

UNIVERSITY OF SOUTHAMPTON

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**ENDOMETRIOSIS AND ASSISTED REPRODUCTION
TECHNOLOGY**

A modern insight into an ancient disease

by

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ABSTRACT

ENDOMETRIOSIS AND ASSISTED REPRODUCTION

TECHNOLOGY: A MODERN INSIGHT INTO AN ANCIENT DISEASE

Endometriosis is a disease that is historically known to present in many guises. Since 13th century it has been metaphorically regarded as an animalistic womb causing female hysteria. Albeit widely researched, significant controversies that surround the disease remain unsolved, from the clinical presentations, diagnosis, right through to its management strategies. Despite the rapid advancement of technology in modern medicine, the ideal diagnostic and investigative tool of the disease remains elusive, and hence the management of the disease remains a challenge to clinicians. Whilst it is long known that endometriosis is detrimental to fertility, it is unclear if Assisted Reproductive Technology (ART) obliterates that risk.

This thesis aims to investigate the impact of different stages and types of endometriosis on ART outcomes, and how various surgical interventions influence the reproductive outcomes. The thesis will also focus on answering two important questions in endometriosis research, that of whether and how endometriosis impacts on 1) the developing oocytes and embryo, and 2) the endometrium.

The thesis begins with the introduction of two robust systematic reviews and meta-analyses, which concluded that women with endometriosis had a lower pregnancy rate and fewer oocytes retrieved during ART. Worse reproductive outcomes were found in those with more severe disease. Surgical treatment, which traditionally is thought to be beneficial, was found to result similar reproductive outcome in diseased and controls, and possibly more harm. Gathering evidence retrospectively from the IVF databases supported the former conclusion that women with endometriosis undertaking ART had lower number of oocytes collected from matured follicles, and further investigation into the laboratory details showed that women with endometriosis had a higher proportion of early embryo arrest. A mouse oocyte model was then utilised to explore the influence of follicular fluid of women with endometriosis on oocyte development. The study found that follicular fluid retrieved from women with endometriosis results in the activation of DNA damage response pathway, which in turn prevented normal oocyte maturation. These effects were found reversed by the agent resveratrol.

The thesis concludes with a biomarker discovery study on the endometrium of women with endometriosis compared to controls using a highly sensitive and

specific state of the art proteomics analysis method (liquid chromatography mass spectrometry with isobaric tag for relative and absolute quantitation (LC-MS/MS iTRAQ®) which uncovered unique proteins not yet described in the literature.

Endometriosis is a disease that is detrimental to the reproductive outcomes of those undergoing ART. Its influence on reproduction is complex, but it is now clear that its impact on reproduction does not stop at the traditionally viewed anatomical distortion with resultant subfertility; but have far reaching consequences including that of defective oocyte and embryo development, with a differential impact on the endometrial proteome. The solution to this challenging disease partly lies in the early diagnosis and treatment of the condition; as such notwithstanding pitfalls and fallacies surrounding biomarker discovery research, the ultimate validation of a diagnostic panel of biomarkers for the non-invasive diagnosis of endometriosis is now urgently needed.

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List of publications and proceedings

Publications

1. **M Hamdan, Omar, SZ. Dunselman, G. Cheong, Y.** Influence of Endometriosis on Assisted Reproductive Technology Outcomes: A Systematic Review and Meta-analysis. *Obstetrics & Gynecology*, 2015. 125(1): p.79-88.
2. **M Hamdan, Dunselman G, Li TC, and Cheong Y.** The impact of endometrioma on IVF/ICSI outcomes: a systematic review and meta-analysis. *Human Reproduction Update* 2015: 21; p.809-825.
3. **M Hamdan and Y Cheong.** In reply. *Obstetrics & Gynecology* 2015: 125; p.1499.

Proceedings

4. **M Hamdan, Macklon, N., & Cheong, Y.** Does surgery prior to ART affect IVF/ICSI outcome in women with endometrioma or deep infiltrating endometriosis (DIE) - a result from meta-analysis and systematic review. *Human Reproduction*; 2014, July; Vol. 29, p.361-361.
5. **Y Cheong, M Hamdan, G Dunselman.** Should surgery be performed in women with endometriosis prior to ART to improve reproductive outcome. *Human Reproduction*; 2014, July; Vol. 29, p.358-359.
6. **M Hamdan, Lane, S., Jones, K., & Cheong, Y.** Effect of follicular fluid from infertile women with endometriosis on oocyte maturation, meiotic spindle morphology and markers of DNA damage in mouse oocytes. *Human Reproduction*; 2015, June; Vol. 30, p.305-306.
7. **M Hamdan, Lane, S., Yell, D., & Cheong, Y.** Does endometriosis affect oocyte and embryo quality: perspectives from in vitro fertilisation cycles. *Human Reproduction*; 2015, June; Vol. 30, p.221-221.
8. **Cheong, Y., Hamdan, M., Dunselman, G. D., & Li, T. C.** Endometrioma and Artificial Reproductive Techniques (ART) outcomes: Results from Meta-Analysis and Systematic review. *Human Reproduction*; 2015, June; Vol. 30, p.255-255.

Declaration of authorship

I, Mukhri Hamdan, declare that this thesis and the work presented in it are my own and have been generated by me as the result of my own original research.

Endometriosis and Assisted Reproductive Technology: Ancient disease affecting modern treatment.

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:

M Hamdan, Omar, SZ Dunselman, G Cheong Y. Influence of Endometriosis on Assisted Reproductive Technology Outcomes: A Systematic Review and Meta-analysis. Obstet Gynecol, 2015. 125(1): p.79-88

M Hamdan, Dunselman G, Li TC, and Cheong Y. The impact of endometrioma on IVF/ICSI outcomes: a systematic review and meta-analysis. Human Reproduction Update 2015: 21; p.809-825.

Signed:.....

Date:

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Definitions and Abbreviation

AFC	Antral follicle count	PF	Peritoneal Fluid
AMH	Anti-Mullerian Hormone	PGC	Primordial Germ Cell
APC	Anaphase Promoting Complex	PGS	Pre-implantation genetic screening
ART	Assisted Reproductive Technology	PR	Pregnancy rate
BFSH	Baseline FSH	RCT	Randomized control trial
CDK	Cyclin Dependent Kinase	RR	Relative risk
CGH	Comparative genomic hybridization	SAC	Spindle Assembly Checkpoint
CA	Cancer Antigen	SMD	Standard means difference
CAM	Cellular Adhesions Molecules	TNF	Tumour Necrosis Factor
CI	Confidence interval	TVOR	Trans-vaginal Oocyte Retrieval
CN	Control		
CN-FF	Control group		
COS	Controlled ovarian stimulation		
CPR	Clinical pregnancy rate		
DFSHT	Total FSH dose		
FISH	Fluorescence <i>in situ</i> hybridization		
AFS	American Fertility Society		
EN-FF	Endometriosis group		
EN	Endometriosis		
FR	Fertilisation rate		
FF	Follicular Fluid		
FSH	Follicle stimulating hormone		
GVBD	Germinal Vesicle Breakdown		
HCG	Human chorionic gonadotropin		
HMG	Human menopausal gonadotropin		
HRT	Hormone replacement therapy		
ICC	Immunocytochemistry		
ICSI	Intra-cytoplasmic sperm Injection		
IL	Interleukin		
IR	Implantation rate		
IUI	Intrauterine insemination		
IVF	In-Vitro Fertilization		
LBR	Live birth rate		
LH	Luteinizing hormone		
MCC	Mitotic Checkpoint Complex		
MD	Mean difference		
MI	Meiosis I		
MII	Meiosis II		
MF	Male factor		
MF-FF	Male Factor group		
MR	Miscarriage rate		
MRI	Magnetic resonance imaging		
NA	Not applicable		
ND	Not documented		
NOR	Number of oocytes retrieved		
OCP	Oral contraceptive pill		
OR	Odds ratio		
PB	Polar Body		
PBE	Polar Body Extrusion		

Chapter 1: Introduction

1.1 Endometriosis – three centuries later

“This is a female disorder, characteristic of those who are sexually maturing”

Daniel Shroen, 1690

There have been very few scholarly attempts to trace the historical trail of endometriosis, but there is evidence that this disease existed in Europe as far back as 300 years ago. Once described as ‘the forgotten disease’ [1], it was not until the German researcher, Carl von Rokitansky, detailed the description of the pathogenesis of endometriosis that in the ensuing decades, Von Recklinghausen, Cullen and Sampson followed up his work.

Back in 1690, a German physician, Daniel Shroen, in his book *Disputatio Inauguralis Medica de Ulceribus Ulceri* has written a brief description on ulcers that in their native form were distributed throughout the stomach and were located in the bladder, the intestines, the broad ligament, and the outside of the uterus, believed to be endometriosis [1]. These ulcers today, represents ectopic endometrial tissues that are often found within the pelvis.

Despite using less precise scientific language that exists today, 18th century physicians had already described the pathology in some detail and perceived it as having an association with subfertility. Modern historical updates of the disease found evidence as early as the 13th century of women describing ‘pelvic pain’ disorders and how it was commonly described as “strangulation or suffocation of the womb” at that time [2]. It is now believed that the unsolved mysteries of hysteria could be most likely attributed to endometriosis in the majority of the cases. Even though hysteria was generally discredited in modern times, it has continued to exert a tremendous influence on attitudes on women and illnesses for most of the 20th century. Until now, even with a significant integrated history on the pathogenesis of the disease, endometriosis remains an enigmatic disease.

1.2 Objectives

This chapter aims to provide an overview of endometriosis and discuss the controversies surrounding its diagnosis and management, particularly for those who require Assisted Reproductive Technology (ART). The discussion will include the existing evidence of how the disease affects fertility, and highlight the knowledge gap between the clinical and basic science studies. This introductory chapter will lead on to the objectives of the whole thesis and the approach that will be taken to achieve these objectives.

1.3 Endometriosis and reproduction: A Synopsis

Endometriosis is defined as the presence of ectopic endometrial cells including its stroma and epithelium. Unlike adenomyosis, these cells are outside the uterus and are commonly found in the peritoneum and ovaries. Endometriosis can inflict debilitating chronic pelvic pain and subfertility to many. Its heterogeneous nature means that often the exact aetiology, symptoms, incidence and diagnosis are variably documented and described.

1.3.1 Aetiology

Despite several theories, the understanding on the aetiology of endometriosis remains limited [3, 4]. The retrograde menstruation theory and the coelomic metaplasia theory has been the top two theories that are highly cited and remain the hallmark hypotheses over the years. Sampson suggested that endometriosis occurs following retrograde flow of menstruation through the Fallopian tube into the peritoneal cavity, where the endometrial tissue subsequently implants [5]. However, this mechanism is unable to explain why endometriosis only occurs in some women when retrograde menstruation is a common occurrence in most women [6]. Another common hypothesis is coelomic metaplasia, which suggested transformation of pelvic epithelium into endometrium by metaplasia, influenced by hormonal changes. The implantation theory suggested that endometrial tissue undergoes the process of adhesion, invasion and proliferation, before it is established as endometriosis in the

peritoneal cavity. Despite the fact that all these theories appear plausible, none of them were actually proven.

1.3.2 Signs and Symptoms

Women with endometriosis may be asymptomatic [7]. Paradoxically they can also presents with multiple non-specific signs and symptoms, but none of these can accurately diagnose the presence of endometriosis. Evidence gathered from existing publications fail to point to any specific diagnostic symptom(s) [8]. In practice, clinicians will consider the differential diagnosis of endometriosis in the presence of non-specific symptoms such as dysmenorrhoea, non-cyclical pelvic pain, deep dyspareunia, subfertility and fatigue, dyschesia, dysuria, haematuria, rectal bleeding and shoulder pain. Nevertheless, the presence of pain and subfertility are strongly associated with the presence of endometriosis and vice-versa. Women who presented with either dysmenorrhoea or subfertility are 8 times more likely to have endometriosis [9]. Women presenting with dysmenorrhoea and abdomino-pelvic pains are 7 and 5 times more likely to have endometriosis respectively [10]. Those who have initially presented to the gynaecologist before diagnosis of endometriosis is made are more likely to have a shorter time-to-diagnosis duration and would have seen fewer physicians. Another study reported women who were diagnosed to have endometriosis have better patient experience if they were seen by a gynaecologist [11], demonstrating that the clinician's specialty and experience at first point of contact can make a significant difference to the patient's experience.

1.4 Incidence and burden of the disease

Generally endometriosis affects 4-5% of women in the reproductive age but this increases to 40-45% in women who present with subfertility. The incidence quoted varies between 2-10% [12-16]. The varying incidence is likely to be the result of the advancement in higher definition imaging available during laparoscopy, and the changing threshold in performing laparoscopic investigations on patients with pelvic pain and subfertility.

Endometriosis is associated with a significant cost and social burden to the healthcare system [17, 18]. For instance, the EndoCost study by the World Endometriosis Research Foundation (WERF) [19] reported that the cost involved in the management of women with endometriosis and women with chronic diseases is similar, with an average of €9,579 annual total cost per woman. A greater cost implication was shown in more severe endometriosis, in the presence of pelvic pain and/or subfertility. The health care costs for these women were mainly due to surgery (29%), monitoring tests (19%), hospitalisation (18%) and physician visits (16%). Apart from that, endometriosis also has severe social and emotional consequences [18, 20].

1.5 Controversies in the current ways of diagnosing endometriosis

Establishing the diagnosis of endometriosis is often not straightforward and this has caused significant delay from when the woman first presents to when the diagnosis is reached. Delay in diagnosis range from 4-5 years (Ireland and Belgium) [21, 22], 8 years (UK and Spain) [22] to 10.4 years (Germany and Austria) [23]. This is largely due to the variation of clinical presentation, non-standard protocols of management, the non-definitive clinical assessment and diagnosis currently available, and a complex biosocial construct that the disorder is associated with. Women with endometriosis, even on presentation to their doctors, are often subjected to multiple tests before being diagnosed.

1.5.1 Clinical examination

Pelvic and abdominal examination can be useful to facilitate the diagnosis of endometriosis. For example, vaginal examination can detect infiltration or nodules of the vagina and cervix (Figure 1-1A, Figure1-1B), uterosacral ligaments or Pouch of Douglas. Recto-vaginal digital examination allows the detection of mass involving recto-sigmoidal colon and adnexal mass. Diagnosis made from clinical examination is often limited by the experience and skill of the attending clinician. Additionally, such invasive examinations are also not appropriate for younger patients, or women who are not sexually active and is generally acknowledged to be highly subjective.

Clinical examination is equivalent to transvaginal ultrasound scan (TVS) for diagnosing endometriosis in the vaginal and recto-vaginal space. But it is inferior to TVS in the case of ovarian, uterosacral ligament and recto sigmoidal endometriosis. A combination of clinical examination and TVS has been shown to have a high predictive value in the diagnosis of Pouch of Douglas (POD) obliteration when the 'sliding sign' (a sign demonstrated by assessing the mobility of the rectum against the uterus and the posterior vaginal fornix) is utilised [24-26]. The conundrum in clinical practice is that even in the absence of any of these signs and symptoms, one is not able to completely rule out the diagnosis of endometriosis [27].

1.5.2 Radiology

Radiological means have long been used to assess women with endometriosis. Although the majority of procedures are relatively non-invasive, it may not be suitable and/or acceptable for all women. As mentioned in the earlier sections, ultrasonography is one of the commonest techniques used to diagnose endometriosis and can be combined with clinical examination. It is a radiation-free technique that is widely available, less time consuming, cost effective and repeatable [28]. However it is operator dependent and only produces limited overview images. Endometriosis/endometrioma is usually an incidental finding during routine scan as part of fertility investigations or during ovarian stimulation cycle (Figure 1-1K).

Recently, Magnetic Resonance Imaging (MRI) has become one of the routine investigations in endometriosis especially for women with a more severe disease who require surgical treatment. This technique produces good overview of the entire pelvis and enables detection of rectal and ureter invasion (Figure 1-1L). The availability of MRI can assist reproductive surgeons in planning ahead of their surgery. Other methods available are radiological examinations such as Computed Tomography (CT) scan and fluoroscopy utilise radiation, however the associated radiation risks of the latter two investigations to women's fertility are not desirable for women of reproductive age.

Although radiological techniques offer high diagnostic accuracy, the gold standard for definitive diagnosis of endometriosis is still via surgical diagnosis (e.g. laparoscopy/laparotomy) with/without histological examination confirmation.

1.5.3 Surgical

The gold standard for diagnosing endometriosis is by visual inspection during laparoscopy (Figure 1-1F, Figure 1-1G). This is however very subjective and highly dependent on the experience and the skills of the surgeons. Laparoscopy is found to be more reliable in excluding endometriosis if the laparoscopic finding was negative compared to positively diagnosing the condition if the findings are positive (positive likelihood ratio (LR+) (95% CI) is 4.30 (2.45–7.55), and the negative likelihood ratio (LR–) is 0.06 (0.01–0.47)) [29]. Some lesions of endometriosis are atypical or microscopic (Figure 1-1H-J) and hence some lesions of endometriosis can be missed during laparoscopic inspection [3]

1.5.4 Histology

Histological diagnosis obtained from tissue samples retrieved during surgery can assist in the confirmation of endometriosis, although having a negative histology does not definitively exclude the presence of the condition [8]. Thus best practice requires the surgeon to obtain tissue samples for confirmation of the diagnosis of endometriosis and it is not possible for one to sample the entire peritoneal cavity for potential disease.

1.6 Stages of endometriosis

The ideal classification system for a disease should 1) describe the condition well, 2) relate to history, 3) facilitate the decision for treatment and 4) is easy to use. It should serve as a common language for the description of the disease. Despite various available staging for endometriosis [30, 31], staging by American Fertility Society (AFS) has been the most frequently used. As in any other classifications, there are several pitfalls such as arbitrariness of scoring, potential observational errors, lack of morphologic components and poor correlation to pain.

Throughout the entire thesis, AFS staging will be used, as it is currently the most popular scoring system used in the literature and so will be of more relevance in the comparative analysis performed in this thesis. AFS staging categorises endometriosis into four stages (ASRM I, minimal; ASRM II, mild; ASRM III, moderate and ASRM IV, severe). The staging is in according to the score given depending on location, extent, and depth of endometriosis implants; presence and severity of adhesions; and presence and size of ovarian endometriomas. Majority of women with endometriosis are at minimal or mild stages, which is characterised by superficial implants and mild adhesions. Chocolate cysts and more severe pelvic adhesions are usually seen in moderate and severe stages of endometriosis. None of the components describes deep infiltrating endometriosis, and therefore ENZIAN classification was published to describe deep infiltrating endometriosis (DIE) as a supplement of the AFS score [30].

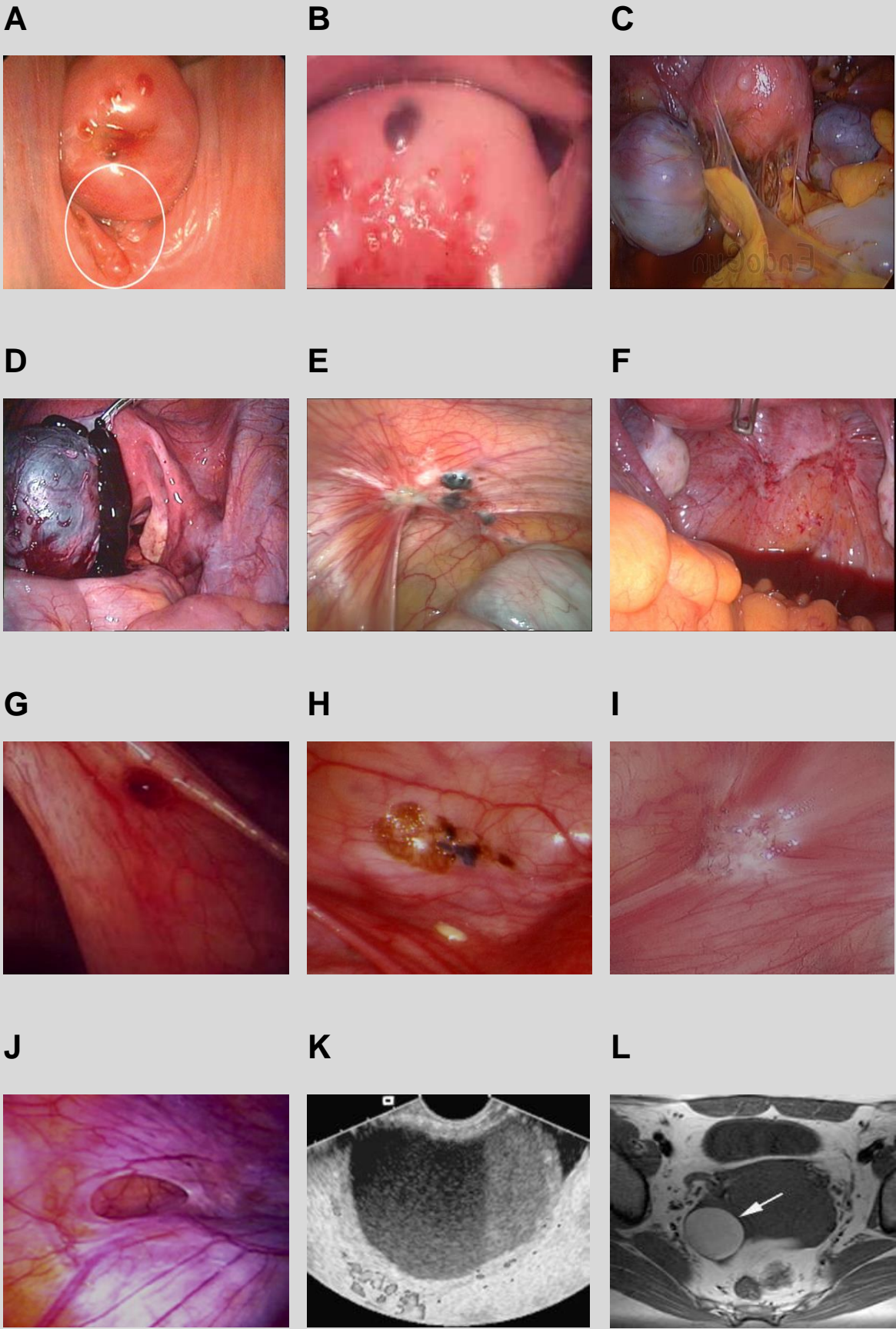


Figure 1-1 Atlas of endometriosis

A: Vaginal endometriosis seen during pelvic examination, which is diagnostic of endometriosis (<http://endopaedia.info/origin1.html>), **B:** Cervical endometriosis seen via speculum examination (<http://get-pregnant.info/tag/cervical-endometriosis>). **C:** Laparoscopic image showing pelvic adhesions, **D:** Laparoscopic image of endometrioma with chocolate material seeping out from the cyst, **E:** Deep Infiltrating endometriosis with overlying adhesions, **F:** Peritoneal endometriosis at Pouch of Douglas **G:** Typical early Red Vesicular lesion [3], **H:** Powder burn lesion at the peritoneum [3], **I:** Fibrotic lesion from repeated bleed [3], **J:** Allen-Masters peritoneal defect [3], **K:** Transvaginal Ultrasonography (TVUS) scan showing ground glass appearance of an endometrioma and **L:** Magnetic Resonance Image indicating a left endometrioma.

1.7 Types of endometriosis

In this section, the different types of endometriosis differentiated via their location will be discussed. The same patient may have endometriosis located in several locations within their pelvic abdominal cavity. The types of endometriosis are often used interchangeably to describe the disease severity.

1.7.1 Peritoneal endometriosis

Peritoneal endometriosis is the commonest type of endometriosis and is usually regarded as an earlier and milder stage of the disease although research is still required to confirm or refute the progressive nature of the disease. In general, reference to endometriosis commonly refers to peritoneal disease rather than disease confined to the ovaries or the recto-vaginal space. The clinical significance of peritoneal disease is still unclear, but its presence usually does not involve anatomical disturbance of the reproductive tract. Although in the absence of structural abnormalities, this type of endometriosis can still exert its impact on reproductive health through systemic and localised route.

1.7.2 Endometrioma

Endometrioma is an ovarian mass arising from growth of ectopic endometrial tissue within the ovary [32]. It is generally believed that endometrioma results from the deposited endometrium that has passed from the Fallopian tubes into the peritoneal cavity, and later caused adherence of the ovary to the pelvic peritoneum and its progressive invagination. According to this theory, an endometrioma is a pseudocyst, which has an inverted ovarian cortex, and hence its removal might involve the removal of normal ovarian tissue with adverse implications on the ovarian reserve. Collections of the non-resorbed bloods from repeated haemorrhage are the potential source of toxicity to the surrounding healthy tissue [33]. Up to 25% of patients undergoing IVF are estimated that have endometriosis and 17-44% of those also have ovarian endometrioma [34, 35].

1.7.3 Deep infiltrating endometriosis (DIE)

DIE is a specific entity of endometriosis and histologically defined in an arbitrary manner when endometriotic lesions extend more than 5 mm underneath the peritoneum. For bowel endometriosis, DIE involves at least the muscular layer of the intestine wall. With regards to the histology, DIE nodules differ from peritoneal and ovarian implants in that they contain a high proportion of fibro-conjunctive tissue (41%), smooth muscular fibres (35%) when compared to that of the endometrial epithelium and stroma (24%). Hence, some consider this form of endometriosis as a separate disease entity, although this is still an area of controversy.

1.8 Endometriosis and subfertility

The mechanisms involved in explaining how endometriosis affects fertility have been controversial (Figure 1-2). Although a large body of evidence is available, none of the mechanisms hypothesised have been proven [36]. The following sections will examine the possible causative mechanisms of subfertility resulting from endometriosis.

1.8.1 Ovarian function and reserve

Women with endometriosis, particularly those with endometrioma are hypothesised to have reduced ovarian function and ovarian reserve (the capacity of the ovary to provide egg cells) [36]. Endometrioma has also been shown to negatively impact on the patient's ovarian response to ovarian stimulation drugs [37, 38]. Recently it is found that there is poorer follicular growth within ovarian tissue adjacent to the endometrioma, which is speculated to be due to the toxic milieu from the cyst [33]. There is a growing interest in examining the hypothesis that iron content from the endometrioma can generate reactive oxygen species (ROS) via Fenton reaction [39, 40]. Two identical researches by Sanchez et al in 2014 [40] and Benaglia et al in 2015 [39] reported higher iron concentration in follicular fluid aspirated from ovary with endometrioma, and more worryingly, one of the papers [39] reported higher iron

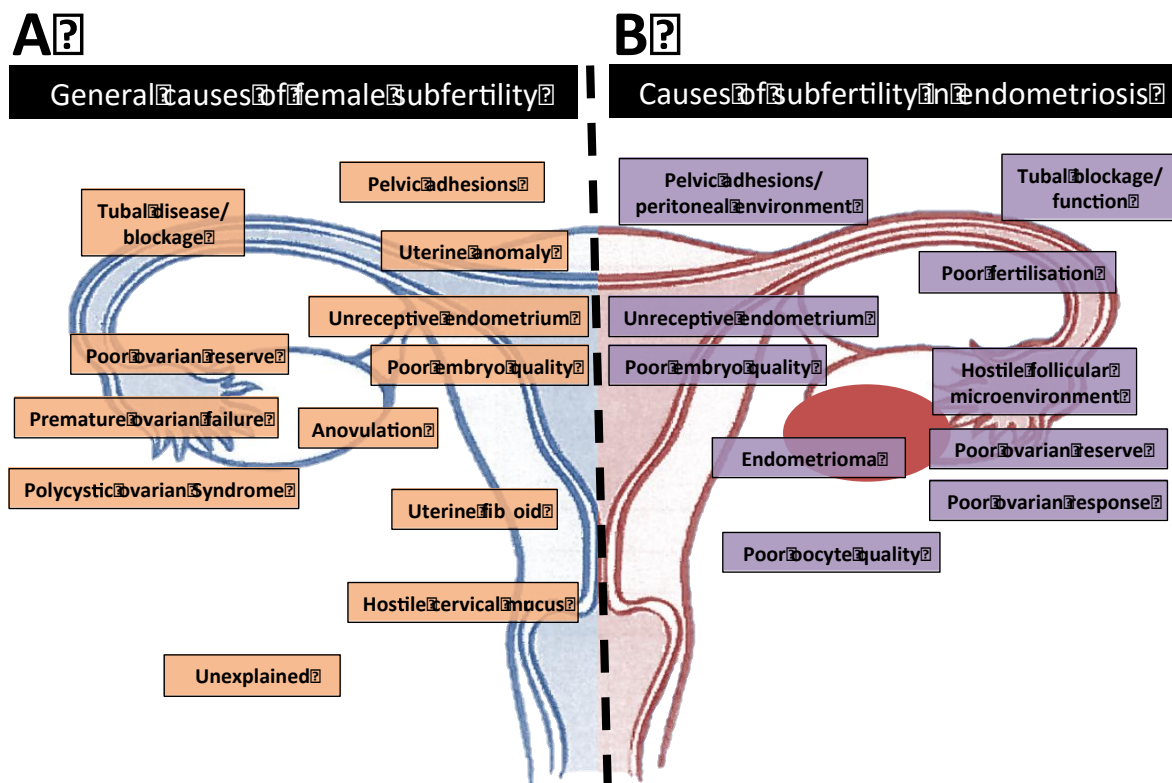


Figure 1-2 Endometriosis potential causes of subfertility

A: Figure showing the causes of subfertility in general female population according to the pelvic structure/organ. Ovulatory dysfunction can be due to hypo-gonadotropic anovulation secondary to hypothalamic and pituitary abnormalities, hyper-gonadotropic anovulation secondary to ovarian failure or eugonadotropic anovulation secondary to polycystic ovarian syndrome. Poor ovarian reserve and poor oocyte quality can result in subfertility.. Pelvic infection such as Gonorrhoea and Chlamydia infection can damage the Fallopian tube and cause tubal occlusion. Uterine abnormalities such as uterine didelphys, uterine septum and bicornuate or unicornuate uterus are associated with subfertility. Unreceptive endometrium can also result in subfertility particularly for those who had recurrent uterine procedure with intrauterine adhesions. Women with unknown cause of subfertility are categorised as unexplained subfertility. **B:** Potential causes of subfertility in women with endometriosis.

concentration in follicles at closer proximity to the cyst. Later in Chapter 4, these issues will be discussed in greater detail.

None of the available evidence has shown that endometrioma and/or endometriosis accelerate deterioration of the ovarian reserve compared to that of those with no disease. However several meta-analyses [41-43] have shown that surgical procedure can negatively impact the ovarian reserve although the effects can also be directly related to the disease process. Surgical treatment, which was traditionally thought to restore ovarian function, can be detrimental to ovarian reserve. This is probably because of the inadvertent removal of the normal ovarian tissues together with the diseased tissues during ovarian cystectomy. When excised endometriotic cyst wall specimens were examined, up to 81% of the specimens (n=70) contained normal ovarian tissues and its containing follicles [44]. Evidence for routine ovarian cystectomy prior to ART remains unclear, and needs to be addressed. The impact of surgical treatment to ART outcome will be presented in Chapter 2 in the form of two systematic reviews and meta-analyses.

1.8.2 Oocyte/embryo Quality

Whether or not, and how endometriosis influences the quality of oocyte/embryo is still a controversial subject. However, data from oocyte donation programme suggested that women with endometriosis had poor oocyte/embryo, evidenced by similar implantation rate of women with endometriosis who received oocytes from women with no endometriosis [45] but recipients who received oocytes from women with endometriosis had a lower implantation rate [46].

The potential adverse impact on oocyte quality by endometriosis was found in several publications [47-49]. Endometriosis and/or endometrioma can invoke inflammatory [50, 51] and oxidative damage [52, 53] on the oocytes and their environment resulting in poorer quality oocytes [54]. For instance inflammatory markers such as PGE2 were found to be higher in FF of women with

endometriosis compared to control (n=29, $P<0.001$, 203.9ng/L vs. 70.8ng/L), and was associated with lower number of oocytes and poorer quality [55].

On the other hand, some of the inflammatory markers such as IL-1B, TGFB2 and PGF2A were not differentially expressed in endometriosis. When examined under electron microscopy, the mitochondria of oocytes from women with endometriosis was found to be abnormal and contain small and blurred vacuoles (n=18, $P<0.05$, 50% vs. 6%), when compared to those with tubal factor [56]. In addition, meiotic spindle morphology of oocytes from women with endometriosis was found to be more disorganised compared to control (n=23, $P<0.01$, 45% vs. 4%,) [57], although another study reported higher percentage of oocyte with normal spindle morphology (n=56, $P<0.001$, 62.3% vs. 56.4%) [58]. Oxidative stress has a pathological impact on human reproduction, oocyte maturation [59] and ROS were found to be increased in granulosa cell of oocytes from women with endometriosis (n=28, $P<0.05$, 191.75 vs. 53.04) [60]. Another recent publication examined the chromosomes from polar body showed that the risk of aneuploidy in women with endometriosis was not dissimilar to those of advance maternal age [61]. Quality of oocyte in women with endometriosis and its assessment will be further discussed in Chapter 3 and potential impact of follicular fluid to oocyte quality will be presented in Chapter 4.

1.8.3 Peritoneal environment and adhesions

The majority of women with endometriosis have an increased volume of peritoneal fluid, increased concentration of activated macrophages and cytokines (interleukin, tumour necrosis factor and ROS) [62-64]. These factors can create a hostile environment for the oocytes and developing embryos and can be potentially detrimental to fertility. As the ovaries are also in constant contact with the peritoneal fluid within the abdominal cavity, damaging effects can also be exerted on primordial cells from birth. As these primordial follicles may only be recruited for completion of meiotic cell cycle after at least two decades, this long duration of exposure may cause an irreversible impact on fertility.

Inflammation as a result of endometriosis can lead to the formation of adhesions [65]. This can significantly distort normal anatomy causing mechanical blockage to the Fallopian tubes. It has been described that ciliary malfunction of the Fallopian tube [66] can cause pathological tubal function. In an animal study, the Fallopian tubes of a baboon were found to have less fimbrial activity when exposed to peritoneal fluid of women with endometriosis. This was speculated to prevent effective oocytes capture due to the presence of 'oocyte capture inhibitor' [67]. In addition to that, peri-tubal adhesions may also prevent the important physiological peristaltic movement of the Fallopian tubes that facilitates the transport of fertilised oocyte/s to a receptive endometrium.

The ovulated oocytes spend a short period of time exposed to the peritoneal environment and the peritoneal fluid within the pelvis, prior to its journey into the Fallopian tube. Peritoneal fluid of women with endometriosis was found to induce microtubules and chromosomal damage to mouse oocytes (n=100) [57].

1.8.4 Fertilisation

Results pooled from a meta-analysis showed that the fertilisation rate in subfertile women with endometriosis was lowered by 7% compared to those with no endometriosis undergoing IVF (relative risk [RR] = 0.93, 95% confidence interval [95% CI] 0.87–0.99, P = 0.03) [68]. This finding however needs to be interpreted with caution in view of the high heterogeneity between the studies. In women with endometriosis, there is some evidence to suggest a better fertilisation rate in sibling oocytes that have undergone ICSI compared to IVF (73.3% vs. 31.9%), suggesting a possible oocyte factor accounting for the poorer fertilisation rate [69]. In a separate study, longer zona pellucida dissolution time was found in women with endometriosis compared to controls (133.8 ±9.4 s vs. 90.5±5.8 s) and this was thought to interfere with fertilisation [70].

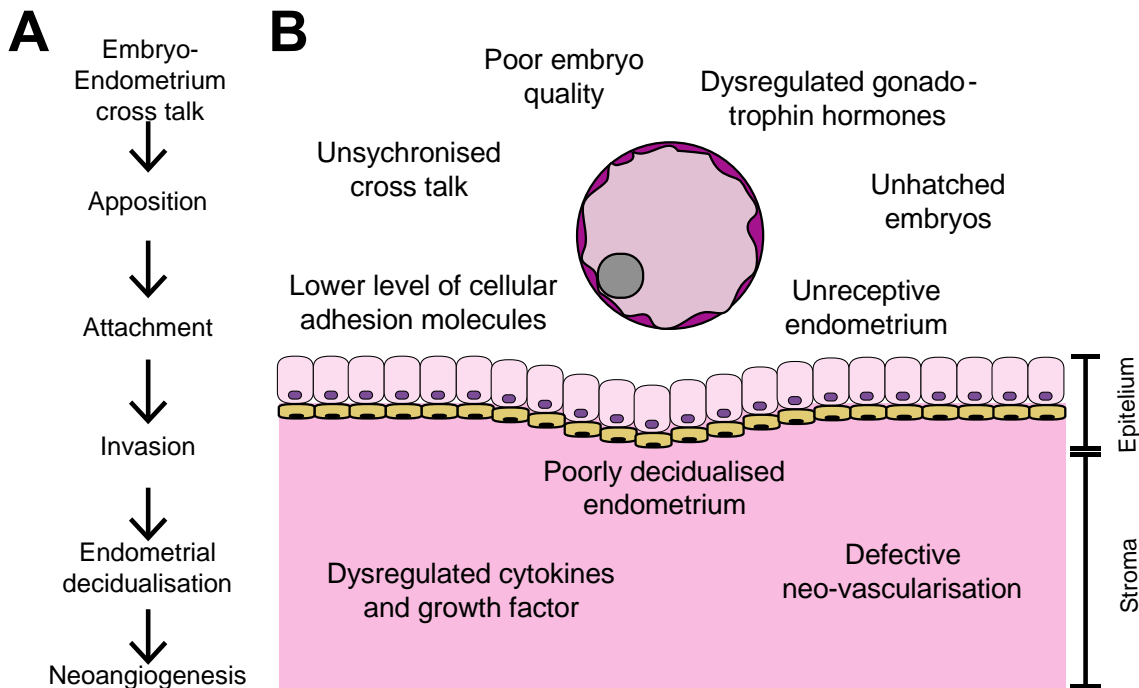


Figure 1-3 Factors contributing to implantation failure in women with endometriosis

A: Steps involved in implantation process. Successful implantation process requires a healthy embryo and a receptive endometrium. This cascading process starts with a cross talk between the free-floating embryo and the endometrium. Signals transduced from the embryo are received by the endometrium where the embryo then apposes itself, and attaches onto the endometrium. Following the attachment, the embryo invades the endometrial stroma and initiates endometrial decidualisation and neo-angiogenesis. **B:** In endometriosis, implantation failure can be due to absent of one or more of the factors involved in the process.

1.8.5 Endometrial receptivity

Receptive endometrium plays an important role in implantation. The cross talk between receptive endometrium and healthy mature quality embryo is needed for a successful implantation process (Figure 1-3). Women with endometriosis, particularly those with more severe disease have lower implantation rate compared to those with no endometriosis (RR = 0.79, 95% CI 0.67–0.93, P = 0.006) [68]. This may well be the result of a detrimental impact of the disease on both the endometrium and/or the embryo. Various studies have identified distinct differences between the endometrium of women with endometriosis and those without at receptor [71] level, mRNA and protein level [72], and on a more global scale, at the proteomics [73-75], secretomics [76, 77] and genomics level [78]. These differences are hypothesized to impact on the embryo apposition, attachment, invasion and implantation. As patients with endometriosis consist of a rather heterogeneous population, from very mild disease, to those with severe pelvic disease, one may expect that the endometrium may reflect these differences. There is currently no diagnostic/investigative tool to help distinguish those with endometriosis from those without. The distinct differences between the endometrium of women with endometriosis as opposed to those without will be explored further in Chapter 5 and 6, where the results of various biomarker discovery studies will also be discussed and critically appraised,

1.9 Treatment

Strategies for treating endometriosis depend largely on the presenting complaint; one of where pain is the main symptom requires a rather different treatment strategy from that of subfertility. As such, this thesis will only deal with those that relate to subfertility.

1.9.1 Medical treatment

Medical management of endometriosis is limited to hormonal therapy, which is contraindicated in women who wish to conceive. A Cochrane review by Hughes et al in [79] has concluded that medical treatment for women with endometriosis and subfertility is not beneficial and clinicians are not recommended to prescribe hormonal treatment for suppression of ovarian function to improve fertility.

1.9.2 Surgical treatment

In subfertile women with minimal and mild endometriosis, operative laparoscopy including adhesiolysis is effective in increasing the spontaneous pregnancy and live birth rate compared to diagnostic scope alone. This is based on moderate evidence from two randomized controlled trials (n= 382) [80]. One further randomized controlled trial published by Italian Group [81], which was excluded in the meta-analysis [80] due to the use medical therapy following surgery, reported no significant difference in live birth rate. With regard to the surgical techniques, one study [82] reported that the treatment with CO₂ laser has higher cumulative birth rate compared to monopolar electrocoagulation in women with mild endometriosis.

In subfertile women with more severe endometriosis, there is currently limited quality data examining the impact of surgical treatment on spontaneous pregnancy. However, two independent prospective cohort studies [83, 84] reported higher crude spontaneous pregnancy rate in women with moderate (57-69%) and severe (52-68%) endometriosis who had surgical treatment comparing the same outcome of those with severe endometriosis that only had expectative management. In women with severe endometriosis with endometrioma, there is evidence that ovarian cystectomy has greater

advantage than aspiration and ablation in terms of spontaneous pregnancy in women who had documented prior subfertility (OR 5.21, 95% CI 2.04 to 13.29), disease recurrence and pain [85] .

The recent ESHRE guideline [8] on endometriosis has recommended that reproductive clinicians consider operative laparoscopy instead of expectant management in subfertile women with more severe endometriosis to improve spontaneous pregnancy [8]. However this recommendation was formulated based on younger patients with a normal ovarian reserve, and should be applied with caution, especially when relating to the reproductive outcomes for the older age group of patients who generally have a lower ovarian reserve. These recommendations may appeal more to certain groups of reproductive practitioners with a particular interest in surgery, but may not be acceptable to all practicing gynaecologists. In addition, one should balance the risk of surgery and its complication rate, with the added benefit gained. ESHRE guideline [8] was developed by reproductive clinicians with a mixture backgrounds, recommendations in the guideline may be biased and only reflect personal views according to the interests and skills.

1.9.3 Medically Assisted Reproduction

By definition, the term of medically assisted reproduction includes ovulation induction, IUI and Artificial Reproductive Technology (ART). With varying degrees of endometriosis and risk of coexisting tubal disease, ART can be the only option.

In ART, IVF treatment (with and without ICSI) is recommended in women with endometriosis [8]. Although sporadic reports showed poorer pregnancy outcomes, two published meta-analyses [68, 86] reported similar LBR in women with endometriosis when compared to other indications of ART. However in more severe disease, the ART reproductive outcomes were consistently poorer [86]. The existing literature on this topic is rather dated, and they were confounded by the inclusion of women who were undergoing oocyte donation/recipient cycle.

The notion of whether surgery should be performed before ART is still a controversial subject. There is only one retrospective study conducted that has

concluded that in women with less severe endometriosis (ASRM stages I-II), surgical treatment prior to ART resulted in a higher LBR (OR 1.47 95% CI [1.01 to 2.13], n=661) when compared to those who did not undergo surgery. Clearly, more RCTs need to be performed to confirm this finding [87].

There is no published data available evaluating the impact of surgical treatment on ART outcomes in women with more severe disease. This is not surprising as it is clearly a challenge to recruit patients to a randomised controlled study whereby there is a possibility that identified endometriosis is left untreated. The two published meta-analyses [68, 86] previously mentioned did not examine the effect of surgical treatment prior to the ART. They also did not separate the outcomes of women with endometrioma compared to those who have no endometriosis or those who only have peritoneal endometriosis. In a separate meta-analysis, surgical treatment of endometriotic cyst (endometrioma) did not alter ART outcomes [88].

1.10 Thesis aims

This thesis is set out to investigate how endometriosis affects women's fertility focussing on those who require ART. In order to achieve this main objective, this thesis is divided into several chapters with their own separate objectives.

1.10.1 The impact of endometriosis and endometrioma on ART outcomes

There are many published data on ART outcomes in women with and without endometriosis since the early 1980s. This includes the benefit of surgical treatment of endometriosis and endometrioma. The results were somehow conflicting and no meaningful conclusion was achieved. Therefore this thesis aims to update the available evidence and determine the impact of endometriosis and endometrioma on ART outcomes in a format of a meta-analysis and systematic review. This will be presented in Chapter 2.

1.10.2 The laboratory findings on oocyte and embryo development in women with endometriosis compared to those without

In this chapter, a comparison study was performed to assess the embryo quality of women with and without endometriosis. With the hypothesis that women with endometriosis have a poorer embryo quality, a database review from two IVF centres in the UK collated and analysed with the results presented in Chapter 3.

1.10.3 The effect of follicular fluid on the oocyte maturation rate of women with endometriosis

This chapter aims to evaluate the factors that contribute to a lower number of oocytes retrieved during IVF cycles of women with endometriosis. In this chapter, immature mouse oocytes were incubated with the follicular fluid of women with and without endometriosis; the working hypothesis is that follicular fluid of women with endometriosis causes maturation arrest by activating DNA damage pathway. This will be presented in Chapter 4.

1.10.4 Proteomic profile of endometrium in women with endometriosis

This chapter aims to identify novel biomarkers in the diagnosis of endometriosis. A state of the art proteomic analysis using Liquid Chromatography with tandem mass spectrometry and isobaric labelling (LC-MS/MS-iTRAQ) of endometrium from women with and without endometriosis was analysed and the study will be presented in Chapter 5 and 6.

Chapter 2: Effect of endometriosis on ART outcomes

Endometriosis and endometriomas can influence fertility differently. Molecularly, one group of thinking is that they are in fact of potentially different disease pathogenesis [3]. Hence, it is crucial that the clinical outcome of the impact of these two entities on reproduction, where possible, be examined separately. This chapter set out to examine the separate question of the influence of endometriosis on ART and the impact of endometrioma on IVF/ICSI outcomes.

For the first part of this chapter, a systematic review and meta-analysis of all available published data on ART outcomes was performed, comparing women with and without endometriosis. To optimise the ART outcomes in women with endometriosis, reproductive practitioners sometimes resort to surgical treatment, although the evidence supporting the need for surgical treatment of endometriosis prior to ART is sparse. The evidence around the impact of surgery on the reproductive outcomes will also be examined.

2.1 Influence of Endometriosis on Assisted Reproductive Technology Outcomes: A Systematic Review and Meta-analysis

2.1.1 Introduction

Endometriosis is associated with subfertility, [89-91] and up to 50% of women with endometriosis have difficulty conceiving naturally [92]. The cause and effect of endometriosis on subfertility remains controversial although it is known that without intervention, women with more severe disease are less likely to conceive [92]. There are conflicting results regarding the reproductive outcomes associated with subfertile women with varying severity of endometriosis undergoing assisted reproductive technology (ART) [46, 93-96]. As discussed previously (Chapter 1), many clinicians will now recommend surgical treatment to improve subfertility in women with mild and moderate endometriosis albeit that this recommendation is based on moderate evidence from two randomized controlled trials (n= 382) [80]. One earlier randomized controlled trial by an Italian Group [81] however reported no significant difference in pregnancy rate

in treatment (24%) and no treatment group (24%). This later study offered adjuvant treatment following surgery and therefore was not included in the meta-analysis by Duffy et al 2014 [80].

There is no consensus as to whether surgical treatment should be offered to women undergoing ART to improve their reproductive outcome. A meta-analysis [86] performed over 10 years ago indicated that the pregnancy rate is halved in women with endometriosis undergoing ART treatment. A more recent meta-analysis [97] reported on the detrimental effect of endometriosis on implantation and clinical pregnancy rates but did not examine the effect of surgery on the reproductive outcome of ART in women with endometriosis. Guidelines from national authorities appear conflicting [8, 98, 99] and based on scarce evidence.

Surgical intervention is generally thought to be beneficial although the drawbacks of surgical complications can be significant [42, 100]. Hence, surgical treatment of endometriosis prior to fertility treatment may improve the outcome although it may potentially cause harm.

2.1.2 Objectives

The objectives of this systematic review and meta-analysis are to investigate the association of endometriosis on the reproductive outcomes of women undergoing ART, and to evaluate the association of surgery prior to the ART treatment on reproductive outcomes.

2.1.3 Criteria for considering studies for this review

2.1.3.1 Type of studies

Published studies (nonrandomised and randomised studies) were eligible for inclusion. Studies were only included once if they had overlapping data. For the purpose of this review, I have excluded publications that only included patients with endometrioma(s) during the course of treatment.

2.1.3.2 Type of participants and interventions

Studies comparing the ART (this is defined as IVF with or without ICSI) outcomes of women with endometriosis to women with no endometriosis were

included. Studies were excluded if the participants received any known non-surgical treatment (medical management, alternative treatment), were involved with donor/recipient oocyte treatment, or if the study did not have any non-endometriosis control group. The revised American Fertility Society staging of endometriosis was used to grade the disease severity. Studies that specified 'surgical treatment' on women with endometriosis were categorised as 'surgical treatment specified' and others were categorised as 'surgical treatment not specified'.

2.1.3.3 Type of outcome measures

The primary outcome measure is the live birth rate (LBR) per woman defined as the number of deliveries that resulted in at least one live born baby expressed per 100 patients.

Secondary outcome measures are i) clinical pregnancy rate (CPR) per woman (defined as pregnancy diagnosed by ultrasonographic visualization of one or more gestational sacs or definitive clinical signs of pregnancy, and was expressed per 100 patients [101]; ii) miscarriage rate defined as any loss of pregnancy before 24 week of gestation; and iii) mean number of oocytes retrieved per cycle (MNOR).

2.1.4 Data collection and analysis

2.1.4.1 Study Selection

Studies published between 1980 and 2014 on endometriosis and ART outcomes, without language restriction were searched. Electronic databases, trial registers and websites including MEDLINE, EMBASE, Cochrane, Central register of Controlled Trials, ClinicalTrial.gov and Web of Science were searched based on keywords and/or medical subject heading (MeSH) terminology (ART, endometriosis, endometrioma, IVF, ICSI, In vitro fertilisation, Intra Cytoplasmic Sperm Injection, outcome, pregnancy, live birth). The reference lists of all known primary and review articles were examined to identify cited articles and abstracts not captured by the electronic search.

2.1.4.2 Data extraction and management

Two review authors (MH, YC) independently examined these full text articles for compliance with the inclusion criteria, select studies eligible for inclusion in the review and the methodological quality of the studies and extracted relevant data. The quality of individual studies was assessed in accordance to the MOOSE criteria and the Newcastle Ottawa Scale [102]. Study investigators were corresponded if clarification needed for study eligibility. The process is documented in the PRISMA chart.

2.1.4.3 Subgroup analysis

Where data was available, the association of 1) the different stages of endometriosis (ASRM I-II and ASRM III-IV) and 2) surgical treatment on the outcome measures specified were analysed.

2.1.4.4 Assessment of heterogeneity

Studies that were collated together in a systematic review will inevitably differ from one to another. Heterogeneity is variability found among studies in a systematic review, including clinical variability (participants, interventions and outcomes studies), statistical heterogeneity (intervention effects evaluated in the different studies) and methodological heterogeneity (variability in study design and risk of bias). Methodological and clinical heterogeneity among the included studies were assessed by measuring the I^2 value. Heterogeneity was

also explored by means of sensitivity analysis. Dichotomous data (e.g. clinical pregnancy rate) and continuous data were analysed using Mantel-Hansel odds ratio and the mean difference between treatments groups respectively.

2.1.4.5 Data synthesis

As previously described in a Cochrane Review, a fixed-effect model was used and heterogeneity between the results of different studies was examined by inspecting the scatter in the data points, the overlap in their CIs, and more formally by checking the results of the χ^2 test and the I^2 statistic. An I^2 greater than 50% was taken to indicate substantial heterogeneity and in that case a random-effects analysis was used.

2.1.4.6 Quality assessment of the data

Two authors (MH, YC) assessed the methodological quality of the studies and extracted relevant data such as diagnosis of endometriosis, surgical treatment, staging of the disease, selection of control and definition of primary and secondary outcome. Where available, statistical data from the original papers or calculated missing parameter by using data provided were extracted. The quality of individual studies was assessed in accordance to the MOOSE criteria and the Newcastle Ottawa Scale [102]. By using the Newcastle–Ottawa scale, non-randomised studies were rated according to eight items categorized in three domains: study group selection, comparability of the groups, and ascertainment of outcome (maximum scores of 4, 2, and 3, respectively). Score were represented with stars for each quality item to provide a visual assessment. Studies were awarded up to nine stars if they fulfilled all the quality items.

2.1.4.7 Measures of treatment effect

For dichotomous data (e.g. clinical pregnancy rate), the number of events in the control and intervention groups of each study keyed in Review Manager 5 and analysed using Mantel-Hansel odds ratio. And for continuous data, mean difference between treatments groups was calculated.

2.1.5 Results

2.1.5.1 Result of search

The search strategy yielded 1346 articles, out of which 1231 were excluded because it was clear from the title or abstract that they did not fulfil the selection criteria (Figure 2-1). One hundred and fifteen full texts retrieved and 53 potential studies were extracted for analysis. Studies with no control groups (n=5) [96, 103-106], only specific to the presence of intact endometrioma(s) during IVF treatment (n=5)[107-111], using donor oocyte cycle (n=2)[45, 112], had prior non-surgical treatment (n=2)[113, 114] and overlapping data (n=3)[115-117] were excluded. A final number of 36 retrospective studies were included in this review and none were RCTs.

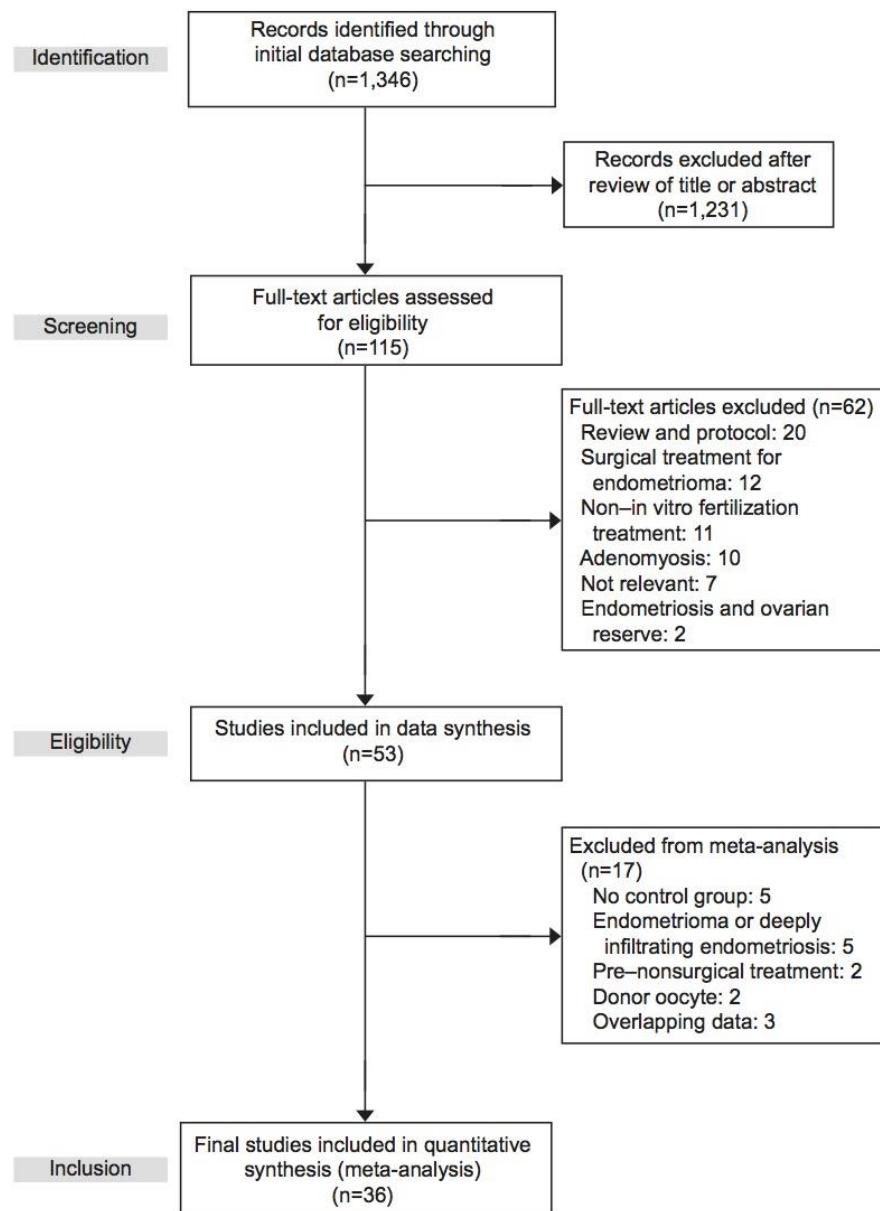


Figure 2-1 Prisma flow chart

Prisma flow chart outlining the process for data extraction, beginning with database search for record identification, followed by screening for full text articles for eligibility and final studies included in the analysis. The boxes to the right indicate number of studies that were excluded with their reason for exclusion stated.

2.1.5.2 Description of studies and participants

All thirty-six retrospective observational studies (n=29,454) were included. Out of which 4,852 women had endometriosis. The majority of control groups (23/36) had tubal factor. Most of the included studies stated the stage of the disease (n=22). Almost all of the included studies reported CPR (33/36) as primary outcome with thirteen (13/36) reported LBR. Twelve studies from this meta-analysis included participants who received surgical treatment for endometriosis whilst the others did not specify. None of these studies provided the duration from the surgical procedure to the ART. Eight studies included patients with endometrioma, out of which 3 studies [118-120] presented data pertaining to women with endometriomas separately that were used. All the studies except one specified that they have excluded participants who have had any medical treatment prior to the ART [119]. The study characteristics are depicted in Table 2-1.

Table 2-1 Characteristics table of all studies included in the review.

No	Reference	Country	Study Design	Study dates	Patient type, Stage(s)	No of patients	Control type	No of patients	Treated	Outcome measures	Protocol	IVF/ ICSI
1	Dong et al 2013	China	Retrospective Cohort	2011-2012	Endo, Stage I-II, III-IV	431	Tubal factor	596	Yes	LBR, CPR	Long Protocol	IVF/ ICSI
2	Mekaru et al 2013	Japan	Retrospective Cohort	2004-2008	Endo, Stage I-II	18	Unexplained	17	Yes	LBR, CPR	Long protocol	IVF ICSI
3	Opoein et al 2012	Norway	Retrospective Cohort	1996-2011	Endo, Stage I-II, III-IV	1074	Tubal factor	1171	Yes	LBR, CPR	Long Protocol	IVF ICSI
4	Lin et al 2012	China	Retrospective Cohort	2006-2010	Endo, Stage I-II, III-IV	177	All other infertility	4267	NM	LBR	Mix protocol	IVF ICSI
5	May et al 2012	UK	Retrospective Cohort	2004-2005	Endo, All stages	4	Tubal factor	13	NM	CPR	NA	IVF ICSI
6	Coccia et al 2011	Italy	Retrospective Cohort	2001-2007	Endo, Stage I-II, III-IV	148	Tubal factor	72	Yes	CPR	Long Protocol	IVF
7	Kim et al 2011	China	Retrospective Cohort	NA	Endo, Stage III-IV	20	Tubal factor	20	NM	LBR, CPR	Long Protocol	IVF
8	Mohammed et al 2011	UK	Retrospective Cohort	2000-2008	Endo, All stages	415	All other infertility	6871	NM	LBR, CPR	Long protocol	IVF
9	Falconer et al 2009	Sweden	Prospective Cohort	NA	Endo, Stage I-II	34	Tubal factor	38	NM	CPR	Long protocol	IVF ICSI
10	Nejad et al 2009	Iran	Retrospective Cohort	2005-2006	Endo, stage I-II, III-IV	80	Tubal factor	57	NM	CPR, IR	Long protocol	IVF ICSI
11	Kuroda et al 2009	Japan	Retrospective case control	2006-2008	Endo, All stages	7	Tubal factor	21	Yes	CPR	Mix protocol	IVF ICSI

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12	Al-Fadhli et al 2007	Canada	Retrospective case control	1999-2003	Endo, All stages	87	All other infertility	87	Yes	CPR	Long protocol	IVF
13	Kuivasari et al 2005	Finland	Retrospective cohort	1996-2003	Endo, Stage I-II, III-IV	98	Tubal factor	87	Yes	LBR, CPR	Long protocol	IVF ICSI
14	Loo et al 2005	Taiwan	Retrospective cohort	2000-2002	Endo, All stages	85	Tubal Factor	71	Yes	CPR	Long protocol	IVF
15	Aboulghar et al 2003	Egypt	Retrospective case control	1999-2001	Endo, Stage IV	85	Tubal Factor	177	Yes	CPR	Long protocol	IVF
16	Hickman et al 2002	USA	Retrospective cohort	1996-1999	Endo, Stage I-II, III-IV	27	Tubal Factor	104	NM	LBR, CPR	Long protocol	IVF
17	Bukulmez et al 2001	Turkey	Retrospective case control	1996-1999	Endo, Stage I-II, III-IV	44	Tubal Factor	588	No	LBR, CPR	Long Protocol	ICSI
18	Meden-Vrtovec 2000	Slovenia	Retrospective cohort	1990-1999	Endo, Stage I-II	612	Tubal factor	7339	NM	LBR, CPR	Long protocol	IVF
19	Bergendal et al 1998	Canada	Retrospective cohort	1994-1997	Endo, All stages	48	Tubal factor	98	NM	LBR	Long protocol	IVF
20	Hull et al 1998	UK	Retrospective cohort	1990-1996	Endo, All stages	194	Tubal factor, Unexplained	836	NM	CPR	NA	IVF
21	Pellicer et al 1998	Spain	Case control study	NA	Endo, All stages	12	Tubal factor	11	NM	CPR, IR	Long protocol	IVF
22	Huang et al 1997	Taiwan	Retrospective cohort	1993 (Jan-Dec)	Endo, Stage I-II, III-IV	75(C)	Tubal factor	60(C)	NM	CPR	Long protocol	IVF
23	Padigas et al 1996	Canada	Retrospective analysis	1990-1994	Endo, stage III-IV	23	Tubal factor	312	Yes	CPR	Long protocol	IVF
24	Arici et al 1996	USA	Retrospective case control	1988-1994	Endo, Stage I-II, III-IV	35	Tubal, unexplained	85	NM	LBR, CPR	Long protocol	IVF

25	Tanbo et al 1995	Norway	Retrospective analysis	1986-1994	Endo, Stage I (mild)	265	Tubal, unexplained	690	NM	CPR	Long protocol	IVF
26	Olivennes et al 1995	USA	Retrospective cohort	1988-1992	Endo, All stages	90	Tubal factor	111	NM	CPR, LBR	Long protocol	IVF
27	Gerber et al 1995	UK	Retrospective study	1988-1991	Endo, Stage I-II, III-IV	129 (C)	Tubal, unexplained, male factor	1377 (C)	NM	CPR	Long protocol	IVF
28	Dmowski et al 1995	USA	Retrospective analysis	1991-1993	Endo, Stage I-II, III-IV	84	All other infertility	109	NM	CPR	Long protocol	IVF
29	Simon et al 1994	Spain	Retrospective analysis	1990-1993	Endo, Stage I-II, III-IV	59	Tubal factor	78	NM	CPR	Long protocol	IVF
30	Mills et al 1992	UK	Retrospective study	1988-1990	Endo, All stages	67	Tubal, unexplained	198	NM	LBR, CPR	Long protocol	IVF
31	Inoue et al 1992	Japan	Retrospective study	Up to Dec 1988	Endo, Stage I-II, III-IV	309	All other infertility	372	Yes	CPR	Long protocol	IVF
32	Mahmood et al 1991	Scotland	Prospective study	NA	Endo, All stages	20	Tubal factor	10	NM	CPR	NA	IVF
33	Frydman et 1987	France	Retrospective Cohort	1984- 1986	Endo, All stages	34(C)	Tubal factor	544(C)	NM	CPR	NA	IVF
34	Matson et al 1986	Australia	Retrospective Cohort	NA	Endo, Stage I, II, III, IV	96	Tubal factor	28	NM	CPR	NA	IVF
35	Wardle et al 1985	UK	Retrospective cohort	NA	Endo, All stages	47	Tubal, unexplained	68	NM	CPR	NA	IVF
36	Mahadevan et al 1983	Australia	Retrospective cohort	1981-1982	Endo, All stages	14(C)	Tubal, unexplained, male factor	414(C)	NM	CPR	NA	IVF

2.1.5.3 Quality of study

The quality of each paper was assessed and scored according to Newcastle-Ottawa Assessment scale (described earlier) as shown in Table 2. Majority of the included studies scored more than 7 and eight studies scored the highest possible score of 9 (Table 2-2)

Table 2-2 Newcastle-Ottawa Scoring scale

Table showing Newcastle-Ottawa Scoring system that was used to assess the quality of each included study in the analysis. This scale was developed to assess the quality of non-randomised studies with its design, content and ease of use directed to the task of incorporating the quality assessments in the interpretation of meta-analytic results. () indicates that the feature is present; x, that the feature is absent. But for comparability by design this checklist awards maximum of two stars (**), one (*) or none if the feature is completely absent.*

No	Reference	Case-cohort representative	Selection of non-exposed control	Ascertainment of exposure	Outcome negative at start	Comparability by design	Comparability by analysis	Outcome assessment	Duration of follow-up	Score
1	Dong	*	*	*	*	*	*	*	*	8
2	Mekaru	*	*	*	*	*	*	*	*	8
3	Opoein	*	*	*	*	**	*	*	*	9
4	Lin	*	*	*	*	*	*	*	*	8
5	May	*	*	*	*	*	*	*	*	8
6	Coccia	*	*	*	*	**	*	*	*	9
7	Kim	X	*	*	*	*	*	*	*	7
8	Mohammed	*	*	*	*	*	*	*	*	8
9	Falconer	*	*	*	*	*	*	*	*	8
10	Nejad	*	*	*	*	*	*	*	*	8
11	Kuroda	*	*	*	*	*	*	*	*	8
12	Al-Fadhli	*	*	*	*	*	*	*	*	8
13	Kuivasari	*	*	*	*	**	*	*	*	9
14	Loo	*	*	*	*	*	*	*	*	8
15	Aboulghar	*	*	*	*	*	*	*	*	8
16	Hickman	*	*	*	*	**	*	*	*	9
17	Bukulmez	*	*	*	*	**	*	*	*	9
18	M-Vrtovec	*	*	*	*	*	*	*	X	7
19	Bergendal	*	*	*	*	*	*	*	*	8
20	Hull	X	X	*	*	**	*	*	*	7
21	Pellicer	*	*	*	*	*	*	*	*	8
22	Huang	*	*	*	*	*	*	*	*	8
23	Padigas	*	*	*	*	*	*	*	*	8
24	Arici	*	*	*	*	**	*	*	*	9
25	Tanbo	*	*	*	*	*	*	*	*	8
26	Olivennes	X	X	*	*	*	*	*	*	6
27	Gerber	*	*	*	*	*	*	*	*	8
28	Dmowski	*	*	*	*	*	*	*	*	8
29	Simon	*	*	*	*	**	*	*	*	9
30	Mills	*	*	*	*	*	*	*	*	8
31	Inoue	*	*	*	*	*	*	*	*	8
32	Mahmood	*	*	*	*	*	*	*	*	8
33	Frydman	*	*	*	*	*	*	*	*	8
34	Matson	*	*	*	*	X	*	*	*	7
35	Wardle	*	*	*	*	**	*	*	*	9
36	Mahadevan	*	*	*	*	*	*	*	*	8

2.1.5.4 Impact of endometriosis

The overall LBR was not statistically different between women with endometriosis and women with no endometriosis (OR 0.94 95% CI [0.84 to 1.06], 13 studies, 12,682 patients, $I^2=35\%$, Figure 2-2). Compared to women with no endometriosis, women with endometriosis had a lower CPR (OR 0.78, 95% CI [0.65, 0.94], 24 studies, 20,757 patients, $I^2 =66\%$, Figure 2-3) lower MNOR per cycle (Mean Difference -1.98, 95%CI [-2.87 to -1.09], 17 studies, 17,593 cycles, $I^2=97\%$, Figure 2-4) and no difference in the miscarriage rate (OR 1.26, 95% CI [0.92, 1.70], 9 studies, 1,259 patients, $I^2 0\%$, Figure 2-5).

