The role of lipid mediators in the aetiology of endometriosis

A thesis submitted to the University of Manchester for the degree of Doctor of Philosophy in the Faculty of Biology, Medicine and Health

2018

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FREQUENTLY USED ABBREVIATIONS

17βHSD	17β-hydroxysteroid dehydrogenases
AA	arachidonic acid
ABC	avidin-biotin complex
ALA	alfa linolenic acid
AOI	areas of interest
Bcl-2	B-cell lymphoma 2
BMI	body mass index
cDNA	complementary deoxyribonucleic acid
CI	confidence interval
CID	collision-induced dissociation
CK8	cytokeratin 8
COCs	combined oral contraceptives
COX	cyclooxygenase
CPP	chronic pelvic pain
Ct	threshold cycle
CYP	cytochrome 450 epoxygenase
DGLA	dihomo-γ-linolenic acid
DHA	hydroxydocosahexaeonic acid
DHET	dihydroxyeicosatrienoic acids
DIE	deep infiltrating endometriosis
DiHDPA	dihydroxydocosapentaenoic acid
DiHETE	dihydroxyeicosatetraenoic acid
DiHOME	dihydroxyoctadecenoic acid
Ec	ectopic lesion
EET	epoxyeicosatrienoic acid
ELISA	enzyme-linked immunosorbent assay
ELOVL	elongase enzyme
EM	endometriosis
EP	Prostaglandin E receptor
EPA	eicosapentaenoic acid
EpDPE	epoxydocosapentaenoic acid
EpETE	epoxyeicosatetraenoic acid
EpOME	epoxyoctadecenoic acid
ER	oestogen receptor
Eu	eutopic endometrium
FA	fatty acid
FGF-9	fibroblast growth factor-9
FLAP	5-lipoxygenes-activating protein
FN1	fibronectin 1
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
GnRH	Gonadotropin-releasing hormone
GREB1	growth regulation by oestrogen in breast cancer

GWAS	genome-wide association studies
H&E	haematoxylin and eosin staining
HDHA	hydroxydocosahexaeonic acid
HEPE	hydroxyeicosapentaeonic acid
HETE	hydroxyeicosatetraeonic acid
HETrE	hydroxyeicosatrieonic acid
HODE	hydroxyoctadecadienoic acid
HOTrE	hydrooctadecatrienoic acid
HpDHA	hydroperoxydocosahexaenoic acid
HpEPE	hydroperoxyeicosapentaeonic acid
HpETE	hydroperoxyeicosatetraeonic acid
HpETrE	hydroperoxyeicosatrienoic acid
HPLC	high performance liquid chromatography
HpODE	hydroperoxyoctadecadieonic acid
HpOTrE	hydroperoxyoctadecatrienoic acid
HRP	horse radish peroxidase
HRT	hormone replacement therapy
IBD	inflammatory bowel disease
ICAM-1	intercellular adhesion molecule 1
IFITM-1	interferon-induced transmembrane protein 1
IFNγ	interferon gamma
IHC	immunohistochemistry
IL	interleukin
KO	knock out
LA	linoleic acid
LC/ESI-MS/MS	liquid chromatography tandem mass spectrometry
LM	lipid mediator
LOX	lipoxygenase
LT	leukotrienes
LX	lipoxin
m/z	mass-to-charge
M1	M1 macrophage
M2	M2 macrophage
MaR	maresin
MCP-1	monocyte chemotactic protein 1
MMP	matrix metalloproteinases
MRM	multiple reaction monitoring
mRNA	messenger ribonucleic acid
MS	mass spectrometry
NEM	non-endometriosis
NF-κB	nuclear factor kappa B
NK	natural killer cells
oxLDL	oxidised low density lipoprotein
oxoETE	oxoeicosatetraeonic acid
oxoODE	oxohydroxyoctadecadienoic acid
P450arom	aromatase cytochrome P450
PBS	phosphate-buffered saline

PDX	protectin
PF	peritoneal fluid
PFA	paraformaldehyde
PG	prostaglandin
PGD	prostaglandin D
PGE	Prostaglandin E
PGF	prostaglandin F
PGH	prostaglandin H
PGI	prostacyclin I
PL	blood plasma
PLA_2	phospholipase A_2
PNM	polymorphonuclear leukocytes
PPAR	peroxisome proliferator-activated receptor
PPRE	peroxisome proliferator response element
PR	progesterone receptor
PTGS	prostaglandin-endoperoxide synthase
PUFA	polyunsaturated fatty acid
PW	peritoneal washes
Q	quadrupole
qRT-PCR	qualitative Real-Time Polymerase Chain Reaction
RA	rheumatoid arthritis
RANTES	Regulated on Activation, Normal T Cell Expressed and Secreted
RNA	ribonucleic acid
RR	relative risk
Rv	resolvins
RXR	retinoid X receptor
sEH	soluble epoxide hydrolase
SNP	single nucleotide polymorphism
SPE	solid phase extraction
SPMs	specialised pro-resolving mediators
SRM	selected Reaction Monitoring
StAR	steroidogenic acute response protein
TGFβ	transforming growth factor β
Th	helper T cells
TIMP	tissue inhibitor of metalloproteinases
TNFα	tumour necrosis factor alpha
trans-EKODE	trans-epoxy-keto-octadecenoic acid
T_{reg}	regulatory T cells
TSP	thrombosponin
TXA	thromboxane
TZD	thiazolidinedione
VCAM-1	vascular cell adhesion molecule 1
VEGF	vascular endothelial growth factor

The University of Manchester

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Doctor of Philosophy (PhD)

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ABSTRACT

Endometriosis is one of the most common, chronic gynaecological disorders characterised by the histological presence of endometrial-like tissues outside the uterus. The most frequent location of ectopic endometrial lesions is the pelvic cavity causing chronic inflammation, fertility problems and a wide range of pain symptoms. Despite extensive research efforts, reliable diagnostic biomarkers still do not exist and the aetiology and underlying pathophysiology of the disease have not yet been completely elucidated.

Eicosanoids and related hydroxy fatty acids are a rich class of biologically active oxygenated metabolites derived from omega-6 and omega-3 polyunsaturated fatty acids (PUFA). These lipid mediators are produced locally in cells through biosynthetic pathways of cyclooxygenase (COX), lipoxygenase (LOX) and cytochrome P450 (CYP) enzyme pathways as well as in a non-enzymatic manner and regulate an array of physiological and pathological processes. Biological fluid specimens, such as plasma and peritoneal fluid, are a rich source of oxygenated lipid metabolites. So far, only a limited number of lipid mediators have been studied in endometriosis. The main aim of this study was to simultaneously measure 79 oxygenated lipid mediators in plasma and peritoneal fluid specimens obtained from consenting women with and without endometriosis using liquid chromatography coupled to electrospray ionisation tandem mass spectrometry (LC/ESI-MS/MS). Lipid mediator profiles in peritoneal specimens would offer insights into the local pathophysiological processes in the peritoneum and analysis of plasma samples would indicate their systemic effects.

Lipid mediators did not show clear trends in plasma with regard to any biosynthetic pathway from women with endometriosis compared to those without. In contrast to plasma, although significant changes were not detected, the lipid mediator profiles in peritoneal fluid showed apparent alterations. Nearly all tested metabolites were present in decreased concentrations in the peritoneal fluid from women diagnosed with endometriosis. The most prominent reductions were observed for 15-LOX derived metabolites. Since 15-LOX metabolites are endogenous ligands for peroxisome proliferator-activated receptor γ (PPARγ) it was hypothesised that the

low concentration of PPARγ ligands could lead to the disturbed regulation of PPARγ mediated pathways, such as downregulating scavenger receptor CD36 expression in peritoneal macrophages from women with endometriosis.

To extend our knowledge about the role of PPARγ in the pathomechanism of endometriosis, expression of 15-LOX, PPARγ and CD36 was investigated in ectopic lesions, eutopic endometrium, peritoneal cells and peritoneal wall samples from women with endometriosis and in eutopic endometrium and peritoneal cells from women without endometriosis using qRT-PCR and immunohistochemistry methods.

In conclusion, data presented in this thesis did not support the theory that PPARγ mediated processes were disturbed in endometriosis. However, results highlight the role of PPARγ in reducing the rate of proliferation and steroidogenesis of ectopic lesions. In addition this research also points out that there are complex, multilevel relationships within the peritoneal environment. A better understanding of these interactions is necessary to elucidate the aetiology and pathomechanisms of endometriosis to improve management of this unmet clinical need.

Acknowledgement

First and foremost I would like to thank my supervisor Professor Kay Marshall for taking me on as her student and for guiding me through my PhD at the University of Manchester. I would also like to extend my gratitude to my second supervisor Professor Anna Nicolaou for her critical appraisal and scientific input. I cannot have had better supervisors than Kay and Anna. I cannot thank you enough for your support, patience, guidance and also for giving me great amount of professional freedom to develop invaluable scientific skills.

My very special thanks goes to Dr Debbie Fisher for her advice and accurate correction on the writing of this thesis. It would not be the same without your kindness and care. I wish also to extend my heartfelt thanks to Dr Alexandra Kendall, Dr Maria Camacho-Munoz, Dr Magda Kiezel-Tsugunova and Luke Brownbridge for their endless patience and technical support.

A huge thanks to everyone that has played a part in making the past 4 years in the lab enjoyable, in particular to Dr Sara Santorelli, Dr Matthew Rosser, Dr Osman Zarroug, Ammar Brifkani, Kathryn McGurk, Marta Koszyczarek, Megan Uttley, Anggit Listyacahyani, Maryam Shubbar and Adel Alghaith.

This project would not have been possible without the donation of tissues from the Central Manchester University Hospitals. I would like to thank Professor Emma Crosbie, Dr Sarah Kitson, Dr Nikolas Tsampras and Dr Edmond Edi-Osagie for obtaining consent from donors and collecting human tissues on our behalf. I am also grateful to Dr Ian Crocker and Dr Clare Tower who provided plasma samples for this project. In addition, I would like to thank every single woman who donated their samples for this study. In addition, my acknowledgements would not be complete without thanking to the staff at the university's Bioimaging facility who provided the help and support to the histology section.

Last but not least, the most significant thanks of course go to my family, who have always been there to support me when needed, in particular to my Dad and Zsolti. None of this would have been possible without your unconditional love, encouragement and patience.

Finally, I would like to dedicate this thesis to all women who are faced with endometriosis every day and their only choice is to be strong.

DECLARATION

I declare that no portion of the work referred to in the thesis has been submitted in support of an application for another degree or qualification of this or any other university or other institute of learning.

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1 CHAPTER: Introduction and Literature Review

1.1 Overview of Endometriosis

Endometriosis is a common, painful gynaecological condition which affects up to 10 percent of women of reproductive age (Eskenazi and Warner, 1997). The exact prevalence of the disease is difficult to determine due to the lack of non-invasive diagnostic techniques and lack of reliable indicator substances. The aetiology of endometriosis and the underlying pathophysiology are still largely unknown. The pathophysiological processes can cause fertility problems, the pain symptoms reduce the quality of life of patients and the cost of the disease creates a profound economic burden on society (Simoens et al., 2012).

Endometriosis is an oestrogen-dependent disorder defined by the histological presence of endometrial-like glandular and stromal tissue outside the uterine cavity. This extra-uterine tissue, also called ectopic endometrium or an ectopic lesion, has similarities but is not identical to the normal endometrium (Acién and Velasco, 2013). The normal endometrium, also referred as eutopic endometrium, is the inner lining of the uterus. This layer is a dynamic, mucous, glandular tissue which responds to ovarian stimulation and undergoes repeated cycles of growth throughout the reproductive life of women from menarche to menopause. The ectopic lesions, also response to exogenous or endogenous hormonal stimuli and go through similar periodic changes and this causes bleeding into the pelvic cavity or their locale (Katorza et al., 2007). The stasis of this blood enhances the local inflammatory process and can cause pain, scarring and adhesion formation which leads to anatomical disruption of the affected organ (Martin et al., 2002). The endometrial implants are found most frequently in the peritoneum, but lesions have also been reported rarely in extra-pelvic organs for example lungs, brain, skin, and external genitalia (Amer, 2008). Figure 1.1.1 shows the female reproductive system and the frequent location of endometriosis in the peritoneal cavity.

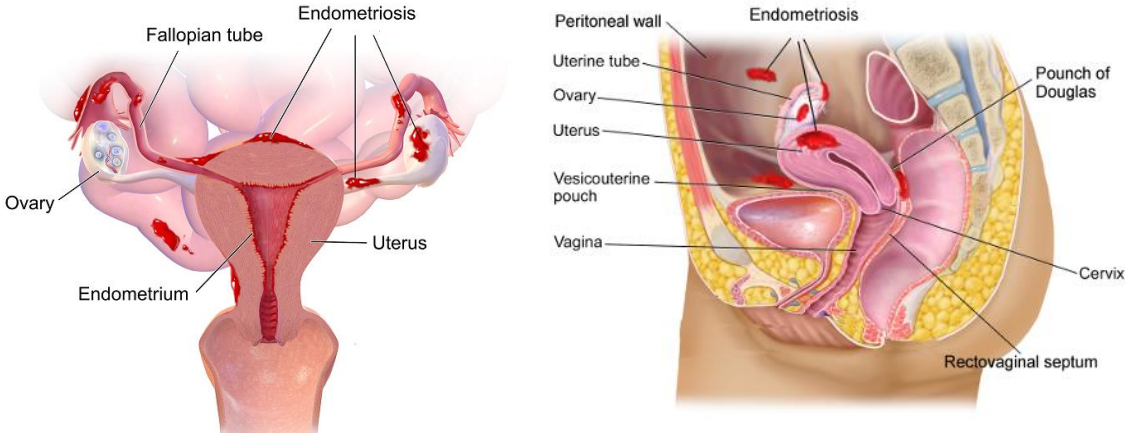


Figure 1.1.1. Female reproductive system and the frequent sites of endometriosis. (Adapted from https://commons.wikimedia.org)

Endometriosis encompasses a wide variety of symptoms. The main presenting symptoms are chronic pelvic pain, dysmenorrhoea (painful menstruation), dyspareunia (painful sexual intercourse) and infertility. The most debilitating symptom is the pelvic pain (Sinaii, 2002). In most cases, pelvic pain is associated with menstruation but it can also be independent of menses. The degree of the pain does not always relate to the seriousness and extent of the disease and can cause a variety of symptoms (Acién and Velasco, 2013). Patients frequently complain of headache, chronic exhaustion and urinary or gastro-intestinal symptoms (Ballweg, 2004).

Endometriosis affects women of reproductive age; thus, the first symptoms occur after the menarche and generally disappear after the menopause. However, studies have reported pelvic pain caused by endometriosis in girls before menarche (Gogacz et al., 2012) and likewise ectopic lesions rarely could reactivate after menopause in women with the disease who use oestrogen only hormone replacement therapy (HRT) (Oxholm et al., 2007).

1.2 Pathophysiology

1.2.1 Histology of endometrium

The human uterus is of mesodermal origin and develops from the Müllerian duct during the embryogenesis. It consists three main histological layers, namely perimetrium, myometrium and endometrium. Perimetrium is the serous layer of visceral peritoneum and lining the outer surface of the uterus. Myometrium is the muscular layer of the uterus consisting mostly of smooth muscle. Endometrium is the inner, mucosal epithelial layer of the uterus. Endometrium is composed of two main cell types the epithelial and stromal cells. The latter cell type is also referred to as mesenchymal cells. The epithelial cells line the uterine cavity and form the glands of the uterus. The stromal cells are a type of connective tissue which support the function of glands. Both cell types are hormone sensitive and respond to the release of ovarian hormones by undergoing periodic changes during the menstrual cycle (Figure 1.2.1A).

1.2.2 Histology of ectopic lesions

The clinical definition of endometriosis characterises the disease in the presence of endometrial-like gland and stroma outside the cavity of uterus. Indeed, in that case when both components are present the confirmation of diagnosis is straightforward. However, the histological appearance of endometriosis can vary widely. In 2003, Abrao *et al.* evaluated 412 biopsies from 241 endometriosis patients. They found that

only 165 out of 421 (40%) of the specimens were well-differentiated and showed the classical endometriotic histology. In the case of 113 (27%) of the samples the glands were sparse or even absent therefore this pattern was classified as stromal endometriosis. Ninety five (23%) out of the total number of sections showed a mixed differentiated pattern. In these cases the well-differentiated endometrial-like cells were present along with undifferentiated epithelial cells. The morphology of the undifferentiated cells resembled the mesothelial lining rather than endometrial epithelium. In 10% (39 out of 412) of the specimens undifferentiated glandular pattern were present (Abrao et al., 2003). Clement (2007) in his review also pointed out that the histology of ectopic lesions is widely divergent and the gland or the stromal components could be absent. The appearance of the glands depend on the actual hormone state and metaplastic changes, whilst the stromal component is frequently infiltrated by fibroids, smooth muscle metaplasia or altered by decidual changes (Fox, 1983). Figure 1.2.1B shows a micrograph of an ectopic lesion.

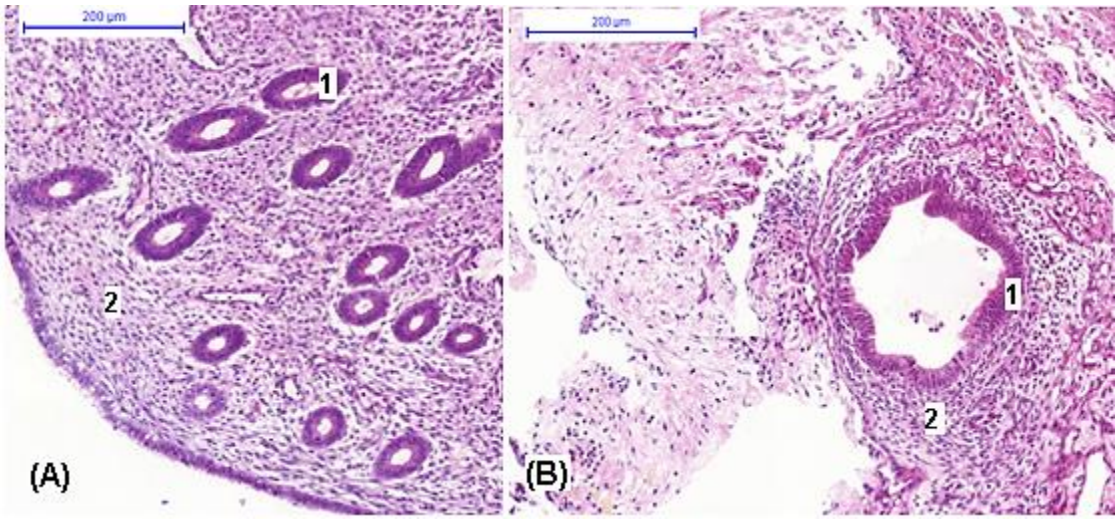


Figure 1.2.1. Histological comparison of eutopic endometrium and ectopic lesion.
Typical cell types can be seen on both micrographs. (A) Eutopic endometrium of patient HP32, H&E stain. (B) Ectopic lesion from patient HP34, H&E stain.
1. Endometrial glands; 2. Endometrial stroma

1.2.3 Morphology of the lesions

The macroscopic appearance of endometrial lesions is also diverse and complex. They are classified into two groups: classical implants and atypical lesions. The classical implants are blue or black in colour and show powder-like burn patterns (Figure 1.2.2.A). They are a combination of glandular and stromal tissues with entrapped menstrual debris with white scar margins around the lesions (Donnez and Van Langendonckt, 2004). Atypical or subtle lesions are variable in colour. They can be red, pink, clear, yellow-brown and white. The red flame-like lesions have an extensive vascular network with increased vascularised stroma and sub-peritoneal area. The

clear, vesicular form is caused by fluid accumulation between the surface of the implant and the overlying peritoneum. The white and yellow-brown subtle patches have a cystic glandular structures with accumulation of secretory products. The healed implants are also white in colour and appear as white fibrotic scarred areas Figure 1.2.21.2.2 (Martin et al., 2002).

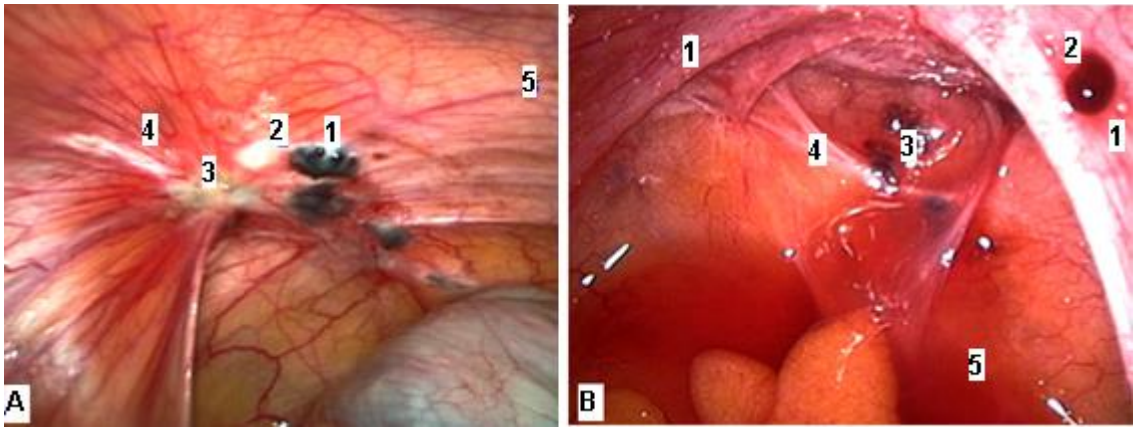


Figure 1.2.2. Phenotypes of ectopic endometrial lesions.
Laparoscopy view of the peritoneum. Different phenotypes of the ectopic lesion can be present at the same time point. A. (1) Classic powder burn lesion with entrapped menstrual debris and white scar margins around the lesions. (2) White lesion. (3) White fibrotic lesion. (4) Extended, highly vascularised area. (5) Yellow-brown hemosiderin decolourization of the peritoneum.
B. (1) Clear vesicles. (2) Red vesicular lesion with red, inflamed surrounded area. (3) Blue lesions. (4) Adhesions. (5) Pouch of Douglas with peritoneal fluid.
(Adapted from https://commons.wikimedia.org)

1.2.4 Evolution of the lesions

Nisolle & Donnez (1997) hypothesised that the lesions change with time and the different morphology correlates with different activity. They suggested the red flame-like lesions are similar to the eutopic endometrium and these represent the first stage of endometriosis. They are active and highly vascularised. The growing and shedding of these lesions induces inflammatory reactions and provokes scarification. The encapsulated lesion is symbolic of the advanced stage, these lesions become blue or black. As the scarification progresses, the vascularization of the lesions reduces and they become latent, white, healed implants (Nisolle and Donnez, 1997).

Khan *et al.* (2014) also proposed a similar evolution of the lesions. They related the colour of the implants to different proliferative stages. They suggest that the initial lesions are the clear, transparent papules with or without serous or mucinous secretin. As the lesions proliferate and new vessels established the colour of the lesions become red due to the overproliferation of microvessels. Then blood accumulates within the implant and it becomes blue before the healing process resulting to turn it into white (Khan et al., 2014).

Although lesion kinetics are hard to study in patients due to the invasiveness of the diagnosis, some demographic data and animal models support this hypothesis. Davis *et al.* in 1993 compared the characteristics of lesions between the adolescent and adult population. They found that the red lesions were the dominant implant type in adolescents, and the classical, fibrous, black lesions were found in adults (Davis et al., 1993). Also, a recent study in a non-human primate model of endometriosis has confirmed that the implants were able to progress and returned after surgical excision. The red lesions were the early stage of the disease and changed their colour from red through blue to white. The time course of this progression depended on the morphology of the lesions (Harirchian et al., 2012). It must be noted that different sub-types of lesions can coexist and the kinetic changes of the lesions are not necessary equivalent to the disease progression. Figure 1.2.21.2.2 illustrates different lesion sub-types in the same patients.

1.2.5 Phenotypes of lesions

Despite the diversity of implants, endometrial lesions can be classified into three major entities as follows: peritoneal, ovarian and deep infiltrating endometriosis (DIE). To date, it is strongly debated whether peritoneal, ovarian and DIE result from the same or different pathological processes. It remains controversial whether the three phenotypes have the same aetiology (Brosens et al., 1993; Brosens and Garry, 2004) or are three different entities with different pathogenesis (Koninckx et al., 1994; Nisolle and Donnez, 1997).

1. Peritoneal endometriosis, also known as superficial endometriosis, is characterised by the endometrial surface epithelium being embedded into the intraepithelial or sub-mesothelial layers of the peritoneal cavity. The atypical or subtle lesions are located on the surface of the peritoneum while the typical, black powder-like lesions infiltrate a few millimetres into the peritoneum (Koninckx et al., 1994). This type of endometriosis is often considered as a dynamic but self-limiting condition (Koninckx, 1994). Figure 1.2.31.2.3 demonstrates the classic histological character of peritoneal lesion.

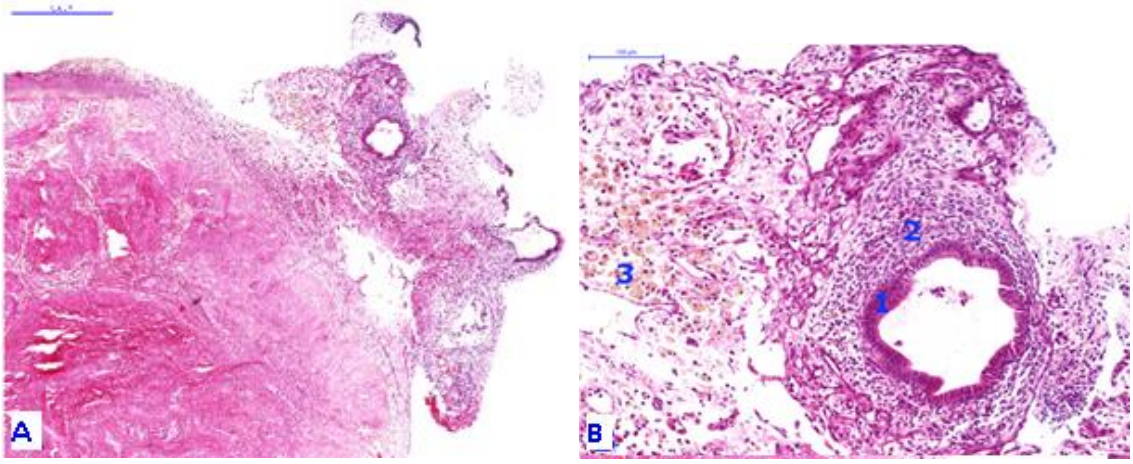


Figure 1.2.3. Peritoneal endometriosis. Histology micrograph of ectopic lesion.
(A) 5x and (B) 20x magnification, H&E stain; Patient HP45. 1. Endometrial glands; 2. Endometrial stroma; (3) hemosiderin-laden

2. Ovarian endometriosis, also referred to as endometrioma or chocolate cysts. This type of endometriosis is frequently accompanied by pelvic adhesions and may take the form of a superficial implant on the surface of the ovary or form haemorrhagic cysts by the invagination of the ovarian cortex (Koninckx et al., 1991). Hughesdon proved that the endometrioma is a pseudocyst, it does not erode the ovary (Hughesdon 1957). The cysts are lined by endometriotic tissue and accumulate menstrual debris. This menstrual bleeding causes the typical chocolate brown colour of the cyst. Figure 1.2.41.2.4 shows the endometrioma in situ and the histology of the cyst with sparse gland and extended stroma.

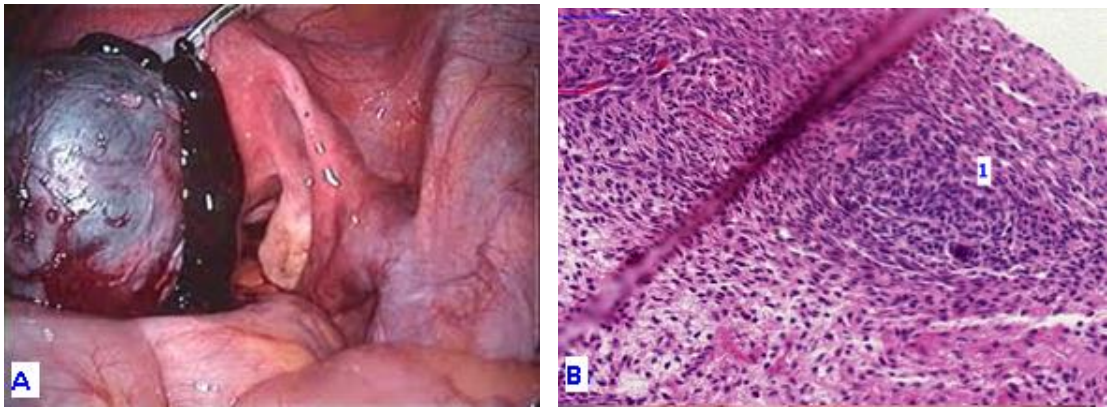


Figure 1.2.4. Ovarian endometriosis.
(A) In situ endoscopic image of a ruptured chocolate cyst in left ovary (https://commons.wikimedia.org). (B) Micrograph of ovarian cyst shows an extended stroma (1); glands cannot be identified. 40x magnification, H&E stain; Patient HP47.

3. Deep infiltrating endometriosis (DIE) can be defined as lesions penetrating deeper than 5 millimetres into the fibromuscular pelvic structures (Koninckx and Martin, 1992). The most frequent locations being the uterosacral ligament, rectovaginal septum and the wall of abdominal organs. Histologically two subtypes can be distinguished. The endometriotic subtype shows the morphology of an ectopic lesion with the presence of glandular and stromal components. While the other subtype can be classified as adenomyosis externa (Koninckx et al., 2012). The characteristics of this lesion are different from ovarian and peritoneal implants. This subtype takes the form of a solid, compact, non-haemorrhagic adenomyotic nodule with proliferative fibrous and smooth muscle cells with sparse endometrial glands and stroma. There is also a lack of evidence of cyclic menstrual shedding. The nodule often infiltrates into the rectovaginal septum causing severe pelvic pain and dyspareunia (Brosens and Brosens, 2000) (Figure 1.2.51.2.5).

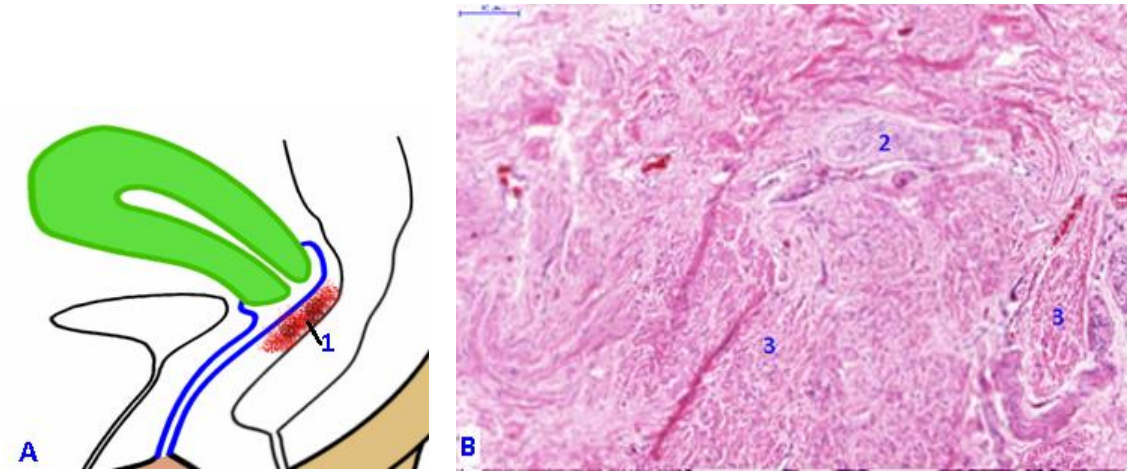


Figure 1.2.5. Deep infiltrating endometriosis (DIE)
(A) Schematic drawing of a typical location of deep infiltrating endometriosis (1) (adapted from https://commons.wikimedia.org). (B) Micrograph of rectovaginal nodular DIE from patient HP53. Extended fibroid (2) and smooth muscle areas (3), glandular and stromal cells cannot be identified. 15x magnification, H&E stain.

1.3 Aetiological Theories

Endometriosis is often referred to as a disease of theories as several theories exist regarding its aetiology. However, none of them are able to explain exclusively all cases. The hypothesis can be classified in three groups based on the origin of cells. For a better overview Figure 1.3.11.3.1 summarizes the theories and indicates the relationships between them, in addition shows the possible induction steps of the pathogenesis.

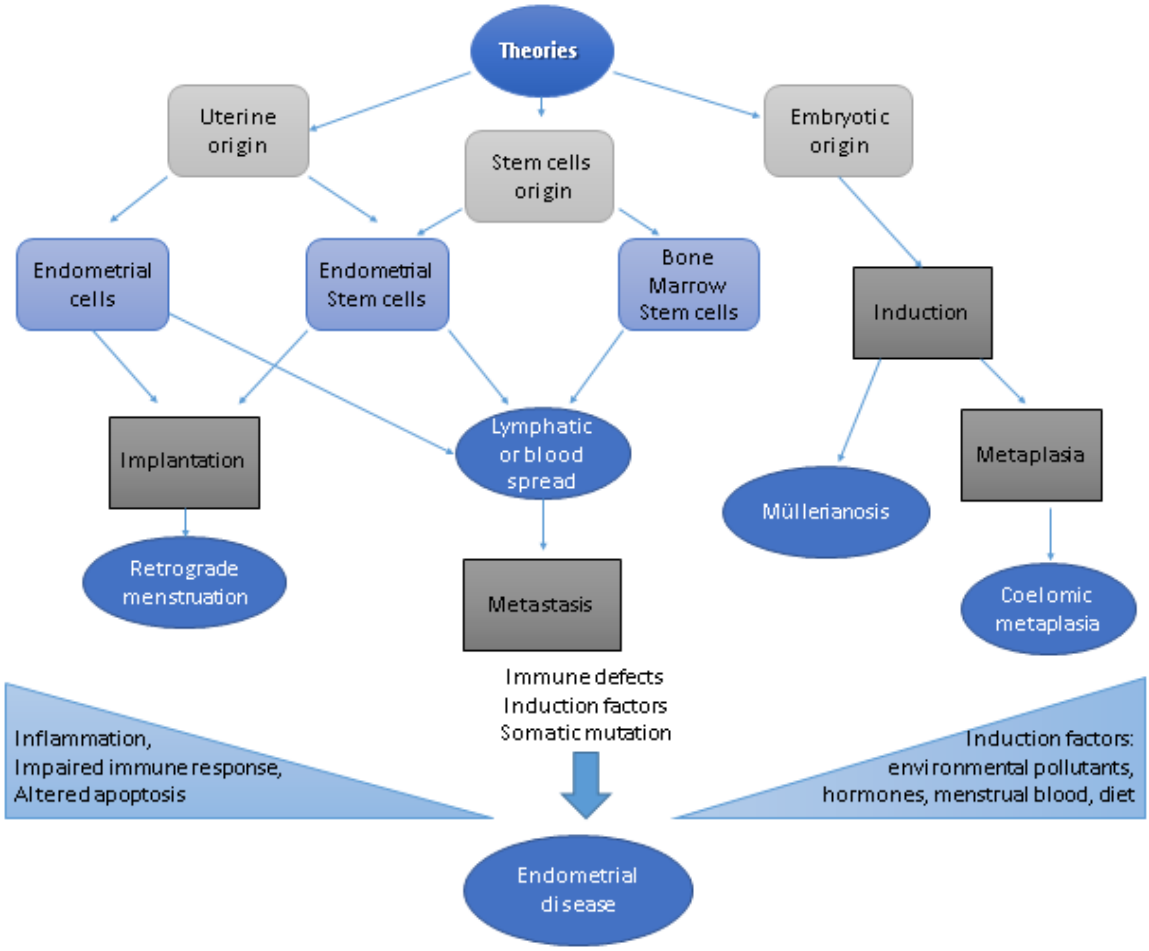


Figure 1.3.1. Overview of the endometriosis theories.
Flow chart illustrates the main theories with the main hypothesised factors involved in the initiation and progression of pathogenesis.

1.3.1 Uterine origin

The most widely accepted theory is that of retrograde menstruation, which originated from Sampson (Sampson, 1927). According to this theory, retrograde menstrual flow transports endometrial fragments through the fallopian tubes into the peritoneal cavity where these cells survive, adhere and implant into the peritoneum and form ectopic endometrial lesions (Sampson, 1927). Several observational and experimental findings support this theory. Retrograde menstruation has been

observed in 90 percent of women (Halme et al., 1984). The ectopic endometrial cells are viable, capable of proliferating in the abdomen, express adhesion molecules on their surface and are able to invade into the surface of peritoneum (Kruitwagen et al., 1991; Spuijbroek et al., 1992; van der Linder et al., 1995). The anatomical distribution of the endometrial lesions also support Sampson's theory, they are more frequent in the posterior and in the recto-vaginal cavity (Jenkins et al., 1986). It is more frequent in adolescents with congenital obstruction, women with abnormal pelvic anatomy and those with heavy, short menstrual cycles (Sanfilippo et al., 1986; Cramer and Missmer, 2002).

Although retrograde menstruation is the most acknowledged theory, it has some flaws. It is a suitable model for peritoneal endometriosis but it fails to explain the extra-peritoneal lesions and it is not able to explain why only 10 percent of women develop the disease whilst retrograde menstruation is a common phenomenon and occurs in nearly all women.

1.3.2 Embryonic origin

Alternative theories propose that endometrial implants do not originate from the uterine endometrium. They assume that the implants have an embryonic origin. Embryonic cells have a multipotent capacity and are able to differentiate into several cell types. Based on this fact two main hypotheses exist namely Müllerianosis and coelomic metaplasia.

According to the Müllerianosis theory, misplaced Müllerian tissue is the source of endometriosis. The female reproductive track develops from the Müllerian duct. Aberrant development occurs during embryogenesis and results in misplacement of Müllerian tissues. The exposure to endogenous hormones activates these tissues after puberty, and leads to endometriosis (Batt et al., 1987). Although, undifferentiated Müllerian tissue has not yet been found in adult women; Signorile *et al.* (2010) reported the presence of primitive ectopic endometrium in female foetuses (Signorile et al., 2010). An interesting finding was the distribution of lesions were similar to that in adults. The foetal ectopic endometrial tissues were found mainly in the cul-de-sac area. These findings have led to the speculation that the aetiology of rectovaginal endometriosis might be explained by Müllerianosis. The results of the team are impressive but raise some questions. Organogenesis in the foetus had not been completed, therefore there is a possibility of the lesions disappearing later. They did not detail the cause of the death of the foetus, nor did they investigate the mother.

The other well-known endometrial theory is coelomic metaplasia, proposed by Meyer in 1919. Coelomic epithelium is the common ancestor of the endometrium and peritoneal mesothelium. Coelomic epithelium is the lining of the coelomic (abdominal) cavity during the embryogenesis, which forms the surface of the peritoneum and abdominal organs. It also constitutes the germinal epithelium of the gonads. Therefore, it could be relevant in the forming of ovarian endometrioma. The theory presumed that mesothelial cells of the peritoneum are able to dedifferentiate by metaplasia and form ectopic endometrial lesions anywhere in the body where the coelomic epithelium can be found.

The main advantages of the coelomic metaplasia and the Müllerianosis theories are that they provide a better explanation of the extra pelvic endometriosis and endometriosis in patients without menstruation i.e. premenarchal girls or even in men. However, coelomic metaplasia has failed to explain why the frequency of the disease does not increase with age if its mechanism is similar to common metaplasia (Vinatier et al., 2001) and Müllerianosis cannot explain the high recurrence after the surgical excision, particularly the recurrence of deep infiltrating endometriosis (Busacca et al., 2006).

The mutual point in these two hypotheses is induction being the key step in pathogenesis. The differentiation of the cells is strictly controlled. Thus, induction factors are necessary for initiating the dedifferentiation processes. The induction factors can be endogenous or exogenous factors such as inflammatory mediators, hormones, pollutants or even the refluxed menstrual blood. The hypotheses state that these factors activate the dormant Müllerian remnants or induce the metaplasia in coelomic epithelium.

Endometriosis is reported in men rarely. Only one case concluded that the lesions had been caused by Müllerianosis. In this case the induction factors could not be identified (Giannarini et al., 2006). The other cases result from oestrogen therapy due to cancer treatment. These cases suggest the importance of the induction factors and that an altered hormonal environment are able to induce the pathological processes which lead to the disease. These cases might provide an opportunity to learn more about disease establishment. However, these cases are rare and the used hormone dose was not physiological. Transgender people who change their gender from male to female could be a better group to study. They obviously do not have endometrium nor a menstrual cycle but receive female hormone therapy long-term. Moreover, abnormal gender development might be more frequent in this group. Therefore, the presence of endometriosis could be anticipated and these cases could

be an evidence for embryotic origin. However, endometriosis in transgender patients has not been reported and not even investigated as yet.

1.3.3 Stem cells origin

The third possible source of endometriosis are stem cells. Adult stem/progenitor cells are responsible for tissue maintenance, regeneration and repair (Mutlu et al., 2015). Of note, adult stem/progenitor cells should not be confused by embryonic stem cells which are pluripotent and capable of differentiating into cells from all three germ layers. Adult stem cells are also clonogenic cells with self-renewing capability but their lineage is more restricted and they have multipotent characteristic only (Gargett et al., 2016).

The human endometrium is a dynamic tissue with high regenerative capacity therefore it was suspected that adult stem/progenitor cells could be found in the basalis layer of the endometrium. Leyendecker *et al.* (2002) found that in women with endometriosis, the basal layer shed during menstruation (Leyendecker et al., 2002). Chan *et al.* have found putative endometrial stem cells in eutopic endometrium (Chan et al., 2004).

The other possible source of adult stem/progenitor cell is the bone marrow. Bone marrow-derived cells could reach any part of the body by the blood stream. Genetic analysis of the endometrium of bone marrow transplanted patients suggests that the bone marrow-derived stem cells take part in the repopulation of the endometrium (Taylor, 2004).

Based on this evidence Figueira *et al.* (2011) speculated that endometriotic progenitor cells from the basal layer of endometrium could be source of endometriosis. They could reach their location by retrograde menstruation or by the blood stream (Figueira et al., 2011). Although, their hypothesis is not supported by direct evidence, the monoclonality of some lesions could support their theory, namely (Yano et al., 1999).

1.3.4 Lymphatic spread

The role of the lymphatic system in the pathology of endometriosis has not been well established. Sampson proposed that the lymphatic spread of endometrial cells also could be responsible for ectopic lesions (Sampson, 1927). Lymphatic spread is able to provide an explanation of endometrial lesions in unusual sites such as brain or lung and a possible explanation of disease recurrence. Recent studies have found endometrial-like cells in the peritoneal lymph nodes from women with endometriosis

(Mechsner et al., 2008; Tempfer et al., 2011), also increased expression of lymphatic growth factors and lymphatic vessel density surrounded the lesions (Reichelt et al., 2012). These findings suggest the existence of lymphatic spread and led to the hypothesis that lymph nodes can be the source of recurrence of the disease (Gong and Tempfer, 2011).

Overall, the theories of aetiology concentrate on the origin of disease but fail to explain the whole pathophysiological process from the origin to the disease onset. It is very likely endometriosis is a multi-factorial disease where several internal and external factors take part in the pathogenesis, also there are the possibilities that the different phenotypes of the disease, such as peritoneal, ovarian and deep infiltrating endometriosis have different aetiology. Hence, further work is required to complete the unanswered questions of the aetiology of endometriosis.

1.4 Scope of Endometriosis

Although, a large and growing body of literature has been published on endometriosis, the underlying pathogenesis of this enigmatic disorder remains elusive. Epidemiological studies investigate what factors and circumstances contribute to the disease, while clinical studies search better treatments and solutions for the patients. This chapter provides an overview about the risk factors and epidemiology of endometriosis, also summarize the actual states and problems of the diagnosis and treatment.

1.4.1 Risk factors

1.4.1.1 Anthropological elements

Several factors may predispose women to endometriosis. It is generally agreed that early menarche, nulliparous women and women with heavy menses have an increased risk of developing of the disease (Eskenazi and Warner, 1997). The role of the demographic factors such as age, ethnicity and social class are controversial. For example there is no direct relationship between the age at the diagnosis and the disease severity (Viganò et al., 2004). Although, it seems adolescents with dysmenorrhoea and chronic pelvic pain have a considerably higher risk of endometriosis. Furthermore, it is likely these adolescent women would have been diagnosed with more advanced disease later in their life (Brosens et al., 2013). Some studies suggest that white women from higher social classes are more predisposed. However, Mangtani and Booth indicated that these findings might be caused by sampling bias because the data are mainly based on private clinic databases (Mangtani and Booth, 1993). Some body characteristics may be associated with the

endometriosis. Interestingly, epidemiological studies have found that body weight, body mass index (BMI) and the waist-to-hip ratio inversely correlate with the disease risk (Missmer et al., 2004; Hediger et al., 2005; Backonja et al., 2017).

Genetic factors also could have a role in the aetiology. Genome-wide association studies (GWAS) suggest a polygenetic inheritance for endometriosis (Rahmioglu et al., 2014). A recent meta-analysis identified 12 single nucleotide polymorphisms (SNP) at 10 independent genetic loci. Two out of 12 SNPs were located within genes (*GREB1*-growth regulation by oestrogen in breast cancer; *FN1*-fibronectin 1) whereas the remainder were located in the intergenetic regions with varying distance from known genes. Of interest, nearly all (11) SNPs were more strongly associated with severe endometriosis (rAFS stage lll/lV) (Zondervan et al., 2016). Although these genetic variants were identified, their roles in the pathophysiology of endometriosis have not been elucidated yet.

Family history analysis and twin studies also suggest a polygenic/multifactorial background for endometriosis (Bischoff and Simpson, 2000). For instance, greater occurrence of endometriosis in monozygotic twins and in their family members were reported (Moen, 1994; Saha et al., 2015). First degree relatives of diagnosed patients have more than a seven fold increased risk of developing endometriosis, and it is likely they will experience a more serious form of the disease (Moen and Magnus, 1993). Saha *et al.* in a large cross-sectional cohort of the Sweden nationwide twin registry investigated the relative contribution of genetic and environmental factors on endometriosis. The best-fit model revealed additive genetic factors might be liable in 47% of cases (95% confidence interval (CI), 36%-57%) for the disease susceptibility suggesting a complex polygenic inheritance. While, the remaining 53% (95% CI, 43%-64%) was unique (nonshared) environmental influences. This study indicates a complex, multifactorial aetiology for endometriosis with strong genetic and environmental effects on the manifestation of the disease (Saha et al., 2015).

1.4.1.2 Lifestyle elements

Endometriosis is a hormone dependent, inflammatory condition. Diet and lifestyle may have impact on the inflammatory or hormone level of the body. These factors can be modified, therefore it would be of benefit to gain knowledge about the relationship to the disease because the inadequate levels of nutrients and vitamins could promote disease progression.

Today, vitamin D is in the focus of research interest as evidence suggest it is an important modulator of human reproductive processes and immune responses, it also has a significant effect on gene regulation (Baeke et al., 2010; Borges et al., 2011; Lerchbaum and Rabe, 2014). Epidemiological studies attempted to explore the role

of vitamin D in the pathophysiology of endometriosis (Parazzini et al., 2013; Sayegh et al., 2014; Skowrońska et al., 2016). Although, an unequivocal link has not yet been found, more data may establish the importance of this vitamin.

The role of fatty acids also has become a topic of interest, particularly the unsaturated and *trans* fatty acids. The long-chain omega-3 polyunsaturated fatty acids (PUFA) are considered to be the most immunosuppressive fatty acids (Puertollano et al., 2007). They have an impact on prostaglandin and cytokine physiology (Calder, 2003). In contrast, the *trans* fatty acids are responsible for systemic low-grade inflammation (Kuipers et al., 2011). The intake of *trans* fatty acids positively associated with several inflammatory markers, such as C-reactive protein or interleukin 6 (IL-6) (Mozaffarian et al., 2004). Therefore, these fatty acids might have role in the pathological process of endometriosis. Missmer *et al.* (2010) in the Nurses' Health Study II, have found that the long-chain omega-3 PUFA reduced the endometriosis risk by 23% while the *trans* fatty acids increased it by 48% (Missmer et al., 2010). Contrary to these findings Trabert *et al.* (2011) reported a reverse association between endometriosis risk and *trans* fatty acids intake (Trabert et al., 2011), whilst Parazzini *et al.* could not find a correlation between the long-chain omega-3 PUFA intake and endometriosis (Parazzini et al., 2004).

Other life style factors such as smoking, exercise, alcohol or caffeine consumption were also objects of studies however evidence of links remain equivocal (Cramer et al., 1986; Bérubé et al., 1998; Saha et al., 2017).

1.4.1.3 Environmental elements

Environmental pollutants are suspected to increase the risk of endometriosis. The endocrine-disrupting compounds (EDCs) are able to mimic endogenous hormones and their effects on the reproductive systems (Balabanič et al., 2011). Hence, they may be responsible for decreasing the age of puberty in adolescents (Buttke et al., 2012). The most frequently investigated compounds are polychlorinated biphenyls (PCB), dioxins and bisphenol A. They imitate oestradiols therefore have been suggested to have role in the aetiology of endometriosis as possible inducing factors. For instance, PCB and dioxin were shown to cause endometriosis in Rhesus monkeys (Rier et al., 2001).

1.4.1.4 Association with other diseases

Epidemiological studies suggest that endometriosis may predispose women to other disorders. Due to the inflammatory nature of the disease associations are suspected with other chronic, autoimmune and malignant conditions. For instance, in a nationwide cohort study the overall cancer risk was nearly 20 percent higher in women with endometriosis compared to the non-endometriosis patient group. The

risks of non-Hodgkin's lymphoma, ovarian cancer and breast cancer were also significantly increased (Brinton et al., 1997). A more recent population-based study found that endometriosis was associated with 80% increased risk for all cancers. Ovarian endometriosis was a subsequent risk for ovarian and endometrial cancer with more than 4-fold and 3-fold, respectively (Kok et al., 2015). Heidenmann *et al.* (2014) also have found link between endometriosis and ovarian cancer, as well as Poole *et al.* (2017) confirmed elevated risk of ovarian cancer (relative risk (RR): 1.82; CI: 1.26-2.58) but did not find association between endometriosis and endometrial cancer (RR: 0.78; CI: 0.42-1.44) (Heidemann et al., 2014; Poole et al., 2017).

The gastro-intestinal tract could be affected by endometriosis. The symptoms and the aetiological factors could overlap between endometriosis and inflammatory bowel disease (IBD). Due to the similarity of the symptoms, differential diagnosis is necessary to distinguish endometriosis, particularly gastrointestinal endometriosis, and IBD. In a large cohort study it was shown that the endometriosis patients have an increased risk of IBDs. It was concluded that the women with endometriosis, compared to the normal population, had at least a 50% increased risk of ulcerative colitis or Crohn's disease after their diagnosis of endometriosis. Although, there is a possibility of an unconfirmed diagnosis due to the similarity of symptoms; strict diagnostic criteria minimalize the ascertainment biases. These findings suggest there is association between endometriosis and inflammatory bowel diseases (Jess et al., 2012).

Clinical case series suggest association between endometriosis and autoimmune disease (Matarese et al., 2003; Thomson and Redwine, 2005). An American cross-sectional survey found a robust, 7-24 fold, increased risk of multiple sclerosis (MS), systemic lupus erythematous (SLE) and Sjögren's syndrome (SS) in women with endometriosis (Sinaii, 2002). Nielsen et al. in 2011, attempted to repeat the same findings in a Danish cohort study. Although, a modestly (20-60%) elevated risk of MS, SLE and SS in women with endometriosis was reported; overall they failed to confirm the previous findings (Nielsen et al., 2011).

Further studies indicate that endometriosis related inflammation could predispose to chronic diseases such as cardiovascular and coronary heart diseases. Mu *et al.* (2016) reported increased risk of women with endometriosis to coronary heart disease (CHD). Association was particularly robust among the younger age group (≤40 years) (RR: 3.08, CI: 2.02-4.70) suggesting endometriosis promotes chronic cardiovascular diseases (Mu et al., 2016).

1.4.2 Epidemiology

Endometriosis affects around 176 million women worldwide (Adamson et al., 2010). The estimated frequency in the overall population of women of childbearing age is about 8-10% (Eskenazi and Warner, 1997). Endometriosis has been found in up to 60% of women with dysmenorrhoea and 40-50% of women with pelvic pain and dyspareunia (Eskenazi et al., 2001). Studies show the prevalence of endometriosis could be as high as 70% in adolescents with chronic pelvic pain (Laufer et al., 2003) and 50% in infertile women (Nesbitt-Hawes and Ledger, 2015). However, endometriosis has also been diagnosed in 2-18% of asymptomatic women undergoing sterilization (Luttinger, 1916). The exact prevalence of the disease is unknown and there are no reliable epidemiological data regarding the onset and latency of the disease. On average, patients suffer a 7.96±7.92 year delay in the UK and 11.73±9.05 year in the USA from the first symptoms to diagnosis (Hadfield et al., 1996). Unfortunately, very recent studies confirmed that the delay is still between 7 to 10 years on average (Staal et al., 2016; Ahn et al., 2017). The delayed diagnosis limits obtaining information about the onset and the early stages of the disease, and introduces uncertainty into the epidemiological data.

1.4.3 Burden of the disease

The symptoms of endometriosis have significant impact on the physical and mental life of women with endometriosis. The most often reported symptoms are pain and fatigue. The Endometriosis Association questioned 3680 patients in the USA and Canada. Almost all responders reported pain (98.5%) and 87% chronic tiredness. Sixty two percent of the sufferers categorised their pain as moderate to severe or severe. Eighty one percent of the women were not able to work or do their daily tasks due to pain (Sinaii, 2002). These symptoms negatively affect several aspects of the life. They have influence on daily activities, work performance, social and sexual life. Nnoaham *et al.* (2011) revealed that the impact of endometriosis on physical health-related quality of life (HRQoL) was similar to women with cancer (Nnoaham et al., 2011). Facchin *et al.* (2015) investigated the mental health, including anxiety and depression of endometriosis patients with and without pelvic pain and compared it to non-endometriosis women without pelvic pain. It was found that endometriosis patients with pelvic pain have a poorer quality of life and worse mental health. Pelvic pain was strongly associated with higher anxiety and depression in endometriosis patients with pelvic pain compared to pain-free groups (Facchin et al., 2015).

In the wider context, the debilitating nature of endometriosis creates a profound economic burden on society. Affected women lost on average 10.8 hours of work weekly due to reduced effectiveness or inability to work (Nnoaham et al., 2011). The

calculated average annual productivity loss is £4988 (€6298) per woman. This amount coupled with the healthcare cost £2465 (€3113) per woman equates to £7586 (€9579) endometriosis-associated cost per woman per annum in Europe (Simoens et al., 2012). The economic burden of the disease is comparable to the annual cost of other chronic disorders. For instance, the estimated global cost of the endometriosis was £12.8 billion ($22 billion) in the USA in 2002. This cost is substantially higher than Crohn's disease £507 million ($865 million) or migraine £7.6-10 million ($13-17 million) (Simoens et al., 2007).

These facts highlight that early diagnosis and effective therapies are crucial in reducing the disease related cost and improving the quality of life for patients. In addition, a systemic review, investigating the patient requirements to develop a patient-centeredness care for endometriosis, also pointed out that from the patient's perspective the main improvement objectives were reduction of the diagnostics delay and more attention and respect from the healthcare professionals. Development of these areas might reduce the disease burden on the patients (Dancet et al., 2014).

1.4.4 Diagnosis

Diagnosis of endometriosis is difficult to confirm because of the wide variety of symptoms and comorbidity with other diseases such as irritable bowel syndrome or pelvic inflammatory disease (Kennedy et al., 2005). Due to the high frequency in the normal population, endometriosis should be considered in any woman of reproductive age who has abdominal or lower back pain that worsens during menstruation (Wellbery, 1999). However, diagnosis based exclusively on symptoms is not reliable. Nnoaham *et al.* in 2012 developed a symptom based screening method to predict endometriosis (Nnoaham et al., 2012). The model accuracy was reliable for severe cases but failed to diagnose minimal or mild endometriosis. The only method for accurate diagnosis involves the use of invasive surgical techniques.

1.4.4.1 Surgical methods

Laparoscopy has become the gold standard in diagnosis. It has been widely used from the 1980s and now dominates gynaecological procedures. This surgical technique has several advantages and disadvantages. It allows to the surgeon to inspect the pelvic cavity, detect lesion morphology, estimate the stage of the disease, take biopsies for histological confirmation and as a main goal it can be used to remove visible endometrial lesions. However its own disadvantages which are as follows: firstly, it is expensive and requires highly skilled and experienced surgeons. Secondly, the diagnosis made by laparoscopy is subjective because sensitivity and specificity depend on the experience of the surgeon and also the wide variety of the lesions in

some cases may lead to misdiagnosis (Barbieri and Missmer, 2002). Therefore the histological confirmation of the lesions are recommended to exclude the presence of malignant and other benign lesions (Brosens et al., 2004). Thirdly, some lesions are not detectable by this technique because they are too small, sometimes termed microscopic endometriosis, can be hidden by adhesions or organs, or infiltrate into the wall of the organs (Brosens et al., 2004). As an invasive method, it also carries risk of postoperative complications. Even so this risk and the recovery period are negligible compared to laparotomy, which is a more invasive method and is used in the most severe cases (Bateman et al., 1994). Lack of better techniques may have made laparoscopy the gold standard but it is not perfect tool. Therefore significant research efforts are needed to establish a reliable non-invasive diagnostic method.

1.4.4.2 Imaging

Imaging methods are candidates of diagnostic tools for endometriosis as they have been considered as non-invasive techniques. The most often used techniques are transvaginal ultrasound and magnetic resonance imagining (MRI). They are mainly relevant in the preoperative assessment to clarify diagnosis, reduce the risk of unexpected findings and avoid unnecessary surgery. The diagnostic accuracy depends on the type, size and the localisation of the lesions. Detection of small or superficial implants is really poor due to the lack of necessary resolution of imaging techniques (Brosens et al., 2003).

1.4.4.3 Biomarkers

The early diagnosis of endometriosis urgently requires development of non-invasive methods. To date, there are no clinically relevant screening tests to detect endometriosis. The most ideal tool would be urine or blood tests. A reliable method must have high sensitivity and specificity, be able to detect endometriosis in early stage, and correlate with the stages of the disease. It also should be able to monitor the effectiveness of the treatment and recurrence. The main candidate markers are glycoproteins, cytokines, adhesion molecules and angiogenic and growth factors because they are highly relevant to pathogenesis and lesion formation. Endometriosis is believed to be a multifactorial disease with different aetiologies. Therefore, different indicator substances known as biomarkers, may be required for different stages or types of endometriosis. It is also possible, that a panel of biomarkers, as a ‘‘fingerprint’’, rather than individual molecules will probably be required (Rogers et al., 2009). May *et al.* in 2010 identified and systematically reviewed more than 100 peripheral biomarkers. They could not identify a sensitive single or panel of markers which would have been clinically relevant as a diagnostic tool (May et al., 2010). Finding the right biomarker is a problematic challenge. Most of the proposed biomarkers could be associated with numerous other conditions and not exclusively

endometriosis. For instance, the cancer antigen 125 (CA-125) has been used in clinical practice but the elevated level of the CA-125 also can be associated with other malignant disease such as ovarian cancer (Markman et al., 2006).

Molecular biology techniques have opened a field for novel biomarkers. Proteomic, genomic and metabolomic methods are capable of screening a wide panel of molecules. For instance the genomic and proteomic analysis of eutopic endometrium are really promising; particularly in the diagnosis for minimal-to-mild endometriosis. Meola *et al.* (2013) compared CALD1 gene expression and caldesmon protein level in eutopic endometrium from patients with and without endometriosis. The diagnostic sensitivity reached 95% and 100% specificity in minimal-to-mild endometriosis (Meola et al., 2013). In another study, the protein expression of eutopic endometrium was assessed by surface-enhancer laser desorption/ionization time-of-flight mass spectrometry (SELDI-TOF MS). The proteomic analysis combined with bioinformatics reach maximal sensitivity (100%) and specificity (100%) of diagnostic accuracy in the minimal-to-mild endometriosis group (Kyama et al., 2011). Although, it must be noted that, these data are only preliminary and must be confirmed in a larger, independent patient population. Proteomic, genomic and metabolomic methods are promising, they provide cost effective approaches and enhanced with bioinformatics they could be ideal diagnostics tools.

1.4.4.4 Classification

Numerous classifications have been proposed for endometriosis. However, none of them are able to cover all aspects of disease. The main goal of the systems is to provide comparable and reproducible descriptions of the pathological findings. The other requirements are to enable monitoring of the treatment and disease progression; also to provide comparable data for research application. The most often used scoring system is American Society for Reproductive Medicine revised classification (ASRM) (Theron et al., 2007). This system describes the location, size, depth, and the morphology of the lesions and extent of adhesions. It is based on scores and distinguishes minimal (I), mild (II), moderate (III) and severe (IV) stages. The main criticism against this system is that the stages of the disease do not correlate with pain or fertility. Also, it is not suitable to predict fertility and detailed classification of DIE (Adamson, 2011). The reliability and reproducibility is also questionable. Only fair-to-good agreement has been reported by Rock in 1995 (Rock, 1995).

1.4.5 Management

The medical management of the endometriosis is a source of ongoing controversy as the aetiology of the disease is not fully established yet. Clinical guidelines from professional societies, such as The European Society of Human Reproduction and Embryology (ESHRE) and The American Society for Reproductive Medicine (ASRM), were established to offer good clinical practice for healthcare professionals who care for women with endometriosis. To date, there are no treatments which can permanently eliminate the disease, hence management of symptoms is the main objective of therapies.

According to ESHRE guidelines, empirical treatment should be considered in women with endometriosis-associated pain as the first line of medical treatment. Non-steroidal anti-inflammatory drugs (NSAIDs) (e.g. aspirin, ibuprofen and naproxen) reduce inflammation and might provide effective analgesics for pelvic pain. The drawback of these drugs is that the long term administration is not recommended due to the risk of gastric ulceration and cardiovascular disease (Kennedy et al., 2005).

Contraceptive drugs such as progestins (*"minipills"*) and the combined oral contraceptives (COCs) which consist of a combination of ethinylestradiol and a progestin, are also widely used to control endometriosis-associated pain. The practical advantages of these contraceptive drugs are to control the menstrual cycle including dysmenorrhea, are safe in the long-term, well-tolerated and cost effective (Vercellini et al., 2011; Tafi et al., 2015).

If the symptoms are not improved, gonadotropin-releasing hormone (GnRH) agonists are used as second line medications (Ferrero et al., 2010). These compounds cause desensitisation of the pituitary by the downregulation of GnRH receptors. This desensitisation leads to gonadotropin suppression and development of a hypo-oestrogenic state (Arya and Shaw, 2006). The long term use of GnRH agonists requires a careful approach because the hypo-oestrogenic state is associated with substantial bone mineral density loss. To compensate for this effect, a small amount of steroid hormone is necessary as an 'add back' therapy, to reduce the loss of bone mineral density (Kennedy et al., 2005; Ferrero et al., 2010).

Alternative hormonal treatment can also be applied in endometriosis treatment. Danazol is a synthetic androgen without oestrogenic and progestational properties. It suppresses the hypothalamic-pituitary-ovarian (HPO) axis by decreasing the frequency of GnRH pulses, which inhibits follicle stimulating hormone (FSH) and luteinising hormone (LH) release. It directly inhibits steroidogenesis in the ovaries

(Dmowski, 1982) and competitively antagonises the androgen, oestrogen and progesterone receptors in the endometrium (Arya and Shaw, 2006). These processes result in the atrophy of endometrial tissues (Dmowski, 1982). Danazol was proposed to treat endometriosis (Wellbery, 1999). However, the use of this drug nowadays is strongly decreased, due to its androgenic side effects and reduced tolerance, compared to GnRH agonists (Tafi et al., 2015).

If pharmacotherapy proved unsuccessful or the hormonal therapy contraindicated because of desired pregnancy or the therapy was not tolerated, surgical treatment should be considered (Dunselman et al., 2014). The aim of surgery, besides confirming the diagnosis, is to remove the lesions and restore normal anatomy. The restoration of disrupted pelvic anatomy in advanced stages is challenging. Sometimes, whole or partial removal of the reproductive organs is necessary. These cases result in iatrogenic infertility, disturbed endocrine homeostasis or early menopause. Ideally, surgery could increase fertility and decrease pain symptoms. Case control studies have proved the excision of the lesions reduces the pain. Sutton *et al.* evaluated the effectiveness of laser laparoscopy in pain relief and compared it to expectant management. 62.5% of the treated patients reported improvement or relief in their pain symptoms. Unexpectedly, 22.6% of the patients in the untreated group also reported improvement in their pain. The outcomes of the surgery were better in the mild or moderate stages of the disease and less effective in minimal endometriosis. Although the result proved that the laparoscopy was effective in pain relief and reduced the pain in around two-thirds of the patients, it had lower efficiency in the early stage of disease. Moreover, the pain was also reduced in the expectant group. This raised the question of usefulness of surgery in minimal and asymptotic endometriosis (Sutton et al., 1994). The results of this study also suggested that the endometriotic lesions might regress on their own. A randomized, blinded study also investigated the effectiveness of laparoscopy in pain relief, and compared it to diagnostic surgery. The pain was reduced in 80% of the patients in the excisional group, but also by 32% of the patients in the diagnostic group. The reduction of pain in the diagnostic group may be explained by the placebo effect or self-regression. However, the patients in the diagnostic group had a second laparoscopy 6 months later. Interestingly, although the disease progressed in 45% of the patients, it was static in 33% and improved in 22% (Abbott et al., 2004). These findings suggest that endometriosis is not a linearly progressive disease. To date, we lack of the knowledge to understand fully the reasons of this and it will be a great step forward to learn more about these processes in the future.

1.5 Elements of Pathophysiology

One of the biggest challenges for endometriosis is to unravel the pathological processes of the disease. Extensive knowledge of pathogenesis is crucial to targeted therapy. Some mechanisms are known however, most of the pathophysiological processes are still elusive. It is widely accepted that endometriosis is an oestrogen dependent, inflammatory disease. This chapter summarizes the factors which could have a role in the pathophysiology.

1.5.1 Steroid hormones

The family of steroid hormones consists of two classes: the corticosteroids and the sex steroids. All steroid hormones are derived from cholesterol having the same structure of tetracyclic hydrocarbon ring. Sex steroids are regulators of reproductive function in both genders acting via nuclear receptors. Nuclear receptors belong to transcription factor family and initiate the transcription of genes by binding to the promoter sequence (Katzenellenbogen and Katzenellenbogen, 1996). Oestradiol and progesterone are the main female sexual hormones regulating the menstrual cycle and gametogenesis. However, beside the regulation of the menstrual cycle, both hormones have pleiotropic actions in the body through regulation of hundreds of genes (Kao et al., 2002). These hormones also take part in the modulation of immune responses and other physiological processes. For instance, oestradiol is a mitogen has anti-apoptotic properties and promotes inflammation, while progesterone is anti-inflammatory and induces apoptosis (Reis et al., 2013).

1.5.1.1 Oestrogens

Naturally occurring oestrogens in women are oestrone, oestradiol and oestriol. 17β-oestradiol is the most potent natural oestrogen and predominant in women during their reproductive years. Oestrone is dominant after the menopause, while oestriol is important in pregnancy.

One of the most important roles of oestradiol is renewing of endometrium after menstruation. The synthesis of oestrogens regulated by the steroidogenic cascade includes two rate-limiting steps. One step is the entry of cholesterol into the mitochondria regulated by steroidogenic acute response protein (StAR), the other is the conversion of androstenedione to oestrone by aromatase cytochrome P450 (P450arom) (Bulun, 2005). After the rate-limiting steps oestrone is subsequently converted to oestradiol by 17β-hydroxysteroid dehydrogenases (17βHSDs).

Endometriosis is an oestrogen-dependent disease. Oestrogen receptor expression and oestrogen synthesis are disturbed in patients with endometriosis (Bulun et al., 2012). In disease-free women P450arom expression is absent in endometrium and myometrium (Bulun et al., 1993, 1994). The expression of P450arom is limited to granulosa cells and is lacking in the theca and other stromal cells of the ovary (Sasano et al., 1989).

In contrast, in women with endometriosis the endometriotic tissue expresses a complete set of steroidogenic genes (Bulun, 2005). As a result, these tissues are able to synthesise oestradiol from cholesterol at the location of ectopic lesion. This oestrogen supports the growth of endometrial lesions (Bulun, 2005). Oestradiol binds with high affinity to its nuclear receptors (ERα and ERβ) then the oestrogen-receptor complex binds to the promoter of genes and initiate the transcription of these genes (Katzenellenbogen and Katzenellenbogen, 1996). Oestrogens do not induce mitosis directly, they activate and regulate transcription of growth factors, which promote the proliferation of cells.

For instance, fibroblast growth factor-9 (FGF-9) is a steroid regulated mitogen. It regulates the endometrial stromal cell proliferation in the normal uterus. The expression of FGF-9 depends on the serum level of oestradiol. It is highest in the late follicular phase of the menstrual cycle (Tsai et al., 2002). In endometriosis the high oestradiol level in the peritoneum also induces the expression of FGF-9 which is able to support survival and progression of ectopic endometrial lesions (Wing et al., 2003).

1.5.1.2 Progesterone

A key role of progesterone is the regulation of endometrium in non-pregnant women. The main site of the progesterone synthesis is the corpus luteum in the ovary. After ovulation, the biosynthesis in the remaining follicle changes. The theca cells become small lutein cells, while the granulosa cells become large luteal cells (Niswender, 2002). Both cell types produce and release progesterone into the blood stream. The luteal cells, beside the progesterone synthesis also synthesise androgens and oestrogens. The small luteal cells, due to the lack of P450arom, produce androgens which are converted to oestrogen in the large luteal cells (Hanukoglu, 1992).

Clinical observations suggest that progesterone resistance may contribute to the pathological processes of endometriosis since progestin treatment was not effective at the 9% (95% [CI], 5.3% to 13.6%) of endometriosis patients with pelvic pain (Vercellini et al., 1997). The effect of progesterone is mediated via intracellular progesterone receptors (PRs). Progesterone receptor B (PR-B) is a strong activator of progesterone targeted genes while progesterone receptor A (PR-A) acts as a suppressor of PR-B (Tung et al., 1993; Vegeto et al., 1993). Attia *et al.* compared PR

expression in eutopic and ectopic endometrium of women with endometriosis. They found that the expression of the PRs were disturbed in ectopic endometrium. The PR-A level was low, and the PR-B was not detectable in the ectopic lesions. This finding proves that the progesterone targeted genes cannot be activated due to lack of PR-B expression in the endometrial tissue (Attia et al., 2000).

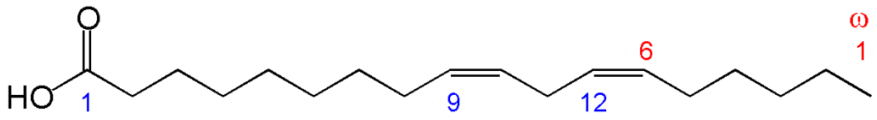
An important role of progesterone is to inhibit the oestrogen-induced growth in the endometrium during the secretory phase. The crucial step of this mechanism is to convert potent oestradiol to the less bioactive oestrone. This step supresses the oestradiol-induced cell growth of endometrium. This conversion is regulated by the progesterone-dependent 17β-HSD2 (Tseng and Gurpide, 1975). 17β-HSD2 is expressed at high levels in the glandular epithelial cells during the secretory phase in the endometrium (Casey et al., 1994). However as a result of progesterone resistance the 17β-HSD2 is down regulated in ectopic endometrial tissues (Zeitoun et al., 1998). The lack of 17β-HSD2 means the effect of oestradiol has not been controlled in endometrial tissue, thus favouring the development of endometriosis.

In summary, decreased expression of progesterone receptors in ectopic endometrial lesions suggest that progesterone resistance exacerbates the pathological processes in endometriosis. The lack of progesterone effect enhances inflammatory processes and the effect of oestrogen in patient with endometriosis (Bulun 2009).

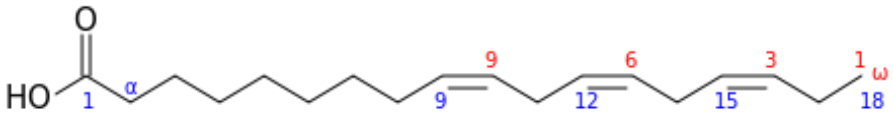
1.5.2 Lipid mediators

Lipid mediators are potent bioactive lipids with hormone-like functions. They are produced locally through biosynthetic pathways in the cells and act in an autocrine and paracrine manner on physiological processes.

Polyunsaturated fatty acids (PUFA) are substrates that form a myriad of oxygenated bioactive lipid mediators. PUFAs are straight chain hydrocarbons contain a carboxyl group and more than one double bond. Their nomenclature is based on the length of the carbon side chains and the position of the first double bond from the omega carbon which is the farthest carbon from the carboxyl group. Biologically the most relevant PUFA groups are the omega-6 and omega-3 families. Figure 1.5.1 illustrates the representative structure of omega-6 and omega-3 PUFAs.



A) omega-6, Linoleic acid (LA)



B) omega-3, α-Linolenic acid (ALA)

Figure 1.5.1. Structure and nomenclature of polyunsaturated fatty acids
(A) omega-6, Linoleic acid (LA) (B) omega-3, α-Linolenic acid (ALA), Carbons are numbered from carboxyl end (blue numbers). Omega carbon is the farthest carbon from the carboxylate. Red numbers indicate the position of double bond relative to omega carbon.
(Taken from https://commons.wikimedia.org)

PUFAs are synthetized in the body and are taken from dietary sources. Mammals are not able to produce C18 PUFAs from precursors. Therefore, omega-6 linoleic acid (LA) and the omega-3 α-linolenic acid (ALA) must be obtained through the diet. These fatty acids are also referred to as essential fatty acids.

Essential fatty acids are precursor of other PUFAs. Omega-6 LA is the precursor of dihomo-γ-linolenic acid (DGLA) and arachidonic acid (AA), whilst the omega-3 ALA is the parent fatty acid of eicosapentaenoic acid (EPA) and hydroxy docosahexaenoic acid (DHA). The biosynthesis of PUFAs occur in the liver through a series of desaturation and elongation. Then, the synthesized PUFAs are transported to the target tissues via circulation and esterified into the phospholipid membrane. Esterified PUFAs contribute to the membrane function and provide a source for lipid mediator synthesis. In response to cell stimulation the activated phospholipase A_2 (PLA_2) releases PUFAs from the cell membrane. These free fatty acids are oxygenated and converted through three major enzymatic pathways, namely cyclooxygenase (COX), lipoxygenase (LOX), cytochrome 450 epoxygenases (CYP) but also via non-enzymatic reactions. Figure 1.5.2 demonstrates schematic diagram of the synthesis of PUFAs in liver and the main enzymatic pathways of lipid mediators. Appendix 8 details the oxygenated lipid species according to the biosynthetic pathways and precursor fatty acids.

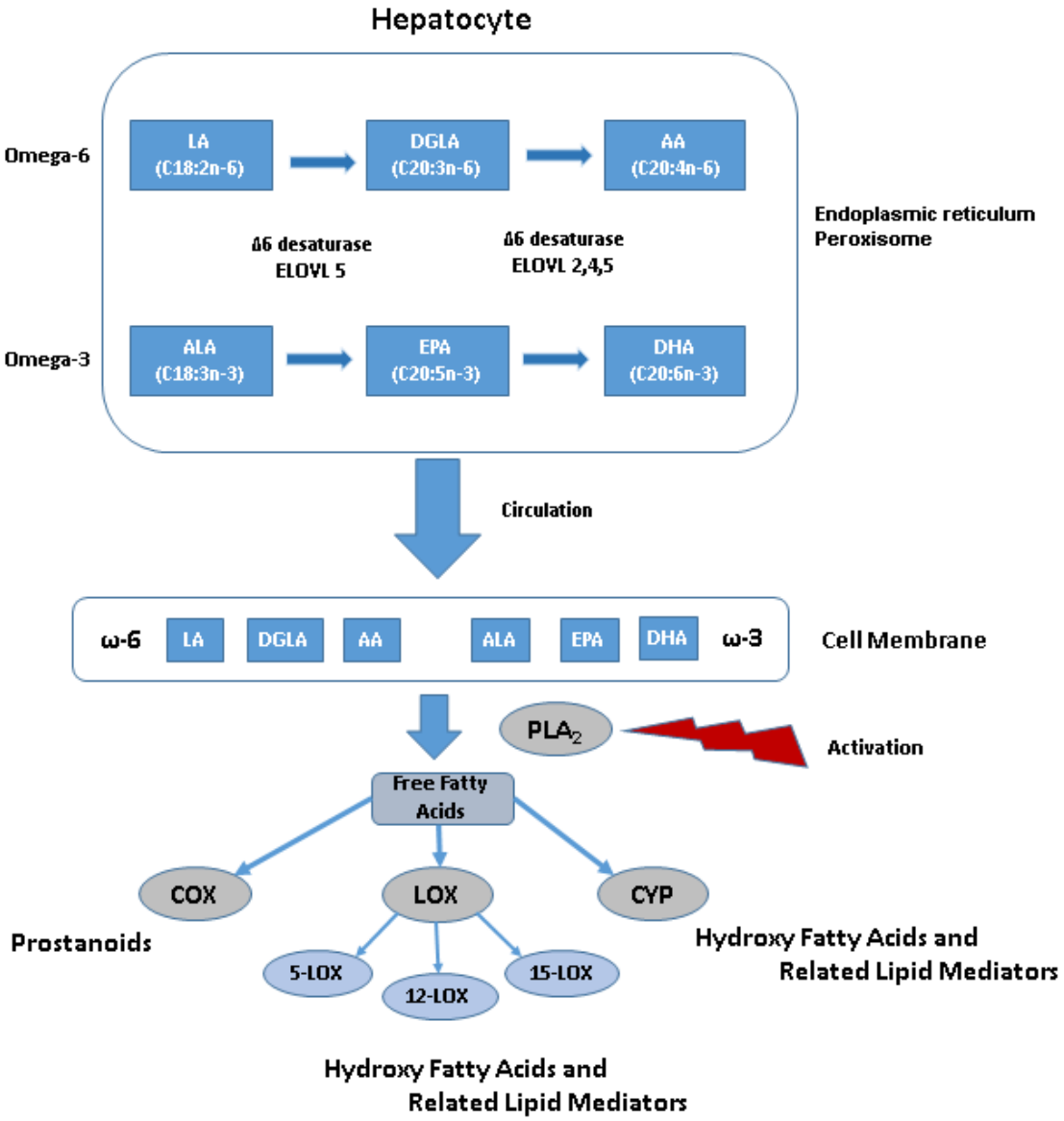


Figure 1.5.2. Synthesis of polyunsaturated fatty acids (PUFA) and bioactive lipid mediators. Anabolism of PUFA in hepatocytes from essential fatty acids are mediated by a series of elongases (ELOVL) and desaturases. Synthetized PUFAs reach the target tissue via blood stream, esterified and stored into the cell membrane. Response to cell stimulation the activated phospholipase A_2 (PLA_2) release fatty acids from the cell membrane. Free fatty acids are derived by cyclooxygenase (COX), lipoxygenase (LOX), cytochrome 450 epoxygenases (CYP) pathways resulting bioactive lipid mediators such as prostanoids, hydroxy fatty acids and related lipid mediators.

1.5.2.1 Cyclooxygenase (COX) pathway

The COX enzyme, also known as prostaglandin-endoperoxide synthase (PTGS), is a bifunctional haem containing dioxygenase with cyclooxygenase and peroxidase activity. COX catalyses the first two steps of prostanoid synthesis from free fatty acids through prostaglandin (PG) G to PGH. The subsequent metabolism of PGH yields the class of prostanoids, which includes PGs, prostacyclin (PGI) and thromboxane (TXA). The series-1, series-2 and series-3 prostanoids are the derivates of DGLA, AA

and EPA, respectively. Figure 1.5.3 illustrates the action of COX isoenzymes and the biosynthesis of series-2 prostanoids. Table 1.5.1 summarizes the precursor fatty acids and the representative prostanoids.

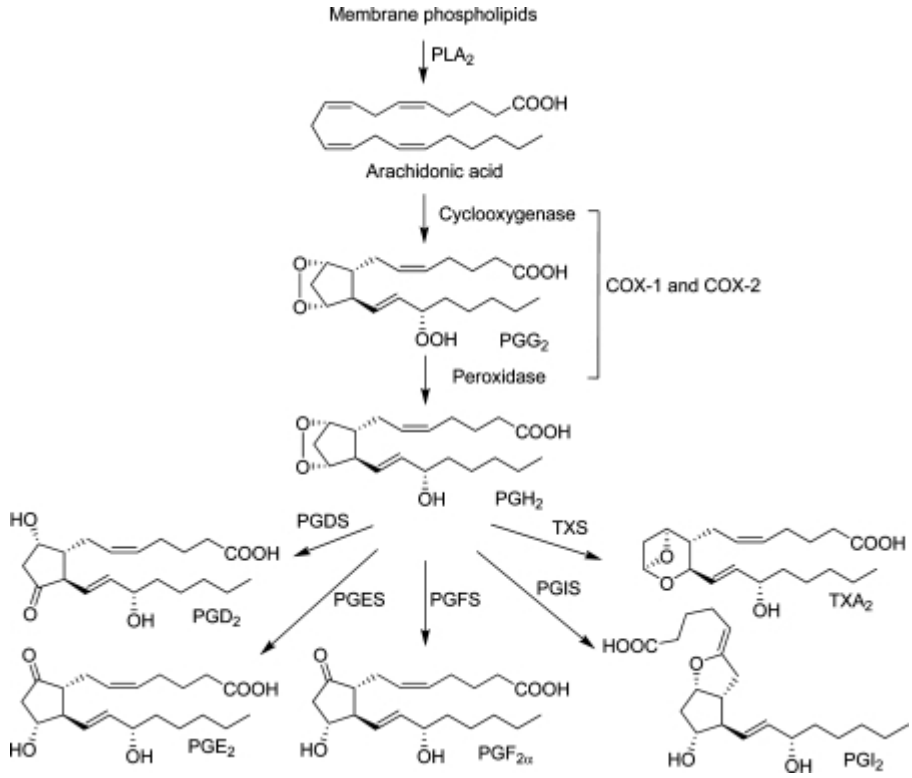


Figure 1.5.3. Biosynthesis of series-2 prostanoids.
(PLA_2) phospholipase A_2; (COX) cyclooxygenase; (PG) prostaglandin; (PGDS), prostaglandin D_2 synthase; (PGES) prostaglandin E_2 synthase; (PGFS) prostaglandin $F_{2\alpha}$ synthase; (PGIS) prostaglandin I_2 synthase; (TXS) thromboxane A_2 synthase; (TXA_2) thromboxane A_2. (open access licence).

Table 1.5.1. Summary of precursor fatty acids and representative prostanoids metabolised by cyclooxygenase (COX).

Precursor fatty acids				**Main products**
ω-6	DGLA	**COX + O_2**	series-1 prostanoids	PGE_1, $PGF_{1\alpha}$, PGD_1, TXA_1
	AA		series-2 prostanoids	PGE_2, $PGF_{2\alpha}$, PGD_2, PGI_2,TXA_2
ω-3	EPA		series-3 prostanoids	PGE_3, $PGF_{3\alpha}$, PGD_3, PGI_3,TXA_3

(DGLA) dihomo-γ-linolenic acid; (AA) arachidonic acid; (EPA) eicosapentaenoic acid; (PG) prostaglandin, (TX) thromboxane

COX is expressed in two isoforms in human. COX-1 is the product of *PTGS1* gene and constitutively expressed in most tissues, while COX-2 is encoded by *PTGS2* gene and its expression is induced by external signals for instance growth factors and pro-inflammatory cytokines. Both isoenzymes are located in the endoplasmic reticulum and nuclear envelope (Spencer et al., 1998). Although, the three-dimensional structure of these isoenzymes are nearly identical, their functions are different. It is well accepted that COX-1 mainly regulates homeostasis, whilst COX-2 plays a crucial

role in the promotion of inflammation. However, increasing evidence suggest that the function of these isoenzymes are actually more complex. In certain circumstances COX-1 contributes to inflammation, whereas COX-2 is also constitutively expressed in kidney, brain and female reproductive tract including endometrial glands and stroma, and indispensable for the normal function of these organs (Langenbach et al., 1995; Lipsky et al., 2000).

In amongst the COX products the most abundant and potent mediators are the AA derived series-2 prostanoids, such as PGE_2, PGD_2, $PGF_{2\alpha}$ and PGI_2. Their homeostatic profiles and levels are dramatically changed in response to inflammatory stimuli. Inflammation is the response of the immune system and its purpose is the elimination of inflammatory stimuli, for instance infection or injury, and restoration of homeostasis. PGs, particularly PGE_2, are the master regulators in the initiation of inflammation and key contributors to the inflammatory-related symptoms such as pain, redness, fever and oedema. Dysregulated PGE_2 synthesis was described in several inflammatory-related diseases for example cancer, metabolic disease, arthrosclerosis and endometriosis (Ricciotti and Fitzgerald, 2011). There is a growing body of evidence suggesting that PGE_2 is a key regulator of the pathological processes of endometriosis. PGE_2 takes part in the regulation of cell proliferation, immune suppression and angiogenesis. Furthermore, PGE_2 along with $PGF_{2\alpha}$ are involved in the endometriosis associated pelvic pain (Ray et al., 2015). Studies found that the PGE_2 level is elevated in the peritoneum of women with endometriosis and COX-2 is overexpressed in ectopic endometrial cells and peritoneal macrophages (Ota et al., 2001; Wu et al., 2002; Ray et al., 2015).

1.5.2.2 Lipoxygenases (LOXs)

LOXs are a heterogeneous class of non-haem, iron-containing dioxygenase enzymes which catalyse the production of hydroperoxy metabolites of PUFA by incorporate a molecular oxygen into the carbon chain of fatty acids. The hydroperoxy products subsequently undergo a number of metabolic transformations resulting a large array of bioactive lipid mediators. LOX occurs in all eukaryotes. The human genome contains six functional LOX genes which encode six different LOX-isoforms (Funk et al., 2002). The isoforms are expressed in a tissue dependent manner. In mammals, the predominant LOX substrate is AA therefore the members of the enzyme family are named according the oxygenated position in AA. Table 1.5.2 shows the genes, the corresponding isoforms and the main expression sites in human. LOXs have a wide substrate specificity resulting a broad variety of bioactive lipid mediators. Table 1.5.3 summarizes the precursor fatty acids along with the intermediate and main final products of LOX enzymes. Figure 1.5.4 illustrates the biosynthesis of AA derived products via 5-, 12- and 15 lipoxygenase pathways.

Table 1.5.2. Summary of LOX genes, the encoded isoforms and the major expression sites in human.
Adapted from Kühn et al. (Kühn et al. 2015).

Gene	Isoenzyme	Main expression sites
ALOX5	5-LOX	leukocytes, macrophages, dendritic cells
ALOX15	15-LOX-1	eosinophils, reticulocytes, bronchial epithelium
ALOX15B	15-LOX-2	hair root, skin, prostate
ALOX12	12-LOX	thrombocytes, skin
ALOX12B	12R type LOX (12R-LOX)	skin
ALOXE3	Epidermal LOX 3 (eLOX-3)	skin

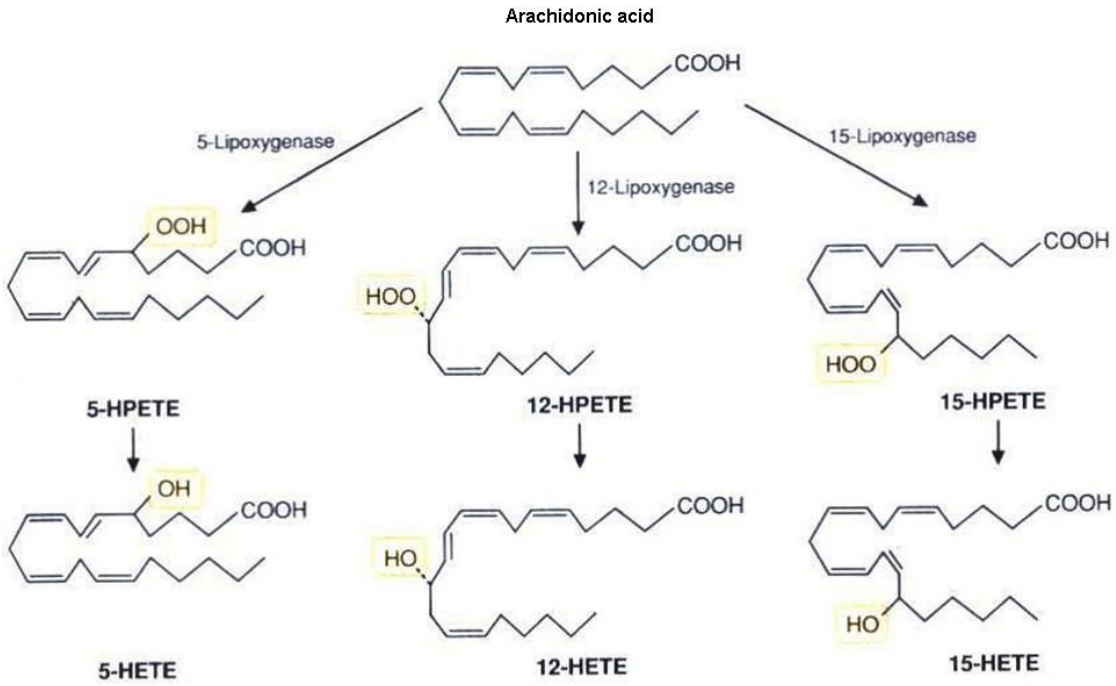


Figure 1.5.4. Lipoxygenase pathways.
Biosynthesis of AA derived hydroperoxyoctadecatrienoic acid (HETE).
Taken from: http://slideplayer.com/slide/7039683/24/images/15/Lipoxygenase+pathway.jpg

Table 1.5.3. Summary of parent fatty acids and their main derivates converted by lipoxygenase pathways (LOX).

Precursor fatty acids			Intermediate derivates	Main products by pathways		
				5-LOX	12-LOX	15-LOX
ω-6	AA	LOX + O_2	HpETE	5-HETE Leukotrienes	12-HETE	15-HETE
	DGLA		HpETrE	-	-	15-HETrE
	LA		HpODE	-	-	13-HODE
ω-3	DHA		HpDHA	4-HDHA 7-HDHA	11-HDHA 14-HDHA	17-HDHA
	EPA		HpEPE	5-HEPE	12-HEPE	15-HEPE
	ALA		HpOTrE	9-HOTrE	-	13-HOTrE

(AA) arachidonic acid; (DGLA) dihomo-γ-linolenic acid; (LA) linoleic acid; (DHA) docosahexaenoic acid; (EPA) eicosapentaenoic acid; (ALA) α-linolenic acid; (HpETE) hydroperoxyeicosatetraenoic acids; (HpETrE) hydroperoxyeicosatrienoic acid; (HpODE) hydroperoxyoctadecadieonic; (HpDHA) hydroperoxydocosahexaenoic acid; (HpEPE) hydroperoxyeicosapentaenoic acids; (HpOTrE) hydroperoxyoctadecatrienoic acid; (HETE) hydroeicosatetraenoic acids; (HETrE) hydroeicosatrienoic acid; (HODE) hydrooctadecadienoic; (HDHA) hydrodocosahexaenoic acid; (HEPE) hydroeicosapentaenoic acids; (HOTrE) hydrooctadecatrienoic acid

1.5.2.2.1 5-Lipoxygenase pathway

5-LOX is the most different and distant relative of LOX family. This isoform is the product of the *ALOX5* gene which is encoded on chromosome 10 in humans, while the other LOX genes are clustered together on chromosome 17. The mechanism of action of this isoenzyme is more complex and diverse from other LOX isoenzymes. The activation of 5-LOX requires interaction with another protein, called 5-lipoxygenase-activating protein (FLAP). FLAP is essential to the appropriate functioning of 5-LOX. Although, the exact role of this protein has not been fully elucidated, it has been suggested it acts as a scaffold protein that facilitates assembly of 5-LOX and PLA_2 at the nuclear envelope and support the binding of the enzyme substrates (Rouzer et al., 1990).

5-LOX is highly expressed in mature leukocytes such as granulocytes, monocytes, macrophages, mast cells, dendritic cells and B lymphocytes. Fibroblast, smooth muscle and endothelial cells expressed it at a lower level while T cells, platelet and erythrocytes have a lack of 5-LOX (Rådmark and Samuelsson, 2007).

5-LOX, similar to other LOX isoenzymes, catalyses the oxygenation of different PUFA species such as AA, EPA and DHA, resulting 5-hydroperoxyeicosatetraenoic acids (HpETE), 5-hydroperoxyeicosapentaenoic acids (HpEPE) and 4- or 7-hydroperoxydocosahexaenoic acid (HpDHA), respectively. Biologically the AA derived 5-HpETE is the most important product. The subsequent metabolism of 5-HpETE results leukotrienes (LT), 5-hydroxyeicosatetraenoic acid (HETE) and 5-oxo-eicosatetraenoic acid (oxoETE). These metabolites play an important role in leukocyte

stimulation and the regulation of granulocyte chemotaxis. For instance, LTB_4 is known as one of the most potent chemotactic agents for granulocytes (Ford-Hutchinson et al., 1980), while 5-oxoETE is one of the most potent activators of eosinophils (Powell and Rokach, 2013). 5-LOX products, similar to prostaglandins, are also key mediators in the initialisation of inflammation and immune response. A number of studies suggest that the AA derived 5-LOX products are proinflammatory and implicated in the pathogenesis of asthma, rheumatoid arthritis, atherosclerosis, allergies, inflammatory bowel disease and cancer (Ochs et al., 2014; Powell and Rokach, 2015).

Despite the known proinflammatory properties of 5-LOX mediators, a very limited number of studies have investigated their role in the pathophysiology of endometriosis. Lousse *et al.* in 2009 investigated the expression of 5-LOX in endometrium, ectopic lesions and peritoneal macrophages using real-time PCR and immunohistochemistry methods. They compared 5-LOX expression in ectopic lesions and peritoneal macrophages to matched eutopic endometrium in endometriosis, as well as peritoneal macrophages and endometrium to women without endometriosis. No significant difference was found in the 5-LOX expression between different specimens within the disease group nor between the disease and disease-free groups (Lousse et al., 2010). However, a more recent study suggested a possible role of cysteinyl leukotrienes in endometriosis. The effect of Montelukast, a cysteinyl leukotriene receptor antagonist, was investigated, in a surgically induced endometriosis rat model. Montelukast significantly reduced the size of the endometrial lesions in the treated group suggesting a possible involvement of cysteinyl leukotriene in the growth of endometrial lesion (Altinbas et al. 2015).

1.5.2.2.2 12-Lipoxygenase pathway

The 12-LOX pathway consists of two 12-LOX isoforms. 12-LOX (*ALOX12*) is highly expressed in platelets and leukocytes (Funk et al., 2002), whereas 12R-LOX is encoded by *ALOX12B* gene and expressed in skin (Mashima and Okuyama, 2015). The main oxygenated product of the 12-LOX pathway is the AA derived 12 HETE.

It has been established a long time that the abnormal expression of 12-LOX (*ALOX12*) and the increased level of 12-HETE are associated with cancer. 12-LOX is upregulated in several epithelial cancers such as oesophageal, breast, lung, skin, prostate and colon cancer (Fürstenberger et al., 2006). A number of studies reported that 12-HETE promotes carcinogenesis via different routes. It is capable of inhibiting apoptosis, inducing invasion and metastasis and stimulating angiogenesis (Tang et al., 1997; Nie et al., 1998, 2003, 2006).

Although, endometriosis is a benign disorder it shares similar pathophysiological processes with cancers i.e. reduced apoptosis, increased cell proliferation and angiogenesis. Thus, there is a possibility that 12-LOX could have a role in the progression of the disease however, this route has not been discovered yet.

1.5.2.2.3 15-Lipoxygenase pathway

The human genome includes two 15-LOX genes. 15-LOX-1 is encoded on *ALOX15*, while 15-LOX-2 is the product of *ALOX15B*. The two isoenzymes show different tissue expressions and substrate specificities. 15-LOX-1 is highly expressed in reticulocytes and a certain population of leukocytes (Nadel et al., 1991; Kühn and O'Donnell, 2006), whereas 15-LOX-2 is mainly expressed in skin and prostate (Brash et al., 1997). The latter isoform, similar to other LOX isoenzymes, prefers AA as substrate and converts it exclusively to 15-HpETE (Brash et al., 1997), whist 15-LOX-1 shows different substrate specificity.

15-LOX-1 is unique in several ways compared to other LOXs. Firstly, this isoform is able to oxidize AA however, it shows a high preference for LA. Since LA is the most abundant PUFA in the body the main 15-LOX-1 product is 13-hydroperoxyoctadecadienoic (HpODE) which is metabolized rapidly to the more stable 13-hydroxyoctadecadienoic acid (HODE) and 13-oxohydroxyoctadecadienoic acid (oxoODE) (Kühn et al., 1993). Secondly, although the main substrates of this isoform are the free fatty acids, it is also capable of oxidizing esterified phospholipids and cholesterol esters or even more complex substrates such as bio membranes and lipoprotein. Of note, the oxygenation efficiency of these substrate is not as effective as the conversation of free fatty acids (Kühn et al., 1993). Lastly, 15-LOX-1 can oxygenate AA in two positions, at carbon 12 and carbon 15, resulting a mixture product of 12- and 15-HpETE (Kühn et al., 1993). This capability leads to confusion in the literature. As it was mentioned above the nomenclature of LOX isoforms is based on the oxygenated position of AA, therefore this isoform frequently termed as 12/15-LOX or 12/15-LO in the literature. Moreover, animal studies increase the confusion further. For example, *alox15* is the orthologue mouse gene of human *ALOX15*. This isoform is highly expressed in murine leukocytes and converts AA to 12-HpETE therefore, it is often called leukocyte-type 12-LOX although, the isoenzyme is the product of the *alox15* gene not alox12. Kühn suggests that the corresponding 15-LOX isoform of other species should be classified as the 12-lipoxygenating *ALOX15* isoform avoiding the confusion with the isoenzymes of 12-LOX pathway (Kühn 1996; Kühn et al. 2015).

15-LOX-1 is the focus of the research because it is involved in several pathophysiological processes such as atherosclerosis, carcinogenesis and

inflammation (Kühn et al. 2015). Accumulating evidence suggests that 15-LOX-1 has an opposite effect to 5- and 12-LOX. While, 5- and 12-LOX promote inflammation and carcinogenesis 15-LOX-1 is anti-inflammatory and promotes the resolution phase of inflammation, it also promotes apoptosis and inhibits cell proliferation in epithelial cancers such as colon, breast and lung cancers (Shureiqi and Lippman, 2001; Yuan et al., 2010). For instance, the expression of 15-LOX-1 was significantly lower in colon and lung cancer compared to normal tissue. Moreover, 15-LOX-1 expression subsequently decreased with the progression of colon cancer suggesting an important anti-tumorigenic role (Yuan et al., 2010; Lee et al., 2011).

Wittwer *et al.* (2006) reported a functional polymorphism in the promoter region of *ALOX15*. A C to T substitution was observed at position 292, which upregulated the transcription of *ALOX15* in macrophages because it had been created a new biding site for transcription factor SPI1 (Wittwer et al., 2006). Borghese *et al.* (2009) investigated this polymorphism (*ALOX15*-292 C/T) in endometriosis patients with and without fertility problems, but found no association between this polymorphism and endometriosis or endometriosis-related infertility (Borghese et al., 2009).

The main 15-LOX products, such as 13-HODE and 15-HETE, are endogenous ligands of peroxisome proliferator-activated receptor gamma (PPARγ) (Sasaki et al., 2006). PPARγ, likewise steroid receptors, is the member of nuclear receptor family and regulates gene expression and has a pleiotropic effect on several physiological and pathological processes. The activation of the receptor by its ligands exerts potent anti-inflammatory and tumour suppressor effects, such as promoting macrophage differentiation and phagocytosis, inhibition of cell adhesion and migration, inducing apoptosis, enhancing cell differentiation and down regulation of cell proliferation (Glass and Ogawa, 2006; Youssef and Badr, 2011). These mechanisms are also important elements of endometriosis pathology. However, a very few researches focusing to elucidate the role of 15-LOX pathway in endometriosis. Tomio *et al.* investigated the effect of an EPA rich diet on endometriosis using 12/15-LOX–knock out (KO) and wild type mice as animal models. EPA administration significantly reduced the number of lesions in the wild type mice compared to 12/15-LOX-KO mice; whereas it significantly increased the concentrations of EPA derived lipid mediators in the peritoneal fluid from wild type but not from 12/15-LOX-KO mice. These findings suggest that EPA administration and EPA derived lipid mediators may have inhibitory roles on the development of endometriosis (Tomio et al., 2013).

1.5.2.3 Cytochrome 450 (CYP) epoxygenases pathway

CYP epoxygenases are members of the CYP450 superfamily. They are haem-containing monooxygenase enzymes which catalyse the metabolism of PUFAs to epoxide products by incorporating an oxygen atom into a double bond of the carbon chain of fatty acids. These epoxy derivates are short-lived but potent lipid mediators which are rapidly esterified into the phospholipid membrane or subsequently hydrolysed by soluble epoxide hydrolase (sEH) to the corresponding diol metabolites (Spector and Kim, 2015). Figure 1.5.5 illustrates the biosynthesis of AA derived products via CYP450 epoxygenase pathway. Table 1.5.4 summarizes the biologically relevant precursor fatty acids along with the representative epoxy and diol products of CYP epoxygenase pathway.

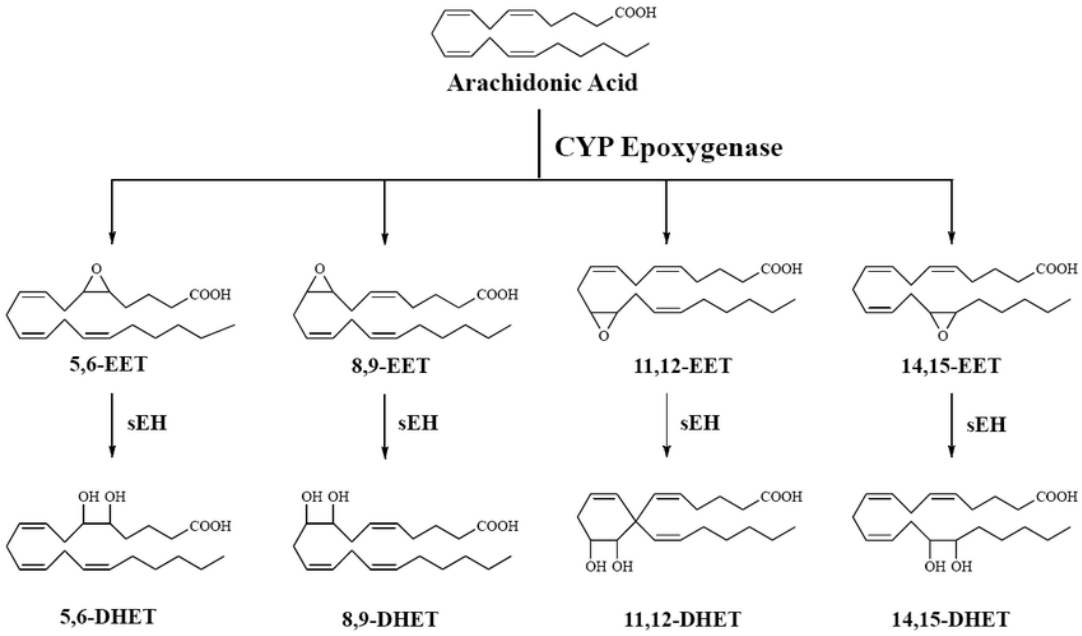


Figure 1.5.5. CYP450 epoxygenase pathway.
Biosynthesis of AA derived eicosapentaenoic acid (EET). Taken from (Sudhahar et al., 2010)

Table 1.5.4. Summary of precursor fatty acids and the representative epoxy and diol product of CYP pathways.

