University of Southampton

Faculty of Medicine

Human Development & Health

Endometriosis – It's Influence on Obstetric and Birth Outcomes and the Potential Role of Reactive Oxygen Species

by

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Thesis for the degree of Doctor of Philosophy

April 2024

University of Southampton

ABSTRACT

Faculty of Medicine, Human Development and Health

Doctor of Philosophy

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The gynaecological conditions of endometriosis and adenomyosis are believed to affect up to 10% of women of child-bearing age in the UK and can cause debilitating pain, infertility and significant burden on healthcare economics. Many important aspects of the pathogenesis and health impacts of the diseases remain elusive. One more recently discovered culprit is that of reactive oxygen species which has been linked not only to the pathogenic process but also to progression of the diseases and their impact on both pain and fertility. Reactive oxygen species are difficult to measure and study in vivo and their relationship with endometriosis therefore remains undetermined.

What remains lacking in our knowledge of two prevalent and debilitating diseases is a full understanding of the pathogenesis and disease progression, how they cause pain and infertility, how they impact on pregnancy and health of any offspring and a reliable non-invasive test and effective treatment. Reactive oxygen species may play a key role across all of these themes.

This thesis sets out to investigate 1) the influence of endometriosis on the reproductive process from fertility parameters to perinatal outcomes and explore a possible role of Developmental Origins of Health and Disease theory in women with the disease 2) whether reactive oxygen species levels are different in women with endometriosis compared to women without endometriosis and whether levels of reactive oxygen species are improved following surgical treatment of disease and 3) how reactive oxygen species affect the oocyte using a reproductive fluid of women affected by endometriosis in an ethically approved human study and murine model.

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Declaration of Authorship

Dr Joanne Horton

Endometriosis – It's Influence on Obstetric and Birth Outcomes and the Potential Role of Reactive Oxygen Species

I declare that this thesis and the work presented in it is my own and has been generated by me as the result of my own original research.

I confirm that:

1. This work was done wholly or mainly while in candidature for a research degree at this University;
2. Where any part of this thesis has previously been submitted for a degree or any other qualification at this University or any other institution, this has been clearly stated;
3. Where I have consulted the published work of others, this is always clearly attributed;
4. Where I have quoted from the work of others, the source is always given. With the exception of such quotations, this thesis is entirely my own work;
5. I have acknowledged all main sources of help;
6. Where the thesis is based on work done by myself jointly with others, I have made clear exactly what was done by others and what I have contributed myself;
7. Parts of this work have been published as:

   J Horton, M Sterrenburg, S Lane, A Maheshwari, TC Li, Y Cheong. Reproductive, obstetric and perinatal outcomes of women with adenomyosis and endometriosis: a systematic review and meta-analysis. Hum Reprod Update, 2019. 25(5):592-632

Signed:

Date: 7th April 2024

Acknowledgements

I would like to thank Professor Ying Cheong for giving me this research opportunity, for all of her brilliant ideas, unending advice and guidance and clinical training in the area of reproductive medicine and surgery. Under her mentorship I have developed as a clinician, academic and as a person and found a career pathway that excites and fulfills me. I admire many things about her but her patience and positivity through this thesis has been infinite.

To Professor Nisreen Alwan MBE who has diligently guided me through my foray into the world of public health and epidemiology. As well as sharing with me her wealth of knowledge and advice, her support and kind words have encouraged me to continue through some challenging times during my research. I could not have completed my work without the help of Prof Alwan's team, particularly Dr Nida Ziaudeen who has lead me through countless floundering attempts at STATA coding and for whom nothing has seemed too much trouble.

Very special thanks to Dr Simon Lane, my steadfast supervisor in all laboratory work. Always approachable and polite enough to hide any disappointment upon multiple occasions of my ineptitude. I am also grateful to Professor Keith Jones and his team (Steph, Larissa, Tianyu and Jess) for allowing me to invade their lab. They have all offered help as well as camaraderie. My gratitude to Professor Martin Feelisch for his expertise in reactive oxygen species and for sharing some of his team's valuable work.

To new friends I have made along the way especially Bonnie, Jenny, Sarah and Karen with whom I have shared everything from tea breaks and our research offices to weddings, babies, new jobs and house moves. To old friends who have put up with 5 years of my PhD ramblings.

Finally, my biggest thank you is to my amazing family. To my parents without whose countless hours of childcare I would still be knee-deep in data analysis. The toughest part of this process has been returning to clinical work after 2 years and 3 months equivalent research time with a 1-year old and trying to maintain the role of mother, wife, clinical trainee and PhD candidate. It simply would not have been possible without the love and support of my family. To my daughter Cora who has had a somewhat absent-minded mother for the last 2 years. And to my long-suffering husband who has held the household and my mental health together while receiving little in return. I shall never be able to repay him but he will be pleased to know…I will try.

Definitions and Abbreviations

ABTS 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid
AE Pregnancy after diagnosis of endometriosis
AP All pregnancies
APH Antepartum haemorrhage
ATP adenosine triphosphate
ART Assisted reproductive technology
ASPA Animals Scientific Procedures Act
ASRM American society for reproductive medicine
BMI Body mass index
CAT Catalase
CI Confidence interval
CPR Clinical pregnancy rate
CR Cancellation rate
CS Caesarean section
DAG Directed acyclic graph
DAPI 4',6-diamidino-2-phenylindole
ddH2O Double distilled water
DIE Deep infiltrating endometriosis
DNP 2,4-Dinitrophenol
DNPH 2,4-Dinitrophenylhydrazine
D-O Diagnosis-outcome model
DOHaD Developmental origin of health and disease
DTNB 5,5'-dithio-bis-(2-nitrobenzoic acid)
DTT Dithiothreitol
ECD Electron-capture dissociation
ELISA Enzyme-linked immunosorbant assay
E-O Exposure-outcome model
ETT Ergothioneine transporter
FOX Ferrous oxidation−xylenol orange
FP First pregnancies
FR Fertilisation rate
FRAP Fluorescence recovery after photobleaching
FSH Follicle stimulating hormone
GDM Gestational diabetes
GnRH Gonadotrophin releasing hormone
GPO Glutathione peroxidase
GR Glutathione reductase
GrX1 Glutaredoxin-1
GSH Glutathione
GSSG Glutathione disulfide
GV Germinal vesicle
hCG Human chorionic gonadotropin
HELLP Haemolysis elevated liver enzyme low platelets
HICSS Hospital integrated clinical support system
hMG Human menopausal gondadotropin
HPLC High performance liquid chromatography
HRA Health research authority
ICD-10 International classification of diseases
ICSI Intra-cytoplasmic sperm injection
IR Implantation rate
IUD Intrauterine death
IUGR Intrauterine growth restriction
LBR Live birth rate
LH Luteinizing hormone
LSCS Lower segment caesarean section
MD Mean deviation
MDA Malondialdehyde
MII Meiosis II
MQ Milli-Q water
MR Miscarriage rate
MRI Magnetic resonance imaging
NADPH Nicotinamide adenine dinucleotide phosphate
NBT Nitroblue tetrazolium
NC Natural conception
NND Neonatal death
NNU Neonatal unit
OC Obstetric cholestasis
OHSS Ovarian hyperstimulation syndrome
OR Odds ratio
PA Placental abruption
PCOS Polycystic ovarian syndrome
PCR Polymerase chain reaction
PET Pre-eclampsia
PGF-2a Prostaglandin2alpha
PIH Pregnancy induced hypertension
PIL Personal Liscence
PP Placenta praevia
PPH Postpartum haemorrhage
PTD Preterm delivery
RR Relative risk
SGA Small for gestational age
SOD Superoxide dismutase
SPD Symphysis pubis dysfunction
TAC Total antioxidant capacity
TAE Tris-acetate-EDTA buffer
TBARS Thiobarbituric acid reactive substances
TBE Tris-borate-EDTA buffer
TRAP Telomerase repeated amplification protocol
TNF Tumour necrosis factor
TVUS Transvaginal ultrasound
USS Ultrasound scan
VTE venous thromboembolism
WERF-EPHect World endometriosis research foundation - the endometriosis phenome and biobanking harmonisation project

Chapter 1 Introduction: The Impact and Burden of Endometriosis and Adenomyosis

The gynaecological conditions of endometriosis and adenomyosis are believed to affect up to 10% of women between menarche and menopause in the UK and can cause significant pain and infertility. Despite knowledge of these conditions arising as early as 1690 [1] many aspects of the pathogenesis and health impacts of the diseases remain elusive. One more recently discovered association is that of reactive oxygen species which have been linked not only to the pathogenic process but also to progression of the diseases and their impact on both pain and fertility [2,3,4,5]. Due to innate difficulties in measuring reactive oxygen species however, this is a theory of ongoing controversy.

The varying symptomatology and lack of reliable non-invasive diagnostic investigations and treatment for the conditions pose a significant burden on gynaecological services and on affected women in the UK. The average time from presentation to diagnosis given these hurdles is 4-10 years[6] in which time affected women can suffer a significant reduction in quality of life and infertility. With increasing demands for assisted conception, understanding the effect of endometriosis and adenomyosis on fertility is of growing importance and more recently evidence has also demonstrated an impact of these diseases reaching beyond conception, to pregnancy complications and the health of the offspring. The exact pregnancy and delivery risks posed are still unclear but with growing interest in the theory of the developmental origin of health and disease this is an area which should be explored fully.

What remains lacking in our knowledge of two prevalent and debilitating diseases is a comprehensive understanding of the pathogenesis and disease progression, how they cause pain and infertility, how they impact on pregnancy and health of any offspring and a reliable non-invasive test and treatment. Reactive oxygen species may play a key role across all of these themes and will be explored in this body of work.

1.1 Basic Anatomy of the Normal Reproductive Tract, the Endometrial Cycle and Folliculogenesis

1.1.1 External genitalia and vagina

The female reproductive tract begins with the external reproductive structures collectively termed the vulva. The vagina then extends internally from the introitus and consists of an outer fibrous adventitia, intermediate smooth muscle layers (an inner circular muscle layer and outer longitudinal layer) and inner mucosal tissue (stratified squamous epithelium and elastic lamina propria). The vagina is closely related to some internal pelvic structures – anterior to the vagina lies the urethra and bladder, laterally to the vagina lies the ureters and uterine arteries and posteriorly lies the rectum and anus[7]. The upper vagina meets the uterine cervix (the narrow lower 2-3 cm of the uterus) which has a central canal with an internal and external opening called the internal and external os.

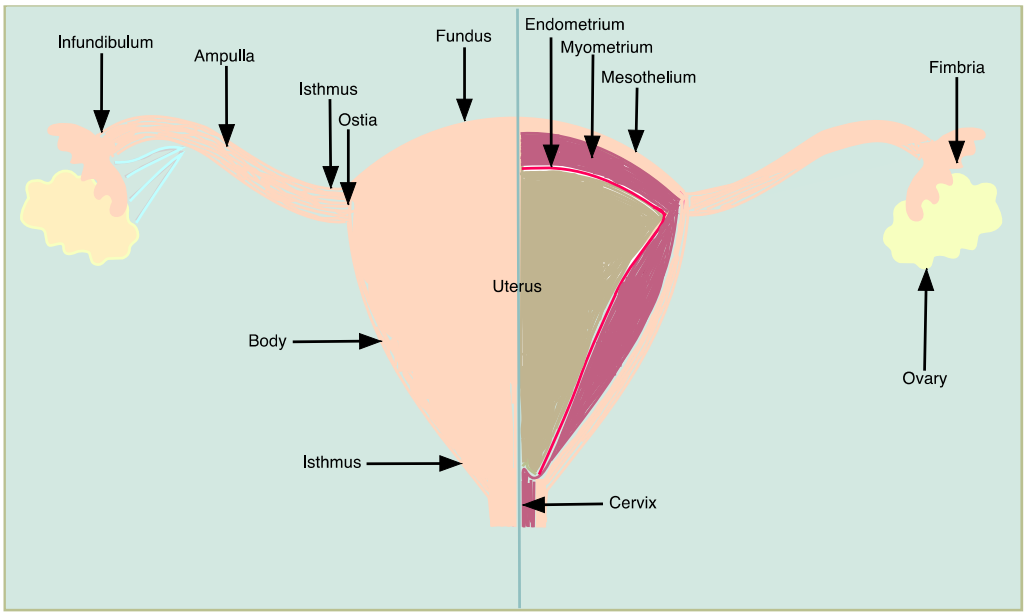


Figure 1: Basic Anatomy of the Female Reproductive Tract

1.1.2 Cervix and uterus

The cervix joins the body of the uterus at an area called the isthmus. The body of the uterus extends superiorly to the opening of the fallopian tubes (ostia) above which the uterus ends in the area known as the fundus[7] (Figure 1).

The body and fundus of the uterus is the predominantly muscular part of the organ with an internal layer (endometrium), middle layer of dense smooth muscles running longitudinally, obliquely, and horizontally (myometrium) and an outer serous membrane (perimetrium). The junctional zone refers to the innermost layer of myometrium just beneath the endometrium.

1.1.3 Endometrium and the endometrial cycle

The endometrium is made up of epithelial cells, glandular cells and blood vessels in two main layers; the stratum basalis and stratum functionalis[7] (Figure 2). The endometrium responds to the hormones luteinising hormone (LH), follicle stimulating hormone (FSH), oestrogen and progesterone. In a normal menstrual cycle the endometrium thickens and increases in vascularity in the proliferative phase under the influence of FSH and oestrogen, the endometrial glands then produce a glycogen rich secretion and become cork-screw shaped during a secretory phase under the influence of LH and progesterone, finally the spiral arterioles in the functional layer of the endometrium contract causing tissue ischaemia and degeneration in response to reduced LH and progesterone and menstruation occurs (Figure 2). The correct function of the endometrium and its response to hormones is not only important for the normal functioning of the menstrual cycle but also crucial for successful implantation of an embryo and thereafter for supporting the developing pregnancy[7].

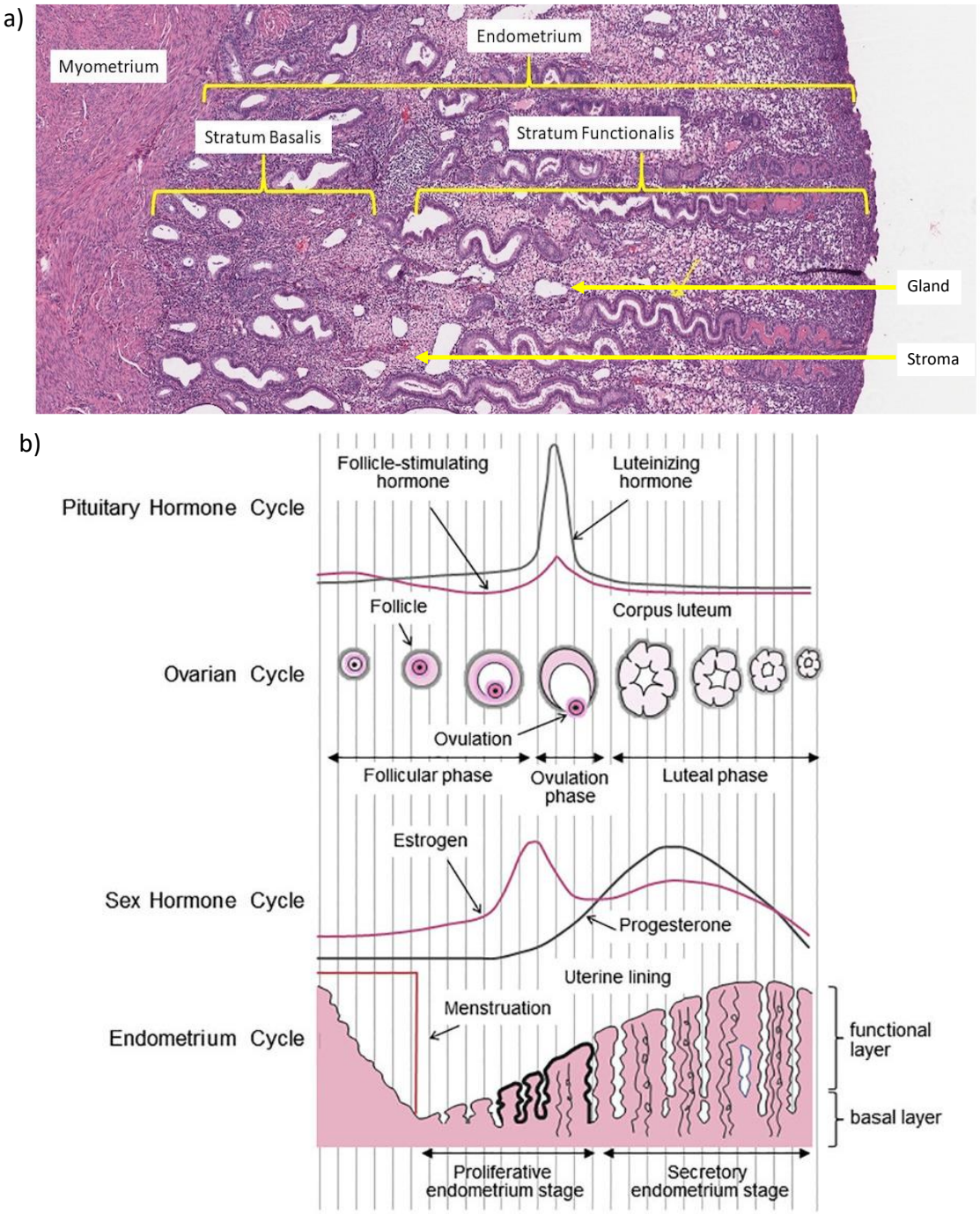


Figure 2: a) Histological image of the endometrium depicting its layers and cell types (Kyle D, Schwartz L. Anatomy & histology. PathologyOutlines.com website. (https://www.pathologyoutlines.com/topic/uterusnormal.html) and b) diagram representing the hormones governing the menstrual cycle and their effect on the ovarian and endometrial cycle (image from Mizutani, S. Matsumoto, K. Kato, Y. et al. New insights into human endometrial aminopeptidases in both implantation and menstruation. J.bbapap 2019: 1868 (2) licence https://creativecommons.org/licenses/by-nc-nd/4.0/)

1.1.4 Fallopian tubes

Arising laterally from the uterus from their opening (ostia), the fallopian tubes project into the pelvis and allow transport of an ovulated oocyte from the ovary to the uterus. It has four anatomical parts, a narrow area where it connects to the uterus (isthmus), the main length of the tube (ampulla), a wide opening close to the ovary (infundibulum) and finger-like, ciliated projections to pick up an ovulated oocyte (fimbrial end)[7] (Figure 1). The basic histological layers are comprised of an outer serosal surface (mesothelium), a smooth muscle layer (arranged obliquely, circularly and longitudinally) via which contractions assist with transportation of an oocyte or embryo and an inner mucosal layer lined with secretory cells and ciliated columnar cells which beat to aid transport of an oocyte or embryo[7].

1.1.5 Ovaries

The ovaries are situated in close proximity to the fimbrial end of the fallopian tubes (Figure 1). The ovarian surface is covered with cuboidal epithelium beneath which lies a connective tissue layer called the tunica albuginea. Internal to the tunica albuginea is the cortex of the ovary consisting of the stroma and follicles and beneath the cortex (the innermost part of the ovary) is known as the medulla through which the ovary receives its blood supply, lymphatic drainage and innervation[7].

1.1.6 The pelvis

The reproductive tract is continuous with the pelvic area of the abdominal cavity via the fimbrial end of the fallopian tube. The pelvis also contains the pelvic attachments of the reproductive tract (broad ligament, ovarian ligament, and round ligament through which the blood supply and lymphatics of reproductive organs run), the bladder (anterior to the uterus), rectum, sigmoid colon, and a portion of the large and small bowel (posterior to the uterus) and peritoneal fluid. Several other structures such as the ureters and major blood vessels and nerves run retroperitoneally in the pelvis. Peritoneal fluid is produced by transudation from blood vessels of the submesothelial layer of the peritoneum and is an ultrafiltrate of plasma. It is removed from the abdominal cavity by the lymphatics at approximately the same rate it is produced therefore a level of <50 mls is usually found but increased volumes can exist in disease states and in late pregnancy. It acts as a lubrication for abdominal organs and allows the transfer of electrolytes and

other molecules across serosal surfaces of the abdominal cavity. It contains electrolytes, glucose, neutrophils, mononuclear cells, eosinophils, macrophages, lymphocytes, mesothelial cells, and an average of 3.0 g/mL of protein.

1.1.7 Folliculogenesis

The ovary is a major site of oestrogen and progesterone production through steroidogenesis and its other main functions are oogenesis (production of oocytes) and folliculogenesis (development of ovarian follicles) (see Figure 2). The ovarian follicles consist of an oocyte, their supporting cells (granulosa cells and follicular cells) and fluid. Primary oocytes are formed within the female fetus from mitosis of ovarian stem cells in gametogenesis[7]. They undergo meiosis I and are arrested in prophase I until the appropriate hormonal signalling occurs pre-ovulation. They are surrounded by a single layer of granulosa cells in a primordial follicle. Following puberty with the production of LH and FSH from the anterior pituitary, primordial follicles are recruited in groups to undergo further development until menopause takes place. Recruited follicles will either develop successfully and eventually rupture at ovulation or will undergo atresia. Folliculogenesis continues under the influence of FSH when the single layer granulosa cells proliferate and become columnar in shape, they contain a primary oocyte which produces a zona pellucida (outer layer) and are then classified as primary follicles. The granulosa cells of the primary follicles become larger and develop an outer layer of theca cells and become secondary follicles[7]. LH signals the theca cells to produce oestrogen. Following this stage, follicular fluid collects in the cavity of the follicles causing a further increase in their size. Within the oocyte meiosis I is resumed, and secondary oocytes are formed when they arrest once more at metaphase II and together the tertiary (antral) follicle is formed. As granulosa and theca cells proliferate during growth of the follicles, oestrogen levels rise and cause a negative feedback loop at the level of the anterior pituitary and as a consequence, FSH and LH levels drop. This leads to the atresia of many tertiary follicles, but a large late-tertiary follicle is able to survive reduction in FSH and continue growing. This follicle produces a large amount of oestrogen due to its size which overcomes the negative feedback loop and signals the release of a higher level of FSH and LH from the anterior pituitary. The surge of LH signals ovulation to occur and the late tertiary follicle ruptures and releases the secondary oocyte[7]. The ruptured follicle becomes a corpus luteum which produces a high level of progesterone. If fertilised, the oocyte completes meiosis II and embryogenesis ensues. With

implantation human chorionic gonadotrophin is released and causes the corpus luteum to persist and continue producing progesterone. If hCG is not released the corpus luteum degenerates and progesterone levels drop. The normal process of folliculogenesis takes 2 months and the final stage of growth of a group of tertiary follicles to ovulation of one late-tertiary follicle takes an average of 28 days[7].

1.2 The Pathology of Endometriosis and Adenomyosis

Endometriosis

Endometriosis is a gynaecological condition characterised by the presence of endometrial tissue (stromal and epithelial cells) outside the uterine cavity. The ectopic endometrial tissue is commonly found in the female pelvis (see Figure 7), but occasionally can occur in distant sites such as abdominal scar sites, the diaphragm and lungs. It is understood to be an oestrogen dependant disease therefore affecting women between menarche and menopause. The incidence is believed to be 1.46 per 1000 person-years in the UK[8] (2.37-2.49 per 1000 person-years generally) and prevalence is as high as 35-50% of women with infertility or pelvic pain[9].

The exact pathogenesis is unknown, but a number of theories have arisen since the condition was first described in 1860 by Karl Freiherr von Rokitansky[10]. Perhaps the first theory put forward in 1919 by Mayer was that of metaplasia. Either coelomic metaplasia, whereby cells of normal peritoneal tissue undergo transformation to endometrial stromal and epithelial cells under the influence of hormonal or immunologic factors [11], or metaplasia of residual cells of the Mullerian duct (embryological origin of the urogenital tract) which maintain the capacity to differentiate into endometrial cells under the influence of oestrogen. Following Mayer's theory another followed almost a decade later in 1927 claiming the disease is a result of retrograde menstruation through which menstrual fluid and endometrial stromal and epithelial cells flow back through the fallopian tubes leading to endometrial cells implanting in the pelvis. However, retrograde menstruation is a common physiological occurrence, but endometriosis will only occur in 10% of women, moreover this theory does not explain cases of endometriosis distant to the pelvis. A more recent proposal is that progenitor cells originating from bone marrow may differentiate into endometrial cells[12]. Benign metastasis is the foundation of other theories that conclude ectopic endometrial tissue is a result of haematological or lymphatic spread [11]. This is supported by

microvascular studies examining lymph flow from the uterus to the ovary and the presence of endometrial cells found in lymph nodes [13]. The pathogenesis of endometriosis remains an active area of research and new findings are continuously emerging. A possible association of gut microbiota and endometriosis has been found demonstrating gut microorganisms that can secrete oestrogen-degrading products (β-glucuronidase and β-glucosidase) leading to an increased level of circulating oestrogen[14]. Gut microbiota could also interact with the abnormally expressed cytokines of endometriosis and many immune-modulating components of gut microbials have already been linked to other inflammatory conditions[14].

However, as endometrial cells are found in the pelvis of many women who do not have endometriosis, for development of the disease to arise there are a number of mechanisms which must take place such as implantation into the peritoneum/epithelium, vascularisation, growth etc. This may be where genetic predisposition, acquired defective immune-mediated clearance or alteration in hormonal responsiveness become important in disease progression [11,15]. Altered gene expression (such as upregulated matrix metalloproteinase and insulin-like-growth-factor)[16] within the endometrial cells, immune cell dysfunction (including different macrophage phenotype)[17,18] or increased levels of bioavailable oestrogen and reduced progesterone receptors may influence their propensity to implant in ectopic sites[19].

The ectopic endometrial cells give rise to the symptoms of the disease most commonly being pelvic pain associated with menstruation (dysmenorrhoea). Other symptoms include pain on sexual intercourse (dyspareunia), painful defaecation with menstruation (dyschezia), lower back pain, painful micturition (cystitis), pain in multiple sites, fatigue and infertility. Women may present with symptoms related to endometriosis in distant sites such as a tender lump occurring cyclically in an abdominal scar site or symptoms of catamenial pneumothorax/haemothorax in cases of thoracic endometriosis[20,21,22].

Adenomyosis

The closely related condition of adenomyosis is characterised by the presence of ectopic endometrial tissue within the myometrium of the uterus (at least 2.5 mm beneath the endometrial-myometrial junction) (see Figure 7). The ectopic tissue leads to myometrial hypertrophy and hyperplasia as well as differences in cytoplasmic organelles, nuclear structures and intercellular junctions [23]. The prevalence is believed to be 8-27% [24].

It is described that endometrial glands and stroma from the stratum basalis of the endometrium invaginate into the myometrium. Theories regarding the pathogenic process suggest that the invasion of endometrial cells may occur due to a traumatised endometrial-myometrial interface or following changes to the junctional zone during pregnancy subsequent to trophoblastic invasion and angiogenesis. These are supported by an observed higher incidence of adenomyosis in parous women and in women who have had uterine curettage procedures. It is also believed that there may be several genetic, hormonal or immunological factors that predispose women to developing adenomyosis as it can rarely occur in nulliparous women and in women with no documented uterine procedures[23]. Similarly to endometriosis, increased local oestrogen secondary to increased aromatase enzyme (key enzyme in steroidogenesis of oestrogen which is upregulated in these conditions) may play a role. The endometrial cells and myocytes (myometrial muscle cells) of women with adenomyosis display greater invasive tendencies when grown on a collagen matrix [23]. Endometrial stromal cells and myometrial cells of adenomyosis have different proteomic profiles indicating altered gene expression. Another theory of pathogenesis is that of de-novo metaplasia based on the junctional zone myometrium and endometrium both originating from pluripotent cells of the Mullerian duct. A degree of plasticity of the basal endometrial cells has also been demonstrated and similarities to myofibroblasts and immature smooth muscle cells in different phases of the menstrual cycle which would indicate an ability to undergo metaplasia [23]. Lymphatic spread has also been stipulated owing to the occasional finding of endometrial nodules in the intra-myometrial lymphatic system [23]. Some environmental influences have been noted with a significantly higher incidence of adenomyosis occurring in post-menopausal women who have received Tamoxifen treatment where the oestrogen receptor antagonist behaves as an agonist in endometrial tissue. This also supports the theory of a hormonal factor influencing development and progression of adenomyosis [25]. As with endometriosis there is no consensus on the exact pathogenesis of the disease.

Symptoms of adenomyosis include dysmenorrhoea, chronic pelvic pain and menorrhagia and it is most commonly diagnosed in women's fourth and fifth decade of life due to the fact that it is still mostly diagnosed at hysterectomy [24,25].

Some may argue that endometriosis and adenomyosis are two distinctly separate conditions, although evidence alluded to thus far suggest the two entities are interlinked. The incidence of adenomyosis is higher in women with endometriosis and there are certainly similarities in the

postulated pathogeneses of the two conditions and the thickness and irregularity of the junctional zone on MRI imaging has been correlated with the presence of endometriosis and its severity by researchers who propose the conditions could be two phenotypes of the same underlying disease process [26].

1.3 Classification of Endometriosis and Adenomyosis

1.3.1 Endometriosis

Endometriosis can be classified by type or stage with some aspects of crossover. Both pose a challenge due to the many facets of the disease. There are different anatomical manifestations, different symptom profiles, uncertain response to treatment and unknown recurrence rates.

Staging and Classification Tools

The most widely used classification system is the revised American Society for Reproductive Medicine classification (r-ASRM) which was published in 1997. It stages the severity of endometriosis based on the amount of disease (lesions, endometrioma and adhesions), size and depth of invasion of lesions and anatomical distortion. See r-ASRM staging table in Appendix 20. Additional classification systems become useful depending on the type of endometriosis or whether a patient is concerned about pain versus fertility. The ENZIAN classification tool is used to grade deep infiltrating endometriosis. The Endometriosis Fertility Index grades the disease on a scale of likely dysfunction of the pelvic anatomy and encompasses fertility history and the r-ASRM score to reliably link the disease to pregnancy rates which could be more meaningful to some patients. One of the significant draw-backs of these diagnostic tools is that they can only be used at surgery [27].

Subtypes of Endometriosis

There are three main subtypes of endometriosis: peritoneal, deep infiltrating, and ovarian.

Peritoneal endometriosis is the most common form of the disease and is characterised by endometriotic lesions of the peritoneum which do not invade the sub-peritoneal space. They can appear as red or black lesions of hormonally active tissue or white opaque areas consistent with

fibrosis of an inactive lesion. Red lesions are morphologically more similar to eutopic endometrium and are considered to be earlier stage lesions [28]. Progression of peritoneal endometriosis is unconfirmed and remains a controversial topic in the field.

Deep infiltrating endometriosis (DIE) was first defined by Koninckx and Martin in the 1990's as endometriosis infiltrating >5 mm into the peritoneum and they have since sub-classified the condition by character of the lesions. Type I lesions are conical and result from peritoneal infiltration, type II are deeper, likely due to retraction and associated with extensive adhesions and type III are spherical with the largest dimension lying beneath the peritoneum [29]. Histological similarities between rectovaginal DIE and adenomyosis have led to a belief that DIE may be a case of adenomyosis externa (or external adenomyosis). DIE lesions contain more fibro-connective tissue and smooth muscle fibres compared to endometrial tissue. Type II and III lesions which display these histological characteristics are now widely considered to be true deep infiltrating endometriosis or "adenomyosis externa" and are not deemed to be a result of the growth/progression of peritoneal endometriosis whereas type I lesions are likely to be peritoneal endometriosis with a deeper invasion. Deep endometriosis has a prevalence of 1-2% and is usually characterised by one large nodule associated with the lower 20 cm of bowel or the vesico-uterine fold and can involve the bowel, the bladder or ureters. Recurrence rate of confirmed deep endometriosis following surgical resection is as low as <5% [30]. Other sub-classifications have been proposed such as the Adamyan's four stages of rectocervical endometriosis (stage 1: confined to rectovaginal tissue, stage 2: invading the cervix, stage 3: spread to the uterosacral ligaments and rectal serosa, stage 4: invasion of the rectal wall and rectouterine peritoneum) [31].

Ovarian endometriosis is characterised by ovarian cysts consisting of blood and endometrial cells called endometriomas.

Although not described as subtypes, extra-abdominal endometriosis (endometriotic lesions at distant sites such as thoracic endometriosis) and iatrogenic endometriosis (endometriosis disseminated by via surgery) are also encountered[22].

1.3.2 Adenomyosis

Adenomyosis has two main pathologic types; diffuse (diffuse non-capsulated involvement of the anterior and posterior walls of the uterus, accounts for 2/3 of cases) and focal (an area of

adenomyosis which may have a pseudocapsule, which is called an adenomyoma, and causes asymmetry of the uterus, accounts for 1/3 of cases) [32]. It can also be defined on MRI as superficial or deep based on whether the lesions are limited to the junctional zone of the myometrium (<1/3 depth of the myometrium) or extend beyond than the junctional zone (>1/3 depth of the myometrium). Both of these classifications are difficult to diagnose on imaging alone, without histo-pathological examination of the uterus. This has contributed to a recent theory that adenomyoma can result from endometriotic lesions of the pelvis invading the uterus from the outside [30,33].

While recognising that disease classification by both type or staging does not predict response to treatment, recurrence rate, prognosis, symptom severity or quality of life, it is important to strive to classify this disease to be able to better inform patients of what the disease may mean to them in terms of treatment options, extent of surgery required and fertility outcomes. A unified classification is also crucial for researchers attempting to further understand the condition.

1.4 Diagnosis of Endometriosis and Adenomyosis

Endometriosis

Endometriosis can be suspected based on symptoms and clinical examination, however 2-50% of endometriosis is asymptomatic [34] and the symptoms can be non-specific. Potential diagnostic factors in the examination include site and type of pain elicited, palpable nodules of the vagina, uterosacral ligaments or pouch of Douglas, adnexal masses, palpation of a rectovaginal mass[20]. On speculum examination, vaginal nodules in deep infiltrating endometriosis can be seen. Examination for endometriosis in distant sites will vary based on where the suspected lesions are, for example scar site lesions may be detected with a palpable mass[21] whereas diaphragmatic lesions will not be diagnosed on examination.

Biomarkers may be used to indicate endometriosis. The tumour marker, CA125, when elevated >35 IU/ml can be consistent with endometriosis but is non-specific and with a low sensitivity for milder forms of the disease. Other biomarkers (such as immunological markers, proteomics, metabolomics and glycoproteins) [35] are becoming popular in experimental fields but are not used in clinical practice. Gene expression profiles and proteomics are among the latest techniques to determine a non-invasive diagnostic test. Ultrasound imaging of the pelvis either vaginally,

abdominally or rectally can diagnose endometriosis. Transvaginal ultrasound (TVUS) can identify endometriomas as ovarian cysts with a typically "ground-glass" heterogenous echotexture. Deep infiltrating endometriosis (endometriotic tissue which penetrates the retroperitoneal space ≥5 mm) and endometriotic nodules can also be identified by this imaging technique. The negative sliding sign as well as tenderness during the procedure while not exclusive to endometriosis can suggest the presence of pelvic adhesions. The accuracy of TVUS is enhanced by bowel preparation and rectal water contrast [34]. Pelvic MRI can image endometriomas and deep nodules and can be useful in determining the extent of deep endometriosis involving the bowel, bladder or ureter [36]. 3.0T MRI and MRI jelly method (with ultrasound jelly injected into the rectum and vagina) were more accurate than other MRI modalities [34]. A Cochrane Review found both ultrasound and MRI imaging to be useful but they lacked the specificity for a SpPin triage test (when a Specificity test is Positive the diagnosis is ruled in) and transvaginal ultrasound was closer to meeting the SpPin criteria than MRI [34]. Transvaginal ultrasonography in a comparative study with MRI was found to have higher sensitivity (95% vs 76%) and greater specificity (98% vs 68%) [28]. A number of other imaging techniques have been used, such as rectal endoscopic ultrasonography and computed tomography colonography, for more specialised investigation of deep infiltrating endometriosis of the bowel[37]. Direct visualisation of endometriotic lesions at laparoscopy or laparotomy is currently the most reliable method of diagnosis which in combination with histology result is the accepted 'gold standard' practice. This technique also allows for disease staging (most commonly with the revised American Society for Reproductive Medicine classification as discussed in Chapter 1, section 1.4.1) and treatment simultaneously. See Figure 3 for a laparoscopic view of endometriosis in the female pelvis and how lesions are visually diagnosed and classified for staging.

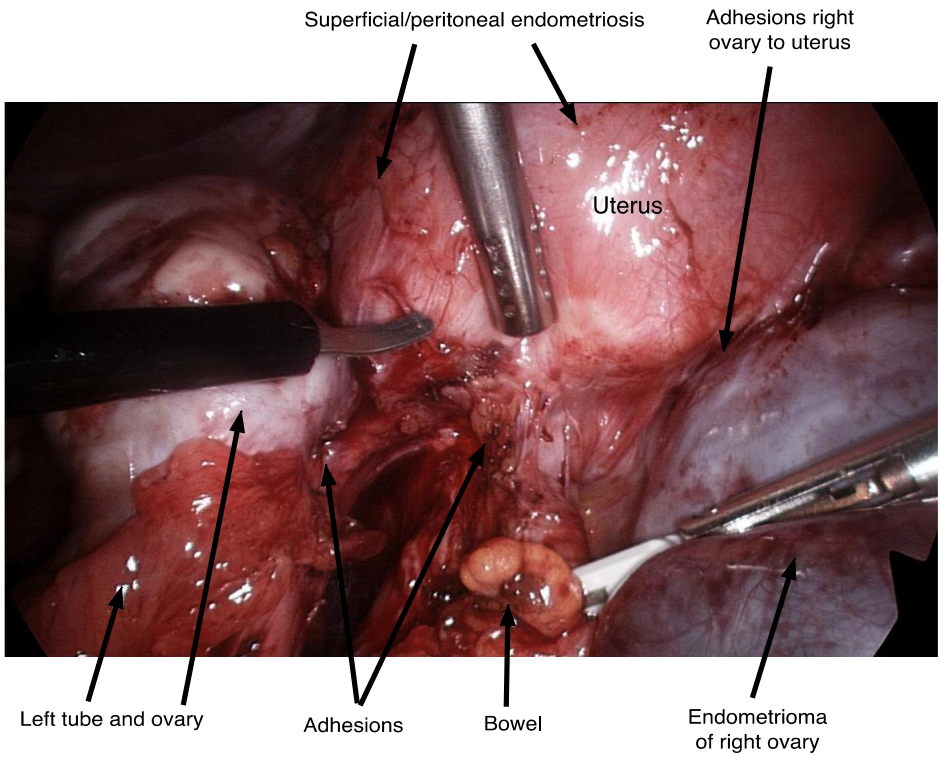


Figure 3: View of the female pelvis at laparoscopy indicating stage IV endometriosis (image from "Dr Kenneth Leong, IVF and Infertility" http://www.kenleong.com.au/fertility.html)

Adenomyosis

Adenomyosis similarly to endometriosis can be suspected clinically but the accuracy of a clinical diagnosis is reported to be a range of 2.6-26% [38]. Symptoms include dysmenorrhoea and menorrhagia. In cases of very large adenomyoma symptoms relating to pressure of the enlarged uterus on surrounding structures such as the bowel and bladder may occur. On examination an enlarged, bulky uterus may be palpated[33].

Ultrasound is used to help diagnose adenomyosis with the best technique being transvaginal. Transvaginal ultrasound can identify several features suggestive of adenomyosis; uterine enlargement without distinct masses, asymmetrical thickening of the anterior or posterior walls, heterogenous myometrium with increased echotexture linear striations and poorly defined hypoechogenic cystic areas within the myometrium as demonstrated in Figure 4 [33]. Adenomyosis on MRI appears as diffuse or focal thickening of the junctional zone (a junctional zone thickness of >12 mm is usually predictive and <8 mm usually excludes the disease) with poorly defined areas

of low signal intensity representing muscle hyperplasia. Using T1 and T2 weighted images (use of different radiofrequency pulse sequences to highlight either fat or both water and fat) adenomyotic foci can be seen. It can have bright foci on T2-weighted images (cystic dilation of glands and ectopic endometrial tissue). These foci can be seen as bright foci on T1-weighted images during menstruation due to the occurrence of bleeding [39]. See Figure 5 (a) and (b) for MRI images of the adenomyotic (a) and normal (b) uterus. Sensitivity and specificity for TVUS has been reported as 0.68 and 0.65 respectively whereas for MRI these are believed to be 0.70 and 0.86 respectively [40]. Adenomyotic cysts or lesions can sometimes be seen hysteroscopically. The definitive diagnosis of adenomyosis is with histological examination and is usually following hysterectomy, though some hysteroscopic microsurgical techniques are increasing in popularity and it is possible to gain a histological diagnosis through this surgical method.

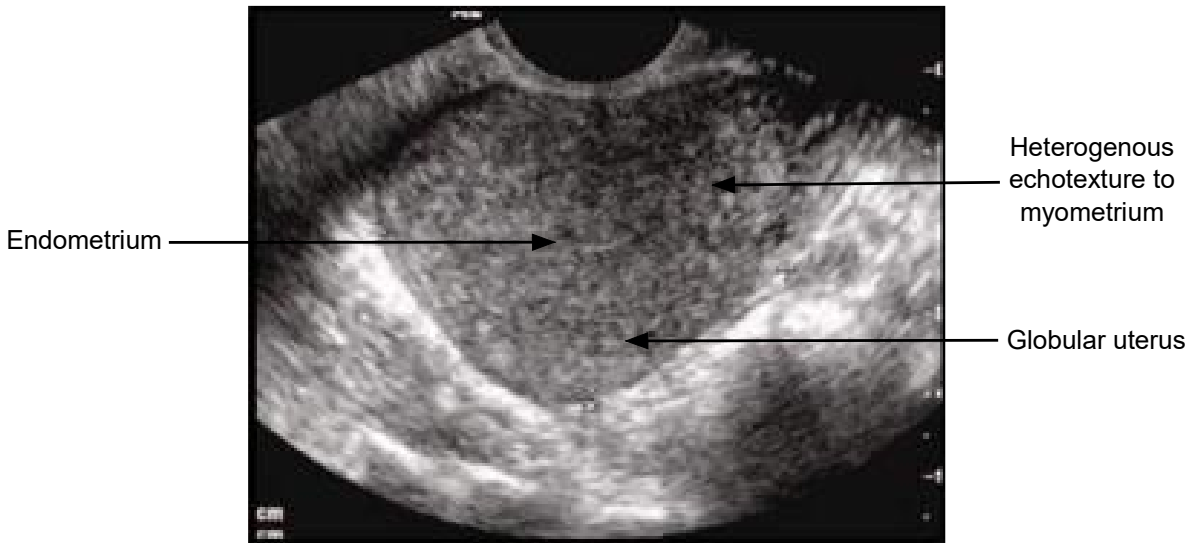


Figure 4: Trans-vaginal US image of the adenomyotic uterus in transverse section. Heterogenous echotexture and enlarged/globular uterus (image from "Progressive Radiology" https://progressiveradiology.com/news/articles/dr-aimee-maceda-on-adenomyosis)

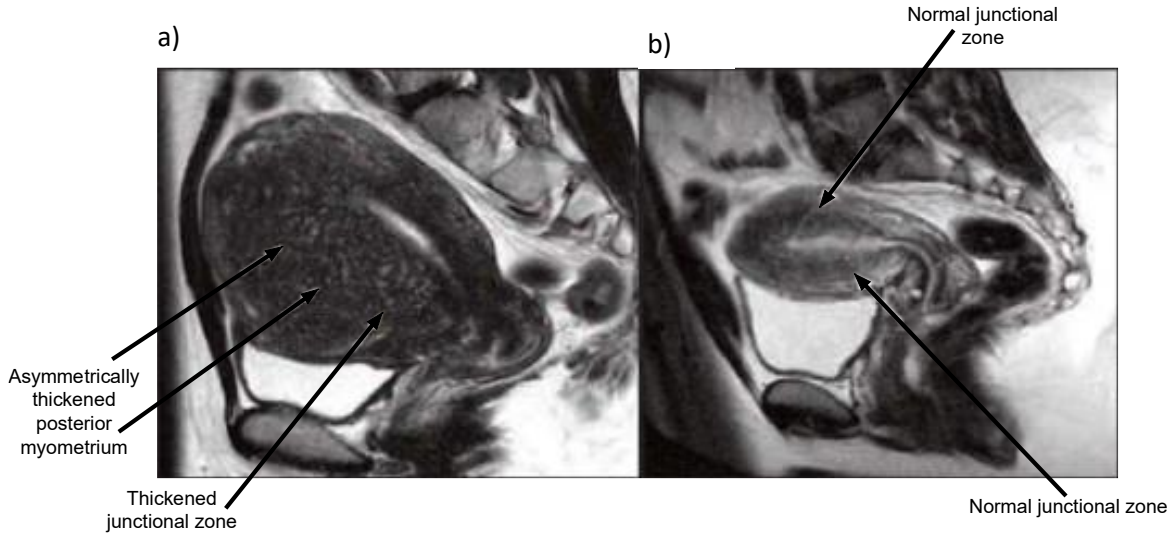


Figure 5: MRI images of the female pelvis: Image (a) demonstrates a thickened Junctional Zone and asymmetric posterior myometrial thickness of an adenomyotic uterus, image (b) shows the appearance of a normal uterus (image from "Progressive Radiology" https://progressiveradiology.com/news/articles/dr-aimee-maceda-on-adenomyosis)

1.5 Treatment of Endometriosis and Adenomyosis

Treatment of endometriosis and adenomyosis are not usually curative (unless surgical pelvic clearance is performed) but aims to alleviate symptoms and improve fertility. Treatment options depend on age, symptoms to improve, previous treatment, type/stage of endometriosis, risk of surgery and the woman's preferences.

1.5.1 Empirical

The least invasive treatment is analgesic medication such as non-steroidal anti-inflammatories, paracetamol and opioid based medications. Neuro-modulatory analgesic drugs are also sometimes used such as gabapentin and amitriptyline. Although not evidence based, increasingly women are turning to alternative therapies to manage their pain such as acupuncture and herbal supplements.

Hormonal suppression of the disease is a useful form of treatment if aiming to avoid surgery, prevent return of the disease following surgical treatment or supress the disease before surgical

treatment to optimise the surgical results. Hormonal treatment is suitable as long as the woman is not trying to conceive. Current hormonal treatments include the combined oral contraceptive pill, progesterone contraceptives, anti-progestogens, gonadotrophin releasing hormone (GnRH) agonists and aromatase inhibitors[41,42]. There is currently insufficient evidence to support the use of selective oestrogen receptor modulators, selective progesterone receptor modulators or anti-TNF-α drugs. Most hormonal treatments have been shown to be equally effective in improving endometriosis-associated pain and adenomyosis. A disadvantage is that symptoms usually recur when treatments are stopped.

1.5.2 Non-Surgical Procedures

Two non-surgical procedures exist for treating adenomyosis. Uterine artery embolization has been utilised as a therapy for adenomyosis with as much as 70% chance of symptom improvement[43]. High-intensity focused ultrasound induces focal thermocoagulation to destroy adenomyotic cysts and lesions of 3-10 cm diameter within the myometrium. A strict eligibility criteria includes body weight <100 kg, no suspected pelvic adhesions or previous lower abdominal surgery and <5 cm abdominal wall depth. Symptom relief is believed to be as high as 88% at 12-24 months follow up. Due to the degree of associated myometrial necrosis following these procedures (necrosis is a high as >90% following embolisation) these approaches are not suitable for women who have not completed their family [44].

1.5.3 Surgical

The most widely used surgical treatment of endometriosis aims to remove endometriotic lesions or deep nodules, divide adhesions and remove any endometriotic ovarian cysts. Removal of lesions may be achieved by laser vaporisation, diathermy or excision, all methods are equally effective, but excision is desirable if a histological diagnosis is to be made. Excision of a deep nodule associated with the bowel may require the assistance of colorectal surgeons and can carry a risk of bowel resection. Other deep nodules can involve the ureters and bladder which may involve stenting of the ureters or bladder resection. Endometrioma can be treated with drainage, fenestration and ablation or cystectomy. Simple drainage carries a high recurrence rate of 80-100% [45] but damages ovarian tissue the least, ablation has a recurrence rate of 20% and cystectomy is the most effective with only a 7% recurrence rate but these techniques are more

damaging to ovarian tissue [46]. Surgery can be performed with open laparotomy or laparoscopically. Laparoscopy is more common practice due to faster recovery times and reduced risk of post-operative complications.

The most common surgical option for women with adenomyosis is hysterectomy, either abdominal, laparoscopic or vaginal depending on accessibility to the uterus from each route, size of the uterus, surgical risks etc. To manage symptoms of menorrhagia, endometrial ablation techniques may be useful but concomitant hormonal or analgesic treatment is often needed to treat dysmenorrhoea. However, these procedures are not an option for women wishing to preserve or improve their fertility. For this group of women, the effectiveness and risk/benefit profile of surgery are controversial. If few, large adenomyoma are present adenomyomectomy of the masses can be performed by laparotomy or laparoscopy. Myomectomy is often performed for leiomyomas but unlike leiomyomas, adenomyomas are more surgically challenging to remove due to the indistinct borders with normal tissue and the soft, friable nature of the lesion. This approach leads to increased risks in future pregnancies due to the incision made on the uterus as well as an operative risk of haemorrhage and hysterectomy. Adenomyomectomy is not appropriate for diffuse adenomyosis without distinct adenomyoma, or where adenomyoma are so numerous that limited normal myometrium would be preserved. Since the development of microsurgery, excision of the adenomyotic tissue can be performed laparoscopically with this method which minimises injury to surrounding myometrium and avoids large incisions of the uterus [47]. An alternative approach to resection of adenomyotic tissue is with the hysteroscopic route. Cysts can be mechanically resected or opened and coagulated with diathermy at hysteroscopy with or without ultrasound guidance [44].

Laparoscopic uterosacral nerve ablation and presacral neurectomy aim to disrupt pelvic nerve pathways are further surgical approaches to reduce endometriosis or adenomyosis pain but are not considered more beneficial than conservative laparoscopic surgery[48].

Though not to be approached without serious consideration hysterectomy with or without oophorectomy (with oophorectomy has a higher chance of improving pain) is sometimes effective for women who do not wish to retain their fertility[48].

Adhesion prevention techniques have been developed for surgical treatment modalities for example hyaluronic acid products, oxidised regenerated cellulose, carboxymethycellulose gel and

steroids. They aim to reduce adhesion formation rate due to peritoneal/viscera healing following surgery[49].

Treatment for infertility associated endometriosis and adenomyosis include the conservative surgical options discussed and in vitro fertilisation with or without long downregulation protocols which aim to supress the disease prior to an IVF cycle[41]. Things to consider include the woman's age, other symptoms, concomitant infertility factors such as male factor, and ovarian reserve should be carefully considered before surgical options for endometrioma are embarked upon.

1.6 The Impact of Endometriosis and Adenomyosis on Fertility

It is known that endometriosis and adenomyosis negatively impact on fertility via multiple pathophysiological effects that are not yet fully understood.

1.6.1 Anatomical dysfunction

At the anatomical level, adhesions affecting pelvic organs and Fallopian tubes in endometriosis can cause a mechanical interference with oocyte pick up and transportation. Figure 6 (a) and (b) below demonstrate the difference between right pelvic structures of a normal pelvis (Figure 6(a)) compared to those of a patient with endometriosis, in Figure 6 (b) the bowel and fallopian tube are fused to the uterus and the ovary is adherent to the right pelvic side wall.

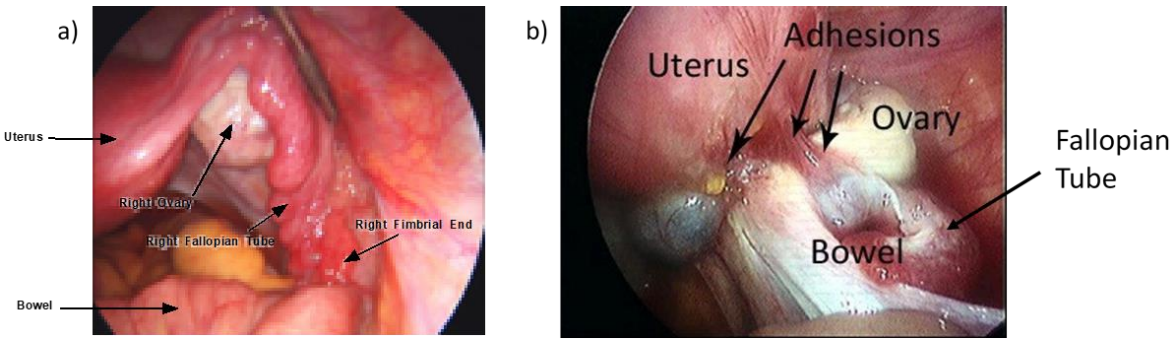


Figure 6: (a) Laparoscopic view of the normal right pelvic structures (image from "Dallas IVF" https://www.dallasivf.com/fertility-treatment/infertility-surgery/) (b) Laparoscopic view of right pelvic structures affected by adhesions from endometriosis (image from "Endometriosis Australia" https://www.endometriosisaustralia.org/single-post/2016/09/03/Can-you-diagnose-Endometriosis-via-Ultrasound)

The development of adhesions is caused by local and systemic inflammatory changes which are known to be dysregulated in endometriosis due to the level of reactive oxygen species and hyperoestrogenism. The normal process of healing involves inflammation, fibroblasts building collagen and a remodelling phase. Development of excessive scar tissue and adhesions can occur in endometriosis due to reactive oxygen species (unstable oxygen molecules missing an electron in their outer shell released during many physiological and pathological processes – see section 1.9), altered fibrinolysis, increased angiogenesis and the protease/protease inhibitor system interfering with the normal wound healing process [51]. In endometriosis and adenomyosis, the peristaltic activity of the uterus is affected. The normal pattern of peristalsis serves to improve sperm transport to the fallopian tubes in the follicular phase with increasing cervico-fundal peristalsis and reducing fundo-cervical peristalsis. The pattern then changes to aid implantation in the fundal area during the luteal phase with reduced cervico-fundal peristalsis and increasing isthmical peristalsis, characterised by contractions from the isthmus to the lower-mid uterine level. Uterine peristaltic activity is controlled by cyclical oestradiol and progesterone levels of the dominant follicle and corpus luteum. A state of hyperoestrogenism exists in women with endometriosis and adenomyosis and oestrogen receptors are overexpressed in the basalis of the endometrium and adenomyotic foci. It is observed in the uterus of these women that hyperperistalsis occurs in the follicular phase with peristaltic activity doubled in frequency and also increased amplitude [50]. This is replaced with dysperistalsis in the mid-cycle when uterine activity of a convulsive nature occurs rather than regular rhythmic contractions. An increase in the intrauterine pressure is also noted in women with endometriosis and adenomyosis. These changes can be induced in women undergoing controlled ovarian stimulation with resulting high endogenous oestrogen produced by ovarian follicles and also replicated in women given intravenous oxytocin. One clear impact of these differences is a significant reduction in directional sperm transport detected on hysterosalpingoscintigraphy (to the ipsilateral fallopian tube of the ovary containing the dominant follicle) compared to women with no disease [52,50,53]. Other anatomical differences in women with adenomyosis are those of hypertrophic myocytes and altered nerve fibres at the endometrium-myometrium interface which may also impact on the function of the myometrium.

1.6.2 Hormonal imbalance

Endometriosis and adenomyosis are conditions of hyperoestrogenism. Although the systemic levels of oestradiol tend to be similar, they measure high in the menstrual blood of women with endometriosis and adenomyosis. Abnormally high levels of P450 aromatase are thought to be responsible as it is the enzyme responsible for the conversion of androstenedione and testosterone to oestrone and oestradiol and its expression is increased in both the ectopic and eutopic endometrial tissue in these diseases. Dysregulation of progesterone receptors and progesterone resistance also feature in women with endometriosis and adenomyosis. Progesterone plays a key role in decidualisation of the endometrium and where receptors are known to be down regulated prior to implantation, gene expression of aromatase is increased in the endometrium of these conditions, leading to changes in local oestrogen production and response to progesterone which could impact on folliculogenesis and endometrial function [54].

1.6.3 Endometrial function

There is evidence that the function of the eutopic endometrium in the conditions of endometriosis and adenomyosis is affected which may lead to implantation failure[55]. Changes to the expression of implantation markers such as glycodelin and osteopontin have been implicated as well as aberrant cyclical expression of HOXA 10 gene, which is not expressed as highly in women with endometriosis at its usual peak in the mid-luteal phase of the menstrual cycle, could also affect the receptivity of the endometrium[56]. Similarly, in adenomyosis, dysregulated proteins within the myometrium indicate altered gene expression and increased activity of vascular endothelial like growth factor and matrix metalloproteinases together with altered vascular distribution (total surface capillaries per mm^2 of endometrium can be up to 10x greater in adenomyosis) could impact on the function of the endometrium.

1.6.4 Inflammation

Endometriosis and adenomyosis are chronic inflammatory conditions. A proposed pathway involves an acute inflammatory reaction from ectopic endometrial deposits leading to recruitment and activation of T Helper cells and, over time, a chronic reaction is maintained by macrophages. Patients with endometriosis and adenomyosis are known to have increased levels

of macrophages, cytokines, prostaglandins, vasoactive substances and reactive oxygen species in peritoneal fluid, follicular fluid endometrial fluid and endometrium[55,57]. The presence of these inflammatory markers can impair fertility in a number of ways including the formation of pelvic adhesions as previously described. Inflammation also reduces tubal motility and interleukin 6 (IL-6), found to be high in the peritoneal fluid of endometriosis patients, can inhibit sperm motility[58]. Other interleukins known to be higher in endometriosis (IL-1, 8 and 10) and TNF- α are high in follicles adjacent to endometriomas, which has been shown to impact on ovarian reserve[59]. Reactive oxygen species which may be higher in endometriosis patients have been implicated in DNA damage to oocytes[60] which could affect the competence of the oocyte in the process of fertilisation and embryogenesis.

In the disease of endometriosis, peritoneal fluid is in contact with endometriotic lesions. It contains higher levels of reactive oxygen species, growth factors, cytokines, macrophages, monocytes, T lymphocytes, endometrial and mesothelial cells. Cytokines and growth factors can induce or suppress angiogenesis, cell survival, proliferation, differentiation and the inflammatory response which play a role in the pathogenesis of endometriosis and its impact on fertility [61]. Peritoneal fluid is the microenvironment for several important events in the reproductive process such as ovulation, oocyte pick-up and fertilisation. Follicular fluid and peritoneal fluid constitute a large proportion of fluid found in the fallopian tubes where early embryo development takes place [62].

1.7 The Impact on Offspring due to Placentation in the Altered Uterine Environment of Endometriosis and Adenomyosis

The work of David Barker and his colleagues in 1990 first drew observations that environmental factors in-utero can lead to adult cardiovascular disease. It gave rise to the developmental origins of health and disease (DOHaD) theory which has gathered firm evidence that these environmental factors have profound effect on the programming of metabolic pathways and cell-signalling which are now known to cause childhood and adult diseases including obesity, metabolic disorders, hypertension, coronary artery disease, behavioural outcomes and some cancers through epigenetics [63].

If the disease processes underlying endometriosis and adenomyosis affect placentation and cause a disturbed uterine environment during implantation and fetal development, there could possibly be a link to childhood and adult diseases via the DOHaD hypothesis.

Evidence is increasing that the impact of these conditions reaches beyond fertility and they are associated with a number of obstetric, post-partum and neonatal complications. Some proposed mechanisms underlying the association are changes in the myometrial junctional zone leading to defective remodelling of the spiral arteries at placentation, changes to the receptivity of the endometrium affecting placentation and changes to prostaglandins [16]. Given the nature of the differences known to exist between women with these conditions and those without (discussed in section 1.2), it is logical that placentation would be affected. Altered placentation could have detrimental impact on fetal development and the course of the pregnancy with conditions such as pre-eclampsia, intra-uterine growth restriction and placenta praevia originating from placentation. Changes to hormone regulation and prostaglandin levels may be the causal link to preterm delivery and changes in the myometrium could theoretically lead to atonic post-partum haemorrhage. Evidence also suggests that the suboptimal intrauterine environment created by an imbalance between embryotrophic and embryo toxic factors, in the context of a uterine and peritoneal inflammatory condition, influences embryo programming and alters fetal development and growth trajectory after birth [64]. Mechanistically, such influence may be via embryo bio-sensing interacting with the secretome of the reproductive tract [65,66], coupled with uterine selectivity for implantation [67].

1.8 Summary of the Reproductive Impacts of Endometriosis and Adenomyosis

Endometriosis and adenomyosis can lead to distortion of the pelvic anatomy, poor folliculogenesis, oocyte DNA damage, a pathological inflammatory process in the pelvis and reproductive fluids and tissues, altered endometrial response to hormones and receptivity to an embryo and dysfunctional uterine peristalsis (see Figure 7). More recent evidence is also suggesting these conditions may be associated with aberrant placentation causing complications for the antenatal woman and her offspring following birth. Reactive oxygen species (discussed in detail in Chapter 1, section 1.9) are implicated in several of the pathological processes affecting fertility in women with endometriosis and adenomyosis but the extent of their role and whether they can be reduced is unknown.

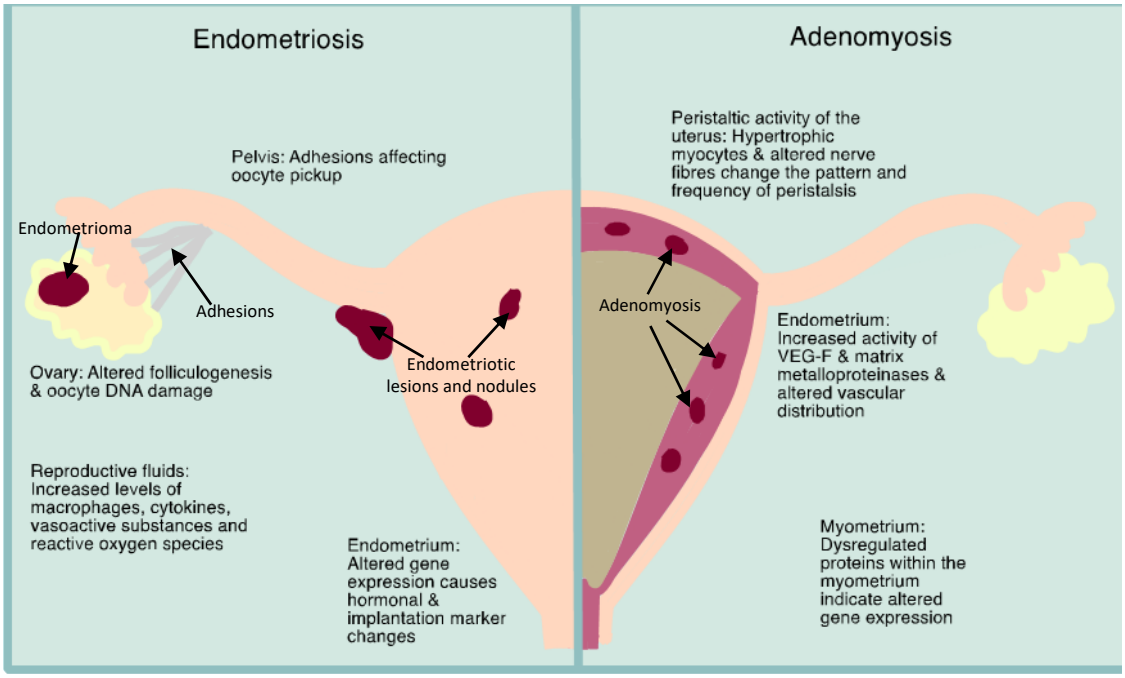


Figure 7: Diagram representing how endometriosis and adenomyosis can macroscopically affect the female reproductive tract and a summary of the impact of endometriosis and adenomyosis on fertility at the anatomical and molecular level

1.9 Reactive Oxygen Species

Reactive oxygen species (ROS) are a number of reactive molecules or "free radicals" mostly generated as a by-product of mitochondrial oxidative phosphorylation where they are released during the mitochondrial electron transport of aerobic metabolism to generate energy in the form of ATP. ROS and its closely related reactive nitrogen species can also be formed by catalytic/oxidative enzymes (e.g. NADPH oxidase, lipoxygenases and peroxisomes) and metal cations (e.g. copper and iron) in the body[68]. As well as these endogenous sources, ROS is also produced by some exogenous sources; oxidising drugs (e.g. chemotherapeutics), environmental toxins (e.g. bleach, smoking) and ionising radiation (e.g. UV or gamma radiation)[69]. Inflammatory conditions lead to a higher level of ROS production due to its effect on mitochondria and oxidative enzymes. Summarised in Figure 10(a).

These reactive oxygen molecules (Table 1) contain atoms with one or more unpaired electrons in their outer shell. They play a role as intra and intercellular messengers for processes including

gene expression and cell signalling cascades [70] and they also form a cellular immune response to cytokines and microbes. They can interact with signalling molecules such as protein tyrosine phosphatise, PI3 kinase, apoptosis signal-regulated kinase 1, redox-factor 1 and iron regulatory protein. Via these signalling molecules they contribute to cell survival, proliferation, metabolism and differentiation, anti-inflammatory and anti-oxidant defence responses and iron homeostasis.

Reactive Oxygen Species	
Superoxide	O_2^-
Singlet Oxygen	$^1O_2$
Hydroxyl	HO
Perhydroxyl radicals	HO_2
Hydrogen peroxide	H_2O_2
Alkoxy	RO
Peroxyl	ROO

Table 1: List of known reactive oxygen species

1.9.1 Oxidative stress and the impact of ROS in vivo

Reactive oxygen species are detoxified by the action of antioxidants which can scavenge and reduce these oxidised molecules (Figure 10(b)). Superoxide dismutase (SOD) converts two superoxide anions to hydrogen peroxide which can then be catalysed into water and oxygen. Glutathione peroxidase enzymes containing selenium can also catalyse hydrogen peroxide to alcohol. Glutathione, an intracellular tri-peptide molecule has an exposed sulfhydryl group which when oxidised by ROS can be reduced in a redox reaction by an NADPH-dependent reductase thus reducing levels of ROS. Many vitamins are also capable of reducing ROS such as Vitamin C and Vitamin E [71]. In this way, there can be a balance between ROS production and removal. Oxidative stress occurs when there is an imbalance towards the pro-oxidative state either by increase in ROS or by reduction in antioxidant defence mechanism. Oxidative stress results in direct or indirect damage of proteins, lipids and nucleic acids and in this way, contributes to the pathogenesis of many diseases. See Figure 10(c). Direct action of ROS has been implicated in carcinogenesis through double stranded DNA breaks, neurodegenerative diseases, cardiovascular

disease via the process of atherosclerosis and diabetes. Indirect action via its cell-signalling pathways has been found for example in promoting tumour metastasis through altered gene expression [72]. Oxidative modifications of cysteine on proteins result in a change in their structure and function [70] and oxidative side chains of amino acids and lipids lead to protein modifications and lipid peroxidation. Excess ROS increases release of Ca^{2+} from endoplasmic reticulum which causes mitochondrial permeability and cessation of ATP production [73].

In summary, a balance between reactive oxygen species and the antioxidants exists in the normal physiological state, when there is an imbalance in favour of reactive oxygen species a state of oxidative stress can occur and lead to disease as a result of damage to DNA, proteins, lipids, cell structures including those of mitochondria and by altering important cell signalling pathways.

1.9.2 Reactive oxygen species in endometriosis

Oxidative stress has been implicated in one of the pathophysiological processes involved in endometriosis. Peritoneal production of ROS and macrophage degradation of erythrocytes releasing heme and iron have been suggested causes of elevated levels of ROS. Hydroxyl molecules are produced by the Fenton reaction of iron with hydrogen peroxide ($Fe^{2+} + H_2O_2 \rightarrow FE^{3+} +OH^- +OH$)[74]. Another consequence of elevated levels of iron is an increase in nuclear factor-kappa B (NF-Kappa B) in peritoneal macrophages which as a transcriptional factor causes increased prostaglandin and affects angiogenesis [75]. Macrophages which appear to be higher in endometriosis patients also release growth factors, cytokines and other enzymes responsible for progression of the disease [37]. Demonstrated pictorially in Figure 10(a).

Oxidative stress can damage peritoneal mesothelium contributing to adhesion formation. The proliferation of epithelial cells in endometriotic lesions has been shown to increase following injection of erythrocytes in a murine model and administering an iron chelator inhibits the proliferative activity [76].

Markers of oxidative stress have been found to be elevated in endometriosis patients in serum, urine, peritoneal fluid, follicular fluid, ovarian cortex and both ectopic and eutopic endometrial tissue [4]. In follicular fluid it is theorised that ROS causes damage to the cytoskeleton, cell membrane and DNA of an oocyte affecting its development and quality which contribute to the effect of endometriosis on fertility [4,60]. Reactive oxygen and nitrogen species are found to be at a

higher level in the endometrium, particularly in the glandular epithelium. Markers of lipid peroxidation (caused by ROS) are also elevated in this tissue and levels of superoxide dismutase have been shown to be reduced [77]. Decidualisation (essential for successful embryo implantation) is mediated by progesterone and the antioxidant enzyme superoxide dismutase. In the mid-luteal phase of healthy endometrium, progesterone and copper-zinc superoxide dismutase levels are high and reactive oxygen species levels are low. Following implantation, the decidua maintains this balance of progesterone, ROS and antioxidants to sustain an early pregnancy. Before menstruation, progesterone levels and superoxide dismutase activity falls and ROS levels increase leading to endometrial shedding. This is assisted by the production of prostaglandin PGF2-α in endometrial stromal cells which causes vasoconstriction of spiral blood vessels and uterine contractions. PGF2-α production is increased by lipid peroxides which are known to be in higher concentration in the endometrium of women with endometriosis [77–79]. It is clear that a balance of reactive oxygen species and antioxidant enzymes in the endometrium are important for supporting implantation and early pregnancy and may be compromised in the condition of endometriosis where levels of reactive oxygen species and lipid peroxidation are elevated, and antioxidant enzymes are reduced. The effects of oxidative stress extend further into the pregnancy where it is known to cause placental tissue damage and contribute to poor oxygen exchange across the feto-maternal vasculature. See Figure 10(c). This mechanism has been explored in conditions such as pre-eclampsia but it has not yet been investigated whether the level of oxidative stress in endometriosis has a similar effect [80,81].

1.10 Measuring Reactive Oxygen Species and Oxidative Stress

Measuring ROS can either be performed directly, by end point markers or by surrogate markers such as an effect on DNA, intracellular redox state or antioxidant levels and can pose many difficulties in research due its dynamic nature and sensitivity in reflecting oxidative stress. Stability of ROS levels both in vivo and in biospecimens together with the large number of factors that can affect ROS levels e.g. age, concurrent stress, smoking status, inflammatory states and many different disease processes make research in this area difficult. Figure 10(d) and Figure 8 displays many of the techniques currently used to measure ROS and the main techniques will be discussed.

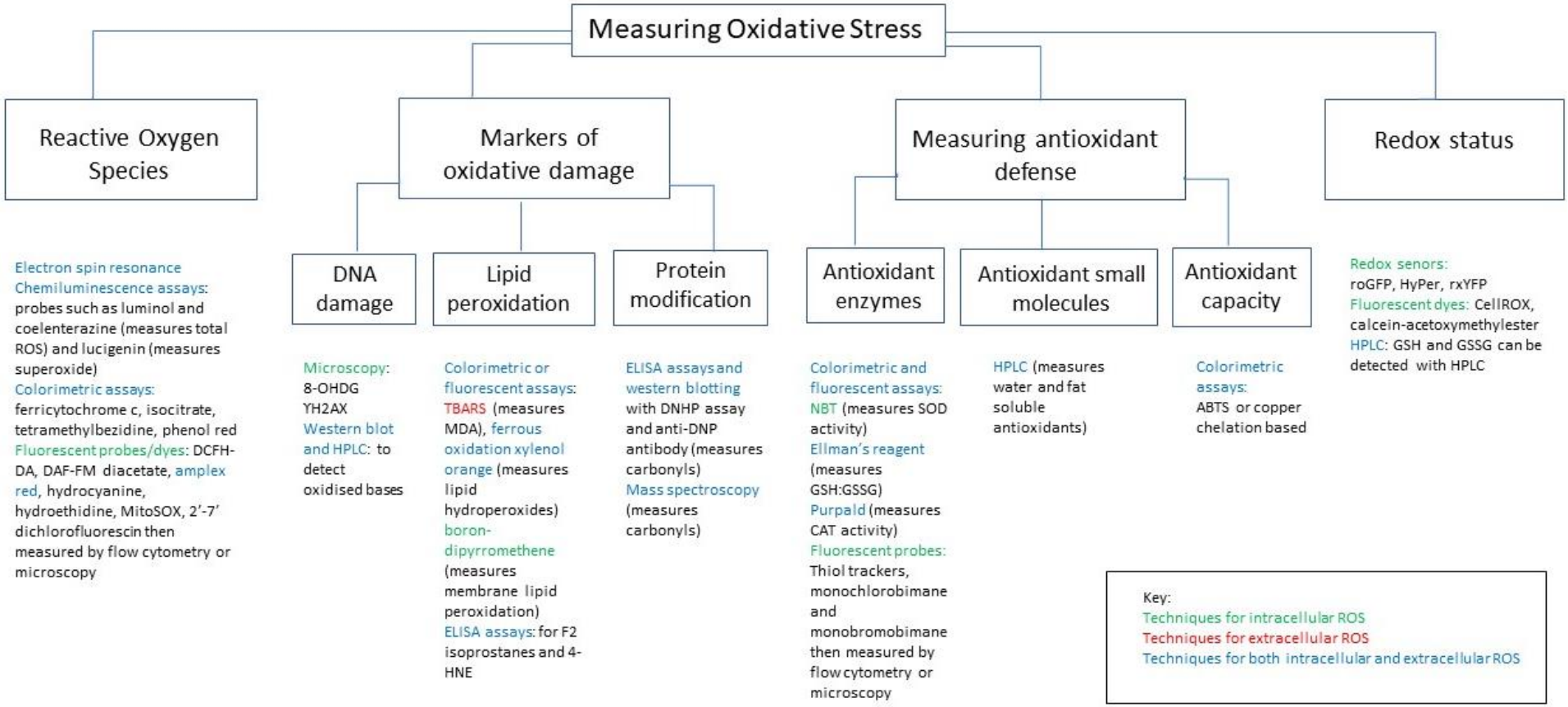


Figure 8: Summary of techniques currently used to measure ROS

1.10.1 Direct Measurement

Direct measurement of ROS has been achieved with chemiluminescence assays. Probes such as luminal (5-amino-2,3 dihydro-1,4 phthalazinedione) react with many intercellular and extracellular free radicals and produces photons of light in these reactions which can be measured with a luminometer (either current luminometers or photon counting luminometers/luminescence plate readers) and expressed as photons per minute, millivolts/second or relative light units. This technique will measure global ROS and not individual reactive oxygen or nitrogen species [82]. Alternatively, some chemiluminescent assays and fluorogenic dyes have been developed for individual ROS such as superoxide, hydroxyl and hydrogen peroxide.

ROS can also be measured with electron spin resonance, flow cytometry and fluorescent probes. Oxidation of DCFH-DA (2,7 dichlorofluorescin diacetate) by hydrogen peroxide produces fluorescence and similarly hydroethidine is oxidised to ethidium bromide (emitting red fluorescence) by superoxide free radicals. These processes are intracellular and can therefore be measured by flow cytometry techniques or microscopy which measure fluorescence.

1.10.2 Indirect measurement

Antioxidant Enzyme Activity:

Superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPO) are important antioxidant enzymes. SOD converts superoxide radicals into hydrogen peroxide and oxygen, catalase and glutathione peroxidase convert hydrogen peroxide to oxygen and water [83] (Figure 9). The amount of these enzymes can be measured with Western blot or immunofluorescent staining, or derived less directly from mRNA levels, but this does not necessarily indicate enzyme activity. The activity can be measured with assays such as nitroblue tetrazolium (NBT). Both NBT and SOD react with superoxide radical and these enzymes will compete with each other in this process. NBT produces a blue precipitate on reaction with superoxide radical. The percentage inhibition of NBT reduction relates to the amount of SOD activity [83]. GPO activity can be measured by decreasing levels of NADPH, as NADPH is consumed during H_2O_2 reduction by GPO. Oxidised glutathione (GSSG) is produced by the enzymatic action of GPO. Glutathione reductase (GR) and

oxidation of its co-substrate NADPH regenerate GSH from the GSSG. NADPH oxidation causes a decrease of absorbance at 340 nm wavelength which is directly proportional to GPO activity [82,83]. CAT, as well as catalysing H_2O_2 to oxygen and water, also has a peroxidase activity with methanol in the presence of H_2O_2 where formaldehyde is produced. This process can be measured with 4-amino-3-hydrazino-5-mercapto-1,2,4-traizole (Purpald) which binds to aldehydes and in solution turns purple from colourless when oxidised by CAT [82].

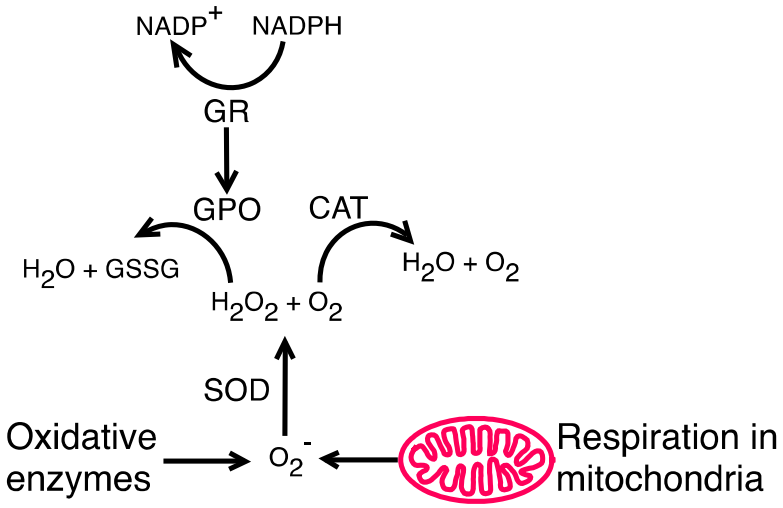


Figure 9: Antioxidant enzyme activity of superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPO).

Total Antioxidant Capacity

The combined effect of all anti-oxidants (proteins, lipids, glutathione, vitamins etc) in the extracellular fluids of the body can be measured. Anti-oxidants are able to inhibit metmyoglobin from oxidising 2,2'azino-bis(3-ethylbenzthizoline-6-sulphonate) (ABTS) to the radical cation $ABTS^+$ which is blue in colour and absorbs light at 734 nm. Another similar assay utilises copper ions which are chelated by a colorimetric probe and is reduced by anti-oxidants. These reactions can be detected by absorbance plate readers and compared to a measurable standard such as Trolox (powerful water-soluble analogue of Vitamin E).

Fat Soluble Antioxidants

Fat soluble antioxidants can be measured with high performance liquid chromatography (HPLC). Samples are mixed with ethyl alcohol containing a standard such as a-tocopherol acetate (ester of Vitamin E) and extracted with hexane. The organic phase is dried and reconstituted and separated by absorbance in HPLC columns [82].

Measurement of by-products

Proteins are susceptible to oxidation induced modifications of amino acid side chains into carbonyls which then alters the protein structure. Lipids similarly undergo peroxidation due to ROS and these are metabolised to malondialdehyde (MDA) and 4-hydroxynonenal (4-HNE). MDA formation can be measured with thiobarbituric acid-reacting substances (TBARS). MDA adducts with two thiobarbituric acid molecules to produce a pink precipitate which absorbs at 532-535 nm and is measured with absorbance spectrophotometry. Many other compounds can interfere with this assay such as aldehydes, oxidised protein breakdown products and urea. Additional techniques such as chromatographic separation of malondialdehyde-thiobarbituric acid adduct or HPLC-based methods can improve the accuracy [84]. A urinary stable metabolite of 4-HNE, 1-4-dihydroxynonane-mercapturic acid can also be measured. In addition, lipid hydroperoxide can also be measured with assays such as FOX reagents (ammonium sulphate, sulphuric acid, butylated hydroxytoluene and xylenol orange) [84]. Lipid hydroperoxide is estimated indirectly by peroxide-mediated oxidation of iron in the acidic conditions of the assay and the oxidised iron forms an orange complex with the xylenol which has absorbance at 560 nm wavelength [82]. Protein carbonyls can be measured both in fluids and tissue in a number of ways including enzyme linked immunosorbent assay (ELISA), Western blotting and mass spectrometry. They can also be measured using a di-nitrophenol hydrazine (DNPH) assay. DNPH reacts with the carbonyls to form a Schiff base, dinitrophenol hydrazine (DNP)-carbonyl which can be detected either with antibodies to DNP and an immunoassay or with spectrophotometry by measuring absorbance at 360 nm wavelength [85].

Measurement of damage – Detecting Oxidative DNA damage

Oxidative damage to DNA bases can be assessed with immunofluorescence by using antibodies to specific oxidised bases such as 8-hydroxy 2-deoxyguanosine, or markers of DNA damage such as

γH2AX. Oxidised bases can also be measured with Western blotting, ELISA and HPLC with electrochemical detection (HPLC-ECD) and gas chromatography-mass spectrometry. ELISA is based on the antigens which detect proteins damaged by ROS. A fluid is analysed for the amount of targeted proteins by spectrophotometry of the desired antibody [82,86].

Detecting Oxidative RNA damage

Modifications of RNA and DNA occur when ROS reacts with nucleobases. Guanine bases are particularly vulnerable and more reactive with ROS such as hydroxyl and singlet oxygen. Common products of this reaction include 8-oxo-2'-deoxyguanosine (DNA damage) and 8-hydroxyguanosine (RNA damage). They are used extensively as biomarkers of oxidative stress and can be measured with high-performance liquid chromatography or gas chromatography (with electrochemical detection or mass spectrometry) and immunocytochemistry [87].

Immunofluorescence is a cell or tissue imaging technique. Antibodies that are chemically conjugated to fluorophores are used to visualise specific target antigens with fluorescent microscopy. Direct immunofluorescence uses an antibody conjugated to a fluorescent dye to probe the antigen of interest, whereas the indirect method uses a primary antibody to target the antigen of interest, with a secondary fluorescently conjugated antibody to target the primary antibody. Indirect immunofluorescence increases the sensitivity of the test but can increase background fluorescence. It can be used to localise and quantify expression levels of target antigens.

An antibody for an altered base pair 8-oxodeoxyguanoisine can be identified indicating base pair alteration RNA damage has occurred.

HPLC, developed from column chromatography, uses a pump to pressurise a sample dissolved in liquid solvent through a column containing adsorbent material. In this process molecules from the sample adhere to the surface of the material in the column and their interactions will differ. The flow rate for each component of the sample through the column will therefore be used to differentiate them by signal peak patterns of the detector and quantities of each component may be determined by amplitude of the signal. In gas chromatography the sample of interest is carried by an inert gas rather than a solvent through the column coated most commonly with a liquid adsorbent surface. Various methods of detection can be coupled with HPLC or gas

chromatography including electrochemical detection and mass spectrometry. ECD interprets an electrical current generated by oxidation/reduction reactions. Any electrochemically active molecules separated onto the chromatographic column can be detected when a current is applied. Mass spectrometry identifies molecules by ionising them, accelerating the resulting charged fragments and sorting them by mass-charge ratio with an electric or magnetic field. The original molecules can then be determined by characteristic fragmentation patterns.

Detecting Other RNA Damage

Other forms of RNA damage can occur through oxidation and by other mechanisms such as ultraviolet radiation. Similarly to oxidation, alkylation, chlorination and nitration can occur as well as crosslinking, photochemical modification and strand breakage, which can all lead to translational disturbance and altered protein synthesis or gene expression [88]. Polymerase chain reaction (PCR) is a technique used to generate copies of targeted sequences exponentially by the action of DNA polymerase and use of selected primers. The primers are short pieces of single stranded DNA complementary to the desired target sequence, which anneal to the area of DNA for replication when the DNA strand is denatured. DNA polymerase adds nucleotides (single units of DNA bases) from the 3'-OH group at the end of the primer onwards in an elongation/extension step, thus synthesizing new DNA strands. Reverse transcription PCR (RT-PCR) is PCR preceded by conversion of RNA to cDNA via reverse transcription. In this way either DNA or RNA sequences can be analysed with PCR methods. Real time quantitative PCR (qPCR) measures the generated products during each cycle of the PCR by detecting a fluorescent dye on an oligonucleotide probe. The probe is labelled with a reported fluorophore which emits at a wavelength absorbed by a quencher fluorophore of the same probe. When the probe is cleaved from the single strand of DNA during extension of the PCR cycle the reporter is released from the quencher and emits fluorescence. PCR products are analysed with gel electrophoresis.

Redox state

The ratio of reduced glutathione (GSH) to the oxidised glutathione (GSSG) indicates the oxidative state of an organism, lower ratios suggesting oxidative stress. Glutathione regenerates a number of important antioxidants (e.g., α-tocopherol and ascorbic acid). GSH and GSSG levels can be measured by HPLC, capillary electrophoresis, or assays which use reagents such as Ellman's reagent (DTNB) and glutathione reductase (see antioxidant enzyme activity regarding GPx),

Luciferin-NT for luminescence (Promega GSH/GSSG-Glo Assay) or o-phthalaldehyde probe (OPA) for fluorescence (Biovision Glutathione Fluorometric Assay Kit). Another test for redox potential is the redox sensitive green fluorescent proteins (roGFP1 and 2). The green fluorescent protein (GFP) has been engineered with the addition of two cysteines to amino acids at position 147/204. At these positions the cysteines can form disulphide bonds when oxidised giving the GFP different fluorescent properties when reduced (as dithiol) or oxidised (as disulphide). RoGFP has two excitation peaks at ~425 nm and ~490 nm with a fluorescence emission wavelength of ~510 nm. When the added cysteines are reduced the excitation is 480 nm and when oxidised excitation is at 400 nm allowing the redox state of the roGFP's environment to be assessed[89,90].

