subsequent analysis. Profile plots were examined for those samples passing 7 or more metrics (Figure 2-11). Stromal profiles appeared inherently noisier than epithelial samples. Although some 'mirroring' was evident in some samples this was not as pronounced and limited to a small number of entities, with outliers not identified by PCA. Therefore all remaining samples were included in subsequent analyses.

Sample	Number of metrics passed
Dioestrus 1	7
Dioestrus 2	7
Dioestrus 3	10
Oestrus 1	10
Oestrus 2	11
Oestrus 3	8
Day 1-1	11
Day 1-2	7
Day 1-3	7
Day 2-1	2
Day 2-2	8
Day 2-3	11
Day 3-1	7
Day 3-2	8
Day 3-3	6
Day 4-1	11
Day 4-2	11
Day 4-3	9

Table 2-6: Quality control metrics for stromal samples.

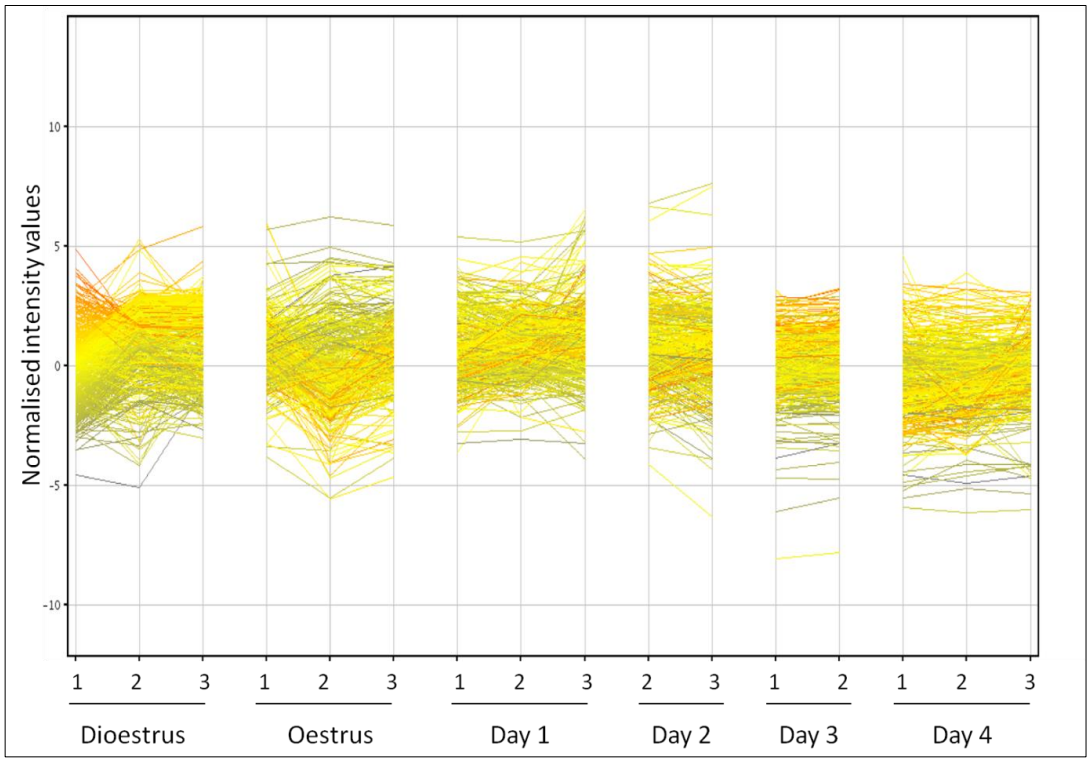


Figure 2-11: Endometrial stromal cell sample profile plot. Normalised intensity values for each entity are displayed according to sample grouping.

2.4.2.3 Sample filtering

Of 55821 entities, 39516 displayed a 5-fold change between at least one condition/time point. These entities were used for subsequent analyses.

2.4.3 Pathway analysis

Pathway analysis within Genespring revealed 217 curated pathways active within the murine uterine tract (Appendix I: Non-significant pathways identified by pathway analysis. Of these pathways, 46 were significant at p=0.05 (Table 2-7), with significance defined by an in-built algorithm which created a composite score of the number of entities matched and the extent of the response of those entities. As the theme of research was focussed on the role of immune pathways in the reproductive environment, only pathways relevant to the immune function were selected and examined in epithelial and stromal samples (highlighted in Table 2-7).

Pathway	WikiPathways ID	p-value	Number of Matched Entities	Number of Pathway Entities
GPCRs, Class A Rhodopsin-like	WP189 62834	0.00	208	231
GPCRs, Other	WP41 54691	0.00	150	210
GPCRs, Other	WP41 62667	0.00	150	210
Cytoplasmic Ribosomal Proteins	WP163 62833	0.00	77	80
Peptide GPCRs	WP234 41308	0.00	63	70
Metapathway biotransformation	WP1251 41349	0.00	117	143
Monoamine GPCRs	WP570 48232	0.00	32	33
Monoamine GPCRs	WP570 60231	0.00	32	33
Calcium Regulation in the Cardiac Cell	WP553 47774	0.00	120	150
Striated Muscle Contraction	WP216 41273	0.00	40	45
Complement and Coagulation Cascades	WP449 41301	0.00	53	62
Prostaglandin Synthesis and Regulation	WP374 41394	0.00	29	31
Small Ligand GPCRs	WP353 41279	0.00	18	18
Selenium	WP1272 58514	0.00	22	31
Cytokines and Inflammatory Response (BioCarta)	WP222 53571	0.00	26	30
Selenium	WP1272 59028	0.00	21	31
Complement Activation, Classical Pathway	WP200 47967	0.00	16	17
Cytochrome P450	WP1274 48227	0.00	35	40
Myometrial Relaxation and Contraction Pathways	WP385 47969	0.00	120	157
Blood Clotting Cascade	WP460 62696	0.00	19	20
Glucocorticoid & Mineralcorticoid Metabolism	WP495 62838	0.00	13	13
SIDS Susceptibility Pathways	WP1266 58254	0.00	49	61
SIDS Susceptibility Pathways	WP1266_58281	0.00	49	61
GPCRs, Class B Secretin-like	WP456 41317	0.01	20	22
Nuclear receptors in lipid metabolism and toxicity	WP431 47744	0.01	26	30
Glucuronidation	WP1241 59029	0.01	11	18
Irinotecan Pathway	WP475 48258	0.01	11	11
Matrix Metalloproteinases	WP441 41300	0.01	25	29
Macrophage markers	WP2271 53132	0.01	10	10
XPodNet - protein-protein interactions in the podocyte expanded by STRING	WP2309 58142	0.01	573	836
XPodNet - protein-protein interactions in the podocyte expanded by STRING	WP2309 58143	0.01	573	836
Oxidative Stress	WP412 41381	0.02	24	29
GPCRs, Class C Metabotropic glutamate, pheromone	WP327 41361	0.02	14	15
Folic Acid Network	WP1273 48256	0.02	20	27
Inflammatory Response Pathway	WP458 57463	0.03	25	30
TGFβ Signaling Pathway	WP113 41270	0.03	41	52
Metapathway biotransformation	WP1251 59060	0.03	22	143
Hedgehog Signaling Pathway	WP116 41332	0.03	19	22
Folic Acid Network	WP1273 60224	0.03	19	27
Glycolysis and Gluconeogenesis	WP157 43573	0.03	38	48
Endochondral Ossification	WP1270 41292	0.03	48	62
Serotonin and anxiety-related events	WP2140 58159	0.03	12	13
Glycolysis and Gluconeogenesis	WP157 51735	0.04	39	50
ESC Pluripotency Pathways	WP339 42897	0.04	81	111
Retinol metabolism	WP1259 51364	0.04	31	39
Oestrogen metabolism	WP1264 48250	0.04	11	13

Table 2-7: Pathways in the murine endometrium which were identified by pathway analysis to be significant ($p<0.05$). Pathways highlighted were selected for examination in epithelial and stromal samples. P values are presented to 2 decimal places.

2.4.4 Endometrial response to seminal plasma – pathway analysis

Pathway analysis within Genespring was based on pre-defined pathways described in disease models. For the sake of clarity and readability, these models are presented in Appendix II for results relating to endometrial epithelium, and Appendix III: Pathway analysis for LCM captured endometrial stromal cells. for pathways relating to endometrial stromal cells.

2.4.5 Epithelial response to seminal plasma – cluster analysis

mRNA entities encoding cytokine proteins and matrix metalloproteinases highlighted in the pathway analysis were selected for subsequent cluster analysis. These elements fell into 8 clusters, as shown in Figure 2-12. Cluster 1 was defined by peaks in expression on days 1 and 3 post mating with reduced expression on day 2 (*Cxcl3, Il10, Il12a, Mmp9, Mmp20, Mmp25, Tnf).* Cluster 2 exhibited a similar pattern of peaks on days 1 and 3, with a less dramatic reduction in expression on day 2 (*Bmp4, Egf, Il7, Il13, Mmp1a, Mmp3, Mmp16, Mmp28).* Cluster 3 generally showed a peak in expression at oestrus and day 2 post mating with a reduction in expression on day 1 (*Ccl5, Csf3, Il1b, Il15, Inhb1, Mmp11, Mmp14, Mmp23, Thbs*). Cluster 4 demonstrated a slight peak at dioestrus and day 1 post mating (*Ifng, Mcp1, Mmp2, Mmp19, Tgfb1, Tgfb2, Tgfb3).* Cluster 5 showed a dip in expression at oestrus, while remaining relatively constant at other time points (*Csf2, Cxcl2, Il2, Il4, Il5, Il12b, Itbp1, Mmp10, Mmp12, Mmp13, Mmp21, Mmp24).* Cluster 6 exhibited minor peaks on days 2 and 4 post mating, although many elements showed large variation (*Cxcl1, Il1a, Lif, Mmp8, Mmp27).* Cluster 7 was characterised by peaks at dioestrus, day 1 and day 4 post mating, with a dramatic reduction in expression at day 2 and 3 (*Csf1t1, Csf1t3, Mmp7).* Finally, cluster 8 elements exhibited huge variations in expression at each time point, and therefore could not be described according to any particular pattern.

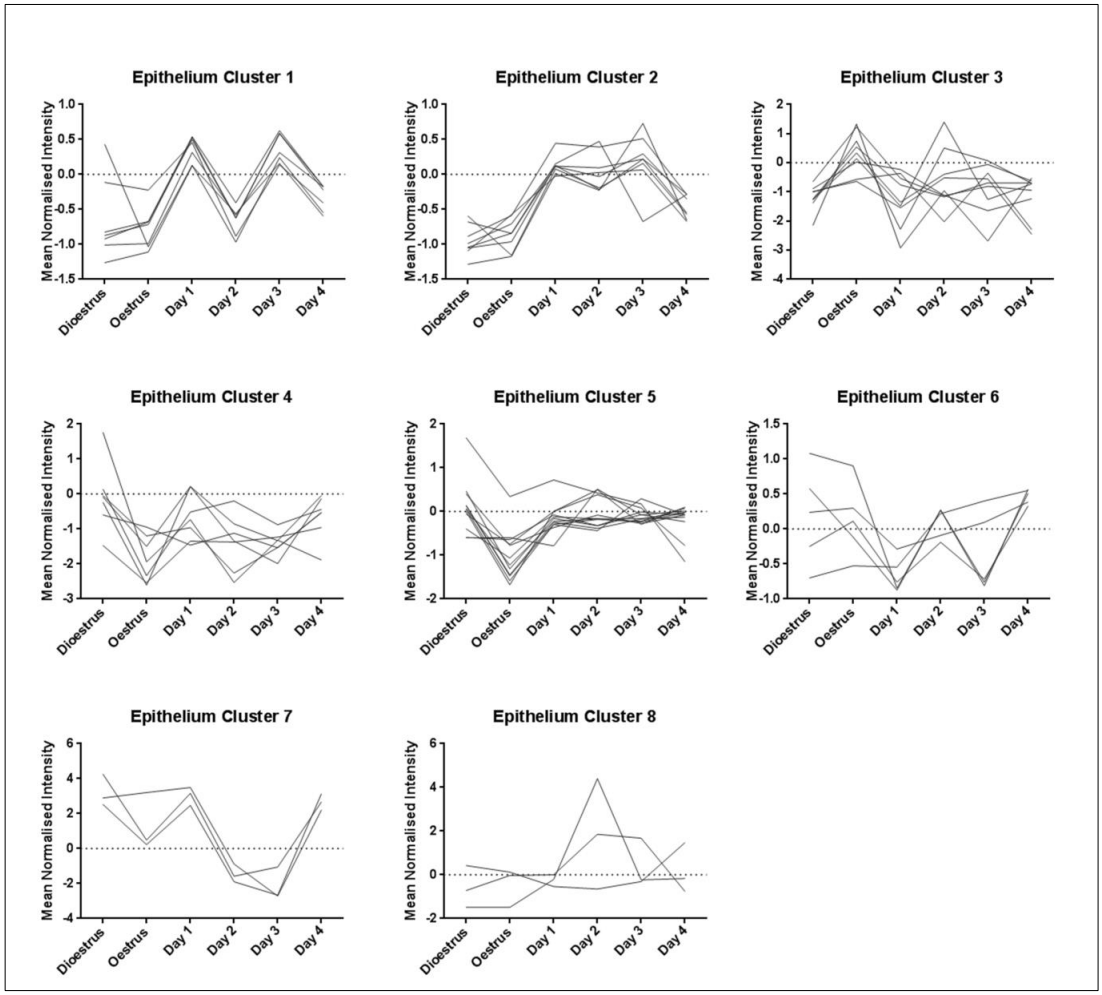


Figure 2-12: Cluster analysis of mRNA encoding cytokine and MMP proteins in uterine epithelial cells. Cluster membership: Cluster 1 - *Cxcl3*, *Il10*, *Il12a*, *Mmp9*, *Mmp20*, *Mmp25*, *Tnf*; Cluster 2 - *Bmp4*, *Egf*, *Il7*, *Il13*, *Mmp1a*, *Mmp3*, *Mmp16*, *Mmp28*; Cluster 3 - *Ccl5*, *Csf3*, *Il1b*, *Il15*, *Inhb1*, *Mmp11*, *Mmp14*, *Mmp23*, *Thbs*; Cluster 4 - *Ifng*, *Mcp1*, *Mmp2*, *Mmp19*, *Tgfb1*, *Tgfb2*, *Tgfb3*; Cluster 5 - *Csf2*, *Cxcl2*, *Il2*, *Il4*, *Il5*, *Il12b*, *Itbp1*, *Mmp10*, *Mmp12*, *Mmp13*, *Mmp21*, *Mmp24*; Cluster 6 - *Cxcl1*, *Il1a*, *Lif*, *Mmp8*, *Mmp27*; Cluster 7 - *Csf1t1*, *Csf1t3*, *Mmp7*; Cluster 8 - *Ifnb*, *Il6*, *Il11*.

2.4.6 Stromal response to seminal plasma: cluster analysis

mRNA entities encoding cytokine proteins and MMPs highlighted in the pathway analysis were also selected for subsequent cluster analysis. These species fell into 7 clusters, as shown in Figure 2-13. Cluster 1 comprised three mRNA species (*Csf1t1, Csf3, Mmp23)* which exhibited a peak at oestrus. Cluster 2 was characterised by a slight increase in expression on day 1 post mating, with a subsequent decrease on days 2, 3 and 4 (*Bmp4, Csf1t3, Csf2, Cxcl2, Cxcl3, Egf, Ifnb1, Il1a, Il2, Il4, Il5, Il6, Il7, Il10, Il11, Il12a, Il12b, Il13, Mmp1a, Mmp3, Mmp8, Mmp10, Mmp13, Mmp12, Mmp16, Mmp20, Mmp21, Mmp24, Mmp25, Mmp28, Tnf*). Cluster 3 comprised only of Mmp7, which showed highest expression at oestrus with lowest expression on day 3 post mating. Cluster 4 increased in expression post mating and remained high on days 2, 3 and 4 (*Ifng, Il15, Mmp2, Mmp19, Tgfb1, Tgfb2, Tgfb3*). Cluster 5 peaked on days 1 and 2 post mating with a subsequent decease on days 3 and 4 (*Ccl5, Inhb1, Il1b, Mcp1, Mmp9, Thbs1*). Cluster 6 peaked on day 1 with a subsequent fall to baseline levels of expression (*Mmp11, Mmp14*). Cluster 7 was characterised by peaks in expression at dioestrus and day 3 post mating (*Cxcl1, Itbp1, Lif, Mmp27*).

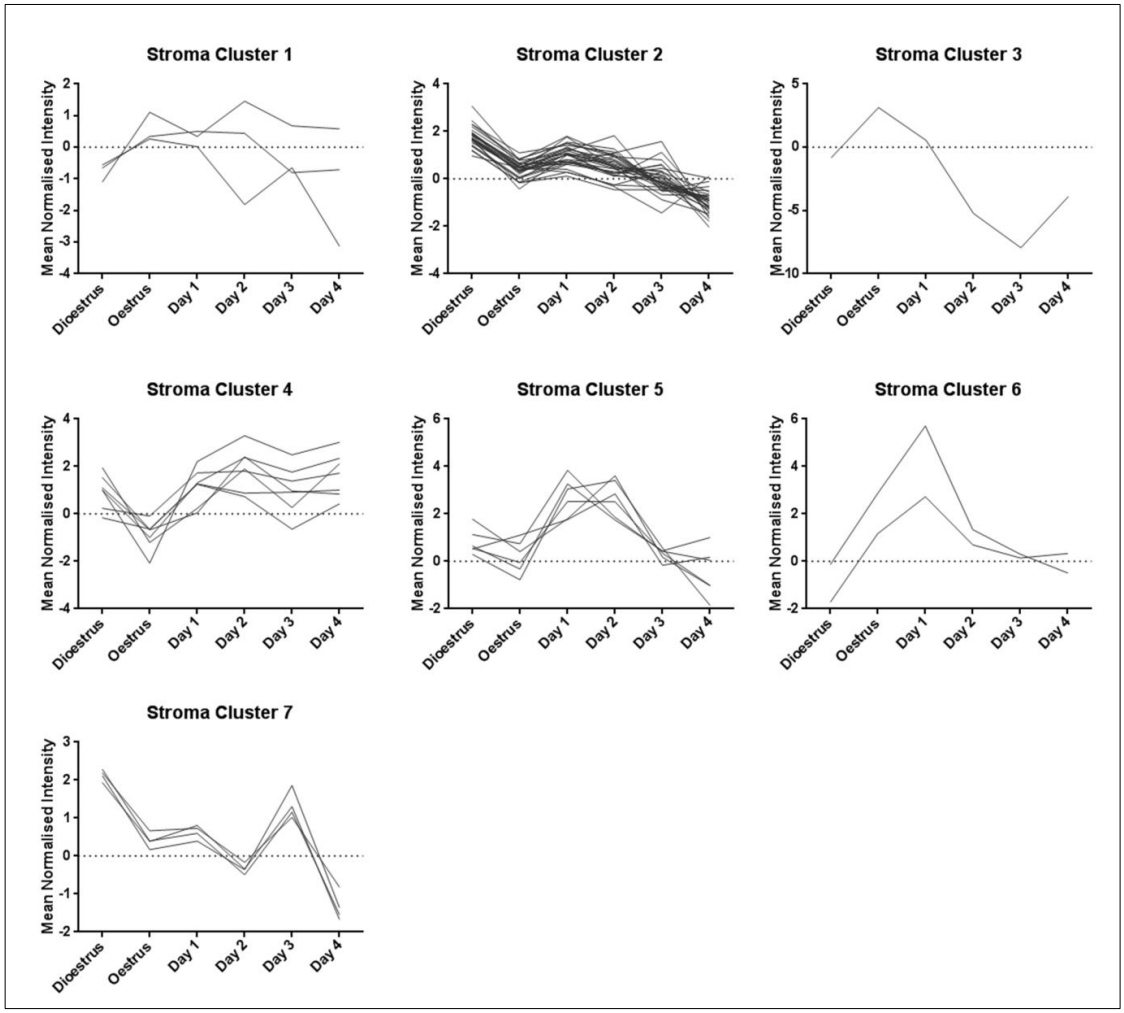


Figure 2-13: Cluster analysis of mRNA encoding cytokines and MMPs in uterine endometrial stromal cells. Cluster membership: Cluster 1 - *Csf1t1, Csf3, Mmp23;* Cluster 2 - *Bmp4, Csf1t3, Csf2, Cxcl2, Cxcl3, Egf, Ifnb1, Il1a, Il2, Il4, Il5, Il6, Il7, Il10, Il11, Il12a, Il12b, Il13, Mmp1a, Mmp3, Mmp8, Mmp10, Mmp13, Mmp12, Mmp16, Mmp20, Mmp21, Mmp24, Mmp25, Mmp28, Tnf;* Cluster 3 - *Mmp7;* Cluster 4 - *Ifng, Il15, Mmp2, Mmp19, Tgfb1, Tgfb2, Tgfb3;* Cluster 5 - *Ccl5, Inhb1, Il1b, Mcp1, Mmp9, Thbs1;* Cluster 6 - *Mmp11, Mmp14;* Cluster 7 - *Cxcl1, Itbp1, Lif, Mmp27.*

2.4.7 Endometrial epithelial and stromal differences

Many mRNA species exhibited differences in expression between endometrial epithelial and stromal cells. The majority showed greater relative expression in stromal cells as compared to epithelial cells, with the exception of *Mmp7* and elements of the prostaglandin pathway (as detailed below) which were consistently more highly expressed in epithelial cells.

2.4.7.1 Colony stimulating factors

The colony stimulating factors varied in expression in each cell type (Figure 2-14) with notable differences in both transcript variants of *Csf1*. This mRNA peaked on days 1 and 4 post mating in the epithelium, while expression in the stroma remained relatively constant. *Csf2* showed a slight peak in expression on day 1 post mating in both epithelium and stroma, with epithelial expression peaking on day 4 while stromal expression slowly declined. Of the colony stimulating factors, *Csf3* showed the greatest difference in expression between epithelium and stroma, with a dramatic drop in epithelial expression on day 1 followed by a peak on day 2 post mating.

2.4.7.2 Chemokines

With the exception of *Ccl5*, the expression of chemokines rose in epithelial cells above the levels seen in stroma on day 4 post mating (Figure 2-15). *Cxcl1* appeared to have opposite expression profiles in epithelial and stromal cells. *Ccl5* exhibited a strong peak in stromal cell expression on day 2 post mating, with a subsequent dramatic fall on day 3. *Mcp1* peaked in stroma on days 1 and 2 post mating.

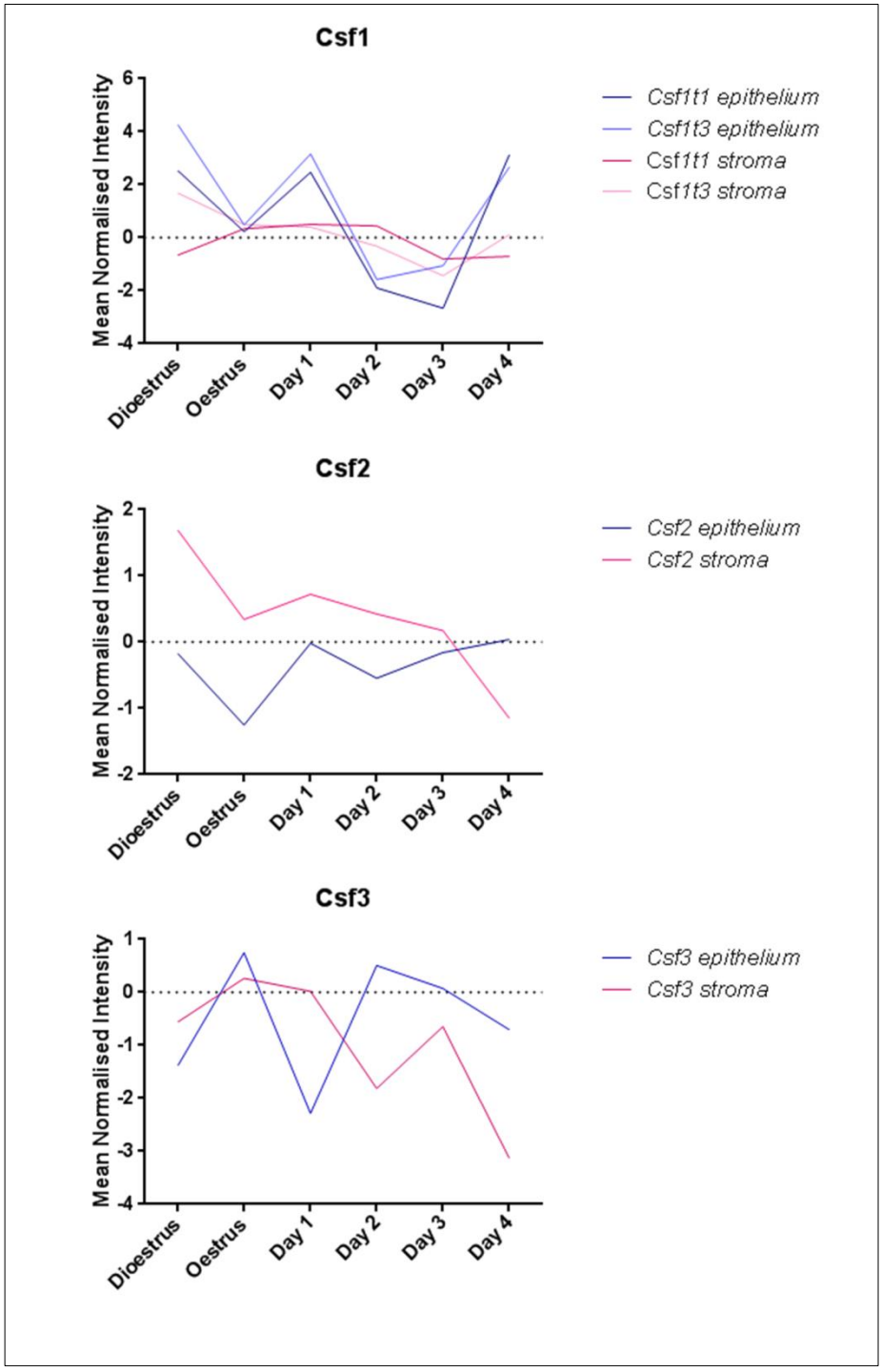


Figure 2-14: Mean CSF mRNA normalised intensity in endometrial epithelial and stromal cells *post coitum.*

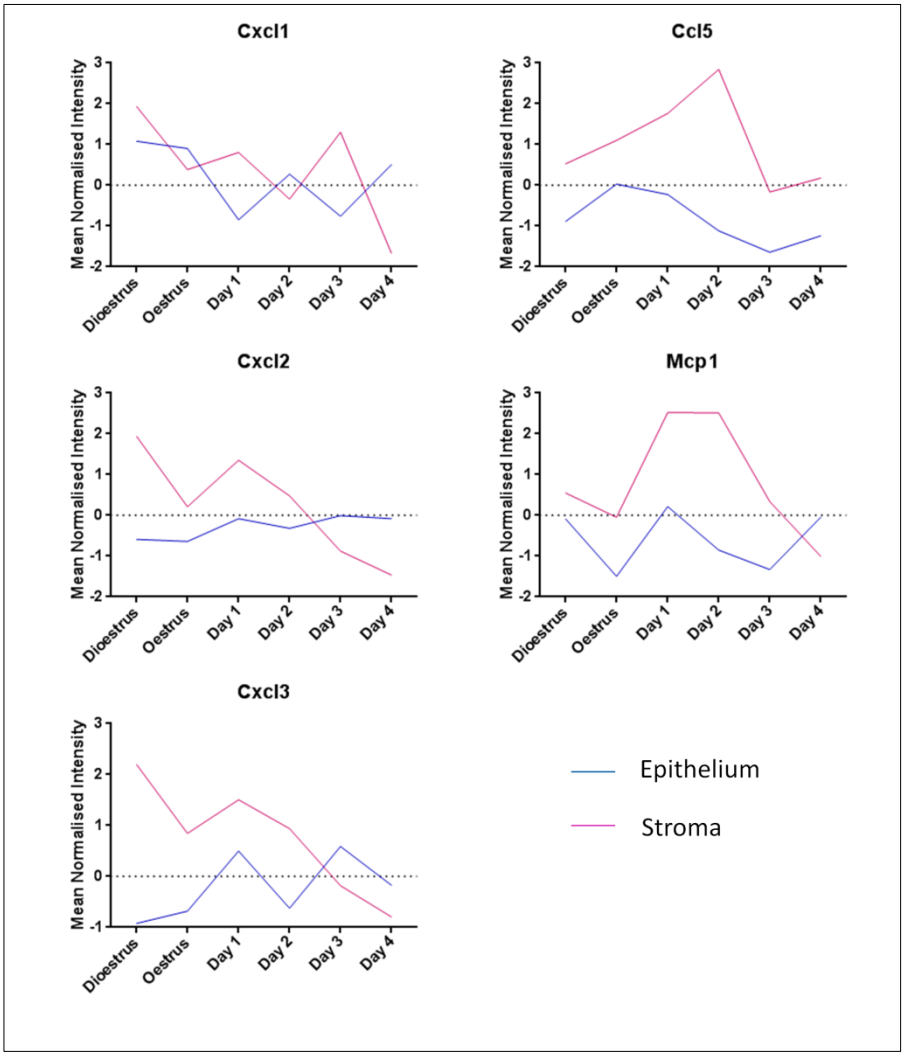


Figure 2-15: mRNA expression of chemokines in endometrial epithelial and stromal cells *post coitum*.

2.4.7.3 Interleukins

Many interleukins followed a similar pattern of expression in epithelium and stromal cells, being initially relatively higher in the stroma with a subsequent decrease in stromal expression below that seen in epithelium on days 3 and/or 4 post mating (Figure 2-16). Interleukins that did not follow this pattern can be seen in Figure 2-17.

Of particular interest was *Il1b*, which demonstrated a sharp increase in stromal expression on days 1 and 2 post mating (Figure 2-17) with a subsequent fall below relative epithelial expression at days 3 and 4. This dramatic fall in stromal expression was also seen in *Il5* and *Il6* expression levels. *Il11* demonstrated a strong peak in epithelial expression on day 4, while stromal expression dramatically decreased. *Il15* remained low in the epithelium post mating, with peaks in stromal expression on days 2 and 4.

2.4.7.4 Lif

Lif expression varied little throughout the post mating period in endometrial epithelial cells, demonstrating a slight dip on day 1 (Figure 2-18). Stromal expression varied more, with the highest expression profiles seen in cycling endometrium at dioestrus and the lowest on day 4 post mating.

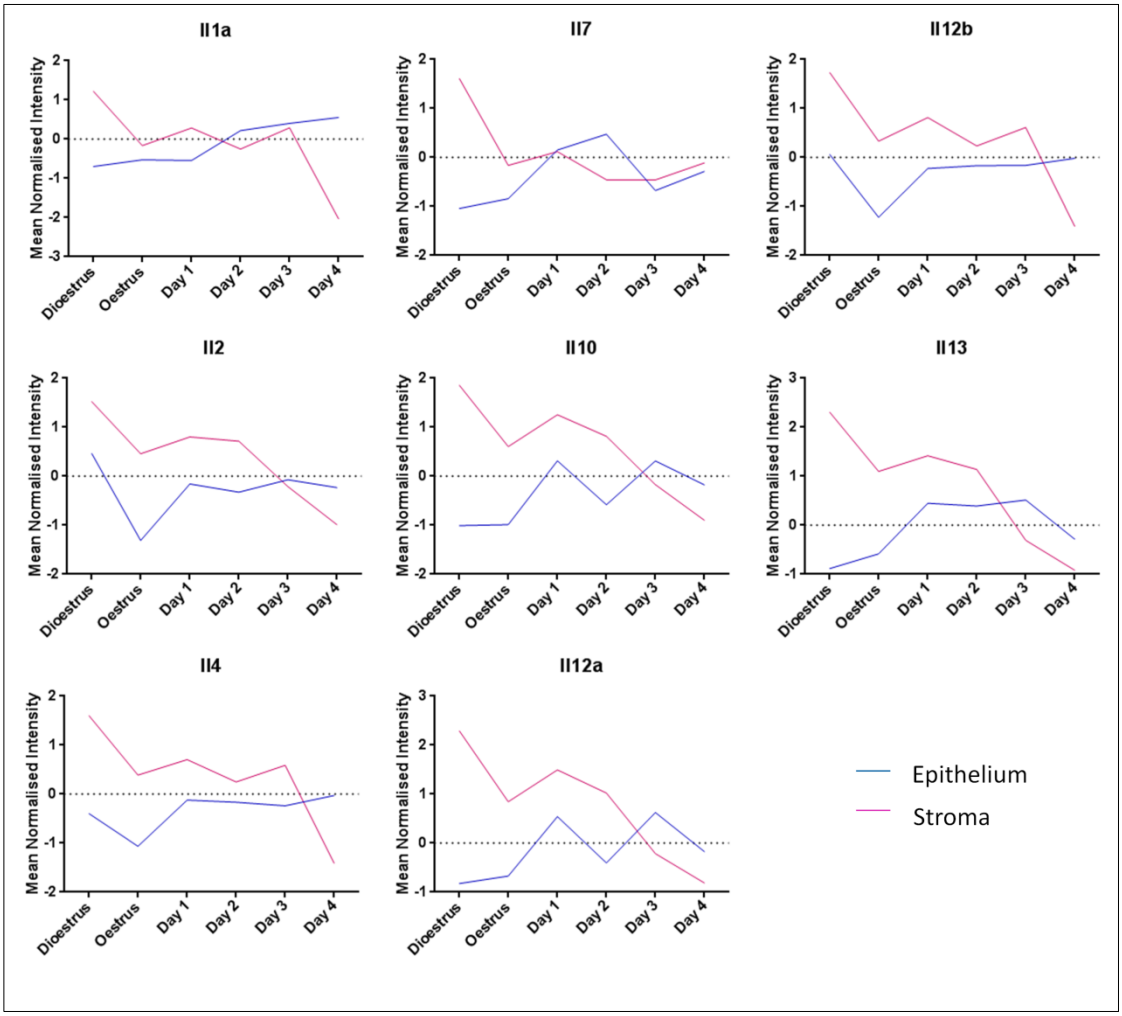


Figure 2-16: Interleukin expression in endometrial epithelium and stromal cells *post coitum*.

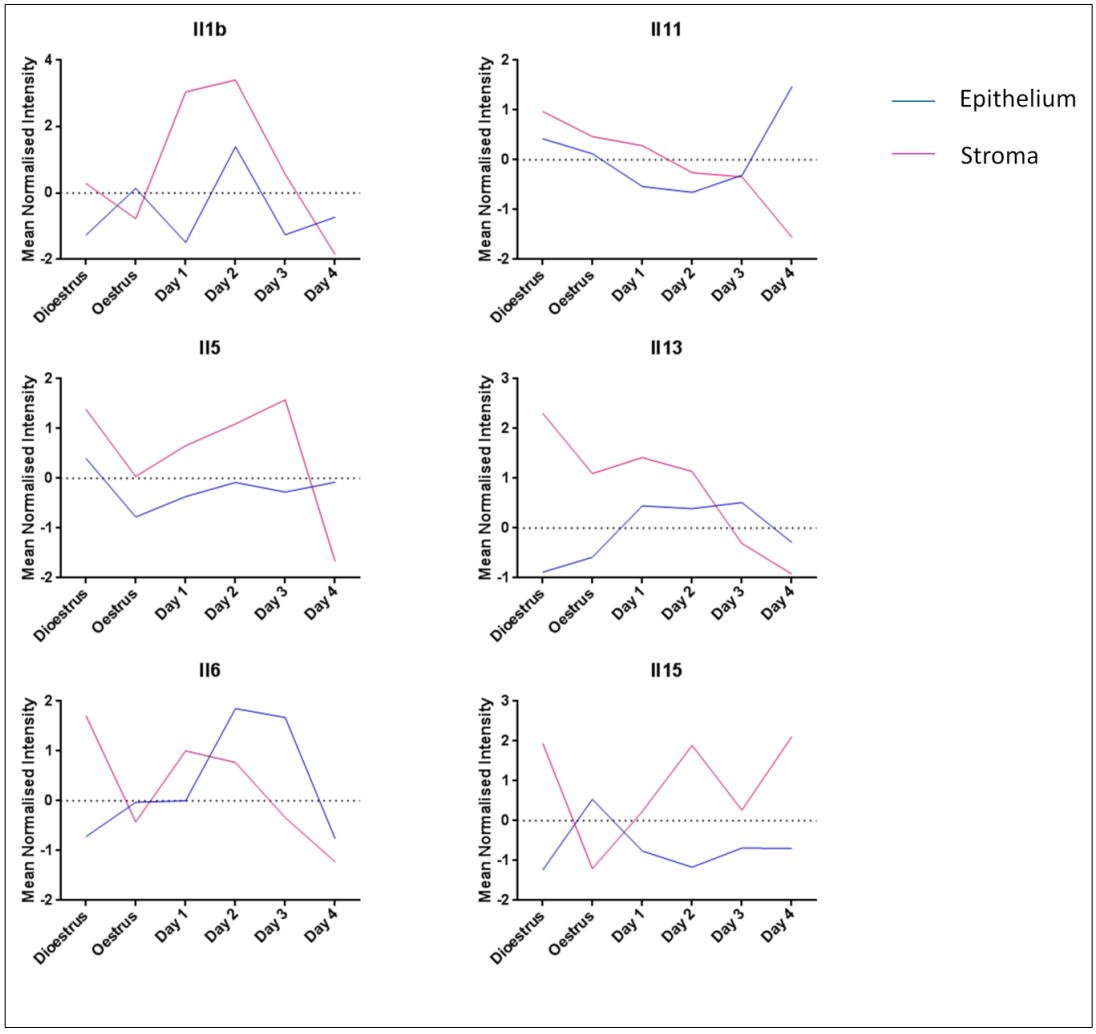


Figure 2-17: Interleukin expression in endometrial epithelial and stromal cells *post coitum* (2).

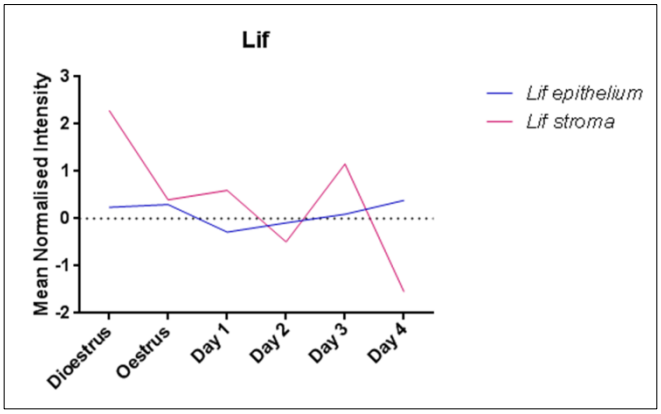


Figure 2-18: *Lif* expression in endometrial epithelial and stromal cells post mating

2.4.7.5 Matrix metalloproteinases

Many MMPs followed a similar pattern of relative expression in epithelium and stromal cells, initially being higher in the stroma with a subsequent increase in epithelial expression on days 3 and/or 4 post mating, mirroring a number of interleukins (Figure 2-19). Three MMP transcript profiles (*Mmp2, Mmp9* and *Mmp13*) showed a divergence by day 2 post mating, with a peak in stromal expression while epithelial expression dropped (Figure 2-20). *Mmp27* was the only metalloproteinase to show a divergence by day 3 post mating (Figure 2-21). *Mmp7* was the only MMP which exhibited consistently relatively higher expression in epithelial cells compared to stroma (Figure 2-22).

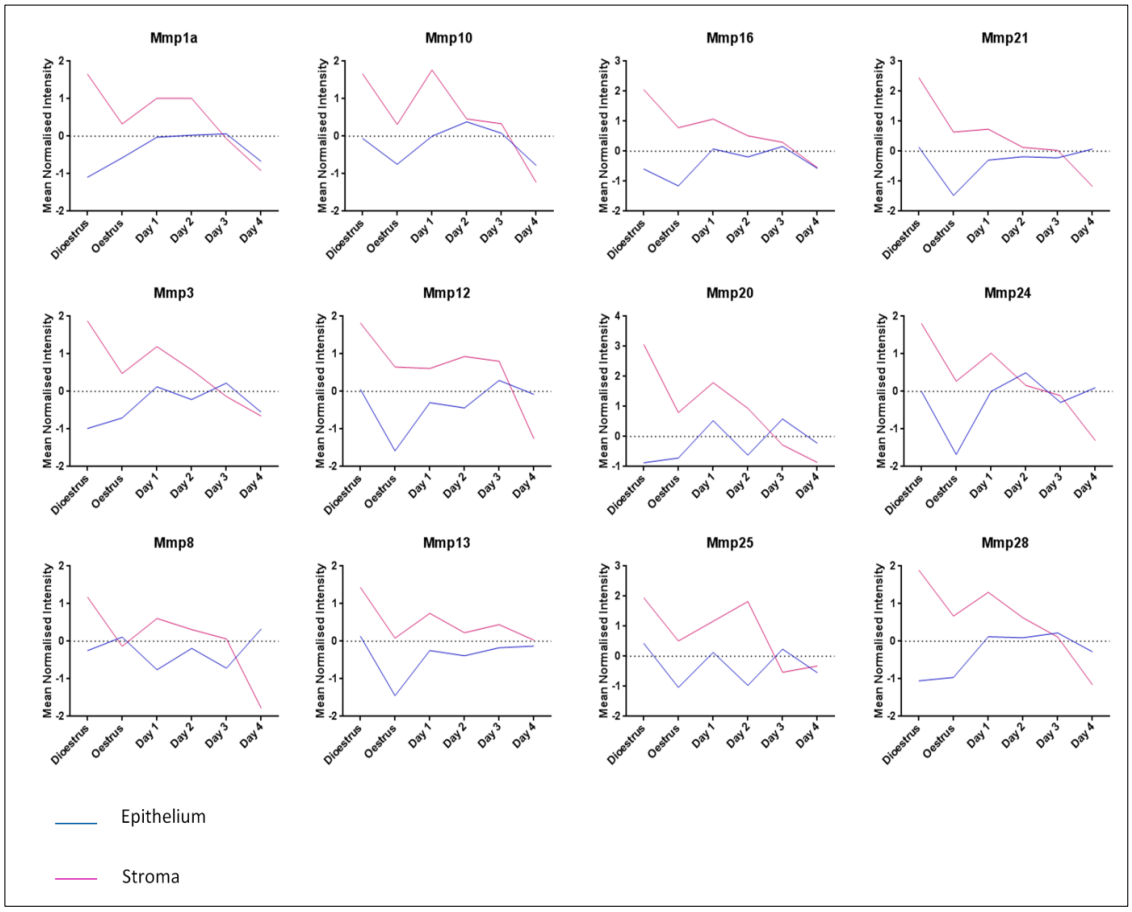


Figure 2-19: Matrix metalloproteinase mRNA expression in endometrial epithelial and stromal cells *post coitum* (1).

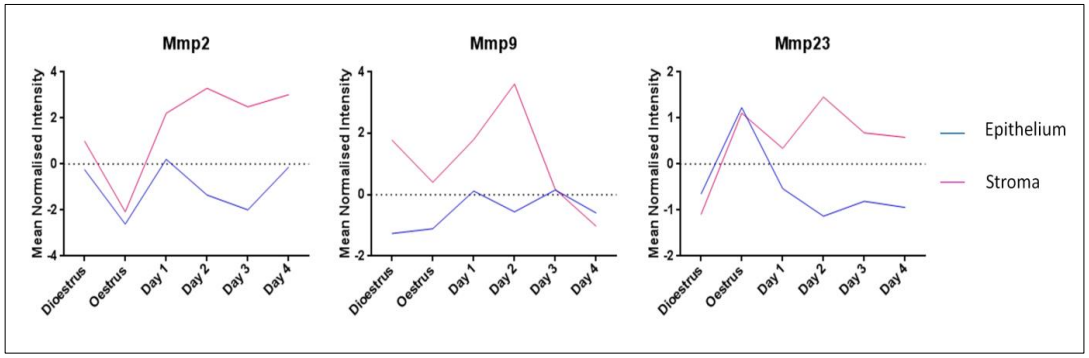


Figure 2-20: Matrix metalloproteinases demonstrating a divergence in expression on day 2 post mating.

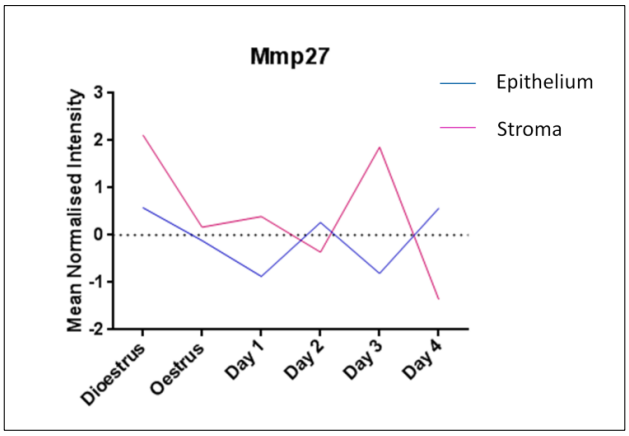


Figure 2-21: *Mmp27* expression in endometrial epithelial and stromal cells *post coitum*.

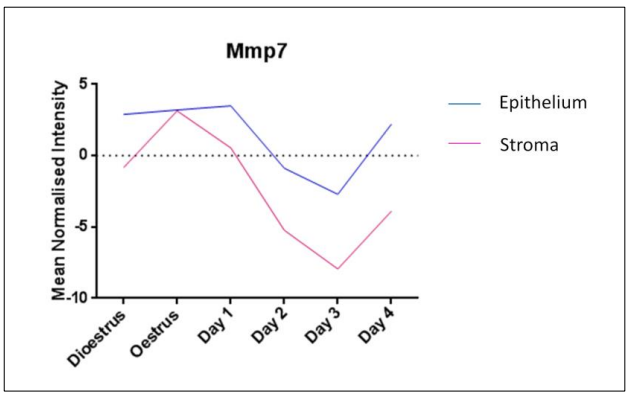


Figure 2-22: *Mmp7* expression in endometrial epithelial and stromal cells *post coitum.*

2.4.7.6 Transforming growth factors

The transforming growth factors were consistently more highly expressed (relatively) in stroma than in epithelium (Figure 2-23). *Tgfb1* exhibited a divergence between stromal and epithelial expression by day 1 post mating, with stromal expression increasing while epithelial expression decreased. *Tgfb2* showed a similar divergence by day 2 post mating.

2.4.7.7 Interferons

Ifnb and *Ifng* were both detectable in endometrial epithelial and stromal cells. *Ifnb* exhibited a sharp peak in epithelial expression on day 2 post mating, elevating its relative expression above that noted in stromal cells (Figure 2-24). *Ifng* peaked on day 2 post mating in both tissue compartments.

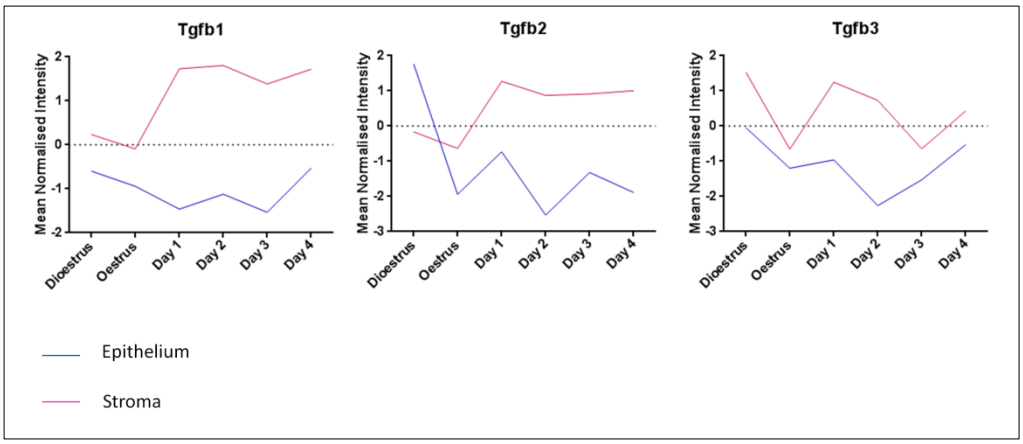


Figure 2-23: Transforming growth factor expression in endometrial epithelial and stromal cells *post coitum*.

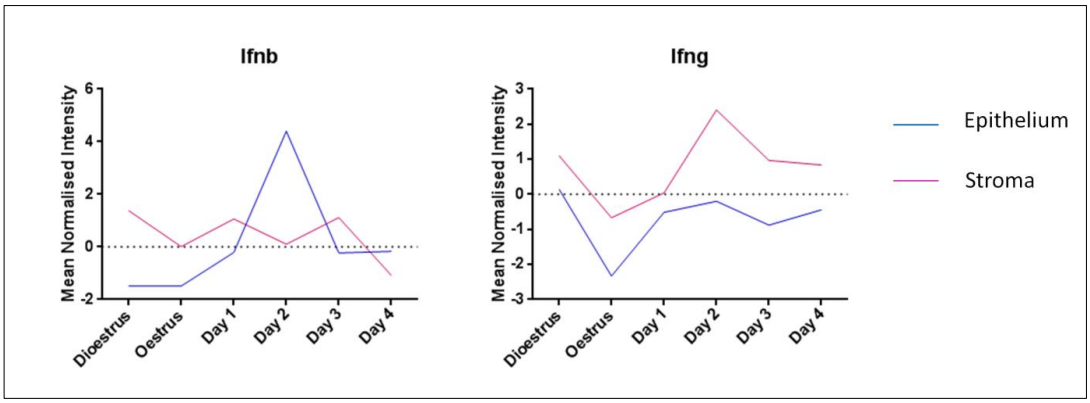


Figure 2-24: Expression of interferons in endometrial epithelium and stroma following mating.

2.4.7.8 Prostaglandin synthases

Prostaglandin synthase mRNA expression was detected in both endometrial epithelial and stromal cells (Figure 2-25). *Ptgs1* demonstrated a dramatic increase in epithelial expression above stromal expression on day 2 post mating, which was sustained until a drop on day 4. *Ptgs2* peaked in the epithelium on day 2 post mating.

2.4.7.9 Prostaglandin receptors

The prostaglandin E receptors *Ptger1*, *Ptger2*, *Ptger3* and *Ptger4* exhibited relative differences in expression between endometrial epithelial and stromal cells (Figure 2-26). Both *Ptger1* and *Ptger3* were relatively more highly expressed in stroma than in epithelium. *Ptger1* showed an increase in stromal expression with a concurrent decrease in epithelial expression on day 2 post mating. *Ptger2* and *Ptger4* were consistently proportionally more highly expressed in epithelium than in stroma. *Ptger2* peaked on days 1 and 4 post mating, while *Ptger4* peaked on day 3.

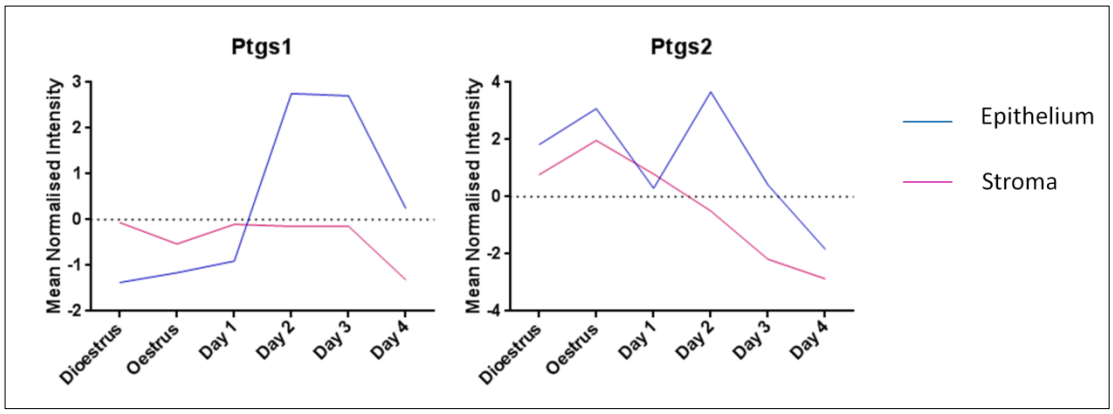


Figure 2-25: Prostaglandin synthase mRNA expression in endometrial epithelial and stromal cells *post coitum*.

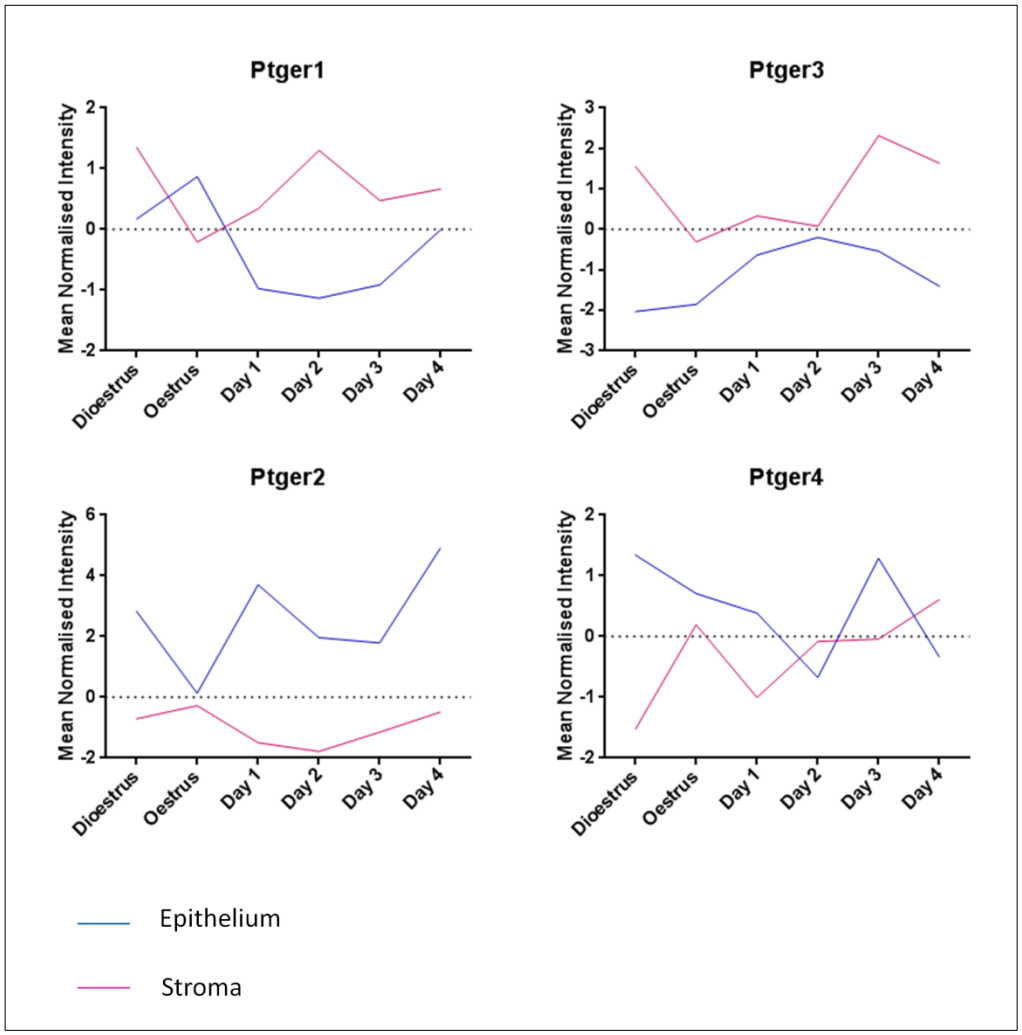


Figure 2-26: Prostaglandin receptor expression in endometrial epithelial and stromal cells following mating.

2.5 Discussion

The response of the endometrium to paternal antigens and immunolological mediators helps to prepare the uterus for implantation and induces maternal tolerance of pregnancy. However, the molecular basis behind the interaction between seminal plasma and the endometrium as well as the subsequent immunological changes which it orchestrates prior to implantation remain poorly understood. This study sought to explore the response of the endometrium to seminal plasma throughout the preimplantation period.

2.5.1 Immune networks within the murine endometrium following mating

Many curated pathways identified within the murine endometrium showed differential expression patterns between the epithelial and stromal compartments. As might be expected, the *Cytokines and Inflammatory Response* pathway (WP222 53571) and the *Inflammatory Response Pathway* (WP458 57463) were activated in the murine endometrium post mating (p=0.00 and p=0.03 respectively). Although most commonly examined as a constituent of seminal plasma influencing the maternal reproductive tract, the TGF-β signalling pathway was identified as being active within the murine endometrium itself post mating, indicating a local role for TGF-β in the maternal response to seminal plasma (p=0.03). Other pathways linked to the immune system were also identified; *Prostaglandin Synthesis and Regulation* (p=0.00), *Matrix Metalloproteinases* (p=0.01), *Macrophage Markers* (p=0.01) and *Oxidative Stress* (p=0.02). Surprisingly, the chemokine pathways WP2292_53116 and WP2292_51127 were not identified as being active in the murine endometrium (both p=0.08, Appendix I). In addition, the *Complement Activation* and the *Blood clotting Cascade* pathways (both p=0.00) were activated, indicating complement activation. Taken together, these pathways indicate that seminal plasma induced an immune response within the murine endometrium. For subsequent analyses, data relating to key molecular mediators (cytokines, MMPs and PGs) within these pathways were selected for further exploration.

2.5.2 Cluster analysis of immune mediators

2.5.2.1 Clusters within the endometrial epithelium

A logical step beyond pathway analysis was to identify interactions between immune mediators via cluster analysis, grouping mediators into expression patterns throughout the pre-implantation period. Within the epithelium, eight patterns were identified, each

of which included elements from multiple pathways, which highlights the likely cross-talk between each pathway. For example, the stroma cluster 2 contained elements from the *Cytokine and Inflammatory Response*, the Inflammatory response, the *TGF-β Signalling* and *Matrix Metalloproteinase* pathways, all of which responded to seminal plasma analogously with the same pattern of decreased expression over the pre-implantation period. This likely reflects the arbitrary nature of pathway design.

Of particular interest within the epithelium was cluster 2 (*Bmp4*, *Egf*, *Il7*, *Il13*, *Mmp1a*, *Mmp3*, *Mmp16* and *Mmp28*), a group of mediators which rose in relative expression post mating above levels seen in cycling mice, and broadly remained high until day 4. EGF and IL-7 share a similar function, in that they induce proliferation and differentiation of immune effector cells [338], while BMP-4 acts as a survival factor for primordial follicles within the ovary [339]. IL-13 modulates the function of monocytes and B cells in humans [340], and also induces (and is induced by) MMPs, as highlighted by studies in airway disease [341-343]. The timing of expression of these mRNA species supports their purported role in the induction of immune tolerance as well as providing a signal to the underlying stroma to begin tissue remodelling, especially given the fact that this response is sustained over the preimplantation period.

The mRNA species falling into cluster 1 (*Cxcl3*, *Il10*, *Il12a*, *Mmp9*, *Mmp20*, *Mmp25* and *Tnf*) peaked at days 1 and 4 post mating; these were again above the relative expression levels seen in cycling mice. TNF-α is known to be involved in the acute inflammatory response [344], so it is unsurprising that its expression is increased early following semen deposition. *Cxcl3* (also known as macrophage inflammatory protein, (MIP)-2β) controls the migration and adhesion of monocytes [345], therefore likely serving as a mediator of the macrophage reaction to seminal plasma. *Cxcl3* has been shown to be induced by IL-13 [346], so it is perhaps surprising that it does not cluster with this cytokine despite exhibiting similar expression patterns. Again, MMPs feature heavily in the initial response to seminal plasma in the epithelium, indicating that the epithelium undergoes extensive remodelling and/or signals to the stroma to begin preparation for implantation in terms of neoangiogenesis to support an implanting blastocyst [347]. There is an inherent difficulty in interpreting MMP mRNA data, however. The vast majority of these proteases are secreted in an inactive form with a pro-peptide domain which must be cleaved in order for the protein to become active [348-351] such that mRNA levels may not faithfully reflect the levels of physiologically

active protein. The active protein is further modulated by TIMPs, creating additional issues in interpretation.

Cluster 3 (*Ccl5*, *Csf3*, *Il1b*, *Il15*, *Inhb1*, *Mmp11*, *Mmp14*, *Mmp23*, and *Thbs1*) and cluster 6 (*Cxcl1*, *Il1a*, *Lif*, *Mmp8*, and *Mmp27*) exhibited a downregulation in expression on day 1 post mating. Interestingly, these two clusters include factors which are considered to be chemotactic/differentiation agents for leukocytes (*Ccl5*, *Csf3*, *Cxcl1*, and *Il15*). It is surprising that the expression of these elements is downregulated, given that leukocyte activity is evident in the endometrium post mating [252, 352-355]. This could be indicative of one feature of cytokine biology, that of functional redundancy, where many cytokines share similar actions [356]. Functional redundancy is due to the biology of cytokine receptors, where receptors for a family of cytokines share a common subunit. For example, a deficiency of IL-2 is not fatal as other cytokines in the same family (IL-4, IL-7, IL-9, IL-15 and IL-21) can signal through the IL-2 receptor, binding to the common γ-chain [357, 358]. Within the current data, this family of interleukins fall into clusters 3 and 5, clusters which show downregulation post mating or a lack of elevation above baseline levels. The exception to this is IL-7 which falls into cluster 2 (exhibiting a peak on days 1, 2, and 3), indicating perhaps that this cytokine performs the functions discussed above, rendering the remaining cytokines within the family functionally redundant. An alternative hypothesis is that other cytokines within the endometrium act as antagonists of *Il4*, *Il9*, *Il15* and *Il21*, although this cannot be determined from the present data.

The downregulation of *Il1a* and *Il1b* on day 1 post coitum could reflect one of two scenarios: firstly, that the immediate inflammatory response invoked by seminal plasma exposure peaks and falls prior to day 1 post mating, lying outside of the sample measurement in the present study. Cytokine expression has been shown to increase within hours of the mating stimulus, with IL-1β expression increasing within 3 hours in human endometrial epithelial cells *in vitro* [262]. Secondly, it is possible that the inflammatory reaction in the endometrium is modified. The function of the immune response to seminal plasma is to induce maternal tolerance to paternal antigens in preparation for implantation such that the traditional acute phase cytokine cascade may not accurately describe events following semen exposure. Indeed, the immune response may be “muted”, reflecting the physiological role of cytokines in conferring immune tolerance rather than a response to infection.

2.5.2.2 Clustering of immune mediators within the stromal compartment

Of particular interest within the endometrial stromal cells was cluster 5 (*Ccl5*, *Il1b*, *Inhb1*, *Mcp1*, *Mmp9* and *Thbs1*). These cytokines and MMPs peaked on days 1 and 2 post mating with a subsequent fall on days 3 and 4. As previously mentioned, *Il1b* was downregulated at day 1 post mating in the epithelium, reflecting a difference in the epithelial and stromal compartments' responses. The function of this cytokine within the endometrial stroma may not be that of a traditional acute inflammatory response – IL-1β performs other functions such as cell proliferation and differentiation, the induction of COX-2 and the regulation of MMPs [359]. Indeed, within the present data, *Ptgs2* (COX-2) rises on day 2 post mating within the stromal compartment, possibly induced by cytokines such as IL-1β. However, it is very likely that *Il1b* also influences the steroid hormone microenviroment, as IL-1β has been shown to suppress steroid sulphatase (which convert E_2 sulphates into active E_2) activity in cycling human endometrial stromal cells *in vitro* [360]. The implication of this is that IL-1β could reduce the effects of E_2 within the stroma, in this case perhaps preventing the progression of the oestrus cycle in preparation for implantation and pregnancy. The remaining members of this cluster mediate leukocyte recruitment and extracellular remodelling in preparation for implantation as previously discussed.

Expression levels in cluster 4 (*Ifng*, *Il15*, *Mmp2*, *Mmp19*, *Tgfb1*, *Tgfb2*, and *Tgfb3*) overall rose on day 1 and remained high throughout the post mating period in the stromal compartment. Interestingly, these elements also clustered together in the endometrial epithelium but did not exhibit a strong temporal pattern. This is surprising given that many studies have shown their involvement in epithelial proliferation and remodelling during the human menstrual cycle [323, 361]. One murine study showed that *Tgfb2* is expressed in epithelial but not stromal cells in the preimplantation period [325], which is in contrast with the present data, where TGF-β mRNA is consistently more highly expressed in the stromal compartment. TGF-β1 has been shown to inhibit proliferation and induce the expression of COX-2 and PRL in human endometrial stromal cells *in vitro* [331], indicating a role in tissue remodelling in preparation for decidualisation, which fits with the present data. In conjunction with the MMPs within this cluster, members of the TGF-β pathway may mediate the extracellular matrix remodelling in preparation for implantation and decidualisation. At the protein level, MMP-2 and MMP-9 are known to cleave latent TGF-β into its activated form [362], and *Mmp2* clusters with all three TGF-β isoforms within both endometrial compartments, indicating that MMPs may in part interact with the TGF-β signalling pathway to create an immunopermissive environment.

Both *Ifng* and the TGF-β isoform transcripts cluster together, which is expected given that in a number of other systems these cytokines demonstrate an antagonistic relationship, for example in lung and dermal fibroblasts [363-365]. Within murine and human endometrial epithelial cells, IFN-γ has been shown to exhibit a potent inhibitory effect on TGF-β [147], although this research focused on the high levels of IFN-γ found in seminal plasma rather than physiologically relevant intra-endometrial levels. The antagonistic effect of IFN-γ on TGF-β is due to downregulation of the TGF-β type II receptor (*Tgfb2*) [365], which in the present study is upregulated at day 1 post mating in the stroma (data not shown). If *Ifng* were performing an autocrine antagonistic role within this cell type, the expected outcome would be a downregulation of *Tgfbr2.* There is, however, a reduction in expression of *Tgfbr2* at day 1 within the epithelium, which could indicate a paracrine interaction. One likely role of both of these cytokines within the endometrium revolves around regulation of T cells. IFN-γ induces apoptosis in T cells and inhibits proliferation, while TGF-β promotes the differentiation of naïve T cells into Tregs [366]. IFN-γ has been shown to have an anti-proliferative effect on human endometrial epithelial cells *in vitro* [367], and an inhibitory effect on VEGF production by endometrial stromal cells [368], which seems in conflict with the events occurring within the endometrium post mating. IFN-γ has also been shown to antagonise prostaglandin responses in the endometrium, inhibiting P_4 receptor-dependent transcription [369]. Taken together, this may indicate that the *in vitro* responses of endometrial cells does not reflect the *in vivo* situation, and therefore merits further investigation in the physiological setting.

2.5.3 Previously identified mediators of immune tolerance and implantation

2.5.3.1 CSFs

Within the literature, several mediators of the uterine response to seminal plasma have been suggested, as highlighted in the introduction. GM-CSF (CSF-2) is known to be released by endometrial epithelial cells in response to seminal plasma within hours of coitus [240, 241]. Indeed, in the present study, *Csf2* rises between oestrus and day 1 post mating in both epithelial and stromal compartments. However, stromal expression of *Csf2* exceeds the relative epithelial expression at all time points except day 4, which raises the question as to whether the stroma is the primary source for the lumenal secretion of this protein. The notion that the epithelium is the primary source of GM-CSF arises from the fact that the studies which have examined this have been mostly conducted *in vitro* with trypsinised cell cultures which consist mostly of epithelial cells,

or panning techniques involving separation of cell types using antibodies – these are all techniques which potentially alter the phenotype and response of the cells collected [238, 240, 241]. One study in primary cultures of epithelial and stromal cells from human endometrium found that GM-CSF mRNA was more highly expressed in epithelium than in stroma, although this was examined in cycling uteri and not in response to mating [370]. When mRNA was collected from intact murine uteri following mating, *Csf2* was upregulated on days 1 and 2 post coitum [213], while the present study demonstrated a decline on day 2 post mating. It is feasible that this difference in response could be explained by the strain of mice used in the experiments: the present study used CD1 mice, an outbred strain with high fertility while the Robertson (1996) study [213] used (Balb/c x C57B1) females mated to (CBA x C57B1), hybrid strains which minimize genetic variability. To the author's knowledge, there have been no studies comparing the response to seminal plasma in mice from different strains which presents an opportunity for future study.

GM-CSF exhibits species differences at the biological level, which may explain the disparity between studies – human and mouse GM-CSF share 70% sequence homology at the nucleotide level and 60% homology at the protein level [371, 372]. This is highlighted by the lack of inter-specific cross-reactivity of these proteins and the differential gene expression in response to GM-CSF stimulation in mice and humans [373]. GM-CSF exhibited a peak on day 1 post mating in both epithelial and stromal cells, indicating a role in the response of the endometrium to seminal plasma. However, the epithelial expression of GM-CSF was highest on day 4 post coitum, indicating a potential role in endometrial receptivity to implantation. In human *in vitro* models, endometrial cell lines produce GM-CSF in response to hCG in a dose-dependent manner [374]. Under normal conditions GM-CSF recruits leukocytes to the endometrium in order to facilitate implantation, with DCs, Tregs and NK cells both directing and limiting the invasion of the trophoblast [375-382]. Although GM-CSF may not be essential for implantation in mice as demonstrated by experiments conducted in GM-CSF null mice, these animals exhibit late gestational pregnancy loss and retarded foetal growth, indicating potential placentation defects [383]. The cellular expression of GM-CSF in the endometrium within this study was unexpected; however, the temporal expression patterns are consistent with findings in the literature.

Csf1 is thought to be a low potency recruitment agent for macrophages to the endometrial epithelium [252], and M-CSF protein levels correlate with macrophage numbers [250]. In the present study, *Csf1* peaked in the epithelium on days 1 and 4

post mating, suggesting that macrophages are recruited to the murine endometrial epithelium in two distinct waves. The main role of macrophages in the endometrium is thought to be tissue remodelling and increasing epithelial cell adhesiveness in preparation for implantation [384]. This notion is supported by a rise in *Csf1* levels on day 4 post mating, where tissue remodelling would take place just prior to implantation. By contrast, the rise on day 1 post mating is most likely indicative of the role of macrophages as APCs in the induction of maternal tolerance to pregnancy, alongside the phagocytosis of semen constituents within the uterine lumen [385].

2.5.3.2 Interleukins

Although IL-6 has been shown to be induced by seminal plasma constituents in human cervical epithelial cells *in vitro* [211, 223, 261], the *in vivo* response remains to be characterised. Interestingly, in the present study, *Il6* was shown to rise on day 1 in the stromal compartment while epithelial cells lagged in their response, rising on day 2. IL-6 expression has been shown to be induced by IL1-β in human endometrial epithelial cells [386-388], and in the present study the pattern of expression of *Il1b* and *Il6* were similar in stromal cells, albeit with differences in epithelial cells. *Il6* is induced very early in the response to seminal plasma (within 4 hours) in human ectocervical cell lines [223], but the present data indicates that this response is maintained for days following mating in mice. IL-6 is a known mediator of the acute inflammatory response, and switches the immune response from a neutrophil-mediated to a monocyte/macrophage mediated event via induction of ICAM-1 and MCP-1 [257, 389]. Indeed, in the present data *Mcp1* follows a similar expression pattern to *Il6*. In the current context, the rise in epithelial and stromal *Il6* is consistent with the resolution of the initial neutrophil response prior to implantation.

Another member of the IL-6 family, LIF, has been shown to be essential for successful implantation in mice [263, 390], and is induced by seminal plasma in human endometrial epithelial cells *in vitro* [262]. However, the present data do not support the findings in the literature, with endometrial expression of *Lif* showing little variation in response to mating, and relative stromal expression exceeding that of the epithelium at most time points. Stromal expression peaked on day 3, potentially initiating the start of decidualisation in preparation for implantation. However, one study showed that murine endometrial stromal cells produce LIF protein with higher concentrations inhibiting decidualisation [391]. Within the present study, a fall in stromal expression of *Lif* on day 4 could indicate a release of the inhibition on decidualisation, thereby enabling successful implantation. This paradox in findings across different studies could be

explained by the differences between *in vitro* and *in vivo* environments, as well as the localised nature of the decidual reaction.

Interestingly, the murine IL-8 homologues *Cxcl1* and *Cxcl2* did not demonstrate dramatic changes in expression post mating, despite their postulated role in the recruitment of neutrophils and granulocytes to the endometrium [276]. This may be due in part to the timing of sample collection since cytokines have been shown to rise almost immediately in response to seminal plasma. *Cxcl5* was not detected within the present study. This may be due to the restricted power of this experiment, as only mRNA demonstrating a minimum of a five-fold change between at least one of the conditions/time points were included in the analysis. However, other murine cytokines may perform the functions that IL-8 carries out in the human, and no direct homologue has been characterised in the murine genome [392]. The present data should be re-examined once further knowledge regarding murine IL-8 homologues is attained.

2.5.3.3 Chemokines

The remaining chemokines previously identified within the literature were MCP-1 and RANTES (CCL5). *Mcp1* was upregulated on days 1 and 2 in the stroma, but only on day 1 in the epithelium. This is consistent with its purported role as a potent chemoattractant for monocytes/macrophages [278], although previous studies have identified this chemokine as highly expressed in murine endometrial epithelium throughout the peri-implantation period [251, 252]. *Ccl5* peaked in the stromal compartment on day 2 post mating, whereas previous studies have demonstrated a peak in expression on day 1 [252]. The reasons for this discrepancy are not immediately clear, and the findings of the present data require validation.

2.5.3.4 Elements of the prostaglandin pathway

The prostaglandin synthases *Ptgs1* and *Ptgs2* were expressed in the murine endometrium and broadly localised to the epithelium post mating which is in agreement with the literature [291, 292]. In contrast to previous findings where *Ptgs2* was seen to increase in expression on day 1 in human endometrium [292], this fell on day 1 post mating, with a peak in expression on day 2 in the present study. It is possible that this may represent species-specific differences in the response to seminal plasma with regards to prostaglandin pathways.

2.5.4 Limitations of the study and future directions

Examination of the transcriptome can be highly informative, indicating the global response of a system to an external stimulus. However, there are many steps between the transcription of mRNA and the translation into protein, and these steps define whether the concentration of mRNA correlates with the concentration of bioactive protein. Those mRNA species which vary in concentration throughout the cell cycle are more likely to be controlled at the transcriptional level, and therefore correlate strongly with protein concentration and functional effect, while those which vary little may or may not be controlled at the post-translational level [393]. It is known that many cytokines are controlled at the post-transcriptional level. For example IL-1β, granulocyte colony stimulating factor (G-CSF) and IFN-γ are regulated by adenine and uridine-rich element (ARE)-mediated mRNA decay after export through the nuclear envelope [394]. In terms of post-translational modification, mechanisms include glycosylation, -NH_2 and/or COOH-terminal truncation, as seen in MCP-1 and -2 [395], IL-6 [396], and RANTES [397]. This does not preclude the fact that a particular gene may be controlled at both the level of transcription and translation, in addition to post-translational modification [398]. In the present study, there were many mRNA species which did not vary between conditions, and it is not possible to determine whether these species are important in the response of the uterine endometrium to seminal plasma.

Pathway analysis is a useful tool for identifying 'system' level changes in mRNA expression. The Genespring microarray analysis tool enables the researcher to explore these pathways by mapping a set of identified mRNA species to known pathways and then determine which pathways are represented within a specific model. However, this approach has several limitations: firstly, the mRNA species are only able to be mapped to known curated pathways. Within the mouse, this amounts to 217 pathways within the wikipathways database, undoubtedly limited to present knowledge, with many pathways yet to be defined. Secondly, these pathways are rigidly defined structures without the flexibility to discover previously unknown interactions. Although there have been efforts to create new analytical tools to discover new pathways and interactions within microarray datasets, this has mainly been aimed at the level of the genome rather than the transcriptome [399]. As previously discussed, it is arguably more informative to examine interactions at the protein level, although again this kind of analysis is limited at present in terms of the ability to detect low abundancy proteins without amplification, and the relative insensitivity of techniques such as mass spectrometry [400].

This study has identified potential new markers of the endometrial response to seminal plasma and the coordinated preparation for implantation. However, these findings require validation with individual entities examined at the mRNA and protein level. Laser capture microscopy to enable examination of the endometrial epithelial and stromal compartments is time consuming and costly in terms of equipment/operator time and sample usage, especially with the limited material available from murine uteri. Therefore these findings present an opportunity for future work to explore the interactions between immune mediators, endometrial compartments and the developing embryo.

In terms of this thesis, the next section will therefore focus on the development of a novel analytical tool to explore immune mediator networks at the protein level in a reproductive context.

3. Bayesian modelling of cytokine interactions *in vivo*

3.1 Introduction

3.1.1 Why model cytokine interactions?

Traditional approaches to the interpretation the biological properties of cytokines have relied on treating these mediators as individual entities, commonly ascribing discrete physiological effects to each. However, the value of this strategy is limited by the key physiological features displayed by cytokines. It is increasingly recognised that these mediators function as networks, where physiological processes are governed by the combined influence of many cytokines. Within these networks, cytokines exhibit pleiotropy, synergy, antagonism and functional redundancy, further complicating the analysis of their influence on physiological processes [91, 92, 152-154]. Furthermore, in some cases cytokines exhibit different effects over a range of concentrations or are dependent upon the local hormonal environment, meaning that *in vitro* studies seldom replicate the *in vivo* situation meaningfully, leading to difficulties in interpreting their roles [95, 401].

3.1.1.1 Pleiotropism

Many cytokines exhibit pleiotropism, in that they exert multiple actions on an array of different target cell types. Pleiotropic actions may be governed by the presence of specific cytokine receptors across multiple cell types, or by the ability of a cytokine to activate multiple receptor signalling pathways within a single lineage. There are several mechanisms by which this can occur. For example, by forming a complex with its soluble IL-6 receptor-α, IL-6 utilises the gp130 subunit to activate signal transduction pathways which would otherwise be unresponsive to IL-6 alone [402]. This mechanism has been dubbed the receptor conversion model, and can be applied to many other cytokines [403]. Moreover, certain cytokines have the ability to send both positive and negative signals simultaneously via the same receptor signalling pathway. For example, TNF-α can induce both apoptotic and anti-apoptotic signals via TNFR-1 [404]. In this case it appears likely that the combined influence of the signals and their relative balance is important, a mechanism coined the orchestrating model [403].

3.1.1.2 Synergy

Synergy is the mechanism by which different cytokines act in concert to produce cellular responses greater than those resulting from individual cytokine types alone, or responses which neither cytokine could achieve in isolation. Cytokines often synergise to induce the production of other cytokines. For example, the combined effect of IL-1β and IFN-γ induces the production of MCP-3 in fibroblasts despite the fact that neither of these mediators can induce this response individually [153, 405]. Synergistic interactions are most likely to occur where the cytokines involved are functionally closely related, although this does not preclude interactions between cytokines with very different action mechanisms. Synergy may be mediated by shared signalling pathway components, as in the case of TNF-α and IL-1β which are both potent activators of the transcription factor NF-κB [406, 407].

3.1.1.3 Antagonism

Any cytokines which exhibit opposing effects within the same system can be considered to be mutually antagonistic. In many cell types IFNs inhibit proliferation, the simultaneous presence of a mitogenic cytokine may result in mutual antagonism [408]. Two cytokines which exhibit mutual antagonism are IFN-γ and IL-4 [409-411]. In this respect, IL-4-induced Th2 T cell differentiation and stability is antagonised by IFN-γ, while IFN-γ induced Th1 T cell differentiation and stability is antagonised by IL-4 [412-415]. This antagonism provides a mechanism for maintaining balance within the immune system, ensuring that neither Th1 nor Th2 responses become uncontrolled. Indeed, where this balance is disrupted, tissue damage can occur, as seen in diseases such as multiple sclerosis and Crohn's disease (Th1 dominant), as well as atopic asthma and allergy (Th2 dominant) [416-419]. Antagonism can occur at any point in the signalling cascade, from interactions at the receptor level to modified patterns of gene activation, and range from subtle modulatory responses to the complete abrogation of an effect [409, 420, 421].

3.1.1.4 Functional redundancy

Given that cytokine networks are crucial to the regulation of many physiological processes, it is unsurprising that there is an in-built redundancy in the system. Many distinct cytokines perform the same biological function; such that the lack of a given cytokine does not always confer a lack of response. The molecular basis for functional redundancy lies in shared receptor components in a manner not dissimilar to pleiotropy [422]. For example, the IL-6 family of cytokines exhibits functional redundancy between

cardiotrophin (CT)-1, ciliary neurotrophic factor (CNTF), IL-6, IL-11, leukaemia inhibitory factor (LIF) and oncostatin M (OSM) [423]. All of these cytokines feature a similar helical structure, and share the gp30 subunit which enables compensation within the network [424-429]. Murine knockout models have demonstrated that mice null for IL-6 develop into normal healthy adults, while gp30 knockout mice fail to survive gestation [430].

3.1.2 Common approaches to modelling gene/protein interactions

To date, many of the studies detailing the spectrum of actions displayed by cytokines have been based on the analysis of recombinant cytokine interactions in *in vitro* systems. Whilst providing a useful starting point, this approach does not provide a realistic representation of cytokine networks either in the whole organism nor at a cellular level. Along with pleiotropy and redundancy, cytokine action is contextual, influenced by the surrounding milieu containing other biologically active agents (for example other cytokines, hormones and prostaglandins) in a synergistic or antagonistic manner [431, 432]. Furthermore, many cytokines can act indirectly, either by stimulating or inhibiting other cytokines via receptor transmodulation. For example, IL-1β-induced MMP-1 expression is mediated by transmodulation of the EGF receptor [433]. Incorporating such complex interactions provides a significant challenge to current modelling techniques aimed at understanding the regulation of cytokine networks.

With the advent of high throughput analytical platforms (e.g. fluid-phase multiplex immunoassays and microarrays) which can profile large numbers of analytes in a small sample, the potential has arisen to examine complex mediator interactions. Many studies have utilised statistical methods to identify groups of mediators which work together either to result in a particular effect, or to distinguish between clinical groupings. Techniques such as correlation, Discriminant Factor Analysis (DFA), cluster analysis and PCA have proved useful in identifying groups of analytes which best discriminate between clinical groups/outcomes, but these provide scant information about how those mediators interact and function as a network [434-439].

Recent studies analysing microarray data have utilised weight matrices and Boolean networks in order to elucidate underlying gene expression profiles, with varying degrees of success [440, 441]. However, these methods operate on the basis that all interactions can be treated as independent events. This is clearly an assumption which

is untrue in the case of cytokines, rendering these approaches unsuitable for modelling cytokine interactions.

In terms of defining cytokine networks at the protein level, very few studies have attempted the complex analysis required to account for multiple interactions. The majority have either focussed on qualitatively and visually comparing a relatively small number of network components [442], or on the very broad descriptive analysis of shifts in overall network structure [443]. Neither of these approaches provides quantitative or directional (i.e. which factor influences the other) information regarding individual cytokine interactions and, as such, have fallen short of providing a method to understand their networks.

Broderick *et al* [444] performed a study in chronic fatigue syndrome in humans which extended the above methods by employing mutual information (MI) criteria association networks to construct a visual representation of cytokine interactions. This technique enables quantitative comparison of networks generated from experimentally derived protein data, with the analytes (in this case cytokines) displayed as 'nodes' connected by 'edges' (Figure 3-1).

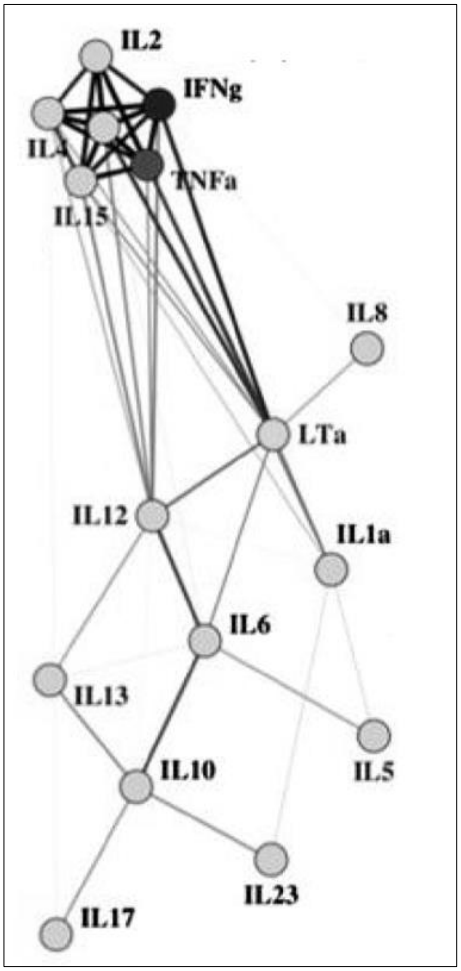


Figure 3-1: Example network generated using mutual information (MI) criteria association networks. Cytokines are displayed as nodes (circles), interconnected by edges (lines). The depth of colour in the edges demonstrates the strength of the association between the connected nodes (adapted from Broderick *et al* (2010)) [444].

In this particular method, the edges are shaded according to the strength of the association (the deeper the colour, the stronger the association). Although this is a step forward in terms of visually representing cytokine interactions, there are significant limitations to this approach. Firstly, the edges simply represent associations, with no indication of the direction of the interaction. Secondly, it is not possible to define the nature of the interaction. As stated above, cytokines exhibit synergy, antagonism and functional redundancy, all features which cannot be defined by utilising an association method to discover underlying networks; pairwise mutual information method simply does not allow for multivariate analysis in order to explore these essential aspects of cytokine function [444].

3.1.3 Bayesian approaches to cytokine network analysis

3.1.3.1 Frequentist and Bayesian approaches

Statistical inference approaches to the analysis of complex data sets fall broadly into two categories: frequentist and Bayesian [445]. The methods discussed so far have fallen into the former category. The frequentist approach is the method taught throughout scientific education where significance tests, unbiased estimators and confidence intervals are used to test a null hypothesis against an alternative. These tests are based on repetition, i.e. the frequency of a particular result if the experiment is repeated multiple times. This approach treats parameters as fixed but unknown quantities, without a distribution.

By contrast, the Bayesian approach treats parameters as random variables which have a probability distribution. It differs from the frequentist approach by taking into account prior knowledge about the parameters – either in the form of known values (e.g. from previous experiments) or as a strength belief that a certain event will happen (personal uncertainty). The use of prior knowledge is controversial in the frequentist approach, given that there is the belief that prior knowledge is subjective and therefore has no place in scientific analyses. However, whilst the Bayesian approach agrees that prior knowledge is subjective, it argues that its use is essential to the interpretation of results providing that the knowledge used is as objective as possible, which can be ensured by robust literature searching [445].

3.1.3.2 Basic Bayesian analysis

Bayesian analysis follows several core steps:

- Capture the data of interest
- Link the data to parameters via a statistical model
- Collect prior information about the parameters
- Combine the statistical model and prior information using Bayes' theorem to form a posterior distribution
- Utilise the resulting posterior distribution to make inference about the parameters

The first two steps are common to both Bayesian and frequentist approaches, with the remaining steps being unique to the Bayesian method.

There are many ways in which to collect data for the prior information, and the quality of this information influences the strength of the posterior distribution [446]. Prior information can be qualitative, for example the belief of a particular doctor that a treatment will have an effect on disease duration, or quantitative where knowledge/data gained from other experiments is incorporated. Bayesian methodology states that no genuinely informative prior knowledge should be discarded, although there must be an endeavour to ensure that this prior knowledge used is as objective as practicable.