Precursor fatty acids			**Epoxy derivates**		**Diol products**
ω-6	AA	**CYP + O_2 + NADPH**	EET	**sEH**	DHET
	LA		EpOME		DiHOME
ω-3	DHA		EpDPE		DiHDPA
	EPA		EpETE		DiHETE

(AA) arachidonic acid; (LA) linoleic acid; (DHA) docosahexaenoic acid; (EPA) eicosapentaenoic acid; (EET) epoxyeicosatrienoic acid; (EpOME) epoxyoctadecenoic acid; (EpDPE) epoxydocosapentaenoic acid; (EpETE) epoxyeicosatetraenoic acid; (DHET) dihydroxyeicosatrienoic acids; (DiHOME) dihydroxyoctadecenoic acid; (DiHDPA) dihydroxydocosapentaenoic acid; (DiHETE) dihydroxyeicosatetraenoic acid

CYPs are highly expressed by the endothelium of vascular system. The role of AA derived epoxyeicosatrienoic acids (EET) are extensively studied in cardiovascular diseases (Fleming, 2011). In recent years, an increasing number of studies indicate that the epoxy products of PUFAs also play a role in tumour biology (Panigrahy et al., 2010). Although, the exact role of the metabolites it not completely elucidated yet, it seems the omega-6 and omega-3 derived AA and EPA epoxy products have an antagonistic effect in cancer physiology. *In vitro* studies suggest that AA derived EETs are pro-tumorigenic promoting metastasis, apoptosis and angiogenesis, whilst the EPA derived epoxyeicosatetraenoic acids (EpETE) have an opposite effect on same processes (Spector and Kim, 2015). These findings suggest that it might be worth paying attention to the CYP pathway in endometriosis research, however, this area has not been investigated yet.

1.5.2.4 Alternative pathways of lipid mediator synthesis

The main lipid mediator pathways and their primary products were illustrated in the previous sections. As it has been shown, the dysregulation of these pathways have cardinal roles in several chronic inflammatory diseases and cancer. The regulatory actions of individual lipid mediators, such as PGE_2, are intensively studied. However, the complex regulation and relations between pathways have not been completely understood. Increasing numbers of researchers suggest that alternative lipid mediator pathways also exist and their products have anti-inflammatory properties which are particularly important in the resolution phase of inflammation. For that reason these mediators are often referred as specialised pro-resolving mediators (SPMs).

SPMs are a relatively new members of lipid mediator family. They are also derivative from PUFAs and classified by their precursor fatty acids, such as AA derived lipoxins (LXs), EPA derived series-E resolvins (RvE_{1-4}) and DHA-derived series-D resolvins (RvD_{1-6}), maresins (MaR_1) and protectins (PDX, PD_1). The synthesis of these mediators is complex since more than one biosynthetic pathways are involved and consisted a transcellular step where the intermediate products are transferred into another cell (Marcus and Hajjar, 1993; Gronert et al., 1999). For instance, AA derived lipoxins (LXs) are the products of combined action of LOX pathways (Serhan and Samuelsson, 1988). Neutrophils produce and secrets LTA_4 by its 5-LOX cascade from AA. The secreted LTA_4 is taken up by platelets and converted further by its own 12-LO into LXs (Serhan and Sheppard, 1990). Figure 1.5.6 illustrates some combinations of alternative pathways and their representative products.

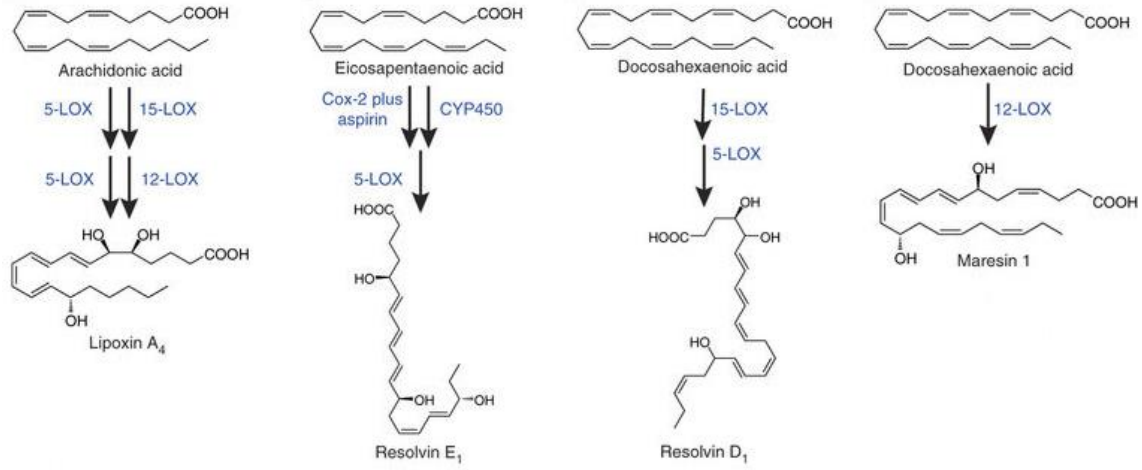


Figure 1.5.6. Alternative lipid mediator pathways and their representative products. Aspirin partial inhibitors for COX. The peroxidase function of the enzyme stays active producing LOX-like products which subsequently metabolites by other LOX pathways resulting specialised pro-resolving mediators (SPMs). Taken from (Piomelli and Sasso, 2014)
(LOX) lipoxygenase, (COX) cyclooxygenase, (CYP450) cytochrome 450 epoxygenase

This example above is a good representation demonstrating that the biosynthesis of lipid mediators is not a one-way route. It is a complex, sophisticated cascade where the pathways work as a network rather than a single line.

To date, a few studies investigated the role of SPMs in endometriosis. For instance, Tomio *et al.* (2012) using an endometriosis mouse model reported that an EPA rich diet increase the RvD_3 concentration in the peritoneal fluid and reduce the lesion size (Tomio et al., 2013). RvD_1 and 17(R)-RvD_1 treatment exerted anti-inflammatory effects and reduced signs of inflammation, including pain and oedema, in an endometrial rat model (Dmitrieva et al., 2014). Wu *et al.* (2014) investigated the effect of LXA_4 on endometrial stromal cells. They revealed that the LXA_4 treatment inhibited pro-inflammatory pathways and cell proliferation, as well suppressed the invasive capability of these cells *in vitro* (Wu et al., 2014). They also reported very recently, that LXA_4 treatment reduced the lesion size in an endometrial mouse model and had an inhibitory effect on the oestrogen-induced epithelial–mesenchymal transition of human eutopic endometrial epithelial cells (Wu et al., 2018). Kumar *et al.* (2014) found similar effects of LXA_4 on endometriosis using a mouse model. LXA_4 treatment reduced the lesion size, attenuated pro-inflammatory and angiogenic pathways and downregulated aromatase expression and oestrogen-regulated genes (Kumar et al., 2014).

1.5.3 Peritoneum as microenvironment for endometriosis

1.5.3.1 Peritoneum and peritoneal fluid

The peritoneum and peritoneal fluid provide a specific microenvironment for the pathological processes of endometriosis.

The peritoneum is derived from the mesoderm that lines the coelom (body cavity) of the embryo. Peritoneum can be described as a single layer of simple, stratified, squamous mesothelial cells which lines the inner surface of the abdominal cavity (parietal layer) and the surface of abdominal organs (visceral layer). The potential space between the parietal and visceral layers is called peritoneal cavity. Mesothelial cells of peritoneum rest on a thin basalis membrane and supported by a variable thickness, irregular connective tissue. Peritoneum along with its connective tissue also refer to as serosa or peritoneal wall (Blackburn and Stanton, 2014).

The well-recognised roles of peritoneum include providing a protective barrier and a frictionless surface for the abdominal organs as well as to contributing the homeostasis in the abdominal cavity by regulating serosal response to injury, infections and diseases. Disturbed regulation of these physiological processes might promote the establishment and the progression of endometriosis. Aetiological theories propose that mesothelial cells might have a key role in the onset of endometriosis since they are actively interact with other cells of the peritoneum, e.g. leukocytes, secreting mediators, growth factors, cytokines as well expressing receptors and adhesion molecules to promote the serosal immune response (Mutsaers, 2002). For instance, the adhesion molecules, such as intercellular adhesion molecule 1 (ICAM-1) and the vascular cell adhesion molecule 1 (VCAM-1) are members of integrin adhesion proteins and they are essential in cell-cell interactions, also in the activation of immune responses by binding and activating leukocytes and promoting the leukocyte recruitment into the abdominal cavity (Young et al., 2013). The expression of these adhesion molecules increases in the inflammatory environment. Jonjic *et al.* (1998) found that proinflammatory cytokines, such as interferon γ (IFNγ), tumour necrosis factor α (TNFα) upregulate the expression of ICAM-1 and VCAM-1 in mesothelial cells *in vitro* (Jonjić et al., 1992). Wu et al (2004) also demonstrated that visually normal peritoneum and the ovarian endometrioma from endometriosis patients highly express ICAM-1 mRNA. Moreover, these peritoneal cells also secrete soluble ICAM-1 (sICAM-1) (Wu et al., 2004). The overexpression of sICAM-1 in peritoneal fluid from women with endometriosis could interfere the cytotoxic reaction of natural killer cells (NK) resulting the escape under the immune surveillance and promoting the disease establishment (Tariverdian et al., 2007).

Mesothelial cells are a source of a variety of extracellular matrix molecules including matrix metalloproteinases (MMPs) and their inhibitors called tissue inhibitor of metalloproteinases (TIMPs). These proteinases, comprise collagenases and gelatinases and are pivotal to endometrial turnover, menstruation, tissue repair and remodelling of the extracellular matrix, also for the activation of a number of bioactive molecules, receptors and ligands which have an effect on the cell behaviour (Sternlicht and Werb, 2001). Studies reported impaired MMPs expression in endometriosis (Osteen et al., 2003). For instance, MMP-1 was expressed in red peritoneal lesions and ovarian endometrioma, but not in black lesions and DIE, suggesting the roles of MMP-1 in the early stage of the disease and different pathomechanisms for the types of endometriosis (Kokorine et al., 1997).

Overall, dysregulation of adhesion molecules and MMPs could promote angiogenesis, cell proliferation as well as the invasion of the mesothelium, thus they could be important factors in the attachment of endometriotic tissues to the peritoneal surfaces (Young et al., 2013).

Mesothelial cells produce peritoneal fluid from plasma as a transudate. Peritoneal fluid is responsible for reducing the friction between peritoneal surfaces and the exchange of nutrients. In women, beside the mesothelial cells peritoneal fluid is produced by ovarian exudation and increased vascular permeability. The volume of peritoneal fluid depends on the local oestrogen concentration and changes with the menstrual cycle. It progressively increases during the follicular phase and reaches maximum (20 ± 6.3 ml) in the early luteal phase (Koninckx et al. 1980). The peritoneal fluid contains steroid hormones, enzymes, immune cells, cytokines, growth and angiogenic factors. The concentration of these factors in the peritoneum often differs from the plasma concentration. For example, at ovulation the oestradiol concentration could be 100 fold higher in the peritoneal fluid than in the plasma. The progesterone concentration, similar to oestrogen is also much higher in the peritoneal fluid (Koninckx et al. 1980). Several factors are being produced locally therefore they have higher concentration in the peritoneal fluid than in the plasma. They also act locally and have a major impact in autocrine and paracrine regulation.

1.5.3.2 Peritoneal fluid cells

Peritoneal fluid contains a wide variety of free floating cells, including macrophages, mesothelial cells and lymphocytes (Oral et al., 1996). Up to 85-90% of peritoneal leukocytes are macrophages (Haney et al. 1981; Syrop & Halme 1987). The macrophages have central role in the immune homeostasis of peritoneum. They activate the natural and adaptive immune systems and remove cellular debris and pathogens. Peritoneal macrophages are increased in women with endometriosis

compared to healthy or infertile controls (Haney et al., 1981). Hill *et al.* found that the total leukocytes, particularly macrophages, NK cells and helper T cells (Th) significantly increased in the early stage of the disease suggesting an active immune response and immunologically dynamic peritoneal environment (Hill et al., 1988).

1.5.3.3 Peritoneal cytokines

The peritoneal leukocytes are a key source of several cytokines, prostaglandins, proteolytic enzymes, growth and chemotactic factors. The most important peritoneal factors are IL-1, IL-6, IL-8, IFNγ, TNFα, transforming growth factor β (TGFβ), vascular endothelial growth factor (VEGF), Regulated on Activation, Normal T Cell Expressed and Secreted (RANTES) and monocyte chemotactic protein 1 (MCP-1) secreted by endometriotic stromal cells, neutrophils and macrophages (Khorram et al., 1993; Akoum et al., 1996; Donnez et al., 1998). These factors are responsible for the regulation of local inflammatory processes and angiogenesis. Beside the immune regulation they also influence the local steroidogenic and prostaglandin synthesis. For instance IL-1, IFNγ and TNFα directly stimulate the ovarian steroidogenesis (Adashi, 1990; Fukuoka et al., 1992). IL-6 increases aromatase expression *in vitro* in stromal and adipose cell cultures from women with endometriosis (Velasco et al., 2006). The high level of IL-1β and TNFα increase prostaglandin levels by stimulating the overexpression of COX-2 in peritoneal macrophages (Wu et al., 2002). In addition, the steroid hormones, such as oestrogen, regulate the gene expression of cytokines.

1.6 Pathomechanism

Endometriosis is often refered to as an enigmatic disease. This is largely because all aspects of the pathogenesis are still not known. Several underlying pathomechanisms are now identified and used as therapeutic targets but a complete understanding is still necessary.

The most plausible theory is Sampson's retrograde menstruation. Based on this theory the main steps of establishment of the disease are: viable cells or tissues that reach the abdominal cavity, attach to the surface of peritoneum or ovary, invade into the deeper layers, proliferate and establish a neovascular system to support survival of the lesions. Parallel with these steps, the endometrial cells constantly escape under the immune surveillance and induce inflammation.

Retrograde menstruation provides a good explanation of how the endometrial cells reach the peritoneum, however along with these cells other cells, such as red blood cells or bacteria also reach the abdominal cavity (Sampson, 1927; Khan et al., 2016). Haemoglobin from lysed red blood cells or bacterial endotoxins, such as lipopolysaccharides (LPS) from gram negative bacteria, could induce oxidative stress and inflammation in the peritoneum. Moreover, long-term exposure to these factors might be able to induce metaplasia in mesothelial cells leading to the establishment of endometrial lesions. Studies have reported elevated levels of haemoglobin in peritoneal fluid from women with endometriosis compared to those without (Van Langendonckt et al., 2002). Also increased concentrations of bacterial endotoxin in peritoneal and menstrual fluid specimens from endometriosis patients has been reported. The highest level of endotoxin was observed during menstruation but a low concentration of bacterial endotoxin was persistently present in the peritoneal fluid from women with endometriosis suggesting a constant exposure to induction factors for inflammation or metaplasia (Khan et al., 2010) (Figure 1.6.11.6.1).

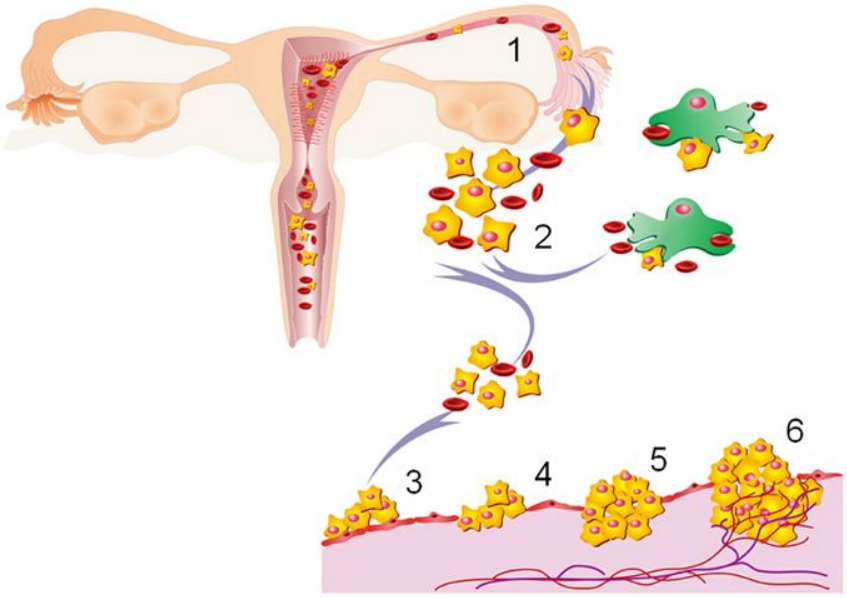


Figure 1.6.1. Schematic picture of retrograde menstruation.
(1) Endometrial cells and tissue along with red blood cells and bacteria reach the peritoneal cavity through the fallopian tubes. (2) Endometrial cells and tissue evade the immune surveillance system. (3) Ectopic lesions established by endometrial cells attach to the peritoneal mesothelial lining or via coelomic metaplasia induced by inflammation and external factors. (4) Ectopic lesions invade into the surface, (5) proliferate (6) and acquire a blood supply. Picture taken from Lousse et al., 2012 (Donnez, 2012).

1.6.1 Peritoneal cells with impaired functions

Dysfunction of the innate immune system is now recognised in the pathogenesis of endometriosis since the immune system fails to eliminate the misplaced endometrial cells, tissues or lesions from the peritoneum and to restore the homeostasis (Oral et al., 1996; Matarese et al., 2003). An increased number of activated macrophages were reported in the peritoneum of women with endometriosis (Haney et al., 1981; Hill et al., 1988; Oosterlynck et al., 1993). Moreover, it was also observed that the effector functions, such as phagocytosis and cytolysis of these cells were impaired (Koninckx et al., 1998).

Peritoneal macrophages from women with endometriosis could promote the establishment and the progression of the disease in several ways. Studies reported that the phagocytotic activities of these cells against endometrial cells were decreased. For instance, macrophages express scavenger receptors to strengthen their phagocytosic activity. CD36 also known as thrombosponin-1 (TSP-1) receptor or fatty acid translocase, is a particulary important multi-ligand scavenger receptor on macrophages (Febbraio et al., 2001). Chuang *et al.* (2009) found that the expression of CD36 was reduced in peritoneal macrophages derived from patients with endometriosis. The decreased expression of CD36 significantly suppressed the phagocytotic activity of these macrophages (Chuang et al., 2009). They also

demonstrated that PGE_2 was able to inhibit the expression of CD36, thus this prostaglandin might be responsible for the decreased phagocytotic activity of macrophages (Chuang et al., 2010).

Macrophages also secret several types of enzymes, such as MMPs, breaking down the extracellular matrix of cells and facilitate their elimination by phagocytosis (Sternlicht and Werb, 2001). For example MMP-9 is an important gelatinase produced by macrophages since it degrades the basal membrane between stromal and epithelial cells during the menstruation. Wu *et al.* (2005) found that the peritoneal macrophages, isolated from women with endometriosis, have decreased levels of protein and enzyme activity of MMP-9 and the treatment of peritoneal macrophages with PGE_2 significantly inhibited MMP-9 activity. Therefore, they speculated that the decreased expression and activity of MMP-9 in peritoneal macrophages might lead to suppressed phagocytotic capability in women with endometriosis because the dysfunctional enzyme may not be able to degrade the targets for phagocytosis (Wu et al., 2005).

Studies have also found that peritoneal macrophages have an increased resistance to apoptosis. Mclaren *et al.* (1997) reported that Bcl-2, an antiapoptotic protein was overexpressed in peritoneal macrophages from women with endometriosis suggesting a possible escape mechanism from programmed cell death for these cells (McLaren et al., 1997). As a consequence, activated macrophages with an extended life time could participate in the disease establishment and the maintance of the inflammatory enviroment by secreting a plethora of immune modulators, growth and angiogenic factors (Vinatier et al., 2001).

1.6.2 Proliferation of endometrial lesions

Endometriosis is considered as a benign disorder however its pathophysiology demonstrates multiple similarities to the pathomechanism of cancer. Like cancer cells, endometrial cells escape under the immune survillance, attach, invade into the surface where they adapt and grow in a new microenviroment.

To date, the mechanism of disease onset is not known. Aetiological theories proposed that endometrial cells can reach the peritoneum by several routes, such as retrograde menstruation, blood or lymphatic spread or going through metaplasia *in situ*. On the other hand it is known that endometrial cells, likewise cancer cells, also establish their own microenviroment, referred to as a niche, and acquire metabolomic changes promoting their own proliferation and blood supply.

As was mentioned earlier, the main metabolomic difference between ectopic and eutopic endometrial tissue is the capability of oestrogen synthesis. Thus, similarly to some cancers, such as uterine or breast cancer, endometrioid ectopic lesions also can be considered as oestrogen-dependent neoplasms (Varma et al., 2004).

Steroid hormones are potent mediators therefore their biosynthesis is strictly regulated. The normal eutopic endometrium is not capable of synthesising oestrogen, moreover it cannot be induced by PGE_2 or other inflammatory stimulants (Tsai et al., 2001). In contrast, the ectopic endometrial lesions express the whole complement of enzymes to produce oestradiol from cholesterol. Additionally they are lacking of the 17β-HSD2 enzyme. As a result of the lack of this enzyme they maximaze the local effect of oestradiol as they not capable to suppress the effect of oestradiol by the conversion to the less potent oestrone (Bulun 2009).

One of the consequences of the abnormal oestrogen synthesis is to support the proliferation and angiogenesis of the implants by activating and regulating growth factors, e.g. FGF-9 and VEGF (Wing et al., 2003; Lin et al., 2006). The other significant event would be to increase the resistance of cells against apoptosis. Apoptosis has a key role to regulate the homeostasis by eliminating the excess or dysfunctional cells (Harada, 2004). The anti-apoptotic effect of oestradiol is well established. Under physiological conditions the expression of anti-apoptotic *BCL2* gene in eutopic endometrium correlates with the level of oestradiol suggesting a oestrogen dependent regulation (Gompel et al., 1994; Otsuki et al., 1994). Studies reported increased Bcl-2 in eutopic endometrium from women with endometriosis also in ectopic lesions (Watanabe et al., 1997; Jones et al., 1998). The reduced apoptotic capability of endometrial cells could contribute to the survival of endometrial cells in the peritoneum. Zubor *et al.* (2009) found that the mRNA expression of *BCL* genes in eutopic endometrium was significantly increased in patients with endometriosis, moreover it was correlated with the severity of the disease (Zubor et al., 2009). While Watanabe *et al.* (1997) reported that the cyclic changes of Bcl-2 were not observed in ectopic lesions (Watanabe et al., 1997).

As was demonstrated ectopic lesions support their own proliferation by produce oestradiol locally. However, the pro-inflammatory enviroment also has a pivotal role to enhance the local oestrogen synthesis further by regulating the rate-liming steps of the synthesis. Studies found that PGE_2 is a potent inducer for StAR and P450arom in endometriotic stromal cells (Noble et al., 1997; Tsai et al., 2001); thus PGE_2 can be one of the main promoters of oestrogen synthesis in ectopic lesions.

1.6.3 PGE_2 as the master regulator for the proliferation of ectopic lesions

A number of studies reported elevated concentration of PGE_2 in peritoneal fluid from women with endometriosis (Dawood et al., 1984; De Leon et al., 1986; Li et al., 2005). High levels of PGE_2 are caused by the overexpression of COX-2 in endometrial stroma and peritoneal macrophages (Ota et al., 2001; Wu et al., 2002). These studies found that COX-2 expression in peripherial blood-derived macrophages was not elevated in women with endometriosis. Therefore, the peritoneal environment must be responsible for the overexpression of COX-2 (Wu et al., 2002).

The transcription of the *COX-2* gene is strictly regulated and induced by cytokines. Macrophages release proinflammatory cytokines, such as IL-1β or TNF-α (Keenan et al., 1995). These cytokines induce the expression of COX-2 in macrophages in an autocrine and paracrine manner. Thus, the macrophages are partly responsible for the high PGE_2 level. On the other hand autotriggering positive feedback loops also exist and induce COX-2. These loops keep the concentrations of PGE_2 and oestradiol high in the peritoneum and favour the survival and progression of endometrial lesions. (Figure 1.6.21.6.2).

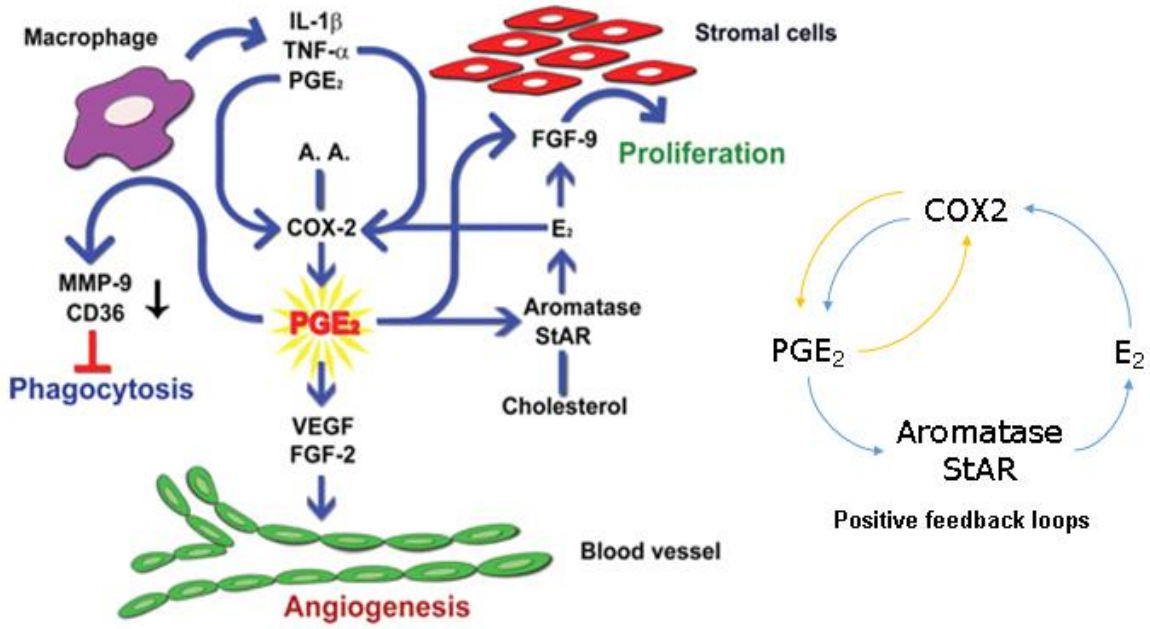


Figure 1.6.2. Proposed model of the pathogenesis to endometriosis with the positive feedback loops.
(PGE_2) prostaglandin E_2; (IL-1β) interleukin-1 beta (TNFα) tumour necrosis factor-alpha; (COX-2) cyclooxygenase-2; (MMP) matrix metalloproteinase; (VEGF) vascular endothelial growth factor; (FGF) fibroblast growth factor; (E_2) oestradiol; (StAR) steroidogenic acute response protein. Taken from Wu et al. (2010) (Wu et al., 2010).

There are two loops. The first loop is the PGE_2 – oestradiol – COX-2 - PGE_2. The high level of PGE_2 induces the expression of StAR and P450arom promoting the biosynthesis of oestradiol. While the elevated oestrogen levels increase the transcription of COX-2 via its nuclear receptors, which increases the PGE_2 level further (Xue et al., 2007; Bulun et al., 2012). The other positive feedback loop is PGE_2 – COX-2 - PGE_2, due to PGE_2 being capable inducing the expression of COX-2 by an autocrine/paracrine manner (Tamura et al., 2002). Thus the high PGE_2 level itself is responsible for the overexpression of COX-2. This model composes the main pathological processes of endometriosis and emphazises the central role of PGE_2. The high level of PGE_2 has an impact on immune respones, as well as on the progression of the lesions. These processes, at least partly, explain how the immune system fails to clear the tissue fragment from the peritoneum.

1.6.4 Role of lipid mediators in the inflammation process

Inflammation is the key element of endometriosis pathology. As was demonstrated above, autotriggering positive feedback loops to maintain the pro-inflammatory enviroment leading to chronic inflammatory status. Study of acute inflammation is necessary for better understanding of chronic inflammatory processes.

Pathologists divide the acute inflammatory response into two parts such as inititation and resolution. Resolution was belived to be a passive process. However, increasing data suggest that the resolution is an active pathway where the specialised pro-resolving mediators (SPMs) such as lipoxin, resolvin, protectin and maresin have key roles (Serhan, 2007).

Prostanoids and leukotrienes induce the initiation of an acute inflammatory response causing the cardinal signs, i.e. rubor (redness), calor (heat), tumor (swelling) and dolor (pain) of inflammation. Briefly, in response to an injury or trauma, cells release eicosanoids into extracellular enviroment. TXA_2 and CysLTs cause vasoconctriction isolating the damaged area, while other eicosanoids, such as PGE_2 and PGI_2 have opposite effects causing vasodilatation and increased vascular permeability. LTB_4 recruits polymorphonuclear leukocytes (PMNs), such as neutrophil granulocytes to the scene of inflammation. These leukocytes and the surrounded cells, produce further mediators to amplify the initial inflammatory response, activate the monocyte-derived macrophages and adaptive immune cells (Buckley et al., 2014).

The resolution pathway settles down the inflammation by clearing initiating stimuli, suppression of pro-inflammatory signals, inactivation and elimination of PMNs by apoptosis and non-inflammation induced phagocytosis (efferocytosis). In addition it activates the class of pro-resolving macrophages, also refer to as M2 macrophage,

and other resolving cell types such as regulatory T cells (Treg); as well as promote the tissue repair and normalisation of homeostasis (Tabas and Glass, 2013). Figure 1.6.31.6.3 illustrates the stages of inflammatory response.

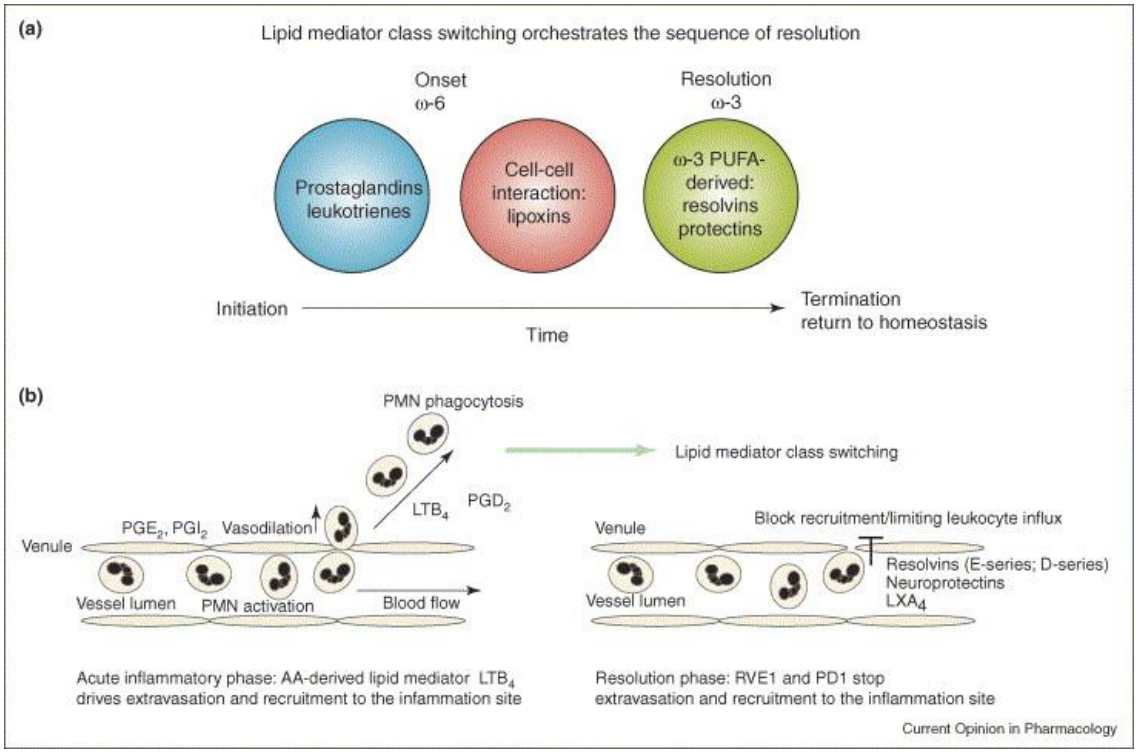


Figure 1.6.3. Schematic flow of acute inflammatory response.
(a) Proposed sequence of bioactive lipid mediators in acute inflammation: onset (initiation, acute phase of inflammation) led by omega-6 prostanoids and leukotrienes towards resolution (termination, return to homeostasis) regulated by omega-3 derived specialised pro-resolving mediators (SPMs). (b) Outline of the main steps of acute inflammatory response: switching from pro-inflammatory (PGE_2, PGI_2, LTB_4) to anti-inflammatory and pro-resolution. Picture taken from Schwab & Serhan 2006 (Schwab and Serhan, 2006).

The starting point of the resolution is a lipid mediator class switch. The emphasis from the omega-6 prostanoids and leukotriene production in neutrophil shifts to SPMs. The presence of PGE_2 is essential in this class switch because it induces the LOX pathways in neutrophils to produce SPMs (Levy et al., 2001). Blocking of COX-2 with selective inhibitors delays the resolution of inflammation. Without PGE_2 the resolution is disturbed and the inflammation more likely to be perpetuated. Therefore this finding suggests that the administration of COX-2 inhibitors must be considered in the later phage of inflammation because the lack of PGE_2 during the class switch could lead to disturbed and delayed resolution (Chan and Moore, 2010).

After the class switch SPMs dominates and actively lead the resolution phase (Serhan, 2007). In the first instance, SPMs inhibit the neutrophil influx and stimulate the non-phlogistic monocytes recruitment to the scene of inflammation (Serhan et al., 2002, 2009; Bannenberg et al., 2005); this step promotes the phenotype switch of macrophage from inflammatory (M1) to non-inflammatory (M2) (Maderna and

Godson, 2009). The resolving macrophages are necessary in the clearance of apoptotic neutrophil granulocytes and cellular debris (Mitchell et al., 2002). This clearance promotes the repair and regeneration of tissues and is crucial to restoring homeostasis. Failures in this process delay the resolution of inflammation and may lead to chronic diseases (Serhan, 2007). Therefore the better understanding of resolution pathway is necessary to the development of new treatments in chronic inflammatory condition like endometriosis.

1.6.5 Peroxisome proliferator-activated receptor γ (PPARγ)

Peroxisome proliferator-activated receptor γ (PPARγ) is a ligand dependent transcripton factor that belongs to the nuclear receptor superfamily. Oxygenated lipid mediators, such as 15d-PGJ_2, HETEs, HODEs and oxoODEs serve as endogenous ligands for PPARγ (Forman et al. 1995; Nagy et al. 1998). PPARγ acts as a heterodimer with retinoid X receptor (RXR). In a steady state, the heterodimer of PPARγ and RXR binds to the peroxisome proliferator response elements (PPREs) in the nucleus. Upon ligand binding it activates or represses the transcripton of their target genes by recruiting further coregulator transcription factors (Nolte et al., 1998; Glass and Ogawa, 2006).

PPARγ was originally described as the central regulator for adipocyte differentiation (Tontonoz et al., 1994). Later on it was found that insulin-sensitizer drugs, such as thiazolidinediones (TZDs), act through PPARγ proving the importance of this receptor in the glucose metabolism (Lehmann et al., 1995). Then, it was reported as the key regulator for monocyte and macrophages differentiation and anti-inflammammatory pathways (Nagy et al. 1998; Ricote et al. 1998; Tontonoz et al. 1998).

Today, PPARγ is in the focus of research due to it being a promising target for chronic inflammation and cancer treatment. Large numbers of studies demonstrated that PPARγ activation inhibits cell proliferation and angiogenesis but promote cell differentiation and apoptosis in a variety of cancers (Youssef and Badr, 2011). Furthermore, its own anti-inflammatory properties suppress the pro-inflammatory cytokine expression via nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) in chronic conditions such as colitis or multiple sclerosis (Su et al., 1999; Genolet et al., 2004; Storer et al., 2005). These findings indicate that PPARγ could be a promising target for endometriosis treatment. However, only a few studies have, to date, investigated the effects of synthetic PPARγ agonists in the pathophysisology of endometriosis. Nevertheless the results were encouraging since the synthetic PPARγ agonists significantly reduced the numbers and size of endometrial-like lesions in rat and baboon models (Lebovic et al., 2004, 2010; Aytan et al., 2007).

1.7 Aims and Objectives

The overall aim of this project is to investigate the inflammatory peritoneal microenvironment of women with and without endometriosis. A large number of studies concentrate on the proinflammatory environment of the disease (Wu and Ho, 2003; Wu et al., 2010; Manolova et al., 2011; Donnez, 2012). The role of prostanoids, particularly PGE_2 are well established in these proinflammatory processes. However, little attention has been paid to other lipid mediators. To date, there is a complete lack of research about the role of CYP products apart from a few animal experiments on the role of LOX metabolites in the endometriosis pathology. In addition, what is not yet understood in the pathophysiology of endometriosis is the effect of the chronic pro-inflammatory environment on the anti-inflammatory mechanisms. In particular how these anti-inflammatory mechanisms fail to downregulate inflammatory processes, eliminate the endometrial lesions and restore the homeostasis of the peritoneum. As a result, the aims and objectives of this thesis were:

1.) Mass spectrometry study:

To simultaneously measure 79 oxygenated lipid mediators derived from COX, LOX, CYP and non-enzymatic pathways in peritoneal fluid, peritoneal washes and in venous plasma samples from women diagnosed with endometriosis compared to those without the disease. Lipid mediator profiles of peritoneal specimens will offer insight into the local pathophysiological processes in the peritoneum and their systemic effects will be assessed by analysing lipid mediator profiles in plasma. Furthermore, potential differences in plasma profiles between patients with and without endometriosis could lead to the identification of a new, non-invasive biomarkers for diagnosing endometriosis.

2.) Measuring pro-inflammatory cytokines in the peritoneum and vascular system:

Pro-inflammatory cytokines are some of the key players in the pathomechanism of endometriosis. To gain information about the endometriosis-associated inflammatory milieu and possible biomarkers, pro-inflammatory cytokines were measured in peritoneal fluid, washes and venous plasma from women with and without endometriosis.

3.) Gene expression study:

To study the expression of PPARγ and its relationship to COX and 15-LOX pathways in the peritoneum, peritoneal cells, ectopic lesions, eutopic endometrium also in

vascular system. Metabolites of COX and 15-LOX pathways are natural ligands for PPARγ. Dysregulation of PPARγ could promote the progression of endometriosis in several ways since PPARγ, a key regulator of macrophage functions, has a protective role in the regulation of inflammation also a key inhibitor for uncontrolled cell proliferation. This would indicate whether PPARγ contributes to the establishment, progression or risk of developing the disease.

4.) Immunohistochemical analysis:

To confirm the findings of the gene expression study by studying the proteins of genes of interest using immunohistochemical staining and investigating the expression of these proteins in the peritoneum, ectopic lesions and in eutopic endometrium.

2 CHAPTER: Materials and Methods

2.1 Clinical specimens

Clinical specimens were collected from consented women (age range: 18-60 years) with and without endometriosis who were undergoing laparoscopic surgery at the Central Manchester University Hospital. All human tissue collected from donors had full ethical approval and was handled in accordance with the requirements of the Human Tissue Act 2004. Ethical approval was obtained from the Local Regionals Ethics Committee: NRES Committee South West - Cornwall & Plymouth (REC ref no. 13/SW/0123). Each donor gave informed written consent prior to their surgical procedure (Appendix 1). Patients were asked about their period, pain symptoms, medical history, including recurrence and pharmacotherapy by the clinicians and all information was recorded on the Patient information sheet (Appendix 2). A summary of patient information is detailed in the appendix (Appendix 3).

Blood samples were collected in sodium heparin vacutainers (BD vacutainers®, Nu-Care) and Tempus™ blood RNA tubes (Applied Biosystems) in the morning of the surgery. Blood tubes were kept on ice and transferred into the laboratory within an hour. Heparin vacutainers were centrifuged at the speed of 2500g for 10 minutes at 4°C to separate plasma. Plasma was aliquoted and snap frozen on dry ice. The leukocyte contained buffy coat was collected with a Pasteur pipette from the top of red blood cells, centrifuged at 1500g for 10 minutes at 4°C and snap frozen. Tempus™ blood RNA tubes were subjected to RNA extraction on the same day. For the RNA extraction procedure see section 2.3.3.3.

Peritoneal fluid was aspirated from the pouch of Douglas at the beginning of the surgical procedure. Where peritoneal fluid was not present, the peritoneal cavity was flushed with isotonic buffer solution and collected as a peritoneal wash. Specimens were kept on ice and transferred into the laboratory after the surgery. Peritoneal fluid and washes were centrifuged at 1500g for 10 minutes at 4°C, separating the fluid specimen and the pellet of peritoneal cells. The supernatant was aliquoted and snap frozen. The peritoneal cell pellet was resuspended in RNAlater® (Ambion) incubated for 2 hours at room temperature, centrifuged at 1500g for 10 minutes then was frozen on dry ice.

The following solid tissue specimens were collected: pipelle biopsy from the functional layer of the eutopic endometrium, omental fat from all participants, and ectopic lesions and peritoneal wall biopsies from women with endometriosis. The type and the location of ectopic lesions were reported by the surgeon. Tissue specimens were dissected during the surgery and placed into phenol free DEME/F12 media (Gibco, USA) containing 1% of penicillin-streptomycin (Sigma-Aldrich, Poole, United

Kingdom). All specimens were kept on ice and transferred into the laboratory after the surgery.

In the laboratory, the tissues specimens were subsampled under aseptic condition by the laboratory staff. Samples were taken for the following methods: immunohistochemistry (the tissue preparation are detailed in section 2.4.2), mass spectrometry, gas chromatography and qPCR, or were used fresh as whole tissue for cell culture. Every method required at least 0.5 to 2g of tissue. For mass spectrometry and gas chromatography the subsamples were placed in an Eppendorf tube and snap frozen on dry ice. Tissues for qPCR were incubated in RNAlater® overnight at 4°C and were snap frozen on the following day. Of note, due to the size of the biopsies and the numbers of method specimens were not tested by all methods. The specimens used for this study are summarized according to assays in the Appendix 4.

All specimens were stored until assayed at -80°C in human tissue dedicated freezer.

2.2 Mass Spectrometry Analysis of Lipid Mediators

2.2.1 Overview of mass spectrometry

Electrospray ionisation liquid chromatography tandem mass spectrometry (LC/ESI-MS/MS) is frequently used in lipidomics studies since it is a highly sensitive, robust tool for the simultaneous analysis of multiple lipid mediators in various biological materials. Mass spectrometry (MS) methods measure the mass-to-charge (*m*/*z*) ratio to identify molecules and provide structural information for the compound of interest. MS is often combined with high performance liquid chromatography (HPLC). HPLC is a powerful technique that separates the components in a complex mixture prior to the MS analysis. Although this separation increases the specificity of the method, it also gives rise to some problems. The separated components are in soluble phase and are not well ionized. Therefore a third system, the electrospray ionisation (ESI) links HPLC to MS by ionising molecules and evaporating the solvent. ESI is a mild ionization method which is suitable for ionization of large biological molecules *i.e.* lipids and lipid mediators without causes major fragmentation before the MS analysis (Chatfield, 2004).

The sensitivity and specificity of the method can be increased by pairing of the mass analysers. This spectrometry is referred as tandem MS (MS/MS). The spectrometer can operate in two main mode. In full scan mode, the mass range is set to wide and provides qualitative information about the components of a complex material. In selected decomposition monitoring, a narrow mass range is scanned and the fragmented ions are detected. This mode gives structural and quantitative information of the tested materials. The selected decomposition monitoring can be performed in different ways. Selected Reaction Monitoring (SRM) is the most conventional MS technique for quantitative determination of the analyte of interest. SRM scans a selected mass, typically the molecular mass of the analyte and detects the product ions. In brief, the principle is as follows: the ionisation source of mass spectrometer ionises the molecules. These ions travel through the analyser. In MS/MS the analyser is a linear triple quadrupole system where the first and the third quadrupoles operate as mass filters. The first quadrupole (MS1 or Q1) selects the analyte of interest. The second quadrupole (Q2) is a collision cell where the selected ion, also called precursor ion, undergoes further fragmentation by collision-induced dissociation (CID). The third quadrupole (MS2 or Q3) identifies the pre-selected daughter ion from CID. This ion arrives at a different part of the detector and is detected according to its *m/z* ratio. The generated signal is converted by the computer and appears as a mass spectra on the display. Multiple Reaction Monitoring (MRM) is the specific application of SRM. In that case, one or more precursor ions

are selected by MS1 and multiple products monitored by MS2 (Chatfield, 2004). This application provides the highest sensitivity and specificity for the quantification of multiple analytes. Figure 2.2.1 illustrates the schematic overview of LC/ESI-MS/MS system.

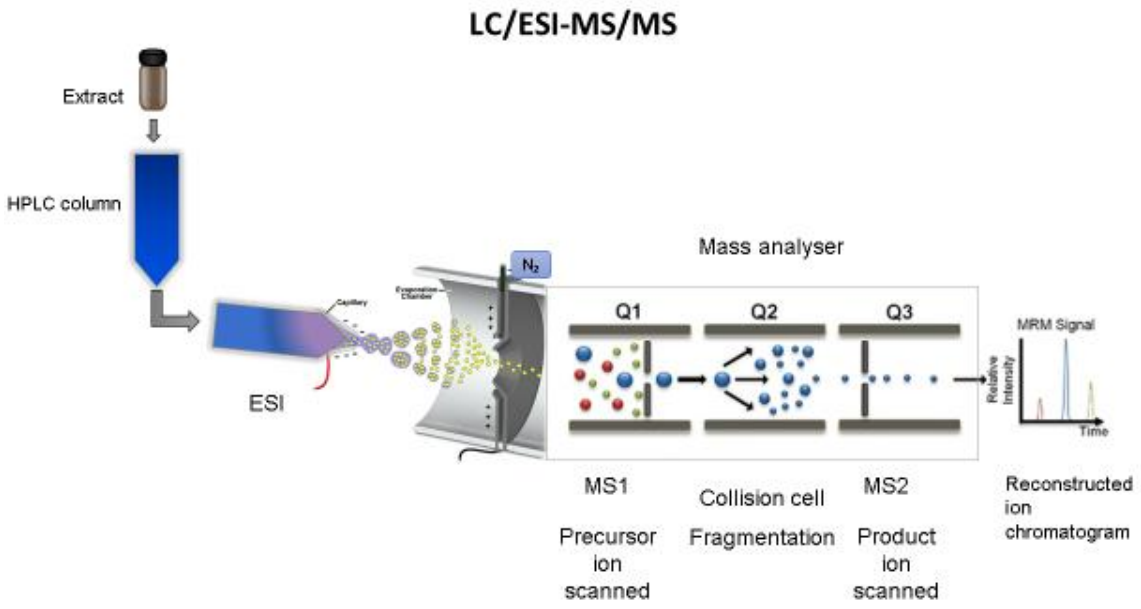


Figure 2.2.1. Schematic overview of electrospray ionisation liquid chromatography tandem mass spectrometry (LC/ESI-MS/MS) system in selected reaction monitoring (SRM) mode. High performance liquid chromatography (HPLC) separates the components of lipid extract. Separated components are vaporised and ionised by electrospray ionisation (ESI) system. The ionised components passed through the mass analyser. The mass analyser consists three quadrupoles. The first quadrupole is the first mass spectrometer (MS1 or Q1) selecting the specific ion masses of analyte and pass through the selected ion into the second quadrupole (Q2) where the precursor ion fragmented by collision-induced dissociation. The third quadrupole is the second mass spectrometer (MS2 or Q3) selects the specific ion masses of the targeted fragment. This product ion hits the detector and appears as a spectra on the screen.

2.2.2 Experimental description

The protocol developed by Masoodi and Nicolaou to measure oxygenated lipid mediators and their related species in brain, liver and plasma specimens was adapted for this study (Masoodi and Nicolaou, 2006; Masoodi et al., 2008). 79 lipid species were measured in biological fluid specimens. Table 2.2.1 shows the list of tested mediators classified by precursor fatty acids.

Table 2.2.1. Prostanoids, leukotrienes, hydroxy fatty acids and related lipid mediators identified by LC/ESI-MS/MS.

LA/ALA/DGLA-derived	AA-derived	EPA-derived	DHA-derived
LA:	PGD_2	RvE1	RvD1
9-HODE	PGE_2	PGD_3	RvD2
13-HODE	$PGF_{2\alpha}$	PGE_3	MaR1
9-oxoODE	PGI_2 (as 6-keto $PGF_{1\alpha}$)	TXB_3	PDX
13-oxoODE	15-keto PGE_2	± 5-HEPE	± 4-HDHA
9(10)-EpOME	15-keto $PGF_{2\alpha}$	± 8-HEPE	± 7-HDHA
12(13)-EpOME	13,14-dihydro $PGF_{2\alpha}$	± 9-HEPE	± 10-HDHA
9,10-DiHOME	13,14-dihydro-15-keto PGE_2	± 11-HEPE	± 11-HDHA
12,13-DiHOME	13,14-dihydro-15-keto $PGF_{2\alpha}$	± 12-HEPE	± 13-HDHA
Trans-EKODE	8-iso $PGF_{2\alpha}$	± 15-HEPE	± 14-HDHA
	PGJ_2	± 18-HEPE	± 17-HDHA
ALA:	Δ^{12}-PGJ_2		± 20-HDHA
9-HOTrE	15-deoxy-$\Delta^{12,14}$-PGJ_2		19,20-DiHDPA
13-HOTrE	TXB_2		16(17) EpDPE
	± 5-HETE		19(20) EpDPE
DGLA:	± 8-HETE		
15-HETrE	± 9-HETE		
PGD_1	± 11-HETE		
PGE_1	± 12-HETE		
$PGF_{1\alpha}$	± 15-HETE		
13,14-dihydro PGE_1	± 20-HETE		
13,14-dihydro $PGF_{1\alpha}$	LTB_4		
13,14-dihydro-15-keto PGE_1	± 14,15-DHET		
13,14-dihydro-15-keto $PGF_{1\alpha}$	± 11,12-DHET		
	± 8,9-DHET		
	± 5,6-DHET		
	± 5(6)-EET		
	± 11(12)-EET		
	± 14(15)-EET		
	± 8(9)-EET		
	5-oxoETE		
	HXA3		
	5,15 DiHETE		
	8,15 DiHETE		

(HODE) hydroxyoctadecadienoic acid; (oxoODE) oxooctadecadieonic acid; (EpOME) epoxyoctadecenoic acid; (DiHOME) dihydroxyoctadecenoic acid; (trans-EKODE) transepoxyketooctadecenoic acid; (HOTrE) hydroxyoctadecatrienoic acid; (HETrE) hydroxyeicosatrienoic acid; (PG) prostaglandin; (HETE) hydroxyeicosatetraenoic acid; (LT) leukotriene; (DHET) dihydroxyeicosatrienoic acid; (EET) epoxyeicosatrienoic acid; (HX) hepoxilin; (DiHETE) dihydroxyeicosatetraenoic acid; (Rv) resolvin; (MaR) maresin; (PDX) protectin; (TX) thromboxane; (HEPE) hydroxyeicosapentaenoic acid; (HDHA) hydroxydocosahexaenoic acid; (DiHDPA) dihydroxydocosapentaenoic acid; (EpDPE) epoxydocosapentaenoic acid

Plasma, peritoneal fluid and washes were tested from women with and without endometriosis in two separated LC/ESI-MS/MS protocols. COX assay involved 24 prostanoids and related lipid mediators, while LOX/CYP assay consisted 55 metabolites of lipoxygenase and cytochrome P450 derived hydroxy fatty acids and related compounds. The flow chart shows the main steps of the LC/ESI-MS/MS (Figure 2.2.22.2.2).

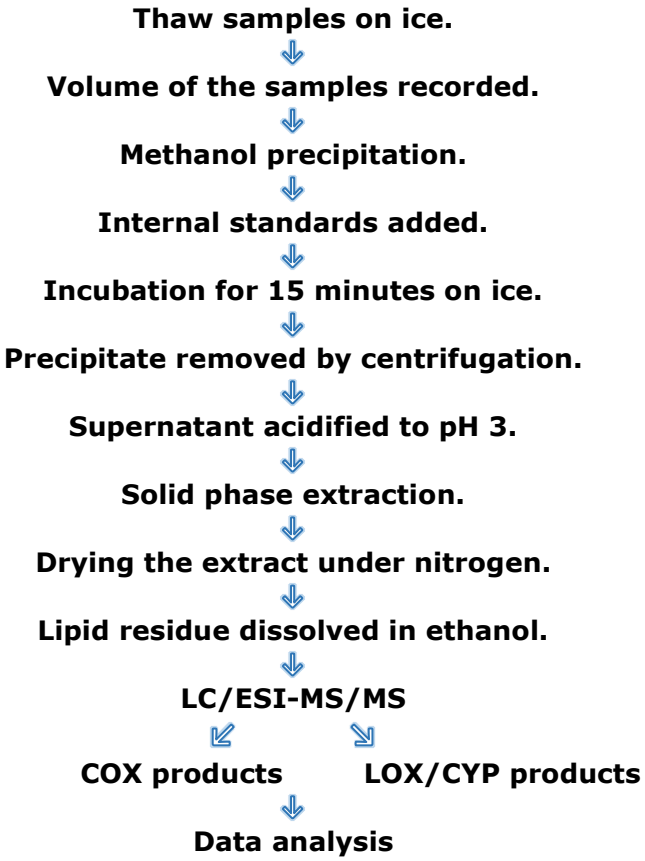


Figure 2.2.2. Overview of extraction and lipidomics analysis of fluid specimens for eicosanoids and related hydroxy fatty acids.

2.2.3 Materials for LC/ESI-MS/MS

1. Standards

The following commercially available lipid mediators and deuterated internal standards were purchased from Cayman Chemicals (Ann Arbor, Michigan, USA) or Santa Cruz Biotechnology (Santa Cruz, California, USA):

PGE_2; PGD_2; 15-keto PGE_2; 15-deoxy-$\Delta^{12,14}$ PGJ_2; PGJ_2; Δ12-PGJ_2; 13,14-dihydro-15-keto PGE_2; 13,14-dihydro-15-keto $PGF_{2\alpha}$; $PGF_{2\alpha}$; 8-iso $PGF_{2\alpha}$; 6-keto-$PGF_{1\alpha}$; TXB_2; PGE_1; PGD_1; 13,14-dihydro-15-keto PGE_1; 13,14-dihydro $PGF_{1\alpha}$; $PGF_{1\alpha}$; 13,14-dihydro $PGF_{2\alpha}$; 13,14-dihydro PGE_1; 13,14-dihydro $PGF_{1\alpha}$; PGE_3; PGD_3; PGF_{α}

TXB_3; resolvin (Rv)E1; RvD1; RvD2; maresin (MaR)1; protectin (PDX); ± 11-hydroxydocosahexaenoic acid (HDHA); ± 4-HDHA; ± 7-HDHA; ± 10-HDHA; ± 13-HDHA; ± 14-HDHA; ± 17-HDHA; ± 20-HDHA; leukotriene (LT) B4; ± 14,15-dihydroxyeicosatrienoic acid (DHET); ± 11,12-DHET; ± 8,9-DHET; ± 5,6-DHET; ± 5(6)-epoxyeicosatrienoic acid (EET); ± 11(12)-EET; ± 14(15)-EET; ± 8(9)-EET; 5-oxoETE; 5-hydroxyeicosatetraenoic acid (HETE); 8-HETE; ± 9-HETE; 11-HETE; 12-HETE; 15-HETE; 20-HETE; 5-hydroxyeicosapentaenoic acid (HEPE); ± 8-HEPE; ± 9-HEPE; ± 11-HEPE; ± 15-HEPE; ± 18-HEPE; 12-HEPE, 9-hydroxyoctadecatrienoic acid (HOTrE); 13-HOTrE; 15-hydroxyeicosatrienoic acid (HETrE); hepoxilin (HX) A3; 19(20)-dihydroxydocosapentaenoic acid (DiHDPA); 9(10) epoxyoctadecenoic acid (EpOME); 12(13) EpOME; 9- hydroxyoctadecadienoic acid (HODE); 13-HODE; 9-oxo-octadecadieonic acid (oxoODE); 13-oxoODE; 5(15)-dihydroxyeicosatetraenoic acid (DiHETE); 8(15)-DiHETE; 19(20)-epoxydocosapentaenoic acid (EpDPE); 16(17) EpDPE; transepoxyketooctadecenoic acid (trans-EKODE); 9,10-dihydroxyoctadecenoic acid (DiHOME); 12,13-DiHOME and PGB_2-*d*4; 12-HETE-*d*8; 8(9) EET-*d11*; 8,9-DHET-*d11* for internal standards.

All mediators and internal standards were reconstituted to 10 ng/µl stock in ethanol and stored at -80 ºC for up to a year.

2. Solvents

Solvents used for solid phase extraction, HPLC and mass spectrometry were purchased as follows: methanol (LC-MS grade, ≥99.9%), acetonitrile (LC-MS grade, ≥99.0%), acetic acid, glacial (HPLC grade, ≥99.7%), methyl formate (HPLC grade, ≥99.7%) and hydrochloric acid (ACS grade, ≥36.5-38%) were purchased from Sigma-Aldrich (Poole, United Kingdom). Ethanol (HPLC grade, ≥99.8%) and hexane (HPLC grade, 97.0%) were obtain from Fisher Scientific (Loughborough, United Kingdom). Tap water was filtered using PURELAB Flex (Elga LabWater, High Wycombe, United Kingdom) purification water system to reach the ultrapure water standards (resistivity: 18.2MΩ-cm, pH: neutral, bacteria <0.1 CFU/cm^3).

3. Equipment

10ml round and flat-bottomed glass tubes with lids and glass, unplugged Pasteur pipettes were purchased from Fisher Scientific (Loughborough, United Kingdom). 50, 100, 250, 500 µl Hamilton glass syringes were obtained from SGE (Australia). P10, P20, P200, P1000 and P5000 single channels pipettors were procured from Gilson, Inc. (Middleton, Wisconsin, USA). SPE cartridges (C18-E 500mg, 6ml) and vacuum manifold, amber glass vials, insert vials, screw caps and septa were supplied by Phenomenex (Macclesfield, United Kingdom). Narrow range (1.7-3.8) pH indicator strips was bought from Merck (Hoddesdon, United Kingdom). Sorvall refrigerated

centrifuge (Sorvall), vortex mixer (Fisher Scientific), nitrogen-drying cabinet with nitrogen supply (custom made, University of Bradford), and vacuum pump (1c Vacuumbrand, Wertheim, Germany) were used for lipid extractions.

LC/ESI-MS/MS analysis was performed on a Xevo TQ-S electrospray ionisation triple quadrupole mass spectrometer (Waters, Elstree, United Kingdom) coupled to a Waters Alliance 2695 ultrahigh-performance liquid chromatography (UPLC) pump (Acquity, Waters, Elstree, United Kingdom). C18 columns (Acquity UPLC BEH, 1.7µm, 2.1 x 50mm;) and the VanGuard pre-column filter (Acquity UPLC BEH, 1.7µm, 2.1 x 5mm) for liquid chromatography were obtained from Waters (Elstree,United Kingdom) The system was operated with MassLynx 4.0 software (Waters, Elstree, United Kingdom).

2.2.4 Preparations

1. 1ng/µl PGB_2-*d*4; 12-HETE-*d*8; 8(9)EET-*d11* and 8,9-DHET-*d11* internal standard cocktail

- Using a glass Hamilton syringe, 100µl of 10ng/µl stock of each of the four internal standards was added to an amber vial.
- Syringes were washed 6 times between each components.
- 600µl ethanol was added to make up to 1ml of 1ng/µl internal standard cocktail.
- Standards were stored at -20ºC for up to three months.

2. 100pg/µl stock cocktail for COX calibration line

- Using a glass Hamilton syringe, 10µl of the 10ng/µl PGE_2; PGD_2; 15-keto PGE_2; 15-deoxy-$\Delta^{12,14}$ PGJ_2; PGJ_2; Δ12-PGJ_2; 13,14-dihydro-15-keto PGE_2; 13,14-dihydro-15-keto $PGF_{2\alpha}$; $PGF_{2\alpha}$; 8-iso $PGF_{2\alpha}$; 6-keto-$PGF_{1\alpha}$; TXB_2; PGE_1; PGD_1; 13,14-dihydro-15-keto PGE_1; 13,14-dihydro $PGF_{1\alpha}$; $PGF_{1\alpha}$; 13,14-dihydro $PGF_{2\alpha}$; 13,14-dihydro PGE_1; 13,14-dihydro $PGF_{1\alpha}$; PGE_3; PGD_3; $PGF_{3\alpha}$;TXB_3 stock were added to an amber vial.
- Syringes were washed 6 times between each components.
- 760µl ethanol was added to make up to 1ml of 100pg/µl COX standard cocktail.
- Standards were stored at -80ºC for up to three months.

3. 100pg/µl stock cocktail for LOX/CYP calibration line

- Using a glass Hamilton syringe, 10µl of the 10ng/µl RvE1; RvD1; RvD2; MaR1; PDX; ± 11-HDHA; ± 4-HDHA; ± 7-HDHA; ± 10-HDHA; ± 13-HDHA; ± 14-HDHA; ± 17-HDHA; ± 20-HDHA; LTB4; ± 14,15-DHET; ± 11,12-DHET; ± 8,9-DHET; ± 5,6-DHET; ± 5(6)-EET; ± 11(12)-EET; ± 14(15)-EET; ± 8(9)-EET; 5-oxoETE; 5-HETE; 8-HETE; ± 9-HETE; 11-HETE; 12-HETE; 15-HETE; 20-HETE; 5-HEPE; ± 8-HEPE; ± 9-HEPE; ± 11-HEPE; ± 15-HEPE; ± 18-HEPE; 12-HEPE; 9-HOTrE; 13-HOTrE; 15-HETrE; HXA3; 19(20)-DiHDPA; 9(10)-EpOME; 12(13)-EpOME; 9-HODE; 13-HODE; 9-oxoODE; 13-oxoODE; 5(15)-DiHETE; 8(15)-DiHETE; 19(20)-EpDPE; 16(17) EpDPE; trans-EKODE; 9,10-DiHOME stock were added to an amber vial. Currently 12,13-DiHOME

standard is not available therefore 9,10-DiHOME standard was used for the quantification.
- Syringes were washed 6 times between each components.
- 460µl ethanol was added to make up to 1ml of 100pg/µl LOX/CYP standard cocktail.
- Standards were stored at -80ºC for up to three months.

4. Mobile Phase A for LC analysis

- 400µl glacial acetic acid was added to 2000ml ultrapure water.

5. Mobile Phase B for LC analysis and strong needle wash

- 500µl glacial acetic acid (HPLC grade) was added to a 2.5L bottle of acetonitrile.

6. Seal wash/weak needle wash

- It was made fresh at the beginning of each run.
- 500ml methanol was measured into a 1L measuring cylinder.
- Ultrapure water was added to make up to 1L solution.

2.2.5 Performance of LC/ESI-MS/MS

2.2.5.1 Lipid extraction from fluid specimens

Lipid mediators are particularly sensitive to a light exposure oxidation, therefore care was taken during the extraction and drying processes to minimize the degradation of the analytes. Where it was possible samples were kept on ice and protected from light. Samples were slowly thawed on ice in a dark place. Defrosted samples were transferred into flat-bottomed, glass sample vials. The original volumes of samples were carefully measured and recorded using. 700µl of ice cold, absolute methanol was added to each sample to facilitate the precipitation of the proteins. Using a Hamilton syringe, 20µl of 1ng/µl internal standards cocktail was pipetted into the extraction tubes. Appropriate volume of ice cold water was added to bring the solutions to 15% methanol (v/v) concentration to a final volume of 4.7ml. Tubes were gently mixed by rolling and incubate on ice in the dark for 15 minutes. At the end of incubation the extraction tubes were centrifuged at 2000g; for 10 minutes at 4ºC.

2.2.5.2 Solid phase extraction (SPE)

During the centrifugation, SPE cartridges were preconditioned with 6ml absolute methanol and 6ml ultrapure water under a low vacuum. The conditioning was completed no more than 10min before samples were added to cartridges. Following the centrifugation, the lipid contained supernatants were transferred into new extraction tubes using glass Pasteur pipettes. The pH of samples were adjusted to pH3 with 1M hydrochloric acid (4-5 drops). The pH was checked using pH indicator strips prior adding the acidified samples to SPE cartridges. Then, samples were

allowed to pass through the cartridges drop wise. After the samples had run through the columns each cartridge was washed with 6ml 15% (v/v) methanol under a low vacuum, with 6ml ultrapure water under a low vacuum and with 6ml hexane under a higher vacuum. Following the last washing step, the lipid extracts were eluted in 6ml methyl formate using moderate vacuum and collected into round-bottomed glass tubes.

2.2.5.3 Drying and reconstituting samples

Tubes were transferred to the drying cabinet and dried under a gentle nitrogen stream. When the solvent had completely evaporated, the pellet was resuspended in 100µl absolute ethanol. The tubes were vortexed briefly and centrifuged for 30 seconds in order to collect the whole samples at the bottom. Finally, the lipid extracts were transferred into amber sample vials with a 100µl glass insert using a glass Hamilton syringe and stored at -20ºC up to a week until LC/ESI-MS/MS analysis.

2.2.5.4 Preparation standards for quantification

To allow accurate determination of the quantity of each analyte of interest calibration lines were generated during the assay. 100pg/µl COX and LOX/CYP cocktails were used to construct a minimum 5-point calibration lines by a twofold-serial dilution for each assay. The standard points were the follows: 10pg/µl – 5pg/µl – 2.5pg/µl – 1.25pg/µl- 0.625pg/µl. Internal standards were added to each standard points and went through the same drying process as the samples. The dried standards were reconstituted in 100 µl ethanol and stored at -20ºC up to a week until LC/ESI-MS/MS analysis.

2.2.5.5 Liquid chromatography

Chromatographic analysis was carried on a C18 column (Acuity UPLC BEH, 1.7µm, 2.1x50mm; Waters). The column temperature was set at 25ºC. Sample injections were performed using an autosampler (Waters, Elstree, United Kingdom). The temperature of autosampler chamber was set at 8ºC to keep the analytes refrigerated during the assay. COX and the LOX/CYP assays were performed by two separated chromatographic runs. The total running time was 5.8 and 5.0 minutes for COX and LOX/CYP, respectively. Standards and analytes were injected in duplicate with the injection volumes at 3µl. To minimise the contamination between samples ethanol blanks were run after each sample. The analytes were separated at a flow rate of 0.6ml/min according to the gradient of water-acetonitrile. Mobile phase A was water:acetic acid (100:0.02), mobile phase B was acetonitrile:acetic acid (100:0.02) solutions. The proportions of gradients in timewise are described in Table 2.2.22.2.2 and Table 2.2.32.2.3

Table 2.2.2. Solvent gradient for COX assay.

Time (min)	Mobile phase A (%)	Mobile phase B (%)	Curve
0.0	80	20	-
0.5	80	20	6
0.6	60	40	6
2.5	60	40	6
4.0	35	65	6
4.1	80	20	6
5.8	80	20	6

Table 2.2.3. Solvent gradient for LOX/CYP assay.

Time (min)	Mobile phase A (%)	Mobile phase B (%)	Curve
0.0	75	25	-
3.0	20	80	6
3.2	75	25	6
5.0	75	25	6

2.2.5.6 ESI-MS/MS

The instrument was operated in a negative ionisation mode. Argon gas was used to facilitate the collision-induced dissociation in the collision cell. COX and LOX/CYP assays were required different MS/MS settings. The parameters are detailed in Table 2.2.42.2.4. The MRM transitions, cone voltage and collision energy for each compound were determined using the IntelliStart protocol and detailed in Tables Table 2.2.52.2.5 for COX and in Table 2.2.62.2.6 for LOX assay.

Table 2.2.4. MS/MS setting for COX and LOX/CYP assays

Assay	Capillary voltage (kV)	Source temperature (°C)	Desolvation temperature (°C)	Dwell time (s)
COX	3.1	150	500	0.007
LOX/CYP	1.5	150	600	0.003

Table 2.2.5. Summary of individual MRM transition, cone voltage, collision energy and indicative retention times for COX assay.

Compound	MRM (m/z)	Cone voltage (V)	Collision energy (eV)	Retention time (min)
PGD_1	353→317	12	12	1.33
PGE_1	353→317	12	12	1.28
6-keto $PGF_{1\alpha}$	369→163	12	24	0.99
13,14-dihydro-15-keto $PGF_{1\alpha}$	355→193	20	30	1.62
$PGF_{1\alpha}$	355→311	14	24	1.17
13,14-dihydro PGE_1	355→337	18	16	1.40
13,14-dihydro-15-keto PGE_1	353→335	12	14	1.69
PGB_2-d4	337→179	12	20	2.06
PGD_2	351→271	24	16	134
PGE_2	351→271	24	16	1.25
15-keto PGE_2	349→113	14	20	1.40
13,14-dihydro $PGF_{2\alpha}$	355→311	14	24	1.32
13,14-dihydro-15-keto $PGF_{2\alpha}$	353→113	10	26	1.69

$PGF_{2\alpha}$	353→193	12	24	1.18
8-iso $PGF_{2\alpha}$	353→193	12	24	1.10
PGJ_2	333→271	14	16	1.99
Δ12-PGJ_2	333→271	14	16	2.03
15-deoxy-Δ12,14-PGJ_2	315→271	12	14	3.90
TXB2	369→169	18	18	1.11
13,14-dihydro $PGF_{1\alpha}$	357→113	4	32	1.37
13,14-dihydro-15-keto PGE_2	351→333	12	12	1.57
TXB_3	367→169	16	14	1.03
PGD_3	349→269	10	16	1.18
PGE_3	349→269	10	16	1.13
$PGF_{3\alpha}$	351→193	2	22	1.08

Table 2.2.6. Summary of individual MRM transition, cone voltage, collision energy and indicative retention times for LOX/CYP assay.

Compound	MRM (m/z)	Cone voltage (V)	Collision energy (eV)	Retention time (min)
9-HODE	295→171	16	16	2.47
13-HODE	295→195	2	18	2.49
15-HETrE	321→303	2	14	2.68
5-HETE	319→115	14	14	2.75
8-HETE	319→155	10	14	2.66
9-HETE	319→123	16	14	2.71
11-HETE	319→167	14	14	2.61
12-HETE	319→179	20	14	2.66
12-HETE-d8	327→184	20	16	2.64
15-HETE	319→175	4	14	2.54
20 HETE	319→245	4	14	2.31
5(6)-EET	319→191	4	10	3.03
8(9)-EET	319→155	10	14	2.66
11(12)-EET	319→167	14	14	2.61
14(15)-EET	319→113	4	14	2.54
5-HEPE	317→115	16	12	2.46
8-HEPE	317→155	26	12	2.38
9-HEPE	317→149	20	14	2.42
11-HEPE	317→167	12	12	2.35
12-HEPE	317→179	28	12	2.39
15-HEPE	317→175	8	14	2.33
18-HEPE	317→215	12	14	2.24
5,6-DHET	337→145	8	16	2.33
8,9-DHET	337→127	8	16	2.22
11,12-DHET	337→167	2	18	2.14
14, 15-DHET	337→207	18	16	2.04
5-oxoETE	317→203	14	18	2.94
LTB_4	335→195	12	14	1.87
RvE1	349→195	14	16	0.81

RvD1	375→141	18	12	1.39
RvD2	375→175	2	22	1.26
4-HDHA	343→101	8	12	2.80
7-HDHA	343→141	6	14	2.68
10-HDHA	343→153	2	16	2.61
11-HDHA	343→193.87	2	12	2.65
13-HDHA	343→193.15	2	12	2.58
14-HDHA	343→161	12	14	2.61
17-HDHA	343→201	14	14	2.55
20-HDHA	343→241	2	12	2.48
PDX †	359→206	18	16	1.81
MaR1†	359→177	16	16	1.82
9 OxoODE	293→185	14	18	2.67
13 OxoODE	293→113	16	20	2.59
9 HOTrE	293→171	20	16	2.23
13 HOTrE	293→195	12	16	2.27
9(10) EpOME	295→171	16	16	2.89
12(13)EpOME	295→195	2	18	2.86
Trans EKODE	309→209	16	10	2.31
9,10 DiHOME	313→201	16	20	1.98
12,13 DiHOME	313→183	16	20	1.91
8(9) EET-d11	330→155	14	12	2.97
8,9 DHET-d11	348→127	16	24	2.23
HXA_3	335→273	16	12	2.29
5,15 DiHETE	335→115	12	12	1.81
8,15 DiHETE	335→155	22	16	1.76
16(17) EpDPE	343→233	14	12	2.89
19(20) EpDPE	343→285	18	12	2.79
19,20 DiHDPA	361→273	18	16	2.04

† PDX and MaR1 both appear in the transition of the other and cannot be separated by retention time. This means that a peak appearing in either transition could be either compound or a combination of both.

2.2.5.7 Quantification of compounds

TargetLynx extension (Waters, Elstree, United Kingdom) running under MassLynx 4.0 software was used to process data and calculate the concentration of the analyte of interests in biological specimens. Utilization of deuterated internal standards, added to samples and standards allowed normalisation of peak integrals against the designated internal standard. In every case, the most related internal standard was used for the normalization, i.e. 12-HETE-d8 was used for the quantification of hydroxy fatty acid metabolites. A calibration line was constructed by plotting normalised peak area versus concentration. The generated calibration line was used to accurately quantify the compounds of interest in biological samples. The calculated concentrations were expressed in pg/µl. In order to calculate the concentration in each extract, the mean of the two duplicate injections was calculated, this was multiplied by 100 (dilution factor of reconstitution after nitrogen drying) to give the total concentration of each compound in the extract. This total concentration (in pg) was normalised against the volume of original sample. The concentration of analytes were reported in pg analyte in a millilitre of specimen (pg/ml). The limit of detection was set to a signal to noise ratio of 3, whist the limit of quantification was set to a signal to noise ratio of 7. If the peak area of the samples were below 150 the signal was considered as noise. Appendix 7 illustrates the standard curve and a representative chromatogram of an analyte of interest in TargetLynx.

Statistical analysis was performed by GraphPad Prism 7 (Graphpad Software Inc). The distribution of data was assessed by D'Agostino & Pearson omnibus normality test ($n \geq 8$/group) or Shapiro-Wilk test ($n < 8$/group). Data that followed a normal distribution was analysed using a student's t-test or one-way analysis of variance (ANOVA) followed by Tukey *post hoc* analysis; whereas non-parametric data were analysed using a Mann-Whitney U test or Kruskal-Wallis test with Dunn's post-test where appropriate. In all cases a p value of less than 0.05 was considered statistically significant.

2.3 Qualitative Real-Time Polymerase Chain Reaction (qRT-PCR)

2.3.1 Overview of qRT-PCR

Qualitative Real-Time Polymerase Chain Reaction (qRT-PCR) is the most advanced form of PCR techniques. This technique is particularly suited for gene expression studies as it allows monitoring the amplification of the nucleic acid of interest in real time and determination of the initial quantity of the sequence with accuracy and high sensitivity. During the PCR reaction, ideally the amplified DNA exponentially doubles with each cycle. Utilisation of fluorescent intercalating dye (e.g. SYBR® Green) or a fluorescent probe (e.g. Taqman®) means that the amplification of DNA can be monitored in real time and data collected at the end of each cycle. There is a direct proportion between the fluorescent signal and the amplified product, called the amplicon. By plotting the fluorescent signal against the cycle number an amplification plot can be generated. The original amount of template can be calculated using a reference point. The commonly used reference point, referred to as the threshold cycle (Ct) or crossing point (Cp) is the cycle when the amplification plot crosses the background fluorescent signal. The Ct value inversely correlates with the initial amount of targeted nucleic acid. Thus, a low Ct value represents a highly expressed gene of interest, while the high (>30) Ct suggests low expression of a targeted gene.

Gene expression studies investigate quantities of messenger RNA (mRNA) since the concentration of mRNA is positively correlated with the activity of the gene of interest. One of the easier and most convenient ways to compare the gene expression between two subjects is to calculate the relative gene expression of the target gene versus an endogenous reference gene, also called a housekeeping gene. Relative quantification measures the relative change (ΔCt) by calculating the difference between the Ct value of the target gene and the Ct value of reference gene. Application of an endogenous reference gene makes possible the comparison of relative gene expression between subjects and results provide information about the potential role of the targeted gene in the investigated condition. However, it is very important to note that the expression of the housekeeping gene should not differ between samples and be effected by the investigated conditions or disease. Using an endogenous reference gene has further advantages, for instance, normalizing the variation in RNA content and the efficiency of reverse transcription, also correcting the difference in sample handling and RNA quality such as possible RNA degradation or presence of inhibitor (Fleige and Pfaffl, 2006).

As was mentioned above gene expression studies use mRNA studying the activity of the gene of interest. PCR applies thermostable DNAase enzymes to amplify the target. These DNAase enzymes require double-stranded nucleic acids as a template. Therefore, prior to the PCR reaction the RNA needs to be transcribed into double-stranded complementary DNA (cDNA) by a reverse transcriptase reaction. Reverse transcription can be performed along with the PCR assay in the same tube in one-step or two-steps by two separated reactions. Although, the one-step PCR is less time consuming the two-step PCR is highly sensitive, requires less starting material and is more flexible since reverse transcription and PCR reaction can be optimised separately, therefore two-step qRT-PCR was used in this study. The flowchart shows the main steps of qRT-PCR study (Figure 2.3.12.3.1):

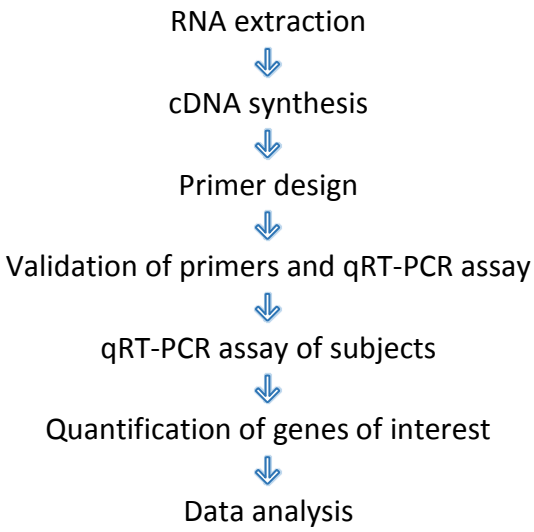


Figure 2.3.1. Outline of qRT-PCR methodology.
The flowchart details the main steps of qRT-PCR experiment studying gene expression.

2.3.2 Materials

MirVana™, RiboPure™ Blood, Tempus™ Spin RNA isolation kits and isopropanol (molecular grade, ≥99.9%) were obtained from Thermo Fisher Scientific (Cramlington, United Kingdom). QuantiTect® Reverse Transcription (cat.: #205311) and QuantiTect® SYBR® Green PCR kits (cat.: #204143) were bought from Qiagen, Germany. DNAase, RNAase free plastic wares, 384-well microplate and filtered pipette tips were supplied by SLS Ltd. (Wilford, United Kingdom).Tri Reagent®, 1-bromo-3 chloropropane, ethanol (molecular grade, ≥99.9%) and Tris-EDTA (TE) buffer (molecular grade, pH8.0) were purchased from Sigma-Aldrich (Poole, United Kingdom). RNaseZap® (Ambion, USA) was used to eliminate any RNase contamination of bench surface and laboratory equipment. Single channel pipettors (Biopette, Labnet, USA) were dedicated to PCR work only. Laboratory equipment

used for this study was as follows: Rotor-stator homogeniser (X-1020, The Scientific Instrument Centre, Germany), vortex mixer (VariMix, SciQuip Ltd., Wem, United Kingdom), bench top centrifuge (Heraeus Fresco 17, Thermo Fisher Scientific, Cramlington, United Kingdom) and block heater (Eppendorf Thermomixer, Sigma-Aldrich, Poole, United Kingdom).

Materials for gel electrophoresis were obtained as follows: agarose gel was purchased from Invitrogen (USA). 20bp DNA ladder and gel loading buffer were bought from Generon (Slough, United Kingdom). 0.5X Tri-borate-EDTA (TBE) buffer was procured from Ambion, USA, ethidium bromide 10mg/ml was purchased from Sigma-Aldrich (Poole, United Kingdom).

2.3.3 RNA extraction

Extracted RNA is particularly susceptible to RNase mediated degradation. These ribonucleases stay active on the surface of the laboratory equipment or in buffers. They are fairly robust in nature therefore challenging to eliminate. The main source of RNase contamination comes from environmental microorganisms and human skin. In order to avoid RNase contamination of the isolated RNAs all necessary precautions were taken.

2.3.3.1 Solid tissue

Solid tissue specimens, such as eutopic endometrium, ectopic lesion and peritoneal wall were subject to RNA extraction. A combination of TRI Reagent® with mirVana™ protocol was used where the lysis buffer of mirVana™ kit was replaced with TRI Reagent®. This modification increased the yield and the purity of the isolated RNA.

Prior to the extraction, tissues were stabilized in RNAlater® and stored at -80°C. Tissues were thawed and placed in 1ml of TRI Reagent® then homogenised using rotor-stator homogenizer. The disrupted samples were incubated at room temperature for 5 minutes to ensure the complete dissociation of nucleoprotein complexes. Following the incubation, the samples were transferred into 2ml Eppendorf tubes, 150µl 1-bromo-3 chloropropane was added, mixed and allowed to stand for 15 minutes at room temperature. Tubes were centrifuged at 12,000g for 15 minutes at 4°C to separate the mixture into three layers. The RNA contained an upper, aqueous layer which was carefully removed and transferred into a new tube. The volume of the aqueous layer was noted and 1.25 times of the aqueous layer's volumes of ethanol was added to precipitate the RNA. Then, the extracted RNA was purified using the spin-column of mirVana kit. A filter cartridge was placed into a collection tube and the RNA-ethanol mixture was pipetted onto the cartridge. Tubes were centrifuged at 10,000g for 30 second to pass the sample through the column.

Cartridges were washed once with 700µl of Wash Solution 1 and twice with 700µl of Wash Solution 2/3. Between the washing steps the tubes were centrifuged at 10,000g for 30 seconds at 4°C and the flow-through was discarded. After the last washing step, the cartridges were centrifuged at 10,000g for 1 minutes to remove the residual fluid from the filter. The cartridges were transferred into fresh collection tubes. To eluate the RNA 30µl of pre-heated (95°C) TE buffer was added to the filters, incubated for 2 minutes at room temperature and was centrifuged at maximum speed (17,000g) for 30 seconds. Finally, the RNA elute was pipette back onto the filter and centrifuged again for 1 minute at maximum speed to collect the extracted RNA.

2.3.3.2 Peritoneal cells

The RioboPure™ blood kit (Thermo Fisher Scientific, Cramlington, UK) was used to isolate RNA from peritoneal cells. The extraction protocol was performed according to the manufacturer manual. In brief, the method was as follows: sample were thawed on ice, then centrifuged at 1500g for 10 minutes at 4°C pelleting the cells. RNAlater® was removed and 800µl lysis buffer and 50µl sodium acetate were added to each tube. Samples were vortex mixed vigorously for a minute to facilitate the lysis of the cells and the dissociation of nucleoprotein complexes. 300µl acid-phenol:chloroform was dispensed into the tubes and incubated for 5 minutes at room temperature. After the incubation, the tubes were centrifuged at 16,000 g for 1 min at 4°C to separate the layers. The upper, aqueous layer was transferred in a new 2ml Eppendorf tube. The volume of the aqueous layer was noted and 1.5 times the volume of the aqueous layer of ethanol was added. The mixture was pipetted on a filter cartridge. The same washing process was performed as was described in the mirVana protocol (see section 2.3.3.1). The RNA was eluted in 30µl of pre-heated (75°C) TE buffer.

2.3.3.3 Venous Blood

Blood was collected into a Tempus™ Blood RNA tube (Thermo Fisher Scientific, Cramlington, UK) on the day of the surgery. RNA extraction was performed using a Tempus™ Spin RNA isolation kit (Thermo Fisher Scientific, Cramlington, UK) according to the manufacturer's protocol. In brief, the protocol was as follows: stabilized blood was transferred from the blood taking tube into a 50ml falcon tube and brought up to a total volume of 12 ml with PBS. Samples were vortexed vigorously for a minute, then centrifuged at 3000g for 30min at 4°C. After the centrifugation, the supernatant was discarded and the RNA pellet was resuspended in 400µl Resuspension Solution. Resuspended RNA was pipetted onto the purification cartridge, cartridges were provided with the kit, and washed three times as was described in the mirVana protocol (see section 2.3.3.1). Following the washing, filters were transferred into a new collection tube. 50µl of pre-heated (70°C) TE buffer was

added onto the filters and incubated in a block heater for 2 minutes at 70°C. After the incubation tubes were centrifuged at the maximum speed (17,000g) for 30 seconds to collect the eluted RNA. The RNA elute was pipette back into the filter and centrifuged again at the maximum speed (17,000g) for 2 minutes at 4°C. 40µl of RNA eluate was transferred into a fresh tube without touching the pellet at the bottom.

2.3.3.4 Quantification of RNA

Following the extraction, the concentration and the purity of the extracted RNA was determined using a Nanodrop 2000c spectrophotometer (Thermo Fisher Scientific, Cramlington, UK). 1µl of RNA was loaded onto the pedestal after blanking with 1µl TE buffer. A ratio of 1.8 or above for A260/A280 measurement was considered sufficient purity for gene expression assay.

2.3.4 cDNA synthesis

A QuantiTect® Reverse Transcriptase kit (cat.: #205311, Qiagen, Germany) was used to synthetize cDNA for qRT-PCR. The protocol comprises a genomic elimination step prior the reverse transcription reducing the non-specific amplification and detection of genomic DNA (gDNA) during the qRT-PCR. To degrade the possible genomic DNA contamination, 2µl of gDNA Wipeout Buffer was added to a total volume of 14µl of containing 360ng RNA and incubated at 42°C for 5 minutes. Following the incubation, the mixtures were placed on ice and the reverse transcriptase master mix, containing RT Primer Mix and Quantiscript RT buffer in a proportion of 1:4 and 1µl Quantiscript Reverse Transcriptase were added to the mixture of up to 14µl. To test the efficiency of the elimination of genomic DNA no reverse transcriptase (-RT) control was applied where Quantiscript Reverse Transcriptase were not added. The reaction mixture was incubated for 20 minutes at 42°C for synthesis of cDNA. The synthesis was terminated by inactivating the reverse transcriptase at 95°C for 3 minutes. cDNA was diluted 1:10 in RNase free water and stored at -20°C until use.

2.3.5 Primer design

Primers are short (18-22 bases) single-stranded nucleic acids which are essential to the activity of DNA polymerase. Primers must be unique to the template in order to achieve high yields of specific PCR products. Careful design of primers is crucial to successful PCR particularly when using SYBR® Green for detection. SYBR® Green dye has a lack of specificity and binds to any double-stranded nucleic acid, emitting a fluorescent signal on binding. Non-optimal and less specific primer pairs can result in the formation of a primer dimer or the amplification of non-specific PCR products. These products are also double-stranded and generate a fluorescent signal. The

detection of nonspecific products reduces the sensitivity of the assay and leads to inaccurate quantification of the target.

A web-based primer designing tool "Primer-BLAST" was used for designing primers [http://www.ncbi.nlm.nih.gov/tools/primer-blast/]. The following criteria were applied:

1. Primers spun exon-exon junction.
2. Product size less than 150 base pair (bp).
3. Primer length between 18-24bp.
4. Primer melting temperature between 55-60^{o}C, with the optimum of 59^{o}C and 2^{o}C difference.
5. GC content of the primer between 40-60%.
6. No 3' CG clamp on primers, maximum two GC in the last 5 bases of the 3' end.

The custom-made sequences were synthetized by Eurofins Genomics UK (Wolverhampton, United Kingdom) or predesigned primers (KiCqStart® SYBR® Green primers) were purchased from Sigma-Aldrich. Primers were reconstituted in TE buffer to 100µM stock solution and stored at -20^{o}C.

2.3.6 Qualitative Real-Time Polymerase Chain Reaction

QuantiTect® SYBR® Green PCR kit (cat.: #204143 Qiagen, Germany) was used for qRT-PCR assay. The 2x QuantiTect SYBR Green PCR Master Mix contained HotStartTaq DNA Polimerase, QuantiTect SYBR Green PCR Buffer with an optimized concentration of SYBR® Green I, ROX passive reference dye, deoxyribonucleotide triphosphate (dNTP) and $MgCl_2$. The reaction volume was set to 10µl which consisted 1µl cDNA, 0.25µl (100µM) forward and 0.25µl (100µM) reverse primer, 5µl 2x SYBR Green PCR Master Mix and 3.5µl nuclease free water. Samples were tested in triplicate on a 384-well microplate. Negative controls such as no template control (NTC) and no reverse transcriptase (-RT) control were included in every experiment. The experiments were performed on a Quantstudio 12K flex real-time PCR system (Applied Biosystems, USA). The reaction was setup for 1 activation and 45 PCR cycles. The temperature changing rate was 1.6^{o}C/sec. The intra and inter-assay variation were in the range of 0.5-2.9% (CV%). Table 2.3.12.3.1 details the cycling conditions of the assay.

Table 2.3.1. The qRT-PCR parameters for Quantstudio 12K flex real-time PCR instrument.

	Step	Time	Temperature
Hold Stage	Activation of DNA polymerase	15 min	95°C
PCR Stage	Denaturation	15 sec	95°C
	Annealing	30 sec	60°C
	Extension	15 sec	72°C
Melt Curve Stage	Denaturation	15 sec	95°C
	Annealing	60 sec	60°C
	Denaturation	15 sec	95°C

2.3.7 Validation of primers and qRT-PCR assay

2.3.7.1 Testing primers for dimer formation

Primer pairs were designed to target COX-2, 15-LOX-1, 15-LOX-2, PPAR-γ, cytokeratin 8 (CK8), interferon-induced transmembrane protein 1 (IFITM-1), CD36, glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and β-actin. Melting curve analysis was performed to check the formation of primer dimers. Double peaks or skewed peaks can suggest the presence of dimerised primers. In the case of 15-LOX-1, 15-LOX-2, PPAR-γ, CK8, CD36 and IFITM-1 the analysis indicated the presence of primer dimer in the NTC control (Figure 2.3.22.3.2) therefore predesigned KiCqStart® SYBR® Green primers were purchased from Sigma-Aldrich. Two or three pair of KiCqStart® primers were tested for each target. Figure 2.3.32.3.3 shows the representative melting curves and Table 2.3.22.3.2 summarises the details of primers were chosen for this study.

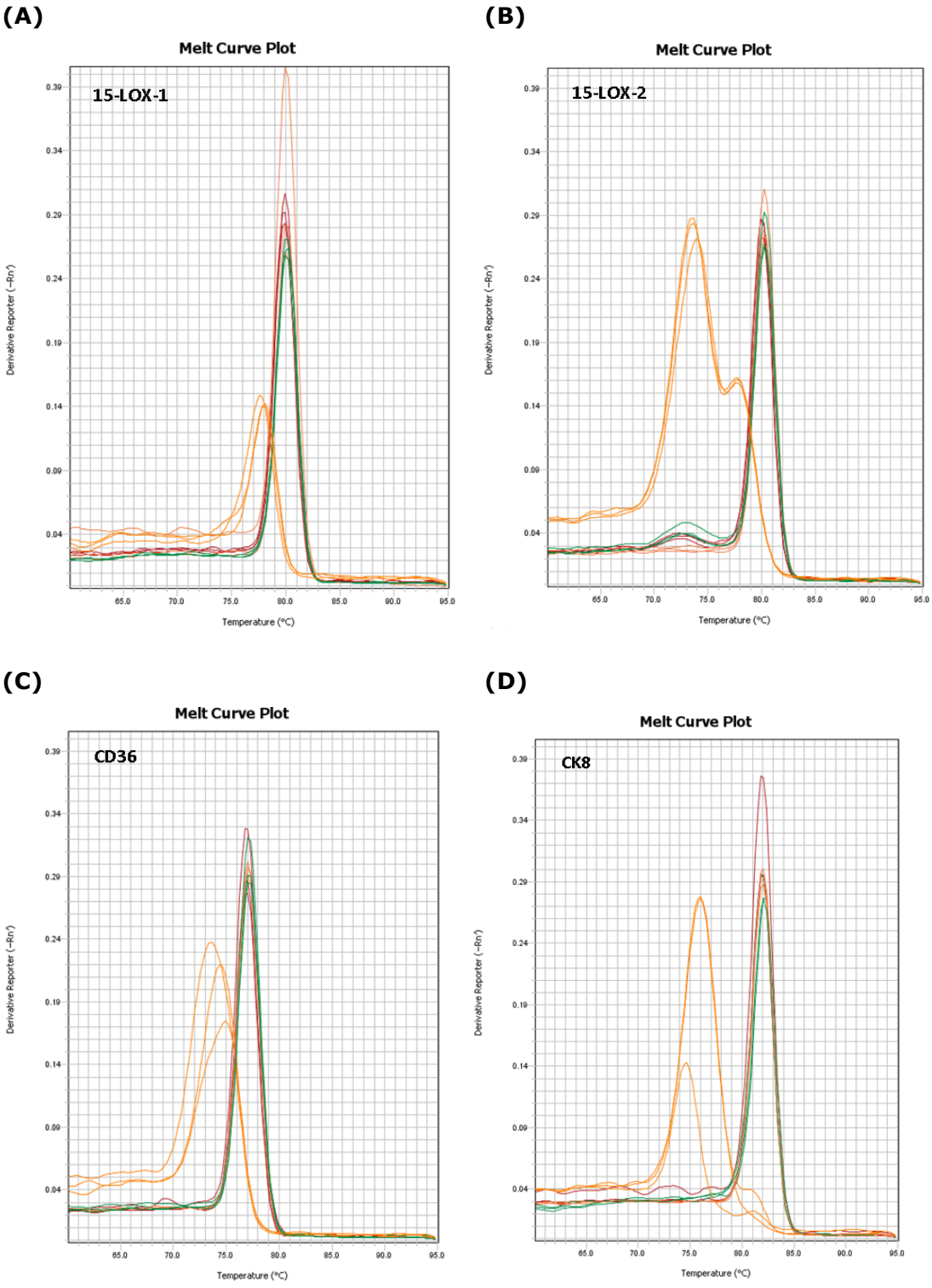
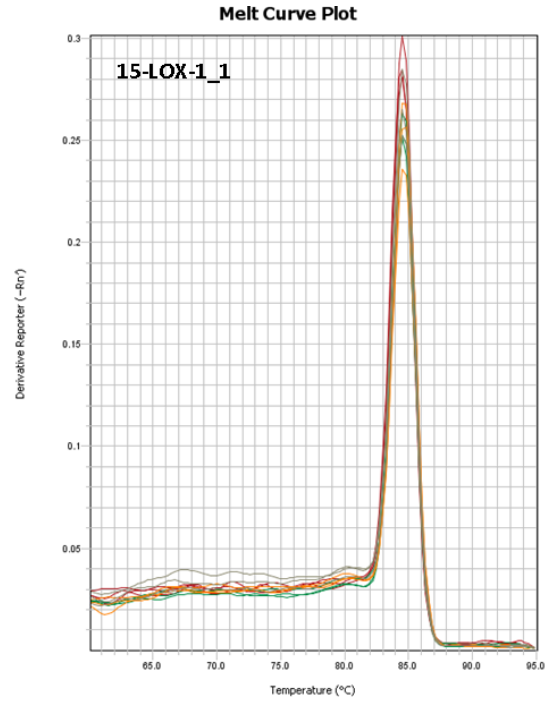
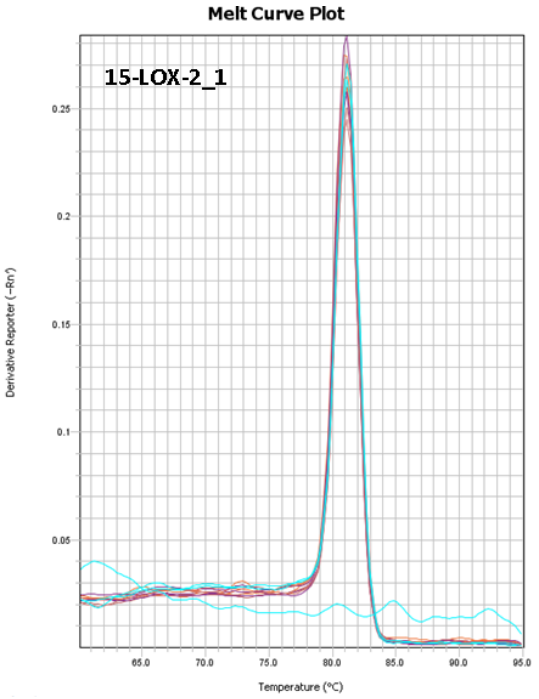


Figure 2.3.2. Melt curves analysis of custom-made primers.
Double peaks indicate the presence of nonspecific products. Due to the lack of template of non-template control (NTC) the formation of primer dimer is more apparent (orange peaks). (A) 15-lipoxigenase 1 (15-LOX-1); (B) 15-LOX-2; (C) CD36; (D) cytokeratin 8 (CK8).

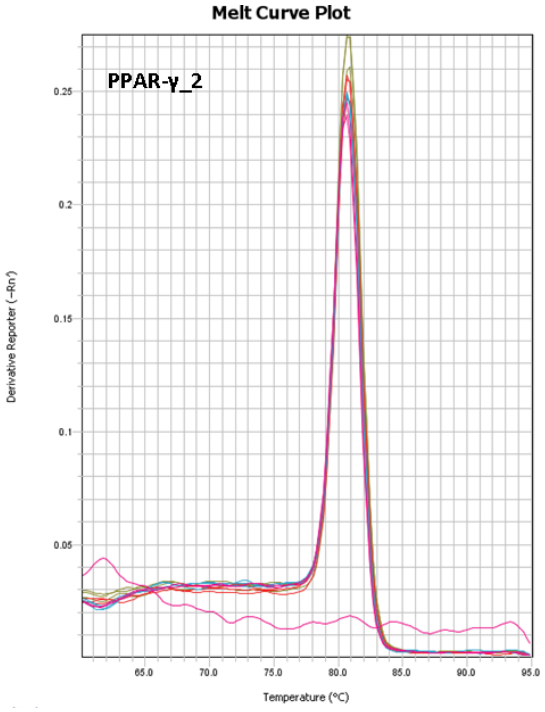
(A)


Melt Curve Plot
15-LOX-1_1
Derivative Reporter (-Rn')
Temperature (°C)

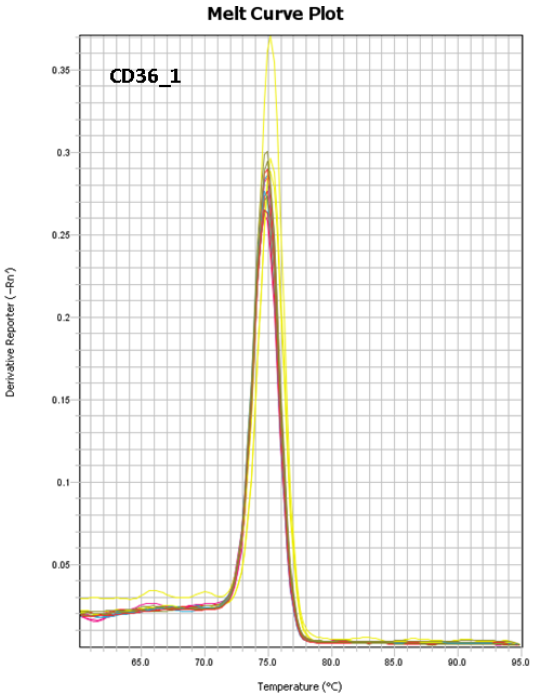
(B)


Melt Curve Plot
15-LOX-2_1
Derivative Reporter (-Rn')
Temperature (°C)

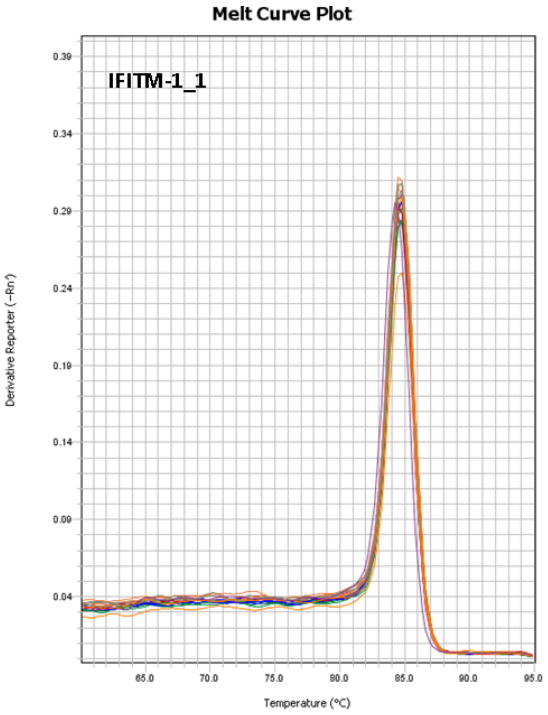
(C)


Melt Curve Plot
PPAR-γ_2
Derivative Reporter (-Rn')
Temperature (°C)

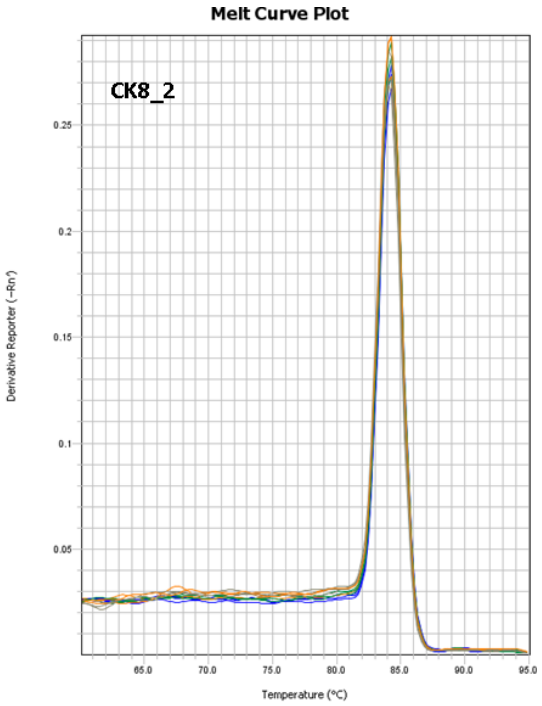
(D)


Melt Curve Plot
CD36_1
Derivative Reporter (-Rn')
Temperature (°C)

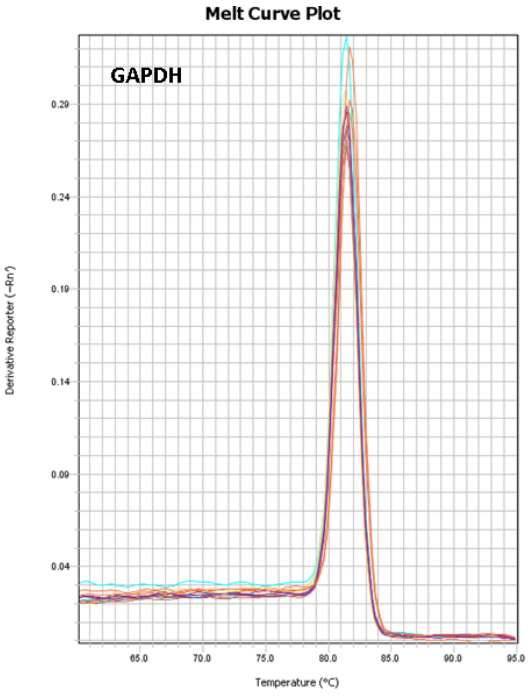
(E)


Melt Curve Plot
IFITM-1_1
Derivative Reporter (-Rn')
Temperature (°C)

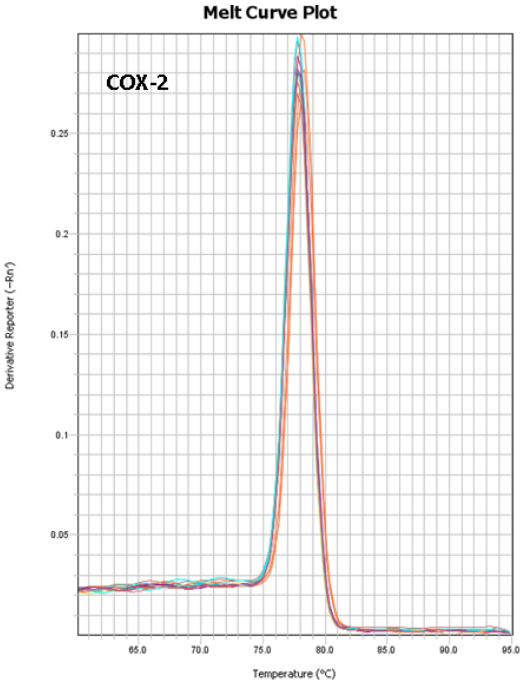
(F)


Melt Curve Plot
CK8_2
Derivative Reporter (-Rn')
Temperature (°C)

(G)


Melt Curve Plot
GAPDH
Derivative Reporter (-Rn')
Temperature (°C)

(H)


Melt Curve Plot
COX-2
Derivative Reporter (-Rn')
Temperature (°C)

(I)

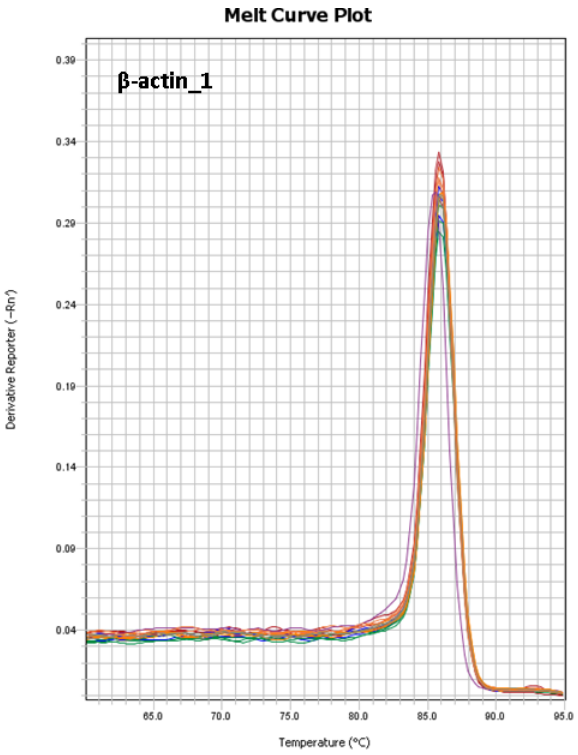


Figure 2.3.3. Melt curves of KiCqStart® SYBR® Green primers.
Narrow single peak proves the presence of the target without additional non-specific product. (A) 15-lipoxygenase 1 (15-LOX-1); (B) 15-LOX-2; (C) Peroxisome proliferator-activated receptor gamma (PPARγ); (D) CD36; (E) Interferon induced transmembrane protein 1 (IFITM-1); (F) Cytokeratin 8 (CK8); (G) Glyceraldehyde-3-phosphate dehydrogenase (GAPDH); (H) Cyclooxygenase-2 (COX-2); (I) β actin

Table 2.3.2. Summary of details of primer pairs used for qRT-PCR.