1.11 The Questions Left Unanswered and Difficulties of Research in This Area

Endometriosis and adenomyosis continue to be enigmatic diseases in terms of the pathophysiology, classification and their clinical impact and management. Their impact on fertility may be far reaching, including the health of the offspring of women with these conditions. Adenomyosis appears to be the most elusive in terms of diagnosis and non-consensus on its classification, thus posing a diagnostic problem for clinicians. The non-unified approach to diagnosis and management often makes it problematic to research. Considering these difficulties this body of work beyond Chapter 3 will focus mainly on endometriosis which can be diagnosed more reliably.

Reactive oxygen species are potentially an important cause of reproductive health problems in this group of women as they may affect many aspects of the reproductive process. ROS remain a particularly poorly understood mechanism in endometriosis yet is an aspect that may produce a non-invasive marker of disease and a possible therapy in the evolving area of antioxidants. They are problematic in research due to their dynamic nature and inconsistencies in methods used to measure them. There is some doubt about sensitivity of many currently used assays in detecting ROS levels. A reliable assay for both systemic bodily fluids and reproductive fluids will need to be explored further. The impact of ROS on DNA of the oocyte has been demonstrated but its impact on the more relevant RNA (since the oocyte is transcriptionally silent for most of its cellular lifespan and during this time RNA can be damaged but not replaced) has not been considered. One reason for this may be the ethical and practical difficulties in obtaining human oocytes for research. A possible solution would be use of an animal model.

The mouse (mus musculus) is a commonly used model for research in fertility both historically and in current research. Although the mouse has an estrous cycle rather than a menstrual cycle there are many similarities between the two and similarities in folliculogenesis which make it a popular animal model for fertility experimental work[91,92]. The mouse provides a mammalian model with several advantages. Mice are easy to breed, handle and care for in terms of time and cost-efficiency as a small, resilient mammal with a short oestrous (reproductive) cycle. It is also relatively easy to keep their respective environments well controlled and stable or to make intentional manipulations according to specific experimental needs. They respond well to ovarian stimulation and have many genetic and physiological similarities to homo sapiens. The haploid genome of the mouse consists of 20 chromosomes and in the human haploid genome is 23 chromosomes and they are similar in size in terms of picograms and megabases (mus musculus: 2689.66 Mb median length and homo sapiens: 2994.26 Mb median length). Given the size of the mouse compared to humans, its oocytes are surprisingly similar in size (60-70 μm compared to ~100 μm human oocyte) and cytoskeletal structure.

The extent of mouse model research is beneficial as the genome of many strains are reliably known and due to thorough and strict in-breeding there are over 450 strains which have a fixed genetic background with good reproducibility and limited spontaneous changes through time. In addition, mice can be genetically engineered for a range of research purposes. Transgenic and knockout mice can be very useful to mimic different human diseases and endometriosis has been replicated in the mouse by simple intraabdominal injection of endometrial cells.

1.12 Thesis Synopsis

1.12.1 Aims

1. This thesis sets out to investigate the influence of endometriosis and adenomyosis on the reproductive process from fertility parameters to perinatal outcomes and explore a possible link to DOHaD in women with the disease.
2. To investigate whether reactive oxygen species levels are different in women with endometriosis compared to women without endometriosis and whether levels of reactive oxygen species are improved following surgical treatment of disease using an ethically approved human study.

3. To explore how reactive oxygen species affect the oocyte using a reproductive fluid of women affected by endometriosis from an ethically approved human study and oocytes in a mouse model.

1.12.2 Outline

Chapter 2 details all materials and methods for experimental work of the thesis. Chapters 3 & 4 address the first aim. Chapter 3 investigates what is already known about the impact of endometriosis and adenomyosis and their subtypes on the reproductive process with an extensive systematic review and meta-analysis. Chapter 4 builds on current knowledge and pioneers observational research in this area by exploring a possible role of DOHaD in a large population-based cohort. Chapter 5 addresses the second aim of the thesis by exploring the local (in peritoneal fluid) and systemic (serum and urine) levels of ROS in women with endometriosis compared to women without endometriosis and investigates whether levels systemically are improved with surgical treatment of the disease. Chapter 6 focuses on the third aim of the thesis and examines a possible effect of ROS on oocyte quality, from which many reproductive problems stem, by studying the redox level in the oocyte and consequential RNA damage.

1.12.3 Hypotheses

This work hypothesises that endometriosis and adenomyosis have a significant impact on every aspect of the reproductive process to the detriment of women affected and that problems associated with antenatal and neonatal course will point towards a link with DOHaD theory. With an investigation into levels of ROS before and after surgery, this work hypothesises that ROS are increased in women with endometriosis both locally and systemically and levels show improvement systemically following surgical treatment. It hypothesises that ROS can be implicated in poorer oocyte quality in women with endometriosis through damage to maternal RNA. This work and its hypotheses are represented on a schematic diagram (Figure 10).

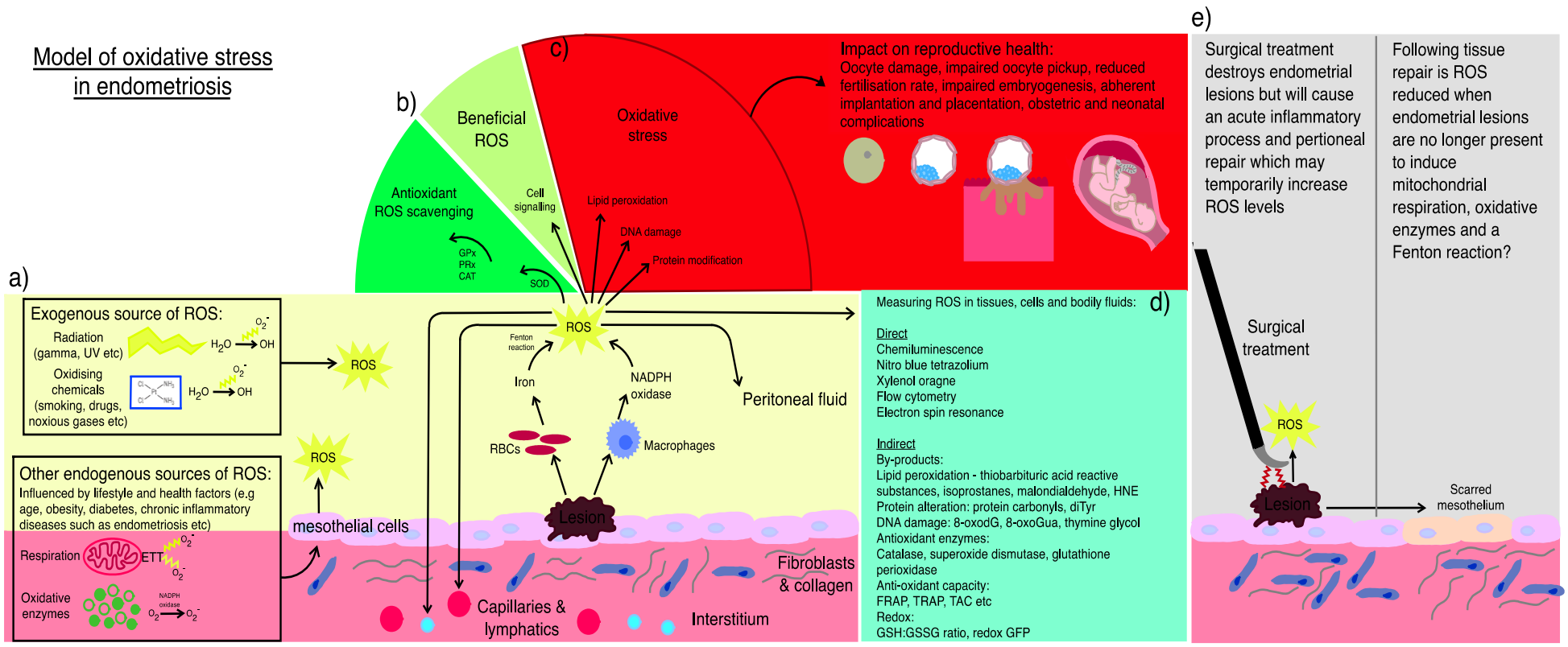


Figure 10: A model of oxidative stress in endometriosis and concepts of the thesis

Chapter 2 Materials and Methods

All methodologies in this Chapter have been carried out by the thesis author as part of a PhD qualification unless stated otherwise. Where any methodology or work has been carried out by someone other than the author, their name and the nature of the work is declared.

2.1 Human Studies

In order to investigate the levels of oxidative stress in women with endometriosis compared to women without endometriosis human samples of serum, urine and peritoneal fluid from endometriosis "cases" and non-endometriosis "controls" were obtained. Serum and urine are potential sources of non-invasive biomarkers of disease [93] due to their ease of collection, a range of markers of endometriosis can be measured in these biospecimens and in some diseases, markers of oxidative stress has correlated between serum and urine [94]. The reproductive fluid of peritoneal fluid and systemic fluids of blood and urine were investigated as these can be easily collected at surgery for endometriosis and oocytes are significantly exposed to peritoneal fluid between ovulation and oocyte pickup. Peritoneal fluid also contributes to a high percentage of fallopian tube fluid. Other reproductive fluids which could be obtained are follicular fluid, fallopian tube fluid and uterine fluid. Fallopian and uterine fluid could be collected at the time of surgery for endometriosis however the volumes of fluid would be limited and may not be adequate for the full spectrum of intended assays and experiments of this body of work. The disadvantage of follicular fluid is that fertile women could not be included for subgroup analysis of the effects of ROS levels on fertility, the endometriosis is most likely to be historical to varying degrees and confirmation of current endometriosis or details such as ASRM score would not be available.

2.1.1 Ethical approval

A previously approved study "Peritoneal fluid biology in health and disease" was used for initial sample collection to develop protocols for assays. This study was commenced in 2008 by Miss Fowziya Sheekh-Hussein, at which time it met regional ethics committee approval, University of Southampton ethics committee approval and was registered with the Research and Development

department of Southampton General Hospital. For use in this work the study was re-registered with the current Integrated Research Application System (IRAS) and two amendments were approved by the aforementioned bodies. All patients gave informed written consent to take part in the study (Appendices 5-8: Peritoneal Fluid Biology study documents).

For ongoing work patients gave informed written consent to take part in the study "XSESS: oXidative Stress in women with Endometriosis pre and poSt Surgery" (IRAS project ID 246994). This study was set up and met ethical approval with a national ethics committee, the Faculty of Medicine ethics committee of the University of Southampton and was sponsored by the Research and Development Department of Southampton General Hospital (Appendices 9-12 XSESS study documents).

2.1.2 Study population

The case and control cohorts for both studies were from a population of women with known or suspected endometriosis undergoing investigation and treatment for the disease at the gynaecology department of Princess Anne Hospital, Southampton. "XSESS" study also included women undergoing tubal sterilisation at Princess Anne Hospital. This is a secondary referral centre for gynaecological conditions and a tertiary referral centre for managing advanced endometriosis.

2.1.3 Inclusion and exclusion criteria

Inclusion:

- Women undergoing surgery (laparoscopy or laparotomy) for possible endometriosis
- Age 18-65 years
- Women undergoing tubal sterilisation additionally included in "XSESS" study

Exclusion:

- Menopausal women
- Active infective diseases
- Pregnancy

2.1.4 Recruitment

Eligible participants were recruited from the gynaecology department at Princess Anne Hospital. The legal age to consent to research in the UK is 16 years but at Princess Anne Hospital, young women <18 years are generally seen in paediatric gynaecology and were therefore not recruited to our study. An upper age limit of 65 years was set to be as inclusive as possible to women who may be receiving treatment for endometriosis. Screening was performed and women fulfilling the inclusion/exclusion criteria were sent an information leaflet and letter of invitation prior to their surgery. Those women were approached on the day of their surgery and if interested in taking part, were able to discuss the study. Women who decided to participate completed a consent form and were asked questions pertaining to medical history to complete the WERF-EPHect (World Endometriosis Research Foundation Endometriosis Phenome and biobanking harmonisation project) study proforma (page 24-30 of Appendix 1 within Appendix 20). For XSESS study these consenting women also completed a pelvic pain questionnaire. Recruited participants then underwent their surgical treatment as planned and attended a follow up appointment with their clinical team, at which time they repeated a pelvic pain questionnaire and had a blood sample and urine sample taken for research.

A power calculation for required sample size to achieve 95% confidence intervals in differences of reactive oxygen species levels before and after surgery was performed based upon available literature of reactive oxygen species levels in endometriosis [4,95] and a similar study measuring reactive oxygen species in seminal fluid pre and post varicocelectomy surgery [96]. This power calculation concluded that 31 cases and 31 controls would be required to achieve significance of <0.05 which provided the recruitment target.

2.1.5 Equipment for sample collection and processing

- red top SST vacutainer collection tube (Becton Dickinson, US)
- conical urine tube with no additive (Becton Dickinson, US)
- Wallace Assisted Reproduction Embryo Replacement Catheter (Cooper Surgical, US)
- Plastipak 20 ml hypodermic luer slip syringe (Becton Dickinson, US)
- 14 French gauge Ryles tube (Pennine Healthcare, UK)
- Multisticks 10SG urine test strips (Siemens, Germany)

- Sterile Extended Fine Tip Pipette with bulb draw (Alpha Laboratories, UK)
- 2 ml Cryogenic vial with internal thread (Alpha Laboratories, UK)
- 2 ml reaction tubes (Greiner Bio-one, Austria)
- 0.5 ml Nunc Cryotube vials with internal thread (Thermo Fisher Scientific, US)
- MyFuge 5 Microcentrifuge with combination rotor (Sigma-Aldrich, US)
- Sterile microlance 23-gauge needle (Becton Dickinson, US)
- Plastipak 5 ml hypodermic luer slip syringe (Becton Dickinson, US)
- 5x5 cm 4ply non-woven sterile swab (Unisurge International, UK)
- V-Grip single use latex-free tourniquet (Richardson Healthcare, UK)

2.1.6 Sample Collection and processing

Peritoneal Fluid Biology Study

When the anaesthetist sited a venous cannula for a recruited patient, a 4 ml sample of blood was obtained in a red top SST vacutainer collection tube, allowed to clot at room temperature for 30 minutes and then placed on wet ice. A general anaesthetic was then given, and the patient was transferred into the operating theatre. The perineum and abdomen were cleaned with betadine and the patient draped with surgical drapes. The bladder was emptied with a sterile urinary catheter and an 8 ml sample collected into a conical urine tube with no additive and placed on wet ice. The surgeon then made a 1 cm incision at the base of the umbilicus and either performed a veress needle entry or a cut down entry into the abdomen which was insufflated to 15-20 mmHg with carbon dioxide. A 10 mm endoscopic trocar was inserted in the umbilicus and patient placed in Trendelenburg position. Between one and three 5 mm ports were inserted in the suprapubic +/or left iliac fossa +/or right iliac fossa areas based on anticipated extent of ongoing laparoscopic surgery required. If one port was being used a Wallace Assisted Reproduction Embryo Replacement Catheter was inserted under vision into an area of pooled peritoneal fluid in the pelvis. Fluid was withdrawn with a 20 ml hypodermic luer slip syringe and emptied into a conical urine collection tube with no additive and placed on wet ice. If multiple ports were inserted the peritoneal fluid was collected with a 14 French gauge Ryles tube with a cut tip and 20 ml syringe. If there were no visible pools of peritoneal fluid the pelvis was washed with up to 20

mls of normal saline and the washings collected using the aforementioned technique. A WERF-EPHect surgical form was completed (Appendix 20) to describe any pelvic disease.

Samples were taken on wet ice to the laboratory at the Complete Fertility Centre (Princess Anne Hospital) or to the molecular biology laboratory at the Institute of Developmental Sciences (University Hospital Southampton). Dipstick urinalysis of the urine was performed using Multisticks 10SG urine test strips, if specific gravity was <1.001 or >1.032 it was retested for accuracy, results were recorded. 2 ml of unprocessed urine was aliquoted into one 2 ml cryogenic vial with internal thread. The remaining urine and the blood sample was centrifuged in their collection tubes at 2500 xg at 4°C for 10 minutes and placed upright on wet ice. The supernatant was aliquoted using sterile extended fine tip pipette with bulb draw into 100 µl samples in 2 ml cryogenic vials. If a white blood cell layer was also present beneath the plasma supernatant (buffy coat layer), this would be aliquoted separately into a cryogenic vial. Red blood cells (the sediment/pellet of the blood collection tube) and the pellet of the urine was aliquoted into separate cryogenic vials. Peritoneal fluid was centrifuged at 900 xg at 4°C for 5 minutes and the supernatant aliquoted into 100 µl samples in cryogenic vials. The pellet was aliquoted into a cryogenic vial. All samples were labelled with study ID number, description of sample type, date of processing and volume of sample. They were stored immediately at -80°C in the Complete Fertility Centre research freezer. Protocol was followed as per WERF-EPHect Standard Operating Procedures for collection, processing and storage of fluid biospecimens in endometriosis research (Appendix 20).

XSESS Study

The above protocol was followed with modifications as per the advice and guidance of Professor Martin Feelisch, Professor of Experimental Medicine and Integrative Biology at the University of Southampton. These modifications were to minimise any ongoing oxidative or reducing processes in the sample once removed from the participant and reduce the amount of oxidation from sample being exposed to atmosphere within a cryovial in storage thus more accurately reflecting the in vivo ROS levels.

Collection of pre-operative samples

When the anaesthetist sited a venous cannula for a recruited patient, a 5-10 ml sample of blood was obtained in a 10 ml hypodermic luer slip syringe and transferred immediately to 2 ml reaction tubes and centrifuged in MyFuge5 at 2000 xg for 3 minutes. The supernatant (serum) was aliquoted using a sterile extended fine tip pipette with bulb draw into six 100 µl samples in 0.5 ml cryogenic vials, any remaining supernatant was aliquoted into 2 ml cryogenic vials. If a white blood cell layer was also present beneath the plasma supernatant (buffy coat layer), this would be aliquoted separately into a 2 ml cryogenic vial. Red blood cells (the sediment/pellet of the blood collection tube) were aliquoted into separate 2 ml cryogenic vials. All were labelled with study ID, date of collection and type of sample and placed on dry ice. A general anaesthetic was then given, and the patient was transferred into the operating theatre. The perineum and abdomen were cleaned with betadine and the patient draped with surgical drapes. The bladder was emptied with a sterile urinary catheter and an 8 ml sample collected into a conical urine tube with no additive. Dipstick urinalysis of the urine was performed using Multisticks 10SG urine test strips, if specific gravity was <1.001 or >1.032 it was retested for accuracy, results were recorded and urine then transferred immediately to 2 ml reaction tubes, then centrifuged in MyFuge5 at 2000 xg for 3 minutes. The supernatant was aliquoted using into six 100 µl samples in 0.5 ml cryogenic vials, any remaining supernatant was aliquoted into 2 ml cryogenic vials. All were labelled with study ID, date of collection and type of sample and placed on dry ice. The surgeon then made a 1 cm incision at the base of the umbilicus and either performed a veress needle entry or a cut down entry into the abdomen which was insufflated to 15-20 mmHg with carbon dioxide. A 10 mm endoscopic trocar was inserted in the umbilicus and patient placed in Trendelenburg position. Between one and three 5 mm ports were inserted in the suprapubic +/or left iliac fossa +/or right iliac fossa areas based on anticipated extent of ongoing laparoscopic surgery required. If one port was being used a Wallace Assisted Reproduction Embryo Replacement Catheter was inserted under vision into an area of pooled peritoneal fluid in the pelvis. Fluid was withdrawn with a 20 ml hypodermic luer slip syringe and emptied into 2 ml reaction tubes. If multiple ports were inserted the peritoneal fluid was collected with a 14 French gauge Ryles tube with a cut tip and 20 ml syringe and transferred into 2 ml reaction tubes and centrifuged in MyFuge5 at 2000 xg for 3 minutes. The supernatant was aliquoted into six 100 µl samples in 0.5 ml cryogenic vials, any remaining supernatant was aliquoted into 2 ml cryogenic vials. All were labelled with study ID,

date of collection and type of sample and placed on dry ice. A WERF-EPHect surgical form was completed (Appendix 20) to describe any pelvic disease.

Samples were taken immediately on dry ice to the molecular biology laboratory at the Institute of Developmental Sciences (University Hospital Southampton) and snap frozen in liquid nitrogen. They were then stored at -80°C in the Complete Fertility Centre research freezer.

Collection of follow up samples

For the additional collection of blood and urine samples at follow-up, blood was taken via standard venepuncture technique and participants produced a urine sample into a sterile pot. These samples were immediately centrifuged in 2 ml reaction tubes at 2000 xg for 3 minutes in MyFuge5. Supernatants were aliquoted into six 100 µl samples in 0.5 ml cryogenic vials, any remaining supernatant was aliquoted into 2 ml cryogenic vials. All were labelled with study ID, date of collection and type of sample and placed on dry ice. Samples were taken immediately on dry ice to the molecular biology laboratory at the Institute of Developmental Sciences (University Hospital Southampton) and snap frozen in liquid nitrogen. They were then stored at -80°C in the Complete Fertility Centre research freezer. Dr Gayatri Upsani and Dr Bonnie Ng assisted with collection of some follow up samples during a period of maternity leave.

2.2 Reactive Oxygen Species Measurement

A number of widely used commercial assays for exploring oxidative stress levels were selected from techniques covering direct measurement, antioxidant levels and by-product measurement.

2.2.1 Total ROS assay

Luminol assays detect peroxidase conjugates (peroxide free radicals) via a reaction involving enzymatic generation of acridinium ester intermediates which react with peroxide to produce chemiluminescence. A commercial assay kit used in this chapter detects peroxide catalysed oxidation of Luminol substrate as it generates thousands of acridinium ester intermediates per minute which produce a sustained, high intensity chemiluminescence.

Materials & Equipment:

- Amersham ECL Prime Western Blotting Detection Reagent Kit (GE Healthcare, UK)
  - Solution A - Luminol Enhancer Solution (Cyclic Diacylhydrazide luminol)
  - Solution B - Peroxide Solution (H_2O_2 in Tris buffer)
- Horse radish peroxidase (HRP) (Sigma Aldrich, US)
- 1x Phosphate buffered solution (PBS) (see Appendix 21)
- 99% Xanthine (Thermo Fisher Scientific, US)
- Xanthine Oxidase (Roche, Switzerland)
- 5 M NaOH
- 2 ml reaction tubes (Greiner Bio-One, Austria)
- Pipettes and sterile pipette tips
- Biolite 96 well flat-bottomed plate (Thermo Fisher Scientific, US)
- Vortex Genie (Scientific Industries)
- Glomax Discover plate reader (Promega, US)

Protocol

500 µl of "mastermix" required to prime the Glomax injectors. Additional 50 µl of "mastermix" produced for each standard, positive control and sample in each experiment. For example, 750 µl of "mastermix" required for testing 5 samples. 750 µl "mastermix" made by aliquoting 733.88 µl of luminol enhancer solution (Amersham ECL Prime Western Blotting Detection Reagent Kit) and 16.12 µl peroxide solution (Amersham ECL Prime Western Blotting Detection Reagent Kit) and mixing with vortex genie 2 (Scientific Industries, US) (45.5:1 ratio).

Producing a standard curve and positive control: 1000 µM xanthine made by dissolving 1 mg of xanthine in 50 µl of 5 M NaOH on a vortex genie. 12.5 ml of PBS then added and mixed on vortex genie. Dilutions of xanthine for 0 µM, 500 µm, 700 µm and 1000 µM made as follows; 50 µl of PBS, 25 µl of 1000 µM xanthine in 25 µl PBS, 34.99 µl of 1000 µM xanthine in 15.01 µl PBS and 50 µl of 1000 µM xanthine. Dilutions of xanthine added to alternate wells of a 96 well plate. Alternate wells used to avoid contamination luminescence from neighbouring wells when reading the plate. 2.5 µl of xanthine oxidase added to 47.5 µl of PBS and mixed on vortex genie to make 50 µl of 1 unit/ml xanthine oxidase. 10 µl of 1 unit/ml xanthine oxidase added to wells of a 96 well

plate containing xanthine serial dilutions. HRP positive control made with 0.5 µl HRP in 499.5 µl PBS. 10 µl of 1:10^3 HRP added to a well of the 96 well plate.

10 µl of samples (urine, serum and peritoneal fluid from patients with and without endometriosis) were pipetted into alternate wells of the 96-well plate.

Literature using a similar assay performed 5 minute incubations of their assay prior to luminescence measurement [97] therefore measuring chemiluminescence from total ROS assay was initially performed with a confocal microscope. However, luminescence was observed with the naked eye to occur instantly, and the reaction was observed to last <1 second, no luminescence was detected with the confocal microscope after transfer of the 96-well plate to the stage. Total ROS assays were therefore measured using Glomax Discover plate reader with inbuilt injectors to read luminescence instantaneously and at further defined time-points following injection of the reagent into samples.

The 96-well plate transferred to Glomax Discover plate reader and an injector flushed and primed with 500 µl luminol mastermix.

A luminescence protocol was created with Glomax Discover software to read all relevant wells at baseline (0.3 seconds) followed by injection of 50 µl luminol mastermix into a well containing sample, standard or control followed by a measurement of luminescence at 0.2 seconds, 0.5 seconds, 0.8 seconds, 1.1 seconds and 1.4 seconds, 1.7 seconds and 2 seconds post injection. Injection step and plate read sequence was repeated for all other wells containing sample, standard or control of the 96-well plate.

2.2.2 TAC assay

The total antioxidant capacity kit used in this chapter measures small molecule antioxidants in fluid biospecimens (Vitamin C, Vitamin E, glutathione etc) and compares their antioxidant capacity to a trolox (a strong antioxidant) standard curve. The small molecule antioxidants reduce copper ions which are then chelated by a colorimetric probe in the assay. The degree of chelated probe will affect the amount of absorbance of the fluids when measured at its peak wavelength of 570 nm.

Materials & equipment:

- Abcam TAC assay kit (Abcam, UK):
  - Trolox 1 mM standard (1 µmol trolox in 20 µl of anhydrous DMSO and diluted in 980 µl ddH_2O)
  - Copper ion (Cu^{2+}) reagent
  - Protein mask
  - Assay diluent
- Eppendorf tubes of various sizes, pipettes and pipette tips
- Biolite 96 well flat-bottomed plate (Thermo Fisher Scientific, US)
- Fluostar plate reader with optima control software (BMG Labtech, Germany)
- . Vortex Genie (Scientific Industries)

Protocol

A trolox standard dilution was made from 1 mM stock and ddH_2O with 0 nmol, 4 nmol, 8 nmol, 12 nmol, 16 nmol and 20 nmol of trolox in 100 µl volumes. Due to the protein content, the human samples were mixed 1:1 with protein mask which prevents Cu^{2+} reduction by protein and ensures only small molecule antioxidant is measured. 1 µl of serum, peritoneal fluid and urine samples from endometriosis patients and non-endometriosis control patients were mixed with 1 µl protein mask on vortex genie and then diluted with 998 µl ddH_2O on vortex genie (based on protocol refinement experiments finding concentration of sample greater than 1:1000 resulting in readings of absorbance beyond those of the standard curve). Cu^{2+} working solution was made from the Cu reagent with assay diluent 1:49 based on using 100 µl of the Cu^{2+} solution for every well of the TAC assay and protected from the light with tin foil.

100 µl of each trolox dilution standard was aliquoted into sequential wells of a 96 well flat-bottomed plate, a negative control of 100 µl ddH_2O was aliquoted into a well of the plate. Prepared human biospecimen dilutions were aliquoted to sequential wells of the 96 well flat-bottomed plate. 100 µl of Cu^{2+} working solution was added to each standard, sample and control well in the dark room and the plate was protected from light with tin foil and incubated at room temperature on an orbital shaker for 90 minutes.

A protocol for absorbance at 570 nm wavelength with 20 flashes per well was designed with Optima Control software for Fluostar plate reader.

2.2.3 GSH/GSSG assay

This assay contains a non-fluorescent dye which becomes fluorescent when reacting with the antioxidant reduced glutathione (GSH) or the oxidised glutathione (GSSG) with excitation at 490 nm wavelength and emission at 520 nm wavelength. When cells experience higher oxidative stress, there is increased GSSG to GSH ratio. This assay therefore aims to measure the redox status of the cells. Samples must be deproteinised, proteins are precipitated by lowering the pH followed by centrifugation, once the protein pellet is removed the sample pH can be neutralised.

Materials & Equipment

- Abcam GSH:GSSG ratio detection assay kit II (Abcam, UK):
  - Thiol green indicator
  - GSH standard
  - GSSG indicator
  - GSSG probe
  - Assay buffer
- Abcam Deproteinising sample preparation kit (Abcam, UK)
  - Trichloroacetic acid (TCA)
  - Neutralisation buffer
- Eppendorf tubes of various sizes, pipettes and pipette tips
- ddH_20 or 1x PBS
- Tin foil
- Biolite 96 well flat-bottomed plate (Thermo Fisher Scientific, US)
- Fluostar plate reader with optima control software (BMG Labtech, Germany)
- Vortex Genie (Scientific Industries)

Protocol

Human biospecimens of 100 µl serum, peritoneal fluid and urine of endometriosis patients and non-endometriosis control patients were deproteinised by adding 15 µl of chilled TCA and placing

on ice for 15 minutes followed by centrifugation at 12000 xg for 5 minutes. The supernatant (75 µl) was collected, and pellet discarded. 10 µl of neutralising buffer was added to the supernatant of the samples in open Eppendorf tubes and placed on wet ice for 5 minutes. The samples are now 76% concentration due to added reagents. 50 µl of each sample was diluted in 50 µl of ddH_2O. 50 µl of this sample was aliquoted into suitable wells of a Thermofisher Biolite 96 well plate for a GSH reaction and 50 µl into other suitable wells for a total GSH and GSSG reaction (38% concentration of original sample following deproteinising process and dilution), the remaining 25 µl of each sample was diluted in 75 µl of ddH_2O and 50 µl volumes of 19% concentration were aliquoted into the 96 well plate for GSH reaction and total GSH and GSSG reaction.

Separate GSH and GSSG standard dilutions were prepared from 10 mM GSH or GSSG in assay buffer with 0 µM, 0.1563 µM, 0.3125 µM, 0.625 µM, 1.25 µM, 2.5 µM, 5 µM and 10 µM of GSH or GSSG in 100 µl volumes. 50 µl of each standard was aliquoted into the 96 well plate in ascending order for GSH and GSSG standards separately.

GSH assay mixture was made with 50 µl of 100x thiol green in 5 ml assay buffer protected from light with tin foil. Total GSH assay mixture was made with 100 µl of 25x GSSG probe in 2.5 ml of GSH assay mixture protected from light with tin foil. 50 µl of GSH assay mixture was added to each well of GSH standard and the samples intended for GSH reaction in the dark room. 50 µl of total GSH assay mixture was added to each well of GSSG standard and the samples intended for total GSH and GSSG reactions in the dark room. The plate was incubated in the dark at room temperature for 30 minutes and fluorescence was measured at 490 nm excitation wavelength and 520 nm emission wavelength on a protocol designed with Optima Control software for Fluostar plate reader.

2.2.4 TBARS Assay

This assay measures Malondialdehyde (MDA) which is the end product of lipid peroxidation and occurs through oxidative modification of lipids. Thiobarbituric Acid Reactive Substances (TBARS) assay is a widely accepted method of detecting MDA levels and therefore determining oxidative damage. It can be used for biological fluids, tissues and cells. Thiobarbituric acid reacts with MDA to form an adduct which is red in colour and therefore measured either colorimetrically (with absorbance at 532 nm) or flourometrically (excitation/emission profile 532/553 nm).

An assay developed by Professor Feelisch's laboratory for measuring MDA in serum and refined by his team to require low volumes of sample has been followed (no further protocol development required for the work of this thesis).

Materials and Equipment

- Malondialdehyde tetra-butylammonium salt (Sigma Aldrich, US)
- 2-Thiobarbituric acid (Sigma Aldrich, US)
- Acetic acid (Sigma Aldrich, US)
- Milli-Q Water
- Clear flat-bottom nonsterile 384-well plate (Thermo Fisher Scientific, US)
- LC-MS grade 100% methanol (Sigma Aldrich, US)
- Eppendorf tubes of various sizes, pipettes and pipette tips
- 15 ml Falcon tube (Corning, US)
- Fluostar plate reader with optima control software (BMG Labtech, Germany)
- Vortex Genie (Scientific Industries)

Protocol

A water bath was created by placing 2 ml cryovials filled with 1.5 ml MQ water in the wells of a heat block at 90°C temperature. 20% Acetic acid was made using 40 ml Acetic Acid in 160 ml MQ water. TBARS colour reagent was made by weighing 0.26 g of Thiobarbituric Acid and dissolving in 50 ml of 20% Acetic Acid. For assay work this was brought to room temperature before use as precipitation occurs during storage at 4°C. A 500 µM stock solution of MDA standard was made by dissolving 0.00157 g of MDA salt in 10 ml of MQ water. From this a working solution of 333 µM concentration was made diluting 200 µl of MDA standard stock in 100 µl of MQ water. Nine standards were then made of the following MDA concentrations from the working solution and diluting with MQ water; 33.4 µM, 24.5 µM, 16.7 µM, 8.36 µM, 4.18 µM, 2.08 µM, 1.04 µM, 0.52 µM and 0 µM. 50 µl of each standard was mixed with 50 µl of Methanol in 0.5 ml PCR tubes giving final MDA standard concentrations of; 16.7 µM, 12.25 µM, 8.35 µM, 4.18 µM, 2.09 µM, 1.04 µM, 0.52 µM, 0.26 µM and 0 µM.

Samples were prepared with a protein precipitation step as follows. 65 µl of samples (serum, urine or peritoneal fluid) were mixed with 65 µl of Methanol and centrifuged at >16000g for 20 minutes at 4°C. The supernatant was removed from the pellet and aliquoted in 0.5 ml PCR tubes.

For each MDA standard, 50 µl were mixed with 50 µl of Methanol and 50 µl of TBARS colour reagent in a 0.5 ml PCR tube. For each sample 80 µl were mixed with 40 µl of TBARS colour reagent in the 0.5 ml PCR tube. PCR tubes were sealed and placed in the cryovials of the water bath to be incubated for 30 minutes. Following incubation, the PCR tubes were placed on ice for 10 minutes. 50 µl of each were pipetted into a 384 well flat-bottomed plate in duplicate and absorbance at 532 nm wavelength was measured with a Fluostar plate reader using Optima Control software.

2.3 Animal Studies for Redox Status and RNA Damage of the Oocyte

To elucidate if higher levels of oxidative stress could potentially affect the oocyte and cause RNA damage, an impact of the ROS needs to be proven. This can be investigated with a technique to measure the redox status of the oocyte. From there potential RNA damage can be explored. A mouse model is deemed a biologically and scientifically justified approach to overcoming ethical and practical difficulties in exploring this with the use of human oocytes. GV oocytes are required for investigations into the redox status and RNA damage of oocytes to mimic the physiological time-point of greatest vulnerability of the oocytes to oxidative stress and RNA damage. GV oocytes collected from women for the process of IVF will all be used for their treatment, GV human oocytes from in-vitro maturation cultures (using the woman's immature oocytes) are not competent in fertilisation which is why this process is not undertaken in IVF. This indicates an inadequate/abnormal maturation process occurs in-vitro for human oocytes. It would be difficult to acquire human GV oocytes and therefore a compromise with mouse oocytes is commonly made for work in this area. Based on significant findings, the research may warrant future replication with human oocytes in a well-designed and ethically stringent study.

2.3.1 Animal Ethics

The study met approval of the regional and University of Southampton ethics committees. All animal work has been carried out in adherence to the Animals Scientific Procedures Act of 1986 (ASPA) under project and personal licences granted by the Home Office (PIL number I02BABE9E)

2.3.2 Mouse Ovarian Stimulation

Three- to four-week-old C57BL6 mice (Charles River, UK) were inbred and supplied by the Biomedical Research Facility (BRF), University of Southampton. They were housed initially at the BRF and for a maximum of 5 days in the animal holding room in the Biological Sciences Laboratory. Temperature was controlled at 18-23°C and humidity at 40-60% in scantainers set to 12 hours light and dark cycles. They received an ASPA approved diet which was available with water ad libitum. Enrichment was provided in their cages. They were injected via intra-peritoneal route with 10 IU pregnant mare serum gonadotrophin (PMSG, Centaur Services, UK) for follicle stimulation to increase the number of oocytes obtained from each mouse and reduce mouse numbers used for experiments. Mice were monitored post injection for signs of distress and discomfort and culled ~48 hours post injection by cervical dislocation.

2.3.3 Oocyte Collection

Collections were performed in M2 media (see Appendix 20) made from individual components by the laboratory technician every 14 days and quality checked with oocyte maturation test. M2 media was used as a handling media for oocyte collection due to its suitability for short-term cultures and being used outside of a 5% CO_2–controlled atmosphere. Spontaneous maturation of GV oocytes was prevented by addition of 1 µM of milrinone (Sigma-Aldrich, US) to the media. A layer of mineral oil was used to prevent evaporation and dishes were heated to 37°C on a heat block. Ovaries were harvested and placed in 150 µl M2 media containing 1 µM of milrinone under mineral oil heated to 37°C. Under a microscope (SZ40, Olympus, Japan) on a 37°C heated mounting stage (MATS-U4020WF, Tokai Hit, Japan), follicles were disrupted with a 27-gauge needle (Beckton-Dickinson, US) on a 1 ml syringe (Beckton-Dickinson, US).

Fully grown GV arrested oocytes were collected from the dish using glass mouth pipettes hand-made from 150 mm glass pipettes (Fisherbrand, UK) that were softened over ethanol burner and stretched to a length that gave an appropriate internal diameter. The pipette was used to manually remove the cumulus cells from the GV oocytes by 2-3 brief movements of the oocyte into the pipette tip (Figure 11). Exposure to the light of the microscope was kept to a minimum and the room was dark to reduce damage to the oocytes.

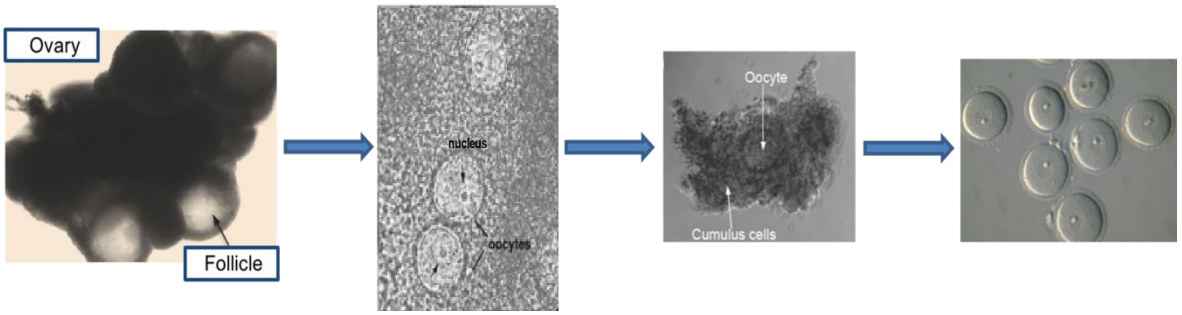


Figure 11: The process of collecting and denuding mouse oocytes from ovarian follicles (image (a) and (c) from Choi et al 228 image (b) from Lees-Murdock et al 229 image (d) from Nasiri et al 230).

2.4 Redox Studies

2.4.1 Gibson Cloning Protocol

The Gibson cloning technique is used to assemble fragments of DNA into a plasmid and the following protocol utilises PCR and the Gibson reaction to design Grx1-roGFP2 RNA to be microinjected into mouse oocytes in order to measure the intracellular redox status of the cell which can then be exposed to the reproductive fluids of women from the XSESS study.

Dr Simon Lane used snapGENE to design a suitable plasmid in silico which contained a pRN3 backbone and inserts for Grx1 and roGFP2, he then designed primers from Eurofins (Eurofins, Luxembourg) to span the intersections of inserts within the plasmid. His protocol was followed for the work in this thesis and no further protocol development was required.

PCR for sections of the construct

- 33.5 µl nuclease free H_2O (Sigma Aldrich, US)
- 10 µl 5x Q5 reaction buffer (New England Biolabs, US)
- 2.5 µl of 10 µM forward primer GOI pRN3 5' for Grx1 (Eurofins Genomics, Luxembourg)
- 2.5 µl of 10 µM reverse primer XFP Cterm2 3' for roGFP2 (Eurofins Genomics, Luxembourg)
- 1 µl 10mM oligonucleotides (Promega, US)

The above reagents were added in order, to a 250 µl PCR reaction tube on ice and mixed briefly on a vortex. Template DNA from ampicillin cultured E-Coli from Grx1-roGFP2 colony was added to the PCR reagents by dipping a pipette tip (sterilised in Bunsen burner flame) in a Grx1-roGFP2 positive E-Coli glycerol stock and agitating the pipette tip in the PCR reagents. PCR tube was transferred to a Bio-Rad T100 Thermo Cycler PCR machine (Bio Rad Laboratories, US) and incubated at 98°C for 30 seconds followed by 35 cycles of incubation at 98°C for 10 seconds, then 70°C for 20 seconds and 72°C for 1 minute 15 seconds. A final incubation at 72°C was performed for 2 minutes and products cooled to 4°C.

0.5 µl of PCR product was mixed with 3.5 µl of blue/orange loading dye (Promega, US) and run with a 5 µl of 1Kb DNA ladder (Promega, US) on a 2% agarose gel (see Chapter 4, section 4.5.6 for method of preparing an agarose gel and performing gel electrophoresis). A band of the expected size ~1175bp indicated presence of Grx1-roGFP2 DNA in the PCR product. Concentration of the DNA was quantified by measuring the integrated density of 1000bp band from the DNA ladder using ImageJ software and subtracting the integrated density of a blank area of gel. The 1000bp DNA ladder band has a known concentration of 120 ng/µl. The integrated density of the PCR product band was then calculated, and a blank area integrated density subtracted. The following analysis was then performed with these values:

Integrated density of the PCR product band / Integrated density of 1000bp DNA ladder band = A

(A x 120) x 2 = final concentration of PCR product in ng/µl

Grx1-roGFP DNA was then diluted to working concentration of 0.1 pmol with nuclease free water. (pmols/µl = (ng/µl x1000) / (base pairs of DNA product x 650 daltons)). DpnI digest was performed to degrade the methylated DNA by preparing on ice the following reagents:

- 1 µl Grx1-roGFP2 DNA product
- 1 µl of pRN3 plasmid backbone
- 5 µl x10 Cut smart buffer (New England Biolabs, US)
- 1 µl DpnI digest (New England Biolabs, US)
- 48 µl nuclease free water (Sigma Aldrich, US)

The reagents were mixed on a vortex genie and incubated in a PCR machine for 30 minutes at 37^{o}C followed by inactivation of the enzyme at 80^{o}C for 20 minutes.

PCR product was then cleaned by transferring into a 1.5 ml Eppendorf, adding 2 µl of pellet paint (Sigma Aldrich, US), 5 µl of 3M sodium acetate (Sigma Aldrich, US) and 500 µl of 100% ethanol BioUltra (Sigma Aldrich, US). The reagents were flicked to mix and left to stand at room temperature for 2 minutes. Centrifugation was then performed at >13000 rpm for 5 minutes. Supernatant was removed and the pellet washed twice by pipetting up and down with 200 µl of 70% ethanol (100% BioUltra ethanol diluted to 70% with nuclease free water). Ethanol was removed and pellet air-dried by leaving the Eppendorf lid open at room temperature for 2 minutes. The pellet was then resuspended in 5 µl of nuclease free water.

Gibson reaction

The resuspended DNA pellet was placed on wet ice and 5 µl of NEBuilder hifi reaction master mix (New England Biolabs, US) was added and incubated in PCR machine at 50°C for 1 hour. 1 µl of Gibson reaction product was mixed with 3 µl blue/orange loading dye and tested on a 2% agarose gel with gel electrophoresis against 0.25 µl pRN3 plasmid backbone mixed with 3.75 µl blue/orange loading dye, 0.25 µl Grx1-roGFP gene insert mixed with 3.75 µl blue/orange loading dye and 5 µl of 1kb DNA ladder (Promega, US). Presence of new plasmid DNA from the individual PCR products was indicated on the gel with an additional band at the size of the combined band sizes of pRN3 and Grx1-roGFP2 genes.

Transformation

5 µl of Gibson reaction plasmid product was added to 50 µl of New England Biolabs 5 alpha competent E-Coli in a 1.5 ml Eppendorf tube on wet ice, flicked to mix and left on ice for 30 minutes. For DNA to be taken up by the bacteria they were heat shock treated for 30 seconds at 42°C on a PCH-1 heat block (Grant Industries, UK) and then placed back on ice for 5 minutes. 950 µl of Super Optimal broth with catabolite repression (SOC broth) (Sigma Aldrich, US) was added to the mixture with a pipette sterilised in Bunsen burner flame and the Eppendorf was placed in an incubator shaker at 37°C and 250 rpm for 1 hour. 100 µl of transformed E-coli mixture spread on an ampicillin agar plate (prepared by laboratory colleague, Tianyu Wu) in sterile conditions (pipette tip sterilised by Bunsen burner flame and mixture spread with L-shaped stirrer kept in 70% ethanol and sterilised in Bunsen burner flame). The agar plate was covered with a lid and incubated at 37°C for 12-15 hours. Colonies were tested for presence of the desired plasmid with the following GoTaq PCR reagents in PCR reaction tubes:

- 10 µl 5x GoTaq green reaction buffer (Promega, US)
- 1 µl 10 mM oligonucleotides (Promega, US)
- 1 µl 10 µM T3 primer (Eurofins Genomics, Luxembourg)
- 1 µl 10 µM T7 primer (Eurofins Genomics, Luxembourg)
- 0.5 µl GoTaq DNA polymerase (Promega, US)
- 11.5 µl nuclease free water (Sigma Aldrich, US)

The reagents were mixed briefly on vortex genie and bacteria from colonies added to separate PCR tubes by sterilising a pipette tip in a Bunsen burner flame, wiping it in a bacterial colony and stirring into the desired PCR tube. PCR reaction was performed in Bio-rad T100 Thermo-cycler PCR machine (Bio Rad Laboratories, US) with incubation for 2 minutes at 95°C followed by 25 cycles of incubation at 95°C for 30 seconds, then 60°C for 30 seconds and 72°C for 1 minute 15 seconds. A final incubation at 72°C for 5 minutes completed the PCR reaction and the samples were then cooled to 4°C. 0.5 µl of products were mixed with 3.5 µl blue/orange loading dye and tested against 5 µl of 1Kb DNA ladder (Promega, US) on 2% agarose gel using methodology as per section 4.5.6. Bands of the expected size (1472bp) were used for confirmation of the Grx1-roGFP2 plasmid in the corresponding transformed E-Coli colony.

In-Vitro Transcription Template Generation

The following PCR mixture was made in order on wet ice in a PCR reaction tube:

- 33.5 µl nuclease free water (Sigma Aldrich, US)
- 10 µl 5x Q5 reaction buffer (Invitrogen, US)
- 2.5 µl 10 µM T3 primer (Eurofins Genomics, Luxembourg)
- 2.5 µl 10 µM T7 primer (Eurofins Genomics, Luxembourg)
- 1 µl Oligonucleotides (Promega, US)
- 0.5 µl Q5 HiFidelity DNA polymerase (Invitrogen, US)

PCR reagents were mixed briefly on vortex genie and sterilised pipette tip was wiped in the desired E-Coli colony and stirred in the PCR reagents. PCR tube was transferred to Bio Rad T100 Thermo-Cycler PCR machine (Bio Rad Laboratories, US) and incubated at 98°C for 30 seconds followed by 35 cycles of incubation at 98°C for 10 seconds, then 70°C for 20 seconds and 72°C for 1 minute 15 seconds. A final incubation at 72°C was performed for 2 minutes and products cooled to 4°C.

The PCR products were cleaned with the following DpnI digest protocol prepared in order on wet ice:

- 50 µl of PCR products
- 38 µl nuclease free water (Sigma Aldrich, US)
- 10 µl x10 NEB Cut smart buffer (New England Biolabs, US)
- 2 µl DpnI digest (New England Biolabs, US)

The reagents were mixed briefly on vortex genie and incubated at 37°C for 30 minutes and then at 80°C for 20 minutes. Products were transferred to a 1.5 ml Eppendorf tube and 350 µl of nuclease free water added. In a fume hood, 450 µl of Phenol-Chloroform (Sigma Aldrich, US) was added and mixed by pipetting up and down. Mixture was centrifuged at >13000 rpm for 10 minutes. In the fume hood, supernatant was aliquoted into a clean 1.5 ml Eppendorf and 450 µl of Chloroform (Sigma Aldrich, US) added and mixed by pipetting up and down. This mixture was also centrifuged at >13000 rpm for 2 minutes and under the fume hood, supernatant was again aliquoted into a clean 1.5 ml Eppendorf (400 µl extracted DNA product expected to be retained).

In the RNA hood, 1 µl pellet paint (Sigma Aldrich, US), 400 µl of 3M Sodium Acetate (Sigma Aldrich, US), and 1000 µl of 100% BioUltra Ethanol (Sigma Aldrich, US) were mixed in the 400 µl extracted DNA by pipetting up and down. The reagents were left to stand at room temperature for 2 minutes and centrifuged at >13000 for 5 minutes. Supernatant was removed in the RNA hood and the pellet was washed twice with 200 µl of 70% ethanol by pipetting up and down and removing excess ethanol. The pellet was air dried at room temperature for 2 minutes and resuspended in 10 µl nuclease free water. 0.5 µl of products were mixed with 3.5 µl blue/orange loading dye and tested against 5 µl of 1Kb DNA ladder (Promega, US) on 2% agarose gel using methodology as per section 4.5.6. Concentration was calculated as described in section 4.4.1 and DNA was diluted to 2x 15 µl volume of 150 ng/µl concentration. To one, 2 µl of T3 primer was added and to the other, 2 µl XFP Cterm2 3' primer was added. These were then sent for DNA sequencing to Eurofins Genomics. If result demonstrated suitable generated Grx1-roGFP2 DNA it was used for in-vitro transcription to RNA and the E-Coli colony was made into a glycerol stock by wiping a sterilised pipette tip into the colony from agar plate and stirring into 2 ml of LB broth with ampicillin (ampicillin 100 mg/ml concentration in LB broth in 1:1000 ratio) in a falcon tube with loose fitting lid and placed in incubator shaker at 37°C and 250 rpm for 12-15 hours. The tube was then flicked, contents transferred into 1.5 ml Eppendorf tube and centrifuged at 3000 rpm for 2 minutes. 1 ml of supernatant was removed, and the pellet resuspended in remaining 0.5 ml by pipetting up and down. 0.5 ml was then transferred to a 2 ml cryovial, and 0.5 ml of glycerol added. Contents were mixed by repeatedly inverting the tube for 10 seconds and stored at -80°C.

<u>In Vitro Transcription and RNA Recovery</u>

The following reagents were added in order on ice in an RNA hood:

- 5 µl nuclease free water (Sigma Aldrich, US)
- 10 µl 2x T3mMessage NTP/CAP (Invitrogen, US)
- 2 µl 10x T3mMessage buffer (Invitrogen, US)
- 1 µl of DNA template (100-200 ng/µl of template required)
- 2 µl T3mMessage enzyme mix (Invitrogen, US)

Reagents were mixed briefly on vortex genie and incubated at 37°C in Bio Rad T100 Thermo-cycler PCR machine for 2 hours. RNA was then recovered by adding 30 µl of Lithium Chloride precipitation solution (Invitrogen, US) to the RNA product and freezing at -20°C for 12-15 hours.

Product was centrifuged at >13000 rpm at 4°C for 15 minutes. Pellet was cleaned in the RNA hood by washing with 70% ethanol twice by pipetting up and down and removing excess ethanol. The pellet was then air dried at room temperature for 2 minutes and resuspended in 10 µl of nuclease free water. 0.5 µl was added to 1.5 µl of nuclease free water and tested for concentration of RNA on Nanodrop ND-1000 spectrophotometer (Thermo Fisher Scientific, US). This concentration was used to dilute RNA to 500 ng/µl concentration suitable for microinjection and was aliquoted into 10x 0.5 µl volumes to be stored for use at -20°C while the remaining stock was stored for longer at -80°C.

2.4.2 Redox GFP (roGFP) Microinjection

Oocytes were microinjected by Dr Simon Lane and laboratory colleague Tianyu Wu in a 37°C heated chamber (Intracel, UK) on the stage of an inverted TE300 microscope (Nikon, Japan) with micromanipulators (Narishige, Japan). A 0.1–0.3% volume of 500 ng/µl concentration roGFP2 and H2B-mCherry was injected using a timed pulse on a Pneumatic Picopump (World Precision Instruments, UK). Micro-injected oocytes were transferred back into a milrinone supplemented M2 media holding dish for 20 minutes at which time oocytes were assessed for viability and live cells selected for ongoing experimentation.

2.4.3 Redox GFP (roGFP) Measurement

Microinjected oocytes were transferred in groups of 4-5 into 180 µl drops of milrinone supplemented M2 media in wells of a flat-bottomed glass 96-well imaging plate. The oocytes were then imaged at 37°C on a Leica SP8 confocal microscope fitted with hybrid detectors and a ×40/1.3NA plan apochromat oil immersion lens. Appropriate gain and power settings for 405 nm, 488 nm and 595 nm laser lines were selected with detection emission at 530 nm. Pinhole used was 2.5-5 and positions were marked for each oocyte focusing on the middle of the nucleus. Sequential imaging of channels was used to prevent bleedthrough from the multiple lasers to incorrect detection channels.

Oocytes were imaged at 1-minute intervals for 10 minutes to establish a baseline, then either an oxidising (hydrogen peroxide) or reducing (resveratrol or dithiothreitol) control agent, or

peritoneal fluid sample, were added to the media. A further timelapse with 1-minute intervals was used to monitor the effect on roGFP2 fluorescence.

2.4.4 Source of Samples

The case and control cohorts for samples used in this chapter were from a population of women with known or suspected endometriosis undergoing investigation and treatment for the disease at the gynaecology department of Princess Anne Hospital, Southampton. This is a secondary referral centre for gynaecological conditions and a tertiary referral centre for managing advanced endometriosis. The women were aged between 18 and 65 years and were all pre-menopausal in the absence of pregnancy and with no evidence of active infection.

2.5 RNA Damage – qPCR Methods

2.5.1 RNA Extraction from Oocytes

Method 1: Denuded oocytes were transferred in groups of 3-4 to a drop of acid tyrodes under mineral oil for a few seconds. When the zona pellucida dissolved the oocytes were washed in M2 containing milrinone. When all oocytes had undergone this process, they were washed in 100 µl of 1x concentration of PVP in PBS. Lysis buffer was prepared with 5 µl of 1 M DTT, 10 µl of 10% Triton X and 0.5 µl of RNase inhibitor in 984.5 µl of nuclease free water. The oocytes were then placed into 5 µl of lysis buffer in a small Eppendorf tube under the microscope. The lysis buffer was frozen, thawed on ice and mixed for 10 seconds on a vortex genie. The freeze/thaw/spin process was repeated 3 times and lysis of the oocytes confirmed under the microscope. RNA concentration was measured on a nanometer and could be stored at -80°C or used to make cDNA. As a positive control for oxidative damage, oocytes were briefly exposed to hydrogen peroxide (0.5% H_2O_2 for 5 minutes) before the zona pellucida was removed.

Method 2: Qiagen RNeasy Plus Micro Kit (Qiagen, Netherlands). Working solutions were made up as per manufacturer instructions; 20 µl 2M DTT (Sigma Aldrich, US) was added to 1 ml Buffer RLT Plus, 44 mls of 100% ethanol (Sigma Aldrich, US) was added to 11 mls of Buffer RPE. 310 µl of RNA carrier was diluted in 1 ml RNAse free water (Sigma Aldrich, US), 5 µl of diluted RNA carrier was

further diluted in 34 µl of Buffer RLT and 6 µl of RNA carrier/Buffer RLT solution was added to 54 µl Buffer RLT for final concentration 4 ng/µl RNA carrier working solution.

5 µl of 4 ng/µl RNA carrier solution was added to 75 µl Buffer RLT. Denuded oocytes in numbers of 60-70 were transferred by mouth pipette in a small volume of PBS to the RNA carrier/Buffer RLT solution, mixed on a vortex genie 2 (Scientific Industries, US) for 2 minutes and centrifuged at full speed for 3 minutes. Supernatant was pipetted into a gDNA eliminator spin column and centrifuged at 8000 xg for 30 seconds. The column was discarded and 75 µl of 70% ethanol was added to the lysate in the collection tube. The lysate/ethanol solution was transferred to a RNeasy min elute column (Qiagen, Netherlands) and centrifuged for 15 seconds at 8000 xg (10,000 rpm). Flow through was discarded and 350 µl of RW1 buffer was added to the spin column before centrifuging at 8000 xg for another 15 seconds. Flow through was again discarded and 500 µl of RPE buffer added to the column. The column was centrifuged for 15 seconds at 8000 xg and flow through discarded. 500 µl of 80% ethanol was pipetted into the spin column and centrifuged for 2 minutes at 8000 xg. Both flow through and collection tube were now discarded, and the spin column placed in a new collection tube. The spin column was dried by centrifuging with the lid open for 5 minutes at full speed. The collection tube was discarded, and spin column placed into a new 1.5 ml collection tube. 14 µl of RNase free water was pipetted directly onto the spin column membrane and centrifuged with the lid closed for 1 minute at full speed. The spin column was discarded and flow through stored in its collection tube at -80°C or used to make cDNA.

2.5.2 RNA Extraction from Other Murine Tissue

Control RNA (of high quality and high RNA concentration) was also obtained from either the liver or testes of C57BL6 mice. Liver obtained when harvesting ovaries, sample of testis tissue donated by laboratory colleague Stephanie Morgan. The first method used was with trizol. 50 mg of tissue briefly washed in PBS was lysed and homogenised in 1 ml of trizol with a tissue homogenizer under a fume hood. The trizol (with cells) was pipetted into a new Eppendorf tube and remaining tissue discarded. 200 µl of chloroform was added under a fume hood and incubated at room temperature for 2-3 minutes and then centrifuged at 12000 xg at 4°C for 15 minutes. The aqueous phase was pipetted into a new Eppendorf tube to which 500 µL of isopropanol was added. This solution was centrifuged at 12000 xg 4°C for 10 minutes and the supernatant collected with a

pipette and discarded. The pellet was washed in 1 ml of 75% pure ethanol by pipetting up and down and spinning on a vortex genie. When the pellet dissolved, the solution was centrifuged 7500 xg 4°C for 5 minutes and supernatant was removed before air drying the pellet for 5 minutes in an open Eppendorf tube. The pellet was re-suspended in 50 µl of RNase free water and incubated on a heat block at 55-60°C for 10-15 minutes. The concentration of RNA was calculated on a Nanodrop ND-1000 spectrophotometer (Thermo Fisher Scientific, US) (see method in 4.5.3 Testing RNA quality). When the concentration was determined the sample was divided into 8 µl volumes. To remove DNA 1 µl of RQ1 10x reaction buffer followed by RQ1 DNase (1 unit/µg of RNA) was added to each sample. This was incubated at 37°C for 30 minutes and 1µl of RQ1 stop solution was then added followed by another 10-minute incubation at 65°C. RNA was stored at -80°C or used to make cDNA.

The second method used was with Qiagen RNeasy Plus micro kit (Qiagen, Netherlands). 5 mg of liver or testicular tissue was added to 350 µl of buffer RLT Plus and homogenised with tissue homogeniser. Once tissue was dissolved the solution was centrifuged for 3 minutes at full speed and supernatant was pipetted into a gDNA eliminator spin column. The remaining protocol followed steps as per section 4.5.1 RNA extraction from oocytes.

2.5.3 Testing RNA Quality

RNA quality was tested by gel electrophoresis on a formaldehyde agarose gel. A 1% agarose gel was made with 1 g agarose dissolved in 100 mls of Tris-borate-EDTA (TBE, which has a higher buffering capacity and is a better conductive medium than TAE) by heating in a microwave for 1 min. 14 µl of gel red was added and when slightly cooled the agarose solution was poured into a gel electrophoresis cast with well comb. When the gel had set (~30 minutes), the comb was removed and the gel on its casting tray was placed in the centre of the electrophoresis unit with wells closest to the negative electrode. TBE was poured into the upper and lower buffer chambers to the fill line ensuring the gel was covered. Samples were prepared on parafilm with 2 µl of RNA or 2 µl of 100bp ladder with 2 µl of blue-orange loading dye, 2 µl of deionised formaldehyde and 6 µl ddH_2O. (2 µl of RNA was used for high concentrations >1000 ng/µl, but 3-4 µl of RNA was tested if concentration was 1-200 ng/µl and ddH_2O adjusted accordingly to make 12 µl total volumes). 5-6 µl of the prepared ladder and RNA sample was pipetted into two wells of the gel. A lid was secured and connected to the power supply and the electrophoresis was run at 100 V for 60

minutes. When complete, the gel was analysed in a UV imager and good quality RNA was considered to display two clear bands corresponding to the 28S and 18S rRNAs at about 1000bp and 700bp with no smearing (signs of degradation).

RNA quality was also tested with NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific, US) by pipetting 0.5 µL of RNA sample into 1.5 µl of nuclease free water. Nucleic acid detection was selected using NanoDrop 1000.3.7.1 software (Thermo Fisher Scientific, US). The analyser was cleaned by pipetting 2 µl of nuclease free water on the analyser and lid and the NanoDrop was initialised. Water was then removed from the lid and analyser with lint free tissue. A blank reference reading was then obtained by repeating the cleaning process for a "blank" background measurement. Water was removed with lint free tissue and 2 µl of the RNA sample was pipetted onto the analyser. The RNA concentration was then measured, and reasonable quality accepted if 260/280 band ratio >1.8.

2.5.4 Reverse Transcription PCR

In a PCR cabinet, for every 5-7 µL of RNA sample, 2 µL of Promega 'oligo dT' primers (primers designed to bind the poly A 3' UTR of mRNAs) were added and heated to 70°C for 5 minutes in a PCR machine (Bio-Rad T100 Thermo Cycler, Bio Rad laboratories, US). The solution was then cooled on ice for 5 minutes to allow formation of 3'-OH ends. In the PCR cabinet, for every 7-9 µL of RNA and oligo primer solution the following reagents are added and mixed on vortex genie;

- 5 µL of 5x M-MLV reverse transcription buffer (Promega, US)
- 6 µL of 10 mM nucleotide mix (Promega, US)
- 25 units of ribonuclease inhibitor (Promega, US)
- 200 units of M-MLV reverse transcriptase (Promega, US)
- Nuclease free water to make a 25 µL volume solution (Sigma Aldrich, US)

RT-PCR protocol of 20°C for 10 minutes to activate the reverse transcriptase, 37°C incubation for 50 minutes, 70°C for 15minutes (to inactivate reverse transcriptase) and cooling to 12°C was followed and resulting cDNA was stored at -20°C.

2.5.5 PCR and qPCR

Primers were designed using Primer-Blast (National Center for Biotechnology Information) and PCR reactions were performed with the following master mix (which could be multiplied as necessary as per number of desired reactions):

- 10 µL 5x GoTaq green reaction buffer (Promega, US)
- 1 µL nucleotide mix 10mM (Promega, US)
- 1 µL forward and 1µL reverse 10mM primer (Eurofins, Luxembourg)
- 33.5 µL nuclease free water
- 0.5 µL DNA polymerase (Promega, US)

Mastermix was divided into Eppendorfs (23 µL in each) to which 2 µL of cDNA template (for final concentration <0.5 µg) was added. Into one mastermix a further 2 µL of nuclease free water was added instead of cDNA template to serve as a no template control. A PCR protocol of 95°C for 2 minutes "hot start" followed by 40 cycles of 95°C for 30 seconds, 57°C annealing step for 1 minute and 72°C extension step for 5 minutes and then cooling to 12°C was performed in Bio-Rad T100 Thermo Cycler. Products were tested on a 2% agarose gel with Tris-acetate EDTA (TAE).

qPCR reactions were performed with fluorescent dye: 12.5 µL Sybr green fluorescent dye Jumpstart ready mix (Sigma-Aldrich, UK), 0.25 µL reference dye, 1 µL forward and reverse 10 mM primer, 2 µL template and 8.25 µL nuclease free water were mixed and aliquoted into wells of a 96 well PCR plate (Star Lab, Germany). qPCR protocol was performed in Chromo4 (MJ Research PTC-200 Thermal Cycler, USA) of 94°C for 2 minutes and 45 cycles of 94°C for 30 seconds, 56°C for 1 minute and 72°C for 30 seconds (with plate read after the extension step in each cycle) followed by a melting curve between 50 and 90°C at 0.2°C intervals for 0.2 seconds.

qPCR was also performed with primers and fluorescent hydrolysis probes designed with Roche Universal Probe Library: 2.5 µL 10x PCR buffer (Thermo Fisher Scientific, US), 1 µL of 50 mM $MgSO_4$ (Thermo Fisher Scientific, US), 0.5 µL nucleotide mix (Promega, US), 0.5 µL of forward and reverse 10 µM primers (Roche, Switzerland), 1 µL template cDNA (for final concentration 10-100 ng), 0.5 µL hydrolysis probe, 0.2 µL Platinum Taq high fidelity DNA polymerase (Thermo Fisher Scientific, US) and 19.15 µL nuclease free water were mixed and aliquoted into wells of a 96 well PCR plate (Star Lab, Germany). Normal PCR master-mix with Promega Taqman products prepared

as above and fluorescent probe primers added. qPCR performed with StepOne Real Time PCR System (Thermo Fisher Scientific, US) and output obtained from StepOne software (Thermo Fisher Scientific, US). Products from all qPCR reactions were validated on a 2% agarose gel (adding Promega blue-orange loading dye to the samples).

2.5.6 Gel Electrophoresis

PCR products were examined with gel electrophoresis. A 2% agarose gel was made with 2 g agarose dissolved in 100 mls of Tris-acetate EDTA (TAE) by heating in beaker in a microwave for 50 seconds. 5 µL of gel red (1:20,0000) was added to enhance bands in UV light and solution was aggravated to mix. When agarose solution had slightly cooled it was poured into an Owl Easycast gel electrophoresis cast (Thermo Fisher Scientific, US) with well comb. When the gel had set (~30 minutes), the comb was removed and the gel on its casting tray was placed in the centre of the Easycast electrophoresis box with wells closest to the negative electrode. TAE was poured into the upper and lower buffer chambers to the fill line ensuring the gel was covered. 3 µL of 100bp ladder from stock (5 µL 100bp ladder with 4 µL green GoTaq buffer in 11 µL nuclease free water) was pipetted into the first well. 5 µL of each PCR product was pipetted into subsequent wells of the gel. If testing PCR products from qPCR reaction 5 µL of blue-orange loading dye (Promega, US) was added to the PCR product and mixed with vortex genie before pipetting sample into the well of the gel. A lid was secured and connected to the Bio-Rad power supply and the electrophoresis was run at 120 V for 50 minutes. When complete, the gel was analysed in SynGene G: Box UV imager (SynGene Inc, US) and analysed for DNA bands with GeneSnap software (SynGene Inc, US).

2.6 RNA Damage Studies - Immunohistochemistry

2.6.1 Oocyte Culture

For immunofluorescence studies oocytes were either cultured in M2 media supplemented with milrinone 1 µM (negative control), incubated for 15 minutes in 3.7% H_2O_2 (Sigma-Aldrich, US) in 1x PBS supplemented with 1% PVP (positive control), or incubated for 6-15 hours in samples of peritoneal fluid supplemented with milrinone 1 µM.

For maturation studies oocytes were either cultured in M2 media or peritoneal fluid concentrations ranging from 100% to 15% by diluting with M2 media. In these studies, no milrinone was added as GV arrest was not desired. Incubation period was 12-16 hours.

All incubations of longer than 1 hour duration were carried out in 1 ml closed Eppendorf tubes with 10 µL of mineral oil (Sigma-Aldrich, US) pipetted gently above the incubation fluid. The Eppendorfs were then placed in small wells of a 37°C heat block in a dark room protected from light. For incubations <1-hour duration oocytes were placed in 100 µL drops of the respective solution in wells of a glass culture slide under a coverslip to prevent evaporation. The glass slide was then placed on a 37°C heat block in a dark room protected from light.

2.6.2 Oocyte Fixation and Permeabilisation

PBS with 1% PVP was made for washing steps. 2.5 g PVP (Sigma-Aldrich, US) was dissolved in 10 ml PBS to make 25x concentration stock. 200 µL of 25x stock was then diluted in 4.8 ml of PBS to make 1% PVP/PBS solution. Fixing solution was made with 27 µL of 37% formaldehyde (Sigma-Aldrich, US) and 25 µL 10% Triton X in 448 µL 1% PVP/PBS. Permeabilising solution was made with 25 µL 10% Triton X in 475 µL 1% PVP/PBS.

The oocytes were separated into exposure groups for incubation in different media as per experiment design. Groups were subsequently processed in parallel using an 8-well glass culture slide. Oocytes were washed in their groups through three separate 100 µL drops of 1% PVP/PBS to remove proteins and treatment drugs from the incubation media and placed in separate 100 µL drops of fixing solution for 20 minutes in the dark at 37°C under a cover slip. Fixing solution was removed by moving oocytes in their groups through three 100 µL 1% PVP/PBS wash drops and they were then transferred to separate 100 µL drops of permeabilising solution for 20 minutes in the dark at 37°C under a cover slip. Following permeabilisation the oocytes were washed in their groups in three 100 µL drops of 1% PVP/PBS.

2.6.3 Oocyte Blocking

Blocking solution was made by dissolving 0.12 g Bovine Serum Albumin (Sigma-Aldrich, US) in 6 ml PBS using a vortex genie. 60 µL 1% Tween 20 (Sigma Aldrich, US) was then added as a detergent.

The oocytes were bathed in 100 µL drops of blocking solution under a coverslip for 1 hour in the dark at 37°C.

2.6.4 Antibody Incubation and Dye Labelling

1 µL of the primary antibody of interest (anti-8-OHG) was mixed in 99 µL of blocking solution and the oocytes were incubated in 50 µL drops in the dark at 37°C for 1 hour under a coverslip. The oocytes were then washed in their groups through three 100 µL drops of 1% PVP/PBS. The incubation was repeated for the secondary antibody (goat anti-rabbit) (1:1000 antibody in blocking solution). Staining of the DNA was then performed by washing the groups of oocytes in 100 µL drops of Hoechst or DAPI at 1:1000 concentration (0.5 µL of Hoechst or DAPI in 500 µL of blocking solution) for 15 minutes in the dark at 37°C under a cover slip.

2.6.5 Immunofluorescence Imaging

Small drops of glycerol with citifluor antifade were placed onto an imaging dish and covered with mineral oil. The oocytes were then distributed to the drops in numbers of 6-7 according to their exposure group. Although all imaging was performed on the day on immunofluorescence to minimise loss of fluorescence over time, if longer-term storage of the oocytes was desired glycerol with citifluor was placed into wells of a microscope mounting slide and oocytes distributed as before. A coverslip was then secured in place with a small amount of wax around each well such that the glycerol drop met the coverslip. Edges of the coverslip were sealed with nail varnish.

Oocytes were imaged under a confocal microscope in a 37°C chamber (Leica SP8 fitted with hybrid detectors and a ×63 oil immersion lens). Confocal microscopy was preferred for this work due to its ability to produce clear z-stack images with distinguishable intra-cellular structures. Appropriate gain and power settings for UV and argon lasers were selected and capture wavelengths suitable for the secondary antibody and chromosome dye of interest were designated and remained constant for imaging all oocytes per experiment. The nucleus of the oocyte was brought into focus and nine 1024x1024-pixel images at 5 µm z-steps were captured.

Chapter 3 Fertility and Beyond – the Further Reaching Influence of Endometriosis and Adenomyosis

3.1 Introduction

As discussed in Chapter 1, impaired fertility in endometriosis and adenomyosis has featured highly in literature with many studies on these conditions being carried out in the assisted reproductive setting and in the area of biological science to find causes of this association. Despite the amount of work in this area, the impact of the disease severity on processes of folliculogenesis, oocyte quality, fertilization, implantation and embryo quality are still under debate. There is also little attention paid to whether different subtypes of the disease have specific impacts on the individual stages of the reproductive cycle. It would seem logical that ovarian endometriosis would have a higher impact on oocyte quality, ovarian reserve and response to ovarian stimulation than perhaps minimal peritoneal endometriosis and adenomyosis may have a stronger association with implantation complications than ovarian endometriosis, for example.

Potential impact on post-implantation stages of reproduction is theorised but not understood and longer follow-up for obstetric and neonatal outcomes is not often undertaken in the current literature. With growing interest in developmental origin of health and disease theory and knowledge that aberrant decidualisation and placentation within a disturbed uterine environment can be linked not only to problems relating to placental insufficiency but also childhood and adult diseases, the conditions of endometriosis and adenomyosis in this context have not yet been explored. It is unclear if and how such early aberrant reproductive development relates to late pregnancy and neonatal or early childhood outcomes in adenomyosis and endometriosis.

To further our understanding of the reproductive impacts of these complex conditions it is first sensible to investigate what current clinical literature can conclude regarding the relationship.

3.2 Objective

To address aim 1 of the thesis this chapter will investigate the association of adenomyosis and endometriosis on the reproductive, obstetric and neonatal outcomes of women through both ART

and natural conception and determine whether endometriosis disease subtypes have specific impacts on different stages of the reproductive course.

3.3 Hypothesis

Women with adenomyosis and endometriosis are more likely to have fertility problems and poorer outcomes in recognised fertility parameters (such as ovarian response and fertilisation rate) compared to women without the conditions. They are also more likely to have complications during pregnancy and delivery which impact negatively on the offspring neonatally. In the case of endometriosis, the risk of complications will be dependent to a certain extent on the subtype/severity of the disease.

3.4 Methods

A systematic review was performed according to a predetermined protocol and data was extracted for a meta-analysis.

3.4.1 Study criteria and Literature Search Strategy

Types of studies

Cohort, case-control and observational studies with an appropriate control group were included in the search. Systematic reviews and meta-analyses were included for qualitative data where appropriate. Non-English papers were translated and relevant case studies and material such as abstracts for conference, or other personal communication were also included.

Types of participants

Studies that examined the reproductive outcomes of women with adenomyosis or endometriosis who conceived naturally (NC) or through in-vitro fertilisation with or without intra-cytoplasmic sperm injection (IVF/ICSI) were included. Studies, where the mode of conception could not be differentiated to be exclusively NC as they were likely to include a subgroup of women undergoing ART, were analysed separately (NC/ART).

Studies on the impact of adenomyosis were included if the diagnosis of adenomyosis was made by imaging modalities or by ICD 10 coded medical records. Studies on endometriosis and its subtypes were included if the diagnosis was made by visualization of lesions at laparoscopy/laparotomy, histology, imaging modalities where endometrioma was diagnosed or ICD 10 coded medical records. Studies involving donor or recipient oocyte treatments, or women with known poor ovarian response were excluded.

The control group consisted of women with a negative laparoscopy or no known adenomyotic or endometriotic disease including those with tubal infertility, male factor infertility, unexplained infertility or mixed aetiology infertility.

Types of outcome measures

The primary outcome was healthy baby rate, defined as a live singleton birth at term of appropriate birthweight for gestation. Secondary outcomes were live birth rate (LBR) and clinical pregnancy rate (CPR) defined as a viable intrauterine pregnancy on ultrasound scan (USS). Secondary outcomes pertaining to pregnancy/delivery complications and IVF/ICSI parameters were:

- Early pregnancy complications - miscarriage rate (MR) (spontaneous pregnancy loss before 24 weeks gestation)

- Late pregnancy complications - pre-eclampsia (PET), pregnancy induced hypertension (PIH), antepartum haemorrhage (APH) (any bleeding per vagina after 24 weeks pregnancy), placenta praevia (PP), placental abruption (PA), small for gestational age fetus (SGA) (defined as birthweight <10th centile for gestational age), preterm delivery (PTD) (delivery >24 weeks and <37 weeks' gestation), lower segment caesarean section delivery (LSCS), intrauterine death (IUD), postpartum haemorrhage (PPH) (excessive bleeding following delivery).

- Neonatal complications - admission to the neonatal unit for any reason (NNU) (admission between birth and 28 days old) and neonatal death (NND) (death from birth to 28 days old).

- Parameters of IVF/ICSI treatment were oocyte yield (number of oocytes retrieved per cycle), number of mature oocytes per cycle (meiosis II oocytes suitable for fertilisation),

fertilisation rate (FR) (total number of fertilised oocytes), implantation rate (IR) (number of clinical pregnancies per embryo transferred) and cycle cancellation rate (CR).

Search Strategy

A systematic search of all published and unpublished studies from January 1980 to December 2018 with no language restriction was performed.

Electronic searches:

NHS evidence healthcare databases; AMED, EMBASE, HMIC, BNI, Medline, CINAHL and Health Business Elite as well the Cochrane electronic database were searched using the keywords adenomyosis, endometriosis, endometrioma, deep infiltrating endometriosis, stage I, stage II, stage III, stage IV and mild, moderate and severe together with 23 search terms (Appendix 1) covering fertility, obstetric and neonatal outcomes.

Other resources:

Systematic reviews, meta-analyses and literature reviews found in the search were hand searched and cross-referenced for relevant articles.

Selection of Studies

Following an initial screen of titles and abstracts retrieved by the search, the full text of all potentially eligible studies were retrieved. The full texts were examined for eligibility and articles satisfying the aforementioned inclusion criteria were selected (Appendix 2: Table of excluded studies). The results of this search are presented (Figure 12).

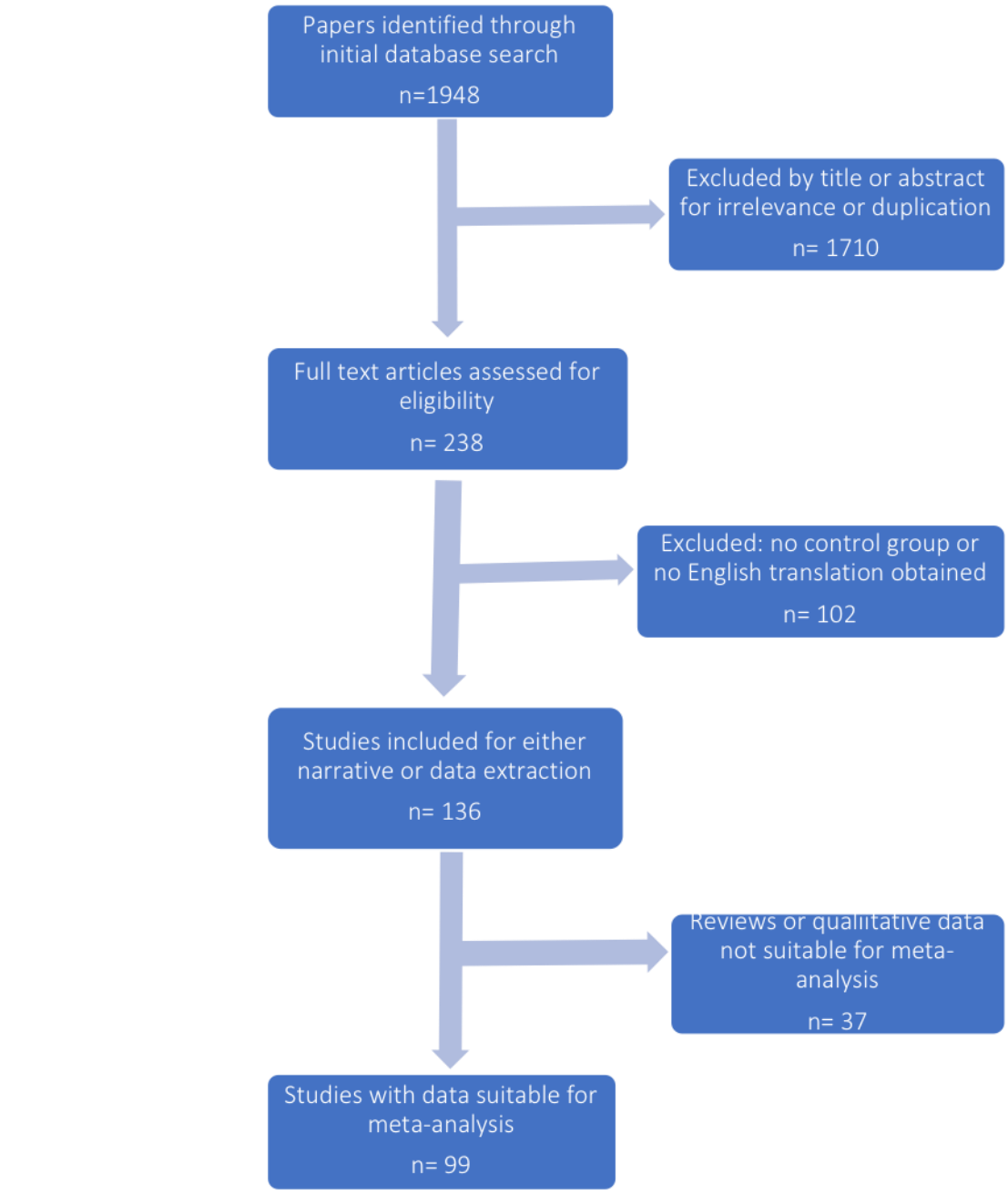


Figure 12: Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) flow chart of study selection process

3.4.2 Data Extraction and analysis

Data was extracted using predefined criteria and a second reviewer independently performed a search and data extraction on a sample of included studies (those published between October

2000 and October 2010). Any discrepancies were resolved after discussion between reviewers and if agreement was not met, discussion involved a third reviewer. Reviewers were selected based on their expertise in the subspecialty of endometriosis, reproductive medicine and methodology in performing meta-analyses. Data extraction included study characteristics and outcome data. (Appendix 3: Data Extraction Form).

Comparative Analysis

Outcomes (primary, secondary and those pertaining to pregnancy and neonatal complications and IVF/ICSI parameters) were examined according to mode of conception (NC, IVF/ICSI and NC/ART) compared to controls as follows:

1) Adenomyosis

2) Endometriosis overall (subtype/severity unspecified)

a. Treated (surgically and/or medically)

b. Untreated

3) Subtypes of endometriosis

a. ASRM stage I-II endometriosis

b. ASRM stage III-IV endometriosis

c. Endometrioma

d. Deep infiltrating endometriosis (DIE)

Data Analysis

Meta-analysis was performed using Review Manager version 5.3 and Preferred Reporting Items for Systematic Reviews guidance was followed where possible. Included studies are presented in Table 2. Statistical data was drawn from the original papers or calculated when suitable raw data was presented.

Data was analysed by outcome in different modes of conception for each disease subgroup. Dichotomous data and continuous data were analysed using Mantel-Hansel odds ratio and the mean difference or confidence intervals between groups, respectively. Relative risk was not used as outcomes were considered rare rather than probable. Publication bias was tested with funnel plot analysis. Sensitivity analyses were performed first by combining any mode of conception subgroup data, if no mode of conception subgroups existed for an outcome, data from excluded papers was used for secondary analysis. If there was no suitable data from excluded papers for an outcome the outlying data was removed for secondary analysis (Appendix 4).

Assessment of Heterogeneity

Included studies were scrutinised for clinical and methodological similarity and suitability of data for clinically meaningful meta-analysis. Statistical heterogeneity among included studies was measured by I^2 with an accepted limit of less than 50%. I^2 scores below this indicated that data could be analysed by a fixed effects model whereas scores equal to or above 50% were analysed by a random-effects model assuming that the effects being analysed in the different studies were not identical but follow similar distributions.

Assessment of Study Quality

Methodological quality of the studies was checked using the Down and Blacks standardised checklist for non-randomised controlled trials and quality of the individual studies which rates 27 items across the domains of study quality, external validity, study bias and confounding and selection bias. Items pertaining to power, blinding, randomising and intervention adverse events were removed from the checklist as they were not relevant to the included studies and when data is combined in meta-analysis (Appendix 2: Data Extraction Form)

3.5 Results

Primary Outcome

No studies reported healthy baby rate (live birth at term of appropriate birthweight for gestation) or presented data allowing a healthy baby rate to be determined.

Description of Studies and Participants

The systematic search retrieved 1948 articles, 238 studies were potentially eligible, and their full texts were reviewed (Figure 12). 99 studies met the inclusion criteria and 95 presented data suitable for inclusion in a meta-analysis (Table 2: Included Studies). The remaining four studies were included for qualitative data.

Ten papers compared fertility and obstetric outcomes in women with adenomyosis diagnosed by USS or magnetic resonance imaging (MRI) features to a control group (Table 2 - studies labelled AD). One paper used uterine enlargement without distinct masses [98] rather than the full spectrum of USS diagnostic features. Five of the studies involved patients who also had endometriosis in the case and control groups [99–103]. The ten papers addressing adenomyosis were grouped as follows: IVF/ICSI (n=6), NC/ART (n=4), NC (n=0). All papers had data that could be used in meta-analysis.

Fifty-eight papers where either the subtype or severity of endometriosis was unspecified or where data was presented for endometriosis as one cohort rather than by subtype or severity were included (Table 2 – studies labelled EN). Thirteen of these papers were NC/ART studies, two papers were NC studies and forty-three were IVF/ICSI studies.

