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MOLECULAR CHARACTERISATION AND PROPHYLACTIC TREATMENT STRATEGIES AMONG WOMEN WITH BENIGN GYNECOLOGICAL DISORDERS

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Cover page: Illustration of human uterus indicating the gynaecological disorders addressed in this thesis. Picture was coloured by Dhriti.V and Drish.V, 3.5 years old twins.

Molecular characterization and prophylactic treatment strategies among women with benign gynaecological disorders

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“The art of clinical diagnosis lies in the ability to ask the right questions.” -Harriet B. Braiker

Dedicated to wonderful ladies in my life

ABSTRACT

Background: Female reproduction is controlled by hormones in a timed and well-coordinated manner by the hypothalamus- pituitary - ovarian axis. In response to the ovarian hormones such as estrogen, and progesterone, endometrium, the inner lining of the uterus, undergoes repeated cycles of tissue growth, differentiation, tissue shedding and remodelling. However, dysregulation of those hormones, may generally cause uncontrolled cell proliferation, alteration of its invasive, migratory or angiogenic characteristics, leading to the displacement of endometrial tissues either outside of uterine cavity, for instance on ovary forming ovarian endometriosis; or inwardly into the myometrium causing adenomyosis. Several epidemiological studies as well as histological evidences have shown an increased risk of developing ovarian cancer among women with endometriosis known as endometriosis associated ovarian cancers (EAOC). Similarly, the risk of ovarian cancer is increased among carriers of *BRCA1* or *BRCA2* germline mutations due to error-prone DNA repair mechanisms.

Aim: We aimed to investigate on early molecular alterations associated with cancer development among women with ovarian endometriosis. In addition, treatment or preventive strategies for reducing disease symptoms were explored among women with ovarian endometriosis, *BRCA1* or *BRCA2* mutations and adenomyosis, respectively.

Methods and Results: *Study I,* we explored if there was a molecular link between endometriosis and ovarian cancer development, by analysing multipotent stem/stromal cells and tissues of endometrium and endometrioma among women with ovarian endometriosis. We investigated for intra-patient heterogeneity within stem- and cancer- cell- pathways using targeted PCR array as well as validated for their tumour initiating characteristics. We observed that a subgroup of women with endometriosis (4/30 endometrioma) exhibited dysregulation in estrogen receptor expression, upregulation of molecules related to epithelial-mesenchymal transition pathway such as *KIT, HIF2a* and *E-cadherin* as well as downregulation of tumour suppressor genes *PTEN* and *ARID1A*, thus supporting a link between above molecular changes and potential risk of EAOC.

Study II, we investigated the molecular regulation of Syndecan-1 (SDC-1) and -4 (SDC-4) upon induced activation of TGF-β signalling in another cohort of ovarian endometrioma, to understand their interactions in the pathophysiology of endometriosis and potential EAOCs. Similar to Study I, we also identified molecular heterogeneity with aberrant activation of TGF-β signalling as well as confirmed their anomalous behaviour using 3D spheroid and invasion assays *in vitro*. Interestingly, the above invasive phenotype could be altered by transient gene

knockdown of either SDC-1 or SDC-4 during active TGF-β signalling. Moreover, we showed that the presence of high levels of TGF-β ligands control endometriotic cell proliferation and reduce its 3D-spheroid invasive potential *in vitro*. Thus, inhibition of SDCs among subjects with aberrant TGF-β signalling could be suggested as a potential treatment strategy to reduce inherent risk towards EAOC.

In *Study III*, we examined the molecular action of selective progesterone receptor modulator, mifepristone among women with *BRCA1* or *BRCA2* mutations. Through *in vitro* studies, we confirmed the anti-proliferative action of mifepristone with inherent levels of progesterone among above cohort of *BRCA1* or *BRCA2* women, thus providing as an alternative preventive approach to avoid/delay the use of salpingo-oophorectomy in reducing ovarian cancer risk.

In *Study IV*, we demonstrated the mechanism of action for bromocriptine in the first known human clinical trial for the management of adenomyosis. Bromocriptine provided a prolactin mediated potent growth inhibition, reduced heavy menstrual bleeding as well as exhibited reversal of fibrosis, thus could be further explored for reversing the pathogenesis of adenomyosis.

Conclusion: To summarize, this thesis has demonstrated important molecular links underlying endometriosis and risk of EAOC. Also, this work has shown the possibilities for early detection and potential treatment strategies to reduce disease symptoms and/or inherent cancer risk.

LIST OF SCIENTIFIC PAPERS

I. **Aberrant expression of genes associated with stemness and cancer in endometria and endometrioma in a subset of women with endometriosis**
Ponandai-Srinivasan S, Andersson KA, Nister M, Saare M, Hassan HA, Varghese SJ, Peters M, Salumets A, Gemzell-Danielsson K* and Lalitkumar PGL*
Human Reproduction, Vol.33, No.10 pp1924-1938, 2018

II. **Syndecan inhibition reverses the pre-malignant phenotype of endometrioma with molecular heterogeneity through TGF-β signalling**
Ponandai-Srinivasan S, Saare M, Bogavarappu NR, Ehrström S, Garcìa-Uribe PA, Rettkowski J, Iyengar A, Salumets A, Götte M, Lalitkumar PGL and Gemzell-Danielsson K.
Manuscript

III. **Mifepristone mediates anti-proliferative effect on ovarian mesenchymal stem/stromal cells from female BRCA1−/2− carriers.**
Ponandai-Srinivasan S, Lalitkumar PGL, Garcia L, Varghese SJ, Carlson JW, Gemzell-Danielsson K*, Floter Radestad A*.
Acta Obstet Gynecol Scand. 2019 Feb;98(2):250-261.

IV. **Bromocriptine induces endometrial growth inhibition in women with Adenomyosis.**
Andersson J*, *Ponandai-Srinivasan S*, Pavone D, Lalitkumar PGL, Bogavarappu NR, Gemzell-Danielsson.
Manuscript

* Equal contribution

CONTENTS

LIST OF ABBREVIATIONS

3D	Three dimensional
aHR	Adjusted hazard ratio
ASRM	American society for reproductive medicine
BRCA1 or BRCA2	Breast cancer gene- 1 or 2
CHC	Combined hormonal contraceptives
CI	Confidence Interval
CSC	Cancer stem like cells
DNA	Deoxy ribo-nucleic acid
DE	Differentially expressed
E	Estrogens
E2	Estradiol
E2 cells	ER+ first-generation progenitors
E2-P cells	ER+ and PR+ second generation progenitors
EAOC	Endometriosis associated ovarian cancer
ECM	extracellular matrix
EMT	Epithelial mesenchymal transition
Endo	Endometrioma from women with endometriosis
EndoSC	Stem/stromal cells from endometrioma or endometriotic cyst
ER	Estrogen Receptor
ER-α	Estrogen receptor alpha
ER-β	Estrogen receptor beta
G2/M	cell cycle Gap2/Mitosis phase
H-En	Endometrium from healthy volunteers
HC	Hydrocortisone
HLA	Human Leukocyte Antigen
HMB	Heavy menstrual bleeding

HR	Hazard ratio
IUS	Intra uterine system
JZ	Junctional Zone
LH	Luteinising hormone
LNG	Levenogesterol
LS	Laparoscopic surgery
MIFE	Mifepristone
ml	millilitre
MRI	Magnetic resonance imaging
ng	nanogram
OR	Odds Ratio
P	Progesterone
P cells	PR+ third generation progenitors
P-En	Endometrium from women with endometriosis
P-EnSC	Stem/stromal cells from endometrium of endometriosis
P/LP	Pathogenic/less pathogenic variants
PBLAC	pictorial blood loss assessment chart
PCA	Principle component analysis
PCR	Polymerase chain reaction
PID	Pelvic inflammatory disease
Poly-A	Poly-adenylated sequence
PR	Progesterone Receptor
PRL	Prolactin
PRL-R	Prolactin receptor
rhTGF-β	Recombinant Transforming growth factor beta
RNA	Ribonucleic acid
RR	Relative risk
RRSO	Risk reducing salpingo-oophorectomy

SC	Stem cells or progenitors
SC^-	Negatively sorted cells for makers CD90, CD73, CD105
SC^+	Positively sorted stem/stromal cells for CD90, CD73, CD105
SDC-4	Syndecan-4
SDC1	Syndecan-1
SIR	Standardized incidence ratio
SMART-seq2	RNA sequencing protocol designed for single cell sequencing
SP	Side populations
SPRM	Selective progesterone receptor modulator
TGF-β	Transforming growth factor beta
TIAR	Tissue injury and repair mechanism
TVUS	Transvaginal ultrasound
μM	micro-molar

1 INTRODUCTION

1.1 THE ENDOMETRIUM AND THE UTERINE CYCLE

The inner lining of the uterus, known as the endometrium, has drawn increasing research attention in recent years due to its distinct role in women's health and disease. It provides a cycle-dependent environment for the implantation of the blastocyst into the uterus to support foetal development (Guyton and Hall 2006). In women of reproductive age, the endometrium undergoes more than 400 cycles of well-coordinated events involving growth and differentiation as well as subsequent tissue shedding and remodelling, as depicted in Fig. 1. Histologically, the endometrium comprises two distinct layers: the upper functionalis layer and the lower basalis layer. The functionalis layer comprises the luminal and glandular epithelium, surrounded by loose stroma. It regenerates to a thickness of about 4–10 mm at the follicular (or proliferative) phase due to a rise in estrogen (E) levels. The basalis layer is richly supplied with spiral arteries that have tubular glands and dense stroma (Guyton and Hall 2006). It has been reported that the amazing regenerating potential of the endometrium is due to the existence of active stem cells within the basalis layer, which repopulate the denuded functionalis layer during every menstrual cycle (Gargett and Masuda 2010; Schwab *et al.* 2005).

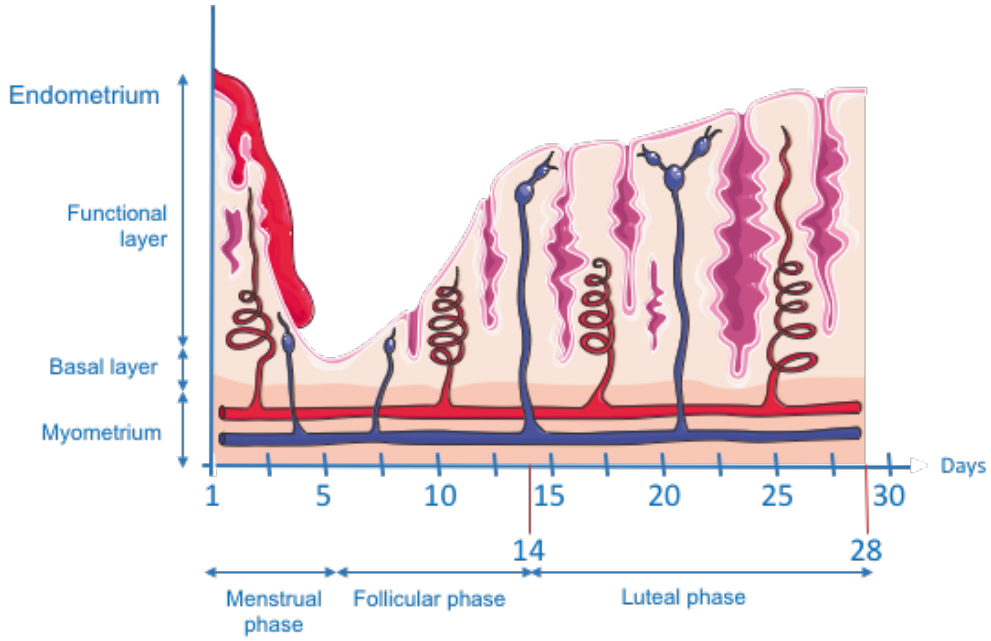

Fig. 1 Schematic diagram on different phases in uterine cycle
Endometrium undergoes a repetitive uterine cycles of tissue regeneration, differentiation, shedding and remodelling. E promote endometrial regeneration during follicular phase while P cause differentiation and prepares endometrium for implantation during luteal phase. In absence of successful implantation, menstrual phase occurs resulting in tissue shedding and remodelling. This image is reused with permission from Servier medical art, licenced by Creative Commons Licence 4.0.

In response to the surge in luteinising hormone (LH) from the pituitary gland, there is a shift from the follicular to the luteal (secretory) phase. The LH surge triggers ovulation from the

dominant ovarian follicle, which releases an oocyte. Consequently, the empty ovarian follicle develops into a hormone-secreting body called the corpus luteum, the role of which is to secrete and release progesterone (P). The rise in P levels alternates with a fall in E levels in the endometrial functionalis layer, priming it to a secretory decidualised phenotype. The endometrium becomes receptive during the mid-luteal phase (6-8 days after ovulation) known as the 'window of implantation', wherein it allows an embryo to implant. The implantation of a human embryo involves sequential events such as apposition, attachment and invasion of embryonic trophoblast cells through the uterine luminal epithelial cells. This process involves an exchange between the endometrium and the embryo of many molecules belonging to the family of cytokines, growth factors and hormones. Glands and blood vessels further grow in size; vascular spaces between them become interconnected to form the placenta, which supplies oxygen and nutrition to the developing foetus. If there is no fertilised oocyte or blastocyst implantation is unsuccessful, there is a rapid fall in P levels. This triggers a cascade of events involving constriction of blood vessels, necrosis and desquamation of the endometrial functionalis layer, leading to the onset of menstruation. The functionalis layer is shed from the uterine cavity during menstruation, leaving the basalis layer intact. (Guyton and Hall 2006; Padykula *et al.* 1984; Jabbour *et al.* 2006).

1.2 BENIGN GYNECOLOGICAL DISORDERS

Women's reproductive health may be hampered by several gynaecological conditions that can place a heavy burden on quality of life, health and wellbeing. Although most benign disorders can be treated, a lack of knowledge about their specific symptoms or risk factors may prevent women from seeking help at the early stage of the disease. This drives the need for better understanding of symptoms and aetiologies for early diagnosis as well as the provision of suitable diagnostic, prophylactic and/or treatment measures. Benign gynaecological disorders can be broadly categorised into three groups: menstrual disorders, pelvic inflammatory disease and benign tumours/cysts.