Target	Gene	Accession No.	Primer ID	Sequence (5'-3')	Tm (°C)	GC%	Amplicon length (bp)	Supplier
15-LOX-1	*ALOX15*	NM_001140	15-LOX-1_1_F	CTCTGACATGGGAATTTTCG	62.1	45.0	199	Sigma
			15-LOX-1_1_R	ATACCGATAGATGATTTCCCAG	60.6	40.9		
15-LOX-2	*ALOX15B*	NM_001039130	15-LOX-2_1_F	GATACAGAGGAACATGAAGC	56.2	45.0	156	Sigma
			15-LOX-2_1_R	CCGATGATTTCAGAGACAAAG	61.2	42.8		
CD36	*CD36*	NM_000072	CD36_1_F	AGCTTTCCAATGATTAGACG	58.4	40.0	111	Sigma
			CD36_1_R	GTTTCTACAAGCTCTGGTTC	55.6	45.0		
PPAR-γ	*PPARG*	NM_138712	PPAR-γ_2_F	TCATAATGCCATCAGGTTTG	61.0	40.0	82	Sigma
			PPAR-γ_2_R	CTGGTCGATATCACTGGAG	58.2	52.6		
CK8	*KRT8*	NM_002273	CK8_2_F	GCTATATGAAGAGGAGATCCG	59.3	47.6	189	Sigma
			CK8_2_R	AGCTCCTCATACTTGATCTG	56.0	45.0		
IFITM-1	*IFITM-1*	NM_003641	IFITM-1_1_F	CTACTCCGTGAAGTCTAGG	53.8	52.6	114	Sigma
			IFITM-1_1_R	ATGAGGATGCCCAGAATC	60.0	50.0		
β-actin	*ACTB*	NM_001101	β-actin_1_F	GACGACATGGAGAAAATCTG	59.7	45.0	131	Sigma
			β-actin_1_R	ATGATCTGGGTCATCTTCTC	58.0	45.0		
GAPDH	*GAPDH*	NM_002046	GAPDH_F	TGTTCGTCATGGGTGTGA	57.3	50.0	143	Eurofins
			GAPDH_R	TGTGGTCATGAGTCCTTC	57.3	50.0		
COX-2	*PTGS2*	NM_000963	COX-2_F	CCATGTCAAAACCGAGGTGTA	58.2	47.9	104	Eurofins
			COX-2_R	AATTCCGGTGTTGAGCAGTTTT	59.3	40.9		

(15-LOX) 15-lipoxygenase; (PPARγ) peroxisome proliferator-activated receptor gamma; (CK8) cytokeratin 8; (IFITM-1) interferon induced transmembrane protein 1; (GAPDH) glyceraldehyde-3-phosphate dehydrogenase; (COX) cyclooxygenase; (Tm) melting temperature

2.3.7.2 Testing primers for target specificity

Target specificity of primers is the key to a successful qRT-PCR experiment. To confirm the specificity of the primer sets primer-BLAST was run on all pairs of primer. The software determined the size of the targeted products, also the length of other predicted products. Except for CK8_2, the primer-BLAST did not predicted same or similar size of alternative product other than the targeted products. The CK8_2 primer might amplify a transcript variant of PPARα however, the size of this product is 221bp, while the length of CK8_2 product is 189bp.

Gel electrophoresis was performed to visualize the PCR products and check the length of amplicons. Table 2.3.2 details the expected length of the products. 4% (w/v) agarose gel was prepared in 0.5X TBE buffer. Followed the heating of the agarose solution in a microwave, ethidium bromide was added to the mixture at a concentration of 0.1µl/ml. The gel was poured and allowed to set solid. PCR products were taken out from the PCR microplate, pooled by targets and mixed with DNA loading buffer in a portion of 4:1. 20µl sample and 5µl of 20bp DNA ladder were loaded in the appropriate wells. The samples were run on the gel with a speed of 5V/cm for 1 hour and visualized under UV light (Figure 2.3.42.3.4).

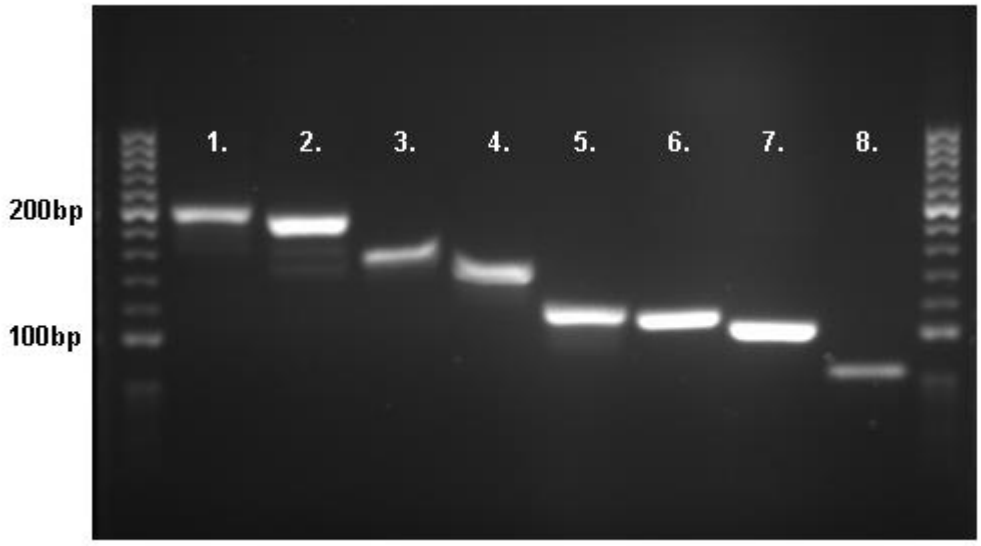


Figure 2.3.4. Gel electrogram of PCR products.
PCR products were subject to gel electrophoreses after qRT-PCR reaction. DNA ladder indicate the size of the amplicons. The expected length of the product were as the follows: 1) 15-LOX-1_1 (199bp); 2) CK8_2 (189bp); 3) 15-LOX-2_1 (156bp); 4) GAPDH (143bp); 5) IFITM-1_1 (114bp); 6) CD36_1 (111bp); 7) COX-2 (104bp); 8) PPAR-γ (82bp).

2.3.7.3 Determining the amplification efficiency

Validation of a qRT-PCR assay is the last step to determine the reaction efficiency. Theoretically the target doubling with each cycle corresponds to 100% efficiency. The simplest calculations, such as comparative Ct method assume that the efficiency is maximal with target doubling with each cycle. It also assumes that the efficiency of the housekeeping gene and the gene of interest are similar. However, several factors could have an effect on the productivity of amplification. Different size and GC-content of the amplicon, different annealing efficiency of the primers, presence of an inhibitor or a not optimal reaction temperature could all change the reaction efficiency and cause bias of the results. Thus, the determination of amplification efficiency is crucial for data analysis. The efficiency of the PCR reaction can be determined in different ways. Serial dilution is one of the most widely used method to determent the reaction efficiency.

a) Serial dilution method

cDNA was diluted in 1:10 over a 5-log range. SYBR® Green qRT-PCR was performed for each target (Figure 2.3.52.3.5). Efficiency curves were generated by plotting Ct values against the dilution factor (Figure 2.3.62.3.6). The slope and the goodness of fit (r^2) was determined by linear regression, whilst the efficiency (E) was calculated according the following equation:

$$E=10^{(-1/slope)}$$

The slope between -3.10 and -3.58 corresponds to 90 to 110% efficiency and indicates the amplification rate is between 1.9 and 2.1.

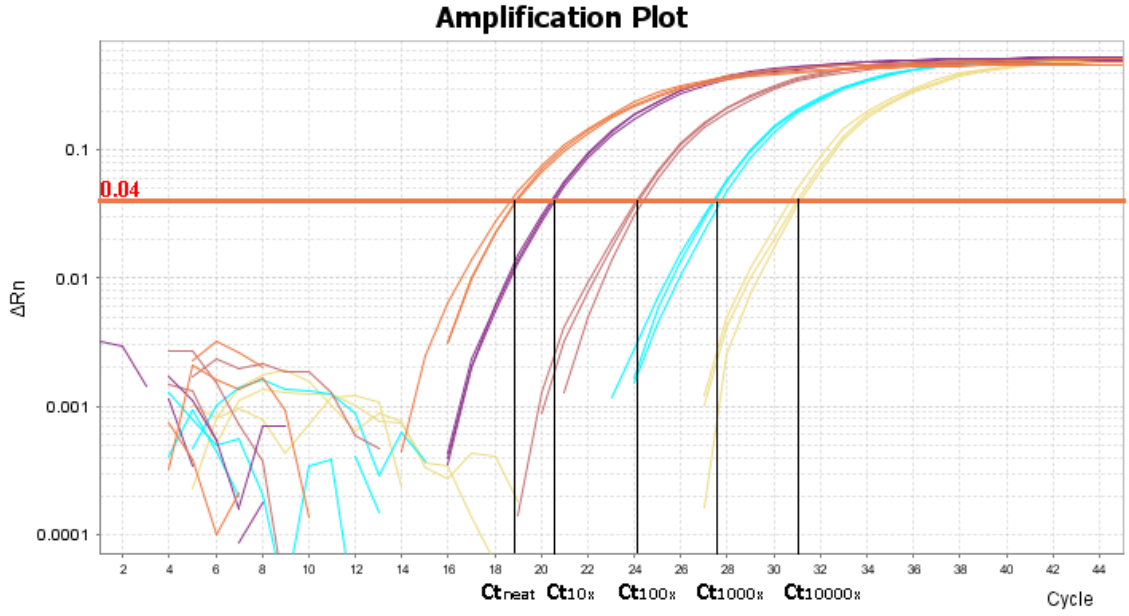


Figure 2.3.5. Representative amplification curves of serially diluted cDNA.
Cycle threshold (Ct) gradually increase by the degree of dilution. (ΔRn) normalized fluorescent signal.

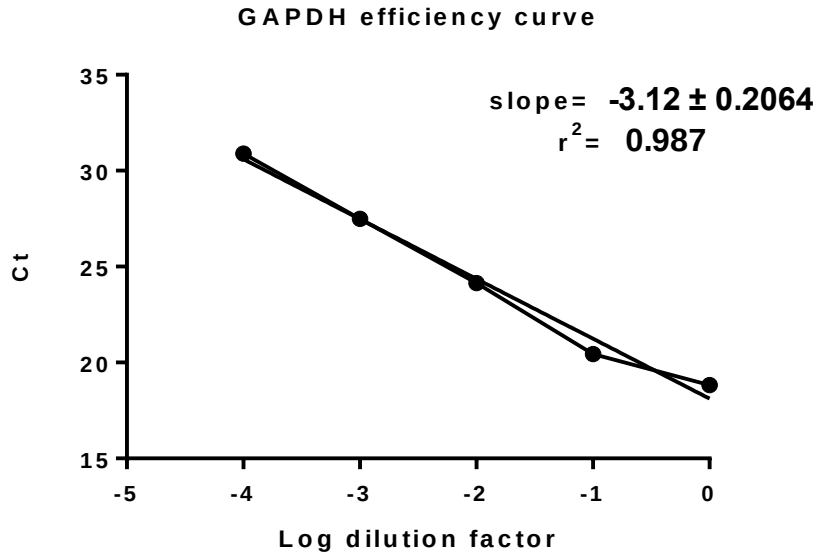


Figure 2.3.6. Representative efficiency curves for GAPDH.
Ct values were plotted against the logarithms of dilution factors. Linear regression was applied to determine the slope of the curve, r^2 represent the best fit value of regression line.

Although the serial dilution method is widely used it has limitations. The determination of reaction efficiency of less expressed genes is challenging or cannot be performed by this method because of the detection limit of the instrument. Also, it is based on the testing results of a low number of samples or standard, thus it does not provide information about the efficiencies of the samples which could be different from the standard.

b) Determining the amplification efficiency from PCR kinetics

Liu and Saint (2002) developed a new method using PCR kinetics to define the amplification efficiency (Liu and Saint, 2002). This method is cost-effective and less time-consuming than serial dilution method as it does not require further experiments. A further advantage of this method is that the efficiency can be calculated for every sample. The Ct value of the analyte was determined on two thresholds along the exponential phase of the amplification curve (Figure 2.3.72.3.7) and the following equation was used to calculate the efficiency:

$$E = \left(\frac{\Delta RnA}{\Delta RnB}\right)^{\left(\frac{1}{CtA-CtB}\right)}$$

Where E is the efficiency, Rn is the normalized reporter fluorescent signal at threshold A or B, Ct is cycle value at threshold A or B. Table 2.3.3 summarizes the reaction efficiencies.

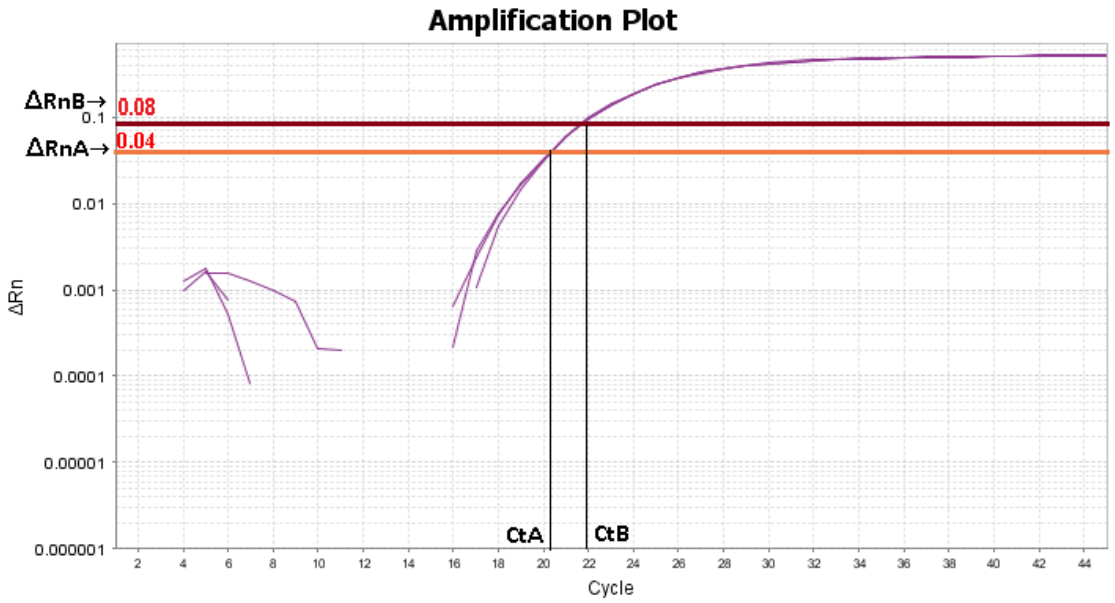


Figure 2.3.7. Calculation of reaction efficiency using amplification curve.
Threshold cycle (Ct) of the analyte was determined at two fluorescent signal levels (ΔRn) along the exponential phase of the reaction. CtA and CtB were used to calculate the efficiency of PCR reaction.

Table 2.3.3. Reaction efficiencies determined by PCR kinetic.
Mean of efficiencies were calculated for each targets and specimens (mean ± SD).

	PCR Efficiency of Gene of Interest (mean ± SD)								
	15-LOX-1_1	**15-LOX-2_1**	**PPAR-γ_2**	**CD36_1**	**COX-2**	**IFITM-1_1**	**CK8_2**	**GAPDH**	**β-actin_1**
Endometrium	1.90 ± 0.03	1.90 ± 0.04	1.80 ± 0.03	1.85 ± 0.03	1.91 ± 0.03	1.66 ± 0.04	1.86 ± 0.03	1.72 ± 0.04	1.87 ± 0.03
Ectopic lesion	1.90 ± 0.03	1.89 ± 0.05	1.82 ± 0.03	1.86 ± 0.02	1.91 ± 0.04	1.68 ± 0.05	1.87 ± 0.03	1.76 ± 0.05	1.88 ± 0.02
Peritoneal wall	1.89 ± 0.01	1.89 ± 0.01	1.80 ± 0.01	1.84 ± 0.04	1.92 ± 0.02	1.67 ± 0.02	1.87 ± 0.02	1.78 ± 0.03	1.88 ± 0.02
Peritoneal cells	1.89 ± 0.02	1.90 ± 0.02	1.78 ± 0.03	1.84 ± 0.02	1.95 ± 0.01	1.64 ± 0.03	1.78 ± 0.02	1.73 ± 0.02	1.88 ± 0.02
Blood	1.89 ± 0.06	1.90 ± 0.02	1.82 ± 0.03	1.83 ± 0.03	1.93 ± 0.02	1.66 ± 0.03	1.86 ± 0.02	1.72 ± 0.04	1.86 ± 0.02
Σ	**1.89** ± 0.04	**1.90** ± 0.03	**1.81** ± 0.03	**1.85** ± 0.03	**1.92** ± 0.03	**1.66** ± 0.04	**1.86** ± 0.03	**1.74** ± 0.04	**1.87** ± 0.02

(15-LOX) 15-lipoxygenase; (PPARγ) peroxisome proliferator-activated receptor gamma; (CK8) cytokeratin 8; (IFITM-1) interferon induced transmembrane protein 1; (GAPDH) glyceraldehyde-3-phosphate dehydrogenase; (COX) cyclooxygenase

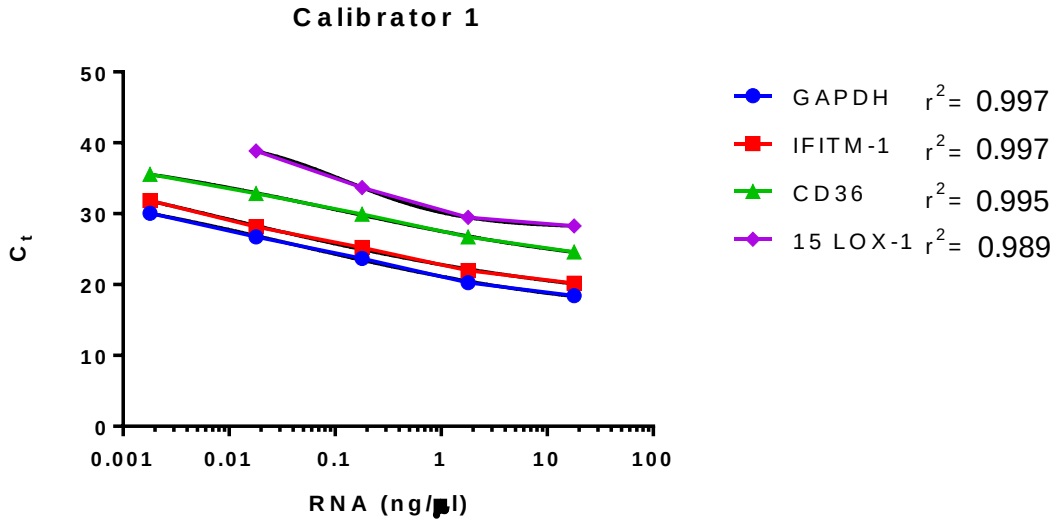
2.3.8 Quantification of genes of interest

The relative standard curve method was applied for the quantification of genes of interest because the amplification efficiencies and dynamic ranges were varied for the studied genes. Using mathematical models for the calculation of PCR data would cause bias in the results since these equations do not take into account the dimensions of the dynamic range and assume the same efficiency over the dynamic range of the assay.

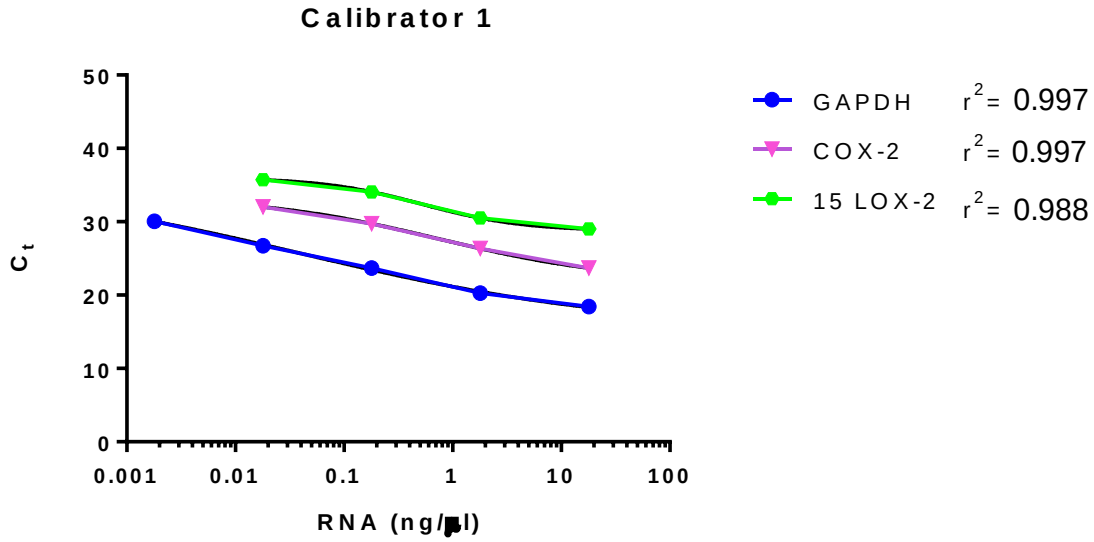
To construct standard curves two samples were chosen. Calibrator 1 was used to determine the mRNA concentration for 15-LOX-1 and 2, IFITM-1, COX-2 and CD36, whereas calibrator 2 was used to determine the mRNA concentration for PPARγ and cytokeratin 8 in samples of interest. Furthermore, standard curves were constructed with both calibrators for the endogenous reference genes and the appropriate curve was used for the calculation of relative gene expressions of genes of interest.

cDNA of the calibrators were diluted in 1:10 with RNAse-free water over a 5-log scale for each target and run in triplicate using QuantiTect® SYBR® Green PCR kit as described in section 2.2.6. Following the qRT-PCR assay the Ct values of the standard points were plotted against the log of template amounts. The highest point was equivalent with 18ng/µl RNA. 4-parameter non-linear regression model was applied for interpolating the concentration of analytes using GraphPad Prism 7 software. Figure 2.3.82.3.8 illustrates the standard curves of both calibrator samples for the target gene of interest.

(A)



(B)



(C)

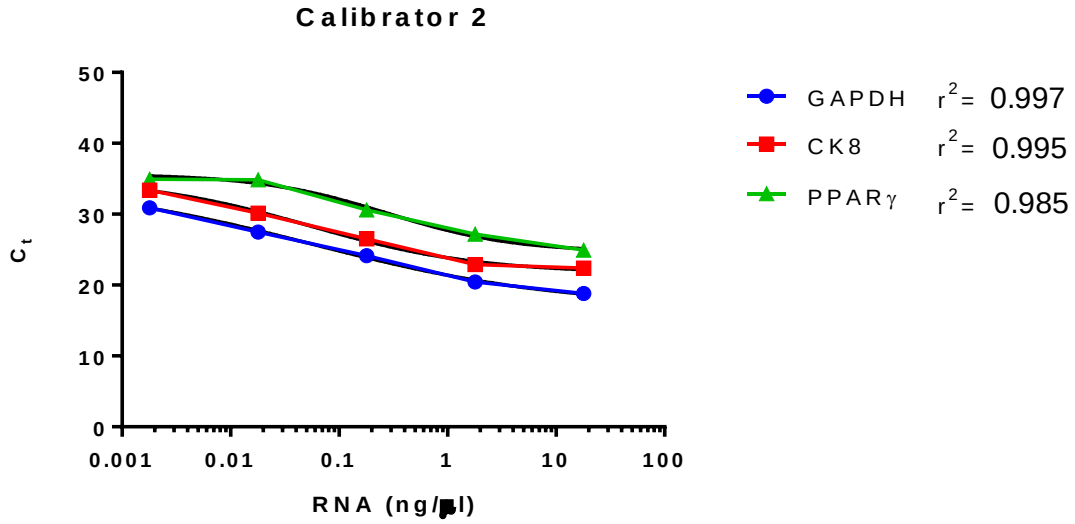


Figure 2.3.8. Representative standard curves of calibrator 1 (A-B) and 2 (C) for the target genes of interest.
Black line and r^2 represent the best-fit regression. Assay was performed using SYBR Green qRT-PCR method normalized to 360ng total RNA. cDNA of the calibrators were serially diluted in 1:10 with RNAse-free water over a 5-log scale. The top standard point was equivalent with 18ng/µl RNA and the range of concentration was 18-0.0018ng/µl in five standard points.

2.3.9 Validation of the endogenous reference gene

GAPDH and β-actin were nominated for endogenous reference genes. The main requirements for an ideal reference gene are that the expression of the gene should not be affected by the investigated condition and the expression levels do not differ between samples. RefFinder [http://150.216.56.64/referencegene.php] was used to test the stability of the possible reference genes. RefFinder is a comprehensive web-based tool which incorporates the currently available four, main reference finder programs such as geNorm, Normfinder, BestKeeper and comparative ΔΔCt method. Based on the results of the four computational methods RefFinder compared and ranked the candidate reference genes. The overall rankings indicate that the GAPDH was the most stabile gene and β-actin was not, completes the requirement of endogenous reference gene. Hence, GAPDH was used for the normalization of the expression of the targeted genes. Figure 2.3.92.3.9 shows the ranks of the investigated genes.

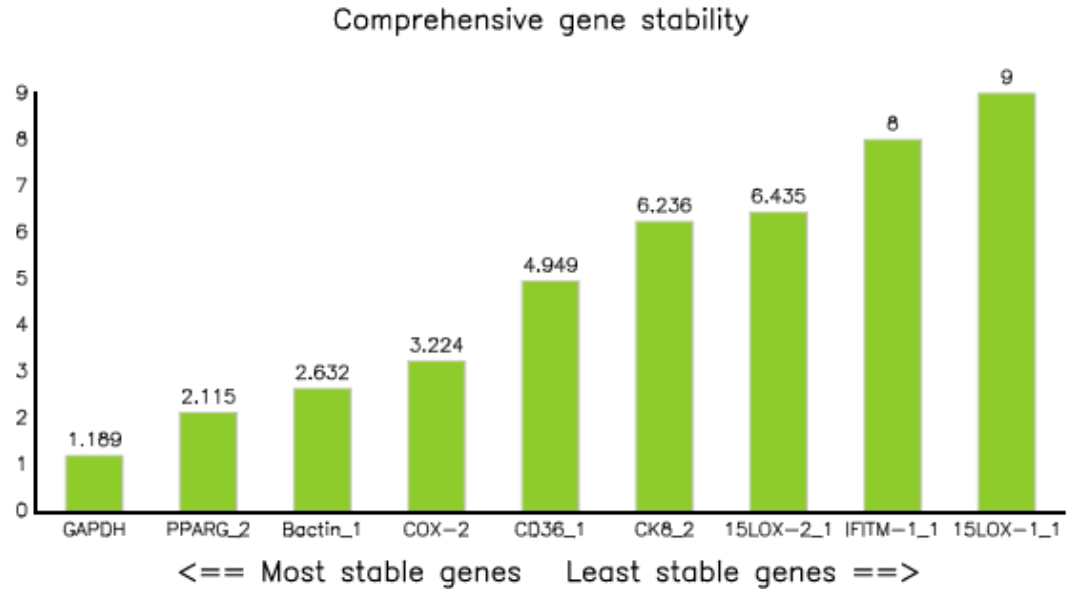


Figure 2.3.9. Comprehensive gene stability of the targeted genes.
Overall gene stability was calculated using four computational methods by RefFinder. GAPDH was ranked as the most stable gene therefore it was appropriate for endogenous reference gene. RefFinder is available on the following website: http://150.216.56.64/referencegene.php.

2.3.10 Data analysis

To determine relative gene expressions the target gene was normalized to the endogenous reference gene for each sample therefore the interpolated mRNA concentrations of samples were divided by the interpolated mRNA concentrations of their respective endogenous control as it shown in the equation below.

$$Relative\ gene\ expression = \frac{Relative\ mRNA\ concentration\ of\ Target\ in\ Unknown}{Relative\ mRNA\ concentration\ of\ Endogenous\ Reference\ gene\ in\ Unknown}$$

The expression fold change for the genes of interest were performed by calculating a ratio between the disease and non-disease groups using the means of relative gene expressions of the study groups as is shown in the equation below.

$$Expression\ fold\ change\ of\ target = \frac{Mean\ of\ Relative\ Gene\ Expression\ in\ Disease\ Group}{Mean\ of\ Relative\ Gene\ Expression\ in\ Non-Disease\ Group}$$

Relative gene expressions and mRNA concentrations were compared between study groups. Statistical analysis was performed by GraphPad Prism 7 (Graphpad Software Inc). The distribution of data was assessed by D'Agostino & Pearson omnibus normality test (n≥8/group) or Shapiro-Wilk test (n<8/group). Data that followed a normal distribution was analysed using a student's t-test or one-way analysis of variance (ANOVA) followed by Tukey *post hoc* analysis; whereas non-parametric data were analysed using a Mann-Whitney U test or Kruskal-Wallis test with Dunn's post-test where appropriate. In all cases a *p* value of less than 0.05 was considered statistically significant.

2.4 Histological methods

Histology studies the morphology and micro anatomy of cells and tissue to facilitate better understanding of function. The flow chart below demonstrates the process of histological examination for paraffin embedded specimens (Figure 2.4.12.4.1).

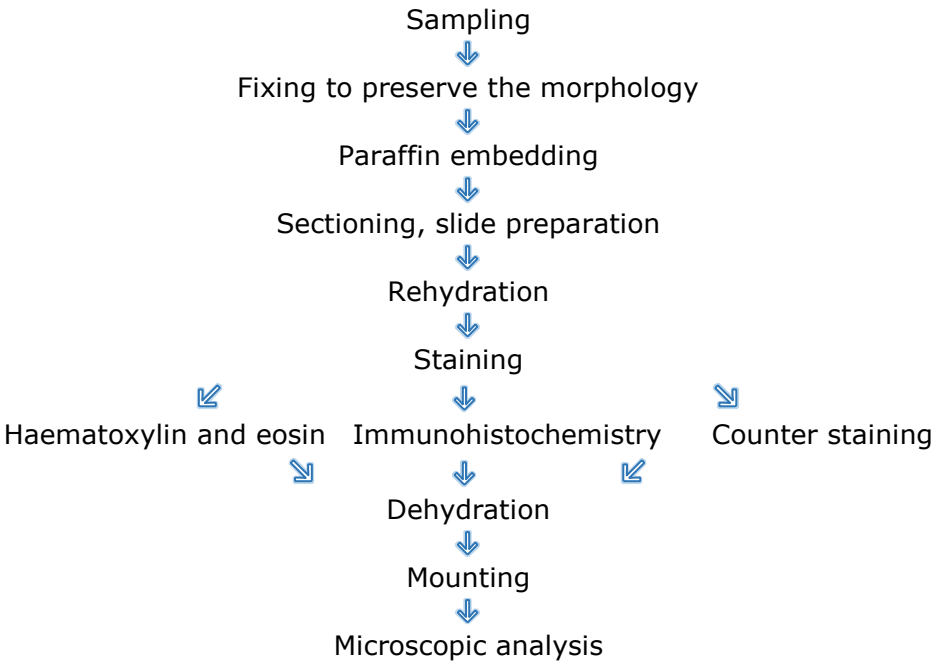


Figure 2.4.1. Outline of the preparation of paraffin embedded specimens for histological examination.

2.4.1 Materials

Solvents, chemicals and consumables used for histological staining and immunohistochemistry were purchased as follows: ethanol (ACS grade, ≥99.5%), phosphate-buffered saline (PBS), 10X, pH7.4, hydrochloric acid (ACS grade, ≥36.5-38%), hydrogen peroxide (ACS grade, 30%), paraformaldehyde powder (pure, >90%), paraffin wax (Lamb wax VA5, cat#8349R2010), embedding cassette (Histosette®), Harris haematoxylin (Shandon, cat#6765001), Eosin Y alcoholic solution (Shandon, cat#6766007), Superfrost™ Plus slides, glass cover slips (50 x 24 x 0.1 mm) and PAP pen were purchased Fisher Scientific (Loughborough, United Kingdom). Histo-Clear II and Omnimount mounting media were obtain from National Diagnostics, USA.

2.4.2 Sample preparation

2.4.2.1 Tissue processing

All tissue samples were transferred in a histological embedding cassette, washed in (Fisher Scientific, Loughborough, United Kingdom) and fixed overnight in 4% (w/v) paraformaldehyde-PBS solution. Next morning the cassettes were washed in PBS and placed in the tissue processor machine (Citadel 2000, Shandon, Thermo Electron Corporation) where the samples were dehydrated through a series of graded ethanol baths and infiltrated with paraffin wax (Lamb wax VA5). The infiltrated tissues were transferred in molten wax and placed in a vacuum chamber (Lindberg/Blue, Thermo Scientific) for 45 minutes then embedded into paraffin blocks on a tissue embedding station (Histocentre 3, Shandon, Thermo Electron Corporation).

2.4.2.2 Sectioning

Blocks were cooled in the freezer prior the sectioning. Serial 4 µm sections were cut using a rotary microtome (1512, Leitz Wetzlar). Two or three serial sections were transferred onto Superfrost™ Plus slides in a 42°C water bath. Slides were air dried and stored at room temperature for further use.

2.4.2.3 Dewaxing, rehydrating

Slides were incubated at 65°C for 20 minutes. The warm slides were immersed into Histo-Clear II for 5 minutes. If the wax was not dissolved completely the process was repeated. Sections were rehydrated in a descending concentration of ethanol baths (100-100-95-80-70% v/v) for 3 minutes in each and soaked in water for 5 minutes.

2.4.3 Haematoxylin and eosin staining

2.4.3.1 Haematoxylin and Eosin staining (H&E) overview

Haematoxylin and eosin (H&E) staining was used to characterise the morphology of tissues. Haematoxylin colours the nuclei of cells in blue, while eosin stains the basic part of the cells such as cytoplasm, collagen and muscle fibres in varying shades of pink to red. Hydrophobic structures such as adipocytes, membrane basalis, Golgi apparatus or reticular fibres do not stain with this method. Haematoxylin in it is own was used as a nuclear counter stain at the end of immunohistochemistry.

2.4.3.2 Performance of H&E staining

H&E staining described by Fischer *et al.* (2008) was used for this study (Fischer et al., 2008). The incubation times of staining steps were optimized to the dye solutions

prior the experiment and for safety reason xylene was replaced by the less toxic Histo-Clear II solution.

Rehydrated paraffin sections were immersed in Harry's haematoxylin for 4 minutes. The slides were washed in cold, running tap water to remove the excess of solution then dipped 3 times in 1% (v/v) acid alcohol (1% (v/v) HCl in 70% (v/v) ethanol) and wash with hot tap water for a minute. Under acetic conditions the haematoxylin is oxidised to haematin which is the active product of haematoxylin and forms a metallic ion complex with aluminium. This haematin-aluminium complex, called haemalum, binds to the lysine and arginine residues of nuclear histones. The slightly alkaline tap water (pH8-8.8) fixes this complex in situ by neutralizing the acid and resulting in an insoluble blue colour of the nucleus. After nuclear stain the slides were passed through alcoholic eosin Y solution for 20 seconds. Eosin is a negatively charged acidic dye thus reacts with positively charged acidophilic components of the tissue. Eosin stains these components in various shades of red, pink or orange.

After the eosin stain the slides were dipped in two baths of water and dehydrated through ascending concentrations of alcohol (70-80-95-100-100% v/v) for 2 minutes in each and submerged in Histo-Clear II twice for 3 minutes. Sections were mounted using Omnimount mounting media and covered with cover slips.

Haematoxylin stain was used as a counter stain after immunohistochemistry. In that case the slides were incubated 4 minutes in haematoxylin, washed in cold tap water, dipped in 1% (v/v) acid alcohol (1% (v/v) HCl in 70% (v/v) ethanol) and washed in hot tap water for a minute. Before mounting the sections were dehydrated in ascending concentration of alcohol and mounted.

2.4.4 Immunohistochemistry

2.4.4.1 Immunohistochemistry overview

Immunohistochemistry (IHC) is a powerful, widely used method to identify and visualize cellular components at their location. The technique is based on immunological and biochemical reactions where the targeted antigen of interest interacts with antigen specific antibodies tagged with a visible label. Positive staining proves the presence and reveals the location of target of interest within the cells and tissues.

A wide variety of methods exists to perform IHC staining. A classical, enzyme-based IHC assay is illustrated in Figure 2.4.22.4.2. The basic steps of the assay are in brief: the primary antibody specifically recognises one part of the antigen, called the

epitope and attaches to this with high affinity. In the second step the primary specific secondary antibody tagged with a biotin molecule and binds to the primary antibody. In the third step a reporter system is added which adheres to the secondary antibody through the biotin molecule. In the case of enzyme-based IHC assay the reporter system contains an enzyme for instant horseradish peroxidase (HRP). This enzyme reacts with a chemical substrate resulting in colour change at the site of antigen. In this study an ABC (avidin-biotin complex) was applied to detect the target of interest. The ABC system uses the specific connection between biotin and avidin giving a strong, reliable signal with a low background.

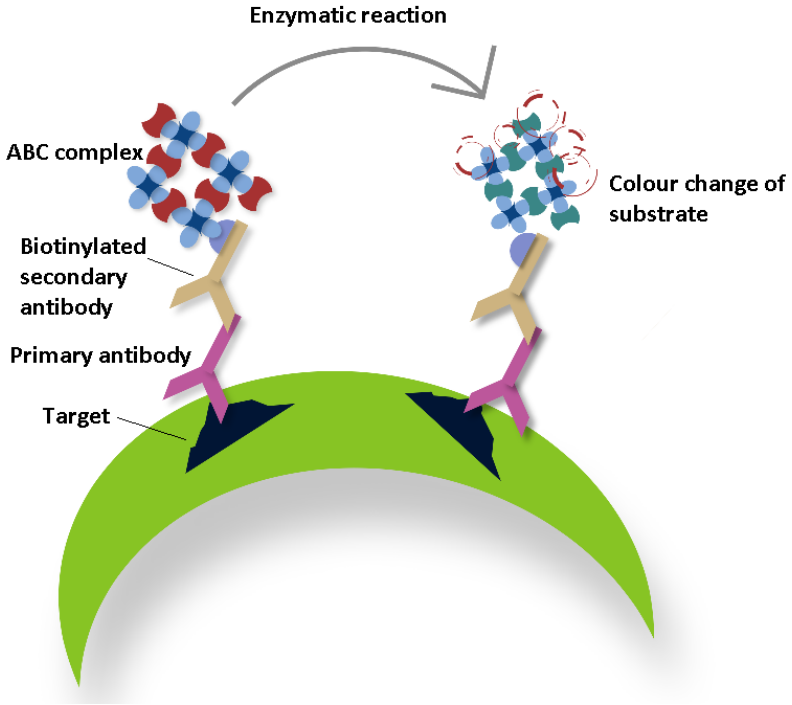


Figure 2.4.2. Antigen antibody complex detected by IHC method.
Immunohistochemistry sequence of reagent application consist of: the primary antibody binds to antigen of interest in the tissue; primary specific biotinylated secondary antibody attaches to the primary antibody; horseradish peroxide enzyme (HRP) coupled avidin biotin complex (ABC) binds to the secondary antibody through biotin molecule; peroxidase activity of HRP causes colour change at the location of antigen which can be visualize under bright field microscope.

2.4.4.2 Performance of IHC

Microscope slides were prepared with two or three serial sections. One or two sections were used for the experiment and one of them for negative control. All specimens were stained in the same experiment to avoid the inter assay variation. Sections were deparaffinised and rehydrated as described in section 2.4.2.3. Antigen retrieval was performed using a heat induced epitope retrieval method developed by Syrbu & Cohen (Syrbu and Cohen, 2011). According to their protocol sections were heated in

Tris-EDTA-SDS buffer (25 mM Tris-HCl, 1mM EDTA, 0.05% SDS, pH 8.5) for 40 minutes at 97 ±1°C. The sections were allowed to cool to room temperature and subsequently immersed in 3%v/v hydrogen peroxide for 10 minutes to block the endogen peroxidase activity of the cells. Sections were placed in a humidified chamber and blocked with 2.5% horse serum (Vectastain Elite ABC HRP Kit, Vector Laboratories, USA) for 20 minutes at room temperature. After the blocking steps, appropriate primary antibody was used at optimized dilution in antibody diluent buffer (0.25% v/v horse serum in TBS Tween 0.1% v/v) and incubated overnight at 4°C. To test the non-specific binding of the secondary antibody negative control was applied where the primary antibody was replaced with antibody diluent buffer.

Next morning the slides were washed three times in Tris-Buffered Saline (TBS) (50mM Tris-Cl, 150mM NaCl, pH7.5) Tween washing buffer (TBS-Tween 0.1% v/v) for 3 minutes with gentle shaking (50 rpm). Biotinylated secondary antibody (#PK-7200, Vectastain Elite ABC HRP Kit, Vector Laboratories) was added to the sections which were then incubated for 2 hours at room temperature in a humidified chamber. Sections were washed 3 times with TBS-Tween washing buffer and then ABC reagent (#PK-7200, Vectastain Elite ABC HRP Kit, Vector Laboratories) was applied for 2 hours at room temperature. Following exposure to the ABC reagent the slides were washed 3 times with washing buffer and incubated with NovaRed peroxidase substrate (#SK-4800, VECTOR NovaRED Peroxidase (HRP) Substrate Kit, Vector Laboratories) for 10 minutes then washed in distilled water for 5 minutes. Sections were counter stained with haematoxylin and mounted as described in section 2.4.3.2. Flow chart demonstrates the main steps of IHC protocol (Figure 2.4.32.4.3).

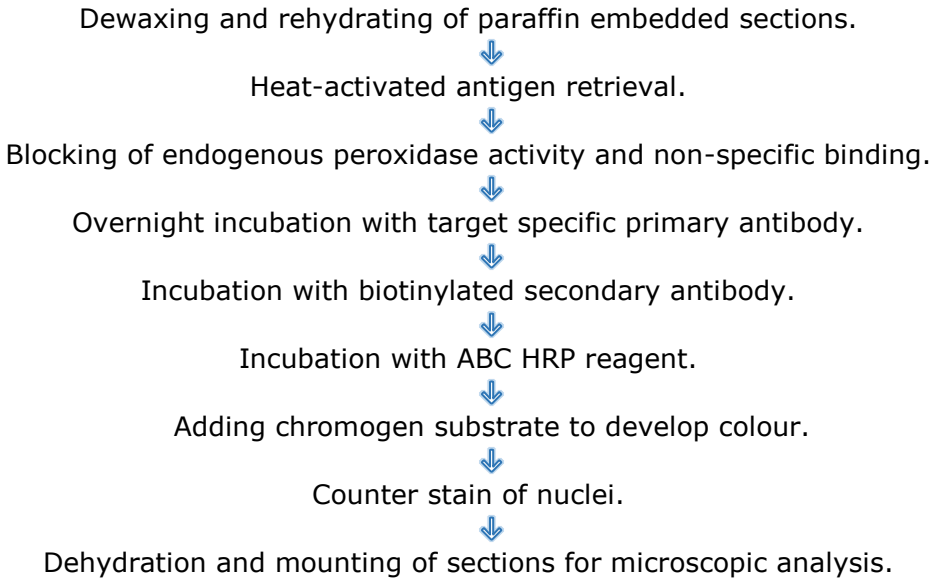


Figure 2.4.3. Outline of immunohistochemistry method for paraffin embedded sections.

2.4.4.3 Optimizing of primary antibodies

Optimal primary antibody dilutions were determined by titration method. Antibodies were applied in a wide range of dilutions as follows: Cytokeratin 8, 18 (1:50-1:100-1:150-1:200-1:250-1:300-1:500); IFITM-1, Abcam (1:250-1:500-1:1000-1:1500); IFITM-1, Sigma (1:500-1:600-1:1000-1:1500-1:2000); COX-2 (1:250-1:500-1:1000-1:1500); 15-LOX-1 (1:100-1:200-1:300); PPARγ (1:100-1:200-1:400); CD36 (1:50-1:100-1:150-1:200-1:400-1:800). Positive and negative controls were included in all experiments. Eutopic endometrium was used as positive control for all targets. In addition, placenta for cytokeratin and omental fat specimens for CD36 were also used as further positive controls. For a negative control, primary antibodies were replaced with antibody diluent buffer. Trial runs always included eutopic endometrium and ectopic lesion specimens from at least three different donors as well as positive and negative controls. Table 2.4.1 summarizes the chosen dilutions was used for this study.

Table 2.4.1. List of primary antibodies were used for this study. The optimal concentrations were titrated by trial runs prior the staining.

Target	Antibody	Supplier	Dilution suggested by supplier	Dilution used in this study
Cytokeratin 8, 18	Mouse monoclonal (K8.8 + DC10)	#MA5-12281, Thermo Fisher Scientific	1:100	**1:250**
IFITM-1	Rabbit polyclonal	#ab106265, Abcam	1:100	**1:500**
IFITM-1	Rabbit polyclonal	#HPA004810, Sigma	1:500	**1:600**
COX-2	Rabbit polyclonal	#ab15191, Abcam	1:400	**1:500**
15-LOX-1	Mouse monoclonal (3G8)	#ab119774, Abcam	1:150	**1:200**
PPARγ	Rabbit monoclonal (K.242.9)	#MA5-14889, Thermo Fisher Scientific	1:400	**1:100**
CD36	Rabbit monoclonal (EPR6573)	#ab133625, Abcam	1:100	**1:400**

(LOX) lipoxygenase; (PPARγ) peroxisome proliferator-activated receptor gamma; (COX) cyclooxygenase; (IFITM-1) interferon induced transmembrane protein 1

2.4.4.4 Image analysis

Mounted slides were scanned on a 3D-Histech Pannoramic-250 microscope slide-scanner using a [*20x/ 0.80 na, Plan Achromat*] objective (Zeiss) and 4M resolution CMOS colour camera (VCC FC60FR19CL, Cis Corporation).

Where a eutopic endometrial biopsy was available, the stage of the period was confirmed on H&E stained sections using "Atlas of Endometrial Histopathology" from Lowe as a reference (Lowe, 1985). The same book and the eutopic endometrial sections were used as references for the identification of endometrial-like glands and stroma in the ectopic lesions. In addition, glandular and stromal cell markers were also applied to confirm the presence of endometrial-like tissue in these specimens.

The IHC analysis was performed with a semi-quantitative scoring method. Three 600 µm diameter representative areas of interest (AOI) were chosen on H&E section using Pannoramic Viewer (version 1.15.4.) software (3D-Histech). The selection of AOIs was not random. For eutopic endometrium, the AOIs must have covered glands and stroma, whereas the AOIs covered the endometrial-like cells within the ectopic lesions. After AOIs were selected on the H&E sections the same areas were identified on the IHC stained sections, and the intensity and distribution were scored. Figure 2.4.42.4.4 illustrates the method of selecting area of interest on the tissue sections.

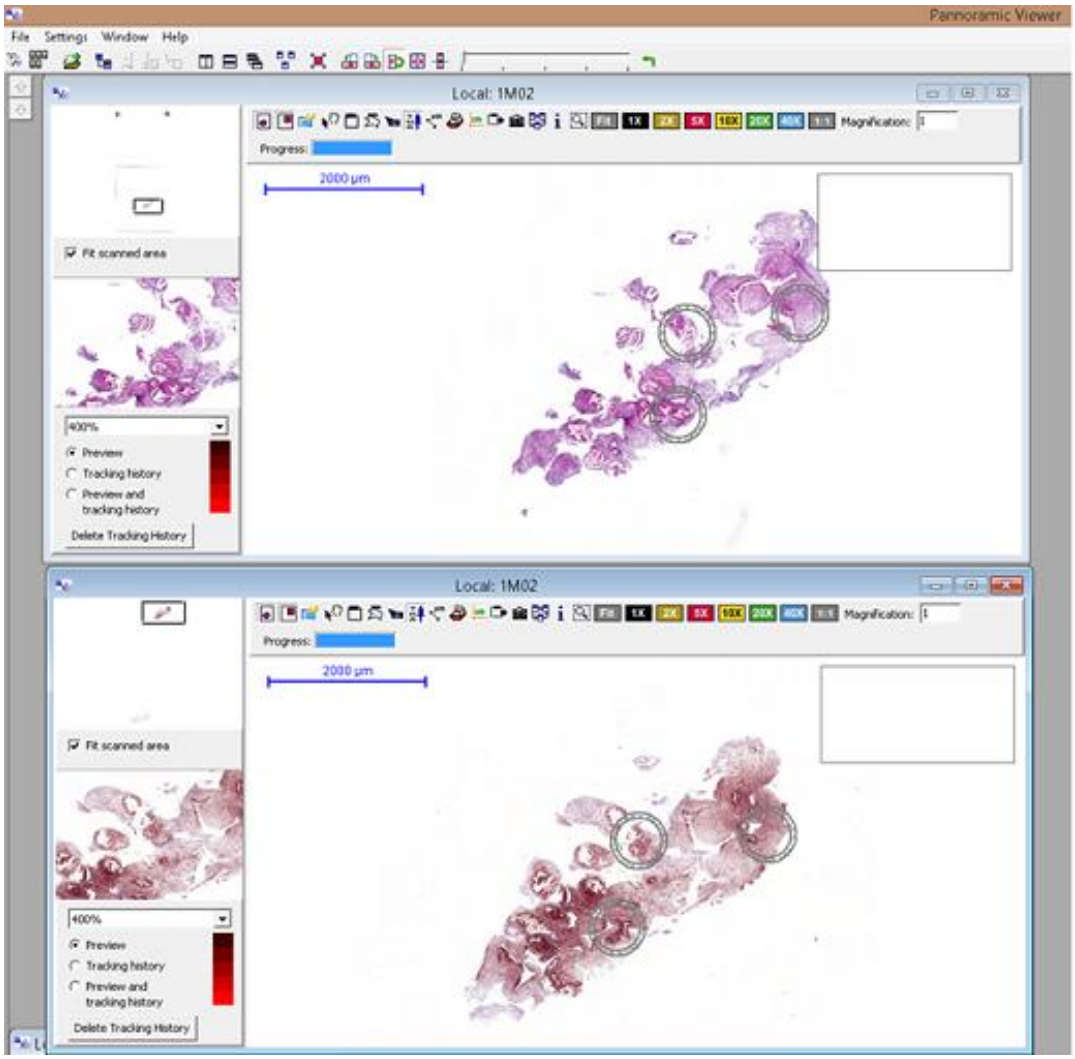


Figure 2.4.4. Selecting areas of interest (AOI) using the Pannoramic viewer.
To ensure that the same areas were analysed for all targets, firstly three 600 µm diameter areas of interest were chosen on an H&E stained section, then the same areas were identified on the immunostained serial sections and used for analysis.

Distribution and staining intensity were scored as described by Busca *et al.* in 2016 and adapted for this study (Busca et al., 2016). The distribution was considered 0 if less than 5% of cells stained, 1 if less than 50% were positive, marked 2 if 50% to 75% were positive and 3 if more than the 75% of the cells were stained. The intensity also was scored from zero to three as follows: 0 absence of staining, 1 weak staining, 2 moderate staining and 3 strong staining. To make a decision between weak and moderate and moderate and strong intensity was challenging. To overcome this problem, reference pictures were chosen prior the analysis to minimize the subjectivity for scoring of intensity. For the same reason sections were randomly scored. Figure 2.4.52.4.5 shows the reference pictures for mild, moderate and strong intensity.

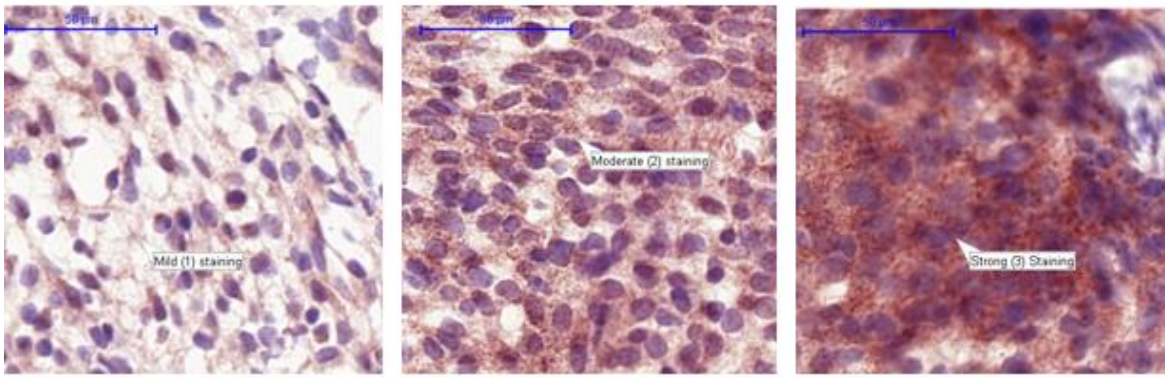


Figure 2.4.5. References pictures for scoring of intensity.
(1) mild; (2) moderate; (3) strong staining. Snapshots of the slide-scans were taken using the Pannoramic Viewer software (3D-Histech). 40x magnification

2.4.4.5 Statistical analysis of IHC results

The mean of intensity and the mean of distribution were calculated for three areas of each section then the staining scores were calculated by adding the mean of intensity and mean of distribution for each section ranging from 0 to 6. In the case of eutopic endometrium, the glands and stroma were scored and analysed separately, then the mean of staining scores of glands and stroma were calculated to make it comparable with ectopic lesion and peritoneal wall. Statistical analysis was performed by GraphPad Prism 7 (Graphpad Software Inc). The distribution of data was assessed by D'Agostino & Pearson omnibus normality test ($n \geq 8$/group) or Shapiro-Wilk test ($n < 8$/group). Data that followed a normal distribution was analysed using a student's t-test or one-way analysis of variance (ANOVA) followed by Tukey *post hoc* analysis; whereas non-parametric data were analysed using a Mann-Whitney U test or Kruskal-Wallis test with Dunn's post-test where appropriate. In all cases a p value of less than 0.05 was considered statistically significant.

2.5 Enzyme-linked Immunosorbent Assay

2.5.1 Overview of Enzyme-linked Immunosorbent Assay

Enzyme-linked immunosorbent assay (ELISA) is a broadly used biochemistry technique designed for detecting an analyte of interest in fluid specimens. The method, similar to other immunological assays, is based on the specific interaction between antibody and antigen. ELISA is popular because it easy to use, flexible and highly sensitive. It is capable of quantifying targets in the picogram range in different biological matrixes and relatively easily can be adapted to the requirements thus several types were developed. One of the most commonly used type is the sandwich ELISA. This method is suitable the detection of biomolecules, hormones, cytokines and growth factors using a target specific capture and detection antibody pair. The "sandwich" refers to the position of the analyte which is being bound between the capture and detection antibody. Figure 2.5.12.5.1 shows the outline of the sandwich ELISA.

Microtiter plate is coated with a capture antibody.

⇩

Blocking of the unspecific binding.

⇩

Incubation of the sample. Analyte of interest binds to the capture antibody.

⇩

Biotinylated detection antibody attaches to the immobilised target.

⇩

HRP coupled avidin binds to the immunocomplex via biotin.

⇩

Chromogen substrate is added and converted by the HRP enzyme.

⇩

Stopping of enzymatic reaction.

⇩

Optical density recorded by a plate reader.

⇩

Calculation of the unknown concentration using a calibration line.

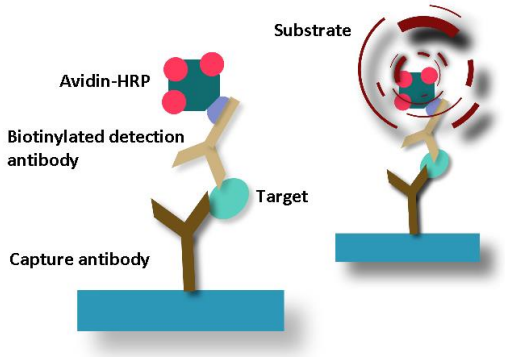


Figure 2.5.1. Outline of sandwich ELISA.
The flow chart summarize the main steps of the assay, whilst cartoon shows the binding sequence of reagents. (HRP) horseradish peroxidase

2.5.2 Materials

Uncoated human TNFα (#88-73-46, Invitrogen) and IL-1β (#88-72-61, Invitrogen) ELISA kits and the stop solution (#1857852) were purchased from Thermo Fisher Scientific (Cramlington, United Kingdom). PBS (#P-3744) and Tween20 (#P-9416) were obtained from Sigma-Aldrich (Poole, United Kingdom).

High binding microtitre plates and plastic wares were supplied by Greiner bio-one Ltd. (Stonehouse, United Kingdom). A benchtop centrifuge (Heraeus Fresco 17, Thermo Scientific) was used for sample preparation. P10, P20, P200, P1000 and P5000 single and P300 12 channels pipettors (Biopette, Labnet, USA) and a vortex mixer were used for the dilution of the reagents. The optical density was recorded by BioTek Elx800 plate reader and Gen 5.1 software.

2.5.3 Preparation

Components of the assay kits were pre-titrated and validated by the supplier therefore the manufacturer instructions were followed. The dilution factors of the antibodies and the incubation periods were identical in both assays hence the assay protocols are not detailed separately.

2.5.3.1 Sensitizing of reaction plates

Capture antibodies were diluted in 1:250 in 1X coating buffer. 100µl of diluted antibody were pipetted per well and incubated overnight at 4°C. Next day, the plates were aspirated and washed 3 times using 300µl wash buffer (PBS-Tween20 0.1% v/v) per well. To avoid the non-specific binding the plates were blocked with 300µl 1X ELISA/ELISAPOT diluent and incubated for an hour at room temperature. After the incubation the blocking solution was removed, the plates were washed once and air dried. The dry plates were sealed and stored at 4°C.

2.5.3.2 Dilution of standards

Standards were reconstituted in 1ml deionized water to give the stock concentration of 15ng/ml and left at room temperature for 15 minutes to dissolve completely. The standards were diluted in 1X ELISA/ELISAPOT dilution buffer. The top standard concentrations were 1000 and 300pg/ml for TNFα and IL-1β, respectively. Then, 2-fold serial dilution of the top standards were performed to make the standard curve for a total of 9 points. The standard points were the following: 1000 – 500 – 250 – 125 - 62.5 - 31.25 - 15.6 - 7.8 - 3.9 pg/ml for TNFα and 300 – 150 – 75 – 37.5 – 18.75 – 9.4 – 4.7 – 2.3 – 1.2 pg/ml for IL-1β.

2.5.3.3 Specimens

Plasma, peritoneal fluid and washes were tested. Samples were thawed on ice and centrifuged at 10000g for 10 minutes at 4°C to remove the precipitation.

Prior to the assay protocols were started all kit components were allowed to reach the room temperature.

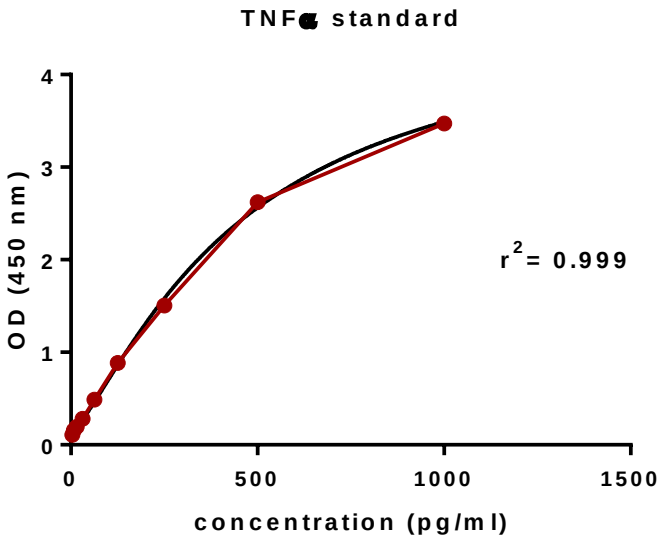
2.5.4 Performance of TNFα and IL-1β ELISA assay

According the manufacturers protocol, 100µl of standard or samples were pipetted in the appropriate wells in duplicate. Plates were incubated at room temperature for 2 hours. At the end of the incubation, wells were emptied and washed 3 times with 300µl wash buffer per wells. After the third wash, the plates were firmly tapped against an absorbent paper to remove the excess of wash buffer. Detection antibodies were diluted in 1X ELISA/ELISAPOT buffer at the dilution of 1:250. 100µl antibody solution was pipetted in every well and incubated at room temperature for one hour. Plates were blotted and the washed three times. Avidin-HRP was added to each well at the concertation of 1:250 and incubated at room temperature for 30 minutes. The incubation was followed by three wash step and 100µl TMB substrate was added each well. The plates were incubated for 15 minutes in the dark. The reaction was stopped by addition of 50 µl stop solution per well and the absorbance was recorded at 450nm.

2.5.5 Determination of analyte concentration

Average absorbance values of standards and analytes were calculated. Standard curves were generated by plotting optical density against the concentration. A 4-parameter non-linear regression model was used for interpolating the concentration of analytes. The calculation was performed by GraphPad Prism 7 software. Figure 2.5.22.5.2 illustrates the representative standard curves of TNFα and IL-1β.

(A)



(B)

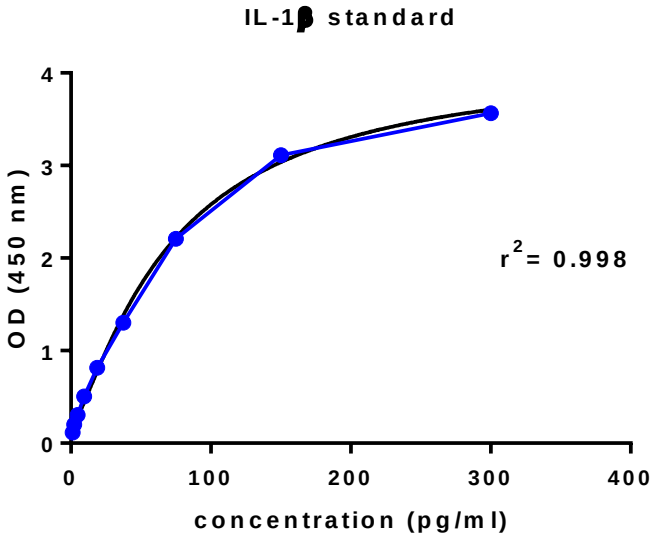


Figure 2.5.2. Representative standard curves of (A) TNFα and (B) IL-1β ELISA. Black line and r^2 represent the best-fit regression.

2.5.6 Glucose concentration determination in peritoneal washes

2.5.6.1 Overview of enzymatic glucose detection assay

The principle of the assay is based on the oxidation of D-glucose by glucose oxidase to D-gluconolactone and H_2O_2. In the presence of HRP the peroxidase substrate reacts with H_2O_2 resulting a colorimetric product where the colour intensity is proportional to the glucose concentration.

2.5.6.2 Materials

A glucose colorimetric detection kit (#EIAGLUC, Invitrogen) was purchased from Thermo Fisher Scientific (Cramlington, United Kingdom). Plastic wares were supplied by Greiner bio-one Ltd. (Stonehouse, United Kingdom). P10, P20, P200, P1000 and P5000 single and P300 12 channels pipettors (Biopette, Labnet, USA) and a vortex mixer were used for the dilution of the reagents and dispensing of samples and reagents. The optical density was recorded by BioTek Elx800 plate reader using Gen 5.1 software.

2.5.6.3 Performing of glucose colorimetric assay

The assay was performed according to the manufacturers instructions. In brief it was as follows: glucose standards were diluted in assay buffer to produce a standard curve from 32mg/dL to 0.5mg/dL for a total of 7 points. 20µl of standards and peritoneal washes were dispensed in duplicate into the appropriate wells of the 96wells microtitre plate. 25µl 1X HRP solution, substrate and 1x glucose oxidase were added into each wells, respectively. The plate was incubated at room temperature for 30 minutes then read the absorbance at 570nm.

2.5.6.4 Determination of analyte concentration

Average absorbance values of standards and analytes were calculated. Standard curves were generated by plotting optical density against the concentration. A 4-parameter non-linear regression model was used for interpolating the concentration of analytes. The calculation was performed by GraphPad Prism 7 software. Figure 2.5.32.5.3 illustrates the representative standard curves of glucose assay.

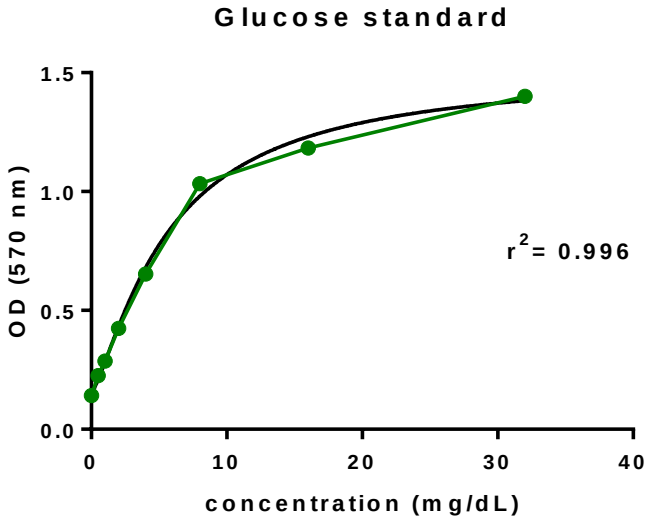


Figure 2.5.3. Representative standard curves of colorimetric glucose assay. Black line and r^2 represent the best-fit regression.

3 CHAPTER: Lipid Mediators in Biological Fluid Specimens

3.1 Introduction

Oxygenated lipid mediators exhibit a range of potent bioactivities in many physiological and pathological processes. Although the oxygenated lipid mediators are a rich class of lipids, only limited sub-groups, such as prostaglandins and leukotrienes, are the focus of research. As was outlined in section 1.6, the role of series-2 prostanoids, particularly PGE_2 is well established in the pathophysiology of endometriosis whereas the function of other COX, LOX and CYP derived lipid mediators remain largely unknown.

For this reason, LC/ESI-MS/MS was applied to define the oxygenated lipid mediator profile of biological fluids from the peritoneum of women with and without endometriosis. This advanced MS technique provides a high sensitivity, specificity and throughput screening of lipid molecules simultaneously in a small specimen volume. Although some recent studies using a similar approach have already examined the alterations of lipid profiles in endometriosis, those studies focused on different classes of the lipids, such as phospho- and sphingolipids (Lee et al., 2014; Chagovets et al., 2017; Domínguez et al., 2017; Adamyan et al., 2018). Thus far, this is the first study measuring 79 oxygenated lipid mediators simultaneously in biological fluid specimens from women with and without endometriosis to explore possible new aspects for the pathology of the disease.

3.2 Participant characteristics

A total of 77 individuals were recruited for this study (Appendix 3); 45 women were diagnosed with endometriosis (case group, EM), 32 patients with other gynaecological disorder (control group, NEM). The main indications of laparoscopic surgery in the non-endometriosis groups were as follows: 9 (28.2%) patients had leiomyoma, 5 (15.6%) ladies suffered from chronic pelvic pain, another 5 participants had benign ovarian cysts and also 5 women were subject to prophylactic surgery due to carrying *BRCA* genes. Four (12.5%) patients had surgery because of heavy menstrual bleeding whereas the last 4 non-endometriosis women had mixed indications for the surgery.

Of the 45 endometriosis patients 31 (68.8%) were diagnosed with peritoneal endometriosis, 3 (6.7%) of them with endometrioma and only one (2.2%) woman suffered from DIE. In the 10 remaining cases of mixed endometrial entities were present. Seven (15.6%) ladies had peritoneal and ovarian endometriosis, whilst 3

participants suffered from peritoneal endometriosis and DIE. The severity of the disease was not classified by ASRM scoring system.

All endometriosis and non-endometriosis participants were asked about their symptoms, menstrual cycle, medications and disease history by the clinicians (see questionnaire in the appendix). Table 3.2.1 illustrates the most common symptoms and Table 3.2.2 summarises the menstrual state for the participants.

Table 3.2.1. Table Summary of the most common gynaecological symptoms of participants. Figures indicate the numbers of sufferers within the study groups.

	Endometriosis (n=45)	Non-endometriosis(n=32)
Menorrhagia	19/45 (42.2%)	14/32 (41.7%)
Dysmenorrhoea	29/45 (64.4%)	12/32 (37.5%)
Pelvic pain	30/45 (66.7%)	12/32 (37.5%)
Fertility problems	19/45 (42.2%)	10/32 (31.2%)
Fibroids	2/45 (4.4%)	10/32 (31.2%)

Table 3.2.2. Summary of menstrual cycle stage and hormone usage of participants. Hormone usage illustrates the numbers of participants who took hormones as a treatment or contraception. (N) numbers of participants; (EM) group of endometriosis; (NEM) group of non-endometriosis patients.

		Age (years)	Stage of Menstrual cycle					Hormone usage
	N	mean ± SD	Non-cycling	Non-regular	Regular			
EM	**45**	34.7 ± 8.4	25 (55.6%)	3 (6.7%)	17 (37.7%)	Menstrual: Proliferative: Secretory:	4 (23.5%) 7 (41.2%) 6 (35.3%)	29 / 45 (64.4%)
NEM	**32**	38.3 ± 8.5	6 (18.8%)	8 (25.0%)	18 (56.2%)	Menstrual: Proliferative: Secretory:	2 (11.1%) 9 (50.0%) 7 (38.9%)	1 / 32 (3.1%)

Table 3.2.1 demonstrates that pain was one of the most commonly reported symptoms for endometriosis. More than 60% of endometriosis patients complained about pelvic pain (66.7%) and/or dysmenorrhoea (64.4%) compared to less than 40% of women without endometriosis. The menstrual cycle also was disturbed in the majority (62.3%) of endometriosis sufferers compared to 43.8% in the non-endometriosis group.

Data from Table 3.2.2 indicates that nearly two-third of endometriosis patients were taking medical therapy. Twenty nine (64.4%) of the participants took hormones as treatment or contraception in the endometriosis group compared to only one (3.1%) individual in the non-endometriosis group. With regards to pharmacotherapy for endometriosis participants 24 (82.7%) women received GnRH analogues. Four patients were subjected to additional HRT or HRT only, whereas only two patients used hormonal contraceptives and one person took an aromatase inhibitor.

From the patient questionnaires it was also apparent that the vast majority of endometriosis patients received medical therapy for the disease. Despite the combined treatment, i.e. surgery and medical treatment, further surgeries were necessary in 17 (37.8%) cases due to disease recurrence.

Five recruited patients were not included in this study due to the following reasons: two patients were out of age range, one patient was diagnosed with endometrial cancer, one patient withdrew her consent, whilst one participant was found pregnant prior the surgery.

Patient information and specimens used for this study are summarized in the appendix 3 and 4.

3.3 Normalisation of peritoneal washes

One of the most challenging problems of the current study was the normalisation of peritoneal wash specimens to make them comparable with peritoneal fluid.

During the laparoscopic surgery the abdominal cavity was flushed with isotonic buffer, which was collected as a peritoneal wash. However, the volume of the buffer used for the lavage was variable depending on the buffer that remained in the tubing. Thus, the dilution factor cannot be calculated and used for the normalisation of MS data.

To overcome this problem, an analyte was sought, the concentration of which was identical in plasma and peritoneal fluid. The normalizing factor could then be calculated based on the ratio of the two identical values of selected analyte in plasma and peritoneal washes.

Glucose was chosen for this purpose since it can be measured easily, its concentration is approximately the same in blood and peritoneal fluid and only severe acute infections, such as sepsis, lower the concentration in the peritoneal fluid (Verrina, 2009).

Firstly, the glucose concentrations of available peritoneal fluid samples were compared to their paired plasma specimens using a handheld glucose monitor and strips (Contour, Bayer plc., UK). It was found that the glucose concentrations in plasma were the same or lower than in the peritoneal fluids. A possible explanation was that, the blood had been taken prior to the surgery and patients were fasted causing low glucose levels, whereas the peritoneal fluid was collected during the

surgery and by that time the glucose level was normalised by intravenous infusion. For that reason, the plasma glucose concentration was not used as a reference point and the dilution factors could not have been calculated based on the plasma glucose values. However, it was noted that the glucose concentrations of peritoneal fluid samples showed low variation. It was in the range of 5.4 ± 0.30 mmol/L (mean ± SD, n=10). Therefore, 5.4 mmol/L was chosen as the reference glucose concentration and used subsequently to calculate the normalizing factors for peritoneal washes.

After determination of the reference point, this normalisation method was tested. In five cases the peritoneal fluid and washes were available from the same subjects. MS analysis was performed on all sample pairs. Normalizing factors were calculated using the glucose concentration of peritoneal washes and the reference concentration of 5.4mmol/L. Glucose concentrations were determined as was described in section 2.6 of the Materials and Methods. Then, percent recovery was calculated between peritoneal fluid and wash pairs for each detected lipid mediators. Table 3.3.1 summarises the arithmetic means of percent recovery for peritoneal washes correlated to peritoneal fluid specimens from the same subjects.

Table 3.3.1. Summary of percent recovery of peritoneal washes correlated to peritoneal fluid. Mass spectrometry was performed for peritoneal fluid and washes from the same subjects. Data of peritoneal washes were normalized by glucose concentration. Percent recoveries were calculated for each detected lipid mediators. Table represents the arithmetic means ± SD of percent recoveries for the subjects.

Patient ID	No. detected lipid mediators	Normalizing factor	Recovery% in peritoneal washes correlated to peritoneal fluid (mean±SD)
HP29	28	3.8	109% ± 35%
HP26	19	6	160% ± 76%
HP58	9	27	283% ± 123%
HP23	14	54	959% ± 619%
HP60	14	54	1130% ± 656%

Data in Table 3.3.1 indicate that the correlation between peritoneal fluid and washes were not always linear and the dynamic range of linear correlation was depend on the dilution factor. Therefore peritoneal washes with higher than a normalizing factor of 27 were excluded from the subsequent analysis.

3.4 Results

In total, 41 endometriosis and 32 non-endometriosis women were included in the MS study and 79 oxygenated lipid species were tested in peritoneal fluid, peritoneal washes and plasma specimens. Table 3.4.1 summarises the number of tested samples by study groups.

Table 3.4.1. Summary of tested biological fluid specimens using LC/ESI-MS/MS. (N) numbers of participants; (EM) group of endometriosis; (NEM) group of non-endometriosis patients.

	N	Peritoneal fluid	Peritoneal washes	Plasma
EM	**41**	7	20	41
NEM	**32**	9	19	29

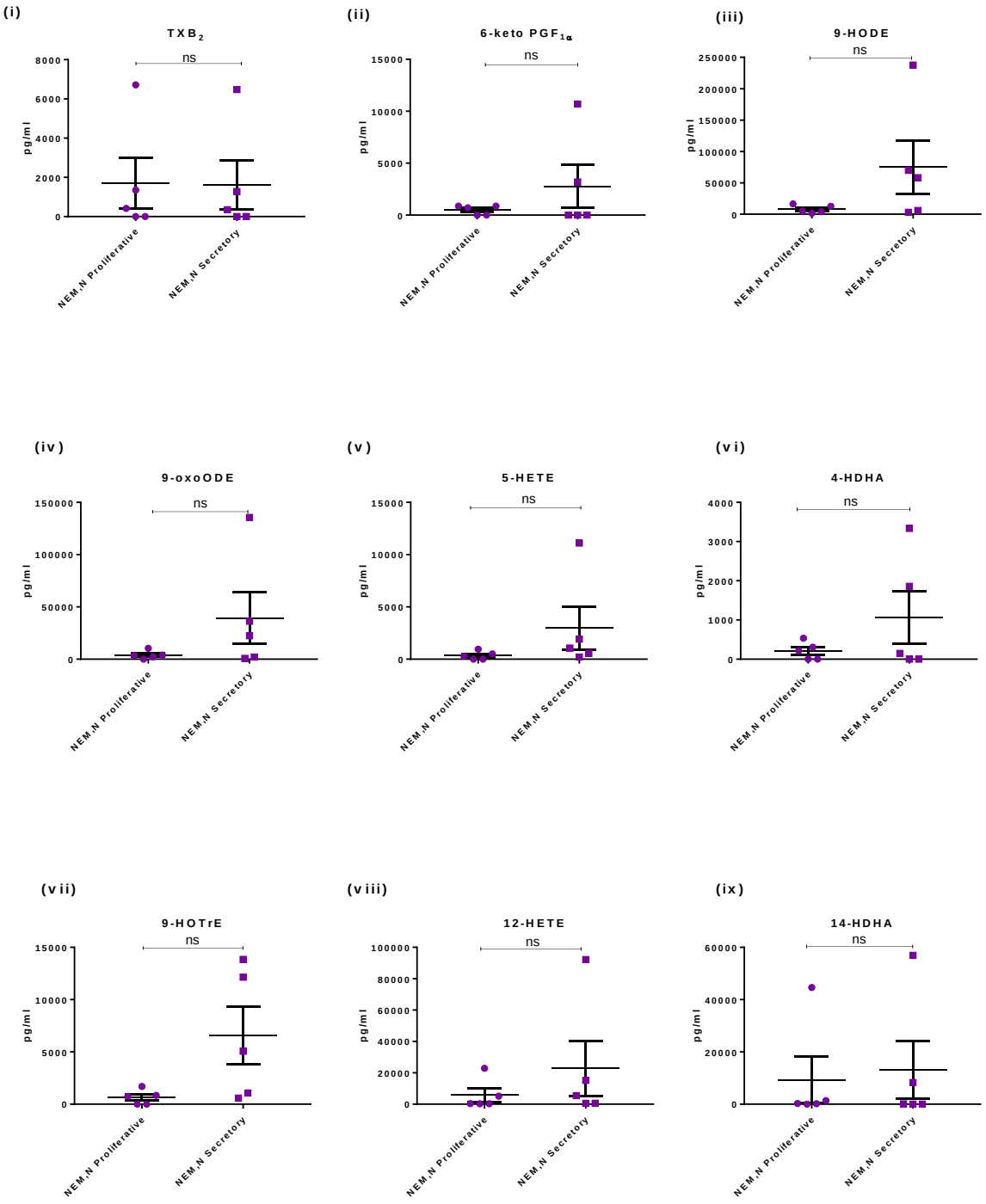
3.4.1 Lipid mediators in peritoneal fluid

Following the normalisation 15 peritoneal fluid (NEM n= 9; EM n= 5) and 17 peritoneal washes (NEM n= 6; EM n= 12) were included in the data analysis. Fluid and normalized washes specimens were subsequently analysed together. A total of 25 species were detected out of 79 tested lipid mediators. Of those species detected and presented here, the analyte was quantified at least in half of the samples. A complete list of all detected mediators is detailed in Appendix 5. Comparisons were performed according to menstrual stages, medical treatment and the presence or absence of endometriosis.

3.4.1.1 Variation of peritoneal fluid lipid mediators by menstrual cycle

Reproductive hormones, such as oestradiol and progesterone show periodic changes with menstrual cycle in premenopausal women. These steroid hormones alongside other autocoids regulate the oogenesis and reproductive function in females as well as the expression of hundreds of genes via their nuclear receptors. Reproductive hormones are present in biological fluids therefore they could have had an effect on lipid mediator synthesis. To determine the variation of lipid signature with the stages of menstrual cycle in peritoneal fluid, lipid mediators were compared according to the stages of the menstrual cycle from women with regular cycle and without using hormonal treatment or hormonal contraceptive medication. Ten non-endometriosis (NEM,N) and seven endometriosis (EM,N) patients reported regular cycle and not taken hormonal medications. Firstly, women without endometriosis were compared. Five non-endometriosis patients were in proliferative, also five in secretory and none in menstrual phases (Figure 3.4.1). Afterwards, lipid mediators form endometriosis patients were plotted according to the stages of the menstrual cycle. Four and three

endometriosis patients were in proliferative and menstrual phases, respectively. Secretory phase were not reported in this group. Lipids from endometriosis patients were compared as follows: EM,N proliferative vs. EM,N menstrual and NEM,N proliferative vs. EM,N proliferative using student t-test or Mann-Whitney U test where appropriate (Figure 3.4.2).



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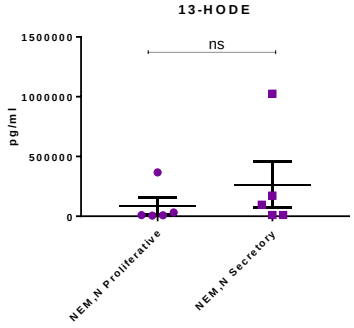

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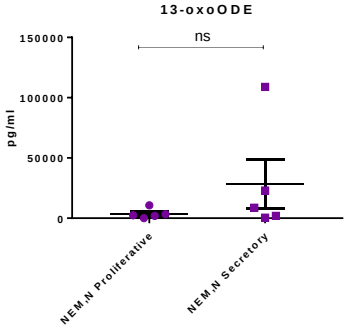

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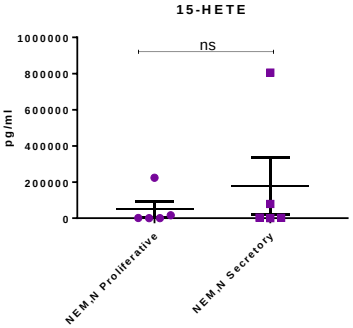

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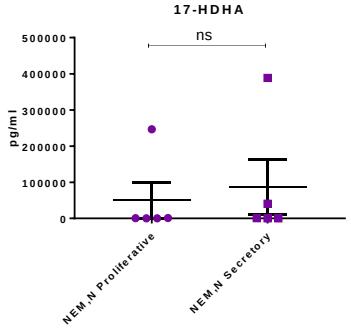

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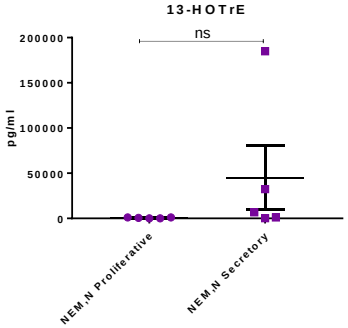

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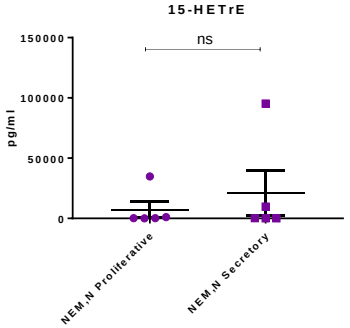

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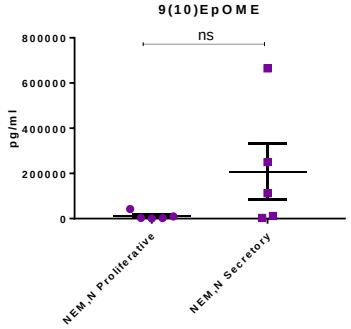

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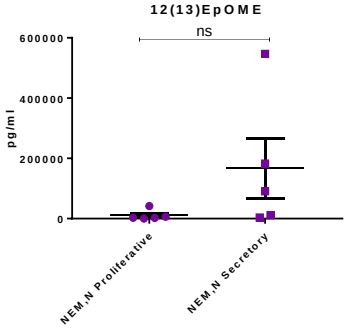

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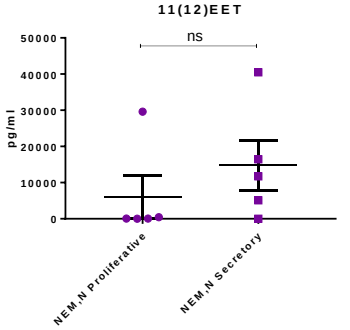


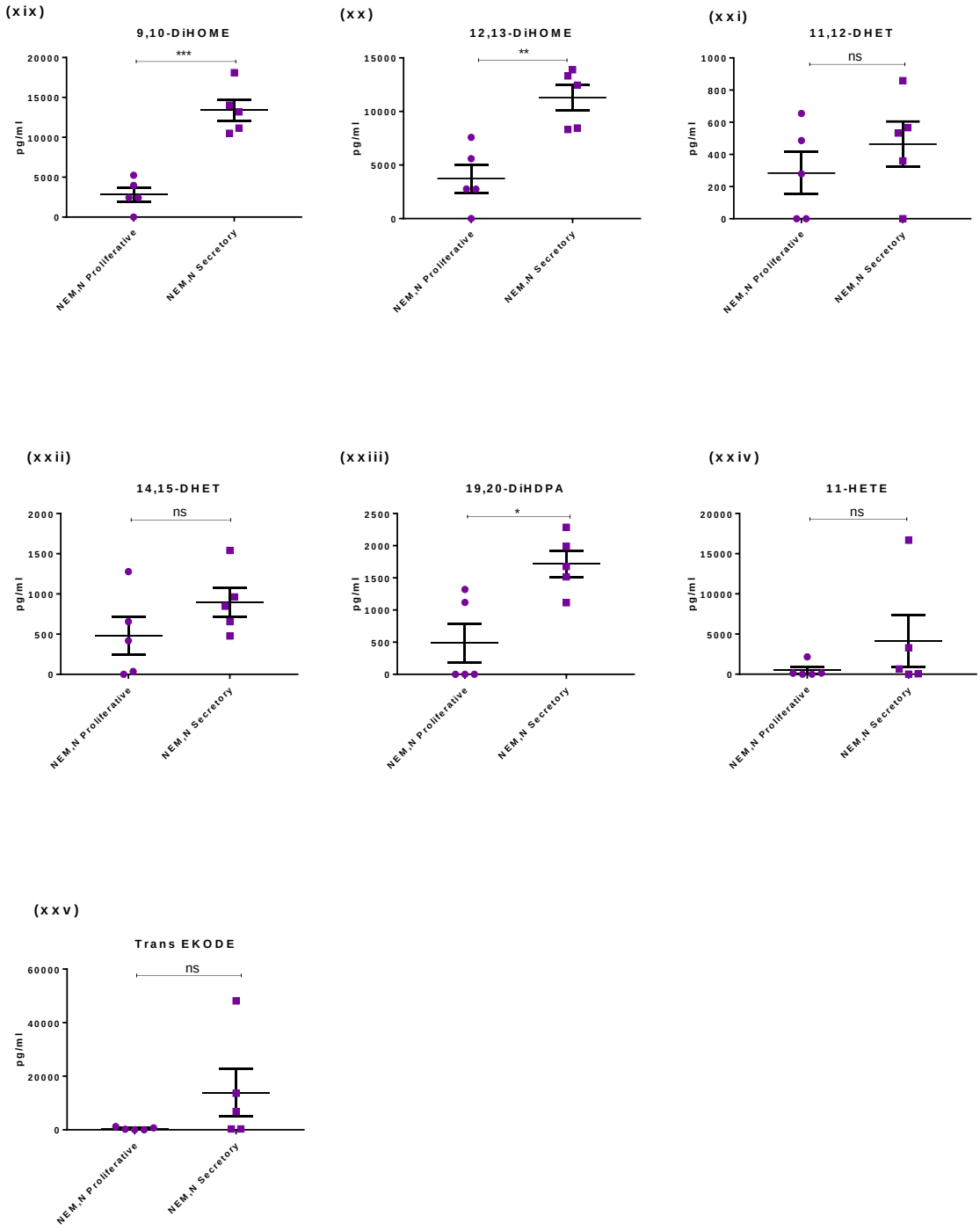
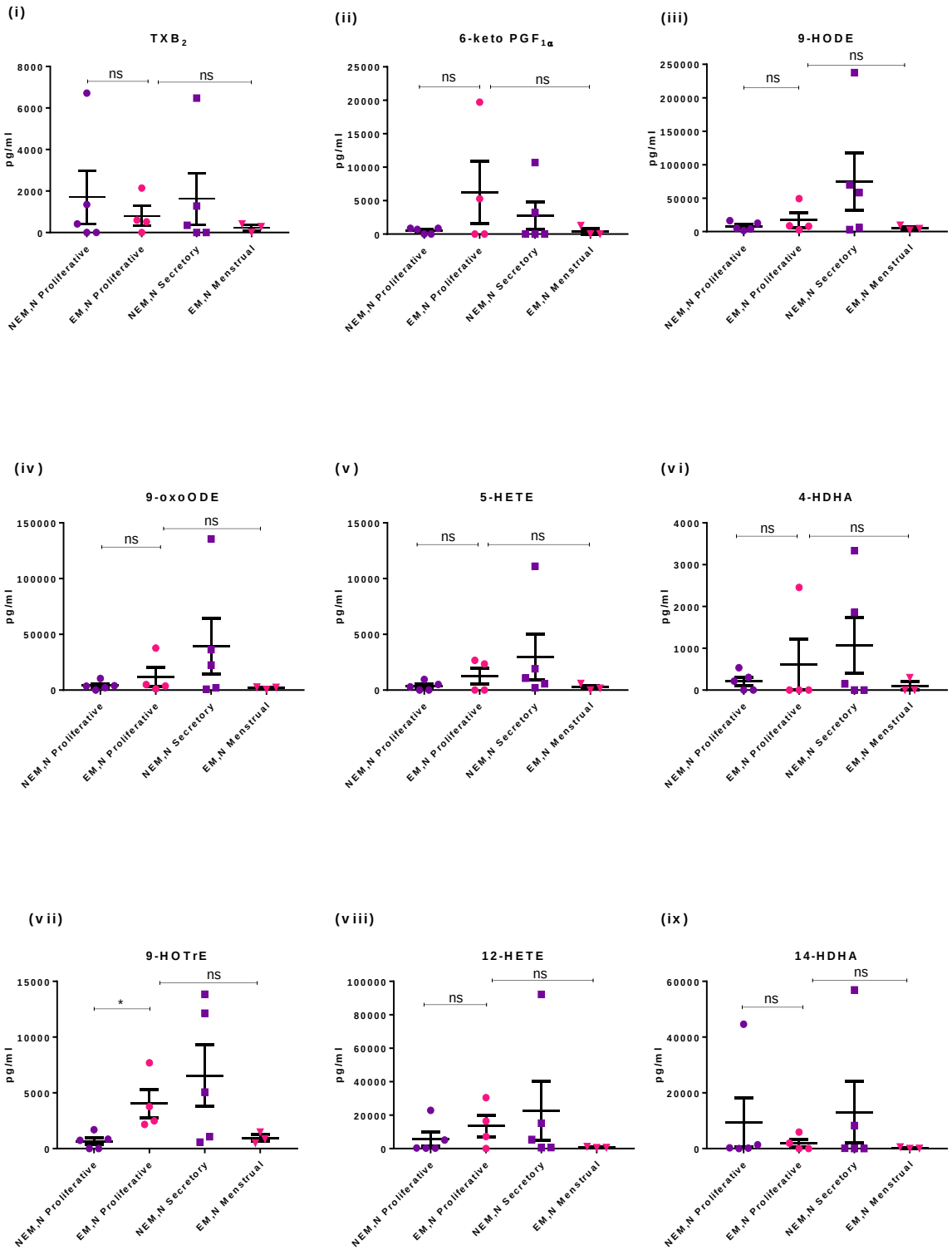


Figure 3.4.1. Lipid mediators in peritoneal fluid from women without endometriosis with regular cycle
*and without taking hormonal medications (NEM,N n=10; Proliferative n=5; Secretory n=5) Measurements were performed using LC/ESI-MS/MS. Data are expressed as arithmetic means of metabolite (pg/ml) ± SEM. Statistical comparisons were performed using student t-test or Mann-Whitney U test where appropriate. (ns) not significant; *p<0.05; **p<0.01; ***p<0.001*


(i)
TXB2
pg/ml
8000
6000
4000
2000
0
ns
ns
NEM,N Proliferative
EM,N Proliferative
NEM,N Secretory
EM,N Menstrual
(ii)
6-keto PGF1α
pg/ml
25000
20000
15000
10000
5000
0
ns
ns
NEM,N Proliferative
EM,N Proliferative
NEM,N Secretory
EM,N Menstrual
(iii)
9-HODE
pg/ml
250000
200000
150000
100000
50000
0
ns
ns
NEM,N Proliferative
EM,N Proliferative
NEM,N Secretory
EM,N Menstrual
(iv)
9-oxoODE
pg/ml
150000
100000
50000
0
ns
ns
NEM,N Proliferative
EM,N Proliferative
NEM,N Secretory
EM,N Menstrual
(v)
5-HETE
pg/ml
15000
10000
5000
0
ns
ns
NEM,N Proliferative
EM,N Proliferative
NEM,N Secretory
EM,N Menstrual
(vi)
4-HDHA
pg/ml
4000
3000
2000
1000
0
ns
ns
NEM,N Proliferative
EM,N Proliferative
NEM,N Secretory
EM,N Menstrual
(vii)
9-HOTrE
pg/ml
15000
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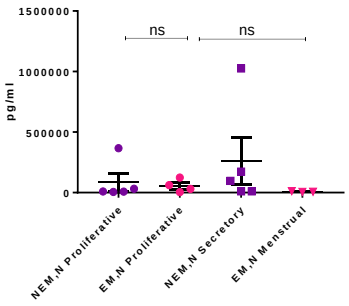
*
ns
NEM,N Proliferative
EM,N Proliferative
NEM,N Secretory
EM,N Menstrual
(viii)
12-HETE
pg/ml
100000
80000
60000
40000
20000
0
ns
ns
NEM,N Proliferative
EM,N Proliferative
NEM,N Secretory
EM,N Menstrual
(ix)
14-HDHA
pg/ml
60000
40000
20000
0
ns
ns
NEM,N Proliferative
EM,N Proliferative
NEM,N Secretory
EM,N Menstrual

(x)



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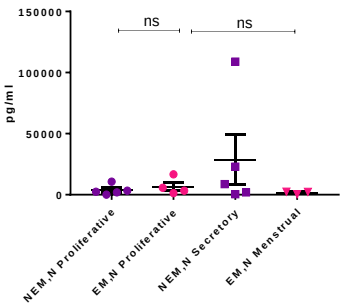

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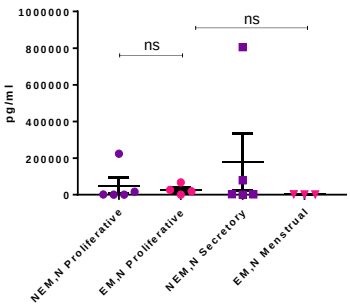

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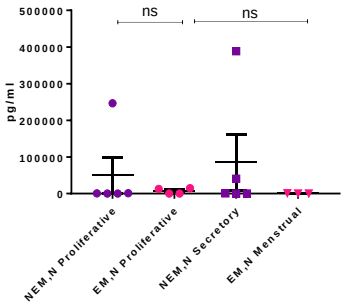

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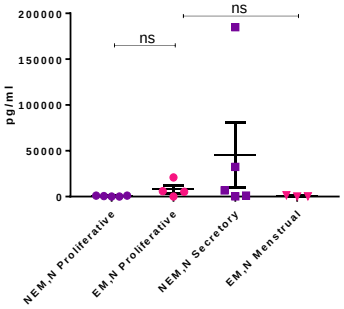

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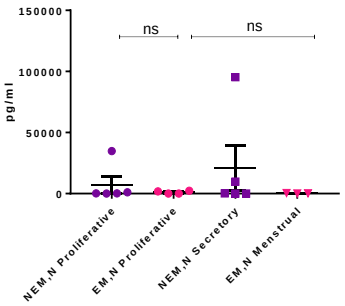

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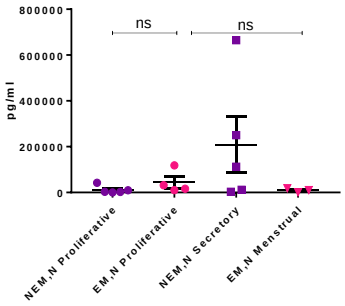

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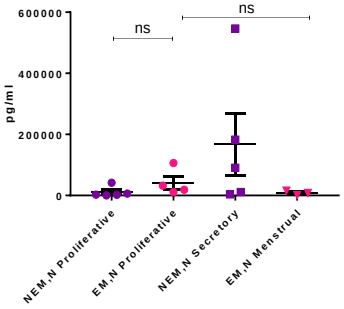

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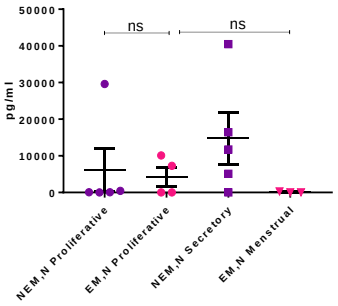


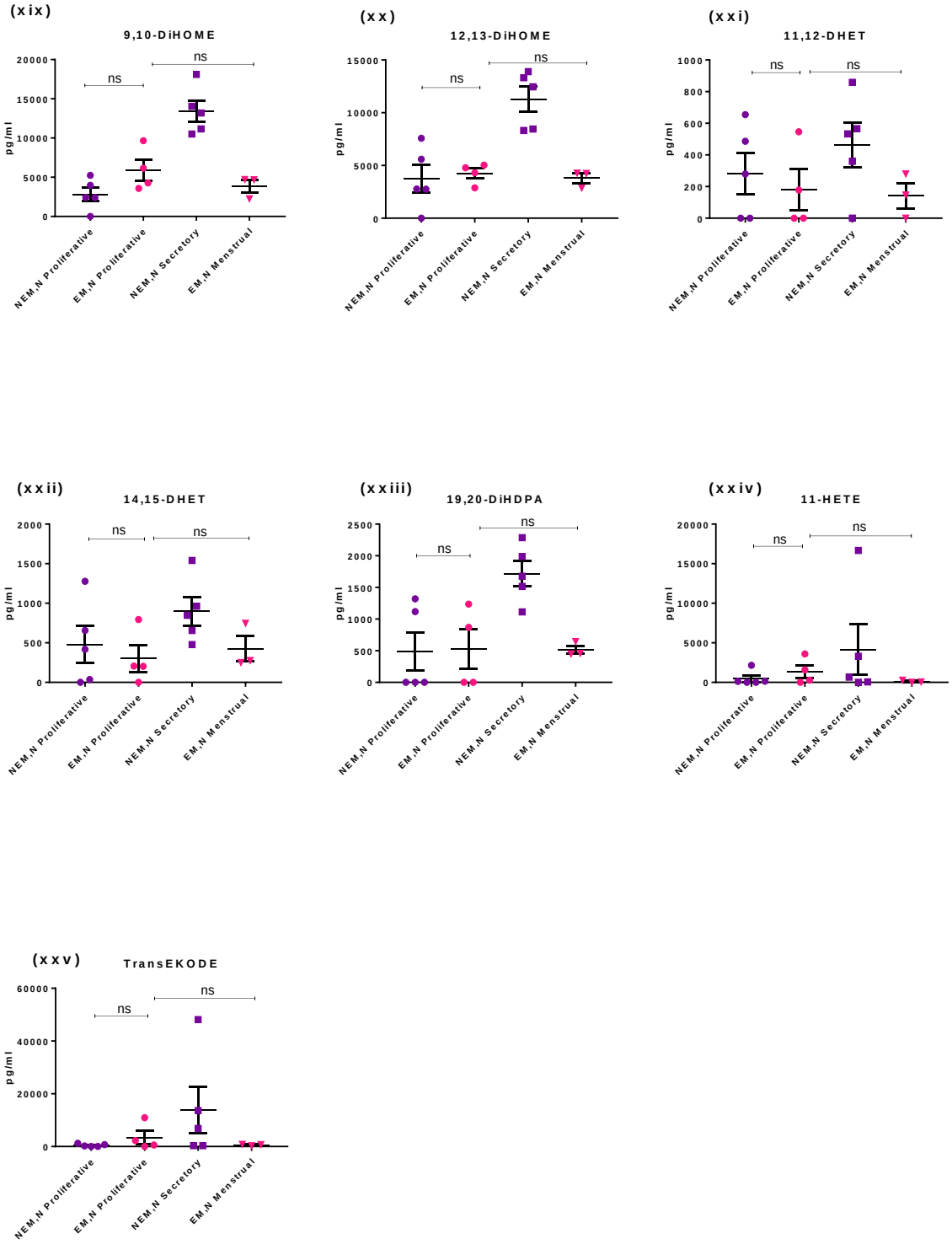


*Figure 3.4.2. Lipid mediators in peritoneal fluid from women with (EM,N n=7) and without endometriosis (NEM,N n=10) with regular cycle and without taking hormonal medications. (NEM,N Proliferative n=5; Secretory n=5) (EM,N Proliferative n=4; Menstrual n=3) Measurements were performed using LC/ESI-MS/MS. Data are expressed as arithmetic means of metabolite (pg/ml) ± SEM. Statistical comparisons were performed between NEM,N Proliferative vs. EM,N Proliferative and EM,N Proliferative vs. EM,N Menstrual using student t-test or Mann-Whitney U test where appropriate. (ns) not significant; *p<0.05*

Apart from TXB_2, all detected lipid mediators were elevated in secretory phase compared to proliferative phase in the peritoneal fluid from non-endometriosis women with regular cycle and without hormonal medication (NEM,N). Of those, significant increase was observed for three sEH derived products, namely 9,10-DiHOME (***p<0.001), 12,13-DiHOME (**p<0.01) and 19, 20-DiHDPA (*p<0.05) (Figure 3.4.1 xix, xx, xxiii).

Although peritoneal fluid from NEM,N patients in menstrual stages was not available, in the EM,N group, apart from 14,15-DHET, an increasing trend was observed from the menstrual stage to the proliferative stage (Figure 3.4.2). However, the statistical analysis did not reveal statistically significant alterations between menstrual and proliferative phases in this group.