Eighteen papers analysed treated endometriosis patients specifically (Table 2 – studies labelled Tx EN), one paper met inclusion criteria but did not present outcome data in a format that could be used in our meta-analysis [104]. The papers included in the meta-analysis were grouped as follows: IVF/ICSI (n= 14), NC/ART (n= 4), NC (n= 0). One paper examined the effects of untreated endometriosis compared to controls [105].

Twenty-six papers examined stage I&II endometriosis separately from other forms of endometriosis (Table 2 – studies labelled I-II). All papers in this part of the analysis reported

endometriosis staging by ASRM at laparoscopy or laparotomy but did not comment on whether the endometriosis was treated. Two studies examined women who conceived naturally or with ART (NC/ART) and all other studies were carried in the IVF/ICSI treatment setting.

Twenty-three papers which analysed fertility and reproductive outcome for ASRM stage III-IV endometriosis were included in the review (Table 2 – studies labelled III-IV). Two papers were NC/ART studies, all other studies were in the IVF/ICSI setting and there were no NC studies.

Eighteen studies addressed endometrioma alone (Table 2 – studies labelled OMA). In six studies, the diagnosis of endometrioma and peritoneal endometriosis was made at laparoscopy/laparotomy. The mode of conception in all studies was IVF/ICSI. Thirteen studies diagnosed endometrioma either on cyst aspiration or on ultrasound scan and had no peritoneal and deep infiltrating endometriosis based on ultrasound findings.

Three studies examined the effects of deep infiltrating endometriosis (DIE) (Table 2 – studies labelled DIE) and did not present data suitable for meta-analysis. The findings of these studies have been reviewed.

Table 2: Details of Studies Meeting the Inclusion Criteria for Meta-Analysis

IVF/ICSI Conception Studies							
Author & Year	Country	Study	Study Group	Control Group	Mode of Conception	Outcomes	Subgroup of analysis
Al-Azemi 2000[106]	UK	Single centre prospective case-control	Surgically proven ovarian endometriosis and had ≥3cycles of ICSI N=40	Laparoscopically proven tubal infertility and had ≥3 cycles of ICSI N=80	ICSI	↑CR ↓oocytes ↑dose HMG ↔cumulative pregnancy and live birth rate	OMA
Al-Fadhli 2006[107]	Canada	Single centre matched case-control	Primary/secondary infertility due to endometriosis mixed stages treated at laparoscopy N=87	Age matched control primary/secondary infertility tubal (n=22), male (n=28), PCOS (n=5), unexplained (n=32) N=87	IVF	↓FR ↑dose of FSH ↔oocytes ↔MII oocytes ↔CPR ↔IR ↔duration of stimulation	EN TxEN
AlKudmani 2018[108]	Canada	Single centre case control	Infertility and surgically treated endometriosis N= 216	Infertility with no endometriosis at laparoscopy N= 209	IVF/ICSI	↓oocytes ↓CPR ↔duration of stimulation ↔dose FSH ↔MII oocytes ↔FR ↔IR	TxEN
Arici 1996[109]	USA	Single centre case-control	Infertility secondary to endometriosis diagnosed and staged at laparoscopy N= 35 (89 cycles)	Tubal factor infertility diagnosed at laparoscopy N= 70 (147 cycles) Unexplained infertility with normal laparoscopy N= 15 (48 cycles)	IVF	↓oocytes ↓FR ↓IR ↓CPR ↓LBR ↓no of embryos transferred ↔MR ↔oocyte quality ↔embryo quality	EN I-II III-IV
Ashrafi 2014[110]	Iran	Single centre prospective cohort	Infertility and untreated endometriomas <3cm on USS N=47	Mild male factor infertility N= 57	IVF/ICSI	↓oocytes ↓MII oocytes ↑FR ↑no of embryos transferred ↔follicles ↔ IR ↔CPR ↔good quality embryos ↔CR	OMA

Benaglia 2013[111]	Italy	Multicentre age matched case-control	First cycle of IVF/ICSI with endometriomas on USS N= 39	First cycle of IVF/ICSI normal USS no history of endometriosis N= 78	IVF/ICSI	↓oocytes ↓follicles ↓MII oocytes ↔CR ↔CPR ↔LBR ↔IR ↔no of embryos transferred ↔high grade embryos ↔Dose FSH	OMA
Benaglia/Bermejo 2012[112]	Italy	Multicentre cohort	Singleton IVF/ICSI pregnancy with endometriomas on USS N= 78	Singleton IVF/ICSI pregnancy with normal USS N= 156	IVF/ICSI	↓Oocytes ↑Dose FSH ↔no of embryos transferred ↔LBR ↔MR ↔PTD ↔SGA ↔LSCS	OMA
Benaglia/Candotti/Busnelli 2015[113]	Italy	Single centre cohort	Infertility with untreated endometriomas on USS N= 46 Infertility with surgically treated endometriomas N= 55 Infertility with recurrent endometriomas on USS (previous surgical treatment) N= 23	Infertility with no endometriomas on USS and no history of endometriomas N= 42	IVF	↓Follicles ↔duration of stimulation ↔dose FSH ↔CR	EN OMA
Benaglia/Candotti 2016[114]	Italy	Multicentre matched case-control	Singleton IVF/ICSI pregnancy with history of surgery for endometriosis or endometrioma on USS N= 239	Singleton IVF/ICSI pregnancy, no history/symptoms of endometriosis and normal USS N= 239	IVF/ICSI	↓Oocytes ↑PP ↔dose FSH ↔no of embryos transferred ↔no of blastocysts ↔LBR ↔MR ↔SGA ↔PTD ↔LGA ↔PIH ↔PET ↔NNU admission ↔PA ↔LSCS ↔IUD ↔GDM ↔PPROM	EN
Bergendal 1998[115]	Sweden	Single centre retrospective matched case-control	Infertility secondary to endometriosis diagnosed at laparoscopy N=48 (65 cycles)	Tubal factor infertility matched to endometriosis patients by oocyte retrieval +/- 1 week N=98 (98 cycles)	IVF	↓FR ↓cleaved oocytes ↔oocytes ↔dose FSH ↔duration of stimulation ↔embryo score at transfer ↔follicles >10mm ↔IR ↔CPR ↔MR	EN TxEN
Bongioanni 2011[116]	Italy	Multicentre case-control	Endometrioma diagnosed on USS untreated (+/- male factor) N=142 Endometrioma with laparoscopic cystectomy (+/- male factor) N=112	Tubal infertility (+/- male factor) diagnosed at laparoscopy N=174	IVF/ICSI	↓oocytes (untreated)↑dose FSH (untreated) ↑CR (treated &untreated) ↔FR ↔IR ↔CPR ↔LBR ↔MII oocytes ↔no of embryos transferred	OMA

Borges 2015[117]	Brazil	Single centre cohort	Infertility with stage III-IV endometriosis N= 431	Mixed aetiology infertility no known endometriosis N= 2510	ICSI	↓follicles ↓oocytes ↓IR ↑dose FSH ↓no of embryos transferred ↓high quality embryos ↔FR ↔CPR ↔CR ↔MR ↔MII oocytes ↔blastocysts	EN III-IV
Brosens 2007[118]	Belgium	Multicentre case-control	IVF pregnancy in endometriosis related infertility diagnosed at laparoscopy N= 245	IVF pregnancy with male factor infertility N= 274	IVF	↓PET	EN
Bukulmez 2001[119]	Turkey	Single centre case-control	Male factor infertility and endometriosis stage I-II at laparoscopy N= 25 (49 cycles) Male factor infertility and stage III-IV endometriosis at laparoscopy or endometrioma on USS N= 19 (29 cycles)	Male factor infertility with negative laparoscopy N= 588 (895 cycles)	ICSI	↔oocytes ↔dose of GnRH ↔MII oocytes ↔FR ↔IR ↔no of embryos transferred ↔CPR	EN I-II III-IV
Canis 2001[120]	?	Single centre cohort	Endometrioma with laparoscopic cystectomy N=39 Endometriosis but no endometrioma treated laparoscopically N=128	Tubal infertility N=59	IVF	↔oocytes ↔no of embryos ↔CPR	TxEN
Chang 1997[121]	Taiwan	Single centre cohort	Stage I-II endometriosis at laparoscopy N= 60 Stage III-IV endometriosis at laparoscopy N= 48	Infertility of mixed aetiology – no endometriosis at laparoscopy N= 156	GIFT (COH data used)	↓oocytes ↓follicles ↓no. of embryos frozen ↔dose of FSH ↔FR	III-IV
Chiang 1999[98]	Taiwan	Single centre age matched case-control	Infertility with adenomyosis uterine enlargement with no distinct masses on USS N= 19	Infertility (cause not specified) with normal USS N= 144	IVF	↑MR ↔oocytes ↔dose of FSH ↔follicles ↔FR ↔LBR ↔no of embryos transferred ↔CPR	AD

Coccia 2011[122]	Italy	Single centre cohort	Stage I-II endometriosis at surgery N= 54 (55 cycles) Stage III-IV endometriosis at surgery N= 94 (109 cycles)	Tubal infertility N= 72 (80 cycles)	IVF	↓oocytes ↓follicles ↓CPR ↓no. of embryos ↓no. of embryos transferred ↑dose FSH/hMG ↔CR ↔FR ↔IR ↔duration of stimulation	EN TxEN
Coelho Neto 2015[123]	Brazil	Single centre cohort	Main study group: Pregnancy achieved via IVF cycle N= 184 Subgroup: women with endometriomas N= 39	No pregnancy achieved with IVF cycle N=333 Subgroup: women without endometrioma N= 478	IVF/ICSI	Age and no. of oocytes were independent predictors of pregnancy ↔oocytes ↔CPR ↔no. of embryos	EN
Coelho Neto/Martins 2016[124]	Brazil	Single centre cohort	Infertility undergoing first cycle IVF with endometriosis diagnosed on USS +/- confirmed at laparoscopy N= 241	Infertility undergoing first cycle IVF with no endometriosis on USS +/- negative laparoscopy N= 546	IVF/ICSI	↓oocytes ↑CR ↔CPR ↔LBR	OMA
Costello 2011[99]	Australia	Single centre cohort	Infertility of any cause other than ovarian failure and adenomyosis on USS N= 37	Infertility of any cause other than ovarian failure and no adenomyosis on USS N= 164	IVF/ICSI	↔dose FSH ↔oocytes ↔CR ↔FR ↔no of embryos transferred ↔CPR ↔LBR ↔IR ↔MR	AD
Dong 2013[125]	China	Single centre cohort	Infertility due to stage I-II endometriosis N= 152 Infertility due to stage III-IV endometriosis N= 279	Tubal infertility diagnosed at laparoscopy N= 596	IVF/ICSI	↓oocytes ↓no. of embryos ↓ FR ↑dose FSH ↓IR ↓high grade embryo rate ↔CPR ↔LBR ↔MR ↔ectopic ↔CR	EN TxEN
Falconer 2009[126]	Sweden	Single centre cohort	Infertility due to stage I-II endometriosis (+/-male factor) diagnosed at surgery N= 34	Tubal factor infertility (+/-male factor) diagnosed at laparoscopy N= 38	IVF/ICSI	↓FR ↓no. of embryos ↔follicles ↔oocytes ↔dose FSH ↔CR ↔CPR	I-II
Fernando 2009[127]	Australia	Single centre cohort	IVF singleton births with peritoneal endometriosis N= 535 IVF singleton births with endometrioma N= 95	IVF singleton births with mixed aetiology infertility but no history of endometriosis N= 1201 Fertile natural conception singleton births matched by age to study groups N= 1260 & 1140 respectively	IVF/ICSI	Endometrioma group: ↑PTD ↑SGA Endometriosis group: ↔PTD ↔SGA	OMA

Frydman 1987[128]	France	Multicentre observational cohort	Infertility due to endometriosis entered in database N= 53	Tubal infertility entered in database N= 933	IVF/ICSI	↓FR ↓embryo transfers ↔no. of embryos transferred ↔CPR	EN OMA
Frydman 1987[128]	France	Multicentre observational cohort	Infertility due to endometriosis entered in database N= 8 Infertility with endometriomas on database N= 26	Tubal infertility entered in database N= 544	IVF/ICSI	↔FR ↔no. of embryos transferred	EN OMA
Fuji 2016[129]	Japan	Single centre cohort	Singleton IVF delivery with endometriosis diagnosed and staged at laparoscopy N= 92	Singleton IVF delivery with normal USS +/- negative laparoscopy N= 512	IVF/ICSI	↑PTD ↑PP ↔SGA	EN III-IV
Geber 1995[105]	UK	Single centre age matched case-control	Infertility due to endometriosis diagnosed at laparoscopy N= 140 (182 cycles)	Male factor infertility N= 44 (45 cycles) Unexplained infertility N= 161(196 cycles) Tubal factor infertility N= 885 (1139 cycles)	IVF/ICSI	↓no. of embryo transfers (endometriosis to tubal group) ↔FR ↔IR ↔CPR ↔MR ↔ectopic	EN
González-Comadran 2017[130]	Spain	Multicentre prospective cohort	Infertility associated with endometriosis in the medical records N= 3583	Tubal, endocrine or unexplained infertility N= 18833	IVF/ICSI	↓oocytes ↑CR ↔FR ↔CPR ↔MR ↔LBR	EN
Gonsález-Foruria 2016[131]	Spain	Single centre cohort	Infertility due to endometriosis N= 101 (326 cycles)	Male factor infertility N= 68 (202cycles) Tubal factor infertility N= 44 (125 cycles) Unexplained infertility N= 81 (243 cycles) Mixed aetiology N= 26 (51 cycles)	IVF	↔oocytes ↔CR ↔MII oocytes ↔FR ↔no.of embryos ↔CPR ↔MR	EN

Guler 2017[132]	Turkey	Cohort	Peritoneal endometriosis treated laparoscopically N= 48 (91 cycles) Endometrioma treated laparoscopically N= 25 (57 cycles) Endometrioma untreated N= 53 (65 cycles)	Tubal infertility diagnosed laparoscopically N= 24 (44 cycles)	ICSI	↓oocytes ↓MII oocytes ↓dominant follicles ↓FR ↓CR ↓CPR ↔LBR ↔dose FSH	OMA
Healy 2010[133]	Australia	Multicentre cohort	IVF/ICSI singleton delivery N= 6730 Subgroup analysis IVF/ICSI with (N= 1265) or without history of endometriosis (N= 5465)	General population NC/ART singleton delivery N= 24619 General population non-ART singleton delivery N= 2167	IVF/ICSI	PP, APH, PPH	EN
Hickman 2002[134]	USA	Single centre cohort	Infertility due to endometriosis diagnosed at laparoscopy N= 27 (31 cycles) Stage I-II N= 18 (20 cycles) Stage III-IV N=9 (11 cycles)	Tubal factor infertility diagnosed at laparoscopy N= 104 (118 cycles)	IVF	↔oocytes ↔FR ↔IR ↔CPR ↔LBR ↔MR	EN I-II III-IV
Hull 1998[135]	UK	Cohort	Infertility due to stage I-II endometriosis diagnosed at laparoscopy N= 194 (219 cycles)	Unexplained infertility negative laparoscopy N= 327 (343 cycles) Tubal factor infertility without hydrosalpinx N= 509 (680 cycles)	IVF	↓oocytes ↓FR ↓no. of embryos and no.of embryos transferred ↔IR ↔CPR	EN I-II
Jacques 2016[136]	France	Single centre age matched case-control	IVF/ICSI pregnancies >22/40 associated with endometriosis diagnosed at laparoscopy N= 113 Subgroups: stage I-II endometriosis N=59 Stage III-IV endometriosis N= 52	IVF/ICSI pregnancies >22/40 associated with male factor infertility diagnosed at laparoscopy N= 113	IVF/ICSI	↑1st trimester bleeding ↑PTD ↑threatened PTD ↑PET ↓birthweight ↑LSCS ↑NNU admission ↔PROM ↔IUGR ↔GDM ↔OC ↔PP ↔PPH	EN I-II III-IV

Kim 2011[137]	Korea	Single centre cohort	Stage III-IV endometriosis related infertility N= 20 (20 cycles)	Tubal factor infertility N= 20 (20 cycles)	IVF	↓IR ↔oocytes ↔MII oocytes ↔no. of embryos transferred ↔CPR ↔LBR ↔MR ↔duration of stimulation ↔dose FSH ↔no. of high-grade embryos	III-IV
Kiran 2012[138]	Turkey	Single centre cohort	Endometrioma on USS at IVF cycle N= 29	Unexplained infertility with no endometrioma on USS at IVF cycle N= 51	IVF	↔oocytes ↔MII oocytes	OMA
Kuivasaari 2005[139]	Finland	Single centre prospective observational	Stage I-II endometriosis related infertility diagnosed and treated at laparoscopy N= 31 (58 cycles) Stage III-IV endometriosis related infertility diagnosed and treated at laparoscopy N= 67 (150 cycles)	Tubal factor infertility diagnosed surgically or HSG N= 87 (184 cycles)	IVF/ICSI	↓IR ↓LBR ↓ectopic ↔oocytes ↔FR ↔MR ↔no. of high-grade embryos ↔no. of embryos transferred	EN I-II III-IV
Kuivasaari-Pirinen 2012[140]	Finland	Single centre cohort	Endometriosis related infertility N= 49	Tubal factor infertility N= 38 Male factor infertility N= 43 Anovulation infertility N= 68 Unexplained infertility N= 30	IVF/ICSI	↑PTD ↓SGA ↓birthweight ↑PP ↑NNU admission ↔IUGR ↔GDM ↔PET ↔PA	EN
Kuroda 2009[141]	Japan	Single centre case-control	Endometrioma on USS or MRI at time of cycle N= 18 (31 cycles) Endometrioma treated laparoscopically N= 36 (51 cycles) Endometriosis without endometrioma treated laparoscopically N= 7 (15 cycles)	Tubal infertility N= 21 (27 cycles)	IVF/ICSI	↓no. of embryos transferred ↓follicles ↔oocytes ↔FR ↔IR ↔CPR ↔LBR ↔MR ↔high grade embryo rate	EN TxEN OMA

Leonardi 2016[142]	Italy	Multicentre matched case-control	Singleton pregnancy with history of endometriosis diagnosed at surgery or endometrioma on USS N= 313	Singleton pregnancy with no endometriosis at laparoscopy N= 313	IVF/ICSI	↓oocytes ↑dose FSH ↔duration of stimulation ↔no. of embryos transferred ↔MR ↔day of embryo transfer	EN TxEN
Lin 2012[143]	China	Single centre cohort	Infertility with endometriosis diagnosed surgically Stage I-II N= 64 Stage III-IV N= 113	Mixed aetiology infertility N= 4267	IVF/ICSI	↔FR ↔IR ↔CPR ↔cleavage rate ↔high quality embryo rate	EN I-II III-IV
Matalliotakis 2011[144]	USA	Single centre cohort	Infertility due to endometriosis diagnosed at laparoscopy Stage I-II N= 75 (144 cycles) Stage III-IV N= 55 (114 cycles)	Tubal infertility diagnosed at laparoscopy N= 104 (206 cycles) Male factor infertility negative laparoscopy N= 59 (133 cycles)	IVF/ICSI	↓oocyte ↔CR ↔FR ↔IR ↔CPR ↔LBR ↔MR ↔ectopic ↔no. of embryos transferred per cycle ↔no. of embryos	EN I-II III-IV
Matson 1986[145]	Australia	Cohort	Endometriosis diagnosed at surgery Stage I-II N= 40 (61 cycles) Stage III-IV N= 56 (93 cycles)	Tubal infertility N= 28 (40 cycles)	IVF	↓CPR ↔oocytes ↔follicles ↔FR ↔no. of embryos transferred	EN TxEN
Meden-Vrtovec 2000[146]	Slovenia	Cohort	Infertility with stage I-II endometriosis diagnosed at laparoscopy N= 7339 cycles	Tubal infertility N= 612 cycles	IVF	↓no. of embryos ↑CPR ↑LBR ↑MR ↑dose hMG ↔oocytes ↔FR ↔ectopic	I-II
Mekaru 2013[147]	Japan	Single centre case-control	Infertility with stage I-II endometriosis diagnosed at laparoscopy N= 18 (39 cycles)	Unexplained infertility with negative laparoscopy N= 17 (41 cycles)	IVF	↓grade 1 embryos ↑dose hMG ↔oocytes ↔no. of embryos transferred ↔FR ↔IR ↔CPR ↔LBR ↔OHSS	EN TxEN
Mohamed 2011[148]	UK	Single centre cohort	Infertility with endometriosis diagnosed at laparoscopy N= 415 cycles	Mixed aetiology infertility N= 6871 cycles	IVF	↔CPR ↔LBR ↔MR	EN

Motte 2016[149]	France	Single centre matched case-control	Infertility with endometriosis treated laparoscopically N= 37 (63 cycles)	Mixed aetiology infertility N= 74 (177 cycles)	IVF/ICSI	↓oocytes ↓CPR ↓LBR ↔CR ↔FR ↔IR ↔MR ↔ectopic ↔dose FSH ↔follicles ↔MII oocytes ↔cleavage rate ↔no. of embryos transferred ↔ embryo quality by grade	EN TxEN
Nakagawa 2016[150]	Japan	Single centre prospective cohort	Endometrioma on USS & MRI +/- male factor N= 26	Male factor or unexplained infertility normal USS +/- negative laparoscopy N= 29	IVF/ICSI	↔dose FSH ↔oocytes ↔FR ↔MR ↔CPR	OMA
Nejad 2009[151]	Iran	Single centre cohort	Infertility due to endometriosis diagnosed at laparoscopy Stage I-II N= 32 Stage III-IV N= 48	Tubal infertility diagnosed at laparoscopy N= 57	IVF/ICSI	↔oocytes ↔IR ↔CPR ↔MR ↔dose FSH ↔duration of stimulation ↔OHSS ↔no. of embryos transferred	EN I-II III-IV
Oehninger 1988[152]	USA	Single centre cohort	Mixed aetiology infertility with stage I-II endometriosis diagnosed at laparoscopy N= 91 (191 cycles) Mixed aetiology infertility with stage III-IV endometriosis diagnosed at laparoscopy N= 22 (35 cycles)	Tubal Infertility N= 447 (917 cycles)	IVF	Outcomes: Oocytes, immature oocytes, FR, no. of embryos transferred, CPR, MR but no significances given	EN TxEN I-II III-IV
Olivennes 1995[153]	USA	Single centre cohort	Infertility due to endometriosis diagnosed at laparoscopy N= 147 (236 cycles) Stage I-II N= 81 (196 cycles) Stage III-IV N= 9 (29 cycles) Endometrioma N= 57 (11 cycles)	Tubal infertility N= 111 (160 cycles)	IVF	Outcomes: Oocytes, CR, FR, CPR, LBR, MR, no. of embryos transferred, dose FSH, significances not given	EN I-II III-IV OMA
Omland 2006[154]	Norway	Single centre cohort	Undergoing ICSI for failed IVF cycle with infertility due to stage I endometriosis diagnosed at laparoscopy N= 43	Male factor N= 91 Unexplained infertility with negative laparoscopy and >1 failed IVF cycle N= 48	ICSI	↓FR ↔oocytes ↔dose FSH ↔IR ↔CPR ↔MR ↔no.of embryos transferred ↔duration of stimulation ↔MII oocytes ↔molar pregnancy	I-II

Opøien 2012[155]	Norway	Single centre cohort	Infertility due to endometriosis diagnosed at laparoscopy Stage I-II N= 724 Stage III-IV N= 350	Tubal infertility diagnosed at laparoscopy/laparotomy N= 1171	IVF/ICSI	↓oocytes ↓FR ↑dose FSH ↔CR ↔IR ↔CPR ↔MR ↔MII oocytes	EN I-II III-IV
Ozgur 2017[156]	Turkey	Single centre matched case-control	Infertility and untreated endometrioma >10mm on USS N= 30	Infertility with no normal USS and no history of endometriosis N= 60	IVF/FET	↑duration of stimulation ↔oocytes ↔MII oocytes ↔FR ↔blastocysts grade ↔IR ↔biochemical pregnancy rate ↔ongoing pregnancy >14/40	OMA
Pabuccu 2004[157]	Turkey	Single centre prospective case control	Endometrioma drained transvaginally at beginning of cycle N= 41 Endometrioma untreated at beginning of cycle N= 40 Previous surgery for endometrioma none seen on USS at cycle N= 44	Tubal infertility but no hydrosalpinx, no endometriosis at laparoscopy N= 46	ICSI	↓follicles ↓MII oocytes↑duration of stimulation dose ↔FSH ↔FR ↔IR ↔CPR ↔MR	OMA
Pellicer 1998[158]	Spain	Single centre case-control	Infertility due to endometriosis diagnosed at laparoscopy N= 17 Stage I-II N= 5 Stage III-IV N= 12	Tubal infertility diagnosed at laparoscopy N= 19	IVF	↔oocytes ↔duration of stimulation ↔no. of fertilised oocytes ↔no. of embryos transferred ↔IR ↔follicle volume	EN I-II III-IV
Polat 2014[159]	Turkey	Single centre case-control	Infertility due to endometriosis stage I-II diagnosed at laparoscopy N= 72 Stage III-IV diagnosed at laparoscopy or USS with endometrioma >3cm N= 413	Tubal infertility diagnosed at laparoscopy N= 131	IVF	↓oocytes, no. of embryos transferred & ↑dose FSH (stage III-IV only) ↓MII oocytes ↑CR ↔FR ↔IR ↔CPR ↔LBR ↔MR ↔duration of stimulation ↔no. good quality embryos	EN I-II III-IV
Pop-Trajkovic 2014[160]	Serbia	Multicentre cohort	Infertility with stage I-II endometriosis treated laparoscopically N= 40 Infertility with stage III-IV endometriosis treated laparoscopically N= 38	Tubal Infertility diagnosed at laparoscopy N= 157	IVF	↓Oocytes ↓follicles ↓FR ↓IR ↓cumulative pregnancy rate ↓LBR ↓no. of embryos ↑dose of FSH ↑CR ↑duration of stimulation ↔MR	EN TxEN

Salim 2012[101]	UK	Single centre prospective observational	Infertility of any cause other than ovarian failure and adenomyosis on USS N= 19	Infertility of any cause other than ovarian failure and no adenomyosis on USS N= 256	IVF/ICSI	↓IR ↓CPR ↑MR ↔dose GnRH ↔oocytes ↔no. of embryos transferred ↔MII oocytes	AD
Saucedo-de-la Llata 2004[161]	Mexico	Single centre cohort	Infertility due to endometriosis Stage I-II N= 111 Stage III-IV N= 132	Tubal infertility N= 268 Male Factor N= 261	IVF/ICSI	↓MII oocytes (stage I-II only) ↓FR ↔oocytes ↔CPR ↔follicles	I-II III-IV
Scarselli 2011[162]	Italy	Single centre cohort	Infertility due to endometriosis diagnosed surgically or endometriosis at IVF cycle N= 144	Tubal infertility N= 70	IVF	↓follicles ↓no. of embryos ↓no. of embryos transferred ↑CPR ↑dose FSH ↔FR ↔CR ↔oocytes	EN I-II OMA
Senapati 2016[163]	USA	Multicentre population-based cohort	Infertility due to endometriosis N= 12335	Tubal infertility N= 22778 Unexplained infertility N= 38713	IVF/ICSI	↓oocyte ↓IR ↓blastocyst transfer ↓LBR ↔CR ↔FR ↔MR ↔ectopic	EN
Shebl 2017[164]	Austria	Single centre matched case-control	Infertility with endometriosis diagnosed at laparoscopy +/- male factor or PCOS N= 114 (129 cycles)	Mixed aetiology infertility with a negative laparoscopy N= 119 (129 cycles)	IVF/ICSI	↓FR ↓MII oocytes ↔IR ↔biochemical pregnancy rate ↔LBR ↔MR ↔gestation at delivery ↔birthweight ↔malformations ↔dose FSH ↔grade I&2 embryos	EN I-II III-IV
Simon 1994[165]	Spain	Single centre case control	Infertility secondary to endometriosis diagnosed at laparoscopy or by endometrioma on USS Stage I-II N= 9 (14 cycles) Stage III-IV N= 50 (82 cycles)	Tubal infertility N= 78 (96 cycles)	IVF	↓CPR ↓IR ↓no. of grade 1 embryos transferred ↔oocytes ↔FR	EN I-II III-IV

Suzuki 2005[166]	Japan	Single centre cohort	Infertility due to endometriosis diagnosed at laparoscopy N= 248 cycles Infertility and endometrioma aspiration at IVF cycle N= 80 cycles	Tubal infertility diagnosed at laparoscopy N= 283 cycles	IVF	↓oocytes ↓no. of embryos transferred ↔FR ↔IR ↔biochemical pregnancy ↔LBR ↔grade 1&2 embryos	OMA
Tanbo 1995[167]	Norway	Single centre cohort	Infertility due to endometriosis stage I diagnosed at laparoscopy N= 143 (285 cycles)	Tubal infertility diagnosed at laparoscopy N= 180 (353 cycles) Unexplained infertility negative laparoscopy N= 215 (385 cycles)	IVF/ICSI	↓cleavage rate ↔oocytes ↔no. of embryos transferred ↔CPR	I-II
Thalluri 2012[102]	Australia	Single centre cohort	Mixed aetiology infertility with adenomyosis on USS N= 38	Mixed aetiology infertility with no adenomyosis on USS N= 175	IVF	↓biochemical pregnancy ↓CPR ↔oocytes ↔FR ↔miscarriage&ectopic ↔embryo grade	AD
Vaz 2017[168]	Brazil	Single centre cohort	Deep infiltrating endometriosis diagnosed at laparoscopy or on MRI N= 27	Tubal infertility N= 51 Male factor infertility N= 65 PCOS N= 20 Unexplained infertility N= 18	FET	↔pregnancy rate ↔MR	DIE
Wardle 1985[169]	UK	Single centre cohort	Infertility with stage I-II endometriosis at laparoscopy N= 17	Tubal infertility, no endometriosis at laparoscopy N= 47 Unexplained infertility, negative laparoscopy N= 21	IVF	↓FR ↓no. of embryo transfer procedures ↔Oocytes ↔CPR	I-II

Wyns 2003[104]	Belgium	Single centre case control	Infertility due to endometriosis treated surgically Peritoneal endometriosis N= 42 (71 cycles) Endometrioma N= 85 (187 cycles)	Tubal infertility N= 193 (422 cycles) Unexplained infertility N= 135 (275 cycles)	IVF	↔dose hCG ↔follicles ↔MII oocytes ↔no. of embryos transferred ↔FR ↔IR ↔CPR	TxEN
Yamamoto 2017[170]	USA	Single centre cohort	Undergoing first cycle IVF with diagnosis of endometriosis on USS or laparoscopically N= 68	Undergoing first cycle IVF with no diagnosis of endometriosis N= 649	IVF	↔oocytes ↔FR ↔CPR ↔CR	EN
Yan 2014[103]	China	Single centre cohort	Infertility with adenomyosis diagnosed on USS N= 77	Infertility with normal pelvic USS N= 77	IVF/ICSI	↓LBR ↔MR ↔CPR	AD
Youm 2011[100]	Korea	Single centre case-control	Mixed aetiology infertility myometrial thickness 2-2.49cm on USS N= 63 (81 cycles) Myometrial thickness >2.5cm N= 48 (73 cycles) No other uterine abnormalities	Mixed aetiology infertility myometrial thickness <2cm on USS no other uterine abnormalities N= 302 (397 cycles)	IVF	↓IR ↓CPR ↑MR ↓LBR ↔oocytes ↔no. of embryos transferred ↔FR ↔ectopic ↔no. of embryos transferred	AD
Yovich 1990[171]	Australia	Single centre cohort	Subgroup analysis: infertility with endometriosis at laparoscopy Stage I-II N= 40 (61 cycles) Stage III-IV N= 56 (93 cycles)	Subgroup analysis: mixed aetiology infertility, no endometriosis at laparoscopy N= 35 (49 cycles)	IVF	↓CPR ↔oocyte per follicle rate ↔FR	I-II III-IV

Natural Conception and ART Population Studies							
Aris 2014[172]	Canada	Multicentre population-based cohort	Delivery with diagnosis of endometriosis by ICD10 in medical records N= 784	Delivery with no diagnosis of endometriosis by ICD10 in medical records N= 30284	NC/ART	↑MR ↑IUD ↔PTD ↔PIH ↔GDM ↔PET ↔IUGR	EN
Berlac 2017[173]	Denmark	Multicentre population-based cohort	Endometriosis in medical records N= 11,739 (19331 births) Endometriosis treated surgically in medical records N= 4465 (3926 births)	No diagnosis of endometriosis on medical records N= 615,533 (1071920 births)	NC/ART	↑PET ↑abruption ↑PP ↑APH ↓PPH ↑LSCS ↑SGA ↑Apgar<7 at 5 mins ↑NND ↑IUD ↑PTD (<34/40), ↔PIH other complications in labour	EN TxEN
Chen 2018[174]	China	Multicentre population-based cohort	Singleton delivery with surgically diagnosed endometriosis by ICD 10 code in medical records N= 469	Singleton delivery with no history of endometriosis in medical records N= 51733	NC/ART	↑MR ↑PP ↑LSCS ↔PIH/PET ↔PA ↔PROM ↔PPH ↔PTD ↔SGA ↔NNU	EN
Conti 2015[175]	Italy	Multicentre cohort	Singleton delivery with history of laparoscopically treated endometriosis N= 316	Singleton delivery, no history of endometriosis N= 1923	NC/ART	↑PTD ↑SGA ↑PPROM ↑GDM ↑NNU admission ↔PIH ↔PET ↔PPH ↔mode of delivery ↔ PROM ↔duration of NNU admission	EN TxEN
Glavind 2017[176]	Denmark	Multicentre population-based cohort	Singleton delivery with endometriosis by ICD 10 code in medical records N= 1719 deliveries (1213 patients)	Singleton delivery no endometriosis by ICD 10 code in medical records N= 81074 deliveries (54616 patients)	NC/ART	↑LSCS ↑PTD ↑PET ↔PPH ↔SGA ↔IUD	EN
Hadfield 2009[177]	Australia	Population based longitudinal	First singleton delivery ICD10 coded endometriosis on medical records N= 3239 Subgroup ovarian endometriosis N= 846	First singleton delivery no ICD10 coded endometriosis on medical records N= 205640 Subgroup no endometriosis IVF pregnancy N= 841	NC/ART	↔PIH ↔PET	EN

Harada 2016[178]	Japan	Multicentre prospective cohort	Singleton pregnancy with history of endometriosis N= 330	Singleton pregnancy with no history of endometriosis N= 8856	NC/ART	↑PTD ↑LSCS ↑PP ↑PA ↑threatened PTD ↔PET ↔GDM ↔IUGR ↔PROM ↔birthweight	EN
Hashimoto 2018[179]	Japan	Multicentre cohort	Singleton pregnancy with adenomyosis diagnosed on USS or MRI N= 49	Singleton pregnancies with normal pelvic USS N= 245	NC/ART	↑2nd trimester miscarriage ↑PET ↑PIH ↑PP ↑PTD ↑GDM ↑LSCS ↑SGA ↔malpresentation ↔PPH ↔Apgar <7 at 5mins	AD
Juang 2007[180]	Taiwan	Single centre nested case-control	Preterm delivery with pre-pregnancy USS/MRI N= 104	Term delivery with pre-pregnancy USS/MRI N= 208	NC/ART	Adenomyosis associated with risk ↑PTD ↑PPROM	AD
Kohl Schwartz 2017[181]	Switzerland	Multicentre observational cohort	Multiparous women with history of surgically treated endometriosis N= 143 (240 pregnancies)	Multiparous women with no history of endometriosis at annual gynaecology check-up N= 143 (268 pregnancies)	NC/ART	↑MR	TxEN
Li 2017[182]	China	Single centre cohort	Pregnancy with history of surgically diagnosed endometriosis Stage I-II N= 45 Stage III-IV N= 30	Pregnancy with no history of gynaecological diseases N= 300	NC/ART	↑PPH ↔GDM ↔PA ↔PIH/PET ↔PP ↔LSCS ↔Apgar score <7 at 5mins ↔birthweight	EN
Manini 2017[183]	Italy	Single centre cohort	Pregnancy with history of surgically treated endometriosis DIE N= 40 Non-DIE N= 222	Pregnancy with no history or USS signs of endometriosis N= 524	NC/ART	↔LSCS ↔PP ↔IUGR ↔PIH ↔GDM ↔PTD ↔PPH	TxEN
Mardanian 2016[184]	Iran	Single centre cohort	First pregnancy following laparoscopic findings of endometriosis N= 101	First pregnancy following negative laparoscopy N= 101	NC/ART	↔PIH ↔PET	EN
Mochimaru 2015[185]	Japan	Single centre age matched case-control	Adenomyosis on USS or MRI +/- fibroids N= 36	No adenomyosis on USS N= 144	NC/ART	↑PTD ↑SGA ↑PPROM ↑severe PPH ↑LSCS ↔PET ↔extreme prematurity ↔IUD ↔NNU ↔Apgar scores	AD

Santulli 2016[186]	France	Single centre cohort	Laparoscopy/laparotomy for pelvic pain, infertility or benign pelvic mass and endometriosis diagnosed N= 284	Laparoscopy/laparotomy for pelvic pain, infertility or benign pelvic mass and no endometriosis found N= 466	NC/ART	↑MR	I-II III-IV DIE
Saraswat 2017[187]	UK	Multicentre population-based cohort	First singleton delivery following surgical diagnosis of endometriosis N= 5375	Singleton delivery no history or symptoms of endometriosis N= 8280	NC/ART	↑MR ↑ectopic ↑PTD ↑hypertensive disorders ↑APH ↑PP ↑PPH ↑LSCS ↑instrumental delivery ↔IUGR ↔IUD ↔PA ↔NND	EN
Shin 2018[188]	Korea	Single centre case control	Singleton delivery >20/40 with adenomyosis diagnosed on USS at 7/40 N= 72	Singleton delivery >20/40 with no adenomyosis on USS at 7/40 N= 8244	NC/ART	↑PTD ↓birthweight ↔LSCS NC subgroup analysis: ↔PTD ↓birthweight	AD
Shmueli 2017[189]	Israel	Single centre cohort	Singleton delivery with history of endometriosis N= 135	Singleton delivery with no history of endometriosis N= 61400	NC/ART	↑LSCS ↑PP ↑PPH ↑NNU ↑neonatal comorbidities analysed e.g. sepsis, HIE etc ↓birthweight ↔PIH ↔PET ↔GDM ↔Apgar <7 at 5mins	EN
Stephansson 2009[190]	Sweden	Multicentre population-based cohort	Singleton delivery history of endometriosis by ICD 10 code in medical records N= 13090 deliveries	Singleton delivery no history of endometriosis by ICD 10 code in medical records N= 1429585 deliveries	NC/ART	↑PTD ↑PET ↑APH ↑LSCS ↔SGA ↔IUD	EN
Tzur 2018[191]	Israel	Single centre population-based cohort	Singleton delivery with surgically diagnosed endometriosis N= 35	Singleton delivery with no history of endometriosis N= 467	NC/ART	↑PTD ↑LSCS ↔PPH ↔PA ↔Apgar <7 at 5 mins ↔hypertensive disorders ↔IUGR ↔birthweight	EN I-II III-IV

Natural Conception Studies							
Exacoustos 2016[192]	Italy	Multicentre observational cohort	Pregnancy with incomplete surgical treatment for DIE with remaining posterior DIE nodule N= 200	Pregnancy with no history of endometriosis or adenomyosis N= 300	NC	↑PTD ↑PP ↑PA ↑LSCS ↓NVD ↑PIH ↔GDM ↔SGA ↔instrumental delivery	DIE
Lin/Leng 2015[187]	China	Single centre cohort	Singleton first pregnancy with history of endometriosis diagnosed at laparoscopy N= 249	Singleton first pregnancy with normal USS and no clinical or surgical history of endometriosis N= 249	NC	↑PTD ↑PP ↑LSCS ↔SGA ↔IUGR ↔PIH ↔PA	EN
Stern 2015[193]	USA	Multicentre population-based cohort	Live birth with diagnosis of endometriosis by ICD10 code in medical records N= 590	Live birth with no diagnosis of endometriosis by ICD10 code in medical records N= 297,987	NC (NC/ART data had no control)	↑primary LSCS ↑PTD ↓birthweight ↔SGA ↔PIH ↔GDM	EN

Key:

NC = Natural conception studies

NC/ART = Natural conception and assisted reproduction therapy studies

IVF/ICSI = IVF/ICSI studies

AD = Adenomyosis studies

EN = Endometriosis (unspecified) studies

TxEn = Treated endometriosis studies

OMA = Endometrioma studies

I-II = Endometriosis stage I-II studies

III-IV = Endometriosis stage III-IV studies

DIE = Deep infiltrating endometriosis studies

Study Design and Setting

Studies examined the reproductive outcomes of spontaneously conceived pregnancies alone (NC)(n=3) or as a result of IVF/ICSI using their own gametes (n=75). Twenty population-based studies examined reproductive outcomes of all types of conception including those conceived through assisted reproduction (NC/ART) (n=20).

In the majority of studies, endometriosis or absence of endometriosis is diagnosed at laparoscopy. Some studies used ultrasound scans to guide diagnosis where endometrioma were identified. The control groups were women with tubal infertility (n=41), male factor infertility (n=6), unexplained infertility (n= 2) or infertility of mixed aetiology (n= 26) where endometriosis was excluded at laparoscopy or was not indicated in clinical history in combination with a normal pelvic ultrasound scan.

Treatment of endometriosis was surgical (excision/ablation of lesions, adhesiolysis, cystectomy/drainage of endometrioma) (n= 12), medical (gonadotrophin releasing hormone analogues, continuous combined contraceptive (n=1), surgical “or” medical (n=2) or a surgical and medical treatment (gonadotrophin releasing hormone analogues, use of continuous combined contraceptive pill or androgens) (n= 3).

Quality of Included Studies and Risk of Bias

The Downs and Black scores pertaining to blinding of participants to treatment were excluded from the tool, as this was not possible when assisted reproduction or spontaneous conception was occurring. Downs and Black scores are shown in Table 3: Risk of Bias Downs and Black Scores.

Table 3: Risk of Bias Downs and Black Scores for Each Study Included in Meta-analysis

IVF/ICSI Conception Studies					
Study	Study quality & reporting /8	External validity /3	Study bias /4	Confounding & selection bias /3	Total score /18
Al-Azemi 2000	5	2	3	2	12
Al-Fadhli 2006	6	2	3	3	14
AlKudmani 2017	6	2	3	2	13
Arici 1996	5	2	3	2	12
Ashrafi 2014	7	1	4	3	15
Benaglia 2013	7	2	3	3	15
Benaglia/Bermejo 2012	8	2	3	3	16
Benaglia/Candotti/Busnelli 2015	7	2	3	3	15
Benaglia/Candotti 2016	7	2	3	2	14
Bergendal 1998	6	2	3	2	13
Bongioanni 2011	6	2	2	1	11
Borges 2015	6	2	3	2	13
Brosens 2007	5	2	3	2	12
Bukulmez 2000	4	2	3	2	11
Canis 2001	4	2	3	2	11
Chang 1997	6	2	3	2	13
Chiang 1998	7	2	3	3	13
Coccia 2011	7	2	3	2	12
Coelho Neto 2015	7	2	3	2	12
Coelho Neto/Martins 2016	7	2	3	3	13
Costello 2011	7	3	4	2	16
Dong 2013	6	2	3	3	14
Falconer 2009	5	2	3	2	12
Fernando 2009	5	2	3	2	12

Frydman 1987	2	1	2	2	7
Frydman 1987	2	1	2	2	7
Fuji 2016	7	2	3	3	15
Geber 1995	3	1	3	2	9
González-Comadran 2017	6	2	4	2	14
Gonsález-Foruria 2016	6	2	3	2	13
Guler 2017	5	2	2	1	10
Healy 2010	6	3	3	2	14
Hickman 2002	5	2	3	2	12
Hull 1998	6	2	3	2	13
Jacques 2016	6	2	3	1	12
Kim 2007	6	2	3	1	12
Kiran 2012	6	2	2	1	11
Kuivasaari 2005	5	2	3	2	12
Kuivasaari-Pirinen 2012	5	2	3	3	13
Kuroda 2009	6	2	3	2	13
Leonardi 2016	7	2	3	3	15
Lin 2012	5	2	3	2	12
Matalliotakis 2011	6	2	3	2	13
Matson 1986	3	0	2	0	5
Meden-Vrtovec 2000	5	2	3	3	13
Mekaru 2013	6	1	3	2	12
Mohamed 2011	7	2	3	3	15
Motte 2016	7	3	3	2	15
Nakagawa 2016	5	2	3	2	12
Nejad 2009	6	2	3	2	13
Oehninger 1988	3	1	4	3	11
Olivennes 1995	6	2	3	3	14

Omland 2006	6	2	3	2	13
Opøien 2012	6	2	2	2	12
Ozgur 2017	7	2	4	2	15
Pabucco 2004	5	2	3	2	12
Pellicer 1998	4	2	3	0	9
Polat 2014	6	2	3	2	13
Pop-Trajkovic 2014	6	2	3	1	12
Salim 2012	5	3	4	2	14
Saucedo-de-la Llata 2004	6	2	3	2	13
Scarselli 2011	5	2	3	2	12
Senapati 2016	6	3	3	4	16
Shebl 2016	7	2	3	3	15
Simon 1994	5	2	3	2	12
Suzuki 2005	5	2	3	2	12
Tanbo 1995	5	2	3	3	13
Thalluri 2012	7	2	3	3	15
Vaz 2017	5	2	4	2	13
Wardle 1985	5	2	3	1	11
Wyns 2003	4	2	3	2	11
Yamamoto 2017	6	3	4	3	16
Yan 2014	8	3	3	3	17
Youm 2011	6	2	3	3	14
Yovich 1990	2	1	3	1	7
Natural Conception and ART Population Studies					
Study	Study quality & reporting /8	External validity /3	Study bias /4	Confounding & selection bias /3	Total score /18
Aris 2014	5	3	3	2	13
Berlac 2017	5	2	3	2	12

Chen 2018	7	3	3	3	16
Conti 2015	6	2	3	2	13
Glavind 2016	8	2	2	3	15
Hadfield 2009	5	2	3	2	12
Harada 2016	5	3	4	3	15
Hashimoto 2018	7	3	3	3	16
Juang 2006	7	2	3	3	15
Koh Schwartz 2017	8	3	3	3	17
Li 2017	7	2	4	3	16
Manini 2017	7	1	4	3	15
Mardanian 2016	5	2	2	2	11
Mochimaru 2015	7	2	3	2	14
Santulli 2016	7	2	3	4	16
Saraswat 2016	7	3	3	2	15
Shin 2017	8	3	4	3	18
Shmueli 2017	7	2	3	3	15
Stephansson 2009	6	2	3	2	13
Tzur 2018	7	2	3	3	15
Natural Conception Studies					
Study	Study quality & reporting /8	External validity /3	Study bias /4	Confounding & selection bias /3	Total score /18
Exacoustos 2016	5	2	4	1	12
Lin/Leng 2015	7	3	3	2	15
Stern 2015	5	2	3	2	12

Outcomes of Adenomyosis Studies

Secondary outcomes were reported in the following study groups for women with adenomyosis compared to controls:

No NC or NC/ART studies reported CPR, LBR or MR. In IVF/ICSI studies clinical pregnancy rate was reduced (0.59, CI 0.36-0.97, p=0.04) (n=6 studies), live birth rate was not affected (n=4) and there was an increased risk of miscarriage (3.40, CI 1.09-10.64, p=0.04) (n=5) (Figure 13).

Late Pregnancy and Neonatal Complications

No NC or IVF/ICSI studies reported late pregnancy or neonatal complications.

NC/ART studies found an increased risk of PTD (OR 2.74, CI 1.89-3.97, p <0.00001) (n=5), SGA (OR 3.90, CI 2.10-7.25, p <0.0001) (n=2), LSCS (OR 2.62, CI 1.00-6.89, p=0.05) (n=3) and PET (OR 7.87, CI 1.26-49.20, p= 0.03) (n=2) (Figure 13). One study found an increased risk of PP and PPH, no increased risk of PIH and reduced risk of GDM. One study found women with adenomyosis had no increased risk of IUD but did have an increased risk of NNU admissions following delivery.

IVF/ICSI Treatment Outcomes

Implantation rate was reduced (0.56, CI 0.39-0.8, p=0.001) (n=3) (Appendix 5a). There was no difference in oocyte yield (n=3) or CR (n=2). One study found no difference in FR, no other outcomes were reported.

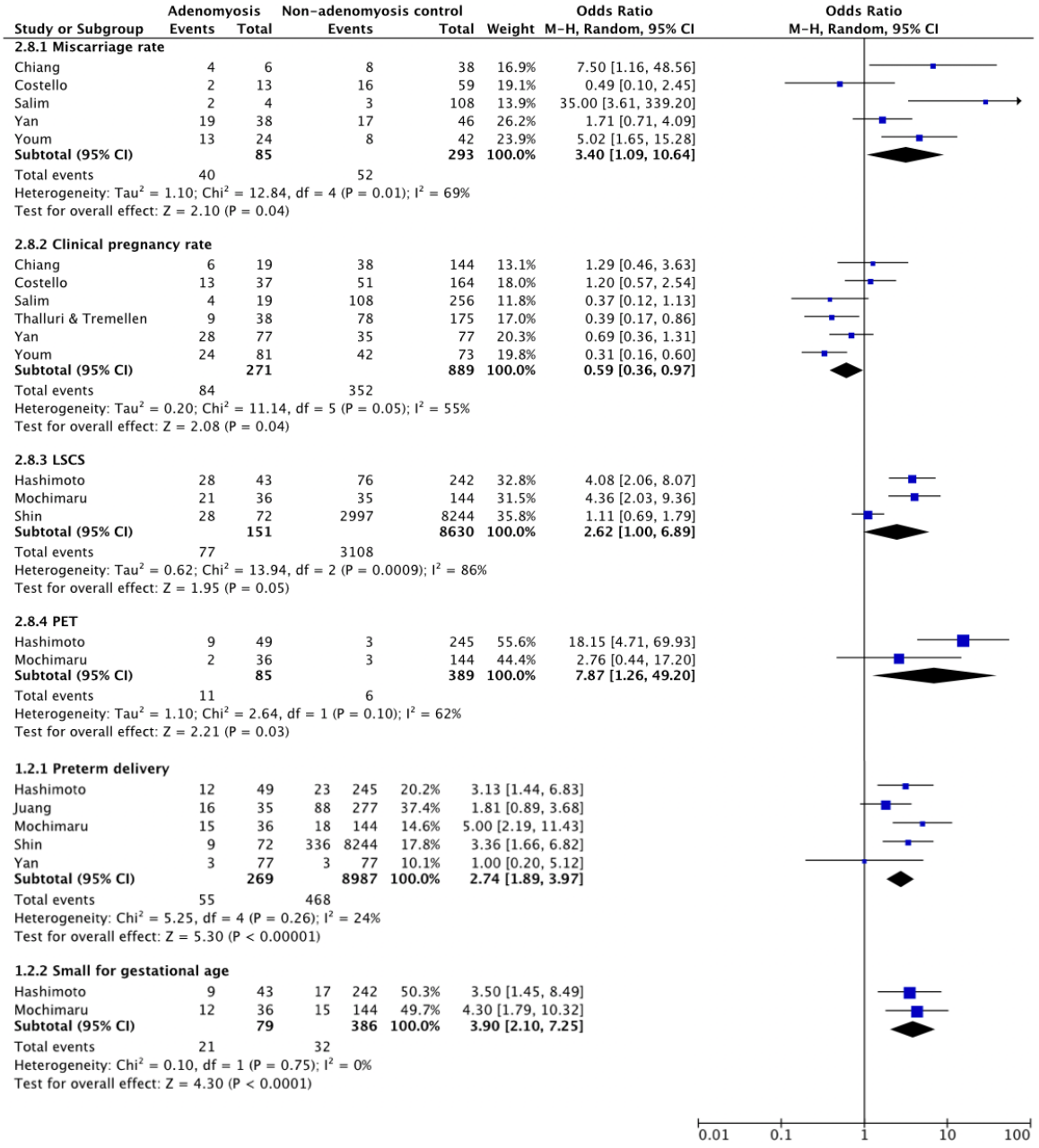


Figure 13: Forest plots demonstrating secondary outcomes (miscarriage rate OR 3.40, n= 5 and clinical pregnancy rate OR 0.59, n= 6) and late pregnancy outcomes (LSCS OR 2.62, n= 3, PET OR 7.87, n= 2), preterm delivery OR 2.74, n= 5 and small for gestational age OR 3.90, n=2) for women with adenomyosis compared to non-adenomyosis controls

Outcome of Endometriosis (overall) Studies

Secondary outcomes were reported in the following study groups for women with endometriosis (no subtype, severity unspecified) compared to controls (Figure 14 and Figure 15):

No NC or NC/ART studies reported CPR or LBR. IVF/ICSI studies demonstrate a reduced CPR (OR 0.93, CI 0.87-0.98, p= 0.01) (n= 27) and LBR (OR 0.83, CI 0.74-0.95, p= 0.005) (n= 14) and increased MR (OR 1.27, CI 1.06-1.53, p=0.009) (n= 16) in women with endometriosis compared to non-endometriosis controls. NC/ART studies also found increased MR (OR 1.83, CI 1.15-2.91, p= 0.01) (n= 3).

Late Pregnancy and Neonatal Complications (Table 4)

NC studies found the risk of PIH (OR 1.29, CI 1.01-1.66, p= 0.04) (n= 2), PTD (OR 1.42, CI 1.31-1.53, p <0.00001) (n= 3), and LSCS (OR 1.82, CI 1.56-2.13, p <0.00001) (n=2) were increased but not the risk of SGA (n=2). No other late pregnancy outcomes were reported. They did not report neonatal outcomes.

NC/ART studies demonstrated an increased risk of PTD (OR 1.41, CI 1.02-1.95) (n= 10), PP (OR 3.25, CI 2.11-4.99, p <0.00001) (n= 8), LSCS (OR 2.07 CI 1.71-2.51, p <0.00001) (n= 9) (Figure 14), PET (OR 1.21, CI 1.05-1.38, p= 0.006) (n=10) (Appendix 5 b), abruption (OR 1.87, CI 1.65-2.12, p <0.00001) (n=7) (Figure 14) and IUD (OR 1.25, CI 1.07-1.45, p= 0.004) (n= 4) (Appendix 5 c) whilst the risk of GDM (n=6), PIH (n=6), PPH (n= 9) and SGA (n=5) were not increased. An increased risk of NNU admission was demonstrated (OR 1.49, CI 1.07-2.09, p= 0.02) (n= 4). NND was increased in one study.

In IVF/ICSI studies there was increased risk of PTD (OR 1.61, CI 1.14-2.26, p= 0.006) (n=5) and LSCS (OR 1.73, CI 1.00-3.00, p= 0.05) (n=3). There was no difference in risk of SGA (n=3), PP (n=3), PPH (n=3), PET (n=6) or PIH (n=3). One study reported no difference in risk of abruption or GDM. Risk of IUD was not reported. There was an increased risk of NNU admissions (OR 1.91, CI 1.12-3.26, p= 0.02) (n=2) but NND rates were not reported.

IVF/ICSI Treatment Outcomes

There was a reduced oocyte yield (MD -1.42, CI -1.94, -0.90, p <0.00001) (n= 17) with lower mature oocytes (MD -1.23, CI -1.41, -1.05, p <0.00001) (n= 5), reduced FR per oocyte (OR 0.92, CI 0.86-0.99, p= 0.03) (n=2) and reduced IR (OR 0.82, CI 0.74-0.92, p= 0.0004) (n= 12). We also found an increased CR (OR 1.50, CI 1.22-1.84, p= 0.0001) (n= 12) (Appendix 6 a-e).

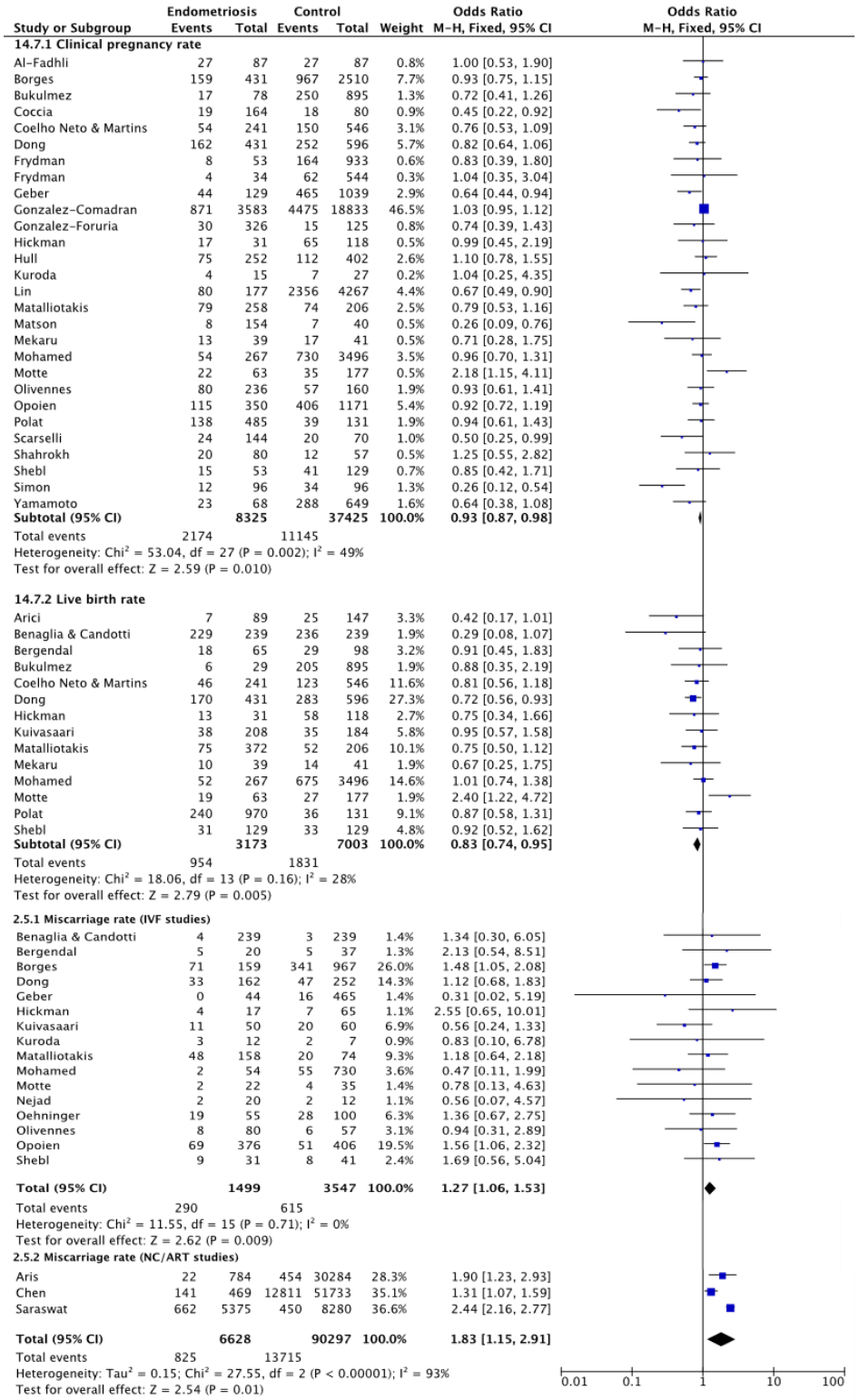


Figure 14: Forest plots demonstrating secondary outcomes in IVF/ICSI studies (clinical pregnancy rate OR 0.93, n= 27, live birth rate OR 0.83, n= 14, miscarriage rate OR1.27, n= 16) and in NC/ART studies (miscarriage rate OR 1.49, n=2) for women with endometriosis compared to non-endometriosis controls

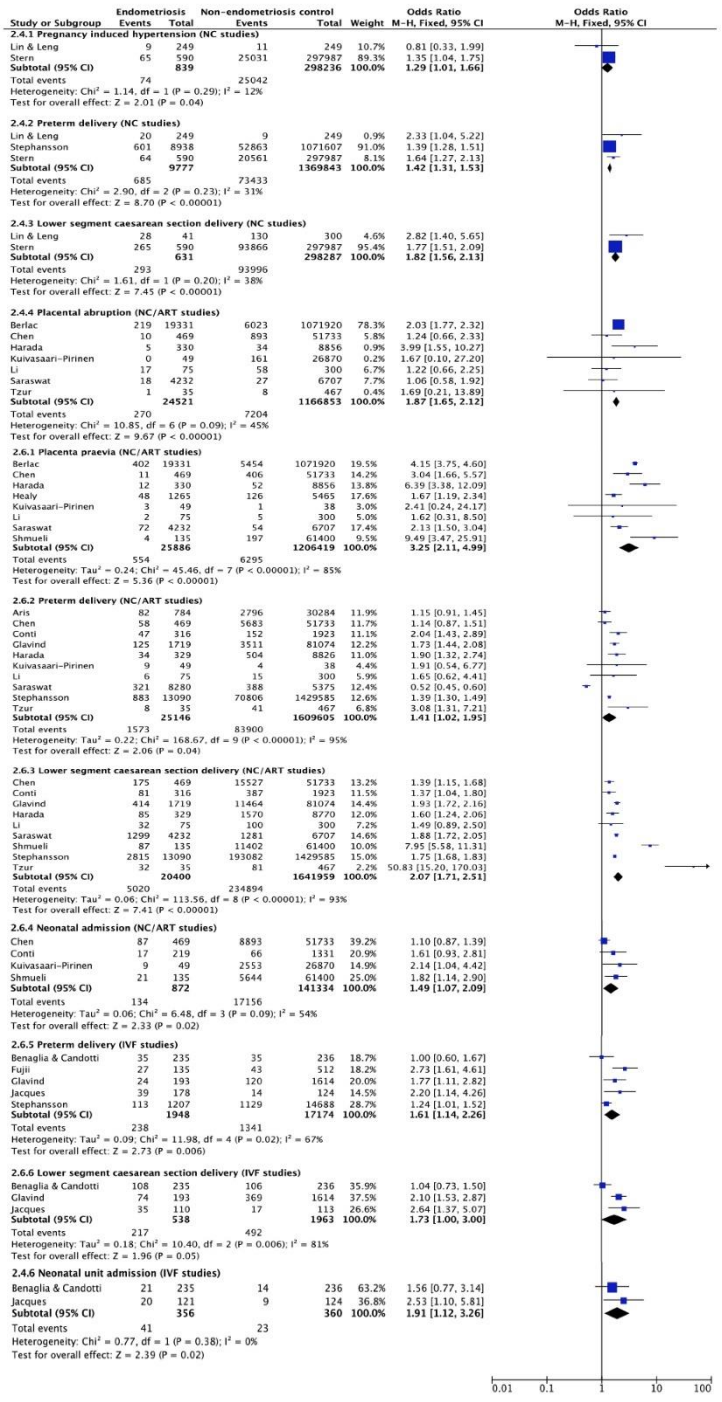


Figure 15: Forest plots of late pregnancy outcomes in NC studies (PIH OR 1.29, n= 2, PTD OR 1.42, n= 3, LSCS OR 1.82, n= 2), NC/ART studies (abruption OR 1.87, n= 7, PP OR 3.25, n= 8, PTD OR 1.41, n= 10, LSCS OR 2.07, n= 9, NNU admission OR 1.49, n= 4) and IVF/ICSI studies (PTD OR 1.61, n= 5, LSCS OR 1.73, n= 3 and NNU admission OR 1.91, n= 2) for women with endometriosis compared to non-endometriosis controls

Table 4: Summary of the risk of late pregnancy and neonatal complications in endometriosis compared to controls according to mode of conception as found in meta-analysis

Endometriosis	NC studies	NC/ART studies	IVF/ICSI studies
Preterm delivery	↑	↑	↑
Small for gestational age	↔	↔	↔
Lower segment caesarean section rate	↑	↑	↑
Placenta previa		↑	↔
Placental abruption		↑	
Gestational diabetes		↔	
Post-partum haemorrhage		↔	↔
Pregnancy induced hypertension	↑	↔	↔
Pre-eclampsia		↑	↔
Intrauterine death		↑	
Neonatal admission		↑	↑
Neonatal death			

Key:

↑ = Risk significantly increased in endometriosis ↔= No difference in risk blank = No data for meta-analysis

Treated Endometriosis

Secondary outcomes were reported in the following study groups for women with treated endometriosis compared to controls.

No NC or NC/ART studies reported CPR, LBR or MR. In IVF/ICSI studies there was no difference in CPR (n= 8), LBR (n= 4), or MR (n= 5).

Late Pregnancy Complications

No NC or IVF/ICSI studies reported late pregnancy or neonatal complications.

Three NC/ART studies reported late pregnancy complications. There was no increased risk of LSCS (n= 3). Individual studies reported other late pregnancy outcomes and found increased risk of GDM (OR 2.13, CI 1.36-3.33, p= 0.0009), increased risk of PTD, PP, PPH, PIH, PET, abruption and SGA and no difference in risk of IUD. One study reported an increased risk of NND (OR 1.90, CI 1.26-2.87, p= 0.002).

IVF/ICSI Treatment Outcomes

Oocyte yield (MD -1.21, CI -1.40, -1.02, p <0.00001) (n= 6), mature oocyte yield (MD -1.27, CI -1.45, -1.08, p <0.00001) (n= 3) and FR were reduced (OR 0.92, CI 0.86-0.99, p= 0.03) (n=2) (Appendix 7 a and b). There was no difference in IR (n=3) or CR (n=3).

Untreated Endometriosis

One study examined the effect of untreated endometriosis on fertility and reproductive outcomes, which found no difference in CPR, MR, oocyte yield, FR or IR.

Endometriosis Subtypes Analysis

Stage I-II endometriosis

Secondary outcomes were reported in the following study groups for women with stage I-II endometriosis compared to controls.

No NC studies reported any of our secondary, late pregnancy or neonatal outcomes. One NC/ART study reported an increased MR (OR 1.68, CI 1.20-2.35, p=0.002) and no other secondary, late pregnancy or neonatal outcomes were reported. IVF/ICSI studies showed no difference in CPR (n=14) or LBR (n=8) but demonstrated an increased MR (OR 1.39, CI 1.05-1.85, p= 0.02), (n=10) (Appendix 8 a).

Late Pregnancy and Neonatal Complications

One study IVF/ICSI study found no increased risk of LSCS, PPH, GDM, PET, PP, PTD or NNU admission. No other studies examined late pregnancy complications.

IVF/ICSI Treatment Outcomes

There was a reduced FR (OR 0.77, CI 0.63-0.93, p= 0.007) (n= 8) and IR (OR 0.76, CI 0.62-0.93, p= 0.008) (n=8) and an increased CR (OR 1.74, CI 1.13-2.67, p=0.01) (n= 4) (Appendix 8 b-d). There was no difference in oocyte yield (n=11) or number of mature oocytes (n= 3).

Stage III-IV Endometriosis

Secondary outcomes were reported in the following study groups for women with stage I-II endometriosis compared to controls.

No NC studies reported any of our secondary, late pregnancy or neonatal outcomes. One NC/ART study reported an increased MR (OR 1.72, CI 1.26-2.34, p=0.0006) and no other secondary, late pregnancy or neonatal outcomes were reported. In IVF/ICSI studies there was reduced CPR (OR 0.86, CI 0.76-0.98, p= 0.02) (n= 13) and reduced LBR (OR 0.70, CI 0.54-0.89, p=0.004) (n= 9). Studies also revealed an increased MR (OR 1.34, CI 1.03-1.74, p=0.03) (n=9) (Appendix 9 a-c).

Late Pregnancy and Neonatal Complications

One IVF/ICSI study reported an increased risk of PET, LSCS, PTD and risk of NNU admission but no difference in risk of PP, GDM and PPH.

IVF/ICSI Treatment Outcomes

There was a significant reduction in oocyte yield (MD -1.69, CI -2.45, -0.92, p <0.0001) (n= 11), mature oocyte yield (MD -1.12, CI -1.84, -0.40, p=0.002) (n=3) and IR (OR 0.80, CI 0.70-0.92, p=0.001) (n= 11) (Appendix 10 a-c). There was no difference in FR (n= 7) or CR (n= 4).

Endometrioma

Secondary outcomes were reported in the following study groups for women with endometrioma compared to controls.

There were no NC or NC/ART studies eligible for inclusion. Studies in IVF/ICSI conceived pregnancies found no difference in CPR (n=9), LBR (n=5) or MR (n=4).

Late Pregnancy and Neonatal Complications

There was no difference in risk of PTD or SGA (n=2) in IVF/ICSI studies. One study demonstrated no increased risk of LSCS. No other late pregnancy or neonatal complications were reported.

IVF/ICSI Treatment Outcomes

There was lower oocyte yield (MD -1.22, CI -1.96, -0.49 p=0.001) (n= 12) and lower mature oocyte yield (MD -2.24, CI -3.40, -1.09, p <0.00001) (n=4) (Appendix 11). There was no difference in IR (n=3) or CR (n=5). Two studies reported fertilisation rate but data could not be combined. One study found increased FR (OR 1.88, CI 1.34-2.64, p= 0.0003) and the other found no difference in FR.

Deep Infiltrating Endometriosis

Only three studies met inclusion criteria for the presence of deep infiltrating endometriosis (DIE). One study was an IVF/ICSI study (Vaz et al)[168], one was NC/ART (Santulli et al)[186] and the other NC (Exacoustos et al.)[192] and therefore no data could be combined in meta-analysis. Santulli et al

reported MR was higher in patients with DIE. Vaz et al found no difference in CPR or MR. Exacoustos et al reported an increased risk of PTD, PP, PA and LSCS in women with DIE but no difference in risk of PIH, GDM and SGA.

3.6 Discussion

This systematic review and meta-analysis investigated the reproductive, obstetric and neonatal outcomes of women with endometriosis and adenomyosis on the reproductive outcomes from gametes to birth. The data on the impact of the disease on gametes and fertilisation was derived from studies with a population of women undergoing IVF/ICSI treatment, where data pertaining to fertilisation and embryo development can be obtained from routinely recorded laboratory observations; whilst the outcomes on early and late pregnancy complications were obtained from the collation of data from a combination of epidemiological data as well as case-control studies.

3.6.1 Main Findings

All comparative analyses of endometriosis in IVF/ICSI studies of this meta-analysis demonstrate a negative impact of the disease on various IVF parameters, in agreement with current evidence, and give us insight into the effect on early gamete and embryo development (Figure 16 and Table 5). It was found that endometriosis consistently leads to reduced oocyte yield and a reduction in mature oocytes perhaps in the milder subtype. This is indicative of altered folliculogenesis and oocyte development the cause of which may be due to altered steroidogenesis and raised inflammatory markers in the follicular environment. Dysfunctional steroidogenesis in endometriosis patients results in oestrogen levels that are increased in the peritoneal fluid but decreased in the follicular fluid [194,195]. Elevated interleukins seen in endometriosis patients can cause cell cycle abnormalities such as those preventing p27 breakdown leading to G0 arrest [194] and follicles with higher levels of interleukins are more likely to contain an immature oocyte [196]. A reduced fertilisation rate was found implicating poorer oocyte quality in line with findings of reactive oxygen species-induced DNA damage, spindle abnormalities and reduced membrane integrity in endometriosis which contribute to oocyte damage, degradation or apoptosis [194]. During the ICSI process, reactive oxygen species can also induce embryonic fragmentation and result in fewer blastocysts [194]. Morphological differences in oocytes have also been noted in

endometriosis patients including increased cytoplasmic granulation, increased zona pellucida hardening, lower mitochondrial content and a higher proportion of abnormal mitochondria which may have a negative impact on fertilisation [196]. In all stages of endometriosis, reduced implantation rate was found, demonstrating a potential clinical impact of changes at the molecular level in endometrial gene expression [197–199], adhesion molecules [200–202] implantation markers and local response to progesterone [203]. The IVF/ICSI studies also reveal an increased risk of miscarriage is associated with adenomyosis, and endometriosis of all ASRM stages, further supporting a theory of suboptimal implantation and early development (Figure 16 and Table 5).

There was over three-fold increased risk of miscarriage in adenomyosis patients with IVF pregnancy and this miscarriage risk was not commonly reported in NC studies. The risk of miscarriage for women with endometriosis was 27% higher than controls with IVF pregnancy and 49% higher in pregnancy by any mode of conception. The results show that endometriosis can be associated with a range of obstetric and fetal complications in IVF pregnancies compared to non-endometriosis IVF controls including preterm delivery (61% higher risk than controls), caesarean section delivery (73% higher risk), neonatal unit admission following delivery (approximately two-fold increased risk) (Figure 16 and Table 5). It was found that similar complications are associated with endometriosis in pregnancies by any mode of conception (NC/ART) compared to non-endometriosis controls including a 23 % higher risk of PET, 85% higher risk of placental abruption, 49% higher risk of neonatal unit admission following delivery, 25% higher risk of IUD, over two-fold increase in caesarean section delivery and nearly three-fold increased risk of placenta praevia (Figure 16 and Table 5). Women with endometriosis conceiving naturally were shown to have an increased risk of preterm delivery (42% higher risk) and pregnancy induced hypertension (29% increased risk) compared to controls (Figure 16 and Table 5). These findings suggest possible implantation and placentation abnormalities but data on individual endometriosis subtypes was lacking to draw conclusions regarding subtype specific complications. Implantation and early placentation is differentially modulated in the endometrium of women with endometriosis compared to those without for example in the differential expression of key factors in decidualisation and implantation by way of aberrant angiogenesis, immune remodelling, alternations in cell adhesion molecules, matrix remodelling, and immune signalling [202–204]and the overexpression of vascular endothelial growth factor, angiopoietins and their receptor. Several changes found in endometriosis could be implicated in the association with placental insufficiency disorders. The thickness of the junctional zone (JZ) has been shown to be increased [205],

endometrial blood perfusion is increased [203,206] and there may be suppression of HOXA-10 upregulation which regulates endometrium receptivity to implantation [203]. Suboptimal placentation can also result from defective spiral artery remodelling at the JZ of the myometrium-endometrium interface together with the size of placental bed and distribution of spiral artery transformation within the placental bed favouring the centre to the periphery [203,207], although this has not been investigated specifically in endometriosis or adenomyosis. Whether the presence of these abnormalities in women with endometriosis and adenomyosis are responsible for the increased risk of early miscarriages and/or later obstetrics complications will need to be borne out by future longitudinal large cohort studies.

Disease and subtype specific outcomes are also observed in this meta-analysis and systematic review although sensitivity analysis for these subgroups revealed a number of findings must be viewed with caution due to results being influenced by small numbers of studies in these areas (Appendix 4). Milder forms of endometriosis are more likely to affect the fertilisation and earlier implantation processes and impact on miscarriage risk as depicted in Figure 16. The more severe disease (ASRM III and IV) influences all stages of reproduction, from the stages of oocyte and gamete development to early and later pregnancy complications (Figure 16). Ovarian endometriosis negatively affects the oocyte yield and number of mature oocytes per IVF/ICSI cycle compared to controls. This work and the work of others have shown that conditions with elevated reactive oxidative species such as endometriosis can detrimentally impact on follicular maturation with resultant meiotic spindle and oocyte DNA damage [60,194]. The evidence that can be collated on DIE is less complete due to the lack of studies with suitable control groups, and many studies did not differentiate DIE from ASRM stages III-IV disease. It is however, observed that DIE is associated with an increased miscarriage risk, and a reduced cumulative pregnancy rate [208], with associated complications ante-natally such as those late pregnancy outcomes of our analysis (Table 5). There is also a growing number of case reports highlighting uncommon ante-natal complications which pose significant morbidity and mortality risks to both mother and fetus. In this systematic literature search I identified 10 case reports [209–218]. The reports included uterine rupture, ovarian cyst accidents requiring surgery in pregnancy, spontaneous haemoperitoneum and spontaneous bowel perforation. Furthermore, deep infiltrating endometriosis and severe endometriosis in several case reports were associated with fourth degree tears with intact anal sphincter due to rectovaginal endometriotic lesions and increased surgical complications at caesarean section delivery including bladder injury, bowel injury and peri-partum hysterectomy.

Many difficulties of analysing observational data have been encountered in this study. The gold standard process for diagnosing endometriosis is visualising disease at surgery and gaining histological confirmation. Adenomyosis is notoriously challenging to diagnose without histological confirmation following a hysterectomy. Imaging modalities and visualising the uterus at surgery is less sensitive for diagnosing adenomyosis compared to endometriosis. Only studies that stated concomitant adenomyosis with endometriosis in their case groups were excluded. Many studies did not explicitly mention if one disease was mutually exclusive from the other in their case group. It is therefore conceivable that endometriosis groups within the included studies were confounded by cases of adenomyosis and vice versa. Furthermore, studies defining case and control group based on ICD 10 coded medical records were also included. It is possible that these diagnoses have been made based on symptoms/clinical examination/imaging and not with surgical confirmation. These diagnoses will inevitably be less reliable and control groups could contain asymptomatic women with endometriosis and endometriosis groups could contain women with convincing symptomatology of endometriosis but no disease. Several of the early and late pregnancy outcomes are uncommon and therefore need to be investigated with high numbers of studies and high sample sizes within studies. Balancing quality of studies with a number of studies to strengthen a meta-analysis measuring uncommon outcomes is complex.

Quality of studies was assessed with a modified Down and Blacks checklist (with points pertaining to RCTs removed from the tool). This could affect the validity of the tool as it has been validated for use in its entirety. The Down and Blacks checklist was selected for its ease of use when there was a large number of included studies to assess. It was decided that a more demanding checklist could introduce user error when used in high numbers repetitively. Scores for quality of the included studies were included in the study but a threshold for inclusion based on quality was not used as it would be set at an arbitrary level. Sensitivity analysis was performed, and it is discussed in this work whether a result should be interpreted with caution or not. However, this meta-analysis could be strengthened by rejecting studies of lower quality and by removing outcome data where sensitivity analysis indicates that results should be interpreted with caution.

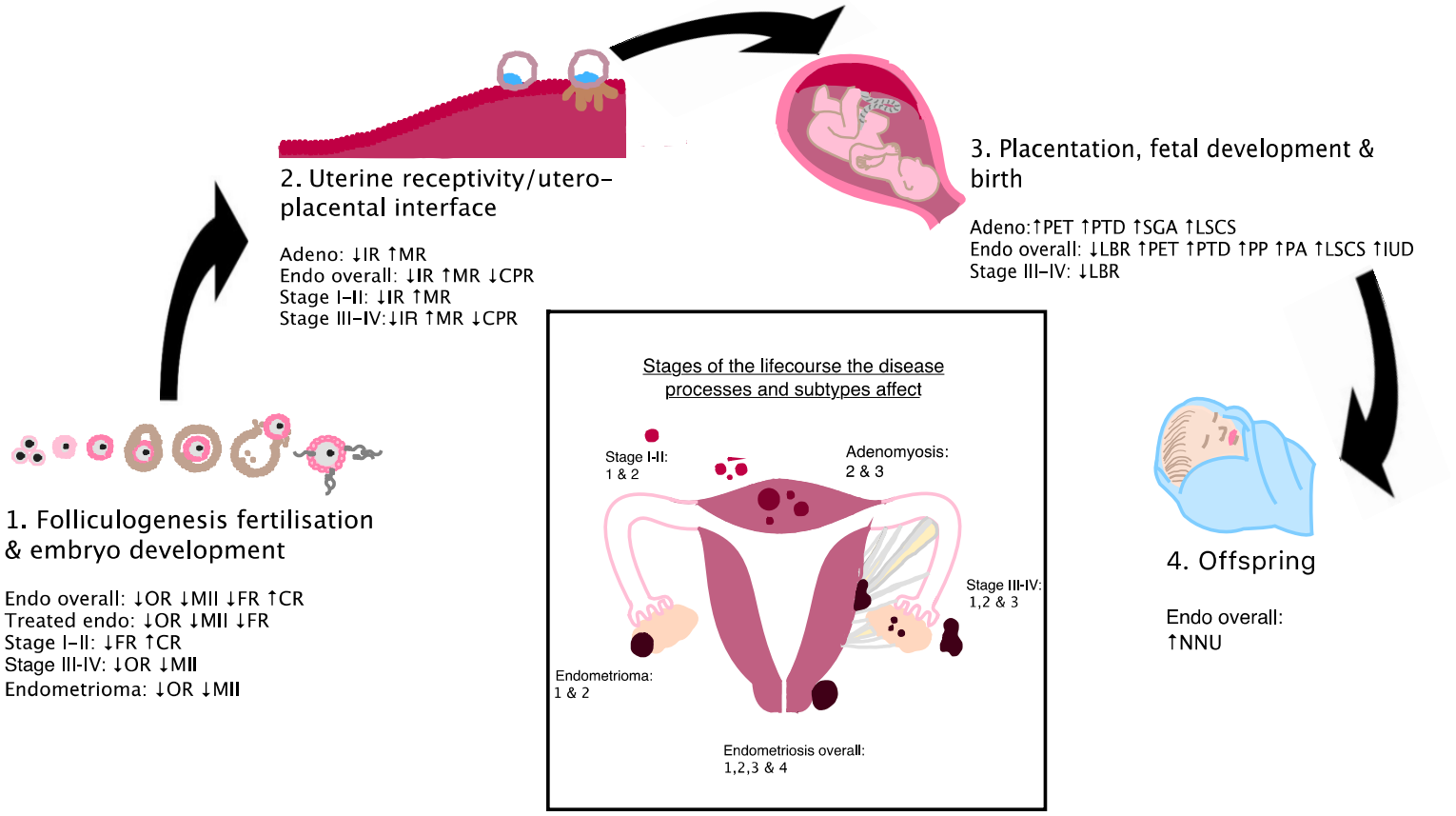


Figure 16: Summary of findings: Impact of endometriosis and adenomyosis on fertility, obstetric and neonatal outcomes

Table 5: Summary of findings

	Adenomyosis			Endometriosis Overall			Treated Endometriosis			Stage I-II Endometriosis			Stage III-IV Endometriosis			Endometrioma			DIE		
	NC	NC/ART	IVF/ICSI	NC	NC/ART	IVF/ICSI	NC	NC/ART	IVF/ICSI	NC	NC/ART	IVF/ICSI	NC	NC/ART	IVF/ICSI	NC	NC/ART	IVF/ICSI	NC	NC/ART	IVF/ICSI
Primary Outcome																					
HBR																					
Secondary Outcomes																					
CPR			↓ n=6			↓ n=26			↔ n=8			↔ n=14			↓ n=13			↔ n=9			↔ n=1
LBR			↔ n=4			↓ n=14			↔ n=3			↔ n=6			↓ n=9			↔ n=4			
Early Pregnancy Complications																					
Miscarriage			↑ n=5		↑ n=2	↑ n=16	↔ n=1	↑ n=1	↔ n=5		↑ n=1	↑ n=10		↑ n=1	↑ n=9	↑ n=1		↔ n=4		↑ n=1	↔ n=1
Late Pregnancy Complications																					
PIH		↔ n= 1		↑ n=2	↔ n=6	↔ n=3		↑ n=1												↔ n=1		
PET		↑ n= 2			↑ n=9	↔ n=6		↑ n=1				↔ n=1			↑ n=1						
GDM		↓ n=1			↔ n=5	↔ n=1		↑ n=1				↔ n=1			↔ n=1					↔ n=1	

PTD		↑ n=5		↑ n=3	↔ n=8	↑ n=5		↑ n=1				↔ n=1			↑ n=2			↔ n=2	↑ n=1		
SGA		↑ n=2		↔ n=2	↔ n=5	↔ n=3		↑ n=1							↑ n=1			↔ n=2	↔ n=1		
PP		↑ n=1			↑ n=7	↔ n=3		↑ n=1				↔ n=1			↔ n=2				↑ n=1		
PA					↑ n=6	↔ n=1		↑ n=1											↑ n=1		
PPH		↑ n=1			↔ n=8	↔ n=3		↑ n=1				↔ n=1			↔ n=1						
LSCS		↑ n= 3		↑ n=2	↑ n=8	↑ n=3		↔ n=3				↔ n=1			↑ n=1			↔ n=1	↑ n=1		
IUD		↔ n=1			↑ n=4			↔ n=1													
Neonatal Complications																					
NNU		↑ n=1			↑ n=4	↑ n=2						↔ n=1			↑ n=1						
NND					↑ n=1			↑ n=1													
Fertility Outcomes																					
OR			↔ n=3			↓ n=17			↓ n=6			↔ n=11			↓ n=11			↓ n=12			
MII						↓ n=5			↓ n=3			↔ n=3			↓ n=3			↓ n=4			
CR			↔ n=2			↑ n=12			↔ n=3			↑ n=4			↔ n=4			↔ n=5			

FR			↔ n=1			↓ n=2			↓ n=2			↓ n=8			↔ n=7			↑ ↔ n=1 n=1			
IR			↓ n=3			↓ n=12			↔ n=3			↔ n=7			↓ n=11			↔ n=3			

Key:

HBR = healthy baby rate
SGA = small for gestational age
PA = placental abruption
NNU = neonatal admissions
OR = ovarian response (oocyte yield)
LSCS = lower segment caesarean section

PPH = post-partum haemorrhage
FR = fertilisation rate
LBR = live birth rate
PIH = pregnancy induced hypertension
GDM = gestational diabetes
↑ = Significantly increased in meta-analysis

PTD = preterm delivery
PP = placenta praevia
IUD = intra-uterine death
NND = Neonatal death
M2 = mature (MII) oocytes
↓ = result decreased but insufficient data for meta-analysis

CR = cycle cancellation rate
IR = implantation rate
MR = miscarriage rate
PET = pre-eclampsia
CPR = clinical pregnancy rate

↓ = Significantly decreased in meta-analysis
↑ = result increased but insufficient data for meta-analysis
↔ = result equivocal
Blank = no data

3.6.2 Implications to Clinical Practice

Although collation of all data into a thorough and conclusive meta-analysis to fully explore the impact of endometriosis and adenomyosis on obstetric and fetal complications is hindered by heterogeneity of current studies, evidence of the disease-outcome link is broad. Therefore, the evidence is such that a paradigm shift may be required towards an increased awareness of the impact of the disease on preimplantation embryo programming, the obstetric impact on the mother and the longer-term impact on the health of the children born. Other conditions linked to placental insufficiency warrant recommendation of high dose aspirin ante-natally and obstetric care includes either serial growth scans or a 16-22 week gestation uterine artery doppler screening ultrasound. Women at increased risk of pre-eclampsia are offered increased blood pressure monitoring through the pregnancy as well as high dose aspirin and uterine artery doppler screening. As resources within obstetric units are strained, the increased risk of women with endometriosis may not currently be such to offer all antenatal screening and intervention, however blood pressure monitoring and high dose aspirin are relatively cheap and non-invasive interventions which may be implemented easily. Many of the potentially associated risks of endometriosis are not modifiable antenatally, such as post-partum haemorrhage, placental abruption and need for lower segment caesarean section delivery. However maternal and neonatal co-morbidity can be improved with early management of intra-partum complications and if endometriosis is considered a risk factor intra-partum women may receive better counselling regarding place of delivery and clinicians and midwives caring for these women would have a higher index of suspicion for certain complications facilitating earlier intervention.

