Aus der Klinik für Gynäkologie und Geburtshilfe
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DISSERTATION

Investigation of invasion factors in deep-infiltrating endometriosis

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“And may you always remember that obstacles in the path are not obstacles, they ARE the path”

Jane Lotter

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Abstract

INTRODUCTION: Endometriosis is considered a chronic disease of benign nature with high prevalence, characterised by the presence of endometrial tissue outside the uterine cavity. The main symptoms include chronic pelvic pain, dysmenorrhea and infertility; clinically, it may appear in three distinct ways: peritoneal endometriosis, endometriomas and deep infiltrating endometriosis (DIE). DIE presents a behavioural pattern in many ways similar to that of malignancies. Endometriotic lesions have already been identified in pelvic lymph nodes, showing the possibility of lymphatic dissemination of endometriosis. In malignancies, chemokines play a sovereign role in the process of metastasis and lymphatic spread of tumour cells. Thus, the aims of this study are: 1) to evaluate the expression of cancer-related chemokines – CXCL12-CXCR4, CCL19/CCL21-CCR7 – in rectovaginal DIE and the matched pelvic sentinel lymph node (PSLN); 2) to evaluate the concentration levels of those chemokines in the peritoneal fluid (PF) of patients with and without endometriosis; 3) to evaluate the possibility of malignant transformation of rectovaginal DIE. METHODOLOGY: 123 patients were enrolled in this study. We performed immunohistochemical staining to assess the expression of all chemokines – ligands and receptors – in rectovaginal DIE (n=27), PSLN (n=27) and eutopic endometrium (EE) from patients without endometriosis as controls (n=20); chemokine concentration in the PF was assessed with multiplexing technology (Luminex® x-MAP® Technology) in patients with (n=36) and without (n=27) endometriosis; the possibility of malignant transformation was also assessed by means of immunohistochemistry to evaluate the expression of BAF250a protein among endometriosis lesions – endometrioma (n=20), rectovaginal DIE (n=30), compromised PSLN (n=7), extragenital endometrial stromal sarcoma (EESS) affecting the bowel (n=2) and EE from controls (n=20).

RESULTS: the staining pattern of cancer-related chemokines was characterised for the first time in rectovaginal DIE and lesions compromising the PSLN; CXCR4 expression was directly correlated to the size of the DIE lesions; CCL19, CCL2 and CXCL8 presented higher statistically significant PF concentrations in women with endometriosis compared with controls and their association improved the likelihood of identifying patients with endometriosis; furthermore, we identified the clonal loss of BAF250a expression in 36% (9/25) of DIE, 40% (2/5) of endometriotic lesions in the PSLN, 30% (6/20) of endometriomas and in 25% (5/20) of EE from controls.

CONCLUSION: Chemokines might be involved in the mechanism of dissemination of disease in endometriosis and the clonal loss of protein BAF250a expression in DIE might represent a marker for malignant transformation. The value of these findings needs to be clarified in further analysis.

Zusammenfassung

EINLEITUNG: Endometriose ist eine benigne, chronische Erkrankung, die mit einer hohen Prävalenz auftritt. Sie ist durch das Vorkommen von endometriumartigen Gewebe außerhalb der Gebärmutterhöhle charakterisiert. Klinisch können drei Manifestationsformen unterschieden werden: peritoneale Endometriose, ovarielle Endometriose (Endometriome) und tief infiltrierende Endometriose (TIE). Die Letztere weist im Wachstumsverhalten viele Parallelen zu malignen Tumoren auf. So wächst sie ebenfalls destruierend-infiltrierend und scheint sich über Lymphgefäße in die regionären Lymphknoten verbreiten zu können. Ziel dieser Studie war es, die Wachstumseigenschaften der TIE weiter zu charakterisieren. Da Chemokine als Invasions und Homingfaktoren im Prozess der Metastatisierung maligner Tumoren aber auch im Pathogeneseprozess der Endometriose eine wichtige Rolle spielen, sollten die Invasionsassoziierten-Chemokine Rezeptoren (CXCR4, CCR7) und deren Liganden (CXCL12, CCL19, CCL21) in rectovaginaler TIE und in den gepaarten pelvinen Sentinel-Lymphknoten (PSLK) untersucht werden; sowie eine Bewertung der Konzentrationen dieser Chemokine in der Douglasflüssigkeit (DF) von Patienten mit und ohne Endometriose erfolgen. Weiterhin sollten die Mechanismen der malignen Transformation von rectovaginaler TIE mittels der Analyse von BAF250a (ARID1A-Tumorsupressorgen) untersucht werden.

METHODEN: 123 Patientinnen wurden in diese Studie eingeschlossen. Expressionsanalysen wurden mittels immunhistochemischer Färbung gegen die entsprechenden Chemokine und deren Rezeptoren durchgeführt. Es wurden rectovaginale TIE und PSLK (n = 27) sowie Endometriumsproben von Patientinnen ohne Endometriose als Kontrollen (n = 20) analysiert. Auch wurde die Chemokine-Konzentration in der DF von Patientinnen mit (n = 36) und ohne (n = 27) Endometriose mittels Multiplextechnik bewertet. Die Expressionsanalysen von BAF250a wurde ebenfalls immunhistochemischer an Endometriomen (n = 20), rectovaginaler TIE (n = 30), Endometriose-positive PSLK (n = 5), intestinale Endometrialen Stromasarkomen (n = 2) und Endometrium als Kontrolle (n = 20) analysiert.

ERGEBNISSE: Invasionsassoziierten-Chemokine zeigten eine starke Expression sowohl in den rectovaginalen TIE und den Endometriose-positiven PSLK; CXCR4-Expression scheint dabei eine größenabhängige Korrelation zu zeigen. CCL19, CCL2 und CXCL8 zeigen eine signifikant höhere Konzentrationen in der DF von

Frauen mit Endometriose im Vergleich zu den Kontrollen; BAF250a konnte in allen Proben nachgewiesen werden, zeigt dabei aber ein heterogenes Expressionsprofil mit partiellen Expressionsverlust in manchen Epithelzellen innerhalb einer Drüse. Dies konnte in 36% (9/25) der TIE, in 40% (2/5) der Endometrioseherde der PSLK, in 30% (6/20) der Endometriome und in 25% (5/20) der Endometriumsproben nachgewiesen werden.

SCHLUSSFOLGERUNG: Erstmals konnten die Expression von Chemokinen, die im Homingprozess der Metastasierungsmechanismen wichtig sind auch in TIE nachgewiesen werden. Besonders der partielle Verlust von BAF250a scheint möglicherweise ein Prädiktor für Prozesse der maligne Transformation auch in Endometriose zu sein.

Abbreviations

ARID1A – AT rich interactive domain 1A
ASRM – American Society for Reproductive Medicine
AUC – area under the curve
BAF250a – BRG-associated factor 250a
DIE – deep-infiltrating endometriosis
EAOC – endometriosis-associated ovarian cancer
EC – endometrioid carcinoma
EE – eutopic endometrium
EESS – extragenital endometrial stromal sarcoma
EL – endometriotic lesions
FFPE – formalin-fixed paraffin-embedded
IHC – immunohistochemistry
IRS – immunoreactivity score
LSAB – labelled streptavidin-biotin
MRI – magnet resonance imaging
NBF – neutral buffered formalin
OE – ovarian endometriosis
PF – peritoneal fluid
PSLN – pelvic sentinel lymph node
ROC – receiver operating characteristic
RVS – rectovaginal septum

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1 INTRODUCTION

1.1 Endometriosis

1.1.1 Definition and history

Endometriosis is a benign chronic disease and it is defined by the occurrence of ectopic tissue, i.e. endometrial glands and/or stroma outside the uterine cavity, resulting in a chronic inflammatory process in all the affected sites [1-3]. Its exact prevalence is not clear, but it is estimated to affect 10 to 15% of women of reproductive age and therefore represents one of the most common gynaecological diseases [4,5].

Most literature citations identify the German researcher and pathologist Carl von Rokitansky as the person responsible for the first detailed description of endometriosis during the mid-nineteenth century (in 1860); however, two recently published papers contradict this fact [6,7]. After detailed analysis of historical documents from the seventeenth and eighteenth centuries found at the National Library of Medicine in Bethesda, Maryland, Knapp (1999) [6] and, later, Nezhat et al (2012) [7], notes that the first detailed description of that which we now call endometriosis was reported by the German physician Daniel Shrön in 1690 in his book *Disputatio Inauguralis Medica de Ulceribus Ulceri*. There he described the presence of ‘ulcers’, which in their primary form were distributed through the peritoneum and pre-eminently localised in the bladder, bowel, large ligament and external parts of the uterus and uterine cervix [6-8]. According to Knapp, the precise organic description of the disease published by Shrön in 1690 was followed in the eighteenth century by another 11 studies from different European countries: Scotland, England, the Netherlands and Germany. Those reports, despite using a scientific language not as precise as today's, suggest that it was a common disease at that time, very well known by clinicians as well as by women, since none of those authors saw their work as original and all of them clearly showed the major organic damage caused by and the main symptoms of endometriosis; moreover, they understood that it was a disease exclusive to women, and only those post-puberty, and that in some cases it was related to recurrent miscarriage and sterility [6,9].

Despite the historical findings, the German pathologist Carl von Rokitansky is credited with pioneering the cellular or histological description of endometriosis with the advent and use of microscopy at the University of Vienna. His description dates

from 1860 and serves as the basis for the current histological definition of the disease; moreover, von Rokitansky was also responsible for naming the variants that we know today as adenomyosis, adenomyoma and ovarian endometriosis, to which he gave descriptive names of Latin origin: *Sarcoma Adenoids Uterinum, Cystosarcoma Adenoids Uterinum* and *Ovarial Cystosarcom* or *Cystosarcoma adenoids ovarii uterinum*, respectively [10]. Thirty years later, in the last decade of the nineteenth century, the microtome, an instrument for obtaining thin microscopic tissue slices, came into use. At the time, it was most utilised by Thomas Cullen at Johns Hopkins Hospital, Baltimore and Robert Meyer at the University of Berlin, highlighting the scientific progress in the field of endometriosis research. In the early twentieth century, Robert Meyer (1903,1919) published his work on the pathogenesis of endometriosis and the coelomatic epithelium metaplasia theory [11,12]. However, it was the American John Sampson, the publisher in 1927 of one of the most influential papers in this field so far, who proposed for the first time the name ‘endometriosis’ and introduced his famous ‘theory of retrograde menstruation’ to explain the presence of ectopic foci of endometrial tissue in the abdominal cavity [13].