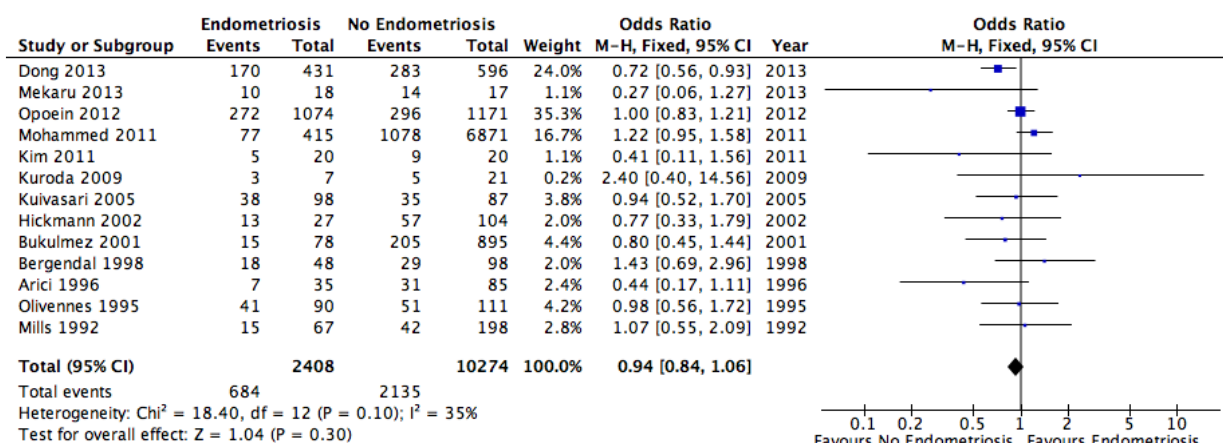


Figure 2-2 Impact of endometriosis on LBR

Forest plot comparing women with and without endometriosis to evaluate the impact of endometriosis on live birth rate (LBR).

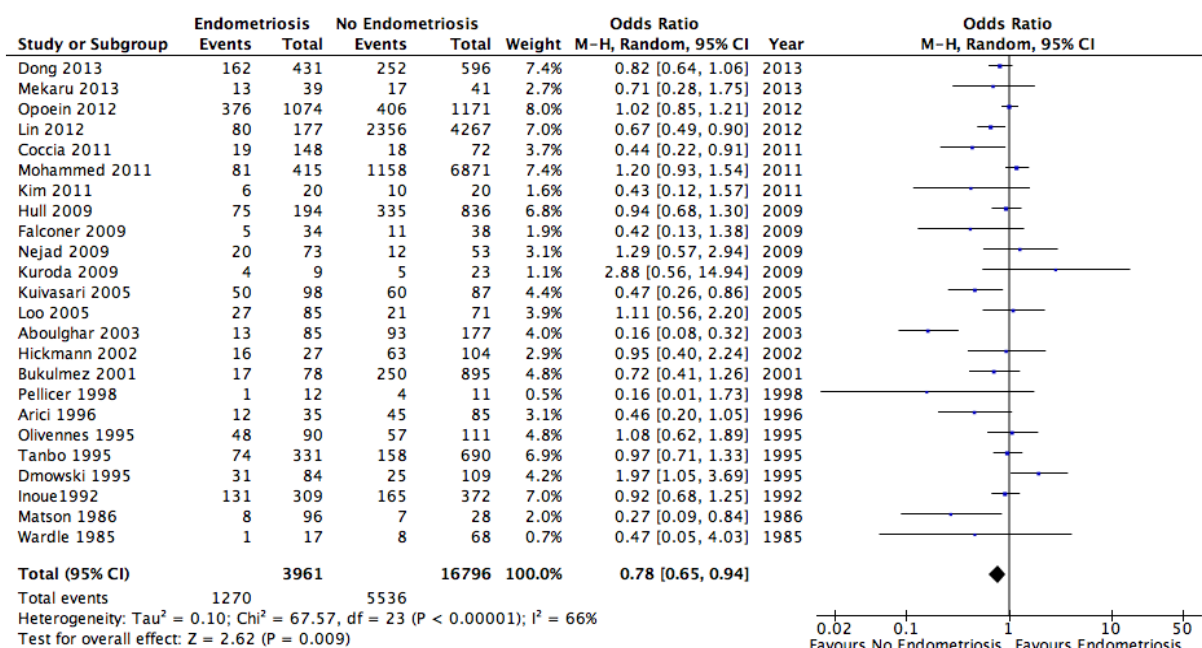


Figure 2-3 Impact of endometriosis on CPR

Forest plot comparing women with and without endometriosis to evaluate the impact of endometriosis on clinical pregnancy rate (CPR).

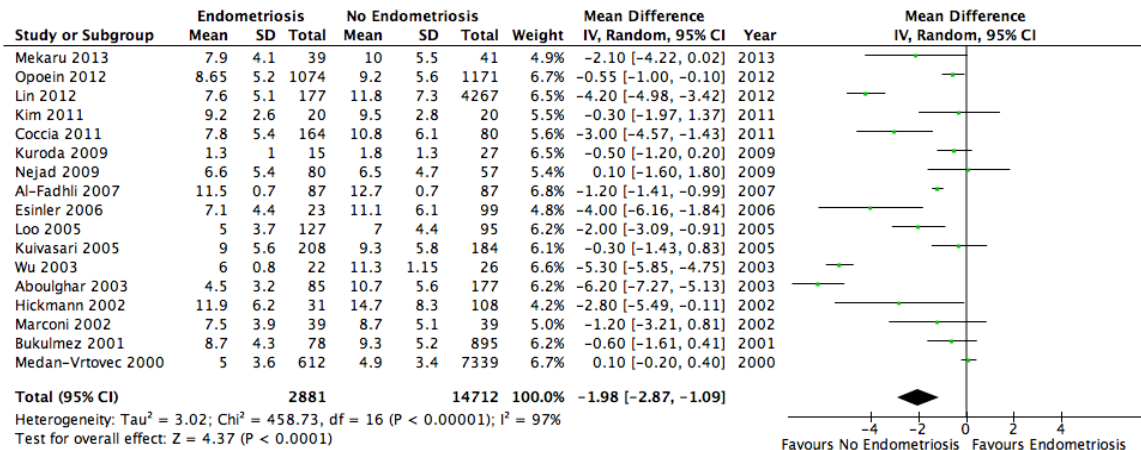


Figure 2-4 Impact of endometriosis on MNOR

Forest plot comparing women with and without endometriosis to evaluate the impact of endometriosis on mean number of oocytes retrieved (MNOR).

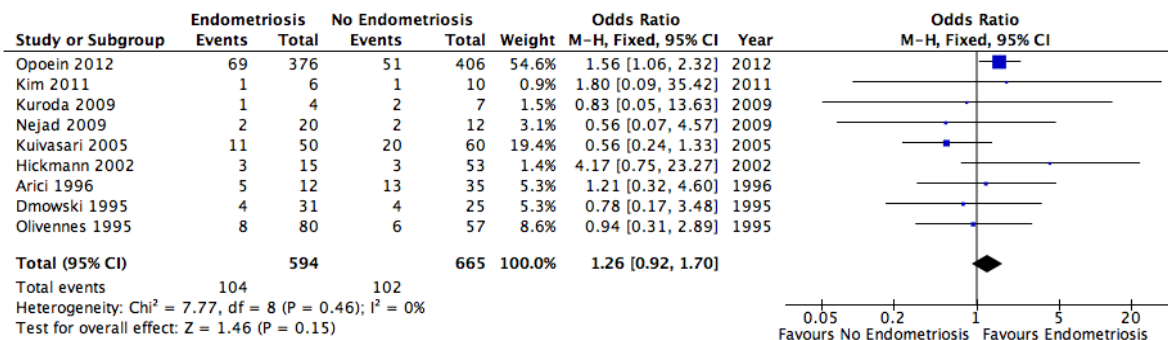


Figure 2-5 Impact of endometriosis on MNOR

Forest plot comparing women with and without endometriosis to evaluate the impact of endometriosis on miscarriage rate.

2.1.5.5 Severity of disease

A subgroup analysis in accordance to ASRM stages (I-II and III-IV) was done. In women with less severe disease (ASRM I-II), all of the outcomes were comparable to women with no endometriosis; this included LBR (OR 0.96, 95% CI [0.82 to 1.12], 8 studies, 4157 patients, $I^2 = 6\%$, Figure 2-6), CPR (OR 0.84, 95% CI [0.69 to 1.03] 15 studies, 9692 patients, $I^2 = 37\%$, Figure 2-7), and MNOR (MD -0.58 [-1.16, 0.01], 11 studies, $I^2 = 70\%$, Figure 2-8). In contrast, women with more severe disease (ASRM III-IV) had a lower LBR (OR 0.77, 95% CI [0.64 to 0.92], 8 studies, 3849 patients, $I^2 = 0\%$, Figure 2-6), lower CPR (OR 0.60, 95% CI [0.44 to 0.81], 15 studies, 9471 patients, $I^2 = 71\%$, Figure 2-7), and lower MNOR (MD -1.76, 95% CI [-2.73 to 0.79], 14 cycles, 9172 patients, $I^2 = 92\%$, Figure 2-8), when compared to women with no endometriosis.

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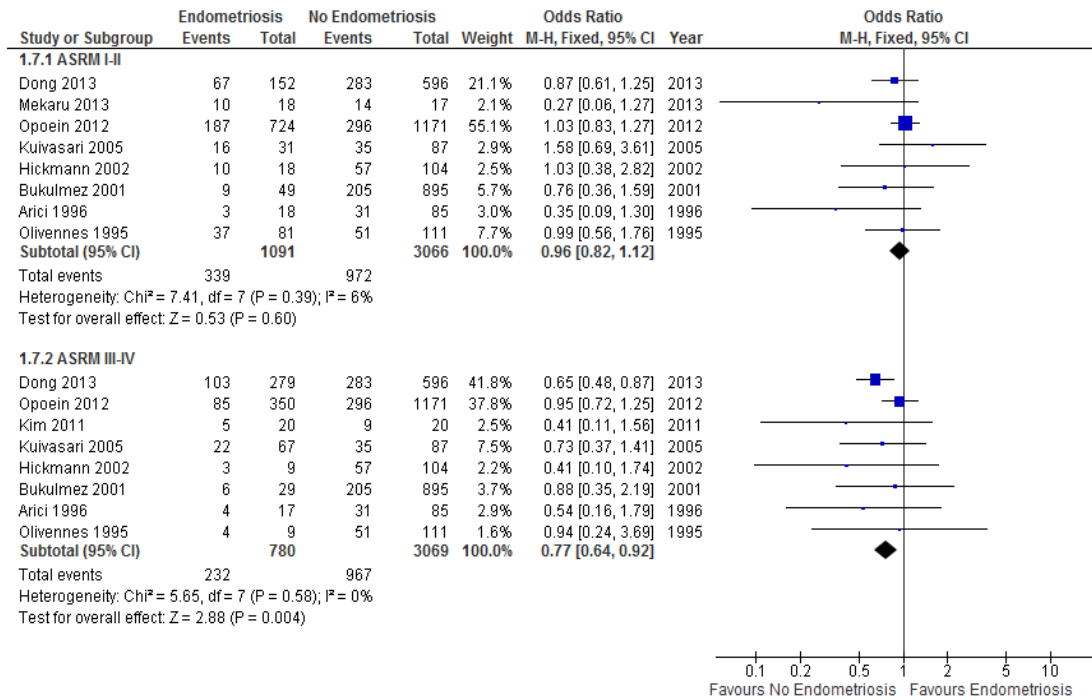


Figure 2-6 Impact of disease severity on LBR

Forest plots comparing women with and without endometriosis according to disease severity, to evaluate the impact severity of endometriosis on Live Birth Rate (LBR).

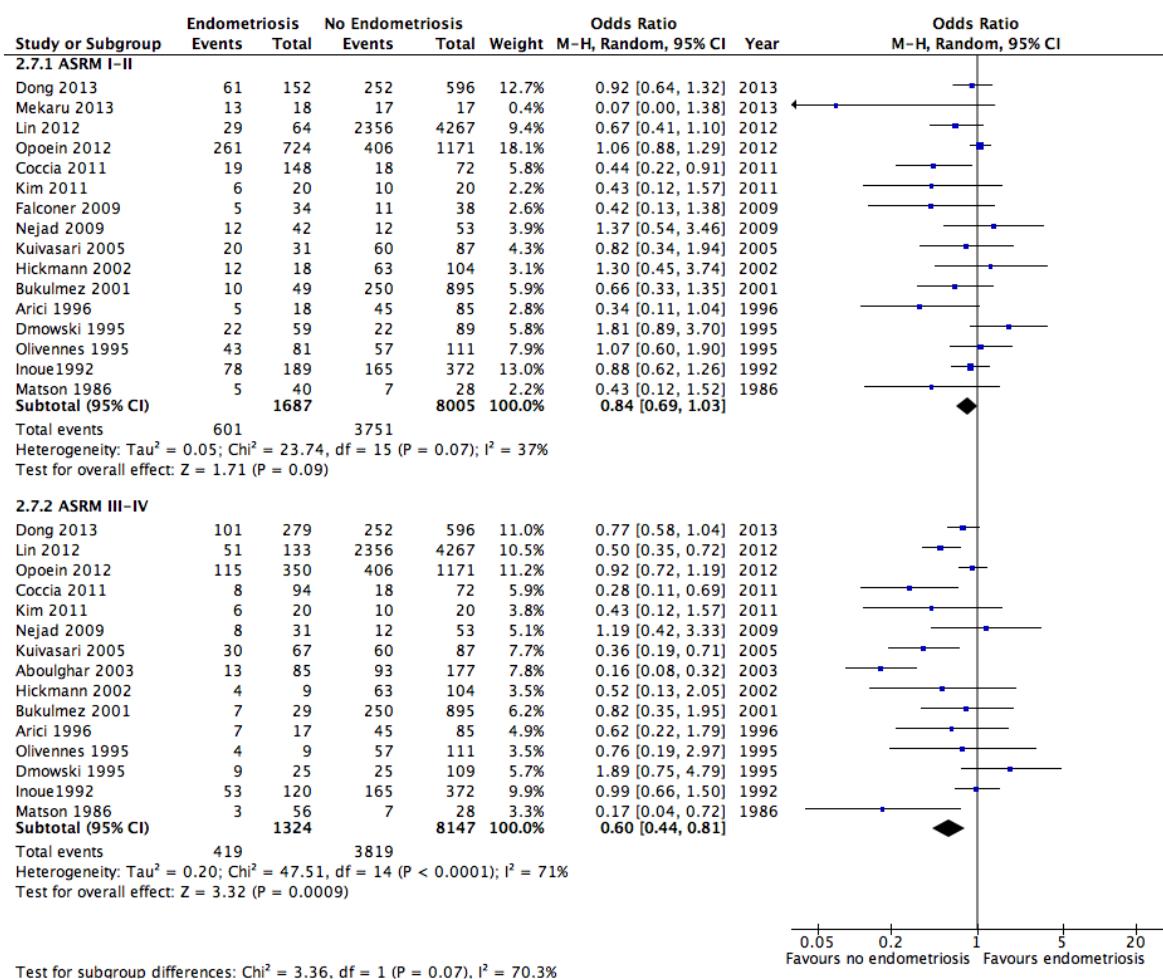


Figure 2-7 Impact of disease severity on CPR

Forest plots comparing women with and without endometriosis according to disease severity, to evaluate the impact severity of endometriosis on clinical pregnancy rate (CPR).

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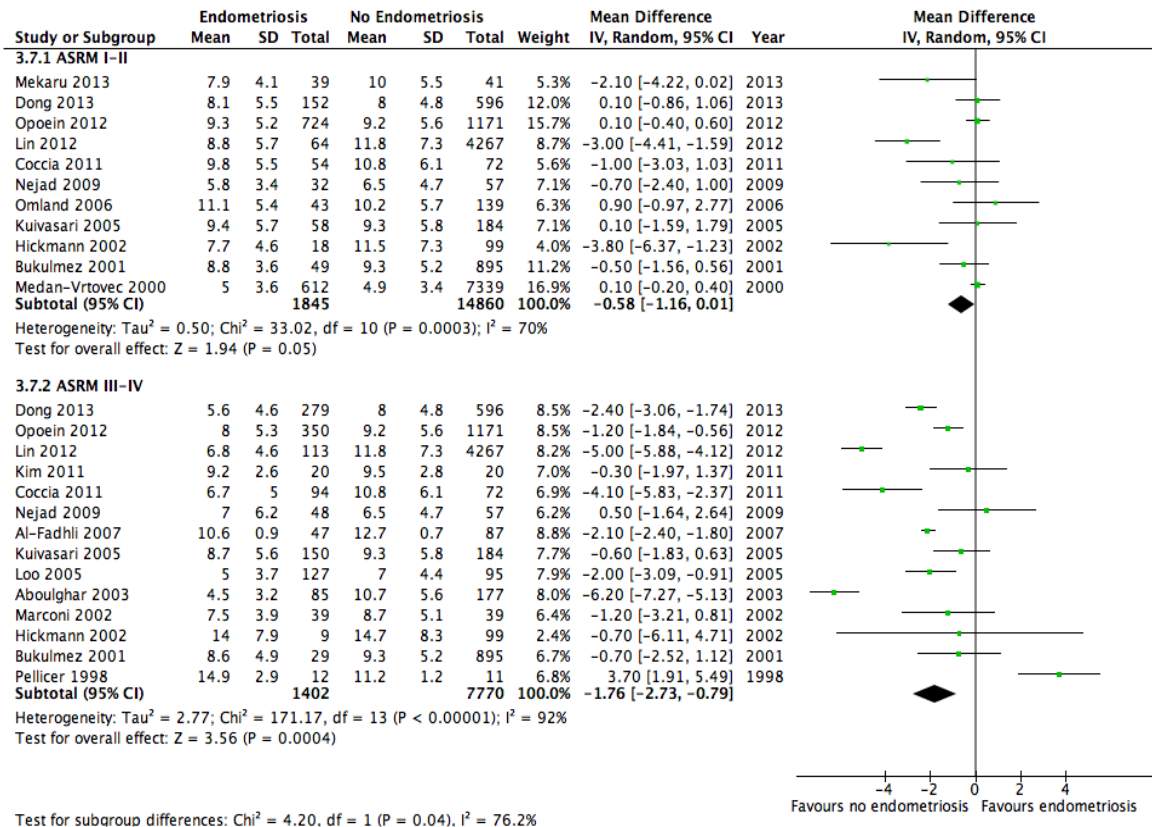


Figure 2-8 Impact of disease severity on MNOR

Forest plots comparing women with and without endometriosis according to disease severity, to evaluate the impact severity of endometriosis on mean Number of Oocytes Retrieved (MNOR).

2.1.5.6 Impact of surgical treatment

The effect of surgery would have been best assessed between women with endometriosis who had received surgical treatment with those who had not received the treatment. However, there was only one study [121] published with this comparison. The authors concluded that in women with less severe endometriosis (ASRM stages I-II), a higher LBR (OR 1.47 95% CI [1.01 to 2.13], 661 women) were recorded in women who had surgical treatment when compared to those who had not.

2.1.5.7 Subgroup analysis

The effect of surgery was evaluated by analysing studies that specified that their participants had surgical treatment prior to ART and those that did not specify surgical treatment separately. There was no difference in the LBR (OR 0.88 95% CI [0.76, 1.02] 4 studies, 3492 patients, $I^2=54\%$, Figure 2-9), a lower CPR (OR 0.69 95% CI [0.50 to 0.96] 9 studies, 4,888 patients, $I^2=79\%$, Figure 2-10), and MNOR (MD -2.37 95% CI [-3.55 to -1.20] 11 studies, 3909 cycles, $I^2=97\%$, Figure 2-11) in studies where participants had prior surgical treatment when compared to women with no endometriosis.

In studies where women had prior surgical treatment, whilst there was no difference in ART outcomes in those with ASRM I-II [LBR (OR 0.99 95% CI [0.83, 1.18], 4 studies, 2796 patients, $I^2=34\%$), CPR (OR 0.87 95% CI [0.61, 1.23], 5 studies, 3016 patients, $I^2=54\%$) and MNOR (MD -0.03 95% CI [-0.44 to 0.38] 5 studies, 3091 cycles, $I^2=18\%$), those with more severe disease (ASRM III-IV) had a lower LBR (OR 0.78 95% CI [0.65 to 0.95], 3 studies, 2550 patients, $I^2=43\%$), CPR (OR 0.53 95% CI [0.33 to 0.84], 6 studies, 3470 patients, $I^2=85\%$), and a lower MNOR (MD -2.46 95% CI [-3.42 to -1.51], 8 studies, 3592 cycles, $I^2=91\%$).

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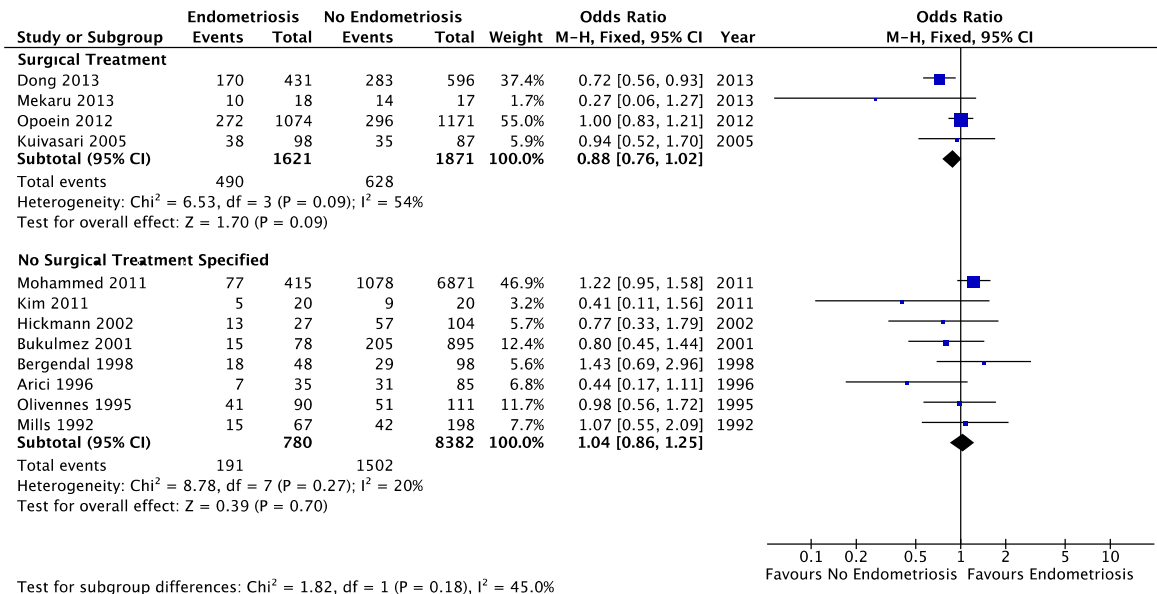


Figure 2-9 LBR of women treated and no surgical treatment specified

Forest plots comparing women with and without endometriosis according to surgical treatment, to evaluate surgical treatment on live birth rate (LBR)

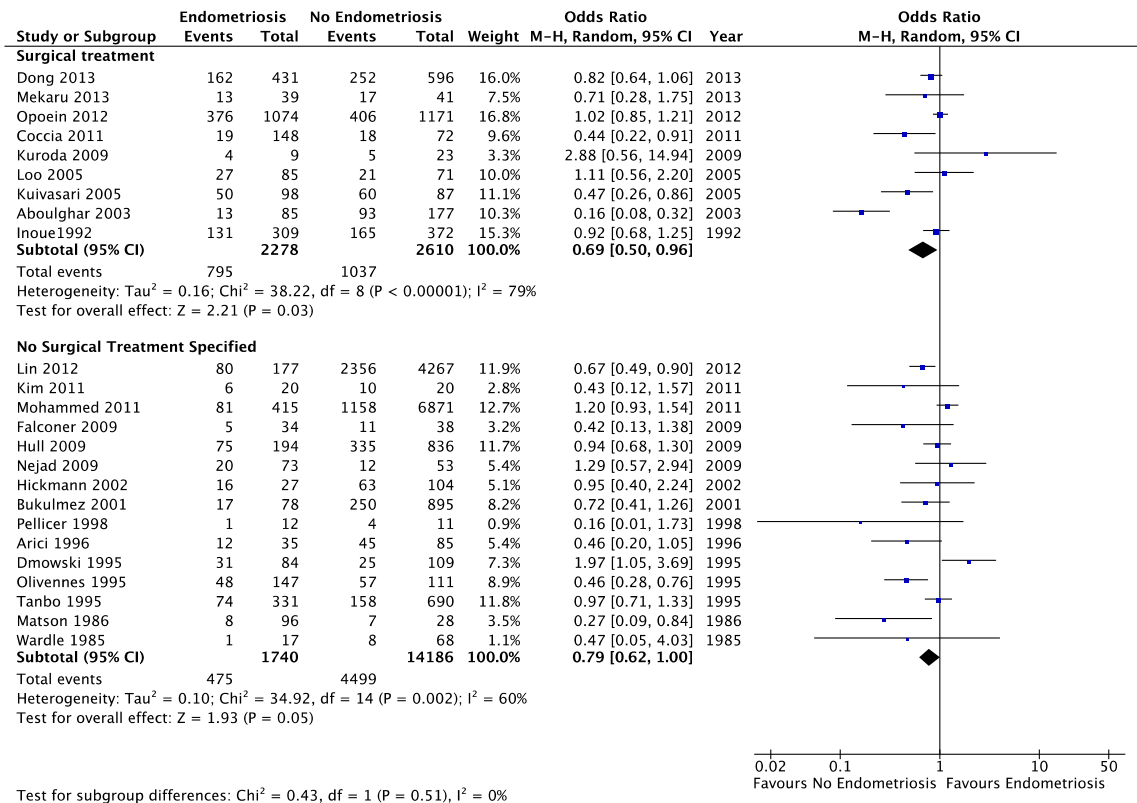


Figure 2-10 CPR of women treated and no surgical treatment specified

Forest plots comparing women with and without endometriosis according to surgical treatment, to evaluate surgical treatment on clinical pregnancy rate (CPR).

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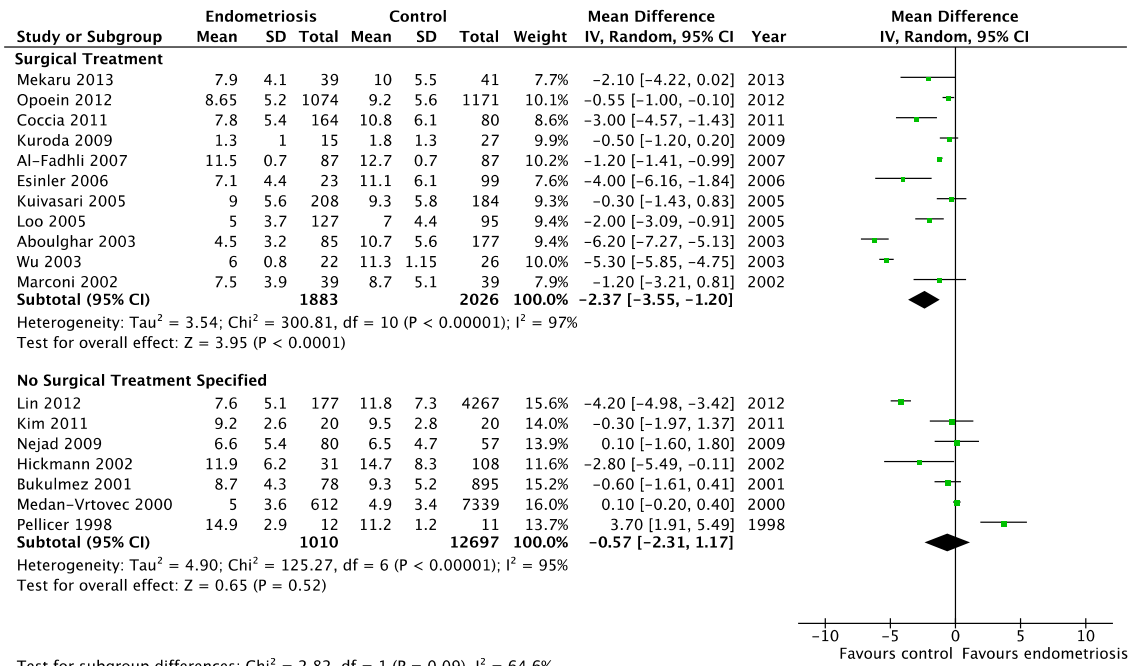


Figure 2-11 MNOR of women treated and no surgical treatment specified

Forest plots comparing women with and without endometriosis according to surgical treatment, to evaluate surgical treatment on mean number of oocytes retrieved (MNOR).

2.1.6 Discussion

This systematic review and meta-analysis shows that women with endometriosis undertaking ART have a similar LBR, a lower CPR and lower MNOR when compared to those without endometriosis. Whilst women with less severe disease (ASRM I-II) have a similar reproductive outcomes compared to those with no endometriosis, women with more severe disease (ASRM III-IV) had a 30% lower LBR, 40% lower CPR and lower mean number of oocytes retrieved when compared to women with no endometriosis.

With regards to the effect of surgery, one study [121] showed some evidence of benefit for treating less severe endometriosis prior to ART (ASRM I-II). Given that this is the only available study comparing groups of women who had surgical treatment to those who had no treatment; this result needs to be interpreted with caution. The nature of the study does not allow a high level of recommendation and does not imply laparoscopy should be performed in all asymptomatic patients prior to ART only to diagnose and treat less severe endometriosis in order to improve the result of the ART treatment [99]. Subgroup analysis according to disease severity, however suggests that surgical treatment prior to ART in women with more severe endometriosis (ASRM III-IV) is associated with a lower LBR, CPR and mean number of oocytes retrieved. However, there is insufficient evidence to recommend surgery routinely prior to ART.

The mechanisms accounting for a poorer reproductive outcome in women with endometriosis is largely unknown. The disease process, with a largely inflammatory component, can directly affect the oocyte quality, quantity and the endometrial receptivity [122, 123]. Surgical removal of the disease just prior to ART, whilst beneficial for the reduction of symptoms [124] and the reduction of the bulk of endometriotic disease, can damage ovarian tissue, diminish ovarian reserve and induce adhesion formation/reformation.

Given the paucity of data currently available, the definitive evidence based strategy for the management of moderate to severe endometriotic disease prior to ART is still unavailable [99]. Surgical treatment of severe endometriosis is associated with a significant surgical morbidity [125]. As women with more

severe stages of endometriotic disease have a significantly lower mean egg yield per cycle, surgical intervention for them is to be contemplated only after careful consideration and appropriate counselling and discussion, especially in those who already may have limited ovarian reserve [42]. Given that women with endometriosis have the same LBR as those without endometriosis, and this is in spite of a lower mean oocyte yield per cycle, the question remains as to whether women with endometriosis may require more cycles of ART treatment in order to achieve a similar live birth rate per woman. The impact of this disease on the ovary and the endometrium will be discussed in the subsequent chapters of this thesis (Chapter 3: and Chapter 4:).

One of the drawbacks of this meta-analysis is the clinical heterogeneity in particular that relating to the variation in surgical skills, learning curve, techniques and the lag time since the surgical intervention. Until the results of current ongoing randomized controlled trials are made [126], the assumption that the treatment efficacy of various surgical treatment modalities such as thermal versus laser ablation or surgical techniques such as excision versus ablation techniques to be similar is a potential confounder of our meta-analysis. However, current available studies do not provide this level of details for analysis. Other clinical parameters, which can potentially contribute to the clinical heterogeneity of the data, included the lack of information on the patients' ovarian reserve, variation in stimulation protocol and the improvement in general of the ART success rate with time. In order to limit heterogeneity caused by the advancement in surgical equipment and assisted conception technology, a sensitivity analysis was performed to assess the outcomes on only studies published after the year 2000; the latter analysis did not alter the findings.

This study was undertaken using extensive search strategies and stringent inclusion/exclusion criteria had enable collation of a comprehensive number of studies with a total number of participants 29,454 women. The majority of studies were of moderate/high quality. This study did not specifically address the effects of endometriomas or deep-infiltrating disease as these have been reviewed separately in previous review [32, 88, 127, 128].

2.1.7 Conclusion

Women with endometriosis have comparable LBR per woman to those without endometriosis although women with more severe endometriosis have poorer reproductive outcomes than those with milder disease. There is insufficient evidence to recommend surgery routinely prior to ART.

2.2 The impact of endometrioma on ART outcomes: A systematic review and meta-analysis

As mentioned in the introductory chapter, endometrioma, which is found in the ovary, is usually a more severe form of endometriosis as classified by ASRM. Endometrioma is long speculated to have a more detrimental impact on IVF treatment outcomes than the other forms of endometriosis because as the disease is closest to the developing oocytes within the ovary, theoretically the detrimental biological impact is likely to be worst. This review will examine and discuss the consequences of leaving the endometrioma intact, and the benefits of its surgical removal in women undergoing ART.

This review will first determine the impact of endometrioma on ART reproductive outcomes; secondly, to determine the impact of surgery on endometrioma on ART outcomes and lastly, to determine the effect of different surgical techniques on ART outcomes.

2.2.1 Should surgeons operate on all endometrioma(s)?

Between 17-44% of women with endometriosis will have endometrioma [34, 35]. As discussed in the first chapter of this thesis, the exact pathophysiology of endometrioma related to infertility is still unknown. It can be detrimental to fertility directly by distorting tubo-ovarian anatomy [4], or indirectly by invoking inflammatory [50, 51] and oxidative damage [52, 53] on the oocytes resulting in poorer quality oocytes [54].

Evidence from the previous section in this chapter, together with other earlier meta-analysis, showed that without differentiating severity, overall, the presence of endometriosis did not adversely affect IVF outcomes in terms of live birth even though women with endometriosis have lower oocytes yield per cycle compared to those without endometriosis [68, 86, 129]. The latter finding is somewhat counterintuitive given that pregnancy rate increases proportionately to the number of oocytes collected until a threshold [130, 131].

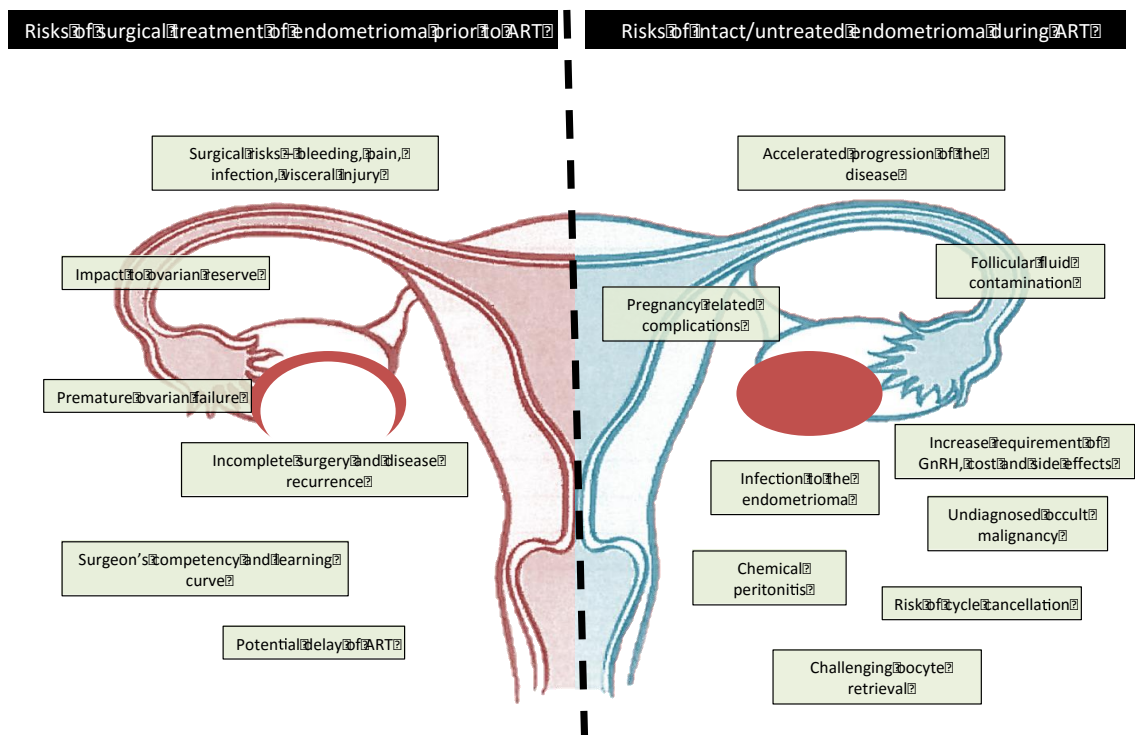


Figure 2-12 Risks of surgical treatment of endometrioma before ART and risks of intact endometrioma during ART

The above figure summarises the risks of surgical treatment for endometrioma (ovarian cystectomy) and the risk of leaving the endometrioma intact during ART. Reproductive medicine practitioner need consider all these factors before subjecting the patients to surgery first or ART treatment.

The impact of endometriosis is likely to be more profound in those with reduced ovarian reserve although this has not been specifically investigated. Furthermore, the differential impact of the presence or absence of endometrioma was not specifically examined in the above-mentioned studies.

There is now molecular, histological and morphological evidence to suggest that endometriosis is detrimental to the ovaries [33]. Toxic content from an endometrioma may lead to unfavourable events such as increased oxidative stress, increase fibrosis, loss of cortex specific stroma, smooth muscle cell metaplasia, vascularization defect and later may reduce follicular maturation. Whether this vicious cycle of damage can be ameliorated by surgical treatment or ART is still controversial.

Surgical treatment of endometriosis and endometrioma prior to ART is widely practiced [127] even though very little evidence exists to provide robust guidance to clinicians [8, 132]. More recent studies have generated some concern that the surgical treatment on endometrioma could be detrimental to ovarian reserve [41-43] and subsequently adversely affect ART reproductive outcomes [32, 88]. The possible adverse outcomes associated with the presence of endometrioma during ART have also not been studied. The risks of surgery and its potential damage to ovarian reserve have to be balanced with the complications associated with the persistence of the endometrioma during ART [133]. As such, this area of management often poses a clinical conundrum for health care practitioners.

The specific impact of endometrioma alone, the differential influences of the disease entity - that of endometrioma rather than endometriosis per se and the impact of surgical intervention of endometriosis on the reproductive outcome of women undergoing ART are areas that require further clarification. This systematic review and meta-analysis has the following objectives: 1) to determine the impact of endometrioma on ART outcomes 2) to determine the impact of surgery on endometrioma on ART outcomes and 3) to determine the effect of different surgical techniques on ART outcomes. The primary outcome is live birth rate (LBR); the secondary outcomes are clinical pregnancy rate (CPR), miscarriage rate (MR), mean number of oocyte retrieved (MNOR), and

any adverse effects such as cancellation rate and associated complications during the ART treatment.

2.2.2 Criteria for considering studies for this review

2.2.2.1 Type of studies

Published cohort or case-control studies (retrospective or prospective), and randomised controlled trials were eligible for inclusion. Where studies reported similar or overlapping data, only the latest or those with the largest dataset were considered for this review.

2.2.2.2 Type of participants

The included studies had 1) women who underwent ART study 2) the presence of endometrioma identified in women in the study group, and 3) a control group. Studies that satisfy the above criteria were included whether or not the participants had prior surgical treatment to their endometrioma. The diagnosis of endometrioma could be by laparoscopy or imaging modalities if 1) the participants had ovarian cysts other than endometrioma, 2) the participants had received any known non-surgical treatment (medical management, alternative treatment) prior to IVF/ICSI, 3) were involved with donor/recipient oocytes treatment, or 4) did not include an appropriate control group.

This review considered the appropriate control groups to include participants who underwent ART 1) for other indications not related to endometriosis, 2) women with endometriosis in the absence of endometrioma, or 3) women with endometrioma that were left untreated 4) women who had endometrioma treated by different surgical techniques.

2.2.2.3 Type of interventions

Surgical treatment for endometriomas includes drainage of the endometrioma without removal of the cyst wall, with or without coagulation of the cyst wall (laparoscopic or transvaginal ultrasound guided), or via cystectomy, either with drainage and excision/stripping of the cyst wall (laparoscopy/laparotomy or both).

Aspiration of endometrioma during oocyte retrieval was not considered an operative surgical treatment prior to IVF/ICSI. Participants who either underwent

IVF or ICSI or both were included. Participants who underwent Gamete Intra-Fallopian Transfer (GIFT) or In Vitro Maturation (IVM) were excluded.

2.2.2.4 Type of outcome measures

Primary outcome measure is live birth rate (LBR) per woman defined as the number of deliveries that resulted in at least one live born baby expressed per 100 patients.

Secondary outcome measures are 1) clinical pregnancy rate (CPR) per woman (defined as pregnancy diagnosed by ultrasonographic visualization of one or more gestational sacs or definitive clinical signs of pregnancy, and was expressed per 100 patients [101]; 2) mean number of oocyte retrieved per cycle; 3) miscarriage rate; 4) fertilisation rate; 5) implantation rate; 6) adverse outcomes including cycle cancellation, and surgical complications such as infection, bleeding and pain during ART. Where available, comparison was also made between participants' characteristics of ovarian reserve (antral follicle count (AFC), Follicle Stimulating Hormone (FSH) and Anti Mullerian Hormones (AMH)).

2.2.3 Search Methods for identification of studies

All published studies from January 1980- December 2014 on surgical treatment of endometrioma and ART outcomes, without language restriction were searched.

2.2.3.1 Electronic searches

The following electronic databases, trial registers and websites were searched. This includes MEDLINE, EMBASE, Cochrane Central register of Controlled Trials, and Web of Science. A search strategy was carried out based on the following keywords and/or medical subject heading (MeSH) terminology: ART, endometriosis, endometrioma, IVF, ICSI, In vitro fertilisation, Intra Cytoplasmic Sperm Injection, outcome, pregnancy, live birth. The reference lists of all known primary and review articles were examined to identify cited articles not captured by electronic search.

2.2.3.2 Searching other resources

Reference lists of articles were hand searched and experts in the field were contacted to obtain additional data. Relevant journal and conference abstract that are not covered in the database were also hand searched.

2.2.4 Data collection and analysis

2.2.4.1 Selection of studies

After a primary screen of the titles and abstracts retrieved by the search, the full texts of all potentially eligible studies were retrieved. Two reviewers (MH, YC) independently examined these full text articles for compliance with the inclusion criteria and selected studies eligible for inclusion in the review. Study investigators were contacted if clarification needed for study eligibility. Disagreement as to study eligibility was resolved after discussion by both reviewers. The process is documented in the PRISMA chart (Figure 2-13)

2.2.4.2 Data extraction and management

Two review authors independently extracted the data using a data extraction form designed and pilot-tested by the authors on two independent occasions. Any disagreements were resolved by discussion between both review authors. Data retrieved included study characteristics and their various outcomes data. Both reviewers counterchecked these extracted data repeatedly. Where studies had multiple publications or were using the same database, the latest and main trial report was used as the reference and the additional details were scanned through from the secondary or earlier papers. Authors were contacted for further data and/or results, as required. All the available data were extracted into Review Manager 5 (RevMan, Mac OS X, Version 5.3. Copenhagen: The Nordic Cochrane Centre, The Cochrane Collaboration, 2014) for further analysis.

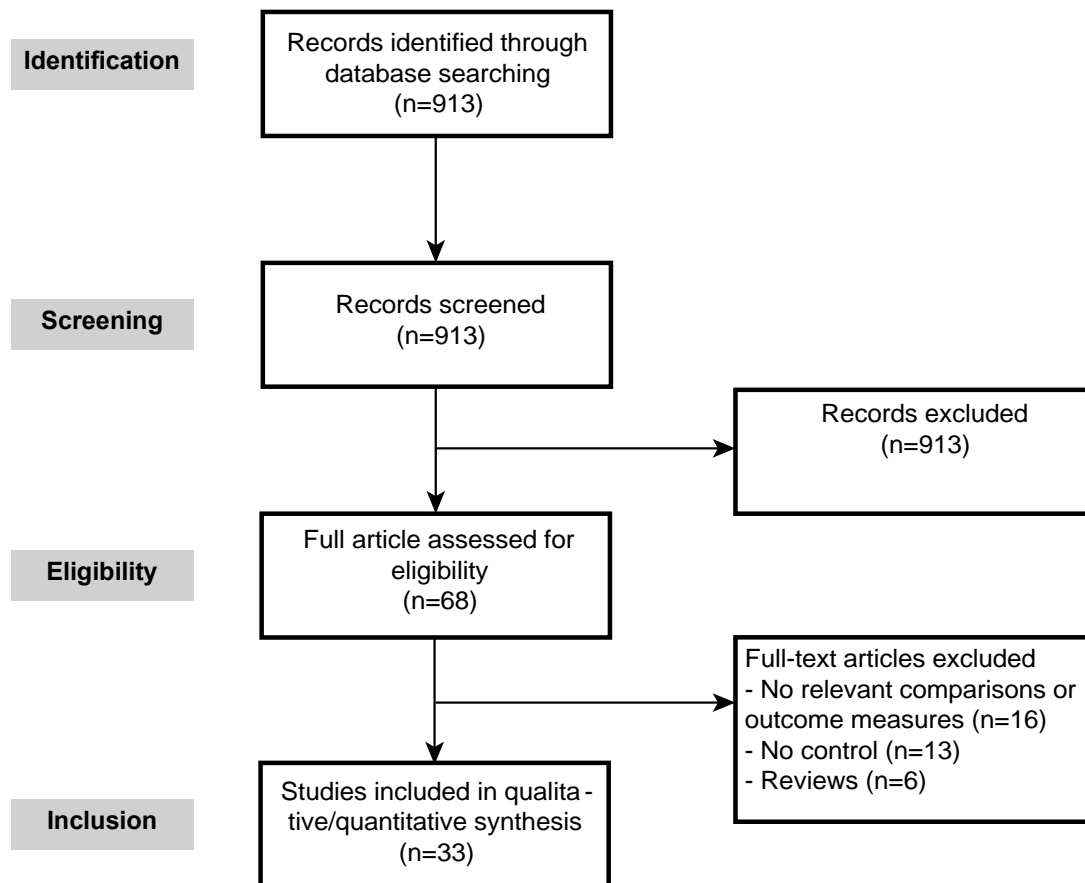


Figure 2-13 Prisma Flow Chart

Prisma flow chart outlining the process for data extraction, beginning with database search for record identification, followed by screening for full text articles for eligibility and final studies included in the analysis. The boxes to the right indicate number of studies that were excluded with their reason for exclusion

2.2.5 Comparative analysis

Comparative analysis was performed to answer different questions and hypothesis. These comparative analyses were presented at the result section accordingly. Intact endometrioma during ART versus those with:

This was performed in studies comparing ART outcomes in women with

- No endometriosis (section: 2.2.9.4; page: 67)
- Peritoneal endometriosis (section: 2.2.9.5; page: 70)

Surgical treatment to their endometrioma prior to IVF versus those with

- Untreated endometrioma (section:2.2.9.6; page: 72)
- Peritoneal endometriosis (section: 2.2.9.7; page: 75)
- Normal unaffected contralateral ovary (section: 2.2.9.8; page:77)

Head to head comparison of different ovarian cystectomy surgical techniques

- Laparoscopic and transvaginal aspiration (section:2.2.9.9; page:77)
- Different laparoscopic cystectomy techniques (section: 2.2.9.10, page: 79)

2.2.6 Assessment of heterogeneity

The clinical and methodological characteristics of the included studies were considered sufficiently similar for meta-analysis to provide a clinically meaningful summary. Statistical heterogeneity was evaluated by the measure of the I^2 . Fixed-effect model and examined heterogeneity between the results of different studies were performed by inspecting the scatter in the data points, the overlap in their CIs, and more formally by checking the results of the χ^2 test and the I^2 statistic. The threshold for the interpretation of I^2 varies and inconsistencies depend on several factors. Scores below 50% were considered to represent low or moderate heterogeneity whereas, I^2 greater than 50% was taken to indicate substantial heterogeneity and in that case a random-effects analysis was used. Incorporation of a random-effects meta-analysis model involves an assumption that the effects being estimated in the different studies are not identical, but follow some distribution. Where there are at least 10 studies in the comparative analysis, funnel plots were generated for the comparison to inspect for small study effects.

2.2.7 Quality assessment of the data

Two reviewers (MH, YC) assessed the methodological quality of the studies and extracted relevant data such as diagnosis of endometriosis, surgical treatment, staging of the disease, selection of control and definition of primary and secondary outcome. Where available, statistical data from the original papers were extracted. Missing parameters were calculated by using data provided if required. The quality of individual studies was assessed in accordance to the MOOSE criteria and the Newcastle Ottawa Scale[134] . By using the Newcastle–Ottawa scale, non-randomised studies were rated according to eight items categorized in three domains: study group selection, comparability of the groups, and ascertainment of outcome (maximum scores of 4, 2, and 3, respectively). Score were represented with stars for each quality item to provide a visual assessment. Studies were awarded up to nine stars if they fulfilled all the quality items. Randomised controlled trials were assessed on the risk of methodological bias [135].

2.2.8 Measures of treatment effect

For dichotomous data (e.g. clinical pregnancy rate), the number of events in the control and intervention groups of each study keyed in Review Manager 5 and analysed using Mantel-Hansel odds ratio. For continuous data, mean difference between treatments groups was calculated.

2.2.9 Results

2.2.9.1 Result of search

The search strategy yielded 913 studies; however 845 studies were excluded because it was clear from the title or abstract that they did not fulfil the selection criteria. Out of 68 potential studies for the analysis, we further excluded 16 studies that had no relevant comparisons (non-endometrioma), 13 studies that had no available control groups and 6 publications were reviews [32, 88, 136-139]. The final number of 33 studies was included for the meta-analysis.

2.2.9.2 Description of studies and participants

The majority of the included studies (Table 2-3, Page: 60) was non-RCT [38, 45, 49, 107, 140-165] (n=30) and the remaining (n=3) were RCTs [166-168].

Thirty studies included women with endometriosis who had surgical treatment to their endometrioma prior to IVF/ICSI, 12 studies included more than one comparative group and 13 studies included women with intact endometrioma (either as study or control group). From the included studies, 18 indicated the laterality of the disease, (bilateral disease = 2/18, unilateral disease n=6/18 and unilateral and/or bilateral n=10/18). Less than half (n=12) of the included studies specified the size of endometrioma as their inclusion criteria which ranged from 1 cm or more, to size greater than 6 cm. Eighteen other studies did not specify the size of the endometrioma (Table 2-3).

All studies except one [107] stated the stimulation protocol; long protocol (n=28/33), followed by mix protocols (n=3/33) and short agonist protocol (1/33). Women in the majority of the studies underwent ovarian cystectomy (n= 27) either laparoscopically (25/27) or laparoscopically and/or laparotomy (2/27) whilst in 3 studies women had transvaginal cyst aspiration.

In five studies where women with endometrioma had no surgical treatment, the comparative controls included women with no endometriosis. Studies examining the outcome of surgical treatment in women with endometriomas included various comparative control groups: 1) untreated endometrioma (n=11), 2) endometriosis with no previous endometrioma (n=7), 3) tubal factor (n=10) and 4) normal contra-lateral ovary (n=4). Three studies compared ovarian cystectomy with trans-vaginal aspiration prior to ART whilst three other studies made head to head comparison of different ovarian cystectomy surgical techniques.

Amongst all the included studies, eleven studies reported outcomes of LBR (11/33), CPR (29/33), MNOR (33/33), miscarriage rate (9/33), implantation rate (14/33), fertilisation rate (19/33), FSH dose requirement (17/33) and cycle cancellation rate (7/33), including baseline characteristics of baseline FSH level (14/33) and antral follicle count (7/33). None of the included studies reported baseline characteristics of AMH levels or any clinical adverse outcomes related to infection, bleeding or pain. Papers that reported IR and FR have presented the data in percentage and none provide the raw data. None of these studies exclusively examined women with recurrent endometrioma although some [107, 146, 164] included women with endometrioma who had prior surgical treatment.

Table 2-3 Characteristic table of studies included in the meta-analysis

No	Study (Year)	Location	Duration	Design	Intervention/ Protocol	Study Group	Type of surgery	N	Control Group	N	Cyst size (cm)	Side	Outcomes
1	Lee et al 2014	Korea	2008- 2012	Retrospective Cohort	IVF/ICSI Long Protocol Antagonist	Surgically treated endometrioma	Laparoscopic cystectomy Transvaginal Aspiration (+Ethanol)	36 29	Non-Treated endometrioma	36	>3cm	ND	LBR, CPR, NOR, MR, FR, DFSH, CR, AFC
2	Benaglia et al 2013	Italy & Spain	2006- 2010	Retrospective cohort study	IVF/ICSI Long Protocol	Non treated endometrioma	Intact endometrioma	39	Non endometriosis	78	>1	BL	LBR, CPR, NOR, FR, DFSH, CR, AMH, AFC
3	Takebayashi 2013	Japan	1997- 2011	Retrospective case control	IVF Long protocol	Surgically treated endometrioma	Laparoscopic cystectomy	12	Laparoscopic laser ablation	15	2-7cm	Either	LBR, CPR, NOR, IR, FR, DFSH
4	Takashima 2013	Japan	2008- 2010	Retrospective case control	IVF Long Protocol	Surgically treated endometrioma	Laparoscopic cystectomy (+Coagulation)	21	Laparoscopic cystectomy (+Suture)	23	ND	UL	CPR, NOR, DFSH, AMH, BFSH, AFC
5	Bongioanni 2011	Italy	ND	Retrospective case control	IVF Long Protocol	Surgically treated endometrioma	Laparoscopic cystectomy	112	Non-Treated endometrioma Tubal factor	142 174	<6	Either	LBR, CPR, NOR, IR, FR, DFSH, CR, BFSH, AFC
6	Barri et al 2010	Spain	2001- 2005	Observational study	IVF ND	Surgically treated endometrioma	Laparoscopic cystectomy	483	Non-Treated endometrioma Male Factor	173 334	ND	ND	CPR, NOR

7	Almog et al 2010	Canada	1998- 2008	Retrospective case control	IVF Long Protocol	Surgically treated endometrioma	Laparoscopic cystectomy	80	Normal contralateral ovary	80	ND	UL	NOR
8	Kuroda et al 2009	Japan	2006- 2008	Retrospective case control	IVF/ICSI Mix protocol	Surgically treated endometrioma	Laparoscopic cystectomy	18	Non-Treated endometrioma Peritoneal Endometriosis Tubal factor	36 15 21	ND	ND	LBR, CPR, NOR, MR, IR, CR, BFSH
9	Yamamoto et al 2009	Japan	2000- 2008	Retrospective case control	IVF/ICSI Long Protocol	Surgically treated endometrioma	Laparoscopic cystectomy Trans-vaginal aspiration	41 11	Peritoneal Endometriosis	50	>2	Either	CPR, NOR, FR, DFSH
10	Somigliana et al 2009	Italy	2002- 2007	Retrospective cohort	IVF/ICSI Long Protocol	Surgically treated endometrioma	Laparoscopic/la parotomy cystectomy	68	Male, tubal, unexplained, combination	136	ND	BL	LBR, CPR, NOR, IR, CR, BFSH
11	Nakagawa et al 2007	Japan	2002- 2006	Retrospective study	IVF Long Protocol	Surgically treated endometrioma	Laparoscopic cystectomy	10	All non- endometriosis indications	70	>4	ND	CPR, NOR, FR, DFSH
12	Pabuccu et al 2007	Turkey	2002- 2006	Randomized control trial	IVF/ICSI Long Protocol Antagonist	Surgically treated endometrioma	Laparoscopic cystectomy	81	Non-Treated endometrioma Peritoneal Endometriosis	67 98	ND	Either	CPR, LBR, NOR, MR, IR, FR, DFSH, BFSH, AFC
13	Duru et al 2007	Turkey	ND	Retrospective case control	ICSI Long Protocol	Surgically treated endometrioma	Laparoscopic cystectomy Laparotomy cystectomy+ Reconstruction	28 10	Peritoneal Endometriosis	10	ND	UL	CPR, NOR, FR, DFSH, BFSH

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14	Demiroglu et al 2006	Turkey	2001- 2005	Randomized control trial	ICSI Long Protocol	Surgically treated endometrioma	Laparoscopic cystectomy	49	Non-Treated endometrioma	50	3-6	UL	CPR, NOR, IR, FR, DFSH, BFSH
15	Esinler et al 2006	Turkey	NA	Retrospective case control	ICSI Long Protocol	Surgically treated endometrioma	Laparoscopic cystectomy	23	Tubal factor	99	>3	Either	CPR, NOR, MR, IR, DFSH, BFSH, AFC
16	Matalliotakis 2006	USA	1996- 2002	Retrospective case control	IVF Long Protocol	Surgically treated endometrioma	Laparoscopic cystectomy	68	Tubal factor	106	ND	ND	LBR, CPR, MR, NOR, FR, CR, BFSH
17	Yazbeck et al 2006	France	1998- 2001	Retrospective controlled study	IVF Long protocol	Surgically treated endometrioma	Laparoscopic cystectomy	63	Peritoneal Endometriosis	50	ND	Either	CPR, NOR, DFSH
18	Loo et al 2005	Taiwan	2000- 2002	Retrospective case control	IVF Long Protocol	Surgically treated endometrioma	Laparoscopic cystectomy	127	Tubal factor	95	>3	ND	CPR, NOR, IR, FR, DFSH, BFSH
19	Ragni et al 2005	Italy	2002- 2004	Retrospective case control	IVF/ICSI Long Protocol	Surgically treated endometrioma	Laparoscopic cystectomy	17	Normal contralateral ovary	17	<3, >3	UL	NOR
20	Suzuki et al 2005	Japan	1996- 2002	Retrospective cohort study	IVF Short Protocol	Non treated endometrioma	Intact endometrioma	80	Peritoneal Endometriosis Tubal factor	 283	ND	Either	LBR, CPR, NOR, IR, FR
21	Pabuccu et al 2004	Turkey	1999- 2002	Randomized control trial	ICSI Long Protocol	Surgically treated endometrioma	Laparoscopic cystectomy Trans-vaginal aspiration	44 41	Non -Treated endometrioma Tubal factor	40 46	ND	Either	CPR, NOR, MR, IR, FR, DFSH, BFSH, AFC
22	Wong et al 2004	USA	1995- 2002	Retrospective cohort	IVF/ICSI Long Protocol	Surgically treated endometrioma	Laparoscopic cystectomy	45	Non-Treated endometrioma	29	ND	ND	CPR, NOR, MR, IR, FR, DFSH, BFSH

23	Garcia-Velasco 2004	Spain	1997-2001	Retrospective case control	IVF Long Protocol	Surgically treated endometrioma	Laparoscopic cystectomy	133	Non-Treated endometrioma	56	ND	ND	CPR, NOR, MR, IR, FR, DFSH, CR, BFSH
24	Wynn and Doney 2003	Belgium	1997-2002	Retrospective case control	IVF Long protocol	Surgically treated endometrioma	Laparoscopic cystectomy	85	Tubal factor Unexplained	193 135	<3, >3	ND	CPR, NOR, IR, FR
25	Wu et al 2003	Taiwan	NA	Retrospective case control	IVF Long Protocol	Surgically treated endometrioma	Laparoscopic cystectomy	22	Tubal factor Normal contralateral ovary	20 10	>6	ND	LBR, CPR, NOR, FR, BFSH
26	Suganuma et al 2002	Japan	NA	Retrospective case control	IVF Long Protocol	Surgically treated endometrioma	Laparoscopic cystectomy Trans-vaginal aspiration	36 23	Non-Treated endometrioma	30	ND	ND	CPR, NOR, FR
27	Marconi et al 2002	Argentina	1999-2000	Retrospective cohort	IVF Long Protocol	Surgically treated endometrioma	Laparoscopic Cystectomy	39	Tubal factor	39	ND	Either	CPR, NOR, DFSH
28	Takuma et al 2002	Japan	NA	Retrospective case control	IVF Long protocol	Surgically treated endometrioma	Laparoscopic Cystectomy	36	Laparoscopic aspiration only (+Electro therapy) (+Alcohol therapy)	41	ND	ND	CPR, NOR,
29	Canis et al 2001	France	NA	Retrospective case control	IVF Long protocol	Surgically treated endometrioma	Laparoscopic cystectomy	41	Tubal factor Peritoneal Endometriosis	59 139	>3	Either	CPR, NOR
30	Tinkanen et al 2000	Finland	1994-1998	Retrospective analysis	IVF/ICSI Long Protocol	Surgically treated endometrioma	Laparoscopic cystectomy	55	Non-Treated endometrioma	45	ND	Either	CPR, NOR

31	Ho et al 2000	Taiwan	1996- 2001	Retrospective case control	IVF Long Protocol	Surgically treated endometrioma	Laparoscopic cystectomy	38	Normal contralateral ovary	38	ND	UL	NOR
32	Yanupolsky et al 1998	USA	1994- 1995	Retrospective cohort study	IVF Long Protocol	Non treated endometrioma	Intact endometrioma	37	Non endometriosis	56	>1	Either	LBR, CPR, NOR, MR, IR, FR
33	Nargund et al 1995	United Kingdom	1988- 1994	Retrospective case control	IVF Long Protocol	Surgically treated endometrioma	Laparoscopic cystectomy	36	Surgically treated benign cyst	54	ND	UL	NOR

LBR=Live birth rate, CPR= Clinical pregnancy rate, NOR=Number of oocytes retrieved, MR=Miscarriage rate, IR=Implantation rate, FR=Fertilisation rate, DFSH=Total FSH dose, BFSH=Baseline FSH, AMH=Anti-Mullerian Hormone, AFC=Antral follicle count, ND=Not documented, IVF=In Vitro Fertilization, RCT= Randomized control trial, NA- Not applicable.

2.2.9.3 Quality assessment of studies

Systematic risk assessment of methodological bias[135] of three included RCTs revealed all studies to have high risk of reporting bias [166-168] and two studies [166, 167] have risk of blinding bias Table 2-4. By assessment using the NOS [134], majority of the non-randomized studies was awarded with 8 stars whilst 2 studies graded to highest possible score (Table 2-5).

Table 2-4 Risk of bias using Cochrane risk assessment tool

Bias	Selection		Performance	Attrition	Reporting
Studies (Year)	Random sequence generation	Allocation concealment	Blinding	Incomplete outcome data	Selective reporting
Pabuccu 2007	Low Risk	Low Risk	Low Risk	Low Risk	High Risk
Demirol 2006	Low Risk	Low Risk	High Risk	Low Risk	High Risk
Pabuccu 2004	Low Risk	Low Risk	High Risk	Low Risk	High Risk

Table 2-5 Newcastle-Ottawa Score

No	Reference	Case-cohort representative	Selection of non-exposed control	Ascertainment of exposure	Outcome negative at start	Comparability by design	Comparability by analysis	Outcome assessment	Duration of follow-up	Score
1	Lee 2014	*	*	*	*	*	*	*	*	8
2	Benaglia 2013	*	*	*	*	*	*	*	*	8
3	Takebayashi 2013	*	*	*	*	*	*	*	*	8
4	Takashima 2013	*	*	*	*	*	*	*	*	8
5	Bongioanni 2011	*	*	*	*	*	*	*	*	8
6	Barri 2010	*	*	*	*	*	*	*	*	8
7	Almog 2010	*	*	*	*	*	*	*	*	8
8	Kuroda 2009	*	*	*	*	**	*	*	*	9
9	Yamamoto 2009	*	*	*	*	**	*	*	*	9
10	Somigliana 2009	*	*	*	*	*	*	*	*	8
11	Nakagawa 2007	*	*	*	*	*	*	*	*	8
12	Pabuccu 2007	*	*	*	*	*	*	*	*	8
13	Duru 2007	*	*	*	*	*	*	*	*	8
14	Demiröl 2006	*	*	*	*	*	*	*	*	8
15	Esinler 2006	*	*	*	*	*	*	*	*	8
16	Mataiotakis 2006	*	*	*	*	*	*	*	*	8
17	Yazbeck 2006	*	*	*	*	*	*	*	*	8
18	Loo 2005	*	*	*	*	*	*	*	*	8
19	Ragni 2005	*	*	*	*	*	*	*	*	8
20	Suzuki 2005	*	*	*	*	*	*	*	*	8
21	Pabuccu 2004	*	*	*	*	**	*	*	*	9
22	Wong 2004	*	*	*	*	*	*	*	*	8
23	Garcia-V 2004	*	*	*	*	*	*	*	*	8
24	Wynn & Don 2003	*	*	*	*	*	*	*	*	8
25	Wu 2003	*	*	*	*	*	*	*	*	8
26	Suganuma 2002	*	*	*	*	*	*	*	*	8
27	Marconi 2002	*	*	*	*	*	*	*	*	8
28	Takuma 2002	*	*	*	*	*	*	*	*	8
29	Canis 2001	*	*	*	*	*	*	*	*	8
30	Tinkanen 2000	*	*	*	*	*	*	*	*	8
31	Ho 2000	*	*	*	*	*	*	*	*	8
32	Yanushpol 1998	*	*	*	*	*	*	*	*	8
33	Nargund 1995	*	*	*	*	*	*	*	*	8

2.2.9.4 Endometrioma (intact) versus women with no endometriosis

When compared to women with no endometriosis, women with intact endometrioma had similar LBR (OR 0.98 95% CI [0.71, 1.36], 5 studies, 928 women, $I^2=0\%$, Figure 2-14), CPR (OR 1.17 95% CI [0.87, 1.58], 5 studies, 928 women, $I^2=0\%$, Figure 2-14), miscarriage rate (OR 1.70 95% CI [0.86, 3.35], 3 studies, 171 pregnancies, $I^2=37\%$, Figure 2-14), but a lower MNOR (SMD -0.23 95% CI [-0.37, -0.10] 5 studies, 941 cycles, $I^2=37\%$). There was a higher cancellation rate (OR 2.83 95% CI [1.32, 6.06], 3 studies, 491 women, $I^2=0\%$, Figure 2-14) in women with intact endometrioma. None of the studies reported other adverse events such as bleeding, infection or pain (Figure 2-14).

Baseline FSH level in women with endometrioma was higher when compared to women with no endometriosis (SMD 0.20 95% CI 0.02, 0.38] 3 studies, 491 cycles, $I^2=57\%$, Figure 2-15). Other parameters such as AFC (SMD -0.02 95% CI [-0.21, 0.18] 2 studies, 433 cycles, $I^2=0\%$, Figure 2-15) and total stimulation dose (SMD -0.07 95% CI [-0.27, 0.12] 2 studies, 433 cycles, $I^2=65\%$, Figure 2-15) were comparable between both groups.

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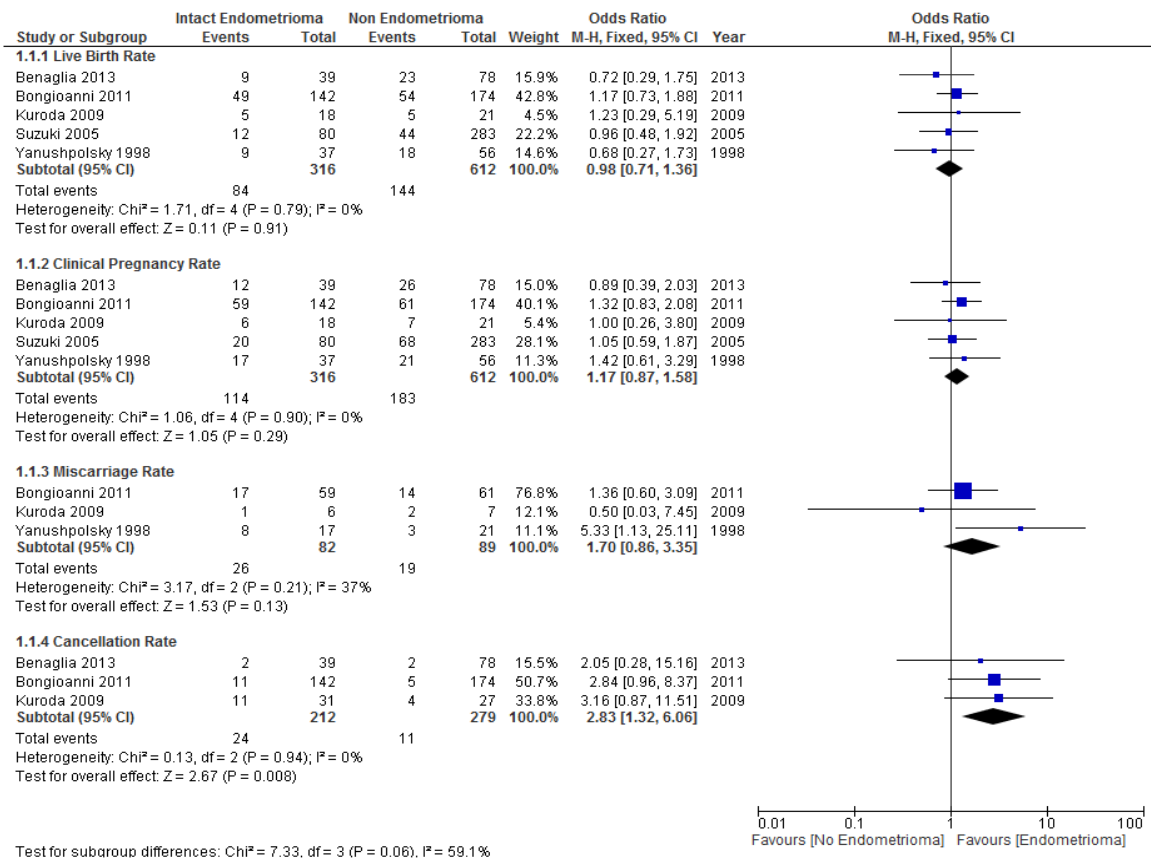


Figure 2-14 Endometrioma (intact) vs. women with no endometriosis

Forest plot of dichotomous data for the comparison of women with endometrioma compared to women with no endometriosis (Tubal factor, Male Factor, Unexplained). The dichotomous outcome measures include Live Birth Rate (LBR), Clinical Pregnancy Rate (CPR), Miscarriage rate (MR) and Cancellation rate.