(i) Menstrual disorders include amenorrhoea (no menstruation, which may also be a desired consequence of treatment), menorrhagia (abnormal bleeding at irregular intervals) and heavy menstrual bleeding (HMB, heavy but regular). Other symptoms related to benign gynaecological disorders may include dysmenorrhea (painful menstruation), vaginal discharge and infertility. Both menorrhagia and HMB can lead to anaemia and severe iron deficiency, which result in chronic symptoms such as fatigue, poor wound healing and risk of infections (Black and Fraser 2012). Treatment may involve the use of hormonal

contraceptives, which might help in reducing excessive or irregular bleeding and dysmenorrhea.

(ii) Pelvic inflammatory disease (PID) is an acute microbial infection primarily caused by the disruption of the mucosal lining of the endocervical wall, which provides the means for potential pathogens to enter into the upper genital tract. As a consequence, these infections may spread to any of the pelvic organs such as the endometrium (endometritis), ovary (oophoritis) or uterine wall (myometritis) (Boyle and Torrealday 2008). PID typically appears as an indurated and oedematous uterus, with the presence of purulent material, ovarian abscess or tenderness, severe abdominal pain during motion/intercourse, HMB and vaginal discharge and fever. Oral or parenteral antibiotic regimens and sometimes surgery may be used for the treatment of PID and tubal-ovarian abscess (Wiesenfeld and Sweet 1993).

(iii) Benign tumours or cysts are formed as cellular outgrowth due to hormonal dysregulation (for instance, aberrant estrogen signalling) within the uterus or ovary. They proliferate slowly and rarely become cancerous (Boyle and Torrealday 2008). The most common types are uterine polyps and fibroids, which are formed in the endometrium or myometrium, respectively. On the other hand, a benign cyst can be formed on the ovary due to either an unruptured persistent follicle or corpus luteum (functional ovary cyst) or as a consequence of retrograde shedding of ectopic endometrial lesions on to the ovary (endometriosis or endometriotic cyst (endometrioma)).

In the context of this thesis, I will focus on two benign gynaecological conditions originating in the endometrium, namely (i) endometriosis and (ii) adenomyosis.

1.3 ENDOMETRIOSIS

Endometriosis is an estrogen-dependent, chronic inflammatory gynaecological disorder, characterised by the presence of endometrial-like tissue outside the uterine cavity that forms ectopic lesions or cysts. These lesions and cysts can be found in a wide variety of locations in the pelvis; however, they are most frequently found on the ovary, forming endometrioma or endometriotic cysts. Sometimes these lesions can penetrate the surface of the peritoneum, forming either superficial or deep infiltrating endometriosis. On rare occasion, they may be found on other distant anatomical sites such as the bladder, the kidneys, the lungs and even the brain (Starzinski-Powitz *et al.* 2001; Zhao *et al.* 2015; Pritts and Taylor 2003). Most women suffering from endometriosis experience symptoms such as dysmenorrhoea (painful menstruation), dyspareunia (painful intercourse), pelvic pain and infertility (Vigano *et al.* 2004); there are a few asymptomatic (no symptoms) cases as well. Moreover, the risk of

endometriosis increases with reproductive health issues related to menstruation such as shorter cycle length, longer duration of menstrual flow and reduced parity. Conversely, the risk of endometriosis is increased with alcohol (Parazzini *et al.* 2013) and coffee consumption (Saha *et al.* 2017) and is decreased with other lifestyle factors such as exercise (Eskenazi and Warner 1997).

1.3.1 Prevalence of endometriosis

The exact prevalence of endometriosis is unknown since the clinical presentation can vary from asymptomatic and unexplained infertility to severe symptoms such as dysmenorrhoea and chronic pain. The prevalence has been estimated to be around 2%–10% among women of reproductive age and about 50–60% of women with either chronic pain or infertility or both (Eskenazi and Warner 1997; Goldstein *et al.* 1980; Meuleman *et al.* 2009). Moreover, endometriosis has a substantial effect on the psychological and social wellbeing of affected women and imposes a huge economic burden both on individuals and on society. For instance, in the United States, the economic burden has been estimated to be about 12,419 US dollars per woman among endometriosis patients (Simoens *et al.* 2012). In addition, a delay of 6.7 years has been projected between the onset of symptoms and diagnosis as well as around 10.7 hours lost per week per woman affected, in terms of productivity due to chronic disease symptoms (Rogers *et al.* 2013).

1.3.2 Diagnosis of endometriosis

There are no validated, non-invasive biomarkers for endometriosis. Currently, diagnosis is made through laparoscopic surgery and subsequently verified by histological or pathological examination. In addition, transvaginal ultrasound (TVUS) and/or magnetic resonance imaging (MRI) can be suggested. The American Society for Reproductive Medicine (ASRM) has put forth some guidelines for the diagnosis of endometriosis (American Society for Reproductive Medicine: Revised classification of endometriosis 1997), including the presence of endometrial-like cells on target tissues, accompanied by specific features such as adhesion of lesions on the peritoneal wall or ovarian surface, and cyst diameter as well as the presence of thick, chocolate-coloured fluid content within the cyst. Based on the above features, ASRM has categorised the severity of endometriosis into several stages: minimal (stage 1), mild (stage 2), moderate (stage 3) and severe (stage 4).

1.3.3 Theories on the pathogenesis of endometriosis

Endometriosis is considered a multi-factorial disease, the aetiology of which is not fully understood. Several predisposing factors have been proposed regarding its pathogenesis, which

could be explained using the 'two-stage' theory: initiation and promotion (Parazzini *et al.* 2016). The initiation phase of the disease is triggered by factors such as early menarche, menstrual cycle length, duration/volume of menstrual flow and parity. Later, promotion factors such as immune dysfunction, haeme, free iron-induced oxidative stress, impaired progesterone synthesis and apoptosis suppression together with aberrant estrogen secretion, support vascularisation and promote growth (Kobayashi *et al.* 2008). The above hypothetical model for endometriotic lesion development is illustrated in Fig. 2.

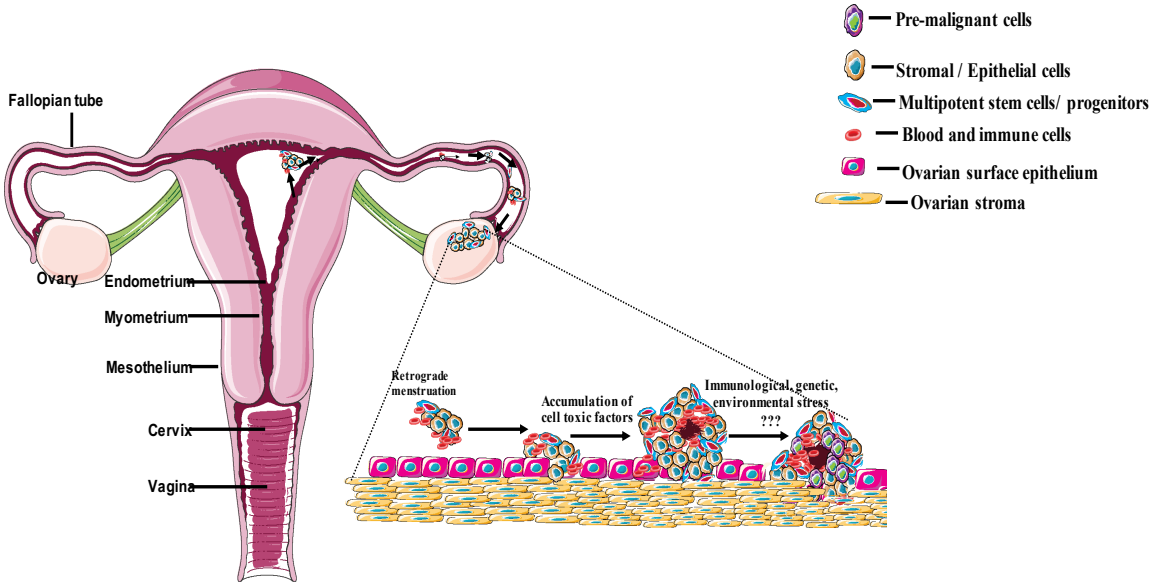

Fig. 2. Schematic diagram of the role of stem cells in the pathogenesis of ovarian endometriosis. Endometrial cells including SCs were shed by retrograde menstruation to peritoneal cavity, where they get adhered, implanted and establish endometriotic lesions. Due to immunological, genetic and microenvironmental factors, some of the endometriotic cells might gain mutations or gene alterations causing altered cellular phenotype. This picture was originally self-drawn, now modified and reprinted from Paper-I (Ponandai-Srinivasan *et al.* 2018). Permission to reuse was provided by Rights Link Copyright clearance centre.

Two important theories will be discussed in relation to the studies involved in this thesis.

A. Retrograde menstruation theory

Samson's retrograde menstruation was the oldest and most accepted theory for the pathogenesis of endometriosis. According to this theory, as a consequence of occasional retrograde menstrual flow, endometrial tissue was shed into the peritoneal cavity and later adhered to the surface of the peritoneum or ovaries, forming ectopic endometrial lesions (Sampson 1927). Experimental evidence from non-human primates showed that endometriosis could possibly be induced by inoculating autologous menstrual products into the peritoneal cavity (D'Hooghe *et al.* 1994). In addition, the lesions developed from these models were histologically and clinically similar to the endometriotic lesions occurring on the ovary or peritoneal sites of humans (D'Hooghe 1997). It was suggested that obstructive menstrual disorders such as iatrogenic cervical stenosis (narrowing of the passageway through the cervix)

or congenital menstrual disorders might also increase the risk of retrograde menstruation (Burney and Giudice 2012). However, this theory fails to explain why only 10% of women have endometriosis even though retrograde menstruation has been observed among 76–90% of women (Sasson and Taylor 2008). Furthermore, it does not explain how endometriotic lesions can be localised in the lungs, brain, etc.

B. Stem cell theory

The immense regenerative potential of the endometrium during menstrual bleeding and the re-epithelisation of the endometrium after childbirth or surgical curettage support the theoretical existence of stem cells (SC) in the endometrium. Adult SC are undifferentiated cells, which have the capacity to self-renew upon external stimuli as well as induce differentiation to attain a cell-type-specific phenotype with a designated function (Gargett and Masuda 2010). Accordingly, researchers have identified adult SC within a highly regenerative endometrium (Meng *et al.* 2007; Gargett *et al.* 2016), menstrual fluid, peritoneal fluid (O *et al.* 2017) and endometriotic lesions (Gargett and Chan 2006; Gargett *et al.* 2014; Silveira *et al.* 2012). Clonogenic cells have been identified that express markers of stemness in a long-term culture derived from endometriotic lesions, which supports to their role in the pathogenesis of endometriosis (Silveira *et al.* 2012). Moreover, it has been suggested that hyperperistalsis in the uterus is associated with the development of endometriosis (Leyendecker *et al.* 2004). As a consequence of hyperperistalsis, endometrial basalis tissue is shed abnormally by retrograde menstruation into the peritoneal cavity; once there, the fragments exhibit increased potential to implant and proliferate due to higher E levels as well as estrogen receptor (ER) and progesterone receptors (PR). In addition, it has been shown that differentiated stromal fibroblasts from women with endometriosis inherit the progesterone resistance and pro-inflammatory phenotype from their SCs (Barragan *et al.* 2016). It has further been suggested that ectopic endometriotic SCs have more invasive and migratory capacity relative to eutopic endometrial SCs, studied using both *in vitro* (Sundqvist *et al.* 2012) and *in vivo* models (Kao *et al.* 2011); angiogenesis has also been stimulated. Experts in the field recently proposed that the endometrial SC might be shed during uterine bleeding among neonatal girls and that the SC remain quiescent within the peritoneal cavity for several years (Brosens *et al.* 2013). Later, if these quiescent cells get reactivated during adolescence due to the function of ovarian hormones, it may also facilitate implantation and establishment of endometriotic lesions (Gargett *et al.* 2014; Puttemans *et al.* 2016). However, certain SC populations may develop somatic mutations and gene deregulations due to prolonged exposure to immunological and environmental stress, to attain pre-malignant potential (Gadducci *et al.* 2014; Gargett *et al.* 2014).

1.4. ADENOMYOSIS

Adenomyosis, previously called endometriosis interna, is a common, benign gynaecological condition, observed in 19.5% of women in reproductive age (Devlieger *et al.* 2003; Garcia-Solares *et al.* 2018). Women with adenomyosis present non-specific symptoms similar to those of endometriosis or uterine fibroids such as abdominal pain, HMB and infertility. At the beginning of the nineteenth century, all abdominal disorders involving mucosal invasion were considered to be 'adenomyoma' (Benagiano *et al.* 2009) since they share several features in terms of symptomology, histology and molecular alterations (Leyendecker *et al.* 2015). Later, Frankl (Frankl 1925) created the word 'adenomyosis' to describe the presence of ectopic endometrial tissue inside the uterine myometrium (Ferenczy 1998). Two years later, Sampson (Sampson 1927) proposed retrograde menstruation theory to describe the mechanism of peritoneal endometriosis. However, since Samson's theory does not explain the mechanism of endometrial invasion inside the myometrium, adenomyosis and endometriosis have been considered to be two separate conditions ever since (Benagiano and Brosens 2006, 2011). Women with adenomyosis usually have early menarche, shorter menstrual cycles, increased body mass index, are multiparous, relatively older in age and have a history of induced abortion (Parazzini *et al.* 2009; Templeman *et al.* 2008).