1.1.2 Clinical presentation and propaedeutics

The major associated symptoms are, because of the chronic inflammatory process, chronic pelvic pain, severe dysmenorrhoea, and deep dyspareunia as well as non-cyclic pelvic pain, cyclic urinary and bowel symptoms such as dysuria, haematuria and dyschezia; additionally, endometriosis usually leads to infertility [14-16]. The disease can be divided into three subsets of clinical presentations: superficial or peritoneal disease, ovarian endometriosis (OE) or ovarian endometriotic cysts (endometriomas) and deep-infiltrating endometriosis (DIE) [17].

The DIE lesions were first described by Cornillie et al (1990) and are characterised when the lesion’s infiltration is greater than or equal to 5 mm depth at the peritoneum from affected structures or organs, such as uterosacral ligaments, ureter, bladder, vagina, bowel or rectovaginal septum (RVS) [18-21]. It is estimated that DIE affects 20% of women with endometriosis [22]. Among all these presentations, the most aggressive subset is certainly DIE, which compromises the bowel or the RVS, when sometimes the progression of the disease leads to subileus and/or ileus as well as ureteral stenosis and risk of secondary renal failure. Several

studies have already shown the association of this kind of endometriosis and the loss of quality of life in affected patients [23,24].

The diagnosis of endometriosis is based on clinical symptoms and clinical history first as well a good clinical examination. Complementary exams such as transvaginal ultrasound with bowel preparation and magnetic resonance imaging (MRI) are helpful before indication of surgery [25,26]. The treatment will depend on the patient's symptoms – pain or infertility – although sometimes both are present.

Clinical treatment is based on hormone therapies and pain relief agents. Surgery is indicated when clinical treatment fails but also in severe cases of DIE and infertility. However, even after radical excision recurrences have been related in up to 13 to 15% of cases [27].

1.1.3 Classification

The classification system most widely used worldwide is that revised by the American Society for Reproductive Medicine (rASRM, 1996) [28], wherein lesions are classified during surgery by a system of points following stages I to IV, as shown in Supplementary Table 1. However, this scoring system has some drawbacks, such as the variability of inter- and intra-observers which can affect the reproducibility of this classification as well as the lack of a clear correlation between the stages of the disease and pregnancy rate or pelvic pain [29]. The German Foundation for Endometriosis Research and the endometriosis work group from Villach, Austria published in 2005 a new proposal to classify DIE, the ENZIAN score [30], which was revised in 2010 and 2011 as represented in Supplementary Table 2 [31,32]. Although this new system complements the rASRM score system, it has current a poor level of international acceptance and is mainly used in the German-speaking countries [33].

1.1.4 Aetiopathogenesis

The pathogenesis of endometriosis remains unclear to date, despite all the hypotheses and theories proposed to clarify its aetiopathological mechanisms, and remains the main challenge to research in this field. It is not well understood why some patients stay within the early stages of the disease whereas others develop the most advanced ones, suggesting the existence of different spectrums of this disease, each one with a particular aetiopathogenesis, as represented in Figure 1 [20,34-36].

The most acceptable theory up to now is the old theory of 'retrograde menstruation' (Sampson, 1927), in which the flow through the uterine tubes during

the menstrual period results in implantation and proliferation of endometrial cells within the pelvic cavity [13]; this theory may explain the physical displacement of the endometrial fragments into the peritoneal cavity, but other factors are required to trigger the development of endometriotic implants [37].

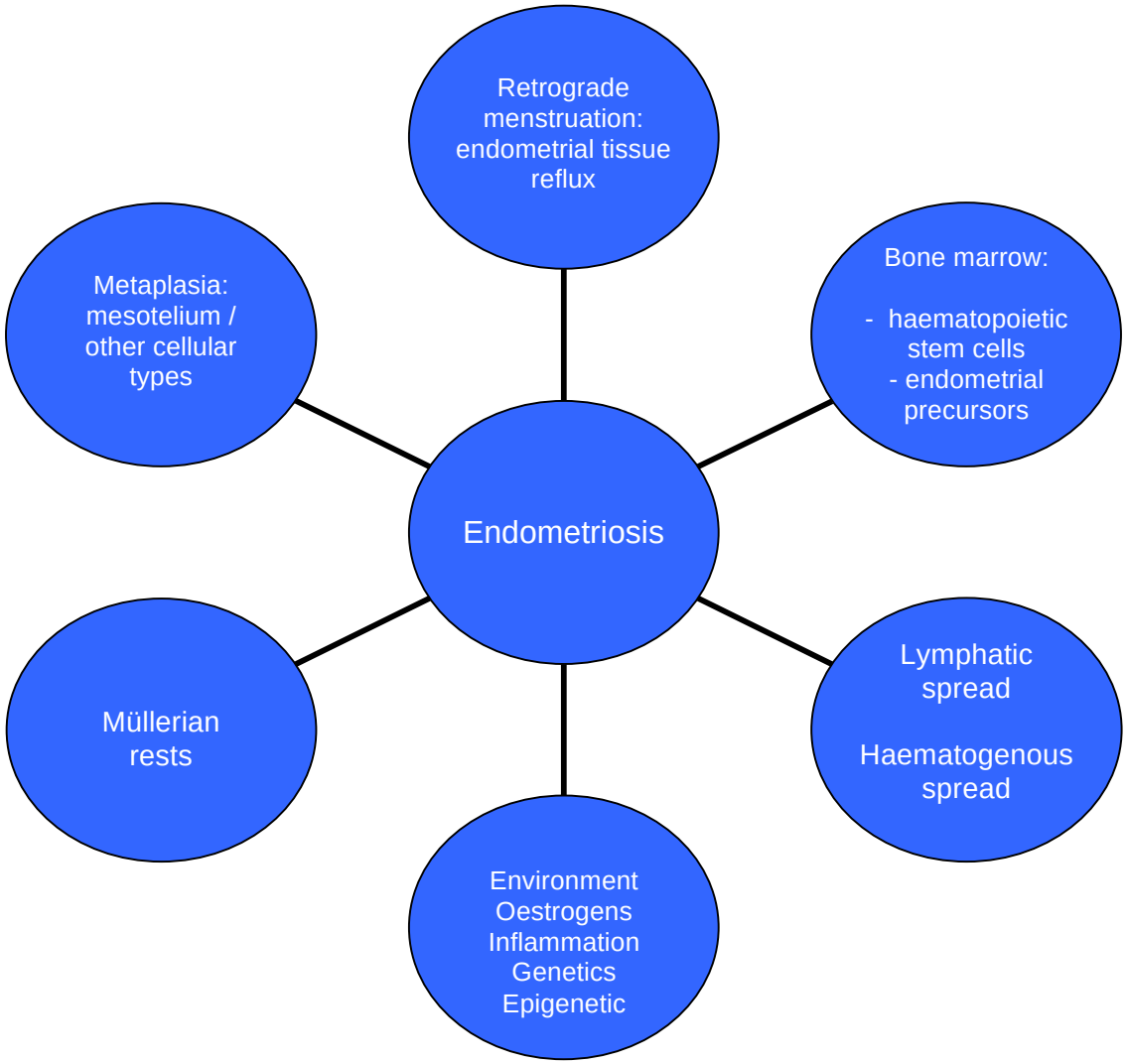

Figure 1. Theories regarding the pathogenesis of endometriosis. Figure adapted from Burney and Giudice, 2012 [37].

Several studies also showed that the immunological system has an important role in the pathogenesis of endometriosis and the lack of *immunovigilance* in the peritoneum is probably one of the causal factors of the disease. After establishment, endometriotic lesions release many pro-inflammatory molecules. In patients with endometriosis, there is an increase in the production and release of several cytokines, the growth factor, and the angiogenic factor, all originating from both the own lesion and also from other cells of the immunological system. There are already data in the literature showing that the peritoneal fluid (PF) of patients with

endometriosis yield many cytokines because of macrophage activation resulting in higher levels of those proteins in the PF of patients compared with controls. It is feasible that disturbances in the immunological homoeostasis could facilitate implantation, proliferation and angiogenesis of endometrial tissue [38,39].

Of all the theories shown in Figure 1, our study will focus on the lymphatic spread of endometrial cells or benign metastasis theory. This theory is documented in the literature, with evidence of endometriosis affecting the pelvic lymph nodes in animal models [40] as well as in women subjected to lymph node removal [41,42]. The presence of endometriotic lesions histologically proven in organs distant from the uterus such as bone, lung and brain could constitute strong evidence to support this theory [43].

1.2 Endometrial capacity of invasion in endometriosis and its similarity to cancer

Although endometriosis is considered a benign disease, it has the capacity of infiltrative and destructive growth, besides occurring in many organs and tissues distant from the uterus, and showing behaviour very similar to that of malignant processes. Interestingly, endometriotic lesions have been detected within incidentally excised mesorectal lymph nodes of patients with DIE (bowel and RVS) who have been submitted to surgical therapy, including resection of the affected bowel segment with adjacent adipose tissue [44-46].

