INVESTIGATIONS INTO THE LIVED EXPERIENCE AND AETIOLOGY OF DYSMENORRHOEA AND PELVIC PAIN IN YOUNG WOMEN

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ABSTRACT

Almost every woman will experience dysmenorrhoea at some time in her life, although the severity, duration and persistence of dysmenorrhoea vary widely. This thesis investigates the lived experience of women with severe dysmenorrhoea through observational studies of women's symptoms, through laboratory studies investigating aetiologies for dysmenorrhoea, and by linking these studies to develop conclusions with strong translational relevance. While dysmenorrhoea may be associated with the more extensively researched medical condition endometriosis, this thesis is intentionally *pain-focused* rather than *endometriosis lesion-focused* to ensure maximal translational potential to address the unmet needs of women with pain.

In summary, this thesis addresses the differences between the one in five young women who suffer severe menstrual pain, and those women who are unaffected by pain. It investigates whether there is evidence for activation of the innate immune system in pelvic pain, and specifically Toll-Like Receptors (TLRs), and whether the hormonal environment influences this immune activation. It concludes with the novel hypothesis that a common aetiological factor linked to activation of Toll-Like Receptors within the uterus underlies the pain experience in women with dysmenorrhoea, chronic pelvic pain and endometriosis.

DECLARATION

I certify that this work contains no material which has been accepted for the award of any other degree or diploma in my name, in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text. In addition, I certify that no part of this work will, in the future, be used in a submission in my name, for any other degree or diploma in any university or other tertiary institution without the prior approval of the University of Adelaide and where applicable, any partner institution responsible for the joint award of this degree.

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Susan Florence Evans Date 4th February 2021

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Science builds on the knowledge of those before us, and I would like to acknowledge the work of the following scientists: Dr Heillie Kwok for her dedicated laboratory work, trial management skills, and her own innovative research into the role of Toll-Like Receptors and pain; Professor Mark Hutchinson, who is a constant source of inspiration; Dr Annie Solterbeck who made statistical sense of the complex laboratory data; Professor Adrian Esterman for his statistical analysis of the clinical audit data; Ms Tiffany Brooks, my collaborator for the initial clinical audit study; Dr Matilda Darling and Dr Carmen Pyragius for their statistical support for the Toll-Like Receptor, steroid hormone and C-reactive protein data; and Dr Tania Crotti for her administrative guidance. I would especially like to thank Professor Roly Sussex for his expert advice during the writing-up phases of the PhD, for all things editorial, and for his personal support.

I've been privileged to grow up and live in a family that nurtures and supports a love of learning, and a free spirit. And there are few individuals as free in spirit than my parents, Topsy and David Evans. To John, Phoebe, Jack and Robert Allison, thank you for allowing me the freedom to take my own path.

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DEDICATION

This PhD is dedicated to the girls and women who suffer pelvic pain, often without a diagnosis, over many years. Their generosity and altruistic motivation to improve the lives of other women is my constant inspiration.

LIST OF ABBREVIATIONS

AR	Androgen receptor
CD4+	T-Helper cells
CD8+	T-cytotoxic cells
CD3+	T-cell co-receptor cells
COX2	Cyclooxygenase-2
CPP	Chronic pelvic pain
DC	Dendritic cell
DIE	Deep infiltrating endometriosis
DRG	Dorsal root ganglion
ELISA	Enzyme-linked immunosorbent assay
EMT	Epithelial-mesenchymal transition
ERα	Estrogen receptor alpha
ERβ	Estrogen receptor beta
fMRI	Functional magnetic resonance imaging
GFAP	Glial fibrillary acidic protein
GPER	G protein-coupled estrogen receptors
HSP	Heat shock protein
IBS	Irritable Bowel Syndrome
IFN-β	Interferon-beta
IL-1β	Interleukin-1 Beta
LPS	Lipopolysaccharide
MAPK	Mitogen activated protein kinase
MET	Mesenchymal-epithelial transition
MD-2	Myeloid differentiation factor 2
MIP-2	Macrophage inflammatory protein 2
MMP-9	Matrix metallopeptidase 9
MyD88	Myeloid differentiation primary response gene 88
NK cells	Natural killer cells
NMDA	N-methyl D-aspartate;
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells

NGF	Nerve growth factor
NRS	Numerical rating scale
PAMPs	Pathogen-associated molecular patterns
PBMCs	Peripheral blood mononuclear cells
PBS	Painful Bladder Syndrome
PGE2	Prostaglandin E2
PR	Progesterone receptor
Th1	T-helper-1
Th2	T-helper-2
TIR	Toll Interleukin-1
TLR	Toll-Like Receptor
TLR4	Toll-Like Receptor 4
TNF-α	Tumour necrosis factor alpha
TRIF	TIR-domain-containing adapter-inducing interferon-β
VAS	Visual analogue scale

PERSONAL STATEMENT

Twenty years working as a gynaecologist caring for young women with endometriosis and pelvic pain have left me with one burning question:

> What is different about the one in five young women with severe dysmenorrhoea that sets them apart from unaffected women, and puts them at risk of a life of pain?

In 1996 I completed my specialist training in Gynaecology with the primary aim of honing my surgical skills and becoming the best laparoscopic surgeon I could be. In those days, major advances in surgical technique were made frequently, and we had the naive idea that if we could just improve our surgery sufficiently, we could thereby cure pelvic pain. Over the following twenty years I realised that despite high quality surgery, many of my patients still had pain, with suffering that involved multiple symptoms both within and outside the pelvis. The gap between what was found during a laparoscopic examination and the symptoms women described was obvious. Girls who started their lives with severe dysmenorrhoea often progressed to a life of educational, personal, and financial disadvantage, in addition to their continued pain.

Pain in women has been under-researched when compared to its life impact on those affected. This thesis seeks to advance knowledge of the mechanisms behind a hidden problem, a taboo subject overlain with stigma, cultural and gender discrimination: pain in women.

CHAPTER 1: INTRODUCTION

In the middle of the flanks of women lies the womb, a female viscus resembling an animal; for it is moved of itself hither and thither in the flanks, also upward in direct line to below the cartilage of the thorax and also obliquely to right or left, either to the liver or spleen; and it likewise is subject to prolapses downwards, and, in a word, is altogether erratic. It delights also in fragrant smells and advances towards them; and it has an aversion to foetid smells and flees from them and, on the whole the womb is like an animal, within an animal.

Arateus of Cappadocia. 2nd century AD

Jenks EW. (1877) pp.10

Menstrual difficulties, including dysmenorrhoea of varying severity, have been a woman's lot through the ages. Metaphors for menstruation, such as 'the curse', aptly describe both the pain experience for a proportion of women, and the negative social and religious views of menstruation throughout history.

The word *pain* derives from the Latin word *poena* i.e. punishment. This association between pain and wrongdoing has been the view across many societies for centuries, as outlined in the book *'The story of pain: From prayer to painkillers'* by Joanna Bourke (2014). Pain was heaven sent and a punishment to accept. While the symptoms originally ascribed to the condition *hysteria* by the Ancient Greeks (Merskey, 1997), and now variably ascribed to the modern day Somatic Symptom Disorder (Diagnostic and statistical manual of mental disorders, 2013) do include non-pain symptoms, pain and menstrual maladies feature prominently. Indeed, the term *hysteria* derives from the Greek word *hysteros* meaning womb, and somatic symptom disorder (SSD) is a DSM-5 diagnosis that describes a cluster of patients who have distressing somatic symptoms along with abnormal thoughts, feelings, and behaviours in response to these symptoms.

Consistent with this association of pain with wrongdoing has been the shame associated with menstrual bleeding. The Abrahamic Holy books (Bible, Torah and Qur'an) describe menstruating women as *unclean*:

> When a woman has her regular flow of blood, the impurity of her monthly period will last seven days, and anyone who touches her will be unclean till evening.

> Anything she lies on during her period will be unclean, and anything she sits on will be unclean. (Holy Bible, King James Version, Leviticus: 15:19)

The combination of pain and bleeding in women has attracted only modest research interest over the years. Despite its severity, long history, and high prevalence, dysmenorrhoea has been under-researched, under-resourced and under-recognised when compared with many other medical or pain conditions (Coco, 1999; Proctor & Farquhar, 2007). The issues behind the lack of attention to pain conditions in women have been elegantly outlined in the recently published book *Pain and prejudice* by Gabrielle Jackson (2019). Jackson concludes that:

> Chronic pain works differently in male versus female brains, which is a problem because almost all of the pain research has traditionally been done using male cell lines, and male rodent and human participants (Jackson, 2009, p9)

Jackson then quotes Janine Austin Clayton, associate director for women's health research at the United States National Institutes of Health, in an interview for the New York Times (Rabin, 2014):

> The result is that we literally know less about every aspect of female biology compared to male biology (Rabin, 2014, p14)

The present thesis seeks to address this large unmet human need by investigating the lived experience and aetiological mechanisms behind dysmenorrhoea and pelvic pain. It does so by considering the patient's presenting symptom profile, the role of Toll-Like Receptor 4 (TLR4) activation of the innate immune system, and the role of the hormonal milieu of women with dysmenorrhoea and chronic pelvic pain. While the interplay of pain with the closely associated, and more thoroughly researched, medical condition endometriosis is considered, this thesis specifically investigates dysmenorrhoea from a pain-based, rather than an endometriosis lesion-based, perspective. The majority of the current literature in this field describes the experience of women with surgically demonstrated endometriosis lesions. However, as dysmenorrhoea and pelvic pain may also be present in women without endometriosis lesions, this has limited application to the clinical management of women with pelvic pain in primary care settings.

The topic is approached from a clinically relevant, pain-based perspective that is designed to provide the information required to support clinicians in their day-to-day management of girls and women with dysmenorrhoea in the real world. It addresses clinically relevant gaps in

current knowledge that can prevent one in five young women reaching their full life potential. Although chronic pelvic pain may also occur in the absence of dysmenorrhoea, in pregnant women, or rarely even in men (Al-Obaidy & Idrees, 2019), this thesis will consider only pelvic pain associated with dysmenorrhoea in non-pregnant women. The laboratory research within this thesis considers girls aged 16-17 years and women aged 18-35 years, to ensure its relevance to the needs of young women.

The present chapter defines dysmenorrhoea and chronic pelvic pain, provides a summary of the epidemiological data available, a description of the human and fiscal burden of dysmenorrhoea-related pelvic pain to individuals and society, and outlines the current theories for the mechanisms behind dysmenorrhoea and chronic pelvic pain. It justifies the need for research into the causes and implications of dysmenorrhoea in our society and throughout the world. Comparisons are made with the more extensively investigated condition endometriosis where appropriate.

1.1 DEFINITIONS

1.1.1 THE DEFINITION AND CLASSIFICATION OF DYSMENORRHOEA

Dysmenorrhoea is defined as pain associated with menstruation. Where no underlying medical condition has been diagnosed, it typically presents as painful spasmodic cramping in the lower abdomen at the time of menstruation, and is classified as *primary dysmenorrhoea* (*Dysmenorrhea: Painful periods - ACOG*, 2019). Where an underlying medical condition such as endometriosis has been diagnosed, pain symptoms are classified as *secondary dysmenorrhoea* (Figure 1.1, Figure 1.2). In particular, secondary causes of dysmenorrhoea include the medical condition endometriosis, where tissue similar to endometrium is present outside the uterus, and the related condition adenomyosis, where endometrial glands and stroma invade the myometrium of the uterus (Figure 1.3).

However, in clinical practice, the differentiation between primary and secondary dysmenorrhoea is less useful. Dysmenorrhoea is a subjective symptom, and the pain symptom profiles associated with the different causes of secondary dysmenorrhoea may overlap within an individual. For example, a woman with uterine dysmenorrhoea may develop endometriosis lesions close to the rectum, resulting in pain on defecation during menstruation. In addition, the comprehensive exclusion of secondary causes of dysmenorrhoea requires assessment and examination by a skilled clinician, high quality imaging of the uterus to exclude adenomyosis, and visualisation or exclusion of endometriosis at a laparoscopic surgical intervention requiring general anaesthesia (Figure 1.4). This approach may not be available or acceptable to a young woman or her family. Consequently, clinical management when offered and where available for women is generally initiated empirically without knowledge of the presence or absence of underlying conditions. For the purpose of this thesis, we will take as a working hypothesis that dysmenorrhoea is pain associated with menstruation.

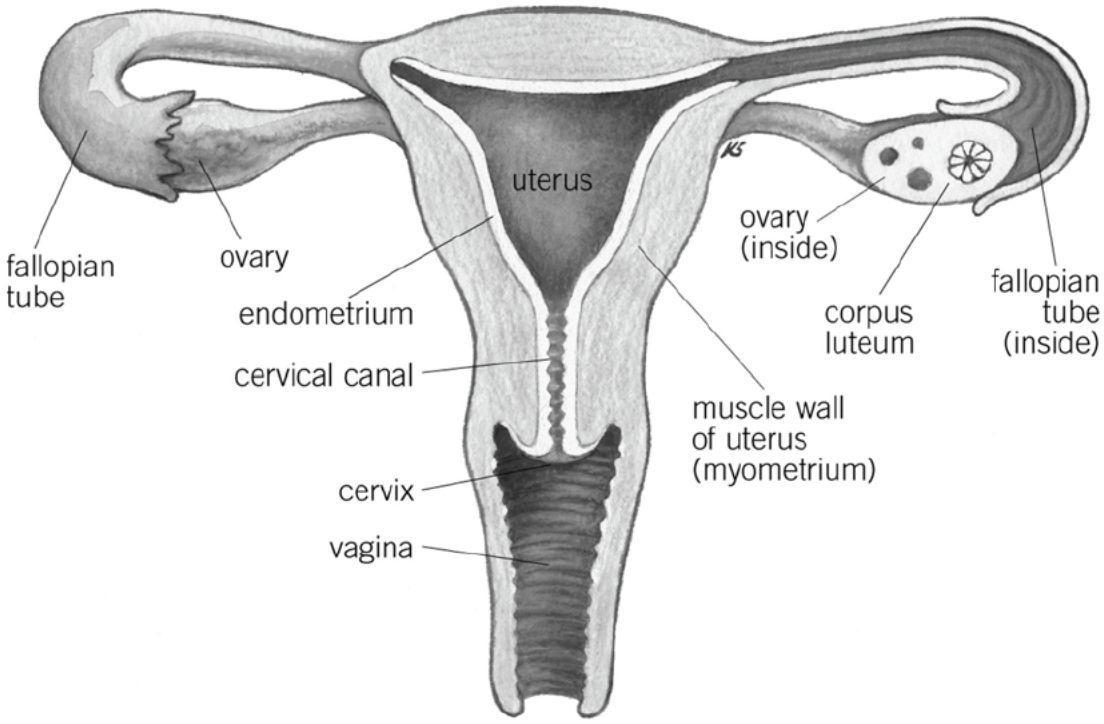

Figure 1.1: The gynaecological organs of the pelvis.
Reproduced with permission Ms K Skelsey

The Causes of Secondary Dysmenorrhoea
Endometriosis
Adenomyosis
Uterine fibroids
Ovarian cysts
Uterine polyps
Congenital malformations
Cervical stenosis
Pelvic Congestion Syndrome
Pelvic Inflammatory Disease
Pelvic adhesions

Figure 1.2: The causes of secondary dysmenorrhoea

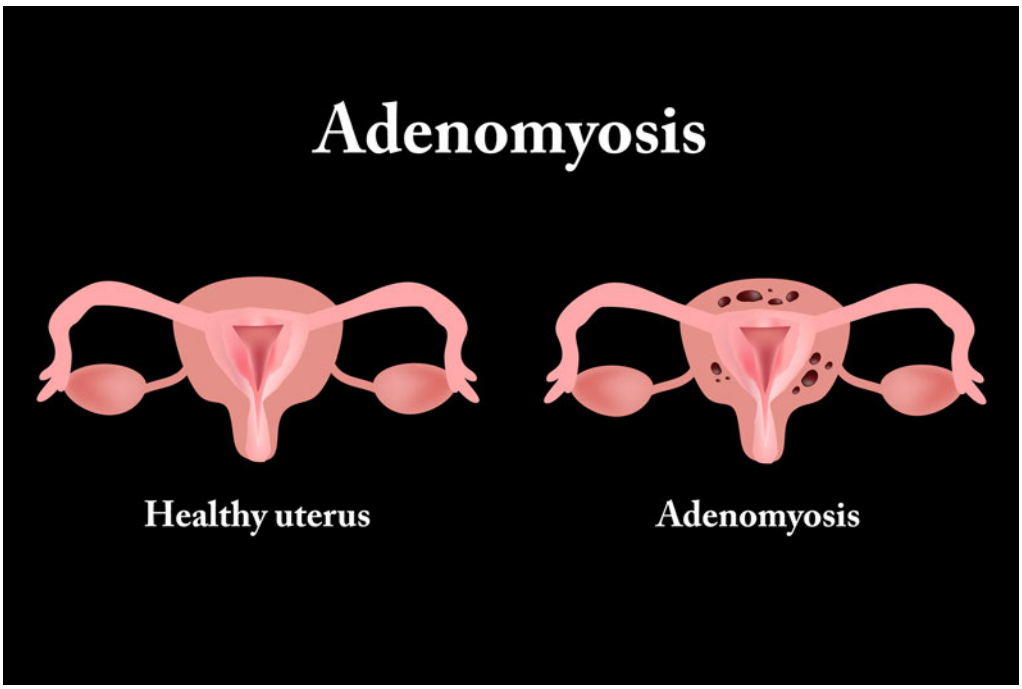

Figure 1.3: Illustration of adenomyosis with endometrial glands and stroma sited within the myometrium. Source: www.bigstockphoto.com

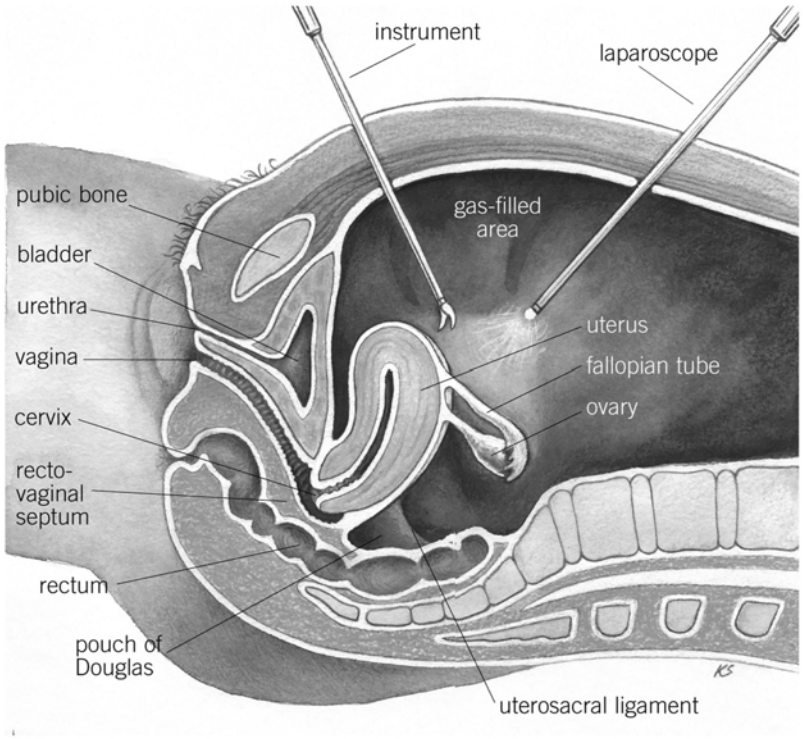

Fig 1.4: Illustration of a laparoscopic surgical procedure where a telescope (laparoscope) is inserted through the umbilicus to view the pelvic organs.
Reproduced with permission Ms K Skelsey

1.1.2 THE DEFINITION OF CHRONIC PELVIC PAIN

Chronic pelvic pain (CPP) is variably defined as pain in the pelvis that has been either continuous or recurrent for longer than either three (Royal Australian College of Obstetrics and Gynaecology, 2020) or six months (European Association of Urology, 2015). In particular, there is disagreement in the literature as to whether or not pain that is experienced exclusively during menstruation (dysmenorrhoea) should be included within the classifications of chronic pain conditions. For example, in 2012, The Royal College of Obstetricians and Gynaecologists (RCOG), in their *Green-top guideline No.41* (RCOG, 2012), specifically excludes pain felt exclusively with menstruation from classification as a chronic pelvic pain. However, while the American College of Obstetricians and Gynaecologists (ACOG Committee, 2004) initially excluded pain experienced exclusively during menstruation from classification as a chronic pain, this guideline was later withdrawn and has yet to be replaced. The current ACOG online consumer information, first published in 2011, includes dysmenorrhoea as a chronic pelvic pain condition (Chronic pelvic pain - ACOG, 2018).

For the purpose of this thesis, CPP is defined as *pain perceived in structures related to the pelvis, which has been continuous or recurrent for more than six months, including pain experienced exclusively during menstruation*.

Multiple medical conditions in both men and women are associated with the development of chronic pelvic pain. However, this thesis will consider only chronic pelvic pain in women where the symptom profile includes dysmenorrhoea, and where pain has been present on most days for more than 6 months. For convenience, this subset of pelvic pain will be described as *dysmenorrhoea-related pelvic pain (DRPP)*.

Insert Fig 1.5 and Fig 1.7

1.1.3 THE DEFINITION AND CLASSIFICATION OF ENDOMETRIOSIS

Endometriosis is an estrogen-dependent, common, chronic inflammatory condition characterised by the presence of tissue displaying the histological features of endometrial glands and stroma outside the uterus. It is frequently, but not consistently, associated with symptoms of dysmenorrhoea and pelvic pain (Moen & Stokstad, 2002). When visualised at a laparoscopic surgical procedure, endometriosis lesions may appear clear, white, pink, red, yellow, brown or bluish-black (Donnez et al., 2003). The American Society of Reproductive Medicine (ASRM) describes 4 stages of endometriosis according to the severity and extent of lesions present (American Society for Reproductive Medicine, 1997), with Stage 1-2 being relatively mild, and Stages 3-4 more severe (Figure 1.5). However, this classification was developed for the purpose of estimating the chance of subsequent pregnancy, rather than as a way to determine the presence or severity of pain experienced by the women affected.

Endometriosis lesions display a wide range of appearances (Figures 1.6a,1.6b and 1.6c). More recent studies have suggested that multiple aetiologies may contribute to variations in endometriosis phenotype with peritoneal lesions, deeply infiltrating lesions, and ovarian cystic lesions, potentially representing three separate disease processes (Gordts et al., 2017; Koninckx et al., 2019).

AMERICAN SOCIETY FOR REPRODUCTIVE MEDICINE
REVISED CLASSIFICATION OF ENDOMETRIOSIS

Patient's Name _____ Date _____

Stage I (Minimal) - 1-5
Stage II (Mild) - 6-15
Stage III (Moderate) - 16-40
Stage IV (Severe) - >40
Total _____

Laparoscopy _____ Laparotomy _____ Photography _____
Recommended Treatment _____
_____
Prognosis _____

PERITONEUM	**ENDOMETRIOSIS**	<1cm	1-3cm	>3cm
	Superficial	1	2	4
	Deep	2	4	6
OVARY	R Superficial	1	2	4
	Deep	4	16	20
	L Superficial	1	2	4
	Deep	4	16	20
	POSTERIOR CULDESAC OBLITERATION	Partial	Complete	
		4	40	
OVARY	**ADHESIONS**	<1/3 Enclosure	1/3-2/3 Enclosure	>2/3 Enclosure
	R Filmy	1	2	4
	Dense	4	8	16
	L Filmy	1	2	4
	Dense	4	8	16
TUBE	R Filmy	1	2	4
	Dense	4*	8*	16
	L Filmy	1	2	4
	Dense	4*	8*	16

*If the fimbriated end of the fallopian tube is completely enclosed, change the point assignment to 16.

Denote appearance of superficial implant types as red [(R), red, red-pink, flamelike, vesicular blobs, clear vesicles], white [(W), opacifications, peritoneal defects, yellow-brown], or black [(B) black, hemosiderin deposits, blue]. Denote percent of total described as R___%, W___% and B___%. Total should equal 100%.

Figure 1.5: Revised American Society for Reproductive Medicine Classification for endometriosis lesions (American Society for Reproductive Medicine, 1997)

1.1.4 CLINICAL IMPLICATIONS

In clinical practice, dysmenorrhoea and chronic pelvic pain have traditionally been associated with the presence of endometriosis. However, the association of endometriosis with dysmenorrhoea and pelvic pain is inconsistent. Women may have endometriosis without pain, or may have pain without endometriosis (Fauconnier & Chapron, 2005). The American Society for Reproductive Medicine classification for the staging of endometriotic lesions during surgical procedures (American Society for Reproductive Medicine, 1997) correlates poorly with the severity of pain experienced (Vercellini et al., 2006). In addition, the removal of endometriosis lesions does not reliably reduce pain symptoms (Abbott et al., 2003). The lack of correlation between the presence of endometriosis and the experience of pain is further discussed in section 1.4. For these reasons, the association of endometriosis with dysmenorrhoea should not be considered to imply causation.

While an endometriosis lesion-based approach to pelvic pain in women has made it easier to achieve consensus among researchers and clinicians, and has facilitated the development of management guidelines, this is of little use to a primary care clinician, caring for a girl or woman with dysmenorrhoea-related pelvic pain in medical practice. Laparoscopic surgery to diagnose or exclude endometriosis may or may not have been performed, and may or may not relieve symptoms (Abbott et al., 2003). In addition, there is currently no sufficiently reliable clinical or non-surgical biomarker to indicate which women may have endometriosis and might benefit most from surgery (Nisenblat et al., 2016), and which women would best be managed without surgery. Initial studies suggest that multiple symptomatology may be common in women with dysmenorrhoea (Ju et al., 2014b; Saidi et al., 2020; Smorgick et al., 2013; Zondervan et al., 2001). The subjective nature of dysmenorrhoea, the variation in presenting symptoms, and the lack of a clinical pain biomarker for endometriosis have hampered research into novel treatment options. Knowledge of the mechanisms behind her symptoms is incomplete. Grace and Zondervan found that 29% of 1160 women with chronic pelvic pain in a community-based study had received no diagnosis for their pain (Grace & Zondervan, 2006).

There is substantial overlap between the clinical presentations of dysmenorrhoea, chronic pelvic pain and endometriosis (Figure 1.7). Dysmenorrhoea may be present as a sole symptom in an otherwise well woman, as one of many symptoms in a woman with chronic pelvic pain, or may be present in association with endometriosis lesions. This thesis has chosen a *pain-focused* approach to dysmenorrhoea-related pelvic pain to better address gaps

in the current literature, and the needs of girls and women with pain in a primary care setting. This contrasts with an *endometriosis lesion-focused* approach, which excludes women with dysmenorrhoea or chronic pelvic pain who do not have endometriosis lesions.

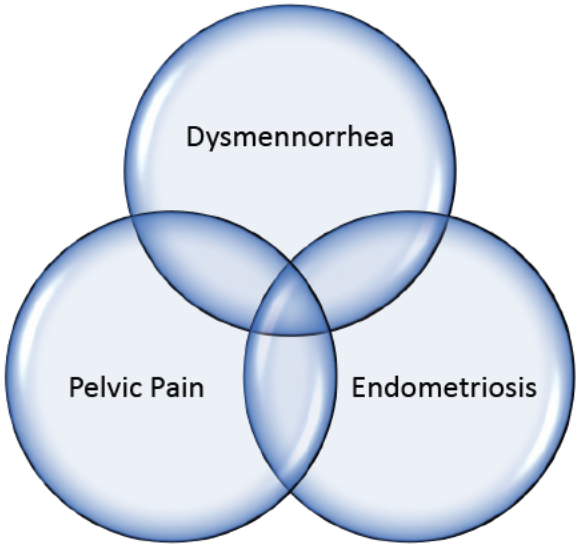

Figure 1.7: Diagramatic representation of the overlap in clinical presentation among women with dysmenorrhoea, chronic pelvic pain or endometriosis

1.2 The Epidemiology of Dysmenorrhoea, Chronic Pelvic Pain and Endometriosis

1.2.1 The prevalence of dysmenorrhoea

Dysmenorrhoea is the commonest gynaecological condition in young women (Coco, 1999). A meta-analysis of 15 community-based studies by Ju found the prevalence of dysmenorrhoea to be between 16% and 91% in women of reproductive age, with severe pain in between 2% and 29% of the populations studied (Ju et al., 2014a). Populations studied included women from Japan, China, the United States of America, Canada, Hungary, India, Mexico, Australia, the United Kingdom, Iran and Turkey. Societal, familial, medical and religious attitudes to menstruation mean that dysmenorrhoea may be disregarded or considered as normal, contributing to the above variations in reported prevalence. These attitudes may also have contributed to the scarcity of research to support the delivery of health care to women with dysmenorrhoea that is informed by basic science and clinical trials. Within an Australian setting, a study of 1051 girls aged 16-18 showed that while 93% of these girls experienced some pain with menstruation, 21% experienced severe pain, frequently associated with disruption of life activities and school absence (Parker et al., 2010). This prevalence far exceeds the prevalence of certain common health conditions for which there are long running and substantial government policy initiatives. For example, the frequency of asthma in Australia is 11%, and the frequency of diabetes is approximately 7% (*Diabetes in Australia*, 2019).

Dysmenorrhoea is more common where there is a family history of the condition, and less common with increasing years towards menopause (Sundell et al., 1990), increasing parity and increased use of the oral contraceptive pill (Andersch & Milsom, 1982; Ju et al., 2014a).

1.2.2 The prevalence of chronic pelvic pain

The prevalence of chronic pelvic pain in women is unknown. International community-based estimates vary between 15% of 5,263 women aged 18-50 years in the USA (Mathias et al., 1996), to 25.4% of women aged 18-50 years in New Zealand (Grace & Zondervan, 2006). This difference may reflect variation in the cohorts studied. Mathias et al. contacted potential participants through a Gallup Organisation telephone survey, whereas Grace and Zondervan used a questionnaire posted to a random sample of women on the New Zealand electoral roll,

with a 66% response rate. Saunders et al. (2007) investigated chronic pain conditions among 2,466 children aged 11-17 years, and found that both the presence of multiple pain sites, and the presence of abdominal pain in a mother to be positive predictors for these symptoms in a child. This suggests that common genetic, environmental or socio-cultural factors may influence the prevalence of chronic pain. While chronic pelvic pain may be associated with a wide range of medical conditions, this thesis investigates only chronic pelvic pain (CPP) in non-pregnant women where the symptom profile includes dysmenorrhoea.

1.2.3 THE PREVALENCE OF ENDOMETRIOSIS

A definitive diagnosis of endometriosis requires visualisation of endometriosis lesions at a laparoscopic surgical intervention. The invasive nature, requirement for general anaesthesia, and variable availability of this intervention throughout the world, mean that prevalence estimates for endometriosis vary according to the population studied, their access to laparoscopic surgery, and their presenting symptoms. Data obtained through the Australian Longitudinal Study on Women's Health found that 11% of women born between 1973 and 1978 were diagnosed with endometriosis by the age of 40-44 years (*Endometriosis in Australia: Prevalence and hospitalisations, Table of Contents - Australian Institute of Health and Welfare*, 2019). In a study of 1,418 women undergoing their first laparoscopic surgical procedure for a variety of indications across ten countries and 19 centres, Nnoahem et al. (2009) found an incidence of endometriosis ranging from 35% in Oxford, England to 100% in Siena, Italy. Mueleman et al. (2009) diagnosed endometriosis in 47% of 221 women undergoing laparoscopy for infertility, where ovulatory function was normal and their partner had a normal semen analysis. Overall, estimates suggest that endometriosis affects approximately 5%-10% of girls and women during their reproductive years, which in 2011 translated to represent 176 million women worldwide (Eskenazi & Warner, 1997). In a study of particular relevance to the pain focus of this thesis, Janssen found endometriosis lesions to be present in 62% of adolescent girls undergoing laparoscopy for the investigation of pain symptoms: 75% of 314 girls with chronic pelvic pain resistant to medical treatments, 70% of 146 girls with dysmenorrhoea alone and 49% of 420 girls with chronic pelvic pain who had yet to try all medical management options (Janssen et al., 2013).

While knowledge of the presence or absence of endometriosis can assist medical management, it does not necessarily result in interventions that resolve pain in an individual. High quality laparoscopic care may not be available or acceptable to young women and their families. In many parts of the world, clinicians are required to manage dysmenorrhoea and

pelvic pain without the potential benefits of easily accessible surgical interventions, or under circumstances where access to surgery is affected by a patient's socioeconomic status (Fourquet et al., 2019). Where surgery is available, clinicians are required to advise on whether surgery is applicable in each individual's case. The lack of an indicative symptom profile for endometriosis, and a reliable laboratory biomarker for either endometriosis (Gupta et al., 2016; Liu et al., 2015; Nisenblat et al., 2016a; Nisenblat et al., 2016b Nisenblat et al., 2016c) or visceral pain, hampers clinical management of girls and women with dysmenorrhoea, as well as the development of new therapeutic interventions.

1.3 HUMAN AND FISCAL BURDENS OF PELVIC PAIN

1.3.1 THE HUMAN BURDEN

Multiple studies have documented the dramatic effect of severe dysmenorrhoea on the lives of the girls and women affected, their families, their partners, their children, and their societies (Bajalan et al., 2018; Patel et al., 2006). The communities studied include girls and women from Europe, the United States, China, Japan, Australia, Iran, Saudi Arabia, Turkey, India, South Korea, Switzerland, Ethiopia, Israel and Italy. These effects include reduced school attendance, reduced quality-of-life, impaired daily functioning, an increased prevalence of mental health conditions, socio-economic disadvantage, and disrupted sleep. To date, there has been limited documentation of the long-term implications of dysmenorrhoea on wellbeing, and the potential for dysmenorrhoea to precede the development of chronic pain conditions.

The severity of the pain experienced by women with dysmenorrhoea should not be under-estimated. Zannoni found that girls with dysmenorrhoea were 28 times as likely to miss school during menses than girls without dysmenorrhoea.

Grandi et al. (2012) investigated the prevalence of dysmenorrhoea and its impact on daily functioning in a cross-sectional study of 408 Italian students. Their study found that 84.1% of these female students reported dysmenorrhoea, 55.2% reported dysmenorrhoea that required medication, 31.9% reported dysmenorrhoea resulting in absenteeism, and 25.3% reported dysmenorrhoea requiring both medication and school absenteeism. Estimated rates of school absenteeism at the time of menstruation range from 20% to 50% (Andersch & Milsom, 1982; Parker et al., 2010; Sundell et al., 1990).

A strong and complex association between dysmenorrhoea and anxiety, stress and depression has also been found (Barnard et al., 2003; Chen & Chen, 2005; Dorn et al., 2009; Iacovides et al., 2015b; Reynolds, 1969). It has yet to be determined whether the mood disorder pre-dates dysmenorrhoea or is consequent upon the pain experience and its impact on quality-of-life. Facchin et al. (2015) used psychometric scoring to assess the quality-of-life, anxiety and depression scores of 110 women with endometriosis and 61 healthy controls. Of the women with endometriosis, 78 had pelvic pain, and 32 had no pain. Facchin et al. determined that it is the presence of pain rather than the presence of mental health issues that determines the impact of endometriosis on quality-of-life measures.

Similarly, the relationships between dysmenorrhoea, sleep and fatigue are multi-faceted. Woosley and Lichstein (2013) investigated the sleep patterns of 89 women aged 18-24 years using daily sleep diaries, and found that reduced sleep efficiency, as measured by a longer waking time after sleep onset and increased number of awakenings, was common both during menstruation and throughout the menstrual cycle in women with dysmenorrhoea. They found that those women with the most severe dysmenorrhoea suffered significantly higher Insomnia Severity Index scores ($p<0.001$) (Morin et al., 2011), when compared to women with mild dysmenorrhoea. Through its effect on absenteeism, sleep, and mood disorders, dysmenorrhoea may confer a profound educational disadvantage on those girls and women affected. Interestingly, despite these demonstrated dramatic reductions in quality-of-life, the perception of society that dysmenorrhoea is normal results in under-reporting of the condition in young women (Coco, 1999; Wong & Khoo, 2010). Wong studied 1,295 Malaysian female students and found that 76.1% of students believed that dysmenorrhoea was a normal part of the menstrual cycle. For 62.3% of these students, their mother was the primary source of information on menstruation.

The severe clinical situation of chronic pelvic pain is also common and of long duration, as shown in the New Zealand study by Grace and Zondervan (2006). Chronic pain from any cause has a major effect on an individual's quality-of-life, with wellbeing indicators progressively diminishing as the duration of pain increases, and with increasing pain severity (Laursen et al., 2005). Sleep disturbance was present in 80% of 157 women with pelvic pain in a study by Cosar et al. (2014).

In contrast to its high impact on individuals and society, dysmenorrhoea has only recently begun to come to the attention of health policy makers. Australia's National Women's Health Policy (2010) was developed as "a guide for the next 20 years to improving the health and wellbeing of all women in Australia, especially those at risk of poor health". It makes recommendations on eight specific areas, including five areas of direct relevance to dysmenorrhoea and pelvic pain: sexual and reproductive health, mental health, chronic conditions, risk factors for depression and anxiety, and the impact of comorbid conditions on physical and psychological health and social function. However, this Policy does not include the words *dysmenorrhoea* or *period pain*, and mentions pelvic pain only as a potential consequence of chlamydial pelvic infection (*National Women's Health Policy 2010*). This discrepancy between impact and health policy attention has been partially addressed in 2018 with the development of Australia's National Action Plan for Endometriosis (*National Action Plan for Endometriosis, 2018*). In 2019 Australia's *National Womens Health Strategy 2020-2030* was released, with the inclusion of the stated objective "to reduce the prevalence and

impact of endometriosis and chronic pelvic pain" (National Australian Womens Health Strategy, 2020)

When considering the qualitative experiences of 18 women after a diagnosis of endometriosis in their article "A life shaped by pain", Huntington and Gilmour (2005) describe impacts affecting a woman's ability to work, her family relationships and her self-esteem. The diagnostic process had typically taken five to ten years, and there had been no formal pain management follow up after the diagnosis was made, and treatment was undertaken.

Gallagher compared Short Form-36 (SF-36) (Ware & Sherbourne, 1992) survey responses from 360 adolescents with endometriosis, and 207 healthy controls within the Women's Health Study (Gallagher, DiVasta, et al., 2018). They found significantly lower physical health (43.4 vs 53.8, $p<0.0001$) and mental health scores (43.3 vs 46.3, $p=0.008$) in the adolescents with endometriosis. Affected girls reported increased rates of mental health diagnoses, use of pain medication and avoiding exercise during menstruation.

Pelvic symptoms associated with endometriosis include recurrent painful periods, painful intercourse, painful defecation during menstruation, chronic lower abdominal pain and hypersensitivity, chronic lower back pain, and infertility (Proctor & Farquhar, 2007). However, the symptoms experienced extend beyond the pelvis. Increasingly, the presence of extra-pelvic symptoms such as migraine, fatigue and mental health in women with endometriosis have been recognised (Tietjen et al., 2007; Yosef et al., 2016). Simoens et al. (2014) estimated a 0.81 reduction in quality adjusted life years in 909 European women with endometriosis treated at tertiary referral centres.

Predictors of depression, anxiety and stress in women with pelvic pain have been investigated in 168 Australian women by Brooks et al. (2020). Higher depression scores were associated with a higher current pain severity score, a history of stabbing pain, the prior experience of a sexually distressing event, having experienced pain as a child, and being nulliparous. Higher anxiety scores were associated with a higher current pain severity score, younger age, a history of stabbing pelvic pain, the prior experience of a sexually distressing event, and a younger age at menarche. Higher stress scores were predicted by a higher current pain severity, a younger age, a history of stabbing pains, and prior experience of a sexually distressing event.

In summary, dysmenorrhoea and pelvic pain, regardless of the presence or absence of endometriosis, are associated with a reduced quality-of-life, increased prevalence of mental

health conditions, and severe sleep disruption. Section 1.4.4 will discuss the growing evidence that recurrent episodes of dysmenorrhoea may predispose women to the future development of chronic pain conditions, potentially through the mechanism of increased central pain sensitisation. If proven, this argues against the presence of dysmenorrhoea as a benign condition.

1.3.2 THE FISCAL BURDEN

The fiscal burden of dysmenorrhoea and pelvic pain affects both individuals and society as a whole. As outlined below, affected individuals suffer losses in educational achievement, incur direct health related expenses, and underachieve in workplace participation. Absolute costs are dependent on the inclusion criteria of the study, and whether or not both the direct medical costs, and the indirect personal costs borne by the individual, are included.

In 1988, Dawood estimated that with an estimated 10%–30% of all working or studying women in the USA losing 1–2 working days per month due to dysmenorrhoea, this equated to an annual loss of 600 million working hours, or up to $US2 billion in business revenue (Dawood, 1988). Personal costs borne by the individual include the impact on family income through reduced workplace participation, the direct costs of health care, impaired personal relationships, and reduced physical and emotional wellbeing (De Graaff et al., 2013). A US-based study in 1996 estimated that annual direct medical costs for outpatient visits for chronic pelvic pain in women aged 18-50 years was $US881.5 million. Among 548 employed respondents, 15% reported time lost from paid work and 45% reported reduced workplace productivity (Mathias et al., 1996).

In addition to the direct costs for providing health care to those affected, society suffers economic losses due to lost workplace productivity, lost caregiver workplace participation, and increased reliance of the individual on living assistance programs. The 2011 study by Bush, Evans and Vancaillie entitled *The $6 billion woman and the $600 million girl: The Pelvic pain report* estimated that pelvic pain costs the Australian economy $6.6 Billion annually (Bush et al., 2011).

Pelvic pain is a frequent cause of emergency department presentation and hospital admission. However, accurate information regarding the utilisation of inpatient and emergency health services by girls and women with pelvic pain is hampered by poor data collection. For example, the justification for hospital admission recorded in health records may be for the

exclusion of pelvic inflammatory disease, or for the management of menstrual disorders, uterine fibroids, or ovarian cysts, rather than for the management of CPP (Velebil et al., 1995; Whiteman et al., 2010). Once in hospital, the reason for admission may be categorised according to the surgical procedure undertaken, rather than the medical condition diagnosed.

In a 2011 European study of 909 women attending an endometriosis referral centre, Simoens et al. (2014) found that a diagnosis of endometriosis is associated with an average of 11 hours of lost workplace productivity per week. This study estimated an average annual cost per woman with endometriosis of €9,579 which included €6,298 in lost productivity and €3,113 in health care costs. Health care costs included surgery (29%), monitoring tests (19%), hospitalisation (18%) and physician visits (16%). In a 2017 US-based prospective study of 113,506 women with endometriosis and 927,599 controls, the direct and indirect costs associated with a diagnosis of endometriosis were assessed. This study showed that in the 12 months following study enrolment, costs associated with endometriosis patients ($16,573) were over three times higher than for matched non-endometriosis patients ($4,733) (Soliman et al., 2018).

With regard to the direct costs for endometriosis-related inpatient hospital care in Australia, Medicare records reveal that over the 2016-2017 financial year, there were 34,200 endometriosis-related hospital admissions (*Endometriosis in Australia: Australian Institute of Health and Welfare*, 2018). Seventy-nine percent of these inpatient care episodes were among girls and women aged 15-44 years. In 2019, accounting firm Ernst and Young released the comprehensive report entitled *The cost of endometriosis in Australia* (2019.), prepared in collaboration with endometriosis advocacy group EndoActive. This estimated the cost of endometriosis in Australia for the 2017/2018 financial year at AUD 7.4 Billion.

In summary, dysmenorrhoea, pelvic pain and endometriosis are conditions with substantial worldwide economic impact, both on the individual affected and society as a whole (Hofmeyr, 1996; Jones, 2004). When considering the age of those affected and the potential impact that health policy changes might achieve, the effective management of dysmenorrhoea-related pelvic pain offers an extraordinary opportunity to improve a country’s economy and workplace productivity.

1.4 The Aetiology of Dysmenorrhoea, Chronic Pelvic Pain and Endometriosis

Overlapping, yet distinct, aetiologies have been advanced for the development of dysmenorrhoea, chronic pelvic pain and endometriosis.

1.4.1 The aetiology of dysmenorrhoea

The pain of dysmenorrhoea is commonly believed to be due to contraction of the uterine myometrium causing pain associated with ischaemia. Moir (1934) demonstrated that an increase in intrauterine pressure to 120mm of mercury triggered feelings of heaviness and discomfort, with a loss of uterine vascular pulsations, while an increase in intrauterine pressure to 150mm of mercury was associated with severe pain. The discovery of prostaglandins in 1935, and the subsequent research by Dawood et al. (1988), provided a mechanism for these contractions. Subsequent research has refined this concept. The enzyme cyclo-oxygenase that catalyses the formation of prostaglandins within the endometrium is inhibited by progesterone during the secretory phase of the menstrual cycle (Evans and Salamonsen, 2012). Progesterone withdrawal following the resolution of the corpus luteum allows levels of cyclo-oxygenase to rise, enhancing prostaglandin production from its precursors. The release of prostaglandins and the closely related leukotrienes from within the uterus stimulates uterine contractions, myometrial ischaemia and pain.

Ample clinical evidence supports this theory. Firstly, women with dysmenorrhoea have higher levels of prostaglandins in both the endometrium and their systemic circulation than women without dysmenorrhoea (Lundström et al., 1979). Secondly, women with dysmenorrhoea display higher levels of uterine contractility, and an increased number of uncoordinated contractions at menstruation, than women without dysmenorrhoea (Åkerlund, 1979; Dawood, 1990). Thirdly, these uncoordinated uterine contractions are associated with reduced myometrial blood flow and ischaemia (Altunyurt et al., 2005). Fourthly, the release of prostaglandins at menstruation is consistent with additional systemic symptoms commonly reported by women during menstruation, including an elevated body temperature, diarrhoea, headaches, and sleep disruption (Hayaishi & Matsumura, 1995). Fifthly, the administration of prostaglandins vaginally or systemically induces uterine contractions, and systemic symptoms such as nausea, diarrhoea and vomiting (Coco, 1999; Dawood & Huang, 1998; Proctor & Farquhar, 2006). Finally, drugs known to block the conversion of arachidonic acid to prostaglandins, by inhibiting the enzyme cyclo-oxygenase, are effective in reducing menstrual

fluid prostaglandin levels (Dawood & Khan-Dawood, 2007), menstrual pain (Marjoribanks et al., 2015), and sleep disruption (Iacovides et al., 2009). These commonly prescribed non-steroidal anti-inflammatory drugs (NSAIDs) include ibuprofen, naproxen, diclofenac, mefenamic acid, and celecoxib amongst others (Chantler et al., 2008, 2009). While NSAIDs are effective treatments for dysmenorrhoea in some, but not all, women with dysmenorrhoea, they may be associated with adverse effects including gastrointestinal bleeding (Campbell & Mcgrath, 1999; Oladosu et al., 2018; Owen, 1984).

1.4.2 THE AETIOLOGY OF CHRONIC PELVIC PAIN

Multiple medical conditions, some present in men as well as women, may pre-date CPP (Moore & Kennedy, 2000). This thesis investigates only those women with pelvic pain where dysmenorrhoea is a symptom. In a study of 3,916 women from the United Kingdom, Zondervan compared the rate of dysmenorrhoea among women who menstruated. Dysmenorrhoea was present in 81% of women with CPP, compared with only 58% of women without CPP (Zondervan et al., 2001). Anecdotally, women with CPP frequently describe a progression of symptoms over their menstrual life, with increasing days per month of pain, an increasing complexity of pain symptoms, and with dysmenorrhoea as their first pain symptom. The potential for severe dysmenorrhoea to progress to chronic pelvic pain over time has been considered by Hardi et al. (2014) using a retrospective questionnaire. One hundred women with CPP, where dysmenorrhoea was a symptom, were asked to report their age at menarche, their age when dysmenorrhoea began, and their age when pain transitioned from pain exclusively with menstruation (dysmenorrhoea alone) to pain on most days (CPP). Their study found that 16% of women transitioned from dysmenorrhoea to CPP within 12 months of developing severe dysmenorrhoea, while over 50% had transitioned within 12 years (Figures 1.8a and 1.8b).

In a survey questionnaire of 1,012 reproductive-aged women, Westling et al. (2013) considered a range of pain-related symptoms including dysmenorrhoea, non-cyclic pelvic pain, mood, fatigue and somatic complaints. They concluded that dysmenorrhoea is an aetiological factor in non-cyclic pelvic pain.

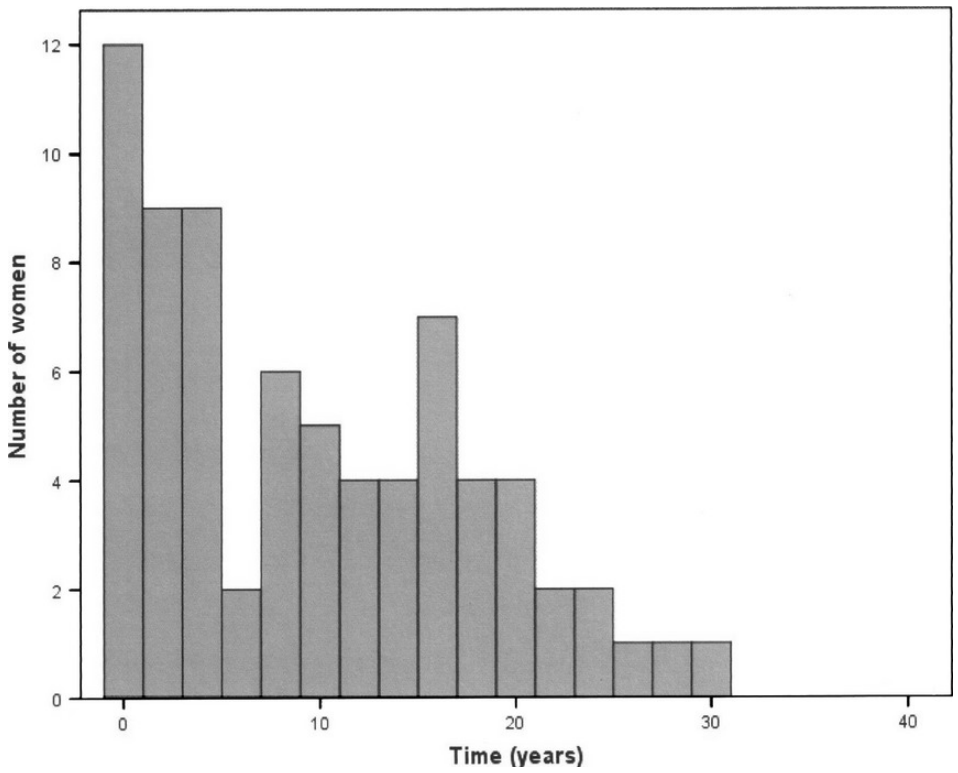

Figure 1.8a: Time in years from first dysmenorrhoea to transition to chronic pelvic pain.