Whilst super-specialisation is increasingly polarising obstetrics and gynaecology, the care of women with adenomyosis and endometriosis undoubtedly warrants a more joined-up approach in gynaecological, preconception and antenatal management. These women, particularly those with more severe stages of disease or following extensive abdominal surgery, should be counselled regarding the risks beyond difficulty trying to conceive. They should be informed of the increased risks of early and late pregnancy complications and the potential morbidity involved, especially in the sphere of assisted reproduction where the risks may be higher, and women are medically assisted to achieve higher risk pregnancies. Unfortunately, there are few risk factors for endometriosis which are proven to be modifiable, however counselling could include lifestyle and

health recommendations alongside treatment of endometriosis ahead of conception to ameliorate the risk of disease recurrence or progression and modify the risk of obstetric complications overall. For example, smoking is not proven to cause or worsen endometriosis, but smoking cessation will reduce the risk of several pregnancy complications which may also be increased by endometriosis, such as preterm delivery, in-utero growth restriction and placental abruption.

In conclusion, the shift in perception of risk with these women should also precipitate into their antenatal and peri-partum management.

3.6.3 Explanation of Findings

There is no doubt that the reproductive impact of the aforementioned disorders starts at the early stages of gamete and embryo development and that the impact is throughout the life course of reproduction. The Barker's hypothesis, where adverse events during the peri-implantation period may program development and influence disease later in life [219], is extensively studied in relation to overt over and under-nutrition in animal models and human studies. The concept of Barker's hypothesis in the context of endometriosis has only been explored pertaining to the aetiology and how in utero exposure to environmental factors may influence the development of endometriosis in the offspring [220]. However, the abnormally placed endometrial glands and stroma in adenomyosis and endometriosis creates a suboptimal developmental environment for the conceptus within the reproductive tract [221,222], and hence has implications that warrant exploration in the context of developmental programming, where aberrant decidualisation and placentation within the perturbed uterine environment can be linked not only to problems relating to placental insufficiency but also childhood and adult diseases. Many obstetric complications such as abnormal placentation, pre-eclampsia, preterm birth and preterm rupture of membranes have complex aetiology, and studies thus far have primarily focussed on the stages of later pregnancy and birth at which point disease has already been established. Arguably, the fate of the pregnancy may have been determined much earlier on, although how the related aberrant uterine environment perturbs the progression of fertilisation, implantation and later pregnancy progression and birth outcomes in terms of a take-home healthy baby warrants further investigation. No papers currently report on the 'heathy baby rate', defined as a live singleton birth at term of appropriate birthweight for gestation, or the health of the offspring in the context

of endometriosis and this review highlights the need for future studies to consider these key reproductive outcomes and the health of the offspring.

3.6.4 Strengths and Weaknesses of the Study

This is an extensive review and has attempted to examine all published work on the reproductive impacts of endometriosis and adenomyosis to emphasise the need for a holistic rather than a polarised view of the conditions. The papers included demonstrate low publication bias by funnel plot analysis (Appendix 4), however a minimal level of bias may exist towards studies published in English, while five studies were successfully translated for inclusion, this was not possible in 2 other studies. Due to the nature of systematic reviews this meta-analysis is confounded by heterogeneity of the clinical studies included although strict criteria were applied to minimize this. The gold standard for diagnosis of endometriosis and its subtypes is laparoscopy, where studies use database medical records or imaging it is possible that false positive and false negative error is occurring, and this reduces the reliability of observed results. Control cohorts in IVF/ICSI studies vary widely between mixed aetiology infertility, male factor, tubal factor or unexplained infertility. These causes of infertility may also impact on the fertility and reproductive outcomes of interest and may not represent a consistent control in this analysis. Individual protocols for controlled ovarian hyperstimulation, and other factors in the assisted reproduction treatment between units, countries and across the time period included in our meta-analysis introduces heterogeneity.

3.6.5 Implications for future research

The heterogeneity of studies is difficult to overcome in a review of 99 papers but this meta-analysis highlights that a more unified approach to studying fertility and reproductive outcomes in these patients is essential in improving knowledge in this area and making a real impact on managing subfertility, the ante-natal and intra-partum course and potentially reducing some modifiable health risks in future generations of offspring in this cohort of women.

3.7 Conclusion

From the current literature, it can be concluded that adenomyosis and endometriosis have a negative impact on parameters pertaining to the whole reproductive course from oocyte number and quality to neonatal outcomes. Compared to women without endometriosis, pregnancy outcomes in IVF, ART pregnancies and spontaneously conceived pregnancies are negatively affected with emerging evidence of an increased risk of preterm delivery, pre-eclampsia, placenta praevia, caesarean section delivery and need for neonatal admission. These complications could be caused by dysfunctional uterine changes impairing the decidualisation and placentation process and therefore these conditions could potentially have far reaching consequences as suggested by Barker's hypothesis. Studies in this area lack longer term follow up into the neonatal period and beyond to verify this theory. There is insufficient data on the effect of adenomyosis in IVF parameters and intra-uterine death and neonatal death were under-reported in the available literature.

Subtypes of endometriosis and the disease adenomyosis have specific impacts on different fertility and reproductive outcomes but these are subtle, and the outcome profiles of each subtype are not fully revealed due to the quality and heterogeneity of the studies available.

A more unified and consistent approach to studying fertility and reproductive outcomes in the area of endometriosis and adenomyosis with longer term follow up of the offspring and attention to the subtype of disease is necessary to explore a possible link with developmental programming and the complication profiles of disease subtypes.

It is concluded in this Chapter that from current available literature true levels of obstetric and neonatal risks in women with endometriosis and links to DOHaD theory cannot be discovered as there is heterogeneity and a lack of well-designed population-based cohort studies examining all prudent obstetric and neonatal complications and longer term follow up of the offspring. The intention is to address these issues with a large cohort study in Chapter 4.

Chapter 4 Endometriosis as a Life Course Health Determinant

4.1 Introduction

It is clear from published literature that endometriosis has historically been investigated more than the under-represented condition of adenomyosis. This may be due to endometriosis being inherently easier to diagnose as endometriotic lesions can be directly visualised whereas adenomyotic lesions are hidden within the myometrium of the uterus making the diagnosis less robust with external visual inspection of the uterus and imaging techniques. I have therefore focussed on the condition of endometriosis for ongoing work. Growing evidence explored in Chapters 1 and 2, demonstrates that endometriosis may lead to significant obstetric and perinatal complications, but the level of risk remains unclear due to heterogeneity and poor quality of available studies. Chapter 2 highlighted that endometriosis negatively impacts on all stages of reproduction and is also associated with a range of obstetric and fetal complications including preterm delivery, caesarean section and neonatal unit admission [223]. Such negative obstetric outcomes are further supported by others [224–226] but longer follow-up studies for obstetric and neonatal outcomes have not often been undertaken, therefore, the true temporal impact of the disease on late pregnancy and health of the offspring, and its associated health determinants, remains unclear. We should be concerned that the presence of this condition does not currently alter clinical management of women pre-conceptually, ante-natally and intra-partum in the UK but without better quality evidence, clinical management will not change. Studies on the reproductive impact of endometriosis is often polarised, with either an obstetric or gynaecology focus, without providing insight into the potential impact of disease subtypes or more importantly, the longitudinal impact. Given the prevalence and associative morbidity of endometriosis, it is desirable that the reproductive impact of the disease is better understood.

One of the difficulties in examining the health impacts of endometriosis is the elusive symptom profile of the disease which precludes timely diagnosis. The gold standard investigation for diagnosis is visualising the disease at surgery and tissue biopsy. The invasive nature of that process and risks involved leads to a lengthy process for the woman from symptom to diagnosis. It is known that barriers to accessing appropriate healthcare pathways are faced by ethnic

minorities and those of lower socioeconomic groups. Availability and cost of transport, education and health literacy, language barriers, perceptions and attitudes towards health services and cultural differences involved in seeking health care and treatment are but a few of the proposed barriers facing people in these groups. It is therefore important to understand which demographic factors may directly influence the pathological processes of the disease, and which are a consequence of factors linked to healthcare access. For example, although studies show that endometriosis is often concurrently present with other diseases an important confounder may relate to socioeconomic background. Social and economic disadvantage may be associated with increased risk of comorbidity secondary to diet and nutrition, smoking and other environmental and behavioural characteristics. In addition, women from disadvantaged socioeconomic groups and ethnic minority groups can also face barriers to accessing healthcare services [227]. In the absence of physical signs of pelvic endometriosis on examination or imaging, NICE guidance stipulates the need for severe, persistent or recurrent symptoms of endometriosis or for initial management to have been ineffective, not tolerated or contraindicated to consider referring women to a gynaecology service [6]. To fulfil the criteria, women may need to present to primary care several times and express their concerns repeatedly. Language barriers and cultural stereotypes in healthcare may affect ethnic minorities' access to healthcare services, particularly in relation to gynaecological health [228]. Conversely, women with high health literacy may be more likely to access timely healthcare for symptoms of endometriosis and request referral for diagnosis. Only when confounders of the disease are accounted for can the true extent of relationship of endometriosis to its health outcomes be revealed.

I therefore performed secondary data analysis of a large healthcare-based cohort study with the aim of addressing some of these issues. I particularly wanted to examine neonatal factors relating to wellbeing of the offspring for example prematurity, birthweight, Apgar score and neonatal death as this data was limited in my meta-analysis (Chapter 2). The main concern regarding quality of current collective evidence was that many studies were limited by small cohort sizes and where some of the pregnancy outcomes of interest are uncommon, larger study sizes are required to investigate them. In addition, while the majority of studies attempted to adjust for confounders, details regarding the process of selecting of confounders were often lacking in the methodologies. One of the main drawbacks of observational studies is the inability to determine true causality due to the impact of confounding variables. I endeavoured to address confounders in a thorough and objective way.

Two of the late pregnancy complications more consistently found across the subgroup analyses of the meta-analysis were those of caesarean section delivery and preterm delivery. These complications potentially have a significant impact on the woman and offspring. Preterm delivery would also carry risk of significant health complications through childhood and beyond. Although small for gestational age was only identified as a risk of adenomyosis in the meta-analysis, this measurable pregnancy outcome most closely represents the impact of the intra-uterine environment on the developing fetus and is a surrogate marker for investigating diseases with developmental origins. The primary outcomes of this study will therefore be caesarean section delivery, preterm delivery and small-for-gestational age.

4.2 Objectives

This research aims to contribute to thesis aim 1 and increase our understanding of the implications of endometriosis on the obstetric and neonatal period of a woman and her offspring.

Primary Objective: To examine the association of endometriosis with obstetric and neonatal adverse outcomes including mode of delivery, preterm delivery and small for gestational age birth. These associations will be investigated using two models, the first, accounting for confounders hypothesised to directly impact the disease, and the second, accounting for confounders potentially impacting healthcare access leading to an endometriosis diagnosis.

Secondary objective: To explore sociodemographic and health characteristics of women with endometriosis.

4.3 Hypothesis

Endometriosis is associated with obstetric complications and neonatal adverse outcomes independent of confounding gynaecological and medical co-morbidities and maternal demographic and lifestyle factors. These associations may substantiate the role of endometriosis in developmental origins of health and disease (DOHaD) by demonstrating the effects of endometriosis could extend to fetal programming related obstetric problems and neonatal health. Diagnosis of endometriosis will be associated with characteristics known to physiologically

influence the disease and also characteristics likely to influence a woman's ability to access secondary and tertiary care diagnostic services.

4.4 Design

A population-based cohort study involving anonymised healthcare record linkage of women diagnosed with endometriosis at surgery with their antenatal and birth records.

4.5 Ethics

This study was approved by ethical review with the HRA and University of Southampton faculty ethics committee. It has been sponsored by the University of Southampton and is supported by the Research and Development Department of University Hospitals Southampton NHS Foundation Trust (IRAS ID: 250339, REC reference: 19/HRA/0530).

4.6 Methods

4.6.1 Data

This anonymised population-based cohort study utilises data recorded in electronic routine healthcare records between 1st January 2003 and 30th September 2018, at a UK tertiary National Health Service (NHS) university hospital with approximately 6,000 deliveries per year. The maternity healthcare database on Hospital Integrated Clinical Support Systems (HICSS) was linked to the surgical database on HICSS where a diagnosis of endometriosis was documented by the operating surgeon. The maternity database contains antenatal booking information, conditions diagnosed in the pregnancy and clinical details regarding labour, delivery and immediate post-partum course. Data was recorded by doctors and midwives involved directly in the woman's care. Measurements such as body mass index (BMI) and blood pressure were performed and recorded by medical professionals. The surgical database includes details of any gynaecological surgery performed entered by a medical member of the operating team including operation date, admission and discharge date, type of procedure and diagnosis at surgery documented by International Statistical Classification of Diseases and Related Health Problems 10th Revision code (ICD 10 code). Data regarding women's pregnancies and deliveries were linked to gynaecological

surgical procedures. Data linkage and anonymization was performed by the data holder. The data was cleaned, processed and analysed in its entirety, inclusive of all pregnancies, whether endometriosis is diagnosed before or after the pregnancy (all pregnancies, AP). The first subgroup inclusive of only women in their first pregnancies (first pregnancies, FP), and a second subgroup including only pregnancies after a diagnosis of endometriosis (pregnancies occurring after endometriosis diagnosis, AE) (Figure 17).

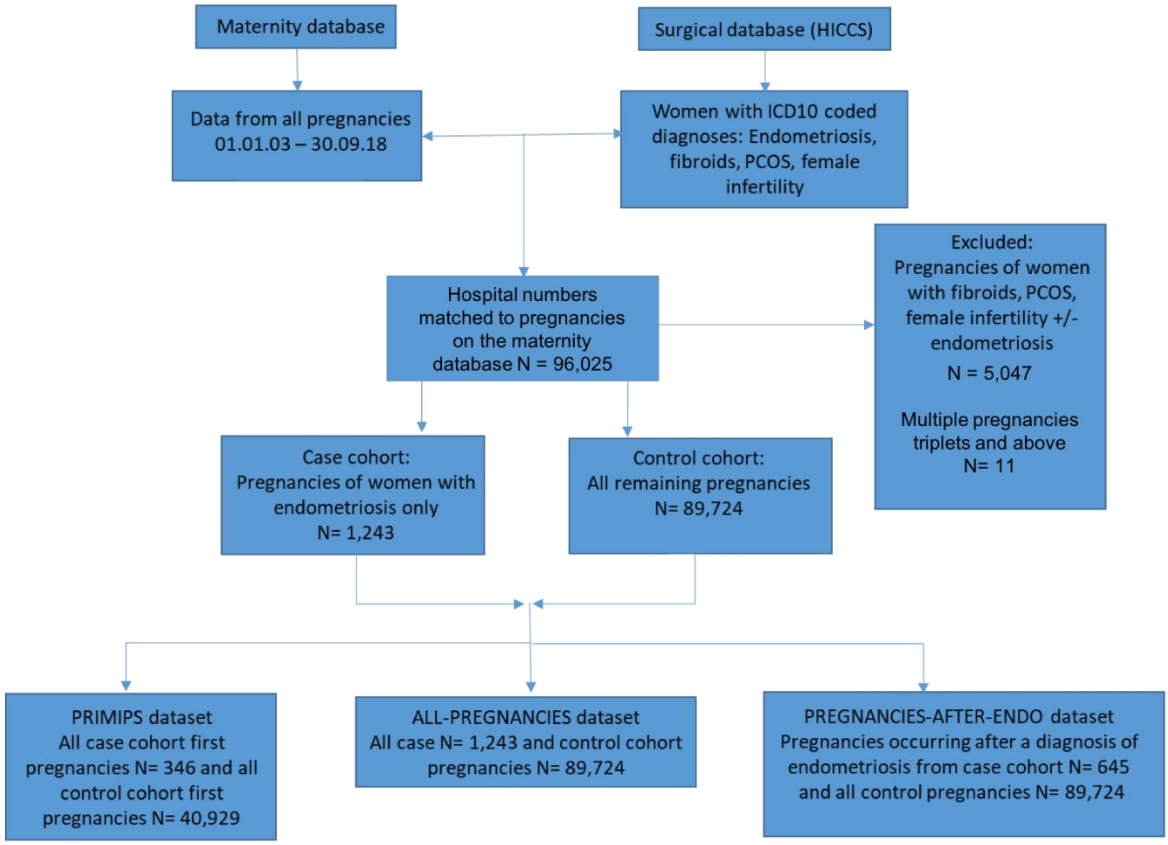


Figure 17: Flow diagram of data collection methodology, inclusion/exclusion criteria and final datasets

4.6.2 Exposure Assessment

Women with a diagnosis of fibroids, polycystic ovary syndrome (PCOS) or female infertility by the relevant ICD 10 codes (Table 6) were excluded as these conditions can independently affect fertility and reproductive outcomes and can be reliably and accurately diagnosed. High order pregnancies (>2 fetuses) were excluded. Women with a diagnosis of endometriosis by ICD 10 code (Table 6) were compared to pregnancies in women who have no recorded aforementioned

gynaecological comorbidities. ICD 10 codes for endometriosis include N80.0 “Endometriosis of the uterus” which can sometimes be interpreted as adenomyosis. This code was included in the endometriosis cohort, as endometriosis affecting the serosa of the uterus could also be coded in this way. We therefore cannot exclude the condition of adenomyosis from this group.

Table 6: ICD 10 Codes for exclusion and inclusion criteria

ICD10-Code	**Condition**	**ICD10-Code**	**Condition**
D25.0	Submucous leiomyoma	E282	Polycystic ovarian syndrome
D25.1	Intramural leiomyoma	N80.0	Endometriosis of uterus
D25.2	Subserosal leiomyoma	N80.1	Endometriosis of ovary
D25.9	Leiomyoma of the uterus (corpus or cervix)	N80.2	Endometriosis of fallopian tube
N97.0	Female infertility associated with anovulation	N80.3	Endometriosis of pelvic peritoneum
N97.1	Female infertility of tubal origin	N80.4	Endometriosis of rectovaginal septum & vagina
N97.2	Female infertility of uterine origin	N80.5	Endometriosis of intestine
N97.3	Female infertility of cervical origin	N80.6	Endometriosis in cutaneous scar
N97.8	Female infertility of other origin	N80.8	Other endometriosis
N97.9	Female infertility unspecified	N80.9	Endometriosis, unspecified

4.6.3 Outcome Assessment

Primary outcomes were caesarean section delivery (CS), which included both elective and emergency caesarean sections, preterm delivery (PTD, gestational age <259 days) and small for gestational age (SGA, calculated by birth weight centile for gestational age using reference values for England and Wales in 2015 and categorising size as SGA if <10th centile, large for gestational age if >90th centile and normal if >10th centile and <90th centile) [229,230]. All outcomes were assessed and recorded by healthcare professionals.

4.6.4 Assessment of confounders and mediators

Maternal age was calculated using date of birth before extraction of the dataset to maintain anonymity. Systolic blood pressure and maternal weight was measured by healthcare professional at the pregnancy first antenatal (booking) appointment. Smoking status, level of education, employment status, partner's employment status, ethnicity and mode of conception were self-reported. Existing medical co-morbidities were self-reported and categorised by the data-gatherer as hypertension, cardiac disease, respiratory disease, autoimmune disease, gastro-intestinal disease, haematological disease, thromboembolic disease, renal disease, endocrine disease, cancer, mental health disease, musculoskeletal disease and diabetes. A binary variable was then created for if a woman had an existing medical co-morbidity or not. Obstetric co-morbidities included previous livebirths, stillbirths, miscarriages, placental abruption, uterine rupture, puerperal psychosis and extensive perineal trauma (defined as 3rd degree tears, 4th degree tears, extensive vaginal tears or cervical tears).

4.6.5 Conceptual framework and statistical analysis

Based on previous evidence, confounding and mediation of the exposure-outcome relationships were conceptualised apriori in two models: an exposure-outcome model (E-O) and diagnosis-outcome model (D-O). The E-O model only considers factors known by previous evidence to be directly linked to the aetiology of endometriosis, for example advancing age and infertility. In the D-O model, additional factors which are associated with a healthcare diagnosis of endometriosis

potentially due to differing health-seeking behaviour and healthcare access barriers are also included, for example ethnicity and socioeconomic background[231–237].

Directed acyclic graphs (DAGs) were produced using DAGitty v2.5. These graphical models demonstrated how variables within the data influence the exposure (endometriosis) and outcome (PTD, CS, SGA) [238]. DAGs for both models (E-O and D-O) were constructed for each outcome and for multiple pregnancy within same woman datasets and first pregnancy dataset (Figure 18 and Figure 19). A minimal set of adjustments for the total (confounder-adjusted) and direct (confounder and mediator-adjusted) effect were used in multivariate analyses for the three main outcomes (SGA, CS, PTD). For SGA multivariate analysis the reference group were babies >10th centile, for CS analysis the reference group were spontaneous vaginal, instrumental vaginal and breech vaginal deliveries and for PTD analysis the reference group were term deliveries (>259 days gestation).

Total effect adjustments for all outcomes for the E-O model were mother's age, BMI and smoking status. Direct effect adjustments for SGA outcome for the E-O model were mother's age, BMI, smoking status, ethnicity, mode of conception, maternal comorbidity and previous live births. Direct effect adjustments for CS outcome for the E-O model were mother's age, BMI, smoking status, ethnicity, level of education, mode of conception, maternal comorbidity, PTD, previous livebirths, SGA and substance use. Direct effect adjustments for PTD outcome for the E-O model were mother's age, BMI, smoking status, mode of conception and maternal comorbidity.

Total effect adjustments for all outcomes for the D-O model were mother's age, BMI, smoking status, ethnicity, level of education, employment status and maternal comorbidity. Direct effect adjustments for SGA outcome for the D-O model were mother's age, BMI, smoking status, ethnicity, level of education, employment status, maternal comorbidity and mode of conception. Direct effect adjustments for CS outcome for the D-O model were mother's age, BMI, smoking status, ethnicity, maternal comorbidity, mode of conception, SGA, PTD and substance use. Direct effect adjustments for PTD outcome for the D-O model were mother's age, BMI, smoking status, ethnicity, level of education, employment status, maternal comorbidity, mode of conception and SGA.

Statistical analysis was performed using Stata version 15. Generalised linear model with log link [239] was applied to examine the relationship between endometriosis and maternal characteristics,

antenatal outcomes, obstetric outcomes and neonatal outcomes. A statistical significance level of 0.05 with 95% CI was used.

In both the endometriosis and comparison groups, women could have more than one pregnancy within the dataset. All analyses were adjusted for clustering to produce robust standard errors.

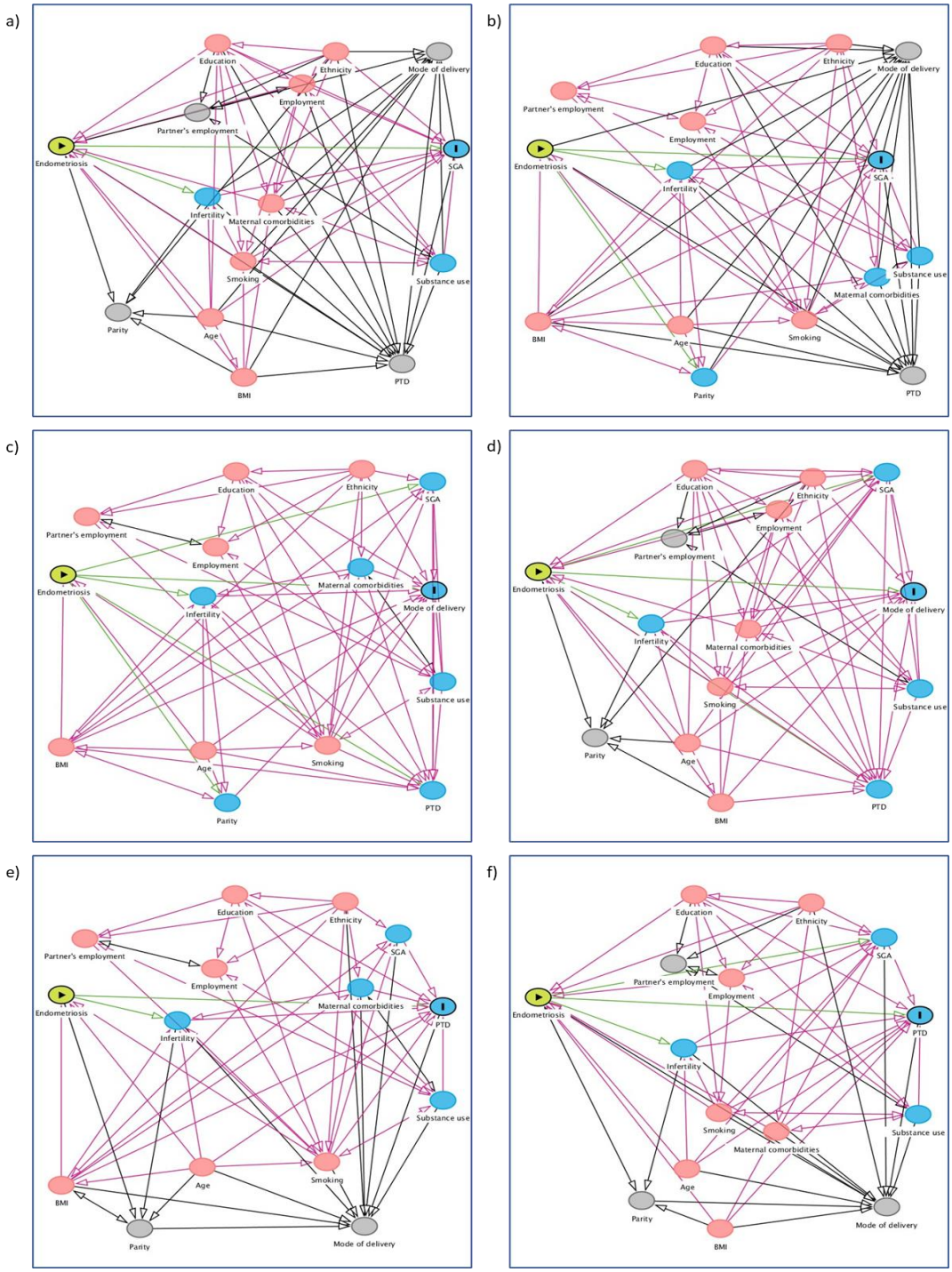


Figure 18:Directed acyclic graphical models demonstrating how variables within the data influence the exposure (endometriosis) and outcomes, SGA, CS and PTD in two models exposure-outcome and diagnosis-outcome a) SGA outcome, E-O model b) SGA outcome, D-O model c) CS outcome (by mode of delivery variable), E-O model d) CS outcome (by mode of delivery variable), D-O model e) PTD outcome, E-O model f) PTD outcome, D-O model

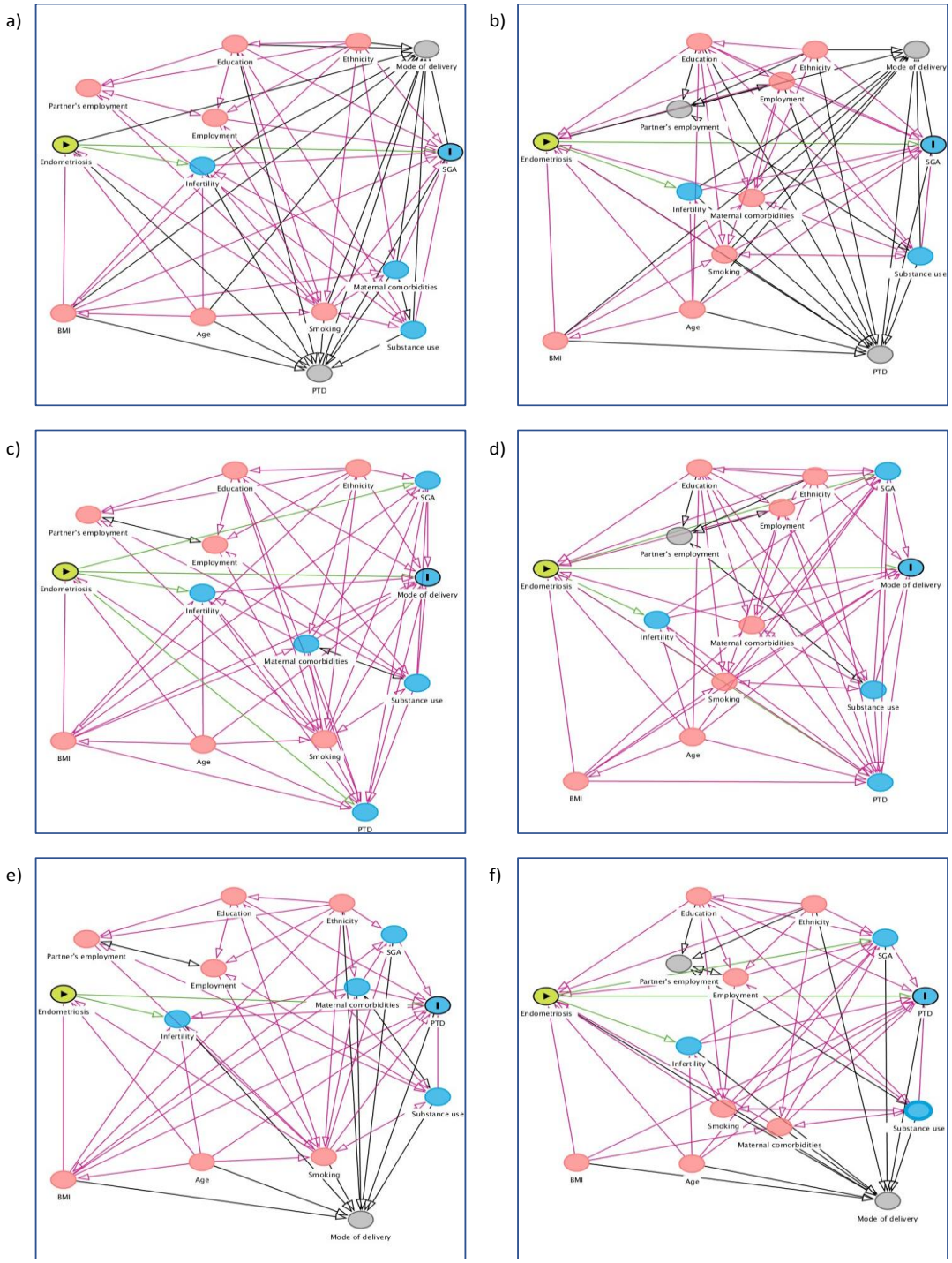


Figure 19: Directed acyclic graphical models demonstrating how variables within the primiparous data influence the exposure (endometriosis) and outcomes SGA, CS and PTD in two models exposure-outcome and diagnosis-outcome a) SGA outcome, E-O model b) SGA outcome, D-O model c) CS outcome (by mode of delivery variable), E-O model d) CS outcome (by mode of delivery variable), D-O model e) PTD outcome, E-O model f) PTD outcome, D-O model

4.7 Results

4.7.1 All pregnancies dataset

There was a total of 96,025 pregnancies included in this 15-year cohort study. Any pregnancy in women with other gynaecological co-morbidities were excluded (n= 5,047). There were 1,243 pregnancies in women with a record of endometriosis without any other recorded gynaecological morbidity (1.4%) and 89,724 in women without (Figure 17). Characteristics associated with endometriosis were older age (increasing risk by 10-year age group, RR 1.63, 95% CI 1.22-2.16 in >40 age group), higher BMI (increasing risk up to RR 1.85, 95% CI 1.11-3.11 in BMI 40+), infertility (RR 5.88, 95% CI 4.90-7.06 of having IVF and RR 3.45, 95% CI 2.62-4.53 of requiring other fertility treatment), recurrent miscarriages (RR 1.79, 95% CI 1.45-2.20) and medical co-morbidities (RR 1.38, 95% CI 1.23-1.55). (Table 7).

Women were less likely to have a diagnosis of endometriosis if they were of Asian (RR 0.58, 95% CI 0.44-0.76) or black African/Caribbean (RR 0.32, 95% CI 0.16-0.61) ethnicity, education level of secondary and below (RR 0.80, 95% CI 0.70-0.92) and unemployed or a student (RR 0.72, 95% CI 0.64-0.82) (Table 7). Women with endometriosis were more likely to be affected by antenatal complications (RR 1.40, 95% CI 1.14-1.71) including PIH, severe PET, OC, GDM, APH and SPD (Table 7).

Table 7: Unadjusted analysis of maternal characteristics and antenatal complications in women with endometriosis compared to controls in "all pregnancies" dataset

	Endometriosis		*Control*				
Descriptive	N (1243)	%	N (89724)	%	RR	95% CI	P value
Maternal Characteristics							
Age	*1243*		*89724*				
<25	296	23.8	23385	26.1			
25-34	687	55.3	51659	57.6	1.05	0.92-1.20	0.480
35-39	205	16.5	12031	13.4	1.34	1.12-1.60	0.001
>40	55	4.4	2649	2.9	1.63	1.22-2.16	0.001
BMI	*1232*		*87289*				
<18.5	25	2.03	2756	3.16			
18.5-24.9	599	48.62	46077	52.79	1.43	0.96-2.12	0.080
25-29.9	375	30.44	23236	26.62	1.77	1.18-2.64	0.006
30-39.9	200	16.23	13274	15.21	1.65	1.09-2.50	0.018
40+	33	2.68	1946	2.23	1.85	1.11-3.11	0.019
Smoking status	*1232*		*87361*				
Non-Smoker	907	73.6	64546	73.9			
Stopped in pregnancy	119	9.7	8085	9.2	1.05	0.87-1.26	0.637
Current Smoker	206	16.7	14730	16.9	0.99	0.86-1.16	0.951
Level of Education	*1232*		*87345*				

Secondary level and below	284	23.05	23765	27.21			
College and above	948	76.95	63580	72.79	0.80	0.70-0.92	0.001
Employment Status	*1224*		*86637*				
Employed	887	72.5	56755	65.5			
Unemployed or Student	337	27.5	29882	34.5	0.72	0.64-0.82	<0.001
Partner's Employment Status	*1154*		*80954*				
Employed	1054	91.3	72237	89.2			
Unemployed or Student	100	8.7	8717	10.8	0.79	0.64-0.97	0.022
Ethnicity	*1238*		*82656*				
White	1135	91.7	71550	86.6			
Asian	55	4.4	6044	7.3	0.58	0.44-0.76	<0.001
Black/African/Caribbean	9	0.7	1802	2.2	0.32	0.16-0.61	0.001
Other	39	3.2	3260	3.9	0.76	0.55-1.04	0.086
Booking Systolic Blood Pressure	*1219*		*86050*				
<140 mmHg	1203	98.69	84885	98.65			
≥140 mmHg	16	1.31	1165	1.35	0.97	0.59-1.58	0.901
Mode of Conception	*1232*		*87345*				
Natural	1060	86.1	84647	96.9			
IVF/GIFT	120	9.7	1530	1.8	5.88	4.90-7.06	<0.001
Other Fertility Treatment (surgical/hormonal	52	4.2	1168	1.3	3.45	2.62-4.53	<0.001
Maternal Co-morbidities	*1243*		*89724*				

No comorbidity	849	68.3	68188	76.0			
Comorbidity	394	31.7	21536	24.0	1.38	1.23-1.55	<0.001
HTN	4	0.3	820	0.9			
Cardiac Disease	13	1.0	838	0.9			
Respiratory Disease	0	-	27	0.03			
Autoimmune Disease	7	0.6	362	0.4			
Gastro-intestinal Disease	15	1.2	703	0.8			
Haematological Disease	13	1.0	648	0.7			
Thromboembolic Disease	8	0.6	262	0.3			
Renal Disease	15	1.2	900	1.0			
Endocrine Disease	19	1.5	1207	1.3			
Cancer	0	-	6	0.006			
Mental Health Disease	290	23.3	14791	16.5			
Musculoskeletal Disease	5	0.4	68	0.07			
Diabetes	5	0.4	855	0.9			
Previous Livebirth	*1243*		*89716*				
1-3	646	51.97	46168	51.5			
0	566	45.53	40929	45.6	0.99	0.88-1.11	0.839
>3	31	2.49	2619	2.9	0.85	0.59-1.21	0.366
Previous Stillbirth	13	1.05	855	0.95	1.10	0.64-1.89	0.738
Previous Miscarriages	*1242*		*89705*				
0	766	61.7	58477	65.2			
1-2	380	30.6	27152	30.3	1.07	0.94-1.21	0.295
3+	96	7.7	4076	4.5	1.79	1.45-2.20	<0.001

	Mean	SD	Mean	SD			
Age	29.38	6.11	28.57	5.88	1.02	1.01-1.04	<0.001
BMI	25.86	5.26	25.49	5.49	1.01	1.00-1.01	0.012
	Endometriosis		Controls		RR	95% CI	P value
	N	%	N. %				
Antenatal Complications	*1243*		*89724*				
No complication	1152	92.96	85198	94.96			
Complication	91	7.04	4526	5.04	1.40	1.14-1.71	0.001
Severe PET (requiring preterm delivery)	8	0.64	439	0.49			
HELLP	0	-	55	0.06			
Eclampsia	0	-	44	0.05			
Puerperal psychosis	0	-	0	-			
Obstetric Cholestasis	11	0.88	735	0.82			
GDM	35	2.82	1800	2.01			
PIH	19	1.53	1353	1.51			
Antepartum Haemorrhage	17	1.37	693	0.77			
VTE	0	-	4	0.004			
Placental Abruption	0	-	15	0.02			
Uterine rupture	0	-	0	-			
Extensive perineal trauma (3^{rd}/4^{th} degree tears, extensive vaginal/cervical tears)	0	-	0	-			
SPD	15	1.21	235	0.26			
Placenta praevia	0	-	36	0.04			

PET	0	-	25	0.03

Women with endometriosis had pregnancies that were more likely to result in a miscarriage (RR 5.20, 95% CI 2.67-10.13) or termination between the first antenatal appointment and 24 weeks completed gestation (RR 4.32, 95% CI 2.49-7.48). The women were more likely to require caesarean section delivery (RR 1.72, 95% CI 1.52-1.94), suffer a retained placenta post-delivery (RR 1.38, 95% CI 1.07-1.80) and were more likely to deliver preterm (RR 1.53, 95% CI 1.28-1.83) (Table 8).

Table 8: Unadjusted analysis of birth outcomes and neonatal complications in women with endometriosis compared to controls in "all pregnancies" dataset

Descriptive	*Endometriosis*		*Controls*				
	N	*%*	*N*	*%*	*RR*	*95%CI*	*P value*
	(1243)		*(89724)*				
Birth characteristics and Neonatal Complications							
Mode of Delivery	*1237*		*76938*				
Spontaneous Vaginal	659	53.3	47876	62.2			
Instrumental Vaginal and Breech	157	12.7	11421	14.9	1.00	0.84-1.19	0.988
Caesarean Section	421	34.0	17641	22.9	1.72	1.52-1.94	<0.001
(Elective	158		6028				
Emergency)	263		11613				
Labour	*1243*		*89724*				
Spontaneous	1157	93.4	84540	94.2			
Induction	86	6.6	5184	5.8	1.16	0.93-1.44	0.179

Retained Placenta	59	6.1	2867	4.4	1.38	1.07-1.80	0.014
Apgar at 5 Minutes							
7-10	1177	96.9	74551	97.37			
4-6	30	2.5	1622	2.12	1.17	0.81-1.67	0.400
0-3	8	0.6	393	0.51	1.28	0.64-2.55	0.478
Gestation at Delivery	*1220*		*77562*				
Term	1088	89.2	71960	92.8			
Preterm	132	10.8	5602	7.2	1.53	1.28-1.83	<0.001
Birthweight	*1209*		*76871*				
Normal	971	80.3	61022	79.4			
LGA	133	11.0	7932	10.3	1.05	0.88-1.26	0.574
SGA	105	8.7	7917	10.3	0.84	0.68-1.02	0.079
Shoulder Dystocia	20	1.6	962	1.2	1.44	0.93-2.23	0.101
Cord Prolapse	3	0.2	80	0.1	2.55	0.84-7.75	0.099
Birth Outcome	*1243*		*77920*				
Livebirth	1219	98.1	77266	99.2			
Stillbirth	4	0.3	396	0.5	0.64	0.24-1.71	0.377
Termination<24/40	12	1.0	167	0.2	4.32	2.49-7.48	<0.001
Miscarriage post booking	8	0.6	91	0.1	5.20	2.67-10.13	<0.001
Neonatal Death	4	0.3	224	0.2	1.24	0.47-3.27	0.669
Maternal Death	2	0.15	183	0.2	0.76	0.19-3.02	0.698

	Mean	*SD*	*Mean*	*SD*	*RR*	*95% CI*	*P Value*
Birthweight	3261.7	760.0	3348.9	649.6	1.00	0.9997-0.9999	<0.001
Gestation	272.7	24.0	277	17.8	0.99	0.988-0.99	<0.001

In the E-O model, the risk of caesarean section and preterm delivery remained elevated in women with endometriosis compared to those without (RR 1.37, 95% CI 1.23-1.53 and RR 1.50, 95% CI 1.23-1.84 respectively). The relationship was unaffected when mediators were adjusted for. In the D-O model the risk of caesarean section and preterm delivery also remained elevated in women with endometriosis (RR 1.33, 95% CI 1.19-1.50 and RR1.52, 95% CI 1.24-1.87 respectively) and the relationship was unaffected when adjusting for mediators (Table 9 and Table 10).

Table 9: Primary obstetric outcomes adjusted for confounders in exposure to outcome (E-O) model in "all pregnancies" dataset

	SGA				CS**				PTD			
	N	RR	95% CI	P value	N	RR	95% CI	P value	N	RR	95% CI	P value
Unadjusted	78080	0.84	0.68-1.02	0.079	78175	1.72	1.52-1.94	<0.001	78782	1.53	1.28-3.83	<0.001
Model 1*	76871	0.91	0.74-1.13	0.411	76987	1.37	1.23-1.53	<0.001	77565	1.50	1.23-1.84	<0.001
Model 2*	72027	0.97	0.79-1.20	0.808	71211	1.09	1.06-1.13	<0.001	77565	1.30	1.04-1.62	0.022

*All models are adjusted for clustering

** Elective and emergency caesarean section delivery compared to all vaginal deliveries including spontaneous, instrumental and breech

Model 1 adjusts for mother's age, BMI and smoking status

Model 2

SGA (small for gestational age): adjusts for mother's age, BMI, smoking status, ethnicity, previous livebirths, mode of conception and maternal comorbidity (if any of the following exist; hypertension, cardiac disease, respiratory disease, autoimmune disease, gastro-intestinal disease, haematological disease, thromboembolic disease, renal disease, endocrine disease, cancer, mental health disease, musculoskeletal disease and diabetes)

CS (caesarean section delivery): adjusts for mother's age, BMI, smoking status, level of education, ethnicity, mode of conception, maternal comorbidity, preterm delivery, small for gestational age, substance use and previous livebirths

PTD (preterm delivery): adjusts for mother's age, BMI, smoking status, maternal comorbidity and mode of conception

Table 10: Primary obstetric outcomes adjusted for confounders in diagnosis to outcome (D-O) model in "all pregnancies" dataset

	SGA				CS**				PTD			
	N	RR	95% CI	P value	N	RR	95% CI	P value	N	RR	95% CI	P value
Unadjusted	78080	0.84	0.68-1.02	0.079	78175	1.72	1.52-1.94	<0.001	78782	1.53	1.28-3.83	<0.001
Model 1*	71536	0.96	0.78-1.19	0.734	71613	1.33	1.19-1.50	<0.001	72149	1.52	1.24-1.87	<0.001
Model 2*	71536	0.95	0.76-1.17	0.606	71218	1.26	1.13-1.40	<0.001	71536	1.33	1.06-1.67	0.012

*All models are adjusted for clustering

** Elective and emergency caesarean section delivery compared to all vaginal deliveries including spontaneous, instrumental and breech

Model 1 adjusts for mother's age, body mass index (BMI), education level, employment status, ethnicity, maternal comorbidity and smoking status

Model 2

SGA (small for gestational age): adjusts for mother's age, BMI, education level, employment status, ethnicity, smoking status, mode of conception and maternal comorbidity (if any of the following exist; hypertension, cardiac disease, respiratory disease, autoimmune disease, gastro-intestinal disease, haematological disease, thromboembolic disease, renal disease, endocrine disease, cancer, mental health disease, musculoskeletal disease and diabetes).

CS (caesarean section): adjusts for mother's age, BMI, smoking status, ethnicity, maternal comorbidity, mode of conception, small for gestational age, preterm delivery and substance use

PTD (preterm birth): adjusts for mother's age, BMI, education level, employment status, ethnicity, maternal comorbidity, smoking status, small for gestational age and mode of conception

4.7.2 First pregnancy dataset

In the first pregnancies (FP) subgroup analysis, there were 346 primigravids who suffered with endometriosis and 40,929 first pregnancies in the comparative group (Figure 17). Primiparous women with endometriosis were more likely to have experienced infertility (RR 3.17, 95% CI 2.10-4.80 for having IVF and RR 2.32, 95% CI 1.23-4.30 for requiring other fertility treatment) and recurrent miscarriages (RR 2.01, 95% CI 1.15-3.50) and were more likely to have medical co-morbidities (RR 1.40, 95% CI 1.10-1.78) and less likely to be of Asian (RR 0.42, 95% CI 0.21-0.81) (Table 11). But there were no differences in age, BMI, level of education or employment status or risk of antenatal complications (Table 11).

Table 11: Unadjusted analysis of maternal characteristics and antenatal complications in women with endometriosis compared to controls in their first pregnancy

	Endometriosis		*Control*				
Descriptive	*N*	*%*	*N*	*%*	*RR*	*95% CI*	*P value*
	(346)		*(40929)*				
Maternal Characteristics							
Age	*317*		*40929*				
<25	107	33.75	14564	35.58			
25-34	171	53.94	22216	54.28	1.05	0.82-1.33	0.707
35-39	29	9.15	3432	8.39	1.15	0.76-1.73	0.506
>40	10	3.15	717	1.75	1.89	0.99-3.59	0.053
BMI	*310*		*39766*				
<18.5	9	2.90	1499	3.77			
18.5-24.9	171	55.16	22658	56.98	1.25	0.64-2.45	0.506
25-29.9	84	27.10	9909	24.92	1.41	0.71-2.79	0.328

30-39.9	40	12.90	5073	12.76	1.31	0.64-2.69	0.462
40+	6	1.94	627	1.58	1.59	0.57-4.44	0.378
Smoking status	*310*		*39800*				
Non-Smoker	230	74.2	29408	73.9			
Stopped in Pregnancy	43	13.9	4776	12.0	1.15	0.83-1.59	0.399
Current Smoker	37	11.9	5616	14.1	0.84	0.60-1.19	0.335
Level of Education	*310*		*39794*				
Secondary level and below	54	17.42	8712	21.89	0.75	0.56-1.01	0.059
College and above	256	82.58	31082	78.11	1.33	0.99-1.78	0.059
Employment Status	*309*		*39559*				
Employed	251	81.23	31052	78.50			
Unemployed or Student	58	18.77	8507	21.50	0.84	0.63-1.12	0.245
Partner's Employment Status	*281*		*36844*				
Employed	263	93.6	33309	90.41			
Unemployed	18	6.41	3535	9.59	0.65	0.40-1.04	0.073
Ethnicity	*314*		*37269*				
White	297	94.59	32663	87.64			
Asian	9	2.87	2382	6.39	0.42	0.21-0.81	0.010
Black/African/Caribbean	0	0	709	1.90	0.00	0.00-0.00	<0.001
Other	8	2.55	1515	4.07	0.58	0.29-1.17	0.131
Booking Systolic Blood Pressure	*308*		*39248*				
<140 mmHg	305	99.03	38747	98.72			
≥140 mmHg	3	0.97	501	1.28	0.76	0.24-2.37	0.639

Mode of Conception	*310*		*39794*				
Natural	276	90.32	38169	95.92			
IVF/GIFT	24	7.74	1029	2.59	3.17	2.10-4.80	<0.001
Other Fertility Treatment (Surgical/Hormonal)	10	3.23	596	1.50	2.32	1.23-4.30	0.009
Maternal Co-morbidities	*346*		*40929*				
No co-morbidity	255	73.7	32638	79.7			
Co-morbidity	91	26.3	8291	20.3	1.40	1.10-1.78	0.006
HTN	3	0.9	181	0.4			
Cardiac Disease	8	2.5	448	1.1			
Respiratory Disease	0	-	10	0.02			
Autoimmune Disease	10	3.1	163	0.4			
Gastro-intestinal Disease	11	3.5	372	0.9			
Haematological Disease	6	1.9	238	0.6			
Thromboembolic Disease	4	1.3	83	0.2			
Renal Disease	6	1.9	477	1.2			
Endocrine Disease	10	3.1	505	1.2			
Cancer	0	-	5	0.5			
Mental Health Disease	75	23.7	5637	13.8			
Musculoskeletal Disease	4	1.3	42	0.1			
Diabetes	1	0.3	159	0.4			
Previous Stillbirth	3	0.95	232	0.56	1.67	0.54-5.16	0.375
Previous Miscarriages	*317*		*40929*				
0	223	64.45	30280	73.98			
1-2	81	23.41	9777	23.89	1.12	0.87-1.45	0.366

3+	13	3.76	872	2.13	2.01	1.15-3.50	0.014
	Mean	SD	Mean	SD			P value
Age	27.53	6.12	26.89	5.89	1.02	1.00-1.04	0.067
BMI	25.12	4.82	24.88	5.16	1.01	0.99-1.02	0.377
	Endometriosis		*Control*		*RR*	*95% CI*	*P value*
	N	*%*	*N*	*%*			
Antenatal Complications	*346*		*40929*				
No complication	328	94.8	38775	94.7			
Complication	18	5.2	2154	5.3	0.99	0.62-1.58	0.960
PIH	5	1.6	868	2.12			
PET	0	-	18	0.04			
Eclampsia	0	-	26	0.06			
HELLP	0	-	61	0.15			
Severe PET (requiring preterm delivery)	2	0.6	294	0.72			
Placenta Praevia	0	-	12	0.03			
GDM	4	1.3	717	1.75			
Obstetric Cholestasis	4	1.3	337	0.82			
APH	5	1.6	322	0.79			
SPD	2	0.6	42	0.10			
Placental abruption	0	-	5	0.01			
VTE	0	-	1	0.002			

Primigravids with endometriosis were more likely to have preterm delivery (RR 1.60, 95% CI 1.15-2.25, p = 0.006) and require a caesarean section delivery (RR 1.80, 95% CI 1.41-2.29, p <0.001) (Table 12). Their risk of PTD and CS remained elevated after adjusting for confounders and disease-seeking behaviour traits (Table 13 and Table 14).

Table 12: Unadjusted analysis of birth outcomes and neonatal outcomes in women with endometriosis compared to controls in their first pregnancy

	Endometriosis		*Controls*		*RR*	*95% CI*	*P value*
Descriptive	*N*	*%*	*N*	*%*			
	(346)		*(40929)*				
Birth Characteristics and Neonatal Complications							
Mode of Delivery	*316*		*34722*				
Spontaneous Vaginal	131	41.4	16805	48.4			
Instrumental Vaginal and breech	59	18.7	8973	25.8	0.84	0.62-1.15	0.279
Caesarean Section	126	39.9	8944	25.7	1.80	1.41-2.29	<0.001
(Elective	24	7.6	1430	4.1			
Emergency)	102	32.3	7514	21.6			
Labour	*317*		*34828*				
Spontaneous	302	95.3	32136	92.3			
Induction	15	4.7	2692	7.7	0.65	0.38-1.08	0.097
Retained Placenta	20	6.3	1673	5.4	1.42	0.90-2.24	0.127
Apgar at 5 Minutes	*314*		*34481*				
7-10	300	95.5	33434	97.0			
4-6	12	3.8	845	2.4	1.57	0.89-2.79	0.120

0-3	2	0.6	202	0.6	1.10	0.28-4.40	0.890
Gestation at delivery	*321*		*34726*				
Term	279	86.9	32021	92.2			
Preterm	42	13.1	2705	7.8	1.60	1.15-2.25	0.006
Birthweight	*312*		*34408*				
Normal	254	81.4	27634	80.3			
SGA	34	10.9	4408	12.8	0.84	0.59-1.20	0.339
LGA	24	7.7	2366	6.9	1.10	0.73-1.67	0.646
Shoulder Dystocia	4	1.3	352	0.9	1.34	0.50-3.58	0.554
Cord Prolapse	1	0.3	25	0.06	4.60	0.67-31.52	0.120
Birth Outcome	*317*		*34888*				
Livebirth	315	99.4	34586	99.1			
Stillbirth	2	0.6	186	0.5	1.18	0.30-4.70	0.816
Termination<24/40	0	0	69	0.2	0.0003	0.0003-0.0004	0.000
Miscarriage post booking	0	0	47	0.1	0.0003	0.0003-0.0004	0.000
Stillbirth	2	0.6	186	0.5	1.18	0.30-4.70	0.816
Neonatal Death	1	0.29	104	0.25	1.14	0.16-8.02	0.898
Maternal Death	1	0.29	67	0.16	1.76	0.25-12.32	0.571
	Mean	*SD*	*Mean*	*SD*	*RR*	*95% CI*	*P value*
Birthweight	3282	682.71	3279.49	647.77	1.00	0.99-1.00	0.947
Gestation	277	19.26	277	18.83	1.00	0.99-1.00	0.445

Table 13: Primary obstetric outcomes adjusted for confounders in exposure to outcome (E-O) model for first pregnancy subgroup

	SGA				CS*				PTD			
	N	RR	95% CI	P value	N	RR	95% CI	P value		RR	95% CI	P value
Unadjusted	34720	0.84	0.59-1.20	0.339	35038	1.80	1.41-2.29	<0.001	35047	1.60	1.15-2.25	0.006
Model 1	34086	1.00	0.73-1.38	0.978	34402	1.46	1.28-1.67	<0.001	34405	1.61	1.17-2.21	0.003
Model 2	31736	1.07	0.78-1.47	0.657	31611	1.14	1.08-1.20	<0.001	34405	1.56	1.12-2.17	0.008

* Elective and emergency caesarean section delivery compared to all vaginal deliveries including spontaneous, instrumental and breech

Model 1 adjusts for mother's age, body mass index (BMI) and smoking status

Model 2

SGA (small for gestational age): adjusts for mother's age, BMI, smoking status, ethnicity, previous livebirths, mode of conception and maternal comorbidity (if any of the following exist; hypertension, cardiac disease, respiratory disease, autoimmune disease, gastro-intestinal disease, haematological disease, thromboembolic disease, renal disease, endocrine disease, cancer, mental health disease, musculoskeletal disease and diabetes).

CS (caesarean section): adjusts for mother's age, BMI, smoking status, level of education, ethnicity, mode of conception, maternal comorbidity, preterm delivery, small for gestational age, substance use and previous livebirths

PTD (preterm delivery): adjusts for mother's age, BMI, smoking status, maternal comorbidity and mode of conception

Table 14: Primary obstetric outcomes adjusted for confounders in diagnosis to outcome (D-O) model for first pregnancy subgroup

	SGA				CS*				PTD			
	N	RR	95% CI	P value	N	RR	95% CI	P value	N	RR	95% CI	P value
Unadjusted	34720	0.84	0.59-1.20	0.339	35038	1.80	1.41-2.29	<0.001	35047	1.60	1.15-2.25	0.006
Model 1	31576	1.06	0.78-1.46	0.696	31847	1.46	1.27-1.67	<0.001	31851	1.66	1.21-2.28	0.002
Model 2	31576	1.06	0.77-1.45	0.716	31611	1.39	1.23-1.58	<0.001	31576	1.66	1.20-2.31	0.002

* Elective and emergency caesarean section delivery compared to all vaginal deliveries including spontaneous, instrumental and breech

Model 1 adjusts for mother's age, body mass index (BMI), education level, employment status, ethnicity, maternal comorbidity and smoking status

Model 2

SGA (small for gestational age): adjusts for mother's age, BMI, education level, employment status, ethnicity, smoking status, mode of conception and maternal comorbidity (if any of the following exist; hypertension, cardiac disease, respiratory disease, autoimmune disease, gastro-intestinal disease, haematological disease, thromboembolic disease, renal disease, endocrine disease, cancer, mental health disease, musculoskeletal disease and diabetes).

CS (caesarean section): adjusts for mother's age, BMI, smoking status, ethnicity, maternal comorbidity, mode of conception, small for gestational age, preterm delivery and substance use

PTD (preterm birth): adjusts for mother's age, BMI, education level, employment status, ethnicity, maternal comorbidity, smoking status, small for gestational age and mode of conception

4.7.3 Pregnancy after a diagnosis of endometriosis dataset

In subgroup analysis, 645 pregnancies (0.7%) occurred after a recorded diagnosis of endometriosis (Figure 17). In unadjusted analysis in this group, compared to the no endometriosis group, a diagnosis of endometriosis is associated with increasing age (RR increases by age group to 4.98, 95% CI 3.39-7.34 in the >40 age group compared to <25 age group), infertility (RR 8.23, 95% CI 6.26-10.82 of having IVF and RR 13.52, 95% CI 11.14-16.40 for needing other fertility treatment compared to natural conception), recurrent miscarriages (three or more) (RR 1.96, 95%CI 1.48-2.59) and having other co-morbidities (RR 1.37, 95% CI 1.16-1.62). A diagnosis of endometriosis was also associated with being overweight (BMI 25-29.9 kg/m^2) RR 1.33, 95% CI 1.12-1.59, compared to normal weight (BMI 18.5-24.9) and being primiparous compared to women with 1-3 previous live births (RR 1.17, 95% CI 1.01-1.37) (Table 15). Women of Asian or Black/African/Caribbean ethnicity (RR 0.61, 9%% CI 0.42-0.88 and RR 0.41, 95% CI 0.18-0.91 respectively compared to women of White ethnicity), lower educational qualification (RR 0.58, 95% CI 0.47-0.71 if secondary level education and below compared to those with university degree), women who were unemployed or a student (RR 0.51, 95% CI 0.42-0.61), those whose partner is unemployed or a student (RR 0.57, 95% CI 0.41-0.78) and those smoking compared to non-smokers (RR 0.63, 95% CI 0.49-0.80) were less likely to have a recorded diagnosis of endometriosis (Table 15).

Table 15: Unadjusted analysis of maternal characteristics, antenatal complications, birth outcomes and neonatal outcomes in women with endometriosis compared to controls in pregnancies after diagnosis subgroup

Descriptive	*Endometriosis*		*Control*				
	N	*%*	*N*	*%*	*RR*	*95% CI*	*P value*
	(645)		*(89724)*		*unadjusted*		
Maternal Characteristics							
Age	*645*		*89724*				
<25	70	10.85	23385	26.06			
25-34	392	60.78	51659	57.58	2.52	1.96-3.25	<0.001
35-39	143	22.17	12031	13.41	3.94	2.96-5.23	<0.001
40+	40	6.20	2649	2.95	4.98	3.39-7.34	<0.001
BMI	*642*		*87289*				
<18.5	14	2.18	2756	3.16	0.76	0.45-1.30	0.32
18.5-24.9	307	47.82	46077	52.79			
25-29.9	207	32.24	23236	26.62	1.33	1.12-1.59	0.001
30-39.9	104	16.20	13274	15.21	1.17	0.94-1.47	0.16
40+	10	1.56	1946	2.23	0.77	0.41-1.45	0.42
Smoking status	*642*		*87361*				
Non-smoker	512	79.75	64546	73.88			
Stopped smoking in pregnancy	57	8.88	8085	9.25	0.89	0.68-1.17	0.40
Current Smoker	73	11.37	14730	16.86	0.63	0.49-0.80	<0.001
Level of Education	*642*		*87345*				
College and above	528	82.24	63580	72.79			

Secondary and below	114	17.76	23765	27.21	0.58	0.47-0.71	<0.001
Employment Status	*639*		*86637*				
Employed	505	79.03	56755	65.51			
Unemployed or student	134	20.97	29882	34.49	0.51	0.42-0.61	<0.001
Partner's Employment Status	*612*		*80954*				
Employed	573	93.63	72237	89.23			
Unemployed or student	39	6.37	8717	10.77	0.57	0.41-0.78	0.001
Ethnicity	*642*		*82656*				
White	587	91.43	71550	86.56			
Asian	30	4.67	6044	7.31	0.61	0.42-0.88	0.007
Black/African/Caribbean	6	0.93	1802	2.18	0.41	0.18-0.91	0.03
Other	19	2.96	3260	3.94	0.71	0.45-1.12	0.14
Booking Systolic Blood Pressure	*631*		*86050*				
<140 mmHg	626	99.21	84885	98.65			
≥140 mmHg	5	0.79	1165	1.35	0.58	0.24-1.40	0.23
Mode of Conception	*642*		*87345*				
Natural	465	72.43	84647	96.91			
IVF/GIFT	55	8.57	1168	1.34	8.23	6.26-10.82	<0.001
Other Fertility Treatment (surgical/hormonal)	122	19.00	1530	1.75	13.52	11.14-16.40	<0.001
Maternal Co-morbidities	*645*		*89724*				
No co-morbidities	450	69.77	68206	76.02			

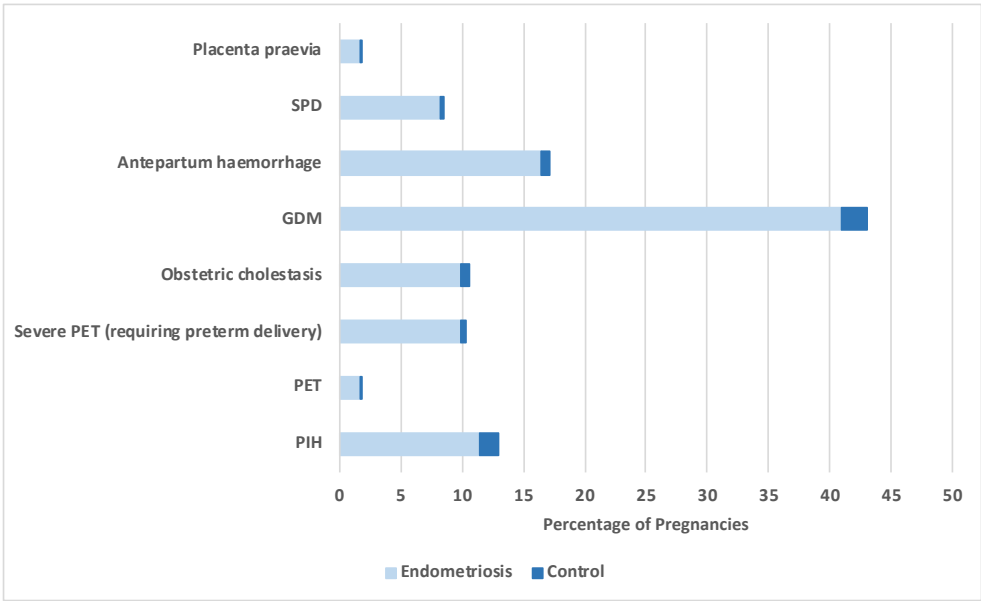
Co-morbidities	195	30.23	21518	23.98	1.37	1.16-1.62	<0.001
(HTN	11	0.34	820	0.9			
Cardiac Disease	16	1.03	838	0.9			
Respiratory Disease	0	-	27	0.03			
Autoimmune Disease	16	0.48	362	0.4			
Gastro-intestinal Disease	21	1.17	703	0.8			
Haematological Disease	26	0.96	648	0.7			
Thromboembolic Disease	11	0.55	262	0.3			
Renal Disease	14	1.44	900	1.0			
Endocrine Disease	23	1.44	1207	1.3			
Cancer	0	-	6	0.006			
Mental Health Disease	186	21.73	14791	16.5			
Musculoskeletal Disease	2	0.34	68	0.07			
Diabetes)	10	0.41	855	0.9			
Previous Livebirth	*645*		*89716*				
1-3	311	48.22	46168	51.46			
0	324	50.23	40929	45.62	1.17	1.01-1.37	0.04
>3	10	1.55	2619	2.92	0.57	0.30-1.07	0.08
Previous Stillbirth	7	1.09	855	0.95	1.14	0.54-2.39	0.73
Previous Miscarriages	*645*		*89724*				
0	399	61.86	58477	65.17			
1-2	191	29.61	27152	30.26	1.03	0.87-1.22	0.73
3+	55	8.53	4095	4.56	1.96	1.48-2.59	<0.001
	Mean	SD	Mean	SD			

Age	31.39	5.29	28.57	5.88	1.07	1.06-1.09	<0.001
BMI	25.72	4.92	25.49	5.49	1.01	1.00-1.02	0.24
Antenatal Complications	*645*		*89724*				
No complication	590	91.47	85198	94.96			
Complication	55	8.53	4526	5.04	1.75	1.33-2.30	<0.001
Birth Characteristics and Neonatal Complications							
Mode of Delivery	*639*		*76938*				
Spontaneous Vaginal	296	46.32	47876	62.23			
Instrumental Vaginal and Breech	107	16.74	11421	14.84	1.51	1.21-1.88	<0.001
Caesarean Section	236	36.93	17641	22.93	2.15	1.81-2.55	<0.001
(Elective	98		6028				
Emergency)	138		11613				
Labour							
Induction	66	10.23	5184	5.78	1.85	1.43-2.38	<0.001
Retained Placenta	36	7.19	2867	4.41	1.67	1.19-2.34	0.003
Apgar at 5 Minutes	*638*		*76455*				
0-3	4	0.63	393	0.51	1.23	0.46-3.26	0.68
4-6	18	2.82	1622	2.12	1.34	0.84-2.13	0.22
7-10	616	96.55	74440	97.36			
Gestation at delivery	*640*		*77562*				
Term (≥37 weeks)	564	88.13	71960	92.78			

Preterm (<37 weeks)	76	11.88	5602	7.22	1.72	1.36-2.18	<0.001
Birthweight	*637*		*76871*				
Normal	530	83.20	61022	79.38			
LGA	62	9.73	7932	10.32	0.90	0.69-1.17	0.43
SGA	45	7.06	7917	10.30	0.66	0.48-0.89	0.007
Shoulder Dystocia	8	1.24	962	1.07	1.16	0.58-2.32	0.68
Cord Prolapse	1	0.16	80	0.09	1.73	0.25-12.16	0.58
Birth Outcome	*645*		*77920*				
Livebirth	641	99.38	77266	99.16			
Stillbirth	2	0.31	396	0.51	0.61	0.15-2.44	0.49
Termination<24/40	0	0	167	0.21	-	-	-
Miscarriage post booking	2	0.31	91	0.12	2.62	0.66-10.30	0.17
	Mean	*SD*	*Mean*	*SD*	*RR*	*95% CI*	*P value*
Birthweight (grams)	3293	675	3349	650	1.00	1.00-1.00	0.03
Gestation (days)	273	20	277	18	0.99	0.99-1.00	<0.001

In unadjusted analyses, a diagnosis of endometriosis before pregnancy was associated with antenatal complications (RR 1.75, 95% CI 1.33-2.30) including PIH, PET and severe PET, placenta praevia and GDM (Figure 20 and Table 15). There was also an increased risk of induction of labour compared to spontaneous labour (RR 1.85, 95% CI 1.43-2.38), instrumental and breech vaginal delivery compared to spontaneous vaginal delivery (RR 1.51, 95% CI 1.21-1.88) and retained placenta post-delivery (RR 1.67, 95% CI 1.19-2.34) (Table 15). Primary outcomes of caesarean

section delivery (RR 2.15, 95% CI 1.81-2.55) and preterm delivery (RR 1.72, 95% CI 1.36-2.18) were associated with pregnancies of women with endometriosis.



SPD = symphysis pubis dysfunction GDM = gestational diabetes

PET = pre-eclampsia PIH = pregnancy induced hypertension

Figure 20: Unadjusted analysis of antenatal complications in pregnancies of women after a diagnosis of endometriosis compared to control pregnancies

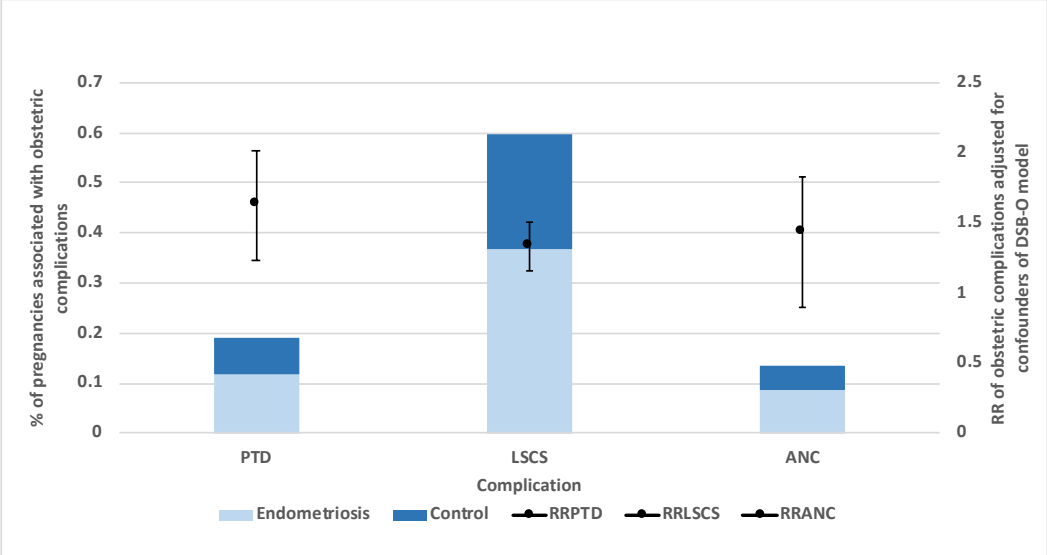


Figure 21: Graphical representation of unadjusted analysis results of the risk of preterm delivery, caesarean section delivery and antenatal complications in pregnancies of women after a diagnosis of endometriosis compared to control pregnancies (% and RR)

Although pregnancies with a diagnosis of endometriosis prior to conception were less likely to result in an SGA baby (RR 0.66, 95% CI 0.48-0.89), there was no evidence of association in the adjusted model, and mean birthweight was lower by 56 g (mean 3293g ± 675 in women endometriosis vs mean 3349 ± 650 in control). Risk of small for gestational age babies amongst women with endometriosis was not significantly associated with endometriosis in the fully adjusted E-O and D-O models (RR 0.84, 95% CI 0.60-1.17 and RR 0.83, 95% CI 0.59-1.18 respectively) (Table 16 and Table 17).

Mean gestation at delivery was three days earlier in women with endometriosis compared to controls (mean 273 days ± 20 vs 276 days ± 18) (Table 15). The risk of preterm delivery remained elevated in women with a diagnosis of endometriosis before pregnancy compared to those without after adjusting for maternal age, BMI and smoking status at the start of pregnancy (RR 1.66, 95% CI 1.27-2.17) (Table 16 and Table 17). However, the risk was attenuated after adding mode of conception and maternal comorbidity in the model (RR 1.20, 95% CI 0.89-1.63). In the D-O model, the relationship remained when additionally adjusting for level of education, employment status, ethnicity and maternal comorbidity (RR 1.70, 95% CI 1.29-2.23), but also attenuated when adding mode of conception and SGA to the model (RR 1.28, 95% CI 0.94-1.73) (Table 16 and Table 17).

Endometriosis was associated with a higher risk of caesarean section delivery compared to women without endometriosis (E-O model RR 1.43, 95% CI 1.27-1.61, D-O model RR 1.40, 95% CI 1.24-1.58). Risk of caesarean section also remained significant in E-O and D-O models when mediators were adjusted for (RR 1.07, 95% CI 1.02-1.12 and RR 1.22, 95% CI 1.08-1.38 respectively) (Table 16 and Table 17).

Table 16: Primary obstetric outcomes adjusted for confounders in exposure to outcome (E-O) model in pregnancies after a diagnosis of endometriosis

	SGA				CS**				PTD			
	N	RR	95% CI	P value	N	RR	95% CI	P value	N	RR	95% CI	P value
Unadjusted	77508	0.66	0.48-0.89	0.007	77577	2.15	1.81-2.55	<0.001	78202	1.72	1.36-2.18	<0.001
Model 1*	76300	0.83	0.59-1.17	0.29	76397	1.43	1.27-1.61	<0.001	76994	1.66	1.27-2.17	<0.001
Model 2*	71470	0.84	0.60-1.17	0.31	70649	1.07	1.02-1.12	0.002	76994	1.20	0.89-1.63	0.23

*Models are adjusted for clustering

** Elective and emergency caesarean section delivery compared to all vaginal deliveries including spontaneous, instrumental and breech

Model 1 adjusts for mother's age, body mass index (BMI) and smoking status

Model 2

SGA (small for gestational age): adjusts for mother's age, BMI, smoking status, ethnicity, previous livebirths, mode of conception and maternal comorbidity (if any of the following exist; hypertension, cardiac disease, respiratory disease, autoimmune disease, gastro-intestinal disease, haematological disease, thromboembolic disease, renal disease, endocrine disease, cancer, mental health disease, musculoskeletal disease and diabetes).

CS (caesarean section): adjusts for mother's age, BMI, smoking status, level of education, ethnicity, mode of conception, maternal comorbidity, preterm delivery, small for gestational age, substance use and previous livebirths

PTD (preterm delivery): adjusts for mother's age, BMI, smoking status, maternal comorbidity and mode of conception

Table 17: Primary obstetric outcomes adjusted for confounders in diagnosis to outcome (D-O) model in pregnancies after a diagnosis of endometriosis

	SGA				CS**				PTD			
	N	RR	95% CI	P value	N	RR	95% CI	P value	N	RR	95% CI	P value
Unadjusted	77508	0.66	0.48-0.89	0.007	77577	2.15	1.81-2.55	<0.001	78202	1.72	1.36-2.18	<0.001
Model 1*	70970	0.86	0.60-1.23	0.41	71029	1.40	1.24-1.58	<0.001	71584	1.70	1.29-2.23	<0.001
Model 2*	70970	0.83	0.59-1.18	0.29	70656	1.22	1.08-1.38	0.002	70978	1.28	0.94-1.73	0.12

*Models are adjusted for clustering

** Elective and emergency caesarean section delivery compared to all vaginal deliveries including spontaneous, instrumental and breech

Model 1 adjusts for mother's age, body mass index (BMI), education level, employment status, ethnicity, maternal comorbidity and smoking status

Model 2

SGA (small for gestational age): adjusts for mother's age, BMI, education level, employment status, ethnicity, smoking status, mode of conception and maternal comorbidity (if any of the following exist; hypertension, cardiac disease, respiratory disease, autoimmune disease, gastro-intestinal disease, haematological disease, thromboembolic disease, renal disease, endocrine disease, cancer, mental health disease, musculoskeletal disease and diabetes).

CS (caesarean section): adjusts for mother's age, BMI, smoking status, ethnicity, maternal comorbidity, mode of conception, small for gestational age, preterm delivery and substance use

PTD (preterm birth): adjusts for mother's age, BMI, education level, employment status, ethnicity, maternal comorbidity, smoking status, small for gestational age and mode of conception

4.8 Discussion

Analysis of this longitudinal cohort dataset spans over 15 years and utilises linked prospective healthcare records to investigate the impact of endometriosis on reproductive outcomes. It showed that endometriosis was directly associated with the risk of caesarean section delivery even after adjusting for confounders and mediators. It was also associated with preterm delivery after adjusting for confounders, with the risk attenuated when including mode of conception in the model. Mode of conception is therefore a potential mediator in the relationship between endometriosis and preterm birth.

Unadjusted analysis was performed for non-primary outcomes to assess for further potential associated risks of endometriosis in pregnancy. In unadjusted analyses, infertility, recurrent miscarriage, and concurrent co-morbidities were associated with endometriosis. Increasing age and being overweight were associated risk factors in women with endometriosis, consistent with findings from previous studies [240–242]. Women of Asian or Black/African/Caribbean ethnicity, those attaining a lower level of education and being unemployed or a student were associated with a lower risk of endometriosis although these findings may be reflecting healthcare inequalities rather than biologically protective effects on endometriosis. As described in previous studies, these characteristics are associated with difficulties and barriers to the timely diagnosis of endometriosis [243,244]. Conversely, White women, a higher level of education and a position of employment are likely more able to access the required investigations and tertiary centre care [243,244].

In the initial analysis inclusive of the entire cohort, as well as in the subgroup analysis of first pregnancies, the data demonstrated that endometriosis increases the early pregnancy, obstetric and perinatal risks, including the risk of preterm delivery, delivery by caesarean section and a number of antenatal complications including PIH, severe PET and GDM. The data also showed that endometriosis prior to pregnancy was associated with induction of labour, preterm delivery, lower birthweight, delivery by caesarean section, instrumental and breech vaginal delivery, retained placenta and a number of antenatal complications including PIH, PET, severe PET, GDM and placenta praevia in unadjusted analysis. Difference in risk of small for gestational age babies was not significant in adjusted analysis. The study supports the findings from the questionnaire-

based Nurse's Health Study II, which found obstetric and perinatal risks associated with endometriosis to be pregnancy loss, GDM, PIH, preterm delivery and lower birth weight [245].

Endometriosis is believed to occur in 1-10% of women of reproductive age and up to 50% of women with subfertility [246,247]. This cohort identified 1.4% of the pregnancies occurred in women who had a diagnosis of endometriosis attributed to them, with a lower prevalence of 0.7% of pregnancies occurring after the diagnosis of endometriosis. The lower prevalence may be due to the exclusion of women diagnosed with other gynaecological conditions (polycystic ovarian disease, infertility and fibroids) who may concurrently have endometriosis. The prevalence in this dataset may also reflect the diagnostic challenge of endometriosis and that the duration of data collection with the database has a limited window of accurately diagnosing women. It is now known that the average time from symptoms to diagnosis is 8 years in the UK [246] and in a population of women conceiving, completing their families, recovering from a post-natal period and often having further pregnancies the opportunity to seek investigation and treatment for endometriosis may be prolonged.

A number of obstetric and perinatal risks were associated with pregnancies following a surgical diagnosis and treatment of endometriosis. One can extrapolate that the effects of endometriosis may endure following surgery, whether some of the disease itself remains and is microscopic, recurs or continues to have an impact on pregnancy regardless of complete resection. Notably, this does somewhat challenge the dogma of surgical practice where widespread excision is performed for macroscopic disease, whilst many have shown that the disease can be microscopic and disseminated [248] and can continue to adversely impact on obstetric and perinatal outcomes.

The increased obstetric and perinatal risks are biologically plausible given that implantation and early placentation is differentially modulated in the endometrium of women with endometriosis compared to those without and several changes found in endometriosis can be implicated in the association with placental insufficiency disorders. In endometriosis, there exists differential expression of key factors in decidualisation and implantation by way of aberrant angiogenesis, immune remodelling, alterations in cell adhesion molecules, matrix remodelling, and immune signalling [202–204] and the overexpression of vascular endothelial growth factor, angiopoietins, and their receptor [11,194,203]. The thickness of the junctional zone is shown to be increased [205], endometrial blood perfusion is increased [203,206], and a number of important implantation markers

differ in women with endometriosis [194,198,203]. Defective spiral artery remodelling at the junctional zone of the myometrium–endometrium interface, as well as the size of placental bed and distribution of spiral artery transformation within the placental bed favouring the centre to the periphery can also interfere with placentation [203,205,207], although this has not been investigated specifically in endometriosis. These known pathological processes could give rise to the range of early pregnancy, obstetric and perinatal complications observed here.

4.8.1 Implications

Findings such as these and other recent reviews suggest that women with endometriosis may need to be considered at increased risk of early and late pregnancy complications, with health risks that potentially extend to their offspring. The prospective morbidity involved for this patient group could justify better pre-conception management of the disease and in time may warrant a more cautious approach to antenatal care and management in the peri-partum. Furthermore, there are some modifiable risk factors such as BMI although more research is required to investigate if weight management has a favourable impact on disease management. Increasing age has an increased association with endometriosis, in addition to the known detrimental impact of age on fertility; hence efforts for earlier diagnosis of the disease must be supported [249,250]. The findings also highlight possible healthcare inequalities in diagnosing endometriosis, with women from minority groups, less advantaged socioeconomic groups and lower levels of education possibly being underdiagnosed and undertreated. This carries implications in how healthcare is delivered and how healthcare professionals interact with women of these groups. It would possibly lead to the risks of these women in pregnancy being unacknowledged.

4.8.2 Strengths and limitations

This is a large, longitudinal cohort study investigating the health determinants, fertility, early pregnancy, obstetric and perinatal outcomes of endometriosis. It utilises linkage between two large hospital databases, in a region of the UK which serves a diverse population and is a good representation of the UK population, with detailed data input by healthcare professionals including maternal height, weight, blood pressure, birthweight, gestation at birth, Apgar scores, and maternal characteristics such as smoking status, previous obstetric history, past medical history and education level, documented from information held by the woman's GP or from the

history provided by the women during their antenatal care. The database has a low number of outlying and unrealistic data and the majority of the data entered is binary and therefore less open to interpretation of the data-gatherer. However, in a database this large, missing data is a common problem. Where data was missing in the inclusion and exclusion data the subject was excluded. Where outcome and covariate data were missing in the maternity dataset, these were included, and complete-case analysis is performed. This was to maintain higher cohort numbers where the case cohort was small, and outcomes of interest are uncommon. Where data was missing it has been demonstrated in the results (*n* numbers stated). Data was missing across the majority of variables. The assumption is that data is missing at random as there were potentially hundreds of different data-gatherers across time and clinical setting. In this preliminary work I sought to start with as large a data set as possible given how uncommon many of the outcomes are. Moving forward in future work and in publishing data, women with missing data will be excluded to deal with this more robustly.

At the time data collection was performed there was no specific ICD 10 code for adenomyosis (code N80.03 has since come into effect in October 2023). The ICD10 code N80.0 “Endometriosis of the uterus” could be applied to adenomyosis but also to endometriosis of the uterine serosa. A diagnosis of adenomyosis without histopathology is not sensitive. It was therefore decided to include women diagnosed with N80.0 in the endometriosis group. Adenomyosis is therefore not completely excluded from the case group. With the application of the new ICD 10 code pertaining specifically to adenomyosis it would now be possible to refine the case and control group. This is a limitation of the study and a recognised difficulty of observational studies examining reproductive impacts of adenomysosis and endometriosis.

There were not as many outcomes pertaining to the heath of the neonate as anticipated and variables like stillbirth and neonatal death had small numbers, therefore associations with endometriosis cannot be drawn in a case cohort of this size. This preliminary work can however be used to strengthen an ethical case for linking mother and baby record databases in ongoing work. Information about early miscarriage would also have been useful. However, maternity data in this study is first collected at pregnancy booking appointment which occurs at about 10 weeks’ gestation, therefore early miscarriage data was unknown. Information about previous miscarriage was collected and was investigated as a characteristic of the disease cohort compared to controls.

A surgical database is utilised and hence the diagnosis of endometriosis is reliable, perhaps more so than when questionnaire/symptom based. However, data extracted from the surgical database does not include free text surgical operation notes and therefore severity of endometriosis by revised American Society for Reproductive Medicine stage cannot be elucidated for useful sub-analysis. This study aimed to remove gynaecological co-morbidities from the study cohort and create as pure a study cohort as methodologically possible. This was done by scrutinising the surgical database and excluding gynaecological co-morbidities which may affect obstetric outcomes within the study cohort. However, women with a diagnosis of PCOS, fibroids and female infertility may exist in the maternity database as these diagnoses can be made without surgery and cannot all be methodologically excluded from the data set. As evidenced by the fact that some of the pregnancies were conceived by assisted conception. In the case of assisted conception pregnancies, this was adjusted for in multivariate analysis. Similarly, women in the control cohort may have had undiagnosed endometriosis and gynaecological co-morbidity. As "endometriosis of the uterus" is included in ICD 10 coding for endometriosis conditions, this was included in the study cohort as a spectrum of endometriosis.

Confounders were approached in a thorough and objective way by utilising DAGs generated by an online tool. All possible confounding or mediating variables of the maternity dataset were entered into a DAG rather than subjectively selecting variables to adjust for. This approach was however limited to the variables available in the dataset. There may be some confounders and mediators not available in the dataset to adjust for. Analysing data of this nature can make it difficult to infer a true causality between exposure and outcome owing to the many confounding factors however this study addresses for the first time the factors which may directly influence endometriosis via a pathophysiological process and those which affect reaching a diagnosis of the disease by healthcare seeking behaviour.