Furthermore, a prospective pilot study in patients with rectovaginal DIE using the technique of colour-labelled pelvic sentinel lymph nodes (PSLN) showed not only endometriotic lesions affecting the PSLN in 21% of the cases but also disseminated endometriotic cells positive for progesterone and/or oestrogen receptors in 83.3% of pelvic lymph nodes, representing for the first time clear evidence of lymphatic dissemination of endometriotic cells [42]. As endometriosis is considered a benign disease process, lymphatic dissemination of endometriotic cells has not been viewed as clinically relevant for the course of the disease. Moreover, recurrences of endometriosis or singular deposits of endometriotic cells in regional lymph nodes have not been reported. Therefore, it seems to be rather different from the general behaviour of malignant processes.

In addition to these data, the expression of VEGF-C and D, both important growth factors, by endometriotic epithelial and stromal cells suggests the presence of

lymphangiogenesis in DIE [47]. The occurrence of lymphatic spread of endometriotic cells as well as lymphangiogenesis suggests that DIE is not only a local process but may be a systemic disease.

Some proteins play a major role in the processes of metastasis and tumour invasion in malignant diseases and, eventually, they might play a similar role in endometriosis. In our study we pay particular attention to specific proteins called chemokines.

1.2.1 Chemokines

Chemokines represent a family of small cytokines or proteins released by cells, especially lymphocytes, and are able to induce *chemotaxis* (*directed movement through the chemicals of the microenvironment*) in nearby responsive cells, directing cellular migration through a concentration gradient (Figure 2) [48]. Proteins are classified as chemokines according to shared structural characteristics such as small molecular size (7-12 kDa or 70-90 aminoacids of extension) and the presence of four cystein residues in specific localisations, which are essential for framing its three-dimensional spatial structure [49].

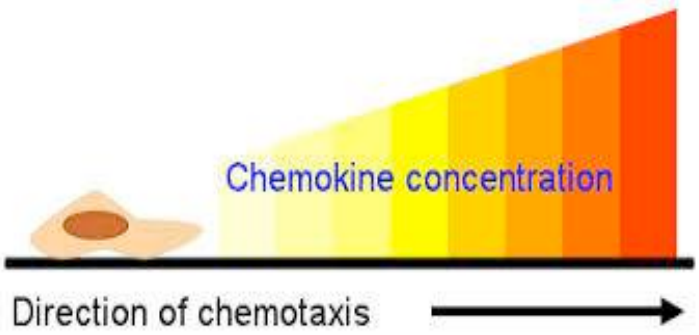

Figure 2. Characterisation of chemotaxis toward a concentration gradient. Source: Image adapted and derivative from Kohidai, L. 2008 (Own work. Based on File:Chtxphenomen1.png.)

Some chemokines control cells of the immune system, directing lymphocytes to lymph nodes (homoeostatic chemokines). Others are inflammatory chemokines and are released from a great variety of cells in response to bacterial infections, virus and other pathogenic agents. There are also some chemokines, which promote angiogenesis or direct cells to tissues, which provide special signs harmful to cell maturation [49,50].

Chemokines interact with G protein-linked transmembrane receptors called chemokine receptors, which are selectively found on the surfaces of their target cells. More than 20 are known to date. The chemokines are divided into four subfamilies:

CC chemokines (β-chemokines), CXC chemokines (α-chemokine), XC chemokines (γ-chemokines) and CX_3C chemokines (δ-chemokines) [50]. In the standardised nomenclature, all chemokines have the suffix 'L' characterising them as 'ligands', and receptors have the suffix 'R' [51,52]. Table 1 shows examples of this nomenclature.

Table 1. Chemokine receptors and their respective ligands; examples of the standardised nomenclature

Receptors	Primary Ligands
α-chemokines	
CXCR1	CXCL1, CXCL6 and CXCL8
CXCR2	CXCL1, CXCL2, CXCL3, CXCL5, CXCL6, CXCL7, CXCL8
CXCR3-A	CXCL9, CXCL10 and CXCL11
CXCR3-B	CXCL4, CXCL9, CXCL10 and CXCL11
CXCR4	CXCL12
CXCR5	CXCL13
CXCR6	CXCL16
CXCR7	CXCL12
β-chemokines	
CCR1	CCL2, CCL3, CCL4, CCL5, CCL7, CCL14, CCL15, CCL23
CCR2	CCL2, CCL7, CCL8, CCL11 and CCL13
CCR3	CCL2, CCL5, CCL7, CCL13, CCL15, CCL16, CCL24, CCL26
CCR4	CCL17 and CCL22
CCR5	CCL2, CCL3, CCL4, CCL5, CCL8, CCL11, CCL13, CCL14
CCR6	CCL20
CCR7	CCL19 and CCL21
CCR8	CCL1
CCR9	CCL25
CCR10	CCL27 and CCL28

Borrelli et al, 2013 [64]

Although chemokines were characterised initially as leucocyte-attractive, it is now recognised that any cell, including tumour cells, may express chemokines and/or their receptors [53]. Tumours have developed many ways of using the multifunctional characteristics of the chemokines to promote their own surveillance and growth, through the control of tumour infiltration by leucocytes and the suppression of antitumour immune response, regulating angiogenesis and influencing the formation and dissemination of metastasis [54].

Chemokines and their receptors also have an important role in the development and maintenance of innate and adaptative immunity. Moreover, they act in the wound-healing processes and angiogenesis. When the physiological role of chemokines is subverted or chronically enlarged, disease will appear. As they are involved in chronic inflammation pathobiology, tumorigenesis and metastasis, as well

as autoimmune diseases, the potential of chemokine antagonists has been assessed for appropriate target therapies [55].

Table 2 shows the complete list of all chemokines divided into four families and the standardised nomenclature with the respective names used in the non-standardised nomenclature for each chemokine, which are usually utilised in studies or related products.

Table 2. Nomenclature of chemokines – standardised and non-standardised

Standardised Nomenclature	Alternative Nomenclature
CXCL1	GRO-1 or GRO- α (growth regulated oncogene α)
CXCL2	GRO-2 or GRO- β (growth regulated oncogene β)
CXCL3	GRO-3 or GRO- γ (growth regulated oncogene γ)
CXCL4	Platelet Factor 4
CXCL5	Epithelial-derived neutrophil-activating peptide 78
CXCL6	Granulocyte chemotactic protein
CXCL7	NAP-2
CXCL8	IL-8 (Interleukin-8)
CXCL9	MIG (Monokine induced by IFN-g), CRG-10
CXCL10	IP-10 / Small-inducible cytokine B10
CXCL11	IP-9 / Interferon-inducible T-cell α chemokine
CXCL12	SDF-1 α and β (Stromal cell-derived factor)
CXCL13	B lymphocyte chemoattractant or B cell-attracting chemokine 1
CXCL14	Breast and kidney-expressed chemokine
CXCL15	Lungkine
CXCL16	SR-PSOX
CCL1	I-309, TCA-3
CCL2	MCP-1 (Monocyte chemotactic protein -1)
CCL3	MIP-1 α (Macrophage inflammatory protein-alpha)
CCL4	MIP-1 β (Macrophage inflammatory protein-beta)
CCL5	RANTES (Regulated upon Activation, Normal T cell Expressed and Secreted)
CCL6	C10, MRP-2
CCL7	MCP-3 (Monocyte chemotactic protein – 3), MARC
CCL8	MCP-2 (Monocyte chemotactic protein – 2)
CCL9	MRP-2, CCF18, MIP-1 γ
CCL10	MRP-2, CCF18, MIP-1 γ
CCL11	Eotaxin-1
CCL12	MCP-5 (Monocyte chemotactic protein – 5)
CCL13	MCP-4 (Monocyte chemotactic protein – 4)
CCL14	HCC-1 (Haemofiltrate CC chemokines - 1)
CCL15	HCC-2 (Haemofiltrate CC chemokines - 2) / MIP-5
CCL16	Liver - expressed chemokine / Monotactin-1
CCL17	TARC (Thymus and activation regulated chemokine)
CCL18	Pulmonary and activation-regulated chemokine
CCL19	MIP-3β (Macrophage inflammatory protein-3β)
CCL20	Liver activation-regulated chemokine / MIP-3α
CCL21	6 Ckine / Exodus-2 / Secondary lymphoid-tissue chemokine
CCL22	Macrophage-derived chemokine (MDC)
CCL23	MPIF-1
CCL24	Eotaxin-2

CCL25	Thymus-expressed chemokine
CCL26	Eotaxin-3/IMAC/MIP-4 α (Macrophage inflammatory protein-4α)
CCL27	Cutaneous T-cell-attracting chemokine / ESkine / IL-11
XCL1	Lymphotactin alpha, SCM-1 alpha, ATAC
XCL2	Lymphotactin beta, SCM-1beta
CX_3CL1	Fractalkine / Neurotactin / ABCD-3

Borrelli et al, 2013 [64]

1.2.2 Chemokines and cancer

Chemokines play a sovereign role in tumour progression. Chronic inflammatory processes promote tumour formation; and not only the tumour cells but also the stroma cells produce chemokines and cytokines. These proteins act in an autocrine and/or paracrine manner to support tumour cell growth, induce angiogenesis and decrease immunovigilance [56].

There are many chemokine receptors which are often expressed in tumour cells, but the receptor CXCR4 is the one most often found among malignancies; up to now it has been shown to be expressed in cells from 23 different types of cancer [54]. Studies have shown that in tumours where this receptor is strongly expressed, metastasis is facilitated in specific distant organs such as lung, liver, bone marrow and lymph nodes, which in turn express the specific ligand CXCL12 (SDF-1 – stromal derived factor-1), resulting in the active axis CXCR4-CXCL12 [57]. Together, they act in a paracrine manner in cancer, promoting tumour progression and growth, angiogenesis, lymphangiogenesis and metastasis in target tissues [56,57].