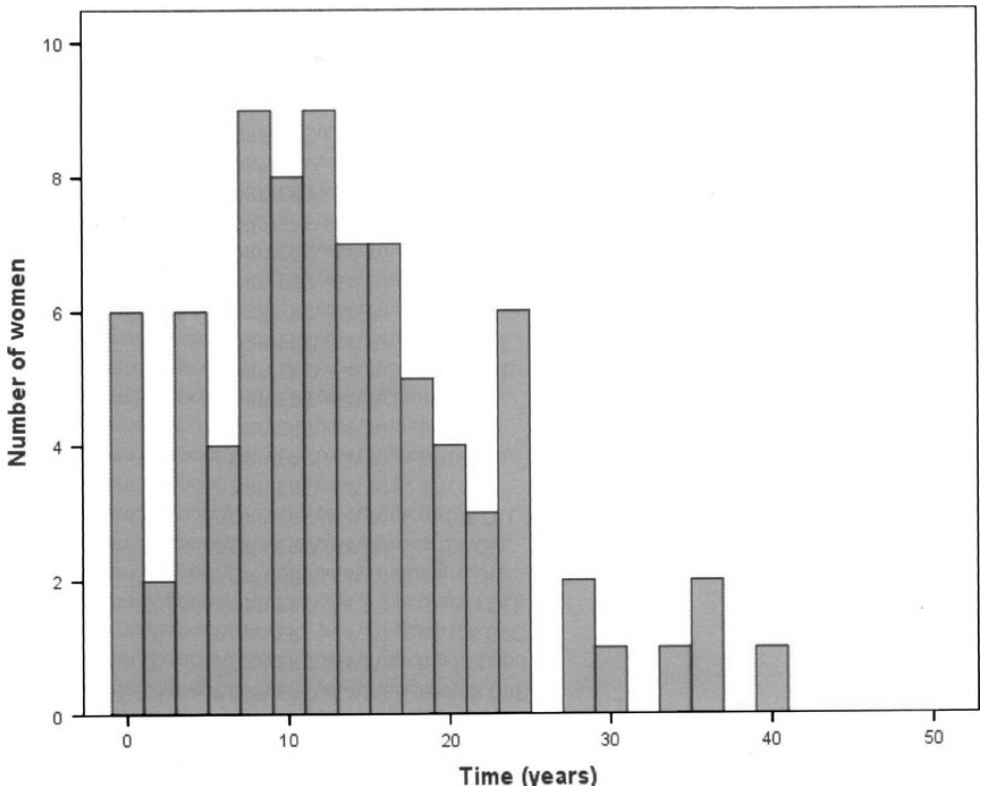

Figure 1.8b: Time in years from menarche to transition to chronic pelvic pain

The mechanism by which dysmenorrhoea predisposes women to chronic pelvic pain remains unknown. The combination of abnormal visceral sensations across a range of pelvic viscera is consistent with viscero-visceral hyperalgesia, a phenomenon of central sensitisation (Giamberardino et al., 2010), and is discussed further in section 1.4.4. However, the mechanism by which this occurs requires further investigation. Jarrell and Arendt-Nielsen (2016) considered the development of CPP following dysmenorrhoea from an evolutionary perspective as a consequence of reduced fertility and recurrent menstruation. The increase in menstrual cycles experienced by women in the modern era is presented in Figure 1.9. Anecdotally, girls with severe dysmenorrhoea often report severe pain from an early age or even from their first period, when they have had few menstrual cycles.

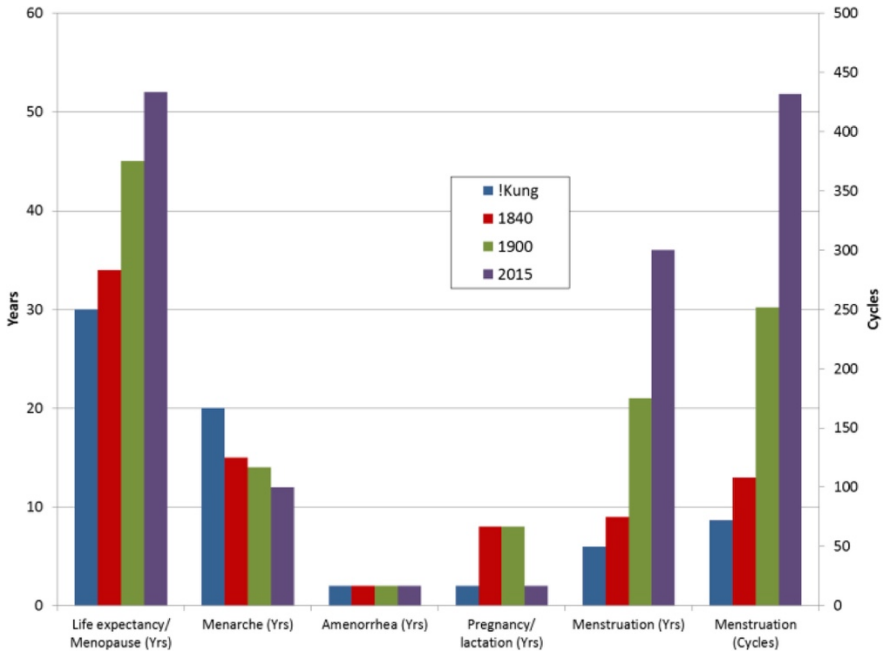

Figure 1.9: Estimation of total number of menstrual cycles and additional menstrual parameters experienced by women in modern and ancient times. Reproduced with permission (Jarrell & Arendt-Nielsen, 2016).

In medical practice, dysmenorrhoea may be present as a primary condition, or in association with a range of diagnosable medical conditions, including endometriosis, adenomyosis, pelvic inflammatory disease, cervical stenosis, sub-mucosal uterine fibroids, and the use of intra-uterine contraceptive devices. The clinical overlap of symptoms between these causes of secondary dysmenorrhoea is common. For example, adenomyosis, a condition where

endometrial glands and stroma are found within the myometrium of the uterus, shares similar symptomatology with endometriosis (Bird et al., 1972). In practice, despite the desirability of full clinical assessment, many women currently receive no diagnosis for their pain despite attending multiple medical consultations (Grace & Zondervan, 2006). Their management is empirical, based on their presenting symptoms, clinical examination, and the preference of their treating doctor.

Of particular interest for the current thesis is the possibility that prolonged, repeated episodes of dysmenorrhoea, often with associated sleep disturbance, may have important long-term implications for the development of chronic pain symptoms in the future. Hunter et al. (2001) demonstrated that repeated episodes of pain are predictive of future pain experiences, and may predispose the patient to a range of pain conditions, including post-surgical pain, over the individual's lifetime (Katz & Seltzer, 2009). Schuh-Hofer et al. (2013), found that one night of sleep deprivation in healthy volunteers was sufficient to induce generalised hyperalgesia and increased anxiety scores, a finding confirmed by Moldofsky, Scarisbrick, and McBeth (McBeth et al., 2015; Moldofsky & Scarisbrick, 1976).

1.4.3 THE AETIOLOGY OF ENDOMETRIOSIS

Endometriosis is an ancient disease, and one which was described in European texts as early as 300 years ago (Knapp, 1999). It may also represent one of the medical conditions underlying the symptoms of hysteria, first described in the second millennium BC, the first mental health condition solely attributed to women (Tasca et al., 2012).

Retrograde menstruation, where menstrual blood passes in a retrograde fashion along the fallopian tubes carrying viable endometrial cells to the peritoneal cavity, has been considered an important factor in the development of endometriosis since it was first described by Sampson in 1927 (Sampson, 1927). "Sampson's Theory" postulates that endometrial cells that reach the peritoneal cavity are deposited, implant, and develop into endometriotic lesions. This theory is supported by the higher prevalence of endometriosis in women with increased peritoneal exposure to menstrual fluid, through heavier menstrual loss, shorter menstrual cycles, or earlier menarche (Missmer & Cramer, 2003). However, retrograde menstruation does not fully explain the presence of endometriosis in all women. Retrograde menstruation occurs in up to 90% of women (Halme et al., 1984), yet endometriosis affects only an estimated 5%-10% of women. In addition, retrograde menstruation does not explain the rare

finding of endometriosis lesions present outside the abdominal cavity (Goldberg & Davis, 2016).

As a variant of Sampson's theory, it has recently been suggested that pluripotent endometrial stem cells may spread to the peritoneal cavity following retrograde bleeding in the neonatal period with the withdrawal of maternal steroid hormones (Brosens et al., 2013; Gargett et al., 2014). While only 5% of female neonates exhibit vaginal bleeding, the presence of a cervical mucus plug may result in occult bleeding, with endometrial cells passed in a retrograde fashion to the peritoneal cavity without the passage of blood from the vagina. This potentially explains the uncommon situation of premenarchal endometriosis in girls with a normal reproductive tract, as documented by Marsh and Laufer (2005).

An alternative "Theory of Coelomic Metaplasia" postulates that pluripotent mesenchymal cells present in the abdominal peritoneum exhibit endometrial differentiation. This theory, first proposed by Meyer (1924), has been further investigated by Ferguson and Figueira et al. (Ferguson et al., 1969; Figueira et al., 2011). This theory provides an explanation for the development of endometriosis lesions outside the abdominal cavity, within the rectovaginal septum, in women with congenital absence of the uterus, and in the rare situation of endometriosis in men (Taguchi et al., 2012).

The "Theory of Metastatic Lymphatic Spread", where viable endometrial cells spread to distant sites via lymphatic channels, was first proposed by Halban in 1925, and discussed together with other theories in an article published by the *Journal of the American Medical Association* (1929) soon afterwards. It is supported by the finding of endometriotic tissue in lymph nodes by Mechsner and Berbic (Berbic et al., 2013; Mechsner et al., 2008). Intriguingly, the potential for lymphatic spread offers a rich opportunity for interaction between the endometrium and the innate immune system. The relationship between endometriosis and immune function is further outlined in Section 1.4.5.

Genome-wide association studies have identified a large number of potential genetic loci associated with the presence of endometriosis (Borghese et al., 2017; Rahmioglu et al., 2015; Treloar et al., 2005), with the largest meta-analysis identifying 14 common genetic loci of particular interest (Sapkota et al., 2017). Associated processes implicate cell adhesion, angiogenesis, inflammation and hormonal pathways in endometriosis development, with the strongest genetic loci associations found in women with more severe lesions (Sanchez et al., 2015).

Epigenetic mechanisms acting via methylation may promote epithelial-mesenchymal transition (EMT) in endometrial lesions (Hsiao et al., 2017), which is further discussed below in section 1.5.3. However, current studies suggest that a large number of genetic loci may predispose women to endometriosis, with each genetic locus affecting risk to a small degree, but with no single gene responsible. Unfortunately, genetic studies have rarely been correlated with reported symptoms, including pain (Sapkota et al., 2017). Gao et al. (2019a) investigated a cohort of 3,406 Swedish women born between 1933 and 1972, and found a lower birth weight for gestational age among the 111 women subsequently diagnosed with endometriosis (hazard ratio 1.35 per standard deviation decrease; 95% CI 1.08 to 1.67), providing support for the presence of developmental aetiological factors.

Irrespective of the origin of endometrial cells, the presence of other factors appears necessary to allow endometriosis lesions to develop in individual women. Other factors potentially of importance include a favourable endocrine, immune or inflammatory environment, or reduced clearance and increased persistence of endometrial cells within the peritoneal cavity.

Environmental factors, including increased levels of organochlorines, have been proposed to increase the prevalence of endometriosis lesions. Heilier et al. (2005) investigated 25 women with peritoneal endometriosis, 25 women with deep endometriotic nodules and 21 control women. They found increased levels of polychlorinated dibenzo-*p*-dioxin (PCDD), polychlorinated dibenzofuran (PCDF), and polychlorinated biphenyl (PCB) serum concentrations in women with endometriosis than controls, with an odds ratio of 3.3. However, other studies have reported conflicting results, as summarised by Heilier et al. (2007). They observe that the presence of these organochlorines is not sufficient for the development of endometriosis, as endometriosis was present through history prior to the development of these chemicals, and that not all women exposed to high levels of organochlorines develop endometriosis.

In summary, while mechanisms for each condition have been proposed, much remains unknown regarding the aetiology of dysmenorrhoea, chronic pelvic pain, and endometriosis, and the relationship between these conditions.

1.4.4 THE ROLE OF PERIPHERAL AND CENTRAL PAIN SENSITISATION IN DYSMENORRHOEA, CHRONIC PELVIC PAIN AND ENDOMETRIOSIS

Central sensitisation represents a heightened responsiveness to normal peripheral inputs (Woolf & Salter, 2000; Woolf, 2007), presenting as an amplified pain response. It may develop in the absence of peripheral tissue injury or inflammation, as in the central pain sensitisation of fibromyalgia or tension headache syndromes (Yunus, 2007, 2008), or develop following repeated episodes of peripheral pain (Apkarian et al., 2005; Bajaj et al., 2002; Hermann et al., 2008). The repeated monthly experience of dysmenorrhoea, with peripheral prostaglandin release, severe stress, disrupted sleep, and reduced quality-of-life, provides a potent environment for the development of central sensitisation, and the evidence that this occurs is compelling.

Women with dysmenorrhoea display increased pain perception throughout the menstrual cycle at sites both within and outside the pelvis, to a variety of stimuli, when compared with controls (Bajaj et al., 2002; Granot et al., 2001; Iacovides et al., 2013; Jarrell & Arendt-Nielsen, 2016; Slater et al., 2015; Smorgick et al., 2013). The central nature of pain in women with dysmenorrhoea has also been suggested by initial functional magnetic resonance imaging (fMRI) studies, where changes to gray matter within the brain of women affected by pain were noted (Vincent et al., 2011).

Vincent et al. investigated 10 women with dysmenorrhoea and 10 pain-free controls. During menstruation, deactivation of brain regions in response to noxious stimulation was observed in control women but not in women with dysmenorrhoea (Figures 1.10a and 1.10b). When the women were not menstruating, activity in the entorhinal cortex appeared to mediate increased pain responses in women suffering from dysmenorrhoea.

Figure 1.10a: Comparison of response to a noxious thermal stimulus of the left arm and midline lower abdomen during menstruation in women with dysmenorrhoea and control women with no pain. Areas of activation are shown in yellow, and areas of deactivation are shown in blue. Reproduced with permission (Vincent et al., 2011)

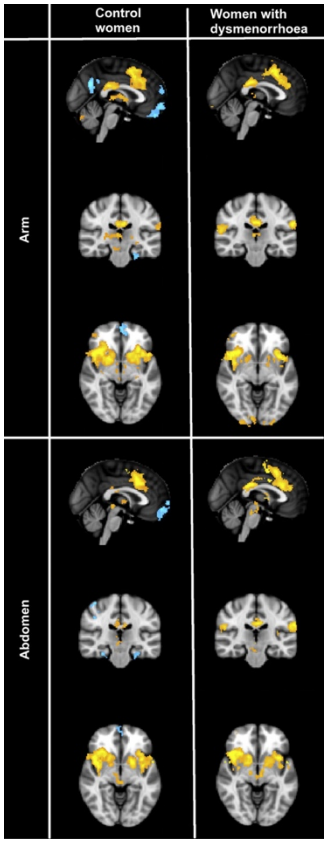

Figure 1.10b: Summary of regions deactivated by noxious thermal stimulation of the left arm and midline lower abdomen in women with and without dysmenorrhoea during the menstrual phase. Reproduced with permission (Vincent et al., 2011)

Region of deactivation	Arm		Abdomen	
	Control women (z score)	Women with dysmenorrhoea (z score)	Control women (z score)	Women with dysmenorrhoea (z score)
Precuneous	4.31	–	–	–
Frontal medial cortex	4.28	–	3.98	–
Left temporal fusiform cortex	4.32	–	4.22	–
Right temporal fusiform cortex	3.57	–	4.26	–
Left occipital cortex	4.33	–	4.01	4.23
Right occipital cortex	3.89	–	–	3.80
Left entorhinal cortex	3.62	–	3.58	–
Right entorhinal cortex	–	–	3.32	–
Right primary somatosensory cortex	–	–	3.74	–

Results are expressed as peak z scores derived from a mixed-effects analysis with outlier deweighting and a threshold at $z > 3.0$, $P < .05$.
–, No significant activations in this region.

Jarrell & Arendt-Nielsen (2016) considered that evolutionary human reproductive factors associated with increased menstrual exposure, including early menarche and delayed childbearing, may predispose women with dysmenorrhoea to transition to chronic pelvic pain through the repeated experience of menstrual pain. Sinaii et al. (2002) found a higher prevalence of both fibromyalgia symptoms and chronic fatigue syndrome in women with endometriosis when compared with controls. Fibromyalgia and chronic fatigue syndrome are both medical conditions that are associated with central pain sensitisation (Fleming & Volcheck, 2015; Woolf, 2011).

Clinically, the presence of central sensitisation provides a rationale for multiple and complex symptom profiles, possibly via the twin mechanisms of viscero-visceral and viscero-somatic hyperalgesia. Viscero-visceral hyperalgesia occurs where pain conditions in one visceral organ enhance the likelihood of pain symptoms in a second visceral organ with which it shares a common central projection pathway in the spinal cord (viscera-visceral convergent neurons). The uterus, colon and bladder share central projection pathways via the dorsal horn of T10-L1 spinal segments (Giamberardino et al., 2006), and an association between pain symptoms in these organs has been demonstrated. Hellman found significantly more urinary symptoms and bladder filling pain in women with severe dysmenorrhoea when compared with dysmenorrhoea-free controls (Hellman et al., 2018). Brinkert et al. (2007) used anal manometry to demonstrate significantly lower colonic distension volume pain thresholds in women with severe dysmenorrhoea when compared with dysmenorrhoea-free controls In addition, Chaban et al. (2012) used retrograde labelling of sensory afferent nerves in a rat model to demonstrate that in a proportion of cases, a single sensory afferent nerve may innervate both the uterus and the bowel, providing even more opportunity for trans-visceral sensitisation (Figure 1.11).

Viscero-somatic hyperalgesia occurs where pain conditions in a visceral organ are associated with pain symptoms in the somatic tissues (skin, muscle, tendon or joint) with which it shares a common central projection pathway in the spinal cord (viscera-somatic convergent neurons). Long-lasting somatic pain sensitisation has been demonstrated in women with severe dysmenorrhoea by Iacovides et al. (2015a). Women experienced a significantly increased sensitivity to experimental deep muscle pain in both pelvic (lower abdomen) and extra-pelvic (forearm) areas.

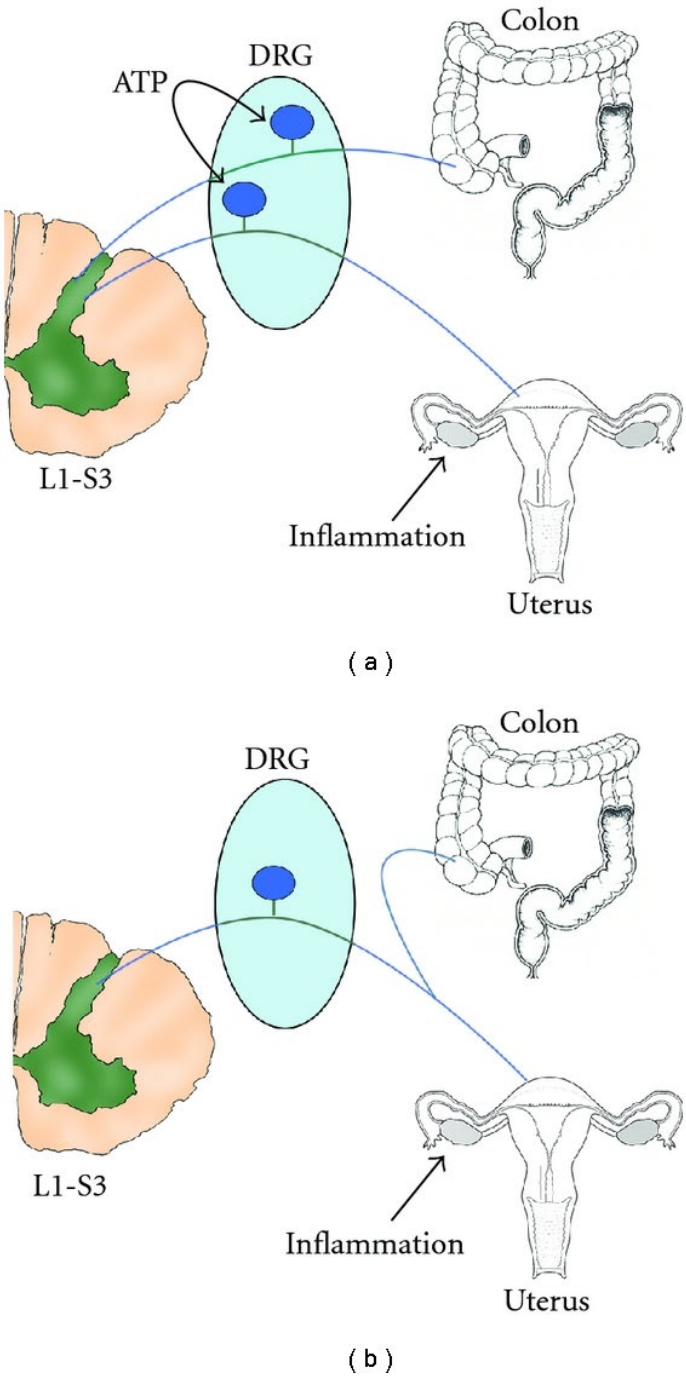

Figure 1.11: Models of alternative possibilities for viscero-visceral cross-sensitisation in the DRG neuron. (a) ATP released by a neuron innervating the inflamed uterus acts on a neighbouring neuron sensitising its responses to colonic distention. (b) The same neuron innervates both the uterus and the colon. Reproduced with permission Professor Victor Chaban

Viscero-visceral and viscera-somatic hyperalgesia fit well with the potential for additional pain, or pain-related, symptoms in women with dysmenorrhoea (Chaban, 2013, 2015; Giamberardino, 1999, 2003; Giamberardino et al., 2010). Researchers have sought to elucidate whether sensitisation predates the transition from dysmenorrhoea alone to chronic pain, or develops during the transition period. Dysmenorrhoea alone is variably classified or excluded as a chronic pain condition in clinical practice, due to variable interpretations of the definitions of chronic pain. Despite the clinical ambiguity, somatic muscle pressure testing (Tu et al., 2009; Tu et al., 2012), and fMRI (Vincent et al., 2011) support the presence of central sensitisation in women with dysmenorrhoea who are otherwise well. Tu et al. compared 19 women with CPP and 12 pain-free controls, and demonstrated a reduced pressure pain threshold at external sites such as hip, knee or shoulder, in women with pelvic pain. Vincent (2011) found that control women, but not those with dysmenorrhoea, displayed deactivation of areas of the brain during menstruation that may be protective from dysmenorrhoea. These fMRI findings suggest that women with dysmenorrhoea have both amplified pain facilitation and impaired pain inhibition. Similar fMRI findings have been found in patients with Irritable Bowel Syndrome by Chen et al. (2011) and Blackstein et al. (2010), and by Woodworth et al. (2018) in patients with urologically-based chronic pelvic pain. These studies further support a role for central sensitisation in visceral pain syndromes. In an elegant study of cutaneous temporal pain summation in 18 women with chronic pelvic pain and 18 pain free controls, Thompson et al. (2019) assessed the pain scores reported following 3 strokes of a cotton tip swab on the abdomen. Women with pelvic pain and allodynia showed significantly increasing pain with successive strokes of the cotton tip swab: ($p = 0.012$ for strokes 1 vs. 2, $p = 0.026$ for strokes 2 vs. 3, and $p = 0.005$ for strokes 1 vs. 3). This effect is consistent with the wind-up phenomenon found in association with central pain sensitization, where there is an increase in pain intensity over time when a given stimulus is delivered repeatedly above a critical rate.

With regard to the presence of central sensitisation in women with endometriosis, As-Sanie et al. (2012) used functional MRI to investigate the relationship between the presence of endometriosis lesions and the experience of chronic pelvic pain in 61 women divided into four groups: 17 with endometriosis and CPP, 15 with endometriosis but no CPP, 6 with CPP but no endometriosis, and 23 healthy controls. When the volumetric assessment of brain gray matter was compared between the groups, women with CPP showed decreases in gray matter volume in the left thalamus, regardless of whether or not endometriosis was present. Women with endometriosis but without CPP showed similar gray matter volumes when compared with normal controls. This finding further dissociates the presence of endometriosis lesions with the experience of chronic pelvic pain. While the conditions are commonly associated, they should not be considered as causal in nature.

At the level of peripheral tissues, there are several mechanisms by which chronic peripheral nociceptive input (e.g. dysmenorrhoea) may increase responsiveness of dorsal horn neurons and promote peripheral or central sensitisation. Normally present nociceptors may become activated, silent nociceptors may be sensitised by inflammation, or there may be a sprouting of new nociceptors resulting in a greater total number of nociceptors (Evans et al., 2007; Mckinnon et al., 2015).

The central mechanisms contributing to central sensitisation are less easy to assess. Extensive investigation of pain thresholds to a variety of painful stimuli in women with dysmenorrhoea have been inconclusive. Iacovides (2015b) summarised these studies and the possible reasons for their inconsistent outcomes. Variations were seen in menstrual cycle phase, the measurement of gonadal hormones, the presence or absence of dysmenorrhoea at the time of testing, and the choice of pain stimuli used. Despite the difficulties encountered, Iacovides concludes that:

> When taken together, the majority of recent studies that have considered tissue depth, areas within and outside of the referred area of pain and controlled for menstrual cycle phase, have shown that across the menstrual cycle, dysmenorrheic women are hypersensitive to experimental pain compared with controls (Iacovides et al., 2015b, p766)

Whether sleep disruption pre-dates the development of dysmenorrhoea or chronic pain is yet to be determined. While pain may disrupt sleep, it is also recognised that a poor night's sleep may pre-date a day of increased pain (Cosar et al., 2014; Gupta et al., 2014; Harrison et al., 2016), and that the relationship of pain to sleep may be bi-directional. Schwertner found a 39% reduction in daily pain scores and a 38% reduction in dysmenorrhoea in women with endometriosis treated with melatonin to improve sleep quality, when compared with untreated controls (Schwertner et al., 2013).

Despite the compelling evidence for central and peripheral sensitisation of pain and the beginnings of a multi-disciplinary approach (Jarrell et al., 2005, 2018), clinical practice continues to rely almost exclusively on either the surgical excision of endometriosis lesions, or hormonal suppression when managing dysmenorrhoea or pelvic pain (Johnson et al., 2013; Leyland et al., 2010; Taylor et al., 2012).

1.4.5 THE ROLE OF INFLAMMATION AND THE IMMUNE SYSTEM IN DYSMENORRHOEA, CHRONIC PELVIC PAIN AND ENDOMETRIOSIS

This thesis seeks to describe and understand new mechanisms for dysmenorrhoea and pelvic pain. The role of inflammation offers a potential new approach to our understanding of these conditions, with an evolving, but not yet established, evidence base. Normal menstruation has been described as an inflammatory event (Evans & Salamonsen, 2012), due to the endometrial edema, influx of leukocytes (Berbic et al., 2014), and subsequent breakdown of tissue that occurs prior to and during menstruation. The leukocytes found within the endometrium include macrophages, mast cells, dendritic cells, neutrophils, eosinophils and regulatory T-cells (Berbic et al., 2014): all cells of the innate or adaptive immune system with the ability to secrete neurostimulatory factors that may sensitise neurons and modulate the experience of pain (Evans & Salamonsen, 2012). Following extensive research, Evans and Salamonsen conclude that with normal menstruation:

> A cascade of events is initiated by progesterone withdrawal achieving a threshold of inflammation after which tissue destruction is inevitable. Progesterone withdrawal sits at the summit of this cascade leading to removal of protective mechanisms, induction of cytotoxic signals, activation of inflammatory signaling pathways (NF-κB) proceeding to production of inflammatory factors (chemokines, cytokines and prostaglandins), recruitment of inflammatory leukocytes and protease activity (Evans & Salamonsen, 2012, pp284-285)

However, while all women menstruate, and therefore experience a certain degree of inflammation within the uterus on a monthly basis, only a proportion of women suffer severe pain. The potential for excessive inflammation to cause menstrual dysfunction, including dysmenorrhoea, must involve additional factors.

Human studies consistently demonstrate evidence of an association between inflammation, immune factors, and the presence of dysmenorrhoea, pelvic pain or endometriosis. Clinically, this is reflected in the ubiquitous use of non-steroidal anti-inflammatory medications for the clinical management of symptoms. In a Cochrane meta-analysis of 80 randomised controlled trials with 5,820 women, Marjoribanks et al. (2015) found that between 38% and 45% of women using a non-steroidal anti-inflammatory drug (NSAID) received moderate or excellent relief of dysmenorrhoea, compared with only 18% of women taking placebo.

Inflammation acts via the increased cell secretion of cytokines, small peptides or proteins that affect the function of other cells. Cells able to secrete cytokines include, among others, the glandular cells of the endometrium (Hannan et al., 2011), glial cells of the central nervous system, and the macrophages found in peritoneal fluid and peripheral blood. Cytokines regulate T-cell functions including the initiation and maintenance of pain (Scholz & Woolf, 2007; Sommer et al., 2018; Sommer & Kress, 2004). Altered cytokine production in peripheral tissues, infiltrating leukocytes, and non-neuronal (glial) components of the CNS can become drivers for activation of the immune system (Bayas et al., 2002; Ledeboer et al. 2005; Cao & DeLeo 2008; Milligan & Watkins 2009). Ma et al. (2013) studied monocytes in the peripheral blood of women with dysmenorrhoea, and found up-regulation of genes coding for pro-inflammatory cytokines, and down-regulation of genes coding for anti-inflammatory responses. These changes were present throughout the menstrual cycle, and not only at menstruation.

Immune system changes in women with endometriosis were first considered in the 1980s, when abnormal polyclonal B-cell activation (Gleicher et al., 1987), and increased numbers of T- and B- lymphocytes in peritoneal fluid and peripheral blood compared with controls, were discovered (Badawy et al., 1987). High serum concentrations of IgG and IgM autoantibodies to endometrial cells (Wild & Shivers, 1985) in women with endometriosis were described. Epidemiologically, women with endometriosis are known to have a higher risk of developing auto-immune inflammatory disorders including systemic lupus erythematosis (SLE), Sjögren's syndrome, multiple sclerosis, and rheumatoid arthritis (Harris et al., 2016; Nielsen et al., 2011). These autoimmune conditions are all associated with the presence of pain and elevated levels of cytokines (Nothnick, 2001).

Trabert et al. (2011) found a 22% reduction in a subsequent diagnosis of endometriosis over the next ten years in women who ate a diet high in anti-inflammatory omega-3 fatty acids, when compared with a diet low in omega-3 fatty acids. However, inflammation and pain may be present without endometriosis lesions. Thomson and Redwine (2005a) investigated the histological findings from peritoneal biopsies in 3,238 women undergoing laparoscopy. In the 24% of women where no endometriosis was found, chronic peritoneal inflammation was present in 15.7% of women with pain, compared with 0.0% of women without pain. Thomson further described the clinical findings in a subsequent group of 40 women with chronic pain, endometriosis excluded and either histological or serological evidence of inflammation Of these women, eleven women were treated with the immune-modulator medication hydroxychloroquine, with 9 describing a resolution of pain symptoms, and 2 describing an 80% reduction in pain symptoms.

Endometriosis is now described as a chronic inflammatory disorder (Lousse et al., 2012). Endometriosis is associated with an increased presence of macrophages within the peritoneal cavity of affected women, higher levels of macrophage derived cytokines, and impaired macrophage phagocytic activity (Wu et al., 2005). In a further association between endometriosis and inflammation, Cicinelli found a higher prevalence of chronic endometritis in women with endometriosis compared to healthy controls. Thirty-three of 78 (42.3%) affected women vs. 12 of 78 (15.4%) healthy controls showed signs of endometritis according to hysteroscopy. Thirty of 78 (38.5%) affected women vs. 11 of 78 (14.1%) healthy controls showed signs of endometritis according to histology (Cicinelli et al., 2017).

The cytokines and neurotrophic factors that have been most researched and associated with dysmenorrhoea, pelvic pain and endometriosis include Interleukin 1-beta (IL-1β), tumour necrosis factor-alpha (TNF-α), and Nerve Growth Factor (NGF). Interleukin 1-beta (IL-1β) is an acute phase inflammatory cytokine released by mononuclear and epithelial cells in response to injury that leads to inflammation. Scholl et al. (2009) investigated peritoneal fluid sampled at laparoscopy, and found a correlation between levels of IL-1β and both the severity of endometriosis lesions present, and the severity of pain reported. Sikora et al. (2012, 2015) found higher levels of IL-1β in the peritoneal fluid and the blood of women with endometriosis.

Tumour necrosis factor alpha (TNF-α) is produced by activated macrophages, natural killer (NK) cells, and T-helper-1 (Th1) cells (Cameron & Kelvin, 2003). It acts synergistically with IL-1β to activate the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) inflammatory pathway. The ability of TNF-α to induce neuropathic hyperalgesia (Sommer & Kress, 2004) and to contribute to chronic inflammatory pain (Pezet & McMahon, 2006) is well established. It has been suggested that TNF-α is one of the essential factors for the pathogenesis and maintenance of endometriosis (Bullimore, 2003). Consistently raised levels of TNF-α have been found in the peritoneal fluid and eutopic endometrium of women with endometriosis (Eisermann et al., 1988; Pizzo et al., 2003; Salmeri et al., 2015).

Nerve Growth Factor (NGF) is a neuropeptide involved in the growth and survival of neurons. Alterations in NGF expression are known to contribute to chronic inflammatory pain (Pezet & McMahon, 2006). An increase in NGF expression has been demonstrated in patients with painful bladder symptoms (Jacobs et al., 2009), and intense immune-reactivity for NGF has been reported in both eutopic endometrium and peritoneal endometriotic lesions (Tokushige et al., 2006; 2007; 2008; 2010; 2011; Wang et al., 2009).

In a further potential mechanism associating cytokines with dysmenorrhoea, both TNF-α and IL-1β are capable of inducing cyclooxygenase-2 (COX-2) expression, with potential for increased prostaglandin E2 (PGE2) production (Wu et al., 2010). A positive feedback cycle then results, with increasing PGE2 inducing COX-2 expression, resulting in further increases in PGE2 with inflammation and pain. Despite the consistent findings relating IL-1β, TNF-α and NGF to both pain and endometriosis, levels of these cytokines in blood have not reliably correlated with the severity of pain symptoms or the severity of endometriosis (Overton et al., 1996).

In summary, studies to date have investigated inflammation and immunity in the periphery by investigating peripheral blood, peritoneal fluid, eutopic endometrium, and endometriotic lesions. Measurable changes have been found. However, these do not reliably correlate with a woman's experience of pain, nor provide a reliable way of predicting the presence or absence of endometriosis. Additional factors apart from the presence or absence of endometriosis appear to drive a woman's lived experience of pain. Research has been **endometriosis lesion-focused** rather than **pain-focused**, and new treatments specifically targeting inflammation in pelvic pain are yet to be developed. The potential for novel clinical management discoveries to modulate inflammation and reduce the impact of pain on a woman's quality-of-life remains under-researched.

1.4.6 THE RELATIONSHIP BETWEEN DYSMENORRHOEA, PELVIC PAIN AND ENDOMETRIOSIS

Traditionally, dysmenorrhoea and pelvic pain have been linked to the presence or absence of visible abnormalities found at laparoscopy, most commonly endometriosis lesions. Where no visible cause for pain was found during laparoscopic examination, pain was considered unexplained. Certainly, as explained in Section 1.2.3, the prevalence of endometriosis lesions in women undergoing laparoscopy for the investigation of pain is high. In a study of 4,334 members of the United States-based Endometriosis Association who completed a questionnaire on their experience of diagnosis, 98.4% reported pain. Of these women, 84% reported menstrual pain and 67% reported non-menstrual pain (Greene et al., 2009). However, while this visual explanation for pain is convenient and easily accepted by patients and clinicians alike, the relationship between endometriosis and pain is far from clear. Many factors argue against a direct causal relationship between endometriosis lesions and pain.

Firstly, women may have endometriosis without pain. In a study of fertile women undergoing laparoscopy for tubal sterilisation purposes, 4% were found to have endometriosis (Eskenazi & Warner, 1997). Abbott et al. (2003) found that of 254 women with pelvic pain, only 176 had histologically proven endometriosis at laparoscopy. Even where endometriosis lesions are present, the severity of the condition according to the American Society of Reproductive Medicine classification illustrated in Figure 1.5 correlates poorly with the severity of pain (Vercellini et al., 2006; Vercellini, 1996).

Secondly, women may have pain without endometriosis. The studies of Eskenazi, Abbott, and Vercellini equally demonstrate that not all women with pain have endometriosis found at laparoscopic surgical assessment (Abbott et al., 2003; Eskenazi & Warner, 1997; Fauconnier & Chapron, 2005; Vercellini, 1996). This has been demonstrated previously in large studies, including those of Redwine and Janssen. Redwine studied 3,238 women undergoing laparoscopy for chronic pain, and found no endometriosis in 17.9% of women (Thomson & Redwine, 2005b). Janssen collated 15 studies of adolescent girls with dysmenorrhoea undergoing laparoscopy, and found no endometriosis in 38% of 880 girls (Janssen et al., 2013).

Thirdly, the site of endometriosis lesions correlates poorly with the site of pain. Among patients with endometriosis, the site and severity of pain are variably correlated with laparoscopic findings. Most consistently, deep infiltrating endometriosis (DIE) lesions of the uterosacral ligaments is associated with more severe pain, and DIE Lesions within the rectovaginal septum are associated with dyspareunia and dyschezia (Chapron et al., 2012). Roman found no correlation between the site of endometriosis and the presence of bowel symptoms in a study of 116 women (Roman et al., 2012).

Fourthly, both dysmenorrhoea and endometriosis are associated with extra-pelvic symptoms. Dysmenorrhoea is over-represented in women with chronic pain conditions outside the pelvis when compared to controls. These conditions include fibromyalgia, tension-type headaches (Yunus, 2007; 2008), migraine (Tietjen, 2007), Irritable Bowel Syndrome (Olafsdottir et al., 2012), and Painful Bladder Syndrome (PBS). Olafsdottir used a postal survey to investigate the frequency of dysmenorrhoea and irritable bowel syndrome in the Icelandic population. While the prevalence of IBS is estimated at between 10% and 15% in western societies, (Hungin et al., 2005; Müller-Lissner et al., 2001), Olafsdottir found a prevalence of 42% for IBS in women with dysmenorrhoea using the Manning criteria (Manning et al., 1978) in a random sample of 2000 participants.

Fifthly, pelvic pain may occur in men in the absence of dysmenorrhoea or endometriosis. In possibly the ultimate evidence that the presence of pain need not be associated with endometriosis, Clemens et al. (2014) of the Multidisciplinary Approach to the Study of Chronic Pelvic Pain (MAPP) Research Network, describes pelvic pain in men, where menstruation, and hormonal cycles do not exist. Despite these observations, Fauconnier and Chapron state that:

> Based on randomized trials against placebo, endometriosis appears to be responsible for chronic pelvic pain symptoms in more than half of confirmed cases. A causal association between severe dysmenorrhoea and endometriosis is very probable (Fauconnier & Chapron, 2005, p595)

However, based on the information provided in this thesis, the relationship between dysmenorrhoea, chronic pelvic pain and endometriosis should be considered as an association and not to imply causality.

1.5 The role of Hormonal Factors in Dysmenorrhoea, Chronic Pelvic Pain and Endometriosis

1.5.1 Gender and pain

Ample evidence, as summarised by Mogil et al. (2012) describes the higher prevalence of chronic pain conditions in women when compared to men. This gender pain disadvantage begins with puberty and persists throughout a woman's life. Perquin et al. (2000) studied the prevalence of chronic pain in 5,336 Dutch children aged 4-18 years. Before puberty the prevalence of chronic pain conditions in their study was approximately equal between boys and girls. However, by the age of 12-14 years girls were over-represented, and by the age of 16-18 years girls were four times more likely to report a chronic pain condition than boys (Figure 1.12).

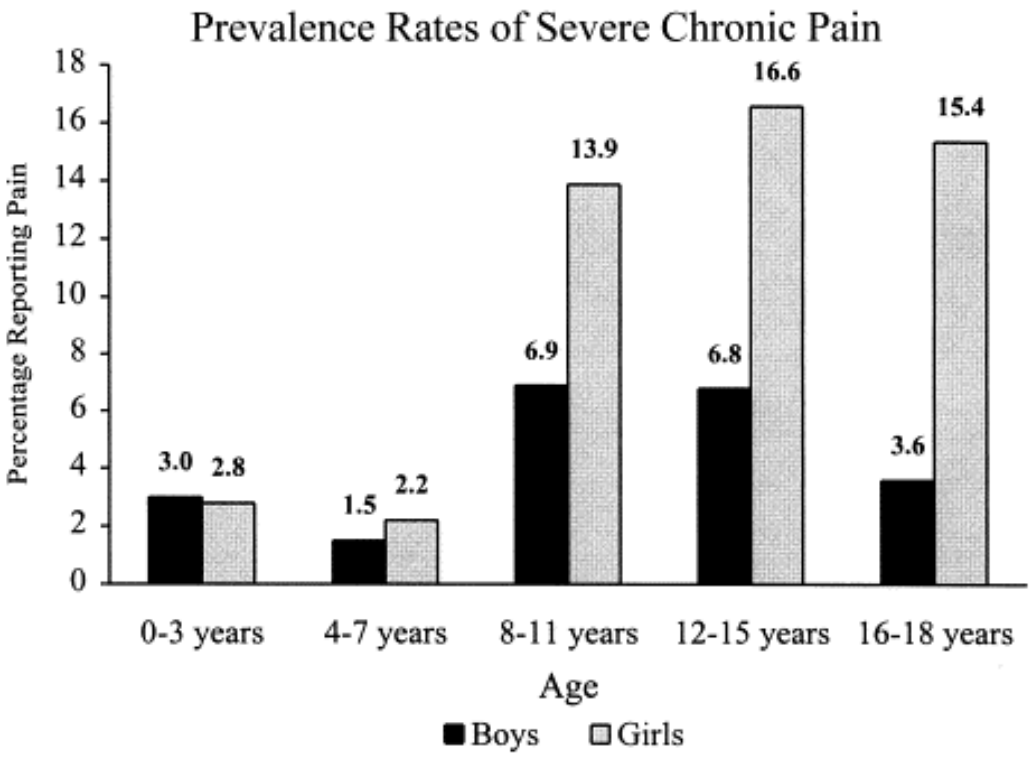

Figure 1.12: Three-month prevalence rates of severe chronic pain (chronic pain with intensities of more than 50 mm on the VAS and occurring weekly) by age groups and gender. Data were based on one pain report per child (Perquin et al., 2000).

The most frequently reported chronic pain grouping was a combination of abdominal pain and headache in 16-18-year-old girls. Girls reported multiple pain sites twice as commonly as boys. In further evidence for an association between hormonal factors and the experience of pain, LeResche studied 3,101 children aged 11-17 years, and showed that it is the stage of puberty rather than the chronological age of children that most accurately predicts the presence of a chronic pain condition in children (LeResche, 1995).

The increased prevalence of chronic pain in women includes medical conditions both within and outside the gynaecological sphere. Greenspan et al. (2007) studied 54 non-gynaecological chronic pain conditions and found a higher female prevalence in 39 of 54 pain conditions, and a higher male prevalence in only 19 of 54 pain conditions. Interestingly, for both sexes, the presence of chronic pain is also associated with the potential for altered gender identity perceptions. Bernardes et al. found a public perception of men with chronic pain as being less masculine, and a public perception of women with chronic pain as being less feminine, when considering a list of 33 personality traits (Bernardes & Lima, 2010).

Research into the implications of sex on medical conditions and pharmaceutical development was hampered by the 1977 US Food and Drug Administration (FDA) guidelines (Greenspan et al., 2007; Houghton et al., 2016), advising that women of childbearing potential should be excluded from drug trials. While these guidelines sought to avoid the potential for adverse drug effects on the foetus in the event of unrecognised pregnancy, they resulted in the inadequate representation of women in clinical trials, and the development of pharmaceutical products and dosing regimens best suited to males. Since the 1990s, the FDA and the National Institutes of Health (NIH) in the USA, have recommended that clinical trials should include female subjects. However, while it is now common for trials to consider whether outcomes differ between females and males, this trial analysis is not required. In 2015 the US Government Accountability Office (GAO) found that 8 out of 10 drugs withdrawn from the US market from 1997-2000 had greater adverse effects in women. While the NIH does not ensure that current studies are designed to identify differences between men and women in disease processes and responses to treatment, new guidelines require that chromosomal sex is considered as a biological variable in preclinical research (Fish, 2008).

Despite the evident effect of chromosomal sex, and substantial effort on the part of researchers since the 1990s (Geller et al., 2011), research to date has not provided an adequate mechanism for the increased prevalence of chronic pain in women. While both sexes produce estrogen and testosterone, estrogen is dominant in females and testosterone is dominant in males. As outlined by Geller, research to date has investigated whether the

relative dominance of estrogen may predispose women to pain. This research has proved inconclusive. An alternative theory might consider the potential for testosterone to protect males from pain, and will be considered further in Chapter 5.

Approaches have considered biological differences between men and women (Mogil, 2012; Sorge et al., 2015), the differences in the way that men and women describe pain (Strong et al., 2009), and the different psychological responses to pain in males and females (Archey et al., 2018).

1.5.2 HORMONAL FACTORS AFFECTING DYSMENORRHOEA

As was discussed in section 1.4.1, it is prostaglandin release within the uterus that has been most consistently associated with dysmenorrhoea. Hormonal factors are well placed to affect the release of prostaglandins from the endometrium, with the potential to either aggravate or ameliorate menstrual pain. Arachidonic acid, the precursor of prostaglandins (Evans and Salamonsen, 2012), is formed from endometrial cell membrane phospholipids following the release of the enzyme phospholipase A from endometrial cell lysosomes. The stability of lysosomes is regulated by several factors, particularly the levels of progesterone: high progesterone levels tend to stabilise, while falling levels tend to destabilise, endometrial lysosomes (Dawood, 1995; Hofmeyr, 1996). In the high progesterone, luteal phase of the menstrual cycle, lysosomes are stable, phospholipase A is not released, and arachidonic acid production is low. As menstruation approaches, progesterone levels fall, lysosomes become unstable, phospholipase A is released, and arachidonic acid is formed (Chan et al., 1981; Dawood & Huang, 1998).

Clinically, use of the combined oral contraceptive pill (OC), which includes both estrogenic and progestogenic components, reduces both prostaglandin levels and dysmenorrhoea in a proportion of women (Hauksson et al., 1989; Lindh & Milsom, 2013). In an evaluation of contraceptive options, Lindh followed a random sample of 656 19-year-old Swedish women over 5 years, and found a significant reduction in dysmenorrhoea among users of the OC, or a levonorgestrel-releasing intra-uterine device, but not among users of a non-hormonal intra-uterine device or other non-hormonal contraceptive methods that included barrier methods, sterilisation, withdrawal or natural family planning (Lindh & Milsom, 2013). Although not evaluated in the Swedish study, the continuous use of an oral progestogen medication, with induction of endometrial atrophy, has been shown to be equally effective as the oral contraceptive pill at reducing dysmenorrhoea in a group of 38 Jordanian women (Al-Jefout &

Nawaiseh, 2016). That the effect of the progestogen on dysmenorrhoea is predominantly uterine is demonstrated by the effectiveness of the levonorgestrel-releasing intra-uterine device for reducing dysmenorrhoea, despite the lack of suppression of ovarian function, and the low systemic dose of levonorgestrel delivered (Backman, 2004).

The hormonal environment may also impact on chronic pain through its association with impaired sleep quality. Baker et al. (1999) found both self-reported sleep disturbance during the pre-menstrual phases of the cycle, and higher morning levels of estrogen, in women with dysmenorrhoea. Sleep disruption has been shown to increase pain sensitivity the following day, even in healthy, young, normally pain-free women (Iacovides et al., 2017), so whether sleep disruption precedes or follows the development of dysmenorrhoea is yet to be determined (Baker et al., 1999, 2008; Baker & Lee, 2018).

1.5.3 HORMONAL FACTORS AFFECTING CHRONIC PAIN

A woman's experience of pelvic pain may include pain associated with pelvic viscera beyond the uterus, such as the bladder or bowel. The assertion that pelvic visceral afferent nerves are sensitised by estrogen is supported by several factors. Firstly, functional disorders of the pelvic viscera are more common in women than men (Greenspan et al., 2007). A meta-analysis of 139 research articles found a frequency of between 7% and 21% for Irritable Bowel Syndrome, with 2 in 3 of those affected being female (Canavan et al., 2014). Painful Bladder Syndrome is estimated to affect between 3 million and 8 million women in the United States, depending on the diagnostic criteria used. It is estimated that 5 in 6 of those affected are women (Berry et al., 2011). Gender differences in patient presentation, societal attitudes, and treatment outcomes are further explored from a clinician's perspective by Houghton et al., and with regard to deep tissue pain perception by Traub and Ji (Houghton et al., 2016; Traub & Ji, 2013).

Secondly, clinical treatments that reduce systemic levels of estrogen, such as gonadotrophin-releasing hormone analogues (GnRHa), are associated with a reduction in pain. Gallagher et al. (2018) followed 51 girls and women aged 15-22 years treated with a combination of GnRH analogue leuprolide and add-back hormonal therapy comprising norethisterone 5mg and conjugated equine estrogen 0.625mg daily. 63% of these women described an improvement in their pain, 28% described no improvement in their pain, and 8% described worse pain than pre-treatment Thirdly, visceral pain symptoms vary in pre-menopausal women across the menstrual cycle (Riley et al., 1999). Pain may be further modified by levels of progesterone,

or the use of synthetic progestogens. In a trial considering the severity of pelvic pain in 111 women with endometriosis over 12 months of use, the synthetic progestogen dienogest reduced the visual analogue score (VAS) for pelvic pain from 8.9 to 0.9 (Maiorana et al., 2017).