1.4.1. Prevalence of adenomyosis

Several reports have suggested a strong association between endometriosis and adenomyosis. Both conditions have been closely linked with molecular changes or abnormalities occurring either on the inner portion of the myometrium or at the junctional zone (JZ) between the endo- and the myometrium, leading to a highly migratory and invasive eutopic endometrium (Benagiano *et al.* 2014). Adenomyosis has been observed in 34.6% of cases having deep infiltrating endometriosis while a reference group without endometriosis had an incidence of only 19.4% (Bazot *et al.* 2004). In another study, 40% of endometriosis cases showed irregular JZ, as opposed to 22.5% in the non-endometriosis group. In terms of older women (40–50 years) undergoing surgery for either adenomyosis or uterine fibroids, 34.1% showed co-existence of endometriosis (Naphatthalung and Cheewadhanaraks 2012). In addition, there was a specific correlation reported between adenomyosis and deep infiltrating endometriosis (Gonzales *et al.* 2012) as well as with a group possessing concomitant endometriosis and infertility (Kunz *et al.* 2005). However, it is important to note that most of the above studies included cases of co-existing endometriosis with adenomyosis due to a lack of clear guidelines or diagnostic criteria for distinguishing between them. As a result, the reported frequency of adenomyosis were higher than the real incidence.

1.4.2. Diagnosis of adenomyosis

Adenomyosis is traditionally diagnosed by histological examination following hysterectomy (Morassutto *et al.* 2016). The pathological examination may present as an enlarged, globular uterus with areas of hypertrophic and hyperplastic myometrial smooth muscle, along with the presence of dark cysts within the myometrium. Histologically, it is visualised by the invagination of endometrial glands and stroma through the JZ into the myometrium, as well as adjacent myometrial hyperplasia, which causes globular and cystic enlargements in the myometrium (Donnez *et al.* 2018). With the advent of non-invasive imaging techniques such as TVUS and MRI, it is now possible to differentiate features specific to adenomyosis and not endometriosis, such as diffuse thickening of the inner myometrium, irregular JZ, the presence of localised lesions or an increased JZ-to-outer-myometrial ratio (Levy *et al.* 2013). Moreover, adenomyosis may present in two forms, focal or as a diffused, tumour-like growth. A focal lesion, also called adenomyoma, might be observed when a circumscribed nodular formation appears on the myometrium, while a diffuse form shows a uniform spreading of endometriotic glands and stroma throughout the surface and the depth of the myometrium (Gordts *et al.* 2018).

1.4.3. Theories on the pathogenesis of adenomyosis

Similar to endometriosis, the aetiology and pathogenesis of adenomyosis remains largely unknown. Here, I will discuss three important theories, namely: Invagination theory, metaplasia theory, and De novo stem cell theory.

A. *Invagination theory*

Invagination theory is the most widely accepted theory for the pathogenesis of adenomyosis. It suggests that adenomyosis may occur due to the invagination of endometrial glands from the basalis layer of endometrium into a traumatised endometrial-myometrial interface via tissue injury and repair (TIAR) mechanism, as depicted in Fig. 3A. Several studies have been performed to understand the cause and consequences of this mechanism. First, uterine peristalsis cause microtrauma to the JZ; however due to hypoestrogenism, there is an elevated oxytocin-mediated uterine peristalsis, which further cause auto-traumatisation and induce TIAR mechanism in a repetitive cycle (Leyendecker *et al.* 2015; Leyendecker *et al.* 2009). In addition, small nerve fibre injury inside the uterus during difficult intrapartum episodes has also been suggested as the cause of this disease (Quinn 2011). Studies measuring intrauterine sinusoidal pressure waves of varying frequencies during menstruation have suggested that the highest level of stress prevails on the endometrial-myometrial interface, which might induce molecular alteration; this, in turn, leads to the invasion of endometrial cells from the basal layer

(Shaked *et al.* 2015). It has been claimed that the use of repeated sharp curettage causes disruption of the endometrial-myometrial border, thereby increasing the incidence of adenomyosis (Curtis *et al.* 2002; Levgur *et al.* 2000).

B. Metaplasia theory

This theory suggests that metaplastic changes occur on embryonic pluripotent Mullerian remnants could potentially establish de novo ectopic endometrial lesions at the intra-myometrial sites (Garcia-Solares *et al.* 2018), as shown in Fig. 3B.

C. Stem cell theory

According to this theory, repeated tissue injury and microtrauma at the JZ, induces activation of stem cells and an alteration in its niche (Vannuccini *et al.* 2017; Gargett *et al.* 2016). As a consequence, progenitor cells are allowed to differentiate in a retrograde direction by breaching the endometrial-myometrial interface and establish de novo adenomyosis lesions, as shown in Fig. 3C. It has also been postulated that multipotent cells from bone marrow and other sources may form de novo focal lesions inside the myometrium. Even though the invagination theory is the most widely accepted, de novo lesions from different sources might be a plausible mechanism. Hence, more studies are required to further establish the role of endometrial SC or Müllerian remnants in the initiation of this disease.

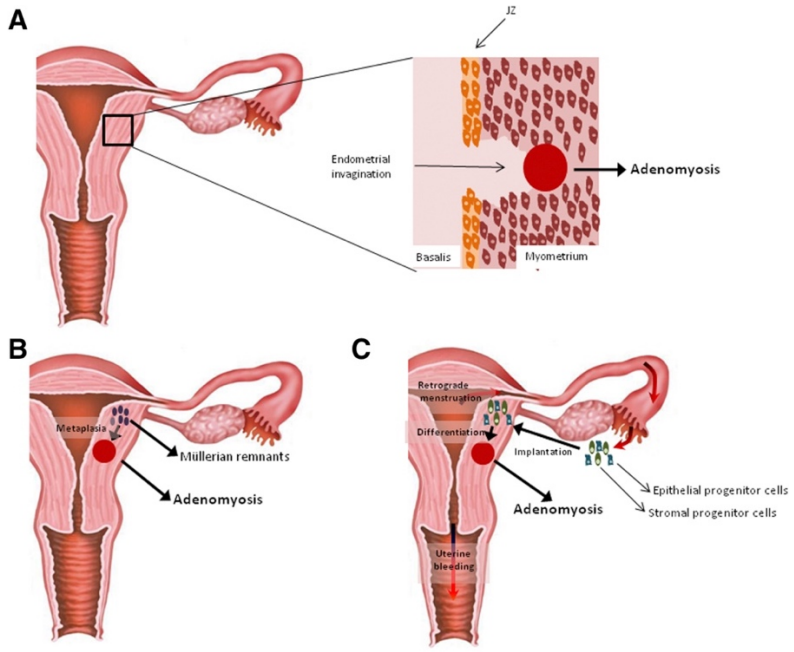

Fig. 3. Theories on the pathogenesis of endometriosis. Adenomyosis are formed due to displacement of endometrial-like cells or tissues into uterine myometrium, which might be explained by (A) Invagination theory (B) Metaplasia theory (C) stem cell theory (reprinted from (Garcia-Solares *et al.* 2018) and permission for reuse provided by RightsLink Copyright clearance centre).

1.5. TREATMENT STRATEGIES FOR ENDOMETRIOSIS/ADENOMYOSIS

There are no disease-specific treatment options available for either endometriosis or adenomyosis. The treatments suggested so far have focused on alleviating the disease symptoms such as pelvic pain, abdominal cramps and HMB. Both disorders are estrogen-dependent, implying that the treatment should be targeted at suppressing excess E production and overcoming P resistance (Vercellini *et al.* 2014), thereby promoting fertility (Tsui *et al.* 2014). It is important to note that adenomyosis has not been as extensively researched as endometriosis.

1.5.1. Laparoscopic surgery

Laparoscopic surgery (LS) is the most common mode of treatment for the management of endometriosis. LS is performed to remove visible regions of endometriotic lesions or cysts either by using an ablation technique, which involves the destruction of a lesion by burning, or an excision technique, which implies cutting the lesion out surgically. A Cochrane review suggests that LS is moderately associated with reducing overall pain (odds ratio (OR): 6.58, 95%; confidence interval (CI): 3.31 to 13.10), improving live birth (OR: 1.94, 95%; CI: 1.20 to 3.16) and clinical pregnancy rate (OR: 1.89, 95%; CI: 1.25 to 2.86) (Duffy *et al.* 2014). Thus, it is very useful to provide relief for pain symptoms and sub-fertility. It is also important to remove active deposits of the ectopic lesions that caused pain symptoms. Unfortunately, treatment by LS alone may not be sufficient as these lesions tend to recur in cases of severe endometriosis (ASRM II/IV).

1.5.2. Hormonal contraception

Combined hormonal contraception (CHC) are widely used to treat women with pain associated with endometriosis (Jensen *et al.* 2018). To be effective, they should be used without a break (the so called 'long cycle treatment') and can be administered in the form of a pill, a patch or a vaginal ring. Continuous administration of CHC significantly reduces bleeding, dysmenorrhea, pelvic pain, dyspareunia and postoperative disease recurrence; and improve the overall quality of life (Vercellini *et al.* 2014; Grandi *et al.* 2019). The disadvantages of such treatment include contraindications to estrogen such as the increased risk of venous thromboembolism (Ferrero *et al.* 2010).

Treatment with progestins results in dose-dependent effects on follicular development and ovulation; as well as cervical mucus. They also act on all organs with PR, including the endometrium. Progestins promote decidualisation and atrophy of the endometrium and endometrial implants. Progestins in various forms reduce vaginal bleeding and provide

resolution from pain symptoms associated with endometriosis (Brown *et al.* 2012). The levonorgestrel-releasing intrauterine system (52 mg LNG-IUS) is a safe, highly effective and accepted reversible, long term contraceptive method as well as an effective treatment for uterine disorders such as endometriosis or adenomyosis related pain and HMB (Gemzell-Danielsson *et al.* 2011). High local concentrations of LNG provided by the IUS in the endometrium and to a lesser degree in the myometrium results in decidualisation and endometrial atrophy, usually without suppressing ovulation (dose-dependent effect).

Selective progesterone receptor modulators (SPRM, such as ulipristal acetate, mifepristone, and vilaprisan, exert an agonist, antagonist or mixed effect due to their competitive action with P on PRs. SPRMs were developed for the treatment of endometriosis and studied for the treatment of adenomyosis; they are also available for the short-term treatment of uterine fibroids (Whitaker *et al.* 2017; Schutt *et al.* 2018; DeManno *et al.* 2003).

Other treatment possibilities include aromatase inhibitors (Ferrero *et al.* 2011) and selective estrogen receptor modulators. The latter act as antagonists on ER, thereby inhibiting endometrial proliferation and inducing atrophy (Tsui *et al.* 2014). Also, gonadotropin releasing hormone agonists (such as buserelin, goserelin, leuprolide, nafarelin, and triptorelin), are effective in inducing amenorrhea and reducing pain. However, hypoestrogenic side effects limit their use unless an add-back therapy is given (Tsui *et al.* 2014).

1.6. ENDOMETRIOSIS-ASSOCIATED OVARIAN CANCERS (EAOC)

Endometriosis is generally considered a benign disorder; however, on rare occasion, it may exhibit certain characteristics of malignancy such as uncontrolled growth, neo-angiogenesis, local invasion and migration (Munksgaard and Blaakaer 2012; Starzinski-Powitz *et al.* 2001). Several epidemiological studies have consistently shown endometriosis carries an increased risk for various malignancies (Kok *et al.* 2015; Munksgaard and Blaakaer 2012); the strongest association was observed for ovarian cancer, known as endometriosis-associated ovarian cancer (EAOC) (Melin *et al.* 2006; Lundberg *et al.* 2019; Saavalainen *et al.* 2018; Pearce *et al.* 2012). A meta-analysis conducted on EAOC estimated that about 80% of reported cases were of ovarian origin (Heidemann *et al.* 2014; Kim *et al.* 2014) and the remaining 20–25% were on extragonadal sites such as a peritoneal cavity, lower pelvis, gastrointestinal tract, abdominal wall, umbilicus, recto-vaginal septum, colon, pleura and others (Heaps *et al.* 1990; Irvin *et al.* 1998; Brooks and Wheeler 1977).

1.6.1. Prevalence of EAOC

A recent population-based cohort study showed an increased incidence of EAOC among women diagnosed with both endometriosis and infertility (adjusted Hazard risk (aHR) ratio: 2.19, 95%CI 1.70 – 2.82) compared to women diagnosed with only endometriosis (aHR: 1.77, 95%CI 1.53 – 2.05) or infertility (aHR: 1.53, 95%CI 1.36 – 1.72) (Lundberg *et al.* 2019). Moreover, endometriosis shows an increased risk, specifically towards the endometrioid (standardised incidence ratio (SIR): 2.26) and clear cell (SIR: 3.95) subtypes of ovarian cancers (Brinton *et al.* 2005; Saavalainen *et al.* 2018; Pearce *et al.* 2012). Moreover, Saavalainen et al. (2018) performed a sub-analysis based on the type of endometriosis (ovarian, peritoneal and deep infiltrating) and the independent risk of each endometriosis type towards different types of genital cancers (ovary, cervix, endometrial, etc.) (Saavalainen *et al.* 2018). Interestingly, there was a strong association reported between incidence of ovarian cancer among women with ovarian endometriosis (SIR: 2.56 (1.98–3.27)), specifically for the endometrioid (SIR: 4.72 (2.75–7.56)) and clear cell (SIR: 10.1 (5.5-16.9)) sub–types. However, women with both peritoneal endometriosis (SIR: 1.32 (0.99–1.72)) and deep infiltrating endometriosis (SIR: 1.41 (0.29–4.1)) showed no increase in the overall risk for ovarian cancer. In addition, the risk of endometrial cancer was not altered in any type of endometriosis.