Furthermore, the expression of the receptor CXCR4 is related to the tumour metastasis potential, severity of disease, recurrence risk and prognosis in several types of cancer, especially in breast cancer [58]. The ligand CXCL12 was previously related to breast cancer metastasis; however the high expression identified in the miofibroblasts of DCIS (ductal carcinoma in situ), a pre-invasive tumour, suggests that this chemokine might have an additional role in the early stages of the breast tumorigenesis [59]. In accordance with this hypothesis, the ligand CXCL12 was also identified as a transcription target of the oestrogen receptor in breast and ovarian cancer cells [60]. The organs already mentioned that express the highest levels of CXCL12 represent the most common locals of metastasis in breast cancer, supporting its role of keeping tumour cells in lymph nodes [61].

The second chemokine receptor most frequently associated with tumour cell migration in malignancies is receptor 7 of the beta-chemokines family – CCR7. Through its specific ligands CCL19 and CCL21, which are highly expressed in

regional lymph nodes, the receptor CCR7 is responsible for facilitating the cell migration from the primary tumour to the lymph nodes [57,62]. Moreover, the receptor CCR7 is highly expressed in the cells of CLL (chronic lymphocytic leukaemia) as well as in the tumour cells of patients with lymphadenopathy; in vitro, it exhibits a high migratory response through its homoeostatic ligands CCL19 and CCL21 [62].

Together, these two systems of chemokine ligand receptors (CXCR4-CXCL12 and CCR7-CCL19 / CCR7-CCL21) are common mediators of metastasis processes in several tumours and thus have been used as targets for chemotherapy, since preliminary laboratory data have shown that chemokine-receptor axis antagonists inhibit the macrophage infiltration's potential, induce the paralysation of tumour growth or apoptosis and, finally, prevent metastatic dissemination [54,56].

1.2.3 Chemokines and endometriosis

Chemokines were first related to endometriosis by Khorram et al (1993), who assessed beta-chemokine CCL5 (RANTES) and concluded that its concentration in the peritoneal fluid of patients with endometriosis was significantly higher compared with controls without the disease, and also that these levels were positively correlated with the stage of disease [63]. During the 20 years since this publication, several authors have evaluated different chemokines in patients with endometriosis and/or infertility in order to elucidate the real role of those proteins in these two associated diseases, as we showed recently in a review of the literature [64]. As most studies looked at the chemokines as a possible marker for endometriosis, we investigated this possibility through a systematic review and concluded that of all the 27 chemokines tested in endometriosis patients for this purpose, three of them have the potential to play this role: CXCL8 (IL-8), CCL2 (MCP-1) and CCL5 (RANTES) [65].

As mentioned before, the main chemokine receptor involved in the process of malignant cell dissemination, invasion and metastasis, the receptor CXCR4 was already identified in the human endometrium as well as in the ovarian lesions of endometriosis or endometriomas [66,67], but not yet in the most aggressive form of endometriosis, DIE in the rectovaginal site or in the bowel. Hence, putting together the knowledge of the role of the chemokines in cancer and the data showing the lymphatic spread of endometriotic cells in patients with rectovaginal DIE, we

hypothesised that the same receptors and their respective ligands responsible for cell migration within malignancies – CXCR4/CXCL12 and CCR7/CCL19-CCL21 – could also play a role in endometriosis, especially DIE, by compromising the rectovaginal site or the bowel. As there is nothing on this in the literature so far, investigating it was one of the aims of our study.

1.2.4 Capacity of malignant transformation of endometriotic lesions

We will also evaluate the presence of a possible factor related to the malignant transformation of endometriotic lesions, with emphasis on rectovaginal DIE, since this risk has been suggested for endometriomas [68-70]. Wiegand et al (2010) evaluated mutations on the tumour-suppressor gene *ARID1A* (AT-rich interactive domain 1A), which encodes the protein BAF250a (BRG-associated factor 250a) among epithelial ovarian carcinomas; mutations on the gene *ARID1A* were identified in 46% (55/119) of clear cell carcinomas, in 30% (10/33) of endometrioid carcinomas and in none of the high-grade serous carcinomas (0/76). Through immunohistochemical evaluation of BAF250a protein in those samples they also found that the loss of expression of BAF250a is strongly correlated with the clear-cell and endometrioid carcinoma subtypes and *ARID1A* mutations as well; in this analysis the authors also identified two cases where *ARID1A* mutation and the loss of BAF250a protein were present not only in ovarian tumour cells but also in atypical endometriosis contiguous to the tumour, suggesting that those alterations might represent an early event in the transformation of endometriosis into cancer; however, this was not found in the distant (‘benign’) lesions of endometriosis [70].

Two years later Samartzis et al (2012) published an interesting paper evaluating the expression of BAF250a protein in distant endometriosis lesions; the complete loss of expression was observed by immunohistochemistry in 15% (3/20) of endometriomas and 5% (1/20) of DIE, however neither the peritoneal endometriosis cases (0/16) nor the eutopic endometrium from controls (0/30) presented such alterations. Moreover, the authors reported the partial loss of BAF250a expression in groups of cells in their samples and they referred to them as ‘cell clusters’ or ‘clonal loss’ but they did not specify in which lesions this phenomenon happened [71]. This phenomenon of clonal loss of BAF250a was first described in uterine endometrioid carcinomas [72]. Despite these findings among endometriosis patients, it is not clear yet what the real risk of malignant transformation is among distant or isolated

endometriosis lesions and more studies are necessary. Hence, we will assess this factor among rectovaginal DIE, the most aggressive form of the disease, as well as in the endometriotic lesions affecting the PSLN of these patients. Furthermore, there are already data in the literature suggesting a possible link between rectovaginal DIE and certain histologic subtypes of cancer. The former publication reported 13 cases of carcinomas of müllerian origin – endometrioid carcinoma (*n*=6), mixed papillary serous and endometrioid carcinoma (*n*=4), malignant mixed müllerian tumour (*n*=2) and undifferentiated (*n*=1) – presenting as colorectal cancer. Interestingly, they identified the presence of typical endometriosis in the bowel adjacent to the tumour cells in nine cases and endosalpingiosis in another one case, so 10 out of the 13 cases showed benign müllerian tissue near the malignant müllerian tumours. The latter was a review of 79 cases of endometrial stromal sarcoma arising in endometriosis, where 19% (15/79) of cases were extragenital endometrial stromal sarcomas (EESS) localised in the bowel. The authors of both papers suggested the possibility of malignant transformation of rectovaginal DIE in those cases [73,74].

The purpose of this study is to better understand the relationship between endometriosis and cancer in two ways: endometriosis ‘mimicking’ cancer in the mechanism of dissemination of disease and the possibility of endometriosis ‘evolving’ into cancer. The objectives of this study are listed below:

1. Investigate the immunohistochemical staining of cancer-related chemokines (ligands and their receptors; CXCR4-CXCL12 and CCR7-CCL19 /CCR7-CCL21) in rectovaginal DIE and compromised PSLN.

1.1. Correlate the expression of the chemokine receptors (CXCR4 and CCR7) in rectovaginal DIE lesions with the expression of their ligands (CXCL12 and CCL19/CCL21) in the respective associated PSLN.

1.2. Correlate the expression of all chemokines and receptors with the rASRM stage of disease, the size of the lesion, the use of hormone medications and the cycle phase.

2. Assess the concentration levels of these chemokines – CCL19, CCL21, CXCL12 – as well as the levels of the systematically selected chemokines – CCL2, CCL5 and CXCL8 – in PF of patients with and without endometriosis using the multiplexing assay technology (Luminex® X-MAP® Technology).

3. Evaluate the potential of malignant transformation of rectovaginal DIE by assessing protein BAF250a (*ARID1A* gene) expression among endometriosis lesions, normal endometrium and EESS.

2 METHODOLOGIES

2.1 Study locale and approval

The present study was performed at the Charité University Hospital, Berlin at the Endometriosis Research Centre, Campus Benjamin Franklin in collaboration with the Institute of Pathology, Campus Mitte. The local Institution Review Board (Ethics Committee) approved the study - number EA4/038/07 – Supplementary Figure 1.

2.2 Patients and samples

A total of 123 patients surgically treated between 2007 and 2014 at Charité University Hospital, Berlin were enrolled in this retrospective study. All patients were told about the study proposals and then read and signed the consent form. The inclusion of patients and controls followed the criteria listed below.

Inclusion criteria (patients with endometriosis)

- Confirmation of endometriosis by histology.
- Absence of concomitant malignant disease at the time of the diagnosis of endometriosis through anamnesis, physical examination, surgical inspection and subsidiary exams when necessary.
- Absence of pelvic inflammatory disease, endometritis.

Inclusion criteria (controls)

- Confirmation during surgery of the non-existence of endometriosis.
- Histological confirmation of the non-existence of adenomyosis in patients subjected to hysterectomy.
- Women should be in the premenopausal period.

Exclusion criteria (patients and controls)

- Confirmation of concomitant malignancies.
- Confirmation of pelvic inflammatory disease during the surgical procedure of patients or controls.

The patients were divided into three subgroups according to the methods to which the samples were submitted and the aim of each assessment. Figure 3 shows this division.