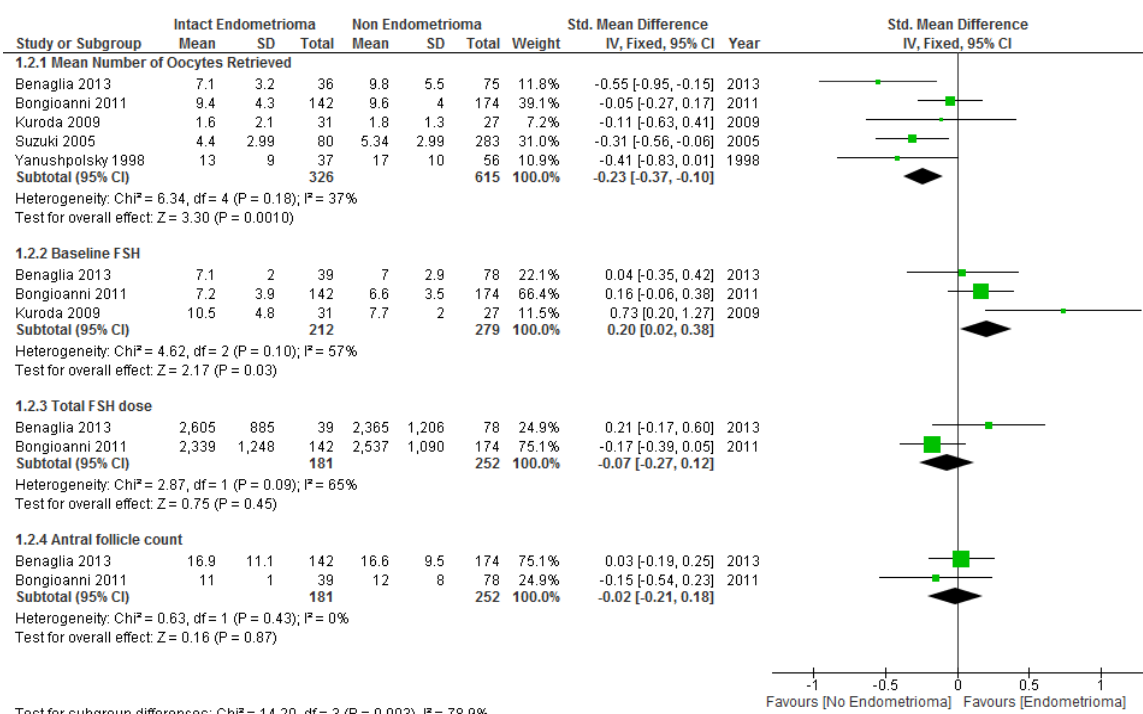


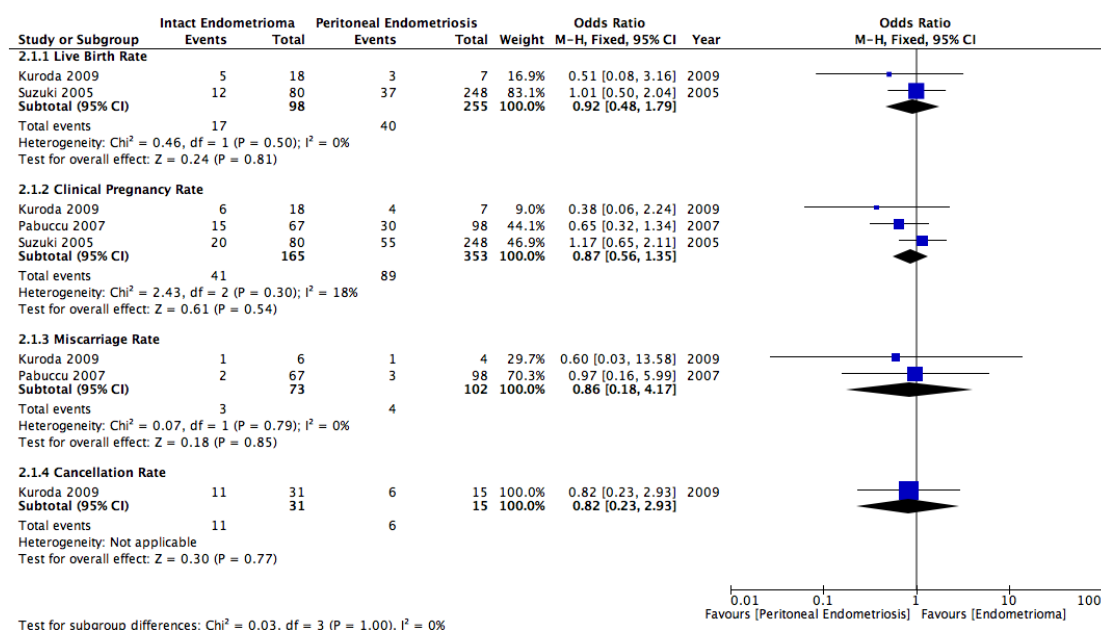
Figure 2-15 Endometrioma (intact) vs. women with no endometriosis

Forest plot of continuous data for the comparison of women with endometrioma compared to women with no endometriosis (Tubal factor, Male Factor, Unexplained). The continuous outcome measures include mean number of oocytes retrieved (MNOR), baseline FSH, Total FSH dose and Antral follicle count.

2.2.9.5 Endometrioma (intact) versus women with peritoneal endometriosis

When compared to women with peritoneal endometriosis, women with intact endometrioma had similar LBR (OR 0.92 95% CI [0.92, 1.79], 2 studies, 353 women, $I^2=0\%$, Figure 2-16A), CPR (OR 0.87 95% CI [0.56, 1.35], 3 studies, 518 women, $I^2=18\%$, Figure 2-16A), miscarriage rate (OR 0.86 95% CI [0.18, 4.17], 2 studies, 175 pregnancies, $I^2=0\%$, Figure 2-16A) and similar MNOR (SMD -0.31 95% CI [-1.03, 0.42] 3 studies, 539 cycles, $I^2=91\%$, Figure 2-16A).

Other parameter such as cancellation rate (OR 0.82 95% CI [0.23, 2.93], 1 study, 46 cycles, Figure 2-16B) was similar in women with intact endometrioma. Baseline FSH level (SMD 0.41 95% CI [-0.29, 1.10] 2 studies, 190 patients, $I^2=60\%$, Figure 2-16B) and AFC (SMD -0.81 95% CI [-0.13, -0.49] 1 study, 165 cycles, Figure 2-16B) in women with endometrioma was comparable to women with peritoneal endometriosis. None of the studies reported total stimulation dose, and other adverse events such as bleeding, infection or pain.

A

Test for subaroup differences: $\chi^2 = 0.03$, $df = 3$ ($P = 1.00$), $I^2 = 0\%$

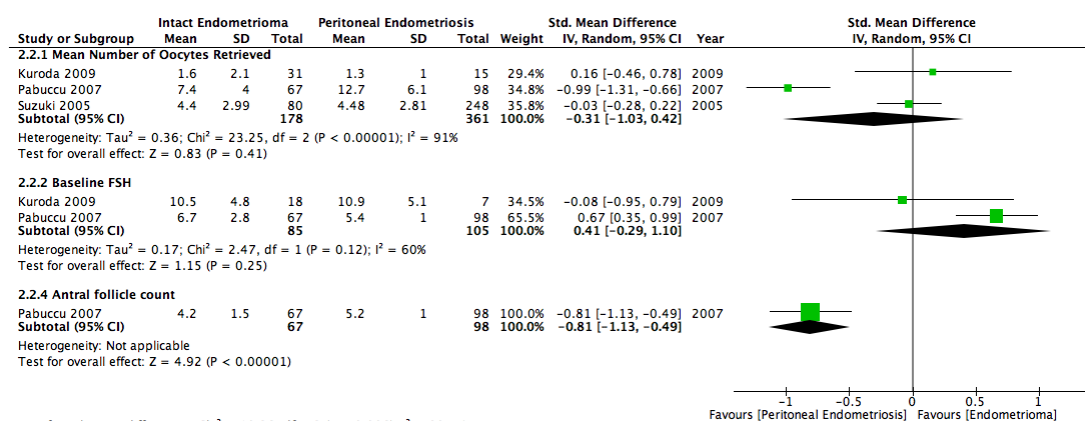
B

Figure 2-16 Endometrioma (intact) vs. women with peritoneal endometriosis

Forest plots comparing women with intact endometrioma and women with peritoneal endometriosis. **A:** The dichotomous outcome measures include Live Birth Rate (LBR), Clinical Pregnancy Rate (CPR), Miscarriage rate (MR) and Cancellation rate. **B:** The continuous outcome measures include mean number of oocytes retrieved (MNOR), baseline FSH (BFSH), and Antral follicle count (AFC).

2.2.9.6 Endometrioma (surgically treated) versus intact endometrioma

In women with endometrioma, those who had surgical treatment before ART, had similar LBR (OR 0.90 95% CI [0.63, 1.28] 5 studies, 655 women, $I^2=32\%$), CPR (OR 0.97 95% CI [0.78, 1.20], 11 studies, 1512 women, $I^2=0\%$), miscarriage rate (OR 1.32 95% CI [0.66, 2.65], 4 studies, 195 pregnancies, $I^2=0\%$), MNOR (SMD -0.17 95% CI [-0.38, 0.05] 9 studies, 810 cycles, $I^2=63\%$, Figure 2-17) and cancellation rate per cycle (OR 1.17 CI 95% [0.69, 2.00] 4 studies, 647 cycles, $I^2=0\%$, Figure 2-17) compared to those with untreated endometrioma.

Women with endometrioma who had surgical treatment had a lower AFC (SMD -0.53 [-0.88, -0.18] 4 studies, 558 cycles, $I^2=73\%$, Figure 2-18) and required a higher dose of FSH (SMD 1.45 [0.23, 2.68] 4 studies, 635 cycles, $I^2=98\%$, Figure 2-18). Both comparison groups had similar baseline FSH (SMD 0.11 [-0.36, 0.57] 7 studies, 951 cycles, $I^2=73\%$, Figure 2-18). None of the studies reported other adverse outcomes such as pain, infection and bleeding during the course of treatment.

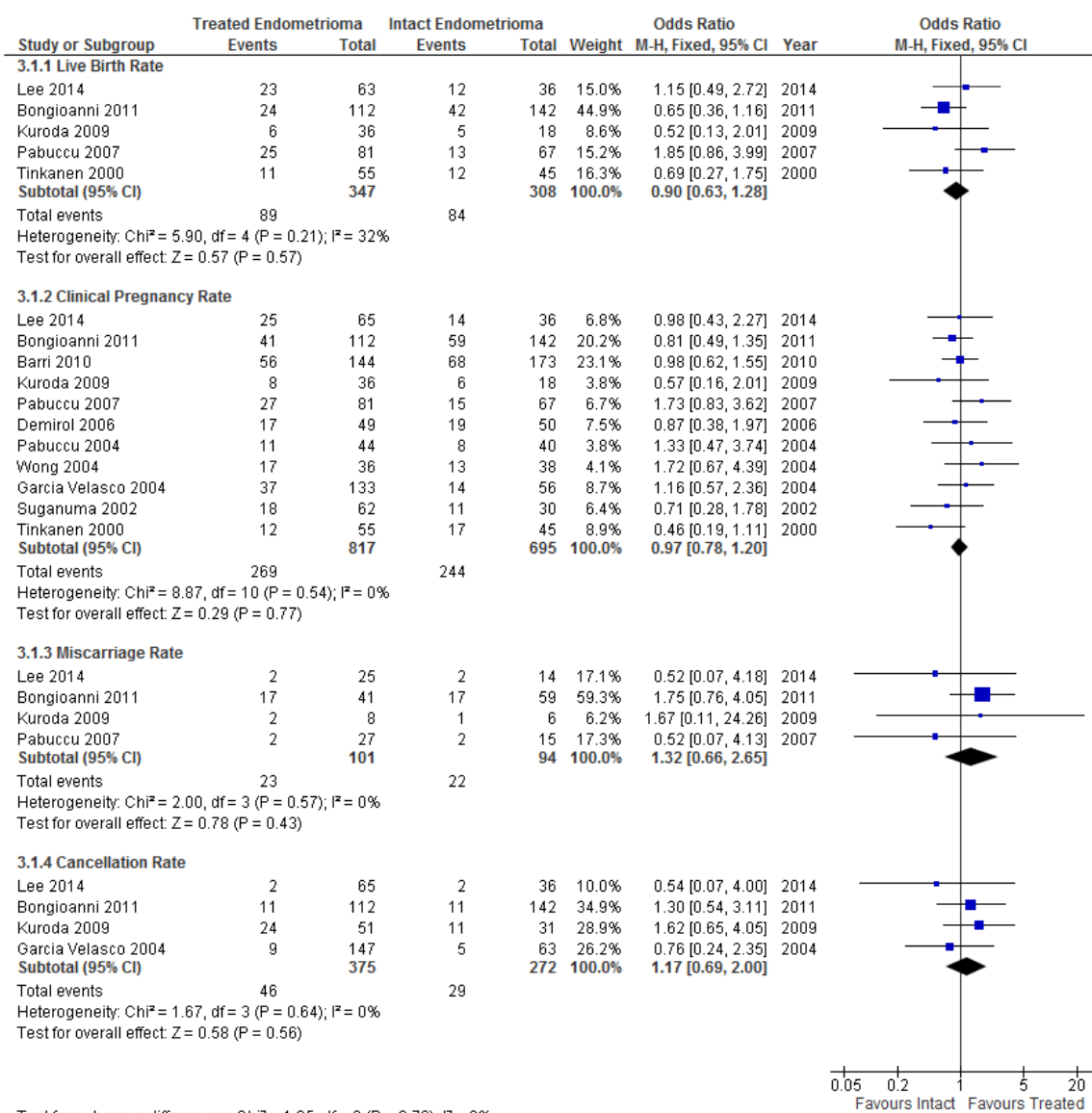


Figure 2-17 Endometrioma (surgically treated) vs. intact endometrioma

Forest plot comparing women with surgically treated endometrioma and women with intact endometrioma, showing dichotomous outcome measures include Live Birth Rate (LBR), Clinical Pregnancy Rate (CPR), Miscarriage Rate (MR) and Cancellation rate.

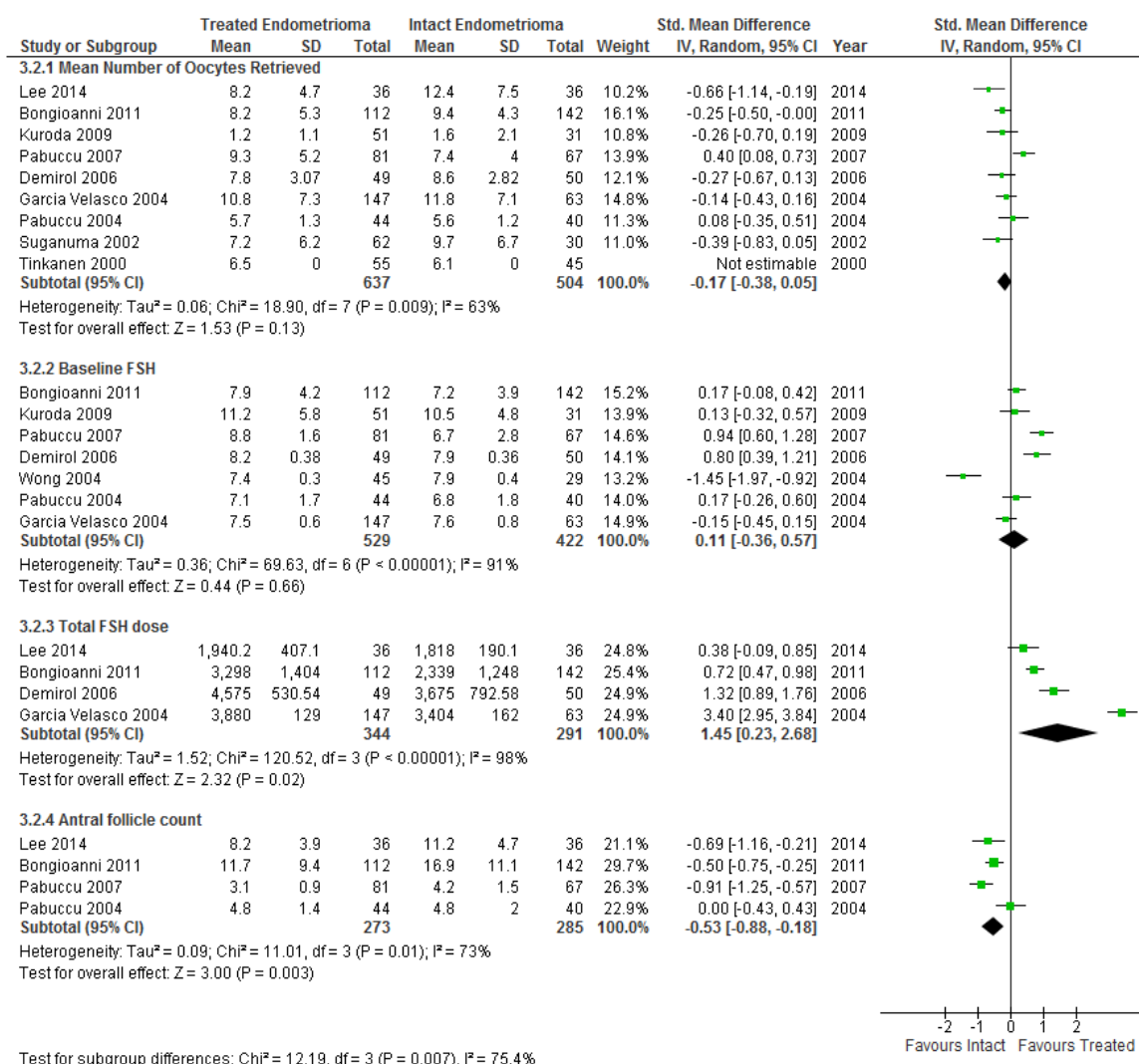


Figure 2-18 Endometrioma (surgically treated) vs. intact endometrioma

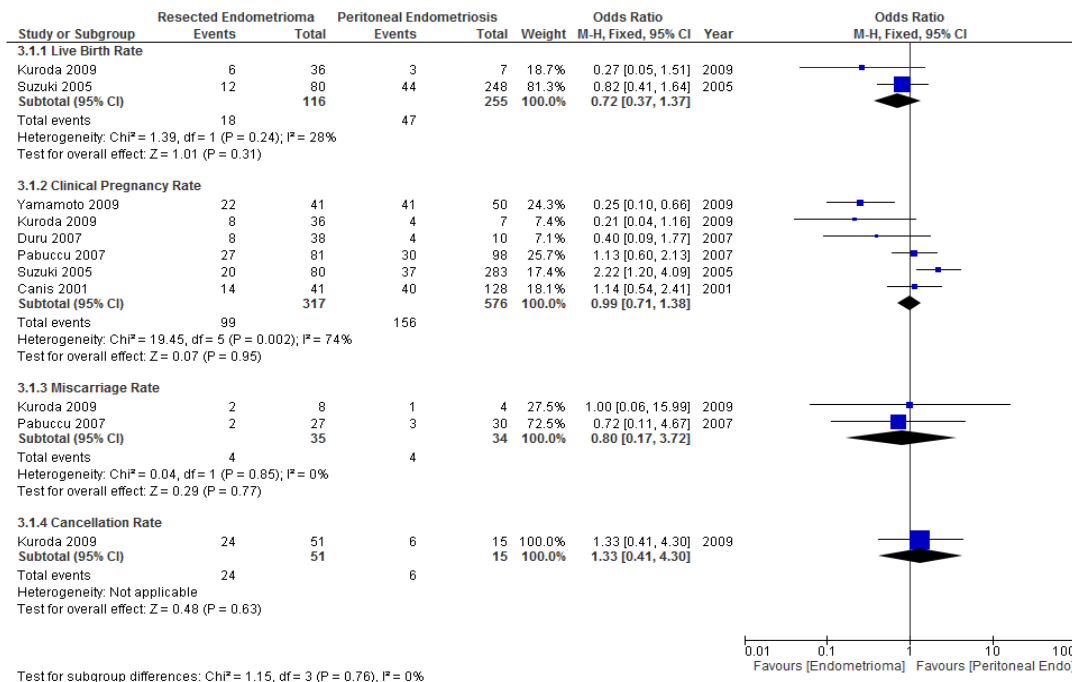
Forest plot comparing women with surgically treated endometrioma and women with intact endometrioma, showing continuous outcome measures include mean number of oocytes retrieved (MNOR), baseline FSH, Total FSH dose and Antral Follicle Count (AFC).

2.2.9.7 Endometrioma (surgically treated) versus women with peritoneal endometriosis alone

Compared to women with only peritoneal endometriosis, those with surgically treated endometrioma had similar LBR (OR 0.72 CI 95% [0.37, 1.37], 2 studies, 371 women, $I^2=28\%$, Figure 2-19A), CPR (OR 0.99 CI 95% [0.71, 1.38] 6 studies, 893 women, $I^2=74\%$, Figure 2-19A), miscarriage rate (OR 0.80 95% CI [0.17, 3.72], 2 studies, 69 pregnancies, $I^2=0\%$, Figure 2-19A), but a lower MNOR (SMD -0.33 95% CI [-0.53, -0.13] 7 studies, 1101 cycles, $I^2=51\%$, Figure 2-19A).

There was no difference in baseline FSH (SMD 1.25 [-0.43, 2.93] 2 studies, 283 cycles, $I^2=96\%$, Figure 2-19B) and total dose of FSH requirement for stimulation (SMD 0.18 [-0.25, 0.61] 2 studies, 167 cycles, $I^2=23\%$, Figure 2-19B). Only one study reported AFC and cancellation rate respectively, however no difference was found between the groups compared.

A



B

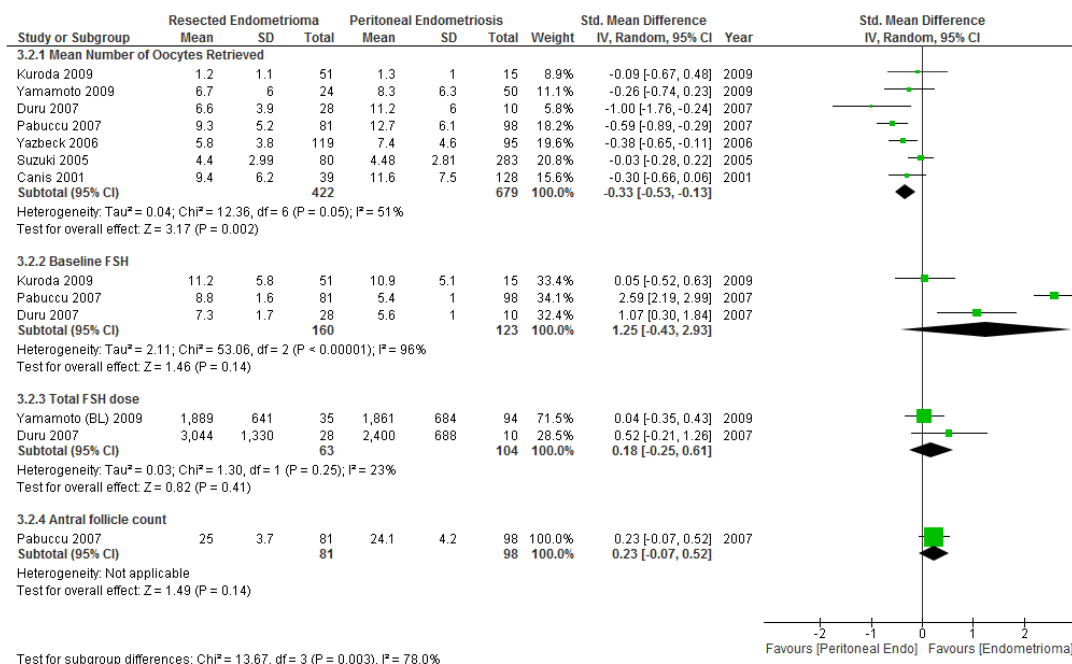


Figure 2-19 Endometrioma (surgically treated) vs. women with peritoneal endometriosis alone

Forest plot comparing women with surgically treated endometrioma and women with peritoneal endometriosis. **A:** Dichotomous and **B:** continuous outcome measures.

2.2.9.8 Endometrioma (surgically treated) versus normal contralateral ovary

In women who had surgical treatment in one ovary, a lower number of oocytes were retrieved (MD -2.59 [-4.13, -1.05] 4 studies, 222 cycles, $I^2=83\%$) compared to the contralateral normal ovary without endometrioma of the same patient.

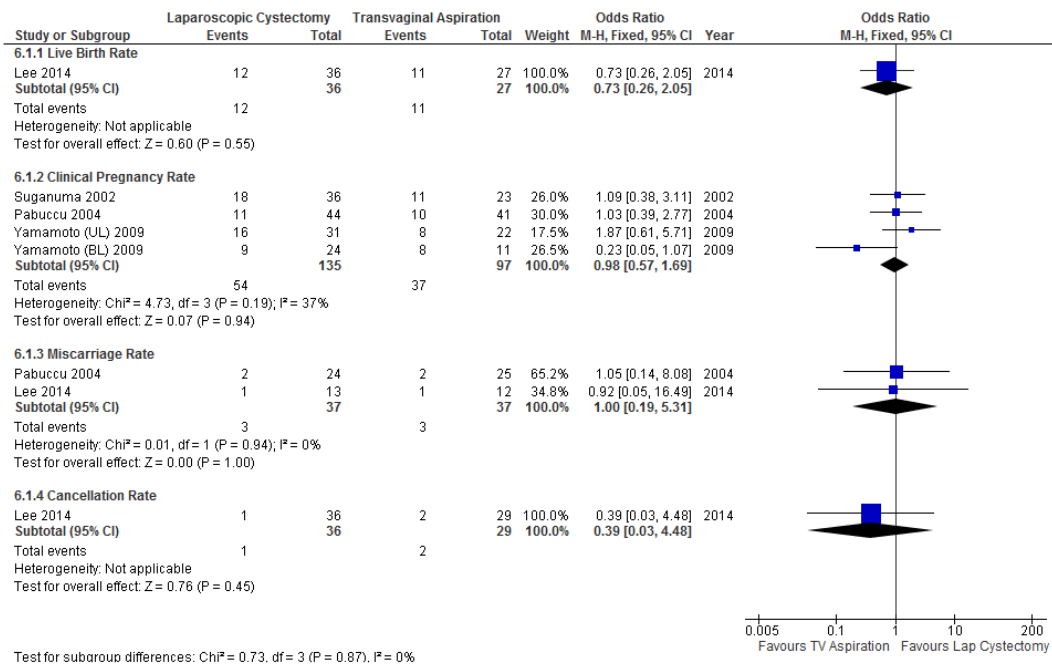
2.2.9.9 Laparoscopic cystectomy versus transvaginal aspiration prior to ART

In women with endometrioma who had surgical treatment, those who underwent laparoscopic/laparotomy cystectomy had similar CPR (OR 0.98 CI 95% [0.57, 1.69] 3 studies, 232 women, $I^2=37\%$), miscarriage rate (OR 1.00 95% CI [0.19, 5.31], 2 studies, 74 pregnancies, $I^2=0\%$) and MNOR (SMD -0.17 [-0.56, 0.22] 4 studies, 289 cycles, $I^2=55\%$, Figure 2-20)

Total FSH dose (SMD -0.02 [-0.42, 0.38] 2 studies, 100 cycles, $I^2=0\%$) and antral follicle count (SMD -0.13 [-1.31, 1.05] 2 studies, 150 cycles, $I^2=92\%$, Figure 2-20) was similar compared to those who had transvaginal aspiration. Only one study reported LBR, cancellation rate and baseline FSH level showing no difference between the groups.

Chapter 2

A



B

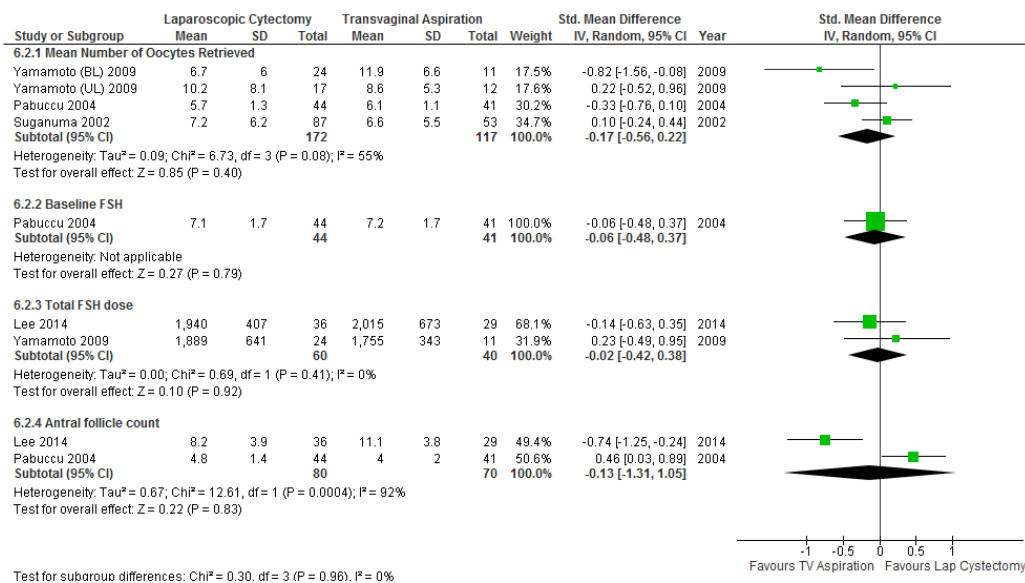


Figure 2-20 Laparoscopic cystectomy vs. transvaginal aspiration prior to ART

A: Forest plot of dichotomous data including Live Birth Rate, Clinical Pregnancy Rate, Miscarriage rate and Cancellation rate. B: Forest plot of continuous data including mean number of oocytes retrieved, baseline FSH, Total FSH dose and Antral follicle count

2.2.9.10 Different laparoscopic cystectomy surgical techniques

This review found three studies comparing the effect of different laparoscopic surgical techniques to IVF outcomes. One study [140] compared conventional laparoscopic cystectomy technique to laser ablation technique; the authors reported no difference in pregnancy rates and MNOR. Another study [144] examined different haemostatic techniques following laparoscopic cystectomy between coagulation and suture reporting no difference in pregnancy rate, the authors however only reported number of oocytes retrieved from the treated ovary compared to contralateral ovary without disease. An earlier study [145] examined 4 different techniques namely laparoscopic cystectomy, laparoscopic aspiration and sclerotherapy, laparoscopic aspiration with and without coagulation. They found no difference in the MNOR but a higher pregnancy rate in-group that had laparoscopic aspiration with coagulation of the cyst wall. None of the studies reported adverse outcomes during ART. Meta-analysis of these available data was not possible as the comparison groups were not similar.

2.2.10 Discussion

This review demonstrated similar LBR, CPR and miscarriage rate, but a lower mean number of oocytes retrieved in women with intact endometrioma when compared to women without endometrioma. Women with endometrioma however, were nearly three times as likely to have a cancelled cycle compared to those without the disease. Amongst those with endometrioma, women who had surgical treatment prior to ART had similar LBR, CPR, MNOR and miscarriage rate compared to those women with intact endometrioma. However these women required a higher total gonadotropin stimulation dose and had a lower AFC compared to those who had no surgery (Figure 2-21).

From previous section and publication [129], women with more severe disease (Stage III and Stage IV) had a poorer reproductive outcome. Severe endometriosis exists in varied forms and is a rather heterogeneous group [3]. In this review we have exclusively examined a defined group of women with endometrioma that will inevitably be overlapped with those categorized at stage III/IV endometriosis. The observation of poorer reproductive outcomes of stage III and IV endometriosis overall but not endometrioma on its own, suggests that endometrioma alone is unlikely to be the major contributory cause, at least in the context of ART. The poorer reproductive outcomes with severe disease may be more closely linked with factors such as the non-ovarian aspects of the disease entity, presence or absence of surgical interventions and the baseline ovarian reserve.

The diminished number of eggs retrieved and the higher baseline levels of FSH in women with endometrioma compared to women with no endometriosis allows speculation that the ovarian endometriotic disease per se exerts some detrimental impact on the ovary. The impact of the disease may not be solely on diminished oocyte numbers but more importantly on oocyte quality, with supportive evidence drawn from oocyte donor recipient studies where recipients of egg donors with endometriosis achieved a lower pregnancy rates than their non-endometriosis donor counterpart [45] in addition to studies that have found the peritoneal [4] and follicular environment [169] of women with endometriosis to hostile to the integrity and intrinsic functions of the oocyte [170] and subsequent embryo development [33]. However, other studies examining the

basic morphology of oocytes and embryo development in women with and without endometriosis did not find any differences in the two groups [47-49, 171]. There were no embryo development data, which we could utilize in this review for comparison. The question that has arisen but yet unanswered is if treatment, be it medical or surgical, should be established at the earliest opportunity to reduce the adverse impact of the disease on the ovary. Given that the diagnosis of endometriosis is often delayed [9, 23, 172], there is a clear need for more effective non-invasive diagnostic clinical tools, and innovative fertility preserving treatments for this condition.

There is no doubt, as revealed by this study and studies on ovarian reserve markers by others that surgery on endometrioma has a detrimental impact on ovarian reserve [41-43]. Arguably, the most reliable data where conclusions can be drawn would be those relating specifically to women with bilateral endometriomas, however it is not possible to extrapolate such data from the current available published studies. The physiological functional compensation of one ovary in the presence of a compromised contra-lateral ovary, coupled with the use of stronger gonadotropin ovarian stimulation, as shown by the higher dose of FSH required for ovarian stimulation in women who has surgery prior to their ART, may well account for the observation that surgery did not have any apparent impact on live birth rate. However, such compensatory mechanisms may not be present in those with already a lower ovarian reserve, where an even lower than usual cumulative live birth rate may be pre-empted given the additive impact of lower oocyte yield in these patients and the presumptive effect on reducing the number of embryos and the number of embryos potentially available for frozen embryo transfers. Hence, the presence of endometrioma would be a justifiable indication for the assessment of ovarian reserve prior to surgery even in the younger patients. It is hence important to consider individualizing the care of women with endometriomas prior to ART, adopting a more conservative approach in those who are asymptomatic, older or with established low ovarian reserve. The advantages of pituitary down regulation prior to ART may in this case be helpful [173].

This study has highlighted the lack of clinical studies examining the complications associated with the surgical treatment of endometrioma [8] and

the complication rate during the course of the IVF treatment and ovum pickup such as pain, infection or fever. Whilst the exact reasons for the high IVF cancellation rate in women with endometrioma compared to those without the disease as found in this study is yet to be determined, and that this finding could be attributed to chance. From the paucity of data available for analysis, this current review recommends that future studies to include outcome measures which examine adverse events including cancellation rates as such events forms crucial aspects of patients' ART journey, as such information will help in the counselling process.

Due to the heterogeneity of data, reproductive outcomes pertaining to the size of the endometrioma were unable to be evaluated. The patients' symptoms in addition to the accessibility of the ovaries for oocyte retrieval are logical reasons to justify the consideration for their removal prior to ART. The latter recommendation is in line with the recent ESHRE guidance on the management of the condition [8].

As with many other meta-analyses, this review may be confounded by the high clinical heterogeneity of the included studies, as inevitably, studies brought together in a systematic review will differ. The majority, with the exception of 3 studies, was all non-randomised controlled trials. Some of the comparisons were only based on non-randomised studies, and therefore will limit the robustness of the findings. I note that differences identified from analysis of too few studies can be due to chance, and also are subjected to confounders such as age and body mass index. Of relevance however, whilst the Newcastle Ottawa Scoring assessment provided a means to assess non-randomized studies, the scoring system itself is not without its drawbacks and criticisms [102]. Overall, the conclusions drawn from this review represents a current collation of best evidence.

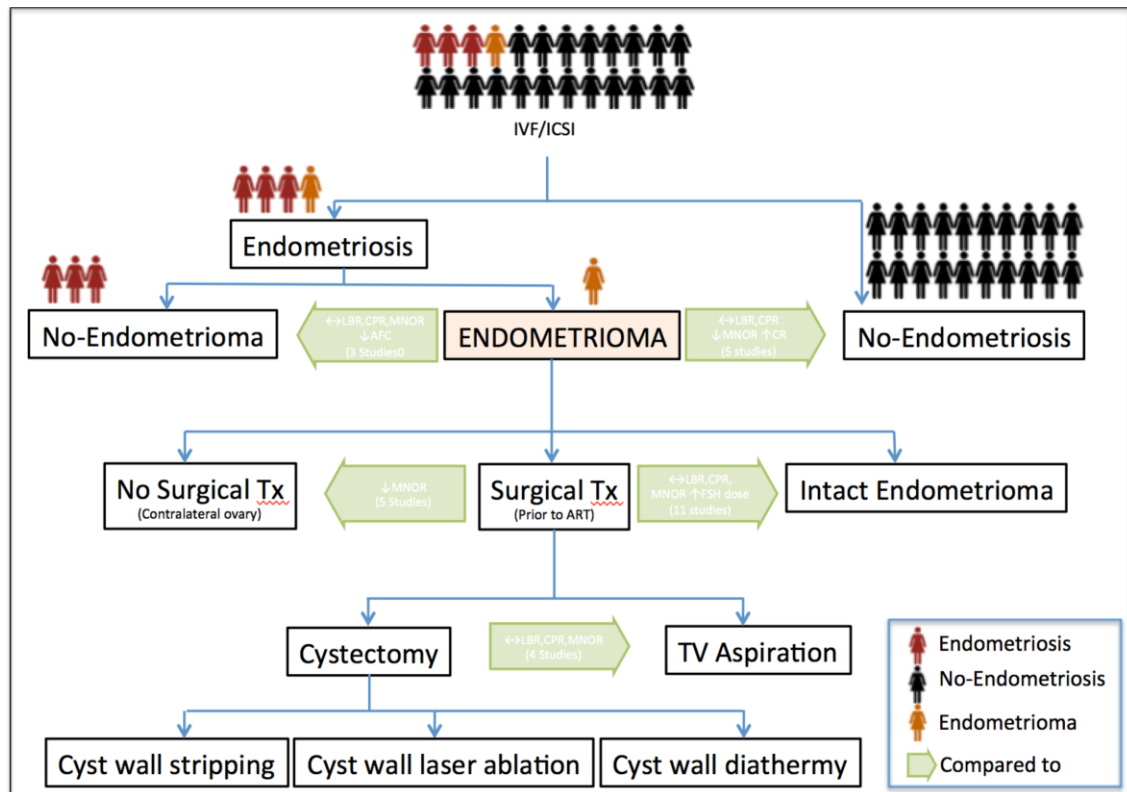


Figure 2-21 Flow chart on the summary of results for each comparisons of this review

Summary of findings between the comparison groups for this review. Arrows in green show what it is comparing to. And arrow next to the mention comparison indicates higher (\uparrow) or lower (\downarrow).

2.2.11 Conclusions

Compared to women without the disease, women with endometrioma have a similar live birth rate, clinical pregnancy rate and miscarriage rate although they have a lower mean number of oocyte retrieved, require higher FSH dosage for ovarian stimulation and have a lower AFC, suggesting that their ovarian reserve is diminished prior to ART. Women with endometrioma should be counselled regarding their increased risk of cycle cancellation. Whilst surgery did not seem to influence live birth rate, surgical treatment of endometrioma prior to ART could exert a further detrimental impact on ovarian reserve. There is therefore not one dogmatic recommendation as to whether women with endometrioma should or should not have surgical intervention prior to ART, but that based on current evidence, consideration should be given to individualise these patients' care.

Chapter 3: Oocyte and embryo quality in women with endometriosis: gathering evidence from IVF/ICSI data

Two preceding chapters in this thesis have emphasised the impact of endometriosis on IVF outcomes. This chapter will further investigate the hypothesis that endometriosis may have an effect (directly or indirectly) on oocytes and/or embryos quality. This chapter will focus on a retrospective study examining the quality of embryos of women with and without endometriosis.

3.1 Does endometriosis influence oocyte quality and/or endometrial receptivity

A significant number of infertile women with endometriosis may eventually require artificial reproductive techniques (ART). According to HFEA data, a significant number of women undertaking IVF have endometriosis (Figure 1-1). Endometriosis is a known associative factor of subfertility although it is not known how mechanistically it is detrimental to fertility. Mechanisms of detrimental impact could be at the level of the endometrium, oocyte or/and embryo quality [47, 48]. The impact of endometriosis on the endometrium will be discussed in later chapters in this thesis (Chapter 5:).

Very few studies examined the impact of the disease on the oocyte or the embryo. Animal studies suggest that oocyte and embryo development is poorer when exposed to follicular and peritoneal fluid of women with endometriosis [170, 174] and studies on oocyte donors and recipients with endometriosis points more to the detrimental role of endometriosis on oocytes and embryo development than that of a defective endometrium [45]. When examined under electron microscopy, the mitochondria of oocytes from women with endometriosis was found to be abnormal and contain small and blurred vacuoles, when compared to the oocytes from those with tubal factor [56]. In addition, meiotic spindle morphology of oocytes from women with endometriosis was found to be more disorganised [57], although this was reported to be no

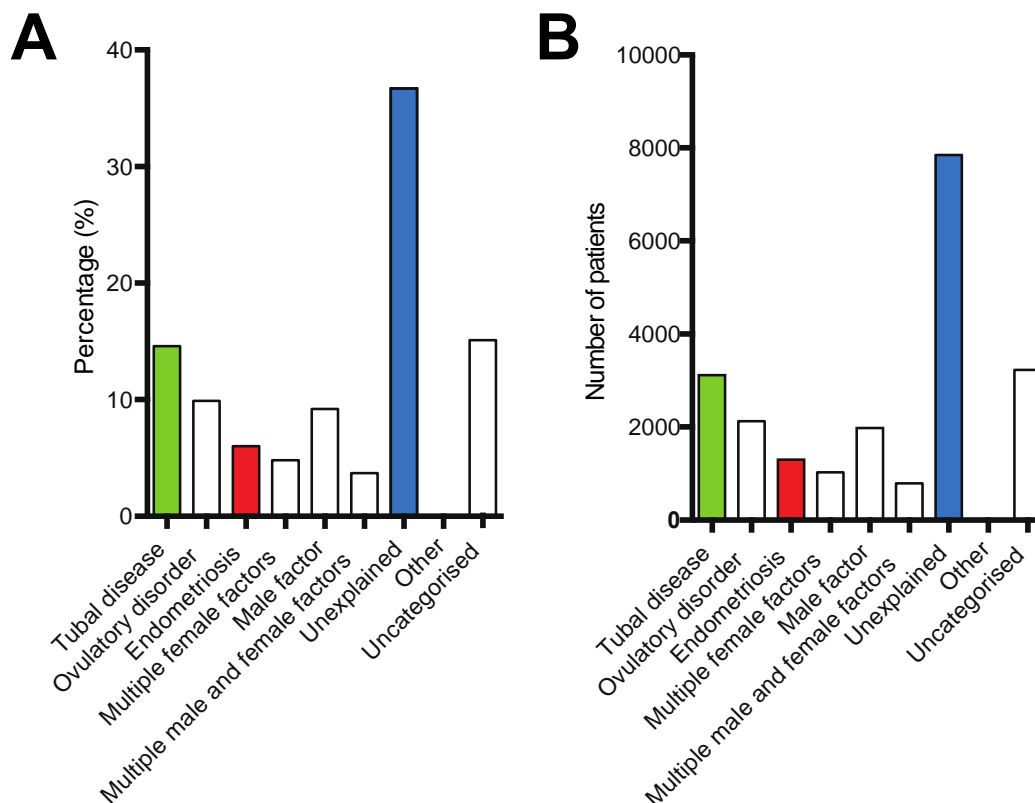


Figure 3-1 HFEA data on indications for IVF treatment

Bar chart showing the **A**: percentages and the **B**: actual number of patients treated with IVF according to their indications. Endometriosis (shown in red bar) is the 5th most common indication for women to undergo IVF treatment, constituting approximately 7% of all patients undergoing IVF. Tubal disease (Green Bar) and Unexplained subfertility (blue Bar) are two other main indications for IVF, which were included in this analysis as control groups. Source: HFEA: Trend and Figures 2013.

different in another [58]. Furthermore, there is recent evidence to suggest that women with endometriosis have a higher chance of aneuploidy not dissimilar to that of women with advance maternal age [61].

From the meta-analysis presented in the earlier chapters of this thesis (see 2.1), women with endometriosis were found to have lower number of oocytes retrieved [129]. Moreover, those women with endometriomas (see 2.2) not only have fewer eggs retrieved, but also require a higher dose of FSH stimulation, and suffer from a higher cancellation rate of their IVF cycles [175]. The evidence from the previous chapters, together with evidence drawn from studies on oocyte recipient cycles that showed poorer reproductive outcome in those who received oocytes from women with endometriosis, suggest that the oocyte quality of women with endometriosis is compromised [45, 46].

However, recent publications that examined the association between embryo quality and the presence of endometriosis or endometrioma are scarce. The majority of the older studies, which examine whether endometriosis and/or endometrioma impact on oocyte and embryo quality fail to conclude that endometriosis detrimentally impact on oocyte and/or embryo quality (Table 3-1).

In 2014, Dong and colleagues [176] analysed 431 women with endometriosis and 596 women with tubal disease. Using their local criteria for embryo assessment, the authors reported a lower ($P<0.01$) percentage of good quality embryo on D3 in endometriosis group (ASRM I-II, 70.6%; ASRM III-IV, 70.1%) when compared to controls (tubal disease, 78.4%). A smaller study by Mekaru et al [177] also reported a lower percentage of grade 1 embryo on D3 per fertilised embryo in endometriosis group ($P=0.044$, endometriosis, 9%, $n=39$; unexplained, 16.3%, $n=41$). This study however included women with all grades of severity of disease and did not analyse them separately. Conversely, Lin et al [95] found no significant difference in number of high quality embryo ($P>0.05$, Endometriosis 43.7%, $n=177$; Control, 43.9%, $n=4267$). By calculating cumulative embryo scores using their local criteria for embryo assessment, Al-Fadhli et al [178] reported no significant difference in the embryo quality between women with and with endometriosis ($P>0.05$, Endometriosis, $n=87$;

Control, n=87). None of the studies mentioned earlier has specifically examined women with endometrioma.

However, Reinblatt et al [48] reported comparable number of good quality embryos between women with bilateral endometriomas compared with controls ($p=0.32$, endometrioma, n=13, 47.2%; tubal/male factor subfertility, n=39, 41.1%). Another larger study [49] analysed women (n=80) with and without endometrioma and showed no difference in the embryo quality rate when compared to control (Table 3-1).

Filippi and colleagues [47] performed the only prospective study (n=29) to date, comparing the embryo quality according to whether the oocytes were derived from the ovary with or without endometrioma of the same patient. The authors concluded that endometrioma has no effect on oocytes development competence and produces similar rate of high quality embryo ($P=0.13$; endometrioma, 31% and without endometrioma, 21%). A significant drawback of the study was that it did not have a non-endometriosis control group. However, an earlier study by Suzuki et al [49] has separately analysed women with endometriosis (with endometrioma, n=80, and without endometrioma, n=248) compared to control (tubal factor, n=283) and found no difference in percentage of good quality embryo between all groups (67.2% vs. 63.0% vs. 58.1% respectively).

The current available studies examining the oocyte/embryo quality for women with and without endometriosis suffer from the following limitations. Embryo morphological assessment [179], is traditionally subjected to a high intra and inter-observer variability when considering factors associated with morphological dysmorphisms such as fragmentation, asymmetry and multinucleation. It is not until recently that a consensus to standardised embryo assessment by Alpha group in Reproductive Medicine and ESHRE Special interest group was published [180].

Table 3-1 Table of published studies on embryo quality of women with endometriosis and endometrioma

Study (Year)	N	Study Design	Stages/ Laterality	Control Group	Conclusion	Embryo Grading
Endometriosis						
Dong et al. 2014	431	Retrospective	I-II, III-IV	Tubal Factor	Lower percentage of good quality D3 embryo in endo	Local protocol*
Mekaru et al. 2013	39	Retrospective	All stages	Unexplained	Lower percentage of grade 1 embryo at D3 in endo	Local protocol*
Lin et al. 2012	177	Retrospective	I-II, III-IV	Tubal Factor	No significant difference in embryo quality	Local protocol*
Al-Fadhli et al. 2006	87	Retrospective	All stages	Non-Endometriosis	No significant difference in embryo quality	Embryo scoring No details
Kuivasari et al. 2005	98	Retrospective	I-II, III-IV	Tubal Factor	No significant difference in embryo quality	Local protocol*
Endometrioma						
Filippi et al. 2014	29	Prospective	Unilateral	Contralateral ovary	No effect on embryo development competence	ESHRE-Istanbul consensus
Reinblatt et al. 2011	13	Retrospective	Bilateral	Male Factor/ Tubal factor	Not associated with reduce embryo quality	Local protocol*
Suzuki et al. 2005	80	Retrospective	Either	Tubal Factor	No effect on embryo quality and pregnancy outcomes	Local protocol*
Loo et al. 2005	127	Retrospective	Not defined	Tubal Factor	Lower embryo cumulative score in endometriosis	Local protocol*

*** Embryo Assessment score**

Dong et al. Class 1: Embryo with 4-6 cells on day 2, 8-10 cells on day 3, equal, and fragmentation of <10%, and no multinucleated blastomeres. Class 2: 3 or >6 cells on day 2, 6-7 or >10 cells on day 3, equal or less equal, fragmentation 10-20%, and no multinucleated blastomeres. Class 3-4: 0-2 cells on day 2, 1-5 cells on day 3, unequal, fragmentation >25%, with or without multinucleated blastomeres.

Mekaru et al. Grade 1: 7 or 8 mono-nucleated blastomeres that were approximately equal in size without fragmentation. Grade 2: similar mono-nucleated blastomeres and less than 10% fragmentation. Grade 3: Embryos containing fewer than 7 or unequal blastomeres without fragmentation. Grade 4: Unequal blastomeres and more than 15% fragmentation.

Lin et al. Grade 1: Blastomeres of equal size and no cytoplasmic fragmentation; Grade 2: blastomeres of equal or unequal size and cytoplasmic fragmentation of less than 20%; Grade 3: blastomeres of equal or unequal size and 20%–50% overall cytoplasmic fragmentation; and Grade 4: blastomeres of equal or unequal size and cytoplasmic fragmentation of more than 50% of the embryo surface.

Kuivasari et al. Class 1: Embryo cells were evenly sized, the blastomeres regularly shaped without fragments and the zona is intact. Class 2: the embryos were slightly non-homogeneous, the maximum fragmentation rate was 20% and the zona is solid. Class 3: the blastomeres were uneven in size and the fragmentation rate was 20–50%. Class 4: the fragmentation rate was >50%.

Filippi et al. European Society for Human Reproduction and Embryology Istanbul Consensus Conference

Reinblatt et al. Grade 1: Embryos with four cells on day 2 or six to eight cells on day 3. Grade 2 - 3: Embryos with 0%–20% fragmentation. Fair-quality: embryos at two cells on day 2 or three to five cells on day 3, grade 2 to 3 or grade 3 (embryo fragmentation 20%–50%). Poor-quality: embryos at lower than three-cell stage by day 3 or any grade 4 embryos (embryo fragmentation >50%)

Suzuki et al. Embryos were graded from 1 to 5 (grade 1 being the best quality), according to the symmetry of the blastomeres and the presence or absence of fragmentation under an inverted microscope.

Loo et al. Grade 1: Even number of blastomeres without any fragmentation was scaled (4 points). Grade 2: uneven number of blastomeres and fragmentation less than 10% (3 points). Grade 3: uneven number of blastomeres and fragmentation percentage between 10–50% (2 points). Grade 4: uneven number of blastomeres and fragmentation greater than 50% (1 point) in score calculation. Each embryo was scored as its blastomeres number multiplied its grading point.

Only one out of all the studies [47] utilised a standard embryo morphological assessment [180]. Moreover, most made their assessments on D3 rather than the now more frequently used D5 embryo assessment and none of the studies utilised data obtained from time-lapse technology. The majority of the studies were also assessing the embryo quality as a secondary outcome rather than as their primary outcome, thus subjecting the data to bias. Five out of the nine studies in Table 3-1 examined women with endometriosis and/or endometrioma; the latter strategy made the bold and erroneous assumption that pelvic endometriosis has the same impact on oocyte/embryo quality as endometrioma(s). Whilst classed under the umbrella as the 'endometriosis' entity, clinically, peritoneal endometriosis has very distinct appearance and presentation from endometrioma, with some suggesting a different mechanism of pathogenesis for the two entities.

Given the advancement in laboratory equipment and incubators, culture media, the recent introduction of standardise criteria for the assessment of embryo development, an analysis on more recent treatment cycles may provide new insight into this subject. For these reasons, the ideal study to examine if the presence of endometriosis impacts on the oocyte or embryo quality would be in depth analysis of embryo using the recent consensus of embryo scoring. The analysis should be up to D5 or blastocyst stage as the embryo quality can be distinctively compared. A two-centre approach in the analysis also will provide a larger number of patients and more consistent findings.

3.2 Hypothesis

Oocytes and embryos quality of women with endometriosis is poorer compared to those with no endometriosis.

3.3 Objective

This study intends to evaluate the oocyte and embryo quality of women with endometriosis compared to women without endometriosis.

3.4 Methods

3.4.1 Data extraction

This study was a retrospective case control study, performed in two tertiary referral fertility centres in the United Kingdom (Complete Fertility Centre and Wessex Fertility Centre, Southampton). Anonymised data from the IDEAS V.6™ (Electronic IVF database, Mellowood Medical, USA) from both fertility centres was extracted and reviewed from January 2011 to December 2014. Data obtained from the database were exported to Excel (Microsoft, USA) before data analysis was performed using statistical analysis package.

3.4.2 Study group and inclusion/exclusion criteria

This study included women who were under 40 years old and underwent IVF treatment using their own gametes. Oocyte donor cycles (altruistic or sharer) or couples with co-existing male factor subfertility that requires intra-cytoplasmic sperm injection (ICSI) was excluded.

Study group (Endometriosis, EN) consisted of women who were subfertile for at least 1 year with endometriosis, diagnosed of having endometriosis laparoscopically and/or sonographically irrespective of the disease severity, duration or surgical treatment received.

Our control groups included women with no known history of endometriosis, but a known history of tubal factor (TF) or unexplained subfertility. Unexplained subfertility was defined as being subfertile for at least 1 year with a normal hormonal profile, patent Fallopian tube(s) and a normal semen analysis. Women were considered as being in the tubal factor group (TF), if they were subfertile for at least 1 year, with known tubal disease in one or both tubes identified through Hysterosalpingography, Hysterosalpingo-contrast-sonography (HyCoSy) or laparoscopy, although we do not have the breakdown of the actual numbers in each of these investigations. If the tubes were blocked resulting in hydrosalpinges, the normal practice in the UK would be to remove or clip the tube(s).

Ovarian stimulation protocols were limited to the antagonist cycle and agonist cycle. Short flare agonist cycles were excluded. However, there was no

restriction on the type of stimulation drugs used and the embryo culture protocol.

3.4.3 Oocytes and Embryos assessment

Qualified and trained embryologists from each centre performed the oocytes and embryo assessment. This included all the procedures that require handling of oocytes and embryos. Following oocyte retrieval procedure, oocytes were incubated in the culture media until the in vitro fertilisation procedure. Where possible, collected oocytes were scored according to the appearance of cumulus cells, zona pellucida and cytoplasm [180].

Irrespective of the oocytes score, insemination was performed at approximately 1400H in the afternoon of the same day of egg collection. Fertilisation check was done 17 ± 1 hours post insemination (HPI). Normal fertilisation was defined as presence of 2 polar bodies with two centrally located pronuclei that are evenly sized (Figure 3.2).

At 68 ± 1 HPI, all embryos were reassessed and scored using standardised scoring system [180] according to the cell number, fragmentation rate and multinucleation. The scoring was documented in the embryology record electronically. At this stage, fertilised oocytes that have not cleaved were categorised as arrested at D1 and were discarded.

At D3, selected women underwent embryo transfer procedure, which was decided according to the respective unit's embryo transfer criteria and protocol. Embryos that were not transferred at day 3 were cultured to blastocyst stage. At D5 of culture, embryos were rescored. Suitable good quality blastocysts were usually frozen on the same day or the day after (D6) if the criteria were met. Embryo assessment and scoring protocol is in Appendix J.

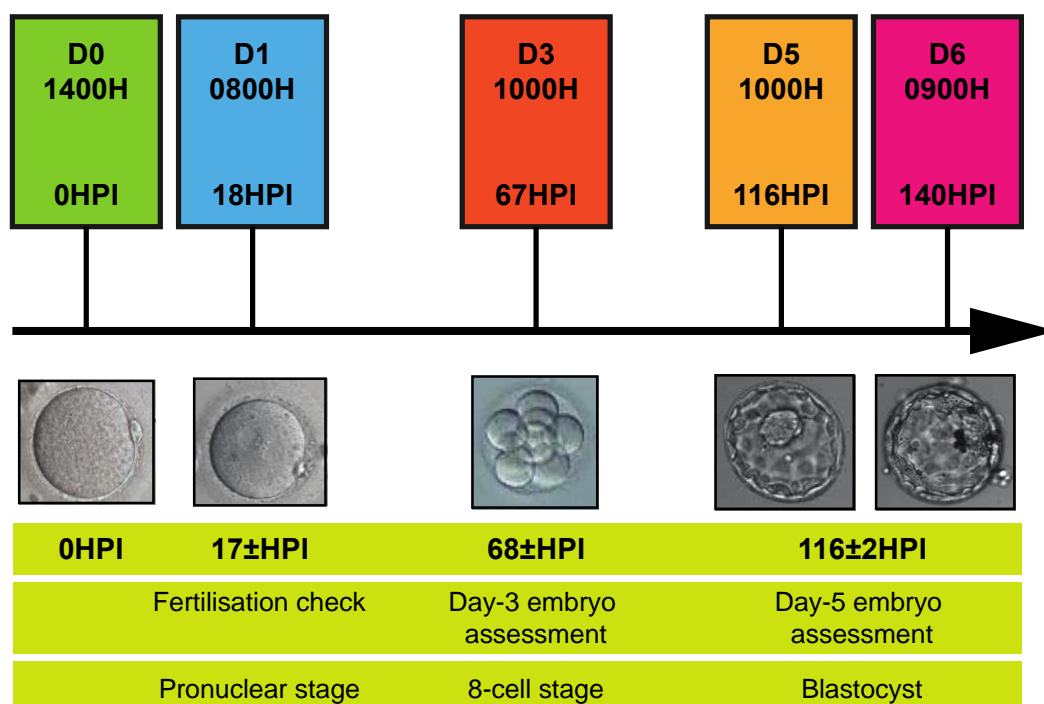


Figure 3-2 Embryo assessment timing

Figure outlining the 1) timing for observation of fertilised oocytes and embryos, and 2) expected stage of development at each time point. The first row represents the timing adopted by both fertility centres. Three lower rows (green) show the consensus regarding recommendations for oocyte and embryo scoring, including the timing (hours post insemination), type of observation and expected stage of development. Source: The Istanbul Consensus workshop on embryo assessment; proceedings of an expert meeting (April 2011). Photo source: ATLAS OF HUMAN EMBRYOLOGY: From Oocytes To Preimplantation Embryos

3.4.4 Outcome measures definitions

Number of oocytes retrieved is defined as total number of oocytes collected per oocyte collection procedure. Percentage of oocytes per mature follicles is defined as total number of oocytes collected over total number of mature follicles (measuring >14mm on the day of trigger). Number of fertilised oocytes is defined as number of normally fertilised oocytes per number of patient. Fertilisation rate is defined as number of normally fertilised oocytes per number of inseminated oocytes. Day 1 arrested embryo is defined as fertilised oocytes in which development has arrested and has not reached cleavage stage. At Day 3, individual embryo was scored into 4 grades (1, 2, 3, and 4). Grade 1 and Grade 2 embryos at day three were grouped as poor quality oocytes, whereas Grade 3 and Grade 4 as good quality oocytes. Number of embryo transferred and cryopreserved are define as number of embryo transferred into the uterus and frozen respectively, over number of patients. Utilisation rate is defined as percentage of embryos transferred or cryopreserved, over number of oocytes retrieved. Blastulation rate is defined as percentage of blastocysts over number of fertilised oocytes. Fully expanded and hatching blastocyst (FEHB) is defined as good quality blastocysts at grade 4 and above. Clinical pregnancy is defined as presence of at least one fetal heart.

3.4.5 Statistical analysis and data representation

Statistical analysis was performed using unpaired t-test for parametric data. Fischer exact test or chi square test was used for categorical data. A p-value <0.05 was considered to be significant. One-way ANOVA was used for comparison of more than 2 groups with the normally distributed data. If the ANOVA showed statistically significant difference, post hoc analysis was done using Tukey's post-hoc test. Kruskal-Wallis test was used for comparison of more than 2 groups with non-parametric data. All statistical analyses were performed using the Statistical Package for Social Sciences software, version 18 (SPSS Inc., Chicago, IL, USA) or Prism6 (GraphPad, USA) except effect size, which was calculated using the Cohen d Test (G*Power, USA). Data is expressed as means \pm standard deviation, or Median (Lower and upper quadrant) or percentages.

3.5 Results

3.5.1 Demographic characteristics of the patients between groups

Total of 678 women who had IVF treatment were analysed, comprising of 3 comparison groups (EN, n=89; TF, n=214; UE, n=375, Table 3-2). The mean age for each group was EN, 34.4 ± 3.3 ; TF, 33.5 ± 3.7 and 34.6 ± 3.2 respectively (Table 3-2). Data for age was normally distributed and there was no difference found between EN with the other groups (TF, $P=0.17$; UE, $P=0.78$, Table 3-2). Women in TF group were however younger than UE groups ($P=0.002$), with small effect size, $d = 0.3214$ (Cohen d Test).

Fifty-nine women in the study group (Endometriosis, EN) diagnosed to have endometriosis laparoscopically, sonographically (n=1) and 29 women did not have any documentation on diagnosis of endometriosis. Twenty-five women had documented surgical treatment, whilst 34 women did not have documented surgical treatment, and 29 women did not have any documentation on their surgical treatment.

On the other hand, AMH levels were not normally distributed and therefore non-parametric test was performed, showing no significant ($P=0.432$, Kruskal-Wallis test) between the three groups [Median: EN, 11.7 (6.16 to 20.15); 15.7 (7.27 to 24.3) and UE, 12.28 (6.70 to 22.93), Table 3-2].

	Endometriosis (n=89)	Tubal Factor (n=214)	Unexplained (n=375)	P-value
Age (years)	34.3 ± 3.4	33.5 ± 3.6*	34.6 ± 3.7*	0.001
AMH (pMol)	11.7 (6.2 to 20.2)	15.7 (7.3 to 24.3)	12.3 (6.7 to 22.9)	0.432
Number of oocytes retrieved	8.6 ± 4.7	9.5 ± 5.2	9.2 ± 4.8	0.311
Number of follicles at trigger	12 ± 5	13 ± 6	13 ± 6	0.746
Oocyte retrieval rate per follicle (%)	65 ± 23**	76 ± 20**	71 ± 24	0.008
Number of fertilised oocytes	5.7 ± 3.6	6.4 ± 3.6	6.3 ± 3.9	0.310
Fertilisation rate (%)	68 ± 21	70 ± 21	69 ± 23	0.758
Number of embryo arrested at D1	0.14 ± 0.4	0.08 ± 0.3	0.11 ± 0.7	0.688
Rate of embryo arrested at D1(%)	4.0 ± 15***	1.2 ± 5.1***	1.5 ± 7.3***	0.020

Table 3-2 Demographic data on patients at different comparison groups

The table shows demographic information of women analysed according to their groups (Endometriosis, Tubal Disease and Unexplained). There was no different in age ($P>0.05$) of women with endometriosis compared to tubal factor and unexplained group. Women with tubal disease were younger than those in unexplained group (, $P=0.0001$). There was lower percentage of oocytes retrieved per number of follicles. Percentage of embryos arrested at D1 is significantly higher in Endometriosis compared to Tubal disease group and unexplained (***, $P=0.021$, $P=0.028$).*

3.5.2 Women with endometriosis have lower number of oocytes retrieved per mature follicle

Number of mature follicles (>14mm) on the day of trigger injection was similar ($P=0.746$, one-way ANOVA) between all groups (EN, 12 ± 5 ; TF, 13 ± 6 ; UE, 13 ± 6 , Table 3-2). Number of oocytes collected at oocyte retrieval procedure was similar ($P=0.31$, one-way ANOVA) but has a trend towards lower yield between the three groups (EN, 8.59 ± 4.7 ; TF, 9.52 ± 5.2 ; UE, 9.16 ± 13 , Table 3-2). Percentage of oocytes collected per mature follicle was found to be significantly lower ($P=0.008$, one-way ANOVA) between all groups (EN, 65 ± 23 ; TF, 76 ± 20 ; UE, 71 ± 24 , Table 3-2). Post hoc analysis showed significantly different percentage of oocytes collected per mature follicle between endometriosis and tubal factor groups ($P=0.017$, Tukey's test).

3.5.3 Endometriosis results similar fertilisation rate compared to controls

All the collected oocytes were inseminated irrespective of their maturity, and the number of fertilised oocytes per woman was found to be similar ($P=0.31$, one-way ANOVA) between groups (EN, 5.7 ± 3.6 ; TF, 6.4 ± 3.6 ; UE, 6.3 ± 3.9 , Table 3-2). The percentages of fertilised oocytes were calculated over the number of oocytes inseminated, to represent the actual fertilisation rate. There was no differences ($P=0.758$, one-way ANOVA) in the percentage of oocytes fertilised or per number of oocytes inseminated, across the three groups, (EN, 67.5 ± 21 ; TF, 69.6 ± 21 ; UE, 69 ± 24 , Table 3-2).

At this stage, unfertilised oocytes were discarded, and fertilised oocytes were incubated and cultured until another routine embryo assessment at D3. The number and the rate of fertilised embryo that have successfully cleaved were examined.

	Endometriosis (n=89)	Tubal Factor (n=214)	Unexplained (n=375)	P-value
Number of D3 embryos	4.9 ± 4	5.8 ± 4	5.8 ± 4	0.136
Quality of embryo at D3 (%) *				
Grade 1	23 ± 29	20 ± 26	22 ± 28	0.651
Grade 2	42 ± 28	43 ± 27	39 ± 27	0.242
Grade 3	24 ± 24	26 ± 25	26 ± 25	0.768
Grade 4	12 ± 21	12 ± 19	13 ± 23	0.607
Number of blastocysts**	4.2 ± 2.5	3.8 ± 2.2	3.7 ± 2.2	0.620
Blastulation rate (%)***	51 ± 22	49 ± 22	46 ± 23	0.501
FEHB**	2.8 ± 2.1	3.0 ± 2.0	2.6 ± 1.9	0.384
FEHB rate (%)***	35 ± 20	38 ± 23	34 ± 26	0.343

Table 3-3 Quality of embryos in the different groups

This table shows an identical quantity and quality of embryos at day three (D3) across all grading amongst Endometriosis, Tubal Disease and Unexplained. (, Mean percentages per total D3 embryos; **, mean number of per patient; ***, mean percentages per fertilised oocytes).*

3.5.4 Women with endometriosis have a higher early embryo arrest and poorer progression to 8 cell cleavage rate

Following the fertilisation check, fertilised oocytes were cultured and incubated before reassessed at 68 ± 1 HPI. During the assessment, fertilised oocytes were expected to cleave and progress to the 8-cell stage. At this stage of the assessment, the majority of the oocytes were found to have further developed, but some were arrested and failed to cleave. The number of arrested embryo per woman was similar ($P=0.688$, one-way ANOVA) amongst the three groups (EN, 0.14 ± 0.43 ; TF, 0.08 ± 0.28 ; UE, 0.11 ± 0.71). However, there were statistically significant differences ($P=0.02$, one-way ANOVA) in the percentages of arrested embryos, per number of fertilised oocytes, across the three groups, (EN, 4.0 ± 15 ; TF, 1.2 ± 5.1 ; UE, 1.5 ± 7.3). Post hoc analysis showed there were higher percentages of arrested embryos, between EN and TF groups [$P=0.02$, MD 2.77, 95%CI (0.33, 5.21, Table 3.1] and between EN and UE groups [$P=0.03$, MD 2.51, 95%CI (0.22, 4.79), Table 3.1].