Combining the data collected (the evidence or 'likelihood') and the prior knowledge (the prior distribution) via the Bayes' theorem results in the posterior (post-evidence) distribution [447]. In reality, Bayes' theorem provides a compromise between the two sets of data, with the posterior distribution falling between the two (Figure 3-2). The posterior distribution reflects the strength of either the prior or the likelihood: if the data are very strong (with large sample size and a narrow distribution) then the posterior will be very close to the likelihood; however, if the data are weak with low sample numbers and a wide distribution, then the posterior distribution will be closer to the prior distribution. In simple terms, Bayes' theorem states that the posterior distribution is proportional to the prior multiplied by the likelihood distribution, which is expressed as:

$$p\left(\frac{A}{B}\right) = \frac{p\left(\frac{B}{A}\right)p(A)}{p(B)}$$

Where, for proposition (A) and data/likelihood (B):

- *p*(A) - the prior distribution, is the initial degree of belief in/evidence supporting the proposition
- *p*(A/B) - the posterior distribution, is the degree of belief in/evidence supporting the proposition having accounted for the data
- p(B/A)/p(B) - represents the amount of support the data provides for the prior knowledge

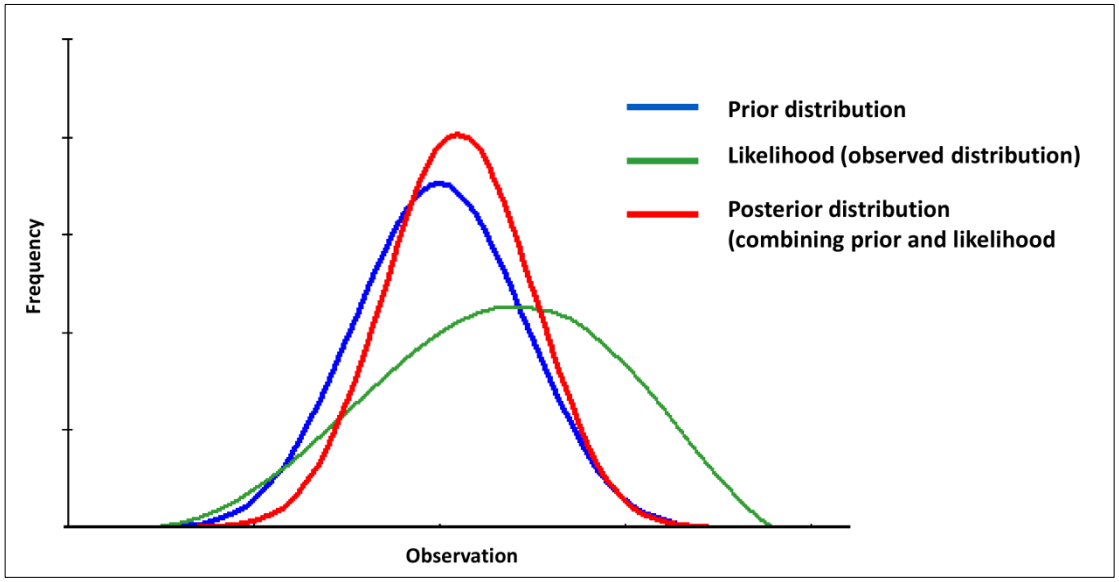


Figure 3-2: Formation of the posterior distribution. Graph demonstrating the effect of combining the prior distribution (blue) with the likelihood/observed data (green) to discover the posterior distribution (red). Adapted from Bink *et al* (2008) [448].

3.1.3.3 Advanced Bayesian analysis with multiple parameters

Although Bayes' theorem provides a simple equation for situations with single variables, it becomes mathematically more challenging to take into account multiple parameters. The majority of biological situations involve multiple variables, and in this case, the posterior distribution for a particular variable is influenced by one or more other variables, such that advanced algorithms are required to integrate the effects of large numbers of parameters into a network. Although recent advances in computational technology have enabled multiple variables to be taken into account, Bayesian analysis remains computationally very expensive and is therefore limited to researchers with appropriate resources [399].

Computational approaches such as machine learning algorithms and data/text mining tools enable the exploration of previously unknown relationships within biological systems in order to allow for influences on posterior distributions. These approaches have proved particularly useful in genomics and proteomics [449, 450]. Within genomics, the location and structure of genes can be extracted from the rapidly expanding database of sequences [451]. Other areas of genomics being tackled from a computational perspective are the identification of regulatory elements [452-454] and non-coding RNA genes [455]. Protein structure prediction remains a key outcome in

terms of proteomics [449], and the methods used provide a basis for the exploration of protein interactions at a functional level.

3.1.3.4 Probabilistic graphical models

A probabilistic graphical model represents joint probabilities between multiple variables in a visual and intuitive manner [456]. These models may be undirected, as in the case of Markov networks and region-based approximation, where interactions are limited to associated probability in a similar way to the MI criteria association networks discussed above, without an indication of the direction of that interaction [457-459]. However, more informative models are generated using directed graphs - so-called directed acyclic graphs (DAGs) – where the probabilities between each variable are conditional upon the state of other variables and give directionality to the interaction. This gives rise to a Bayesian network which provides an efficient model to conduct probabilistic inference [449]. The network itself comprises a series of overlapping Markov blankets, defined as the connectivity between a parent node, it's children and any subsequent descendants.

3.1.3.5 Probabilistic graphical model Bayesian network analysis from experimental data

The pioneers of Bayesian network analysis, Nir Friedman and Dana Pe'er, first applied probabilistic graphical models to experimentally-derived gene expression data in order to analyse the yeast cell cycle, thereby creating a predictive model of cell cycle machinery [460]. However, Djebbari and Quackenbush report that subsequent (unpublished) application to clinically related questions (for example treatment group versus control) proved less insightful and resulted in this form of analysis falling out of favour [399]. Moreover, many of the early studies used general purpose search algorithms which resulted in a failure to learn accurate or realistic Bayesian networks [399]. Such limitations have been overcome by introducing network 'seeds', wherein preliminary topologies discovered through prior knowledge are introduced as soft constraints in order to bias the search for the best Bayesian network [461, 462]. Although providing slight restrictions on the learned Bayesian network, this approach does not preclude the discovery of new interactions between mediators [463], and provides an optimal approach for network generation [464].

Learning probabilistic graphical models from experimental data provides its own challenges. Firstly, as outlined previously, learning Bayesian networks is

computationally expensive. Secondly, the causality of interactions between variables has proved an interesting problem requiring the development of advanced algorithms which aim to accurately represent the biological interactions involved [465, 466]. This approach must therefore be adapted for each experimental scenario, and as such requires expert knowledge of algorithm development.

Bayesian analysis has been most widely applied to experimentally-derived expression microarray data. In this case, the principal outcomes have been the identification of expression patterns and the construction of resultant genetic networks [399, 467-471]. However, while analysis of the genome and transcriptome are useful, functional cytokine effects are at the level of the proteome. Significantly, many genes, including those encoding cytokines, are subject to post-transcriptional and post-translational modifications which restricts the functional interpretation of genomic and transcriptomic analyses [472-475]. To date, there has been no attempt to utilise Bayesian techniques to model *in vivo* protein interactions and effects within a hormonally driven environment, thus development of a Bayesian network solution to examine *in vivo* cytokine networks provides an opportunity to expand on existing statistical techniques and extend the current knowledge surrounding cytokine interactions.

3.1.4 Murine lactation as a model to investigate cytokine networks

Murine lactation provides a unique opportunity to explore the interrelationships between cytokines on a background of hormonal changes. Upon receiving the suckling stimulus, the lactating mother produces PRL and oxytocin via the hypothalamus and the pituitary gland (Figure 3-3). PRL has documented immunoregulatory effects on Th1/Th2-type cytokine responses and the survival/differentiation of immune effector cells in both mice and humans [476-479], and also acts as a cytokine in certain physiological states such as haematopoiesis [480]. This physiological perturbation of cytokine networks therefore provides the opportunity to explore interrelationships without a disease background which may in itself alter cytokine responses.

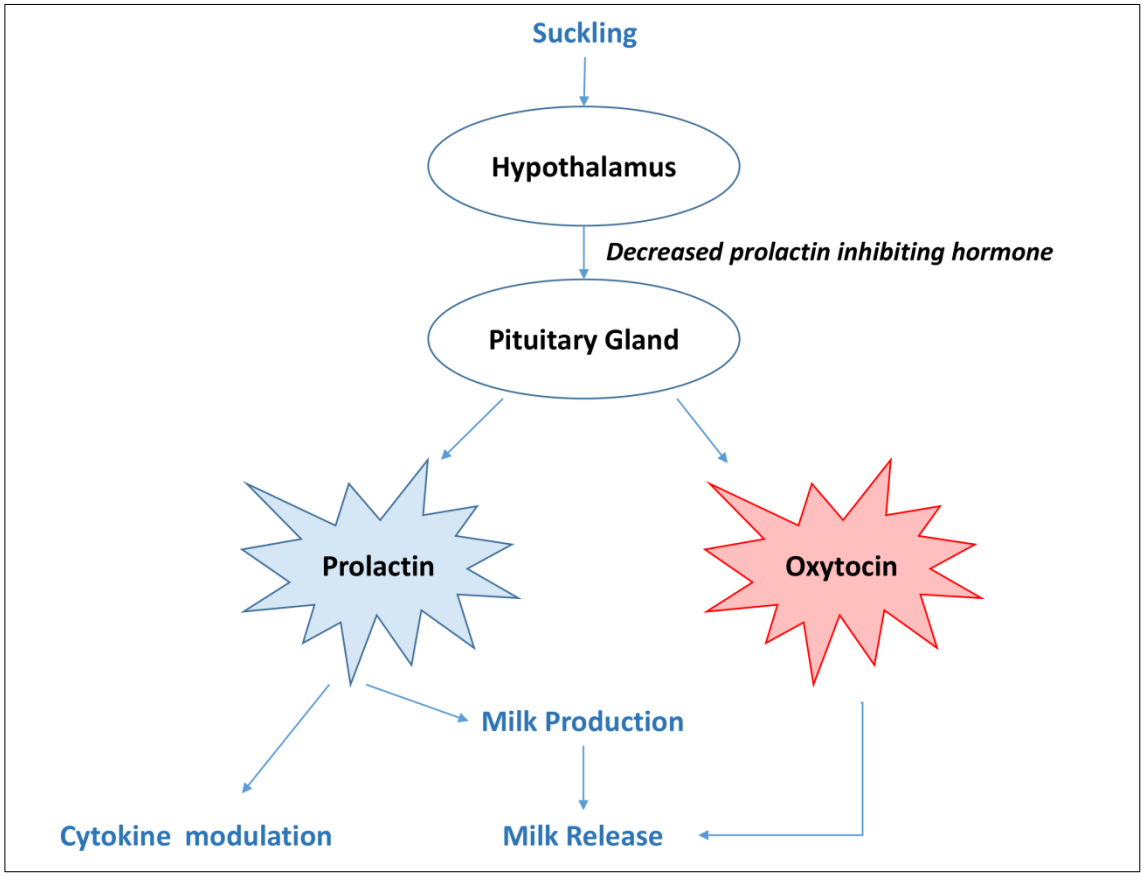


Figure 3-3: Hormones involved in lactation. Stimulation provided by suckling results in the reduction of PRL inhibiting hormone and the release of PRL and oxytocin.

3.2 Aims

The aims of this chapter were:

- To generate cytokine data at the protein level using murine lactation as a physiological system where cytokine/hormone interactions can be examined *in vivo*
- To develop a Bayesian network solution suitable for the interpretation of cytokine interactions at the protein level
- To describe causal relationships between cytokines and other inflammatory mediators (hormones) in murine lactation
- To utilise *in silico* perturbations to predict and validate changes in cytokine networks when lactation is prevented from becoming established.

3.3 Methods

3.3.1 Animals

3.3.1.1 Data set 1

Eight to ten-week old virgin CD1 female mice were group housed (10 per cage) with *ad libitum* access to water and Standard Expanded Beekay diet (B&K, Grimston, Aldborough, UK). The lighting cycle was 14h:10h light:dark, (05:30 on; 19:30 off). Humidity and temperature were maintained at 55-65% and 21.5±1°C. Oestrus synchronisation was achieved via the Whitten effect, whereby bedding impregnated with male urine was introduced to the female's cage 48 hours prior to mating. Oestrous females were identified by vaginal cytology [176]. Females in oestrous were naturally mated with 12 week old CD1 stud males of proven fertility (1 female:1 male), with pregnancy confirmed on day 15 by manual palpation. Females were group housed until late pregnancy, at which point they were caged individually to litter down and nurse their pups Each dam received 8 pups (via culling/cross-fostering) in order to standardise the suckling stimulus. Animals were sacrificed under Schedule 1 of the Animals (Scientific Procedures) Act, 1986 on the following days post partum:

- Day 1 (<24h of littering), n=8
- Day 2, n=8
- Day 4, n=8
- Day 10, n=7
- Day 16, n=8
- Day 21, n=7
- Day 24 (post lactation), n=7

Weaning was performed on day 21 (at the light cycle midpoint), when the independent pups were removed from their mothers.

3.3.1.2 Data set 2

Lactation was abrogated in a further two groups of dams whose pups were all culled/allocated to different mothers at birth; these females were then sacrificed on days 2 and 4 (time-matched to lactating females) (n = 8 in both groups).

3.3.1.3 Negative controls

Negative (baseline) controls were provided by naturally cycling virgin females of the same age and strain (*n*=7).

3.3.2 Sample collection and analysis

3.3.2.1 Sample collection

Mice were sacrificed at 12.00pm ±1h (half way through the lighting cycle) to minimise the impact of circadian rhythms on any of the analytes measured. Whole blood samples were collected by cardiac puncture using a 23G needle mounted on a 2ml syringe. Blood was transferred into Eppendorfs and placed on ice to clot. Serum was isolated by centrifugation at 5000rpm for 3 minutes, and then stored in aliquots at -80^{o}C for subsequent analysis.

3.3.2.2 Cytokine analysis

Serum was assayed for IL-1α, IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-9, IL-10, IL-12 (p40), IL-12 (p70), IL-13, IL-17, eotaxin, G-CSF, GM-CSF, IFN-γ, keratinocyte chemoattractant (KC), MCP-1, MIP-1α, MIP-1β, RANTES and TNF-α by fluid-phase multiplex immunoassay as per manufacturer's instructions (Bio-Rad Laboratories, Hemel Hempstead, Hertfordshire, UK). Samples were run on a Luminex-100 cytometer (Luminex Corporation, Austin, Texas), equipped with StarStation software (Version 2; Applied Cytometry Systems, Dinnington, UK). Serum diluent was used in all cases to avoid false positive/negatives and the serum:diluent ratio was reduced to 1:1 in order to maximise sensitivity [481]. All samples were run on one plate to avoid batch variation.

3.3.2.3 Hormone analysis

E_2 and P_4 were assayed by commercial enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's instructions (Alpha Diagnostic, San Antonio, Texas). Absorbance was measured at 450nm using a MultiScan Ascent plate reader (ThermoFisher Scientific, Loughborough, UK) with standard curves produced by the plate reader Ascent software programme using a 4 parameter logistic curve.

PRL assays were outsourced to a specialist external laboratory and levels were determined by homologous specific radioimmunoassay, as previously described [482].

3.3.3 Data analysis and presentation

3.3.3.1 Basic data analysis

All data were expressed as pg/ml (cytokines, E_2) or ng/ml (PRL, P_4) ±SEM. Data distributions were assessed for normality using Anderson-Darling tests. Basic analytical approaches were based on subsequent Kruskall-Wallis/analysis of variance with *post hoc* Mann-Whitney-*U*/Fisher's LSD tests, as appropriate. Pup removal data were similarly compared using t-tests or Mann-Whitney-*U* tests. Corrections for multiple comparisons were then applied using Holm's modified Bonferroni method. Statistical analyses were performed in SPSS (Version 15), GraphPad Prism (Version 5.04) and in the freeware 'R'.

3.3.3.2 Bayesian hierarchical cluster (BHC) analysis

BHC analysis was performed as previously described to allow the identification of distinct groupings of analytes based solely on input data using BHC via a flexible, non-parametric Gaussian Process model [483]. Briefly*, z*-scores were computed for each time-series, using SEMs for each measurement, with an assumed null hypothesis that the variable is constant at the weighted mean value. Resultant *z*-score *P* values (with 0.05 threshold) were corrected using Benjamini–Hochberg false discovery rate [484]. Time series were assigned to a specific cluster on strictly probabilistic grounds, wherein each cluster contained time series which behaved similarly throughout lactation.

3.3.3.3 Generation of heatmaps

Correlations between cytokine, steroid hormone and PRL profiles were determined using Pearson's product-moment correlations, which were used as a basis for the generation of a correlation heat map using the freeware 'R'.

3.3.4 Construction of Bayesian network directed acyclic graphs

In Bayesian network formalism, a network of interacting variables (e.g. genes or proteins) is represented as a graphical model in which the variables are nodes and their interactions are represented by directed edges [485]. The edge between two nodes (for example P_1 and P_2), is associated with a conditional probability, i.e. the probability of the state of P_2 given the state of P_1.

Construction of the network involved several stages:

- Establishment of a prior network based on information from the literature and/or existing datasets
- Application of the experimental data to the prior network

3.3.4.1 Construction of the prior network.

In order to establish a 'prior' network containing the proteins from the present analytical target set, a 'seed' network was learned from the biomedical literature and protein-protein interaction databases, principally using 'MetaCore' (GeneGo Inc., Thomson Reuters, http://www.genego.com) and 'Predictionet' (https://compbio.dfci.harvard.edu/predictivenetworks) [486]. The seed network was species-restricted to the mouse. Any conflicting edges in the prior network causing feedback cycles were removed due to the acyclic nature of the DAG.

3.3.4.2 Combination of the prior network with the experimental data.

Prior to performing the Bayesian network analysis, *z*-score normalisations were applied to the raw data in Matlab in order to account for the wide variation in concentration dynamic ranges. A machine learning algorithm (implemented in the WEKA-based open-source package MeV (http://www.tm4.org/mev/ [487]) was used to refine the seed network in conjunction with the experimental data derived from post-partum lactating and non-lactating (pup-free) mice in order to predict two high-confidence networks [399, 488].

Following z-score normalisation, cytokine and hormone concentrations were converted from continuous to discrete data, and assigned to three mutually exclusive bins (low, medium and high concentrations) using an equal width binning approach. These discretised data were then used to learn the Bayesian network. Both the network topology and the conditional probabilities associated with each edge were learned from the data, starting from the initial seeded prior network. Only nodes with three parents were selected (as per convention in the field) as searching for the best possible network for a given set of moderately sized proteins is computationally expensive [399]. Standard non-parametric bootstrapping (100 bootstrap operations) was applied in order to address potential over-fitting in the Bayesian analysis [489], wherein multiple data sets were created by re-sampling with replacement to estimate the confidence in the various network features learned. For each iteration, the Tabu Search algorithm was used to optimise the BDe score as the Bayesian metric [490]. Altering the Bayesian metric to a Euclidean algorithm gave comparable results. The network directed acyclic

graph was then visualised using Cytoscape (http://www.cytoscape.org) [491]. Bayesian networks represent a ‘snapshot’ at any given time, however the networks generated herein reflect sustained effects or causal events seen across multiple time points. For Bayesian network generation, the interaction matrix between each node for each time point was learned and then used to initialise the inference process for the next time point. Therefore, the final Bayesian network visualized from the final time point reflected the dynamic process encoded by the previous time step.

3.3.4.3 In silico *perturbation of Bayesian networks*

In order to explore the DAG and the relationship between the nodes, artificial perturbation of the concentration of selected nodes was performed. *In silico* perturbation of eotaxin, IL-3, IL-12 (p40), IL-13, MCP-1 and PRL was performed in order to determine the relative importance of these nodes in the determination of cytokine network structure. This was achieved by artificially altering the conditional probability associated with the selected node and observing the effects on downstream nodes.

3.3.4.4 In vivo *perturbation of Bayesian network.*

In vivo perturbation of cytokine and hormone networks was achieved by the removal of pups at birth. This approach removed the suckling stimuli and therefore prevented the establishment of lactation. The *in vivo* perturbation provided a physiological validation for the predictions made through *in silico* perturbation.

3.3.4.5 Variational Bayesian state space modelling (VBSSM)

In order to further validate the robustness of this model, a VBSSM network was constructed. VBSSM does not take into account prior information (the ‘seed’) in the reconstruction of a given network, generating a network based solely on observed data, whilst having the advantage of taking into account ‘hidden’ nodes [492]. An implementation of the VBSSM method in Matlab was used in the present reconstruction, and the results compared to the Bayesian network generated above [486]. The VBSSM algorithm employed utilized linear Gaussian state-space models to reverse engineer interactions between proteins from time series data, providing distributions over the model parameters leading to an inference of the underlying structure of the state-space. The algorithm allowed for the selection of a high significance level (measured in terms of *z*-score) of the network interactions.

3.3.4.6 Comparison of network structure

In order to compare the two physiological networks, categorical Bayesian network inference was used to compute the F-score, which represents the harmonic average of specificity and sensitivity, and accounts for edge appearance and disappearance. The F-score is expressed in terms of the number of true and false positive and false negative edges, and provides a statistical score of similarity between any two networks. The F-score falls between the values of 0 and 1, with 1 representing an identical network. The comparative complexity of the two networks was assessed and compared via Catnet (http://cran.r-project.org/web/packages/catnet/index.html).

3.4 Results

3.4.1 Circulatory cytokines and hormone concentrations during lactation

Cytokine, E_2, P_4 and PRL concentrations were profiled across lactation and compared to those of virgin, naturally cycling animals (Figure 3-4 and Figure 3-5). Significant changes in concentration were noted for IL-1α ($P<0.001$), IL-2 ($P<0.001$), IL-3 ($P<0.01$), IL-5 ($P<0.01$), IL-9 ($P<0.001$), IL-10 ($P<0.01$) IL-12 (p40) ($P<0.001$), IL-12 (p70) ($P<0.001$), IFN-γ ($P<0.01$), G-CSF ($P<0.05$), GM-CSF ($P<0.05$), KC ($P<0.01$), MCP-1 ($P<0.05$), MIP-1α ($P<0.05$), MIP-1β ($P<0.001$) and RANTES ($P<0.05$) (Figure 3-4). IL-13 approached significance ($P<0.075$). IL-1β, IL-4, IL-6, eotaxin, TNF-α and E_2 differed from NC animals, but did not vary across lactation.

Cytokine levels on the first day of lactation were either moderately raised or comparable to naturally cycling levels. The concentration of over half of the cytokines investigated (eotaxin, G-CSF, GM-CSF, IFN-γ, IL-1α, IL-9, IL-12 (p40), IL-12 (p70), IL-13, KC, MIP-1α, and RANTES) decreased on day 2 of lactation, a change which was significant for MCP-1 ($P<0.001$).

Most cytokine concentrations peaked on day 10 of lactation, which was particularly reflected in the significantly higher levels of IL-2 and MCP-1 at this time point over all others ($P<0.05$). The timing of peak levels differed for IL-1α (plateau on days 16-24), IL-9 (plateau on days 4-16), IL-12 (p40) (peak day 24), KC (peak day 21) and RANTES (peak day 16). There followed a significant decrease (IL-2, IFN-γ, MCP-1, MIP-1α ($P<0.05$) or trend towards decrease (eotaxin, G-CSF, GM-CSF, IL-3, IL-5, IL-6, IL-10, IL-12 (p70), IL-13, KC, MIP-1β, TNF-α) in cytokine profiles on day 16. By contrast, IL-1β and IL-4 levels varied little throughout lactation and did not differ from naturally cycling levels. Whilst the levels of eotaxin, IL-5, IL-9, IL-12 (p70), IL-13, IL-17, RANTES and TNF-α did not differ significantly between time points, their levels did, however, vary in support of the above trends.

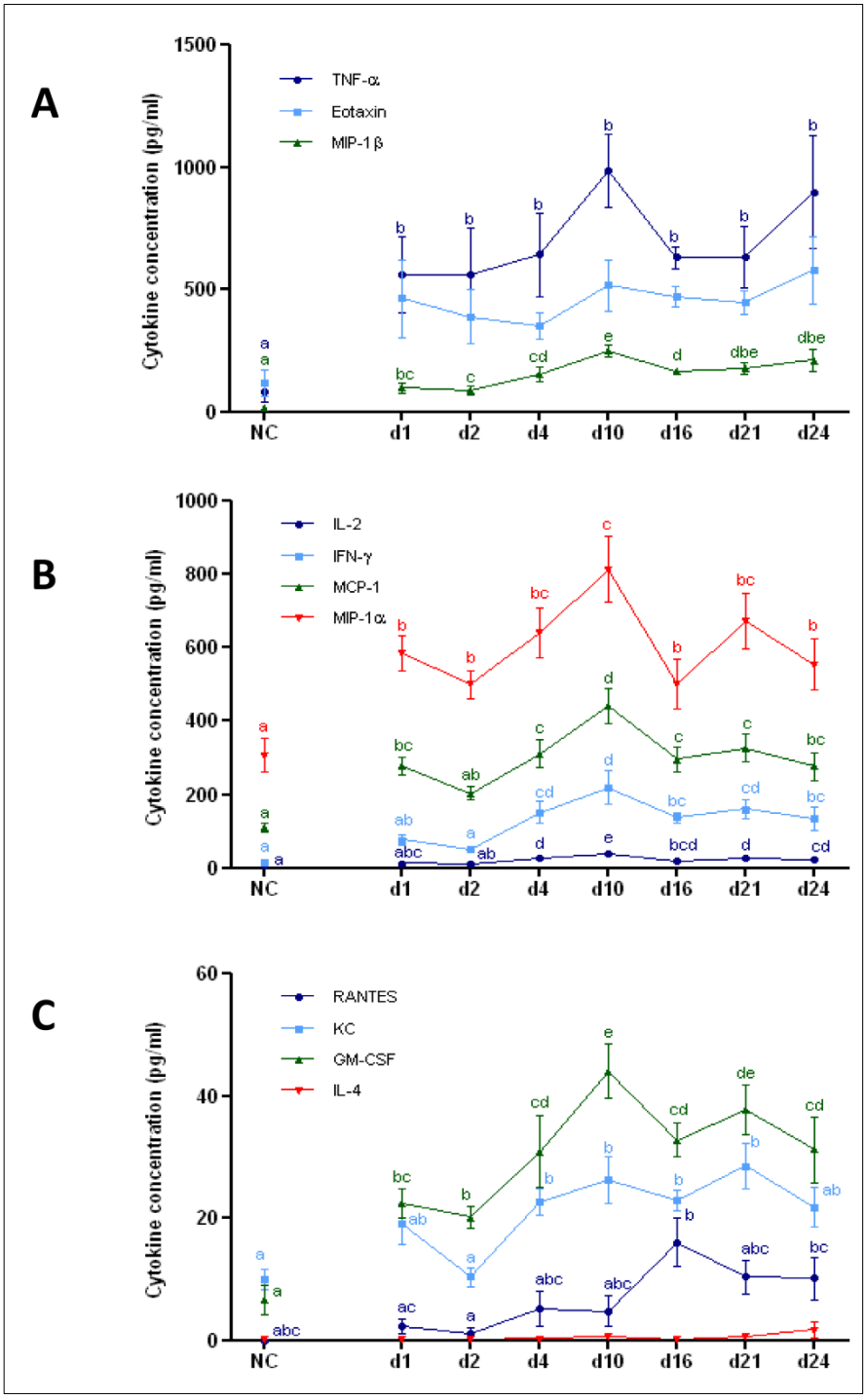


Figure 3-4: Circulatory cytokine concentrations during lactation (1). Line graphs charting the progression of circulatory cytokine concentration (pg/ml) throughout lactation. Panel A – TNF-α, eotaxin and MIP-1β. Panel B – IL-2, IFN-γ, MCP-1 and MIP-1α. Panel C – RANTES, KC, GM-CSF and IL-4. Significant differences between the time points are indicated by lowercase letters, where 'a' is significantly different to 'b' and 'c' etc.

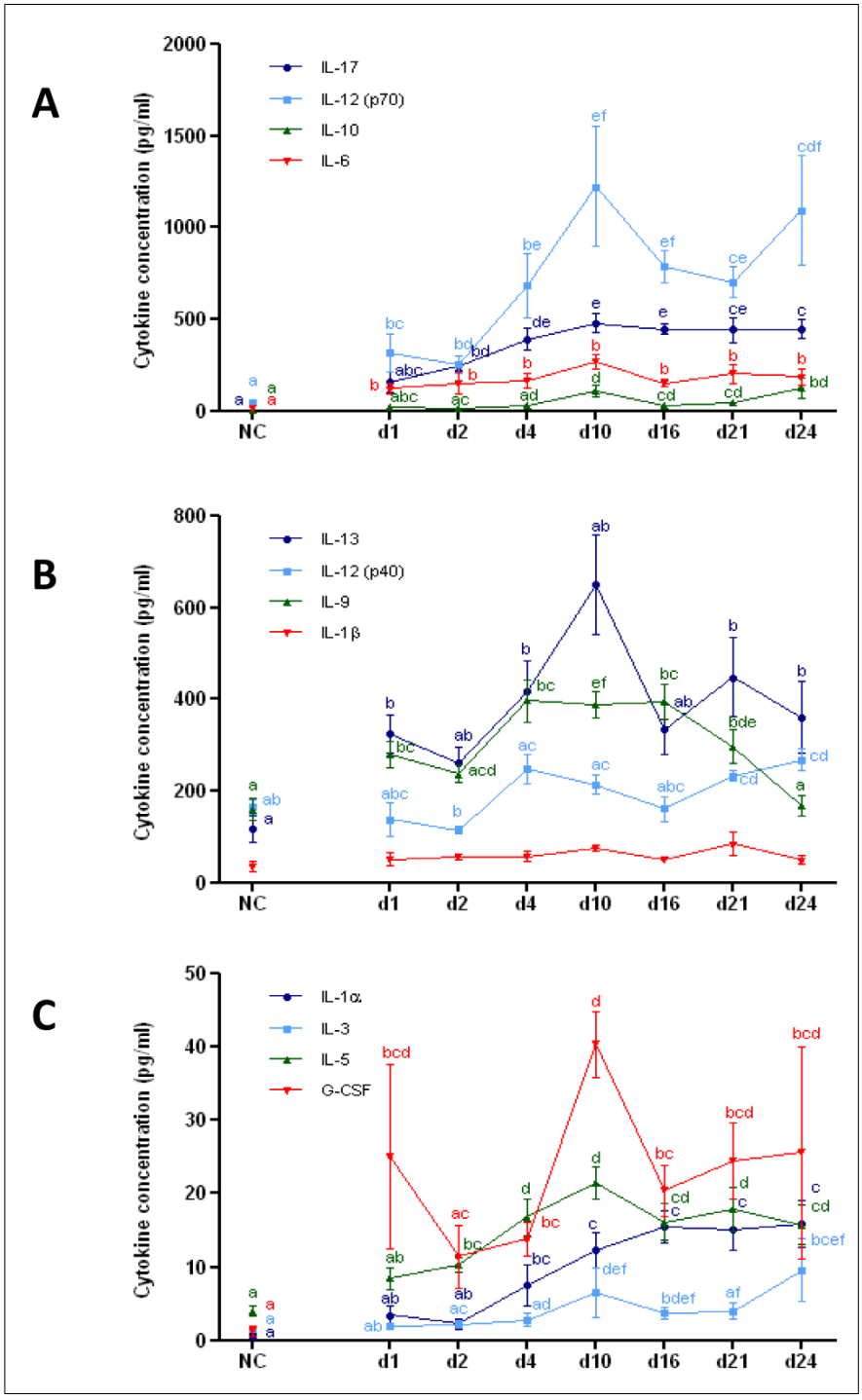


Figure 3-5: Circulatory cytokine concentrations during lactation (2). Line graphs charting the progression of circulatory cytokine concentration (pg/ml) throughout lactation. Panel A – IL-17, IL-12 (p70), IL-10 and IL-6. Panel B – IL-13, IL-12 (p40), IL-9 and IL-1β. Panel C – IL-1α, IL-3, IL-5 and G-CSF. Significant differences between the time points are indicated by lowercase letters, where 'a' is significantly different to 'b' and 'c' etc.

Hormone profiles are displayed in Figure 3-6. PRL varied significantly across lactation, exhibiting a peak on day 4 ($P<0.001$). E_2 concentrations fell early in lactation and then remained relatively steady, except for a small peak at the time of weaning. P_4 concentrations increased over early lactation, peaking on day 4 and falling to very low levels by day 16, with a subsequent increase towards weaning ($P<0.001$). Concentrations at the time of weaning for all three hormones were very similar to those of naturally cycling virgin animals.

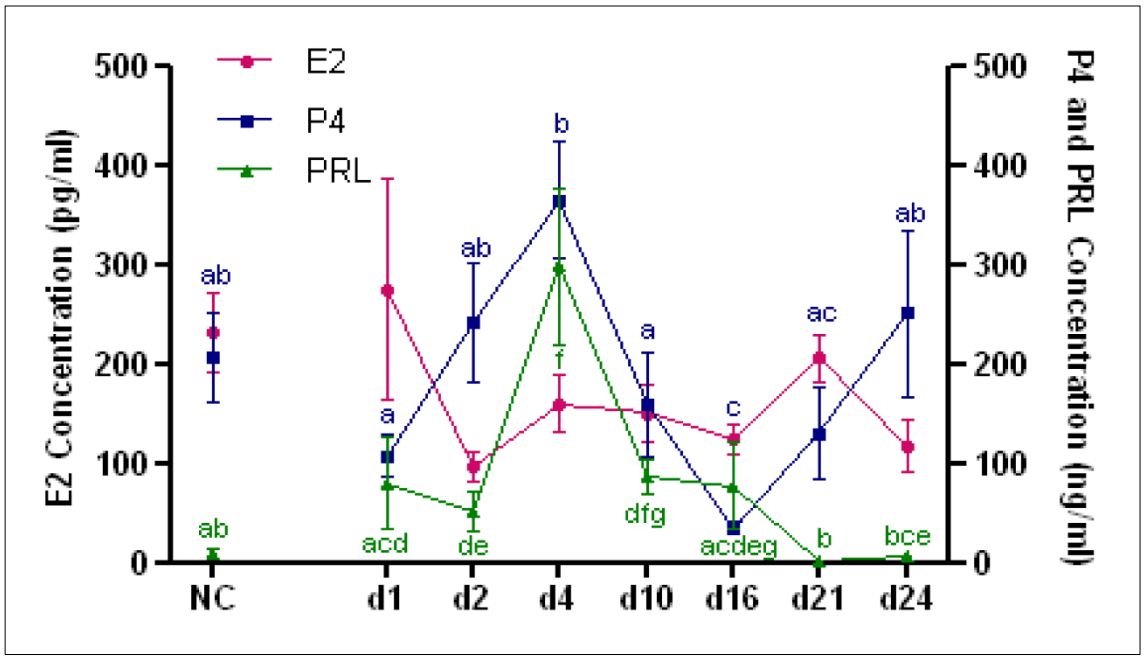


Figure 3-6: Circulatory hormone profiles (E_2, P_4 and PRL) across lactation. Significant differences between the time points are indicated by lowercase letters, where 'a' is significantly different to 'b' and 'c' etc.

3.4.2 Cluster analysis

Data clusters generated by BHC revealed analytes which followed similar patterns throughout lactation (Figure 3-7). Cytokine and hormone profiles fell into three distinct time-series clusters. Cluster 1 (eotaxin, G-CSF, GM-CSF, IL-2, IL-3, IL-4, IL-5, IL-6, IL-10, IL-13, IFN-γ, KC, MCP-1, MIP-1α and TNF-α) were initially low in concentration, followed by a broad peak centred on day 10 of lactation. Cluster 2 (IL-1α, IL-1β, IL-12 (p40), IL-12 (p70), IL-17, MIP-1β and RANTES) showed a similar pattern with the exception of the lack of a day 10 peak. Cluster 3 (IL-9, E_2, P_4, and PRL) peaked around day 5, and tailed off steadily.

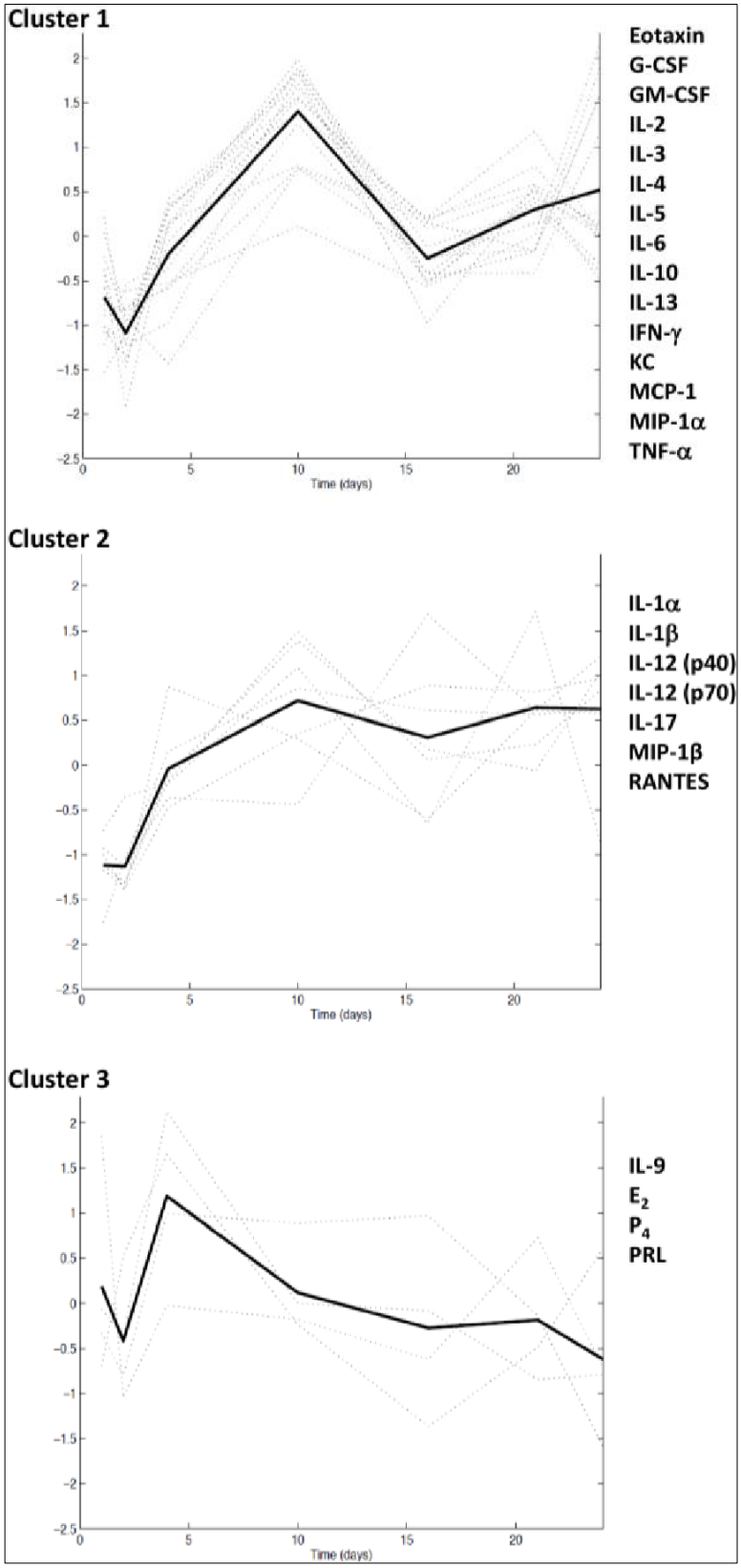


Figure 3-7: Bayesian Hierarchical Cluster (BHC) analysis of lactation data. Cytokines and hormones clustered into three patterns as described in the text.

3.4.3 Correlations between mediators

Significant positive correlations were noted across the array of mediators investigated, except for E_2, IL-1β, P_4 and PRL, which were negatively correlated with the majority of other analytes (Figure 3-8). Interestingly, these mediators also clustered together in the cluster analysis (Figure 3-7).

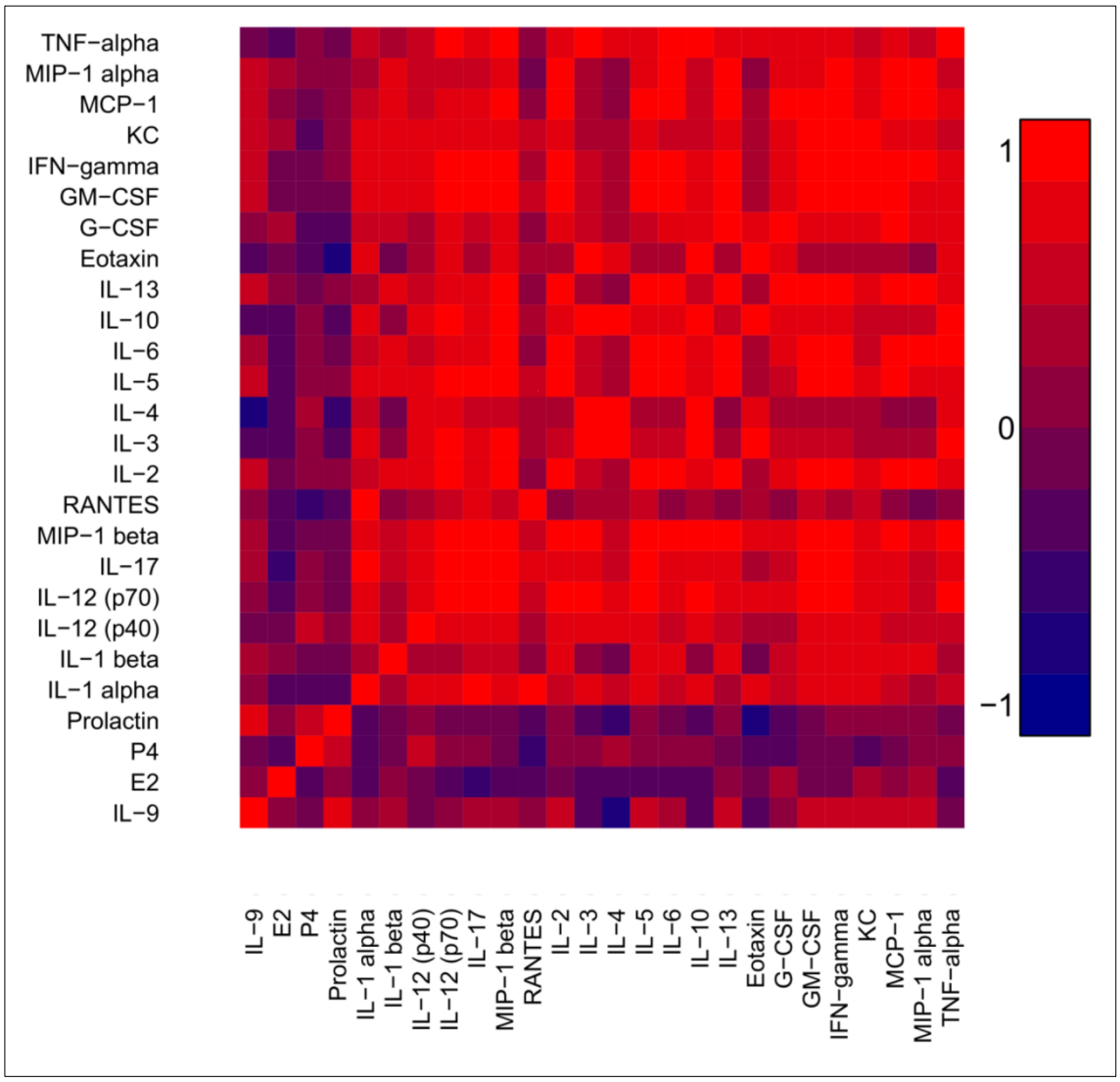


Figure 3-8: Heatmap demonstrating the correlations between mediators across murine lactation. Red indicates a positive correlation while blue indicates a negative correlation (P=0.05).

3.4.4 Cytokine and hormone Bayesian networks during lactation

3.4.4.1 Prior network structure

A prior network was derived from the literature pertaining to murine cytokine interactions as described in the methodology. The prior network is displayed in Figure 3-9, and shows the cytokines as 'nodes' connected by edges. Due to the nature of the generation of the prior network, the direction of the interaction is not known. As the knowledge within the literature was primarily limited to individual interactions, many cytokines (Eotaxin, GM-CSF, IL-1α, IL-1β, IL-3, IL-9, IL-10, IL-12 (p70), IL-17 and KC) featured edges connecting them to only one or two cytokines.

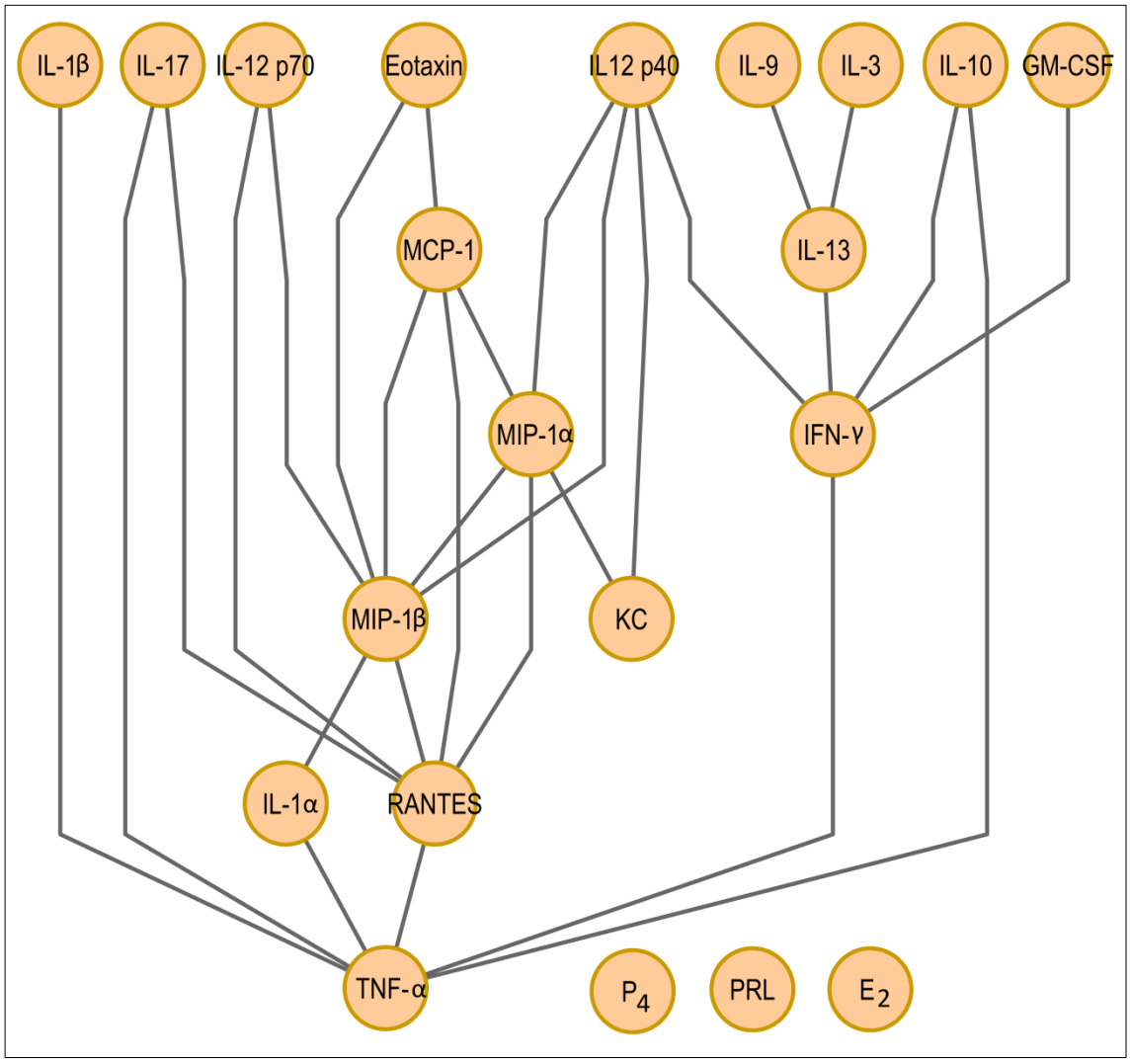


Figure 3-9: Prior network indicating known interactions between cytokines in the murine environment.

3.4.4.2 Bayesian network structure

Networks were generated in a probabilistic graphical format with analytes (cytokines and hormones) depicted as ‘nodes’ (circles) and the interactions between them represented by ‘edges’ (lines). Each analyte was classified according to the probability of being in artificially defined concentration ranges or ‘bins’, and the nodes were colour coded to depict the probability of being in a low (red), medium (white) or high (green) concentration bin given the state of their parent nodes (thereby making the probability of being in a particular concentration bin ‘conditional’). Where a node had equal conditional probabilities across all concentration bins the node was coloured white. Probability ranged between 0 and 1. Each node had 3 associated probabilities (one per concentration bin), the sum of which added to 1 (Figure 3-10).

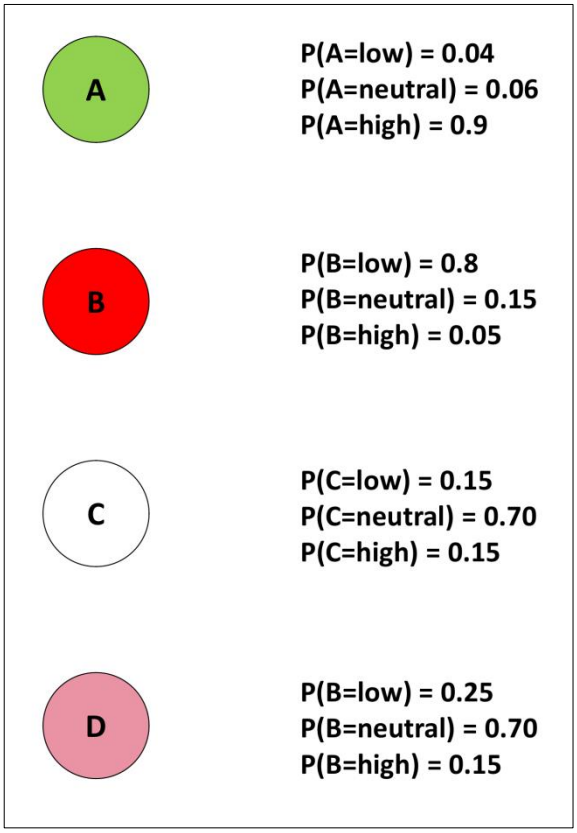


Figure 3-10: Schematic describing node colours and conditional probabilities. Node A has a high probability of being in the high concentration bin (0.9) therefore is coloured green. Node B has a high probability of being in a low concentration bin and is therefore coloured red. Node C has a high probability of being in a medium concentration bin and is therefore coloured white. In the case of equal probabilities across all concentration bins, the node would be coloured white. Node D has a slightly higher probability of being in a low concentration bin than a high concentration bin and is therefore coloured pink.

Intensity of colour (for example red versus pink) was determined by the level of conditional probability, where a stronger colour represented a higher probability of being of low or high concentration. Edges were directional, indicating which nodes were parental nodes, terminal nodes and key 'hubs' (defined as nodes featuring at least 2 incoming and 1 outgoing edge) (Figure 3-11). High confidence edges (i.e. features appearing in more than 90% of iterations) were coloured green and lower confidence edges (appearing in 70-90% of iterations) were coloured grey. When nodes were 'perturbed' (artificially changed to a different conditional probability profile) the conditional probabilities were displayed next to the node as a bar chart relating to the

three concentration bins. The Bayesian networks were directed (indicating the direction of the interaction between nodes), acyclic (without feedback loops) graphs (DAGs).

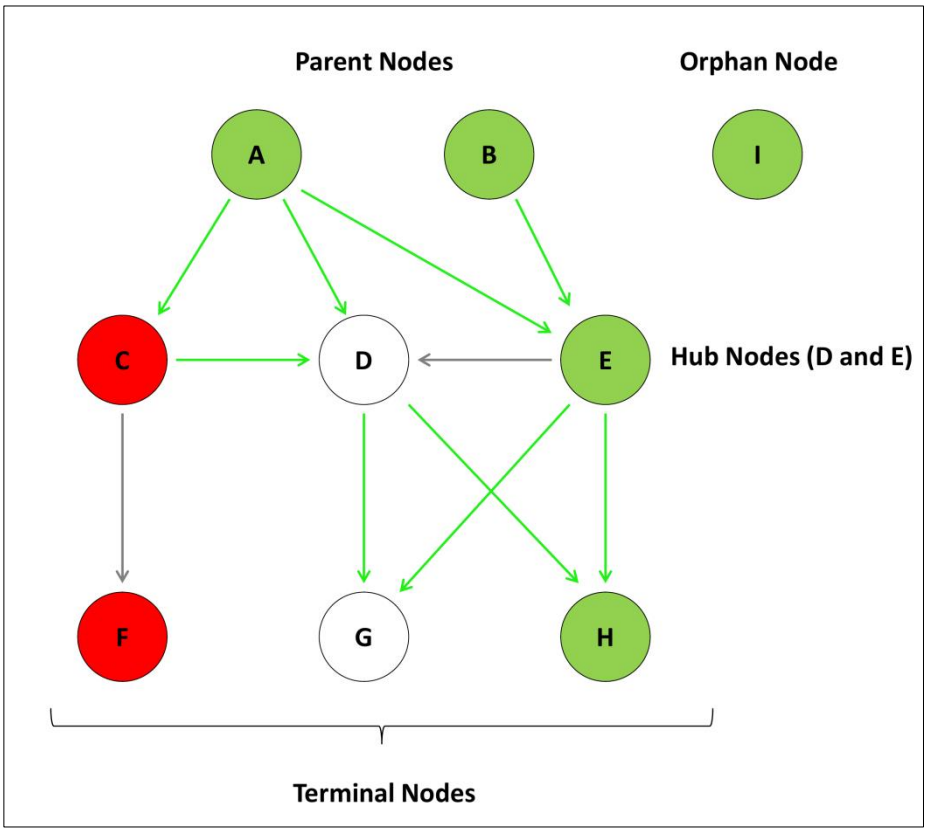


Figure 3-11: Schematic diagram depicting Bayesian network structure and nomenclature. Nodes A and B are defined as ‘parent’ nodes as they only feature outgoing edges. Nodes D and E feature at least 2 incoming and 1 outgoing edge, therefore are defined as ‘hub’ nodes. Nodes F, G and H only feature incoming edges and are therefore defined as terminal nodes. Node I is defined as an orphan node as it is unconnected to the network. Edges coloured green indicate high confidence, while edges coloured grey indicate low confidence interactions.

3.4.4.3 Core network features

The Bayesian network generated using data from lactating mice appeared to form two main branches; the first with IL-3, E2 and eotaxin as parents, and the second with IL-12 (p40) as the principal parent node (Figure 3-12). Within this structure, six core hubs (defined as those nodes with two or more input edges and one or more output edges) were identified, comprising IFN-γ, IL-13, MCP-1, MIP-1α, MIP-1β and RANTES. TNF-α was displayed as a major terminal node (defined as a node with parents but no children) and was connected, either directly or indirectly, to each of the core hubs. Minor terminal nodes were G-CSF, IL-2, IL-6 and KC. 42 edges (35 of high confidence) connected 25 of the 26 nodes, with IL-4 being the only orphan node. During lactation, E_2, P_4 and PRL demonstrated a high probability of being present at high concentration (green nodes) relative to the low concentration (red nodes) of cytokine network components, with the exception of eotaxin and IL-9.

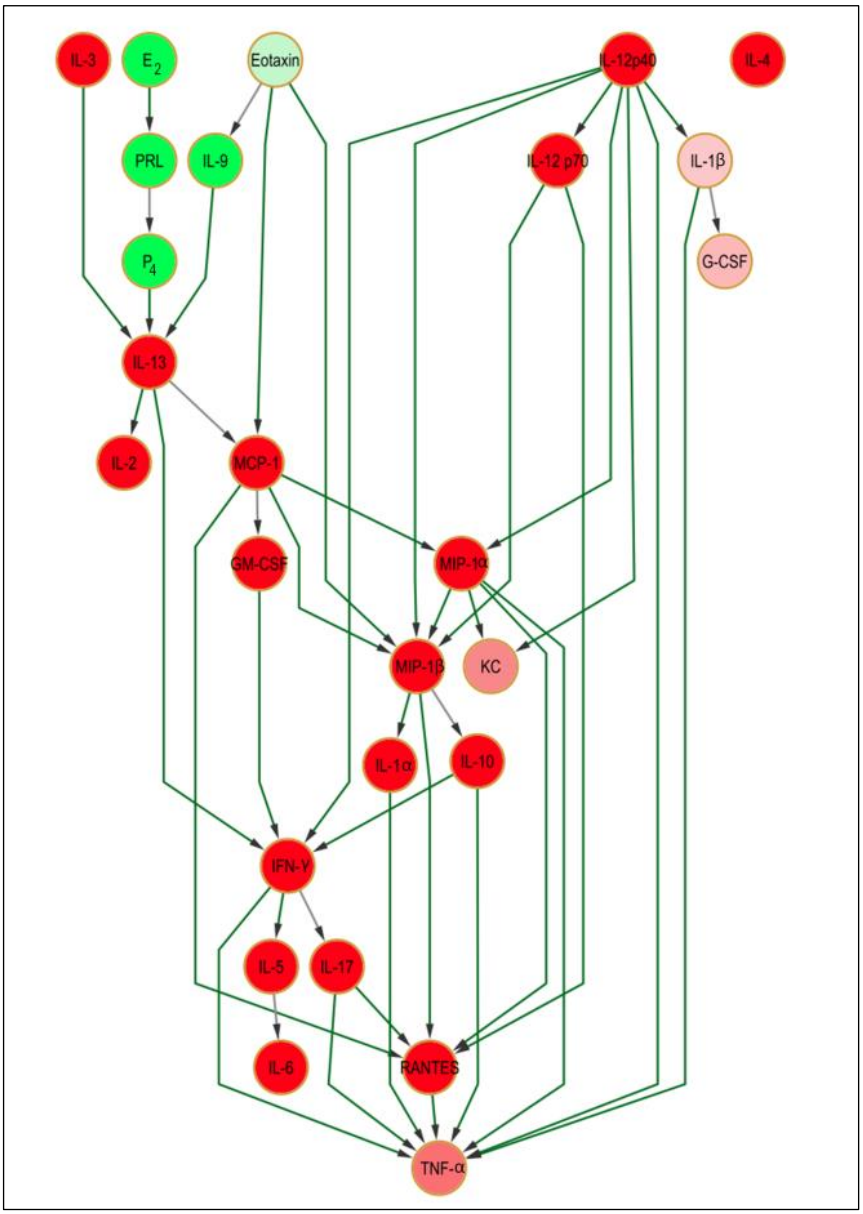


Figure 3-12: Hierarchical Bayesian network DAG generated using data from lactating mice. The nodes (cytokines and hormones) are colour coded depicting the conditional probability of the node concentration being in a high (green), low (red) or medium (white) concentration bin given the state of their parent nodes. The depth of colour indicates the level of the probability of that node being in the stated concentration bin (e.g. red indicates a high probability of being in a low concentration bin, while pink indicates a slightly lower probability of being in that bin). Green edges represent those interactions being present in 90% of iterations, while grey edges were present in 70% of iterations.

3.4.4.4 Robustness of the network

The Bayesian network generated from the lactating mice data set was robust, with the features displayed changing minimally when the bootstrapping confidence level was varied between 70% and 90% of the total number of iterations conducted. Furthermore, very few edges between nodes were of low confidence level.

3.4.4.5 Variational Bayesian state space modelling (VBSSM) of lactation data

The VBSSM (i.e. not based on prior knowledge) results generated a conserved core structure network similar to that obtained from the seeded Bayesian network analyses (Figure 3-13). Features not seen in the lactation Bayesian network were IL-10 and IL-12 (p70) as parents, and IL-1α as a new terminal node. However, IL-2, RANTES and TNF-α were terminal nodes as seen in the Bayesian network. IL-13, IFN-γ and MIP-1β were key hubs with numerous incoming and outgoing edges as seen in the Bayesian network. Statistical comparison of the VBSSM and Bayesian networks for similarity showed high specificity (high true negatives and low false positives) and medium sensitivity and F-score, and this alongside retention of hub nodes suggests a high level of similarity between the two networks (Table 3-1).

	VBSSM compared to seeded Bayesian network in lactation
Specificity	0.94
Sensitivity	0.30
F-Score	0.46

Table 3-1: Statistical comparison of VBSSM and Bayesian networks in lactation.

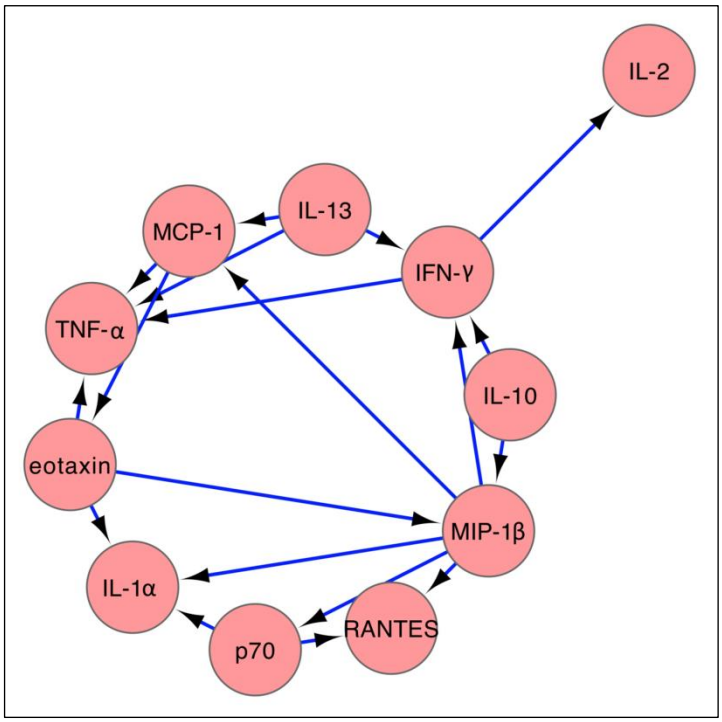


Figure 3-13: VBSSM model of data derived from lactating mice. Network was derived from data only without the use of prior knowledge. Due to the method of analysis, the colour of the nodes and edges in this method bear no relation to the concentration bin of the node or the strength of the relationship between nodes. IL-10 and IL-13 were parental nodes (those without incoming edges), while MIP-1β was a 'hub' (numerous incoming and outgoing edges). IL-1α, IL-2, RANTES and TNF-α were terminal nodes, only featuring incoming edges.

3.4.5 In silico *perturbation of cytokine and hormone Bayesian network*

3.4.5.1 PRL perturbation

The first perturbation performed *in silico* was the reduction in PRL concentration, given the evidence that PRL is a critical immunomodulator of lactation and that its levels were expected to fail to rise physiologically in dams which had had their pups removed at birth. Perturbation was achieved by allocating a conditional probability of 1 in the low concentration bin, thereby reducing the conditional probability in the medium and high concentration bins to 0. Apart from a fall in P_4 profile, *in silico* perturbation of PRL did not markedly affect downstream nodes, with the exception of IL-2 and IL-13 which both demonstrated a shift in conditional probability towards a medium/high concentration profile bin (Table 3-2). This suggests that PRL exerts a negative regulatory effect on these cytokines.

Cytokine/Hormone	Low	Medium	High
PRL	0.065	0.692	0.243
P_4	0.066	0.786	0.148
P_4 -PRL	0.778	0.111	0.111
IL-13	0.400	0.499	0.101
IL-13 -PRL	0.379	0.348	0.272
IL-2	0.274	0.602	0.124
IL-2 -PRL	0.274	0.481	0.246
MCP-1	0.431	0.458	0.111
MCP-1 -PRL	0.418	0.386	0.196
GM-CSF	0.359	0.526	0.115
GM-CSF -PRL	0.353	0.469	0.178
MIP-1α	0.235	0.687	0.078
MIP-1α -PRL	0.250	0.644	0.106
MIP-1β	0.607	0.208	0.186
MIP-1β -PRL	0.570	0.224	0.205
KC	0.165	0.706	0.129
KC -PRL	0.168	0.692	0.140
IL-1α	0.626	0.223	0.151
IL-1α -PRL	0.605	0.230	0.165
IL-10	0.648	0.230	0.122
IL-10 -PRL	0.622	0.245	0.133
IFN-γ	0.504	0.299	0.197
IFN-γ -PRL	0.465	0.299	0.236
IL-5	0.517	0.327	0.156
IL-5 -PRL	0.487	0.336	0.177
IL-17	0.513	0.391	0.096
IL-17 -PRL	0.486	0.406	0.108
IL-6	0.608	0.306	0.086
IL-6 -PRL	0.585	0.319	0.096
RANTES	0.483	0.276	0.241
RANTES -PRL	0.454	0.287	0.259
TNF-α	0.372	0.317	0.311
TNF-α -PRL	0.364	0.321	0.316

Table 3-2: Table indicating the shift in conditional probability upon PRL perturbation into a lower concentration bin. (Subscript indicates perturbation).

3.4.5.2 Perturbation of IL-13 and MCP-1

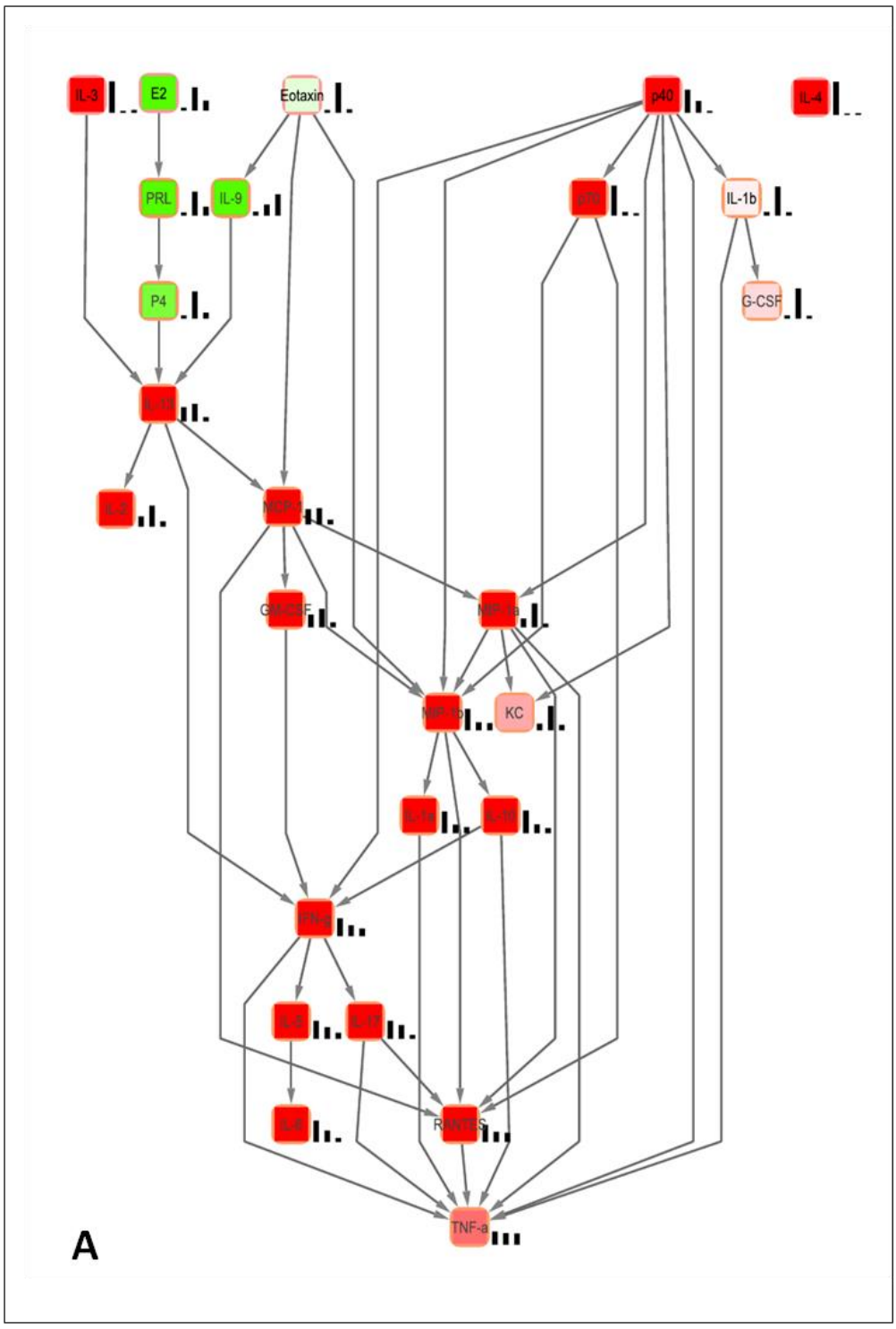
As PRL appeared to mediate only slight changes in the downstream network, the core hubs immediately downstream of PRL (IL-13 and MCP-1) were selected for perturbation. IL-13 was in a low concentration bin in the lactation network, therefore perturbing this cytokine into the high concentration bin resulted in significant downstream network changes, with GM-CSF and MCP-1 shifting towards the high concentration bin, and IFN-γ becoming neutral (Table 3-3). More minor effects extended down to the terminal node.

In a similar manner to IL-13, increasing MCP-1 concentration resulted in comparable downstream changes, with GM-CSF shifting towards a high concentration bin and MIP-1β and RANTES towards a neutral profile (Table 3-3). Further downstream, effects included MIP-1α shifting to a high concentration bin, KC becoming medium/high, and IFN-γ and TNF-α becoming neutral.

Combined IL-13 and MCP-1 perturbation was performed to examine the joint effects of these cytokines. This perturbation (on a background of low PRL) resulted in a significant number of downstream nodes changing colour compared to the lactation network (i.e. shifting from low to high concentration bins). The remaining nodes demonstrated a shift in conditional probability towards the medium/high bins (Figure 3-14). Within this combined perturbation, IFN-γ demonstrated the greatest shift in conditional probability – greater than the individual perturbations of IL-13 and MCP-1. This is suggestive of a likely synergistic effect by IL-13 and MCP-1.

Cytokine	Low	Medium	High	Cytokine	Low	Medium	High
P_4	0.066	0.786	0.148	IL-10	0.648	0.230	0.122
$P_{4\ -PRL+IL-13}$	0.778	0.111	0.111	IL-10 $_{-PRL+IL-13}$	0.526	0.301	0.173
$P_{4\ -PRL+MCP-1}$	0.778	0.111	0.111	IL-10 $_{-PRL+MCP-1}$	0.448	0.348	0.204
$P_{4\ -PRL+IL-13+MCP-1}$	0.778	0.111	0.111	IL-10 $_{-PRL+IL-13+MCP-1}$	0.448	0.348	0.204
IL-13	0.400	0.499	0.101	IFN-γ	0.504	0.299	0.197
IL-13 $_{-PRL+IL-13}$	0.000	0.000	1.000	IFN-$\gamma_{PRL+IL-13}$	0.325	0.337	0.338
IL-13 $_{-PRL+MCP-1}$	0.379	0.348	0.272	IFN-γ $_{-PRL+MCP-1}$	0.352	0.325	0.323
IL-13 $_{-PRL+IL-13+MCP-1}$	0.000	0.000	1.000	IFN-γ $_{-PRL+IL-13+MCP-1}$	0.316	0.341	0.343
IL-2	0.274	0.602	0.124	IL-5	0.517	0.327	0.156
IL-2 $_{-PRL+IL-13}$	0.111	0.111	0.778	IL-5 $_{-PRL+IL-13}$	0.377	0.386	0.237
IL-2 $_{-PRL+MCP-1}$	0.274	0.481	0.246	IL-5 $_{-PRL+MCP-1}$	0.398	0.375	0.228
IL-2 $_{-PRL+IL-13+MCP-1}$	0.111	0.111	0.778	IL-5 $_{-PRL+IL-13+MCP-1}$	0.370	0.390	0.240
MCP-1	0.431	0.458	0.111	IL-17	0.513	0.391	0.096
MCP-1 $_{-PRL+IL-13}$	0.200	0.229	0.571	IL-17 $_{-PRL+IL-13}$	0.390	0.467	0.144
MCP-1 $_{-PRL+MCP-1}$	0.000	0.000	1.000	IL-17 $_{-PRL+MCP-1}$	0.407	0.454	0.138
MCP-$1_{PRL+IL-13+MCP-1}$	0.000	0.000	1.000	IL-17 $_{-PRL+IL-13+MCP-1}$	0.384	0.470	0.146
GM-CSF	0.359	0.526	0.115	IL-6	0.608	0.306	0.086
GM-CSF $_{-PRL+IL-13}$	0.225	0.316	0.458	IL-6 $_{-PRL+IL-13}$	0.506	0.371	0.123
GM-CSF $_{-PRL+MCP-1}$	0.111	0.111	0.778	IL-6 $_{-PRL+MCP-1}$	0.521	0.361	0.119
GM-CSF $_{-PRL+IL-13+MCP-1}$	0.111	0.111	0.778	IL-6 $_{-PRL+IL-13+MCP-1}$	0.501	0.374	0.124
MIP-1α	0.235	0.687	0.078	RANTES	0.483	0.276	0.241
MIP-1α $_{-PRL+IL-13}$	0.250	0.521	0.229	RANTES $_{-PRL+IL-13}$	0.373	0.322	0.305
MIP-1α $_{-PRL+MCP-1}$	0.270	0.360	0.370	RANTES $_{-PRL+MCP-1}$	0.328	0.347	0.326
MIP-1α $_{-PRL+IL-13+MCP-1}$	0.270	0.360	0.370	RANTES $_{-PRL+IL-13+MCP-1}$	0.327	0.347	0.326
MIP-1β	0.607	0.208	0.186	TNF-α	0.372	0.317	0.311
MIP-1β $_{-PRL+IL-13}$	0.434	0.293	0.273	TNF-α $_{-PRL+IL-13}$	0.346	0.328	0.325
MIP-1β $_{-PRL+MCP-1}$	0.321	0.357	0.321	TNF-α $_{-PRL+MCP-1}$	0.341	0.331	0.329
MIP-$1β_{-PRL+IL-13+MCP-1}$	0.321	0.357	0.321	TNF-α $_{-PRL+IL-13+MCP-1}$	0.340	0.331	0.329
KC	0.165	0.706	0.129	IL-1α	0.626	0.223	0.151
KC $_{-PRL+IL-13}$	0.183	0.635	0.181	IL-1α $_{-PRL+IL-13}$	0.528	0.256	0.216
KC $_{-PRL+MCP-1}$	0.201	0.569	0.230	IL-1α $_{-PRL+MCP-1}$	0.465	0.281	0.254
KC $_{-PRL+IL-13+MCP-1}$	0.201	0.569	0.230	IL-1α $_{-PRL+IL-13+MCP-1}$	0.465	0.281	0.254

Table 3-3: Table indicating the shift in conditional probability upon PRL/IL-13/MCP-1 perturbation. Subscripts indicate the direction of perturbation: - indicates perturbation to a low concentration bin, + indicates perturbation to a high concentration bin.


IL-3
E2
Eotaxin
p40
IL-4
PRL
IL-9
p70
IL-1b
P4
G-CSF
IL-12
IL-2
MCP-1
GM-CSF
MIP-1a
MIP-1b
KC
IL-1a
IL-1ra
IFN-g
IL-5
IL-17
IL-6
RANTES
TNF-a
A

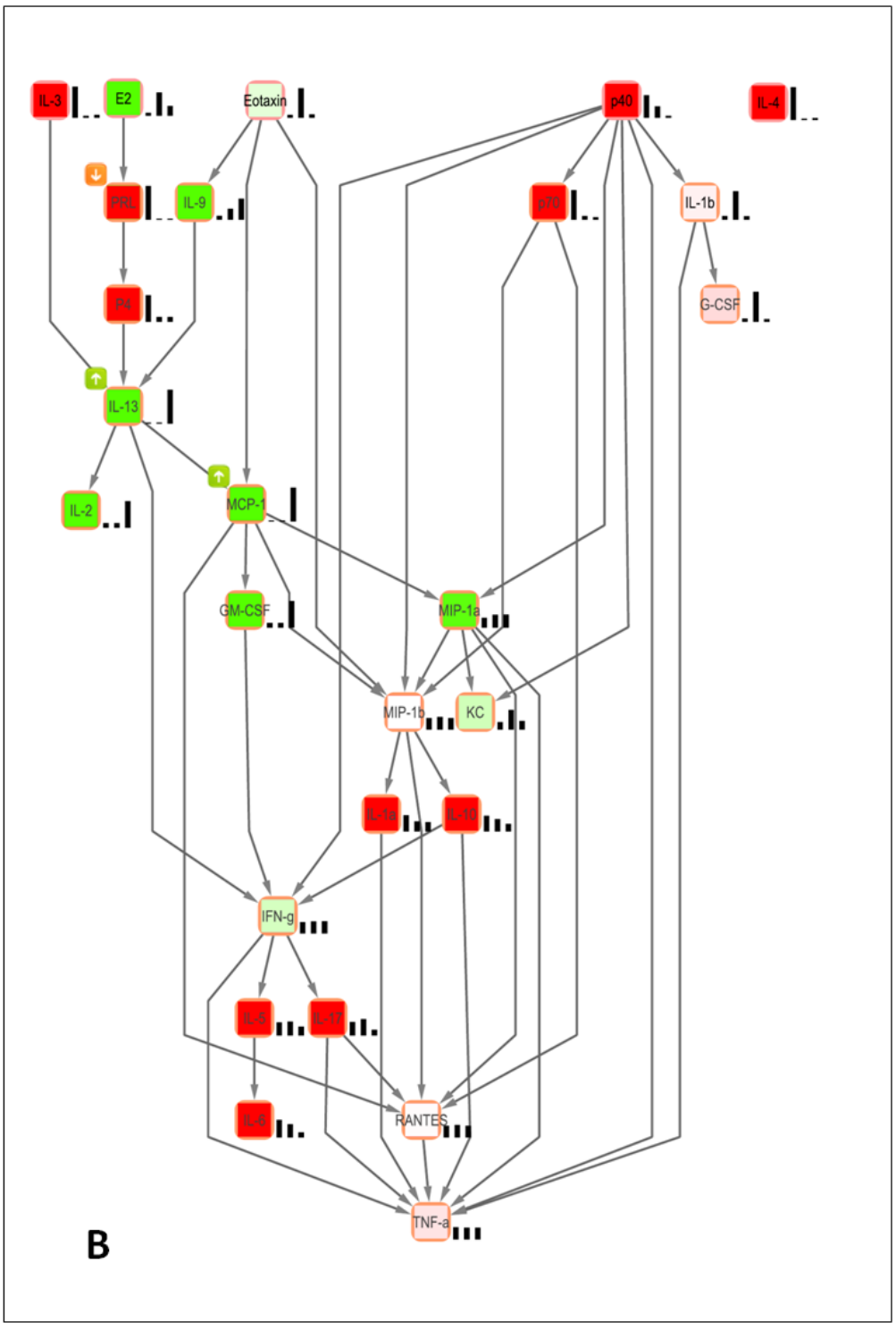
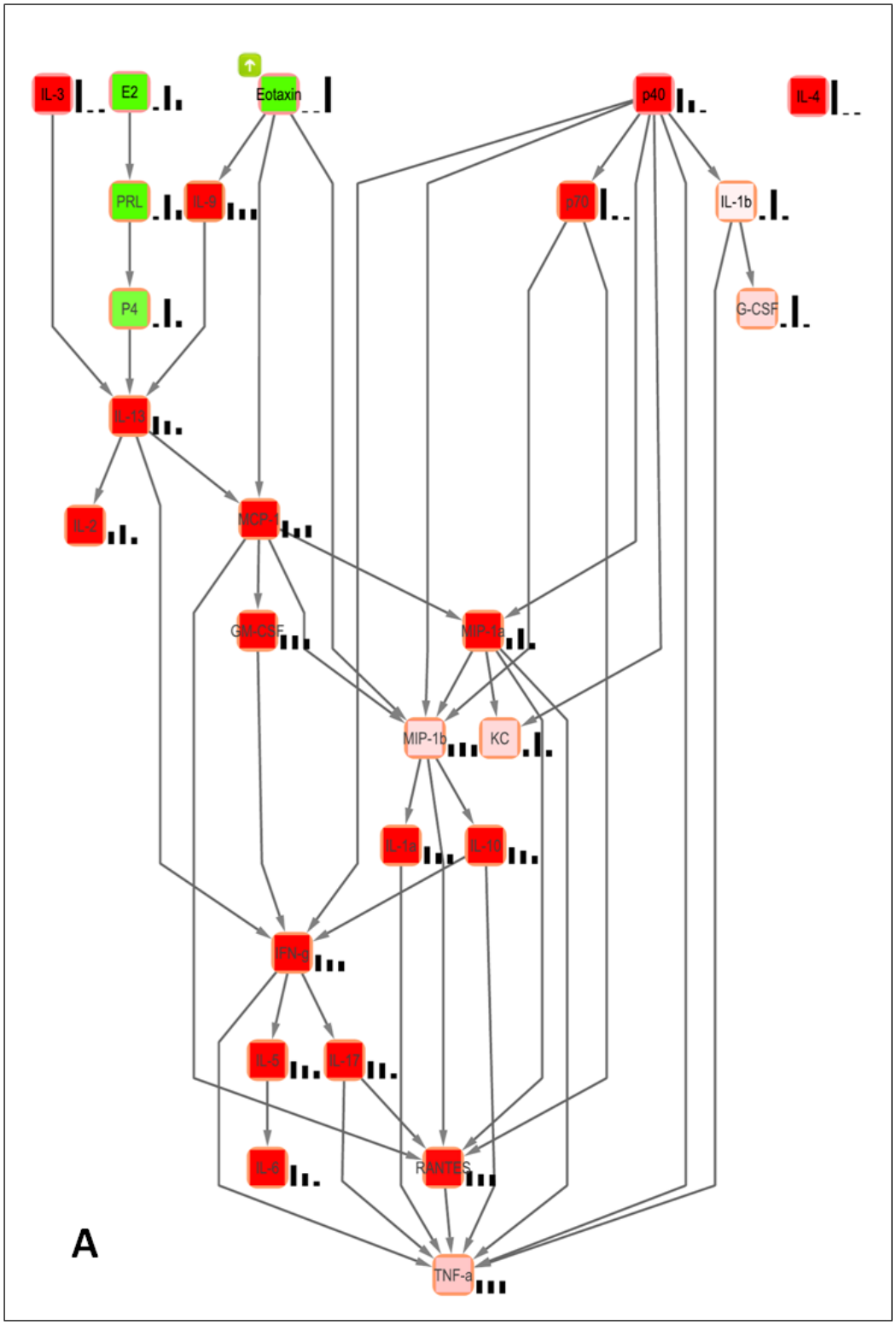


Figure 3-14: Change in conditional probability following IL-13, MCP-1 and PRL perturbation. Panel A shows the cytokine and hormone Bayesian network during lactation with conditional probability indicators showing the allocation to low, medium or high concentration bins. Panel B shows the network after perturbation of IL-13, MCP-1 and PRL, with the conditional probability indicators (small bar charts representing conditional probability values in low-to-high concentration bins) and node colour indicating the relevant shift.

3.4.5.3 Perturbation of network parent eotaxin

As eotaxin in the lactating Bayesian network was in the medium/high bin, perturbation was taken to the extremes of high and low (Figure 3-15). Increasing eotaxin caused a mild shift in MIP-1β into a higher concentration bin, while decreasing eotaxin caused markedly greater effects. In this case IL-9 shifted to a lower concentration bin, while MIP-1β and MCP-1 became more medium. Most interestingly, MIP-1β shifted into a higher concentration bin regardless of the direction of eotaxin perturbation.


IL-3
E2
Eotaxin
p40
IL-4
PRL
IL-9
p70
IL-1b
P4
G-CSF
IL-13
IL-2
MCP-1
GM-CSF
MIP-1a
MIP-1b
KC
IL-1a
IL-10
IFN-g
IL-5
IL-17
IL-6
RANTES
TNF-a
A

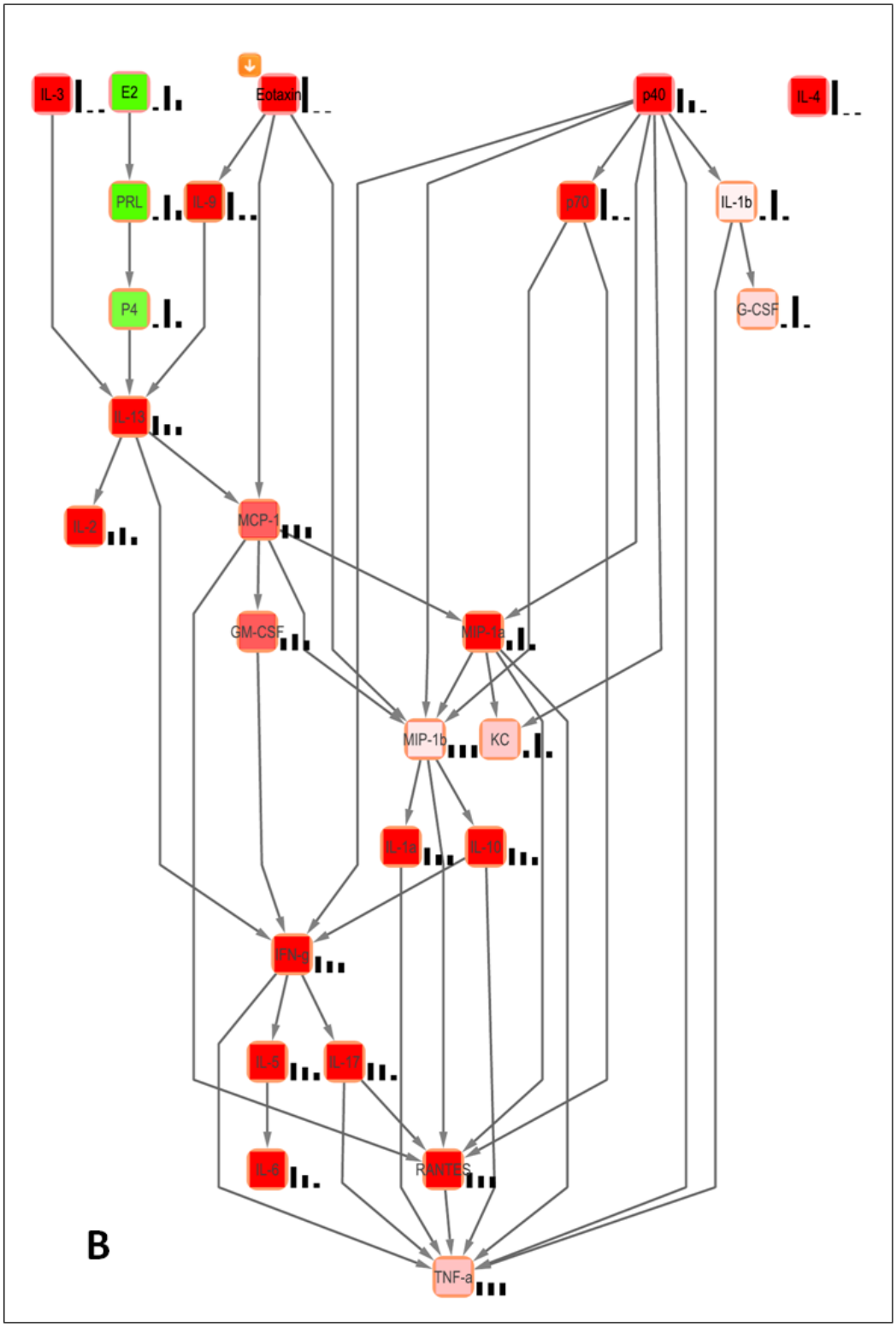


Figure 3-15: Eotaxin perturbation within the murine lactation network. Panel A shows eotaxin perturbed to a high concentration bin. Panel B shows eotaxin perturbed to a low concentration bin.