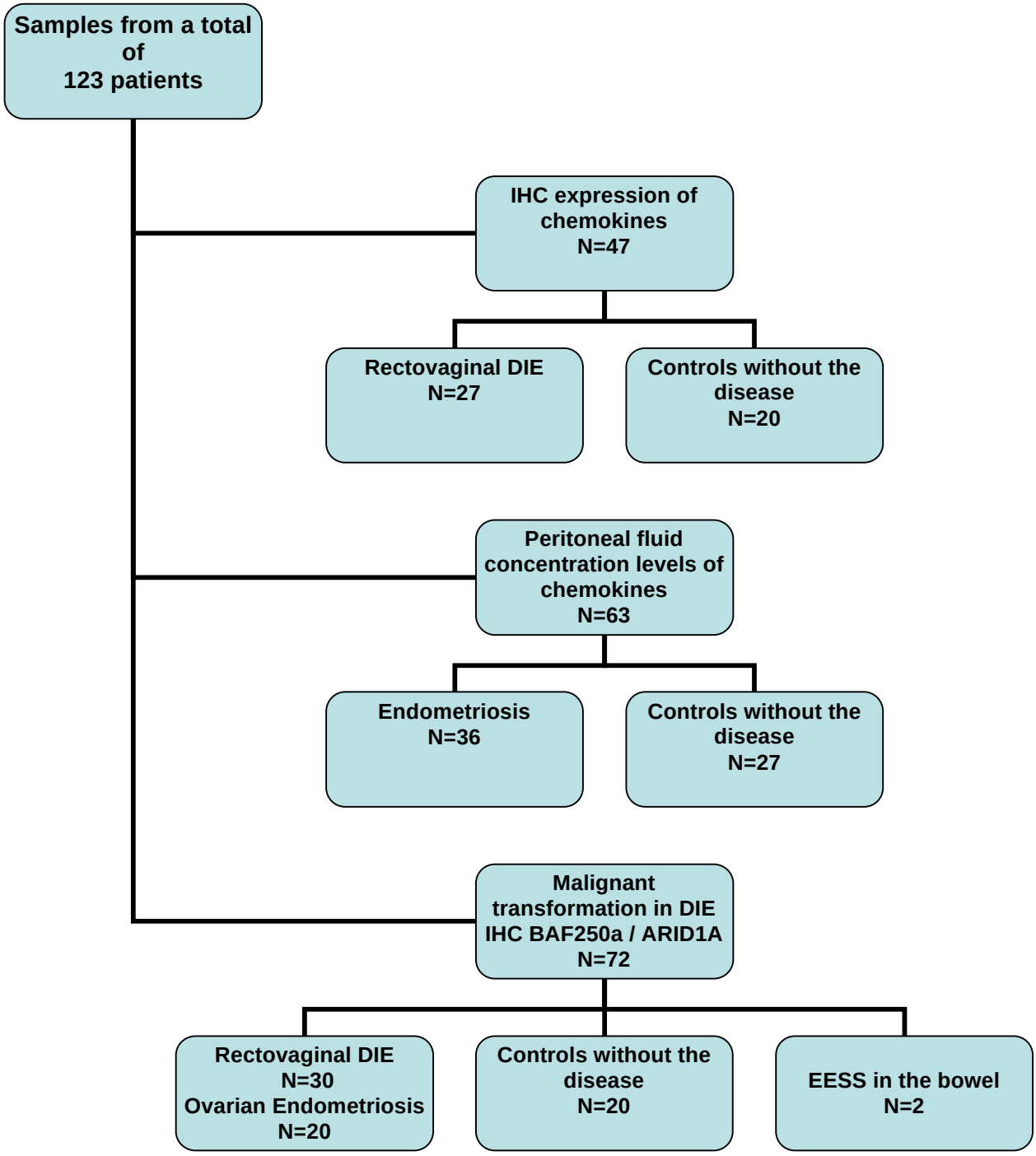

Figure 3. Subgroups of patients included in the study; IHC: immunohistochemistry; DIE: deep-infiltrating endometriosis; PSLN: pelvic sentinel lymph node; EESS: extragenital endometrial stromal sarcoma; EC: endometrioid carcinoma

We collected from patients with endometriosis the peritoneal fluid (PF), the lesions compromising the ovaries, the DIE lesions affecting the rectovaginal site or the bowel and the pelvic sentinel lymph nodes from the patients with DIE; from controls we collected the PF and the eutopic endometrium.

Sample collection, processing and storage

- Peritoneal fluid (PF): the peritoneal fluid from the pouch of Douglas was aspirated at the beginning of the laparoscopic procedure, immediately dropped into a dried conic tube of polystyrene (15ml DB Falcon^{TM}) and transported to the lab within one hour. After centrifugation (x 2,000 rpm at room temperature) the supernatants were removed, divided into 1.5ml aliquots and stored at –80^{o}C.
- Endometriotic lesions (EL): during surgical procedure, all endometriosis lesions (DIE or ovarian endometriosis) were removed and put in 10 % neutral buffered formalin (NBF) solution and sent to the pathology division. There, the tissues were fixed by immersion with NBF, dehydrated and embedded in paraffin. They were stored in formalin-fixed paraffin-embedded (FFPE) blocks until usage.
- Sentinel lymph nodes (SLN): we assessed the iliac lymph nodes using the sentinel lymph node technique, which was performed with patent blue. At the beginning of the procedure, after anaesthesia, with the patient in position, 4 ml of patent blue was injected around the visible or touchable nodule in the posterior vaginal fornix (Figure 4). During the subsequent laparoscopy, the retroperitoneal space was opened laterally to the infundibulopelvic (IP) ligament and the coloured SLN removed from the iliac region (Figure 5). The samples were stored in FFPE blocks after the same procedures as those for the endometriotic lesions already discussed.
- Eutopic endometrium (EE): the endometrial samples from controls were obtained after hysterectomy or curettage for benign gynaecological interventions and the tissues were stored in FFPE blocks as previously described.
- Tumour cells: the samples from endometrial stromal sarcoma in the bowel were removed during surgery and the tissues were stored in FFPE blocks as previously described.

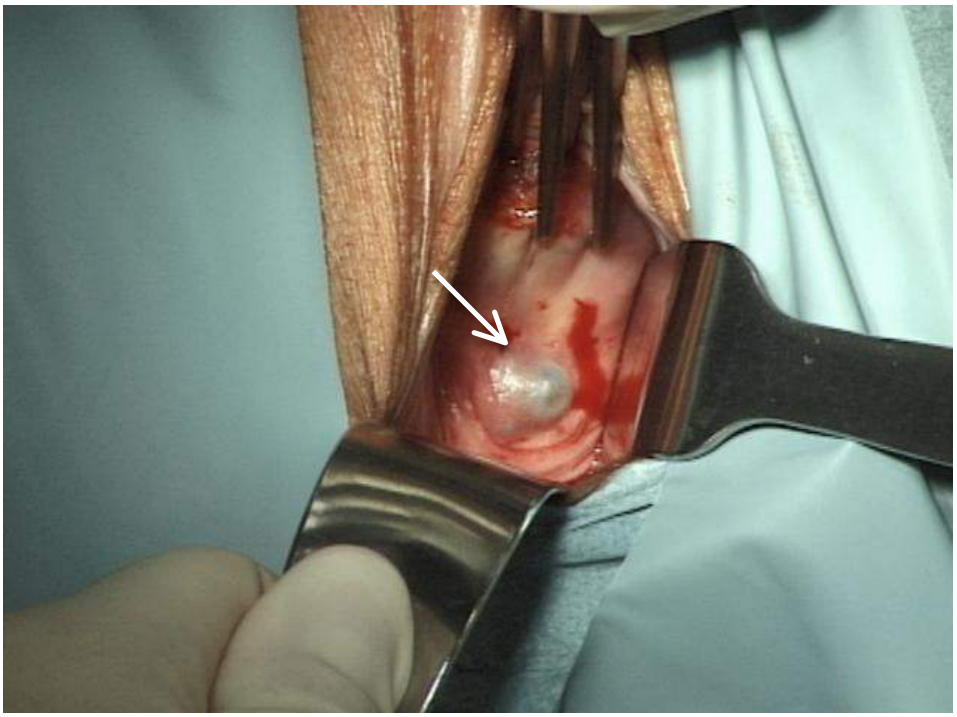

Figure 4. Identification of the nodule at the posterior vaginal wall – pouch of Douglas – for the injection of patent blue®; Mechsner et al, 2008 [42]

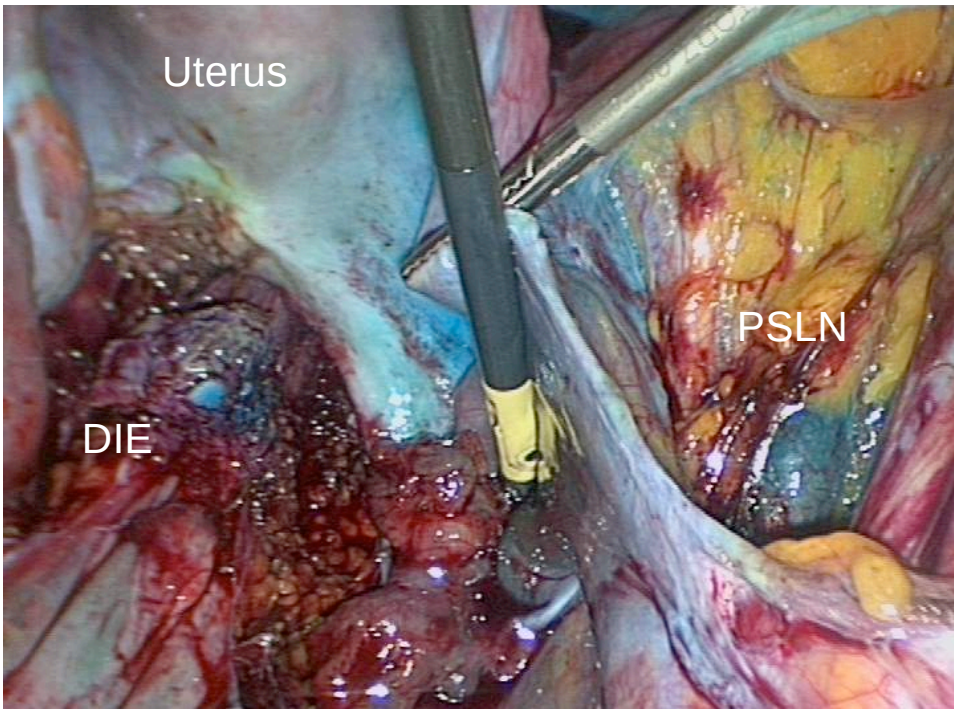

Figure 5. Localisation of the pelvic sentinel lymph node (PSLN) during laparoscopy; the deep endometriotic lesion (DIE) is also blue-stained; Mechsner et al, 2008 [42]

2.3 Methods

2.3.1 Immunohistochemistry

The immunohistochemistry (IHC) technique allows the detection or localisation of antigens (proteins) in the tissues, based on the principle of the specific binding between antibodies and antigens within the biological tissues, and to that end it uses antibodies produced against the proteins of interest. There are many systems or methods of detection or staining and in our study the immunohistochemical staining was based on the labelled streptavidin-biotin (LSAB) method (Figure 6) and followed the protocol of the Institute of Pathology at Charité Hospital, University of Medicine of Berlin, Campus Mitte.