3.5.5 Embryos development and quality at D3 were unaffected by endometriosis

Embryo assessment at D3 was usually performed to monitor the development of individual embryos, to enable the decision for choosing suitable embryo/s for transfer or for extended culture until blastocyst stage, with the clinical circumstances taken into account. In this study, percentages of embryos at all grades per women were similar between the comparison groups; grade 1 embryos ($P=0.651$, one-way ANOVA, EN, 23 ± 29 ; TF, 20 ± 26 ; UE, 22 ± 28 , Table 3.2), grade 2 embryos ($P=0.242$, one-way ANOVA, EN, 42 ± 28 ; TF, 43 ± 27 ; UE, 39 ± 27 , Table 3.2), grade 3 embryos ($P=0.768$, one-way ANOVA, EN, 24 ± 24 ; TF, 26 ± 25 ; UE, 26 ± 25 , Table 3.2), and grade 4 embryos ($P=0.607$, one-way ANOVA, EN, 12 ± 21 ; TF, 12 ± 19 ; UE, 13 ± 23 , Table 3.2).

Considering the embryo assessment tool was subjective and have high inter and intra observer variability, embryos graded as 1 and 2 were pooled as 'good quality' embryos, whilst embryos graded as 3 and 4 pooled as 'poor quality'.

	Endometriosis (n=89)	Tubal Factor (n=214)	Unexplained (n=375)	P-value
Number of embryo transferred *	1 ± 0.5	1 ± 0.5	1 ± 0.6	0.787
Number of embryos cryopreserved *	1.6 ± 2.3	1.5 ± 2.3	1.6 ± 2.3	0.433
Cryopreservation rate (%)**	17 ± 18	23 ± 21	26 ± 23	0.110
Number of utilised embryos *	2.8 ± 2.1	2.9 ± 2.1	3.0 ± 2.2	0.301
Utilisation rate (%) **	27 ± 14	30 ± 18	33 ± 20	0.168

Figure 3-3 Utilisation of embryos per patients at different comparison groups

The above table shows the comparison between number of embryos transferred per patient, percentage of transferred embryos per fertilised oocyte, the number of embryos suitable for cryopreservation per patient, the percentages of cryopreserved embryos per fertilised oocyte and utilisation rate between endometriosis, tubal factor and unexplained. (, Mean percentages per total D3 embryos; **, mean number of embryos per patient; ***, mean percentages per fertilised oocytes).*

There was no difference across the three comparison groups whether the comparison was made with good quality embryos ($P=0.59$, one-way ANOVA, EN, 64 ± 34 ; TF, 63 ± 33 ; UE, 61 ± 33) or the poor quality embryos ($P=0.59$, one-way ANOVA, EN, 36 ± 34 ; TF, 37 ± 33 ; UE, 39 ± 33).

Following day 3 assessments, these embryos were either selected to be transferred or continue to be cultured to D5, and some may be of sufficient quality to be suitable for freezing. Next, the number and quality of the embryos that progressed to blastocyst was examined.

3.5.6 Endometriosis did not impair blastocyst development or the development of Fully Expanded Hatching Blastocysts (FEHB)

In this section, only women who had blastocyst transfer at D5 or D6 were included for calculation of blastocyst number and blastulation rate. Those who already had embryo transfer before D5 (e.g. D2, D3, and D4) were excluded in this analysis.

The number of embryos at blastocyst stage per woman was similar ($P=0.62$, one-way ANOVA) amongst all the three groups (EN, 4.2 ± 2.5 ; TF, 3.8 ± 2.2 ; UE, 3.7 ± 2.2) and the finding mirrored blastulation rate, which also found to be similar across all the groups ($P=0.50$, one-way ANOVA, EN, 51 ± 22 ; TF, 49 ± 22 ; UE, 46 ± 23).

Within the embryos in blastocyst stage, number of good quality blastocyst that have fully expanded and hatching were analysed. FEHB per women was found to be similar ($P=0.38$, one-way ANOVA) amongst the three groups (EN, 2.8 ± 2.1 ; TF, 3.0 ± 2.0 ; UE, 2.6 ± 1.9), and FEHB rate per fertilised oocytes was no statistically different ($P=0.34$, one-way ANOVA) across the three groups (EN, 35 ± 20 ; TF, 38 ± 23 ; UE, 34 ± 26).

3.5.7 Utilisation of embryos were similar between women with and without endometriosis

In this section, the number of embryos transferred and number of embryos frozen per cycle individually, and also in combination representing the utilisation rate was analysed. 'Utilised embryos' as defined in the method section, were the total number of embryos that were transferred, or frozen in each cycle. Therefore utilisation rate was the percentage of total embryos transferred and frozen per woman over total number of oocytes retrieved per woman.

The number of embryos transferred per-cycle was the same ($P=0.18$, one-way ANOVA) among the comparison groups (EN, 1.5 ± 0.66 ; TF, 1.5 ± 0.60 ; UE, 1.5 ± 0.63 , Table 3.2). The number of embryos cryopreserved per-cycle was also the same ($P=0.86$, one-way ANOVA) among the comparison groups (EN, 1.6 ± 2.3 ; TF, 1.5 ± 2.3 ; UE, 1.6 ± 2.3 , Table 3.2).

The number of embryos utilised were similar ($P=0.53$, one-way ANOVA) among the comparison groups (EN, 2.8 ± 2.1 ; TF, 2.9 ± 2.1 ; UE, 3.0 ± 2.2 , Table 3.2). The utilisation rate was also similar ($P=0.91$, one-way ANOVA) among the comparison groups (EN, 36 ± 21 ; TF, 35 ± 22 ; UE, 37 ± 2.3 , Table 3.2).

3.5.8 Pregnancy outcomes were not affected by endometriosis

The clinical pregnancy rate in women with endometriosis (39.7%) was similar ($P=0.254$, Chi-Square) when compared to those with no endometriosis (TF, 43.6; UE, 38.7%).

3.6 Discussion

From this study, the number of oocytes retrieved per mature follicle was found to be lower in women with endometriosis compared to controls. This finding is in line with what was presented in Chapter 2, namely that fewer oocytes were retrieved in women with endometriosis undergoing IVF when compared to control. The disparity between the number of collected oocytes and the number of mature follicles may be due to the failure of oocytes to mature within the developed follicles, and were therefore not available for retrieval. The oocyte maturation process and follicular growth do not occur simultaneously [181] and the final maturation for the oocyte usually takes place within 36 following LH surge (natural cycle) or after trigger injection (artificial cycle).

Oocyte maturation, whereby fully-grown oocytes become fertilizable eggs, occurs in the follicular fluid (FF) of antral follicles just prior to ovulation [182]. The lower number of oocytes retrieved per follicle suggests the possible detrimental effect of the endometriotic disease for oocyte development. There are several factors affecting the physiological oocyte maturation process [183], intrinsically by high level of oxidative stress [59, 184], or extrinsically by cigarette smoking, ultraviolet (UV) radiation or toxic drugs [185]. Aberrant follicular microenvironment in women with endometriosis provides a direct effect on the developing oocyte and one biological plausible hypothesis is free radical damage via oxidative stress, which may then mediate damage to the ovary and the developing oocytes [33]. This hypothesis will be examined later in the thesis where the impact of the follicular fluid on the oocyte maturation process will be investigated (Chapter 4).

The fertilisation rate was lower in women with endometriosis but was not statistically significant across the groups, which is in contrast with the results from previous meta-analyses [68, 86]. However, the fertilised oocytes in endometriosis group were statistically more likely to arrest and suffer from failure to cleave from fertilisation to the 8 cells stage. The higher number of early embryo arrest at D1 suggests the presence of poor oocyte quality. This is because following the oocyte and sperm interaction during fertilisation, a dynamic process is triggered initiating maternal to zygotic transition. The

success of this transition very much depends on the oocyte quality, rather than the sperm [186].

Despite poorer fertilisation rate and a higher rate of D1 arrest, embryos of women from endometriosis group that have successfully cleaved was shown in this study to have similar developmental potential. At D3 assessment, majority of the embryos achieved comparable quality compared to the control groups. The results were unaffected when the graded embryos were re-categorised into 2 groups of good and poor quality embryo. This finding is not unexpected, as the poorer quality oocytes, which failed to develop beyond the 8-cell stage, were already excluded. The latter may represent a natural biological triaging quality control process that prevents poor quality oocytes from developing further, and it therefore follows that the pregnancy rate is not dissimilar when compared to the control groups.

Embryos that were not transferred at day 3 had an extended culture up to day 5, when they were further assessed. At this stage, suitable blastocysts were transferred or cryopreserved for future use. In this study the blastulation rate and FEHB rate were similar in the endometriosis group compared to the control groups, which suggests that the increased aneuploidy rate reported by previous studies may not be apparent at embryological assessment [61, 187]. This current study also found similar utilisation rate of embryo between the groups indicated by equal number of embryos transferred and frozen per collected oocytes. This is counter-intuitive as one expects a higher rate of arrest prior to the 8-cell stage in the endometriosis group to lead to a lower number of embryos available for freezing. This study may not be sufficiently large in sample size to delineate the difference or that the difference may become more apparent in older women or in women with low ovarian reserve, which are two groups of women not specifically examined in this study.

One of the weaknesses of this study was that not all women allocated to the endometriosis group had laparoscopically diagnosed endometriosis. Out of 89 women in the study group, 35 had laparoscopically confirmed endometriosis with surgical treatment, and 29 had laparoscopically diagnosed disease although surgical treatment was not documented and some had ultrasound

confirmation of endometriosis. The latter group of women with what was presumed to be small endometrioma did not have a confirmatory laparoscopy or treatment due to patient choice, as there is still uncertainty to the clinical benefit of operating on these endometrioma. On the other hand, women included in the control group were allocated to the group based on the presence of tubal factors and had laparoscopic confirmation of the absence of endometriosis. Women in this group had no signs, symptoms or history of endometriosis and had normal scans. There is of course a possibility these women may have endometriosis although we envisage this number will be small given that the majority had laparoscopically diagnosed tubal disease without the diagnosis of endometriosis.

Embryology data gathered from this study was retrospective and was based on time specific, morphological assessment, which is known to have high inter- and intra-observer variability. As an alternative, it is now possible to use a non-invasive, time-lapse imaging of the embryos within an undisturbed condition. By using this technology, critical and subtle differences in cell division can be recorded and analysed in greater detail. This will provide more reliable data compared to the former. One of the follow on research study will be to examine the records from time-lapse incubators.

This study is suffers from the following confounders: 1) restriction associated with a retrospective study, 2) subjective embryo assessment process despite using the newest assessment criteria, 3) incomplete assessment of the oocyte due to the intact cumulus cell layers required in IVF, and 4) unavailability of subgroups for the differential assessment of severity of disease.

3.7 Conclusions

This study demonstrated that women with endometriosis have a lower number of oocyte collected per mature follicle. Although fertilisation rate was unaffected, the presence of endometriosis is associated with a higher rate of early embryo arrest, which implicates poor oocyte quality. This study demonstrated that endometriosis has no effect on the embryo quality beyond the 8-cell-stage of embryo development and on the overall clinical pregnancy rate.

Chapter 4: The impact of the follicular environment on maturation and oocyte quality

In the preceding chapters, endometriosis was consistently shown to result in a lower oocyte yield for women undergoing assisted conception, and in severe cases, a lower clinical pregnancy rate. This chapter aims to explore, investigate and discuss the notion that the latter clinical outcomes are secondary to 'oocyte factors', namely that the lower oocyte yield in women with endometriosis is a result of defective oocyte maturation process precipitated by unsuitable intra-follicular conditions secondary to the disease. The chapter will examine factors affecting oocyte maturation, concentrating on the influence of follicular fluid, the main constitute of the oocyte microenvironment, on oocyte maturation.

4.1 Follicular fluid as physiological environment

Follicular fluid has an important role in both follicle and oocyte development, from antral to pre-ovulatory follicles (Figure 4-1). This fluid fills the follicular antrum and surrounds the ovum in an ovarian follicle. In human, a single pre-ovulatory follicle can contain up to 15 ml of fluid and provides a conducive microenvironment for the developing follicle and it's oocyte.

Follicular fluid is produced from secreted plasma of blood vessels and capillaries, which are usually found within the thecal layer [183]. It begins with a simple capillary network surrounding the follicle at the antral stage, and then turns to a more complicated network as the follicle grows. Follicular fluid production is also facilitated by hydrostatic forces, generated by contraction of cilia on individual cells. Moreover, osmotic pressure created by the differences in the solute concentrations between the two areas facilitates the mechanism [182]. Lastly, secretions from the granulosa and theca cells contribute to the production of follicular fluid [182].

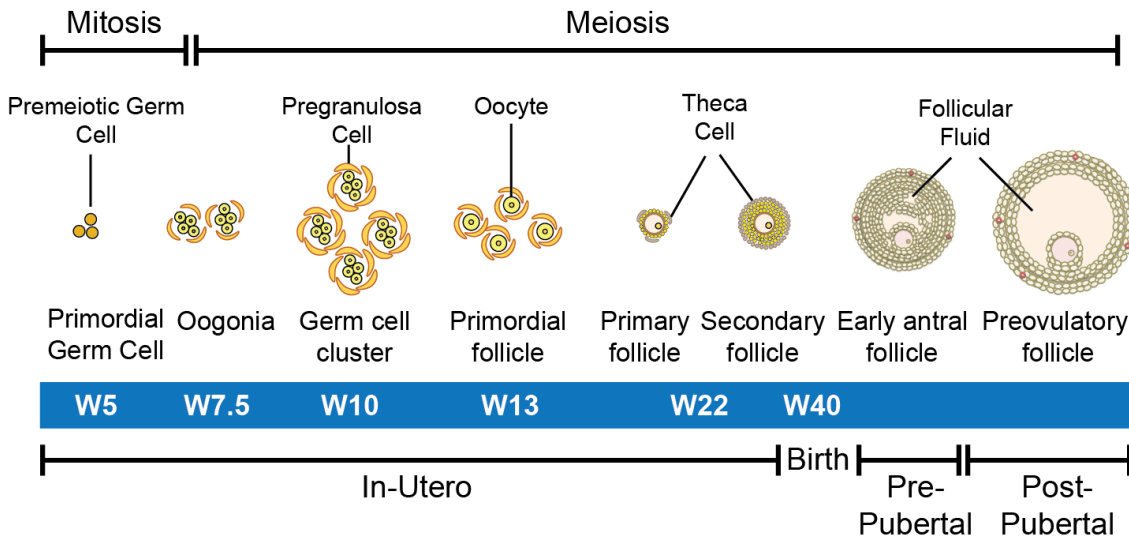


Figure 4-1 Life journey of a follicle

At the 5th week following a conception, the primordial germ cells (PGC) migrated into the genital ridge and formed a colony. Between 7-8 weeks, surviving PGC will proliferate (Mitosis) and undergo sex determination. Then at 10 weeks, these cells begin Meiosis but arrest at Prophase I stage until meiosis resumes after puberty. Individual primordial oocytes are recruited to grow and produce mature oocytes through the process of folliculogenesis. The oocytes grow as the theca and granulosa cell layers expand. When the follicle reaches the antral stage, it forms a fluid filled cavity resulting in differentiation of granulosa cell layers into mural or cumulus cells. After reaching puberty, follicles become sensitive to gonadotropin. Following the luteinising hormone LH surge, the oocytes resume meiosis and becoming arrested at metaphase II (MII), while cumulus cells expand and the follicular wall tension changes, ready for ovulation. W= week, indicating number of week in utero when the process take place.

The formation of the follicular cavity is unique. It starts with development of multiple foci of fluid within the granulosa layers. These foci will expand and coalesce, forming a larger, centrally located antrum. The lumen is also formed by cell death or/and apoptosis. To contain and accommodate more fluid in a growing follicle, the surrounding theca and granulosa cells will become oedematous and swell to form a tight seal. Increasing fluid volume within the antral cavity results in differentiation of granulosa cell layers into mural or cumulus cells (Figure 4-2).

Physiologically, FF is important for steroidogenesis, follicular growth, oocyte maturation, the ovulation process, and transportation into the oviduct. This viscous, straw-coloured fluid mainly contains sodium and potassium, which plays an important role for osmotic balance. Apart from that, hormones, interleukins, protein, Glutathione (GSH), reactive oxygen species (ROS), sugar and anti-apoptotic factors are also found in follicular fluid [188]. Anti-oxidant such as GSH is required to ensure the redox balance within the follicular fluid and is fundamental for oocyte growth. However imbalance of oxidant and antioxidant can be pathological and is known to affect meiosis negatively, for instance by causing DNA damage [189-191].

A comprehensive review by Lord et al in 2013 [192] shows that oxidative stress is responsible as the initiator of cascade of events in aged oocytes. The review highlighted the capability of oxidative stress to suppress the levels of critical cell cycle factors such as maturation-promoting factor. This review examined the mechanism of the oxidative stress in the aging oocyte (post ovulated) resulting in the deterioration of oocyte quality. Many effects on the oocyte were reported, including zona hardening and partial exocytosis of cortical granules, elongation of the meiosis II spindle, loss of chromosome integrity, misaligned chromosomes and premature centromere separation, as well as epigenetic changes. The exact mechanism on how the oxidative stress causes such far-reaching effects is still being elucidated. It is however suggested that oxidative stress has the capacity to directly damage multiple intracellular compartments of the oocyte such as lipids, proteins and DNA [192].

Inferring from published papers on oocyte aging, there is evidence suggesting accumulation of ROS concentration in oocytes over time and rendered these oocytes in a state of oxidative stress, which then affect multiple aspects of oocyte biochemistry and functionality including mitochondrial function [192-194].

Wakayama et al in 2004 [195] reported that there is evidence that microtubule organization is affected by ROS in post mature oocytes. The commonly observed abnormalities were misaligned chromosomes, or dispersed, elongated or completely disrupted spindles. However there is no clear mechanism how ROS intersects with the process and this will form an area of further research.

Hormones in FF such as FSH, hCG and LH are essential to promote oocyte maturation by interaction between somatic and endocrine cells [183]. Gonadotropins within FF also play an important role in the secretion of several substances by granulosa cells. Other hormones such as growth hormone, prolactin, oestradiol and progesterone are also found in normal follicular fluid. When compared to women who were unstimulated, FF from those who undergone stimulated cycle has significantly lower level of oestradiol, luteinising hormone and androstendione [196]. These hormones are not routinely measured except for research purposes to find suitable biomarkers for oocyte quality.

Since both maturation and ovulation are inflammatory process, pro inflammatory markers such as interleukins and cytokines are commonly found in FF [197]. The majority of proteins in FF were discovered from proteomic analysis done to find markers for oocyte quality assessment [188]. Additionally reactive oxygen species, which have the ability to react with and damage proteins, lipids and nucleic acid, can be found in the FF. It is known that supra physiological ROS has a detrimental effect on oocytes.

Follicular vascularity, intra-follicular oxygen content and mitochondrial activity are physiological mechanisms, which are involved in maintaining the optimal ROS level. Additionally, enzymatic (superoxide dismutase, catalase, glutathione peroxidase glutathione-S-transferase) and non-enzymatic (N-acetyl-cysteine) antioxidants can neutralise the excess ROS. The failure of anti-oxidant defence mechanisms to counteract oxidative stress allows free radicals to alter the

cellular redox state. Studies (human and animal) showed that exposure of human oocytes to high levels of ROS affects the fertilisation and pregnancy outcomes [198], more specifically affecting the meiotic progression [199], chromosomal division [184], spindle integrity [197] and DNA structure [200].

Sugar is an important constituent in follicular fluid. During follicular growth, different glucose metabolisms take place at different levels. The major pathway for glucose metabolism in a follicle is anaerobic glycolysis, consuming glucose and producing lactate as the growing follicles undergo luteinisation. The oocyte however, has poor glycolytic activity and depends on the granulosa to supply the products of glycolysis (pyruvate) for its energy needs [201].

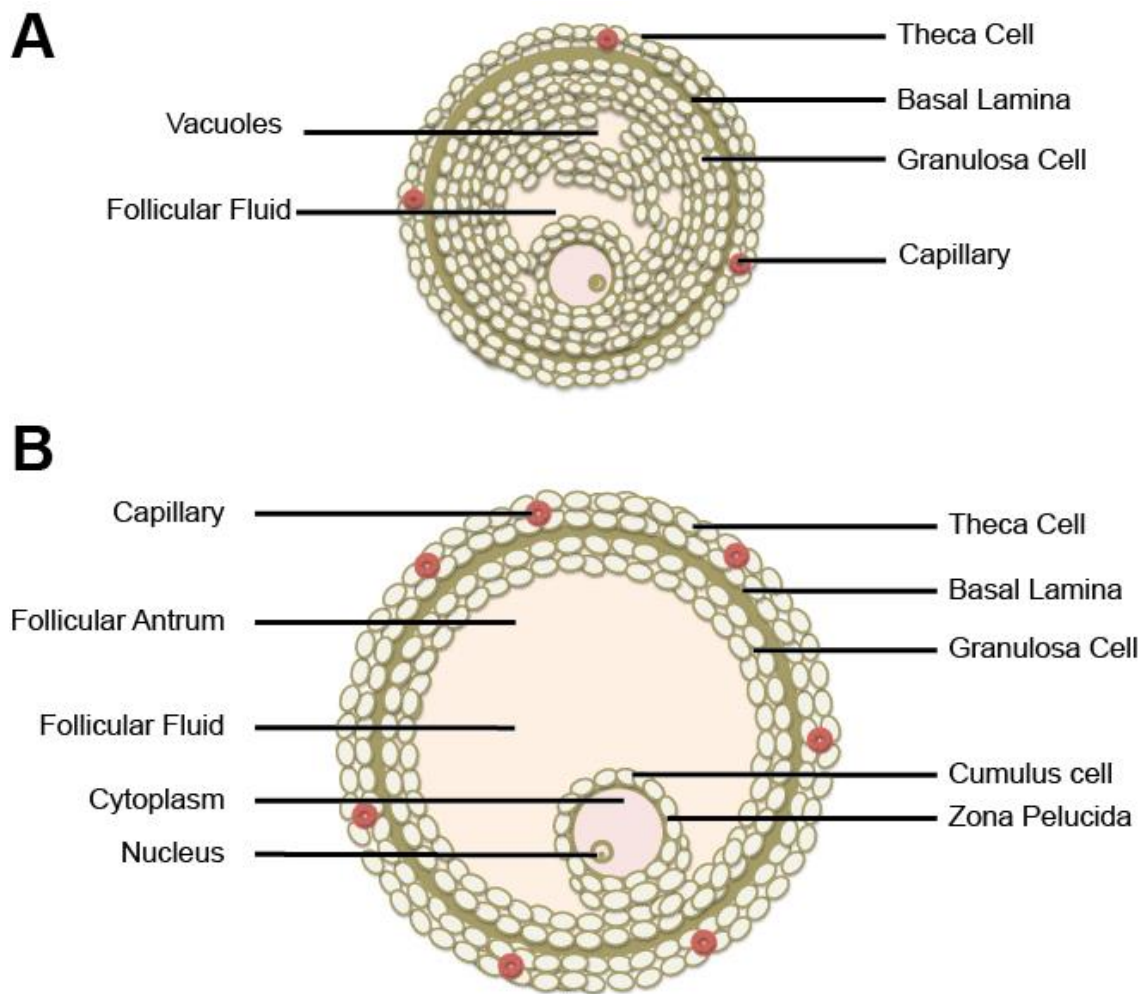


Figure 4-2 Follicular fluid formation

A: Antral follicle: No distinct follicular cavity is seen. Multiples vacuoles exist within the layers of granulosa epithelium, which later coalesce to form the follicular antrum. The cavity is created by cell death/apoptosis to provide more space for the fluid. B: Fully-grown follicle: More complex capillary network develops as the follicle grows. Surrounding theca cells swell and form a tight layer to contain the FF. A single follicle may contain up to 15 ml of fluid.

4.2 Oocyte development within the follicular environment

Oocyte development occurs within the growing follicle, and later in the presence of follicular fluid. During this process, primordial germ cells mature and specialises into a fully functional eggs, which are then ovulated from follicles in preparation for fertilization. It begins when primordial germ cells undergo oogenesis and become primary oocytes around the third trimester [202]. These primary oocytes then undergo ootidogenesis to form ootids by starting meiosis. This meiotic division starts before birth but is arrested at prophase I until puberty, at which point up to 20 primary follicles containing these ootids begin to develop and grow every ovarian cycle (Figure 4-3). At this point, the oocytes will be in constant contact with follicular fluid and it is during this time the meiotic spindle is formed and chromosome segregation takes place. This intimate relationship of the oocyte with the follicular fluid continues until it is ovulated spontaneously (natural cycle) or surgically retrieved (artificial reproductive technique). Usually in unstimulated, normal menstrual cycling women will have one dominant follicle that outgrow the primary follicles cohort and ovulate [203]. However women in an artificially stimulated IVF cycle will have more follicles that grow, overriding the natural selection and therefore generate more oocytes for fertility treatment. Meiosis I is completed when the first polar body is extruded, which is the stage when oocytes are collected during IVF treatment cycle (Figure 4-3). Regardless of the follicular growth process, oocyte yield is known to inversely correlated to the nuclear maturation process [61].

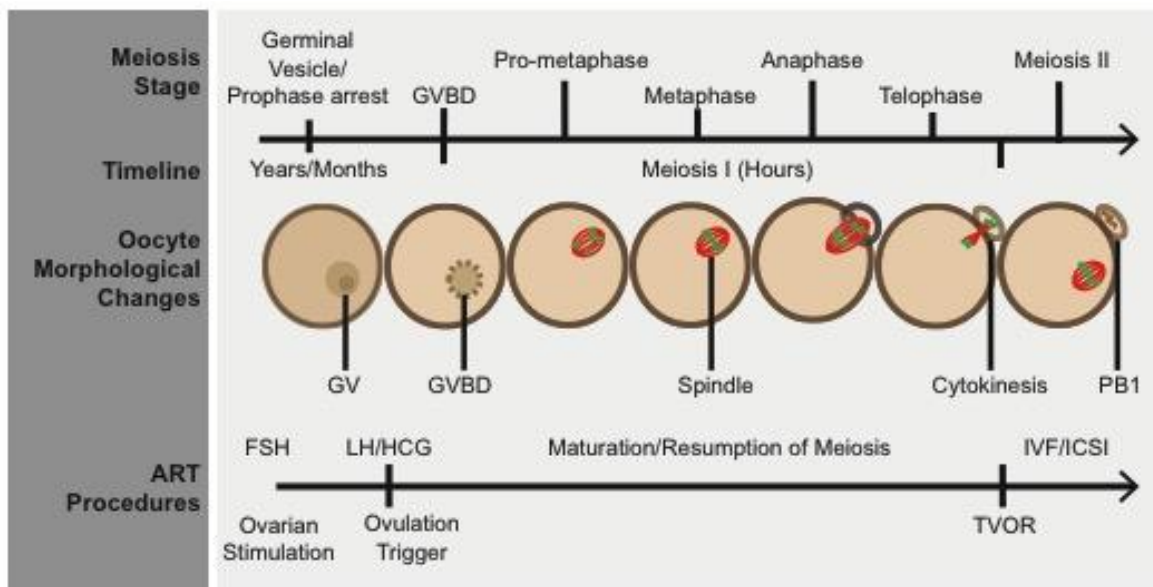


Figure 4-3 Summary of progression of oocyte maturation following prophase arrest

Oocytes arrest in prophase of meiosis I for long periods of time. Resumption of meiosis is morphologically evident by the breaking down of the germinal vesicle (GVBD). The oocyte then assembles a bipolar spindle (MI spindle) and segregates bivalent chromosomes. The cytokinesis is asymmetrical, resulting in the extrusion of a small polar body (Polar body 1) which marks the end of meiosis I. Oocytes then assemble a second, smaller spindle (MII spindle) and arrest, awaiting fertilisation. (GV, Germinal Vesicle; GVBD, Germinal Vesicle Breakdown; PB, Polar body).

4.3 Follicular fluid of women with endometriosis

Whilst the majority of publications have examined serum, peritoneal fluid and endometrium of women with endometriosis, limited studies have analysed FF and its effect on the oocyte maturation process. Available studies on FF however, were using samples collected during a stimulated cycle of IVF treatment. This may not be representative, and cannot be generalised to unstimulated women. The hypothesis of this study was built on two meta-analysis (Chapter 2) that included participants who have endometriosis and ovarian stimulation, and compared with a control group who had similar treatment.

In FF, inflammatory markers IL-6 [55, 197, 204, 205], IL9 [197] and TNF α [197, 205] were found higher concentrations in endometriosis compared to women with no endometriosis. This is self-explanatory as endometriosis is an inflammatory disease. However, there are reports [197, 204] that have shown IL-1 β levels were not different in women with and without endometriosis.

Free radical species, which include reactive oxygen species (ROS) and reactive nitrogen species (RNS) require electrons from nucleic acid, lipids, proteins, carbohydrates or any nearby molecules to achieve stability. This will initiate a cascading reaction, resulting in damage to vulnerable tissue, including oocytes. In studies on endometriosis, ROS capacity is generally quantified using two techniques, either directly by measuring the ROS or indirectly by measuring anti-oxidant concentration. Lower antioxidant may reflect high ROS content, as the antioxidant may have been utilised to counterbalance the circulating ROS and vice versa. But it is also possible that antioxidant concentration is lower due to lower production of antioxidants. In a comparative analysis between endometriosis (n= 23) and controls (n=68), antioxidant defences (Vitamin C and superoxide dismutase) were found to be lower in FF and serum of women with endometriosis, [206]. This finding is in keeping with a more recent publication [197], which found lower glutathione, suggesting a lower anti-oxidant capacity in subfertile women with endometriosis.

A subgroup of women with endometrioma (condition where endometriosis found within the ovary, Chapter 1), have now become the interest of many IVF

clinicians. This is surrounding the concern that the molecular milieu inside the cyst could be a potential source of toxicity to an adjacent developing follicle and oocyte [33]. In contrast to the theory, Opoein et al in 2013 who have examined cytokine level in FF of women with endometriosis, reported similar levels of IL-1B, IL-6, IL-8, IL-10, IL-12 and TNF- α between FF of both ovaries from the same women with endometriosis, where only one ovary has an endometrioma [207].

4.4 Follicular fluid and the impact on oocyte quality

In humans, data with regards to the relationship between FF and quality of oocyte is limited. However, it has recently emerged to be an area of interest since it has become increasingly popular oocyte assessment tool for embryologists. FF assessment can compliment or provide an alternative to the existing morphological assessment for oocyte and embryo quality [188]. Hormones, TGF- β , interleukins and reactive oxygen species, proteins and peptides are among the markers that have been measured. Proteomic analysis of FF collected from follicles containing oocytes that fail or were successfully fertilised showed differentially expressed proteins [208]. Additionally, ROS at supra-physiological level is also associated with poor oocyte and embryo quality, as demonstrated in a study (n=803) correlating ROS level in FF samples during IVF cycle to morphological embryo assessment [209].

Due to limited availability of human oocytes, the impact of FF to oocyte quality has been evaluated using animal studies. Da Broi and colleagues reported abnormal meiotic spindle structure of bovine oocytes after exposure to FF of women with endometriosis [170]. The same group recently reported that the effect was reversed when an anti-oxidant (N-acetyl cysteine) was added into the culture media containing FF of women with endometriosis [210]. In a porcine oocyte model study, reduction of DNA fragmentation was observed following supplementation of antioxidant in maturation media [200], suggesting reactivity of ROS to the DNA, resulting in damage.

To this end, it is established that a normal FF is important to provide a suitable condition for oocyte maturation. Altered FF content in endometriosis maybe responsible for maturation defects, however it is not known whether it is caused

by ROS contributing to DNA damage, which then disrupts the maturation process.

4.5 Hypothesis

Follicular fluid of women with endometriosis has a detrimental effect on oocyte maturation and spindle morphology and causes more DNA damage.

4.6 Objective

This chapter aims to determine the effect of follicular fluid from women with endometriosis on the oocyte maturation rate. This chapter will address the cause of poor oocyte yield in women with endometriosis.

4.7 Method and Material

This subsection is divided into clinical and experimental parts. The clinical part will describe how the samples were obtained and stored (4.6.1 until 4.6.8). The experimental part will describe how the laboratory works were undertaken throughout the experiments (4.6.9 until 4.6.20). The experimental workflow described in this section is summarised in Figure 4-4.

4.7.1 Human and clinical ethics

This study received institutional and regional review board approval by Southampton University Hospital NHS Foundation Trust and Hampshire B ethical committee respectively. All procedures conducted in this study requiring human involvement are subjected to the ethical committee guidelines (Study on Human Oocyte, RHMO&G0213, REC13/SC/0551).

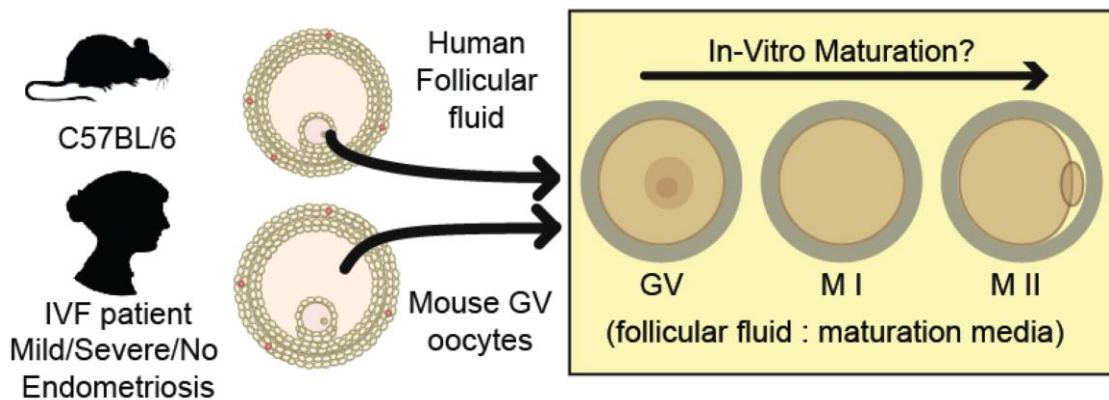


Figure 4-4 Experimental workflow

Experimental workflow: GV oocytes collected from C57BL/6 mice were incubated in media with (Severe Endometriosis, SE-FF; Mild Endometriosis, ME-FF; Male factor, MF-FF) or without (No-FF) follicular fluid. After overnight (14-16 hours) incubation, oocytes were assessed for completion of maturation.

4.7.2 Inclusion and exclusion criteria

Women are classified as being in the endometriosis group if they are subfertile for at least 1 year and have laparoscopic diagnosis of endometriosis. Where possible, the disease stage is documented and recorded according to the ASRM classification [211]. Women are classified as being in the control group if they are subfertile for at least 1 year and have laparoscopy with no documented endometriosis. This group includes those with a diagnosis of male factor subfertility, unexplained, tubal factor or in a same sex relationship.

For both groups, women at the age of 40 years old and above, with BMI more than 30 or a current smoker were excluded. With the exception of endometrioma, we excluded women with ovarian cysts seen at the baseline scan at the beginning of the stimulation cycle (see 4.7.5).

4.7.3 Patient recruitment

This study was performed at the Princess Anne Hospital Southampton (Appendix C). Patients were recruited from Complete Fertility Centre (level G). Suitable candidates for the study were screened and invited to participate. Women who were interested to participate were given an information sheet outlining the study (Appendix D). Patients were guided through the participant information sheet by the researcher who explained the details of the study. Those who agree to participate were required to sign the consent form (Appendix E) in 3 copies (a copy was given to the patient, a copy was filed in the notes and a copy was kept by the research team). The consent was given a study ID number (OVUM001, OVUM002, OVUM003....) that was subsequently used for research data. Patient identifiers were only kept on these consent forms, stored separately from the research data. Participants were asked several questions according to the clinical record form for record purposes. Participants' course of treatment was not affected by this recruitment procedure and they commenced their treatment as scheduled.

4.7.4 Treatment regime and protocol

Treatment regime, protocol and stimulation drug dosing is individualised according to the clinicians discretion. The stimulation regime was usually limited to short antagonist or long agonist protocol, with some deviation from the protocol according to clinical circumstances.

4.7.5 Controlled ovarian stimulation and ovulation trigger

As for any other patients, women who agreed to participate were assessed at the beginning of their cycle before starting the stimulation protocol. Controlled ovarian stimulation was started as per the protocol (Appendix G) providing the assessment (between Day 2 to Day 4 of menstrual cycle) was normal. On the 5th day of stimulation, participants were rescanned to monitor ovarian response following the administration of the stimulation drugs. From then onwards, every two days, participants were required to come for further surveillance scan. If needed, the dose of stimulation drug was adjusted or changed according to the multidisciplinary teams decision in a meeting held daily. The scan was repeated until participants have met the criteria for oocyte retrieval (3 or more follicles, measuring 17mm or larger in size). They were then injected with trigger injections (BHCG or buserelin, Appendix H) to start the ovulation process. Trans-vaginal oocytes retrieval (TVOR) was done 36 hours after the injection (Appendix I).

4.7.6 Collection and processing of follicular fluid sample

Follicular fluid samples from participants were collected during the TVOR procedure. FF was collected in individual sterile tubes, pre-heated to 37°C that contained no culture medium. The entire content of the first follicle with mean diameter of at least >15mm from the first punctured ovary was aspirated. Only follicular fluid free of blood contamination upon visual inspection and having an oocyte were used. The fluid samples were centrifuged at 1200g for 20 min to separate cell remnants, and the supernatant was snap frozen at minus 80°C, in at minimum of 3 aliquots of at least 200µl in each Cryovial, for later use.

4.7.7 Sample identification and data recording

Linked anonymisation was used to protect confidentiality. Samples were coded OVUM001, OVUM002, OVUM003... etc. in order of their collection, which is similar to their ID study number. A record of the sample code and relating patient recruitment number was updated and stored by the researchers in a database. The patient information was subsequently correlated to the samples after the experiment was finished. Basic demographics were collected from the patient notes and recorded on the proforma. This contained anonymised data such as the patient's medical history, age, parity and date of last menstrual period. Any notable findings at the surgery were recorded (Appendix F).

4.7.8 Sample Transportation

Follicular fluid was transported on solid carbon dioxide (CO₂, dry ice) inside a polystyrene box from Princess Anne Hospital to the Centre of Biological Sciences at the University of Southampton. The average journey time was approximately 15 minutes. On arrival, the FF was stored immediately in -80°C freezers or thawed to room temperature for use on the same day.

4.7.9 Mouse handling and dissection

4.7.9.1 Animal Ethics

All experiments were carried out at Centre of Biological Sciences, University of Southampton. Procedures performed adhered to Home Office Animal Procedure Committee rules and regulation, which is governed by the Animals (Scientific Procedures) Act 1986.

4.7.9.2 Breeding

Experimental procedures were performed using young (4-6 week) female mice produced from a C57BL/6 breeding pair (Charles River, UK). These mice were inbred by the University of Southampton animal lab facility, based at Southampton General Hospital.

4.7.9.3 Hormonal priming

Mice were hormonally primed to increase the number of oocytes collected per mouse, so as to reduce the number of animals sacrificed for experiments. Mice

were primed with Pregnant Mare Serum Gonadotrophin (PMSG, Centaur Services, UK), 48-hours before use. 0.2ml of PMSG solution (10IU) was injected into peritoneal cavity of the mice. Immediately after injection and 24 hours later, mice were examined for sign of distress and discomfort.

4.7.9.4 Dissection

Mice were sacrificed by cervical dislocation. Before cutting open the abdomen with tissue scissors, abdominal skin was washed with 75% ethanol. The ovaries were identified and removed using small dissecting scissors to cut away the oviduct, connective tissue and surrounding fat. Ovaries were cleaned from blood with lint free tissue (KimTech, Kimberly Clarke Professional, USA) before transfer into a Petri dish containing pre-warmed M2 media supplemented with 1mM milrinone (Sigma-Aldrich, UK).

4.7.10 Oocyte collection and handling

4.7.10.1 Handling pipette

For the whole experiment, oocytes were handled using a mouth pipette. It was made up of a mouthpiece, a finely drawn glass pipette (150mm, Fisherbrand, UK) and a plastic tube (~30cm), which connects them together. The glass pipettes were made by heating the tapered end (~2cm from the shoulder), before pulling the 2 ends apart as soon as the heat from the flame of ethanol burner has sufficiently softened it. The glass was then broken approximately 10cm from the shoulder and stored. Prior to use, the pulled pipettes were broken again approximately 2-3cm from the shoulder to the desired diameter. For curved pipettes, similar steps were taken except the pipettes were bend to approximately 90 degree, prior to pulling the two ends apart.

4.7.10.2 Oocyte collection

Oocyte collection was performed in dark room to minimise the exposure of oocytes to light. It was done on a stereomicroscope with x80 variable zoom (SZ40, Olympus, Japan) and a transmitted illumination attachment (SZ2-ILA, Olympus, Japan). A heated stage (MATS-U4020WF, Tokai Hit, Japan) was used to maintain a 37°C environment for oocytes during the procedure.

Under stereomicroscope view, the ovaries were repeatedly punctured using 30 gauges, 1/2-inch hypodermic needle (Becton-Dickinson, UK) to rupture follicles and release the cumulus-oocyte-complexes (COCs) into the media. The cumulus layers were stripped off by gently sucking the COC in and out using the appropriately sized hand-pulled glass pipette. The denuded oocytes were pooled into a holding dish. The entire procedure was completed within 20 minutes to reduce stress caused to the oocytes.

4.7.11 Oocyte culture

4.7.11.1 M2 Media

M2 media was made fresh from individual component in the lab every 14 days. For this thesis, M2 media was mainly used as a handling media during experiments particularly for oocyte collection. Although not preferable for long-term culture, M2 media can provide an alternative culture media for experiments where there was no option of providing a 5% CO₂ atmosphere.

4.7.11.2 MEM Media

Minimum Essential Media (MEM) was made fresh from powdered sachets (Life Technologies, UK), and supplemented with 20% fetal bovine serum. Bacteriostatic and bactericidal solution, Pen/strep, was added to protect against microbial infection. MEM media require equilibration in 5% CO₂ to attain physiological pH and so were incubating for several hours prior to use. Mineral oil used to overlay MEM during experiments was also pre-incubated in 5% CO₂. Stock media was made in the lab every 14 days.

4.7.11.3 Preparation and addition of follicular fluid to the media

When FFs from identified samples (Severe Endometriosis; SE-FF, Mild Endometriosis, ME-FF, Male Factor, MF-FF) were fully thawed, they were added into prepared media (M2 or MEM). All experiments were conducted alongside a negative control group with culture media in the absence of FF (Media only; No-FF). All groups were prepared in 150µl droplets in a 30mm dish (Falcon, UK) or in a separate well of a 96-well plate (Costar 3599, UK). To prevent evaporation, media were covered with mineral oil (30 µl for 96-well plate).

Drug	Stock Concentration	Working Concentration	Manufacture
Milrinone	10 mM	1.0 μ M	Sigma-Aldrich, UK
Reversine	0.1 mM	0.1 μ M	Sigma-Aldrich, UK
Resveratrol	50 mM	2.0 μ M	Sigma-Aldrich, UK
ATMi	4 mM	40 μ M	Calbiochem via Merck, UK
2,7, DCDHFDA	10 mM	20 μ M	Sigma-Aldrich, UK

Table 4-1 Drug concentration and preparation

The above table lists the drugs used in various experiments including their stock and working concentration. Milrinone was suspended in DMSO and Ethanol at 1:1 ratio, the stock was divided into 20 μ l aliquots in 0.5ml Eppendorfs and stored at -4°C. All other drugs were suspended in DMSO, the stock was divided into 1 μ l aliquots in 0.5ml Eppendorfs and stored at -20°C. 2,7 DCDHFDA was stored protected from light. Warmed media was added into the drug, prior adding to FF. (ATMi = Ataxia Telangiectasia Mutated Inhibitor; 2,7 DCDHFDA = 2', 7'-dichlorodihydrofluro-acetate)

4.7.11.4 Preparation and addition of drugs to the media

Stock drug were prepared prior to use for all the experiments within this thesis. Except Milrinone, all drugs were suspended with Dimethyl Sulfoxide, DMSO (Sigma Aldrich, UK), usually at a concentration 1000 times that of the desired working concentration. The stock was aliquoted at 1 μ l into 0.5ml Eppendorfs and stored at -20°C. For Milrinone, it was suspended with DMSO and Ethanol at 1:1 ratio, the stock was divided into 20 μ l aliquots in 0.5ml Eppendorfs and stored at -4°C.

All the drugs were made to the desired working concentration by adding warmed media to the aliquoted stock drug, before adding to FF. Drugs and their concentration used in this experiments were: Milrinone (Stock, 10mM; Working, 1 μ M), Resveratrol, (Stock, 50mM; Working, 2 μ M), Reversine (Stock, 0.1mM; Working, 0.1 μ M), ATMi (Stock, 4mM; Working, 40 μ M), 2,7,DHDCF-DA (Stock, 10mM; Working, 20 μ M, Table 4-1).

4.7.11.5 Milrinone and control of GV arrest

To maintain the oocytes in prophase I (GV) arrested stage, oocytes were kept in media containing Milrinone (1 μ M) from the time of collection. In order to commence maturation (GVBD), Milrinone was removed by moving oocytes through 5 droplets of Milrinone-free media.

4.7.11.6 Oocyte Incubation

Oocytes were randomly selected from the holding dish and divided equally into several groups depending on the experiment. Oocytes were incubated in the incubator at 37°C, 95% humidity and 5% CO₂ concentration in the culture system covered with mineral oil. When oocytes were cultured in M2 media, the incubation was performed on hot block with the temperature set to 37°C under room air, shielded from light. Generally oocytes were incubated for 14-16 hours for overnight maturation experiments, however timing for incubation varied for different experiments depending on the outcome measure.

In experiments where oocytes need to be maintained at GV stage, they were held in culture media containing milrinone with or without FF. In some

experiments, GV oocytes were pretreated with drug or DNA damaging agent (e.g. UVB) according to protocol, prior to oocyte incubation.

4.7.12 Oocyte fixation and permeabilisation

Oocytes were fixed to preserve them for immunofluorescence study. They were then permeabilised to allow antibody access to the inside of the cells during immunostaining. For fixation, the oocytes were briefly washed through 3 drops of PHEM-PVP (Pipes 60mM, Hepes 35mM, EGTA 25mM, MgSO₄·7 H₂O 4mM, 1% PVP, Appendix VII), and incubated with fixation solution (PHEM, Paraformaldehyde (2%), Triton X-100 (0.05%, Appendix K) at room temperature in a humidified chamber for 30 minutes. Although the fixation solution contains detergent to help permeabilisation, these oocytes were incubated in permeabilisation buffer again (PBS-PVP, Triton X-100 (0.05%)) for 15 minutes but this time without formaldehyde. Oocytes were washed three times in PBS-PVP (Appendix K) and were further washed overnight at 4°C. Oocytes could then be stored in this solution for up to two weeks at 4°C.

4.7.13 Oocyte blocking

Before immunofluorescence process, fixed oocytes were incubated in blocking solution (Appendix K) overnight at 4°C or for 1 hour, at room temperature. This is to prevent the oocytes to have non-specific binding of the antibodies. Preferably the blocking solution used matches the species of secondary antibody and blocking solution was made fresh prior to use.

4.7.14 Immunofluorescence

For immunofluorescence, fixed oocytes were first labelled with primary antibodies (which recognise the target protein) and then detected by fluorescently tagged secondary antibodies (which recognise the primary antibody). If double staining required (e.g. CREST and Mad2), antibodies for 2 different proteins were labelled in tandem before detection of both secondary antibodies simultaneously. In this case, antibodies derived from different species were used to enable species-specific secondary antibodies to target a particular fluorescent dye to each protein of interest.

Antibody/Fluorescent	Solvent/ Solution	Species	Dilution	Laser Wavelength (nm)
Primary Antibody				
Anti- γ H2AX	Blocking	Rabbit	1:200	-
Anti-B Tubulin	Blocking	Mouse	1:400	-
Anti-Centromere Antigen	Blocking	Human	1:400	-
Anti-MAD2	Blocking	Rabbit	1:1000	-
Secondary Antibody				
Anti-Rabbit Alexafluor 633	Blocking	Goat	1:1000	633
Anti-Mouse Alexafluor 488	Blocking	Goat	1:1000	488
Anti-Human Alexafluor 555	Blocking	Goat	1:1000	561
Dyes/Fluorescent				
Hoechst 33288	PBS-PVP 1%	-	1:1000	405
2,7, DCDHFDA	DPBS-PVP 1%	-	20 mM	488

Table 4-2 Antibody/dye dilution and preparation

Table listing antibody and its solvent used in various experiments. Blocking solution comprises of 880 μ l PBS, 70 μ l GOAT serum, 50 μ l 1% Tween 20.

Oocytes were incubated in primary antibody (diluted in blocking solution) for 1 hour at room temperature. Oocytes were washed 3 times with washing solution (Appendix K) for 5 minutes each to remove any unbound primary antibody. If needed the procedure was repeated with another primary antibody. These oocytes were incubated with secondary antibody/ies (diluted in blocking solution) for another 1 hour at room temperature, protected from light.

Following incubation with primary and secondary antibodies, oocytes were washed with washing solution and finally, incubated with Hoechst (1:1000 in PBS-PVP) to label the chromosomes. The oocytes were then mounted in small drops of Citifluor on a correctly labelled glass slide. If possible not more than 5 oocytes were mounted in each drop. Any excessive Citifluor was removed. Mounted oocytes were covered with coverslips and carefully sealed with nail varnish along the edges. The slides were left to dry before being imaged.

4.7.15 Antibodies and chromosome dyes used in this thesis

Chromosomes were stained with Hoechst 33288 (Sigma Aldrich, UK). Sites of DNA damage were labelled with rabbit anti- γ H2AX (AMSBio, UK), microtubules were labelled using bovine anti-mouse α -tubulin (Abcam, UK), Mad2 proteins were labelled using rabbit anti-Xenopus Mad-2 (kind gift from R.H Chen, Taiwan) and kinetochores were labelled using human anti-human centromere antigen (ImmunoVision, USA).

The above primary antibodies were detected using appropriate secondary antibodies raised in goat and labelled with Alexa-633, Alexa-488, or Alexa-555 (Table 4-2). All secondary antibodies were purchased from Abcam, UK.

4.7.16 DNA damage measurement

In this experiment nuclear γ H2AX was used as a DNA damage marker, because γ H2AX foci represent Double Strand Breaks in a 1:1 manner. Hence, it is more representative of DNA damage, compared to other DNA damage markers. Only oocytes at GV stage were used to quantify the DNA damage signal because I found that chromosomes were ubiquitously labelled during meiosis regardless of DNA damage.

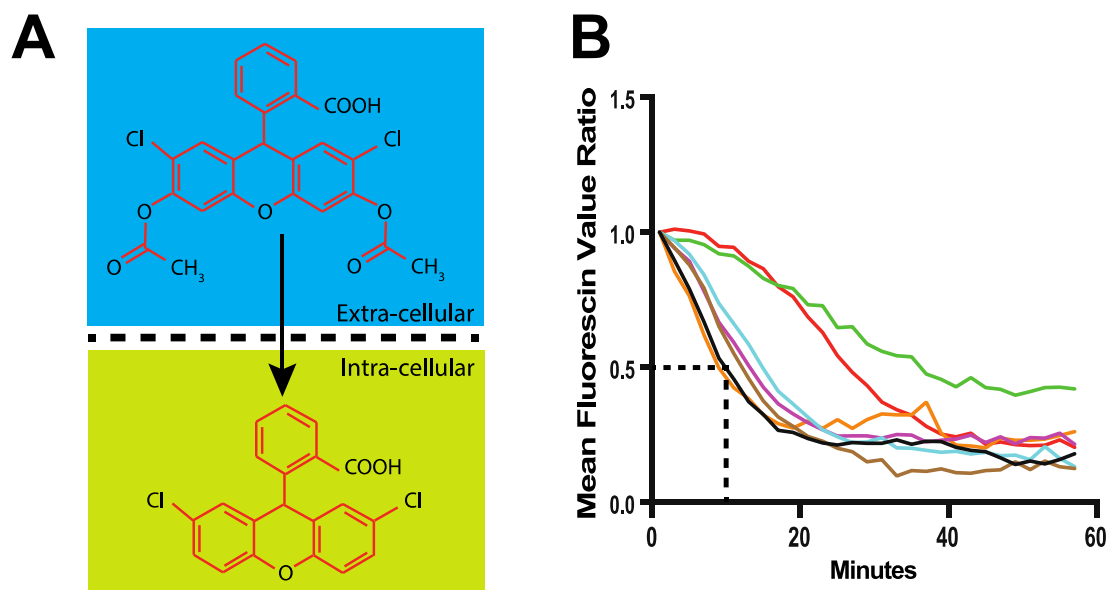


Figure 4-5 Intracellular ROS uptake after incubation with FF

A: 2',7'-Dichloro-dichloro-fluoroacetate was rendered impermeable following removal of acetate from esterase when entering the oocytes. B: Graph representing loss of fluorescence timing showed half-life is approximately 10 minutes.

4.7.17 Intracellular ROS measurement

Intracellular ROS was measured by using 2', 7'-dichlorodihydrofluorescein-diacetate, (2,7,DHDCF-DA, Sigma Aldrich, UK) which is cell-permeable in its esterised form. When 2,7,DHDCF-DA entered the cell, esterase activity

Oocytes were fixed and immunostained with DNA damage marker (γ H2AX) following one-hour incubation with media only (negative control) or media with FF (SE-FF or MF-FF). For positive control, GV oocytes were exposed to Ultra Violet [185] using UV trans-illuminator (MacroVue UV-20, Hoefer inc, US) for 20 seconds before being incubated in the media in the absence of FF. cleaves the acetate groups and turn into a lipid insoluble compound, which is trapped inside the cell (Figure 4-5A). Oxidation of the compound transforms it to the highly fluorescent form, thus fluorescence is proportional to the rate of oxidation in the cell.

Prior to use, 2,7,DHDCF-DA was prepared in two steps. First, the stock was prepared with anhydrated Dimethyl Sulfoxide. Secondly, it was diluted in Dulbecco's Phosphate Buffered Saline (DPBS) with 1% Polyvinylpyrrolidone (PVP) to get the working solution (40 μ M). DPBS was used because it contains pyruvate. Hydrogen Peroxide (H₂O₂) solution (Sigma Aldrich, UK) (0.5% w/v) was used for positive control and water as negative control. Collected GVs were carefully washed through 3 drops of DPBS-1%PVP to remove any esterases that might be in the media. Washed GVs were incubated in 40 μ M DHDCF-DA in DPBS-PVP for 20 minutes to load the fluorescein into the oocytes. After 20 minutes, oocytes were quickly incubated in a media with or without FF for 15 minutes, according to groups and depending on the experiments. Oocytes were immediately transferred to an imaging dish for image capture on the confocal microscope. Images were captured within 5 minutes, to avoid the rapid loss of signal that was observed over tens of minutes following removal of oocytes from the dye (Figure 4-5B). Identical settings appropriate for FITC (488nm laser) were used for each group. The pinhole was opened (4 A.U.) to capture more signals, as a narrow confocal plane was not needed.

4.7.18 Iron measurement

In this experiment, both iron assay kit (MAK025-1KT, Sigma Aldrich, UK) and in-lab iron detection kit were used. The iron assay kit provides a simple convenient means of measuring iron in a variety of biological samples including follicular fluid. In principle, released iron is reacted with a chromagen resulting in a colorimetric (593nm) product, proportional to the iron present. In this experiment total iron (Fe^{2+} and Fe^{3+}) was measured.

Iron standards (provided in the kit) were prepared for colorimetric detection. 10 μL of the 100mM iron standard was diluted with 990 μL of water to generate a 1mM standard solution. Then 0, 2, 4, 6, 8, and 10 μL of the 1mM standard solution was added into a 96 well plate to generate 0, 2, 4, 6, 8, and 10nmol per well standards. Iron Assay Buffer was added to each well to bring the volume to 100 μL and then 5 μL of the Iron Reducer was added to each standard well.

For sample preparation, 10 μL FF was pipetted into the well, and 90 μL iron assay buffer was added to bring samples to a final volume of 100 μL . Then, 5 μL of iron reducer was added to each of the sample wells to reduce Fe^{3+} to Fe^{2+} for measuring total iron concentration.

The mixture was mixed well using a horizontal shaker, and was incubated for 30 minutes at room temperature. During the incubation, the plate was protected from light. Following incubation, 100 μL of iron probe was added to each well containing standard or test samples. Again, the mixture was mixed well and incubated for 60 minutes at room temperature. After one hour, the plate was read using absorbance microplate reader (Biotek, ELx800TM, UK) at an absorbance wavelength of 550nm.

The in-lab iron detection kit is a ferrozine-based colorimetric assay [212]. 100 μL aliquot of FF (or standard solution) was added to 100 μL of 10mM HCL, and 100 μL fresh iron-releasing reagent (1.4M HCL and 4.5% w/v KMnO_4 in H_2O). This mixture was incubated for 2 hours at 60°C under the fume hood and then cooled to room temperature. Following incubation, 30 μL Iron detection solution (6.5mM ferrozine, 6.5mM neocuproine, 2.5M ammonium acetate, 1M ascorbic acid) was then added into the mixture and further incubated for 30 minutes. Finally, 280 μL of the solution was transferred to 96-well-plate and the plate was

read using absorbance microplate reader (Biotek, ELx800™, UK) at an absorbance wavelength of 550nm.

4.7.19 Oocyte imaging

Fixed samples were imaged by confocal microscopy (Leica SP8, Leica Microsystem, USA) using the 63x oil immersion objective lens with 6x digital zoom. Z-stacks were compiled with 1µm slice separation in the z-axis and an x and y image size of at least 512 x 512 pixels. Common arrangements for imaging included Hoechst with Alexa555 labelled CREST and either Mad2 or tubulin labelled with Alexa488 or Alexa633. The spectral separation of these fluorochromes never resulted in observable bleed-through between channels at the laser powers that were used.

During experiments where the fluorescent intensity was of importance (e.g. γH2AX or studying relative Mad2 levels at different time points) the laser power and other confocal parameters were not adjusted between acquisitions. Additionally, all oocytes were imaged on the same day to minimize any loss of fluorescence that might occur with time.

4.7.19.1 Inverted microscopy

Inverted microscopy was used where 2D information would be sufficient and when imaging large numbers of oocytes simultaneously (~30 at 20x). In this thesis, this was mainly used to identify the number of oocytes with or without polar body extruded (labelled with Hoechst).

4.7.19.2 Confocal microscopy

Confocal microscopy was used to get clearer and more detailed images, for both fixed specimens and live oocytes. In confocal microscopy, out-of-focus light is blocked, allowing only in-focus light to reach the detector, via a pinhole. This produces much clearer images, and makes small structures more distinguishable. It has automated stage with movable objective lens, which enables images to be captured in stacks and later be reconstructed to provide 3D images. With confocal microscopy, detailed z-stacks could be captured in fixed specimens, as there was no time limitation to image them, but this has to balance with the risk of bleaching the fluorescence. As opposed to inverted

microscopy, it has limited ability to image large numbers of oocytes simultaneously.

4.7.20 Experiments endpoints and image analysis

4.7.20.1 Oocytes maturation rate

After overnight incubation (14-16hours), live oocytes were labelled with Hoechst (DNA stained) and imaged under inverted microscopy in 2 views; bright field and Hoechst. Analysis was performed using ImageJ, with or without image processing (e.g. adjustment for brightness and contrast). By examining individual oocytes in detail, they were classified as being matured at metaphase II (MII) stage when there was one polar body seen in either view and classified as being immature in the absence of polar body in both views. No oocytes fixation and permeabilisation were involved.

When the maturation rate for control group (No-FF) is lower than 65% for respective media (MEM or M2) then data for the experiment was not included for analysis. The oocytes were discarded and not used for further experiments (e.g. fixed for immunostaining).

4.7.20.2 Meiosis I maturity stage determination

Oocytes were fixed and permeabilised to determine the maturity stage of individual oocytes. Chromosomes and spindles were stained with Hoechst and anti-Tubulin antibodies respectively. Oocytes were termed analysable when the spindles were present in a lateral or sagittal view and non-analysable when their spindles were poorly/not stained, or have unclear borders. Oocytes were classified as GV stage if the germinal vesicles was still intact. Oocytes were classified as Prometaphase I stage if they presented with condensed chromosome without specific orientation to the spindle, Metaphase I stage if chromosomes were lined up at the metaphase plate with spindle clearly visualised, Anaphase stage if chromosomes have separated toward opposite pole directions with elongated spindle, and Telophase I if chromosomes reached the opposite pole and spindle started to break down to begin cytokinesis. Oocytes were collectively grouped as Meiosis II (MII), if chromosomes are aligned in a compact metaphase plate at the equator of the

spindle, barrel shaped meiotic spindle formed by organized microtubules crossing from one pole to another, and in a presence of one polar body.

4.7.20.3 Calculating spindle dimension

To get accurate measurements of spindle dimensions, images were taken in multiple slices extending through the whole spindle structure, and these images were reconstructed. The analysis was done in 3D (Z-resolution), whereby spindle poles were marked in the stack, and distances between the marked poles were calculated using 3D Pythagoras ($a^2 + b^2 + c^2 = d^2$).

4.7.20.4 DNA damage (γ H2AX) quantification

DNA damage signal was calculated using ImageJ. First the border of nuclear envelope of each oocyte was marked using the oval tool. The mean signal within the nucleus (a) was subtracted from the mean cytoplasmic background signal (b). The resulting value was multiplied by the area of the nucleus (c) to obtain total nuclear signal. Total nuclear signal was thus calculated using the formula of $(a-b)*c$. This calculation was performed by an Image-J macro to ensure the steps were standardized and reproducible (Appendix L).

4.7.20.5 Intracellular ROS quantification

ROS measurement was done using the ImageJ application. Images for ROS measurement captured in 2 channels showing bright field and 2'7'DHDCF-DA channel. To measure the intracellular ROS uptake, the cytoplasm border was traced using the oval tool, and the mean grey value of the cytoplasm was calculated. When the border of the oocyte not discernable due to too low signal in 2'7'DHDCF-DA channel, the measurement was taken according to the tracing done on the brightfield view (Figure 4-6).

4.7.20.6 Iron quantification

Total absorbance of transmitted light of each FF sample detected by microplate reader was plotted against the standard curve. Standard curve was generated according to standard iron solution, as described earlier (4.7.18). Different standard curves were generated for different experiments. The standard curve graph was plotted using Microsoft excel application.

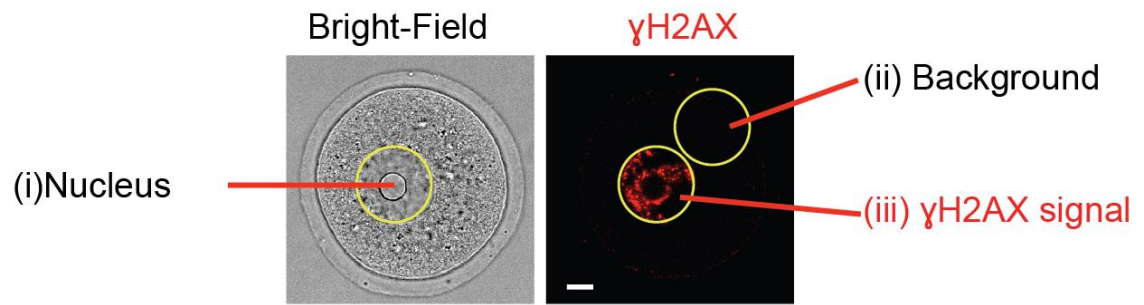


Figure 4-6 DNA damage quantification

(i) Circle outlining the nucleus was determined in the Bright-field channel. In Z-Projection of γ H2AX channel, (ii) the same circle was positioned at the nucleus to quantify the γ H2AX signal and (iii) the same size circle was marked at the cytoplasm to represent the background signal. Total γ H2AX signal is calculated by subtracting the signal from the nucleus (ii) with the background (iii) and multiply with nuclear area. Scale bar represents 20 μ m.

4.7.20.7 Mad2/Crest Analysis

The analysis of Mad2 levels on kinetochores was performed using an ImageJ macro developed by the research group that I was working with. The original writer of the macro, Dr Simon Lane has kindly given me the permission to use it in this experiment. In this macro the user opens the confocal images in ImageJ as a virtual stack using the 'LOCI Bio-formats' plugin available from (<http://loci.wisc.edu/bio-formats/imageJ>). The macro displays the channels containing CREST and chromatin signals, and the user logs the positions of the kinetochores by clicking on them in pairs, so that both kinetochores of each bivalent are recorded consecutively.

The macro then marks the image with open circles (displayed in all planes), and a solid circle (displayed in the plane in which the kinetochore is clicked) to differentiate overlapping kinetochore. To prevent logging the same kinetochore repeatedly, the circle was numbered according to the number of the bivalent. For every logged circle, intensity of the CREST and Mad2 signal on the kinetochore were computed automatically. Background subtraction (kinetochore – background signal) for both Mad2 and CREST, and the ratios between the two (Mad2/CREST) were also calculated. Generated data (kinetochore and background readings, and ratios) together with the positions of the kinetochores and the inter kinetochore distances were automatically recorded to an excel spread sheet before being analysed using the statistical package.

4.7.21 Figure preparation

Data obtained from ImageJ application were saved in Excel (Microsoft, USA), then copied and pasted into Prism 6 (GraphPad Software, Inc. USA), where statistical analyses were performed. Using this software, graphs were made and then copied into Adobe Illustrator. Images were adjusted to improve colour contrast only where appropriate, and images within one experiment were processed in the same manner.

4.7.22 Statistical analysis

Fischer's-exact tests were used for dichotomous data (e.g. polar body extrusion), with the level of significance set at $P < 0.05$. Analysis of variance (ANOVA) tests were used for continuous data (e.g. mean grey area, spindle width and length) comparing more than two groups, with Tukey's test for post hoc analysis. Data was keyed in to Prism 6 software (GraphPad, Software.Inc) and statistical analysis was performed using the same software.

4.8 Results

4.8.1 Clinical data between groups

Sixteen follicular fluid samples (Endometriosis, $n=10$; Male factor, $n=6$), collected from January 2013 to January 2015 were used for various experiments carried out in this chapter. The demographic background of the women from whom the samples were obtained showed no significant difference ($P > 0.05$) in age [$P=0.47$, 95%CI (-3.78, 1.85),], body mass index [$P=0.66$, 95%CI (-5.26, 3.46), t-test], length of infertility [$P=0.41$, 95%CI (-17.35, 39.85), t-test], baseline FSH [$P=0.31$, 95%CI (-4.69, 1.70), t-test] and antral follicle count [$P=0.16$, 95%CI (-1.99, 10.79), t-test] between endometriosis and male factor group. However a significantly [$P=0.04$ 95%CI (0.29, 12.64), t-test] lower number of oocytes were retrieved from women with endometriosis (8.2 ± 5.6) compared to male factor (14.7 ± 2.2 ; Table 4-3), which is consistent with the meta-analyses done and discussed earlier in this thesis (Chapter 2).

In terms of the respective operative findings, all women in the endometriosis group have at least one laparoscopic procedure. All, except one woman with severe endometriosis had endometrioma during the laparoscopic procedure. One fifth (2/10) of these women were also found to have bilateral endometrioma during laparoscopy and two women (2/10) were recorded to have endometrioma present during the transvaginal oocyte retrieval procedure. The information and description of the operative finding of all women with endometriosis is listed in Table 4-4.