Further Work

Main findings of this work suggest that endometriosis is associated with preterm birth and caesarean section delivery both of which have far-reaching health impacts. Epidemiological studies suggest risks for the offspring including childhood respiratory tract infection, immune disorders such as asthma, and obesity are associated with caesarean section delivery compared to vaginal delivery [251]. Cardiovascular disease, obstructive lung disease, kidney disease, neurological

conditions, diabetes and obesity are just some of the sequelae of preterm birth [252] which are known to carry economic burden [253]. If further research can identify modifiable risk factors of endometriosis, a significant improvement in both direct and indirect health economic burden of the disease could be made.

As well as the primary outcome findings, several possible associated impacts of women with endometriosis in pregnancy were suggested in unadjusted analysis. These included instrumental and breech vaginal delivery, pregnancy induced hypertension, pre-eclampsia, gestational diabetes and placenta praevia which can carry significant maternal and fetal risk. Investigation of the association with adjusted analysis is therefore warranted for these outcomes.

One data subset explored pregnancies that occurred after a diagnosis of endometriosis. This begins to explore the temporal effect of endometriosis on pregnancy and neonatal outcome. However further work to examine outcomes of pregnancies before compared to after diagnosis, measuring time from diagnosis to the pregnancies in question and primiparous pregnancy after diagnosis of endometriosis to control for effect of parity would all give clarity to the temporality of the observed associated risks. The size of the case cohort in this study would limit these analyses currently but the surgical and maternity databases continue to gather data and when spanning a longer timeframe, may support this work in the future.

The maternal database contained limited variables to explore neonatal outcomes. Data was limited to Apgar scores at delivery, admission to the neonatal unit and neonatal death. Future work aims to link the databases used in this study to a paediatric database and investigate the longer-term impact on the offspring of women with endometriosis compared to those without gynaecological co-morbidity.

This study has highlighted that additionally to factors known to physiologically increase a woman's risk of endometriosis, racial and social factors which affect a woman's access to healthcare and reduce their likelihood of investigation need to be considered in multivariate analysis modelling in future work.

Chapter 5 Reactive Oxygen Species Levels in Women with Endometriosis

5.1 Introduction

Reactive oxygen species are a range of chemicals thought to play key roles in numerous physiological and pathophysiological biological functions. They are free radicals (a group of atoms with one or more unpaired electrons) occurring in biological systems derived from oxygen. Reactive oxygen species (ROS) can result in oxidative stress (OS) where there is an imbalance in the body's natural ability to combat free radicals and this is often the case in a number of disease processes including endometriosis [254,255]. Whilst ROS are essential for a whole spectrum of inter and intracellular signalling, OS can have negative effect on fertility in women with endometriosis through damage to macromolecules inclusive of lipids, proteins and nucleic acids essential for reproduction. The global impact within the pelvis is the result of adhesion formation with distortion of the tubo-ovarian anatomy but ROS is also implicated in modulating folliculogenesis, oocyte quality, oocyte transport, fertilisation and implantation by its actions in reproductive fluids and the endometrium.

ROS is known to be increased locally and systemically in women with endometriosis; however, the best technique to assess OS has been matter of debate, because ROS generation and turnover is significantly transient and there is a lack of consistency in assays and type of fluid/tissue used in current studies[255]. Hence, in order to accurately reflect the in vivo oxidative stress environment, the methodology used for ROS measurement needs to be verified and validated in order to be precise and accurate and studies need to be adequately powered. Therefore, in the current literature, findings are often inconsistent and researching the best methods to capture ROS activity remains a challenge. To tackle some of these limitations this work will use an assay, or method of measurement, from each of the known measurement approaches (direct, indirect – measurement of oxidative damage, redox status and antioxidant defence levels), rather than focussing on just one. The literature has been reviewed for suitable assays and techniques and assay product guides have been followed for suitability of different assays with the biospecimens collected in human studies of this work. The advice of a leading chemist in reactive species has been sort, Professor Martin Feelisch, and one of his assays has been adopted. Finally, the work has been powered using the most suitable available literature.

5.2 Objective

The objective of this chapter is to address the second aim of the thesis; To investigate whether reactive oxygen species levels are different in women with endometriosis compared to women without endometriosis and whether levels demonstrate a difference in reproductive fluids (local to the disease) and systemically (distant to the disease) and if they are improved following surgical treatment of disease.

The reproductive fluid of peritoneal fluid and systemic fluids of blood and urine will be investigated as these can be easily collected at surgery and oocytes are significantly exposed to peritoneal fluid between ovulation and oocyte pickup. Peritoneal fluid also contributes to a high percentage of fallopian tube fluid and is the only reproductive fluid in direct contact with endometriotic lesions. Correlation between the peritoneal fluid (in contact with endometriotic lesions) and systemic fluids of blood and urine (more distant to the disease) will be determined to indicate if systemic fluids are potentially useful biomarkers. It will then be investigated whether surgical treatment of endometriosis alters the levels of systemic ROS in women with endometriosis compared to those without to indicate if current gold standard treatment of endometriosis reduces levels of fertility-damaging ROS.

5.3 Hypothesis

ROS levels are higher both in reproductive fluids (peritoneal and follicular fluid) and systemic fluids (urine and serum) in women with endometriosis compared to women without endometriosis and these systemic levels are improved following surgical treatment of the disease.

5.4 Methods

5.4.1 Sample collection

The serum, urine and peritoneal fluid, clinical information and pelvic pain questionnaire results for women with and without endometriosis were collected and processed under the ethically approved studies “Peritoneal fluid biology in health and disease” and “XSESS: oXidative Stress in women with Endometriosis pre and poSt Surgery”. For patients recruited to “XSESS” their blood

and urine was also collected 2-3 months post-surgery. Participants were recruited to "Peritoneal fluid biology in health and disease" between July 2017 and June 2018. Participants were recruited to "XSESS: oXidative Stress in women with Endometriosis pre and poSt Surgery" between November 2018 and July 2019. The case and control cohorts for both studies were from a population of women with known or suspected endometriosis undergoing investigation and treatment for symptoms of pain, recurrent miscarriage or infertility at the gynaecology department of Princess Anne Hospital, Southampton. "XSESS" study also included women undergoing tubal sterilisation at Princess Anne Hospital to increase the number of control cases. Menopausal women, those with known active infective diseases and pregnancy were exclusion criteria. Peritoneal fluid was collected at the time of surgery following general anaesthetic and insertion of laparoscopic ports. Either a Wallace Assisted Reproduction Embryo Replacement Catheter or a 14 French gauge Ryles tube with a cut tip were placed into a pool of peritoneal fluid in the pelvis and extracted using a 20 ml syringe. Samples were transferred into reaction tubes and centrifuged in MyFuge5 at 2000 xg for 3 minutes. The supernatant was aliquoted into six 100 µL samples in 0.5 ml cryogenic vials, any remaining supernatant was aliquoted into 2 ml cryogenic vial and placed on dry ice before being stored at -80^{o}C. For the additional collection of blood and urine samples at follow-up, blood was taken via standard venepuncture technique and participants produced a urine sample into a sterile pot. These samples were immediately centrifuged in 2 ml reaction tubes at 2000 xg for 3 minutes in MyFuge5. Supernatants were aliquoted into 100 µL samples in 0.5 ml cryogenic vials, any remaining supernatant was aliquoted into 2 ml cryogenic vials and placed on dry ice before being stored at -80^{o}C.

5.4.2 Assays

The correlation between reproductive fluid levels of ROS and systemic levels and correlation of ROS levels in women with and without endometriosis were analysed. To determine whether ROS levels are higher in systemic and reproductive fluid in women with endometriosis compared to women without endometriosis three widely used commercial assays for exploring oxidative stress levels were selected from techniques covering direct measurement (Amersham Lumigen assay, GE Healthcare, US), anti-oxidant measurement (Total Antioxidant Capacity assay, AbCam, UK) and redox status measurement (GSH/GSSG assay, AbCam, UK). An assay for by-product measurement (TBARS assay) developed by Professor Feelisch's laboratory was also adopted. These same assays

were utilised to determine whether systemic levels of ROS in women with endometriosis are reduced following surgical treatment.

Total ROS Assay

An assay protocol was developed to detect peroxidase conjugates (peroxide free radicals) via a reaction involving enzymatic generation of acridinium ester intermediates which react with peroxide to produce chemiluminescence.

Amersham ECL Prime Western Blotting Detection Reagent Kit (GE Healthcare, UK) was first tested with serial dilution concentrations of HRP enzyme in PBS in sequential wells of a 96-well plate to establish a standard curve. Luminol mastermix was prepared in a 45.5:1ratio of luminol enhancer solution to peroxide solution from Amersham ECL Prime Western Blotting Detection Reagent Kit. The 96-well plate was transferred to Glomax Discover plate reader and an injector flushed and primed with 500 µL luminol mastermix.

A luminescence protocol was created with Glomax Discover software to read all relevant wells at baseline (0.3 seconds) followed by injection of 50 µL luminol mastermix into a well containing standard followed by a measurement of luminescence at 0.2 seconds, 0.5 seconds, 0.8 seconds, 1.1 seconds and 1.4 seconds, 1.7 seconds and 2 seconds post injection.

Results suggested that luminescence from neighbouring wells may be interfering with plate-reading as baseline luminescence were all 100-500 moles of luciferase but after injection of luminol to the first horseradish peroxidase (HRP) sample, that sample and all other samples gave a luminescence level of 900,000-10,000,000 moles of luciferase. The assay was therefore repeated with samples placed in wells distant from each other and results demonstrated that HRP reacted with the peroxide component of the assay to produce chemiluminescence which increased with increasing HRP concentration however a suitable standard curve was not achieved due to a significant increase at 1:1000 concentration HRP (Figure 23a). On removing higher concentration HRP solutions a more suitable standard curve was produced indicating that lower concentration dilutions were needed (from 1:1,000,000 for example) and that HRP is a suitable positive control. HRP 1:10,000 concentration was selected as a positive control and PBS was selected as a negative control.

With a suitable positive and negative control established, the assay was tested with samples of peritoneal fluid, serum and urine from patients with and without endometriosis (cases and controls respectively) from "Peritoneal Fluid Study". 10 µl of samples (urine, serum and peritoneal fluid from patients with and without endometriosis) and 10 µl of HRP standards were pipetted into alternate wells of the 96-well plate and Glomax Discover software luminescence protocol repeated. Provisional results demonstrated higher levels of luminescence in samples of endometriosis patient fluids compared to non-endometriosis control patient fluids with an exception of urine which produced similar levels between the two groups.

While horseradish peroxidase was an effective positive control for use alongside the total ROS assay, it could not be used for calibration. A more suitable standard curve with luminescence covering a range similar to that of human samples was required. The work of Uy [97] on optimising this assay for determining ROS levels in cell culture media developed a useful calibration method with use of xanthine and xanthine oxidase and the total ROS assay validated by an NBT assay. 1000 µM of xanthine was made by dissolving 1 mg of xanthine in 50 µl of 5M NaOH and 12.5 ml of PBS added. Dilutions of xanthine for 0 µM, 500 µM, 700 µM and 1000 µM were then made. Dilutions of xanthine were added to alternate wells of a 96 well plate. 10 µl of 1 unit/ml xanthine oxidase added to wells of a 96 well plate containing xanthine serial dilutions. 10 µl of $1:10^3$ of HRP in PBS was added to a well as a positive control. 10 µl of samples (urine, serum and peritoneal fluid from patients with and without endometriosis) were also pipetted into alternate wells of the 96-well plate. The luminescence protocol was repeated on the Glomax Discover plate reader (baseline readings from wells followed by injection of 50 µl luminol mastermix into a well containing sample, standard or control followed by a measurement of luminescence at 0.2 seconds, 0.5 seconds, 0.8 seconds, 1.1 seconds and 1.4 seconds, 1.7 seconds and 2 seconds post injection). Increasing xanthine-xanthine oxidase showed a linear increase in luminescence. A reliable standard curve was generated with levels of luminescence similar in range to that generated by a number of human samples from "Peritoneal fluid biology" and "XSESS" study (Figure 22 b).

The chemiluminescence reaction over time showed an ongoing reaction in HRP positive control sample up until 1.5 seconds from injection with luminol master-mix whereas in human fluid samples luminescence levels peaked at 0.3 seconds (0.2 seconds post-injection) with the exception of non-endometriosis control urine sample. This assay displayed a second peak at 1.2

seconds (1.1 seconds post-injection) indicating possible contamination of luminescence from another reaction well, which would also account for erroneously higher levels of luminescence (Figure 23 c and d). Going forward a 96-well plate with clear bottom and opaque walls was used to prevent well-well photometric contamination.

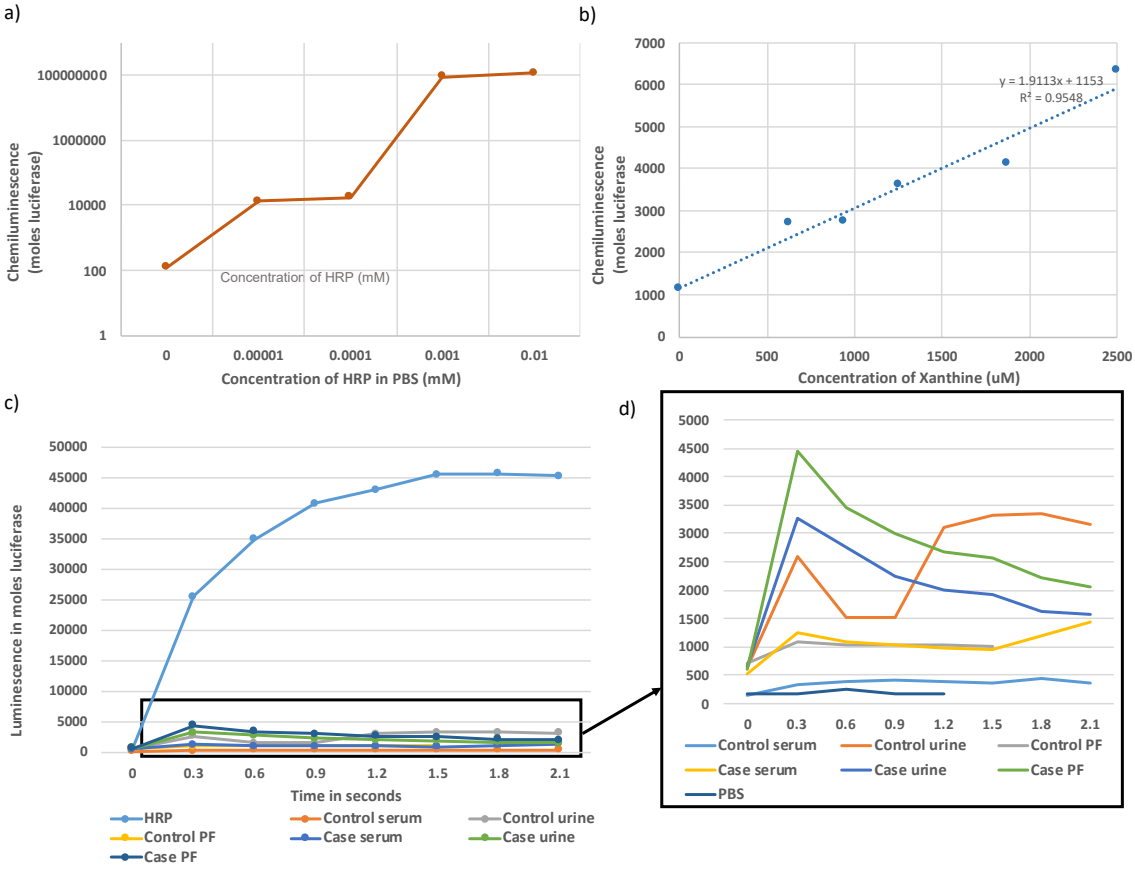


Figure 22: a) Graphical representation of results of Luminol assay measuring total ROS in serial dilutions of HRP enzyme (0 mM, 0.00001 mM, 0.0001 mM, 0.001 mM and 0.01 mM) demonstrating a non-linear relationship. b) Standard curve generated with xanthine serial concentration dilutions and xanthine oxidase reacting with Luminol assay. c) Results of total ROS in urine, serum and peritoneal fluid of endometriosis (case) and non-endometriosis (control) patients of "peritoneal fluid biology" study compared to a positive (HRP enzyme 1:10,000 with PBS) and negative (PBS) control. d) Demonstrates the luminescence from total ROS in the same samples without HRP positive control value

Total Antioxidant Capacity

A commercial total antioxidant capacity assay was used to measure small molecule antioxidants (Vitamin C, Vitamin E, glutathione etc) in the serum, urine and peritoneal fluid of women and compare their antioxidant capacity to a trolox (a strong antioxidant) standard curve. The small molecule antioxidants reduce copper ions which are then chelated by a colorimetric probe in the assay. The degree of chelated probe affects the amount of absorbance of the fluids when measured at its peak wavelength of 570 nm.

A trolox standard dilution was made from 1 mM stock and ddH_2O with 0 nmol, 4 nmol, 8 nmol, 12 nmol, 16 nmol and 20 nmol of trolox in 100 µl volumes. Cu^{2+} working solution was made from mixing Cu reagent with assay diluent in a 1:49 ratio protected from the light. Samples of serum and peritoneal fluid were diluted with ddH_2O in 1:1, 1:4 and 1:10 ratio to a volume of 100 µl. Samples of urine were first diluted with protein mask in 1:1, 1:4 and 1:10 ratio and then made to a volume of 100 µl with ddH_2O. 100 µl of each trolox dilution standard was aliquoted into sequential wells of a 96 well flat-bottomed plate, a negative control of 100 µl ddH_2O was aliquoted into a well of the plate. Prepared human biospecimen dilutions were aliquoted to sequential wells of the 96 well flat-bottomed plate. 100 µl of Cu^{2+} working solution was added to each standard, sample and control well in the dark room and the plate was protected from light and incubated at room temperature on an orbital shaker for 90 minutes. A protocol for absorbance at 570 nm wavelength with 20 flashes per well was designed with Optima Control software for Fluostar plate reader.

Due to quantities of samples needed, for initial protocol development some samples were kindly donated by Dr Mukhri Hamdan from “Peritoneal Fluid Study” collected in November 2014 and January 2015, which had been stored at -80^{o}C. One case patients’ samples collected recently for this thesis were also tested to check for a potential effect of long-term storage. Results demonstrated a good standard curve indicating correct dilutions, preparation of Cu^{2+} working solution and absorbance protocol (Figure 23 a) however all sample dilutions except a control urine 1:10 dilution gave absorbance levels exceeding the highest level trolox standard and therefore could not be interpreted. The assay was repeated with lower sample concentrations.

The samples were prepared as before to achieve dilutions of 1:10, 1:100, 1:1000, 1:10,000 and 1:100,000. Two patients’ samples were again donated by Dr Mukhri Hamdan of a case and a

control (March 2014 and January 2015 respectively). Samples collected for this thesis in a case patient were also included in the assay, as before. Results demonstrated a suitable standard curve and negative control. There was no significant difference found between samples stored since 2014/2015 and samples collected for this thesis (Figure 23 b). Sample dilutions with concentration >1:1000 produced absorbance levels above the trolox standard curve limits and concentrations <1:1000 produced absorbance levels lower than the limits of the trolox standard and leading to negative total antioxidant capacity (TAC) levels after data analysis. A suitable sample concentration of 1:1000 was selected for ongoing assay work (1 µl of serum, peritoneal fluid and urine samples from endometriosis patients and non-endometriosis control patients were mixed with 1 µl protein mask and then diluted with 998 µl ddH_2O).

The protocol was tested with a greater sample number. The urine, serum and peritoneal fluid of 3 women without endometriosis (controls), 7 women with endometriosis (cases) and 3 women with endometriosis at their 3-month post-treatment follow-up who had been recruited to "XSESS" study were tested with the TAC assay. A suitable standard curve was again obtained, and all sample results fell within the range of that standard curve (Figure 23 c and d).

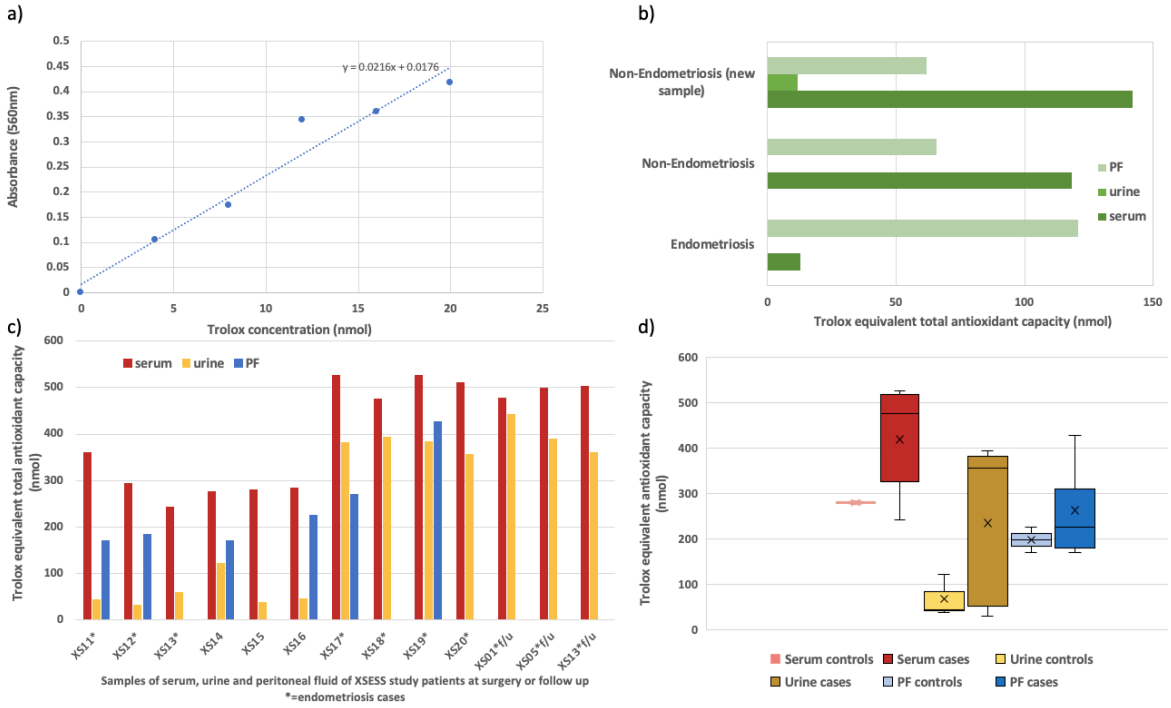


Figure 23: a) Total antioxidant capacity of Trolox standard curve b) Total antioxidant capacity of test samples from women with and without endometriosis collected in 2014 compared to samples collected within 3 months of testing. c) Total antioxidant capacity in the urine, serum and peritoneal fluid of patients with endometriosis (cases n=6), without (controls n=3) and endometriosis cases at follow up (n=3) from "XSESS" study. d) Mean levels of total antioxidant capacity of serum, urine and peritoneal fluid in endometriosis cases and non-endometriosis controls with 95% confidence level error bars.

Glutathione Assay

A commercial assay was used containing a non-fluorescent dye, which becomes fluorescent when reacting with the antioxidant reduced glutathione (GSH) or the oxidised glutathione (GSSG) with excitation at 490 nm wavelength and emission at 520 nm wavelength. When cells experience higher oxidative stress, there is increased GSSG to GSH ratio. This assay therefore measures the redox status of the cells. Suitable sample volumes and dilutions were first investigated.

Human biospecimens of 100 µl serum, peritoneal fluid and urine of endometriosis patients and non-endometriosis control patients were deproteinised by adding 15 µl of chilled TCA and placing on ice for 15 minutes followed by centrifugation at 12000 xg for 5 minutes. The supernatant was collected, and pellet discarded. 10 µl of neutralising buffer was added to the supernatant of the samples in open Eppendorf tubes and placed on wet ice for 5 minutes. 50 µl of each sample was diluted 1:1 in ddH_2O and 50 µl of this sample was aliquoted in duplicate into wells of a 96 well plate (for a GSH reaction and a total GSH and GSSG reaction). The remaining 25 µl of each sample was diluted in 75 µl of ddH_2O and 50 µl volumes were aliquoted into the 96 well plate for GSH reaction and total GSH and GSSG reaction.

Separate GSH and GSSG standard dilutions were prepared from 10 mM GSH or GSSG in assay buffer with 0 µM, 0.1563 µM, 0.3125 µM, 0.625 µM, 1.25 µM, 2.5 µM, 5 µM and 10 µM of GSH or GSSG in 100 µl volumes. 50 µl of each standard was aliquoted into the 96 well plate in ascending order for GSH and GSSG standards separately.

GSH assay mixture was made with 100x thiol green assay buffer protected from light. Total GSH assay mixture was made with 25x GSSG probe in GSH assay mixture protected from light. 50 µl of GSH assay mixture was added to each well of GSH standard and the samples intended for GSH reaction in a dark room. 50 µl of total GSH assay mixture was added to each well of GSSG standard and the samples intended for total GSH and GSSG reactions in a dark room. The plate was incubated in the dark at room temperature for 30 minutes and fluorescence was measured at 490 nm excitation wavelength and 520 nm emission wavelength on a protocol designed with Optima Control software for Fluostar plate reader.

Deproteinising the samples resulted in variable amounts of supernatant sample and reduced the final sample volume considerably. There was not enough sample following deproteinising to fulfil

the number of intended dilutions in the first assay when 100 µL starting sample used. The results however demonstrated a good linear fit of the standard curve. Fluorescence in the sample wells of duplicates were consistent and fluorescence also correlated well with their dilution except one sample of 20% urine which showed possible dilution error. Fluorescence from sample concentrations of 20% were too low to measure GSH: GSSG levels reliably. The assay was repeated using a starting volume of 150 µL of sample before deproteinising and no further dilutions were performed following deproteinising therefore the sample concentration was 82%. The standard curves for reduced glutathione and total oxidised and reduced glutathione gave suitable linear best fit values of fluorescence which indicated no pipetting errors or problems with quality of assay reagents. Levels of fluorescence for the majority of samples in total glutathione assay wells were either very similar or lower than fluorescence levels in reduced glutathione well leading to uninterpretable final GSH: GSSG result.

There was a long assay preparation time involved in processing 39 samples and it was considered possible that samples were being oxidised during this time period leading to the observed results. The assay was repeated with serum, urine and peritoneal fluid from one case patient and one control (6 samples) and incubation period reduced by half. Similar results were again observed. It was decided that the glutathione assay should not be used for this particular group of samples.

TBARS Assay

Following difficulty with glutathione assay protocol development an alternative assay was pursued. The expertise of a renowned chemist in the area of reactive oxygen and nitrogen species was sought regarding the work in this chapter. With Professor Martin Feelisch, a number of other ROS assay techniques were explored and thiobarbituric acid reactive substance (TBARS) assay was recommended. This assay measures Malondialdehyde (MDA) which is the end product of lipid peroxidation and occurs through oxidative modification of lipids. Thiobarbituric Acid Reactive Substances (TBARS) assay is a widely accepted method of detecting MDA levels and therefore determining oxidative damage. It can be used for biological fluids, tissues and cells. Thiobarbituric acid reacts with MDA to form an adduct which is red in colour and therefore measured either colorimetrically (with absorbance at 532 nm) or flourometrically (excitation/emission profile 532/553 nm).

A protocol developed by his laboratory, which they were using to good effect with serum, was followed. A water bath was created by placing cryovials filled with MQ water in the wells of a heat block at 90^{o}C temperature. 20% Acetic acid was made using Acetic Acid in MQ water. TBARS colour reagent was made by dissolving Thiobarbituric Acid in 20% Acetic Acid. A 500 µM stock solution of MDA standard was made by dissolving MDA salt in MQ water. From this a working solution of 333 µM concentration was made diluting MDA standard stock in MQ water. Nine standards were then made of the following MDA concentrations from the working solution and diluting with MQ water; 33.4 µM, 24.5 µM, 16.7 µM, 8.36 µM, 4.18 µM, 2.08 µM, 1.04 µM, 0.52 µM and 0 µM. 50 µl of each standard was mixed with 50 µl of Methanol in PCR tubes giving final MDA standard concentrations of; 16.7 µM, 12.25 µM, 8.35 µM, 4.18 µM, 2.09 µM, 1.04 µM, 0.52 µM, 0.26 µM and 0 µM.

Serum, urine and peritoneal fluid samples were prepared with 65 µl of samples mixed 1:1 with Methanol and centrifuged at >16000g for 20 minutes at 4^{o}C. The supernatant was removed from the pellet and aliquoted in 0.5 ml PCR tubes.

For each MDA standard, 50 µl were mixed 1:1 with Methanol and 50 µl of TBARS colour reagent in a 0.5 ml PCR tube. For each sample 80 µl were mixed with 40 µl of TBARS colour reagent in a 0.5 ml PCR tube. PCR tubes were sealed and placed in the cryovials of the water bath to be incubated for 30 minutes. Following incubation, the PCR tubes were placed on ice for 10 minutes. 50 µl of each were pipetted into a 384 well flat-bottomed plate in duplicate and absorbance at 532 nm wavelength was measured with a Fluostar plate reader using Optima Control software.

5.5 Data Analysis

Total ROS assay analysis

Luminescence readings on a logarithmic scale in units of moles of luciferase were taken from each well at multiple timepoints following injection of each sample (samples, positive and negative controls) with lumigen mastermix. Mean levels of luminescence were taken from time-points 0.2 seconds post injection to 2 seconds post-injection and background luminescence from well pre-injection was subtracted. Standard curve was plotted with luminescence levels of the xanthine-xanthine oxidase standards and gradient of the line of best fit calculated with the equation x=(y-b)/slope (where x is xanthine concentration, y is luminescence and b is the y-axis intercept). The same equation was then applied using the luminescence levels from test samples to calculate the equivalent xanthine concentration.

Total Antioxidant analysis

Absorbance value from the blank (ddH_2O) standard was subtracted from all standard and sample absorbance levels. Absorbance levels of the standards was plotted against the respective concentration of trolox (nmol) on standard calibration curve graph with line of best fit. Gradient of the line of best fit was then calculated with the equation x=(y-b)/slope (where x is trolox concentration, y is absorbance and b is the y intercept). The value of the slope could then be applied to the same formula using the absorbance levels from test samples to calculate trolox concentrations (nmol). Final total antioxidant capacity of the test samples was then calculated as a trolox equivalent capacity as follows:

Sample Total Antioxidant Capacity = (Ts/Sv) *D

Ts = TAC amount in the sample calculated from the standard curve (nmol)

Sv = sample volume in the well (µL)

D = sample dilution factor

Glutathione analysis

Fluorescence value from the blank (ddH_2O) standard was subtracted from all standard and sample fluorescence levels. Fluorescence levels of the standards was plotted against the respective concentration of GSH (reduced glutathione) (µM) or total GSH and GSSG (oxidised and reduced glutathione) (µM) on standard calibration curve graph with line of best fit. Gradient of the line of best fit was then calculated with the equation x=(y-b)/slope (where x is GSH or total GSH and GSSG concentration, y is absorbance and b is the y intercept). The value of the slope was applied to the same formula using the fluorescence levels from test samples to calculate GSH concentrations and total GSH and GSSG concentrations respectively (µM). Final concentration of GSH in the test samples was calculated as follows:

GSH = (A/B) *D

A = Amount of GSH in the sample (µM)

B = Sample volume (µL)

D = Sample dilution factor

Final concentration of GSH and GSSG in the test samples was calculated as follows:

Total glutathione = (A/B) *D

A = Amount of GSH and GSSG in the sample (µM)

B = Sample volume (µL)

D = Dilution factor

Oxidised glutathione was then deduced by subtracting the known reduced glutathione concentration from the total glutathione concentration and a ratio of reduced to oxidised was found (ratio = GSH/GSSG).

TBARS analysis

Absorbance levels measured at 750nm were subtracted from absorbance levels measured at 532nm for all standards and samples. Corrected absorbance levels of the standards were plotted against the respective concentration of MDA (µM) on standard calibration curve graph with line of

best fit. Gradient of the line of best fit was then calculated. MDA equivalent values in µM of the corrected absorbance of the samples were then calculated with the equation x=(y-b)/slope (where x is MDA equivalent, y is corrected absorbance, b is the y intercept and slope is the value of the gradient of the slope).

Statistical Analysis

Experiments in this chapter yielded results in the form of continuous data therefore they were reported as means with standard deviations. Tests for outlying data and normality were performed. Where data was normally distributed, mean and standard deviation is interpreted and where data was not normally distributed, median and IQ range were interpreted. For all results a p value <0.05 was deemed statistically significant.

Software GraphPad Prism version 7 (GraphPad Software Inc., US) and SPSS version 25 (IBM, UK, 2017) were used for all analyses.

For assay analysis independent samples t-test was used to scrutinise inter-group differences of women with and without endometriosis where data was normally distributed, and ANOVA was used for multiple group comparisons. Where data was not normally distributed, Mann-Whitney and Kruskal-Wallis tests were used respectively. Bonferroni correction was applied to all multiple group comparison tests. To compare samples taken at the time of surgery to follow-up samples paired sample t-test for normally distributed data and Wilcoxin signed rank tests were used for non-normally distributed data.

5.6 Results

5.6.1 Patient samples

Summary characteristics of participants recruited to “Peritoneal fluid biology in health and disease” are described in Table 18 and the characteristics of “XSESS” study recruited patients are described in Table 19. Full participant details recruited to “Peritoneal fluid biology in health and disease” are described in Appendix 22 and the full participant details recruited to “XSESS” study are described in Appendix 23.

Univariate analysis of the main characteristics of the women recruited to “XSESS” revealed that the control group and endometriosis group were similar by age, BMI and smoking status but that control group were more likely to have medical co-morbidities and more likely to have other gynaecological pathology noted at surgery. The endometriosis group were more likely to be nulliparous (Table 20).

Table 18: Characteristics of patients recruited to study "Peritoneal Fluid Biology in Health and Disease"

Study No.	Age	BMI	Smoking Status	Pathology	ASRM stage	Reason for surgery
PF 248	23	22.2	Non-smoker	Nil	-	Pain
PF 249	19	21.4	Non-smoker	Nil	-	Pain
PF 250	38	25.8	Smoker	Endometriosis	I	Pain
PF 251	37	38.1	Non-smoker	Endometriosis	IV	Pain
PF 252	26	21.6	Smoker	Endometriosis	I	Infertility
PF 253	41	25	Non-smoker	Endometriosis	IV	Pain
PF 254	33	43.3	Smoker	Endometriosis	III	Pain
PF 255	34	40.1	Non-smoker	Endometriosis	II	Pain
PF 256	32	20.2	Non-smoker	Endometriosis	III	Pain
PF 257	30	26.5	Non-smoker	PID adhesions	-	Pain
PF 258	25	23	Non-smoker	Nil	-	Pain
PF 259	30	26.2	Non-smoker	Endometriosis	III	Pain & infertility
PF 260	27	39.6	Non-smoker	Endometriosis	-	Pain & infertility
PF 261	32	22.1	Non-smoker	Endometriosis adenomyosis	I	Pain & infertility
PF 262	42	36.5	Non-smoker	Endometriosis	IV	Pain

Table 19: Characteristics of patients recruited to study "XSESS: oXidative Stress in women with Endometriosis pre and poSt Surgery"

Study No.	Age	BMI	Smoking Status	Pathology	ASRM stage	Reason for surgery
XS 1	29	19.8	Non-smoker	Endometriosis, unicornuate uterus & non-communicating rudimentary horn	III	Pain
XS 2	43	26.7	Non-smoker	Adhesions old endo, adenomyosis	I	Pain
XS 3	42	25.4	Non-smoker	Nil	-	Pain
XS 4	30	32.2	Non-smoker	Endometriosis and dermoid cyst	II	Pain
XS 5	30	22.7	Non-smoker	Endometriosis & fibroids	II	Pain
XS 6	45	22.2	Non-smoker	Endometriosis	II	Infertility
XS 7	37	31.8	Non-smoker	Nil	-	Pain
XS 8	30	27.7	Non-smoker	Adenomyosis, uterine septum, PID adhesions	-	Infertility
XS 9	28	20.7	Smoker	Scarred peritoneum from previous surgery no adhesions	-	Pain
XS 10	26	26.2	Non-smoker	Endometriosis	I	Pain
XS 11	33	21.8	Non-smoker	Endometriosis	IV	Infertility
XS 12	41	34	Non-smoker	Endometriosis	IV	
XS 13	39	25.8	Non-smoker	Endometriosis	III	Pain
XS 14	44	29.5	Non-smoker	Endometriosis	IV	Pain
XS 15	36	24.2		Endometriosis, fibroid and hydrosalpinx	III	Pain
XS 16	38	26.4	Non-smoker	Endometriosis	II	Recurrent miscarriage
XS 17	37	22.2	Non-smoker	Endometriosis, arcuate uterus	II	Recurrent miscarriage
XS 18	38	25	Vapes	Endometriosis	III	Pain
XS 19	35	24.2	Non-smoker	Endometriosis	III	Pain

XS 20	29	21.3	Non-smoker	Endometriosis	IV	Pain
XS 21	31	32.4	Smoker	Omental adhesions from previous surgery	-	Pain
XS 22	36	28.7	Non-smoker	Frozen pelvis - adhesions from previous surgery	-	Recurrent miscarriage
XS 23	43	26	Non-smoker	Endometriosis	IV	Pain
XS 24	31	25.9	Non-smoker	Endometriosis	II	Pain
XS 25	31	23.3	Non-smoker	Endometriosis	II	Pain
XS 26	21	22.5	Non-smoker	Endometriosis	I	Pain
XS 27	37	19.2	Non-smoker	Filmy adhesions, hydrosalpinx	-	Infertility
XS 28	41	24.4	Non-smoker	Adenomyosis	-	Pain
XS 29	48	19.3	Non-smoker	Nil	-	Pain
XS 30	33	26.2	Non-smoker	Endometriosis	IV	Infertility
XS 31	29	32.1	Non-smoker	Endometriosis, adhesions from previous surgery	III	Recurrent miscarriage
XS 32	32	23.9	Smoker	Endometriosis	IV	Infertility
XS 33	24	24.3	Non-smoker	Endometriosis	III	Pain
XS 34	45	25.7	Non-smoker	Adenomyosis	-	Pain
XS 35	30	37.5	Smoker	Endometriosis	II	Pain
XS 36	49	25.7	Non-smoker	Adenomyosis	-	Pain
XS 37	40	31.1	Smoker	Fibroid, physiological cyst	-	Pain
XS 38	34	22.5	Non-smoker	Endometriosis	III	Pain
XS 39	20	28.9	Non-smoker	Endometriosis	III	USS finding
XS 40	36	26.1	Smoker	Endometriosis	I	Pain
XS 41	47	25	Non-smoker	-	-	Pain
XS 42	38	29.7	Non-smoker	Ruptured haemorrhagic cyst or corpus luteum	-	Infertility

XS 43	34	21.3	Non-smoker	Endometriosis	III	Infertility
XS 44	29	23.8	Smoker	Adhesions from PID	-	Pain
XS 45	40	21.8	Non-smoker	Adhesions from previous surgery	-	Pain
XS 46	50	26.6	Non-smoker	Endometriosis and fibroid	I	Pain
XS 47	46	40.2	Smoker	Endometriosis and adenomyosis	I	Pain
XS 48	34	28.6	Non-smoker	Endometriosis	IV	Infertility
XS 49	51	34.1	Non-smoker	Endometriosis	III	Pain
XS 50	44	27.2	Non-smoker	Simple cyst	-	Pain
XS 51	19	22.6	Smoker	Nil	-	Pain
XS 52	39	34.9	Non-smoker	Nil	-	Pain
XS 53	31	22	Smoker	Adhesions from caesarean section	-	Sterilisation
XS 54	44	26.3	Non-smoker	Endometriosis	IV	Infertility
XS 55	33	22.3	Non-smoker	Scarring from inactive endometriosis	-	Recurrent miscarriage
XS 56	37	28.7	Non-smoker	Haematosalpinx, hydrosalpinx, fibroid, pelvic adhesions	-	Infertility
XS 57	38	24.4	Non-smoker	Endometriosis	I	Infertility
XS 58	36	29.7	Smoker	Adhesions from appendicectomy	-	Infertility
XS 59	40	28	Non-smoker	Endometriosis	I	Infertility
XS 60	37	21.6	Non-smoker	Nil	-	Sterilisation
XS 61	54	41.5	Non-smoker	Dermoid cyst and adhesions	-	Dermoid cyst
XS 62	29	32.1	Non-smoker	Pelvic adhesions	-	Infertility

Table 20: Univariate analysis of characteristics of women recruited to XSESS study

Characteristic	***Endometriosis n=36***		***Control n=26***		***OR***	***95% CI***	***P value***
	Mean	***SD***	***Mean***	***SD***			
Age	35.39	7.47	37.58	7.73	0.96	0.90-1.03	0.264
BMI	26.47	4.64	26.73	5.28	0.52	0.89-1.10	0.834
	N	***%***	***N***	***%***	***OR***	***95% CI***	***P value***
Parity					0.63	0.40-1.00	0.048
0	23	63.9	11	42.3			
1	6	16.7	2	7.7			
2	3	8.3	9	34.6			
3+	4	11.1	4	15.4			
Smoking Status							
Smoker	17	47.2	19	73.1			
Non-smoker	19	52.8	7	26.9	0.44	0.12-1.58	0.207
Co-Morbidity							
No	17	47.2	5	19.2			
Yes	19	52.8	21	80.8	0.27	0.82-0.86	0.027
Additional Pathology							
No	30	83.8	14	53.8			
Yes	6	16.7	12	46.2	0.23	0.07-0.75	0.015
Reason for Surgery							
Other	1	2.8	3	11.5			
Pain	22	61.1	15	57.7	4.4	0.42-46.4	0.218
Infertility	10	27.8	6	23.1	4.5	0.25-80.6	0.307
Recurrent miscarriage	3	8.3	2	7.7	5.0	0.42-59.7	0.203
ASRM score							
I	8	22.2	-				
II	8	22.2	-				
III	11	30.6	-				
IV	9	25.0	-				

5.6.2 Total ROS Assay with Human Samples of Peritoneal Fluid, Urine and Serum

The total ROS assay protocol was applied to samples of serum, urine and peritoneal fluid collected from patients recruited to "XSESS" study. Two outlier results were identified, using a z score cut-off of +/- 2.5, and were removed from the data analysis. Data was normally distributed. Total reactive oxygen species (ROS) measured as an equivalent to xanthine/xanthine oxidase reaction in moles luciferase in serum specimens were higher in women with endometriosis (mean 2182.2, S.D. 2834.42, n=35), compared to women without endometriosis (mean 1657.42, S.D. 2511.74, n=24). ROS levels in urine in women with endometriosis were also higher compared to those without endometriosis (mean 1261.51, S.D. 1762.49, n=35 and mean 879.3, S.D. 1352.44, n=23 respectively). ROS in peritoneal fluid was higher in women with endometriosis (mean 2401.89, S.D. 2778.56, n=28) compared to women without (mean 1028.41, S.D. 1863.32, n=17) (Figure 24).

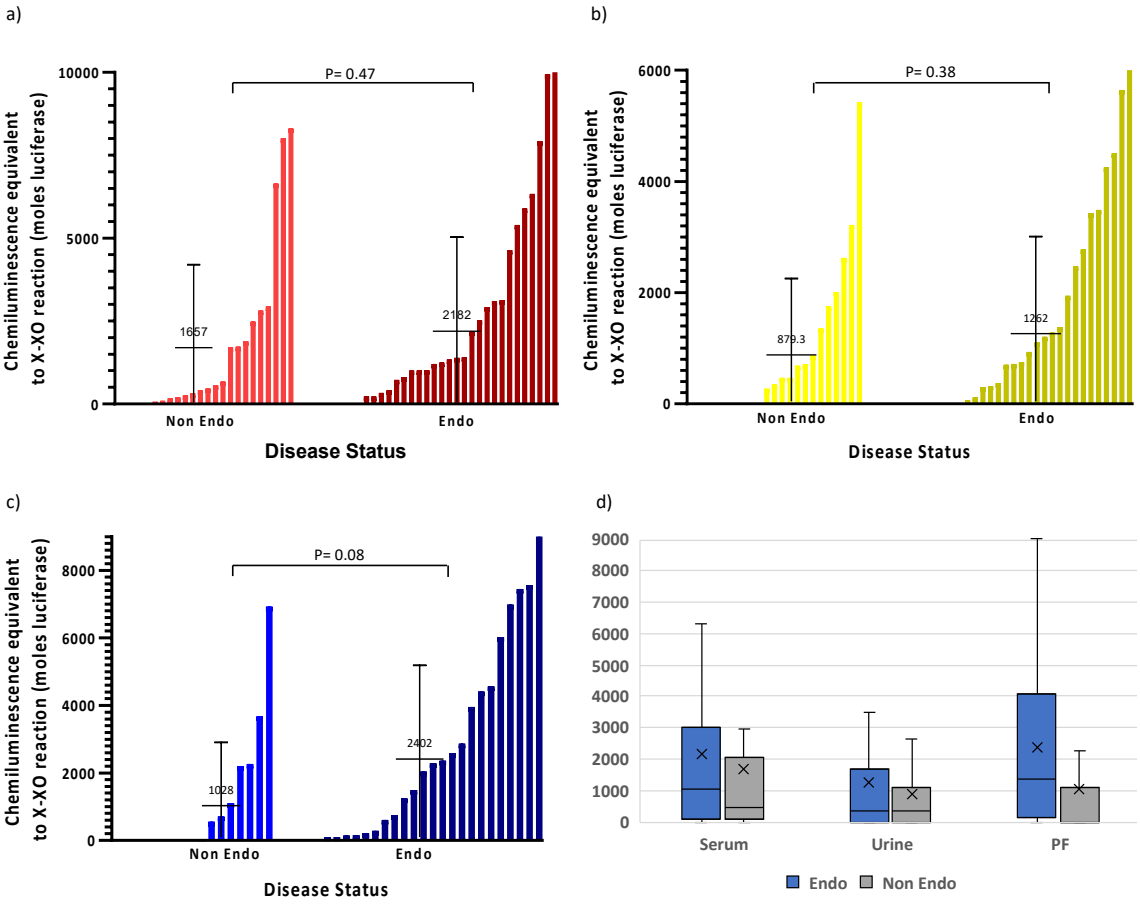


Figure 24: a) Bar chart representing total reactive oxygen species in the serum of women with endometriosis and women without endometriosis b) Bar chart representing total reactive oxygen species in the urine of women with endometriosis and women without endometriosis c) Bar chart representing total reactive oxygen species in the peritoneal fluid of women with endometriosis and women without endometriosis d) Box and whisker plot demonstrating the total reactive oxygen species in serum, urine and peritoneal fluid of women with and without endometriosis.

In sub-group analysis the subgroups were a combination of normally distributed and non-normally distributed data, therefore comparison analysis was performed with non-parametric tests adjusted for multiple comparisons. None of the observed relationships were statistically significant.

ROS levels, measured as an equivalent to xanthine/xanthine oxidase reaction in moles luciferase, were analysed by severity of disease (stage I n= 9, stage II n= 8, stage III n=11, stage IV n=9) compared to controls (n=24). There was no observed trend in ROS levels when examining samples from women with endometriosis by severity of the disease (Figure 25 a). Serum was similar across disease severity (stage I mean 2393.78, S.D. 3535.00, stage II mean 2176.5, S.D. 1579.33, stage III mean 3055.73, S.D. 3566.15 and stage IV 427.5, S.D. 398.48). Urine was no different across disease severity (stage I mean 838.33, S.D. 1492.00, stage II mean 2235.37, S.D. 2488.27, stage III mean 1358.09, S.D. 1706.81 and stage IV mean 405.9, S.D. 565.00). Peritoneal fluid ROS levels were also no different across disease severity (stage I 2709, S.D. 3515.23, stage II mean 1853.25, S.D. 1930.25, stage III 2258.86, S.D. 3291.53 and stage IV 2498.57, S.D. 2782.93). (Figure 25 a).

ROS levels, measured as an equivalent to xanthine/xanthine oxidase reaction in moles luciferase, were analysed by reason surgery was undertaken (pain n= 38, infertility n=14, recurrent miscarriage n= 4 and asymptomatic women undergoing sterilisation n= 3). Although ROS levels appeared to be higher in serum of women with recurrent miscarriage compared to those with pain, infertility or those who were asymptomatic, this difference was not significant. Similarly, although ROS levels appeared to be lowest across all fluid types in women who were asymptomatic compared to those undergoing surgery for pain, infertility or recurrent miscarriage, this was not significant. It was found that ROS levels were no different in serum, urine and peritoneal fluid of women undergoing surgery for pain (mean 1953.78, S.D. 2676.36, mean 1895.56, S.D. 4003.1 and mean 2957.75, S.D. 3901.45 respectively), infertility (mean 1535.54, S.D. 2855.98, mean 918.03, S.D. 1572.65 and mean 721.47, S.D. 1253.22 respectively), recurrent miscarriage (mean 2124.05, S.D. 1966.46, mean 665.12, S.D. 916.75 and mean 1890.48, S.D. 2809.88 respectively) and asymptomatic women (mean 350.79, S.D. 365.03, mean 155.56, S.D. 269.43 and mean 0.0, S.D. 0.0). (Figure 25 b).

Samples were analysed by pathology found at surgery (pure endometriosis n= 29, endometriosis and other pathology n= 9, no endometriosis but other pathology n= 16 and no pathology n= 8).

Other pelvic pathology included adhesions, fibroids and ovarian cysts. It seemed ROS levels, measured as an equivalent to xanthine/xanthine oxidase reaction in moles luciferase, may be higher in peritoneal fluid of women found to have only endometriosis and that ROS levels may be lowest in serum, urine and peritoneal fluid in women with no pathology, however this observed difference was not significant. ROS levels in serum, urine and peritoneal fluid were not different between groups of women with endometriosis (mean 1845.88, S.D. 2887.9, mean 1145.88, S.D. 1669.28 and mean 2497.38, S.D. 2828.63 respectively), endometriosis and additional pathology (mean 2538.41, S.D. 2487.9, mean 1213.76, S.D. 1998.72 and mean 1758.37, S.D. 2680.12 respectively), other pathology (mean 2121.85, S.D. 2910.66, mean 1082.47, S.D. 1535.28 and mean 1273.57, S.D. 2115.42 respectively) and a normal pelvis (mean 728.43, S.D. 1046.52, mean 3317.04, S.D. 8232.97 and mean 366.83, S.D. 898.53 respectively). (Figure 26 a).

ROS levels, measured as an equivalent to xanthine/xanthine oxidase reaction in moles luciferase, in serum and urine at the time of surgery were compared to levels at follow up (matched serum samples n= 42, matched urine samples n= 43). In both women with (n= 29) and without endometriosis (n= 14), ROS levels appeared to be higher at post-operative follow up than at the time of surgery but this was not significant (Figure 26 b). There was no difference in ROS levels of serum and urine in women with endometriosis at the time of surgery (mean 2301.45, S.D. 3065.09 and mean 985.33, S.D. 1537.61 respectively) compared to at follow up (mean 2904.55, S.D. 3084.94 and mean 1936.08, S.D. 2557.74 respectively). There was also no difference in ROS levels of serum and urine in women without endometriosis at surgery (mean 2136.45, S.D. 3083.59 and mean 2736.61, S.D. 6200.70 respectively) compared to at follow up (mean 2909.71, S.D. 3631.21 and mean 2147.28, S.D. 2745.77 respectively) (Figure 26 b).

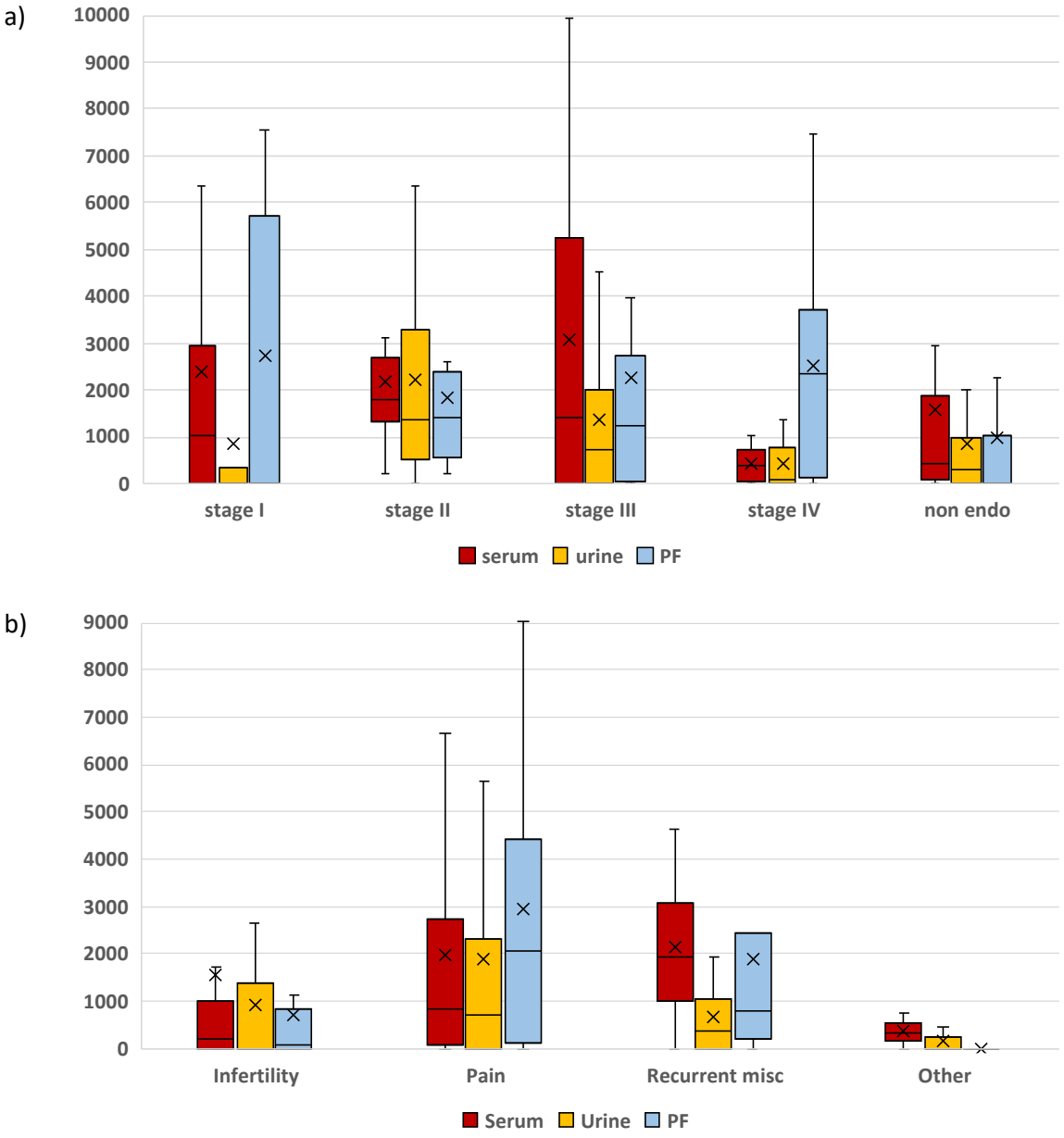


Figure 25: a) Box and whisker plot displaying the total reactive oxygen species levels in serum, urine and peritoneal fluid of women with stages I-IV endometriosis and women without endometriosis. b) Box and whisker plot of total reactive oxygen species in serum, urine and peritoneal fluid of women undergoing surgery for different symptoms.

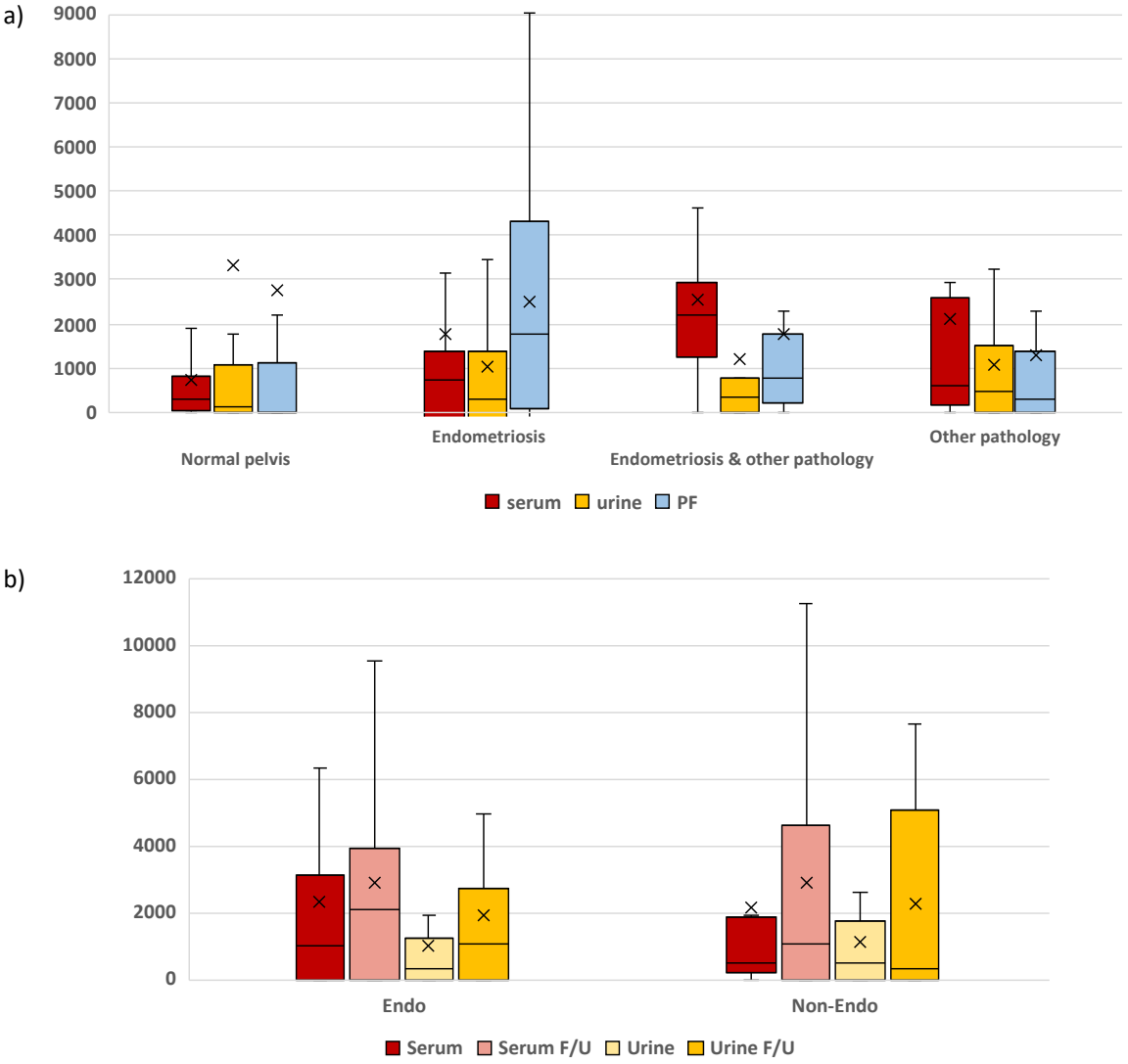


Figure 26: a) Box and whisker plot of total reactive oxygen species in serum, urine and peritoneal fluid of women with different pathology types at time of surgery. b) Box and whisker plot showing total reactive oxygen species in serum and urine obtained at surgery and at follow up of women with and without endometriosis.

5.6.3 Total Antioxidant Assay with Human Samples of Peritoneal Fluid, Urine and Serum

The TAC assay protocol was applied to all samples of women recruited to "XSESS" study. Two high outlier data points were determined with z-score in the endometriosis case group and removed from the dataset. Data was normally distributed. TAC levels in urine of 46% of endometriosis cases and 33% of controls produced absorbance levels lower than the limits of the trolox standard and leading to negative TAC levels after data analysis. The TAC assay may not be suitable for urine samples in this study and caution is taken in interpreting the results of urine samples.

On parametric comparison testing, total antioxidant capacity levels (Trolox equivalent in nmols) were no different in serum of women with and without endometriosis (mean 139.43, S.D. 98.56, n= 35 and mean 138.21, S.D. 104.33, n= 24 respectively) (p= 0.561). In urine TAC was higher in women without endometriosis (mean 102.08, S.D. 168.43, n= 24) compared to women with endometriosis (mean 54.15, S.D. 114, n=36) (p= 0.016) and in peritoneal fluid TAC was observed to be higher in women with endometriosis (mean 96.73, S.D. 89.06, n=24) compared to those without (mean 52.86, S.D. 50.27, n= 18) (p= 0.027) (Figure 27 a).

Samples were examined by ASRM stage of disease and compared to the control group (stage I n= 7, stage II n= 7, stage III n= 11, stage IV n= 9). The majority of results groups were normally distributed but some results groups in serum and peritoneal fluid were not normally distributed. Nonparametric comparison testing revealed no differences were found in TAC levels (Trolox equivalent in nmols) of serum in women with stage I endometriosis (mean 149.02, S.D. 207.46), stage II endometriosis (mean 215.33, S.D. 146.97), stage III endometriosis (mean 111.29, S.D. 67.58), stage IV endometriosis (mean 138.92, S.D. 84.53) and women without endometriosis (mean 138.21, S.D. 104.33). There was no difference in the TAC levels of urine in women with stage I endometriosis (mean 117.47, S.D. 181.27), stage II endometriosis (mean 87.63, S.D. 229.49), stage III endometriosis (mean 44.70, S.D. 78.34), stage IV endometriosis (mean 17.29, S.D. 47.39) and women without endometriosis (mean 102.08, S.D. 34.38). There was also no difference in TAC levels of peritoneal fluid in women with stage I endometriosis (mean 65.07, S.D. 77.58), stage II endometriosis (mean 138.28, S.D. 127.90), stage III endometriosis (mean 46.03, S.D. 31.88), stage IV endometriosis (mean 97.38, S.D. 17.66) and women without endometriosis (mean 52.86, S.D. 11.85). (Figure 27 b).

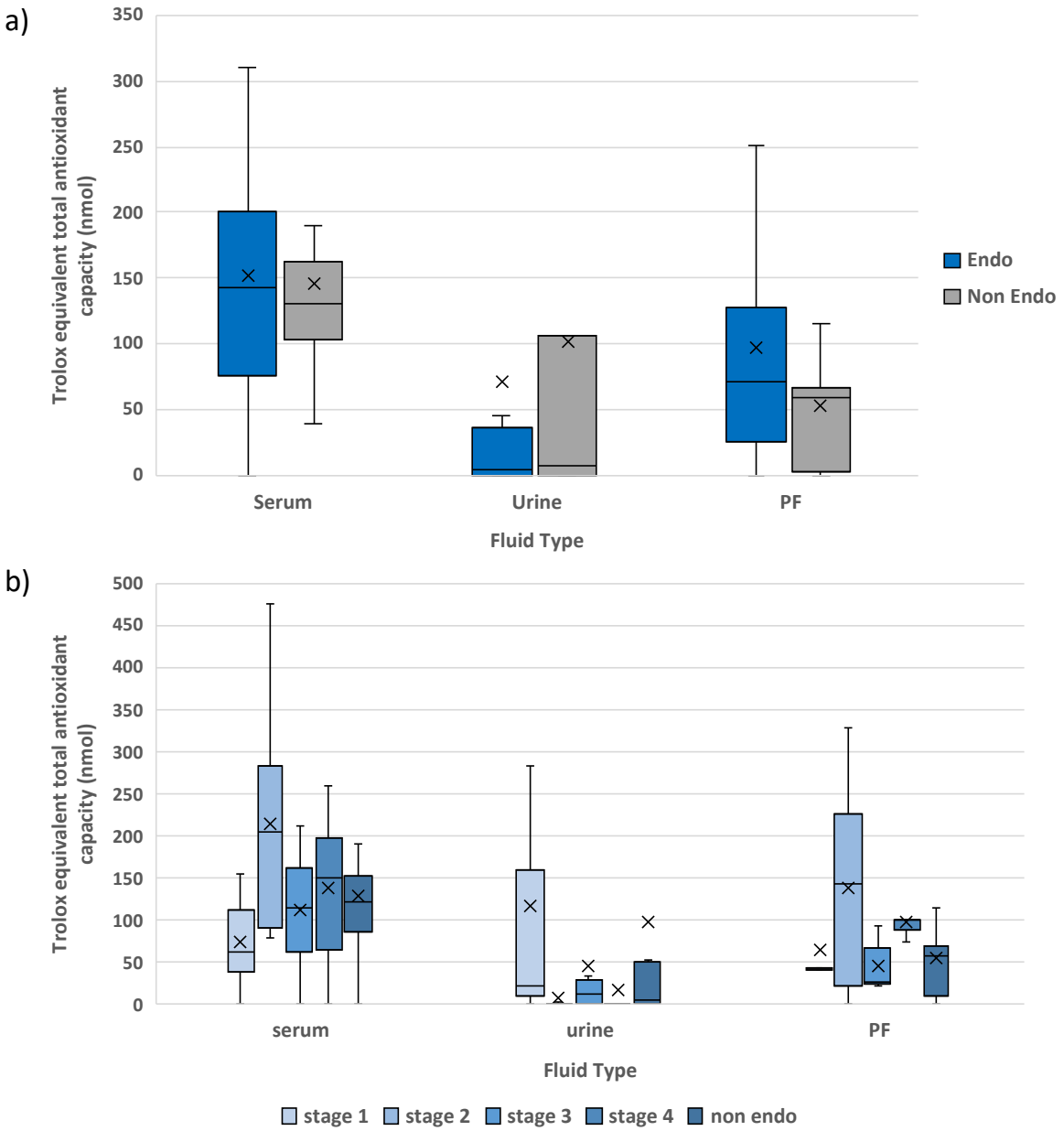


Figure 27: Findings of Total antioxidant capacity (TAC) as equivalent to Trolox standard in serum, urine and peritoneal fluid samples of women recruited to "XSESS" study. a) Box and whisker plot of TAC of samples from women with and without endometriosis. b) Box and Whisker plot of samples by severity of disease

In sub-group analysis the subgroups were a combination of normally distributed and non-normally distributed data, therefore comparison analysis was performed with non-parametric tests and adjusted for multiple comparisons.

All samples were analysed by reason for the woman's surgery. The levels of TAC (Trolox equivalent in nmols) in urine of women with infertility were demonstrated to be higher than asymptomatic women (mean 175.59, S.D. 170.34 and mean 2.41, S.D. 4.82 respectively p= 0.009) and women with pelvic pain (mean 55.62, S.D. 133.72, p= 0.002). TAC was no different in serum and peritoneal fluid of women with infertility (mean 163.16, S.D. 136.10, n= 14 and mean 79.32, S.D. 78.75, n= 8 respectively), recurrent miscarriage (mean 213.55, S.D. 83.24, n= 4 and mean 127.60, S.D. 120.69, n = 3 respectively), pain (mean 135.81, S.D. 112.13, n= 37 and mean 74.98, S.D. 77.39, n = 28 respectively) and asymptomatic women undergoing surgery for sterilisation (mean 122.77, S.D. 6.09, n= 3 and mean 47.80, S.D. 33.97, n =2 respectively). (Figure 28 a).

Subgroup analysis by pathology found at surgery was performed comparing TAC in women with no pelvic pathology (n= 7), endometriosis only (n= 28), endometriosis with other pelvic pathology (n= 9) and women with no endometriosis but presence of other pelvic pathology (n= 16). Other pelvic pathology included adhesions, fibroids and ovarian cysts. No differences were found between groups. TAC levels (Trolox equivalent in nmols) in serum of women with no pathology (mean 215.85, S.D. 151.92), only endometriosis (mean 119.31, S.D. 77.95), endometriosis with other pathology (mean 201.92, S.D. 140.75) and other pathology (mean 116.02, S.D. 49.61) were similar. TAC levels in urine of women with no pathology (mean 131.58, S.D. 227.85), only endometriosis (mean 57.63, S.D. 111.63), endometriosis with other pathology (mean 5.96, S.D. 9.18) and other pathology (mean 101.08, S.D. 150.37) were similar. TAC levels in peritoneal fluid of women with no pathology (mean 70.39, S.D. 66.50), only endometriosis (mean 65.25, S.D. 60.87), endometriosis with other pathology (mean 146.71, S.D. 107.91) and other pathology (mean 39.8, S.D. 37.29) were also not different. (Figure 28 b).

Serum and urine collected at the time of a woman's surgery was matched to the serum and urine collected at their post-operative follow up consultation (matched serum samples n= 40, matched urine samples n= 41). Samples were from women without endometriosis (n= 14) and in women with endometriosis (serum n= 26 and urine n= 27). There was no difference in TAC (Trolox equivalent in nmols) in the serum of women with and without endometriosis at the time of their

surgery when disease was present (mean 168.22, S.D. 107.88 and mean 167.79, S.D. 137.44 respectively) compared to post-operatively when disease had been treated (mean 137.42, S.D. 95.51 and mean 136.17, S.D. 121.90 respectively). There was also no difference in TAC in the urine of women with and without endometriosis at the time of surgery (mean 88.01, S.D. 137.40 and mean 132.82, S.D. 155.0 respectively) compared to post-operatively (mean 34.85, S.D. 69.31 and mean 67.11, S.D. 111.04 respectively). (Figure 28 c).

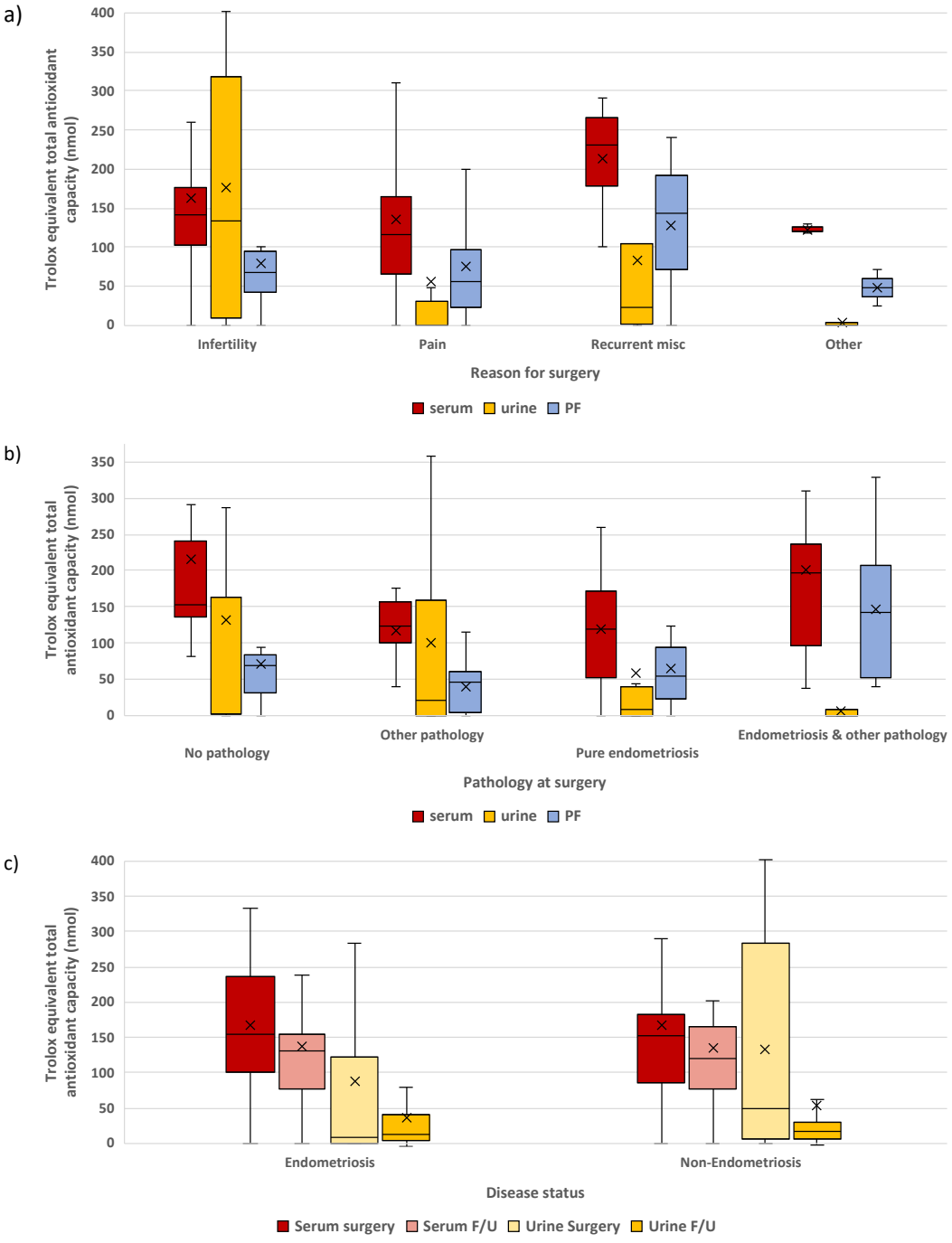


Figure 28: Findings of Total antioxidant capacity (TAC) as equivalent to Trolox standard in serum, urine and peritoneal fluid samples of women recruited to "XSESS" study. a) Box and whisker plot of TAC in samples of women undergoing surgery for different symptoms. b) Box and whisker plot of TAC in samples of women with different pathology types at time of surgery. c) Box and whisker plot showing TAC in samples obtained at surgery and at follow up of women with and without endometriosis.

5.6.4 Thiobarbituric Acid Reactive Substance Assay with Human Samples of Peritoneal Fluid, Urine and Serum

TBARS assay was applied to human samples collected in "XSESS" study. No outliers were found in the dataset. Some groups within the data were normally distributed and some were not normally distributed. Lipid peroxidation biomarker, malondialdehyde (MDA) levels were quantified by its reaction with TBARS in serum, urine and peritoneal fluid of women found to have endometriosis at surgery and compared to serum, urine and peritoneal fluid of women found not to have endometriosis at surgery with non-parametric comparison analysis. MDA levels (absorbance of TBARS-MDA adduct at 532nm) were no different in serum (mean 4.63, S.D. 4.44, n=37), urine (mean 5.37, S.D. 4.3, n=36) and peritoneal fluid (mean 5.00, S.D. 4.14, n=24) in women with endometriosis compared to the control group (mean 3.87, S.D. 3.33, n=24 in serum, mean 3.93, S.D. 3.22, n=24 in urine and mean 3.86, S.D. 3.56, n=18 in peritoneal fluid) (Figure 29 a).

Samples were analysed by severity of disease (stage I n= 9, stage II n= 9, stage III n= 11, stage IV n= 8). There was no difference in MDA levels (absorbance of TBARS-MDA adduct at 532nm) of serum in women with stage I endometriosis (mean 1.91, S.D. 3.35), stage II endometriosis (mean 6.57, S.D. 4.36), stage III endometriosis (mean 5.46, S.D. 4.83), stage IV endometriosis (mean 4.35, S.D. 4.22) and women without endometriosis (mean 3.87, S.D. 3.33). There was no difference in MDA levels of urine in women with stage I endometriosis (mean 3.68, S.D. 3.00), stage II endometriosis (mean 7.22, S.D. 4.69), stage III endometriosis (mean 5.22, S.D. 4.78), stage IV endometriosis (mean 5.42, S.D. 4.37) and women without endometriosis (mean 3.93, S.D. 3.22). There was also no difference in MDA levels of peritoneal fluid in women with stage I endometriosis (mean 4.42, S.D. 4.43), stage II endometriosis (mean 8.17, S.D. 4.22), stage III endometriosis (mean 2.95, S.D. 1.58), stage IV endometriosis (mean 3.61, S.D. 3.85) and women without endometriosis (mean 3.86, S.D. 3.56). (Figure 29 b).

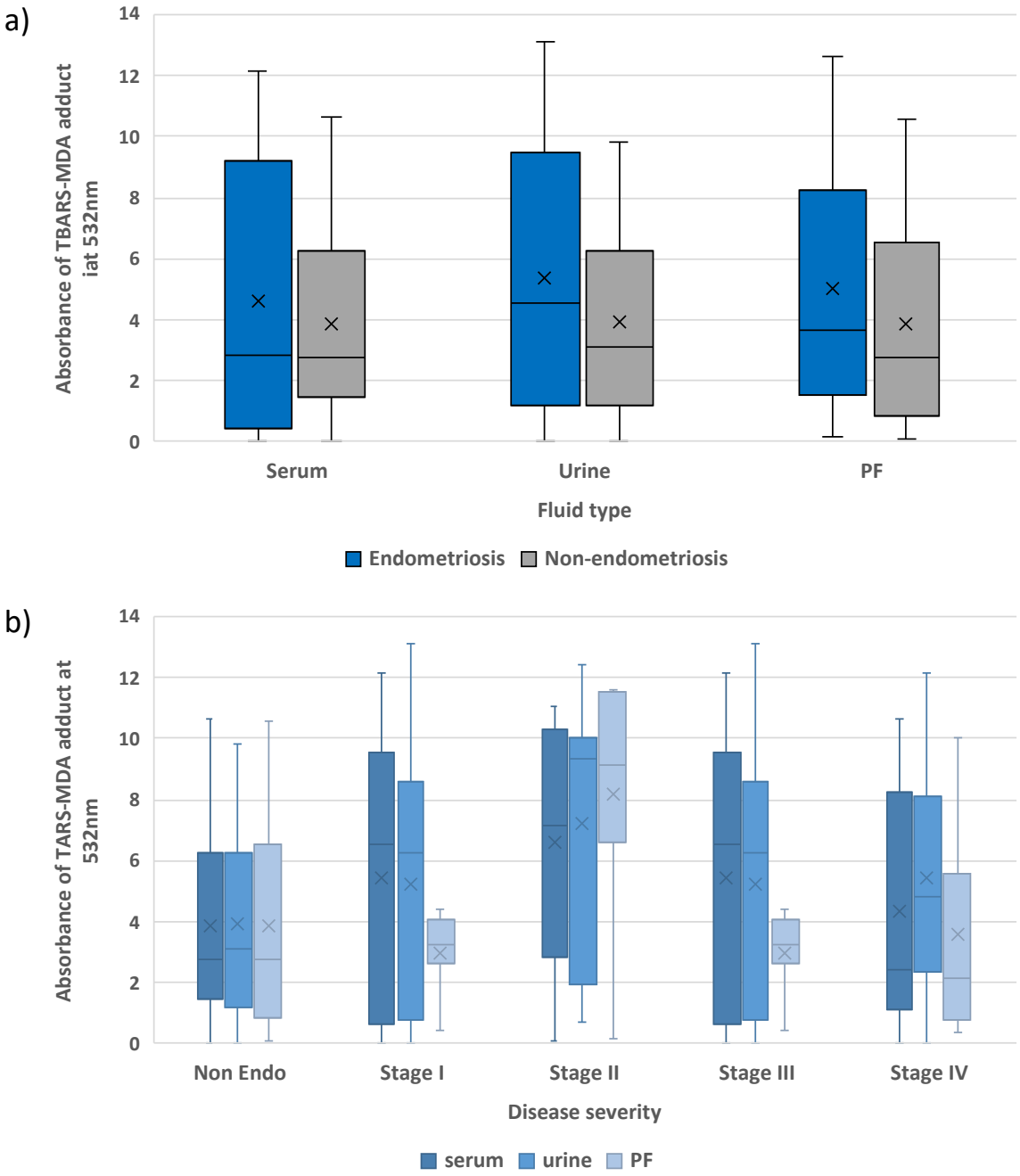


Figure 29: Findings of Malondialdehyde (MDA) levels by TBARS-MDA adduct absorbance levels in serum, urine and peritoneal fluid samples of women recruited to "XSESS" study. a) Box and whisker plot of MDA levels in samples of women with and without endometriosis. b) Box and Whisker plot of MDA levels in samples by severity of disease

Sub-group analysis was also performed to compare MDA levels (absorbance of TBARS-MDA adduct at 532nm) according to whether surgery was performed for infertility, recurrent miscarriage, pelvic pain or in asymptomatic women undergoing sterilisation. There were no differences in MDA levels of serum in women with infertility (mean 4.89, S.D. 4.00, n= 13), recurrent miscarriage (mean 7.81, S.D. 4.63, n= 5), pain (mean 3.61, S.D. 3.86, n= 38) and asymptomatic women (mean 4.83, S.D. 3.78, n= 5). There were no differences in MDA levels of urine in women with infertility (mean 4.93, S.D. 3.69, n= 13), recurrent miscarriage (mean 9.32, S.D. 5.37, n= 5), pain (mean 4.29, S.D. 3.55, n= 37) and asymptomatic women (mean 3.70, S.D. 4.03, n= 5). There were no differences in MDA levels of peritoneal fluid in women with infertility (mean 4.27, S.D. 4.74, n= 8), recurrent miscarriage (mean 11.00, S.D. 0.93, n= 3), pain (mean 3.71, S.D. 3.21, n= 28) and asymptomatic women (mean 6.19, S.D. 4.05, n= 3). (Figure 30 a). Although not significant, MDA levels appeared to be higher in all fluids in women suffering recurrent miscarriage.

In analysis according to type of pathology seen at surgery, MDA levels (absorbance of TBARS-MDA adduct at 532nm) were no different in serum, urine and peritoneal fluid of women with endometriosis (mean 4.25, S.D. 4.37, mean 4.83, S.D. 4.10 and mean 4.53, S.D. 3.89 respectively n= 28), endometriosis with additional pathology (mean 5.79, S.D. 4.72, mean 7.01, S.D. 4.71 and mean 6.42, S.D. 4.91 respectively, n= 9), no endometriosis but other pelvic pathology (mean 3.79, S.D. 3.17, mean 3.69, S.D. 3.12 and mean 3.3, S.D. 3.55 respectively, n= 15) and those with a normal pelvis (mean 4.00, S.D. 3.79, mean 4.34, S.D. 3.54 and mean 4.58, S.D. 3.69 respectively, n= 9). (Figure 30 b). Although not significant, MDA levels appeared to be higher in all fluids of women with endometriosis and additional pathology compared to the other pathology groups.

Analysis of MDA levels (absorbance of TBARS-MDA adduct at 532nm) in matched samples of serum and urine in women at the time of their surgery compared to at their follow up consultation was performed. MDA levels were no different at surgery (serum mean 4.99, S.D. 4.7, n= 27 and urine mean 5.68, S.D. 4.52, n= 26) compared to follow up (serum mean 5.91, S.D. 3.74 and urine mean 6.10, S.D. 4.43) in women with endometriosis and no different at surgery (serum mean 3.41, S.D. 2.97, n= 14 and urine mean 3.46,S.D. 2.83, n= 14) compared to follow up (serum 4.35, S.D. 3.92 and urine mean 4.22,S.D. 3.19) in women without endometriosis. (*Figure 30* c). Although the differences were not significant, MDA levels appeared to be higher at follow up in women with endometriosis compared to at surgery and lower at follow up in women without endometriosis compared to at surgery.

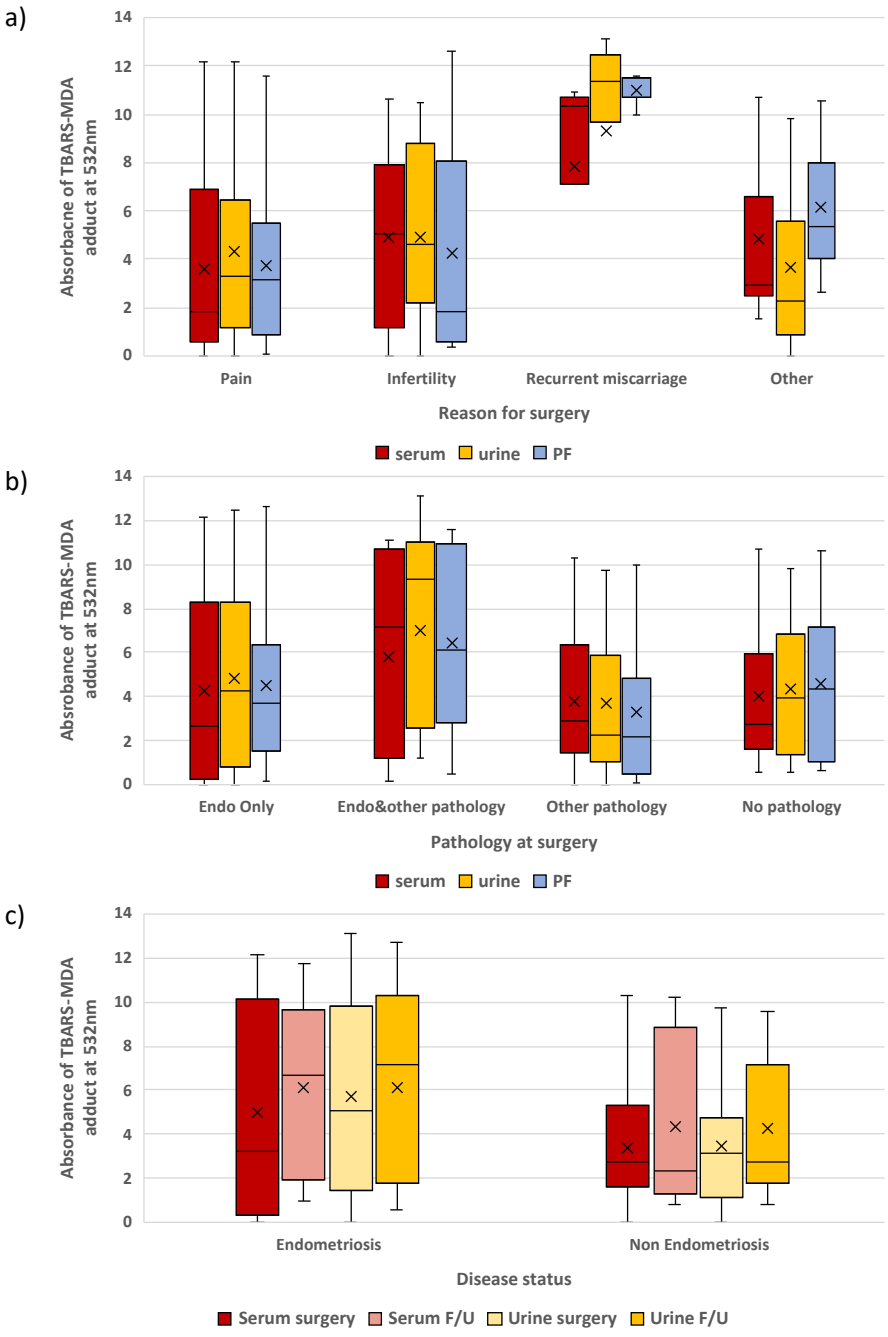


Figure 30: Findings of Malondialdehyde (MDA) levels by TBARS-MDA adduct absorbance levels in serum, urine and peritoneal fluid samples of women recruited to "XSESS" study. a) Box and whisker plot of MDA levels in samples of women undergoing surgery for different symptoms. b) Box and whisker plot of MDA levels in samples of women with different pathology types at time of surgery. c) Box and whisker plot showing MDA levels in samples obtained at surgery and at follow up of women with and without endometriosis.