1.6.2. Histological markers of EAOC

Several chronological events have been documented that support a strong link between endometriosis and the development of ovarian cancer. Based on microscopic observations, Sampson (1925) proposed a set of criteria for the diagnosis of ovarian cancer in women with endometriosis; these criteria were as follows: (i) clear evidence of endometriosis close to tumour; (ii) demonstration of cancer arising within or from endometrioma, but not from anywhere else; and (iii) histological identification of tissue resembling endometrial stroma surrounding characteristic epithelial glands. is decreased with lifestyle factors such as exercise (Eskenazi and Warner 1997). Later, Scott (1953) elucidated an additional criterion: evaluation of the morphological changes that continuously occur within the epithelium during transformation from benign to malignant in endometriosis (Scott 1953). Currently, the above four histological criteria are used for diagnosing the transition from benign to malignant conversion in ovarian endometriosis (Tanase *et al.* 2013).

Several histological pieces of evidence suggest that EAOC may originate from atypical endometriosis of the ovary (Fukunaga *et al.* 1997; Czernobilsky and Morris 1979). Atypical endometriosis has been characterised as being similar to hyperplasia of the endometrial glands with cytological atypia (Clement 2007; Seidman 1996) and has been detected in 80% of EAOC

cases (Fukunaga *et al.* 1997; Worley *et al.* 2013). It possesses some unique features, such as eosinophilic cytoplasm, large hyperchromatic or pale nuclei with moderately marked pleomorphism, an increased nuclear-to-cytoplasmic ratio, cellular crowding and stratification or tufting. It has also been suggested that other factors, such as genetic, immunological, environmental (including microenvironmental) and cytokines play a key role in the malignant transformation of endometriosis to EAOC (Worley *et al.* 2013; Varma *et al.* 2004).

1.6.3. Molecular markers of EAOC

In the past two decades, numerous studies have consistently shown that EAOC may arise from genetic alterations that occur within an endometrioma due to the altered peritoneal microenvironment. One possible explanation might be the accumulation of haeme, free iron and reactive oxygen species from menstrual reflux, which may induce oxidative stress and develop mutations in key genes. Yamaguchi et al. (2008) showed that the concentration of free iron was significantly higher in endometriotic cysts compared to non-endometriotic cysts. In addition, endometriotic cyst fluid also expressed high levels of reactive oxygen species such as 8-hydroxy-deoxyguanosine similar to the levels observed in carcinomas (Yamaguchi *et al.* 2008) and their higher levels were associated with poor prognosis in epithelial ovarian cancer (Pylvas *et al.* 2011). Furthermore, an impaired immune response may also cause chronic inflammation and macrophage or aberrant cytokine activation, which would trigger mutations within endometrioma (Gazvani and Templeton 2002).

Alternatively, there might be inherent mutations in eutopic endometrial cells among susceptible subjects due to family history or impaired hormonal activity. They may also add more mutations as a consequence of local inflammation or altered niche within endometriotic cysts during retrograde flow. Some of the common somatic mutations and genomic aberrations reported during malignant transformation of EAOC include: silencing of the key tumour suppressor genes *TP53* (Sainz de la Cuesta *et al.* 2004; Akahane *et al.* 2007), *PTEN* (Govatati *et al.* 2014) and *ARID1A* (Wiegand *et al.* 2010); oncogene activation of *KRAS* (Amemiya *et al.* 2004; Stewart *et al.* 2012), *PIK3CA* and *CTNNB1* (McConechy *et al.* 2014) and downregulation of *BCL2* (Nezhat *et al.* 2002; Akahane *et al.* 2007). A study analysing deep infiltrating endometriotic lesions with no risk of EAOC demonstrated several mutations in cancer-driver genes, such as *ARID1A* and *PI3KCA* (Anglesio *et al.* 2017), that were commonly mutated in both clear cell ovarian carcinomas and concurrent endometriotic lesions (Anglesio *et al.* 2015). Moreover, EAOC cases also show loss of BAF250a expression, a protein encoded by *ARID1A,* especially within areas of contiguous endometriosis or atypical endometriosis

surrounding the tumour; this implies their expression has a role in the early stages of malignant transformation of endometriosis (Stamp *et al.* 2016).

Furthermore, the DNA mismatch repair genes *hMLH1 or hMLH2* were also inactivated by hypermethylation within endometriotic tissue (Esteller *et al.* 1999). In some cases, aneuploidy in the advanced stages of endometriosis was reported, particularly along chromosome 17, on which tumour suppressor gene *TP53* resides (Kosugi *et al.* 1999). Moreover, microsatellite analysis performed with EAOC patients showed a loss of heterozygosity among both cancerous and benign endometriotic lesions from the same patient, indicating endometriosis might be a clonal precursor to a subtype of ovarian cancer (Sato *et al.* 2000). This also implies that the molecular mechanisms that accompany the changes in EAOC might be different from ovarian cancer developed without a history of endometriosis. Hence, there is a need for a better understanding of its pathogenic mechanisms in order to develop specific prophylactic measures and avoid potential tumour growth among EAOC patients.

1.7. BRCA1 OR BRCA 2 MUTATIONS AND OVARIAN CANCER RISK

The breast cancer gene 1 (*BRCA1*) and gene 2 (*BRCA2*), are tumour suppressor genes that play a key role in monitoring DNA damage response and repair of DNA double-strand breaks by homologous recombination. However, cells that bear mutations in *BRCA1* or *BRCA2* repair DNA lesions via an alternative error-prone mechanism that leads to genomic instability and increased risk of breast and ovarian cancers (Stoppa-Lyonnet 2016).

1.7.1. Prevalence of *BRCA1* or *BRCA2* mutations

Hereditary mutations constitute around 15–20% of all cases of ovarian cancers; mutations on *BRCA1* or *BRCA2* are the major contributors (65–85%) within that category (Norquist *et al.* 2016). Pathogenic *BRCA1* or *BRCA2* mutations have been observed in 20% of high grade serous cancers, and 8% with clear and endometrioid histological subtypes of ovarian cancers (Alsop *et al.* 2012). Moreover, the lifetime risk for developing breast cancer due to *BRCA1 or BRCA2* mutations has been estimated to be around 50–80%. With regard to ovarian cancer, the lifetime risk has been individually estimated to be around 30–60% and 10–25% due to *BRCA1* and *BRCA2* mutations (Kuchenbaecker *et al.* 2017). In addition, it has been observed that the age at diagnosis for ovarian cancer with *BRCA1 or BRCA2* mutations is older than that of breast cancer; for instance, the mean age for *BRCA1* and *BRCA2* mutation is around 40–60 years and 50–70 years respectively (Finch *et al.* 2014). In addition, both these mutations increase the risk of other cancers such as BRCA1 for fallopian tube cancer and primary peritoneal cancer, and pancreatic cancer, prostate and breast cancer among *BRCA2* mutation carriers.

In a general, unselected ovarian carcinoma cohort, 19% of cases were observed to have pathogenic and less/likely pathogenic (P/LP) germline variants of the *BRCA1* or *BRCA2* mutations (Maistro *et al.* 2016). Another cross-sectional study that screened for the prevalence of P/LP variants showed five times more individuals with P/LP variants by next-generation sequencing than previously detected based on personal or family history (Manickam *et al.* 2018); this indicates the need for genetic screening to identify *BRCA1* or *BRCA2* mutation carriers with potential cancer risk.

1.7.2. Theories on the pathogenesis of *BRCA1* or *BRCA2* mutations and cancer development

The association between *BRCA1* or *BRCA2* germline mutations and its potential cancer risk can be explained based on Knudson's 'two-hit' hypothesis (Knudson 1971). According to the hypothesis, each tumour suppressor gene must undergo two hits (mutations) in its allelic pair in order to develop cancer (Knudson *et al.* 1975). With hereditary cancers, one of the hits is a germline mutation observed in all somatic cells, while the occurrence of the second hit is a deletion of a wild-type allele within the somatic cells of the target tissue. Accordingly, it has been shown that women with the *BRCA1* mutation bear one inherited mutant allele and another wild-type *BRCA1* allele. However, in most cancers, the wild-type allele is deleted, leaving no functional *BRCA1* gene (Merajver *et al.* 1995). In this model, the inheritance pattern is autosomal-dominant, meaning that the mutant *BRCA1* or *BRCA2* allele was inherited from the previous generation. With respect to molecular expression, however, the *BRCA1* or *BRCA2* gene exhibits a recessive pattern since both the alleles need to be downregulated or inactivated to form a tumour (Rosen 2013).

Moreover, the relationship between *BRCA1* or *BRCA2* and *TP53* also plays a critical role in the pathogenesis of breast and ovarian cancers. *BRCA1 or BRCA2* interacts with tumour suppressor *TP53* and regulates DNA damage response as well as cell-cycle checkpoint activation, especially through *CHK1*, which blocks cell-cycle progression in the Gap 2/Mitosis (G2/M) phase. With *BRCA1* or *BRCA2* deficiency, it has been suggested that *TP53* and *p21* are activated to regulate genomic instability, resulting in cell-cycle arrest or senescence. As expected, the incidence of *TP53* mutations is higher among *BRCA1* mutated cancers (>80%) compared to sporadic cancers (25%) (Holstege *et al.* 2009). Jiang et. al. (2011) reported that *TP53* mediates the nuclear transport of *BRCA1* by disrupting the *BRCA1-BARD* complex; hence, *TP53* mutations among *BRCA*1 mutated carriers might amplify their chromosomal abnormalities due to repeated error-prone DNA damage response, which leads to increased incidence of breast or ovarian cancer.

1.8. STRATEGIES FOR REDUCING OVARIAN CANCER RISK AMONG HIGH-RISK POPULATIONS

Women with high risk for ovarian cancer were suggested with various risk-reducing strategies, which might vary in their effectiveness and potential side effects. Here, I will be discussing the important prevention strategies, namely prophylactic surgery and chemoprevention.

1.8.1. Prophylactic surgery

Risk reducing Salpingo-oophorectomy (RRSO) shows decreased risk of breast and ovarian cancer (Domchek *et al.* 2010; Rebbeck *et al.* 2009) as well as improved overall survival (Eleje *et al.* 2018) among women with *BRCA1* or *BRCA2* mutations. However, RRSO were accompanied by adverse effects such as infertility, sexual dysfunction, premature menopause and increased morbidity; in addition, they had a negative impact on body image and quality of life (Domchek *et al.* 2010; Madalinska *et al.* 2005; Rocca *et al.* 2006). Alternatively, reduced cancer risk with limited side effects was reported with the use of prophylactic salpingectomy with ovarian retention (Carcangiu *et al.* 2006; Finch *et al.* 2006) and tubal ligation (sterilisation) (Kjaer *et al.* 2004; Sieh *et al.* 2013). In addition, it has been suggested that combination strategies such as hysterectomy plus RRSO (Hazard ratio (HR): 0.06) provide better protection compared to the use of either tubal ligation (HR: 0.72) or hysterectomy (HR: 0.79).

1.8.2. Chemoprevention

It has been suggested that CHCs reduce the risk of epithelial ovarian cancers among both the general (EAOC) and the high-risk population (*BRCA1 or BRCA2* carriers) and that their protective effect is improved with long-term use (Havrilesky *et al.* 2013; McLaughlin *et al.* 2007; Iodice *et al.* 2010). SPRMs such as mifepristone (MIFE) also show promising results in experimental settings for reducing or delaying the use of RRSO or inherent cancer risk. For instance, mifepristone (MIFE) has been shown to prevent mammary tumorigenesis in an animal model of the *BRCA1* mutation (Poole *et al.* 2006). In addition, clinical studies examining a low dose of MIFE exhibited a favourable, anti-proliferative effect on breast tissue in premenopausal women (Engman *et al.* 2008). On other hand, the use of non-steroidal anti-inflammatory drugs and aspirin show diverse effects in several epidemiological studies.

1.9. ENDOMETRIAL STEM CELLS

The human endometrium is a highly regenerative organ which regrows from a thickness of 1–2 mm just after menstrual shedding to its full thickness of more than 14 mm during the secretory phase of the menstrual cycle. Considering the immense regeneration potential, it has been suggested that endometrial tissue might contain a population of SC with a capacity to self-

renew and produce differentiated cells indefinitely, in order to restore the endometrium after every menstrual cycle.

1.9.1. Historical overview of endometrial SC

Prianishnikov was the first to postulate the existence of SC (Prianishnikov 1978). Over a decade ago, a rare clonogenic population was identified within pure epithelial and stromal cells that has the capacity to form colonies and differentiate into mesenchymal lineages (Chan *et al.* 2004). Subsequently, a putative cell population, known as label-retaining cells were identified, that can retain bromodeoxyuridine after their incorporation into a mouse endometrium (Chan and Gargett 2006). Consequently, another form of clonogenic cells known as endometrial side populations (SP) were identified, which efficiently efflux Hoechst dye (Cervello *et al.* 2011). These SP cells were able to exhibit self-renewal and multi-lineage differentiation capacities *in vitro* as well as undergo tissue reconstitution *in vivo*. Concurrently, Taylor (2004) reported that the HLA-mismatched bone-marrow derived stem cells from donors were successfully engrafted into the recipient endometrium, suggesting that the endometrium could be regenerated from non-uterine stem cells (Taylor 2004). Subsequently, the putative stem cells were suggested to be of hematopoietic origin and postulated their key role in endometrial regeneration and disease pathologies (Du and Taylor 2007).

1.9.2. Endometrial SC in regeneration and diseases

Prianishnikov suggested a model on hierarchical hormone responsiveness for the regeneration of endometrial cells during each uterine cycle (Prianishnikov 1978). According to the model, endometrial SCs are located deep inside the epithelium of the basalis endometrium and may not be subjected to any destruction during menstrual bleeding. These SC are a very small pool of hormone-independent cells (Ferenczy 1976) and their proliferation is chiefly regulated by microenvironmental factors as a consequence of tissue injury. Upon SC division, first-generation progenitor cells (E2-cells) are formed, which express ER in response to raising E2 levels in the uterine cavity. Concurrently, the second-generation daughter cells (E2-P cells), unlike E2-cells, are formed during the later stage of the proliferative phase and become responsive to both P and E2 by expressing both ER and PR. Subsequently, E2-P cells further divide in response to higher P levels in the secretory phase; they later undergo terminally differentiated P cells with decidualised cellular phenotype. During menstruation, only P-cells residing in the functionalis layer are denuded from the endometrial surface, leaving their predecessors intact. The above model is illustrated in Fig. 4.