	Endometriosis (n=10)	Male Factor (n=6)	P-value
Age (years)	33.8±2.7	32.8±2.3	0.47
BMI (kg/m²)	24.4±4.1	23.5±2.3	0.66
Duration of infertility (Months)	42.8±12.6	54.0±34.57	0.41
Basal serum FSH	8.13±2.3	6.63±0.9	0.31
Antral Follicle Count (AFC)	11.6±5.1	16.0±6.8	0.16
Number of oocytes retrieved	8.2±5.6	14.7±5.3	0.04*

Table 4-3 Clinical data of samples used in the experiments

Table showing clinical information of women included in endometriosis or male factor group. The demographic detail includes age, body mass index (BMI), baseline Follicular Stimulating Hormones (FSH), Antral Follicle Count (AFC) and number of oocytes retrieved. Statistically significant comparison ($P < 0.05$, student T-Test) indicated with asterisk ().*

Patient	Laparoscopy findings	Endometrioma at laparoscopy	Endometrioma at TVOR	ASRM
01	Endometrioma and DIE with rectal involvement. Cystectomy done	Yes	No	III-IV
02	Severe endometriosis with adhesions. No endometrioma documented.	No	Yes	III-IV
03	Endometrioma (4cm) at the right ovary and severe adhesions at the left ovarian fossa	Yes	No	III-IV
04	Severe endometriosis with ovarian endometrioma. Salpingo-oophorectomy done.	Yes	No	III-IV
05	Bilateral endometrioma (8 and 4cm) and cystectomy done. Adhesiolysis done to release severe adhesions	Yes	Yes	III-IV
06	Severe endometriosis, bilateral endometrioma and cystectomy done. Complete obliteration of Pouch of Douglas.	Yes	No	III-IV
07	Small endometrioma seen bilaterally, ruptured but cystectomy not done. Endometriosis at ovarian fossa.	Yes	No	III-IV
08	Endometriosis noted bilaterally at utero sacral ligament. Both fimbrial ends are free and adhesion seen at the both ovarian fossa	No	No	I-II
09	Mild endometriosis and ablated. 3 laparoscopies done with the same findings of mild endometriosis.	No	No	I-II
10	Multiple endometriotic lesions at pelvic side wall. No adhesions seen.	No	No	I-II

Table 4-4 Operative findings for patients in endometriosis group

Table showing clinical information of women included in endometriosis group. The laparoscopic findings column is the summary of operative findings including either bilateral or unilateral involvement and surgical treatment performed. The next two columns describe presence of endometrioma at laparoscopy and trans vaginal oocyte retrieval (TVOR). The last column indicates the stage of the disease according to ASRM staging.

4.8.2 Follicular fluid of women with endometriosis causes MI arrest

Oogenesis is a process that takes place within a developing follicle. Although the maturation process between Prophase I arrest to MII stage is relatively short compared to the entire duration of meiosis, oocytes are constantly exposed and immersed within FF during the course of this event, which take approximately 14 days from the beginning of the antral follicle formation until ovulation. Therefore follicular fluid provides the environment in which the oocyte grows and matures and is thought to influence the process [170, 210]. In a previous study, the maturation rate of bovine oocytes was compromised when cultured in FF from women with mild endometriosis [170]. This has developed the hypothesis that FF of women with endometriosis interferes with the maturation process. This detrimental impact to the maturation process during oogenesis in women with endometriosis has added a new insight of pathogenesis of endometriosis for subfertility [61]. Here the role of FF from women with endometriosis on the ability of mouse oocytes to complete maturation was tested.

In these experiments, 1,668 GV stage mouse oocytes were incubated in 2 different FF concentrations. I started with 15% FF concentration as guided by the previous publication [170]. These oocytes were incubated in culture media comprised of 15% FF and 85% of standard maturation media (M2 or MEM; Appendix K). In this first experiment, 586 immature oocytes were incubated for 14-16 hours in media with 15% FF (MF-FF, n=132; ME-FF, n=97; SE-FF, n=167), or 0% FF as a control (No-FF, n= 190). Following this incubation, oocytes were assessed for the completion of meiosis I by the presence of a polar body. Polar bodies were confirmed by brightfield and also a second signal from the DNA dye Hoechst 33288 (Sigma-Aldrich, UK, Figure 4-7A). When incubated in SE-FF, 92 out of 167 (55.1%) oocytes extruded a polar body. This maturation rate was significantly lower compared to No-FF (132/190, 69.5%, $P=0.001$, 95%CI, Fisher's exact test) and MF-FF (88/132, 66.7%, $P=0.0442$, 95%CI, Fisher's exact test). However the same result was not seen with ME-FF, as 64 out of 97 (65.9%) oocytes extruded a polar body, which was similar to oocytes incubated with No-FF, (132/190, 69.5%, $P=0.1728$, Fisher's exact test)

and MF-FF, (88/130, 67.7%, $P=1.00$, 95%CI, Fisher's exact test). The maturation rate was also not significantly different between severe and mild endometriosis (55.1% vs. 65.9%, $P=0.0923$, 95%CI, Fisher's exact test; Figure 4-7B)

Following the experiment above, it was hypothesised that maturation rate correlated to the FF concentration. Therefore the experiment was repeated by incubating 1,082 immature oocytes in 50% FF (MF-FF, $n=242$; ME-FF, $n=245$; SE-FF, $n=309$), or 0% FF as a control (No-FF, $n=286$) In this experiment a more marked and significant lower maturation rate was seen when oocytes were incubated in SE-FF (150/309, 48.5%) when compared to media with no FF or with Male Factor (No-FF, 216/286, 75.5%, $P<0.0001$; MF-FF, 172/242, 68.4%, $P=0.0001$, 95%CI, Fisher's exact test). Additionally with higher concentration, poorer maturation rate was seen in the ME-FF (56/140, 40%) group compared to all groups (No-FF, 216/286, 75.5%, $P<0.0001$; MF-FF, 172/242, 71.1%, $P<0.0001$; SE-FF, 150/309, 48.5%, $P=0.3937$, 95%CI, Fisher's exact test; Figure 4-7C).

To allow for comparison between the different FF concentrations, in each experiment the experimental groups were normalised against the control group (No-FF) to account for day-to-day changes in maturation rate. After normalising the data, it was found that maturation of oocytes incubated in FF from the same women at different concentrations was markedly reduced ($P<0.05$, 95% CI) at the higher concentration of FF for both groups (Figure 4-7D).

With these experiments, it was found that FF of women with both mild and severe endometriosis causes MI arrest and was dose dependent. The effect is not seen in male factor, which was similar to the control group. At this point the finding from previous publication was reproduced using a mouse oocyte model. Experiments performed following this section were done using 50% concentration of FF and 50% standard incubation media.

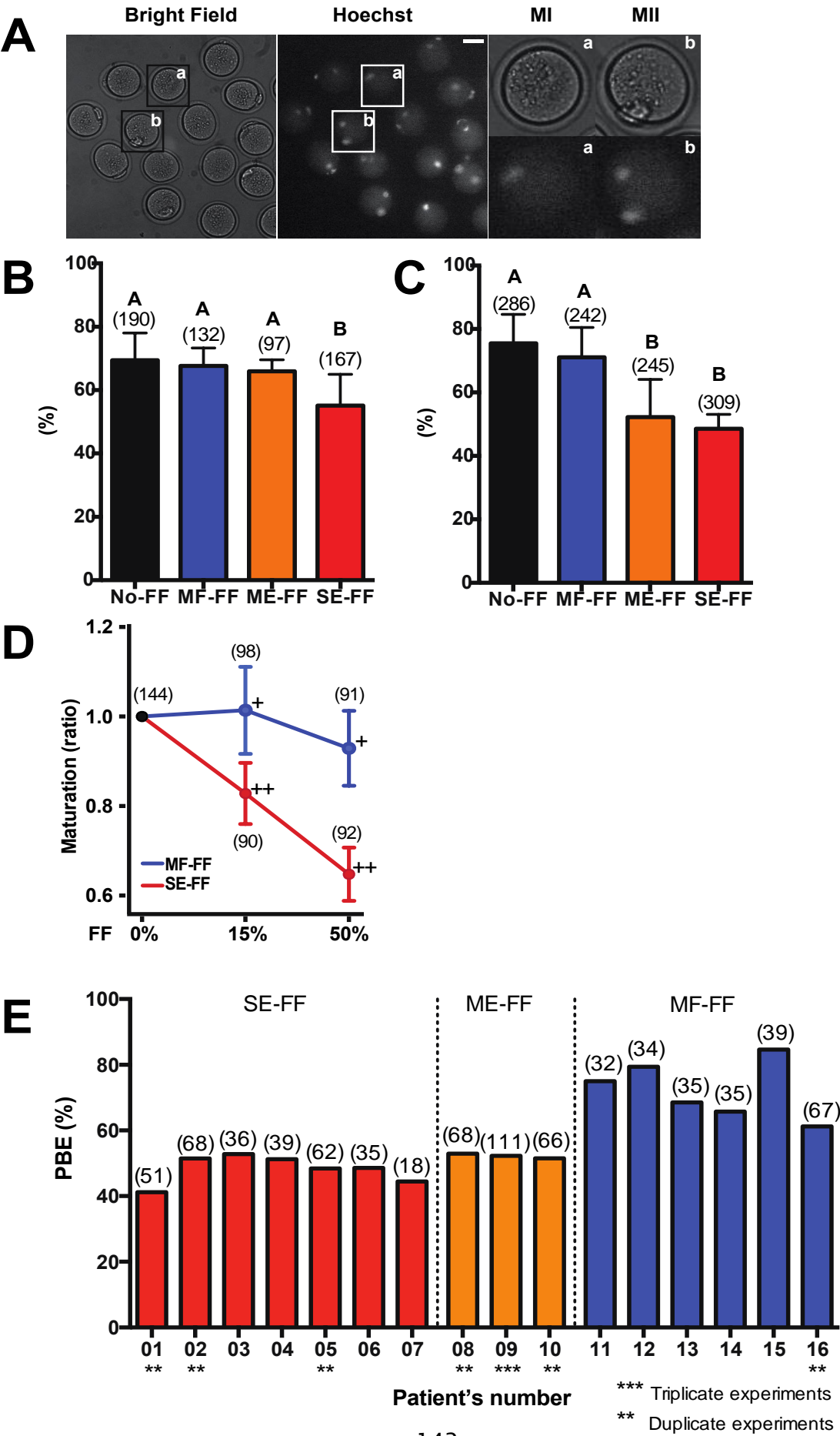


Figure 4-7 Maturation rate after overnight incubation with media

A: Inverted microscopy images showing mouse oocytes after maturation in FF, Bright field and stained with Hoechst. **(a)** Represent MI and **(b)** MII stage oocytes evidenced by polar body extrusion. Scale bar showing 20 μm . **B:** Maturation rate after in-vitro maturation with 15% FF, and **C:** with 50% FF. Significantly different ($P < 0.05$) groups are indicated by different letters. **D:** Maturation rate (normalised to control) of paired samples at different concentration of FF, + and ++ indicate $P < 0.05$. **E:** Bar chart showing maturation rate (at 50% FF concentration) for each patient's sample. The (**) and (***) indicate the % comprises data from 2 or 3 separate experiments respectively. Number of oocytes is included in the parenthesis. **(B-D):** Error bars are standard deviation; Number of oocytes is indicated in parenthesis.

4.8.3 Endometriosis causes delayed polar body extrusion and often leads to MI arrest

To find the mechanism behind lower maturation rate in endometriosis, time-lapse experiments were next performed. At this point, I hypothesised that the low maturation could be due to a discrepancy in the time taken for germinal vesicle breakdown (GVB) and/or polar body extrusion (PBE). The first polar body contains redundant genetic material, which will no longer be involved with the rest of meiosis process, but is still an important morphologic indicator of the end of Meiosis I. The maturation process usually occurs following complete attachment of spindles to the chromosomes, division of sister chromatids and cytokinesis).

In this experiment the collected oocytes that were released from Milrinone were then incubated with culture media with and without 50% FF (Male Factor or Endometriosis). Oocytes were left undisturbed on the confocal microscope for 16 hours, with imaging at 5 minutes intervals. The series of images for each group were analysed and the time of GVB and PBE were recorded.

GVB timing (time taken for complete loss of nuclear envelope) was similar in all the groups. Nearly 100% of oocytes incubated with media, with and without FF had GVB within 90 minutes after being released from Milrinone. However, PBE timing (time taken for completion of cytokinesis) was significantly delayed in oocytes incubated with SE-FF. The mean timing for oocytes in SE-FF group to extrude polar body was 13.5 hours after GVB to extrude PB, compared to 9.3 and 9.25 hours for No-FF and MF-FF respectively (Figure 4-8).

This experiment demonstrated important additional information, whereby FF was found not to affect GVB and had a modest effect on PBE timing, but in the presence of endometriosis, FF causes a significant delay to PBE timing and often led to maturation arrest. Next I set out to investigate the stage of meiosis at which these oocytes arrested.

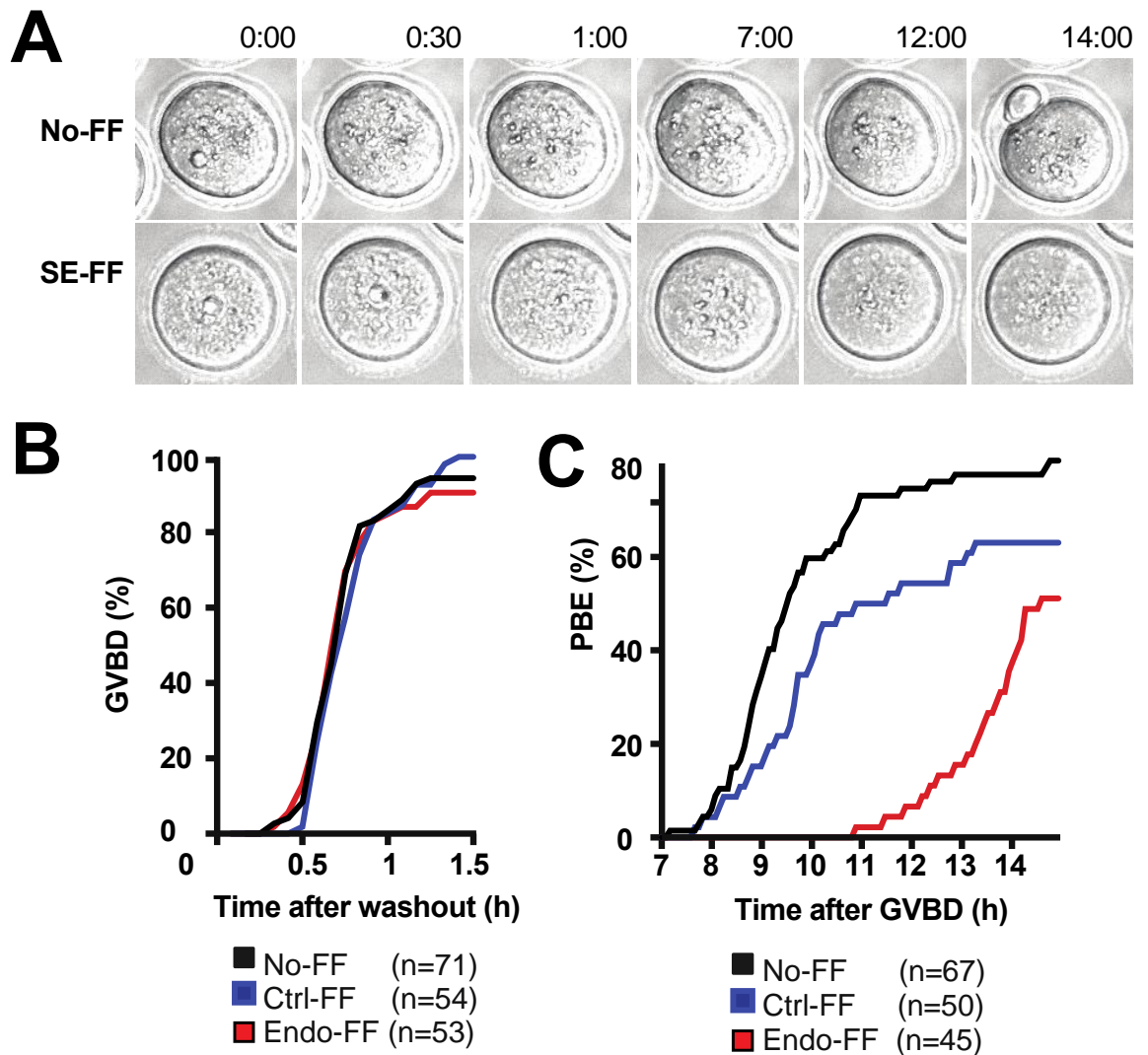


Figure 4-8 Germinal Vesicle breakdown and Polar Body extrusion timing

A: Time lapse imaging using confocal microscopy of oocytes incubated with media with and without endometriosis at the times indicate (h:mm) time after washout **B:** Germinal Vesicle Breakdown (GVBD) timing **C:** Polar Body Extrusion (PBE) timing.

4.8.4 Non-maturing oocytes are Metaphase I arrested

Nuclear maturity depends on nucleus modifications starting with oocytes arrested at prophase I of meiosis where the prominent visible nucleus is referred to as the germinal vesicle (GV) (Figure 4-9). Upon resuming meiosis, the oocyte undergoes germinal vesicle breakdown (GVBD) and the nuclear envelope dissolves. The oocyte is now said to be in prometaphase. In the following hours the oocyte will build its spindle structure whereby the chromosomes will align to [213]. In Metaphase I, homologous chromosome pairs move together to form the metaphase plate and these homologous chromosomes are pulled apart in Anaphase I [181]. Once separated, the spindle structure depolymerizes in Telophase I, and will extrude the polar body containing half the oocyte's genetic material but very little of the cytoplasmic contents will be extruded to mark the end of the meiosis I. Next I investigated at what stage of meiosis I these oocytes stop maturing.

In this experiment, 190 oocytes (No-FF, n=77; MF-FF, n= 32; SE-FF, n= 81) were fixed and chromosomes and tubulin were visualised with Hoechst and anti-Tubulin antibodies to determine the stage of the arrested oocytes as described in the methods section. When incubated with SE-FF, there were significantly more oocytes arrested at Metaphase I stage, compared oocytes incubated in No-FF ($P<0.0001$) and MF-FF ($P=0.0008$). There was a corresponding reduction in the number of oocytes arrested at metaphase II when compared to No-FF ($P<0.0001$) and MF-FF ($P=0.0009$). There were no differences seen in other stages: GV (No-FF, $P=1$; MF-FF, $P=0.2936$), Prometaphase (No-FF, $P<0.4971$; MF-FF $P=1$), Anaphase No-FF, $P=0.627$; MF-FF, $P=0.1$) and Telophase (No-FF, $P=0.3293$; MF-FF, $P=0.554$, Figure 4-9)

From this experiment it was determined that the majority of the non-maturing oocytes were arrested in Metaphase I. Next, the morphometric measurement of the spindles at MI stage will be examined.

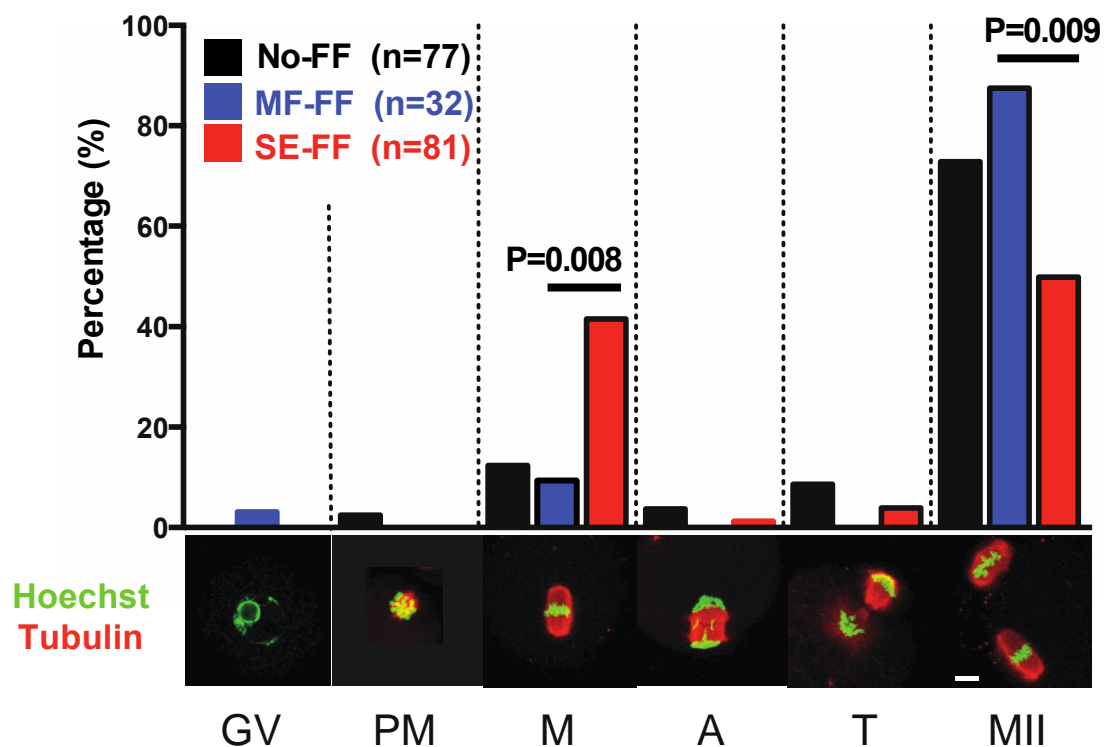


Figure 4-9 Arrest stage and spindle dimensions of MI and MII oocytes after overnight incubation

Percentages of stages of arrest after overnight incubation in 50% FF. Number of oocytes is shown in the parenthesis. Confocal microscopy images depict oocytes at the indicated stages. Chromosomes were stained with Hoechst and spindles were stained with anti- β Tubulin. **GV**; Germinal Vesicle; **PM**, Prometaphase; **M**, Metaphase I; **A**, Anaphase; **T**, Telophase and **MII**, Metaphase II. Scale bar represent 20 μ m.

4.8.5 MI arrest cannot be explained by differences in spindle morphology

The meiotic spindle is the structure that moves, aligns, and segregates the chromosomes during meiosis. There are over 100 proteins associated with the spindle apparatus and its movement but its main structural component is a dimer consisting of α - and β -tubulin subunits. The structural and orientation-related modifications during meiosis depend on the close interaction of microfilaments and microtubules.

The spindle apparatus is absent at the GV stage when the chromosomes are contained in the germinal vesicle, and the centrosomal material is located close to the cortex of the cell. During GVBD, the centrosomes also disintegrate, and the centrosomal material forms multiple foci around the cytosol, closely associated with the condensing chromatin. Microtubules are observed to nucleate at these centrosomal masses and start to form in Prometaphase [213, 214]. The oocyte then assembles a bipolar spindle (MI spindle) and segregates the bivalent chromosomes. The cytokinesis is asymmetrical, resulting in the extrusion of a small polar body (PB I), which marks the end of meiosis, I. At metaphase II, the centrosomes are reformed, and the spindle apparatus is fully formed. It is known that meiotic spindle of the oocyte is very sensitive to internal and external factors such as oxidative stress and inflammatory markers [215]

Barcelos et al in 2010 [216] in a comparative analysis of the spindle and chromosome configurations of in vitro matured oocytes from patients with endometriosis and from control subjects did not demonstrate an increase percentage of meiotic abnormalities for oocytes obtained from stimulated cycles of endometriosis when compared to controls. The authors however reported a tendency toward higher proportion of Telophase I in apparently matured oocytes observed in endometriosis patients that may suggest impairment or delay in completion of meiosis I.

Da Broi et al [170] examined spindle morphology, chromosome appearance and nuclear maturation rate in bovine oocytes exposed to follicular fluid of women

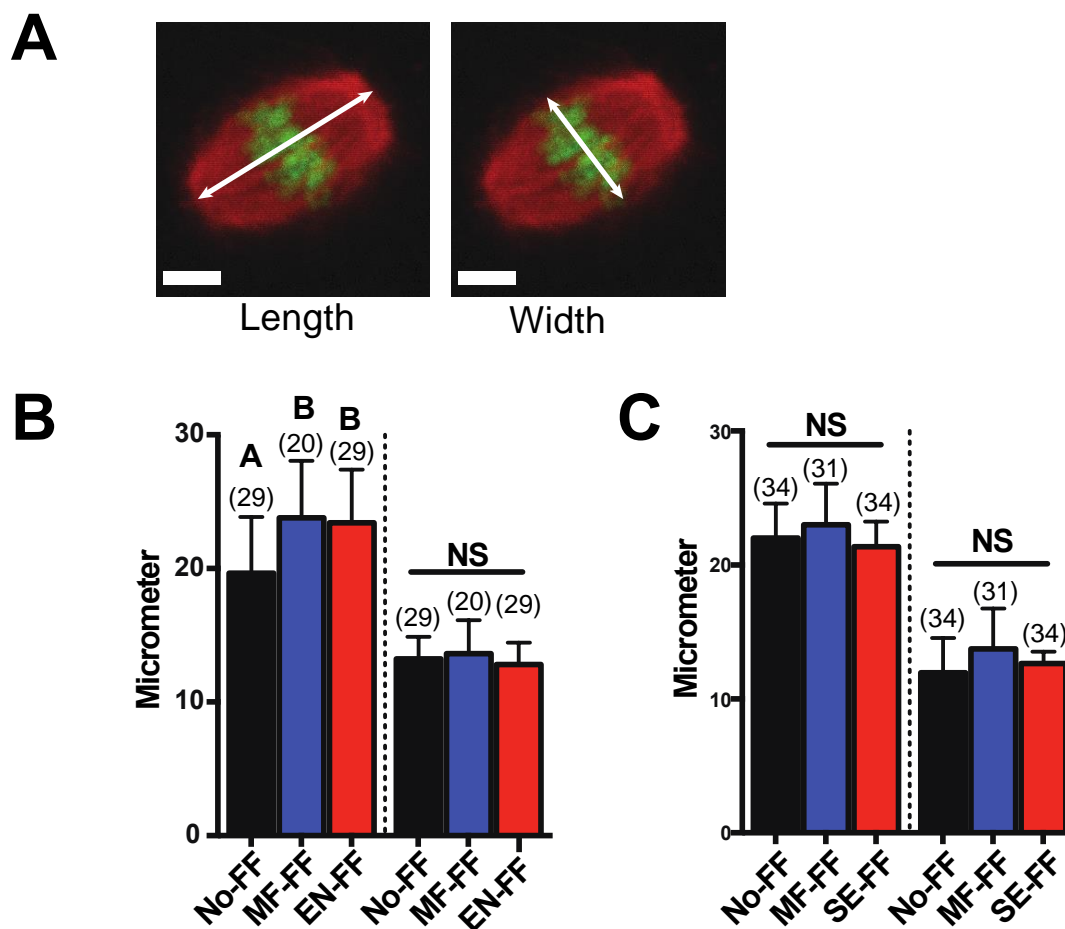


Figure 4-10 Spindle dimensions of MI and MII oocytes after overnight incubation

A: Confocal microscopy images of spindle after incubation with MF-FF or SE-FF with measurement of its dimension. (Tubulin, Red; Chromatin, green) **B:** Length and width of spindle of non-maturing oocytes and **C:** MII oocytes. Error bars are standard deviation. Number of oocytes is indicated in the parenthesis. Significantly different ($P < 0.05$) groups are indicated by different letters. Scale bar represent $10\mu\text{m}$. NS: not significant ($P > 0.05$)

with endometriosis compared to follicular fluid from women with no endometriosis and concluded that the adverse effects of the FF of women with endometriosis on oocytes may be linked to an aberrant meiosis process.

Because of its important role in reproduction, spindle morphology can potentially become a useful marker to determine oocyte quality. Recent technological advances have made visualization of the spindle apparatus possible whereby microtubules and chromosomes can be visualised and scored. Currently these visible structures are scored according to their morphology and location from polar body [217], which has showed in human oocytes the nuclear maturation rate is not affected by the location and morphology of the spindle. This scoring criteria is however very subjective and potentially have wide intra and inter observer variation and make it less reliable. Some of these techniques also require fixing and immunostaining of the oocytes, which prevent further use. This is not desirable in IVF treatment.

In this experiments, the spindle length and width was measured (Figure 4-10A) for both MI and MII stages. After overnight (14-16 hours) incubation, all oocytes were fixed and immunostained with tubulin and imaged as described in the method section. Following incubation with SE-FF (n=29), oocytes at arrested MI stage have longer spindle length compared to those incubated in No-FF [MD -3.78 CI95% (-6.4, -1.18) n=29], but similar length when compared to MF-FF [MD -0.37 CI95% (-3.25, 2.50) n=20]. When incubated in SE-FF these MI oocytes also have no difference (P=0.685) in spindle width, compared to No-FF [MD 0.4147, CI95% (-0.7782, 1.608)] and MF-FF [MD -0.814, CI95% (-2.135, 0.5061), Figure 4-10B)].

In addition, oocytes incubated in SE-FF have no difference in spindle dimensions at metaphase II between the groups tested for both length [(P=0.1612, One way Annova; No-FF, MD 0.6534, CI95% (-1.428, 2.734)] and MF-FF [MD 1.638, CI95% (-0.4602, 3.736), Figure 4-10C] and width [(P=0.0534, One way Annova; MF-FF, MD 1.080, CI95% (-0.9084, 3.608)] and No-FF [MD -0.7010, CI95% (-2.657, 1.255, Figure 4-10C)].

This experiment excluded the hypothesis of abnormal spindle morphology as the cause of MI arrest.

4.8.6 MI arrest is not associated with displaced or non-biorientated chromosomes

Although the preceding experiment has found no difference in spindle morphology between all groups, it did not specifically examine the attachment and orientation of the chromosomes to the spindle equator. It was hypothesised that non-biorientated chromosomes could activate the SAC, which in turn could account for the delay in MI. During this process, chromosomes need to move and orientate themselves on the metaphase plate in preparation for anaphase I [218]. Correct attachment of sister kinetochores to the spindle is in an amphitelic arrangement, a configuration that generate tension across the bivalent. In non-amphitelic arrangement (monotelic, syntelic or merotelic), the inter-kinetochore distance is shorter and each kinetochore will be at random position in relation to the spindle equator. As the working hypothesis is that MI arrest in endometriosis is caused by a defect in achieving the fundamental amphitelic configuration, and therefore in this experiment inter-kinetochore stretch (stretch), distance from the spindle equator (displacement) and the angle of intersection with the spindle equator (θ) was measured.

Following 8 hours of incubation in media (no-FF) and with FF (SE-FF, MF-FF), oocytes were fixed. For this experiment, kinetochore and chromosomes were immunolabelled and imaged in 3 dimensional. Using ImageJ application, 3D images were reconstructed. Kinetochore pairs were identified and numbered in pairs (Figure 4-11A). An ImageJ macro was then used to calculate the position of the spindle plane so as to minimise the displacement and maximise the angle of intersection of each bivalent in the oocyte. Subsequently, the inter-kinetochore distance, displacement and angle of intersection with the equator were calculated for each bivalent.

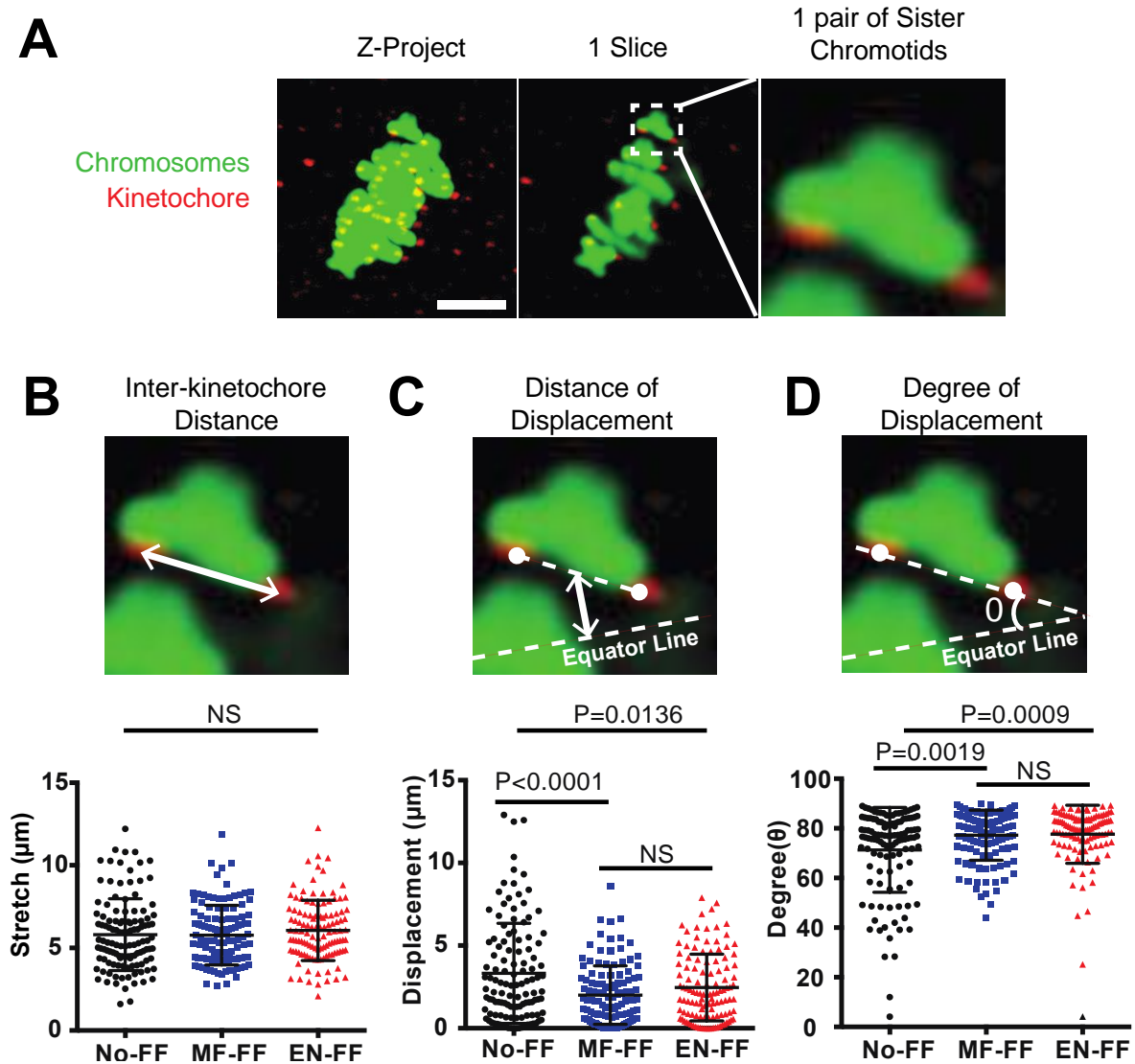


Figure 4-11 Inter-kinetochore distance and displacement

A: Following 7h post GVBD oocytes incubated in media (with and without FF), were fixed and kinetochores were immuno-labelled with CREST. Subsequently, immunostained oocytes were imaged and each kinetochore-pair was marked using ImageJ software. Inter-kinetochore distance, displacement from the spindle equator and the angle of intersection with the equator were calculated. Scatter plot graph showing the **B:** stretch **C:** distance of displacement from spindle equator and **D:** angle of intersection with the equator. Solid arrows represent the measurement taken for respective outcomes. Error bars represent the mean and standard deviation. $P < 0.05$ is considered significant.

The inter-kinetochore distance was found similar between all groups ($P=0.4680$, one-way ANOVA Figure 4-11B). However there was significant displacement ($P<0.0136$, one-way ANOVA,) between all groups and post hoc test however did not find any difference between EN-FF and MF-FF ($P=0.2884$, Tukey's Test). Similarly, there was significant degree of deviation found ($P=0.0009$, one-way ANOVA, Figure 4-11C) between all groups, but post hoc test did not find any difference between EN-FF and MF-FF ($P= 0.9761$, Tukey's Post Hoc Test, Figure 4-11D) degree of deviation of the chromosomes between endometriosis and male factor.

From this experiment, amphitelic configuration and position were unaltered in oocytes incubated in FF of women with and without endometriosis. Therefore this experiment excluded the hypothesis suggesting incorrect assembly of meiotic spindle and bivalent biorientation as the cause of MI arrest.

4.8.7 MI arrest is reversed by inhibiting SAC function

At this point we know that FF from women with endometriosis causes maturation arrest and the majority are halted at the Metaphase I stage. Subsequent experiments performed in this chapter will try to examine the mechanism of this arrest, according to a proposed pathway, which is based on existing cell cycle knowledge [219] (Figure 4-12A).

Progression of an oocyte from metaphase to anaphase requires a cascading process. This is mainly orchestrated by Spindle Assembly Checkpoint (SAC), whereby during meiosis (or mitosis), it prevents separation of the duplicated chromosomes until each chromosome is properly attached to the spindle apparatus. Activated SAC will inhibit the Anaphase Promoting Complex (APC) preventing the transition from Metaphase I to Anaphase. In both mitosis and meiosis, the SAC is activated during Prometaphase, but it gets switched off by complete kinetochore attachment in mitosis (defined as metaphase). In meiosis however, it is less clear when it gets switched off and complete kinetochore attachment is not required [218].

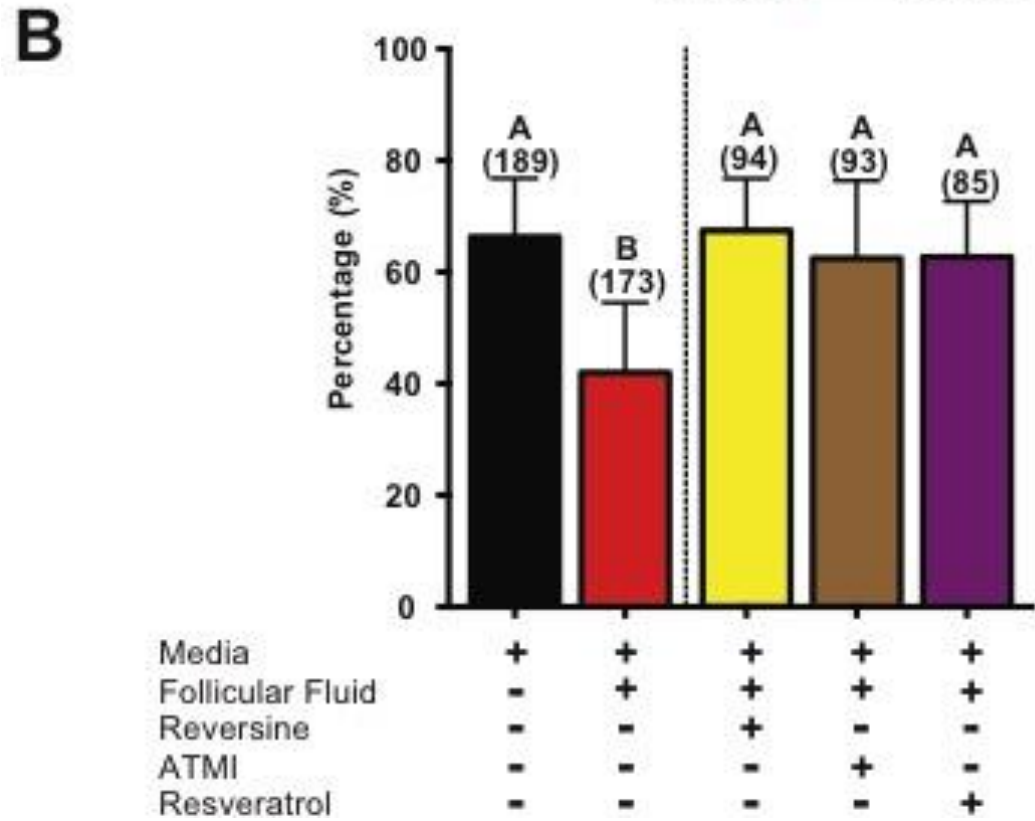
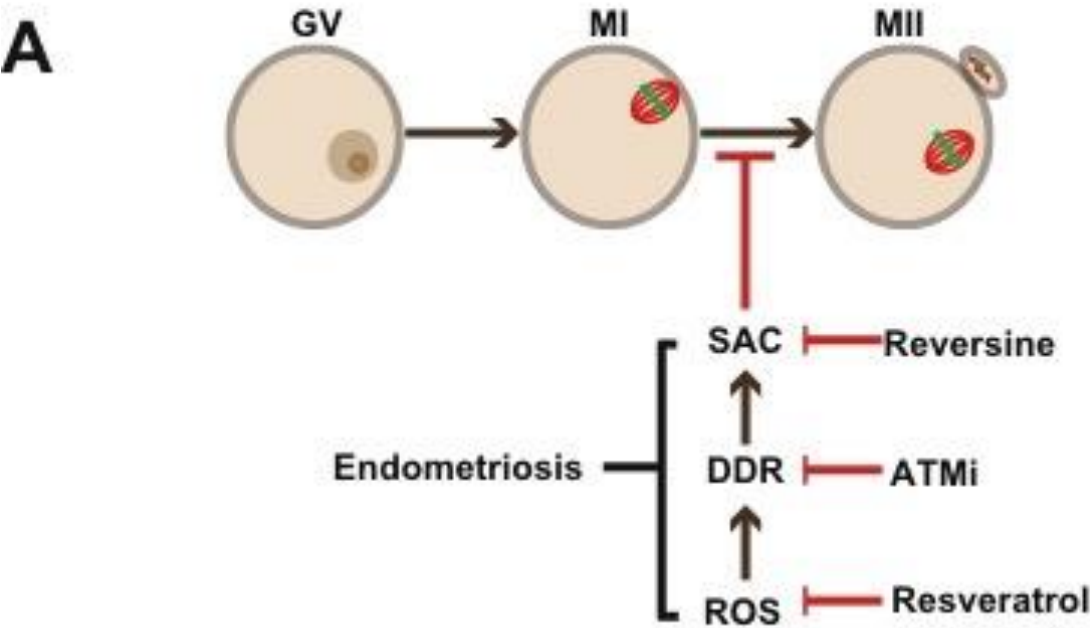


Figure 4-12 Maturation rate following addition of drugs to the SE-FF

A: Flowchart of the proposed pathway on how endometriosis causes Meiosis I arrest. Progression of oocytes from Metaphase I to Anaphase I is controlled by the Spindle Assembly Checkpoint (SAC). The SAC remains activated until all kinetochores have attached to the spindle. The checkpoint is however inhibited by MPS1 inhibitor, Reversine (RVN). The SAC will remain activated in response to DNA Damage (DDR) [185, 220]. However the DDR is inactivated by ATMi, allowing maturation to occur in the presence of DNA damage. Reactive Oxygen Species (ROS) can cause DNA damage and activate DDR. By adding Resveratrol (RTL), it is hypothesised that free ROS are scavenged and will not activate the DDR. Inactivated DDR will not activate the SAC, and this will improve the maturation rate. B: Maturation rate following incubation with media or SE-FF alone or with Resveratrol (RTL), Reversine (RVN) or ATMi. Significantly different groups ($P < 0.05$) are indicated by different letters.

During prometaphase, several important steps are regulated by MPS1 protein kinase. Apart from activities in centrosomes, MPS1 functions involve activities at the kinetochore in both chromosome attachment and the spindle assembly checkpoint. In this section I analysed the effect of reversine, an inhibitor of the Mps1 kinase whose activity is essential for the SAC. To test whether metaphase arrest by SE-FF is due to continuous SAC activity, the same maturation experiments were repeated with Reversine (Sigma-Aldrich, UK) added in the culture media.

By adding 0.1 μ M Reversine (Sigma Aldrich, UK), a dose shown to inhibit the SAC [221] into culture media with 50% SE-FF throughout the incubation, the majority of the incubated oocytes progressed to MII, significantly more than in SE-FF alone ($P=0.0002$, 95%CI, Fisher's exact test, Figure 4-12B). The maturation rate was also comparable ($P=0.8933$, 95%CI, Fisher's exact test) to those incubated in media only with no FF (Figure 4-12B).

From this experiment, inhibiting the SAC rescues the maturation rate. Given that the meiotic spindle appears normal, this raises the question of what factors contribute to the activation of the SAC when incubated in SE-FF.

4.8.8 Mad2 level was not altered in oocyte incubated in FF of women with endometriosis

Before further dissecting the cause of SAC activation, I attempted to validate the above finding by measuring the MAD2 signal, which is one of SAC proteins on kinetochores. Presence of MAD2 protein on the kinetochore indicates an activated SAC, and it usually disappears when the SAC has been deactivated. I speculated that MAD2 would still be present at Metaphase I (8 hours post GVB) and confirmed the finding that SAC remain activated.

In this experiment, oocytes were fixed and immunolabelled with MAD2 and CREST antibody, following incubation in media with and without endometriosis for 8 hours after GVB. These oocytes were imaged in 3D and analysed using ImageJ software with in house macro function. For each bivalent the, CREST signals were identified to represent the Kinetochore location, and both CREST and MAD2 signals were measured in their respective channels (Figure 4-13A).

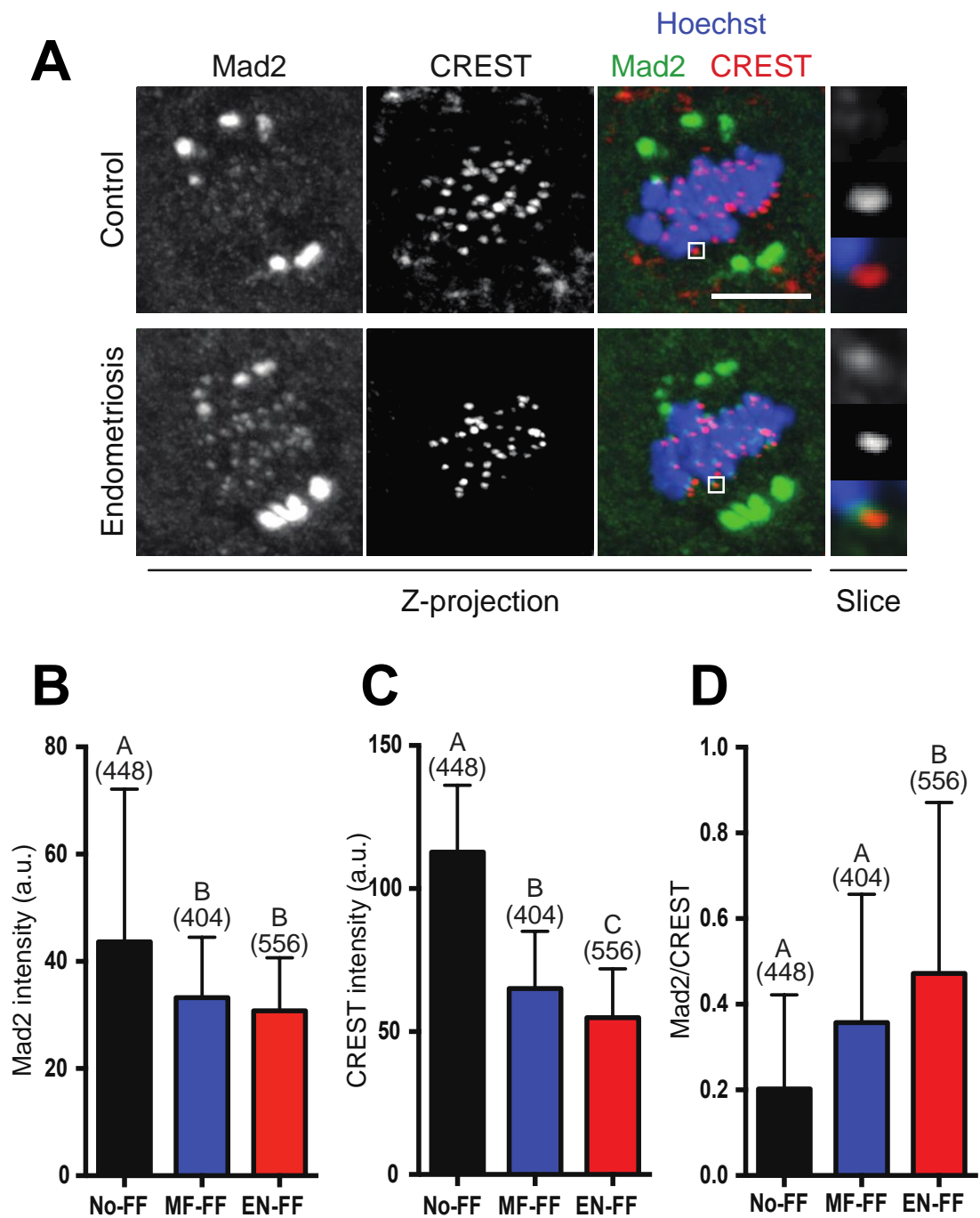


Figure 4-13 Mad2

A: Confocal images of Mad2 and CREST in Z-projection. **B:** Bar chart of Mad2 intensity **C:** CREST intensity and the ratio of Mad2 and CREST. Error bar represent standard deviation. Number of oocytes indicated in the parentheses.

From this experiment, MAD2 levels were found to be similar between SE-FF and MF-FF groups ($P=0.9761$, Tukey's post hoc test, Figure 4-13B). However, this it was also found that the CREST signal was greatly diminished in the SE-FF group compared to the MF-FF group (stats). When the Mad2 signal was normalised using the CREST signal, it was found that there was more Mad2 per unit CREST in the SE-FF group (stats), however, in light of the different apparent sizes of the kinetochores it was not possible to draw meaningful conclusions about the levels of Mad2 and the checkpoint.

4.8.9 MI arrest is reversed by inhibiting the DNA damage response pathway

One recently proposed pathway that maintains SAC activity in oocyte meiosis I is the DNA damage response (DDR) pathway [185, 220]. In response to DNA damage, oocytes meiotic progression will be delayed. This is to ensure faithful and perfect cell division. DNA damage can take many forms ranging from mild changes in nucleotide base to breaks in both strands (Double Strand Break). DNA damage is centred on a pair of related protein kinases called ATM (Ataxia Telangiectasia Mutated) and ATR (ATM and Rad3-related). ATM is recruited and activated by DNA damage and maintains SAC function and therefore prevents progression from Metaphase to Anaphase. I hypothesised that if inhibiting the DDR pathway by ATMi, reversed the low rate of polar body extrusion caused by Endometriosis FF, the arrest process may be due to DNA damage.

In order to conduct this experiment, 40 μM of ATM inhibitor (ATMi, Sigma-Aldrich), a dose shown to inhibit ATM in mouse oocytes [222], was added in the culture media with 50% of SE-FF at the beginning of the experiment for the entire incubation. By inhibiting ATM, most of the incubated oocytes progressed to MII, significantly more than SE-FF alone ($P=0.005$, 95%CI, Fisher's exact test, Figure 4-12B) and this maturation rate was comparable ($P=1$, 95%CI, Fisher's exact test) to those incubated in media only with no FF (Figure 4-12B). This experiment suggested that SE-FF mediated its arrest through ATM kinase, possibly because it can directly or indirectly damage the DNA structure. In the next section I therefore try to quantify the DNA damage.

4.8.10 Follicular fluid of women with severe endometriosis induces more DNA damage

DNA comprises of two strands and therefore, DNA damage can take the form of either a single or double strand break (SSB or DSB). These breaks are recognised by a collection of proteins known as the MRN complex that in turn recruit ATM and ATR. ATM and ATR are the major enzymes responsible for signalling the DNA damage checkpoint and for recruiting further proteins to repair the damaged DNA. The checkpoint is signalled through proteins Chk1 and Chk2 (checkpoint 1 and 2). Central to the response to DNA damage is the production of γ H2AX by phosphorylation of H2AX protein [223]. H2AX has not been found to be concentrated at specific regions of the DNA, but is randomly incorporated into histones throughout the DNA [224] .Figure 4-14A.

DSBs and SSBs can be induced exogenously by ionizing radiation or cytotoxic agents, and endogenously by continuous exposure of reactive oxygen species and by products of lipid metabolism. Although there are many publications available demonstrating the detrimental impact of various insults to the chromosomes (UV, etoposide), there were however no available publications that have investigated the effect of the FF towards the DNA structure. Therefore the experiment was conducted to examine the impact of FF to the DNA.

In this experiment we found that more DNA damage (γ H2AX was induced after one-hour incubation with FF from women with endometriosis compared to No-FF ($P<0.0001$, MD 0.9 [0.49 to 1.32] 95%CI, One-way Anova, Tukey's multiple comparison test, Figure 4-14B) and MF-FF ($P=0.023$, MD -0.45 [-0.88 to -0.03] 95%CI, One-way Anova, Tukey's multiple comparison test, Figure 4-14B). Although it was observed that MF-FF also causes DNA damage compared to No-FF ($P=0.014$, MD 0.45 [0.05 to 0.85] 95%CI, One-way

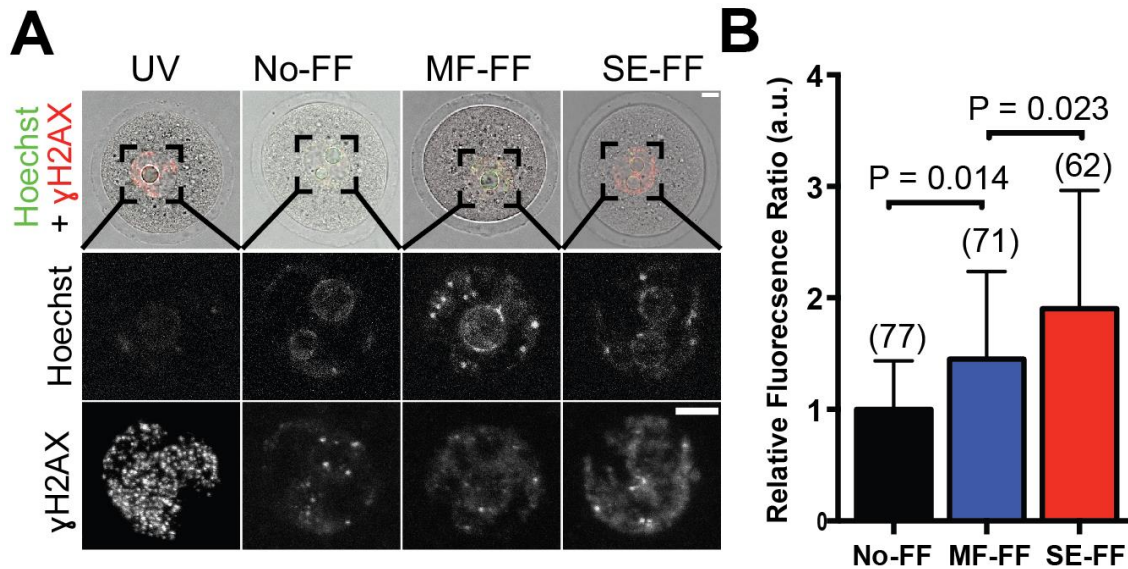


Figure 4-14 DNA damage quantification after incubation with FF

A: Confocal images of γ H2AX nuclear signal between positive controls (UVB), negative control (No-FF), severe endometriosis (SE-FF) and Male Factor (MF-FF) groups. Oocytes were fixed and immunostained with DNA damage marker (γ H2AX) following one-hour incubation with media only (negative control) or media with FF. For positive control, oocytes were exposed to Ultra Violet [185] using UV trans-illuminator (MacroVue UV-20, Hoefer inc, US) for 20 seconds before incubation. Scale bar represent 20 μ m. **B:** Bar chart comparing nuclear γ H2AX signal following one hour incubation with FF derived from A. Number of oocytes are indicated in the parenthesis. Error bars are standard deviation.

Annova, Tukey's multiple comparison test, Figure 4-14B), the effect seen was less than demonstrated by SE-FF. This experiment has demonstrated that FF from women with endometriosis induces DNA damage in oocytes. Taken together with the finding that the maturation rate is normalised by adding ATMi, an inhibitor to the DNA damage pathway, it suggests that oocytes may arrest in meiosis I because of this DNA damage. Consequently, I next investigate what factor in the FF causes DNA damage.

4.8.11 MI arrest is reversed by adding resveratrol

Reactive Oxygen Species (ROS) can cause DNA damage and I speculate this may further activate the DNA damage response pathway, (Figure 4-12A). Following the preceding section, I hypothesised that the poor maturation rate caused by incubation in FF from patients with Endometriosis could be rescued by adding Resveratrol (3,5,4'-trihydroxytranstilbene), a substance that is synthesized de novo by certain plants and usually found in the fruits. Resveratrol can exert numerous biological activities including potent antioxidant effects [225] With the Resveratrol supplementation, it can exert an antioxidative effect and scavenged excessive ROS. In this section I hypothesised that this beneficial effect will restore redox balance within the oocytes incubated in SE-FF, and prevent maturation arrest.

Several publications were found to have investigated the beneficial impact of supplementing Resveratrol in culture media (with no FF). In a study using porcine oocytes, anti oxidant effect was seen when 2 μ M of Resveratrol were added to the culture media, and the authors also found reduction of dead tissue during IVM and following vitrification [226]. Another study using 1120 bovine oocytes found more PB were extruded with better cumulus expansion when supplemented with resveratrol (1 μ M) [227]. This is in agreement with an earlier study reported improvement of developmental potential by adding resveratrol (2 μ M) during in vitro maturation of bovine oocytes [228]. This is by increasing the intracellular GSH level, decreasing ROS level and regulating gene expression during oocyte maturation.

In this experiment, 2 μ M of Resveratrol was added in the culture media along with SE-FF at the beginning of the experiment for the entire incubation period. I

found significantly more oocytes with extruded PB when incubated in culture media that have been supplemented with Resveratrol, compared to SE-FF only ($P=0.001$, 95%CI, Fisher's exact test, Figure 4-12B) and this maturation rate is comparable ($P=0.891$, 95%CI, Fisher's exact test, Figure 4.9A) to those incubated in media only with no FF.

4.8.12 Follicular fluid from women with severe endometriosis causes higher intracellular ROS

At supra-physiological level, reactive oxygen species are known to have a detrimental effect on the body and commonly found raised in women with endometriosis [229]. Imbalance in the anti-oxidative ability to neutralise the free reactive oxidative species circulating within the FF may initiate many adverse effects [230] to oocyte fertilisation, meiotic process, chromosomal division and pregnancy (see. 4.1 page 107). Apart from that, oxidative stress has pathological impact on human reproduction including oocyte maturation [59]. Lower ROS levels in follicular fluid of women undergoing IVF correlated with normal spindle morphology, good embryo quality and pregnancy outcome [58]. However little is known on the direct effect on oocyte maturation process.

I therefore wanted to test the effect of En-FF on ROS activity in mouse oocytes. I first preloaded immature oocytes with 40 μM of 2'7'DHDCFDA for 30 minutes before exposing these oocytes to media with and without FF for 15 minutes (or 0.5% H_2O_2 for 5 minutes as positive control). Oocytes were then immediately imaged using confocal microscopy (Figure 4.11A). Mean grey area of the signal calculated with ImageJ application represent the intracellular ROS Figure 4-15A. In this experiment higher intracellular ROS was found in oocytes preloaded with 2'7'DHDCFDA when incubated with SE-FF compared to No-FF ($P<0.0001$, MD 0.81 [0.41 to 1.21] 95%CI, One-way Anova, Tukey's multiple comparison test, Figure 4-15B) and MF-FF ($P=0<0.0001$, MD -1.01 [-1.38 to -0.64] 95%CI, One-way Anova, Tukey's multiple comparison test, Figure 4-15B). There was no significant difference in the uptake between oocytes incubated in M2 (No-FF) and MF-FF ($P=0.44$, MD 0.81 [-0.59 to 0.19] 95%CI, One-way Anova, Tukey's multiple comparison test, Figure 4-15B).

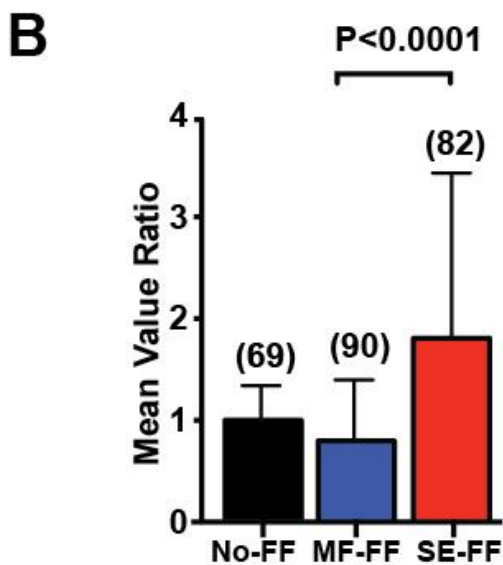
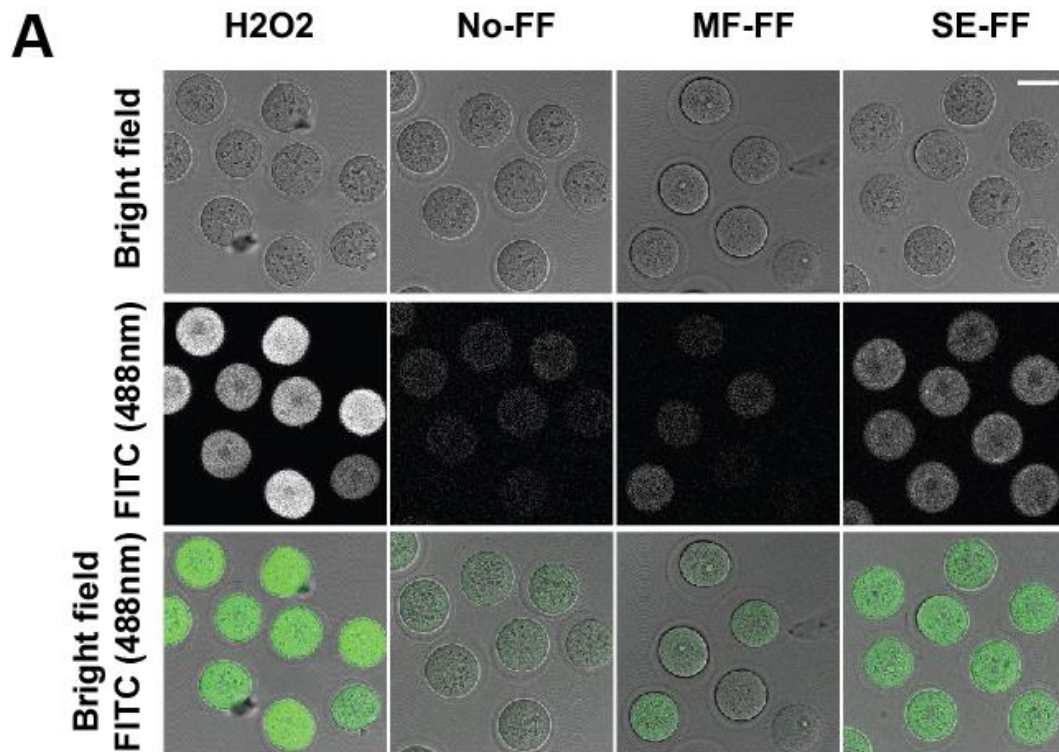


Figure 4-15 Intracellular ROS uptake after incubation with FF

A: Confocal imaging on intracellular ROS with positive control using hydrogen peroxide (H2O2), media only (No-FF), male factor (MF-FF) and Severe endometriosis (SE-FF). **B:** Bar graph showing higher intracellular uptake in oocytes incubated with FF from women with endometriosis compared to controls. Error bars are standard deviation. Scale bar represent 20 μ m.

This experiment demonstrated that FF from women with endometriosis causes higher intracellular ROS in exposed oocytes. This finding was in line with the previous experiment (Section 1.7.8) when anti-oxidative effect from Resveratrol was found to restore maturation rate. With some extrapolation, these results suggest that in the absence of exogenous anti-oxidative substance (such as Resveratrol), intracellular ROS induced by FF of women with endometriosis is responsible to lower maturation rate. I further queried the source of ROS in endometriosis group, and next I have quantified iron concentration of FF from both groups with the hypothesis of higher iron level in endometriosis group that will generate more ROS.

4.8.13 Follicular fluid from women with severe endometriosis have iron content similar to those with no endometriosis

Iron is essential for many physiological processes including electron transport and carrying oxygen to the tissues. In women, iron content in the body is regulated by intestinal absorption at the duodenum and iron loss from menstruation, pregnancy and others [231]. In endometriosis however, it was suggested that iron within the pelvic cavity is from retrograde menstruation and hemorrhaging foci of ectopic endometrium, which a proportion of them are phagocytosed by the macrophages [232]. The exact iron/heme scavenging and sequestration mechanisms available to the ovary have not been defined. None of these proteins involved in the heme metabolism has been assayed in other endometriosis research.

Whilst iron is important for various biological activities, it is known to be a source of ROS as part of its metabolism, by means of the Fenton Reaction (Figure 4-16A). In the Fenton reaction, iron in Ferrous (Fe^{2+}) state were changed to Ferritin (Fe^{3+}) and produce reactive by-products such as hydroxyl radicals, which can directly damage the DNA [233]. Interestingly, iron has long been associated with endometriosis, especially women with endometrioma. According to Sampson's theory, endometriosis is believed to be originated from retrograde menstruation (blood and tissues), which further accumulate and can implant within the pelvis and/or ovary (endometrioma). Menstrual blood that is rich in haemoglobin is the main source of haem. Accumulation of 'old blood'

within the cavity of ovary means there will be an abundant supply of haem that may undergo Fenton Reaction and produce more hydroxyl radicals.

Based on this, two recent publications have examined the content of iron and ferritin in FF with relation to the endometriosis/endometrioma [39, 40]. They found higher iron concentration in FF obtained from ovaries containing endometrioma [39] particularly in close proximity of the cyst [40]. In this experiment, I have quantified total iron concentration (Fe^{2+} and Fe^{3+}) in FF from both groups, to correlate with the ROS level and individual maturation rate.

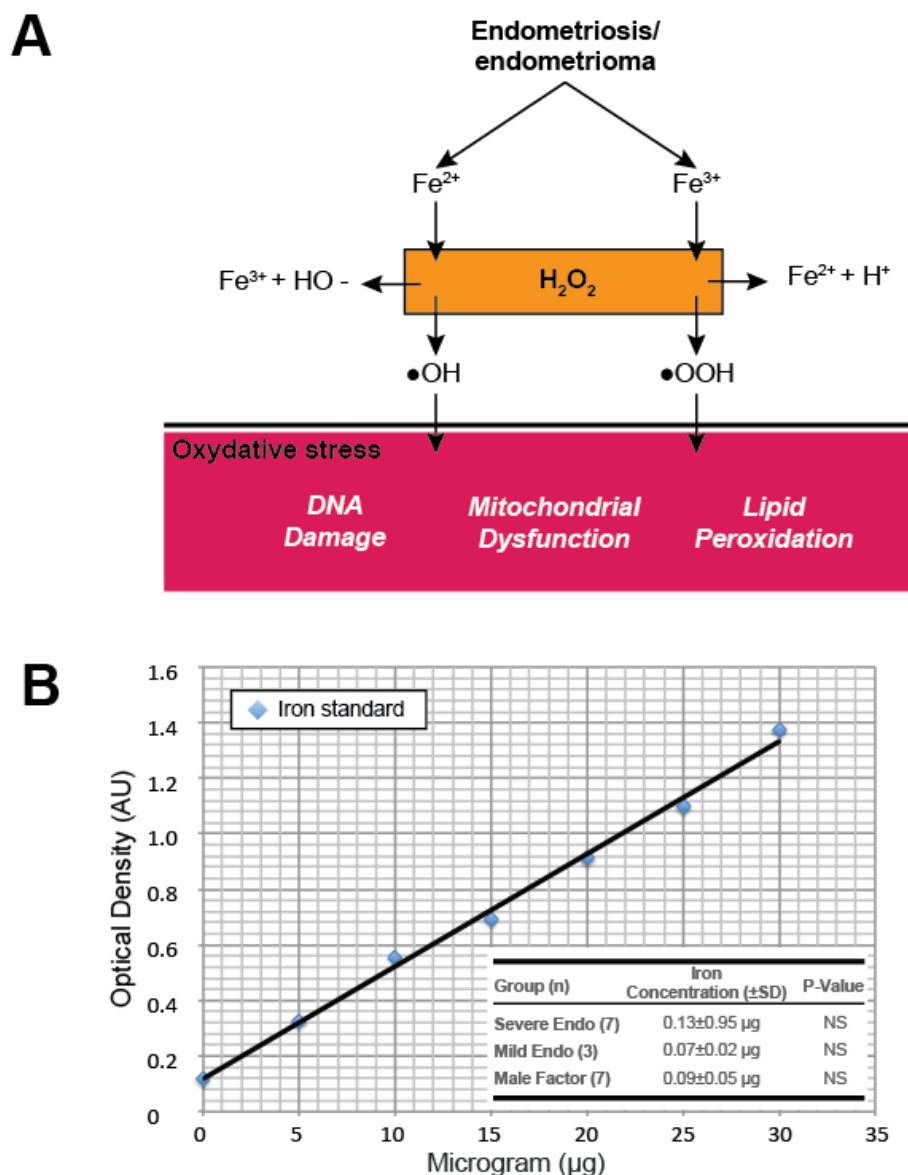


Figure 4-16 Fenton reaction hypothesis and Iron Quantification

A: Women with endometriosis/endometrioma are hypothesised to have high abundance of Haem (Fe^{2+} and Fe^{3+}), which will undergo Fenton reaction (Orange circle) producing Fe^{3+} and reactive/damaging hydroxyl particles ($\cdot\text{OH}$ and $\cdot\text{OOH}$). These particles can cause oxidative stress induced nucleic acid disruption and lead to DNA damage. It also causes mitochondrial dysfunction and lipid peroxidation. **B:** Standard curve of standard iron measurement, with the inset table showing mean iron concentration of FF from different groups. All concentration was below detectable value. P-Value calculated using Analysis of Variance (ANOVA).