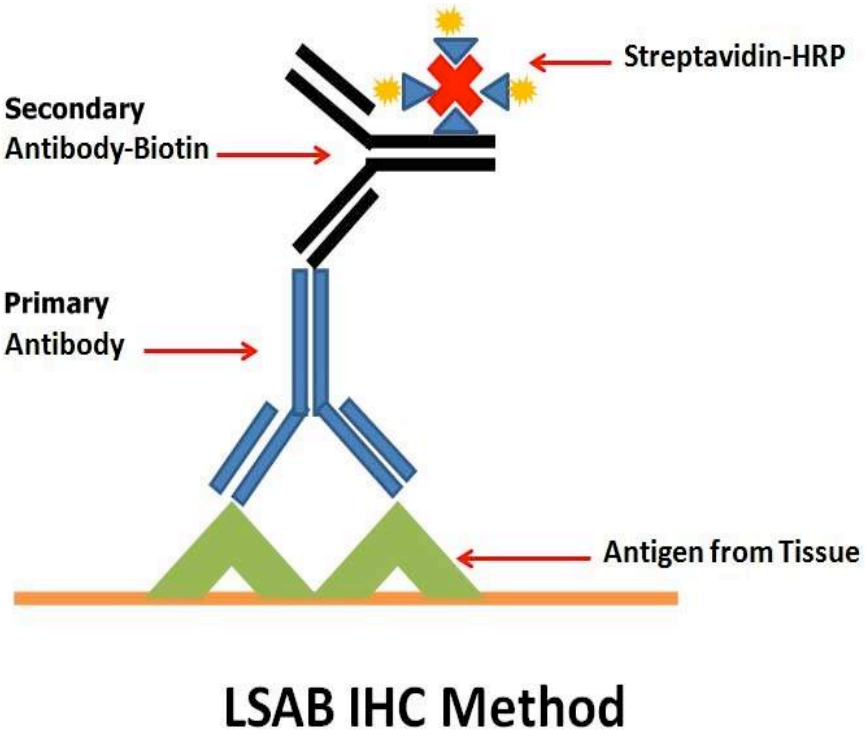

Figure 6. Illustration of the immunohistochemistry (IHC) method – Labelled Streptavidin-Biotin (LSAB); HRP – Horseradish Peroxidase; Source: http://www.genecopoeia.com/product/vitroview-lsab-immunohistochemistry-detection-system/

The paraffin blocks from all samples were sliced into 2 µm whole sections with the manual rotating Microtome (LEICA®-RM2125 RT; Leica Microsystems) and mounted on SuperFrost® glass slides (Menzel-Gläser, Braunschweig, Germany) or Labsolute® glass slides (Th. Geyer GmbH & Co. KG, Germany). After deparaffinisation and rehydration (xylene 2x10min; 3xethanol 100%; 1xethanol 96%; 1xethanol 90%; 1xethanol 80%; 3xethanol 70%; distilled water) we performed the antigen retrieval using the HIER (heat induced epitope retrieval) method with citrate

buffer (pH = 6) for five minutes. The endogenous peroxidase was blocked with the Dako-blocker S2023 for 15 minutes before incubation with the primary antibodies (polyclonal rabbit antibody Anti-ARID1A HPA005456, Sigma-Aldrich® - Prestige Antibodies®, dilution 1:700; monoclonal mouse anti-human CXCR4 – clone #44716, MAB172 R&D Systems, dilution 1:200; monoclonal mouse anti-human CCR7 – clone CCR7.6B3, MAB71278 Covalab, R&D Biotechnology, dilution 1:200; monoclonal mouse anti-human CXCL12/SDF-1 – clone #79018, MAB350 R&D Systems, dilution 1:100; polyclonal rabbit anti-human CCL19/MIP-3 beta – bs-2454R Bioss, dilution 1:100; polyclonal rabbit anti-human CCL21/6Ckine – bs-1666R Bioss, dilution 1:100) for one hour. Next, we performed the secondary antibody incubation (Dako REAL™ Detection System, Peroxidase/DAB+, Rabbit/Mouse – Code K5001) for another 30 minutes and the streptavidin-peroxidase (HRP- Horseradish Peroxidase) was added for an additional 15 minutes before the DAB (3,3' – Diaminobenzidine) Chromogen was given. The slides were counterstained with haematoxylin, dehydrated and mounted. We used human tonsil and/or lymph node samples as positive controls for all chemokine antibodies and for the anti-ARID1A antibody we used a TMA (tumour micro array) of epithelial ovarian carcinomas (including high-grade serous, clear-cell and endometrioid subtypes) as positive and negative controls.

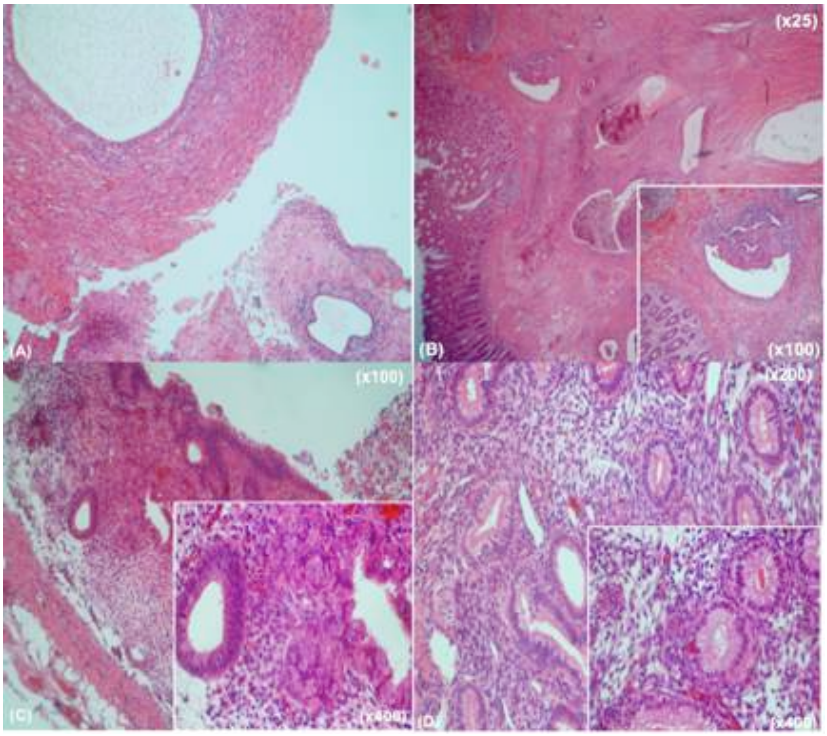

Figure 7. Haematoxylin-eosin (HE) staining. (A) ovarian endometriosis (x100); (B) rectovaginal deep infiltrating endometriosis (DIE); (C) endometriosis lesions compromising the pelvic sentinel lymph node (PSLN); and (D) eutopic endometrium.

All the microscope slides mounted for IHC evaluation were previously stained with haematoxylin-eosin (HE) in order to identify the tissues of interest (endometriotic lesions in all sites, including glands and stroma as well as the eutopic endometrium), as Figure 7 shows.

2.3.2 Luminex® xMAP® technology

The chemokines of interest in this study – CXCL12, CCL19 and CCL21 – were assessed in PF of women with and without endometriosis using the multiplex Luminex® xMAP® Technology. The chemokines with the best potential as possible markers for endometriosis – CCL2, CCL5 and CXCL8 – selected from 27 different chemokines in our recent systematic review [65] were also evaluated with Luminex® and the comparison of all six chemokines was possible in an unbiased fashion. Luminex assays are based on xMAP® technology (multi-analyte profiling beads) enabling the detection and quantisation of multiple RNA or protein targets simultaneously. The xMAP system combines a flow cytometer, fluorescent-dyed microspheres (beads), lasers and digital signal processing to allow efficient multiplexing of up to 100 unique assays within a single sample. Multiplexing technology has become an important tool in cytokine detection. Assays can be performed with great speed and accuracy by making use of hundreds of specially prepared magnetic beads, or microspheres. The microspheres are dyed internally with a mixture of dyes to provide a unique spectral address; when mixed with a sample, molecules on the outside of the spheres react to any molecules that they are designed to capture.

In our study, instead of using one kit with the six chemokines included, we had to use two kits with three chemokines each because RANTES (CCL5) should not be mixed with all the others, as the provider advises. Hence, we used the kits from two groups of the Bio-Plex assays as proposed by Bio-Rad®: 1) the ‘Human Chemokine Panel’ comprising CXCL12 (SDF-1), CCL19 (MIP-3beta) and CCL21 (6Ckine) and 2) the ‘Human Cytokine Panel Group I’ comprising CCL2 (MCP-1), CCL5 (RANTES) and CXCL8 (IL-8).

This method was first validated and calibrated for serum and culture media and recently a unique paper published data using peritoneal fluid [75]. As they did, we used a modified protocol from the one established for serum and the PF was

diluted 1:2 using the standard sample diluent provided with the kit. The diluted sample volume added to the plate was 50µl per well.

2.4 Immunoreactivity score and statistical analysis

The score of immunoreaction for the immunohistochemical analysis of all slides was based on a previously published score [71,76]. In this score we assessed the percentage of positive cells as well as the staining intensity for both epithelial and stromal cells of endometriosis lesions and eutopic endometrium. The percentage of positive cells was classified as 0 (0%), 1 (<10%), 2 (11-50%), 3 (51-80%) and 4 (>80%), and the staining intensity was graded as 0 (negative), 1 (weak), 2 (moderate) and 3 (strong). In order to get the final score of immunoreaction or the immunoreactivity score (IRS), the percentage of cells was multiplied by the staining intensity, resulting in a value between zero and 12. The cases in which it was not possible to assess both the epithelial and the stroma cells were excluded from the statistical analysis. The final score used for statistical analysis was the mean value obtained from two independent observers who evaluated the microscope slides after IHC and applied the scoring system previously described.