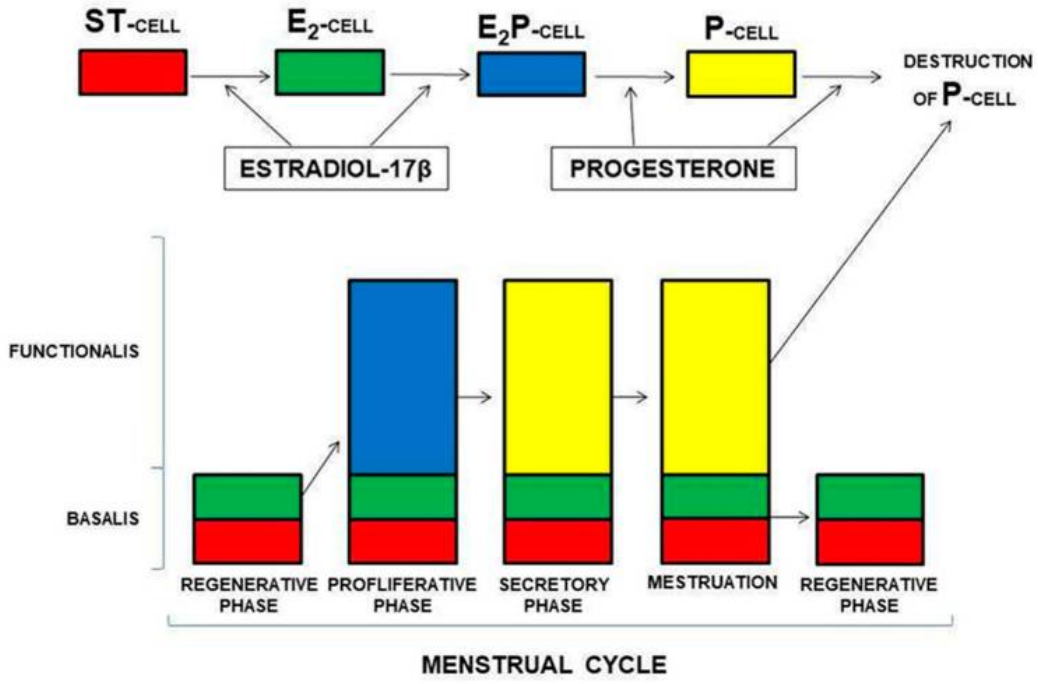

Fig. 4. Postulated model on the involvement of stem cells in endometrial regeneration Stem cells and its progenitors are influenced by estrodiol (E2) and progesterone (P) resulting in the formation and distribution of various cell types at different stages of uterine cycle. Abbreviations (ST-cell=stem cell; E_2-cell=ER+ progenitors; E_2P-cell= both ER+ and PR+ progenitors; P-cell= PR+ differentiated cells). The image is reused from (Tempest, Maclean*, et al.* 2018) and permission for reuse provided by Creative Commons 4.0.

However, due to constant and prolonged stimulation of E2, which alternates with insufficient P levels in the uterus, the equilibrium of the above processes may be disturbed; this may have potential implications in proliferative uterine disorders such as endometriosis or adenomyosis. Also, it was proposed that the functionalis endometrium in women with endometriosis possesses a high percentage of 'basalis-like cells' that are subsequently shed via retrograde menstruation and contribute to the initiation of ectopic endometriotic implants (Leyendecker *et al.* 2002). The above theory remains unproven since it is not possible to differentiate between cells originating in the basalis layer and those originating in the functionalis layer.

1.9.3. Markers for identifying endometrial SC

It has been suggested that putative SC from the endometrium are formed from heterogeneous cell clusters, including stromal, epithelial and endothelial progenitors, as depicted in Fig. 5. It has been hypothesised that epithelial progenitors in the basal compartment initiate the regeneration to form differentiated luminal and glandular epithelial cells. However, it has been challenging to identify endometrial epithelial SC due to a lack of specific markers as well as techniques to culture and maintain epithelial cells for longer duration *in vitro*. As of now, epithelial SC activity has been demonstrated for markers SSEA-1 (Hapangama *et al.* 2019; Valentijn *et al.* 2013), SOX9 (Tempest, Maclean*, et al.* 2018; Hapangama *et al.* 2019), N-

cadherin (Nguyen *et al.* 2017), Musachi-1 (Gotte *et al.* 2008; Tempest, Baker*, et al.* 2018), LGR5 (Tempest, Baker*, et al.* 2018) and in a small proportion of previously characterised SP cells (Cervello *et al.* 2011; Masuda *et al.* 2010). In addition, it has been suggested that characterised SP cells are predominantly of endothelial origin (Cervello *et al.* 2011; Masuda *et al.* 2010). On the other hand, stem cells of stromal/mesenchymal origin are usually fibroblast-like and reside within the perivascular space along with loose stroma in the basalis layer of the endometrium. Some suggested markers with confirmed stem cell properties are as follows: CD146+, PDGF-Rβ+ (Schwab and Gargett 2007), W5C5/SUSD2 (Masuda *et al.* 2012), CD44, CD29, CD73, CD90 and CD105 (Meng *et al.* 2007; Gargett *et al.* 2009; Dominici *et al.* 2006).

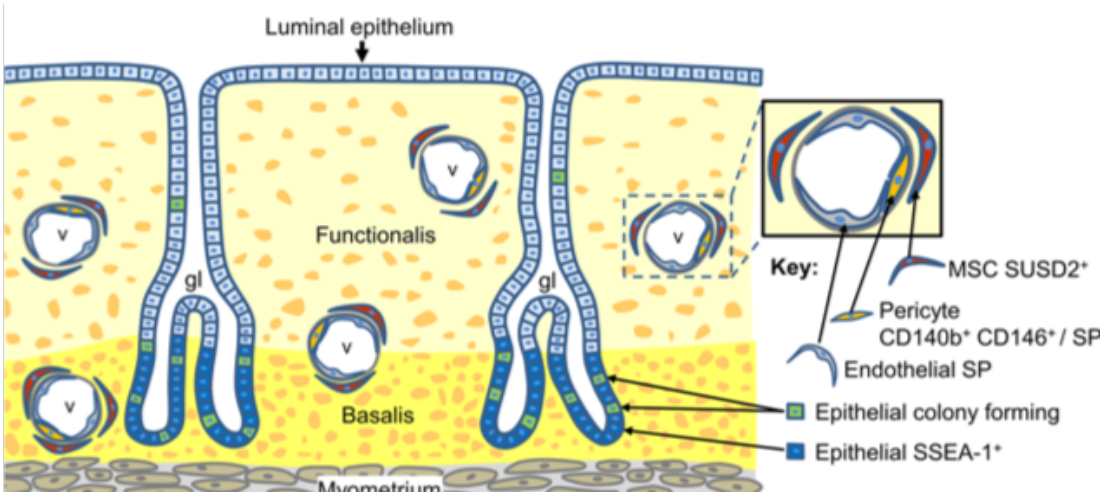

Fig.5. Schematic diagram on the postulated location of endometrial progenitors in the human endometrium. Putative stem cells were supposed to be formed in basalis region of endometrium and composed of stromal (CD140b+CD146+ /W5C5/ SP), epithelial (SSEA-1+ SOX9+ / SOX9 / N-cadherin) and endothelial (SP) cell clusters. The image is reprinted from (Gargett *et al.* 2016) and permission for reuse provided by Rights Link Copyright clearance centre.

2. AIM AND OBJECTIVES

This study aimed to investigate early molecular alterations associated with the development of EAOC among women with ovarian endometriosis. In addition, we wanted to explore the possibilities for early detection and potential treatment strategies to reduce disease symptoms and/or inherent cancer risk among women with ovarian endometriosis, *BRCA1 or BRCA2* mutations and adenomyosis.

The specific objectives of the thesis were as follows:

- To understand whether there is a molecular link between endometriosis and ovarian cancer by exploring the gene deregulations within the endometrium and endometrioma of women with ovarian endometriosis.

- To explore the role and regulation of syndecan -1 (SDC-1) and -4 (SDC-4) in Transforming growth factor-β (TGF-β) signalling on endometriotic stem/stromal cells *in vitro*, in order to understand interactions and involvement in the pathophysiology of endometriosis-associated cancer.

- To evaluate the mechanism of action for the selective progesterone receptor modulator, mifepristone, on *BRCA1* or *BRCA2* mutated ovarian stem/stromal cells *in vitro*, as a preventive option to reduce inherent ovarian cancer risk.

- To decipher the mechanism of action for the dopamine agonist, bromocriptine, on the endometrium of women with adenomyosis, as a prophylactic treatment option to reduce heavy menstrual bleeding.

3. METHODOLOGICAL CONSIDERATIONS

3.1. PATIENT COHORTS AND STUDY DESIGN

Characteristics	Study I	Study II	Study III	Study IV
Tissue biopsies of interest	Endometrial (En)-healthy (H), Eutopic (P) and Endometrioma (Endo) biopsies	Endometrioma biopsies	*BRCA1* or *BRCA2* mutated ovarian punch biopsies	Endometrial biopsies before and after bromocriptine treatment
Sample size (n)	H-En-14, P-En-37, Endo-30	15	9	8
Inclusion criteria	Surgically verified endometriosis (ASRM III-IV) with no indications of cancer		Positivefor *BRCA1* or *BRCA2* mutation	Diffuse adenomyosis
	Volunteers—fertile age, regular menstruation, parity, clinically examined for the absence of hormonal disorders	Not applicable		
Exclusion criteria	Subjects had not received any hormonal medications for at least three months before surgery. Median age <45 years		No ovarian cancer	Subjects diagnosed for endometriosis by TVUS and MRI
Median age (range) in years	H-En ≤40; P-En & Endo:33 (23-43)	34 (23-43)	42 (37-65)	45 (39-50)
BMI (Kg/m^2)	22.3 ± 3.5	unknown	23.2 ± 2.5	24.4 ± 3.6
Menopausal status	Premenopausal		Postmenopausal-4; Premenopausal- 5	Premenopausal
Menstrual cycle phase	H-En: secretory phase; P-En-proliferative (19), secretory (17); Endo (proliferative–19, secretory–11)	Proliferative–4, secretory–2, others-unknown	Unknown	Proliferative phase

3.2. ETHICAL CONSIDERATIONS

All studies were performed in accordance with the ethical permits approved by the institutional ethical review committee at Karolinska Institutet, Sweden (Study I and II (2008/1566-31/3 and 2016/95-31/4), III (2010/661-31/1), IV (2013/2060-31/12)), University of Tartu, Estonia (Study I) and the Mayo Clinic, USA (Study IV). Study IV was approved by the Medical products agency as well as registered at clinicaltrials.gov (NCT01821001) and Eudract.ema.europa.eu (EudraCT 2013-004409-14). Informed consent was obtained from all cases and controls, prior to their inclusion in the study.

3.3. ISOLATION OF $CD90^+CD73^+CD105^+$ STEM/STROMAL CELLS

$CD90^+CD73^+CD105^+$ stem/stromal cells (SC^+) were isolated from healthy volunteers (Study I), eutopic endometrium (Study I), and ectopic endometrioma (Paper I and II) from women with endometriosis, as well as 4 mm ovarian punch biopsies in women with *BRCA1 or BRCA2* mutations (Paper III). We opted for the most commonly used mesenchymal markers, CD90, CD73 and CD105, suggested by the International Society for Cell Therapy (Dominici *et al*. 2006), the expression patterns of which have been reported to remain unaltered during prolonged culturing conditions (Jones *et al*. 2010; Pittenger *et al*. 1999). Alternatively, other markers have been suggested in the literature, such as CD146, PDGFRβ (Schwab and Gargett 2007) and SUSD2/W5C5 (Masuda *et al*. 2012), the expression patterns of which are altered during prolonged culturing conditions *in vitro* due to low oxygen tension (Tormin *et al*. 2011) or spontaneous differentiation (Yang *et al*. 2018). We restricted our analysis to sorted SC^+ until passage 6 and adopted physiologically relevant culturing conditions such as 3D-spheroid cultures. This approach enabled us to avoid discrepancies with colony formation and multipotent differentiation capacities, reported previously on later-passaged cells (Digirolamo *et al*. 1999; Pittenger *et al*. 1999).

3.4. TARGETED PCR ARRAY AND CLUSTERING ANALYSIS

We used a targeted gene array with a customised panel of markers, previously reported for its role in premalignancy in endometriosis and/or EAOC. The list of genes is summarised in Table S1 (Paper I). This approach was preferred over whole transcriptome analysis as the expression differences observed among cancer-associated markers might be small and hence could be masked by other known genes involved in disease pathogenesis or hormone-dependent regulation.

Initially, we screened for a panel of 42 preselected genes on cultured SC+ and validated the observations with only 25 out of 42 genes on frozen tissues of both eutopic endometrium and ectopic cyst (Table S1 and marked with *). As a form of method development, we also used the above panel of genes in paraformaldehyde-fixed ectopic cyst tissues (Paper II). Furthermore, intra-patient heterogeneity based on the gene expression patterns from the above panel of genes was explored using hierarchical clustering on heatmap and principle component analysis (PCA). Using both these methods, we were able to identify samples with higher gene expression variability or molecular heterogeneity, which was later validated using functional analysis such as 3D spheroid suspension and invasion cultures.

3.5. TREATMENT DOSES

For Study II, we explored the consequences of high levels of TGF-β ligands secreted in peritoneal fluid in the context of pathogenesis of endometriosis. However, the reported TGF-β levels in the literature were ambiguous, ranging among 2 ng/ml, 250 ng/ml and 900ng/ml in women with infertility, endometriosis at stage I–II and endometriosis at stage II–IV, respectively (Pizzo *et al*. 2002). In contrast, only a smaller difference of approximately 10 ng/ml vs. 1 ng/ml was observed by (Oosterlynck *et al*. 1994) between women having endometriosis vs. control women with no benign pathology. Hence, we selected a wide range of rhTGF-β doses such as 2 ng/ml, 10 ng/ml or 250 ng/ml and an equivalent dose of transforming growth factor receptor beta-I/II (TGFRBI/II) inhibitor Ly2109761 to either activate or downregulate TGF-β signalling *in vitro*.