For this experiment, I have performed 2 different techniques (method section) of measuring iron; 1) using the Iron detection kit (Sigma Aldrich), and 2) using ferrozine based lab-made kit. Using both techniques, iron was not detectable in any of the male factor or endometriosis follicular fluids and there was no difference ($P < 0.05$) between groups (SE-FF, 0.13 ± 0.95 ; ME-FF, 0.07 ± 0.02 ; MF-FF, 0.09 ± 0.05 , Figure 4-16B). From this experiment, I was not able to replicate the findings from the previous publications. Therefore my secondary aim for this experiment, to correlate with iron level to ROS content was abandoned.

4.9 Discussion

FF of women with endometriosis was found to have a detrimental impact on the maturation rate of mouse oocytes and caused Metaphase I arrest. To date, this is the first study that has used a mouse model to examine the effect of FF on maturation rate, similar findings were previously reported using bovine oocytes [170]. In this study, oocyte maturation rate was found to be concentration dependent. This is in contrast to the earlier publication, by which the authors reported similar maturation rate in 1%, 5% 10% and 15% concentration of FF, and this guided me, at the beginning of my experiments, to use 15% of FF. This current study found markedly reduced maturation at 50% concentration and therefore it was used as the working concentration for the rest of my experiments, to obtain the maximum effect.

Although the maturation rate was low, no effect on the spindle morphology was demonstrated. After overnight incubation, it was found that both arrested and matured oocytes have identical spindle dimensions compared to control groups. This is consistent with studies using human oocytes whereby the authors reported no difference in spindle structure and spindle location in relation to polar body of oocytes from women with and without endometriosis. In animal studies however, several groups reported altered spindle morphology for the same comparison [170, 174]. The main criticism in these studies is that both were using subjective description to assess and grade the spindle. This will potentially result high inter and intra observer variability. In this study the width and length of the spindle was objectively measured, which is more robust and accurate.

At this point the hypothesis that the abnormal spindle structure causes poor maturation rate is excluded. An alternative hypothesis was therefore sought and a series of experiments were then performed to examine the mechanism behind the maturation arrest in the endometriosis group. Based on the earlier findings, the working hypothesis was that FF in women with endometriosis affects the progression from Metaphase I to Anaphase I by the continuous activation of the Spindle Assembly Checkpoint (SAC). By switching off SAC function, using MPS1 inhibitor, Reversine, the maturation rate was found to return to normal and similar to control group. This has led to the conclusion that the SAC remained activated in women with endometriosis, which prevented the transition from Metaphase to Anaphase stage. It is known that SAC will be deactivated in the absence of signal from unattached kinetochore. This mechanism is ubiquitously present in both mitosis and meiosis, although the mechanism is less effective in meiosis [218].

One known factor that can influence the SAC in oocytes is the DNA damage Response, machinery that is activated in response to DNA damage [185, 220]. Rescue of the maturation rate to control levels was found after inhibiting the DNA damage response pathway by adding ATMi. In keeping with this finding, FF of women with endometriosis was also found to cause more DNA damage to mouse oocytes compared to control FF. No previous study had quantified the DNA damage caused by FF in oocytes.

Damage to the DNA can be induced exogenously and/or endogenously [220]. It is known that DNA damage can be instigated by ROS, which is strongly associated with endometriosis [230]. In agreement to this, a higher intracellular ROS in oocytes incubated in FF of women with endometriosis was found. Although many studies have directly examined the content of ROS in the FF, there is however limited study examining intracellular ROS. Previously, several human studies have quantified ROS within granulosa cell [60, 234, 235], out of which only one study [60] compared women with and without endometriosis. This study found higher ROS in granulosa cell of women with endometriosis and was associated with low fertilisation rate and poorer embryo quality. To my

knowledge, this study reports novel findings that have not been reported before, albeit the findings were based on experiments conducted in an animal model.

Whilst there are established association between endometriosis and ROS and the effect of anti-oxidative treatment, this is the first study to use Resveratrol in the culture media with FF. In an animal study, mice injected daily with Resveratrol were found to have a higher response to ovarian stimulation and produced better quality oocytes [236]. Apart from direct injections, Resveratrol was found to improve embryonic development when supplemented in the culture media [228]. A recent study using N-Acetyl cysteine (NAC) as an addition to the incubation media has also shown to restore normal spindle structure [210] induced by endometriosis. But this later study reported similar oocyte maturation rate when incubated with media added with FF of women with and without endometriosis.

One potential contributor to ROS production in FF is iron. Contrary to my hypothesis, iron content was found to be undetectable both in controls and the experimental groups. I speculated that iron in FF would trigger the Fenton Reaction and produce ROS. The findings, that there was no difference found in iron levels between endometriosis and control groups, conflict with work by Benaglia and colleagues [39], who found higher ferritin, but not iron concentration in FF collected from ovaries with no endometrioma (N=39, Ferritin $p=0.026$, total iron $p=0.77$) compared to the contralateral ovary without endometrioma. FF collected in the mentioned study however was pooled collectively during oocyte retrieval procedure, and may have been contaminated with blood. Whereas in this study, I collected FF aspirated from the first follicle and minimised the exposure to blood. Moreover only one patient in this study had an existing endometrioma found during the TVOR procedure. In a study [40] which has examined individual follicles aspirates, higher total iron and ferritin were found in women with endometriomas (N=13, Ferritin $P=0.027$, total iron $P=0.009$) especially in close proximity to the endometrioma. They also reported a lower number of oocytes retrieved adjacent to the endometrioma.

The work presented here has several translational potentials. The utilisation of techniques such as in-vitro-maturation (IVM), whereby immature oocytes are

collected and cultured in vitro, could theoretically eliminate the risks of prolonged exposure of developing oocytes to the FF which is now known to have a detrimental effect on DNA and hence maturation. Clearly such a strategy will need to be balanced against the availability of laboratory expertise and skills, given that IVM is not a widely practiced method in most IVF laboratories, and the number of non-stimulated cycles that would be required could be financially onerous, this approach will have to be assessed through future cohort as well as randomised controlled trials. The supplementation of activators and inhibitors within the media for the culture of oocyte and embryos is an active area of research. The addition of Resveratrol has been shown in this thesis to be effective in reversing adverse effects of ROS on the mouse oocyte [236], but clearly, for translational purposes, this work will need to be evaluated on human oocytes prior to being used clinically.

A further strand of future work could explore the use of maternal dietary supplementation such as anti-oxidants to alleviate the impact of the disease. Dovetailing the concept of the Development of Health and Disease (DOHaD) [237], dietary supplementation could have a far reaching role in the integrity and health of the oocyte from at the time of conception. Several studies have inconsistently shown positive impact of anti-oxidants on reproductive outcome in women with endometriosis and this concept should be further explored [238, 239].

This study has stringent criteria for patient recruitment and therefore has low number of patients recruited. Additionally, FF was collected exclusively from the first follicle punctured from each oocyte retrieval procedure to avoid contamination with blood and flushing media. Therefore limited fluids were collected, hence only several aliquots were stored per patient. Because of limited availability of FF, repeat experiments using the same patient sample were limited. However the maturation rate was similar across different patients within the same group. As factors in FF can mediate the resumption of meiosis [240] irrespective PDE3A inhibition by Milrinone, some oocytes managed to move on into meiosis I stage. Therefore experiments that required assessment at GV stage (i.e. DNA damage, or ROS assessment) were repeated to get an

acceptable sample size. Finally, as in any animal model experiment, results from this study cannot necessarily be extrapolated to human, and further study using human oocytes is required.

4.10 Conclusion

In conclusion, incubation of mouse oocytes in FF from women with endometriosis causes increased metaphase I arrest. MI arrest is reversible by inhibitors of oxidative stress, the DNA damage response pathway or the SAC. I have demonstrated a pathway involving ROS mediated activation of the SAC by increased DNA damage as the cause of lower oocyte maturation in endometriosis.

Chapter 5: Proteomics as a diagnostic tool for endometriosis

The search for a reliable biomarker to enable the early diagnosis of endometriosis has been the focus of research for many years [73, 74, 241-250]. The advent of new, reliable and promising technology in the proteomics arena has brought the field of biomarker discovery one step closer to an array of markers that could eventually be validated and sufficiently robust to be translated into clinical use. The first half of this chapter will provide an overview of the current literature on biomarkers for endometriosis, and critically appraise the literature in this field. The techniques utilised for the proteomics analysis will also be discussed. This will form the theoretical basis of the subsequent half, where the results of the proteomic analysis of the endometrium will be presented.

5.1 Biomarkers in endometriosis

Women with endometriosis are usually present with multiple non-specific presenting complaints. Endometriosis is often diagnosed several years after the patients present with symptoms [9, 23, 172]. The delay of the diagnosis is a significant financial burden to the health system [19], and is detrimental to the reproductive health of women (e.g. longer term impact on oocyte, embryo quality and implantation), on which some of the biological impacts are potentially irreversible.

A new and reliable diagnostic tool is now crucially required to facilitate the accurate and timely diagnosis of endometriosis. In the field of modern medicine, much focus is now placed on biomarker discovery in order to find a reliable diagnostic or screening tool for endometriosis. A biomarker is a biological measure that is objectively quantified and evaluated as an indicator of normal biological or pathogenic processes, which also includes pharmacological responses to a therapeutic intervention [251]. An ideal biomarker should provide high sensitivity and specificity of a disease that can reliably be used for both prognostic and predictive purposes [252]. No such biomarker currently exists in the field of endometriosis.

A biomarker discovery process can be divided into four main phases [253]. Phase 1 is the preclinical discovery phase whereby the potential candidates for biomarkers are identified, which are then subsequently developed and validated in phase 2. In this second phase, non-invasive diagnostic tests are performed and the diagnostic power of selected biomarkers is validated in an independent data set. Phase 3 is the prospective clinical validation phase whereby the proposed test is translated and evaluated in the clinical setting. In this phase, diagnostic accuracy and predictive value are established using an independent set of patients, which include involvement of patients across different health care and geographical setting. Lastly, commercial assays are developed and tested by the industry in phase 4. The studies available in the literature are mainly in phase 1 or in the early stage of phase 2.

5.2 Serum and plasma biomarkers

Research has been performed on several tissue and biological fluid types. As the endometrium is closely related to the aetiology of the endometriosis, it is highly plausible that the endometrial tissue can be used for biomarkers discovery, even though patients need to undergo an outpatient procedure in order to have their endometrium sampled. Conversely, whilst serum or urine are subjected to systemic circulation (blood or urine) before being detected peripherally, they are considered fairly non-specific and are often more difficult to interpret, although they can be obtained relatively non-invasively.

The proposed biomarkers from the literature for endometriosis from the serum and plasma samples are often grouped according to their biological role(s). As an inflammatory disease, biomarkers for endometriosis were predominantly markers of inflammation. Pro-inflammatory cytokine such as interleukin-6 (IL-6) is linked with endometriosis [204, 254-257] although some publications did not find the linkage [258-260]. The discrepancies between the findings may be due to the variation of laboratory assays, and the differences in control group selection ranging from unexplained to tubal factor, and those with proven fertile to healthy control. There are conflicting opinions whether interleukin-8 (IL-8), a chemokine responsible for recruiting and activating neutrophil, can be associated with endometriosis. Some published studies showed that there is no

difference [255, 261] in IL-8 levels between women with and without endometriosis and even though other studies reported increased level in endometriosis group [262, 263]. Other cytokines that were associated with endometriosis include Tumour Necrosis Factor- α (TNF- α) [255, 258, 262, 264-266], Monocyte Chemotactic Protein I (MUC-I) [262, 267-269], Interferon gamma [267, 270, 271], and Interleukin-1 (IL-1) [204, 254, 258].

Although it is hypothesised that women with endometriosis have a different level of immune cells and response to controls, the total immunoglobulin levels in women with endometriosis was found to be similar to those without endometriosis [272, 273]. One study found lower IgA and IgG level in endometriosis [274]. In addition, anti-endometrial antibodies were shown to be present at a higher level in women with endometriosis [274-279], although the sensitivity and specificity were low negating its routine use clinically. The levels of T-cell [271, 275], B-cells [280-283], Natural Killer cells [280, 282, 283], macrophages [282] and polymorphonuclear neutrophils [282, 283], were not significantly different in women with or without endometriosis. Other diagnostic markers such as the glycoprotein CA-125 has been associated with endometriosis [259, 267, 284, 285], but it's diagnostic sensitivity and specificity were low [257, 286, 287], even though its levels were found to be higher in women with more severe disease and those with co-existing endometrioma [288]. Other glycoproteins that had been investigated include CA19-9, CA15-3 and CA-72.

The factors involved in cell adhesion had been widely studied too. In the receptive endometrium, the presence of cellular adhesion molecules are important for implantation process, however, excessive levels of these molecules in blood and serum may suggest the invasiveness of the disease. The increased levels of intracellular adhesion molecules-1 are not inconsistent to be associated with endometriosis [270, 289, 290]. Pro-angiogenic factors such as vascular endothelial growth factor (VEGF) were uniformly found to be higher in the serum of women with endometriosis compared to those without endometriosis in some studies [291, 292] but not significantly different in others [293]. Serum soluble class I and II Human Leukocyte Antigen (HLA) levels

were found lower in women with endometriosis (with and without the family history of endometriosis) when compared to healthy controls [292].

Vodolazkaia et al. 2012, in an analysis of 28 selected biomarkers using multiplex and single immunoassay technologies found four diagnostic biomarkers of endometriosis (Annexin-V, VEGF, CA-125 and sICAM-1/or Glycodelin) from plasma taken during menstruation phase [294]. The authors quoted the sensitivity of 81–90% and a specificity of 63–81%. The findings from this study support the literature that certain glycoproteins such as CA-125 is known to be elevated during the menstrual phase of women with endometriosis [295]. The study, however, did not evaluate if these markers vary during the rest of the menstrual cycle (follicular and luteal phases), according to hormonal use or with the time of the day. It is known that serum IL-6, amongst many other cytokines and inflammatory markers exhibit significant diurnal variability [296]. Furthermore, even if verified and validated, a test that can only be performed during the menstrual phase is likely to pose substantial practical and logistical implementation issues, potentially deterring its widespread use. The control group of the study was also only based on the laparoscopic exclusion of endometriosis without histological evaluation, subjecting the study to further bias. Despite these limitations, the group proposed using the same model to predict endometriosis in patients scheduled for laparoscopy in the absence of any ultrasound findings of endometriosis, however, it was acknowledged that more robust validation studies are required prior to its clinical introduction.

More than 100 putative peripheral (blood, serum, urine) biomarkers from 161 published studies were reviewed by May et al in 2010 [62] and the authors did not manage to identify a single biomarker or a panel of biomarkers that have unequivocally been shown to be clinically useful. The review paper, however has recommended further research before biomarkers able to be implemented in routine clinical care and suggested the use of panels of markers rather than a single marker, to increase the sensitivity and specificity of the diagnostic test for endometriosis.

Despite the exponential increase in the number of publications exploring the usefulness of peripheral biomarkers (serum, plasma and urine) in the diagnosis of endometriosis, researchers are still unable to arrive at any consensus [8, 62].

The varying and conflicting results of biomarker studies to date are attributed to the lack of well-characterised clinical data, non-standardised clinical and laboratory protocols and small sample sizes [252]. A recent global initiative spear headed by the World Endometriosis Research Foundation (WERF) provided some harmonisation guidelines in improving the comparability of endometriosis studies by providing standardisation and recommendations for surgical data and sample collection, non-clinical and epidemiological data collection, and biological fluid, tissue and sample collection processing, and long-term storage to enable later sample analysis [297, 298]. The recommendations form the basis of the methods used in the following chapters of this thesis.

Although a huge selection of potential markers had been investigated in the serum and plasma domain, many are too non-specific whilst others require verification and validation prior to clinical use. Given the issues discussed above related to the high level of heterogeneity in the studies on biological fluids (serum, plasma and urine) to date, it is unlikely that a reliable and accurate non invasive biomarker derived from the studies examining the serum or plasma alone will be imminently available to aid the diagnosis, elucidate the natural history or predict treatment efficacy related to lesions or symptoms of endometriosis. In the next section, the available markers from endometrium tissues analysis will be discussed.

5.3 Endometrial biomarkers and implantation in patients with endometriosis

A successful implantation process requires the successful crosstalk between good quality embryo and a receptive endometrium. Absence of one or more factors during the process may lead to implantation failure. In endometriosis, many key implantation markers within the endometrium were consistently found to be dysregulated (Table 5-1) and hence, the analysis of the endometrium is proposed to be a closer reflection of the condition than other biological fluids. Furthermore, an endometrial biopsy is a common gynaecological outpatient procedure. The details of the procedure are described later in this chapter. The process of implantation begins with a dialogue between the embryo and the

endometrium, followed by the apposition and attachment of the embryo to the receptive endometrium. The attached embryo subsequently invades the stromal layer, before the endometrium start to decidualise [299, 300].

Endometrial-embryo crosstalk

Several studies have reported conflicting results on the levels of the cytokines, in particular interleukins (IL-6 and IL-8), which are considered important factors in the key endometrial signalling pathways of implantation [246, 301-303]. For instance, Kyama et al, [246] found that whilst IL-8 levels were reported elevated, but IL-6 levels were not. However, other factors that are involved in endometrial-embryo cross talk were consistently found lower in endometriosis. These include Apolipoprotein-AI [304], an important factor for embryo signalling, and Activin A that binds to the receptors at the blastocyst level during implantation process [305].

Attachment

Cellular adhesions molecules (CAM) such as osteopontin and its integrin, $\alpha v \beta 3$ facilitate embryo and endometrium attachment. Both factors were reported to be lower [306-308] in eutopic endometrium of women with endometriosis although several other publications reported a higher expression [309, 310]. Miller et al. 2010 [311] found an altered glycosylation in selectins ligands, which plays an important role in ligand–receptor interaction between the blastocyst and the endometrium during the implantation process. This study however has exclusively examined women with more severe stages of endometriosis (Stage III-IV) and only analysed nine samples from women with endometriosis. Apart from CAM, factors that involved in embryo attachment such as MUC1 was also found lower in endometriosis. MUC1-gene, which encoded cell surface associated (MUC1) or polymorphic epithelial mucins (PEM) were found downregulated in the endometrium of women with endometriosis [312].

Implantation Stage	Molecular Group	Molecular Markers	Findings in endometriosis	Reference (Year)
Embryo-Endometrium Dialogue	Activin	Activin A	↓	Stoikos 2010
	Apolipoprotein	Apolipoprotein-E	↓	Sundqvist 2012
		Apolipoprotein-A	↑	Brosens 2010
	Cytokine	IL-8	↑	Kyama 2006, Ulukus 2009
			↔	Luk 2005
		IL-6	↓	Ponce 2009
			↔	Kyama 2009
Embryo Attachment	Cellular Adhesion Molecules	αvβ3	↓ or absent	Donaghay 2007, Khoram 2002
			↑	Cassal 2012, Kyama 2008, Hii 1998
		Osteopontin	↓	Giudice 2001, Wei 2009
			↑	D'Amico 2014
	Mucins	MUC 1	↓	Burney 2006
Embryo Invasion	Glycoprotein	Glycodelin A	↓	Giudice 2001, Wei 2009, Burney 2006
	Cytokine	LIF	↓	Dimitriadis 2006, Giudice 2001, Wei 2009
		MMP	↑	Chung 2002, Uzan 2004, Di Carlo 2009, Sotnikova 2010
Endometrial Decidualisation	Gap Junction protein	Connexin-43	↓	Yu 2014
	Growth factor	IGFBP-1	↓	Burney 2006
	Homeobox protein	HOXA10	↓	Matsuzaki 2010, Sczepenska 2010, Donaghay 2007, Eun Kwoon 2004, Vitello 2007, Taylor 1990
	Neuronal protein	PGP9.5, VR1, NGFp75	↔	Newman 2013
	Cytokine	IL-11	↓	Dimitriadis 2006
		TIMP	↔	Collette 2006, Ramon 2005, Uzan 2004

Table 5-1 Table of analysis of endometrium

The above table shows the various markers and factors that have been found either up or down regulated in endometrium of women with endometriosis. Cell surface associated (MUC 1), Homeobox 10 (HOXA10), and Insulin-like growth factor-binding protein, Leukocyte Inhibitory Factor (LIF1). Interleukin (IL), Matrix Metalloproteinase (MMP).

Invasion and endometrial decidualisation

The factors involved in embryo invasion were also found to be dysregulated. For instance, Leucocyte Inhibiting Factor (LIF), a key implantation factor in human, was found to be lower in the endometrium of women with endometriosis [307, 308]. Glycodelin A, a key immune response regulator for implantation and embryo invasion was also found to be lower in endometriosis, which is in contrast to the findings from the serum whereby it was found to be higher.

Connexin-43, a protein that is involved in endometrial decidualisation process was found to be significantly lower in the endometrium of women with endometriosis compared to controls [313]. IGFBP-1, a gene that encoded Insulin-like growth factor-binding protein 1 (IBP-1) was also found to be lower in women with endometriosis. A potent marker for endometrial receptivity, Homeobox A10 (HOXA10) marker was consistently reported lower in several publications.

The analysis of endometrial biopsy samples have shown morphological, cellular and molecular distinctiveness in the endometrium of women with endometriosis compared to those without endometriosis, although researchers have so far are not successful in translating the latter knowledge into clinical practice. For example, the aberrant neuronal growth within the uterus was identified as a factor that may contribute to abnormal fertility and uterine dysfunction [314]. It was proposed that the analysis of innervation in endometrial biopsy samples as a less invasive diagnostic tool for pelvic pathology such as adenomyosis and/or endometriosis [315]. However, whilst the results of this test were initially promising, more recent studies have shown that an endometrial biopsy alone is an inaccurate and unreliable diagnostic tool for endometriosis [244, 249].

Hence, surgical intervention such as laparoscopy remains the only definitive way of diagnosing endometriosis although surgical inspection of the pelvis is still subject to considerably high false positive, even coupled with histological examination via a peritoneal biopsy [8].

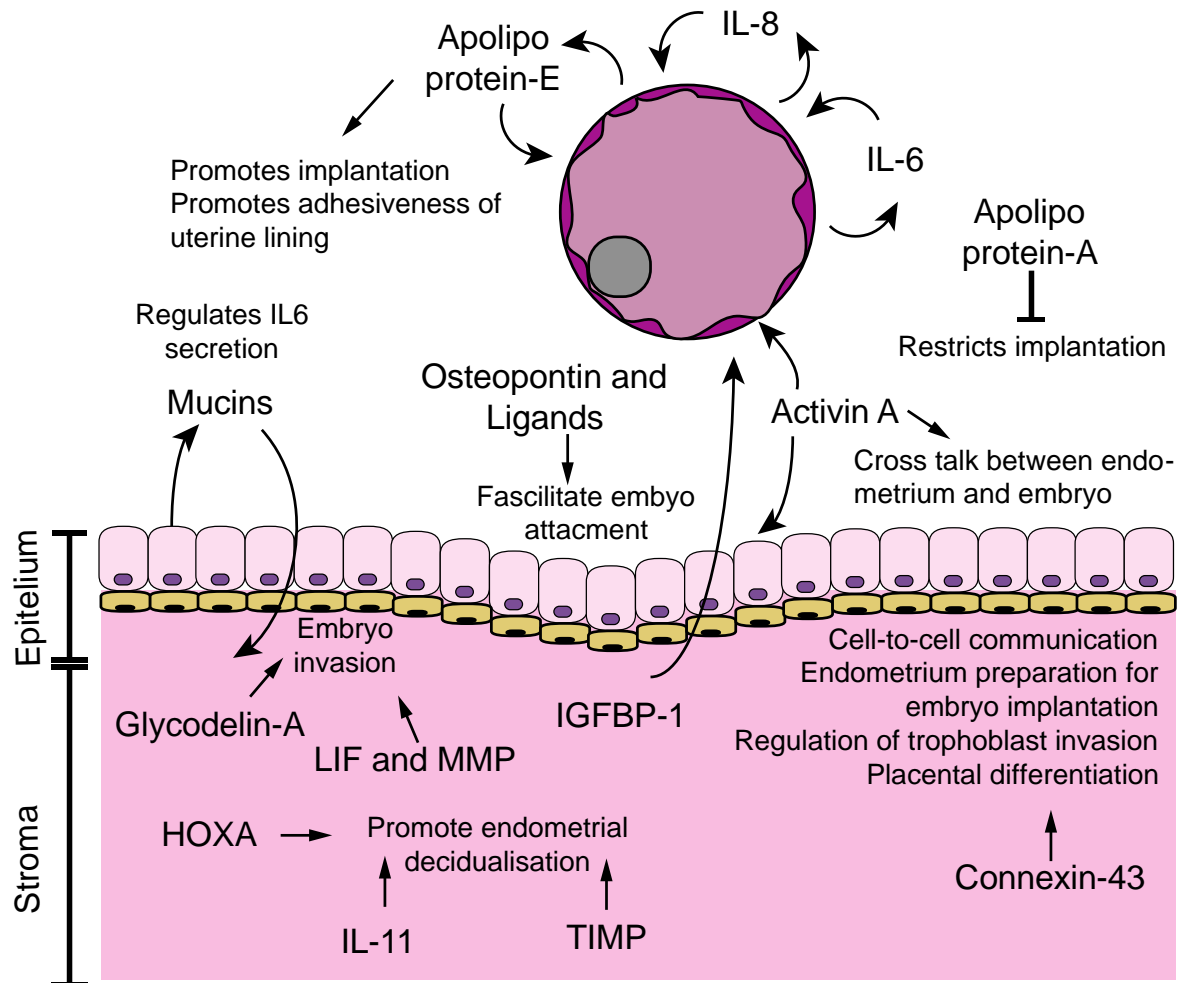


Figure 5-1 Potential factors contributing to implantation failure in women with endometriosis

In a normal implantation process, a cascade of biochemical reaction takes place within embryo and the endometrium during the implantation window. However in endometriosis, implantation failure can be due to dysregulated one or more of the factors involved in the process. This interaction involves cytokines, hormones, growth factors, cellular adhesion molecules, and gap junction protein.

Despite decades of effort, single biomarkers generally lack the specificity and sensitivity required for clinical routine use. Due to the clinical heterogeneity of endometriosis, biomarker discovery is moving away from seeking one idealised endometriosis-specific biomarker in favour of a panel of markers. The –omics technology offers a more global and comprehensive analysis of whole tissue or blood and has gain popularity in the recent years as a biomarker discovery tool [183]. In the next part of this thesis, a systematic review of the various proteomic studies on endometriosis for biomarker discovery in endometriosis will be presented.

5.4 Objective

This chapter is set out to systematically review the available literature on proteomics as a possible diagnostic tool for endometriosis.

5.5 Method

PUBMED was searched for articles published in the English language up to December 2015, including both clinical and experimental studies, using the following MeSH terms: ‘endometriosis’ or ‘endometrioma’ AND ‘proteomics’ or ‘protein study’ OR ‘biomarkers’. Initial screening of the title and abstract was performed, and published studies/articles considered irrelevant to this review were excluded. The reference lists of the selected articles and from other review were also evaluated. Data were extracted and documented systematically in the form of a table.

5.6 Overview of proteomic analysis

Proteins are important because they are the direct bio-functional molecules in living organisms. The term “proteomics” was coined from merging “protein” and “genomics” in the 1990s. Proteomics describes efforts to identify and quantify all the proteins of a proteome, including expression, cellular localisation, interactions, post-translational modifications (PTMs), and turnover as a function of time, space, and cell type, thus making the full investigation of a proteome more challenging than sequencing a genome.

Proteomic is a large-scale study of proteins that enable qualitative and quantitative examination of the total protein profile in a tissue or fluid. Up to 10,000 proteins (2,000-5,000 in serum/plasma; 7,000-10,000 in tissues) can be identified in a single sample. However, it is recently possible to segregate lower abundance protein for more targeted analysis.

Whilst complementary to genomic and transcriptomic, proteomic has the potential to provide considerably more data compared to the former. A gene (or its sequence) does not always transcribe to a protein. The impact of epigenetic on post-translational protein production is also significant. Genetic analysis is therefore less representative as diagnostic markers than proteomic analysis because in proteomic studies, the actual production of protein is measured and will be more indicative of the disease. On the other hand, metabolomics, as the final downstream product of gene transcription provide the closest to the phenotype of the biological system studied. Metabolomes are however, more diverse, containing many different biological molecules causing more complex analysis and interpretation issues [316].

5.7 Proteomic analysis techniques

Currently, there are several methods used for the study of proteins. Proteins are identified using antibody (immunoassays) or antibody-free techniques (mass spectrometry). By using antibody technique, proteins are detected quantitatively through enzyme-linked immunosorbent assay (ELISA) or individually through Western blot technique. By using antibody-free techniques, proteins are detected by mass spectrometry with or without prior segregation of samples using one/two-dimensional separation, or through chromatography.

The progress of proteomics has been steered by the development of new technologies for peptide/protein separation, mass spectrometry analysis, isotope labelling for quantification, and bioinformatics data analysis. Mass spectrometry is an analytical chemistry technique that can distinguish the quantity and the type of chemicals content in a sample by measuring the mass-to-charge ratio and abundance of gas-phase ions, and has emerged as a core tool for large-scale protein analysis [317]. In the past decade, there has been a

rapid advancement in the resolution, mass accuracy, sensitivity, and scan rate of mass spectrometers used to analyse proteins.

Proteomics analysis strategies are generally divided into bottom-up, top down and middle down techniques. Bottom-up proteomics (also known as shotgun proteomics) is used to distinguish a protein by analysing its peptides or the peptide sequence. It is an indirect measurement of proteins using peptides extracted from digested proteins. In this technique, peptide mixture is usually fractionated and subjected to LC-MS/MS analysis. Tandem mass spectra generated from peptide fragmentation are compared with theoretical spectra generated from in silico digestion of a protein database. Identified peptide sequences are then assigned to proteins for protein inference [318]. In contrast, top-down proteomics utilised intact proteins for protein analysis, without prior proteins digestion. This technique provides a more representative analysis of post-translational modification of a protein. It has been reported that more than a thousand proteins can be identified using multidimensional separations from complex samples. As this technique uses undigested protein, it has a limited analytical ability because in the gas phase of a spectrometer, an intact protein is more difficult to be fractionated, ionised, and fragmented [318].

To balance the pro and cons of both techniques, middle-down proteomics is then introduced. This technique analyses larger peptide fragments than bottom-up proteomics to minimise peptide redundancy between proteins. With the larger peptide fragments, it is more representative of post-translational modifications and avoid the analytical challenges of examining intact proteins [318]. Among all the three techniques, bottom-up proteomics has become the main technique for the analysis of proteins and their modifications.

5.7.1 Preparation of complex protein mixture

Prior to protein analysis by mass spectrometry, samples need to be procured by several steps in a specific sequence. This process begins with extraction and isolation of protein from chemical and physical interactions with other biomolecules. Application of this subcellular isolation technique allows global analysis of proteins within the subcellular compartment. Subsequently, the extracted and isolated proteins are selectively depleted to known high or low abundance protein using chemical-based approach (e.g. ethanol, acetonitrile

and TCEP) or antibody based, in which the latter is more expensive, although relatively more effective. Alternatively, the protein abundance dynamic range can be adjusted using combinatorial ligand libraries, which can provide a cheaper and more holistic approach [318]. The depleted proteins are then digested by a proteolytic digestion step, whereby biochemical heterogeneity of proteins within a sample as peptides is normalised and compartmentalised. Theoretically, this will create a less heterogeneous mixture when protein splices isoforms and post-translational modifications.

5.7.2 Separation and fractionation of complex protein mixture

The complex mixture of protein is subsequently separated using either gel-based or gel-free protein separation techniques. Although gel-free is a well-established technique for proteomic analysis, due to high gel-to-gel discrepancy and labour intensiveness, this technique has been gradually superseded by gel-free techniques namely protein chip array and liquid chromatography based protein/peptide separation techniques.

For gel-based technique, proteins can be separated by polyacrylamide gel electrophoresis (2D-PAGE) based on its molecular mass and charge. Due to the low dynamic range and significant gel-to-gel variation, this technique has now been largely replaced by 2D difference gel electrophoresis (2D-DIGE), which provides more accurate and sensitive results. 2D-DIGE can run multiple samples in one gel and avoid running differences between gels. It can also differentially label the protein with fluorescent tags in gel-based techniques and strategies of isotopically coded affinity tag (ICAT). Another frequently used gel-based method is 1D-SDS-PAGE that can separate protein based on their molecular weight. Although gel-based techniques can differentiate splice isoforms and are able to remove low molecular weight impurities, this however limit the sensitivity. Although the analysis is more focus on those important for cell regulatory function [319, 320], the excluded lower abundance proteins can contain important markers and may be missed. Therefore gel-based techniques have become a less popular technique.

The gel-free technique on the other hand has increased the comprehensiveness of proteomic analysis by using chromatofocussing and size exclusion chromatography (SEC). Protein can also be separated and

fractionated by liquid chromatography based on its charge, size and hydrophobicity. This technique can be coupled with Mass Spectrometry (online) or fractions collected for later analysis (offline). Even though these established protein separation techniques are cheap and quick, they have poor sensitivity and are lacking in reproducibility.

Protein microarrays (protein chip) represent a recent advancement in technology in this field. This technique has replaced traditional proteomics techniques such as 2D gel electrophoresis that were time-consuming, labour-intensive and not suitable for the analysis of low abundant proteins. It is a separation step for the protein according to the surface and is usually coupled with MALDI. Using different surfaces (Surface Enhance Laser Desorption Ionisation-Time of Flight, SELDI), some protein will be bound, while the others will be removed by washing. The commonly available surfaces used in the proteomic analysis include CM10 (weak-positive ion exchange), H50 (hydrophobic surface, similar to C₆-C₁₂ reverse phase chromatography), IMAC30 (metal-binding surface), and Q10 (strong anion exchanger).

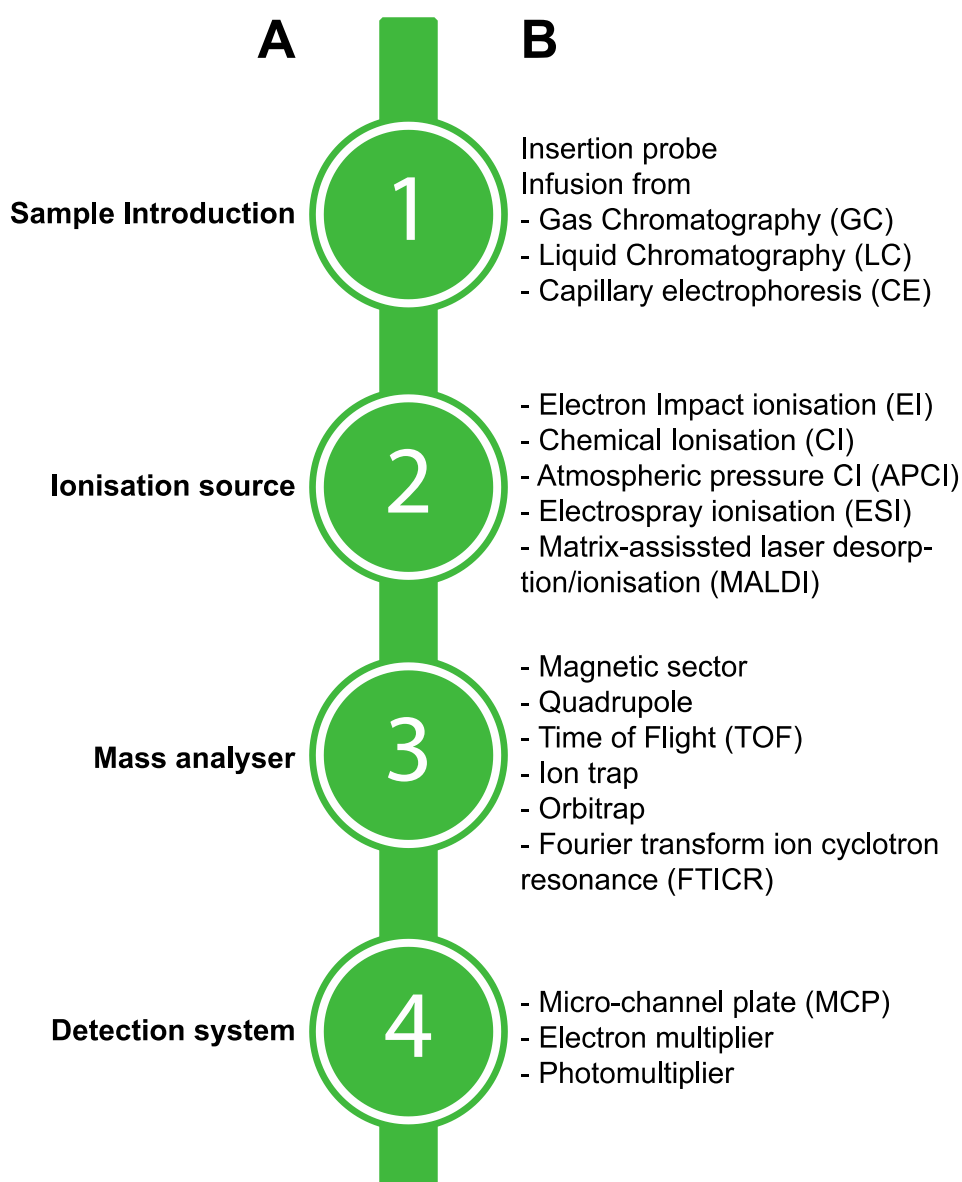


Figure 5-2 Flow chart of Mass Spectrometry

A: Four important steps of mass spectrometry **B:** Various options of techniques and analysis for each step. With the availability of various options, there is a spectrum of different combinations of the components to choose from for analysis.

5.7.3 Mass Spectrometry Analysis

In mass spectrometry analysis, the mass-charge ratio of charged particles is measured. It has several important elements (Figure 5-2). The first element involves sample transformation to gaseous phase from solid/liquid samples, which depends on the sample introduction technique. The second element involves the ionisation of the diffused and vaporised samples by ionisation device. Ionisation sources include electrospray ionisation (ESI), matrix-assisted laser desorption/ionization (MALDI), electron impact ionisation (EI), chemical ionisation (CI), and atmospheric pressure chemical ionisation (APCI). The third element involves the passage of the ionised sample from the atmospheric pressure to the high vacuum and movement towards the detector by electrostatic force or called mass analyser. The mass analyser can be quantified using time-of-flight (TOF), ion trap, orbitrap or Fourier transform ion cyclotron resonance (FTICR). The final element involves the detection of ionised sample by ion detector for ion quantification (Figure 5-3).

In addition, there is also a variation on how the samples can be delivered to the device without affecting the vacuum pressure. This includes firstly, the direct insertion technique using an insertion probes, whereby the sample undergoes desorption process such as direct heating or laser desorption to assist the vaporisation and ionisation. Alternatively, the direct infusion technique uses simple capillary column, which has the advantage of using only small amount of sample.

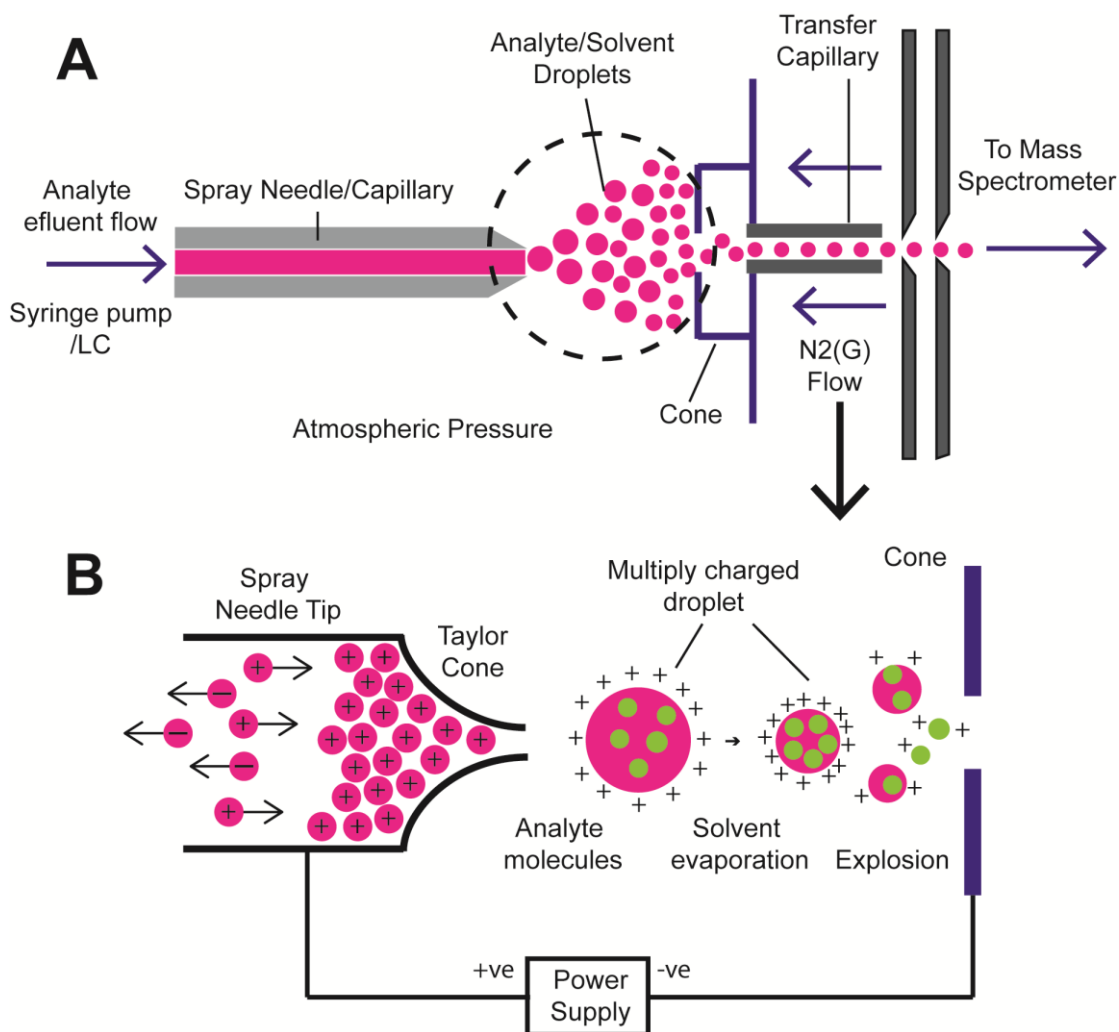


Figure 5-3 Electrospray ionisation schematic diagram

A: In electrospray ionisation the analytes is introduced to the source and passes through the electrospray needle that has a high potential difference applied to it. This forces the spraying of charged droplets from the needle with a surface charge of the same polarity to the charge on the needle. The droplets are repelled from the needle towards the source-sampling cone on the counter electrode (shown in purple). Solvent evaporation occurs as the droplets traverse the space between the needle tip and the cone. **B:** Proteins and peptides are usually analysed under positive ionisation conditions and saccharides and oligonucleotides under negative ionisation conditions.

5.7.4 Quantification methods

Proteome quantification was classically performed using 2-DE, a technique that quantifies proteome by comparing staining densities of proteins on two or more gel. It provides a measure of relative quantification. This method has several disadvantages such as the limited resolution for a large number of proteins, is unsuitable for membrane proteins, and ineffective in analysing low abundant proteins in a high dynamic range of samples. This technique has been substantially substituted by MS-based methods using either stable isotope labelling; by measuring individual sample based on ion intensity of peptides, or label-free approaches, by measuring absolute or relative protein abundance based on the intensities of different isotope coded peaks, which is differentiated by mass spectrometry.

Using isobaric labelling, peptides were labelled for relative quantification during analysis. This tags consists of three functional groups; 1) Amine reactive group, 2) isotopic reporter group, which both are linked up by 3) isotopic balancer group. There are several features of isobaric labelling which have led to its widespread use in proteomics. For instance, an isobaric labelling strategy is capable of analyzing multiple labeled pools of peptides, up to 6-plex for tandem mass tags (TMT) and 8-plex for isobaric tags for relative and absolute quantification (iTRAQ®), in a single analysis, reducing analytical time significantly. In contrast, all other isotope-labelling approaches are limited to two or three isotope comparisons. Another benefit of using an isobaric label is that it does not increase the complexity of the mass spectrometry scan and does not decrease the precursor signal sensitivity as in stable isotope labelling of amino acids in cell culture (SILAC) and stable isotope labelling of amino acids in mammals (SILAM), since all the tags lead to the same mass increase for each labelled peptide [318].

When compared to other stable isotope labelling, iTRAQ® has the capability of high throughput quantification since multiple samples can be processed simultaneously. Not only more samples can be processed simultaneously, it also avoids comparative analysis across many datasets, hence reduces the analysis timing, inconsistency and the variation [321]. iTRAQ® is an LC-based method offering the flexibility of choosing a wide range of stationary and mobile

phases available to resolve complex biological samples at the protein and peptide level. It is a multiplex, and therefore able to process more sample in one experiment. Although it has good technical reproducibility, labelling at the peptide level is found to cause potential sources of error in earlier sample handling particularly in variable degrees of trypsin action.

5.7.5 Bioinformatics

Bioinformatics is an important aspect of proteomic analysis, particularly with the increasingly complex datasets generated from a more comprehensive MS-based proteomic analysis. However, the integration of bioinformatics tools into proteomics and software packages has simplified and standardised the proteomic analysis [318].

Using bioinformatics, peptide fragmentation spectra generated from the mass spectrometry is matched to the theoretically generated peptide fragmentation spectra based on the database search. This will create a list of peptide candidates for each experimental spectrum, which then ranked and filtered to create peptide spectrum matches (PSMs). PSM are filtered using a statistical calculation of the correlation of the theoretical and experimental spectra, and the incorrect reverse PSMs is used to calculate the false discovery rate (FDR). Identified peptides are assigned to proteins by inference to create a list of proteins present in the sample. The relative protein quantification is then performed by averaging the peptide ratio measurements for peptides assigned to the protein [318].

An ongoing challenge for proteomics is to correctly map the identified peptide sequences to protein sequences. The ability to properly assign peptides to proteins has become even more challenging as proteomics methods/techniques have become more sensitive and comprehensive, with the growing protein databases size. Proteomics experiments have also become more biology-driven, which increases the importance to correctly identify and quantify proteins from peptides. These challenges include the stochastic sampling and identification of unique peptides from protein isoforms (homologous proteins) or unrelated proteins, which share no unique tryptic peptide sequences (redundant proteins) [318].

5.8 Common proteomic analysis techniques used in endometriosis research

Due to multiple options of each element of mass spectrometry and proteomic analysis, there is a spectrum of different combinations of the components to choose from in any one set of experiments (Figure 5-2). Several methods of proteomic analysis in endometriosis that have been described including Two-dimensional Gel Electrophoresis-Time-of-Flight (2DIGE-TOF) which has the advantage of analysing thousands of polypeptide in a single run but on the other hand, requires the handling of a large amount of samples all at once, has limited reproducibility and has not been automated for high throughput analysis.

Matrix Assisted Laser Desorption/Ionisation Time-of-Flight (MALDI-TOF), on the other hand is a mass analysis technique that can rapidly identify proteins isolated by gel electrophoresis, size exclusion chromatography, strong/weak ion exchange, isotope-coded protein labelling (ICPL), and 2D-GEL which offers a quick and easy method of mass analysis using a minimal amount of sample. However, this technique lacks reproducibility where the same analysis protocol can potentially yield different results, especially over time.

Surface Enhance Laser Desorption Ionisation-Time-of-Flight (SELDI-TOF) is a technique that enables analysis of protein mixture, from body fluids or tissue. There are multiple surfaces that enable different protein separation capabilities according to strong/weak ion exchange. Several studies have used more than one surface to improve protein detection. Although SELDI-TOF is a simple and fast technique, it is expensive and requires the removal of the low abundant proteins prior to analysis.

The newer method of proteomic analysis utilises a gel-free technique in combination with liquid chromatography (LC) and tandem mass spectrometry (MS/MS). LC-MS/MS employs a combination of atmospheric pressure ionisation source, ion focusing component, collision chamber, a second mass filtering device, and ion impact detector; the latter is a high throughput, a sensitive and specific method for protein analysis, without the need to exclude the novel low abundance protein [322].

In the field of biomarker discovery, particularly using the proteomic technique, the verification of biomarkers will require 100–1000 samples, whereas the validation of biomarkers requires analysis of even larger numbers (thousands to tens of thousands) [323]. The same assays are used for verification and clinical validation of biomarkers. ELISA is regarded as a general assay method for the later phases. However, the development of an ELISA methods for one or a panel of biomarker(s) candidate is expensive and time-consuming, and the specific antibodies are sometimes unavailable [318].

5.9 Endometriosis-related proteomics studies for biomarker discovery

In endometriosis, proteomic analysis has been performed on several tissue types including the serum, plasma, urine, endometrium, peritoneum, and follicular fluid, however most of these biomarker panels have not been verified or clinically validated [62]. Despite that, the authors of several published reviews on proteomic analysis [253, 324] concluded that proteomic analysis might be used in infertile women with or without pelvic pain to predict the presence of the disease [325].

Blood is an important source of biomarkers as it can be obtained in a non-invasive manner and analysed via high throughput measurements [326]. In this section, proteomic studies using serum, plasma and follicular fluid will be discussed. Since a decade ago till date, there are 13 available publications that have examined serum and plasma using proteomic techniques. Less than half [241, 327-330] of the proteomic analysis used gel-based methods whereas 8 publications [75, 248, 267, 324, 331-333] used protein chip analytic techniques.

Faserl et al. 2011 [327], by using 2D-DIGE MALDI-MS/MS, discovered 25 protein spots with significantly ($P < 0.02$) higher vitamin-D binding protein in women with compared with those without endometriosis. They also found a higher GC*2 allele product in endometriosis and speculated that this gene was unable to activate the phagocytic function to facilitate the implantation of endometrial tissue within the peritoneal cavity.

Techniques	Reference (Year)	Sample size	Cycle phase	Isobaric labelling	Verification
2D-DIGE-MALDI-MS/MS	Faserl 2012	N = 76 EN: 56, (ME: 20, SE: 36) Control: 20	Not mentioned	No	No
2D-MALDI-TOF-MS	Gajbhiye 2012	N = 70 EN: 40, (ME: 17, SE: 23) Control: 30	Not mentioned	No	Yes (ELISA)
	Nabeta 2012	N = 151 EN: 69 (ME: 21, SE: 48) Control: 82	Not mentioned	No	No
	Zhang 2006*	N = 12 EN: 6 Control: 6	Secretory: 12	No	No
Protein chip array-SELDI-TOF-MS	Fassbender 2012	N = 302 EN: 113 (ME: 81, SE: 32) Control: 89	Follicular phase: 76 Luteal phase: 69 Menstrual phase: 52	No	No
	Jing 2009	N = 120 EN: 59, (ME: 29, SE: 30) Control: 61	Not mentioned	No	Yes
	Wolfler 2009	N = 90 EN: 36, (ME: 19, SE: 32) Control: 39	Luteal: 39 Follicular: 51	No	No
	Zhang 2009	N = 80 EN: 48 Control: 32	Not mentioned	No	Yes
	Wang 2008	N = 66 EN: 36, (ME: 22, SE: 14) Control: 30	Not mentioned	No	No
	Liu 2007	N = 68 EN: 36, (ME: 9, SE: 10) Control: 32	Not mentioned	No	No
	Seeber 2008	N = 197 EN: 53, (ME: 22, SE: 41) Control: 78	Not mentioned	No	No
	Wang 2007	N = 32 EN: 16, (ME: 10, SE: 6) Control: 16	Not mentioned	No	No
	Zheng 2011	N = 246 EN: 126, (ME: 50, SE: 76) Control: 120	Not mentioned	No	No
Nano sized magnetic beads MALDI-TOF-MS					
LC-MS/MS-iTRAQ	NA	NA	NA	NA	NA

Table 5-2 Table of various proteomic analysis of serum and plasma

Table showing the summary of available published studies on proteomic analysis according to the techniques used, the number of samples and the stage of the sampling. WB: Western Blotting 2D: 2 dimensional, DIGE: Difference in gel electrophoresis, TOF: time of Light, MS: Mass Spectrometry, SELDI: Surface-enhanced laser desorption or ionisation, MALDI: Matrix assisted laser desorption or ionisation, N: total number of samples, LC: Liquid chromatography, EN: Total number of endometriosis patient, ME: Mild endometriosis, SE: severe endometriosis, MF: Male factor. NA: Not available.

By using 2D-MALDI-TOF MS, verified by Western Blot (using different set of serum samples $n=60$), Gajbhiye et al [328] have identified three endometrial antigens, Topomyosin 3 (TPM3), Stomatin-like protein 2 (SLP2) and Tropomodulin 3 (TMOD 3), and they proposed using these markers for early diagnosis of endometriosis. Using the same technique, Nabeta et al in 2011 [329], found high anti-syntaxin 5 (STX5), which have 53.6% sensitivity and 72.2% specificity for the diagnosis of endometriosis, which was better than using CA-125 alone. However, when STX5 and CA-125 were combined, they reported that the sensitivity was increased to 69.6%. The authors acknowledged that their discoveries require further clinical validation. Almost 10 years ago, Zhang et al 2006 [241], the only study that has combined serum and endometrium in their analysis using 2D-MaDI-TOF-MS, have identified 13 protein spots, which were differentially expressed, out of which 11 were known proteins. Some of the proteins were involved in the regulation of cell cycle, signal transduction or immunological function.

The rest of serum proteomic studies used gel free mass spectrometry, and these studies reported a variable sensitivity and specificity for endometriosis. For instance, Fassbender et al in 2011 [73] reported a sensitivity of 75% and specificity of 86% for the diagnosis of mild endometriosis using a model based on five peptides and protein peaks (range 4.898-14.698 m/z). However, an earlier study done by Seeber et al 2008 [267], found six differentially expressed proteins and by combining monocyte chemoattractant protein-1, migration inhibitory factor leptin and CA-125, the author reported the diagnostic capability could be up to 73% with a 94% specificity.

There are a number of proteomic studies that have analysed follicular fluid (FF) [334-337], however, only one [337] has compared FF of women with and without endometriosis. This study has analysed samples from 21 patients undergoing IVF treatment (Control: 9, Endometriosis: 12). The FF proteins were first separated using 2D electrophoresis and the spots were compared using LC-ESI-MS/MS. This study found 29 differentially expressed spots, out of which 21 proteins were identified. Interestingly, similar to this thesis, they found that the proteins that were discovered showed some functional enrichment for

response to oxidative oxygen species and positive regulation of apoptosis. This was observed regardless of whether the women achieved a pregnancy or not.

Proteomic analysis of blood and serum has resulted in a diverse spectrum of results even when similar technology and techniques were used. This can be largely attributed to heterogeneity in study design, method of analysis, technical variation as well as clinical differences. As studies utilising gel separation technique are now deemed obsolete and have been heavily criticised for being imprecise due to their exclusion of low abundance proteins, more modern techniques such as LC-MS/MS have now largely surpassed the previous proteomics techniques as the next generation biomarker discovery technology. In the next section, the proteomic studies using endometrial tissue will be discussed.

5.10 Proteomic studies on endometrium

To date, 15 publications (Table 5-3) have analysed eutopic endometrium of women with endometriosis for proteomic research. More than half of the studies (n=9) used gel base techniques whereas the rest used the protein chip technique (n=6). One study combined gel and liquid chromatography techniques (Zhang 2010). Only one study used isobaric labelling for the analysis [245]. None of the studies used liquid chromatography with tandem mass spectrometry. All studies, except one [241] had at least one control group for comparison. The majority of the studies have indicated the timing of endometrial sampling according to the phase of menstrual cycle, and only 4 studies have verified their result with less than 100 samples (ranging from 6 to 48 samples, Table 5-4). The majority of the studies have performed immunohistochemistry [72, 243, 250] and/or Western Blotting [243, 250, 338]. None of the study used ELISA for verification/validation and none has used more than 1000 samples, which is the suggested benchmark for biomarker validation [318].

Techniques	Reference (Year)	Sample size	Cycle phase	Isobaric labelling	Verification
2D-DIGE-TOF-MS	Stephens 2009	N = 32 Endometriosis: 18 Control: 14	Not mentioned	No	Yes (IHC)
	Zhang 2007	N = 12 EN: 6, (ME: 2, SE: 3) Control: 6	Secretory: 12 Proliferative: 0	No	No
2D-MALDI-TOF-MS	Jiang 2012	N = 20 EN: 10 Control: 10	Window of implantation	No	No
	Rai 2010	N = 116 EN: 57, (ME: 12, SE: 45) Control: 59	Secretory: 42 Proliferative: 17	No	Yes (WB/IHC)
	Chehna-Patel 2010	N = 20 EN: 9 Control: 11	Not mentioned	No	Yes (WB/IHC)
	Fowler 2007	N = 24 EN: 12 Control: 12	Secretory: 12 Proliferative: 12	No	No
	ten Have 2007	N = 17 EN: 11, (ME: 11) Control: 6	Secretory: 17 Proliferative: 0	No	No
Protein chip array-SELDI-TOF-MS	Fassbender 2012	N = 49 EN: 31, (ME: 16, SE: 15) Control: 18	Secretory: 27 Menstrual phase: 22	No	No
	Kyama 2011	N = 29 EN: 19, (ME: 9, SE: 10) Control: 10	Secretory: 29	No	No
	Ding 2010	N = 53 EN: 24, (ME: 19, SE: 5) Control: 29	Not mentioned	No	No
	Wang 2010	N = 26 EN: 13, (ME: 8, SE: 5) Control: 13	Not mentioned	No	No
	Fassbender 2007	N = 16 EN: 10, (ME: 5, SE: 5) Control: 6	Secretory: 16	No	No
	Kyama 2006	N = 9 EN: 6, (ME: 3, SE: 3) Control: 3	Secretory: 9	No	No
2D-DIGE-MS/MS iTRAQ	Marinowski 2013	N = 8 EN: 7, (SE: 7) Control: 1	Proliferative: 9	Yes	No
1D-LC-MS/MS	Zhang 2010	N = 5 EN: 5, (ME: 3, SE: 2) Control: 0	Secretory: 5 Proliferative: 0	No	Yes (WB)
LC-MS/MS-iTRAQ	NA	NA	NA	NA	NA

Table 5-3 Table of various proteomic techniques on the endometrium

The above table shows a summary of the available published studies on proteomic analysis according to the techniques used. WB: Western Blot, IHC: Immunohistochemistry, DIGE: Difference in gel electrophoresis, TOF: Time of Flight, MS: Mass Spectrometry, SELDI: Surface-enhanced laser desorption or ionisation, MALDI: Matrix assisted laser desorption or ionisation, LC: Liquid Chromatography, EN: Total number of endometriosis patient, ME: Mild endometriosis, SE: severe endometriosis, MF: Male factor. NA: Not available.

The studies using gel-based proteomic studies are usually combined with MALDI-TOF-MS [242, 243, 247, 250] or DIGE-TOF-MS [72, 241]. By using the gel-based technique, protein were first identified and then isolated before being quantified using mass spectrometry. One of the studies using DIGE-TOF-MS was Zhang et al in 2007 [241], which included both the endometrial and serum in their analysis. The group discovered 13 and 11 protein spots from serum and endometrium respectively that were differently expressed between women with and without endometriosis. Out of that, 11 were known proteins including vimentin, B-actin, and ATP synthase subunit. Using the same technique, Stephens et al in 2009 [72] also discovered vimentin was differentially expressed, alongside with peroxiredoxin 6, and ribonuclease/ angiogenin inhibitor 1 (RNH1). However these two studies with similar methodology did not reach consensus on a large body of proteins discovered.

Apart from that, four studies [242, 243, 247, 250] used 2D MALDI-TOF-MS technique. Rai et al in 2010 [243] analysed the eutopic endometrium according to the different stages of endometriosis. They identified upregulated proteins (MVP, HSP90-beta, GRP78, HSP70, HSP60, HSP27, and DJ-1) and downregulated protein (ERp57), which later were verified by Western blotting and immunohistochemistry. An earlier study by Fowler et al. 2007 [247] identified dysregulated proteins in women with endometriosis which involved molecular chaperones (Heat Shock Protein 90 and Annexin A2), proteins involved in cellular redox state (peroxiredoxin 2), proteins involved in protein and DNA formation/breakdown (Ribonucleoside-diphosphate Reductase, Prohibitin and Prolyl 4-hydroxylase), and secreted proteins (Apolipoprotein A1). Chehna-Patel et al. 2010 [250] discovered 11 spots, out of which 4 (Haptoglobin, Rho-GDIa, SM-22a, and Rab37) have been verified (n=6) by both immunohistochemistry and western blotting. Ten Have and colleagues [242] discovered 21 up-regulated proteins in the endometriosis and pathway analysis that showed the proteins that were involved in apoptosis, immune reaction, glycolytic pathway, cell structure and transcription factors. The most recent publication [245], which is also the only study that used isobaric labelling technique, included the combination of the eutopic endometrium and the endometrioma cyst wall in their analysis. This study interestingly found that

Vimentin was upregulated, which is in keeping with one of the previous publications [72, 241].

In the proteomic analysis of eutopic endometrium, a gel-free technique with protein chip has been used in Surface Enhance Laser Desorption Ionisation-Time of Flight (SELDI-TOF). This technique provides distinctive proteomic profiles in the form of mass/charge (m/z). Published studies on proteomic analysis of endometrial tissue using SELDI-TOF [73-75, 246, 248, 339] have started as early as 2009 but now have been superseded by newer techniques. The number of samples used in the studies utilising this technique varies from 9 to 53 and some studies did not indicate the respective menstrual cycle phases where the samples were collected from [73, 74, 246, 248]. Multiple surfaces (see Separation and fractionation of complex protein mixture, page: 185) of the protein chip [73, 74, 246, 248] have been used in this technique to maximise protein detection. None of the studies using this technique has been verified or validated.

5.11 Summary of the literature review

One can currently conclude that there are no reliable biomarkers for the diagnosis of endometriosis. In the review of the biomarkers studies thus far, it is apparent that the majority of studies in the literature used proteomics techniques that are now dated and their results lack verification and validation, coupled with the incomplete clinical characterisation of the patient and control groups. As a result, researchers are yet unable to reach consensus on a potentially reliable biomarker or biomarker panel for the diagnosis of endometriosis.

Whilst the biomarker discovery phase is the most exciting and promising part of the search for a diagnostic marker for endometriosis, there are few, if any, new biomarkers that have yet to be translated into clinical use by completing the proteomic process chain of identification, validation in clinical trials, and approval by regulatory agencies. Such poor progress in biomarker diagnostic breakthrough is not solely specific to the field of endometriosis, but is common throughout medicine. The latter can be attributed to many factors; nevertheless,

a robust first step at the biomarker discovery phase with well-established and defined techniques and technology will form a strong platform going forward.

There is currently no study that has combined a gel-free technique with liquid chromatography and isobaric labelling. This is because this technology is relatively new and costly. In addition to this technique, isobaric peptide labelling using iTRAQ[®], which provides a complementary efficient tool for quantitative proteomic analysis. This robust technique requires stringent tissue procurement and had been utilised and validated in the field of oncology [319, 320]. The raw data generated from the analysis is further empowered by complementary validated bioinformatics analysis for pathway mapping. The next section will describe the use of liquid chromatography mass spectrometry (LC-MS/MS) together with isobaric peptide labelling (iTRAQ[®]), a method which has been verified and validated [319, 320] as a key method of proteomics in modern medicine. As described in the previous chapter, LC-MS/MS together with isobaric peptide labelling (iTRAQ[®]) is a high throughput proteomic analysis strategy that utilises liquid chromatography technology for effective protein segregation. In this chapter, this technique is combined with tandem mass spectrometry together with the isobaric tag for a comprehensive quantitative proteomics analysis.