Hormonal factors may influence the pain experience via peripheral or central effects, with central effects further divided between central neural mechanisms, and central effects following activation of the innate immune system. From a neural perspective, estrogens modulate the voltage-gated calcium channels (VGCCs) and purinoreceptors (P2Xs) associated with neuronal sensitivity of visceral afferents either directly, or via the enhanced expression of P2X receptors associated with inflammation (Chaban, 2013). The impact of hormonal factors on the innate immune system and the actions of glial cells will be discussed further in Section 1.6.

1.5.4 HORMONAL FACTORS AFFECTING ENDOMETRIOSIS

Endometriosis is a clinical condition rarely diagnosed outside the reproductive years, and ample evidence links the development or progression of endometriosis lesions with steroid hormones (Bulun et al., 2002; 2010; Monsivais et al., 2014). Modification of the hormonal environment with a reduction in estrogen effect is a major focus of medical management of the condition worldwide (Taylor et al., 2012), although its effectiveness in managing pain is variable. For example, a Cochrane analysis (Brown et al., 2010) investigated the effectiveness of estrogen suppression using GnRH analogues for pain management across 41 trials involving 4,935 women. Brown et al. confirmed that GnRH-a was more effective than placebo at reducing pain in women with endometriosis. However, GnRH analogues were no more effective than danazol ($p=0.53$) or levonorgestrel ($p=0.46$) for pain reduction. GnRH analogues carried a significant higher risk of adverse effects, including vaginal dryness ($p<0.00001$) and hot flushes ($p<0.00001$) than danazol, a synthetic steroid medication that combines anti-estrogenic, weak androgenic and weak progestogenic activity.

Estrogen affects the phenotype of endometrial cells present within the endometrium and their response to inflammation. This presents as an increase in the ratio of epithelial-mesenchymal transition (EMT) cells to mesenchymal-epithelial transition (MET) cells. EMT cells acquire an invasive phenotype in the presence of chronic inflammation. This change promotes cellular proliferation and fibrosis (Wu et al., 2007), with the potential to enhance endometriosis lesion formation. In contrast, MET cells promote the decidualisation of the endometrium (Yu et al., 2016). The ratio of EMT to MET within the endometrium is also affected by the presence of

seminal plasma (Ibrahim et al., 2019). McGuane et al. (2015) demonstrated that exposure to seminal plasma induces an eight-fold increase in the growth of endometrial xenografts in a mouse model of endometriosis.

The ratio of the two estrogen receptors, ERα and ERβ, is altered in the presence of endometriosis. ERβ is more highly expressed in ectopic endometriotic tissue than ERα, and has been shown to promote endometriosis lesion growth (Han et al., 2015). This mechanism is described further in Section 1.6.3.

Since hormones are modulators of gene transcription, the pattern of modulated genes also depends on the additional signalling pathways currently active within the cell at the time of hormone exposure (Katzenellenbogen & Katzenellenbogen, 2002). The alteration of estrogen signalling also provides a potential mechanism for environmental factors, including toxins, to enhance the growth of endometriosis lesions (Smarr et al., 2016).

Multiple enzyme mechanisms within endometriosis lesions further alter the intra-lesional hormonal equilibrium, providing an additional mechanism for lesion growth and development. Delvoux et al. described an increased expression of the enzyme 17β-hydroxysteroid-dehydrogenase Type 1 (17β-HSD Type 1), but not 17β-HSD types 2, 4, 5, 7 or 12, within endometriosis lesions, compared with eutopic endometrium (Delvoux et al., 2009). This favours the production of the more potent 17β-estradiol (E2) over two of its metabolites, estrone and estriol, which display weaker ER agonist activity. In further research Delvoux demonstrated that 17β-HSD Type 1 could be selectively inhibited using 3-[15b-estronyl]-N-(5-methyl-thiazol-2-yl)-propionamide *in vitro* (Delvoux et al., 2014).

Zeitoun et al. demonstrated the increased expression of the enzyme aromatase within endometriosis lesions. Aromatase favours the conversion of androgens to estrogens (Zeitoun & Bulun, 1999) and is found in multiple tissues including the granulosa cells of the ovary, brain, adipose tissue, placenta, skin, and bone. It is also expressed in certain pathological conditions including uterine fibroids, breast cancer and endometrial cancer. Importantly, it is found in endometriosis lesions, but not in eutopic endometrium. The ability of aromatase inhibitors, such as letrozole, to reduce the size of endometriosis lesions has been demonstrated (Bulun et al., 2001), but their use is limited by adverse side effects associated with low estrogen, including hot flushes, dry vagina and reduced bone density.

Importantly, positive feedback between inflammation and estrogen within the ectopic endometrial stromal cells of women with endometriosis results in increased expression of

PGE2, which is itself a potent inducer of aromatase (Noble et al., 1997). The subsequent conversion of androgens to estrogen results in increased estrogen levels, which stimulate the production of cyclo-oxygenase-2, resulting in an increase in PGE2 (Huang et al., 1998). Hormonal manipulation thus has the potential to act in multiple ways in women with endometriosis, including via modulation of inflammation.

1.6 The Interplay of Chromosomal Sex and Hormonal Factors on the Innate Immune System

An area of increasing research interest since the 1990s is the role of the immune system in the initiation and maintenance of chronic pain in females. While Section 1.5 demonstrates the well supported belief that hormonal factors play a role in the experience of pain, chromosomal sex provides an additional mechanism for gender sex differences, particularly with regard to immune function.

Immune differences between males and females present clinically as two broad scenarios: the sex differential in susceptibility to infectious disease, and the sex differential in inflammatory or autoimmune conditions, potentially including dysmenorrhoea, endometriosis or pelvic pain. Certain immune differences between the sexes are evident from birth and suggestive of a chromosomal sex mechanism. Others, such as the increased frequency of chronic pain conditions in females post puberty are adaptive, and suggestive of a hormonal mechanism. Section 1.6.1 provides an overview of human immunity prior to the further discussion of the impact of chromosomal sex on immune function in Section 1.6.2.

1.6.1 Introduction to the innate immune cells in peripheral blood

The innate immune response is the body's first line of defence against pathogens. Its purpose is to detect the presence of infectious agents and to initiate mechanisms to eliminate potential infectious threats. It achieves this through a flexible network of cells that travel via the circulation to tissues where the initiation of an inflammatory response is required. Prominent among immune cells are the haematopoietically-derived peripheral blood mononuclear cells (PBMCs). PBMCs are peripheral blood cells with a round nucleus that are found in the buffy coat of centrifuged blood. In contrast, erythrocytes and platelets have no nucleus, and neutrophils, basophils and eosinophils have a multi-lobed nucleus. The majority (70%-90%) of PBMCs are lymphocytes, which can be further subdivided into T-cells, B-cells and Natural Killer (NK) cells. Monocytes represent 5%-10% of PBMCs, and are further subdivided into macrophages and dendritic cells (DCs). Circulating macrophages respond to immune inflammatory challenges by infiltrating the affected tissue and differentiating to fulfil the immune response required (Ginhoux & Jung, 2014).

Dendritic cells comprise 1%-2% of PBMCs, and are further divided into plasmacytoid DCs and myeloid DCs. Myeloid DCs are known for their role as antigen-presenting cells (Kleiveland,

2015). They process antigen material and present it to T-cells (Ueno et al., 2007). DCs capture microbes, present their antigens, and provide signals necessary for the T-cell expansion and differentiation necessary to mount an immune response. They thus act as messengers between the innate and adaptive immune systems. DCs are found in blood and in tissues that are in contact with the external environment, such as the skin, intestines, and endometrium (Schulke et al., 2009). Once activated, DCs migrate to draining lymph nodes, where they initiate and modify the adaptive immune response through their interaction with T- and B- cells.

T-cells are formed in the thymus and differentiate to multiple subtypes classified according to their surface receptor that include T-cell co-receptor cells (CD3+), T-helper cells (CD4+) and cytotoxic T-cells (CD8+). Females have higher numbers of $CD3^+$, $CD4^+$ and CD8+ expressing cells than males, and a different balance between subsets of T-helper (CD4+) Cells. T-helper cells differentiate into two major cell subtypes known as T_h1 and T_h2 cells (Villacres et al., 2004). T_h1 helper cells facilitate an increased cell-mediated immune response typically involving macrophages and cytotoxic T-Cells (CD8+), resulting in the secretion of pro-inflammatory cytokines such as interferon gamma (IFN-γ) and tumour necrosis factor alpha (TNF-α). T_h2 helper cells facilitate a humoral immune response typically involving B-Cells, resulting in the secretion of anti-inflammatory cytokines such as interleukin 4 (IL-4), IL-10, and IL-13 (Mosmann & Sad, 1996). An increase in the T_h1/T_h2 ratio is considered pro-inflammatory, while a decrease in the T_h1/T_h2 ratio is considered anti-inflammatory. During pregnancy a bias towards T-helper 2 (Th2) protects the foetus by inhibiting the production of proinflammatory cytokines (Sykes et al., 2012). Females exhibit upregulated expression of proinflammatory genes compared to males, with many of these genes incorporating estrogen response elements in their gene promoters (Hewagama et al., 2008).

The cytokine interleukin-1beta (IL-1β) plays a pivotal role in the implementation of innate and adaptive immune responses. IL-1β is a pro-inflammatory cytokine and is expressed by many cells, including macrophages, NK cells, monocytes, and neutrophils. IL-1β is released in response to a wide variety of pathogen-associated molecular patterns (PAMPs). Under the influence of E2, monocytes differentiate into inflammatory DCs, with increased production of IFNα and pro-inflammatory cytokines (Seillet et al., 2013). Importantly, PBMCs offer an easily accessible source of immune cells suitable for repeated sampling through venepuncture.

1.6.2 CHROMOSOMAL SEX AND THE IMMUNE SYSTEM

Males and females differ in their innate and adaptive immune responses, with adult females generally mounting stronger antibody and cell-mediated immune responses to antigenic stimulation, vaccination, and infection than males (Fish, 2008; Jaillon et al., 2019; Klein & Flanagan, 2016a; Marriott & Huet-Hudson, 2006). This sexual dimorphism is associated with a well-documented survival advantage in females through reduced bacterial, viral, parasitic and fungal infections (Fish, 2008; Roberts et al., 2001; Steeg & Klein, 2016), and an enhanced response and benefit for females from public health vaccination programs (Klein et al., 2010). The increased immune response in females offers protection against infection during childbirth, and enhanced survival of offspring through the non-genetic passive transfer of immunity from mother to child during pregnancy and breast feeding. In contrast, the relatively reduced immune response in males is associated with a higher mortality rate from infections and cancer (Figure 1.13).

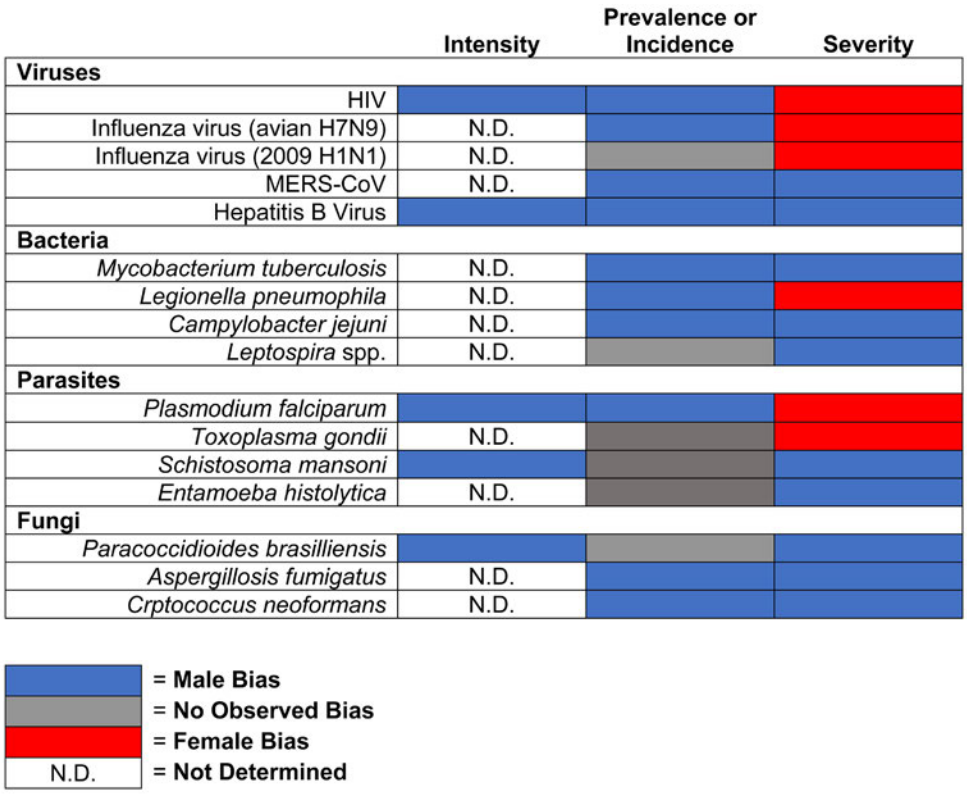

Figure 1.13: Sex differences in the intensity (i.e., pathogen load), prevalence (i.e., proportion of population with disease), incidence (i.e., new cases of disease), and severity (i.e., hospitalisation or progression of disease state) of disease following microbial infections in humans (Steeg & Klein, 2016).

However, while a stronger inflammatory immune response may help women resist infections, this may come at the expense of an increased susceptibility to inflammatory and autoimmune disease conditions, potentially including dysmenorrhoea, endometriosis and pelvic pain. As an example, Cooper and Stroehla (2003) reviewed the prevalence of autoimmune disease in the United States of America, and found that 85% of Americans with thyroiditis, systemic sclerosis, systemic lupus erythematosis and Sjögren's Disease were female.

Hewagama et al. demonstrated that when T-cells are repeatedly stimulated, T-cells from women overexpressed a greater number of genes than T-cells from men. Estrogen response elements were identified in the promoters of half of the overexpressed immune genes in women, but less than 10% of the overexpressed immune genes in males. The inflammatory/cytotoxic effector genes overexpressed in women, with effects 1.4-1.8 times higher in women than men, included interferon gamma (IFNG), lymphotoxin beta (LTB), granzyme A (GZMA), interleukin-12 receptor beta2 (IL12RB2), and granulysin (GNLY).

In a further association between chromosomal sex and inflammatory disorders, Amadori et al. (1995) studied the CD4 (T Helper cells) /CD8 (T cytotoxic cells) ratio between males and females in 46 families. They confirmed the known sex-dependent ratio described by Hewagama, with the additional finding that the CD4/CD8 ratio of parents significantly influenced the CD4/CD8 ratio in offspring. This is consistent with either genetic factors or common environmental factors as a cause for the ratio difference between males and females. Many genes on the X chromosome regulate immune function (Libert et al., 2010), and skewed X chromosome activation has been proposed as a potential cause of the higher prevalence of autoimmune disease among females. While evidence for skewed activation has been found in Hashimoto's Thyroiditis (Simmonds et al., 2014), none of the highly expressed gender-biased genes detected in Hewagama's study were encoded on the X chromosome.

Despite the findings of Hewagama, the X chromosome is known to contain the largest number of immune-related genes within the human genome (Bianchi et al., 2012). Humans with chromosomal anomalies offer a unique opportunity to investigate the relative importance of chromosomal versus hormonal factors in the development of immune-mediated medical conditions. Males with Klinefelter syndrome (XXY) display lower testosterone levels, higher oestrogen levels, and respond immunologically more like females to immune challenges. They exhibit higher immunoglobulin concentrations, higher $CD4^+$ T-cell numbers, higher CD4/CD8 T-cell ratios and higher B-cell numbers than XY males. In comparison, females with Turner syndrome (XO) display lower immunoglobulin concentrations, lower T-cell and lower B-Cell numbers than XX females (Cacciari et al., 1981). Both males with Klinefelter syndrome and

females with Turner syndrome show an increased prevalence of autoimmune disease (Bianchi et al., 2012). In showing that this effect could be reversed by treatment with testosterone, Kocar et al. (2000), provided evidence that both chromosomal sex, including the X chromosome, and hormonal factors are involved in immune function.

Jarrell and Arendt-Nielsen (2016) have proposed that recurrent episodes of painful menstruation predispose women to the development of endometriosis and chronic pelvic pain through increased immune activation. Recurrent ovulatory cycles result in a repetitive cycle of hormonal fluctuations and menstruation inflammation, predispose females to a higher prevalence of autoimmune conditions, inflammatory conditions (including endometriosis), and the development of chronic pain. Multiple clinical studies support an association between endometriosis and immune-mediated medical conditions. An increased prevalence of endometriosis has been found in women with multiple sclerosis, systemic lupus erythematosis, rheumatoid arthritis, Sjögren's Syndrome, and fibromyalgia (Brandt et al., 2015; Greenbaum et al., 2019; Harris et al., 2016; M. D. Lockshin, 2005; Miclea et al., 2019; Sinaii et al., 2002). Conversely, Porpora et al. (2019) found an increased prevalence of inflammatory bowel disease ($p<0.07$), coeliac disease ($p<0.0001$), autoimmune thyroiditis ($p<0.0001$), and systemic lupus erythematosis ($p=0.01$) among 148 women with endometriosis when compared with 150 controls.

1.6.3 SEX STEROID HORMONES AND THE IMMUNE SYSTEM

Endogenous estrogens include estrone (E1), 17β-estradiol (E2), and estriol (E3), with E2 being the major form in females of reproductive age. E3 is produced in high levels during pregnancy. Immune cells express estrogen receptors (ERs), progesterone receptors (PRs), and androgen receptors (ARs), implying that endogenous sex hormones are directly involved in the activation, development or functional responses of immune cells (Whitacre et al., 1999). The enzymatic pathways of the precursors and metabolites of sex hormones are governed by complex enzyme interactions (Figure 1.14).

Estrogen receptors display three distinct subtypes: ERα, ERβ, and G protein-coupled estrogen receptors (GPER). ERα and ERβ belong to the nuclear hormone receptor class of transcription factors that regulate gene transcription by translocating to the cell nucleus and binding to DNA. GPER is a member of the G protein-coupled receptor (GPCR) family, localised in the endoplasmic reticulum, which binds with high affinity to estradiol, and is responsible for the rapid non-genomic actions of estradiol (Vrtacnik et al., 2014).

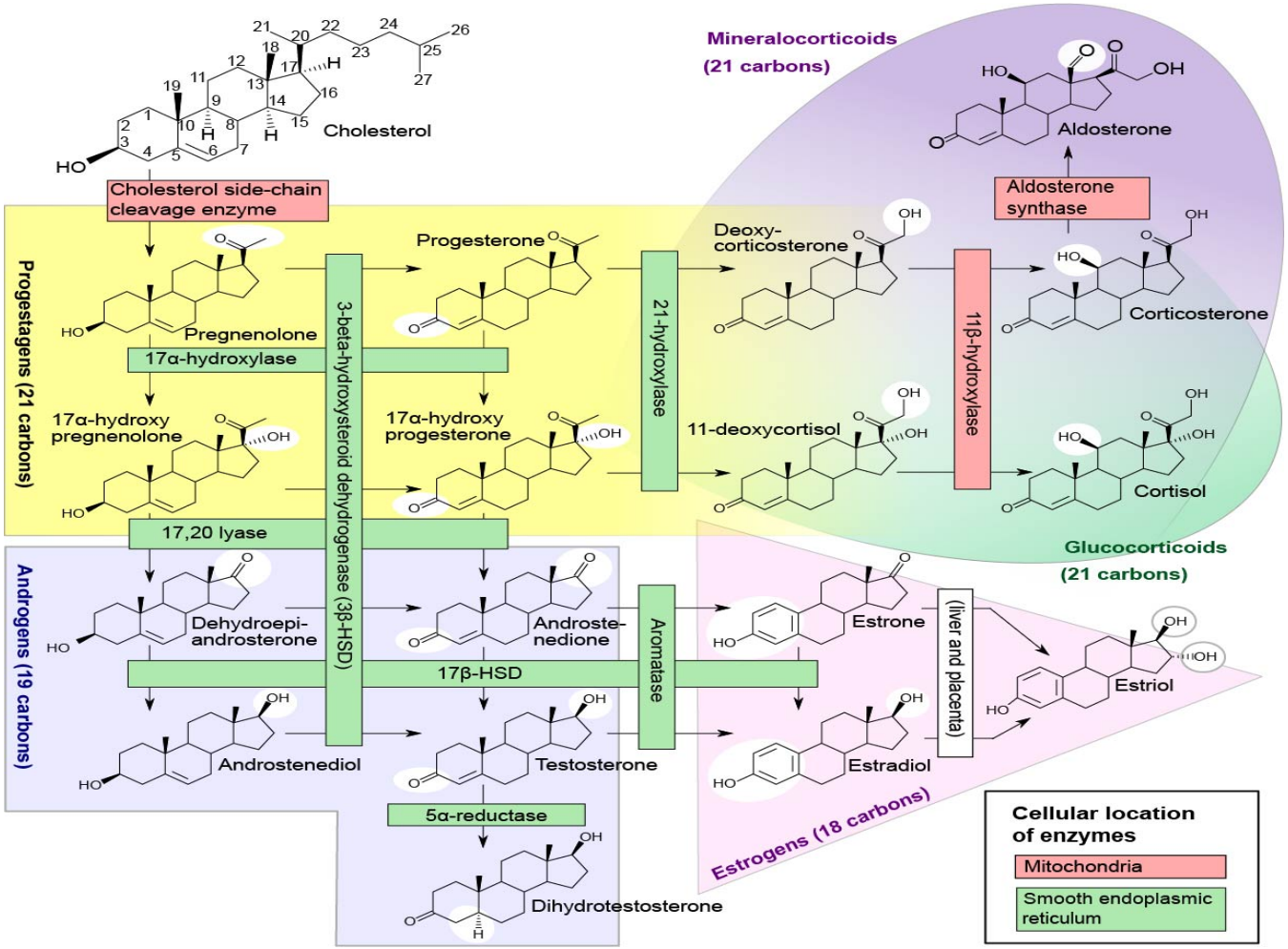

Figure 1.14: Diagram of the pathways of human steroidogenesis (2014). Reproduced with permission D Richfield and M. Häggström. Derived from previous version by Hoffmeier and Setters.

Estrogens modify the function of macrophages, the differentiation of DCs, and the expression of cytokines (Bachy et al., 2008; Cunningham et al., 2014; Khan & Ahmed, 2016; Liu et al., 2002; Liu et al., 2002; Papenfuss et al., 2011). While estrogen receptors are common across populations of immune cells, they are not ubiquitous. In the periphery, CD4+ T-cells express more ERα than ERβ, B-cells express more ERβ than ERα, and CD8+ T-cells and monocytes express low amounts of ER (Phiel et al., 2005). However, in general, ERα plays a larger role in immune processes than ERβ (Liu et al., 2002; Liu et al., 2002; Maret et al., 2003).

Estrogen effects may be tissue specific, and display both agonist and antagonist functions within cells (Heldring et al., 2007), or have effects which are estrogen level dependent. For example, low doses of E2 increase the production of pro-inflammatory cytokines including IL-1β, IL-6, and TNF-α from monocytes and macrophages, whereas high concentrations reduce the production of these cytokines (Bouman et al., 2004). ER signalling may be anti-inflammatory in certain cell types including monocytes and macrophages (Härkönen & Väänänen, 2006), as measured by the inhibition of NF-kB, yet be pro-inflammatory with an increase in NF-kB proteins in other tissues (Karpuzoglu et al., 2006). Notably, Karpuzoglu et al. investigated the response of splenocytes to T-cell stimulation with and without the presence of estrogen. Estrogen-treated mice were found to have an up-regulated expression of cyclo-oxygenase 2 compared with controls. As COX-2 facilitates the production of PGE2, this has potential relevance for the severity of dysmenorrhoea. Estrogens modulate visceral nociception and opioid receptor function within minutes of administration (Gintzler & Liu, 2012), consistent with a role for GPER in addition to the nuclear estrogen receptors ERα and ERβ. In a study of 31 patients with IBS and 30 normal controls, Jacenik demonstrated a statistically significant overexpression of ERα in women with diarrhoea-predominant IBS under the age of 50 years (Jacenik et al., 2018).

Multiple endocrine disruptors, both plant-based and pollutant in origin, can affect immune function through action on the ER. Ren described over 20 dietary phytoestrogens, including seasonings (garlic, aniseed, fennel, caraway, parsley), legumes (soybeans, chick peas, clover), grains (wheat, barley, rye, rice and oats), vegetables/herbs (carrots, potatoes, alfalfa, red clover), fruits (apples, pears, grapes, dates, pomegranates, cherries), and drinks (beer, coffee), which could act as either ER agonists or antagonists, with variable binding to either ERα or ERβ (Ren et al., 2001). Environmental pollutants with ER activity are found in plastics (bisphenol-A, phthalates), detergents and surfactants (octylphenol, nonylphenol), organochlorine pesticides (methoxychlor, dichlorodiphenyl-trichloroethane or DDT, hexachlorobenzene, and dieldrin), and industrial chemicals (polychlorinated biphenyls or

PCBs, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin or TCDD) (Ahmed, 2000). Bisphenol-A (BPA) exposure increases pro-inflammatory cytokines and reduces anti-inflammatory cytokine secretion in rodents, and is immunotoxic to human macrophages (Chen et al., 2018). Bisphenol exposure has been associated with a higher prevalence of medical conditions, including endometriosis (Kim et al., 2019; Rashidi et al., 2017). Rashidi compared BPA levels in 50 women with ovarian endometriomata and 50 women with no endometriosis. While the percentage of urine samples containing BPA was similar in both groups (86% of cases and 82.4% of controls), higher concentrations of BPA were significantly associated with the presence of endometriosis. The mean concentration of BPA was 5.53 ± 3.47 ng/mL in women with endometriosis, and 1.43 ± 1.57 ng/mL in women without endometriosis ($p < 0.0001$).

Progesterone (P4) is a steroid hormone secreted primarily by the ovarian corpus luteum following ovulation, and by the adrenal cortex in non-pregnant women. Progesterone receptors (PR) bind the hormone progesterone, are found within cells, and display 2 main subtypes: PRα and PRβ. Human P4 levels are low in the follicular phase of the menstrual cycle (1-2nM), and rise following ovulation to a peak (20-40nM) in the late luteal phase. During pregnancy, placental production of P4 increases serum progesterone levels to 100–500nM (Stites & Siiteri, 1983). PRβ exerts the tissue effects of progesterone, while PRα regulates the activity of PRβ. Both T-lymphocytes and macrophages express PR (Khan et al., 2005). Progesterone in blood is bound to transcortin (corticosteroid-binding globulin), and at physiological levels displays similar effects on leukocytes as glucocorticoids (Bamberger et al., 1999; Kontula et al., 1983). Reducing inflammatory responses may be an important role of P4. P4-exposed macrophages and DCs in rodents have a lower state of activation and produce lower amounts of IL-1β and TNF-α compared with untreated cells (Butts et al., 2007).

Androgens are a family of sex steroid hormones that bind to the androgen receptor. The major androgen in both males and females is testosterone, which is around 50% formed in the periphery from its precursors (androstenedione, dehydroepiandrostenedione (DHEA) and the sulphated form DHEA-S), 25% secreted by the ovary, and 25% secreted by the adrenal gland. Testosterone is metabolised to the more potent dihydrotestosterone (DHT) within peripheral tissues under the influence of 5-alpha-reductase (Figure 1.14). Ovarian androgen production is suppressed by the use of the oral contraceptive pill (OC). Women with normal ovarian function have levels of testosterone that vary between 0.42 and 2.94nmol/l (Pesant et al., 2012). Androgen receptors (ARs) bind with nuclear DNA and exist in 2 forms: ARα and ARβ. Androgens are considered to be generally immunosuppressive, resulting in decreased T- and B-cell proliferation, and decreased immunoglobulin and cytokine production (Bebo et al., 1998; Miller & Hunt, 1996; Olsen & Kovacs, 2001).

In peripheral blood, estrogens and androgens are predominantly bound to either albumen or Sex Hormone Binding Globulin (SHBG), with only 1%-2% in the unbound or free form. Only the unbound fraction is biologically active and able to activate the nuclear receptor. SHBG levels are decreased by androgens, polycystic ovarian syndrome, hypothyroidism and obesity (Dunn et al., 1981; Grasa et al., 2017). SHBG levels are increased by estrogens, the oral contraceptive pill, pregnancy, and hyperthyroidism. Through hormone binding, a higher level of SHBG inhibits the actions of both androgens and estrogens.

Importantly, androgens are converted to estrogens by the enzyme aromatase in a range of tissues, such as ovarian granulosa cells where aromatase is present, providing a secondary estrogen effect. Individuals with increased aromatase activity show accelerated estrogen conversion, and thus lower testosterone levels, within affected tissues. Capellino et al. and Folomeev et al. demonstrated that individuals with rheumatoid arthritis (Capellino et al., 2014; Castagnetta et al., 2003; Cutolo et al., 2004, 2006) and SLE (Folomeev et al., 1992) show increased aromatase, reduced testosterone, and an increase in the pro-inflammatory cytokines TNF-α, IL-1β and IL-6 in affected tissues. Interestingly, aromatase activity is downregulated by Vitamin D (Capellino et al., 2014), offering the potential for Vitamin D supplementation as an androgen-enhancing treatment option.

As discussed in Section 1.5, the hormonal profile of endometriosis lesions differs substantially from the profile of eutopic endometrium. Firstly, endometriosis lesions contain abundant aromatase, allowing local androgen conversion to estradiol. Aromatase production is stimulated by prostaglandin E_2 (PGE_2), resulting in the local production of estrogen, which further stimulates the formation of PGE_2 inducing a positive feedback cycle (Bulun et al., 2000; Noble et al., 1997; Zeitoun & Bulun, 1999). Secondly, low levels of PR in endometriotic stromal cells reduce the induction of the enzyme 17β-hydroxysteroid dehydrogenase, reducing the conversion of estradiol to the less potent estrone (Bulun et al., 2010; Cheng et al., 2007). Thirdly, ERβ levels are significantly higher in endometriosis lesions than in eutopic endometrial tissue (Carli et al., 2008). Bulun has proposed that a dramatically higher ERβ-to-ERα ratio in endometriotic stromal cells, combined with suppression of the PR progesterone receptor, increases cyclo-oxygenase-2 levels, contributing to progesterone resistance and inflammation (Bulun et al., 2012).

These factors result in high levels of intra-lesion estradiol, relative progesterone resistance, and the ability of endometriosis lesions to maintain high estrogen concentrations, even following ovarian removal or suppression of circulating estrogen levels.

1.7 TOLL-LIKE RECEPTORS (TLRS)

The complex interaction between the immune system, hormonal factors, and the presence of dysmenorrhoea, endometriosis or chronic pelvic pain has been described in Sections 1.5 and 1.6. However, they do not provide a specific mechanism by which the immune system becomes activated in some, but not all, women. The discovery of the Toll gene by Christiane Nüsslein-Volhard and Eric Wieschaus in drosophila (fruit fly) in 1995, and the subsequent discovery of a human homologue of the drosophila toll protein now known as TLR4, offered a new mechanism for immune activation, with potential implications across a range of human conditions. TLR4 was found to induce genes involved in inflammatory responses (Medzhitov et al., 1997), and a multitude of research papers have since considered the role of TLR4 in inflammation, sepsis and chronic pain.

TLRs are pattern-recognition receptors that respond to different components of invading microbes. Each TLR responds to a different component (Figure 1.15). To date, 13 TLRs have been described, with TLR 1-10 present in mammalian species, each responding to different components of potential invading pathogens. TLR4 is the quintessential, and most investigated TLR: a transmembrane protein encoded by the TLR4 gene. It is present on the surface of both circulating immune cells such as macrophages and monocytes, and glial cells such as astrocytes and microglia, within the brain and spinal cord.

Recognised ligands for TLR4 include lipopolysaccharide (LPS), a component present in the cell wall of many gram-negative bacteria (e.g. *Neisseria* spp.), various gram-positive bacteria, certain viruses, polysaccharides, low-density lipoproteins, beta-defensins, and heat shock protein (Brubaker et al., 2015). TLR4 also recognizes components of pathogens and endogenous molecules produced during tissue damage (Vaure & Liu, 2014). The ability of TLR4 activation to enhance the circulating immune response has led to its development as a vaccine adjuvant (Zariri & van der Ley, 2015), where a stronger immune response is desired.

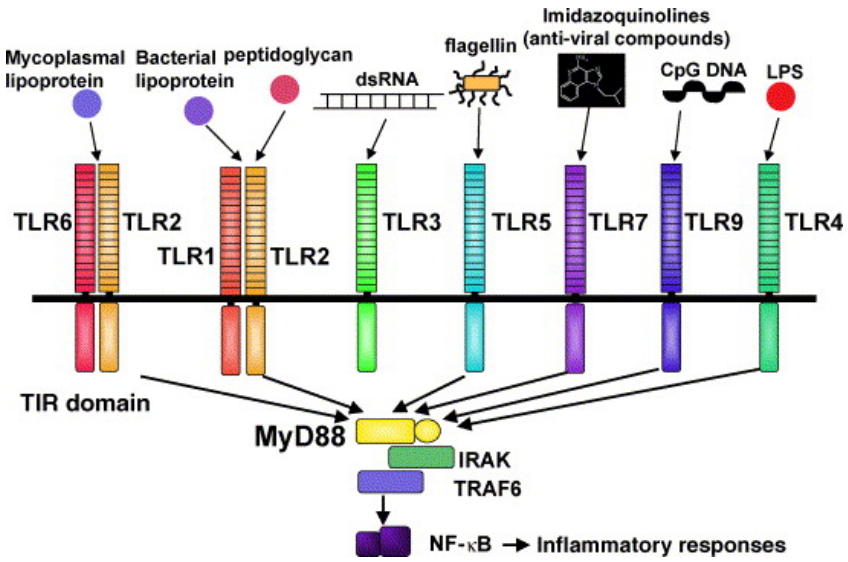

Figure 1.15: Pictorial representation of human TLRs and their specific ligands: triacylated lipoprotein for TLR1; peptidoglycan for TLR2; double-stranded RNA for TLR3; lipopolysaccharide (LPS) for TLR4; flagellin for TLR5; diacylated lipoprotein for TLR6; imidazoquinoline and its derivative R-848 for TLR7; and bacterial unmethylated CpG DNA for TLR9. MyD88 associates with the TIR domain of TLRs and transduces signals to induce immune responses. Reproduced with permission: (Yamamoto et al., 2004).

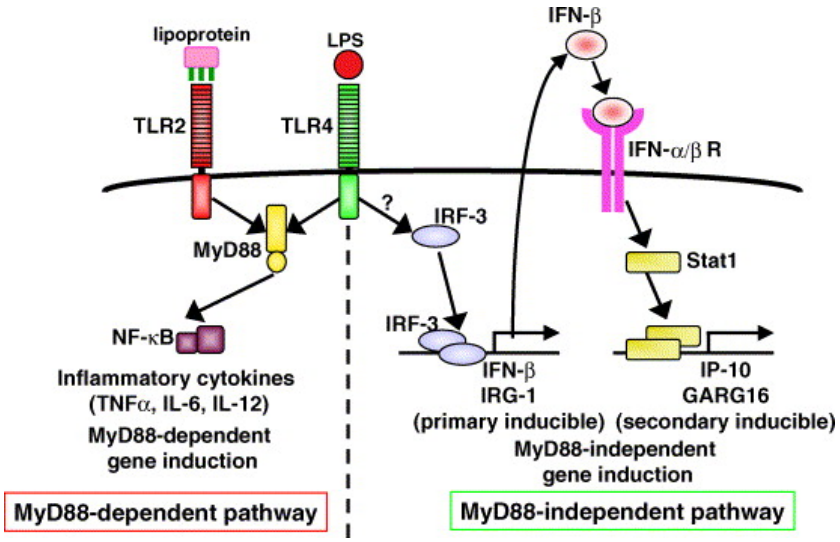

Figure 1.16: Pictorial description TLR signalling pathways: MyD88-dependent pathway that leads to the production of pro-inflammatory cytokines with quick activation of NF-κB and MAPK; and MyD88-independent pathway associated with the induction of IFN-β and IFN-inducible genes, and maturation of dendritic cells with slow activation of NF-κB and MAPK. Reproduced with permission (Yamamoto et al., 2004).

1.7.1 TLR4 AND ACTIVATION OF CIRCULATING IMMUNE CELLS

Activation of TLR4 triggers one of two intracellular signalling pathways (Figure 1.16). The first pathway is mediated by myeloid differentiation primary response gene 88 adaptor protein (MyD88), and is termed the MyD88-dependent pathway. The importance of the MYD88 pathway in LPS-induced inflammation has been illustrated by Kawaii et al. who found that MYD88-deficient macrophages in mice were unable to produce pro-inflammatory cytokines (Kawai & Akira, 2007). The second pathway is mediated by TIR-domain-containing adapter-inducing interferon-β (TRIF), and is termed the TRIF-dependent pathway (Takeda & Akira, 2004). It acts via NF-κB (nuclear factor kappa-light-chain-enhancer of activated B cells) to control DNA transcription. Following LPS binding to TLR4, this pathway results in the production of interferon (IFN) and the development of antigen-specific adaptive immunity (Martin & Wesche, 2002).

TLR4 is the only TLR currently described that can signal via both MYD88-dependent and MYD88-independent pathways. Both TLR2 and TLR4 are able to trigger the intracellular MYD88 pathway. Hoshino demonstrated that TLR4 deficient mice are hyporesponsive to LPS (Hoshino et al., 2016). LPS binds to the TLR4 receptor by collaborating at the cell surface with several molecules including CD14, a protein made by macrophages, and MD-2, a lymphocyte antigen that provides a link between the TLR4 receptor and LPS signalling (Figure 1.17). Further details regarding the binding of LPS to TLR4 are outlined in a comprehensive review by Lu et al. (2008).

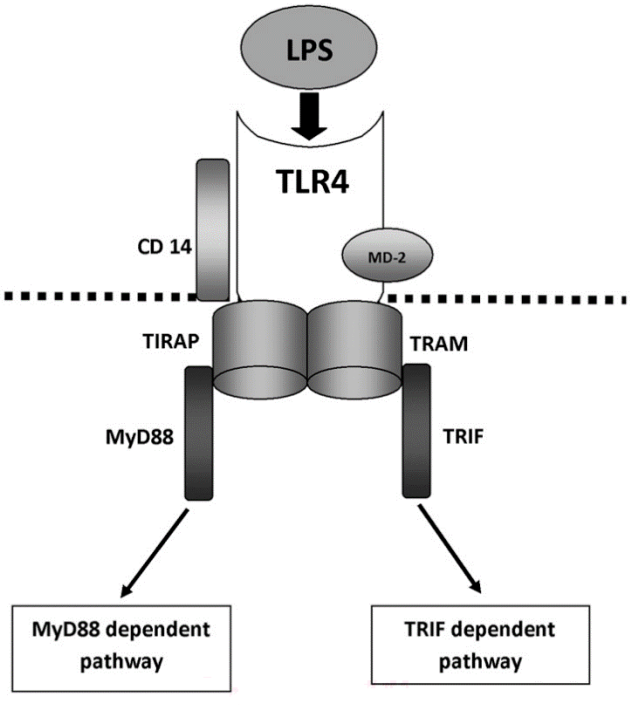

Figure 1.17: TLR4 signaling through MYD88-dependent and TRIF-dependent pathways. Reproduced with permission: (Ahmed et al., 2013).

1.7.2 TLR4 AND ACTIVATION OF GLIA WITHIN THE SPINAL CORD AND BRAIN

Glial cells are non-neuronal cells of the brain and spinal cord that do not produce electrical impulses, but play a role in neurotransmission, synaptic connections, learning and memory (Fields et al., 2014). Until recently, glial cells were considered to be purely structural in function: the support for the neuronal conduction of pain impulses from the periphery to the brain. However, in the last decade, the recognition that glia of the spinal cord and brain express TLRs, including TLR4, and that TLR4 activation of glia contributes to both the initiation and maintenance of chronic pain, has provided a potential new mechanism with which to study female pelvic pain. Glia play an active role within the innate immune system (Baaklini et al., 2019; Lehnardt, 2010) with the formation of a wide range of cytokines including IL-1β following immune recognition of the presence of an infective pathogen or damaged tissues.

There are three major glial cell types within the central nervous system (CNS): astrocytes, oligodendrocytes and microglia, which communicate with each other, and modulate the activity of neuronal synapses, using neurotransmitters including glutamate, ions including potassium and calcium, and small molecules. Astrocytes are derived from neuronal stem cells (Mujtaba et al., 1998). Their functions include the regulation of cerebral blood flow, which is believed to be the basis for changes observed during functional MRI imaging (Ogawa et al., 1990). Oxygen delivery exceeds the rate of oxygen utilisation in areas of the brain with increased neuronal activity, which in turn increases the MRI signal. Glia can affect both the encoding and the consolidation of memory (Steadman et al., 2020). Interestingly, Han et al. who replaced forebrain mouse astrocytes in humanised chimeric mice with human astrocytes, demonstrated faster learning among these mice than controls (Han et al., 2013).

The association of astrocytes with memory and learning has potential implications for the development of chronic pain. Microglia are the macrophages of the CNS. They invade the developing brain from the fetal yolk sac, the same tissue of origin as macrophages within the systemic circulation (Ginhoux et al., 2010; Li & Barres, 2018). Microglia are involved in neuronal pruning, the phagocytosis of pathogens and debris, and are the first glia activated following acute injury. Oligodendrocytes are derived from neuronal stem cells (Mujtaba et al., 1998). They support and restore myelin sheath formation through the formation of synaptic proteins (Hughes & Appel, 2019). The transition from an early microglial activation response to a later astrocytic activation response has been suggested as the mechanism behind the development of chronic pain following acute injury (Cairns et al., 2015).

The traditionally accepted 'bipartite" neural synapse model (Figure 1.18a), with two neurons and a single neurotransmitter (noradrenaline), has now become the 'tetrapartite' synapse model (Figure 1.18b) with two neurons, surrounding glial cells (commonly astrocytes and microglia), and a plethora of neurotransmitters, each able to modulate the pain impulse transmitted. Critical to the tetrapartite synapse are Toll-like receptors, particularly TLR2 and TLR4. Activation of the innate immune system has a mechanism to modulate or amplify pain impulses at the level of the synapse, through the activation of glial cells (Lacagnina et al., 2018; Nicotra et al., 2012; Grace et al., 2014). The discovery in 2017 that the brain has lymphatic vessels providing easy access for circulating immune cells to interact with glial cells within the central nervous system, further supports the ability of immune activation to influence central pain sensitisation (Absinta et al., 2017; Ha et al., 2018).

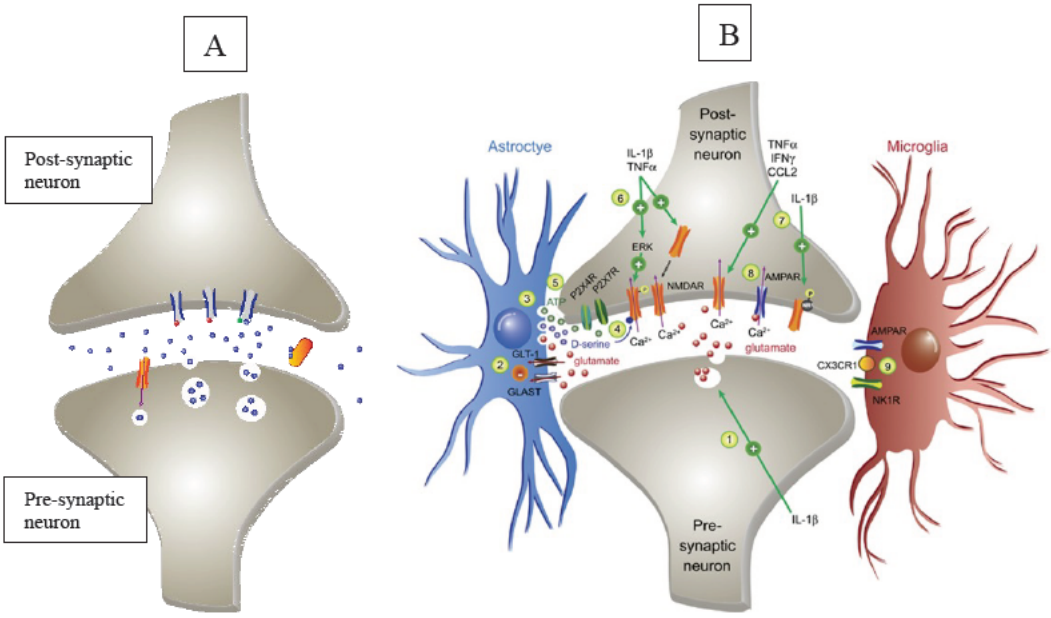

Figure 1.18a: Diagram outlining the structure of a simple bipartate synapse, comprising a pre-synaptic, a post-synaptic neuron and a single neurotransmitter. Reproduced with permission Dr K Dodds

Figure 1.18b: Diagram outlining the structure of the tetrapartate synapse, comprising a pre-synaptic, post-synaptic neuron, glial cells (astrocyte and microglia), and the release of multiple neurotransmitters. Reproduced with permission (Dodds et al., 2016).

1.7.3 TLR4 AGONISTS AND ANTAGONISTS IN CURRENT MEDICAL PRACTICE

Multiple drugs in current clinical practice have demonstrated activity at TLR receptors, either as agonists or antagonists. TLR4 agonists include commonly prescribed medications such as opioids (morphine, buprenorphine, fentanyl, methadone, oxycodone, pethidine), carbamazepine, ethanol, and oxcarbazepine (Hutchinson et al., 2010; Hutchinson et al., 2010). Tapentadol is a mixed agonist/antagonist. TLR4 antagonists include amitriptyline, cyclobenzaprine, ketotifen, imipramine, mianserin, ibudilast, naloxone, naltrexone, propentofylline, palmitoylethanolamide (Impellizzeri et al., 2015), (+)-naloxone (Hutchinson et al., 2008), melatonin, and eritoran (TAK-242).

As an example of a drug affecting TLR activity, melatonin is an indolamine derived from the amino acid tryptophan that is formed in the pineal gland. It has multiple physiological effects, including anti-angiogenesis, anti-proliferation, and anti-inflammation, and acts through signalling pathways that include nuclear factor-κB(NF-κB) (Shrestha et al., 2017; Yao et al., 2019). Of relevance to this thesis, melatonin reduces both the activation and production of cyclo-oxygenase-2 from microglia (Yao et al., 2019). As described in section 1.4.3, levels of melatonin are lower in women with dysmenorrhoea than in normal controls (Schwertner et al., 2013). In another study, Berkiks et al. (2018) showed that melatonin mediated significant protective effect against LPS-induced depression and anxiety, both common symptoms in humans with inflammatory conditions (Hardeland, 2019; Yao et al., 2019).

As an example of foodstuffs affecting TLR activity, curcumin, the active ingredient in turmeric, has known TLR4 antagonist activity (Boozari et al., 2019; Gupta et al., 2012; Islam et al., 2019; Momtazi-Borojeni et al., 2019; Zhang & Zeng, 2019). Unitt and Hornigold (2011) demonstrated that phytohaemagglutin (in green beans) enhanced the release of inflammatory cytokines from peripheral blood mononuclear cells (PBMCs) through a pure TLR4 mechanism. TLR agonists have been proposed as adjuvants for vaccines against viruses or bacteria through their ability to enhance the inflammatory immune response (Behzad et al., 2012; Liu et al., 2006). Lipopolysaccharide (endotoxin) is a large molecule found in the outer membrane of gram-negative bacteria, that induces a strong immune response via TLR2 and TLR4 receptors. LPS is widespread in the human environment, even present at low levels as a contaminant in intravenous saline, where LPS is acceptable provided its concentration does not exceed the pyrogenic threshold of 5 EU/kg/hr (Research, 2019)

1.7.4 TLR4 AND DYSMENORRHOEA, PELVIC PAIN AND ENDOMETRIOSIS

Since their discovery, the role of Toll-Like Receptors, particularly TLR4, in infective and malignant conditions of the thoracoabdominal viscera, has been an intense research focus. Preclinical research has demonstrated a benefit of TLR4 inhibition in inflammatory or malignant conditions of the heart and lung (Wang et al., 2016), kidney (Fenhammar et al., 2011; Zhang et al., 2015), liver (Yu et al., 2013), and pancreas (Pan et al., 2017). Toll-like receptor antagonists such as eritoran (TAK-242) have been developed and investigated in human trials of sepsis (Rice et al., 2010).

However, the inflammatory role of TLR4 in female sex-specific pelvic pain and reproductive health has attracted less interest. A search of Pubmed (accessed 03/01/2020) found that while 5,642 articles were available when combining the search terms TLR4 and infection, only 18 were found when searching ‘TLR4’ with ‘Endometriosis’, 7 were available searching ‘TLR4’ with ‘pelvic pain’, and 2 were available when searching ‘TLR4’ with ‘dysmenorrhoea’, both utilising a rodent model.

Despite this lack of prior research activity, multiple lines of evidence support a strong role for TLRs in female pain. TLR4 receptors may be activated following exposure to certain bacteria (presence of LPS) or where there are damaged or degenerating cells present (Gong et al., 2020; Molteni et al., 2016). The process of menstruation involves cells undergoing tissue breakdown (Evans & Salamonsen, 2012; Salamonsen et al., 2002) and the potential for bacterial contamination of the endometrial cavity. The activation of TLR4 receptors triggers a cascade of cellular processes acting via the NF-kb signaling pathway, and resulting in the productions of COX-2, and cytokines IL-1β, IL-6 and TNF-α (Shih et al., 2018; Saito et al., 2010; Uematsu et al., 2002) (Figure 1.19). With regard to dysmenorrhoea, a TLR4 -mediated rise in COX-2, and subsequent increase in PGE2 production offers a potential mechanism for increased myometrial contractions and an increase in the intensity of dysmenorrhoea. Seillet et al. demonstrated that estrogen enhanced the TLR4 response of human dendritic cells (Seillet et al., 2012a, 2013), providing a mechanism for an increase in symptoms as estrogen rises with puberty. Seillet’s study found that DCs from premenopausal women exhibited increased TLR4 responses when compared to men, while DCs from postmenopausal women did not.