5.7 Discussion

Although few analyses in this study yielded significant results, several interesting trends were observed in the data from assay work in this chapter. Reactive oxygen species (ROS) appeared to be higher in serum, urine and peritoneal fluid of women with endometriosis compared to those without endometriosis. This is in agreement with the association between ROS levels found in other inflammatory chronic health conditions [256–258]. ROS did not appear to be associated with severity of the disease, but subgroup analysis numbers were small which may have hindered a pattern of results being revealed. Women with endometriosis and endometriosis with concomitant pelvic pathology (e.g., fibroids, ovarian cyst, adhesions etc) displayed higher levels of ROS compared to women with a normal pelvis or pelvic pathology excluding endometriosis. This suggests that amount of pelvic disease present may correlate with ROS, and that pelvic conditions other than endometriosis may give rise to oxidative stress but to a lesser degree. ROS was consistently lowest in women who were asymptomatic and undergoing surgery for sterilisation. ROS was higher in women undergoing surgery for symptoms of pain, infertility or recurrent miscarriage compared to asymptomatic women. In serum, ROS was highest in women having surgery for recurrent miscarriage and in peritoneal fluid and urine, it was highest in women with pelvic pain. This interestingly may demonstrate a relationship between physiological symptoms of the disease rather than its objective severity. Or it may reflect that subgroup analysis numbers are too low to demonstrate a clear relationship. In both women with and without endometriosis,

ROS levels were higher at post-operative follow up than at the time of surgery. It was hypothesised that surgical treatment of endometriosis would reduce ROS levels. As the levels followed the same pattern in both cases and controls, it is possible that this reflects the inflammatory process of healing and recovery as both groups underwent surgery. Within the case group a number of women may have commenced a hormonal treatment following surgery to further treat/prevent disease. Oxidative stress may be increased by some hormonal contraceptives [259] however this may not be true of the hormonal intrauterine device which is commonly used to manage to endometriosis [260]. One study has found that hormonal contraceptive increased the antioxidant defence in women with endometriosis compared to controls, bringing their oxidative stress balance to a level similar to controls [261]. This may have affected post-operative levels of ROS in this study and caused ROS levels post-operatively to be

similar to women without endometriosis. This information was collected in the pain questionnaire of this study and qualitative analysis of this data may clarify this. The increase in ROS levels may also be due to the reduced time taken to collect samples from the patient and a level of ROS being more reflective of the "in-vivo state". At surgery blood is collected by the anaesthetist on siting a cannula before being processed, similarly urine is collected after a catheterisation process rather than by simple micturition. Peritoneal fluid takes the longest to obtain through its method of extraction. ROS may be more volatile and easily affected by its environment than some of the markers of ROS such as lipid peroxidation and total antioxidant capacity which are downstream factors and may take longer to respond to the reduction/oxidation status. This observation does however call into question the usefulness of ROS as a potential biomarker of endometriosis as the levels may be impacted by other physiological processes, potential use of hormones post-operatively as an adjunct to surgical treatment and sample collection and processing methodology.

Total antioxidant capacity (TAC) levels were similar in serum of women with and without endometriosis, lower in urine of women with endometriosis and higher in peritoneal fluid of women with endometriosis compared to women without endometriosis. TAC levels were higher in the serum, urine and peritoneal fluid of women having surgery for infertility and recurrent miscarriage compared to women with pain or those requesting sterilisation. TAC in serum and peritoneal fluid were highest in women found to have endometriosis and additional pelvic pathology, in urine TAC was higher in women with pelvic pathology excluding endometriosis. TAC was found to be higher in serum in women at the time of surgery compared to post-operative follow-up in both case and control groups. A lot of these findings reflect that TAC levels may be higher in women with pelvic disease and symptoms of that disease.

An observation in these results is that the fluid closely related to the disease has higher levels of TAC where a reduction would have been expected, and in systemic fluids the TAC levels were similar or reduced. The literature discussing conditions of high oxidative stress generally observe a reduction in antioxidant capacity. It is noted that studies around oxidative stress and antioxidant capacity balance in other diseases test systemic fluids such as serum. This work has measured TAC in the fluid immediately in contact with the disease process which is slightly unique, and the data may be reflecting a compensatory mechanism to increase TAC locally and diminish TAC systemically. Another consideration would be that ROS in the immediate proximity to the disease

may be created more continuously leading to the response of a compensatory mechanism (antioxidant activity) being in a more continuous turnover, whereas systemically the ROS to antioxidant relationship observed may be more stable and have more permanence. Similar studies with testing of "local" fluid for comparison is lacking. This observation that systemic compared to local fluids may differ in ROS markers needs further investigation if considering a peripheral biomarker for endometriosis. It questions whether a peripheral biomarker would reflect ROS levels in the pelvis. What also may need to be considered when observing TAC in urine is that urine is a filtrate of the body and continuously differs in concentration of solute and water content. Although the least invasive biospecimen to collect from a patient, ROS markers within it may be more difficult to interpret. What also needs to be examined is the component parts of TAC i.e., antioxidant enzymes, chain breaking antioxidants and transition metal binding proteins [257]. This assay encompasses all small molecule antioxidants (mainly vitamins), but some subtypes may be diminished while others increased leading to the overall observation. A systematic review on the impact of anti-oxidants on fertility has concluded that both ROS and antioxidants contribute to healthy oocyte maturation and ovulation and that some antioxidants in too high levels in the reproductive fluids act as pro-oxidants and can contribute to infertility [262]. A greater understanding of the results needs to be sought.

MDA levels were higher in serum, urine and peritoneal fluid in women with endometriosis compared to women without endometriosis reflecting an increased level of lipid peroxidation as a result of higher reactive oxidative species. The higher levels of MDA in women with endometriosis did not show an association with disease severity, although mean MDA levels appeared to be higher in stage II endometriosis compared to other stages and mean levels in stage I, III and IV were all very similar. This may indicate that ROS while affected by the disease process may not differ by "amount" of disease present. However, small sample sizes in this subgroup analysis warrants caution in interpreting any observed trends. MDA levels were highest in women having surgery for recurrent miscarriage compared to other symptoms and highest where surgery revealed endometriosis together with other pelvic pathology. Signs of oxidative stress being higher in women with recurrent miscarriage and associated with endometriosis in addition to other pelvic pathology have been consistent findings across the assays. Oxidative stress may have more of an impact on miscarriage rate than fertility or the true relationship may be impeded by low subgroup analysis numbers. The pathology status of the woman does seem to reflect the amount of oxidative stress present in the pelvic environment and systemically. MDA levels were

higher at post-operative follow up in women with endometriosis compared to levels at the time of surgery and lower at follow up in women without endometriosis. This possibly reflects the fact that women without endometriosis mostly underwent a diagnostic laparoscopy compared to an operative laparoscopy and the healing and recovery process would be less significant.

Results of significance were those within TAC assay work. In urine TAC was significantly higher in women without endometriosis than women with endometriosis and in peritoneal fluid TAC was significantly higher in women with endometriosis compared to those without. The levels of TAC were significantly higher in women with infertility compared to women with pelvic pain. Given the findings of some significant differences between endometriosis and non-endometriosis groups and in some sub-group analysis work, sample size is possibly almost reaching the required level to observe true differences for TAC assay. It also highlights that sample size may be inadequate for total ROS and TBARS assays. Unfortunately, sample size calculations were based on a very limited amount of published evidence in women with endometriosis and a lack of studies utilising the exact assays this study has used, it is unsurprising therefore that the results are underpowered. This work would give useful data for re-calculating a more appropriate sample size in future work.

This study was powered for detecting a difference in ROS pre and post-surgical treatment. Therefore, subgroup analyses, while interesting must be viewed with caution. A further limitation of subgroup analysis examining the reason a woman was having surgery, was that women undergoing surgery for infertility and recurrent miscarriage would also have concomitant dysmenorrhoea (painful periods), hence warranting the investigative laparoscopy. These women were presenting to the relevant specialist clinic with either recurrent miscarriage or infertility as their primary symptom and pain would only have been elicited on direct questioning, it was therefore considered for the purpose of this study that their pain was not of a significant level. Women undergoing surgery specifically for pain were listed for surgery from the pelvic pain clinic where a suspicion of endometriosis would be significantly higher. As the aim of this thesis was to investigate endometriosis from a point of view of reproductive impact, it was decided to include women with recurrent miscarriage and infertility undergoing investigation for endometriosis and examine them as a subgroup. Results from this particular subgroup analysis would however need to be interpreted carefully in view of some symptom crossover. A much larger sample size would be desirable for all subgroup analyses.

Results from the total ROS, total antioxidant capacity and TBARS assays indicate that these techniques, once refined, may be appropriate for investigating ROS levels in the human samples of serum, urine and peritoneal fluid. When TAC assay is applied to urine samples in the patient group, the high number of invalidated results (those falling below zero after analysis as an equivalent to Trolox) raises an issue for analysis and interpretation of results. One reason may be that TAC levels in urine vary more than in serum and peritoneal fluid. Solute and water concentration can physiologically vary greatly compared to serum and peritoneal fluid. It may be extrapolated that the same is true of the level of small molecule antioxidants within urine. As human samples were all diluted in the initial protocol (designed on a much smaller scale of sample numbers), it may be that the assay should be applied to 100% concentration of urine. The risk would then be that some TAC levels extend beyond the absorbance of the standard curve. A Trolox standard curve would need to also be extended to higher concentrations in this case. Time constraints did not allow for protocol development in this area. An accepted level of negative results was returned in the ROS and TBARS assay work, but the same protocol development should be applied to these assays to allow for higher concentrations of human samples to be used alongside standard curves with increased range. This would reduce negative results. Glutathione assay results, suggest it may not be suitable for work with biospecimens in this patient group most likely due to an ongoing oxidation reaction within the thawed samples during the assay preparation time or a range of data produced by human samples extending too far outside the range of the standard curve.

Within all assay results a large spread of data was noted with a number of negative results due to dilution of samples in the methodology, up to high outliers. To address this, a more stringent Z-score could be applied. The significant spread of data may reflect the complex nature of reactive oxygen species in vivo, as an extremely high number of factors play a role in the balance of reactive oxygen species within the body [263–265]. The results of the assay in this work are applied to just one main disease process of endometriosis but the variability in results may be due to age, smoking status, BMI, diet, other disease processes, the physiological process of surgery and recovery, the collection and storage process, thawing and preparing samples for assay work…this list is not exhaustive. ROS is too dynamic and multifactorial to apply to a simplistic protocol and analysis. Therefore, inferences will be drawn from the results but for any more specific data, the assays would require further protocol refinement, a much larger sample size and a complex multivariate analysis adjusting for total and direct confounders and mediators.

5.7.1 Further work

Data collected with a pain questionnaire pre and post-surgery could be used in a qualitative analysis and interpreted in conjunction with ROS levels to see if there is a correlation with severity of symptoms before and after treatment. This study has not revealed an association between ROS levels and disease severity but observed a possible relationship between ROS levels and symptomatology of the disease. It is possible that ROS may be linked to severity of symptoms.

There is scope to further refine the current protocols for luminol, TAC and TBARS assay. By extending the range of standard concentrations used for calibration of assay results, human sample dilution may not be required, and negative results reduced. An extended data analysis to adjust for confounders and mediators including BMI, smoking status, age, diet, comorbidities, use of hormones/other medication etc would also be warranted and subgroup analysis would benefit from a much greater sample size. There is a broad range of techniques and assays for measuring ROS, this work has attempted to cover a number of methods of determining ROS levels and three out of four have been applicable to the human samples but ideally more assays/techniques should be utilised to increase the reliability of results.

This work examined processed, cell-free samples to limit ongoing oxidative reaction and more accurately reflect the ROS levels in vivo. However, a comparison with analysing more physiological samples reflecting their full cell component would be interesting.

In this body of work, the assay preparation time was kept consistent. Assays were all prepared under the same conditions and a balance was struck between keeping assay preparation time low to reduce influences on the ROS status of the samples and the need for increased use of consumables and time required to perform greater numbers of assays of smaller sample groups. This allows comparisons to be made between samples and groups in this work but stringent absolute levels of ROS and its markers in women with endometriosis and women without endometriosis cannot be drawn. Performing the assays with smaller sample groups to reduce assay preparation time should also be explored if more accurate absolute levels of ROS and its markers for the condition of endometriosis are desired, for example if ROS assays were to be used as biomarkers of disease. The diversity of data in this work currently does not indicate a ROS assay would be a suitable non-invasive biomarker of disease.

Extending this work to include more types of reproductive and systemic fluids would increase knowledge in this area. Another non-invasive biospecimen is saliva. This has benefits in its ease of collection but was not considered for this work due to its physiological distance to site of disease of interest. The reproductive fluid examined in these experiments has been peritoneal fluid which is an incredibly important fluid for giving information about ROS that an oocyte would be exposed to at ovulation and fertilisation. Another highly valuable fluid to examine would be follicular fluid due to its significance to the health of the quiescent and maturing oocyte pre-ovulation, and tubular fluid in which the early embryo develops.

Chapter 6 The Impact of Endometriosis on Mouse Oocyte RNA Damage

6.1 Introduction

Peritoneal fluid is an important reproductive fluid as it is in continuous contact with the reproductive organs in the pelvis and the oocyte is exposed to this fluid following ovulation. Peritoneal fluid, together with follicular fluid, also constitutes a high proportion of the fluid in the fallopian tube where oocyte transportation takes place as well as fertilisation and early embryogenesis. In the disease of endometriosis, peritoneal fluid is in direct contact with endometriotic lesions unlike other reproductive fluids (follicular fluid, fallopian tube fluid and uterine fluid). Peritoneal fluid has been collected in human studies for use in this body of work due to its direct contact with endometriotic lesions and its relative ease of collection in experimentally appropriate volumes of fluid compared to other reproductive fluids which can be more technically challenging to collect or are in microlitre volumes. The samples have been collected during the course of endometriosis surgery when severity and confirmed active disease can be documented.

As shown in current literature and investigated in Chapter 5, ROS may be higher in women with endometriosis throughout the reproductive fluid, including peritoneal fluid, and systemically compared to women without the disease[4]. It has not been demonstrated as to whether elevated ROS impacts on the oocyte intracellularly. It is, however, known that oocyte exposure to follicular fluid with higher ROS levels can damage DNA [60], but this has not been explored with peritoneal fluid. It stands to reason that ROS in these fluids could damage the RNA too. Fully grown GV oocytes are interesting in that they are transcriptionally quiescent, meaning that they rely solely on transcription of stockpiled mRNAs in their large cytoplasms for synthesis of new proteins. The period of quiescence lasts until zygotic genome activation. The oocyte at the GV stage through to early embryogenesis may therefore be particularly sensitive to RNA damage, as it has no recourse to replace the damaged RNA, and this will in turn effect the quality of the protein pool and consequently the function of the cell(s). There is an ethical barrier to using human GV oocytes in potential research into RNA damage. GV oocytes collected from women for the process of IVF will all be used for their treatment, GV human oocytes from in-vitro maturation cultures (using the woman's immature oocytes) are not competent at fertilisation which is why this process is not

undertaken in IVF. This indicates an inadequate/abnormal maturation process occurs in-vitro for human oocytes making them difficult to use for controlled RNA damage investigations. It would be difficult to acquire human GV oocytes and therefore a compromise of using mouse oocytes is commonly made for work in this area and will be adopted in the experimental work in this Chapter.

What is currently unknown is whether elevated levels of ROS in peritoneal fluid can directly influence the intracellular redox status of the oocyte. It is also unknown whether, similarly to the impact of higher ROS in follicular fluid on oocyte DNA, ROS in peritoneal fluid can damage the far more vulnerable oocyte RNA. To explore this, mouse oocytes will be exposed to the peritoneal fluid of women with and without endometriosis. Based on significant findings, the research may warrant future replication with human oocytes in a well-designed and ethically stringent study with low n numbers.

6.2 Objective

This chapter will address aim three of the thesis; To explore how reactive oxygen species affect the oocyte using a reproductive fluid of women affected by endometriosis from an ethically approved human study and oocytes in a mouse model. Firstly, it will be investigated whether the ROS levels affect the redox status within the mouse oocyte to demonstrate a direct link of ROS to a potential impact on the oocyte intracellularly. A number of techniques will then be employed to investigate if RNA damage occurs within the mouse oocyte (as a substitute for human oocytes) following exposure to peritoneal fluid (an important reproductive fluid) of women with endometriosis compared to non-endometriosis control fluid.

6.3 Hypothesis

High levels of ROS in peritoneal fluid of women with endometriosis can alter the redox status within oocytes. As a consequence, increased levels of RNA damage occur within the mouse oocyte when exposed to peritoneal fluid of women with endometriosis compared to women without endometriosis.

6.4 Methods

Sample collection

Peritoneal fluid obtained through the ethically approved studies "Peritoneal fluid biology in health and disease" and "XSESS: oXidative Stress in women with Endometriosis pre and poSt Surgery" was used to expose mouse oocytes to a reproductive fluid of women with endometriosis and without endometriosis. The case and control cohorts for both studies were from a population of women with known or suspected endometriosis undergoing investigation and treatment for the disease at the gynaecology department of Princess Anne Hospital, Southampton. "XSESS" study also included women undergoing tubal sterilisation at Princess Anne Hospital. Menopausal women, those with known active infective diseases and pregnancy were exclusion criteria. Peritoneal fluid was collected at the time of surgery following general anaesthetic and insertion of laparoscopic ports. Either a Wallace Assisted Reproduction Embryo Replacement Catheter or a 14 French gauge Ryles tube with a cut tip were placed into a pool of peritoneal fluid in the pelvis and extracted using a 20 ml syringe. Samples were transferred into reaction tubes and centrifuged in MyFuge5 at 2000 xg for 3 minutes. The supernatant was aliquoted into six 100 µl samples in 0.5 ml cryogenic vials, any remaining supernatant was aliquoted into 2 ml cryogenic vial and placed on dry ice before being stored at -80^{o}C.

Mouse oocyte harvesting

To investigate whether ROS levels in reproductive fluids (in this case, peritoneal fluid) can alter the redox status of an exposed oocyte, a mouse model was applied. Use of mouse oocytes met approval of the regional and University of Southampton ethics committees. All animal work has been carried out in adherence to the Animals (Scientific Procedures) Act of 1986 under project and personal licences granted by the Home Office. Three/four-week-old C57BL6 mice were injected via intra-peritoneal route with 10 IU pregnant mare serum gonadotrophin and culled ~48 hours post injection by cervical dislocation. Ovaries were harvested and placed in M2 media containing milrinone under mineral oil heated to 37^{o}C. Follicles were disrupted with a 27-gauge needle on a syringe under a microscope on a 37^{o}C heated mounting stage and fully grown GV arrested oocytes were collected from the dish using glass mouth pipettes. The pipette was used to manually remove the cumulus cells from the GV oocytes by 2-3 brief movements of the oocyte

into the pipette tip. Exposure to the light of the microscope was kept to a minimum and the room was dark to reduce damage to the oocytes.

Gibson cloning protocol

Green Fluorescent Protein (GFP) has been adapted into an intracellular redox biosensor by introducing two exposed cysteines which can be oxidised or reduced. The roGFP2 protein fluorescence is one such adapted GFP which preferentially interacts with gutaredoxins and detects the intracellular glutathione redox status. It is excited at both 405 nm, and 488 nm with a single emission peak at 510 nm. Oxidation causes an increase of the 405 nm excitation peak and reduction causes an increase in the 488 nm excitation peak. Therefore, in this chapter, experiments measuring the ratio of fluorescence in the reduced roGFP2 channel and the oxidised roGFP channel aimed to show the relative reduction-oxidation state of the intracellular environment.

roGFP2 RNA construct was made by Dr Simon Lane and used in protocol development work. A roGFP2 construct with the addition of glutaredoxin-1 (Grx1) was then developed to extend the range of redox potential to -320 mV (from the usual range of redox potential of roGFP2 -280 mV to -290 mV), as known physiological glutathione redox potentials of <-310 mV occur in vivo [266]. Grx-1-roGFP2 RNA construct was made using Gibson cloning protocol. A suitable plasmid in silico and suitable primers to span the intersections of inserts within the plasmid were designed by Dr Simon Lane for the Gibson cloning protocol. Remaining protocol then carried out. DNA of Grx1-roGFP2 inserts were generated and purified using a PCR reaction, the pRN3 plasmid backbone was then combined with the Gibson reaction. Gel electrophoresis was used to confirm that the backbone and inserts had been combined. The plasmid with Grx1-roGFP2 inserts was then taken up by E-Coli by transformation and grown on agar plate. Colonies of E-Coli were tested for the presence of the plasmid by in-vitro transcription and PCR reaction and products were tested with gel electrophoresis. The tested E-Coli colonies were then used and DNA for Grx1-roGFP2 extracted by PCR, cleaned and sent for DNA testing at Eurofins Genomics to ensure that sequences for Grx1-roGFP2 were generated. In vitro transcription and recovery of Grx1-roGFP2 RNA construct was then performed to use in redox experiments and glycerol stock was made of successful E-Coli colonies for future work. A 0.1-0.3% volume of 500 ng/µL concentration of Grx1-roGFP2 RNA construct and H2B-mCherry was microinjected by Dr Simon Lane and Tianyu Wu in a

37°C heated chamber on the stage of an inverted TE300 microscope with micromanipulators using a timed pulse on a Pneumatic Picopump. Redox status was then measured by sequential imaging of the microinjected oocytes in milrinone supplemented M2 media at 37°C on a confocal microscope using the two excitation wavelengths of roGFP in its oxidised (405 nm) and reduced (488 nm) states and an emission wavelength of 530 nm.

RNA extraction from tissue and oocytes

To detect RNA damage two methodologies were applied, real time quantitative PCR and immunofluorescence. For qPCR protocol development, RNA was extracted from murine oocytes and control murine tissue by either homogenising in trizol followed by separation of protein, organic and aqueous phase with chloroform and concentrating and de-salting nucleic acid with ethanol. With this method, the RNA was then re-suspended in RNase free water and DNase used to remove any remaining DNA. The other technique used to improve RNA quality was Qiagen RNeasy Plus micro kit with genomic DNA eliminator spin columns, ethanol wash to concentrate the RNA and resuspension of RNA in RNase free water.

For experimental work RNA was also extracted from mouse oocytes by either dissolving the zona pellucida in acid tyrodes, followed by freeze/thaw/spin cycles in a lysis buffer of DTT with Triton X or with Qiagen RNeasy Plus Micro Kit. RNA quality was tested by gel electrophoresis on a formaldehyde agarose gel. A 1% agarose gel was made with 1 g agarose dissolved in 100 mls of Tris-borate-EDTA and 14 µL of gel red was added. The gel in its casting tray was placed in the centre of the electrophoresis unit with wells closest to the negative electrode. TBE was poured into the upper and lower buffer chambers ensuring the gel was covered. Samples were prepared on parafilm with 2 µL of RNA or 2 µL of 100bp ladder with 2 µL of blue-orange loading dye, 2 µL of deionised formaldehyde and 6 µL ddH_2O. Prepared ladder and RNA sample was pipetted into two wells of the gel. Electrophoresis was run at 100 V for 60 minutes. When complete, the gel was analysed in a UV imager. RNA quality was also tested with NanoDrop ND-1000 spectrophotometer.

PCR and qPCR

Reverse transcription was then performed taking 5-7 µL of RNA sample, adding 2 µL of 'oligo dT' primers, heating to 70°C for 5 minutes in a PCR machine and cooling on ice for 5 minutes. Then, 5x

M-MLV reverse transcription buffer, 10 mM nucleotide mix, ribonuclease inhibitor, M-MLV reverse transcriptase and nuclease free water were added. RT-PCR protocol of 20^{o}C for 10 minutes, 37^{o}C incubation for 50 minutes, 70^{o}C for 15 minutes and cooling to 12^{o}C was followed and resulting in cDNA for qPCR experiments.

PCR technique was adopted to investigate if RNA in the oocyte is damaged by exposure to peritoneal fluid of women with endometriosis. As the oocyte is transcriptionally quiescent the damaged RNA cannot be replaced and could be detected using qPCR following reverse transcription to cDNA. On the basis that reverse transcription will terminate at an area of RNA damage and damaged RNA will give rise to shorter DNA, a PCR protocol was developed to measure extent of RNA damage. PCR reactions performed with a primer pair at proximal and distal positions on a long gene aimed to highlight RNA damage by comparing quantities of the proximal gene PCR product to the distal gene PCR product. If RNA damage had occurred, the proximal PCR would have more product after PCR compared to the distal, and the ratio of the two would be proportional to the amount of damage to the RNA (Figure 31).

PCR protocol was performed with the master mix of 5x GoTaq green reaction buffer, 10 mM nucleotide mix, forward and reverse 10 mM designed primers, nuclease free water and DNA polymerase to which cDNA template to be tested was added. Into one mastermix no cDNA was added to serve as a negative control. A PCR protocol of 95^{o}C for 2 minutes, followed by 40 cycles of 95^{o}C for 30 seconds, 57^{o}C annealing step for 1 minute and 72^{o}C extension step for 5 minutes and then cooling to 12^{o}C was performed.

qPCR reactions were performed in a 96- well plate from a mastermix of Sybr green fluorescent dye Jumpstart ready mix, reference dye, forward and reverse 10mM primer, cDNA template and nuclease free water. qPCR protocol was performed in Chromo4 of 94^{o}C for 2 minutes and 45 cycles of 94^{o}C for 30 seconds, 56^{o}C for 1 minute and 72^{o}C for 30 seconds (with plate read after the extension step in each cycle) followed by a melting curve between 50 and 90^{o}C at 0.2^{o}C intervals for 0.2 seconds.

In protocol development qPCR was also performed with primers and fluorescent hydrolysis probes by mixing 10x PCR buffer, 50 mM $MgSO_4$, 0.5 µL nucleotide mix, forward and reverse 10 µM primers, Platinum Taq high fidelity DNA polymerase, nuclease free water, cDNA template and a designed hydrolysis probe. Normal PCR master-mix with Promega Taqman products prepared as

above and fluorescent probe primers added. qPCR performed with StepOne Real Time PCR System and output obtained from StepOne software.

Products from all PCR and qPCR reactions were validated on a 2% agarose gel made with 2 g agarose dissolved in 100 mls of Tris-acetate EDTA (TAE) with 5 µL of gel red (1:20,0000). Blue-orange loading dye was added to the samples and a 100 bp DNA ladder. Electrophoresis was run at 120 V for 50 minutes. When complete, the gel was analysed in SynGene G: Box UV imager and analysed for DNA bands with GeneSnap software.

Primer pairs were designed using Primer-Blast (National Center for Biotechnology Information). Primers based on genes known to be expressed in the oocyte were found where suitable proximal and distal pairs had a large number of nucleobases between them (>150 bp). Suitability was established based upon similar annealing temperature for primer pairs and low self-recognition to reduce the risk of primer dimer formation. Primer pairs for gene tubulin beta 1 (Tubb 1) and Bmp7 were designed.

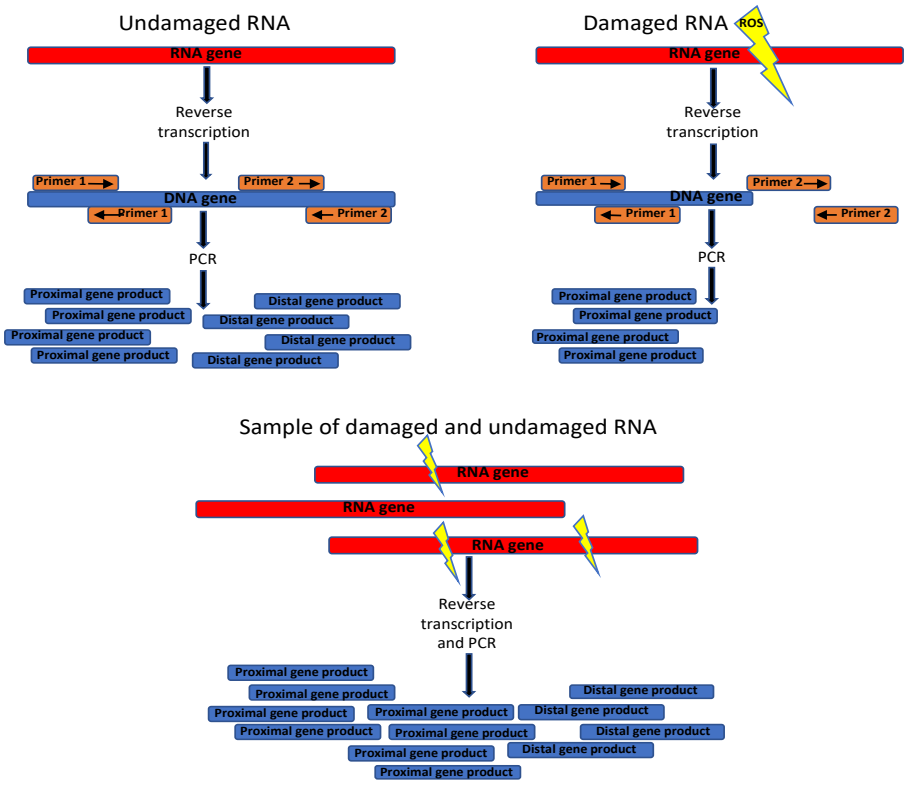


Figure 31: Use of reverse transcription and PCR to measure RNA damage.

Immunofluorescence

Due to the limitations of qPCR in examining RNA damage, a fluorescence protocol was developed to detect RNA damage in oocytes. For immunofluorescence, oocytes were incubated in media, H_2O_2, or peritoneal fluid with milrinone. Incubations >1 hour were carried out in 1 ml closed Eppendorf tubes with 10 µL of mineral oil pipetted above the incubation fluid and placed in wells of a 37^{o}C heat block in a dark room. Incubations <1 hour were carried out in 100 µL of incubation fluid in wells of a glass culture slide under a coverslip placed on a 37 ^{o}C heat block in a dark room. Following incubations in experimental groups, oocytes were then fixed (with formaldehyde, Triton X and 1% PVP/PBS) for 20 minutes, permeabilised (with Triton X and 1% PVP/PBS) for 20 minutes, blocked (with Bovine Serum Albumin in PBS and 1% Tween 20) for 1 hour and incubated with primary and secondary antibody (anti-8-OHG and goat anti-rabbit respectively in blocking solution) for 1 hour followed by a DNA dye (Hoechst or DAPI at 1:1000 concentration in blocking solution) for 15 minutes before imaging. Oocytes were imaged under a confocal microscope in small drops of glycerol with citifluor anti-fade in an imaging dish covered with mineral oil.

The immunofluorescence methodology was first tested with positive (H_2O_2) control and negative (M2 media) control. Increased RNA damage in oocytes exposed to H_2O_2 compared to negative M2 media controls was observed. The protocol was then applied to oocytes incubated in peritoneal fluid for 15 minutes (parallel to oocytes exposed to H_2O_2 and media controls). While the positive and negative controls continued to show expected levels of RNA damage, the peritoneal fluid oocyte groups were similar to media controls. It was considered more physiological to increase the incubation time for oocytes in peritoneal fluid and the immunofluorescence protocol was applied to oocytes following incubations of 16 hours to establish if increasing RNA damage was observed. Oocytes were exposed to the peritoneal fluid of 6 recruited patients for 14-16 hours. At this longer, more physiological incubation period a high rate of oocyte death was observed.

To determine a concentration of peritoneal fluid more suitable for long incubation period, maturation studies were performed whereby GV oocytes were incubated without milrinone in peritoneal fluid of decreasing concentration and a media control. Without milrinone to arrest development, healthy GV oocytes are expected to progress through meiosis I to the point of polar body extrusion and then arrest at metaphase of meiosis II until fertilisation takes place. This should usually take place in ~12 hours and expected in vitro maturation rates are 70-80% in media

controls. This was also in keeping with the physiology of ovulation, oocyte pick-up and transportation in the fallopian tube where oocytes are not exposed to 100% concentration of peritoneal fluid in vivo. The follicular fluid released with the oocyte dilutes the peritoneal fluid at ovulation and during transport of the oocyte in the fallopian tube infundibulum over 12-24 hours, it is exposed to a mixture of follicular fluid, peritoneal fluid and fallopian tube fluid. Maturation studies revealed a high oocyte death rate in 100% peritoneal fluid, a good survival rate in 50% peritoneal fluid and good survival with comparable maturation rates to media control in 15% peritoneal fluid (Figure 32).

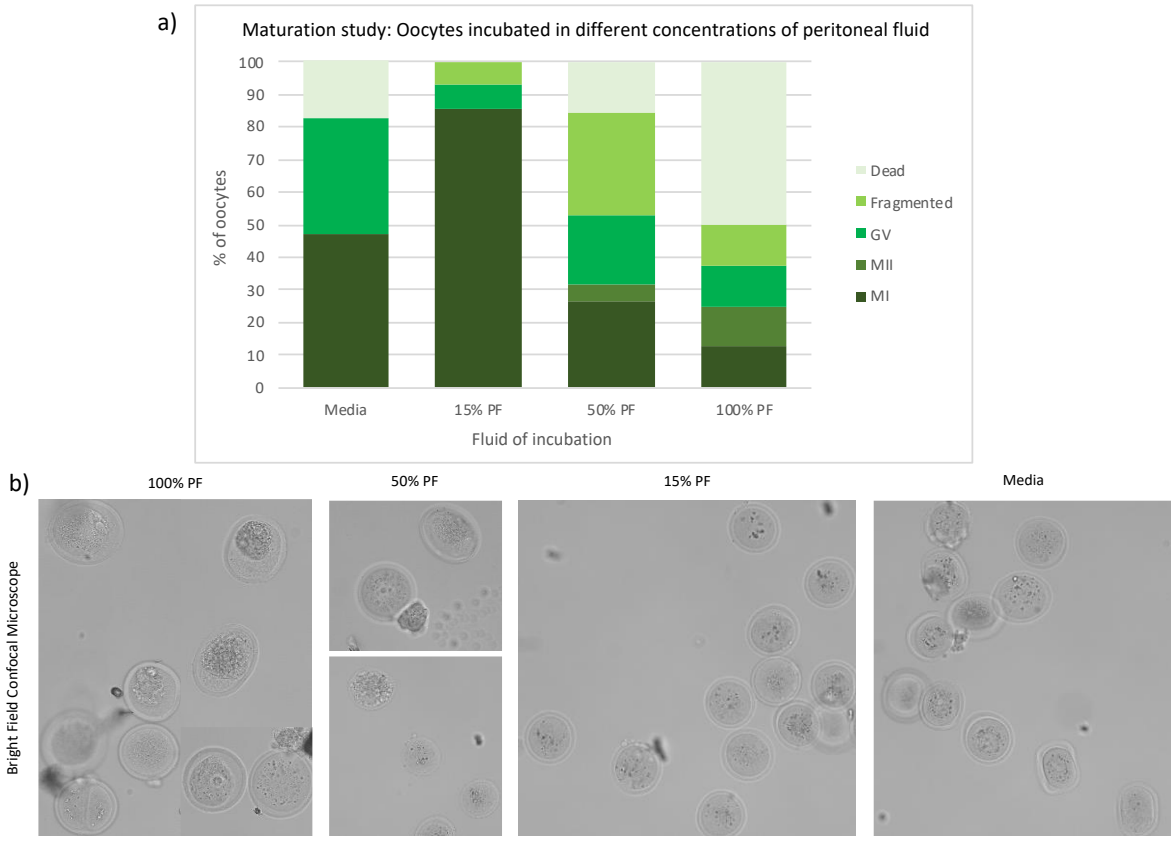


Figure 32: a) Results of maturation study of oocytes incubated in media compared to 100% human peritoneal fluid, 50% peritoneal fluid and 50% media, 15% peritoneal fluid and 85% media. Number of damaged oocytes and number at different stages of meiosis measured and represented graphically b) Sample of oocytes from the maturation study imaged in the bright field of the confocal microscope demonstrating the graphical information.

6.5 Data Analysis

6.5.1 Redox GFP (roGFP) analysis

Images of the oocytes were analysed with software ImageJ. Images of each individual oocyte at the level of the nucleus taken in 3 channels (bright field, 488 and 595 emission) across 10 timepoints were opened in ImageJ and a hyperstack was created using 3 channels and 10 slices. An image summation of all the slices in 3 channels was created using a z project function for each oocyte. The area of these oocytes (in cross-section) was isolated with an oval selection tool placed around the plasma membrane. Mean integrated density of this area was then measured by the software for the emission channels. When fluorescence at these wavelengths were analysed in oocytes exposed to control or sample media a comparison in redox states was made.

6.5.2 PCR

qPCR was analysed by examining amplification plots. Threshold was determined by the linear phase of the plot and Ct was taken from where the PCR curve crossed the amplification threshold. Gradient of the linear component of the curve was interpreted to analyse efficiency of the qPCR with the equation E = -1+10(-1/slope) and to ensure efficiencies were similar between positive controls and oocyte samples. Comparative Ct analysis would be performed using the Ct values for the treated oocyte cDNA samples compared to the untreated oocyte cDNA and liver cDNA using the following formula:

$$\Delta\Delta Ct = \Delta Ct(sample) - \Delta Ct(reference)$$

Melting curve was interpreted in qPCR reactions where intercalated dye was used (Sybr green) to ensure absence of primer-dimers or contaminant DNA if multiple peaks were present, data was interpreted with caution. Gel electrophoresis was also performed on all qPCR products to identify if the expected band was seen (number of bases equivalent to those of the gene identified) and to confirm no multiple bands indicating primer-dimers or DNA contaminant.

6.5.3 Immunofluorescence

Images were analysed with ImageJ software. Z stack images were examined and image representing the nucleus in cross section was selected for analysis. The area of the oocyte (in cross-section) was isolated with an oval selection tool placed around the plasma membrane. Mean integrated density of this area was then measured. The mean integrated density of the area of the nucleus was calculated by the same method and subtracted from the mean integrated density of the oocyte. These levels correlated to the detected fluorescence from the antibody to oxidised guanosine bases of RNA in the cytoplasm. A comparison of the level of this fluorescence was compared across oocytes from different exposure groups.

6.5.4 Statistical analysis

qPCR did not yield results to be analysed. Immunofluorescence and roGFP experiments yielded results in the form of continuous data and were reported as means with standard deviations. Tests were performed for determining if data in comparison groups were normally distributed (Kolmogorov-Smirnova and Shapiro-Wilk). In experiments comparing two groups of data, either independent samples t-test or Wilcoxin signed rank test based on whether the data was normally distributed. For comparison of multiple groups an analysis of variance test (ANOVA or Kruskal-Wallis) was used and adjusted for multiple comparisons (Bonferroni). For all results a p value <0.05 was deemed statistically significant.

Software GraphPad Prism version 7 (GraphPad Software Inc., USA) and SPSS version 25 (IBM, UK, 2017) were used for all analyses.

6.6 Results

6.6.1 qPCR to Detect RNA Damage

PCR was first performed with cDNA from RNA extracted from ovarian, liver, and testicular tissue to establish a suitable PCR protocol due to high quantities of RNA in these tissues. PCR protocol optimised by testing annealing temperature gradients, concentration of template and primer in the PCR solution and by altering thermal cycling protocol from 35 cycles to 40 cycles. Both Bmp7 and Tubb1 isolated cDNA (by adding these primer pairs to RT-PCR step) and cDNA from whole tissue RNA extraction was tested. Best results by gel electrophoresis interpretation were observed with Bmp7 primer pairs, an increased template concentration of 8%, primer concentration of 4%, increased thermal cycling of 40 cycles and cDNA from whole tissue RNA.

QPCR was then performed with the optimised PCR protocol however primer dimer formation was suspected due to similar amplification curves in negative control PCR wells compared to those with template.

QPCR was therefore performed with primer pair concentrations of 2%, 4% and 8% and qPCR reactions also prepared with and without DMSO 1.25 µL (5% concentration of a 25 µL PCR solution).

This revealed good amplification curves with increasing products above 35 cycles, but multiple bands persisted on agarose gel. New primers were designed for alpha tubulin and Bmp7 which were again optimised with liver cDNA. On testing the new primers with qPCR, the proximal alpha tubulin and distal Bmp7 primers yielded good signal from amplification with efficiency >90% but distal alpha tubulin and proximal Bmp7 primers did not (Figure 33 a). Proximal alpha tubulin had an acceptable cycle threshold whereas distal Bmp7 had a cycle threshold >30. The only primer demonstrating a plateau region of the PCR curve was distal alpha tubulin indicating the reaction was incomplete for the other primers. Finding a suitable pair of primers was not successful and possibility of primer-dimers persisted with two peaks consistently appearing on the melting curve rather than a single distinct peak (the amplified DNA products) (Figure 33 b) though not evident on gel electrophoresis of PCR products (Figure 33 c).

Advice was sought from a research group with extensive experience of PCR work and as the MgSO4 used in extracting liver RNA can affect the PCR reaction and there was risk of cDNA contamination, another method of RNA extraction with a commercial kit (Qiagen RNeasy Plus micro kit) was adopted. It was also advised to test RNA quality extraction which was achieved with 2% agarose gel electrophoresis (Figure 33 d) and interpretation of 260/280 band ratio on nanometer. For ongoing PCR work only cDNA from confirmed quality RNA was used.

New primer pairs with quencher fluorescent probe emitting at 530 nm wavelength were advised and suitable primers were designed with Roche universal probe library. Primer pairs for gene alpha tubulin (αTub) with corresponding hydrolysis probe were designed and PCR protocol was optimised with annealing temperature gradients, primer and template concentrations. Positive control was cDNA from liver RNA, test samples were cDNA from oocyte RNA (damaged with H_2O_2 or undamaged prior to RNA extraction) and negative controls were master mix with no cDNA template. A high efficiency of the qPCR reaction would need to be achieved in order to detect RNA damage in oocytes, where RNA quantity is very low.

qPCR demonstrated that there were small levels of RNA in oocyte samples due to Ct being consistently high (35 cycles). Amplification plots frequently did not plateau despite increasing cycle number to 45 suggesting the reactions had not completed. Data was often irreproducible, with PCR efficiency of the reactions and evidence of PCR products in gels varying. The overall poor efficiency of PCR reactions could not be overcome with replacing all reagents, improving the quality of RNA, testing annealing temperatures, increasing the extension stage and increasing the template. The main concerns were of RNA quantity and quality and suitable primer design. Due to the specific needs of two primer pair designs at the beginning and end of a long gene consistently found in the mouse oocyte with similar annealing temperatures, low self-recognition and as great a distance between pairs as possible, primer design was very limited.

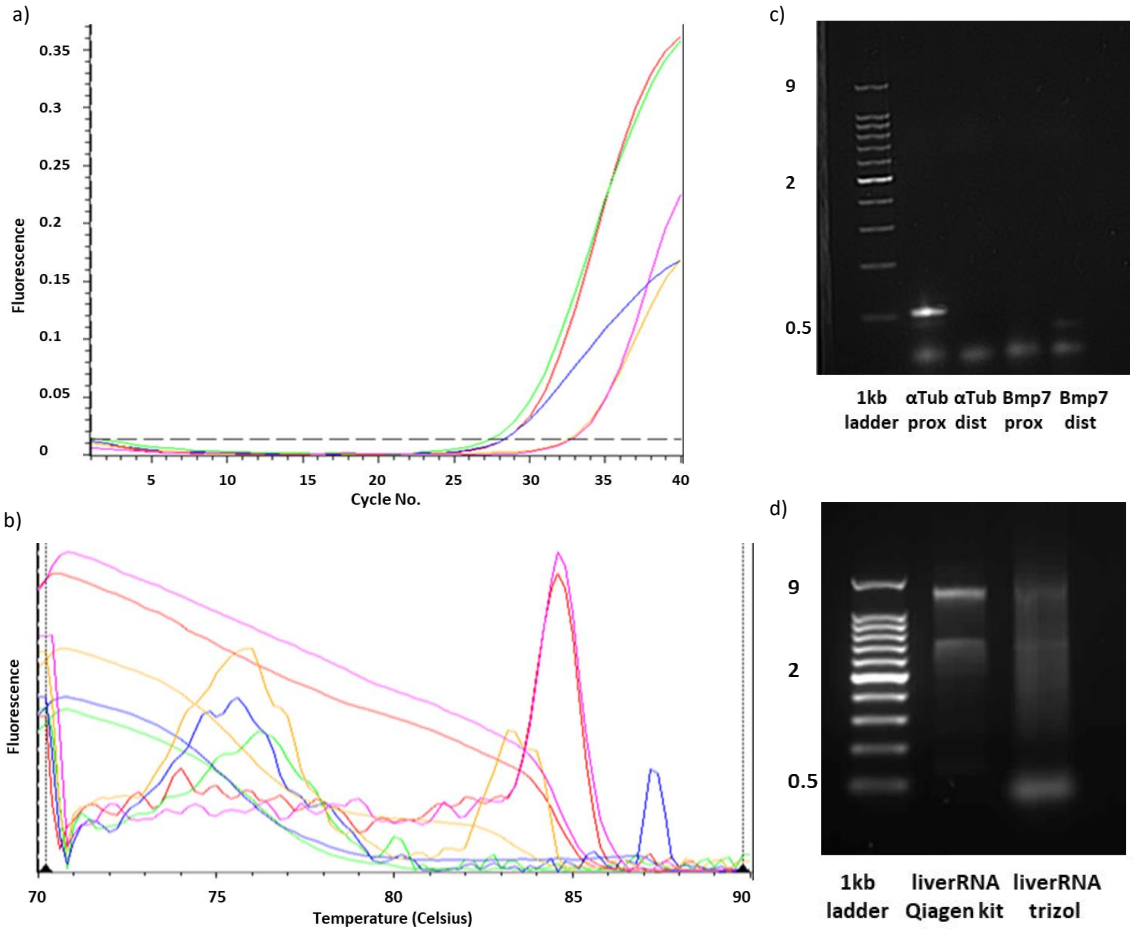


Figure 33: a) qPCR quantitative fluorescence amplification plot of designed primers alpha tubulin proximal (green & red), alpha tubulin distal (blue), Bmp7 proximal (yellow) and Bmp7 distal (pink) with liver cDNA. b) Melting curve of qPCR products showing possible primer dimers. c) Gel electrophoresis result showing gene bands from above qPCR, signal seen with alpha tubulin proximal and Bmp7 distal but no signal with alpha tubulin distal or Bmp7 proximal. d) Formaldehyde-agarose gel showing good quality (left lane, obtained with Qiagen RNeasy Plus Microkit) and poor quality (right lane with Trizol method) RNA from mouse liver.

6.6.2 Immunofluorescence to Detect RNA Damage

The immunofluorescence protocol was applied to oocytes exposed for 14-16 hours to 15% peritoneal fluid of patients recruited to "Peritoneal Fluid Biology" study and compared against positive and negative controls and results for respective levels of RNA damage analysed.

The use of anti-8-OHG antibody with immunofluorescence technique has never been used to determine a physiological level of RNA damage in oocytes. Groups of 10-20 oocytes were incubated for 16 hours in media (negative control) or in 7 different peritoneal fluid samples (3 non endometriosis controls and 4 endometriosis cases) and compared to oocytes fixed and imaged immediately with no incubation period (second negative control) or oocytes incubated in H_2O_2 (positive control). Data in all groups were normally distributed.

Mean fluorescence of anti-8-OHG antibody measured by integrated density in pixels in oocytes incubated in media with oxidising agent H_2O_2 was 4514263.9 (S.D. 6102798.3). Mean fluorescence of anti-8-OHG antibody in media incubated oocytes was 1660599.4 (S.D. 3511593) and in oocytes incubated in peritoneal fluid of women with endometriosis was 777589.3 (S.D 1681373.5). Mean fluorescence of anti-8-OHG antibody in oocytes incubated in peritoneal fluid of women with a normal pelvis was 86495.1 (S.D. 69219.5). Mean fluorescence of anti-8-OHG antibody in oocytes fixed immediately in the immunofluorescence process rather than being incubated was 295469.1 (S.D. 304826.9) (Figure 34 a and b).

On parametric statistical testing to compare these groups, RNA damage in oocytes exposed to H_2O_2 was significantly higher than oocytes fixed immediately (no incubation) ($p < 0.001$), incubated in media ($p < 0.001$) and incubated in peritoneal fluid of women with ($p < 0.001$) and without endometriosis ($p < 0.001$). RNA damage was significantly lower in oocytes incubated in peritoneal fluid of women without endometriosis compared to those incubated in media ($p = 0.049$). RNA damage by anti-8-OHG antibody fluorescence was not higher in oocytes exposed to the peritoneal fluid of women with endometriosis compared to without endometriosis (mean integrated density of pixels 777589.3 and 86495.1 respectively, $p = 0.721$) and RNA damage was not higher in oocytes exposed to the peritoneal fluid of women with endometriosis compared to oocytes fixed immediately (mean integrated density of pixels 777589.3 and 295469.1 respectively, $p = 0.93$) (Figure 34 a and b).

Following recruitment to the XSESS study, the anti-8-OHG antibody immunofluorescence methodology was applied to groups of 10-20 oocytes incubated for 16 hours in media control and different peritoneal fluid samples (6 non endometriosis controls and 6 endometriosis cases) and compared to a group of oocytes fixed and imaged immediately or incubated in H_2O_2. Data in all groups were normally distributed apart from the media control group. Oocytes in the H_2O_2 group unfortunately did not survive and were too damaged to analyse.

Using non-parametric statistical tests and adjusting for multiple tests, RNA damage was higher in oocytes incubated in peritoneal fluid of women with endometriosis (mean integrated density of pixels 340925.5, S.D 134427.4) and without endometriosis (mean integrated density of pixels 419344.37, S.D. 270934.79) compared to oocytes fixed immediately (mean integrated density of pixels 191805, S.D 111153.14) ($p<0.001$ and $p<0.001$ respectively) (Figure 34 c). RNA damage in oocytes incubated in media compared to other incubation groups was not significant (Figure 34 c). RNA damage was higher in oocytes incubated in the peritoneal fluid of women undergoing surgery for infertility (mean integrated density of pixels 571708.22, S.D. 255954.17) compared to those with pain (mean integrated density of pixels 321924.99, S.D 171072.10) ($p<0.001$) or who were asymptomatic and having tubal sterilisation procedure (mean integrated density of pixels 305879.64, S.D. 104802.35) ($p=0.027$) (Figure 34 d). Oocytes fixed immediately had lower levels of RNA damage compared to those incubated in peritoneal fluid of women with infertility ($p=0.004$), pain ($p=0.005$) or those without symptoms ($p<0.001$) (Figure 34 d).

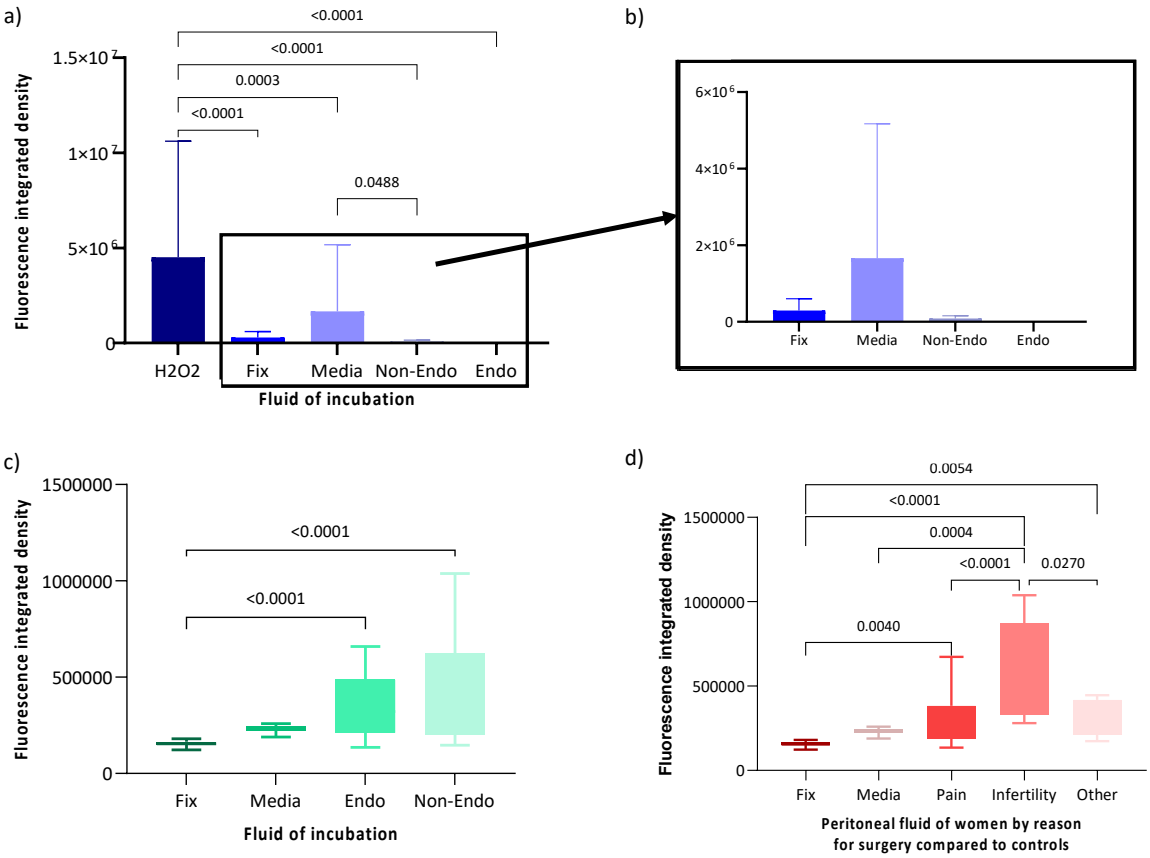


Figure 34: Results of two immunofluorescence studies measuring fluorescence of anti-8-OHG antibody in oocytes fixed immediately or incubated in media, oxidising agent or peritoneal fluid of women recruited to the "peritoneal fluid biology" study (images a and b) or the "XSESS" study (images c and d) measured in integrated density (pixels) displayed graphically by a) Box and whisker plot representation of anti-8-OHG fluorescence levels in oocytes within each group. b) Magnified box and whisker plot of the fixed, media and peritoneal fluid oocyte groups. c) Box and whisker plot of anti-8-OHG fluorescence levels within each group in the XSESS study. d) Box and whisker plot of anti-8-OHG fluorescence levels within each group in the XSESS study by reason for surgery.

6.6.3 Redox GFP (roGFP) to Measure the Redox Status in Mouse Oocytes Exposed to Peritoneal Fluid of Endometriosis Cases and Non-Endometriosis Controls

RoGFP2 mRNA was tested. Oocytes were microinjected with mRNA to express the roGFP2 construct, and then exposed to different media. As a positive control for microinjection, a Histone2B-mCherry construct was co-injected. First the oocytes were imaged in 180 µl M2 media, 20 µl of 100 µM H_2O_2 was then added, and oocytes were imaged every minute for 10 minutes. To the same well 20 µl of 10 mM DTT was added and oocytes were imaged every minute for 10 minutes. Levels of fluorescence from the channels for reduced roGFP2 compared to oxidised roGFP2 the oocytes appeared to demonstrate greater oxidation when exposed to H_2O_2 compared to media (Figure 35 a). Ongoing mild oxidation was seen following exposure to dithiothreitol (DTT) which may be due to the concentration of reducing agent not overcoming the oxidising influence of H_2O_2 or it may be due to the insensitivity of roGFP2 to physiological levels of reduction which is a recognised weakness of using roGFP2 to replicate in vivo redox status (Figure 35 a).

RoGFP2 was then tested by exposing mouse oocytes to 180 µl M2 media with 20 µl of 100 µM H_2O_2 and imaging every minute for 10 minutes alongside a second group of oocytes exposed to resveratrol. Levels of fluorescence from the channels for reduced roGFP2 compared to oxidised roGFP2 demonstrated minimal oxidation when exposed to H_2O_2 (Figure 35 b, c and d) and minimal reduction when exposed to resveratrol (Figure 36).

Results indicated that while it is a sensor of intra-cellular oxidation, roGFP2 may not be suitable for measuring reduction at the physiological level.

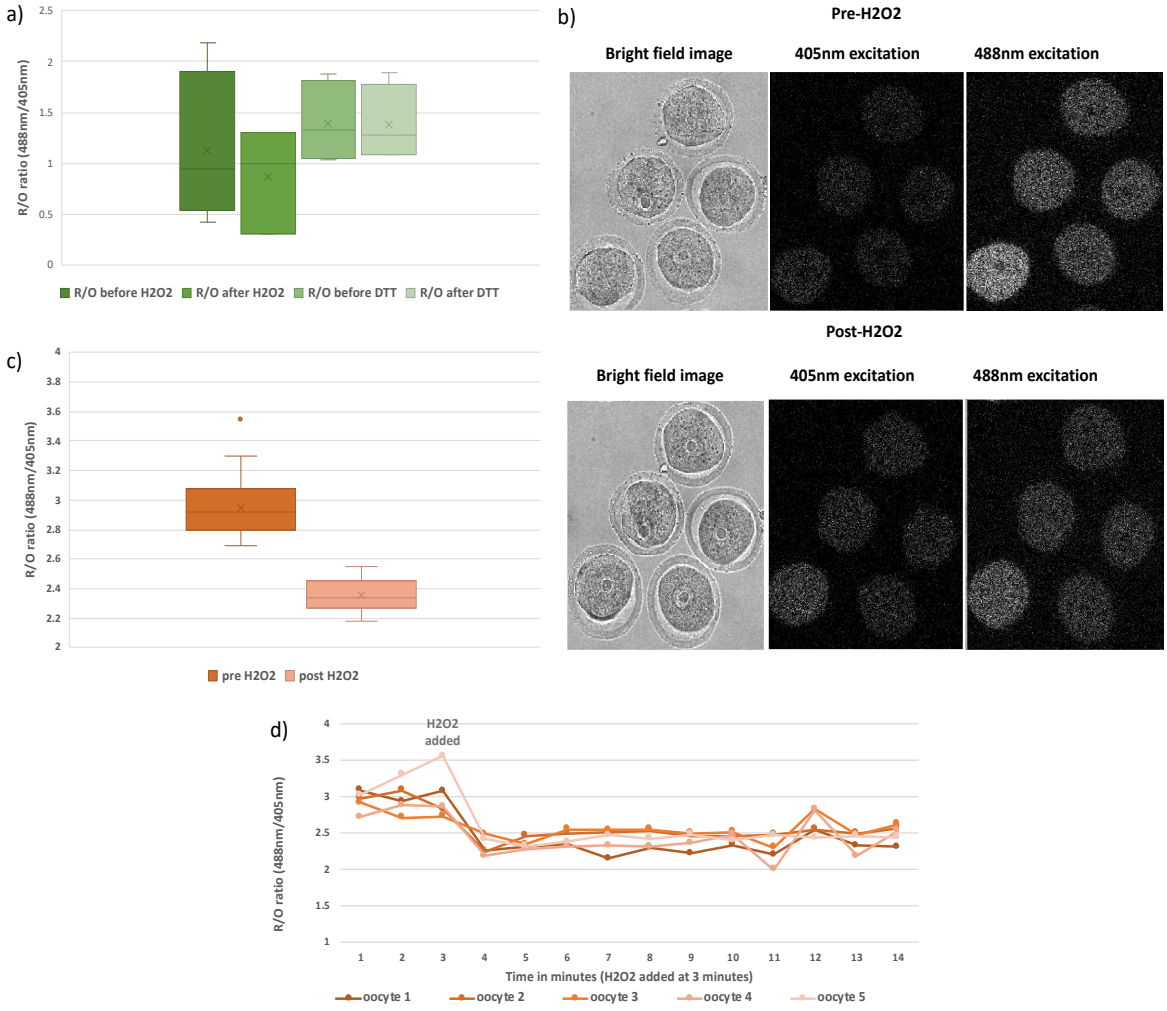


Figure 35: Intracellular redox status of oocytes microinjected with roGFP2 and exposed to an oxidising agent and reducing agents and reduction/oxidation ratio measured by fluorescence following excitation at 488 nm and 405 nm wavelengths a) Box and whisker plots of the R/O ratio measured within oocytes exposed to H_2O_2 followed by DTT. b) Example confocal images of oocytes exposed to H_2O_2 with fluorescence of roGFP2 seen within the cytoplasm when excited at the 488 nm and 405 nm wavelengths. c) Graphical display of redox status within oocytes oxidising after exposure to H_2O_2. d) Box and whisker plot representation of R/O ratios of oocytes before and after exposure to H_2O_2.

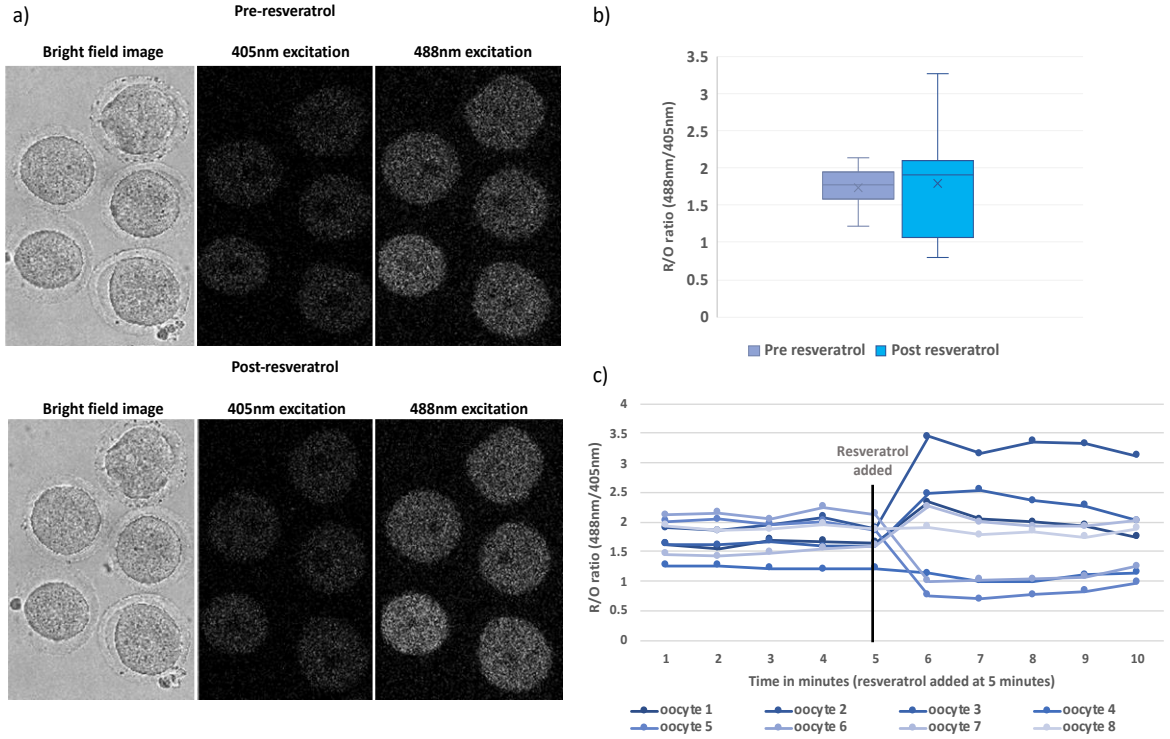


Figure 36: a) Example confocal images of oocytes exposed to resveratrol with fluorescence of roGFP2 seen within the cytoplasm when excited at the 488 nm and 405 nm wavelengths. b) Box and whisker plot of representation of R/O ratios of oocytes before and after exposure to resveratrol. c) Graphical display of redox status within oocytes reducing after exposure to resveratrol.

Grx1-roGFP2 mRNA was microinjected into groups of mouse oocytes to be tested with human samples from patients with and without endometriosis. To observe a subtle physiological effect, 4 samples of peritoneal fluid from patients with stage III and IV endometriosis were selected along with 5 samples of peritoneal fluid from control patients from the XSESS study. An overnight incubation was performed in peritoneal fluid with a media control but on analysis of images, a constituent of the peritoneal fluid interfered with the measurement of fluorescence. Maturation studies previously performed in immunofluorescence work was referred to, to optimise ongoing incubation studies.

The Grx1-roGFP2 incubations were repeated with at a lower concentration of 20% peritoneal fluid in media. On analysis of the images a maximum change in the fluorescence was noted at around 4 hours after which levels either plateaued or demonstrated significant oxidation and deterioration in the quality of the oocyte (loss of spherical shape, shrinking of cytoplasm etc). The incubation and imaging process inevitably expose the oocyte to damaging effects including atmospheric oxygen levels, fluctuations in temperature, regular exposure to laser radiation etc.

Over three incubation experiments a total of 141 oocytes were incubated in media or 20% peritoneal fluid from 9 women taking part in the XSESS study (4 with stage III or IV endometriosis and 5 with a normal pelvis). Change in r/o ratio in oocytes incubated in media was not significant (mean change in r/o ratio 0.06, $p= 0.26$) (Figure 37 a and d). There was a change in r/o ratio in oocytes incubated in the peritoneal fluid of women with and without endometriosis. A shift in intra-cellular redox status of oocytes towards reduction was observed in these groups with the least reduction occurring in peritoneal fluid of women with endometriosis (mean change in r/o ratio 0.07, $p <0.001$) (Figure 37 b and d) and the most reduction occurring in non-endometriosis peritoneal fluid (mean change in r/o ratio 0.17, $p <0.001$) (Figure 37 c and d).

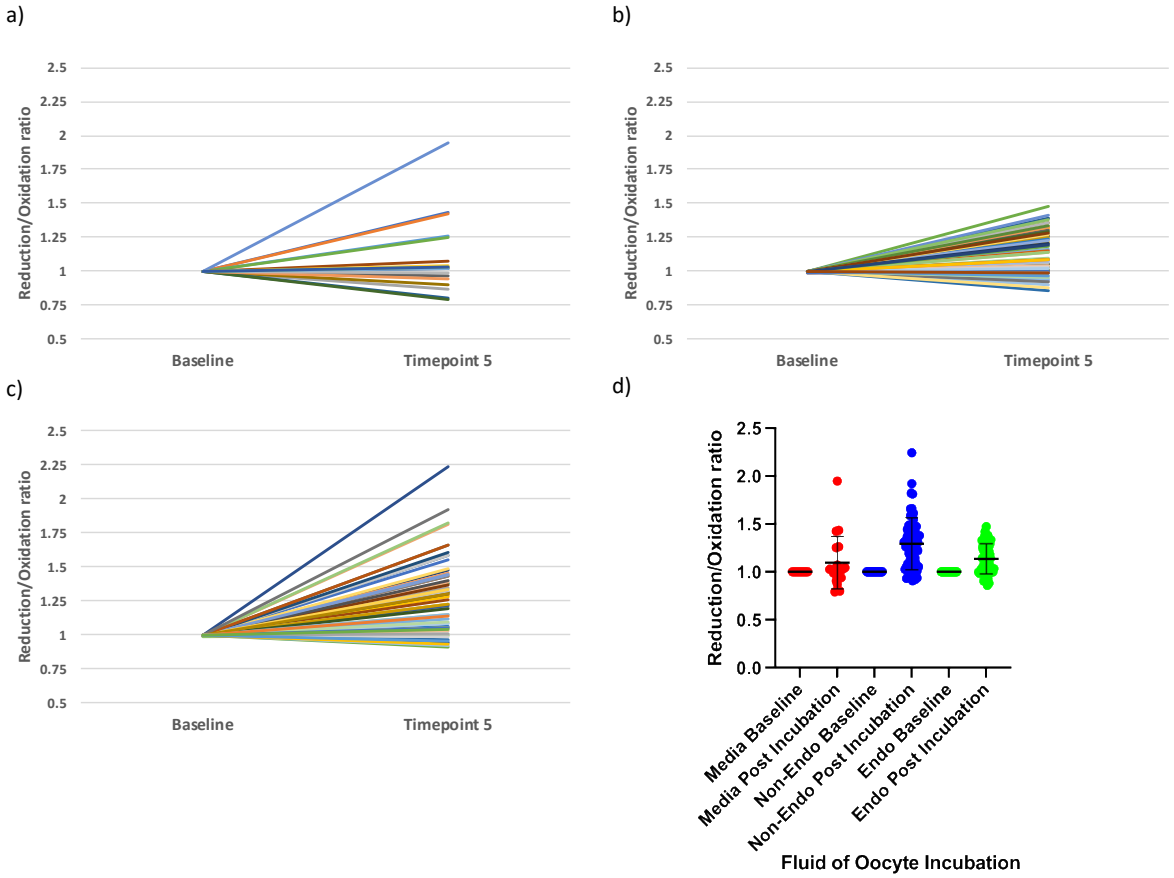


Figure 37: Change in reduction/oxidation ratio in mouse oocytes injected with Grx1-roGFP2 and incubated over 4 hours in a) media b) peritoneal fluid of women with a normal pelvis c) peritoneal fluid of women with endometriosis. d) Grouped scatter plot of R/O ratios of oocytes incubated in media control and the peritoneal fluid of women with and without endometriosis.

Investigating the impact of exposure to peritoneal fluid on the mouse oocyte, by severity of endometriosis revealed an inverse relationship between severity and degree of reduction. No difference in reduction within oocytes incubated in peritoneal fluid of stage I endometriosis (mean r/o at baseline 0.99 and at 4 hours 1.01, p = 0.234), but a significant change in reduction for oocytes exposed to peritoneal fluid of stage III endometriosis (mean r/o at baseline 0.68 and at 4 hours 0.73, p = 0.016) and stage IV endometriosis (mean r/o ratio at baseline 0.58 and at 4 hours 0.72, p <0.001) was seen. Oocytes incubated in the peritoneal fluid of stage I endometriosis showed the least reduction and those incubated in stage IV endometriosis peritoneal fluid demonstrated the highest reduction (Figure 38 a and b). After adjusting for multiple comparisons, analysis found a significant difference in change in r/o ratio within oocytes incubated in peritoneal fluid of stage I endometriosis compared to stage IV endometriosis (p = 0.021), stage I endometriosis compared to normal pelvis (p <0.001), stage III endometriosis compared to normal pelvis (p = 0.002).

The peritoneal fluid of this intra-cellular redox incubation experiment was obtained at surgery for women with a range of medical problems. On analysis of the redox status of oocytes incubated in the peritoneal fluid of women with pelvic pain, infertility, recurrent miscarriage or who were asymptomatic (undergoing surgery for other reasons such as sterilisation) it was found that reduction was significant in oocytes incubated in the peritoneal fluid of women with pain (mean change in r/o ratio 0.13, p <0.001), infertility (mean change in r/o ratio 0.05, p <0.001), and asymptomatic women (mean change in r/o 0.29, p <0.001) but there was no change in redox status in oocytes exposed to peritoneal fluid of women with recurrent miscarriage (mean change in r/o 0.02, p = 0.234) (Figure 38 c and d). In comparison analysis, once adjusted for multiple comparison groups, reduction was significantly less in oocytes exposed to the peritoneal fluid of women with infertility compared to pain and asymptomatic women (p = 0.003 and p <0.001 respectively) and recurrent miscarriage compared to pain and asymptomatic women (p = 0.005 and p <0.001 respectively). Reduction was significantly higher in oocytes exposed to peritoneal fluid of asymptomatic women compared to those with pain (p = 0.001).

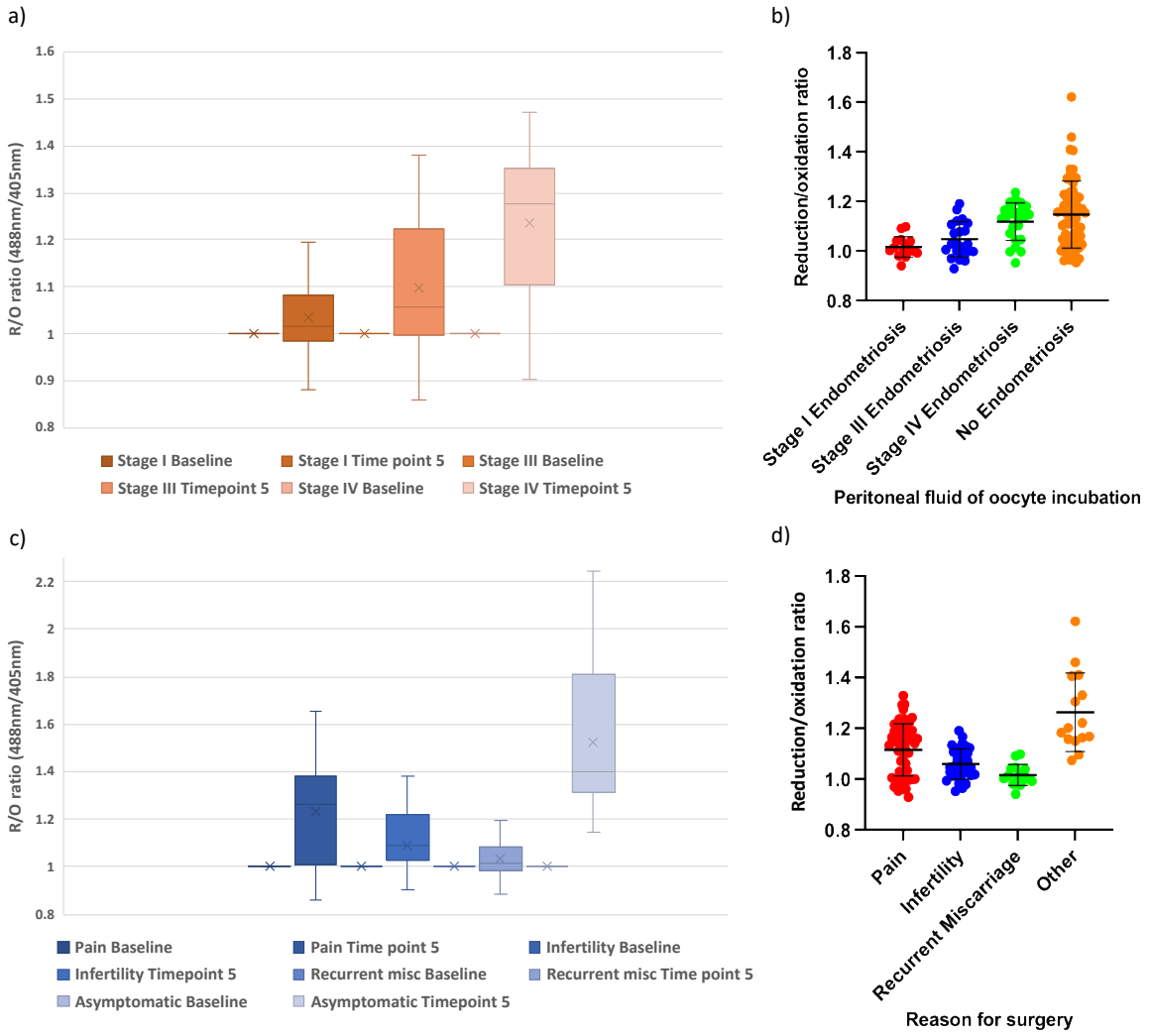


Figure 38: Change in reduction/oxidation ratio in mouse oocytes injected with Grx1-roGFP2 and incubated over 4 hours a) Box and whisker plot and b) grouped scatter plot of R/O ratio of oocytes incubated in peritoneal fluid of women with different severity of endometriosis (by ASRM staging) c) Box and whisker plot and d) grouped scatter plot of R/O ratio of oocytes incubated in peritoneal fluid of women with pelvic pain, infertility, recurrent miscarriage or who were asymptomatic (having surgery for "other" reasons).

6.6.4 Discussion

This chapter has explored RNA damage within oocytes at the physiological level when exposed to the peritoneal fluid of women with and without endometriosis using three experimental techniques. To date, there has been no published work in the area of oocyte RNA damage as a result of endometriosis using these techniques. The first technique attempted to use qPCR to measure the amount of oxidised RNA bases. The main limitations of this work have been in the quantity of RNA extracted from mouse oocytes and the limited number of genes on which to design a suitable primer pair. This work has highlighted that the technique of qPCR for detecting oxidised RNA bases may be unsuitable for use with RNA in mouse oocytes.

It may be surmised that the amount of RNA in groups of 30-60 oocytes (average harvested number from one to two mice) is too low to be suitable for use with qPCR. Although a fully grown mouse oocyte has up to 27 million mRNA molecules compared to the average 100,000-600,000 mRNA molecules in a somatic cell [267], the number of GV oocytes collected from a stimulated mouse (2 ovaries) is ~20-30 and the number of granulosa cells in a single murine antral follicle is >400 [268]. For a qPCR reaction using somatic cells of 50 mg of tissue, a significantly larger amount of mRNA is extracted. A compounding factor for RNA damage is a degree of cytoskeletal, DNA and RNA damage which is caused during oocyte collection from handling, exposure to temperature and atmospheric fluctuations and light, this is kept to a minimum by shortening the collection time. Increasing oocyte collection beyond two mice would therefore compromise on the quality of oocyte RNA due to length of time required for harvesting and preparing the oocytes unless multiple people were able to work on this technique at one time which has not been explored. RNA is “lost” and compromised during RNA extraction technique. Further work in finding a method of extracting higher quality/quantity RNA from mouse oocytes would need to be undertaken. Two techniques were utilised in this work, a manufactured kit and self-made technique, but it is likely that the starting concentration of RNA has impacted the use of both techniques as neither resulted in high quality RNA. This is another experimental step that could be developed in the future. The design of more suitable primers and probes would also benefit from further investigation if future work in this area were to be undertaken. One possible solution may be to find an alternative mammalian model with oocytes containing higher amounts of RNA and a greater range of long housekeeping genes.

The second technique applied was the use of an antibody to detect altered RNA bases in an immunofluorescence protocol. The protocol developed clearly and consistently indicated the anti-8-OHG antibody works well with non-physiological levels of RNA damage when using oxidising agent and comparing damage to two negative controls (oocytes undergoing fixation and imaging immediately and those undergoing the same incubation period as the positive control but with M2 media). RNA damage was significantly lower in oocytes incubated in peritoneal fluid of women without endometriosis compared to those incubated in media. RNA damage could be higher in oocytes exposed to the peritoneal fluid of women with endometriosis compared to those without endometriosis, but results were conflicting and the difference was not statistically significant. Higher sample groups may reveal a stronger relationship between endometriosis and RNA damage and further work to achieve this would be reasonable.

The main limitation in immunofluorescence work is that the level of physiological damage to RNA may be too low to be distinguished with anti-8-OHG antibody. The level of RNA damage in oocytes exposed to peritoneal fluid may be too subtle for the technique of immunofluorescence to accurately measure.

The third technique applied to measure RNA damage within the oocyte was with an intracellular reduction/oxidation biosensor. Two biosensors were constructed using an adapted Gibson cloning protocol. RoGFP2 worked well with positive and negative controls including oxidising and reducing agents and media but a concern about the degree of oxidation and reduction detected was established and a second sensor was constructed with the addition of glutaredoxin-1 to extend the range of redox potential of the probe to -320 mV (from the usual range of redox potential of roGFP2 -280 mV to -290 mV) covering a more physiological range [266]. Microinjection of Grx1-roGFP2 into mouse oocytes was followed by imaging during an incubation period with either negative or positive control media (M2 media and M2 media with added oxidising agent H_2O_2) or the peritoneal fluid of women with and without endometriosis. It demonstrated that the redox status of oocytes in media did not significantly change but those in peritoneal fluid of women all showed a significant shift towards reduction. Reduction was greater in the peritoneal fluid of women without endometriosis compared to those with endometriosis. This either indicates that media is not as nurturing or protective for the oocyte as their physiological environmental fluids, or that physiological fluids are more reducing to the oocyte. It also indicates that the fluids they are exposed to in endometriosis differ to those without endometriosis and

lead to less reduction within the oocyte. Something within the peritoneal fluid of women with endometriosis is producing this effect and higher reactive oxygen species levels or lower antioxidant capacity levels may be responsible among other factors. Reactive oxygen species and its balance with antioxidants may be influential on the quality of oocytes.

The redox status of oocytes incubated in the peritoneal fluid of women with infertility and recurrent miscarriage showed significantly less reduction than those incubated in peritoneal fluid of women with pelvic pain and asymptomatic women. An environment with less reduction may cause oocyte damage if it is due to higher ROS or lower antioxidant capacity. This may point towards a connection between oocyte quality in women with endometriosis and fertility problems. On analysing the redox status of oocytes by severity of endometriosis an inverse relationship between severity and level of reduction was emerging. Indeed, a significant difference was found between the reduction levels caused by stage I endometriosis compared to stage IV endometriosis. Explanation for this, in view of the fact that peritoneal fluid of women without endometriosis causes a higher level of reduction, may be due to small number of samples used leading to subgroup analysis limitation or it may support findings of other studies which have found some antioxidants in too high a level act as reducing agents and impair fertility [262]. More severe disease may be inducing a response to increase levels of antioxidants which in excess then act as reducing agents. It may simply be that the observed relationship requires a greater range of peritoneal fluid samples and greater numbers of oocytes to demonstrate a true correlation. A systematic review into studies examining ROS and antioxidants in the reproductive tract and their impact on fertility has concluded that ROS and antioxidants both contribute to the quality of oocytes, oocyte maturation process and ovulation in a very delicate balance [262]. Any imbalances may have a negative impact on fertility and the results of this chapter suggest there may be a significant difference in the redox status of women with endometriosis compared to those without.

An interesting observation from immunofluorescence and roGFP work was that where RNA damage was seen to be lower, the reduction levels tended to be higher. RNA damage was lower in oocytes incubated in peritoneal fluid compared to media and reduction was greater in oocytes incubated in peritoneal fluid compared to media. RNA damage was highest in the oocytes exposed to peritoneal fluid of women undergoing surgery for infertility and reduction within the oocyte was lower in women undergoing surgery for infertility compared to other causes. This may

give an insight into a mechanism of damage to oocytes in women with infertility. Unfortunately, a difference in RNA damage from peritoneal fluid of women with and without endometriosis was not found in this work to reflect the difference in reduction found in redox study results. The relationship between reactive oxygen species, antioxidant defence and the health of the oocyte is highly complex, and this work gives an intriguing glimpse into this relationship but is by no means robust or thorough enough to draw conclusions.

6.6.5 Further work

More thorough investigation of oxidative RNA damage in the oocyte as a result of endometriosis is indicated by this work. This could add to our understanding of how endometriosis impacts on fertility. A large amount of time was dedicated to protocol development across a range of previously unapplied experimental techniques. A suitable technique has been identified with microinjection of the biosensor Grx1-roGFP2 and another potential technique in immunofluorescence has been identified. Possible RNA damage and change in intracellular redox status have been indicated in this work. Larger scale experiments with a greater number and range of human peritoneal fluid samples and bigger oocyte groups would now be beneficial in understanding the impact further and performing more robust sub-group analyses. Due to use of mouse oocytes, qPCR would not be used in future work as the technique still requires optimisation and the requirement of high number of oocytes to obtain useful RNA for a single experiment should be ethically justified. Downstream effects could also be explored, with protein synthesis studies to demonstrate a tangible biological impact of the RNA damage and altered redox state within the oocyte.

Chapter 7 General Discussion and Future Research

7.1 Discussion and conclusions

This thesis explores the impact of endometriosis on the reproductive process as a whole where evidence is currently polarised to fertility or pregnancy outcomes. Despite the amount of clinical research in this area, the impact of the disease severity on processes of folliculogenesis, oocyte quality, fertilisation, implantation and embryo quality are still under debate. Potential impact on post-implantation stages of reproduction is theorised but not understood and longer follow-up for obstetric and neonatal outcomes is not often undertaken in the current literature.

In a systematic review and meta-analysis of 99 papers, the association between endometriosis and adenomyosis on the reproductive, obstetric and neonatal outcomes of women conceiving with ART and naturally was investigated and whether disease subtypes have specific impacts on different stages of reproduction was explored. It was found that endometriosis consistently leads to reduced oocyte yield and fertilisation rate indicative of altered folliculogenesis, oocyte development and quality. All stages of endometriosis were associated with reduced implantation rate suggesting there is a negative impact on the endometrium. In IVF/ICSI conceiving women it was revealed that adenomyosis and endometriosis of all stages are linked to an increased risk of miscarriage (over three-fold increased risk in adenomyosis). A range of obstetric and fetal complications were associated with endometriosis in IVF pregnancies compared to IVF control pregnancies including preterm delivery, caesarean section delivery and admission to the neonatal unit. In pregnancies conceived either by IVF or naturally, it was found that women with endometriosis had a higher risk of pre-eclampsia, placental abruption, placenta praevia, stillbirth, caesarean section delivery and neonatal unit admission. This may indicate implantation and placentation abnormalities. Milder forms of endometriosis were more likely to affect fertilisation, early implantation processes and risk of miscarriage, whereas more severe disease appeared to influence all stages of reproduction from oocyte and gamete development to early and late pregnancy complications. Disease limited to the ovary negatively affected the oocyte yield and mature oocyte yield in IVF/ICSI treatment.