3.4.5.4 Perturbation of network parent IL-3

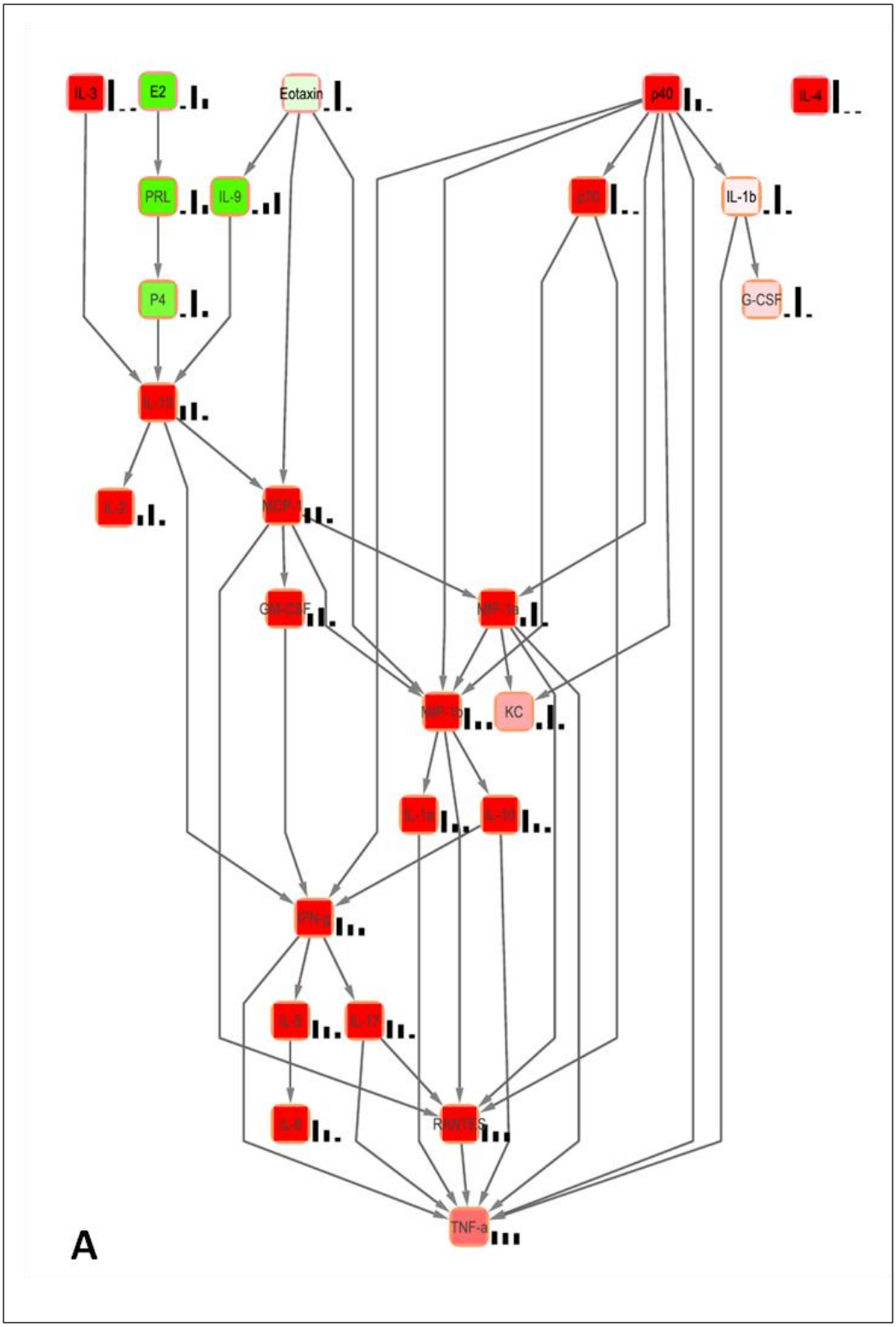
In the lactating network, IL-3 was classified into a low concentration bin, with a conditional probability of 0.919 in this regard. When allocated to a high concentration bin, there were resultant changes in the conditional probability status of IL-2, IL-13 and MCP-1 to a small degree (Table 3-4), with IL-13, IL-2 and MCP-1 shifting to a slightly higher concentration bin. However, the greatest effects were seen when IL-3 was perturbed to a medium concentration bin status, creating a larger shift in these cytokines to a high concentration bin, suggesting that the effects of IL-3 were not related to concentration in a linear fashion.

	Low	Medium	High
IL-3	0.919	0.024	0.057
IL-13	0.400	0.499	0.101
IL-13$_{IL\text{-}3_L}$	0.410	0.507	0.083
IL-13$_{IL\text{-}3_M}$	0.333	0.333	0.335
IL-13$_{IL\text{-}3_H}$	0.273	0.446	0.281
IL-2	0.274	0.602	0.124
IL-2$_{IL\text{-}3_L}$	0.278	0.610	0.111
IL-2$_{IL\text{-}3_M}$	0.252	0.456	0.292
IL-2$_{IL\text{-}3_H}$	0.215	0.526	0.258
MCP-1	0.431	0.458	0.111
MCP-1$_{IL\text{-}3_L}$	0.437	0.462	0.102
MCP-1$_{IL\text{-}3_M}$	0.391	0.380	0.229
MCP-1$_{IL\text{-}3_H}$	0.359	0.436	0.205

Table 3-4: Conditional probabilities associated with IL-3 perturbation. L-Low, M-Medium, H-High, indicates the direction of IL-3 perturbation.

3.4.5.5 Perturbation of network parent IL-12 (p40)

IL-12 (p40) was selected for perturbation due to being a parent of the second branch. Perturbation of IL-12 (p40) to a high concentration bin resulted in a large number of downstream changes (Figure 3-16). IL-1β, IL-12 (p70) and KC made major shifts to a high concentration bin, while MIP-1α exhibited a minor shift in this direction. MIP-1β, IFN-γ, RANTES and TNF-α became medium.


IL-3
E2
Eotaxin
p40
IL-4
PRL
IL-9
p70
IL-1b
P4
G-CSF
IL-12
IL-2
MCP-1
GM-CSF
MIP-1a
MIP-1b
KC
IL-1a
IL-10
IFN-g
IL-5
IL-17
IL-6
RANTES
TNF-a
A

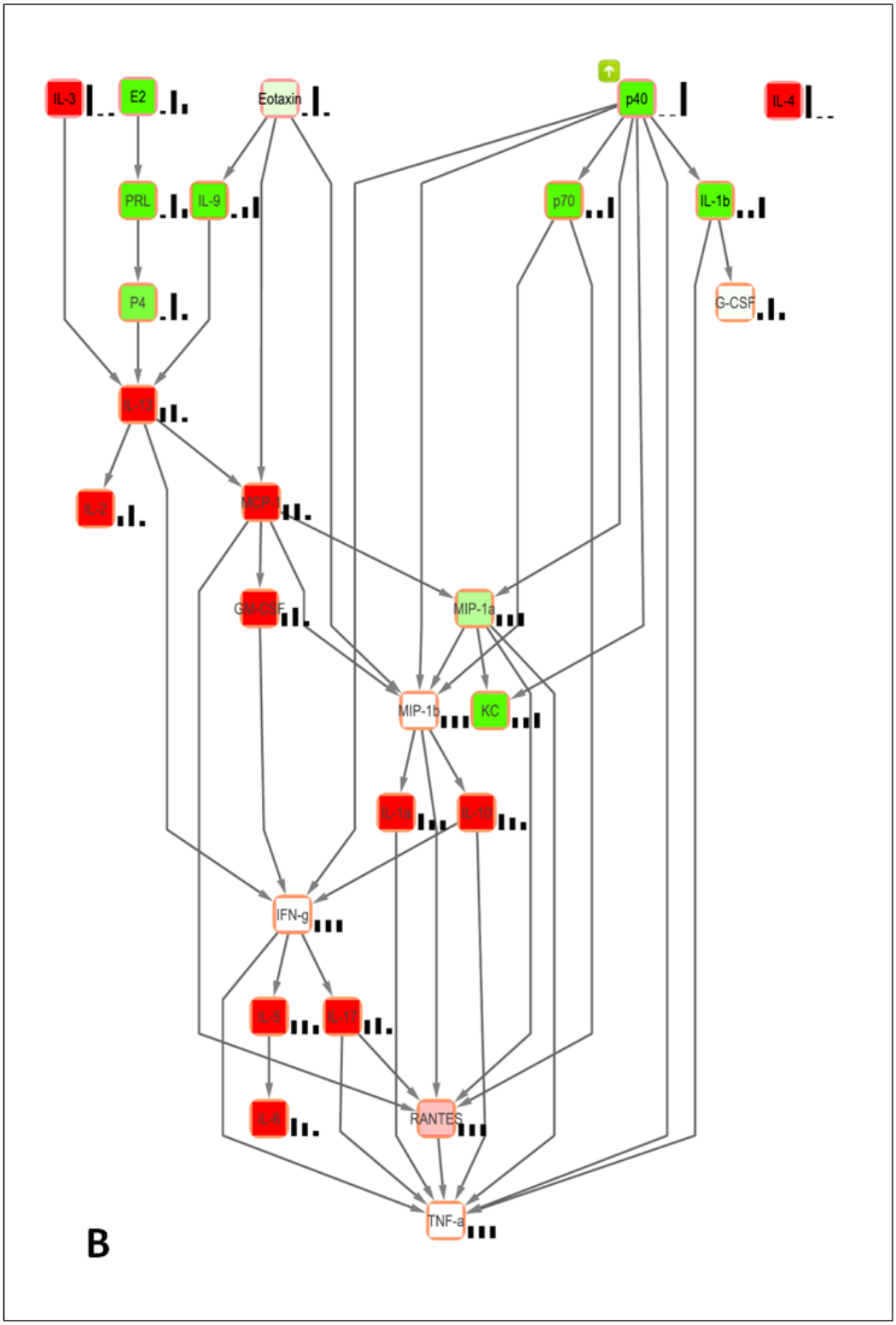


Figure 3-16: IL-12 (p40) perturbation within the murine lactation network. Panel A shows the lactation Bayesian network with conditional probability indicators. Panel B shows the network after IL-12 (p40) perturbation. Changes in node classification were seen for MIP-1α (red to green), KC (pink to green), MIP-1β (red to white), IFN-γ (red to white), RANTES (red to pink) and TNF-α (pink to white)

3.4.5.6 Combined branch perturbations

As IL-13, MCP-1, and IL-12 (p40) were identified as important driver nodes in their respective branches, combined perturbation of these nodes was performed. This perturbation affected the majority of their downstream nodes, of particular interest was the combined effect of IL-12 (p40) and MCP-1 on MIP-1α (Table 3-5). Combined perturbation of IL-12 (p40) and MCP-1 exhibited the largest shift in MIP-1α conditional probability towards a high concentration bin.

Cytokines	**Low**	**Medium**	**High**
MIP-1	0.235	0.687	0.078
MIP-1$_{IL\text{-}12\ (p40)}$	0.319	0.319	0.363
MIP-1$_{MCP\text{-}1}$	0.270	0.360	0.370
MIP-1$_{IL\text{-}12\ (p40)+MCP\text{-}1}$	0.200	0.200	0.600

Table 3-5: Conditional probabilities indicating the synergistic relationship between IL-12 (p40) and MCP-1.

3.4.6 Physiological perturbation of lactation networks by pup removal

3.4.6.1 Cytokine and hormone profile changes

Pup removal at birth resulted in a significant fall in maternal serum concentrations of IL-17 on day 2 ($P<0.05$) (Figure 3-17). By contrast, KC concentrations were significantly higher in mice without pups on day 2 ($P<0.05$). By day 4 of lactation, the differences in cytokine profiles between females with and without pups were more pronounced: IL-1α, IL-12 (p40), IL-17, IFN-γ, G-CSF, E_2 and PRL concentrations were significantly higher in nursing dams ($P<0.05$). Similar trends were noted for IL-2, IL-5, IL-9, and IL-12 (p70).

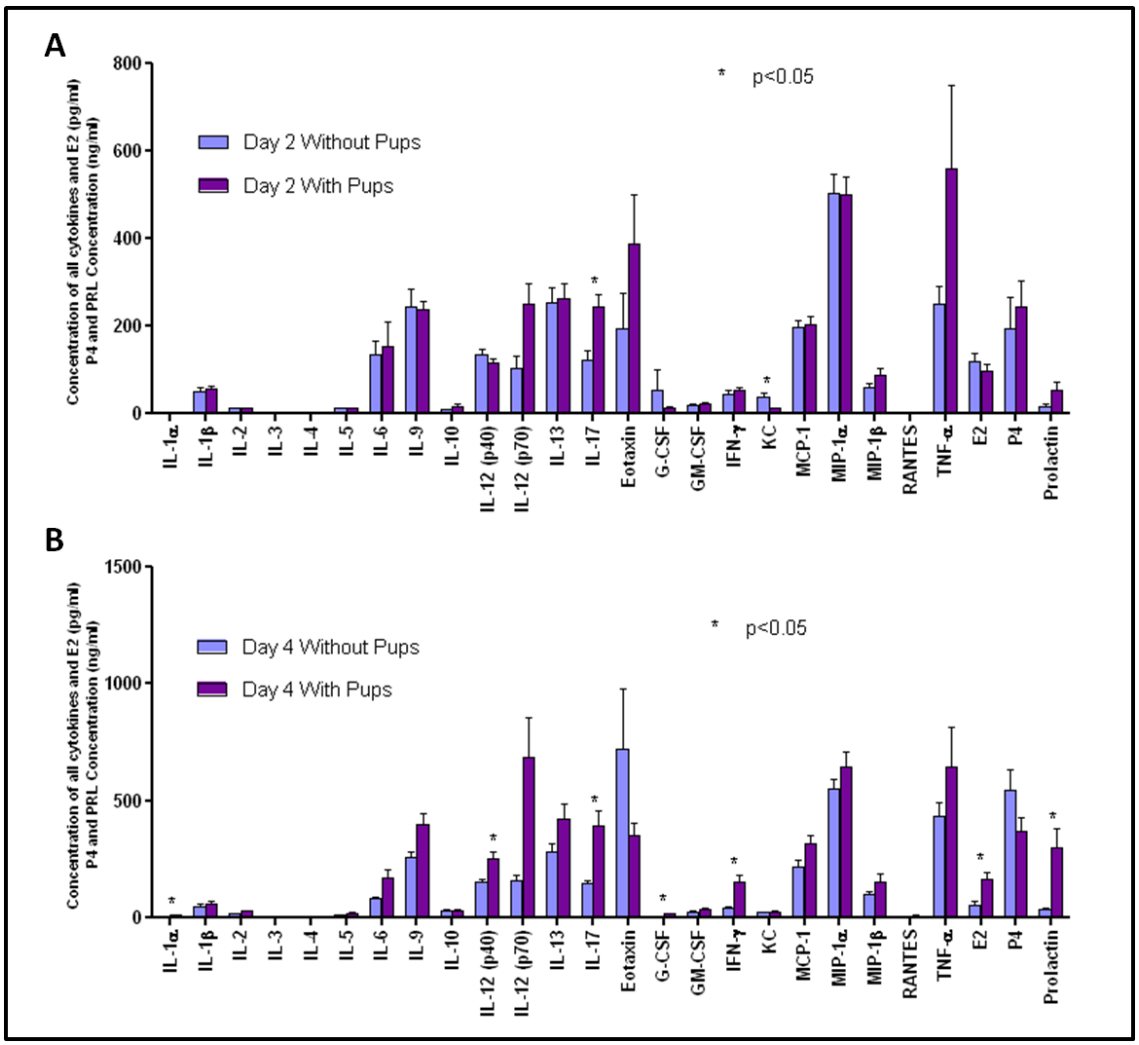


Figure 3-17: Graphs depicting the change in cytokine and hormone concentration following pup removal. Panel A shows cytokine profiles at day 2 with/without pups, while panel B shows cytokine profiles at day 4 with/without pups.

3.4.6.2 Bayesian network generated from pup free dams

The Bayesian network generated from pup free dams (Figure 3-18) retained the same core hubs as the lactation network (IFN-γ, IL-13, MCP-1, MIP-1α, MIP-1β and RANTES), IL-12 (p40) remained as the principal parent and TNF-α as the terminal node, while IL-10 became an additional parent. 42 edges (32 of high confidence) connected 23 of the 26 nodes with G-CSF and IL-6 as orphan nodes. PRL became a terminal node.

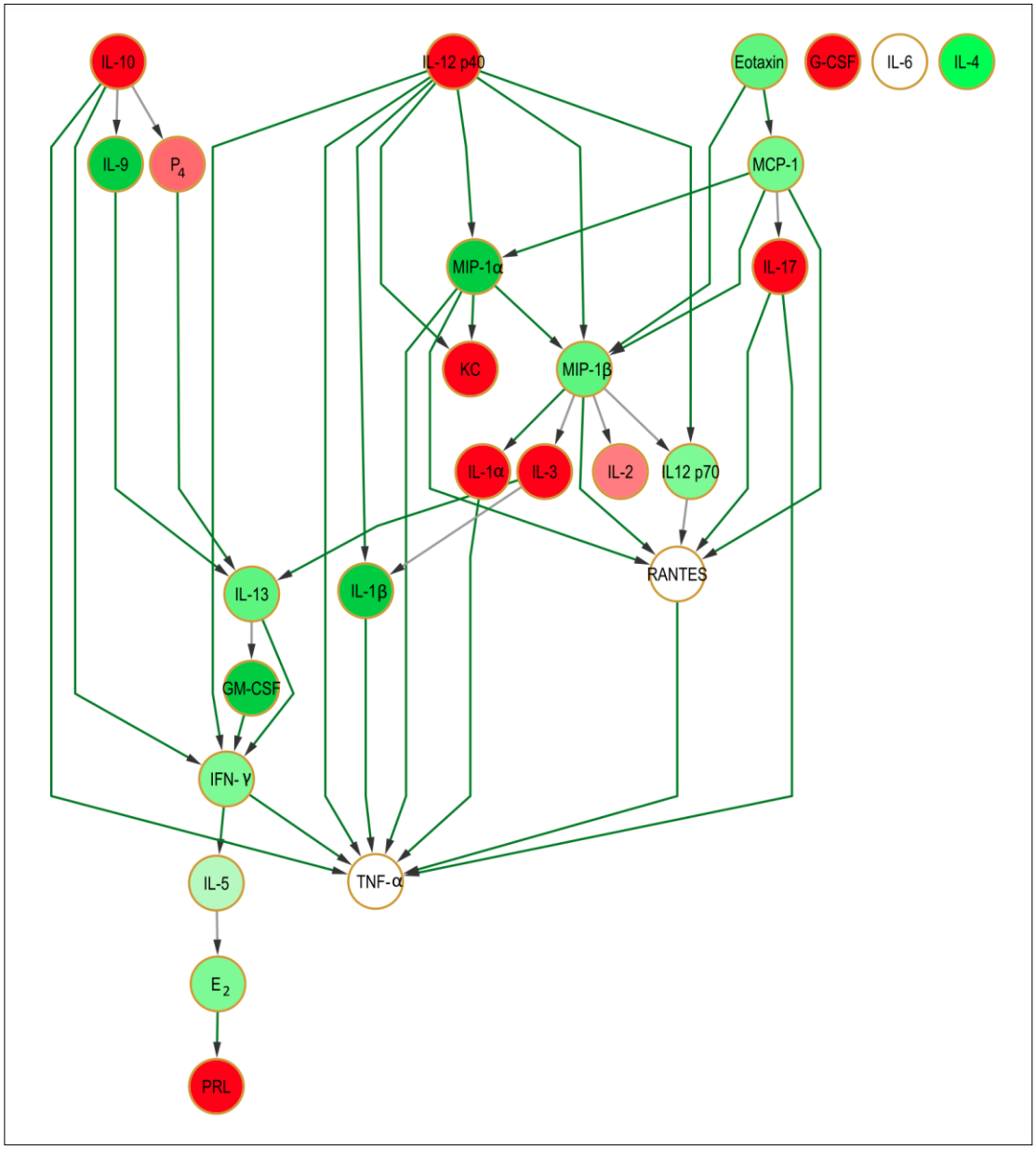


Figure 3-18: Bayesian network generated using data from pup free dams. Colour coding is as described for Figure 3-12.

3.4.6.3 Variational Bayesian state space modelling (VBSSM) of pup-free data

VBSSM modelling of the pup free data revealed a network of striking similarity to the pup-free Bayesian network. IL-10 and IL-12 (p40) featured as parent nodes, while PRL became a terminal node. When compared statistically, the networks demonstrated high specificity (high true negatives and low false positives), however sensitivity (high true positives and low false negatives) and the corresponding F-score were low. This led to a high level of confidence that the networks were structurally similar.

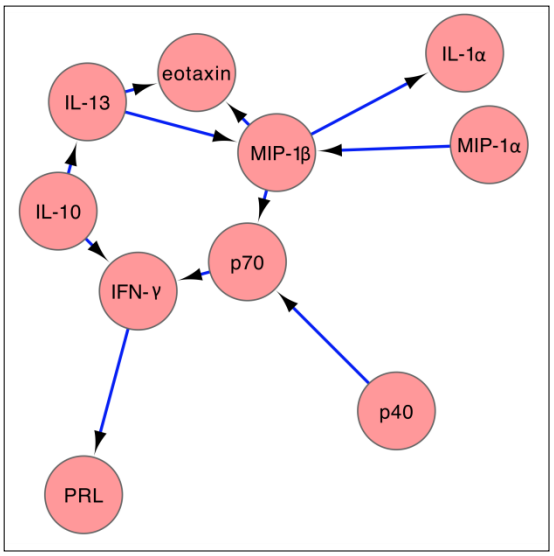


Figure 3-19: VBSSM model of pup-free data.

	VBSSM compared with seeded Bayesian Pup-free
Specificity	0.95
Sensitivity	0.14
F-Score	0.25

Table 3-6: Statistical comparison of VBSSM and Bayesian networks in pup-free scenario.

3.4.6.4 Comparison of the physiological Bayesian networks

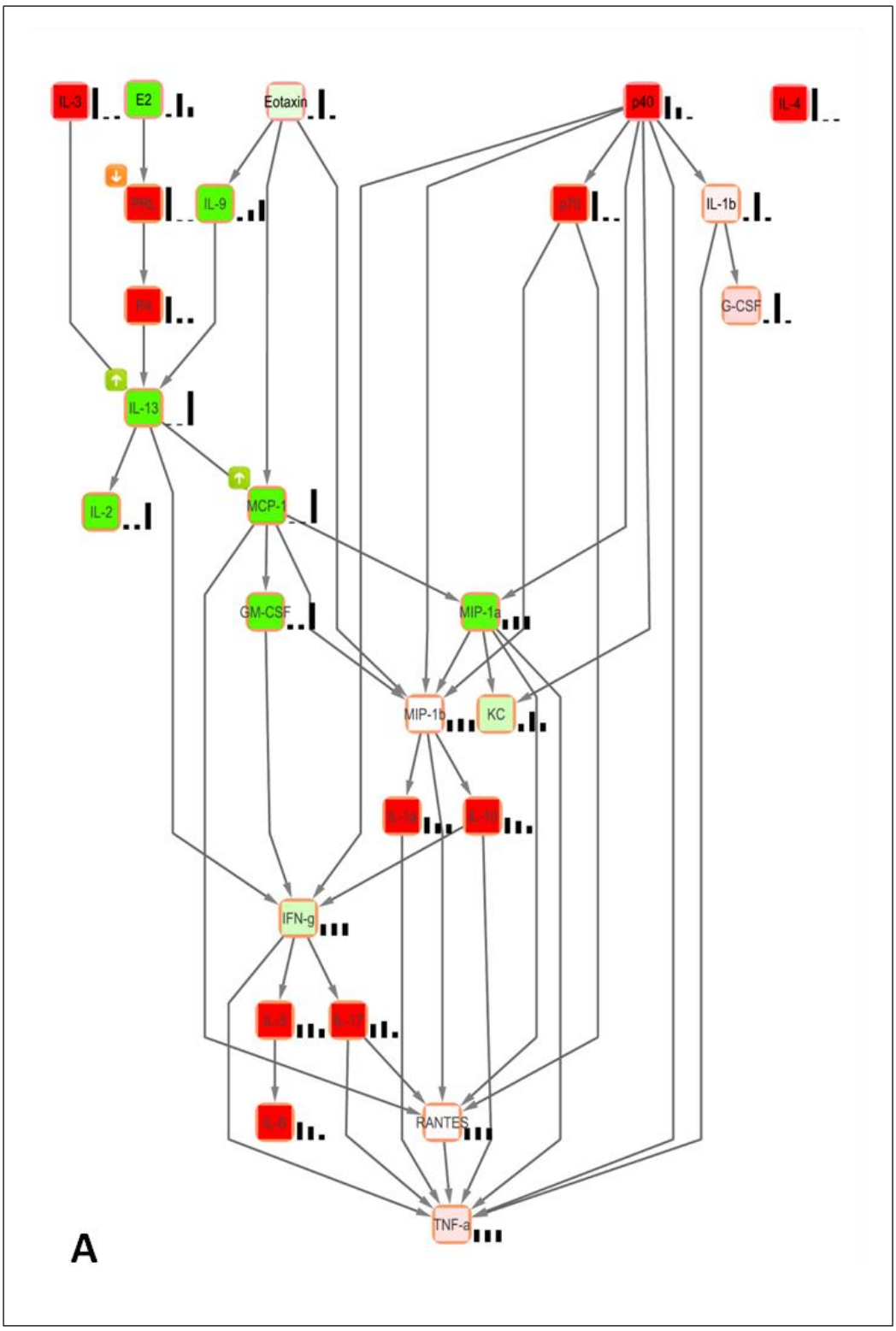
Structural comparisons of the two physiological networks (lactation and pup free) were performed to assess the closeness of fit. Despite being produced from two independent data sets, the two networks shared striking similarity, with an F-score of 0.861. The total complexities of the lactation and pup free dam networks were 379 and 375 respectively, indicating a close match despite some differences in topology. The most striking difference was that over half of the cytokines (GM-CSF, IFN-γ, IL-1β, IL-4, IL-5, IL-8, IL-12 (p70), IL-13, MCP-1, MIP-1α, MIP-1β, RANTES and TNF-α) moved to higher concentration bins in the pup free dam setting.

3.4.6.5 Comparison of predicted (in silico) and monitored (in vivo) effects

The node status shown in the pup free dam network closely resembles the cytokine concentration predicted by combined PRL/IL-13/MCP-1 perturbation, despite

differences in network topology (Figure 3-20). This perturbation correctly predicted the nodal status of IL-1α, IL-10, RANTES, TNF-α, IFN-γ, and GM-CSF. In the case of IL-5, IL-6, IL-17, MIP-1α and MIP-1β close categorisation was noted.

MIP-1α, MIP-1β and IFN-γ featured as common children of IL-12 (p40) and MCP-1 in the lactation network. Combined perturbation of IL-12 (P40) and MCP-1 led to correct prediction of all three. Therefore, perturbation of key cytokines within the lactation network *in silico* predicted the vast majority of cytokine concentration ranges within the pup-free network *in vivo*. Only KC could not be correctly predicted via *in silico* perturbation.


IL-3
E2
Eotaxin
p40
IL-4
IL-9
IL-1b
G-CSF
IL-13
IL-2
MCP-1
GM-CSF
MIP-1a
MIP-1b
KC
IFN-g
RANTES
TNF-a
A

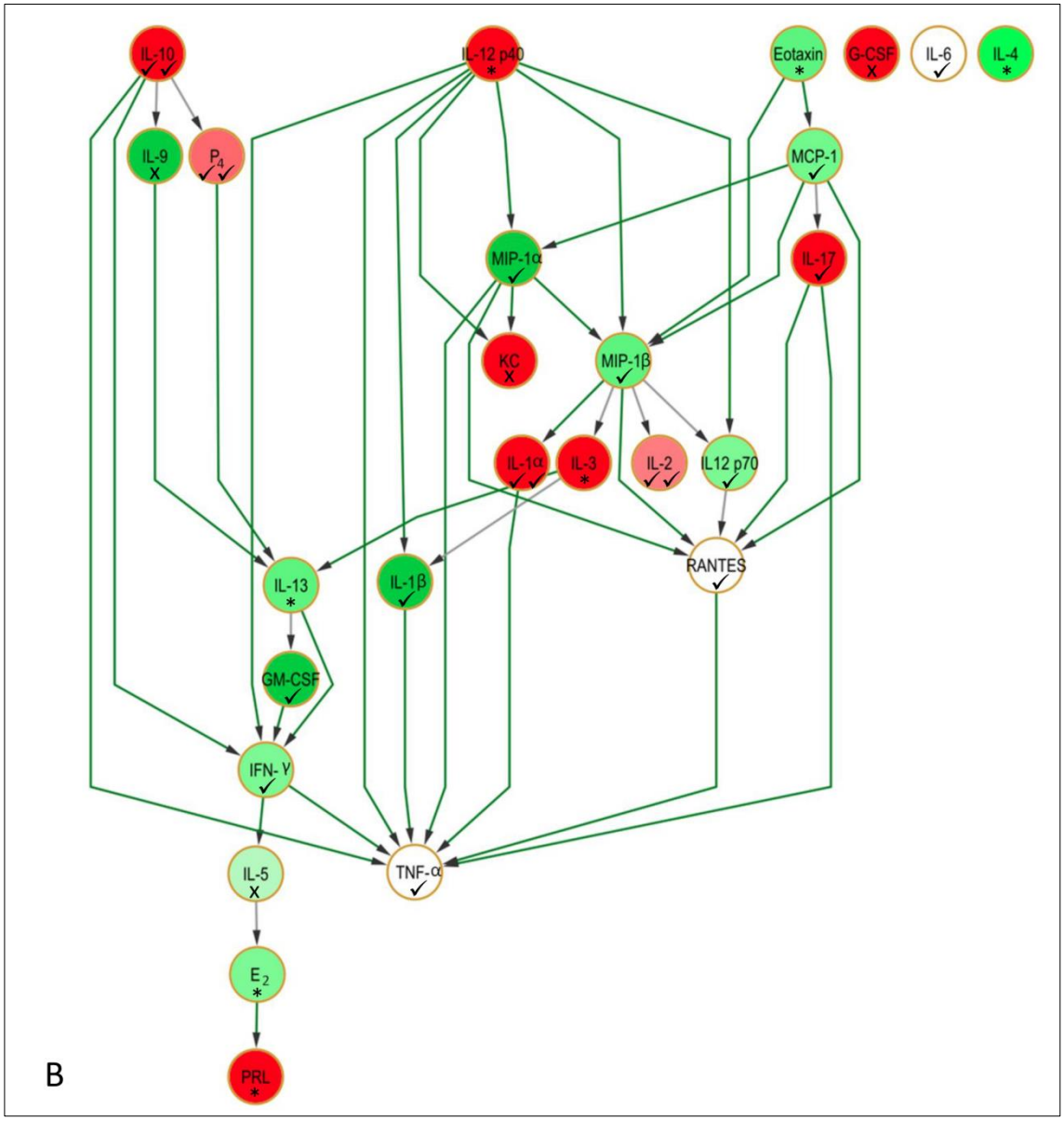


Figure 3-20: Comparison of *in silico* and *in vivo* perturbations. Panel A depicts the lactation network with IL-13, MCP-1 and PRL perturbation. Panel B depicts the pup-free *in vivo* network. For panel B, symbols indicate if the nodal concentration bin was correctly categorized by *in silico* perturbation. ✓✓ - Correctly/closely categorized by PRL perturbation alone. ✓ - Correctly/closely categorized by PRL perturbation in combination with IL-12 (p40), IL-13 and MCP-1, or by any other single mediator perturbation. X – Not correctly categorized by any perturbation. * - Could not be evaluated due to being upstream of the perturbations performed.

3.5 Discussion

3.5.1 Bayesian network analysis is a robust method

The networks generated were statistically robust. Despite changing the search algorithm and scoring metric with which the network was learned from BDe to a Euclidean algorithm, the networks generated proved to be consistent even with bootstrap confidence set to 0.9 (i.e. displayed features occurring in ≥90% of iterations). In comparison to other studies, this level of confidence is particularly high, as 50-70% is typically used [399]. Edges between nodes were also consistently of high confidence. This robustness was further demonstrated by the fact that VBSSM networks (i.e. those which did not take prior knowledge into account in their construction), despite comprising fewer nodes, revealed core nodes and an overall structure similar to those obtained from the seeded models.

3.5.2 Key cytokine identification using Bayesian networks

The lactation Bayesian network identified principal parents (IL-3, IL-12 (p40) and eotaxin), core hubs (IFN-γ, IL-13, MCP-1, MIP-1α, MIP-1β and RANTES) and TNF-α as a terminal node. These core cytokines were also evident in the pup-free dam network which was generated from an independent data set, indicating that they may form an integral network common to the lactating and non-lactating systemic environment. Several of these nodal relationships were preserved when the networks were generated through a VBSSM approach which does not take into account prior knowledge. These observations suggest that these relationships are robust and are likely reflect the physiological environment.

Perhaps the most unexpected result was that PRL did not feature either as a parent or a core hub, despite evidence accrued since the early 1980s which portrays PRL as a critical immunomodulator [476, 493-495]. For example, hypophysectomised rats exhibit restored immune cell function upon administration of PRL [496, 497], and enhances mitogenesis in T and B cells [498]. However, these immunomodulatory properties may not be fully cytokine-mediated. The cluster analysis in the present study revealed that IL-9 was the only cytokine clustering with the hormones investigated. IL-9 is a pleiotropic cytokine, produced by $CD4^+$ T helper cells [499], and demonstrates pro-inflammatory actions in murine models of inflammation [500, 501]. Although there are no documented links between PRL and IL-9, it is feasible that this cytokine in part mediates the immunomodulatory properties of PRL in inflammation. Within the lactation network, IL-9 and PRL did not feature a direct link, but they were connected via a

downstream node, IL-13, which is suggestive of an indirect relationship between these mediators, and may merit further investigation.

The observation that many cytokine profiles peaked at a later time point post-partum than PRL, and the fact that the latter was connected to the lactation network by a low confidence edge via P_4, suggested that this hormone may have a more peripheral role to play in modulating cytokine networks. Nevertheless, perturbation of PRL did influence some downstream cytokines including IL-2 and IL-13, indicating that although this hormone is not a key driver of the cytokine networks in lactation it does influence the networks at a lower level.

3.5.3 Bayesian network portrayal of established and novel cytokine/hormone relationships

The highest cytokine concentrations generally coincided with maximal suckling/milk production, and their subsequent decrease followed the reduction in suckling/milk yield as lactation neared completion. This trend is supported in the literature, where mammary gland tissue expands and differentiates towards maximal suckling, followed by a sharp decline as lactation draws to an end [502, 503]. However, although cytokine profiles during murine pregnancy are believed to be regulated by the coordinated activities of PRL, E_2 and P_4 [504], the absence of correlation between cytokines and these hormones highlighted in the heatmap suggests that hormones do not have as major a role in the regulation of cytokine profiles during lactation as anticipated. The failure of PRL perturbation to affect global Bayesian network cytokine profiles suggests that serum cytokine profiles may be governed through other lactation-related agents.

In this respect, other mediators associated with suckling, such as oxytocin, may be involved with the regulation of cytokine networks [505-507]. Oxytocin exhibits cytokine-like properties, including mimicking IL-2 activity in T-cell induction of IFN-γ [508], mediation of IL-1β induction of adrenocorticotrophic hormone [509], and modulation of immune responsiveness [510-512]. Oxytocin also contributes to lactational amenorrhoea in humans [83, 513], potentially via IFN-γ producing T-cells [514], supporting the notion that oxytocin may affect ovarian function through cytokine network alterations. Sample volume restricted the ability to assess the role of oxytocin within this model system, although it remains a potential investigative target for the future.

The lactation Bayesian network constructed highlighted the presence of IL-12 (p40) as a parent to one of the main branches. IL-12 (p40) acts through IL-12 (p70) to modulate MIP-1β, a relationship which has been well documented, whilst also antagonising IL-12 (p70) heterodimer activity as part of a self-regulating cytokine response [515-518]. Although this relationship was not particularly evident in the present Bayesian networks, there were high confidence edges between all these mediators in the pup-free network. In turn, IL-12 (p70) has been shown to modulate RANTES expression through the induction of MIP-1β [519], as illustrated in the present Bayesian network by strong edges between IL-12 (p70), MIP-1β and RANTES. The interactions between IL-12 (p40) and (p70), MIP-1β and RANTES induce the differentiation and recruitment of T cells and other leukocytes [520]. Given that T cells account for a significant proportion of the cellular composition of breast milk, it is unsurprising to note that this system is activated during lactation [521].

IL-13 appears to have a specific role in integrating the influence of all three hormones measured, as well as eotaxin (via IL-9) and IL-3. The markedly divergent hormonal (E_2, P_4 and PRL) and IL-13 profiles suggest that elevated levels of the former may contribute to IL-13 underexpression. E_2 and IL-13 have been shown to have opposing effects on macrophages, downregulating and upregulating MHC II molecule expression, respectively [522]. Furthermore, PRL induces PRL-induced protein (PIP), which is co-stimulated by IL-13 and its close relative, IL-4 [523, 524]. Through PIP, IL-13 has been shown to inhibit E_2-induced biological effects, as highlighted by *in vitro* studies on breast cancer cell proliferation [523]. In humans, E_2 and P_4 induce IL-13 in peripheral blood cells, indicating that regulation of IL-13 profile may be species-specific and/or relate to physiological context (and thus the balance of regulatory mediators governing its expression) [525]. Interestingly, the 'traditional' role of IL-13 in inhibiting inflammatory cytokine production is not supported by the present data, although this may simply reflect its broader range of physiological functions which are increasingly being recognised [526-528].

Both IFN-γ and GM-CSF appeared to be key molecules in the cytokine network involved in lactation. Despite the fact that cytokine concentrations are relatively high at the end of pregnancy, most cytokine concentrations during lactation were similar to those of cycling mice suggesting a relatively rapid correction of high peripartum levels. GM-CSF concentrations, however, were higher and akin to those reported in mid-pregnancy [101]. GM-CSF is embryoprotective, potentially supporting the viability of diapausing embryos, a common reproductive strategy in rodents [529, 530]. By

contrast, high systemic IFN-γ levels have been shown to be detrimental to embryo survival in mice [531]. They have also been linked with recurrent miscarriage in women [532], although conversely IFN-γ has been shown to contribute to the maintenance of early gestation in mice [533, 534]. Within the current networks, IFN-γ appeared to be antagonised by IL-10, a finding reflected in the literature [535, 536].

Eotaxin is reported to antagonise MCP-1, as indicated by its action resulting in a decreased response of monocytes to MCP-1 signalling [537]. This relationship is upheld by the present network, wherein moderately elevated eotaxin is connected by a high confidence edge to low MCP-1. MCP-1 and GM-CSF (linked by a low confidence edge in the lactation network) both recruit macrophages to sites of inflammation, and correlate with IL-6, TNF-α and IL-1β [538]. This relationship is in part supported by the present data, which highlight a strong positive correlation between IL-6 and TNF-α. High confidence edges between MCP-1 and MIP-1α/RANTES are also noted in both of the present principal networks, which may reflect the role of these cytokines in recruiting T cells, as demonstrated in both humans and murine models [539-543]. Thus, many of the nodal interrelationships highlighted by this study have been independently reported within the literature, lending credence to the biological relevance of these Bayesian networks. Although this begs the question as to whether the incorporation of prior networks in terms of the seeding approach introduces bias towards the discovery of nodal interrelationships, the presence of many of these relationships in the unseeded VBSSM model indicates that seeding did not bias the present results unduly. As outlined, the aim of seeding was to enrich the data with a view to providing a more complete network with which to identify key mediators.

3.5.4 Identification of synergy, antagonism and functional redundancy

3.5.4.1 Synergy

Despite a previous report that IL-13 is an inducer of MCP-1 [544], the synergistic effect of IL-13 and MCP-1 on IFN-γ highlighted during this study is previously unidentified. When IL-13 and MCP-1 were perturbed together, IFN-γ exhibited a greater increase in concentration compared to individual perturbations. Interestingly, IFN-γ has been shown to influence both IL-13 and MCP-1, a situation not reflected in the present network. IFN-γ modulates of IL-13 responses via IL-13Rα2 in human primary cells and cell lines [545], and induces MCP-1 in human astrocytoma cells [546, 547]. However, these relationships may reflect a feedback loop in response to the synergistic relationship, a feature which cannot be demonstrated within a Bayesian network.

Further previously unidentified synergistic relationships were noted through *in silico* perturbation between IL-12 (p40) and MCP-1 (synergising to boost MIP-1α concentrations), and IL-12 (p40) and IL-13 (synergising to raise IFN-γ concentrations). Although many studies report that these cytokines co-exist in biological fluids, there are no studies exploring the direct relationships between them. However, IL-12 has been shown to synergise with mycobacteria in a murine model of inflammation to drastically increase IFN-γ production by macrophages [548], highlighting that synergy identified within the present networks my involve intermediary processes.

3.5.4.2 Antagonism

Within the Bayesian networks generated, PRL and IL-3 were identified as having opposing/modulatory effects on IL-13, and when perturbed together, resulted in intermediate effects on concentration compared to individual perturbations. PRL and IL-3 both induce the STAT5 signalling pathway, which is evident in the lactating mouse [549, 550]. This may provide a mechanism for antagonism if there is competition for the signalling pathway and the cytokines providing the initial signal exhibit opposing effects despite utilising the same signalling pathway. However, the nature of the antagonistic effect of PRL and IL-3 on IL-13 is puzzling, as signalling by both of these cytokines via STAT5 results in proliferation and differentiation of cells in numerous systems [551-559]. These highlighted antagonistic relationships merit further investigation to identify if they are direct or mediated by cytokines not included in the present study, beyond which mechanisms can be derived.

3.5.4.3 Functional redundancy

In both the Bayesian networks generated, IL-4 remained peripheral and unconnected to any other node. Despite this, IL-4 tracked IL-13 levels (as demonstrated by the cluster analysis), a feature which may be indicative of the functional redundancy in the system given that IL-4 and IL-13 are known to operate through the same receptor [91, 560, 561]. Functional redundancy between these two cytokines is widely reported in the literature [358, 562], and the fact that this relationship was highlighted in the Bayesian network is indicative of its relevance to the physiological situation.

3.5.5 In vivo *perturbation of PRL*

Pup removal at birth was associated with a failure in the circulatory concentrations of all the cytokines studied to rise as seen in lactating dams which, while evident by day

2, was most marked by day 4. The exception to this global inflammatory depression was eotaxin, whose levels actually increased in the first 4 days post partum. The reasons for this are unclear, although this may reflect a process of eosinophil recruitment to the uterus and/or mammary glands [4, 563]. The overall depression in cytokine levels in pup-free dams was largely independent of PRL concentrations, which failed to rise due to the absence of suckling stimulus, again suggesting an independent regulation of PRL and cytokines. Interestingly, even by day 4 post partum, cytokine levels had not entirely returned to the pre-gestational levels of the naturally cycling group, suggesting that cytokine profiles are to some degree maintained post partum, independent of whether dams are nursing. The reasons for this are unclear, and may possibly reflect ongoing uterine involution in conjunction with oxytocin and prostaglandin (PG)$F_{2\alpha}$ [564-566]. Although embryos ensuing from post partum oestrus matings would risk developing in an environment where cytokine levels are higher than those encountered in virgin animals, a combination of immunological compartmentalisation of the intrauterine environment and embryo diapause may contribute to overcoming this problem [567]. Dams that had their pups removed post partum had lower levels of abortifacient mediators such as IFN-γ [101], in line with a notion of return to cyclicity (as in the present case) or preparation for implantation of diapaused embryos (as would occur in the wild/presence of a stud male).

3.5.6 In silico *perturbation and prediction of physiological changes*

In silico perturbation is a valuable tool for exploring cytokine interactions as shown above. However, it also has the potential to predict responses to altered physiological scenarios. Despite the original premise that PRL would feature as a key regulator of cytokine networks, *in silico* perturbation of this hormone (to reflect a non-suckling environment) resulted in correct categorisation of only three nodes (IL-1α, IL-2 and IL-10). More detailed perturbation of other nodes in combination with PRL was required in order to correctly categorise the majority of nodes to the concentration bins seen in the pup-free network. Allocation of the driver nodes IL-13 and MCP-1 to high concentration bins *in silico* correctly (or closely) predicted the concentration status of 13 out of their 14 downstream cytokines *in vivo* when pups were removed. MIP-1α and MIP-1β, whose conditional probabilities were less accurately predicted by IL-13 and MCP-1 perturbation, were correctly categorised when their direct driver node parents (IL-12 (p40) and MCP-1) were perturbed. This shows a high level of predictive capability by *in silico* perturbation which, in future, may reduce the need for experimental validation, with early and less costly identification of research targets. The only cytokine which

could not be correctly categorised by *in silico* perturbation was the peripheral terminal cytokine KC. This could be explained by the fact that KC may be modulated by other mediators not included in this study and therefore not featured in the presented network, or as a potential artefact of Bayesian network analysis.

Although the cytokine concentration status between the lactation and pup removed networks may have differed markedly, the essential six network core hubs around which both networks assembled were conserved, suggesting that these structural elements form an integral part of cytokine network regulation. Much of this structure was retained when the analytical process was repeated using a VBSSM approach, indicating that the hierarchical structure established in the standard Bayesian model was not due to introduced bias in the seeding process. The identification of core hubs infers the possibility of identifying lynchpins within a given physiological scenario which may be relevant to the identification of therapeutic targets.

3.5.7 Study limitations and future directions

Bayesian networks have proved useful in revealing the structure of cytokine and hormone networks, and in describing the likely causal relationships between mediators. However, bringing order to these causal inferences comes at a price. For example, the networks generated within this study present TNF-α as the terminal node, despite studies indicating that, for example, TNF-α can induce MIP-1β expression in order to increase the number of activated leukocytes present in milk [568-570]. Thus, the biological interpretation of these relationships must be made with the *caveat* that Bayesian networks are necessarily incomplete by virtue of the fact that that they cannot possibly represent all possible edges between nodes as the methodology precludes the portrayal of structural loops [571]. As such, they are also unable to account for mediators which have autoregulatory function on their own production, either in isolation or through the intermediary of other signalling molecules. Furthermore, although this study was based on the broadest commercially available cytokine panel available for multiplex immunoassay, the resultant networks will inevitably have failed to include all possible mediators. However, despite these considerations, Bayesian networks represent a significant step forward in terms of defining the nature of cytokine and hormone interactions when compared to the traditional and increasingly outmoded practice of defining cytokine interactions according to the Th1:Th2 dichotomy. The present methodology also precluded the incorporation of the influence of ‘hidden’ Markov blankets, unmeasured nodes which could change the topology of the networks

discovered. This could be achieved by using a VBSSM approach with modifications. VBSSM incorporates the advantage of taking into account hidden nodes, but traditionally this algorithm does not incorporate a prior network. Development of such a method forms the basis of the networks in Chapter 4.

Key areas for future development include exploring whether the generic network structure (i.e. the nature/composition of core hubs) and the previously unreported synergistic/antagonistic cytokine interactions revealed as part of this study, are conserved across multiple physiological processes and/or species. This is particularly valuable in light of the fact that the present networks have been studied *in vivo*, in contrast to most modelling endeavours which are typically performed on cell lines *in vitro*. While *in vitro* approaches may provide a wealth of information on the functional regulation of one or more cell types under highly controlled conditions, they commonly fall short of presenting a realistic depiction of physiological events *in vivo*. The 'top down' method developed herein will also prove useful in investigating immune privileged organs (e.g. the eye, testes and conceptus), solid tumours, specific body compartments (e.g. ovarian follicles and uterus), although it can just as readily be applied to tissue explants, organ cultures and cell co-/monocultures. Furthermore, focussing on protein levels rather than differential gene expression profiles (as most other studies) offers the added benefit of overcoming the inferential interpretation problems ensuing from the fact that many signalling proteins such as cytokines are known to be extensively regulated at the post-transcriptional level as previously discussed [91]. Since the interactions identified within this chapter have been identified *in silico*, *in vivo* validation is still required as a gold standard validation in order to be confident that these are both real and biologically meaningful. However, the application of Bayesian networks to immunological disorders could identify those cytokines which orchestrate the imbalance which creates tissue destruction, enabling targeting of therapies. Current work is undertaking the application of these networks to human endometrial cancer in order to identify novel molecular mediators.

In terms of this thesis, the next section will apply the Bayesian network methodology developed herein to the physiological environment of human oocyte maturation. This approach will aim to identify candidate regulators of oocyte maturation, alongside a 'fingerprint' of cytokines which identify the oocytes most likely to fertilise, developing the Bayesian methodology to include classifier analysis.

4. Modelling follicular fluid cytokines in relation to oocyte maturation

4.1 Introduction

4.1.1 Overview of folliculogenesis

In order to appreciate the nature of the impact of assisted conception treatment regimes on oocyte maturation and viability, it is first necessary to understand the development of the oocyte in relation to the physiological process of folliculogenesis. For the purposes of this Chapter, follicular development and oocyte maturation will be considered from a human perspective, with inferences drawn from animal models where species-specific data are wanting. Follicular development in humans is essential for the ovary to fulfil its two main functions: (i) to generate a single dominant follicle containing an oocyte with competence for fertilisation and development, and (ii) to secrete hormones required to prepare the reproductive tract for pregnancy. Follicle development up to the point ovulation is a complex series of events, including: primordial follicle recruitment; proliferation of granulosa and theca cells; oocyte maturation; and steroidogenesis, as detailed in the following sections.

The generation of mature, competent oocytes is a complex, dynamic process involving an intricate dialogue between the oocyte and its surrounding somatic cells. In humans, folliculogenesis involves recruitment of follicles from the primordial pool, growth of the follicle/development of a fluid filled antrum, and concurrent oocyte maturation, a process lasting approximately 290 days in humans [572]. Folliculogenesis occurs within the ovarian cortex, and is generally considered to be divided into two stages (Figure 4-1). The first, or ‘pre-antral’, stage is where the primordial follicle develops into a larger, non-cavitated follicle, with concurrent oocyte growth. This stage is governed by local mediators and is gonadotrophin-independent. The second, ‘antral’ stage features the development of a fluid-filled antrum and further growth which is follicle stimulating hormone (FSH)-dependent.

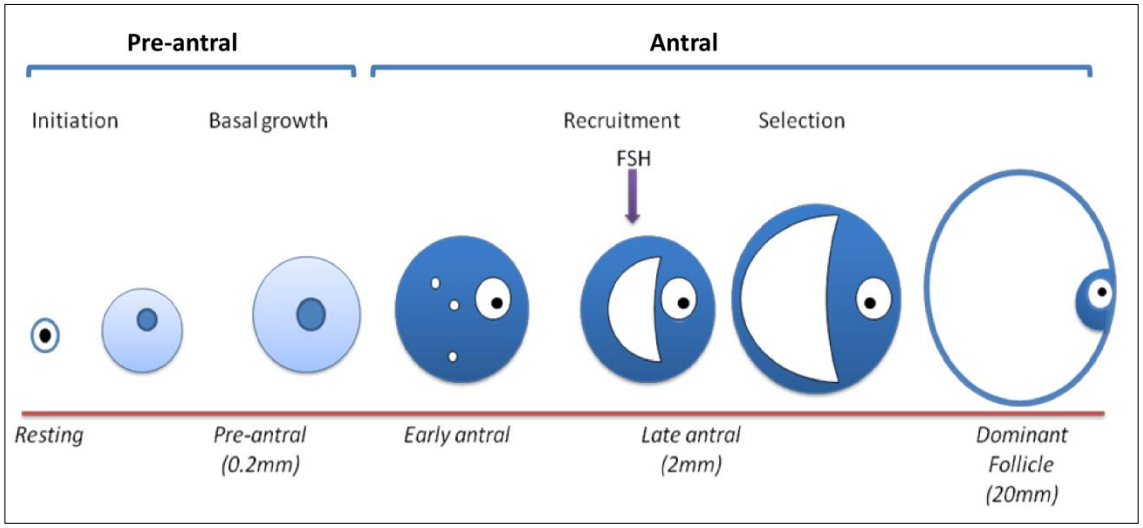


Figure 4-1: Development of the ovarian follicle from resting to the dominant preovulatory (Graafian) stage.

4.1.2 Pre-antral follicle development

4.1.2.1 The primordial follicle

The primordial follicle consists of a small (25μm) oocyte which is arrested in the dictyate stage of meiosis (prophase I), surrounded by a single layer of flattened/squamous pre-granulosa cells (GCs) and a basal lamina which creates a barrier between the follicle and other surrounding stromal cell types. Multiple activator and repressor signalling pathways are active in the primordial follicle in order to balance dormancy and activation. For example, neurotropin and nerve growth factor (NGF) are important in follicle activation [573], yet the phosphatidylinositol 3 kinase (PI3K) pathway acts as a repressor [574]. Primordial follicles do not have their own vascular supply such that the external influence from the endocrine system remains low [575-577]. Indeed, circulatory gonadotrophins do not influence recruitment as primordial follicles do not express follicle stimulating hormone (FSH) receptors [578]. Upon recruitment from the primordial pool, GC morphology changes from a squamous to cuboidal phenotype. Continuous recruitment of follicles in this manner continues throughout the first four decades of life, until the onset of menopause. Initially, the vast majority of follicles leave the resting pool due to atresia, with small numbers entering the growth phase [579]. After the age of 30, more follicles enter the growth phase, a process which accelerates from approximately 38 years of age, resulting in an overall loss of ovarian reserve [579, 580]. This decline in ovarian reserve is indicated by falling anti-Müllerian hormone (AMH), a mediator produced by small growing follicles which

inhibits the recruitment of primordial follicles, such that lower AMH levels are indicative of a reduced ovarian reserve [581].

4.1.2.2 The primary follicle

The primary follicle consists of a layer of proliferating cuboidal GCs surrounding the oocyte. At this stage, growth remains gonadotrophin-independent as follicle development progresses in the absence of FSH [582] and FSH receptors [583]. The oocyte continues its growth, increasing in size to 120 µm, and develops a glycoproteinaceous zona pellucida (ZP) which separates it from the GC layer. At this stage, transcriptional activation within the oocyte facilitates communication with surrounding somatic cells and allows it to support its own growth [572, 584, 585]. The development of an intimate paracellular dialogue between the oocyte and GCs via mediators such as cytokines facilitates follicle growth and oocyte development [586, 587]. Moreover, cell-cell contacts are established between the oocyte and GC cells via cytoplasmic projections and microvilli in order to allow diffusion of ions, metabolites and signalling molecules [588, 589]. Once the follicle has attained a layer of GCs approximately 4 cells thick, FSH receptors are expressed on their surface in preparation for the gonadotrophin responsiveness of the antral phase [578].

4.1.2.3 The secondary follicle

Secondary follicles acquire a layer of theca cells (TC) which surround the oocyte and GC, which subsequently differentiates into two primary layers: the theca interna (closest to the GCs), which form interstitial cells; and the theca externa, which forms a smooth muscle cell-like layer that facilitates ovulation [590]. Alongside TC development, perifollicular blood flow is improved by neoangiogenesis, while GCs proliferate [591]. The oocyte completes its growth during the pre-antral stage, but does not resume meiosis. This is potentially due to the inhibitory effects of oocyte- and GC-derived cyclic nucleotides, since a decrease in the concentration of cAMP in the oocyte is associated with resumed meiosis [592, 593].

4.1.3 Antral follicle development

4.1.3.1 The antral follicle

The antral follicle is characterised by a cavity (antrum) which contains follicular fluid (FF). FF is an ultrafiltrate, containing both free and protein-bound steroid hormones,

plasma and locally derived proteins, proteoglycans and electrolytes [594, 595]. A recent review of the theories behind the formation of follicular fluid postulates that the formation of hyaluronan and chondroitin sulphate proteoglycan by granulosa cells provides the osmotic gradient required to draw fluid from surrounding extracellular matrix and, in turn, local blood capillaries [596]. This may involve remodelling of cell-cell junctions in the stromal and thecal layers, although little is known about the process. Initially, small pockets of fluid form within the GC layer, eventually coalescing to form a single antrum. Antral follicles are heterogeneous in size, ranging between 0.4 - 25 mm in diameter, which is largely determined by the volume of FF within the antrum (0.2 - 7ml) [597, 598]. In parallel with antral growth, GCs and TCs proliferate extensively, with the former differentiating into distinct phenotypes: the membrana, the periantral area and the cumulus oophorus. The cumulus oophorus extends to surround the oocyte within the antrum (Figure 4-2).

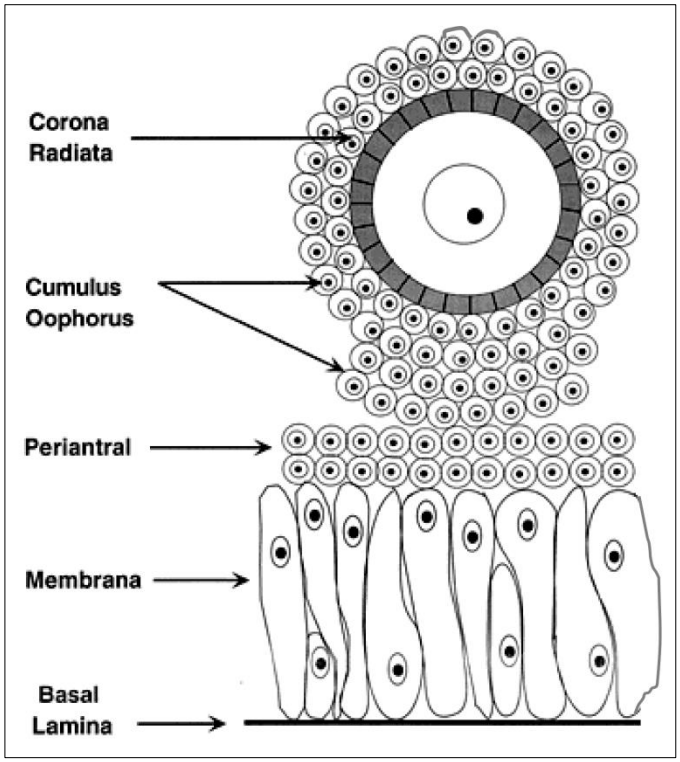


Figure 4-2: Structure of granulosa cells within an antral follicle. Adapted from Erickson (2004) [599]

Within antral follicles, the oocyte resumes meiosis in response to the pre-ovulatory LH surge, progressing to metaphase I and arresting at this point [600]. LH binds to receptors on GC cells, reversing the inhibition of meiosis by cAMP and cGMP [592,

593], and initiating a cascade of communication between the oocyte and the surrounding granulosa to induce meiotic maturation [601, 602]. The antral phase also is characterised by the presence of a large number of atretic follicles, wherein the formation of FF and proliferation of GCs is vastly reduced or non-existent.

Throughout the antral phase, follicles are recruited for growth in subsequent cycles. Recruitment can be considered as the rescue of a cohort of antral follicles from atresia [603], and it is accepted that follicles reaching 2-5mm in diameter are recruited for further growth at the end of each luteal phase. It has been estimated that the cohort of recruited follicles is 3-11 follicles per ovary [603], from a potential pool of 6-46 antral follicles per ovary [604]. From this pool of recruited follicles a single follicle is selected for preferential growth and subsequent ovulation, while the others undergo atresia.

4.1.3.2 Dominant follicle selection

Following the decrease in E_2 at the end of the luteal phase, there is a second rise in FSH. This marks the selection of a dominant follicle from the available pool of antral follicles for development in the follicular phase [572], with the mechanism of selection relying on the follicle's sensitivity to FSH [605]. The selected dominant follicle then grows rapidly in contrast to its remaining cohort counterparts; this growth can typically be monitored by ultrasound [606]. As many as 99.9% of all available follicles in the ovary are destined to undergo atresia [607], a process is thought to be initiated by GC apoptosis that is controlled by a delicate balance between pro- and anti-apoptotic mediators [608]. Growth factors and gonadotrophins support the dominant follicle through to ovulation. Concurrent with this stage of follicle development, the oocyte completes meiosis I and progresses to meiosis II, where it remains arrested until fertilisation.

4.1.4 Ovulation

The process of folliculogenesis culminates in the ovulation of the cumulus-oocyte complex (COC) from the dominant follicle, making the mature oocyte available for fertilisation. Ovulation involves active remodelling of the extracellular matrix in order to facilitate follicle rupture prior to luteinisation, alterations to follicular microcirculation, formation of an avascular area at the follicular apex and extrusion of the oocyte-cumulus complex [609]. This process is considered to be an inflammatory, cytokine-mediated event associated with well-recognised histological markers such as an influx of leukocytes, which are believed to be derived from the spleen [16, 610-612]. These

leukocytes (comprising lymphocytes, granulocytes and macrophages) favour ovulation through their secretion of an array of proteases and vasoactive agents [2, 610]. Macrophages perform a number of functions in ovulation, including the secretion of MMPs to mediate extracellular matrix remodelling [112, 613] and the phagocytosis of GCs from atretic follicles [25, 614, 615]. Lymphocytes also contribute to follicular function through their selective production of an array of cytokines [16].

4.1.5 The corpus luteum

The follicular life cycle is completed by luteinisation, resulting in a highly vascularised corpus luteum (CL) which serves to support the resulting pregnancy. Latin for “yellow body”, the CL is a temporary endocrine structure producing high levels of P_4 and moderate levels of E_2 and inhibin. The E_2 component serves to inhibit GnRH production [616], thereby reducing FSH and LH production to halt further follicular growth and ovulation in preparation for an impending pregnancy, while P_4 helps establish and maintain a pregnancy. The formation, function and regression of the CL is mediated by the large resident and temporary populations of leukocytes, the composition of which changes according to the stage of CL development. In the initial formation stages, granulocytes such as neutrophils and eosinophils and various phenotypes of T cells are abundant, facilitating angiogenesis [617, 618]. Mid-cycle up to the point of luteal regression, an influx of macrophages dominates and the granulocyte population diminishes, these immune cells serving to modulate steriodogenesis within the CL [619-621]. In women, the CL regresses to become the corpus albicans approximately 14 day after ovulation in the absence of pregnancy

4.1.6 Oocyte maturation

4.1.6.1 Nuclear maturation

Concurrent with folliculogenesis, the oocyte undergoes meiotic and maturation (Figure 4-3). In contrast to spermatozoa, oocytes arrest at various points in the meiotic process, awaiting signals to progress to the next stage in development, under the control of maturation promoting factor (MPF). Meiotic maturation, stimulated by LH and mediated by MPF, has been defined as the progression of the oocyte from the dictyate phase of prophase I to the metaphase I stage of meiosis, and is accompanied by germinal vesicle/nuclear envelope breakdown [622], cytoskeletal rearrangement and spindle assembly [623, 624]. In humans, a second meiotic arrest occurs between metaphase I and metaphase II, with the metaphase II oocyte being capable of

fertilisation. Subsequent fertilisation initiates the transition from metaphase II to anaphase II, completing meiosis and initiating the first mitotic divisions in the embryo.

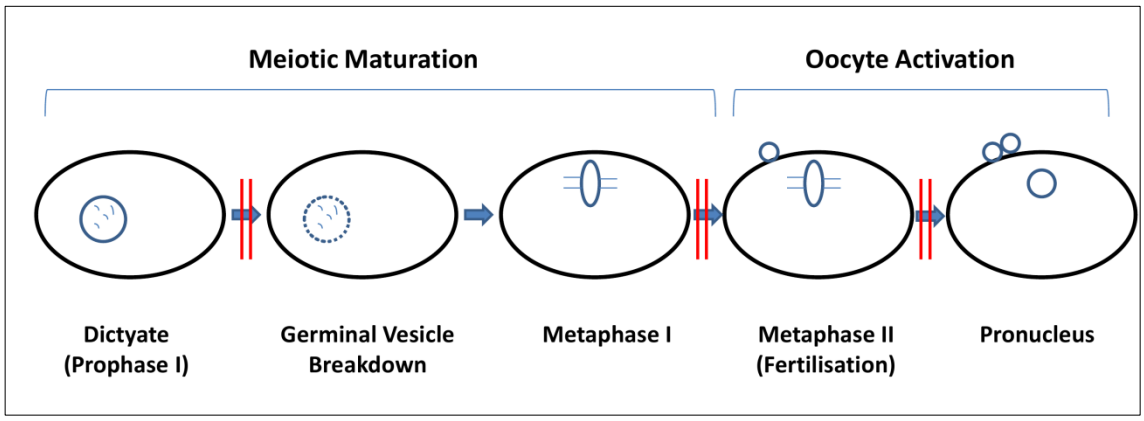


Figure 4-3: Nuclear maturation of the human oocyte. Red lines indicate points of arrest.

4.1.6.2 Cytoplasmic maturation

In general terms, oocyte cytoplasmic maturation comprises the accumulation of mRNA, proteins, substrates, and nutrients that are required to achieve oocyte developmental competence and successful embryo development [625], alongside redistribution of cytoplasmic organelles and cytoskeletal filaments [626-628]. Prior to germinal vesicle breakdown, the oocyte accumulates maternal mRNA through transcription in order to support the oocyte through the early stages of embryogenesis [629, 630]. This pauses until the maternal-zygotic transition, when a burst of transcription signals the activation of the embryonic genome [631].

Structural changes within the cytoplasm, such as the migration of mitochondria to areas of high energy consumption [632], alongside a peak in protein synthesis at the metaphase I stage [633] occur concurrently with but distinct from nuclear maturation. Relocation of cortical granules, uniquely present in an oocyte, from a diffuse cytoplasmic distribution at the germinal vesicle stage to primarily underlying the inner surface of the plasma membrane at metaphase II indicates that the oocyte is ready to ward off polyspermy via alterations to the oocyte ECM [634, 635]. However, much of this work has been completed in bovine oocytes which may not translate to the human situation. Human cytoplasmic maturation remains undefined, and reliable markers of this aspect are scarce.

4.1.7 Molecular regulators of folliculogenesis

Historically, it was thought that ovarian somatic cells such as GCs drove folliculogenesis, with the oocyte merely being a passenger and receiving signals to develop at the appropriate time. More recently, however, it has become apparent that the oocyte participates in a complex dialogue with its GC complement, adjacent theca/interstitial cells, as well as with other surrounding follicles [636]; the nature of this dialogue changes according to the stage of follicular development [637]. This interplay is essential not only for oocyte maturation, but also for follicular development, from the primordial to primary follicle transition, and subsequent growth and differentiation through to ovulation [587]. The nature of this dialogue influences the quality of the resultant oocyte, which impacts on subsequent embryo viability [638]. Mediators of this process include cytokines, hormones, growth factors, eicosanoids and MMPs, although many more agents are still probably unidentified [586, 636, 639-644]. These mediators are both intra- and inter-follicular, and are influenced by the steroidal milieu [645]. They may influence the oocyte either directly or indirectly via the intermediary of both cumulus and GCs [646]. For the purposes of this thesis, the mediators which will be focussed upon are cytokines.

4.1.8 The role of cytokines in folliculogenesis

The importance of cytokines in ovarian physiology is increasingly being recognised, although our understanding of their precise roles and interactions remains limited. Specific signalling pathways/paracrine dialogues have been identified for a number of these mediators [647], although much of the data are fragmented and relate to individual aspects of ovarian function. Cytokines such as interleukins and those of the TGF-β family participate in the intercellular communication between the oocyte and its surrounding somatic cells, as well as in the regulation of follicle survival and apoptosis [104, 648-653]. Ovarian immune effector cells such as macrophages and lymphocytes also secrete cytokines, including IFN-γ, TNF-α, G-CSF, GM-CSF, IL-1, IL-6, IL-8 and MCP-1, all of which have been implicated in oocyte development, ovulation and steroidogenesis [2, 16, 637, 654]. Cytokines within the ovarian follicle are for the most part locally produced, often at low concentrations where, once secreted, they diffuse to create chemotactic gradients or to act locally in a paracrine/autocrine manner. Cytokines have a short half-life, the implication of this being that even major local inflammatory perturbations are unlikely to result in marked systemic effects affecting circulatory profiles. In this regard, the physiological concentration of intrafollicular cytokines typically exceeds that of the circulation, although this is not always the case

with reproductive disorders such as endometriosis and pelvic inflammatory disease [149, 655-658]. A review of the known roles of cytokines in folliculogenesis will follow, with inferences drawn from animal models where human data are not available.

4.1.8.1 The primordial to primary follicle transition

Follicular recruitment from the primordial pool is driven by a complex interplay between the oocyte, pre-theca and pre-GCs, mostly mediated by cytokines and growth factors [584, 659]. Pre-granulosa cells express many cytokines, such as stem cell factor (SCF; kit ligand), basic fibroblast growth factor (bFGF, a.k.a. FGF-2) and LIF, which have been shown to promote the primordial to primary follicle transition *in vitro* in goats, hamsters and rodents [339, 660-665]. FGF-7 (produced by pre-theca cells and the oocyte) and LIF have been shown to upregulate pre-granulosa cell SCF expression and promote the transition from primordial to primary follicles in the mouse [660, 666]. However, species-specific differences are apparent: for example, SCF promotes recruitment from the primordial follicle pool in mice but not in rabbits [667-669].

Another group of cytokines, bone morphogenic proteins (BMPs), modulates gonadotrophin receptor expression, follicular development and steroid production [104, 650, 670]. BMPs utilise the SMAD signalling cascade [671], which leads to the activation and nuclear translocation of an array of transcriptional co-factors, subsequently mediating cellular responses to BMPs, such as the regulation of TGF-β expression [672, 673]. During folliculogenesis, regulation of this signalling cascade determines cell responsiveness to different BMPs, thereby providing a mechanism for recruitment of specific primordial follicles [673]. Rodent models suggest that pre-theca cell derived BMP-4 and BMP-7 promote primordial to primary follicle transition via stimulation of pre-granulosa cell proliferation, as demonstrated *in vitro* by a pre-mitotic increase in DNA synthesis [339, 674, 675]. In humans, both the primordial follicles and corpora lutea of the adult ovary express BMP-15 while foetal and peri-pubertal ovary primordial follicles do not, suggesting that it may participate in recruitment from the primordial pool [676, 677]. Moreover, mutations in the BMP-15 gene are associated with hypergonadotrophic ovarian failure in women, suggesting that this cytokine is essential for the progression of folliculogenesis [678].

Stromal cell-derived factor (SDF)-1α and its receptor (C-X-C chemokine receptor type 4; CXCR4) have been detected in immature oocytes and pre-granulosa of mouse neonatal ovaries [679]. *In vitro* exposure of these ovaries to SDF-1α results in an increase in follicular recruitment, although this occurs at the expense of follicular

diameter and follicular activation at the primary stage [679]. SDF-1α has a chemotactic function, as highlighted by its involvement in assisting primordial germ cell gonad colonisation [680]. In the mature ovary, SDF-1α is highly chemotactic for lymphocytes which produce cytokines, proteases and various vasoactive substances throughout folliculogenesis [353, 681-683].

Oocyte-derived growth differentiation factor (GDF)-9 is closely related to BMPs, and is critical for oocyte, granulosa and theca cell development [684-686]. Mice with *gdf-9* mutations exhibit a failure of folliculogenesis to progress beyond the primary follicle stage coupled with an incomplete development of the TC layer which renders them infertile [687, 688]. The early expression of GDF-9 appears to be consistent across all species studied to date, including humans [676, 689, 690]. Ovarian culture techniques employ GDF-9 to rescue human primordial follicles from atresia based on the evidence that GDF-9 is involved in early folliculogenesis [691, 692].

By contrast, AMH has been shown to inhibit primordial follicle growth [693, 694]. Primordial follicles are recruited at a higher rate in the absence of AMH in murine models, causing a premature depletion of the ovarian primordial follicle pool [694]. Human AMH levels fall with age, reaching undetectable levels by the menopause concurrent with a depletion of the primordial follicle population [695-697]. However, AMH receptors are expressed on primordial follicle pre-GCs, suggesting that growing follicles may use AMH as a signalling molecule to suppress resting primordial follicles [694, 698-700].

4.1.8.2 Primary to antral follicle development

Cytokines involved in regulating preantral follicle growth include GDF-9 and BMP-15 of oocyte origin, GC-derived activins, BMP-4 and BMP-7 of TC origin, and TGF-β produced by both theca and GCs [104, 339, 675, 691, 700-702]. Of these mediators, BMP-15 promotes GC proliferation in rat primary follicles in conjunction with granulosa-derived SCF [703]. These mediators are believed to govern GC expansion via a negative feedback loop, providing a mechanism for the oocyte to regulate its own follicular growth [701, 703]. Selection for further growth and development from the primary follicle pool is dependent upon GDF-9, which increases the number of medium-sized antral follicles in mice, especially in conjunction with VEGF [704-706]. These cytokines synergise when administered together, as highlighted by the fact that combined intra-ovarian injections of each cytokine cDNA results in more oocytes being ovulated [704]. GDF-9 also stimulates preantral follicle growth, promoting granulosa-

cumulus cell phenotypic transition and, suppressing GC P_4 production in mice, rats and cattle [686, 707-711]. These effects may be achieved through an upregulation of COX-2, hyaluronanan synthase (Has)-2 and steroidogenic acute regulatory protein (StAR) expression, which in turn induce/produce steriodogenic factors and prostaglandins [709, 712].

Neoangiogenesis accompanies theca development in secondary follicles prior to antrum formation. This is promoted by follicular mediators such as bFGF, leptin and follicular and circulatory VEGF [713-717]. In this respect, angiogenesis is regulated independently across different follicles, possibly via the selective local production of pro- and anti-angiogenic isoforms of VEGF, a mechanism achieved through alternative mRNA splicing [718, 719].

EGF, in addition to its role in angiogenesis, appears to mediate primary to antral follicle development [720]. Moreover, EGF regulation of TGF-β via alterations in receptor levels in hamster and human ovaries at the primary to antral stage of development, suggests a role for TGF-β in primary follicle selection for antrum development [721, 722]. Both GC-derived EGF and FGFs increase GC SCF expression and, in turn, promote proliferation of thecal interstitial cells and oocyte growth via the mitogen activated protein kinase (MAPK) signalling pathway, as demonstrated in rats [721, 723-726]. In bovine models, FGF-18 is thought to inhibit theca-induced GC proliferation via alterations in cell cycle progression, thereby participating in follicle atresia [715]. Oocyte-derived FGF-8 modulates cumulus expansion [727], while FGF-7 and FGF-10 mediate GC/TC proliferation [728]. bFGF has been shown to promote pre-antral follicle growth in humans and goats [729-731]. However, FGFs are known to have disparate roles in mono- and polyovulatory species, which may lead to functional misinterpretations [732].

The primary to antral follicle transition may also be partially mediated by macrophage migration inhibitory factor (MIF). Intraperitoneal injections of anti-MIF antibodies *in vivo* reduce GC and TC proliferation in mice, an effect potentially mediated by modulation of macrophage cytokine production [733]. Macrophages are found in the ovary throughout folliculogenesis, localising to the TC layer in murine and human primary follicles where the array of cytokines they produce (IL-1α/β, IL-6, GM-CSF and TNF-α amongst others) stimulates cell proliferation and inhibits apoptosis [654, 734, 735].

4.1.8.3 Antral follicle growth and selection

In addition to the FSH-dependence of the antral stage, growth and selection of antral follicles may also involve autocrine/paracrine roles for GC-derived activin and BMP-6, and a paracrine role for oocyte-derived GDF-9, BMP-6 and BMP-15. These agents promote GC proliferation and modulate FSH-dependent follicle development [104, 736, 737]. In contrast to the positive effects exerted by these cytokines in dominant follicle selection, AMH plays an inhibitory role by reducing the sensitivity of pre-antral and small antral follicles to FSH [738].

BMP-15 supports follicular development through to the point of ovulation [707, 739]. *In vivo* inhibition of BMP-15 and GDF-9 in mice results in fewer antral/preovulatory follicles, larger oocyte size and decreased GC mitogenesis [712, 740]. Moreover, BMP-15 plays a role in follicle selection, reducing the recruitable pool of follicles in rats by inhibiting the expression of GC FSH receptors [701, 741]. By contrast, BMP-2, 5 and 6, appear to support follicle survival by maintaining GC and TC proliferation rates in bovine follicles [742]. Theca-derived BMP-4 and BMP-7 act as species-specific paracrine regulators of GC function, interacting with the activin/inhibin system and E_2 to select and rescue antral follicles from atresia [104, 743, 744]. Oocyte-derived IL-7 promotes oocyte maturation in rats concurrently with the suppression of GC apoptosis, lending further credence to the notion that the oocyte governs its own fate [745].

All three isoforms of TGF-β are expressed in the ovary with specific intrafollicular localisation. TGF-β1 and 2 expressions localise to the GC and TC compartments respectively in rats and pigs [746, 747]. Mice express both TGF-β1 and 2 in GCs, with TC expression of both isoforms decreasing with advancing maturity [748]. TGF-β3 localises to the theca interna throughout the oestrus cycle in swine, increasing towards ovulation [749]. These isoforms have similar effects although their potency differs according to their target cell [750]. GC FSH-receptor expression is upregulated by TGF-β1 and 2, and the former synergises with FSH to stimulate VEGF thereby promoting angiogenesis [751, 752]. Furthermore, TGF-β1 inhibits E_2 production in FSH-stimulated follicles in bovine ovaries, with a reduction in this inhibition enabling the selection of a single dominant follicle [753]. TGF-β3 mRNA expression increases post-hCG injection in pigs, modulating preovulatory follicle development [746].

SCF is involved in preovulatory follicle growth, stimulation of oocyte nuclear maturation and steroidogenesis [754-756]. Indeed, the interplay between theca and GC compartments may be modulated by the concerted action of SCF and hepatocyte

growth factor (HGF), promoting TC proliferation and GC steroid hormone production [757, 758]. SCF is a requisite factor for antrum formation and subsequent expansion, its pivotal role highlighted by a murine study [759].

TNF-α is believed to promote GC proliferation in the mouse through the insulin-like growth factor (IGF) system and/or the acute phase protein serum amyloid A3 [760, 761]. However, the follicular response to TNF-α is complex. TNF-α knock-out mice exhibit reduced oocyte apoptosis as well as increased GC proliferation [762]. The effects of TNF-α likely involve the input of other mediators, as highlighted by the fact that nerve growth factor (NGF) levels stimulate TNF-α-mediated apoptosis of murine GCs [761]. The mechanism by which TNF-α balances proliferation versus apoptosis and atresia remains unclear, although several hypotheses have been put forward: TNF-α is expressed in the human oocyte from the primary stage, increasing as the follicle reaches ovulation [763], suggesting that the follicular response may be dose-dependent. This may be further refined by the ratio of receptors TNF-R1 and 2 since TNF-R1 activation results in cytotoxicity while TNF-R2 results in proliferation [764]. This may also explain theeffects of TNF-α induction of nitric oxide (NO), which have a dichotomous effect on the apoptosis pathway [765-768]. Indeed, TNF-α induces NO nitric oxide synthase (NOS)-II, leading to NO-induced GC proliferation and/or differentiation via the EGF signalling pathway [769]. Interestingly, both TNF-related apoptosis-inducing ligand (TRAIL) and its pro- and anti-apoptotic receptors are expressed in GCs, thereby potentially modulating their apoptotic rate and, in turn, follicular atresia [770].

The importance of IL-6 signalling in folliculogenesis has been noted in swine, where IL-6 receptors have been shown to promote GC survival [771]. IL-6 and its soluble receptor IL-6(sR) have been proposed to be essential in the regulation of follicle growth and atresia in humans [772]. Leporine models have shown that IL-8 mediates follicular maturation via granulocyte chemotaxis and activation [773], and that its production is upregulated by both EGF and TGF-α in humans [774]. IL-8 is a CXC chemokine, a potent chemoattractant, particularly for neutrophils, and an angiogenic factor [775-777]. IL-8 is induced by hypoxia, potentially influencing angiogenesis given that ovarian follicles can represent a hypoxic environment [778]. In line with this observation, rat models have shown that IL-8 may act as an angiogenic factor during late folliculogenesis [778-781]. IL-8 is stimulated by VEGF in bovine TCs, promoting angiogenesis and improving perifollicular blood flow, and thus potentially contributing to supporting oocyte viability [782, 783].

Recently classified as a cytokine [784], leptin appears to play an role in reproductive function, since leptin/leptin receptor deficient mice are infertile [785]. Produced by adipocytes, leptin influences the hypothalamo-pituitary-gonadal axis, stimulating GnRH and luteinising hormone (LH) production and providing a metabolic signal to the reproductive system [786, 787]. Leptin inhibits antral follicle steroidogenesis in GC and TCs in human and animal *in vitro* models, although these findings should be interpreted with caution given that these studies were conducted using supraphysiological levels of leptin [788-793]. This is underscored by a porcine study demonstrating that the STAT-3 mediated response of GCs to leptin is dose-dependent: physiological levels increase steroidogenesis while artificially increased concentrations have the opposite effect [794].

4.1.8.4 Peri-ovulatory oocyte maturation

The events involved in folliculogenesis culminate in ovulation which, as outlined earlier, involves active remodelling of the extracellular matrix, microcirculatory vasomotion, formation of an avascular area at the follicular apex and expulsion of the oocyte-cumulus complex [795]. Although the process of ovulation falls outwith the scope of this thesis, the accumulation of cytokines within FF immediately prior to ovulation likely continues to influence oocyte viability and, by inference, ability to fertilise and generate a successful pregnancy.

IL-1β mRNA levels increase in FF macrophages and GCs in mice and humans as the follicle nears rupture, most likely in response to changing gonadotrophin profiles [796-803]. This points to a role for IL-1β in ovulation - a notion supported by *in vivo* studies in horses and humans [799, 804, 805]. IL-1β reduces LH receptor expression in rat GCs *in vitro* [806], and signals for GCs to switch from proliferation to differentiation [807], indicating that this cytokine participates in determining whether follicles undergo atresia or progress to ovulation. IL-1β also upregulates macrophage NO production, thus mediating the growth/apoptosis decision [767-769, 808], and improves perifollicular blood flow via an upregulation in VEGF gene expression [800, 809, 810].

The resumption of oocyte meiosis, cumulus cell expansion and ovulation are events mediated by several members of the EGF-like cytokine family, stimulated by the pre-ovulatory LH surge [811]. LH induces the expression of amphiregulin (AREG), epiregulin (EREG), and betacellulin (BTC) in mural GCs in mice [812, 813]. These factors induce the expression of prostaglandin-endoperoxide synthase 2 (PTGS2) in

mural GCs and cumulus cells, leading to an increase in PGE_2 production [814-816]. PGE_2 then exhibits positive feedback by stimulating AREG and EREG production in cumulus cells, which bind in an autocrine/paracrine manner to the cumulus cells' EGF receptor (EGFR) to stimulate cumulus expansion directly, and meiotic resumption indirectly [816]. Cumulus cell EGFR expression is stimulated by oocyte-derived GDF-9 and BMP-15 in the mouse, effectively enabling the cumulus oophorus to respond to LH-induced EGF-like peptides [817, 818].

Several interleukins are thought to mediate pre-ovulatory changes in the oocyte. IL-6 is an autocrine regulator of ovarian function, responsible for orchestrating cumulus cell expansion in mice by the induction of genes specifically involved in extracellular matrix formation/stability (*Has2, Ptx3, Ptgs2, Tnfaip6* [819]. Monocytes/macrophages as well as mast, theca and GCs have all been reported to produce another interleukin, IL-8 [820, 821]. IL-8 levels are highest in the TC of late follicular/early ovulatory follicles, followed by an increase in the GC layer close to the time of ovulation [780, 822, 823]. Neutrophil numbers rise in the TC layer with increasing IL-8 concentrations towards ovulation, consistent with a chemotactic role for this cytokine in leukocyte recruitment [2, 3, 681, 824].

4.1.9 Ovarian Stimulation

Most treatment regimes for COH rely on gonadotrophins in order to compensate for low pregnancy rates, overriding the natural selection of one dominant follicle. Multiple follicles are recruited each cycle, in theory maximising the number of oocytes available for fertilisation and therefore increasing the likelihood of a pregnancy. However, ovarian stimulation may in itself be detrimental to oogenesis, embryo quality and endometrial receptivity [825].

The influence of exogenous gonadotrophins promotes the growth of multiple follicles to the pre-ovulatory stage by bypassing physiological regulatory mechanisms. Urinary-derived or recombinant FSH are administered, occasionally in conjunction with LH, to increase serum FSH concentrations above the threshold required for follicle development, ensuring that the entire cohort of recruited follicles for that cycle attains pre-ovulatory status [826]. In the long protocol (Figure 4-4), GnRH agonists from cycle day (CD) 21 suppress pituitary FSH and LH in order to promote even follicle growth and prevent premature luteinisation. This is followed by administration of exogenous gonadotrophins at CD2, which is continued for 8-12 days depending on follicle growth. Once at least two follicles have attained at least 18mm diameter, a single dose of hCG

is administered 36 hours before oocyte retrieval to 'trigger' the final stages of oocyte maturation. After oocyte retrieval, fertilisation is attempted via IVF or ICSI, with resultant embryos being typically (albeit not exclusively) transferred on day 3 or day 5.

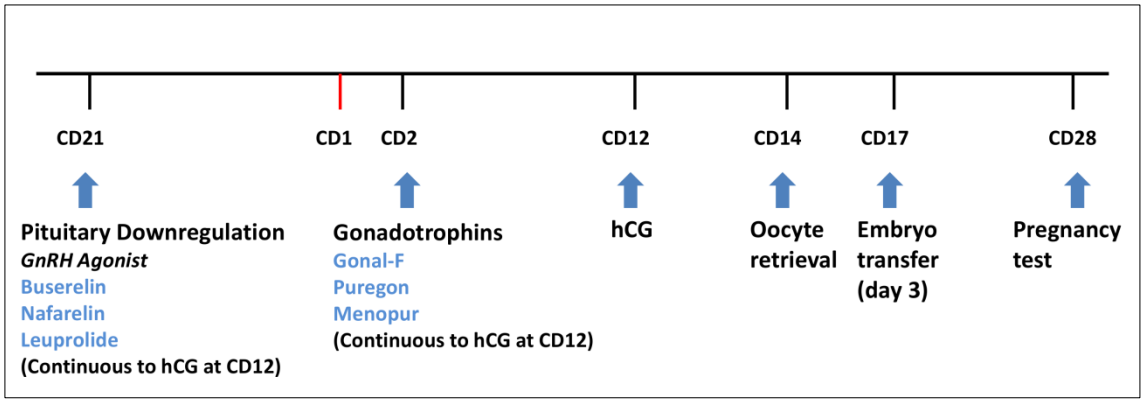


Figure 4-4: Ovarian stimulation utilising the long protocol. CD indicates the cycle day at which interventions occur, with CD1 being the first day of menses.

4.1.10 Assessment of oocyte maturation in the clinic

Oocyte maturity/quality is typically evaluated using morphological criteria in assisted conception laboratories, despite evidence that this method of assessment may not be optimal in its current form [827]. The methodology for assessing oocyte morphology varies between laboratories, but often includes assessment of appearance of the cumulus oocyte complex, zona pellucida and the meiotic spindle (Table 4-1). Although the reliability of morphological assessment can be improved by denuding the oocyte, a process which is essential in ICSI treatment, allied assessment techniques remain highly subjective, open to inaccuracy, and inherently render the oocyte incapable of further development by removing its cumulus support [828-832].

Despite improving the identification of mature oocytes, denuding the oocyte and observation of the polar body does not guarantee fertilisation. IVF and ICSI success rates in terms of live births remain very similar, despite overcoming the physical barriers to fertilisation. An average ICSI fertilisation rate of 67.5% has recently been reported [833], meaning that even oocytes identified as mature do not always fertilise. This, in conjunction with failure of live birth rates to rise rapidly in assisted conception protocols, has lead researchers to search for alternative methods to determine which are the best oocytes from a collected cohort.

Identifying novel molecular markers of oocyte maturation presents an opportunity for the improvement of assisted conception success rates. However the technology and expertise required are often out of the reach of clinics outside of a research situation. For example, examination of the first polar body using fluorescence in-situ hybridisation (FISH), comparative genomic hybridisation (array-CGH) and gene expression microarray may yield cytogenetic information but these approaches are cumbersome, rely heavily on expertise and may not give results in a time frame which is clinically informative [834, 835]. By contrast, profiling the FF which provides the oocyte with its developmental milieu remains an attractive proposition for the discovery of markers of oocyte maturation given that it is non-invasive beyond routine assisted conception interventions and therefore presents no developmental risk to the oocyte itself.

	Factor	Marker	Outcome	References
Non-invasive	Cumulus oocyte complex	Expansion of cumulus and corona radiata	Correlation between dense corona radiata and immature oocytes	[836, 837]
	Zona pellucida (ZP)	Thickness and darkness of ZP	Reduced ZP thickness correlates with increased fertilisation rates. Increased birefringence associated with increase fertilisation rates	[838-841]
Invasive	Perivitelline space	Size of perivitelline space/granularity	Increased space and granularity correlates with embryo quality. Presence of coarse granules predicts lower implantation rates	[842-844]
	First polar body	Shape, fragmentation and size	Correlation between abnormal shape, enlarged size and low fertilisation rates	[844, 845]
	Ooplasm appearance	Density and granulation	'Dark' ooplasm associated with compromised embryo quality. Absence of granularity correlates with low fertilisation rates	[638, 846-848]
	Vacuoles and/or cytoplasmic inclusions	Presence of absence of vacuoles	Decreased clinical pregnancy rates following transfer of embryos derived from oocytes containing vacuoles. Cytoplasmic inclusions may affect fertilisation, although this is controversial	[847, 849-853]
	Meiotic spindle	Presence and appearance of the meiotic spindle	Presence of meiotic spindle and proximity to the first polar body associated with increased fertilisation rates.	[854-857]

Table 4-1: Commonly used morphological criteria for the assessment of oocyte maturity/quality.

4.1.11 Impact of ovarian stimulation

4.1.11.1 Oocyte maturation

The fact that that COH has a negative impact on oocyte development has been increasingly recognised in recent years [858, 859]. Based on the appearance of the COC, fertilisation rate, embryo morphology and implantation rate, one study found that

higher egg retrieval rates correlated with lower quality oocytes [860]. Similarly, another study reported that increasing the number of oocytes collected above 13 resulted in a fall in pregnancy rates [861]. In this respect, excessively high numbers of poor quality oocytes are seen in ovarian hyperstimulation syndrome (OHSS), which suggests that overriding the physiological mechanism for follicle selection may be counterproductive. Studies in rodents demonstrate a delay in embryo development and impaired implantation following gonadotrophin stimulation [862, 863].

COH may have an impact on the ability of the oocyte to complete meiosis, leading to aneuploid oocytes and embryo mosaicism [139, 864]. Previous studies have shown that as many as 20% of all oocytes retrieved in these cycles are classed as immature, being at a nuclear maturation stage of metaphase I or earlier, despite the follicle having Graafian appearance [865]. Mouse embryos from stimulated cycles show evidence of induced-DNA lesion [866], and zygotes show an increased rate of chromosomal defects in the pronucleus with corresponding compromised embryo development [867]. Similarly, *in vitro* matured oocytes exposed to FSH exhibit accelerated nuclear maturation with significant levels of aneuploidy [868].