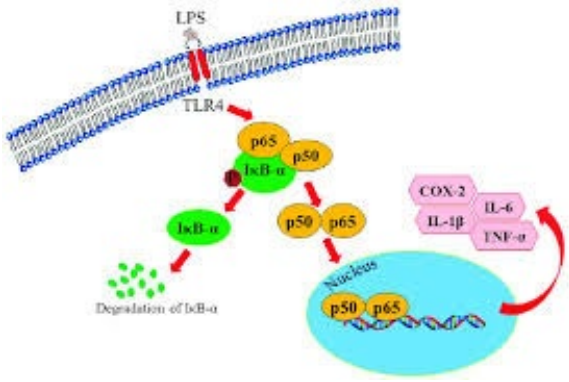

Figure 1.19. TLR4-mediated NF-κB signaling pathway with enhanced production of COX-2, IL-1β, IL-6 and TNF-α following LPS stimulation (Shih et al. 2018)

The wide variation in hormonal levels across the menstrual cycle, and among individuals, offers addition ways in which activation of the immune system can impact female pain. Nicotra et al. used a graded severity model of sciatic nerve pain in rats (Nicotra et al., 2014), developed by Grace, to demonstrate its importance in chronic pain. Female rats were more allodynic than males following sciatic nerve injury, and female rats were more allodynic during the higher estrogenic phases of the rat oestrus cycle (Nicotra et al., 2014). This was further supported by Soucy et al. who determined that 17β-estradiol (E2) acting via ERα is required for a full microglial response to LPS in the brain of female mice (Soucy et al., 2005). Rettew et al. (2008) showed that E2 enhanced the expression of TLR4 on the surface of peritoneal macrophages in rodents, while testosterone reduced TLR4 expression consistent with an immunosuppressive effect. Recent research has found sex-specific variation in glial cell response between males and females after acute injury, with microglial activation prominent in males but not females, and astrocytic activation common to both sexes (Chen et al., 2018).

Use of the oral contraceptive pill (OC) substantially alters the inflammatory response, including the release of inflammatory cytokines. Sikora et al. (2015) measured levels of IL-1β and neopterin (a biomarker for systemic inflammation) in serum and monocyte culture in 4 groups of women: 25 women with a normal menstrual cycle, 25 women using the oral contraceptive pill, 25 women with endometriosis and 20 post-menopausal women where estrogen levels are low. Serum IL-1β levels were lowest in women on the oral contraceptive pill and highest in the women with endometriosis. Serum neopterin levels were lowest in women with normal menstrual cycles and highest in women with endometriosis.

With regard to endometriosis, TLR4 is expressed on macrophages, the cells associated with the clearance of menstrual debris from the peritoneal cavity. Khan et al. have provided a solid and compelling body of work supporting a role for the immune system and TLR's in endometriosis through a series of research projects. Their findings have demonstrated:

a. A higher prevalence of endotoxin in the peritoneal fluid of 54 women with endometriosis when compared with 25 healthy controls. Within this group, higher levels of *E.Coli* in the menstrual blood of women with endometriosis were observed, potentially contributing to the higher levels of E.coli within the peritoneal fluid samples. Macrophages from the fluid of women with endometriosis induced higher expression of IL-6 and TNF-α when treated with LPS (Khan et al., 2006)

b. The demonstration of more severe endometritis ($p<0.0001$), but no higher frequency of the condition in a histological study comparing the endometrium of 73 women with endometriosis and 55 healthy controls (Khan et al., 2014).

c. The additive effect of 17β-estradiol and LPS on the *in vitro* production of IL-6 and TNF-α by macrophages and endometrial stromal cells obtained by women with endometriosis (Khan et al., 2015). The peritoneal fluid from 46 women with endometriosis and 30 control women was collected at laparoscopy. Macrophages and endometrial stromal cells isolated from women were then exposed to either 17β-estradiol or LPS or a combination of E2 and LPS.

Khan's research culminated in the publication of the "bacterial contamination theory for the development of endometriosis" (Khan et al., 2018). However, the potential implications of these findings on the presence or severity of dysmenorrhoea or pelvic pain do not seem to have been further explored.

In summary, these studies indicate that roles exist for genetic, hormonal and environmental factors to explain the increased immune activation in females compared to males. In addition, initial evidence exists that immune activation in females may involve a TLR based mechanism.

1.8 THE EVIDENCE FOR A COMMON AETIOLOGICAL MECHANISM ACROSS DYSMENORRHOEA, CHRONIC PELVIC PAIN AND ENDOMETRIOSIS

As has been described in sections 1.1, 1.2, 1.3, 1.4, and 1.5, despite their varied clinical presentations, dysmenorrhoea, chronic pelvic pain and endometriosis display substantial overlap with regard to epidemiology, human and fiscal burdens, and potential aetiologies.

1.8.1 THE COMMON ASSOCIATION WITH MENSTRUATION

Exposure of an individual to menstruation is associated with the presence or progression of each of these conditions. While there are many causes of female pelvic pain, dysmenorrhoea is by far the most frequent symptom reported. In a study by Jarrell of 181 women with CPP, 62% of women described dysmenorrhoea (Jarrell & Arendt-Nielsen, 2016). Hardi described the transition from dysmenorrhoea alone to CPP in a retrospective study of 100 women, and found that transition occurred in 16% of women within 12 months of severe dysmenorrhoea, and in over 50% of women within 12 years (Hardi et al., 2014). Risk factors for the development of endometriosis include an earlier age at menarche (Nnoaham et al., 2012), shorter menstrual cycles and fewer pregnancies (Missmer & Cramer, 2003; Treloar et al., 2005).

In 2016, Jarrell and Arendt-Nielsen (Jarrell & Arendt-Nielsen, 2016) proposed that the high frequency of chronic pelvic pain in modern communities may be due to the increased number of menstrual cycles experienced in modern times. Girls and women now experience many more episodes of menstruation than women in previous generations (Figure 1.9). Menarche is earlier, pregnancy is delayed, fewer pregnancies occur and breast-feeding is reduced. They proposed that dysmenorrhoea may have developed to enhance reproduction in unknown ways, at a time where there were fewer menses experienced and therefore fewer implications of pain to the individual.

However, while the transition from dysmenorrhoea to CPP may occur over time, as proposed by Jarrell and supported by Hardi et al (Hardi et al., 2014), severe dysmenorrhoea itself frequently presents at, or soon after, menarche. Adolescents with severe dysmenorrhoea display a high prevalence of laparoscopically-visualised endometriosis despite their brief exposure to menstruation (Harel, 2008; Janssen et al., 2013; Marsh & Laufer, 2005; Zannoni et al., 2014). This strongly suggests that other factors apart from the number of menstrual

cycles experienced are involved in the generation of dysmenorrhoea, endometriosis and chronic pelvic pain.

With regard to endometriosis, suppression of menstruation reduces the likelihood of lesion recurrence following endometriosis excision when compared with women in whom menstruation is not suppressed (Unger & Laufer, 2011). Vercellini (2003) found a lower frequency of dysmenorrhoea in women who suppressed menstrual bleeding using a levonorgestrel-releasing intra-uterine device following laparoscopic removal of endometriosis lesions when compared with women who underwent surgery alone.

These factors confirm an association between dysmenorrhoea, pelvic pain, and endometriosis with menstruation, but do not imply causation.

1.8.2 A COMMON EPIDEMIOLOGY AND EARLY AGE OF SYMPTOM ONSET

Consistent with their association with menstruation, dysmenorrhoea, CPP and endometriosis are most frequently diagnosed during a woman's reproductive years, beginning at or close to menarche. Jarrell & Arendt-Nielsen (2016) proposed an evolutionary aetiology that attributes much of the risk of dysmenorrhoea and endometriosis to the more numerous menstrual cycles experienced by first-world girls and women in the modern era. However, we know that dysmenorrhoea can be severe from menarche, which argues against this conclusion.

Pain symptoms also present at an early age in women who are later diagnosed with endometriosis. In 1998, Ballweg et al. (2004) reviewed the histories of 3020 women in the 1980s and 4000 women in 1998 with surgically confirmed endometriosis. Forty-one percent in the 1980s and 66% in the 1998 cohort described pain symptoms that began before the age of 20. A comparison of these two patient cohorts is presented in Figure 1.20.

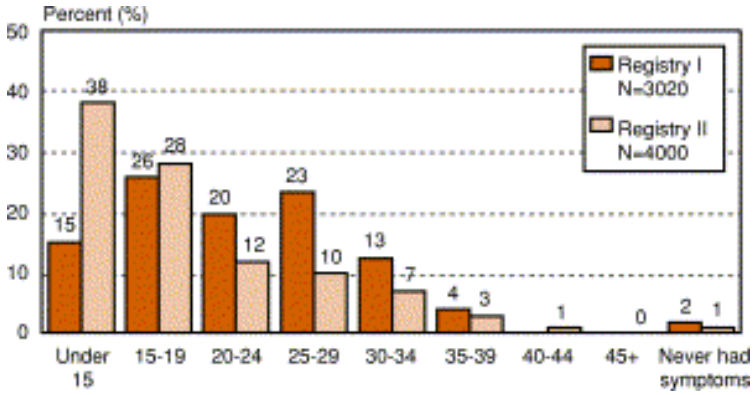

Figure 1.20: Age of first pelvic symptoms across two patient cohorts sampled 10 years apart (Ballweg et al., 2004).

Marsh & Laufer (2005) describe five premenarchal girls with unexplained pelvic pain and with histologically-confirmed endometriosis lesions visualised at laparoscopy and histologically confirmed despite an otherwise normal reproductive tract. This demonstrates that endometriosis lesions may be present from a young age, and in the absence of menstruation.

The early age for the development of all three conditions suggests a common premenarchal mechanism predisposing to the development of one or more of dysmenorrhoea, chronic pelvic pain or endometriosis. These findings are inconsistent with a mechanism for endometriosis relying on the cumulative effect of retrograde menstruation over progressive menstrual cycles.

1.8.3 A COMMON SYMPTOM PROFILE SUGGESTING CENTRAL PAIN SENSITISATION

The presence of endometriosis lesions is increasingly associated with an increased prevalence of non-gynaecological pain symptoms within and beyond the pelvis that include migraine, Painful Bladder Syndrome, Irritable Bowel Syndrome, or pelvic muscle dysfunction (Tietjen, 2007; Smorgick et al., 2013; Surrey et al., 2018; Vannuccini et al., 2018). However, this should not be seen as implying causality between endometriosis lesions and these additional symptoms. The presence of pain is a common feature across these conditions, and central pain sensitisation provides a potential mechanism for this effect.

The co-occurrence of dysmenorrhoea with additional pain symptoms within the pelvis may be ascribed to viscero-visceral hyperalgesia (Section 1.4.4), where pain from one visceral organ affects the pain reactivity in a second visceral organ (Giamberardino, 1999; Giamberardino et al., 2010). This phenomenon is attributed to the convergence of afferent neurons from both visceral organs in the dorsal horn of the spinal cord. Inflammation in the first organ results in central pain sensitisation with amplification of pain sensations from the second organ (Brumovsky & Gebhart, 2010). Li et al. (2008) used retrograde tracing dyes to demonstrate that while some sensory afferent neurons of the dorsal root ganglion are specific to either the uterus or the colon, a proportion of sensory afferent neurons receive sensory input from both uterus and bowel (Li et al., 2008). Sensory afferent neurons innervating the bladder, uterus and bowel ascend within the hypogastric nerve to similar spinal cord segments (Figure 1.21).

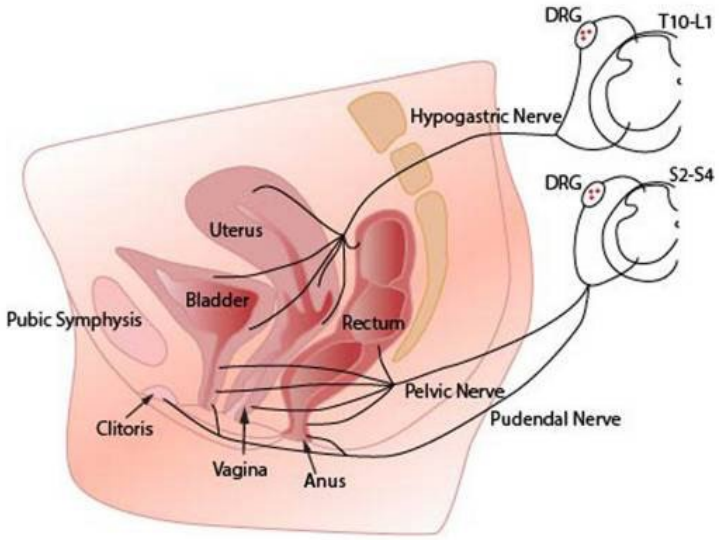

Figure 1.21: The common innervation pathways for pelvic viscera in women through the hypogastric nerve. Reproduced with permission (Jobling et al., 2014).

Pain sensitisation also provides a mechanism for pain symptoms outside the pelvis, including migraine, through the release of systemic cytokines, or activation of glial immune cells within the CNS, as described in Section 1.7. In contrast to the traditional view that neurogenic inflammation, passes purely from periphery to central locations, Sorkin et al. (2018) have presented evidence that neuroinflammation within the CNS may travel in an antidromic direction to induce inflammation in the periphery.

1.8.4 The common association with inflammation, TLR4, and the immune system

Menstruation provides ample opportunity for activation of the immune system. Breakdown of the endometrium during menses is associated with edema, leukocyte infiltration (Salamonsen et al., 2002), activation of cytotoxic T-Cells of the endometrium (H. D. White et al., 1997), and an increase in intrauterine chemokines (Roomruangwong et al., 2020).

The ability of endometrial cells to reach draining lymph nodes during menstruation, and thus present endometrial cells to T-Cells, has been confirmed in both a baboon (Papio anubis) model (Hey-Cunningham et al., 2011) and in humans (Berbic et al., 2013; Javert, 1950, 1952a, 1952b). Berbic et al. reviewed the obturator lymph node histology of seven women with endometriosis and nine women without endometriosis at the time of hysterectomy for gynaecological malignancy (cervical or ovarian). CD10+ endometrial stromal cells were found

across the menstrual cycle, with numbers peaking during menstruation. Any situations where lymphocyte activation is impaired has the potential to result in the reduced clearing of menstrual debris from the peritoneal cavity, prolonged exposure to both endometrial cells and micro-organisms, and increased potential for immune activation.

In women with endometriosis, the numbers of various immune cell populations, including macrophages, uterine NK cells and immature dendritic cells within the endometrium and peritoneal fluid, are chronically increased (Berbic et al., 2009; Schulke et al., 2009). Macrophages comprise 50% of peritoneal cavity leukocytes (Kubicka et al., 1996) and have a central role in the development of endometriosis (Hogg et al., 2020). Macrophages infiltrate endometriosis lesions (Greaves et al., 2014), and exhibit disrupted function with complex alterations in pro- and anti-inflammatory cytokines (Beste et al., 2014) associated with a transition from the classical M1 macrophage activity to the more pro-inflammatory M2 profile (Johan et al., 2019).

The dysregulation of immune cells, including macrophages, has been demonstrated in women with endometriosis, and particularly in women where pain is a major symptom (Akoum et al., 2005; Akoum et al., 2006; Berbic et al., 2009; Schulke et al., 2008; 2009; 2009). This disruption includes deficiencies in macrophage phagocytic activity (Sharpe-Timms et al., 1998), activation of macrophages, and increased synthesis of cytokines. Macrophages are present in almost all tissues, displaying varied physiology in response to their environment, and an ability to transition to an inflammatory phenotype with tissue infiltration in the presence of inflammation (Mosser & Edwards, 2010; Gordon & Taylor, 2005).

Activation of the innate immune system within the uterus, potentially involving TLR4 mechanisms, offers a unifying theory between the enhanced immune activation in females, the increased female exposure to infectious organisms via the reproductive tract, and inflammatory conditions including dysmenorrhoea, endometriosis and chronic pain.

1.8.5 UNANSWERED QUESTIONS IN AN AREA OF UNMET NEED

To date, medical practice has considered dysmenorrhoea to be due to either biochemical changes within a normal uterus, or to secondary pelvic conditions such as endometriosis. This ‘peripheral’ approach to dysmenorrhoea provides the rationale for current medical management: drugs to reduce prostaglandin release, drugs to reduce estrogen levels, or surgery to remove endometriosis. While these techniques are successful in a proportion of

cases, there remain girls and women for whom these treatments are ineffective, unacceptable, or associated with adverse effects.

This literature review has outlined substantial gaps in current knowledge regarding dysmenorrhoea and its relationship to chronic pelvic pain and endometriosis. Clinically, gynaecologists who care for girls and women with pain must do so without the benefit of high-quality research to guide their recommendations. They offer laparoscopic investigation to remove endometriosis, without the benefit of reliable, non-invasive ways to predetermine whether endometriosis will be present, or evidence that the lesions are wholly responsible for their patient's pain. They offer hormonal management and non-surgical treatments without the benefit of a clear knowledge of the underlying mechanisms underlying their patient's pain.

Dysmenorrhoea is clearly a condition with important educational, employment, quality-of-life, financial and health economic implications, worthy of further research. Many questions remain, despite convincing evidence that the end mechanism behind uterine dysmenorrhoea is prostaglandin-mediated, hormone-facilitated excessive contraction of the uterus, with resulting myometrial ischaemia.

With approximately one in five young women throughout the world affected, dysmenorrhoea has been under-researched with regard to its frequency and human impact. Dysmenorrhoea dramatically affects a woman's ability to live to her full potential, by affecting her ability to engage in educational, social, workplace and personal opportunities.

This thesis has five aims, to address knowledge gaps that will support researchers, women and clinicians in their quest to lessen the impact of DRPP:

Aim 1: To further knowledge of the symptoms associated with dysmenorrhoea-related pelvic pain, as described by women themselves. This knowledge seeks to determine whether these experiences are consistent with a purely pelvic condition, or a systemic condition with symptoms both within and outside the pelvis. These findings will determine whether a specific symptom profile is associated with the presence or absence of endometriosis, to refine where surgical management is appropriate.

Aim 2: To determine whether activation of the innate immune system, via Toll-Like Receptors, provides an explanation for the symptoms associated with DRPP. These findings will guide clinicians and researchers in their quest to develop new and effective non-surgical management options for women with DRPP.

Aim 3: To determine whether levels of high sensitivity C-Reactive Protein (hsCRP), an easily measurable blood test, may act as a biomarker for pain and immune activation in women with DRPP. These findings, if positive, will support researchers with a clinically objective endpoint for clinical trials of novel therapies.

Aim 4: To determine whether levels of sex steroid hormones, or cortisol, influence activation of the innate immune system in women with DRPP. These findings will guide clinicians towards prescribing hormonal medication optimially suited to the minimisation of pain and pain-related symptoms.

Aim 5: To determine whether it is possible to develop a Unifying Theory for the relationship between dysmenorrhoea, endometriosis and chronic pelvic pain.

CHAPTER 2: THE LIVED EXPERIENCE OF DYSMENORRHOEA

2.1 INTRODUCTION

This chapter aims to further knowledge in an area of medicine that has been under-researched with regard to its impact on the wellbeing of women, their families and our society: the lived experience of women with dysmenorrhoea, as described by women themselves. Through the use of a clinical symptom questionnaire developed over many years within a multi-disciplinary pelvic pain clinic, it has four aims, all of high translational importance for treating clinicians and women affected by dysmenorrhoea-related pelvic pain.

2.2 AIMS

This chapter has four aims:

Aim 1: To investigate the frequency of 14 symptoms, both within and outside the pelvis, in a group of 168 women with dysmenorrhoea-related pelvic pain. This aim considers whether the range of symptoms present in women with dysmenorrhoea is consistent with purely peripheral mechanisms within the pelvis. A high prevalence of symptoms outside the pelvis would support the presence of central mechanisms, as a component of the pain experience in these women.

Aim 2: To determine whether a specific profile of pain-related symptoms can differentiate between women with or without laparoscopically confirmed endometriosis. The development of an indicative symptom profile would dramatically improve the ability of health practitioners to target invasive surgical procedures, including laparoscopy, for those women most likely to obtain benefit from the procedure. This finding would have implications for health economics, through allowing the better targeting of health dollars and hospital resources. A comparison of the symptom profiles of women with and without endometriosis lesions, provides additional insights into whether the relationship between endometriosis lesions and pain is one of causation or association. A similar symptom profile for women both with and without endometriosis would support an associative relationship, potentially with a common underlying aetiology.

Aim 3: To document the range of symptoms commonly experienced by women with dysmenorrhoea-related pelvic pain, and determine whether certain symptoms cluster with dysmenorrhoea. Where clustering of symptoms is found, this supports the presence of a common underlying mechanism. A clustering of certain symptoms would also form the basis for a new clinical syndrome: Dysmenorrhoea-Related Pelvic Pain Syndrome (DRPPS).

Aim 4: To investigate whether a history of distressing sexual events alters the frequency, or severity of clinical symptoms in women with dysmenorrhoea and related symptoms.

This chapter introduces the manuscript entitled "The co-morbidities of dysmenorrhoea: A clinical survey comparing symptom profile in women with and without endometriosis" which has been published in the Journal of Pain Research.

2.3 HYPOTHESES

This chapter investigates four hypotheses:

Hypothesis 1: That women with dysmenorrhoea suffer a high frequency of additional symptoms both within and outside the pelvis that contribute to their lived experience of dysmenorrhoea.

Hypothesis 2: That the reported symptom profile can differentiate between women with or without a laparoscopically proven diagnosis of endometriosis.

Hypothesis 3: That the presence of endometriosis is a common, but non-essential, feature of DRPPS.

Hypothesis 4: That a history of distressing sexual events is associated with an alteration in the symptom profile in women with dysmenorrhoea-related pelvic pain.

2.4 MANUSCRIPT AND STATEMENT OF AUTHORSHIP

The following manuscript was published in the Journal of Pain Research on 13th December 2018. All authors gave final approval of the version to be published, and agree to be accountable for all aspects of the work:

All authors gave final approval of the version to be published, and agree to be accountable for all aspects of the work.

Statement of Authorship

Title of Paper	The Co-Morbidities of dysmenorrhea: A clinical survey comparing symptom profile with and without endometriosis
Publication Status	☐ Published
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Principal Author

Name of Principal Author (Candidate)	Susan F Evans		
Contr bution to the Paper	Study design, acquisition of data, preparation of data, analysis of data, interpretation of data, preparation of article.		
Overall percentage (%)	80		
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.		
Signature		Date	12/09/2020

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);

ii. permission is granted for the candidate in include the publication in the thesis; and

iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contr bution.

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Signature		Date	12/09/20

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Contr bution to the Paper	Study design, interpretation of data, preparation of article		
Overall percentage (%)	5		
Signature		Date	28/09/2020

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ORIGINAL RESEARCH

The comorbidities of dysmenorrhea: a clinical survey comparing symptom profile in women with and without endometriosis

This article was published in the following Dove Press journal:
Journal of Pain Research

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Purpose: Dysmenorrhea is a common disorder that substantially disrupts the lives of young women. The frequency of 14 associated symptoms both within and outside the pelvis was determined.

Patients and methods: Symptom questionnaires were completed by 168 women with dysmenorrhea, allocated to three groups based on their diagnostic status for endometriosis confirmed (Endo+), endometriosis excluded (Endo−), or endometriosis diagnosis unknown (No Lap). Those with endometriosis confirmed were further divided into current users (Endo+ Hx+) and non-users of hormonal treatments (Endo+ Hx−). Users of hormonal treatments were further divided into users (Endo+ Hx+ LIUCD+) and non-users (Endo+ Hx+ LIUCD−) of a levonorgestrel-releasing intra-uterine contraceptive device (LIUCD). The frequency and number of symptoms within groups and the effect of previous distressing sexual events were sought.

Results: Women with and without endometriosis lesions had similar symptom profiles, with a mean of 8.5 symptoms per woman. Only 0.6% of women reported dysmenorrhea alone. The presence of stabbing pelvic pains was associated with more severe dysmenorrhea (P=0.006), more days per month of dysmenorrhea (P=0.003), more days per month of pelvic pain (P=0.016), and a diagnosis of migraine (P=0.054). The symptom profiles of the Endo+ Hx+ and Endo+ Hx− groups were similar. A history of distressing sexual events was associated with an increased number of pain symptoms (P=0.003).

Conclusion: Additional symptoms are common in women with dysmenorrhea, and do not correlate with the presence or absence of endometriosis lesions. Our study supports the role of central sensitization in the pain of dysmenorrhea. The presence of stabbing pelvic pains was associated with increased severity of dysmenorrhea, days per month of dysmenorrhea, days per month of pelvic pain, and a diagnosis of migraine headache. A past history of distressing sexual events is associated with an increased number of pain symptoms.

Keywords: dysmenorrhea, endometriosis, headache, bladder pain syndrome, chronic pain, pelvic pain, levonorgestrel-releasing intra-uterine device, stabbing pain

Introduction

Dysmenorrhea, the experience of painful menstruation, is common.[1] A study of Australian girls aged 16–18 years showed that while 93% experienced some pain with menstruation, 21% experienced severe pain, frequently associated with disruption of life activities and school absence.[2] While dysmenorrhea may be present as a sole symptom without evidence of disease, it may also be associated with the medical condition endometriosis, or may pre-date chronic pelvic pain.[3–6] Endometriosis is associated with an average of 11 hours of lost workplace productivity per week,[7] and pelvic pain has

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been estimated to cost the Australian economy over US$6 billion per year.[8] Dysmenorrhea in isolation, whether as a symptom of endometriosis or when associated with chronic pelvic pain, is clearly a condition with important educational, employment, quality of life, financial, and health economic implications.

Developing effective clinical guidelines for the management of dysmenorrhea, endometriosis, and chronic pelvic pain is complicated due to their overlapping symptomatology, a lack of biomarkers, and the requirement that women undergo laparoscopic surgery to determine whether endometriosis lesions are present.[9] In addition, confounding factors, potentially including a past history of sexually distressing events, may modify the pain experience.[10]

While several studies have linked a laparoscopic diagnosis of endometriosis with a wide range of additional symptoms at a rate higher than the general population,[9,11–15] few papers have investigated the frequency of these symptoms in women presenting with dysmenorrhea in clinical practice. In addition, they have not determined whether a specific symptom profile is associated with the presence of endometriosis lesions.

This study investigates the prevalence of 14 symptoms in women with dysmenorrhea who were referred to a pelvic pain and endometriosis unit with ready access to laparoscopic surgery. It compares the symptom profile of women with and without a laparoscopic diagnosis of endometriosis, and the severity of symptoms in women with and without a history of distressing sexual events. Three specific hypotheses were investigated:

1. That the prevalence of additional symptoms is high in women with dysmenorrhea,
2. That a specific symptom profile may indicate the presence of endometriosis lesions, and
3. That experience of distressing sexual events may alter the symptom profile of women with dysmenorrhea.

This study provides an insight into both the pain experience and the symptom profile of women presenting to gynecologists and laparoscopic surgeons in real-life clinical practice.

Patients and methods

Study design

This is a cross-sectional analytical study.

Participants

The target population consisted of girls and women over 16 years of age, with dysmenorrhea rated as more than 3 on a 0–10 numerical scale, and with sufficient written English language skills to complete a symptom questionnaire. All participants were given an informed opportunity, to give or withhold consent for the use of their de-identified health information for research purposes, by marking one of two boxes on the questionnaire (see the section "Test methods" for details). Figure 1 shows the CONSORT flow diagram for study inclusion.

Permission to study the symptoms of those women who had indicated their consent on the questionnaire was approved by the Royal Adelaide Hospital Ethics Committee, HREC Reference No: R20170604. The study was conducted in accordance with the Declaration of Helsinki.

Test methods

The symptom questionnaire (displayed in Appendix 1) was posted to all new patients (301 women) referred to a pelvic pain and endometriosis unit, with ready access to laparoscopic surgery for the period January 1, 2015 to June 30, 2016. The questionnaire was developed by a pain specialist gynecologist (SFE) to assist with patient management. It was refined over 18 years to ensure ease of use by patients, minimize unanswered questions, and facilitate a full clinical assessment of relevant symptoms by the clinician.

The dysmenorrhea-associated symptoms investigated included stabbing pains in the pelvis, bowel symptoms, food intolerances, bladder symptoms, headaches, pain with sexual activity, vulval pain, fatigue, poor sleep, nausea, sweating, dizziness, anxiety, and low mood. The presence of anxiety or low mood was assessed using a Depression Anxiety Stress Scale-21 questionnaire.[16]

Additional questions recorded patient age, parity, use of hormonal treatments for menstrual symptoms, days of pain per month, duration of dysmenorrhea, and whether there was a history of distressing sexual events, including sexual assault.

Six months following the closure of study enrolment, notes for each patient were retrieved and reviewed by the principal investigator, who was blinded to patient symptoms. Each de-identified patient was allocated to groups according to whether a diagnosis of endometriosis had been confirmed (Endo+), excluded (Endo–), or could not be determined as no laparoscopy had been performed (No Lap). Endometriosis was considered confirmed when endometriotic lesions had been confidently observed during laparoscopy at any time in their life, or where endometriomas were confidently visualized on ultrasound at the time of clinical assessment. An ultrasound diagnosis of endometrioma has known high specificity for pathological confirmation at histology.[17] Endometriosis was

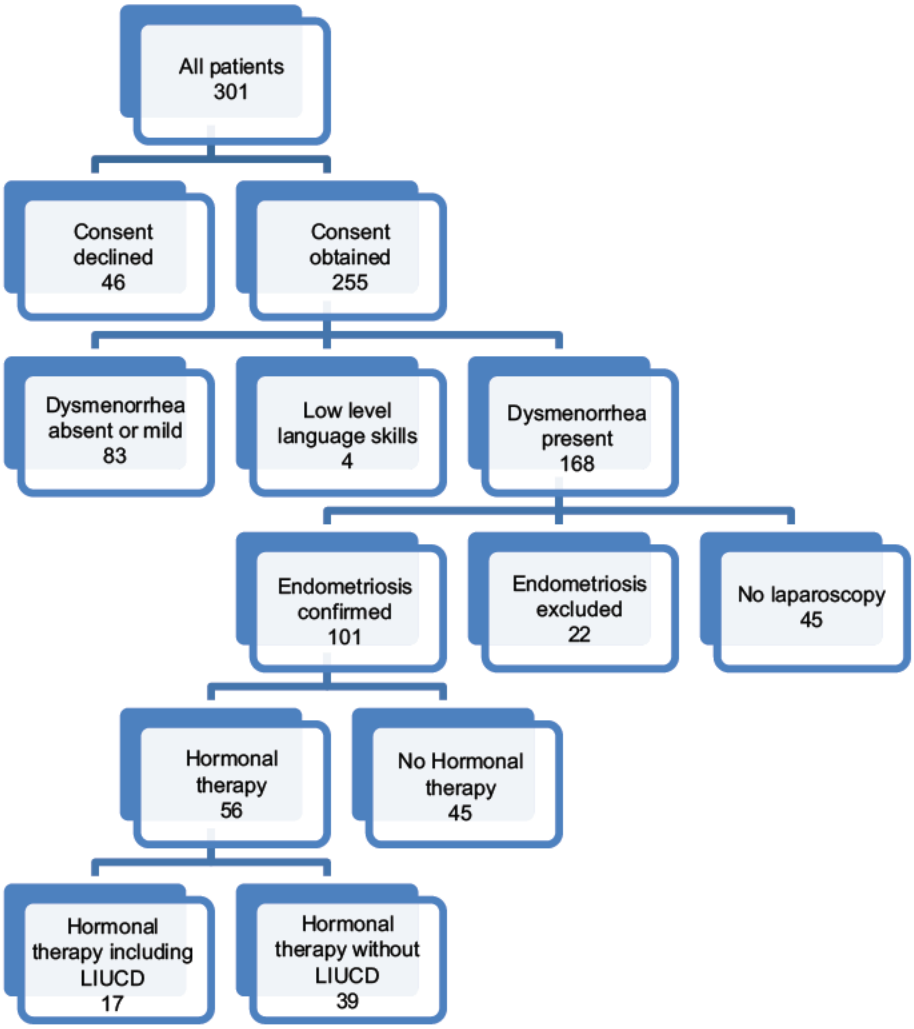

Figure 1 Consort diagram displaying the study selection process and method of group allocation.
Abbreviation: LIUCD, a levonorgestrel-releasing intra-uterine device.

considered as excluded where at least one laparoscopy had been performed during their life, and there had never been evidence of endometriotic lesions.

Statistical analysis

Following consent, the questionnaire was de-identified, each subject was allocated a numerical code, and the de-identified data were entered into SPSS statistics 23 software (IBM Corp, Armonk, NY, USA). Questionnaire data and data analysis were undertaken by investigators blinded to endometriosis diagnosis. Statistics for each table are based on all cases with valid data in the specified range(s) for all variables.

Descriptive statistics included means and SDs for continuous variables, and counts and percentages for categorical variables. Mean and SDs were compared using independent samples *t*-tests and proportions by chi-squared tests. Predictors of stabbing pain were determined by multivariable logistic regression. Differences were considered significant at a P-value of <0.05.

Missing data

As questionnaires were completed independently, prior to clinic attendance, it was not possible to ensure that all questions were completed. Thus, for each symptom, some data were missing. The missing data rate for the 14 symptoms excluding "pain with intercourse" averaged 1.1% of responses. Missing data rate for the symptom "pain with intercourse" was 15% of responses, and missing data rate regarding history of "distressing sexual events, including sexual assault" was 18%.

Results

Details of patient numbers overall and in each group are provided in Figure 1. Of 301 women who completed the questionnaire, informed consent was provided by 255 women and declined by 25 women. Twenty-one women neglected to complete the request for consent and were deemed to have declined. Where the patient was under the age of 18 years, inclusion required the presence of a parent during the medical consultation to confirm consent.

From this cohort of 255 women, 168 patients suffered dysmenorrhea. Eighty-seven women were excluded from the study as their pain from dysmenorrhea was rated as less than 3 on a numerical 0–10 score (n=83), or written English language skills were poor (n=4), and consent could not reasonably be obtained.

From this cohort of 168 women, those patients with endometriosis confirmed (Endo+, n=101), endometriosis excluded (Endo–, n=22), and where a diagnosis could neither be confirmed nor excluded (No Lap, n=45), were identified. Of those women with endometriosis confirmed, 16% were classified as stage 1–2 and 84% were classified as stage 3–4, using the revised American Society for Reproductive Medicine Classification.[18]

The cohort of 101 women with endometriosis confirmed was then divided into those patients using hormonal therapies (Endo+ Hx+, n=56) and those patients not using hormonal therapies (Endo+ Hx−, n=45). The hormonal therapies utilized by participants included the oral contraceptive pill (n=33), a levonorgestrel-loaded intra-uterine contraceptive device (LIUCD, n=16), an oral contraceptive pill with LIUCD in combination (n=3), an etonorgestrel implant (n=6), depot medroxyprogesterone acetate (n=1), oral norethisterone (n=2), and oral estradiol (n=1).

From the cohort of 56 women with endometriosis confirmed who were using hormonal therapies, those women currently using hormonal treatments were further divided into those women whose hormonal therapy included a LIUCD (Endo+ Hx+ LIUCD+, n=17), and those women on hormonal therapy that did not include a LIUCD (Endo+ Hx+ LIUCD–, n=39).

Results were collated for eight patient groupings:

1. All women with dysmenorrhea (All Dys, n=168)
2. Women with endometriosis confirmed (Endo+, n=101)
3. Women with endometriosis excluded (Endo–, n=22)
4. Women with endometriosis confirmed who were current users of hormonal therapy (Endo+ Hx+, n=56)
5. Women with endometriosis confirmed who were non-users of hormonal therapy (Endo+ Hx–, n=45)
6. Women with endometriosis confirmed who were using hormonal therapy that included a LIUCD (Endo+ Hx+ LIUCD+, n=17)
7. Women with endometriosis confirmed who were using hormonal therapy without a LIUCD (Endo+ Hx+ LIUCD–, n=39)
8. Women on whom no laparoscopy had been performed (No Lap, n=45)

Additional symptoms present per woman with dysmenorrhea

Figure 2 displays the number of additional symptoms experienced per woman across the whole group with dysmenorrhea. Dysmenorrhea was the sole symptom in 0.6% of women, and 2.4% of women reported that all 14 symptoms were present. The mean number of additional symptoms was 8.5.

Demographic, pain, and menstrual data by endometriosis diagnosis

Patient groups All Dys, Endo+, Endo–, and No Lap were broadly similar with regard to age, age of menarche, age when dysmenorrhea commenced, frequency of previous pregnancy, and parity (Table 1). The age of participants ranged from 13 to 50 years with a mean age of 29.2 years (SD 8.4 years). Across all women, the mean severity of dysmenorrhea was 7.9 on a 0–10 numerical scale with an SD of 1.7.

Compared to women without endometriosis (Endo–), women with a confirmed diagnosis of endometriosis (Endo+) showed no statistically significant differences for severity of dysmenorrhea (8.1 vs 8.3, P=0.528), days per month of

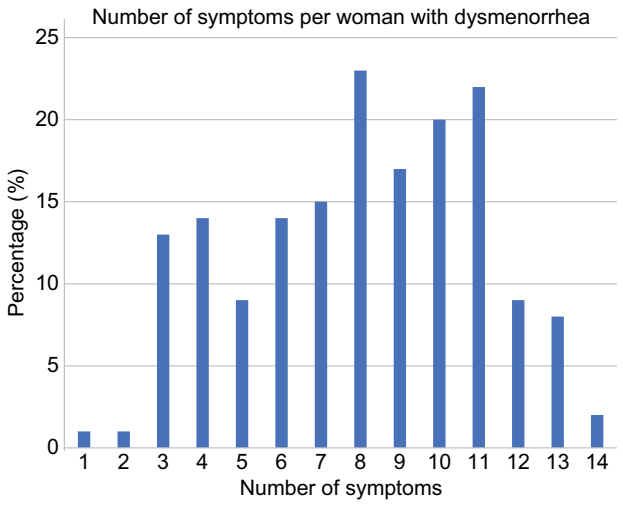

Figure 2 The percentage of women reporting between one and 14 symptoms in addition to dysmenorrhea.

dysmenorrhea (8.1 vs 6.1, *P*=0.235), days per month of pelvic pain (18.3 vs 21.1, *P*=0.177), and parity (38.6% vs 45.5%, *P*=0.588). Table 1 displays the demographic, pain, and menstrual data for each patient group by endometriosis diagnosis.

Symptom profile by endometriosis diagnosis

Table 2 displays the prevalence of each of the following symptoms across the four patient groups: stabbing pelvic pains, bowel symptoms, food intolerances, bladder symptoms, pain with sexual activity, headaches, vulval pain, fatigue, poor sleep, nausea, sweating, dizziness, anxiety, and low mood. One hundred percent of participants reported dysmenorrhea, consistent with the study inclusion criteria.

The frequency of each symptom was compared in women with endometriosis confirmed (Endo+) and women with endometriosis excluded (Endo–). The frequency of individual symptoms was similar in both groups, apart from a significantly higher frequency of bladder symptoms (*P*=0.005) in women with endometriosis excluded. Figure 3 displays the prevalence of each symptom across the four patient groups.

Demographic, pain, and menstrual data by use of hormonal therapies in women with confirmed endometriosis

Women with confirmed endometriosis were more frequent users of a LIUCD (15.8% vs 7.1%, *P*=0.106), but this difference was not statistically significant. Table 3 displays the

Table 1 Demographic, pain, and menstrual data by endometriosis diagnosis

General demographics	All women with dysmenorrhea (All Dys) (n=168)	No laparoscopy (No Lap) (n=45)	Endometriosis confirmed (Endo+) (n=101)	Endometriosis excluded (Endo–) (n=22)	Significance Endo+ vs Endo– (*P*-value)*
Mean age (SD)	29.2 (8.4)	29.3 (8.3)	29.2 (8.3)	28.8 (9.7)	0.848
Mean age menarche (SD)	12.7 (1.7)	12.7 (2.0)	12.8 (1.7)	12.6 (1.5)	0.753
Previous pregnancy (%)	39.9	40	38.6	45.5	0.553
Parous (%)	32.7	31.8	32.0	38.1	0.588
Menstrual and pain data					
Severity dysmenorrhea 0–10 scale (SD)	7.9 (1.7)	7.2 (2.0)	8.1 (1.5)	8.3 (1.6)	0.528
Age dysmenorrhea began (SD)	16.2 (6.1)	16.7 (6.4)	15.9 (5.8)	16.48 (6.9)	0.699
Dysmenorrhea days per month (SD)	7.2 (6.0)	5.7 (5.1)	8.1 (6.6)	6.1 (4.0)	0.235
Days per month pelvic pain (SD)	17.9 (9.1)	15.5 (9.2)	18.3 (9.1)	21.1 (8.0)	0.177

Notes: All Dys, all women with dysmenorrhea, chi-squared test; Endo+, women with endometriosis confirmed; Endo–, women with endometriosis excluded; No Lap, women where no laparoscopy had been performed; *comparison of mean by independent samples *t*-test.

Table 2 Symptom prevalence by endometriosis diagnosis

Symptom	All women with dysmenorrhea (All Dys) (n=168)	No laparoscopy (No Lap) (n=45)	Endometriosis confirmed (Endo+) (n=101)	Endometriosis excluded (Endo–) (n=22)	Significance Endo+ vs endo– (*P*-value)*
Dysmenorrhea (%)	100	100	100	100	
Stabbing pain (%)	63.9	47.7	69	72.7	0.731
Bowel problems (%)	47.3	40.9	49.5	50.0	0.966
Food intolerances (%)	66.1	62.8	65	77.3	0.267
Bladder problems (%)	23.2	18.2	20	50	0.005
Headaches (%)	56	46.7	58.4	63.6	0.651
Sexual pain (%)	37.8	36.1	38.2	38.9	0.956
Vulval pain (%)	38.6	35.9	41.2	31.8	0.415
Fatigue (%)	74.4	68.9	77.2	72.7	0.652
Poor sleep (%)	57.7	57.8	58.4	54.5	0.739
Nausea (%)	49.1	42.9	48	66.7	0.12
Sweating (%)	33.9	28.9	33.7	45.5	0.296
Dizziness/faint (%)	58.9	48.9	62.4	63.6	0.912
Anxiety (%)	58.7	61.4	56.4	63.6	0.536
Low mood (%)	57.7	60	58.4	50	0.47

Notes: All Dys, all women with dysmenorrhea; Endo+, women with endometriosis confirmed; Endo–, women with endometriosis excluded; No Lap, women where no laparoscopy had been performed; *chi-squared tests.

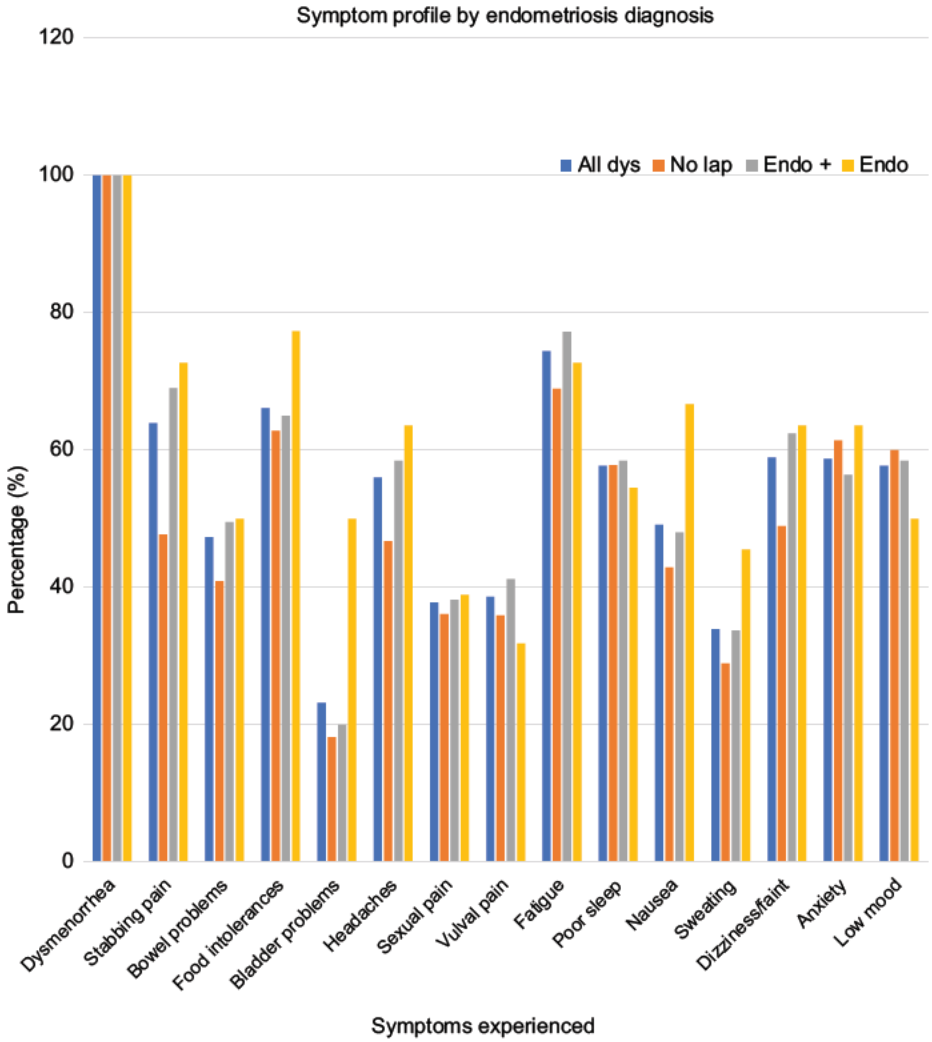

Figure 3 The frequency of specific symptoms by endometriosis diagnosis.
Notes: All Dys, all women with dysmenorrhea; Endo+, women with endometriosis confirmed; Endo–, women with endometriosis excluded; No Lap, women where no laparoscopy had been performed.

Table 3 Demographic, pain, and menstrual data in women with endometriosis according to use or non-use of hormonal therapies and use or non-use of a levonorgestrel-releasing intra-uterine device

General demographics	Endo+ Hx+ (n=56)	Endo+ Hx– (n=45)	Significance Endo+ Hx+ vs Endo+ Hx– (*P*-value)*	Endo+ Hx+ LIUCD+ (n=17)	Endo+ Hx+ LIUCD– (n=39)	Significance Endo+ Hx+ LIUCD+ vs Endo+ Hx+ LIUCD– (*P*-value)*
Mean age, years (SD)	28.2 (7.9)	30.3 (8.7)	0.221	31.1 (7.0)	27.0 (8.1)	0.073
Mean age at menarche, years (SD)	12.8 (1.8)	12.7 (1.6)	0.888	12.9 (1.6)	12.7 (1.6)	0.658
Previous pregnancy (%)	28.6	51.1	0.021	41.2	23.1	0.168
Parous (%)	22.6	43.2	0.026	35.3	16.7	0.13
Menstrual and pain data						
Severity on dysmenorrhea 0–10 scale (SD)	8.3 (1.5)	7.8 (1.4)	0.167	8.1 (1.9)	8.311 (1.4)	0.743
Age dysmenorrhea began, years (SD)	14.9 (3.7)	17.2 (7.4)	0.054	14.8 (3.4)	14.9 (3.9)	0.949
Dysmenorrhea days per month (SD)	9.4 (7.5)	6.5 (4.9)	0.037	12.9 (9.1)	8.1 (6.5)	0.05
Pelvic pain days per month (SD)	20.1 (9.2)	16.0 (8.4)	0.027	20.8 (7.6)	19.8 (10.0)	0.714

Notes: Endo+ Hx+, women with endometriosis confirmed on hormonal therapy; Endo+ Hx+ LIUCD+, women with endometriosis confirmed using hormonal therapy that included a LIUCD; Endo+ Hx+ LIUCD–, women with endometriosis confirmed using hormonal therapy excluding a LIUCD; Endo+ Hx–, women with endometriosis confirmed on no hormonal therapy; *comparison of mean by independent samples *t*-test, comparison of proportions by chi-squared tests.
Abbreviation: LIUCD, levonorgestrel-releasing intra-uterine contraceptive device.

demographic, pain, and menstrual data in women with endometriosis according to use or non-use of hormonal therapies including a LIUCD.

Compared to women with endometriosis who were non-users of hormonal therapy (Endo+ Hx−), women with endometriosis who were current users of hormonal therapies (Endo+ Hx+) reported significantly more days of dysmenorrhea per month (P=0.037), more days of pelvic pain per month (P=0.027), were more likely to have had a previous pregnancy (P=0.021), and were more likely to be parous (P=0.026).

Compared to women with endometriosis using hormonal therapies that excluded a LIUCD (Endo+ Hx+ LIUCD–), women with endometriosis using hormonal therapies that included a LIUCD (Endo+ Hx+ LIUCD+) reported significantly more days of dysmenorrhea per month (P=0.05).

Symptom profile by use of hormonal therapies in women with confirmed endometriosis

A comparison was undertaken between women with endometriosis who used (Endo+ Hx+) or did not use (Endo+ Hx−) hormonal therapy, to determine if hormonal treatment was associated with a change in symptoms (Table 4). The frequency of individual symptoms was similar in both groups apart from an increase in sweating among those not receiving hormonal therapy (P=0.009). Figure 4 displays the symptom profile in women with endometriosis by use of hormonal therapy.

A further comparison was undertaken between women with endometriosis using hormonal therapy that included a LIUCD (Endo+ Hx+ LIUCD+), and women with endometriosis using hormonal therapy without a LIUCD (Endo+ Hx+ LIUCD–). Those women using a LIUCD trended to a higher frequency of stabbing pains in the pelvis (82.4% vs 63.2%, P=0.155) and sweating (58.8% vs 38.5%, P=0.159) than those women using hormonal therapies without a LIUCD, but these differences were not statistically significant.