In Study III, a MIFE dose for *in vitro* treatment was selected based on the effect of dose dependency for proliferation and apoptosis markers. However, the optimal dose of 10 μM MIFE was equivalent to an *in vivo* oral dose of 200 mg MIFE, which is the (single)dose used for emergency contraceptives. Moreover, P concentration was five times greater than MIFE, in order to balance the difference between their binding affinity with PR.

In Study IV, the *in vivo* effects of bromocriptine on the endometrium were explored by providing 5 mg of vaginal bromocriptine treatment every day for six months to women with adenomyosis. The above dose was selected based on previous literature for reducing disease symptoms associated with hyperprolactinemia (Kletzky and Vermesh 1989).

3.6. SPHEROID CULTURES

Three-dimensional spheroid suspension and invasion cultures were used to provide a physiologically relevant condition for sorted SC^+ where they exhibit their proliferation and

invasion capacity in three-dimensional space. Spheroid cultures allow cells with higher colony-forming capacity to form only multicellular spheroids during serum-starved special growth conditions; cells in the later stages of differentiation exhibit low colony-forming capacity (Kunz-Schughart *et al*. 1998; Lin and Chang 2008). Spheroid suspension cultures were previously used to enrich the population with cancer-initiating characteristics, known as cancer stem cells (CSC) (Dey *et al*. 2009; Kunz-Schughart *et al*. 1998). Hence, we used a specifically designed spheroid enrichment media comprising DMEM/F12 with growth factors such as FGFb, EGF, B27, Insulin-Transferrin-selenium and allowed the SC^+ to form suspension 3Dspheroids by growing them in ultra-low attachment plates/flasks. The above culturing system was able to evaluate whether the aberrant gene expression within certain samples correlates with the expression profile of CSC markers. In addition, we used a 3D-spheroid invasion assay to allow SC^+ from eutopic and ectopic endometrial cells to migrate within the extracellular matrix in the presence of chemotherapeutic agents such as paclitaxel to evaluate chemo-resistance *in vitro* (Paper I). Furthermore, we challenged endometriotic SC^+ with TGF-β and/or transient gene silencing of SDC-1 or SDC-4 and evaluated for their invasive behaviour using an *in vitro* 3D-invasion assay, 3D spheroid formation assays and transcriptomic analysis.

3.7. TRANSCRIPTOMIC SEQUENCING AND ANALYSIS

We performed transcriptomic analysis to evaluate differentially expressed (DE) genes from invaded 3D-spheroids that were transiently silenced for genes SDC-1 or SDC-4 in combination with or without rhTGF-β (Paper III). In addition, transcriptomic profiles were assessed on endometrial tissues that were obtained before and after bromocriptine treatment in women with adenomyosis (Paper IV). Since it was required to measure transcriptome from a few thousand cells, we used a SMART-seq2 protocol for library preparation in both Studies III and IV (preparations were made in accordance with (Picelli *et al*. 2014)); the protocol has the sensitivity to detect signals from single cells. However, it is very selective for poly-adenylated (poly-A+) RNA; hence, information on poly-A- RNA was not detected in the analysis. Furthermore, the above protocol lacks strand specificity of mRNAs, implying that the information on copy number variability between differentially regulated genes might be lost. The Partek analysis tool (Partek Inc., CA) was used to screen for differentially expressed genes and annotate those gene expression patterns towards specific pathways involved in disease development.

4. RESULTS AND DISCUSSION

4.1. PAPER I

In this study, we hypothesised that there might be aberrant activation or silencing of genes relevant to pathways in cancer within stem/stromal cells of endometrioma, which may have a potential link with the onset/development of EAOC.

4.1.1. Eutopic and ectopic SC^+ did not express ovarian CSC markers

We evaluated the gene deregulations between endometrium and endometrioma using two independent cohorts, namely, the SC^+ cohort and the Tissue cohort, which involve sorted stem/stromal cells and whole tissues, respectively. We first observed that the sorted SC^+ samples from both healthy and endometriosis (eutopic endometrium and ectopic endometrioma) samples in the SC^- cohort exhibited enrichment of the suggested endometrial stem/stromal markers (W5C5/SUSD2$^+$ and MCAM/CD146$^+$), expressed significantly high levels of pluripotent markers (*OCT3/4*, *SOX2* and *NANOG*) and successfully differentiated into all three mesenchymal lineages (adipocytes, osteocytes and chondrocytes) in comparison with negatively sorted SC^-, hence providing substantial pieces of evidence for its multipotentiality and stem cell phenotype.

Moreover, we compared the above endometriosis and healthy control groups for expression of CSC-associated markers (ALDH1$^+$ (Ginestier *et al*. 2007; Penumatsa *et al*. 2010; Silva *et al*. 2011), CD133$^+$ (Curley *et al*. 2009; Kusumbe *et al*. 2009; Silva *et al*. 2011) and CD44v6$^+$ (Gun *et al*. 2012; Shi *et al*. 2013)), which were previously characterised for their aberrant expression in ovarian cancer. As expected, CSC markers showed no significant group-wise differences between the healthy and patient sample groups, when evaluated for marker expression individually or in combination with other markers, using both real-time PCR and flow cytometry, respectively.

4.1.2. A subset of endometriomas aberrantly expressed stem- and cancer-related genes

Interestingly, one endometrioma SC^+ (#36) showed an exceptionally aberrant increase in gene expression for dedifferentiation markers (*SOX2, NANOG*), hypoxia-induced oxidative stress markers (*HIF1α*) and higher percentage in co-expression of CD44$^+$CD133$^+$ by flow cytometry; it also exhibited increased colocalization of CD44v6 and CD133 with immunofluorescent imaging analysis in comparison to all other endometrioma, eutopic as well as healthy

endometrial SC^+ samples (Paper I, Fig. 3B–D). Furthermore, gene expression datasets from the targeted PCR array were analysed for intra-patient heterogeneity using hierarchical heatmap clustering and PCA. A few samples of endometrioma (such as patient nos. #36 and #24 in the SC cohort as well as E238.3 in the tissue cohort, hereafter referred to as Endo-hi) specifically showed higher molecular heterogeneity compared to other endometrioma samples (referred to as Endo-lo) such as upregulation of cancer-associated genes (*E-cadherin*, *EPAS1* and *KIT)* and downregulation of tumour suppressor genes (*PTEN* and *ARID1A* which were incidentally mutated in EAOC cases (Ayhan *et al*. 2012; Govatati *et al*. 2014)). Previously, the loss of BAF250a expression (the protein encoded by *ARID1A*) was correlated with the early onset of EAOC (Xiao *et al.* 2012). Considering a lower expression pattern for *ARID1A* specifically in Endo-hi samples, it would be beneficial to measure the encoded BAF250a protein levels on such unique samples in the future. Moreover, Endo-hi samples from both cohorts exclusively expressed higher levels of the ER-β-to-ER-α ratio (Lazennec 2006; Pujol *et al*. 1998), indicating a molecular shift to compensate for the observed aberrant expression phenotype among cancer-driven genes. Incidentally, we also observed high a *E-cadherin* to *N-cadherin* ratio specifically among Endo-hi tissue (E238.3) compared to the remaining low-expression samples in the Tissue cohort.

4.1.3. An aberrantly expressed endometriotic SC^+ exhibited chemo-resistance

We were further interested in understanding the physiological relevance of the above premalignant phenotype and correlating it with its cancer initiation capacity and chemo-resistance. Hence, we examined the above samples from the SC^+ cohort for its invasion potential in the presence and absence of 10 μM paclitaxel treatment using a 3D-spheroid invasion system. Paclitaxel, a chemotherapeutic drug, is currently used as a first-line chemotherapy regimen for ovarian cancer (Ledermann 2018). As expected, the endometrioma SC^+ group showed a significant decrease in the *in vitro* invasion area upon treatment with 10nM paclitaxel (1.33 μm2 vs. 2.47 μm2, $P<0.05$; Fig. 6) compared to no-treatment cells when cultured for an extended duration within a 3D-spheroid invasion assay. Interestingly, one of the Endo-hi samples (#36) in the paclitaxel-treated group exclusively exhibited higher *in vitro* invasion potential compared to other samples, indicating the possibility of the existence of cancer stem-like cells in such rare endometrioma. However, *in vivo* tumour formation capacity needs to be explored before further confirming the existence of CSC in such unique samples. From the above findings, we speculate there might be a very rare quiescent population with premalignant and reprogrammed characteristics, attributed to cancer initiation for samples such

as #36 (and to a lesser extent, #24), which might be clonally expanded upon activation by treatment with a high dose of chemotherapy drugs for prolonged periods of culture. Further investigation on such rare patient characteristics could potentially result in a better understanding of EAOC.

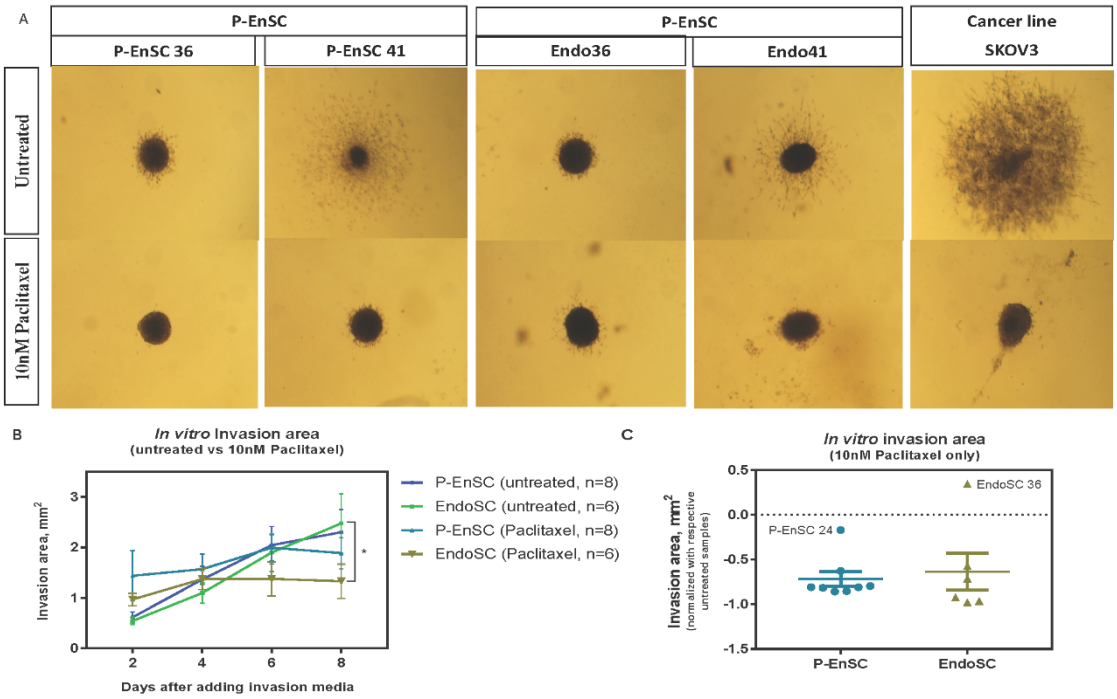

Fig 6. Functional assessment of chemo sensitivity to paclitaxel treatment among high- and low-expression variability samples in an endometriosis SC cohort using *in vitro* 3D-spheroid invasion assay. (A) Representative images of 3D spheroids, cultured in an extracellular matrix along with invasion media, chemoattractant MCP-1, in the presence or absence of 10 nM paclitaxel, and evaluated for its *in vitro* invasion capacity using 3D-spheroid invasion assay. B) Trend curves representing the invasion area of 3D-spheroids in the absence or presence of 10 nM paclitaxel treatment (day 0–8), measured using ImageJ software, between patient samples (i) P-EnSC and (ii) EndoSC (n=8 both). (C) Scatter plot shows the increase in invasion fold area measured at the end of 10 nM paclitaxel treatment (day 8) for individual samples within each patient subgroup, normalised against respective untreated samples (dotted line = baseline). Symbols '*' P<0.05.

4.1.4. An aberrantly expressed endometriotic tissue had previous incidence of non-gynaecological cancers

In the Tissue cohort, the E238.3 sample was separated as an outlier (CI>95%) as it showed a unique higher variability trend among 22/25 analysed genes. Having identified some unique molecular profiles for that patient, we looked into the medical records of patients in the Tissue cohort for the risk factors associated with the disease such as stage of endometriosis, infertility, recurrent endometriosis, smoking and gynaecological/non-gynaecological comorbidities. Surprisingly, we found that patient E238.3 had been diagnosed with stage IV recurrent endometriosis at the time of surgery and had a prior history of melanoma. Though it is of a non-gynaecological cancer type, there are a few convincing epidemiological and clinical

reports on an association between endometriosis and the risk of melanoma (Kvaskoff *et al.* 2009; Kvaskoff *et al.* 2007); however, the molecular link between them is not known.

4.2. PAPER II

In this follow-up study, we intended to use the validated panel of molecular markers from Study I to identify patient samples with higher molecular heterogeneity and explore a potential treatment strategy to reverse their invasive behaviour *in vitro*.