4.1.11.2 Cytokine profiles

During ovarian stimulation, FF composition changes as individual cells of the ovarian follicle respond to exogenous gonadotrophins by secreting altered profiles of signalling molecules, likely including cytokines. As FF bathes the oocyte and provides the milieu in which it matures, such changes may have profound effects of maturation, fertilisation and early embryo development [869]. There is a suggestion that FF composition differs between the long protocol and the short protocol for ovarian stimulation, with the implication that the choice of stimulation regime could improve maturation rates and oocyte quality [870]. One recent study characterised the cytokine profiles in natural cycles, and demonstrated that cytokines are compartmentalised between plasma and FF, with certain cytokines such as IL-8 and VEGF varying between the follicular and luteal phases [871]. However, direct comparisons between ovarian stimulation and natural cycles are lacking, such that it is not possible to say how the cytokine profile is altered compared to normal physiology. Although this area of research falls outwith the remit of this thesis, it warrants further investigation.

4.1.12 Potential markers of oocyte maturity

As cytokines mediate many stages of folliculogenesis, they present attractive targets for the discovery of biomarkers of oocyte maturation and quality. Analysing the cytokine

profiles of FF collected (and usually discarded) at the time of oocyte retrieval offers a non-invasive analytical strategy for assessing oocyte developmental potential. Although several research groups have adopted this strategy, few have analysed the fate of individual follicles, or focussed on a wide panel of cytokines. Nevertheless, FF cytokines have been associated with fertilisation potential, oocyte maturational status and embryo quality as discussed below.

4.1.12.1 FF cytokines associated with positive outcomes

FF G-CSF concentration is positively correlated with oocyte quality/maturity, fertilisation success and subsequent pregnancy [872-875]. Ageing patients and higher responders to ovarian stimulation exhibit lower FF G-CSF levels, factors which are associated with decreased oocyte quality based on fertilisation rates and embryo development [151]. High FF G-CSF concentrations, in conjunction with IL-15, have been linked to increased implantation potential and a successful live birth [151, 876-878]. Related to G-CSF, FF M-CSF levels appear to follow the same patterns, with circulatory levels being touted as a marker of successful outcome in assisted conception cycles across a range of studies [875, 879-882].

Increased FF SCF concentrations at the time of oocyte retrieval have been associated with increased pregnancy rates following IVF [883], although this has not been demonstrated in ICSI programmes [884]. SCF is critical at various stages of murine oocyte development, correlating with normal oocyte nuclear maturation and polar body extrusion [754, 885]. Similar observations have been made for SDF-1α, whose FF concentrations increase in line with follicle diameter [886].

EGF and EGF-like peptides have been implicated in oocyte maturation *in vivo*, and these cytokines are frequently used as culture medium supplements to promote *in vitro* maturation of GV stage oocytes [887-897]. Given their role in oocyte maturation, AREG, EREG and BTC may thus serve as suitable markers of oocyte maturation [897]. AREG is the most abundant EGF-like protein in FF from stimulated ovaries, and although the evidence in favour of its potential role as a biomarker remains inconclusive at present, FF AREG may prove valuable in predicting oocyte maturational status [898-900].

Follicles containing mature oocytes have higher FF concentrations of MIP-1α, IL-6 and IL-6sR [772, 901]. However, IL-6 concentrations are increased following ovarian stimulation compared with natural cycles, such that this apparent increase could be

artefactual [902, 903]. LIF, a member if the IL-6 family modulates cumulus expansion in humans and mice [904], and enhances fertilisation of ovine oocytes *in vitro* [905]. LIF is present at much higher concentrations in Graafian compared to secondary follicles [906], and although FF LIF levels correlate with oocyte quality and implantation potential in humans [907], this observation is inconsistent across studies [908, 909].

Of the TGF-β family, FF TGF-β1 levels correlate positively with fertilisation and pregnancy rates following ovarian stimulation in humans [910, 911]. TGF-β1 production is sensitive to different ovarian stimulation regimes: stimulation with pure FSH results in higher FF TGF-β1 concentrations than stimulation with human menopausal gonadotrophins (HMG), suggesting differential regulation by FSH and LH [911]. BMP-15 and GDF-9 are also detectable in human FF. Oocytes from follicles with high FF BMP-15 concentrations exhibit higher fertilisation rates, with improved cleavage and preimplantation development [912]. Similarly, high concentrations of GDF-9 are positively associated with oocyte nuclear maturation and embryo development [913].

Embryo cleavage and implantation rates may be improved by higher FF IL-1β concentrations, and several observational studies in humans have linked FF IL-1β concentrations with successful pregnancy following assisted conception [149, 914-916]. However, other studies in humans and horses have found no correlation between FF IL-1β and oocyte maturation, fertilisation or embryo cleavage rates, such that further studies are very much needed to determine if this is indeed a potential marker of oocyte maturity [804, 916-918].

4.1.12.2 FF cytokines associated with negative outcomes

High TNF-α concentrations in FF may inhibit oocyte development and maturation [919, 920], although the clinical value of this remains to be demonstrated. Elevated FF TNF-α levels in swine result in fewer oocytes progressing from GV to MII, with increased incidence of chromosomal abnormalities at the MII stage [921, 922]. This has implications for women with inflammatory disorders such as endometriosis, who have elevated FF TNF-α concentrations and lower success rates in assisted conception [923-927]. There are also implications for assisted conception generally, as there is evidence that exogenous gonadotrophins raise FF TNF-α concentrations and perturb the profile of other cytokines, thereby reducing the fertilisation potential of oocytes when compared to natural cycles [139, 140, 799, 865, 928-932]. The detrimental effects of TNF-α appear to be determined by its concentration in combination with the local cytokine milieu [401, 933].

Poor outcomes following assisted conception, higher rates of OHSS and premature luteinisation correlate with high FF VEGF concentrations [706, 925, 934-947]. Although low FF VEGF levels have been associated with improved maturity status at the time of oocyte recovery [948], the presence of VEGF is known to positively correlate with perifollicular blood, and oocytes from well vascularised follicles exhibit increased fertilisation and pregnancy rates in humans [949-954]. Animal studies have shown that VEGF is beneficial to oocyte maturation both *in vivo* and *in vitro* [955-959]. This seemingly dichotomous situation could reflect three scenarios: (i) that there is a 'window' of VEGF concentration, below which there is inadequate angiogenesis and above which pathological processes develop; (ii) that the physiological effects of VEGF depend on the contribution of other cytokines; and/or (iii) that high VEGF levels are a marker of a poor vascularisation, with the follicle attempting to compensate. It is also possible that the varying effects are mediated by different isoforms of VEGF, although the majority of studies have not distinguished between its pro- and anti-angiogenic forms [718]. As noted for TNF-α, the ovarian stimulation regimes used in assisted conception are reported to alter VEGF production [706, 938, 960].

Elevated FF leptin concentrations in humans have been associated with a reduced ovarian response, poor embryo quality and reduced likelihood of pregnancy [925, 943, 946, 961-963], despite the fact that leptin is positively correlated with oocyte nuclear maturation and fertilisation success in humans, mice and cattle [964]. Other studies, however, have shown no correlation between FF leptin concentration and assisted reproduction outcome [655, 656, 965-971]. The ovary has been shown to exhibit a biphasic response to leptin, and FF concentrations may by altered by its complex interaction with the metabolome given that many studies do not control for weight/BMI or metabolic status [972-975].

Many other cytokines (including GM-CSF, HGF, IFN-α, IFN-γ, IL-2, IL-4, IL-7, IL10, IL-12, IL-18, MCP-1, MIP-1β, and platelet derived growth factor (PDGF)) have been identified in FF [151, 877, 935, 948, 976-988]. One recent study showed that IL-8, IL-18 and MIP-1β positively correlated with pregnancy outcome in IVF cycles, while total IL-12 reflected oocyte fertilisation and subsequent embryo development [985]. By contrast, IL-4 and IL-7 have been associated with poor outcomes following assisted conception [978]. However, given the general paucity of information regarding these cytokines, their role and effectiveness as biomarkers remains to be conclusively demonstrated.

4.1.13 Identified opportunities

Most studies to date are based on pooled FF rather than relating individual follicle profiles to oocyte maturity or pregnancy outcome. While this may be informative in terms of the immunological environment, this approach only allows general conclusions to be reached regarding the impact of ovarian stimulation on oocyte maturation, and ignores the follicle-specific physiology described above [989]. This potentially impacts on treatment cycle outcome, as there is significant variation in the developmental potential of oocytes retrieved from the same patient during the same cycle.

Many groups looking for markers of oocyte maturation have focussed on nuclear maturation, i.e. the presence/absence of a polar body and germinal vesicle [844]. Suitable markers of cytoplasmic maturation remain scarce, and the cytokines within follicular fluid offer a non-invasive milieu to explore for potential indicators that an oocyte is ready for fertilisation.

Although many studies have examined either individual or small groups of cytokines within the ovarian follicle, no studies have examined the networks which influence oocyte maturatity status in assisted conception cycles. It is clear from the review above that cytokines influence oocyte quality, but there is much still to be learned about how cytokine networks change within the follicle following ovarian stimulation, and whether any changes within these networks can identify good quality mature oocytes which will subsequently fertilise. This will form the basis of this Chapter, by using modelling techniques akin to those employed in Chapter 3. However, one of the limitations identified within that approach was the inability of the algorithm to take in to account ‘hidden’ Markov blankets. A potential strategy to address the limitations identified involves utilising VBSSM networks with the novel application of prior networks, and will be explored within this experimental chapter.

4.2 Aims

The aims of this section of the thesis were therefore:

- to use the mathematical methodologies developed in Chapter 3 as a basis for improved modelling strategies
- to discover, display and explore cytokine networks within human follicular fluid surrounding oocytes in follicles subjected to ovarian stimulation, thereby highlighting their likely involvement in maturation
- to identify potential markers of cytoplasmic maturation
- to classify maturation and the developmental viability of those oocytes based on their follicular fluid cytokine profile.

4.3 Materials and Methods

4.3.1 Subjects

4.3.1.1 Ethical approval

Ethical approval was obtained from the Leeds (East) Regional Ethics Committee and all recruited participants gave written informed consent. Inclusion criteria were broad, and included any age group, subfertility aetiology and ethnicity who complied with Assisted Conception Unit (ACU) protocols and any IVF or IVF/ICSI cycle.

4.3.1.2 Patient selection

Patients were selected from the MPD ICSI category as during the ICSI process the maturity of the oocyte can be accurately assessed. Exclusion criteria were applied in order to eliminate patients with identified inflammatory conditions which may affect results. Exclusion criteria included non-Caucasian ethnicity, polycystic ovary syndrome (determined using Rotterdam Consensus Group criteria), pelvic inflammatory disease >Grade 1, tubal disease >Grade 1 (French Tubal Score), endometriosis >Grade 1 (American Society for Reproductive Medicine Revised Classification of Endometriosis), smoking, extremes of BMI (<19 and >30kg/m2) and any other identified medical condition/medication potentially affecting patient inflammatory/immune responses.

4.3.1.3 Ovarian stimulation and oocyte collection

Ovarian stimulation, oocyte collection and embryo transfer were performed by the clinical team at St James's University Hospital ACU under the direction of Mrs Vinay Sharma. The stimulation protocol utilised was unchanged from the unit standard and included pituitary down regulation with leuprorelin acetate SR (Prostap®) followed by stimulation with daily human menopausal gonadotrophin (hMG; Menopur®). Follicles were identified 35 to 37 hours after hCG injection via transvaginal ultrasound guidance. Each follicle was individually aspirated and flushed, and FF examined for an oocyte.

4.3.1.4 Oocyte preparation and identification of maturity

Once retrieved, the oocytes were mechanically stripped of the cumulus oophorus and corona radiata cells by pipetting and assessed for maturity. Mature (metaphase II (MII)), immature (germinal vesicle (GV) or metaphase I (MI)) were distinguished using the following criteria:

- MII oocytes – presence of an extruded polar body

- MI oocytes – determined by default, displaying neither a polar body or germinal vesicle
- GV oocytes – presence of a germinal vesicle containing a single and large nucleolus, eccentrically located.

Only mature MII oocytes were subjected to ICSI, and were injected with a single sperm according to unit protocols for ICSI. Fertilisation success was assessed 16-20 hours after ICSI. Fertilisation was considered normal when the oocytes contained 2 pro-nuclei (PN). For data analysis, MII oocytes were further differentiated into MII-F (those which fertilised) and MII-NF (those which did not fertilise).

4.3.1.5 Data collection

FFs were processed and labelled with their unique follicle and oocyte number which enabled longitudinal tracking (i.e. fertilised/non-fertilised, transferred/frozen as an embryo, developmental stage attained and embryo grade). All patients had pregnancy tests (biochemistry, 2 weeks) and monitoring of early pregnancy (ultrasound scan, 6 weeks) as per unit protocols. A comprehensive database of 110 clinical parameters was completed for each participant (by Drs Nadia Gopichandran and Ellissa Baskind), detailing specific demographics, lifestyle, treatment regime and the outcome for each individual follicle, including the presence of an oocyte, its maturity and fate.

4.3.2 Preparation of FF and serum/plasma

FF aspirates were collected from individual follicles and examined for the presence of an oocyte. The aspirate was transferred into a 15ml centrifuge tube (Greiner Bio, UK) on ice and labelled with the corresponding follicle number. Each follicle aspirated and each oocyte retrieved was assigned a sequential number in the order of aspiration and retrieval. The follicle/oocyte number was carried forward to the embryo and linked to its ultimate fate (e.g. fertilised or not, pregnant or not). Blood was collected in 6ml lithium heparin tubes (Beckton, Dickinson and Company, UK) for plasma separation and 6ml EDTA tubes for serum separation (Beckton, Dickinson and Company, UK).

FF aspirates were centrifuged at 2,000 rpm for 5 minutes at 4^{o}C in a Sanyo Harrier 18/80 MSE refrigerated Centrifuge (Sanyo, UK). The supernatant fluid was then divided into 1ml aliquots and stored in 1.5ml microfuge tubes (Starlab, UK). Whole blood was centrifuged at 2,000 rpm for 10 minutes and the supernatant serum and plasma were

placed in aliquots in 1.5ml microfuge tubes. All samples were processed and stored at -80^{o}C within 1 hour of collection.

4.3.3 Sample analysis

4.3.3.1 Cytokines

Cytokines were measured by fluid phase multiplex immunoassay using 23-plex and 27-plex kits (Bio-Rad, Hercules, CA, USA) according to the manufacturer's instructions with minor modifications (original instructions available at http://tinyurl.com/korjr9s). Briefly, FF and plasma samples were thawed on ice, then microfuged for 2 minutes at 8000rpm to remove any possible debris that may affect the assay filter plates. The supernatant was then diluted 1:1 with assay buffer and mixed with the relevant bead sets in a 96 well filter plate (Millipore, UK). Recombinant cytokines were serially diluted according to kit instructions and provided a standard curve. Following 30 minutes' incubation at room temperature, the plate was washed 3 times with vacuum extraction of the wash buffer. Biotinylated secondary antibodies were added followed by a 30 minute incubation and 3 washes. The streptavidin-phycoerythrin detection complex was added and incubated for 10 minutes, followed by a further 3 washes. The wells were then resuspended in 125μl assay buffer ready for analysis. All incubations were performed on an orbital shaker at 300rpm with an initial 30 seconds at 1000rpm. The plate was shaken for 30 seconds at 1000rpm before analysis. Fluorescence was measured on a twin laser Luminex 100 cytometer platform (Luminex, Texas, USA). Unknown cytokine concentrations were automatically calculated by Bio-Plex Manager software (version 4.1) using a 5 parameter logistic calibration curve.

4.3.3.2 von Willebrand Factor (vWF)

The concentration of vWF in plasma and follicular fluid samples was measured using a commercially available enzyme-linked immunosorbent assay (ELISA) kit (Diagnostica Stago UK LTD, Reading, UK) following the manufacturer's instructions. Absorbance was measured at 450nm using a MultiScan Ascent plate reader (ThermoFisher Scientific, Loughborough, UK) with standard curves produced by the plate reader Ascent software programme using a 4 parameter logistic curve. vWF was solely used for the purposes of accounting for the occasional blood contamination of FF that occurs during oocyte collection.

4.3.3.3 C-reactive protein (CRP)

CRP is one of the most sensitive acute phase proteins, and its raised concentrations in blood are traditionally associated with inflammatory processes. The analysis of CRP utilised an enhanced latex-turbidometric immunoassay performed on an automated clinical chemical autoanalyser (COBAS MIRA, Roche, Basel, Switzerland) using a commercially available kit (Thermo Electron Corporation, Alpha Laboratories, Hampshire, UK). 20µl of follicular fluid or plasma was aliquoted into a MIRA sample cup (Alpha Laboratories, Hampshire, UK). Calibration curves and samples were run according to the reaction parameters detailed in Table 4-2. Calibration was performed at each start up using the Thermo Extended Range Calibration set (Thermo Electron Corporation, Alpha Laboratories, Hampshire, UK).

Variable	Defined condition
Temperature	37^{o}C
Primary wavelength	570nm
Secondary wavelength	800nm
Assay type	2 point endpoint
Direction	Increase
Sample:Reagent ratio	1:100
Delay time	300 seconds
Incubation time	265 seconds
Reagent blank limits (570nm / 800nm, 1cm light path)	Low 0.000 AU High 0.015 AU
Linearity	0.12 – 320 mg/ml
Limit of quantification (570nm / 800nm, 1cm light path)	0.12 mg/ml
Final absorbance	Absorbance (test) – absorbance (blank)

Table 4-2: Reaction conditions for CRP measurement.

4.3.3.4 Protein analysis

The concentration of protein was measured using a commercially available kit (Bio-Rad DC-Protein assay, Bio-Rad) in all FF and plasma samples in order to standardise for the protein-free medium flush component within collected fluids. All cytokine concentrations were expressed in pg cytokine/mg protein to account for any dilution effects. Prior pilot analysis of clean catch FF indicated that clean catch fluid protein levels were subject to minimal variability, making protein-based standardisation a reasonable correction method. Absorbance was measured at 630nm using a MultiScan

Ascent plate reader (ThermoFisher Scientific, Loughborough, UK) with standard curves produced by the plate reader Ascent software programme using a linear curve.

4.3.4 Statistics and data analysis

4.3.4.1 Pre-processing of FF cytokine concentrations

When collecting FF samples, a needle is required to traverse the vaginal mucosa and ovarian tunica albuginea and cortex to aspirate the follicles. During this process, vasculature may be disrupted and blood, either macroscopic or microscopic may contaminate the FF. Furthermore, washes between follicular aspirations with protein free flush medium formulated with Gentamicin (Enhanced HTF Culture Medium with HEPES, Conception Technologies, San Diego, California, USA) had the potential to provide a mild dilution factor. The cytokine concentrations measured in the FF may therefore not be an accurate representation of the genuine follicular content and therefore such contamination must be accounted for and factored in to the analysis. This entailed cytokine analysis of both FF and plasma together with the measurement of von Willebrand Factor (vWF) and protein concentrations within blood and FF.

As vWF is present in the circulation but not in FF, an equation was designed (by Drs Michele Cummings and Nic Orsi) to allow the relative blood contamination of FF to be taken into account, and therefore adjust the cytokine values based on their blood profiles. Equation 1 corrected the FF sample for blood contamination.

$$FF\ cytokine\ concentration = \frac{(cytokine\ in\ FF\ x\ vWF\ in\ plasma) - (cytokine\ in\ plasma\ x\ vWF\ in\ FF)}{plasma\ vWF - FF\ vWF}$$

Equation 1: Correction of cytokine values for blood contamination

The resulting corrected FF cytokine concentrations were then expressed as a function of total protein to mitigate dilution effects (Equation 2).

$$Dilution\ corrected\ FF\ cytokine\ concentration\ \left(\frac{pg}{mg}\right) = \frac{corrected\ FF\ cytokine\ concentration}{protein\ concentration}$$

Equation 2: Correction of FF cytokine concentrations for dilution effects

4.3.4.2 Missing data

Forty eight out of the 8,651 data points were missing (circa 0.55% of the total) and, as such, these values were imputed using the regularized expectation-maximization (RegEM) algorithm [990] via Matlab (Tapio Schneider, California Institute of Technology, California, USA), wherein the algorithm was used to compute iteratively maximum log-likelihood estimates of missing values based on presumptive relationships between the complete and incomplete data sets. This was deemed to be a fair assumption based on the recognised physiological cytokine relationships highlighted by *in vivo* studies [481, 560, 991, 992]. Each iteration was based on a two (E and M) step process, where the algorithm determines the expectation of log-likelihood of the complete data based on the incomplete data and the current parameter in the first step:

$$Q(\Theta|\Theta^{(t)}) = E\ (\log p(X,Y|\Theta)|X,\Theta^{(t)})$$

where the available observations X are drawn from a distribution parameterized by Θ, and Y corresponds to the missing (but additional) data (i.e. the values to impute). The second step involves the algorithm determining a new parameter maximising Q (which then converges to a likelihood function local maximum with subsequent iterations):

$$\Theta^{(t+1)} = \arg\max_{\Theta} Q(\Theta|\Theta^{(t)})$$

4.3.4.3 Data analysis

Data were analysed using GraphPad Prism version 5 (GraphPad Software Inc., San Diego, California, USA). Normality was determined by visualising normal Q-Q plots and testing for normality using Shapiro-Wilk. As the data was not normally distributed non-parametric analysis was applied. Mann-Whitney *U* was applied, using a significance level of p=0.05, for initial data exploration. P values were adjusted for multiple comparisons using Holm's sequentially selective Bonferroni modification.

4.3.5 Advanced analysis

In order to mitigate the effects of uneven sample size on the advanced analysis techniques described below, data were randomly truncated (via a random number

generator) to meet the smallest sample size. In the present dataset this was equivalent to the 26 samples in the GV category. This truncated dataset was used in the PCA analysis and in the generation of the modified VBSSM networks.

4.3.6 Principal components analysis

Data were normalised prior to PCA in order to reduce the effect of the dynamic range of each cytokine. First, each data point was divided by the maximum value in that particular dataset. Then data were then scaled by subtracting the mean. This approach rendered the data dimensionless and removed outliers. Subsequent individual and pairwise PCA was performed in Matlab (http://www.mathworks.com/products/matlab/) using Singular Value Decomposition (SVD).

4.3.7 Modified variational Bayesian network analysis

Data from each of the maturation categories were selected and randomly truncated in order to create equal sample sizes in each group. Variational Bayesian state space models (VBSSM) were used as described by Beal *et al* [486] with modifications. The original VBSSM algorithm utilised data only in order to approximate the underlying network. In the present study, a prior network was generated and applied to the VBSSM structure in order to refine the search process. The prior network was generated using information from human models only and was based on a text learning algorithm developed by Benjamin Haibe-Kains in conjunction with the Quackenbush laboratory (available at https://compbio.dfci.harvard.edu/predictivenetworks/). The initialisation fields were set to 10 seeds, with the number of iterations set to 1000. As VBSSM takes into account hidden Markov models with latent variables, the maximum hidden state dimension was limited to 20 [486]. A confidence check was performed, wherein a z score of 2.33 was selected (equivalent to a significance threshold of 98%) in order to demonstrate the number of edges reaching significance [492]. Cytokines not reaching the required level of significance were considered orphaned from the network and therefore not represented in the final visualisation.

Networks were visualised using the open graph Viz platform Gephi version 0.8.2-beta (available at https://gephi.github.io/). The spatialisation technique selected was the force-directed Yifan Hu algorithm [993]. This algorithm is both efficient and high quality in combining a multilevel approach (aimed at effectively overcoming local minima); it uses the Barnes and Hut octree technique, which approximates short- and long-range force efficiently. A community detection algorithm was used to detect the modular

structure in the graph topology, as previously described [994, 995]. Communities were delineated by colour within the final graphical representation, and represented groups of cytokines which demonstrated the strongest relationships.

4.3.8 Classifier analysis

4.3.8.1 Multinomial modelling and Akaike Information Criterion (AIC)

Multinomial modelling (logistic regression) and Akaike Information Criterion (AIC) were performed in '*R*' with the categories defined as GV, MI, MII-NF and MII-F. The correct classification rate (CRC) was determined by removing the data for one oocyte, building the multinomial/AIC model then using the model to predict the correct maturity status of the the removed oocyte based on its cytokine profile. Accordingly, each oocyte was ascribed a probability of being allocated to each of the GV, MI, MII-NF and MII-F categories.

4.4 Results

4.4.1 Demographics

Demographics and relevant clinical data for participants in the ICSI only oocyte maturity data exploration are shown in (Table 4-3). After applying the exclusion criteria, 212 representative FF samples were collected. Following cumulus cell stripping prior to ICSI, oocytes were allocated to the following groups: GV, (*n*=26), MI, (*n*=51), metaphase II not fertilised (MII-NF, *n*=51) and metaphase II fertilised (MII-F, *n*=84).

Parameter	Average (Range/±SEM)
Age (years)	34 (21–42)
MPD starting dose (IU)	300 (150–750)
Total ampoules MPD	45 (24–100)
Total follicles collected	15.3±0.86
FSH (IU/l)	6.78±0.23
LH (IU/l)	4.49±0.23
Oestradiol (pmol/l)	158.0±12.54

Table 4-3: Demographics and clinical data for included participants. Age, MPD starting dose and total ampules are presented as median (range). All other data presented as mean±SEM.

4.4.2 Cytokine analysis

4.4.2.1 Exclusions from study

Upon analysis of the standard curves for each cytokine, the concentrations of several cytokines fell outwith the linear portion of the standard curve (Table 4-4). These cytokines were excluded from any further analysis.

Below limits of detection	Above linear portion of standard curve
IL-1β	HGF
IL-2	VCAM-1
IL-4	ICAM-1
IL-5	
IL-17	
MIP-1α	
IL-1α	

Table 4-4: Cytokines in FF lying outwith the detection range of the assay

4.4.2.2 Differences between plasma and FF

FF cytokine concentrations were measured and compared to plasma values. Of the 40 cytokines measured, 16 demonstrated significant differences between plasma and FF (Figure 4-5). PDGF, IL-12 (p70), IL-9, IL-6 and GM-CSF were significantly lower in FF compared to plasma ($P<0.001$). However VEGF, CTACK, GRO-α, M-CSF, SDF-1α, TRAIL, IP-10, G-CSF, IL-1ra, MIF and SCGF-β all showed concentrations above those noted in plasma ($P<0.001$).

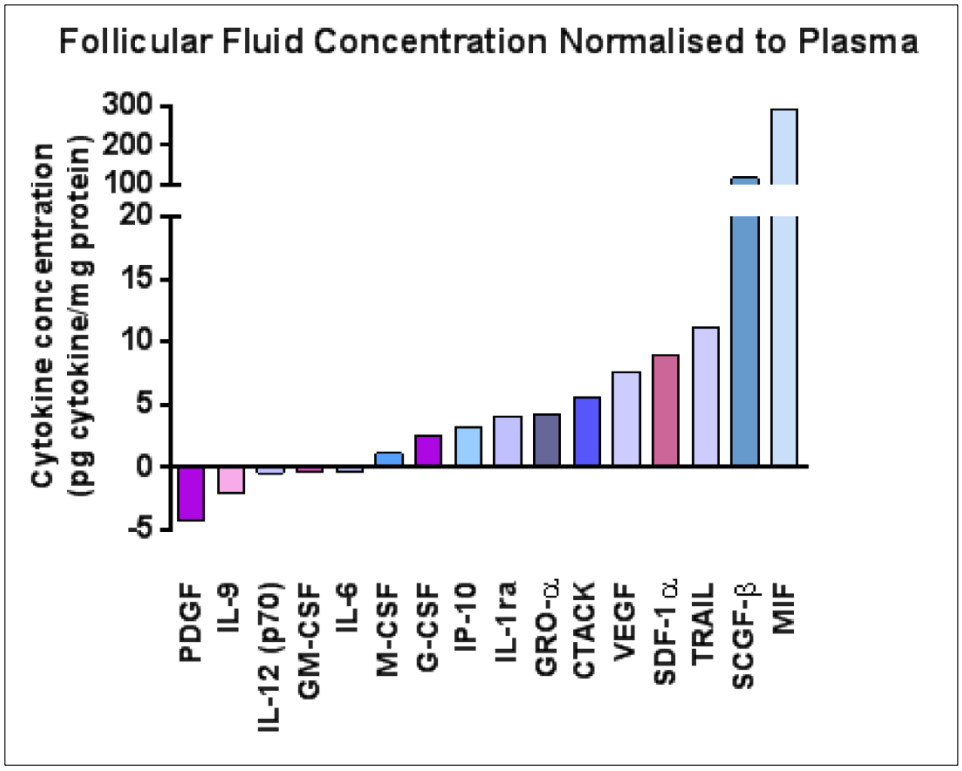


Figure 4-5: Concentration of cytokines in FF normalised to plasma.

4.4.3 Cytokine profiles in follicles containing oocytes of differing maturity

4.4.3.1 Cytokines increasing in concentration with advanced maturity

FF eotaxin, VEGF and IL-8 levels demonstrated trends towards increasing with advancing oocyte maturity (Figure 4-6). Both VEGF and IL-8 were high in FF from GV oocytes, decreasing at the MI stage and then increasing to MII-F. Significant differences were noted across maturation statuses, as indicated in Figure 4-6. Data for all cytokines not demonstrating significant differences between maturity states are presented in Table 4-5.

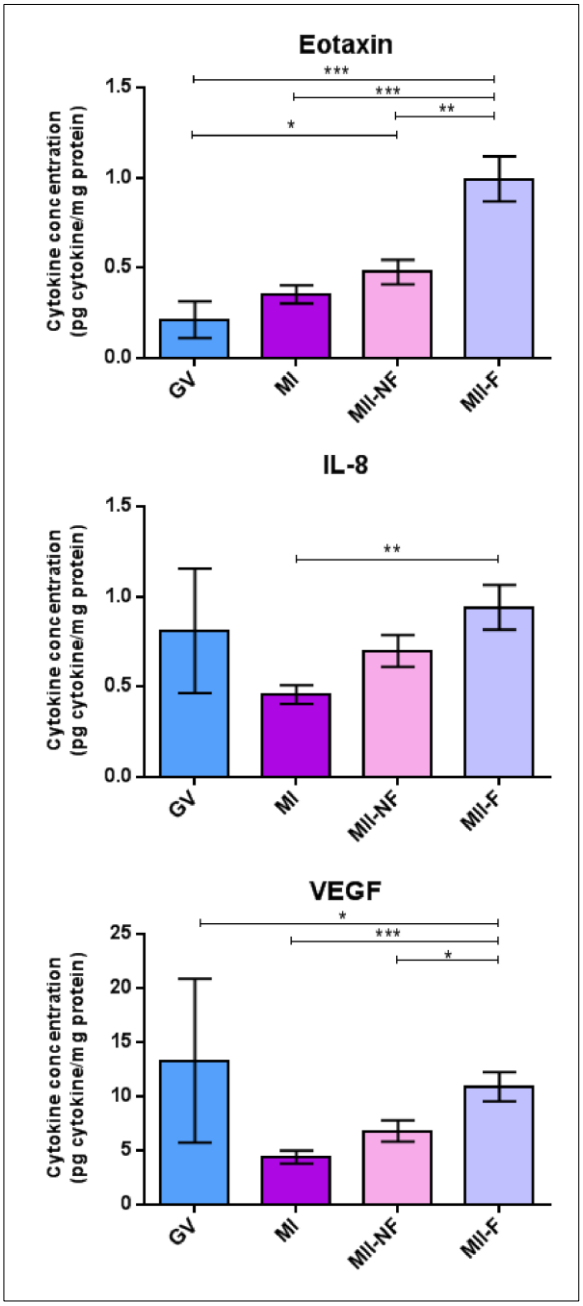


Figure 4-6: Cytokines showing an increasing FF concentration trend with advancing oocyte maturity status. Asterisks indicate significant differences between follicles containing oocytes of differing maturity (*p<0.05, **p<0.01, ***p<0.001).

	GV	MI	MII-NF	MII-F
bFGF	6.61±5.10	3.46±2.25	1.03±0.58	1.31±1.10
GM-CSF	-0.20±0.48	0.08±0.04	0.06±0.03	0.74±0.57
IFN-γ	0.09±0.39	0.42±0.16	0.25±0.11	0.14±0.05
IL-1ra	10.15±10.33	1.49±0.53	16.41±8.42	4.69±1.20
IL-6	-0.12±0.44	0.15±0.05	0.05±0.01	0.05±0.01
IL-7	1.91±1.13	0.50±0.21	0.33±0.25	0.10±0.03
IL-8	0.81±0.35	0.46±0.05	0.70±0.09	0.94±0.12
IL-9	1.80±1.25	0.60±0.37	0.11±0.08	0.00±0.01
IL-10	0.03±0.02	0.01±0.00	0.01±0.00	0.02±0.00
IL-12 (p70)	0.41±0.23	0.22±0.12	0.03±0.03	0.07±0.02
IL-13	0.05±0.02	0.03±0.01	0.02±0.00	0.02±0.01
IL-15	0.23±0.23	0.11±0.11	0.02±0.02	0.17±0.13
IL-18	0.92±0.25	0.71±0.10	0.61±0.05	0.78±0.10
IP-10	4.56±1.34	5.69±0.83	5.95±0.78	4.67±0.62
LIF	3.18±3.00	3.16±3.06	0.11±0.03	0.70±0.60
MCP-1	1.16±0.28	0.76±0.08	0.82±0.12	0.85±0.09
MIG	3.54±0.54	4.32±0.57	3.50±0.39	4.29±0.61
MIP-1β	0.43±0.13	0.36±0.05	0.46±0.07	0.34±0.03
PDGF	4.93±2.76	8.18±3.31	3.62±1.89	1.37±0.32
SCF	1.22±0.16	1.58±0.19	1.50±0.14	1.46±0.23
SCGF-β	233.31±41.84	237.00±50.44	271.48±32.04	349.68±40.10

Table 4-5: Concentrations of cytokines without significant differences according to oocyte maturation stage. Data presented as pg cytokine/mg protein ±SEM.

4.4.3.2 Cytokines decreasing in concentration with advanced maturity

The remaining FF cytokines demonstrated a trend to decrease in concentration relative to oocyte advancing maturity status. Figure 4-7 shows cytokines (CTACK, MCP-3, β-NGF, M-CSF, TRAIL, SDF-1α, IL-12 (p40), IFN-α2, and IL-2ra) which were lowest in concentration in follicles containing MII-F oocytes, which were significantly different to follicles containing less mature oocytes. Similarly, Figure 4-8 shows other cytokines which decreased in FF with advancing oocyte maturity status, albeit less significantly (IL-3, TNF-β, IL-16, G-CSF, RANTES, GRO-α, MIF, IL-7 and TNF-α).

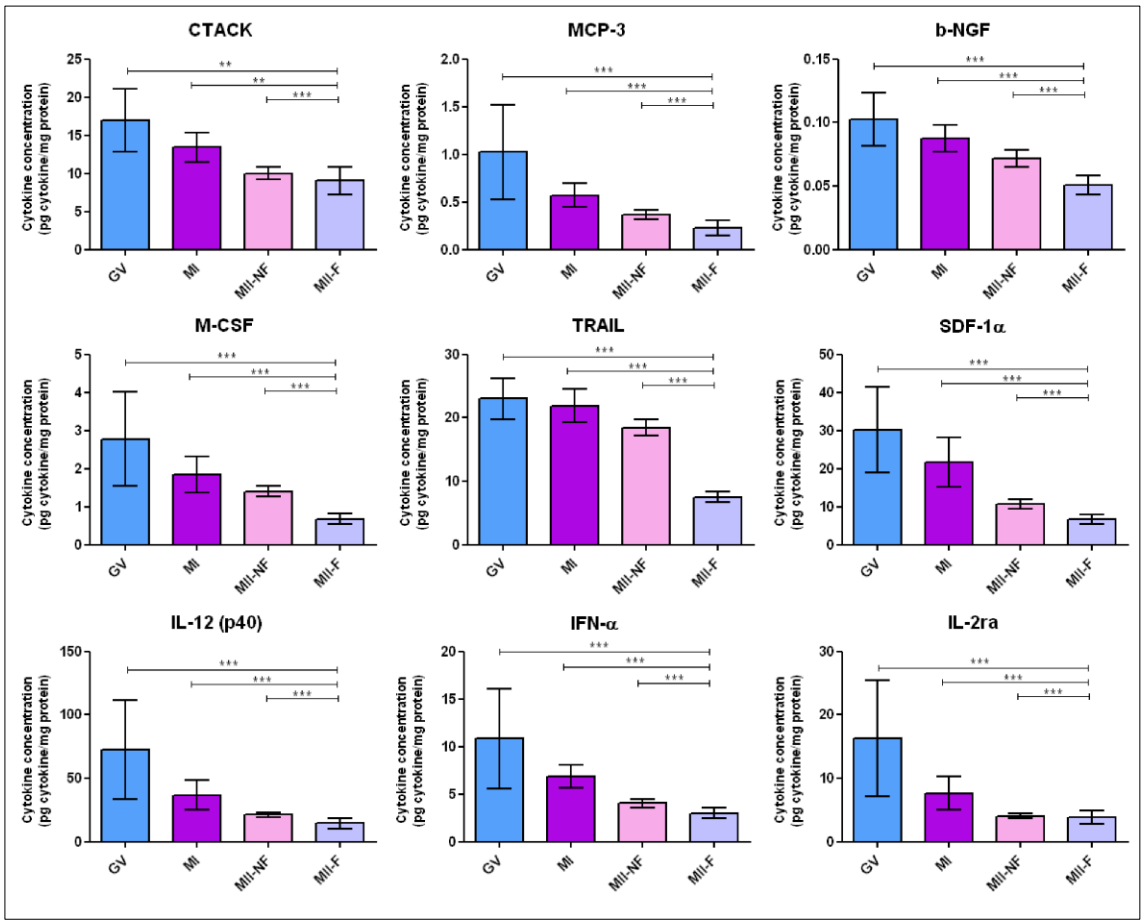


Figure 4-7: Cytokines showing a decreasing concentration trend with increasing maturity status.

Asterisks indicate significant differences between follicles containing oocytes of differing maturity (*p<0.05, **p<0.01, ***p<0.001).

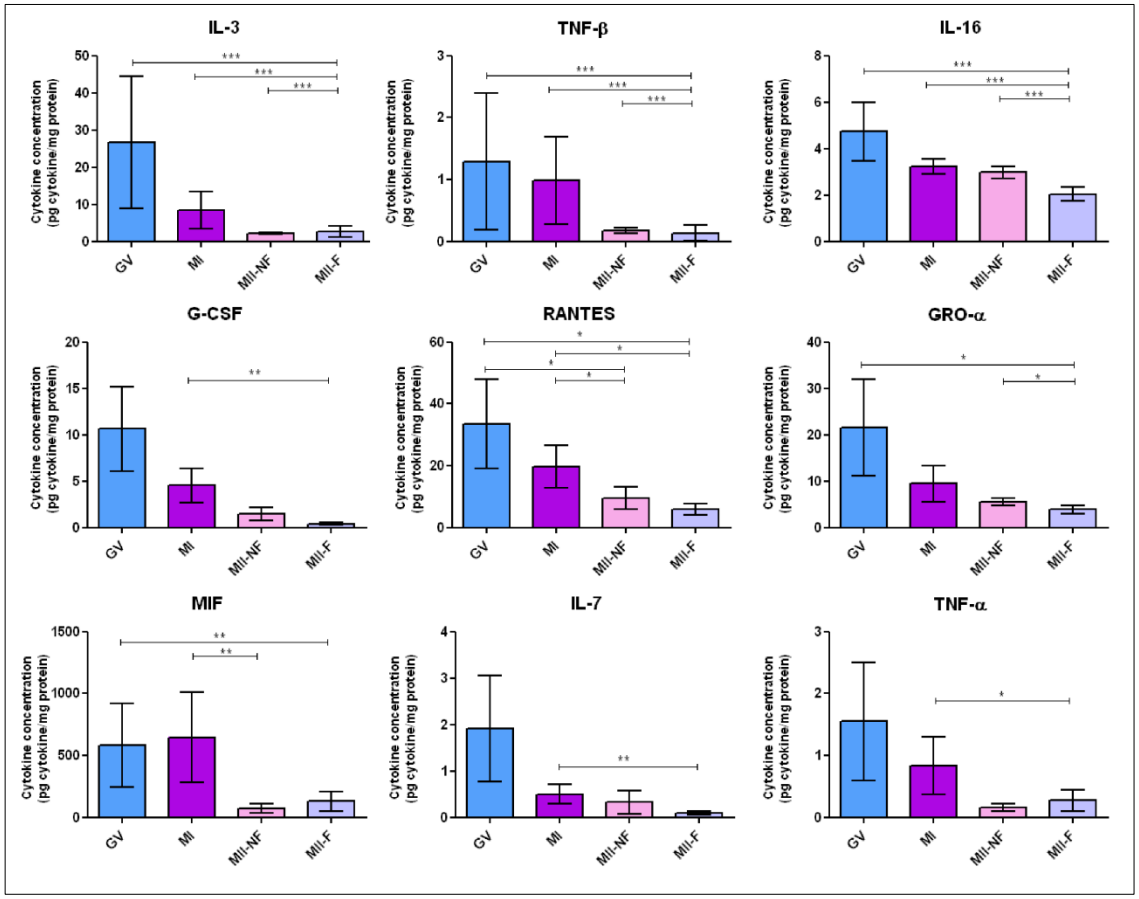


Figure 4-8: Further cytokines showing a decreasing concentration trend with maturity.

Asterisks indicate significant differences between follicles containing oocytes of differing maturity (*p<0.05, **p<0.01, ***p<0.001).

4.4.4 CRP

Variation in CRP concentration was large across follicles (Figure 4-9). CRP was significantly lower in follicles containing MII-F oocytes when compared with the other maturity levels (P<0.001). Plasma CRP levels were similar to those in FF in follicles yielding a MII-F oocyte.

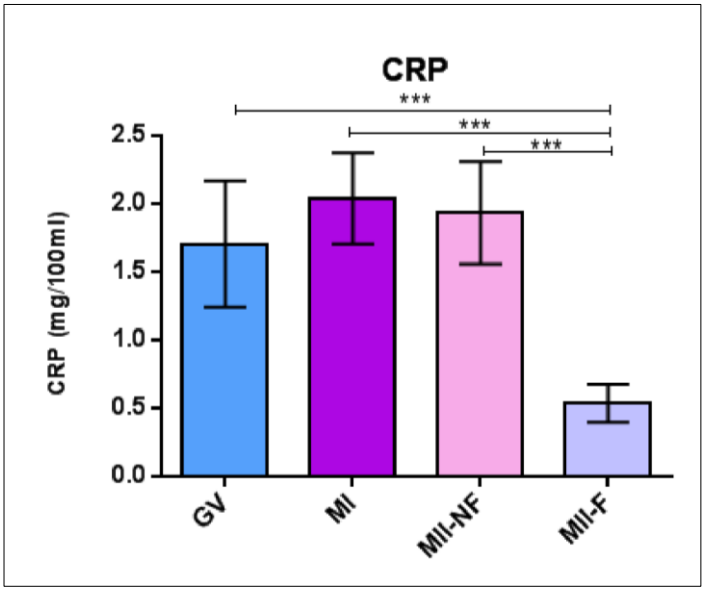


Figure 4-9: CRP concentration in follicles yielding oocytes of different maturity status.

4.4.5 Principal components analysis

Data were processed by principal components analysis (PCA). Each individual stage was analysed, then subsequently each maturity status was compared to the others, i.e. MII-F versus MII-NF, MII-F vs MI, MII-NF versus MI, MI versus GV.

4.4.5.1 GV

PCA of the GV stage revealed 7 principal components (PCs), with the first 2 PCs describing 75% of the variance (Appendix V). Within these PCs, GM-CSF and IL-6 were highlighted as important by PC1 (50% of variance), and G-CSF, MCP-1 and CTACK by PC2 (25% of variance, Figure 4-10).

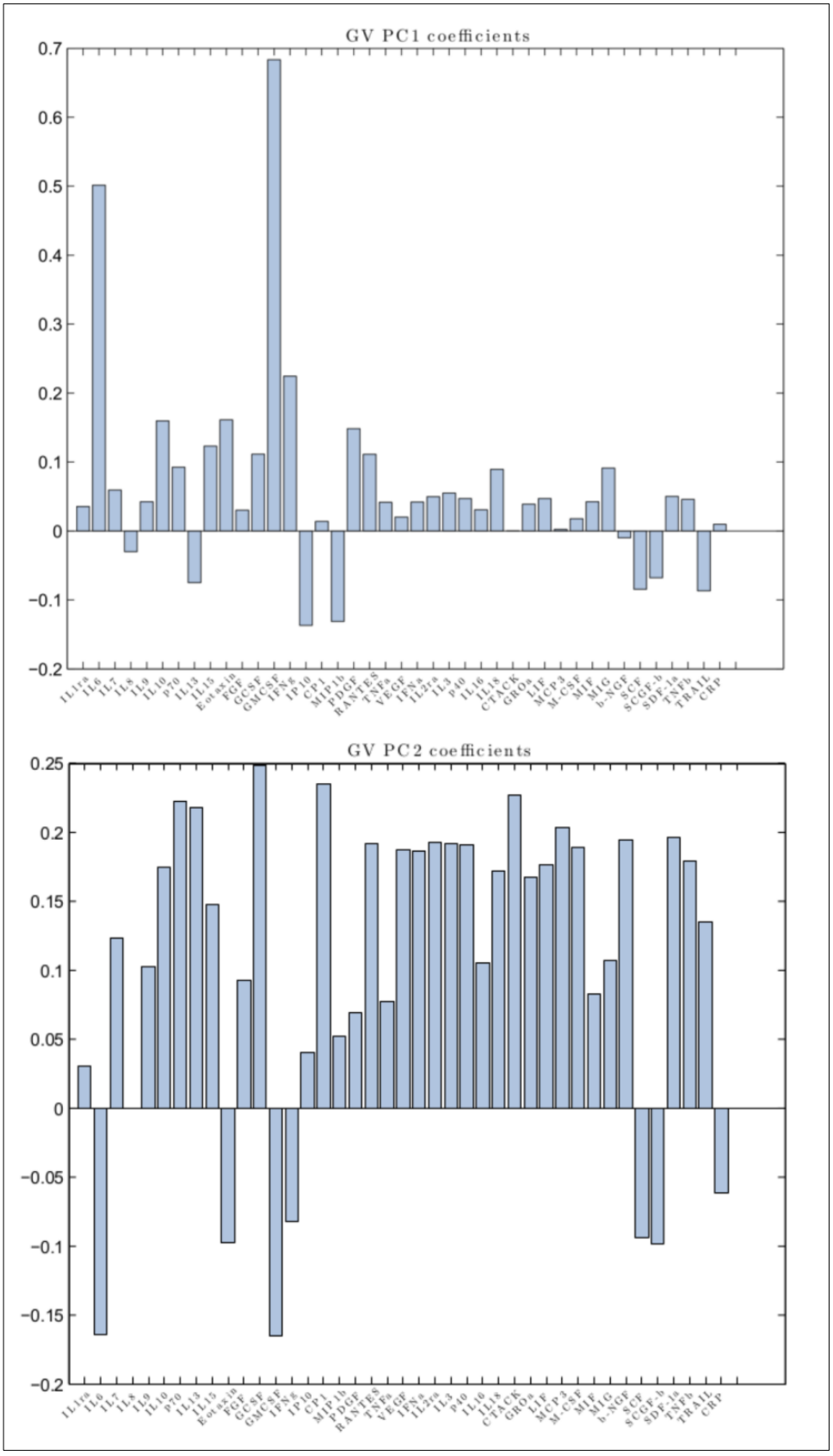


Figure 4-10: PC Coefficients for the GV stage of oocyte maturity.

4.4.5.2 MI

In follicles containing an MI oocyte, PCA revealed 10 PCs, with 65% of variance accounted for by the first 3 PCs (Appendix V). PC1 (35% of variance) denoted MIF, bFGF, TRAIL and IFN-α2 as important; PC2 (20% of variance) pointed to IL-16, βNGF, SCF and IL-18 while PC3 highlighted VEGF, IL-10, IL-8 and MIP-1β.

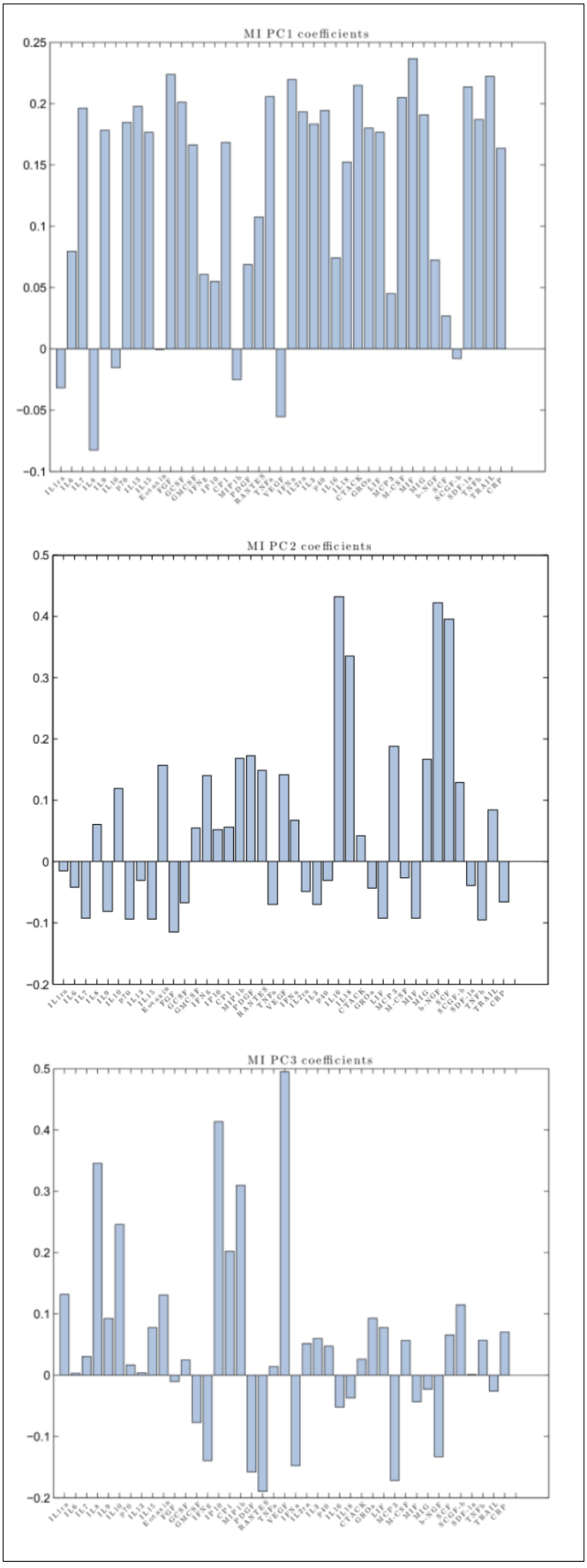


Figure 4-11: PC coefficients for oocytes at the MI stage of maturity.

4.4.5.3 MII-NF

Variation within the PCA for FF containing MII-NF oocytes was explained by the first 2 PCs of 10 generated (60%). PC1 revealed several cytokines which explained the variation to a greater degree than others (IL-12 (p40), M-CSF, IL-2ra, β-NGF, SDF-1α, IFN-α2 and IL-6) while PC2 highlighted just LIF and VEGF (Figure 4-12).

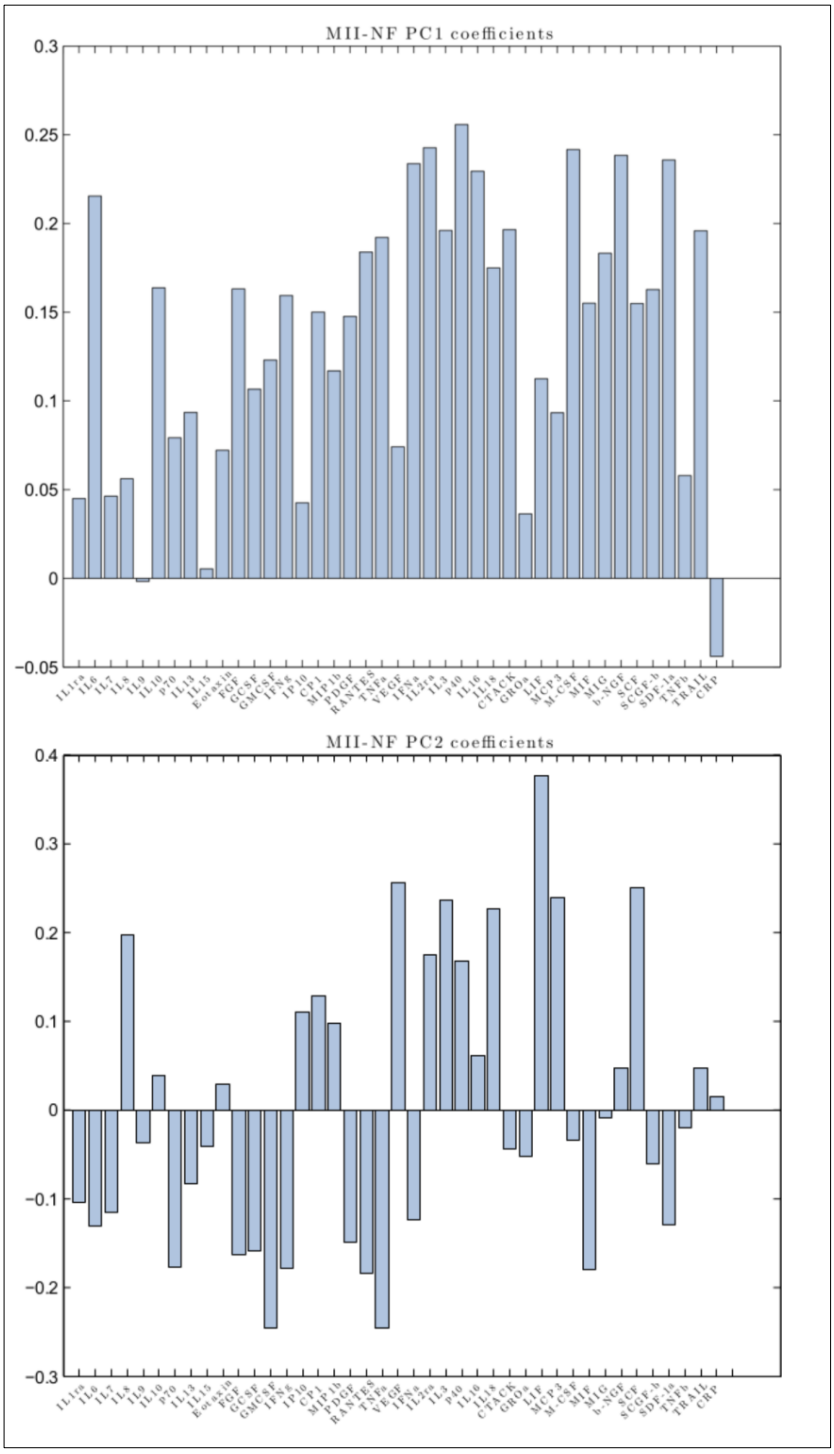


Figure 4-12: PC Coefficients for MII-NF oocyte FF

4.4.5.4 MII-F

60% of the variation within FF containing an MII-F oocyte was explained by PCs 1, 2 and 3. IFN-α2, IL-13, MCP-3 and TRAIL were highlighted by PC1, with PDGF, IL-7 and IL-12 (p70) dominating in PC2. PC3 showed the importance of IP-10 and MIP-1β.

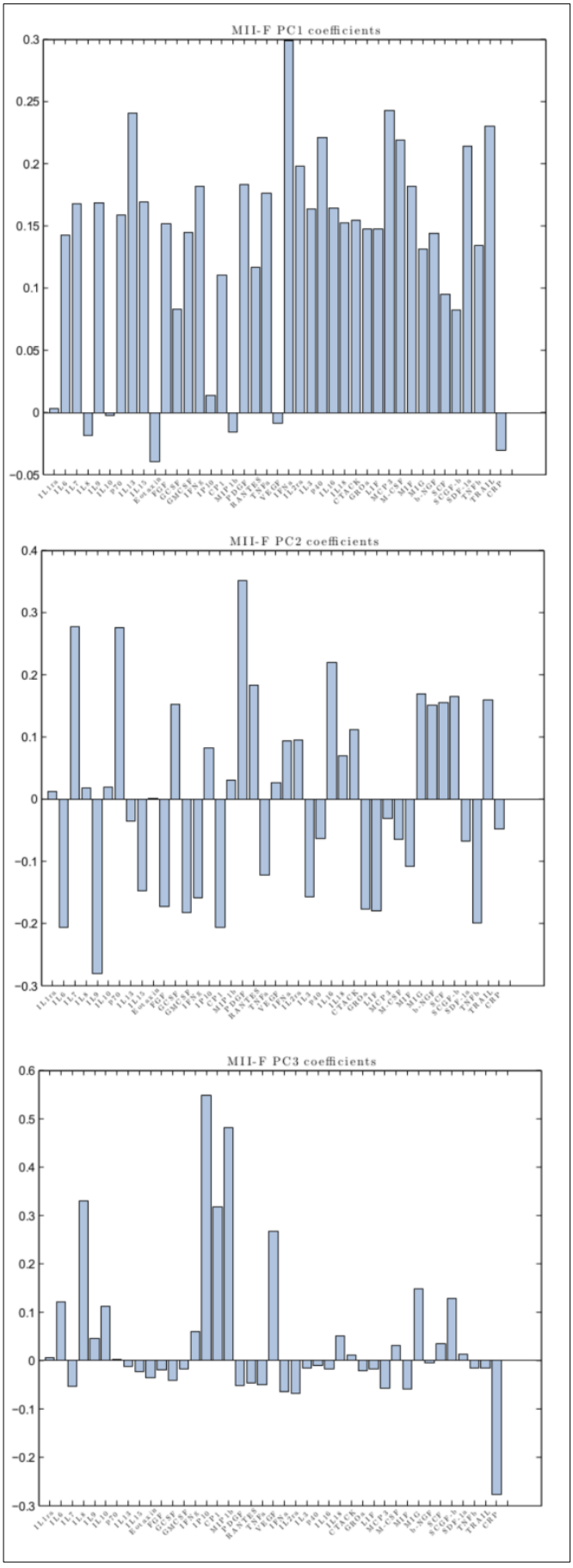


Figure 4-13: MII-F oocyte FF PC coeffiecients

4.4.5.5 MII-F versus MII-NF

The analysis of MII-F versus MII-NF revealed ten PCs, the first three describing 60% of the total variance (Appendix VI). Discrimination was observed between MII-NF and MII-F oocytes, when plotting PC1 versus PC2 (Figure 4-14), and to a much lesser extent when plotting PC1 versus PC3 and PC2 versus PC3 (Appendix VI). Principal component 1, which described 36% of the total variance, highlighted IFN-α2, TRAIL, M-CSF, SDF-1α and IL-13 (in order of importance) as explaining a large proportion of the variation (Figure 4-15). PC2 included IL-12 (p70), TRAIL, GM-CSF, LIF and TNF-β.

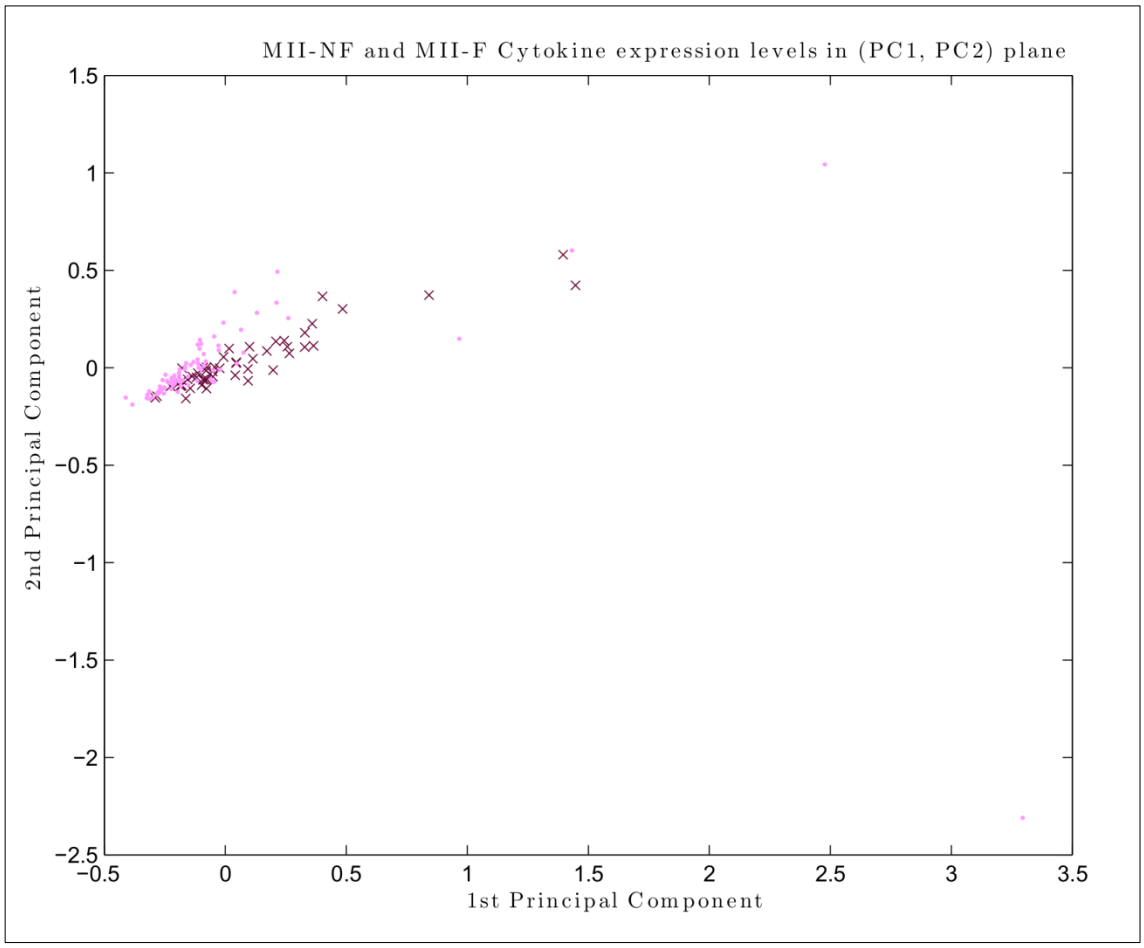


Figure 4-14: Principal components analysis plot for MII-F versus MII-NF oocytes.

MII-F oocytes are displayed as purple spots, while MII-NF oocytes are displayed as black crosses. A marginal separation can be seen between MII-F and MII-NF oocytes.

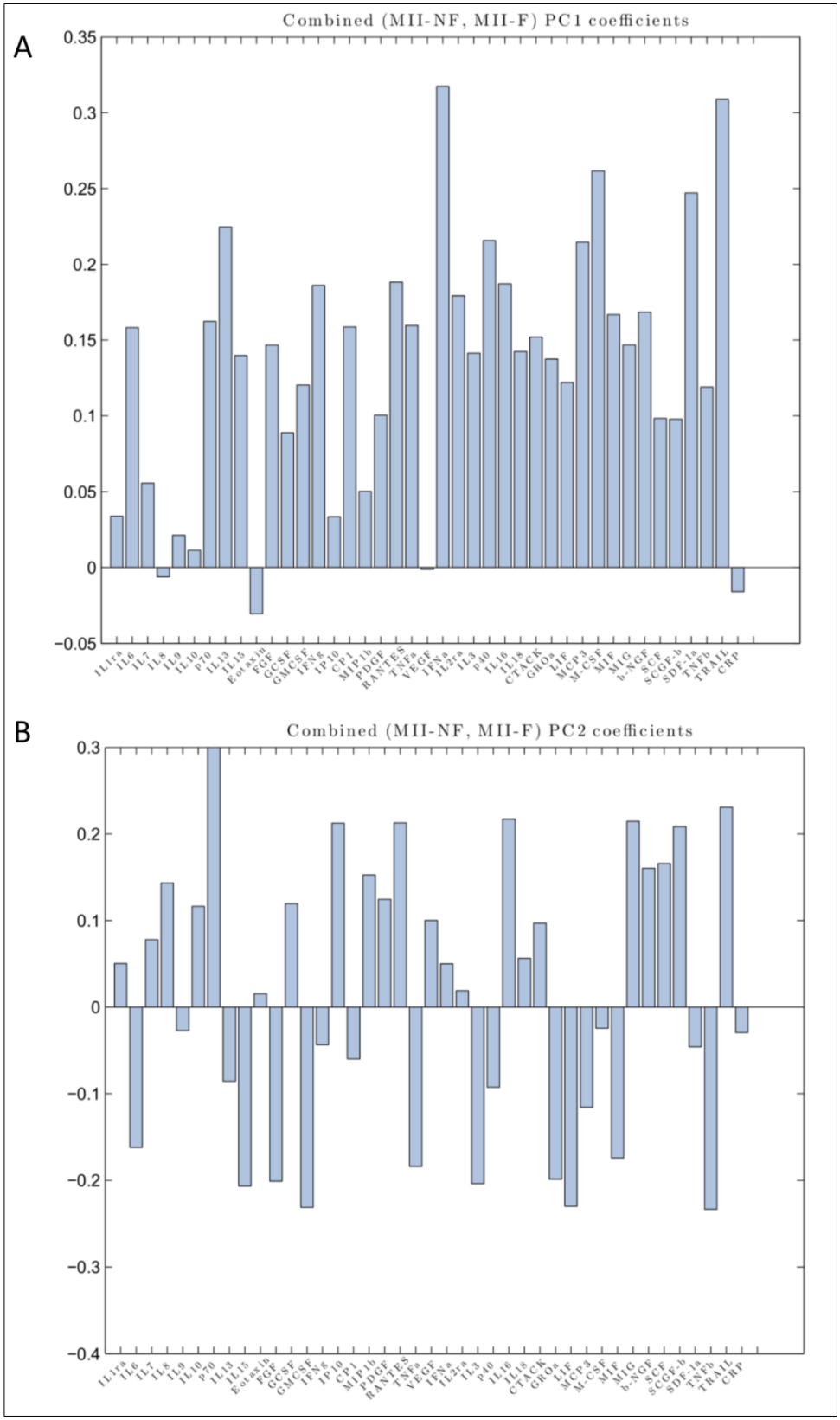


Figure 4-15: Combined coefficients for MII-NF and MII-F oocytes.

Relative importance of cytokines within PC1 (Panel A) and PC2 (Panel B).

4.4.5.6 MII-F versus MI

Pairwise comparisons between FF containing MII-F and MI oocytes revealed a separation between these maturity states (Figure 4-16). Of the 10 PCs, 60% of the variance was explained by PC 1, 2 and 3 (Appendix VI). PC1 revealed that IFN-α2, IL-13, TRAIL and FGF basic were influential in the separation of these maturity states (Figure 4-17). PC2 highlighted MIP-1β, SCGF-β and IP-10, while PC3 yielded IP-10 as its most influential cytokine.

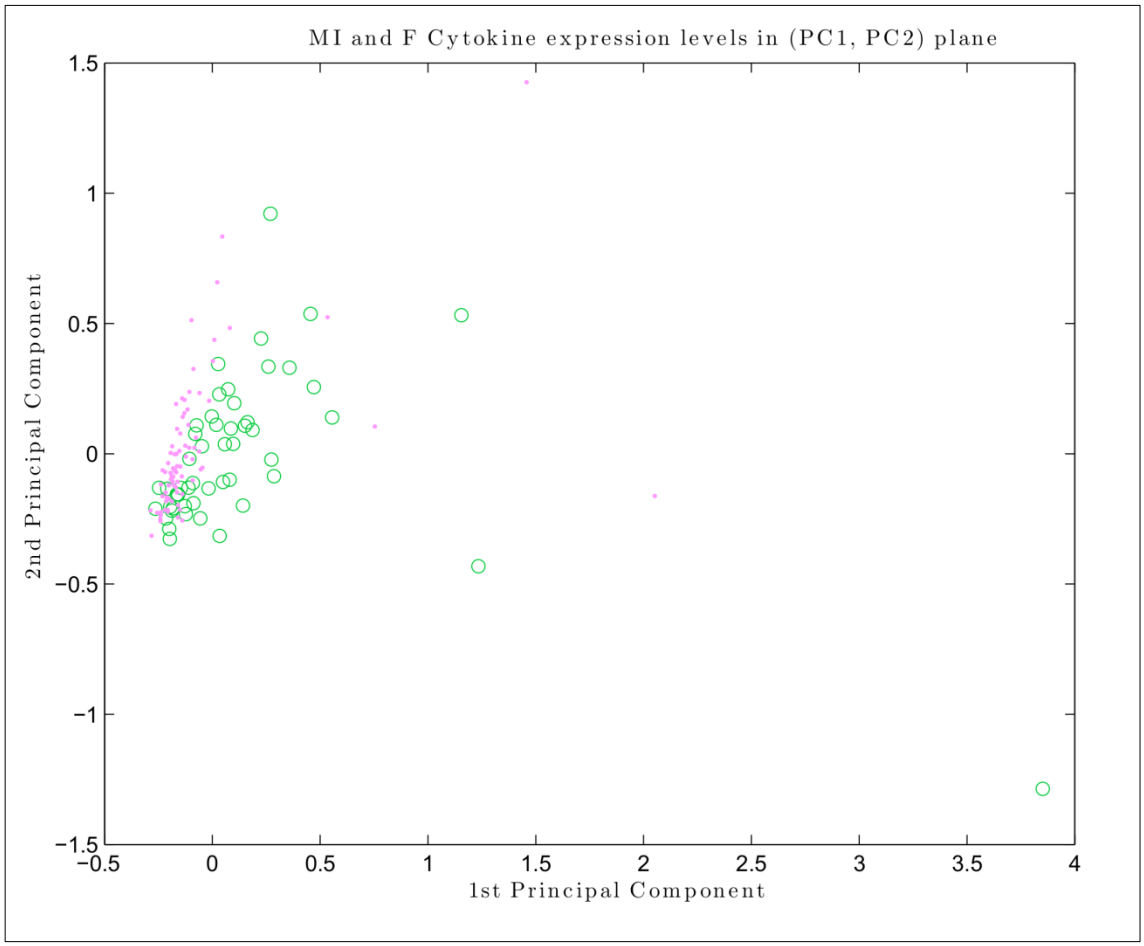


Figure 4-16: Pairwise PCA of FF containing MII-F and MI oocytes.

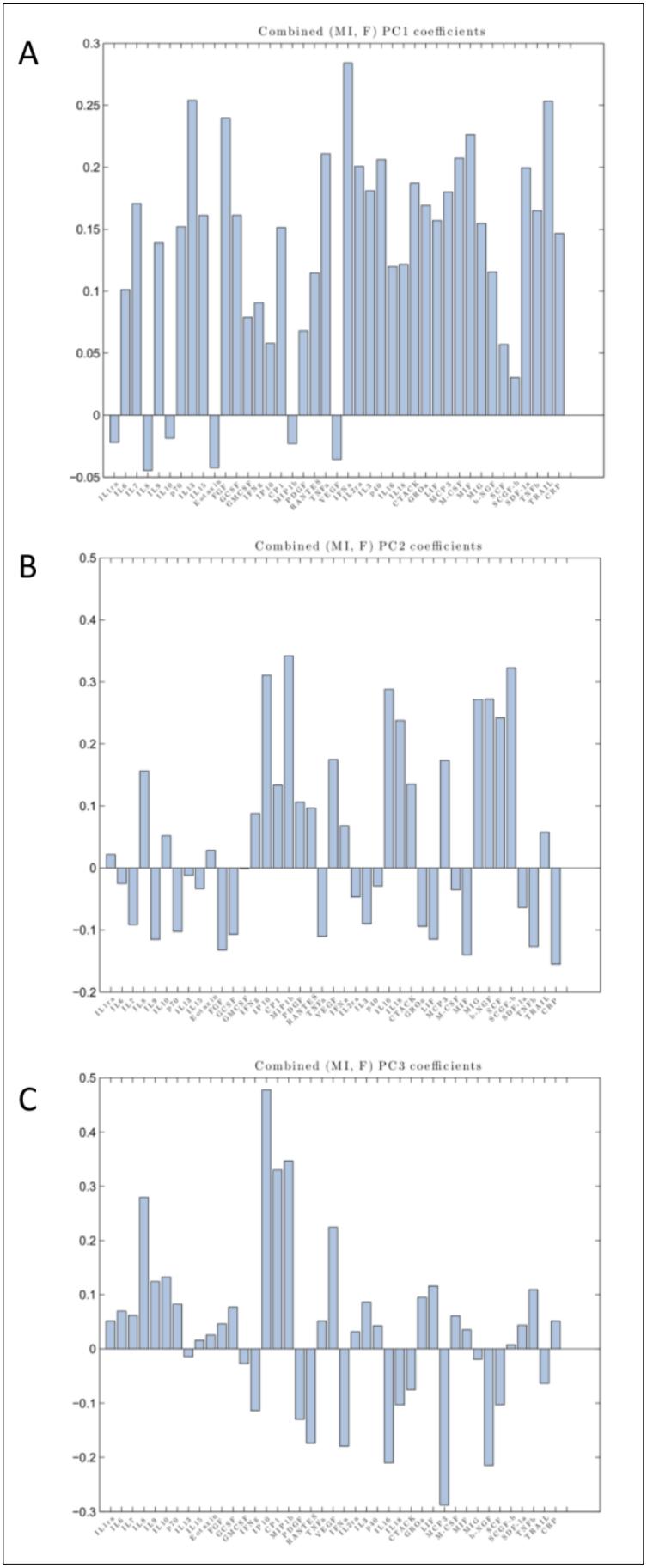


Figure 4-17: PCs associated with the pairwise comparison of MII-F and MI oocytes

4.4.5.7 MII-NF versus MI

PCA was unable to differentiate between MII-NF and MI oocytes (Figure 4-18). Of the ten principal components, 62% of the variation was explained by PC1, PC2 and PC3 (Appendix VI). Samples were equally distributed when plotting PC1 versus PC2, suggesting that these maturity states share a similar cytokine profile (Figure 4-18). Plotting PC3 did not improve separation (Appendix VI).

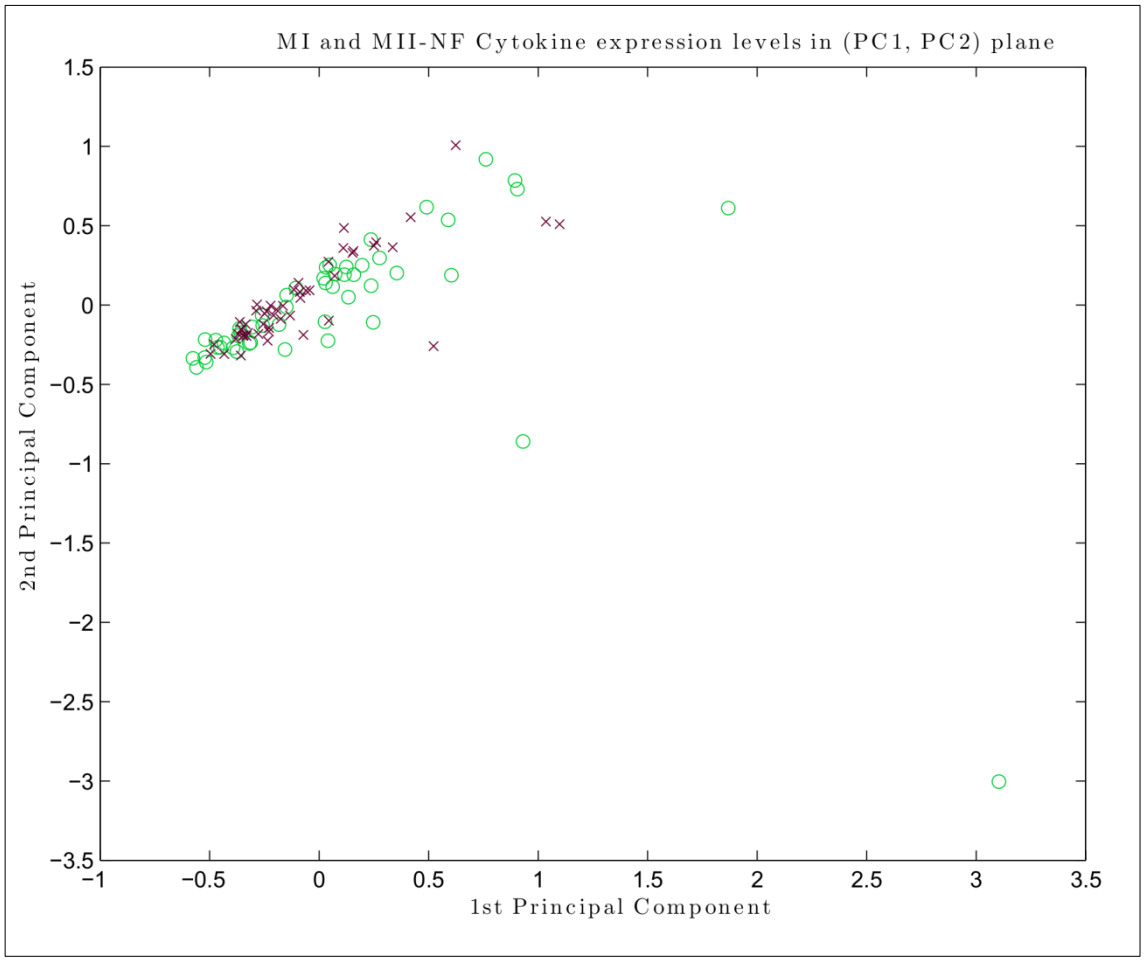


Figure 4-18: MI versus MII-NF principal components analysis.

Principal components analysis plots for MII-NF (black crosses) versus MI (green circles) oocytes. No distinct separation was noted between these maturity states.

4.4.5.8 MII-NF versus GV

PCA was unable to differentiate between the cytokine profiles of FF containing MII-NF and GV oocytes (Figure 4-19). 65% of the variation was explained by PCs 1 and 2 (Appendix VI).

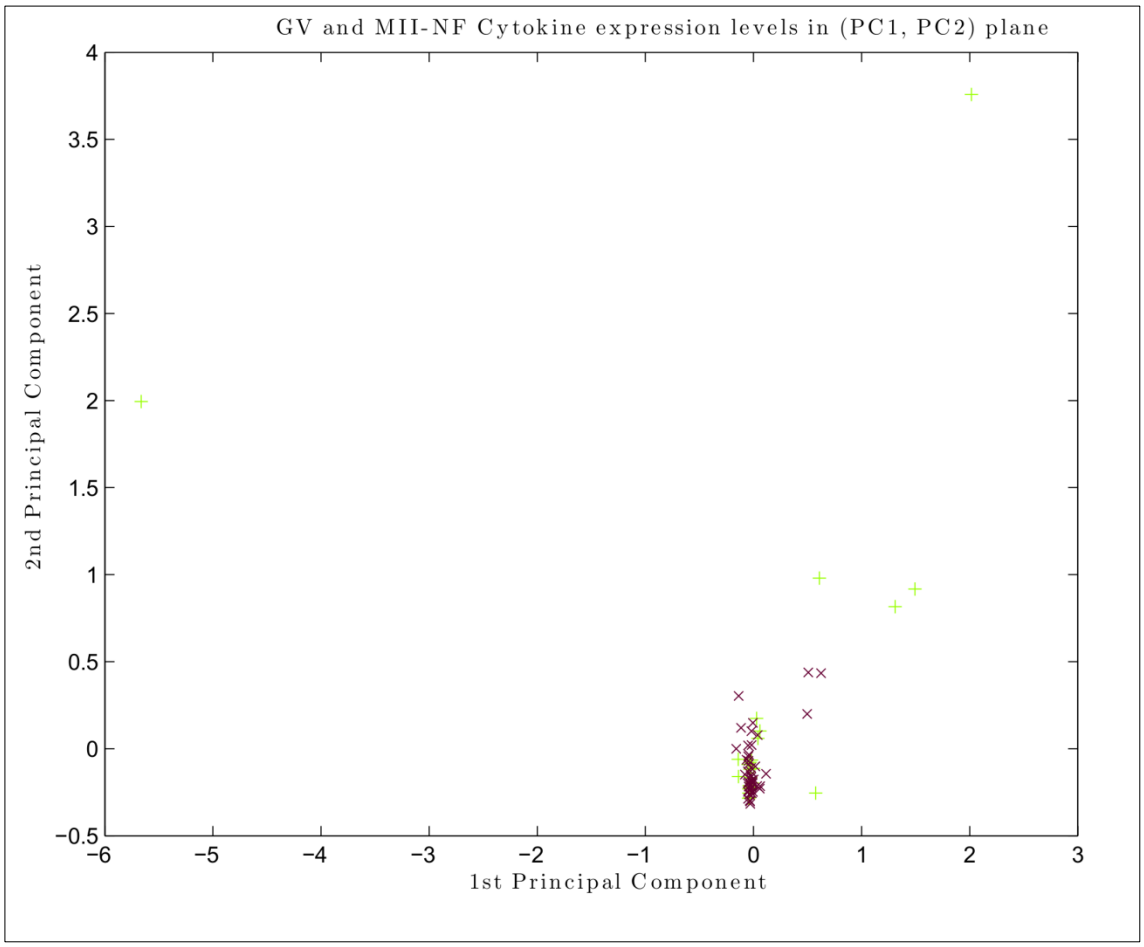


Figure 4-19: PCA plot of FF containing MII-NF versus GV oocytes.

No separation was seen between MII-NF (purple crosses) and GV oocytes (green plusses).

4.4.5.9 MI versus GV

As for MII-NF and MI, PCA was unable to differentiate between MI and GV oocytes (Figure 4-20). Of the ten principal components, 65% of the variation was explained by PC1 and PC2 (Appendix VI). Equal distribution was noted for both maturity states suggesting that these samples shared a similar cytokine profile.

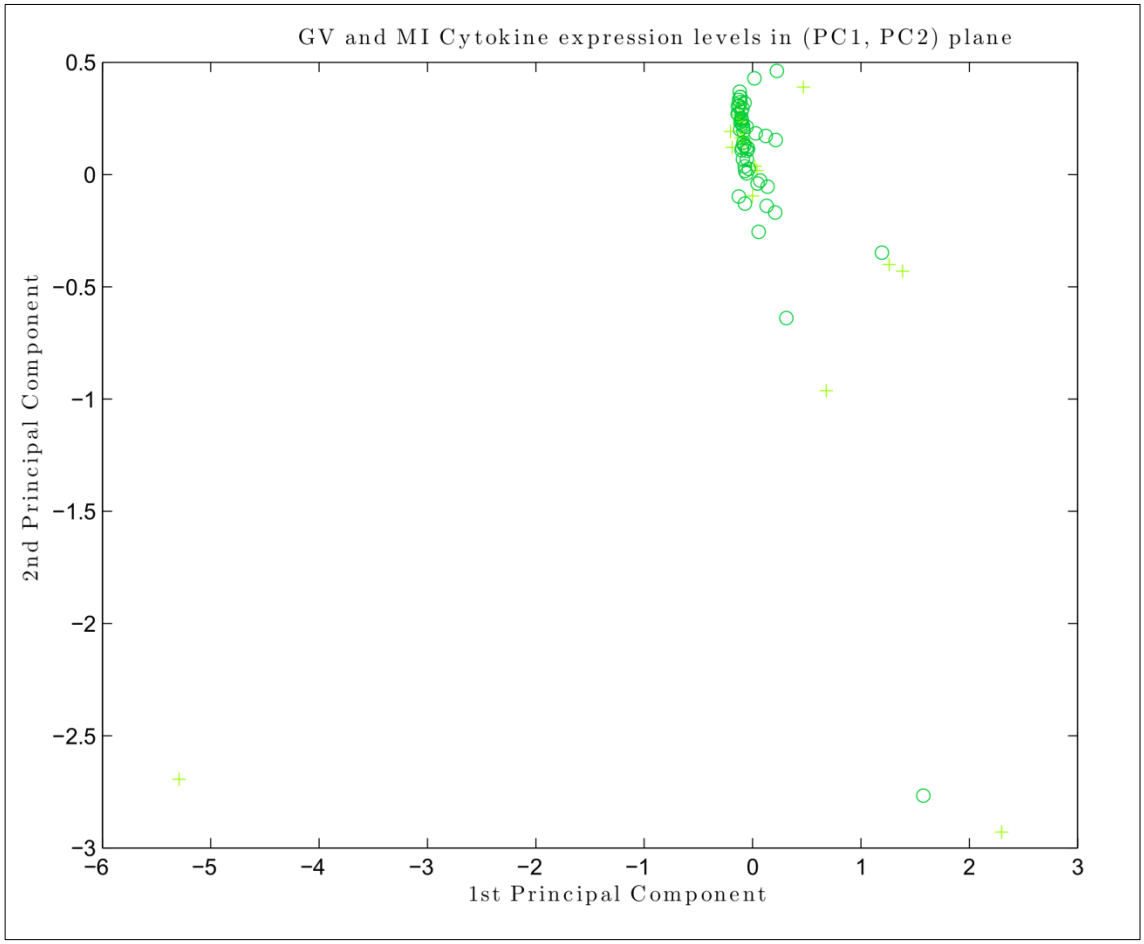


Figure 4-20: MI versus GV principal components analysis.

PCA for MI (green circles) versus GV (green plusses) oocytes. No distinct separation was noted between these maturity states.

4.4.6 Cytokine networks within FF

4.4.6.1 Networks in follicles containing a GV oocyte

A modified VBSSM network was generated from the data acquired from FF containing a GV oocyte (Figure 4-21). The network contained 30 cytokines, grouped into 5 communities. IL-1ra, IL-9, IL-13, IL-18, IP-10, M-CSF, MCP-3, MIF, β-NGF, RANTES and TNF-β appeared as parents within the different communities. IL-10 and VEGF appeared as hubs. Terminal nodes for each of the communities were represented by G-CSF, IFN-γ, TNF-α and TRAIL. Cytokines orphaned from this network were CTACK, eotaxin, bFGF, GM-CSF, GRO-α, IFN-α2, MIP-1β, monokine induced by gamma-interferon (MIG), PDGF, SCGF-β and SDF-1α.

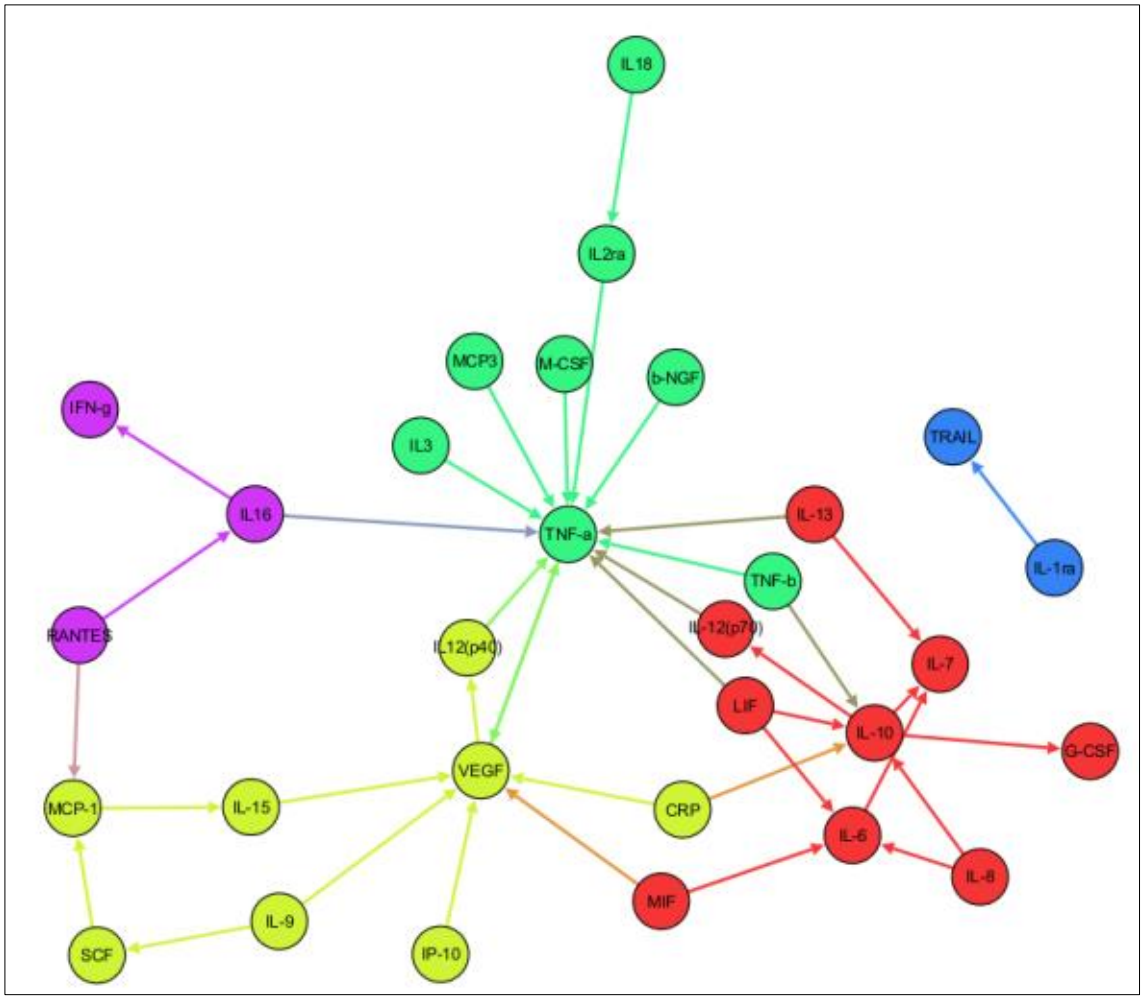


Figure 4-21: Gephi network generated from cytokines within the FF of follicles containing a GV oocyte.