The women's age was described according to group by summary measures (mean, standard deviation, median, minimum and maximum) and compared between the groups with Student's t-test for the first two subgroups of patients (analysis of chemokines by IHC and Luminex, respectively) and analysis of variance (ANOVA), followed by Bonferroni's multiple comparisons test in order to verify between which groups the differences occurred in the third subgroup of patients (analysis of ARID1A). The use of hormone medications and the menstrual cycle phase were described as groups by means of absolute and relative frequencies and to verify the existence of association we used the chi-square test and the likelihood ratio, respectively. Moreover, the stage and the site of the disease as well as the size of the lesion were described among the women with endometriosis by means of absolute and relative frequencies. The chemokine concentration levels in the PF were described according to group, use of hormones, cycle phase, stage and site of the disease by using summary measures and compared between categories with the Mann-Whitney test; only for the site of the disease was the comparison of the chemokine PF levels performed with the Kruskal-Wallis test, followed by Dunn's multiple comparisons test. For the proteins that showed differences between the

groups, receiver operating characteristic (ROC) curves were created in order to determine the cut-off points with better sensitivity and specificity and to discriminate patients with endometriosis. Using the established cut-offs, we created a model of multiple logistic regressions to estimate the probabilities of endometriosis for each combination of the protein's 'positivity'.

For the qualitative characteristics in the subgroup #3 (ARID1A), absolute and relative frequencies were presented and the association between the groups was verified with the chi-square test or Fisher's exact test or likelihood ratio test. The association of the clonal loss of BAF250a occurrence was verified by the chi-square test. The IRSs for *ARID1A* were described according to group by means of summary measures and compared between the groups with the Kruskal-Wallis test.

The IRS for chemokines was described according to group, use of hormone medications, cycle phase, size of the lesion and stage of the disease with the use of summary measures and we compared the categories with Mann-Whitney's test or the Kruskal-Wallis test, the latter followed by the Dunn multiple comparisons test when there was significance in order to identify between which categories the difference occurred. Scattergrams were created between the scores of chemokine receptors and their respective ligands to estimate the Spearman's correlations between the scores.

All tests were performed with a significance level of 5% (0.05). The software SPSS (version 20.0, Chicago, IL, USA) and Excel 2003 were used.

3 RESULTS

The results are presented according to objective.

3.1 Immunohistochemical expression of cancer metastasis-related chemokines in rectovaginal deep infiltrating endometriosis (DIE) and the corresponding pelvic sentinel lymph nodes (PSLN)

In this subgroup of patients, the women's age in the group of DIE was statistically lower compared with the women in the control group ($p<0.001$); however, all patients were at the premenopausal stage; the use of hormone medications and the phase of the menstrual cycle did not present a statistically significant association within the group of women ($p>0.05$), as shown in Table 3.

Table 3. Clinical features of patients included in subgroup #1

Variable	**Group**		**Total (N = 47)**	
	Control (N = 20)	DIE (N = 27)		
Age (years)				**<0.001**
Mean (SD)	42.5 (4.0)	32.5 (7.2)	36.8 (7.8)	
Median (min.; max.)	42.5 (34; 50)	31 (21; 46)	39 (21; 50)	
Hormone				0.808*
No	14 (70.0)	18 (66.7)	32 (68.1)	
Yes	6 (30.0)	9 (33.3)	15 (31.9)	
Cycle Phase				0.126**
No cycle	0 (0.0)	2 (7.4)	2 (4.3)	
Follicular	3 (15.0)	7 (25.9)	10 (21.3)	
Luteal	10 (50.0)	6 (22.2)	16 (34)	
Unknown	7 (35.0)	12 (44.4)	19 (40.4)	
Lesion's Size (DIE)				
< 2cm		3 (11.1)	3 (11.1)	
2-3 cm		13 (48.1)	13 (48.1)	
> 3cm		11 (40.7)	11 (40.7)	
Localization (DIE)				
Left		4 (14.8)	4 (14.8)	
Right		2 (7.4)	2 (7.4)	
Central		21 (77.8)	21 (77.8)	
PSLN				
Left		7 (25.9)	7 (25.9)	
Right		7 (25.9)	7 (25.9)	
Bilateral		13 (48.1)	13 (48.1)	
Bowel resection				
No		11 (40.7)	11 (40.7)	
Yes		16 (59.3)	16 (59.3)	

Student's t-test; * Chi-square test; ** Likelihood ratio test

The immunolocalisation of all chemokines was interpreted in both epithelial and stromal cells of DIE lesions, endometriotic lesions compromising the PSLN and the eutopic endometrium from control subjects. Endometriotic lesions affecting the

PSLN were identified in 5/27 (18.5%) of the rectovaginal DIE patients included in this part of the study. The immunohistochemical staining of the chemokines in the epithelium as well as in the stroma was not always possible for all tested samples because of the lack of adequate tissue in the slides after IHC was performed and therefore the IRS was only considered for those with both expressions assessed. The epithelial and stromal expression was assessable in 23/27 (85%) of DIE cases, 4/5 (80%) cases of endometriotic lesions affecting the PSLN for all five chemokines tested; for control subjects the epithelial and stromal expression was assessable in the EE of 20/20 (100%) cases of all chemokines tested, except for CXCR4, whose expression was assessable in 19/20 (95%) cases.

Cytoplasmic and/or nuclear expression was considered positive for the chemokine receptor CXCR4, and a cell membrane staining was considered positive for its specific ligand CXCL12, as clearly represented in Figure 8. The expression pattern of the receptor CCR7 was basically in the cell membrane but in some cases the nuclear expression was also identified; the ligand CCL19 presented a cytoplasmic and cell membrane expression pattern whereas the ligand CCL21 mainly disclosed a nuclear staining pattern. Representative images are shown in Figure 9.

The IRS of all chemokines – receptors and ligands – according to each group is represented in Table 4. Although the median IRS for CXCR4, CXCL12, CCR7 and CCL21 was higher in DIE lesions and lesions compromising the PSLN compared with the score in the EE from controls, there was no statistically significant difference between them ($p>0.05$); for CCL19 the median IRS was lower in the DIE lesions than in the others but again this was not statistically significant ($p>0.05$).

Regarding the use of hormone medications only the ligand CCL19 presented an IRS statistically higher in women who had used hormone medications prior to the surgical procedure ($p=0.045$), and the receptors CXCR4 and CCR7 as well as the ligands CXCL12 and CCL21 did not show any influence of the use of hormones and the respective IRS, once there was no statistically significant difference between the groups ($p>0.05$), as shown in Supplementary Table 3. The phase of the menstrual cycle did not influence the IRS of the receptors CXCR4 and CCR7 or the ligands CXCL12 and CCL21, showing no statistically significant difference between the groups ($p>0.05$), whereas the ligand CCL19 revealed a higher IRS during the luteal phase ($p=0.041$), as presented in Supplementary Table 4.