4.2.1. A subgroup of endometriomas aberrantly expressed syndecans and molecules of TGF-β signalling

First, we included patient #36 from the previous study as we wanted to correlate whether the premalignant characteristics observed in Paper I could be further established with *in vitro* functional analysis. We performed gene expression clustering analysis on a new cohort of endometriosis samples (15 endometriotic samples, including sample #36) using 20 genes in a targeted PCR array. Two of the 15 samples (patient #36 (included from the previous study) and #23, Endo-hi) were clustered outside the ellipse in a PCA plot, implying variability with more than 95% CI. Eleven of the 15 samples (Endo-lo) had homogenous gene expression patterns. The remaining two samples (#44 and #18) showed a certain level of gene expression variability; however, it was non-significant (Fig. 1B Paper II). The two Endo-hi, when compared to the remaining samples, showed aberrant expression of molecules involved in TGF-β signalling such as *TGF-β1*, *ESR1*, *CTNNB1*, *SNAI1* and *BMI1*. Incidentally, also in Paper I, we observed higher mRNA levels of TGF-β1 and molecules of TGF-β signalling (*BMI1* and *CTNNB1*) among high variability samples.

Previous studies have shown that higher TGF-β1 levels were secreted in peritoneal fluid (Oosterlynck *et al*. 1994; Pizzo *et al*. 2002; Young *et al*. 2014) and peritoneal and endometriotic lesions (Akl *et al*. 2015; Tamura *et al*. 1999); they were also associated with increased severity of endometriosis (Young *et al*. 2014; Pizzo *et al*. 2002; Oosterlynck *et al*. 1994) as well as an increase its metastatic potential in the ovary (Lamouille *et al*. 2014; Bierie and Moses 2006). On a similar note, heparan sulphate proteoglycan coreceptors SDC-1 and SDC-4 (Chelariu-Raicu *et al*. 2016) have been reported to be upregulated in endometriotic cells and their deregulation was reported in several solid tumours (Akl *et al*. 2015; Chen *et al*. 2018). In our cohort, we observed higher expression levels of SDC-4 and SDC-1 for Endo-hi compared to Endo-lo samples. Thus, by linking both these lines of thought, we explored their interactions

in the context of the pathophysiology of endometriosis and the potential risk for developing EAOC.

4.2.2. In vitro activation of TGF-β signalling inhibited endometriotic cell growth and invasion

We showed that activation of TGF-β signalling by treatment with 2 ng/ml rhTGF-β1 reduced the *in vitro* proliferation and invasion potential in endometriotic cell line 12Z as well as patient-derived endometriotic SC^+. Alternatively, treatment with an equivalent dose (0.06 μM) of TGFRBI/II inhibitor Ly2109761 displayed increased invasion potential, thus confirming a tumour-suppressive role for TGF-β towards the pathogenesis of endometriosis (Paper II, Fig. 2). However, we did not observe any difference between the selected low (2 ng/ml) or high (250 ng/ml) rhTGF-β1 doses with respect to the key transcription factor of TGF-β signalling (*SNAI*1), protein levels of pSMAD3 as well as *in vitro* proliferation or invasion potential of endometriotic SC^+ spheroids. It might be possible that the above non-significant increase upon *in vitro* rhTGF-β1 treatment might be due to the fact that endometriotic samples recruited for this study were already diagnosed with a severe stage of endometriosis (ASRM stage -3 or -4) and might therefore be prone to higher TGF-β1 levels in their peritoneal fluid and hence they were already primed to a mesenchymal phenotype via epithelial mesenchymal transition (EMT).

4.2.3. A subset of endometriotic SC^+ exhibited resistance to TGF-β induced growth inhibition

Activation of TGF-β signalling by rhTGF- β1 on 12Z endometriotic SC^+ showed significant upregulation of SDC-1 and downregulation of SDC-4 at both the mRNA and protein levels. We also observed the reversal of the above effect upon its inhibition with Ly2109761. Similar to the above response, patient-derived Endo-hi SC^+ (samples #36 and #23) exclusively showed upregulation of SDC-1 compared to their respective controls, while no such difference was observed with Endo-lo samples. Likewise, Endo-hi samples also exhibited higher invasion potential upon activation of TGF-β signalling. Knowing that the aberrant expression of SDC-1 was reported in several solid tumours (Akl *et al*. 2015), we hypothesised the invasion potential of Endo-hi SC^+ might be regulated by high SDC-1 expression and lead to TGF-β induced tumour suppression.

4.2.4. Transient knockdown of SDC-1 and SDC-4 reversed the premalignant characteristics during active TGF-β signalling

In line with that hypothesis, transient gene knockdown of both SDC-1 and SDC-4 reduced downstream targets of TGF- β signalling (*SNAI1* and pSMAD3) at both the mRNA and the protein levels. We investigated whether reducing the expression of SDC-1 and SDC-4 in the presence and the absence of active TGF-β signalling had an impact on premalignant characteristics of endometriotic SC^+. Interestingly, the resistance towards TGF-β-induced tumour suppression, shown previously by Endo-hi SC^+ spheroids, was reversed to a significant level only upon SDC-1 inhibition. Moreover, transcriptomic signatures from the above invaded spheroids (gene silenced for SDC-1 together with activated TGF-β signalling) showed downregulation of several cancer-associated growth signalling pathways (Paper II, Table I). Thus, we found a direct relationship between SDC-1 levels and TGF-β signalling towards regulating premalignant characteristics. Therefore, we postulate that the inhibition of high SDC-1 levels in the presence of active inherent TGF-β signalling might have an onco-protective effect on endometriomas exhibiting high molecular heterogeneity.

From the findings of Studies, I and II, we speculated on the potential mechanisms of EAOC development among women with ovarian endometriosis, in two possible steps. (A) Cells from eutopic endometrial SC^+ may be activated due to dysregulated endometrial niche signals such as aberrant estrogen synthesis and progesterone resistance. Retrograde menstruation occurs as a consequence of uterine hyperperistalsis and other risk factors; this disperses the inherently active endometrial tissues onto the peritoneal cavity as implants/lesions. Later, aberrant estrogen signalling within these lesions stimulates SC^+ to express high levels of markers related to epithelial mesenchymal transition (EMT) and promotes implantation of lesions on the peritoneum, ovary or other sites. (B) Upon exposure to high levels of peritoneal fluid factors or insults from chronic inflammation, ectopic endometriotic SC might develop stochastic somatic mutations within cancer driver genes; these may later cause dedifferentiation and lead to malignant transformation of cells on the ovary.

4.3. PAPER III

In this study, we focused on exploring the mechanism by which the SPRM, MIFE, acts on healthy ovaries from *BRCA1* or *BRCA2* mutated carriers. Currently, mastectomy and salpingo-oophorectomy are considered to be the first line of prophylactic treatment measures to reduce the incidence of breast and ovarian/fallopian cancer (Domchek *et al*. 2010; Kauff *et al*. 2008; Rebbeck *et al*. 2009). Previously, MIFE has been shown to inhibit cancer growth *in vivo* and

in vitro on ovarian cancer cell lines (Goyeneche *et al*. 2007). However, its molecular action on *BRCA1* or *BRCA2* mutated healthy ovaries still needs to be explored. Here, we hypothesised that MIFE may control cell growth on *BRCA1* or *BRCA2* mutated ovaries and might thereby provide an alternative treatment strategy for reducing or delaying ovarian cancer risk.

4.3.1. Heterogeneity in PR levels and their clinical impact

As we know that MIFE binds competitively with PR (Chabbert-Buffet *et al.* 2005), we explored whether the recruited samples possess sufficient levels of PR to have the desired drug action. We isolated the previously characterised mesenchymal stem/stromal markers CD90, CD73 and CD105 and evaluated for the expression of PR and *OCT3/4* between SC^+ and SC^- from *BRCA1* or *BRCA2* mutated ovarian cells. As we expected, SC^+ showed significantly higher *OCT3/4* expression compared to SC^- while PR showed a non-significant trend, indicating the relevance of this study for multipotent stem/stromal cells. Interestingly, five out of nine patients showed higher PR expression compared to the remaining samples, and the expression of the latter samples was comparatively similar to their respective sorted negative fractions SC^- (Fig. 1E). The above observation allowed us to question whether the higher PR levels may have a potential cancer risk. Hence, we performed a principle component analysis to correlate the heterogeneity in PR levels with their respective clinical characteristics. We categorised them into high- and low-PR expression subgroups based on the above differences and observed three distinctly separate clusters. *Cluster 1* had two high-PR expressed samples, B57 and B28, marked in blue spots. *Cluster 2* had one low-PR and one high-PR expressed sample, B50 and B54 respectively, marked in pink and blue spots respectively. *Cluster 3* had three low-PR expression samples, B53, B45 and B26, marked in pink, and two high PR expression samples, B49 and B15, marked in blue. When analysing these cluster distributions with respect to contributing clinical factors (Figs. 7A–B), Cluster 1 comprised two high-variability PR patients who were previously reported with an incidence of breast cancer and ongoing concomitant hormonal medication, such as tamoxifen. Alternatively, Cluster 3, which has predominately more low-variability PR samples, had no previous cancer history and was not under any hormonal or concomitant medication.

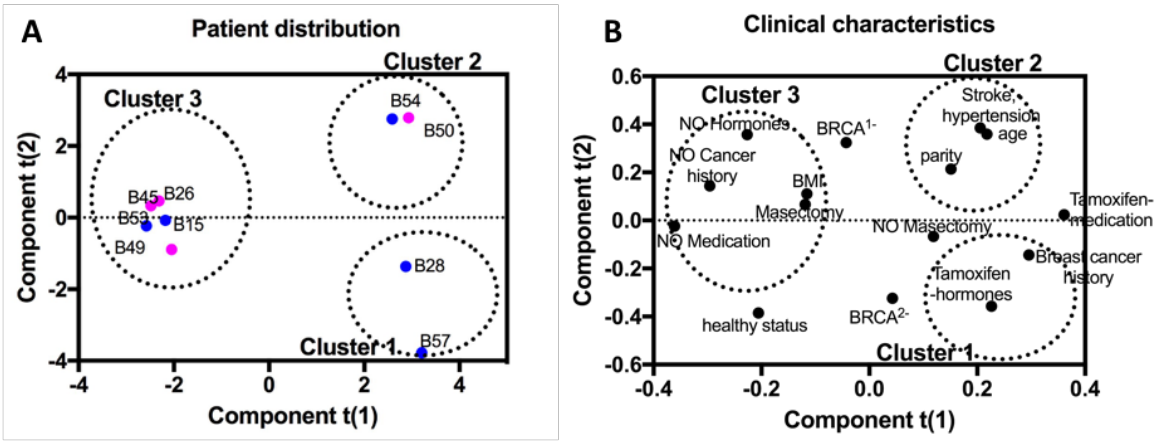

Fig. 7. Correlation of PR levels with patients' clinical characteristics using principle component analysis. The graph shows (A) Clustering of *BRCA1* or *BRCA2* mutated samples. (B) corresponding distribution of clinical characteristics. The data was analysed using SIMCA 14 software (Sartorious Stedim Biotech) and illustrated using Prism 7 (Graphpad Inc.).

4.3.2. Anti-proliferative action for MIFE on *BRCA1 or BRCA2* mutated ovaries

To study MIFE's mechanism of action on *BRCA1* or *BRCA2* mutated healthy ovaries, we challenged those ovarian SC^{+} with increasing doses of MIFE (0.1, 1, 10, 25 and 100 µM) *in vitro.* We observed a dose-dependent anti-proliferative effect, with reduced expression of proliferation marker *ki67* and BrdU as well as an increase in doubling time for cell growth. However, 10 µM was considered an optimal dose compared to 25 µM, as the latter does exhibit activation of BCL2 and a MAPK growth signalling pathway as well as upregulation of tumour suppressor gene TP53. Since our cohort already possesses the *BRCA1* or *BRCA2* mutation that induces genomic instability, the suboptimal concentration of MIFE may further disturb the growth signalling; thus, TP53 were activated to enforce regulation between survival and apoptosis machinery.

4.3.3. Combined MIFE and P treatment reduced proliferation and p21 mediated cell cycle arrest

We performed a combination treatment of MIFE with an agonist of PR and glucocorticoid receptor (GCR), namely, P or hydrocortisone (HC), in order to evaluate MIFE's drug action with inherent P levels. In line with our hypothesis, combination treatment with P and MIFE showed reduced proliferation compared to combination treatment with MIFE and HC. Specifically, the P and MIFE combination treatment lowered the expression of proliferation (*ki67* and BrdU), increased its doubling time and reducing the expression of tumour suppressor genes (*TP53* and PTEN). On the other hand, *BCL2* was upregulated with MIFE and P treatment. It was previously shown that *BCL2* regulates pro-apoptotic or anti-apoptotic factors

based on its interaction with *BAX* or *BCL-XL* respectively (Adams and Cory 2018). Interestingly, we neither detected *BAX* nor cleaved caspase-3 at protein levels. In addition, the gene expression for *BAX* showed no difference for any of the treatments, indicating that MIFE in the presence of inherent P expression does not induce apoptosis. This also allowed us to speculate as to whether *BCL2* in the absence or lower expression of *TP53* may induce cell survival mechanisms, including a transient state of cell cycle arrest. As observed previously with ovarian cancer cell lines (Goyeneche *et al*. 2007), our data also indicated that the combination treatment of MIFE and P on *BRCA1* or *BRCA2* mutated ovarian cells induced cell cycle arrest at the G1/S phase, with upregulation of $p21^{CIP1}$ and corresponding lower levels of CDK2 (a downstream target of p21). In addition, it presented with a higher percentage of pro-apoptotic cells. Finally, we investigated whether the drug action might cause a permanent cell cycle arrest. A kinetic proliferation assay suggested that MIFE induces only a temporary halt in growth signalling until the termination of treatment, implying the need for prolonged usage of this drug to effectively control tumour growth. The postulated mechanism of action for mifepristone from the above observations is presented in Fig. 8.