In performing the systematic review and meta-analysis, many difficulties of analysing observational data were encountered. Quality of studies were scrutinised but a threshold for high

quality studies was not applied, as the tool used does not assign a score to high vs low quality. Sensitivity analysis for the disease subgroups revealed a number of findings that must be viewed with caution due to results being influenced by small numbers of studies in these areas. The strength of evidence presented in this meta-analysis could be improved by setting a threshold for lower quality studies and results not meeting sensitivity analysis. The meta-analysis found low publication bias but due to the nature of systematic reviews, the analysis is confounded by heterogeneity of included studies. Outcomes extending to late pregnancy and neonatal complications were fewer in number to those focusing on aspects of fertility and early pregnancy and the studies lacked a longitudinal approach to investigating disease impact. Several of the late pregnancy outcomes of the meta-analysis are relatively uncommon and a high number of studies with large sample sizes are therefore needed to draw more accurate conclusions. There is also a concern about the reliability of observed results given the difficult nature of diagnosing endometriosis and adenomyosis, the heterogeneity of how studies approached diagnosing these diseases and the possibility that endometriosis case groups could be confounded by concomitant adenomyosis and vica versa. Many studies could not be included in the meta-analysis due to the presentation of data within the published work and due to the pre-set inclusion and exclusion criteria of the systematic review. This work could include many more crucial studies if the inclusion/exclusion criteria was adjusted, and authors contacted for raw data. Many of the aforementioned limitations in interpreting observational data prompted a large population-based cohort study.

An anonymised record linkage cohort study aimed to increase our understanding of the temporal association of endometriosis with obstetric and neonatal adverse outcomes. It also explored the sociodemographic and health characteristics of women with the disease and used two models to address the confounders believed to physiologically increase the risk of endometriosis and confounders potentially impacting healthcare access and acquisition of a diagnosis of endometriosis. 96,025 pregnancies over 15 years were analysed and demonstrated an increased risk of endometriosis in women with infertility, recurrent miscarriage, concurrent comorbidities, increasing age and being overweight. There was a reduced risk amongst women of Asian or Black/African/Caribbean ethnicity, those attaining a lower level of education and being unemployed or a student which may reflect healthcare inequalities rather than biologically protective effects.

In univariate analysis, the study found an association between endometriosis and increased early pregnancy, obstetric and perinatal risks, including the risk of preterm delivery, delivery by caesarean section and a number of antenatal complications including PIH, severe PET and GDM. Endometriosis was found to be directly associated with caesarean section delivery after adjusting for confounders and mediators. Risk of preterm delivery was significant after adjusting for confounders, but the relationship may be mediated by mode of conception. In a subgroup analysis, these risks were demonstrated following surgical diagnosis at which time treatment was likely to have occurred. This indicates the chronic nature of endometriosis post-treatment and that endometriosis either recurs or the effects may endure following surgical treatment of the macroscopic disease.

This is a large longitudinal cohort study investigating the health determinants, fertility, early pregnancy, obstetric and perinatal outcomes of endometriosis by linking two large hospital databases. Interpretation by the data gatherer was limited due to the majority of data being binary and displayed extremely low outlying data. Missing data within the outcomes and covariates was assumed to be random due to the length of data collection, number of data-gatherers and the fact that data was collected for clinical reasons with a low likelihood of bias. A complete case-analysis was performed but exclusion of missing data may increase reliability of results. Unfortunately, data extracted from the surgical database did not include free text surgical operation notes and therefore severity of endometriosis or presence of adenomyosis could not be elucidated for useful sub-analysis. Additionally, there was no ICD 10 code specifically for adenomyosis as code N80.0 “Endometriosis of the uterus” could be applied to adenomyosis but also to endometriosis of the uterine serosa. Data pertaining to women with endometriosis in this study could therefore be confounded by the condition of adenomyosis. Women also may have symptoms of endometriosis, but no formal diagnosis and these could not be excluded. This further emphasises the difficulty in identifying a pure case group in observational studies, particularly with closely related and diagnostically challenging conditions. 1.4% of the pregnancies within the cohort had endometriosis whereas endometriosis is believed to occur in 1-10% of women of reproductive age. The prevalence found in this cohort may reflect the exclusion of women with other gynaecological comorbidity (polycystic ovarian disease, infertility and fibroids) and the diagnostic challenge of the disease. This has undoubtably impacted on the strength of the results of this work and many findings have been affected by small case group sample size. Much of the data pertaining to more uncommon pregnancy complications had small numbers and

associations with endometriosis cannot be confidently drawn in a case cohort of this size. The lack of variables reflecting the health of the neonate was disappointing and further work linking maternity, surgical and paediatric databases will be needed to investigate whether endometriosis has an impact on the longer-term health of the offspring. Analysing data of this nature can make it difficult to infer a true causality between exposure and outcome owing to the many confounding factors but a robust methodological approach to this was taken and accounting for confounders was objective with the use of software-generated DAGs.

This work addresses for the first time, the factors which may directly influence endometriosis via a pathophysiological process and those which affect reaching a diagnosis of the disease by healthcare seeking behaviour. Potential healthcare inequalities are exposed with women of ethnic minorities, lower level of education and less advantaged socio-economic groups less likely to be investigated and treated for endometriosis. The findings overall do suggest that women with endometriosis may need to be considered at greater risk for a number of pregnancy and intrapartum complications and that health risks may extend to their offspring where issues such as prematurity are concerned.

A plausible biological connection between disease severity and the findings of related reduction in oocyte yield, fertilisation rate, miscarriage risk and the cohort study findings of endometriosis associated late pregnancy and neonatal complications was considered. Many physiological differences occurring in endometriosis have been implicated in its impact on fertility and pregnancy including dysfunctional steroidogenesis, endometrial gene expression, adhesion molecules, implantation markers, response of the endometrium to progesterone and elevated interleukins.

Reactive oxygen and nitrogen species and their balance with antioxidants also carries biological plausibility and may have a disease-severity relationship. ROS is implicated in modulating folliculogenesis, oocyte quality, oocyte transport, fertilisation and implantation by its actions in reproductive fluids and the endometrium. There are known difficulties in measuring ROS which not only has a transient nature but is also influenced by a multitude of intrinsic and extrinsic factors which effect its generation and turnover within the body. There is also a significant number of techniques and assays to measure ROS which are not often verified and validated to suitable reproductive fluids/tissues of endometriosis. Therefore, in the current literature, findings

are often inconsistent and elucidating the best methods to capture ROS activity remains a challenge.

To tackle some of these limitations and explore a pathophysiological relationship between endometriosis and the findings of the meta-analysis and cohort study, a study utilising ROS assays was performed. Samples of serum, urine and peritoneal fluid of women with and without endometriosis were collected at surgery to diagnose and treat the disease, if present, and serum and urine were again collected at a post-operative follow up consultation. An assay in direct measurement of ROS (total ROS measured with lumgien assay), indirect measurement of ROS (TBARS assay to measure MDA as a biproduct of lipid peroxidation) and antioxidant defence levels (total antioxidant capacity assay) were selected to compare levels of ROS in biospecimens of women with and without endometriosis and determine whether these levels may be altered by surgical treatment.

Total ROS assay work yielded no positive results. Total ROS levels appeared to be higher in serum, urine and peritoneal fluid of women with endometriosis compared to those without endometriosis, but this was not a true finding on statistical analysis. Women with endometriosis and endometriosis with concomitant pelvic pathology (e.g., fibroids, ovarian cyst, adhesions etc) displayed potentially higher levels of ROS compared to women with a normal pelvis or pelvic pathology excluding endometriosis but this was not significant. ROS seemed to be consistently lowest in women who were asymptomatic and undergoing surgery for other reasons but on anlsysis therewas no difference. An observed trend was that ROS levels were higher at post-operative follow up than at the time of surgery. This was not found to be a positive result on analysis.

TAC levels were higher in the serum, urine and peritoneal fluid of women having surgery for infertility and recurrent miscarriage compared to women with pain or those requesting sterilisation. TAC in serum and peritoneal fluid were highest in women found to have endometriosis and additional pelvic pathology, in urine TAC was higher in women with pelvic pathology excluding endometriosis. TAC was found to be higher in serum in women at the time of surgery compared to post-operative follow up in both case and control groups.

TBARS assay yielded no positive results but there were some interesting observed trends within the data. MDA levels seemed higher in serum, urine and peritoneal fluid in women with

endometriosis compared to women without endometriosis reflecting a possible increased level of lipid peroxidation as a result of higher reactive oxidative species. MDA levels appeared higher in women having surgery for recurrent miscarriage compared to other symptoms and higher where surgery revealed endometriosis together with other pelvic pathology. MDA levels appeared higher at post-operative follow up in women with endometriosis compared to levels at the time of surgery and lower at follow up in women without endometriosis.

None of the assays found a relationship between ROS levels and disease severity. Results reaching significance were those within TAC assay work. Sample size may be inadequate for Lumigen and TBARS assays and for subgroup analysis. Unfortunately, current literature in this niche area is limited and robust power calculation was lacking. Within all assay results a large spread of data was noted and a number of negative results due to dilution of samples as a consequence of protocol development. The significant spread of data may reflect the complex nature of reactive oxygen species and a high number of factors that contribute to the balance of reactive oxygen species within the body. The results may also reflect the inadequacy of investigating a very complex physiological process with a narrow selection of assays. However, results from the lumigen, total antioxidant capacity and TBARS assays indicated that these techniques, once refined, may be appropriate for investigating ROS levels in the human samples of serum, urine and peritoneal fluid in the disease of endometriosis.

The results suggest ROS may be higher in the disease of endometriosis and also higher when other pelvic pathology concomitantly exists, but higher sample numbers are needed to determine a relationship. While this would be anticipated as ROS is known to be higher in inflammatory and chronic health conditions, it demonstrates the complexity of measuring ROS in vivo. A potential difference between assay results in the surgically acquired samples compared to samples collected at post-operative follow up was consistently observed. ROS levels may be higher in women with endometriosis at postoperative follow up due to a recovery process of the body but also may reflect ROS levels being affected by the process of sample collection and processing as post-operative follow up occurred months after surgery. Women found to have endometriosis at the time of surgery may have been advised to commence hormonal treatment to reduce disease recurrence and this too may have an impact on oxidative stress levels. Analysis of the pain questionnaires obtained from each participant at surgery and at follow up would be interesting for comparison of markers of oxidative stress with perceived pain.

A surprising finding of higher antioxidant levels in biological fluid closest to the disease (peritoneal fluid) and lower levels in systemic fluids deserves consideration. It may reflect a compensatory mechanism in response to continuous high levels of ROS or a higher turnover of antioxidants in proximity to the disease versus a more stable level of ROS and antioxidant systemically. The assay detected small molecule antioxidants overall and cannot determine the complex balance of numerous subtypes such as vitamins. There are also a great many more antioxidant enzymes, chain-breaking antioxidants and transition metal binding proteins which are beyond the scope of this assay. As with other findings, it may purely reflect differences in collecting and processing the fluids where this is quicker with systemic fluids and a longer process with fluids local to the disease. Across the assays, ROS seems to have a relationship with infertility and/or recurrent miscarriage rather than disease severity which interestingly points towards the significance of ROS in physiological symptoms of the disease.

As total ROS and lipid peroxidation may be higher in serum, urine and peritoneal fluid of women with endometriosis and be higher in those undergoing surgery for infertility and recurrent miscarriages, its impact on fertility was explored. Peritoneal fluid surrounds the female reproductive organs. Following ovulation, the oocyte is exposed to the peritoneal fluid, and it constitutes a significant proportion of the fallopian tube fluid towards the fimbrial end where fertilisation takes place. ROS is known to damage RNA and DNA within cells by causing breaks in the single and double strands. It was therefore explored whether there was evidence of RNA damage within the oocyte when exposed to the peritoneal fluid of women with and without endometriosis by a number of techniques.

The first technique attempted to use qPCR to measure oxidised RNA bases. Significant limitations in the ability to extract a large enough quantity of quality RNA from mouse oocytes and the limited number of genes on which to design a suitable primer pair highlighted this technique may be unsuitable for detecting physiological RNA damage in mouse oocytes.

Anti-8-OHG antibody was then used with immunofluorescence to detect altered RNA bases. RNA damage was found to be significantly lower in oocytes incubated in peritoneal fluid of women without endometriosis compared to those incubated in media. The difference in RNA damage in oocytes exposed to the peritoneal fluid of women with endometriosis compared to those without endometriosis was not significant. Use of the anti-8-OHG antibody clearly detected non-

physiological levels of RNA damage but damage in physiological fluids may be more subtle. Higher sample groups may therefore be required to investigate the relationship between endometriosis and RNA damage and the technique warrants further work.

An intracellular reduction/oxidation biosensor was constructed to determine whether the redox status of the oocyte differs when exposed to the peritoneal fluid of women with and without endometriosis. Grx1-roGFP2 was microinjected into mouse oocytes before incubation in different peritoneal fluid samples and the redox status investigated. Results showed redox status of oocytes in media did not significantly change but those in peritoneal fluid of women demonstrated a significant shift towards reduction and this was more pronounced in the peritoneal fluid of women without endometriosis compared to those with endometriosis. A number of conclusions could be drawn from this observation. It could be considered that media is not as nurturing or protective for the oocyte as their physiological environmental fluids. It could be concluded that physiological fluids are more reducing for the oocyte. Higher antioxidant capacity in physiological fluids and particularly in those of women without endometriosis may be responsible for the results. An interesting observation was that where RNA damage was seen to be lower in immunofluorescence work, the reduction levels tended to be higher. RNA damage was lower in oocytes incubated in peritoneal fluid compared to media and reduction was greater in oocytes incubated in peritoneal fluid compared to media. RNA damage was highest in the oocytes exposed to peritoneal fluid of women undergoing surgery for infertility and reduction within the oocyte was lower in women undergoing surgery for infertility.

The results indicate that the fluids oocytes are exposed to in endometriosis do differ to those without endometriosis and lead to less reduction within the oocyte. The redox status of oocytes incubated in the peritoneal fluid of women with infertility and recurrent miscarriage also showed significantly less reduction than those incubated in peritoneal fluid of women with pelvic pain and asymptomatic women. This may point towards a connection between oocyte compromise in women with endometriosis and fertility problems. Reactive oxygen species may be responsible among other factors and therefore reactive oxygen species may influence the quality of oocytes.

7.2 Future research

7.2.1 Record linkage cohort study to explore the impact of maternal endometriosis on neonatal and early childhood outcomes of the offspring

The linkage cohort study in this thesis utilised two large hospital databases and found a number of obstetric and perinatal risks in women with endometriosis. To further investigate the impact of maternal endometriosis on the offspring, a further paediatric database will be interrogated. With anonymised child data linked to the maternal and surgical data, outcomes for the offspring of women with endometriosis will be explored beyond their delivery and early neonatal details to determine whether there is a negative impact extending into childhood.

7.2.2 Reactive oxygen species in severity of endometriosis and its symptoms

The ROS assay work in this thesis demonstrated higher levels of ROS in systemic and reproductive fluid of women with endometriosis. Higher numbers of samples are required to understand the strength of this relationship and to perform sub-group analyses by severity of disease. The provisional work will form the basis of a stronger power calculation. XSESS study design included a detailed questionnaire by participants pre and post-surgery. This included a range of symptoms of endometriosis and their severity. To date there has been no evidence of a relationship between ROS and severity of endometriosis. To determine if ROS levels are associated with severity of physical disease and/or symptom severity a qualitative study based on the XSESS questionnaires will be carried out. Following the appropriate ethical approvals, continuation of recruitment to XSESS study based on a new power calculation, ROS and total antioxidant capacity in samples from women without endometriosis and with endometriosis of different stages will be compared.

7.2.3 Endometriosis and oxidative RNA damage in the oocyte

Possible RNA damage and change in intracellular redox status have been indicated in this work but findings have not reached significance. Larger scale experiments with a greater number and range of human peritoneal fluid samples and bigger oocyte groups would now be beneficial in understanding the impact further and performing more robust sub-group analyses. Techniques of immunofluorescence with anti-8-OHG to determine oxidised RNA bases together with Grx1-

roGFP2 microinjection for intracellular redox status will be used with a larger number of peritoneal fluid samples and larger groups of mouse oocytes. It may also be possible to implement these techniques with a much smaller cohort of human oocytes given the appropriate ethical approvals on the OVUM study to explore a link between findings within the mouse model and humans. Downstream effects of RNA damage such as protein synthesis will also be desirable to observe a functional impact of any observed damage.

Appendices

Appendix 1: Search Terms for Systematic Review and Meta-analysis

Outcomes	preterm, small for gestational age, growth restriction, hypertension, pre-eclampsia, cholestasis, caesarean, haemorrhage, placenta praevia, placental abruption, prematur*, miscarriage, fertilization rate, fertilisation rate, implantation rate, livebirth rate, clinical pregnancy rate, mid-trimester loss, cycle cancellation, ovarian response, reproductive outcome, obstetric outcome, pregnancy outcome, fertility outcome
Diseases	Endometriosis Adenomyosis Deep infiltrating endometriosis Endometrioma* OR ovarian endometriosis Mild, Moderate, Stage 1, Stage 2 AND endometriosis Moderate, Severe, Stage 3, Stage 4 AND endometriosis

Appendix 2: Table of Excluded Studies

Author	Year of Publication	Country	Reason for Exclusion
Aboulghar[269]	2003	Egypt	Case group is stage IV endometriosis
Abuelghar[270]	2014	Egypt	No outcomes pertaining to the meta-analysis
Allerstorfer[271]	2016	Austria	No control group
Al Jama[272]	2011	Saudi Arabia	No non-adenomyosis control
Badawy[273]	1988	USA	No control group
Baggio[274]	2015	Italy	Case group is treated DIE +/- endometrioma
Ballester[275]	2012	France	No non-endometriosis control group
Ballester/Oppenheimer[276]	2011	France	No non-endometriosis control group
Ballester/Oppenheimer/ d'Argent[277]	2012	France	No non-endometriosis control
Bayram[278]	2013	Turkey	Case group chronic pelvic pain
Benaglia[279]	2014	Italy	Adenomyosis group and control group had concomitant endometriosis
Benaglia/Cardellicchio[280]	2014	Italy	No non-endometriosis control group
Berube[281]	1998	Canada	No outcomes pertaining to the meta-analysis
Braza-Boils[282]	2015	Spain	Should have been excluded on title and abstract screen
Carassou-Maillan[283]	2014	France	Includes IUI treatment
Centini[284]	2015	France	No non-endometriosis control group
Darai[285]	2010	France	No control group
Diaz[286]	2000	Spain	Donor egg treatment
Dmowski[287]	2002	USA	No control group
Esinler[288]	2006	Turkey	Case group is treated endometrioma
Filippi[289]	2014	Italy	No non-endometriosis control group

Fitzsimmons[290]	1987	USA	Infertile patient group but mode of conception not stated
Fujishita[291]	2002	Japan	No control group
Geber[292]	2002	Brazil	Case group treated stage III-IV endometriosis
Göker[293]	2002	Turkey	IUI treatment
Göker[293]	2002	Turkey	Translated duplicate paper
Guo[294]	2016	China	Case group it treated stage III-IV endometriosis
Harada[295]	2015	Japan	No control group
Hasdemir[296]	2016	Turkey	Adenomyosis group and control group had concomitant endometriosis
Hirano[297]	1995	Japan	No translation obtained
Hjordt Hansen[298]	2014	Denmark	Cases diagnosed with ICD 8 code
Ho[299]	2002	Taiwan	No control group
Huang[300]	2014	China	Should have been excluded on title and abstract screen
Inoue[301]	1992	Japan	Mixed IUI and IVF treatment
Inoue[301]	1992	Japan	Duplicate paper
Isaksson[302]	1997	Finland	No control group
Jayakrishnan[303]	2016	India	No control group
Kahyaoglu[304]	2008	Turkey	Case group treated stage IV endometriosis with endometrioma
Kawwass[305]	2015	USA	Case and control group had concomitant poor ovarian reserve
Kereszturi[306]	2015	Hungary	No non-endometriosis control group
Khamsi[307]	2000	Canada	No non-endometriosis control group
Kortelahti[308]	2003	Finland	Conception was mixed natural conception and IVF
Kucera[309]	2005	Czech Republic	No translation obtained

Lind[310]	2015	Sweden	No outcomes pertaining to the meta-analysis
Littman[311]	2005	USA	No non-endometriosis control
Loo[312]	2005	Taiwan	Case group is treated endometrioma +/- endometriosis
Luke[313]	2015	USA	Assisted reproductive technology treatment not defined
Mahadevan[314]	1983	Australia	Not stated how endometriosis was diagnosed
Mahmood[315]	1991	UK	No control group
Mao[316]	2009	France	Case group is treated endometriomas and stage IV endometriosis
Marconi[317]	2002	Argentina	Case group is treated endometriomas
Martinez-Conejero[318]	2011	Italy	Donor egg treatment
Matorras[319]	2010	Spain	Donor insemination treatment
May[320]	2012	UK	No outcomes pertaining to the meta-analysis
Maubon[321]	2010	France	Adenomyosis group had concomitant endometriosis
Mavrelos[322]	2017	UK	Adenomyosis group stratified by score of USS features, unable to extrapolate outcome data for adenomyosis group overall
Mekaru[323]	2014	Japan	IUI or ovulation induction treatment
Miller[324]	2016	USA	Treatment was monitored cycles and superovulation cycles
Mills[325]	1992	UK	Not stated how endometriosis was diagnosed
Montanaro Gauci[326]	2001	South Africa	IUI treatment
Murta[327]	2018	Brazil	Outcome data cannot be used as percentages and pregnancy and cycle numbers not given
Nakagawa[328]	2007	Japan	No non-endometriosis control group
Nieweglowska[329]	2015	Poland	No outcomes pertaining to the meta-analysis
Nirgianakis[330]	2018	Switzerland	Case group was treated DIE
Nur Azurah[331]	2014	Malaysia	No non-endometriosis control group

Omland[332]	1998	Norway	Artificial insemination treatment
Oppenheimer[333]	2013	France	No control group
Pagidas[334]	1996	Canada	Case group is treated Stage III-IV endometriosis
Pal[335]	1998	USA	No non-endometriosis control
Papaleo[336]	2011	Italy	No non-endometriosis control
Piver[337]	2005	France	Case and control group based upon thickness of junctional zone
Puente[338]	2016	Spain	Assisted reproductive technology treatment not defined and no outcomes of the meta-analysis
Roustan[339]	2015	France	Case group was treated endometriomas with poor ovarian function
Ruiz Anguas[340]	2007	Mexico	No non-endometriosis control group
Ruiz Anguas[340]	2007	Mexico	Translated duplicate paper
Salamun[341]	2017	Slovenia	No non-endometriosis control group
Santulli/Lamau[342]	2016	France	No non-endometriosis control group
Scala[343]	2018	Italy	No control group
Sharma[344]	2018	India	Case group had endometrioma >4 cm excluded and adenomyosis group excluded uterine size >12/40 size
Sinaii[345]	2008	UK	No control group
Singh[346]	2014	India	Case group is treated Stage III-IV endometriosis
Singh/Goldberg[347]	2001	USA	IUI treatment
Somigliana[348]	2008	Italy	Case group is treated endometriomas
Stepniewska[349]	2009	Italy	No control group
Sung[350]	1997	USA	Donor egg treatment
Takemura[351]	2013	Japan	No suitable case and control group

Taniguchi[352]	2016	Japan	No outcomes pertaining to the meta-analysis
Thomas[353]	1986	UK	No control group
Thomin[354]	2016	France	No control group
Thomin[354]	2016	France	Duplicate
Tremellen[355]	2011	Australia	No control group
Van Loendersloot[356]	2013	Netherlands	Not stated how endometriosis was diagnosed
Vercellini[357]	2012	Italy	No control group
Werbrouck[358]	2006	Belgium	IUI treatment
Yamaguchi[359]	2018	Japan	Adenomyosis diagnosed with a questionnaire
Yamamoto[360]	2017	USA	No non-endometriosis control group
Yan[361]	2013	China	Adenomyosis group had concomitant endometriosis
Yang[362]	2016	China	Case and control groups were participants with poor ovarian response +/- endometriosis
Yanushpolsky[363]	1998	USA	Control group has concomitant endometriosis
Zhang[364]	2016	China	Case group is treated endometriomas
Zhang[364]	2016	China	Translated duplicate paper
Ziller[365]	2015	Germany	No outcomes pertaining to the meta-analysis

Appendix 3: Data Extraction Form

Study Details

Title					
Author					
Date					
Institution					
Journal					
Extracted by	J Horton				
Study start date					
Follow up period					
Recruitment method					
Single Centre			Multicentre		
Design	RCT	Quasi RCT	Retrospective Cohort	Prospective Cohort	Other

Participants

Control Group:

	Mean	Median	Range	SD
Age				
BMI				

Parity					
Social Class					
Ethnicity					
Smokers	Yes	No	Mix	Unknown	
Partner's smoking status	Yes	No	Mix	Unknown	
Semen analysis	Normal	Abnormal (ICSI)	Donor	Mix	Unknown
Partnership	Heterosexual	Same sex	Single female	Mix	Unknown
Type of Infertility	Male	Tubal	PCOS	Unexplained	Endometriosis
	Other	Mix	Unknown		
Previous fertility treatment					
Type of endometriosis					
Method of diagnosis					
Staging system used					

Previous treatment for endometriosis	
Inclusion Criteria	
Exclusion criteria	
Recruitment method	
Treatment	
Ovulation induction method	

Test Group 1:

	Mean	Median	Range	SD
Age				

BMI					
Parity					
Social Class					
Ethnicity					
Smokers	Yes	No	Mix	Unknown	
Partner's smoking status	Yes	No	Mix	Unknown	
Semen analysis	Normal	Abnormal (ICSI)	Donor	Mix	Unknown
Partnership	Heterosexual	Same sex	Single female	Mix	Unknown
Type of Infertility	Male	Tubal	PCOS	Unexplained	Endometriosis
	Other	Mix	Unknown		
Previous fertility treatment					
Type of endometriosis					
Method of diagnosis					
Staging system used					

Previous treatment for endometriosis	
Inclusion Criteria	
Exclusion criteria	
Recruitment method	
Treatment	
Ovulation induction method	

Test Group 2 (if applicable):

	Mean	Median	Range	SD
Age				

BMI					
Parity					
Social Class					
Ethnicity					
Smokers	Yes	No	Mix	Unknown	
Partner's smoking status	Yes	No	Mix	Unknown	
Semen analysis	Normal	Abnormal (ICSI)	Donor	Mix	Unknown
Partnership	Heterosexual	Same sex	Single female	Mix	Unknown
Type of Infertility	Male	Tubal	PCOS	Unexplained	Endometriosis
	Other	Mix	Unknown		
Previous fertility treatment					
Type of endometriosis					
Method of diagnosis					
Staging system used					

Previous treatment for endometriosis	
Inclusion Criteria	
Exclusion criteria	
Recruitment method	
Treatment	
Ovulation induction method	

Outcomes:

	Control	Group 1	Group 2	Group 3	Significance
Ovarian Response					

Cycle cancellation					
Fertilisation rate					
Implantation rate					
Clinical pregnancy rate					
Live birth rate					
Miscarriage					
Mid-trimester loss					
Prematurity					
Preterm					
SGA					
IUGR					
Hypertension					
Pre-eclampsia					
Cholestasis					
Placenta previa					
Placental abruption					
Haemorrhage					
Caesarean					
Reproductive outcome					
Details					
Obstetric outcome					
Details					

Pregnancy outcome					
Details					
Fertility outcome					
Details					
Other					

Statistics:

Power	Yes		No	Details:	
Tests	Students T	Chi	Chi^2	Wilcoxin	Regression
Other					
P value used					
Are all results given	Yes		No		
Bias					
Confounders					

Authors conclusions:

Main conclusions	

Ethics:

Funding	
Sponsorship	
Declarations	
Others	

ALL CRITERIA	DESCRIPTION OF CRITERIA (with additional explanation as required, determined by consensus of raters)	POSSIBLE ANSWERS
1	**Is the hypothesis/aim/objective of the study clearly described?** Must be explicit	Yes/No
2	**Are the main outcomes to be measured clearly described in the Introduction or Methods section?** If the main outcomes are first mentioned in the Results section, the question should be answered no. **ALL** primary outcomes should be described for YES	Yes/No
3	**Are the characteristics of the patients included in the study clearly described?** In cohort studies and trials, inclusion and/or exclusion criteria should be given. In case-control studies, a case-definition and the source for controls should be given. Single case studies must state source of patient	Yes/No
4	**Are the interventions of interest clearly described?** Treatments and placebo (where relevant) that are to be compared should be clearly described.	Yes/No
5	**Are the distributions of principal confounders in each group of subjects to be compared clearly described?** A list of principal confounders is provided. YES = age, severity	Yes/No
6	**Are the main findings of the study clearly described?** Simple outcome data (including denominators and numerators) should be reported for all major findings so that the reader can check the major analyses and conclusions.	Yes/No
7	**Does the study provide estimates of the random variability in the data for the main outcomes?** In non normally distributed data the inter-quartile range of results should be reported. In normally distributed data the standard error, standard deviation or confidence intervals should be reported	Yes/No
8	**Have all important adverse events that may be a consequence of the intervention been reported?** This should be answered yes if the study demonstrates that there was a comprehensive attempt to measure adverse events (COMPLICATIONS BUT NOT AN INCREASE IN PAIN).	Yes/No
9	**Have the characteristics of patients lost to follow-up been described?** If not explicit = NO. RETROSPECTIVE – if not described = UTD; if not explicit re: numbers agreeing to participate = NO. Needs to be >85%	Yes/No
10	**Have actual probability values been reported (e.g. 0.035 rather than <0.05) for the main outcomes except where the probability value is less than 0.001?**	Yes/No
11	**Were the subjects asked to participate in the study representative of the entire population from which they were recruited?** The study must identify the source population for patients and describe how the patients were selected.	Yes/No/UTD
12	**Were those subjects who were prepared to participate representative of the entire population from which they were recruited?** The proportion of those asked who agreed should be stated.	Yes/No/UTD
13	**Were the staff, places, and facilities where the patients were treated, representative of the treatment the majority of patients receive?** For the question to be answered yes the study should demonstrate that the intervention was representative of that in use in the source population. Must state type of hospital and country for YES.	Yes/No/UTD
14	**Was an attempt made to blind study subjects to the intervention they have received?** For studies where the patients would have no way of knowing which intervention they received, this should be answered yes. Retrospective, single group = NO; UTD if > 1 group and blinding not explicitly stated	Yes/No/UTD
15	**Was an attempt made to blind those measuring the main outcomes of the intervention?** Must be explicit	Yes/No/UTD
16	**If any of the results of the study were based on "data dredging", was this made clear?** Any analyses that had not been planned at the outset of the study should be clearly indicated. Retrospective = NO. Prospective = YES	Yes/No/UTD
17	**In trials and cohort studies, do the analyses adjust for different lengths of follow-up of patients, or in case-control studies, is the time period between the intervention and outcome the same for cases and controls?** Where follow-up was the same for all study patients the answer should yes. Studies where differences in follow-up are ignored should be answered no. Acceptable range 1 yr follow up = 1 month each way; 2 years follow up = 2 months; 3 years follow up = 3months.....10years follow up = 10 months	Yes/No/UTD
18	**Were the statistical tests used to assess the main outcomes appropriate?** The statistical techniques used must be appropriate to the data. If no tests done, but would have been appropriate to do = NO	Yes/No/UTD
19	**Was compliance with the intervention/s reliable?** Where there was non compliance with the allocated treatment or where there was contamination of one group, the question should be answered no. Surgical studies will be YES unless procedure not completed.	Yes/No/UTD

20	**Were the main outcome measures used accurate (valid and reliable)?** Where outcome measures are clearly described, which refer to other work or that demonstrates the outcome measures are accurate = YES. ALL primary outcomes valid and reliable for YES	Yes/No/UTD
21	**Were the patients in different intervention groups (trials and cohort studies) or were the cases and controls (case-control studies) recruited from the same population?** Patients for all comparison groups should be selected from the same hospital. The question should be answered UTD for cohort and case control studies where there is no information concerning the source of patients	Yes/No/UTD
22	**Were study subjects in different intervention groups (trials and cohort studies) or were the cases and controls (case-control studies) recruited over the same time?** For a study which does not specify the time period over which patients were recruited, the question should be answered as UTD. Surgical studies must be <10 years for YES, if >10 years then NO	Yes/No/UTD
23	**Were study subjects randomised to intervention groups?** Studies which state that subjects were randomised should be answered yes except where method of randomisation would not ensure random allocation.	Yes/No/UTD
24	**Was the randomised intervention assignment concealed from both patients and health care staff until recruitment was complete and irrevocable?** All non-randomised studies should be answered no. If assignment was concealed from patients but not from staff, it should be answered no.	Yes/No/UTD
25	**Was there adequate adjustment for confounding in the analyses from which the main findings were drawn?** In nonrandomised studies if the effect of the main confounders was not investigated or no adjustment was made in the final analyses the question should be answered as no. If no significant difference between groups shown then YES	Yes/No/UTD
26	**Were losses of patients to follow-up taken into account?** If the numbers of patients lost to follow-up are not reported = unable to determine.	Yes/No/UTD
27	**Did the study have sufficient power to detect a clinically important effect where the probability value for a difference being due to chance <5%** Sample sizes have been calculated to detect a difference of x% and y%.	1-5

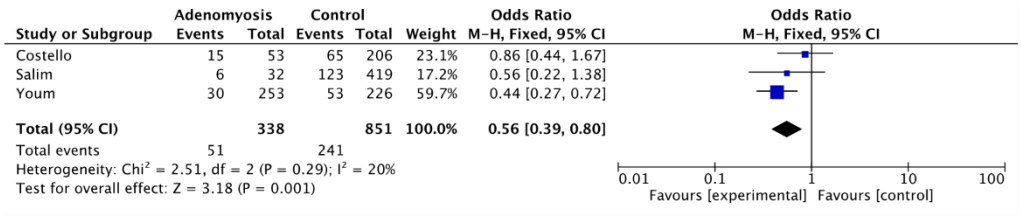
Appendix 4: Results of Analysis for Publication Bias and Sensitivity

Disease	Mode of Conception	Outcome	Publication Bias Risk	Sensitivity Analysis Result
Adenomyosis	IVF/ICSI	CPR	Low	Robust
		MR	Low-moderate	Robust
		IR	Low	Interpret with caution
	NC/ART	PTD	Low	Robust
		SGA	Very Low	Interpret with caution
		LSCS	Low	Interpret with caution
		PET	Low	Interpret with caution
Endometriosis	IVF/ICSI	CPR	Low	Robust
		LBR	Low	Robust
		MR	Low	Robust
		PTD	Low	Robust
		LSCS	Low	Robust
		NNU	Very Low	Robust
		OR	Very Low	Robust

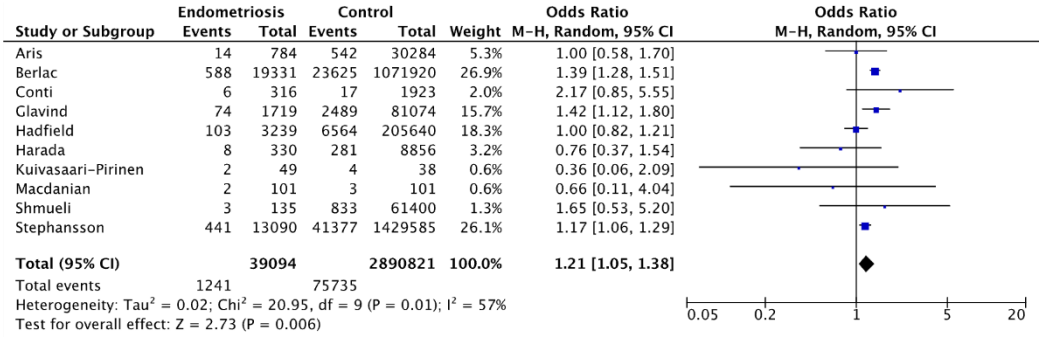
		MII	Very Low	Robust
		FR	Very Low	Robust
		IR	Low	Robust
		CR	Low	Robust
	NC/ART	MR	Very Low	Interpret with caution
		PTD	Low	Robust
		PP	Low	Robust
		LSCS	Moderate	Robust
		PET	Low	Interpret with caution
		PA	Low	Robust
		IUD	Low	Interpret with some caution
		NNU	Low	Robust
	NC	PIH	Low	Interpret with caution
		PTD	Low	Robust
		LSCS	Low	Robust
Treated Endometriosis	IVF/ICSI	OR	Very Low	Robust
		MII	Very Low	Robust
		FR	Low	Robust
Stage I-II Endometriosis	IVF/ICSI	MR	Low	Robust
		FR	Moderate	Interpret with caution
		IR	Low	Interpret with caution
		CR	Low	Robust
Stage III-IV Endometriosis	IVF/ICSI	CPR	Low	Interpret with some caution
		LBR	Low	Interpret with some caution
		MR	Low	Robust
		OR	Very Low	Robust
		MII	Very Low	Interpret with caution
		IR	Low	Interpret with caution
Endometrioma	IVF/ICSI	OR	Low-Moderate	Robust
		MII	Very Low	Robust

Appendix 5: Forest Plots demonstrating a) implantation rate (OR 0.56, n= 3) of women with adenomyosis compared to women without b) PET (OR 1.21, n= 10) in women with endometriosis compared to those without in NC/ART studies and c) IUD (OR 1.25, n= 4) in women with endometriosis compared to women without in NC/ART studies

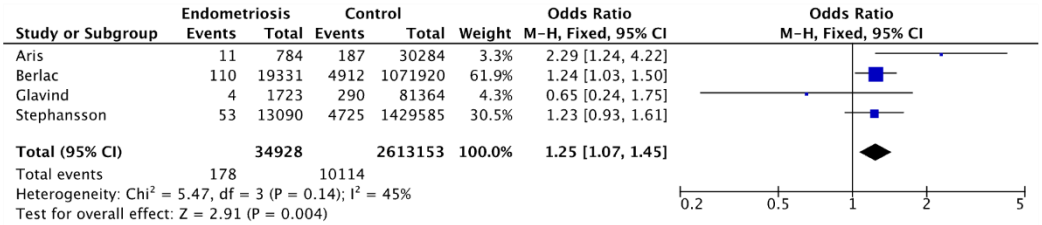
a)



b)

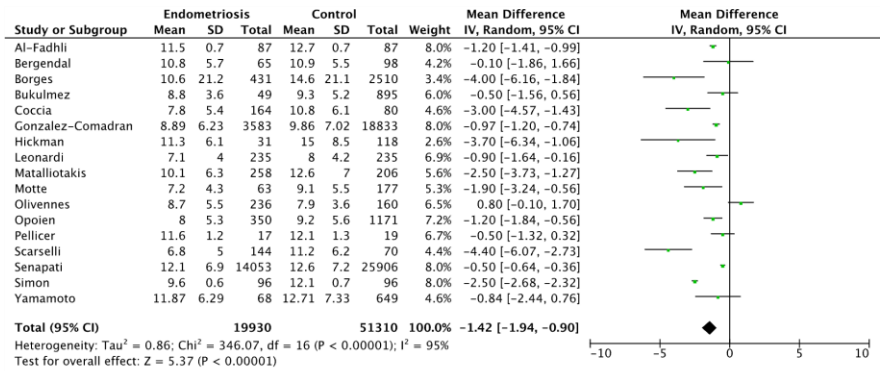


c)

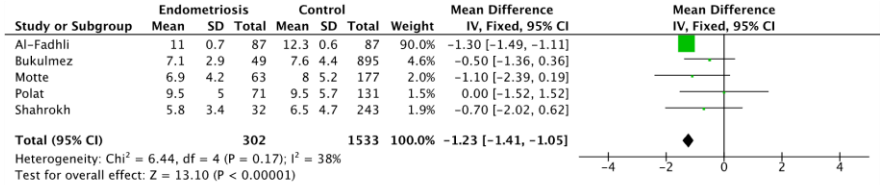


Appendix 6: Forest plots demonstrating IVF/ICSI outcomes in women with endometriosis compared to women without endometriosis a) Oocyte yield (OR -1.42, n= 17) b) MII oocyte yield (OR -1.23, n= 5) c) Fertilisation rate (OR 0.92, n= 2) d) Implantation rate (OR 0.82, n= 12) and e) Cycle cancellation rate (OR 1.50, n= 12)

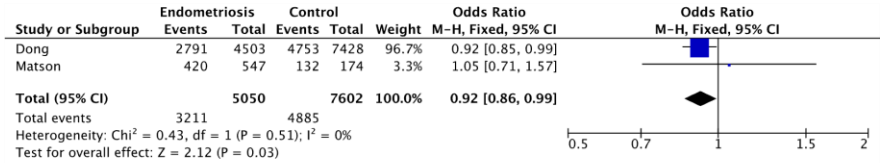
a)



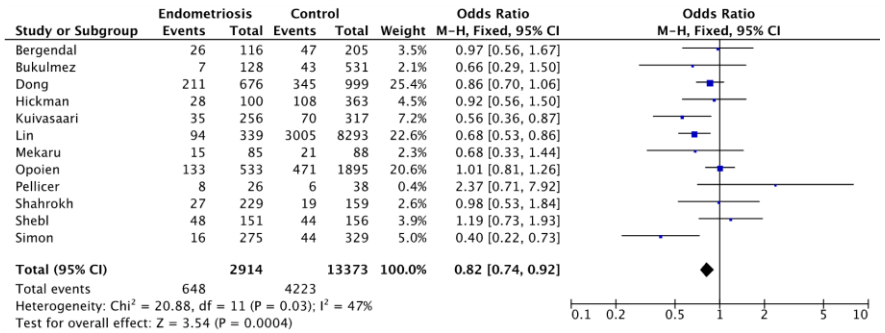
b)



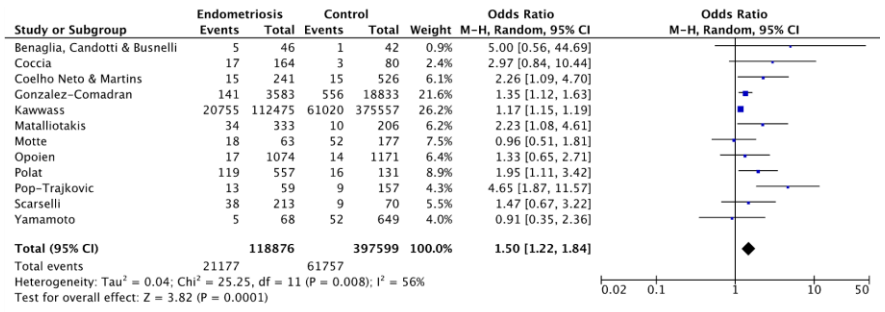
c)



d)

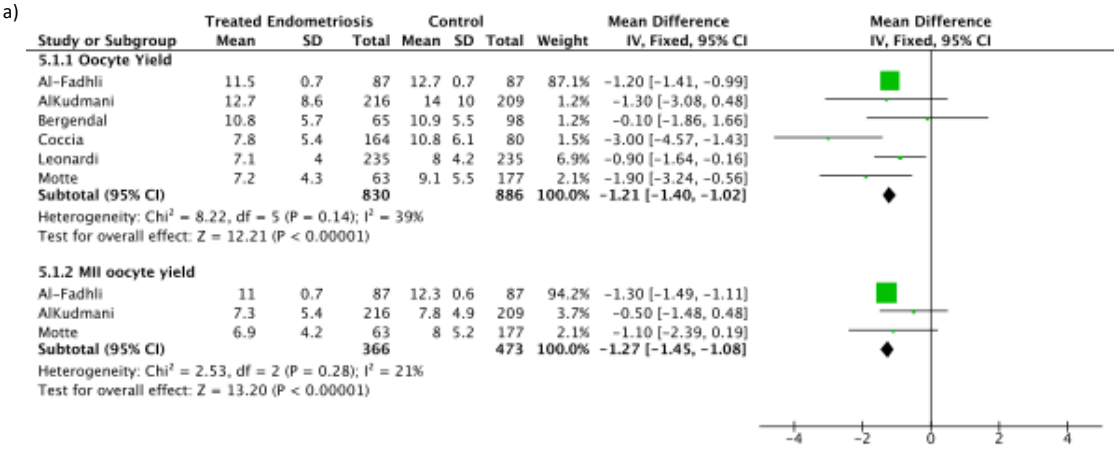


e)

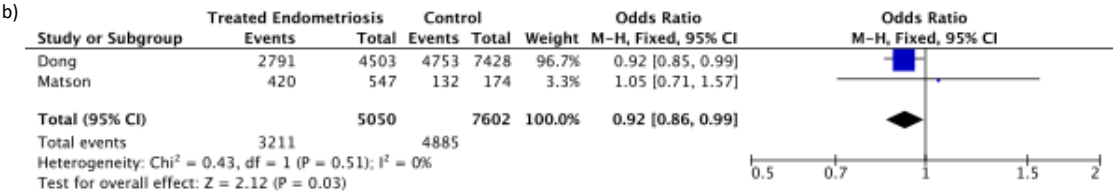


Appendix 7: Forest plots demonstrating IVF/ICSI outcomes in women with treated endometriosis compared to women without endometriosis a) Oocyte yield (OR -1.21, n= 6) and MII oocyte yield (OR -1.27, n= 3) and b) Fertilisation rate (OR 0.92, n= 2)

a)

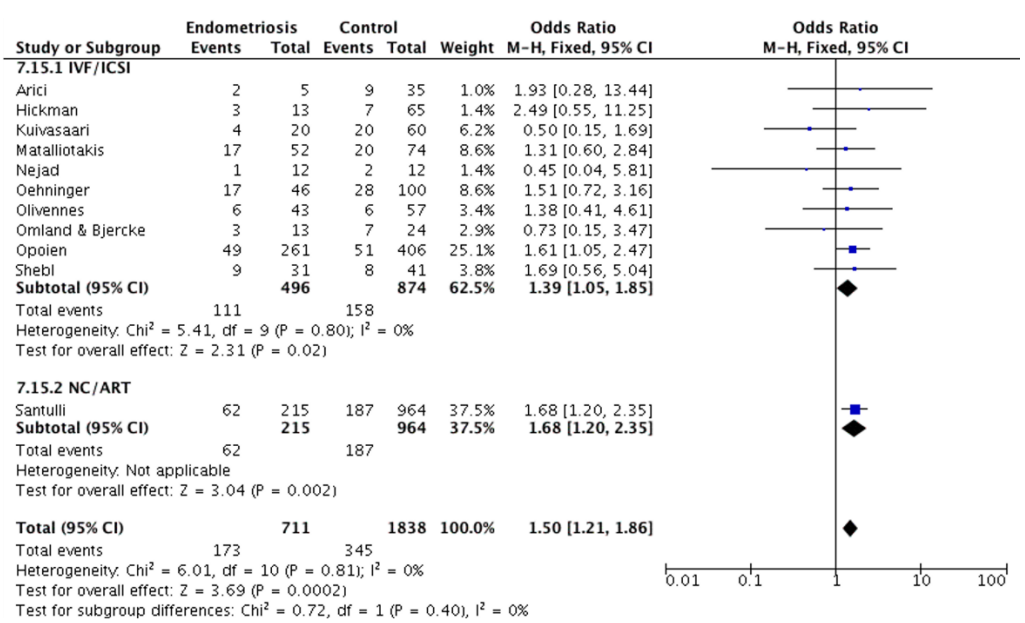


b)

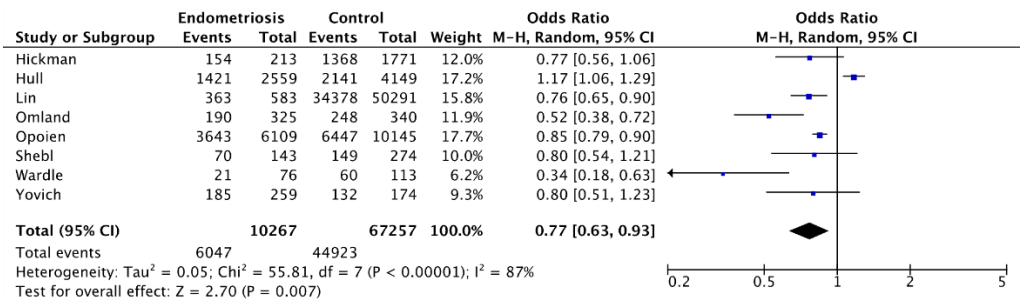


Appendix 8:Forest plots demonstrating outcomes of women with stage I-II endometriosis compared to women without endometriosis in IVF/ICSI studies a) Miscarriage rate (OR 1.39, n= 10) b) Fertilisation rate (OR 0.77, n= 8) c) Implantation rate (OR 0.76, n= 8) and d) cycle cancellation rate (OR 1.74, n= 4)

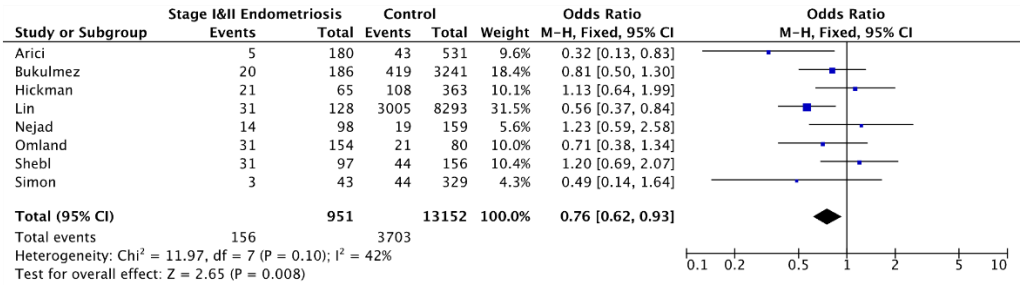
a)



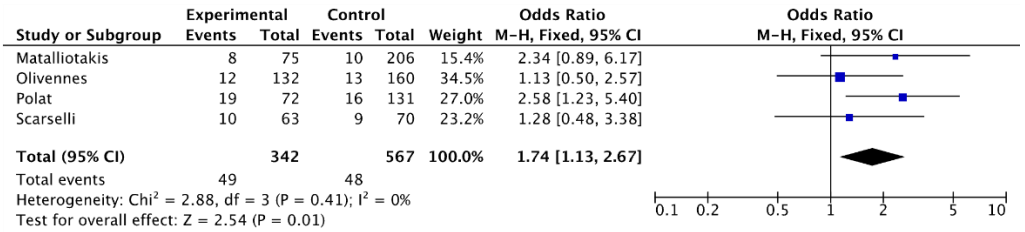
b)



c)

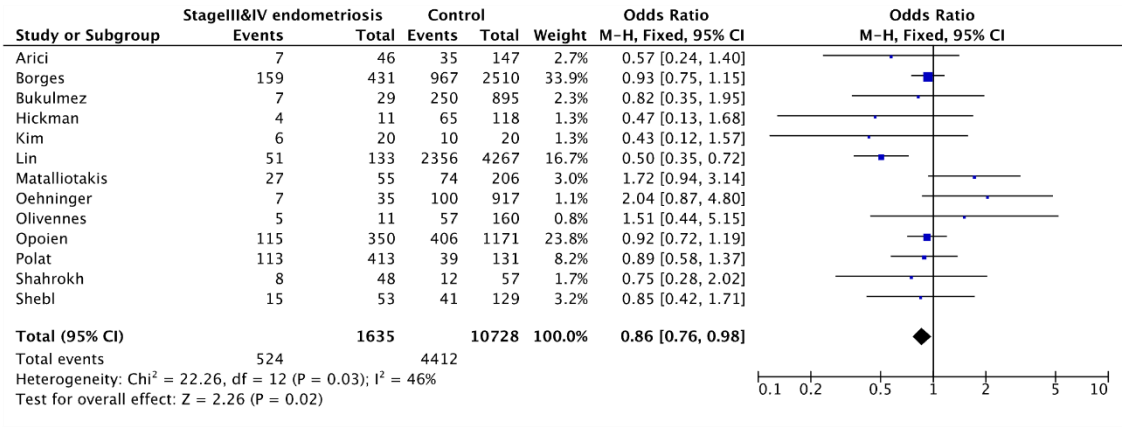


d)

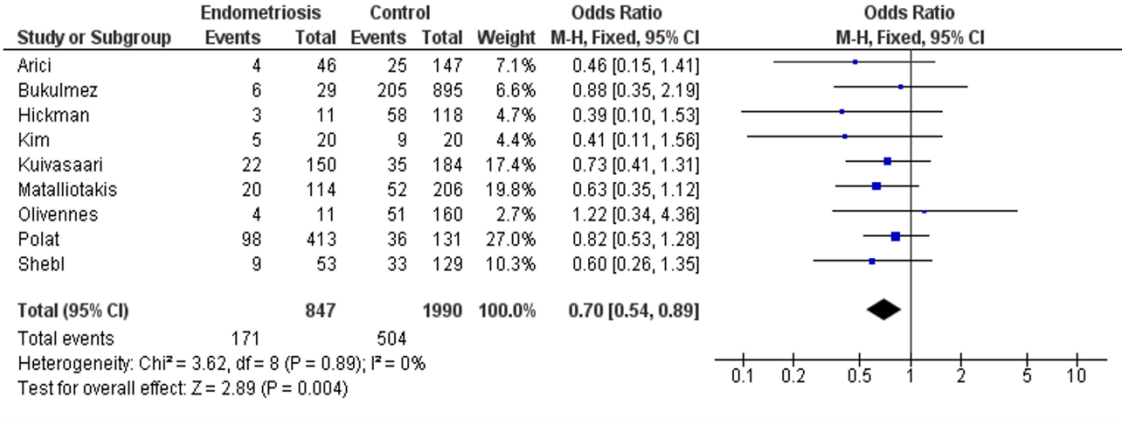


Appendix 9:Forest plots demonstrating secondary outcomes in women with stage III-IV endometriosis compared to women without in IVF/ICSI studies a) CPR (OR 0.86, n= 13) b) LBR (OR 0.70, n= 9) c) Miscarriage rate (OR 1.34, n= 9)

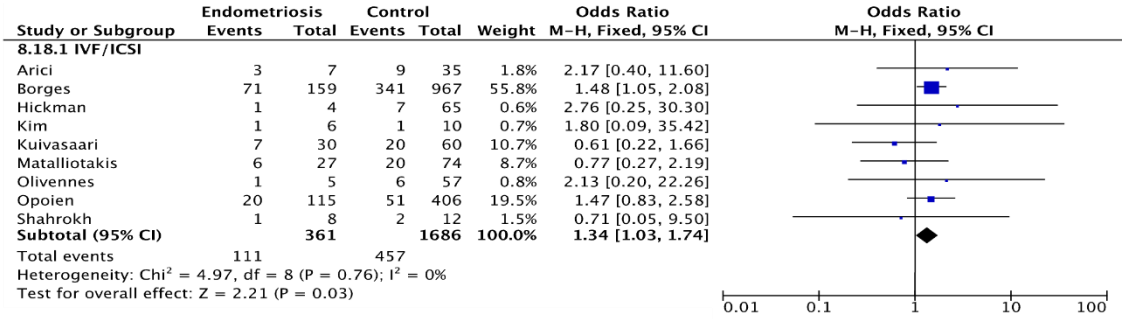
a)



b)

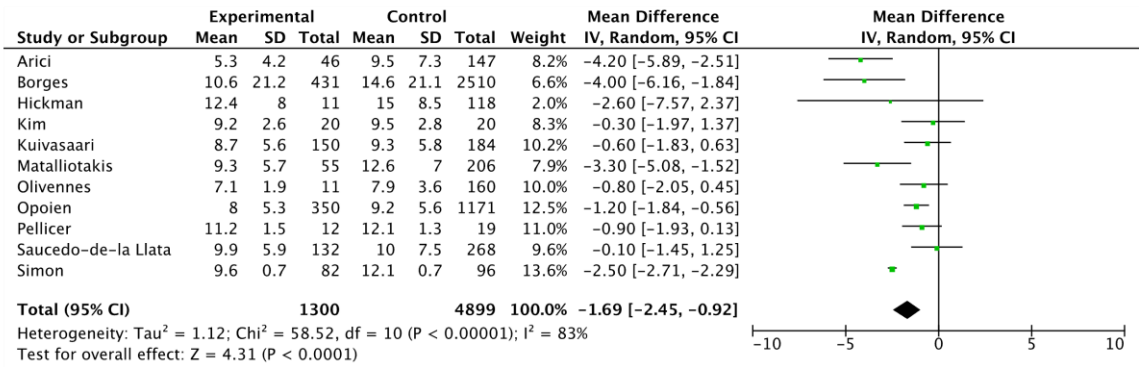


c)



Appendix 10:Forest plots demonstrating IVF/ICSI outcomes in women with stage III-IV endometriosis compared to women without endometriosis a) Oocyte yield (OR -1.69, n= 11) b) MII oocyte yield (OR -1.12, n= 3) and c) Implantation rate (OR 0.80, n= 11)

a)



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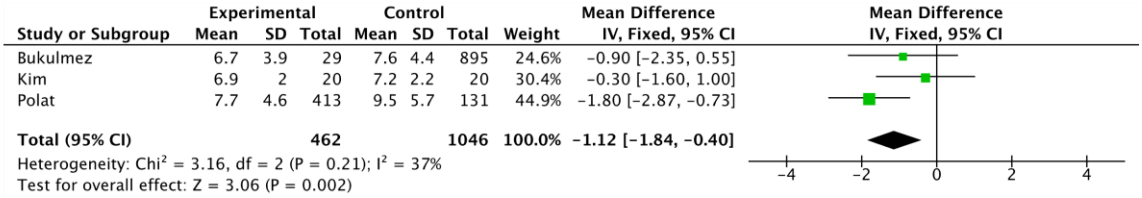

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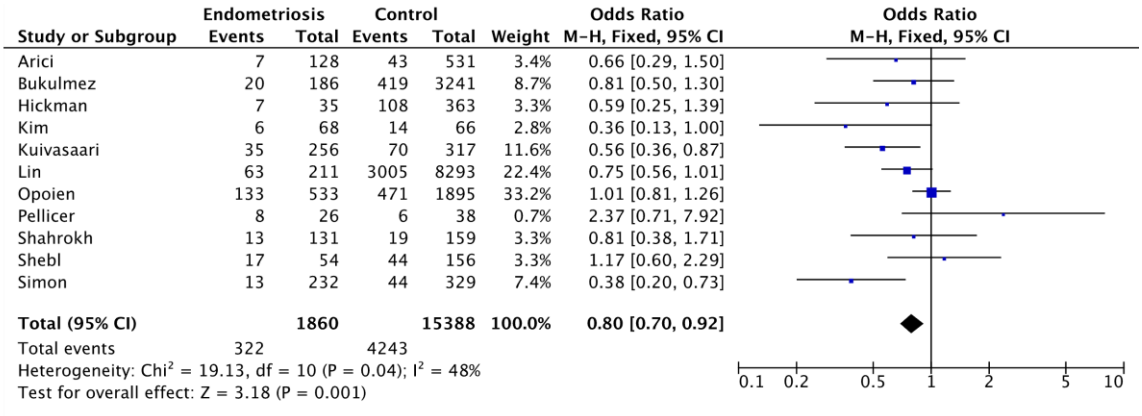
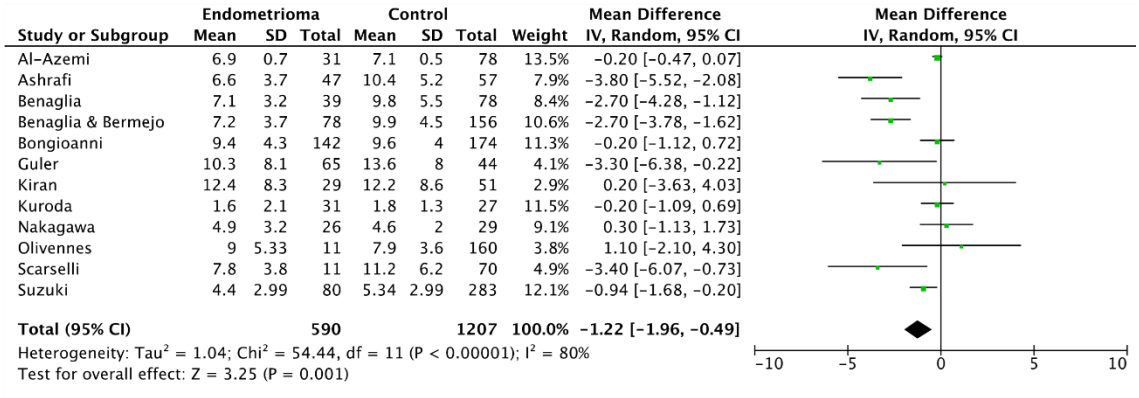
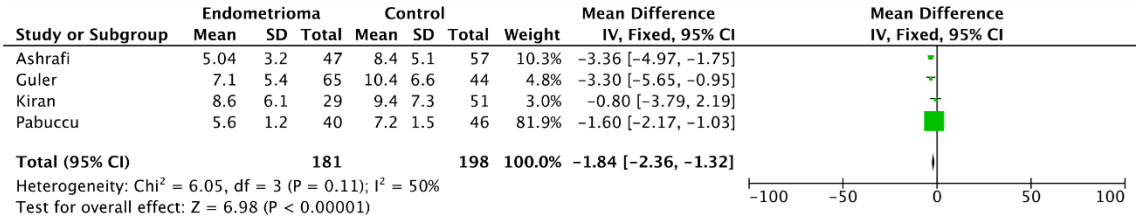


Appendix 11:Forest plots demonstrating IVF/ICSI outcomes in women with endometrioma compared to women without endometriosis a) Oocyte yield (OR -1.22, n= 12) b) MII oocyte yield (OR -2.24, n= 4)

a)



b)



Study Protocol

Research Team

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Miss Nida Ziauddeen, Postgraduate Research Student in Primary Care and Population Sciences, N.Ziauddeen@soton.ac.uk

Study Site

Princess Anne Hospital, University Hospitals of Southampton Foundation Trust.

Short Title:

Endometriosis as a Life Course Health Determinant.

Long Title:

Examining the life course health associations of endometriosis from fertility to early offspring outcomes in a population-based cohort.

Summary

The study aims to investigate the relationship between endometriosis and mode of conception, pregnancy complications and birth outcomes for mother and offspring.

Background & Study Rationale

Endometriosis is a chronic inflammatory disease defined by the presence of endometrial stroma and glands outside the uterine cavity. It affects up to 10% of reproductive age women and is present in 30-50% of women with infertility[1].

This condition negatively impacts on fertility via multiple pathophysiological effects that are not yet fully understood. At the anatomical level adhesions affecting pelvic organs and fallopian tubes in endometriosis can cause a mechanical interference with oocyte pick up and transportation[2]. Endometriosis patients are known to have a "pro-inflammatory" tendency from increased levels of macrophages, cytokines, vasoactive substances and reactive oxygen species in peritoneal fluid, follicular fluid and endometrial fluid. Folliculogenesis is also affected in endometriosis and abnormal gene expression in the endometrium causes changes in local oestrogen production and response to progesterone, which alter endometrial function [2,3]. Changes to the expression of implantation markers such as glycodelin and osteopontin could also affect the receptivity of the endometrium.

Fertility outcome has featured highly in literature with many studies on endometriosis being carried out in the assisted reproductive setting. Despite the amount of work in this area the impact of the disease on processes of folliculogenesis, oocyte quality, fertilization, implantation and embryo quality are still under debate.

Potential impact on post-implantation stages of reproduction is also less understood and longer follow-up for obstetric and neonatal outcomes is not often undertaken in the current literature. There is growing interest in developmental origin of health and disease (DOHaD) theory and knowledge that aberrant decidualisation and placentation within a disturbed uterine environment can be linked not only to problems relating to placental insufficiency but also childhood and adult diseases[4]. Evidence is increasing that the impact of endometriosis reaches beyond fertility and is associated with a number of obstetric, post-partum and neonatal complications. Some proposed mechanisms underlying the association are changes in the myometrial junctional zone leading to defective remodelling of the spiral arteries at placentation, changes to the receptivity of the endometrium affecting placentation and changes to prostaglandins[5]. Given the nature of the differences at the level of the endometrium known to exist between women with endometriosis, it is logical that placentation would be affected. The condition of endometriosis in the context of DOHaD has not yet been explored.

Aim & Objectives

This research aims to increase our understanding of the wider implications of endometriosis on the whole reproductive life course of a woman and her offspring.

Primary Objective: To investigate whether previous diagnosis of endometriosis is associated with later fertility complications, the antenatal/intra-uterine course, birth and the neonatal delivery outcome i.e. Apgar scores, for the woman and her offspring.

Secondary objectives:

- To examine whether other obstetric risks (maternal health, lifestyle and demographic characteristics) of women with history of endometriosis differ from those without endometriosis.
- To examine possible confounding gynaecological conditions known to impact on fertility and pregnancy outcomes to gain a better understanding of the relationship of these conditions to endometriosis and to fertility and pregnancy outcomes (recurrent miscarriages, fibroid uterus, infertility, polycystic ovary syndrome etc.).

Design

A population-based cohort study involving anonymised record linkage of women diagnosed with endometriosis at surgery with their subsequent antenatal and birth records.

Population

Women delivering in the maternity unit at Princess Anne Hospital, Southampton and women diagnosed with endometriosis at surgery performed at Princess Anne Hospital, Southampton. Women diagnosed with possible confounding gynaecological conditions of fibroid uterus, polycystic ovary syndrome, female infertility, miscarriage and recurrent miscarriage.

Sample Size

Endometriosis is known to affect ~10% of women of reproductive age and the whole population of maternity database is 96,489 women (84,219 records with complete data). We anticipate ~3,000 matched records between a surgical diagnosis of endometriosis and a maternity database delivery as some women may move out of area for maternity care or may not conceive and surgical HICSS database may not capture all those diagnosed with endometriosis if data was input as free text rather than by ICD 10 code. Non-endometriosis controls will be randomly selected in a 2:1 ratio based on power calculations and our expected number of cases.

For our secondary outcome analysis, records for confounding gynaecological conditions are anticipated to be 320-8000 per condition based on incidence of these conditions being 25% (fibroids), 14%(infertility), 2% (PCOS) and 1% (recurrent miscarriage) of the UK population and surgical HICSS database may not capture all those diagnosed.

Inclusion Criteria:

- All maternity records with birth outcome data between 2003 and 2018 at Princess Anne Hospital, Southampton.
- All gynaecological surgery records with a diagnosis of endometriosis entered between 2003 and 2018 at Princess Anne Hospital, Southampton
- All gynaecological surgery records with a diagnosis of recurrent miscarriages, miscarriage, fibroid uterus, polycystic ovary syndrome and infertility entered between 2003 and 2018 at Princess Anne Hospital, Southampton

Exclusion Criteria:

- Incomplete maternity or surgical records.

Screening

Assessment of eligibility will be performed by the data holder based on strict database search criteria.

Recruitment

Participants will not be recruited as this is a retrospective population-based cohort study using routinely-collected healthcare data.

Consent

Consent is not applicable as data will be fully anonymised by the data holder before releasing to the research team.

Data Collection

The data will be collected from a healthcare database routinely used for patient records including surgical and maternity notes by the data holder (university Hospitals of Southampton – UHS). Analysis will make use of routine healthcare care data collected by Gynaecological Surgery and Maternity Services at Princess Anne Hospital in Southampton. The anonymised dataset will be extracted from the HICSS (Hospital Integrated Clinical Support System) database for the period January 2003 to September 2018. The Princess Anne Hospital is a regional centre for gynaecology and fetal and maternal medicine covering Southampton and parts of Hampshire. It is a lower super output area but the hospital in this area covers a wide area of Hampshire for tertiary care services and has a reliable surgical and maternity database

Data Processing and Storage

Data is stored within the hospital site on a secure healthcare database. Data extraction will only be performed by the data holder (the UHS data informatics team). The research team will only have access to anonymised data. Access to the anonymised dataset will then be restricted to the research team and stored electronically on a password protected University of Southampton University of Southampton network drive. All paperwork, if any, will also be stored in secure research offices.

The dataset will be kept by the Academic Unit of Primary Care and Population Sciences for 15 years in line with Faculty of Medicine research guidelines.

Data analysis

Analysis of the anonymised dataset will be performed by the research team using Stata software. Parameters for fertility, antenatal, delivery and neonatal complications in women with endometriosis will be compared to those of women without endometriosis in univariable analyses with t-test for continuous variables and chi square test for categorical variables. These parameters will include parity, mode of conception, miscarriages, pre-eclampsia, intra-uterine growth restriction, preterm delivery, Apgar scores, mode of delivery etc.

Multivariable analysis will be performed to adjust for potential confounders of the endometriosis and pregnancy/birth outcome relationship e.g. smoking, alcohol intake and BMI at booking. Regression analysis, both linear and logistic regression, will be used to adjust for confounding factors in gynaecological conditions and maternal health and lifestyle characteristics.

Insurance matters

The research sponsor is the University of Southampton and their Insurance Policy will apply.

User involvement

Public and patient involvement in study design has not been sought due to the use of fully anonymised routinely-collected healthcare data and patients and public will not be directly involved or impacted upon by this study.

Dissemination of Research Findings

The results of this study will be published in medical and scientific journals as a source of information for other researchers, scientists and doctors. The results may also be presented at academic meetings or be published within scientific thesis such as a PhD.

Conflicts of Interest

None to declare

Funding

National Institute for Health Research (NIHR) and Clinical Research Network (CRN) funding has supported this study.

Study contact

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023 8120 6033

References

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Appendix 13: Peritoneal fluid biology in health and disease patient information leaflet

Study Information Sheet

Project Title:

Peritoneal fluid biology in health and disease

You are being invited to take part in a study because you may have the condition "endometriosis" and will be undergoing surgery as part of your routine medical care. We are currently running a research project looking at factors which may influence scar formation in the abdomen and female fertility. Before you decide whether or not you wish to take part, please read this information sheet which will explain the purpose of the study and what it will involve. Thank you for your time and please contact us for further information or questions you may have.

Purpose of the study

The study will aim to provide us with a better understanding of factors that may influence scar formation inside your abdomen and how fertility can be affected. This particular study is looking at the composition of peritoneal fluid in people with and without a condition called endometriosis. We are looking at how the peritoneal fluid and some other bodily fluids (such as blood and urine) differ in people with the condition and its effects on scar tissue and fertility.

Background information

The peritoneum is a membrane which surrounds the organs of the abdomen and pelvic cavity. The main role of the peritoneum is to regulate the normal function of the abdominal cavity by allowing the exchange of molecules and production of peritoneal fluid. It is this fluid that helps to maintain a protective environment in which intra-abdominal organs can function properly. When the peritoneum is injured (for example by surgery or from endometriosis), a series of responses involving cellular mediators such as macrophages and mesothelial cells attempt to restore the injured part of the peritoneum. These cells are present in the peritoneal fluid and the peritoneum. Adhesion formation typically occurs as a result of the peritoneal wound healing response in the first five to seven days following the injury. This can affect fertility if adhesions form around the

tubes and ovaries. The peritoneal fluid also surrounds the ovaries and tubes and can affect the quality of eggs when they have been released from the ovary (at ovulation). We want to look at different molecules and cells involved in these two damaging processes. We want to measure them in peritoneal fluid and other fluids of the body (urine/blood) and examine how they increase scar tissue and affect fertility.

What does participating in the study involve?

We may gather some health-related information from your medical records and may need to ask you some short questions about your menstrual cycle and medical history. This should take no more than 5 minutes.

On the day of your surgery the nurses will need to test a sample of your urine for signs of infection and possibly for pregnancy, we will use the rest of the sample that would usually be disposed of for our study. When you are having your surgery a cannula (small tube) will be put into a vein by your anaesthetist for giving you important medication throughout the operation, this is routine. We will take a blood sample from this tube. At the beginning of your operation a small sample of peritoneal fluid and sometimes peritoneum will be taken. Peritoneum is often removed as part of this surgery as a biopsy (to test for endometriosis) and peritoneal fluid is often removed and disposed of during this surgery to clean the surgical field or wash blood out from the surgery. The composition of the samples and their genetics as well as their effect on adhesions and fertility can then be analysed in the laboratory.

Will my details be confidential?

All of the findings of this study will be kept confidential in accordance with standards followed by medical researchers and in compliance with national law. Your samples will be anonymous so that anybody looking at the results of the study will not know that you were involved.

What will happen to my samples at the end of the study?

At the end of the study all of your samples will be destroyed in line with hospital and laboratory policy.

Do I have to take part?

It is entirely your choice whether or not you take part in this study. If you would like to take part you will be asked to sign a consent form.

What will happen to me if I take part?

If you decide to take part, we will ask you to give consent for us to take peritoneal fluid and tissue during your surgery. You do not need to have any additional cuts or delay your surgery in any way. Very often this fluid and scar tissue is removed during your surgery and thrown away as part of routine surgery. We will also ask for your consent to use some of your routine pre-surgery urine sample and for your consent to take a blood sample from the cannula (small tube) used to give you routine medication throughout the operation.

What will happen if I don't want to continue taking part after I have consented?

You may withdraw from the study at any time and this will not affect your future care in any way. If you withdraw from the study, we will destroy the samples of fluid/tissue taken from you but we would use the data collected up to the point of your withdrawal.

What are the benefits of taking part?

From this study we hope to gain better understanding of the mechanism involved in scar formation and reduced fertility in some people. In the long term we hope this work will contribute to treatment strategies to minimise the adverse effects of some components of peritoneal fluid.

What are the disadvantages to taking part?

The study will not affect the treatment you receive or the risks of your operation in any way. We may take 5 minutes of your time for some health-based questions and consenting.

What will happen to the information gathered by this study?

It is hoped that this information will be printed in a medical journal as a source of information for other researchers, scientists and doctors. The results may be presented at academic meetings and any information used in analysing the results would not be linked to you in any way.

What if there is a problem?

Any concerns or complaints you have about the way you have been approached or treated during the study will be taken seriously and addressed. Contact information is available from departmental staff in the first instance and you can find the contact information for the researchers below.

If you have concerns about the care provided to you or the Trust services, the Patient Support Service (PSS) may be contacted by telephone 023 8079 8498 or email PSS@uhs.nhs.uk. If you wish to make a formal complaint you may contact the Complaints Team in writing or by telephone on 023 8079 6361/023 8079 6299.

What will happen to my samples at the end of the study?

At the end of the study your samples will be destroyed in line with hospital and laboratory policy.

Who is organising the funding the research?

It is organised and funded jointly by University of Southampton and University Hospitals of Southampton NHS Foundation Trust.

Who has approved this study?

All research in the NHS is looked at by an independent group of people call a research ethics committee to protect your safety, rights, wellbeing and dignity. This study has been given a favourable opinion by South Central – Southampton ethics committee.

Who can I speak to for more information or if I have questions?

Main investigator:
Professor Ying Cheong, Gynaecology Consultant and Professor of Reproductive Medicine, University of Southampton
E-mail: y.cheong@soton.ac.uk
Telephone: 02380 796033
Co-investigator:
Dr Joanne Horton, Clinical Research Fellow and Obstetrics and Gynaecology Specialist Registrar.
E-mail: J.M.Horton@soton.ac.uk
Telephone: 02380 796044

Appendix 14: Peritoneal fluid biology in health and disease patient invitation letter

Research Offices
F Level
Princess Anne Hospital
Coxford Road
Southampton
SO50 AYZ
Date: _____

Patient name
Patient Address

Dear *Miss/Mrs _____,*

The gynaecology department is keen to improve medical knowledge and patient care and is therefore active in medical research. You will soon be having your planned gynaecological surgery at the Princess Anne Hospital and we would like to invite you to take part one of our research studies.

This study is for women who may have a condition called endometriosis. If taking part in research is something you would like to consider please read the information sheet enclosed.

On the day of your surgery your doctor will ask if you would like to see a member of the research team. If you would like to take part a researcher will be on hand to answer any questions you may have. Your care will not be affected in any way whether you decide to participate or not and the study will not take up any of your own time.

Kind Regards,

Dr Joanne Horton
Clinical Research Fellow to Professor Ying Cheong
Consultant Gynaecologist and Specialist in Reproductive Medicine

Appendix 15: Peritoneal fluid biology in health and disease patient consent form

University of Southampton

University Hospital Southampton NHS
NHS Foundation Trust

CONSENT FORM

Title of study: Peritoneal Fluid Biology in Health and Disease

Study number:

Name of researcher: Dr Joanne Horton

Name of Principal Investigator: Prof Ying Cheong

Participant ID:

Thank you for reading the information about our research project. If you would like to take part, please read and sign this form.

PLEASE INITIAL THE BOXES IF YOU AGREE WITH EACH SECTION:

1. I have read the information sheet version 8 dated 24/03/2018 for the above study. I have had the opportunity to consider the information, ask questions and have had these answered satisfactorily. ☐

2. I understand that my participation is voluntary and that I am free to withdraw at any time without giving any reason, without my medical care or legal rights being affected. ☐

3. I agree to a sample of urine, blood and peritoneal fluid to be taken for research in this study. I understand how the samples will be collected and that the samples will be destroyed on completion of the study. ☐

4. I agree to have a biopsy of adhesion and peritoneal tissue taken for research in this study. I agree for the tissue to be genetically analysed. I understand how the biopsies will be taken and that the tissue will be destroyed on completion of the study. ☐

5. I understand that relevant sections of my medical notes and data collected during the study may be looked at by individuals from University Hospital Southampton, NHS Foundation Trust where it is relevant to me taking part in this research. I give permission for these individuals to have access to my records. ☐

6. I agree to participate in this study ☐

Name of Participant	Date	Signature

Name of researcher taking consent:	Date	Signature

Appendix 16: Peritoneal fluid biology in health and disease study protocol

Peritoneal Fluid Biology in Health and Disease

Aims and objective:

Characterise the composition of peritoneal fluid PLs in women with various adhesive pelvic pathology

Examine the PL composition of peritoneal macrophages and mesothelial cells

Develop an *in vitro* wound healing model to examine the PLs dependent wound healing potential of mesothelial cells.

Characterise the composition of peritoneal fluid and adhesion tissue in patients with adhesions and endometriosis and its effects on fertility.

This study will aim to provide us with a better understanding of how the composition of peritoneal fluid and adhesion tissue differs in healthy and diseased individuals; and can aid in the future assessment of possible therapeutic mixes on fertility and intra-abdominal wound healing in benign and malignant intra-abdominal conditions.

Background and literature review:

Peritoneal adhesion formation and reformation is a consequence of intra-abdominal surgery and is associated with a significant morbidity postoperatively. The most common consequences of peritoneal includes, recurrent episodes of chronic abdominal and pelvic pain, small-bowel obstruction that frequently require hospitalisation and infertility[1,2,3]. Although there are many causes of adhesions, including bacterial peritonitis, radiotherapy, chemical peritonitis, long-term continuous ambulatory peritoneal dialysis, foreign body reaction, endometriosis and pelvic inflammation. The majority of adhesions are produced following surgery, occurring in up to 90% of patients following a major abdominal surgery and in 55-100% of women undergoing pelvic surgery[1]. Much interest has been focused on reducing the incidence of postoperative adhesion, however the lack of understanding of the complex molecular mechanisms involved in adhesion formation and reformation hinders the search for effective treatment strategies[1]. There is growing evidence that the composition of peritoneal fluid in patients with pelvic pathology differs in a vast number of ways for example the levels of many different glycoproteins, hormones, growth factors and immunological cells[4]. Reactive oxygen species have often been implicated in both endometriosis and as a consequence of surgery and wound healing [5], but their stability in bodily fluids has made them difficult to accurately study. It is well known that reactive oxygen species damage the integrity of DNA and disrupt the normal function of a variety of cell types. There is evidence that reactive oxygen species can in a similar way, affect fertility[6].

The peritoneum and the peritoneal fluid:

The peritoneum is a serous membrane surrounding the organs of the abdomen and pelvic cavity. The visceral peritoneum lines over the organs whilst the parietal pericardium lines the wall of the abdominal cavity. The main role of the peritoneum is to maintain and control the normal homeostasis of the abdominal cavity by allowing, the exchange of molecules and production of

peritoneal fluid. This fluid circulates within the abdominal cavity and helps to maintain a protective environment in which intra-abdominal organs can function efficiently[1].The peritoneal fluid contains macrophages, lymphocytes, polymorphonuclear leukocytes and a continuous sheet of mesothelial cells which are attached to the basement membrane. The submesothelial layers consist of extracellular matrix (ECM), made up of collagen, glycoproteins (i.e. laminin and fibronectin), glycosaminoglycans and proteoglycacans[7].

When the peritoneum is injured, whether by surgery or as a result of inflammatory processes such as endometriosis a series of events take place, to help restore the injured part of the peritoneum. It is likely that the activity of cellular mediators found in the peritoneal fluid, such as macrophages and mesothelial cells may have an important role to play in peritoneal healing. In the normal female pelvic, the volume and composition of peritoneal fluid changes during the menstrual cycle, reaching maximum level after ovulation. Furthermore, the amount of peritoneal fluid also increases in pelvic pathology, during postsurgical repair or following and inflammatory insult[7].

Adhesion formation typically occurs as a result of peritoneal wound healing response in the first five to seven days following injury. Several studies have demonstrated that adhesion formation involves important components such as acute inflammatory response, fibrinolysis and metalloproteinases and their tissue inhibitors. The initiation of adhesion formation takes place during coagulation which initiates a cascade of events resulting in the formation of a fibrin matrix[7]. The fibrin matrix connects two injured peritoneal surfaces forming fibrin bands. These fibrin bands can be broken down by fibrinolytic enzyme systems of the peritoneum, such as the plasmin system, however surgery dramatically diminishes fibrinolytic activity, resulting in persistent formation of fibrin bands. Current literature studies suggests that pro-inflammatory cytokines such as interleukin (IL)-1, IL-6 and tumour necrosis factor (TNF)- α are involved in adhesion formation and reformation[1]. Many studies have also shown that simple factors such as disrupted sleep patterns can impact on the mechanisms related to the adhesion formation/ healing pathways [8, 9, 10, 11,12]. Therefore it is important to document these as part of the demographic details of the participants as part of the study.

<u>Phospholipids and their effect on peritoneal healing</u>

Phospholipids (PLs) play an important role in many biochemicals, physiological and pathological processes. In humans, PLs lines the mesothelium of healthy individuals and functions like a liquid barrier to prevent surfaces from adhering to each other. These lipids also play an important role in extracellular cells signalling molecules. Furthermore, PLs have been shown to be modifiers of macrophage differentiation and function, and thus can have an impact on peritoneal immune function and peritoneal healing[13].

By some mechanisms yet unknown, the phospholipids content of the peritoneal fluid changes in intra-abdominal pathological conditions such as endometriosis and ovarian malignancy[2]. These changes can be a result of an intrinsic change in PLs secretion by the mesothelial cells or an increase in filtration via the serum. In women with pelvic pathology such as advance endometriosis or malignancy, extensive adhesions often occur. Adhesion formation is a known consequence of aberrant healing of the peritoneum. It is yet unknown how PLs contribute to this aberrant intra-abdominal healing.

A number of previous studies have examined factors which may influence adhesion formation/reformation such as cytokine concentration[14], however no studies have yet investigated the relationship between the concentration of peritoneal fluid PLs and pelvic adhesion formation.

Materials and Methods:

Study:

This study will be performed in the Princess Anne Hospital, Southampton. It is a pilot study, which involves both clinical and laboratory work. The study will involve women undergoing laparoscopy and laparotomy for gynaecological conditions in the following subgroups:

endometriosis,

normal pelvises

adhesions

gynaecological cancers (specifically ovarian cancer)

subfertility

The study will be completed when n=500 is reached, or when the laboratory experiments are completed.

Recruitment methods:

The subjects will be recruited from the gynaecological departments in Princess Anne Hospital.

Inclusion criteria

Women undergoing surgery (laparoscopy or laparotomy) for gynaecological conditions

Ages between 18-65 years.

Exclusion criteria:

Subjects will be excluded if they've had:

Any acute abdominal symptoms indicative of acute pelvic pathology (for example: If they have a clinical history or laboratory evidence of pelvic inflammatory disease).

Consent:

Patients will receive a patient information leaflet prior to their operation and will have the opportunity to discuss participation with a named investigator for the study. They will have the opportunity to consider participating in the study and will have adequate time to ask any questions they may have which will be answered by a named investigator. Informed written consent will be obtained according to Good Clinical Practice policies.

Process:

Prior to surgery, the pelvic cavity will be inspected for adhesion formation/reformation and the adhesion will be scored using the well – validated adhesion scoring method (MCASM)[9]. The pelvis will also be examined for endometriosis and if present will be assessed by validated ARSM staging system.

We may also require the patients to fill up two short validated sleep questionnaires to assess the possibility of disrupted circadian rhythm on adhesion formation and healing. If indicated these will be completed at the patients' leisure as part of the recruitment process.

Specimen preparation

Peritoneal fluid will be collected during laparoscopy using a laparoscopic aspiration needle or other 20ml suction device under direct vision, at the beginning of the procedure, prior to the commencement of any pelvic surgery as per WERF-EPHect Standard Operating Procedure for collecting, processing and storage of biospecimens[15].

In some cases, a blunt probe may be used to move away bowel to expose the pouch of Douglas for collection of the peritoneal fluid.

Care must be taken not to contaminate the peritoneal fluid with blood.

The collected fluid will be centrifuged at 900g x 5 minutes and the supernatant and pellet frozen in liquid nitrogen and stored at -80^{0}C until analysis as per WERF-EPHect SOPs[15].

Peritoneal macrophages and mesothelial cells will be separated by differential adherence in short term culture.