Dysmenorrhea and stabbing pelvic pains

One hundred and six of the 166 women (63.9%) who answered the question about stabbing pains in the pelvic region reported that stabbing pains were present. Based on univariate logistic regression, variables associated with stabbing pain included: severity of dysmenorrhea (OR =1.60, 95% CI =1.27–2.01, P<0.001), number of additional symptoms (OR =1.26, 95% CI =1.12–1.41, P<0.001), diagnosis of migraine (OR =2.30, 95% CI =0.97–5.46, P=0.058), number of days per month of dysmenorrhea (OR =1.15, 95% CI =1.04–1.28, P=0.007), and number of days per month

Table 4 Symptom prevalence across women with endometriosis, according to use or non-use of hormonal therapies, and use or non-use of a levonorgestrel-releasing intra-uterine device

Symptom	Endo+ Hx+ (n=56)	Endo+ Hx– (n=45)	Endo+ Hx+ LIUCD+ (n=17)	Endo+ Hx+ LIUCD– (n=39)	Significance Endo+ Hx+ vs Endo+ Hx– (*P*-value)*	Significance Endo+ Hx+ LIUCD+ vs Endo+ Hx+ LIUCD– (*P*-value)*
Dysmenorrhea (%)	100	100	100	100	1	1
Stabbing pain (%)	69.1	68.9	82.4	63.2	0.983	0.155
Bowel problems (%)	48.9	50	47.1	51.3	0.912	0.771
Food intolerances (%)	66.7	63.6	52.9	68.4	0.752	0.270
Bladder problems (%)	20.0	20.0	17.6	21.1	1.000	0.770
Headaches (%)	55.6	60.7	64.7	59	0.601	0.686
Sexual pain (%)	35.9	40	37.5	41.2	0.693	0.804
Vulval pain (%)	38.6	43.3	35.3	47.2	0.635	0.413
Fatigue (%)	68.9	83.9	88.2	82.1	0.073	0.562
Poor sleep (%)	55.6	60.7	70.6	56.4	0.601	0.318
Nausea (%)	50	46.4	58.8	41	0.723	0.219
Sweating (%)	20	44.6	58.8	38.5	0.009	0.159
Dizziness/faint (%)	62.2	62.5	52.9	66.7	0.977	0.329
Anxiety (%)	57.8	55.4	52.9	56.4	0.807	0.810
Low mood (%)	57.8	58.9	64.7	56.4	0.907	0.562

Notes: Endo+ Hx+, women with endometriosis confirmed on hormonal therapy; Endo+ Hx+ LIUCD+, women with endometriosis confirmed using hormonal therapy that included a LIUCD; Endo+ Hx+ LIUCD–, women with endometriosis confirmed using hormonal therapy excluding a LIUCD; Endo+ Hx–, women with endometriosis confirmed on no hormonal therapy; * chi-squared tests.

Abbreviation: LIUCD, levonorgestrel-releasing intra-uterine contraceptive device.

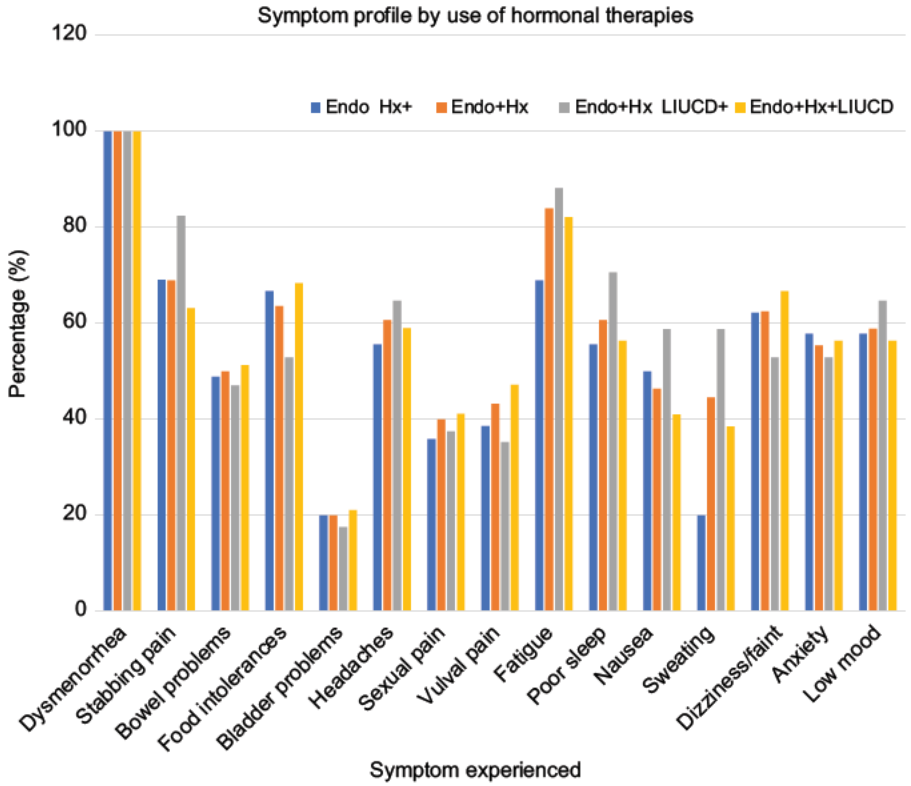

Figure 4 The frequency of specific symptoms by use or non-use of hormonal therapies, and use or non-use of a levonorgestrel-releasing intra-uterine device.
Notes: Endo+ Hx+, women with endometriosis confirmed using hormonal therapy; Endo+ Hx–, women with endometriosis confirmed not using hormonal therapy; Endo+ Hx+ LIUCD+, women with endometriosis confirmed using hormonal therapy that included a LIUCD; Endo+ Hx+ LIUCD–, women with endometriosis confirmed using hormonal therapy excluding a LIUCD.
Abbreviation: LIUCD, levonorgestrel-releasing intra-uterine contraceptive device.

of pelvic pain (OR =1.05, 95% CI =1.01–1.08, P=0.017). Multivariable logistic regression found that the best joint predictors of stabbing pain were the severity of period pain (OR =1.71, 95% CI =1.31–2.24, P<0.001), the number of additional symptoms (OR =1.30, 95% CI =1.13–1.49, P<0.001), and a diagnosis of migraine (OR =4.34, 95% CI =1.46–12.87, P=0.008).

Dysmenorrhea and headache

One hundred and twenty-three of the 152 women (80.9%) with dysmenorrhea who answered the question "Do you have headaches?" reported headache on at least 1 day per month. Of these, 43 women (40.2%) reported the presence of headache on 10 or more days per month, and 26 women (21.1%) on 15 or more days per month. The average number of days of reported headache was 10 days per month.

Analysis of symptoms by past history of distressing sexual events

Participants were asked whether they had "experienced distressing sexual events during their life, including sexual assault", with the opportunity to answer "Yes", "No", or "I prefer not to answer this question". This question was answered by 138 (82.1%) of women, with 23 (16.7%) answering "Yes", and 115 (83.3%) answering "No". Figure 5 displays the dysmenorrhea score, days per month of pelvic pain, and number of symptoms in women with and without a history of distressing sexual events. Women with a history of distressing sexual events reported a significantly higher number of symptoms (P=0.003) than women without a history of these events (Table 5). There was a trend toward significance for the days per month of pain (25 vs 20 days, P=0.053).

Discussion

Our study has demonstrated four clinical findings. First is that additional symptoms are common in women with dysmenorrhea. Second is that the symptom profile in our study was generally independent of the presence or absence of endometriosis lesions, and independent of the use or non-use of hormonal therapy in those with confirmed endometriosis. Third is that the presence of stabbing pains in women

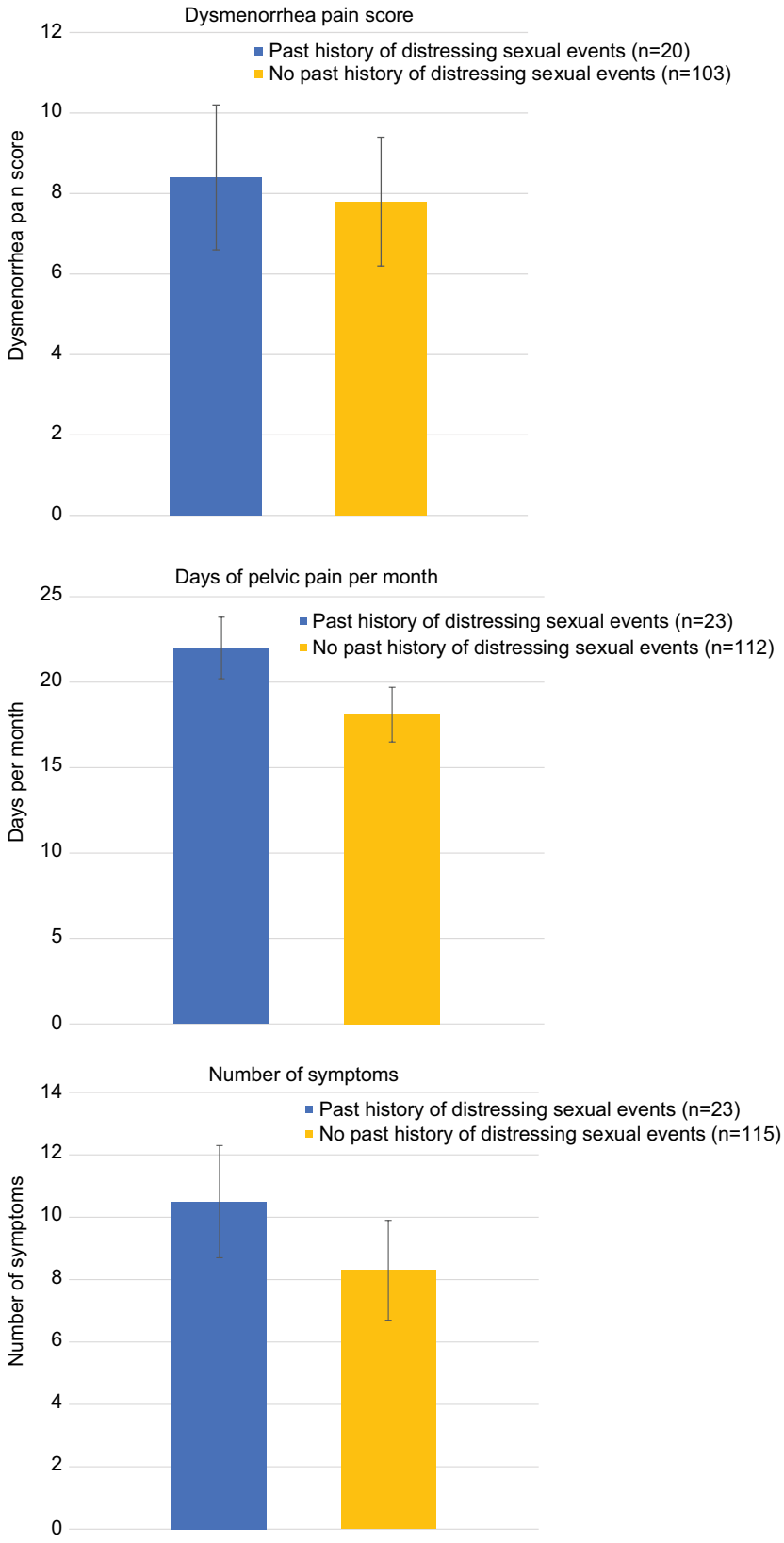

Figure 5 Dysmenorrhea pain score, days per month of pelvic pain, and number of symptoms reported in women with, and without, a history of distressing sexual events.

Table 5 Dysmenorrhea score, days per month of pelvic pain, and number of symptoms in women with, and without, a history of distressing sexual events

	Past history of distressing sexual events			No past history of distressing sexual events			Significance*
	N	Mean	SD	N	Mean	SD	
Dysmenorrhea pain score	20	8.4	1.8	103	7.8	1.6	0.112
Pelvic pain days per month	23	22.0	8.4	112	18.1	9.0	0.055
Number of symptoms	23	10.5	2.6	115	8.3	3.2	0.003

Note: *Independent samples *t*-tests.

with endometriosis is highly associated with the severity of dysmenorrhea, days per month of dysmenorrhea, days per month of pelvic pain, and diagnosis of migraine headache. And finally that a past history of distressing sexual events is associated with an increase in the severity and number of pain symptoms.

A woman's experience of dysmenorrhea includes all symptoms experienced at menstruation, both pelvic and extra-pelvic. A major strength of this study is its symptom-based applicability to the management of women presenting with dysmenorrhea and its associated symptoms, regardless of endometriosis status. While our group may represent a more complex subset of women with dysmenorrhea than is found in the general population, it is likely that they are similar to women referred to other gynecologists providing care for women with endometriosis and pelvic pain.

The lack of association between pain severity, presenting symptom profile and the presence of endometriotic lesions

Dysmenorrhea and pelvic pain have traditionally been associated with the presence of laparoscopically diagnosed endometriosis lesions. While these conditions commonly coexist, the relationship between the clinical symptoms with which a girl or woman presents to her health practitioner (dysmenorrhea) and the medical condition that may or may not be found at laparoscopy (endometriosis) remains controversial. A literature review by Janssen et al[4] considered laparoscopic outcomes in adolescent girls aged 10–21 years presenting with pain. They found endometriosis lesions to be present in 62% of adolescents overall, in 75% of girls with chronic pelvic pain resistant to treatment with the oral contraceptive pill and anti-inflammatory medications, in 70% of girls with dysmenorrhea alone, and in 49% of girls with chronic pelvic pain not necessarily resistant to treatment. Additional factors affecting the experience of dysmenorrhea appear likely. A consensus paper published in 2013 included majority but not universal support for a statement describing a "philosophical shift to consideration of endometriosis and pelvic pain as a spectrum or continuum of disease".[9] It was recognized that this approach would avoid excluding women who lack laparoscopic confirmation of a diagnosis of endometriosis, yet have similar symptoms and associated diagnostic and therapeutic interventions.

Robust evidence demonstrates that the presence of endometriosis lesions does not determine the severity of pelvic pain. In 656 women with chronic pelvic pain, Yosef et al found significant correlations between the severity of pain and the presence of dysmenorrhea, irritable bowel syndrome, painful bladder syndrome, pelvic muscle pain, or abdominal wall pain, but not with the presence of endometriotic lesions.[19] As-Sanie et al used functional MRI to demonstrate that the experience of chronic pain was associated with anatomical changes in the brain rather than the presence of endometriosis lesions,[20] and Vercellini et al confirmed the lack of association between the severity of laparoscopically-staged endometriosis lesions and the severity of pain experienced.[21] With regard to the reduction in quality of life and increase in anxiety and depression common in women with endometriosis,[22] Facchin et al demonstrated that it is the presence of pain, rather than the presence of lesions, that is of importance.[23] Women with asymptomatic endometriosis enjoyed the same quality of life and mental health outcomes as healthy controls. In our study, women with endometriosis excluded had a significantly higher frequency of bladder symptoms (50% vs 20%, P=0.005) than women with endometriosis confirmed. This may reflect the presence of coexisting painful bladder syndrome, a common cause of pelvic pain that clusters with other pelvic pain diagnoses.[24] By choosing to study women presenting with dysmenorrhea, rather than purely those with confirmed endometriosis, our findings further separate the pain of dysmenorrhea and the presence of endometriosis lesions. Our study also provides clinically relevant information to assist the management of women with dysmenorrhea where their endometriosis status is unknown.

The association between pain symptoms and central sensitization

Two mechanisms consistently implicated with both the development of endometriotic lesions[25] and a range of persistent visceral pain conditions[26] are inflammation and activation of the innate immune system resulting in central pain sensitization. Inflammation elicits pain via inflammatory mediators and peripheral sensitization of nociceptors.[27] Central sensitization may arise following continuous or recurrent nociceptive input from the periphery, resulting in enhanced excitability of spinal projection neurons in the spinal cord and central nociceptor terminals within the brain.[28] Enhanced neurotransmission with long lasting molecular changes in both the spinal cord and brain may result in pain perception that no longer reflects, and is independent of, peripheral pain nociceptive signaling.[26] Altered central processing of nociception in women with dysmenorrhea, both during menses and on pain-free days, as measured by serum cortisol, response to thermal stimuli, and functional MRI investigation, has been demonstrated by Vincent et al, Arendt-Nielssen et al, and Iacovides et al,[29–31] with literature review by Payne et al.[32] Central sensitization has the potential to affect both pelvic and extra-pelvic pain symptomatology.

With regard to pelvic symptoms, there is ample evidence for the convergence of sensory information from discrete pelvic organs of the gastrointestinal or genitourinary tract in the dorsal root ganglia, spinal cord, and brain,[33,34] resulting in viscero-visceral hyperalgesia and the experience of multiple pain symptoms across pelvic organs. The women in our study demonstrated a high frequency of bowel (47.3%) and bladder (23.2%) symptoms. Both irritable bowel syndrome and painful bladder syndrome cluster in women with pelvic pain, and are associated with central sensitization and inflammation[13,24,35]

There is also evidence for the convergence of sensory information from visceral and somatic pelvic structures in the dorsal horn of the spinal cord. This results in visceral-somatic hyperalgesia and the experience of reflex muscle contraction and spasm associated with visceral pain as a manifestation of central sensitization.[36–38] Our study demonstrated a high frequency of stabbing pain (63.9%), a symptom associated with musculoskeletal pain[39] in women with dysmenorrhea. The presence of stabbing pelvic pain was highly associated with the severity of dysmenorrhea, days per month of dysmenorrhea, and days per month of pelvic pain.

With regard to extra-pelvic symptoms, central pain mechanisms have been implicated in the experience of fatigue, poor sleep, fibromyalgia, and headache.[40] The high frequency of chronic headache in our study is striking. About 80.9% of participants reported headache, with 21.1% reporting headaches on 15 or more days per month. In comparison, a population-based study in Italy found 42.8% of people reported headache in the previous year, and only 3.4% reported headache on 15 or more days per month.[41] These findings are consistent with growing evidence for the role of central sensitization[20] and extra-pelvic symptoms in the lived pain experience of women with dysmenorrhea.

Endometriosis as a common comorbidity of pelvic pain syndrome (PPS)

Endometriosis and dysmenorrhea frequently coexist.[4] However, the high frequency of symptoms in our study, both within and outside the pelvis, regardless of endometriosis status, supports the view that endometriosis and pain may be associated, rather than etiologically linked, conditions. They may share a similar underlying mechanism, yet represent distinct clinical entities within a larger syndrome of associated symptoms and conditions. Both endometriosis and dysmenorrhea would then be common, but non-essential features of female PPS, much as polycystic ovaries are common, but non-essential, features of the systemic metabolic disorder, polycystic ovarian syndrome (PCOS).[42]

Further support for considering endometriosis and dysmenorrhea as common features within female PPS comes from Clemens et al, who found similar pain symptoms in male PPS, where endometriosis and dysmenorrhea do not occur.[43] Sutcliffe et al described similar clinical symptoms during pelvic pain flares in men and women with urologically based PPS, although flares were more frequent and of longer duration in women.[44]

The association between pain symptoms and use of hormonal therapies including a LIUCD

A LIUCD is a long-acting, reversible contraceptive that releases 20 μg of levonorgestrel per day over 5 years of use. It offers effective contraception, reduced menstrual blood flow, reduced dysmenorrhea, and high levels of patient satisfaction in the general population,[45,46] or women with adenomyosis,[47] particularly in those women who have chosen to continue using a LIUCD for more than 12 months.[48] However, a proportion of women request removal of the device in the first year of use, with the commonest reasons for premature device removal being pain and irregular bleed-

ing.[49] In women with endometriosis, use of a LIUCD has the added benefit of reducing the recurrence of endometriosis lesions following laparoscopic surgery.[50] However, despite these known benefits of a LIUCD, Lockhat et al[51] found that only 68% of women continued to use a LIUCD at 12 months post-insertion, with 11.8% of women requesting removal of the LIUCD due to "abdominal pain". Our study found that women with endometriosis using hormonal therapies reported significantly more days per month of pelvic pain, and that those using a LIUCD had a higher, but not statistically significant, increase in stabbing pains (82.4% vs 63.2%, P=0.155) when compared with non-users of hormonal therapies. This may reflect the referral of women with pain for whom these therapies had been tried but found unsuccessful, potentially in women with more severe central pain sensitization. Research to review whether the presence of stabbing pains may indicate a subgroup of women more likely to report persistent or unresolved pain following LIUCD insertion should be considered. Our study did not include sufficient patients to investigate the relative benefits of individual oral hormonal therapies or regimes, as has been proposed in other studies.[52,53]

The effect of previously distressing sexual events on symptom severity

Our finding that those women who reported a history of distressing sexual events suffered more pelvic pain supports similar findings by Yosef et al.[19] Their study found that a history of sexual abuse was significantly associated with increased severity of pelvic pain. Ballina et al described enhanced recovery and reduced pain severity following sexual assault in women with opioid receptor polymorphisms, further linking pain mechanisms to pain severity.[54] Schliep et al found no increase in the frequency of endometriosis in women with prior history of sexual assault.[10]

Despite our finding that a history of distressing sexual events was associated with more severe pain, the majority of our patients reported no sexual assault. The frequency of distressing sexual events (16.7%) is consistent with population data described in the Australian Bureau of Statistics Personal Safety Survey[55] (2016), where 18% of Australian women had experienced sexual violence since the age of 15 years. With a 15% missing data rate for this question in the present study, it is possible that adverse sexual events were under-reported in our sample. However, by analyzing only those women who reported either "yes" or "no", the potential for error has been minimized.

Translational significance for clinical practice

Our study investigated 14 dysmenorrhea-associated symptoms in a group of women presenting to a gynecology clinic with particular interest in endometriosis, dysmenorrhea, and pelvic pain. This has wide-ranging clinical applications. Firstly, our study shows that the full assessment of a woman with dysmenorrhea should proceed with the expectation that multiple symptoms, both within and outside the pelvis, are likely to be present. Secondly, we found that the presenting symptom profile was an unreliable indicator of the presence of endometriosis, as similar pain profiles were found in women both with and without endometriosis lesions. Thirdly, our findings have implications for the training of health practitioners. The wide range of symptoms included within dysmenorrhea-associated PPS extends beyond the traditional teaching of individual medical and allied health care domains. There are consequently similarities between the changing management of PPS and the evolved management of PCOS. Comprehensive care of the pelvic and extra-pelvic symptoms of PCOS has moved beyond management of ovarian cysts to include the assessment of blood lipid and sugar profiles, the management of hirsuitism and acne, and lifestyle advice to manage obesity and reduce inactivity.

The high frequency of the covarying of migraine, stabbing pain, and dysmenorrhea supports further research investigating a potential common mechanism involving acquired central pain sensitization for these conditions. A pilot study by Sator-Katzenschlager et al[56] using amitriptyline or gabapentin in 56 women with chronic pelvic pain found a reduction in pain among both treatment groups. If proven, this would support the use of therapeutic interventions directed at central sensitization with effects on both pelvic and extra-pelvic symptoms in women with dysmenorrhea.

Conclusion

This study aims to extend our knowledge in an area that has been under-researched with regard to its impact on the well-being of women, their families, and our society: the lived experience of women with dysmenorrhea.

While the lack of a control group in this study limits our ability to make firm conclusions, our findings indicate that additional symptoms are common in these women, and that the presence of endometriosis lesions cannot reliably be predicted by the presenting symptom profile. Of the symptoms investigated, the presence of stabbing pelvic pains and migraine headaches were most closely correlated with the severity of dysmenorrhea. The presence of both pelvic and

extra-pelvic symptoms is consistent with central sensitization as a component mechanism in female PPS. A history of distressing sexual events was associated with an increased number of pain symptoms, but was not more common than in the general population.

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Author contributions

Study design, acquisition of data, preparation of data: SFE, TAB, PER, MLH. Analysis of data: SFE, AJE. Interpretation of data: SFE, PER, MLH. Preparation of article: SFE, PER, MLH. All authors contributed to data analysis, drafting and revising the article, gave final approval of the version to be published, and agree to be accountable for all aspects of the work.

Disclosure

SFE received royalties from book authorship, has received payment from Pfizer and Bayer for educational presentations, and is involved in the development of novel treatments for pelvic pain. PER is a shareholder in Havah Therapeutics and iXBiopharma, director and shareholder of Lipotek, consultant to Bionomics and Novartis, and has received payment for educational presentations from Novartis and Sequirus. The other authors report no conflicts of interest in this work.

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2.5 CONCLUSIONS

The manuscript entitled “The co-morbidities of dysmenorrhoea: A clinical survey comparing symptom profile in women with and without endometriosis”, has increased our knowledge of the lived experience of women with dysmenorrhoea-related pelvic pain presenting to a gynaecologist for medical care.

Hypothesis 1, that women with dysmenorrhoea report a high frequency of additional symptoms, both pelvic and extra-pelvic, was confirmed. We determined that women presenting to a gynaecologist with dysmenorrhoea-related pain suffered an average of eight symptoms in addition to dysmenorrhoea, and that these symptoms included both pelvic and extra-pelvic symptoms, consistent with the presence of a central pain mechanism. While the lack of a control group in this study limits our ability to make firm conclusions, our findings indicate that additional symptoms are common in these women. Of the symptoms investigated, the presence of stabbing pelvic pains, and migraine headaches was most closely correlated with the severity of dysmenorrhoea.

Hypothesis 2, that the symptom profile may be used as a guide for clinicians to the presence or absence of endometriosis lesions was not supported. While the frequency of bladder symptoms was significantly higher in women without endometriosis, other symptoms were of similar frequency between the two groups.

Hypothesis 3, that the presence of endometriosis is a common, but non-essential, feature of DRPPS was supported. Similar symptoms were found in women with and without a diagnosis of endometriosis. This finding supports a relationship of association rather than causation between the presence of endometriosis lesions and the presence of pain.

Hypothesis 4, that a history of distressing sexual events would modify the symptom profile was confirmed. Those women affected reported an increase in pain severity, and an increase in the number of pain symptoms reported. However, a report of distressing sexual events was no more common among our study cohort than among the general population of young women in Australia. This suggests that while these events may worsen symptoms, they are not a major aetiological factor for the development of DRPP.

Chronic pelvic pain has been variably associated with a history of sexual abuse in the pain literature, with the most consistent finding being an association with sexual abuse in childhood.

Rapkin et al. (1990) found that while the frequency of reported sexual assault was not significantly different between 31 adult women with chronic pelvic pain (19.4%), 32 women with chronic pain of alternate aetiology (16.4%), and 142 pain-free controls (12.5%), those women with chronic pelvic pain were more likely to report sexual abuse occurring during childhood (39.0% versus 18.4% and 9.4% respectively). Lampe et al. (2000) investigated 36 women with chronic pelvic pain, 23 women with chronic low back pain, and 20 pain-free controls with similar findings. They found that while the frequency of reported sexual abuse did not vary significantly across groups, the women with pelvic pain reported a higher frequency of abuse before the age of 15 years.

Our documentation of 14 symptoms commonly associated with dysmenorrhoea-related pelvic pain, regardless of the presence or absence of endometriosis lesions, further develops the clinical concept of Dysmenorrhoea-Related Pelvic Pain Syndrome. However, the mechanism by which inflammation develops remains unknown. Why is it that one in five girls develop severe dysmenorrhoea at or soon after menarche, while four in five do not? Chapter 3 investigates the potential of innate immune system activation to act as the common mechanism in dysmenorrhoea-related pelvic pain.

CHAPTER 3: DYSMENORRHOEA, THE INNATE IMMUNE SYSTEM AND A POSSIBLE NEW BIOMARKER

To have great pain is to have certainty; to hear that another person has pain is to have doubt.

Elaine Scarry, 1985

3.1 INTRODUCTION

Chapter 2 investigated a cohort of women with dysmenorrhoea-related pelvic pain, and found a high prevalence of additional symptoms, both pelvic and extra-pelvic. These findings are consistent with a systemic, rather than purely pelvic, mechanism for the presence of pain-related symptoms. However, they do not inform the nature of the mechanism involved.

As described in Sections 1.4.4 and 1.8.3, women with dysmenorrhoea, chronic pelvic pain and endometriosis show an increased inflammatory phenotype. They describe the strong association between inflammation and central pain mechanisms, and the increasing evidence of a role for Toll-Like Receptors (TLRs) in the genesis of inflammation. A series of research findings by Khan et al. (Khan et al., 2008, 2010, 2013, 2015) describe an increased expression of TLR4 activity, and an increased colonisation of the uterus by *E.Coli*, in the uterus of women with endometriosis when compared with healthy controls. They proposed a bacterial contamination theory for the development of endometriosis lesions. However, the implications of these findings for women with dysmenorrhoea, or CPP, remain unclear. Not all the women with *E.coli* colonisation within their study displayed endometriosis, and their research did not report the implications of TLR4 activation within the uterus on the reported experience of pain.

Further research in this area would be facilitated by the identification of an effective biomarker for pain, and or, endometriosis. To date, a range of approaches have been used to investigate potential biomarkers for the presence of endometriosis with the aim of early diagnosis, and more effective targeting of laparoscopic surgical procedures. Nisenblat et al.(2016b) investigated a panel of blood cytokines and concluded that:

> Of the biomarkers that were subjected to meta-analysis, none consistently met the criteria for a triage diagnostic test. Nisenblat et al. (2016b) p2

A further study investigated 78 plasma microRNA markers and found 49 markers that were differentially expressed in women with endometriosis. While three microRNA markers (miR-155, miR-574-3p and miR139-3p) could be independently validated, when combined together as a prognostic test (Nisenblat et al., 2019) these markers demonstrated a sensitivity and specificity for the presence of endometriosis of 83% and 51% respectively, which was considered insufficient to guide clinical practice. More recently, the potential for deletions in mitochondrial DNA (1.2 and 3.7kb) has been investigated by Creed et al. (2019), who found a sensitivity and specificity of 81.8% and 72.2% for the 1.2kb deletion, and 85.1% and 57.9% for the 3.7kb deletion, with a test combining the two markers currently under consideration for commercial development.

Unfortunately, even where a highly sensitive and specific biomarker for the presence of endometriosis is determined, its use as a clinical biomarker for the presence of pain may be limited. The lack of a biomarker for the subjective symptom of pain has hampered the development and evaluation of pain therapies across a range of pain conditions. An effective biomarker would enhance the clinical assessment of pain and inform data enrichment of clinical trials designed to test the effectiveness of pain therapies. In a novel approach, recent studies at the University of Adelaide suggest that an increased responsiveness of mononuclear cells, with subsequent release of IL-1β, within the peripheral blood can provide an objective measurement of the presence of chronic pain (Kwok et al. 2013). As outlined in Section 1.6.1. PBMCs are cells of the innate immune system within the peripheral blood that activate glial cells within the dorsal horn of the spinal cord, resulting in central pain sensitisation and the symptomatic experience of pain. Using pre-clinical models, Kwok et al. (2012, 2013) demonstrated that activation of PBMCs mirror changes within the brain and spinal cord. In subsequent studies using this model, Kwok et al. were able to accurately predict the presence of pain in 28 out 34 patients with chronic pain. This finding suggests that the responsiveness of PBMCs to TLR2 or TLR4 stimulation has significant potential as an in vitro biomarker for the presence of chronic pain, and this approach warrants further investigation. Kwok's cohort of patients was confounded by the inclusion of a heterogeneous population of males and females with varied chronic pain conditions, an average age of 46 years, multiple co-morbidities, and the concurrent use of potentially immune-modifying medications. In contrast, young women with dysmenorrhoea-related pelvic pain offer an opportunity to investigate the potential of Kwok's test in a consistent cohort of young, otherwise healthy women without additional comorbidities or the use of confounding medications. If proven to be effective, such a biomarker could support the use and development of entirely new classes of medications for the management of this common and debilitating condition.

As described in section 1.5.2. the oral contraceptive pill is commonly used to manage dysmenorrhoea and has demonstrated efficacy in a proportion of women. The mechanism of benefit of the oral contraceptive (OC) is unknown. As discussed in section 1.7.4., the innate immune system, as mediated by TLRs is modified by the hormonal environment.

Chapter 3 seeks to investigates the role of Toll-Like Receptors, specifically TLR 2 and TLR4, in women with dysmenorrhoea-related pelvic pain. We also seek to determine whether the pain-modulating effect of the oral contraceptive pill is mediated via TLRs. Chapter 3 utilises laboratory-based tests and clinical symptoms to investigate a new cohort of young women.

3.2 AIMS

Chapter 3 has four aims:

Aim 1: To determine whether there is evidence of activation of the innate immune system, via Toll-Like Receptors 2 and 4, in young women with dysmenorrhoea-related pelvic pain.

Aim 2: To investigate the potential of an in vitro test for the responsiveness of PBMCs to TLR stimulation as a pain biomarker in young women with dysmenorrhoea-related pelvic pain.

Aim 3: To determine whether the effectiveness of this test as a biomarker for pain is affected by the presence or absence of pain at the time of sampling.

Aim 4: To determine whether use of the oral contraceptive pill modifies the innate immune system in women with dysmenorrhoea-related pelvic pain.

3.3 HYPOTHESES

This chapter investigates four hypotheses:

Hypothesis 1: That women with dysmenorrhoea-related pelvic pain exhibit activation of the innate immune system via Toll-Like Receptor mechanisms compared with pain-free controls.

Hypothesis 2: That the responsiveness of PBMCs to TLR2 or TLR4 stimulation, as measured by release of the cytokine IL-1β, can act as a pain biomarker for dysmenorrhoea-related pelvic pain.

Hypothesis 3: That IL-1β release from PBMCs is higher on days where reported pain is severe than on days when pain is absent or mild.

Hypothesis 4: That IL-1β release from PBMCs is modified by the use or non-use of the oral contraceptive pill.

This is the first study to investigate an *in vitro* immune relationship in young women with pain at an early stage of their chronic pain condition, and in response to a dynamic, natural pain stimulus – their own menstrual cycle.

This chapter introduces the manuscript entitled “Toll-Like Receptor Responsiveness of Peripheral Blood Mononuclear Cells in Young Women with Dysmenorrhea” which has been published in the Journal of Pain Research.

3.4 Manuscript and Statement of Authorship

The study was part-funded by the Australia New Zealand College of Anaesthetists (ANZCA) Research Foundation (Grant 15/013). Its contents are solely the responsibility of the authors and the funders had no role in the study design, data collection and analysis, decision to publish, or preparation of the manuscript.

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Toll-like receptor responsiveness of peripheral blood mononuclear cells in young women with dysmenorrhoea

All authors gave final approval of the version to be published, and agree to be accountable for all aspects of the work.

Statement of Authorship

Title of Paper	Toll-L ke Receptor Responsiveness of Peripheral Blood Mononuclear Cells in Young Women with Dysmenorrhea
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Principal Author

Name of Principal Author (Candidate)	Susan F Evans		
Contr bution to the Paper	Study design, acquisition of data, preparation of data, analysis of data, interpretation of data, preparation of article		
Overall percentage (%)	80		
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.		
Signature		Date	7th September, 2020

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);

ii. permission is granted for the candidate in include the publication in the thesis; and

iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contr bution.

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Overall percentage (%)	2		
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Contr bution to the Paper	Study design, interpretation of data, preparation of article		
Overall percentage (%)	2		
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Contr bution to the Paper	Study design, interpretation of data, preparation of article		
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ORIGINAL RESEARCH

Toll-Like Receptor Responsiveness of Peripheral Blood Mononuclear Cells in Young Women with Dysmenorrhea

This article was published in the following Dove Press journal:
Journal of Pain Research

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Purpose: Dysmenorrhea is a common disorder that substantially disrupts the lives of young women. To determine whether there is evidence of activation of the innate immune system in dysmenorrhea and whether the degree of activation may be used as a biomarker for pain, we compared the responsiveness of peripheral blood mononuclear cells (PBMCs) to toll like receptor (TLR) 2 or 4 stimulation. We also investigated whether this effect is modulated by the use of the oral contraceptive pill (OC).

Patients and Methods: Fifty six women aged 16 35 years, with either severe or minimal dysmenorrhea, and use or non use of the OC, were enrolled. PBMCs were collected on two occasions in a single menstrual cycle: the menstrual phase and the mid follicular phase. PBMCs were exposed to lipopolysaccharide (LPS), a TLR4 agonist, and PAM3CSK4 (PAM), a TLR2 agonist, and the resulting interleukin 1beta (IL 1β) output was determined. Statistical analysis compared the EC50 between groups as a measure of TLR responsiveness of PBMCs.

Results: The key finding following LPS stimulation was a pain effect of dysmenorrhea (p 0.042) that was independent of use or non use of OC, and independent of day of testing. Women with dysmenorrhea showed a large 2.15 fold (95% CI 4.69, 0.09) increase in IL 1β release when compared with pain free participants across both days.

Conclusion: This is the first study to demonstrate an ex vivo immune relationship in women with dysmenorrhea related pelvic pain. It provides evidence for the potential of immune modulation as a novel pharmacological target for future drug development in the management of dysmenorrhea.

Keywords: pain, chronic pain, oral contraceptive pill, endometriosis, pelvic pain, IL 1β

Introduction

Dysmenorrhea, or period pain, is a distressing condition that has a global medical, personal, societal and economic impact. Almost every woman will experience dysmenorrhea at some time in her life,[1] although the severity, duration and persistence will vary widely. A meta-analysis of 15 studies worldwide[2] revealed the substantial life impact of dysmenorrhea in young women. Pain sufficient to cause distress or absenteeism affected between 2% and 29% of young women, depending on study design and the severity of dysmenorrhea considered. In addition, dysmenorrhea commonly resulted in emergency department presentation, hospital admission,[3] reduced educational achievement, impaired mental health,[4,5] reduced workplace productivity,[6] disturbed sleep,[7] and reduced quality of life.[8] Dysmenorrhea frequently precedes,[9] and is believed to be an etiological factor in, the development of persistent pelvic pain in some

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women.[10–12] Hardi et al[9] retrospectively reviewed 100 women with dysmenorrhea-predominant persistent pelvic pain, and found that dysmenorrhea predated the development of persistent pain by between 1 and 30 years.

Current medical management options include the oral contraceptive pill (OC), non-steroidal anti–inflammatory medications (NSAIDs), or progestogens. However, many young women find these options to be ineffective, contra-indicated or unacceptable.[13] There is an unmet need for new, effective treatments for dysmenorrhea.

Traditionally, dysmenorrhea in women has been classified as either primary, where no organic cause has been identified, and secondary, where an associated condition, such as endometriosis is present. However, despite the recognized association and common co-existence of endometriosis with dysmenorrhea, there is no reliable correlation between the severity of endometriosis lesions and the severity of pain.[14] In previous work, our group has demonstrated the strong association between dysmenorrhea and a range of symptoms, both within and outside the pelvis, that include: headache, fatigue, anxiety, low mood, bladder or bowel symptoms, and nausea.[15] These factors strongly suggest the presence of additional elements affecting the experience of dysmenorrhea. Compelling epidemiological, clinical and experimental evidence in both human and animal studies demonstrates that increased peripheral and central nervous system (CNS; glial) immune system activity, via Toll-like receptors (TLRs), is involved in the development of persistent pain conditions.[16] In this paper, we propose that immune pathology may also be present in women affected by dysmenorrhea, whether or not an associated condition such as endometriosis is present, and that activation of the immune system in these women occurs via TLRs. Therefore, the innate immune reactivity of an individual's peripheral blood immune cells may provide insights into the mechanism behind their disease state.

TLRs are a family of receptors found on the surface membrane of cells of the innate immune system, such as macrophages, glia and astrocytes. They recognize molecular patterns typically associated with microbial pathogens and facilitate an immune response with the release of cytokines, including interleukin-1beta (IL-1β), both peripherally and in the spinal cord. Lipopolysaccharide (LPS), also known as endotoxin, is a component of the cell wall of gram-negative bacteria, which elicits robust immune responses in animals via TLR4 receptors. PAM3CSK4 (PAM) is a synthetic lipopeptide that elicits strong immune responses via TLR2 receptors. Altered TLR inflammatory responses have been demonstrated in visceral medical conditions characterized by persistent pain, such as inflammatory bowel disease and painful bladder syndrome.[17,18] Macrophages are cells of the innate immune system that originate from peripheral blood monocytes and are present in almost all tissues. These cells alter their physiology, infiltrate tissues and secrete cytokines including IL-1β[19,20] in response to inflammatory mediators.

We hypothesize that peripheral blood mononuclear cells (PBMCs) from women with severe dysmenorrhea exhibit enhanced IL-1β release following stimulation of either TLR4 or TLR2 receptors when compared with normal (control) women with minimal, or no, dysmenorrhea. Where confirmed, this offers the potential for the responsiveness of PBMCs to TLR stimulation to be used as a biomarker for pain in future research of dysmenorrhea treatment options. We also hypothesize that TLR responsiveness is modulated by use of the OC, and by the presence or absence of severe pain at the time of testing.

Materials and Methods

Study Design

This is an observational clinical and laboratory study, with samples taken from women at two stages of their menstrual cycle. This study was approved by the Human Research Ethics Committee of the Royal Adelaide Hospital, Adelaide, South Australia. HREC/14/RAH/63. RAH Approval No. 140217. The study was conducted in accordance with the Declaration of Helsinki.[21]

Participants

The target population consisted of girls and women aged between 16 and 35 years of age. Potential participants were invited to complete a Survey Monkey Questionnaire when they responded to recruitment notices displayed to the general public or following an invitation to enroll through a private medical clinic, Pelvic Pain SA, Adelaide, Australia. After selection based on the eligibility criteria, participants were provided with a study information sheet. Study exclusion criteria (Table 1) ensured that women possessing factors that might influence inflammation, TLR receptor response, the immune system, or the severity of dysmenorrhea were excluded from the study. Eligible participants from the general public were offered the opportunity of an initial phone interview to provide further information about the study, to obtain consent for further participation in

Table 1 Study Exclusion Criteria

Dysmenorrhoea on Day 1 2 reported as 4 6 on a 10-point scale
Menstrual cycle length less than 26 or more than 30 days
Irregular menstrual cycles
Previous pregnancy
Use of reproductive hormones (apart from OC)
Use of levonorgestrel-releasing intrauterine device
Use of thyroxine, insulin or corticosteroids
BMI less than 16 or more than 30
Inflammatory process, surgical procedure or infection in previous 4 weeks
Renal, hepatic, cardiac or auto-immune disease
Use of immunosuppressant medications
Use of medications affecting TLR responsiveness, including amitriptyline and minocycline
Use of analgesics including anti inflammatory drugs, opioids or paracetamol for 5 drug half-lives prior to testing
Use of alcohol for 24 hrs prior to testing
Use of opioids or marijuana for 30 days prior to testing
Inability to read or comprehend written information provided

the study, and to arrange a screening assessment visit with the Principal Investigator (PI). Figure 1 shows the Enrolment Flow Chart for study inclusion.

Three hundred and sixty women completed the online questionnaire, with 105 women choosing to provide their contact details for consideration for study inclusion. Phone interview assessment by the Principal Investigator further excluded 44 women based on the presence of exclusion criteria. Sixty-one women recruited from the general public and five additional women recruited through Pelvic Pain SA, Adelaide, Australia proceeded to the Screening Visit, where they underwent a full clinical assessment, and the appropriate study group allocation was accurately determined. Participants below the age of 18 were interviewed in the presence of their parent or guardian, who co-signed the consent form with the participant. One participant was excluded due to previous pregnancy. Thus, a total of 65 women were enrolled in the study. Nine women were excluded during the study due to a high pre-test level of C-reactive protein (1), use of marijuana (1), irregular menstrual cycles (3), non-attendance for testing (2), insufficiently severe pain (1), and the development of an unrelated neurological illness (1). Fifty-six women satisfied all inclusion and exclusion criteria and completed the testing procedures.

Group Allocation

Participants were allocated to seven clinical groups at the Screening Visit (Table 2; groups 2A and B combined). Group allocation was determined by the self-reported presence or absence of severe dysmenorrhea, the use or non-use of the OC, and the self-reported presence of pelvic pain for more, or less, than 15 days per month, as reported at the Screening Visit. Controls were women with self-reported dysmenorrhea of between 0 and 3 on an 11-point numerical scale (NRS)[22] using anchor points of 0 and 10, where 0 represented no pain and 10 represented the most severe pain imaginable on the worst day of their menstrual period (Groups 1 and 2). Controls who were OC users (Group 2) were further divided into women who were pain-free prior to use of the OC (Group 2A), and women who had self-reported dysmenorrhea of between 4 and 10 prior to OC use (Group 2B). However, as study results for Group 2A and Group 2B were found to be similar, these groups were combined to form an enlarged Group 2, as illustrated in Table 2.

Dysmenorrhea-based pelvic pain participants (Groups 3, 4, 5 and 6) had self-reported dysmenorrhea of 7–10 on an 11-point NRS on the worst day of their menstrual period. As the number of days affected by pain varied throughout the severe dysmenorrhea cohort, participants with dysmenorrhea were further divided into those with pelvic pain for less than 15 days per month (Groups 3 and 4), and those with pelvic pain for more than 15 days per month (Groups 5 and 6). This provided an estimate of the severity of life impact of their dysmenorrhea-based pelvic pain condition. Dysmenorrhea-based pelvic pain participants (Groups 3,4,5 and 6) were further divided based on OC use (non-OC users in Groups 3 and 5; OC users in Groups 4 and 6).

Study Visit Schedule

Participants were assessed on two occasions during a single menstrual cycle: the menstrual phase (Day 1–2 of their menstrual cycle), a phase associated with high pain in women with dysmenorrhea; and the mid-follicular phase (Day 7–10 of their menstrual cycle), a time associated with low pain in women with dysmenorrhea. At Study Visit 1, participants attended the Pain and Anaesthesia Research Clinic (PARC) of the Royal Adelaide Hospital, Adelaide, Australia on either day 1 or 2 of their menstrual cycle.

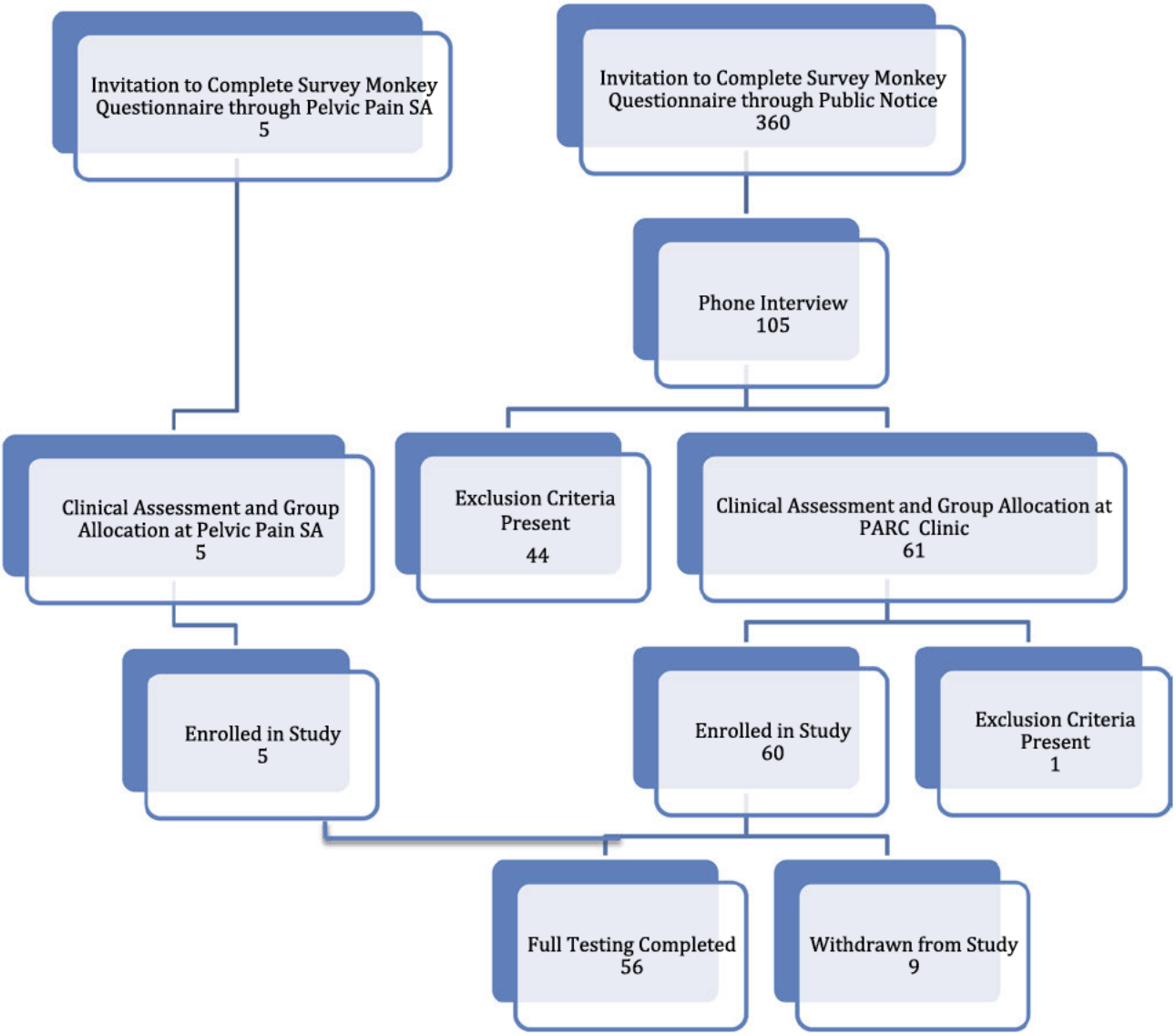

Figure 1 Enrolment flow chart for participant recruitment and study inclusion.

Participants were expressly requested not to use potentially confounding medications, including non-steroidal anti–inflammatory medications, for at least five half-lives of the drug prior to presentation for testing. At their visit, participants were asked to confirm the absence of exclusion criteria including use of confounding medications, report their current pain score at the time of testing, complete a survey of pain symptoms, and provide a blood sample for analysis. Analysis of blood samples included: TLR2 and TLR4 responsiveness of PBMCs, a measurement of C-reactive protein (CRP) levels to exclude the presence of un-recognized pre-test inflammation, and a quantitative assessment of luteinizing hormone (LH) and follicle-stimulating hormone (FSH) blood levels to confirm baseline hormonal status on day 1–2. All blood samples were taken between the hours of 0830 and 1200. At Study Visit 2, participants attended PARC on one of day 7, 8, 9 or 10 of their menstrual cycle. The testing regime was undertaken as specified above (study visit 1).

Laboratory Methods

Specimen Collection

At each study visit, 35 mL of blood was collected. Eight mL of blood was collected into a tube containing clot activator and serum gel separator for the analysis of CRP, LH and FSH levels (Healthscope Laboratories, Adelaide, Australia). Twenty-seven mL of blood was collected into tubes containing ethylenediaminetetraacetic

Table 2 Summary of Group Allocation Criteria and Demographics

Group	Description	Number of Women	Pain Reported ≥ 7 of 10	Pelvic Pain >15 Days/ Month	OC Use	Mean Age (Years)	Mean BMI	Pain Score at Testing Day 1–2	Pain Score at Testing Day 7–10
1	Controls: No OC	8	-	-	-	23.6	23.9	1.25	0.25
2	Controls: OC	15	-	-	+	21.9	22.3	0.92	0.31
3	Dysmenorrhea: No OC	7	+	-	-	25.7	23.1	6.4	0.4
4	Dysmenorrhea: OC	8	+	-	+	21.3	22.4	5.75	0.25
5	Pelvic Pain: No OC	8	+	+	-	22.6	22.2	6.9	1.25
6	Pelvic Pain: OC	9	+	+	+	22.9	20.4	6.9	3.9

Notes: + = present, - = absent.
Abbreviation: OC, oral contraception.

acid (EDTA), and PBMCs isolated using Optiprep (Sigma-Aldrich, Castle Hill, NSW, Australia) as directed by the manufacturer, using the mixer flotation method.