Table 5-4 Table of published proteomic studies on endometriosis using plasma, serum, endometrium and follicular fluid

Reference (Year)	Techniques	Sample Type (N)	Cycle Phase (N)	Surface	Results	Sensitivity (%)	Specificity (%)	Verification
Serum/Plasma								
Fassbender 2012	Protein Chip Array-SELDI-TOF-MS	Plasma: 254	Menstrual: 68	Q10	Minimal to mild compared with control group: 4,898 m/z; 5,715 m/z; 8,328 m/z; 9,926 m/z; 14,698 m/z	75	86	No
		Minimal to mild: 89 Moderate to severe: 76 Control group: 89	Follicular: 98 Luteal: 88	H50 CM10	Moderate to severe compared with control group: 3,192 m/z; 4,519 m/z; 2,189 m/z; 4,373 m/z; 7,457 m/z	98	81	
Seeber 2009	Protein Chip Array-SELDI-TOF-MS	Serum: 141	Cycle day <14: 91	CM10	Mild to severe compared with control group: 1,629 m/z; 3,047 m/z; 3,526 m/z; 3,774 m/z; 5,046 m/z; 5,086 m/z	66	99	No
		Mild: 22 Moderate to Severe: 41 Control Group: 78	Cycle day >14: 25 Unknown: 25		Minimal to mild; moderate to severe compared with control group: not mentioned			
Jing 2009	Protein Chip Array-SELDI-TOF-MS	Serum: 120	Not mentioned	Immobilised metallic affinity capture 30	Minimal to severe compared with control group: 5,830 m/z, 8,865 m/z	89.66	96.67	Yes,
		Minimal to mild: 29 Moderate to severe: 30 Control group: 61			Minimal to mild; moderate to severe compared with control group: not mentioned	89.67 (After blinded test)	96.77 (After blinded test)	Blinded test performed Endo: 30 Controls: 31
Wolfler 2009	Protein Chip Array-SELDI-TOF-MS	Serum: 90	Luteal: 39	Q10	Minimal to severe compared with control group: 4,159 m/z; 5,264 m/z; 5,603 m/z; 9,861 m/z; 10,533 m/z	81.3	60.3	No
		Minimal to mild: 19 Moderate to severe: 32 Control group: 39	Follicular: 51		Minimal to mild compared with control group: 4,161 m/z; 4,597 m/z; 6,895 m/z; 6,955 m/z; 7,034 m/z	89	66.7	

Reference (Year)	Techniques	Sample Type (N)	Cycle Phase (N)	Surface	Results	Sensitivity (%)	Specificity (%)	Verification
					Moderate to severe compared with control group: 4,157 m/z; 6,239 m/z; 6,318 m/z; 7,029 m/z; 12,449 m/z	56.9	48.5	
Zhang 2009	Protein Chip Array-SELDI-TOF-MS	Serum: 80 Endometriosis Not Mentioned: 48 Control group: 32	Not mentioned	CM10	Endometriosis compared with control group: 4,974 m/z; 5,813 m/z; 4,290 m/z Minimal to mild; moderate- to-severe compared with control group: not mentioned	91.7 (training test) 91.7 (after blinded test)	95.8 (training test) 75 (after blinded test)	Yes Blinded test. Endo: 12 Controls: 8
Wang 2008	Protein Chip Array-SELDI-TOF-MS	Serum: 66 Minimal to mild: 22 Moderate to severe: 14 Control group: 30	Not mentioned	H4	Minimal to severe compared with control group: 8,142 m/z; 5,640 m/z; 5,847 m/z; 8,940 m/z; 3,269 m/z Minimal to mild; moderate to severe compared with control group: not mentioned	91.7	90	No
Liu 2007	Protein Chip Array-SELDI-TOF-MS	Plasma: 87 Endometriosis (stages not mentioned): 52 Control group: 46	Not mentioned	CM 10	Endometriosis compared with control group: 3,956 m/z; 11,710 m/z; 6,986 m/z Minimal to mild; moderate to severe compared with control group: not mentioned	87.5	85.7	No
Wang 2007	Protein Chip Array-SELDI-TOF-MS	Serum: 32 Minimal to mild: 10 Moderate to severe: 6 Control group: 16	Not mentioned	H4	Minimal to severe compared with control group: 3,269 m/z; 6,096 m/z; 5,894 m/z; 8,141 m/z Minimal to mild; moderate to severe compared with control group: not mentioned	NA	NA	No
Faserl 2012	2D-DIGE-MALDI-MS/MS	Serum: 76 Minimal to Mild: 20 Moderate to severe: 36	Not Mentioned	NA	Vitamin D-Binding protein GC*2 allele	NA	NA	No

Reference (Year)	Techniques	Sample Type (N)	Cycle Phase (N)	Surface	Results	Sensitivity (%)	Specificity (%)	Verification
Gajbhiye 2012	2D-MALDI-TOF-MS	Controls: 20 Serum: 70 Minimal to Mild: 17 Moderate to severe: 23 Controls: 30	Not Mentioned	NA	Tropomyosin 3 (TPM3)[antiTPM3a-autoAB, antiTPM3c-autoAB, antiTPM3d-autoAB] Stomatin like protein 2 (SLP2) [anti-SLP2a-autoAb, antiTPM3c-autoAB] Tropomodulin 3 (TMOD3) [anti-TMOD3b-autoAB, anti-TMOD3c-autoAB, anti-TMOD3d-autoAB]	61, 44, 78 50, 61 61, 78, 78	93, 93, 89 96, 93 96, 93, 96	Yes Elisa Minimal to mild: 18 Moderate to severe: 32 Controls: 27
Nabeta 2012	2D-MALDI-TOF-MS	Serum: 151 Minimal to Mild: 21 Moderate to severe: 48 Controls: 82	Not Mentioned	NA	All endometriosis compared to control. Anti-syntaxin 5 (STX5) autoAb Stage II compared to control. Anti-syntaxin 5 (STX5) autoAb	53.6 80	72.2 NA	No
Zhang 2006	2D-MALDI-TOF-MS	Serum: 12 All stages Controls: 6	Secretory: 12	NA	Vimentin B-actin ATP Syntase B subunit	NA	NA	No
Zheng 2011	Nano sized beads-MALDI-TOFMS	Serum: 246 Minimal to Mild: 50 Moderate to severe: 76 Controls: 120	Not Mentioned	NA	All endometriosis compared to controls 5988.7; m/z; 7185.3 m/z; 8929.8 m/z	89.3	90.0	No
Endometrium								
Fassbender 2011	Protein Chip Array-SELDI-TOF-MS	Endometrium: 59 Minimal to mild: 16 Moderate to severe: 5 Controls: 18	Early luteal phase: 27 Menstrual phase: 22	IMAC 30; CM10; Q10; H50	Minimal–severe vs. controls: 2072 m/z; 2973 m/z; 3623 m/z; 3680 m/z; 21133 m/z Minimal – mild versus controls: 2071 m/z; 2166 m/z; 2228 m/z; 3649 m/z; 40367 m/z Moderate–severe vs. controls: 3274 m/z; 7455 m/z; 13552 m/z; 39889 m/z; 42108	91 94 92	80 100 84	No

Reference (Year)	Techniques	Sample Type (N)	Cycle Phase (N)	Surface	Results	Sensitivity (%)	Specificity (%)	Verification
Kyama 2011	Protein Chip Array-SELDI-TOF-MS	Endometrium: 29 Minimal to mild: 9 Moderate to severe: 10 Controls: 10	Luteal phase: 29	CM10; H50; IMAC 30; Q10	Minimal–severe vs. controls: 8.650 m/z; 8.659 m/z; No 13.910 m/z; 5.183 m/z; 1.949 m/z	89.5	90	No
						100	100	
					Minimal–mild vs. control: 1.924 m/z; 2.504 m/z; 90.675 m/z, T-Plastin; 39.956 m/z, Annexin 5	80	70	
					Moderate–severe vs. controls: 10.110 m/z, 5.828 m/z; 12.172 m/z; 4.279 m/z			
Ding 2010	Protein Chip Array-SELDI-TOF-MS	Endometrium: 53 Minimal to mild: 9 Moderate to severe: 5 Controls: 29	Not mentioned	CM10	Minimal–severe vs. controls: 15.334 m/z; 15.128 m/z; 87.5 86.2 No 16.069 m/z	87.5	86.2	No
					Minimal–mild; moderate–severe vs. controls			
Wang 2010	Protein Chip Array-SELDI-TOF-MS	Endometrium: 26 Minimal to mild: 8 Moderate to severe: 5 Controls: 13	Not mentioned	H4	Minimal–severe vs. controls: 6.898 m/z; 5.891 m/z; 91.7 90 No 5.385 m/z; 6.448 m/z; 5.425 m/z	91.7	90	No
					Minimal–mild; moderate–severe vs. controls not mentioned			
Fassbender 2010	Protein Chip Array-SELDI-TOF-MS	Endometrium: 16 Minimal to mild: 5 Moderate to severe: 5 Controls: 6	Luteal phase: 16	CM10; IMAC 30	Minimal–severe vs. controls: 32 peaks differentially expressed	NA	NA	No
					Minimal–mild vs. controls: 23 peaks Moderate–severe vs. controls: 25 peaks			
Kyama 2006	Protein Chip Array-SELDI-TOF-MS	Endometrium: 9 Eutopic EM mild: 3 Paired eutopic EM and peritoneum and peritoneal endometriotic lesion controls: 3	Luteal phase: 9	CM10; H50; IMAC 30; Q10	Mild vs. control: 2.8–12.3 kDa was 3–24 times lower in the eutopic endometrium of women with endometriosis than controls	NA	NA	No

Reference (Year)	Techniques	Sample Type (N)	Cycle Phase (N)	Surface	Results	Sensitivity (%)	Specificity (%)	Verification
Jiang 2012	2D-MALDI-TOF/TOF-MS	Endometrium: 20 All stages: 10 Controls: 10	WOI	NA	Down regulated: Anxin A4	NA	NA	Yes Western Blotting
Stephens 2009	2D-DIGE-MALDI-TOF-MS	Endometrium: 32 Endometriosis: 18 Control: 14	Not mentioned	NA	Vimentin (VIM), Peroxiredoxin 6 (PRDX6), and Ribonuclease/angiogenin inhibitor 1 (RNH1) Could not be confirmed for coronin 1A (CORO1A) or transgelin (TAGLN2)	NA	NA	Yes Immunohistochemistry Endo: 18 Controls: 14
Zhang 2007	2D-DIGE-TOF-MS	Endometrium: 12 Minimal to Mild: 2 Moderate to severe: 4 Controls: 6	Secretory: 12	NA	11 proteins spots identified Vimentin B-actin ATP Syntase B subunit	NA	NA	No
Rai 2010	2D-MALDI-TOF-MS or MS/MS	Endometrium: 116 Minimal to Mild: 12 Moderate to severe: 45 Controls: 59	Secretory: 42 Proliferative: 17	NA	Upregulated: MVP, HSP90 beta, GRP78, HSP70, HSP60, HSP27, and DJ-1 Downregulated: ERp57	NA	NA	Yes Western Blotting, Immunohistochemistry Endo: 24 Controls: 24
Chehna-Patel 2010	2D-DIGE-TOF-MS	Endometrium: 20 All stages: 9 Controls: 11	Not Mentioned	NA	Haptoglobin, Rho-GDIa, SM-22a, and Rab37	NA	NA	Yes Western Blotting, Immunohistochemistry Endo: 3 Controls: 3
Fowler 2007	2D-DIGE-TOF-MS	Endometrium: 24 All stages: 12 Controls: 12	Secretory: 12 Proliferative: 12	NA	Heat shock protein 90; Annexin A2; Peroxiredoxin 2; Ribonucleoside-diphosphate reductase; prohibitin; prolyl 4-hydroxylase Apolipoprotein A1	NA	NA	No
Ten-Have 2011	2D-DIGE-TOF-MS	Endometrium: 17 Minimal to Mild: 11 Controls: 6	Secretory: 17	NA	Vimentin Heat Shock Protein (HSP) 70, 90B Protein hypothetical protein ARPC2	NA	NA	No

Reference (Year)	Techniques	Sample Type (N)	Cycle Phase (N)	Surface	Results	Sensitivity (%)	Specificity (%)	Verification
					CLL-associated antigen KW13 tumour antigen			
Marianowski 2013	2D-DIGE-MS/MS	Endometrium: 8 Moderate to Severe: 7 Controls: 1	Proliferative : 8	NA	Collagen α 1 (XIV), Calmodulin, Collagen α (VI), Plexin, Integrin α V β 3, Transgelin, Desmin, and Vimentin	NA	NA	No Isobaric tag (iTRAQ) used
Zhang 2010	1D-LC-MS/MS	Endometrium: 5 Minimal to Mild: 3 Moderate to severe: 2	Secretory: 5	NA	Collagen, α -1, α -2, α -3 (VI), α -1 (XIV) chain, actin, Annexin A2, EMILIN-1, Ferritin light polypeptide variant, fucosyl-transferase 10, myosin-9, protein S100- A9, KIAA1783	NA	NA	Yes Western Blotting Number not mentioned
Follicular fluid								
Lu Turco 2013	2D-LC ESI-MS/MS	Follicular Fluid: 21 Endometriosis (Pregnant): 4 Endometriosis (not pregnant): 8 Control: 9	During IVF treatment	NA	Endometriosis (Pregnant): Serum albumin, Ig alpha-1 chain C region Ceruloplasmin, Hemopexin Complement factor H, Focal adhesion kinase 1 Angiotensinogen Endometriosis (Not pregnant): Complement factor I, Serum albumin, Proteasome subunit alpha type-5, Alpha-1-antitrypsin, Angiotensinogen, Vitronectin, Kininogen-1	NA	NA	No

5.12 Objective

The objective of this study is to evaluate the proteome of the endometrium of women with and those without endometriosis using LC-MS/MS with iTRAQ®.

5.13 Selection of sample

5.13.1 Tissue type

Researchers have tested several forms and types of biological tissue, namely plasma, urine, endometrial fluid, peritoneal fluid, follicular fluid as well as the endometrial tissue. The ideal biomarker needs to be easily accessible from the patient and fully reflects, in this case, the presence or absence of the disease. Biological fluids such as the peritoneal fluid or follicular fluid are generally not easily accessible. Whilst the serum and urine, are easily obtainable, they reflect the disease only if the biomarkers have gained access into the systemic circulation from the peritoneal cavity, which may or may not be the case in endometriosis. The endometrial cavity is in close contact with the peritoneal cavity, sharing closely networked vascular and lymphatic structures, as well as being in direct contact with the peritoneal fluid through the Fallopian tubes. As previously discussed, many molecular factors have already been found to be distinctly expressed in the endometrium of women with endometriosis compared with controls. The retrograde menstruation hypothesis further supports the close relationship of the endometrium to the endometriosis deposits, although there is still some debate over the validity of this hypothesis [5].

5.13.2 Selection of menstrual phase

For this study, the endometrial samples were obtained during the entire menstrual cycle.

5.14 Sample size

The experiment was run in two sets of samples, to confirm or to reject selections based on a single set of a sample. This also avoided the over interpretation of the single set of data when extrapolated to a generalised cohort [340]. In this study, each set of sample has eight different patients for two

respective experiments, with a total of sixteen patients. The sample number was based on the number of samples used for other proteomic analysis that has led to biomarker discovery [341].

From the two sets of experiments, endometrium samples from women with and without endometriosis were initially analysed individually according to the menstrual phase (follicular or luteal). Samples from women with endometriosis were also initially analysed separately according to disease severity (minimal/mild or moderate/severe). Subsequent to the initial analysis, data from women with endometriosis was pooled irrespective of the disease severity and menstrual phase, as no difference was found when independently analysed. Therefore, all further analysis was performed collectively according to controls and endometriosis group, irrespective of the disease severity and menstrual phase.

5.15 Method and Material

This subsection is divided into the clinical and experimental aspects of the study. The clinical part will describe how the sample was obtained and stored while the experimental part will describe the laboratory work undertaken throughout the study. Data recording, sample collection and tissue storage were performed according to World Endometriosis Research Foundation (WERF) Endometriosis Phenome and Biobanking Harmonisation Project (EPHect), unless otherwise stated.

5.16 Human and clinical ethics

This study received institutional and regional review board approval by University Hospital Southampton (RHMO&G160) and Hampshire B ethical committee (MREC08/HO502/162) respectively.

5.16.1 Inclusion and exclusion criteria

Women in the endometriosis group had the laparoscopic diagnosis of endometriosis (laparoscopy or laparotomy) with the disease stage documented according to the ASRM classification. The control group consisted of women with no endometriosis as diagnosed by a negative laparoscopy. Women were

excluded from the study if they were age 45 years old and above, on hormonal treatment within three months prior to the procedure, had a BMI of more than 30, or a current smoker.

5.16.2 Patient recruitment

This study was performed at the Princess Anne Hospital Southampton. Patients were recruited from the outpatients and pre-assessment clinics. The suitable candidates for the study were screened and invited to participate in the study. Women who were interested in participating in the study were given an information sheet outlining the study. Patients were guided through the participant information sheet by the researcher who explained the details of the study. Those who agree to participate were required to sign the consent form in 3 copies (a copy was given to the patient, a copy was filed in the notes and a copy was kept by the research team). The consent was assigned a study ID number (BC001, BC002, BC003....). Patient identifiers were only kept on these consent forms and were stored separately from the research data. Participants were asked several questions according to the proforma for record purposes. The participants' course of treatment were not affected by this recruitment procedure and they had their surgery as planned.

5.16.3 Sample identification and data recording

Linked anonymisation was used to protect confidentiality. Samples were coded BC001, BC002, BC003... etc. in order of their collection, which is similar to their ID study number. A record of the sample code and related patient recruitment number was updated and stored by the researchers in a database. The patient information was subsequently correlated to the samples on completion of the experiments. Anonymised patients' data including the patients' demographic, medical history, age, parity and date of last menstrual period were recorded on a case report form (CRF). Any notable findings at the surgery were recorded on the same CRF.

5.16.4 Laparoscopic diagnosis

Laparoscopy, in our centre, is indicated (but not limited to) for women who 1) have intractable pelvic pain, 2) require pelvic procedure such as cystectomy,

ovarian drilling or adhesiolysis, 3) need alternative options for tubal assessment e.g. contraindicated for HyCoSy, or 4) for confirmation of abnormal findings seen during HyCoSy procedure. Patients were grouped into those with endometriosis (study group) and those without (control), in accordance with the findings during laparoscopy.

The findings of the laparoscopy were documented in the proforma. Details of size, location and number of sites of all types (superficial, endometrioma, deep infiltrating) and severity (minimal, mild, moderate and severe according to ASRM criteria) of endometriosis were recorded. Whenever possible photographic evidence was obtained.

5.16.5 Endometrial tissue collection

Endometrial tissue was collected using endometrium sampler (Endocell®, Wallach, USA). Sample collection was performed before any uterine manipulation or procedure. Then, the endometrial sampler was inserted gently through the cervix and then advanced inward until resistance was felt. The inner piston of the device was then withdrawn to create suction and the endometrial sample was obtained by moving the endometrial sampler up and down within the uterine cavity. Endometrial tissues that were suctioned in the tube were collected in individual falcon tubes containing normal saline. The procedure was repeated for at least 2 times or until an adequate tissue sample was obtained.

5.16.6 Processing of endometrium sample and storage

The collected tissue samples were processed up to 4 hours from the collection. Tissues were transferred into a petri dish and were gently teased apart with a tissue forceps and then washed repeatedly with Phosphate Buffered Saline (PBS) to remove any blood. Healthy tissues that were free from blood were cut into smaller pieces (approximately 15mm in length) using a pair of tissue scissors. The processed tissues were then transferred into at least 3 separate Cryovials (Greiner, UK). These vials were snap frozen in -80°C freezer located at G level (Complete Fertility Centre) for later use.

5.16.7 Sample Transportation

Endometrium was transported on solid carbon dioxide (dry ice) inside a polystyrene box from Princess Anne Hospital to the Centre of Biological Sciences, University of Southampton. The average journey time was approximately 15 minutes. On arrival, the endometrium was stored immediately to -80 freezers (B Level) or thawed on ice for use on the same day.

5.16.8 Laboratory assay

All laboratory procedures were performed in the Proteomic Unit, Centre of Biological Sciences located in Building 85, University Of Southampton. A standard written protocol was followed to maintain the consistency of the result. The experiment workflow is illustrated in Figure 5-4.

5.16.9 Tissues handling and Lysate preparation

Samples from the identified patients were thawed from the -80 freezer on ice. Each sample was transferred from the Cryovials (Greiner, UK) to 30 mm Petri dish. Healthy tissue samples (free from blood) were picked with forceps, put into an Eppendorf tube, and dissolved in 200uL 0.5M Triethyl Ammonium Bicarbonate (TEAB) with 0.05% Sodium Dodecyl Sulphate (SDS) using vials containing ceramic beads. TEAB acts as the phase-transfer catalyst to dissolve the samples, whereas SDS disrupts non-covalent bonds (hydrogen, hydrophobic, and electrostatics) in the proteins, denaturing them, and causing the molecules to lose their original shape. Samples were then homogenised using the FastPrep® (Qbiogene, USA) system. Thorough tissue homogenisation was performed to ensure that the protein content of tissue from all subcellular compartments was released. The homogenised tissues were then sonicated, a technique for lysing cells by liquid shear and cavitation. Tissue sonication was done using sonicator (Misonic XL2020, USA) on ice at an amplitude of 20% for 15 seconds burst, for 2 times. These tissues were then centrifuged at 16,000g for 10 minutes at 4°C. The supernatant was then transferred to a fresh Eppendorfs tube and kept on ice.

5.16.10 Infra-red protein quantification

After preparation of the lysate, total protein was quantified using an infrared (IR)-based spectrometry system reader (Direct detect, Merck Millipore, UK) to ensure sufficient protein was present in the homogenised sample for further analysis. Two microlitres of each lysate was aliquoted onto a membrane card that employs a hydrophilic polytetrafluoroethylene (PTFE) membrane that is transparent in most of the infrared spectral region. The card was then inserted into the slot on the reader. The automated reading will run and the results were displayed in the programme and were compared to the standard curve.

Protein content should ideally be between 2.5-5 µg/µl for an optimal analysis. In total 100ug of protein was used per sample, the lysates were of the same volume and each sample was matched up to the largest volume with TEAB.

5.16.11 Reduction, alkylation and digestions

For this procedure, proteins were reduced to disintegrate the disulphide bonds, alkylated to irreversibly block the cysteine group and finally digested into peptides. All three steps were done serially, before iTRAQ® labelling. To reduce the lysate, 2µl of reducing reagent, 50mM of Tris-2-carboxymethyl phosphine (TCEP) was added for every 20µl of protein. The sample was vortexed and spun down to mix. The mixture was then incubated at 60°C for 1 hour and a metal block was placed on these tubes to minimise condensation.

After one-hour incubation, 1µl of an alkylating agent, 200mM of Methylmethane Thiosulphonate (MMTS), was added for every 20µl of protein added. The sample was again vortexed and spun down to mix. The sample was then incubated at room temperature for one hour. Finally, to digest the protein, 6µl of 500ng/ml trypsin was added into the samples and were left incubated overnight in the dark at room temperature.

5.16.12 iTRAQ® labelling

To begin the iTRAQ® labelling process, 50µl of isopropanol was added into each iTRAQ® 8-plex reagents (thawed to room temperature). Then, iTRAQ® labels were added into their individual digest and incubated in the dark, at room temperature for 2 hours. After 2 hours, 8 µl of 5% hydroxylamine was added to

neutralise the reaction. The mixture then vortexed and spun down to mix. Individual sample was then lyophilised using a SpeedVac concentrator (Thermo-Scientific, USA). Samples were then stored at -20°C for later use.

5.16.13 Offline C8 HPLC

For this stage, all 8 samples were combined into one Eppendorf tube. To pool the samples, 80µl of mobile phase [98% mobile phase A (99.9% H₂O, 0.1 % NH₄OH) and 2% mobile phase B (99.9% Acetonitrile, 0.1% NH₄OH)] was added to sample 1 and then vortexed for two minutes before transferring the full content to sample number 2. This was repeated until all 8 samples were reconstituted in a common pool. If required, the final volume was adjusted to 100µl with mobile phase solution. The final sample was then centrifuged at 13,000 per min for 10 minutes.

The samples were subsequently analysed using offline high pH, reversed phase, high-performance liquid chromatography (HPLC). To begin the HPLC, 100µl of the reconstituted sample was injected into the loop. Fractions were collected in a peak dependent fashion and the fractions were lyophilised using a SpeedVac concentrator (Thermo-Scientific, USA) and stored at -20 °C until further analysis.

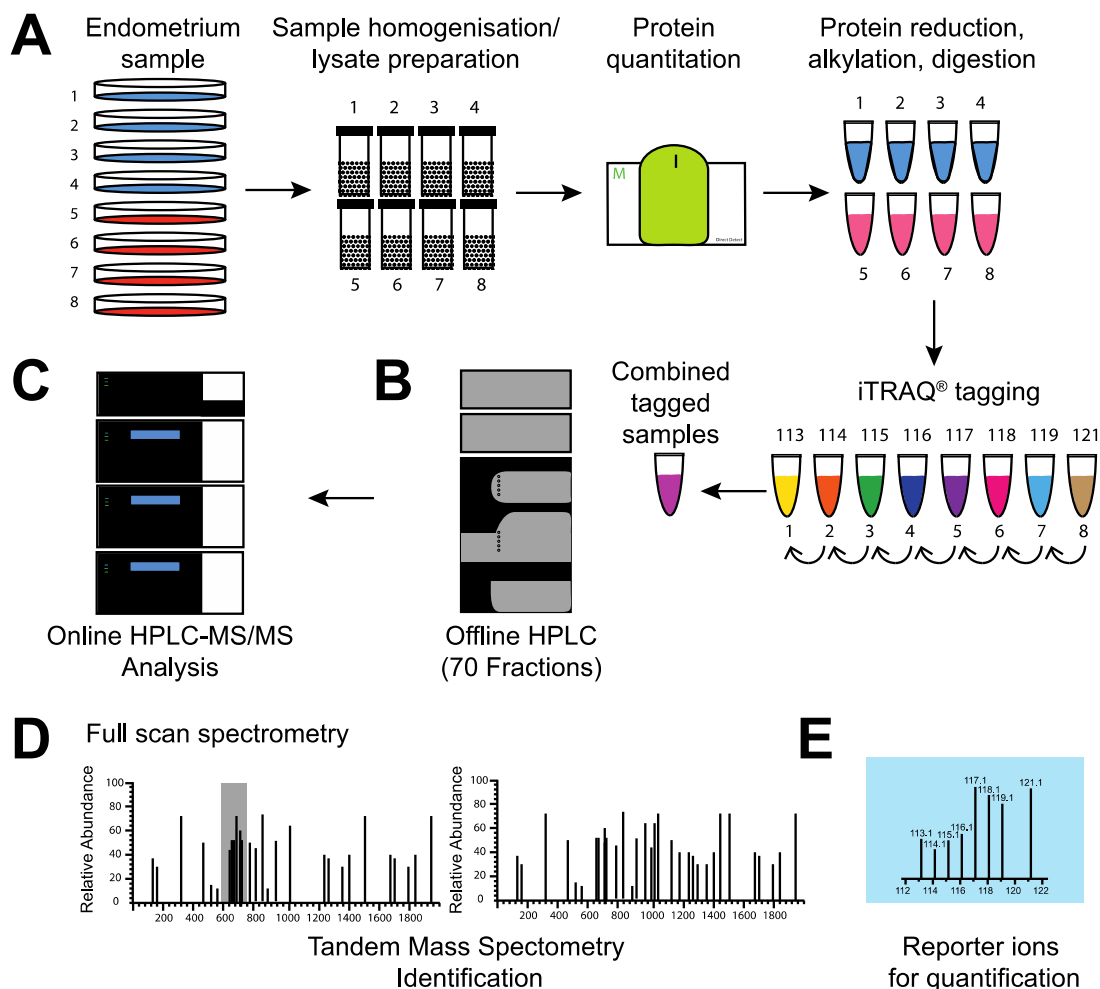


Figure 5-4 Experimental workflow

A: Schematic representation of experimental workflow beginning with sample procurement including tissue homogenisation using ceramic beads. Homogenised lysate derived from the sample was tested for protein content and was then reduced, alkylated and digested. Peptides from individual sample were then tagged using iTRAQ® tags before all samples reconstituted into one common vial. **B:** The pooled samples were subsequently analysed using offline high pH, reversed phase, high performance liquid chromatography (HPLC) for 70 fractions. Fractions were collected in peak dependent manner. **C:** Each collected fractions were further analysed by online low pH, reverse phase C18, for separation of tryptic peptide hyphenated with **D:** Tandem mass spectrometry ran for approximately 3-4 hours. **E:** The reporter ion intensity were quantified, which indicated the relative amount of peptide in the mixture that was labelled with the corresponding iTRAQ® tags.

5.16.14 Online C8 HPLC

Online low pH, reverse phase C8 were performed next. Before starting the analysis, the mass spectrometry system was fully calibrated. Eppendorf containing each fraction collected earlier (Section 5.6.14) were dissolved in 30 μ l of loading phase (2% acetonitrile, 0.1% formic acid). Each sample was then vortexed to mix and further centrifuged using the mini-centrifuge. By using 96-well plate, 30 μ l mobile phase solution was pipetted into wells A1 to A12. Then 30 μ l of the first sample into well B1, making sure all the peptides around the Eppendorf tube was also taken up. This was repeated for the next samples by pipetting into B2, B3, B4 and so on. The 96-well plate will then be placed in the mass spectrometry.

5.16.15 Tandem Mass Spectrometry

Peptides were electrosprayed directly from a LC separation (5.16.14) into the heated inlet source where desolvated peptide ions were focused by a stacked ring ion guide (S-lens), then focused, filtered (between 300–2000 m/z), and transferred by the square quadrupole and octupole to the dual linear ion trap (LIT). Peptides ions were collected, isolated, and fragmented by collision induced dissociation (CID) in the high-pressure cell. Isolated precursor and fragment ions were passed to the low-pressure trap for detection. For higher resolution and mass accuracy detection, precursor or fragment ions can be passed to the Orbitrap mass analyser via the second quadrupole and C-trap. Beam-type collision was performed in the higher energy collisional dissociation (HCD) collision cell instead of the ion trap for detection with Orbitrap. The Orbitrap detects ion currents of peptide ions that process around an orbital electrode. A Fourier transform is used to convert the frequency-based ion current to an m/z value.

5.16.16 Bioinformatics and statistical analysis:

Unprocessed raw files were submitted to Proteome Discoverer™ 1.4 (Thermo-Scientific, USA) for target decoy searching with Sequest HT. Quantification ratios were median-normalized and log₂ transformed. As described previously (see section 5.7.5), the raw data was matched to the theoretically generated peptide fragmentation spectra based on the database search automated by

Proteome Discoverer™. This will create a list of peptide candidates for each experimental spectrum, which then ranked and filtered to create peptide spectrum matches (PSMs). PSMs were filtered using a statistical calculation of the correlation of the theoretical and the experimental spectra. Incorrect reverse PSMs were used to calculate the false discovery rate (FDR). A confidence score was calculated for each of the protein groups using local FDR [342].

A protein was considered differentially expressed in the endometrium from endometriosis patients compared to healthy controls when its 1-group t-test p-value was below 0.05. Identified up and down regulated proteins were presented in heat map format for visual representation. The heat map was constructed by using Cluster version 3.0 software available online. To view the clustering results generated by a software called Cluster 3.0, Java TreeView (<http://jtreeview.sourceforge.net>) was used, which displayed the hierarchical clustering results.

The up and down-regulated proteins were analysed separately and in a combination of both data sets. Relation of exclusivity and interconnection of proteins found in both group was represented in Venn diagram. The pathway and biological processes analysis were performed using Ingenuity Pathway Analysis (IPA, Qiagen, USA) and MetaCore™ (Thomson Reuters, USA) respectively.

IPA is a web-based software application for the analysis, integration, and interpretation of data derived from -omics experiments including proteomics. This application offers a comprehensive analysis and search tools to uncover the significance of data and identify new targets or candidate biomarkers within the context of biological systems. Using IPA, key regulators and activities were identified and expression pattern was described. This application is able to make a prediction of down-stream effects of biological and disease processes, by building the interactive models of experimental systems.

MetaCore™ is an integrated software suite for functional analysis of various experiments including proteomics. MetaCore™ is based on a high quality, manually curated database of transcription factors, receptors, ligands, kinases, drugs, and endogenous metabolites as well as other molecular classes. Species-specific directional interactions between protein-protein, protein-DNA

and protein-RNA, drug targeting, and bioactive molecules and their effects. Signalling and metabolic pathways are represented on maps and networks with rich ontologies for diseases and processes with a hierarchical or graphic output.

Results of analysis were presented in p-value, and to eliminate type I error, the p-value was corrected for false discovery rate (FDR). The Z-score analysis was also done for identification of significant differential protein expression. FDR corrected p-value $p < 0.05$ and Z-score of $Z \geq 2$ was considered statistically significant. The Z-score was expressed as a unit of SD from the normalised mean of zero. Corrections were done before sample-to-sample comparison and was therefore comparison-independent [343].

5.17 Results

5.17.1 Clinical Data between groups

Sixteen endometrial samples (endometriosis, $n=8$; control, $n=8$) were collected between September 2013 and September 2015. There was no significant difference ($P > 0.05$) in age ($P=0.829$, Endometriosis, 33.1 ± 4.35 ; Control, 32.5 ± 6.63), body mass index ($P=0.88$, Endometriosis, 25.4 ± 4.60 ; Control, 25.7 ± 4.17) and baseline FSH [$P=0.51$, Endometriosis, 6.9 ± 1.90 ; Control, 7.5 ± 1.83] between the two groups.

All women (8/8) included in endometriosis group suffered from subfertility and only three women (3/8) had infertility in the control groups. The majority of these women were nulliparous Endometriosis, 7/8; Controls, 4/8. Six out of eight women with endometriosis had laparoscopic indicated for tubal patency test and the other two women indicated for ovarian cyst (Table 6.1). Four out of eight women in endometriosis group had visual and histological confirmation of the disease. The majority of patients were in the follicular phase (13/16) except three who were in the luteal phase (3/16). Of the eight patients in endometriosis group, three had stage I/II endometriosis and five women had stage III/IV (5/8). Two out of eight women with endometriosis had endometrioma ($n=1$ unilateral, $n=1$ bilateral, Table 6.1).

Table 5-5 Clinical data of women included in the analysis

Table showing clinical data and surgical descriptions of all women included for the proteomic analysis according to the group. The clinical data includes age, Body Mass Index (BMI), Parity (P), Miscarriage (M), menstrual phase, and subfertility status (SF). Surgical description includes indication for the surgery of each woman, the presence of endometrioma during the surgery, stage of the disease (ASRM staging), and the availability of the histological confirmation (HPE). (NA, Not applicable; NS, not sent).

No	Age	BMI	P	M	SF	Indication	Day	Phase	Laparoscopy findings	Endome- trioma	HPE	ASRM
Control group (No Endometriosis)												
01	30	19.7	0	0	Yes	Tubal check	D3	Follicular	No adhesions or endometriosis. Bilateral patent tubes.	NA	NA	NA
02	28	30.3	3	1	Yes	Tubal check	D3	Follicular	Bicornuate uterus no endometriosis.	NA	NA	NA
03	32	27.2	0	1	Yes	Ovarian drilling	D7	Follicular	Bilateral polycystic ovary	NA	NA	NA
04	30	24.9	0	0	No	Tubal check	D7	Follicular	Normal findings, no endometriosis. Unilateral patent tube.	NA	NA	NA
05	24	21.7	0	1	No	Ovarian Cyst	D14	Follicular	Dermoid cyst.	NA	NA	NA
06	31	29.3	0	0	No	Dysmenorrhoea	D8	Follicular	Normal findings. No endometriosis. Bilateral patent tubes.	NA	NA	NA
07	43	22.4	1	0	No	Dysmenorrhoea	D21	Luteal	Normal findings. No endometriosis. Bilateral patent tubes.	NA	NA	NA
08	42	30.4	1	1	No	Dysmenorrhoea	D5	Follicular	Normal findings. No endometriosis. Bilateral patent tubes.	NA	NA	NA
Study group (Endometriosis)												
09	33	30.5	0	0	Yes	Tubal check	D3	Follicular	Nodule of endometriosis below USL. Superficial spots at L ovarian fossa	No	NS	I-II
10	25	24.7	0	0	Yes	Tubal check	D13	Follicular	Single endometriotic spot at USL.	No	Yes	I-II
11	29	19.5	0	0	Yes	Ovarian cyst	D5	Follicular	Bilateral endometriomas (>3 cm). Adhesions at POD and ovarian fossa.	Yes	Yes	III-IV
12	34	29.3	0	0	Yes	Ovarian cyst	D12	Follicular	Endometrioma at left ovary (5cm). Superficial endo at L ovary and USL.	Yes	Yes	III-IV
13	38	21.6	0	0	Yes	Tubal check	D20	Luteal	One spot of endometriosis at Left ovarian fossa. No endometrioma.	No	NS	I-II
14	34	29.3	0	0	Yes	Tubal check	D8	Follicular	Endometriotic spots at left ovarian fossa. No endometrioma.	No	NS	I-II
15	38	28.5	1	0	Yes	Tubal check	D14	Follicular	Endometriotic spots at right ovarian fossa. No endometrioma.	No	NS	I-II
16	34	19.7	0	0	Yes	Tubal check	D19	Luteal	Nodule at ovary and widespred endometriosis at peritoneum and USL	No	Yes	III-IV

5.17.2 Up and Down-regulated Proteins

The first and second data set profiled a total of 9,112 and 7,212 proteins respectively (confidence interval 99%; Figure 6-2). Approximately 210 proteins were commonly up-regulated and 235 proteins were commonly down-regulated (Figure 5-5). The menstrual phases (follicular vs. luteal) or severity of the disease (minimal/mild or moderate/severe) did not significantly influence the variation in the level of up and down regulated proteins. Hence, all further analysis was then performed collectively into two groups, controls and endometriosis.

The commonly modulated proteins from the two groups were further utilised for in-silico bio-informatics interpretation. Up and down-regulated proteins are presented in a heat-map format (Figure 5-6) for a visual representation of the data. In both datasets, Liver carboxylesterase-1 (CES1) was significantly up regulated (PSM=63, Log₂Ratio= 0.5) and was the most highly expressed protein. Other highly up-regulated proteins were Homeobox protein-B6 (HOXB6, PSM=13, Log₂Ratio= 0.5) Synaptotagmin-like protein 1 (SYTL-1, PSM=9, Log₂Ratio= 0.4), Tumour associated signal transducer 2 (TACSTD2, PSM=30, Log₂Ratio= 0.5), Microsomal Glutathione S-transferase 2 (MGST2, PSM=8, Log₂Ratio= 0.5) and Cluster Differentiation 99 (CD99, PSM=15, Log₂Ratio= 0.5). Adiponectin (ADIPOQ, PSM=33, Log₂Ratio= -0.6) was significantly down regulated and was the lowest expressed protein. Other proteins that were also found highly down regulated from both datasets were protein phosphatase 1 regulatory subunit 12A (PPP1R12A, PSM=201, Log₂Ratio= 0.2) and 12B (PPP1R12B, PSM=10, Log₂Ratio= -0.8), Voltage-gated potassium channel subunit beta-2 (KCNAB2, PSM=19, Log₂Ratio= -0.6), Sushi domain containing protein 2 (SUSD2, PSM=24, Log₂Ratio= -0.8) and plexin-D1 (PLXD1, PSM=50, Log₂Ratio= -0.2). The data for up and down regulated proteins was represented in PSM and Log₂-Ratio (Table 5-6). When compared to the literature, the majority of up or down-regulated proteins were novel proteins, which were not well described in the field of endometriosis in prior publications.

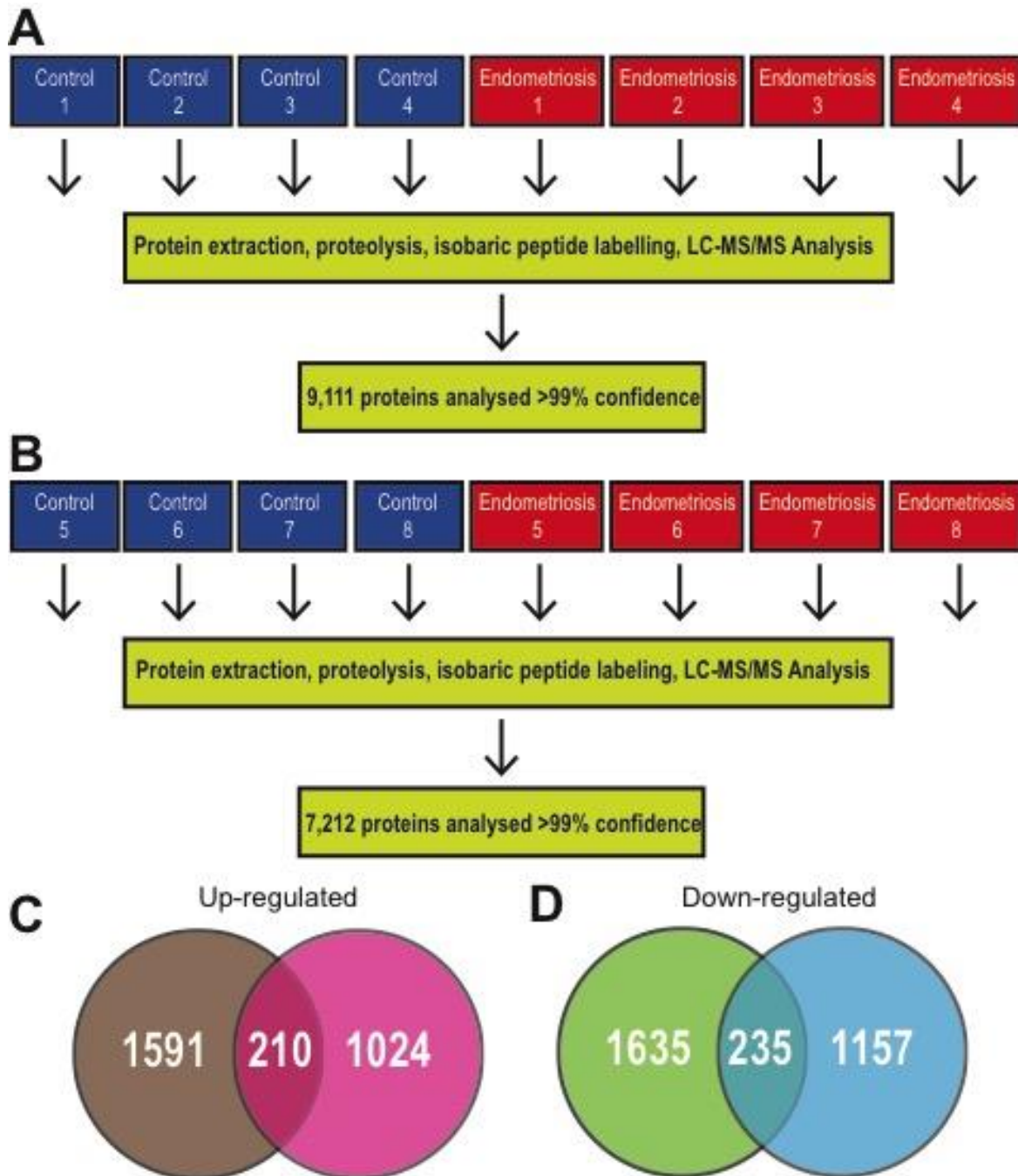


Figure 5-5 Summary of result in both experiments

A: First and **B:** Second set of experiments consists of 8 different samples of controls and 8 samples of endometriosis (Total of 16 samples). Total number of proteins profiled from both experiments were shown in the Venn diagrams (>99% confidence) **C:** Up-regulated and **D:** Down-Regulated proteins from first (pink) and second (orange) experiments, with commonly up and down regulated proteins (respectively) represented by overlapping area between both experiments.

	#PSMs (Exp1)	#PSMs (Exp2)	Average \log_2 -ratio (Exp1)	Average \log_2 -ratio (Exp2)
Up-regulated proteins				
Homeobox protein-B6 (HOXB6)	13	3	0.5	0.4
Liver carboxylesterase-1 (CES1)	63	29	0.5	0.6
Synaptotagmin-like protein 1 (SYTL-1)	9	7	0.4	0.3
Tumour associated signal transducer 2 (TACSTD2)	30	21	0.5	0.4
Microsomal glutathione S-transferase 2 (MGST2)	8	9	0.5	0.4
Cluster Differentiation 99 (CD99)	15	5	0.5	0.7
Down-regulated proteins				
Adiponectin (ADIPOQ)	33	13	-0.6	-0.8
Protein phosphatase 1 regulatory subunit 12A (PPP1R12A)	201	95	-0.2	-0.1
Protein phosphatase 1 regulatory subunit 12B (PPP1R12AB)	10	6	-0.8	-0.4
Voltage-gated potassium channel subunit beta-2 (KCNAB2)	19	5	-0.6	-0.2
Sushi domain containing protein 2 (SUSD2)	24	10	-0.8	-0.9
Plexin-D1 (PLXD1)	50	12	-0.2	-0.1

Table 5-6 Highest and lowest expressed protein

Table shows the highest and lowest expressed proteins across 2 datasets. Peptide spectrum match (PSM) and average \log_2 -ratio for both experiments were calculated.

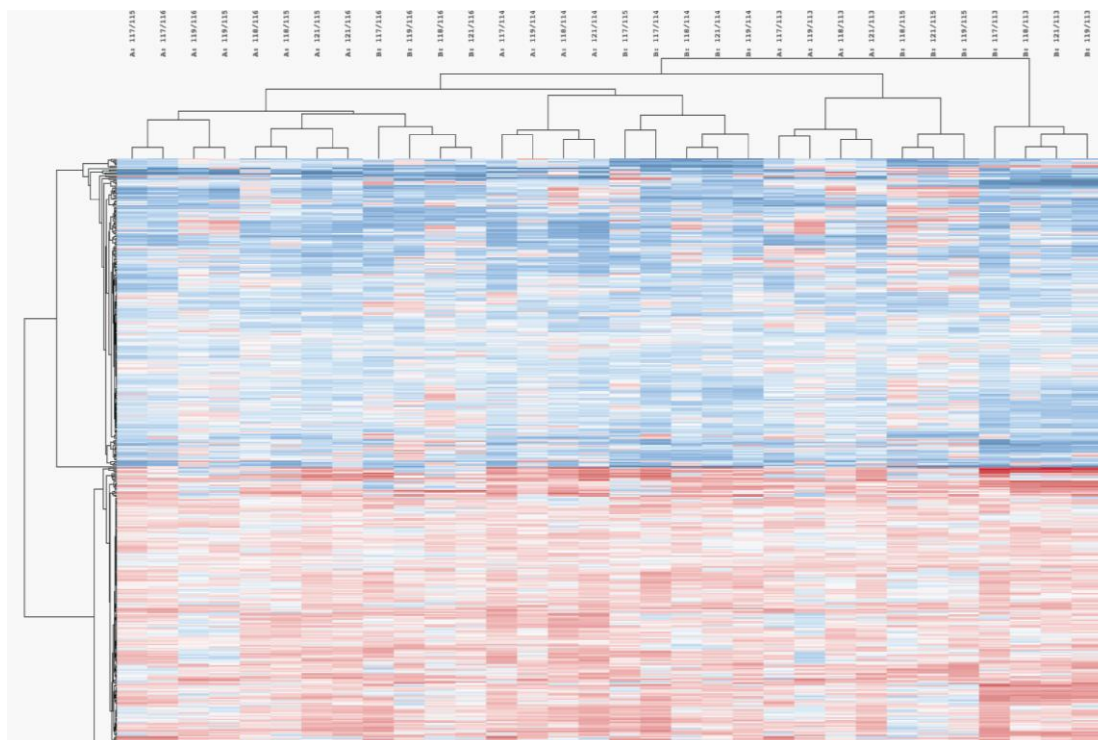


Figure 5-6 Heatmap of common differentially expressed protein

Heatmap shows up and down regulated proteins after protein modulation. Heatmap was generated for visual representation. The up regulated and down regulated proteins have a good level of agreement, although some degree of inter-individual variation was observed. Boxes in blue colour represent up regulated proteins, and boxes in red colour represent down regulated protein. For both colours, the higher intensity (darker) boxes represent higher differentially expressed proteins. Dendrogram shows hierarchal clustering, performed by using Cluster V3.0 and viewed through Java TreeView (available online).

5.17.3 Pathway map analysis

Pathway map analysis of IPA predicted the phagocytosis pathway to be significantly inhibited $p=0.0013$ and $Z=2.2$ (Figure 5-7). There were 11 out of 13 commonly modulated proteins that were down-regulated. Amongst the proteins that were down-regulated along the phagocytosis pathway were C-Reactive Protein (CRP); GRB2-associated-binding protein 2 (GAB2); IQ Motif And Sec7 Domain 1 (IQSEC1); Interferon regulatory factor 8 (IRF8); Lumican (LUM); Lymphocyte antigen-75 (LY75); MAP kinase activated protein kinase 2 (MAPKAP2); Mannose Receptor, C Type 1 (MRC1); Protein Kinase C Alpha (PRKCE); Active BCR-related (ABR); Adiponectin Collagen Domain Containing (ADIPOQ); Complement component 1 q subcomponent, A (C1QA). On the other hand, CCAAT/enhancer-binding protein beta (CEBPB) and Lymphocyte antigen 75 (LY75) were both found to be significantly down regulated although these findings contradicted the phagocytosis pathway.

Using IPA analysis, the pathway for migration of macrophages was also predicted to be inhibited $p=0.01$ and $Z=2.0$ (Figure 5-8). In this pathway, six proteins were found commonly modulated. All proteins FMS-Like Tyrosine Kinase 1 (FILT1); MAP kinase activated protein kinase 2 (MAPKAP2); Myosin-IXB (MYO9B), Plexin D1 (PLXND1); Adiponectin C1Q (ADIPOQ) except one [RAS Homolog gene family, member B (RHOB)] were down regulated and were predicted to contribute to the inhibition of migration of macrophages.

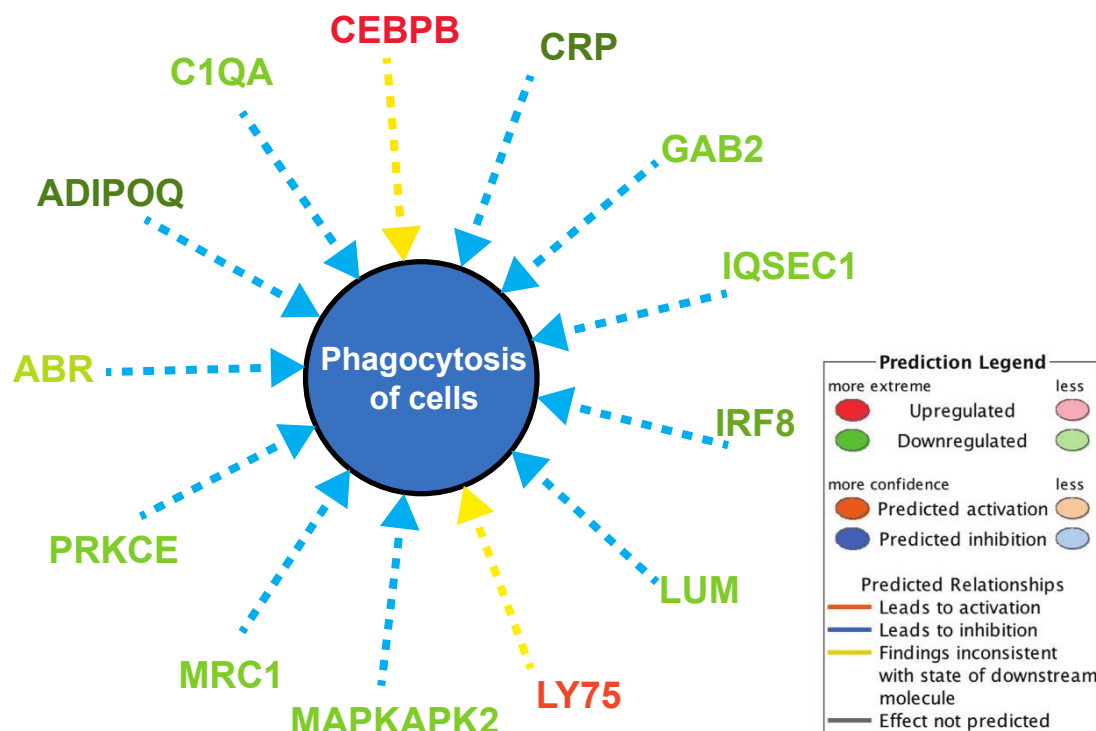


Figure 5-7 Phagocytosis of cells pathway analysis

The figure shows the result of the Ingenuity Pathway Analysis, using disease function module, on the phagocytosis of cells. This pathway is significantly inhibited in commonly modulated proteins compared to healthy controls. FDR corrected P value=0.013 and activation Z score=2.2. Significant value is considered if $P < 0.05$ or/and $Z \geq 2$. Down regulated proteins (green) and up-regulated proteins (red) are shown in the phagocytosis pathway, with darker shades (green or red) indicating more profound effect. Arrows represent pathways predicted to cause inhibition (blue) or inconsistent with state of downstream molecules (yellow) are shown. CCAAT/enhancer-binding protein beta (CEBPB); C-Reactive Protein (CRP); GRB2-associated-binding protein 2 (GAB2); IQ Motif And Sec7 Domain 1 (IQSEC1); Interferon regulatory factor 8 (IRF8); Lumican (LUM); Lymphocyte antigen 75 (LY75); MAP kinase activated protein kinase 2 (MAPKAPK2); Mannose Receptor, C Type 1 (MRC1); Protein Kinase C, Alpha (PRKCE); Active BCR related (ABR); Adiponectin, C1Q And Collagen Domain Containing (ADIPOQ); Complement component 1, q subcomponent, A (C1QA).

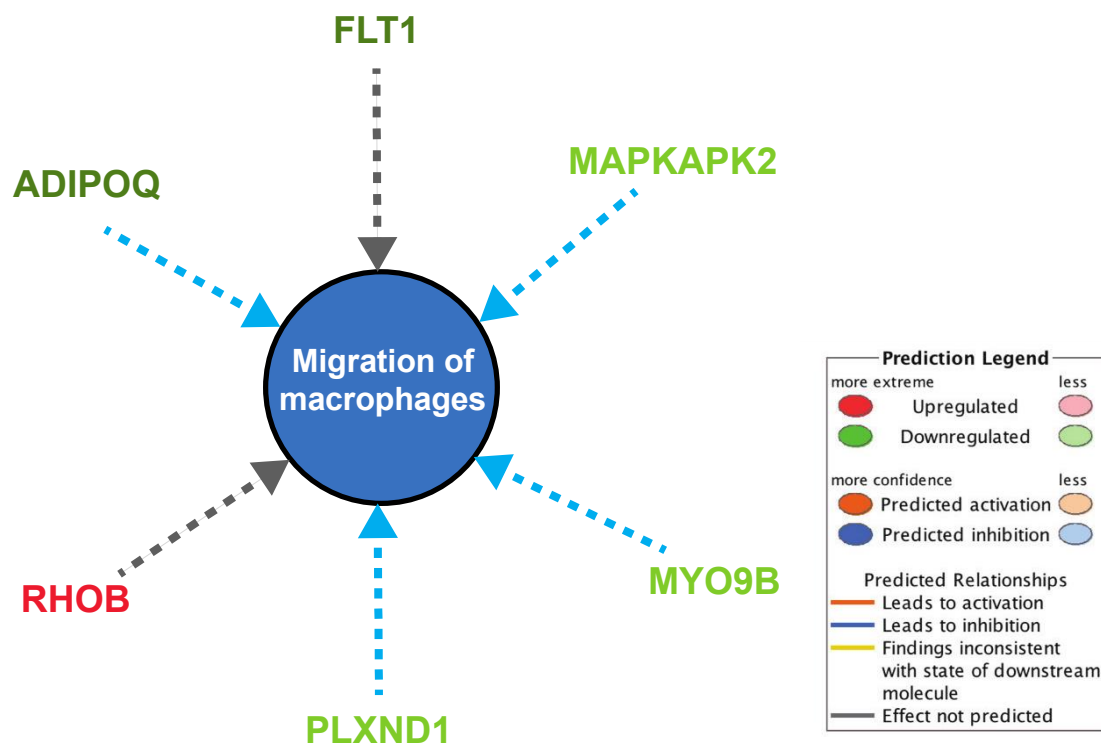


Figure 5-8 Migration of macrophages pathway analysis

The figure shows the result of the Ingenuity Pathway Analysis, using disease function module, on the migration of the macrophages. This pathway is significantly inhibited in commonly modulated proteins compared to healthy controls. FDR corrected p value=0.01 and activation Z score=2.0. Significant value is considered if $p < 0.05$, $Z \geq 2$. Down regulated proteins (green) and up-regulated proteins (red) are shown in the migration of macrophages pathway, with darker shades (green or red) indicating more profound effect. Arrows represent pathways predicted to cause inhibition (blue) or pathways not predicted to be related (Grey). Fms-Like Tyrosine Kinase 1 (FLT1); MAP kinase activated protein kinase 2 (MAPKAP2); Myosin-IXB (MYO9B), Plexin D1 (PLXND1); RAS Homolog gene family, member B (RHOB); Adiponectin C1Q (ADIPOQ).

5.17.4 Biological process analysis

Using the disease and function module in MetaCore™, apoptosis and survival pathways were significantly enriched in commonly modulated proteins with FDR corrected value of $p=0.046$ (Figure 5-9). Up regulated proteins were NADH ubiquinone oxidoreductase core subunit S3 (NDUFS3), Ku70, Ku80 and Ku70/80, and their protein functions are listed in Table 5-7.

Another pathway that was found significantly enriched was the oxidative phosphorylation biological process ($p=0.001$, Figure 5-10). Up-regulated proteins were NADH Dehydrogenase (Ubiquinone) Fe-S (NDUF) S3, NDUFS4, NDUF5, NDUF6, NDUF8, NDUF9, Cytochrome C Oxidase Subunit Va and Vb (COXVb, COXVa), ATP Synthase, H⁺ Transporting, Mitochondrial F1 Complex, Beta Polypeptide (ATP5B), Ubiquinol-Cytochrome C Reductase Binding Protein (UQCRCB), Cytochrome C-1 (CYC1), and their functions described in Table 5-7.

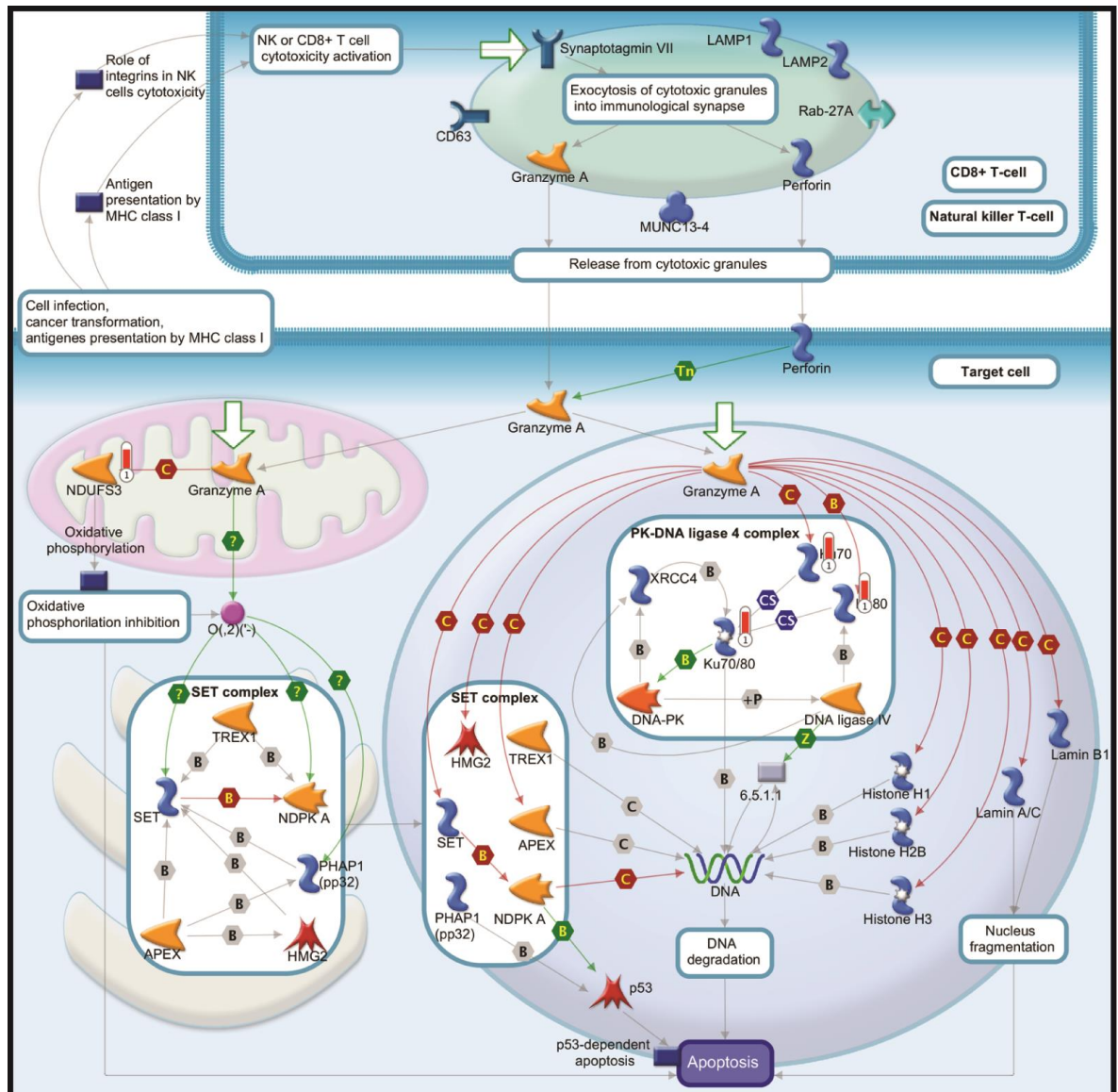


Figure 5-9 Apoptosis and survival pathway

Schematic representation of apoptosis and survival pathway analysis performed using MetaCore™, which is significantly enriched (over-represented) pathway compared to commonly modulated proteins. FDR corrected value is $p=0.046$. Protein-proteins interaction is represented with arrow either resulting an activation (green) or inhibition (red). Arrow direction shows the directionality of the interaction. Up-regulated proteins are NDUFS3 (NADH:ubiquinone oxidoreductase core subunit S3) , Ku70, Ku80 and Ku70/80. p value of <0.05 is considered to be significant. The up-regulated protein is represented by mini red colour thermometer next to the protein and blue thermometer represent down regulated protein.

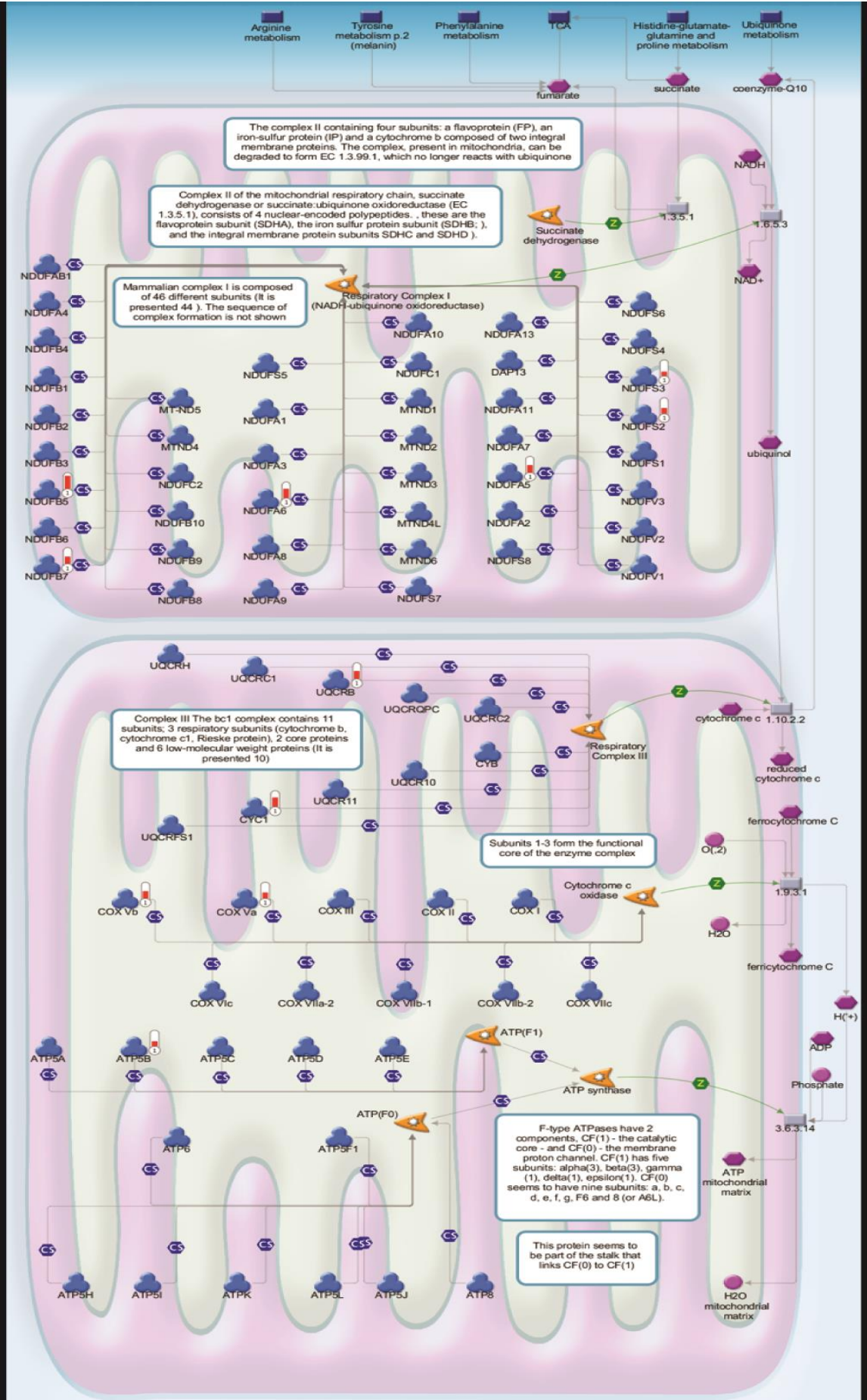


Figure 5-10 Oxidative phosphorylation pathway

Schematic representation of the oxidative phosphorylation pathway analysis performed using MetaCore™ is significantly enriched (over-represented) compared to commonly modulated proteins. FDR corrected value is $P=0.046$. Protein-proteins interaction is represented with arrows either resulting an activation (green) or inhibition (red). Arrow direction shows the directionality of the interaction. The up regulated proteins are NADH Dehydrogenase (Ubiquinone) Fe-S (NDUF) S3, NDUFS4, NDUF A5, NDUF A6, NDUF B5, NDUF B6, Cytochrome C Oxidase Subunit Va and Vb (COXVb, COXVa), ATP Synthase, H⁺ Transporting, Mitochondrial F1 Complex, Beta Polypeptide (ATP5B), Ubiquinol-Cytochrome C Reductase Binding Protein (UQCRB), Cytochrome C-1 (CYC1). p value of <0.05 is considered significant. The up-regulated protein is represented by mini red colour thermometer next to the protein and blue thermometer represents down-regulated protein.

5.18 Discussion

Nearly ten thousand proteins were profiled from each of the 2 datasets in this study. From that, 210 and 235 proteins were commonly up and down-regulated respectively. Inter-individual variations were observed in the heat map representation, but the majority proteins reached agreement in terms of either being up or down-regulated. Some of the proteins were found to be more markedly up-regulated and down-regulated compared to others.

Homeobox B6 (HOXB6), a Homeobox protein family that is involved in endometrial receptivity, was found to be markedly up regulated. HOXB6 protein is specifically involved in development, including that of lung and skin, and is localised to both the nucleus and cytoplasm [344]. Its function within the endometrium remains unknown. Unlike HOXA-10, the differential expression of HOXB6 has not been reported in endometriosis although a HOX-centric network has been associated with serous ovarian cancer risk [345], and the latter being associated with endometriosis in several studies [346, 347]. Sushi domain containing protein 2 (SUSD2) was significantly down-regulated, this protein has not been described in endometriosis and has an unknown significance in relation to endometriosis, although its reduced expression has been associated with the progression of non small cell lung cancer [348] .

It is interesting that the two pathways, namely ones associated with phagocytosis and macrophage migration were inhibited according to the ingenuity pathway analysis. Whilst proteins involved in the immune and inflammation signalling pathways appear to be down regulated (CRP, ADIPOQ, GAB2, IQSEC1, IRF8), the overall directional effect on phagocytosis and macrophage migration was an inhibitory one. Increased macrophage activation and activity have been reported in ectopic endometrium [349, 350], although one of the studies has extrapolated macrophage activity through an increased expression of macrophage markers from immunohistochemistry experiments rather than actually observed activity or its markers. Consistent with the findings from this study, several publications have shown that ectopic endometrium in women with endometriosis had a higher macrophage inhibiting factor [351, 352]. Theoretically, ectopic endometrium has progressed to 'disease', whilst

eutopic endometrium is a 'pre-disease' state; hence it is quite plausible that the macrophage behaviour within the endometrium of women with endometriosis is different from the active disease within the peritoneum. The literature however supports the down regulation of phagocytosis in peritoneal endometriotic lesions [353].

The oxidative and phosphorylation pathway was significantly enriched, although this finding is not surprising. Whilst oxidative phosphorylation is a vital part of metabolism, it produces reactive oxygen species (ROS) such as superoxide and hydrogen peroxide, which lead to the propagation of free radicals, damaging cells and contribute to the disease. ROS is a well-known detrimental factor in the pathogenesis of endometriosis [229, 230, 238]. Several components in the respiratory complex 1 (NADH- ubiquinone oxidoreductase), III and cytochrome oxidase are significantly up-regulated supporting the notion that endometriosis is a condition of high oxidative stress.

The apoptosis and survival pathway was also significantly enriched. Endometriosis is increasingly being recognised as a condition in which ectopic endometrial cells exhibit abnormal proliferative and apoptotic regulation in response to appropriate stimuli [354]. Eutopic endometrium of women with endometriosis does not undergo the same increased level of apoptosis as those in women with no endometriosis [355, 356], with a lower number of apoptotic cells in the epithelium and stroma [357], and a higher proliferation capacity [358]. The apoptosis and survival enriched pathway shows several proteins within the PK DNA-Ligase IV complex to be up-regulated. DNA-ligase joins single-strand breaks in a double-stranded poly deoxynucleotide in an ATP-dependent reaction. It is an important protein for DNA repair [359]. The up regulated DNA mechanism is coupled with an up regulation of NADH:ubiquinone oxidoreductase core subunit S3 within the mitochondria. Oxidative damage and mitochondria DNA mutations in endometriotic lesions has been reported [360], although these observations have not been described within the eutopic endometrium.

Due to the variation in methodology in the published studies on proteomic analysis of the endometrium, conflicting results have been reported. Whilst most

researchers adopt the same principles in their analysis, the chosen variable options for each step of the analysis, such as protein separation (gel-based or non-gel-based), variation in sample introduction, ionisation and ion detection methods, has resulted in numerous permutations of techniques used in any one study/experiment. The gel-based approaches are cumbersome in general, requires high sample availability, have limited sensitivity, and only allow one to deal with a limited subset of proteins at any one time [361]. As a result, there is now an increasing interest in gel-free techniques. These approaches may or may not utilise MS. The development of these gel-free strategies [112] will inevitably focus on performing large-scale quantitative (using labelled and label-free MS-based methods, immunoassays/protein microarrays) and functional (MS-based, protein microarrays) proteomics [325].

One of the commonly used gel-free techniques, particularly in endometrial tissue proteomic studies, is the combination of protein microarray (protein chip, see Chapter 5) with Surface Enhanced Laser Desorption or Ionisation and Time of light (Protein chip-SELDI-TOF). Although this technique provides rapid analysis turnover, it lacks reproducibility. Therefore, this thesis focused on the combination of liquid chromatography with tandem mass spectrometry (LC-MS/MS). LC-MS/MS is a modern advanced gel-free technique that facilitates high throughput analysis, enabling analysis of a significantly increased number of samples when compared to former techniques. LC-MS/MS technique also avoids excluding low/high abundance protein in the analysis that can potentially contain a novel marker. An isobaric tag such as tandem mass tags (TMT) or isobaric tags for relative and absolute quantification (iTRAQ) can be used concomitantly with this technique, and provide a powerful tool for quantitative proteomics.

This study has been analysed using statistical analysis described in the methods section (see section 5.16.16) with a 99% confidence interval (CI), which only allow very low false discovery rate. The other strength of the study is that experiments were done using a working and proven standard operating procedure. Tissue procurement prior to the experiment used a multistep technique. System suitability check was done regularly and the machines

(including liquid chromatographer and mass spectrometer) were fully calibrated and optimised prior to use. Discovery of the proteins in this study will be uploaded to PRIDE (<http://www.ebi.ac.uk/pride/archive/>) for public proteomic data repository and will be independently cross-verified for transparency.

By using 8-plex ITRAQ LC MS/MS with deep phenotyping in accordance to the World Endometriosis Research Foundation (WERF) Endometriosis Phenome and Biobanking Harmonisation Project (EPHect) guidance, we were able to perform cluster analysis independent of the menstrual phase, and were able to delineate the biomarkers, which are non-menstrual phase dependent. This study has also determined the fold change of each putative biomarker. This study was not powered to examine for correlation between the putative biomarkers and the disease severity, however this could be the focus of future investigation if particular proteins correlate well with the presence or absence of the disease.

The number of samples used for this study is small but sufficient for a biomarker discovery phase 1 [318]. Future studies will need to verify and validate the panel of up and down-regulated proteins using an independent sample set. Although the samples were thoroughly washed and cleaned during tissue procurement, there is always a possibility of contamination with blood that will interfere with the protein analysis.