Lipid mediators in proliferative phase from endometriosis and non-endometriosis women with regular cycle and without hormonal medication were compared. Sixteen out of 25 lipids were higher in the EM, N group compared to NEM,N group. However, only 9-HOTrE (*p<0.05) was significantly higher in proliferative stage from women with endometriosis compared to non-endometriosis women in the same cycle phase; whereas TXB_2, 14-HDHA, 13-HODE, 15-HETE, 17-HDHA, 15-HETrE, 11(12)EET, 11,12-DHET and 14,15-DHET were lower in proliferative phage of EM,N compared to proliferative phage of NEM,N.

3.4.1.2 Variation of peritoneal fluid lipid mediators by GnRH treatment

From this analysis, women without endometriosis (NEM,N; n=10) with a regular menstrual cycle and without hormone treatments or hormonal contraceptives were selected and compared to endometriosis patients with regular periods, without hormone treatment or hormonal contraceptives (EM,N; n=7) and amenorrhoeal endometriosis patients using GnRH agonists (EM,T; n=6). Patients using drugs other than GnRH agonists, such as aromatase inhibitors or HRT, were excluded from the treatment group. Table 3.4.2 summarises the concentration of arithmetic means (ng/ml) ± SEM for the detected lipid mediators by biosynthetic pathways and study groups.

*Table 3.4.2. Comparison of lipid mediators detected using LC/ESI-MS/MS in peritoneal fluid. Non-endometriosis (NEM,N; n=10) women with regular menstrual cycles and without hormone treatments or hormonal contraceptives were compared to endometriosis patients with regular period, without hormonal pharmacotherapy (EM,N; n=7) and amenorrhoeal endometriosis patients using GnRH agonists (EM,T; n=6) Data are expressed as arithmetic means of metabolite (ng/ml) ± SEM. Ratios were calculated using arithmetic means. Statistical comparisons were performed using Kruskal-Wallis test with Dunn's post-hoc test. p<0.05 was considered to be significant. (*p<0.05)*

		NEM,N n=10	EM,N n=7	EM,T n=6	p-value	$\frac{EM,N}{NEM,N}$	$\frac{EM,T}{NEM,N}$
Pathway	**Lipid mediator**	**Mean ± SEM (ng/ml)**					
COX	**TXB_2**	**1.66** ± 0.84	**0.56** ± 0.28	**ND**	0.0446 *	0.3	ND
	6-keto $PGF_{1\alpha}$	**1.63** ± 1.05	**3.75** ± 2.76	**0.57** ± 0.43	0.7333	2.3	0.3
	9-HODE	**41.39** ± 23.13	**12.14** ± 6.30	**7.55** ± 2.48	0.5973	0.3	0.2
	9-oxoODE	**21.74** ± 13.18	**7.58** ± 5.06	**3.92** ± 1.18	0.8828	0.3	0.2
5-LOX	**5-HETE**	**1.66** ± 1.07	**0.83** ± 0.44	**0.18** ± 0.14	0.1959	0.5	0.1
	9-HOTrE	**3.60** ± 1.64	**2.71** ± 0.93	**3.43** ± 0.85	0.5048	0.8	1.0
	4-HDHA	**0.64** ± 0.35	**0.39** ± 0.35	**0.03** ± 0.03	0.1719	0.6	0.05
12-LOX	**12-HETE**	**14.29** ± 8.98	**8.07** ± 4.36	**5.16** ± 2.62	0.8676	0.6	0.4
	14-HDHA	**11.18** ± 6.71	**1.22** ± 0.83	**0.28** ± 0.28	0.1624	0.1	0.02
15-LOX	**13-HODE**	**173.89** ± 101.42	**35.15** ± 16.90	**33.91** ± 17.46	0.6160	0.2	0.2
	13-oxoODE	**16.15** ± 10.55	**4.59** ± 2.11	**3.73** ± 1.06	0.8791	0.3	0.2
	15-HETE	**113.35** ± 80.11	**16.71** ± 9.23	**14.26** ± 9.28	0.9902	0.1	0.1
	15-HETrE	**14.18** ± 9.66	**0.68** ± 0.37	**1.67** ± 1.25	0.7357	0.05	0.1
	13-HOTrE	**22.82** ± 18.28	**4.81** ± 2.84	**7.21** ± 5.22	0.6981	0.2	0.3
	17-HDHA	**67.77** ± 43.12	**4.10** ± 2.48	**3.78** ± 1.72	0.7007	0.1	0.1
CYP	**9(10)EpOME**	**109.95** ± 66.56	**29.46** ± 15.24	**18.23** ± 4.38	0.6418	0.3	0.2
	12(13)EpOME	**88.82 ±** 54.14	**27.72** ± 13.58	**16.77** ± 2.96	0.6394	0.3	0.2
	11(12)EET	**10.39** ± 4.56	**2.52** ± 1.62	**8.44** ± 8.17	0.6767	0.2	0.8
sEH	**9,10-DiHOME**	**8.11** ± 1.92	**5.04** ± 0.89	**10.17** ± 2.75	0.8748	0.6	1.3
	12,13-DiHOME	**7.51** ± 1.51	**4.05** ± 0.33	**10.10** ± 3.29	0.8714	0.5	1.3
	11,12-DHET	**0.37** ± 0.10	**0.16** ± 0.08	**0.23** ± 0.09	0.1319	0.4	0.6
	14,15-DHET	**0.69** ± 0.16	**0.35** ± 0.11	**0.49** ± 0.07	0.8382	0.5	0.7
	19,20-DiHDPA	**1.10** ± 0.27	**0.52** ± 0.17	**1.12** ± 0.39	0.2591	0.5	1.0
NE	**11-HETE**	**2.31** ± 1.64	**0.83** ± 0.51	**0.13** ± 0.07	0.5826	0.4	0.1
	trans-EKODE	**7.13** ± 4.76	**2.22** ± 1.47	**1.71** ± 0.35	0.4918	0.3	0.2

(COX) cyclooxygenase; (LOX) lipoxygenase; (CYP) cytochrome P450 epoxygenases; (sEH) soluble epoxy hydrolase; (NE) non-enzymatic. For the name of lipid mediators please see the section of frequently used abbreviations.

Apart from TXB_2 statistically significant differences were not detected between the three study groups. However, the statistical analysis revealed a significant alteration in TXB_2. It is necessary to point out that the concentration of TXB_2 was under the limit of detection in the endometriosis group with medical treatment. In addition, the Dunn's *post-hoc* multiple comparison has also not confirmed the statistical difference between the study groups.

Overall, except 6-keto $PGF1_\alpha$, 9,10-DiHOME, 12,13-DiHOME and 19,20-DiHDPA, all lipid mediators were suppressed in the endometriosis groups with and without medical treatment, compared to non-endometriosis.

sEH derived products showed slight changes; these mediators were depleted in the endometriosis group without medical treatment, but were elevated in the treatment group. To visualise the proportional changes of lipid mediator concentrations between the three study groups, data were grouped according to biosynthetic pathways and expressed as percentage of total detected lipid mediators (Figure 3.4.3Figure 3.4.2).

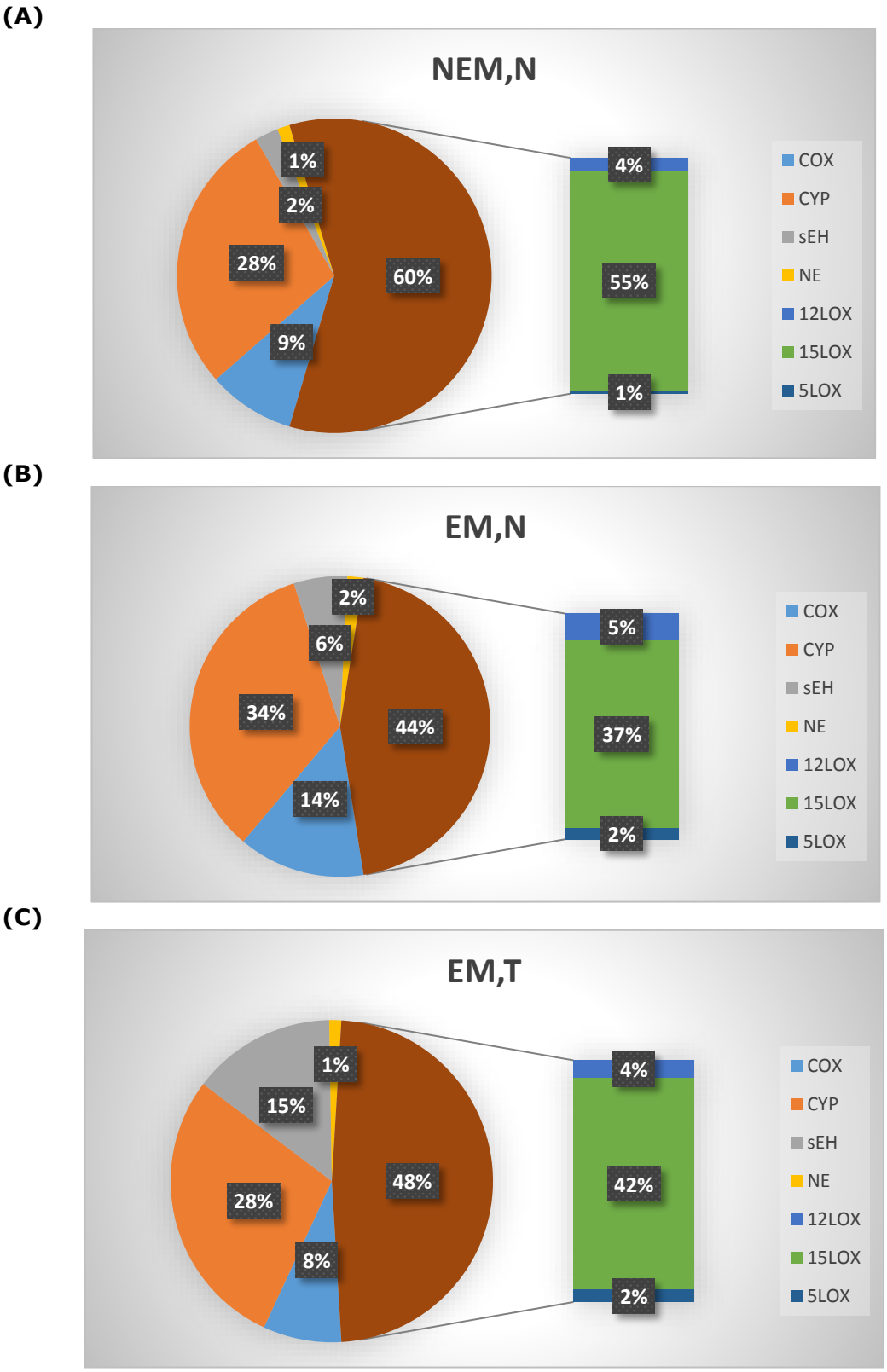


Figure 3.4.3. Proportions of detected lipid mediators in peritoneal fluid demonstrated by biosynthetic pathways.
Lipid mediators from (A) pre-menopausal women without (NEM,N; n=10) and (B) with (EM,N; n=7) endometriosis with regular cycles and not taking any form of hormonal treatments or hormonal contraceptives were compared to (C) endometriosis patients receiving hormonal treatment (EM,T; n=6). Bar of pie chart illustrates the proportions of LOX derived metabolites

according to LOX pathways. Data were obtained using LC/ESI-MS/MS. (COX) cyclooxygenase; (LOX) lipoxygenase; (CYP) cytochrome P450 epoxygenases; (sEH) soluble epoxy hydrolase; (NE) non-enzymatic

Pie charts demonstrate that LOX derived mediators comprise the highest percentage of total lipid mediators in peritoneal fluid for all study groups. The proportion of LOX products was reduced in both endometriosis groups compared to that of the non-endometriosis group. 5 and 12-LOX products were present in approximately same percentages for all three study groups. However, 15-LOX metabolites were lower in the groups of endometriosis with and without medical treatment. Of those, the lowest proportion were observed in the group of EM,N. In contrast, the highest fraction of COX products were also demonstrated in this group. Metabolites of the CYP pathway, consisting CYP and sEH derived products were present in elevated portions in the endometriosis groups (EM,N=40%, EM,T=43% vs. NEM,N=30%). However, the proprotion between CYP and sEH derived metabolites were different in these groups. sEH species were elevated in the endometriosis group with medical treatment compared to those without. Metabolites derived by non-enzymatic pathways did not show any differences between the study groups.

3.4.1.3 Variation of peritoneal fluid lipid mediators by medical condition

Lipid species detected in peritoneal fluid from patients not diagnosed with endometriosis (NEM n=15) were compared to women diagnosed with endometriosis (EM n=17). Table 3.4.3 shows a summary of the concentration of arithmetic means (ng/ml) ± SEM of the detected lipid mediators by biosynthetic pathways.

Table 3.4.3. Comparison of lipid mediators detected by LC/ESI-MS/MS in peritoneal fluid from women not diagnosed with endometriosis (NEM, n=15) and from women diagnosed with endometriosis (EM, n=17). Data are expressed as arithmetic means of metabolite (ng/ml) ± SEM. Statistical comparisons were performed using Mann-Whitney U test. $p<0.05$ was considered to be significant. Ratios were calculated between EM and NEM using arithmetic means.

		NEM n=15	EM n=17		$\frac{EM}{NEM}$
Pathway	**Lipid mediator**	**Mean ± SEM (ng/ml)**		**p-value**	
COX	**TXB_2**	**1.46** ± 0.60	**0.53** ± 0.22	0.429	0.4
	6-keto $PGF_{1\alpha}$	**3.36** ± 1.70	**3.02** ± 1.37	0.866	0.9
	9-HODE	**55.46** ± 22.49	**22.95** ± 12.47	0.433	0.4
	9-oxoODE	**22.59** ± 9.87	**13.71** ± 8.06	0.493	0.6
5-LOX	**5-HETE**	**2.33** ± 1.08	**1.68** ± 1.20	0.106	0.7
	9-HOTrE	**6.29** ± 2.94	**3.75** ± 0.87	0.544	0.6
	4-HDHA	**0.62** ± 0.26	**0.87** ± 0.59	0.233	1.4

12-LOX	**12-HETE**	**13.85** ± 6.26	**34.67** ± 24.53	0.455	2.5
	14-HDHA	**9.04** ± 4.52	**3.76** ± 2.85	0.139	0.4
15-LOX	**13-HODE**	**192.00** ± 81.92	**57.15** ± 26.68	0.370	0.3
	13-oxoODE	**18.74** ± 8.56	**8.20** ± 4.23	0.518	0.4
	15-HETE	**99.95** ± 54.21	**35.31** ± 21.58	0.941	0.4
	15-HETrE	**11.37** ± 6.49	**3.15** ± 1.87	0.651	0.3
	13-HOTrE	**26.29** ± 14.30	**9.18** ± 4.43	0.837	0.3
	17-HDHA	**58.94** ± 29.49	**11.03** ± 7.52	0.815	0.2
CYP	**9(10)EpOME**	**121.49** ± 52.10	**61.97** ± 36.50	0.922	0.5
	12(13)EpOME	**96.95** ± 41.91	**55.18** ± 31.42	0.860	0.6
	11(12)EET	**9.64** ± 3.51	**10.90** ± 5.54	0.409	1.1
sEH	**9,10-DiHOME**	**7.88** ± 1.53	**7.90** ± 1.43	0.830	1.0
	12,13-DiHOME	**7.92** ± 1.62	**6.50** ± 1.38	0.653	0.8
	11,12-DHET	**0.35** ± 0.08	**0.18** ± 0.05	0.145	0.5
	14,15-DHET	**0.58** ± 0.12	**0.42** ± 0.07	0.335	0.7
	19,20-DiHDPA	**1.08** ± 0.22	**0.86** ± 0.21	0.462	0.8
NE	**11-HETE**	**3.25** ± 1.44	**2.09** ± 1.43	0.554	0.6
	trans-EKODE	**7.72** ± 3.78	**4.79** ± 2.67	0.808	0.6

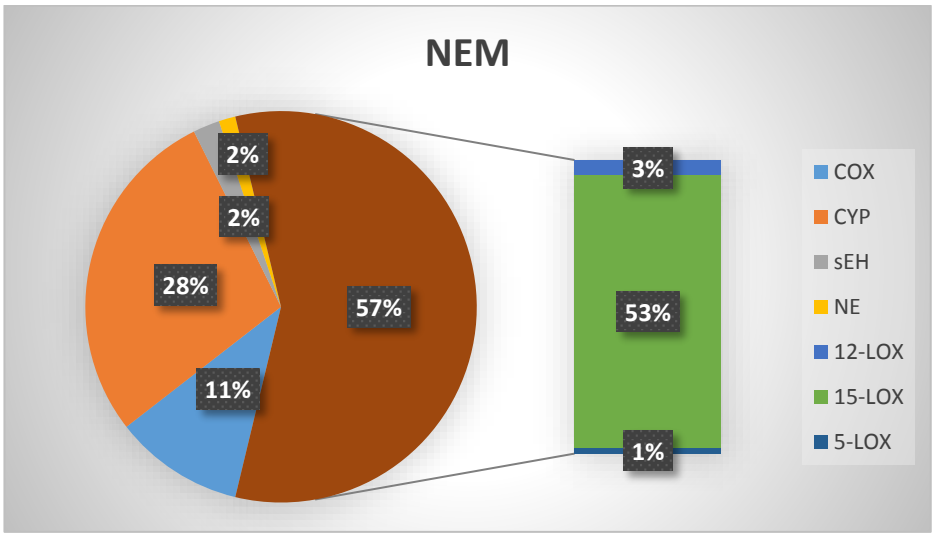
(COX) cyclooxygenase; (LOX) lipoxygenase; (CYP) cytochrome P450 epoxygenases; (sEH) soluble epoxy hydrolase; (NE) non-enzymatic. For the name of lipid mediators please see the section of frequently used abbreviations.

For the detected lipid mediators, statistical differences could not be observed between endometriosis and non-endometriosis samples in peritoneal fluid. The concentrations of 20 analytes were lower in the disease group. The means were about the same between the two study groups for 6-keto PGF1α, 11(12) EET and 9,10-DiHOME, whereas 12-HETE and 4-HDHA concentrations were elevated in peritoneal fluid from women with endometriosis. Subsequent analysis highlighted that those elevations were caused by an outlier. Nearly all detected metabolites were at the highest concentrations in peritoneal fluid from patient HP02. This patient had hysterectomy due to severe stage of endometriosis with a frozen pelvis. She suffered endometriosis more than ten years causing infertility, chronic pelvic pain and dysmenorrhea and used cyclic HRT without GnRH agonist as treatment for her condition.

15-LOX derived metabolites showed the most prominent reductions in the group of endometriosis compared to peritoneal fluid from women without endometriosis. To

demonstrate the proportional changes in lipid mediator concentrations between the study groups, data were arranged according to biosynthetic pathways and expressed as percentage of total detected lipid mediators (Figure 3.4.4Table 3.4.2).

(A)



(B)

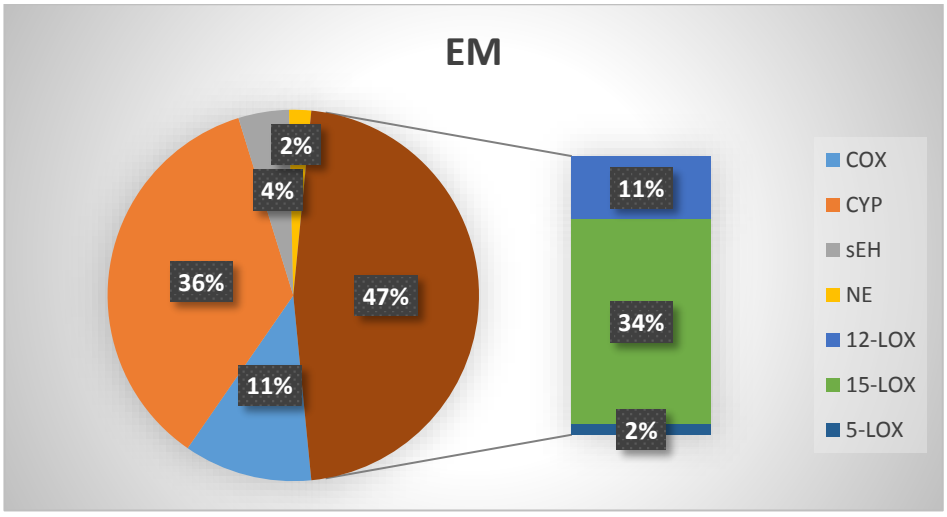


Figure 3.4.4. Proportions of detected lipid mediators in peritoneal fluid
(A) from women not diagnosed with endometriosis (NEM, n=15) and (B) from women diagnosed with endometriosis (EM, n=17). Metabolites were grouped by biosynthetic pathways and expressed as percentage of total detected lipid mediators. Bar of pie chart illustrates the proportions of LOX derived metabolites according to LOX pathways. Data were obtained using LC/ESI-MS/MS. (COX) cyclooxygenase; (LOX) lipoxygenase; (CYP) cytochrome P450 epoxygenases; (sEH) soluble epoxy hydrolase; (NE) non-enzymatic.

Figure 3.4.4Figure 3.4.1 demonstrates that LOX derived mediators comprise the highest percentage of total lipid mediators in peritoneal fluid from women with and without endometriosis. Analysis revealed that the total of LOX metabolites was 10% lower in endometriosis compared to non-endometriosis (NEM=57% vs. EM=47%). The proportions of lipid metabolites derived by the three LOX pathways were also

altered between the two study groups. 15-LOX metabolites were lower in endometriosis (34%) compared to non-endometriosis (53%), whereas 12-LOX products were elevated in endometriosis (11%) versus 3% in non-endometriosis groups. 5-LOX products were present in about the same proportions. There were no differences for COX and non-enzymatic products between the two study groups, whereas the proportions of CYP and sEH products were increased in endometriosis group.

3.4.2 Lipid mediators in plasma

Despite the decades of research looking for reliable biomarker(s), non-invasive diagnostic methods still do not exist for endometriosis. Recent systemic reviews concluded that although some potential biomarkers look promising with respect to diagnostic accuracy, more research effort is still required prior to a reliable biomarker based test being introduced into clinical practice (Nisenblat et al. 2016; Liu et al. 2015; Gupta et al. 2016).

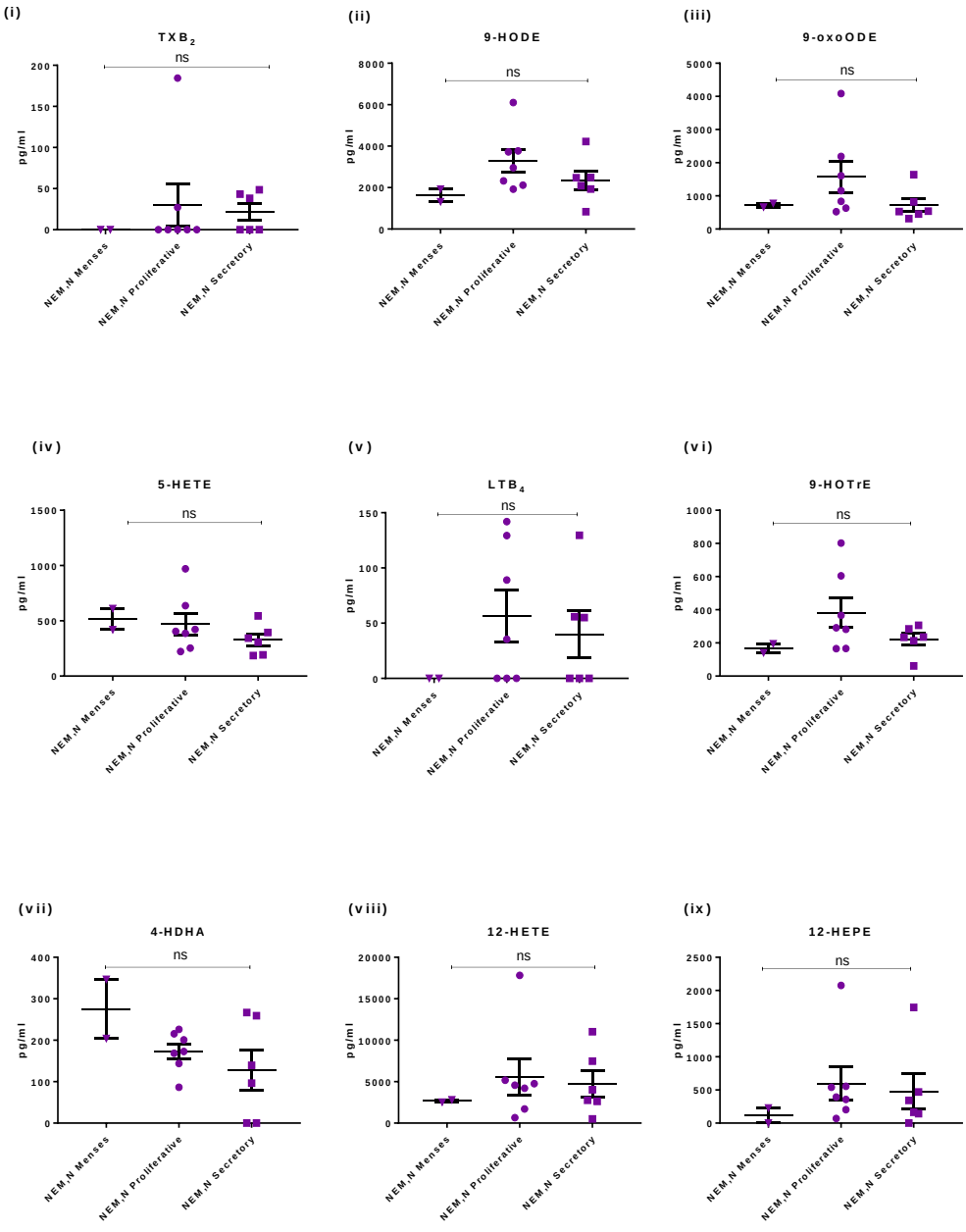
Lipid mediators, potentially in a particular profile or 'signature' may provide a novel group of compounds that could be used as biomarkers for the diagnosis of endometriosis since many lipids are involved in the pathomechanism of the disease. Lipid mediator profiles in plasma were assessed using an LC/ESI-MS/MS method to identify potential differences in plasma profiles between patients diagnosed with and without endometriosis. In addition, plasma and peritoneal fluid concentrations were assessed to evaluate the systemic effect of the disease compared to the local, pathological processes at the lesion sites.

A total of 70 plasma specimen (NEM n=26; EM n=41) were tested using LC/ESI-MS/MS. Twenty six species were detected out of 79 tested lipid mediators. Of those species detected and presented here, the analyte was quantified in at least half of the samples. A complete list of all detected mediators is detailed in Appendix 6. Comparisons were performed according to menstrual stages, medical treatment and the presence or absence of endometriosis.

3.4.2.1 Variation of plasma lipid mediators by menstrual cycle

Data were grouped according to stage of menstrual cycle from women with and without endometriosis who reported having regular menstrual cycles and were not using hormonal contraceptives or hormonal medication. A total of 30 (NEM,N n=15; EM,N n=15) women were included in this analysis. Two non-endometriosis women were in the menstrual, 7 in the proliferative and 6 in the secretory phase; whereas 3 endometriosis participants were in the menstrual, 7 in the proliferative and 5 in the

secretory phase. Figure 3.4.5 demonstrates the detected lipid mediators according to menstrual cycle in non-endometriosis patients with regular cycle and without hormonal medications. Figure 3.4.6 demonstrates some selected lipid species from endometriosis women with a regular cycle and without hormonal medications.



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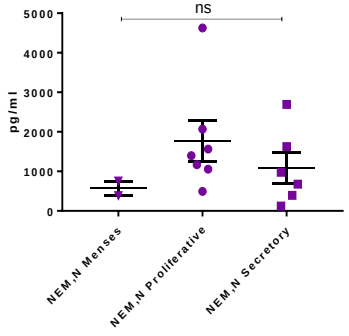

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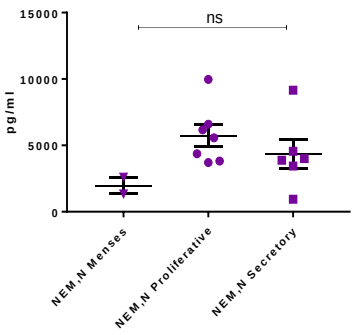

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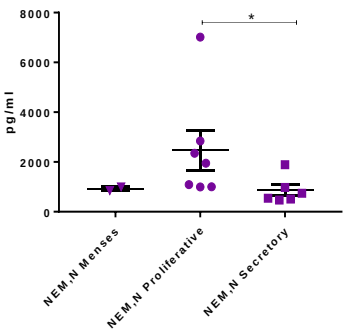

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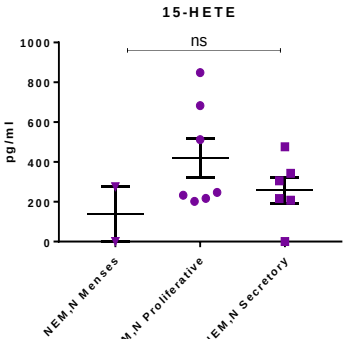

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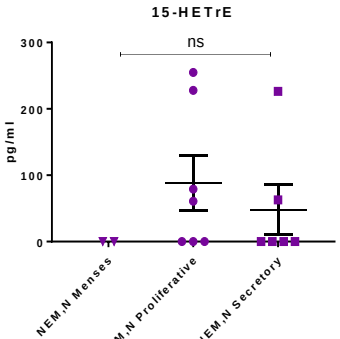

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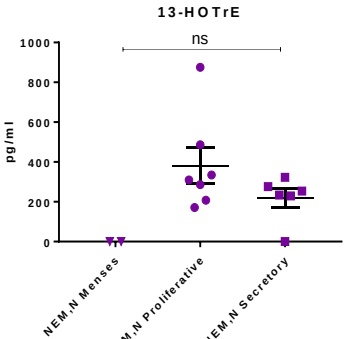

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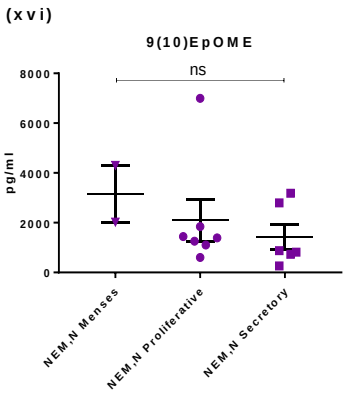

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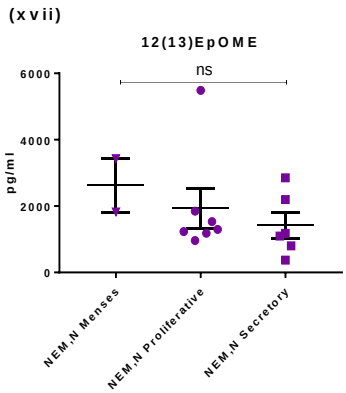

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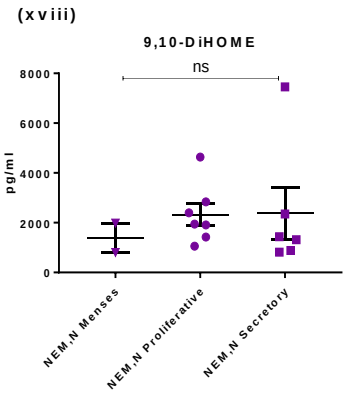


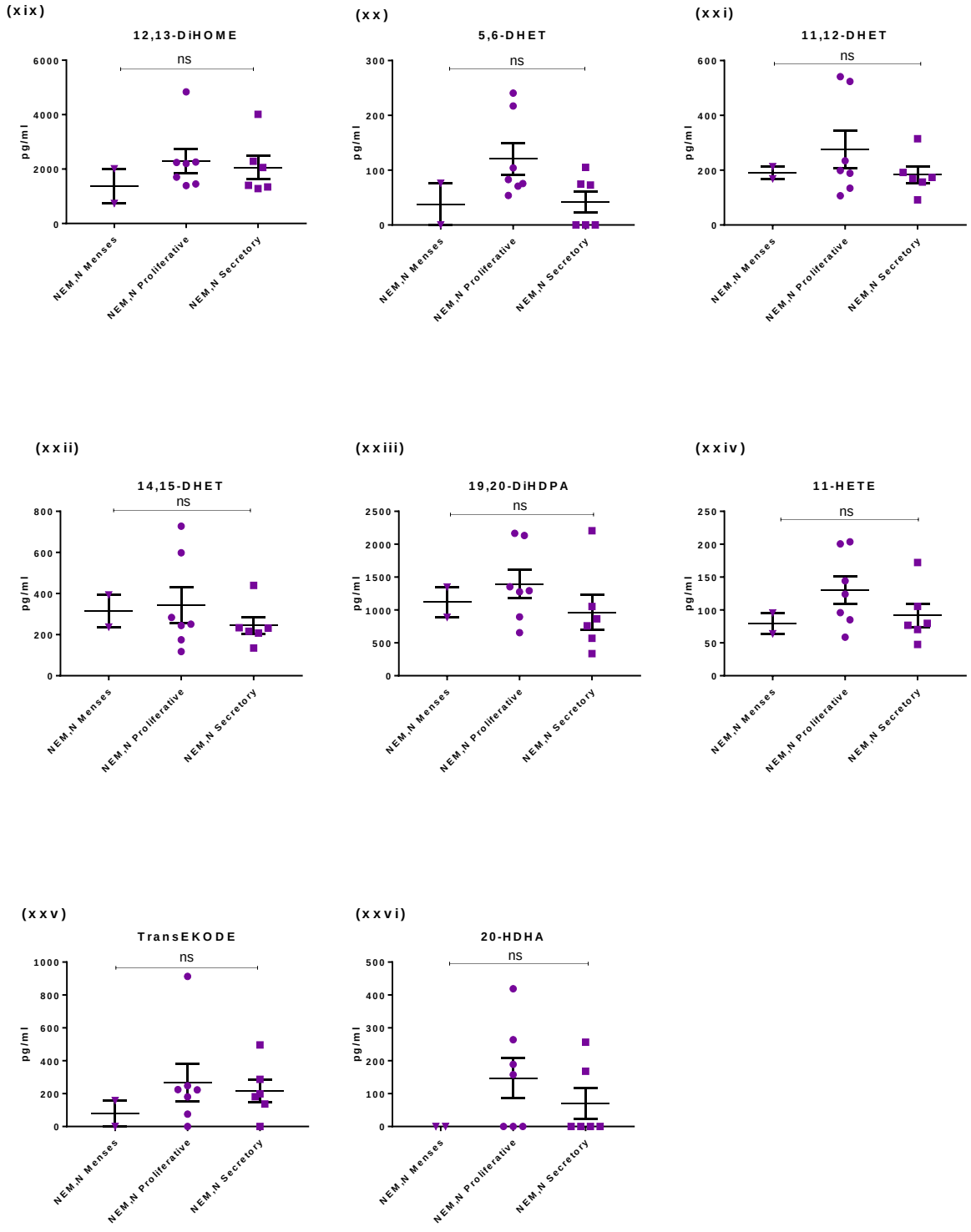


*Figure 3.4.5. Lipid mediators in plasma from women without endometriosis with regular cycle and without taking hormonal medications (NEM,N n=15; Menstrual n=2, Proliferative n=7; Secretory n=6) Measurements were performed using LC/ESI-MS/MS. Data are expressed as arithmetic means of metabolite (pg/ml) ± SEM. Statistical comparisons were performed using one-way ANOVA or Kruskal-Wallis test with Dunn's post-hoc test where appropriate. (ns) not significant; *p<0.05*

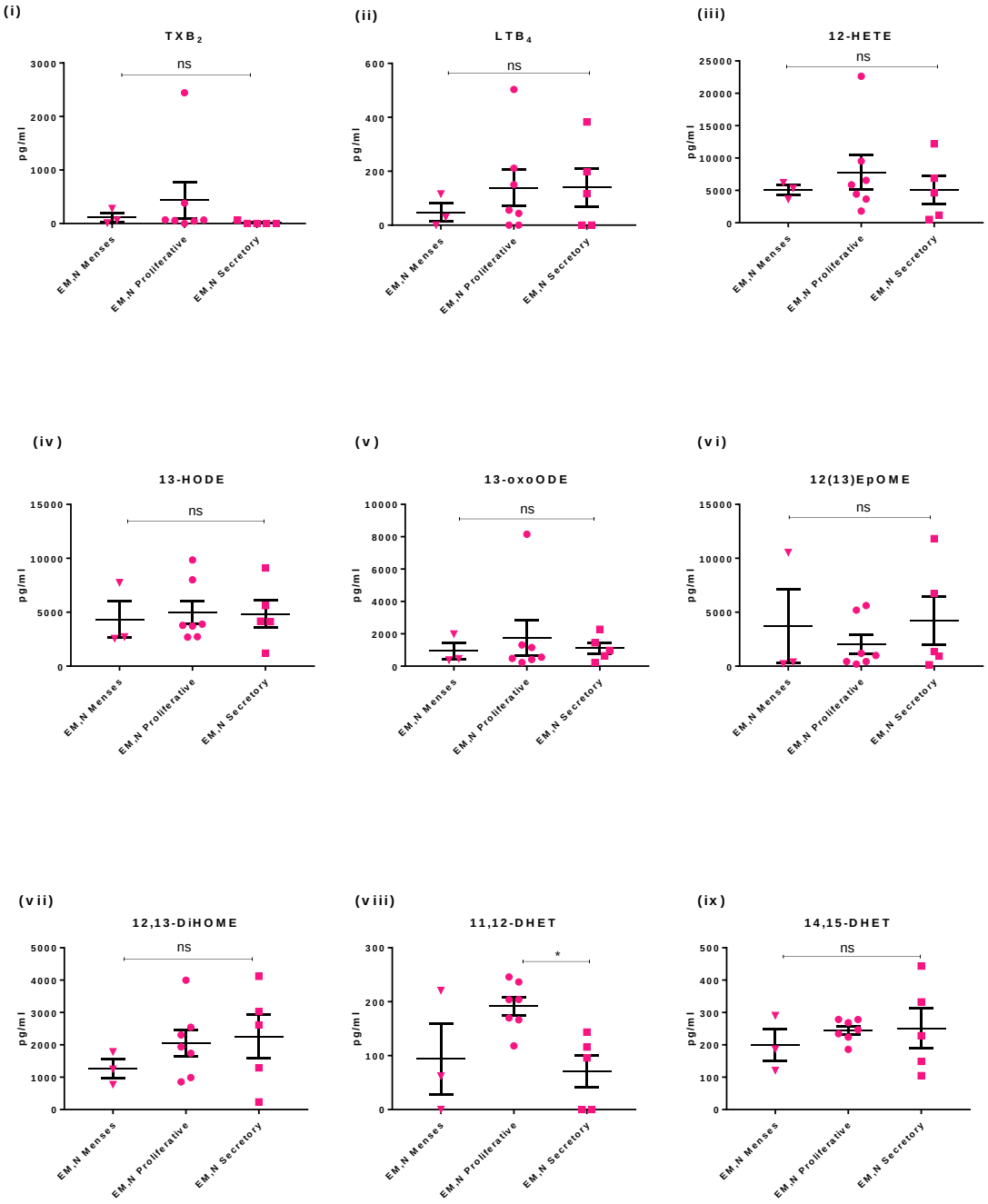
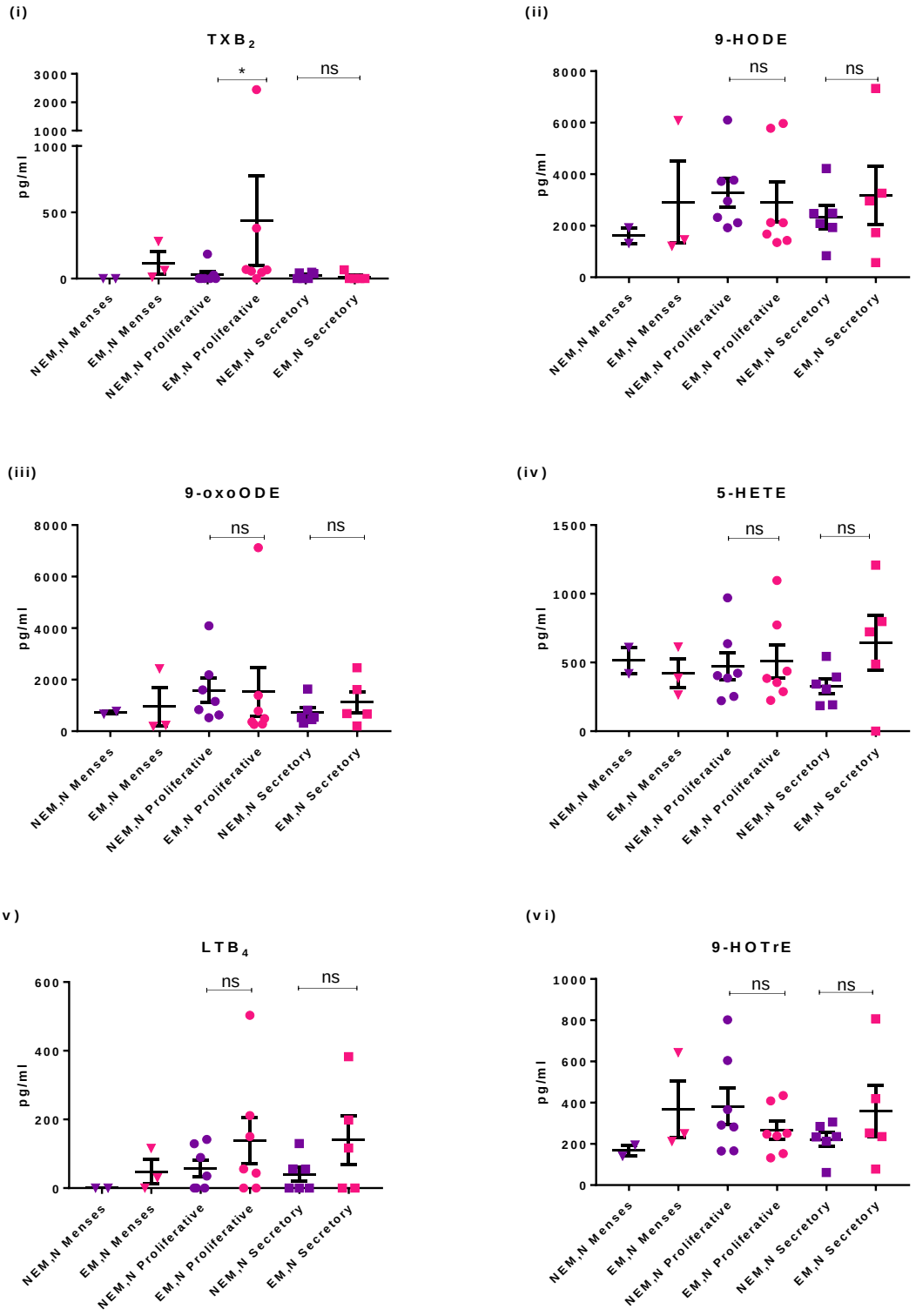


*Figure 3.4.6. Lipid mediators in plasma from women with endometriosis with regular cycle and without taking hormonal medications (EM,N n=15; Menstrual n=3, Proliferative n=7; Secretory n=5) Measurements were performed using LC/ESI-MS/MS. Data are expressed as arithmetic means of metabolite (pg/ml) ± SEM. Statistical comparisons were performed using one-way ANOVA or Kruskal-Wallis test with Dunn's post-hoc test where appropriate. (ns) not significant; *p<0.05*

The statistical comparison of lipid metabolites across the3 stages of menstrual cycle revealed significant difference between proliferative and secretory stages for 13-oxoODE in NEM,N and for 11,12-DHET in EM,N groups. In order to examine the

potential differences between the groups of endometriosis and non-endometriosis women, lipid mediators in proliferative and in secretory phases were compared (Figure 3.4.7). Due to the low number of participants, menstrual stage comparisons were not performed.



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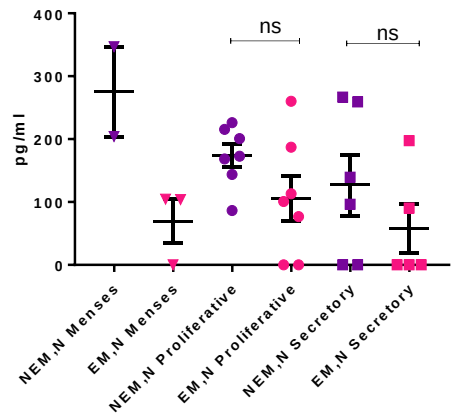

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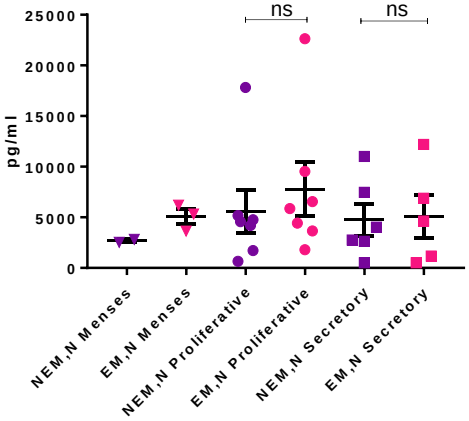

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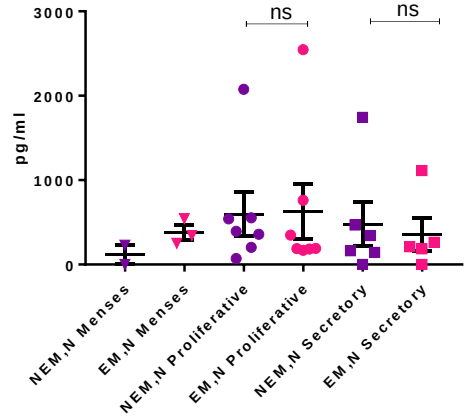

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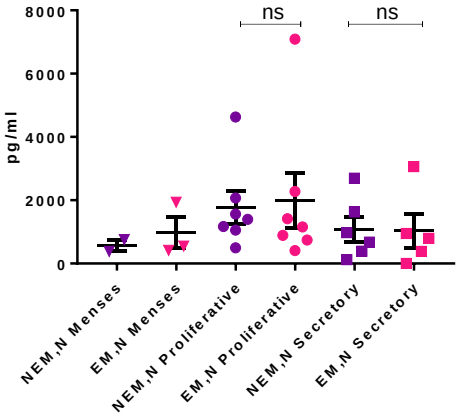

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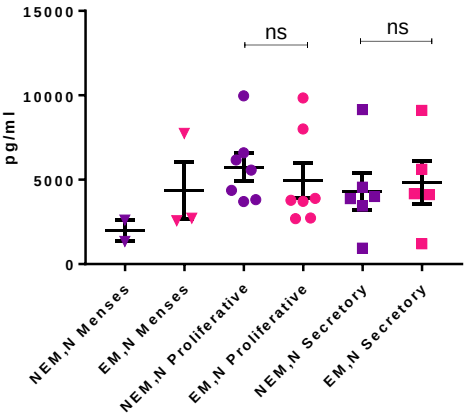

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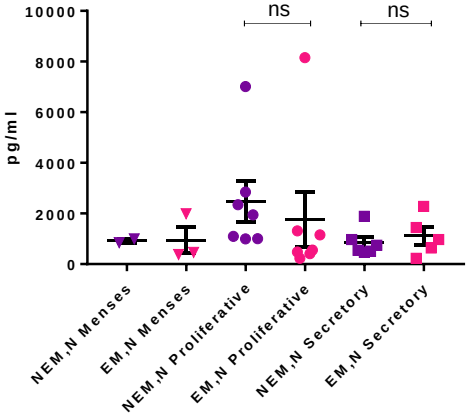

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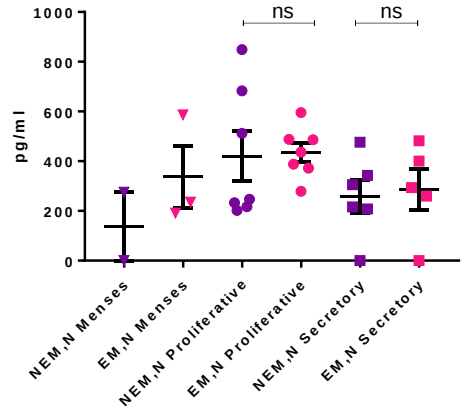

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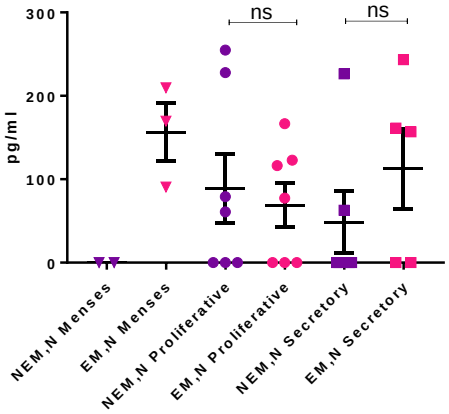

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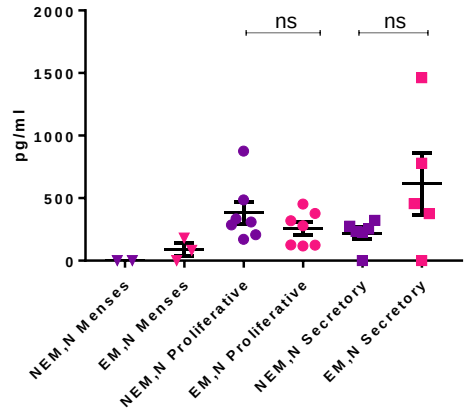

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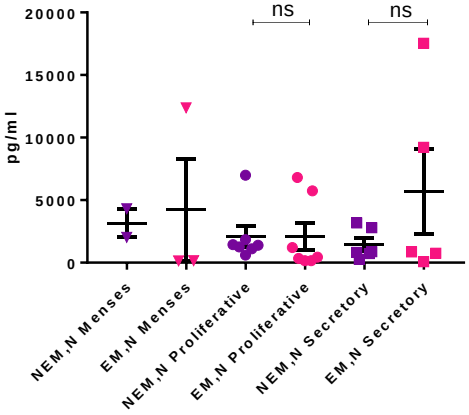

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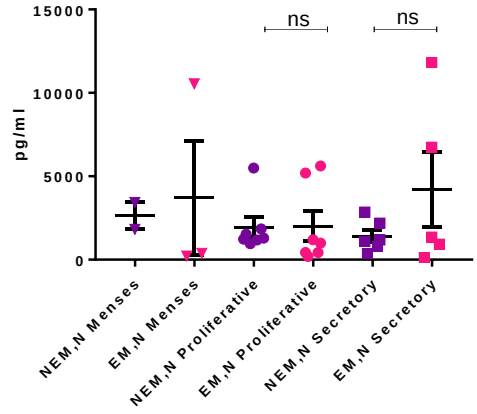

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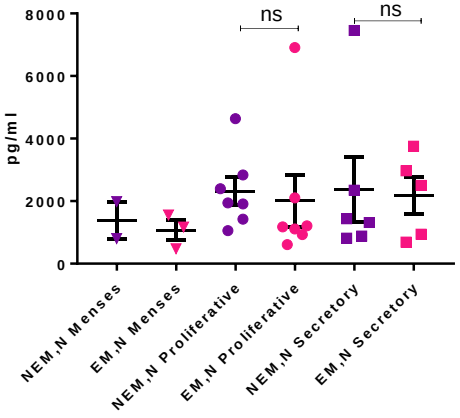

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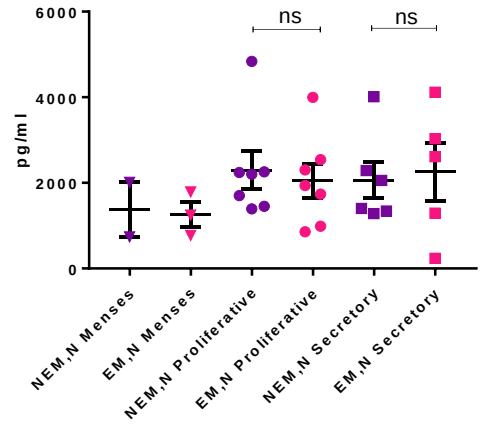

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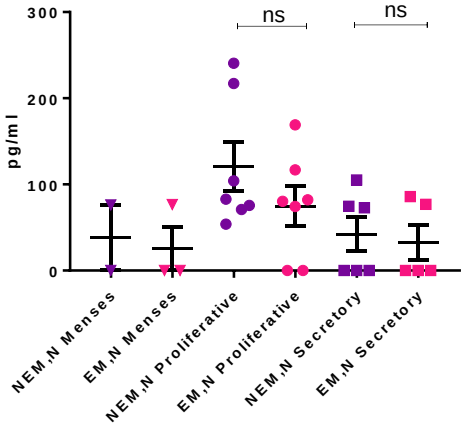

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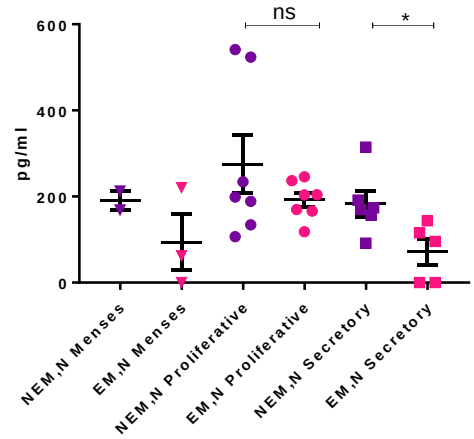

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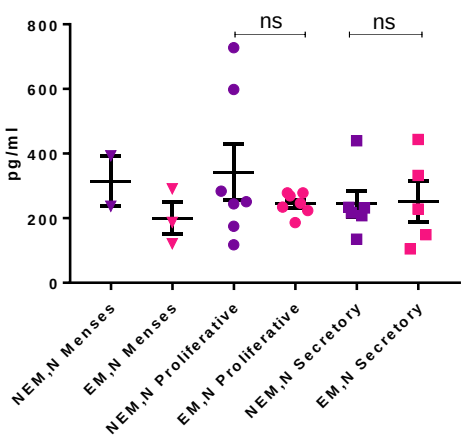

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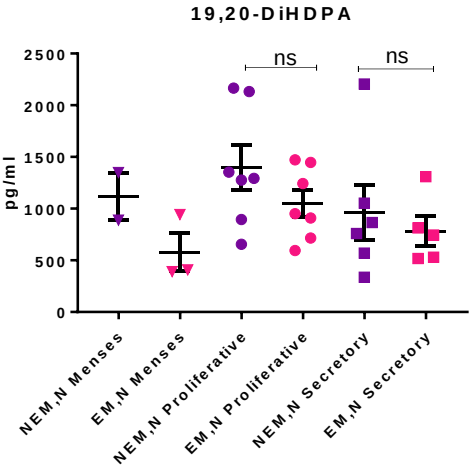

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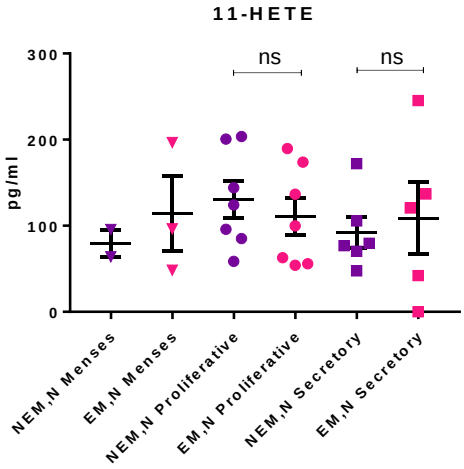


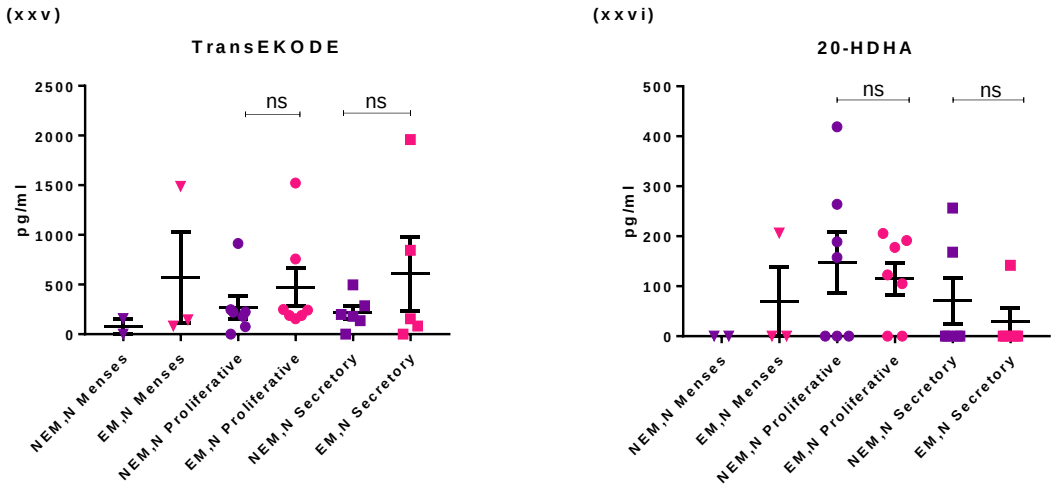


*Figure 3.4.7. Lipid mediators in plasma from women with (EM,N n=15) and without endometriosis (NEM,N n=15) with regular cycle and without taking hormonal medications. (NEM,N Menstrual n=2, Proliferative n=7; Secretory n=6) (EM,N Menstrual n=3, Proliferative n=7;Secretory n=5) Measurements were performed using LC/ESI-MS/MS. Data are expressed as arithmetic means of metabolite (pg/ml) ± SEM. Statistical comparisons were performed between NEM,N Proliferative vs. EM,N Proliferative and NEM,N Secretory vs. EM,N Secretory using student t-test or Mann-Whitney U test where appropriate. (ns) not significant; *p<0.05*

The analysis revealed that TXB_2 level was significantly elevated in the proliferative phase, whereas 11,12-DHET concentration was significantly decreased in the secretory phase in plasma taken from women with endometriosis with a regular cycle, compared to non-endometriosis women with a regular cycle. Although the statistical analysis did not reveal further statistically significant alterations for lipid metabolite synthesis according to menstrual cycle phases, some possible trends could be observed. 12-LOX metabolites showed a peak during the proliferative phase in both study groups. The main products (13-HODE, 15-HETE) of 15-LOX pathways also showed peaks during the proliferative phase in both groups. In contrast, the trend was the opposite for 15-HOTrE in the EM,N group. It was elevated during the menstrual and secretory phases and was low in the proliferative stage. sEH derived 5,6-DHET, 11,12-DHET and 19,20-DiHDPA were also elevated during the proliferative phase in both study groups, whilst the changes were not prominent for 14, 15-DHET across the menstrual cycle for both study groups.

3.4.2.2 Variation in plasma lipid mediators by medical treatment

For this analysis non-endometriosis (NEM,N; n=15) women with regular menstrual cycles without hormone treatments or hormonal contraceptives were selected and compared to endometriosis patients with regular periods, without hormonal pharmacotherapy (EM,N; n=15) and amenorrhoeal endometriosis patients using GnRH agonists (EM,T; n=19). Table 3.4.4Table 3.4.6 summarizes the concentration of the detected lipid mediators by biosynthetic pathways in each study group.

*Table 3.4.4. Comparison of lipid mediators detected using LC/ESI-MS/MS in plasma. Non-endometriosis (NEM,N; n=15) women with regular menstrual cycles, without hormone treatments or hormonal contraceptives were compared to endometriosis patients with regular periods, without pharmacotherapy (EM,N; n=15) and amenorrhoeal endometriosis patients using a GnRH agonists (EM,T; n=19). Data are expressed as arithmetic means of metabolite (ng/ml) ± SEM. Ratios were calculated using arithmetic means. Statistical comparisons were performed using Kruskal-Wallis test with Dunn's post-hoc test. p<0.05 was considered to be significant. (*p<0.05; **p<0.01)*

Plasma Pathway	Lipid mediator	NEM,N n=15	EM,N n=15	EM,T n=19	p-value	$\frac{EM.N}{NEM.N}$	$\frac{EM.T}{NEM.N}$
		Mean ± SEM (ng/ml)					
COX	**TXB_2**	**0.02 ±** 0.01	**0.23 ±** 0.16	**0.10 ±** 0.04	0.3653	10.2	4.2
	9-HODE	**2.68 ±** 0.34	**3.00 ±** 0.56	**3.19 ±** 0.59	0.8201	1.1	1.2
	9-oxoODE	**1.12 ±** 0.25	**1.28 ±** 0.46	**1.67 ±** 0.34	0.2415	1.1	1.5
5-LOX	**5-HETE**	**0.42 ±** 0.05	**0.54 ±** 0.09	**0.58 ±** 0.14	0.5697	1.3	1.4
	LTB_4	**0.04 ±** 0.01	**0.12 ±** 0.04	**0.21 ±** 0.14	0.3335	2.8	4.9
	9-HOTrE	**0.29 ±** 0.05	**0.32 ±** 0.05	**0.36 ±** 0.07	0.7743	1.1	1.2
	4-HDHA	**0.17 ±** 0.02	**0.08 ±** 0.02	**0.13 ±** 0.05	0.0344 *	0.5	0.8
12-LOX	**12-HETE**	**4.85 ±** 1.15	**6.33 ±** 1.40	**3.27 ±** 0.69	0.0476 *	1.3	0.7
	12-HEPE	**0.49 ±** 0.16	**0.49 ±** 0.16	**0.13 ±** 0.05	0.0024**	1.0	0.3
	14-HDHA	**1.33 ±** 0.30	**1.47 ±** 0.45	**0.61 ±** 0.12	0.0146 *	1.1	0.5
15-LOX	**13-HODE**	**4.68 ±** 0.65	**4.79 ±** 0.68	**5.84 ±** 1.04	0.8910	1.0	1.3
	13-oxoODE	**1.62 ±** 0.43	**1.38 ±** 0.51	**1.86 ±** 0.42	0.3813	0.9	1.2
	15-HETE	**0.32 ±** 0.06	**0.37 ±** 0.04	**0.32 ±** 0.05	0.2764	1.2	1.0
	15-HETrE	**0.06 ±** 0.02	**0.10 ±** 0.02	**0.07 ±** 0.02	0.4595	1.7	1.2
	13-HOTrE	**0.27 ±** 0.06	**0.34 ±** 0.10	**0.36 ±** 0.09	0.7976	1.3	1.4
CYP	**9(10)EpOME**	**1.97 ±** 0.46	**3.73 ±** 1.41	**3.82 ±** 0.90	0.2800	1.9	1.9
	12(13)EpOME	**1.82 ±** 0.33	**3.08 ±** 1.02	**3.36 ±** 0.69	0.1995	1.7	1.9
sEH	**9.10-DiHOME**	**2.22 ±** 0.45	**1.88 ±** 0.43	**1.71 ±** 0.35	0.3344	0.9	0.8
	12.13-DiHOME	**2.08 ±** 0.28	**1.96 ±** 0.30	**1.61 ±** 0.26	0.2338	0.9	0.8

	5.6-DHET	**0.08 ±** 0.02	**0.05 ±** 0.01	**0.03 ±** 0.01	0.0450 *	0.7	0.4
	11.12-DHET	**0.23 ±** 0.03	**0.13 ±** 0.02	**0.10 ±** 0.02	0.0107 *	0.6	0.5
	14.15-DHET	**0.30 ±** 0.04	**0.24 ±** 0.02	**0.21 ±** 0.02	0.4001	0.8	0.7
	19.20-DiHDPA	**1.19 ±** 0.15	**0.87 ±** 0.09	**0.67 ±** 0.07	0.0186 *	0.7	0.6
NE	**11-HETE**	**0.11 ±** 0.01	**0.11 ±** 0.02	**0.14 ±** 0.03	0.9313	1.0	1.3
	trans-EKODE	**0.22 ±** 0.06	**0.54 ±** 0.16	**0.47 ±** 0.14	0.4390	2.4	2.1
	20HDHA	**0.10 ±** 0.04	**0.08 ±** 0.02	**0.12 ±** 0.05	0.8247	0.8	1.2

(COX) cyclooxygenase; (LOX) lipoxygenase; (CYP) cytochrome P450 epoxygenases; (sEH) soluble epoxy hydrolase; (NE) non-enzymatic. For the name of lipid mediators please see the section of frequently used abbreviations.

The analysis revealed that TXB_2 levels were increased in plasma taken from women with endometriosis, compared to women without this condition. TXB_2 was highest in the group of endometriosis without medical treatment and showed a reduction in the endometriosis group taking medical treatment. Other detected COX products were unchanged between study groups.

Two of the 5-LOX metabolites, i.e. 5-HETE and 9-HOTrE, were not altered between the three study groups. LTB_4 were elevated in both endometriosis groups whereas 4-HDHA was significantly decreased in these groups, compared to women without endometriosis. 15-LOX products did not show alterations between the study groups. The most prominent variations were observed for the metabolites of the 12-LOX pathway. All 12-LOX species were significantly depleted in the treatment group of women with endometriosis compared to those without. Figure 3.4.8 illustrates the significantly altered LOX products in plasma.

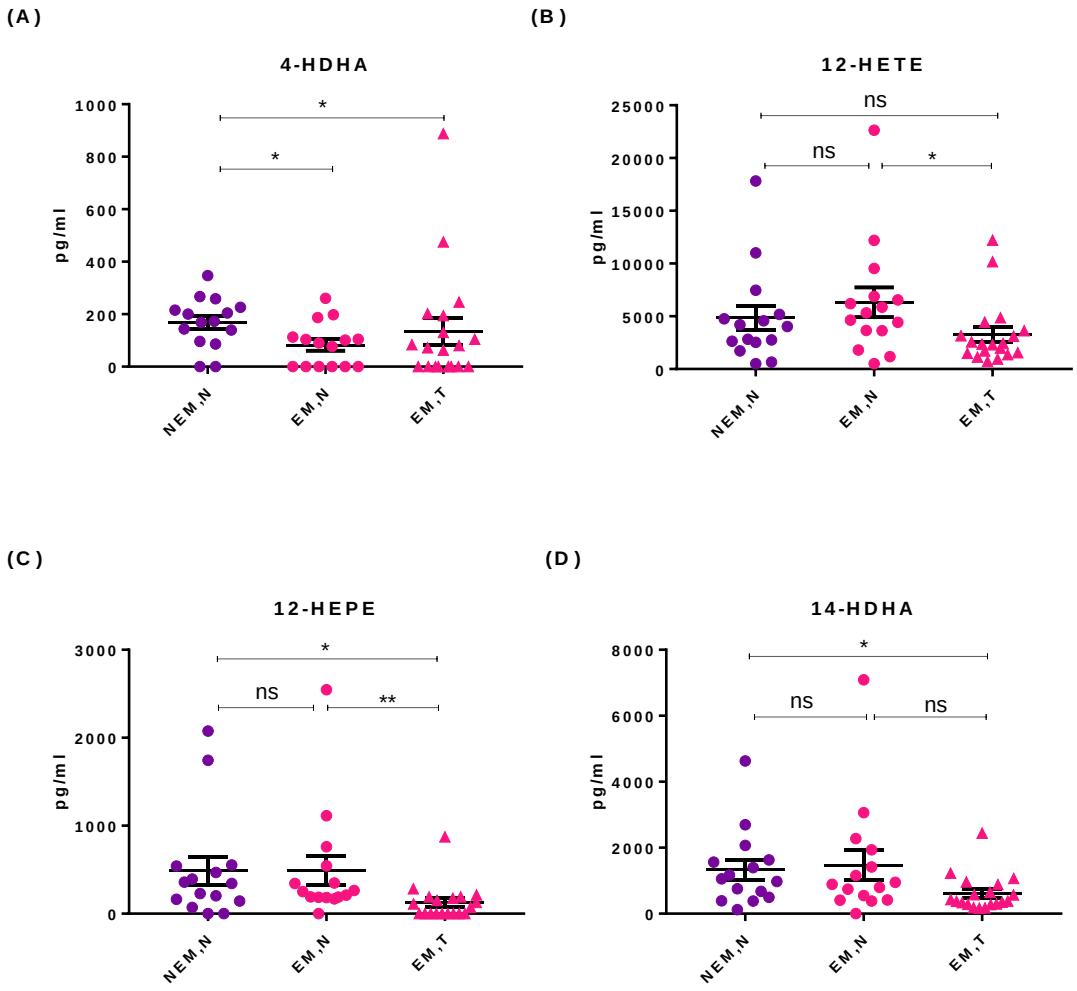


Figure 3.4.8. Lipoxygenase mediators in plasma.
*Non-endometriosis (NEM,N; n=15) women with regular menstrual cycles without hormone treatments or hormonal contraceptives were compared to endometriosis patients with regular periods, without pharmacotherapy (EM,N; n=15) and amenorrhoeal endometriosis patients taking GnRH analogue (EM,T; n=19) Measurements were performed using LC/ESI-MS/MS. Data are expressed as arithmetic means of metabolite (pg/ml) ± SEM. Statistical comparisons were performed using Kruskal-Wallis test with Dunn's post-hoc test. (*p<0.05; **p<0.01)*

Significant alterations were not observed for CYP metabolites or LA derived sEH products. However, other sEH products such as the AA derived DHETs and DHA derived DiHDPA were suppressed in both endometriosis study groups. Moreover, except for 14,15-DHET, these species were significantly suppressed in plasma from women with endometriosis taking GnRH agonist compared to women without endometriosis. Finally, statistically relevant changes were not revealed for non-enzymatic products in plasma. Figure 3.4.9 illustrates representative sEH products in plasma and Figure 3.4.10 demonstrates the proportional changes in lipid mediator concentrations between the study groups. Data were grouped according to biosynthetic pathways and expressed as a percentage of total detected lipid

mediators. Pie charts from Figure 3.4.10 well demonstrate the decreased proportion of 12-LOX metabolites in the treatment group (EM,T=13%) compared to endometriosis (EM,N=25%) and non-endometriosis groups with regular periods (NEM,N=23%). Furthermore the pie charts also demonstrate the reduction in sEH products for this group (EM,T=14%) compared to the non-endometriosis group (NEM,N=21%).

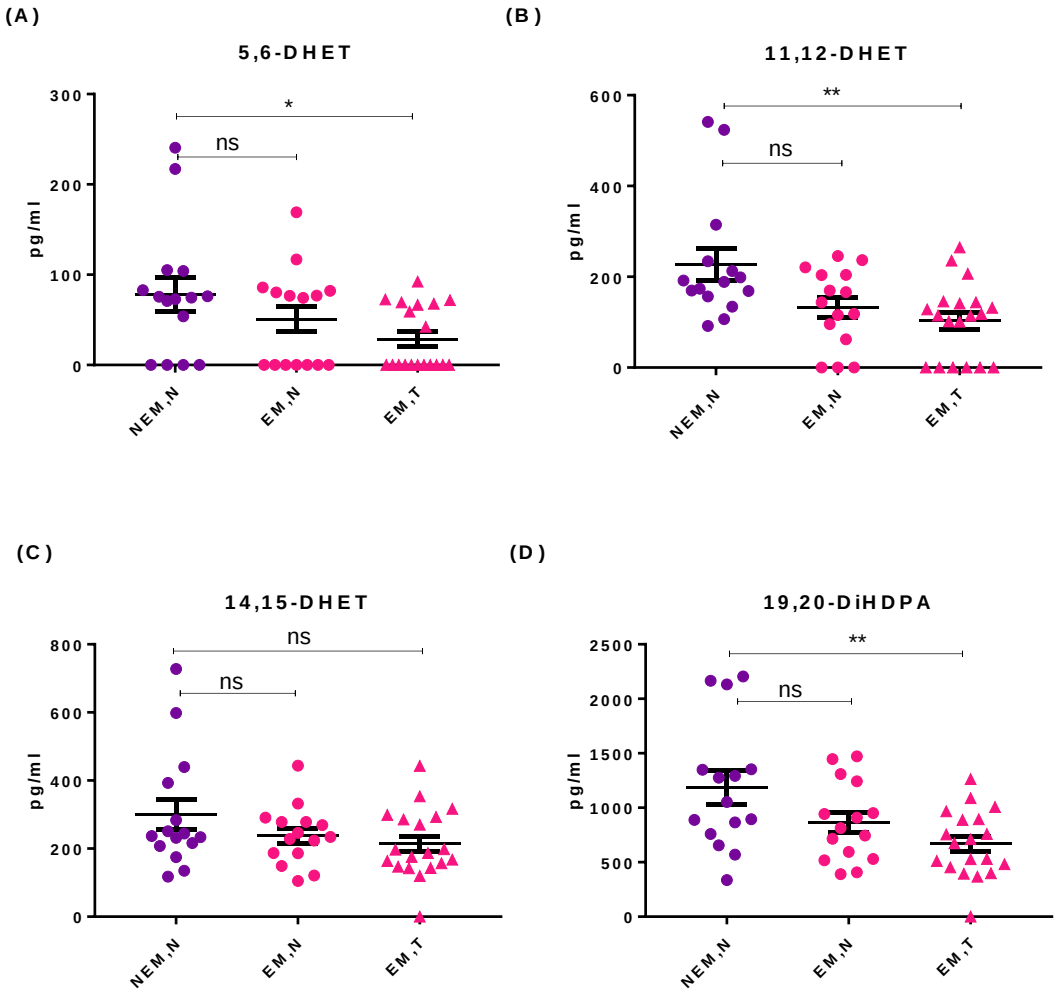


Figure 3.4.9. Soluble epoxyhydrolase products in plasma.
*Non-endometriosis (NEM,N; n=15) women with regular menstrual cycles and without hormone treatments or hormonal contraceptives were compared to endometriosis patients with regular periods, without pharmacotherapy (EM,N; n=15) and amenorrhoeal endometriosis patients using a GnRH analogue (EM,T; n=19) Measurements were performed using LC/ESI-MS/MS. Data are expressed as arithmetic means of metabolite (pg/ml) ± SEM. Statistical comparisons were performed using Kruskal-Wallis test with Dunn's post-hoc test. (ns) not significant; *p<0.05; **p<0.01.*

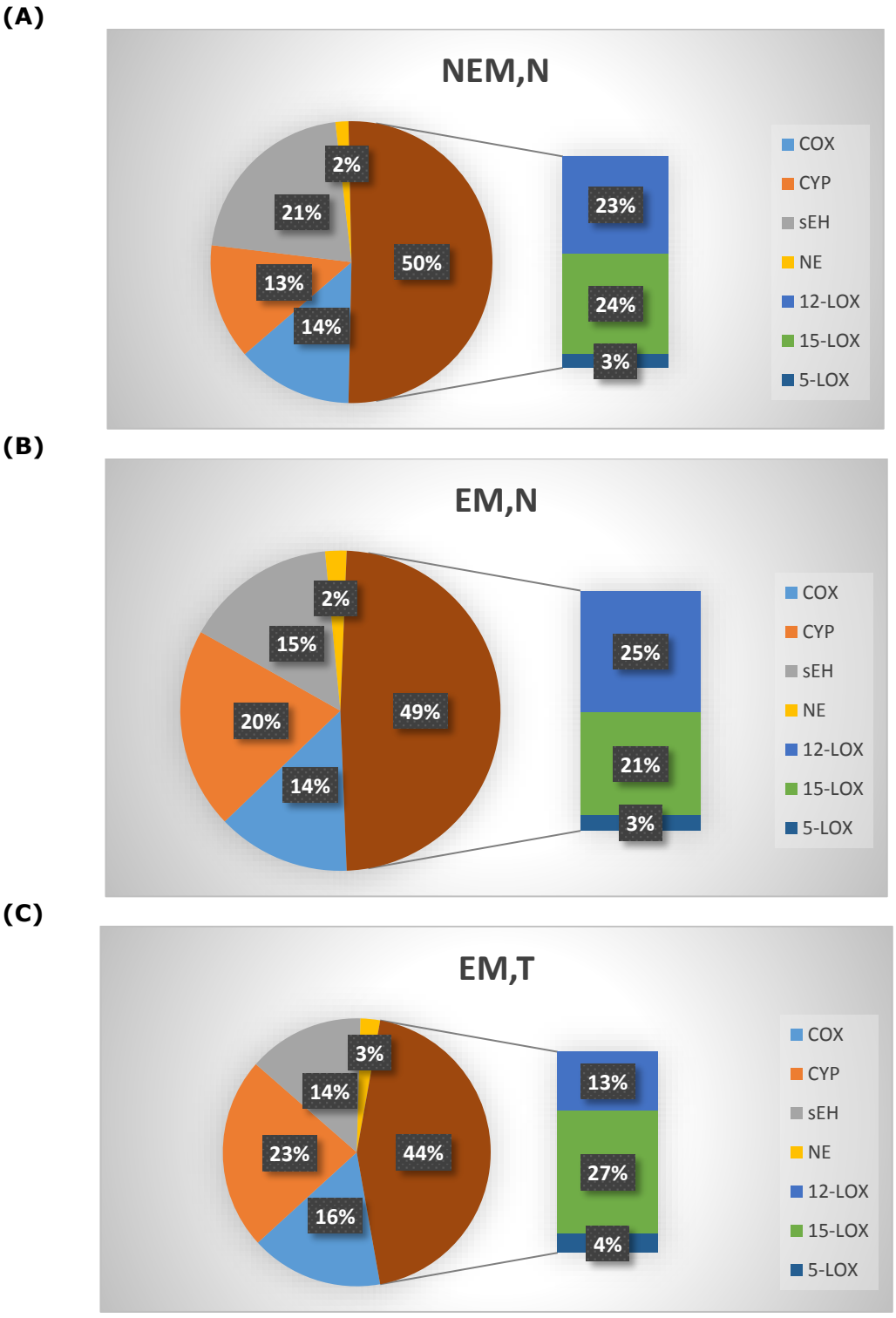


Figure 3.4.10. Proportions of detected lipid mediators in plasma demonstrated by biosynthetic pathways.
Lipid mediators from pre-menopausal women (A) without (NEM,N; n=15) and (B) with (EM,N; n=15) endometriosis with regular cycles and not taking any form of hormonal treatments or hormonal contraceptives were compared to (C) endometriosis patients receiving hormonal treatment (EM,T; n=19). Bar of pie chart illustrates the proportions of LOX derived metabolites according to LOX pathways. Data were obtained using LC/ESI-MS/MS. (COX) cyclooxygenase; (LOX) lipoxygenase; (CYP) cytochrome P450 epoxygenases; (sEH) soluble epoxy hydrolase; (NE) non-enzymatic

3.4.2.3 Variation of plasma lipid mediators by medical condition

Plasma samples were obtained from 70 participants on the day of laparoscopic surgery. Of those, 41 patients were diagnosed with endometriosis and 29 were diagnosed with other gynaecological conditions (Appendix 3). Data obtained from mass spectrometry were compared according to the presence or absence of endometriosis. Table 3.4.5Table 3.4.4 summarizes the concentration of the detected lipid mediators by biosynthetic pathways in plasma.

*Table 3.4.5. Comparison of lipid mediators in plasma from women not diagnosed with endometriosis (NEM, n=29) and from women diagnosed with endometriosis (EM, n=41). Measurements were performed using LC/ESI-MS/MS. Data are expressed as arithmetic means of metabolite (ng/ml) ± SEM. Statistical comparisons were performed using Mann-Whitney U test. (*p<0.05; **p<0.01) Ratios were calculated between EM and NEM using arithmetic means.*

Plasma	Lipid mediator	NEM n=29	EM n=41		$\frac{EM}{NEM}$
Pathway		Mean ± SEM (ng/ml)		p-value	
COX	TXB_2	**0.06 ±** 0.02	**0.20 ±** 0.07	0.0753	3.7
	9-HODE	**2.81 ±** 0.22	**2.85 ±** 0.35	0.3188	1.0
	9-oxoODE	**1.14 ±** 0.19	**1.32 ±** 0.24	0.5857	1.2
5-LOX	5-HETE	**0.44 ±** 0.05	**0.53 ±** 0.07	0.3116	1.2
	LTB_4	**0.05 ±** 0.01	**0.15 ±** 0.06	0.5644	2.8
	9-HOTrE	**0.34 ±** 0.04	**0.32 ±** 0.04	0.4479	1.0
	4-HDHA	**0.17 ±** 0.02	**0.12 ±** 0.03	0.0074 **	0.7
12-LOX	12-HETE	**5.68 ±** 0.98	**4.98 ±** 0.74	0.6187	0.9
	12-HEPE	**0.56 ±** 0.11	**0.39 ±** 0.10	0.0694	0.7
	14-HDHA	**1.50 ±** 0.25	**1.22 ±** 0.25	0.0665	0.8
15-LOX	13-HODE	**5.17 ±** 0.43	**4.99 ±** 0.56	0.2121	1.0
	13-oxoODE	**1.52 ±** 0.28	**1.48 ±** 0.27	0.3547	1.0
	15-HETE	**0.34 ±** 0.04	**0.34 ±** 0.03	0.7246	1.0
	15-HETrE	**0.09 ±** 0.02	**0.08 ±** 0.01	0.7338	0.9
	13-HOTrE	**0.29 ±** 0.03	**0.33 ±** 0.05	0.7023	1.1
CYP	9(10)EpOME	**1.80 ±** 0.35	**3.24 ±** 0.68	0.7762	1.8
	12(13)EpOME	**1.73 ±** 0.26	**2.82 ±** 0.50	0.7135	1.6
sEH	9.10-DiHOME	**2.27 ±** 0.31	**1.81 ±** 0.25	0.0501	0.8
	12.13-DiHOME	**2.19 ±** 0.21	**1.73 ±** 0.17	0.0593	0.8
	5.6-DHET	**0.08 ±** 0.01	**0.05 ±** 0.01	0.0513	0.6

	11.12-DHET	**0.20 ±** 0.02	**0.13 ±** 0.01	0.0164 *	0.7
	14.15-DHET	**0.27 ±** 0.03	**0.23 ±** 0.01	0.2121	0.8
	19.20-DiHDPA	**1.23 ±** 0.15	**0.78 ±** 0.06	0.0087 **	0.6
NE	**11-HETE**	**0.11 ±** 0.01	**0.12 ±** 0.02	0.9245	1.1
	trans-EKODE	**0.31 ±** 0.05	**0.45 ±** 0.09	0.8566	1.4
	20HDHA	**0.15 ±** 0.03	**0.09 ±** 0.03	0.1545	0.6

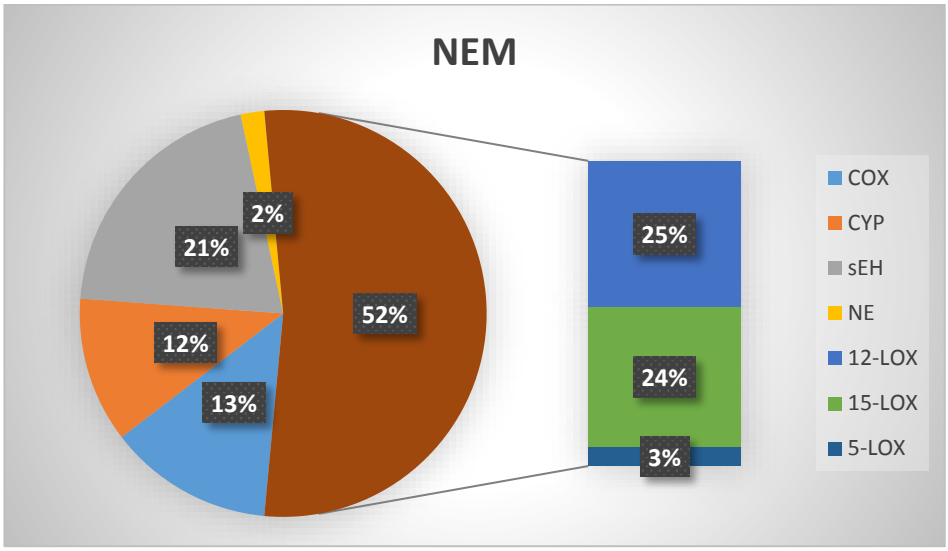
(COX) cyclooxygenase; (LOX) lipoxygenase; (CYP) cytochrome P450 epoxygenases; (sEH) soluble epoxy hydrolase; (NE) non-enzymatic. For the name of lipid mediators please see the section of frequently used abbreviations.

Apart from TXB_2, COX derived prostanoids were below the limit of detection in plasma. TXB_2 was nearly four times higher in the endometriosis than in the non-endometriosis group. The expression of other COX products, namely 9-HODE and 9-oxoODE, were similar between the two study groups.

Amongst the LOX metabolites, the AA derived LTB_4 also showed a 2.8-fold elevation the in endometriosis group whereas the majority of ω-3 LOX products were suppressed in plasma from women diagnosed with endometriosis. Thus, 3 out of 5 ω-3 LOX species were suppressed in the endometriosis group. The means of 9 and 13-HOTrE were very similar between the two study groups, whereas 12-HEPE, 4 and 14-HDHA were present in depleted concentrations in plasma from women with endometriosis. The statistical analysis revealed significant alteration for 4-HDHA (**p=0.0074). In addition, the differences were also close to the significance threshold ($p<0.05$) for 12-HEPE ($p=0.0694$) and 14-HDHA ($p=0.0665$).

The most prominent alterations in plasma were observed for sEH derived products. The arithmetic means were lower for all detected sEH metabolites in the endometriosis group. 11,12-DHET (*p=0.0164) and 19,20-DiHDPA (**p=0.0087) were significantly lower in plasma from patients with endometriosis compared to those without. Furthermore, data from Table 3.4.4Table 3.4.5 also indicate that the differences in three metabolites were very close to the significance threshold ($p<0.05$). These species were as follows: 5,6-DHET ($p=0.0513$), 9,10-DiHOME ($p=0.0501$) and 12,13-DiHOME ($p=0.0593$). To demonstrate the proportional changes of lipid mediators in plasma, data were grouped according to biosynthetic pathways and expressed as percentage of total detected lipid mediators (Figure 3.4.11).

(A)



(B)

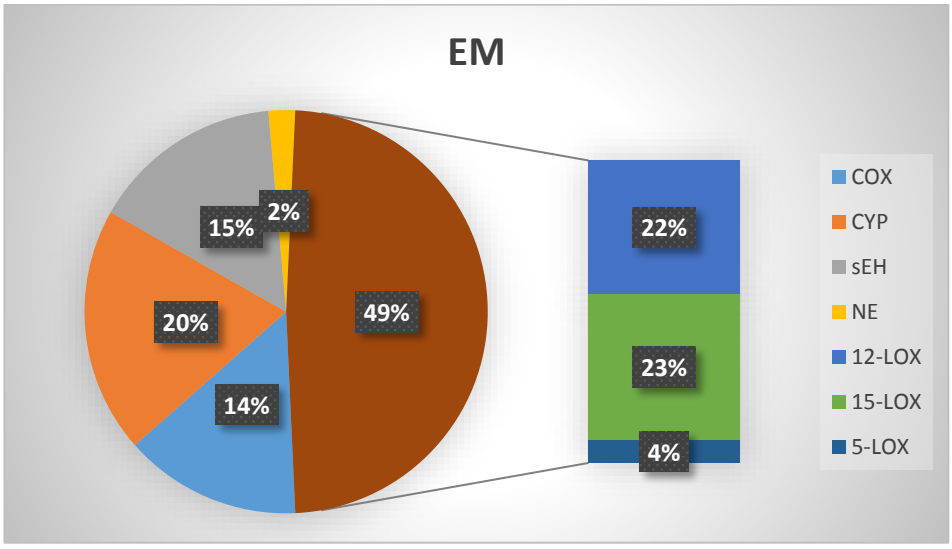


Figure 3.4.11. Proportions of detected lipid mediators in plasma from (A) women without (NEM, n=29) and (B) with (EM, n=41) endometriosis. Metabolites were grouped by biosynthetic pathways and expressed as percentage of total detected lipid mediators. Bar of pie chart illustrates the proportions of LOX derived metabolites according to LOX pathways. Data were obtained using LC/ESI-MS/MS. (COX) cyclooxygenase; (LOX) lipoxygenase; (CYP) cytochrome P450 epoxygenases; (sEH) soluble epoxy hydrolase; (NE) non-enzymatic.

Pie charts illustrate that although the accumulated percentages of CYP and sEH products were similar in the two study groups (NEM=33%; EM=35%) the proportions between them were different, suggesting suppressed sEH activity in women with endometriosis. From the charts above and in Figure 3.4.1 it can also be seen that the activity of LOX pathways were different in the peritoneum and the vascular system. While 15-LOX products dominated in peritoneal fluid, the expression of 12 and 15-LOX metabolites were similar in plasma.

3.5 Discussion

3.5.1 Lipid mediators in peritoneal fluid

Peritoneum and peritoneal fluid is a special microenvironment for the physiological and pathological processes. Peritoneal fluid is in interaction with the surface of the peritoneal organs also with the free-floating cellular components. These organs and free-floating cells take up and release metabolites into the peritoneal fluid. Peritoneal fluid contains cytokines, chemokines, serum proteins, steroid and protein hormones and lipid mediators (Syrop and Halme, 1987). Studying these mediators may provide an insight into the endometriosis-associated pathomechanism(s) within the peritoneum.

The role of pro-inflammatory cytokines and PGE_2 in the pathomechanism of endometriosis are well established (section 1.6.3) and the importance of AA-derived prostanoids in the female reproductive tract is also well documented. Prostanoids are essential for the normal physiological function of the reproductive tract but their impaired production also contributes to menstrual disorders, such as heavy, painful bleeding, and infertility (Jabbour et al., 2006). It is also well known that the prostanoid synthesis in the endometrium changes across the menstrual cycle. For instance, PGE_2 and $PGF_{2\alpha}$ are low in the proliferative phase, increase during the secretory phase and reach the peak in the menstrual phase (Downie et al., 1974). A more recent comprehensive study also investigated the expression of prostanoid enzymes in the endometrium and found that COX-2 expression increased in the menstrual phase, with both COX-1 and COX-2 expression lowest in the proliferative phase and increasing during the early to mid-secretory phase (Catalano et al., 2011). These studies demonstrate that the menstrual cycle has effect on the prostanoid synthesis in the endometrium.

Although the role of prostanoids within the uterus is well studied, relatively little is known about their production within the peritoneum. Moreover, even less is known about the role of other oxygenated lipid mediators in the peritoneum. For this reason, 79 lipid species were investigated in peritoneal fluid specimens from women with and without endometriosis using LC/ESI-MS/MS to identify lipid mediator profile and facilitate the better understanding of endometriosis-associated inflammatory milieu.

A total of 25 metabolites were detected out of the 79 tested lipid species in the peritoneal fluid from women with and without endometriosis, and were compared according to the stages of menstrual cycle, GnRH agonist treatment and the presence or absence of endometriosis.

Firstly, data was evaluated across the menstrual cycle to see whether the menstrual cycle has effect on the lipid mediator synthesis within the peritoneum. However, the conclusions must be considered with care due to the low number of participants in the study groups and the complete lack of some menstrual stages, such as NEM, N menstrual and EM,N secretory stages.

Data obtained from non-endometriosis women with normal menstrual cycle without using hormonal medication or contraceptives (NEM,N) showed that the lipid mediator synthesis was low during the proliferative phase and increased during the secretory phase. Data was not available in menstrual phase. Apart TXB_2, all lipids were elevated in the secretory group compared to proliferative phase in NEM,N group (Figure 3.4.1). Moreover, significant elevation was observed for three sEH products namely 9,10-DiHOME (***p=0.002), 12,13-DiHOME, (**p=0.0028), and 19,20-DiHDPA (*p=0.0317) suggesting the expression of sEH might change with the menstrual cycle. However, also needs to be pointed out that other sEH products such as 11,12-DHET (p=0.377), and 14,15-DHET (p=0.194) were not significantly elevated in the secretory phase, compared to the proliferative phase in the peritoneal fluid from non-endometriosis women with a normal menstrual cycle. To resolve this contradiction, the number of participants should be increased in the future. Increasing the number of participants would be particularly important because no study has reported on the cyclical variation of sEH metabolites in the peritoneal fluid thus far.

Firm conclusions cannot also be drawn about the variation of lipid mediator synthesis in endometriosis women, since peritoneal fluid from endometriosis women with normal menstrual cycle, without using hormonal medication or contraceptives (EM,N) were not collected in the secretory phase. Therefore no data was available to assess whether the lipid mediator synthesis was also elevated during secretory phase in these patients.

As was mentioned above, a limited number of studies have measured lipid mediators in the peritoneal fluid and evaluated their variation across the menstrual cycle. These studies focused on series-2 prostanoids. De Leon *et al*. did not observe a significant cyclic variation of prostanoids in peritoneal fluid under physiological conditions, thus they speculated that the ovarian follicle and corpus luteum might not the primary source of prostanoids measured in the peritoneal fluid (De Leon et al., 1986). Ylikorkala *et al.* also did not find a relationship between the prostanoid concentration in the peritoneal fluid and the menstrual cycle from women with and without endometriosis (Ylikorkala et al., 1984). Syrop and Halme summarized studies investigating the prostanoids levels in the peritoneal fluid from women with and

without endometriosis. However, due to the diverse findings and the lack of cycle information, they could not draw a definitive conclusion with regard to the alteration of prostanoid levels in different pathological conditions (Syrop and Halme, 1987).

In the current study, lipid mediators in the proliferative phase from women with and without endometriosis with normal menstrual cycle, without using hormonal medication or contraceptives were compared and found only a single lipid, namely 9-HOTrE was significantly increased in peritoneal fluid during the proliferative phase from women with endometriosis (Figure 3.4.1 vii). Although significant alteration was observed for 9-HOTrE, it should take into account that the sample numbers (NEM,N n=5; EM,N n=4) were low. On the other hand, biological activity of 9-HOTrE has not been reported so far (Tam, 2013). Thus, it is not possible to derive a conclusion from this finding.

Overall, data obtained in this study did not provided enough evidence to conclude whether lipid mediator synthesis in the peritoneum was affected by the menstrual cycle.

Pharmacotherapy could also have an effect on the lipid mediator synthesis. The partial inhibitory effect of NSAIDs on COX isoenzymes is well known. NSAIDs inhibit the cyclooxygenase function, whilst the peroxidase activity of the COX isoenzymes is not affected by these drugs (Vane, 1971). This partial inhibition of COX shifts the metabolism from prostanoids to HETE, resulting in a reduction in prostanoid synthesis, promotion of anti-inflammatory processes and a change in the lipid profile (Claria and Serhan, 1995). Unfortunately, information about NSAIDs usage by the patients was not recorded, which is a limitation of this study.

However, patients were asked about the usage of hormonal medications. The majority of the endometriosis patients (24/45, 53%) received GnRH agonist treatment for their condition. GnRH agonists are recommended and frequently described to reduce endometriosis-associated pain (Dunselman et al., 2014). Of interest, the classic pain-inducing prostaglandins, such as PGE_2 and $PGF_{2\alpha}$, did not reached the limit of detection in the peritoneal fluid in any group. Only the stable metabolites of PGI_2, 6-keto PGF1α, was detected in all groups. The highest level was found in the non-treated endometriosis (EM,N) and the lowest in the GnRH agonist treated group (EM,T). PGI_2 is also a regulator of pain and inflammation (Murata et al., 1997). For instance Wang *et al.* found that 6-keto PGF1α, was significantly increased in the peritoneal fluid from sterilized women with chronic pelvic pain compared to subject without pelvic pain (Wang et al., 1992).

The exact mechanism of analgesia exerted by GnRH agonist has not been well studied. However, there might be an indirect relationship between GnRH agonist treatment and the prostanoid synthesis. As was discussed in section 1.6.3, a regulatory relationship was proposed between E_2 and the COX-2 expression (see also figure 1.6.2). E_2 upregulates the expression of COX-2 and promotes the prostanoids synthesis. However, GnRH agonists markedly reduce the level of E_2 in the body and induce hypo-oestrogenic state (Arya and Shaw, 2006). As a consequence, the expression of COX-2 might be down regulated in the lack of E_2 resulting low pain-inducting prostanoid levels. The findings of the current study might support this hypothesis, since all COX products were the lowest in the GnRH treated group; what is more, TXB_2 did not reach the limit of detection in this group. Thus, this hypothesis might provide a possible explanation for the analgesic effect of GnRH agonists, however further studies are needed to confirm this.

Finally, data was compared according the presence or absence of endometriosis. Statistically relevant alterations were not observed for the detected metabolites between the endometriosis and non-endometriosis groups. Although statistical differences were not detected, some interesting changes were observed.

The first and the most apparent alteration of lipid mediator profiles in peritoneal fluid was that nearly all metabolites were present in lower concentrations in endometriosis patients regardless of biosynthetic pathways or medical treatments (Table 3.2.3 and 3.2.4). This finding might suggest an impaired step at the common part of the biosynthesis pathway for the metabolites tested.

The initial step of the biosynthesis is regulated by activated PLA_2 isoenzymes which release the precursor fatty acids from the plasma membrane. PLA_2s cleave the ester bond of the glycerophospholipids in the sn-2 position by a hydrolysis. The pro-inflammatory cytokines and growth factors induce, while glucocorticoids inhibit the activation of PLA_2s (Clark et al., 1995).The PLA_2 family consists of more than 30 PLA_2 isoforms in mammals and they are classified into five groups according to primary structure, localisation, substrate preference and Ca^{2+} ion dependency (Schaloske and Dennis, 2006). Mostly two groups, the secreted PLA_2s ($sPLA_2$) and cytosolic PLA_2s ($cPLA_2$) are responsible for the release of the precursor fatty acids from the phospholipid membranes. $sPLA_2$s does not have a particular preference towards the fatty acids whereas $cPLA_2$s preferably act on AA (Uozumi et al., 1997; Murakami et al., 2011).

A few studies have investigated the expression of PLA_2s in endometriosis. A global gene expression analysis screening 53,000 genes and transcribed sequences found that a secreted PLA_2, namely PLA_2IIA was the most up-regulated gene (153-fold) in ectopic lesions compared to their paired eutopic endometrium from endometriosis patients. Expression of the $cPLA_2$ genes, however, was not altered between the two specimens. The immunoblot analysis confirmed the increase protein expression of PLA_2IIA (Eyster et al., 2007). Lousse *et al.* (2010) also reported a significant increase in gene and protein expressions for PLA_2IIA in ectopic lesions compared to matched eutopic endometrium and in peritoneal macrophages from women with endometriosis compared to control (Lousse et al., 2010). However, these findings are somewhat contradictory to other studies. Although the secretory PLA_2IIA was upregulated in ectopic lesions and peritoneal macrophages from endometriosis patients, to date no study reported elevated concentration of PLA_2IIA or other $sPLA_2$s in peritoneal fluid from women diagnosed with endometriosis (Ueki et al., 1994; Kocbek et al., 2015). Henceforth, it can be speculated that the secretion of these isoenzymes from the ectopic lesions into the peritoneal fluid might be disturbed and this disturbed secretion of $sPLA_2$s might explain the low lipid mediator concentrations in the peritoneal fluid from endometriosis patients.

The influence of the peritoneal microenvironment on the activity of PLA_2s has not yet been investigated in endometriosis. However, it was reported that different adenocarcinomas isolated from malignant pleural effusion specimens were capable of inhibiting the PLA_2s activity in polymorphonuclear cells (PMN) from healthy subjects in a cell number-dependent manner (Abe et al., 1997). This finding is particularly appealing since it might be applicable to other pathological conditions, such as endometriosis, where the free-floating endometrial, mesothelial cells and peritoneal macrophages might have a similar inhibitory effect on the activity of PLA_2s and this possible inhibition was responsible for the observed decreased concentrations of lipid mediators in endometriosis.

The dysfunction of PLA_2s could also provide a possible explanation for the low concentration of the classical eicosanoids. Only two metabolites, TXB_2 and 6-keto-$PGF_{1\alpha}$ were detected in the peritoneal fluid specimens. TXB_2 and 6-keto-$PGF_{1\alpha}$ are the biologically inactive forms of TXA_2 and PGI_2, respectively. These metabolites cannot be measured directly since have a very short half-life and convert rapidly to their more stable, inactive metabolites. The main source of TXA_2 is platelets, but it is also produced by other cell types, such as endothelial cells or macrophages, whilst PGI_2 is secreted by endothelial and vascular smooth muscle cells (Nakahata, 2008; Kawabe et al., 2010). These metabolites counteract each other and are considered

the most important regulators of cardiovascular homeostasis but they are also crucial molecules for the initiation of inflammation (Kawabe et al., 2010; Buckley et al., 2014).

6-keto-$PGF_{1\alpha}$ and TXB_2 were not significantly altered in the peritoneal fluid between the study groups. The concentration of these metabolites in endometriosis measured by mass spectrometry has not been reported so far. Therefore the expected values measured by this method are not known. Previous studies used radioimmunoassay (RIA) or ELISA methods and reported contradictory results with regard to both metabolites. For instance, two groups reported significantly increased 6-keto-$PGF_{1\alpha}$ concentration in peritoneal fluid from women with endometriosis (Drake et al., 1981; Dawood et al., 1984), whilst other groups have not found significant changes (Ylikorkala et al., 1984; Mudge et al., 1985). Similar to 6-keto-$PGF_{1\alpha}$, some groups reported significantly elevated TXB_2 concentration in peritoneal fluid from endometriosis patients (Drake et al., 1981; Pungetti et al., 1987), while others have not observed such a change (Dawood et al., 1984; Ylikorkala et al., 1984; Yamaguchi and Mori, 1990). These contradictory results may be explained by the fact that they tested a low number of participants. Another possible explanation for these contradictions is that immunological methods were used. The main issue with these methods is that they are not ideal to measure lipid mediators. Firstly, these molecules are present in low concentrations, secondly they cannot be measured directly since they degrade quickly due to their short half-life and thirdly lipid molecules are not ideal epitopes for antibody recognition. Thus, the results of these immunoassays are less robust compared to mass spectrometry which is a method designed to detect lipids in low concentrations from complex biological samples.

Apart from 6-keto-$PGF_{1\alpha}$ and TXB_2, other series-2 prostaglandins, such as PGE_2, PGD_2 and PGJ_2 were sparse or not detected in the peritoneal fluid. The low concentration of PGE_2 in endometriosis was an unexpected outcome since studies report upregulated COX-2 expression in ectopic lesions and elevated PGE_2 concentrations in the peritoneal fluid from women with endometriosis (Ota et al., 2001; Wu et al., 2002). Moreover, PGE_2 was proposed as the master regulator of endometriosis pathology (Chuang et al., 2010; Wu et al., 2010). Of note, contradictory studies were also published where the PGE_2 concentration was not significantly elevated in the peritoneal fluid from endometriosis patients (Dawood et al., 1984; Pungetti et al., 1987). However, these early studies have not received much scientific attention.

Metabolites of CYP biosynthetic pathway were also not significantly changed in the peritoneal fluid from women with and without endometriosis (Table 3.4.3). Three

epoxy products, namely the AA derived 11(12)EET and the LA derived 9(10)EpOME and 12(13)EpOME were detected in the peritoneal fluid. The EpOME products were decreased in the endometriosis group. The EM/NEM ratios of 9(10)EpOME and 12(13)EpOME were 0.5 and 0.6, respectively, whereas 11(12)EET expression was similar between the two study groups, with the EM/NEM ratio of 1.1. However, it was also noted that 11(12)EET was reduced in the untreated endometriosis (EM,N) group compared to women with endometriosis taking GnRH agonist treatment (EM,T) and women without endometriosis not taking hormonal treatments like contraceptives (NEM,N). The 11(12)EET concentration was similar in the two latter groups (Table 3.4.2).

CYP enzymes form a large family. The classes of CYP2C and 2J isoenzymes metabolize PUFAs and generate epoxy derivatives. These products are biologically potent but unstable molecules. Epoxide hydrolases regulate the level of epoxy derivatives by incorporating a water molecule into the epoxide group resulting in biologically less potent diol products (Fleming, 2011). AA derived EETs are studied extensively since they have important physiological functions including angiogenesis, vasodilatation, apoptosis and regulation of inflammation. EETs express anti-inflammatory effects by down regulating the expression of cell adhesion molecules on endothelial cells and inhibiting the NF-κB pathway (Node et al., 1999). For this reason the low level of 11(12)EET in the not treated endometriosis group might promote disease proliferation in these patients. However, more data and research are needed to confirm these effects.