Quantification of PBMC Responsiveness to TLR Stimulation

To test our hypotheses, we utilized the laboratory technique devised by Kwok et al to measure IL-1β release from PBMCs following TLR stimulation.[23,24] Two analyses were undertaken: one using the TLR4 agonist LPS, and the other using the TLR2 agonist PAM.

Isolated cells were diluted to 1 x 10^6 cells·mL ^{1} in enriched RPMI 1640 (10% (v/v) fetal calf serum and 1% (v/v) penicillin), and plated into 96 well plates (Nunc, Roskilde, Denmark) using 100 µL per well. Sufficient cells were obtained from all participants, and plasma was not collected. As no reference range for LPS or PAM concentration is available for this group, TLR responsiveness was assessed across a range of concentrations. Triplicate wells were treated with a dosage curve of TLR agonists: LPS; 12.5 pg·mL ^{1} to 10 µg·mL; ^{1} and PAM 12.5 pg·mL ^{1} to 1 µg·mL ^{1} (Sigma-Aldrich, Castle Hill, NSW, Australia). Control wells contained no TLR agonist. Plates were incubated for 20 hrs at 37 °C and 5% CO_2 in a humidified environment (Thermoline Scientific, Sydney, Australia). IL-1β levels were determined using a commercially available ELISA kit (IL-1β ELISA; BD Bioscience, Australia) according to the manufacturer's instructions. The absorbance was quantified on a BMG Polarstar microplate reader (BMG Labtechnologies, Offenburg, Germany) at 450 nm. The manufacturer's limit of quantification of 0.8 pg·mL ^{1} was used, with any readings below this removed from further analysis.

Method for Statistical Analysis

To determine the responsiveness of PBMCs to LPS stimulation, represented by the EC50, concentration-response curves were fitted to an E_{max} model using the Hill equation[25] and a non-linear mixed-effects model approach. The slope parameter was fixed to 1 to reduce the number of parameters to be estimated. The model used was of the form:

$$E = E_0 + \frac{E_{max} {}^{*}C}{EC_{50} + C}$$

where E_0 is the response Y at baseline (absence of dose), E_{max} is the asymptotic maximum dose effect (maximum effect attributable to the drug), and EC_{50} is the concentration which produces 50% of the maximal effect.

Individual E_{max} models were fitted for each subject at each timepoint (Day 1–2 or Day 7–10). Before model fitting, individual plots of the concentration-response data collected were used to estimate the EC_{50}, E_{max} and E_{min} for each subject at each timepoint, to assess the applicability of an E_{max} model. As several participants demonstrated a maximum response at well below the maximum concentration tested, potentially associated with reduced cell stability at higher concentrations of LPS, only concentrations up to the observed maximum response were used in model fitting. For doses above the maximum response, the maximum response observed was imputed to force a plateau and facilitate model fit. All model fitting and all analyses were performed using Statistical Analysis Software (SAS v 9.4). Starting values were set with Max representing the maximum response observed for that participant at that timepoint, and Min representing the minimum response for that participant at that timepoint. From the model, estimated values for EC_{50}, E_{max} and E_{min} were obtained for each subject at each timepoint. The results for EC_{50} were analyzed using a mixed model for repeated measures with EC_{50} as the outcome variable and day (1–2 or 7–10), OC use (yes or no) and pain (no pain,

dysmenorrhea <15 days per month, dysmenorrhea >15 days per month) as factors in the model.

The main effects from the model provide information on the differences in PBMC responsiveness according to the presence of pain (irrespective of OC use or menstrual phase), differences in PBMC responsiveness according to OC use (irrespective of the presence of pain or menstrual phase) and differences in PBMC responsiveness according to menstrual phase (irrespective of the presence of pain or OC use). Participant was included as a repeated term using a compound symmetry covariance structure. From the model the differences in responsiveness of PBMCs to LPS stimulation were determined, according to the six clinical groups described in Table 2.

During the analysis, variation in the days per month of pain was noted within groups 3, 4, 5 and 6 between that reported at the Screening Visit and that reported at presentation on study test days. For this reason, further analysis was undertaken combining Groups 3 and 5 (pain; no OC use) and Groups 4 and 6 (pain; OC use) together to include participants experiencing pain regardless of the number of pain days per month. This analysis allowed us to consider the effect of three individual factors within the entire participant group: the presence or absence of pain (Pain vs No Pain), the use or non-use of the OC (OC vs No OC), and the menstrual phase of testing (Day 1–2 vs Day 7–10). From the model the differences in Least Squares (LS) mean values for comparisons of interest were obtained with 95% confidence limits and relevant P-values by factor, as shown in Figure 4.

The model was a reasonable fit, with a few outlier results noted. Outliers were identified through two methods. Using Method 1, the raw mean and standard deviation were obtained, and values outside mean ± 2 standard deviations were flagged as outliers. Using Method 2, once the model was fitted, the residuals were obtained together with their standard error. Any value outside ± 2 was flagged as an outlier. The mixed model was then re-run excluding any value identified by either method as an outlier. The overall results were not substantially changed, and further statistical analysis was continued using the full data set with outliers included.

Results

All participants were aged between 16 and 35 years, had regular menstrual cycles, no previous pregnancies, normal body mass index (BMI) and were in good general health, apart from the presence of dysmenorrhea-predominant pelvic pain. As described in Table 2, and using one-way ANOVA and Kruskal–Wallis post hoc analyses, no significant differences were found for the age (p=0.52), height (p=0.52) or weight (p=0.68) of the women in each group. There were no significant differences in BMI (p=0.38) or age (p=0.60) between those with and without pain, and there were no significant differences in BMI (p=0.21) or age (p=0.11) between users and non-users of the OC. The average menstrual pain score for each group recorded at the time of testing was noted to be less than the maximal pain score predicted by participants during group allocation at the screening visit.

Table 2 summarizes the group allocation criteria and demographics.

IL-1β Release Following Stimulation with PAM and LPS

All participants exhibited minimal IL-1β release in the non-stimulated state. Both TLR4 agonist LPS, and TLR2 agonist PAM3CSK4, induced elevations in IL-1β in the isolated PBMCs from all participants. Inspection of the data showed that the effect size following PAM stimulation was smaller and more variable than the effect size following LPS stimulation (Figures 2 and 3). The IL-1β response to LPS stimulation followed a biphasic distribution across the logarithmic concentration range of 12.5 pg·mL ^{1} to 10 μg·mL ^{1} (Figure 2). A similar direction of response was seen in PBMCs stimulated with PAM across the logarithmic concentration range of 12.5 pg·mL ^{1} to 1 μg·mL ^{1} (Figure 3) but the response followed a monophasic distribution with wide variability. In view of the smaller effect size and higher variability following PAM stimulation, only LPS responses are analysed further.

Analysis of EC50

The Least Squares (LS) mean values for EC_{50} and E_{max} for the variables Pain, OC use and Day of testing were obtained from the mixed model. E_{max} values were variable, consistent with suspected non-specific cellular toxicity at high LPS concentrations. As it is the responsiveness of PBMCs to LPS stimulation that we are investigating, rather than the absolute Il-1β levels, further analysis considers the LS means for EC_{50} rather than E_{max}. EC_{50} is the outcome variable we have used to compare the responsiveness of PBMCs to TLR4 stimulation with LPS between participants.

The LS mean values for EC_{50} by factor with p value are displayed in Table 3 and plotted in Figure 4. The difference

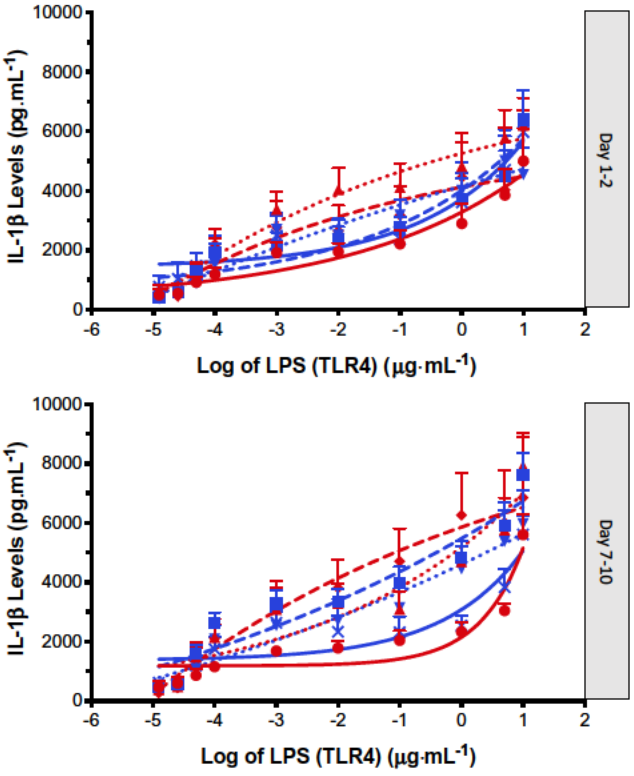

Figure 2 IL-1β release (Mean ± SEM) following LPS (TLR4) stimulation (12.5 pg mL to 10 µg mL) of PBMC's obtained from controls: no OC (red circle, solid line), controls: OC (blue square, solid line), dysmenorrhea: no OC (red triangle, dotted line), dysmenorrhea: OC (blue upside down triangle, dotted line), pelvic pain: no OC (red diamond, dashed line), and pelvic pain: OC (blue cross, dashed line). A four-parameter logistic dose-response curve has been fitted to each graph; data obtained from day 1 2 and day 7 10 of each individuals' menstrual cycle.

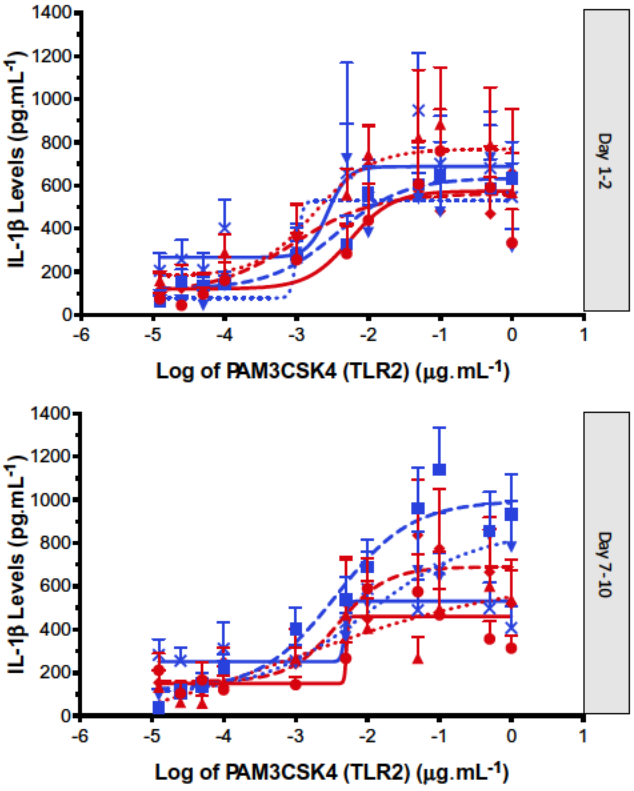

Figure 3 IL-1β release (Mean ± SEM) following PAM3CSK4 (TLR2) stimulation (12.5 pg·mL to 1 µg mL) of PBMC's obtained from controls: no OC (red circle, solid line), controls: OC (blue square, solid line), dysmenorrhea: no OC (red triangle, dotted line), dysmenorrhea: OC (blue upside down triangle, dotted line), pelvic pain: no OC (red diamond, dashed line), and pelvic pain: OC (blue cross, dashed line). A four-parameter logistic dose-response curve has been fitted to each graph; data obtained from day 1 2 and day 7 10 of each individuals' menstrual cycle.

in LS means for each main effect factor analysis for EC_{50} is represented graphically as a forest plot in Figure 5.

Pairwise Comparisons of IL-1β Release by Factor (Pain, OC Use and Day)

Pairwise comparisons allow us to compare the variables across the entire participant cohort. Pairwise comparison of Pain versus No Pain (Table 3; Figure 5, Line 1) among all participants showed a statistically significant effect of pain (p=0.042). The LS mean EC_{50} in the no pain group (control) was 4.47 ng·mL ^{1} compared with 2.08 ng·mL ^{1} in the pain group, representing a difference of 2.39 ng·mL ^{1} (pain – no pain) with 95% confidence limits from −4.69 ng·mL ^{1} to −0.09 ng·mL. ^{1} When considering the impact of this difference, the women with dysmenorrhea showed a large 2.15-fold (95% CI 4.69–0.09) increase in IL–1β release when compared with pain-free participants across both days.

Pairwise comparison of OC versus no OC (Table 3; Figure 5, Line 2) for all participants showed no significant effect of OC use (p=0.98). The LS mean for EC_{50} in the OC user group was 3.29 ng·mL ^{1} compared with 3.26 ng·mL ^{1} in the OC non-user group, representing a difference of 0.03 ng·mL ^{1} (OC – no OC), with 95% confidence limits from 2.26 ng·mL ^{1} to 2.33 ng·mL. ^{1}

Pairwise comparison of Menstrual Phase (Day 1–2) versus Mid Follicular Phase (Day 7–10) (Table 3; Figure 5, Line 3) for all participants showed no significant effect of Day (p=0.22). The LS mean for EC_{50} in the Day 1–2 group, was 2.80 ng·mL ^{1} compared with 3.74 ng·mL ^{1} in the Day 7–10 group, representing a difference of 0.94 ng·mL ^{1} (Day 1–2 – Day 7–10), with 95% confidence limits from 2.46 ng·mL ^{1} to 0.59 ng·mL. ^{1}

Pairwise comparison of Menstrual Phase (Day 1–2) versus Mid-Follicular Phase (Day 7–10) (Table 3; Figure 5, Line 5) among pain-free controls who were non-users of the OC showed no significant effect of Day (p=0.16). The LS mean for EC_{50} in the Day 1–2, no pain group was 3.12 ng·mL ^{1} compared with 5.86 ng·mL ^{1} in the Day 7–10, no pain group, representing a difference of 2.74 ng·mL ^{1} (Day 1–2 – Day 7–10) with 95% confidence limits from 6.55 ng·mL ^{1} to 1.08 ng·mL. ^{1}

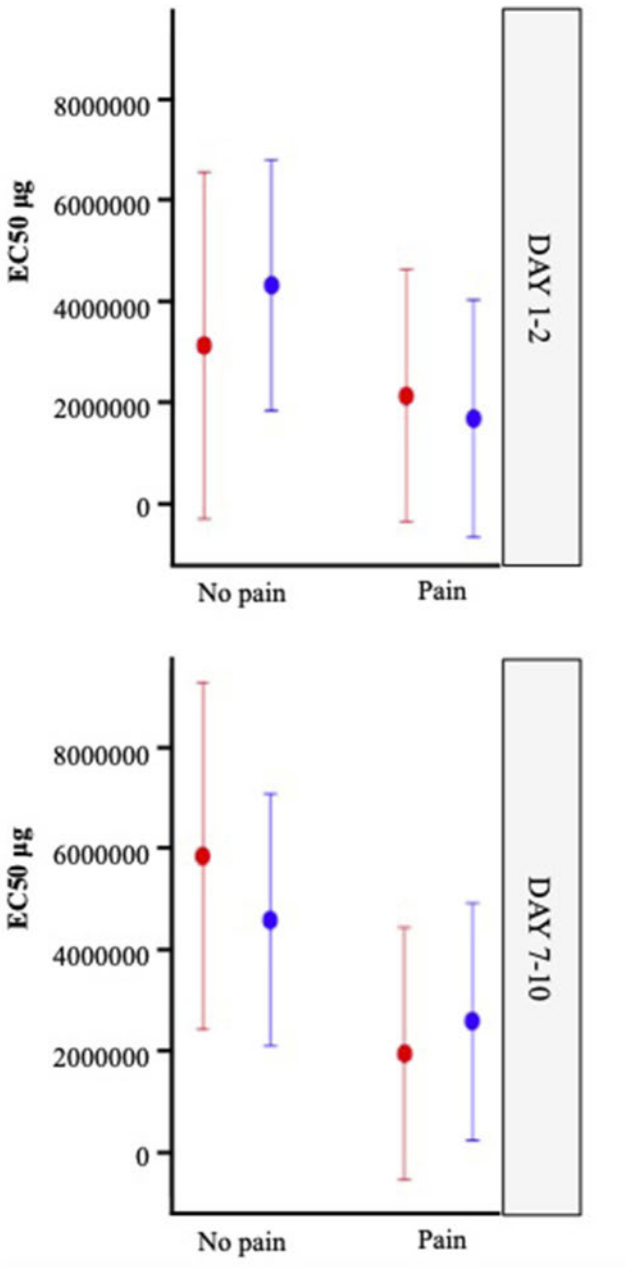

Figure 4 Least Squares means for EC50 with 95% confidence limits by factor using mixed model.

Discussion

To our knowledge, this data is the first to demonstrate an increase in systemic immune responsiveness in young women with dysmenorrhea-related pelvic pain to TLR agonists. This is a preliminary study with potentially wide-ranging clinical implications. Our initial hypothesis, that there would be a "pain response" to severe dysmenorrhea, as evidenced by a dynamic, increased responsiveness of PBMCs following TLR stimulation at the time of painful menstruation, was confirmed. However, the association was less than that demonstrated by Kwok et al[23] in an older, undifferentiated cohort of both male and female chronic pain patients. While the direction of response for both TLR2 and TLR4 stimulation was similar, the effect size was greater with TLR4 stimulation.

Our research demonstrated a significant difference between the responsiveness of PBMCs to TLR4 stimulation (Table 3, p=0.042) in women with and without dysmenorrhea-related pelvic pain. The mechanism behind the upregulation of the innate immune system in these women with pain may involve factors that increase exposure of the uterus or peritoneal cavity to TLR stimulation, or that impair the immune response to normal TLR stimulation.

Recurrent Retrograde Menstruation with Exposure of the Peritoneal Cavity to Endometrial Immune Cells and Microorganisms as a TLR Stimulus to the Innate Immune System

Exposure of the peritoneal cavity to menstrual blood and debris via the process of retrograde menstruation through the fallopian tubes has already been demonstrated in 76–90% of women.[26] During the late secretory phase of the menstrual cycle, an influx of immune cells, including macrophages, arrives in the endometrium following the normal, pre-menstrual fall in progesterone. Menstrual fluid that reaches the peritoneal cavity includes blood, shed endometrial cells, immune cells and bacteria, including the gram-negative bacilli with cell walls comprised of LPS.[27] As such, retrograde menstruation provides multiple opportunities for interaction with the innate immune system.[28] Menstrual stromal cells have been identified in lymph nodes draining the uterus during menstruation providing further evidence of immune clearance of endometrial cells in menstrual fluid.[29] Our research demonstrated an increased responsiveness of the innate immune system independent of the day of testing (Table 3, p=0.22). This supports the presence of immune activation across the menstrual cycle, rather than purely in the presence of severe pain. These findings are consistent with the clinical studies by Slater, Vincent, Payne, Granot and As-Sanie[30–34] who have demonstrated a reduced pain stimuli threshold in women with dysmenorrhea, present throughout the menstrual cycle, at both pelvic and extra-pelvic sites, as measured by pressure pain threshold or functional MRI change.

Table 3 Least Squares Means for EC50 (with 95% Confidence Limits) and Significance (*) by Factor, Derived from Mixed Model Comparing Participants with Pain/No Pain, OC Use/Non OC Use, Day 1–2/Day 7–10 in Controls Who Were Non OC Users, and Day 1–2/Day 7–10 in All Participants

	Pain [1]	**No Pain [2]**	**Difference [1–2]**	**P value***
LS mean EC50 (95% CI)	2.08 (0.63 3.53)	4.47 (2.69 6.25)	−2.39 (−4.69, −0.09)	0.042*
	OC use [1]	**No OC use [2]**		
LS mean EC50 (95% CI)	3.29 (1.85 4.73)	3.26 (1.47 5.05)	0.03 (−2.26, 2.33)	0.98
	Day 1–2, no pain, no OC [1]	**Day 7–10, no pain, no OC [2]**		
LS mean EC50 (95% CI)	3.12 (−0.31 6.55)	5.86 (2.43 9.28)	−2.74 (−6.55, 1.08)	0.16
	Day 1–2 [1]	**Day 7–10 [2]**		
LS mean EC50 (95% CI)	2.80 (1.44, 4.17)	3.74 (2.38, 5.11)	−0.94 (−2.46, 0.59)	0.22

An Impaired Immune Response with Reduced Clearing of Menstrual Debris from the Peritoneal Cavity as a Stimulus to the Innate Immune System

While all women with an intact uterus menstruate, and many exhibit retrograde menstruation, not all women suffer severe dysmenorrhea. A healthy immune response may be required to clear the pelvis of menstrual debris promptly, and an impaired immune response may be associated with reduced clearing of menstrual debris. Persistent chronic inflammation associated with residual menstrual tissue could be a mechanism for increased pain. Such an impaired response would provide prolonged stimulation of TLRs,

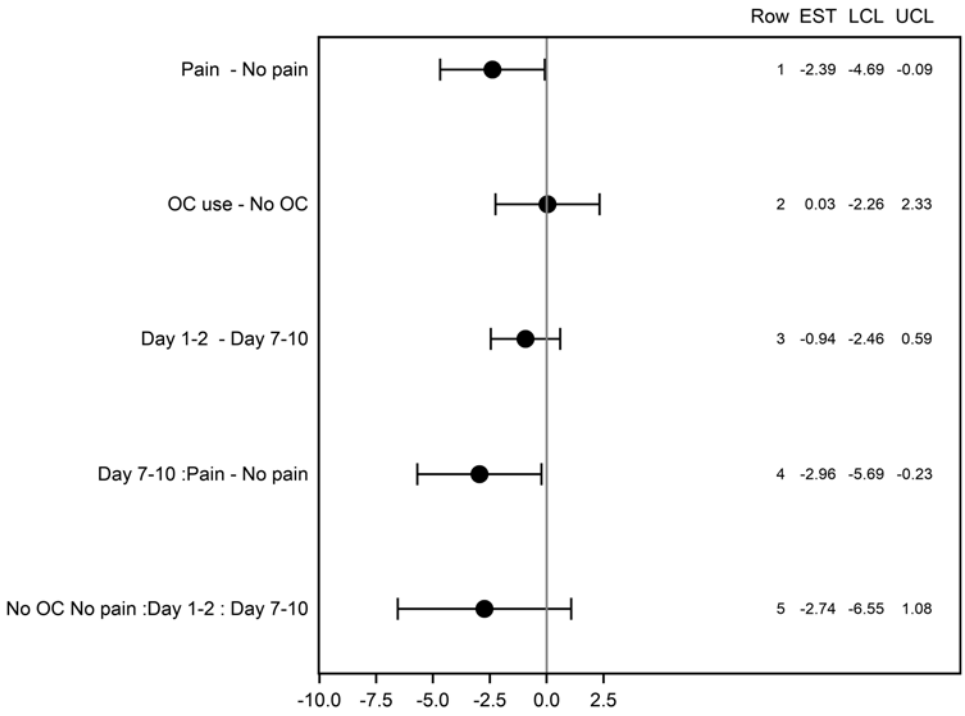

Figure 5 Forest Plot for difference in LS means for EC50 with 95% confidence limits by factor.

with potential for enhanced immune system activation. Our finding that there was no significant difference between TLR4 responsiveness in the menstrual compared with the mid-follicular phase across our entire participant cohort (Figure 5, Line 3) suggests that the process of menstruation itself is not the sole contributor to TLR responsiveness. This finding was further investigated within the group of pain-free controls who were non-OC users (Group 1). There was no significant difference between the responsiveness of PBMCs in the menstrual compared with the mid-follicular phase (Table 3, p=0.16) of pain-free controls who were non-OC users.

Recurrent Stimulation of the Innate Immune System, via TLRs as a Potential Driver of Peripheral and Central Pain Sensitization

It has been proposed that recurrent, monthly, painful menstruation may lead to the development of persistent pain states.[23] Our study provides evidence that this effect may act in part via TLR4 mediated mechanisms. Preclinical models have demonstrated that glia assume a pro-inflammatory reactive state following activation by TLRs, and that TLR4 mediated inflammation contributes to central pain amplification via the dorsal horn of the spinal cord.[35] The established ability of TLR antagonists to significantly reduce experimentally induced neuropathic pain in an animal model further supports these findings.[36] In human studies, dysmenorrhea appears to be a central factor in the development of viscero-visceral hyperalgesia, and pain co-morbidities including migraine.[15,37-39] Multiple studies have shown that even small peripheral immune challenges have a profound effect on pain enhancement, even in healthy subjects.[40–42] Doses of LPS as low as 0.4 ng·kg ^{1} delivered intravenously (IV) can provoke immune enhancement of pain responsiveness, which is below the threshold for a clinical response in healthy adults. This dose is also below the FDA approved level of LPS contamination (1 ng·kg ^{1}) in drug preparations and intravenous fluids. Our data, demonstrating an underlying sensitivity of PBMCs to TLR4 stimulation in women with dysmenorrhea-related pelvic pain provides a plausible mechanism by which cyclical exposure to menstruation engendering recurrent innate immune stimulation may predispose some women to chronic pelvic pain.

The Oral Contraceptive as Treatment for Dysmenorrhea

Despite its widespread use for the clinical management of dysmenorrhea, the exact mechanism by which the OC reduces pain in some, but not all women with dysmenorrhea remains uncertain. Our hypothesis that use of the OC would modulate the responsiveness of PBMCs in women with dysmenorrhea was not supported. Furthermore, we found no significant differences in IL-1β release between women who were users or non-users of the OC (Table 3, p=0.98). It appears that the benefits some women derive from OC treatment for the management of dysmenorrhea is via pathways other than TLR-mediated immune system modulation. Alternatively, it may be that the OC normalizes the increased responsiveness of PBMCs to pain, masking the underlying pain pathology.

While our research found no significant difference between the responsiveness of PBMCs to TLR stimulation in women who were users or non-users of the OC, this requires further research. It is known that the sensitivity of TLR4 receptors is increased by estrogen[35,43]. The OC is widely used by young women for a range of hormonal management and contraceptive purposes apart from dysmenorrhea, and an improved understanding of its immune effects would have wide-ranging implications for clinical practice. It is important to note that the women using the OC in our investigation were not selected on the basis of clinical benefit of OC use for pain. This limits the conclusions that can be drawn from our data.

TLR Stimulation of PBMCs as a Blood Biomarker for Pain in Women with Dysmenorrhea

While the evidence linking TLR mediated neuro-inflammation and chronic pain in animal models is compelling,[35,44] its relevance to human pain conditions has been lacking, largely due to the inaccessibility of the human central nervous system (CNS) and the lack of a reliable, effective, human biomarker for pain or immune activation. Pain in humans has thus been a subjective experience, conventionally assessed by patient reports or rating scales. This lack of suitable biomarker hampers the development of new pain management options. It reduces our ability to predict which women are most at risk of pain progression, our ability to monitor the response to pain therapies and it confounds patient stratification for enriched clinical trials.

However, given the research findings by Kwok et al[24] that the TLR response from PBMCs does indeed mirror similar

changes in the spinal cord, the demonstration by Kwok et al and Schrepf et al[17,23] that the technique we have used can indeed differentiate a human cohort of either chronic pain or bladder pain patients from controls, and our own research findings, we believe that the measurement of innate immune responsiveness has the potential to be a useful biomarker for pain in women with dysmenorrhea. Unlike our study, Kwok et al[24] and Schrepf et al[17] found the response to TLR2 stimulation to be a better discriminator of persistent pain status than the response to TLR4 stimulation. However, the participant cohort in Kwok and Schrepf's studies included confounding diagnostic variables, such as co-morbid medical conditions, drug or hormone use, male patients, and pain conditions outside the pelvis. For the potential of this test as a biomarker for pain to be realized, longitudinal intervention studies, ideally placebo controlled, using the biomarker would be required. This testing can be performed independently of the menstrual cycle stage, simplifying future translational clinical trial development. However, the wide variability between individual responses, and the time and resource intensive requirements of the laboratory procedure we have used, limit its practical use. While repeating the study with larger numbers of participants may further refine the research findings, we do not believe that this should be undertaken. Advances in TLR testing using single point analysis provide improved convenience over our research protocols, despite their lack of ability to measure dynamic PBMC responsiveness in individual patients. Our study has demonstrated that testing can be performed independently of the menstrual cycle stage, simplifying future translational clinical trial development.

The Association Between Dysmenorrhea, Endometriosis and the Innate Immune System

Despite our focus on the role of the innate immune system and the primary care presentation of dysmenorrhea, it is recognised that a proportion of our study participants with pain may have endometriosis. Endometriosis was found in 62% of 880 adolescents undergoing laparoscopy for severe dysmenorrhea in a meta-analysis of 15 studies.[45] However, dysmenorrhea may occur without endometriosis, endometriosis may be present without pain, and pain may persist despite complete surgical excision of endometriotic lesions, showing that the association between endometriosis and dysmenorrhea should not be assumed to imply causality.[14,46]

Endometriosis is recognized as an inflammatory condition, with an increase in IL-1β release both at a local and systemic level.[47,48] Increased *E. Coli* colony formation with increased endotoxin (LPS) levels has been demonstrated in both menstrual blood and the peritoneal fluid of women with endometriosis, with endometrial cell growth enhanced by LPS administration.[27] These findings prompted Khan et al to propose the "Bacterial contamination theory" for the development of endometriosis lesions, whereby contamination of menstrual fluid by *E. Coli* elicits peritoneal inflammation and predisposes to the development of endometriosis lesions.[49] The prolonged presence of endometrial cells in the peritoneal cavity following menstruation has been demonstrated in women with endometriosis, and ascribed to either deficient cell-mediated immunity,[50,51] or enhanced endometrial cell survival. Our research has investigated TLR activation from a pain, rather than an endometriosis lesion perspective. Despite this, it is possible that similar mechanisms, potentially involving impairment of the innate immune system and TLR activation, may predispose an individual to both conditions.

Study Strengths and Weaknesses

A strength of this study is the stringent exclusion criteria used, the lack of clinical co-morbidities, and the lack of confounding medications in this young, otherwise healthy, patient group. Another strength is the sampling at two stages of the menstrual cycle in each participant. This enables estimates of within-subject variability to be distinguished from between-subject and assay variability. Our study's demonstration that testing can be done during both the menstrual and mid-follicular phases of the menstrual cycle further enhances the applicability of this test to research and clinical practice.

Our study is an observational study with small group sizes, rather than an interventional study with large numbers of participants. Therefore, firm conclusions cannot be made. However, our study design was suitable given the exploratory nature of our research. As our participants were not randomized for OC usage, there is potential for selection bias. Future studies may choose to stratify groups according to participant-reported benefit or non-benefit of the OC for pain symptoms.

Conclusion

To our knowledge, this is the first study to demonstrate an ex vivo immune relationship in young women with dysmenorrhea-related pelvic pain. This response was at an early stage

of their pain condition, and in a participant cohort free of confounding medical conditions or medications.

Our study found a significantly increased responsiveness of peripheral blood mononuclear cells to toll-like receptor stimulation using lipopolysaccharide (p=0.042). No difference was found in PBMC responsiveness in women who were users or non-users of the OC (p=0.98). We propose that the mechanism of increased pain responsiveness in young women with dysmenorrhea results from a TLR-mediated activation of the immune system, after impaired clearing and prolonged exposure of the peritoneal cavity to menstrual and bacterial debris. This research provides a basis for the consideration and future development of novel treatments for dysmenorrhea and persistent pelvic pain.

Our study makes substantial progress in expanding our knowledge of the biological basis of dysmenorrhea-related pelvic pain, a field that has been under-researched when compared to its impact on individual women and society. This study identifies key parameters and therapeutic targets for further research and drug development in this area of unquestioned need.

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Author Contributions

All authors contributed to data analysis, drafting and revising the article, gave final approval of the version to be published, and agree to be accountable for all aspects of the work.

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Disclosure

Susan F Evans receives royalties from book authorship, has received payment from Pfizer and Bayer for educational presentations, and is a director of Alyra Biotech. Alyra Biotech is involved in the development of novel treatments for the management of pelvic pain. The submitted work is publication of research that was planned and initiated in November 2013. She also reports a patent (PCT/AU2018/051383) pending to Alyra Biotech. Paul E Rolan is a shareholder in Havah Therapeutics and iXBiopharma, director and shareholder of Lipotek, consultant to Bionomics and Novartis, and has received payment for educational presentations from Novartis and Sequirus. Mark R Hutchinson is director of the Australian Research Council Centre of Excellence for Nanoscale BioPhotonics and the recipient of an ARC Future Fellowship (FT180100565). Dr Ann C Solterbeck is director of Statistical Revelations and receives payment from pharmaceutical companies outside this research area. The authors report no other conflicts of interest in this work.

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3.6 CONCLUSIONS

Hypothesis 1, that young women with severe dysmenorrhoea have enhanced IL-1β release from peripheral blood mononuclear cells following stimulation of TLR 2 and 4 receptors using the TLR4 agonist lipopolysaccharide (LPS) and the TLR2 agonist Pam3CSK4, when compared with normal (control) women with minimal dysmenorrhoea was confirmed. Unlike the studies of Kwok and Schrepf (Kwok et al., 2013; Schrepf et al., 2015) our research found TLR4 to be a better discriminator for pain than TLR2. The studies by Kwok and Schrepf were confounded by medications, diverse pain diagnoses, and medical co-morbidities, while our study enrolled a young, more homogeneous population.

Hypothesis 2, that the responsiveness of PBMCs to TLR2 or TLR4 stimulation, as measured by the release of IL-1β release, has potential as a biomarker for the presence of dysmenorrhoea-related pelvic pain was confirmed. However, while further studies using this technique and a larger sample size may elicit further information, the technique we used is labour intensive and operator dependent. It is likely that simpler, bedside, reproducible tests for immune activation will replace it, and be easier to use as a guide for clinical management.

Hypothesis 3, that increased immune responsiveness of PBMCs to TLR stimulation would be present only on days of high pain was not supported. Women with severe pain showed increased TLR4 responsiveness on both high and low pain days. This finding offers the potential for simplification of specimen timing in future trials.

Hypothesis 4, that the responsiveness of PBMCs to TLR stimulation would be modulated by use of the oral contraceptive pill was not supported. Despite its widespread use for dysmenorrhoea, our findings suggest that the OC reduces dysmenorrhoea-related pelvic pain through mechanisms independent of the innate immune system.

CHAPTER 4: IS HIGH SENSITIVITY C-REACTIVE PROTEIN MEASUREMENT IN BLOOD A RELIABLE PROXY FOR INNATE IMMUNE SENSITISATION IN WOMEN WITH DYSMENORRHOEA?

4.1 INTRODUCTION

As discussed in Chapter 3, the discovery and validation of a blood biomarker for dysmenorrhoea-related pelvic pain would simplify the evaluation and development of novel pelvic pain treatments. A potential simple marker for activation of the immune system is high-sensitivity C-Reactive Protein (hsCRP). Chapter 4 investigates the potential for hsCRP to act as a biomarker for pain and immune activation in our cohort of women with dysmenorrhoea.

C-Reactive Protein is an acute phase protein of the innate immune system found in blood plasma, whose concentration rises in response to inflammation or infection (Gabay & Kushner, 1999; M. Harrison, 2015). CRP is produced in the liver and rises in response to an increase in IL-6 secretion by macrophages or T-cells. The main biologic function of CRP is to recognise pathogens and damaged cells within the body and to facilitate their removal by recruiting phagocytic cells and the complement system. (Volanakis, 2001) A substantially raised CRP is found in women with acute pelvic inflammatory disease, where a bacterial infection of the peritoneal cavity induces a substantial host response. However, a raised CRP is non-specific, and has been associated with a range of both gynaecological (Hemilä et al., 1987), and systemic inflammatory conditions including inflammatory bowel disease (Vermeire et al., 2004), and rheumatoid arthritis (Amos et al., 1977). Ma et al studied monocytes in the peripheral blood of women with dysmenorrhoea, and found an up-regulation of genes coding for pro-inflammatory cytokines, with these changes present throughout the menstrual cycle (Ma et al., 2013).

In response to infection, or acute inflammation, CRP levels rise above the commonly reported normal level of <5 mg/L, usually to an order of magnitude of difference (Lourenço et al., 2014). High levels such as these are easily recognised as abnormal. However, over recent years, there has been increasing interest in the relevance of mildly elevated CRP levels, potentially as a marker for low-grade inflammation and increased risk of inflammatory disease states. This interest has been facilitated by the development of a high-sensitivity test for CRP (hsCRP)

able to measure and distinguish levels below 5mg/L. High sensitivity testing has determined that a normal level of CRP in healthy individuals is <1mg/L, and shown an association between a CRP level of 1-3 mg/L and a range of systemic inflammatory conditions (Dhingra et al., 2008). In this study, based on the Framingham Offspring Study of 3782 participants (mean age 55 years; 52% women) free of cardiovascular disease, the prevalence of inflammatory conditions among women rose from 46.5% where hsCRP<1mg/L, to 51.7% where hsCRP 1-3mg/L, to 56.0% where hsCRP 3-10mg/L, and to 70.1% where hsCRP >10mg/L. The level of hsCRP has already been utilised within a Norwegian Risk Factor Composite score, incorporating body mass index, fibrinogen, C-reactive protein, and triglycerides to predict the presence of chronic pain (Sibille et al., 2016).

Recently, Gold et al investigated 2939 women aged between 42 and 52 years, to determine whether an increased hsCRP level could be used as a biomarker for women reporting a range of premenstrual symptoms, including abdominal cramps and back pain. (Gold et al., 2016) Their research found that an hsCRP level >3 mg/L was significantly positively associated with abdominal cramps or back pain with an adjusted odds ratio of 1.40 (95% CI 1.09-1.80). While this study suggests a possible relationship between hsCRP and pain, research by Mu et al investigating a relationship between hsCRP and the presence of endometriosis was not supportive. Mu et al. (2018) measured the levels of a range of inflammatory blood markers, including IL-β, IL-6, TNF-α receptor 1 and 2, and hsCRP in 350 women (average age 42 years) with endometriosis, and 694 healthy female controls. While they found a correlation between the levels of IL-β and endometriosis, they found no correlation between levels of hsCRP levels and the presence of endometriosis. However, both the study by Mu et al. and the study by Gold et al. investigated middle-aged and older women, without documenting the phase of their menstrual cycle at blood sampling. For this reason, the potential of hsCRP to act as a biomarker for dysmenorrhoea-related pelvic pain in young women remains unanswered.

4.2 Aims

Chapter 4 investigates the potential of hsCRP to act as a simple, rapid and reproducible biomarker for the degree of inflammation, or the severity of pain by further investigating the cohort of young women, with and without dysmenorrhoea-related pelvic pain, described in Chapter 3. It has 2 aims:

Aim 1: To investigate the potential for the blood measurement of hsCRP to act as a biomarker for the severity of dysmenorrhoea-related pelvic pain within an outpatient clinical setting.

Aim 2: To investigate whether blood levels of hsCRP correlate with the number of days of pelvic pain per month (DPelvicPM) reported by participants in Chapter 3.

4.3 HYPOTHESES

This chapter investigates two hypotheses:

Hypothesis 1: That increasing levels of hsCRP correlate with an increasing DPelvicPM, and can thus act as a biomarker for the lived experience of pain.

Hypothesis 2: That increasing levels of hsCRP correlate with an increased responsiveness of PBMCs to TLR4 stimulation, as measured by the EC_{50} for release of IL-1β from PBMCs, in young women with dysmenorrhoea-related pelvic pain, and can thus act as a biomarker for inflammation.

4.4 METHODS

This cohort comprises 56 women stratified into groups according to the presence of pain, measurement of the DPelvicPM, and stratification based on the use or non-use of the oral contraceptive pill.

High-sensitivity CRP measurement was undertaken on blood samples taken on Day 1-2 of the menstrual cycle, and on Day 7-10 of the menstrual cycle, as per the protocol outlined in the published article, *Evans SF, Kwok YH, Solterbeck A, Liu J, Hutchinson MR, Hull ML, & Rolan PE. (2020). Toll-Like Receptor Responsiveness of Peripheral Blood Mononuclear Cells in Young Women with Dysmenorrhea. Journal of Pain Research, 13, 503–516.*

Blood analysis for hsCRP was performed through Healthscope Laboratories.

4.5 RESULTS (UNPUBLISHED)

Multiple comparisons were made between the level of hsCRP and variables including clinical group allocation, day of testing, DPelvicPM, use or non-use of the OC, and the responsiveness

of PBMCs to LPS stimulation as measured by the EC_{50} for release. Comparisons made and figures generated were formed using GraphPad Prism.

A comparison between the level of hsCRP in women who are users, and non-users, of the OC

A two-tailed unpaired t-test was used to investigate the levels of hsCRP among the entire participant cohort divided into users and non-users of the OC. Levels of hsCRP were significantly higher among OC than non-users on both Day 1-2 (p=0.027) and Day 7-10 (p=0.016) (Figure 4.1).

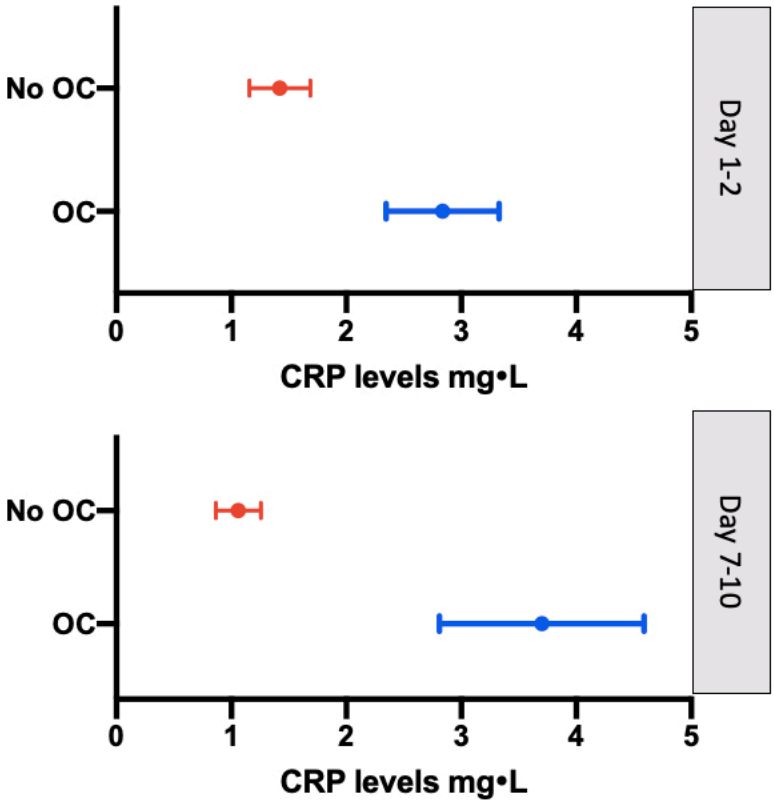

Figure 4.1: Comparison of hsCRP levels for each individual either taking (blue) or not taking (red) the OC on Day 1-2 or Day 7-10.

Further analysis was undertaken to compare hsCRP with clinical grouping according to the presence or absence of pain, whether pain was present for more than 15 or less than 15 days per month, and OC use (Figure 4.2). Using a one-way ANOVA with Tukey's post hoc test, there was no significant difference found when comparing the mean of any group, to the mean of every other group, at either timepoint. A difference was noted between OC and non-OC users, but due to the small sample size in this comparison, significance was not reached.

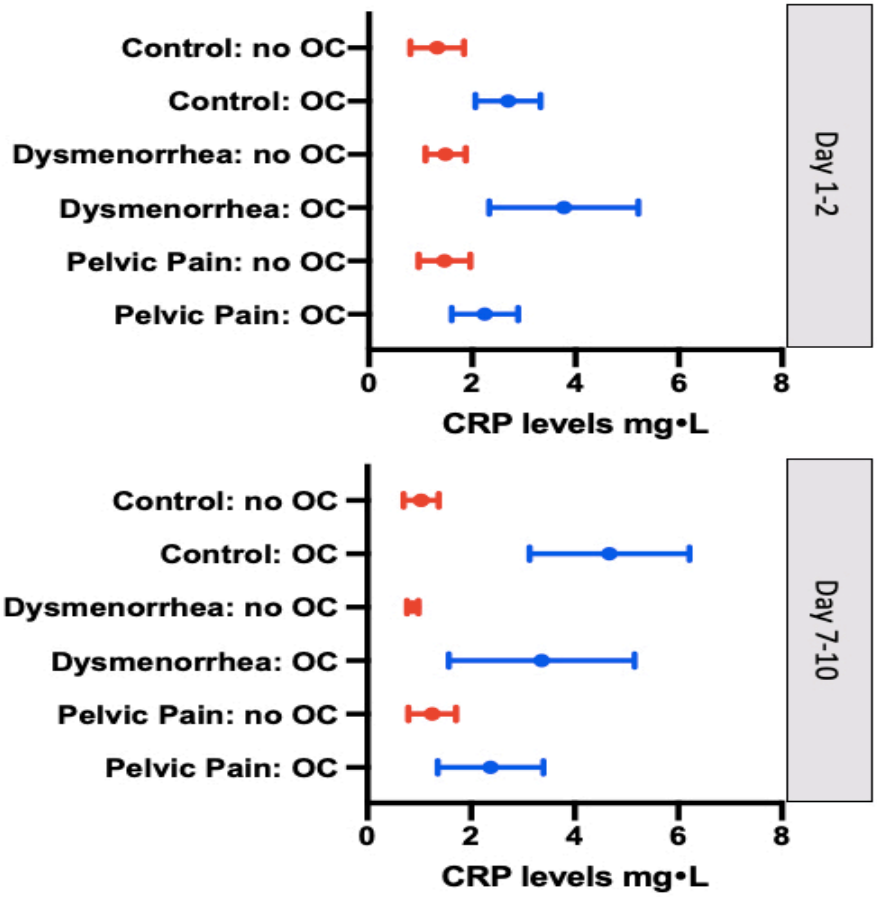

Figure 4.2: Comparison of hsCRP levels (Mean ± SEM) versus Clinical Group

A comparison between the level of hsCRP and DPelvicPM

A two-tailed, non-parametric Spearman's correlation computing r for X, versus every Y dataset, and linear regression analysis was used to investigate the relationship between hsCRP levels and DPelvicPM (Figure 4.3). No statistically significance difference was found when comparing the relationship between DPelvicPM and hsCRP levels on Day 1-2 (no OC;

r= -0.04184, p = 0.9221, OC; r = -0.1745, p = 0.4259, combined; r = -0.02920, p = 0.8324) or Day 7-10 (no OC; r= -0.1429, p = 0.8028, OC; r = -0.3409, p = 0.1413, combined; r = -0.06873, p = 0.6181) for users, non-users and the combined group.

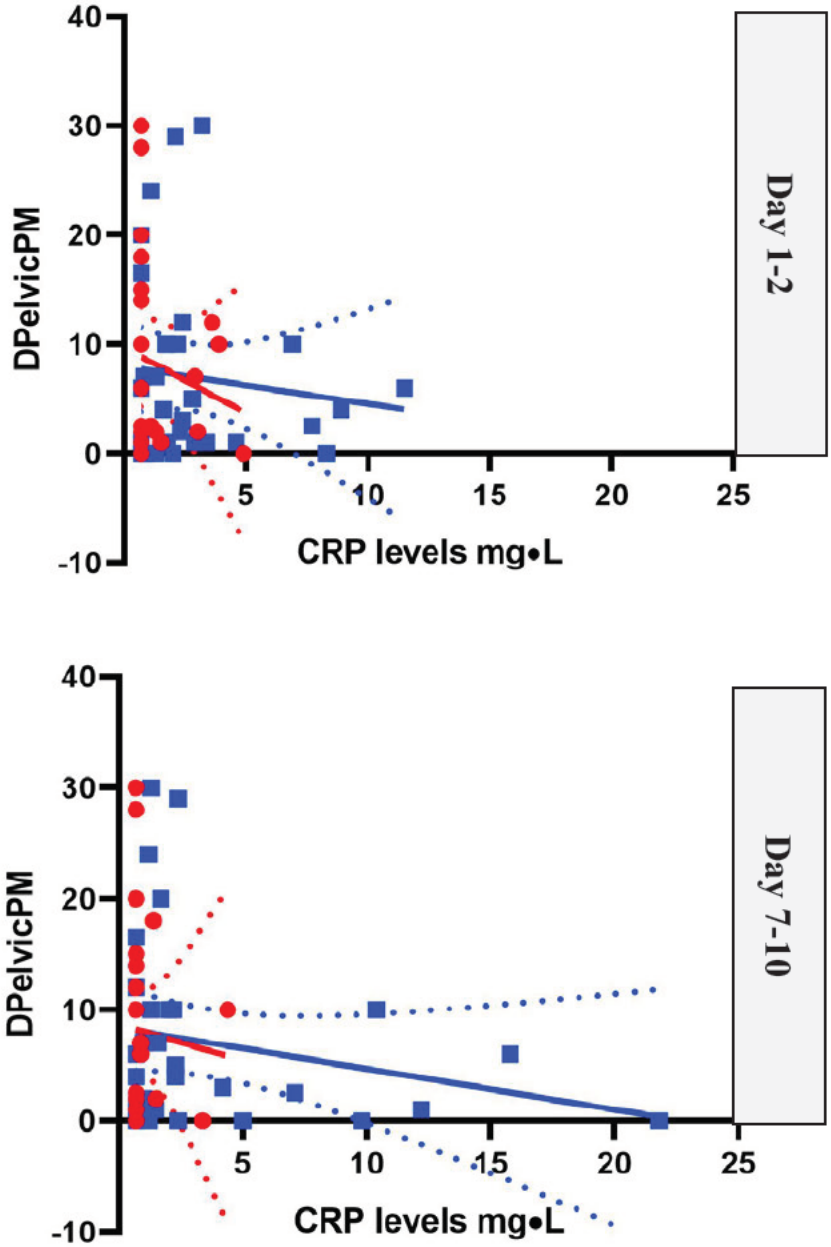

Figure 4.3: Linear regression with 95% confidence intervals comparing levels of hsCRP and DPelvicPM for Day 1-2 and Day 7-10. CRP levels for each individual either taking (blue) or not taking (red) the oral contraception versus days in month of pain on Day 1-2 or Day 7-10. Solid lines represent the linear regression. Dashed lines represent the 95% confidence intervals.