5.19 Conclusion

Proteomic profiling of endometrium in women with and without endometriosis has uncovered distinctly differentially up and down regulated proteins. The global processes borne out by this study is in keeping with the general knowledge around the pathogenesis of endometriosis. Clearly, in a biomarker discovery process, this is only the very initial phase. Subsequent steps will include further verification of a chosen protein panel, followed by large-scale clinical validation studies. It is only when the diagnostic capability of the test is fully proven, can one consider its introduction into clinical practice.

Table 6.3 Protein description of significant up and down regulated proteins according to respective pathway analysis

Protein Name	Gene name	Protein accession	Protein description(s)	Predicted	Reference(s)
Up regulated proteins					
Homeobox protein-B6	HOXB6	P17509	A protein member of the Homeobox family and encodes a protein with a Homeobox DNA-binding domain. It is included in a cluster of Homeobox B genes located on chromosome 17. The encoded protein functions as a sequence-specific transcription factor that is involved in development, including that of lung and skin, and has been localized to both the nucleus and cytoplasm. Altered expression of this gene or a change in the subcellular localization of its protein is associated with some cases of acute myeloid leukaemia and colorectal cancer.	+	Kaur et al 1990, Shen et al 1991, Peverali et al 1990 [362-364]
Liver carboxylesterase-1	CES-1	P23141	This protein is an enzyme that is known to hydrolyze aromatic and aliphatic esters and can manage cellular cholesterol esterification levels. It may also play a role in detoxification in the lung and/or protection of the central nervous system from ester or amide compounds. Carboxylesterase 1 deficiency may be associated with non-Hodgkin lymphoma or B-cell lymphocytic leukaemia. Inhibition of CES1 by in particular organophosphates reduces tumor-killing activity by monocytes.	+	Oertel et al 1985, Markey et al 2011 [365, 366]
Synaptotagmin-like protein 1	SYTL-1	Q8IYJ3	The synaptotagmins are integral membrane proteins of synaptic vesicles thought to serve as Ca(2+) sensors in the process of vesicular trafficking and exocytosis. Calcium binding to synaptotagmin I participates in triggering neurotransmitter release at the synapse. SYT1 is the master switch responsible for allowing the human brain to release neurotransmitters. SYT1 senses calcium concentrations as low as 10 ppm and subsequently signals the SNARE complex to open fusion pores.	+	Lee et al 2010, Perin 1991 [367, 368]
Tumour associated signal transducer 2	TACSTD2	P09758	This encoded protein is a carcinoma-associated antigen defined by the monoclonal antibody GA733. This antigen is a member of a family including at least two type I membrane proteins. It transduces an intracellular calcium signal and acts as a cell surface receptor. Mutations of this gene result in gelatinous drop-like corneal dystrophy, an autosomal recessive disorder characterized by severe corneal amyloidosis leading to blindness.	+	Linnenbach et al, 1992 [369]
Microsomal glutathione S-transferase	MGST2	Q99735	The MAPEG (Membrane-Associated Proteins in Eicosanoid and Glutathione metabolism) family consists of six human proteins, several of which are involved in the production of leukotrienes and prostaglandin E, important mediators of inflammation. This gene encodes a protein that catalyzes the conjugation of leukotriene A4 and reduced glutathione to produce leukotriene C4	+	Jakobsson 1996[370]

Cluster Differentiation 99	CD99	P14209	It is expressed on all leukocytes but highest on thymocytes and is believed to augment T-cell adhesion and apoptosis of double positive t cells. It has been found in endothelial cells and in the periodontium, including gingival fibroblasts and gingival epithelial cells. It also participates in migration and activation. ^[11] There is also experimental evidence that it binds to cyclophilin A. It is found on the cell surface of Ewing's sarcoma tumors ^[13] and is positive in granulosa cell tumors. It is more expressed in malignant gliomas than in the brain, and such overexpression results in higher levels of invasiveness and lower rates of survival.	+	Bernard et al 2000, Oh et al, 2007 [371, 372]
Down regulated proteins					
Adiponectin	ADIPOQ	Q15848	Important adipokine involved in the control of fat metabolism and insulin sensitivity, with direct anti-diabetic, anti-atherogenic and anti-inflammatory activities. Stimulates AMPK phosphorylation and activation in the liver and the skeletal muscle, enhancing glucose utilization and fatty-acid combustion. Antagonizes TNF-alpha by negatively regulating its expression in various tissues such as liver and macrophages, and also by counteracting its effects. Inhibits endothelial NF-kappa-B signaling through a cAMP-dependent pathway. May play a role in cell growth, angiogenesis and tissue remodeling by binding and sequestering various growth factors with distinct binding affinities, depending on the type of complex, LMW, MMW or HMW.	-	Yamauchi et al, 2001 [373]
Protein phosphatase 1 regulatory subunit 12A	PPP1R12A	O14974	Myosin phosphatase target subunit 1, which is also called the myosin-binding subunit of myosin phosphatase, is one of the subunits of myosin phosphatase. Myosin phosphatase regulates the interaction of actin and myosin downstream of the guanosine triphosphatase Rho. The small guanosine triphosphatase Rho is implicated in myosin light chain (MLC) phosphorylation, which results in contraction of smooth muscle ^[3] and interaction of actin and myosin in nonmuscle cells.	-	Bannert et al, 2003; Takahashi et al, 1997 [374, 375]
Protein phosphatase 1 regulatory subunit 12B	PPP1R12B	O60237	Myosin light chain phosphatase (MLCP) consists of three subunits- catalytic subunit, large subunit/myosin binding subunit (MBS) and small subunit (sm-M20). This gene is a multi-functional gene which encodes both MBS and sm-M20. MLCP regulates myosins and the dephosphorylation is enhanced by the presence of MBS. The sm-M20 is suggested to play a regulatory role in muscle contraction by binding to MBS. MBS is also encoded by another gene, myosin light chain phosphatase target subunit 1. sm-M20 shows higher binding affinity to this gene product than to myosin light chain phosphatase target subunit 2-MBS even though the two MBS proteins are highly similar.	-	Bannert et al , 2003; Fujioka 1998 [374, 376]
Voltage-gated potassium channel	KCNAB2	Q13303	Voltage-gated potassium (Kv) channels represent the most complex class of voltage-gated ion channels from both functional and structural standpoints. Their diverse functions include regulating	-	Kaab et al, 1998 [377]

subunit beta -2			neurotransmitter release, heart rate, insulin secretion, neuronal excitability, epithelial electrolyte transport, smooth muscle contraction, and cell volume. Four sequence-related potassium channel genes - shaker, shaw, shab, and shal - have been identified in Drosophila, and each has been shown to have human homolog(s). This gene encodes a member of the potassium channel, voltage-gated, shaker-related subfamily. This member is one of the beta subunits, which are auxiliary proteins associating with functional Kv-alpha subunits. This member alters functional properties of the KCNA4 gene product.		
Sushi domain containing protein 2	SUSD2	Q9UGT4	This gene is a member of the Antp homeobox family and encodes a protein with a homeobox DNA-binding domain. It is included in a cluster of homeobox B genes located on chromosome 17. The encoded protein functions as a sequence-specific transcription factor that is involved in development, including that of lung and skin, and has been localized to both the nucleus and cytoplasm. Altered expression of this gene or a change in the subcellular localization of its protein is associated with some cases of acute myeloid leukemia and colorectal cancer	-	Ferrai et al, 2009 [378]
Plexin-D1	PLXND1	Q9Y4D7	Plays an important role in cell-cell signaling, and in regulating the migration of a wide spectrum of cell types. Regulates the migration of thymocytes in the medulla. Regulates endothelial cell migration. Plays an important role in ensuring the specificity of synapse formation.	-	Sakurai et al, 2010 [379]
Phagocytosis and cell pathway analysis					
CCAAT/enhancer-binding protein beta	CEBPB	P17676	Activity of this protein is important in the regulation of genes involved in immune and inflammatory responses, among other processes.	+	van der Krieken 2015 [380]
C-Reactive Protein	CRP	P02741	Inflammatory marker	-	Pepys et al, 2003 [381]
GRB2-associated-binding protein 2	GAB2	Q9UQC2	Proteins containing pleckstrin homology (PH) domain, and bind SHP2 tyrosine phosphatase and GRB2 adapter protein. They act as adapters for transmitting various signals in response to stimuli through cytokine and growth factor receptors, and T- and B-cell antigen receptors. The protein is the principal activator of phosphatidylinositol-3 kinase in response to activation of the high affinity IgE receptor.	-	Zhao et al, 1999 [382], Nishida et al, 1999 [383]
IQ Motif And Sec7	IQSEC1	Q6DN90	Protein related to endocytosis and Art6 signaling events. Guanine nucleotide exchange factor for ARF1	-	Aizel et al,

Domain 1			and ARF6). Guanine nucleotide exchange factor activity is enhanced by lipid binding Accelerates GTP binding by ARFs of all three classes. Guanine nucleotide exchange protein for ARF6, mediating internalisation of beta-1 integrin.		2013 [384]
Interferon regulatory factor 8	IRF8	Q02556	Proteins from family that composed of a conserved DNA-binding domain in the N-terminal region and a divergent C-terminal region that serves as the regulatory domain. The IRF family proteins bind to the IFN-stimulated response element (ISRE) and regulate expression of genes stimulated by type I IFNs, namely IFN-alpha and IFN-beta. IRF family proteins also control expression of IFN-alpha and IFN-beta-regulated genes that are induced by viral infection.	-	Tamura et al, 2015 [385]
Lumican	LUM	P51884	Lumican is a major keratin sulfate proteoglycan of the cornea but is ubiquitously distributed in most mesenchymal tissues throughout the body. Lumican is involved in collagen fibril organization and circumferential growth, corneal transparency, and epithelial cell migration and tissue repair.	-	Chakravarti et al, 2002 [386]
Lymphocyte antigen 75	LY75	O60449	This protein is also known as CD 205 or DEC-205. It is one of the major endocytosis receptors on dendritic cells and has been widely used for vaccine generation against viruses and tumors. This protein involves in antigen uptake and can induce either tolerance or immunity in the absence or presence of inflammatory stimulus. It has also been suggested that DEC205 may bind apoptotic and necrotic cells and oligonucleotides.	+	Cao et al, 2015 [387]
MAP kinase activated protein kinase 2	MAPKAP2	P49137	Stress-activated serine/threonine-protein kinase protein involved in cytokines production, endocytosis, reorganization of the cytoskeleton, cell migration, cell cycle control, chromatin remodeling, DNA damage response and transcriptional regulation.	-	Sokoe et al. 1993 [388]
Mannose Receptor, C Type 1	MRC1	P22897	Protein involves in the recognition of complex carbohydrate structures on glycoproteins. It is an important part of several biological processes, including cell-cell recognition, serum glycoprotein turnover, and neutralization of pathogens. The protein is a type I membrane receptor that mediates the endocytosis of glycoproteins by macrophages.	-	Andersen 2015 [389]
Protein Kinase C, Alpha	PRKCE	Q02156	This protein is a family of serine- and threonine-specific protein kinases that can be activated by calcium and the second messenger diacylglycerol. PKC family members phosphorylate a wide variety of protein targets and are known to be involved in diverse cellular signaling pathways. PKC family members also serve as major receptors for phorbol esters, a class of tumor promoters.	-	Micol et al, 1999 [390]
Active BCR related	ABR	Q12979	Functional studies in mice determined that this protein plays a role in vestibular morphogenesis	-	Kaartinen et

Adiponectin, C1Q And Collagen Domain Containing	ADIPOQ	Q15848	Important adipokine involved in the control of fat metabolism and insulin sensitivity, with direct anti-diabetic, anti-atherogenic and anti-inflammatory activities. Stimulates AMPK phosphorylation and activation in the liver and the skeletal muscle, enhancing glucose utilization and fatty-acid combustion. Antagonizes TNF-alpha by negatively regulating its expression in various tissues such as liver and macrophages, and also by counteracting its effects. Inhibits endothelial NF-kappa-B signaling through a cAMP-dependent pathway. May play a role in cell growth, angiogenesis and tissue remodeling by binding and sequestering various growth factors with distinct binding affinities, depending on the type of complex, LMW, MMW or HMW.	-	al 2002 [391] Yamauchi et al, 2001 [373]
Complement component 1, q subcomponent, A	C1QA	P02745	C1q protein associates with C1r and C1s in order to yield the first component of the serum complement system. Deficiency of C1q has been associated with lupus erythematosus and glomerulonephritis	-	Mosaad et al, 2015 [392]
Migration of macrophages pathway					
Fms-Like Tyrosine Kinase 1	FLT1	P17948	Also known as VEGF receptor-1. It binds and reduces free circulating levels of the proangiogenic factors VEGF (vascular endothelial growth factor) and PlGF (placental growth factor). sFlt-1 thereby blunts the beneficial effects of these proangiogenic factors on maternal endothelium, with consequent maternal hypertension and proteinuria.	-	Khalil et al, [393]
Myosin-IXB	MYO9B	Q13459	Actin-based motor molecules with ATPase activity. Unconventional myosins serve in intracellular movements. May be involved in the remodeling of the actin cytoskeleton. Binds actin with high affinity both in the absence and presence of ATP and its mechano-chemical activity is inhibited by calcium ions.	-	Wirth et al, 1996 [394]
Plexin D1	PLXND1	Q9Y4D7	Plays an important role in cell-cell signaling, and in regulating the migration of a wide spectrum of cell types. Regulates the migration of thymocytes in the medulla. Regulates endothelial cell migration. Plays an important role in ensuring the specificity of synapse formation.	-	Sakurai et al, 2010 [379]
MAP kinase activated protein kinase 2	MAPKAP2	P49137	Stress-activated serine/threonine-protein kinase involved in cytokines production, endocytosis, reorganization of the cytoskeleton, cell migration, cell cycle control, chromatin remodeling, DNA damage response and transcriptional regulation.	-	Sokoe et al. 1993 [388]

RAS Homolog gene family, member B	RHOB	P62745	Mediates apoptosis in neoplastically-transformed cells after DNA damage. Not essential for development but affects cell adhesion and growth factor signaling in transformed cells. Plays a negative role in tumorigenesis as deletion causes tumor formation. Involved in intracellular protein trafficking of a number of proteins.	+	Mellor et al, 1998 [395], Gampel et al 1999 [396]
Adiponectin, C1Q And Collagen Domain Containing	ADIPOQ	Q15848	An important adipokine that involves in the control of fat metabolism and insulin sensitivity, with direct anti-diabetic, anti-atherogenic and anti-inflammatory activities. Stimulates AMPK phosphorylation and activation in the liver and the skeletal muscle, enhancing glucose utilization and fatty-acid combustion. Antagonizes TNF-alpha by negatively regulating its expression in various tissues such as liver and macrophages, and also by counteracting its effects. Inhibits endothelial NF-kappa-B signaling through a cAMP-dependent pathway. May play a role in cell growth, angiogenesis and tissue remodeling by binding and sequestering various growth factors with distinct binding affinities, depending on the type of complex, LMW, MMW or HMW.	-	Yamauchi et al, 2001 [373]
Apoptosis and survival pathway					
NADH Dehydrogenase (Ubiquinone) Fe-S	NDUF S3	O75489	Core subunit of the mitochondrial membrane respiratory chain NADH dehydrogenase (Complex I) that is believed to belong to the minimal assembly required for catalysis. Complex I functions in the transfer of electrons from NADH to the respiratory chain. The immediate electron acceptor for the enzyme is believed to be ubiquinone. Reduce level of NDUF3 is associated with increase risk of serous ovarian cancer. Suppression of mitochondrial complex I such as NDUF3 influences cell metastatic properties.	-	Wang et al, 2013 [397], He et al, 2013 [398]
X-ray repair cross-complementing protein 6 (XRCC6) X-ray repair cross-complementing protein 5 Ku80, Ku70/80	Ku70, Ku80, Ku70/80	P12956 P13080	ATP-dependent DNA helicase II subunit 1. Involved in non-homologous end joining (NHEJ) DNA double strand break repair. DNA binding is sequence-independent but has a high affinity to nicks in double-stranded DNA and to the ends of duplex DNA. Binds to naturally occurring chromosomal end, and therefore provides chromosomal end protection. Required for telomere recombination to repair telomeric ends in the absence of telomerase. ku70, of the ku70/ku80 heterodimer, binds to the stem loop of tlc1, the RNA component of telomerase. Involved in telomere maintenance. Interacts with telomeric repeats and subtelomeric sequences thereby controlling telomere length and protecting against subtelomeric rearrangement. Maintains telomeric chromatin, which is involved in silencing the expression of genes located at the telomere.	-	Pace et al, 2010 [399], Boulton et al, 1998 [400]
Oxidative phosphorylation pathway					

Chapter 5

NADH Dehydrogenase (Ubiquinone) Fe-S 3	NDUFS3	O75489	Core subunit of the mitochondrial membrane respiratory chain NADH dehydrogenase (Complex I) that is believed to belong to the minimal assembly required for catalysis. Complex I functions in the transfer of electrons from NADH to the respiratory chain. The immediate electron acceptor for the enzyme is believed to be ubiquinone. Reduce level of NDUFS3 is associated with increase risk of serous ovarian cancer. Suppression of mitochondrial complex I such as NDUFS3 influences cell metastatic properties.	-	Wang et al, 2013 [397], He et al, 2013 [398]
NADH Dehydrogenase (Ubiquinone) Fe-S4	NDUFS4	O43181	Accessory subunit of the mitochondrial membrane respiratory chain NADH dehydrogenase (Complex I) that is believed not to be involved in catalysis. Its vital role in cellular ATP production. It removes electrons from NADH and passes them by a series of different protein-coupled redox centers to the electron acceptor ubiquinone. The electron flux leads to ATP generation via the building of a proton gradient across the inner membrane.	-	Emahazion et al, 1998[401]
NADH dehydrogenase [ubiquinone] 1 alpha 5	NDUFA5	Q16718	It transfers electrons from NZDH to ubiquinone. The NDUFA5 protein localizes to the mitochondrial inner membrane and it is thought to aid in this transfer of electrons. It is consistently found reduced expression in brains of autism patients. Mitochondrial dysfunction and impaired ATP synthesis can result in oxidative stress.	-	Anitha et al, 2013 [402]
NADH dehydrogenase [ubiquinone] 1 alpha 6	NDUFA6	P56556	Accessory subunit of the mitochondrial membrane respiratory chain NADH dehydrogenase (Complex I) that is believed to be not involved in catalysis. Complex I functions in the transfer of electrons from NADH to the respiratory chain. The immediate electron acceptor for the enzyme is believed to be ubiquinone	-	Emahazion et al 1998 [403]
NADH dehydrogenase [ubiquinone] 1 beta 5	NDUFB5	O43674	Is a subunit of Complex 1 of the respiratory chain, which transfers electrons from NADH to ubiquinone. However, NDUFB5 is an accessory subunit of the complex that is believed not to be involved in catalysis.	-	Emahazion et al, 1998 [403]
NADH dehydrogenase [ubiquinone] 1 beta 6	NDUFB6	O95139	NDUFB6 is a subunit of the enzyme NADH dehydrogenase (ubiquinone) the largest of the respiratory complexes. The structure is L-shaped with a long, hydrophobic transmembrane domain and a hydrophilic domain for the peripheral arm that includes all the known redox centers and the NADH binding site	-	Emahazion et al 1998 [403]
Cytochrome C Oxidase Subunit Va and Vb	COXVa, COXVb	P20674 P10606	Cytochrome c oxidase (COX) is the terminal enzyme of the mitochondrial respiratory chain. It is a multi-subunit enzyme complex that couples the transfer of electrons from cytochrome c to molecular oxygen and contributes to a proton electrochemical gradient across the inner mitochondrial membrane to drive ATP synthesis via protonmotive force. The mitochondrially-encoded subunits perform the	-	Fornuskova et al, 2007 [404]; Chen et al, 2010

			electron transfer of proton pumping activities. The functions of the nuclear-encoded subunits are unknown but they may play a role in the regulation and assembly of the complex. COX5A and COX5B are involved in the regulation of cancer cell metabolism		[405]
ATP Synthase, H+ Transporting, Mitochondrial F1 Complex, Beta Polypeptide	ATP5B	P06576	This gene encodes a subunit of mitochondrial ATP synthase. Mitochondrial ATP synthase catalyzes ATP synthesis, utilizing an electrochemical gradient of protons across the inner membrane during oxidative phosphorylation. ATP synthase is composed of two linked multi-subunit complexes: the soluble catalytic core, F1, and the membrane-spanning component, Fo, comprising the proton channel	-	Neckleman et al, 1989 [406]
Ubiquinol-Cytochrome C Reductase Binding Protein	UQCRB	P14927	The ubiquinone-binding protein is a nucleus-encoded component of ubiquinol-cytochrome c oxidoreductase (Complex III; EC 1.10.2.2) in the mitochondrial respiratory chain and plays an important role in electron transfer as a complex of ubiquinone and QP-C	-	Suzuki et al , 1990 [407]
Cytochrome C-1	CYC1	P08574	This is the heme-containing component of the cytochrome b-c1 complex, which accepts electrons from Rieske protein and transfers electrons to cytochrome c in the mitochondrial respiratory chain	-	Zhu et al, 2012 [408]

Table 5-7 Table of significant proteins according to respective pathway analysis

Table showing the up (red) and down regulated (green) protein and description of specialized function according to the pathway analysis using Pathway analysis and MetaCore™, the pathways are predicted to be inhibited (-), activated (+).

Chapter 6: General discussion and Conclusions

This thesis provides an overview of the impact of endometriosis on fertility in relation to Assisted Reproductive Technology (ART). In two systematic reviews and meta-analyses, this thesis explored the impact of endometriosis on the reproductive outcomes of women with and without endometriosis. When compared to controls, the clinical pregnancy rate was significantly lower in women with endometriosis, although the live birth rate was not dissimilar. Endometriosis also resulted in a significantly lower number of oocytes retrieved during ovarian stimulation for IVF/ICSI. The effects were more markedly seen in more severe endometriosis. For those undergoing ART, the question of whether surgery helps improve the reproductive outcome of women with endometriosis compared to those without, unfortunately, is still not adequately answered, mainly because of insufficient evidence available to draw firm conclusions.

The management of endometrioma is also still highly controversial. Most practitioners would advocate surgical removal, although evidence drawn from the literature cautioned the adoption of a dogmatic view of either 'removal' or 'non-removal', but recommends individualising each patient's care. This is because the second systematic review and meta-analysis in this thesis were not able to demonstrate a definitive benefit for the surgical removal of endometrioma prior to ART, and on the contrary, has suggested potential harm by way of decreasing the ovarian reserve. Conversely, the review also demonstrated that leaving the endometrioma intact during ART treatment have no significant impact on the live birth rate.

In a retrospective review of two IVF databases of two independent IVF centres demonstrated that women with endometriosis undergoing ART had a lower number of oocytes collected per mature follicle compared to controls. It also found that women with endometriosis have a higher percentage of arrested embryos at D3, which suggested a potential poor oocyte quality issue as opposed to the presence of poor quality spermatozoa. However, the number and percentage of a good quality embryo and utilisation rate were similar, hence the pregnancy rate was unaffected.

The consistent findings of lower oocyte yield in women with endometriosis further prompted the investigation of a biologically plausible cause. The follicular fluid, the main component of the micro follicular environment, was investigated in a mouse oocyte model. The FF obtained from women with endometriosis was shown to have a detrimental effect on the meiosis process. The mouse oocytes incubated in FF of women with endometriosis have lower maturation rate. This finding is consistent with an earlier publication pertaining to bovine oocytes. A stepwise approach was therefore taken to uncover the cause. Oocyte incubated in the FF of women with endometriosis had a slower polar body extrusion rate and a higher rate of developmental arrest, with the majority being arrested at metaphase I stage, with identical meiotic spindle morphometric measurements.

Additionally, FF of women with endometriosis was found to induce a higher level of intracellular ROS, which in turn caused DNA damage. The effect however reverted to normal with the addition of Resveratrol, a potent antioxidant. The reason for the high levels of ROS in the follicular fluid of women with endometriosis was not attributed to the presence of iron. FF is believed to have activated the pathway leading to oocyte developmental arrest, in line with recently found DNA damage pathway, which can be activated by various DNA damaging agents and can cause meiotic arrest. Activation of this pathway kept the spindle assembly checkpoint activated and prevented the extrusion of the first polar body, accounting for the poor oocyte maturation rate in women with endometriosis during ART.

Last but not least, the proteome of the endometrium of those with endometriosis is distinct from those without the disease, affirming that the reproductive impact of endometriosis is not only confined to the detrimental development of the oocyte but also on the endometrium. The enriched molecular pathways found in the proteomic analysis suggests a significant association of endometriosis with the apoptosis and survival, oxidative phosphorylation, phagocytosis and macrophage migration pathway. However, the biomarker discovery process is a relatively long and arduous one; such experiments presented in this thesis,

although robust, will in future require further verification, validation and clinical trials prior to its introduction into clinical practice.

This thesis has combined both clinical and scientific aspect of the disease. The clinical systematic review and meta-analysis provided an overview on the topic of how endometriosis influences the outcome of ART. The overwhelming conclusion of the systematic reviews and meta-analysis as well as the observational study on oocyte and embryo development suggested that the presence or absence of endometriosis is critical to the oocyte maturation and recovery rate in and ART cycle. This is an important clinical point as the number of oocytes retrieved correlates well with the cumulative pregnancy rate of IVF [131]

One of the drawbacks of this meta-analysis is the clinical heterogeneity in particular that relate to the variation in surgical skills, learning curve, techniques and the lag time since the surgical intervention. Until the results of ongoing randomised controlled trials are available, the assumption that the treatment efficacy of various surgical treatment modalities such as thermal versus laser ablation or surgical techniques such as excision versus ablation techniques to be similar is a potential confounder of our meta-analysis. However, the current available studies do not provide data in this level of detail for analysis. Other clinical parameters, which can potentially contribute to the clinical heterogeneity of the data, included the lack of information on the patients' ovarian reserve, variation in stimulation protocol and the improvement in general of the ART success rate with time.

The results of the retrospective database analysis, whilst in line with the findings of the meta-analysis, are subjected to confounding factors due to its retrospective nature, small sample size and hence stratification according to severity was not possible.

Mechanistically, the thesis further explored in a mouse model, using follicular fluid from women with and without endometriosis obtained during ovarian stimulation cycles during IVF, the morphology of oocyte development and ROS content and DNA damage within the oocyte. There was a lower oocyte maturation rate in oocytes incubated overnight within FF of women with

endometriosis compared to those without endometriosis. I postulated that the ROS content within the FF of endometriosis disrupts the spindle formation and chromosomal alignment that cause poor oocyte maturation although experimental results refuted this hypothesis. Mouse oocytes, when exposed transiently to the follicular fluid of women with endometriosis, had a higher level of ROS content and DNA damage compared to controls.

Stringent patient recruitment criteria were applied to this study with FF collected exclusively from the first follicle punctured from each oocyte retrieval procedure to avoid contamination with blood and flushing media. However, due to this stringent methodology, only a limited amount of follicular fluid was collected, which limited the number of repeat experiments possible. As factors in FF can mediate the resumption of meiosis irrespective PDE3A inhibition by Milrinone, some oocytes managed to move on into meiosis I stage. Therefore experiments that required assessment at GV stage (i.e. DNA damage, or ROS assessment) were repeated to get an acceptable sample size. Another weakness is that this study was conducted in-vitro, whereas this process is normally taking place inside a follicle. Thus this system is simplified (there are no granulosa, blood supply, cumulus mass etc.). In addition, oocytes are exposed to light during collection (a possible variable) and exposed to atmospheric oxygen concentrations, which are very different to those experienced in vivo. Finally, as in any animal model experiment, results from this study cannot necessarily be extrapolated to human, and further study using human oocytes is required.

Proteomic analysis of endometrium was performed using LC-MS/MS technique for biomarker discovery. From this analysis, menstrual phase independent biomarkers were discovered, although validation is still awaited. LC-MS/MS is a modern advanced gel-free technique that facilitates high throughput analysis, enabling analysis of a significantly increased number of samples when compared to former techniques. LC-MS/MS technique avoids excluding extremely low and high abundance protein in the analysis that can potentially contain novel markers. This study has been analysed using statistical analysis with a 99% confidence interval, with low false discovery rate. The other strength

of the study is that experiments were performed using a working and proven standard operating procedure with careful tissue procurement techniques. However, as this was a biomarker discovery project based on deep phenotyping, only a small sample size was required, hence stratification in accordance to endometriosis type was not possible. However, such stratification will be required in future larger validation studies.

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Chapter 7: Future research

7.1 Study on human oocyte

The work pertaining to the impact of human follicular fluid of women with endometriosis in this thesis was applied to a mouse oocyte model. Here, the hypothesis is that the poor oocyte yield in women undergoing ovarian stimulation during IVF/ICSI is due to poor oocyte maturation caused by defective meiosis process, as demonstrated in mouse oocytes and that reactive oxygen species contained in the follicular fluid of women with endometriosis could activate the DNA damage pathway and are responsible for the meiosis defect and poor maturation in human oocytes.

Existing studies of human oocytes have demonstrated an increased ROS level within the granulosa tissue of women with endometriosis [60], but no studies have 1) investigated the ROS level within the actual oocytes; 2) quantified the DNA damage level in human oocytes, or 3) investigated the effect of follicular fluid of women with endometriosis on *human* oocytes.

7.1.1 Objective

To determine the effect of the maturation rate following incubation of human oocyte in the follicular fluid of women with endometriosis, and to investigate the cause of poor oocyte yield in women with endometriosis.

7.1.2 Method

Immature human oocytes collected from healthy women (non-endometriosis) will be incubated in the FF of women with endometriosis. Vice versa, immature oocytes collected from women with endometriosis will be incubated in FF of women with no endometriosis. The outcome measures are the maturation rate (polar body extrusion), and DNA damage level (immunohistochemistry). For translational purposes, I would also like demonstrate improvement of the maturation rate of oocyte from women with endometriosis incubated in the culture media supplemented by resveratrol, as shown in experiment in this thesis using mouse oocytes.

7.2 The impact of peritoneal environment on egg and embryo quality

Ovulated oocytes are required to take a short, but important journey across the peritoneal cavity before being captured by the fimbrial end of a Fallopian tube. During this time, these oocytes are exposed to the peritoneal fluid. It is hypothesised that peritoneal fluid of women with endometriosis may be detrimental to the ovulated oocytes and further impair the oocyte and embryo development. There is some evidence of an increased level of structural defect in meiotic spindle of mature oocytes when incubated in peritoneal fluid of women with endometriosis [174], however this study did not quantify the DNA damage.

Primordial cells in an ovary are in constant contact with peritoneal fluid for many years before being selected to grow and finally ovulate. During this prolonged exposure of the ovary, it is hypothesised that peritoneal fluid can induce DNA damage in oocytes whilst they were still in the ovary.

7.2.1 Objective

To investigate the effect of peritoneal fluid on oocyte/embryo quality.

7.2.2 Method

The method chosen to investigate the impact of the peritoneal environment on egg is guided by answering several different questions. The first question is whether the oocyte-damaging agents in endometriosis restrained to the ovary, the abdominal cavity or is it systemic. For this question, mouse GV oocytes will be used to measure ROS and DNA damage when exposed to FF, PF and blood plasma from the same patient with or without Endometriosis.

The next question is does PF/plasma affect the primordial follicle? To answer this question, follicle cultures isolated from mouse ovaries were exposed to PF/plasma from patients with or without endometriosis and after 2 weeks primordial oocytes were assessed for DNA damage.

Appendix A Abstract: Publication 1

Review

Influence of Endometriosis on Assisted Reproductive Technology Outcomes

A Systematic Review and Meta-analysis

Mukhri Hamdan, MObGyn, Siti Z. Omar, MObGyn, Gerard Dunselman, MD, PhD, and Ying Cheong, MD, MRCOG

OBJECTIVE: To investigate the association of endometriosis on assisted reproductive technology (ART) outcomes and to review if surgical treatment of endometriosis before ART affects the outcomes.

DATA SOURCES: We searched studies published between 1980 and 2014 on endometriosis and ART outcome. We searched MEDLINE, PubMed, ClinicalTrials.gov, and Cochrane databases and performed a manual search.

METHODS OF STUDY SELECTION: A total of 1,346 articles were identified, and 36 studies were eligible to be included for data synthesis. We included published cohort studies and randomized controlled trials.

TABULATION, INTEGRATION, AND RESULTS: Compared with women without endometriosis, women with endometriosis undertaking in vitro fertilization and intracytoplasmic sperm injection have a similar live birth rate per woman (odds ratio [OR] 0.94, 95% confidence interval [CI] 0.84–1.06, 13 studies, 12,682 patients, $I^2=35\%$), a lower clinical pregnancy rate per woman (OR 0.78, 95% CI 0.65–0.94), 24 studies, 20,757 patients, $I^2=66\%$), a lower mean number of oocyte retrieved per cycle (mean difference -1.98 , 95% CI -2.87 to -1.09 , 17 studies, 17,593 cycles, $I^2=97\%$), and a similar miscarriage rate per woman (OR 1.26, 95% CI 0.92–1.70, nine studies, 1,259 patients, $I^2=0\%$). Women with more

severe disease (American Society for Reproductive Medicine III–IV) have a lower live birth rate, clinical pregnancy rate, and mean number of oocytes retrieved when compared with women with no endometriosis.

CONCLUSION: Women with and without endometriosis have comparable ART outcomes in terms of live births, whereas those with severe endometriosis have inferior outcomes. There is insufficient evidence to recommend surgery routinely before undergoing ART.

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Endometriosis is associated with subfertility,^{1–3} and up to 50% of women with endometriosis have difficulty conceiving naturally.⁴ The cause and effect of endometriosis on subfertility remains controversial although it is known that without intervention, women with more severe disease are less likely to conceive.⁴ There are conflicting results regarding the reproductive outcomes associated with subfertile women with varying severity of endometriosis undergoing assisted reproductive technology (ART).^{5–9} Many clinicians will recommend surgical treatment to improve subfertility in women with mild and moderate endometriosis albeit that this recommendation is based on moderate evidence from two randomized controlled trials ($n=382$).¹⁰ One further randomized controlled trial¹¹ not included in the meta-analysis by Duffy et al¹⁰ reported conflicting results.

There is currently no consensus as to whether surgical treatment should be offered to women undergoing ART to improve their reproductive outcome. A meta-analysis¹² performed more than 10 years ago indicated that the pregnancy rate is halved in women with endometriosis undergoing ART treatment. A more recent meta-analysis¹³ reported on the detrimental effect of endometriosis on

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Financial Disclosure

The authors report no conflict of interest.

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Appendix B Abstract: Publication 2

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human
reproduction
update

The impact of endometrioma on IVF/ICSI outcomes: a systematic review and meta-analysis

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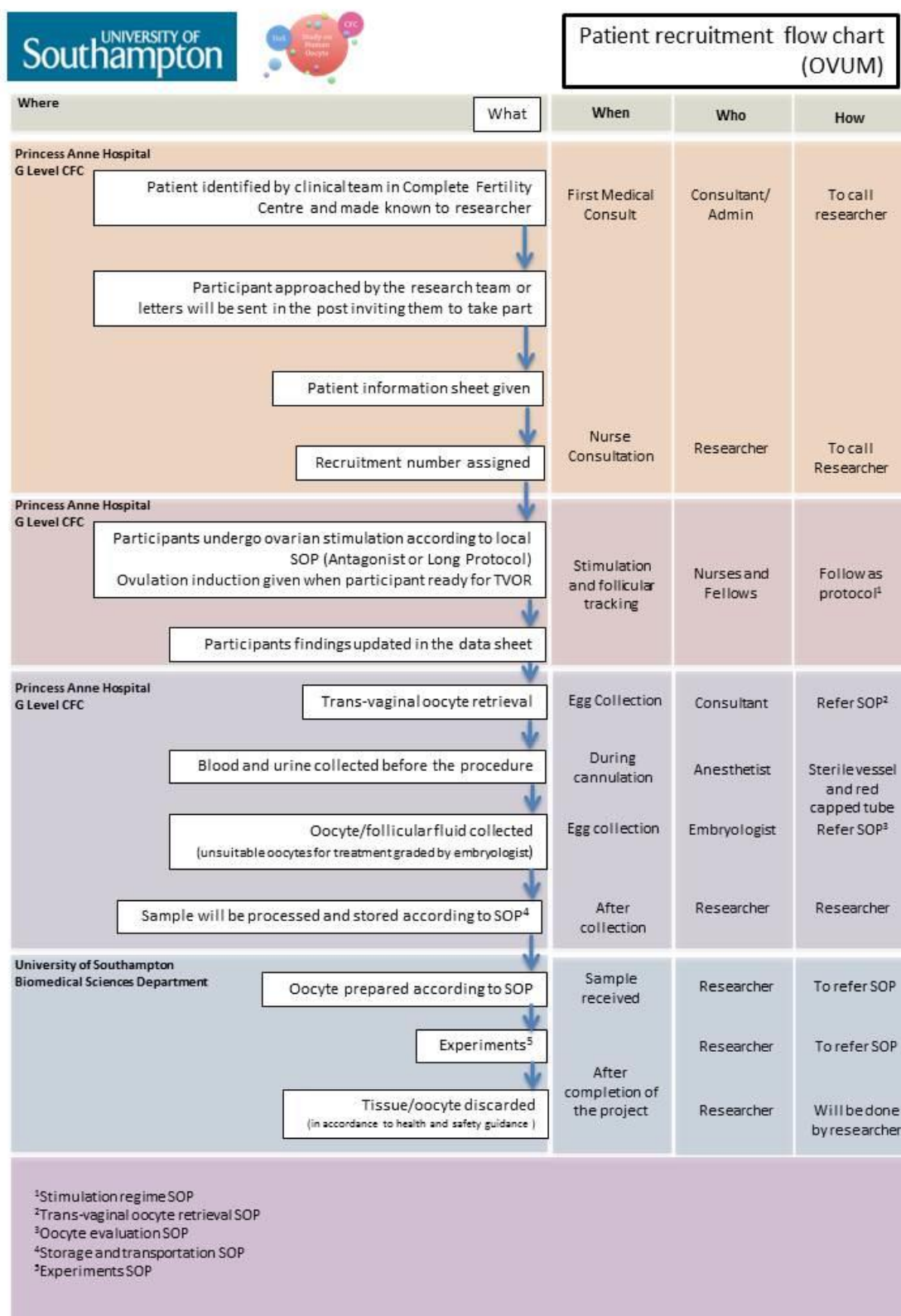
- Introduction
- Methods
 - Criteria for considering studies for this review
 - Search methods for identification of studies
 - Data collection and analysis
 - Data synthesis
- Results
 - Result of search
 - Description of studies and participants
 - Quality assessment of studies
 - Impact of endometrioma without intervention on IVF/ICSI outcomes
 - Impact of surgical intervention of endometrioma on IVF/ICSI outcomes
 - Impact of different surgical techniques to IVF/ICSI outcomes
- Discussion
- Conclusion

BACKGROUND: Endometriosis is a disease known to be detrimental to fertility. Women with endometriosis, and the presence of endometrioma, may require artificial reproductive techniques (ART) to achieve a pregnancy. The specific impact of endometrioma alone and the impact of surgical intervention for endometrioma on the reproductive outcome of women undergoing IVF/ICSI are areas that require further clarification. The objectives of this review were as follows: (i) to determine the impact of endometrioma on IVF/ICSI outcomes, (ii) to determine the impact of surgery for endometrioma on IVF/ICSI outcome and (iii) to determine the effect of different surgical techniques on IVF/ICSI outcomes.

METHODS: We performed a systematic review and meta-analysis examining subfertile women who have endometrioma and are undergoing IVF/ICSI, and who have or have not had any surgical management for endometrioma before IVF/ICSI. The primary outcome was live birth rate (LBR). Our secondary outcomes were clinical pregnancy rate (CPR), mean number of oocyte retrieved (MNOR), miscarriage rate (MR), fertilization rate, implantation rate, antral follicle count (AFC), total stimulating hormone dose, and any rates of adverse effects such as cancellation and associated complications during the IVF/ICSI treatment.

RESULTS: We included 33 studies for the meta-analysis. The majority of the studies were retrospective (30/33), and three were RCTs. Compared with women with no endometrioma undergoing IVF/ICSI, women with endometrioma had a similar LBR (odds ratio [OR] 0.98; 95% CI [0.71, 1.36], 5 studies, 928 women, $I^2 = 0\%$) and a similar CPR (OR 1.17; 95% CI [0.87, 1.58], 5 studies, 928 women, $I^2 = 0\%$), a lower mean

Appendix C Recruitment flowchart



Appendix D Patient Information Sheet

Participant Information Sheet

Project title:

OVUM: Study on human oocyte

We would like to invite you to take part in our research study looking at changes within the DNA that may influence quality and development of oocytes (eggs). We would like you to understand why the research is being done and what it would involve for you. One of our team will go through the information sheet with you and answer any questions you have. We suggest this should take about 5 minutes.

Purpose of the study

This study aims to provide us as clinicians with a better understanding on the factors that influence oocyte quality and development within women who are undergoing IVF treatment. We would like to quantify the incidence of unintended alterations within eggs (scientifically known as DNA damage) in human oocyte and assess the extent of this damage if present. Oocytes will be assessed for changes within the DNA by measuring specific proteins to the DNA, the DNA per se will not be analysed. In other words, this study will not analyse the participants' genetics and is therefore not a genetic study.

Rationale

The research will provide clinicians with information about the severity of changes within the DNA in human eggs, and its ability to repair such changes/damage. By increasing our understanding of the response to DNA damage in oocytes, clinicians and scientists can then start to investigate ways to reduce or prevent these changes occurring in women's eggs.

Background

In female germline (lineage of cells spanning many generations of a female), DNA damage has the potential to induce infertility and even to lead to genetic abnormalities that may be passed on to the resulting embryo. This alteration in the DNA can be caused as a consequence of the metabolic processes continuously active inside a cell, and also by external factors, for example exposure to cigarette smokes. As such cells have evolved mechanisms that detect and then repair this damage to keep the DNA in excellent condition. These mechanisms also act as a 'checkpoint', preventing cells from progressing through the cell cycle in the presence of such damage. As with other cells, oocytes are also susceptible to DNA alteration/damage, and in fact may be more susceptible because of their unique cell division termed meiosis. In the female meiosis involves a prolonged arrest (a resting stage where the

cell essentially goes to sleep) that can last for up to 4 decades. During this time DNA damage can accumulate.

Research questions

To what extent are human oocytes capable of repairing DNA damage as they progress through cell division?

Inclusion criteria

- Only female patients will be included
- Age 18-65 years old

Exclusion criteria

- Menopausal women
- Any active infective disease
- Pregnant women

What does participating in this involve?

You will be asked to sign a consent form. Following this, the study has different parts:

1. You will be asked a few basic questions on your menstrual pattern, quality of life and past medical history
2. You then will continue with your IVF treatment as planned in Complete Fertility Centre in accordance to the unit protocol
3. We will ask you to provide a urine sample before the TVOR procedure (women are routinely asked to empty their bladder before this procedure)
4. Blood will be taken before the procedure during intravenous cannulation (women are normally cannulated during this procedure)
5. Trans-vaginal oocytes retrieval will be done as per unit protocol.
6. Embryologist in charge will grade and decide on the maturity of the oocytes
7. Immature oocyte/s which is/are not suitable for treatment (if present) will be collected and transported to Biomedical Sciences department according to standard operating procedure
8. Samples of blood, urine, and follicular fluid will be then taken to the laboratory and processed by a variety of techniques. They may be frozen and stored for later use
9. Immature oocytes will be let to get mature in vitro and this will be recorded according to the experiment SOP
10. This study will involve the collection of follicular fluid (fluid contained in the follicle surrounding the egg) which normally discarded after the procedure
11. The sample of blood, urine, oocyte and follicular fluid will be analyzed in a laboratory
12. We will perform various tests on these samples, which will include looking at your DNA (the building-blocks) of your gene, which are the way your body is made. We will not be performing genetic tests.

You will not have to make additional visits to the hospital except those needed for your routine care.

Why have I been invited to take part?

You have been invited to take part because you are attending the hospital as part of your routine care and fulfill the criteria for recruitment in to our study.

Will anyone know that I'm taking part?

The data collected will be completely confidential within the hospital. All of the findings will be kept confidential in accordance with standards followed by medical researchers in compliance with national laws. Your sample will be anonymous which means that people looking at the study results will not know that you were involved.

Do I have to take part?

It is your choice whether or not you take part. If you would like to take part you will be asked to sign a consent form. 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 all your identifiable samples, but we would use the data collected up to your withdrawal.

What will happen if I don't want to continue taking part in the study?

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 would use the data collected up to the point of your withdrawal.

What are the benefits to taking part?

We hope that your help will help us to gain better understanding factors that influence eggs quality and development within women who are undergoing IVF treatment. In the long term we hope that this work will contribute to the development of policy and protocol in the clinical setting to minimise the adverse effects of DNA alterations/damage in eggs.

What are the disadvantages to taking part?

The study will not affect the treatment you receive in any way. We may take out 5 minutes of your time for the initial interview and consenting (this can be done while you are waiting to see a consultant or a nurse). Otherwise your plan of IVF treatment not affected at all.

Will any genetic test be done?

We are not looking at specific genetic disorders or diseases. Oocytes will be assessed for DNA alteration/damage by measuring recruitment of specific proteins to the DNA, the DNA per se will not be analysed.

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 and doctors. The results may be presented at academic meetings and a summary and any information used in analyzing the results would not be linked to you in any way. If you would like to be informed as to the outcomes of the study, please provide the researcher with your contact address, and relevant information may be sent to you on completion of the study.

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 listened and dealt with. Contact information is available from departmental staff in the first instance. If you have concerns about the care provided to you or the Trust services, the Patient Support Service (PSS). PSS may be contacted by telephone 023 8079 8498, email PSS@uhs.nhs.uk. If taking part in this research project harms you, there are no special compensation arrangements. If you

are harmed due to someone's negligence, then you may have grounds for a legal action but you may have to pay for it.

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

At the end of the study your sample will be destroyed

Who is organizing the funding the research?

It is organized and funded jointly by University of Southampton and Ministry of Higher Education Malaysia.

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 favorable opinion by South Central – Southampton ethics committee.

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

Main investigator:

Dr Y Cheong, Gynecology consultant and senior lecturer, University of Southampton

E-mail: y.cheong@soton.ac.uk

Telephone: 02380 796033

Co-Investigator:

Dr Mukhri Hamdan, Clinical Research Fellow, University of Southampton

E-mail: mh1v12@soton.ac.uk

Telephone: 02380 796044

Thank you for reading this

Appendix E Consent

INFORMED CONSENT FORM

Title of study: **OVUM: Study on human oocyte**

Name of Principal Investigator: Dr Y.Cheong

Centre/Site number: Princess Anne Hospital

Study number:

REC approval number:

EudraCT number:

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.

PART A: Consent for the current study

(Samples to be destroyed on study completion)

PLEASE INITIAL THE BOXES IF YOU AGREE WITH EACH SECTION:

1. I have read the information sheet version 1.0 dated 07/10/2013 for the above study and have been given a copy to keep. 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 give a sample of (blood / urine/ follicular fluid/ oocyte/s that is/are found not suitable to be used for further treatment) for research in this study. I understand how the sample will be collected, that giving a sample for this research is voluntary and that I am free to withdraw my approval for use of the sample at any time.

--	--

4. 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.

--	--

5. I agree to participate in this study

--	--

Name of Participant

Date

Signature

Name of researcher taking consent:

Date

Signature

Appendix F Patient proforma

OVUM : Patient Proforma		
Recruitment details:		
Recruitment Number		
Date		
Time		
TVOR performed by		
Embryologist		
Samples collected:	Number of samples	Remarks
Oocyte	<input type="checkbox"/> No <input type="checkbox"/> Yes _____	<input type="checkbox"/> Immature <input type="checkbox"/> Mature <input type="checkbox"/> Unfertilized <input type="checkbox"/> Others
Follicular fluid	<input type="checkbox"/> No <input type="checkbox"/> Yes _____	<input type="checkbox"/> Bloody <input type="checkbox"/> Clear <input type="checkbox"/> Pink
Serum	<input type="checkbox"/> No <input type="checkbox"/> Yes _____	
Urine	<input type="checkbox"/> No <input type="checkbox"/> Yes _____	
Others	<input type="checkbox"/> No <input type="checkbox"/> Yes _____	
Demographic Details:		
Age (years)		
Ethnicity		
Height (cm)		
Weight (kg)		
BMI		
Indication for IVF/ICSI:		
<input type="checkbox"/> Endometriosis	D1.1 Symptoms: <input type="checkbox"/> Asymptomatic <input type="checkbox"/> Dysmenorrhea <input type="checkbox"/> Dyspareunia	
	D1.2 Stage of disease (ASRM): <input type="checkbox"/> I <input type="checkbox"/> II <input type="checkbox"/> III <input type="checkbox"/> IV	
	D1.3 Endometrioma: <input type="checkbox"/> Yes <input type="checkbox"/> No	
	D1.4 DIE: <input type="checkbox"/> Yes <input type="checkbox"/> No	
	D1.5 Surgical treatment: <input type="checkbox"/> Yes <input type="checkbox"/> No	
	D1.6 Date of operation:	
	D1.7 What did the operation show:	
<input type="checkbox"/> PCOS	<input type="checkbox"/> Hyperandrogenism <input type="checkbox"/> PCO morphology <input type="checkbox"/> Oligomenorrhea	
<input type="checkbox"/> Male Factor	<input type="checkbox"/> Oligospermia <input type="checkbox"/> Azoospermia <input type="checkbox"/> Asthenospermia <input type="checkbox"/> Teratospermia	
<input type="checkbox"/> Tubal Disease	<input type="checkbox"/> Bilateral salpingectomy <input type="checkbox"/> Blocked tubes <input type="checkbox"/> Hydrosalpinx <input type="checkbox"/> Others	

<input type="checkbox"/> Unexplained			
<input type="checkbox"/> Age/Poor ovarian reserve			
<input type="checkbox"/> Ovulatory disorder	<input type="checkbox"/> Hypogonadotropic Hypogonadism <input type="checkbox"/> Turners Syndrome <input type="checkbox"/> Others		
<input type="checkbox"/> Others (Please specify)			
Current gynecological history:			
Normal length of menstrual cycle:			
Menarche			
Bleed days/Total cycle length			
Menstrual disorder	E4.1 <input type="checkbox"/> Dysmenorrhea		
	E4.2 <input type="checkbox"/> Menorrhagia		
	E4.3 <input type="checkbox"/> Inter-menstrual bleeding		
	E4.4 <input type="checkbox"/> Others _____		
LMP date			
Day of cycle on day sample taken (Days since LMP)			
Past Gynecology/Obstetrics history			
Previous contraceptive use: Type, Duration, When discontinued			
Pregnancies G=Total number of pregnancies P =number of deliveries after 24weeks			
Number of live births Details:			
Previous fertility treatment	Ovulation induction	IUI	IVF/ICSI
Duration of subfertility	Months		
History of S.T.I. (please describe if yes)			
Medical/Surgical information			
Medical Conditions (Including psychiatric conditions)			
Regular Medication: (Include dosage and what it is for)			
Previous surgery	<input type="checkbox"/> No <input type="checkbox"/> Yes _____ _____		
Does the patient smoke?	Yes (ciggs/day)	No	Ex-Smoker

Does patient drink alcohol?	Yes (units/week)			No	
IVF/ICSI treatment regime	<input type="checkbox"/> IVF			<input type="checkbox"/> ICSI	
Attempt number	1	2	3	4	>5
Stimulation Regime	Long	Short	Ant	Natural	Others
AFC at baseline					
Stimulation drug (Type)(Dose)(Total dose)					
Suppression drug (Type)(Dose)(Total dose)					
Endometrial Thickness before trigger	mm				
Ovulation Trigger	HCG		GnRH		
Number of Follicles before trigger	<12mm		>12mm	>17mm	
Remarks on TVOR	Easy		Bloody		Complicated
Remarks on Embryo Transfer	Easy		Bloody		Complicated
Embryology Record					
Number of oocytes retrieved					
Number of immature oocytes					
Grade of oocyte/Best Grade					
Fertilization Rate					
Transferred (Number of embryo/Day)					
Frozen (Number of embryo/Day)					
Number of discarded oocytes					
Semen Source	<input type="checkbox"/> Partner			<input type="checkbox"/> Donor	
Investigations Results					
LH				Date:	
FSH				Date:	
D21 Se Progesterone				Date:	
AMH				Date:	
Chlamydia				Date:	
Ultrasound				Date:	
Seminal Analysis				Date:	
Histology result				Date:	
IVF/ICSI Outcome					
Se HCG level	m/iu			Not pregnant	
Outcome	L2.1 <input type="checkbox"/> Biochemical:				
	L2.2 <input type="checkbox"/> Miscarriage:				
	L2.3 <input type="checkbox"/> Live birth:				
Number of pregnancy	Singleton		Twin		Triplet
Others					

Appendix G Ovarian stimulation protocol

(Adopted from Complete Fertility Centre SOP)

The standard stimulation regime will be an antagonist regime; the decision to use a regime other than the standard antagonist regime should be clearly documented. This is normally the decision made at the clinical team meeting in CFC.

Cycle management - Antagonist

Day 1

Patients contact the unit on the first day of their period. They will commence their baseline scans on day 3 of cycles. Prior to that, all documentation and investigations should be in place. This should have been checked by the nursing team in accordance to the 'to start treatment schedule'. The final 'go ahead' to start should be at the daily MDT.

Day 2

Patients attend the clinic on day 2/3 of the cycle.

If the patient is oligo-/amenorrhoeic a withdrawal bleed may be induced with a progestogen (e.g. medroxyprogesterone acetate 10mg b.d for 5-7 days) after first confirming that a pregnancy test is negative.

The woman has a scan to exclude any problems (e.g. cysts, fibroids, polyps), which may affect the cycle.

The baseline scan should record the following:

An assessment of the number of antral follicles on each ovary (AFC = total of both entered into IDEAS)

- Ovarian morphology
- Uterine morphology and endometrial thickness
- Any other abnormality should be noted and e-photo retained if appropriate

All results are discussed at the **daily MDT meeting** and patients may proceed with treatment if:

- No baseline cyst > 15mm (PCOS morphology should be noted, if present)
- Thin endometrium (<6mm)
- Uterine shape normal, no evidence of fibroids or endometrial polyps

If these conditions are not satisfied, treatment should be deferred, discussed at the MDT or an appointment made for medical review. The nurses should check that the patients have their drugs. If partner's sperm is to be used, the partner is reminded that he will have to attend the clinic on the day of egg collection to produce a semen sample. If a surgical sperm recovery is required the partner should be reminded of the arrangements and timing of the procedure.

If donor sperm is to be used, the embryologists should check if straws are available in clinic or require ordering. If for any reason the cycle is cancelled the embryologists must be informed.

Starting stimulation - Day 1 (Day 3 of cycle)

If the baseline scan is normal on day 2 of their cycle and no other outstanding issues during MDT, the patient will begin gonadotropin stimulation the following day (day 3) in accordance to the following regime.

Exceptions

Severe endometriosis – Consultant discretion

Patients for GA – may need programming with long protocol

Cycle Two (ALL PATIENTS)

Repeat antagonist unless:

- In-coordinate follicle cohort in cycle one (consider long protocol and/or change to Urinary FSH)
- Unexpectedly poor embryos (consider long protocol and/or change to urinary FSH)
- Previous unexpected response
- Hypogonadotrophic Hypogonadism - require a preparation containing LH (e.g. Menopur), starting on 150 IU or as determined by previous response

FSH stimulation

FSH will be administered subcutaneously (SC) with the FSH Pen in the abdominal wall. Injections are usually administered in the morning.

Administration of GnRH antagonist - Day 5 of Stimulation (Day 7 of cycle).

The patient does not need to visit the unit on this day. GnRH antagonist (Cetrotide) is given as a daily s/c injection from pre-filled syringes in the **morning**.

Monitoring days:

An ultrasound scan is performed to monitor response to the drugs. The following parameters are noted on scan: number of follicles on each ovary; mean diameter of follicles (mm); endometrial thickness and quality. It should be checked that there has been no problem with the injection procedure. The results of the scan can be discussed at the MDT meeting. The patient should be phoned after 14.00 with her results and be advised of any changes to her regime and told when to attend again.

Further visits:

The patient then attends the clinic as required to further monitor her progress and to decide on the day to proceed with hCG administration and arrange the time of the egg collection. Usually this will be on alternate days, but may be daily, depending on response.

Criteria for changing dose:

The dose of FSH may be reduced by 12.5iu onwards daily any time from day 5 of stimulation if the patient is at risk of over responding, but the dose should not be reduced until there are follicles larger than 14mm.

The dose should not usually be increased.

Appendix H Ovulation trigger

(Adopted from Complete Fertility Centre SOP)

- 1) Criteria for administration of HCG
 - a) If the following minimum criteria are met then hCG may be given to induce ovulation. (6500 IU s/c – abdominal wall – Ovitrelle).
 - i) ≥ 3 follicles of at least 17 mm in diameter;
 - ii) Endometrial thickness is at least 7 mm;
- 2) If these criteria are met but hCG administration would result in a pick up on Sunday, administration can be delayed by one day.
- 3) Moreover, consideration to one or two days longer stimulation should be given if there are a number of medium sized follicles (14-16mm) to allow these to reach a mature size.
- 4) HCG should be given at night and timed to occur 36 hours before the planned egg collection procedure. 40 hours is normal for release of eggs and 36 hrs allows plenty of time before this occurs.
- 5) If more than one patient is scheduled for collection the hCG should be timed at intervals depending upon the workload in the centre and with discussion with the embryologist present at the meeting. ICSI cases are usually scheduled first.
- 6) NB: In women at increased risk of OHSS who are being permitted to proceed to oocyte pick-up, either a reduced dose of 5000IU hCG or GnRH agonist (triptorelin 0.2mg or Buserelin 0.5mg) should be administered to trigger final oocyte maturation and a 'freeze all' policy may have to be adopted (see OHSS protocol Doc 625).
- 7) For Luteal support, please see Luteal support SOP. PLEASE note that patients with GnRH agonist long protocol CANNOT be triggered by GnRH agonist.








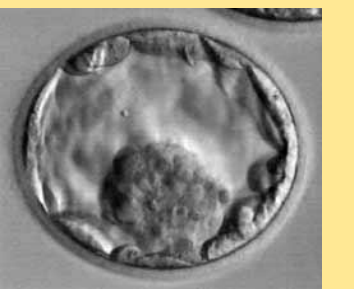

Appendix I Oocyte retrieval

(Adopted from Complete Fertility Centre SOP)

1. Insert the IV cannula in recovery room
2. The patient should empty her bladder just prior to the procedure.
3. Prepare the equipment trolley
4. Show the woman to the theatre and introduce her to all members of the team in theatre. The partner may be present to provide emotional support. If she has brought a music CD or tape this should be played.
5. The identity of the patient and her partner should be checked as per the embryology witnessing protocol
6. Check that the patient understands the procedure and explain what will be done. She should lie flat on the examination couch.
7. The necessary monitoring equipment should be connected. This comprises of oxygen saturation monitor, blood pressure cuff
8. A consultant anaesthetist will administer sedation. It will be titrated to each patient's requirements. It will consist of an infusion of Propofol, and boluses of midazolam and fentanyl.
9. Place the patient's legs in lithotomy position, using the stirrups and drape with sterile towels.
10. The external genitalia should be gently swabbed with warmed Hartmann's and cotton wool balls. A sterile towel is placed under the patient's buttocks.
11. The Cusco speculum is warmed with sterile warmed Hartmann's to provide lubrication. It is gently inserted into the vagina and the blades opened to visualise the cervix.
12. The vagina is gently cleansed with cotton wool balls on a sponge holder soaked in sterile Hartmann's. Excess fluid may be removed with a dry swab.
13. (If general anesthesia is used the vagina may be cleaned simply by inserting the soaked cotton wool balls on a sponge holder and omitting the speculum).
14. Remove the speculum.
15. Prepare the trans-vaginal probe and fit the biopsy guide according to the following procedure:
16. Place a small amount of sterile Hartmann's or sterile gel in the probe cover
17. Cover probe with the cover
18. Clip the plastic biopsy guide onto probe
19. Introduce needle through the guide and check that it runs freely
20. Turn on the scan machine and use the programme showing the needle track.
21. Advance the probe to the vaginal fornix.
22. Line up the ovary on the screen. Ensure the ovary is easily accessible and that no other structures will be penetrated between the vagina and ovary. Gently advance the needle through the vaginal wall and into the ovary. Puncture the first follicle and aspirate the follicular fluid using the foot pedal of the pump. Gently move the needle around to ensure complete drainage of the follicle. The pump pressure should be set to 125mmHg.
23. The collection test tube should be passed to the embryologist by the nurse for checking and a new tube fitted.

24. If an egg is not obtained in the first aspirate consider flushing the follicle with flushing medium through the second channel. This process may be repeated until the egg obtained. However, if repeated flushing is required the eggs are usually of poor quality and it may be better to move to a new follicle.
25. If an egg is obtained, the needle should be gently advanced into the next follicle and the process repeated. The embryologist must be informed when a new follicle is entered.
26. The approach should be planned systematically so that the next follicle can be entered with minimal sideways movement of the needle. This minimises both trauma to the ovary and discomfort. It is also important to ensure that the tip of the needle is visible at all times and that it follows the needle track on screen. In this way all follicles can be drained systematically and the needle withdrawn from the ovary
27. The second ovary can then be dealt with in exactly the same way. The embryologist should be informed when the ovary changes
28. At the end of the procedure the pelvis should be scanned to ensure no evidence of significant bleeding into the follicles or pelvis. The probe may then be gently withdrawn.
29. The Cusco's speculum should then be re-inserted to visualise the vaginal vault and ensure no significant bleeding from the needle punctures. Most bleeding will stop following the application of direct pressure. Occasionally, a small Vicryl suture (2-0) will be required to stop any bleeding. Suture material and sterile instruments (needle holders, forceps and scissors) should be kept in theatre in case required.
30. The woman's legs should be removed from the stirrups and placed flat on the bed. Her blood pressure and pulse should be checked and recorded. When it is established that her condition is satisfactory she should be moved on a trolley to the recovery room.
31. All used equipment should be cleaned and disposed of. **Sharps must be disposed of in the appropriate container.** The condoms should be removed from the probe and everything discarded including the plastic needle guide. The probe and clip-on guide should be thoroughly cleaned with alcohol / disinfectant before the next patient. Any spillage of body fluids must be dealt with according to Trust procedure and the Aspiration Room tidied before the next patient is brought in.
32. The procedure should be documented on the *TVOR Procedure form* (TVOR Procedure). The following should be recorded:
 - Type of anesthesia and drugs administered
 - Number of follicles entered and number of eggs obtained on each side
 - Any adverse events with sedation (i.e. low SaO₂, administration of O₂ etc)
 - An assessment of pain and discomfort
 - Post operative observations
 - The degree of difficulty or any problems encountered
33. If the patient gives a history of pelvic infection or an endometrioma is drained during the TVOR, the patient should receive antibiotic prophylaxis (e.g Augmentin 1.2 gm IV during procedure and 375 mg t.d.s p.o. for 3 days afterwards).
34. The operator should check the labeling on the culture dish as per witnessing protocol

Appendix J Embryo scoring

		
Early blastocyst	Early blastocyst	1AB
Cavity very small = Can't yet grade Inner cell mass = Can't yet grade Trophectoderm = Can't yet grade	Cavity very small = Can't yet grade Inner cell mass = Can't yet grade Trophectoderm = Can't yet grade	Cavity < 1/2 embryo's volume = 1 Inner cell mass = A Trophectoderm = B
		
2BB	2BC	3BB
Cavity > 1/2 embryo's volume = 2 Inner cell mass = B Trophectoderm = B	Cavity > 1/2 embryo's volume = 2 Inner cell mass = B Trophectoderm = C	Cavity completely fills embryo = 3 Inner cell mass = B Trophectoderm = B
		
4AA	4AB	4BB
Well expanded = 4 Inner cell mass = A Trophectoderm = A	Well expanded = 4 Inner cell mass = A Trophectoderm = B	Well expanded = 4 Inner cell mass = B Trophectoderm = B

Appendix K Solutions

GENERAL SOLUTIONS

25x PVP solution

2.5g of PVP dissolved in 10ml ddH₂O

PBS (10x)

NaCl	80g
KCL	2g
Na ₂ HPO ₄ ·7H ₂ O	26.8g
KH ₂ PO ₄	2.4g

pH adjusted to 7.4 with 5M HCl and then made up to 1L with ddH₂O

PBS

10x PBS	5ml
ddH ₂ O	45ml

Filter sterilized and stored at 4°C.

PBS-PVP (PBS, 1% PVP)

10x PBS	600μl
25x PVP	240μl
ddH ₂ O	5.16ml

FIXATION SOLUTIONS

3x PHEM Buffer

Pipes	2.72g
Hepes	0.975g
EGTA	0.57g
MgSO ₄ ·7H ₂ O	0.304g

Dissolved in ddH₂O and adjusted to pH 7.4 with KOH.

Solution was then made up to 50ml with ddH₂O and filtered before storage at 4°C. Final (1x) concentrations: Pipes 60mM, Hepes 35mM, EGTA 25mM, MgSO₄·7H₂O 4mM.

PHEM-PVP (PHEM Buffer, 1% PVP)

3x PHEM buffer	2ml
25x PVP	240μl
ddH ₂ O	3.76ml

Fixing Solution (PHEM buffer, 2% Formaldehyde, 0.5% Triton X-100)

3x PHEM buffer	5ml
ddH ₂ O	8ml
Paraformaldehyde prills	0.3g

Paraformaldehyde prills were dissolved in PHEM buffer to give a 2% solution by heating and stirring and addition of 5M NaOH. This preparation was made in a fume cupboard and then cooled to 4°C. 0.5% volume (75µl) Triton X-100 was added and allowed to dissolve, the solution was then made up to 15ml with ddH₂O, filtered and stored at 4°C.

MEM Solutions

MEM was prepared by dissolving the content of the sachet (stored at 4°C) in 900ml ddH₂O. 2.2g of NaHCO₃ was added and the pH adjusted to 7.4 before making up to 1L with ddH₂O. The Media was stored at 4°C until preparation for use. Before use the media was supplemented with 1/200 volumes of PenStrep solution, and 20% Fetal Calf Serum before being filter sterilized.

IMMUNOFLUORESCENCE SOLUTIONS

Washing Solution (PBS, 1% BSA, 0.1% Tween20)

BSA	100mg
10xPBS	100µl
10% Tween20	100µl
ddH ₂ O	800µl

Blocking Solution (PBS, 7% Goat Serum, 1%BSA, 0.1% Tween20)

Washing Solution	2.79ml
100% goat serum	210µl

Appendix L Image J Macro

```
1  subtitle = split(getInfo("image.subtitle"), "-");
2  imagename = split(subtitle[1], "");

3  rename("temp");
4  run("Split Channels");
5  selectWindow("C3-temp");
6  close();
7  selectWindow("C1-temp");
8  run("Gaussian Blur...", "sigma=2 stack");
9  setOption("BlackBackground", false);
10 run("Make Binary", "method=Default background=Default thresholded
    remaining");
11 setThreshold(1, 255);
12 run("Convert to Mask", "method=MaxEntropy background=Default
    black");
13 rename("mask");
14 selectWindow("C2-temp");
15 rename("gh2ax");
16 imageCalculator("AND create stack", "mask", "gh2ax");
17 selectWindow("Result of mask");
18 run("Z Project...", "projection=[Sum Slices]");
19 run("Measure");
20 close();
21 selectWindow("Result of mask");
22 close();
23 selectWindow("mask");
24 run("Z Project...", "projection=[Sum Slices]");
25 run("Measure");
26 close();
27 selectWindow("mask");
28 close();
29 selectWindow("gh2ax");
30 run("Duplicate...", "title=gh2ax-1 duplicate range=1-19");
31 run("Gaussian Blur...", "sigma=2 stack");
32 run("Make Binary", "method=Percentile background=Default
    thresholded remaining");
33 run("Convert to Mask", "method=MaxEntropy background=Default
    black");
34 imageCalculator("AND create stack", "gh2ax-1", "gh2ax");
35 run("Z Project...", "projection=[Sum Slices]");
36 run("Measure");
37 close();
38 selectWindow("Result of gh2ax-1");
39 close();
40 selectWindow("gh2ax-1");
```

```

41  run("Z Project...", "projection=[Sum Slices]");
42  run("Measure");
43  close();
44  selectWindow("gh2ax-1");
45  close();
46  selectWindow("gh2ax");
47  close();
48  n=nResults;
49  A = getResult("Mean",n-4);
50  B = getResult("Mean",n-3);
51  C = getResult("Mean",n-2);
52  D = getResult("Mean",n-1);

53  Ratio = (A/B)/((C-A)/(D-B))
54  print(imagenam[0] + "\t\t" + Ratio)

```

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