LOX derived metabolites, including 5-, 12- and 15-LOX products, were also measured in the peritoneal fluid from women with and without endometriosis (Table 3.4.3). No significant differences were observed. Except for 12-HETE and 4-HDHA, all LOX metabolites were detected in lower concentrations in the endometriosis group compared to the non-endometriosis group. Although, 12-HETE and 4-HDHA concentrations were apparently higher in endometriosis it should be pointed out that patient data from one individual skewed these results. The 12-HETE concentration in peritoneal fluid from endometriosis patient (HP02) was more than 40 times higher (12-HETE: HP02=422.1ng/ml vs. EM=10.4±4.2ng/ml vs. NEM= 13.6±6.3ng/ml), whereas the 4-HDHA was 33 times higher than the mean of the rest of the group (4-HDHA: HP02=9.9ng/ml vs. EM=0.3±0.17ng/ml vs. NEM= 0.6±0.26ng/ml). Patient HP02 had a severe, recurrent condition causing infertility, chronic pelvic pain and dysmenorrhoea.

In amongst the 5-LOX products 9-HOTrE, an ω-3 ALA metabolite, was the most abundant lipid in the peritoneal fluid. The biological activity of 9-HOTrE has not been explored yet therefore a conclusion could not been drawn from this observation. To date, the AA derived 5-LOX metabolites have received more attention since these lipids are widely considered pro-inflammatory mediators that have a pivotal role in the initiation of inflammation by recruiting PMN cells to the affected site. For instance, LTB_4 is known as one of the most potent chemotactic agents for granulocytes (Ford-Hutchinson et al., 1980), while 5-oxoETE is one of the most potent activator of eosinophils (Powell and Rokach, 2013). However, these metabolites were not detected in the peritoneal fluid suggesting the inflammation was not in the early, initial phase in those patients.

Overexpression of 12-LOX and increased levels of 12-HETE were reported in several adenocarcinomas (Fürstenberger et al., 2006). There is a growing body of evidence to suggest that 12-HETE is capable of inhibiting apoptosis, inducing invasion and metastasis and stimulating angiogenesis (Tang et al., 1997; Nie et al., 1998, 2003, 2006). These processes are also elements of the pathomechanism of endometriosis therefore elevated levels of 12-HETE were expected in the endometriosis samples. Apart from the outlier patient (HP02), elevated 12-HETE concentrations were not observed in the endometriosis group. This result might suggest that different mechanisms may be responsible for the increased resistance of endometrial cells to apoptosis, also for invasion into the peritoneum and for the establishment of neovascular supply to ectopic lesions.

Of all the tested metabolites the most marked reductions were observed for 15-LOX derived products. The percentage reductions were on average between 60 to 90% in the endometriosis compared to non-endometriosis group. The same trends were observed in the treated and non-treated endometriosis groups. The most abundant metabolites were 13-HODE and 15-HETE, which are the main products of 15-LOX-1 and 15-LOX-2 isoenzymes, respectively. With regard to these mediators the mean of 13-HODE was 3.4-fold lower, whereas the mean of 15-HETE was 2.8-fold lower in the endometriosis group compared to non-endometriosis group.

The role of 15-LOX in physiological and pathological processes has not been completely elucidated yet but an increasing number of studies propose that 15-LOX products have an opposite effect to 5- and 12-LOX metabolites. Whilst 5- and 12-LOX derivatives promote inflammation and carcinogenesis, 15-LOX metabolites possess anti-inflammatory and tumour suppressor properties.

15-LOX derived lipids exert their anti-inflammatory effects in two ways. First, 15-HETE and 17-HDHA are the precursor molecules of specialised pro-resolving mediators (SPMs). These molecules control the resolution phase of inflammation and down regulate the pro-inflammatory environment by limiting the infiltration of PMNs to the site of inflammation and enhancing the clearance mechanisms of macrophages (Serhan, 2014). 15-HETE is the precursor of lipoxins, whereas 17-HDHA is the parent fatty acid of two SPMs classes, namely the D-series resolvins and the protectins (Serhan and Samuelsson, 1988; Serhan et al., 2002). Although SPMs were not been detected in the peritoneal fluid either in endometriosis or non-endometriosis groups, the suppressed production of precursors might allow us to speculate that the biosynthesis of these lipids was disturbed or down regulated in endometriosis patients.

The second way the 15-LOX metabolites regulate inflammation is to control gene expression via PPARγ. 15-LOX metabolites are endogenous ligands for PPARγ (Nagy et al. 1998; Pham and Ziboh 2000; Egawa et al. 2016) and PPARγ mediates inflammatory reactions in various ways such as supressing the pro-inflammatory cytokine synthesis (IL-8, IL-12, TNFα) in macrophages, down regulating MMPs and adhesion molecules (ICAM-1) expression in local tissues and endothelial cells and promoting macrophage differentiation at the site of inflammation (Tontonoz et al., 1998; Moraes et al., 2006). These studies indicate that PPARγ activity could be beneficial for endometriosis. A few studies have investigated the effects of synthetic PPARγ agonists on endometrial explant growth in rat and baboon models and found that the tested synthetic agonists effectively reduced the numbers and size of endometrial-like lesions (Lebovic et al., 2004, 2010; Aytan et al., 2007).

The present study demonstrated low 15-LOX metabolite concentrations in the peritoneal fluid from women diagnosed with endometriosis. The low concentration of 15-LOX metabolites might result in disrupting the function of PPARγ which could in turn promote the establishment and proliferation of endometriosis. For that reason, a further aim of this thesis was to assess the expression of 15-LOX and PPARγ in endometriosis.

3.5.2 Lipid mediators in plasma

The primary aim of the evaluation of plasma lipid mediators was to identify a possible new biomarker or a panel of biomarkers for the diagnosis of endometriosis. Additionally, lipid mediator profiles of plasma and peritoneal fluid were also examined to gain insight into the local and systemic pathological processes of endometriosis. Similarly to peritoneal fluid, data obtained from plasma were analysed according to the stages of menstrual cycle, GnRH agonist treatment and the presence or absence of endometriosis.

Plasma data were evaluated across the stages of the menstrual cycle from women with (EM,N) and without (NEM,N) endometriosis who reported having regular menstrual cycles and were not using hormonal contraceptives. Apart 5-HETE, 4-HDHA, 9(10)EpOME, 11(12)EpOME and 19,20-DiHDPA all lipids were the highest during the proliferative phase in the NEM,N group, whereas in the EM,N group only 14 out of 26 species showed peak in the same stage. Only two significant changes were observed across the menstrual cycle. 13-oxoODE in the non-endometriosis and 11,12-DHET in the endometriosis group were reduced significantly from the proliferative stage to the secretory phase. Furthermore, also two significant alterations were observed when data was compared according to the cycle stages. TXB_2 was significantly higher in proliferative, whilst 11,12-DHET was significantly lower in the secretory phase in plasma from women with endometriosis with a regular cycle, compared to those without the condition. In contrast, a recent study using a similar method tested 50 lipid mediators, including TXB_2 and 11,12-DHET, in serum from women with and without endometriosis, did not find a significant difference for any mediators in the proliferative or secretory phases between the study groups (Lee et al., 2016). However, it needs to be pointed out that the ethnicity of the subjects was different from those in the current study, and serum rather than plasma specimens were used. These differences might provide an explanation to the differing outcomes.

Overall, based on the available evidence, lipid levels are likely to vary across the menstrual cycle since 21 out of 26 lipids showed peak in the proliferative phase from women without endometriosis with regular cycle. However, these results must be interpreted with a caution because some mediators showed contradictory trends across the menstrual cycle between the two study groups. 9-HODE, 9-HOTrE and 15-HETrE were the highest during the proliferative phase in the NEM,N whereas the same mediators were the lowest in proliferative phase in the EM,N group. 13-HOTrE decreased from the proliferative phase to the secretory stage in the NEM,N but was

elevated in the EM,N group. And finally, one mediator showed a completely opposite trend. 5-HETE decreased from the menstrual phase to the secretory phase in the non-endometriosis but increased across the menstrual cycle in the endometriosis group.

These finding are challenging to explain because these mediators have not yet been investigated in relation to endometriosis or their cyclical variation across the menstrual cycle. A very limited number of studies have investigated the variation of plasma lipid metabolites across the menstrual cycle in healthy subjects, with a main focus on COX metabolites. However these studies are not in agreement; therefore, a firm conclusion cannot be drawn with regard to the alterations of these lipids across the stage of the menstrual cycle (Jordan and Pokoly, 1977; van Orden et al., 1977; Koullapis and Collins, 1980; Schlegel et al., 1982). Hence, increasing the number of participants, particularly in the menstrual stage, is necessary to reach a conclusion with regard to the cyclic variation of the lipid mediators in the vascular system.

Data were also analysed according to GnRH agonist treatment (Table 3.4.4). Non-endometriosis women with regular menstrual cycles without hormone treatments or hormonal contraceptives (NEM,N) were selected and compared to endometriosis patients with regular periods, without hormonal pharmacotherapy (EM,N) and amenorrhoeal endometriosis patients using GnRH agonists (EM,T). One 5-LOX metabolite, namely 4-HDHA, as well as 12-LOX and sEH derived products were significantly changed between the study groups. 4-HDHA was significantly lower in the endometriosis without hormonal treatment group, whereas all 12-LOX detected metabolites were significantly depleted in the GnRH agonist treated endometriosis group, compared to the not treated endometriosis, or non-endometriosis without hormonal treatment, or hormonal contraceptive groups. 4-HDHA directly inhibits endothelial cell proliferation and angiogenesis via PPARγ (Sapieha et al., 2011), therefore the elevation in the GnRH treated group might be beneficial for the endometriosis patients and might downregulate the neoangiogenesis in the ectopic lesions. The findings also suggest that the GnRH treatment might downregulate 12-LOX, since all of the detected 12-LOX metabolites were significantly reduced in the treatment group. 12-LOX is highly expressed in platelets, therefore it is believed that these metabolites are important in the physiology of the vascular system (Yeung and Holinstat, 2011). On the other hand, overexpression of 12-LOX and elevated level of 12-HETE were reported in cancer (Fürstenberger et al., 2006). 12-HETE was found to be an active regulator of tumour angiogenesis and apoptosis (Pidgeon et al., 2002; Nie et al., 2006). Therefore, the reduction of 12-HETE might also be positive for endometriosis patients who received GnRH treatment.

The sEH derived lipids, namely 5,6-DHET, 11,12-DHET and 19,20-DiHDPA, were also significantly decreased in the EM,T group compared to NEM,N. In addition, 11,12-DHET (*p=0.0164) and 19,20-DiHDPA (**p=0.0087) were significantly reduced when study groups were compared according to the condition (NEM vs. EM; Table 3.4.5). A large cohort study suggests that endometriosis patients are likely to have a higher risk of cardiovascular diseases (Mu et al., 2016). The role of the CYP450 pathway in oxidation, peroxidation, and reduction of steroids, which contribute to the onset and progression of cardiovascular diseases is acknowledged (Fleming, 2011). EETs express favourable effects on the vascular system by their anti-inflammatory, vasodilatory and anti-thrombotic properties (Sudhahar et al., 2010); whereas, 19,20-DiHDPA was found associated with the risk of ventricular arrhythmias recently (Zhang et al., 2016).The EETs and EpDPEs were not detected in plasma, but their stable metabolites namely, 11,12-DHET and 19,20-DiHDPA were significantly reduced in endometriosis. Therefore it could be speculated that the level of EETs and EpDPEs were also supressed in endometriosis, nevertheless it needs to be noted that sEH activity was not investigated in these patients.

When comparing data according to the presence or absence of endometriosis, noticeable differences were found in plasma for TXA_2, LTB_4 and also for trans-EKODE, which is produced by non-enzymatic auto-oxidation; therefore it is considered a marker of oxidative stress (Wang et al., 2009). These metabolites were elevated in the plasma taken from endometriosis women compared to those without, suggesting a low grade systemic inflammation in endometriosis patients. It is well established that the low grade inflammation and oxidative stress are cofounding factors for atherosclerosis and cardiovascular diseases (Danesh, 2000; Hansson, 2005; Bonomini et al., 2008). In general, therefore, it seems the present study strengthens the findings of Mu *et al.* (2016), which suggests that endometriosis patients have a higher risk of cardiovascular diseases.

Lipid mediators obtained from the peritoneum and the vascular system demonstrated different profiles, possibly suggesting different pathological processes locally and at the systemic level. One of the main differences was found for the variance of the lipids species across the menstrual cycle. Lipids were elevated in secretory phase compared to proliferative phase in the peritoneal fluid from women without endometriosis (NEM,N). In contrast, the same metabolites in the same group showed the highest levels during the proliferative phase in plasma. In addition, in the peritoneal fluid the most apparent changes were observed for 15-LOX metabolites comparing women with and without endometriosis, however, the expression of these lipids was not altered between the study groups in the plasma. The other pronounced

change in the peritoneal fluid was that nearly all lipids were downregulated in endometriosis compared to non-endometriosis groups. These changes were not observed in plasma. Moreover, the mean values in plasma were similar for the majority of detected lipids.

In summary, variations in the lipid levels in plasma were identified between the study groups. However, clear trends could not be observed with regard to any biosynthetic pathways. As a consequence of this, it is questionable whether these lipids would be ideal biomarkers for the diagnosis of endometriosis. A possible explanation is that several additional factors, e.g. menstrual cycle stage, severity of the disease, presence of other co-morbidities might also cause variation in the lipid profile of plasma; therefore further studies, which take these variables into account, will need to be undertaken.

4 CHAPTER: Pro-inflammatory cytokines in fluid specimens

4.1 Introduction

The pathomechanisms of endometriosis were outlined in section 1.6 and demonstrated that autotriggering positive feedback loops exist to maintain the pro-inflammatory enviroment (Figure 1.6.2). There is a direct relationship between pro-inflammatory cytokines, namely IL-1β and TNF-α, and the local synthesis of prostanoid and oestrogen produced by peritoneal macrophages or ectopic lesions. To facilitate better understanding of the endometriosis-associated inflammatory milieu, pro-inflammatory cytokines were measured in peritoneal fluid, washes and venous plasma from women with and without endometriosis using a sandwich ELISA method.

A total of 64 plasma (EM n= 37; NEM N= 27), 50 peritoneal washes (EM n= 31; NEM n= 19) and 10 (EM n= 3; NEM n= 7) peritoneal fluid specimens were tested. In the case of IL-1β, the concentrations were below the detection limit of the assay for nearly all specimens, therefore the data have not been presented here.

4.2 Results

4.2.1 Normalisation of peritoneal washes

Data obtained from peritoneal washes were normalized to glucose concentration as it was described in section 3.3. In brief, peritoneal fluid was diluted with unknown volume of isotonic buffer during the laparoscopic surgery and collected as peritoneal wash. The concentration of metabolites decreased with dilution. Therefore to estimate the original concentration of analyte of interest a reference metabolite were chosen which concentration was relatively stable in peritoneal fluid. Glucose was chosen for this purpose. The average glucose concentration was 5.4 ± 0.30 mmol/L (mean ± SD, n=10) in peritoneal fluid and this concentration was used as the reference glucose concentration. The glucose concentrations of peritoneal washes were measured as was described in section 2.6. The ratio of reference concentration and the glucose concentration of peritoneal washes were calculated for each sample and used subsequently as normalizing factors. Values measured in peritoneal washes were multiplied with these factors to estimate the original concentration of the analyte of interest in peritoneal fluid. Similarly to MS data analysis, peritoneal washes with higher than normalizing factor of 27 were excluded from the subsequent analysis, leaving 8 and 2 samples in the groups of endometriosis and non-endometriosis respectively.

4.2.2 TNFα in peritoneal fluid

Peritoneal fluid (NEM n= 7; EM n= 3) and normalised peritoneal washes (NEM n= 2; EM n= 8) were included in this analysis. Data were combined and classified according to the stage of menstrual cycle, GnRH treatment and the presence or absence of endometriosis.

Data were grouped according to the stage of menstrual cycle from women with and without endometriosis to increase the numbers of participants for each stage of the cycle. A total of 10 women were compared who reported regular menstrual cycles and had not used hormonal contraceptives or hormonal medications. Three patients were in the menstrual (NEM=0; EM=3), five in the proliferative (NEM=4; EM=1) and two participants were in the secretory (NEM=2; EM=0) phase. No statistical alterations were observed between the stages of menstrual cycle. TNFα showed peaks during the proliferative phase (Figure 4.2.1).

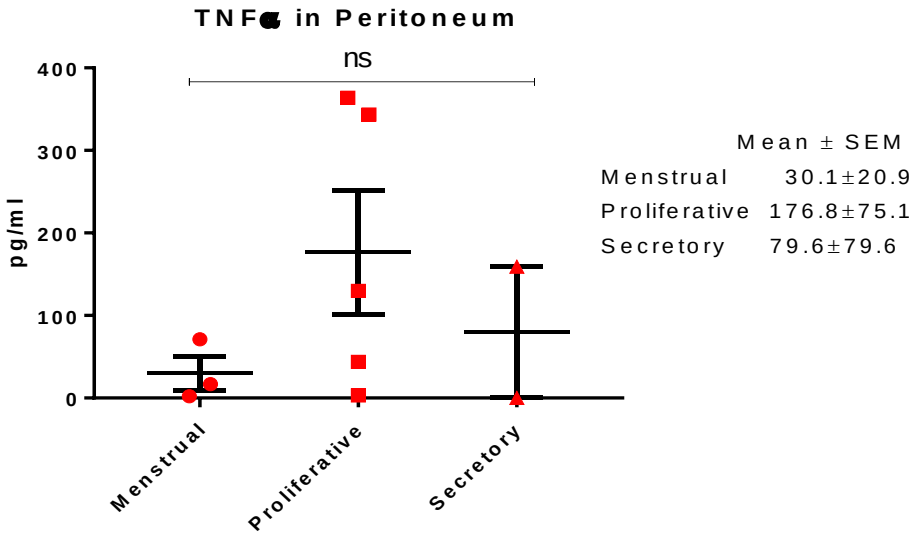


Figure 4.2.1. Concentrations of TNFα in peritoneal fluid from women with and without endometriosis according to the stages of menstrual cycle
(Menstrual n=3; Proliferative n=5; Secretory n=2). Measurements were performed using ELISA method. Dots represent the TNFα concentration (pg/ml) of the tested individuals. Data are expressed as arithmetic means of metabolite (pg/ml) ± SEM. Statistical comparisons were performed using Kruskal-Wallis test with Dunn's post-hoc test. (ns) not significant

Statistical differences were also not observed comparing data according to GnRH agonist treatment. TNFα concentrations were the lowest in the group of endometriosis women with regular cycles, without hormonal contraceptives or taking hormonal medications, and were the highest in the group of endometriosis with GnRH agonist treatment (Figure 4.2.2).

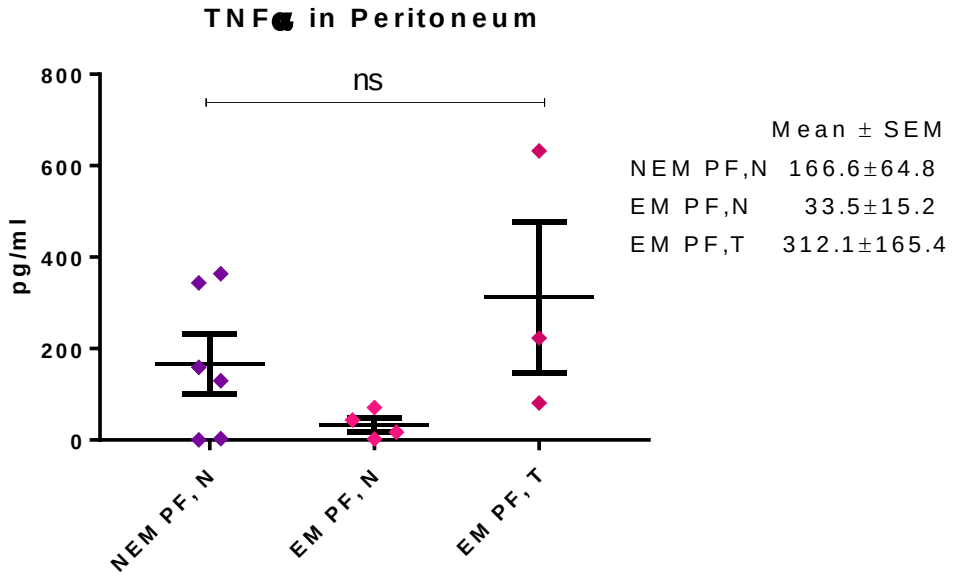


Figure 4.2.2. Concentrations of TNFα in peritoneal fluid according to medical treatment. Concentrations of TNFα in peritoneal fluid (PF) from non-endometriosis (NEM,N; n=6) women with regular menstrual cycles and without hormone treatments or hormonal contraceptives were compared to endometriosis patients with regular cycles, without hormonal pharmacotherapy (EM,N; n=4) and amenorrhoeal endometriosis patients using GnRH agonists (EM,T; n=3). Measurements were performed using ELISA method. Dots represent the TNFα concentration (pg/ml) of the tested individuals. Data are expressed as arithmetic means of metabolite (pg/ml) ± SEM. Statistical comparisons were performed using one-way ANOVA with Tukey post-hoc test. (ns) not significant

The comparison of endometriosis and non-endometriosis groups was also not statistically different. TNFα was elevated in peritoneal fluid from women diagnosed with endometriosis. Figure 4.2.3 illustrates the TNFα concentrations in peritoneal specimens.

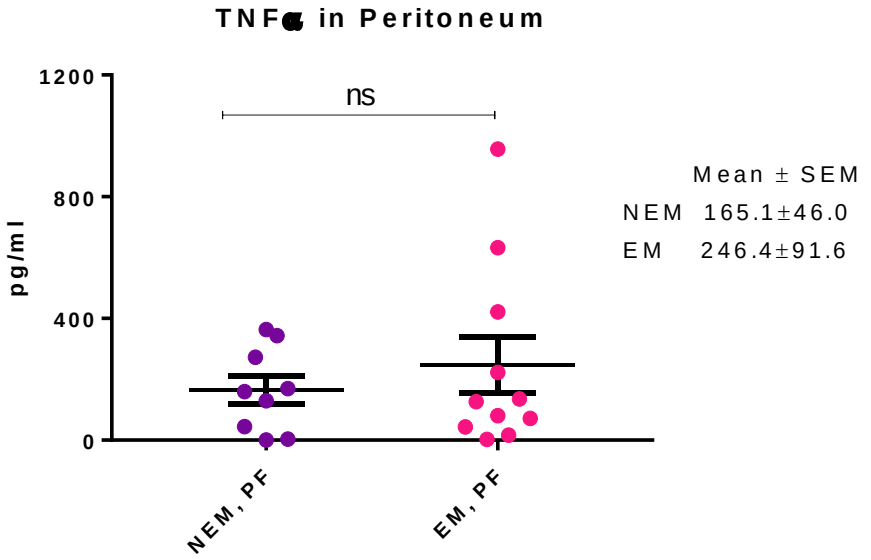


Figure 4.2.3. Concentrations of TNFα in peritoneal fluid from women with and without endometriosis.
Concentrations of TNFα in peritoneal fluid (PF) from women not diagnosed with endometriosis (NEM,PF n=9) were compared to women diagnosed with endometriosis (EM,PF n=11). Measurements were performed using ELISA method. Dots represent the TNFα concentration (pg/ml) of the tested individuals. Data are expressed as arithmetic means of metabolite (pg/ml) ± SEM. Statistical comparisons were performed using Mann-Whitney U-test. (ns) not significant

4.2.3 TNFα in plasma

A total of 64 venous plasma samples (NEM n= 27; EM n= 37) were included in this analysis. Comparisons were performed according to the stage of menstrual cycle, medical treatment and presence or absence of endometriosis.

Data were grouped according to the stage of menstrual cycle from women with and without endometriosis, to obtain sufficient numbers of participants for each stage of the cycle. A total of 25 women were included in this analysis, who reported regular menstrual cycles and have not used hormonal contraceptives or hormonal medications. Five patients were in menstrual (NEM=2; EM=3), 10 in proliferative (NEM=5; EM=5) and 11 participants were in secretory (NEM=6; EM=5) phase. Although the concentration of TNFα did not show significant alteration with the menstrual cycle, an increasing trend from the menstrual phase to the secretory phase was observed (Figure 4.2.4).

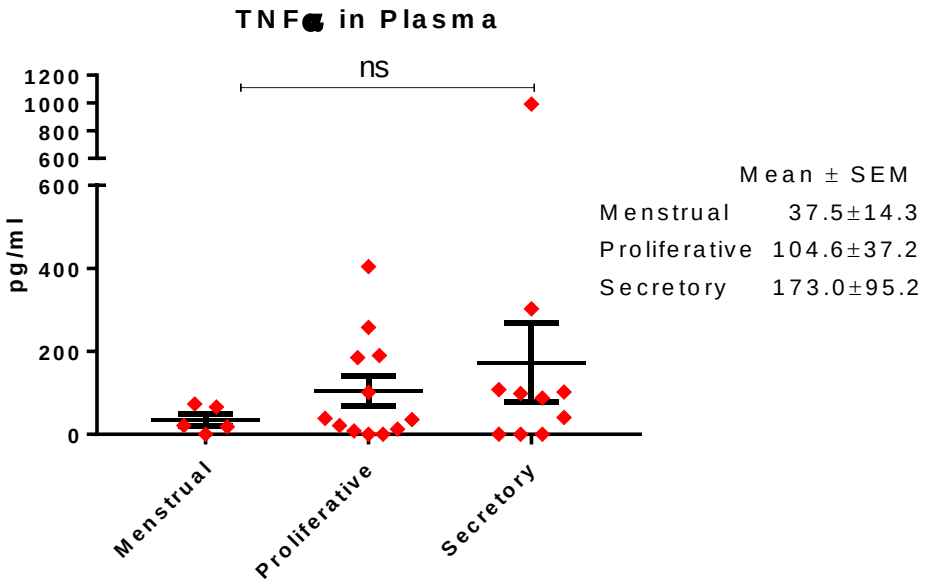


Figure 4.2.4. Concentrations of TNFα in plasma from women with and without endometriosis according to the stages of menstrual cycle.
(Menstrual n=5; Proliferative n=10; Secretory n=11). Measurements were performed using ELISA method. Dots represent the TNFα concentration (pg/ml) of the tested individuals. Data are expressed as arithmetic means of metabolite (pg/ml) ± SEM. Statistical comparisons were performed using Kruskal-Wallis test with Dunn's post-hoc test. (ns) not significant

On comparing and grouping the data according to GnRH agonist treatment, an increasing trend was observed for the three study groups. The TNFα concentrations were the lowest in the group of non-endometriosis women with regular cycles, without hormonal contraceptives or taking hormonal medications; were increased in the group of endometriosis with regular cycles, without GnRH agonist treatment; and was the highest in the group of endometriosis with GnRH agonist treatment (Figure 4.2.5).

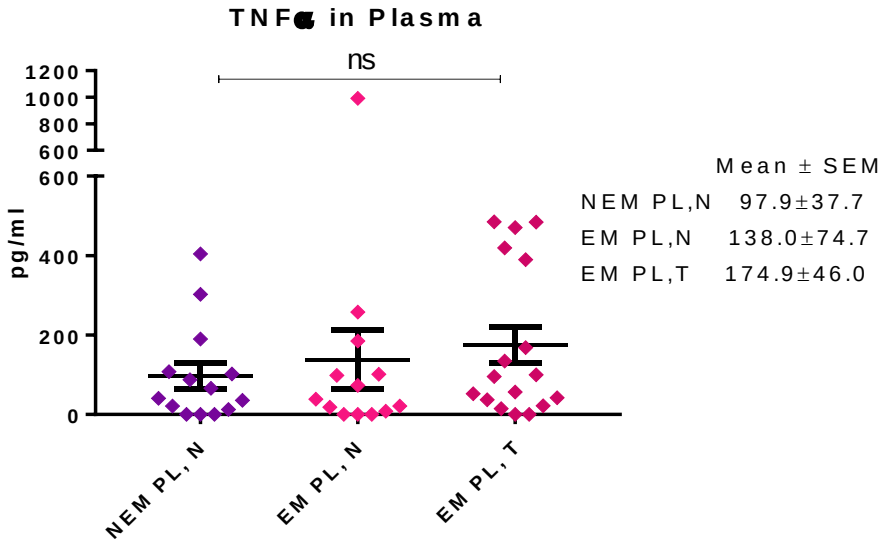


Figure 4.2.5. Concentrations of TNFα in plasma.
Concentrations of TNFα in plasma (PL) from non-endometriosis (NEM,N; n=14) women with regular menstrual cycles and without hormone treatments or hormonal contraceptives were compared to endometriosis patients with regular cycles, without hormonal pharmacotherapy (EM,N; n=13) and amenorrhoeal endometriosis patients using GnRH agonists (EM,T; n=17) Measurements were performed using ELISA method. Dots represent the TNFα concentration (pg/ml) of the tested individuals. Data are expressed as arithmetic means of metabolite (pg/ml) ± SEM. Statistical comparisons were performed using Kruskal-Wallis test with Dunn's post-hoc test. (ns) not significant

The comparison according to the presence or absence of endometriosis did not reveal statistically significant differences. The mean concentrations were found similar for the disease (EM 156.5 ± 45.2, mean ± SEM) and non-disease groups (NEM 148.6 ± 34.9, mean ± SEM) (Figure 4.2.6).

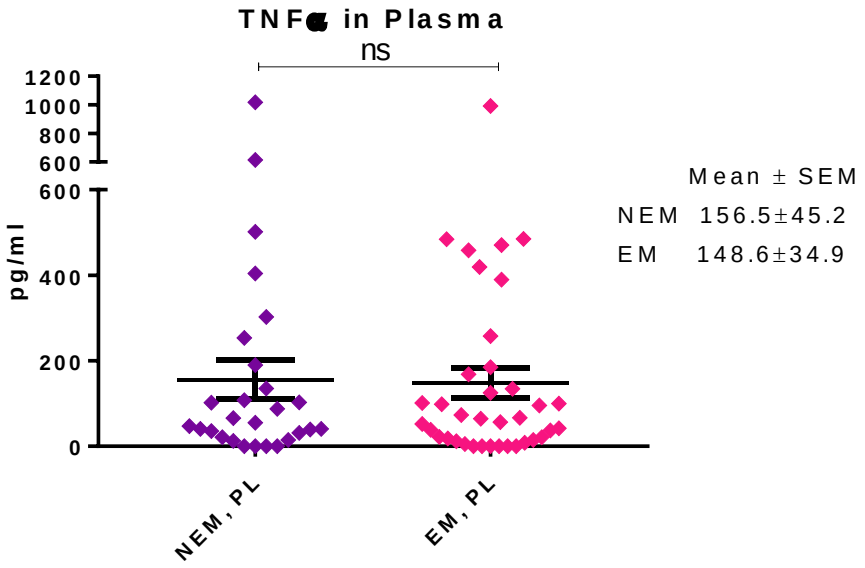


Figure 4.2.6. Concentrations of TNFα in plasma.
Concentrations of TNFα in plasma (PL) from women not diagnosed with endometriosis (NEM,PL n=27) were compared to women diagnosed with endometriosis (EM,PL n=37). Measurements were performed using ELISA method. Dots represent the TNFα concentration (pg/ml) of the tested individuals. Data are expressed as arithmetic means of metabolite (pg/ml) ± SEM. Statistical comparisons were performed using Mann-Whitney U-test. (ns) not significant

4.3 Discussion

4.3.1 TNFα in peritoneal fluid

Cytokines are secreted proteins in the immune system with a pivotal role in the regulation of immune responses. The biological properties of cytokines determine the nature of immune responses. Inflammation is a key process in the pathophysiology of endometriosis (Donnez, 2012). The endometriosis-associated inflammatory response depends on the secretory products of peritoneal cells, including macrophages, granulocytes and mesothelial cells. TNFα and IL-1β are the main pro-inflammatory cytokines leading the initiation of the immune response upon pathogenic invasion or tissue damage. These cytokines are predominantly produced by activated macrophages but are also secreted by other leukocytes such as neutrophil granulocytes, natural killer cells or other cell types such as fibroblasts, smooth muscle cells, endothelial cells or even endometrial cells (Manolova et al., 2011). There are complex relationships between cytokines and lipid mediators. Cytokines could regulate the expression of lipid pathways, e.g. IL-1β and TNFα up-regulate COX-2 (Wu et al., 2002), whilst lipid mediators are pivotal in the proliferation

and differentiation of immune cells. For instance PGE_2 is crucial in T cell differentiation and activation (Kalinski, 2012).

TNFα and IL-1β were measured in the peritoneal fluid samples from women with and without endometriosis. IL-1β concentrations were below the detection limit of the assay for nearly all samples therefore a conclusion cannot be drawn. Likely explanations are that these cytokines were not present in the specimens or that, due to dilution of the samples, the sensitivity of the assay was not sufficient for this purpose.

TNFα was in the detection range of the assay. Firstly, data were compared according to the stages of menstrual cycle. TNFα was elevated in the proliferative phase and decreased in menstrual and secretory phases (Figure 4.2.1). This finding is not in agreement with previous reports. Studies suggest that TNFα directly stimulates ovarian steroidogenesis (Adashi, 1990; Fukuoka et al., 1992) and its concentration correlates with the stage of menstrual cycle. Cheong *et al.* found that TNFα concentration in the peritoneal fluid significantly increases from the proliferative phase to the secretory phase (Cheong et al., 2002). The low number of participants might provide an explanation to this contradiction, since only two participants were in secretory stage.

Secondly, data were compared according to the GnRH agonist treatment. Since TNFα correlates with the menstrual cycle, a depleted TNFα concentration was expected in amenorrhoeal endometriosis women using GnRH agonists. However, the finding was in contradiction with the expectation and previous reports. The statistical comparison did not revealed significant difference between the study groups. Moreover, TNFα concentration was found to be highest in the GnRH treatment group (Figure 4.2.2). Ho *et al.* investigated the effect of 6 months of GnRH treatment on the TNFα concentration in the peritoneal fluid from women with endometriosis and found that GnRH treatment downregulated TNFα (Ho et al., 1996). Again, it is important to point out that only three and four participants were included in the non-treated endometriosis and in the GnRH treated endometriosis groups, respectively. In addition, information was not available about the course and the length of the GnRH agonist treatment.

Finally, data were compared according to the presence or absence of endometriosis (Figure 4.2.3). As was mentioned above, TNFα promotes steroidogenesis, therefore it could be speculated that the increased levels of TNFα in the peritoneum might promote the proliferation and steroidogenesis of the endometrial ectopic lesions.

Previous studies have reported contradictory findings with regard to the TNFα concentration in the peritoneal fluid from endometriosis patients (May et al., 2010). For example, Keenan *et al.* (1995) reported significantly increased TNFα concentrations in the peritoneal fluid, whereas Kalu *et al.* (2007) did not observe such an alteration (Keenan et al., 1995; Kalu et al., 2007). In the present study elevated TNFα concentrations were found in the peritoneal fluid from women diagnosed with endometriosis. Although the statistical analysis did not reveal significant differences between endometriosis and non-endometriosis groups, the elevated TNFα concentration might suggest a moderate pro-inflammatory environment in the peritoneum for endometriosis women.

Furthermore, overexpression of TNFα in the peritoneal fluid may be a cofounding factor for the formation of pelvic adhesions. Endometriosis is frequently accompanied by pelvic adhesions causing chronic pelvic pain and infertility (Viganò et al., 2004). Elevated TNFα concentrations were demonstrated in endometriosis women with adhesions, when compared to endometriosis women without adhesions (Cheong et al., 2002). Although it was not possible to correlate TNFα results with the patient data in all cases in this study, it is interesting that the highest concentration of TNFα was measured in patient HP08 who had moderate endometriosis with extended adhesions. That patient had had five previous laparoscopies where extensive inta-abdominal adhesions were found and the indication of the current surgery was also the division of adhesions.

4.3.2 TNFα in plasma

The TNFα concentration was measured in plasma from women with and without endometriosis to gain information about the systemic inflammatory status of the patients.

Data were assessed according to the stages of the menstrual cycle (Figure 4.2.4) GnRH treatment (Figure 4.2.5) and presence or absence of endometriosis (Figure 4.2.6). Significant differences were not observed between the study groups. Although statistical alterations were not found, the comparison of the concentration of TNFα according to medical treatment demonstrated elevated TNFα levels in the treated and non-treated endometriosis groups compared to non-endometriosis group. In addition, the TNFα level showed variation across the menstrual cycle which gradually increased from the menstrual phase to the secretory phase. This latter finding is consistent with the literature. TNFα influences cell differentiation and tissue remodelling and is an important factor in the preparation of the endometrium for

menstruation. For this reason, the TNFα level gradually increases from the beginning to the end of the cycle in the endometrium and in the plasma (Philippeaux and Piguet, 1993; O'Brien et al., 2007).

As was mentioned above, slight variations were observed between the study groups for TNFα concentration in plasma when the results were analysed according to medical treatment. Since TNFα correlates with the menstrual cycle those results also could be explained by the menstrual cycle changes rather than endometriosis. In conclusion the alteration of TNFα is a good illustration of how the hormonal changes during the menstrual cycle could affect the outcome of the study.

5 CHAPTER: The role of peroxisome proliferator-activated receptor γ (PPARγ) in the pathomechanism of endometriosis

5.1 Introduction

An increasing number of studies have investigated the role of PPARγ in the pathomechanism of endometriosis. Animal studies reported that synthetic PPARγ agonists significantly reduced the numbers and size of endometrial-like lesions in rat and baboon models (Lebovic et al., 2004, 2010; Aytan et al., 2007). Thiazolidinediones (TZDs) also suppressed peritoneal inflammation in a mouse model by downregulating the secretion of the chemotactic RANTES and the pro-inflammatory IL-1β (Hornung et al., 2003). In addition, further studies revealed that activation of PPARγ decreased the attachment of endometrial cells to the mesothelial cells and inhibited the proliferation of endometrial cells by suppressing oestrogen synthesis and PGE_2 signalling via downregulation of P450 aromatase and EP2 and EP4 receptor expression, respectively in *in vitro* models (Kavoussi et al., 2009; Lebovic et al., 2013).

PPARγ is a ligand-dependent transcripton factor which directly regulates gene expression. The PPARγ-mediated upregulation of CD36 on monocytes is well established (Nagy et al. 1998). CD36 was found to be downregulated in peritoneal macrophages from women with endometriosis (see section 1.6.1). However, Chuang *et al.* demonstrated that PGE_2 promoted the downregulation of CD36 in peritoneal macrophages from endometriosis patients (Chuang et al., 2010); the exact mechanism has not been elucidated yet and the possible role of PPARγ in this process has not been investigated so far.

The current study quantified oxygenated lipid mediators in the peritoneum. As was decribed in section 1.6.5, some of the detected lipds, particularly 15-LOX and COX metabolites (15d-PGJ_2, HETEs, HODEs) are potential endogenous ligands of PPARγ. Since PPARγ is a ligand-dependent transcripton factor, alterations in the levels of endogenous agonists maydisturb the function of PPARγ, resulting in aberrant gene expression which might further contribute to the establishment or progression of endometriosis.

For this reason, one of the main objective of this thesis was to investigate the gene and protein expression of COX-2, 15-LOX-1, PPARγ and CD36 in biological specimens from women with and without endometriosis, to gain insight into the role of PPARγ in the pathomechanism of endometriosis. Furthermore, since endometrial lesions vary morphologically and histologically (see section 1.2.2 and 1.2.3), glandular and stromal markers, namely cytokeratin and interferon-induced transmembrane protein

1 (IFITM-1) were also assessed to confirm the presence of endometrial-like gland and stromal compartments in the ectopic lesions.

5.2 Gene expression study of endometriosis

Quantitative real time PCR assays were performed to quantify the expression of genes of interest in eutopic endometrium, peritoneal cells and blood from women with and without endometriosis, and in ectopic lesions and peritoneal wall specimens from women with endometriosis. Gene expression of cytokeratin 8, IFITM-1, COX-2, 15-LOX-1, 15-LOX-2, PPARγ, CD36 and GAPDH were measured using SYBR Green method.

5.2.1 Participant characteristics for gene expression study

A total of 63 individuals were involved in this study; 36 women were diagnosed with endometriosis, 27 patients with other gynaecological disorders. The menstrual cycle was regular in the cases of 15 (41.7%) women and 17 (63.0%) women with and without endometriosis, respectively. 23 out of 36 patients received hormone treatment for endometriosis or taken contraceptive pills compared to only one woman in the non-endometriosis group. Table 5.2.1 summarizes the participant characteristics and their stage of the menstrual cycle whereas Table 5.2.2 summarizes the specimen from patients were used for the gene expression study.

Table 5.2.1. Summary of participant characteristics for gene expression study. Women with (EM) and without (NEM) were recruited for this study. Hormone usage illustrates the numbers of participants who taken hormone as a treatment or contraception. (N) numbers of participants

		Age (years)	Stage of Menstrual cycle					Hormone usage
	N	mean ± SD	Non-cycling	Non-regular	Regular			
EM	**36**	34.3 ± 8.8	20 (55.5%)	1 (2.8%)	15 (41.7%)	Menstrual: Proliferative: Secretory:	3 (0.2%) 7 (20.0%) 5 (33.3%)	23 / 36 (63.9%)
NEM	**27**	38.3 ± 9.0	3 (11.1%)	7 (25.9%)	17 (63.0%)	Menstrual: Proliferative: Secretory:	2 (11.8%) 9 (52.9%) 6 (35.3%)	1 / 27 (3.7%)

Table 5.2.2. Summary of tissue specimens used for gene expression study. Women with (EM) and without (NEM) were recruited for this study and tissue specimens were collected during the laparoscopic surgery. (R) regular menstrual cycle; (NR) non-regular menstrual cycle

	Endometrium		Ectopic lesion		Peritoneal wall		Peritoneal cells		Blood	
EM	27	R: 8 NR:19	14	R: 6 NR:8	8	R: 1 NR: 7	7	R: 3 NR:4	13	R: 3 NR:10
NEM	24	R: 14 NR:10	-		-		8	R: 6 NR:2	7	R: 4 NR:3

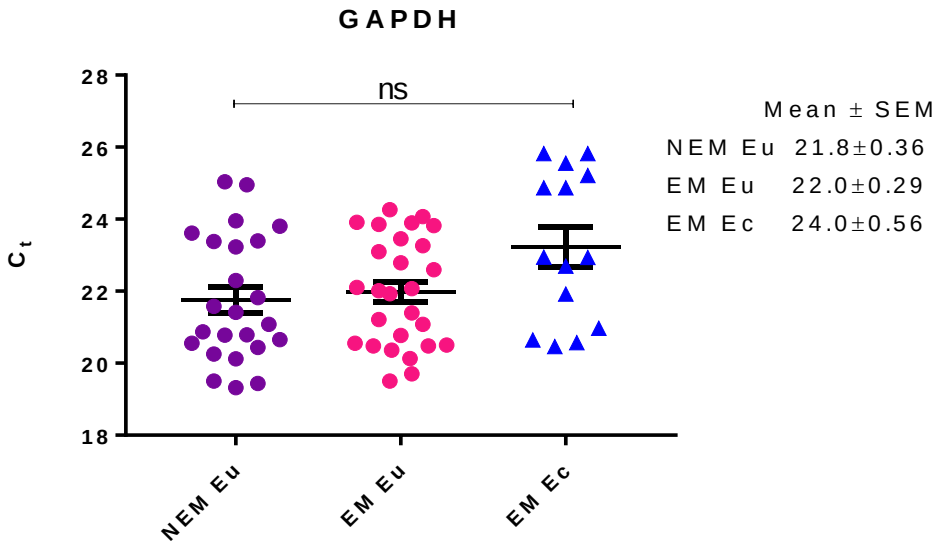
5.2.2 Gene expression study of eutopic endometrium and ectopic lesions

Eutopic endometrium from women with and without endometriosis and ectopic lesions from endometriosis patients were investigated. A total of 51 eutopic endometrial samples were used, involving 27 specimens from women with endometriosis and 24 endometrium from women without endometriosis. Eight out of 27 in the group with endometriosis and 14 out of 24 patients in the group without endometriosis had regular menstrual cycles and did not receive hormone treatments or use hormonal contraceptives. 14 ectopic lesions from endometriosis patients were also studied. Six patients had normal menstrual cycle whereas eight woman had amenorrhoea due to medical treatments. In eight cases the ectopic lesions and eutopic endometrium were paired since they were obtained from the same patients.

5.2.2.1 Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)

Firstly, the expression of GAPDH were compared between the studied specimens. Figure 5.2.13.5.1 demonstrates the Ct values and the relative mRNA concentrations of GAPDH in eutopic endometrium and ectopic lesions. The expression of GAPDH was not significantly different between the three study groups. Neither the Ct values, nor the relative mRNA concentrations showed significant alterations for GAPDH. The means of Ct values were nearly identical in eutopic endometrium from women with (mean EM ± SD= 22.0 ± 1.51) and without endometriosis (mean NEM ± SD= 21.8 ± 1.75). The Ct values of ectopic lesions were elevated (mean EC ± SD= 23.2 ± 2.09) compared to eutopic endometrium, nevertheless the changes have not reached statistical significance. The relative mRNA concentrations of GAPDH also were not statistically different although, it was decreased in ectopic lesions compared to eutopic endometrium. As GAPDH was appointed by RefFinder (see section 2.3.9) as the most stable gene between the tested genes and its expression was not significantly altered between the study groups and specimens GAPDH was subsequently used to calculate the relative gene expression of the genes of interest.

(A)



(B)

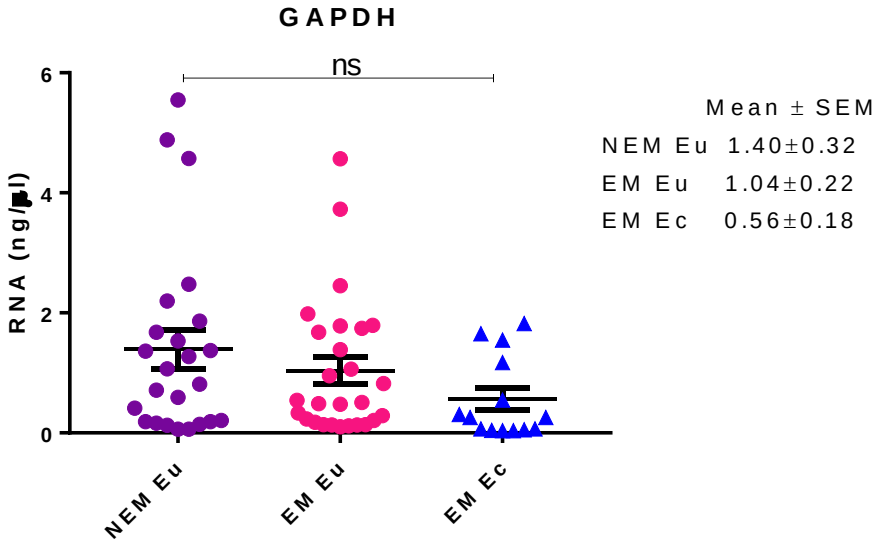


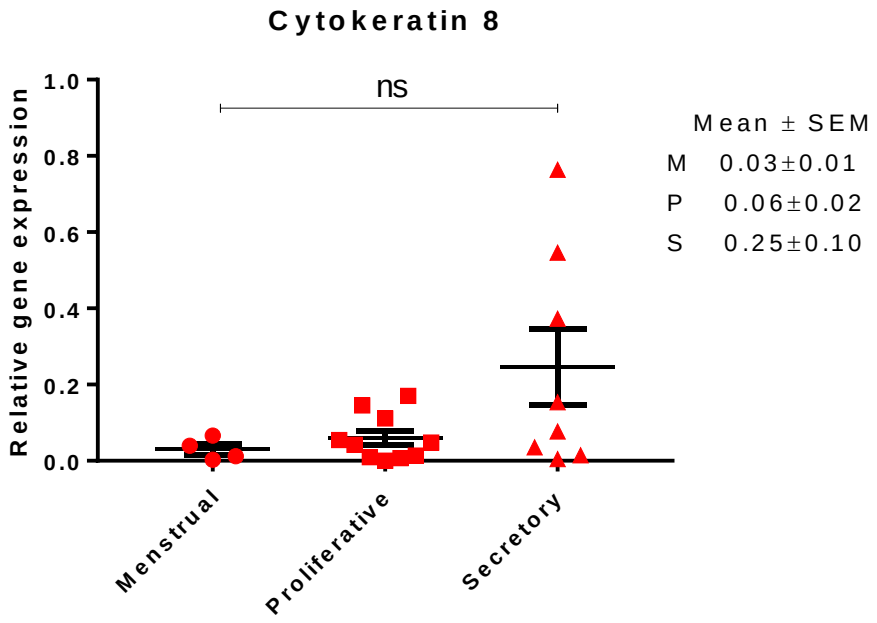
Figure 5.2.1. Gene expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) quantified in eutopic endometrium from women not diagnosed with endometriosis (NEM Eu, n=24), women diagnosed with endometriosis (EM Eu, n=27) and in ectopic lesions (EM EC, n=14) from women with endometriosis. Assays were performed using the SYBR Green qRT-PCR method normalized to 360ng total RNA. (A) Threshold cycle (Ct) values of GAPDH. (B) Relative mRNA concentration of GAPDH (ng/µl). RNA concentrations were determined by interpolated Ct values of unknown from standard curve of calibrator 1 using 4-parameter non-linear regression model. Data are expressed as mean ± SEM. Statistical comparisons were performed using Kruskal-Wallis test with Dunn's post-hoc test. (ns) not significant

5.2.2.2 Variation in gene expression in eutopic endometrium by menstrual cycle

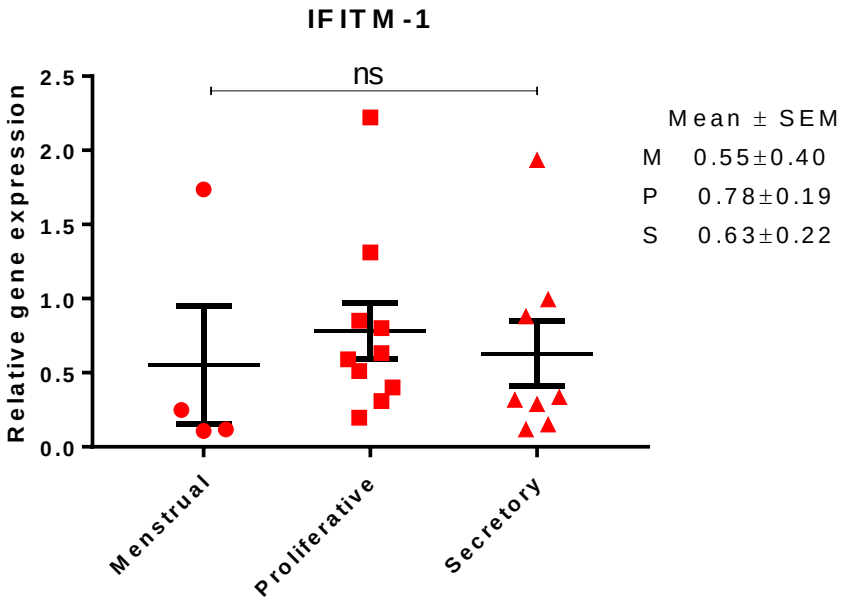
Gene expression was also investigated in eutopic endometrium according to stage of menstrual cycle. Data were grouped by the stage of menstrual cycle from women with and without endometriosis who reported regular menstrual cycle and were not using hormonal contraceptives. A total of 22 women were included in this analysis. Four of women were in the menstrual (NEM=2; EM=2), 10 in the proliferative (NEM=7; EM=3) and 8 in the secretory (NEM=5; EM=3) stage. Figure 5.2.2 illustrates the variation of gene expression according to stage of menstrual cycle.

Genes of interest were not statistically altered with the menstrual cycle. However, some trends could be observed. Cytokeratin 8 expression was gradually increased from the menstrual phase to the secretory phase. The alterations of IFITM-1 were not as obvious as cytokeratin 8, but showed elevation in the proliferative phase. COX-2 and 15-LOX-1 showed consistent expression through the stages of cycle. PPARγ was increased from the menstruation stage to the proliferative stage and stayed elevated during the secretory phase, whereas CD36 expression was low in the first two stages of the cycle but was upregulated in the secretory phase.

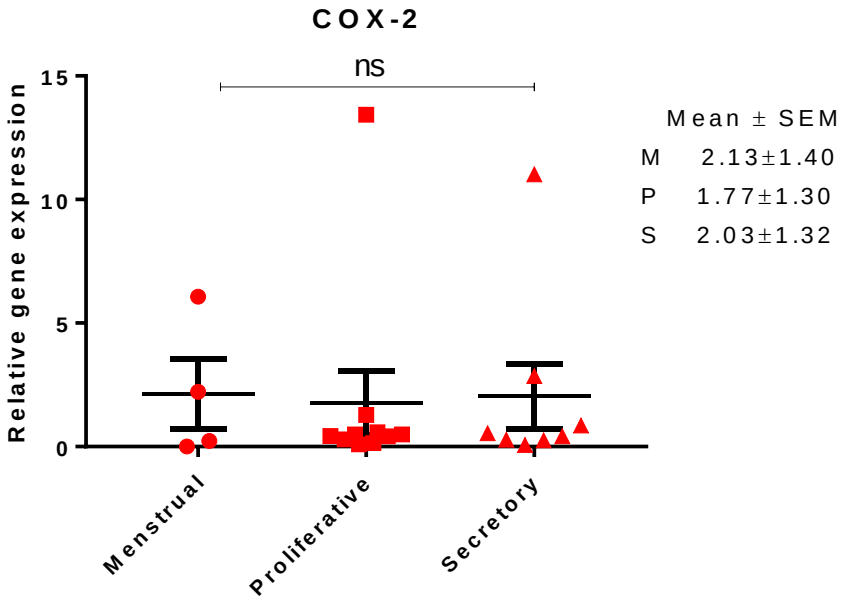
(A)



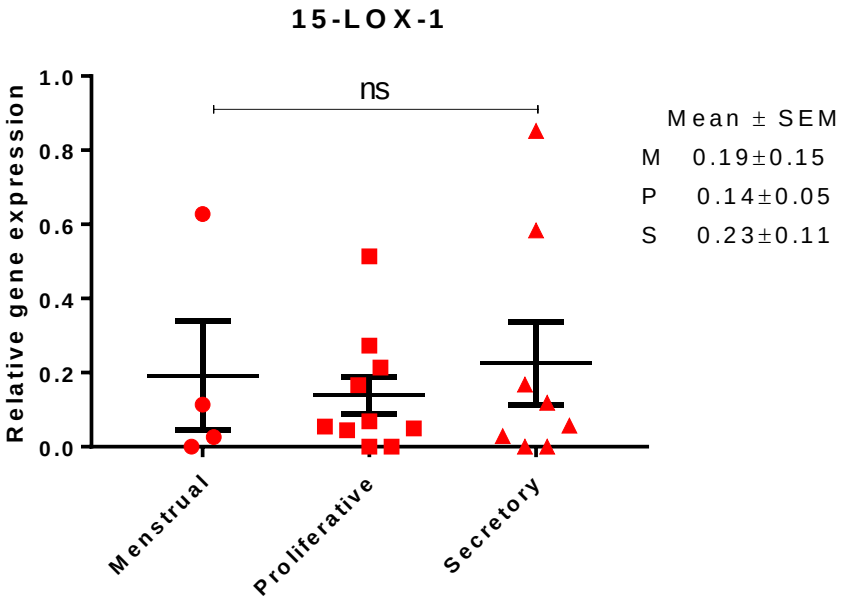
(B)


IFITM-1
ns
Relative gene expression
2.5
2.0
1.5
1.0
0.5
0.0
Menstrual
Proliferative
Secretory
Mean ± SEM
M 0.55±0.40
P 0.78±0.19
S 0.63±0.22

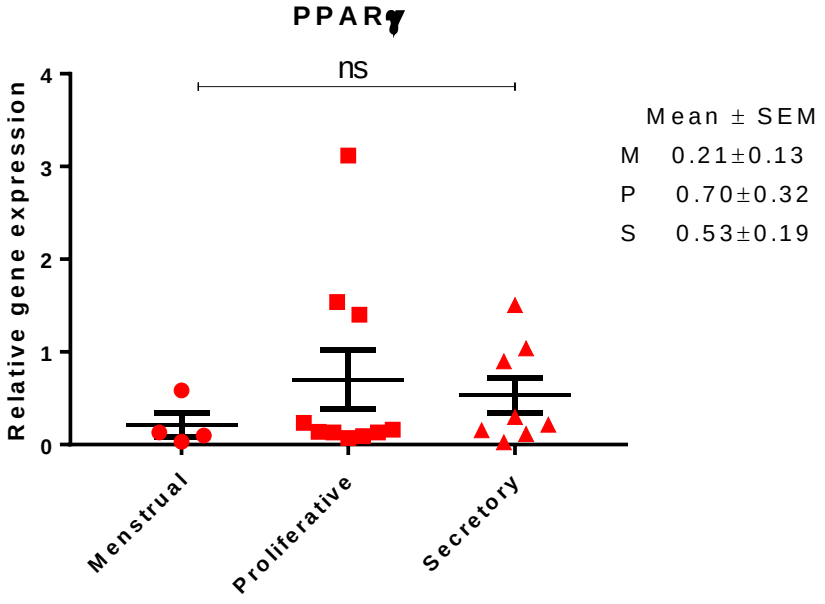
(C)


COX-2
ns
Relative gene expression
15
10
5
0
Menstrual
Proliferative
Secretory
Mean ± SEM
M 2.13±1.40
P 1.77±1.30
S 2.03±1.32

(D)


15-LOX-1
Relative gene expression
1.0
0.8
0.6
0.4
0.2
0.0
ns
Menstrual
Proliferative
Secretory
Mean ± SEM
M 0.19±0.15
P 0.14±0.05
S 0.23±0.11

(E)


PPARγ
Relative gene expression
4
3
2
1
0
ns
Menstrual
Proliferative
Secretory
Mean ± SEM
M 0.21±0.13
P 0.70±0.32
S 0.53±0.19

(F)

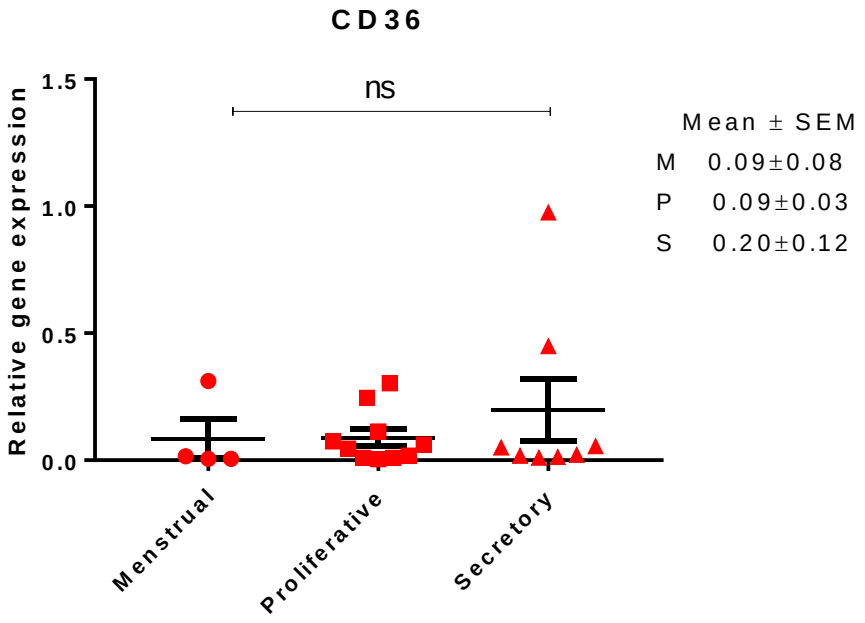


Figure 5.2.2. Gene expression in eutopic endometrium from women with and without endometriosis according to the stages of menstrual cycles.
(Menstrual n=4; Proliferative n=10; Secretory n=8) (A) Cytokeratin 8(CK8); (B) Interferon induced transmembrane protein 1(IFITM-1); (C) Cyclooxygenase-2 (COX-2); (D) 15-lipoxygenase (15-LOX); (E) Peroxisome proliferator-activated receptor gamma (PPARγ) (F) CD36. Assays were performed using the SYBR Green qRT-PCR method normalized to 360ng total RNA. mRNA concentrations of targeted genes were determined by interpolated Ct values of unknowns from standard curve of calibrators 1 and 2 using 4-parameter non-linear regression model. Subsequently mRNA concentrations were used to calculate relative gene expression and normalized to GAPDH endogenous reference gene. Data are expressed as mean ± SEM. Statistical comparisons were performed using Kruskal-Wallis test with Dunn's post-hoc test. (ns) not significant

5.2.2.3 Cytokeratin 8

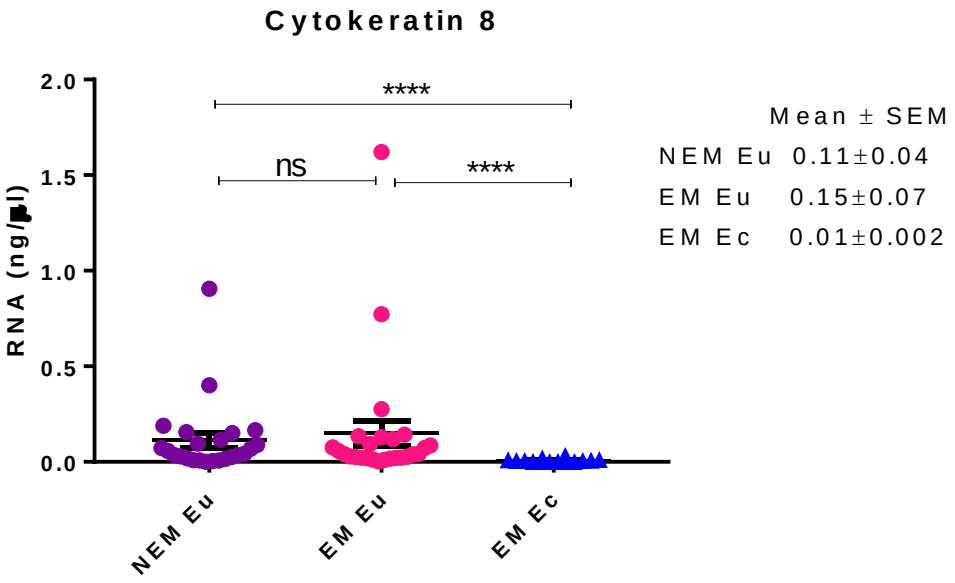
Cytokeratin is a widely used maker of glandular epithelium (Rekhtman and Bishop, 2011). Cytokeratin 8 was used to prove the presence of glandular cells in ectopic lesions. Figure 5.2.3 shows the relative mRNA concentrations and relative gene expression of cytokeratin 8 in eutopic endometrium and ectopic lesions.

As was expected, cytokeratin 8 was highly expressed in eutopic endometrium. The expression in endometrium was very similar and was no significant difference between the groups with endometriosis and without endometriosis. In contrast, the mRNA concentration of cytokeratin 8 in ectopic lesions was strongly depleted (****$p<0.0001$) compared to eutopic endometrium from women with endometriosis. The normalised gene expression data also confirmed a significantly lower expression of cytokeratin 8 in ectopic lesions. The expression was 6-fold lower in ectopic lesions compared to eutopic endometrium form women with endometriosis (Figure 5.2.3B).

The difference lost significance when eutopic endometrium from women with and without endometriosis with regular cycles was compared to ectopic lesions from endometriotic women with normal menstrual cycles (Figure 5.2.4A). The comparison of eutopic endometrium and ectopic lesions from women with endometriosis by menstrual cycle and hormone treatment also not revealed significant alterations for cytokeratin 8 expression. Interestingly, the gene expression of cytokeratin 8 was elevated in eutopic endometrium and ectopic lesions in the group of patients who received hormone treatment for endometriosis (Figure 5.2.4B).

The comparison of cytokeratin 8 expression in paired eutopic endometrium and ectopic lesions showed the same results as was described above namely the cytokeratin 8 expression was lower in ectopic lesions than in eutopic endometrium (Figure 5.2.5).

(A)



(B)

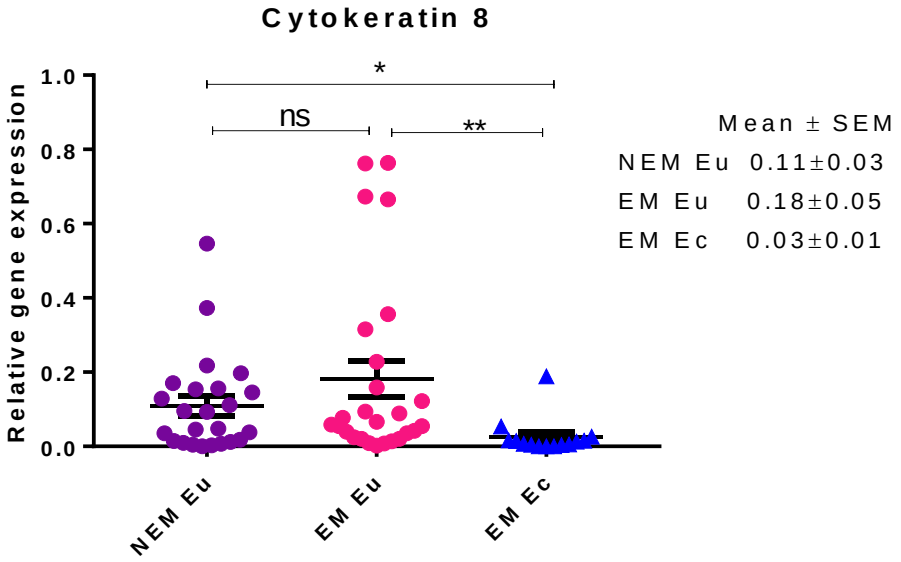
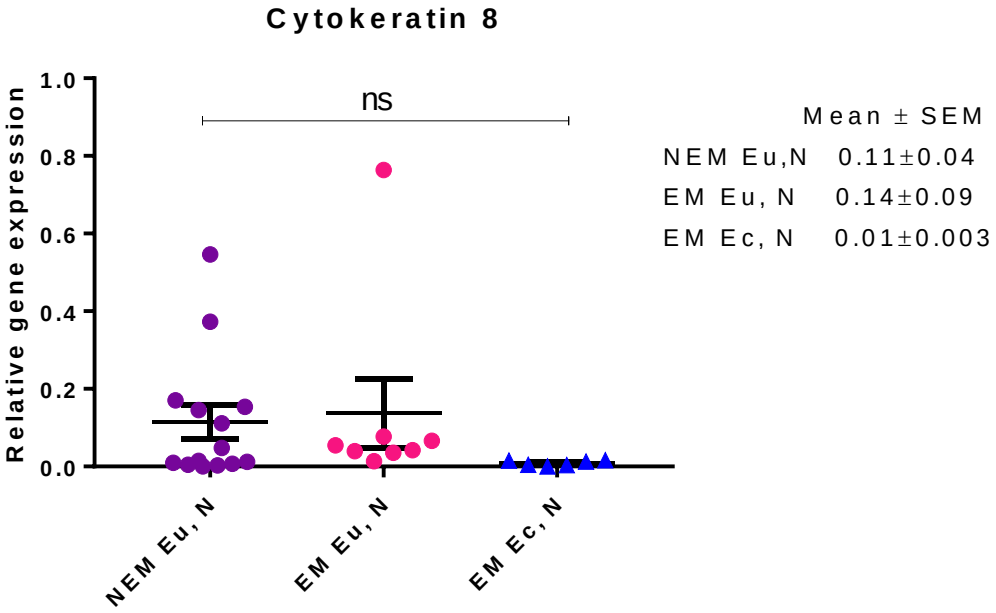


*Figure 5.2.3. Gene expression of cytokeratin 8 quantified in eutopic endometrium from women not diagnosed with endometriosis (NEM Eu, n=24), women diagnosed with endometriosis (EM Eu, n=27) and in ectopic lesions (EM Ec, n=14) from women with endometriosis. Assays were performed using the SYBR Green qRT-PCR method normalized to 360ng total RNA. (A) mRNA concentration of cytokeratin 8 (ng/µl). RNA concentrations were determined by interpolated Ct values of unknowns from standard curve of calibrator 2 using 4-parameter non-linear regression model. (B) Relative gene expression normalized to GAPDH endogenous reference gene. Data are expressed as mean ± SEM. Statistical comparisons were performed using Kruskal-Wallis test with Dunn's post-hoc test. (ns) not significant; *p<0.05; **p<0.01; ****p<0.0001*

(A)



(B)

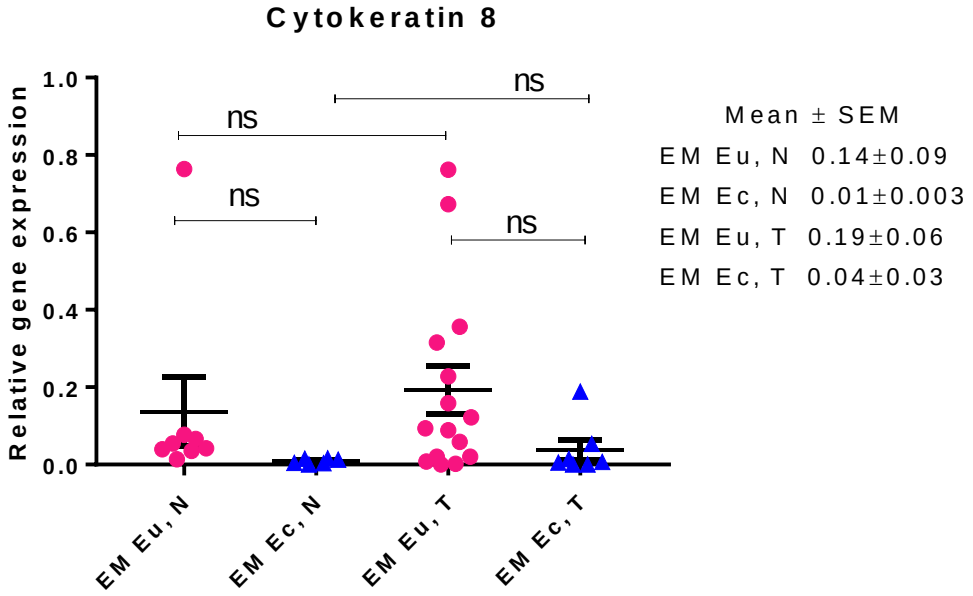


Figure 5.2.4. Relative gene expression of cytokeratin 8 in eutopic endometrium and ectopic lesions with regular menstrual cycle and GnRH treatment.
(A) Comparisons of cytokeratin 8 gene expression in eutopic endometrium from premenopausal women not diagnosed with endometriosis (NEM Eu N, n=14), premenopausal women diagnosed with endometriosis (EM Eu N, n=8) and in ectopic lesions) from premenopausal women with endometriosis (EM Ec N, n=6). All participants had regular menstrual cycles and did not take hormonal contraceptives or received hormone treatments. (B) Comparisons of cytokeratin 8 gene expression according GnRH agonist treatment. Eutopic endometrium and ectopic lesions from endometriosis patients with regular cycles, without hormonal treatments or contraceptives (EM Eu N, n=8; EM Ec N, n=6) were compared to endometriosis patients in amenorrhoea receiving GnRH agonist treatments for endometriosis

(EM Eu T, n=15; EM Ec T, n=7) Assays were performed using the SYBR Green qRT-PCR method normalized to 360ng total RNA. mRNA concentrations of cytokeratin 8 were determined by interpolated Ct values of unknowns from standard curve of calibrator 2 using 4-parameter non-linear regression model. Subsequently mRNA concentrations were used to calculate relative gene expression and normalized to GAPDH endogenous reference gene. Data are expressed as mean ± SEM. Statistical comparisons were performed using Kruskal-Wallis test with Dunn's post- hoc test. (ns) not significant

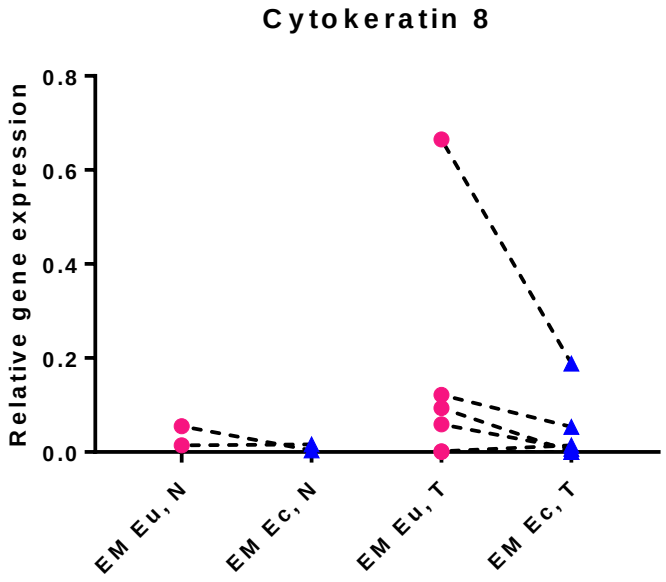


Figure 5.2.5. Relative gene expression of cytokeratin 8 in paired specimens from women with endometriosis. Eutopic endometrium (Eu) and ectopic lesions (Ec) biopsies were obtained from the same individuals. Dotted lines indicates the matched eutopic and ectopic specimens. Data were grouped according to medical treatment such as endometriosis patients with regular cycles without hormonal treatments or contraceptives (EM Eu N; EM Ec N, n=2) and endometriosis patients with GnRH agonist treatment (EM Eu T; EM Ec T, n=6). Assays were performed using the SYBR Green qRT-PCR method normalized to 360ng total RNA. mRNA concentration of cytokeratin 8 were determined by interpolated Ct values of unknowns from standard curve of calibrator 2 using 4-parameter non-linear regression model. Subsequently mRNA concentrations were used to calculate relative gene expression and normalized to GAPDH endogenous reference gene.

5.2.2.4 Interferon induced transmembrane protein 1 (IFITM-1)

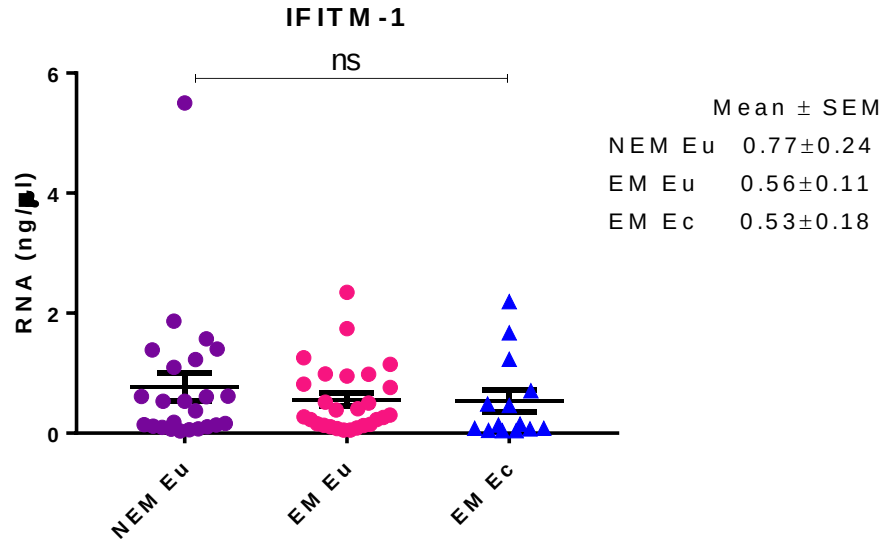
IFITM-1 has been recently described as a potential marker of endometrial stroma (Parra-Herran et al., 2014).

IFITM-1 was detected in eutopic endometrium from women with and without endometriosis and ectopic lesions from endometriosis patients (Figure 5.2.6). The means of the mRNA concentration of IFITM-1 were very similar in the three study groups. Although, no significant differences were observed in mRNA concentrations between the study groups, the comparison of relative gene expression revealed significant elevation in ectopic lesions compared to eutopic endometrium from non-endometriosis patients. (Figure 5.2.6B).

The relative gene expression of IFITM-1 was not significantly altered in eutopic endometrium and ectopic lesions from endometriosis and non-endometriosis patients with regular period (Figure 5.2.7A). The comparison of endometriosis patients by treatment also did not reveal significant changes for IFITM-1 expression. Although, it is worthwhile to note that the expression was increased in ectopic lesions compared to eutopic endometrium in both treated and non-treated groups, and revealed the highest expression in ectopic lesions from women who received hormone treatments for endometriosis (Figure 5.2.7B).

The analysis of matched specimens tend to show an elevated expression for IFITM-1 in ectopic lesions compared to their eutopic endometrium pairs (Figure 5.2.8).

(A)



(B)

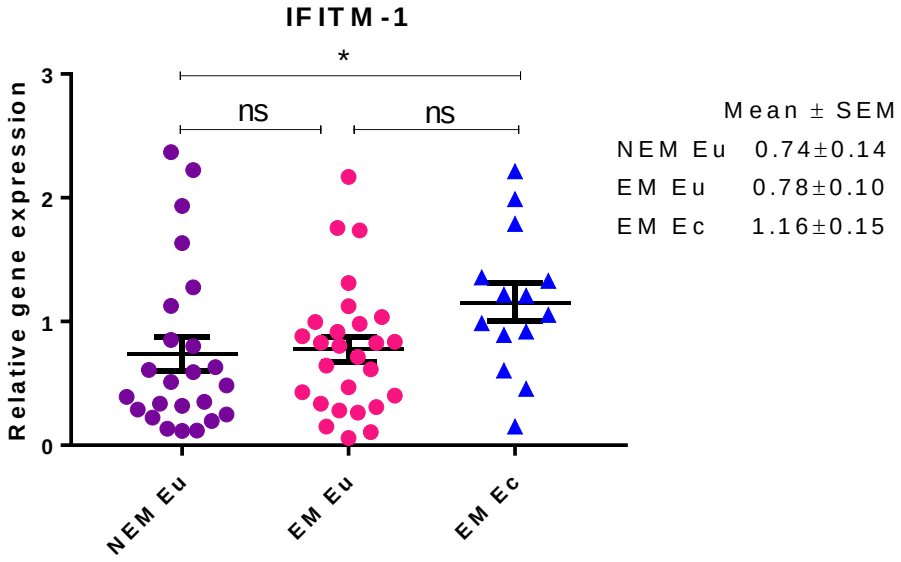
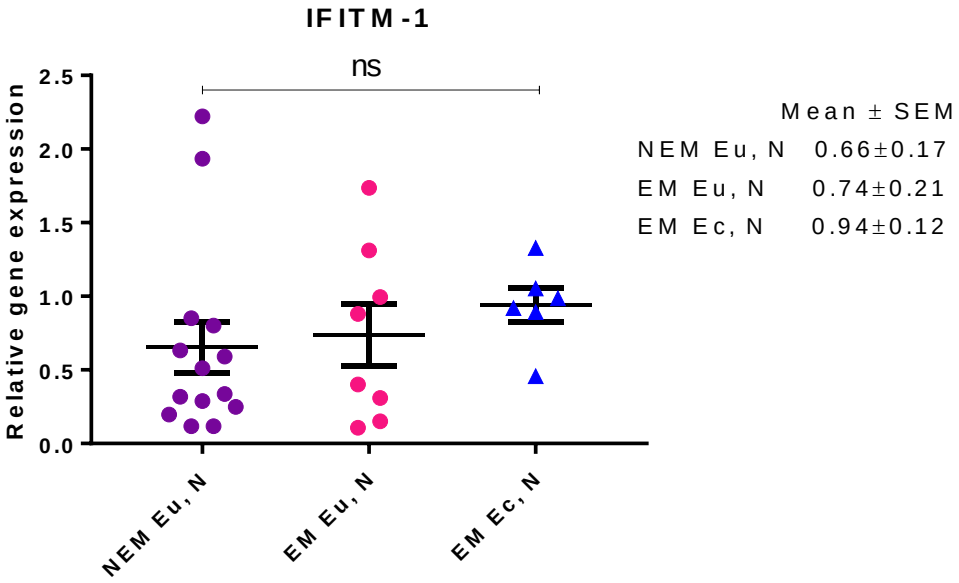


Figure 5.2.6. Gene expression of interferon induced transmembrane protein 1 (IFITM-1) quantified in eutopic endometrium
*from women not diagnosed with endometriosis (NEM Eu, n=24), women diagnosed with endometriosis (EM Eu, n=27) and in ectopic lesions (EM Ec, n=14) from women with endometriosis. Assays were performed using the SYBR Green qRT-PCR method normalized to 360ng total RNA. (A) mRNA concentration of IFITM-1 (ng/µl). RNA concentrations were determined by interpolated Ct values of unknowns from standard curve of calibrator 1 using 4-parameter non-linear regression model. (B) Relative gene expression normalized to GAPDH endogenous reference gene. Data are expressed as mean ± SEM. Statistical comparisons were performed using Kruskal-Wallis test with Dunn's post-hoc test. (ns) not significant; *p<0.05*

(A)



(B)

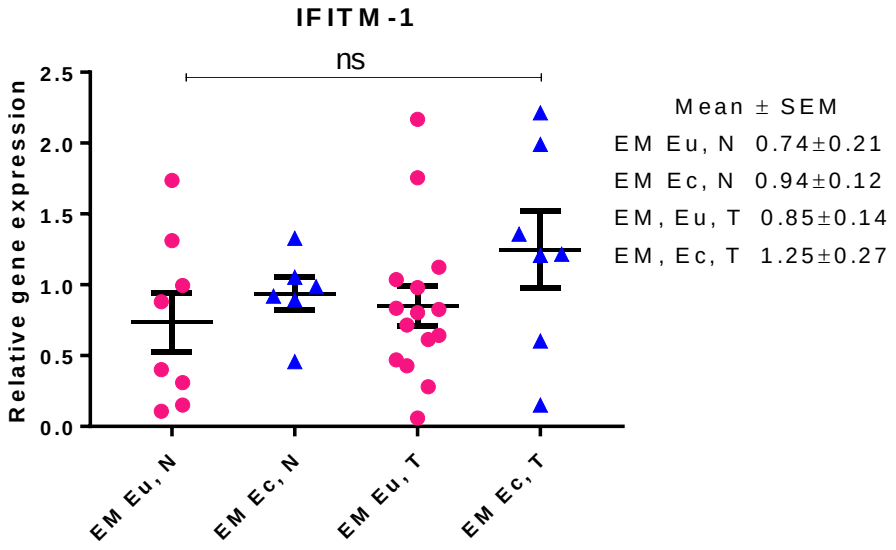


Figure 5.2.7. Relative gene expression of interferon induced transmembrane protein 1 (IFITM-1) in eutopic endometrium and in ectopic lesions with regular menstrual cycle and GnRH treatment.
(A) Comparisons of IFITM-1 gene expression in eutopic endometrium from premenopausal women not diagnosed with endometriosis (NEM Eu N, n=14), premenopausal women diagnosed with endometriosis (EM Eu N, n=8) and in ectopic lesions) from premenopausal women with endometriosis (EM Ec N, n=6). All participants had regular menstrual cycles and did not take hormonal contraceptives or received hormone treatments. (B) Comparisons of IFITM-1 gene expression according GnRH agonist treatment. Eutopic endometrium and ectopic lesions from endometriosis patients with regular cycles, without hormonal treatments or contraceptives (EM Eu N, n=8; EM Ec N, n=6) were compared to endometriosis patients in amenorrhoea receiving GnRH agonist treatments for endometriosis (EM Eu T, n=15; EM Ec T, n=7) Assays were performed using the SYBR Green qRT-PCR method normalized to 360ng total RNA. mRNA concentrations of IFITM-1 were determined by interpolated Ct values of

unknowns from standard curve of calibrator 1 using 4-parameter non-linear regression model. Subsequently mRNA concentrations were used to calculate relative gene expression and normalized to GAPDH endogenous reference gene. Data are expressed as mean ± SEM. Statistical comparisons were performed using Kruskal-Wallis test with Dunn's post- hoc test. (ns) not significant

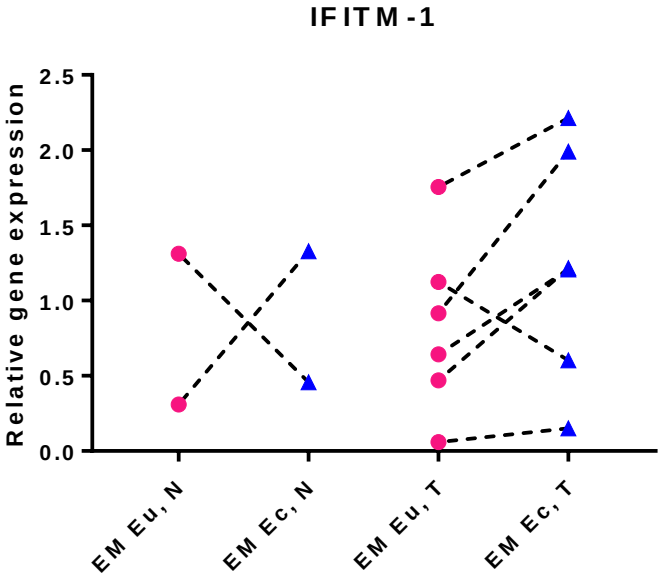


Figure 5.2.8. Gene expression of interferon induced transmembrane protein 1 (IFITM-1) in paired specimens
from women with endometriosis. Eutopic endometrium (Eu) and ectopic lesions (Ec) biopsies were obtained from the same individuals. Dotted lines indicates the matched eutopic and ectopic specimens. Data were grouped according to medical treatment such as endometriosis patients with regular cycles without hormonal treatments or contraceptives (EM Eu N; EM Ec N, n=2) and endometriosis patients with GnRH agonist treatment (EM Eu T; EM Ec T, n=6). Assays were performed using the SYBR Green qRT-PCR method normalized to 360ng total RNA. mRNA concentration of IFITM-1 were determined by interpolated Ct values of unknowns from standard curve of calibrator 1 using 4-parameter non-linear regression model. Subsequently mRNA concentrations were used to calculate relative gene expression and normalized to GAPDH endogenous reference gene.

5.2.2.5 Cyclooxygenase-2 (COX-2)

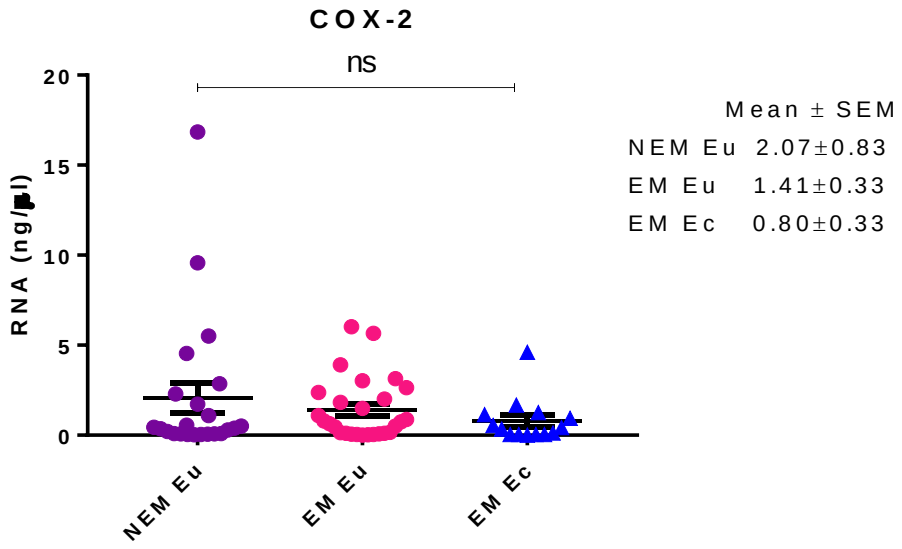
COX-2 expression was investigated in eutopic endometrium from women with and without endometriosis and in ectopic lesions from women with endometriosis to gain knowledge about the prostaglandin biosynthesis in endometriosis.

Figure 5.2.9 presents the summary of COX-2 expression in endometrium from women with and without endometriosis and ectopic lesions from endometriosis patients. Neither the mRNA concentration nor the relative gene expression of COX-2 were significantly altered in any of the study groups. The mean mRNA concentration in ectopic lesions was decreased compared to means of eutopic endometrium from patients with and without endometriosis. The comparison of relative gene expression shown a slightly elevated expression for COX-2 in endometrium and ectopic lesions from patients with endometriosis compared to eutopic endometrium from patients without endometriosis. However, the opposite was observed when women with regular cycles were compared. In that case, the COX-2 expression was lower in eutopic endometrium and ectopic lesions from women with endometriosis compared to eutopic endometrium from women without endometriosis. (Figure 5.2.10A).

The comparison of treated and non-treated endometriosis patients did not reveal significant alteration in COX-2 expression in eutopic endometrium and ectopic lesions. Although, significant alterations have not been found the data showed that the relative gene expression of COX-2 was about 4-fold higher in both specimens in the medically treated group compared to endometriosis patients without hormone therapy (Figure 5.2.10B).

The analysis of matched specimens tend to show a moderately elevated expression of COX-2 in ectopic lesions compared to their paired eutopic endometrium (Figure 5.2.11).

(A)



(B)

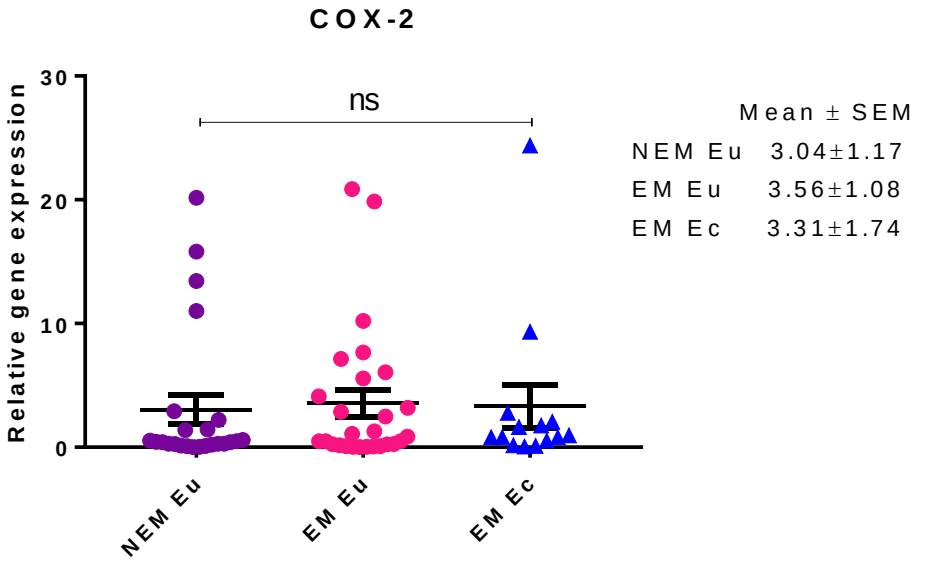
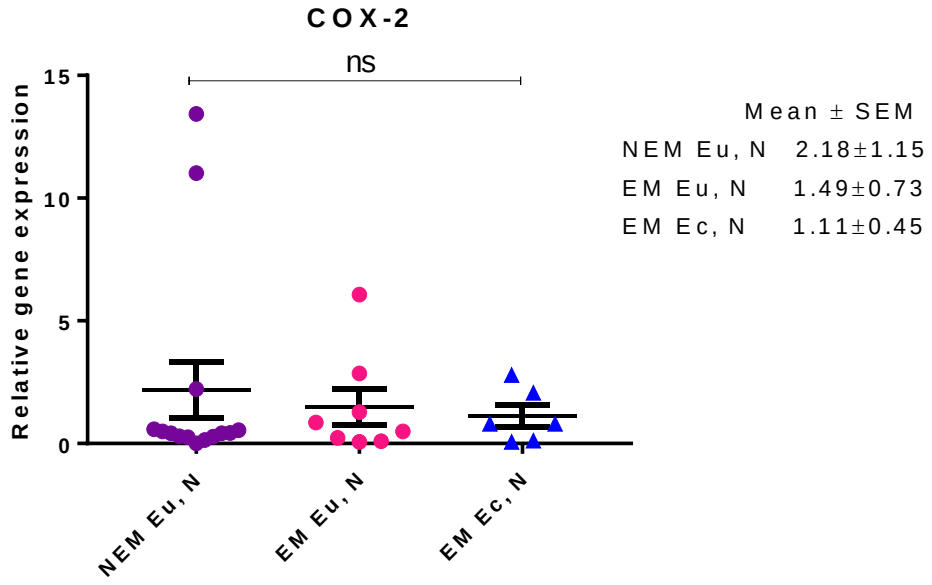


Figure 5.2.9. Gene expression of cyclooxygenase-2 (COX-2) quantified in eutopic endometrium from women not diagnosed with endometriosis (NEM Eu, n=24), women diagnosed with endometriosis (EM Eu, n=27) and in ectopic lesions (EM Ec, n=14) from women with endometriosis. Assays were performed using the SYBR Green qRT-PCR method normalized to 360ng total RNA. (A) mRNA concentration of COX-2 (ng/µl). RNA concentrations were determined by interpolated Ct values of unknowns from standard curve of calibrator 1 using 4-parameter non-linear regression model. (B) Relative gene expression normalized to GAPDH endogenous reference gene. Data are expressed as mean ± SEM. Statistical comparisons were performed using Kruskal-Wallis test with Dunn's post-hoc test. (ns) not significant

(A)



(B)

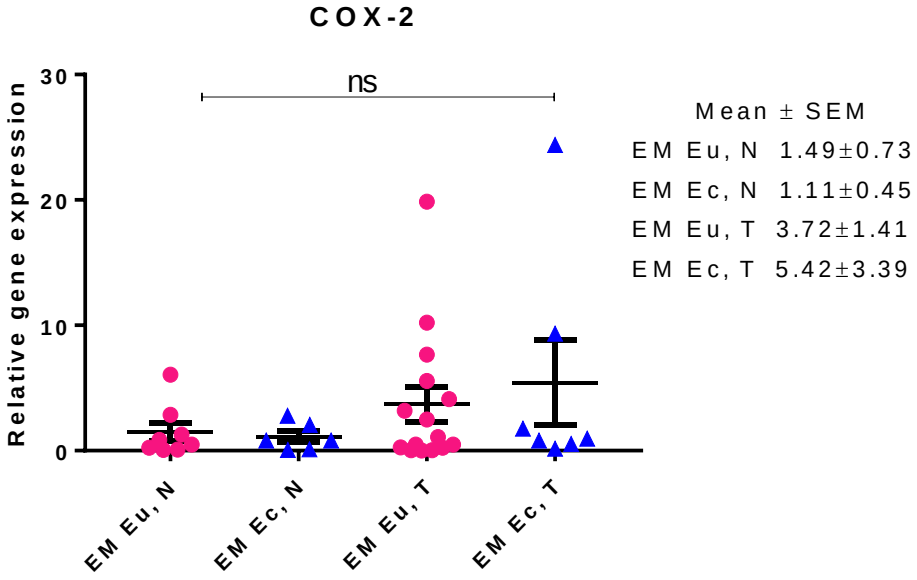


Figure 5.2.10. Relative gene expression of cyclooxygenase-2 (COX-2) in eutopic endometrium and in ectopic lesions with regular menstrual cycle and GnRH treatment.
(A) Comparisons of COX-2 gene expression in eutopic endometrium from premenopausal women not diagnosed with endometriosis (NEM Eu N, n=14), premenopausal women diagnosed with endometriosis (EM Eu N, n=8) and in ectopic lesions from premenopausal women with endometriosis (EM Ec N, n=6). All participants had regular menstrual cycles and did not take hormonal contraceptives or received hormone treatments. (B) Comparisons of COX-2 gene expression according GnRH agonist treatment. Eutopic endometrium and ectopic lesions from endometriosis patients with regular cycles, without hormonal treatments or contraceptives (EM Eu N, n=8; EM Ec N, n=6) were compared to endometriosis patients in amenorrhoea receiving GnRH agonist treatments for endometriosis (EM Eu T, n=15; EM Ec T, n=7) Assays were performed using the SYBR Green qRT-PCR method normalized to 360ng total RNA. mRNA concentrations of COX-2 were determined by interpolated Ct values of

unknowns from standard curve of calibrator 1 using 4-parameter non-linear regression model. Subsequently mRNA concentrations were used to calculate relative gene expression and normalized to GAPDH endogenous reference gene. Data are expressed as mean ± SEM. Statistical comparisons were performed using Kruskal-Wallis test with Dunn's post- hoc test. (ns) not significant

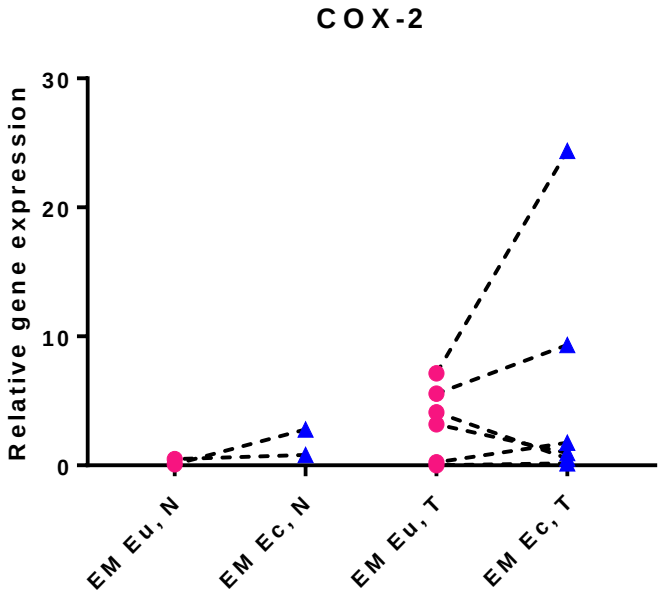


Figure 5.2.11. Gene expression of cyclooxygenase-2 (COX-2) in paired specimens from women with endometriosis. Eutopic endometrium (Eu) and ectopic lesions (Ec) biopsies were obtained from the same individuals. Dotted lines indicates the matched eutopic and ectopic specimens. Data were grouped according to medical treatment such as endometriosis patients with regular cycles without hormonal treatments or contraceptives (EM Eu N; EM Ec N, n=2) and endometriosis patients with GnRH agonist treatment (EM Eu T; EM Ec T, n=6). Assays were performed using the SYBR Green qRT-PCR method normalized to 360ng total RNA. mRNA concentration of COX-2 were determined by interpolated Ct values of unknowns from standard curve of calibrator 1 using 4-parameter non-linear regression model. Subsequently mRNA concentrations were used to calculate relative gene expression and normalized to GAPDH endogenous reference gene.

5.2.2.6 15-Lipoxygenase-1 (15-LOX-1)

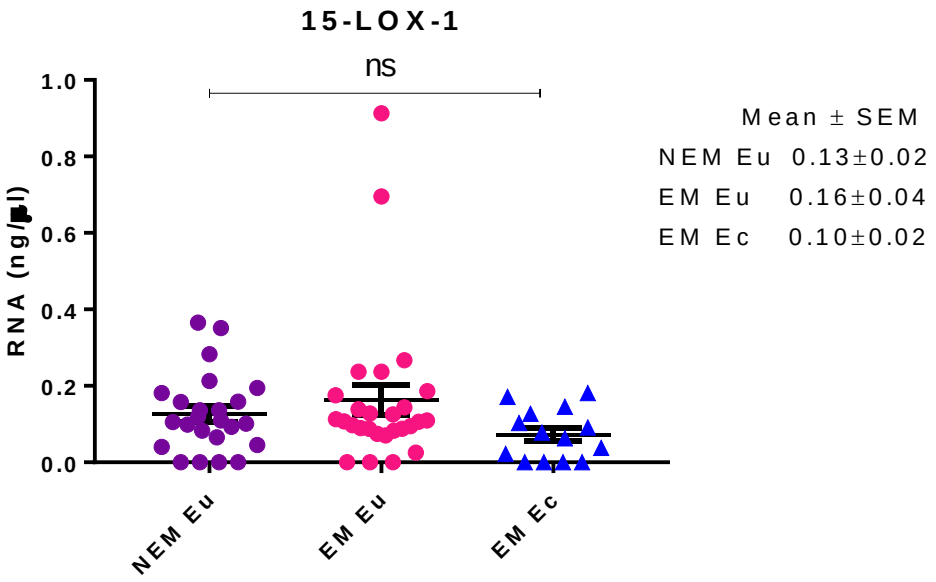
15-LOX-1 expression was investigated in eutopic endometrium from women with and without endometriosis and in ectopic lesions from women with endometriosis to establish a possible role of 15-LOX in the pathology of endometriosis.

Overall, 15-LOX-1 was one of the lowest expressed genes in the genes of interest groups. The gene expression was below the detection limit in three endometriotic and four non-endometriotic eutopic endometrial samples, also in four ectopic lesions. Figure 5.2.12 presents the summary of 15-LOX-1 expression in endometrium and ectopic lesions. Neither the mRNA concentration nor the relative gene expression of 15-LOX-1 were significantly altered in any of the study groups. The mean of mRNA concentration were similar in the three study groups whereas the relative gene expression was approximately half in eutopic endometrium from women without endometriosis compared to eutopic endometrium and ectopic lesions from women diagnosed with endometriosis (Figure 5.2.12).

The comparison of endometriosis and non-endometriosis patients with regular cycles also did not reveal significant alteration for 15-LOX-1 gene expression (Figure 5.2.13A). However, the comparison of the treated and non-treated endometriosis groups revealed more than 5 times higher 15-LOX-1 expression in the medically treated group. Moreover, 15-LOX-1 gene expression was significantly increased in eutopic endometrium in the hormone treated group compared to the eutopic endometrium from the endometriosis patients who did not received medical therapy or used hormonal contraceptives (Figure 5.2.13B).

The comparison of 15-LOX-1 expression in paired specimen showed the same results. 15-LOX-1 expression in the non-treated endometriosis group were the same in the paired eutopic endometrium and ectopic lesions whereas was upregulated in cases of both specimens in the treatment group (Figure 5.2.14).