A comparison between the level of hsCRP and EC_{50} for Day 1-2 and Day 7-10

A two-tailed, non-parametric Spearman's correlation, computing r for X versus every Y dataset and linear regression was used to investigate the relationship between hsCRP and EC50 for participants with pain, without pain, or the combined participant cohort (Figure 4.4). No statistical significance was found when comparing the relationship between EC_{50} and hsCRP levels on days 1-2 (pain; r= 0.06761, p = 0.7131, no pain; r = -0.08455, p = 0.7013, combined; r = -0.008018, p = 0.9537) or days 7-10 (pain; r= 0.03677, p = 0.8417, no pain; r = -0.3806, p = 0.0732, combined; r = -0.1397, p = 0.3092) of their menstural cycle. While the correlation between CRP levels and EC_{50} was not found to be significant, there was a trend towards significance in women without pain on Day 7-10 (p=0.0732). This trend describes an increased EC_{50}, where a lower responsiveness of PBMCs to TLR4 stimulation represents reduced activation of the innate immune system, in women with lower levels of hsCRP.

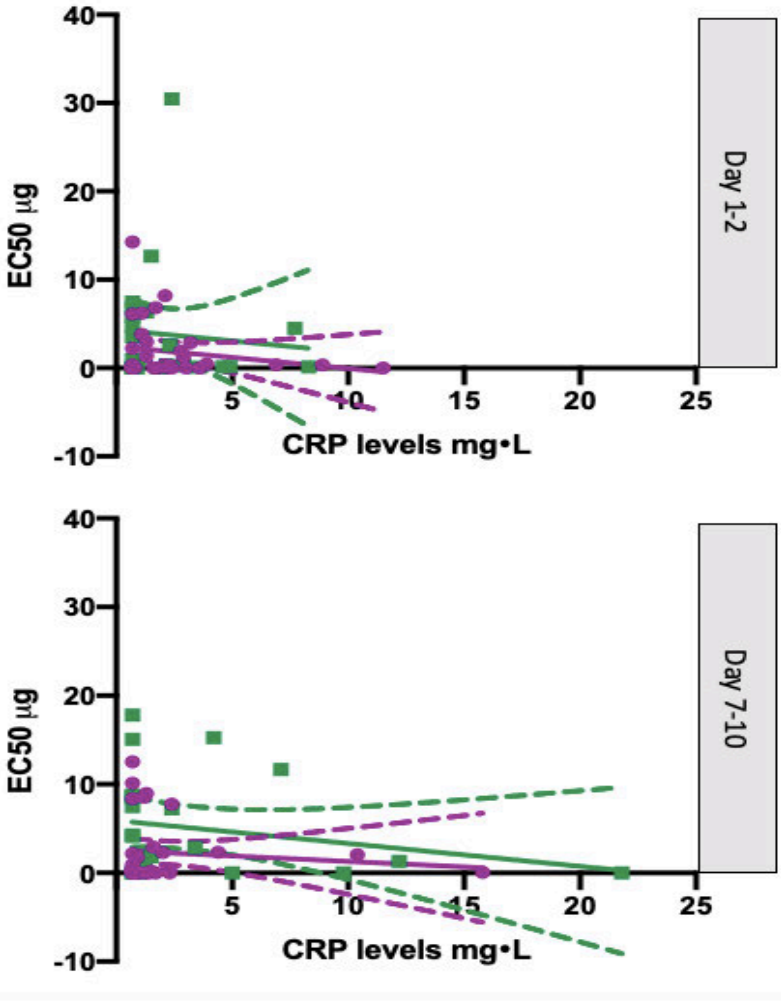

Figure 4.4: CRP levels for each individual either experiencing (purple) or not experiencing (green) pain during their menstral cycle versus EC_{50}. Solid lines represent the linear regression. Dashed lines represent the 95% confidence intervals.

4.6 DISCUSSION

Hypothesis 1, that increasing levels of hsCRP correlate with an increasing DPelvicPM and can thus act as a clinical biomarker for the lived experience of pain was not confirmed. This finding is in contrast to the findings of Gold et al. (2016), who found a relationship between premenstrual symptoms and hsCRP in an older cohort of patients aged between 42 and 52 years, but is consistent with the findings of Mu et al. (2018) who found no correlation between the levels of hsCRP and the presence of endometriosis.

Hypothesis 2, that increasing levels of hsCRP correlate with an increased responsiveness of PBMCs to TLR stimulation (lower EC_{50}) in young women with dysmenorrhoea-related pelvic pain, was not confirmed. Studies into the regulation of CRP have established that interleukin-6 (IL-6) is the principal inducer of the CRP gene, while IL-1β, glucocorticoids and complement activation products synergise this effect (Ganter et al., 1989; Majello et al., 1990). As IL-6 is an important component of the inflammatory cascade, our research suggests that additional non-inflammatory mechanisms play a role in hsCRP levels.

Our study found that levels of hsCRP are increased by use of the OC in this cohort of young women with or without dysmenorrhoea-related pelvic pain. This finding is consistent with current literature. Buchbinder et al. (2008) measured hsCRP levels in a group of 412 unselected female blood donors, and found that the 97th percentile for CRP levels was 7.52 mg/L for non-users of the OC, and 11.95 mg/L in OC users. Donors were not stratified according to the reason for use, or non-use, of the OC. Divani et al. (2015) investigated the effect of OC use in 59 young women using a panel of inflammatory markers, including hsCRP, monocyte chemotactic protein-1, soluble tumor necrosis factor (sTNF), interleukin-6 (IL-6), and soluble CD40 ligand. This study found significantly more women with levels of hsCRP>3 among OC users ($p < 0.0001$), compared to nonusers. However, their small sample size was unable to determine a relationship between hsCRP and other inflammatory markers.

A study of 389 post-menopausal women who were either users, or non-users, of hormone replacement therapy (HRT), found that while use of hormonal replacement therapy was associated with an increase in CRP, it was also associated with reduced levels of other inflammatory markers, including vascular cell adhesion molecule-1 plasma E-selectin (17.8 vs 14.8 ng/mL, $p<0.01$), interleukin-6 (1.51 vs 1.29 pg/mL, $p<0.01$), and s-thrombomodulin plasma (4.8 to 4.3 ng/mL $p<0.01$). They concluded that the discrepancy between increased plasma levels of hsCRP, and reduced plasma levels of other inflammatory markers, suggests

that the increased hsCRP found in HRT users is due to metabolic hepatic activation, rather than an acute-phase response (Silvestri et al., 2003).

Women use the OC for a wide range of purposes: contraception, menstrual suppression, acne management, and the treatment of pain. It is possible that the increased hsCRP in women using the OC, may be, at least in part, a pain effect, rather than purely an OC effect.

4.7 Conclusions

The higher levels of hsCRP among women using the combined oral contraceptive pill, already reported within the literature, was confirmed. Our research does not support the use of hsCRP as a biomarker for pain in this group of young women with dysmenorrhoea-related pelvic pain. In addition, our research does not support the use of hsCRP as a biomarker for inflammation in this group.

CHAPTER 5: DOES THE HORMONAL ENVIRONMENT AFFECT THE INNATE IMMUNE SYSTEM OR THE LIVED EXPERIENCE OF PAIN?

It's so extraordinary that we female humans should be linked to the moon and the tides. It'd sound like science fiction if you made it up. Mysterious planetary forces making us bleed.

Sofka Zinovieff, 2018

5.1 INTRODUCTION

Chapter 2 found a high prevalence of additional symptoms, both within and outside the pelvis, in young women with dysmenorrhoea-related pelvic pain, suggestive of a systemic aetiology. Chapter 3 found evidence that increased activation of the innate immune system may provide a mechanism for this effect. Sections 1.5 and 1.6 described the interplay between hormonal factors, pain and the immune system.

This chapter further interrogates the cohort of young women investigated in Chapter 3. It seeks to assess whether levels of sex steroid hormones, or cortisol, are associated with activation of the innate immune system, as measured by the responsiveness of PBMCs to TLR4 stimulation. It also seeks to assess whether levels of sex steroid hormones, or cortisol, are associated with variation in the lived experience of pain symptoms, as measured by the days per month of pelvic pain (DPelvicPM), the days per month of period pain (DPeriodPM), or the days per month of headache (DHeadachePM). In particular it investigates whether the levels of androgens, including testosterone, in women may either protect or predispose an individual to pain symptoms.

The evidence that hormonal levels influence pain perception in women has been outlined previously (Section 1.5). This is consistent with research within the University of Adelaide that found a relationship between estradiol levels and TLR4 activation in female rodents (Nicotra et al., 2014). This chapter extends this investigation to human females with DRPP. It also further investigates the relationship between androgen levels and TLR4 activation in females with DRPP, an area not yet researched. If the optimal hormonal environment for pain reduction

without increased activation of the immune system can be determined, this would have direct translational importance to the clinical management of women with pain.

The accurate assessment of androgen levels in women is hampered by the relatively low levels of androgens circulating in women, when compared to men for whom the tests were formulated. The most sensitive laboratory method for androgen assessment is liquid chromatography mass spectrometry (LC-MS) (Rosner et al., 2010). These tests are labour-intensive and therefore of limited use in primary health, where radioimmunoassay or direct chemiluminescent immunoassay techniques are commonly used. Our research uses high sensitivity liquid chromatography, mass spectrometry (LC-MS) across a range of 11 steroid hormones, and cortisol, analysed as a single batch, across two phases of the menstrual cycle. The clinical variables chosen (DPelvicPM, DPeriodPM and DHeadachePM) are quantifiable, and easily assessed during clinical assessment. They do not require laboratory testing, and are therefore appropriate for the low-resource, primary care, setting. As not all migraine headaches are classic in symptomatology, and the accurate diagnosis of migraine was not undertaken in this study, we have chosen to measure the DHeadachePM.

5.2 AIMS

Chapter 5 seeks to investigates whether the hormonal environment is associated with the subjective experience of pain symptoms, or the responsiveness of PBMCs to LPS stimulation. It has three aims:

Aim 1: To further clinical understanding of the effect of the steroid hormone blood profile on the subjective experience of dysmenorrhoea-related pelvic pain at two stages of the menstrual cycle. This information has direct translational application to the choice of hormonal therapies offered by clinicians to girls and women with pain.

Aim 2: To further clinical understanding of the relationship between the steroid hormone blood profile, and the degree of immune activation at two stages of the menstrual cycle.

Aim 3: To determine which measurement of androgen activity correlates best with the presence of absence of pain symptoms.

5.3 HYPOTHESES

This chapter investigates three hypotheses:

Hypothesis 1: That the hormonal profile of an individual is associated with the days per month of pain experienced.

Hypothesis 2: That the hormonal profile of an individual is associated with the responsiveness of PBMCs to TLR4 stimulation, as measured by the EC_{50} for IL-1β release.

Hypothesis 3: That the Free Androgen Index (FAI) provides a simple and useful test for assessing androgen activity in women.

This chapter introduces the manuscript entitled “The Relationship between Androgens and Days per Month of Period Pain, Pelvic Pain, Headache, and TLR4 responsiveness of Peripheral Blood Mononuclear Cells in Young Women with Dysmenorrhoea” which has been submitted for publication in the Journal of Pain Research.

5.4 MANUSCRIPT AND STATEMENT OF AUTHORSHIP

The study was part funded by a grant from the Anaesthesia and Pain Medicine Foundation of the Australia and New Zealand College of Anaesthetists.

The following manuscript was accepted for publication to the Journal of Pain Research on 12th January 2021:

The Relationship between Androgens and Days per Month of Period Pain, Pelvic Pain, Headache, and TLR4 responsiveness of Peripheral Blood Mononuclear Cells in Young Women with Dysmenorrhoea

All authors gave final approval of the version to be published, and agree to be accountable for all aspects of the work.

Statement of Authorship

Title of Paper	The Relationship between Androgens and Days per Month of Pelvic Pain, Period Pain, Headache, and TLR4 Responsivenss of Peripheral Blood Mononuclear Cells in Young Women with Dysmenorrhea
Publication Status	☐ Submitted for Publication
Publication Details	(Paper submitted to Journal of Pain Research, September 2020) Susan F Evans, Yuen H. Kwok, Ann Solterbeck, Carmen E Pyragius, Mark R. Hutchinson, M. Louise Hull, Paul E Rolan. (2020) "The relationship between Androgens and Days per Month of Pelvic pain, Period Pain, Headache, and TLR4 responsiveness of Peripheral Blood Mononuclear Cells in Young Women with Dysmenorrhoea".

Principal Author

Name of Principal Author (Candidate)	Susan F Evans		
Contribution to the Paper	Study design, acquisition of data, preparation of data, analysis of data, interpretation of data, preparation of article		
Overall percentage (%)	80		
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.		
Signature		Date	12/09/2020

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);

ii. permission is granted for the candidate in include the publication in the thesis; and

iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

Name of Co-Author	Yuen H Kwok		
Contribution to the Paper	Acquisition of data, laboratory analysis, preparation of data.		
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Contribution to the Paper	Study design, preparation of article.		
Overall percentage (%)	2		
Signature		Date	11/09/2020

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Overall percentage (%)	3		
Signature		Date	11/9/2020

Name of Co-Author	Paul E Rolan		
Contribution to the Paper	Study design, interpretation of data, preparation of article.		
Overall percentage (%)	2		
Signature		Date	28/9/20

The Relationship between Androgens and Days per Month of Period Pain, Pelvic Pain, Headache, and TLR4 responsiveness of Peripheral Blood Mononuclear Cells in Young Women with Dysmenorrhoea

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ABSTRACT

Purpose: Women bear a disproportionate burden of persistent pain conditions when compared to men. To determine whether the hormonal environment affects the clinical experience of pain, as measured by the days per month of pelvic pain (DPelvicPM), period pain (DPeriodPM), headache (DHeadachePM) or the *in vitro* EC_{50} for Interleukin-1β (IL–1β) release following TLR4 stimulation with Lipopolysaccharide from Peripheral Blood Mononuclear Cells (PBMCs). Findings were stratified according to use or non-use of the oral contraceptive pill.

Patients and methods: Fifty-six women aged 16-35 years, with minimal or severe dysmenorrhea, and use or non-use of the OC, were enrolled. Blood was collected on two occasions in a single menstrual cycle: Day 1-2 and Day 7-10. Hormonal analysis for testosterone, dehydrotestosterone, dehydroepiandrosterone, Androstenedione, 3α-Androstanediol, 3β-androstanediol, estradiol, estrone, 17α-hydroxyprogesterone, progesterone, cortisol and sex-hormone binding globulin was undertaken using ultra-sensitive Liquid Chromatography Mass Spectrometry (LC-MS). PBMCs were exposed to lipopolysaccharide (LPS) and the resulting Interleukin-1β output was determined.

Results: Non-users of the OC showed a strongly inverse correlation between a reducing free androgen index (FAI) and increasing DPelvicPM ($p=0.0032$), DPeriodPM ($p=0.013$), DHeadachePM ($p=0.041$) on Day 1-2. Non-users of the OC showed a significant increase in DPelvicPM ($p=0.049$) with increasing estradiol on on Day 7-10. Modestly significant associations were found between reduced androgens and potentiated LPS-induced IL–1β (lower EC_{50}).

Conclusion: This is the first study to investigate the relationship between the hormonal environment and activation of the immune system in young women with dysmenorrhoea-related pain conditions. Low androgen levels were consistently associated with increased pain. Translational implications for the findings are discussed.

Keywords: Pain, testosterone, oral contraceptive pill, dysmenorrhoea, pelvic pain, IL-1β

Funding: The study was part funded by the Australia New Zealand College of Anaesthetists (ANZCA) Research Foundation and the Australian Research Council (FT180100565).

INTRODUCTION

Women bear a disproportionate burden of pain compared with men across the majority of pain conditions (1), and ample evidence suggests a role for both the hormonal and immune environment in the development of female pain. Before puberty, the prevalence of chronic pain conditions is approximately equal in boys and girls (2). However, girls are over-represented by the age of 12-14 years, with the presence of persistent pain correlating more closely with the stage of pubertal development than with age (3). A major contributor to the disparity in chronic pain between girls and boys is the presence of dysmenorrhea and abdominal pain (4). Dysmenorrhea frequently predates (5), and is believed to be an etiological factor in, the development of chronic pelvic pain in women (6).

Compelling epidemiological, clinical and experimental evidence in both human and animal studies demonstrates that increased peripheral and central nervous system immune system activity, via Toll-Like Receptors (TLRs), is involved in the initiation and maintenance of chronic pain conditions (7–9). Our group has already demonstrated that immune pathology, including an increase in TLR4 responsiveness, is present in women affected by dysmenorrhea-related pelvic pain (10).

Toll-like receptors are pattern-recognition receptors on the surface of cells which recognise molecular patterns typically associated with microbial pathogens, and which respond by the release of cytokines including Interleukin-1β (IL-1β) that promote inflammation. Lipopolysaccharide (LPS) within the cell wall of gram-negative bacteria is a potent agonist of TLR4, an antigen on immune cells including mononuclear cells in the peripheral blood, and glial cells within the brain and spinal cord. Altered TLR inflammatory responses have already been demonstrated in visceral medical conditions characterized by persistent pain, such as inflammatory bowel disease and painful bladder syndrome (11,12).

In our previously published work (10), peripheral blood from women with dysmenorrhea-related pelvic pain showed significantly enhanced IL-1β release from peripheral blood mononuclear cells (PBMCs) following *in vitro* TLR4 stimulation with lipopolysaccharide, when compared with pain-free controls. The present study further investigates this cohort of women to determine whether the hormonal environment affects the degree of TLR4-induced immune activation, or the experience of pelvic pain.

In this study, Immune system TLR4-dependent reactivity is measured by determining the dose of LPS required to achieve 50% of maximal IL-1β release response (EC_{50}) from peripheral

blood mononuclear cells (PBMCs). Correlations between the levels of individual steroid hormones and cortisol with the DPeriodPM, the DPelvicPM, and the days of headache per month (DHeadachePM), allow an objective assessment of the relationship between hormone levels and the subjective experience of pain. Correlations between the levels of individual steroid hormones with the EC_{50} allow an assessment of the relationship between hormone levels and the degree of immune system activation. The self-reported number of days of period (DPeriodPM) or pelvic pain per month (DPelvicPM), is used as an indicator of pelvic pain severity.

We investigate the hypothesis that androgens may be protective against the development of chronic pain conditions in women, and discuss the potential role of androgens as a treatment option for women with chronic dysmenorrhea-related pelvic pain.

METHODS

Study design

This is an observational clinical and laboratory study further extending analysis of the patient cohort reported by Evans et al.,(10) and approved by the Human Research Ethics Committee of the Royal Adelaide Hospital, Adelaide, South Australia. HREC/14/RAH/63. RAH Approval No. 140217. The study was conducted in accordance with the Declaration of Helsinki (13). All participants below the age of 18 were interviewed in the presence of a parent or guardian.

Participants

The target population consisted of girls and women aged between 16 and 35 years with either minimal, or severe, dysmenorrhea. Participants with minimal dysmenorrhea had self-reported pain of between 0-3 on an 11-point numerical scale (14) on the worst day of their menstrual period. At the screening visit, participants with severe dysmenorrhea had self-reported pain of between 7-10 on the 11-point numerical scale on the worst day of their menstrual period. Participants were further separated according to use or non-use of the oral contraceptive pill (OC). Potential participants were identified either when they responded to recruitment notices displayed to the general public, which provided a link to an anonymous Survey Monkey questionnaire, or through a private clinic, Pelvic Pain SA. Eligible participants from the general public were offered the opportunity of an initial phone interview to provide further information about the study, to obtain consent for further participation in the study, and to arrange a screening assessment visit with the Principal Investigator (PI). Figure 1 shows the Enrolment Flow Chart for study inclusion. Study exclusion criteria (Table 1) ensured that women

possessing factors that might influence inflammation, TLR receptor response, the immune system, or the severity of dysmenorrhea were excluded from the study.

Box 1: Study exclusion criteria.

Dysmenorrhoea on Day 1-2 reported as 4-6 on a 10-point scale
Menstrual cycle length less than 26 or more than 30 days
Irregular menstrual cycles
Previous pregnancy
Use of reproductive hormones (apart from OC)
Use of levonorgestrel-releasing intrauterine device
Use of thyroxine, insulin or corticosteroids
BMI less than 16 or more than 30
Inflammatory process, surgical procedure or infection in previous 4 weeks
Renal, hepatic, cardiac or auto-immune disease
Use of immunosuppressant medications
Use of medications affecting TLR responsiveness, including amitriptyline and minocycline
Use of analgesics including anti-inflammatory drugs, opioids or paracetamol for 5 drug half-lives prior to testing
Use of alcohol for 24 hours prior to testing
Use of opioids or marijuana for 30 days prior to testing
Inability to read or comprehend written information provided

Three hundred and sixty women completed the online questionnaire, with 105 women choosing to provide their contact details for consideration for study inclusion. Phone interview assessment by the Principal Investigator further excluded 44 women based on the presence of exclusion criteria. Sixty-one women recruited from the general public and 5 additional women recruited through Pelvic Pain SA, Adelaide, Australia proceeded to the Screening Visit, where they underwent a full clinical assessment, and the appropriate study group allocation was accurately determined. Participants below the age of 18 were interviewed in the presence of their parent or guardian, who co-signed the consent form with the participant. One participant was excluded due to previous pregnancy. Thus, a total of 65 women were enrolled in the study. Nine women were excluded during the study due to a high pre-test level of C-reactive protein (1), use of marijuana (1), irregular menstrual cycles (3), non-attendance for testing (2), insufficiently severe pain (1), and the development of an unrelated neurological illness (1). Fifty-six women satisfied all inclusion and exclusion criteria and completed the testing procedures.

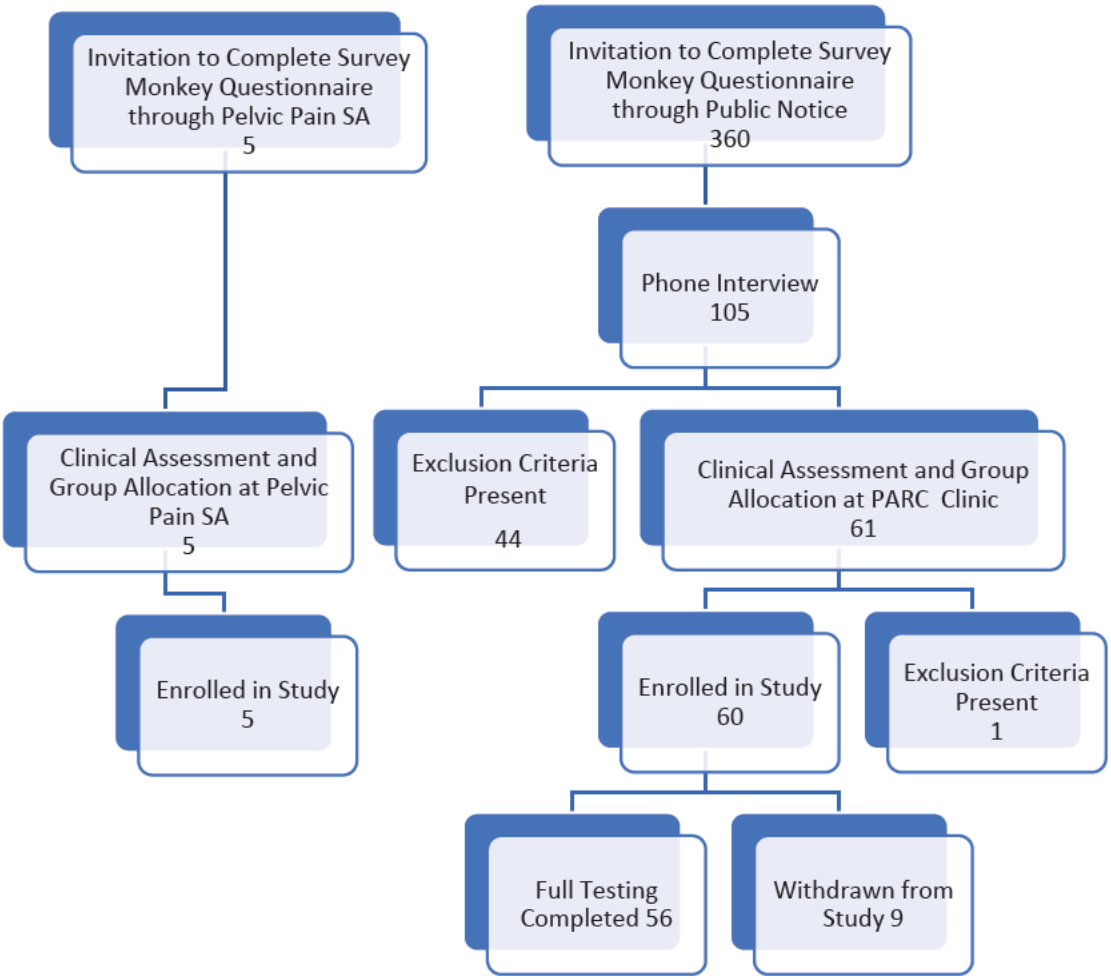

Figure 1. Enrolment flow chart for participant recruitment and study inclusion.

Laboratory Methods

Study visit schedule and specimen collection

Participants were assessed on two occasions during a single menstrual cycle: Day 1-2 and Day 7-10. At each study visit, participants attended the Pain and Anaesthesia Research Centre (PARC) of the Royal Adelaide Hospital, Adelaide, Australia. At their visit, participants were asked to confirm the absence of exclusion criteria, report the number of days of period pain and pelvic pain per month, and provide a blood sample for analysis. The use of anti-inflammatory medications was avoided for 5 drug half-lives prior to blood sampling.

Analysis of blood samples included: a measurement of high sensitivity C-reactive protein (hsCRP) to exclude the presence of un-recognised pre-test inflammation; a quantitative assessment of luteinising hormone (LH) and follicle stimulating hormone (FSH) to confirm baseline hormonal status on Day 1-2; a quantitative assessment of Sex Hormone Binding

Globulin (SHBG) (Healthscope Laboratories, Adelaide, Australia) to allow later calculation of the Free Androgen Index (FAI) and Free Estrogen Index (FEI); and an extended hormonal profile analysed as a single batch, using ultra-sensitive Liquid Chromatography Mass Spectrometry (LC-MS) (Anzac Research Institute, Sydney, Australia). The hormonal analysis included measurement of testosterone (T), dihydrotestosterone (DHT), dehydroepiandrosterone (DHEA), androstenedione (adione), 3α-androstanediol (3αdiol), 3β-androstanediol (3βdiol), estradiol (E2), estrone (E1), 17α-hydroxyprogesterone (17OHP4), progesterone (P4) and cortisol (C). Measurements for T, DHT, DHEA, adione, 3αdiol, 3βdiol, 17OHP4, P4 and C are measured in ng/ml. Measurements for E2 and E1 are in pg/ml. Our use of high-sensitivity liquid chromatography mass spectrometry (LCMS) steroid hormonal assays overcomes difficulties in androgen research in women associated with the low levels of androgens present in women compared to men. Hormonal analysis on both Day 1-2 (menstrual phase) and Day 7-10 (mid follicular phase) of a single menstrual cycle allows estimates of within-subject variability to be distinguished from between-subject and assay variability.

The IL-1β release from PMBCs across a range of concentrations of lipopolysaccharide stimulation of TLR4 receptors was used as a measure of TLR4 immune activation (Figure 2), and was determined using the technique developed by Kwok (15,16) and published in detail by Evans et al. (10). All blood samples were taken between the hours of 0830 and 1200.

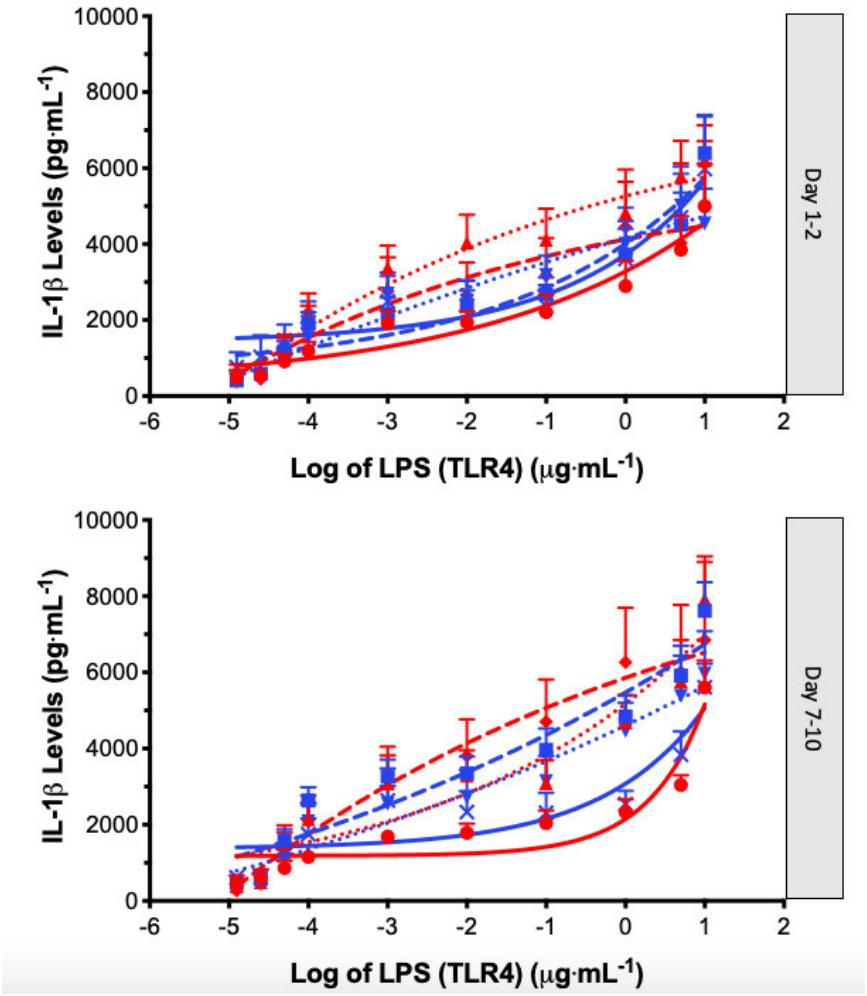

Figure 2. IL-1β release (Mean ± SEM) following LPS (TLR4) stimulation (12.5pg·mL to 10μg·mL) of PBMC's obtained from controls: no OC (red circle, solid line), controls: OC (blue square, solid line), dysmenorrhea: no OC (red triangle, dotted line), dysmenorrhea: OC (blue upside down triangle, dotted line), pelvic pain: no OC (red diamond, dashed line), and pelvic pain: OC (blue cross, dashed line). A four-parameter logistic dose-response curve has been fitted to each graph; data obtained from day 1-2 and day 7-10 of each individuals' menstrual cycle.

Statistical analysis

Determination of EC_{50}

Immune system TLR4-dependent reactivity is measured by determining the dose of LPS required to achieve a 50% of maximal IL-1β release response (EC_{50}) from peripheral blood mononuclear cells (PBMCs). Statistical modelling and a non-linear mixed-effects model approach was used to estimate the EC_{50} for each subject, at each testing timepoint (10). Concentration - response curves to LPS stimulation were fitted to an E_{max} model using the Hill equation (17), with the slope parameter fixed to 1 to reduce the number of parameters to be estimated. The model used was of the form:

$$E = E_0 + \frac{E_{max} * C}{EC_{50} + C}$$

where E_0 is the response Y at baseline (absence of dose), E_{max} is the asymptotic maximum dose effect (maximum effect attributable to the drug) and EC_{50} is the concentration which produces 50% of the maximal effect. Individual E_{max} models were fitted for each subject at each timepoint (Day 1-2 or Day 7-10).

All model fitting and all analyses were performed using Statistical Analysis Software (SAS v 9.4). Starting values were set with Max representing the maximum response observed for that participant at that timepoint, and Min representing the minimum response for that participant at that timepoint. From the model the differences in LS means for contrasts of interest were obtained with 95% confidence limits and relevant P-values by factor. The statistical analysis used is further described in our previous paper by Evans et al. (10).

A lower EC_{50} represents increased responsiveness of PBMCs to stimulation with LPS, a proxy measurement for increased immune system activation. A higher EC_{50} represents reduced responsiveness of PBMCs and reduced negative regulatory pressure and/or controls.

Determination of Free Androgen Index and Free Estrogen Index

The FAI was determined according to the formula: FAI = 100*testosterone/shbg

The FEI was determined according to the formula: FEI = 100*estradiol/shbg

Statistical Comparisons

Two-tailed non-parametric Spearman's correlations were undertaken to determine the p-value and Spearman's Rank Correlation Coefficient (r). Correlations were made between the levels of individual hormones, cortisol, the FAI, and the FEI, with the days per month of pelvic pain (DPelvicPM), period pain, (DPeriodPM), headache (DHeadachePM), and the EC_{50} for IL-1β release from PBMCs (Tables 1, 2, 3 and 4). Results were reported according to the use or non-use of the OC, and testing on Day 1-2 or Day 7-10 of the menstrual cycle.

RESULTS

Participant recruitment

Three hundred and sixty women completed the online Survey Monkey questionnaire. Following application of the exclusion criteria, 65 women were enrolled in the study. Nine women were excluded during the study due to a high pre-test level of C-reactive protein (1), use of marijuana (1), irregular menstrual cycles (3), non-attendance for testing (2), insufficiently severe pain (1), and development of an unrelated neurological illness (1). Fifty-six women aged between 16 and 35 years, with regular menstrual cycles, no previous pregnancy, normal body mass index (BMI) and good general health, apart from the presence of dysmenorrhea-related pelvic pain, completed the study. There were no differences in age ($p=0.52$), height ($p=0.52$) or weight ($p=0.68$) of the women in each group (one-way ANOVA and Kruskal-Wallis analysis).

Comparisons between hormonal levels and variables

Multiple correlations were made between hormone levels, pain indices (DPelvicPM, DPeriodPM, DHeadachePM) and *the* EC_{50} for LPS-induced IL-1β release (EC_{50})

Comparison between hormonal levels and Days of Pelvic Pain per Month (DPelvicPM)

Day 1-2: Women who were non-users of the OC showed a significant inverse correlation between the DPelvicPM and levels of androstenedione ($p=0.036$), DHEA ($p=0.0018$) and FAI ($p=0.0032$) (Table 1, Figure 3). The combined cohort of all participants showed a significant inverse correlation between the DPelvicPM and levels of testosterone ($p=0.021$), DHEA ($p=0.019$) and FAI ($p=0.0026$).

Day 7-10: Women who were non-users of the OC showed a significant inverse correlation between DPelvicPM and the FAI ($p=0.0058$). Women who were users of the OC showed a significant positive correlation between DPelvicPM and levels of estradiol ($p=0.049$) or the FEI ($p=0.049$) (Table 2, Figure 4). There was no effect of cortisol on DPelvicPM.

	DAY 1-2			DAY 7-10		
	No OC	OC	Combined	No OC	OC	Combined
DPELVICPM						
TESTOSTERONE	0.17	0.09*	0.021**	0.99	0.77	0.85
ANDROSTENEDIONE	0.036*	0.65	0.077	0.52	0.68	0.98
DHEA	0.0018**	0.63	0.019*	0.098	0.89	0.24
FREE ANDROGEN INDEX (FAI)	0.0032**	0.098	0.0026**	0.0058**	0.77	0.26
ESTRADIOL (E2)	0.30	0.45	0.15	0.068	0.049*	0.021*
FREE ESTROGEN INDEX (FEI)	0.50	0.34	0.72	0.71	0.049**	0.64
CORTISOL	0.36	0.18	0.27	0.47	0.35	0.40

Table 1: Comparison of significance (p value) between androgen, estrogen and cortisol levels and Days of Pelvic Pain per Month (DPelvicPM) according to Day of testing (Day 1-2 or Day 7-10) and OC use (No OC, OC, or Combined) describing significance (p value). *$p \leq 0.05$, **$p \leq 0.01$

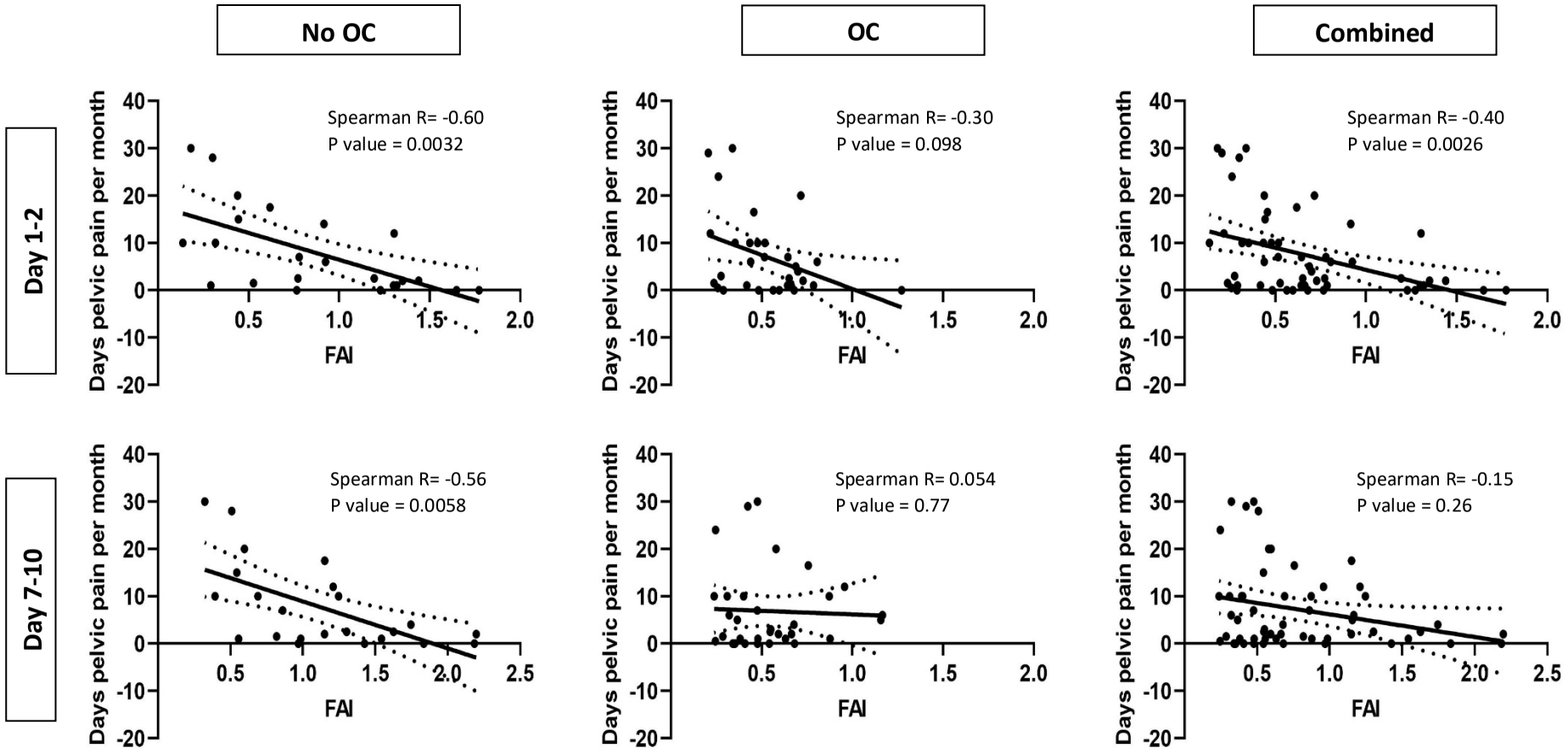

Figure 5.3: Comparison between DPelvicPM and the Free Androgen Index according to Day of testing (Day 1-2 or Day 7-10) and OC use (No OC, OC, or Combined).

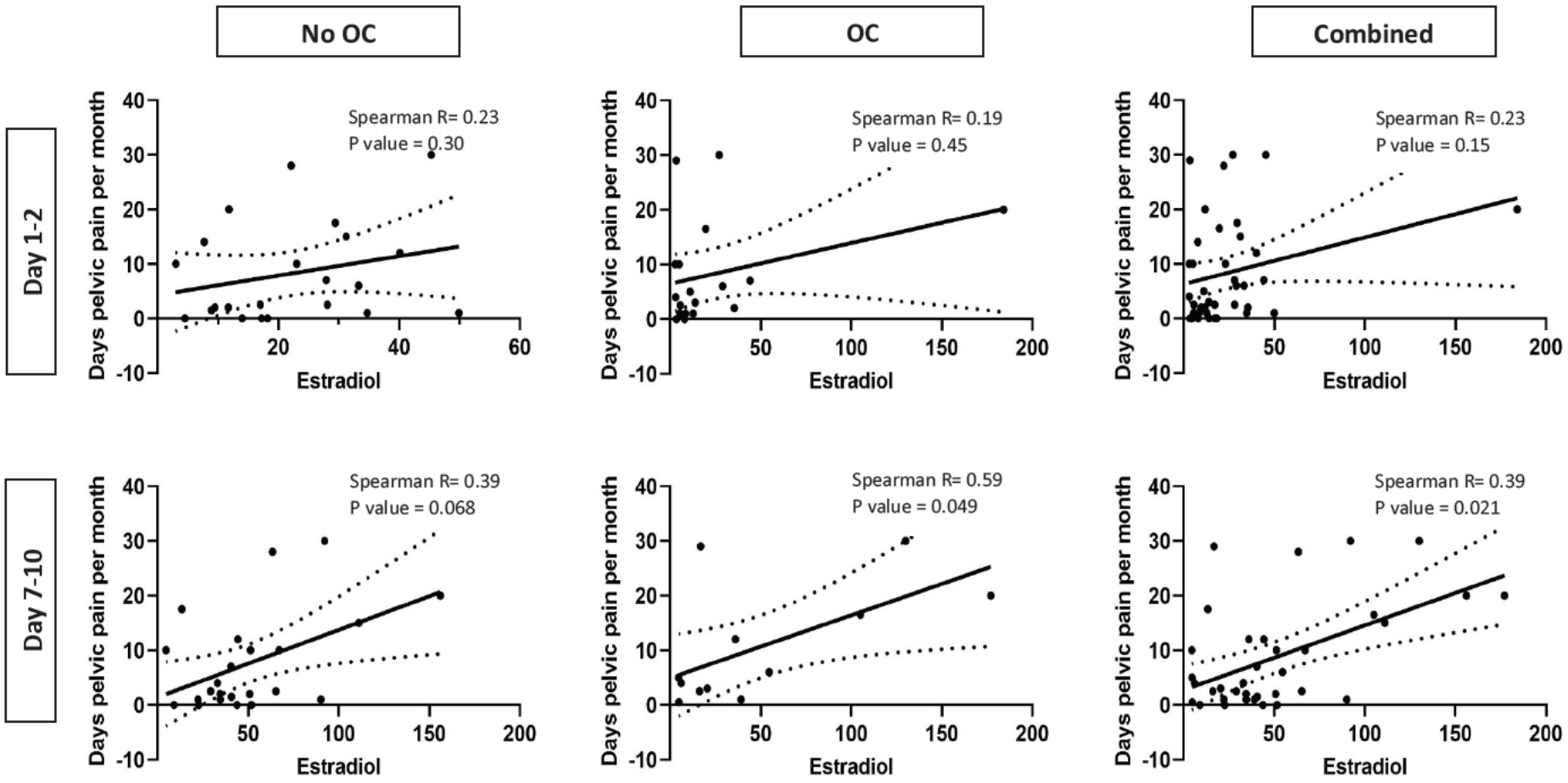

Figure 5.4: Comparison between DPelvicPM and Estradiol according to Day of testing (Day 1-2 or Day 7-10) and OC use (No OC, OC, or Combined).

Comparison between hormonal levels and Days of Period Pain per Month (DPeriodPM)

Day 1-2: Women who were non-users of the OC showed a significant inverse correlation between DPeriodPM and levels of androstenedione (p=0.035), DHEA (p=0.013) and FAI (p=0.013) (Table 2, Figure 5). The combined cohort of all participants showed a significant inverse correlation between DPeriodPM and levels of DHEA (p=0.060) and FAI (p=0.013).

Day 7-10: Women who were non-users of the OC showed a significant inverse correlation between DPeriodPM and FAI (p=0.029).
There was no effect of estradiol, FEI or Cortisol on DPeriodPM.

	DAY 1-2			**Day 7-10**		
	No OC	OC	Combined	No OC	OC	Combined
DPERIODPM						
TESTOSTERONE	0.24	0.13	0.072	0.85	0.44	0.61
ANDROSTENEDIONE	0.035*	0.65	0.077	0.45	0.87	0.62
DHEA	0.013*	0.81	0.060	0.33	0.96	0.53
FREE ANDROGEN INDEX (FAI)	0.013*	0.19	0.013*	0.029*	0.79	0.25
ESTRADIOL (E2)	0.37	0.73	0.34	0.20	0.23	0.17
FREE ESTROGEN INDEX (FEI)	0.98	0.54	0.84	0.74	0.099	0.75
CORTISOL	0.72	0.24	0.68	0.63	0.66	0.79

Table 2: Comparison of significance (p value) between Androgen, estrogen and cortisol levels and Days per Month of Period Pain (DPeriodPM) according to Day of testing (Day 1-2 or Day 7-10 of menstrual cycle) and OC use (No OC, OC, or Combined) describing significance (p value) and spearman coefficient (r value). *$p \leq 0.05$, **$p \leq 0.01$

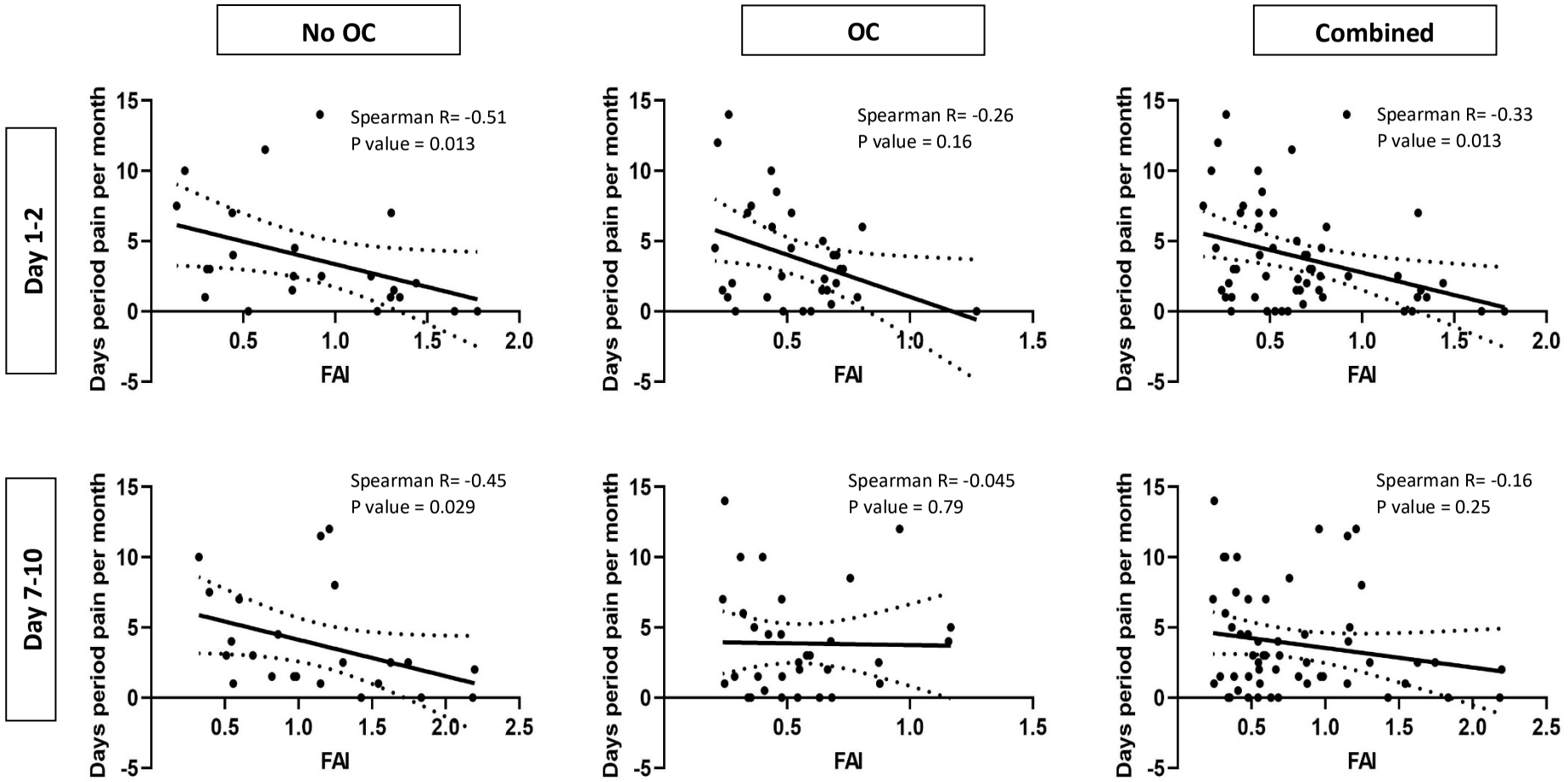

Figure 5.5: Comparison between DPeriodPM and the Free Androgen Index according to Day of testing (Day 1-2 or Day 7-10) and OC use (No OC, OC, or Combined).

Comparison between hormonal levels and Days of Headache per Month (DHeadachePM)

Day 1-2: Women who were users of the OC showed a significant inverse correlation between DHeadachePM and levels of testosterone (p=0.049) (Table 3). The combined cohort of all participants showed a significant inverse correlation between DHeadachePM and levels of testosterone (p=0.010), DHEA (p=0.035), and FAI (p=0.041).

Day 7-10: The combined cohort showed a significant inverse correlation between DHeadachePM and DHEA (p=0.047).
There was no effect of estradiol, FEI or Cortisol on DPeriodPM.

	DAY 1-2			**Day 7-10**		
	No OC	OC	Combined	No OC	OC	Combined
DHEADACHEPM						
TESTOSTERONE	0.13	0.049*	0.010*	0.54	0.99	0.70
ANDROSTENEDIONE	0.26	0.28	0.095	0.39	0.58	0.91
DHEA	0.14	0.23	0.035*	0.077	0.30	0.047*
FREE ANDROGEN INDEX (FAI)	0.20	0.079	0.041*	0.33	0.15	0.82
ESTRADIOL (E2)	0.61	0.93	0.69	0.78	0.38	0.78
FREE ESTROGEN INDEX (FEI)	0.88	0.73	0.42	0.92	0.83	0.68
CORTISOL	0.87	0.44	0.56	0.71	0.42	0.61

Table 3: Comparison of significance (p value) between androgen, estrogen and cortisol levels and Days per Month of Headache (DHeadachePM) according to Day of testing (Day 1-2 or Day 7-10 of menstrual cycle) and OC use (No OC, OC, or Combined) describing significance (p value). *$p \leq 0.05$, **$p \leq 0.01$

Comparison between hormonal levels,cortisol and EC_{50} for LPS-induced IL-1β release (EC_{50})

Day 1-2: The combined cohort of all participants showed a significant positive correlation between the EC_{50} of LPS-induced *in vitro* IL-1β release (increased responsiveness of PBMCs) and androstenedione levels (Table 4).