Nodes are depicted in circles, which are colour coded according to community.

4.4.6.2 Network generated from follicles containing an MI oocyte

The network generated from FF containing an MI oocyte depicted 24 cytokines grouped into 4 communities (Figure 4-22). Eotaxin, IL-9, IL-13, IP-10 and RANTES appeared as parent nodes, while IL-2ra, IL-7, IL-12 (p40), IL-15, IL-16, M-CSF, β-NGF, PDGF, and TRAIL featured as terminal nodes. Hubs were represented by IL-6, IL-10 and VEGF. Orphaned nodes were CTACK, bFGF, G-CSF, GM-CSF, GRO-α, IFN-α2, IFN-γ, IL-3, IL-8, IL-12 (p70), LIF, MCP-3, MIG, SCGF-β, SDF-1α, TNF-α and TNF-β.

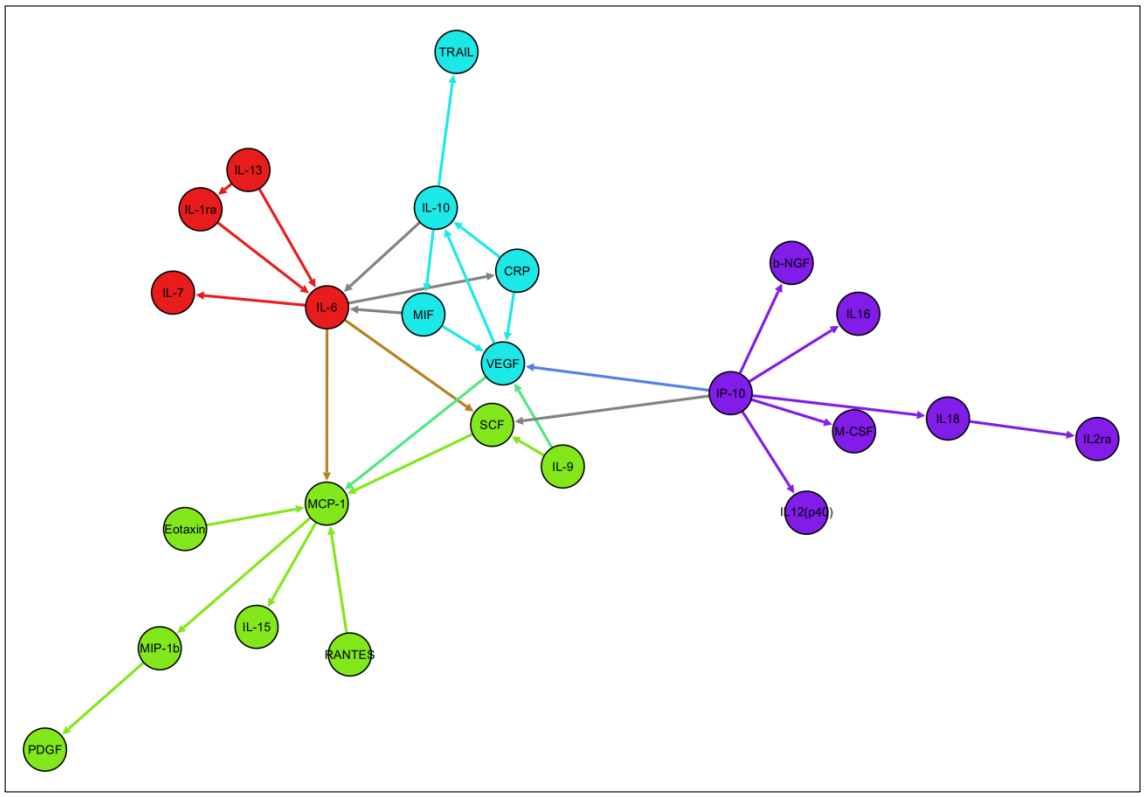


Figure 4-22: Gephi network generated from cytokines in the FF from follicles containing an MI oocyte.

4.4.6.3 Cytokine networks within follicles containing an MII-NF oocyte

The network generated from follicles containing an MII-NF oocyte contained 18 cytokines grouped into 4 communities (Figure 4-23). CRP, IL-9, IL-13, IL-18, IP-10, LIF and RANTES featured as parent nodes. The terminal nodes within each of the communities were represented by IL-2ra, IL-7, IL-15 and TRAIL, with IL-6, IL-10 and VEGF as hubs. Cytokines orphaned from this network were CTACK, eotaxin, bFGF, G-CSF, GRO-α, IFN-α2, IFN-γ, IL-3, IL-8, IL-12 (p40), IL-12 (p70), IL-16, IL-17, M-CSF, MCP-3, MIG, MIP-1β, β-NGF, PDGF, SCGF-β, SDF-1α, TNF-α and TNF-β.

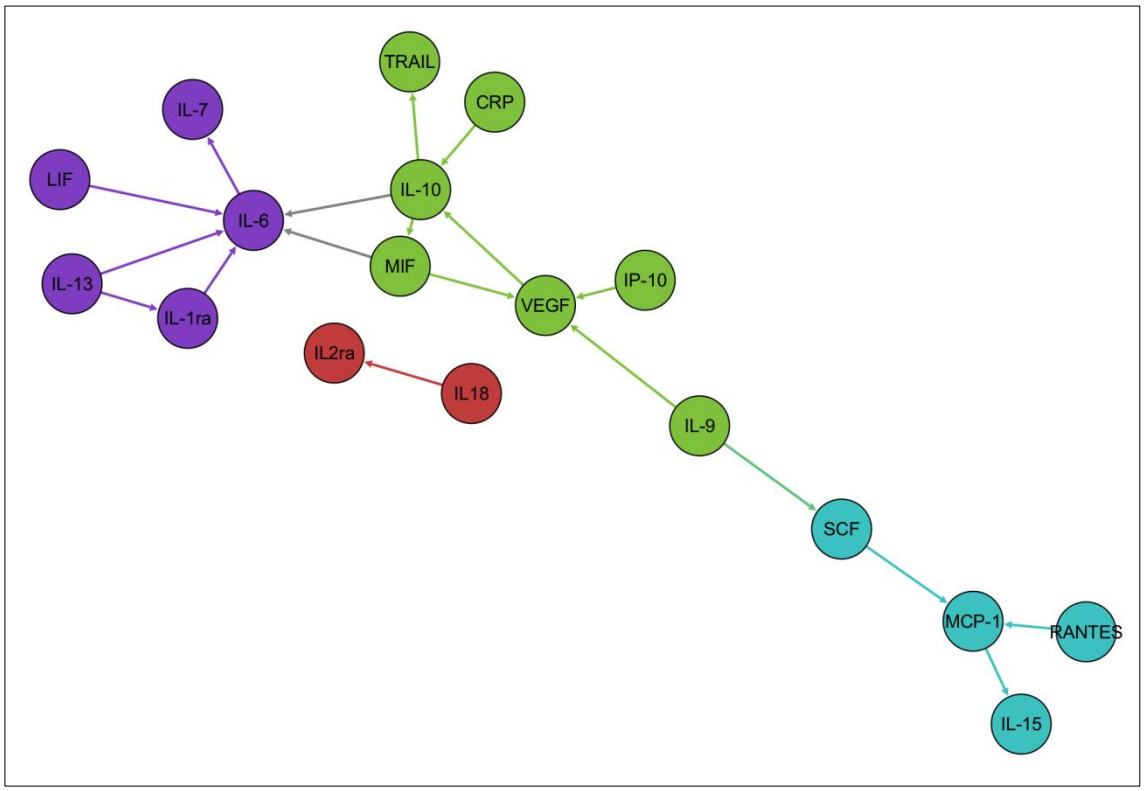


Figure 4-23: Cytokine network generated from follicles containing an MII-NF oocyte.

4.4.6.4 Cytokine networks in follicles containing an MII-F oocyte

The network generated from follicles containing an MII-F oocyte contained 22 cytokines grouped into 5 communities (Figure 4-24). Parent nodes were provided by IL-9, IL-18 and RANTES, with terminal nodes including IFN-α2, IL-3, IL-12 (p40) IL-12 (p70), IL-15, MCP-3, MIG, SCF and TRAIL. CRP, IL-10 and VEGF were hubs, while CTACK, eotaxin, bFGF, G-CSF, GRO-α, IFN-α2, IL-8, IL-16, LIF, M-CSF, MIF, β-NGF, PDGF, SCGF-β, SDF-1α, TNF-α and TNF-β were orphaned from the network.

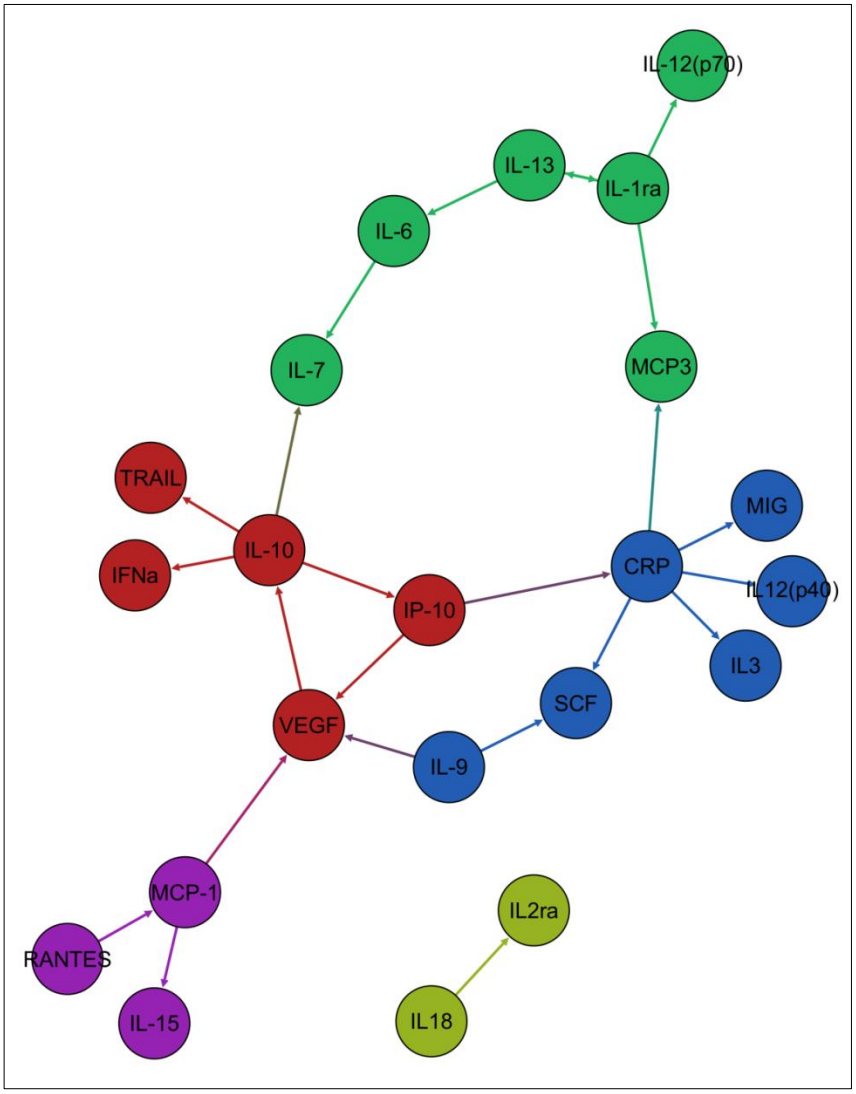


Figure 4-24: Gephi network provided by cytokines within follicles containing an MII-F oocyte.

4.4.6.5 Cytokines preserved across all networks

A number of cytokines were preserved across all networks, with many retaining relationships within communities. IL-1ra, IL-6 and IL-7 were connected with IL-13 in all networks (with the exception of IL-1ra in the GV oocyte containing follicles). IL-9 featured an outgoing edge to VEGF in all networks, and was also connected to SCF via an incoming edge. IL-10 was presented as a hub in all networks. IL-15 was always represented as a child of MCP-1, which also featured a connection with RANTES (a parent node in all networks). IL-2ra was connected with IL-18 in all networks, either as an independent community or embedded into another subnetwork. IP-10 featured as a parent in all networks except for MII-F, with an outgoing edge connecting this cytokine to VEGF. TRAIL featured as a terminal node in all networks.

4.4.6.6 MII-NF and MII-NF network comparison

As the result which may make the most difference clinically was the differentiation between MII-NF and MII-F oocytes, this section will concentrate in the similarities and differences between those networks. The networks generated from FF containing MII-F and MII-NF oocytes displayed both conserved cytokine relationships as well as having unique network component signatures. IL-1ra, IL-2ra, IL-6, IL-7, IL-9, IL-10, IL-13, IL-15, IL-18, IP-10, MCP-1, RANTES, SCF, TRAIL and VEGF were all present in both networks (Table 4-6). Cytokines present in the MII-NF network only were LIF and MIF, while the MII-F network was characterised by IFN-α2, IL-3, IL-12 (p40), IL-12 (p70), MCP-3 and MIG. CRP was present in both networks, as a parent in the MII-NF network, and as a hub in the MII-F network. Cytokines not present in either network were CTACK, eotaxin, bFGF, G-CSF, GRO-α, IFN-γ, IL-16, IL-17, M-CSF, β-NGF, PDGF, SCGF-β, SDF-1α, TNF-α and TNF-β, many of which were also absent from GV and MI networks.

Cytokines common to MII-F and MII-NF Networks	Cytokines present in MII-NF network only	Cytokines present in MII-F network only	Cytokines not present in either network
IL-1ra	LIF	IFN-α2	CTACK
IL-2ra	MIF	IL-3	Eotaxin
IL-6		IL-12 (p40)	bFGF
IL-7		IL-12 (p70)	G-CSF
IL-9		MCP-3	GRO-α
IL-10		MIG	IFN-γ
IL-13			IL-16
IL-15			M-CSF
IL-18			β-NGF
IP-10			PDGF
MCP-1			SCGF-β
RANTES			SDF-1α
SCF			TNF-α
TRAIL			TNF-β
VEGF			

Table 4-6: Cytokines common to and differing across the MII-F and MII-NF networks

4.4.7 Classification of oocytes

4.4.7.1 Multinomial modelling

Using a multinomial modelling approach across all 4 maturity states, a model generated by the inclusion of all cytokines detectable within the standard range correctly predicted 58% of the oocytes according to maturity stage (i.e. when taking into account the concentrations of all analytes). The best prediction was that of MII-F oocytes where 87.34% of oocytes were correctly categorised (Table 4-7).

		Predicted maturity			
		GV	MI	MII-NF	MII-F
Actual maturity	GV	10.53	21.05	57.89	10.53
	MI	14.71	35.29	32.35	17.65
	MII-NF	27.27	20.45	43.18	9.09
	MII-F	1.27	6.33	5.06	87.34

Table 4-7: Classification of oocyte maturity status by multinomial modelling. For each stage, the actual maturity versus predicted maturity is presented as a percentage through the CCR.

4.4.7.2 Cytokine selection

When cytokines were grouped and selected for their ability to predict oocyte maturity status by the Akaike Information Criterion (AIC), several cytokines were retained (CTACK eotaxin, FGF, IFN-α2, IL-1ra, IL-2ra, IL-3, IL-6, IL-7, IL-8, IL-12 (p40), IL-12 (p70), IL-16, MCP-1, MCP-3, M-CSF, MIF, MIP-1β, βNGF, PDGF, RANTES, SCF, SCGF-β, SDF-1α, TNF-α, TNF-β, TRAIL and VEGF). Correct classification was increased for all oocytes to 64%. Again, the best prediction was seen in MII-F oocytes, with 89.87% being correctly predicted as falling into their category (Table 4-8).

		Predicted maturity			
		GV	MI	MII-NF	MII-F
Actual maturity	GV	36.84	10.53	47.37	5.26
	MI	2.94	44.12	29.41	23.53
	MII-NF	27.27	18.18	45.45	9.09
	MII-F	1.27	3.80	5.06	89.87

Table 4-8: Classification of oocytes maturity status by Akaike Information Criterion. For each maturity, the actual maturity versus predicted maturity is presented as a percentage (CCR).

4.4.7.3 Prediction capability of groups of two and three cytokines

Cytokines were grouped into pairs in order to identify which cytokines give the best CCR and therefore may provide the basis for a diagnostic test. When taking pairs of cytokines, VEGF and TRAIL provided the best CCR of 57.3% (Table 4-9). This was comparable to the 58% CCR using the multinomial modelling approach.

Cytokine 1	Cytokine 2	CCR (%)
VEGF	TRAIL	57.3
IL-8	TRAIL	56.5
Eotaxin	TRAIL	55.6
G-CSF	TRAIL	55.6
IL-12 (p70)	TRAIL	55.2
MIP-1β	TRAIL	55.1
RANTES	TRAIL	54.8
IL-3	TRAIL	54.1

Table 4-9: CCR of oocyte maturity status by pairs of cytokines.

When triplets of cytokines were grouped and examined to determine the best CCR, IL-12 (p70), IL-18 and TRAIL provided a CCR of 59% (Table 4-10). This exceeded the CCR provided by multinomial modelling, but did not reach the level of prediction provided by AIC (64%). Further exploration of groups of 4, 5 or 6 cytokines did not improve the CCR.

Cytokine 1	Cytokine 2	Cytokine 3	CCR (%)
IL-12 (p70)	IL-18	TRAIL	59.0
Eotaxin	IL-18	TRAIL	58.9
Eotaxin	IL-8	TRAIL	58.9
IL-8	VEGF	TRAIL	58.7
IL-15	VEGF	TRAIL	58.7
IL-1ra	Eotaxin	TRAIL	58.5
IL-8	GM-CSF	TRAIL	58.4
IL-8	SCF	TRAIL	58.4

Table 4-10: Triplets of cytokines and their CCR of oocyte maturation.

4.5 Discussion

4.5.1 Oocyte maturation

The fact that cytokines are involved in oocyte maturation, folliculogenesis and ovulation is increasingly well-recognised, although likely perturbations in their profiles allied to assisted conception protocols are overlooked despite their putative impact on oocyte viability. Indeed, most methods of ovulation induction aim to maximise oocyte availability, with little regard to the quality of those oocytes and the environment in which they have matured.

In natural cycles, where a woman is monitored through her menstrual cycle for a dominant oocyte which is collected prior to ovulation for ICSI, it is highly unlikely that the oocyte will be assessed to be GV or MI (and therefore immature) in terms of nuclear maturation as the single dominant follicle often contains a mature oocyte [136, 996]. By contrast, in instances when women are subjected to COH regimens, many oocytes are retrieved in one session, a proportion of which will be immature. In the present study, this was highlighted by the high numbers of GV (8%) and MI (17%) oocytes collected from the women included in this study, which slightly exceeds the published combined proportion of between 15-20% [997]. Why not all oocytes respond to the maturation signals of exogenous gonadotrophins in the same manner is unclear, although this may be influenced by age, clinical condition, ovarian reserve and/or perifollicular vascularisation. However, in the present study, exclusion criteria would have limited the impact of many of these potential confounders, which suggests that the proportion of immature oocytes retrieved truly reflects the impact of ovarian hyperstimulation protocols.

To the best of the author's knowledge, this is the first study to examine the FF collected from individual follicles during assisted conception which has considered nuclear maturity as an outcome measure, and discovered a difference between MII oocytes which do/don't fertilise. These follicles used as part of this study appeared to have the features consistent with those yielding a mature oocyte, such as being of large size (>17mm diameter) and ample volume. The fact that many harboured an immature oocyte points to a dysregulation (likely anachronistic) of folliculogenesis and oocyte maturation, processes which are intricately linked in natural cycles.

4.5.2 FF cytokine composition

FF cytokine composition varied according to oocyte maturity. In general terms, cytokine concentrations fell (with the exception of eotaxin, VEGF and IL-8) in parallel with increasing oocyte maturity. This was not due to a dilution effect as all samples were normalised to total protein levels and adjusted for peripheral blood contamination, and therefore represented a physiologically relevant dynamic change. Little is known about the role of eotaxin in the ovary, although the presence of its ovarian transcripts has been reported in the rat [998]. Eotaxin has been shown to rise sharply in ovarian tissues in response to the preovulatory LH surge in mice; this observation supports the present data where those oocytes which exhibited the highest level of nuclear maturity had the highest relative FF eotaxin concentration.

Although low FF VEGF levels have been associated with improved maturational status at the point of oocyte recovery [948], the opposite relationship was found in the present study. The presence of VEGF is essential, however, as this cytokine positively correlates with perifollicular blood flow [949], which contributes to the creation of a favourable environment for oocyte maturation, as demonstrated by the fact that oocytes from well-vascularised follicles exhibit increased fertilisation and pregnancy rates [949-954], which would tie in better with the present findings. However, high VEGF levels can also contribute to pathological follicular developmental processes and correlate with reduced pregnancy rate [944]. This apparent discrepancy likely reflects the delicate balance required between having sufficient VEGF to induce adequate angiogenesis which keeps pace with follicular development, while excessively high levels may simply reflect an inadequate attempt at supporting this process in compromised/hypoxic follicles. The disparate angiogenic properties of the various VEGF isoforms (which are not typically resolved in most studies) likely further contribute to the lack of clarity in this picture [718]. This presents an opportunity for future study, particularly given that the ovarian stimulation regimes utilised in assisted conception reportedly alter VEGF production [706, 938, 960].

The present observation that FF IL-8 levels increase with oocyte maturity is supported in the literature only in relation to follicle size. IL-8 concentrations increase with follicle size, promoting the recruitment of the leukocytes required to mediate ovulation [2, 681, 824]. IL-8 has been shown to be stimulated by VEGF in bovine theca cells, promoting angiogenesis and improving perifollicular blood flow, and the previously discussed rise in VEGF may thus account for the observed concomitant increase in IL-8 [782, 783]. Interestingly, rat models have shown that IL-8 may act as an angiogenic factor during

late folliculogenesis [778-781]. In this regard, IL-8 was only represented within the GV network and not within those of more mature categories. Moreover, IL-8 did not share a community with VEGF. Other pro-angiogenic cytokines include bFGF and TNF-α, which were orphaned from all networks. This may point to a degree of functional redundancy within the system, in that other cytokines may also participate in supporting/regulating angiogenesis. In the present data, RANTES (present in all networks) may offer this redundancy, as this cytokine exhibits pro-angiogenic properties in pathological scenarios such as atherosclerosis [999].

4.5.3 Cytokine profile differences between maturity states

The greatest differences in cytokine profiles were seen between the most mature oocytes (MII) and the other maturity stages, which supports the notion that cytokines are intricately involved in coordinating oocyte maturation. What was most surprising was the striking difference between MII-F and MII-NF oocytes. These oocytes had reached nuclear maturity and were morphologically identical, yet they were distinguished by their FF cytokine profiles as demonstrated by profile plots and PCA. This could be due to a lack of cytoplasmic maturity, since nuclear and cytoplasmic aspects of maturation are considered to be asynchronous in stimulated cycles, with oocytes acquiring the ability to fertilise throughout the MII arrest phase [1000]. Although not formally assessed for cytoplasmic maturity, the operator selecting oocytes for the ICSI procedure made a visual check of the cytoplasm (for ‘graininess’ and irregularity, presence of vacuoles and refractile bodies) and upon mechanical stripping ensured that cumulus cells were not compact and stripped away easily, suggesting that these MII oocytes were all competent for fertilisation. However, the ‘progressive’ nature of the changes in cytokine profiles suggests that perhaps these MII oocytes were not fully competent at the time of ICSI, such that cytokines may present a useful point-of-care biomarker-based assay to aid embryologists in selecting which oocytes most likely to fertilise should undergo ICSI first (since the process is reasonably labour-intensive and exposes denuded oocytes to *in vitro*-related insults, rapid handling of those oocytes most likely to fertilise would likely improve cycle outcome). An alternative hypothesis is, of course, that MII-NF oocytes may never reach a stage where fertilisation is possible; i.e. that their fate has already been predicated earlier in their maturation/development.

Figure 4-25 displays the potential routes open to an oocyte in terms of maturation. Scenario 1 is a linear progression from GV to MII-F, a process which is accepted in present IVF circles and forms the basis of *in vitro* maturation (IVM) of immature oocytes [1001]. However, IVM remains a very inefficient process, with few oocytes fertilising

and implanting to produce a viable pregnancy which has been postulated to be caused by the lack of support from the follicular environment to maintain adequate nuclear and cytoplasmic maturation [1002]. However, these low success rates could also be explained by the fact that some oocytes may never be destined to become competent following COH (either through a flaw in development or through an inherent inability to complete nuclear/cytoplasmic maturation). Scenario 2 proposes instead that development progresses normally to the MI phase, but experiences a divergence at this point to create MII-F and MII-NF oocytes. The implications of this being are that those oocytes which progress down the MII-NF route at this stage would not be able gain the ability to fertilise (and would therefore not benefit from IVM). Indeed, IVM of human oocytes is still considered to be experimental due to the low fertilisation rates [1003, 1004]. Finally, Scenario 3 takes the divergence back even further, in that an oocyte's fertilisation potential may be decided upon at the GV stage. This is highly speculative, and would require extensive research to elucidate which pathway (or pathways) are most likely to be involved.

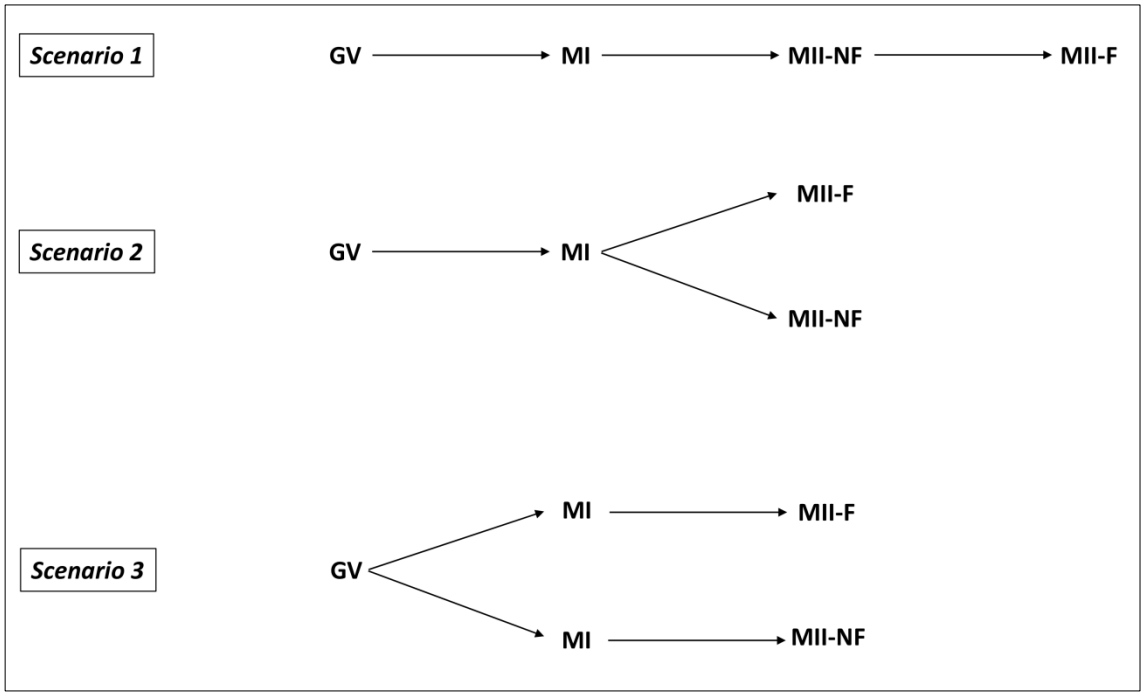


Figure 4-25: Hypothetical oocyte maturation pathways.

4.5.4 Advantages of the variational Bayesian approach

Within the lactation data set presented in Chapter 3, Bayesian networks were constructed using a prior network generated using TabuSearch in order to bias the

heuristic search/optimisation algorithm. These were structurally robust and enabled the exploration of cytokine interactions within the networks themselves. However, further scientific advances since completing that work revealed that other experimental approaches may provide a more accurate approximation of the underlying networks. The VBSSM approach used in Chapter 3 was based on data only. However, a method of combining a VBSSM network with prior knowledge was developed in conjunction with Dr Tathagata Dasgupta (University of Harvard), based upon an extension of the work developed by Dr Matthew J. Beal [486]. This approach offered several advantages over the previous Bayesian approach, chief amongst which that VBSSM takes into consideration 'hidden factors' whereas the previous Bayesian approach did not. In any network, there is the potential that various unmeasured factors could influence the connections between the measured nodes, given that it is experimentally difficult to measure every conceivable factor in any one system. The search algorithm used in this Chapter took into account these 'hidden' nodes, and presented a network which represented their influence but compressed it into a known network of those measured factors. The addition of a prior network to VBSSM network learning is wholly novel, and served to refine the network to give a very strong approximation of the true network. This approach does have limitations, in that VBSSM networks generally only indicate the structure of the network i.e. the direction of the relationships between nodes and the structure of the communities or subnetworks. This novel method is now being modified to include node-specific conditional probabilities with a view to initiating *in silico* network challenges to reveal more about the nature of cytokine interactions.

4.5.5 Cytokine networks within FF containing immature oocytes

The networks discovered by the present study were conserved across all of the stages of maturation, with some communities retaining consistent membership. This suggested that there may be core cytokine interactions which are preserved and tightly regulated within the ovarian follicle, in much the same manner as conserved systemic networks were identified in murine lactation. For example, the present study demonstrated that IL-13, along with IL-1ra, IL-6 and IL-7, did not vary significantly across FF from different maturity states. However, elevated IL-13 concentrations in FF have been shown to correlate with poor pregnancy rates [978], while increased IL-6 and IL-13, and decreased IL-7 are indicative of PCOS [1005-1007]. Therefore, it is perhaps not surprising that this group of cytokines should be under tight regulation and vary little between follicles in women without a tendency towards the PCOS spectrum. Indeed, IL-13 could be considered as a parent node across all networks, and demonstrated connections within a community with IL-1ra, IL-6 and IL-7.

Other examples of cytokines preserved in communities across all networks were IL-9, VEGF and SCF. SCF has been shown to be critical to various stages of murine oocyte development, with recognised associations with normal nuclear maturation and the promotion of polar body extrusion [754, 885]. As described earlier, VEGF plays a central role in folliculogenesis too such that it is not an unexpected finding that these two cytokines should be preserved across networks. Data remain scarce regarding the role played by IL-9 in folliculogenesis, although this mediator is known to promote cell proliferation and repress apoptosis such that it may be involved in follicular growth [1008].

TRAIL was featured in all networks as a terminal node, and was the only cytokine to be present in all cytokine sets when classifying oocytes into their maturation state. TRAIL and its pro- and anti-apoptotic receptors are expressed in GCs where they may regulate apoptosis and follicular atresia [770], as highlighted by porcine studies[1009]. This particular cytokine is known to induce apoptosis (specifically in tumour cells) via the death receptors DR4 and DR5 [1010-1012], although very little is known about its physiological role. However, GC resistance to TRAIL has been shown to be a major factor in GC ovarian cancer, where the regulation of apoptosis is dysregulated, resulting in unchecked proliferation [1013, 1014]. Therefore, it would be anticipated that this cytokine should be tightly regulated across all stages of follicular development in COH (indeed, the risk of developing ovarian cancer following COH is low according to a recent Cochrane review) [1015]. The terminal node status afforded to TRAIL in the networks herein suggests that it is an ‘endpoint’ to a communicative process. As with the networks generated in Chapter 3, these networks preclude the display of feedback loops which may exist within the signalling pathways. Therefore, two possibilities exist, TRAIL could either be the culmination of the signalling process and provide the ultimate regulatory node of follicle growth/atresia, or it could be an artefact of the network assembly. Although the latter is a possibility, evidence from the statistical-based modelling approach which aimed to correctly classify oocyte maturity status based on cytokine profiles also highlighted TRAIL as a significant component present in all cytokine groupings. Thus, this is the first study to demonstrate that TRAIL concentrations are raised in follicles containing immature oocytes or those which have attained nuclear maturity but have a low fertilisation potential. This suggests that raised FF TRAIL concentrations in Graafian follicles during COH could indicate a follicle which may otherwise have been destined for atresia and thus contains an oocyte with reduced viability.

Many other cytokines, such as IL-10 (a hub in all networks), RANTES (in a community with IL-15 and MCP-1), IL-2ra (connected to IL-18) and IP-10 were conserved across the networks. However, the roles of these FF cytokines have yet to be elucidated in their present context. Their conserved nature across the present networks suggests that these agents should be the focus of future study. There were also several cytokines which were orphaned from all of the networks (CTACK, eotaxin, bFGF, G-CSF, GRO-α, IL-16, M-CSF, β-NGF, PDGF, SCGF-β, SDF-1α, TNF-α and TNF-β) which was perhaps surprising given that a number of these showed a statistical trend to differentiating between oocyte maturity status (CTACK, eotaxin, G-CSF, GRO-α, IL-16, M-CSF, β-NGF and SDF-1α). However, as has been noted in many scientific studies, correlation does not always imply causation [1016] such that drawing conclusions from basic statistical inferences can be misleading. This is one particular advantage of using causal networks based on prior information which can 'filter out' those nodes which may essentially be 'noise' in the system [490, 1017].

For each maturation stage, the networks revealed novel relationships between cytokines. The GV network was the most complex, perhaps reflecting the efforts of the follicle to stimulate maturation in an unresponsive oocyte, or indicating that follicular atresia is at an advanced stage. PCA analysis identified GM-CSF and IL-6 as cytokines which are responsible for much of the variance within the data, despite the fact that both of these cytokines did not differ significantly across maturity levels. In fact, GM-CSF is orphaned from all of the networks presented, and this discrepancy could be explained as an artefact of the PCA analysis. PCA is an unsupervised pattern recognition technique which reduces large datasets into a small number of PCs, which in turn represent the variation in the data [1018]. In general, PCA reduces 'noisy' data into components which filter out that noise and highlight important factors [1019], although it does have its limitations. If the variables are not correlated in the first instance, then PCA simply ranks variables according to their variance rather than their relative importance to other variables. PCA is also very sensitive to scale and normalisation, such that the correct preparation of the data is paramount to the outcome. It also does not offer the advantage of taking into account prior knowledge, thus ranking the influence of variables according to known relationships. Despite this, there was a certain level of agreement between PCA and network analysis for each stage of maturation. At the GV stage, some of the most influential variables highlighted by PCA were also represented in the network (IL-6, IL-10 and IP-10). Although IL-6 is known to influence oocyte quality at the later stages (MII and cumulus expansion)

[819], information as to its role in early oocyte maturation is lacking. IL-6 is both anti-inflammatory and anti-apoptotic [1020], so its importance in follicles containing a GV oocyte following COH could reflect a rescue mechanism whereby a follicle which would normally have become atretic is saved by exogenous hormones. Indeed, IL-6 has been shown to be induced in pre-ovulatory follicles in response to gonadotrophins [799]. Similarly, little is known about the role of IP-10 in folliculogenesis, although it featured in all networks and always with an outgoing edge to VEGF. The relationship between IP-10 and VEGF is inhibitory, in that IP-10 is anti-angiogenic [1021-1023], and could indicate that angiogenesis is under tight regulatory control. In the present networks, IP-10 is featured as a parent node at all maturity stages except MII-F, possibly reflecting a release on the control on angiogenesis in follicles which contain oocyte with the highest potential to fertilise.

At the MI stage, both MIF and TRAIL were highlighted as being important in explaining the variance in PCA, and featured prominently within the network. MIF mediates the primary to antral follicle transition via stimulation of GC and TC proliferation, such that it is unsurprising that this cytokine should be prominent at the MI stage [733]. The implications of this cytokine differentiating between immature and MII-F oocytes are discussed below.

4.5.6 Differentiation between MII-NF and MII-NF oocytes

Of particular interest were the network cytokines which differentiated MII-NF and MII-F oocytes. LIF and MIF were only present in the MII-NF network, while IFN-α2, IL-3, IL-12 (p40), IL-12 (p70), MCP-3 and MIG were present in the MII-F network. LIF, highlighted by both PCA and network analyses, is known to promote the primordial to primary follicle transition in the mouse [660, 666], and appears to modulate cumulus expansion in both humans and mice [904]. Previous studies have shown that LIF is present at much higher concentrations in preovulatory follicles compared to immature follicles [906], which is supported by the present data (although the findings are not statistically significant). Although FF LIF levels have been linked with oocyte quality and subsequent implantation potential in humans [907], this correlation remains inconsistent across studies [908, 909], and is not supported by the present networks. MIF is a cytokine which promotes GC proliferation and differentiation [1024, 1025], and is present in all networks except MII-F. Indeed, MIF was measured at higher concentrations in GV and MI follicles, although there was little difference between MII-NF and MII-F stages. The presence of both of these cytokines in the MII-NF network

may suggest that these follicles containing less mature oocytes are still expending energy in order to reach ovulatory status in response to COH.

The MII-F network was more complex and contained more components than its MII-NF counterpart. The presence of IFN-α2, IL-3, IL-12 (p40), IL-12 (p70), MCP-3 and MIG in the MII-F network indicated that additional relationships were at play. Interestingly, IL-3, IL-12 (p70), MCP-1 and MIG also featured in the cytokine selection classification model as part of the pairs and triplets of cytokines giving the highest CCR. Total IL-12 has previously been shown to correlate with successful oocyte fertilisation and subsequent embryo development [985], making the presence of IL-12 (p70) in the MII-F (but not MII-NF) network an interesting observation. IL-12 forms two proteins, IL-12 (p40) which is the monomeric/homodimeric protein product of the *IL-12B* gene, and IL-12 (p70) which is the heterodimeric protein formed from the combination of the *IL-12A* (p35) and *IL-12B* (p40) gene products. Only the p70 entire protein (highlighted in both the network analysis and PCA) has biological activity, while the p40 subunit appears to have an antagonistic effect on the p70 protein by blocking its receptor interactions. IL-12 (p70) has a number of biological effects, but the most interesting in this context is its indirect anti-angiogenic role. This cytokine is known to induce IFN-γ which, in turn, induces IP-10 which is then responsible for mediating its anti-angiogenic effect [1026]. Although IL-12 (p70) and IP-10 are present in the MII-F network, they are not connected: IL-12 (p70) features as a terminal node. However, IP-10 is connected with VEGF in all networks, featuring as a parent node, except in the MII-F network. This could indicate that the angiogenic process in ongoing in follicles containing GV, MI and MII-NF oocytes, but complete in follicles containing MII-F oocytes. Unfortunately, the present networks do not reveal the nature of the interaction (e.g. if there is an antagonistic effect) which would enable evaluation of this hypothesis. Work is ongoing to develop the present modelling method further in order to discover the true nature of these interactions.

4.5.7 Classification based on cytokine profiles

Traditional methods of classification such as multinomial modelling, AIC and CCR revealed that FF cytokines can predict oocyte maturational status with a reasonable degree of accuracy, certainly above that expected by chance. When looking for small groups of cytokines which could predict this difference, the best classification rate was 59% with a group of cytokines comprising IL-12 (p70), IL-18 and TRAIL. While IL-12 (p70) and TRAIL have been discussed above, there is a paucity of information regarding the role played by IL-18 in the ovary. IL-18 has previously been found in FF

[981, 983], and a recent study has shown that together with IL-8 and MIP-1β, IL-18 positively correlated with pregnancy outcome in IVF cycles [985]. These cytokines, alongside those highlighted by modified VBSSM network analysis, may serve as markers of cytoplasmic maturation, thereby adding to the toolkit of assays to identify the best oocytes.

4.5.8 Study limitations and future directions

The network approach used herein represents a very significant step forward in terms of understanding cytokine behaviour in ovarian follicles following COH. There are, however, a number of *caveats*. Firstly, although the present networks represent an efficient method of discovering the structure/topology of cytokine networks (whilst even including underlying 'hidden' nodes), it is not possible to determine the nature of these interactions. The next steps towards addressing this issue involves allocating conditional probabilities to each network node in the same manner as seen in Chapter 3. In turn, this will enable a meaningful *in silico* manipulation of these various networks.

Although the maturation networks were generated with a high level of confidence (as demonstrated by stringency testing), the possibility of further refining them in order to differentiate between background noise and truly important interactions remains. Much of the evidence presented in this Chapter has indicated that the relationships seen in the networks are likely to be biologically representative. Moreover, from a classification perspective, the identified differences between MII-F and MII-NF oocytes may well be definable by using a smaller number of cytokines. Although the classifier analysis achieved this with a degree of success, it is likely that a combination of the classifier and network approaches may improve prediction rates even further. In particular, within-network feature selection may be a method by which this can be achieved, and work is also ongoing to explore this option. The inclusion of other significant cytokines such as additional members of the TGF-β family may also further improve correct classification rates. Once such a classification system has been fully refined, specific FF cytokine signatures could be used to identify MII-NF and MII-F oocytes before ICSI. The drive for the implementation of such a prospective clinical trial rests with improving success rates and reducing wastage in the clinic. The reliable identification of the most viable oocytes for fertilisation and subsequent embryo transfer, and development of a fast, bedside test would be key to achieve that aim.

5. Conclusions

Immune networks are active in a large number of reproductive processes, including the ovarian menstrual/oestrus cycle [6, 43], pregnancy [50-52], parturition [57, 58, 64], lactation [68, 76] and the menopause [84-86]. Within those immune networks, cytokines are key orchestrators of physiological processes ranging from cell proliferation and apoptosis [1027] to the communication between oocytes and their surrounding complement of somatic cells [586, 643, 1028-1030]. Although it is now recognised that cytokines function as networks, very few studies have taken an alternative approach to examining these interactions, preferring instead to attribute given physiological effects to the action of single or small groups of mediators.

The original research hypothesis of this thesis was that cytokine-based immune networks underpin the processes in a number of reproductive physiological scenarios, and that the structure of those networks could be revealed, displayed and explored using both traditional and machine-learning data handing methodologies. In order to explore this in more detail, several physiological reproductive scenarios were employed, namely the response of the murine endometrium to seminal plasma, murine lactation and human oocyte maturation following COH. The aims and findings of each of these is briefly summarised below.

5.1 Pathway analysis of the murine endometrial response to seminal plasma

The aim of the studies contained in Chapter 2 was to characterise the inflammatory/immune-related pathways involved in the endometrial response to seminal plasma in the mouse by using traditional methods of pathway analysis. A number of pathways relevant to this context were found to be transiently activated within the endometrium for up to 4 days *post coitum*. Moreover, there was a degree of specificity in response noted across the endometrium's epithelial and stromal compartments. The multiple curated pathways identified included:

- Cytokines and Inflammatory Response pathway
- Inflammatory Response Pathway
- TGF-β Signalling Pathway
- Prostaglandin Synthesis and Regulation
- Matrix Metalloproteinases

- Macrophage Markers
- Oxidative Stress

The cluster analysis demonstrated that elements of each of these pathways behaved in similar and tightly coordinated manner, as highlighted by the results of the cluster analysis. The use of pathway analysis is an accepted methodology for handling large datasets, particularly microarray work, and in this instance this has been particularly successful in identifying previously unknown modulators of the endometrial response to seminal plasma.

Although this approach offered the benefit of facilitating the identification of the broad areas of physiology/cell biology, curated pathways are by their very nature restrictive and, as such, preclude the discovery of novel interactions between individual mediators (since they operate on the basis of rigidly established and well-characterised interactions). This led on to the next experimental Chapter wherein novel methodologies were developed in order to better characterise the specific interactions between different inflammatory network mediators.

5.2 Bayesian methodology in cytokine network discovery

The studies contained within Chapter 3 aimed to develop a novel approach of discovering, displaying and exploring mediator (specifically cytokine and peptide/steroid hormone) interactions in the *in vivo* setting. In order to address the inherent limitations highlighted in Chapter 2, a machine learning-based method for describing protein interaction networks was developed, applied and biologically validated in a physiological model of murine lactation. This Bayesian network-based method was highly successful in revealing previously unknown interactions between mediators, including their directionality, as well as in providing relative quantitative measures of their effects on downstream mediators. In turn, this enabled the characterisation of some of the more elusive features of cytokine biology. The networks generated were statistically very robust and attached a high level of confidence to the interactions they described. In brief, this approach enabled the identification of key cytokines (principal parents - IL-3, IL-12 (p40) and eotaxin; hubs - IFN-γ, IL-13, MCP-1, MIP-1α, MIP-1β and RANTES; terminal node – TNF-α) around which other cytokines assembled. Many of these relationships were conserved across physiological scenarios (i.e. even with pup removal) and were maintained even when prior knowledge was disregarded during network construction (i.e. using variational

Bayesian state space models). Bayesian networks offered the opportunity to numerically define the very nature of cytokine interactions through *in silico* perturbation of the networks generated. Relationships such as synergy (MCP-1 and IL-13), antagonism (PRL and IL-3) and functional redundancy (IL-4) were revealed, all of which would otherwise have remained in the domain of speculation if a reductionist approach alone had been applied. Perhaps the most surprising conclusion from this work was that PRL did not feature as a key node, despite the widely held belief that PRL is a critical immunomodulator, as highlighted by an array of *in vitro* studies . To the best of the author's knowledge, this is the first instance of protein network modelling ever performed in a whole animal system *in vivo*.

Comparisons between *in vivo* perturbation (i.e. preventing the establishment of lactation) and *in silico* perturbation of the cytokine network showed that the latter perturbation could accurately predict system responses in the former. Should this useful feature of Bayesian network analysis be widely conserved across pathophysiological settings has significant ramifications insofar as *in silico* network manipulation could be used for initial screening assessments of viable experiments prior to their empirical implementation. In the context of work involving *in vivo* models, this could significantly reduce the number of animals used, while in the *in vitro* setting this could streamline experimental plans and offer cost and labour-saving benefits. Despite these advantages, however, Bayesian network analytical methods had some *lacunae*. Firstly, the networks generated precluded the display of feedback loops and, secondly, the methodology did not account for 'hidden' Markov blankets. As such, a further, more refined machine-learning approach to cytokine network modelling was developed, which formed the core of Chapter 4.

5.3 Cytokine networks in FF during COH

In this Chapter, the targets were the ovarian follicular fluid cytokine networks and their relation to oocyte maturation in the assisted conception arena. This model was chosen as it offered a real-life compartmentalised *in vivo* study system wherein the results of this investigation may have a clinical impact. As such, the aims of Chapter 4 were to extend the methodology developed in Chapter 3 to discover, display and explore cytokine networks within human follicular fluid surrounding oocytes exposed to ovarian stimulation, as well as to develop classifier models to ascribe oocyte maturity and fertilisability status based solely on their follicular fluid cytokine profile.

In this final endeavour, machine learning-based network analysis was successfully adapted to include the influence of hidden Markov blankets and applied to determining the role played by inflammatory cytokine/growth factor networks in human oocyte maturation. The networks grouped cytokines into communities or subnetworks based on cytokines which demonstrate a stronger interaction – a significant advance from the methodology utilised in Chapter 3. The application of this methodology successfully revealed complex networks at each stage of oocyte maturation, with a strongly conserved cytokine community membership (IL-1ra, IL-2ra, IL-6, IL-7, IL-9, IL-10, IL-13, IL-15, IL-18, IP-10, MCP-1, RANTES, SCF, TRAIL and VEGF). Moreover, their interrelationships were strongly maintained such that it appeared that these mediators formed a tightly regulated core network within the ovarian follicle. However, there were significant divergences in network composition noted between follicles which contained MII-NF and MII-F oocytes. More specifically, LIF and MIF were present only in the MII-NF network, while IFN-α2, IL-3, IL-12 (p40), IL-12 (p70), MCP-3 and MIG were present in the MII-F network, suggesting that the presence or absence of these cytokines from the final network structure had a bearing on oocyte maturity. This opens up new avenues of research into their specific roles in both cytoplasmic and nuclear maturation.

5.4 Future directions

The various facets of this thesis (semen-induced immunomodulation, hormone-responsive inflammatory network regulation and cytokine/growth factor-dependent oocyte maturation) have highlighted the key role played by cytokines across a breadth of reproductive processes. Bayesian theory-based machine learning methodologies have very much been at the centre of this iterative process which has offered intuitive and biologically relevant insights into the complexity of immune network regulation. While this project has highlighted a sequential process in refining the first application of this methodology to *in vivo* acquired protein data, there remains much work in devising optimised ways for utilising these networks and their underlying algorithms to the best of their potential. Firstly, the methodology used in Chapter 4 requires expanding, to include ways of identifying the relationship between the nodes beyond that which is directional. An ideal network would represent the influence of all nodes, both measured and hidden, at any one snapshot in time. It would be flexible enough to manipulate in order to explore the effects of individual and groups of mediators on hypothetical and real physiological scenarios, with an emphasis on the pathophysiological changes underpinning disease. The accurate representation of feedback loops would be

essential to the structure of the network, informing new biologically relevant discoveries.

Although the application of Bayesian network analysis to the datasets within this thesis involves considerably larger numbers of cytokines than seen in traditional reductionist studies, these remain modest in light of the hundreds involved in many biological processes *in vivo* interactions. Of particular note would be the involvement of cytokine cell-bound receptors, soluble receptors, and receptor antagonists. Although not included in the final draft of this thesis, work to characterise FF eicosanoid profiles was performed to understand the involvement of these non-protein inflammatory mediators in oocyte maturation. It would have potentially been very interesting to include these in the Bayesian network analysis in order to extend the spectrum of agents involved. Similarly, there are known interactions which take place between the metabolome and the proteome: for example, leptin influences the hypothalamo-pituitary-gonadal axis, stimulating GnRH and luteinising hormone (LH) production and providing a metabolic signal to the reproductive system [786, 787]. The networks generated in Chapter 4 indicated communities which may be of use in analysing interactions between metabolic, immune and other molecules within the system, i.e. networking of networks. This may also improve correct classification rates, as it is likely that multiple factors influence oocyte maturation. Expanding this concept even further, interactions between the genome, transcriptome, proteome and metabolome, as well as an array of immune effector cell subtypes and patient demographics could be incorporated into these models with a high level of accuracy. It is only by making inroads into the development of such integrated, complex data modelling that we can hope to get a fuller and biologically more meaningful understanding of inflammatory networks and the various pathophysiologies they underpin. At the dawn of the Big Data era, machine learning-based methodologies are increasingly become commonplace in the analytical steps supporting the new advances of tomorrow’s science. There is no need to establish a timeline for applying these developments to reproductive biology. That time is now.

6. References

1. Best, C.L., et al., *Localization and characterization of white blood cell populations within the human ovary throughout the menstrual cycle and menopause.* Hum Reprod, 1996. **11**(4): p. 790-7.
2. Brannstrom, M. and A. Enskog, *Leukocyte networks and ovulation.* J Reprod Immunol, 2002. **57**(1-2): p. 47-60.
3. Chang, R.J., A. Gougeon, and G.F. Erickson, *Evidence for a neutrophil-interleukin-8 system in human folliculogenesis.* Am J Obstet Gynecol, 1998. **178**(4): p. 650-7.
4. Gouon-Evans, V., E.Y. Lin, and J.W. Pollard, *Requirement of macrophages and eosinophils and their cytokines/chemokines for mammary gland development.* Breast Cancer Res, 2002. **4**(4): p. 155-64.
5. Hunt, J.S. and S.A. Robertson, *Uterine macrophages and environmental programming for pregnancy success.* J Reprod Immunol, 1996. **32**(1): p. 1-25.
6. Abrams, E.T. and E.M. Miller, *The roles of the immune system in women's reproduction: evolutionary constraints and life history trade-offs.* Am J Phys Anthropol, 2011. **146 Suppl 53**: p. 134-54.
7. Robertson, S.A., et al., *Cytokine-leukocyte networks and the establishment of pregnancy.* Am J Reprod Immunol, 1997. **37**(6): p. 438-42.
8. Seamark, R.F., M. Hadjisavas, and S.A. Robertson, *Influence of the immune system on reproductive function.* Animal Reproduction Science, 1992. **28**(1-4): p. 171-178.
9. Muehlenbein, M.P., et al., *Toward quantifying the usage costs of human immunity: Altered metabolic rates and hormone levels during acute immune activation in men.* Am J Hum Biol, 2010. **22**(4): p. 546-56.
10. Baird, D.T. and A. Mitchell, *Hormonal control of folliculogenesis: the key to successful reproduction.* Ernst Schering Res Found Workshop, 2002(41): p. 1-9.
11. Berkholtz, C.B., L.D. Shea, and T.K. Woodruff, *Extracellular matrix functions in follicle maturation.* Semin Reprod Med, 2006. **24**(4): p. 262-9.
12. Drummond, A.E., *The role of steroids in follicular growth.* Reprod Biol Endocrinol, 2006. **4**: p. 16.
13. Field, S.L., et al., *Cytokines in ovarian folliculogenesis, oocyte maturation and luteinisation.* Mol Reprod Dev, 2013. **81**(4): p. 284-314.

14. Fritz, M.A. and L. Speroff, *The endocrinology of the menstrual cycle: the interaction of folliculogenesis and neuroendocrine mechanisms.* Fertil Steril, 1982. **38**(5): p. 509-29.
15. Chua, A.C., et al., *Dual roles for macrophages in ovarian cycle-associated development and remodelling of the mammary gland epithelium.* Development, 2010. **137**(24): p. 4229-38.
16. Bukulmez, O. and A. Arici, *Leukocytes in ovarian function.* Hum Reprod Update, 2000. **6**(1): p. 1-15.
17. Espey, L.L., *Ovulation as an Inflammatory Reaction--A Hypothesis.* Biol Reprod, 1980. **22**(1): p. 73-106.
18. Richards, J.S., et al., *Ovulation: new dimensions and new regulators of the inflammatory-like response.* Annu Rev Physiol, 2002. **64**: p. 69-92.
19. Dahm-Kahler, P., et al., *Monocyte chemotactic protein-1 (MCP-1), its receptor, and macrophages in the perifollicular stroma during the human ovulatory process.* Fertil Steril, 2009. **91**(1): p. 231-9.
20. Johnson, M.L., et al., *Tumor necrosis factor alpha regulates collagenolytic activity in preovulatory ovine follicles: relationship to cytokine secretion by the oocyte-cumulus cell complex.* Biol Reprod, 1999. **61**(6): p. 1581-5.
21. Matousek, M., et al., *Inhibition of ovulation in the rat by a leukotriene B(4) receptor antagonist.* Mol Hum Reprod, 2001. **7**(1): p. 35-42.
22. Guerriero, S., S. Ajossa, and G.B. Melis, *Luteal dynamics during the human menstrual cycle: new insight from imaging.* Ultrasound Obstet Gynecol, 2005. **25**(5): p. 425-7.
23. Milvae, R.A., S.T. Hinckley, and J.C. Carlson, *Luteotropic and luteolytic mechanisms in the bovine corpus luteum.* Theriogenology, 1996. **45**(7): p. 1327-49.
24. Neuvians, T.P., et al., *Involvement of pro-inflammatory cytokines, mediators of inflammation, and basic fibroblast growth factor in prostaglandin F2alpha-induced luteolysis in bovine corpus luteum.* Biol Reprod, 2004. **70**(2): p. 473-80.
25. Pate, J.L. and P. Landis Keyes, *Immune cells in the corpus luteum: friends or foes?* Reproduction, 2001. **122**(5): p. 665-76.
26. Nishimura, R. and K. Okuda, *Hypoxia is important for establishing vascularization during corpus luteum formation in cattle.* J Reprod Dev, 2010. **56**(1): p. 110-6.
27. Antoniazzi, A.Q., et al., *Endocrine delivery of interferon tau protects the corpus luteum from prostaglandin F2 alpha-induced luteolysis in ewes.* Biol Reprod, 2013. **88**(6): p. 144.

28. Galvao, A.M., et al., *Role of tumor necrosis factor-alpha, interferon-gamma and Fas-ligand on in vitro nitric oxide activity in the corpus luteum.* Cytokine, 2013. **64**(1): p. 18-21.
29. Miyamoto, A., et al., *Impact of angiogenic and innate immune systems on the corpus luteum function during its formation and maintenance in ruminants.* Reprod Biol, 2013. **13**(4): p. 272-8.
30. Galvao, A., et al., *Cytokines tumor necrosis factor-alpha and interferon-gamma participate in modulation of the equine corpus luteum as autocrine and paracrine factors.* J Reprod Immunol, 2012. **93**(1): p. 28-37.
31. Szostek, A.Z., et al., *Tumor necrosis factor-alpha inhibits the stimulatory effect of luteinizing hormone and prostaglandin E(2) on progesterone secretion by the bovine corpus luteum.* Domest Anim Endocrinol, 2011. **40**(4): p. 183-91.
32. Levy, C., et al., *Estradiol and progesterone receptors in human endometrium: normal and abnormal menstrual cycles and early pregnancy.* Am J Obstet Gynecol, 1980. **136**(5): p. 646-51.
33. Shah, D. and N. Nagarajan, *Luteal insufficiency in first trimester.* Indian J Endocrinol Metab, 2013. **17**(1): p. 44-9.
34. Siklosi, G., *Luteal insufficiency as the primary cause of habitual abortion--its successful treatment.* Acta Biomed Ateneo Parmense, 1992. **63**(1-2): p. 101-11.
35. Focchi, G.R., et al., *Ultrastructural aspects of the remodeling process of the Corpus albicans in the recent postmenopausal period.* Sao Paulo Med J, 1996. **114**(3): p. 1173-6.
36. Katabuchi, H., et al., *Distribution and fine structure of macrophages in the human ovary during the menstrual cycle, pregnancy and menopause.* Endocr J, 1997. **44**(6): p. 785-95.
37. Whitacre, C.C., S.C. Reingold, and P.A. O'Looney, *A gender gap in autoimmunity.* Science, 1999. **283**(5406): p. 1277-8.
38. Beagley, K.W. and C.M. Gockel, *Regulation of innate and adaptive immunity by the female sex hormones oestradiol and progesterone.* FEMS Immunol Med Microbiol, 2003. **38**(1): p. 13-22.
39. Robertson, S.A., et al., *Peri-conceptual cytokines--setting the trajectory for embryo implantation, pregnancy and beyond.* Am J Reprod Immunol, 2011. **66 Suppl 1**: p. 2-10.
40. Gaide Chevronnay, H.P., et al., *Regulation of matrix metalloproteinases activity studied in human endometrium as a paradigm of cyclic tissue breakdown and regeneration.* Biochim Biophys Acta, 2012. **1824**(1): p. 146-56.

41. Horne, A.W., J.O. White, and E.N. Lalani, *The endometrium and embryo implantation. A receptive endometrium depends on more than hormonal influences.* BMJ, 2000. **321**(7272): p. 1301-2.
42. Marbaix, E., et al., *Menstrual breakdown of human endometrium can be mimicked in vitro and is selectively and reversibly blocked by inhibitors of matrix metalloproteinases.* Proc Natl Acad Sci U S A, 1996. **93**(17): p. 9120-5.
43. Salamonsen, L.A. and L.J. Lathbury, *Endometrial leukocytes and menstruation.* Hum Reprod Update, 2000. **6**(1): p. 16-27.
44. Engelhardt, H., B.A. Croy, and G.J. King, *Role of uterine immune cells in early pregnancy in pigs.* J Reprod Fertil Suppl, 1997. **52**: p. 115-31.
45. Gangnuss, S., et al., *Seminal plasma regulates corpora lutea macrophage populations during early pregnancy in mice.* Biol Reprod, 2004. **71**(4): p. 1135-41.
46. Johansson, M., et al., *Semen activates the female immune response during early pregnancy in mice.* Immunology, 2004. **112**(2): p. 290-300.
47. Moldenhauer, L.M., J.D. Hayball, and S.A. Robertson, *Utilising T cell receptor transgenic mice to define mechanisms of maternal T cell tolerance in pregnancy.* J Reprod Immunol, 2010. **87**(1-2): p. 1-13.
48. Moldenhauer, L.M., et al., *GM-CSF is an essential regulator of T cell activation competence in uterine dendritic cells during early pregnancy in mice.* J Immunol, 2010. **185**(11): p. 7085-96.
49. Robertson, S.A., et al., *Activating T regulatory cells for tolerance in early pregnancy - the contribution of seminal fluid.* J Reprod Immunol, 2009. **83**(1-2): p. 109-16.
50. Trowsdale, J. and A.G. Betz, *Mother's little helpers: mechanisms of maternal-fetal tolerance.* Nat Immunol, 2006. **7**(3): p. 241-6.
51. Adams Waldorf, K.M. and J.L. Nelson, *Autoimmune disease during pregnancy and the microchimerism legacy of pregnancy.* Immunol Invest, 2008. **37**(5): p. 631-44.
52. Khosrotehrani, K. and D.W. Bianchi, *Multi-lineage potential of fetal cells in maternal tissue: a legacy in reverse.* J Cell Sci, 2005. **118**(Pt 8): p. 1559-63.
53. Lissauer, D.M., et al., *Fetal microchimerism: the cellular and immunological legacy of pregnancy.* Expert Rev Mol Med, 2009. **11**: p. e33.
54. Aluvihare, V.R., M. Kallikourdis, and A.G. Betz, *Regulatory T cells mediate maternal tolerance to the fetus.* Nat Immunol, 2004. **5**(3): p. 266-71.
55. Mor, G. and I. Cardenas, *The immune system in pregnancy: a unique complexity.* Am J Reprod Immunol, 2010. **63**(6): p. 425-33.

56. Roberts, C.W., W. Walker, and J. Alexander, *Sex-associated hormones and immunity to protozoan parasites.* Clin Microbiol Rev, 2001. **14**(3): p. 476-88.
57. Ness, R.B., *The consequences for human reproduction of a robust inflammatory response.* Q Rev Biol, 2004. **79**(4): p. 383-93.
58. Golightly, E., H.N. Jabbour, and J.E. Norman, *Endocrine immune interactions in human parturition.* Mol Cell Endocrinol, 2011. **335**(1): p. 52-9.
59. Condon, J.C., et al., *Surfactant protein secreted by the maturing mouse fetal lung acts as a hormone that signals the initiation of parturition.* Proc Natl Acad Sci U S A, 2004. **101**(14): p. 4978-83.
60. Lannon, S.M., et al., *Synergy and Interactions Among Biological Pathways Leading to Preterm Premature Rupture of Membranes.* Reprod Sci, 2014.
61. Behrman, R.E. and A.S. Butler, eds. *Preterm Birth: Causes, Consequences, and Prevention*. 2007, National Academies Press: Washington DC.
62. Whittle, W.L., et al., *Glucocorticoid regulation of human and ovine parturition: the relationship between fetal hypothalamic-pituitary-adrenal axis activation and intrauterine prostaglandin production.* Biol Reprod, 2001. **64**(4): p. 1019-32.
63. Gomez-Lopez, N., et al., *Maternal circulating leukocytes display early chemotactic responsiveness during late gestation.* BMC Pregnancy Childbirth, 2013. **13 Suppl 1**: p. S8.
64. Gomez-Lopez, N., et al., *Evidence for a role for the adaptive immune response in human term parturition.* Am J Reprod Immunol, 2013. **69**(3): p. 212-30.
65. Shynlova, O., et al., *Myometrial immune cells contribute to term parturition, preterm labour and post-partum involution in mice.* J Cell Mol Med, 2013. **17**(1): p. 90-102.
66. Goldenberg, R.L., J.C. Hauth, and W.W. Andrews, *Intrauterine infection and preterm delivery.* N Engl J Med, 2000. **342**(20): p. 1500-7.
67. Goldberg, G.R., et al., *Longitudinal assessment of the components of energy balance in well-nourished lactating women.* Am J Clin Nutr, 1991. **54**(5): p. 788-98.
68. Butte, N.F., W.W. Wong, and J.M. Hopkinson, *Energy requirements of lactating women derived from doubly labeled water and milk energy output.* J Nutr, 2001. **131**(1): p. 53-8.
69. Jason, J.M., P. Nieburg, and J.S. Marks, *Mortality and infectious disease associated with infant-feeding practices in developing countries.* Pediatrics, 1984. **74**(4 Pt 2): p. 702-27.
70. Popkin, B.M., et al., *Breast-feeding and diarrheal morbidity.* Pediatrics, 1990. **86**(6): p. 874-82.

71. Morrow, A.L. and J.M. Rangel, *Human milk protection against infectious diarrhea: implications for prevention and clinical care.* Semin Pediatr Infect Dis, 2004. **15**(4): p. 221-8.
72. van Odijk, J., et al., *Breastfeeding and allergic disease: a multidisciplinary review of the literature (1966-2001) on the mode of early feeding in infancy and its impact on later atopic manifestations.* Allergy, 2003. **58**(9): p. 833-43.
73. Grabenhenrich, L.B., et al., *Early-life determinants of asthma from birth to age 20 years: A German birth cohort study.* J Allergy Clin Immunol, 2014.
74. Guibas, G.V., et al., *Breastfeeding and wheeze prevalence in pre-schoolers and pre-adolescents: the Genesis and Healthy Growth studies.* Pediatr Allergy Immunol, 2013. **24**(8): p. 772-81.
75. Fooladi, A.A., et al., *Impact of altered early infant gut microbiota following breastfeeding and delivery mode on allergic diseases.* Inflamm Allergy Drug Targets, 2013. **12**(6): p. 410-8.
76. Ouwehand, A., E. Isolauri, and S. Salminen, *The role of the intestinal microflora for the development of the immune system in early childhood.* Eur J Nutr, 2002. **41 Suppl 1**: p. I32-7.
77. Jeppesen, D.L., et al., *T-lymphocyte subsets, thymic size and breastfeeding in infancy.* Pediatr Allergy Immunol, 2004. **15**(2): p. 127-32.
78. Ogra, S.S., D. Weintraub, and P.L. Ogra, *Immunologic aspects of human colostrum and milk. III. Fate and absorption of cellular and soluble components in the gastrointestinal tract of the newborn.* J Immunol, 1977. **119**(1): p. 245-8.
79. Gregory, K.E. and W.A. Walker, *Immunologic Factors in Human Milk and Disease Prevention in the Preterm Infant.* Curr Pediatr Rep, 2013. **1**(4).
80. Kainonen, E., S. Rautava, and E. Isolauri, *Immunological programming by breast milk creates an anti-inflammatory cytokine milieu in breast-fed infants compared to formula-fed infants.* Br J Nutr, 2013. **109**(11): p. 1962-70.
81. Fanaro, S., et al., *Intestinal microflora in early infancy: composition and development.* Acta Paediatr Suppl, 2003. **91**(441): p. 48-55.
82. Hasselbalch, H., et al., *Decreased thymus size in formula-fed infants compared with breastfed infants.* Acta Paediatr, 1996. **85**(9): p. 1029-32.
83. McNeilly, A.S., C.C. Tay, and A. Glasier, *Physiological mechanisms underlying lactational amenorrhea.* Ann N Y Acad Sci, 1994. **709**: p. 145-55.
84. Cramer, D.W., et al., *Mumps, menarche, menopause, and ovarian cancer.* Am J Obstet Gynecol, 1983. **147**(1): p. 1-6.
85. Dorman, J.S., et al., *Menopause in type 1 diabetic women: is it premature?* Diabetes, 2001. **50**(8): p. 1857-62.

86. Hadjidakis, D.J., et al., *Diabetes and premature menopause: is their co-existence detrimental to the skeleton?* Eur J Endocrinol, 2005. **152**(3): p. 437-42.

87. Pikwer, M., et al., *Early menopause is an independent predictor of rheumatoid arthritis.* Ann Rheum Dis, 2012. **71**(3): p. 378-81.

88. Pikwer, M., et al., *Early menopause and severity of rheumatoid arthritis in women older than 45 years.* Arthritis Res Ther, 2012. **14**(4): p. R190.

89. Ghosh, M., M. Rodriguez-Garcia, and C.R. Wira, *The immune system in menopause: Pros and cons of hormone therapy.* J Steroid Biochem Mol Biol, 2013. **142**: p. 171-5.

90. Jang, M.H., J.Y. Seoh, and M. Miyasaka, *Cytokines, chemokines, and their receptors: targets for immunomodulation. Conference report: International Cytokine Society Conference 2005.* J Leukoc Biol, 2006. **80**(2): p. 217-9.

91. Orsi, N.M., *Cytokine networks in the establishment and maintenance of pregnancy.* Hum Fertil (Camb), 2008. **11**(4): p. 222-30.

92. Orsi, N.M. and R.M. Tribe, *Cytokine networks and the regulation of uterine function in pregnancy and parturition.* J Neuroendocrinol, 2008. **20**(4): p. 462-9.

93. Crivellato, E., *The role of angiogenic growth factors in organogenesis.* Int J Dev Biol, 2011. **55**(4-5): p. 365-75.

94. Kopf, M., et al., *Averting inflammation by targeting the cytokine environment.* Nat Rev Drug Discov, 2010. **9**(9): p. 703-18.

95. Lopez-Pedrera, C., et al., *Cardiovascular risk in systemic autoimmune diseases: epigenetic mechanisms of immune regulatory functions.* Clin Dev Immunol, 2012. **2012**: p. 9746–9748.

96. Ma, S. and C.C. Ma, *Recent development in pleiotropic effects of statins on cardiovascular disease through regulation of transforming growth factor-beta superfamily.* Cytokine Growth Factor Rev, 2011. **22**(3): p. 167-75.

97. Nowsheen, S., et al., *Molecular markers for cancer prognosis and treatment: have we struck gold?* Cancer Lett, 2011. **327**(1): p. 142-152.

98. Modugno, F., et al., *Inflammation and endometrial cancer: a hypothesis.* Cancer Epidemiol Biomarkers Prev, 2005. **14**(12): p. 2840-7.

99. Oertelt-Prigione, S., *The influence of sex and gender on the immune response.* Autoimmun Rev, 2012. **11**(6-7): p. A479-85.

100. Remmers, N., et al., *Molecular pathology of early pancreatic cancer.* Cancer Biomark, 2011. **9**(1-6): p. 421-40.

101. Orsi, N.M., et al., *Murine serum cytokines throughout the estrous cycle, pregnancy and post partum period.* Anim Reprod Sci, 2006. **96**(1-2): p. 54-65.

102. Rostene, W., et al., *Neurochemokines: a menage a trois providing new insights on the functions of chemokines in the central nervous system.* J Neurochem, 2011. **118**(5): p. 680-94.

103. Pangas, S.A., *Growth factors in ovarian development.* Semin Reprod Med, 2007. **25**(4): p. 225-34.

104. Knight, P.G. and C. Glister, *TGF-beta superfamily members and ovarian follicle development.* Reproduction, 2006. **132**(2): p. 191-206.

105. Arsenescu, R., V. Arsenescu, and W.J. de Villiers, *TNF-alpha and the development of the neonatal immune system: implications for inhibitor use in pregnancy.* Am J Gastroenterol, 2011. **106**(4): p. 559-62.

106. Forbes, K. and M. Westwood, *Maternal growth factor regulation of human placental development and fetal growth.* J Endocrinol, 2010. **207**(1): p. 1-16.

107. Witkin, S.S., et al., *Unique alterations in infection-induced immune activation during pregnancy.* BJOG, 2011. **118**(2): p. 145-53.

108. Olson, D.M., *The role of prostaglandins in the initiation of parturition.* Best Pract Res Clin Obstet Gynaecol, 2003. **17**(5): p. 717-30.

109. Kniss, D.A., et al., *Interleukin-1 receptor antagonist blocks interleukin-1-induced expression of cyclooxygenase-2 in endometrium.* Am J Obstet Gynecol, 1997. **177**(3): p. 559-67.

110. Molnar, M., R. Romero, and F. Hertelendy, *Interleukin-1 and tumor necrosis factor stimulate arachidonic acid release and phospholipid metabolism in human myometrial cells.* Am J Obstet Gynecol, 1993. **169**(4): p. 825-9.

111. Hirsch, E., Y. Filipovich, and M. Mahendroo, *Signaling via the type I IL-1 and TNF receptors is necessary for bacterially induced preterm labor in a murine model.* Am J Obstet Gynecol, 2006. **194**(5): p. 1334-40.

112. Parks, W.C., C.L. Wilson, and Y.S. Lopez-Boado, *Matrix metalloproteinases as modulators of inflammation and innate immunity.* Nat Rev Immunol, 2004. **4**(8): p. 617-29.

113. McQuibban, G.A., et al., *Inflammation dampened by gelatinase A cleavage of monocyte chemoattractant protein-3.* Science, 2000. **289**(5482): p. 1202-6.

114. McQuibban, G.A., et al., *Matrix metalloproteinase processing of monocyte chemoattractant proteins generates CC chemokine receptor antagonists with anti-inflammatory properties in vivo.* Blood, 2002. **100**(4): p. 1160-7.

115. Shull, M.M., et al., *Targeted disruption of the mouse transforming growth factor-beta 1 gene results in multifocal inflammatory disease.* Nature, 1992. **359**(6397): p. 693-9.

116. Schonbeck, U., F. Mach, and P. Libby, *Generation of biologically active IL-1 beta by matrix metalloproteinases: a novel caspase-1-independent pathway of IL-1 beta processing.* J Immunol, 1998. **161**(7): p. 3340-6.
117. Ledger, W.L., et al., *The costs to the NHS of multiple births after IVF treatment in the UK.* BJOG, 2006. **113**(1): p. 21-5.
118. Reddy, U.M., et al., *Infertility, assisted reproductive technology, and adverse pregnancy outcomes: executive summary of a National Institute of Child Health and Human Development workshop.* Obstet Gynecol, 2007. **109**(4): p. 967-77.
119. MacKay, A.P., et al., *Pregnancy-related mortality among women with multifetal pregnancies.* Obstet Gynecol, 2006. **107**(3): p. 563-8.
120. Sutcliffe, A.G. and C. Derom, *Follow-up of twins: health, behaviour, speech, language outcomes and implications for parents.* Early Hum Dev, 2006. **82**(6): p. 379-86.
121. HFEA. *Facts and Figures 2011/12 Fertility Problems and Treatment*. 2012 14/09/2014]; Available from: http://www.hfea.gov.uk/381.Html.
122. Wilkes, S., et al., *Epidemiology and management of infertility: a population-based study in UK primary care.* Fam Pract, 2009. **26**(4): p. 269-74.
123. Hull, M.G., et al., *Population study of causes, treatment, and outcome of infertility.* Br Med J (Clin Res Ed), 1985. **291**(6510): p. 1693-7.
124. Philippov, O.S., et al., *Estimation of the prevalence and causes of infertility in western Siberia.* Bull World Health Organ, 1998. **76**(2): p. 183-7.
125. Chiamchanya, C. and W. Su-angkawatin, *Study of the causes and the results of treatment in infertile couples at Thammasat Hospital between 1999-2004.* J Med Assoc Thai, 2008. **91**(6): p. 805-12.
126. Isaksson, R. and A. Tiitinen, *Present concept of unexplained infertility.* Gynecol Endocrinol, 2004. **18**(5): p. 278-90.
127. Templeton, A., C. Fraser, and B. Thompson, *The epidemiology of infertility in Aberdeen.* BMJ, 1990. **301**(6744): p. 148-52.
128. Sirmans, S.M. and K.A. Pate, *Epidemiology, diagnosis, and management of polycystic ovary syndrome.* Clin Epidemiol, 2013. **6**: p. 1-13.
129. Patrelli, T.S., et al., *Can the impact of pelvic inflammatory disease on fertility be prevented? Epidemiology, clinical features and surgical treatment: evolution over 8 years.* J Reprod Med, 2013. **58**(9-10): p. 425-33.
130. Holoch, K.J. and B.A. Lessey, *Endometriosis and infertility.* Clin Obstet Gynecol, 2010. **53**(2): p. 429-38.
131. Leyland, N., et al., *Endometriosis: diagnosis and management.* J Obstet Gynaecol Can, 2010. **32**(7 Suppl 2): p. S1-32.

132. Sutcliffe, A.G., *Health risks in babies born after assisted reproduction.* BMJ, 2002. **325**(7356): p. 117-8.
133. Kulkarni, A.D., et al., *Fertility treatments and multiple births in the United States.* N Engl J Med, 2013. **369**(23): p. 2218-25.
134. SOGC-CFAS, *Guidelines for the number of embryos to transfer following in vitro fertilization No. 182, September 2006.* Int J Gynaecol Obstet, 2008. **102**(2): p. 203-16.
135. Nargund, G., et al., *The ISMAAR proposal on terminology for ovarian stimulation for IVF.* Hum Reprod, 2007. **22**(11): p. 2801-4.
136. Aboulghar, M.A., et al., *In vitro fertilization in a spontaneous cycle: a successful simple protocol.* J Obstet Gynaecol (Tokyo 1995), 1995. **21**(4): p. 337-40.
137. Paulson, R.J., et al., *In vitro fertilization in unstimulated cycles: the University of Southern California experience.* Fertil Steril, 1992. **57**(2): p. 290-3.
138. Baart, E.B., N.S. Macklon, and B.J. Fauser, *Ovarian stimulation and embryo quality.* Reprod Biomed Online, 2009. **18 Suppl 2**: p. 45-50.
139. Baart, E.B., et al., *Milder ovarian stimulation for in-vitro fertilization reduces aneuploidy in the human preimplantation embryo: a randomized controlled trial.* Hum Reprod, 2007. **22**(4): p. 980-8.
140. Fauser, B.C., et al., *Mild ovarian stimulation for IVF: 10 years later.* Hum Reprod, 2010. **25**(11): p. 2678-84.
141. Verberg, M.F., et al., *Mild ovarian stimulation for IVF.* Hum Reprod Update, 2009. **15**(1): p. 13-29.
142. de Mouzon, J., et al., *Assisted reproductive technology in Europe, 2006: results generated from European registers by ESHRE.* Hum Reprod, 2010. **25**(8): p. 1851-62.
143. Boomsma, C.M., et al., *Endometrial secretion analysis identifies a cytokine profile predictive of pregnancy in IVF.* Hum Reprod, 2009. **24**(6): p. 1427-35.
144. Huisman, G.J., et al., *Implantation rates after in vitro fertilization and transfer of a maximum of two embryos that have undergone three to five days of culture.* Fertil Steril, 2000. **73**(1): p. 117-22.
145. Muasher, S.J., et al., *Controlled preparation of the endometrium with exogenous steroids for the transfer of frozen-thawed pre-embryos in patients with anovulatory or irregular cycles.* Hum Reprod, 1991. **6**(3): p. 443-5.
146. Lelaidier, C., et al., *Controlled preparation of the endometrium with exogenous oestradiol and progesterone: a novel regimen not using a gonadotrophin-releasing hormone agonist.* Hum Reprod, 1992. **7**(10): p. 1353-6.

147. Robertson, S.A., *Seminal plasma and male factor signalling in the female reproductive tract.* Cell and Tissue Research, 2005. **322**(1): p. 43-52.
148. Koot, Y.E., et al., *Molecular aspects of implantation failure.* Biochim Biophys Acta, 2012. **1822**(12): p. 1943-50.
149. Asimakopoulos, B., et al., *Concentrations of inflammatory cytokines and the outcome in ICSI cycles.* In Vivo, 2010. **24**(4): p. 495-500.
150. de Cassia, S.F.R., et al., *Metaphase II human oocyte morphology: contributing factors and effects on fertilization potential and embryo developmental ability in ICSI cycles.* Fertil Steril, 2010. **94**(3): p. 1115-7.
151. Ledee, N., et al., *Cytokines and chemokines in follicular fluids and potential of the corresponding embryo: the role of granulocyte colony-stimulating factor.* Hum Reprod, 2008. **23**(9): p. 2001-9.
152. Balkwill, F.R. and F. Burke, *The cytokine network.* Immunol Today, 1989. **10**(9): p. 299-304.
153. Gouwy, M., et al., *Synergy in cytokine and chemokine networks amplifies the inflammatory response.* Cytokine Growth Factor Rev, 2005. **16**(6): p. 561-80.
154. Kelso, A., *Cytokines: principles and prospects.* Immunol Cell Biol, 1998. **76**(4): p. 300-17.
155. Nip, M.M., et al., *Autoantibodies and antisperm antibodies in sera and follicular fluids of infertile patients; relation to reproductive outcome after in-vitro fertilization.* Hum Reprod, 1995. **10**(10): p. 2564-9.
156. Carroll, M., et al., *Testing for hypersensitivity to seminal fluid-free spermatozoa.* Hum Fertil (Camb), 2013. **16**(2): p. 128-31.
157. Sublett, J.W. and J.A. Bernstein, *Seminal plasma hypersensitivity reactions: an updated review.* Mt Sinai J Med, 2011. **78**(5): p. 803-9.
158. Tan, J. and J.A. Bernstein, *Fertility and human seminal plasma (HSP) hypersensitivity.* Ann Allergy Asthma Immunol, 2013. **111**(2): p. 145-6.
159. Carp, H.J., et al., *Effect of paternal leukocyte immunization on implantation after biochemical pregnancies and repeated failure of embryo transfer.* Am J Reprod Immunol, 1994. **31**(2-3): p. 112-5.
160. Elram, T., et al., *Treatment of recurrent IVF failure and human leukocyte antigen similarity by intravenous immunoglobulin.* Reprod Biomed Online, 2005. **11**(6): p. 745-9.
161. Penn, D.J., *The scent of genetic compatibility: sexual selection and the major histocompatiblity complex.* Ethology, 2002. **801**: p. 1-21.

162. Penn, D.J., K. Damjanovich, and W.K. Potts, *MHC heterozygosity confers a selective advantage against multiple-strain infections.* Proc Natl Acad Sci U S A, 2002. **99**(17): p. 11260-4.

163. Chaix, R., C. Cao, and P. Donnelly, *Is mate choice in humans MHC-dependent?* PLoS Genet, 2008. **4**(9): p. e1000184.

164. Wedekind, C., et al., *MHC-dependent mate preferences in humans.* Proc Biol Sci, 1995. **260**(1359): p. 245-9.

165. Yamazaki, K. and G.K. Beauchamp, *Genetic basis for MHC-dependent mate choice.* Adv Genet, 2007. **59**: p. 129-45.

166. Shufaro, Y. and J.G. Schenker, *Implantation Failure, Etiology, Diagnosis and Treatment.* International Journal of Infertility & Fetal Medicine 2011. **2**(1): p. 1-7.

167. Mohlin, F.C., et al., *Analysis of genes coding for CD46, CD55, and C4b-binding protein in patients with idiopathic, recurrent, spontaneous pregnancy loss.* Eur J Immunol, 2013. **43**(6): p. 1617-29.

168. Wirstlein, P., M. Mikolajczyk, and J. Skrzypczak, *Assessment of the transcription levels for the complement activation control system in eutopic endometrium in women with two or more consecutive miscarriages of unknown etiology.* Folia Histochem Cytobiol, 2010. **48**(3): p. 328-32.

169. Foxman, B., et al., *Mycoplasma, bacterial vaginosis-associated bacteria BVAB3, race, and risk of preterm birth in a high-risk cohort.* Am J Obstet Gynecol, 2014. **210**(3): p. 226 e1-7.

170. Nelson, D.B., et al., *Early pregnancy changes in bacterial vaginosis-associated bacteria and preterm delivery.* Paediatr Perinat Epidemiol, 2014. **28**(2): p. 88-96.

171. Oliver, R.S. and R.F. Lamont, *Infection and antibiotics in the aetiology, prediction and prevention of preterm birth.* J Obstet Gynaecol, 2013. **33**(8): p. 768-75.

172. Urman, B., K. Yakin, and B. Balaban, *Recurrent implantation failure in assisted reproduction: how to counsel and manage. A. General considerations and treatment options that may benefit the couple.* Reprod Biomed Online, 2005. **11**(3): p. 371-81.

173. Salamonsen, L.A., et al., *Society for Reproductive Biology Founders' Lecture 2009. Preparing fertile soil: the importance of endometrial receptivity.* Reprod Fertil Dev, 2009. **21**(7): p. 923-34.

174. Robertson, S.A., et al., *Seminal fluid and the generation of regulatory T cells for embryo implantation.* Am J Reprod Immunol, 2013. **69**(4): p. 315-30.

175. Allen, E., *The oestrus cycle in the mouse.* American Journal of Anatomy, 1922. **30**: p. 297-371.
176. Caligioni, C.S., *Assessing reproductive status/stages in mice.* Curr Protoc Neurosci, 2009. **Appendix 4**: p. Appendix 4I.
177. Champlin, A.K., D.L. Dorr, and A.H. Gates, *Determining the stage of the estrous cycle in the mouse by the appearance of the vagina.* Biol Reprod, 1973. **8**(4): p. 491-4.
178. Evans, G.S., et al., *Proliferative changes in the genital tissue of female mice during the oestrous cycle.* Cell Tissue Kinet, 1990. **23**(6): p. 619-35.
179. Quarmby, V.E. and K.S. Korach, *The influence of 17 beta-estradiol on patterns of cell division in the uterus.* Endocrinology, 1984. **114**(3): p. 694-702.
180. McMaster, M.T., et al., *Lactoferrin in the mouse uterus: analyses of the preimplantation period and regulation by ovarian steroids.* Mol Endocrinol, 1992. **6**(1): p. 101-11.
181. Zheng, Y., et al., *Immunohistochemical characterization of the estrogen-stimulated leukocyte influx in the immature rat uterus.* J Leukoc Biol, 1988. **44**(1): p. 27-32.
182. Tchernitchin, A., et al., *Dramatic early increase in uterine eosinophils after oestrogen administration.* Nature, 1974. **248**(444): p. 142-3.
183. Lee, Y.H., et al., *Estrogen regulation of an eosinophil chemotactic factor in the immature rat uterus.* Endocrinology, 1989. **125**(6): p. 3022-8.
184. Green, E.L., ed. *Biology of the laboratory mouse*. 2nd Ed ed. 1966, DOVER PUBLICATIONS, INC.: New York.
185. Walmer, D.K., et al., *Lactoferrin expression in the mouse reproductive tract during the natural estrous cycle: correlation with circulating estradiol and progesterone.* Endocrinology, 1992. **131**(3): p. 1458-66.
186. Parkening, T.A., T.J. Collins, and E.R. Smith, *Plasma and pituitary concentrations of LH, FSH, and prolactin in aging C57BL/6 mice at various times of the estrous cycle.* Neurobiol Aging, 1982. **3**(1): p. 31-5.
187. Wood, G.A., et al., *Circulating hormones and estrous stage predict cellular and stromal remodeling in murine uterus.* Reproduction, 2007. **133**(5): p. 1035-44.
188. Grant, E.C. and J.H. Mackintosh, *A comparison of the social postures of some common laboratory rodents.* Behavior, 1963. **21**: p. 246-259.
189. McGill, T.E., *Sexual behavior in three inbred strains of mice.* Behaviour, 1962. **19**: p. 341-350.
190. Parkes, A.S., *Observations on the oestrous cycle of the albino mouse.* Proceedings of the Royal Society of Biology 1926. **100**: p. 151-170.

191. Robertson, S.A. *Seminal fluid signaling in the female reproductive tract: Lessons from rodents and pigs*. in *2006 ADSA/ASAS Joint Annual Meeting*. 2006. Minneapolis, MN: Amer Soc Animal Science.

192. Rodriguez-Martinez, H., et al., *Seminal plasma proteins: what role do they play?* Am J Reprod Immunol, 2011. **66 Suppl 1**: p. 11-22.

193. Ramm, S.A. and P. Stockley, *Ejaculate allocation under varying sperm competition risk in the house mouse, Mus musculus domesticus.* Behavioural Ecology, 2007. **18**(2): p. 491-495.

194. Lundwall, A., et al., *Chemical characterization of the predominant proteins secreted by mouse seminal vesicles.* Eur J Biochem, 1997. **249**(1): p. 39-44.

195. Zhang, Y., et al., *Prostaglandin E synthase is regulated in postnatal mouse testis.* Front Biosci (Elite Ed), 2012. **4**: p. 1071-80.