(A)



(B)

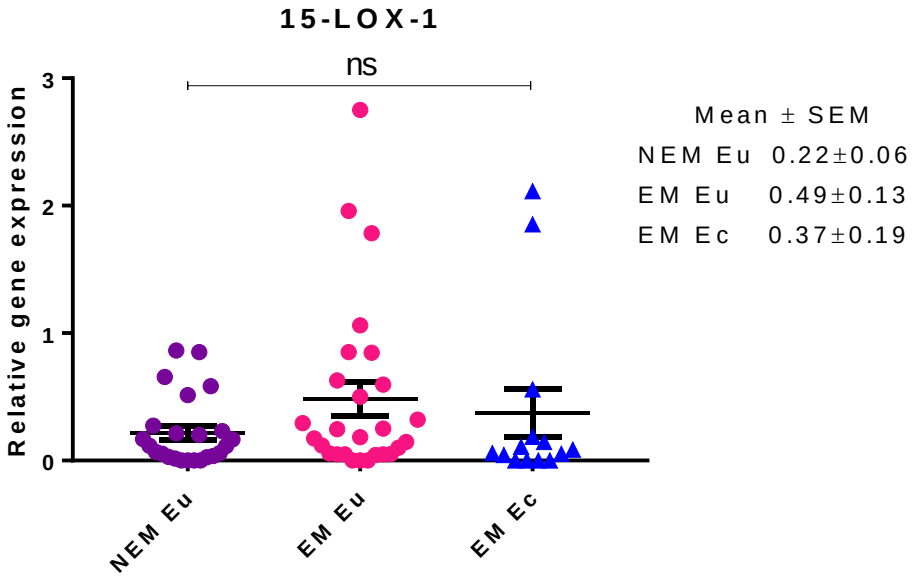
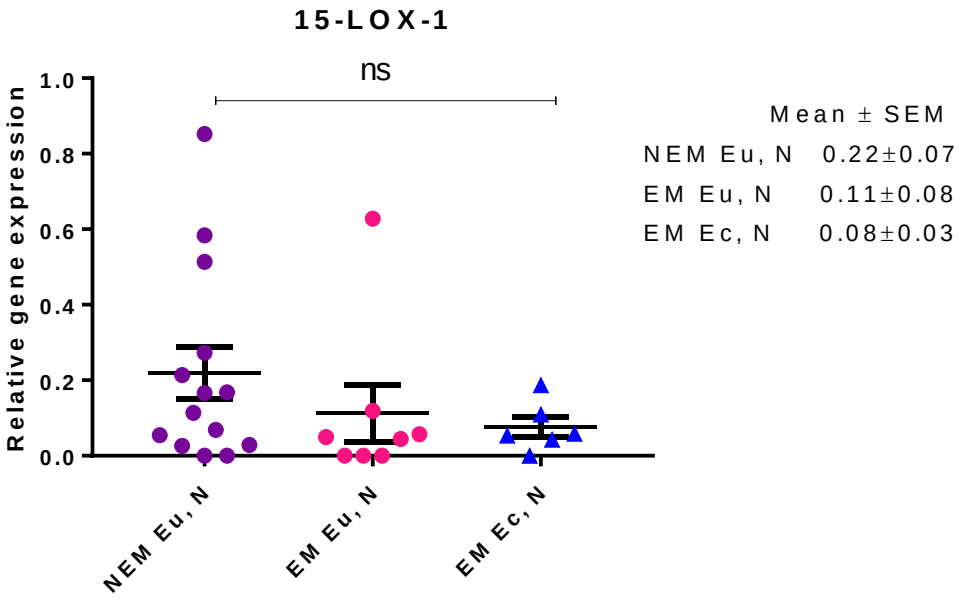


Figure 5.2.12. Gene expression of 15 lipoxygenase-1 (15-LOX-1) quantified in eutopic endometrium
from women not diagnosed with endometriosis (NEM Eu, n=24), women diagnosed with endometriosis (EM Eu, n=27) and in ectopic lesions (EM Ec, n=14) from women with endometriosis. Assays were performed using the SYBR Green qRT-PCR method normalized to 360ng total RNA. (A) mRNA concentration of 15-LOX-1 (ng/µl). RNA concentrations were determined by interpolated Ct values of unknowns from standard curve of calibrator 1 using 4-parameter non-linear regression model. (B) Relative gene expression normalized to GAPDH endogenous reference gene. Data are expressed as mean ± SEM. Statistical comparisons were performed using Kruskal-Wallis testwith Dunn's post-hoc test. (ns) not significant

(A)



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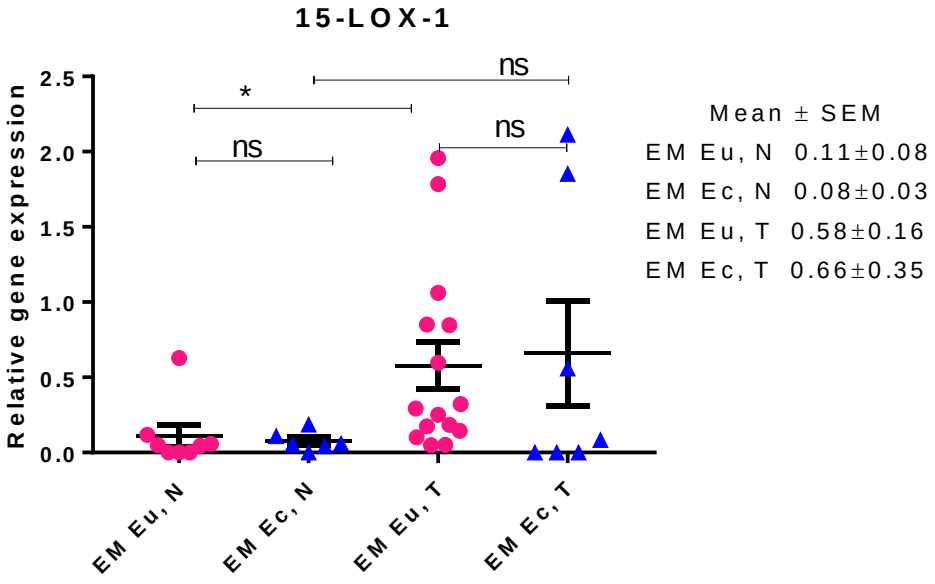


Figure 5.2.13. Relative gene expression of 15 lipoxygenase-1 (15-LOX-1) in eutopic endometrium and in ectopic lesions with regular menstrual cycle and GnRH treatment.
(A) Comparisons of 15-LOX-1 gene expression in eutopic endometrium from premenopausal women not diagnosed with endometriosis (NEM Eu N, n=14), premenopausal women diagnosed with endometriosis (EM Eu N, n=8) and in ectopic lesions) from premenopausal women with endometriosis (EM Ec N, n=6). All participants had regular menstrual cycles and did not take hormonal contraceptives or received hormone treatments. (B) Comparisons of 15-LOX-1 gene expression according GnRH agonist treatment. Eutopic endometrium and ectopic lesions from endometriosis patients with regular cycles, without hormonal treatments or contraceptives (EM Eu N, n=8; EM Ec N, n=6) were compared to endometriosis patients in amenorrhoea receiving GnRH agonist treatments for endometriosis (EM Eu T, n=15; EM Ec T, n=7). Assays were performed using the SYBR Green qRT-PCR method normalized to 360ng

*total RNA. mRNA concentrations of 15-LOX-1 were determined by interpolated Ct values of unknowns from standard curve of calibrator 1 using 4-parameter non-linear regression model. Subsequently mRNA concentrations were used to calculate relative gene expression and normalized to GAPDH endogenous reference gene. Data are expressed as mean ± SEM. Statistical comparisons were performed using Kruskal-Wallis test with Dunn's post- hoc test. (ns) not significant, *p<0.05*

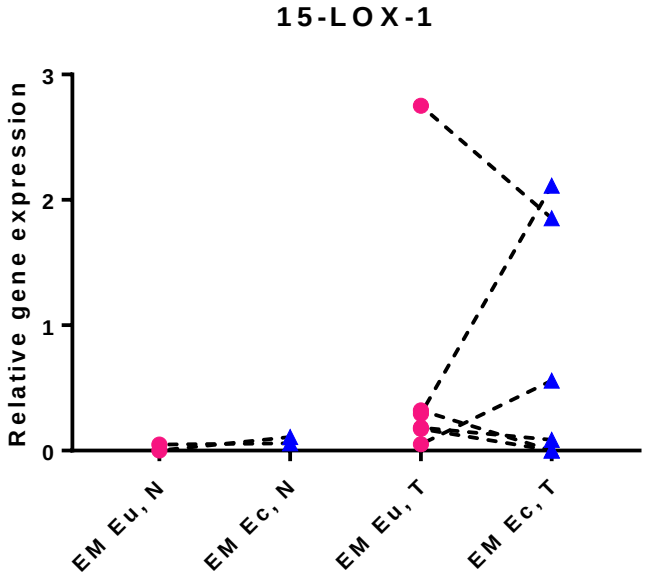


Figure 5.2.14. Relative gene expression of 15 lipoxygenase-1 (15-LOX-1) in paired specimens from women with endometriosis. Eutopic endometrium (Eu) and ectopic lesions (Ec) biopsies were obtained from the same individuals. Dotted lines indicates the matched eutopic and ectopic specimens. Data were grouped according to medical treatment such as endometriosis patients with regular cycles without hormonal treatments or contraceptives (EM Eu N; EM Ec N, n=2) and endometriosis patients with GnRH agonist treatment (EM Eu T; EM Ec T, n=6). Assays were performed using the SYBR Green qRT-PCR method normalized to 360ng total RNA. mRNA concentration of 15-LOX-1 were determined by interpolated Ct values of unknowns from standard curve of calibrator 1 using 4-parameter non-linear regression model. Subsequently mRNA concentrations were used to calculate relative gene expression and normalized to GAPDH endogenous reference gene.

5.2.2.7 15-Lipoxygenase-2 (15-LOX-2)

15-LOX-2 expression was also investigated in eutopic endometrium from women with and without endometriosis and ectopic lesions from women with endometriosis to establish the possible role of 15-LOX in the pathology of endometriosis.

15-LOX-2 was expressed at a very low levels in all of the tested tissue specimens. The gene expression was out of the detection limit in 12 endometriotic and 15 non-endometriotic eutopic endometrium out of 26 and 25 specimens, respectively. The portion samples outside the detection limit was worst in ectopic lesions since only five out of 14 cases were above the limit of detection. Hence, only the eutopic endometrium from endometriosis patients were compared classified by medical treatment. 15-LOX-2 was detected 11 (61.1%) out of 18 endometrium in the hormone treated group whereas 15-LOX-2 was expressed only in 3 (37.5%) out of 8 endometrium in the non-treated group. Although, the statistical comparison not revealed significant alteration between the treated and non-treated groups 15-LOX-2 expression was 10-fold higher in eutopic endometrium from women who received hormonal treatment for endometriosis (Figure 5.2.15).

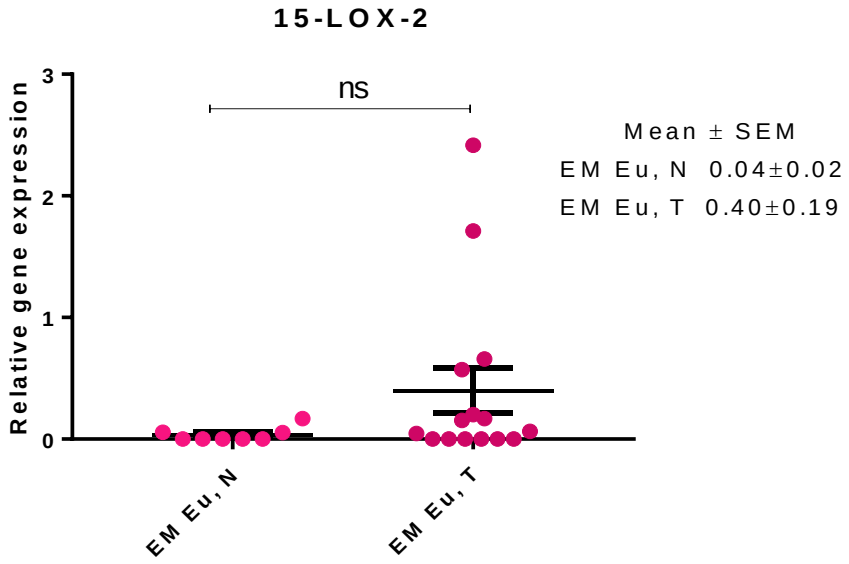


Figure 5.2.15. Relative gene expression of 15 lipoxygenase-2 (15-LOX-2) in eutopic endometrium from women with endometriosis.
Endometriosis patients with regular cycles and without hormonal treatments or contraceptives (EM Eu N, n=8) were compared to endometriosis patients in amenorrhoea receiving hormone treatments (EM Eu T, n=15) for their condition. Assays were performed using the SYBR Green qRT-PCR method normalized to 360ng total RNA. mRNA concentration of 15-LOX-2 were determined by interpolated Ct values of unknowns from standard curve of calibrator 1 using 4-parameter non-linear regression model. Subsequently mRNA concentrations were used to calculate relative gene expression and normalized to GAPDH endogenous reference gene. Data are expressed as mean ± SEM. Statistical comparisons were performed using Mann-Whitney U-test. (ns) not significant

5.2.2.8 Peroxisome proliferator-activated receptor gamma (PPARγ)

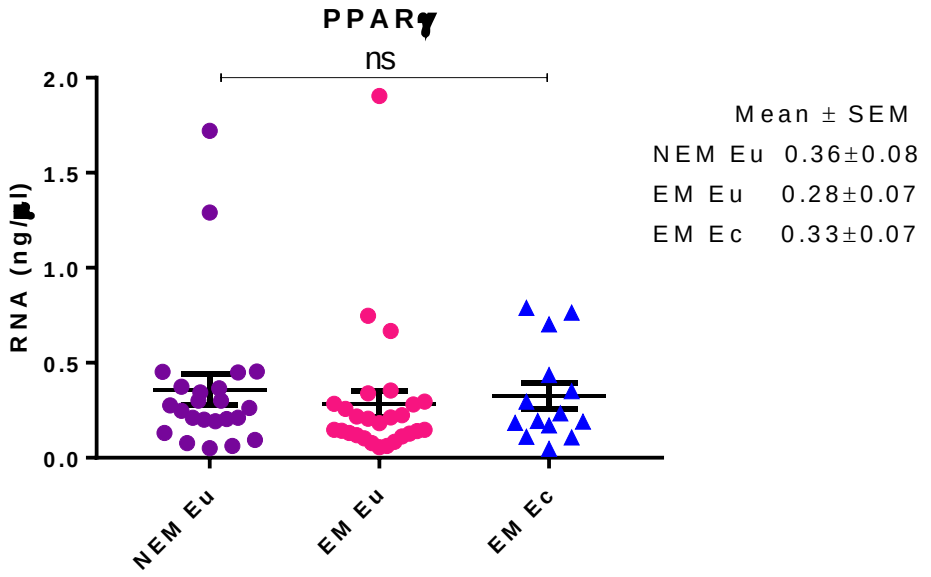
PPARγ has a pleiotropic effect on female reproductive functions. It has a regulatory role in prostaglandin, steroid and cytokine synthesis (Bogacka et al., 2015). PPARγ expression was studied in eutopic endometrium from women with and without endometriosis and ectopic lesions from women with endometriosis.

The mean PPARγ mRNA concentration of was not statistically different in eutopic endometrium from women with and without endometriosis and ectopic lesions from women with endometriosis. However, the comparison of normalised gene expression revealed significant increases for PPARγ in ectopic lesions compared to eutopic endometrium from women with (*p=0.033) and without endometriosis (*p=0.019) (Figure 5.2.16).

The comparison of relative gene expression between endometriosis and non-endometriosis patients with regular cycles showed no significant alteration for PPARγ expression (Figure 5.2.17A). However, the comparison of endometriosis patients by treatments revealed a robust increase for PPARγ expression in ectopic lesions from women undergone medical treatment. The PPARγ expression in this group was significantly elevated compared to the other three study groups (Figure 5.2.17B).

The analysis of paired eutopic endometrium and ectopic lesions specimens also confirmed the increased expression of PPARγ in ectopic lesions for the treatment group. From the chart in Figure 5.2.18 it is apparent that the GnRH agonist treatment could have an effect on PPARγ expression. In the treatment group the PPARγ expression in ectopic lesions showed a robust increase compared to eutopic endometrium, whereas this obvious difference have not been observed between eutopic endometrium and ectopic lesions in the non-treated group.

(A)



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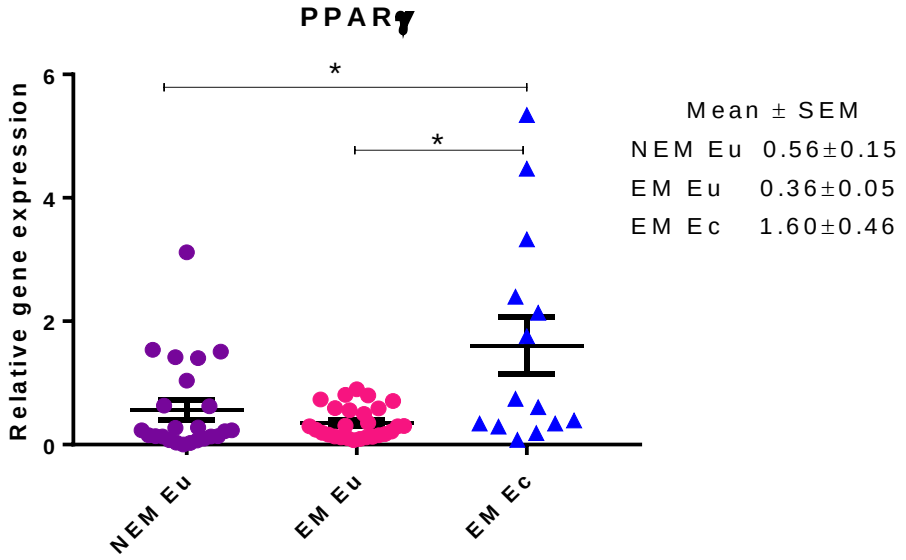
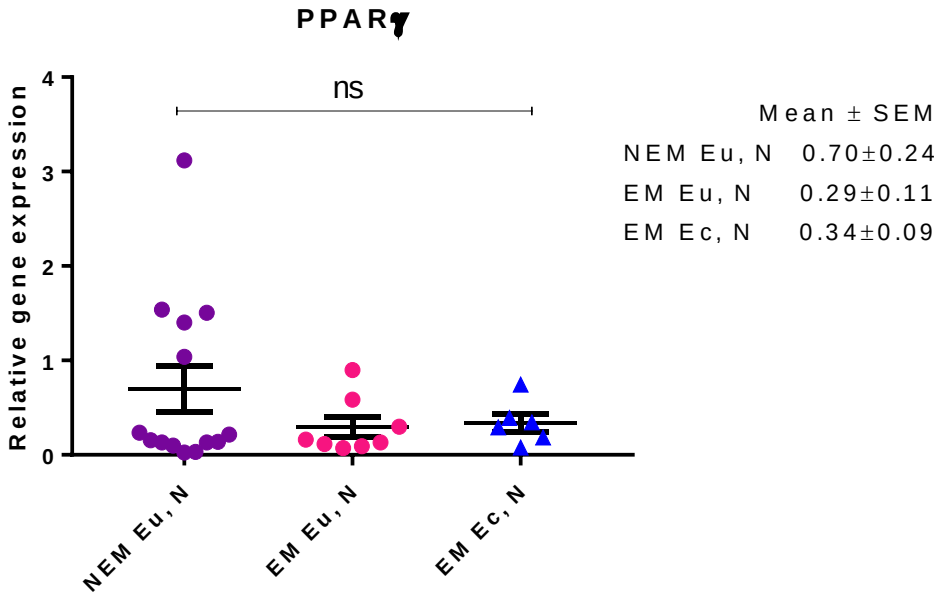


*Figure 5.2.16. Gene expression of peroxisome proliferator-activated receptor gamma (PPARγ) quantified in eutopic endometrium from women not diagnosed with endometriosis (NEM Eu, n=24), women diagnosed with endometriosis (EM Eu, n=27) and in ectopic lesions (EM Ec, n=14) from women with endometriosis. Assays were performed using the SYBR Green qRT-PCR method normalized to 360ng total RNA. (A) mRNA concentration of PPARγ (ng/µl). RNA concentrations were determined by interpolated Ct values of unknowns from standard curve of calibrator 2 using 4-parameter non-linear regression model. (B) Relative gene expression normalized to GAPDH endogenous reference gene. Data are expressed as mean ± SEM. Statistical comparisons were performed using Kruskal-Wallis test with Dunn's post-hoc test. (ns) not significant;*p<0.05*

(A)



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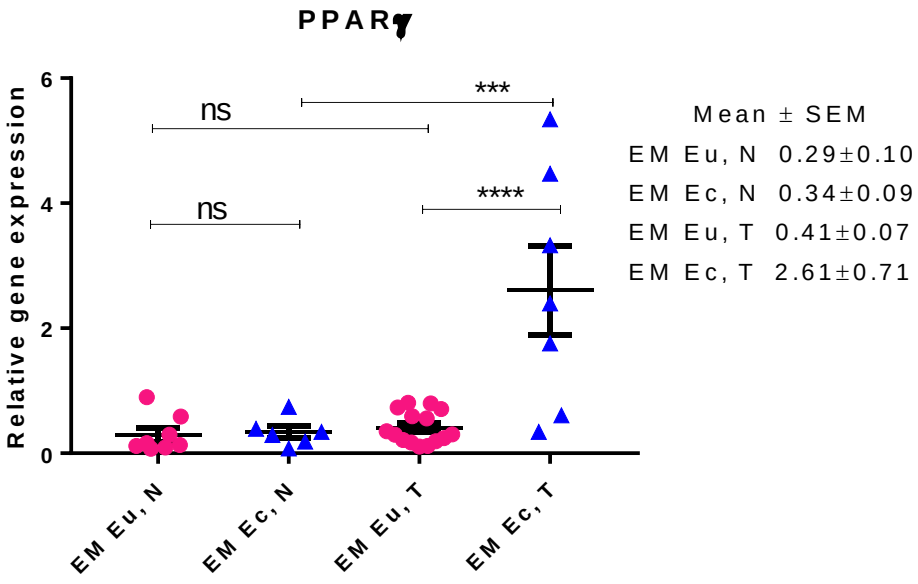


Figure 5.2.17. Relative gene expression of peroxisome proliferator-activated receptor gamma (PPARγ) in eutopic endometrium and in ectopic lesions with regular menstrual cycle and GnRH treatment.
(A) Comparisons of PPARγ gene expression in eutopic endometrium from premenopausal women not diagnosed with endometriosis (NEM Eu N, n=14), premenopausal women diagnosed with endometriosis (EM Eu N, n=8) and in ectopic lesions) from premenopausal women with endometriosis (EM Ec N, n=6). All participants had regular menstrual cycles and did not take hormonal contraceptives or received hormone treatments. (B) Comparisons of PPARγ gene expression according GnRH agonist treatment. Eutopic endometrium and ectopic lesions from endometriosis patients with regular cycles, without hormonal treatments or contraceptives (EM Eu N, n=8; EM Ec N, n=6) were compared to endometriosis patients in amenorrhoea receiving GnRH agonist treatments for endometriosis (EM Eu T, n=15; EM Ec T,

*n=7). Assays were performed using the SYBR Green qRT-PCR method normalized to 360ng total RNA. mRNA concentrations of PPARγ were determined by interpolated Ct values of unknowns from standard curve of calibrator 2 using 4-parameter non-linear regression model. Subsequently mRNA concentrations were used to calculate relative gene expression and normalized to GAPDH endogenous reference gene. Data are expressed as mean ± SEM. Statistical comparisons were performed using Kruskal-Wallis test with Dunn's post-hoc test analysis in figure A and one-way ANOVA with Tukey's post hoc analysis in figure B. (ns) not significant; ***p<0.001; ****p<0.0001*

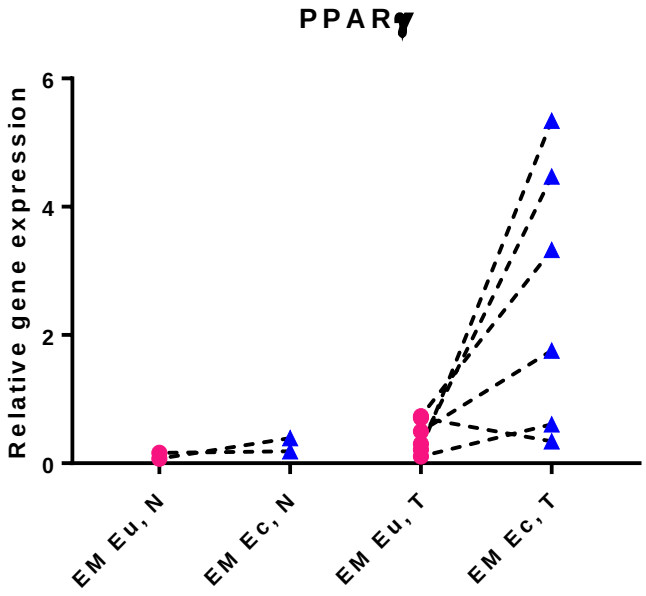


Figure 5.2.18. Relative gene expression of peroxisome proliferator-activated receptor gamma (PPARγ) in paired specimens
from women with endometriosis. Eutopic endometrium (Eu) and ectopic lesions (Ec) biopsies were obtained from the same individuals. Dotted lines indicates the matched eutopic and ectopic specimens. Data were grouped according to medical treatment such as endometriosis patients with regular cycles without hormonal treatments or contraceptives (EM Eu N; EM Ec N, n=2) and endometriosis patients with GnRH agonist treatment (EM Eu T; EM Ec T, n=6). Assays were performed using the SYBR Green qRT-PCR method normalized to 360ng total RNA. mRNA concentration of PPARγ were determined by interpolated Ct values of unknowns from standard curve of calibrator 2 using 4-parameter non-linear regression model. Subsequently mRNA concentrations were used to calculate relative gene expression and normalized to GAPDH endogenous reference gene.

5.2.2.9 CD36

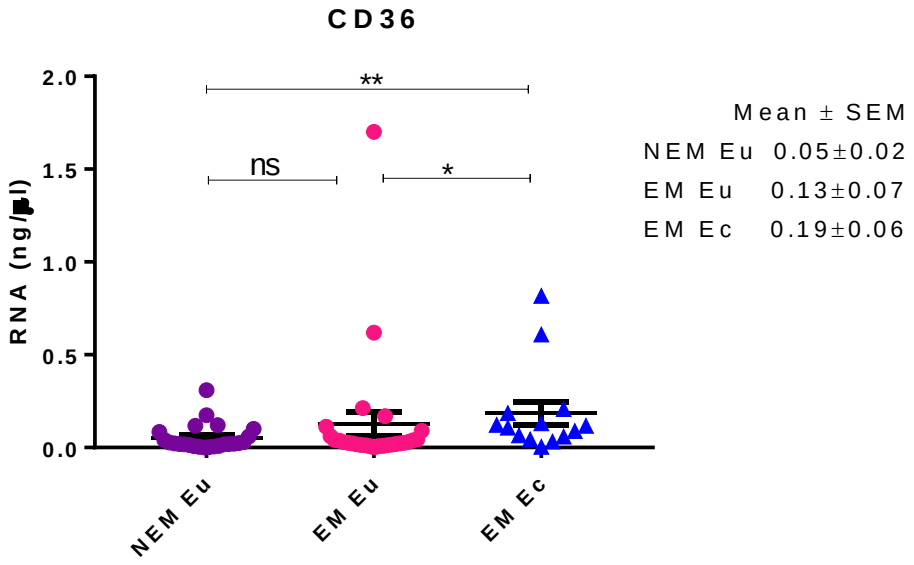
CD36 is the member of the scavenger receptor family with a broad range of ligand specificity. PPARγ is the key regulator of the expression of CD36 (Lippman et al., 1998). The expression of CD36 was studied in eutopic endometrium and ectopic lesions to obtain information about the activity of PPARγ.

The expression of CD36 was not different in eutopic endometrium from patients with and without endometriosis but it was significantly increased in ectopic lesions (Figure 5.2.19). The mRNA concentrations were significantly elevated in ectopic lesions compared to eutopic endometrium from women with (*p=0.024) and without endometriosis (**p=0.005). The relative gene expression also showed elevation in ectopic lesions. The mean of relative gene expression was 2.80±1.38 (mean±SEM) in ectopic lesions compared to a mean of 0.14±0.04 (mean±SEM) in eutopic endometrium from women with endometriosis and the mean of 0.08±0.02 (mean±SEM) from non-endometriosis patients. The statistical analysis confirmed that the CD36 expression was significantly elevated in ectopic lesions compared to eutopic endometrium from endometriosis and non-endometriotic patients.

The further analysis of data did not revealed any statistical alteration for CD36 expression in tissue specimens from women with regular cycles (Figure 5.2.20A). However, the analysis of endometriosis patients' data were more interesting. The expression of CD36, similar to PPARγ, was upregulated in ectopic lesions from women who received hormone treatment for endometriosis. The comparison revealed that the CD36 expression was significantly decreased in eutopic endometrium in the hormone treated group, also in ectopic lesions in the non-treated group compared to ectopic lesions from the hormone treatment group (Figure 5.2.20B).

The analysis of paired eutopic endometrium and ectopic lesions specimens also demonstrated a robust increase for CD36 expression in ectopic lesions from endometriosis women whose received medical therapy for their condition (Figure 5.2.21).

(A)



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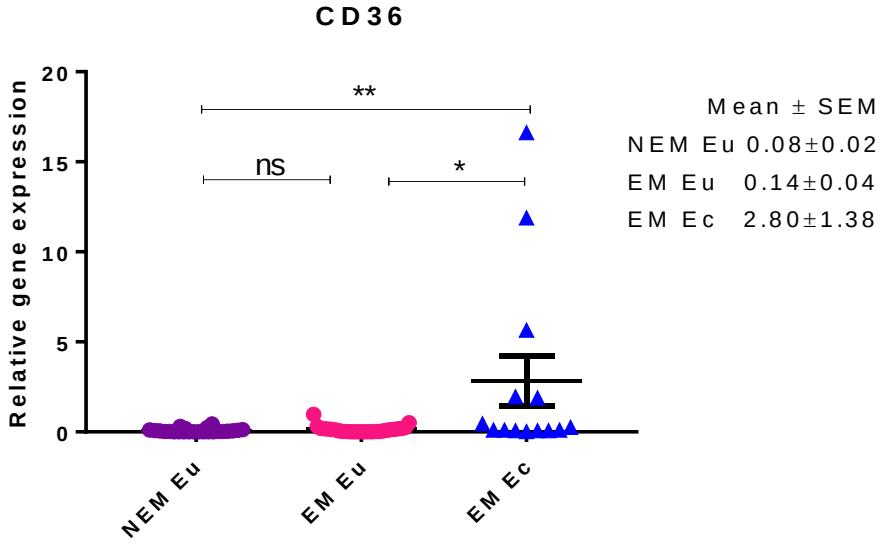
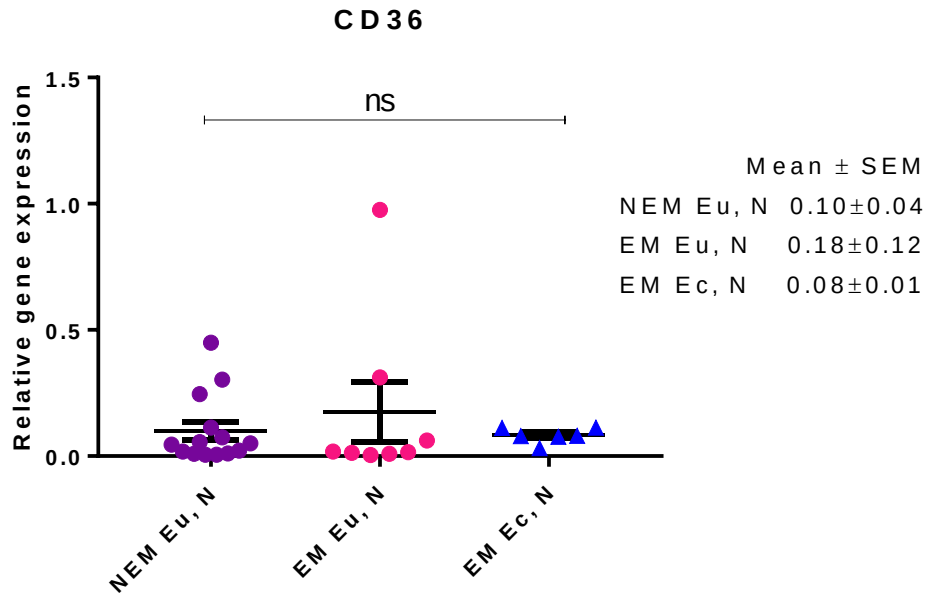


*Figure 5.2.19. Gene expression of CD36 quantified in eutopic endometrium from women not diagnosed with endometriosis (NEM Eu, n=24), women diagnosed with endometriosis (EM Eu, n=27) and in ectopic lesions (EM Ec, n=14) from women with endometriosis. Assays were performed using the SYBR Green qRT-PCR method normalized to 360ng total RNA. (A) mRNA concentration of CD36 (ng/µl). RNA concentrations were determined by interpolated Ct values of unknowns from standard curve of calibrator 1 using 4-parameter non-linear regression model. (B) Relative gene expression normalized to GAPDH endogenous reference gene. Data are expressed as mean ± SEM. Statistical comparisons were performed using Kruskal-Wallis test with Dunn's post-hoc test. (ns) not significant;*p<0.05; **p<0.01*

(A)



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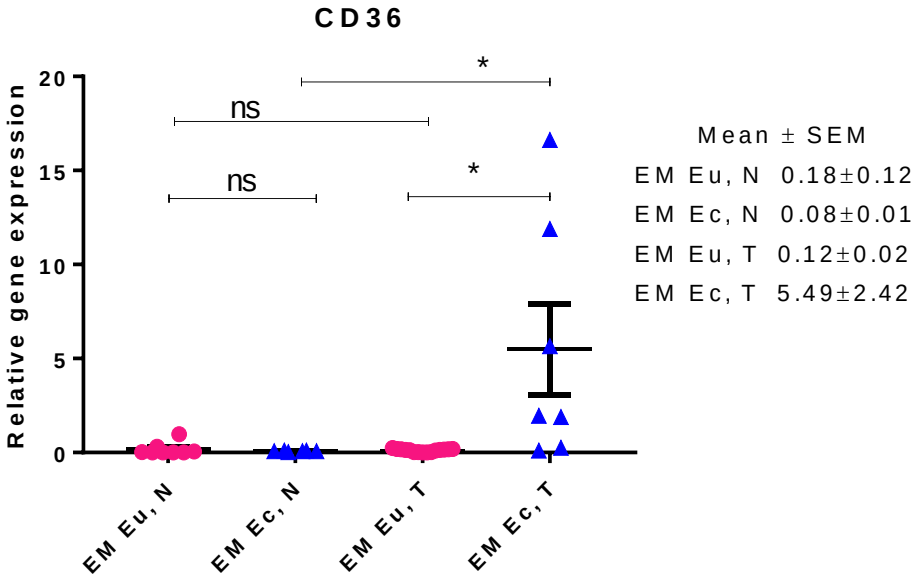


Figure 5.2.20. Relative gene expression of CD36 in eutopic endometrium and in ectopic lesions with regular menstrual cycle and GnRH treatment.
(A) Comparisons of CD36 gene expression in eutopic endometrium from premenopausal women not diagnosed with endometriosis (NEM Eu N, n=14), premenopausal women diagnosed with endometriosis (EM Eu N, n=8) and in ectopic lesions) from premenopausal women with endometriosis (EM Ec N, n=6). All participants had regular menstrual cycles and did not take hormonal contraceptives or received hormone treatments. (B) Comparisons of CD36 gene expression according GnRH agonist treatment. Eutopic endometrium and ectopic lesions from endometriosis patients with regular cycles, without hormonal treatments or contraceptives (EM Eu N, n=8; EM Ec N, n=6) were compared to endometriosis patients in amenorrhoea receiving GnRH agonist treatments for endometriosis (EM Eu T, n=15; EM Ec T, n=7). Assays were performed using the SYBR Green qRT-PCR method normalized to 360ng

*total RNA. mRNA concentrations of CD36 were determined by interpolated Ct values of unknowns from standard curve of calibrator 1 using 4-parameter non-linear regression model. Subsequently mRNA concentrations were used to calculate relative gene expression and normalized to GAPDH endogenous reference gene. Data are expressed as mean ± SEM. Statistical comparisons were performed using Kruskal-Wallis test with Dunn's post- hoc test. (ns) not significant;*p<0.05*

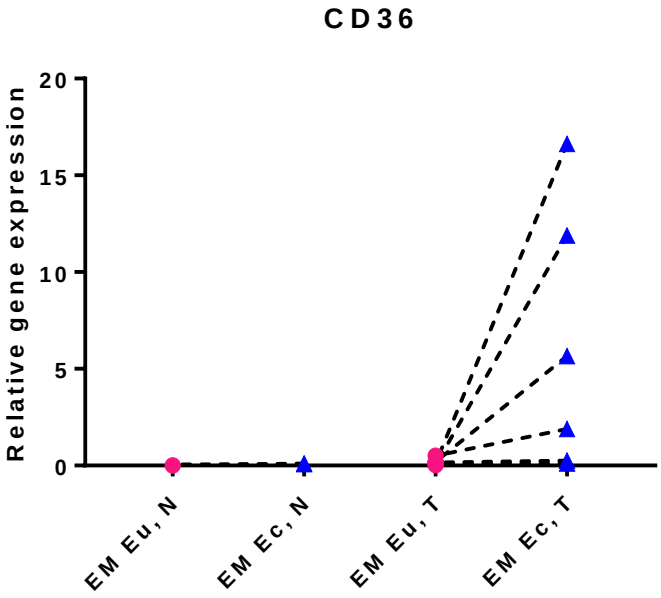


Figure 5.2.21. Relative gene expression of CD36 in paired specimens from women with endometriosis. Eutopic endometrium (Eu) and ectopic lesions (Ec) biopsies were obtained from the same individuals. Dotted lines indicates the matched eutopic and ectopic specimens. Data were grouped according to medical treatment such as endometriosis patients with regular cycles without hormonal treatments or contraceptives (EM Eu N; EM Ec N, n=2) and endometriosis patients with GnRH agonist treatment (EM Eu T; EM Ec T, n=6). Assays were performed using the SYBR Green qRT-PCR method normalized to 360ng total RNA. mRNA concentration of CD36 were determined by interpolated Ct values of unknowns from standard curve of calibrator 1 using 4-parameter non-linear regression model. Subsequently mRNA concentrations were used to calculate relative gene expression and normalized to GAPDH endogenous reference gene.

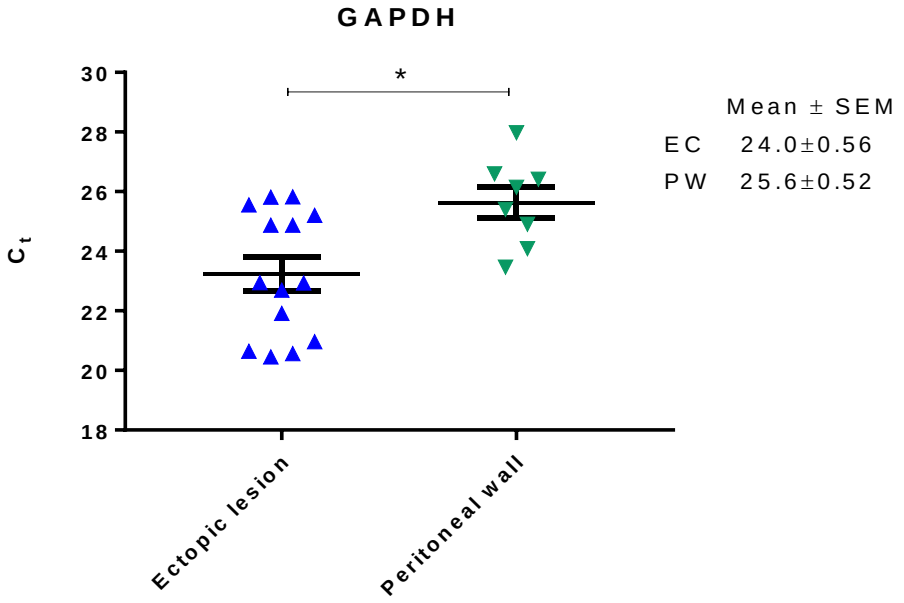
5.2.3 Gene expression study in the peritoneal wall

Biopsies of peritoneal wall from endometriosis patients were investigated using SYBR Green qRT-PCR assay to study the expression of cytokeratin 8, IFITM-1, COX-2, 15-LOX-1, 15-LOX-2, PPARγ, CD36 and GAPDH in the peritoneum. The aim of these experiments was to study the peritoneal environment of endometriosis and to find possible cofounding factors for the pathogenesis of the disease. Hence comparisons were performed for the expression of genes of interest between the peritoneal wall and ectopic lesions.

A total of 14 ectopic lesions as described in the previous section and 8 peritoneal biopsies were collected from endometriosis patients and used for this study. Seven out of eight patients in the group of peritoneal wall had in amenorrhoea attributable to medical treatments. Only one patient not received treatment for endometriosis and had a regular cycles.

Firstly, the expression of GAPDH were compared between ectopic lesions and the peritoneal wall samples. Figure 5.2.22 demonstrates the Ct values and the relative mRNA concentrations of GAPDH in the studied specimens. The expression of GAPDH was more supressed in peritoneal wall compared to ectopic lesions (Figure 5.2.22). The Ct values and the mRNA concentrations were also significantly different between ectopic lesions and peritoneal wall. Although, the RefFinder, see section 2.3.9, suggested that GAPDH was stable in different specimens and was not affected by the experimental conditions, the normalisation of genes of interest with GAPDH in the case of peritoneal wall could obscure real changes due to the significant difference. In order to avoid producing artefactual changes, the peritoneal wall data were not normalised to GAPDH, the relative mRNA concentrations were used for comparison only. Although these data are not as robust as normalised relative gene expressions the comparisons could still be valid. The reason being that the comparison of the relative mRNA concentrations is because the data were normalised at two levels. Besides normalising against the gene of interest to the endogenous reference gene, i.e. GAPDH, the experiment also was normalised to the amount of RNA at the beginning of the experiment. 360ng of extracted RNA was used for each reverse transcription, same volume (1µl) of cDNA was assayed in qRT-PCR and the same standard curve was used to interpolate the mRNA concentrations. Table 5.2.3 summarizes the comparison of mRNA concentrations of gene of interests.

(A)



(B)

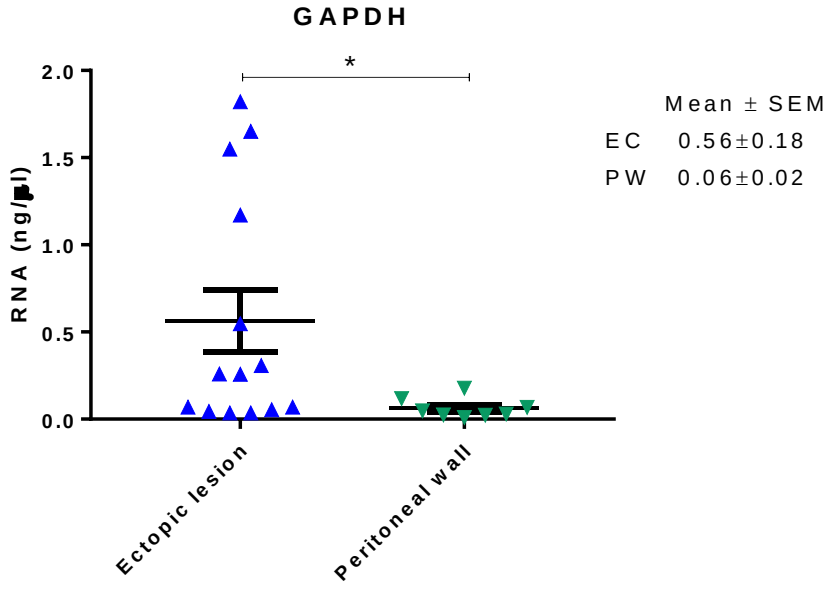


Figure 5.2.22. Gene expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) in ectopic lesions (EC, n=14) and peritoneal wall (PW, n=8)
*from women with endometriosis. Assays were performed using the SYBR Green qRT-PCR method normalized to 360ng total RNA. (A) Threshold cycle (Ct) values of GAPDH. (B) Relative mRNA concentration of GAPDH (ng/µl). RNA concentrations were determined by interpolated Ct values of unknown from standard curve of calibrator 1 using 4-parameter non-linear regression model. Data are expressed as mean ± SEM. Statistical comparisons were performed using Mann-Whitney U test. *p<0.05*

Table 5.2.3. Relative mRNA concentration in peritoneal wall and ectopic lesions from women with endometriosis.
Assay was performed using the SYBR Green qRT-PCR method normalized to 360ng total RNA. RNA concentrations were determined by interpolated Ct values of unknown from standard curve using 4-parameter non-linear regression model. Data are expressed as mean ± SEM. Statistical comparisons were carried out using D'Agostino & Pearson normality test followed with Mann-Whitney U test. (ND) not detected; (a) significant

Peritoneal wall	Relative mRNA concentration (ng/µl) (mean ± SEM)	
Targets	**Ectopic lesions (n=14)**	**Peritoneal wall (n=8)**
GAPDH	0.56 ± 0.18[a]	0.06 ± 0.02[a]
CK8	0.007 ± 0.002	0.003 ± 0.001
IFITM-1	0.53 ± 0.18	0.13 ± 0.05
COX-2	0.80 ± 0.33	0.17 ± 0.05
15-LOX-1	0.10 ± 0.02	0.07 ± 0.02
15-LOX-2	ND	ND
PPARγ	0.33 ± 0.07	0.20 ± 0.05
CD36	0.19 ± 0.06	0.11 ± 0.03

(GAPDH) glyceraldehyde-3-phosphate dehydrogenase; (CK8) cytokeratin 8; (IFITM-1) interferon induced transmembrane protein 1; (COX) cyclooxygenase; (15-LOX) 15-lipoxygenase; PPARγ-peroxisome proliferator-activated receptor gamma

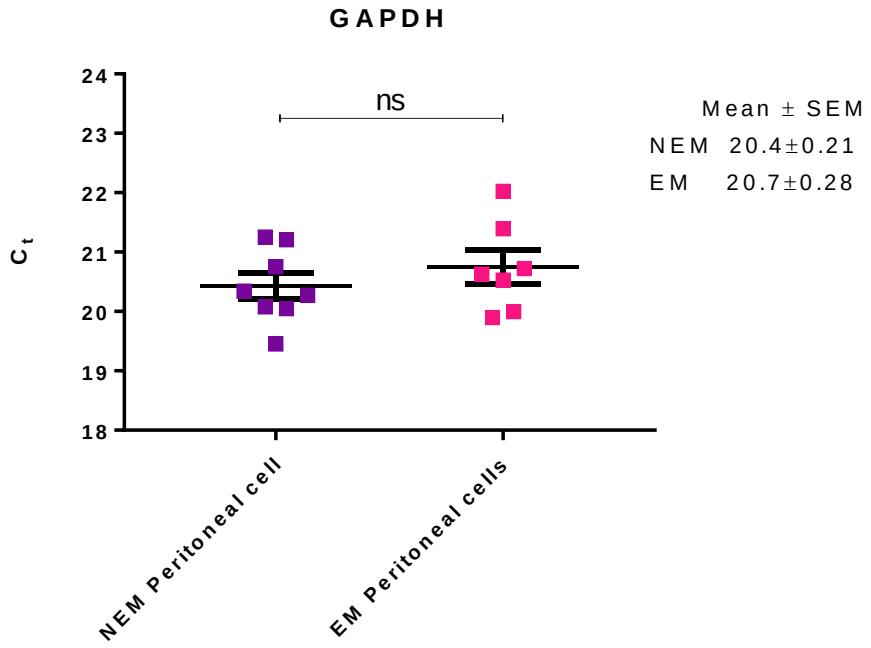
Overall, the studied genes showed lower expression in peritoneal wall compared to ectopic lesions. PPARγ and COX-2 displayed the highest mRNA concentrations in peritoneal wall, whereas 15-LOX-2 was below the limit of detection. Cytokeratin 8 was detected at a very low level in peritoneal wall. The mRNA concentration was approximately half of the mRNA concentration in ectopic lesions. IFITM-1 also was expressed in peritoneal wall at low level. The mean of mRNA concentration of IFITM-1 were 4-fold lower in peritoneal wall compared to ectopic lesions. The mean concentration of COX-2 was nearly identical with IFITM-1 in peritoneal wall and was nearly five-fold lower than in ectopic lesions. The PPARγ and CD36 revealed similar mRNA concentration in peritoneal wall and ectopic lesions. The statistical comparison of peritoneal wall to ectopic lesions, except GAPDH, not revealed significant difference for the expression of gene of interests.

5.2.4 Gene expression study of peritoneal cells

Peritoneal fluid contains a wide variety of free floating cells, including macrophages, mesothelial cells and lymphocytes (Oral et al., 1996). These cells play a pivotal role in the maintenance of homeostasis in the abdominal cavity. Impaired function of peritoneal leukocytes, such as reduced phagocytic activity of macrophages, or decreased cytotoxicity of NK and T cells, could promote the attachment and proliferation of endometrial implants. Therefore, peritoneal cells from women with (EM n=7) and without (NEM n=8) were used to study the gene expression of cytokeratin 8, IFITM-1, COX-2, 15-LOX-1, 15-LOX-2, PPARγ, CD36 and GAPDH using SYBR Green qRT-PCR assay to gain insight the possible role of these genes in the pathomechanism of endometriosis.

Initially, GAPDH expression was analysed and tested whether its expression shows differences between the group of endometriosis and non-endometriosis. Figure 5.2.23 demonstrate the distribution of Ct values and the relative mRNA concentration of the subjects within the studies groups. Neither the Ct values, nor the mRNA concentrations were significantly altered between the two study groups. Hence, GAPDH was subsequently used to calculate the relative gene expression for genes of interests.

(A)



(B)

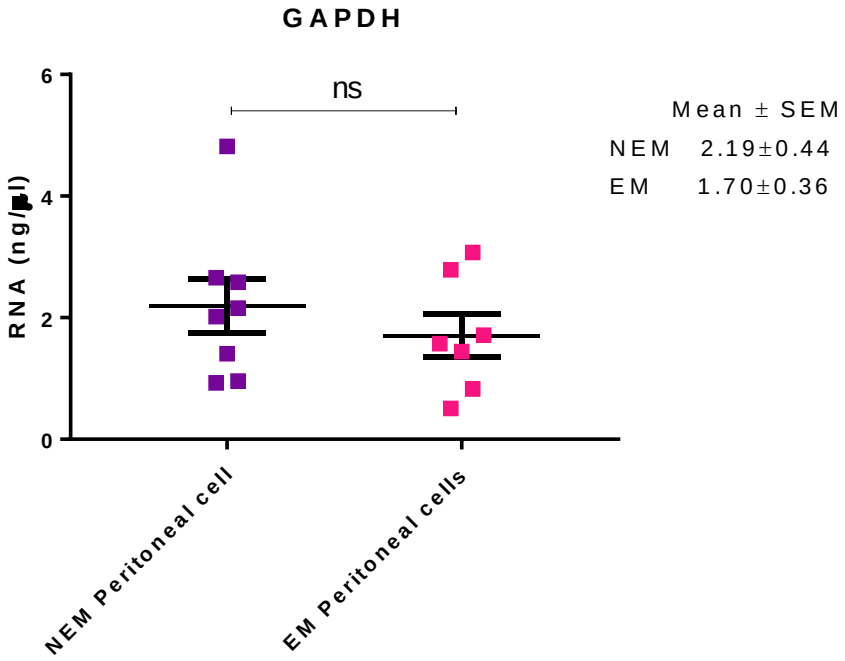


Figure 5.2.23. Gene expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) quantified in peritoneal cells
from women not diagnosed with endometriosis (NEM, n=8), women diagnosed with endometriosis (EM, n=7). Assays were performed using the SYBR Green qRT-PCR method normalized to 360ng total RNA. (A) Threshold cycle (Ct) values of GAPDH. (B) Relative mRNA concentration of GAPDH (ng/µl). RNA concentrations were determined by interpolated Ct values of unknown from standard curve of calibrator 1 using 4-parameter non-linear regression model. Data are expressed as mean ± SEM. Statistical comparisons were performed using Mann-Whitney U test. (ns) not significant

Because of the low numbers of subjects in the study groups, comparisons have not been performed by menstrual cycle and medical treatments. The statistical analysis did not revealed significant alteration for the expression of target genes in peritoneal cells between the group of endometriosis and non-endometriosis. Table 5.2.4 summarize the means of relative mRNA concentration and relative gene expression of gene of interests in peritoneal cells from patients with and without endometriosis.

Table 5.2.4. Gene expression in peritoneal cells from women not diagnosed with endometriosis (NEM, n=8), and from women diagnosed with endometriosis (EM, n=7).
Assay was performed using the SYBR Green qRT-PCR method normalized to 360ng total RNA. RNA concentrations were determined by interpolated Ct values of unknown from standard curve using 4-parameter non-linear regression model. Data are expressed as mean ± SEM. Statistical comparisons were carried out using D'Agostino & Pearson normality test followed with Mann-Whitney U test. None of the genes of interest showed significant alteration between the two study groups. (ND) not detected

Peritoneal cells	Relative mRNA concentration (ng/µl) (mean ± SEM)		Relative gene expression (mean ± SEM)	
Targets	**NEM (n=8)**	**EM (n=7)**	**NEM (n=8)**	**EM (n=7)**
GAPDH	**2.19** ± 0.44	**1.70** ± 0.36	-	-
CK8	**0.04** ± 0.01	**0.02** ± 0.01	**0.03** ± 0.01	**0.02** ± 0.02
IFITM-1	**0.81** ± 0.18	**0.42** ± 0.08	**0.49** ± 0.14	**0.27** ± 0.04
COX-2	**0.37** ± 0.09	**0.31** ± 0.08	**0.21** ± 0.07	**0.19** ± 0.03
15-LOX-1	**1.90** ± 0.88	**2.33** ± 0.94	**1.22** ± 0.64	**2.40** ± 1.45
15-LOX-2	ND	ND	ND	ND
PPARγ	**1.53** ± 0.40	**1.68** ± 0.74	**0.54** ± 0.09	**0.68** ± 0.16
CD36	**4.40** ± 1.70	**4.27** ± 1.85	**1.82** ± 0.49	**2.14** ± 0.68

(GAPDH) glyceraldehyde-3-phosphate dehydrogenase; (CK8) cytokeratin 8; (IFITM-1) interferon induced transmembrane protein 1; (COX) cyclooxygenase; (15-LOX) 15-lipoxygenase; PPARγ-peroxisome proliferator-activated receptor gamma

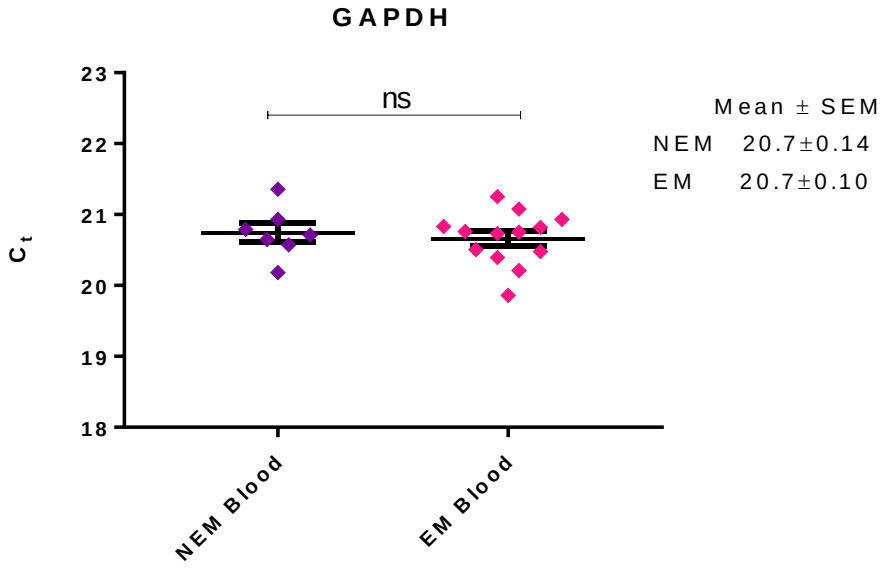
As in solid tissue specimens 15-LOX-2 was under the limit of detection. In amongst the detected genes, cytokeratin 8 showed the lowest expression in peritoneal cells. IFITM-1 and COX-2 were also expressed at low levels. IFITM-1 was approximately two-fold higher in the non-endometriosis group, while COX-2 expression was nearly identical in the two groups. 15-LOX-1 and CD36 showed the highest expressions between the targeted genes. Interestingly, the relative gene expression of 15-LOX-1 was 2-fold increased in the peritoneal cells from women with endometriosis compared to women without endometriosis. CD36 and PPARγ, like other tested genes, did not showed significant alteration between the study groups.

5.2.5 Gene expression study of blood

Gene expression of cytokeratin 8, IFITM-1, COX-2, 15-LOX-1, 15-LOX-2, PPARγ, CD36 and GAPDH were investigated in peripheral blood from women with (n=13) and without (n=7) endometriosis to obtain information about the expression of these genes in leukocytes at systemic level.

The expression of endogenous reference gene was not different between the study groups hence it was used subsequently to calculate the relative gene expression of genes of interests. Figure 5.2.24 demonstrate the distribution of Ct values and the relative mRNA concentration of GAPDH of subjects within the study groups.

(A)



(B)

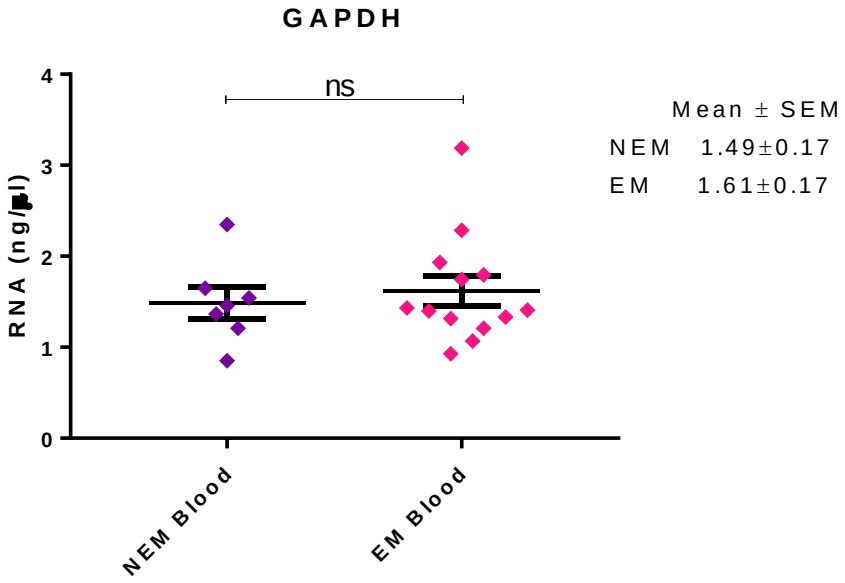


Figure 5.2.24. Gene expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) quantified in peripheral blood from women not diagnosed with endometriosis (NEM, n=7), women diagnosed with endometriosis (EM, n=13). (A) Threshold cycle (Ct) values of GAPDH. (B) Relative mRNA concentration of GAPDH (ng/µl). RNA concentrations were determined by interpolated Ct values of unknown from standard curve of calibrator 1 using 4-parameter non-linear regression model. Data are expressed as mean ± SEM. Statistical comparisons were performed using Mann-Whitney U test. (ns) not significant

Similarly to peritoneal cells, because of the low numbers of subjects in the study groups comparisons have not been performed by menstrual cycle and medical treatments. The statistical analysis did not reveal significant alteration for the expression of genes of interest in blood between the endometriosis and non-endometriosis groups. Furthermore, the means of mRNA concentrations and the means of relative gene expressions were very alike in the two study groups. Table 5.2.5 summarize the means of relative mRNA concentration and relative gene expression of genes of interest in blood from patients with and without endometriosis.

Table 5.2.5. Gene expression in peripheral blood from women not diagnosed with endometriosis (NEM, n=7), and from women diagnosed with endometriosis (EM, n=13). Assay was performed using the SYBR Green qRT-PCR method normalized to 360ng total RNA. RNA concentrations were determined by interpolated Ct values of unknown from standard curve using 4-parameter non-linear regression model. Data are expressed as mean ± SEM. Statistical comparisons were carried out using D'Agostino & Pearson normality test followed with Mann-Whitney U test. None of the genes of interest showed significant alteration between the two study groups. (ND) not detected

Blood	Relative mRNA concentration (ng/µl) (mean±SEM)		Relative gene expression (mean±SEM)	
Targets	**NEM (n=7)**	**EM (n=13)**	**NEM (n=7)**	**EM (n=13)**
GAPDH	**1.49** ± 0.17	**1.61** ± 0.17	-	-
CK8	ND	ND	ND	ND
IFITM-1	**0.90** ± 0.19	**1.10** ± 0.15	**0.63** ± 0.13	**0.78** ± 0.14
COX-2	**1.19** ± 0.19	**1.63** ± 0.20	**0.82** ± 0.12	**1.10** ± 0.19
15-LOX-1	**1.25** ± 0.28	**1.59** ± 0.51	**0.87** ± 0.19	**1.11** ± 0.32
15-LOX-2	**0.58** ± 0.14	**0.61** ± 0.13	**0.41** ± 0.09	**0.38** ± 0.06
PPARγ	**0.04** ± 0.01	**0.05** ± 0.01	**0.02** ± 0.004	**0.02** ± 0.004
CD36	**1.19** ± 0.15	**1.57** ± 0.24	**0.82** ± 0.09	**0.98** ± 0.11

(GAPDH) glyceraldehyde-3-phosphate dehydrogenase; (CK8) cytokeratin 8; (IFITM-1) interferon induced transmembrane protein 1; (COX) cyclooxygenase; (15-LOX) 15-lipoxygenase; PPARγ-peroxisome proliferator-activated receptor gamma

Cytokeratin 8 showed the lowest expression in peritoneal cells whereas in the case of blood, PPARγ showed the lowest expression, whilst cytokeratin 8 did not reach the limit of detection. Surprisingly, 15-LOX-2 was present in every subject, although it was expressed at a low level. 15-LOX-1 and COX-2 showed the highest expressions. CD36 and IFITM-1 were also detected. The expression of COX-2, 15-LOX-1 and IFITM-1 showed slight increases in the endometriosis group compared to non-endometriosis patients however, these alterations were not significant.

5.2.6 Summary of the results of gene expression study

SYBR Green qRT-PCT assays were used to study the expression of target genes in different tissue specimens and cells from women with and without endometriosis to gain insight the possible roles of these genes in the pathomechanism of endometriosis. Table 5.2.6 and 5.2.7 summarize the mRNA concentrations and relative gene expressions for the genes of interests in the studied specimens. Bold figures indicate the highest values between the study groups.

Table 5.2.6. Summary of relative mRNA concentrations of gene of interest in different tissue specimens.
Eutopic endometrium, peritoneal cells and blood from women with (EM) and without endometriosis (NEM) and in ectopic lesions and peritoneal wall specimens from women with endometriosis were used for this study. Assay was performed using SYBR Green qRT-PCR method normalized to 360ng total RNA. RNA concentrations were determined by interpolated Ct values of unknown from standard curve using 4-parameter non-linear regression model. Data are expressed as mean ± SEM. Statistical comparisons were carried out using D'Agostino & Pearson normality test followed with Mann-Whitney U test or Kruskal-Wallis test with Dunn's post-hoc test as appropriate. (ND) not detected; (a;b) significant; bold numbers highlights the highest values

mRNA (ng/ml) mean ± SEM	Eutopic endometrium		Ectopic lesion	Peritoneal wall	Peritoneal cells		Blood	
	NEM	EM	EM	EM	NEM	EM	NEM	EM
Targets	**n=24**	**n=27**	**n=14**	**n=8**	**n=8**	**n=7**	**n=7**	**n=13**
GAPDH	1.40 ± 0.32	1.04 ± 0.22	0.56 [a] ± 0.18	0.06[a] ± 0.02	2.19 ± 0.44	1.70 ± 0.36	1.49 ± 0.17	1.61 ± 0.17
CK8	**0.11** [b] ± 0.04	**0.15** [a] ± 0.07	0.01 [ab] ± 0.002	0.003 ± 0.001	0.04 ± 0.01	0.02 ± 0.01	ND	ND
IFITM-1	0.77 ± 0.24	0.56 ± 0.11	0.53 ± 0.18	0.13 ± 0.05	0.81 ± 0.18	0.42 ± 0.08	**0.90** ± 0.19	**1.10** ± 0.15
COX-2	**2.07** ± 0.83	**1.41** ± 0.33	0.80 ± 0.33	0.17 ± 0.05	0.37 ± 0.09	0.31 ± 0.08	1.19 ± 0.19	**1.63** ± 0.20
15-LOX-1	0.13 ± 0.02	0.16 ± 0.04	0.07 ± 0.02	0.07 ± 0.02	**1.90** ± 0.88	**2.33** ± 0.94	1.25 ± 0.28	1.59 ± 0.51
15-LOX-2	ND	ND	ND	ND	ND	ND	0.58 ± 0.14	0.61 ± 0.13
PPARγ	0.36 ± 0.08	0.28 ± 0.07	0.33 ± 0.07	0.20 ± 0.05	**1.53** ± 0.40	**1.68** ± 0.74	0.04 ± 0.01	0.05 ± 0.01
CD36	0.05 [b] ± 0.02	0.13 [a]± 0.07	0.19 [ab] ± 0.06	0.11 ± 0.03	**4.40** ± 1.70	**4.27** ± 1.85	1.19 ± 0.15	1.57 ± 0.24

(GAPDH) glyceraldehyde-3-phosphate dehydrogenase; (CK8) cytokeratin 8; (IFITM-1) interferon induced transmembrane protein 1; (COX) cyclooxygenase; (15-LOX) 15-lipoxygenase; PPARγ-peroxisome proliferator-activated receptor gamma

Table 5.2.7. Summary of relative gene expression of gene of interest in different tissue specimens.
Eutopic endometrium, peritoneal cells and blood from women with (EM) and without endometriosis (NEM) and in ectopic lesions and peritoneal wall specimens from women with endometriosis were used for this study. Assay was performed using SYBR Green qRT-PCR method normalized to 360 ng total RNA. RNA concentrations were determined by interpolated Ct values of unknown from standard curve using 4-parameter non-linear regression model. Data are expressed as mean ± SEM. Statistical comparisons were carried out using D'Agostino & Pearson normality test followed with Mann-Whitney U test or Kruskal-Wallis test with Dunn's post-hoc test as appropriate. (ND) not detected; (a;b) significant; bold numbers highlights the highest values

Relative gene expression (mean ± SEM)	Eutopic endometrium		Ectopic lesion	Peritoneal wall	Peritoneal cells		Blood	
	NEM	EM	EM	EM	NEM	EM	NEM	EM
Targets	**n=24**	**n=27**	**n=14**	**n=8**	**n=8**	**n=7**	**n=7**	**n=13**
CK8	**0.11** [b] ± 0.03	**0.18** [a] ± 0.05	0.03 [ab] ± 0.01	-	0.03 ± 0.01	0.02 ± 0.02	ND	ND
IFITM-1	0.74 [a] ± 0.14	0.78 ± 0.10	**1.16** [a] ± 0.15	-	0.49 ± 0.14	0.27 ± 0.04	0.63 ± 0.13	0.78 ± 0.14
COX-2	**3.04** ± 1.17	**3.56** ± 1.08	**3.31** ± 1.74	-	0.21 ± 0.07	0.19 ± 0.03	0.82 ± 0.12	1.10 ± 0.19
15-LOX-1	0.22 ± 0.06	0.49 ± 0.13	0.37 ± 0.19	-	**1.22** ± 0.64	**2.40** ± 1.45	0.87 ± 0.19	1.11 ± 0.32
15-LOX-2	ND	ND	ND	-	ND	ND	0.41 ± 0.09	0.38 ± 0.08
PPARγ	0.56 [b] ± 0.15	0.36 [a] ± 0.05	**1.60** [ab] ± 0.46	-	0.54 ± 0.09	0.68 ± 0.16	0.02 ± 0.004	0.02 ± 0.004
CD36	0.08 [b] ± 0.02	0.14 [a] ± 0.04	**2.80** [ab] ± 1.38	-	**1.82** ± 0.49	**2.14** ± 0.68	0.82 ± 0.09	0.98 ± 0.11

(GAPDH) glyceraldehyde-3-phosphate dehydrogenase; (CK8) cytokeratin 8; (IFITM-1) interferon induced transmembrane protein 1; (COX) cyclooxygenase; (15-LOX) 15-lipoxygenase; PPARγ-peroxisome proliferator-activated receptor gamma

Comparing the expressions of gene of interest in ectopic lesions from women with endometriosis to eutopic endometrium from women with and without endometriosis it was found that cytokeratin 8, IFITM-1, PPARγ and CD36 were significantly altered between the study groups.

No significant differences for the targeted gene expression in peritoneal cells and blood specimens were found.

The highest relative gene expression for cytokeratin 8 was detected in eutopic endometrium. The expression was significantly decreased in ectopic lesions compared to eutopic endometrium and the expression was also low in peritoneal wall and peritoneal cell specimens and was below the limit of detection in blood.

The highest relative gene expression for IFITM-1 was found in ectopic lesions whilst the lowest mRNA concentration was measured in peritoneal wall biopsies. The relative expressions were found alike in eutopic endometrium and blood specimens.

The expression of COX-2 was upregulated in eutopic endometrium in both study groups likewise in ectopic lesions. Peritoneal cells and blood cells also expressed COX-2 however statistically significant alterations were not observed between the study groups.

The highest relative gene expression for 15-LOX-1 was found in peritoneal cells from women with endometriosis and the lowest in eutopic endometrium from women without endometriosis. Significant alterations for 15-LOX-1 expression were not found.

Apart from blood, 15-LOX-2 expression was below the limit of detection in all specimens.

The highest relative gene expression for PPARγ was detected in ectopic lesions. The relative expression of PPARγ was significantly elevated in ectopic lesions compared to eutopic endometrium from women with and without endometriosis. The lowest relative expression for PPARγ was observed in blood.

CD36 was significantly upregulated in ectopic lesions compared to eutopic endometrium from women with and without endometriosis. Peritoneal cells also highly expressed CD36. Moreover the highest mRNA concentrations were measured in these specimens.

5.2.7 Key findings of gene expression study

As described above the comparisons of gene expression classified by presence or absence of endometriosis showed significant alterations between eutopic endometrium and ectopic lesions for the targeted genes. Further analysis of data according to the regularity of menstrual cycle and the usage of GnRH agonists also revealed significant variations between the study groups.

To demonstrate and summarize the relative changes of the targeted genes, ratios were calculated using the means of relative gene expression for appropriate targeted genes, patient groups and specimens. Table 5.2.8 presents the relative gene expression ratios for the analysed genes.

What is striking in this table is the robust upregulation of COX-2, 15-LOX-1, PPARγ and CD36 in ectopic lesions from endometriosis women taking GnRH agonists for their condition.

Table 5.2.8. Relative gene expression ratios for targeted genes classified by different conditions.
(A) All data represent the relative gene expression ratios between the groups of endometriosis (EM) and non-endometriosis (NEM) by tissue specimen, such as eutopic endometrium (EU), ectopic lesions (EC), peritoneal cells (PC) and blood (BL). (B) Regular period represents the ratios by targeted genes for patients who reported having a regular period and were not subjected to hormone treatment prior to the surgery. (C) Treatment illustrates the relative changes in gene expressions for genes of interests between endometriosis patients those who received hormonal treatment (T) prior the surgery and endometriosis patients who had not received medical treatment or used hormonal contraceptives and had a normal, regular period (N). Ratios were calculated using means of relative gene expression for appropriate targeted genes, patient groups and specimens. Arrows note the ratios where the relative change showed at least ±80% alteration in gene expression between the compared groups.

(A) Relative Gene Expression Ratios All data	$\frac{\mathbf{EU(EM)}}{\mathbf{EU(NEM)}}$	$\frac{\mathbf{EC\ (EM)}}{\mathbf{EU(EM)}}$	$\frac{\mathbf{EC\ (EM)}}{\mathbf{EU(NEM)}}$	$\frac{\mathbf{PC\ (EM)}}{\mathbf{PC\ (NEM)}}$	$\frac{\mathbf{BL\ (EM)}}{\mathbf{BL\ (NEM)}}$
CK8	1.6	0.2 ↓	0.3	0.7	ND
IFITM-1	1.0	1.5	1.6	0.6	1.2
COX-2	1.2	0.9	1.1	0.9	1.3
15-LOX-1	2.2 ↑	0.8	1.7	2.0 ↑	1.3
15-LOX-2	ND	ND	ND	ND	0.9
PPARγ	0.6	4.4 ↑	2.9 ↑	1.3	1.0
CD36	1.8 ↑	20.0 ↑	35.0 ↑	1.2	1.2
(B) Relative Gene Expression Ratios Regular Period	$\frac{\mathbf{EU_N\ (EM)}}{\mathbf{EU_N\ (NEM)}}$	$\frac{\mathbf{EC_N\ (EM)}}{\mathbf{EU_N\ (EM)}}$	$\frac{\mathbf{EC_N\ (EM)}}{\mathbf{EU_N\ (NEM)}}$		
CK8	1.3	0.1 ↓	0.1 ↓		
IFITM-1	1.1	1.3	1.4		
COX-2	0.7	0.7	0.5		
15-LOX-1	0.5	0.7	0.4		
15-LOX-2	ND	ND	ND		
PPARγ	0.4	1.2	0.5		
CD36	1.8 ↑	0.4	0.8		
(C) Relative Gene Expression Ratios Treatments	$\frac{\mathbf{EU_T\ (EM)}}{\mathbf{EU_N\ (EM)}}$	$\frac{\mathbf{EU_T\ (EM)}}{\mathbf{EU_N\ (NEM)}}$	$\frac{\mathbf{EC_T\ (EM)}}{\mathbf{EC_N\ (EM)}}$	$\frac{\mathbf{EC_T\ (EM)}}{\mathbf{EU_N\ (EM)}}$	$\frac{\mathbf{EC_T\ (EM)}}{\mathbf{EU_N\ (NEM)}}$
CK8	1.4	1.7	4.0 ↑	0.3	0.4
IFITM-1	1.1	1.3	1.3	1.7	1.9 ↑
COX-2	2.5 ↑	1.7	4.9 ↑	3.6 ↑	2.5 ↑
15-LOX-1	5.3 ↑	2.6 ↑	8.3 ↑	6.0 ↑	3.0 ↑
15-LOX-2	ND	ND	ND	ND	ND
PPARγ	1.4	0.6	7.7 ↑	9.0 ↑	3.7 ↑
CD36	0.7	1.2	68.6 ↑	30.5 ↑	54.9 ↑

(ND) not detected; (GAPDH) glyceraldehyde-3-phosphate dehydrogenase; (CK8) cytokeratin 8; (IFITM-1) interferon induced transmembrane protein 1; (COX) cyclooxygenase; (15-LOX) 15-lipoxygenase; PPARγ-peroxisome proliferator-activated receptor gamma

5.3 Immunohistochemical analysis of endometriotic tissue

Immunohistochemical analysis was performed to detect selected targets in endometrium, ectopic lesions and the peritoneal wall. Fifteen endometrial biopsies were obtained from endometriotic (n=7) and non-endometriotic (n=8) women. Six peritoneal wall biopsies and thirteen ectopic endometrial lesions, involving nine peritoneal endometriosis, two ovarian cysts and two DIE specimens were collected during the laparoscopic surgery from women with endometriosis and used in this study. The biopsies included five paired samples where the ectopic lesion and eutopic endometrium were obtained from the same subject. Stage of menstrual cycle was reported by patients and were confirmed histologically where eutopic endometrial samples were available. Table 5.3.1 summarizes the stage of menstrual cycle of patients whose specimens were used for IHC analysis. Expression of COX-2, 15-LOX-1, PPARγ, CD36, cytokeratin 8, 18 and IFITM-1 were investigated using a semi-quantitative scoring method. Anti-cytokeratin 8, 18 and anti-IFITM-1 antibodies were utilised as gland and stromal markers and facilitating the identification of glandular and stromal cells in ectopic lesions. 4μm paraffin embedded sections were stained using IHC method as described in sections 2.4.4.2.

Table 5.3.1. Stage of menstrual cycles of subjects whose specimens were used for immunohistochemical analysis.
Hormone usage illustrates the number of participant who taken hormone as a treatment or contraception. (N) number of participants; (NEM) non-endometriosis; (EM) endometriosis; (Eu) eutopic endometrium; (Ec) ectopic lesion; (Pw) peritoneal wall

		Stage of Menstrual cycle			Hormone usage
	N	Regular	Non-regular	Non-cycling	
NEM Eu	8	4	3	1	0/8
EM Eu	7	2	1	4	5/7
EM Ec	13	6	1	6	7/13
EM Pw	6	1	1	4	5/6

5.3.1 Cytokeratin

Cytokeratin is a reliable and widely used marker for the identification of glandular epithelium as it builds up the intermediate filaments of cytoskeleton in epithelial cells (Rekhtman and Bishop, 2011). A mixture of anti-cytokeratin 8 and 18 monoclonal antibodies were used to detect the two main classes of cyokeratins. Cytokeratin 18 represented the acetic, type I cyokeratins, whereas cytokeratin 8 belongs to the class of basic or neutral type II cyokeratins.

In the first instance, anti-cytokeratin 8 and 18 antibodies were applied on human endometrium and validated to see whether the antibody was capable distinguishing the glandular and stromal cells of endometrium. Seven endometriotic and eight non-endometriotic eutopic endometrial samples were tested. Glands and stroma were analysed separately. Figure 5.3.1 illustrates the cytokeratin staining of eutopic endometrium. Figure 5.3.2 compares the histoscores of glands and stroma for cytokeratin. Scoring method was described in section 2.4.4.2

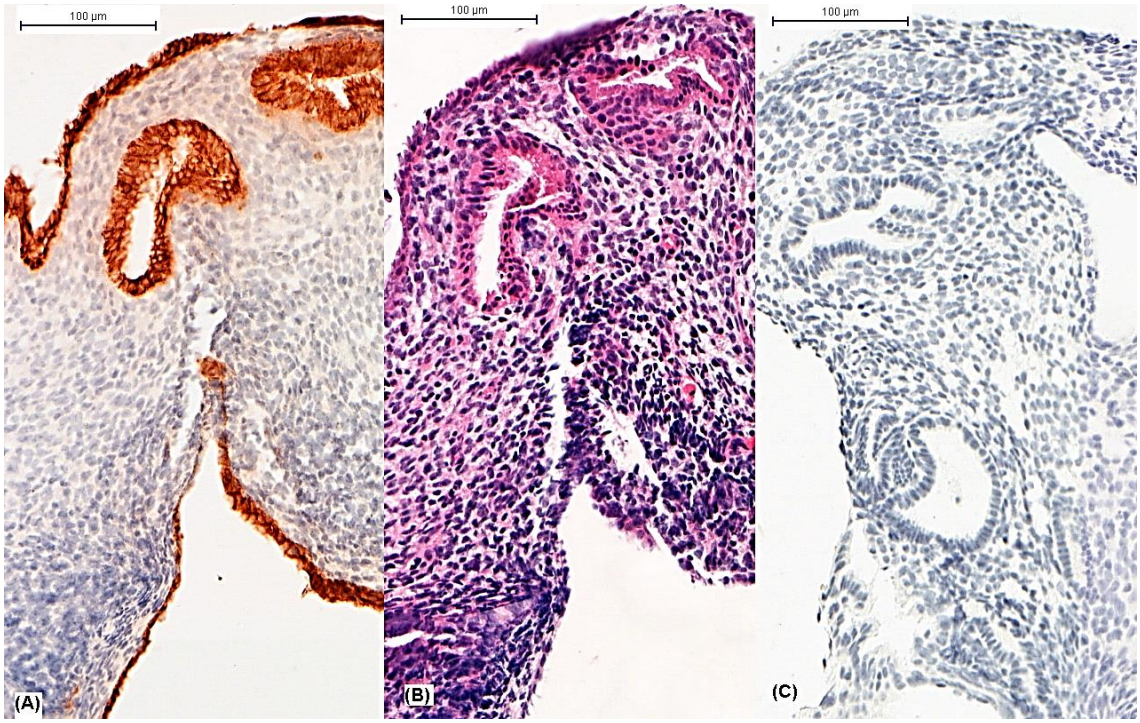


Figure 5.3.1. Cytokeratin expression in endometrium, Patient HP34.
(A) Cytokeratin expression. Glands show a strong immunoreactivity against cytokeratin, whilst stroma was negative. Cytokeratin staining. (B) Morphology of endometrium, H&E. (C) Negative control for cytokeratin staining. No staining was observed in the negative control. Haematoxylin. Scale bar represents 100 µm.

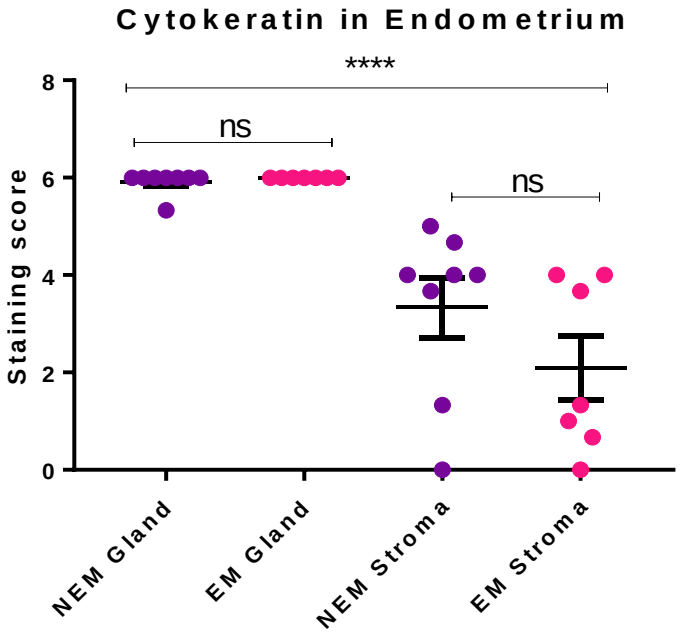


Figure 5.3.2. Immunohistochemistry analysis of cytokeratin expression in eutopic endometrium.
*Glandular and stromal cells were analysed from women not diagnosed with endometriosis (NEM, n=8) and women diagnosed with endometriosis (EM, n=7). Cytokeratin immunoreactivity was significantly higher in glands compared to stroma. Dots present the staining scores of each section. Bars represent mean ± SEM. Comparisons made using Kruskal-Wallis test with Dunn's post-hoc test. ****p<0.0001, (ns) not significant*

Cytokeratin was highly expressed in glands and was low or missing in the stromal cells of the endometrium. Statistical comparison of staining scores confirmed the expression of cytokeratin in glands was significantly higher ($p < 0.0001$) than in stroma. The comparison also indicated that endometriosis has no effect on the expression of cytokeratin. Neither glands nor stroma were significantly different compared to women with and without endometriosis. The experiment confirmed that the antibodies were immunoreactive against cytokeratin and it is an ideal marker to identify the presence of endometrial glands.

Cytokeratin staining was applied on ectopic lesions and the peritoneal wall samples from endometriotic patients to detect glandular cells in the tissue specimens. As described in section 1.2.2 the morphology of ectopic lesions is not always obvious and well-organised. Glands are frequently sparse or even absent. Therefore, cytokeratin staining was used to facilitate the identification of glands in the ectopic lesions. Figure 5.3.3 illustrates a well-differentiated and an undifferentiated lesion.

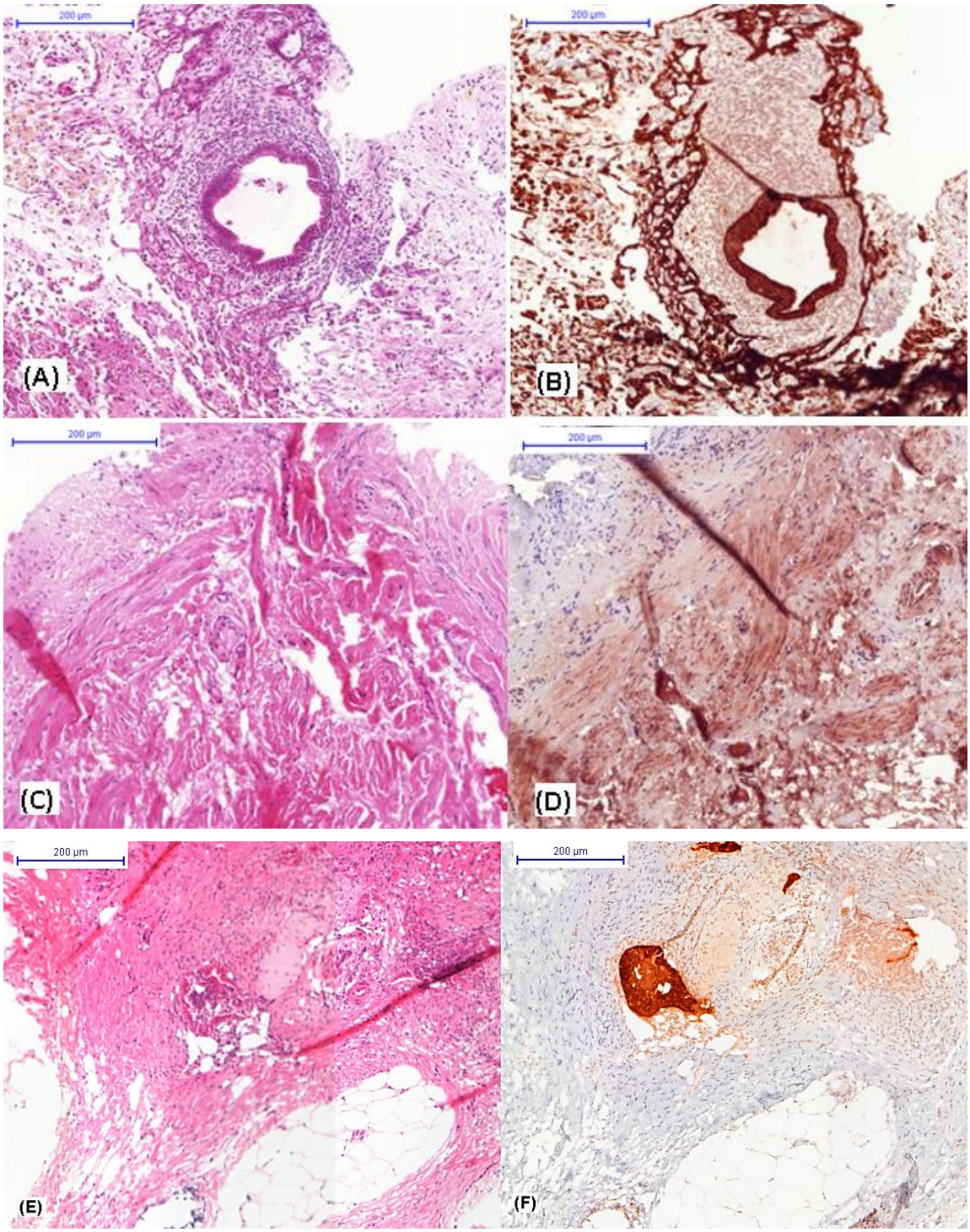


Figure 5.3.3. Cytokeratin staining of ectopic lesions.
(A) Well-differentiated ectopic lesion, Patient HP45. Endometrial-like gland surrounded with stromal cells, H&E staining. (B) Glands were strongly immunoreactive to cytokeratin. Cytokeratin staining. (C) Undifferentiated lesions, Patient HP32. H&E. (D) Cells were moderately positive to cytokeratin and partly showed glandular morphology. (E) Ectopic lesion, Patient HP34, H&E staining. (F) Glands were strongly immunoreactive to cytokeratin. Cytokeratin staining, scale bar represents 200 µm.

Figure 5.3.1 and 5.3.3 demonstrate that the histological morphology of eutopic endometrium and ectopic lesions were very different. Glands and stroma were dominated in eutopic endometrium whereas other cell types, such as fibroblasts or smooth muscle also were present beside the endometrial-like cells in the ectopic lesions therefore, the comparison of eutopic and ectopic lesion was challenging. To overcome this problem the arithmetic mean of histoscores for eutopic gland and stroma were calculated and compared to the histoscore of ectopic lesion or peritoneal wall. Figure 5.3.4 shows the comparison of histoscores of ectopic lesions to eutopic endometrial samples.

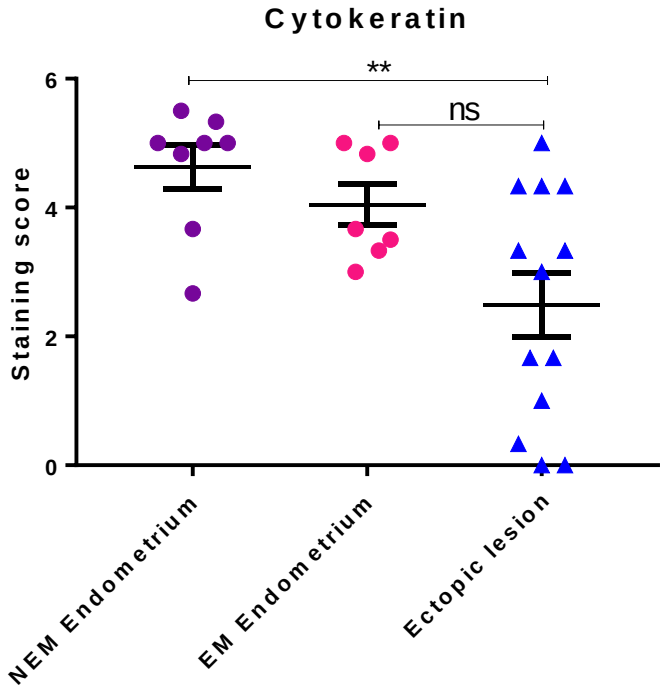
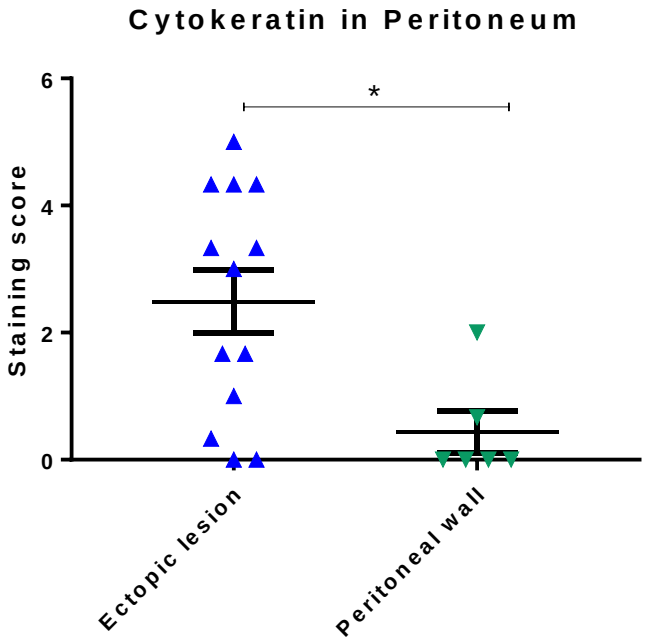


Figure 5.3.4. Immunohistochemistry analysis of cytokeratin expression in eutopic endometrium and ectopic lesions.
*Dots represent the arithmetic mean of histoscores of eutopic gland and stroma from women not diagnosed with endometriosis (NEM, n=8) and women diagnosed with endometriosis (EM, n=7) compared to histoscores of ectopic lesions (blue triangles, n=13). There was no significant difference between eutopic endometrium from women with endometriosis and ectopic lesion with regards of cytokeratin expression. Cytokeratin expression was significantly elevated in endometrium from non-endometriotic subjects compared to ectopic lesions. Comparisons made using Kruskal-Wallis test with Dunn's post-hoc test. **p<0.01, (ns) not significant, bars represent mean ± SEM.*

Cytokeratin expression was significantly higher in eutopic endometrium from women without endometriosis compared to the ectopic lesions. No significant immunoreactivity was demonstrated between eutopic endometrium from women with endometriosis and ectopic lesions. Seven ectopic specimens showed similar immunoreactivity, while six showed decreased cytokeratin expression in the ectopic lesion compared to eutopic endometrium from women with endometriosis. Grouping of ectopic lesions according to lesion types revealed that the cytokeratin expression was lower in endometrioma and DIE specimens compared to peritoneal lesions (Figure 5.3.5B).

Cytokeratin expression also was investigated in peritoneal wall biopsies. As was expected, the immunoreactivity of cytokeratin was very low. Figure 5.3.5A illustrates the cytokeratin expression of peritoneal wall specimens compared to ectopic lesions. Figure 5.3.5B demonstrates the cytokeratin expression in ectopic lesions plotted by lesion types.

(A)



(B)

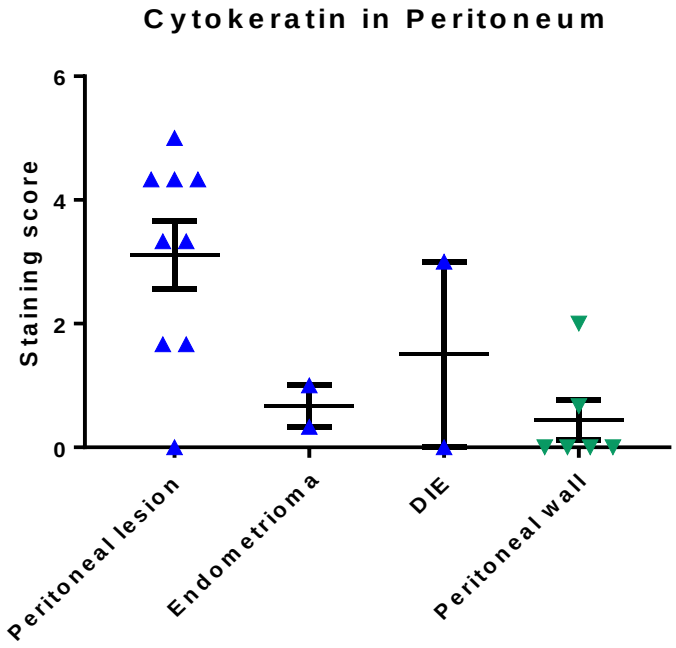


Figure 5.3.5. Immunohistochemistry analysis of cytokeratin expression in ectopic lesions and peritoneal wall.
*(A) Cytokeratin expression was significantly increased in ectopic lesion (n=13) compared to peritoneal wall (n=6). Comparison was made using Mann-Whitney U-test, *p<0.05. (B) Graph illustrates the cytokeratin expression by endometriosis types compared to peritoneal wall (n=6). Peritoneal lesion (n=9); Endometrioma (n=2); Deep infiltrating endometriosis (DIE), (n=2). Due to the low sample numbers in group of endometrioma and DIE statistical analysis has not been performed.*

5.3.2 Interferon induced transmembrane protein 1 (IFITM-1)

IFITM-1 has been recently described as specific marker of endometrial stroma (Parra-Herran et al., 2014). Two antibodies were tested on eutopic endometrium for specificity and their ability to produce optimal immunoreactivity. Polyclonal rabbit antibodies were obtained from Abcam plc (#ab106265, Cambridge, UK) and Sigma-Aldrich (#HPA004810, Pool, UK). Figure 5.3.6 shows the representative micrographs of Abcam and Sigma antibodies on eutopic endometrium. Figure 5.3.7 illustrates the comparison of the staining scores of tested antibodies.

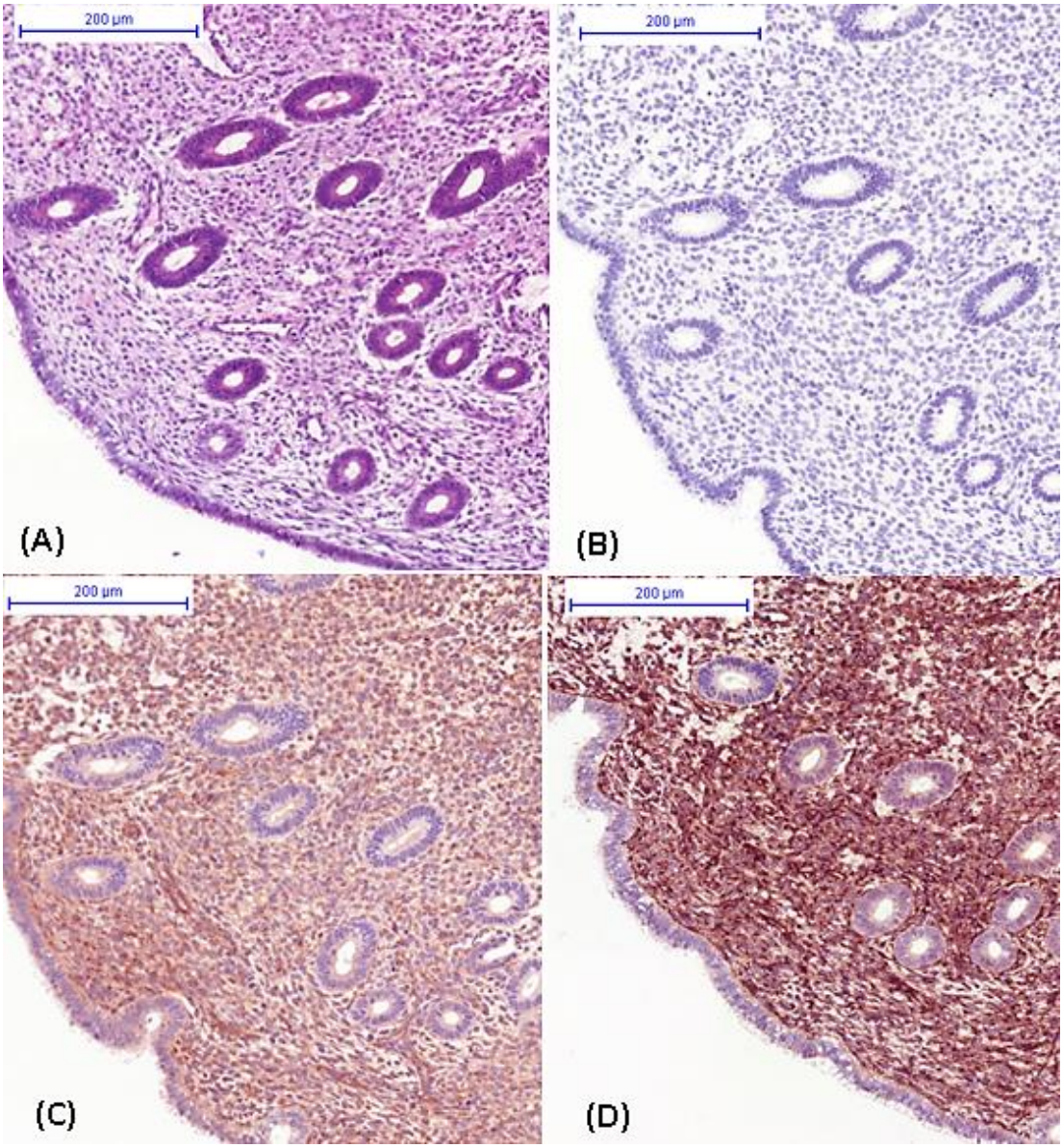
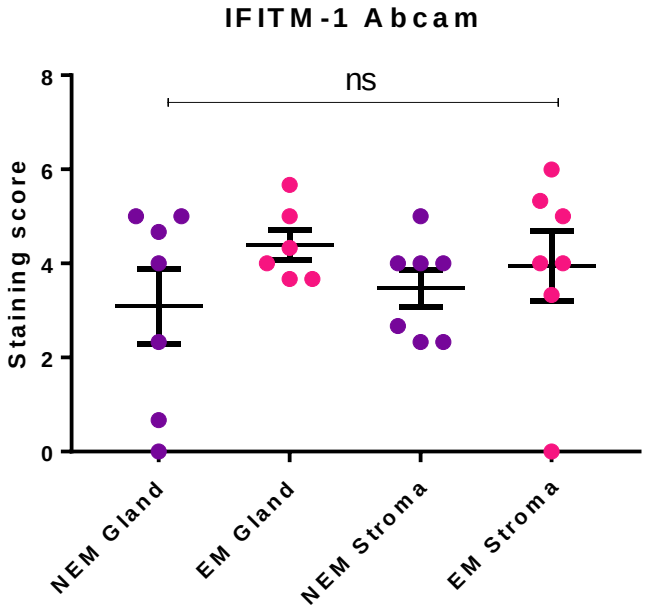


Figure 5.3.6. Staining characteristic of IFITM-1 antibodies.
(A) Morphology of endometrium, Patient HP32, H&E, 10x magnification. (B) Negative control for IFITM-1 staining. No staining was observed in negative control. Haematoxylin staining. (C) Representative micrograph of Abcam antibody. Glands and stroma showed similar immunoreactivity against IFITM-1. (D) Representative micrograph of Sigma antibody. Stroma

strongly immunoreactive, while glands demonstrate weak to moderate staining intensity. IFITM-1 staining, scale bar indicates 200 µm.

(A)



(B)

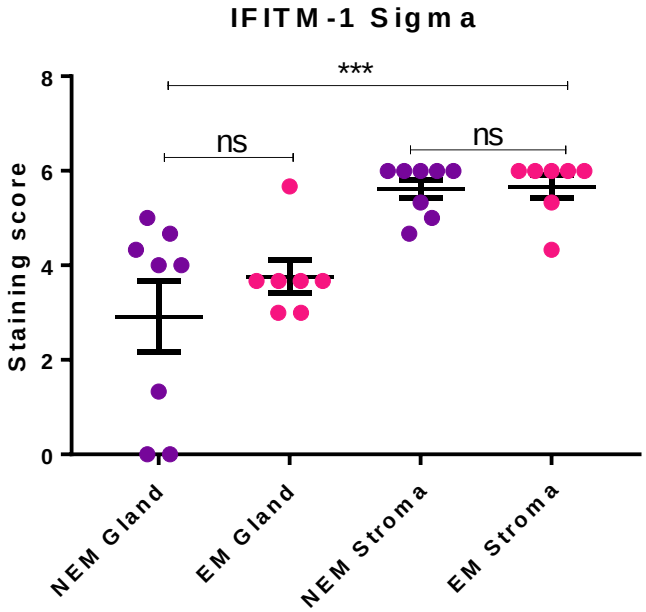


Figure 5.3.7. Comparison of antibody specificity for interferon induced transmembrane protein 1 (IFITM-1) using endometrial specimens.
*(A) Abcam antibody lacked specificity against IFITM-1 in eutopic endometrium. There was no significant difference between gland and stroma from women with (EM, n=7) and without endometriosis (NEM, n=7). (B) Sigma antibody showed high specificity against IFITM-1. IFITM-1 immunoreactivity was strong in stroma and weak or absent in glands. (EM, n=7) (NEM, n=8). Comparisons made using Kruskal-Wallis test with Dunn's post-hoc test. ***p<0.001, (ns) not significant, bars represent mean ± SEM.*

Abcam antibody produced non-specific staining, whilst Sigma antibody showed high specificity against IFITM-1. The immunoreactivity of Sigma antibody was strong in stroma and weak or absent in glandular cells. Abcam antibody failed to demonstrate significant specificity against its target therefore Abcam antibody was eliminated and Sigma antibody was chosen for subsequent use.

IFITM-1 from Sigma was used to detect endometrial-like stromal cells in ectopic lesions and peritoneal wall. Figure 5.3.8 shows representative pictures of immunoreactivity of IFITM-1 on ectopic lesions.

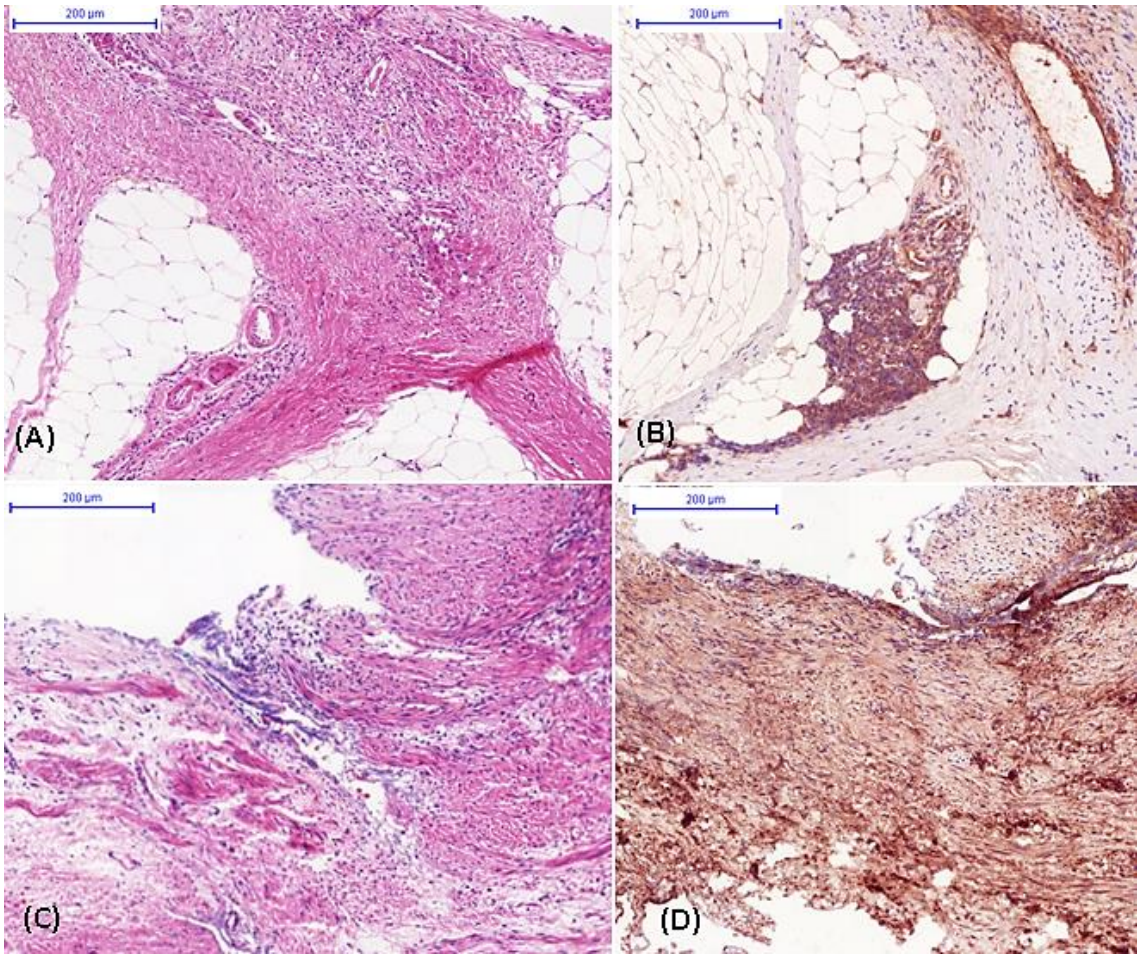


Figure 5.3.8. IFITM-1 staining of ectopic lesions.
(A) Well-differentiated ectopic lesion. Patient HP34, H&E staining. (B) Immunoreactivity of IFITM-1. Stromal cells located around endometrial-like glands. Fibroblast and smooth muscle were negative. Patient HP34, IFITM-1 staining. (C) Undifferentiated ectopic lesion with diffuse staining. Section did not show endometrial morphology. Patient HP24, H&E staining. (D) Immunoreactivity of IFITM-1. Cells showed strong, diffuse immunoreactivity. Patient HP24, IFITM-1 staining. Scale bar represents 200 µm.