Day 7-10: Users of the OC showed a significant positive correlation between the EC_{50} of LPS-induced *in vitro* IL-1β release and levels of testosterone (p=0.042). Non-users of the OC showed a highly significant positive correlation between levels of cortisol and the EC_{50} of LPS-induced *in vitro* IL-1β release increased (p=0.0011, Spearman's coefficient r=+0.63). Users of the OC showed a highly significant inverse correlation between cortisol and the EC_{50} of LPS-induced *in vitro* IL-1β release (p=0.0080, Spearman coefficient r=-0.46) (Table 4, Figure 6). There was no significant effect of DHEA, estradiol, FAI or FEI on EC_{50}.

	DAY 1-2			**Day 7-10**		
	No OC	OC	Combined	No OC	OC	Combined
EC50						
TESTOSTERONE	0.28	0.71	0.29	0.83	0.042*	0.078
ANDROSTENEDIONE	0.080	0.29	0.044*	0.39	0.28	0.75
DHEA	0.39	0.40	0.23	0.66	0.37	0.81
FREE ANDROGEN INDEX (FAI)	0.20	0.87	0.26	0.44	0.13	0.62
ESTRADIOL (E2)	0.54	0.73	0.26	0.81	0.82	0.64
FREE ESTROGEN INDEX (FEI)	0.88	0.73	0.42	0.91	0.83	0.68
CORTISOL	0.87	0.70	0.55	0.0011** (r=+0.63)	0.0080** (r=-0.46)	0.38

Table 4: Comparison of significance (p value) between androgen, estrogen and cortisol levels and EC50 according to Day of testing (Day 1-2 or Day 7-10 of menstrual cycle) and OC use (No OC, OC, or Combined) describing significance (p value) and spearman coefficient (r value). *$p \leq 0.05$, **$p \leq 0.01$

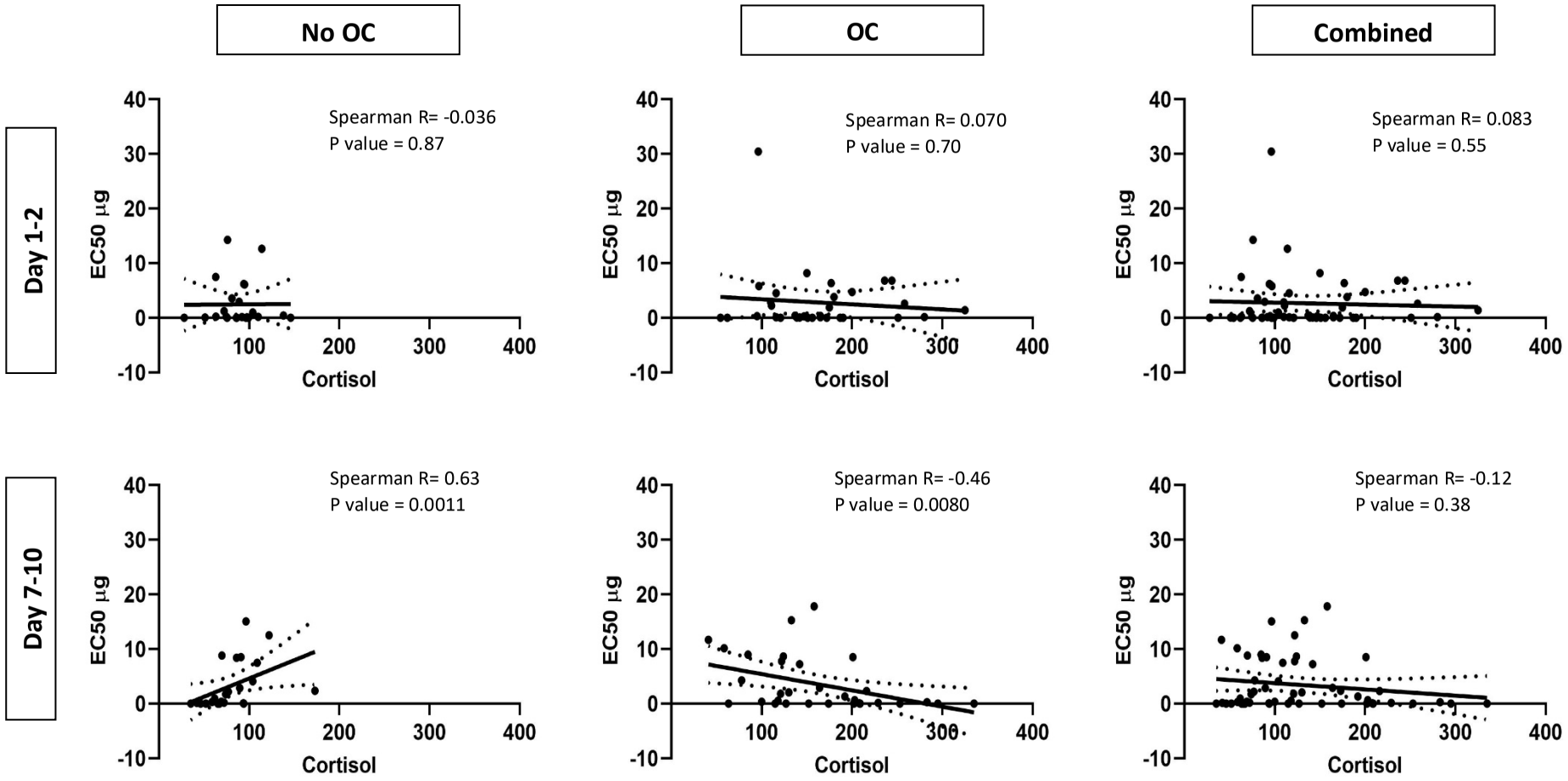

Figure 5.6: Comparison between EC_{50} for LPS-induced IL-1β release (EC_{50}) and Cortisol according to Day of testing (Day 1-2 or Day 7-10) and OC use (No OC, OC, or Combined).

Comparison of hormone and cortisol levels between users or non-users of the OC
A one-way ANOVA with Tukey's post hoc test was used to compare hormone levels in women who were users or non-users of the OC on both Day 1-2 and Day 7-10 (Table 5). In women who use the OC, the pill taken on Day 1-2 contains lactose only (lactose pill days). The pill taken on Day 7-10 contains ethinylestradiol in combination with a synthetic progestogen (hormone pill days).

Day 1-2: (lactose pill days) Use of the OC was associated with a significantly higher level of SHBG (p=0.0001) and cortisol (p=0.0001). Use of the OC was associated with a significantly lower level of DHEA (p=0.0043) and FAI (p=0.0013). There was no significant difference in testosterone, estradiol, or FEI.

Day 7-10: (hormone pill days) Use of the OC was associated with a significantly higher level of SHBG (p=0.0001) and cortisol (p=0.0001). Use of the OC was associated with a significantly lower level of androstenedione (p=0.0019), DHEA (0.0281) and FAI (p=0.0001). There was no significant difference in testosterone, estradiol or FEI.

The following hormones were present at levels too low for statistical analysis: estrone, progesterone, 17-hydroxyprogesterone, 3bdiol and estrone. Levels of DHT and 3alpha-androstanediol were not significantly associated with alteration in either symptoms or the EC_{50}.

When considering these results overall, lower levels of androgenic hormones, and particularly the calculated Free Androgen Index, were inversely associated with increasing DPelvicPM, DPeriodPM, and DHeadachePM. Higher estradiol levels on Day 7-10 (pill days), particularly in OC users, were associated with an increase in the DPelvicPM, but not the DPeriodPM, DHeadachePM. or EC_{50}. Weak associations were found between lower levels of androgen activity, and a lower EC_{50} consistent with a mildy enhanced immune responsiveness. Strong associations were found between cortisol levels and EC_{50} on Day 7-10 (pill days), with divergent direction of action according to use or non-use of the OC.

Discussion

The association between hormone levels and pain symptoms

Multiple lines of evidence (18,19) describe the excess of chronic pain conditions in females when compared to males. Pain research to date has predominantly considered ways in which the relative estrogen dominance in females may predispose females to chronic pain, as opposed to the ways in which the relative androgen dominance in males may protect males from these conditions. The physiological and potentially therapeutic roles of androgens in females has been under-researched. Our research found a highly significant inverse relationship between androgen levels, particularly a low FAI, and increasing pain symptoms, particularly in non-users of the OC. Oral contraceptive use results in both the suppression of testosterone production by the ovaries, and the induction of SHBG by the liver, with a consequently lower FAI (Figure 3).

Multiple mechanisms of action in both the periphery and the central nervous system support an inverse relationship between testosterone levels and pain. Studies of male-to-female transgender patients show an increase in chronic pain conditions following hormonal transition from an androgenic to an estrogenic hormonal environment (20,21), while female-to-male transgender patients show an improvement in pre-existing pain conditions following the administration of testosterone. Our research found a highly significant association between androgen levels and pain symptoms, particularly in women who were non-users of the OC, where the ovarian production of testosterone is unsuppressed and the production of SHBG within the liver is not enhanced.

In patients with Rheumatoid Arthritis, an inflammatory condition associated with reduced androgen levels in both males and females, there is increased production of proinflammatory cytokines within synovial cells (22). Within these cells, increased levels of IL-1β, tumour necrosis factor (TNF-α) and Interleukin-6 (IL-6) enhance the activity of aromatase (23), resulting in the increased conversion of testosterone to estradiol, increased levels of estrogen, and reduced levels of androgens (22,24–26). The disease-modifying effects of anti-TNF medications in patients with RA may act through the local enhancement of androgen activity (27).

Previous human studies have demonstrated the central nature of dysmenorrhea-related pelvic pain, its relationship to viscero-visceral hyperalgesia, and its association with pain co-morbidities including migraine (28–31). Within the dorsal horn of the spinal cord, it has been

proposed that androgens act in concert with estrogen to increase endogenous opioids following a nociceptive stimulus (32). White and Robinson proposed that a low testosterone level fails to induce sufficient endogenous opioids to dampen pain signals (33). Fibromyalgia, a centrally-mediated pain condition, is associated with reduced levels of androgens, and a strong association has been found between the days where symptoms were severe, and the days where testosterone levels were low (34). At the neuronal level, dihydrotestosterone, a metabolite of testosterone, provides neuroprotection against microglial inflammatory responses via suppression of TLR4, and inhibition of TNF-alpha and IL-1β production (35). Within the brain, Vincent et al. (36) found reduced activation of brain centres associated with descending pain inhibition, including the ventral rostral medulla, in healthy women with low androgen levels undergoing a cold thermal pain stimulus, despite no variation in the temperature required to induce pain.

In contrast with the robust association with androgenic activity, our research found a modest correlation between estradiol levels on Day 7-10 and pain symptoms. Our findings are consistent with established clinical practice, where lower estrogen OCs or estrogen suppression is prescribed to reduce the frequency or volume of menstrual bleeding (37). The oral contraceptive pill has known effectiveness at the level of the uterus: reducing menstrual flow (38), and reducing prostaglandin release (39,40). It is yet to be determined whether the OC-induced androgen suppression and SHBG induction may have unintended negative effects on the development of central pain sensitization.

The association between hormone levels and EC_{50} *of LPS-induced IL-1β release from PBMCs*

Activation of the innate immune system via TLR4 is known to be involved in the initiation and maintenance of chronic pain (8,9,41,42), with activation showing as increased responsiveness of immune cells to TLR stimulation. Our group has already demonstrated an increased responsiveness of PBMCs to TLR4 activation with LPS within this group of women with dysmenorrhea-related pelvic pain, compared to pain-free controls (10). Our finding of a modest association between reduced androgen activity and a lower LPS-induced IL-1β EC_{50} (increased immune activation) suggests that activation of the immune system via TLR4 comprises one factor affecting pain symptoms, but that immune mechanisms outside the hormonal environment are also involved. It also suggests that the ability of hormonal manipulation to manage chronic pelvic pain is limited. Our study found no significant effect of estrogens on EC_{50}. This is consistent with the research of Bouman et al. (43) who found that neither estradiol nor progesterone influenced the release of cytokines from monocytes in humans. However, they did not assess the effect of androgens.

The association between cortisol and EC_{50} *of LPS-induced IL-1β release from PBMCs*

Our finding of a highly significant association between increased levels of cortisol (a suppressor of inflammation) and an increased EC_{50} of LPS-induced IL-1β release (reduced activation of the innate immune system) in non-users of the OC is consistent with an inflammation-based model of chronic pain. The divergent effect in OC users, where higher levels of cortisol were associated with a lower EC_{50} of LPS-induced IL-1β release (increased activation of the innate immune system) is a potential cause for concern to health care providers, owing to the known association between immune activation and chronic pain. Our findings are consistent with those of Vincent et al. (44) who described a significantly lower mean level of cortisol in women with dysmenorrhea where all participants were non-users of the OC, suggesting an impairment of the immune response. Use of the OC has already been associated with a reduced cortisol response to cold-pressor testing and emotional memory responses (45–47).

Measurement of the FAI within clinical practice

The majority of testosterone within the circulation is protein-bound to either SHBG or albumin, leaving a relatively small percentage of testosterone in its unbound, bioavailable state. While there are multiple cellular mechanisms involved, it is the unbound testosterone fraction which contributes maximally to its clinical effects. The Free Androgen Index (FAI) provides a simple and easily calculated estimate of androgen activity, requiring only a measurement of total testosterone and SHBG. FAI results are less reliable as an indicator of testosterone activity in populations where SHBG levels are below 30nmol/l, such as in males or women with Polycystic Ovarian Syndrome, obesity, or insulin resistance (48). For these reasons, the Calculated Free Testosterone (cFT) is preferred in some laboratories. The cFT incorporates albumin levels, and utilises the more complex Vermeulen Equation (49), to provide an estimate which more closely matches the results obtained by dynamic equilibrium testing in these groups. Our research excluded women with a BMI > 30, and women with irregular menstrual cycles. The FAI is therefore an appropriate test within our study population.

Translational potential

Our key research finding of an inverse association between androgen levels and pain symptoms, both within and outside the pelvis, offers a new approach to the management of pain in women. Multiple androgen-enhancing options are already available. Progestogen-only contraceptive users avoid the estrogen-induced rise in SHBG associated with combined OCs, and Máximo et al. (50) demonstrated that women using progestogen-only contraception have a higher pain threshold than women using the combined OC. Intrauterine contraceptive

devices maintain natural ovarian testosterone production by avoiding ovarian suppression, and their estrogen-induced rise in SHBG. When considering the further development of OC formulations, an optimal OC for a woman with pain might comprise an estrogen with minimal induction of SHBG, and a progestogen with minimal anti-androgen activity. Conservation of the ovaries at the time of hysterectomy maintains gonadal production of testosterone in women where post-surgical testosterone replacement is not anticipated (51).

Testosterone therapy is recommended to reduce pain perception and fatigue in women with opioid-induced androgen deficiency (OPIAD) (52), and White et al. reduced tender points and stiffness in women with fibromyalgia by using testosterone gel (33). However, the role of androgen replacement in the absence of opioid use in women with low androgen levels and chronic pain is less established. Where levels of estrogen or progestogen are low, these are replaced to conserve bone density and prevent endometrial hyperplasia. The supplementation of testosterone to prevent fatigue, improve sexual function, or reduce chronic pain, anxiety and low mood, has been under-utilised. In women using hormonal replacement, the estrogen-induced rise in SHBG may further reduce androgen activity through increased testosterone binding. Our study also raises potential concerns regarding the profound suppression of androgens associated with the use of gonadotrophin releasing hormone agonist or antagonist medications over prolonged periods of time in women with pain.

Optimal testosterone levels for the reduction of pain have yet to be determined. Huang et al. (53) researched the effect of increasing doses of testosterone over 24 weeks in hysterectomized and oophorectomized women compared to placebo. They observed a significant dose response relationship between free testosterone levels and the Psychological General Well-Being Index (PGWBI) score, lean body mass, and stair climbing power when compared to placebo, but only where supra-physiological levels were achieved. Fasting glucose, lipid profile and liver function tests remained unchanged from baseline following testosterone administration, and did not differ according to dose. Their study considers the use of testosterone as a pharmacological treatment, rather than a physiological replacement. The future development of selective androgen receptor modulators (SARMs) offers the potential of androgen effect without the potential side effect of virilization.

Our study raises substantial further research questions relating to the use of testosterone therapy to reduce pain or enhance quality-of-life in women with current endometriosis lesions. The aromatase-mediated conversion of testosterone to estradiol within endometriosis lesions has the potential to promote lesion growth. The optimal testosterone regime for these women, and the potential for testosterone to be used in combination with an aromatase inhibitor

medication to prevent the conversion of testosterone to estrogen (54), has yet to be fully explored.

Our study has notable strengths, including the stringent exclusion criteria used, the lack of clinical co-morbidities or confounding medications in this young, healthy patient group, and the accurate measurement of steroid hormones at low levels, using liquid chromatography mass spectrometry methods in a single batch. Another strength is the sampling at 2 stages of a single menstrual cycle in each participant. A potential study weakness is the use of blood hormonal assays rather than tissue hormonal assays. As shown in the synovial fluid of patients with rheumatoid arthritis (55), or the intralesional fluid in women with endometriosis lesions, the enzyme aromatase allows a local hormonal concentration of estradiol that may be many times greater than blood levels (56). Another weakness is the heterogeneity of participants within the oral contraceptive group. This group includes participants using a range of different oral contraceptive formulations for a range of clinical indications.

Conclusion

Our study provides new insights into the way the hormonal environment affects the experience of pain in women, using clinical variables that are readily determined in a primary care setting: the DPelvicPM, the DPeriodPM, and the DHeadachePM. Of the tests undertaken in our cohort of young women with normal BMI and regular menstrual cycles, the FAI proved to be a consistent indicator of difference.

Our findings support the concept that the female predominance of chronic pain relates more to the protection men receive from higher testosterone levels, than the risk women incur from higher estradiol levels. The potential for use of the OC to reduce menstrual symptoms, yet increase immune activation, with increased potential for the development of chronic pain, requires further research. If confirmed, this has widespread implications for the management of dysmenorrhoea-related pelvic pain in young women.

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AUTHOR CONTRIBUTIONS

Study design, acquisition of data, preparation of data: SFE, YHK, PR, LH, MH
Analysis of data: SFE, YHK, AS, CEP
Interpretation of data: SFE, PR, LH,
Preparation of article: SFE, CEP, PR, LH, MH

DISCLOSURE

Susan F. Evans receives royalties from book authorship, has received payment from Pfizer and Bayer for educational presentations, is a shareholder in Alyra Biotech and Havah Therapeutics, and is involved in the development of novel treatments for pelvic pain. Paul E. Rolan is a shareholder in Havah Therapeutics, Alyra Biotech, Lipotek and iXBiopharma, a consultant to Bionomics and Novartis, and has received payment for educational presentations from Novartis and Sequirus. Mark R. Hutchinson is director of the Australian Research Council Centre of Excellence for Nanoscale BioPhotonics (CE140100003) and the recipient of an ARC Future Fellowship (FT180100565). The present study was funded in part by the Anaesthesia and Pain Medicine Foundation, Australia and New Zealand College of Anaesthetists (Grant 15/013), and the Australian Research Council (FT180100565). Its contents are solely the responsibility of the authors and the funders had no role in the study design, data collection and analysis, decision to publish, or preparation of the manuscript.

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5.5 CONCLUSIONS

The research outlined in Chapter 5 supports a role for the hormonal environment, particularly androgens, in the subjective experience of pain.

Hypothesis 1, that the hormonal profile of an individual is associated with variation in the days per month of pain experienced was confirmed. These findings were predominantly in women who were non-users of the OC, and thus experiencing their own natural ovarian hormonal cycles. They were also predominantly with regard to androgen, rather than estrogen activity. An increased number of DPelvicPM was inversely correlated with levels of androstenedione, DHEA and FAI on Day 1-2, and the FAI on Day 7-10. An increased number of DPeriodPM was inversely correlated with levels of androstenedione, DHEA and FAI on Day 1-2, and FAI on Day 7-10 in non-users of the OC. An increased number of DHeadachePM was inversely correlated with the FAI on Day 1-2 in users of the OC, and in the larger combined group on both Day 1-2 and Day 7-10. These findings provide a potential novel management option for the reduction of pain symptoms in women: androgen supplementation.

Our findings of an increase in pain symptoms with lower androgen levels is consistent with the study by Aloisi et al. (2007) which investigated pain symptoms in 47 male-to-female, and 26 female-to-male, transgender patients 12 months following gender reassignment. Aloisi assessed the frequency of pain symptoms within individuals under both estrogen-dominant and androgen-dominant hormonal conditions. Fourteen of the 47 male-to-female participants (30%) reported the presence of a painful condition, which was not present prior to the initiation of estrogen hormonal therapy. Sixteen of the 26 female-to-male participants (61%) reported a painful condition prior to the initiation of testosterone therapy, with 11 of these participants reporting an improvement in pain following hormonal transition to an androgen-dominant hormonal environment.

Testosterone supplementation for the clinical management of fibromyalgia, a centrally-mediated condition associated with widespread pain, has been investigated by White and Robinson (2015). They have proposed a potential mechanism for the association between low levels of androgens and pain symptoms. In healthy individuals, the release of Substance P by nociceptive neurons is increased following either a painful or stressful experience, resulting in reduced serotonin levels and impaired wellbeing. The raised Substance P stimulates aromatase within the central nervous system, with increased conversion of testosterone to estrogen, an estrogen-mediated upregulation of endogenous opioids, and a reduction in

symptoms (Figure 5.1.a). In patients with fibromyalgia, reduced levels of testosterone provide insufficient substrate for aromatase following the release of Substance P, resulting in inadequate levels of estradiol, reduced production of endogenous opioids, and the clinical symptom of pain (Figure 5.1.b).

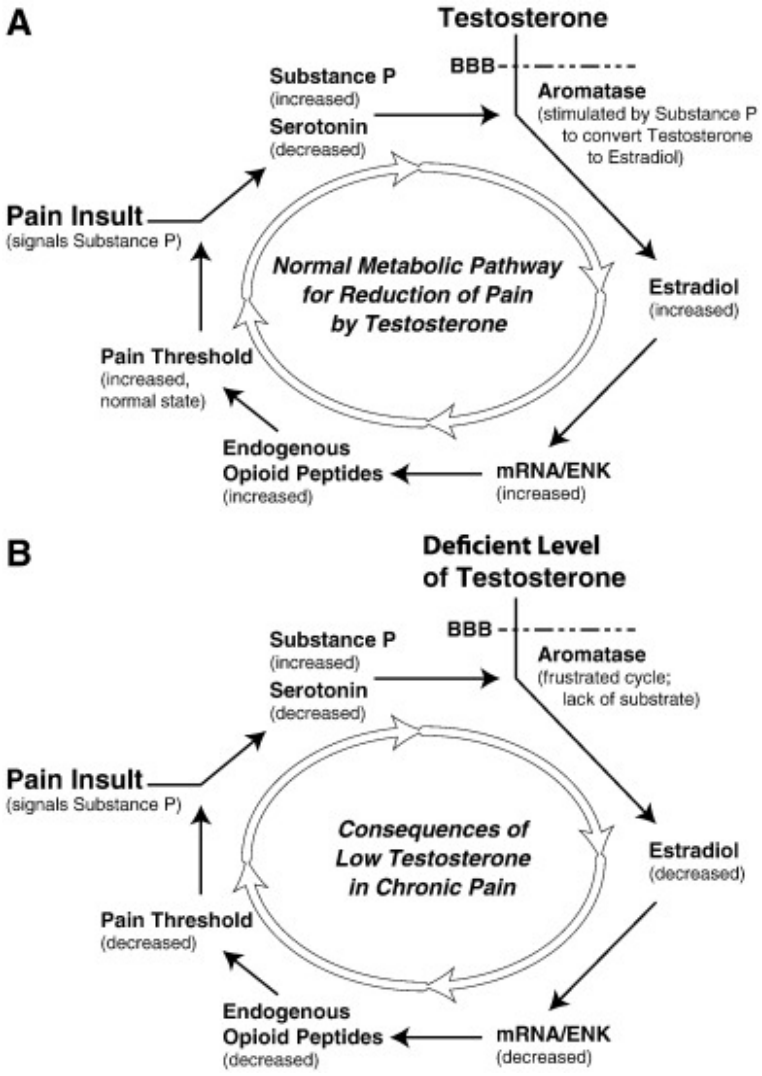

Figure 5.1.a and 5.1.b: Hypothesised signaling pathway for testosterone in relation to nociception in the CNS (White & Robinson, 2015)

The finding that testosterone supplementation can be utilised in the management of opioid overuse (O'Rourke & Wosnitzer, 2016; Smith & Elliott, 2012) further supports a central mechanism for testosterone effect, and an interaction with opioid mechanisms. Individuals who use regular opioids show lower basal testosterone levels than non-users (Abs et al., 2000; Finch, Roberts, Price, Hadlow, & Pullan, 2000; Malik, Khan, Jabbar, & Iqbal, 1992; Rasheed,

& Tareen, 1995), although whether low androgen levels precede, or follow, the use of regular opioids remains unclear.

Hypothesis 2, that the hormonal profile of an individual is associated with changes in the responsiveness of PBMCs to TLR4 stimulation, as measured by the EC_{50} for IL-1β release remains inconclusive. A modest positive correlation between the EC_{50} and levels of androstenedione on Day 1-2 and testosterone on Day 7-10 was found in the combined group of participants. However, the lack of a clear outcome suggests that multiple mechanisms affect immune responsiveness. Androstenedione is a weak androgen, and a precursor for both estrone and testosterone. Our finding of complex results with unclear implications when considering the effect of steroid hormones on the innate immune response is consistent with the experience of other researchers. Hewagama et al. (2008), described both estrogen- and androgen-response elements on the promotor segments of innate immunity genes, including those affecting female T-cells suggesting a role for hormonal mechanisms in pain. However, Asai et al. (2001) found that females have fewer TLR4 receptors on PBMCs than males, and a lower level of TNF-α secretion following LPS stimulation, seemingly inconsistent with the known female preponderance in pain conditions. While this may suggest reduced activation of the immune system in females, Klein et al. (2016b) found higher CD4+ T cell counts, CD4/CD8 ratios and macrophage phagocytic activity in females than males. Bouman et al. (2005) proposed that estradiol has multiple and opposing effects on human immune cells: low doses increase the production of pro-inflammatory cytokines and induce a pro-inflammatory phenotype, whereas high concentrations reduce inflammatory cytokines and induce a low inflammatory phenotype. Asai (2001) postulated that estradiol modulates cytokine secretion in females, and that the mechanism behind the higher prevalence of pain in women involves non-circulatory mechanisms.

Androgens have been clearly shown to reduce immune activation and reduce the production of cytokines in rodent studies. Rettew et al. (2008) demonstrated a reduction in TLR4 expression, and reduced sensitivity to TLR4 stimulation in the macrophages of mice treated with testosterone. Benten et al. (2004) demonstrated that this testosterone-induced, down-regulation of LPS signalling occurs via nongenomic Ca^{2+} signalling, providing a further complexity in the relationship between testosterone, TLR mechanisms and immunity. Yang et al. (2020) showed that DHT (a potent metabolite of testosterone) inhibited the production of prostaglandin E2 through the suppression of cyclo-oxygenase 2. DHT also inhibited the TLR4/NF-κB signaling pathway with a reduction in the production of proinflammatory factors including PGE2, TNF-α and IL-1β from microglial cells in the central nervous system. This

immune suppression with subsequent reduction in the formation of PGE2 provides a potential mechanism for our finding of an inverse relationship between the FAI and DPeriodPM.

Hypothesis 3, that the FAI provides a clinically useful measure of androgen activity, inversely correlated with the presence of pain symptoms, was confirmed. The FAI is a simple laboratory test, suitable for clinical use in primary care provided that the SHBG level is above 30, and obesity is absent. Current clinical management of dysmenorrhoea and pelvic pain relies heavily on the manipulation of the steroid hormone profile to suppress menstruation from an early age (Harada et al., 2008; Larsson et al., 1992; Lindh & Milsom, 2013; Milsom et al., 1990). This results in suppression of the FAI via a reduction in testosterone and an increase in SHBG. From the perspective of a patient, this effect may enhance their perceived benefits. For example, the suppression of androgens is associated with a reduction in acne, and a reduction in unwanted body hair (Fraser & Kovacs, 2003). However, while hormonal suppression of menstruation is effective at reducing the number of menses experienced, or the heaviness of menstrual flow, there exists the possibility that by reducing androgen activity, an individual may increase their risk of developing chronic pain conditions, including CPP.

From the perspective of a primary care clinician, the knowledge that low androgen levels may contribute to an increase in pain symptoms is of immediate translational clinical importance. As outlined within the published article, multiple options currently exist to either maintain endogenous androgen levels, or to supplement testosterone levels, through exogenous hormone therapy.

CHAPTER 6: ACTIVATION OF THE INNATE IMMUNE SYSTEM VIA TOLL-LIKE RECEPTOR4 AS A UNIFYING HYPOTHESIS FOR DYSMENORRHOEA, CHRONIC PELVIC PAIN AND ENDOMETRIOSIS

"One of the greatest pains to human nature is the pain of a new idea"

Walter Bagehot, Physics and Politics, 1869

In view of the shared features of association with menstruation, co-occurrence in time and symptomatology, and activation of TLR4 and the innate immune system, this thesis puts forward a new theory for the relationship between dysmenorrhoea, CPP and endometriosis. It is proposed that these conditions be considered as three phenotypic variants, arising from a common underlying mechanism (Figure 6.1). Our research proposes that this underlying mechanism involves the activation of Toll-Like Receptors within the uterus, with symptoms presenting clinically once hormone levels rise at puberty.

With regard to dysmenorrhoea, it is proposed that pre-menarchal activation of TLR4 primes an individual to experience dysmenorrhoea once menstruation begins, through the TLR4-mediated stimulation of cyclo-oxygenase-2 and consequent PGE2 production. PGE2 results in increased myometrial contraction with an increase in menstrual pain. A pre-menarchal timing for the activation of TLR4 is consistent with the onset of dysmenorrhoea soon after menstruation begins, and the pain symptoms experienced by a proportion of girls before menarche.

With regard to CPP, it is proposed that intrauterine TLR4 activation within the uterus results in an increase in local cytokines, including IL-1β, IL-6 and TNF-α. This has two effects. Firstly, local cytokines sensitise nociceptors associated with sensory afferent neural pathways as described in Figure 1.21. This results in the activation of spinal glia and the transition from dysmenorrhoea to CPP, and is consistent with the research by Kwok et al (2014) in a rodent model. The shared spinal segments involved and the potential for antidromic inflammatory processes provide an explanation for the clustering of bowel and bladder symptoms with dysmenorrhoea described in Chapter 2. The activation of glial cells within the spinal cord provides a potential explanation for the presence of systemic symptoms such as headache, fatigue, anxiety, low mood, poor sleep and nausea.

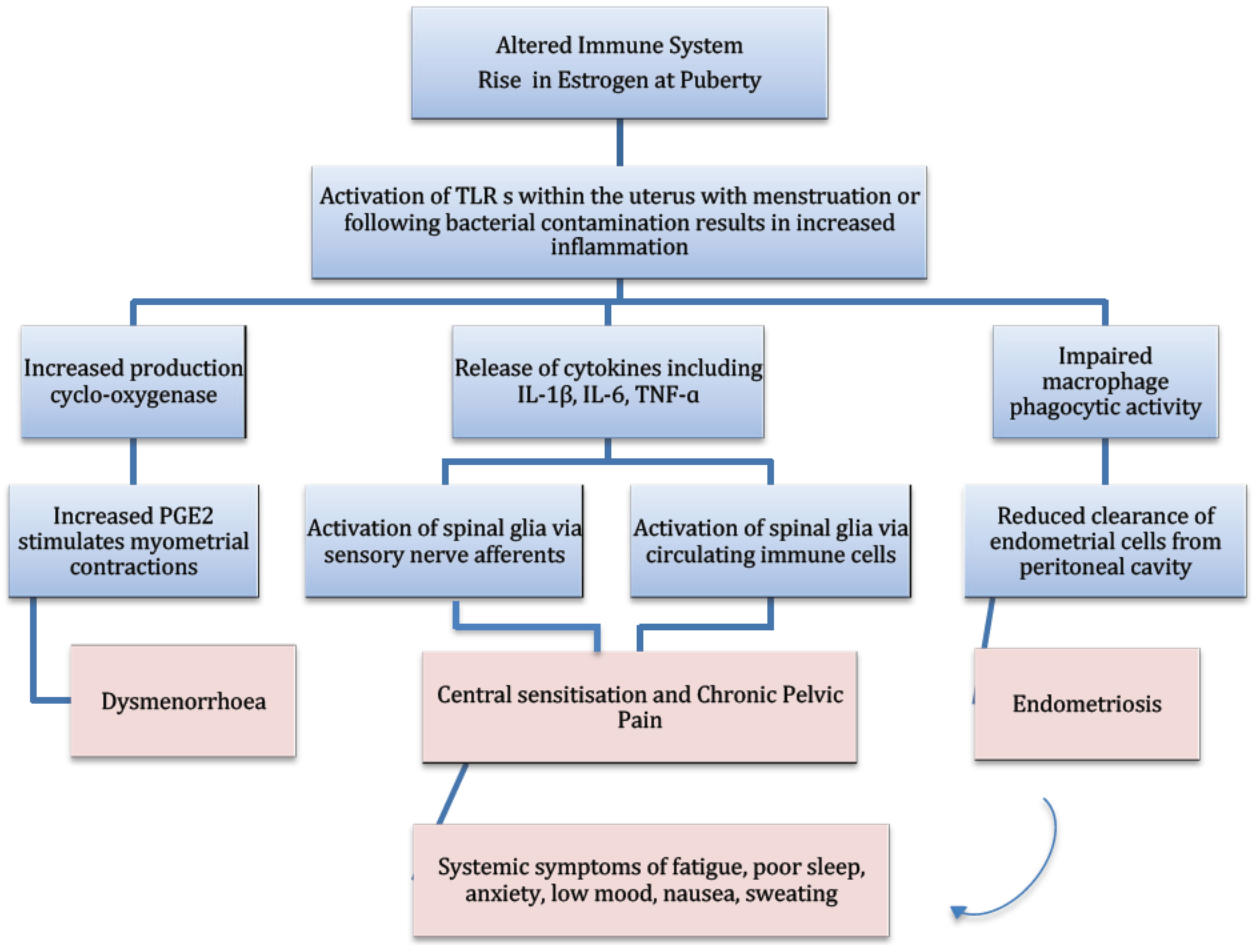

Figure 6.1: Pictorial description of the proposed Unifying Theory for dysmenorrhoea-related pelvic pain where a common underlying inflammatory mechanism, acting via TLR activation, results in one or more phenotypic variants.

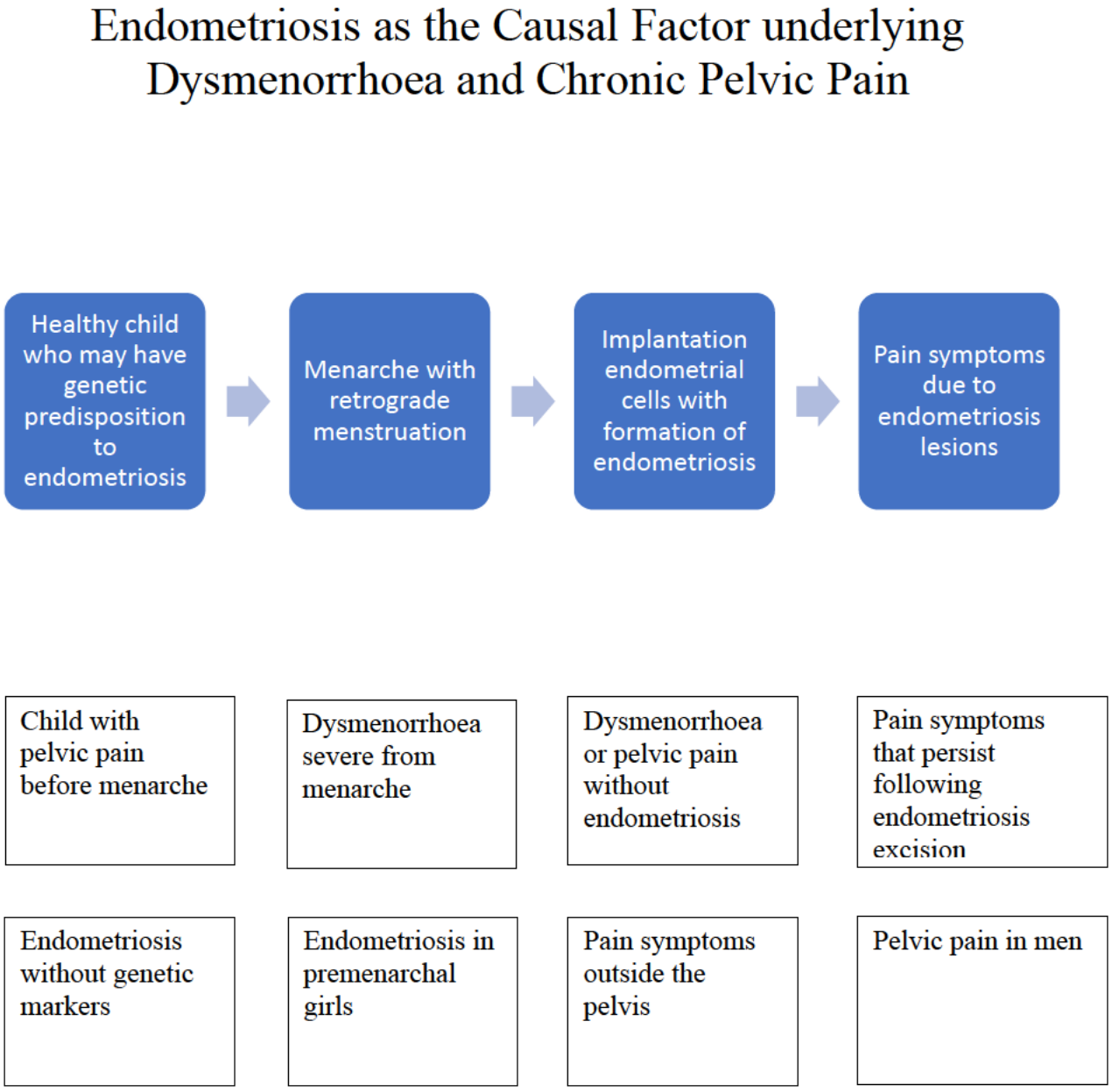

Figure 6.2: Pictorial description of a mechanism for pain where endometriosis lesions are the causative factor (blue boxes). The white boxes describe clinical situations not adequately explained by this theory.

Secondly, local cytokines within the uterus activate circulating immune cells, including PBMCs, increasing their responsiveness to further TLR4 stimulation. This is consistent with our findings in Chapter 3. The free flow of activated immune cells from the circulation to the brain and spinal cord further enhances sensitisation of the central nervous system. Regular menstrual cycles result in repeated episodes of immune activation, and the progression from dysmenorrhoea to CPP. This is consistent with the research by Hardi et al., describing the transition from dysmenorrhoea to CPP in women over time. Factors known to modify the immune response, including higher levels of androgens, modify the pain experience of individuals, as outlined in Chapter 5.

With regard to the formation of endometriosis lesions, it is proposed that a TLR4-mediated impairment of macrophage function results in the reduced clearance of menstrual debris following retrograde menstruation. This facilitates endometriosis lesion formation. Lesion development may be further enhanced by the antidromic spread of central inflammatory processes via neuronal pathways. A pre-menarchal timing for the activation of TLR4 is consistent with Marsh and Laufer's (2005) finding of endometriosis lesions in a proportion of pre-pubertal girls with pain.

Although not addressed in this thesis, TLR4 activation within the uterus with the consequent increase in local inflammation, may also contribute to the known association between the presence of endometriosis, and an increased risk of infertility and miscarriage (Pallacks et al., 2017).

The Unifying Theory offers a distinct change from the current concept of endometriosis lesions as the major causative factor for pain symptoms (Figure 6.2), which is rejected by the findings of this thesis. From a clinical perspective, endometriosis lesions become a common, but non-essential, feature of *Dysmenorrhoea-Related Pelvic Pain Syndrome (DRPPS),* and DRPPS assumes an umbrella role for the cluster of symptoms and conditions commonly experienced by women with DRPP. In this way the relationship between endometriosis and DRPPS may be likened to the relationship between polycystic ovaries and Polycystic Ovarian Syndrome (PCOS). While polycystic ovaries were initially considered to be a purely ovarian condition (Evans & Riley, 1958), PCOS now includes symptoms as diverse as hyperlipidaemia, insulin resistance, hirsuitism, and acne. The symptoms of PCOS cluster together, and polycystic ovaries are frequently found, but the presence of cystic ovaries is no longer essential for the diagnosis (Kauffman et al., 2008).

Our theory easily encompasses the wide range of symptoms found in clinical practice, and therefore has direct, translational, clinical relevance. It explains the situation where pelvic pain is severe, yet endometriosis lesions are minimal or absent, where pain is minimal, yet endometriosis lesions are widespread, and where pain persists following the surgical removal of endometriosis lesions. Individuals may vary in their phenotypic presentation depending on the relative contribution of each of the three pathways described (Figure 6.1).

Evolution reduces the frequency of disadvantageous genetic human traits, particularly those with potential to reduce fertility, such as endometriosis. That these conditions remain frequent in women throughout the world, suggests that a genetic predisposition to these conditions confers certain survival benefits. A reduced mortality from infectious disease, including post-partum sepsis, associated with an enhanced immune response, offers such an advantage. However, this survival advantage comes at the price of increased auto-immune disease and increased chronic pain. In previous generations, the disadvantages of enhanced intrauterine activation of the immune system were modified by a later menarche, early and multiple pregnancies, and thus fewer lifetime menses. Additional environmental factors associated with the modern era, offer an additional mechanism for increased TLR4 stimulation.

This thesis has taken the first steps towards developing and investigating a novel and comprehensive theory linking dysmenorrhoea, CPP and endometriosis.

CHAPTER 7. FUTURE DIRECTIONS

This thesis has progressed the evidence for an entirely new theory for the relationship between dysmenorrhoea, chronic pelvic pain and endometriosis. It has taken the first steps towards establishing a role for TLR4 in the initiation and experience of dysmenorrhoea and CPP.

7.1 RECOMMENDED TOPICS FOR FUTURE STUDY

Female visceral pain remains an under-researched area with regard to its human impact. Multiple potential further lines of research arose while conducting this research. The following topics comprise areas of particular interest for future research.

7.1.1 DETERMINATION OF THE AGE AT WHICH PRIMING OF THE INNATE IMMUNE SYSTEM OCCURS IN FEMALES.

Early life events are known to affect the developing brain (Stolp et al., 2005). An association between exposure to adverse early life events, including infections, and the subsequent development of neuropsychiatric conditions, including pain, anxiety and depression, has been demonstrated in animal and human models (Benmhammed et al., 2019; Giridharan et al., 2019; Kannampalli et al., 2017; Liu et al., 2017; Palma et al., 2016; Schwarz et al., 2011). There exists the potential for early life events, potentially even pre-birth, to predispose to dysmenorrhoea, chronic pelvic pain or endometriosis, by providing a priming event that sensitises the individual to future pain when an appropriate stimulus such as the onset of menstruation occurs.

7.1.2 THE ROLE OF GENETIC TLR4 POLYMORPHISMS IN PREDISPOSING FEMALES TO DYSMENORRHOEA, CPP OR ENDOMETRIOSIS.

TLR polymorphisms are common around the world. Genetic variants are already associated with reduced steroid responsiveness (Kim et al., 2020), and a range of other medical conditions (Ferwerda et al., 2008; Figueroa et al., 2012; Koval et al., 2018; Pirahmadi et al., 2013; Sharma et al., 2016; Sharma et al., 2019; Singh et al., 2015; Vijay, 2018; Witte et al., 2014). An association between TLR4 polymorphism with endometriosis has been demonstrated (Latha et al., 2011), but the relationship with dysmenorrhoea and CPP

remains to be determined. Where an association between TLR4 polymorphisms and female visceral pain is found, do these polymorphisms affect uterine immune activation, the responsiveness PBMCs to LPS stimulation, or the activation of spinal glial cells?

7.1.3 The role of other Toll-Like Receptors in female pain

Each Toll-Like Receptor responds to a unique ligand, as described in Figure 1.15. TLR4, the classical TLR researched in human pathology, is but one of the TLRs capable of immune activation in humans. Recent research has showcased the potential importance of non-TLR4 mechanisms. For example, plasmacytoid dendritic cells produce Type 1 Interferons (IFN-α/β) following the activation of TLR7 and TLR9 by viral nucleic acid (Seillet et al., 2012b). A viral ligand, present at an early life stage, is a potential contender for immune priming in girls. Seillet demonstrated an increased TLR7 response in female mice when compared to male mice, and in post-menopausal women treated with estradiol, demonstrating both gendered and hormonal influences worthy of further investigation. Inhibitors of TLR8 reduce TNF-α production within the synovial cells of patients with rheumatoid arthritis (Sacre et al., 2008).

7.1.4 The role of dendritic cells within the uterus as antigen-presenting cells

Intrauterine immune activation relies on the effective presentation of microbial elements to T-cells. Dendritic cells are a component of the innate immune system, and well placed to efficiently enhance the immune stimulus, through their ability to attach to bacterial particles, and presented them appropriately to T cells (Ueno et al., 2007). An understanding of the intrauterine factors affecting dendritic cell function offers potential for the development of novel, intrauterine, translational, immune-modulating treatment options.

7.1.5 The role of eosinophils in female inflammatory pain

All TLRs, except TLR8, are expressed to a variable degree by eosinophils (Kvarnhammar & Cardell, 2012), and bronchial asthma is more frequent among women with endometriosis (Sinaii et al., 2002; Smorgick et al., 2013). Recent evidence has determined that TLR4 activation of eosinophils promotes an inflammatory phenotype (Yoon et al., 2019), providing an alternative TLR-mediated mechanism for female pain, and a further translational mechanism for novel immune-modifying therapies.

7.1.6 The relative role of circulatory immune cell activation, versus intrauterine sensory neuron activation in TLR-mediated female visceral pain.

Our research investigated the immune response of PBMCs. However, the activation of spinal glia may also occur through the TLR4-mediated activation of sensory afferent neurons. As shown in Figure 1.11 and Figure 1.21, sensory afferents from the uterus converge on the dorsal horn. This provides a mechanism for spinal glial activation that bypasses the circulation. Research to determine the relative importance of these activation mechanisms in female visceral pain would allow targeting of translational treatment options.

7.1.7 Further elucidation of the optimal hormonal environment to prevent the transition from dysmenorrhoea to CPP, and the development of endometriosis in girls

Our research has found a significant association between reduced androgen activity, as measured by the FAI, and increased pain symptoms. Consistent with current literature, the most commonly used treatment for dysmenorrhoea, the OC, influences the FAI through an increase in SHBG, and influences androgen levels directly through ovarian and adrenal suppression. As such, use of the OC in young women may have both advantages, (reduced frequency of menses when used continuously) and disadvantages (increased innate immune activation, and the potential for increased prevalence of chronic pain). A longitudinal observational study following girls from puberty, or before, and determining the treatments undertaken, and the frequency of later chronic pelvic pain, would be optimal. That such studies, for a condition affecting 1 in 5 women, have not already been undertaken is testament to the lack of research attention that this area of health care has received.

7.1.8 Environmental factors, including diet and environmental toxins, that influence Immune activation in females.

Section 1.6.3 outlined dietary and environmental factors that may influence immune activation. Many of these factors are a feature of modern life. Martinez-Garcia et al. demonstrated that fasting and dietary lipids affect the expression of TLR2 and TLR4 on circulating white blood cells, and the release of IL-1β following LPS stimulation (Martínez-

García et al., 2020). Our research did not prescribe specific dietary preparation prior to blood sampling, potentially affecting research results. Future studies would benefit from recording the fasting status, and pre-test diet of participants. The determination of lifestyle factors affecting TLR expression, also provide a potential avenue for the reduction of inflammation in primary care, or low-resource settings.

7.1.9 The relationship between clinical symptom phenotypes, TLR4 response and hormone levels

Further investigation of this relationship would provide additional guidance to health practitioners determining the optimal treatment modalities for individual women

7.2 Concluding Statement

With little research into chronic pelvic pain (CPP) worldwide, current management occurs in a vacuum of knowledge few areas of medicine would accept. Such research neglect provides multiple opportunities for innovation in diagnosis and clinical management. This thesis has provided new insight into the under-researched condition, dysmenorrhoea-related pelvic pain. It has proposed a novel theory that is consistent with the full range of symptoms and presentations found in clinical practice.

The development of the Unifying Theory began with investigating the lived experience of women affected by DRPP, and investigating the range of symptoms reported. This confirmed the inconsistent association between specific symptoms and a lifetime diagnosis of endometriosis, and introduced the concept of Dysmenorrhoea-Related Pelvic Pain Syndrome. This suggested the existance of a systemic, rather than purely pelvic, mechanism for pain.

Evidence for activation of TLR4 and the innate immune system as a mechanism for the systemic syndrome was then sought, with the finding of increased responsiveness of PBMCs to TLR4 stimulation in women with pain, when compared. This built on the preclinical research by Kwok et al. (2012, 2014) demonstrating that an increased responsiveness of PBMCs mirrored TLR4-mediated activation of glia within the spinal cord.

Chapter 5 demonstrated that a factor known to modify immune activation (higher levels of androgens), moderated pain symptoms experienced by women with DRPPS.

This thesis opens a new door to potential clinical management options for women with pain. With an at-risk patient population (teens with severe dysmenorrhoea) easily identified at an early stage of their pain condition, novel treatments that target inflammation in young women have the potential for the secondary prevention of CPP. As such, immune modulation offers the potential for CPP to become one of the few areas of human chronic pain where prevention is a realistic goal. If this can be achieved, the improved management of dysmenorrhoea-related pelvic pain has the potential to alter quality-of-life outcomes for women, and health economics outcomes for society, throughout the world.

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