196. Leblond, E., L. Desnoyers, and P. Manjunath, *Phosphorylcholine-binding proteins from the seminal fluids of different species share antigenic determinants with the major proteins of bovine seminal plasma.* Mol Reprod Dev, 1993. **34**(4): p. 443-9.

197. Tremellen, K.P., R.F. Seamark, and S.A. Robertson, *Seminal transforming growth factor beta1 stimulates granulocyte-macrophage colony-stimulating factor production and inflammatory cell recruitment in the murine uterus.* Biol Reprod, 1998. **58**(5): p. 1217-25.

198. Fujimoto, N., et al., *Identification of prostatic-secreted proteins in mice by mass spectrometric analysis and evaluation of lobe-specific and androgen-dependent mRNA expression.* J Endocrinol, 2006. **190**(3): p. 793-803.

199. Pang, S.F., P.H. Chow, and T.M. Wong, *The role of the seminal vesicles, coagulating glands and prostate glands on the fertility and fecundity of mice.* J Reprod Fertil, 1979. **56**(1): p. 129-32.

200. Beyler, S.A. and L.J.D. Zaneveld, *The male accessory sex glands.*, in *Biochemistry of mammalian reproduction*, L.J.D. Zaneveld and R.T. Chatterton, Editors. 1982, John Wiley & Sons: New York, USA. p. 65-88.

201. Druart, X., et al., *Proteomic characterization and cross species comparison of mammalian seminal plasma.* J Proteomics, 2013. **91C**: p. 13-22.

202. Kelly, V.C., et al., *Characterization of bovine seminal plasma by proteomics.* Proteomics, 2006. **6**(21): p. 5826-33.

203. Watson, J.G., J. Carroll, and S. Chaykin, *Reproduction in mice: The fate of spermatozoa not involved in fertilization.* Gamete Research, 1993. **7**(1): p. 75-84.

204. Bromfield, J.J., C.T. Roberts, and S.A. Robertson. *Seminal plasma programs uterine receptivity and pregnancy outcome*. in *37th Annual Meeting of the Society for the Study of Reproduction*. 2004. Vancouver, Canada: Biology of Reproduction.

205. Carp, H.J., et al., *Influence of insemination on the implantation of transferred rat blastocysts.* Gynecol Obstet Invest, 1984. **18**(4): p. 194-8.

206. Peitz, B. and P. Olds-Clarke, *Effects of seminal vesicle removal on fertility and uterine sperm motility in the house mouse.* Biol Reprod, 1986. **35**(3): p. 608-17.

207. Cukierski, M.A., et al., *Effects of seminal vesicle and coagulating gland ablation on fertility in rats.* Reprod Toxicol, 1991. **5**(4): p. 347-52.

208. Queen, K., C.B. Dhabuwala, and C.G. Pierrepoint, *The effect of the removal of the various accessory sex glands on the fertility of male rats.* J Reprod Fertil, 1981. **62**(2): p. 423-6.

209. O, W.S., H.Q. Chen, and P.H. Chow, *Effects of male accessory sex gland secretions on early embryonic development in the golden hamster.* J Reprod Fertil, 1988. **84**(1): p. 341-4.

210. O'Leary, S., et al., *Seminal plasma regulates endometrial cytokine expression, leukocyte recruitment and embryo development in the pig.* Reproduction, 2004. **128**(2): p. 237-47.

211. Sharkey, D.J., et al., *Seminal fluid induces leukocyte recruitment and cytokine and chemokine mRNA expression in the human cervix after coitus.* J Immunol, 2012. **188**(5): p. 2445-54.

212. Sanford, T.R., M. De, and G.W. Wood, *Expression of colony-stimulating factors and inflammatory cytokines in the uterus of CD1 mice during days 1 to 3 of pregnancy.* J Reprod Fertil, 1992. **94**(1): p. 213-20.

213. Robertson, S.A., et al., *Role of high molecular weight seminal vesicle proteins in eliciting the uterine inflammatory response to semen in mice.* J Reprod Fertil, 1996. **107**(2): p. 265-77.

214. Pandya, I.J. and J. Cohen, *The leukocytic reaction of the human uterine cervix to spermatozoa.* Fertil Steril, 1985. **43**(3): p. 417-21.

215. Taylor, N.J., *Investigation of sperm-induced cervical leucocytosis by a double mating study in rabbits.* J Reprod Fertil, 1982. **66**(1): p. 157-60.

216. McMaster, M.T., et al., *Activation and distribution of inflammatory cells in the mouse uterus during the preimplantation period.* J Immunol, 1992. **148**(6): p. 1699-705.

217. Lovell, J.W. and R. Getty, *Fate of semen in the uterus of the sow: histologic study of endometrium during the 27 hours after natural service.* Am J Vet Res, 1968. **29**(3): p. 609-25.

218. Claus, R., *Physiological role of seminal components in the reproductive tract of the female pig.* J Reprod Fertil Suppl, 1990. **40**: p. 117-31.

219. Rozeboom, K.J., M.H. Troedsson, and B.G. Crabo, *Characterization of uterine leukocyte infiltration in gilts after artificial insemination.* J Reprod Fertil, 1998. **114**(2): p. 195-9.

220. Robertson, S.A., *Seminal fluid signaling in the female reproductive tract: lessons from rodents and pigs.* J Anim Sci, 2007. **85**(13 Suppl): p. E36-44.

221. De, M., R. Choudhuri, and G.W. Wood, *Determination of the number and distribution of macrophages, lymphocytes, and granulocytes in the mouse uterus from mating through implantation.* J Leukoc Biol, 1991. **50**(3): p. 252-62.

222. Bischof, R.J., et al., *Inflammatory response in the pig uterus induced by seminal plasma.* J Reprod Immunol, 1994. **26**(2): p. 131-46.

223. Sharkey, D.J., et al., *Seminal plasma differentially regulates inflammatory cytokine gene expression in human cervical and vaginal epithelial cells.* Mol Hum Reprod, 2007. **13**(7): p. 491-501.

224. Moldenhauer, L.M., et al., *Cross-presentation of male seminal fluid antigens elicits T cell activation to initiate the female immune response to pregnancy.* J Immunol, 2009. **182**(12): p. 8080-93.

225. Robertson, S.A., et al., *Seminal Fluid Drives Expansion of the CD4+CD25+ T Regulatory Cell Pool and Induces Tolerance to Paternal Alloantigens in Mice.* Biol Reprod, 2009. **80**(5): p. 1036-1045.

226. Tafuri, A., et al., *T cell awareness of paternal alloantigens during pregnancy.* Science, 1995. **270**(5236): p. 630-3.

227. Shima, T., et al., *Regulatory T cells are necessary for implantation and maintenance of early pregnancy but not late pregnancy in allogeneic mice.* J Reprod Immunol, 2010. **85**(2): p. 121-9.

228. Politch, J.A., et al., *Concentrations and significance of cytokines and other immunologic factors in semen of healthy fertile men.* Hum Reprod, 2007. **22**(11): p. 2928-35.

229. Chen, W., et al., *Conversion of peripheral CD4+CD25- naive T cells to CD4+CD25+ regulatory T cells by TGF-beta induction of transcription factor Foxp3.* J Exp Med, 2003. **198**(12): p. 1875-86.

230. Baratelli, F., et al., *Prostaglandin E2 induces FOXP3 gene expression and T regulatory cell function in human CD4+ T cells.* J Immunol, 2005. **175**(3): p. 1483-90.

231. Burgess, A.W. and D. Metcalf, *The nature and action of granulocyte-macrophage colony stimulating factors.* Blood, 1980. **56**(6): p. 947-58.

232. Sheridan, J.W. and D. Metcalf, *Studies on the bone marrow colony stimulating factor (CSF): relation of tissue CSF to serum CSF.* J Cell Physiol, 1972. **80**(1): p. 129-40.

233. Kay, A.B., et al., *Messenger RNA expression of the cytokine gene cluster, interleukin 3 (IL-3), IL-4, IL-5, and granulocyte/macrophage colony-stimulating factor, in allergen-induced late-phase cutaneous reactions in atopic subjects.* J Exp Med, 1991. **173**(3): p. 775-8.

234. Williamson, D.J., et al., *The detection and initial characterization of colony-stimulating factors in synovial fluid.* Clin Exp Immunol, 1988. **72**(1): p. 67-73.

235. Leizer, T., et al., *Cytokine regulation of colony-stimulating factor production in cultured human synovial fibroblasts: I. Induction of GM-CSF and G-CSF production by interleukin-1 and tumor necrosis factor.* Blood, 1990. **76**(10): p. 1989-96.

236. Hamilton, J.A., *Rheumatoid arthritis: opposing actions of haemopoietic growth factors and slow-acting anti-rheumatic drugs.* Lancet, 1993. **342**(8870): p. 536-9.

237. Xing, Z., et al., *Human upper airway structural cell-derived cytokines support human peripheral blood monocyte survival: a potential mechanism for monocyte/macrophage accumulation in the tissue.* Am J Respir Cell Mol Biol, 1992. **6**(2): p. 212-8.

238. Robertson, S.A., G. Mayrhofer, and R.F. Seamark, *Uterine epithelial cells synthesize granulocyte-macrophage colony-stimulating factor and interleukin-6 in pregnant and nonpregnant mice.* Biol Reprod, 1992. **46**(6): p. 1069-79.

239. Zhao, Y. and N. Chegini, *The expression of granulocyte macrophage-colony stimulating factor (GM-CSF) and receptors in human endometrium.* Am J Reprod Immunol, 1999. **42**(5): p. 303-11.

240. Robertson, S.A., G. Mayrhofer, and R.F. Seamark, *Ovarian steroid hormones regulate granulocyte-macrophage colony-stimulating factor synthesis by uterine epithelial cells in the mouse.* Biology of Reproduction, 1996. **54**(1): p. 183-196.

241. Robertson, S.A. and R.F. Seamark, *Granulocyte macrophage colony stimulating factor (GM-CSF) in the murine reproductive tract: stimulation by seminal factors.* Reprod Fertil Dev, 1990. **2**(4): p. 359-68.

242. Robertson, S.A., et al., *Granulocyte-macrophage colony-stimulating factor (GM-CSF) targets myeloid leukocytes in the uterus during the post-mating inflammatory response in mice.* J Reprod Immunol, 2000. **46**(2): p. 131-54.

243. Robertson, S.A., et al., *Granulocyte-macrophage colony-stimulating factor promotes glucose transport and blastomere viability in murine preimplantation embryos.* Biol Reprod, 2001. **64**(4): p. 1206-15.

244. Higgins, D.M., et al., *Relative levels of M-CSF and GM-CSF influence the specific generation of macrophage populations during infection with Mycobacterium tuberculosis.* J Immunol, 2008. **180**(7): p. 4892-900.

245. Ozawa, H., et al., *Granulocyte-macrophage colony-stimulating factor gene transfer to dendritic cells or epidermal cells augments their antigen-presenting function including induction of anti-tumor immunity.* J Invest Dermatol, 1999. **113**(6): p. 999-1005.

246. Larsen, C.P., et al., *Regulation of immunostimulatory function and costimulatory molecule (B7-1 and B7-2) expression on murine dendritic cells.* J Immunol, 1994. **152**(11): p. 5208-19.

247. Stumbles, P.A., et al., *Resting respiratory tract dendritic cells preferentially stimulate T helper cell type 2 (Th2) responses and require obligatory cytokine signals for induction of Th1 immunity.* J Exp Med, 1998. **188**(11): p. 2019-31.

248. Askelund, K., et al., *CD83(+)dendritic cells in the decidua of women with recurrent miscarriage and normal pregnancy.* Placenta, 2004. **25**(2-3): p. 140-5.

249. Scholz, C., et al., *Distribution and maturity of dendritic cells in diseases of insufficient placentation.* Am J Reprod Immunol, 2008. **60**(3): p. 238-45.

250. Pollard, J.W., et al., *Apparent role of the macrophage growth factor, CSF-1, in placental development.* Nature, 1987. **330**(6147): p. 484-6.

251. Wood, G.W., E.H. Hausmann, and K. Kanakaraj, *Expression and regulation of chemokine genes in the mouse uterus during pregnancy.* Cytokine, 1999. **11**(12): p. 1038-45.

252. Wood, G.W., E. Hausmann, and R. Choudhuri, *Relative role of CSF-1, MCP-1/JE, and RANTES in macrophage recruitment during successful pregnancy.* Mol Reprod Dev, 1997. **46**(1): p. 62-9; discussion 69-70.

253. Heinrich, P.C., et al., *Principles of interleukin (IL)-6-type cytokine signalling and its regulation.* Biochem J, 2003. **374**(Pt 1): p. 1-20.

254. Dillon, S.R., et al., *Interleukin 31, a cytokine produced by activated T cells, induces dermatitis in mice.* Nat Immunol, 2004. **5**(7): p. 752-60.

255. Zhang, Q., et al., *Structures and biological functions of IL-31 and IL-31 receptors.* Cytokine Growth Factor Rev, 2008. **19**(5-6): p. 347-56.

256. Jacobs, A.L., et al., *Secretion and hormonal regulation of interleukin-6 production by mouse uterine stromal and polarized epithelial cells cultured in vitro.* Endocrinology, 1992. **131**(3): p. 1037-46.

257. Romano, M., et al., *Role of IL-6 and its soluble receptor in induction of chemokines and leukocyte recruitment.* Immunity, 1997. **6**(3): p. 315-25.

258. Bettelli, E., T. Korn, and V.K. Kuchroo, *Th17: the third member of the effector T cell trilogy.* Curr Opin Immunol, 2007. **19**(6): p. 652-7.

259. Bettelli, E., et al., *Reciprocal developmental pathways for the generation of pathogenic effector TH17 and regulatory T cells.* Nature, 2006. **441**(7090): p. 235-8.

260. Yang, X.O., et al., *Molecular antagonism and plasticity of regulatory and inflammatory T cell programs.* Immunity, 2008. **29**(1): p. 44-56.

261. Sharkey, D.J., et al., *TGF-beta mediates proinflammatory seminal fluid signaling in human cervical epithelial cells.* J Immunol, 2012. **189**(2): p. 1024-35.

262. Gutsche, S., et al., *Seminal plasma induces mRNA expression of IL-1beta, IL-6 and LIF in endometrial epithelial cells in vitro.* Mol Hum Reprod, 2003. **9**(12): p. 785-91.

263. Cheng, J.G., C.I. Rodriguez, and C.L. Stewart, *Control of uterine receptivity and embryo implantation by steroid hormone regulation of LIF production and LIF receptor activity: towards a molecular understanding of "the window of implantation".* Rev Endocr Metab Disord, 2002. **3**(2): p. 119-26.

264. Stewart, C.L., *The role of leukemia inhibitory factor (LIF) and other cytokines in regulating implantation in mammals.* Ann N Y Acad Sci, 1994. **734**: p. 157-65.

265. Sherwin, J.R., et al., *Identification of genes regulated by leukemia-inhibitory factor in the mouse uterus at the time of implantation.* Mol Endocrinol, 2004. **18**(9): p. 2185-95.

266. Bhatt, H., L.J. Brunet, and C.L. Stewart, *Uterine expression of leukemia inhibitory factor coincides with the onset of blastocyst implantation.* Proc Natl Acad Sci U S A, 1991. **88**(24): p. 11408-12.

267. Chen, J.R., et al., *Leukemia inhibitory factor can substitute for nidatory estrogen and is essential to inducing a receptive uterus for implantation but is not essential for subsequent embryogenesis.* Endocrinology, 2000. **141**(12): p. 4365-72.

268. Yang, Z.M., et al., *Differential hormonal regulation of leukemia inhibitory factor (LIF) in rabbit and mouse uterus.* Mol Reprod Dev, 1996. **43**(4): p. 470-6.

269. Arici, A., et al., *Interleukin-8 in the human endometrium.* J Clin Endocrinol Metab, 1998. **83**(5): p. 1783-7.

270. Arici, A., et al., *Interleukin-8 induces proliferation of endometrial stromal cells: a potential autocrine growth factor.* J Clin Endocrinol Metab, 1998. **83**(4): p. 1201-5.

271. Arici, A., *Local cytokines in endometrial tissue: the role of interleukin-8 in the pathogenesis of endometriosis.* Ann N Y Acad Sci, 2002. **955**: p. 101-9; discussion 118, 396-406.

272. Kayisli, U.A., N.G. Mahutte, and A. Arici, *Uterine chemokines in reproductive physiology and pathology.* Am J Reprod Immunol, 2002. **47**(4): p. 213-21.

273. Denison, F.C., et al., *Seminal plasma components stimulate interleukin-8 and interleukin-10 release.* Mol Hum Reprod, 1999. **5**(3): p. 220-6.

274. Arici, A., P.C. MacDonald, and M.L. Casey, *Modulation of the levels of interleukin-8 messenger ribonucleic acid and interleukin-8 protein synthesis in human endometrial stromal cells by transforming growth factor-beta 1.* J Clin Endocrinol Metab, 1996. **81**(8): p. 3004-9.

275. Modi, W.S. and T. Yoshimura, *Isolation of novel GRO genes and a phylogenetic analysis of the CXC chemokine subfamily in mammals.* Mol Biol Evol, 1999. **16**(2): p. 180-93.

276. Watanabe, K., et al., *Chemoattractants for neutrophils in lipopolysaccharide-induced inflammatory exudate from rats are not interleukin-8 counterparts but gro-gene-product/melanoma-growth-stimulating-activity-related factors.* Eur J Biochem, 1993. **214**(1): p. 267-70.

277. Meter, R.A., C.R. Wira, and J.V. Fahey, *Secretion of monocyte chemotactic protein-1 by human uterine epithelium directs monocyte migration in culture.* Fertil Steril, 2005. **84**(1): p. 191-201.

278. Deshmane, S.L., et al., *Monocyte chemoattractant protein-1 (MCP-1): an overview.* J Interferon Cytokine Res, 2009. **29**(6): p. 313-26.

279. Alam, R., et al., *RANTES is a chemotactic and activating factor for human eosinophils.* J Immunol, 1993. **150**(8 Pt 1): p. 3442-8.

280. Altman, G.B., et al., *RANTES production by cultured primate endometrial epithelial cells.* Am J Reprod Immunol, 1999. **42**(3): p. 168-74.

281. Arima, K., et al., *Effects of lipopolysaccharide and cytokines on production of RANTES by cultured human endometrial stromal cells.* Mol Hum Reprod, 2000. **6**(3): p. 246-51.

282. Bany, B.M., X. Zhang, and T.G. Kennedy, *Effects of epidermal growth factor and interleukin-1alpha on plasminogen activator secretion and decidualization in rat endometrial stromal cells.* Biol Reprod, 1998. **59**(1): p. 131-5.

283. Ushikubi, F., et al., *Impaired febrile response in mice lacking the prostaglandin E receptor subtype EP3.* Nature, 1998. **395**(6699): p. 281-4.

284. Bany, B.M. and T.G. Kennedy, *Role of interleukin 1 in the regulation of cyclooxygenase gene expression in rat endometrial stromal cells.* J Reprod Fertil, 1999. **115**(1): p. 125-31.

285. Lim, H., et al., *Cyclo-oxygenase-2-derived prostacyclin mediates embryo implantation in the mouse via PPARdelta.* Genes Dev, 1999. **13**(12): p. 1561-74.

286. Coleman, R.A., W.L. Smith, and S. Narumiya, *International Union of Pharmacology classification of prostanoid receptors: properties, distribution, and structure of the receptors and their subtypes.* Pharmacol Rev, 1994. **46**(2): p. 205-29.

287. Wang, H., et al., *Rescue of female infertility from the loss of cyclooxygenase-2 by compensatory up-regulation of cyclooxygenase-1 is a function of genetic makeup.* J Biol Chem, 2004. **279**(11): p. 10649-58.

288. Chakraborty, I., et al., *Developmental expression of the cyclo-oxygenase-1 and cyclo-oxygenase-2 genes in the peri-implantation mouse uterus and their differential regulation by the blastocyst and ovarian steroids.* J Mol Endocrinol, 1996. **16**(2): p. 107-22.

289. Lim, H. and S.K. Dey, *Prostaglandin E2 receptor subtype EP2 gene expression in the mouse uterus coincides with differentiation of the luminal epithelium for implantation.* Endocrinology, 1997. **138**(11): p. 4599-606.

290. Reese, J., et al., *COX-2 compensation in the uterus of COX-1 deficient mice during the pre-implantation period.* Mol Cell Endocrinol, 1999. **150**(1-2): p. 23-31.

291. Rees, M.C., et al., *Immunohistochemical localisation of cyclooxygenase in the human uterus.* Prostaglandins, 1982. **23**(2): p. 207-14.

292. Rees, M.C., et al., *Endometrial and myometrial prostaglandin release during the menstrual cycle in relation to menstrual blood loss.* J Clin Endocrinol Metab, 1984. **58**(5): p. 813-8.

293. Jones, R.L., R.W. Kelly, and H.O. Critchley, *Chemokine and cyclooxygenase-2 expression in human endometrium coincides with leukocyte accumulation.* Hum Reprod, 1997. **12**(6): p. 1300-6.

294. Milne, S.A. and H.N. Jabbour, *Prostaglandin (PG) F(2alpha) receptor expression and signaling in human endometrium: role of PGF(2alpha) in epithelial cell proliferation.* J Clin Endocrinol Metab, 2003. **88**(4): p. 1825-32.

295. Milne, S.A., et al., *Expression, localization, and signaling of PGE(2) and EP2/EP4 receptors in human nonpregnant endometrium across the menstrual cycle.* J Clin Endocrinol Metab, 2001. **86**(9): p. 4453-9.

296. Lopes, F.L., et al., *Transcriptional regulation of uterine vascular endothelial growth factor during early gestation in a carnivore model, Mustela vison.* J Biol Chem, 2006. **281**(34): p. 24602-11.

297. Kaczmarek, M.M., et al., *Assessment of VEGF-receptor system expression in the porcine endometrial stromal cells in response to insulin-like growth factor-I, relaxin, oxytocin and prostaglandin E2.* Mol Cell Endocrinol, 2008. **291**(1-2): p. 33-41.

298. Tasaki, Y., et al., *Expression of VEGF and its receptors in the bovine endometrium throughout the estrous cycle: effects of VEGF on prostaglandin production in endometrial cells.* J Reprod Dev, 2010. **56**(2): p. 223-9.

299. Lefevre, P.L. and B.D. Murphy, *Differential gene expression in the uterus and blastocyst during the reactivation of embryo development in a model of delayed implantation.* Methods Mol Biol, 2009. **550**: p. 11-61.

300. Murphy, B.D., *Embryonic diapause: advances in understanding the enigma of seasonal delayed implantation.* Reprod Domest Anim, 2012. **47 Suppl 6**: p. 121-4.

301. Thom, M.D., D.D. Johnson, and D.W. MacDonald, *The evolution and maintenance of delayed implantation in the mustelidae (mammalia: carnivora).* Evolution, 2004. **58**(1): p. 175-83.

302. Kaczmarek, M.M., et al., *Seminal plasma affects prostaglandin synthesis in the porcine oviduct.* Theriogenology, 2010. **74**(7): p. 1207-20.

303. Kaczmarek, M.M., K. Krawczynski, and J. Filant, *Seminal plasma affects prostaglandin synthesis and angiogenesis in the porcine uterus.* Biol Reprod, 2013. **88**(3): p. 72.

304. Christenson, L.K., et al., *Luteal maintenance during early pregnancy in the pig: role for prostaglandin E2.* Prostaglandins, 1994. **47**(1): p. 61-75.

305. Sales, K.J. and H.N. Jabbour, *Cyclooxygenase enzymes and prostaglandins in pathology of the endometrium.* Reproduction, 2003. **126**(5): p. 559-67.

306. Sales, K.J. and H.N. Jabbour, *Cyclooxygenase enzymes and prostaglandins in reproductive tract physiology and pathology.* Prostaglandins Other Lipid Mediat, 2003. **71**(3-4): p. 97-117.

307. Kennedy, T.G., C. Gillio-Meina, and S.H. Phang, *Prostaglandins and the initiation of blastocyst implantation and decidualization.* Reproduction, 2007. **134**(5): p. 635-43.

308. Van Ly, D., et al., *Prostaglandins but not leukotrienes alter extracellular matrix protein deposition and cytokine release in primary human airway smooth muscle cells and fibroblasts.* Am J Physiol Lung Cell Mol Physiol, 2012. **303**(3): p. L239-50.

309. Weinreb, R.N., et al., *Prostaglandins increase matrix metalloproteinase release from human ciliary smooth muscle cells.* Invest Ophthalmol Vis Sci, 1997. **38**(13): p. 2772-80.

310. Van Lint, P. and C. Libert, *Chemokine and cytokine processing by matrix metalloproteinases and its effect on leukocyte migration and inflammation.* J Leukoc Biol, 2007. **82**(6): p. 1375-81.

311. Mitsiades, N., et al., *Matrix metalloproteinase-7-mediated cleavage of Fas ligand protects tumor cells from chemotherapeutic drug cytotoxicity.* Cancer Res, 2001. **61**(2): p. 577-81.

312. Tang, M., et al., *Lefty is expressed in mouse endometrium in estrous cycle and peri-implantation period.* Hum Reprod, 2005. **20**(4): p. 872-80.

313. Ito, A., et al., *Degradation of interleukin 1beta by matrix metalloproteinases.* J Biol Chem, 1996. **271**(25): p. 14657-60.

314. Singer, C.F., et al., *Local cytokines induce differential expression of matrix metalloproteinases but not their tissue inhibitors in human endometrial fibroblasts.* Eur J Biochem, 1999. **259**(1-2): p. 40-5.

315. Haro, H., et al., *Matrix metalloproteinase-7-dependent release of tumor necrosis factor-alpha in a model of herniated disc resorption.* J Clin Invest, 2000. **105**(2): p. 143-50.

316. Aplin, J.D., *Adhesion molecules in implantation.* Rev Reprod, 1997. **2**(2): p. 84-93.

317. Goetzl, E.J., M.J. Banda, and D. Leppert, *Matrix metalloproteinases in immunity.* J Immunol, 1996. **156**(1): p. 1-4.

318. Feng, J., J.F. Woessner, Jr., and C. Zhu, *Matrilysin activity in the rat uterus during the oestrous cycle and implantation.* J Reprod Fertil, 1998. **114**(2): p. 347-50.

319. Das, S.K., et al., *Expression of matrix metalloproteinases and tissue inhibitors of metalloproteinases in the mouse uterus during the peri-implantation period.* Dev Genet, 1997. **21**(1): p. 44-54.

320. Chegini, N., et al., *Human uterine tissue throughout the menstrual cycle expresses transforming growth factor-beta 1 (TGF beta 1), TGF beta 2, TGF beta 3, and TGF beta type II receptor messenger ribonucleic acid and protein and contains [125I]TGF beta 1-binding sites.* Endocrinology, 1994. **135**(1): p. 439-49.

321. Tang, X.M., et al., *Expression of transforming growth factor-beta (TGF beta) isoforms and TGF beta type II receptor messenger ribonucleic acid and protein, and the effect of TGF beta s on endometrial stromal cell growth and protein degradation in vitro.* Endocrinology, 1994. **135**(1): p. 450-9.

322. Marshburn, P.B., A.M. Arici, and M.L. Casey, *Expression of transforming growth factor-beta 1 messenger ribonucleic acid and the modulation of deoxyribonucleic acid synthesis by transforming growth factor-beta 1 in human endometrial cells.* Am J Obstet Gynecol, 1994. **170**(4): p. 1152-8.

323. Gold, L.I., et al., *Increased expression of transforming growth factor beta isoforms and basic fibroblast growth factor in complex hyperplasia and adenocarcinoma of the endometrium: evidence for paracrine and autocrine action.* Cancer Res, 1994. **54**(9): p. 2347-58.

324. Chegini, N., L.I. Gold, and R.S. Williams, *Localization of transforming growth factor beta isoforms TGF-beta 1, TGF-beta 2, and TGF-beta 3 in surgically induced endometriosis in the rat.* Obstet Gynecol, 1994. **83**(3): p. 455-61.

325. Das, S.K., et al., *Expression of transforming growth factor-beta isoforms (beta 2 and beta 3) in the mouse uterus: analysis of the periimplantation period and effects of ovarian steroids.* Endocrinology, 1992. **130**(6): p. 3459-66.

326. Slager, H.G., et al., *Transforming growth factor-beta in the early mouse embryo: implications for the regulation of muscle formation and implantation.* Dev Genet, 1993. **14**(3): p. 212-24.

327. Lin, H.Y., et al., *Expression of Smad2 and Smad4, transforming growth factor-beta signal transducers in rat endometrium during the estrous cycle, pre-, and peri-implantation.* Anim Reprod Sci, 2004. **80**(3-4): p. 303-16.

328. Zhao, K.Q., et al., *Maternal Smad3 deficiency compromises decidualization in mice.* J Cell Biochem, 2012. **113**(10): p. 3266-75.

329. Bruner, K.L., et al., *Transforming growth factor beta mediates the progesterone suppression of an epithelial metalloproteinase by adjacent stroma in the human endometrium.* Proc Natl Acad Sci U S A, 1995. **92**(16): p. 7362-6.

330. Streuli, C.H., et al., *Extracellular matrix regulates expression of the TGF-beta 1 gene.* J Cell Biol, 1993. **120**(1): p. 253-60.

331. Chang, H.J., et al., *Transforming growth factor (TGF)-beta1-induced human endometrial stromal cell decidualization through extracellular signal-regulated kinase and Smad activation in vitro: peroxisome proliferator-activated receptor gamma acts as a negative regulator of TGF-beta1.* Fertil Steril, 2008. **90**(4 Suppl): p. 1357-65.

332. Riesewijk, A., et al., *Gene expression profiling of human endometrial receptivity on days LH+2 versus LH+7 by microarray technology.* Mol Hum Reprod, 2003. **9**(5): p. 253-64.

333. Kao, L.C., et al., *Global gene profiling in human endometrium during the window of implantation.* Endocrinology, 2002. **143**(6): p. 2119-38.

334. Kao, L.C., et al., *Expression profiling of endometrium from women with endometriosis reveals candidate genes for disease-based implantation failure and infertility.* Endocrinology, 2003. **144**(7): p. 2870-81.

335. Burney, R.O., et al., *Gene expression analysis of endometrium reveals progesterone resistance and candidate susceptibility genes in women with endometriosis.* Endocrinology, 2007. **148**(8): p. 3814-26.

336. Giudice, L.C., *Microarray expression profiling reveals candidate genes for human uterine receptivity.* Am J Pharmacogenomics, 2004. **4**(5): p. 299-312.

337. Cummings, M., et al., *A robust RNA integrity-preserving staining protocol for laser capture microdissection of endometrial cancer tissue.* Anal Biochem, 2011. **416**(1): p. 123-5.

338. Niu, N. and X. Qin, *New insights into IL-7 signaling pathways during early and late T cell development.* Cell Mol Immunol, 2013. **10**(3): p. 187-9.

339. Nilsson, E.E. and M.K. Skinner, *Bone morphogenetic protein-4 acts as an ovarian follicle survival factor and promotes primordial follicle development.* Biol Reprod, 2003. **69**(4): p. 1265-72.

340. McKenzie, A.N., et al., *Interleukin 13, a T-cell-derived cytokine that regulates human monocyte and B-cell function.* Proc Natl Acad Sci U S A, 1993. **90**(8): p. 3735-9.

341. Bailey, J.R., et al., *IL-13 promotes collagen accumulation in Crohn's disease fibrosis by down-regulation of fibroblast MMP synthesis: a role for innate lymphoid cells?* PLoS One, 2012. **7**(12): p. e52332.

342. Firszt, R., et al., *Interleukin-13 induces collagen type-1 expression through matrix metalloproteinase-2 and transforming growth factor-beta1 in airway fibroblasts in asthma.* Eur Respir J, 2013.

343. Wadsworth, S.J., et al., *IL-13 and TH2 cytokine exposure triggers matrix metalloproteinase 7-mediated Fas ligand cleavage from bronchial epithelial cells.* J Allergy Clin Immunol, 2010. **126**(2): p. 366-74, 374 e1-8.

344. Warren, J.S., P.A. Ward, and K.J. Johnson, *Tumor necrosis factor: a plurifunctional mediator of acute inflammation.* Mod Pathol, 1988. **1**(3): p. 242-7.

345. Smith, D.F., et al., *GRO family chemokines are specialized for monocyte arrest from flow.* Am J Physiol Heart Circ Physiol, 2005. **289**(5): p. H1976-84.

346. Ooi, A.T., et al., *Identification of an interleukin 13-induced epigenetic signature in allergic airway inflammation.* Am J Transl Res, 2012. **4**(2): p. 219-28.

347. Rundhaug, J.E., *Matrix metalloproteinases and angiogenesis.* J Cell Mol Med, 2005. **9**(2): p. 267-85.

348. Nagase, H., *Activation mechanisms of matrix metalloproteinases.* Biol Chem, 1997. **378**(3-4): p. 151-60.

349. Nagase, H., et al., *Substrate specificities and activation mechanisms of matrix metalloproteinases.* Biochem Soc Trans, 1991. **19**(3): p. 715-8.

350. Nagase, H., et al., *Stepwise activation mechanisms of the precursors of matrix metalloproteinases 1 (tissue collagenase) and 3 (stromelysin).* Biomed Biochim Acta, 1991. **50**(4-6): p. 749-54.

351. Nagase, H., et al., *Activation mechanisms of the precursors of matrix metalloproteinases 1, 2 and 3.* Matrix Suppl, 1992. **1**: p. 237-44.

352. Schall, T.J., et al., *Selective attraction of monocytes and T lymphocytes of the memory phenotype by cytokine RANTES.* Nature, 1990. **347**(6294): p. 669-71.

353. Bleul, C.C., et al., *A highly efficacious lymphocyte chemoattractant, stromal cell-derived factor 1 (SDF-1).* J Exp Med, 1996. **184**(3): p. 1101-9.

354. Kryczek, I., et al., *The chemokine SDF-1/CXCL12 contributes to T lymphocyte recruitment in human pre-ovulatory follicles and coordinates with lymphocytes to increase granulosa cell survival and embryo quality.* Am J Reprod Immunol, 2005. **54**(5): p. 270-83.

355. Ashkar, A.A., et al., *Assessment of requirements for IL-15 and IFN regulatory factors in uterine NK cell differentiation and function during pregnancy.* J Immunol, 2003. **171**(6): p. 2937-44.

356. Kishimoto, T., T. Taga, and S. Akira, *Cytokine signal transduction.* Cell, 1994. **76**(2): p. 253-62.

357. Ozaki, K. and W.J. Leonard, *Cytokine and cytokine receptor pleiotropy and redundancy.* J Biol Chem, 2002. **277**(33): p. 29355-8.

358. Lin, J.X., et al., *The role of shared receptor motifs and common Stat proteins in the generation of cytokine pleiotropy and redundancy by IL-2, IL-4, IL-7, IL-13, and IL-15.* Immunity, 1995. **2**(4): p. 331-9.

359. Rossi, M., et al., *Identification of genes regulated by interleukin-1beta in human endometrial stromal cells.* Reproduction, 2005. **130**(5): p. 721-9.

360. Matsuoka, R., et al., *Regulation of estrogen activity in human endometrium: effect of IL-1beta on steroid sulfatase activity in human endometrial stromal cells.* Steroids, 2002. **67**(7): p. 655-9.

361. Godkin, J.D. and J.J. Dore, *Transforming growth factor beta and the endometrium.* Rev Reprod, 1998. **3**(1): p. 1-6.

362. Yu, Q. and I. Stamenkovic, *Cell surface-localized matrix metalloproteinase-9 proteolytically activates TGF-beta and promotes tumor invasion and angiogenesis.* Genes Dev, 2000. **14**(2): p. 163-76.

363. Eickelberg, O., et al., *Molecular mechanisms of TGF-(beta) antagonism by interferon (gamma) and cyclosporine A in lung fibroblasts.* FASEB J, 2001. **15**(3): p. 797-806.

364. Higashi, K., et al., *Interferon-gamma interferes with transforming growth factor-beta signaling through direct interaction of YB-1 with Smad3.* J Biol Chem, 2003. **278**(44): p. 43470-9.

365. Yu, J., et al., *Pro- and antiinflammatory cytokine signaling: reciprocal antagonism regulates interferon-gamma production by human natural killer cells.* Immunity, 2006. **24**(5): p. 575-90.

366. Chen, W., *IDO: more than an enzyme.* Nat Immunol, 2011. **12**(9): p. 809-11.

367. Tabibzadeh, S.S., P.G. Satyaswaroop, and P.N. Rao, *Antiproliferative effect of interferon-gamma in human endometrial epithelial cells in vitro: potential local growth modulatory role in endometrium.* J Clin Endocrinol Metab, 1988. **67**(1): p. 131-8.

368. Kawano, Y., et al., *Effects of interferon-gamma on secretion of vascular endothelial growth factor by endometrial stromal cells.* Am J Reprod Immunol, 2000. **43**(1): p. 47-52.

369. Zoumpoulidou, G., et al., *Convergence of interferon-gamma and progesterone signaling pathways in human endometrium: role of PIASy (protein inhibitor of activated signal transducer and activator of transcription-y).* Mol Endocrinol, 2004. **18**(8): p. 1988-99.

370. Chegini, N., X.M. Tang, and Q. Dou, *The expression, activity and regulation of granulocyte macrophage-colony stimulating factor in human endometrial epithelial and stromal cells.* Mol Hum Reprod, 1999. **5**(5): p. 459-66.

371. Shanafelt, A.B., K.E. Johnson, and R.A. Kastelein, *Identification of critical amino acid residues in human and mouse granulocyte-macrophage colony-stimulating factor and their involvement in species specificity.* J Biol Chem, 1991. **266**(21): p. 13804-10.

372. Gough, N.M., et al., *Structure and expression of the mRNA for murine granulocyte-macrophage colony stimulating factor.* EMBO J, 1985. **4**(3): p. 645-53.

373. Lacey, D.C., et al., *Defining GM-CSF- and macrophage-CSF-dependent macrophage responses by in vitro models.* J Immunol, 2012. **188**(11): p. 5752-65.

374. Paiva, P., et al., *Human chorionic gonadotrophin regulates FGF2 and other cytokines produced by human endometrial epithelial cells, providing a mechanism for enhancing endometrial receptivity.* Hum Reprod, 2011. **26**(5): p. 1153-62.

375. Bulmer, J.N., et al., *Granulated lymphocytes in human endometrium: histochemical and immunohistochemical studies.* Hum Reprod, 1991. **6**(6): p. 791-8.

376. Hemberger, M., *Immune balance at the foeto-maternal interface as the fulcrum of reproductive success.* J Reprod Immunol, 2013. **97**(1): p. 36-42.

377. Chaouat, G., *Inflammation, NK cells and implantation: friend and foe (the good, the bad and the ugly?): replacing placental viviparity in an evolutionary perspective.* J Reprod Immunol, 2013. **97**(1): p. 2-13.

378. Erlebacher, A., *Immunology of the maternal-fetal interface.* Annu Rev Immunol, 2013. **31**: p. 387-411.

379. Fu, B., et al., *Natural killer cells promote immune tolerance by regulating inflammatory TH17 cells at the human maternal-fetal interface.* Proc Natl Acad Sci U S A, 2013. **110**(3): p. E231-40.

380. Gonzalez, I.T., et al., *Uterine NK cells are critical in shaping DC immunogenic functions compatible with pregnancy progression.* PLoS One, 2012. **7**(10): p. e46755.

381. Hatta, K., et al., *Emerging themes in uterine natural killer cell heterogeneity and function.* Am J Reprod Immunol, 2012. **68**(4): p. 282-9.

382. Male, V., L. Gardner, and A. Moffett, *Isolation of cells from the feto-maternal interface.* Curr Protoc Immunol, 2012. **Chapter 7**: p. Unit 7 40 1-11.

383. Seymour, J.F., et al., *Mice lacking both granulocyte colony-stimulating factor (CSF) and granulocyte-macrophage CSF have impaired reproductive capacity,*

perturbed neonatal granulopoiesis, lung disease, amyloidosis, and reduced long-term survival. Blood, 1997. **90**(8): p. 3037-49.

384. Lee, J.Y., M. Lee, and S.K. Lee, *Role of endometrial immune cells in implantation.* Clin Exp Reprod Med, 2011. **38**(3): p. 119-25.
385. Olive, D.L., J.B. Weinberg, and A.F. Haney, *Sperm-macrophage interaction in the mouse: a quantitative assay in vitro using 111indium oxine-labeled sperm.* Biol Reprod, 1987. **37**(5): p. 1170-8.
386. Laird, S.M., et al., *Stimulation of human endometrial epithelial cell interleukin 6 production by interleukin 1 and placental protein 14.* Hum Reprod, 1994. **9**(7): p. 1339-43.
387. von Wolff, M., et al., *Endometrial expression and secretion of interleukin-6 throughout the menstrual cycle.* Gynecol Endocrinol, 2002. **16**(2): p. 121-9.
388. von Wolff, M., et al., *Endometrial interleukin-6 in vitro is not regulated directly by female steroid hormones, but by pro-inflammatory cytokines and hypoxia.* Mol Hum Reprod, 2002. **8**(12): p. 1096-102.
389. Hurst, S.M., et al., *Il-6 and its soluble receptor orchestrate a temporal switch in the pattern of leukocyte recruitment seen during acute inflammation.* Immunity, 2001. **14**(6): p. 705-14.
390. Stewart, C.L., *Leukaemia inhibitory factor and the regulation of pre-implantation development of the mammalian embryo.* Mol Reprod Dev, 1994. **39**(2): p. 233-8.
391. Fouladi Nashta, A.A., et al., *Role of leukemia inhibitor factor (LIF) in decidualisation of murine uterine stromal cells in vitro.* J Endocrinol, 2004. **181**(3): p. 477-92.
392. Guenet, J.L., *The mouse genome.* Genome Res, 2005. **15**(12): p. 1729-40.
393. Greenbaum, D., et al., *Comparing protein abundance and mRNA expression levels on a genomic scale.* Genome Biol, 2003. **4**(9): p. 117.
394. Anderson, P., *Post-transcriptional control of cytokine production.* Nat Immunol, 2008. **9**(4): p. 353-9.
395. Proost, P., et al., *Posttranslational modifications affect the activity of the human monocyte chemotactic proteins MCP-1 and MCP-2: identification of MCP-2(6-76) as a natural chemokine inhibitor.* J Immunol, 1998. **160**(8): p. 4034-41.
396. May, L.T., et al., *Synthesis and secretion of multiple forms of beta 2-interferon/B-cell differentiation factor 2/hepatocyte-stimulating factor by human fibroblasts and monocytes.* J Biol Chem, 1988. **263**(16): p. 7760-6.

397. Kameyoshi, Y., et al., *Cytokine RANTES released by thrombin-stimulated platelets is a potent attractant for human eosinophils.* J Exp Med, 1992. **176**(2): p. 587-92.

398. Arava, Y., et al., *Genome-wide analysis of mRNA translation profiles in Saccharomyces cerevisiae.* Proc Natl Acad Sci U S A, 2003. **100**(7): p. 3889-94.

399. Djebbari, A. and J. Quackenbush, *Seeded Bayesian Networks: constructing genetic networks from microarray data.* BMC Syst Biol, 2008. **2**: p. 57.

400. Garbis, S., G. Lubec, and M. Fountoulakis, *Limitations of current proteomics technologies.* J Chromatogr A, 2005. **1077**(1): p. 1-18.

401. Baud, V. and M. Karin, *Signal transduction by tumor necrosis factor and its relatives.* Trends Cell Biol, 2001. **11**(9): p. 372-7.

402. Taga, T., et al., *Interleukin-6 triggers the association of its receptor with a possible signal transducer, gp130.* Cell, 1989. **58**(3): p. 573-81.

403. Hirano, T., *Molecular basis underlying functional pleiotropy of cytokines and growth factors.* Biochem Biophys Res Commun, 1999. **260**(2): p. 303-8.

404. Liu, Z.G., et al., *Dissection of TNF receptor 1 effector functions: JNK activation is not linked to apoptosis while NF-kappaB activation prevents cell death.* Cell, 1996. **87**(3): p. 565-76.

405. Menten, P., et al., *Differential induction of monocyte chemotactic protein-3 in mononuclear leukocytes and fibroblasts by interferon-alpha/beta and interferon-gamma reveals MCP-3 heterogeneity.* Eur J Immunol, 1999. **29**(2): p. 678-85.

406. Lowenthal, J.W., et al., *Tumor necrosis factor-alpha activation of the IL-2 receptor-alpha gene involves the induction of kappa B-specific DNA binding proteins.* J Immunol, 1989. **142**(9): p. 3121-8.

407. Osborn, L., S. Kunkel, and G.J. Nabel, *Tumor necrosis factor alpha and interleukin 1 stimulate the human immunodeficiency virus enhancer by activation of the nuclear factor kappa B.* Proc Natl Acad Sci U S A, 1989. **86**(7): p. 2336-40.

408. Hasbold, J., et al., *Integrating signals from IFN-gamma and IL-4 by B cells: positive and negative effects on CD40 ligand-induced proliferation, survival, and division-linked isotype switching to IgG1, IgE, and IgG2a.* J Immunol, 1999. **163**(8): p. 4175-81.

409. Nguyen, V.T. and E.N. Benveniste, *IL-4-activated STAT-6 inhibits IFN-gamma-induced CD40 gene expression in macrophages/microglia.* J Immunol, 2000. **165**(11): p. 6235-43.

410. Ritter, U. and H. Moll, *Monocyte chemotactic protein-1 stimulates the killing of leishmania major by human monocytes, acts synergistically with IFN-gamma and is antagonized by IL-4.* Eur J Immunol, 2000. **30**(11): p. 3111-20.

411. Paludan, S.R., *Interleukin-4 and interferon-gamma: the quintessence of a mutual antagonistic relationship.* Scand J Immunol, 1998. **48**(5): p. 459-68.

412. Kaplan, M.H., et al., *Stat6 is required for mediating responses to IL-4 and for development of Th2 cells.* Immunity, 1996. **4**(3): p. 313-9.

413. Chatelain, R., K. Varkila, and R.L. Coffman, *IL-4 induces a Th2 response in Leishmania major-infected mice.* J Immunol, 1992. **148**(4): p. 1182-7.

414. Maggi, E., et al., *Reciprocal regulatory effects of IFN-gamma and IL-4 on the in vitro development of human Th1 and Th2 clones.* J Immunol, 1992. **148**(7): p. 2142-7.

415. Parronchi, P., et al., *IL-4 and IFN (alpha and gamma) exert opposite regulatory effects on the development of cytolytic potential by Th1 or Th2 human T cell clones.* J Immunol, 1992. **149**(9): p. 2977-83.

416. Mosmann, T.R. and S. Sad, *The expanding universe of T-cell subsets: Th1, Th2 and more.* Immunol Today, 1996. **17**(3): p. 138-46.

417. Strober, W., *Immunology: The expanding T(H)2 universe.* Nature, 2010. **463**(7280): p. 434-5.

418. Eyerich, K. and N. Novak, *Immunology of atopic eczema: overcoming the Th1/Th2 paradigm.* Allergy, 2013. **68**(8): p. 974-82.

419. Wilczynski, J.R., *Th1/Th2 cytokines balance--yin and yang of reproductive immunology.* Eur J Obstet Gynecol Reprod Biol, 2005. **122**(2): p. 136-43.

420. Deon, D., et al., *Cross-talk between IL-1 and IL-6 signaling pathways in rheumatoid arthritis synovial fibroblasts.* J Immunol, 2001. **167**(9): p. 5395-403.

421. Alvaro-Gracia, J.M., et al., *Mutual antagonism between interferon-gamma and tumor necrosis factor-alpha on fibroblast-like synoviocytes: paradoxical induction of IFN-gamma and TNF-alpha receptor expression.* J Clin Immunol, 1993. **13**(3): p. 212-8.

422. Miyajima, A., T. Hara, and T. Kitamura, *Common subunits of cytokine receptors and the functional redundancy of cytokines.* Trends Biochem Sci, 1992. **17**(10): p. 378-82.

423. Hibi, M., K. Nakajima, and T. Hirano, *IL-6 cytokine family and signal transduction: a model of the cytokine system.* J Mol Med (Berl), 1996. **74**(1): p. 1-12.

424. Bazan, J.F., *Haemopoietic receptors and helical cytokines.* Immunol Today, 1990. **11**(10): p. 350-4.

425. Ip, N.Y., et al., *CNTF and LIF act on neuronal cells via shared signaling pathways that involve the IL-6 signal transducing receptor component gp130.* Cell, 1992. **69**(7): p. 1121-32.

426. Carbia-Nagashima, A. and E. Arzt, *Intracellular proteins and mechanisms involved in the control of gp130/JAK/STAT cytokine signaling.* IUBMB Life, 2004. **56**(2): p. 83-8.

427. Kling, J., et al., *Redundancy of interleukin-6 in the differentiation of T cell and monocyte subsets during cutaneous leishmaniasis.* Exp Parasitol, 2011. **129**(3): p. 270-6.

428. Liu, Z., et al., *Acute obstructive cholangiopathy in interleukin-6 deficient mice: compensation by leukemia inhibitory factor (LIF) suggests importance of gp-130 signaling in the ductular reaction.* Liver, 2000. **20**(2): p. 114-24.

429. Tanaka, T. and T. Kishimoto, *Immunotherapeutic implication of IL-6 blockade.* Immunotherapy, 2012. **4**(1): p. 87-105.

430. Alexander, W.S. *Knockout Mouse Models of Cytokine Action in Hematopoietic Stem Cell Regulation*. Madame Curie Bioscience Database [Internet] 2000 [cited 2014 27/02/2014]; Available from: http://www.ncbi.nlm.nih.gov/books/NBK6511/.

431. Green, A.R., *Peptide regulatory factors: multifunctional mediators of cellular growth and differentiation.* Lancet, 1989. **1**(8640): p. 705-7.

432. Sporn, M.B. and A.B. Roberts, *Peptide growth factors are multifunctional.* Nature, 1988. **332**(6161): p. 217-9.

433. Wan, Y., et al., *Transmodulation of epidermal growth factor receptor mediates IL-1 beta-induced MMP-1 expression in cultured human keratinocytes.* Int J Mol Med, 2001. **7**(3): p. 329-34.

434. Alex, P., et al., *Distinct cytokine patterns identified from multiplex profiles of murine DSS and TNBS-induced colitis.* Inflamm Bowel Dis, 2009. **15**(3): p. 341-52.

435. Szodoray, P., et al., *Circulating cytokines in Norwegian patients with psoriatic arthritis determined by a multiplex cytokine array system.* Rheumatology (Oxford), 2007. **46**(3): p. 417-25.

436. Yan, X.J., et al., *Identification of outcome-correlated cytokine clusters in chronic lymphocytic leukemia.* Blood, 2011. **118**(19): p. 5201-10.

437. van den Ham, H.J., et al., *Differential cytokine profiles in juvenile idiopathic arthritis subtypes revealed by cluster analysis.* Rheumatology (Oxford), 2009. **48**(8): p. 899-905.

438. Lvovschi, V., et al., *Correction: cytokine profiles in sepsis have limited relevance for stratifying patients in the emergency department: a prospective observational study.* PLoS One, 2012. **7**(1).
439. Lvovschi, V., et al., *Cytokine profiles in sepsis have limited relevance for stratifying patients in the emergency department: a prospective observational study.* PLoS One, 2011. **6**(12): p. e28870.
440. Weaver, D.C., C.T. Workman, and G.D. Stormo, *Modeling regulatory networks with weight matrices.* Pac Symp Biocomput, 1999: p. 112-23.
441. Akutsu, T., S. Miyano, and S. Kuhara, *Identification of genetic networks from a small number of gene expression patterns under the Boolean network model.* Pac Symp Biocomput, 1999: p. 17-28.
442. Kerr, J.R., et al., *Gene expression subtypes in patients with chronic fatigue syndrome/myalgic encephalomyelitis.* J Infect Dis, 2008. **197**(8): p. 1171-84.
443. Emmert-Streib, F., *The chronic fatigue syndrome: a comparative pathway analysis.* J Comput Biol, 2007. **14**(7): p. 961-72.
444. Broderick, G., et al., *A formal analysis of cytokine networks in chronic fatigue syndrome.* Brain Behav Immun, 2010. **24**(7): p. 1209-17.
445. O'Hagan, A., *Bayesian principles*, in *Bayesian Methods in Health Economics: The Biomedical & Life Sciences Collection, Henry Stewart Talks*, T. O'Hagan, Editor 2009, Henry Stewart Talks Ltd: London.
446. Stevens, J.W. and A. O'Hagan, *Incorporation of genuine prior information in cost-effectiveness analysis of clinical trial data.* Int J Technol Assess Health Care, 2002. **18**(4): p. 782-90.
447. Lee, P.M., *Bayesian statistics: an introduction*. 2nd ed. 1997, London: Arnold.
448. Bink, M.C.A.M., et al., *Bayesian analysis of complex traits in pedigreed plant populations* Euphytica, 2008. **161**(1): p. 85-96.
449. Larranaga, P., et al., *Machine learning in bioinformatics.* Brief Bioinform, 2006. **7**(1): p. 86-112.
450. Krallinger, M., R.A. Erhardt, and A. Valencia, *Text-mining approaches in molecular biology and biomedicine.* Drug Discov Today, 2005. **10**(6): p. 439-45.
451. Mathe, C., et al., *Current methods of gene prediction, their strengths and weaknesses.* Nucleic Acids Res, 2002. **30**(19): p. 4103-17.
452. Aerts, S., et al., *A genetic algorithm for the detection of new cis-regulatory modules in sets of coregulated genes.* Bioinformatics, 2004. **20**(12): p. 1974-6.
453. Bockhorst, J., et al., *A Bayesian network approach to operon prediction.* Bioinformatics, 2003. **19**(10): p. 1227-35.

454. Won, K.J., A. Prugel-Bennett, and A. Krogh, *Training HMM structure with genetic algorithm for biological sequence analysis.* Bioinformatics, 2004. **20**(18): p. 3613-9.

455. Carter, R.J., I. Dubchak, and S.R. Holbrook, *A computational approach to identify genes for functional RNAs in genomic sequences.* Nucleic Acids Res, 2001. **29**(19): p. 3928-38.

456. Pearl, J., *Graphical Models for Probabilistic and Causal Reasoning*, in *The Computer Science and Engineering Handbook*, A.T. Tucker, Editor 1997, CRC Press: Boca Raton, Florida. p. 699-711.

457. Schafer, J. and K. Strimmer, *An empirical Bayes approach to inferring large-scale gene association networks.* Bioinformatics, 2005. **21**(6): p. 754-64.

458. Jojic, V., et al., *Efficient approximations for learning phylogenetic HMM models from data.* Bioinformatics, 2004. **20 Suppl 1**: p. i161-8.

459. Leone, M. and A. Pagnani, *Predicting protein functions with message passing algorithms.* Bioinformatics, 2005. **21**(2): p. 239-47.

460. Friedman, N., et al., *Using Bayesian networks to analyze expression data.* J Comput Biol, 2000. **7**(3-4): p. 601-20.

461. Le Phillip, P., A. Bahl, and L.H. Ungar, *Using prior knowledge to improve genetic network reconstruction from microarray data.* In Silico Biol, 2004. **4**(3): p. 335-53.

462. Husmeier, D. and A.V. Werhli, *Bayesian integration of biological prior knowledge into the reconstruction of gene regulatory networks with Bayesian networks.* Comput Syst Bioinformatics Conf, 2007. **6**: p. 85-95.

463. Gevaert, O., S. Van Vooren, and B. De Moor, *A framework for elucidating regulatory networks based on prior information and expression data.* Ann N Y Acad Sci, 2007. **1115**: p. 240-8.

464. Wolpurt, D.H., *No free lunch theorems for optimization.* IEEE Transactions on Evolutionary Computation, 1997. **1**(1): p. 67-82.

465. Li, Y., et al., *Critical reasoning on causal inference in genome-wide linkage and association studies.* Trends Genet, 2010. **26**(12): p. 493-8.

466. Pearl, J., *Causality: Models, Reasoning and Inference*. 2nd ed. 2009, Cambridge: Cambridge University Press.

467. Moloshok, T.D., et al., *Application of Bayesian decomposition for analysing microarray data.* Bioinformatics, 2002. **18**(4): p. 566-75.

468. Sha, N., M.G. Tadesse, and M. Vannucci, *Bayesian variable selection for the analysis of microarray data with censored outcomes.* Bioinformatics, 2006. **22**(18): p. 2262-8.

469. Zhao, H., et al., *Multivariate hierarchical Bayesian model for differential gene expression analysis in microarray experiments.* BMC Bioinformatics, 2008. **9 Suppl 1**: p. S9.

470. Imoto, S., et al., *Bayesian network and nonparametric heteroscedastic regression for nonlinear modeling of genetic network.* Proc IEEE Comput Soc Bioinform Conf, 2002. **1**: p. 219-27.

471. Kim, S., S. Imoto, and S. Miyano, *Dynamic Bayesian network and nonparametric regression for nonlinear modeling of gene networks from time series gene expression data.* Biosystems, 2004. **75**(1-3): p. 57-65.

472. Gracias, D.T. and P.D. Katsikis, *MicroRNAs: key components of immune regulation.* Adv Exp Med Biol, 2011. **780**: p. 15-26.

473. Stumpo, D.J., W.S. Lai, and P.J. Blackshear, *Inflammation: cytokines and RNA-based regulation.* Wiley Interdiscip Rev RNA, 2010. **1**(1): p. 60-80.

474. Mortier, A., J. Van Damme, and P. Proost, *Regulation of chemokine activity by posttranslational modification.* Pharmacol Ther, 2008. **120**(2): p. 197-217.

475. Pierre, P., *Immunity and the regulation of protein synthesis: surprising connections.* Curr Opin Immunol, 2009. **21**(1): p. 70-7.

476. Buckley, A.R., *Prolactin, a lymphocyte growth and survival factor.* Lupus, 2001. **10**(10): p. 684-90.

477. Brand, J.M., et al., *Prolactin triggers pro-inflammatory immune responses in peripheral immune cells.* Eur Cytokine Netw, 2004. **15**(2): p. 99-104.

478. Carreno, P.C., et al., *Prolactin affects both survival and differentiation of T-cell progenitors.* J Neuroimmunol, 2005. **160**(1-2): p. 135-45.

479. Lau, J., et al., *Prolactin regulation of the expression of TNF-alpha, IFN-gamma and IL-10 by splenocytes in murine multiple low dose streptozotocin diabetes.* Immunol Lett, 2006. **102**(1): p. 25-30.

480. Richards, S.M. and W.J. Murphy, *Use of human prolactin as a therapeutic protein to potentiate immunohematopoietic function.* J Neuroimmunol, 2000. **109**(1): p. 56-62.

481. Gopichandran, N., et al., *Multiplex determination of murine seminal fluid cytokine profiles.* Reproduction, 2006. **131**(3): p. 613-21.

482. Chandrashekar, V., A. Bartke, and T.E. Wagner, *Endogenous human growth hormone (GH) modulates the effect of gonadotropin-releasing hormone on pituitary function and the gonadotropin response to the negative feedback effect of testosterone in adult male transgenic mice bearing human GH gene.* Endocrinology, 1988. **123**(6): p. 2717-22.

483. Cooke, E., et al. *Bayesian hierarchical clustering for microarray time series data with replicates and outlier measurements*. in *MASAMB 2011*. 2011. Vienna.

484. Benjamini, Y. and Y. Hochberg, *Controlling the false discovery rate: a practical and powerful approach to multiple testing.* J Royal Stat Soc B 1995. **57**: p. 289-300.

485. Pe'er, D., *Bayesian network analysis of signaling networks: a primer.* Sci STKE, 2005. **2005**(281): p. pl4.

486. Beal, M.J., et al., *A Bayesian approach to reconstructing genetic regulatory networks with hidden factors.* Bioinformatics, 2005. **21**(3): p. 349-56.

487. Jenssen, T.K., et al., *A literature network of human genes for high-throughput analysis of gene expression.* Nat Genet, 2001. **28**(1): p. 21-8.

488. Hall, M., et al., *The WEKA Data Mining Software: An Update.* SIGKDD Explorations, 2009. **11**(1): p. 10-18.

489. Friedman, N., M. Goldszmidt, and A. Wyner, *Data analysis with Bayesian networks: a bootstrap approach*, ed. K. Laskey and H. Prade. 1999: Morgan Kaufmann.

490. Heckerman, D., D. Geiger, and D. Chickering, *Learning Bayesian networks: the combination of knowledge and statistical data.* Machine Learning, 1995. **20**(95): p. 197-234.

491. Shannon, P., et al., *Cytoscape: a software environment for integrated models of biomolecular interaction networks.* Genome Research, 2003. **13**(11): p. 2498-2504.

492. Bishop, C., *Pattern Recognition and Machine Learning*. 2nd Edition ed. Information Science and Statistics, ed. M. Jordan, J. Kleinberg, and B. Scholkopf. 2007, Singapore: Springer Science and Business Media.

493. Reber, P.M., *Prolactin and immunomodulation.* Am J Med, 1993. **95**(6): p. 637-44.

494. Cejkova, P., M. Fojtikova, and M. Cerna, *Immunomodulatory role of prolactin in diabetes development.* Autoimmun Rev, 2009. **9**(1): p. 23-7.

495. Saha, S., et al., *Prolactin alters the mechanisms of B cell tolerance induction.* Arthritis Rheum, 2009. **60**(6): p. 1743-52.

496. Berczi, I., et al., *Regulation of humoral immunity in rats by pituitary hormones.* Acta Endocrinol (Copenh), 1981. **98**(4): p. 506-13.

497. Quintanar-Stephano, A., et al., *Hypophysectomy and neurointermediate pituitary lobectomy decrease humoral immune responses to T-independent and T-dependent antigens.* J Physiol Biochem, 2010. **66**(1): p. 7-13.

498. Matera, L., et al., *Modulatory effect of prolactin on the resting and mitogen-induced activity of T, B, and NK lymphocytes.* Brain Behav Immun, 1992. **6**(4): p. 409-17.

499. Uyttenhove, C., R.J. Simpson, and J. Van Snick, *Functional and structural characterization of P40, a mouse glycoprotein with T-cell growth factor activity.* Proc Natl Acad Sci U S A, 1988. **85**(18): p. 6934-8.

500. Cheng, G., et al., *Anti-interleukin-9 antibody treatment inhibits airway inflammation and hyperreactivity in mouse asthma model.* Am J Respir Crit Care Med, 2002. **166**(3): p. 409-16.

501. Angkasekwinai, P., et al., *Regulation of IL-9 expression by IL-25 signaling.* Nat Immunol, 2010. **11**(3): p. 250-6.

502. Shipman, L.J., et al., *Metabolic adaptations in mouse mammary gland during a normal lactation cycle and in extended lactation.* Q J Exp Physiol, 1987. **72**(3): p. 303-11.

503. Wilde, C.J., C.H. Knight, and D.J. Flint, *Control of milk secretion and apoptosis during mammary involution.* J Mammary Gland Biol Neoplasia, 1999. **4**(2): p. 129-36.

504. Basak, S., et al., *Expression of pro-inflammatory cytokines in mouse blastocysts during implantation: modulation by steroid hormones.* Am J Reprod Immunol, 2002. **47**(1): p. 2-11.

505. Nishimori, K., et al., *Oxytocin is required for nursing but is not essential for parturition or reproductive behavior.* Proc Natl Acad Sci U S A, 1996. **93**(21): p. 11699-704.

506. Wagner, K.U., et al., *Oxytocin and milk removal are required for post-partum mammary-gland development.* Genes Funct, 1997. **1**(4): p. 233-44.

507. Young, W.S., 3rd, et al., *Deficiency in mouse oxytocin prevents milk ejection, but not fertility or parturition.* J Neuroendocrinol, 1996. **8**(11): p. 847-53.

508. Johnson, H.M. and B.A. Torres, *Regulation of lymphokine production by arginine vasopressin and oxytocin: modulation of lymphocyte function by neurohypophyseal hormones.* J Immunol, 1985. **135**(2 Suppl): p. 773s-775s.

509. Watanobe, H., S. Sasaki, and K. Takebe, *Involvement of oxytocin and cholecystokinin-8 in interleukin-1 beta-induced adrenocorticotropin secretion in the rat.* Neuroimmunomodulation, 1995. **2**(2): p. 88-91.

510. Clodi, M., et al., *Oxytocin alleviates the neuroendocrine and cytokine response to bacterial endotoxin in healthy men.* Am J Physiol Endocrinol Metab, 2008. **295**(3): p. E686-91.

511. Maccio, A., et al., *Oxytocin both increases proliferative response of peripheral blood lymphomonocytes to phytohemagglutinin and reverses immunosuppressive estrogen activity.* In Vivo, 2010. **24**(2): p. 157-63.

512. Szeto, A., et al., *Oxytocin attenuates NADPH-dependent superoxide activity and IL-6 secretion in macrophages and vascular cells.* Am J Physiol Endocrinol Metab, 2008. **295**(6): p. E1495-501.

513. Heinig, M.J. and K.G. Dewey, *Health effects of breast feeding for mothers: a critical review.* Nutr Res Rev, 1997. **10**(1): p. 35-56.

514. Langer-Gould, A., et al., *Interferon-gamma-producing T cells, pregnancy, and postpartum relapses of multiple sclerosis.* Arch Neurol, 2010. **67**(1): p. 51-7.

515. Hilkens, C.M., et al., *Accessory cell-derived IL-12 and prostaglandin E2 determine the IFN-gamma level of activated human CD4+ T cells.* J Immunol, 1996. **156**(5): p. 1722-27.

516. van der Pouw Kraan, T.C., et al., *Regulation of IL-12 production by human monocytes and the influence of prostaglandin E2.* Ann N Y Acad Sci, 1996. **795**: p. 147-57.

517. Heinzel, F.P., et al., *In vivo production and function of IL-12 p40 homodimers.* J Immunol, 1997. **158**(9): p. 4381-8.

518. Tripathi, A. and A. Sodhi, *Prolactin-induced production of cytokines in macrophages in vitro involves JAK/STAT and JNK MAPK pathways.* Int Immunol, 2008. **20**(3): p. 327-36.

519. Wang, J., et al., *Inhibition of CCR5 expression by IL-12 through induction of beta-chemokines in human T lymphocytes.* J Immunol, 1999. **163**(11): p. 5763-9.

520. Fahey, T.J., 3rd, et al., *Macrophage inflammatory protein 1 modulates macrophage function.* J Immunol, 1992. **148**(9): p. 2764-9.

521. Mosconi, E., et al., *Breast milk immune complexes are potent inducers of oral tolerance in neonates and prevent asthma development.* Mucosal Immunol, 2010. **3**(5): p. 461-74.

522. Sinha, S., et al., *IL-13-mediated gender difference in susceptibility to autoimmune encephalomyelitis.* J Immunol, 2008. **180**(4): p. 2679-85.

523. Blais, Y., et al., *Interleukin-4 and interleukin-13 inhibit estrogen-induced breast cancer cell proliferation and stimulate GCDFP-15 expression in human breast cancer cells.* Mol Cell Endocrinol, 1996. **121**(1): p. 11-8.

524. Gooch, J.L., A.V. Lee, and D. Yee, *Interleukin 4 inhibits growth and induces apoptosis in human breast cancer cells.* Cancer Res, 1998. **58**(18): p. 4199-205.

525. Hamano, N., et al., *Effect of female hormones on the production of IL-4 and IL-13 from peripheral blood mononuclear cells.* Acta Otolaryngol Suppl, 1998. **537**: p. 27-31.
526. Wynn, T.A., *IL-13 effector functions.* Annu Rev Immunol, 2003. **21**: p. 425-56.
527. Joshi, B.H., et al., *Role of interleukin-13 in cancer, pulmonary fibrosis, and other T(H)2-type diseases.* Vitam Horm, 2006. **74**: p. 479-504.
528. Wills-Karp, M. and F.D. Finkelman, *Untangling the complex web of IL-4- and IL-13-mediated signaling pathways.* Sci Signal, 2008. **1**(51): p. pe55.
529. Savion, S., et al., *Cytokine expression in the uterus of mice with pregnancy loss: effect of maternal immunopotentiation with GM-CSF.* Reproduction, 2002. **123**(3): p. 399-409.
530. Sjoblom, C., M. Wikland, and S.A. Robertson, *Granulocyte-macrophage colony-stimulating factor (GM-CSF) acts independently of the beta common subunit of the GM-CSF receptor to prevent inner cell mass apoptosis in human embryos.* Biol Reprod, 2002. **67**(6): p. 1817-23.
531. Haddad, E.K., et al., *Role of interferon-gamma in the priming of decidual macrophages for nitric oxide production and early pregnancy loss.* Cell Immunol, 1997. **181**(1): p. 68-75.
532. Wilson, R., et al., *Abnormal cytokine levels in non-pregnant women with a history of recurrent miscarriage.* Eur J Obstet Gynecol Reprod Biol, 2004. **115**(1): p. 51-4.
533. Ashkar, A.A. and B.A. Croy, *Interferon-gamma contributes to the normalcy of murine pregnancy.* Biol Reprod, 1999. **61**(2): p. 493-502.
534. Murphy, S.P., et al., *Interferon gamma in successful pregnancies.* Biol Reprod, 2009. **80**(5): p. 848-59.
535. D'Andrea, A., et al., *Interleukin 10 (IL-10) inhibits human lymphocyte interferon gamma-production by suppressing natural killer cell stimulatory factor/IL-12 synthesis in accessory cells.* J Exp Med, 1993. **178**(3): p. 1041-8.
536. Naundorf, S., et al., *IL-10 interferes directly with TCR-induced IFN-gamma but not IL-17 production in memory T cells.* Eur J Immunol, 2009. **39**(4): p. 1066-77.
537. Ogilvie, P., et al., *Eotaxin is a natural antagonist for CCR2 and an agonist for CCR5.* Blood, 2001. **97**(7): p. 1920-4.
538. Kobayashi, Y., et al., *Levels of MCP-1 and GM-CSF mRNA correlated with inflammatory cytokines mRNA levels in experimental autoimmune myocarditis in rats.* Autoimmunity, 2002. **35**(2): p. 97-104.
539. Kunstfeld, R., et al., *MCP-1 and MIP-1alpha are most efficient in recruiting T cells into the skin in vivo.* J Invest Dermatol, 1998. **111**(6): p. 1040-4.

540. Alam, R., et al., *Increased MCP-1, RANTES, and MIP-1alpha in bronchoalveolar lavage fluid of allergic asthmatic patients.* Am J Respir Crit Care Med, 1996. **153**(4 Pt 1): p. 1398-404.

541. Yoshida, S., et al., *Role of MCP-1 and MIP-1alpha in retinal neovascularization during postischemic inflammation in a mouse model of retinal neovascularization.* J Leukoc Biol, 2003. **73**(1): p. 137-44.

542. Low, Q.E., et al., *Wound healing in MIP-1alpha(-/-) and MCP-1(-/-) mice.* Am J Pathol, 2001. **159**(2): p. 457-63.

543. Conti, P. and M. DiGioacchino, *MCP-1 and RANTES are mediators of acute and chronic inflammation.* Allergy Asthma Proc, 2001. **22**(3): p. 133-7.

544. Goebeler, M., et al., *Interleukin-13 selectively induces monocyte chemoattractant protein-1 synthesis and secretion by human endothelial cells. Involvement of IL-4R alpha and Stat6 phosphorylation.* Immunology, 1997. **91**(3): p. 450-7.

545. Daines, M.O. and G.K. Hershey, *A novel mechanism by which interferon-gamma can regulate interleukin (IL)-13 responses. Evidence for intracellular stores of IL-13 receptor alpha -2 and their rapid mobilization by interferon-gamma.* J Biol Chem, 2002. **277**(12): p. 10387-93.

546. Zhou, Z.H., et al., *Regulation of monocyte chemoattractant protein (MCP)-1 transcription by interferon-gamma (IFN-gamma) in human astrocytoma cells: postinduction refractory state of the gene, governed by its upstream elements.* FASEB J, 2001. **15**(2): p. 383-92.

547. Yamana, J., L. Santos, and E. Morand, *Enhanced induction of LPS-induced fibroblast MCP-1 by interferon-gamma: involvement of JNK and MAPK phosphatase-1.* Cell Immunol, 2009. **255**(1-2): p. 26-32.

548. Xing, Z., A. Zganiacz, and M. Santosuosso, *Role of IL-12 in macrophage activation during intracellular infection: IL-12 and mycobacteria synergistically release TNF-alpha and nitric oxide from macrophages via IFN-gamma induction.* J Leukoc Biol, 2000. **68**(6): p. 897-902.

549. Pallard, C., et al., *Interleukin-3, erythropoietin, and prolactin activate a STAT5-like factor in lymphoid cells.* J Biol Chem, 1995. **270**(27): p. 15942-5.

550. Watson, C.J. and T.G. Burdon, *Prolactin signal transduction mechanisms in the mammary gland: the role of the Jak/Stat pathway.* Rev Reprod, 1996. **1**(1): p. 1-5.

551. D'Atri, L.P., et al., *The low viability of human CD34+ cells under acidic conditions is improved by exposure to thrombopoietin, stem cell factor,*

interleukin-3, or increased cyclic adenosine monophosphate levels. Transfusion, 2011. **51**(8): p. 1784-95.

552. Hoshino, A., J.A. Hirst, and H. Fujii, *Regulation of cell proliferation by interleukin-3-induced nuclear translocation of pyruvate kinase.* J Biol Chem, 2007. **282**(24): p. 17706-11.
553. Li, X., Y. Wu, and Y. Chen, *Down-regulating the expression of IL-3Rbeta interfered with the proliferation, not differentiation in NB4 cells.* Int J Hematol, 2011. **93**(1): p. 83-90.
554. Luo, X.J., et al., *The interleukin 3 gene (IL3) contributes to human brain volume variation by regulating proliferation and survival of neural progenitors.* PLoS One, 2012. **7**(11): p. e50375.
555. Olazabal, I., et al., *Prolactin (PRL)-PRL receptor system increases cell proliferation involving JNK (c-Jun amino terminal kinase) and AP-1 activation: inhibition by glucocorticoids.* Mol Endocrinol, 2000. **14**(4): p. 564-75.
556. Nitze, L.M., et al., *Reevaluation of the proposed autocrine proliferative function of prolactin in breast cancer.* Breast Cancer Res Treat, 2013. **142**(1): p. 31-44.
557. Sengupta, A. and D.K. Sarkar, *Roles of dopamine 2 receptor isoforms and g proteins in ethanol regulated prolactin synthesis and lactotropic cell proliferation.* PLoS One, 2012. **7**(9): p. e45593.
558. Walker, T.L., et al., *Prolactin stimulates precursor cells in the adult mouse hippocampus.* PLoS One, 2012. **7**(9): p. e44371.
559. Wood, C.E., et al., *Progestin effects on cell proliferation pathways in the postmenopausal mammary gland.* Breast Cancer Res, 2013. **15**(4): p. R62.
560. Orsi, N.M., et al., *Murine serum cytokines throughout the estrous cycle, pregnancy and postpartum period.* Animal Reproduction Science, 2006. **96**(1-2): p. 54-65.
561. Jiang, H., M.B. Harris, and P. Rothman, *IL-4/IL-13 signaling beyond JAK/STAT.* J Allergy Clin Immunol, 2000. **105**(6 Pt 1): p. 1063-70.
562. Fallon, P.G., et al., *IL-4 induces characteristic Th2 responses even in the combined absence of IL-5, IL-9, and IL-13.* Immunity, 2002. **17**(1): p. 7-17.
563. Gouon-Evans, V. and J.W. Pollard, *Eotaxin is required for eosinophil homing into the stroma of the pubertal and cycling uterus.* Endocrinology, 2001. **142**(10): p. 4515-21.
564. Wakasa, T., et al., *Change in morphology and oxytocin receptor expression in the uterine blood vessels during the involution process.* Gynecol Obstet Invest, 2009. **67**(2): p. 137-44.

565. Gunduz, M.C., G. Kasikci, and H.H. Kaya, *The effect of oxytocin and PGF2alpha on the uterine involution and pregnancy rates in postpartum Arabian mares.* Anim Reprod Sci, 2008. **104**(2-4): p. 257-63.

566. Pitzel, L., H. Jarry, and W. Wuttke, *Effects and interactions of prostaglandin F2 alpha, oxytocin, and cytokines on steroidogenesis of porcine luteal cells.* Endocrinology, 1993. **132**(2): p. 751-6.

567. Munoz-Suano, A., A.B. Hamilton, and A.G. Betz, *Gimme shelter: the immune system during pregnancy.* Immunol Rev, 2011. **241**(1): p. 20-38.

568. Glabinski, A.R., et al., *TNF-alpha microinjection upregulates chemokines and chemokine receptors in the central nervous system without inducing leukocyte infiltration.* J Interferon Cytokine Res, 2003. **23**(8): p. 457-66.

569. Liu, B., et al., *Maternal hematopoietic TNF, via milk chemokines, programs hippocampal development and memory.* Nat Neurosci, 2014. **17**(1): p. 97-105.

570. Renaud, S.J., R. Sullivan, and C.H. Graham, *Tumour necrosis factor alpha stimulates the production of monocyte chemoattractants by extravillous trophoblast cells via differential activation of MAPK pathways.* Placenta, 2009. **30**(4): p. 313-9.

571. Gyftodimos, E. and P. Flach. *Hierarchical Bayesian networks: a probabilistic reasoning model for structured domains*. in *Proceedings of the ICML-2002 Workshop on Development of Representations.* . 2002.

572. Gougeon, A., *Dynamics of follicular growth in the human: a model from preliminary results.* Hum Reprod, 1986. **1**(2): p. 81-7.

573. Dissen, G.A., et al., *Neurotrophic control of ovarian development.* Microsc Res Tech, 2002. **59**(6): p. 509-15.

574. John, G.B., et al., *Foxo3 is a PI3K-dependent molecular switch controlling the initiation of oocyte growth.* Dev Biol, 2008. **321**(1): p. 197-204.

575. Brown, H.M. and D.L. Russell, *Blood and lymphatic vasculature in the ovary: development, function and disease.* Hum Reprod Update, 2014. **20**(1): p. 29-39.

576. Robinson, R.S., *The critical importance of ovarian angiogenesis.* Reprod Fertil Dev, 2013. **25**(2): p. iii-v.

577. Siu, M.K. and C.Y. Cheng, *The blood-follicle barrier (BFB) in disease and in ovarian function.* Adv Exp Med Biol, 2012. **763**: p. 186-92.

578. Oktay, K., D. Briggs, and R.G. Gosden, *Ontogeny of follicle-stimulating hormone receptor gene expression in isolated human ovarian follicles.* J Clin Endocrinol Metab, 1997. **82**(11): p. 3748-51.

579. Hansen, K.R., et al., *A new model of reproductive aging: the decline in ovarian non-growing follicle number from birth to menopause.* Hum Reprod, 2008. **23**(3): p. 699-708.

580. Kelsey, T.W., et al., *Data-driven assessment of the human ovarian reserve.* Mol Hum Reprod, 2012. **18**(2): p. 79-87.

581. Anderson, R.A., S.M. Nelson, and W.H. Wallace, *Measuring anti-Mullerian hormone for the assessment of ovarian reserve: when and for whom is it indicated?* Maturitas, 2012. **71**(1): p. 28-33.

582. Barnes, R.B., et al., *The role of LH and FSH in ovarian androgen secretion and ovarian follicular development: clinical studies in a patient with isolated FSH deficiency and multicystic ovaries.* Hum Reprod, 2002. **17**(1): p. 88-91.

583. Dierich, A., et al., *Impairing follicle-stimulating hormone (FSH) signaling in vivo: targeted disruption of the FSH receptor leads to aberrant gametogenesis and hormonal imbalance.* Proc Natl Acad Sci U S A, 1998. **95**(23): p. 13612-7.

584. Gougeon, A., *Regulation of ovarian follicular development in primates: facts and hypotheses.* Endocr Rev, 1996. **17**(2): p. 121-55.

585. Gougeon, A., *Human ovarian follicular development: from activation of resting follicles to preovulatory maturation.* Ann Endocrinol (Paris), 2010. **71**(3): p. 132-43.

586. Wigglesworth, K., et al., *Bidirectional communication between oocytes and ovarian follicular somatic cells is required for meiotic arrest of mammalian oocytes.* Proc Natl Acad Sci U S A, 2013. **110**(39): p. E3723-9.

587. Albertini, D.F., et al., *Cellular basis for paracrine regulation of ovarian follicle development.* Reproduction, 2001. **121**(5): p. 647-53.

588. Makabe, S., T. Naguro, and T. Stallone, *Oocyte-follicle cell interactions during ovarian follicle development, as seen by high resolution scanning and transmission electron microscopy in humans.* Microsc Res Tech, 2006. **69**(6): p. 436-49.

589. Bruzzone, R., T.W. White, and D.L. Paul, *Connections with connexins: the molecular basis of direct intercellular signaling.* Eur J Biochem, 1996. **238**(1): p. 1-27.

590. Erickson, G.F. and D.R. Danforth, *Ovarian control of follicle development.* Am J Obstet Gynecol, 1995. **172**(2 Pt 2): p. 736-47.

591. Robinson, R.S., et al., *Angiogenesis and vascular function in the ovary.* Reproduction, 2009. **138**(6): p. 869-81.

592. Conti, M., et al., *Role of cyclic nucleotide signaling in oocyte maturation.* Mol Cell Endocrinol, 2002. **187**(1-2): p. 153-9.

593. Norris, R.P., et al., *Cyclic GMP from the surrounding somatic cells regulates cyclic AMP and meiosis in the mouse oocyte.* Development, 2009. **136**(11): p. 1869-78.

594. Edwards, R.G., *Follicular fluid.* J Reprod Fertil, 1974. **37**(1): p. 189-219.

595. Edwards, R.G., et al., *Observations on preovulatory human ovarian follicles and their aspirates.* Br J Obstet Gynaecol, 1980. **87**(9): p. 769-79.

596. Rodgers, R.J. and H.F. Irving-Rodgers, *Formation of the Ovarian Follicular Antrum and Follicular Fluid.* Biol Reprod, 2010.

597. Jokubkiene, L., P. Sladkevicius, and L. Valentin, *Number of antral follicles, ovarian volume, and vascular indices in asymptomatic women 20 to 39 years old as assessed by 3-dimensional sonography: a prospective cross-sectional study.* J Ultrasound Med, 2012. **31**(10): p. 1635-49.

598. Erickson, G.F. and S. Shimasaki, *The role of the oocyte in folliculogenesis.* Trends Endocrinol Metab, 2000. **11**(5): p. 193-8.

599. Erickson, G.F. *Follicle growth and development*. Gynaecology and Obstetrics 2004 [cited 2014 09/01/2014]; Available from: http://www.glowm.com/resources/glowm/cd/pages/v5/v5c012.html.

600. Jones, K.T., *Turning it on and off: M-phase promoting factor during meiotic maturation and fertilization.* Mol Hum Reprod, 2004. **10**(1): p. 1-5.

601. Peng, X.R., et al., *Localization of luteinizing hormone receptor messenger ribonucleic acid expression in ovarian cell types during follicle development and ovulation.* Endocrinology, 1991. **129**(6): p. 3200-7.

602. Minegishi, T., et al., *Expression of luteinizing hormone/human chorionic gonadotrophin (LH/HCG) receptor mRNA in the human ovary.* Mol Hum Reprod, 1997. **3**(2): p. 101-7.

603. Pache, T.D., et al., *Growth patterns of nondominant ovarian follicles during the normal menstrual cycle.* Fertil Steril, 1990. **54**(4): p. 638-42.

604. Baird, D.T., *Factors regulating the growth of the preovulatory follicle in the sheep and human.* J Reprod Fertil, 1983. **69**(1): p. 343-52.

605. Oktem, O. and K. Oktay, *The ovary: anatomy and function throughout human life.* Ann N Y Acad Sci, 2008. **1127**: p. 1-9.

606. Birtch, R.L., et al., *Ultrasound image attributes of human ovarian dominant follicles during natural and oral contraceptive cycles.* Reprod Biol Endocrinol, 2005. **3**: p. 12.

607. Celestino, J.J.H., et al., *Mechanisms of atresia in ovarian follicles.* Animal Reproduction, 2009. **6**(4): p. 495-508.

608. Hussein, M.R., *Apoptosis in the ovary: molecular mechanisms.* Hum Reprod Update, 2005. **11**(2): p. 162-77.
609. Zackrisson, U., et al., *Alterations of follicular microcirculation and apex structure during ovulation in the rat.* Eur J Obstet Gynecol Reprod Biol, 2011.
610. Oakley, O.R., et al., *Periovulatory leukocyte infiltration in the rat ovary.* Endocrinology, 2010. **151**(9): p. 4551-9.
611. Richards, J.S., Z. Liu, and M. Shimada, *Immune-like mechanisms in ovulation.* Trends Endocrinol Metab, 2008. **19**(6): p. 191-6.
612. Richards, J.S. and S.A. Pangas, *New insights into ovarian function.* Handb Exp Pharmacol, 2010(198): p. 3-27.
613. Curry, T.E., Jr. and K.G. Osteen, *The matrix metalloproteinase system: changes, regulation, and impact throughout the ovarian and uterine reproductive cycle.* Endocr Rev, 2003. **24**(4): p. 428-65.
614. Penny, L.A., *Monocyte chemoattractant protein 1 in luteolysis.* Rev Reprod, 2000. **5**(2): p. 63-6.
615. Duncan, W.C., *The human corpus luteum: remodelling during luteolysis and maternal recognition of pregnancy.* Rev Reprod, 2000. **5**(1): p. 12-7.
616. Knecht, M., et al., *Gonadotropin-releasing hormone as a modulator of ovarian function.* J Steroid Biochem, 1985. **23**(5B): p. 771-8.
617. Jiemtaweeboon, S., et al., *Evidence that polymorphonuclear neutrophils infiltrate into the developing corpus luteum and promote angiogenesis with interleukin-8 in the cow.* Reprod Biol Endocrinol, 2011. **9**: p. 79.
618. Schruefer, R., et al., *Human neutrophils promote angiogenesis by a paracrine feedforward mechanism involving endothelial interleukin-8.* Am J Physiol Heart Circ Physiol, 2005. **288**(3): p. H1186-92.
619. Penny, L.A., et al., *Immune cells and cytokine production in the bovine corpus luteum throughout the oestrous cycle and after induced luteolysis.* J Reprod Fertil, 1999. **115**(1): p. 87-96.
620. Wang, L.J., et al., *Distribution of leukocyte subpopulations in the human corpus luteum.* Hum Reprod, 1992. **7**(2): p. 197-202.
621. Castro, A., et al., *Luteal leukocytes are modulators of the steroidogenic process of human mid-luteal cells.* Hum Reprod, 1998. **13**(6): p. 1584-9.
622. Fan, H.Y. and Q.Y. Sun, *Involvement of mitogen-activated protein kinase cascade during oocyte maturation and fertilization in mammals.* Biol Reprod, 2004. **70**(3): p. 535-47.

623. Masui, Y., *From oocyte maturation to the in vitro cell cycle: the history of discoveries of Maturation-Promoting Factor (MPF) and Cytostatic Factor (CSF).* Differentiation, 2001. **69**(1): p. 1-17.

624. Greenstein, D., *Control of oocyte meiotic maturation and fertilization.* WormBook, 2005: p. 1-12.

625. Krisher, R.L., *The effect of oocyte quality on development.* J Anim Sci, 2004. **82 E-Suppl**: p. E14-23.

626. Ferreira, E.M., et al., *Cytoplasmic maturation of bovine oocytes: structural and biochemical modifications and acquisition of developmental competence.* Theriogenology, 2009. **71**(5): p. 836-48.

627. Coticchio, G., et al., *Contributions of the actin cytoskeleton to the emergence of polarity during maturation in human oocytes.* Mol Hum Reprod, 2014. **20**(3): p. 200-7.

628. Coticchio, G., et al., *Mechanistic foundations of the metaphase II spindle of human oocytes matured in vivo and in vitro.* Hum Reprod, 2013. **28**(12): p. 3271-82.

629. Brevini-Gandolfi, T.A., et al., *Changes in poly(A) tail length of maternal transcripts during in vitro maturation of bovine oocytes and their relation with developmental competence.* Mol Reprod Dev, 1999. **52**(4): p. 427-33.

630. Piccioni, F., V. Zappavigna, and A.C. Verrotti, *Translational regulation during oogenesis and early development: the cap-poly(A) tail relationship.* C R Biol, 2005. **328**(10-11): p. 863-81.

631. Gosden, R.G., *Oogenesis as a foundation for embryogenesis.* Mol Cell Endocrinol, 2002. **186**(2): p. 149-53.

632. Krisher, R.L. and B.D. Bavister, *Responses of oocytes and embryos to the culture environment.* Theriogenology, 1998. **49**(1): p. 103-14.