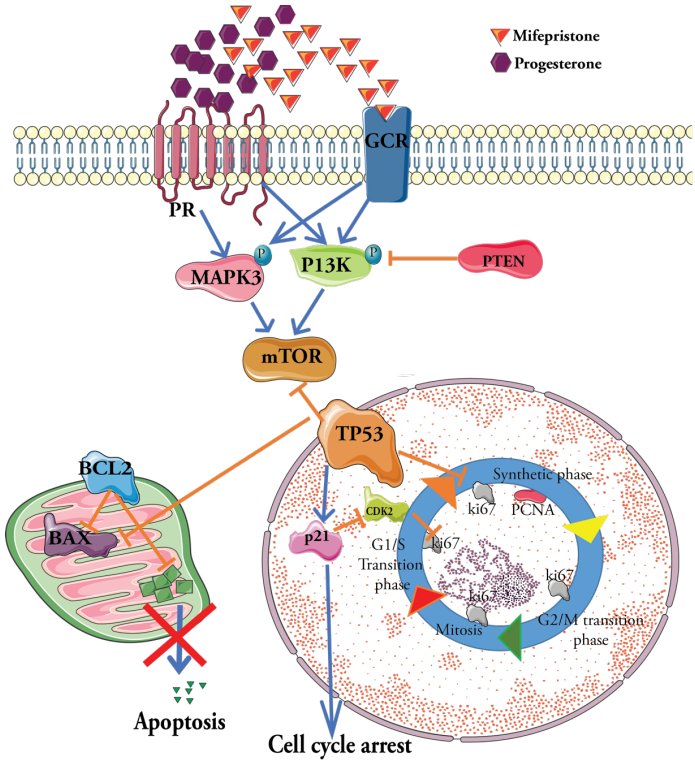

Fig. 8. Postulated molecular mechanism of action for mifepristone among *BRCA*1 or BRCA2 mutation carriers. MIFE acts as a competitive agonist on either PR or GCR (or sometimes both), inducing activation of growth signalling such as MAPK-ERK or PI3K pathway which activate *mTOR* and increases the cellular activity, leading to active cell proliferation. Tumor suppressor gene *TP53* controls the proliferative activity of cells by regulating cell cycle arrest (mediated by p21) or apoptosis (by disrupting *BCL2-BAX* complexes which promotes caspase 3) upon upregulation of growth signalling. This illustration is self-drawn using Adobe Illustrator, with biological arts and shapes obtained from Servier medical art, licenced by Creative Commons 4.0).

Therefore, we confirm that MIFE treatment in the presence of inherent P levels confers a potent anti-proliferative effect via inducing p21 mediated cell cycle arrest. Moreover, an optimal dose of MIFE and other SPRMs could possibly be developed as an alternative preventive strategy to retard tumour cell growth among women with *BRCA1 or BRCA2* mutations.

4.4. PAPER IV

In the fourth study, we explored the mechanism of action for bromocriptine, a dopamine agonist, as a prophylactic treatment strategy for reducing disease symptoms (such as HMB, pelvic pain, etc.) associated with adenomyosis. Adenomyosis, previously known as endometriosis interna, was believed to develop when the endometrium 'invades' the myometrium. Adenomyosis shares several features with other gynaecological disorders (such as endometriosis and uterine fibroids), in terms of symptomology, histology and molecular alterations (Leyendecker *et al.* 2015; Lazzeri *et al.* 2014). Similar to endometriosis, adenomyosis also shows increased proliferative and invasive capacity due to increased estrogen production, progesterone resistance and impaired cytokine expression.

The pathogenesis of this disease is unknown; however, animal studies have shown that the increased prolactin levels (hyperprolactinemia) are regulated by increased levels of estrogen in the uterine cavity (Yamashita *et al*. 1997; Lupicka *et al*. 2017) and the degree of prolactin (PRL) upregulation is correlated with increased severity of the disease (Mori *et al*. 1991; Lupicka *et al*. 2017). Bromocriptine is the gold standard for the treatment of hyperprolactinemia (Kletzky and Vermesh 1989). Administration of bromocriptine vaginally to hyperprolactinaemic women lowered their PRL levels to normal, restored menstrual cyclicity and fertility (Ginsburg *et al.* 1992). Thus, we hypothesised that vaginal administration of bromocriptine may reduce the PRL levels as well as their accompanying disease symptoms in women with adenomyosis.

4.4.1. Bromocriptine treatment reversed tissue injury and reduce cell activity

Adenomyosis tissues were previously shown to undergo repeated tissue injury via the TIAR mechanism and healing processes, resulting in TGF-β-induced collagen deposition and fibrogenesis (Leyendecker *et al*. 2009; Liu *et al*. 2016). Our results indicated that the bromocriptine treatment inhibits fibrosis by downregulating molecules of collagen biosynthesis compared to non-treated samples. Moreover, mitochondrial genes involved in oxidative phosphorylation and active ATP production were downregulated in bromocriptine-treated samples; this was a reversal of the previous report suggesting increased oxidative phosphorylation in women with adenomyosis. Thus, we believe that bromocriptine provides a

favourable outcome by overcoming tissue injury and modulating the overall cellular activity within the endometrium of women with adenomyosis.

4.4.2. Patients with good response to bleeding outcomes exhibited low expression of proliferation markers

We correlated the molecular changes induced by bromocriptine treatment with the bleeding outcomes, measured using a pictorial blood loss assessment chart (PBLAC). As we expected, most of the patients in the cohort had reduced bleeding symptoms; two patients, referred to as good responders, performed exceptionally well with a PBLAC reduction score of more than 50%. We further explored the unique molecular characteristics of those two cases compared to the rest of the samples in the cohort. It was surprising to note that the treated samples did not show any significant pathway alteration between good responders and the rest of the samples. However, when we compared the untreated baseline samples, good responders showed downregulation of molecules involved in apoptosis, cell cycle, mismatch repair pathways and hedgehog signalling. Specifically, *ki67* and *BCL2* showed lower expression within those pathways. However, the previous report suggested that adenomyosis patients possess a higher expression of endometrial *BCL2* during the proliferative phase (Jones *et al.* 1998). Considering that the samples obtained in the study were from the proliferative phase, we suggest the outcome of the treatment was determined based on its inherent cellular phenotype. Moreover, *BCL2* negatively regulates the expression of *BAX* and promotes apoptosis. It was suggested that *BAX* expression is relatively absent among adenomyosis patients with regard to increased *BCL2* expression (Huang *et al.* 2003). However, in our cohort, we observed a closer non-significant trend with upregulation of *BAX* and downregulation of *ki67* for bromocriptine-treated samples, compared to the paired non-treated samples.

4.4.3. PRL expression correlated positively with *ki67* and *BAX*

Previously, it was shown that engraftment of a portion of pituitary gland (which produces high levels of prolactin) within mouse uteri upregulates a 2–10-fold increase of prolactin receptor (PRL-R) and prolactin (PRL) mRNA levels and is associated with pathogenesis of adenomyosis (Yamashita *et al.* 1997). Correspondingly, bromocriptine was shown to reduce hyperprolactinemia (Kletzky and Vermesh 1989). Unfortunately, neither receptor showed any significant reduction upon treatment with bromocriptine compared to non-exposed tissues. Nevertheless, we found a correlation between PRL-R and PRL in relation to molecules regulating cell growth (such as *ki67* and *BAX*). As previously observed, only the baseline treatment showed a positive correlation for *ki67* and *BAX* with PRL. On the other hand, no

correlation was observed in relation to PRL-R either at baseline or at six months of bromocriptine treatment. To confirm whether there was a reduction in PRL secretion levels as a consequence of bromocriptine treatment, it might be worth investigating their levels in the uterine fluid of women who have undergone the above treatment. In addition, we postulate that the bromocriptine induces growth inhibition via a non-*PRL-R* mediated mechanism, which requires further exploration.

Therefore, we confirm that the vaginal administration of bromocriptine induced potent endometrial growth inhibition accompanied by a reversal of tissue injury. In addition, it overcame HMB, pelvic pain and other factors related to the pathogenesis of adenomyosis.

5. CONCLUSIONS

To summarise, this thesis work has shown the importance of understanding the early molecular alterations associated with malignant transformation in the ovary. This emphasises the need for early screening and establishes the possibility of development of some suitable prophylactic or preventive measures. Though the incidence of cancer is rare among individuals with endometriosis (which are usually benign), it is worth being aware of the possible warning signs and available treatment options to reduce any potential risk.

In Study I, we were able to show that the customised panel of markers was sufficient to distinguish molecular heterogeneity among endometriomas. The identified small subgroup exhibited aberrant levels of stem- and cancer-cell-related gene signatures (*KIT, HIF2α, E-cadherin*) dysregulated ER signalling and downregulation of key tumour suppressor genes (*PTEN, ARID1A*).

In Study II, functional analysis of another cohort of ovarian endometriosis enabled us to confirm that the subgroups of patients with aberrantly expressed biomarkers have anomalous behaviour/development, which can be reversed by modulating either SDC-1 or SDC-4 during active TGF-β signalling. With regard to the pathogenesis of the disease, we revealed that the presence of high levels of TGF-β may have an impact on controlling endometriotic cell growth as well as reducing their premalignant potential *in vitro.*

In Study III, we could evaluate the tumour-suppressive role of the SPRM, mifepristone, in controlling *BRCA1* or *BRCA2* mutated ovarian cell growth; this may pose an alternative treatment for reducing ovarian cancer risk as well as avoid/delay risk-reducing salpingo-oophorectomy.

In Study IV, we demonstrated the mechanism of action for bromocriptine in the first human clinical trial for the management of adenomyosis. With regard to reduced bleeding symptoms, bromocriptine provided PRL-mediated potent growth inhibition, reduced cellular activity and reversal of fibrosis demonstrating a potential role in the treatment of adenomyosis.

6. STUDY LIMITATIONS

Studies I and II

- From both the studies, 5/44 (11%) endometrioma samples showed aberrant molecular profiles for stem- and cancer-associated genes and support the potential risk of developing ovarian cancer. Considering the rare occurrence of EAOC (1–2.5%) among women with ovarian endometriosis, the above findings seems to be a bit of an overestimation compared to the actual occurrence of cancer.

- We have adopted a targeted panel of preselected genes to identify samples with higher molecular heterogeneity among women with ovarian endometriosis. This approach might have missed several other differentially regulated genes with its key role in cancer development (for instance *CXCR4*, which was not in our preselected panel of genes, but later identified by transcriptomic analysis on 3D-invasion spheroids in Study II).

- We have identified highly altered gene signatures among certain endometriomas in all three ovarian endometriosis cohorts, performed certain functional analyses (3D spheroids, invasion assays, chemo-resistance, etc.) and correlated their expression patterns with the clinical history of patients to speculate on their potential risk of developing EAOC. However, we may not be able to confirm whether those identified potential risk cases might really develop cancer later in life until a prospective evaluation can be done.

- In Study I, the high expression of CSC-associated makers and chemo-resistance capacity were exclusively observed in one endometrioma sample (#36) in the SC cohort. Incidentally, the same sample also showed reversal of premalignant characteristics upon inhibition of SDC-1 or SDC-4 during active TGF-ß signalling (Paper I). However, the above key observation requires further validation using animal studies focussing on the existence of CSCs as well as possibilities for reducing their tumour initiating capacity *in vivo*.

Study III

- This study involved healthy volunteers with *BRCA1 or BRCA2* mutations. However, it lacked the appropriate controls such as healthy volunteers with a non-mutated *BRCA* gene. Hence, we were not able to assess whether the observed cell cycle arrest was due to the impact of an error-prone DNA-damaged pathway (because of the *BRCA1 or BRCA2* mutations) or the result of a mifepristone-mediated anti-proliferative effect.

- We included patients with varied clinical characteristics (such as two patients with tamoxifen treatment (due to prior incidence of breast cancer), two patients with *BRCA2* and the remaining five patients with *BRCA1* mutations as well as differences in their menopausal status), which may have contributed to differential PR expression and exhibited variability in their anti-proliferative effect.

Study IV

- The small size of the study population (only eight samples out of 18 recruited patients) were included due to occasional patient dropouts from six months' vaginal bromocriptine treatment, poor quality of extracted RNA and stringent inclusion criteria. As a consequence, it was challenging to draw potential conclusions from the study.
- There was a lot of variability with both PRL and PRL-R expression and there is no clear evidence exhibiting reduced expression of the above markers after bromocriptine treatment. Hence, further validation will be needed on the above markers at the protein level, typically using immunohistochemistry or uterine fluid to evaluate PRL levels at baseline and after six months of treatment.

7. FUTURE DIRECTIONS

For the future evaluation of early diagnostic markers towards the onset or development of EAOC, the biomarkers developed from Studies I and II should be validated. This can be achieved using archived samples from women diagnosed with ovarian endometriosis who later developed clear-cell or endometrioid subtypes of ovarian cancer and comparing the samples with a cohort of women (controls) who had ovarian endometriosis without developing ovarian cancer.

One endometrioma SC^+, Endo36, which was used in both Study I and Study II, showed an aberrant molecular profile with >95% CI compared to other samples. It expressed high levels of CSC markers $CD44^+$ $CD133^+$ (both by flow cytometry and immunofluorescence) and dedifferentiation markers (*SOX2, NANOG*). Furthermore, it exhibited chemo-resistance and higher 3D-spheroid invasion potential in the presence of paclitaxel treatment (Study I) and showed resistance to TGF-β-induced growth inhibition and upregulation of SDC-I. However, inhibition of SDC-1 during active TGF-β signalling suppressed its premalignant characteristics (Study II). Evaluation of such rare samples is needed to understand the molecular mechanisms involved in the early stages of cancer development.

With respect to *BRCA1 or BRCA2* mutations and ovarian cancer risk, the safety and efficacy of mifepristone after long-term treatment should be further studied and its outcomes correlated with breast and ovarian cell proliferation markers *in vivo*. Moreover, we should explore the molecular alterations that occur within adnexal sites (fallopian tubes, distal fimbriae, etc.) from women who underwent RRSO for preinvasive conditions such as serous tubal intraepithelial carcinoma and/or *BRCA1 or BRCA2* mutation, to identify early biomarkers for high-grade ovarian cancers.

Finally, it would be interesting to investigate the possibility of using bromocriptine within an intrauterine system among women with adenomyosis, in order to evaluate its safety and efficacy for sustained relief from disease symptoms.

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