Of the ectopic lesions, only 2 out of 13 showed well-organised stromal morphology. The stroma were undifferentiated in 11 specimens. In the case of undifferentiated samples, five sections displayed strong diffuse staining, see Figure 5.3.8 D, three showed patchy staining with a possibility of sparse stromal cells, whilst three were immunoreactive around the vessels, particularly the endothelium of vessels were strongly positive. Interestingly, a similar pattern was observed in peritoneal wall. The endothelium of the vessels in peritoneal wall were strongly positive for IFITM-1. Figure 5.3.9 illustrates a representative micrograph for IFITM-1 immunoreactivity in peritoneal wall.

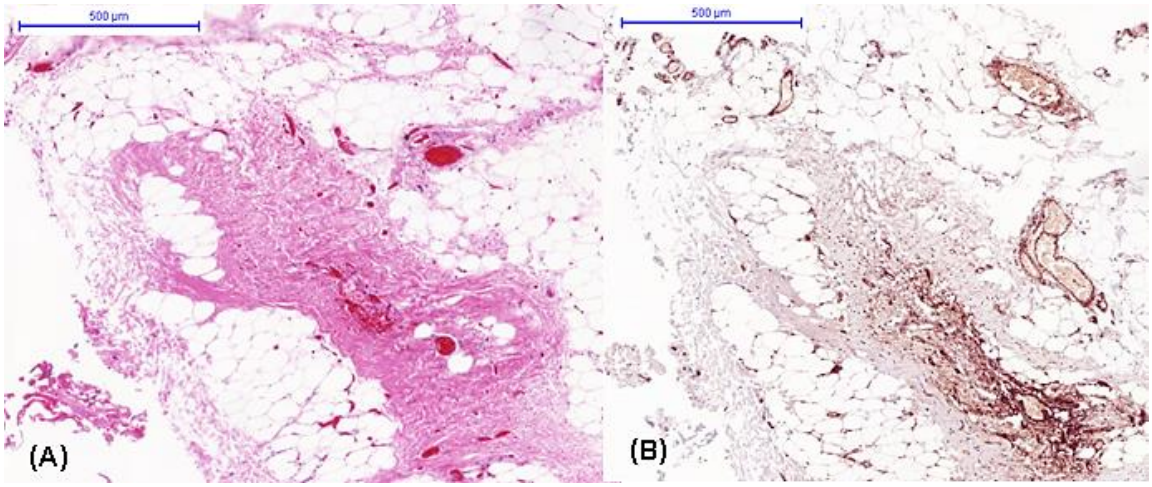


Figure 5.3.9. IFITM-1 staining of peritoneal wall.
(A) Peritoneal wall, Patient HP50, H&E staining. (B) Immunoreactivity of IFITM-1. Vessel endothelium and surrounded connective, reticuloid tissue showed strong immunoreactivity. IFITM-1 staining, scale bar represents 500 µm.

Histoscores of endometrium were compared to histoscores of ectopic lesions and peritoneal wall. Similarly to cytokeratin, the arithmetic mean of histoscores for eutopic gland and stroma were calculated and compared to the histoscores of ectopic lesion or peritoneal wall. Figure 5.3.10 shows the comparison of histoscores between eutopic endometrium and ectopic lesions. Figure 5.3.11 illustrates the comparison of histoscores between ectopic lesions and peritoneal wall.

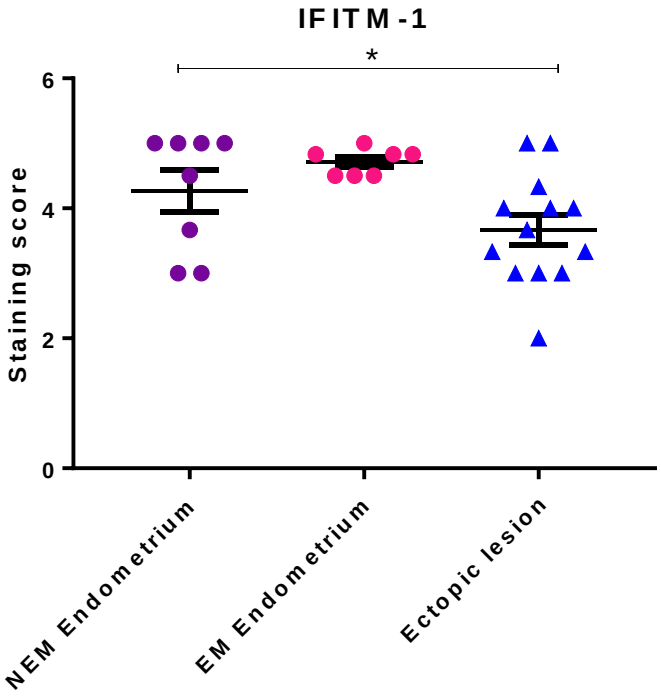
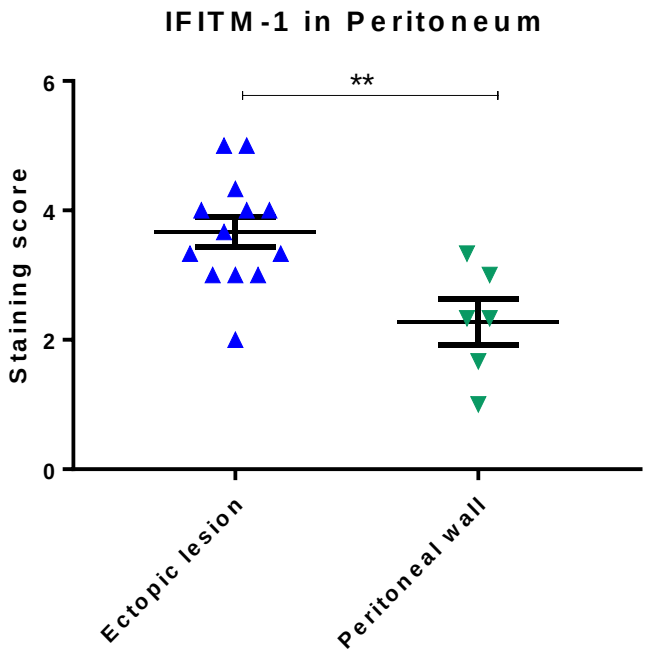


Figure 5.3.10. Immunohistochemistry analysis of interferon induced transmembrane protein 1 (IFITM-1) expression in eutopic endometrium and ectopic lesions.
*Dots represent the arithmetic mean of histoscores of eutopic gland and stroma from women not diagnosed with endometriosis (NEM, n=8) and women diagnosed with endometriosis (EM, n=7) compared to histoscores of ectopic lesions (blue triangles, n=13). Although, the Kruskal-Wallis test revealed significant differences between the three tested groups the Dunn,s post hoc test have not confirmed it. *p<0.05, bars represent mean ± SEM.*

(A)



(B)

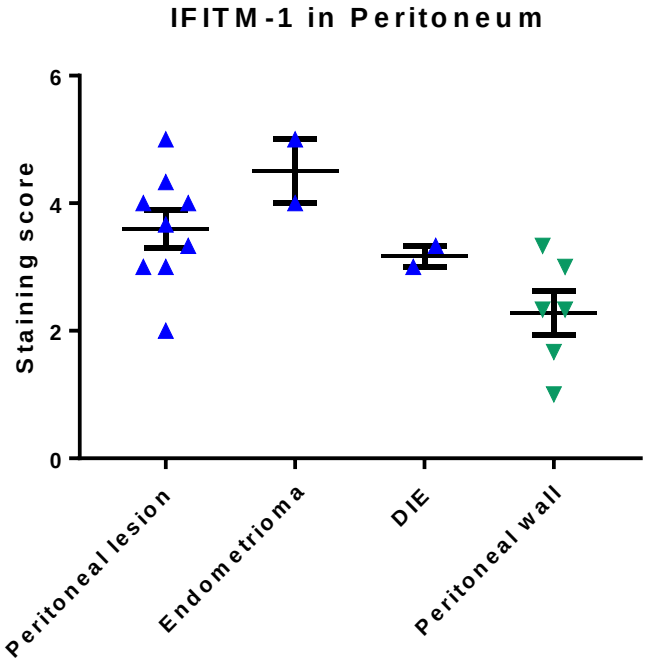


Figure 5.3.11. Immunohistochemistry analysis of interferon induced transmembrane protein 1 (IFITM-1) expression in ectopic lesions and peritoneal wall.
*(A) IFITM-1 expression was significantly increased in ectopic lesions (n=13) compared to peritoneal wall (n=6). Comparison was made using unpaired t-test, **p<0.01. (B) Graph illustrates the IFITM-1 expression by endometriosis types compared to peritoneal wall (n=6). Peritoneal lesion (n=9); Endometrioma (n=2); Deep infiltrating endometriosis (DIE), (n=2). Due to the low sample numbers in group of endometrioma and DIE statistical analysis has not been performed.*

There was no significant difference between IFITM-1 expression in eutopic endometrium from women with and without endometriosis and ectopic lesion. Although, the statistical comparison passed the significance threshold (p=0.0495), the post hoc analysis did not show significant difference between the three groups.

The comparison of ectopic lesions and peritoneal wall biopsies showed that the IFITM-1 expression was significantly higher in ectopic lesions than in peritoneal wall (p=0.0069). Although only 2 samples were assessed, the highest IFITM-1 expression was observed in the endometrioma specimens (Figure 5.3.11B).

5.3.3 Cyclooxygenase 2 (COX-2)

Studies suggest that COX-2 is a key regulator of the pathomechanisms of endometriosis. It is overexpressed in eutopic endometrium and ectopic lesions in women with endometriosis (Ota et al., 2001; Chishima et al., 2002). Hence, COX-2 expression was investigated in eutopic endometrium, ectopic lesion and peritoneal wall specimens. Figure 5.3.12 illustrates the COX-2 staining in eutopic endometrium. Figure 5.3.13 compares the histoscores of glands and stroma for COX-2.

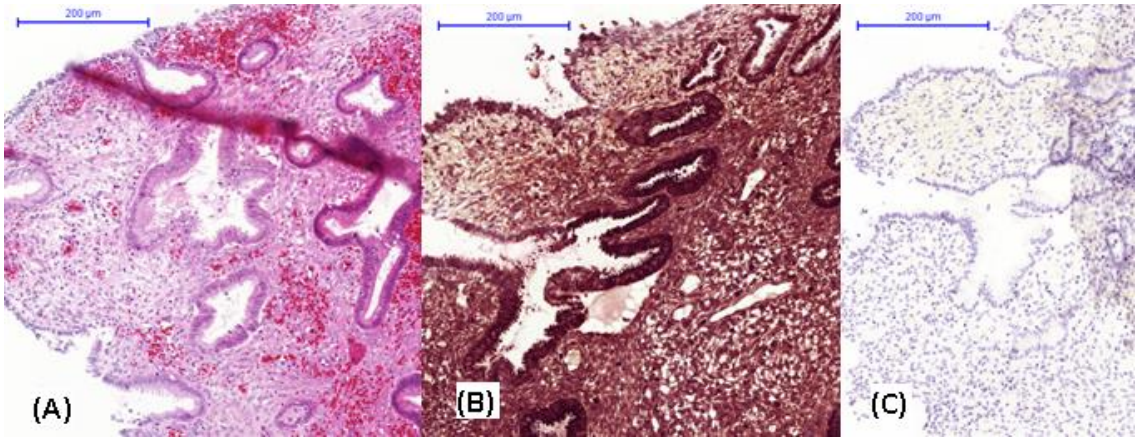


Figure 5.3.12. Representative micrograph of COX-2 expression in eutopic endometrium, Patient HP42.
(A) Morphology of endometrium, H&E staining. (B) COX-2 expression. Glands and stroma show strong, diffuse immunoreactivity against COX-2. COX-2 staining. (C) Negative control for COX-2 staining. No staining was observed in negative control. Haematoxylin. Scale bar represent 200 µm.

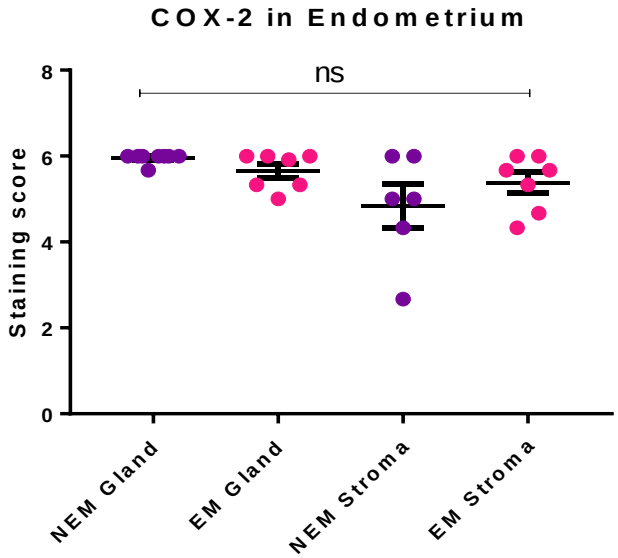


Figure 5.3.13. Immunohistochemistry analysis of cyclooxygenase 2 (COX-2) expression in eutopic endometrium.
Glandular and stromal cells were analysed from women not diagnosed with endometriosis (NEM, n=8) and women diagnosed with endometriosis (EM, n=7). There were no significant

difference between gland and stromal cells. Dots present the staining scores of each section. Bars represent mean ± SEM. Comparisons made using Kruskal-Wallis test with Dunn's post-hoc test. (ns) not significant

COX-2 expression showed consistently strong and diffuse immunoreactivity in eutopic endometrium. There was no different neither between glands and stroma nor between the group of endometriosis and non-endometriosis patients. COX-2 expression also was investigated in ectopic lesions and peritoneal wall. Figure 5.3.14 and 5.3.15 demonstrate the immunoreactivity of COX-2 in ectopic lesions and peritoneal wall. Figure 5.3.16 shows the comparison of histoscores between eutopic endometrium and ectopic lesions. Figure 5.3.17 illustrates the comparison of histoscores between ectopic lesions and peritoneal wall.

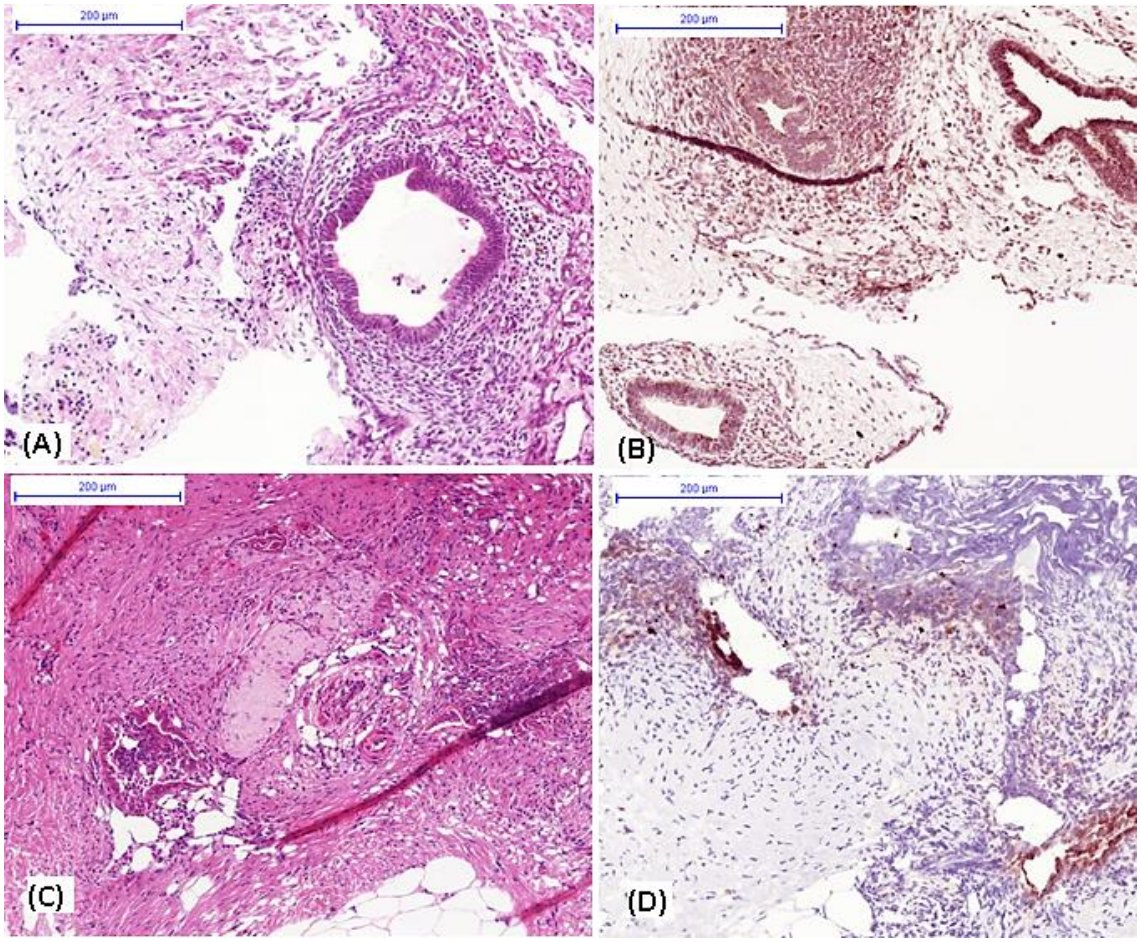


Figure 5.3.14. COX-2 expression in ectopic lesions.
(A) Patient HP45 ectopic lesion, H&E staining. (B) COX-2 expression in ectopic lesion. Gland and stromal cells were moderately immunoreactive against COX-2. (C) Patient HP34 ectopic lesion, H&E. (D) Except some glandular cells and macrophages most of the lesion showed negative staining against COX-2. Scale bar represent 200 µm.

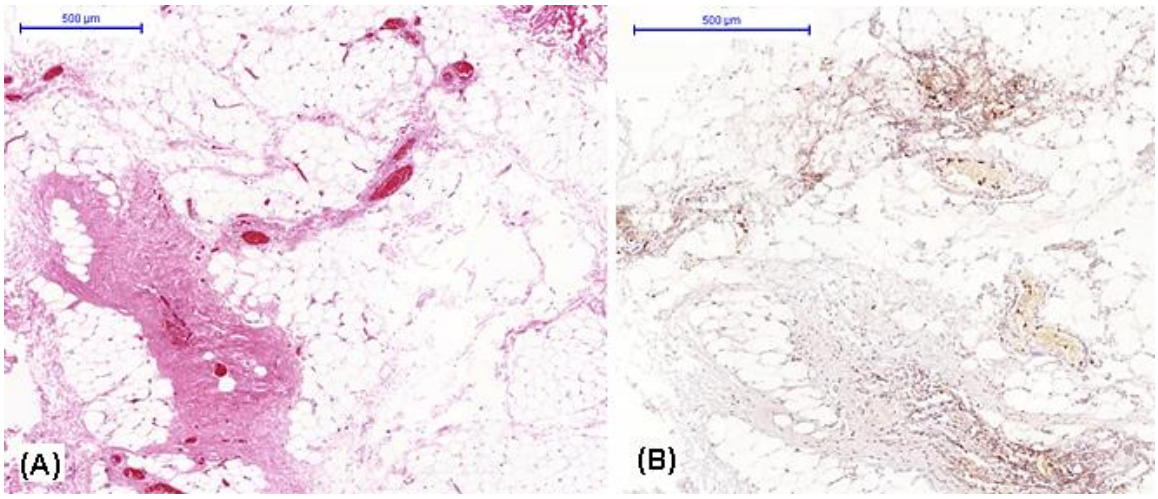


Figure 5.3.15. COX-2 expression in peritoneal wall.
(A) Patient HP50 peritoneal wall, H&E staining 3.47x magnification. (B) COX-2 expression in peritoneal wall. Biopsy of peritoneal wall showed weak immunoreactivity against COX-2. Scale bar represent 500 µm.

(A)

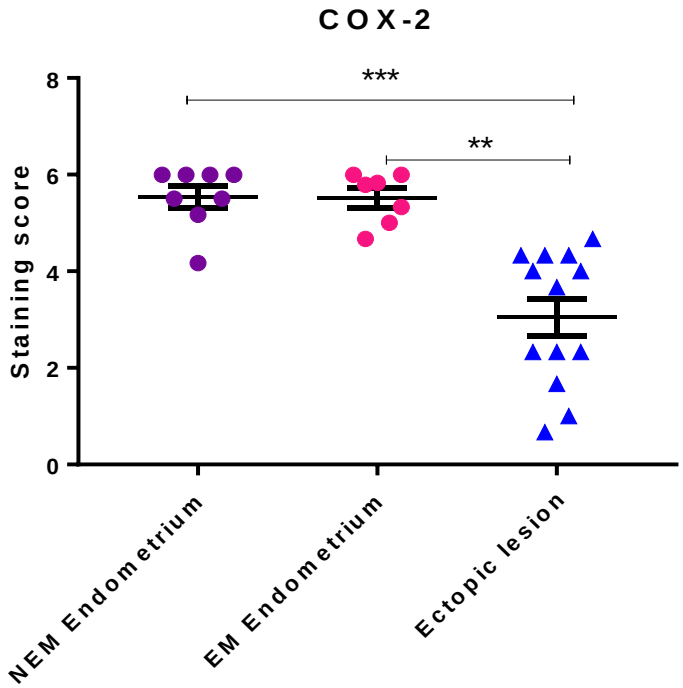
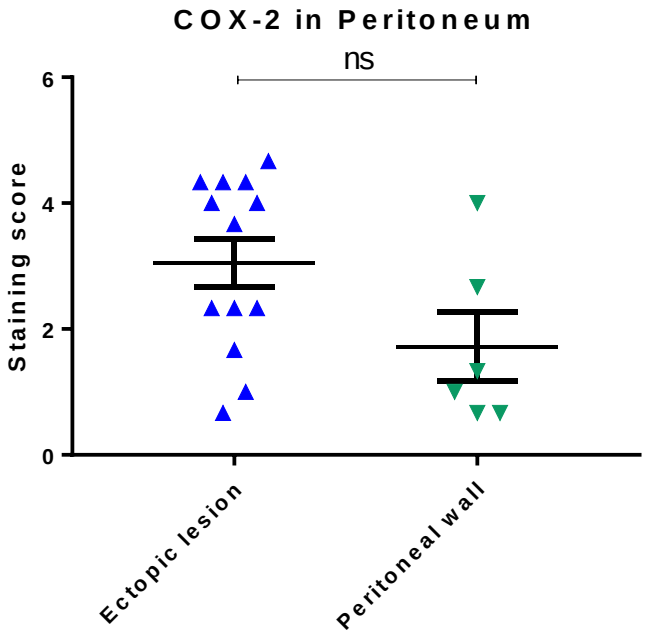


Figure 5.3.16. Immunohistochemistry analysis of cyclooxygenase 2 (COX-2) expression in eutopic endometrium and ectopic lesions.
*Dots represent the arithmetic mean of histoscores of eutopic gland and stroma from women not diagnosed with endometriosis (NEM, n=8) and women diagnosed with endometriosis (EM, n=7) compared to histoscores of ectopic lesions (blue triangles, n=13). COX-2 expression was significantly lower in ectopic lesions compared to eutopic endometrium. Bars represent mean ± SEM. Comparisons made using Kruskal-Wallis test with Dunn's post-hoc test. **p<0.01; ***p<0.001*

(A)



(B)

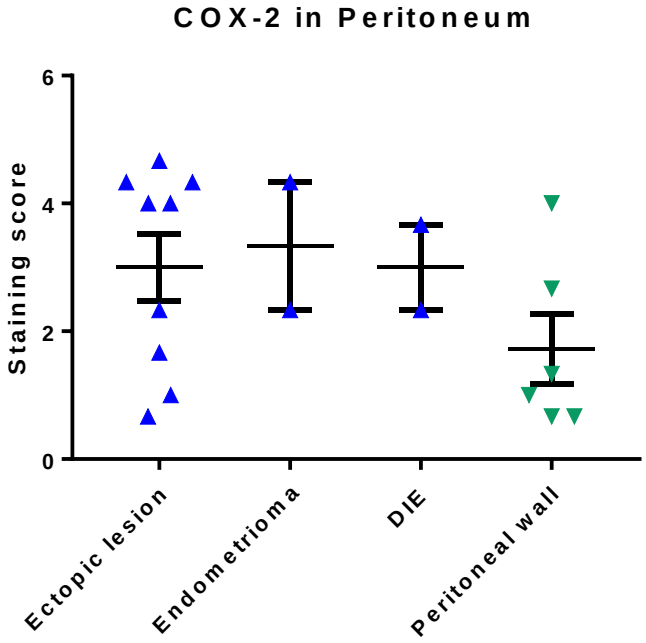


Figure 5.3.17. Immunohistochemistry analysis of cyclooxygenase 2 (COX-2) expression in ectopic lesions and peritoneal wall.
(A) COX-2 expression was not significantly changed in ectopic lesions (n=13) compared to peritoneal wall (n=6). Comparison was made using unpaired t-test, (ns) not significant. (B) Graph illustrates the COX-2 expression by endometriosis types compared to peritoneal wall (n=6). Peritoneal lesion (n=9); Endometrioma (n=2); Deep infiltrating endometriosis (DIE), (n=2). Due to the low sample numbers in group of endometrioma and DIE statistical analysis has not been performed.

Contrary to expectation, COX-2 was significantly low expressed in ectopic lesions compared to eutopic endometrium from women with and without endometriosis. Only 5 out of 13 ectopic lesions displayed strong and diffuse immunoreactivity against COX-2. Three sections were moderately immunoreactive, while six showed weak staining. COX-2 expression was not altered according to lesion types. Significant alteration was not observed for COX-2 expression between ectopic lesion and peritoneal wall.

5.3.4 15 lipoxygenase 1 (15-LOX-1)

15-LOX-1 expression was investigated in eutopic endometrium, ectopic lesions and peritoneal wall biopsies. Figure 5.3.18 shows the representative micrograph of 15-LOX-1 expression in eutopic endometrium. Figure 5.3.19 compares the histoscores of glands and stroma for 15-LOX-1.

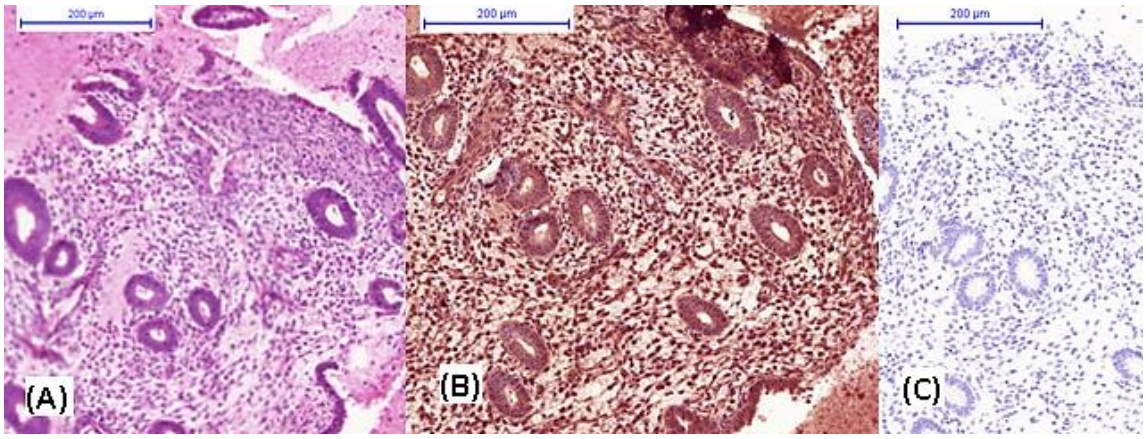


Figure 5.3.18. Representative micrograph of 15-LOX-1 expression in eutopic endometrium, Patient HP32.
(A) Morphology of endometrium. H&E staining. (B) 15-LOX-1 expression. Glands and stroma show strong, diffuse immunoreactivity against 15-LOX-1. 15-LOX-1 staining. (C) Negative control for 15-LOX-1 staining. No staining was observed in negative control. Haematoxylin staining. Scale bar represents 200 µm.

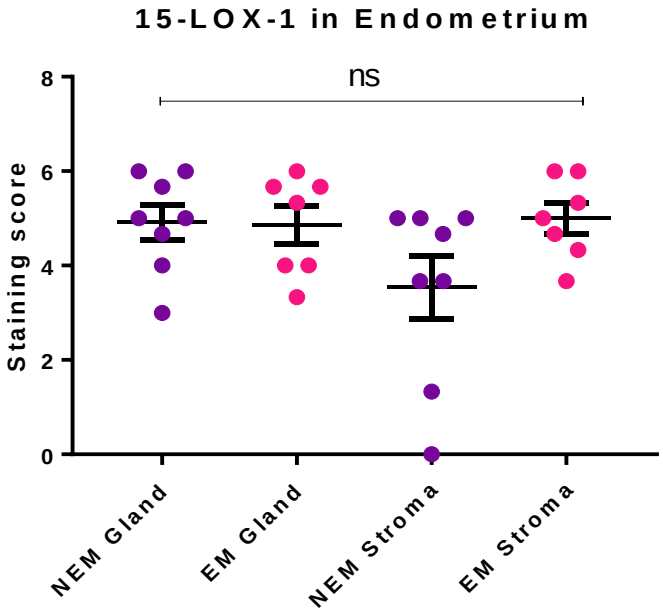


Figure 5.3.19. Immunohistochemistry analysis of 15 lipoxygenase 1 (15-LOX-1) expression in eutopic endometrium.
Glandular and stromal cells were analysed from women not diagnosed with endometriosis (NEM, n=8) and women diagnosed with endometriosis (EM, n=7). There was no significant difference between gland and stromal cells. Dots present the staining scores of each section. Bars represent mean ± SEM. Comparisons made using Kruskal-Wallis test with Dunn's post-hoc test. (ns) not significant

Glands and stroma displayed strong, diffuse 15-LOX-1 expression in eutopic endometrium. There was no significant difference between gland and stromal expression, nor between endometriosis and non-endometriosis samples. An interesting observation was that the 15-LOX-1 expression apparently decreased in stroma and showed cell membrane localisation in glands during menstruation. Figure 5.3.20 illustrates the 15-LOX-1 expression in menstrual phase. Although, only two non-endometriotic subjects were in menstrual phase the reduction in stromal expression and the translocation of 15-LOX-1 into the cell membrane was apparent in both cases.

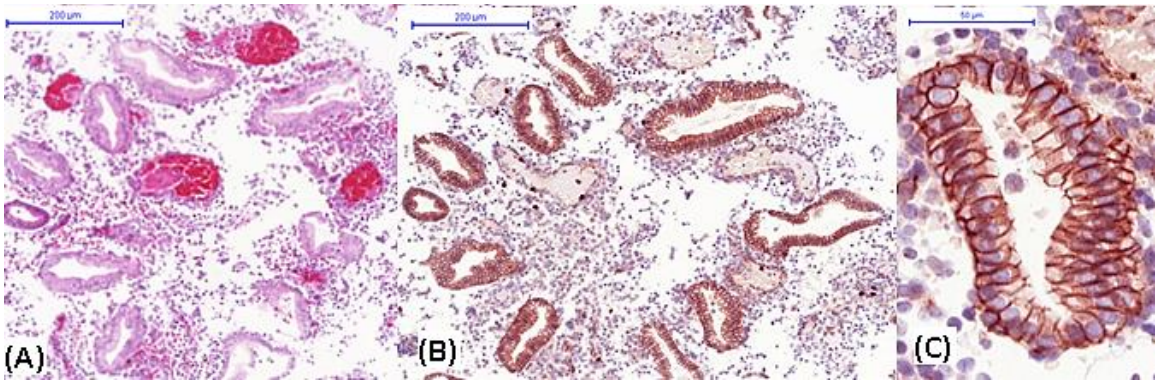


Figure 5.3.20. 15-LOX-1 expression in the phase of menstruation.
(A) Patient HP37 on the first day of the cycle. Stroma shows the sign of degradation and infiltration with red blood cells and macrophages, H&E staining. (B) 15-LOX-1 localised in the cell membrane of glands and not demonstrated in stroma. 15-LOX-1 staining. (C) 15-LOX-1 localised in cell membrane of glands, 15-LOX-1 staining. Scale bar represent 200 μm (A, B) and 50 μm (C).

Micrographs of Figure 5.4.21 illustrate 15-LOX-1 expression in ectopic lesions. Ectopic lesions displayed decreased immunoreactivity compared to eutopic endometrium. The results of IHC analysis confirmed that 15-LOX-1 expression was significantly reduced in ectopic lesions compared to eutopic endometrium from women with endometriosis (***p=0.0008) and without endometriosis (*p=0.0251) (Figure 5.4.22). None of the ectopic sections displayed strong immunoreactivity. Five out of 13 ectopic lesions showed moderate staining, three specimens were weakly positive, while five ectopic lesions did not express 15-LOX-1 (Figure 5.4.22; 5.4.23A). Interestingly, with regards to endometriosis types, endometriomas showed the highest expression, whilst 15-LOX-1 was absent in DIE specimens (Figure 5.4.23 B). 15-LOX-1 had also very low expression or was absent in peritoneal wall biopsies. 15-LOX expression was significantly lower in peritoneal wall compared to ectopic lesions (Figure 5.4.23).

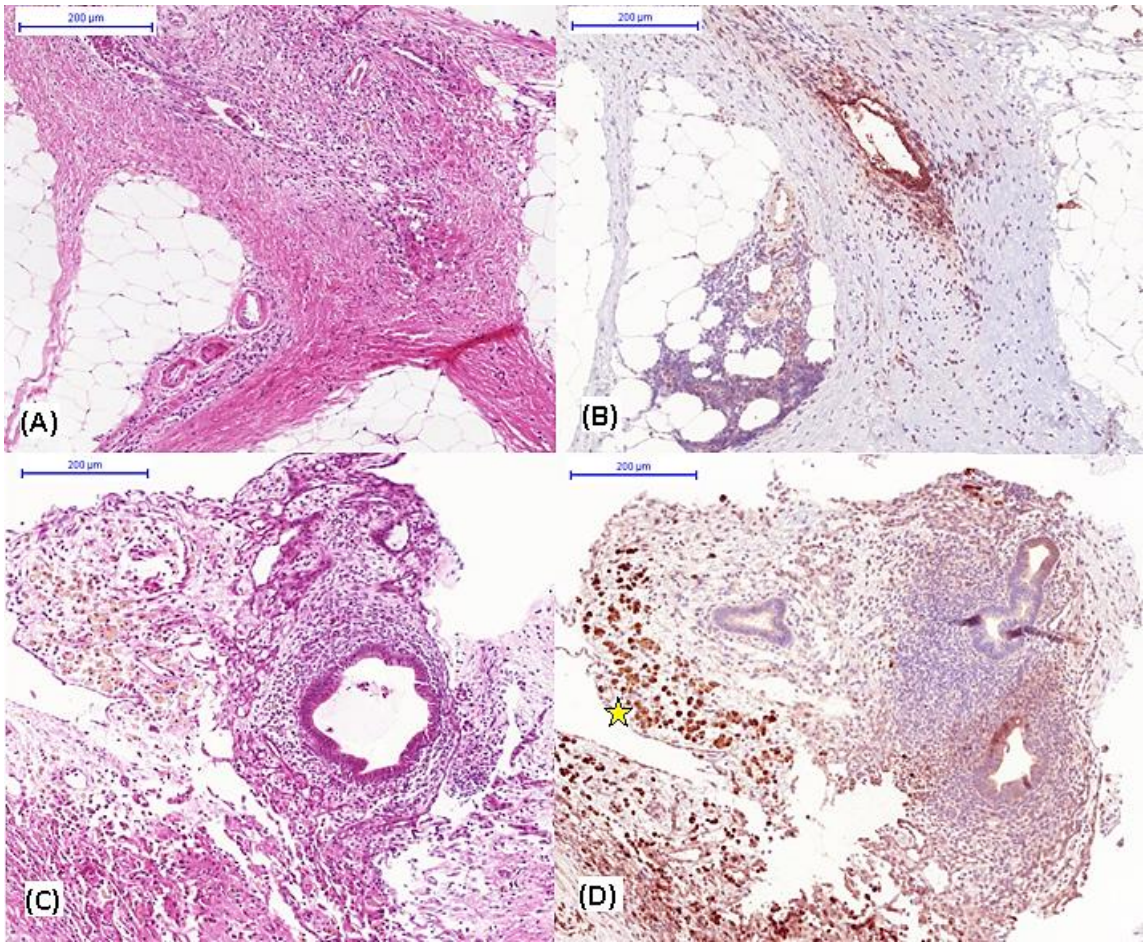


Figure 5.3.21. 15-LOX-1 expression in ectopic lesion.
(A) Patient HP34 ectopic lesion, H&E. (B) 15-LOX-1 expression in ectopic lesion. Glands show strong immunoreactivity, while stromal cells around glands show weak staining against 15-LOX-1. Other parts of the section were not reactive for 15-LOX-1. (C) Patient HP45 ectopic lesion, H&E. (D) Glandular and stromal cells were weakly positive against 15-LOX-1. Hemosiderin macrophages highly expressed 15-LOX-1 (yellow star). 15-LOX-1 staining. Scale bar represent 200 µm.

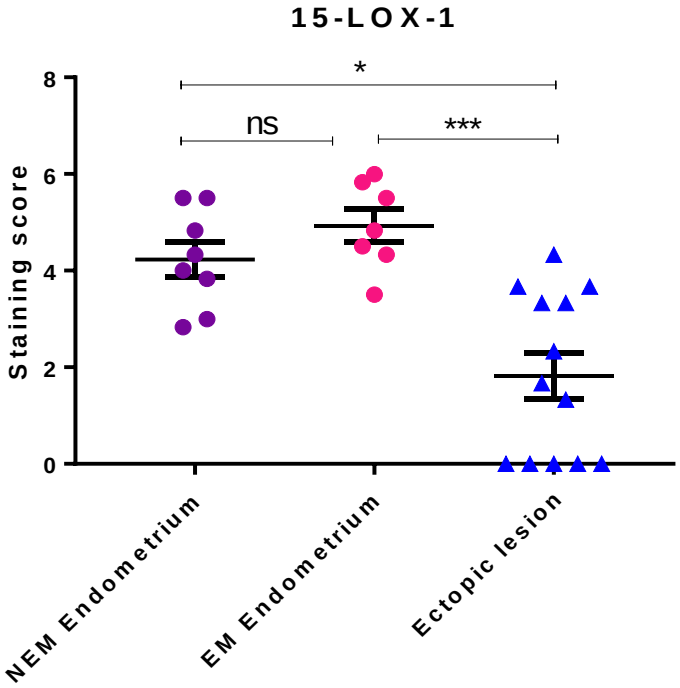
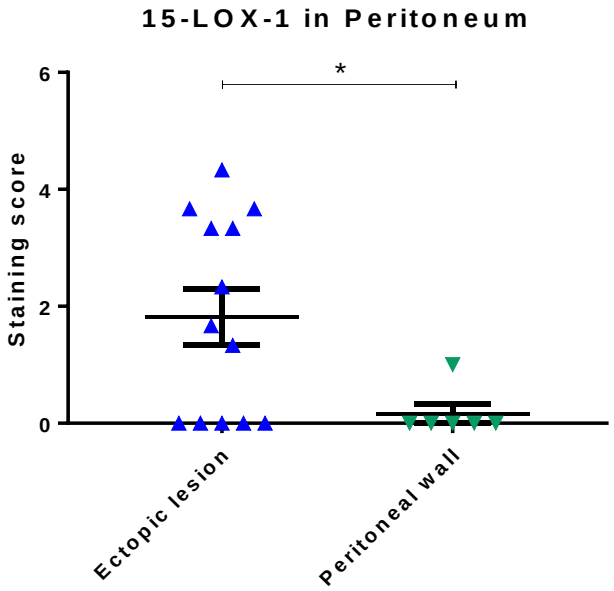


Figure 5.3.22. Immunohistochemistry analysis of 15 lipoxygenase 1 (15-LOX-1) expression in eutopic endometrium and ectopic lesions.
*Dots represent the arithmetic mean of histoscores of eutopic gland and stroma from women not diagnosed with endometriosis (NEM, n=8) and women diagnosed with endometriosis (EM, n=7) compared to histoscores of ectopic lesions (blue triangles, n=13). 15-LOX-1 expression was significantly lower in ectopic lesion compared to eutopic endometrium. Eutopic endometrium from women with and without endometriosis were not showed significant difference. Bars represent mean ± SEM. Comparisons made using Kruskal-Wallis testwith Dunn's post-hoc test. ***p<0.001; *p<0.05; (ns) not significant*

(A)



(B)

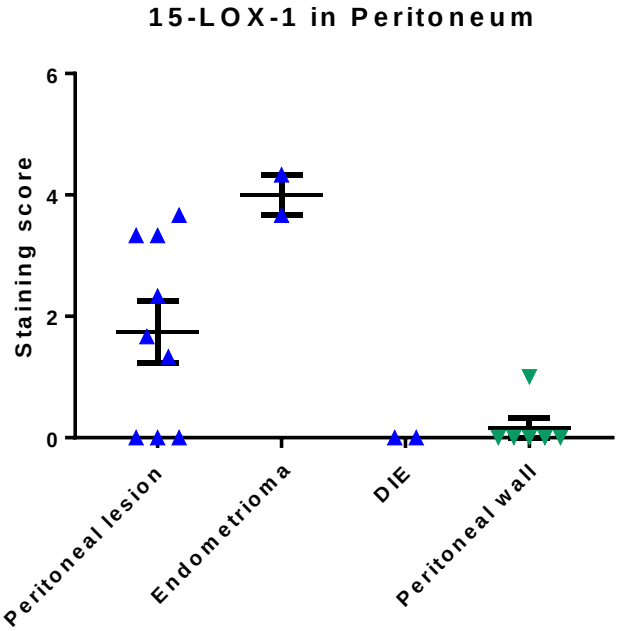


Figure 5.3.23. Immunohistochemistry analysis of 15 lipoxygenase 1 (15-LOX-1) expression in ectopic lesions and peritoneal wall biopsies.
(A) 15-LOX-1 expression was significantly higher in ectopic lesions (n=13) compared to peritoneal wall (n=6). Expression of 15-LOX-1 was very low or absent in peritoneal wall. Comparison was made using Mann-Whitney U-test. (B) Graph illustrates the 15-LOX-1 expression by endometriosis types compared to peritoneal wall (n=6). Peritoneal lesion (n=9); Endometrioma (n=2); Deep infiltrating endometriosis (DIE), (n=2). Due to the low sample numbers in group of endometrioma and DIE statistical analysis has not been performed. (ns) not significant

5.3.5 Peroxisome proliferator-activated receptor gamma (PPARγ)

PPARγ is the member of ligand activated transcription factor of nuclear receptor family. It has pleiotropic effects on several physiological and pathological processes by regulating the expression of genes. 15-LOX-1 products serve as natural ligands for PPARγ therefore. PPARγ expression was studied in eutopic endometrium, ectopic lesions and peritoneal wall. Figure 5.3.24 demonstrates the representative micrograph of PPARγ in eutopic endometrium. Figure 5.3.25 compares the histoscores of glands and stroma for PPARγ.

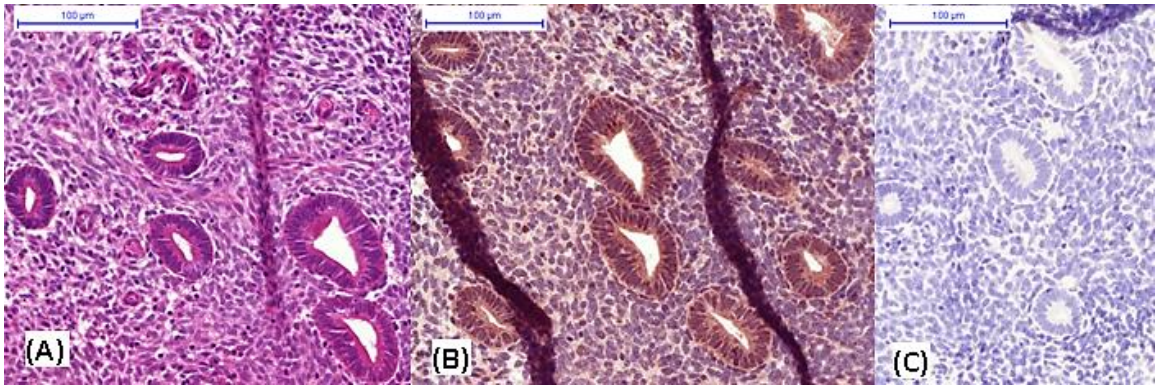


Figure 5.3.24. Representative micrograph of PPARγ expression in eutopic endometrium, Patient HP35.
(A) Morphology of endometrium, H&E. (B) PPARγ expression. Glands showed strong, diffuse immunoreactivity, whilst stroma showed a weak to moderate immunoreactivity against PPARγ. PPARγ staining. (C) Negative control for PPARγ staining. No staining was observed in negative control. Haematoxylin staining. Scale bar represent 100 µm.

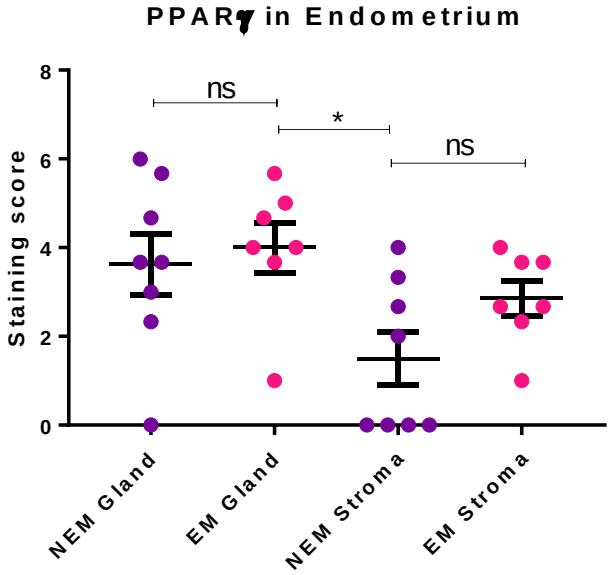


Figure 5.3.25. Immunohistochemistry analysis of peroxisome proliferator-activated receptor gamma (PPARγ) expression in eutopic endometrium.
Glandular and stromal cells were analysed from women not diagnosed with endometriosis (NEM, n=8) and women diagnosed with endometriosis (EM, n=7). PPARγ immunoreactivity

*was elevated in glands compared to stroma. Multiple comparison revelled significant difference between gland from women with endometriosis and stroma from women without endometriosis. Bars represent mean ± SEM. Comparisons made using Kruskal-Wallis test with Dunn's post-hoc test. *p<0.05; (ns) not significant*

The results obtained from the IHC analysis of eutopic endometrium showed the expression of PPARγ was more prominent in the glands than in stroma. The means for the glands were increased in both study groups compared to stroma. PPARγ expression was not demonstrated in the four cases of stroma and in one case of glands in the non-endometriosis group. There were no significant differences between either glands from women with and without endometriosis nor stroma from women with and without endometriosis. Post hoc analysis revealed that the glands from women with endometriosis were significantly different from the stroma derived from women without endometriosis.

PPARγ expression showed wide scale variation in ectopic lesions. Micrographs in Figure 5.3.26 illustrate the PPARγ expression in ectopic lesions. Four out of 13 specimens showed strong immunoreactivity against PPARγ, five samples stained moderately, while PPARγ was not detectable in four ectopic lesions. The comparison of staining scores of eutopic endometrium and ectopic lesions have not found significant differences between the study groups (Figure 5.3.27). PPARγ also had very low expressed or absent in peritoneal wall. PPARγ expression was significantly lower in peritoneal wall compared to ectopic lesions (Figure 5.3.28).

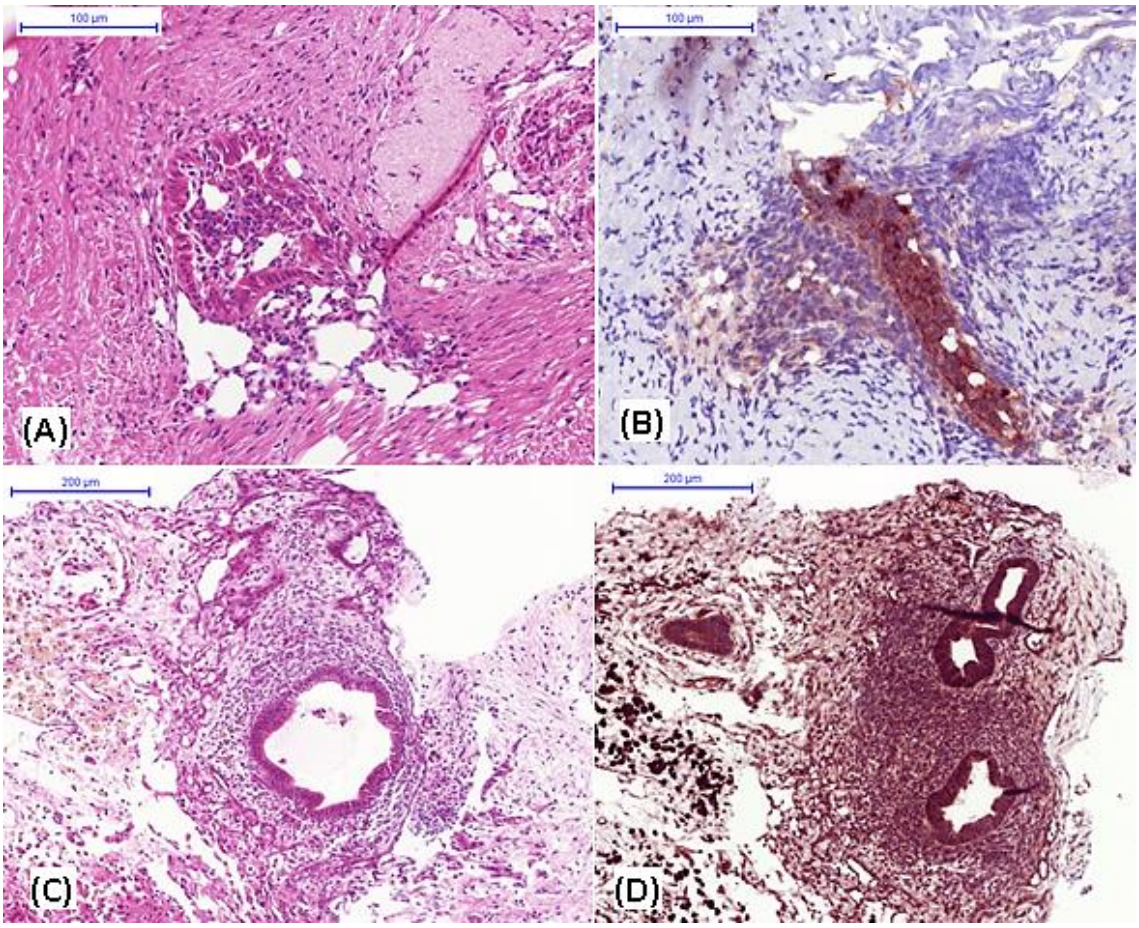


Figure 5.3.26. PPARγ expression in ectopic lesion.
(A) Patient HP34 ectopic lesion. H&E staining. (B) PPARγ expression in ectopic lesion. Glands show strong immunoreactivity, while stromal cells around glands show weak staining against PPARγ. Other parts of the section were not reactive for PPARγ. PPARγ staining. (C) Patient HP45 ectopic lesion, H&E. (D) Glandular and stromal cells were strongly positive against PPARγ. PPARγ staining. Scale bar represent 100 µm and 200 µm.

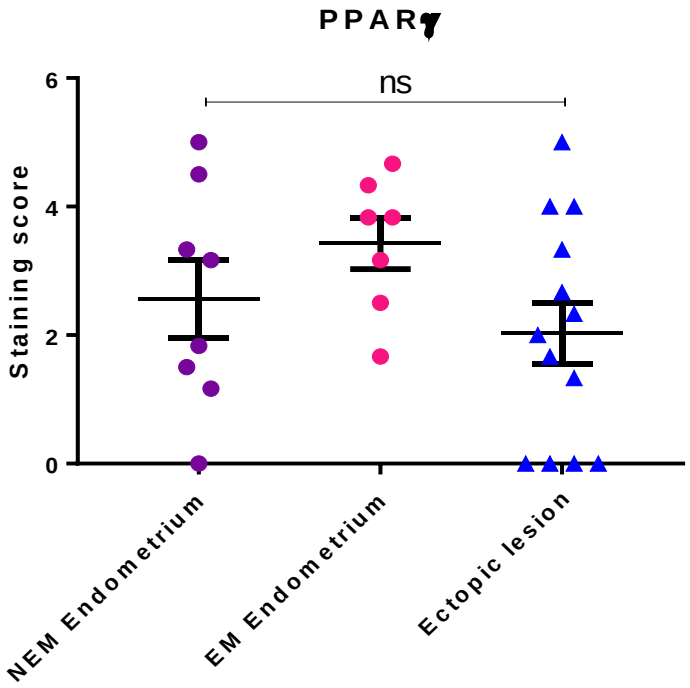
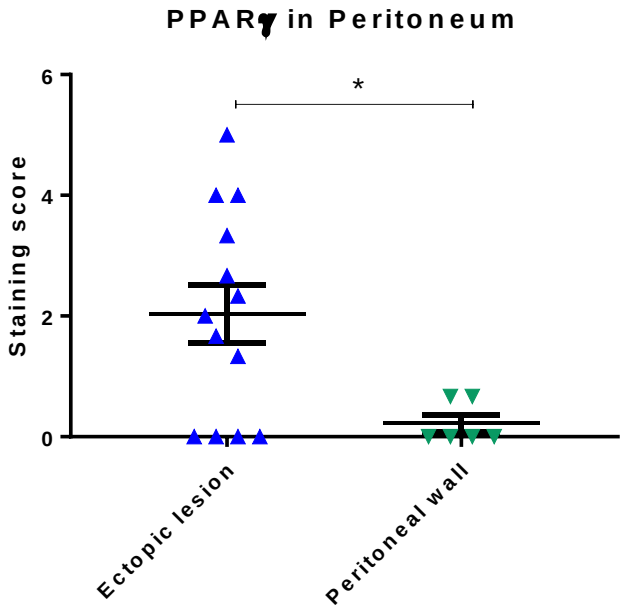


Figure 5.3.27. Immunohistochemistry analysis of Peroxisome proliferator-activated receptor gamma (PPARγ) expression in eutopic endometrium and ectopic lesions.
Dots represent the arithmetic mean of histoscores of eutopic gland and stroma from women not diagnosed with endometriosis (NEM, n=8) and women diagnosed with endometriosis (EM, n=7) compared to histoscores of ectopic lesions (blue triangles, n=13). There were no statistically significant difference between eutopic endometrium from women with and without endometriosis and ectopic lesions with regards to PPARγ expression. Comparisons made using one-way ANOVA with Tukey's post-hoc test. (ns) not significant, bars represent mean ± SEM.

(A)



(B)

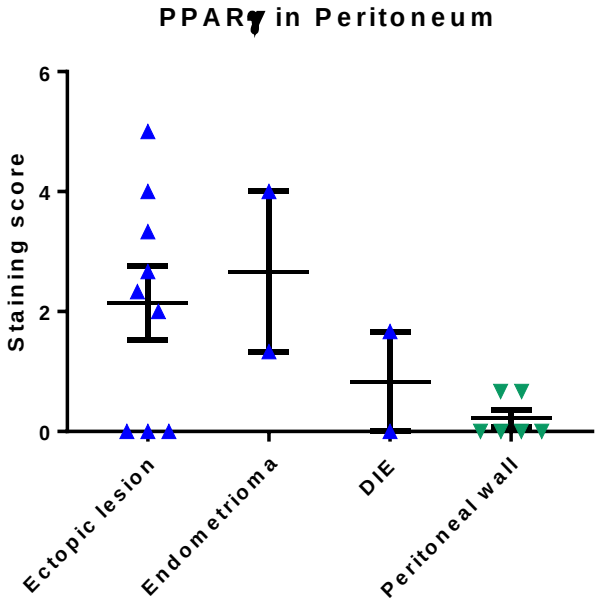


Figure 5.3.28. Immunohistochemistry analysis of Peroxisome proliferator-activated receptor gamma (PPARγ) expression in ectopic lesions and peritoneal wall biopsies.
(A) PPARγ expression was significantly higher in ectopic lesions (n=13) compared to peritoneal wall (n=6). Expression of PPARγ was very low expressed or absent in peritoneal wall. Comparison was made using Mann-Whitney U-test. (B) Graph illustrates the PPARγ expression by endometriosis types compared to peritoneal wall (n=6). Peritoneal lesion (n=9); Endometrioma (n=2); Deep infiltrating endometriosis (DIE), (n=2). Due to the low sample numbers in group of endometrioma and DIE statistical analysis has not been performed. (ns) not significant

5.3.6 CD36

CD36 is the member of scavenger receptor family. It highly is expressed in activated macrophages and essential for successful phagocytosis. CD36 expression was studied in eutopic endometrium, ectopic lesions and peritoneal wall specimens. Figure 5.3.29 demonstrates the representative micrograph of CD36 in eutopic endometrium. Figure 5.3.30 compares the histoscores of glands and stroma for CD36.

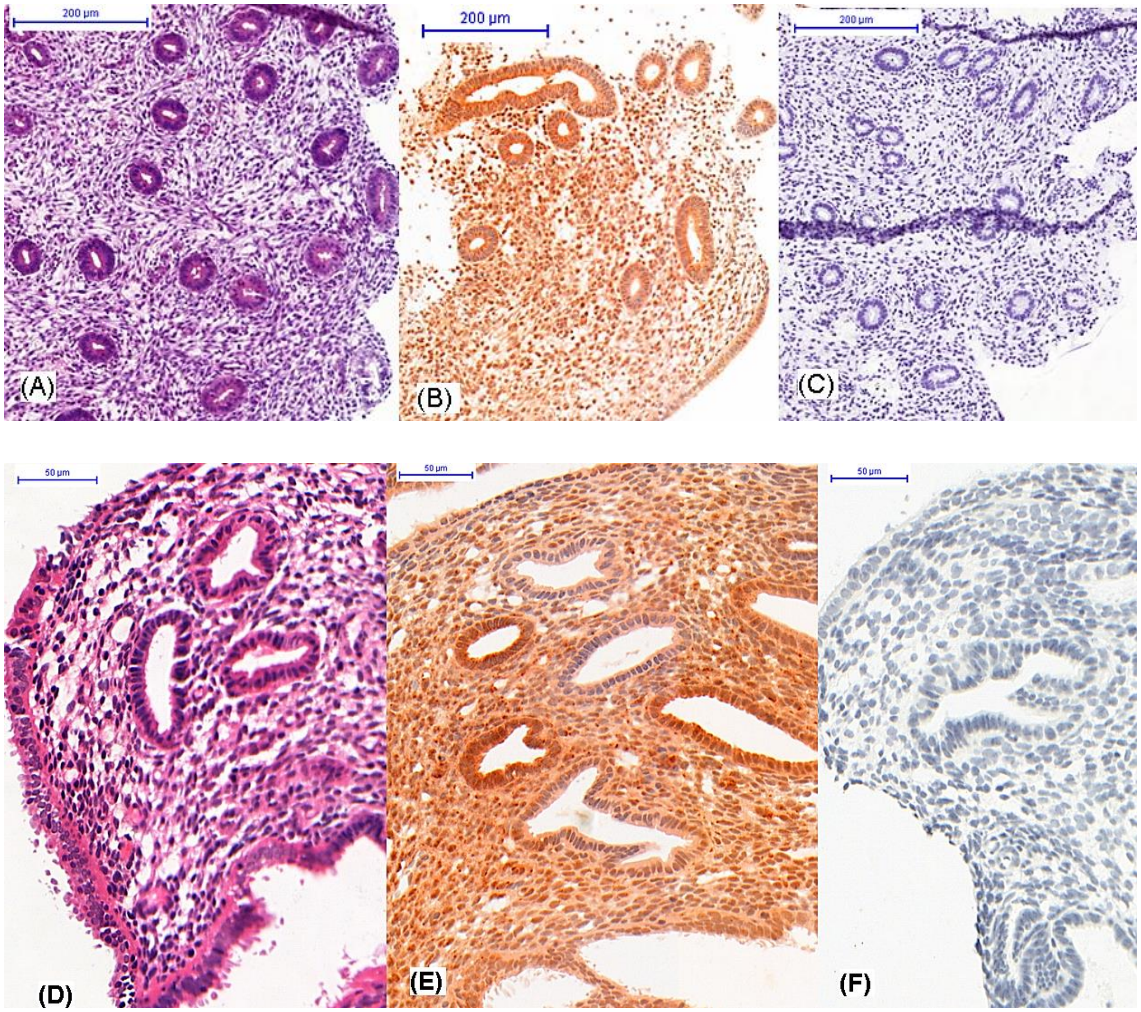


Figure 5.3.29. Representative micrograph of CD36 expression in eutopic endometrium. (A, D) Morphology of endometrium, H&E staining. (B, E) CD36 expression in eutopic endometrium. Glands and stroma show strong, diffuse immunoreactivity against CD36. CD36 staining. (C, F) Negative control for CD36 staining. No staining was observed in negative control. Haematoxylin staining. (A-C) Patient HP47, scale bar represent 200 µm. (D-F) Patient HP34, scale bar represent 50 µm.

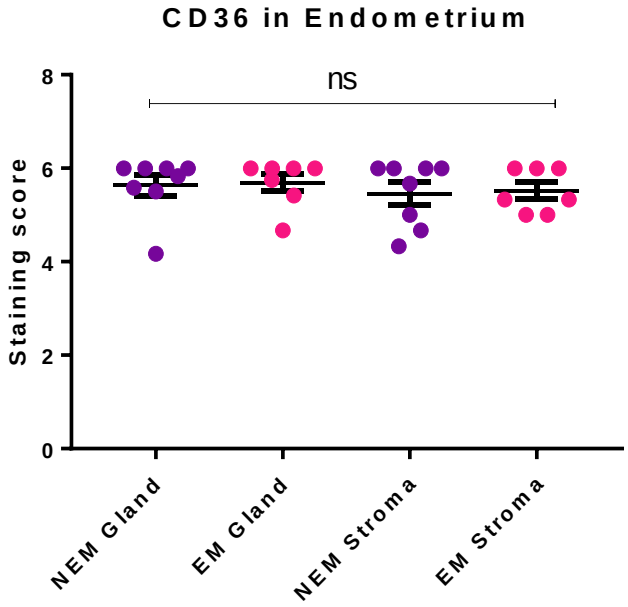


Figure 5.3.30. Immunohistochemistry analysis of CD36 expression in eutopic endometrium. CD36 highly expressed in eutopic endometrium. Glandular and stromal cells were analysed from women not diagnosed with endometriosis (NEM, n=8) and women diagnosed with endometriosis (EM, n=7).Dots present the staining scores of each section. Bars represent mean ± SEM. Comparisons made using Kruskal-Wallis testwith Dunn's post-hoc test. (ns) not significant

CD36, like COX-2, showed consistently strong and diffuse staining in eutopic endometrium. The immunoreactivity of glands and stoma were very similar. There was no significant difference between glands and stroma, or between the group of endometriosis and non-endometriosis patients.

CD36 expression also was investigated in ectopic lesions and peritoneal wall specimens. Figure 5.3.31 and 5.3.33 demonstrates the immunoreactivity of CD36 in ectopic lesions and peritoneal wall. Figure 5.3.32 shows the comparison of histoscores between eutopic endometrium and ectopic lesions. Figure 5.3.34 illustrates the comparison of histoscores between ectopic lesions and peritoneal wall.

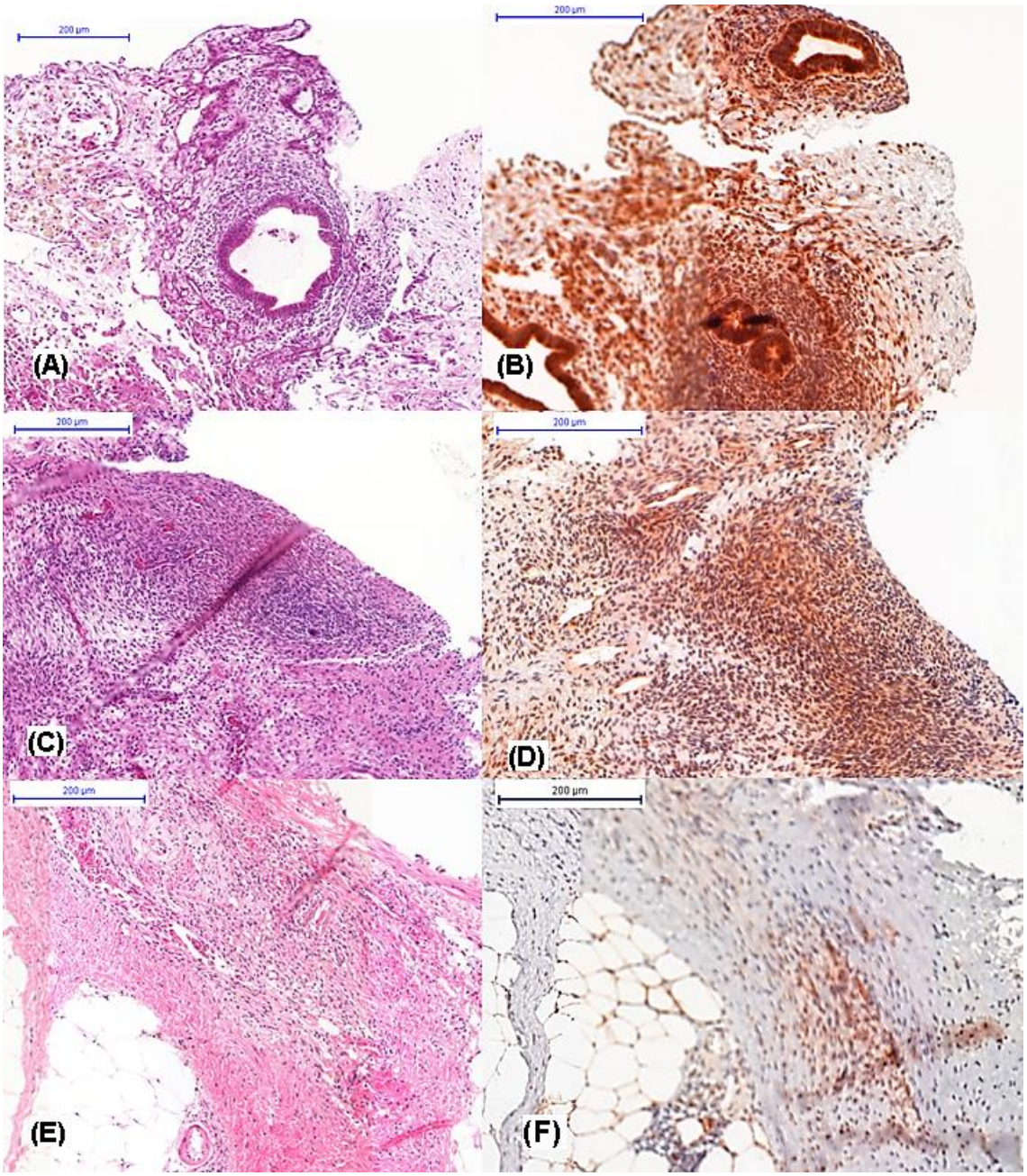


Figure 5.3.31. CD36 expression in ectopic lesion.
(A) Patient HP45 ectopic lesion. H&E staining. (B) CD36 expression in ectopic lesion. Glands and stroma showed immunoreactivity against CD36. CD36 staining. (C) Patient HP47 endometrioma. Glands were sparse, while stromal cells were dominated in the section. H&E. (D) Stromal cells showed strong immunoreactivity against CD36. (E) Patient HP34 ectopic lesion. H&E staining. (F) CD36 expression in ectopic lesion. Fat cells also showed immunoreactivity against CD36. CD36 staining. Scale bar represent 200 µm.

(A)

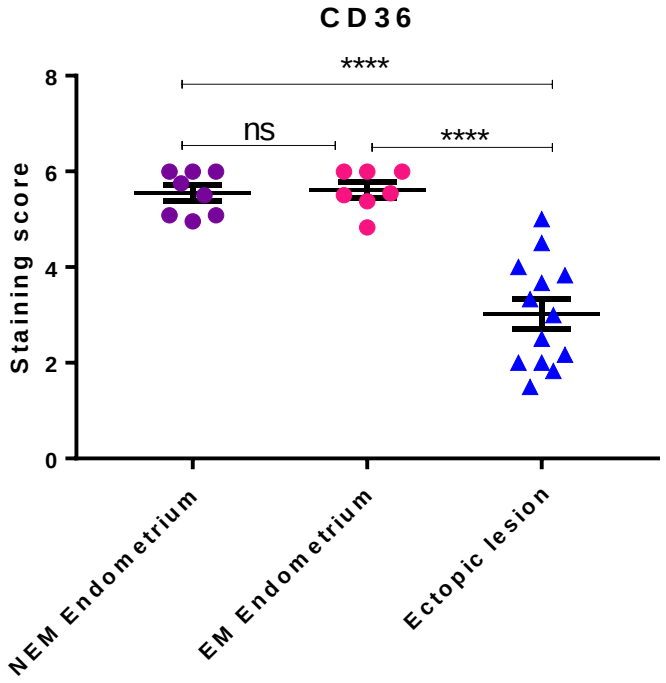


Figure 5.3.32. Immunohistochemistry analysis of CD36 expression in eutopic endometrium and ectopic lesions.
*Dots represent the arithmetic mean of histoscores of eutopic gland and stroma from women not diagnosed with endometriosis (NEM, n=8) and women diagnosed with endometriosis (EM, n=7) compared to histoscores of ectopic lesions (blue triangles, n=13). CD36 expression was significantly lower in ectopic lesions compared to eutopic endometrium. Bars represent mean ± SEM. Comparisons made using one-way ANOVA with Tukey's post-hoc test. ****p<0.0001*

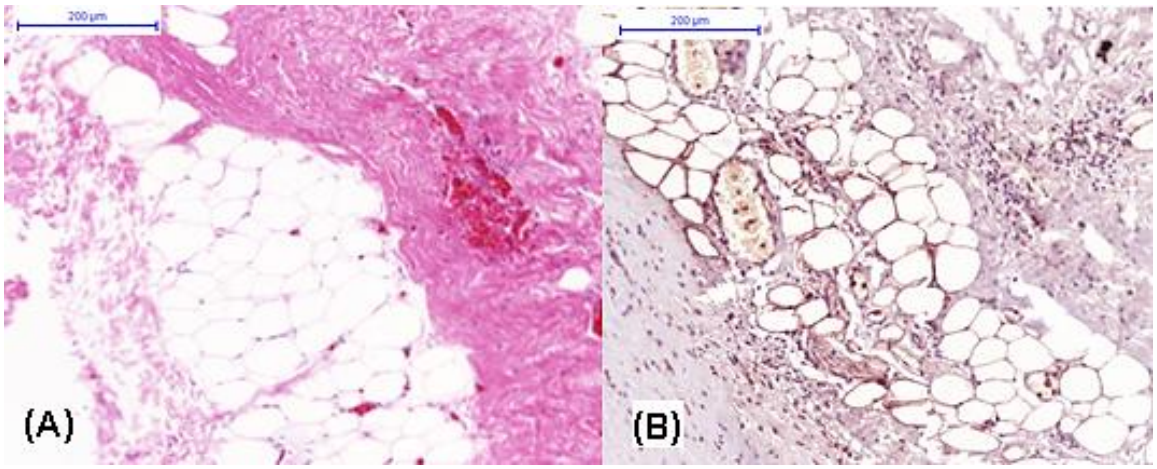
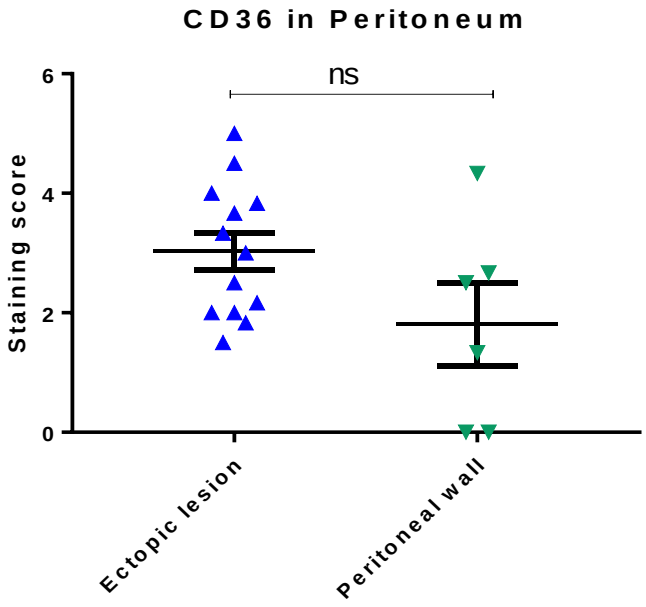


Figure 5.3.33. CD36 expression in peritoneal wall.
(A) Peritoneal wall, Patient HP50, H&E staining. (B) Immunoreactivity of CD36. Fat cells were immunoreactive against CD36. CD36 staining. Scale bar represent 200 µm.

(A)



(B)

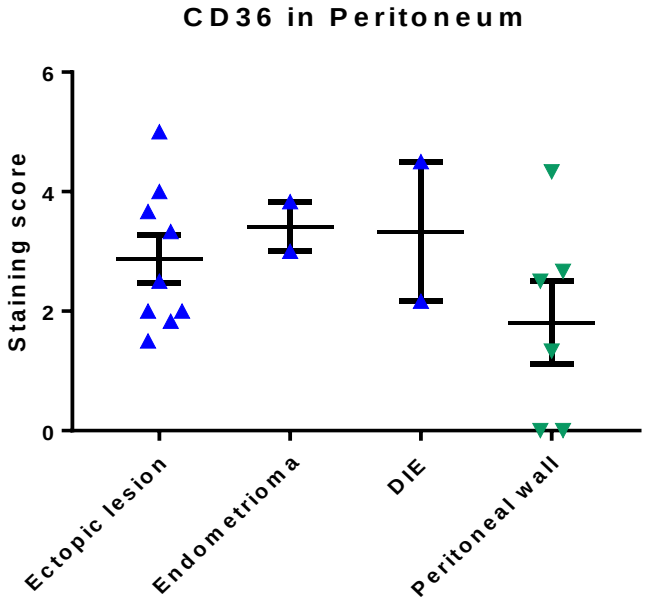


Figure 5.3.34. Immunohistochemistry analysis of CD36 expression in ectopic lesions and peritoneal wall specimens.
(A) CD36 expression was not significantly changed in ectopic lesions (n=13) compared to peritoneal wall (n=6). Comparison was made using unpaired t-test, (ns) not significant. (B) Graph illustrates the CD36 expression by endometriosis types compared to peritoneal wall (n=6). Peritoneal lesion (n=9); Endometrioma (n=2); Deep infiltrating endometriosis (DIE), (n=2). Due to the low sample numbers in group of endometrioma and DIE statistical analysis has not been performed.

CD36 expression was observed in all ectopic lesions. The expression was significantly lower ($p<0.0001$) in ectopic lesions compared to eutopic endometrium from women with and without endometriosis. Comparing ectopic lesions according to lesion type, no significant difference in CD36 expression was observed (Figure 5.3.34B).

CD36 was presented in four out of six peritoneal wall specimens. Mainly fat cells showed immunoreactivity against CD36 (Figure 5.3.33 B). There was no significant difference between ectopic lesions and peritoneal wall with regard to CD36 expression.

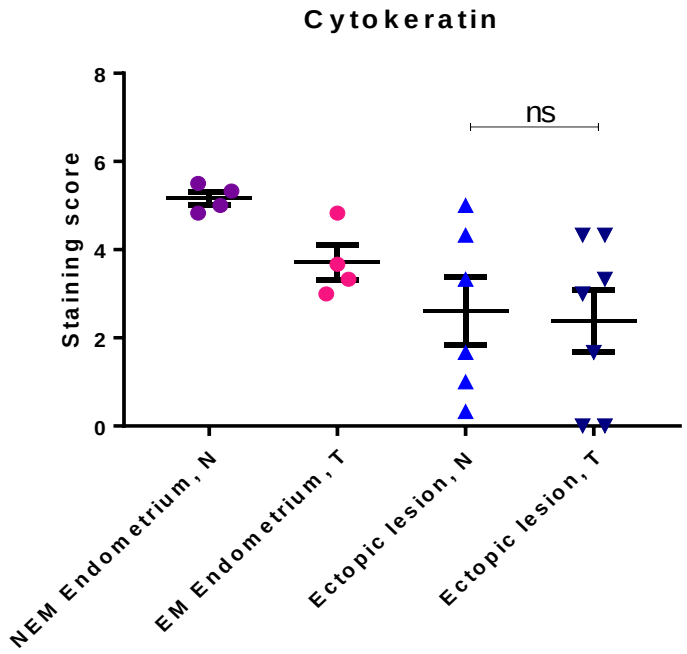
5.3.7 Effects of medical treatment on protein expression in ectopic lesions

Data obtained from this gene expression study revealed that some genes of interest, such as PPARγ and CD36 were upregulated in women with endometriosis. Further analysis highlighted that the most robust upregulations were detected in the group of women who were subject to GnRH treatments. For that reason, the IHC data was assessed and classified by menstrual cycle phase and medical treatment. Specimens derived from women with normal periods, who were not taking hormones for medical or contraceptive reasons were included in the non-treated group (N), whereas samples from patients receiving GnRH agonist treatment for endometriosis were classified in the treatment group (T). For ectopic lesions, the non-treated and treated groups involved six and seven specimens, respectively. Unfortunately, the sample sizes were too small to be included in the statistical analysis of eutopic endometrium since only 2 out of 7 and 4 out of 8 eutopic endometrium biopsies taken from endometriosis and non-endometriosis patients had regular periods and were not taking hormones as contraceptive or medical treatment. For representative purposes, the graphs include the groups of eutopic endometrium from non-endometriosis women with normal menstrual cycle (n=4) and eutopic endometrium from endometriosis women with GnRH agonist treatment (n=4), but these groups were not included in the statistical comparisons. Paired eutopic endometrium and ectopic lesions also were grouped according to menstrual cycles and medical treatment (Figure 5.3.35-40).

The statistical analysis did not reveal any significant differences in cytokeratin and IFITM-1 expression between the treated and non-treated study groups in ectopic lesions. Unexpectedly, the histoscores showed a significant reduction in the expression of COX-2 (*p=0.011), 15-LOX-1 (**p=0.005) and PPARγ (*p=0.039) in the treatment group. The most unexpected outcome was that there was no significant

difference in CD36 expression in ectopic lesions between the treated and non-treated study groups. Moreover, the mean of the treatment group was lower than the mean of the group without treatment. Staining scores of paired specimens tended to be lower in ectopic lesions compared to matched eutopic endometrium.

(A)



(B)

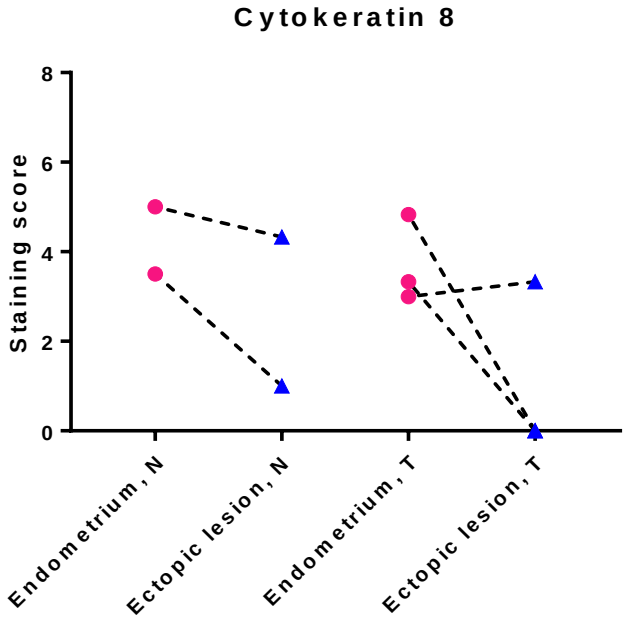
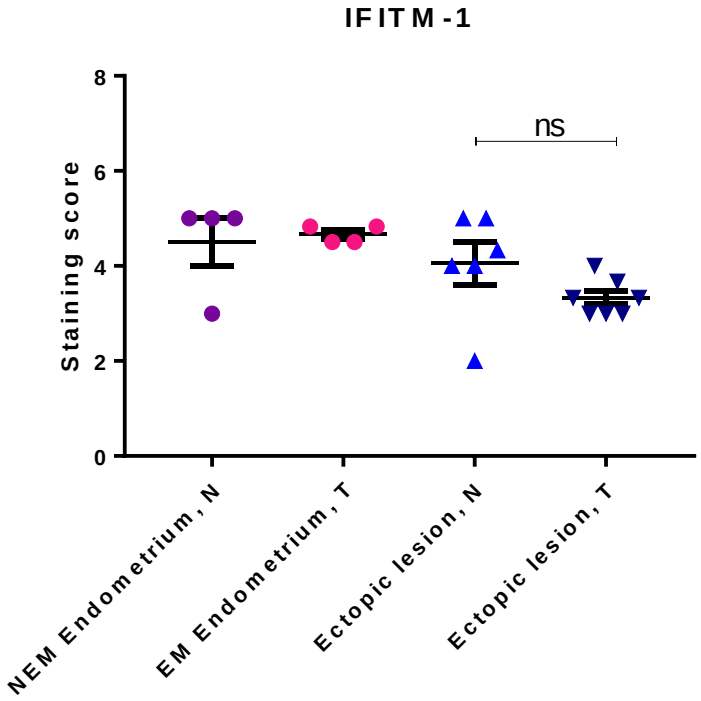


Figure 5.3.35. (A) Immunohistochemistry analysis of cytokeratin expression in eutopic endometrium and ectopic lesions compared by medical treatment.
Dots represent the arithmetic mean of histoscores of eutopic gland and stroma from women not diagnosed with endometriosis with regular cycle (NEM,N n=4), women diagnosed with endometriosis taking GnRH agonist (EM,T n=4) and histoscores of ectopic lesions from endometriosis women without GnRH treatment (EC, N n=6) and with GnRH treatment (EC, N

n=7). Data are expressed as mean±SEM. Statistical comparisons were performed for the groups of ectopic lesions using unpaired t-test, (ns) not significant. (B) Immunohistochemistry analysis of CD36 in paired specimens from women with endometriosis without GnRH treatment (EM Eu N; EM Ec N, n=2) and taking GnRH agonist (EM Eu T; EM Ec T, n=3). Dotted lines indicates the matched eutopic and ectopic specimens.

(A)



(B)

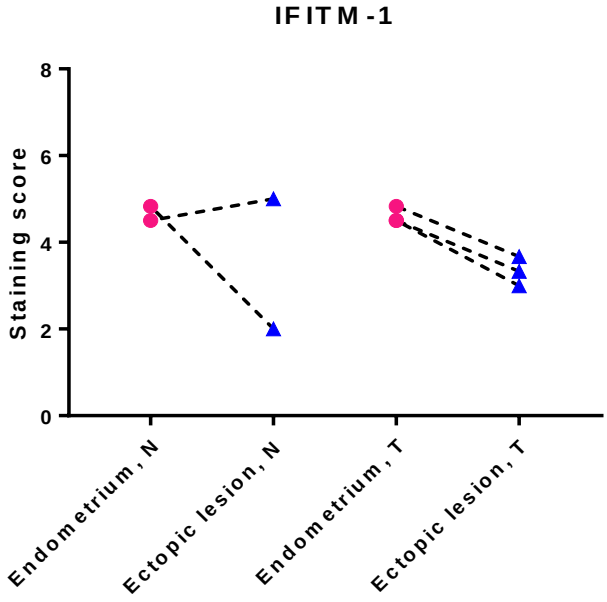
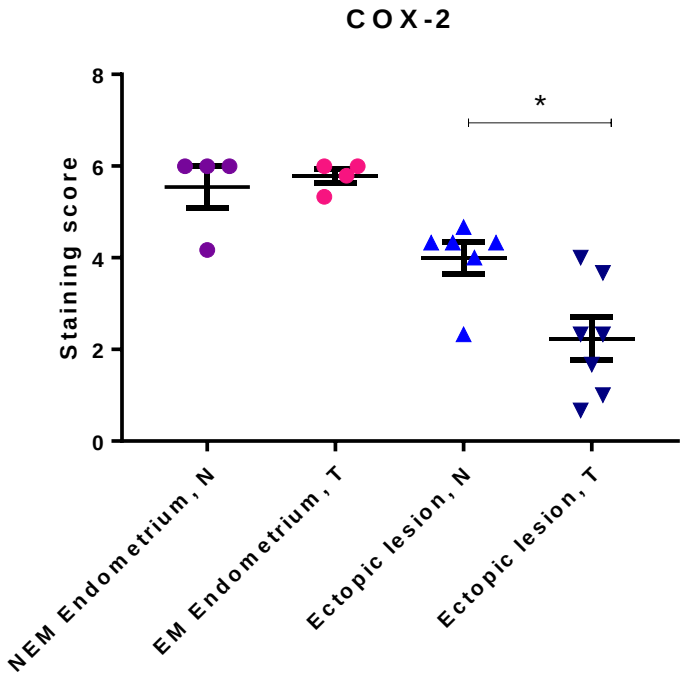


Figure 5.3.36. (A) Immunohistochemistry analysis of interferon induced transmembrane protein 1 (IFITM-1) expression in eutopic endometrium and ectopic lesions compared by medical treatment.
Dots represent the arithmetic mean of histoscores of eutopic gland and stroma from women not diagnosed with endometriosis with regular cycle (NEM,N n=4), women diagnosed with

endometriosis taking GnRH agonist (EM,T n=4) and histoscores of ectopic lesions from endometriosis women without GnRH treatment (EC, N n=6) and with GnRH treatment (EC, N n=7). Data are expressed as mean±SEM. Statistical comparisons were performed for the groups of ectopic lesions using unpaired t-test, (ns) not significant. (B) Immunohistochemistry analysis of IFITM-1 in paired specimens from women with endometriosis without GnRH treatment (EM Eu N; EM Ec N, n=2) and taking GnRH agonist (EM Eu T; EM Ec T, n=3). Dotted lines indicates the matched eutopic and ectopic specimens.

(A)



(B)

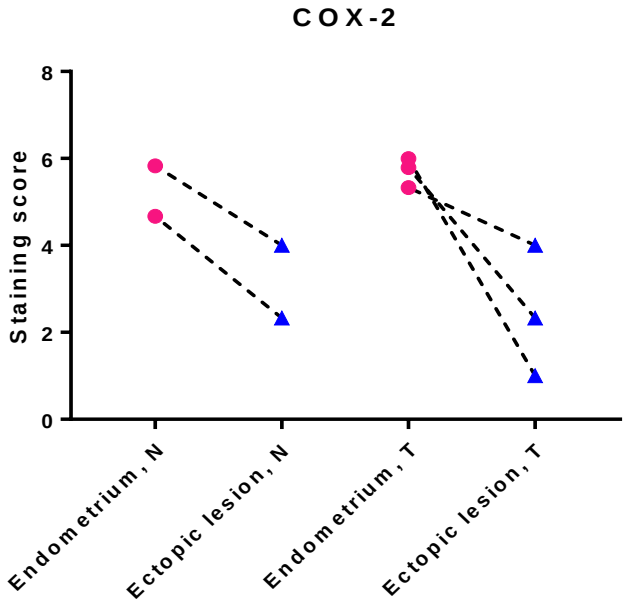
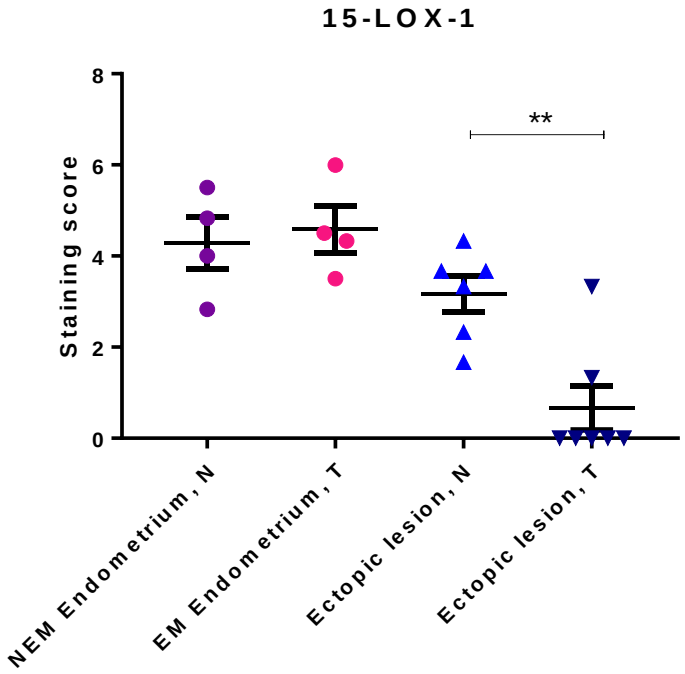


Figure 5.3.37. (A) Immunohistochemistry analysis of Cyclooxygenase 2 (COX-2) expression in eutopic endometrium and ectopic lesions compared by medical treatment.
Dots represent the arithmetic mean of histoscores of eutopic gland and stroma from women not diagnosed with endometriosis with regular cycle (NEM,N n=4), women diagnosed with

*endometriosis taking GnRH agonist (EM,T n=4) and histoscores of ectopic lesions from endometriosis women without GnRH treatment (EC, N n=6) and with GnRH treatment (EC, N n=7). Data are expressed as mean±SEM. Statistical comparisons were performed for the groups of ectopic lesions using Mann-Whitney U test; *p<0.05. (B) Immunohistochemistry analysis of COX-2 in paired specimens from women with endometriosis without GnRH treatment (EM Eu N; EM Ec N, n=2) and taking GnRH agonist (EM Eu T; EM Ec T, n=3). Dotted lines indicates the matched eutopic and ectopic specimens.*

(A)



(B)

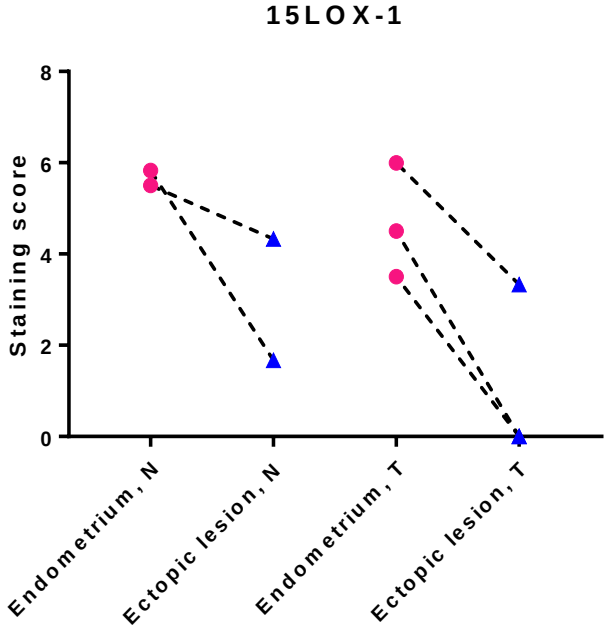
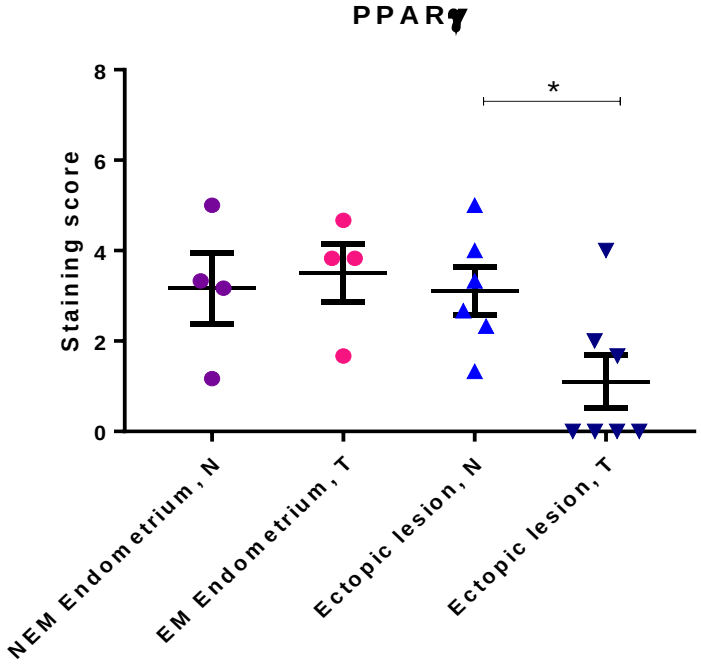


Figure 5.3.38. (A) Immunohistochemistry analysis of 15 lipoxygenase 1 (15-LOX-1) expression in eutopic endometrium and ectopic lesions compared by medical treatment.
Dots represent the arithmetic mean of histoscores of eutopic gland and stroma from women not diagnosed with endometriosis with regular cycle (NEM,N n=4), women diagnosed with

*endometriosis taking GnRH agonist (EM,T n=4) and histoscores of ectopic lesions from endometriosis women without GnRH treatment (EC, N n=6) and with GnRH treatment (EC, N n=7). Data are expressed as mean±SEM. Statistical comparisons were performed for the groups of ectopic lesions using Mann-Whitney U test; **p<0.01. (B) Immunohistochemistry analysis of 15-LOX-1 in paired specimens from women with endometriosis without GnRH treatment (EM Eu N; EM Ec N, n=2) and taking GnRH agonist (EM Eu T; EM Ec T, n=3). Dotted lines indicates the matched eutopic and ectopic specimens.*

(A)



(B)

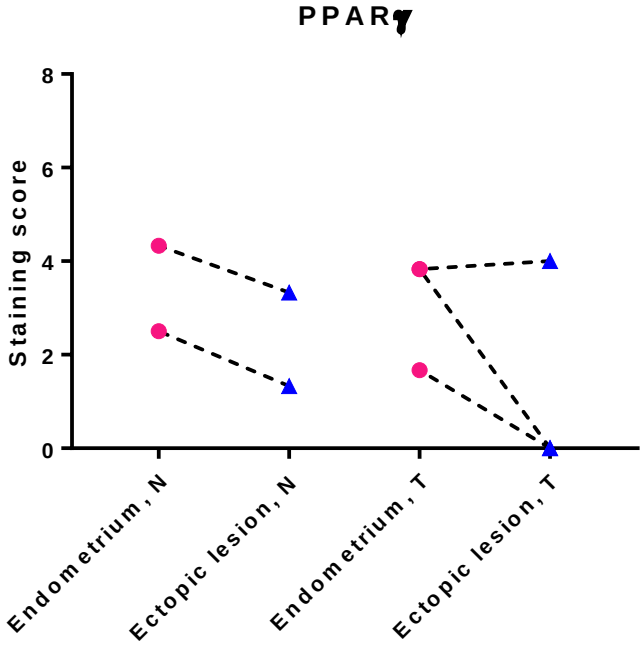
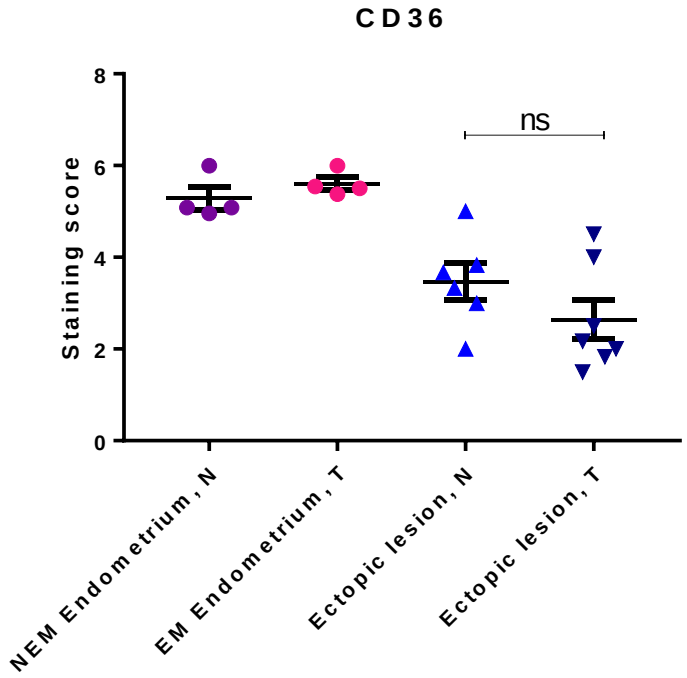


Figure 5.3.39. (A) Immunohistochemistry analysis of peroxisome proliferator-activated receptor gamma (PPARγ) expression in eutopic endometrium and ectopic lesions compared by medical treatment.

*Dots represent the arithmetic mean of histoscores of eutopic gland and stroma from women not diagnosed with endometriosis with regular cycle (NEM,N n=4), women diagnosed with endometriosis taking GnRH agonist (EM,T n=4) and histoscores of ectopic lesions from endometriosis women without GnRH treatment (EC, N n=6) and with GnRH treatment (EC, N n=7). Data are expressed as mean±SEM. Statistical comparisons were performed for the groups of ectopic lesions using Mann-Whitney U test; *p<0.05. (B) Immunohistochemistry analysis of (PPARγ) in paired specimens from women with endometriosis without GnRH treatment (EM Eu N; EM Ec N, n=2) and taking GnRH agonist (EM Eu T; EM Ec T, n=3). Dotted lines indicates the matched eutopic and ectopic specimens.*

(A)



(B)

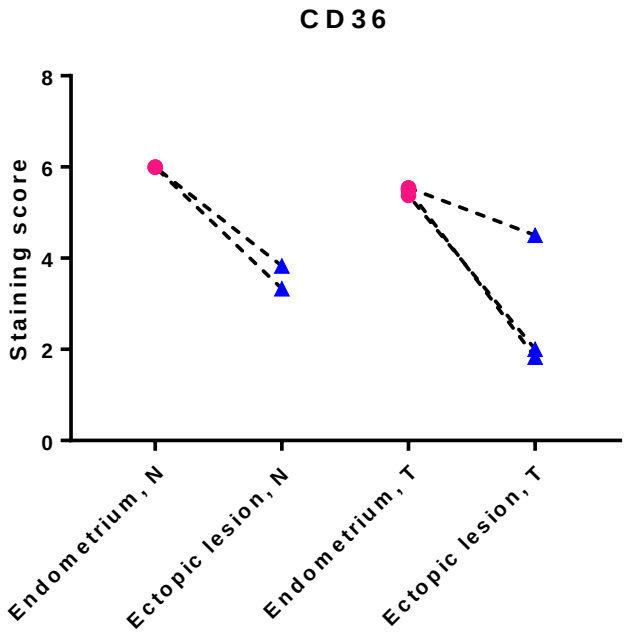


Figure 5.3.40. (A) Immunohistochemistry analysis of CD36 expression in eutopic endometrium and ectopic lesions compared by medical treatment.

Dots represent the arithmetic mean of histoscores of eutopic gland and stroma from women not diagnosed with endometriosis with regular cycle (NEM,N n=4), women diagnosed with endometriosis taking GnRH agonist (EM,T n=4) and histoscores of ectopic lesions from endometriosis women without GnRH treatment (EC, N n=6) and with GnRH treatment (EC, N n=7). Data are expressed as mean±SEM. Statistical comparisons were performed for the groups of ectopic lesions using unpaired t-test, (ns) not significant. (B) Immunohistochemistry analysis of CD36 in paired specimens from women with endometriosis without GnRH treatment (EM Eu N; EM Ec N, n=2) and taking GnRH agonist (EM Eu T; EM Ec T, n=3). Dotted lines indicates the matched eutopic and ectopic specimens.

5.3.8 Summary for the results of the immunohistochemical analysis

Immunohistochemical analysis was performed to confirm the findings of the gene expression study, by assessing protein expression of the genes of interest using immunohistochemical staining, in eutopic endometrium, ectopic lesions and in the peritoneal wall specimens. In addition, the possible effect of GnRH agonist treatment on the expression of cytokeratin, IFITM-1, COX-2, 15-LOX-1, PPARγ and CD36 in ectopic lesions was examined. Table 5.3.2 summarizes the means of histoscores in specimens were used for this study.

Table 5.3.2. Comparison of protein expression in eutopic endometrium (Eu), ectopic lesions (Ec) and in the peritoneal wall (Pw)
from women not diagnosed with endometriosis (NEM) and women diagnosed with endometriosis (EM). Data were obtained using immunohistochemical staining and semi-quantitative scoring method. Statistical comparisons were performed using Kruskal-Wallis testwith Dunn's post-hoc test, one-way ANOVA with Tukey's post-hoc test, unpaired t-test and Mann-Whitney U test where it was appropriate. (a;b) significant

	NEM, Gland n=8	NEM, Stroma n=8	EM, Gland n=7	EM, Stroma n=7	NEM, Eu n=8	EM, Eu n=7	EM, Ec n=13	EM, Pw n=6	EM, Ec,N n=6	EM Ec,T n=7
CK	5.9 ± 0.1[a]	3.3 ± 0.6 [a]	6.0 ± 0.0 [b]	2.1 ± 0.7 [b]	4.6 ± 0.3 [a]	4.1 ± 0.3	2.5 ± 0.5 [ab]	0.4 ± 0.3 [b]	2.6 ± 0.8	2.4 ± 0.7
IFITM-1	2.9 ± 0.7 [a]	5.6 ± 0.2 [a]	2.9 ± 0.3 [b]	5.7 ± 0.2 [b]	4.3 ± 0.3	4.7 ± 0.1	3.7 ± 0.2 [a]	1.7 ± 0.6 [a]	4.1 ± 0.5	3.3 ± 0.2
COX-2	6.0 ± 0.04	4.8 ± 0.5	5.7 ± 0.2	5.4 ± 0.3	5.5 ± 0.2 [a]	5.5 ± 0.2 [b]	3.1 ± 0.4 [ab]	1.7 ± 0.6	4.0 ± 0.3 [a]	2.2 ± 0.5 [a]
15-LOX-1	4.9 ± 0.4	3.5 ± 0.7	4.9 ± 0.4	5.0 ± 0.3	4.2 ± 0.4 [a]	4.9 ± 0.3 [b]	1.8 ± 0.5 [abc]	0.2 ± 0.2 [c]	3.2 ± 0.4 [a]	0.7 ± 0.5 [a]
PPARγ	3.6 ± 0.7	1.5 ± 0.6 [a]	4.0 ± 0.6 [a]	2.9 ± 0.4	2.6 ± 0.6	3.4 ± 0.4	2.0 ± 0.5 [a]	0.2 ± 0.1 [a]	3.1 ± 0.5 [a]	1.1 ± 0.6 [a]
CD36	5.6 ± 0.2	5.5 ± 0.2	5.7 ± 0.2	5.5 ± 0.2	5.6 ± 0.2 [a]	5.6 ± 0.2 [b]	3.0 ± 0.3 [ab]	1.8 ± 0.7	3.5 ± 0.4	2.6 ± 0.4

(G) endometrial gland; (S) endometrial stroma; (N) no hormone treatments or hormonal contraceptives; (T) GnRH treatment; (CK) cytokeratin; (IFITM-1) interferon induced transmembrane protein 1; (COX-2) cyclooxygenase 2; (15-LOX) 15-lipoxygenase; (PPARγ) PPARγ-peroxisome proliferator-activated receptor gamma

5.3.9 Key findings of the immunohistochemical analysis

In summary, the following results have been found with regard of eutopic endometrium: cytokeratin was significantly elevated in glands whereas IFITM-1 was significantly highly expressed in stroma. Statistical differences were not observed between the group of endometriosis and non-endometriosis.