633. Tomek, W., H. Torner, and W. Kanitz, *Comparative analysis of protein synthesis, transcription and cytoplasmic polyadenylation of mRNA during maturation of bovine oocytes in vitro.* Reprod Domest Anim, 2002. **37**(2): p. 86-91.

634. Hosoe, M. and Y. Shioya, *Distribution of cortical granules in bovine oocytes classified by cumulus complex.* Zygote, 1997. **5**(4): p. 371-6.

635. Thibault, C., D. Szollosi, and M. Gerard, *Mammalian oocyte maturation.* Reprod Nutr Dev, 1987. **27**(5): p. 865-96.

636. Eppig, J.J., *Oocyte control of ovarian follicular development and function in mammals.* Reproduction, 2001. **122**(6): p. 829-38.

637. Vinatier, D., et al., *Immunological aspects of ovarian function: role of the cytokines.* Eur J Obstet Gynecol Reprod Biol, 1995. **63**(2): p. 155-68.

638. Wilding, M., et al., *An oocyte score for use in assisted reproduction.* J Assist Reprod Genet, 2007. **24**(8): p. 350-8.

639. Oktay, K., et al., *Interaction of extracellular matrix and activin-A in the initiation of follicle growth in the mouse ovary.* Biol Reprod, 2000. **63**(2): p. 457-61.

640. Skinner, M.K., *Regulation of primordial follicle assembly and development.* Hum Reprod Update, 2005. **11**(5): p. 461-71.

641. Richards, J.S. and L. Hedin, *Molecular aspects of hormone action in ovarian follicular development, ovulation, and luteinization.* Annu Rev Physiol, 1988. **50**: p. 441-63.

642. Buccione, R., A.C. Schroeder, and J.J. Eppig, *Interactions between somatic cells and germ cells throughout mammalian oogenesis.* Biol Reprod, 1990. **43**(4): p. 543-7.

643. Eppig, J.J., *Oocyte-somatic cell interactions during oocyte growth and maturation in the mammal.* Dev Biol (N Y 1985), 1985. **1**: p. 313-47.

644. Su, Y.Q., et al., *Oocyte regulation of metabolic cooperativity between mouse cumulus cells and oocytes: BMP15 and GDF9 control cholesterol biosynthesis in cumulus cells.* Development, 2008. **135**(1): p. 111-21.

645. Schams, D. and B. Berisha, *Steroids as local regulators of ovarian activity in domestic animals.* Domest Anim Endocrinol, 2002. **23**(1-2): p. 53-65.

646. Gilchrist, R.B., L.J. Ritter, and D.T. Armstrong, *Oocyte-somatic cell interactions during follicle development in mammals.* Anim Reprod Sci, 2004. **82-83**: p. 431-46.

647. Sharkey, A., *Cytokines and implantation.* Rev Reprod, 1998. **3**(1): p. 52-61.

648. Kaipia, A. and A.J. Hsueh, *Regulation of ovarian follicle atresia.* Annu Rev Physiol, 1997. **59**: p. 349-63.

649. Bornstein, S.R., H. Rutkowski, and I. Vrezas, *Cytokines and steroidogenesis.* Mol Cell Endocrinol, 2004. **215**(1-2): p. 135-41.

650. Shimasaki, S., et al., *The role of bone morphogenetic proteins in ovarian function.* Reprod Suppl, 2003. **61**: p. 323-37.

651. Drummond, A.E., et al., *Ovarian follicle populations of the rat express TGF-beta signalling pathways.* Mol Cell Endocrinol, 2003. **202**(1-2): p. 53-7.

652. Bristol, S.K. and T.K. Woodruff, *Follicle-restricted compartmentalization of transforming growth factor beta superfamily ligands in the feline ovary.* Biol Reprod, 2004. **70**(3): p. 846-59.

653. McNatty, K.P., et al., *Oocyte-expressed genes affecting ovulation rate.* Mol Cell Endocrinol, 2005. **234**(1-2): p. 57-66.

654. Wu, R., et al., *Macrophage contributions to ovarian function.* Hum Reprod Update, 2004. **10**(2): p. 119-33.

655. Asimakopoulos, B., et al., *Cytokine and hormonal profile in blood serum and follicular fluids during ovarian stimulation with the multidose antagonist or the long agonist protocol.* Hum Reprod, 2006. **21**(12): p. 3091-5.

656. Asimakopoulos, B., et al., *Intrafollicular and circulating concentrations of leptin do not predict the outcome in IVF-ICSI cycles.* Reprod Sci, 2009. **16**(1): p. 113-9.

657. Best, C.L. and J.A. Hill, *Cytokines in ovarian function*, in *Cytokines in human reproduction*, J.A. Hill, Editor 2000, Wiley-Liss: New York. p. pp. 43-77.

658. Tarrant, J.M., *Blood cytokines as biomarkers of in vivo toxicity in preclinical safety assessment: considerations for their use.* Toxicol Sci, 2010. **117**(1): p. 4-16.

659. McLaughlin, E.A. and S.C. McIver, *Awakening the oocyte: controlling primordial follicle development.* Reproduction, 2009. **137**(1): p. 1-11.

660. Nilsson, E.E., P. Kezele, and M.K. Skinner, *Leukemia inhibitory factor (LIF) promotes the primordial to primary follicle transition in rat ovaries.* Mol Cell Endocrinol, 2002. **188**(1-2): p. 65-73.

661. Nilsson, E.E. and M.K. Skinner, *Kit ligand and basic fibroblast growth factor interactions in the induction of ovarian primordial to primary follicle transition.* Mol Cell Endocrinol, 2004. **214**(1-2): p. 19-25.

662. Wang, J. and S.K. Roy, *Growth differentiation factor-9 and stem cell factor promote primordial follicle formation in the hamster: modulation by follicle-stimulating hormone.* Biol Reprod, 2004. **70**(3): p. 577-85.

663. Gougeon, A., et al., *Kit ligand and the somatostatin receptor antagonist, BIM-23627, stimulate in vitro resting follicle growth in the neonatal mouse ovary.* Endocrinology, 2010. **151**(3): p. 1299-309.

664. Parrott, J.A. and M.K. Skinner, *Kit-ligand/stem cell factor induces primordial follicle development and initiates folliculogenesis.* Endocrinology, 1999. **140**(9): p. 4262-71.

665. da Nobrega, J.E., Jr., et al., *Leukemia inhibitory factor stimulates the transition of primordial to primary follicle and supports the goat primordial follicle viability in vitro.* Zygote, 2012. **20**(1): p. 73-8.

666. Kezele, P., E.E. Nilsson, and M.K. Skinner, *Keratinocyte growth factor acts as a mesenchymal factor that promotes ovarian primordial to primary follicle transition.* Biol Reprod, 2005. **73**(5): p. 967-73.

667. Hutt, K.J., E.A. McLaughlin, and M.K. Holland, *KIT/KIT ligand in mammalian oogenesis and folliculogenesis: roles in rabbit and murine ovarian follicle activation and oocyte growth.* Biol Reprod, 2006. **75**(3): p. 421-33.

668. Yoshida, H., et al., *Stepwise requirement of c-kit tyrosine kinase in mouse ovarian follicle development.* Dev Biol, 1997. **184**(1): p. 122-37.

669. Packer, A.I., et al., *The ligand of the c-kit receptor promotes oocyte growth.* Dev Biol, 1994. **161**(1): p. 194-205.

670. Myers, M. and S.A. Pangas, *Regulatory roles of transforming growth factor beta family members in folliculogenesis.* Wiley Interdiscip Rev Syst Biol Med, 2010. **2**(1): p. 117-25.

671. Massague, J. and D. Wotton, *Transcriptional control by the TGF-beta/Smad signaling system.* EMBO J, 2000. **19**(8): p. 1745-54.

672. von Bubnoff, A. and K.W. Cho, *Intracellular BMP signaling regulation in vertebrates: pathway or network?* Dev Biol, 2001. **239**(1): p. 1-14.

673. Kaivo-oja, N., et al., *Smad signalling in the ovary.* Reprod Biol Endocrinol, 2006. **4**: p. 21.

674. Lee, W.S., et al., *Effect of bone morphogenetic protein-7 on folliculogenesis and ovulation in the rat.* Biol Reprod, 2001. **65**(4): p. 994-9.

675. Erickson, G.F. and S. Shimasaki, *The spatiotemporal expression pattern of the bone morphogenetic protein family in rat ovary cell types during the estrous cycle.* Reprod Biol Endocrinol, 2003. **1**: p. 9.

676. Sun, R.Z., et al., *Expression of GDF-9, BMP-15 and their receptors in mammalian ovary follicles.* J Mol Histol, 2010. **41**(6): p. 325-32.

677. Al-Musawi, S.L., et al., *Species differences in the expression and activity of bone morphogenetic protein 15.* Endocrinology, 2013. **154**(2): p. 888-99.

678. Di Pasquale, E., P. Beck-Peccoz, and L. Persani, *Hypergonadotropic ovarian failure associated with an inherited mutation of human bone morphogenetic protein-15 (BMP15) gene.* Am J Hum Genet, 2004. **75**(1): p. 106-11.

679. Holt, J.E., et al., *CXCR4/SDF1 interaction inhibits the primordial to primary follicle transition in the neonatal mouse ovary.* Dev Biol, 2006. **293**(2): p. 449-60.

680. Ara, T., et al., *Impaired colonization of the gonads by primordial germ cells in mice lacking a chemokine, stromal cell-derived factor-1 (SDF-1).* Proc Natl Acad Sci U S A, 2003. **100**(9): p. 5319-23.

681. Brannstrom, M. and R.J. Norman, *Involvement of leukocytes and cytokines in the ovulatory process and corpus luteum function.* Hum Reprod, 1993. **8**(10): p. 1762-75.

682. Norman, R.J. and M. Brannstrom, *White cells and the ovary--incidental invaders or essential effectors?* J Endocrinol, 1994. **140**(3): p. 333-6.

683. Norman, R.J. and M. Brannstrom, *Cytokines in the ovary: pathophysiology and potential for pharmacological intervention.* Pharmacol Ther, 1996. **69**(3): p. 219-36.

684. Mazerbourg, S. and A.J. Hsueh, *Genomic analyses facilitate identification of receptors and signalling pathways for growth differentiation factor 9 and related orphan bone morphogenetic protein/growth differentiation factor ligands.* Hum Reprod Update, 2006. **12**(4): p. 373-83.

685. Vitt, U.A., et al., *Bone morphogenetic protein receptor type II is a receptor for growth differentiation factor-9.* Biol Reprod, 2002. **67**(2): p. 473-80.

686. Hayashi, M., et al., *Recombinant growth differentiation factor-9 (GDF-9) enhances growth and differentiation of cultured early ovarian follicles.* Endocrinology, 1999. **140**(3): p. 1236-44.

687. Carabatsos, M.J., et al., *Characterization of oocyte and follicle development in growth differentiation factor-9-deficient mice.* Dev Biol, 1998. **204**(2): p. 373-84.

688. Vitt, U.A., et al., *In vivo treatment with GDF-9 stimulates primordial and primary follicle progression and theca cell marker CYP17 in ovaries of immature rats.* Endocrinology, 2000. **141**(10): p. 3814-20.

689. Bodensteiner, K.J., et al., *Molecular cloning of the ovine Growth/Differentiation factor-9 gene and expression of growth/differentiation factor-9 in ovine and bovine ovaries.* Biol Reprod, 1999. **60**(2): p. 381-6.

690. Jaatinen, R., et al., *Localization of growth differentiation factor-9 (GDF-9) mRNA and protein in rat ovaries and cDNA cloning of rat GDF-9 and its novel homolog GDF-9B.* Mol Cell Endocrinol, 1999. **156**(1-2): p. 189-93.

691. Hreinsson, J.G., et al., *Growth differentiation factor-9 promotes the growth, development, and survival of human ovarian follicles in organ culture.* J Clin Endocrinol Metab, 2002. **87**(1): p. 316-21.

692. Kedem, A., et al., *Growth differentiating factor 9 (GDF9) and bone morphogenetic protein 15 both activate development of human primordial follicles in vitro, with seemingly more beneficial effects of GDF9.* J Clin Endocrinol Metab, 2011. **96**(8): p. E1246-54.

693. Durlinger, A.L., J.A. Visser, and A.P. Themmen, *Regulation of ovarian function: the role of anti-Mullerian hormone.* Reproduction, 2002. **124**(5): p. 601-9.

694. Durlinger, A.L., et al., *Control of primordial follicle recruitment by anti-Mullerian hormone in the mouse ovary.* Endocrinology, 1999. **140**(12): p. 5789-96.

695. Knauff, E.A., et al., *Anti-Mullerian hormone, inhibin B, and antral follicle count in young women with ovarian failure.* J Clin Endocrinol Metab, 2009. **94**(3): p. 786-92.

696. Hagen, C.P., et al., *Serum levels of anti-Mullerian hormone as a marker of ovarian function in 926 healthy females from birth to adulthood and in 172 Turner syndrome patients.* J Clin Endocrinol Metab, 2010. **95**(11): p. 5003-10.

697. Voorhuis, M., et al., *Genes involved in initial follicle recruitment may be associated with age at menopause.* J Clin Endocrinol Metab, 2011. **96**(3): p. E473-9.

698. Weenen, C., et al., *Anti-Mullerian hormone expression pattern in the human ovary: potential implications for initial and cyclic follicle recruitment.* Mol Hum Reprod, 2004. **10**(2): p. 77-83.

699. Rajpert-De Meyts, E., et al., *Expression of anti-Mullerian hormone during normal and pathological gonadal development: association with differentiation of Sertoli and granulosa cells.* J Clin Endocrinol Metab, 1999. **84**(10): p. 3836-44.

700. Grondahl, M.L., et al., *Anti-Mullerian hormone remains highly expressed in human cumulus cells during the final stages of folliculogenesis.* Reprod Biomed Online, 2011. **22**(4): p. 389-98.

701. Otsuka, F., et al., *Bone morphogenetic protein-15 inhibits follicle-stimulating hormone (FSH) action by suppressing FSH receptor expression.* J Biol Chem, 2001. **276**(14): p. 11387-92.

702. Drummond, A.E., et al., *Expression and localization of activin receptors, Smads, and beta glycan to the postnatal rat ovary.* Endocrinology, 2002. **143**(4): p. 1423-33.

703. Otsuka, F. and S. Shimasaki, *A negative feedback system between oocyte bone morphogenetic protein 15 and granulosa cell kit ligand: its role in regulating granulosa cell mitosis.* Proc Natl Acad Sci U S A, 2002. **99**(12): p. 8060-5.

704. Shimizu, T., et al., *Gene injections of vascular endothelial growth factor and growth differentiation factor-9 stimulate ovarian follicular development in immature female rats.* Fertil Steril, 2008. **89**(5 Suppl): p. 1563-70.

705. Shimizu, T., *Promotion of ovarian follicular development by injecting vascular endothelial growth factor (VEGF) and growth differentiation factor 9 (GDF-9) genes.* J Reprod Dev, 2006. **52**(1): p. 23-32.

706. Cerrillo, M., et al., *Differential regulation of VEGF after final oocyte maturation with GnRH agonist versus hCG: a rationale for OHSS reduction.* Fertil Steril, 2009. **91**(4 Suppl): p. 1526-8.

707. Juengel, J.L., et al., *Physiology of GDF9 and BMP15 signalling molecules.* Anim Reprod Sci, 2004. **82-83**: p. 447-60.

708. Elvin, J.A., et al., *Molecular characterization of the follicle defects in the growth differentiation factor 9-deficient ovary.* Mol Endocrinol, 1999. **13**(6): p. 1018-34.

709. Elvin, J.A., et al., *Paracrine actions of growth differentiation factor-9 in the mammalian ovary.* Mol Endocrinol, 1999. **13**(6): p. 1035-48.

710. Spicer, L.J., et al., *Growth differentiation factor 9 (GDF9) stimulates proliferation and inhibits steroidogenesis by bovine theca cells: influence of follicle size on responses to GDF9.* Biol Reprod, 2008. **78**(2): p. 243-53.

711. Vitt, U.A., et al., *Growth differentiation factor-9 stimulates proliferation but suppresses the follicle-stimulating hormone-induced differentiation of cultured granulosa cells from small antral and preovulatory rat follicles.* Biol Reprod, 2000. **62**(2): p. 370-7.

712. Dragovic, R.A., et al., *Role of oocyte-secreted growth differentiation factor 9 in the regulation of mouse cumulus expansion.* Endocrinology, 2005. **146**(6): p. 2798-806.

713. Gospodarowicz, D., et al., *Corpus luteum angiogenic factor is related to fibroblast growth factor.* Endocrinology, 1985. **117**(6): p. 2383-91.

714. Brown, L.F., et al., *Vascular permeability factor/vascular endothelial growth factor: a multifunctional angiogenic cytokine.* EXS, 1997. **79**: p. 233-69.

715. Chaves, R.N., et al., *The fibroblast growth factor family: involvement in the regulation of folliculogenesis.* Reprod Fertil Dev, 2012. **24**(7): p. 905-15.

716. Joo, J.K., et al., *Role of leptin in improvement of oocyte quality by regulation of ovarian angiogenesis.* Anim Reprod Sci, 2010. **119**(3-4): p. 329-34.

717. Araujo, V.R., et al., *Importance of vascular endothelial growth factor (VEGF) in ovarian physiology of mammals.* Zygote, 2013. **21**(3): p. 295-304.

718. McFee, R.M., T.G. Rozell, and A.S. Cupp, *The balance of proangiogenic and antiangiogenic VEGFA isoforms regulate follicle development.* Cell Tissue Res, 2012. **349**(3): p. 635-47.

719. Fraser, H.M., *Regulation of the ovarian follicular vasculature.* Reprod Biol Endocrinol, 2006. **4**: p. 18.

720. Ryan, K.J. and A. Makris, *Significance of angiogenic and growth factors in ovarian follicular development.* Adv Exp Med Biol, 1987. **219**: p. 203-9.

721. Yang, P. and S.K. Roy, *Epidermal growth factor modulates transforming growth factor receptor messenger RNA and protein levels in hamster preantral follicles in vitro.* Biol Reprod, 2001. **65**(3): p. 847-54.

722. Roy, S.K. and A.R. Kole, *Ovarian transforming growth factor-beta (TGF-beta) receptors: in-vitro effects of follicle stimulating hormone, epidermal growth factor and TGF-beta on receptor expression in human preantral follicles.* Mol Hum Reprod, 1998. **4**(3): p. 207-14.

723. Jin, X., et al., *Signal transduction of stem cell factor in promoting early follicle development.* Mol Cell Endocrinol, 2005. **229**(1-2): p. 3-10.

724. Parrott, J.A. and M.K. Skinner, *Direct actions of kit-ligand on theca cell growth and differentiation during follicle development.* Endocrinology, 1997. **138**(9): p. 3819-27.

725. Goritz, F., K. Jewgenow, and H.H. Meyer, *Epidermal growth factor and epidermal growth factor receptor in the ovary of the domestic cat (Felis catus).* J Reprod Fertil, 1996. **106**(1): p. 117-24.

726. Driancourt, M.A., et al., *Roles of KIT and KIT LIGAND in ovarian function.* Rev Reprod, 2000. **5**(3): p. 143-52.

727. Portela, V.M., et al., *Expression and function of fibroblast growth factor 18 in the ovarian follicle in cattle.* Biol Reprod, 2010. **83**(3): p. 339-46.

728. Buratini, J., Jr., et al., *Expression and function of fibroblast growth factor 10 and its receptor, fibroblast growth factor receptor 2B, in bovine follicles.* Biol Reprod, 2007. **77**(4): p. 743-50.

729. Almeida, A.P., et al., *Gene expression and immunolocalization of fibroblast growth factor 2 in the ovary and its effect on the in vitro culture of caprine preantral ovarian follicles.* Reprod Domest Anim, 2012. **47**(1): p. 20-5.

730. Matos, M.H., et al., *Follicle stimulating hormone and fibroblast growth factor-2 interact and promote goat primordial follicle development in vitro.* Reprod Fertil Dev, 2007. **19**(5): p. 677-84.

731. Matos, M.H., et al., *Effects of fibroblast growth factor-2 on the in vitro culture of caprine preantral follicles.* Cells Tissues Organs, 2007. **186**(2): p. 112-20.

732. Schams, D., et al., *Expression and possible role of fibroblast growth factor family members in porcine antral follicles during final maturation.* Reproduction, 2009. **138**(1): p. 141-9.

733. Matsuura, T., et al., *Anti-macrophage inhibitory factor antibody inhibits PMSG-hCG-induced follicular growth and ovulation in mice.* J Assist Reprod Genet, 2002. **19**(12): p. 591-5.

734. Wu, R., et al., *Ovarian leukocyte distribution and cytokine/chemokine mRNA expression in follicular fluid cells in women with polycystic ovary syndrome.* Hum Reprod, 2007. **22**(2): p. 527-35.

735. Tingen, C.M., et al., *A macrophage and theca cell-enriched stromal cell population influences growth and survival of immature murine follicles in vitro.* Reproduction, 2011. **141**(6): p. 809-20.

736. Gueripel, X., V. Brun, and A. Gougeon, *Oocyte bone morphogenetic protein 15, but not growth differentiation factor 9, is increased during gonadotropin-induced follicular development in the immature mouse and is associated with cumulus oophorus expansion.* Biol Reprod, 2006. **75**(6): p. 836-43.

737. Mottershead, D.G., L.J. Ritter, and R.B. Gilchrist, *Signalling pathways mediating specific synergistic interactions between GDF9 and BMP15.* Mol Hum Reprod, 2012. **18**(3): p. 121-8.

738. Durlinger, A.L., et al., *Anti-Mullerian hormone attenuates the effects of FSH on follicle development in the mouse ovary.* Endocrinology, 2001. **142**(11): p. 4891-9.

739. Juengel, J.L., et al., *Growth differentiation factor 9 and bone morphogenetic protein 15 are essential for ovarian follicular development in sheep.* Biol Reprod, 2002. **67**(6): p. 1777-89.

740. Myllymaa, S., et al., *Inhibition of oocyte growth factors in vivo modulates ovarian folliculogenesis in neonatal and immature mice.* Reproduction, 2010. **139**(3): p. 587-98.

741. Otsuka, F., et al., *Bone morphogenetic protein-15. Identification of target cells and biological functions.* J Biol Chem, 2000. **275**(50): p. 39523-8.

742. Fatehi, A.N., et al., *Expression of bone morphogenetic protein2 (BMP2), BMP4 and BMP receptors in the bovine ovary but absence of effects of BMP2 and BMP4 during IVM on bovine oocyte nuclear maturation and subsequent embryo development.* Theriogenology, 2005. **63**(3): p. 872-89.

743. Adashi, E.Y., *The IGF family and folliculogenesis.* J Reprod Immunol, 1998. **39**(1-2): p. 13-9.

744. Scheele, F. and J. Schoemaker, *The role of follicle-stimulating hormone in the selection of follicles in human ovaries: a survey of the literature and a proposed model.* Gynecol Endocrinol, 1996. **10**(1): p. 55-66.

745. Cheng, Y., et al., *Oocyte-expressed interleukin 7 suppresses granulosa cell apoptosis and promotes oocyte maturation in rats.* Biol Reprod, 2011. **84**(4): p. 707-14.

746. Sriperumbudur, R., L. Zorrilla, and J.E. Gadsby, *Transforming growth factor-beta (TGFbeta) and its signaling components in peri-ovulatory pig follicles.* Anim Reprod Sci, 2010. **120**(1-4): p. 84-94.

747. Teerds, K.J. and J.H. Dorrington, *Immunohistochemical localization of transforming growth factor-beta 1 and -beta 2 during follicular development in the adult rat ovary.* Mol Cell Endocrinol, 1992. **84**(1-2): p. R7-13.

748. Ghiglieri, C., et al., *Immunolocalization of transforming growth factor-beta 1 and transforming growth factor-beta 2 in the mouse ovary during gonadotrophin-induced follicular maturation.* Hum Reprod, 1995. **10**(8): p. 2115-9.

749. Steffl, M., M. Schweiger, and W.M. Amselgruber, *Expression of transforming growth factor-beta3 (TGF-beta3) in the porcine ovary during the oestrus cycle.* Histol Histopathol, 2008. **23**(6): p. 665-71.

750. Juengel, J.L. and K.P. McNatty, *The role of proteins of the transforming growth factor-beta superfamily in the intraovarian regulation of follicular development.* Hum Reprod Update, 2005. **11**(2): p. 143-60.

751. Dunkel, L., et al., *Follicle-stimulating hormone receptor expression in the rat ovary: increases during prepubertal development and regulation by the opposing actions of transforming growth factors beta and alpha.* Biol Reprod, 1994. **50**(4): p. 940-8.

752. Kuo, S.W., et al., *Potential role of follicle-stimulating hormone (FSH) and transforming growth factor (TGFbeta1) in the regulation of ovarian angiogenesis.* J Cell Physiol, 2011. **226**(6): p. 1608-19.

753. Ouellette, Y., C.A. Price, and P.D. Carriere, *Follicular fluid concentration of transforming growth factor-beta1 is negatively correlated with estradiol and follicle size at the early stage of development of the first-wave cohort of bovine ovarian follicles.* Domest Anim Endocrinol, 2005. **29**(4): p. 623-33.

754. Ye, Y., et al., *Kit ligand promotes first polar body extrusion of mouse preovulatory oocytes.* Reprod Biol Endocrinol, 2009. **7**: p. 26.

755. Silva, J.R., et al., *The Kit ligand/c-Kit receptor system in goat ovaries: gene expression and protein localization.* Zygote, 2006. **14**(4): p. 317-28.

756. Miyoshi, T., et al., *Regulatory role of kit ligand-c-kit interaction and oocyte factors in steroidogenesis by rat granulosa cells.* Mol Cell Endocrinol, 2012. **358**(1): p. 18-26.

757. Ito, M., et al., *Hepatocyte growth factor and stem cell factor involvement in paracrine interplays of theca and granulosa cells in the human ovary.* Fertil Steril, 2001. **75**(5): p. 973-9.

758. Tanikawa, M., et al., *Presence of stem cell factor in follicular fluid and its expression in the human ovary.* Fertil Steril, 2000. **73**(6): p. 1259-60.

759. Reynaud, K., et al., *Effects of Kit Ligand and anti-Kit antibody on growth of cultured mouse preantral follicles.* Mol Reprod Dev, 2000. **56**(4): p. 483-94.

760. Son, D.S., et al., *Tumor necrosis factor alpha (TNF) increases granulosa cell proliferation: dependence on c-Jun and TNF receptor type 1.* Endocrinology, 2004. **145**(3): p. 1218-26.

761. Son, D.S., K.F. Roby, and P.F. Terranova, *Tumor necrosis factor-alpha induces serum amyloid A3 in mouse granulosa cells.* Endocrinology, 2004. **145**(5): p. 2245-52.

762. Cui, L.L., et al., *Tumor necrosis factor alpha knockout increases fertility of mice.* Theriogenology, 2011. **75**(5): p. 867-76.

763. Kondo, H., T. Maruo, and M. Mochizuki, *Immunohistochemical evidence for the presence of tumor necrosis factor-alpha in the infant and adult human ovary.* Endocr J, 1995. **42**(6): p. 771-80.

764. Tartaglia, L.A., et al., *The two different receptors for tumor necrosis factor mediate distinct cellular responses.* Proc Natl Acad Sci U S A, 1991. **88**(20): p. 9292-6.

765. Brune, B., *Nitric oxide: NO apoptosis or turning it ON?* Cell Death Differ, 2003. **10**(8): p. 864-9.

766. Brunswig-Spickenheier, B. and A.K. Mukhopadhyay, *Stimulation of nitric oxide-cyclic guanosine monophosphate pathway in bovine ovarian theca cells by tumor necrosis factor alpha (TNF alpha). Is this pathway implicated in the TNF alpha-induced inhibition of luteinizing hormone-stimulated prorenin production?* Biol Reprod, 1997. **57**(4): p. 700-6.

767. Rosselli, M., P.J. Keller, and R.K. Dubey, *Role of nitric oxide in the biology, physiology and pathophysiology of reproduction.* Hum Reprod Update, 1998. **4**(1): p. 3-24.

768. Matsumi, H., et al., *Regulation of nitric oxide synthase to promote cytostasis in ovarian follicular development.* Biol Reprod, 2000. **63**(1): p. 141-6.

769. Hattori, M., et al., *Nitric oxide: a modulator for the epidermal growth factor receptor expression in developing ovarian granulosa cells.* Am J Physiol, 1996. **270**(3 Pt 1): p. C812-8.

770. Jaaskelainen, M., et al., *TRAIL pathway components and their putative role in granulosa cell apoptosis in the human ovary.* Differentiation, 2009. **77**(4): p. 369-76.

771. Maeda, A., et al., *Changes in expression of interleukin-6 receptors in granulosa cells during follicular atresia in pig ovaries.* J Reprod Dev, 2007. **53**(4): p. 727-36.

772. Kawasaki, F., et al., *The clinical role of interleukin-6 and interleukin-6 soluble receptor in human follicular fluids.* Clin Exp Med, 2003. **3**(1): p. 27-31.

773. Belayet, H.M., et al., *Pharmacologic doses of interleukin 8 suppositories induce follicular maturation in rabbits.* Cytokine, 2000. **12**(4): p. 361-7.

774. Kawano, Y., et al., *The effects of epidermal growth factor and transforming growth factor-alpha on secretion of interleukin-8 and growth-regulated oncogene-alpha in human granulosa-lutein cells.* Gynecol Obstet Invest, 2012. **73**(3): p. 189-94.

775. Hebert, C.A. and J.B. Baker, *Interleukin-8: a review.* Cancer Invest, 1993. **11**(6): p. 743-50.

776. Mukaida, N., et al., *Molecular mechanism of interleukin-8 gene expression.* J Leukoc Biol, 1994. **56**(5): p. 554-8.

777. Koch, A.E., et al., *Interleukin-8 as a macrophage-derived mediator of angiogenesis.* Science, 1992. **258**(5089): p. 1798-801.

778. Yoshino, O., et al., *Upregulation of interleukin-8 by hypoxia in human ovaries.* Am J Reprod Immunol, 2003. **50**(4): p. 286-90.

779. Hickey, M.M. and M.C. Simon, *Regulation of angiogenesis by hypoxia and hypoxia-inducible factors.* Curr Top Dev Biol, 2006. **76**: p. 217-57.

780. Arici, A., et al., *Interleukin-8 expression and modulation in human preovulatory follicles and ovarian cells.* Endocrinology, 1996. **137**(9): p. 3762-9.

781. Goto, J., et al., *Morphological analyses of interleukin-8 effects on rat ovarian follicles at ovulation and luteinization in vivo.* Cytokine, 2002. **20**(4): p. 168-73.

782. Murayama, C., et al., *Effect of VEGF (vascular endothelial growth factor) on expression of IL-8 (interleukin-8), IL-1beta and their receptors in bovine theca cells.* Cell Biol Int, 2010. **34**(5): p. 531-6.

783. Pancarci, S.M., et al., *Nitric oxide concentrations, estradiol-17beta progesterone ratio in follicular fluid, and COC quality with respect to perifollicular blood flow in cows.* Anim Reprod Sci, 2012. **130**(1-2): p. 9-15.

784. Lord, G.M., *Leptin as a proinflammatory cytokine.* Contrib Nephrol, 2006. **151**: p. 151-64.

785. Serke, H., et al., *Leptin-deficient (ob/ob) mouse ovaries show fatty degeneration, enhanced apoptosis and decreased expression of steroidogenic acute regulatory enzyme.* Int J Obes (Lond), 2012. **36**(8): p. 1047-53.

786. Magni, P., et al., *Expression of a leptin receptor in immortalized gonadotropin-releasing hormone-secreting neurons.* Endocrinology, 1999. **140**(4): p. 1581-5.

787. De Biasi, S.N., L.I. Apfelbaum, and M.E. Apfelbaum, *In vitro effect of leptin on LH release by anterior pituitary glands from female rats at the time of spontaneous and steroid-induced LH surge.* Eur J Endocrinol, 2001. **145**(5): p. 659-65.

788. Spicer, L.J. and C.C. Francisco, *The adipose obese gene product, leptin: evidence of a direct inhibitory role in ovarian function.* Endocrinology, 1997. **138**(8): p. 3374-9.

789. Spicer, L.J., C.S. Chamberlain, and C.C. Francisco, *Ovarian action of leptin: effects on insulin-like growth factor-I-stimulated function of granulosa and thecal cells.* Endocrine, 2000. **12**(1): p. 53-9.

790. Spicer, L.J. and C.C. Francisco, *Adipose obese gene product, leptin, inhibits bovine ovarian thecal cell steroidogenesis.* Biol Reprod, 1998. **58**(1): p. 207-12.

791. Agarwal, S.K., et al., *Leptin antagonizes the insulin-like growth factor-I augmentation of steroidogenesis in granulosa and theca cells of the human ovary.* J Clin Endocrinol Metab, 1999. **84**(3): p. 1072-6.

792. Brannian, J.D., Y. Zhao, and M. McElroy, *Leptin inhibits gonadotrophin-stimulated granulosa cell progesterone production by antagonizing insulin action.* Hum Reprod, 1999. **14**(6): p. 1445-8.

793. Zachow, R.J. and D.A. Magoffin, *Direct intraovarian effects of leptin: impairment of the synergistic action of insulin-like growth factor-I on follicle-stimulating hormone-dependent estradiol-17 beta production by rat ovarian granulosa cells.* Endocrinology, 1997. **138**(2): p. 847-50.

794. Ruiz-Cortes, Z.T., et al., *Biphasic effects of leptin in porcine granulosa cells.* Biol Reprod, 2003. **68**(3): p. 789-96.

795. Zackrisson, U., et al., *Alterations of follicular microcirculation and apex structure during ovulation in the rat.* Eur J Obstet Gynecol Reprod Biol, 2011. **157**(2): p. 169-74.

796. Martoriati, A. and N. Gerard, *Interleukin-1 (IL-1) system gene expression in granulosa cells: kinetics during terminal preovulatory follicle maturation in the mare.* Reprod Biol Endocrinol, 2003. **1**: p. 42.

797. Martoriati, A., et al., *Inhibition of in vitro maturation of equine oocytes by interleukin 1 beta via specific IL-1 receptors.* Reproduction, 2003. **126**(4): p. 509-15.

798. Martoriati, A., et al., *Expression of interleukin-1 (IL-1) system genes in equine cumulus-oocyte complexes and influence of IL-1beta during in vitro maturation.* Biol Reprod, 2002. **67**(2): p. 630-6.

799. Loret de Mola, J.R., et al., *Gonadotropins induce the release of interleukin-1 beta, interleukin-6 and tumor necrosis factor-alpha from the human preovulatory follicle.* Am J Reprod Immunol, 1998. **39**(6): p. 387-90.

800. Levitas, E., et al., *Periovulatory and interleukin-1 beta-dependent up-regulation of intraovarian vascular endothelial growth factor (VEGF) in the rat: potential role for VEGF in the promotion of periovulatory angiogenesis and vascular permeability.* J Soc Gynecol Investig, 2000. **7**(1): p. 51-60.

801. Adashi, E.Y., *The potential role of interleukin-1 in the ovulatory process: an evolving hypothesis.* Mol Cell Endocrinol, 1998. **140**(1-2): p. 77-81.

802. Machelon, V., et al., *Macrophage and granulosa interleukin-1 beta mRNA in human ovulatory follicles.* Hum Reprod, 1995. **10**(8): p. 2198-203.

803. Wang, X.F., F.Q. Xing, and S.L. Chen, *Interleukin-1beta expression on ovarian granulosa cells and its clinical implication in women undergoing in vitro fertilization.* Di Yi Jun Yi Da Xue Xue Bao, 2002. **22**(10): p. 934-6.

804. Caillaud, M., G. Duchamp, and N. Gerard, *In vivo effect of interleukin-1beta and interleukin-1RA on oocyte cytoplasmic maturation, ovulation, and early embryonic development in the mare.* Reprod Biol Endocrinol, 2005. **3**: p. 26.

805. Martoriati, A., G. Duchamp, and N. Gerard, *In vivo effect of epidermal growth factor, interleukin-1beta, and interleukin-1RA on equine preovulatory follicles.* Biol Reprod, 2003. **68**(5): p. 1748-54.

806. Gottschall, P.E., et al., *Interleukin 1: an inhibitor of luteinizing hormone receptor formation in cultured rat granulosa cells.* FASEB J, 1988. **2**(9): p. 2492-6.

807. Karakji, E.G. and B.K. Tsang, *Regulation of rat granulosa cell plasminogen activator system: influence of interleukin-1 beta and ovarian follicular development.* Biol Reprod, 1995. **53**(6): p. 1302-10.

808. Tao, M., et al., *Possible contribution of follicular interleukin-1beta to nitric oxide generation in human pre-ovulatory follicles.* Hum Reprod, 1997. **12**(10): p. 2220-5.

809. Tanaka, T., et al., *Induction of VEGF gene transcription by IL-1 beta is mediated through stress-activated MAP kinases and Sp1 sites in cardiac myocytes.* J Mol Cell Cardiol, 2000. **32**(11): p. 1955-67.

810. Jung, Y.D., et al., *Vascular endothelial growth factor is upregulated by interleukin-1 beta in human vascular smooth muscle cells via the P38 mitogen-activated protein kinase pathway.* Angiogenesis, 2001. **4**(2): p. 155-62.

811. Richards, J.S., *Hormonal control of gene expression in the ovary.* Endocr Rev, 1994. **15**(6): p. 725-51.

812. Ashkenazi, H., et al., *Epidermal growth factor family members: endogenous mediators of the ovulatory response.* Endocrinology, 2005. **146**(1): p. 77-84.

813. Park, J.Y., et al., *EGF-like growth factors as mediators of LH action in the ovulatory follicle.* Science, 2004. **303**(5658): p. 682-4.

814. Asano, K., et al., *Interferon gamma induces prostaglandin G/H synthase-2 through an autocrine loop via the epidermal growth factor receptor in human bronchial epithelial cells.* J Clin Invest, 1997. **99**(5): p. 1057-63.

815. Ben-Ami, I., et al., *PGE2 up-regulates EGF-like growth factor biosynthesis in human granulosa cells: new insights into the coordination between PGE2 and LH in ovulation.* Mol Hum Reprod, 2006. **12**(10): p. 593-9.

816. Shimada, M., et al., *Paracrine and autocrine regulation of epidermal growth factor-like factors in cumulus oocyte complexes and granulosa cells: key roles for prostaglandin synthase 2 and progesterone receptor.* Mol Endocrinol, 2006. **20**(6): p. 1352-65.

817. Su, Y.Q., et al., *Mouse oocytes enable LH-induced maturation of the cumulus-oocyte complex via promoting EGF receptor-dependent signaling.* Mol Endocrinol, 2010. **24**(6): p. 1230-9.

818. Sugiura, K., et al., *Estrogen promotes the development of mouse cumulus cells in coordination with oocyte-derived GDF9 and BMP15.* Mol Endocrinol, 2010. **24**(12): p. 2303-14.

819. Liu, Z., et al., *Interleukin-6: an autocrine regulator of the mouse cumulus cell-oocyte complex expansion process.* Endocrinology, 2009. **150**(7): p. 3360-8.

820. Szukiewicz, D., et al., *Mast cell-derived interleukin-8 may be involved in the ovarian mechanisms of follicle growth and ovulation.* Inflamm Res, 2007. **56 Suppl 1**: p. S35-6.

821. Runesson, E., et al., *The human preovulatory follicle is a source of the chemotactic cytokine interleukin-8.* Mol Hum Reprod, 1996. **2**(4): p. 245-50.

822. Runesson, E., et al., *Gonadotropin- and cytokine-regulated expression of the chemokine interleukin 8 in the human preovulatory follicle of the menstrual cycle.* J Clin Endocrinol Metab, 2000. **85**(11): p. 4387-95.

823. Ujioka, T., et al., *Interleukin-8 as an essential factor in the human chorionic gonadotropin-induced rabbit ovulatory process: interleukin-8 induces neutrophil accumulation and activation in ovulation.* Biol Reprod, 1998. **58**(2): p. 526-30.

824. Malizia, B.A., et al., *The human ovarian follicular fluid level of interleukin-8 is associated with follicular size and patient age.* Fertil Steril, 2010. **93**(2): p. 537-43.

825. Santos, M.A., E.W. Kuijk, and N.S. Macklon, *The impact of ovarian stimulation for IVF on the developing embryo.* Reproduction, 2009. **139**(1): p. 23-34.

826. Macklon, N.S., et al., *The science behind 25 years of ovarian stimulation for in vitro fertilization.* Endocr Rev, 2006. **27**(2): p. 170-207.

827. Rienzi, L., G. Vajta, and F. Ubaldi, *Predictive value of oocyte morphology in human IVF: a systematic review of the literature.* Hum Reprod Update, 2011. **17**(1): p. 34-45.

828. Veeck, L.L., *Oocyte assessment and biological performance.* Ann N Y Acad Sci, 1988. **541**: p. 259-74.

829. El-Talatini, M.R., et al., *Localisation and function of the endocannabinoid system in the human ovary.* PLoS One, 2009. **4**(2): p. e4579.

830. Liu, S., et al., *Evaluation of the immature human oocytes from unstimulated cycles in polycystic ovary syndrome patients using a novel scoring system.* Fertil Steril, 2010. **93**(7): p. 2202-9.

831. Balaban, B. and B. Urman, *Effect of oocyte morphology on embryo development and implantation.* Reprod Biomed Online, 2006. **12**(5): p. 608-15.

832. Patrizio, P., et al., *Molecular methods for selection of the ideal oocyte.* Reprod Biomed Online, 2007. **15**(3): p. 346-53.

833. Johnson, L.N., et al., *Does intracytoplasmic sperm injection improve the fertilization rate and decrease the total fertilization failure rate in couples with well-defined unexplained infertility? A systematic review and meta-analysis.* Fertil Steril, 2013. **100**(3): p. 704-11.

834. Kuliev, A., et al., *Chromosomal abnormalities in a series of 6,733 human oocytes in preimplantation diagnosis for age-related aneuploidies.* Reprod Biomed Online, 2003. **6**(1): p. 54-9.

835. Bermudez, M.G., et al., *Expression profiles of individual human oocytes using microarray technology.* Reprod Biomed Online, 2004. **8**(3): p. 325-37.

836. Rattanachaiyanont, M., A. Leader, and M.C. Leveille, *Lack of correlation between oocyte-corona-cumulus complex morphology and nuclear maturity of oocytes collected in stimulated cycles for intracytoplasmic sperm injection.* Fertil Steril, 1999. **71**(5): p. 937-40.

837. Ebner, T., et al., *Blood clots in the cumulus-oocyte complex predict poor oocyte quality and post-fertilization development.* Reprod Biomed Online, 2008. **16**(6): p. 801-7.

838. Bertrand, E., M. Van den Bergh, and Y. Englert, *Does zona pellucida thickness influence the fertilization rate?* Hum Reprod, 1995. **10**(5): p. 1189-93.

839. Shen, Y., et al., *High magnitude of light retardation by the zona pellucida is associated with conception cycles.* Hum Reprod, 2005. **20**(6): p. 1596-606.

840. Rama Raju, G.A., et al., *Meiotic spindle and zona pellucida characteristics as predictors of embryonic development: a preliminary study using PolScope imaging.* Reprod Biomed Online, 2007. **14**(2): p. 166-74.

841. Montag, M., et al., *Oocyte zona birefringence intensity is associated with embryonic implantation potential in ICSI cycles.* Reprod Biomed Online, 2008. **16**(2): p. 239-44.

842. Chamayou, S., et al., *Meiotic spindle presence and oocyte morphology do not predict clinical ICSI outcomes: a study of 967 transferred embryos.* Reprod Biomed Online, 2006. **13**(5): p. 661-7.

843. Farhi, J., et al., *Coarse granulation in the perivitelline space and IVF-ICSI outcome.* J Assist Reprod Genet, 2002. **19**(12): p. 545-9.

844. Rienzi, L., et al., *Significance of metaphase II human oocyte morphology on ICSI outcome.* Fertil Steril, 2008. **90**(5): p. 1692-700.

845. Ebner, T., et al., *Prognostic value of first polar body morphology on fertilization rate and embryo quality in intracytoplasmic sperm injection.* Hum Reprod, 2000. **15**(2): p. 427-30.

846. Ten, J., et al., *Donor oocyte dysmorphisms and their influence on fertilization and embryo quality.* Reprod Biomed Online, 2007. **14**(1): p. 40-8.

847. Loutradis, D., et al., *Oocyte morphology correlates with embryo quality and pregnancy rate after intracytoplasmic sperm injection.* Fertil Steril, 1999. **72**(2): p. 240-4.

848. Suppinyopong, S., R. Choavaratana, and C. Karavakul, *Correlation of oocyte morphology with fertilization rate and embryo quality after intracytoplasmic sperm injection.* J Med Assoc Thai, 2000. **83**(6): p. 627-32.

849. Otsuki, J., et al., *The relationship between pregnancy outcome and smooth endoplasmic reticulum clusters in MII human oocytes.* Hum Reprod, 2004. **19**(7): p. 1591-7.

850. De Sutter, P., et al., *Oocyte morphology does not correlate with fertilization rate and embryo quality after intracytoplasmic sperm injection.* Hum Reprod, 1996. **11**(3): p. 595-7.

851. Balaban, B., et al., *Oocyte morphology does not affect fertilization rate, embryo quality and implantation rate after intracytoplasmic sperm injection.* Hum Reprod, 1998. **13**(12): p. 3431-3.

852. Xia, P., *Intracytoplasmic sperm injection: correlation of oocyte grade based on polar body, perivitelline space and cytoplasmic inclusions with fertilization rate and embryo quality.* Hum Reprod, 1997. **12**(8): p. 1750-5.

853. Otsuki, J., Y. Nagai, and K. Chiba, *Lipofuscin bodies in human oocytes as an indicator of oocyte quality.* J Assist Reprod Genet, 2007. **24**(7): p. 263-70.

854. Moon, J.H., et al., *Visualization of the metaphase II meiotic spindle in living human oocytes using the Polscope enables the prediction of embryonic developmental competence after ICSI.* Hum Reprod, 2003. **18**(4): p. 817-20.

855. Cohen, Y., et al., *Spindle imaging: a new marker for optimal timing of ICSI?* Hum Reprod, 2004. **19**(3): p. 649-54.

856. Fang, C., et al., *Visualization of meiotic spindle and subsequent embryonic development in in vitro and in vivo matured human oocytes.* J Assist Reprod Genet, 2007. **24**(11): p. 547-51.

857. Madaschi, C., et al., *Spindle imaging: a marker for embryo development and implantation.* Fertil Steril, 2008. **90**(1): p. 194-8.

858. Sharma, V., et al., *A comparison of treatments with exogenous FSH to promote folliculogenesis in patients with quiescent ovaries due to the continued administration of an LH-RH agonist.* Hum Reprod, 1987. **2**(7): p. 553-6.

859. Sharma, V., et al., *Influence of superovulation on endometrial and embryonic development.* Fertil Steril, 1990. **53**(5): p. 822-9.

860. Pellicer, A., et al., *Is the retrieval of high numbers of oocytes desirable in patients treated with gonadotrophin-releasing hormone analogues (GnRHa) and gonadotrophins?* Hum Reprod, 1989. **4**(5): p. 536-40.

861. van der Gaast, M.H., et al., *Optimum number of oocytes for a successful first IVF treatment cycle.* Reprod Biomed Online, 2006. **13**(4): p. 476-80.

862. Ertzeid, G. and R. Storeng, *Adverse effects of gonadotrophin treatment on pre- and postimplantation development in mice.* J Reprod Fertil, 1992. **96**(2): p. 649-55.

863. Ertzeid, G., R. Storeng, and T. Lyberg, *Treatment with gonadotropins impaired implantation and fetal development in mice.* J Assist Reprod Genet, 1993. **10**(4): p. 286-91.

864. Hodges, C.A., et al., *Experimental evidence that changes in oocyte growth influence meiotic chromosome segregation.* Hum Reprod, 2002. **17**(5): p. 1171-80.

865. Imthurn, B., et al., *Nuclear maturity and oocyte morphology after stimulation with highly purified follicle stimulating hormone compared to human menopausal gonadotrophin.* Hum Reprod, 1996. **11**(11): p. 2387-91.

866. Elbling, L. and M. Colot, *Abnormal development and transport and increased sister-chromatid exchange in preimplantation embryos following superovulation in mice.* Mutat Res, 1985. **147**(4): p. 189-95.

867. Vogel, R. and H. Spielmann, *Genotoxic and embryotoxic effects of gonadotropin-hyperstimulated ovulation of murine oocytes, preimplantation embryos, and term fetuses.* Reprod Toxicol, 1992. **6**(4): p. 329-33.

868. Roberts, R., et al., *Follicle-stimulating hormone affects metaphase I chromosome alignment and increases aneuploidy in mouse oocytes matured in vitro.* Biol Reprod, 2005. **72**(1): p. 107-18.

869. Hammadeh, M.E., et al., *Relationship between cytokine concentration in serum and preovulatory follicular fluid and in vitro fertilization/intracytoplasmic sperm injection outcome.* Chem Immunol Allergy, 2005. **88**: p. 80-97.

870. Choi, Y.S., et al., *Comparison of follicular fluid IGF-I, IGF-II, IGFBP-3, IGFBP-4 and PAPP-A concentrations and their ratios between GnRH agonist and GnRH antagonist protocols for controlled ovarian stimulation in IVF-embryo transfer patients.* Hum Reprod, 2006. **21**(8): p. 2015-21.

871. Baskind, N.E., N.M. Orsi, and V. Sharma, *Follicular-phase ovarian follicular fluid and plasma cytokine profiling of natural cycle in vitro fertilization patients.* Fertil Steril, 2014.

872. Ledee, N., et al., *Impact of follicular G-CSF quantification on subsequent embryo transfer decisions: a proof of concept study.* Hum Reprod, 2013. **28**(2): p. 406-13.

873. Salmassi, A., et al., *Is granulocyte colony-stimulating factor level predictive for human IVF outcome?* Hum Reprod, 2005. **20**(9): p. 2434-40.

874. Salmassi, A., et al., *Detection of granulocyte colony-stimulating factor and its receptor in human follicular luteinized granulosa cells.* Fertil Steril, 2004. **81 Suppl 1**: p. 786-91.

875. Yanagi, K., et al., *Cyclic changes of granulocyte colony-stimulating factor (G-CSF) mRNA in the human follicle during the normal menstrual cycle and immunolocalization of G-CSF protein.* Hum Reprod, 2002. **17**(12): p. 3046-52.

876. Ledee, N., et al., *New pre-conception immune biomarkers for clinical practice: interleukin-18, interleukin-15 and TWEAK on the endometrial side, G-CSF on the follicular side.* J Reprod Immunol, 2011. **88**(2): p. 118-23.

877. Ledee, N., et al., *Levels of follicular G-CSF and interleukin-15 appear as noninvasive biomarkers of subsequent successful birth in modified natural in vitro fertilization/intracytoplasmic sperm injection cycles.* Fertil Steril, 2010.

878. Ledee, N., et al., *Levels of follicular G-CSF and interleukin-15 appear as noninvasive biomarkers of subsequent successful birth in modified natural in vitro fertilization/intracytoplasmic sperm injection cycles.* Fertil Steril, 2011. **95**(1): p. 94-8.

879. Salmassi, A., et al., *Expression of mRNA and protein of macrophage colony-stimulating factor and its receptor in human follicular luteinized granulosa cells.* Fertil Steril, 2005. **83**(2): p. 419-25.

880. Salmassi, A., et al., *Circulating level of macrophage colony-stimulating factor can be predictive for human in vitro fertilization outcome.* Fertil Steril, 2010. **93**(1): p. 116-23.

881. Nishimura, K., et al., *Changes in macrophage colony-stimulating factor concentration in serum and follicular fluid in in-vitro fertilization and embryo transfer cycles.* Fertil Steril, 1998. **69**(1): p. 53-7.

882. Kawano, Y., et al., *The production and clinical evaluation of macrophage colony-stimulating factor and macrophage chemoattractant protein-1 in human follicular fluids.* Am J Reprod Immunol, 2001. **45**(1): p. 1-5.

883. Smikle, C.B., et al., *Elevated ovarian follicular fluid stem cell factor concentrations are associated with improved pregnancy rates in in-vitro fertilization cycles.* Fertil Steril, 1998. **69**(1): p. 70-2.

884. Hammadeh, M.E., et al., *Relationship between cytokine concentrations (FGF, sICAM-1 and SCF) in serum, follicular fluid and ICSI outcome.* Am J Reprod Immunol, 2004. **51**(1): p. 81-5.

885. Klinger, F.G. and M. De Felici, *In vitro development of growing oocytes from fetal mouse oocytes: stage-specific regulation by stem cell factor and granulosa cells.* Dev Biol, 2002. **244**(1): p. 85-95.

886. Nishigaki, A., et al., *Concentrations of stromal cell-derived factor-1 and vascular endothelial growth factor in relation to the diameter of human follicles.* Fertil Steril, 2011. **95**(2): p. 742-6.

887. Goud, P.T., et al., *In-vitro maturation of human germinal vesicle stage oocytes: role of cumulus cells and epidermal growth factor in the culture medium.* Hum Reprod, 1998. **13**(6): p. 1638-44.

888. Merlo, B., et al., *Effect of EGF on in vitro maturation of domestic cat oocytes.* Theriogenology, 2005. **63**(7): p. 2032-9.

889. Merriman, J.A., D.G. Whittingham, and J. Carroll, *The effect of follicle stimulating hormone and epidermal growth factor on the developmental capacity of in-vitro matured mouse oocytes.* Hum Reprod, 1998. **13**(3): p. 690-5.

890. Ben-Ami, I., et al., *In vitro maturation of human germinal vesicle-stage oocytes: role of epidermal growth factor-like growth factors in the culture medium.* Hum Reprod, 2011. **26**(1): p. 76-81.

891. Bolamba, D., et al., *Effects of epidermal growth factor and hormones on granulosa expansion and nuclear maturation of dog oocytes in vitro.* Theriogenology, 2006. **65**(6): p. 1037-47.

892. Oyamada, T., H. Iwayama, and Y. Fukui, *Additional effect of epidermal growth factor during in vitro maturation for individual bovine oocytes using a chemically defined medium.* Zygote, 2004. **12**(2): p. 143-50.

893. Park, K.W., K. Iga, and K. Niwa, *Exposure of bovine oocytes to EGF during maturation allows them to develop to blastocysts in a chemically-defined medium.* Theriogenology, 1997. **48**(7): p. 1127-35.

894. Song, H.J., et al., *Influence of epidermal growth factor supplementation during in vitro maturation on nuclear status and gene expression of canine oocytes.* Res Vet Sci, 2010.

895. Hsieh, M., K. Thao, and M. Conti, *Genetic dissection of epidermal growth factor receptor signaling during luteinizing hormone-induced oocyte maturation.* PLoS One, 2011. **6**(6): p. e21574.

896. Lee, H.J., et al., *In vitro maturation (IVM) of murine and human germinal vesicle (GV)-stage oocytes by coculture with immortalized human fallopian tube epithelial cells.* Fertil Steril, 2011. **95**(4): p. 1344-8.

897. Richani, D., et al., *Mode of oocyte maturation affects EGF-like peptide function and oocyte competence.* Mol Hum Reprod, 2013. **19**(8): p. 500-9.

898. Inoue, Y., et al., *Amphiregulin is much more abundantly expressed than transforming growth factor-alpha and epidermal growth factor in human follicular fluid obtained from patients undergoing in vitro fertilization-embryo transfer.* Fertil Steril, 2009. **91**(4): p. 1035-41.

899. Zamah, A.M., et al., *Human oocyte maturation is dependent on LH-stimulated accumulation of the epidermal growth factor-like growth factor, amphiregulin.* Hum Reprod, 2010. **25**(10): p. 2569-78.

900. Liu, N., et al., *Comparison of follicular fluid amphiregulin and EGF concentrations in patients undergoing IVF with different stimulation protocols.* Endocrine, 2012. **42**(3): p. 708-16.

901. Kawano, Y., et al., *Production of macrophage inflammatory protein-3alpha in human follicular fluid and cultured granulosa cells.* Fertil Steril, 2004. **82 Suppl 3**: p. 1206-11.

902. Loret de Mola, J.R., et al., *Elevated interleukin-6 levels in the ovarian hyperstimulation syndrome: ovarian immunohistochemical localization of interleukin-6 signal.* Obstet Gynecol, 1996. **87**(4): p. 581-7.

903. Geva, E., et al., *Elevated levels of interleukin-6 in the follicular fluid at the time of oocyte retrieval for in vitro fertilization may predict the development of early-form ovarian hyperstimulation syndrome.* Fertil Steril, 1997. **68**(1): p. 133-7.

904. De Matos, D.G., et al., *Leukemia inhibitory factor induces cumulus expansion in immature human and mouse oocytes and improves mouse two-cell rate and delivery rates when it is present during mouse in vitro oocyte maturation.* Fertil Steril, 2008. **90**(6): p. 2367-75.

905. Ptak, G., et al., *Leukaemia inhibitory factor enhances sheep fertilization in vitro via an influence on the oocyte.* Theriogenology, 2006. **65**(9): p. 1891-9.

906. Coskun, S., et al., *Presence of leukemia inhibitory factor and interleukin-12 in human follicular fluid during follicular growth.* Am J Reprod Immunol, 1998. **40**(1): p. 13-8.

907. Ledee-Bataille, N., et al., *Concentration of leukaemia inhibitory factor (LIF) in uterine flushing fluid is highly predictive of embryo implantation.* Hum Reprod, 2002. **17**(1): p. 213-8.

908. Hsieh, Y.Y., et al., *Leukemia inhibitory factor in follicular fluid is not related to the number and quality of embryos as well as implantation and pregnancy rates.* Biochem Genet, 2005. **43**(9-10): p. 501-6.

909. Ozornek, M.H., et al., *Epidermal growth factor and leukemia inhibitory factor levels in follicular fluid. Association with in vitro fertilization outcome.* J Reprod Med, 1999. **44**(4): p. 367-9.

910. Fried, G. and H. Wramsby, *Increase in transforming growth factor beta1 in ovarian follicular fluid following ovarian stimulation and in-vitro fertilization correlates to pregnancy.* Hum Reprod, 1998. **13**(3): p. 656-9.

911. Fried, G., H. Wramsby, and M. Tally, *Transforming growth factor-beta1, insulin-like growth factors, and insulin-like growth factor binding proteins in ovarian follicular fluid are differentially regulated by the type of ovarian hyperstimulation used for in vitro fertilization.* Fertil Steril, 1998. **70**(1): p. 129-34.

912. Wu, Y.T., et al., *High bone morphogenetic protein-15 level in follicular fluid is associated with high quality oocyte and subsequent embryonic development.* Hum Reprod, 2007. **22**(6): p. 1526-31.

913. Gode, F., et al., *Influence of follicular fluid GDF9 and BMP15 on embryo quality.* Fertil Steril, 2011. **95**(7): p. 2274-8.

914. Bonetti, T.C., et al., *Cytokine and hormonal profile in serum samples of patients undergoing controlled ovarian stimulation: interleukin-1beta predicts ongoing pregnancy.* Hum Reprod, 2010. **25**(8): p. 2101-6.

915. Karagouni, E.E., et al., *Interleukin-1beta and interleukin-1alpha may affect the implantation rate of patients undergoing in vitro fertilization-embryo transfer.* Fertil Steril, 1998. **70**(3): p. 553-9.

916. Mendoza, C., et al., *Relationship between fertilization results after intracytoplasmic sperm injection, and intrafollicular steroid, pituitary hormone and cytokine concentrations.* Hum Reprod, 1999. **14**(3): p. 628-35.

917. Caillaud, M., et al., *In vitro equine oocyte maturation in pure follicular fluid plus interleukin-1 and fertilization following ICSI.* Anim Reprod Sci, 2008. **106**(3-4): p. 431-9.

918. Bedaiwy, M., et al., *Differential expression of follicular fluid cytokines: relationship to subsequent pregnancy in IVF cycles.* Reprod Biomed Online, 2007. **15**(3): p. 321-5.

919. Soto, P., R.P. Natzke, and P.J. Hansen, *Actions of tumor necrosis factor-alpha on oocyte maturation and embryonic development in cattle.* Am J Reprod Immunol, 2003. **50**(5): p. 380-8.

920. Mendoza, C., et al., *Follicular fluid markers of oocyte developmental potential.* Hum Reprod, 2002. **17**(4): p. 1017-22.

921. Ma, C.H., et al., *Effects of tumor necrosis factor-alpha on porcine oocyte meiosis progression, spindle organization, and chromosome alignment.* Fertil Steril, 2010. **93**(3): p. 920-6.

922. Lee, K.S., et al., *Relationships between concentrations of tumor necrosis factor-alpha and nitric oxide in follicular fluid and oocyte quality.* J Assist Reprod Genet, 2000. **17**(4): p. 222-8.

923. Falconer, H., et al., *IVF outcome in women with endometriosis in relation to tumour necrosis factor and anti-Mullerian hormone.* Reprod Biomed Online, 2009. **18**(4): p. 582-8.

924. Carlberg, M., et al., *Elevated expression of tumour necrosis factor alpha in cultured granulosa cells from women with endometriosis.* Hum Reprod, 2000. **15**(6): p. 1250-5.

925. Nikolettos, N., et al., *Evaluation of leptin, interleukin-1beta, tumor necrosis factor-alpha and vascular endothelial growth factor in serum and follicular fluids of women undergoing controlled ovarian hyperstimulation as prognostic markers of ICSI outcome.* In Vivo, 2004. **18**(5): p. 667-73.

926. Bedaiwy, M.A., et al., *Prediction of endometriosis with serum and peritoneal fluid markers: a prospective controlled trial.* Hum Reprod, 2002. **17**(2): p. 426-31.

927. Pizzo, A., et al., *Behaviour of cytokine levels in serum and peritoneal fluid of women with endometriosis.* Gynecol Obstet Invest, 2002. **54**(2): p. 82-7.

928. Buscher, U., et al., *Cytokines in the follicular fluid of stimulated and non-stimulated human ovaries; is ovulation a suppressed inflammatory reaction?* Hum Reprod, 1999. **14**(1): p. 162-6.

929. Munne, S., et al., *Treatment-related chromosome abnormalities in human embryos.* Hum Reprod, 1997. **12**(4): p. 780-4.

930. Out, H.J., et al., *A randomized, double-blind, multicentre clinical trial comparing starting doses of 150 and 200 IU of recombinant FSH in women treated with the GnRH antagonist ganirelix for assisted reproduction.* Hum Reprod, 2004. **19**(1): p. 90-5.

931. Lashen, H., et al., *Superovulation with a high gonadotropin dose for in vitro fertilization: is it effective?* J Assist Reprod Genet, 1998. **15**(7): p. 438-43.

932. Das, S., et al., *Follicular fluid expression of alpha-defensins and their role in ovulation.* J Assist Reprod Genet, 2008. **25**(2-3): p. 83-7.

933. Trehan, P., N. Gopichandran, and N. Orsi. *Cytokine preimplantation embryotrophism in the mouse*. in *Fertility 2007*. 2007. York: British Fertility Society.

934. Geva, E., et al., *Follicular fluid levels of vascular endothelial growth factor. Are they predictive markers for ovarian hyperstimulation syndrome?* J Reprod Med, 1999. **44**(2): p. 91-6.

935. Artini, P.G., et al., *Vascular endothelial growth factor, interleukin-6 and interleukin-2 in serum and follicular fluid of patients with ovarian hyperstimulation syndrome.* Eur J Obstet Gynecol Reprod Biol, 2002. **101**(2): p. 169-74.

936. Agrawal, R., et al., *Concentration of vascular endothelial growth factor released by cultured human luteinized granulosa cells is higher in women with polycystic ovaries than in women with normal ovaries.* Fertil Steril, 2002. **78**(6): p. 1164-9.

937. Quintana, R., et al., *Relationship of ovarian stimulation response with vascular endothelial growth factor and degree of granulosa cell apoptosis.* Hum Reprod, 2001. **16**(9): p. 1814-8.

938. Wang, T.H., et al., *Human chorionic gonadotropin-induced ovarian hyperstimulation syndrome is associated with up-regulation of vascular endothelial growth factor.* J Clin Endocrinol Metab, 2002. **87**(7): p. 3300-8.

939. Babayof, R., et al., *Serum inhibin A, VEGF and TNFalpha levels after triggering oocyte maturation with GnRH agonist compared with HCG in women with polycystic ovaries undergoing IVF treatment: a prospective randomized trial.* Hum Reprod, 2006. **21**(5): p. 1260-5.

940. Chen, S.U., et al., *Signal mechanisms of vascular endothelial growth factor and interleukin-8 in ovarian hyperstimulation syndrome: dopamine targets their common pathways.* Hum Reprod, 2010. **25**(3): p. 757-67.

941. Pellicer, A., et al., *The pathogenesis of ovarian hyperstimulation syndrome: in vivo studies investigating the role of interleukin-1beta, interleukin-6, and vascular endothelial growth factor.* Fertil Steril, 1999. **71**(3): p. 482-9.

942. Savchev, S.I., et al., *Follicular fluid-specific distribution of vascular endothelial growth factor isoforms and sFlt-1 in patients undergoing IVF and their correlation with treatment outcomes.* Reprod Sci, 2010. **17**(11): p. 1036-42.

943. Asimakopoulos, B., et al., *Follicular fluid levels of vascular endothelial growth factor and leptin are associated with pregnancy outcome of normal women participating in intracytoplasmic sperm injection cycles.* Physiol Res, 2005. **54**(3): p. 263-70.

944. Friedman, C.I., et al., *Elevated level of follicular fluid vascular endothelial growth factor is a marker of diminished pregnancy potential.* Fertil Steril, 1998. **70**(5): p. 836-9.

945. Tokuyama, O., et al., *Vascular endothelial growth factor concentrations in follicular fluid obtained from IVF-ET patients: a comparison of hMG, clomiphene citrate, and natural cycle.* J Assist Reprod Genet, 2002. **19**(1): p. 19-23.

946. Barroso, G., et al., *Vascular endothelial growth factor, nitric oxide, and leptin follicular fluid levels correlate negatively with embryo quality in IVF patients.* Fertil Steril, 1999. **72**(6): p. 1024-6.

947. Anasti, J.N., et al., *Human follicle fluid vascular endothelial growth factor concentrations are correlated with luteinization in spontaneously developing follicles.* Hum Reprod, 1998. **13**(5): p. 1144-7.

948. Kawano, Y., et al., *Production of vascular endothelial growth factor and angiogenic factor in human follicular fluid.* Mol Cell Endocrinol, 2003. **202**(1-2): p. 19-23.

949. Monteleone, P., et al., *Follicular fluid VEGF levels directly correlate with perifollicular blood flow in normoresponder patients undergoing IVF.* J Assist Reprod Genet, 2008. **25**(5): p. 183-6.

950. Mattioli, M., et al., *Follicle activation involves vascular endothelial growth factor production and increased blood vessel extension.* Biol Reprod, 2001. **65**(4): p. 1014-9.

951. Van Blerkom, J., M. Antczak, and R. Schrader, *The developmental potential of the human oocyte is related to the dissolved oxygen content of follicular fluid: association with vascular endothelial growth factor levels and perifollicular blood flow characteristics.* Hum Reprod, 1997. **12**(5): p. 1047-55.

952. Borini, A., et al., *Perifollicular vascularity and its relationship with oocyte maturity and IVF outcome.* Ann N Y Acad Sci, 2001. **943**: p. 64-7.

953. Abramovich, D., F. Parborell, and M. Tesone, *Effect of a vascular endothelial growth factor (VEGF) inhibitory treatment on the folliculogenesis and ovarian apoptosis in gonadotropin-treated prepubertal rats.* Biol Reprod, 2006. **75**(3): p. 434-41.

954. Yamamoto, S., et al., *Expression of vascular endothelial growth factor (VEGF) during folliculogenesis and corpus luteum formation in the human ovary.* Gynecol Endocrinol, 1997. **11**(6): p. 371-81.

955. Luo, H., et al., *Effect of vascular endothelial growth factor on maturation, fertilization and developmental competence of bovine oocytes.* J Vet Med Sci, 2002. **64**(9): p. 803-6.

956. Luo, H., et al., *Vascular endothelial growth factor (VEGF) promotes the early development of bovine embryo in the presence of cumulus cells.* J Vet Med Sci, 2002. **64**(11): p. 967-71.

957. Einspanier, R., et al., *Expression of the vascular endothelial growth factor and its receptors and effects of VEGF during in vitro maturation of bovine cumulus-oocyte complexes (COC).* Mol Reprod Dev, 2002. **62**(1): p. 29-36.

958. Barboni, B., et al., *Vascular endothelial growth factor production in growing pig antral follicles.* Biol Reprod, 2000. **63**(3): p. 858-64.

959. Koos, R.D., *Increased expression of vascular endothelial growth/permeability factor in the rat ovary following an ovulatory gonadotropin stimulus: potential roles in follicle rupture.* Biol Reprod, 1995. **52**(6): p. 1426-35.

960. Artini, P.G., et al., *Correlation between the amount of follicle-stimulating hormone administered and plasma and follicular fluid vascular endothelial growth factor concentrations in women undergoing in vitro fertilization.* Gynecol Endocrinol, 1998. **12**(4): p. 243-7.

961. Anifandis, G., et al., *Serum and follicular fluid leptin levels are correlated with human embryo quality.* Reproduction, 2005. **130**(6): p. 917-21.

962. Mantzoros, C.S., et al., *Predictive value of serum and follicular fluid leptin concentrations during assisted reproductive cycles in normal women and in women with the polycystic ovarian syndrome.* Hum Reprod, 2000. **15**(3): p. 539-44.

963. Robins, J.C., et al., *Association of leptin with poor ovarian stimulation during in vitro fertilization.* J Reprod Med, 2005. **50**(5): p. 356-60.

964. Suzuki, H., et al., *Ghrelin and leptin did not improve meiotic maturation of porcine oocytes cultured in vitro.* Reprod Domest Anim, 2010. **45**(5): p. 927-30.

965. Almog, B., et al., *Intrafollicular and serum levels of leptin during in vitro fertilization cycles: comparison between the effects of recombinant follicle-stimulating hormones and human menopausal gonadotrophin.* Gynecol Endocrinol, 2011. **27**(9): p. 666-8.

966. Chakrabarti, J., et al., *Overt leptin response to controlled ovarian hyperstimulation negatively correlates with pregnancy outcome in in vitro fertilization--embryo transfer cycle.* J Hum Reprod Sci, 2012. **5**(2): p. 194-9.

967. Dorn, C., et al., *Leptin, VEGF, IGF-1, and IGFBP-3 concentrations in serum and follicular fluid of women undergoing in vitro fertilization.* Arch Gynecol Obstet, 2003. **268**(3): p. 187-93.

968. Hill, M.J., et al., *The utility of serum leptin and follicular fluid leptin, estradiol, and progesterone levels during an in vitro fertilization cycle.* J Assist Reprod Genet, 2007. **24**(5): p. 183-8.

969. Takikawa, S., et al., *Assessment of the predictive value of follicular fluid insulin, leptin and adiponectin in assisted reproductive cycles.* Gynecol Endocrinol, 2010. **26**(7): p. 494-9.

970. Wunder, D.M., R. Kretschmer, and N.A. Bersinger, *Concentrations of leptin and C-reactive protein in serum and follicular fluid during assisted reproductive cycles.* Hum Reprod, 2005. **20**(5): p. 1266-71.

971. Wunder, D.M., et al., *Steroids and protein markers in the follicular fluid as indicators of oocyte quality in patients with and without endometriosis.* J Assist Reprod Genet, 2005. **22**(6): p. 257-64.

972. Butzow, T.L., et al., *Serum and follicular fluid leptin during in vitro fertilization: relationship among leptin increase, body fat mass, and reduced ovarian response.* J Clin Endocrinol Metab, 1999. **84**(9): p. 3135-9.

973. Chen, R., et al., *Serum and follicular fluid leptin levels in patients undergoing controlled ovarian hyperstimulation for in vitro fertilization cycle.* Clin Exp Obstet Gynecol, 2004. **31**(2): p. 103-6.

974. Webb, R., et al., *Control of follicular growth: local interactions and nutritional influences.* J Anim Sci, 2004. **82 E-Suppl**: p. E63-74.

975. Michalakis, K., et al., *The complex interaction between obesity, metabolic syndrome and reproductive axis: a narrative review.* Metabolism, 2013. **62**(4): p. 457-78.

976. Bili, H., et al., *Cytokines in the human ovary: presence in follicular fluid and correlation with leukotriene B4.* J Assist Reprod Genet, 1998. **15**(2): p. 93-8.

977. Orvieto, R., et al., *Interleukin-2 and ovarian hyperstimulation syndrome: a pilot study.* Hum Reprod, 1995. **10**(1): p. 24-7.

978. Ostanin, A.A., et al., *Role of cytokines in the regulation of reproductive function.* Bull Exp Biol Med, 2007. **143**(1): p. 75-9.

979. Boomsma, C.M., et al., *Ovarian stimulation for in vitro fertilization alters the intrauterine cytokine, chemokine, and growth factor milieu encountered by the embryo.* Fertil Steril, 2010. **94**(5): p. 1764-8.

980. Cerkiene, Z., A. Eidukaite, and A. Usoniene, *Follicular fluid levels of interleukin-10 and interferon-gamma do not predict outcome of assisted reproductive technologies.* Am J Reprod Immunol, 2008. **59**(2): p. 118-26.

981. Kilic, S., et al., *IL-18 and IL-18 binding protein concentrations in serum and ovarian follicular fluid do not affect in vitro fertilization outcomes in obese, overweight, and non-obese patients.* J Reprod Immunol, 2009. **79**(2): p. 211-4.

982. Pelzer, E.S., et al., *Microbial colonization of follicular fluid: alterations in cytokine expression and adverse assisted reproduction technology outcomes.* Hum Reprod, 2011. **26**(7): p. 1799-812.

983. Vujisic, S., et al., *Ovarian follicular concentration of IL-12, IL-15, IL-18 and p40 subunit of IL-12 and IL-23.* Hum Reprod, 2006. **21**(10): p. 2650-5.

984. Yang, Y., et al., *Is interleukin-18 associated with polycystic ovary syndrome?* Reprod Biol Endocrinol, 2011. **9**: p. 7.

985. Sarapik, A., et al., *Follicular proinflammatory cytokines and chemokines as markers of IVF success.* Clin Dev Immunol, 2012. **2012**: p. 606459.

986. Dahm-Kahler, P., et al., *Monocyte chemotactic protein-1 in the follicle of the menstrual and IVF cycle.* Mol Hum Reprod, 2006. **12**(1): p. 1-6.

987. Lamaita, R.M., et al., *Inflammatory response patterns in ICSI patients: a comparative study between chronic anovulating and normally ovulating women.* Reprod Sci, 2012. **19**(7): p. 704-11.

988. Hammadeh, M.E., et al., *Comparison between cytokine concentration in follicular fluid of poor and high responder patients and their influence of ICSI-outcome.* Am J Reprod Immunol, 2003. **50**(2): p. 131-6.

989. Gutman, G., et al., *Interleukin-18 is high in the serum of IVF pregnancies with ovarian hyperstimulation syndrome.* Am J Reprod Immunol, 2004. **51**(5): p. 381-4.

990. Li, H., K. Zhang, and T. Jiang. *The regularized EM algorithm*. in *Proceedings of the 20th National Conference on Artificial Intelligence*. 2005.

991. Orsi, N.M., et al., *Uterine and serum cytokine arrays in the mouse during estrus.* Anim Reprod Sci, 2007. **100**(3-4): p. 301-10.

992. Orsi, N.M., et al., *Regulation of maternal serum and amniotic fluid cytokine profiles in the mouse: possible roles in the onset of labour.* J Reprod Immunol, 2007. **75**(2): p. 97-105.

993. Hu, Y., *Efficient and high quality force-directed graph drawing.* The Mathematica Journal, 2005. **10**(1): p. 37-71.

994. Subelj, L. and M. Bajec, *Unfolding communities in large complex networks: combining defensive and offensive label propagation for core extraction.* Phys Rev E Stat Nonlin Soft Matter Phys, 2011. **83**(3 Pt 2): p. 036103.

995. Blondel, V.D., et al., *Fast unfolding of communities in large networks* J. Stat. Mech., 2008: p. P10008.

996. Son, W.Y., et al., *Fertilization, embryo development, and clinical outcome of immature oocytes obtained from natural cycle in vitro fertilization.* J Assist Reprod Genet, 2013. **30**(1): p. 43-7.

997. Cha, K.Y. and R.C. Chian, *Maturation in vitro of immature human oocytes for clinical use.* Hum Reprod Update, 1998. **4**(2): p. 103-20.

998. Wong, K.H., H. Negishi, and E.Y. Adashi, *Expression, hormonal regulation, and cyclic variation of chemokines in the rat ovary: key determinants of the intraovarian residence of representatives of the white blood cell series.* Endocrinology, 2002. **143**(3): p. 784-91.

999. Suffee, N., et al., *RANTES/CCL5-induced pro-angiogenic effects depend on CCR1, CCR5 and glycosaminoglycans.* Angiogenesis, 2012. **15**(4): p. 727-44.

1000. Eppig, J.J., et al., *Relationship between the developmental programs controlling nuclear and cytoplasmic maturation of mouse oocytes.* Dev Biol, 1994. **164**(1): p. 1-9.

1001. Coticchio, G., et al., *Human oocyte maturation in vitro.* Int J Dev Biol, 2012. **56**(10-12): p. 909-18.

1002. Hardy, K., et al., *In vitro maturation of oocytes.* Br Med Bull, 2000. **56**(3): p. 588-602.

1003. Grondahl, C., *Oocyte maturation. Basic and clinical aspects of in vitro maturation (IVM) with special emphasis of the role of FF-MAS.* Dan Med Bull, 2008. **55**(1): p. 1-16.

1004. Piquette, G.N., *The in vitro maturation (IVM) of human oocytes for in vitro fertilization (IVF): is it time yet to switch to IVM-IVF?* Fertil Steril, 2006. **85**(4): p. 833-5; discussion 841.

1005. Gallinelli, A., et al., *Correlations between concentrations of interleukin-12 and interleukin-13 and lymphocyte subsets in the follicular fluid of women with and without polycystic ovary syndrome.* Fertil Steril, 2003. **79**(6): p. 1365-72.

1006. Amato, G., et al., *Serum and follicular fluid cytokines in polycystic ovary syndrome during stimulated cycles.* Obstet Gynecol, 2003. **101**(6): p. 1177-82.

1007. Knebel, B., et al., *Increased low grade inflammatory serum markers in patients with Polycystic ovary syndrome (PCOS) and their relationship to PPARgamma gene variants.* Exp Clin Endocrinol Diabetes, 2008. **116**(8): p. 481-6.

1008. Goswami, R. and M.H. Kaplan, *A brief history of IL-9.* J Immunol, 2011. **186**(6): p. 3283-8.

1009. Inoue, N., et al., *Roles of tumor necrosis factor-related apoptosis-inducing ligand signaling pathway in granulosa cell apoptosis during atresia in pig ovaries.* J Reprod Dev, 2003. **49**(4): p. 313-21.

1010. Song, J.J. and Y.J. Lee, *Differential cleavage of Mst1 by caspase-7/-3 is responsible for TRAIL-induced activation of the MAPK superfamily.* Cell Signal, 2008. **20**(5): p. 892-906.

1011. Kichev, A., et al., *Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) signaling and cell death in the immature central nervous system after hypoxia-ischemia and inflammation.* J Biol Chem, 2014. **289**(13): p. 9430-9.

1012. Falschlehner, C., et al., *TRAIL signalling: decisions between life and death.* Int J Biochem Cell Biol, 2007. **39**(7-8): p. 1462-75.

1013. Lane, D., et al., *Acquired resistance to TRAIL-induced apoptosis in human ovarian cancer cells is conferred by increased turnover of mature caspase-3.* Mol Cancer Ther, 2006. **5**(3): p. 509-21.

1014. Kyronlahti, A., et al., *GATA4 protects granulosa cell tumors from TRAIL-induced apoptosis.* Endocr Relat Cancer, 2010. **17**(3): p. 709-17.

1015. Rizzuto, I., R.F. Behrens, and L.A. Smith, *Risk of ovarian cancer in women treated with ovarian stimulating drugs for infertility.* Cochrane Database Syst Rev, 2013. **8**: p. CD008215.

1016. Aldrich, J., *Correlations Genuine and Spurious in Pearson and Yule.* Statistical Science, 1995. **10**(4): p. 364-376.

1017. Spirtes, P., *Introduction to Causal Inference.* Journal of Machine Learning Research, 2010. **11**: p. 1643-1662.
1018. Manly, B.F.J., *Multivariate Statistical Methods: A Primer*. 2005, Boca Raton, Florida: Chapman and Hall.
1019. Lee, Y.H., et al., *Reducing system noise in copy number data using principal components of self-self hybridizations.* Proc Natl Acad Sci U S A, 2012. **109**(3): p. E103-10.
1020. Chen, R.H., et al., *Interleukin-6 inhibits transforming growth factor-beta-induced apoptosis through the phosphatidylinositol 3-kinase/Akt and signal transducers and activators of transcription 3 pathways.* J Biol Chem, 1999. **274**(33): p. 23013-9.
1021. Yates-Binder, C.C., et al., *An IP-10 (CXCL10)-derived peptide inhibits angiogenesis.* PLoS One, 2012. **7**(7): p. e40812.
1022. Bodnar, R.J., et al., *IP-10 induces dissociation of newly formed blood vessels.* J Cell Sci, 2009. **122**(Pt 12): p. 2064-77.
1023. Sato, E., et al., *Expression of IP-10 related to angiogenesis in uterine cervical cancers.* Br J Cancer, 2007. **96**(11): p. 1735-9.
1024. Wada, S., et al., *Macrophage migration inhibitory factor in the human ovary: presence in the follicular fluids and production by granulosa cells.* Biochem Mol Biol Int, 1997. **41**(4): p. 805-14.
1025. Wada, S., et al., *Induction of macrophage migration inhibitory factor in human ovary by human chorionic gonadotrophin.* Hum Reprod, 1999. **14**(2): p. 395-9.
1026. Sgadari, C., A.L. Angiolillo, and G. Tosato, *Inhibition of angiogenesis by interleukin-12 is mediated by the interferon-inducible protein 10.* Blood, 1996. **87**(9): p. 3877-82.
1027. Goldring, M.B. and S.R. Goldring, *Cytokines and cell growth control.* Crit Rev Eukaryot Gene Expr, 1991. **1**(4): p. 301-26.
1028. Eppig, J.J., et al., *Oocyte control of granulosa cell development: how and why.* Hum Reprod, 1997. **12**(11 Suppl): p. 127-32.
1029. Eppig, J.J., M. O'Brien, and K. Wigglesworth, *Mammalian oocyte growth and development in vitro.* Mol Reprod Dev, 1996. **44**(2): p. 260-73.
1030. Eppig, J.J., K. Wigglesworth, and F.L. Pendola, *The mammalian oocyte orchestrates the rate of ovarian follicular development.* Proc Natl Acad Sci U S A, 2002. **99**(5): p. 2890-4.

7. Appendices

7.1 Appendix I: Non-significant pathways identified by pathway analysis.

Pathway	Adjusted p-value	Matched Entities	Total Entities	Pathway
Fatty Acid Omega Oxidation WP33 59026	0.05	7		7
Nucleotide GPCRs WP207 41362	0.06	10		11
SIDS Susceptibility Pathways WP2086 52939	0.07	31		40
PodNet protein-protein interactions in the podocyte WP2310 58140	0.07	218		315
PodNet protein-protein interactions in the podocyte WP2310 58138	0.07	218		315
Focal Adhesion WP85 59067	0.07	130		191
SHH, FGF8, Stat3 WP1500 42211	0.08	6		6
ErbB signaling pathway WP1261 41378	0.08	35		46
Chemokine signaling pathway WP2292 53116	0.08	129		193
Chemokine signaling pathway WP2292 51127	0.08	129		193
Leptin and adiponectin WP683 45174	0.09	9		10
Glucuronidation WP1241 41277	0.11	14		18
Hypothetical Network for Drug Addiction WP1246 48795	0.11	24		32
Ovarian Infertility Genes WP273 41282	0.11	24		31
Synthesis and Degradation of Ketone Bodies	0.12	5		5
Wnt Signaling Pathway WP403 41302	0.12	44		60
Electron Transport Chain WP295 62836	0.12	72		102
Osteoblast WP238 41255	0.13	8		10
G Protein Signaling Pathways WP232 41275	0.13	65		91
Tryptophan metabolism WP79 54741	0.14	32		44
Tryptophan metabolism WP79 47759	0.14	32		44
Type II interferon signaling (IFNG) WP1253 48389	0.14	25		34
PPAR signaling pathway WP2316 52058	0.14	58		87
PPAR signaling pathway WP2316 53110	0.14	58		87
One carbon metabolism and related pathways WP1770 48487	0.15	36		49
Eicosanoid Synthesis WP318 62829	0.16	15		19
Statin Pathway WP1 53530	0.16	15		20
Statin Pathway WP1 41296	0.16	15		20
Regulation of Actin Cytoskeleton WP523 47746	0.16	105		151
Id Signaling Pathway WP512 41335	0.18	37		52
Estrogen metabolism WP1264 59015	0.18	4		13
Polyol pathway WP1265 41264	0.18	4		4
Glutathione metabolism WP164 48254	0.20	14		19
Wnt Signaling Pathway and Pluripotency WP723 41353	0.21	67		97

Pathway	Adjusted p-value	Matched Entities	Total Pathway Entities
Wnt Signaling Pathway and Pluripotency WP723 59063	0.21	67	97
Notch Signaling Pathway WP29 41252	0.23	33	47
Biogenic Amine Synthesis WP522 41322	0.23	11	15
Urea cycle and metabolism of amino groups WP426 45004	0.26	15	20
Hypertrophy Model WP202 41285	0.26	15	20
Triacylglyceride Synthesis WP386 62840	0.27	17	23
Ptf1a related regulatory pathway WP201 41299	0.27	8	14
Nicotine Activity on Dopaminergic Neurons WP2083 58000	0.27	8	10
S1P pathways and spinal cord injury WP2170 59059	0.28	3	6
miR-1 in cardiac development WP608 41375	0.28	3	5
Arachidonate Epoxygenase Epoxide Hydrolase WP1250 58253	0.28	3	3
Steroid Biosynthesis WP55 62839	0.29	10	13
IL-7 Signaling Pathway WP297 41313	0.30	31	44
Heart Development WP2067 45788	0.30	31	47
Nucleotide Metabolism WP87 41363	0.31	14	19
Prostaglandin pathway WP1439 41354	0.33	5	6
Connective tissue growth factor pathway WP2442 57984	0.33	5	6
Glutathione and one carbon metabolism WP730 41399	0.33	22	31
neural retinal development WP535 43786	0.35	7	9
Alanine and aspartate metabolism WP240 62062	0.36	9	40
Leptin Insulin Overlap WP578 62835	0.36	11	18
Senescence and Autophagy WP1267 48388	0.38	66	98
One Carbon Metabolism WP435 42576	0.42	20	29
Cytochrome P450 WP1274 59077	0.43	2	40
Alzheimers Disease WP2075 59061	0.43	2	77
Phase I, non P450 WP1255 59040	0.43	2	8
Amino acid conjugation of benzoic acid WP1252 41304	0.43	2	2
miRs in Muscle Cell Differentiation WP2076 59033	0.43	2	21
Oxidative Damage WP1496 43789	0.43	12	42
Osteoclast WP454 48251	0.43	10	14
Keap1-Nrf2 WP1245 48229	0.43	10	14
Aflatoxin B1 metabolism WP1262 48257	0.44	4	5
Phase I, non P450 WP1255 41309	0.44	6	8
TLR WP2440 58153	0.44	6	8
Alpha6-Beta4 Integrin Signaling Pathway WP488 41271	0.54	44	67
Wnt Signaling Pathway NetPath WP539 41242	0.56	71	109
Oxidative phosphorylation WP1248 41278	0.62	38	59
Amino acid conjugation of benzoic acid WP1252 59036	0.65	1	2
Aflatoxin B1 metabolism WP1262 59068	0.65	1	5

Pathway	Adjusted p-value	Matched Entities	Total Pathway Entities
Polyol pathway WP1265 59024	0.65	1	4
Cardiac Hypertrophy- miR-208 WP1526 59045	0.65	4	8
Nuclear Receptors WP509 41291	0.68	24	38
Circadian Exercise WP544 41312	0.68	31	49
Splicing factor NOVA regulated synaptic proteins WP1983 59012	0.71	20	42
Amino Acid metabolism WP662 42241	0.72	60	95
G1 to S cell cycle control WP413 41269	0.74	38	62
Purine metabolism WP2185 58151	0.74	107	178
Purine metabolism WP2185 47829	0.74	107	178
miRNAs involved in DNA damage response WP2085 59079	0.76	6	49
Histone modifications WP300 41360	0.77	3	5
Cell cycle WP190 62828	0.80	53	88
Signal Transduction of S1P Receptor WP57 41348	0.80	13	22
Acetylcholine Synthesis WP175 42542	0.81	4	7
Delta-Notch Signaling Pathway WP265 41380	0.81	51	84
IL-4 signaling Pathway WP93 41293	0.82	37	61
IL-1 Signaling Pathway WP37 41331	0.82	22	37
IL-9 Signaling Pathway WP10 43998	0.83	14	24
Splicing factor NOVA regulated synpatic proteins WP1983 42575	0.83	25	42
Integrin-mediated cell adhesion WP6 62831	0.83	60	100
Integrin-mediated cell adhesion WP6 41290	0.83	60	100
Methylation WP1247 58512	0.83	5	9
Mismatch repair WP1257 41257	0.83	5	9
T Cell Receptor Signaling Pathway WP480 41339	0.84	82	133
DNA Replication WP150 41336	0.86	24	41
Apoptosis WP1254 47965	0.86	50	83
TFs Regulate miRNAs related to cardiac hypertrophy WP2080 59071	0.88	2	11
Diurnally regulated genes with circadian orthologs WP1268 41369	0.88	28	48
Serotonin Receptor 2 and STAT3 Signaling WP2079 45981	0.88	1	2
Serotonin and anxiety-related events WP2140 59058	0.88	1	13
EBV LMP1 signaling WP1243 59031	0.88	1	22
Insulin Signaling WP65 57331	0.88	96	159
Toll-like receptor signaling pathway WP1271 49294	0.89	57	97
Proteasome Degradation WP519 62832	0.89	34	59
Cholesterol Biosynthesis WP103 41337	0.89	8	15
Fatty Acid Biosynthesis WP336 41307	0.90	12	22
Selenium metabolism-Selenoproteins WP108 49742	0.90	27	48
Translation Factors WP307 41351	0.91	28	51
Selenium metabolism-Selenoproteins WP108 41386	0.92	26	48

Pathway	Adjusted p-value	Matched Entities	Total Pathway Entities
EPO Receptor Signaling WP1249 41283	0.92	14	26
IL-2 Signaling Pathway WP450 41330	0.93	44	76
IL-5 Signaling Pathway WP151 59065	0.93	39	69
IL-6 signaling Pathway WP387 41281	0.94	58	99
Apoptosis Modulation by HSP70 WP166 41376	0.94	9	18
PluriNetWork WP1763 41345	0.95	177	291
Sterol regulatory element binding protein related WP731 41316	0.95	3	7
Pentose Phosphate Pathway WP63 45643	0.95	3	7
miRNAs and TFs in iPS Cell Generation WP2375 58155	0.95	3	11
Heme Biosynthesis WP18 41372	0.95	4	9
MAPK Cascade WP251 62830	0.96	15	29
Neural Crest Differentiation WP2074 59025	0.96	1	97
B Cell Receptor Signaling Pathway WP274 41374	0.96	92	156
TCA Cycle WP434 57464	0.96	16	31
G13 Signaling Pathway WP298 41392	0.96	20	38
FAS pathway and Stress induction of HSP regulation WP571 47743	0.96	20	38
IL-3 Signaling Pathway WP373 41387	0.97	57	100
Kit Receptor Signaling Pathway WP407 41265	0.97	37	67
Mitochondrial Gene Expression WP1263 47754	0.97	9	19
Mitochondrial Gene Expression WP1263 49535	0.97	9	19
Androgen Receptor Signaling Pathway WP252 47768	0.98	63	112
p38 MAPK Signaling Pathway (BioCarta) WP350 41398	0.98	17	34
EGFR1 Signaling Pathway WP572 41396	0.98	102	176
MAPK signaling pathway WP493 47770	0.99	91	159
Homologous recombination WP1258 41350	0.99	5	13
Fatty acid oxidation WP2318 58160	0.99	4	11
TGF-beta Receptor Signaling Pathway WP258 41259	0.99	85	150
NLR proteins WP1256 41241	0.99	3	9
Signaling of Hepatocyte Growth Factor Receptor WP193 41367	0.99	16	34
Fatty Acid Beta Oxidation WP1269 41314	0.99	16	34
miRNA regulation of DNA Damage Response WP2087 59076	0.99	2	91
MicroRNAs in cardiomyocyte hypertrophy WP1560 41377	0.99	44	104
Toll Like Receptor signaling WP88 41390	0.99	15	33
Mitochondrial LC-Fatty Acid Beta-Oxidation WP401 47761	0.99	6	16
Kennedy pathway WP1771 45176	0.99	5	14
EBV LMP1 signaling WP1243 41295	0.99	9	22
Glycogen Metabolism WP317 43575	1.00	15	34
TNF-alpha NF-kB Signaling Pathway WP246 41391	1.00	98	184
Eukaryotic Transcription Initiation WP567 41326	1.00	13	41

Pathway	Adjusted p-value	Matched Entities	Total Entities	Pathway
Estrogen signalling WP1244 59014	1.00	26	74	
Estrogen signalling WP1244 48233	1.00	27	74	
mRNA processing WP310 62837	1.00	230	551	

7.2 Appendix II: Endometrial epithelial cell response to seminal plasma.

7.2.1.1 Prostaglandin synthesis and regulation pathway

The prostaglandin synthesis and regulation pathway was activated in uterine epithelial cells (Figure 7-1). *Pgh2* synthesis via *Pla2g4a* and *Ptgs1* appeared to increase, with expression of these entities increasing post mating, followed by a subsequent decrease at day 3 and 4. This pattern was also reflected in the PG synthase *Ptgds*. *Prl* increased in expression above controls, most notably at day 1 and day 3 post mating.