Once the initial parameters of the PLs composition have been established, isolated mesothelial cells from peritoneal fluid will be maintained in primary culture to assess the effects of fluid components in a wound healing model.

The extent to which the biochemical composition of the peritoneal fluid is influenced by blood composition as opposed to more local factors will be determined by analysis of the cellular and plasma components of parallel blood samples.

Blood samples will be collected pre-operatively when patients have their routine pre-operative bloods performed or at cannulation for administration of anaesthetic agents. It will be centrifuged at 2500g for 10 minutes and plasma/white blood cell/red blood cell layers aliquoted and frozen at -80^{0}C as per WERF-EPHect SOPs[15].

Urine will be collected as a clean catch sample or from sterile in/out catheter at the beginning of the operation as per routine practice. A urinalysis will be performed and recorded. The urine will be centrifuged at 2500g for 10 minutes and supernatant and pellet aliquoted and stored at -80^{0}C as per WERF-EPHect SOPs[15].

Adhesions, if present and small amount of surrounding peritoneum will be excised using laparoscopic techniques for research purposes. These adhesions will normally be excised for clinical reasons and the tissue will normally be disposed of. This tissue will be disposed of when the research project is complete.

Resected tissues will be sent to pathology department routinely, where they will be processed in usual way by the pathologist

Excess tissues that were archived in paraffin blocks, not necessary for clinical diagnosis will be used for this research.

These tissues will be further processed and stained for various markers to determine the expression of different proteins (e.g. osteopontin, integrin)

Analysis of adhesion tissue will focus on biochemical, molecular and

cellular composition. This will be ascertained through molecular

biological techniques.

Specimen storage:

The collected specimens will be labelled and frozen in a liquid nitrogen and stored at -80^{0}C until analysis as per WERF-EPHect SOPs[15].

Anonymisation process

All subjects will remain anonymous, only subject age and their initial will be used, to ensure patient confidentiality.

Laboratory assay

Electrospray ionization mass spectrometry will be used to analyse the molecular species compositions of both peritoneal fluid supernatants and parallel blood plasma samples.

This comparison will establish both the extent to which peritoneal PL is an ultrafiltrate of blood or is produced locally and the effects of inflammation on peritoneal PL composition

Similar analyses will also determine the effects of inflammation on PL composition of isolated macrophage and mesothelial cells.

Correlation of PL compositions between peritoneal fluid supernatant and mesothelial cells will provide an indication of any precursor: product relationship.

This will be explored further in cultured mesothelial, which will also be used in a wound healing model to assess the effects of any differentially expressed factors identified by biochemical analysis of the peritoneal fluid supernatants.

Reactive oxygen species levels in the peritoneal fluid, urine and serum will be measured using a variety of techniques including chemiluminescence.

Peritoneal fluid will be used for in vitro oocyte (egg) culture using mouse oocytes and its impact on mouse DNA/RNA damage will be assessed.

Tissue undergoing adhesion composition characterization will be subject to RNA extraction techniques and Polymerase Chain Reaction for amplification.

Samples will be stained using immunohistochemistry for different protein for example (but not limited to) osteopontin, integrin, and prostaglandin.

Data recording:

The findings at surgery will be recorded on a WERF-EPHECT standard or minimum surgical form[16]. This will include: The last menstrual period, volume of the peritoneal fluid collected, colour of the peritoneal fluid collected, the presence or absence of endometriosis and adhesions using (MCASM and ASRM)[7].

Data analysis:

The data will be analysed using SPSS

Results will be expressed as the mean and SEM

The difference in the concentrations of the saturated phospholipid will analysed,

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Appendix 17: XSESS study patient information leaflet

Study Information Sheet

Project Title:

XSESS Study (oXidative Stress in women with Endometriosis pre and poSt Surgery)

You are being invited to take part in a study because you may have the condition "endometriosis" and will be undergoing surgery as part of your routine medical care. We are currently running a research project looking at factors which may influence development of the disease and its impact on female fertility. Before you decide whether or not you wish to take part, please read this information sheet which will explain the purpose of the study and what it will involve. Thank you for your time and please contact us for further information or questions you may have.

Purpose of the study

This study is looking at levels of oxidative stress which is an imbalance of "free radicals" (temporarily altered oxygen and nitrogen molecules) in the body, which can occur in many different common conditions including endometriosis. We aim to study oxidative stress (the altered oxygen and nitrogen molecules) in some bodily fluids of women with endometriosis compared to people without the disease and investigate if surgical treatment can reduce the oxidative stress levels. The study will aim to provide us with a better understanding of factors that may cause the disease to develop/progress and how fertility can be affected as a consequence of the disease.

Background information

Endometriosis is a gynaecological condition characterised by the presence of endometrial tissue (tissue lining the womb) developing outside the womb. The extra endometrial tissue is commonly found in the female pelvis but occasionally can occur in other places. We find this condition in 10% of the female population and it can cause pelvic pain and infertility. Oxidative stress (altered oxygen and nitrogen molecules of the body) might be one of the causes of the disease and it is known to damage female fertility through damaging DNA in the oocytes (eggs). Oxidative stress

can also damage the peritoneum (lining of the abdomen) contributing to scar tissue formation which can cause pelvic pain and also reduce fertility. Markers of oxidative stress have been found to be higher in endometriosis patients in blood, urine, peritoneal fluid (fluid inside the abdomen), follicular fluid (fluid inside follicles of the ovary) and tissue of the ovary and lining of the womb. It is unknown whether surgical treatment of endometriosis reduces the levels of oxidative stress but it has been demonstrated in other diseases such as after varicocelectomy (treatment of varicose veins of the testicles).

What does participating in the study involve?

We will gather some health-related information from your medical records which will be relevant to the surgery you are having and your levels of oxidative stress. This information will be your age, current symptoms, where you are in your menstrual cycle, smoking history, other medical conditions you have, pregnancy history, body mass index and medication you are on. We may need to ask you some short questions if this information is not in your medical records. We would also like to give you a short questionnaire about your pelvic pain. This should take no more than 5 minutes.

When you are having your surgery a cannula (small tube) will be put into a vein by your anaesthetist for giving you important medication throughout the operation. We will take a blood sample from this tube. When you are under general anaesthetic the surgeon will routinely empty your bladder with a catheter to prevent injury to the bladder during the surgery, we will take a small amount of this urine for the study. At the beginning of your operation a small sample of peritoneal fluid will be taken. Peritoneal fluid is often removed and disposed of during this surgery to clean the surgical field or wash blood out from the surgery. At your routine follow up appointment 3-4 months after your surgery we would like to repeat your pelvic pain questionnaire and take a small sample of your urine and blood. Oxidative stress levels of all the samples and their effect on fertility can then be analysed in the laboratory.

Will my details be confidential?

All of the findings of this study will be kept confidential in accordance with standards followed by medical researchers and in compliance with national law. Your samples will be anonymous so that anybody looking at the results of the study will not know that you were involved. The samples will

be kept in locked research premises at the Princess Anne Hospital and the University of Southampton laboratory. Personal and medical information and data collected will be stored on a secure, password protected, University of Southampton electronic network and accessed only by the research team. Paperwork for the study including consent forms will be stored in locked research offices at the Princess Anne Hospital.

Do I have to take part?

It is entirely your choice whether or not you take part in this study. Your care will not be affected in any way and you will not be treated any differently if you choose not to take part. If you would like to take part you will be asked to sign a consent form.

What will happen to me if I take part?

If you decide to take part, we will ask you to complete a pelvic pain questionnaire and some short medical questions which will take no longer than 5 minutes. We will ask you to give consent for us to take peritoneal fluid, urine and blood during your surgery and for you to give us a urine sample and blood test at your follow up appointment. Your normal treatment and care will not be affected in any way.

What will happen if I don't want to continue taking part after I have consented?

You may withdraw from the study at any time and this will not affect your future care in any way. If you withdraw from the study, we will destroy the samples of fluid/tissue taken from you but we would use any scientific data collected up to the point of your withdrawal.

What are the benefits of taking part?

From this study we hope to gain better understanding of the wider benefits of endometriosis surgery and provide a further insight into free-radical tissue damage of endometriosis.

What are the disadvantages to taking part?

The study will not affect the treatment you receive or the risks of your operation in any way. We may take 5 minutes of your time for some health-based questions and consenting on the day of

your surgery and we will ask for another 5-minute questionnaire and a blood and urine test at your follow up appointment.

What will happen to the information gathered by this study?

It is hoped that this information will be printed in a medical journal as a source of information for other researchers, scientists and doctors. The results may be presented at academic meetings and any information used in analysing the results would not be linked to you in any way. If you would like to know the results of the study, we will be glad to send you a copy of any written reports and you may discuss the results with us at any time.

What if there is a problem?

Any concerns or complaints you have about the way you have been approached or treated during the study will be taken seriously and addressed. Contact information is available from departmental staff in the first instance and you can find the contact information for the researchers below.

If you have concerns about the care provided to you or the Trust services, the Patient Support Service (PSS) may be contacted by telephone 023 8079 8498 or email PSS@uhs.nhs.uk. If you wish to make a formal complaint you may contact the Complaints Team in writing or by telephone on 023 8079 6361/023 8079 6299.

What will happen to my samples at the end of the study?

At the end of the study your samples will be destroyed in line with hospital and laboratory policy.

Who is organising the funding the research?

It is organised and funded jointly by University of Southampton and University Hospitals of Southampton NHS Foundation Trust.

Who has approved this study?

All research in the NHS is looked at by an independent group of people call a research ethics committee to protect your safety, rights, wellbeing and dignity. This study has been given a favourable opinion by South Central – Hampshire B ethics committee.

What happens to any personal or medical information about me in this study?

University Hospital Southampton NHS Foundation Trust is the sponsor for this study based in the United Kingdom. We will be using information from you and your medical records in order to undertake this study and will act as the data controller for this study. This means that we are responsible for looking after your information and using it properly. University Hospital Southampton NHS Foundation Trust will keep identifiable information about you for 5 years after the study ends.

Your rights to access, change or move your information are limited, as we need to manage your information in specific ways in order for the research to be reliable and accurate. If you withdraw from the study, we will keep the information about you that we have already obtained. To safeguard your rights, we will use the minimum personally-identifiable information possible.

You can find out more about how we use your information at www.uhs.nhs.uk/ClinicalResearchin Southampton/Public-and-patients/Get-involved/General-Data-Protection-Regulation.aspx or by contacting the data protection office on 020 8120 4743.

University Hospital Southampton NHS Foundation Trust will use your name, hospital number, date of birth and contact details to contact you about the research study, and make sure that relevant information about the study is recorded for your care, and to oversee the quality of the study. Individuals from University Hospital Southampton NHS Foundation Trust and regulatory organisations may look at your medical and research records to check the accuracy of the research study. Princess Anne Hospital will pass these details to University Hospital Southampton NHS Foundation Trust along with the information collected from you and your medical records. The only people in University Hospital Southampton NHS Foundation Trust who will have access to information that identifies you will be people who need to contact you to or audit the data collection process. The people who analyse the information will not be able to identify you and will not be able to find out your name, hospital number, date of birth or contact details.

University Hospital Southampton NHS Foundation Trust will collect some information about you for this research study from your medical records. This information will include your name, date of birth, hospital number, address and health information, which is regarded as a special category of information. We will use this information to contact you if you have indicated that you would like results of the study and to record your participation in the study on the Hospital's clinical research management system.

When you agree to take part in a research study, the information about your health and care may be provided to researchers running other research studies in this organisation and in other organisations. These organisations may be universities, NHS organisations or companies involved in health and care research in this country or abroad. Your information will only be used by organisations and researchers to conduct research in accordance with the UK Policy Framework for Health and Social Care Research.

This information will not identify you and will not be combined with other information in a way that could identify you. The information will only be used for the purpose of health and care research, and cannot be used to contact you or to affect your care. It will not be used to make decisions about future services available to you, such as insurance.

Who can I speak to for more information or if I have questions?

Main investigator:

Professor Ying Cheong, Gynaecology Consultant and Professor of Reproductive Medicine, University of Southampton
E-mail: y.cheong@soton.ac.uk
Telephone: 02380 796033

Co-investigator:

Dr Joanne Horton, Clinical Research Fellow and Obstetrics and Gynaecology Specialist Registrar.
E-mail: J.M.Horton@soton.ac.uk
Telephone: 02380 796044

Appendix 18: XSESS study patient invitation letter

Research Offices F Level
Princess Anne Hospital
Coxford Road
Southampton
SO50 AYZ

Date: _____

Patient name
Patient Address

Dear *Miss/Mrs _____,*

The gynaecology department is keen to improve medical knowledge and patient care and is therefore active in medical research. You will soon be having your planned gynaecological surgery at the Princess Anne Hospital and we would like to invite you to take part one of our research studies.

This study is for women who may have a condition called endometriosis. If taking part in research is something you would like to consider please read the information sheet enclosed.

On the day of your surgery your doctor will ask if you would like to see a member of the research team. If you would like to take part a researcher will be on hand to answer any questions you may have. Your care will not be affected in any way whether you decide to participate or not and the study will not take up any of your own time.

Kind Regards,

Dr Joanne Horton

Clinical Research Fellow to Professor Ying Cheong

Consultant Gynaecologist and Specialist in Reproductive Medicine

CONSENT FORM

Title of study: XSESS (o<u>X</u>idative <u>S</u>tress in women with <u>E</u>ndometriosis pre and po<u>S</u>t <u>S</u>urgery)

Study number:
Name of researcher: Dr Joanne Horton
Name of Principal Investigator: Prof Ying Cheong
Participant ID:

Thank you for reading the information about our research project. If you would like to take part, please read and sign this form.

PLEASE <u>INITIAL</u> THE BOXES IF YOU AGREE WITH EACH SECTION:

1. I have read the information sheet version 1 dated 01/05/2018 for the above study. I have had the opportunity to consider the information, ask questions and have had these answered satisfactorily. ☐

2. I understand that my participation is voluntary and that I am free to withdraw at any time without giving any reason, without my medical care or legal rights being affected. ☐

3. I agree to a sample of urine, blood and peritoneal fluid to be taken at the time of my surgery and for urine and blood to be collected at my follow up review for research in this study. ☐

4. I understand how the samples will be collected and that the samples will be destroyed on completion of the study. ☐

5. I understand that relevant sections of my medical notes and data collected during the study may be looked at by individuals from University Hospital Southampton, NHS Foundation Trust where it is relevant to me taking part in this research. I give permission for these individuals to have access to my records. ☐

6. I agree to participate in this study ☐

Name of Participant	Date	Signature

Name of researcher taking consent:	Date	Signature

Study Protocol

Research Team
Lead Investigator: Professor Ying Cheong, Gynaecology Consultant and Professor of Reproductive Medicine, University of Southampton.
Co-investigator: Dr Joanne Horton, Clinical Research Fellow and Obstetrics and Gynaecology Specialist Registrar.

Study Site
Princess Anne Hospital, University Hospitals of Southampton Foundation Trust.

Short Title:
Oxidative stress in endometriosis

Long Title:
OXidative Stress in women with Endometriosis pre and poSt Surgery (XSESS Study)

Summary
The study aims to investigate the impact of surgery on oxidative stress levels before and after surgical treatment in women with endometriosis.

Background & Study Rationale
Endometriosis is a gynaecological condition characterised by the presence of endometrial tissue outside the uterine cavity. The ectopic endometrial tissue is commonly found in the female pelvis but occasionally can occur in distant sites such as abdominal scar sites, the diaphragm and lungs. It is understood to be an oestrogen dependant disease therefore affecting women between menarche and menopause. Prevalence is believed to be up to 10% of women in their reproductive years and prevalence is as high as 35-50% of women with infertility or pelvic pain.

Oxidative stress has been implicated in one of the pathophysiological processes involved in endometriosis. Markers of oxidative stress have been found to be elevated in endometriosis patients in serum, urine, peritoneal fluid, follicular fluid, ovarian cortex and both ectopic and eutopic endometrial tissue [1]. Peritoneal production of ROS and macrophage degradation of erythrocytes releasing heme and iron have been suggested causes of elevated levels of ROS. Oxidative stress can damage peritoneal mesothelium contributing to adhesion formation and it is theorised that ROS causes damage to the cytoskeleton, cell membrane and DNA of an oocyte affecting its development and quality which contribute to the effect of endometriosis on fertility [1,2]. It is unknown whether surgical treatment of endometriosis reduces the levels of ROS but it has been demonstrated in other pathologies such as reduction of ROS in seminal fluid after varicocelectomy (treatment of varicocele) [3].

Objectives
Oxidative stress resulting in free radical tissue injury is a known consequence of endometriosis. One of the free radical mediated injury is that of damage to the oocytes (eggs) within the ovary, resulting in poorer reproductive outcomes, and possibly longer term detrimental impact on the babies born. It is not known if surgery, besides removing

endometriotic lesions, also impact on the levels of oxidative stress. This research will increase our understanding of the wider beneficial impact of endometriosis surgery and provide a further mechanistic insight into free-radical tissue injury in the context of endometriosis.

Primary Objective: To investigate oxidative stress levels in women before and after surgical treatment of endometriosis.

Secondary objective: To investigate whether surgery will improve fertility parameters in women with endometriosis.

Hypotheses: Surgical treatment will reduce oxidative stress and improve fertility parameters in women with endometriosis.

Design
Non-experimental longitudinal study to investigate whether oxidative stress is reduced post-surgery in women having treatment for endometriosis.

Intervention
Laparoscopy or laparotomy for excision, diathermy or laser treatment to endometriotic lesions and/or ovarian cystectomy for endometrioma. This intervention will not be dependent of participation in the study, intervention will have been decided on clinical grounds between the patient and their gynaecologist prior to taking part in the study. Patients will be participants of the study from consent until a follow up outpatient appointment approximately 3 months post-surgery.

Population
Women with known or suspected endometriosis undergoing investigation and treatment for the disease at the gynaecology department of Princess Anne Hospital, Southampton. This is a secondary referral centre for many benign gynaecological conditions and a tertiary referral centre for advanced endometriosis.

Sample Size
Power calculations have been completed based on a range of oxidative stress measurement assays in published literature that will be used in this study. As no current studies have examined oxidative stress pre and post treatment we have used levels in endometriosis patients compared to negative controls. A minimum of 62 patients will be recruited (31 with endometriosis and 31 negative controls) to satisfy a type 1 error of 0.05 (5%) and type 2 error of 0.2 (20%).

Inclusion Criteria:
- Women undergoing surgery (laparoscopy or laparotomy) for possible endometriosis
- Age 18 – 65 years

Exclusion Criteria:

- Menopausal women
- Active infective diseases
- Pregnancy

Recruitment

Women will be recruited from the gynaecology department at Princess Anne Hospital. Screening will be performed by clinical staff and researchers. Women fulfilling the inclusion/exclusion criteria will be approached by clinical staff involved in their care and asked if they would be happy to speak to a member of the research team. Those women who agree will be given an information leaflet outlining details of the study and a researcher will discuss the study with them and answer any questions they may have. Women who decide to participate will complete a consent form with the researcher and be asked a few questions pertaining to medical history to complete the WERF-ePhect (World Endometriosis Research Foundation endometriosis phenome and biobanking harmonization project[4]) study proforma (page 24-30 of Appendix 1) and a pelvic pain questionnaire (appendix 3). At the time of consent, a study number will be assigned so that patient identifiers will only be kept on the consent form. This will be stored separately from the research data in locked research offices. Patients will undergo their surgical treatment as planned. Following their surgical treatment, the patients will be given their routine follow up outpatient appointment. Adherence is likely to be near 80% due to the intervention of interest being elective surgery and study follow up coinciding with their clinical follow up appointment.

Sample Collection

When the anaesthetist sites a venous cannula for a recruited patient, a 4ml sample of blood will be obtained in a Becton Dickinson red top SST vacutainer collection tube, allowed to clot at room temperature for 30 mins and then placed on wet ice. A general anaesthetic will be given and the patient transferred into the operating theatre. The perineum and abdomen will be cleaned with an anti-bacterial skin preparation such as betadine and the patient draped with surgical drapes. The bladder will be emptied with a sterile urinary catheter and an 8ml sample collected into a Becton Dickinson conical urine tube with no additive and placed on wet ice. The surgery will performed as planned. If one laparoscopic port is being used a sterile surgical tubing will be inserted under vision into an area of pooled peritoneal fluid in the pelvis. Fluid will be withdrawn with a Becton Dickinson Plastipak 20ml hypodermic luer slip syringe and emptied into a Becton Dickinson conical urine collection tube with no additive and placed on wet ice. If multiple ports are inserted the peritoneal fluid will be collected with a Pennine 14 French gauge Ryles tube with a cut tip and 20ml syringe. If there are no visible pools of peritoneal fluid the pelvis will be washed with up to 20mls of normal saline and the washings are collected using the aforementioned technique. A WERF-EPHect surgical form will be completed (appendix 2) to describe any pelvic disease.

When a recruited patient attends their follow up outpatients appointment a 4ml sample of blood will be obtained in a Becton Dickinson red top SST vacutainer collection tube, allowed to clot at room temperature for 30 mins and then placed on wet ice. They will be given a Becton Dickinson conical urine tube with no additive to perform a clean catch urine specimen and this will be placed on wet ice.

Sample Processing and Storage

Samples will be taken on wet ice a University of Southampton laboratory or laboratory at the Princess Anne Hospital. Dipstick urinalysis of the urine will be performed using Siemens Multisticks 10SG urine test strips, if specific gravity is <1.001 or >1.032 it is retested for accuracy, if blood is present the sample is discarded. Results are recorded. 2 ml of unprocessed urine will be aliquoted into one Alpha Laboratories 2 ml Cryogenic vial with internal thread. The remaining urine and the blood sample will then be centrifuged in their collection tubes at 2500g at 4°C for 10 minutes and placed upright on wet ice where the supernatant will be aliquoted using an Alpha Laboratories Sterile Extended Fine Tip Pipette with bulb draw into 2 ml cryogenic vials. If a white blood cell layer is also present beneath the plasma supernatant (buffy coat layer) it will be aliquoted separately into a cryogenic vial. Red blood cells (the sediment/pellet of the blood collection tube) and the pellet of the urine will be aliquoted into separate cryogenic vials. Peritoneal fluid will be centrifuged at 900g at 4^0C for 5 minutes and the supernatant aliquoted into cryogenic vials. The pellet will be aliquoted into a cryogenic vial. All samples will be labelled with study ID number, description of sample type, date of processing and volume of sample. They will be stored securely in -80°C freezers in the University of Southampton laboratory or in the Princess Anne Hospital Research Facilities. This protocol follows WERF-EPHect Standard Operating Procedures for collection, processing and storage of fluid biospecimens in endometriosis research (page 1-24 of appendix 1).

Sample analysis

Oxidative stress will be measured in a number of ways e.g. ROS in the serum, urine and peritoneal fluid of endometriosis patients take before and after surgical treatment using techniques of chemilumiescence, fluorescence and absorbance. Commercial assays such as those used in existing literature [5,6] may be used to measure ROS for example Lumigen, GSH:GSSG and TAC assays. Other markers or surrogates of oxidative stress will also be analysed using verified and validated methods. Other valid upstream and downstream cellular and molecular processes will also be interrogated using various assays and culture methods as appropriate.

Schedule of Participation

	Screening	Recruitment & Intervention	Follow Up Visit
Inclusion/Exclusion criteria	X		
Consent Form		X	
Medical History		X	
Pain questionnaire		X	X
Endometriosis Staging Surgical Form		X	
Sample Collection: Blood Urine Peritoneal Fluid		 X X X	 X X -

Screening
Assessment of eligibility will be performed by patients' clinical team or a researcher either at their gynaecology outpatient appointment when they are listed for surgery or at least a week prior to their planned operation. Patient information leaflets will be given to the patient at time of screening or posted to them at least 1 week prior to their planned operation.

Recruitment & Intervention
If patients would like to participate they will be approached by a researcher to discuss the study and any questions they may have on the day of their surgery. They will then complete an informed consent form and a pelvic pain questionnaire with the researcher. They will undergo their planned operation during which samples will be collected as described.

Follow Up Visit
A researcher will visit the patient at their routine gynaecology outpatient follow-up appointment which is usually scheduled 3-4 months post-operatively (subject to clinic waiting lists). A further urine and blood sample will be taken from the patient and they will be asked to repeat a pelvic pain questionnaire.

Data Recording & Management
Linked anonymization will be used to maintain patient confidentiality. A study number will be assigned to each patient at the time of consent. The consent form will be the only record linking a study number to a patient number. All other documents (WERF-EPHect surgical form and collection proforma) will be anonymised by using study number only.

A record of the study number, pertinent patient medical details and sample details will be stored by the researchers in a database with no linked patient identifiers.
All records either electronic or paper (e.g consent form will be stored in locked research offices).

Samples will be labelled with a study number (no patient identifiers) and stored in secure (locked) sites in the Princess Anne research facilities or in University of Southampton Laboratories.

Data Analysis
Standard statistical tests will be performed to compare the groups according to whether data is normally distributed or not. Tests for paired data will be applied when comparing patient's samples before and after surgery and tests for unpaired data will be applied when comparing endometriosis patients to controls (non-endometriosis patients). Where appropriate, subgroup analysis will be performed (e.g by ASRM staging of endometriosis severity etc). Standard statistical software will be used such as SPSS or graph pad. Statistical advice will be obtained from RDS where appropriate.

Institutional Review Board
This study, its protocol and all study documents (e.g. consent form and patient information leaflet) will be reviewed by the Regional Ethics Committee, the University Hospitals of

Southampton Research and Development department and ERGO (Ethics and Research Governance Online) of the University of Southampton for ethical approval.

The study may be discontinued at any time by the REC or government agencies if it is deemed necessary.

Publication of Research Findings

It is hoped that results of this study will be published in a medical and scientific journals as a source of information for other researchers, scientists and doctors. The results may also be presented at academic meetings or be published within scientific thesis such as a PhD.

Appendix

Appendix 1: WERF-EPHect Standard Operating Procedures for collection, processing and storage of fluid biospecimens in endometriosis research pages 1-10, 18-21 & 24-30
Appendix 2: WERF-EPHect surgical form
Appendix 3: Pelvic Pain Questionnaire

References

1. Scutiero, G. *et al.* Oxidative Stress and Endometriosis: A Systematic Review of the Literature. *Oxid. Med. Cell. Longev.* **2017,** 1–7 (2017).
2. Hamdan, M., Jones, K. T., Cheong, Y. & Lane, S. I. R. The sensitivity of the DNA damage checkpoint prevents oocyte maturation in endometriosis. *Sci. Rep.* **6,** 36994 (2016).
3. Keyhan, H., Dadvar, A., Ansari, M. & Rafiee, K. Comparison of before and after varicocelectomy levels of nitric oxide in seminal fluid of infertile men. *Nephrourol. Mon.* **4,** 629–32 (2012).
4. World Endometriosis Research Foundation. Available at: https://endometriosisfoundation.org/ephect/.
5. Sharma, R. K., Reynolds, N., Rakhit, M. & Agarwal, A. Methods for Detection of ROS in the Female Reproductive System. in *Studies on Women's Health* 33–60 (Humana Press, 2013). doi:10.1007/978-1-62703-041-0_2
6. Uy, B., McGlashan, S. & Shaikh, S. Measureent of Reactive Oxygen Species in the Culture Media Using Acridan Lumigen PS-3 Assay. *J Biomol Tech* **22,** 95–107 (2011).

WERF EPHect Standard Operating Procedures:
Collection, processing, and storage of fluid biospecimens in endometriosis research

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Detailed standard operating procedure for the collection, processing and storage of blood specimens

NOTES

- This SOP applies to plasma collection using EDTA or lithium-heparin coagulation tubes, and serum with (SST) or without separator, collected via venepuncture.
- This SOP does not cover blood withdrawal techniques/procedures (qualified personnel should follow standard withdrawal procedures).
- This SOP does not cover safety procedures for the collection and processing of these samples and personnel must follow institutional biosafety guidelines.
- For a summary version of this protocol with side-by-side standard vs. minimal protocol step comparisons, please see "WERF EPHect SOP Fluid Collection Table".
- As this protocol applies to different processing and storage methods (e.g. the use of RNA stabilising fluid), keep a copy of the exact step-by-step protocol used in your lab.

Processing and storage materials

1. Biospecimen form (page 24);
2. Log sheet to record sample-related data;
3. Blood collection tubes with EDTA and heparin for plasma, SST or no SST for serum
4. Crushed ice if a delay is anticipated
5. Appropriate racks to hold tubes in upright position
6. Centrifuge
7. Volume adjustable pipette
8. Transfer pipettes
9. Labels suitable for long-term freezer storage, and IDs printed using 2D barcoding
10. Aliquot vials with screw top gasket closure
11. Freezers: -80C or liquid nitrogen (LN_2)

1. Blood collection

1.1. Blood collection should be performed by a licensed phlebotomist, nurse, anaesthesiologist, or medical doctor.

1.2. Collection should be performed in an adequate setting, e.g. in the phlebotomy room, or on the ward. Blood collection in the operating theatre should be avoided, if possible.

1.3. Time of sample collection in the clinic:

1.3.1. ***Standard collection:*** Collect blood samples before induction of anaesthesia (before pre-med is given).

1.3.2. ***Required minimum:*** Record whether blood is collected (1) prior to pre-medication; (2) after pre-medication but before anaesthesia; or (3) after anaesthesia.

1.4. Fasting status at sample collection:

1.4.1. ***Standard collection:*** Collect only samples after fasting for at least 10 hours. Record on the log sheet the time since the study participant ate or drank anything except plain water (Fasted since: __:__pm/am).

1.4.2. ***Required minimum:*** Record on the log sheet the time since the study participant ate or drank anything except plain water (Fasted since: __:__pm/am).

1.5. Preparation of sample collection tubes:

1.5.1. ***Standard collection:*** Label each blood collection tube with a 2D barcode in addition to a human readable unique identifier, participant ID, date of collection and type of sample. Record on the log sheet the date and time of sample collection (Date: __/__/__ and __:__am/pm).

1.5.2. ***Required minimum:*** Label each blood collection tube with a unique identifier, participant ID, date of collection, and type of sample. Record on the log sheet the date and time of sample collection (Date: __/__/__ and __:__am/pm).

1.6. Order of sample type collection:

1.6.1. ***Standard collection:*** Prioritise: 1) EDTA plasma; 2) SST serum; 3) other tube types, in a pre-determined order of priority. Keep a record of the order in your adapted SOP.

1.6.2. ***Required minimum:*** Collect collection tubes always in the same order of priority and keep a record of the order in your adapted SOP.

1.7. It is important that tubes with anti-coagulants (e.g., EDTA, heparin), which need to be inverted after blood draw, are gently inverted 8-10 times (no vigorous shaking). Place in the collection rack in an upright position.

1.8. Temperature and waiting conditions of samples until processed in the lab:

1.8.1. ***Standard collection:*** Place samples on wet ice/in refrigerator immediately if there will be more than 1 hour before processing (maximum time to processing: 4 hours). If the time to processing is less than 1 hour, samples can be kept at room temperature.

1.8.2. ***Required minimum:*** Place samples on wet ice/in refrigerator if there will be more than 2 hour before processing (maximum time to processing: 4 hours). If the time to processing is less than 2 hours, samples can be kept at room temperature.

2. Sample processing in the laboratory

2.1. Allow any SST tubes (red top) to clot for 30-60 minutes in an upright position at room temperature in the first 30 minutes from collection of the sample. SST tubes should be placed on wet ice after 1 hour if there will be more than 2 hours until processing.

2.2. Time until samples processed in the lab:

2.2.1. ***Standard collection:*** Record on the log sheet the time sample processing started in the laboratory. Blood samples should be centrifuged within 1 hour of blood collection.

2.2.2. ***Required minimum:*** Record on the log sheet the time sample processing started in the laboratory. Blood samples should be centrifuged within 4 hours of blood collection.

2.3. Centrifugation of samples:

2.3.1. ***Standard collection:*** Centrifuge samples for 10 minutes at 2500g at 4°C. Keep a record of the standard time and g in your centre's SOP.

2.3.2. ***Required minimum:*** Centrifuge samples for 10 minutes at 2500g at room temperature. Keep a record of the standard time and g in your centre's SOP.

2.4. After centrifugation of samples:

2.4.1. ***Standard collection:*** Place the spun tubes on a rack in upright position and on wet ice during aliquotting.

2.4.2. ***Required minimum:*** Place the spun tubes on a rack in upright position at room temperature during aliquotting.

2.5. Aliquotting of samples:

2.5.1. ***Standard collection:*** Have a set number of aliquot tubes and sizes for each sample type that will be collected. Pre-label aliquot vials (see section 3). Put empty aliquot vials into a rack that sits on wet ice before aliquotting. Aliquot into small volumes between 100-500uL to minimize later freeze-thaw cycles.

2.5.2. ***Required minimum:*** Have a set number of aliquot tubes and sizes for each sample type that will be collected. Pre-label aliquot vials (see section 3).

2.6. Aspirate plasma/serum using an appropriate transfer pipette (ideally a volume-adjustable pipette) being careful not to disturb the cell layer below, holding the tube at a 45° angle.

2.7. Transfer plasma/serum to appropriately sized aliquot vial with screw top gasket closure (do not use tubes with a push top as they are not airtight) and fill as close to full as possible to minimise exposure to air.

2.8. Repeat steps 2.6 and 2.7 until all the plasma and serum has been transferred. Record volume of sample in each aliquot of plasma or serum.

2.9. If cells are accidentally mixed with the plasma/serum, the aliquot vial can be re-centrifuged as before and the plasma/serum can be transferred to a new aliquot vial.

2.10. White Blood Cell (WBC) aliquotting: Using a transfer pipette take the buffy coat layer from the collection tube. Aspirate slowly and carefully using a circular motion to remove all the visible buffy coat and transfer to an appropriate sized aliquot vial with screw top gasket closure. Record the volume of sample in each aliquot of WBC.

2.11. Red Blood Cell (RBC) aliquotting: Using a transfer pipette, gently mix the remaining erythrocytes and aspirate in another appropriate sized aliquot vial with screw top gasket closure and fill as close to full as possible to minimise surface area. Record the volume of sample in each aliquot of RBC.

3. Labelling aliquots and storage

3.1. Use special labels and ink that do not disintegrate when stored in very low temperature freezers (i.e. do not use laser printers or most ink-printers as they disintegrate when frozen).

3.2. Preparation of sample aliquot tubes:

3.2.1. ***Standard collection:*** Label the aliquot tubes with the participant ID number followed by a unique aliquot ID number. For example: ENDO-123456-U654321-P-01: Center identifier (ENDO), participant ID (123456), unique aliquot vial ID (U654321), sample type (P for plasma), aliquot number (01). Also, include date of sample creation on the label to be able to distinguish samples from the same participant collected at different time points. Furthermore, include the above information in human readable format and in a 2D barcode on the label.

3.2.2. ***Required minimum:*** Label the aliquot tubes with the participant ID followed by a unique aliquot ID number. For example: ENDO-123456-U654321-P-01: Center identifier (ENDO), participant ID (123456), unique aliquot vial ID (U654321), sample type (P for plasma), aliquot number (01). Also, include date of sample creation on the label to be able to distinguish samples from the same participant collected at different time points.

3.3. Time until sample aliquots are put into freezers for storage:

3.3.1. ***Standard collection:*** Samples should be processed and stored into freezers within a maximum of 1 hour and time should be recorded on the log sheet. Also record the type, number and volume of aliquots prepared.

3.3.2. ***Required minimum:*** Samples should be processed and stored into freezers within a maximum of 4 hours, and time of storage should be recorded on the log sheet. Also record the type, number and volume of aliquots prepared.

3.4. Sample storage in freezers:

3.4.1. ***Standard collection:*** Store serum, plasma and WBC/RBC aliquots in liquid nitrogen (LN_2) freezers, which have less temperature fluctuations than -80°C freezers.

3.4.2. ***Required minimum:*** Store serum, plasma and WBC/RBC aliquots in -80°C or lower freezers.

3.5. Record on the log sheet any variations or deviations from the SOP, problems, or issues (e.g., hemolysis, vial cracked during processing).

3.6. Record the location of each sample in the freezer including freezer number, rack, box, and position in the box along with all other sample attributes in a database. If possible avoid using a spreadsheet format, but preferably use a relational database.

4. Freezer check

4.1.1. ***Standard collection:*** Split aliquots from the same sample type and individual between freezers in case of a freezer breaking down. Check freezers bi-weekly and keep a written-log of checks. Have alarm systems setup on all freezers in addition to human bi-weekly checks.

4.1.2. ***Required minimum:*** Manually check freezers bi-weekly and keep a written-log of checks.

5. Data recording check list

5.1. Record protocol, specifying which steps are adhered to (standard or minimum).

5.2. Record the time since the study participant ate or drank anything except plain water (fasted since: __:__pm/am).

5.3. For each sample, record:

5.3.1. Date and time of blood collection (Date: __/__/__ and __:__am/pm).

5.3.2. Start time of sample processing in the laboratory (__:__am/pm).

5.3.3. Type, number and volume of aliquots prepared.
5.3.4. Date and time aliquots stored into freezers (Date: __/__/__ and __:__am/pm).

5.3.5. Any variations or deviations from the SOP, problems, or issues.

5.4. In the long-term, record:

5.4.1. Any freeze-thaw that occurs with a sample for any reason.

5.4.2. Any change of location of a sample, including sending a sample out to an assay lab for processing.

5.4.3. Any new samples created from the original aliquots (i.e., a sub-aliquot) in the same manner as described above.

5.5. Keep a bi-weekly log of freezer checks.

Detailed standard operating procedure for the collection, processing and storage of urine specimens

NOTES

- This SOP does not cover safety procedures for the collection and processing of these samples and personnel must follow institutional biosafety guidelines.
- For a summary version of this protocol with side-by-side standard vs. minimal protocol step comparisons, please see "WERF EPHect SOP Fluid Collection Table".
- As this protocol applies to different processing and storage methods, keep a copy of the exact step-by-step protocol used in your lab.

Processing and storage materials

1. Biospecimen form (page 24);
2. Log sheet to record sample-related data;
3. Sterile urine collection container with a wide mouth and a leak-proof cap
4. Crushed ice if a delay is anticipated
5. Transfer pipette
6. Dipstick for urine analysis
7. Volume adjustable pipette
8. Centrifuge
9. Labels suitable for long-term freezer storage, and IDs printed using 2D barcoding
10. Aliquot vials with screw top gasket closure
11. Freezers: -80C or liquid nitrogen (LN_2)

1. Urine collection

1.1. Sample collection method:

1.1.1. ***Standard collection:*** Obtain a clean catch, mid-stream, first morning void*, urine sample from the patient. Instruct them to collect the urine sample first thing in the morning when they get out of bed. Provide the patient with a sterile specimen container with a wide mouth and a leak-proof cap and written instructions on how to collect clean catch midstream urine sample.

1.1.2. ***Required minimum:*** Obtain a clean catch urine sample from the patient in the clinic or at the patient's home by providing the patient with a sterile specimen container with a wide mouth and a leak-proof cap and written instructions on how to collect clean catch midstream urine sample.

*Patient instruction for first morning clean catch urine sample[1]:

1. Collect the first urine that occurs as the first void after waking up.
2. Wash your hands with soap and warm water.
3. Sit on the toilet with your legs spread apart. Use two fingers to spread open your labia.
4. Use an antiseptic wipe to clean the inner folds of the labia. Wipe from the front to the back.
5. Use a second antiseptic wipe to clean over the opening where urine comes out (urethra), just above the opening of the vagina.
6. Keeping your labia spread open, urinate a small amount into the toilet bowl, then stop the flow of urine.
7. Hold the urine cup a few inches from the urethra and urinate until the cup is about half full.
8. You may finish urinating into the toilet bowl.

[1] http://www.nlm.nih.gov/medlineplus/ency/article/007487.htm

9. Screw the cap on to the collection cup securely and place in the refrigerator until taking the sample to the clinic.

1.2. If the sample is collected in the clinic, the research nurse should record the time of sample collection. If the sample is a first morning void brought into clinic with the patient, the patient should record the time and type of sample collection and report to the research nurse.

1.3. If the sample is collected in clinic, it should be put on wet ice immediately. If the patient collects the sample at home, it should be maintained in a refrigerator and delivered in an ice pack to the clinic (at 4°C).

1.4. Record on the log sheet the time since the study participant ate or drank anything except plain water (Fasted since: __:__pm/am). Record what time the urine sample was collected, whether this was a first morning void or spot urine, and whether the study participant has urinated during the night time if the collected sample is a first morning void.

1.5. Labelling of the collection cup:

1.5.1. ***Standard collection:*** Label the collection cup before giving to the patient for collection, with a 2D barcode in addition to human-readable the unique identifier of the patient and sample identifier.

1.5.2. ***Required minimum:*** Once the collection cup is collected from the patient, label the sample with the unique identifier of the patient and sample identifier.

2. Sample processing in the laboratory, labelling aliquots and storage

2.1. Time until samples processed in the lab:

2.1.1. ***Standard collection:*** Keep the sample refrigerated until processed and complete processing within 2 hours. Record on the log sheet the time sample processing started in the laboratory.

2.1.2. ***Required minimum:*** Keep the sample refrigerated until processed and complete processing within a maximum of 48 hours. Record on the log sheet the time sample processing started in the laboratory.

2.2. Discard the sample if it contains blood, and record.

2.3. Mix the sample by either swirling the cup or pipetting the urine up and down a couple of times.

2.4. Perform dipstick urine analysis and record result including specific gravity. If specific gravity is lower than 1.001 or greater than 1.032, retest for accuracy. Record on the log sheet, the results, including specific gravity and that retest is performed.

2.5. Aspirate required amount of unprocessed urine and label the aliquot vials with screw top gasket closures.

2.6. Preparation of sample aliquot tubes:

2.6.1. ***Standard collection:*** Label the aliquot vials with the participant ID number followed by a unique aliquot ID number. For example: ENDO-123456-U654321-U-01: Center identifier (ENDO), participant ID (123456), unique aliquot vial ID (U654321), sample type (U for urine) and aliquot number (01). Also, include date of sample creation on the label to be able to distinguish samples from the same participant collected at different time points. Further, include the above information in human readable format and in a 2D barcode on the label.

2.6.2. ***Required minimum:*** Label the aliquot vials with the participant ID followed by the sample aliquot number. Also include date of sample creation on the label. For example: ENDO-123456-U-01: Center identifier (ENDO), participant ID (123456), type of sample (U for urine), aliquot number (01). Also, include date of sample creation on the label to be able to distinguish samples from the same participant collected at different time points.

2.7. Sample storage in freezers:

2.7.1. ***Standard collection:*** Store the unprocessed urine aliquots in liquid nitrogen (LN_2) freezers, which have less temperature fluctuations.

2.7.2. ***Required minimum:*** Store the unprocessed urine aliquots at -80°C or lower.

2.8. Fill a sterile tube with the remaining urine in the sample collection container and centrifuge at 1000-3000g at 4°C for 5 minutes.

2.9. Place the sample on wet ice and aspirate the supernatant into required number of aliquots. Label the aliquots as in 2.6. and store the processed urine aliquots as in 2.7.

2.10. Duration until sample aliquots are put into freezers for storage:

2.10.1. ***Standard collection:*** Samples should be processed and stored into freezers within a maximum of 2 hours and time should be recorded on the log sheet. Also record the type, number and volume of aliquots prepared.

2.10.2. ***Required minimum:*** Samples should be processed and stored into freezers within a maximum of 48 hours and time should be recorded on the log sheet. Also record the type, number and volume of aliquots prepared.

2.11. Record on the log sheet any variations or deviations from the SOP, problems, or issues.

2.12. Record the location of each sample in the freezer including freezer number, rack, box, and position in the box along with all other sample attributes in a database. If possible, avoid using a spreadsheet format, but preferably use a relational database.

3. Freezer check

3.1.2. ***Standard collection:*** Split aliquots from the same sample type and individual between freezers in case of a freezer breaking down. Check freezers bi-weekly and keep a written-log of checks. Have alarm systems setup on all freezers in addition to human bi-weekly checks.

3.1.1. ***Required minimum:*** Manually check freezers bi-weekly and keep a written-log of checks.

4. Data recording check list

4.1. Record protocol, specifying which steps are adhered to (standard or minimum).

4.2. Record the time since the study participant ate or drank anything except plain water (Fasted since: __:__pm/am).

4.3. For each sample, record:

4.3.1. Date and time of urine collection (Date: __/__/__ and __:__am/pm).

4.3.2. Type of urine collection (e.g. Clean catch spot urine or clean catch first morning void urine).

4.3.3. Start time of sample processing in the laboratory (__:__am/pm).

4.3.4. Results of dipstick urinalysis including specific gravity and that retest is performed.
4.3.5. Type, number and volume of aliquots prepared.

4.3.6. Date and time aliquots stored into freezers (Date: __/__/__ and __:__am/pm).

4.3.7. Any variations or deviations from the SOP, problems, or issues.

4.4. In the long-term, record:

4.4.1. Any freeze-thaw that occurs with a sample for any reason.

4.4.2. Any change of location of a sample, including sending a sample out to an assay lab for processing.

4.4.3. Any new samples created from the original aliquots (i.e., a sub-aliquot) in the same manner as described above.

4.5. Keep a bi-weekly log of freezer checks.

Detailed standard operating procedure for the collection, processing and storage of saliva specimens

NOTES

- This SOP does not cover safety procedures for the collection and processing of these samples and personnel must follow institutional biosafety guidelines.
- For a summary version of this protocol with side-by-side standard vs. minimal protocol step comparisons, please see Supplemental Table 3.
- As this protocol applies to different processing and storage methods, keep a copy of the exact step-by-step protocol used in your lab.

Processing and storage materials

1. Biospecimen form (Supplemental Appendix VII);
2. Log sheet to record sample-related data;
3. Sterile saliva collection container or is collecting sample for DNA the manufacturer's provided collection container
4. Crushed ice if a delay is anticipated.
5. Transfer pipette
6. Volume adjustable pipette
7. Centrifuge
8. RNA stabilizer fluid *(optional if planning RNA studies)*
9. Labels suitable for long-term freezer storage, and IDs printed using 2D barcoding
10. Aliquot vials with screw top gasket closure
11. Freezers: -80C or liquid nitrogen (LN_2)

1. Peritoneal fluid collection

1.1. The sample is collected after premedication/anesthesia administration.

1.2. Sample collection is performed by either (1) 2 20ml suction devices (2) laparoscopic needle and manual aspiration using a syringe. Always use the same device and record the device used for collection in the SOP.

1.3. If no peritoneal fluid or a very small amount of peritoneal fluid is found, the pelvis can be washed with 20ml sterile normal saline solution using laparoscopic needle and manual aspiration using a syringe under direct visual control. This peritoneal lavage fluid (PLF) can be processed as peritoneal fluid, but the supernatant from PLF should be regarded with caution (see main text for discussion).

1.4. Record the time, date, volume and method of sample collection.

1.5. Sample treatment until transported to the lab:

1.5.1. ***Standard collection:*** Transfer the sample into a screw top vial and put the sample on wet ice and transfer to the lab within 30 minutes.

1.5.2. ***Required minimum:*** Transfer the sample into a screw top vial and put the sample on wet ice and transfer to the lab as soon as possible.

2. Sample processing in the laboratory

2.1. Record on the log sheet the start time of sample processing in the laboratory.

2.2. Record colour, clarity and volume of the sample.

2.3. Centrifugation of samples:

2.3.1. ***Standard collection:*** Centrifuge samples for 5 minutes at 900g at 4°C. Keep a record of the standard time and g in your adapted SOP.

2.3.2. ***Required minimum:*** Centrifuge samples for 5 minutes at 900g at room temperature. Keep a record of the standard time and g in your adapted SOP.

2.4. Transfer the supernatant to appropriate sized aliquot vial with screw top gasket closure (not 'push fit top' as they are not airtight) and fill as close to full as possible to minimise surface area. If PLF method is used for collection, be cautious in downstream analyses of the supernatant (See main text for discussion).

2.5. Transfer the pellet to an appropriate sized aliquot vial with screw top gasket closure (not 'push fit top' as they are not airtight).

3. Labelling aliquots and storage

3.1. Use special labels and ink that do not disintegrate when stored in very low temperature freezers (i.e. do not use laser printers or most ink-printers as they disintegrate when frozen).

3.2. Preparation of sample aliquot tubes:

3.2.1. ***Standard collection:*** Label the aliquot vials with the participant ID number followed by a unique aliquot ID number. For example: ENDO-123456-U654321-PF: Center identifier (ENDO), participant ID (123456), unique aliquot vial ID (U654321), sample type (PF for peritoneal fluid) and aliquot number (01). Also, include date of sample creation on the label to be able to distinguish samples from the same participant collected at different time points. Further, include the above information in human readable format and in a 2D barcode on the label.

3.2.2. ***Required minimum:*** Label the aliquot vials with the participant ID followed by the sample aliquot number. For example: ENDO-123456-PF-01: Center identifier (ENDO), participant ID (123456), type of sample (PF for peritoneal fluid) and aliquot number (01). Also, include date of sample creation on the label to be able to distinguish samples from the same participant collected at different time points.

3.3. Record the time of the sample processing completion/ time put into the freezer. Also record the type, number and volume of aliquots prepared.

3.4. Sample storage in freezers:

3.4.1. ***Standard collection:*** Store fluid aliquots in liquid nitrogen (LN_2) freezers, which have less temperature fluctuations, for long term storage.

3.4.2. ***Required minimum:*** Store fluid aliquots at -80°C or lower freezers for long term storage.

3.5. Record any variations or deviations from the SOP, problems, or issues.

3.6. Record the location of each sample in the freezer including freezer number, rack, box, and position in the box along with all other sample attributes in a database. If possible avoid using a spreadsheet format, but preferably use a relational database.

4. Freezer check

4.1.1. ***Standard collection:*** Split aliquots from the same sample type and individual between freezers in case of a freezer breaking down. Check freezers bi-weekly and keep a written-log of checks. Have alarm systems setup on all freezers in addition to human bi-weekly checks.

4.1.2. ***Required minimum:*** Manually check freezers bi-weekly and keep a written-log of checks.

5. Data recording Check list

5.1. Record protocol, specifying which steps are adhered to (standard or minimum).

5.2. For each sample, record:

5.2.1. Date and time of fluid collection (Date: __/__/__ and __:__am/pm).

5.2.2. Start time of sample processing in the laboratory (__:__am/pm).

5.2.3. Type, number and volume of aliquots prepared.

5.2.4. Date and time aliquots stored into freezers (Date: __/__/__ and __:__am/pm).

5.2.5. Any variations or deviations from the SOP, problems, or issues.

5.3. In the long-term, record:

5.3.1. Any freeze-thaw that occurs with a sample for any reason.

5.3.2. Any change of location of a sample, including sending a sample out to an assay lab for processing.

5.3.3. Any new samples created from the original aliquots (i.e., a sub-aliquot) in the same manner as described above.

5.4. Keep a bi-weekly log of freezer checks.

Detailed standard operating procedure for the collection, processing and storage of menstrual effluent (blood) specimens

NOTES

- This SOP does not cover safety procedures for the collection and processing of these samples and personnel must follow institutional biosafety guidelines.
- For a summary version of this protocol with side-by-side standard vs. minimal protocol step comparisons, please see "WERF EPHect SOP Fluid Collection Table".
- As this protocol applies to different processing and storage methods, keep a copy of the exact step-by-step protocol used in your lab.

Processing and storage materials

1. Biospecimen form (page 24)
2. Log sheet to record sample-related data
3. A diaphragm or a mixing cannula
4. Sterile closed container for transfer of the sample to the lab
5. Crushed ice if a delay is anticipated
6. Transfer pipette
7. Volume adjustable pipette
8. Centrifuge
9. EDTA/heparin tubes if plasma is going to be collected from the sample
10. Labels suitable for long-term freezer storage, and IDs printed using 2D barcoding
11. Aliquot vials with screw top gasket closure
12. Freezers: -80C or liquid nitrogen (LN_2)

1. Menstrual effluent (blood) collection

1.1. Collect menstrual effluent sample with a diaphragm or mixing cannula.

1.2. Record on the log sheet the day of the menstrual cycle of the sample collection (Day __) and the date and time of menstrual effluent collection (Date: __/__/__ and __:__am/pm).

1.3. Labelling of sample collection container:

1.3.1. ***Standard collection:*** Once the collection container is collected from the patient label the sample with a 2D barcode in addition to human-readable the unique identifier of the patient and sample identifier.

1.3.2. ***Required minimum:*** Once the collection container is collected from the patient, label the sample with the unique identifier of the patient and sample identifier.

1.4. Sample treatment until transported to the lab:

1.4.1. ***Standard collection:*** The sample is put in a closed container and transferred and processed in the laboratory within 1 hour on wet ice.

1.4.2. ***Required minimum:*** The sample is put in a closed container and transferred and processed in the laboratory within 1 hour at room temperature.

EPHect BIOSPECIMEN COLLECTION FORM (to be completed by research nurse)

Date and time sample collected: (DD/MM/YYYY) ___ / ___ / _____ Time: ____:____ AM/PM

What was the first day of your last menstrual period? (DD/MM/YYYY) ___ / ___ / _____

Are your periods regular? (Predictable within one week)
☐ Yes ☐ No

Specify range of days :____ [regular range: 21-35days]

If you have not had a menstrual period in the past 90 days, please tell us why:
☐ Taking hormones continuously (*e.g. the Pill, injections, Mirena, HRT*)
☐ Pregnant
☐ Breastfeeding
☐ Unsure
☐ Other (*Please describe*) _____

Are you currently having a menstrual period/vaginal bleeding *(including spotting for which you only need a panty liner)*?
☐ No
☐ Yes, menstrual period
☐ Yes, irregular bleeding/spotting

Do you currently have a coil [IUD] in place?
☐ No
☐ Yes → If yes, what kind of IUD? ☐ Progesterone containing IUD (Mirena)
☐ Other coil/intrauterine device

When was the last time you had something to eat?
____ : ____ am/pm ☐ Today ☐ Yesterday

When was the last time you had something to drink (other than plain water) and what did you drink?
____ : ____ am/pm ☐ Today ☐ Yesterday
_____

Clinical Measurements:

Height: _____ in cm, or _____ in inches

Weight: _____ in kg, or _____ in pounds

Hip circumference: _____ in cm, or _____ in inches

Waist circumference: _____ in cm, or _____ in inches
See WHO guidelines on how to take measurements (also included on page 30):
www.who.int/nutrition/publications/obesity/WHO_report_waistcircumference_and_waisthip_ratio/en/

If <u>saliva</u> samples are being collected:

Please indicate whether or not you have used the following in the last 24 hours and what time you used each item.

Toothpaste	☐ No ☐ Yes →	___ : ___ AM/PM	☐ Today ☐ Yesterday
Gum	☐ No ☐ Yes →	___ : ___ AM/PM	☐ Today ☐ Yesterday
Cigarettes	☐ No ☐ Yes →	___ : ___ AM/PM	☐ Today ☐ Yesterday
Alcohol	☐ No ☐ Yes →	___ : ___ AM/PM	☐ Today ☐ Yesterday

In the past 24 hours have you eaten:

Spicy food? ☐ No ☐ Yes
Fish? ☐ No ☐ Yes

If <u>urine</u> samples are being collected:

When did you last urinate (prior to providing the sample)?
____ : ____ am/pm ☐ Today ☐ Yesterday

What time was the urine sample produced?
____ : ____ am/pm

Is this urine sample your first morning void?
☐ No
☐ Yes → If yes, did you get up during the night to urinate? ☐ No ☐ Yes

In collecting this sample, did you follow a clean catch protocol?
☐ No
☐ Yes

If undergoing an operation:

Was any pre-med taken before blood, urine, saliva, endometrial fluid and eutopic endometrium/ myometrium collection? (NB. EPHect recommends sample taking <u>prior to</u> pre-med administration)
☐ No
☐ Yes

If yes, tick which samples were taken after pre-med administration:
☐ Blood ☐ Urine ☐ Saliva ☐ Endometrial fluid
☐ Eutopic endometrium/ myometrium

Time pre-med was administered: ____am/pm

Please specify the type of pre-med was administered _____

Was anaesthetic administered before blood, endometrial fluid and eutopic endometrium collection?
- ☐ No
- ☐ Yes

If yes, tick which samples were taken after anaesthesia administration:
☐ Blood ☐ Endometrial fluid ☐ Eutopic endometrium

If yes, time anaesthetic was administered: ____am/pm

Please specify the type of pre-med was administered: _____

Method(s) of excision:

Ectopic endometrium
- ☐ Electrosurgery
- ☐ Harmonic scalpel
- ☐ Laser [CO_2, NdYag and others]
- ☐ Cold scissors/scapels

Eutopic endometrium
- ☐ Endometrial sampling device
- ☐ Curettage with cervical dilation
- ☐ Brushing

Myometrium
- ☐ Laser [CO_2, NdYag and others]
- ☐ Electrosurgery
- ☐ Cold scissors/scapels
- ☐ TruCut biopsy

Peritoneum
- ☐ laser [CO_2, NdYag and others]
- ☐ Electrosurgery
- ☐ Ultrasound energy
- ☐ Harmonic scalpel
- ☐ Cold scissors/scapels
- ☐ Brushing

Method(s) of collection:

Peritoneal fluid
- ☐ No lavage. Amount collected ___ml
- ☐ Lavage method with 10ml sterile saline solution. Amount of peritoneal lavage fluid (PLF) ___ml

Endometrial fluid
- ☐ No lavage. Amount collected ___ml
- ☐ Lavage method with 4ml sterile saline solution. Amount of uterine lavage fluid (ULF) ___ml

Use of prescription drugs, non-prescription drugs, vitamins or supplements in the past 30 days.

Type of drug	**Have you ever taken this drug every day for over a month?**	**At what age did you first take this drug every day for over a month?**	**In total, how many years you have taken this drug? Please estimate, and enter "0 total years" if less than 1 year.**	**Are you currently taking this drug every day?**	**Please write down the specific name of the drug you have used most recently if known:**
PRESCRIPTION DRUGS	✓ *if yes*	*Age 1st*	*Years taken:*	✓ *if yes*	*Name of drug:*
a. Hormonal medications	☐	__ __	__ __	☐	
Birth control pill	☐	__ __	__ __	☐	
Progestin injection/shot	☐	__ __	__ __	☐	
Transdermal patch/dot	☐	__ __	__ __	☐	
Vaginal ring	☐	__ __	__ __	☐	
Progesterone containing coil/IUD	☐	__ __	__ __	☐	
Hormonal implant	☐	__ __	__ __	☐	
Oral progestins to regulate cycle	☐	__ __	__ __	☐	
GnRH agonist infection/shot	☐	__ __	__ __	☐	
Norethindrone acetate	☐	__ __	__ __	☐	
Danazol	☐	__ __	__ __	☐	
Hormone replacement therapy (HRT)	☐	__ __	__ __	☐	
Other:	☐	__ __	__ __	☐	
b. Pain medications	☐	__ __	__ __	☐	
Paracetamol/acetaminophen	☐	__ __	__ __	☐	
Aspirin	☐	__ __	__ __	☐	
Ibuprofen	☐	__ __	__ __	☐	
COX-2 inhibitors (e.g. celebrex, vioxx)	☐	__ __	__ __	☐	
Other anti-inflammatory analgesics (e.g. naproxen, mefanamic acid, aleve, naprosyn, relafen, keoprofen, anaprox)	☐	__ __	__ __	☐	
Narcotic analgesics (e.g. hydrocodone+ paracetamol, codeine, morphine)	☐	__ __	__ __	☐	
Muscle relaxants (e.g. diazepam/ temazepam, buscopan)	☐	__ __	__ __	☐	
Other:	☐	__ __	__ __	☐	
c. Diuretic (water pill)	☐	__ __	__ __	☐	
d. Diabetic tablets	☐	__ __	__ __	☐	
e. Insulin	☐	__ __	__ __	☐	
f. Thyroid drugs	☐	__ __	__ __	☐	
g. Drugs for epilepsy	☐	__ __	__ __	☐	
h. Sleeping tablets / tranquilisers	☐	__ __	__ __	☐	
i. Anti-depressants	☐	__ __	__ __	☐	
j. Other drugs to treat mental illness	☐	__ __	__ __	☐	
k. Drugs for osteoporosis ("brittle bones")	☐	__ __	__ __	☐	

l. Drugs for rheumatoid arthritis	☐	__ __	__ __	☐	
m. Antibiotics for a month or more	☐	__ __	__ __	☐	
n. Antacids	☐	__ __	__ __	☐	
o. Drugs for stomach ulcer / gastritis	☐	__ __	__ __	☐	
p. Drugs for high cholesterol	☐	__ __	__ __	☐	
q. Drugs for allergies (antihistamines)	☐	__ __	__ __	☐	
r. Steroids (oral, inhaled, or nasal)	☐	__ __	__ __	☐	
s. Chemotherapy for cancer	☐	__ __	__ __	☐	
t. Tamoxifen for cancer	☐	__ __	__ __	☐	
u. Blood pressure drugs	☐	__ __	__ __	☐	
v. Drugs for angina (chest pain)	☐	__ __	__ __	☐	
w. Other drugs for a heart condition	☐	__ __	__ __	☐	
x. Inhaler for asthma	☐	__ __	__ __	☐	
y. Warfarin / heparin to thin blood	☐	__ __	__ __	☐	
z. Migraine tablets/injections	☐	__ __	__ __	☐	
Other 1:	☐	__ __	__ __	☐	
Other 2:	☐	__ __	__ __	☐	
Other 3:	☐	__ __	__ __	☐	
Other 4:	☐	__ __	__ __	☐	
Other 5:	☐	__ __	__ __	☐	

Type of drug	**Have you taken this drug in the past 30 days?**	**Have you taken this drug in the past 48 hours (2 days)?**	**In the past 30 days, on how many days have you taken this drug?**	**Please write down the specific name of the drug if known:**
NON-PRESCRIPTION DRUGS	✓ *if yes*	✓ *if yes*	*Number of days:*	*Name of drug:*
a. Aspirin	☐	☐	__ __ days	
b. Paracetamol	☐	☐	__ __ days	
c. Ibuprofen	☐	☐	__ __ days	
d. Other anti-inflammatory analgesics (e.g. naproxen)	☐	☐	__ __ days	
e. Herbal pain medication:	☐	☐	__ __ days	
f. Other pain medication:				
g. Migraine tablets	☐	☐	__ __ days	
h. Antihistamine for allergies	☐	☐	__ __ days	
i. Cold medicine / lemsip	☐	☐	__ __ days	
j. Decongestant	☐	☐	__ __ days	
k. Cough syrup	☐	☐	__ __ days	
l. Antacids	☐	☐	__ __ days	
m. Sleeping tablets	☐	☐	__ __ days	
n. Eye drops	☐	☐	__ __ days	
o. Vaginal thrush treatments (cream or tablets)	☐	☐	__ __ days	
p. Cystitis treatments / cymalon	☐	☐	__ __ days	
q. Mouth ulcer treatments	☐	☐	__ __ days	
r. Nicotine replacement treatments	☐	☐	__ __ days	
Other 1:.....	☐	☐	__ __ days	
Other 2:.....	☐	☐	__ __ days	
Other 3:.....	☐	☐	__ __ days	
Other 4:.....	☐	☐	__ __ days	
Other 5:.....	☐	☐	__ __ days	
VITAMINS & SUPPLEMENTS				
#1:	☐	☐	__ __ days	
#2:	☐	☐	__ __ days	
#3:	☐	☐	__ __ days	
#4:	☐	☐	__ __ days	
#5:	☐	☐	__ __ days	
#6:	☐	☐	__ __ days	
#7:	☐	☐	__ __ days	
#8:	☐	☐	__ __ days	
#9:	☐	☐	__ __ days	
#10:	☐	☐	__ __ days	

Measurement of waist and hip circumference

I. Waist circumference should be measured at the midpoint between the lower margin of the least palpable rib and the top of the iliac crest, using a stretch-resistant tape that provides a constant 100 g tension;

II. Hip circumference should be measured around the widest portion of the buttocks, with the tape parallel to the floor.

For both measurements, the subject should stand with feet close together, arms at the side and body weight evenly distributed, and should wear little clothing. The subject should be relaxed, and the measurements should be taken at the end of a normal expiration. Each measurement should be repeated twice; if the measurements are within 1 cm of one another, the average should be calculated. If the difference between the two measurements exceeds 1 cm, the two measurements should be repeated.

WERF EPHect Minimal Surgical Form (EPHect MSF) Supplemental Appendix II

Surgeon ID: _____ Patient ID: _____ Date: __ __ /__ __ /__ __ __ __ (DD MM YYYY)

I. Menses: LMP: __ __ /__ __ /__ __ __ __ (DD MM YYYY) Cycle day: __ __ Currently bleeding? ☐ **No** ☐ **Yes**

II. Current hormonal treatment: ☐ **No** ☐ Do not know ☐ **Yes**

☐ COCP ☐ POP ☐ Depot progestin
☐ GnRH agonist ☐ GnRH antagonist ☐ IUCD
☐ Other _____

Last application: __ __ /__ __ /__ __ __ __ (DD MM YYYY)

III. Previous surgical diagnosis of endometriosis: ☐ **No** ☐ Do not know ☐ **Yes**

IV. Current surgery: Procedure(s): _____

V. Any pathology observed during surgery: ☐ **No** ☐ **Yes** → **If no: end of questionnaire**
Visual diagnosis of endometriosis: ☐ **No** ☐ **Yes** → **If no: go to question VII**

	Endometriosis	<1cm	1-3cm	>3cm
Perito-neum	superficial	1 ☐	2 ☐	4 ☐
	deep	2 ☐	4 ☐	6 ☐
ovary	Left superficial	1 ☐	2 ☐	4 ☐
	deep	4 ☐	16 ☐	20 ☐
	Right superficial	1 ☐	2 ☐	4 ☐
	deep	4 ☐	16 ☐	20 ☐
	Pouch of Douglas obliteration	**Partial**		**Complete**
		4 ☐		40 ☐
	Adhesions	**<1/3 enclosure**	**1/3 – 2/3**	**>2/3 enclosure**
ovary	Left filmy	1 ☐	2 ☐	4 ☐
	dense	4 ☐	8 ☐	16 ☐
	Right filmy	1 ☐	2 ☐	4 ☐
	dense	4 ☐	8 ☐	16 ☐
tube	Left filmy	1 ☐	2 ☐	4 ☐
	dense	4 ☐ *	8 ☐ *	16 ☐
	Right filmy	1 ☐	2 ☐	4 ☐
	dense	4 ☐ *	8 ☐ *	16 ☐

Revised American Fertility Society Score

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

Mark the total area of endometriosis, possibly of multiple lesions, NOT just the largest lesion

VI. Location of endometriosis, number and appearance of lesions:

LEFT SIDE

Location of Endometriosis	Lesion Size (please circle) A = <1cm B = 1 – 3 cm C = >3cm							Adhesions (please check)				
	Vascular	Clear	Yellow	Red	White	Blue/Black	Brown	Filmy	Web	Thin	Dense	Sac Like
Left pelvic sidewall ☐	A B C	A B C	A B C	A B C	A B C	A B C	A B C					
Left utero-sacral ligament ☐	A B C	A B C	A B C	A B C	A B C	A B C	A B C					
Left ovary – serosa ☐	A B C	A B C	A B C	A B C	A B C	A B C	A B C					
Left tube – serosa ☐	A B C	A B C	A B C	A B C	A B C	A B C	A B C					
Others ☐ __ __ __ __ __ __	A B C	A B C	A B C	A B C	A B C	A B C	A B C					
Others ☐ __ __ __ __ __ __	A B C	A B C	A B C	A B C	A B C	A B C	A B C					

RIGHT SIDE

Location of Endometriosis	Lesion Size (please circle) A = <1cm B = 1 – 3 cm C = >3cm							Adhesions (please check)				
	Vascular	Clear	Yellow	Red	White	Blue/Black	Brown	Filmy	Web	Thin	Dense	Sac Like
Right pelvic sidewall ☐	A B C	A B C	A B C	A B C	A B C	A B C	A B C					
Right utero-sacral ligament ☐	A B C	A B C	A B C	A B C	A B C	A B C	A B C					
Right ovary – serosa ☐	A B C	A B C	A B C	A B C	A B C	A B C	A B C					
Right tube – serosa ☐	A B C	A B C	A B C	A B C	A B C	A B C	A B C					
Others ☐ __ __ __ __ __ __	A B C	A B C	A B C	A B C	A B C	A B C	A B C					
Others ☐ __ __ __ __ __ __	A B C	A B C	A B C	A B C	A B C	A B C	A B C					

CENTRAL AREA

Location of Endometriosis	Lesion Size (please circle) A = <1cm B = 1 – 3 cm C = >3cm							Adhesions (please check)				
	Vascular	Clear	Yellow	Red	White	Blue/Black	Brown	Filmy	Web	Thin	Dense	Sac Like
Uterovesical pouch/ Anterior cul-de-sac ☐	A B C	A B C	A B C	A B C	A B C	A B C	A B C					
Pouch of Douglas/ Posterior cul-de-sac ☐	A B C	A B C	A B C	A B C	A B C	A B C	A B C					
Uterus – serosa ☐	A B C	A B C	A B C	A B C	A B C	A B C	A B C					
Bladder – deep infiltrating ☐	A B C	A B C	A B C	A B C	A B C	A B C	A B C					
Bladder – serosa ☐	A B C	A B C	A B C	A B C	A B C	A B C	A B C					
Colon – deep infiltrating ☐	A B C	A B C	A B C	A B C	A B C	A B C	A B C					
Colon – serosa ☐	A B C	A B C	A B C	A B C	A B C	A B C	A B C					
Vagina ☐	A B C	A B C	A B C	A B C	A B C	A B C	A B C					
Others ☐ __ __ __ __ __ __	A B C	A B C	A B C	A B C	A B C	A B C	A B C					
Others ☐ __ __ __ __ __ __	A B C	A B C	A B C	A B C	A B C	A B C	A B C					

Biopsy taken: ☐ **No** ☐ **Yes**
Location(s): 1. _____ 2. _____ 3. _____
4. _____ 5. _____ 6. _____

VII. Endometrioma: ☐ **No** ☐ **Yes**
☐ Left size(s): 1. __ __cm 2. __ __cm 3. __ __cm
☐ Right size(s): 1. __ __cm 2. __ __cm 3. __ __cm
☐ Sent to histology
☐ Sample collected for research: ☐ Left ☐ Right

VIII. Additional findings:

Fibroids (Myoma) ☐ **No** ☐ **Yes**
Adhesions (w/o evidence of endometriosis) ☐ **No** ☐ **Yes**
Non-endometriotic ovarian cyst ☐ **No** ☐ **Yes**
Any other findings _____

WERF EPHect Questionnaire - Minimum (EPHect EPQ-M)

Pain

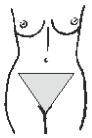
C1. Everyone experiences painful situations at some point in their lives. Such experiences may include headaches, tooth pain, joint, or muscle pain. People are often exposed to situations that may cause pain such as illness, injury, dental procedures, or surgery.

We are interested in the types of thoughts and feelings that you have **when you are in pain**. Listed below are thirteen statements describing different thoughts and feelings that may be associated with pain. Using the scale, please indicate the degree to which you have these thoughts and feelings when you are experiencing pain.

	Not at all	To a slight degree	To a moderate degree	To a great degree	All the time
I worry all the time about whether the pain will end	☐	☐	☐	☐	☐
I feel I can't go on	☐	☐	☐	☐	☐
It's terrible and I think it's never going to get any better	☐	☐	☐	☐	☐
It's awful and I feel that it overwhelms me	☐	☐	☐	☐	☐
I feel I can't stand it anymore	☐	☐	☐	☐	☐
I become afraid that the pain will get worse	☐	☐	☐	☐	☐
I keep thinking of other painful events	☐	☐	☐	☐	☐
I anxiously want the pain to go away	☐	☐	☐	☐	☐
I can't seem to keep it out of my mind	☐	☐	☐	☐	☐
I keep thinking about how much it hurts	☐	☐	☐	☐	☐
I keep thinking about how badly I want the pain to stop	☐	☐	☐	☐	☐
There's nothing I can do to reduce the intensity of the pain	☐	☐	☐	☐	☐
I wonder whether something serious may happen	☐	☐	☐	☐	☐

The following questions ask about pelvic pain <u>with</u> your periods *(including irregular bleeding or bleeding while on hormonal treatments, but not spotting)*.

By 'pelvic pain' we mean any type of pain (cramping, shooting, stabbing, etc.) in the lower part of your belly, as shown by the shaded area in this picture:



C2. Has there been a time in your life when you typically had pelvic pain during your periods?

☐ No pain → **Skip to question C15**
☐ Mild cramps (medication never or rarely needed)
☐ Moderate cramps (medication usually needed)
☐ Severe cramps (medication and bed rest needed)

→ C2.1. At what age did you start having period pain? ___ years

If you have had a period in the last 3 months, please complete the following questions, otherwise, please check here ___ and continue to question C12

Appendix 21: Stock Solutions Used

Stock Solutions Used

PBS (10x)

- 80 g NaCl
- 2 g KCl
- 26.8 g $Na_2HPO_4 \cdot 7H_2O$
- 2.4 g KH_2PO_4

pH adjusted to 7.4 with 5M HCl and solution made up to 1L with ddH_2O

PBS Working Solution (1x)

- 5 ml 10x PBS
- 45 ml ddH_2O

M2 Media

- 4.97 g HEPES
- 0.2 M NaOH
- 0.04 g Penicillin G (potassium salt)
- 0.05 g Streptomycin Sulfate
- 0.25 g $CaCl_2 \cdot 2H_2O$
- 1 g Glucose
- 0.04 g Sodium Pyruvate
- 5.53 g NaCl
- 0.36 g KCl
- 0.16 g KH_2PO_4
- 0.29 g $MgSO_4 \cdot 7H_2O$
- 0.35 g $NaHCO_3$
- 0.01 g Phenol Red

- 2.6 g Sodium Lactate
- 4 g Bovine Serum Albumin
- ddH_2O

HEPES is dissolved in 50-100 ml of ddH_2O and pH adjusted to 7.4 with 0.2 M NaOH. The penicillin and streptomycin are dissolved in a small amount of ddH_2O as is the $CaCl_2$. All other components except BSA and sodium lactate are dissolved in 500 ml of ddH_2O to which the dissolved HEPES, antibiotics and $CaCl_2$ are added. The sodium lactate is then added followed by BSA which is mixed in very gently. The pH is adjusted to between 7.2 and 7.4 with NaOH. Osmolarity is checked (to be 285-287 mols) and the solution is stored for up to 2 weeks in 4^oC.

Appendix 22: Characteristics of patients recruited to study "Peritoneal Fluid Biology in Health and Disease"

Study No.	Date of sample	Age	BMI	Smoking Status	Parity	Co-morbidities	Pathology	ASRM stage	Symptom
PF 248	8/7/2017	23	22.2	Non-smoker	0	-	Nil	-	Pain
PF 249	24/7/2017	19		Non-smoker	0	-	Nil	-	Pain & dyspareunia
PF 250	18/8/2017	38	25.8	Smoker	0	-	Endometriosis	I	Pain & dyspareunia
PF 251	30/08/2017	37				-	Endometriosis	IV	
PF 252	26/09/2017	26	21.6		0+1	Asthma, pompholyx, IBS, bipolar	Endometriosis	I	Infertility
PF 253	16/10/2017	41	25	Non-smoker	0	IDDM	Endometriosis	IV	Pain
PF 254	29/12/2017	33				-	Endometriosis	III	Pain
PF 255	29/12/2017	34				-	Endometriosis	II	Pain
PF 256	31/01/2018		20.2	Non-smoker	0	Raynaud's, eczema, pulmonary stenosis	Endometriosis	III	Pain
PF 257	05/04/2018	30	26.5	Non-smoker	1	psoriasis	PID adhesions	-	Pain
PF 258	01/06/2018	25	23	Non-smoker	0+1	-	Nil	-	Dyspareunia
PF 259	01/06/2018	30	26.2	Non-smoker	0	-	Endometriosis	III	Dyschezia, dysmenorrhoea, PR bleeding & infertility
PF 260	01/06/2018	27	39.6	Non-smoker	0	Asthma, anxiety, depression	Endometriosis	-	Dysmenorrhoea, dyspareunia & infertility
PF 261	15/06/2018	32	22.1	Non-smoker	0+3	Asthma	Endometriosis & adenomyosis	I	Dysmenorrhoea & infertility
PF 262	15/06/2018	42	36.5	Non-smoker	2+1	Intermittent SVT	Endometriosis	IV	Pain

Appendix 23: Characteristics of patients recruited to study "XSESS: oXidative Stress in women with Endometriosis pre and poSt Surgery"

Study No.	Date of sample	Age	BMI	Smoking Status	Parity	Co-morbidity	Pathology	ASRM stage	Symptom	Follow up
XS 1	9/11/18	29	19.8	Non-smoker	0	-	Endometriosis, unicornuate uterus & non-communicating rudimentary horn	III	Pain	3/1/19
XS 2	9/11/18	43	26.7	Non-smoker	1	T2DM, anxiety	Adhesions old endo, adenomyosis	I	Pain	2nd surgery
XS 3	9/11/18	42	25.4	Non-smoker	4+2	Eczema, osteoarthritis, partial thyroidectomy	Nil	-	Pain	10/1/19
XS 4	14/11/18	30	32.2	Non-smoker	0	Asthma, anxiety	Endometriosis and dermoid cyst	II	Pain	16/4/19
XS 5	15/11/18	30	22.7	Non-smoker	0	-	Endometriosis & fibroids	II	Pain	20/12/19
XS 6	16/11/18	45	22.2	Non-smoker	0	Shingles (not active), asthma	Endometriosis	II	Infertility	Missed
XS 7	16/11/18	37	31.8	Non-smoker	2+2	SVT, asthma, dermatitis, migraines	Nil	-	Pain	Missed
XS 8	20/11/18	30	27.7	Non-smoker	0	Asthma, goitre	Adenomyosis, uterine septum, PID adhesions	-	Infertility	9/2/19
XS 9	23/11/18	28	20.7	Smoker	2+3	Asthma, depression	Scarred peritoneum from previous surgery no adhesions	-	Pain	2/5/19
XS 10	27/11/18	26	26.2	Non-smoker	0	Eczema, shingles (not active)	Endometriosis	I	Pain	18/4/19
XS 11	27/11/18	33	21.8	Non-smoker	0	-	Endometriosis	IV	Infertility	6/6/19
XS 12	28/11/18	41	34	Non-smoker	0	Anaemia, migraines	Endometriosis	IV		28/3/19
XS 13	30/11/18	39	25.8	Non-smoker	2+1	-	Endometriosis	III	Pain	4/9/19
XS 14	5/12/18	44	29.5	Non-smoker	0	Asthma, eczema	Endometriosis	IV	Pain	14/3/19
XS 15	5/12/18	36	24.2		0	-	Endometriosis, fibroid and hydrosalpinx	III	Pain	10/1/19
XS 16	18/12/18	38	26.4	Non-smoker	1+3	-	Endometriosis	II	Recurrent miscarriage	18/7/19
XS 17	18/12/18	37	22.2	Non-smoker	0	Asthma, hypothyroid, depression	Endometriosis, arcuate uterus	II	Recurrent miscarriage	26/3/19
XS 18	19/12/18	38	25	Vapes	2	Asthma	Endometriosis	III	Pain	7/3/19

XS 19	20/12/18	35	24.2	Non-smoker	0	-	Endometriosis	III	Pain	Pregnant
XS 20	21/12/18	29	21.3	Non-smoker	0	Raynaud's	Endometriosis	IV	Pain	7/2/19
XS 21	9/2/19	31	32.4	Smoker	3+2	-	Omental adhesions from previous surgery	-	Pain	1/5/19
XS 22	12/3/19	36	28.7	Non-smoker	0+6	Epilepsy	Frozen pelvis - adhesions from previous surgery	-	Recurrent miscarriage	18/4/19
XS 23	13/3/19	43	26	Non-smoker	1	-	Endometriosis	IV	Pain	23/5/19
XS 24	10/4/19	31	25.9	Non-smoker	0	-	Endometriosis	II	Pain	Unable to contact
XS 25	22/3/19	31	23.3	Non-smoker	2	-	Endometriosis	II	Pain	6/11/19
XS 26	2/4/19	21	22.5	Non-smoker	0	Anxiety, PCOS, thrombophilia, IBS	Endometriosis	I	Pain	16/5/19
XS 27	26/3/19	37	19.2	Non-smoker	0	Hypothyroidism, lymphoma	Filmy adhesions, hydrosalpinx	-	Infertility	24/5/19
XS 28	5/4/19	41	24.4	Non-smoker	2+3	Anxiety	Adenomyosis	-	Pain	17/6/19
XS 29	5/4/19	48	19.3	Non-smoker	0	Coeliac, hypothyroidism	Nil	-	Pain	20/5/19
XS 30	9/4/19	33	26.2	Non-smoker	0	Anxiety	Endometriosis	IV	Infertility	8/7/19
XS 31	9/4/19	29	32.1	Non-smoker	1+6	-	Endometriosis, adhesions from previous surgery	III	Recurrent miscarriage	8/7/19
XS 32	10/4/19	32	23.9	Smoker	0	Psoriasis	Endometriosis	IV	Infertility	19/8/19
XS 33	17/4/19	24	24.3	Non-smoker	0+0	-	Endometriosis	III	Pain	8/8/19
XS 34	17/4/19	45	25.7	Non-smoker	2+0	Eczema, IBS, hayfever	Adenomyosis	-	Pain	DNA
XS 35	18/4/19	30	37.5	Smoker	0+0	Eczema, recurrent UTIs	Endometriosis	II	Pain	2/7/19
XS 36	18/4/19	49	25.7	Non-smoker	2+3	Anaemia, asthma, duodenal ulcer	Adenomyosis	-	Pain	2/7/19
XS 37	26/4/19	40	31.1	Smoker	1+0	Anxiety	Fibroid, physiological cyst	-	Pain	3/9/19
XS 38	2/5/19	34	22.5	Non-smoker	3+0	-	Endometriosis	III	Pain	3/7/19
XS 39	15/5/19	20	28.9	Non-smoker	0+0	-	Endometriosis	III	USS finding	30/8/19
XS 40	15/5/19	36	26.1	Smoker	1+1	-	Endometriosis	I	Pain	Declined f/u
XS 41	15/5/19	47	25	Non-smoker	1+0	Hiatus hernia, osteoarthritis	-	-	Pain	11/7/19

XS 42	16/5/19	38	29.7	Non-smoker	0+0	Endometriosis	Ruptured haemorrhagic cyst or corpus luteum	-	Infertility	18/7/19
XS 43	21/5/19	34	21.3	Non-smoker	0+0	Mild asthma, eczema	Endometriosis	III	Infertility	Declined f/u
XS 44	22/5/19	29	23.8	Smoker	3+1	Asthma, platelet disorder	Adhesions from PID	-	Pain	DNA
XS 45	22/5/19	40	21.8	Non-smoker	0+0	Asthma, anxiety, depression, migraines	Adhesions from previous surgery	-	Pain	Not contactable
XS 46	29/5/19	50	26.6	Non-smoker	3+0	Endometriosis	Endometriosis and fibroid	I	Pain	15/8/19
XS 47	29/5/19	46	40.2	Smoker	3+0	Depression, anaemia	Endometriosis and adenomyosis	I	Pain	3/10/19
XS 48	4/6/19	34	28.6	Non-smoker	0	IDDM, mixed personality disorder	Endometriosis	IV	Infertility	23/7/19
XS 49	12/6/19	51	34.1	Non-smoker	3+0	Endometriosis	Endometriosis	III	Pain	11/10/19
XS 50	12/6/19	44	27.2	Non-smoker	2+1	Anaemia	Simple cyst	-	Pain	Not contactable
XS 51	12/6/19	19	22.6	Smoker	0+0	Asthma	Nil	-	Pain	Not contactable
XS 52	10/7/19	39	34.9	Non-smoker	2+0	Endometriosis, adenomyosis	Nil	-	Pain	26/9/19
XS 53	13/6/19	31	22	Smoker	3+1	Gallstones	Adhesions from C/S	-	Sterilisation	Not contactable
XS 54	13/6/19	44	26.3	Non-smoker	0+0	Anxiety, migraines	Endometriosis	IV	Infertility	4/9/19
XS 55	18/6/19	33	22.3	Non-smoker	0+3	Eczema, depression	Scarring from inactive endometriosis	-	Recurrent miscarriage	30/7/19
XS 56	18/6/19	37	28.7	Non-smoker	0+0	-	Haematosalpinx, hydrosalpinx, fibroid, pelvic adhesions	-	Infertility	4/3/20
XS 57	18/6/19	38	24.4	Non-smoker	0+1	Latent TB	Endometriosis	I	Infertility	1/10/19
XS 58	2/7/19	36	29.7	Smoker	0+2	IBS	Adhesions from appendicectomy	-	Infertility	DNA
XS 59	2/7/19	40	28	Non-smoker	1+1	Eczema, asthma, anaemia, depression	Endometriosis	I	Infertility	8/8/19
XS 60	10/7/19	37	21.6	Non-smoker	2	-	Nil	-	Sterilisation	Declined f/u
XS 61	10/7/19	54	41.5	Non-smoker	2+3	Diverticulitis	Dermoid cyst and adhesions	-	Dermoid cyst	30/4/20
XS 62	30/7/19	29	32.1	Non-smoker	0+0	-	Pelvic adhesions	-	Infertility	26/9/19

Human Reproduction Update, Vol.25, No.5, pp. 593–633, 2019
Advance Access Publication on July 18, 2019 doi:10.1093/humupd/dmz012

human reproduction update

Reproductive, obstetric, and perinatal outcomes of women with adenomyosis and endometriosis: a systematic review and meta-analysis

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Submitted on September 28, 2018; resubmitted on January 2, 2019; editorial decision on February 8, 2019

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