The expression of PPARγ was increased in glandular cells compared to stromal cells. The statistical comparison revealed significant difference between gland from women with endometriosis and stroma from women without endometriosis.

There were no statistical alterations in the expression of COX-2, 15-LOX-1 and CD36 between gland and stroma or between the study groups. These targets showed strong diffuse staining in the both cell types of eutopic endometrium.

Suppressed expression was observed for all six targets in ectopic lesions and in the peritoneal wall specimens compared to eutopic endometrium. The expression of cytokeratin, IFITM-1, 15-LOX-1 and PPARγ were statistically significantly decreased in the peritoneal wall compared to ectopic lesions.

The comparisons of ectopic lesions according to treatment showed that the protein expression of all targets was low in ectopic lesions from endometriosis women using GnRH treatment compared to those without. Moreover the alterations were statistically significant for COX-2, 15-LOX-1 and CD36.

5.4 Discussion

5.4.1 Morphology of lesions

There is an ongoing debate about the classification of ectopic lesions. As discussed in the introduction (section 1.2), an increasing number of studies suggest that the morphology of endometrial lesions is complex and the vast majority of them do not show a well-differentiated glandular and stromal structure.

For this reason, histological and IHC analysis were performed to study the morphology of ectopic lesions. Cytokeratin and IFITM-1 were applied as glandular and stromal markers, respectively. In addition, the gene expression of these markers was also tested using quantitative PCR method to confirm the presence of endometrial like tissue in the ectopic lesions.

Cytokeratin is a reliable and widely used marker for the identification of glandular epithelium (Rekhtman and Bishop, 2011). As was expected, the highest cytokeratin gene and protein expressions were found in the eutopic endometrium since endometrial glandular cells are one of the main cell types in eutopic endometrium. Compared to eutopic endometrium, decreased cytokeratin expression was found in ectopic lesions. This difference could be explained by the different proportions of glandular cells within the specimens. As was described previously in sections 1.2.1 and 1.2.2, glands are abundant in endometrium, whereas they could be sparse or even absent in ectopic lesions. For instance, 2 out of 13 ectopic specimens were not immunoreactive for cytokeratin suggesting the lack of glandular cells for these biopsies.

Identification of the stromal cells within the lesions was more challenging. Several markers have been proposed and reported as ideal stromal markers. One of the most widely used marker is neprilysin, also known as CD10. However, the specificity of this marker is questionable since smooth muscle can also be positive for CD10 and, as a result, can cause misidentification particularly in complex specimens, such as endometrial lesions or tumours (Oliva et al., 2002).

IFITM-1 has been recently described as a possible new endometrial stromal marker (Parra-Herran et al, 2014). Although, the specificity of CD10 and IFITM-1 have not been compared, which is a limitation of this study, the immunostaining of the eutopic endometria revealed significant difference for the immunoreactivity between glandular and stromal cells suggesting that IFITM-1 is a potential stromal marker.

IFITM-1 was detected in all ectopic lesions advising the presence of stromal cells. However, it also was observed that the endothelium of blood vessels were strongly positive for IFITM-1. Popson *et al.* (2014) reported that IFITM-1 is crucial for the lumen formation of vessels (Popson et al., 2014). To date, there are no publications on the role of IFITM-1 in endometriosis. However, these findings might suggest a possible role of IFITM-1 in the angiogenesis of the ectopic lesions.

As mentioned above several recent studies demonstrated that the histological appearance of endometriosis can vary widely and the majority of the lesions show mixed and undifferentiated patterns (Oliva et al., 2002; Abrao et al., 2003; Kamergorodsky et al., 2009). Taken together, the findings from this study are in agreement with the literature. A total of 13 ectopic lesions were investigated and only two specimens showed classic morphology with well-organised glandular and stromal components, while the rest of the samples showed mixed and undifferentiated morphologies. It is acknowledged that several factors, such as menstrual cycle stage, medicinal treatment or peritoneal environment, could affect the appearance of the endometrial lesions (Clement, 2007), as well as that the endometrial lesions evolve and progress with time (Nisolle and Donnez, 1997; Khan et al., 2014). Overall, these factors might provide a good explanation for the diversity of the ectopic lesions. However, more research is required to assess their impacts on the morphology of the ectopic lesions.

The IHC analysis of undifferentiated specimens revealed an unexpected observation, namely the cytokeratin and IFITM-1 showed diffuse staining in some of the ectopic specimens (Figure 5.3.2.D; 5.3.8.D). Moreover, the typical glandular and stromal cell morphology could not been observed in those cases. This phenomenon was particularly prominent for IFITM-1 where the protein expression varied on a wide scale in the undifferentiated ectopic lesions. These observations are hard to explain but might be related to the pathophysiology of endometriosis and suggest a metaplastic change within the lesions. Recent studies reported that IFITM-1 is a possible prognostic marker for various cancers as it is overexpressed in breast, lung, oesophageal and colorectal cancers, furthermore it promotes the proliferation and metastasis of these conditions (He et al., 2015; Borg et al., 2016; Sari et al., 2016; Lui et al., 2017). Thus, it could be hypothesised that IFITM-1 has a role in the proliferation and angiogenesis of the endometrial lesions and overexpression of this protein might correlate with the stages of ectopic lesions. However it is important to point out that although IFITM-1 adequately distinguished glandular and stromal cells in the endometrium, further studies are necessary to validate IFITM-1 as a potential stromal marker in ectopic lesions.

5.4.2 Expression of the enzymes from the lipid mediator biosynthetic pathways in the peritoneum and in the eutopic endometrium

Data obtained from the mass spectrometry study revealed that the concentrations of COX and 15-LOX metabolites were lower in the peritoneal fluid specimens from women diagnosed with endometriosis. To elucidate whether the main enzymes were downregulated causing supressed synthesis of these metabolites, gene expression and IHC studies were performed on ectopic lesions, eutopic endometrium, peritoneal cells and peritoneal wall samples from women with endometriosis and on eutopic endometrium and peritoneal cells specimens from women without endometriosis.

5.4.2.1 Cyclooxygenase-2

A large number of published studies report that COX-2 is overexpressed in eutopic endometrium and ectopic lesions from women with endometriosis and the prostaglandins, particularly PGE_2 have a key role in the pathomechanism of the disease (Chishima et al. 2002; Wu et al. 2007; Bulun 2009). In the current study, low prostanoid concentrations were found in the peritoneal fluid from women with endometriosis. When performing qRT-PCR, the gene expression study did not reveal a significant difference for COX-2 between the study groups (Table 5.2.7). Moreover, histological analysis of COX-2 proteins showed significantly lower expression in the ectopic lesions compared to eutopic endometrium from women with and without endometriosis (Figure 5.3.16). The expression of the COX-2 isoenzyme was particularly low in 6 out of 13 ectopic lesions which might explain the low concentrations of COX metabolites in the peritoneal fluid of those endometriosis patients. However, it should be pointed out that a clear conclusion cannot be drawn from the available data since the expression of COX-1 was not investigated in this study and the protein expression of COX-2 was not tested in peritoneal cells, which also secrete these metabolites (Wu et al., 2002). Although it was not possible to investigate the protein expression of COX-2 in peritoneal cells due to the limited availability of samples, the gene expression study revealed very similar relative gene expression for COX-2 (EM: 0.21±0.07; NEM: 0.19±0.03, p=0.613) in the peritoneal cells from women with and without endometriosis. These findings also point towards the endometrial environment not exhibiting the signs of acute inflammation, such as elevated series-2 prostaglandins or pro-inflammatory cytokines.

With respect to the eutopic endometrium, high relative gene expression was observed for COX-2. The IHC staining also showed strong immunoreactivity in the glandular and stromal cells in both study groups (Figure 5.3.13). It is generally accepted that the high expression of COX-2 is a sign of inflammation. Thus, the upregulated COX-

2 in eutopic endometrium indicates active inflammation for both study groups. However, the elevated expression of COX-2 could also be the part of the normal physiology in the uterus. Increasing evidence suggests that COX-2 is constitutively expressed in the endometrium and is indispensable to the normal function of uterus such as menses and implantation (Langenbach et al., 1995; Dubois et al., 1998). However, most of the studies do not take account of this option (Lipsky et al., 2000). In this study, COX-2 expression in the eutopic endometrium was similar in both groups and as far as the physiological role of COX-2 is considered, the most likely explanation of this finding is that the high COX-2 expression can be attributed to physiological rather than pathological processes. However, it is also necessary to recognise that the study groups were small. Thus, grouping of data according to the stages of menstrual cycle was not possible and the participants in both groups suffered different gynaecological conditions, such as leiomyoma, therefore these factors could also have played a role in the high COX-2 expression in the eutopic endometrial samples.

As was mentioned above, the expression of the COX-2 isoenzyme was particularly low in 6 out of 13 ectopic lesions. The comparison of COX-2 immunoreactivity in ectopic lesions according to medical treatment revealed that the COX-2 isoenzyme expression was significantly lower in those patients who received GnRH analogue treatment. The GnRH agonist treatment might provide an explanation to the down regulation of COX-2 in those specimens. Kim *et al.* investigated the effect of GnRH agonist on stromal cells of eutopic endometrium and endometrioma from women with endometriosis and found that the GnRH treatment downregulated COX-2 expression (Kim et al., 2009). Today, it is acknowledged that ectopic lesions are capable of synthesising oestradiol from cholesterol at the location of the ectopic lesion. This oestrogen supports the growth of endometrial lesions (Bulun, 2005). As was discussed in section 1.6.3, this oestradiol also promotes prostanoid synthesis by upregulating COX-2 expression resulting a pro-inflammatory environment (Figure 1.6.2). Studies found that GnRH agonists suppress oestradiol synthesis by inhibition of aromatase expression resulting hypo-osteogenic state (Ishihara et al., 2003; Kim et al., 2009). As a result, the lack of local oestradiol might provide an explanation to the downregulation of COX-2 in those women who received GnRH agonist treatment.

5.4.2.2 15-lipoxygenase

The other investigated biosynthetic enzyme was 15-LOX, because the most marked differences in expression were found for 15-LOX metabolites in the peritoneal fluid. Although statistically significant differences were not observed, all 15-LOX products

were decreased by at least 60% in endometriosis compared to the non-endometriosis group, regardless of medical treatment. Since all metabolites showed the same trend as the main enzymes, 15-LOX-1 and 15-LOX-2 were hypothesised to be downregulated in endometriosis patients.

Apart from in blood, 15-LOX-2 mRNA was not detected in the investigated specimens; therefore it will not be discussed subsequently. To elucidate the source of 15-LOX metabolites, 15-LOX-1 mRNA and protein expression were examined in ectopic lesions, peritoneal cells and peritoneal wall biopsies from endometriosis and in peritoneal cells from non-endometriosis participants. Amongst the tested specimens, the highest relative mRNA expression for 15-LOX-1 was observed in the peritoneal cells (Table 3.3.7). 15-LOX-1 mRNA expression was double in peritoneal cells in the endometriosis than in the group of non-endometriosis (EM: 2.40±1.45; NEM: 1.22±0.64, p=0.613). For comparison, 15-LOX-1 mRNA expression was 0.52±0.25 in ectopic lesions, 0.49±0.13 in endometriosis and 0.22±0.06 in non-endometriosis eutopic endometrium. These findings suggest that the peritoneal cells could be one of the main sources of 15-LOX products. However, firm conclusion cannot be drawn since the population of cell types for peritoneal cells was not defined in this study.

Although the different types of peritoneal cells were not defined in this study, a large volume of previous research reported that the majority of peritoneal cells are macrophages and the portions of macrophages in peritoneal cells are in the range of 80 to 90% (Haney et al., 1981; Syrop and Halme, 1987; Dunselman et al., 1988). Thus, it could be hypothesised that peritoneal macrophages were one of the major sources of the measured 15-LOX-1 mRNA in the peritoneal cell specimens. To date, no reports have published about the 15-LOX-1 expression in human peritoneal macrophages. However, it is know that the murine peritoneal macrophages highly express the orthologue *ALOX15* isoform (Chen et al., 1994). Moreover, murine models of zymosan A-induced peritonitis are used to study the role of 15-LOX-1 in immune response and inflammation (Rostoker et al., 2013). Data obtained from IHC analysis also support that macrophages express 15-LOX-1, since tissue macrophages in ectopic lesions showed strong immunoreactivity for 15-LOX-1 (Figure 5.3.21D). In conclusion, the data reported here did not support the assumption that the 15-LOX expression is downregulated in peritoneal cells from endometriosis subjects, since 15-LOX-1 mRNA expression was higher in peritoneal cells from endometriosis compared to non-endometriosis patients. However, as was mentioned above, protein expression was not investigated in these cases. In addition, peritoneal cells were only one possible source of 15-LOX metabolites.

15-LOX-1 expression was also investigated in ectopic lesions. The mRNA and protein expression varied on a wide scale. A comparison of the mRNA expression according to medical treatment did not reveal any significant difference in expression in ectopic lesions from women with and without GnRH treatment. However, protein expression was significantly lower in women who received GnRH agonist treatment. Similarly to COX-2, GnRH treatment might promote the downregulation of 15-LOX-1 in ectopic lesion, which might explain the low concentrations of LOX metabolites in the peritoneal fluid of those endometriosis patients. The comparison of ectopic lesion according to lesion types showed that 15-LOX-1 was highly expressed in endometrioma, and not expressed in DIE specimens (Figure 5.3.23B). However, further analysis of the data revealed that patients with endometrioma (HP24, HP47) were not on hormonal medication whereas patients with DIE (HP51, HP53) used a GnRH agonist for their condition. The effect of a GnRH agonist on 15-LOX expression has not been investigated so far, therefore firm conclusion cannot be drawn from this finding but these results might allow the speculation that GnRH agonists somehow inhibit 15-LOX-1 expression in ectopic lesions.

Previous studies have not paid much attention to the role of the peritoneal wall in the pathology of endometriosis, even though the theory of coelomic metaplasia proposes the peritoneum as the possible origin of endometriosis. Today, the main research focus is restricted to the expression of adhesion molecules on mesothelial cells and the attachment of endometrial cells to the surface of peritoneum. To avoid this limitation, peritoneal wall biopsies were also included in this study. However, the results are limited since peritoneal specimens were only available from endometriosis patients. For 15-LOX-1, it was found that the mRNA concentration was lower in peritoneal wall biopsies from endometriosis patients compared to ectopic lesions (Table 5.2.3). Except for a weak positive immunostaining, the 15-LOX-1 was not detected in these specimens suggesting that the peritoneum was not the main source of 15-LOX metabolites (Figure 5.3.23).

To date, the role of 15-LOX derivatives has not been elucidated in endometriosis. Moreover, there is a gap of knowledge regarding their role in the female reproductive system. Animal studies demonstrated that 15-HETE is a regulator of the follicle maturation and indispensable to the ovulation (Tanaka et al., 1989; Downey et al., 1998). To improve our understanding about the role of 15-LOX in the female reproductive system for human, 15-LOX-1 expression was investigated in eutopic endometrium from women with and without endometriosis. Strong 15-LOX-1 immunoreactivity was observed in eutopic endometrium from women with and without endometriosis (Figure 5.3.19). There were also no significant differences

between glands and stroma or between endometriosis and non-endometriosis groups. Thus far only a single study has investigated 15-LOX-1 expression in normal eutopic endometrium, as well as in endometrial hyperplasia and endometrial adenocarcinomas. High 15-LOX-1 expression was reported in normal endometrium, which is in agreement with the results of the current study. In addition they also found that the protein expression of 15-LOX-1 was decreased from the normal eutopic endometrium to hyperplasia and was significantly lower in adenocarcinomas compared to normal endometria, suggesting that the downregulation of 15-LOX-1 might play a role in the tumorigenesis (Sak et al., 2016).

The primary aim of these experiments was to assess the 15-LOX expression in women with and without endometriosis and investigate whether downregulation of 15-LOX enzymes correlates with decreased concentrations of 15-LOX derived mediators. Based on this study, firm conclusion cannot be drawn. Different grades of 15-LOX expression were observed in the tested specimens. For instance, elevated gene expression was observed in peritoneal cells from endometriosis subjects, which was contradictory with other research findings. Again, in these cases the protein expression was not measured, thus strong conclusions cannot be drawn. However, the significantly lower expression of 15-LOX-1 isoenzyme in ectopic lesions from endometriosis patients with GnRH treatment might allow speculation that the GnRH agonists downregulate 15-LOX-1 expression.

5.4.3 The role of PPARγ in the pathology of endometriosis

The pathomechanism of endometriosis is not yet completely understood but the dysfunction of immune system is a well-accepted theory (Manolova et al., 2011). The natural function of the body's immune system is to detect and destroy pathogens and aberrant cells. A failure to recognise and eliminate micro-organisms or abnormal cells may lead to disease pathogenesis. Peritoneal macrophages are the sentinels of peritoneal homeostasis and the key regulators of the immune response.

Macrophages express scavenger receptors to enhance their phagocytic ability (Van Berkel et al., 2000). Scavenger receptors are a class of structurally diverse transmembrane receptors with broad ligand specificity including oxidised low density lipoprotein (oxLDL), fatty acids, anionic phospholipids and apoptotic cells (Krieger, 2001; Zeng et al., 2003). Decreased expression of these receptors may disturb the phagocytic ability of macrophages. Reduced expression of CD36, a class B scavenger receptor, was reported in peritoneal macrophages from women with endometriosis resulting in supressed phagocytic activity (Chuang et al., 2009).

5.4.3.1 Expression of PPARγ mediated CD36 expression

CD36 was also identified as a receptor of thrombospondin (TSP)-1 on platelets (Li et al., 1993). Today, CD36 is defined as a multi-ligand scavenger receptor for oxLDL, long-chain fatty acids, apoptotic cells and collagens (Febbraio et al., 2001). CD36 is widely expressed on microvascular endothelial cells, adipocytes, monocytes, macrophages, dendritic cells and on epithelial cells and regulates a broad range of physiological processes (Febbraio et al., 2001). For instance it facilitates the uptake of long-chain fatty acids in adipocytes and muscle cells, important for providing energy, cellular membrane structures and for precursors of lipid mediators (Abumrad et al., 1993; Ibrahimi et al., 1999). However, disturbed expression of CD36 in pathological condition has also been reported. For example, CD36 has been found to regulate oxidative stress and inflammation in many conditions, such as Alzheimer's disease, atherosclerosis and fibrosis in chronic kidney failure (Okamura et al., 2009; Vangaveti et al., 2010). Its pathological role is well-characterised in atherosclerosis.

oxLDL was found to be a potent chemoattractant for circulating monocytes (Quinn et al., 1987). Monocytes bind with a high affinity to oxLDL via CD36 and uptake these lipoproteins by receptor-mediated endocytosis. The uptake of oxLDL by the monocytes promotes monocyte/macrophage differentiation and further upregulates CD36 in the differentiated macrophages. Lipoproteins overloading of macrophages leads to foam cell formation, fat-laden macrophages that serves as a hallmark of early stage atherosclerosis (Tontonoz et al. 1998). The oxLDL mediated upregulation of CD36 is regulated by PPARγ (Nagy et al. 1998). *CD36* gene contains PPARγ responsive elements in macrophages; as a result PPARγ is directly capable to regulate the transcription of CD36 (Febbraio et al., 2001). Nagy et al. (1998) also demonstrated that the main components of oxLDL were 9-HODE and 13-HODE, the endogenous ligands for PPARγ (Nagy et al. 1998). These findings suggest a positive feedback loop occurs in foam cells where a direct regulatory relationship exists between PPARγ and CD36. Figure 5.5.1 illustrates the positive feedback loop in foam cells.

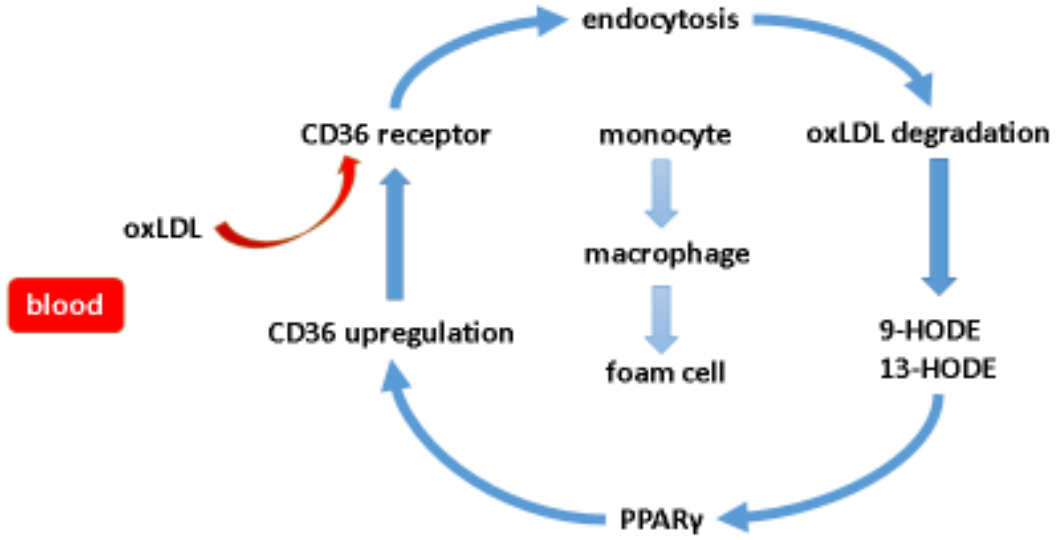


Figure 5.4.1. Schematic drawing of foam cell formation in atherosclerosis
oxLDL binds with the high affinity to scavenger receptor CD36 on vascular monocytes. Monocytes uptake oxLDL via receptor mediated endocytosis. 9-HODE and 13-HODE are released during the degradation of oxLDL and activate PPARγ. PPARγ directly upregulates CD36 transcription by binding to the PPARγ responsive element of CD36. In addition, PPARγ promotes monocyte/macrophage differentiation. The upregulation of CD36 further facilitates the uptake of oxLDL in the differentiated macrophages resulting in pathological, lipid-loaded foam cells. (oxLDL) oxidised low density lipoprotein; (HODE) hydroxyoctadecadienoic acid; (PPARγ) peroxisome proliferator-activated receptor γ

Studies using *in vitro* techniques revealed that cytokines, such as IL-4, are also able to induce the upregulation of CD36 in macrophages. This upregulation was a result of coordinate induction of PPARγ and 15-LOX-1 (Huang et al., 1999). 15-LOX-1 expression has been found to be inducible by Th2 cytokines, such as IL-4 and IL-13 in monocytes, alveolar macrophages and a lung carcinoma cell line (Heydeck et al. 1998; Schnurr et al. 1999; Berry et al., 2007). 15-LOX metabolites activate PPARγ which induces the transcription of CD36 resulting in the upregulated CD36 expression in the cells (Huang et al., 1999). Figure 5.5.2 represents the process of cytokine induced CD36 upregulation in macrophages.

Figure 5.4.2. Schematic drawing of cytokine induced CD36 upregulation in macrophages.
IL-4 and IL-13 induce the expression of 15-LOX-1. 13-HODE, the main product of 15-LOX-1 bind to and activate PPARγ. The activated PPARγ induces the transcription of CD36. (IL) interleukin; (LOX) lipoxygenase; (HODE) hydroxyoctadecadienoic acid; (PPARγ) peroxisome proliferator-activated receptor γ

These studies demonstrated that there is a direct relationship between PPARγ and CD36 in macrophages; furthermore the presence of PPARγ ligands is necessary to induce PPARγ mediated CD36 upregulation. Hence, the presence of CD36 might provide essential information about the activity of PPARγ.

5.4.3.2 Expression of PPARγ and CD36 in the peritoneum and eutopic endometrium

As was discussed in Section 1.6.5, the role of PPARγ in the pathophysiology of endometriosis has been proposed but has so far not been extensively studied. Data obtained from mass spectrometry revealed that endogenous agonists of PPARγ were downregulated in peritoneal fluid from women with endometriosis compared to those without. PPARγ has a relatively large binding pocket, which binds a broad range of natural and synthetic ligands (Itoh et al. 2008). Oxygenated lipid mediators, such as 15d-PGJ_2, HETEs, HODEs and oxoODEs were proposed as endogenous ligands for PPARγ (Forman et al. 1995; Nagy et al. 1998). Although a large number of studies identified lipid metabolites as potential agonists for PPARγ using *in vitro* techniques (Nagy et al. 1998; Egawa et al. 2016), the low physiological availability of these molecules, e.g. 15d-PGJ_2, question their relevance *in vivo* (Powell, 2003). LA derived 13 and 9-HODE and their oxidized forms, 13 and 9-oxoODE were also reported as potent agonists for PPARγ (Nagy et al. 1998). Since these metabolites are abundant in the body it could be assumed that they also act as endogenous ligands for PPARγ. In this study 13-HODE was the most abundant oxygenated lipid mediator (NEM: 192±81.9pg/ml; EM: 57.2±26.7pg/ml, p=0.370) in the peritoneal fluid. It is noteworthy that the 13-HODE concentration was more than three times lower in the endometriosis group compared to non-endometriosis group. The concentration of 13-HODE was 3.5- and 2.5-fold higher than the next most abundant mediator 9-HODE in women with and without endometriosis respectively (NEM: 55.5±22.5pg/ml; EM: 23.0±12.5pg/ml, p=0.433). Hence, it was hypothesised that the insufficient availability of endogenous ligands leads to dysregulation of PPARγ mediated pathways.

To elucidate this question and extend our knowledge about the role of PPARγ in the pathomechanism of endometriosis the gene and protein expression of PPARγ and CD36 were studied in ectopic lesions, eutopic endometrium, peritoneal cells and peritoneal wall samples from women with endometriosis and in eutopic endometrium and peritoneal cells from women without endometriosis.

A large number of studies have demonstrated the presence and significance of PPARs, including PPARγ, in the female reproductive system (Komar 2005; Froment et al. 2006; Toth et al. 2007). PPARs regulate ovarian function, such as follicular development, oocyte maturation, ovulation and steroidogenesis in addition to tissue remodelling and angiogenesis (Komar, 2005; Froment et al., 2006). Chen *et al.* (2009) suggested that PPARγ may be involved in ovulation and luteinisation by downregulating TNFα secretion in granulosa-lutein cells (Chen et al., 2009). PPARs also control tissue remodelling and angiogenesis in ovaries by regulating the expression of proteases such as MMP-3, -9, -13, and angiogenic factors, like VEGF and its receptors (Bogacka et al., 2015). The function of PPARs in the uterus has not been extensively studied but it is very likely that, similar to their role in the ovary, PPARs regulate endometrial tissue remodelling and angiogenesis (Bogacka et al., 2015).

In the current study, PPARγ was highly expressed in glandular cells and moderately expressed in stromal cells of eutopic endometrium (Figure 5.3.24 and 5.3.25). The elevated expression of PPARγ in glandular cells might suggest PPARγ-mediated transcriptional regulation, which may be related to their secretory function. Also strong immunoreactivity was observed for CD36 in eutopic endometrium (Figure 5.3.29). There was no difference in the intensity with regard to CD36 between the glands and stroma, or the endometriosis and non-endometriosis groups (Figure 5.3.30). Although PPARγ-mediated CD36 expression has not yet been studied in eutopic or ectopic endometrium, the presence of PPARγ and the highly expressed CD36 in eutopic endometrium may indicate that the endogenous PPARγ ligands were present in adequate concentrations in both study groups and that the PPARγ activity resulted in the upregulation of CD36. However, PPARγ was not detected in some control endometria (Figure 5.3.25) but CD36 was expressed in all specimens (Figure 5.3.30). This apparent contradiction may be due to basal, PPARγ-independent CD36 expression. For instance, Chawla *et al.* reported that PPARγ deficient macrophages express CD36 at low levels. They also found that the synthetic PPARγ agonist rosiglitazone did not have an effect on CD36 expression in these PPARγ-deficient cells, whereas the same treatment increased CD36 expression in the wild-type macrophages (Chawla et al., 2001). Therefore, it is likely that both PPARγ-dependent and independent pathways were activated in eutopic endometrium.

Gene expression results for peritoneal cells also suggest that PPARγ activity was not disturbed. Similar PPARγ gene expression was observed in both study groups (EM: 0.68±0.16; NEM: 0.54±0.09, p=0.336). Downregulated CD36 expression was expected in the peritoneal cells from women with endometriosis suggesting the

dysfunction of PPARγ. However, increased relative gene expression was found for CD36 in the endometriosis group (EM: 2.14±0.68; NEM: 1.82±0.49, p=0.463), which might suggests the appropriate function of PPARγ. PPARγ directly regulates the transcription of CD36, therefore mRNA data probably sufficiently prove that the transcription regulatory role of PPARγ were not disturbed on these occasions.

The IHC analysis of ectopic lesions showed similar results as eutopic endometrium for PPARγ and CD36. Namely, PPARγ was not detected in 4 out of 13 ectopic specimens (Figure 5.3.27) whereas all sections were positive for CD36 (Figure 5.3.32). Further analysis also highlighted that these patients received a GnRH agonist treatment for their condition. Moreover, the comparison according medical treatment revealed that PPARγ protein expression was significantly lower in ectopic lesions from endometriosis patients receiving GnRH treatment compared to those without (Figure 5.3.39A); whilst the same comparison for CD36 did not show a significant difference between the two study groups (Figure 5.3.40A). With regard to the regulatory relationship between PPARγ and CD36, the same explanation could be applied here that PPARγ-dependent and independent pathways were activated in the ectopic lesions resulting the expression of CD36. However a noteworthy phenomenon was also observed with regard to the negative PPARγ sections. These sections were also negative for 15-LOX-1 (Figure 5.3.22); in addition, three out of four patients had recurrent endometriosis. Overall, these findings might allow the speculation that GnRH treatment inhibits the expression of both targets and as a result of the downregulation of 15-LOX-1 and PPARγ, may promote disease relapse. However, it must pointed out that the study groups were small, hence firm conclusions cannot be drawn from these data.

PPARγ protein expression showed a wider scale variation in ectopic lesions. The altered PPARγ expression could have been associated with the pathomechanism of endometriosis in several ways, but two processes could be particularly important in the pathology of endometriosis. Firstly, PPARγ was reported as negative regulator of fibrogenesis (Mann et al. 2010). In 2017, Liu *et al.* investigated the correlation between PPARγ expression and fibrosis in endometrioma and DIE specimens using the IHC method. They found that PPARγ was involved in the fibrogenesis of endometrial lesions and PPARγ expression was negatively correlated with the extent of fibrosis (Liu et al., 2018). Data presented here are in agreement with this research. Well-differentiated lesions showed high expression of PPARγ (Figure 3.4.27), whereas PPARγ expression was decreased or absent in inactive, fibrotic lesions and DIE specimens (Figure 5.3.28). Taking this study into account, it might provide an alternative explanation for the low expression of PPARγ in ectopic lesions. It is well

known that endometriotic lesions show a kaleidoscopic variation in size, colour and structure, but their proliferation with time is poorly understood. However these findings might suggest the role of PPARγ in lesion kinetics.

Secondly, the regulatory role of PPARγ in steroidogenesis is acknowledged. Numerous studies have investigated the role of PPARγ in mediating steroidogenesis in follicular cells. For instance Fan *et al.* (2005) reported that PPARγ downregulated oestradiol secretion from ovarian granulosa cells by inhibiting aromatase expression via NF-κB (Fan et al. 2005). Chang *et al.* (2013) observed the same inhibitory effect of PPARγ on aromatase in endometrial stromal cells. In addition they also found that the ligand-activated PPARγ downregulated COX-2 resulting in decreased PGE_2 production (Chang et al., 2013). This finding could be relevant to endometriosis since aromatase is aberrantly expressed in ectopic lesions resulting in local oestrogen synthesis (Bulun, 2005). The inhibition of aromatase via PPARγ in ectopic lesions could be beneficial to suppress oestradiol release and shrink lesion size, whilst the downregulation of COX-2 could suppress the pro-inflammatory processes within the lesion microenvironment.

In addition, the study demonstrated that there is a transregulatory action between PPARγ and the also ligand activated nuclear receptor oestrogen receptor (ER) α and ERβ. Ligand-activated PPARγ downregulates the expression of ERα in endometrial cancer cells resulting suppressed migration, invasion and proliferation (Zhang et al., 2015). Furthermore, PPARγ acts as a competitive inhibitor of oestrogen regulated genes by binding to their oestrogen binding elements within their promoter sequence and as a consequence, prevents the transactivation by ER in these genes (Keller et al, 1995). In contrast, oestradiol downregulates CD36 expression in breast cancer cell lines (Uray et al., 2004) and inhibits the translocation of CD36 into the plasma membrane in experimental animal model (Zafirovic et al., 2017). These results shows that oestrogen is capable of regulating PPARγ-mediated gene expression. This relationship is particularly important since it can provide a hypothesis as to why CD36 gene expression was robustly upregulated in the GnRH agonist treated endometriosis group (Table 5.2.8). GnRH treatment suppresses oestrogen production causing a hypo-oestrogen state. In the absence of oestrogen, ERs cannot regulate the PPARγ-mediated transactivation of CD36, resulting an aberrant upregulation of this gene. Although CD36 mRNA expression was nearly 70-fold higher in ectopic lesions from women with GnRH treatment compared to those without, the protein expression could not confirm such an upregulation of CD36 in ectopic lesions from women with GnRH agonist treatment (Figure 5.3.40). A likely explanation of this contradiction is

that post-translation modifications might be responsible for the decreased protein expression in those samples.

The aim of this section was to investigate whether the low concentration of endogenous ligands of PPARγ in endometriosis patients could lead to the dysfunction of this nuclear receptor. Taken together, data presented here are not sufficient to drawn a firm conclusion due to the small and heterogeneous study groups. Downregulation of CD36 was expected in the endometrial specimens compared to control. However, the data do not support this hypothesis, which might allow the assumption that the function of PPARγ was not impaired in endometriosis.

5.4.4 Expression of target genes in the blood

Gene expression of cytokeratin 8, IFITM-1, COX-2, 15-LOX-1, 15-LOX-2, PPARγ and CD36 were investigated in peripheral blood from women with (n=13) and without (n=7) endometriosis using the SYBR Green method. Except for cytokeratin 8, all targeted genes were detected in blood. Gene expression was compared according to presence or absence of endometriosis (Table 5.2.5) but not according to menstrual cycle or medical treatments due to insufficient numbers of subjects in the study groups. Statistical analysis did not reveal any significant differences in the expression of genes of interest in blood between the endometriosis and non-endometriosis groups.

With regard to enzymes of lipid mediator biosynthetic pathways, the mRNA expression of COX-2, 15-LOX-1 and 15-LOX-2 were investigated. As mentioned above, statistically significant differences were not observed for these genes between the study groups (Table 5.2.3). COX and 15-LOX metabolites in plasma (Table 3.4.4 and 3.4.5) from women with and without endometriosis were also not significantly different, which supports the results of the mRNA expression study. However, it necessary to point out that the TXB_2 concentration was 3.7-fold higher in the endometriosis compared to the non-endometriosis group, whereas COX-2 expression showed a slight elevation only in the endometriosis group (EM: 1.10±0.19; NEM: 0.82±0.12, p=0.485). This contradiction may be explained by the fact that COX-1 expression was not investigated, and only blood cells were tested. TXA_2 is predominantly derived from platelet COX-1; in addition vascular endothelial cells are also a rich source of COX metabolites (Félétou et al., 2010). However these sources were not investigated in the current study.

The level of 15-LOX metabolites did not show significant alteration between endometriosis and non-endometriosis groups. Moreover, the mean concentrations

were very similar for all 15-LOX products. The mRNA expression of 15-LOX-1 and 15-LOX-2 were also similar in the two study groups. These findings might suggest that the 15-LOX pathway was not altered in the vascular system in women diagnosed with endometriosis. On the other hand, it is notable that the source of 15-LOX-1 is very likely to be the immature red blood cells rather than monocytes, since 15-LOX-1 is constitutively expressed at high levels in reticulocytes (Nadel et al., 1991) whilst peripheral monocytes do not express 15-LOX-1 in the circulation (Kühn and O'Donnell, 2006). To date, 15-LOX-2 expression has not been reported in blood. Therefore further studies would be necessary to identify the source of the 15-LOX-2 mRNA in the blood.

In this study, IFITM-1 was used as a putative endometrial stromal marker but it is noteworthy that high expression was observed in blood. The relative gene expression was same in eutopic endometrium (EM: 0.74±0.14; NEM: 0.78±0.10, p=0.383) and blood (EM: 0.78 ± 0.14; NEM: 0.63 ± 0.13, p=0.506) specimens. Although the physiological expression of IFITM-1 expression in blood has not been reported so far, this protein plays a crucial role in the inhibition of cellular entry of numerous viruses (Weston et al., 2014). Hence the high expression in blood might relate to leukocytes. In addition an increasing number of studies suggest IFITM-1 is a possible prognostic marker for several cancers and plays an anti-tumorigenic role (Borg et al., 2016; Lui et al., 2017). High IFITM-1 expression was reported in blood leukocytes from low risk chronic myeloid leukaemia patients, which may support that leukocytes constitutively express this protein. Of note, the same study reported that IFITM-1 was a potential prognostic marker for chronic myeloid leukaemia since the IFITM-1 expression was significantly decreased from the low risk group to the high risk group and the gene expression was positively correlated with survival (Akyerli et al., 2005).

The mRNA expression of PPARγ and CD36 were also measured in blood specimens, but differences were not observed between the endometriosis and non-endometriosis groups. (PPARγ EM: 0.02 ± 0.004; NEM: 0.02 ± 0.004, p=0.877; CD36: EM: 0.98 ± 0.11; NEM: 0.82 ± 0.09, p=0.386). As discussed earlier, the ligand activates PPARγ directly, upregulates the transcription of CD36 in monocytes and macrophages as well as facilitating macrophage differentiation. However the low PPARγ and high CD36 expression observed seem to contradict that observation. To explain this apparent contradiction, it is important to take into account two facts. Firstly, CD36 is constitutively highly expressed in platelets (Ghosh et al., 2011). Thus the detected CD36 mRNA was very likely derived from platelets. Secondly, the low expression of PPARγ in the blood related to the inactive state of monocytes. Under physiological conditions, PPARγ expression is low in peripheral leukocytes (Amoruso et al., 2007).

Thus this observation could be explained by a PPARγ-independent regulation. On the other hand, low PPARγ expression may also suggests that systemic inflammation was not present in either endometriosis or non-endometriosis patients, possibly because of its peritoneal-based location. In contrast, rheumatoid arthritis (RA) is a chronic inflammatory condition characterised by systemic inflammation and the presence of pro-inflammatory monocytes (Fu et al., 2018). Ganeb *et al.* (2016) found that the PPARγ expression was significantly increased in peripheral monocytes from RA patients compared to healthy subjects regardless the stage of the disease (Ganeb et al., 2016).

Taken together mRNA expressions of cytokeratin 8, IFITM-1, COX-2, 15-LOX-1, 15-LOX-2, PPARγ and CD36 were very similar in the study groups. Although it was not possible to investigate all enzymes of the lipid mediator pathways, the mRNA expression results were in agreement with and corroborate the results obtained from mass spectrometry.

6 CHAPTER: General discussion

6.1 Introduction

Although endometriosis was first described more than 100 years ago, the aetiology of the disease is still strongly debated. Research seeks to explain what factors or abnormalities make endometrial cells capable of attaching to the surface of the peritoneum to establish ectopic lesions. A possible way of answering this is to study the differences at the molecular level between eutopic endometrium and ectopic lesions from women with endometriosis and make comparisons between these tissues and eutopic endometrium from non-endometriosis subjects. However, the dynamic balance between the ectopic lesions and their microenvironment should not be ignored. Studying the relationships within this special milieu is necessary to the better understanding of the pathomechanisms and the immune response between the endometrial lesions and the members of immune system. A large number of studies focus on the immune response and suggest that the inflammatory response is disturbed in endometriosis and may perpetuate the disease.

Inflammation is a dynamic process where the role of lipid mediators is essential. Pro-inflammatory mediators initiate the elimination, whilst pro-resolving mediators lead the resolution and return to homeostasis. This active process requires active communication between the participants also between participants and the environment. Bioactive mediators are charged to deliver these messages. Cytokines and hormones are the messengers of the immune and the endocrine system, respectively whilst lipid mediators due to their short half-life, are mainly responsible for the local communication. Better understanding of these interactions could provide a deeper insight into the pathological processes of endometriosis.

The primary aim of the current study was to compare the lipid mediator profile of fluid specimens from women with and without endometriosis. For this reason, LC/ESI-MS/MS was applied to define the oxygenated lipid mediator profile of biological fluids from the peritoneum and the vascular system. This advanced MS technique provides a high sensitivity, specificity and throughput screening of lipid molecules simultaneously in a small specimen volume. Although some recent studies using a similar approach have already examined the alterations of lipid profiles in endometriosis those studies focused on different classes of the lipids, such as phospho- and sphingolipids (Lee et al., 2014; Chagovets et al., 2017; Domínguez et al., 2017; Adamyan et al., 2018). Thus far, this is the first study measuring 79 oxygenated lipid mediators simultaneously in biological fluid specimens from women with and without endometriosis to explore possible new aspects for the pathology of the disease.

6.2 Proposed pathomechanism of endometriosis in the peritoneum

The lipids of interest were produced through three biosynthetic pathways, namely COX, LOX and cytochrome P450 as well as in a non-enzymatic manner. Overexpression of COX-2 was reported in ectopic lesions and peritoneal macrophages, along with increased concentration of PGE_2 from women with endometriosis (Ota et al., 2001; Wu et al., 2002; Khan et al., 2012). Hence, elevated series-2 prostaglandin concentrations were expected in the peritoneal fluid from women with endometriosis. Contrary to our hypothesis, this study did not find a significant difference in expression of prostaglandins in the peritoneal fluid between endometriosis and non-endometriosis.. Moreover, the main prostaglandins, such as PGE_2 and $PGF_{2\alpha}$ did not reach the limit of detection in any group. It is somewhat surprising since these metabolites play a cardinal role in the initiation of inflammation and trigger pain. Two thirds of endometriosis patients (66.7%), whereas only 37.5% of non-endometriosis patients reported pelvic pain (Table 3.2.1). To explain this finding, several explanations can be considered here.

Multiple factors could have an effect on the prostanoid concentrations. One of the most well-known is the menstrual cycle phase. Studies have previously reported that the concentration of PGE_2 and $PGF_{2\alpha}$ increased in the endometrium from the proliferative stage through the secretory stage, and reached the peak in the menstrual phase (Downie et al., 1974). Data in this study were analysed according to the stages of menstrual cycle to assess the variation of lipid signals across the cycle. However, due to the insufficient numbers of participants in the study groups, firm conclusion could not been drawn with regard to the variation of prostanoids across the menstrual cycle.

Further explanations of the low prostanoid levels in the peritoneal fluid could be the following: lipid mediators are potent but short-lived molecules, particularly prostanoids which have a short half-life. The majority of the fluid specimens were normalised since they were collected as peritoneal washes. The normalizing method may have had an effect on the concentration of measured metabolites. Furthermore, it is acknowledged that NSAIDs express their inflammatory and analgesic effects by inhibiting the expression of COX. Since the majority of the participants complained about pelvic pain, it is very likely they used NSAIDs since these drugs easily available and they are highly recommended by doctors as the empirical treatment for endometriosis (Kennedy et al., 2005). In addition, more than half of the

endometriosis patients used GnRH agonists for their condition, which also could have an inhibitory effect on the COX expression. Grouping data according to the medical treatment showed that COX metabolites were the lowest in the GnRH treatment group, compared to endometriosis and non-endometriosis women without GnRH agonist treatment.

To define the expression level of COX-2, different specimens from women with and without endometriosis were assessed by qPCR and IHC. Similar relative gene expression was observed for COX-2 in eutopic endometrium and ectopic lesions for the case and the control groups (Table 5.2.7). In contrast, although the protein expression of COX-2 was equally high in the eutopic endometrium from women with and without endometriosis, the expression in ectopic lesions were significantly lower compared to eutopic endometrium from women with and without endometriosis (Figure 5.3.16). Further analysis revealed that COX-2 isoenzyme expression was significantly lower in those patients who received GnRH analogue treatment (Figure 5.3.37). This finding is consistent with the mass spectrometry results, since COX products were the lowest in the GnRH treatment group compared to endometriosis and non-endometriosis subjects without GnRH treatment. Overall, the results may suggest that GnRH agonists suppress the expression of COX-2, and as a results supress the pro-inflammatory environment in the peritoneum.

15-LOX-1 metabolites were also depleted in the peritoneal fluid from women with endometriosis. Therefore, mRNA and protein expression of the biosynthetic enzymes of the 15-LOX pathway were also investigated to clarify whether the low expression of these enzymes resulted in low concentrations of 15-LOX products in the peritoneal fluid in the endometriosis group. The outcomes of 15-LOX study resembled those of the COX results. 15-LOX-1 isoenzyme was highly expressed in the eutopic endometrium and no significant difference was observed between the groups of endometriosis and non-endometriosis women (Figure 5.3.19). However, the protein expression was significantly lower in ectopic lesions compared to eutopic endometrium from women with and without endometriosis (Figure 5.3.22). Grouping data according to medical treatment also revealed that 15-LOX-1 isoenzyme was significantly downregulated in endometriosis women receiving GnRH agonist treatment, compared to those who were not (Figure 5.3.38). These findings might indicate that GnRH agonists suppress the expression of 15-LOX-1. However, it is also important to point out that while the lowest COX metabolite levels were observed in the treatment group, this was not the case for 15-LOX metabolites. The means of the latter lipids were similar in the treated and non-treated endometriosis groups (Table 3.4.2). In addition, mRNA expression of 15-LOX-1 was double in peritoneal cells in

women with endometriosis compared to women without endometriosis (EM: 2.40±1.45; NEM: 1.22±0.64, p=0.613), which is contradictory to expectation.

Taken together, the data presented here is not sufficient to conclude that 15-LOX-1 was downregulated in the peritoneum of endometriosis patients. However, the significantly low expressed 15-LOX-1 isoenzyme in ectopic lesions from endometriosis patients with GnRH agonist treatments might allow the speculation that the GnRH agonists downregulate the expression of 15-LOX-1.

The further objective of the current study was to investigate the possible consequences of the decreased 15-LOX metabolites in the peritoneal fluid from women with endometriosis. It was assumed that the low concentration of 15-LOX metabolites might disturb the PPARγ-mediated pathways. To elucidate this question, the mRNA and protein expression of PPARγ and CD36 were studied.

The most striking results were observed in the treatment group. PPARγ and CD36 were significantly upregulated in ectopic lesions from women receiving pharmacotherapy (Figure 3.2.17B and 3.2.20B). The mRNA expression of PPARγ showed 7.7-fold, whereas the mRNA expression of CD36 showed 68.6-fold upregulation in ectopic lesions from endometriosis patients with GnRH agonist treatment compared to endometriosis patients who did not used GnRH agonists for their condition. Unexpectedly, the protein expression did not confirm the upregulation of these targets. PPARγ was significantly downregulated in ectopic lesions from endometriosis women with GnRH agonist treatment, and there was no significant difference observed for CD36. This discrepancy might be explained by the fact that it were not always possible to use the same specimens for gene and protein expression studies due to the size of the ectopic lesions, or that unknown post-translational modifications might downregulate protein expression.

In summary, it was hypothesised that CD36 expression would be downregulated in the endometriosis groups, supporting the idea that the low concentration of 15-LOX disturbs the function of PPARγ. However, the results of this research do not support this hypothesis, therefore it cannot be concluded that the function of PPARγ was impaired in endometriosis.

6.3 Lipid mediators in the vascular system

The other objective of this study was to evaluate the lipid mediator profiles of plasma from women with and without endometriosis. Recent systemic reviews have highlighted that despite considerable research efforts, reliable diagnostic biomarkers still do not exist and the laparoscopy remains the gold-standard diagnostic method for endometriosis (Gupta et al. 2016; Nisenblat et al. 2016; Liu et al. 2015). Therefore, it was investigated whether any lipid mediator or lipid mediator profiles may provide a novel group of compounds that could be used as biomarkers for the diagnosis of endometriosis. Additionally, studying the lipid mediator signature of plasma for women with the disease may facilitate the better understanding the systemic effects of the pathomechanism of endometriosis.

As was discussed earlier the study groups were heterogeneous, therefore several factors could have had an impact on the plasma levels of the targets. Data presented here could not provide a clear answer as to how these lipids vary across the menstrual cycle. Based on the evidence presented here it is very likely that the menstrual cycle has an effect on synthesis of these mediators, however the numbers of participants in the study groups were not sufficient to draw such a conclusion.

Comparison of data according the medical treatment revealed significant alterations between the study groups. 5-LOX derived 4-HDHA, as well as 12-LOX and sEH derived products were significantly changed between the study groups (Table 3.4.4). 4-HDHA was significantly lower in the non-treated endometriosis group, whereas all 12-LOX detected metabolites were significantly depleted in the GnRH agonist-treated endometriosis group, compared to the non-treated endometriosis, or non-endometriosis groups. The sEH derived lipids, namely 5,6-DHET, 11,12-DHET and 19,20-DiHDPA, were also significantly decreased in the treatment group compared to the non-endometriosis group. Assessment of the expression of these biosynthetic enzymes was not one of the objectives of the current study therefore it cannot provide an explanation for the observed alterations in circulating levels.

However, it is well-established that metabolites of the CYP450 pathway contribute to the onset and progression of cardiovascular diseases (Fleming, 2011). A recent large cohort study concluded that women with endometriosis have an increased risk to the coronary heart diseases (Mu et al., 2016). It also needs to be noted that the comparison of data according to the presence or absence of endometriosis also revealed noticeable increases in plasma TXA_2, LTB_4 and trans-EKODE levels in the endometriosis group. The pro-inflammatory properties of TXA_2, LTB_4 have previously

been acknowledged, whereas trans-EKODE is considered a marker of oxidative stress (Wang et al., 2009). Previous studies also suggest an association between endometriosis and systemic inflammation (Agic et al., 2006; Nothnick and Alali, 2016). The findings from this study support this association, but it is important to point out that the low grade inflammation and oxidative stress are cofounding factors for atherosclerosis and cardiovascular diseases (Danesh, 2000; Hansson, 2005; Bonomini et al., 2008).Therefore, it seems the present study strengthens the findings of Mu *et al.* (2016), which suggests that endometriosis patients have a higher risk of coronary heart diseases.

The current study was able to demonstrate a limited number of statistically relevant alterations, but only for the lipid signature in the plasma. In addition, the evaluation of target gene expression in blood also showed no alteration between the study groups. As a conclusion, therefore it seems the investigated lipid mediators are not likely to be potential biomarkers for endometriosis. On the other hand, the observed alterations may support Mu *et al.'s* study and provide additional evidence with regard to the increased risk of coronary heart disease in women with endometriosis.

6.4 Limitations of the study

Although the experiments of this study were carefully planned and performed, some unavoidable limitations are acknowledged.

One main limitation of the current study concerns the nature of peritoneal fluid specimens. As part of the collection process when no peritoneal fluid could be visualised, the peritoneal cavity was washed with isotonic buffer with variable recovery. To gain usable information from these samples normalization was performed which might have caused bias in the results of mass spectrometry.

Secondly, the small number of samples limited further stratification of the groups. For instance, the expression of protein of interest in the eutopic endometria could not be investigated according to the phase of the menstrual cycle. Consequently the effect of menstrual cycle on protein expression cannot be elucidated. For the same reason, the endometriosis group were not further divided according to the subtypes of endometriosis, i.e. peritoneal, ovarian and DIE. This division would have been particularly beneficial since the aetiology of these subtypes is strongly debated.

In addition, due to the size of ectopic lesions the histological confirmation of the presence of endometrial-like compartment were not always possible. Although glandular and stromal markers were applied in the PCR study they cannot replace

the histological validation. Ideally subsamples should have been taken from each ectopic lesion for histology and then taken further subsamples for other methods. However in reality, the size of the biopsies in most of the cases did not allow division of the same specimen between several methods. This fact may limit the value of the conclusions with regard to the ectopic lesions

Furthermore, it would also have been useful to investigate the targets of interest in the peritoneal wall from women with and without endometriosis. However, peritoneal biopsies were not obtained from non-endometriosis subjects. Thus, comparisons could not be performed between the endometriosis and non-endometriosis groups. Information about the peritoneum could have largely contributed to the better understanding of the endometriosis environment.

Questionnaires were used to collect information about the period, pain symptoms, relapse and medical treatments of the subjects. Unfortunately, this questionnaire did not always provide detailed medication information. Going forward, patients should be asked about their medical treatment in more detail.

Lastly, this study lacked an ideal control group. The control group used included benign gynaecological pathologies such as non-endometrial ovarian cysts, leiomyoma, heavy menstrual bleeding and dysmenorrhoea. The symptomology of these pathologies overlap with endometriosis thus, the pathomechanisms and biochemical changes might have also shown similarities and, as a result, may moderate interpretation of the findings.

6.5 Future work

The findings presented in this thesis have raised a number of questions that would provide interesting topics for future investigations.

First of all, increasing participant numbers could fill the gaps in the current study. Additionally, obtaining more detailed patient information, including diet, pain scores or medical treatments, could help to elucidate their possible effects on endometriosis. For this aim a more complex statistical analysis, e.g. principal component analysis (PCA) would be useful. This method also could be applied to establish the possible association between endometriosis and cardiovascular diseases.

Secondly, the mass spectrometry results of the peritoneal fluid indicated that the activity of PLA_2s might be disturbed in endometriosis patients. It is likely that the peritoneal cells have a paracrine effect on the function of these isoenzymes. Investigation into the activity of PLA_2s could shed more light on the role of lipid

mediators in the pathology of endometriosis. A study into the relationship between peritoneal, endometrial cells and PLA_2s would also be an exciting area for further work. PCR, IHC and ELISA techniques could be ideal methods to gain preliminary data for this research. In addition, these data also revealed that the endogenous ligands of PPARγ, i.e. 15-LOX-metabolites, were present in low concentrations in the peritoneal fluid from endometriosis patients. The low concentration of the natural agonists could lead to dysfunction of this nuclear receptor, resulting in impaired regulation of the inflammatory processes for instance. However PPARγ might regulate several other endometriosis related processes, such as angiogenesis, apoptosis, macrophage differentiation or adhesion molecule expression. This study provides preliminary data but further research is needed to better understand the role of PPARγ in the pathology of endometriosis.

Thirdly, further studies are required to better understand the complex interactions within the peritoneal environment. This study provides preliminary data about the lipid mediators in the peritoneal fluid. However, the investigation of the source of these mediators was not the scope of the current study. Therefore, future research should focus on these questions and examine more closely the biosynthesis and secretion of these lipids by ectopic lesions, peritoneal cells and by the peritoneal wall. Flow cytometry could also be used to identify cell populations in the peritoneal fluid.

Lastly, histological analysis pointed out that the morphology of the endometrial lesions was variable in nature and also suggested that although, IFITM-1 was proposed as stromal marker, it might have a role in the pathomechanisms of endometriosis. Recent studies evaluated IFITM-1 as a potential proliferative molecular marker with a predictive value in several cancers. In addition, it was also reported that IFITM-1 might be an essential factor in angiogenesis. Putting together these findings with the 15-LOX-1, PPARγ results might open a novel and exciting area of research to discover a novel proliferative marker for endometriosis

6.6 Conclusion

The primary aim of this thesis was to determine the oxygenated lipid mediator profiles in plasma and peritoneal fluid specimens from women with and without endometriosis to gain a deeper insight into the pathophysiological processes of endometriosis.

To date, there are no clinically relevant screening tests to detect endometriosis. Since this is the first study to identify a wide array of oxygenated lipid mediators in plasma, it was proposed that these lipids may provide a novel group of compounds that could

be used as biomarkers for the diagnosis of endometriosis. Statistical analysis of the presented data has not identified an endometriosis-related lipid profiles in the plasma of affected women. Therefore, it is unlikely these lipids would be ideal biomarkers for endometriosis diagnosis. On the other hand, the presented data might provide additional evidence with regard to the increased risk of coronary heart disease for women with endometriosis. However, the results also highlight that several factors, e.g. menstrual cycle phase, cytokine levels, and co-morbidities had an impact on the lipid mediator profile of plasma and the elimination of these factors is necessary to reveal the endometriosis-related changes.

A large number of studies have proposed the role of a pro-inflammatory peritoneal environment in the pathophysiology of endometriosis. However, the findings of the current study do not support this idea. Pro-inflammatory cytokines and lipid mediators were not significantly increased in the peritoneal fluid from women with endometriosis. Moreover, nearly all tested metabolites were present in decreased concentrations in the endometriosis group compared to the non-endometriosis group. The 15-LOX derived metabolites showed the most prominent reduction, hence the expression of 15-LOX-1 and 15-LOX-2 were subsequently investigated. Different grades of 15-LOX expression were observed in the tested specimens. These results did not lead to the conclusion that the downregulation of 15-LOX isoenzymes caused the low concentrations of 15-LOX derived metabolites in the peritoneal fluid from women diagnosed with endometriosis.

The role of PPARγ in chronic inflammatory conditions, such as atherosclerosis is well-studied. 15-LOX metabolites serve as endogenous ligands for PPARγ; therefore, it was assumed that the low concentrations of 15-LOX metabolites may disturb the regulation of PPARγ mediated pathways. The results presented in this thesis suggest that PPARγ agonists were present in adequate concentrations to activate PPARγ, therefore it is likely that PPARγ-regulated pathways were not disturbed in endometriosis.

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Appendix 1. Consent form

MANCHESTER 1824

of Manchester

Consent Form. v_2 10/05/13

Consent Form

Investigation into the role of local hormones and prostaglandins in female reproductive tract disorders

Study number/ Patient ID number _____

Hospital number _____

I agree that the following tissues and/or body fluids may be used for biomedical research (please provide details):

Please read each item and initial each box to indicate agreement.

1. I confirm that I have read and understood the information sheet for the above study and have had the opportunity to ask questions. ☐
2. I understand that my participation is voluntary and that I am free to withdraw at any time, without giving any reason, without my medical care or legal rights being affected. ☐
3. I understand that I will be asked to give tissues and/ or body fluids, which may be stored at the University of Manchester to be used for this study and other future studies. ☐
4. I understand that the clinician/ clinical staff overseeing my care may pass on my medical history in an anonymised form to the research group at the University of Manchester. ☐
5. I agree that my anonymised and relevant clinical data may be kept on the research group's database at the University of Manchester. ☐
6. I agree to take part in the above study. ☐
7. I understand that relevant sections of data collected during the study may be looked at by responsible individuals from the University of Manchester, from regulatory authorities or from the NHS Trust, where it is relevant to my taking part in the research. I give permission for these individuals to have access to this data ☐

_____	_____	_____
Name of Participant	**Date**	**Signature**
_____	_____	_____
Name of Person taking consent	**Date**	**Signature**

3 copies: 1 for patient; 1 for clinician; 1 to be kept with hospital notes

Appendix 2. Patient information form

Patient Ethical Tissue Number	ET

Non-pregnant donor info form – Reproduction Research project

Date		Age of patient (yrs)		
Consultant		Time of surgery		
Ethnicity		❑ White European ❑ Asian - Indian ❑ Chinese	❑ Black - African ❑ Asian - Pakistani ❑ Mixed: (Please specify)	❑ Black - Caribbean ❑ Asian - Bangladeshi ❑ Other (Please specify)
First day of last menstrual period		Length of menstrual cycle		
Stage of menstrual cycle		Menstruation	Follicular	Ovulation / Luteal
Type of surgical procedure		laparoscopy	hysterectomy	Other (please state):
List of specimens collected				
Site of endometrial tissue excision (please circle):		Eutopic		Ectopic
Comments on location, size & number of endometrial lesions				
Site of hysterectomy (please ligature fundus end)		Fundus	Lower	Total
Blood pressure (mmHg)				
Body Mass Index				
Number of previous		gravida:	miscarriages:	abortions:
		vaginal births:	Caesarean sections:	
Does the patient smoke?		Y		N
If Yes, how many per day?				
Does the patient have any of the following medical conditions: (please tick where appropriate)		- endometriosis	mild	moderate / severe
		- fibroids		
		- menorrhagia		
		- dysmenorrhoea		
		- pelvic pain		
		- fertility problems	infertility	subfertility
		- diabetes	IDDM	NIDDM
		- recurrent infections		
		- other (please state)		
Medication history and dates				
Any current medication				
Reason for procedure:		- recurrent pathology		
		- severe disease state		
		- other (please specify)		
Date medical condition was diagnosed				
Any other comments ? Planned treatments ? Planned follow up				

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Appendix 3. Participant characteristics

Endometriosis patients (EM)

Patient ID	Group	Age	BMI	Stage of Cycle	Hormonal Medication	Menor-rhagia	Dysmenor-rhoea	Chronic Pelvic pain	Fertility Problem	Fibroids	Recur-rence
HP01	**EM**	53	24,8	S	N	N	N	Y	N	N	N
HP02	**EM**	41	27	A	HRT	N	Y	Y	Y	N	Y
HP06	**EM**	44	24	M	N	Y	Y	Y	Y	Y	Y
HP08	**EM**	36	21	A	Prostap& HRT	Y	Y	Y	Y	N	Y
HP09	**EM**	42	29	A	HRT	Y	Y	Y	N	N	N
HP10	**EM**	40	27	P	N	Y	Y	Y	N	N	N
HP12	**EM**	36	24	P	N	Y	Y	Y	Y	N	N
HP17	**EM**	29	26	A	C	N	Y	Y	N	N	Y
HP20	**EM**	25	27	A	Prostap	Y	Y	Y	Y	N	N
HP23	**EM**	38	28	M	N	Y	N	N	N	N	N
HP24	**EM**	36	19,1	S	N	N	N	Y	Y	N	N
HP27	**EM**	34	27,9	A	Prostap	N	N	Y	Y	N	Y
HP28	**EM**	46	32,8	A	C	N	N	Y	Y	N	N
HP31	**EM**	38	24,7	P	N	N	Y	Y	N	N	N
HP32	**EM**	27	27,8	P	N	N	Y	Y	Y	N	N
HP34	**EM**	33	30,9	A	Prostap	N	N	Y	Y	N	N
HP35	**EM**	35	23	A	Prostap	Y	Y	Y	Y	N	Y
HP40	**EM**	40	24,8	P	N	Y	Y	Y	N	N	Y
HP41	**EM**	34	29,4	A	Prostap	Y	Y	Y	Y	N	Y
HP44	**EM**	29	36,8	A	Prostap	Y	Y	Y	N	N	Y
HP45	**EM**	20	28,9	S	N	Y	Y	Y	N	N	N
HP46	**EM**	37	20,8	A	Prostap	N	N	N	N	N	Y
HP47	**EM**	45	24,8	P	N	N	N	N	N	N	Y
HP49	**EM**	35	27,5	A	N	Y	Y	Y	Y	N	Y
HP50	**EM**	22	22	A	Prostap& HRT	Y	Y	Y	N	N	Y
HP51	**EM**	43	22,7	A	Prostap	N	Y	Y	Y	N	Y
HP52	**EM**	31	21,7	A	Prostap	Y	Y	Y	Y	N	Y
HP53	**EM**	39	36,7	A	Prostap	N	Y	N	Y	N	Y
HP54	**EM**	22	22,7	A	Prostap	N	Y	N	N	N	N
HP56	**EM**	23	24,6	M	Prostap	Y	Y	Y	Y	N	N
HP62	**EM**	41	20,8	P	N	N	N	N	Y	N	N
HP63	**EM**	29	N/A	A	Prostap	N	Y	Y	N	N	N
HP64	**EM**	34	N/A	A	Zoladex	N	Y	N	N	N	Y
HP67	**EM**	N/A	N/A	A	Prostap	N	N	N	Y	N	N
HP68	**EM**	48	N/A	A	Prostap	N	N	N	N	Y	N
HP70	**EM**	51	25	O	N	Y	Y	N	N	N	N
HP71	**EM**	N/A	N/A	A	Prostap	Y	N	N	N	N	N
HP73	**EM**	N/A	N/A	S	N	N	N	N	N	N	N
HP74	**EM**	20	N/A	M	N	N	N	N	N	N	N
HP77	**EM**	35	N/A	A	Prostap	N	N	N	N	N	N
HP78	**EM**	21	N/A	A	Prostap	N	N	N	N	N	N
HP79	**EM**	30	37	A	GnRH agonist	N	Y	Y	N	N	N
HP80	**EM**	N/A	28	S	Arom. Inhib.	N	Y	Y	N	N	N
HP81	**EM**	32	38,1	A	Prostap	Y	Y	Y	N	N	N
HP82	**EM**	30	35	A	Prostap	N	Y	Y	N	N	N

Appendix 3 cont. Participant characteristics

Non-Endometriosis patients (NEM)

Patient ID	Group	Age	BMI	Diagnosis	Stage of Cycle	Hormonal Medicatior	Menor-rhagia	Dysmenor-rhoea	Chronic Pelvic pain	Fertility Problem	Fibroids
HP03	**NEM**	36	21	BENIGN OV CYST	A	N	N	N	N	N	N
HP04	**NEM**	51	32	LEIOMYOMA	A	N	Y	Y	Y	N	Y
HP05	**NEM**	43	24	CHRONIC PELVIC PAIN	P	N	Y	Y	Y	Y	N
HP07	**NEM**	43	29	BENIGN OV CYST	A	N	N	N	N	N	N
HP11	**NEM**	36	37	LEIOMYOMA	S	N	Y	Y	N	Y	Y
HP13	**NEM**	48	28	LEIOMYOMA	P	N	Y	Y	N	N	Y
HP14	**NEM**	25	25	CERVIC EXCISION	A	N	N	N	N	N	N
HP15	**NEM**	56	35	LEIOMYOMA	S	N	Y	N	N	N	Y
HP16	**NEM**	29	25	CHRONIC PELVIC PAIN	P	N	N	N	Y	Y	N
HP18	**NEM**	27	24	BARTHOLIN CYST	P	N	N	N	N	N	N
HP19	**NEM**	23	24	CHRONIC PELVIC PAIN	S	N	N	Y	Y	N	N
HP21	**NEM**	48	31,9	LEIOMYOMA	M	N	Y	Y	N	N	Y
HP25	**NEM**	39	23	BENIGN OV CYST	A	N	Y	N	Y	N	N
HP26	**NEM**	24	23,6	CHRONIC PELVIC PAIN	A	N	N	N	Y	N	N
HP29	**NEM**	43	30,1	MENORRHAGIA	A	N	Y	N	N	N	Y
HP33	**NEM**	47	33	LEIOMYOMA	A	N	N	Y	Y	N	Y
HP37	**NEM**	28	N/A	UTERINE SEPTUM DIV.	M	N	N	N	N	Y	N
HP38	**NEM**	36	20,3	BENIGN OV CYST	S	N	Y	N	Y	N	N
HP39	**NEM**	35	N/A	MENORRHEA	A	N	Y	Y	N	Y	N
HP42	**NEM**	43	32,9	PROPHYLACTIC	S	N	N	N	N	Y	N
HP43	**NEM**	30	25,9	LEIOMYOMA	A	N	Y	Y	Y	Y	Y
HP48	**NEM**	36	N/A	PROPHYLACTIC	A	N	Y	Y	Y	N	N
HP55	**NEM**	43	20	CHRONIC PELVIC PAIN	P	N	N	Y	Y	N	N
HP57	**NEM**	35	40,9	OV CYST	M	N	N	N	N	Y	N
HP58	**NEM**	42	N/A	PROPHYLACTIC	P	N	N	N	N	Y	N
HP60	**NEM**	40	26	PROPHYLACTIC	P	N	N	N	N	N	N
HP61	**NEM**	48	31,3	PROPHYLACTIC	A	N	N	N	N	N	N
HP65	**NEM**	42	N/A	MENORRHAGIA	S	N	Y	N	N	N	N
HP66	**NEM**	37	N/A	LEIOMYOMA	S	N	N	N	N	Y	Y
HP69	**NEM**	28	N/A	CHRONIC PELVIC PAIN	P	N	N	N	Y	N	N
HP72	**NEM**	46	N/A	MENORRHAGIA	P	N	Y	Y	N	N	N
HP75	**NEM**	N/A	N/A	LEIOMYOMA	A	Y	N	N	N	N	Y

EM: endometriosis

NEM: non-endometriosis

Stage of cycle: (P) proliferative; (S) secretory; (A) amenorrhoea

Hormonal Medication: (N) no usage; (HRT) hormone replacement therapy; (C) hormonal contraceptive

(Y) yes; (N) no

Appendix 4 List of specimens used for this study

Endometriosis patients (EM)

Patient		Stage of	Hormonal		MS				PCR				IHC			ELISA	
ID	Group	Cycle	Medication	PF	PW	PL	EU	EC	Pwall	Pcell	Blood	EU	EC	Pwall	PF	PW	PL
HP01	EM	S	N			x	x										x
HP02	EM	A	HRT		x	x	x									x	x
HP06	EM	M	N		x	x	x									x	x
HP08	EM	A	Prostap& HRT		x	x	x									x	
HP09	EM	A	HRT			x										x#	
HP10	EM	P	N			x	x									x#	
HP12	EM	P	N	x		x	x	x									x
HP17	EM	A	C	x		x									x		x
HP20	EM	A	Prostap	x		x	x								x		x
HP23	EM	M	N	x	x	x									x	x#	x
HP24	EM	S	N		x #	x		xx					xx			x#	x
HP27	EM	A	Prostap			x										x#	x
HP28	EM	A	C	x		x	x					x				x	x
HP31	EM	P	N		x #	x	x	x					x			x#	x
HP32	EM	P	N		x	x		x	x			x	x	x		x	x
HP34	EM	A	Prostap		x #	x						x	x			x#	x
HP35	EM	A	Prostap		x	x	x		x			x		x		x#	x
HP40	EM	P	N		x	x				x						x#	
HP41	EM	A	Prostap		x	x		x		x			x			x#	x
HP44	EM	A	Prostap			x											x
HP45	EM	S	N		x #	x	x						x			x#	x
HP46	EM	A	Prostap			x											x
HP47	EM	P	N		x	x		x		x		x	x			x#	x
HP49	EM	A	N			x											x
HP50	EM	A	Prostap& HRT			x		x					x	x		x#	x
HP51	EM	A	Prostap		x	x	x	x		x	x	x	x			x#	x
HP52	EM	A	Prostap		x	x	x	x		x		x	x	x		x	x
HP53	EM	A	Prostap			x	x	x	x		x		x	x		x#	x
HP54	EM	A	Prostap		x	x	x				x					x#	x
HP56	EM	M	Prostap		x #	x	x		x	x	x		x	x		x#	x
HP62	EM	P	N		x #	x					x					x#	x
HP63	EM	A	Prostap		x #	x	x				x					x#	x
HP64	EM	A	Zoladex		x #	x			x		x					x#	x
HP67	EM	A	Prostap				x	x									
HP68	EM	A	Prostap		x #	x	x									x#	x
HP70	EM	O	N			x											x
HP71	EM	A	Prostap		x #	x	x		x							x#	
HP73	EM	S	N			x	x				x						x
HP74	EM	M	N		x	x	x			x	x					x	x
HP77	EM	A	Prostap			x					x						x
HP78	EM	A	Prostap			x	x				x						
HP79	EM	A	GnRH agonist			x	x	x									x
HP80	EM	S	Arom. Inhib.				x	x	x		x					x#	x
HP81	EM	A	Prostap				x		x		x					x	x
HP82	EM	A	Prostap				x										

Appendix 4 cont. List of specimens used for this study

Non-Endometriosis patients (NEM)

Patient ID	Group	Diagnosis	Stage of Cycle	Hormonal Medication	PF	MS PW	PL	EU	PCR Pcell	Blood	IHC EU	PF	ELISA PW	PL
HP03	**NEM**	BENIGN OV CYST	A	N		x #	x						x#	x
HP04	**NEM**	LEIOMYOMA	A	N		x #	x	x					x#	
HP05	**NEM**	CHRONIC PELVIC PAIN	P	N		x #	x	x					x#	
HP07	**NEM**	BENIGN OV CYST	A	N		x #	x						x#	x
HP11	**NEM**	LEIOMYOMA	S	N	x		x							x
HP13	**NEM**	LEIOMYOMA	P	N	x		x	x						x
HP14	**NEM**	CERVIC EXCISION	A	N			x	x						x
HP15	**NEM**	LEIOMYOMA	S	N	x		x	x						x
HP16	**NEM**	CHRONIC PELVIC PAIN	P	N			x	x						x
HP18	**NEM**	BARTHOLIN CYST	P	N			x	x						x
HP19	**NEM**	CHRONIC PELVIC PAIN	S	N	x		x	x				x		x
HP21	**NEM**	LEIOMYOMA	M	N			x	x						x
HP25	**NEM**	BENIGN OV CYST	A	N	x		x	x				x		x
HP26	**NEM**	CHRONIC PELVIC PAIN	A	N	x	x	x	x				x	x#	x
HP29	**NEM**	MENORRHAGIA	A	N	x	x	x	x			x	x	x#	x
HP33	**NEM**	LEIOMYOMA	A	N		x #	x						x#	x
HP37	**NEM**	UTERINE SEPTUM DIV.	M	N			x	x			x			x
HP38	**NEM**	BENIGN OV CYST	S	N		x	x	x			x		x#	x
HP39	**NEM**	MENORRHEA	A	N			x	x			x			x
HP42	**NEM**	PROPHYLACTIC	S	N		x	x	x	x		x	x	x#	x
HP43	**NEM**	LEIOMYOMA	A	N			x				x			x
HP48	**NEM**	PROPHYLACTIC	A	N		x #	x	x	x		x		x#	x
HP55	**NEM**	CHRONIC PELVIC PAIN	P	N		x	x		x	x			x	x
HP57	**NEM**	OV CYST	M	N		x	x	x	x	x			x#	x
HP58	**NEM**	PROPHYLACTIC	P	N	x	x	x	x	x	x	x	x	x#	x
HP60	**NEM**	PROPHYLACTIC	P	N	x	x	x	x	x	x		x	x#	x
HP61	**NEM**	PROPHYLACTIC	A	N		x #	x	x		x			x#	x
HP65	**NEM**	MENORRHAGIA	S	N			x	x		x				x
HP66	**NEM**	LEIOMYOMA	S	N		x			x				x#	
HP69	**NEM**	CHRONIC PELVIC PAIN	P	N		x			x				x	
HP72	**NEM**	MENORRHAGIA	P	N		x #		x					x#	
HP75	**NEM**	LEIOMYOMA	A	Y		x	x	x		x			x#	x

PF: Peritoneal fluid

PW: Peritoneal wash

PL: Plasma

EU: Eutopic endometrium

EC: Ectopic lesion

Pcell: Peritoneal cells

Pwall: Peritoneal wall

Stage of cycle: (P) proliferative; (S) secretory; (A) amenorrhoea

Hormonal Medication: (N) no usage; (HRT) hormone replacement therapy; (C) hormonal contraceptive

(Y) yes; (N) no

Normalization factor >27, excluded from the analysis

Appendix 5. Lipid mediators in normalized peritoneal fluid specimens

			Endometriosis (n=17)				Non-Endometriosis (n=15)			
Lipid mediators	**FA**	**Pathway**	**mean (pg/ml)**		**SEM**	**n(d)/n**	**mean (pg/ml)**		**SEM**	**n(d)/n**
MaR1	DHA	12LOX	**343,4**	±	**286,5**	2	**389,1**	±	**317,3**	3
11 HDHA	DHA	12LOX	**561,3**	±	**412,8**	3	**523,0**	±	**319,0**	4
14 HDHA	DHA	12LOX	**3762,1**	±	**2849,9**	8	**9045,0**	±	**4521,5**	11
12 HETE	AA	12LOX	**34667,4**	±	**24530,5**	16	**13852,7**	±	**6256,2**	15
12 HEPE	EPA	12LOX	**1183,4**	±	**974,7**	4	**686,0**	±	**410,1**	6
HXA3	AA	12LOX	**921,6**	±	**921,6**	1	**338,0**	±	**308,3**	2
17 HDHA	DHA	15LOX	**11029,1**	±	**7516,4**	13	**58940,6**	±	**29491,2**	11
15HETrE	DGLA	15LOX	**3147,3**	±	**1865,2**	12	**11371,5**	±	**6494,3**	11
15 HETE	AA	15LOX	**35307,9**	±	**21584,4**	16	**99946,5**	±	**54208,5**	15
15 HEPE	EPA	15LOX	**1456,6**	±	**1319,8**	4	**9472,0**	±	**5720,6**	5
13 HODE	LA	15LOX	**57145,1**	±	**26677,6**	17	**192001,6**	±	**81917,0**	15
13 HOTrE	ALA	15LOX	**9177,4**	±	**4427,8**	14	**26292,2**	±	**14303,0**	13
13 OxoODE	LA	15LOX	**8201,6**	±	**4231,7**	17	**18741,1**	±	**8560,2**	14
5,15 DiHETE	AA	15LOX	**116,0**	±	**115,9**	1	**295,3**	±	**268,6**	2
8,15 DiHETE	AA	15LOX	**947,1**	±	**636,6**	4	**926,5**	±	**867,8**	2
PDX	DHA	5LOX	**133,1**	±	**124,1**	2	**354,4**	±	**289,2**	3
RvD1	DHA	5LOX				ND				ND
RvD2	DHA	5LOX	**108,4**	±	**108,4**	1	**237,2**	±	**176,2**	3
7 HDHA	DHA	5LOX	**444,2**	±	**265,0**	5	**564,3**	±	**294,0**	5
4 HDHA	DHA	5LOX	**866,3**	±	**589,1**	6	**622,8**	±	**263,0**	10
LTB4	AA	5LOX	**254,7**	±	**220,7**	4	**113,8**	±	**104,1**	2
5 HETE	AA	5LOX	**1681,4**	±	**1198,3**	9	**2329,2**	±	**1075,1**	13
5OXOETE	AA	5LOX	**829,7**	±	**649,1**	4	**324,2**	±	**270,2**	3
5 HEPE	EPA	5LOX	**62,5**	±	**55,7**	2	**44,8**	±	**18,5**	5
9 HOTrE	ALA	5LOX	**3746,7**	±	**866,3**	17	**6287,9**	±	**2943,4**	13
18 HEPE	EPA	COX	**248,9**	±	**193,1**	4	**189,3**	±	**83,1**	7
9 HODE	LA	COX	**22951,6**	±	**12467,6**	17	**55463,8**	±	**22494,9**	15
9 OxoODE	LA	COX	**13714,0**	±	**8055,5**	17	**22588,4**	±	**9865,3**	14
PGE2	AA	COX	**477,6**	±	**438,4**	5	**607,1**	±	**476,9**	7
PGD2	AA	COX	**336,3**	±	**176,0**	6	**909,2**	±	**804,2**	9
PGF2a	AA	COX	**555,9**	±	**386,8**	4	**963,2**	±	**752,8**	5
6keto PGI2	AA	COX	**3021,5**	±	**1370,3**	9	**3361,9**	±	**1696,0**	7
TXB2	AA	COX	**533,3**	±	**220,4**	9	**1455,9**	±	**596,6**	9

Appendix 5 cont. Lipid mediators in normalized peritoneal fluid specimens

			Endometriosis (n=17)				Non-Endometriosis (n=15)			
Lipid mediators	**FA**	**Pathway**	**mean (pg/ml)**		**SEM**	**n(d)/n**	**mean (pg/ml)**		**SEM**	**n(d)/n**
5(6) EET	AA	CYP	**10882,2**	±	**7358,0**	6	**11461,5**	±	**5970,5**	6
20HETE	AA	CYP	**11,8**	±	**11,8**	1	**136,9**	±	**44,4**	7
8(9) EET	AA	CYP	**6650,5**	±	**4621,0**	5	**5142,9**	±	**2610,2**	6
11(12) EET	AA	CYP	**10901,2**	±	**5541,0**	10	**9643,1**	±	**3512,6**	12
14(15) EET	AA	CYP	**7195,6**	±	**4933,8**	7	**6680,7**	±	**3541,8**	6
9(10)EpOME	LA	CYP	**61972,6**	±	**36497,2**	17	**121489,4**	±	**52096,7**	14
12(13)EpOME	LA	CYP	**55182,5**	±	**31418,8**	17	**96945,4**	±	**41910,1**	14
16(17)EpDPE	DHA	CYP	**3610,4**	±	**2552,1**	4	**2951,0**	±	**1742,7**	4
19(20) EpDPE	DHA	CYP	**1740,1**	±	**1249,7**	6	**1236,6**	±	**722,0**	4
RvE1	EPA	SHE				ND	**5,3**	±	**3,6**	2
8,9 DHET	AA	SHE	**47,1**	±	**28,8**	4	**105,2**	±	**26,5**	9
5,6 DHET	AA	SHE	**42,1**	±	**27,5**	4	**61,4**	±	**27,3**	6
14,15 DHET	AA	SHE	**416,2**	±	**68,3**	16	**577,6**	±	**121,0**	12
11,12DHET	AA	SHE	**181,5**	±	**46,9**	11	**349,0**	±	**78,0**	10
12,13DiHOME	LA	SHE	**6504,5**	±	**1376,1**	17	**7921,0**	±	**1620,6**	14
9,10DiHOME	LA	SHE	**7902,4**	±	**1433,0**	17	**7878,1**	±	**1525,5**	14
19,20 DiHDPA	DHA	SHE	**856,2**	±	**210,8**	12	**1078,0**	±	**217,6**	10
13 HDHA	DHA	NENZ	**541,7**	±	**377,8**	3	**1246,1**	±	**554,5**	8
10 HDHA	DHA	NENZ	**412,5**	±	**284,0**	4	**696,7**	±	**341,2**	5
20 HDHA	DHA	NENZ	**675,4**	±	**455,0**	4	**1014,9**	±	**433,1**	11
9 HETE	AA	NENZ	**1094,0**	±	**964,3**	3	**1171,0**	±	**698,0**	5
8 HETE	AA	NENZ	**1720,1**	±	**1041,8**	6	**2124,7**	±	**962,6**	9
11 HETE	AA	NENZ	**2092,8**	±	**1427,5**	12	**3246,8**	±	**1441,5**	12
8 HEPE	EPA	NENZ	**89,5**	±	**74,8**	3	**52,0**	±	**36,5**	4
11 HEPE	EPA	NENZ	**165,6**	±	**135,2**	3	**182,1**	±	**106,7**	4
9 HEPE	EPA	NENZ				ND				ND
Trans EKODE	LA	NENZ	**4794,8**	±	**2668,5**	16	**7716,4**	±	**3777,1**	13

FA Precursor fatty acid

n(d)/n number of detected samples/number of tested samples

ND not detected

Appendix 6. Lipid mediators in plasma

			Endometriosis (n=41)				Non-Endometriosis (n=29)			
Lipid mediators	**FA**	**Pathway**	**mean (pg/ml**		**SEM**	**n(d)/n**	**mean (pg/ml)**		**SEM**	**n(d)/n**
MaR1	DHA	12LOX				ND				ND
11 HDHA	DHA	12LOX	**68,9**	±	**32,6**	9	**71,0**	±	**22,6**	9
14 HDHA	DHA	12LOX	**1222,7**	±	**300,5**	40	**1502,8**	±	**251,7**	28
12 HETE	AA	12LOX	**4984,0**	±	**880,8**	41	**5681,3**	±	**981,5**	29
12 HEPE	EPA	12LOX	**391,5**	±	**121,9**	31	**556,0**	±	**112,1**	25
HXA3	AA	12LOX				ND				ND
17 HDHA	DHA	15LOX	**78,0**	±	**34,5**	10	**139,4**	±	**32,1**	14
15HETrE	DGLA	15LOX	**82,0**	±	**15,7**	22	**89,0**	±	**17,9**	16
15 HETE	AA	15LOX	**336,1**	±	**37,7**	39	**340,5**	±	**36,3**	26
15 HEPE	EPA	15LOX				ND				ND
13 HODE	LA	15LOX	**4989,7**	±	**664,4**	41	**5168,0**	±	**425,1**	29
13 HOTrE	ALA	15LOX	**327,1**	±	**64,9**	35	**293,3**	±	**34,1**	26
13 OxoODE	LA	15LOX	**1477,3**	±	**326,9**	41	**1519,4**	±	**276,0**	29
5,15 DiHETE	AA	15LOX				ND				ND
8,15 DiHETE	AA	15LOX				ND				ND
PDX	DHA	5LOX				ND				ND
RvD1	DHA	5LOX				ND				ND
RvD2	DHA	5LOX				ND				ND
7 HDHA	DHA	5LOX	**5,4**	±	**6,4**	1				ND
4 HDHA	DHA	5LOX	**116,1**	±	**30,7**	25	**169,5**	±	**22,6**	25
LTB4	AA	5LOX	**146,7**	±	**76,5**	22	**51,5**	±	**11,6**	15
5 HETE	AA	5LOX	**534,7**	±	**89,0**	39	**437,6**	±	**51,1**	29
5OXOETE	AA	5LOX	**3,8**	±	**3,0**	2				ND
5 HEPE	EPA	5LOX	**21,0**	±	**10,1**	6	**85,2**	±	**28,3**	11
9 HOTrE	ALA	5LOX	**323,2**	±	**45,4**	40	**337,6**	±	**35,3**	29
18 HEPE	EPA	COX	**28,8**	±	**11,8**	8	**84,7**	±	**17,5**	16
9 HODE	LA	COX	**2853,3**	±	**417,9**	41	**2807,5**	±	**217,2**	29
9 OxoODE	LA	COX	**1316,3**	±	**282,7**	41	**1137,4**	±	**187,6**	29
PGE2	AA	COX	**122,4**	±	**80,4**	16				ND
PGD2	AA	COX	**35,7**	±	**24,5**	5				ND
PGF2a	AA	COX	**120,0**	±	**86,8**	15				ND
6keto PGI2	AA	COX	**684,1**	±	**336,6**	23	**47,9**	±	**47,9**	1
TXB2	AA	COX	**202,2**	±	**87,4**	27	**55,0**	±	**19,8**	14

Appendix 6 cont. Lipid mediators in plasma

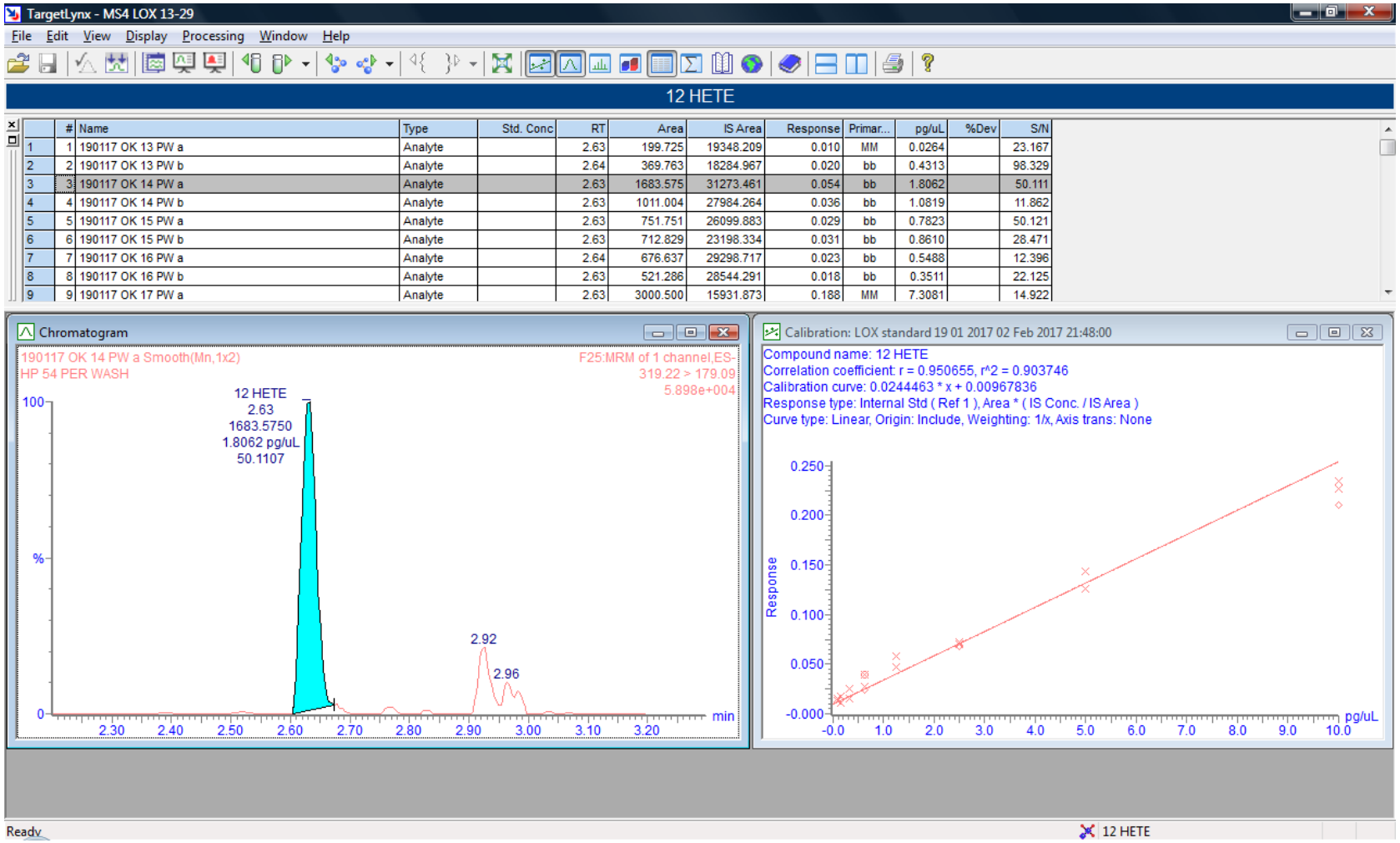
			Endometriosis (n=41)				Non-Endometriosis (n=29)			
Lipid mediators	**FA**	**Pathway**	**mean (pg/ml**		**SEM**	**n(d)/n**	**mean (pg/ml)**		**SEM**	**n(d)/n**
5(6) EET	AA	CYP	**178,4**	±	**98,3**	8	**64,1**	±	**25,2**	6
20HETE	AA	CYP	**173,1**	±	**55,5**	13	**428,5**	±	**82,6**	21
8(9) EET	AA	CYP	**55,0**	±	**44,5**	4	**26,0**	±	**15,2**	3
11(12) EET	AA	CYP	**120,6**	±	**54,2**	11	**55,7**	±	**19,1**	8
14(15) EET	AA	CYP	**145,0**	±	**69,0**	8	**51,2**	±	**26,2**	5
9(10)EpOME	LA	CYP	**3243,9**	±	**809,3**	41	**1798,8**	±	**348,3**	29
12(13)EpOME	LA	CYP	**2820,9**	±	**598,9**	41	**1725,2**	±	**264,6**	29
16(17)EpDPE	DHA	CYP	**98,4**	±	**45,8**	8	**33,8**	±	**21,7**	3
19(20) EpDPE	DHA	CYP	**89,5**	±	**43,3**	7	**9,5**	±	**9,5**	1
13 HDHA	DHA	NENZ	**10,5**	±	**10,6**	2	**19,5**	±	**8,9**	5
10 HDHA	DHA	NENZ	**47,3**	±	**15,3**	13	**84,8**	±	**16,1**	19
20 HDHA	DHA	NENZ	**94,2**	±	**30,3**	17	**153,2**	±	**29,8**	18
9 HETE	AA	NENZ	**0,0**	±	**0,0**	41	**3,5**	±	**3,5**	1
8 HETE	AA	NENZ	**63,3**	±	**16,4**	17	**78,7**	±	**15,1**	17
11 HETE	AA	NENZ	**116,9**	±	**20,7**	38	**110,6**	±	**9,7**	29
8 HEPE	EPA	NENZ	**0,8**	±	**0,9**	1	**6,7**	±	**5,6**	2
11 HEPE	EPA	NENZ				ND				ND
9 HEPE	EPA	NENZ				ND	**6,4**	±	**6,4**	1
Trans EKODE	LA	NENZ	**445,4**	±	**106,1**	35	**307,9**	±	**49,2**	26
RvE1	EPA	SHE	**36,7**	±	**14,7**	12	**14,2**	±	**6,1**	6
8,9 DHET	AA	SHE	**31,6**	±	**9,0**	14	**50,5**	±	**11,2**	14
5,6 DHET	AA	SHE	**47,3**	±	**10,3**	21	**75,5**	±	**13,0**	20
14,15 DHET	AA	SHE	**229,2**	±	**17,3**	40	**274,5**	±	**25,0**	29
11,12DHET	AA	SHE	**131,4**	±	**16,5**	32	**201,7**	±	**20,8**	28
12,13DiHOME	LA	SHE	**1729,2**	±	**196,3**	40	**2192,9**	±	**212,8**	29
9,10DiHOME	LA	SHE	**1811,6**	±	**301,9**	40	**2267,2**	±	**312,7**	29
19,20 DiHDPA	DHA	SHE	**780,4**	±	**66,6**	40	**1229,7**	±	**145,9**	29

FA Precursor fatty acid

n(d)/n number of detected samples/number of tested samples

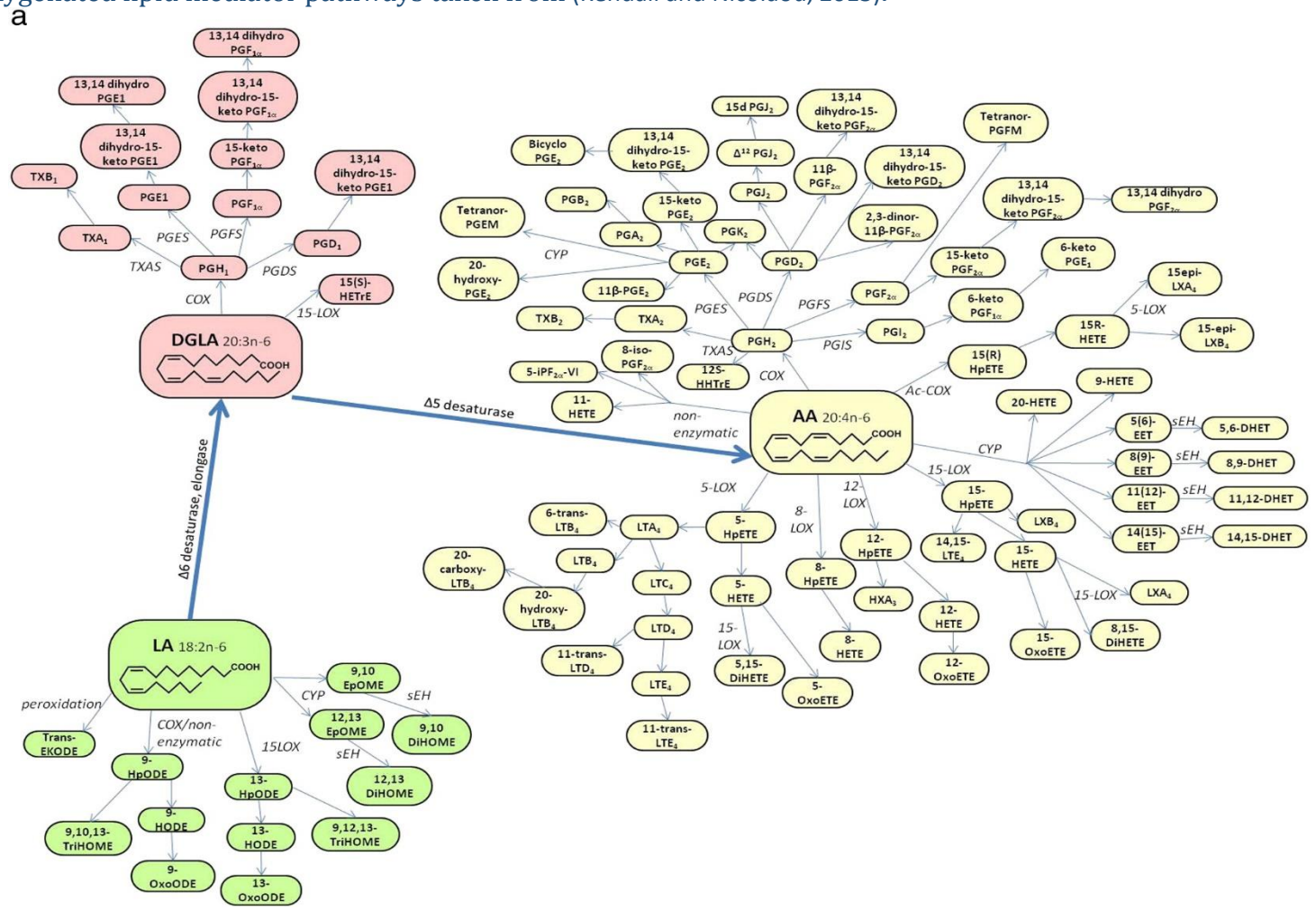
ND not detected

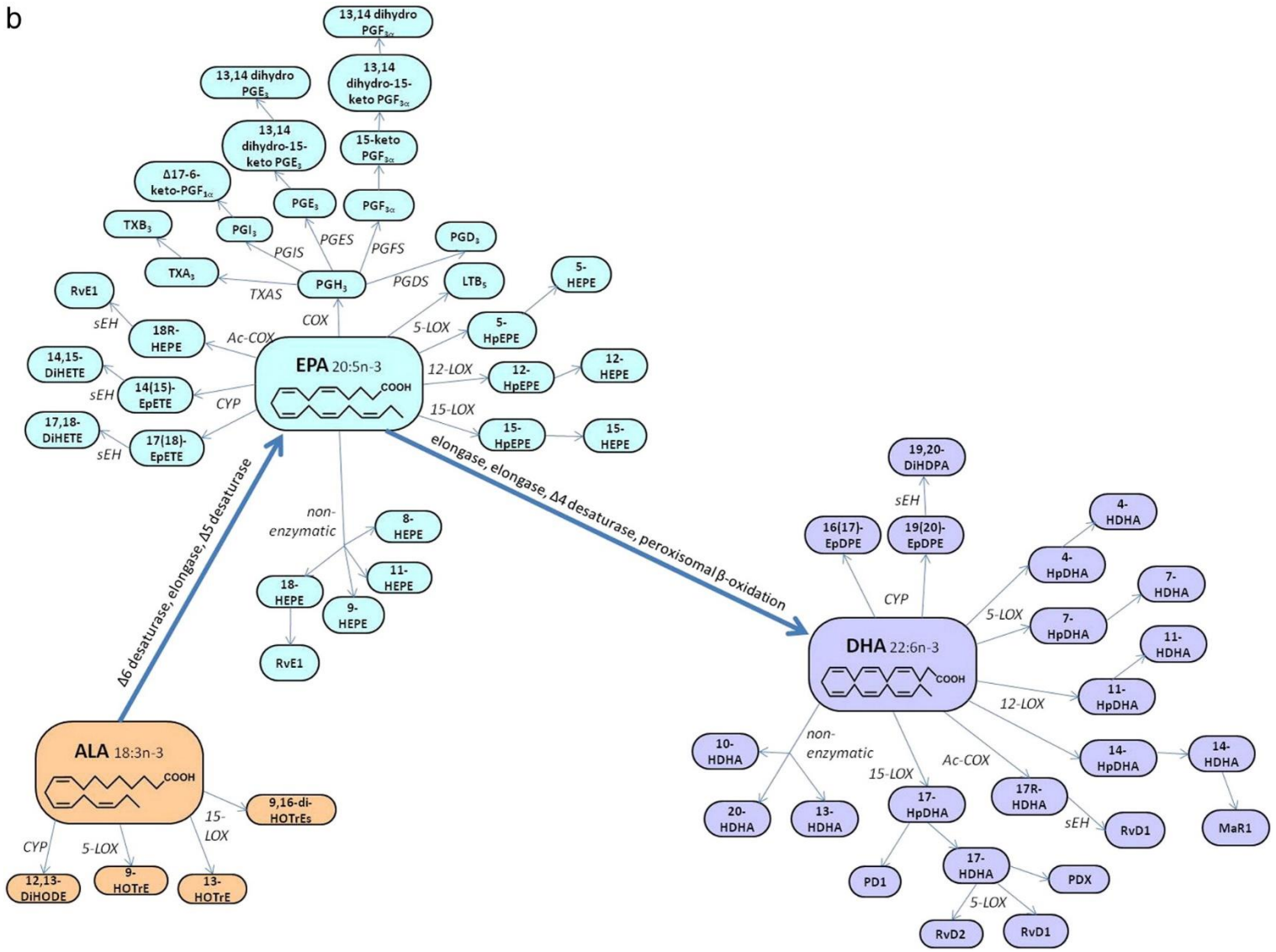
Appendix 7. Representative chromatogram and standard curve in TargetLynx



#	Name	Type	Std. Conc	RT	Area	IS Area	Response	Primar...	pg/uL	%Dev	S/N
1	190117 OK 13 PW a	Analyte		2.63	199.725	19348.209	0.010	MM	0.0264		23.167
2	190117 OK 13 PW b	Analyte		2.64	369.763	18284.967	0.020	bb	0.4313		98.329
3	190117 OK 14 PW a	Analyte		2.63	1683.575	31273.461	0.054	bb	1.8062		50.111
4	190117 OK 14 PW b	Analyte		2.63	1011.004	27984.264	0.036	bb	1.0819		11.862
5	190117 OK 15 PW a	Analyte		2.63	751.751	26099.883	0.029	bb	0.7823		50.121
6	190117 OK 15 PW b	Analyte		2.63	712.829	23198.334	0.031	bb	0.8610		28.471
7	190117 OK 16 PW a	Analyte		2.64	676.637	29298.717	0.023	bb	0.5488		12.396
8	190117 OK 16 PW b	Analyte		2.63	521.286	28544.291	0.018	bb	0.3511		22.125
9	190117 OK 17 PW a	Analyte		2.63	3000.500	15931.873	0.188	MM	7.3081		14.922

Appendix 8. Oxygenated lipid mediator pathways taken from (Kendall and Nicolaou, 2013).




b
13,14 dihydro PGF3α
13,14 dihydro-15-keto PGF3α
13,14 dihydro PGE3
13,14 dihydro-15-keto PGE3
15-keto PGF3α
Δ17-6-keto-PGF1α
PGE3
PGF3α
TXB3
PGI3
PGES
PGFS
PGD3
PGIS
TXA3
PGH3
PGDS
LTB5
5-HEPE
RvE1
TXAS
COX
5-LOX
sEH
18R-HEPE
Ac-COX
5-HpEPE
14,15-DiHETE
EPA 20:5n-3
12-LOX
12-HEPE
12-HpEPE
sEH
14(15)-EpETE
CYP
15-LOX
17,18-DiHETE
15-HpEPE
15-HEPE
sEH
17(18)-EpETE
elongase, elongase, Δ4 desaturase, peroxisomal β-oxidation
Δ6 desaturase, elongase, Δ5 desaturase
non-enzymatic
8-HEPE
11-HEPE
18-HEPE
9-HEPE
RvE1
19,20-DiHDPA
sEH
4-HDHA
16(17)-EpDPE
19(20)-EpDPE
4-HpDHA
7-HDHA
CYP
5-LOX
7-HpDHA
11-HDHA
DHA 22:6n-3
11-HpDHA
12-LOX
14-HpDHA
14-HDHA
non-enzymatic
10-HDHA
Ac-COX
15-LOX
17R-HDHA
17-HpDHA
20-HDHA
13-HDHA
sEH
RvD1
MaR1
PD1
17-HDHA
PDX
5-LOX
RvD2
RvD1
ALA 18:3n-3
9,16-di-HOTrEs
15-LOX
CYP
5-LOX
12,13-DiHODE
9-HOTrE
13-HOTrE