7.2.1.2 Cytokine and inflammatory response pathway

Members of the cytokine and inflammatory response pathway fluctuated post mating (Figure 7-2). *Il1a* demonstrated a clear increase in expression post mating, most notably between days 1 and 2, a relationship also reflected in *Il6* and *Il13* expression levels. *Il10* and *Il12a* also increased post mating, with day 1 and day 3 exhibiting the highest levels. *Csf1* peaked at dioestrus, day 1 and day 4 post mating, *Cxcl3* peaked at days 1 and 3 and *Cd4* levels peaked at dioestrus and day 4. *Il13* remained high at days 1,2 and 3 with a subsequent drop at day 4. *Il1b* and *H2Eb1* peaked at day 2 post mating with all other conditions remaining low. *Tgfb1* decreased post mating, day 4 presenting the lowest levels. *IL-5* dropped from an initially high level at dioestrus to a low level of expression at oestrus and a subsequent recovery post mating. Expression of *Tnfa* peaked at day 1 then dropped at day 2 post mating. The lowest levels of *Ifng* expression were evident at oestrus. *Csf2* was lower at oestrus compared with dioestrus, and remained static throughout the post mating time points.

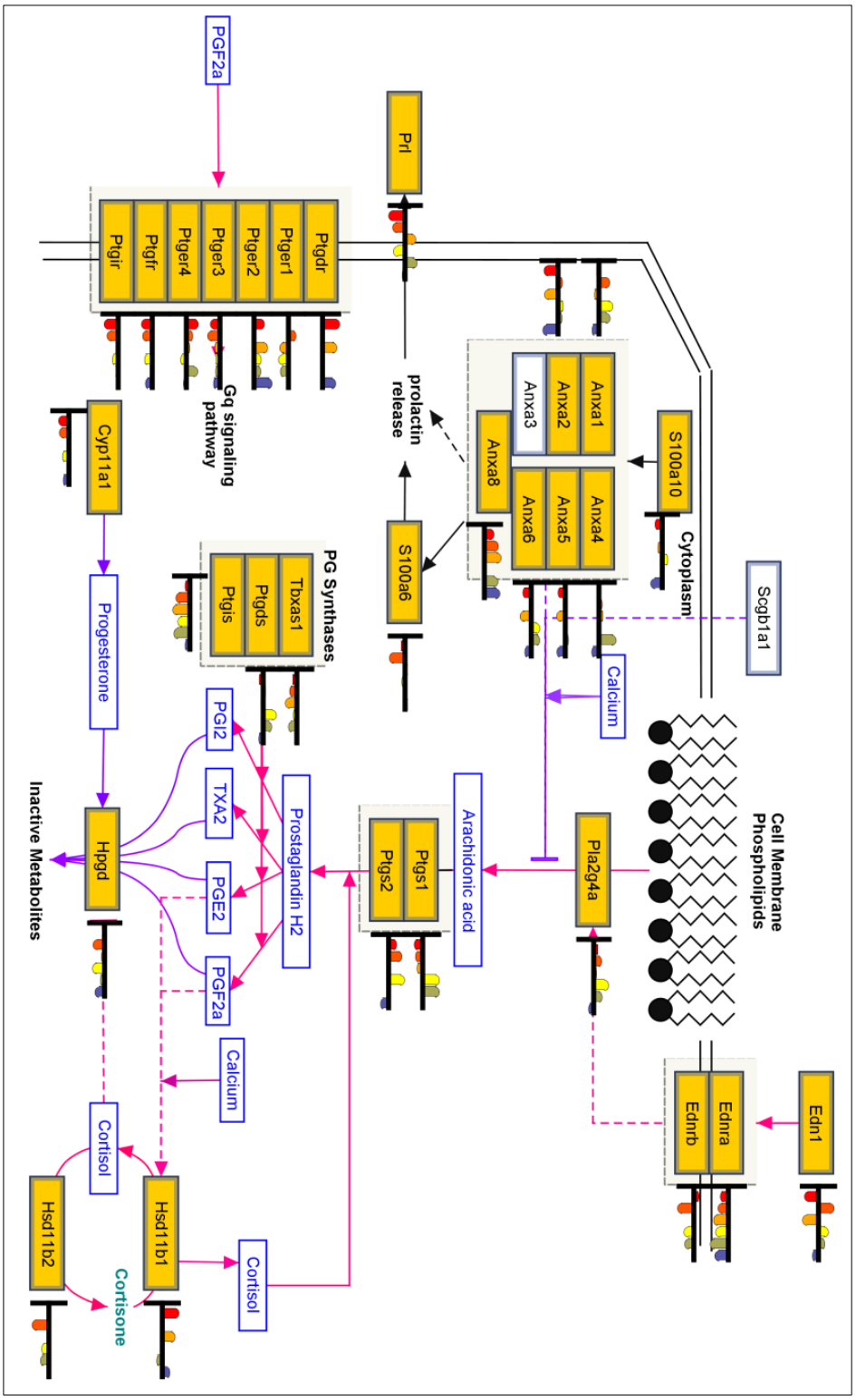


Figure 7-1: Elements of the prostaglandin synthesis and regulation pathway active in uterine epithelial cells.

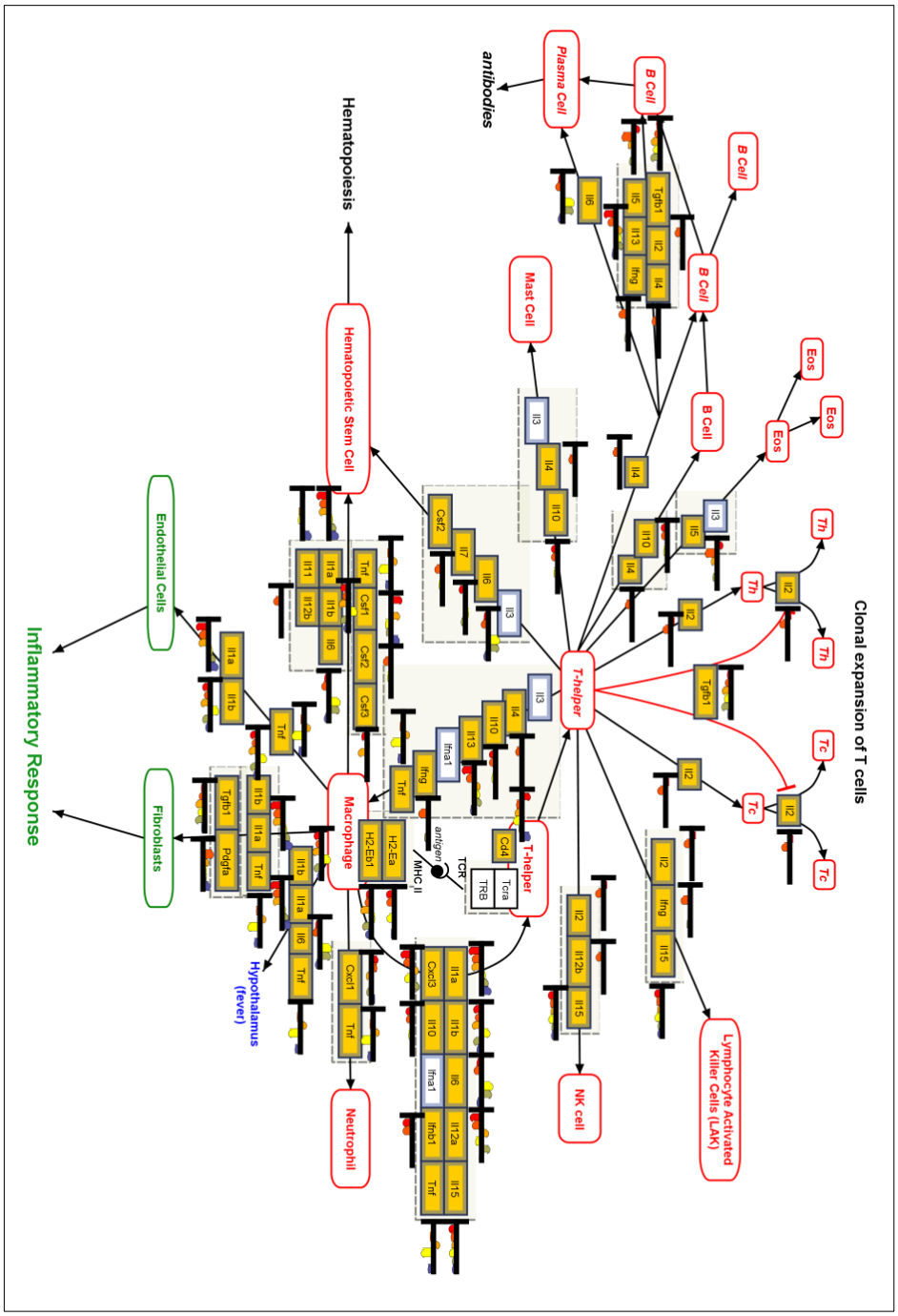


Figure 7-2: Elements of the cytokine and inflammatory response pathway activated in uterine epithelial cells.

7.2.1.3 MMPs

Uterine epithelial MMPs exhibited varied responses to mating (Figure 7-3). Several MMPs exhibited a peak in expression at oestrus (*Mmp8*, *Mmp11*, *Mmp14*, *Mmp23* and the inhibitor *Timp4*). *Mmp7* showed an initially high expression at dioestrus, oestrus and day 1 post mating with a subsequent fall at days 2, 3 and 4. A number of MMPs displayed a drop in expression between dioestrus and oestrus with an ensuing recovery post mating (*Mmp2*, *Mmp10*, *Mmp12*, *Mmp16*, *Mmp19*, *Mmp21*, *Mmp25* and *Mmp27*). The MMP inhibitors *Timp2* and *Timp3* remained broadly low in expression throughout.

7.2.1.4 Macrophage markers

The macrophage markers *Cd14* and *Cd68* exhibited a peak in expression at oestrus with a subsequent drop post mating (Figure 7-4). *F3* was highly expressed at dioestrus and day 1 post mating while *Cd163* showed the lowest expression at oestrus. *Lyz2* remained low throughout.

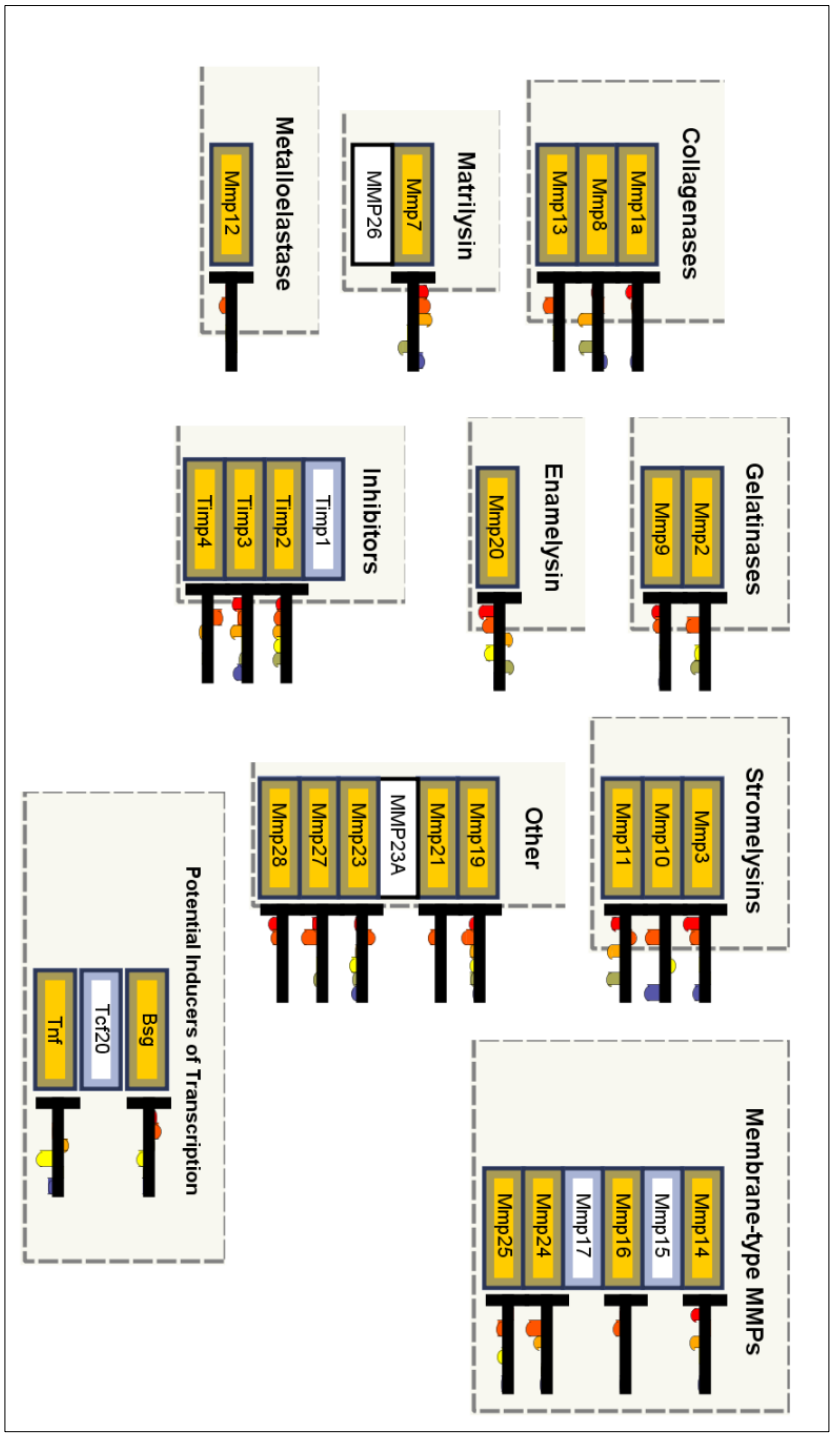


Figure 7-3: MMP response to mating

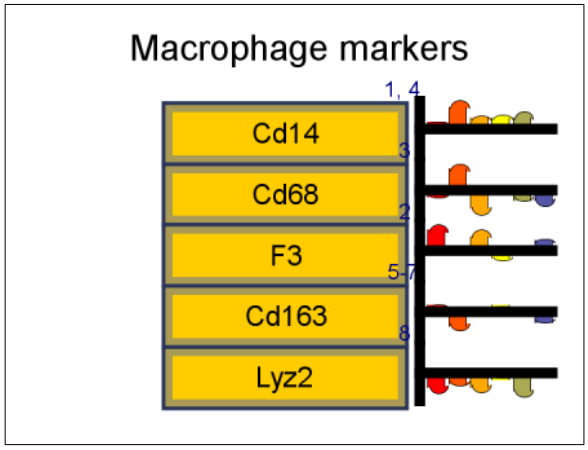


Figure 7-4: Macrophage markers in uterine epithelial cells post mating

7.2.1.5 Inflammatory response pathway

Several elements of the inflammatory response pathway demonstrate their lowest expression at dioestrus (*Cd80*, *Ifng*, *Il2*, *Il2ra*, *Il2rb*, *Il4*, *Il5*, *Il5ra* and *Zap70*, Figure 7-5) while others peaked at dioestrus (*Cd40*, *Cd40lg*, *Il4ra*, *Lama5*, *Lamc2*, *Thbs3* and *Tnfrsf1b*). Fibrotic response factors *Col1a1* and *2* and *Thbs3* initially showed increasing expression post mating followed by a reduction at day 4. *Lamb1-1* reduced post mating which was most evident at day 2.

7.2.1.6 TGF-β signalling pathway

TGF-β receptors *Tgfbr1* and *2* initially showed low expression at dioestrus followed by an increase in expression at oestrus (Figure 7-6). *Tgfbr3* showed high expression at dioestrus with a subsequent reduction at oestrus. Expression of all receptors remained low following mating, with *Tgfbr1* increasing in expression at day 4. *Tgfb1* reduced in expression following mating with a subsequent increase at day 4, while inhibin beta A (*Ihba*) exhibited its lowest expression at day 4. *Lif* showed a dramatic reduction in expression at day 1 post mating, with a recovery towards day 4, and the repressor *Spp1* and co-factor *Foxh1* mirrored this trend at day 2. *Wnt1* and *Egf* showed low expression in cycling mice with a peak at day 1 post mating.

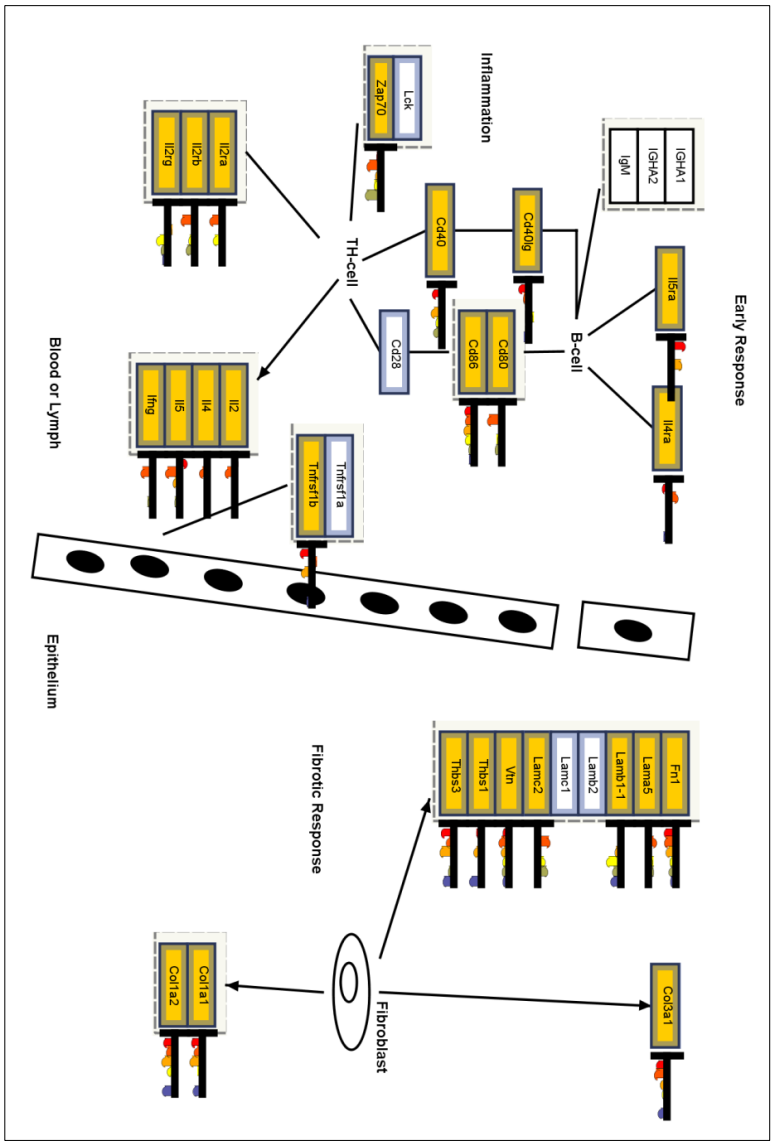


Figure 7-5: Elements of the inflammatory response pathway activated in uterine epithelial cells post mating

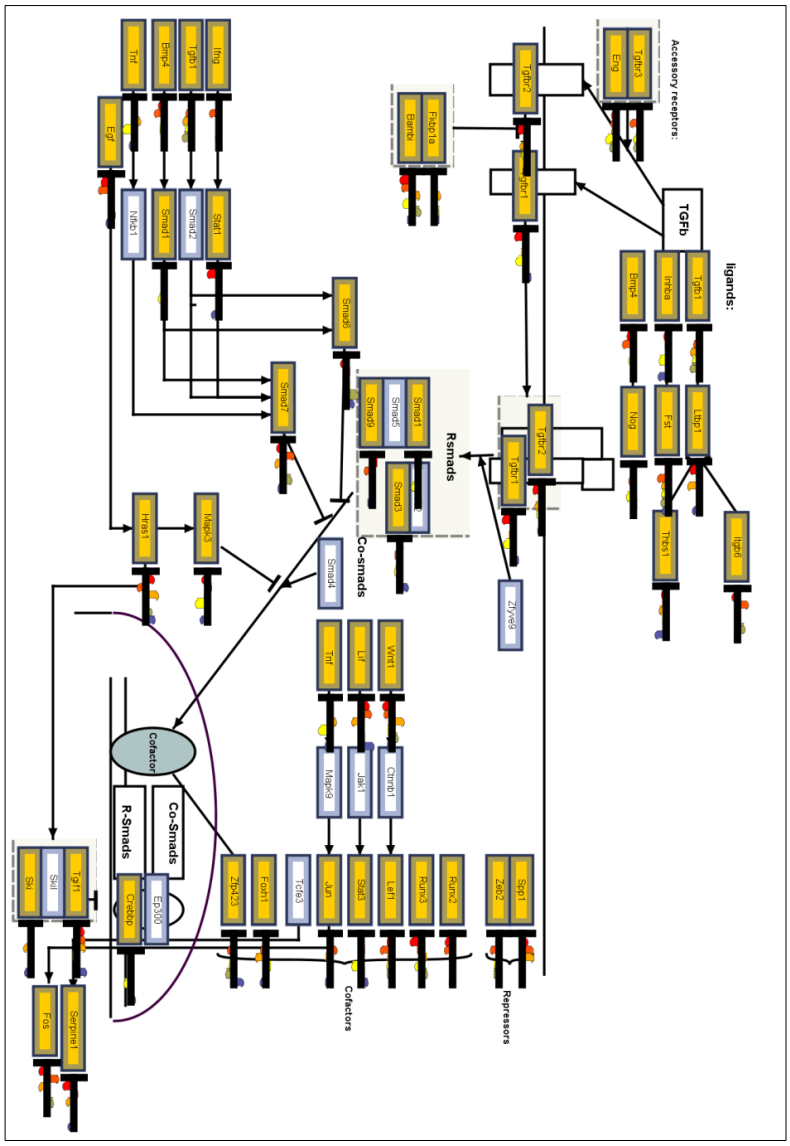


Figure 7-6: TGF-β pathway elements activated in uterine epithelial cells post mating

7.3 Appendix III: Pathway analysis for LCM captured endometrial stromal cells.

7.3.1.1 Prostaglandin synthesis and regulation pathway

Several members of the prostaglandin synthesis and regulation pathway (Figure 7-7-) showed a higher expression at dioestrus compared to oestrus (*Ednra, Ednrb, Hpdg, Hsd11b1, Hsd11b2, Prl, Ptgder, Ptger1, Ptger3, Ptgir, Ptgis* and *S100a6*) while others showed the reverse (*Anxa1, Anxa2, Anxa4, Anxa5, Anxa6, Anxa8, Edn1, Pla2g4a, Ptger2, Ptger4* and *Ptgs2*). *Cyp11a1, Ednra, Hsd11b1, Hpgd Ptgir* and *Tbxas1* exhibited a peak in expression post mating at day 1, while *S100a10* showed the lowest expression at this time point. Elements which peaked at day 2 included *Anxa2, Anxa6, Hsd11b2, Ptger1* and *S100a10*. *Anxa1, Anxa4, Cyp11a1, Pla2g4a, Ptgdr, Ptgs1, Ptgs2,* and *Prl* experienced a reduction in expression post mating with day 4 being the lowest time point.

7.3.1.2 Cytokines and inflammatory response pathway

The vast majority of elements in the cytokine and inflammatory response pathway were more highly expressed at dioestrus compared with oestrus (Figure 7-8), with the notable exception of *Pgdfa* which exhibited the reverse. Many cytokines peaked at day 1 post mating with a subsequent decrease in expression over time to day 4 (*Csf2, Cxcl3, Il2, Il6, Il10, Il11, Il12a, Il12b, Il13, Pgdfa* and *Tnf*) with others exhibiting a peak at day 2 (*H2-eb1, Ifng, Il1b, IL15* and *Pdgfa*) or day 3 (*Cd4*, *H2-ea* and *Il5*). The vast majority of elements showed a marked reduction in expression at day 4 with the exceptions of *Ifng*, *Il15* and *Tgfb*.

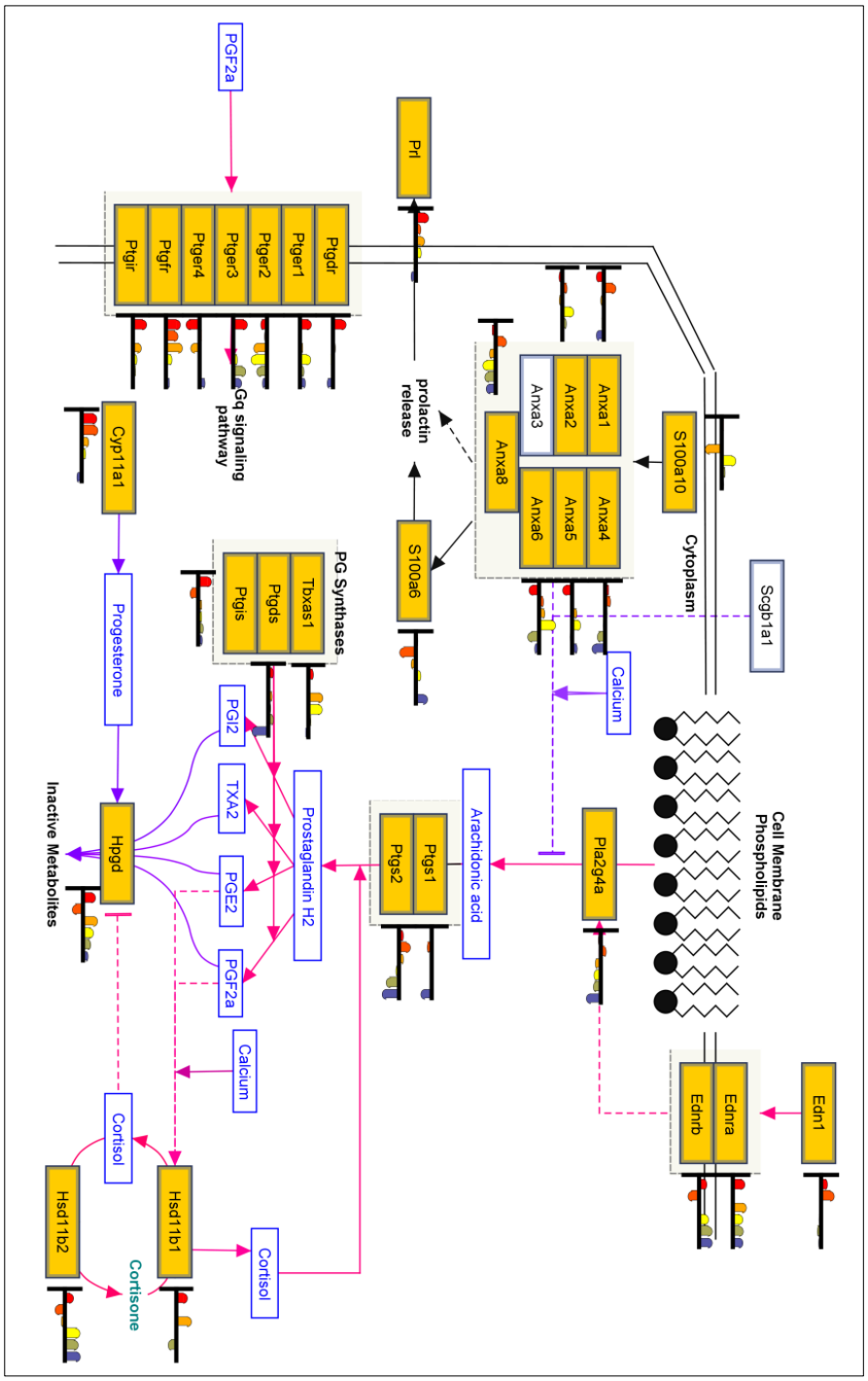


Figure 7-7: Prostaglandin synthesis and regulation pathway elements active in uterine stromal cells

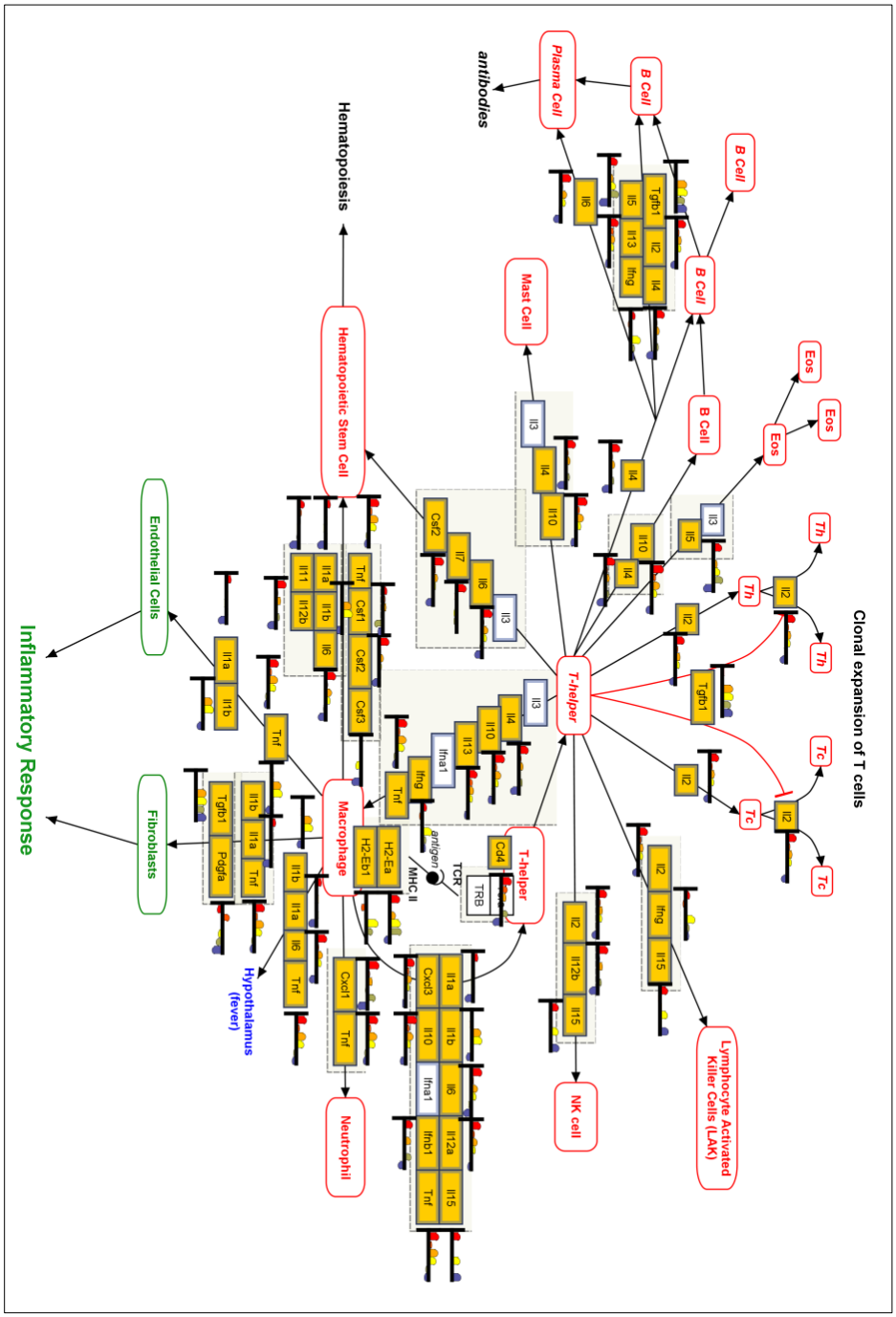


Figure 7-8: The cytokines and inflammatory response pathway in uterine stromal cells

7.3.1.3 MMPs

The stromal MMP response to seminal plasma is shown in Figure 7-9. The majority of MMPs were expressed more highly at dioestrous compared to oestrus (*Mmp1a, Mmp2, Mmp3, Mmp8, Mmp9, Mmp10, Mmp12, Mmp13, Mmp16, Mmp20, Mmp21, Mmp24, Mmp25, Mmp27 and Mmp28*). Post mating, *Mmp1a, Mmp3, Mmp10* and *Mmp14* peaked at day 1, while *Mmp2, Mmp9, Mmp19* and *Mmp25* peaked at day 2. With the exception of *Mmp2, Mmp7* and *Mmp19*, day 4 showed the lowest expression of MMPs.

7.3.1.4 Macrophage markers

Cd14 and *Cd68* were both more highly expressed at oestrus compared to dioestrus, while *F3* and *Cd163* showed the reverse (Figure 7-10). *Cd14* and *Cd163* showed progressively lower expression post mating following a small peak at day1, with day 4 showing the lowest. *Cd68* and *Lyz2* peaked at day 2, with a subsequent reduction in expression, while *F3* was lowest at day 3.

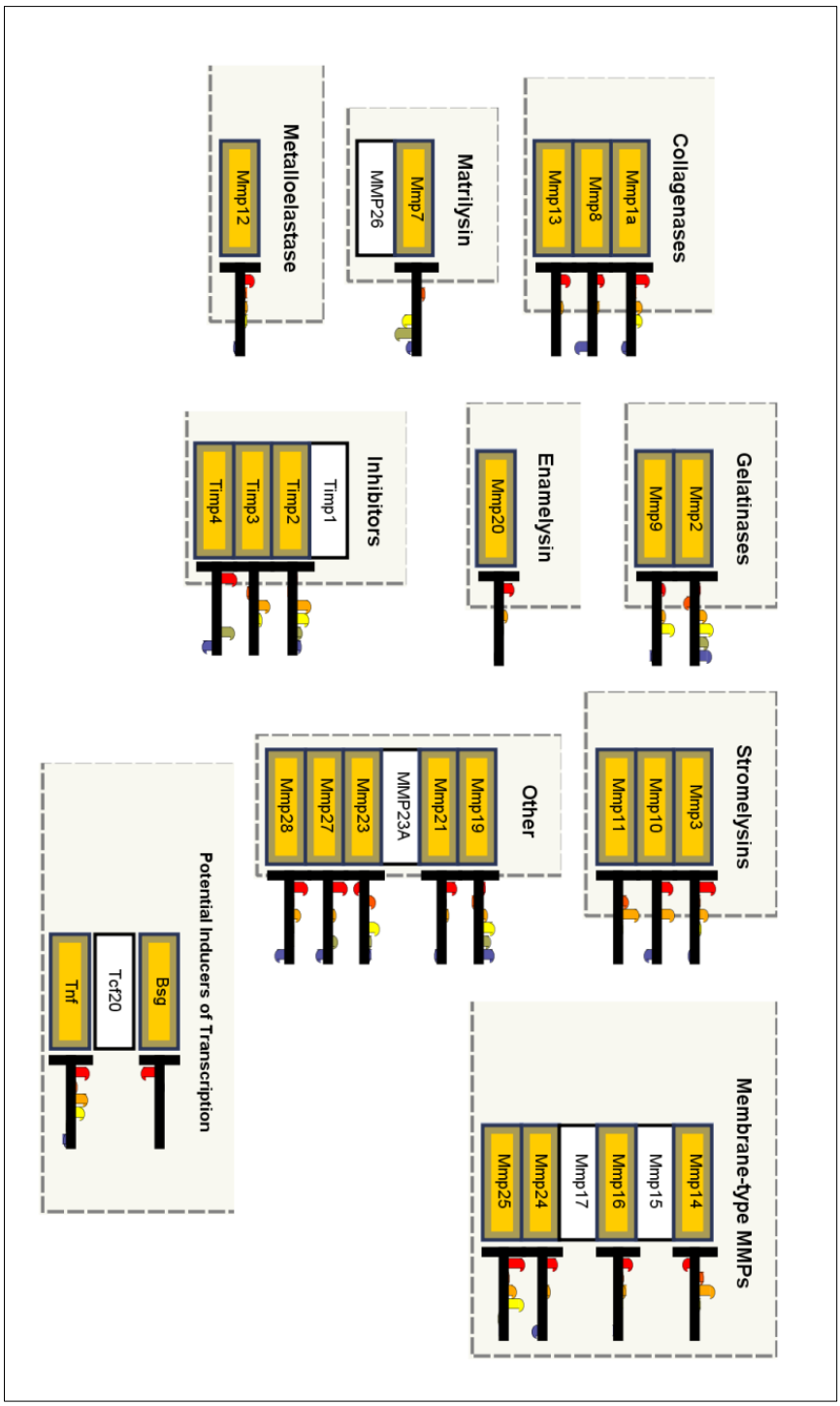


Figure 7-9: Uterine stromal cell MMP response to seminal plasma

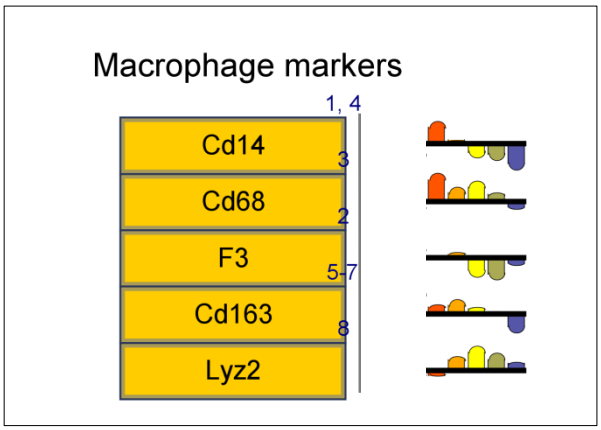


Figure 7-10: Macrophage markers in uterine stromal cells post mating

7.3.1.5 Inflammatory response pathway

With the exception of *Il4ra*, *Lama5* and *Lamc2*, all elements of the inflammatory response pathway were more highly expressed at dioestrus compared to oestrus (Figure 7-11). Post mating, *Cd40, Cd40lg*, *Cd86* and *Thbs1* peaked at day 1, while *Col1a1*, *Col1a2*, *Col3a1*, *Ifng* and *Vtn* peaked at day 2. *Il2ra* was initially high at days 1 and 2 with a subsequent drop in expression at days 3 and 4. *Lama5* and *Lamc2* were progressively downregulated post mating. *Il2b* and *Zap70* peaked at day 4, with *Cd40lg*, *Il2*, *Il4*, *Il5*, *Il5ra* and *Tnfrsf1b* showing their lowest expression at this time point.

7.3.1.6 TGF-β signalling pathway

The complex TGF-β signalling pathway exhibited differential responses to the mating stimulus (Figure 7-12). *Tgfb1* was very low in expression in controls, with a dramatic increase post mating, sustained through days 1-4. Of the TGF-β receptors, *Tgfbr1* and *Tgfbr3* were more highly expressed at dioestrus compared to oestrus.*Tgfbr3* maintained a steady level of expression post mating, while *Tgfbr1* and *Tgfbr2* were initially high at day 1 with a subsequent reduction in expression. *Inhba* and *Thbs1* peaked at day 1 with a subsequent fall in expression, while *Fst* and *Nog* peaked at day 2. Of the signal transducing SMADs, *Smad1* peaked at dioestrus and day 4, *Smad 9* was highly expressed at dioestrus with its lowest expression at day 4, and *Smad3* was low at dioestrus with a peak at day 4. *Smad 7* exhibited a dramatic fall in expression at day 2 post mating with a subsequent recovery at days 3 and 4.

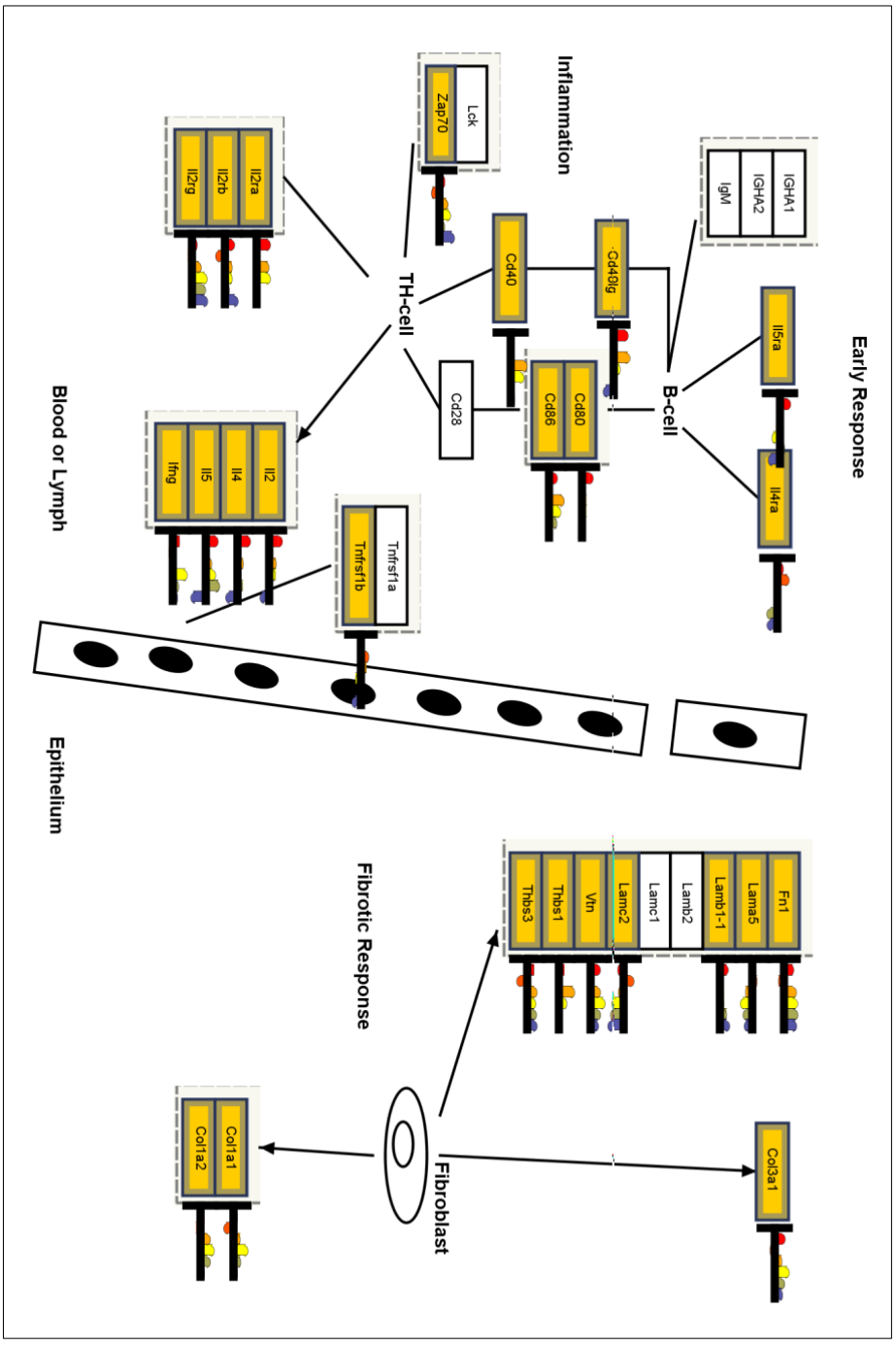


Figure 7-11: Inflammatory response pathway activation in uterine stromal cells

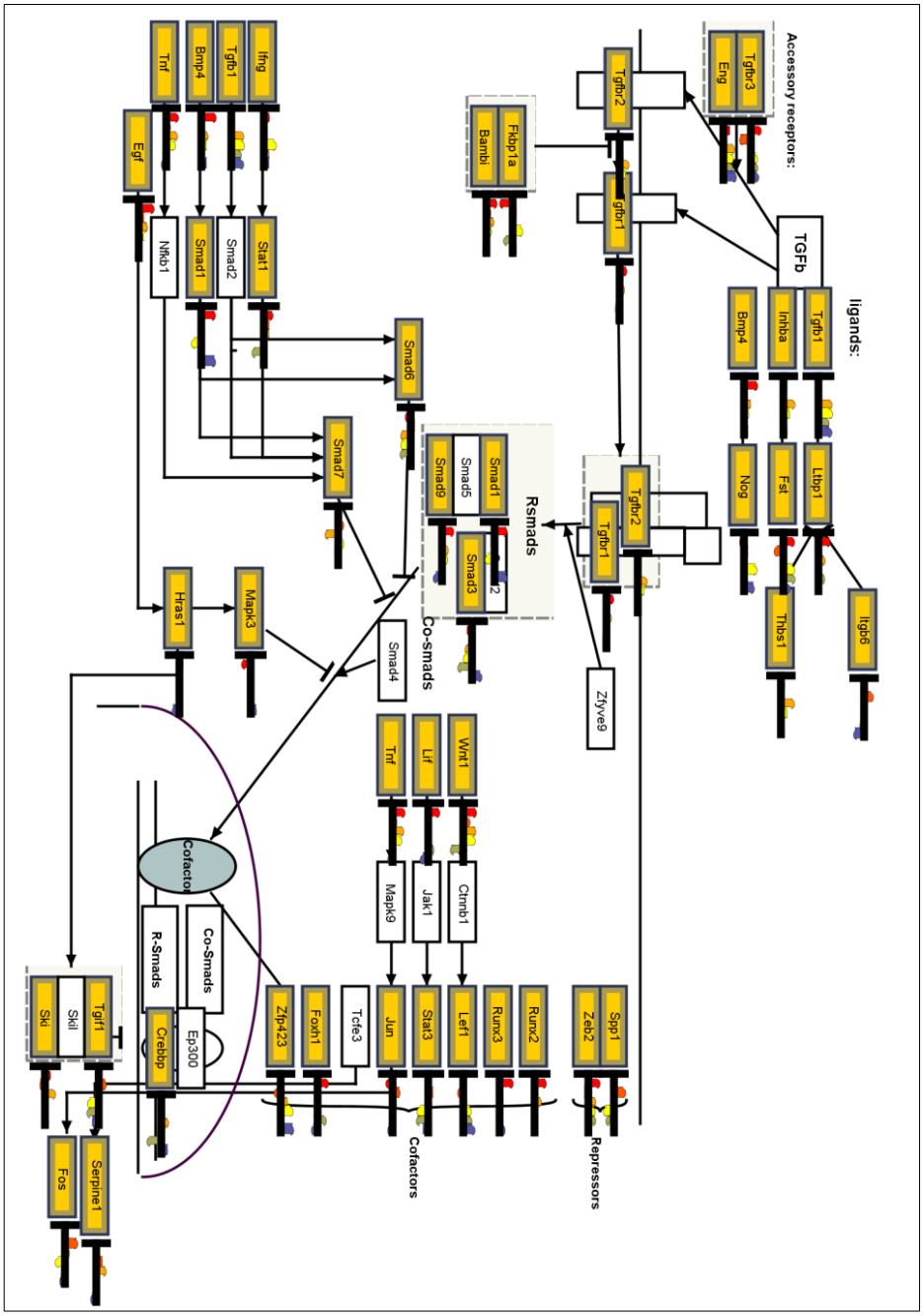


Figure 7-12: TGF-β signalling pathway response to mating in uterine stromal cells

7.4 Appendix IV: Cytokines measured via fluid phase multiplex immunoassay

Cytokine	Symbol
Cutaneous T Cell attracting chemokine	CTACK
Eotaxin	Eotaxin
Fibroblast growth factor basic	FGF basic
Granulocyte colony stimulating factor	G-CSF
Growth regulated oncogene alpha	GRO-α
Hepatocyte growth factor	HGF
Intercellular adhesion molecule	ICAM
Interferon alpha 2	IFN-α2
Interferon gamma	IFN-γ
Interferon gamma induced protein 10	IP-10
Interleukin 12 p40 subunit	IL-12 (p40)
Interleukin 1 alpha	IL-1α
Interleukin 1 receptor antagonist	IL-1ra
Interleukin 10	IL-10
Interleukin 12 p70 subunit	IL-12 (p70)
Interleukin 13	IL-13
Interleukin 15	IL-15
Interleukin 16	IL-16
Interleukin 17	IL-17
Interleukin 18	IL-18
Interleukin 1β	IL-1β
Interleukin 2	IL-2
Interleukin 2 receptor alpha	IL-2rα
Interleukin 3	IL-3
Interleukin 4	IL-4
Interleukin 5	IL-5
Interleukin 6	IL-6
Interleukin 7	IL-7
Interleukin 8	IL-8
Interleukin 9	IL-9
Leukaemia inhibitory factor	LIF
Macrophage colony stimulating factor	M-CSF
Macrophage inhibitory protein 1 alpha	MIP-1α
Macrophage inhibitory protein 1 beta	MIP-1β
Macrophage migration inhibitory factor	MIF
Monocyte chemotactic protein 1	MCP-1
Monocyte chemotactic protein 3	MCP-3
Monokine induced by gamma interferon	MIG
Nerve growth factor beta	βNGF

Cytokine	Symbol
Platelet derived growth factor	PDGF
Regulated upon activation T cell expressed and secreted	RANTES
Stem cell factor	SCF
Stem cell growth factor beta	SCGF-β
Stromal derive factor	SDF-1α
TNF-related apoptosis-inducing ligand	TRAIL
Tumour necrosis factor alpha	TNF-α
Tumour necrosis factor beta	TNF-β
Vascular cell adhesion molecule	VCAM
Vascular endothelial growth factor	VEGF

7.5 Appendix V: Principal components analysis (individual data)

7.5.1 Scree plots for maturity stages

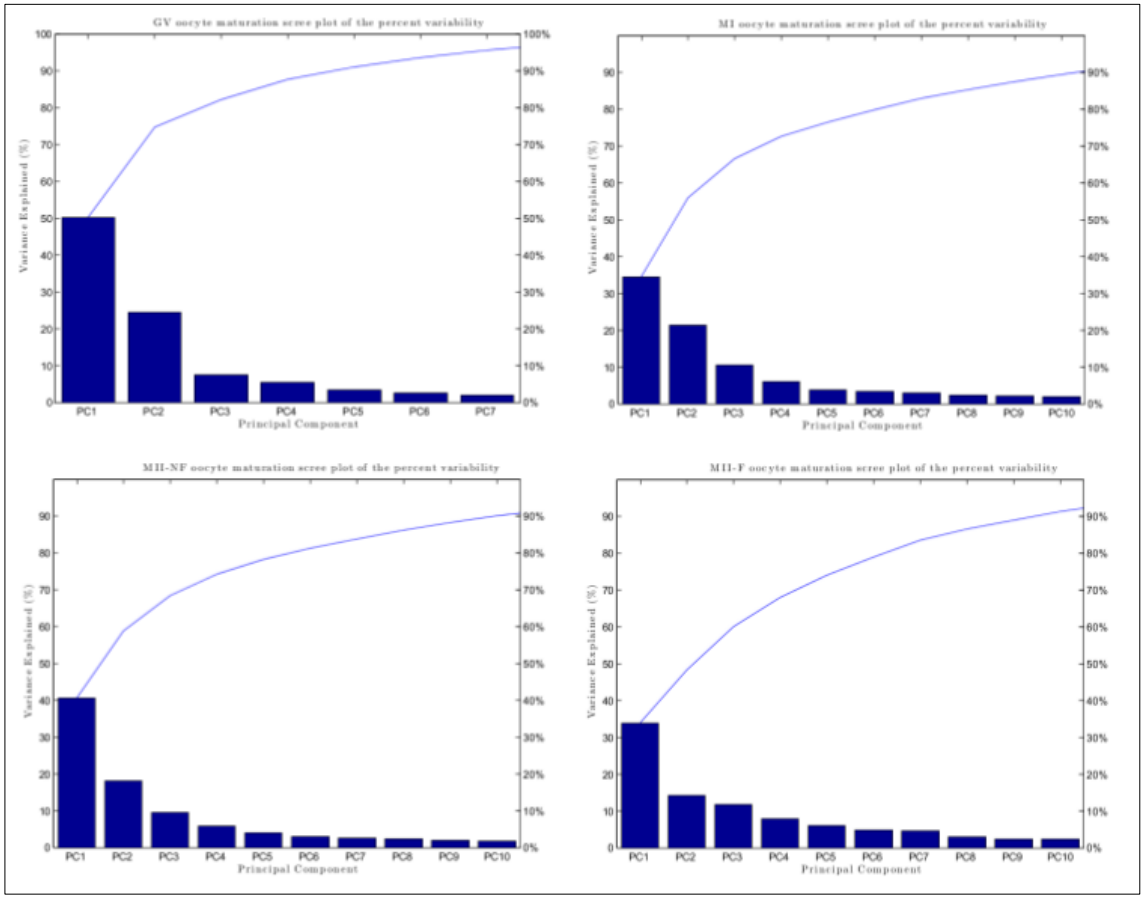


Figure 7-13: Scree plots for the PCA of individual maturity stages

7.6 Appendix VI: Principal Components Analysis (Pairwise Comparisons)

7.6.1 MII-F vs MII-NF Scree plot

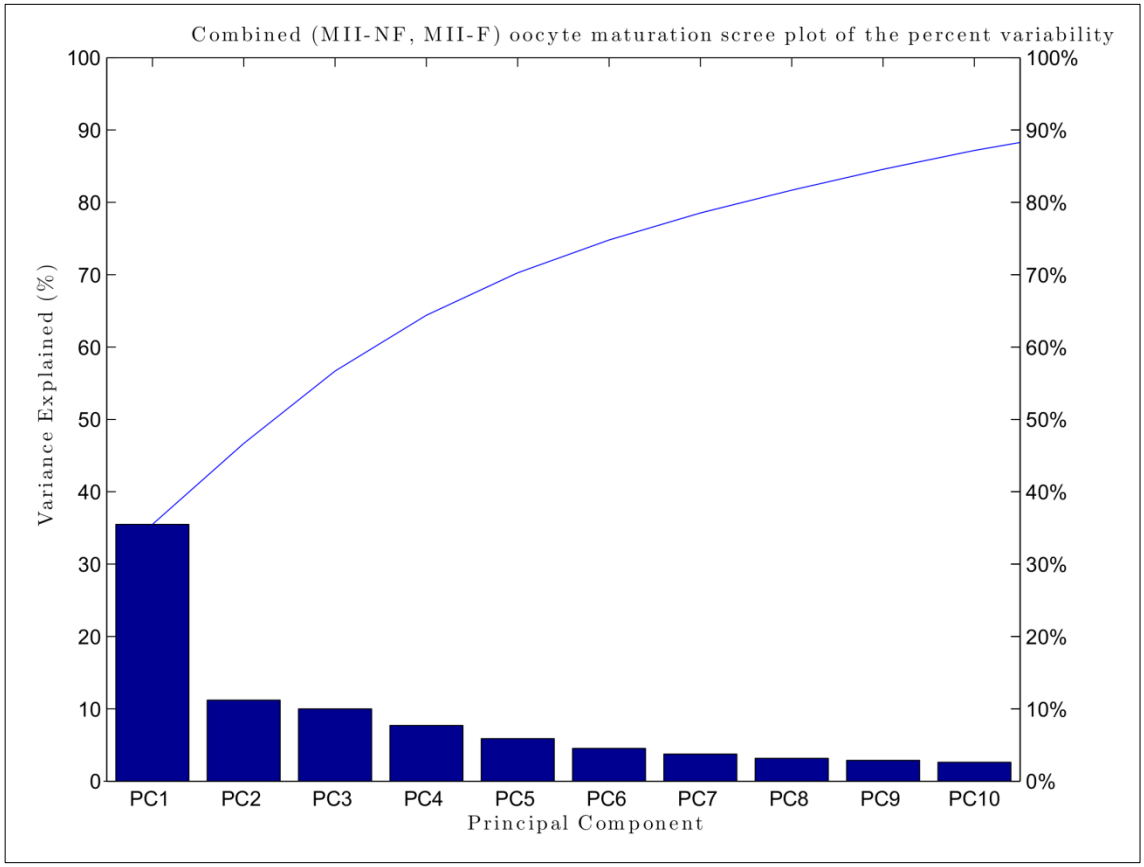


Figure 7-14: Scree plot demonstrating the variance explained by each of the PCs when comparing MII-F and MII-NF oocytes. PCs 1, 2 and 3 represented 60% of the total variation.

7.6.2 MII-F vs MII-NF alternative PC plots

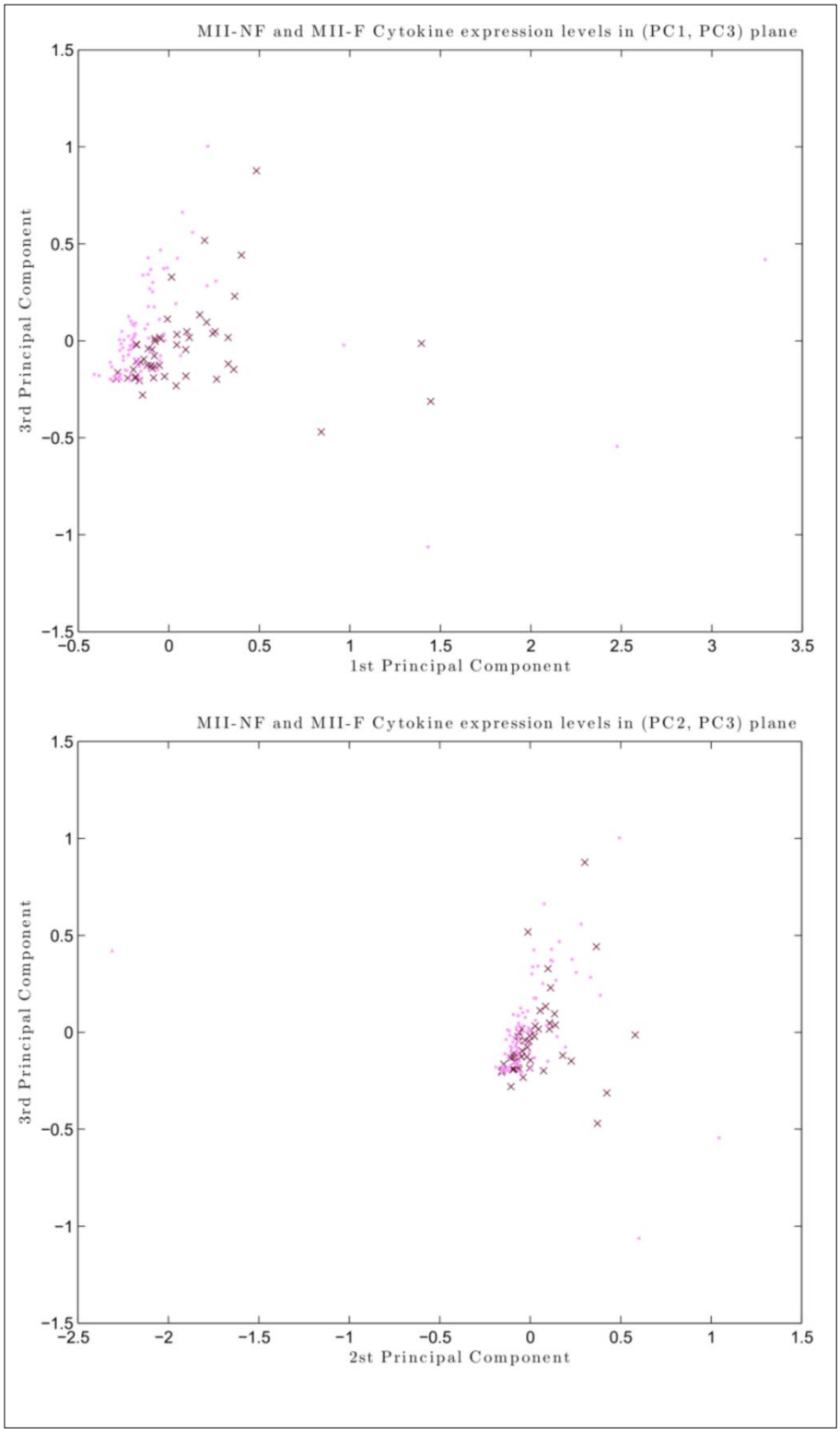


Figure 7-15: Alternative PC plots for MII-F vs MII-NF

7.6.3 PC3 coefficients for MII-F vs MII-NF

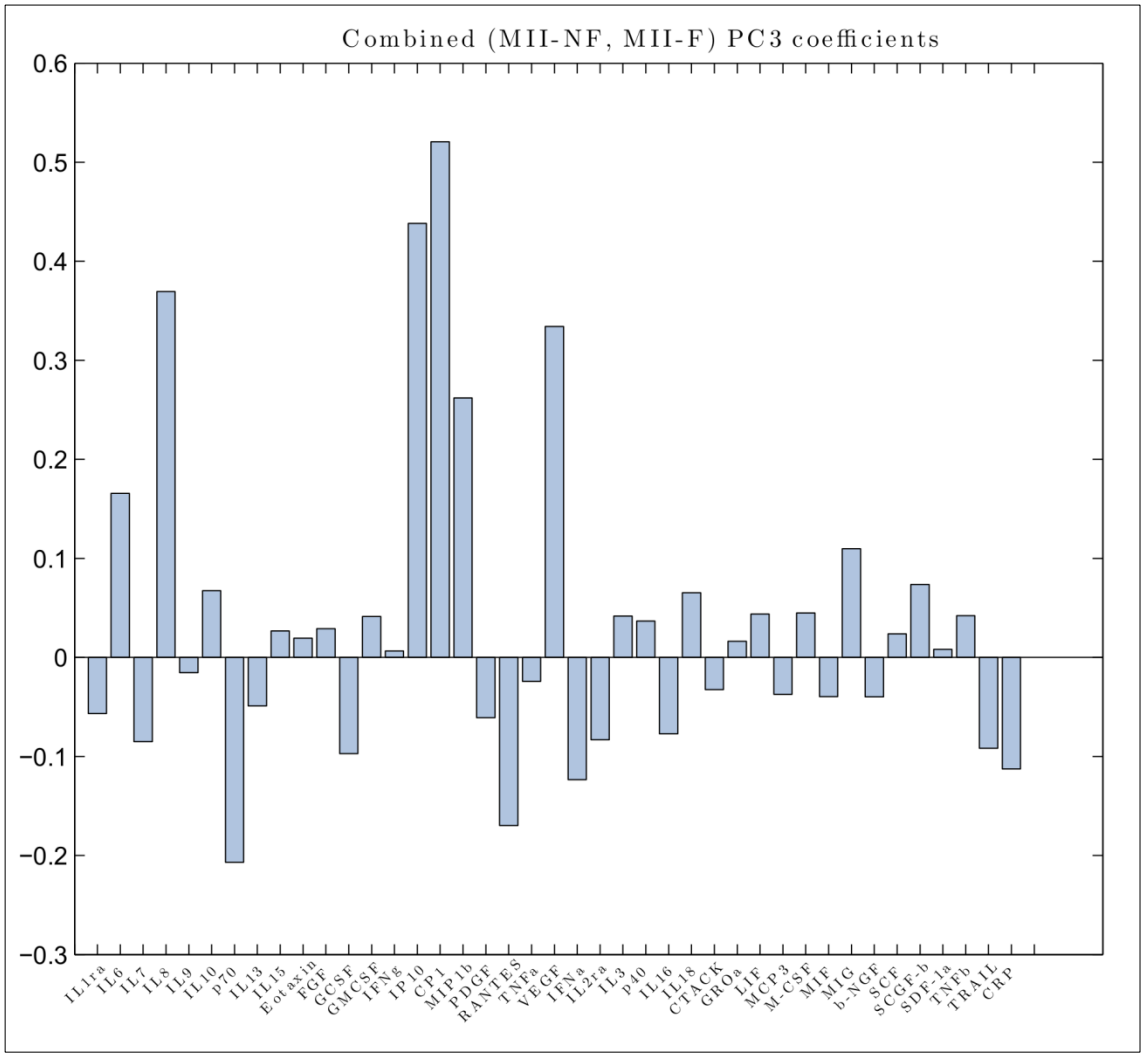


Figure 7-16: Relative contributions of cytokines as determined by PC3

7.6.4 MII-F vs MI Scree Plot

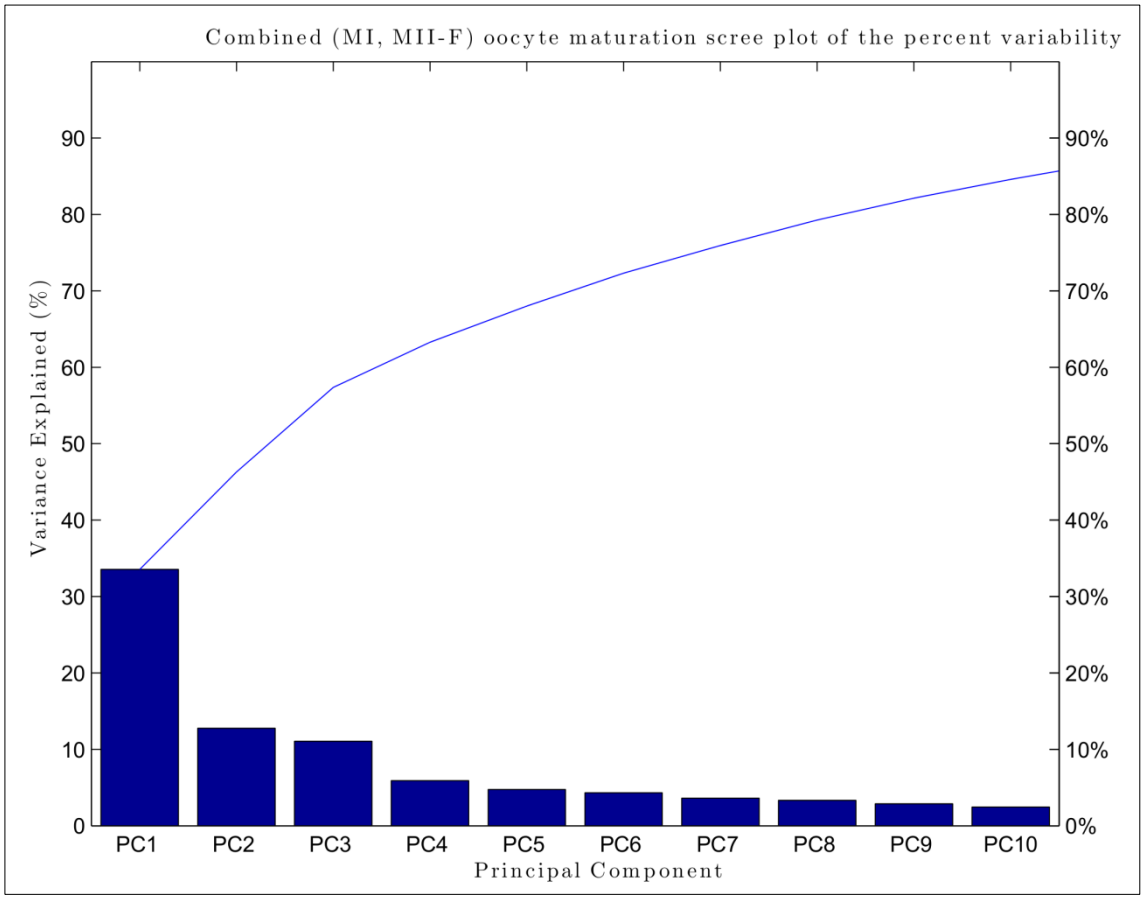


Figure 7-17: Scree plot demonstrating the amout of variance explained by each PC when comparing MII-F and MI oocyte FF

7.6.5 MII-NF vs MI Scree plot

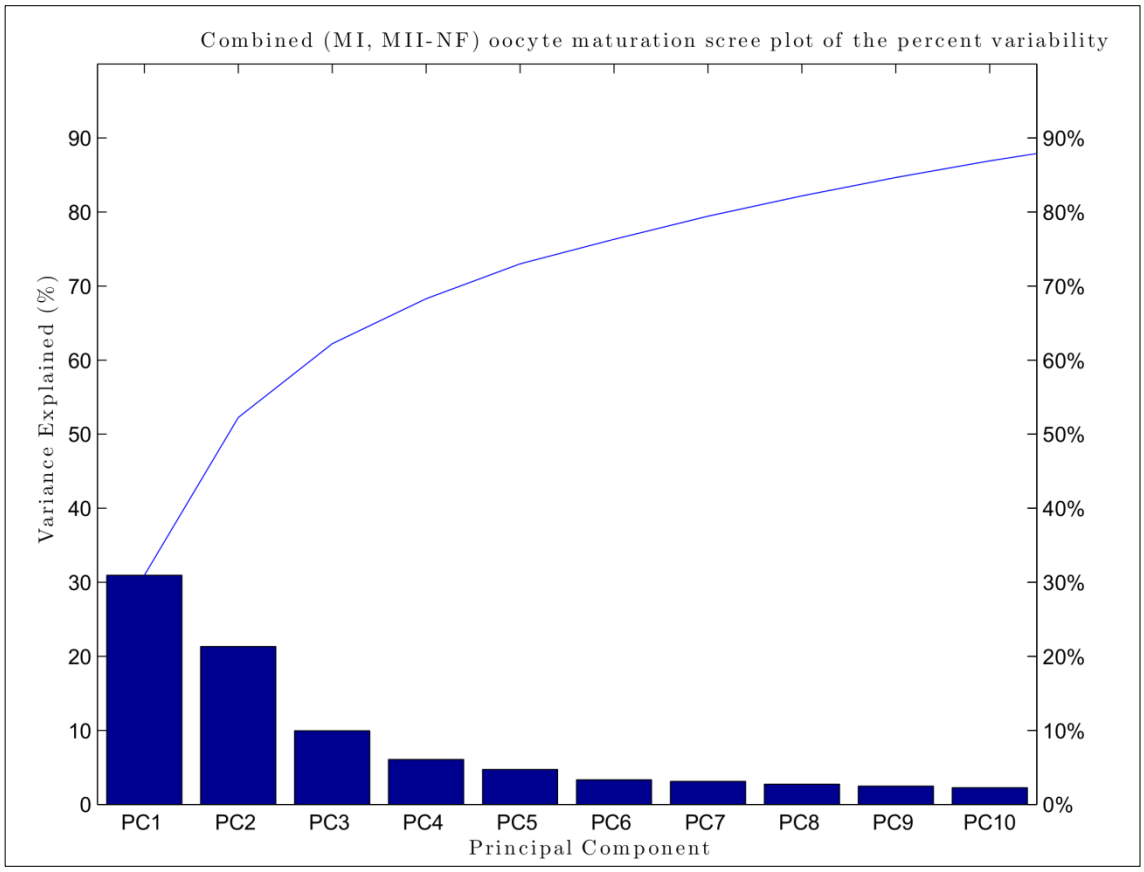


Figure 7-18: Scree plot demonstrating the relative contribution of each PC to total variance.

PCs 1, 2 and 3 explained 60% of the total.

7.6.6 MII-NF and MI PC coefficients

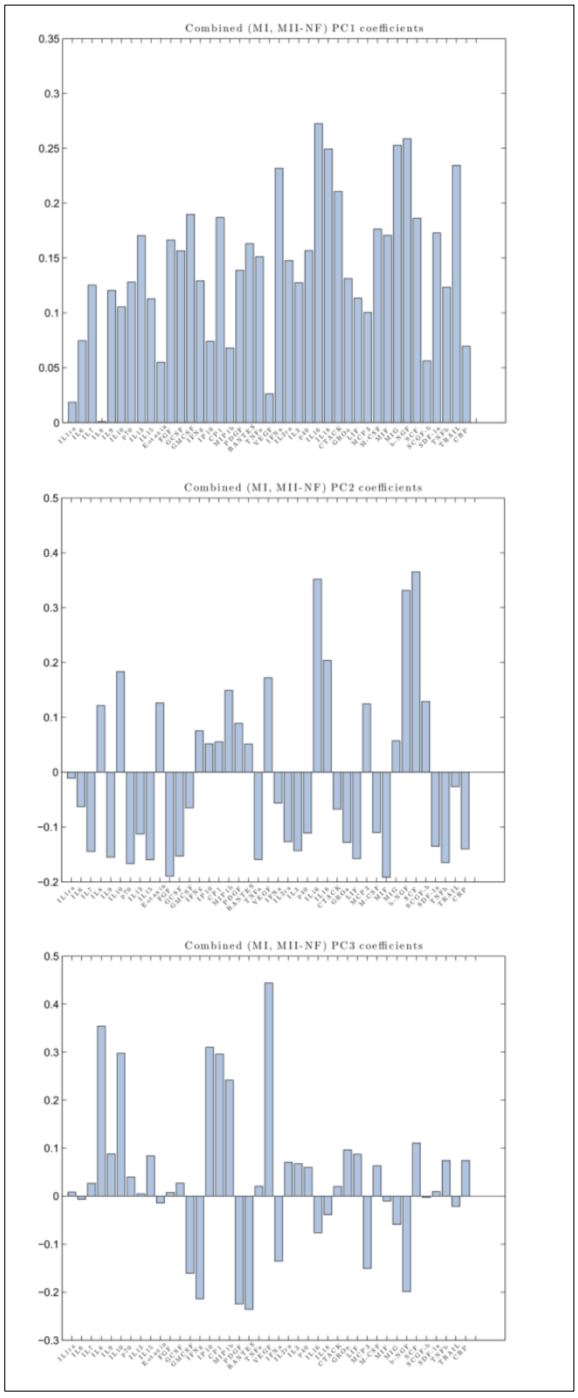


Figure 7-19: PC coefficients for MII-NF vs MI oocytes

7.6.7 MII-NF vs GV Scree Plot

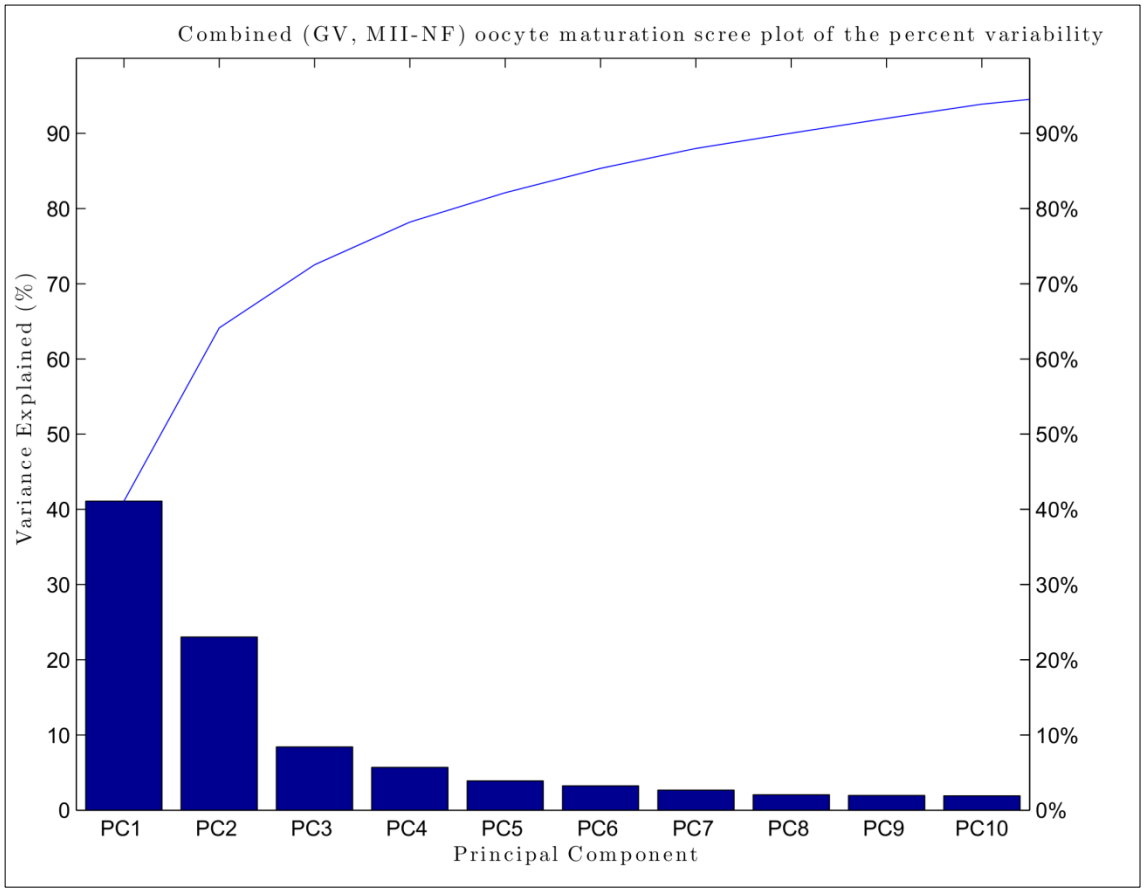
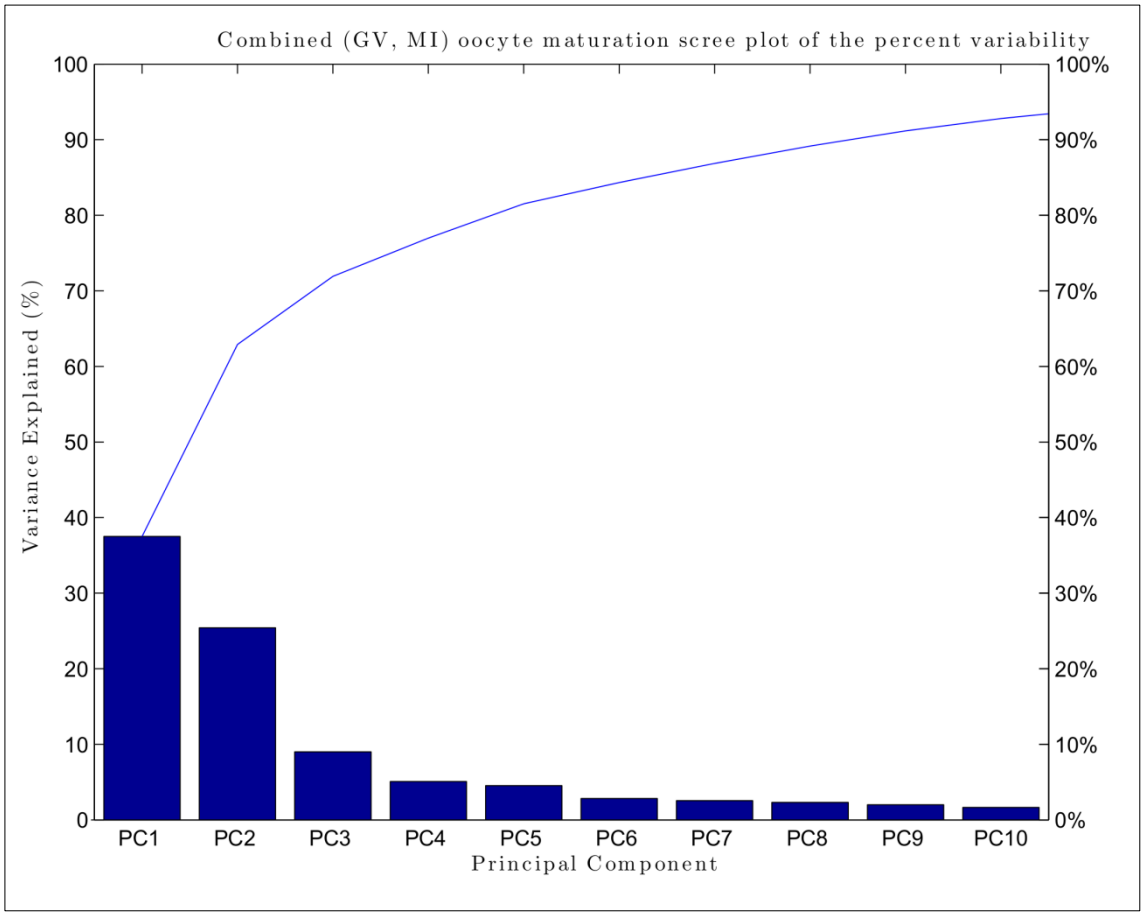


Figure 7-20: Scree plot of combined PCs for MII-NF versus GV oocytes

7.6.8 MI vs GV Scree plot



7.6.9 MI vs GV PC Coefficients

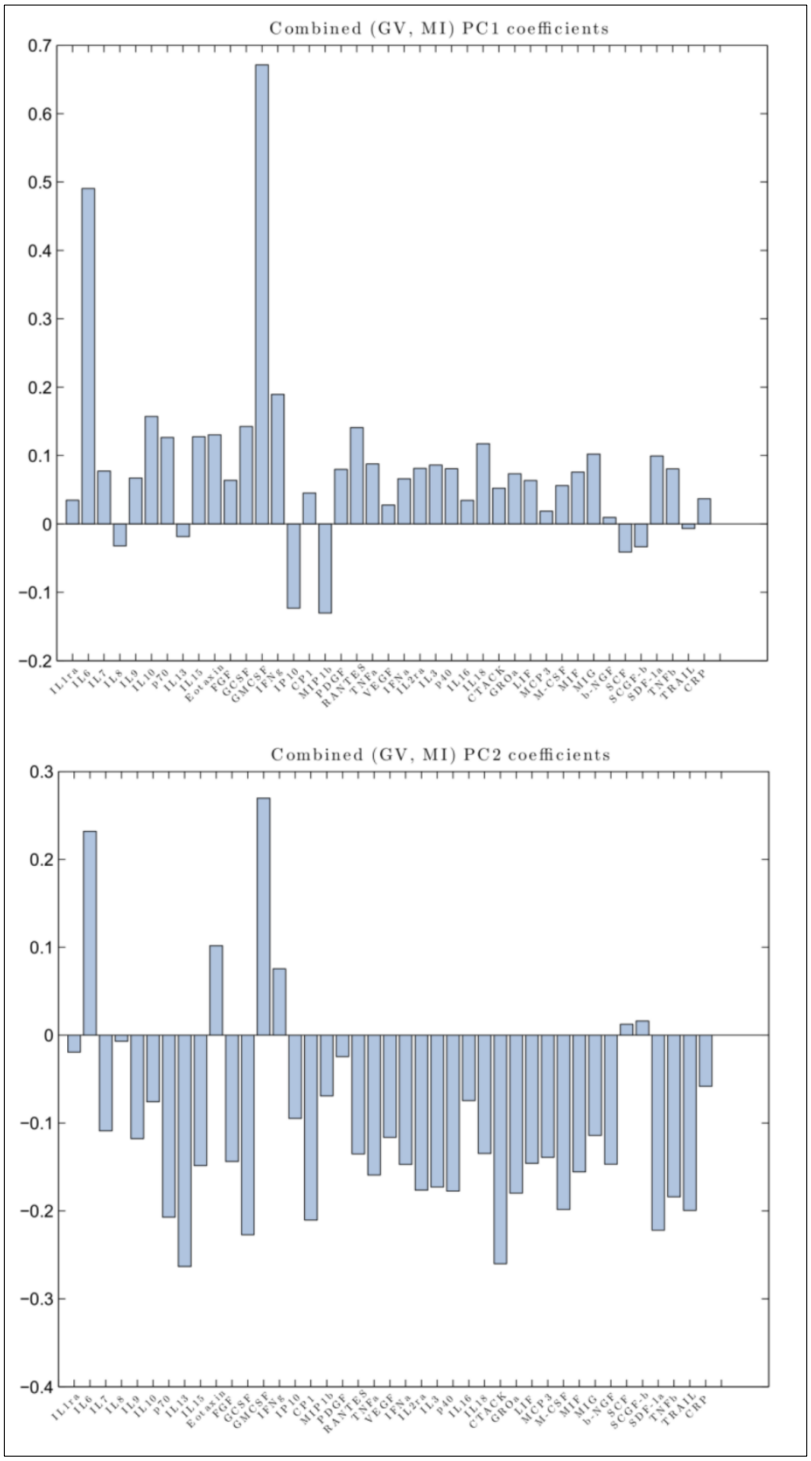


Figure 7-21: PC coefficients for MI and GV oocytes