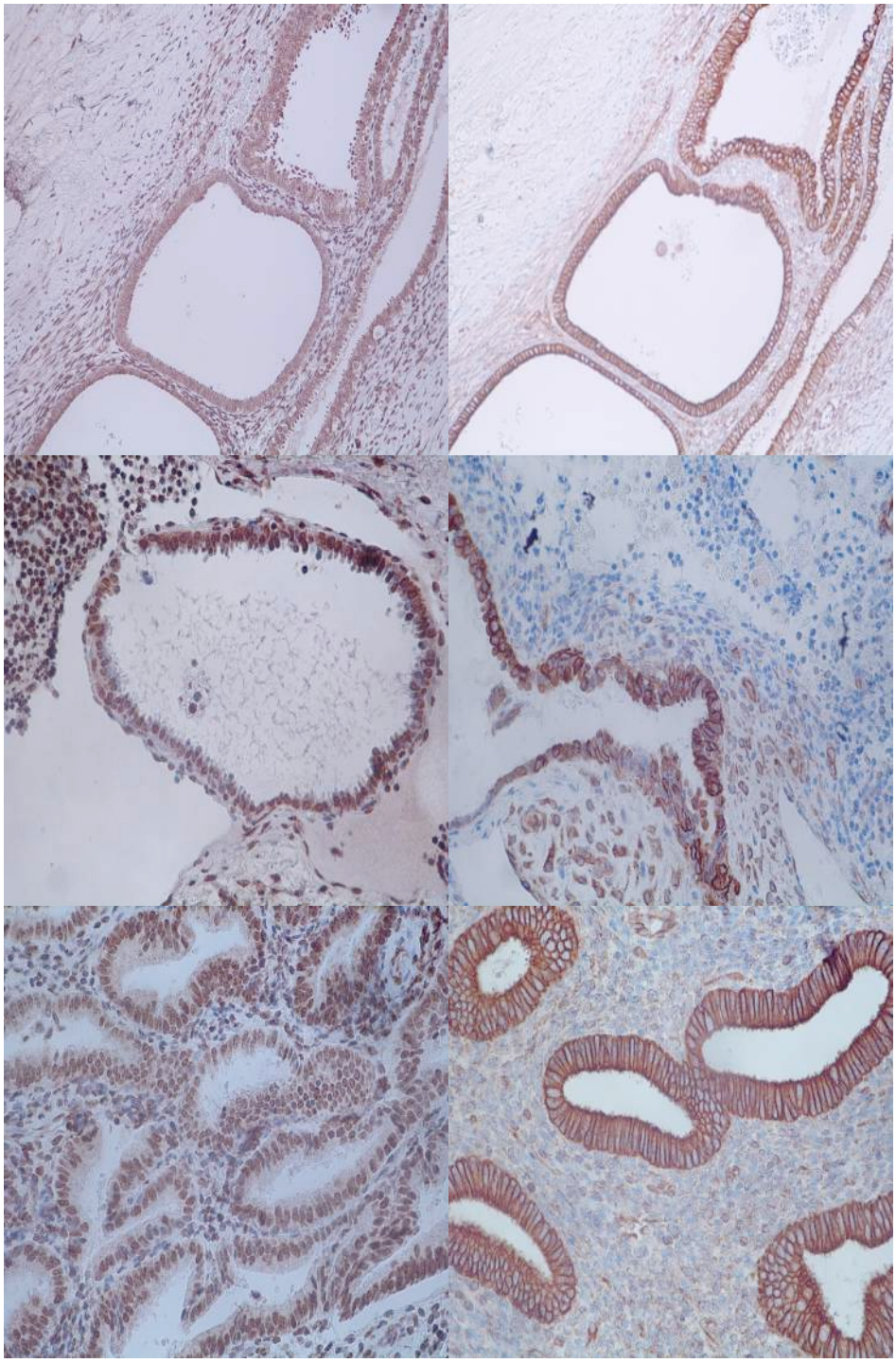

Figure 8. Immunohistochemical staining patterns of the chemokine axis CXCR4-CXCL12. The nuclear and/or cytoplasmic expression of CXCR4 and the cell membrane expression of CXCL12 in (A) DIE lesions (x200); (B) endometriotic lesion affecting the PSLN (x400) and (C) EE from controls (x400).

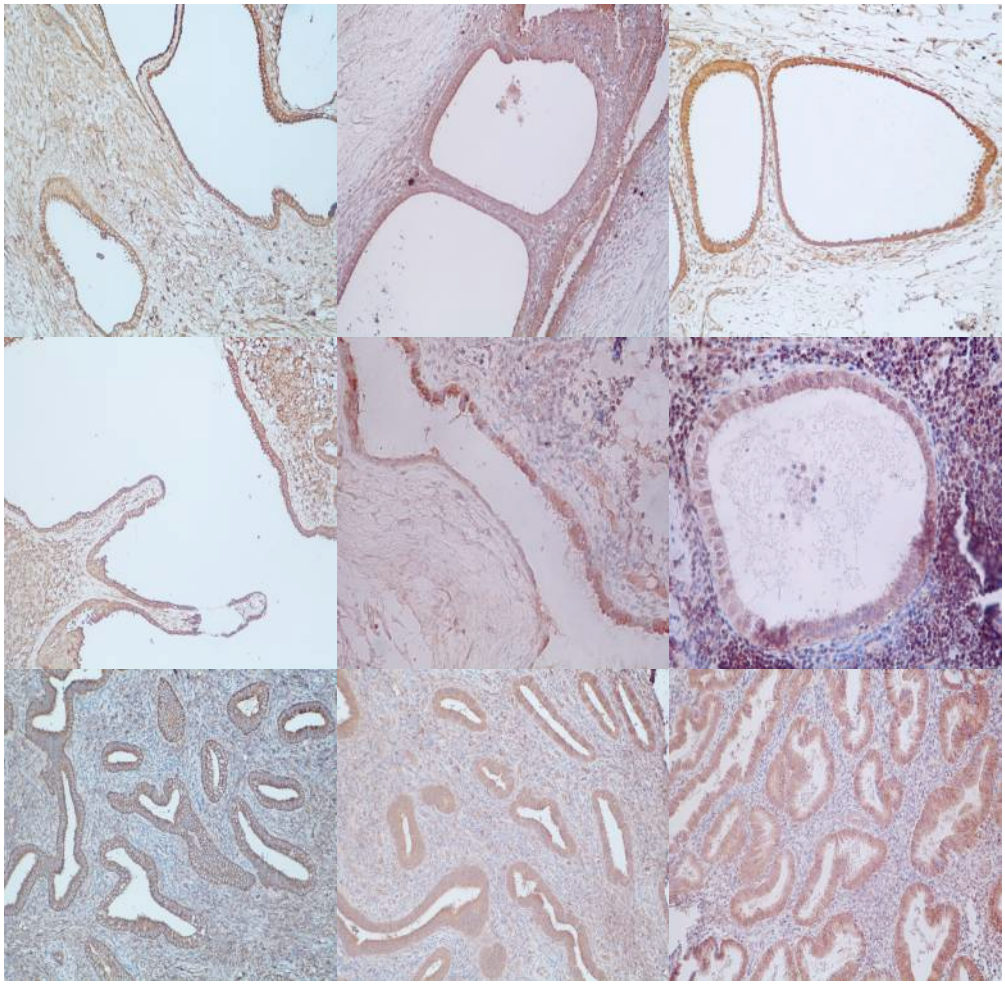

Figure 9. Immunohistochemical staining pattern of the chemokine receptor CCR7 and the ligands CCL19 and CCL21. The cell membrane expression of CCR7 in (A) DIE lesions (x200); (B) endometriotic lesion affecting the PSLN (x200) and (C) EE from controls (x200). The cell membrane staining of CCL19 in (A) DIE lesions (x200); (B) endometriotic lesion affecting the PSLN (x200) and (C) EE from controls (x200). The nuclear expression of CCL21 in (A) DIE lesions (x200); (B) endometriotic lesion affecting the PSLN (x400) and (C) EE from controls (x200).

Table 4. Immunoreactivity score (IRS) of all chemokines according to group and results of comparative tests

Variable	Group	Mean	SD	Median	P25	P75	Min.	Max.	N	p
	Control	7.88	1.57	7.5	7.0	9.0	6	12	20	
CXCL-12	DIE	8.74	2.56	9.0	8.0	10.5	1	12	23	0.057
	PSLN	8.00	1.41	8.5	6.5	9.0	6	9	4	
	Control	9.42	2.92	10.0	9.0	12.0	1	12	19	
CXCR-4	DIE	9.87	2.98	12.0	7.0	12.0	3	12	23	0.443
	PSLN	11.13	1.03	11.3	10.1	12.0	10	12	4	
	Control	8.13	2.29	8.3	7.0	10.5	3	10.5	20	
CCL-19	DIE	7.48	3.28	8.0	6.0	10.5	0	12	23	0.749
	PSLN	8.38	0.48	8.3	8.0	8.9	8	9	4	
	Control	8.88	2.81	9.0	6.0	12.0	5	12	20	
CCL-21	DIE	10.24	1.45	10.5	9.0	12.0	8	12	23	0.370
	PSLN	9.25	3.28	10.3	5.9	11.6	5	12	4	
	Control	8.43	2.06	8.5	7.0	9.0	5	12	20	
CCR-7	DIE	9.63	1.83	9.0	8.0	12.0	7	12	23	0.146
	PSLN	9.38	1.60	10.0	7.8	10.4	7	11	4	

Kruskal-Wallis' test

Looking at the chemokine IRSs according to the stages of the disease among women with endometriosis, we found that CCL19 showed a statistically lower score for advanced stages (III-IV) compared with early stages (I-II) of the disease (p = 0.036). None of the other chemokines – CXCR4, CXCL12, CCR7 and CCL21 – presented statistically significant differences in the IRS between the different stages of disease (p>0.05), as represented in Supplementary Table 5.

The chemokine receptor CXCR4 IRSs were statistically different in terms of the size of the disease (p=0.03), and the IRS from the other chemokines did not disclose any statistically significant difference regarding the size of the disease, as shown in Table 5.

Table 5. Chemokine immunoreactivity score according to lesion sizes

Variable	Lesion Size (DIE)	Mean	SD	Median	P25	P75	Min.	Max.	N	p
CXCL-12	< 2cm	6.75	5.30	6.8	3.0	10.5	3	11	2	0.275
	2-3 cm	8.00	2.64	8.5	8.0	9.0	1	11	10	
	> 3cm	9.77	1.69	10.0	8.0	11.3	8	12	11	
CXCR-4	< 2cm	4.25	2.48	4.3	2.5	6.0	3	6	2	**0.003**
	2-3 cm	8.95	2.82	9.5	7.0	12.0	5	12	10	
	> 3cm	11.73	0.91	12.0	12.0	12.0	9	12	11	
CCL-19	< 2cm	4.00	5.66	4.0	0.0	8.0	0	8	2	0.349
	2-3 cm	7.35	2.89	6.5	6.0	8.5	3	12	10	
	> 3cm	8.23	3.15	8.5	7.0	10.5	3	12	11	
CCL-21	< 2cm	9.75	3.18	9.8	7.5	12.0	8	12	2	0.521
	2-3 cm	9.90	1.20	10.0	9.0	10.5	8	12	10	
	> 3cm	10.64	1.42	10.5	9.0	12.0	9	12	11	
CCR-7	< 2cm	9.00	0.00	9.0	9.0	9.0	9	9	2	0.185
	2-3 cm	8.95	1.95	8.5	7.0	10.5	7	12	10	
	> 3cm	10.36	1.68	10.5	9.0	12.0	8	12	11	

Kruskal-Wallis' test

The IRS from CXCR4 was statistically higher in DIE lesions greater than 3 cm compared with the other lesion sizes, smaller than 2 cm or between 2 cm and 3 cm (p = 0.006 and p = 0.015, respectively), as represented in Table 6.

Table 6. Multiple comparisons of CXCR4 between lesion sizes

Comparisons	Z value	p
< 2cm VS 2-3 cm	-1.35	0.176
< 2cm VS > 3cm	-2.74	**0.006**
2-3 cm VS > 3cm	-2.42	**0.015**

Dunn multiple comparisons

Using scattergrams we tried to correlate the IRS of the chemokine receptors in the DIE lesion and the IRS of their specific ligands in the PSLN lymphatic cells. As regards the charts represented in Figure 10 for each chemokine axis, it was not possible with the findings to establish a correlation between the IRS of the two chemokine receptors evaluated and their respective ligands (p>0.05).

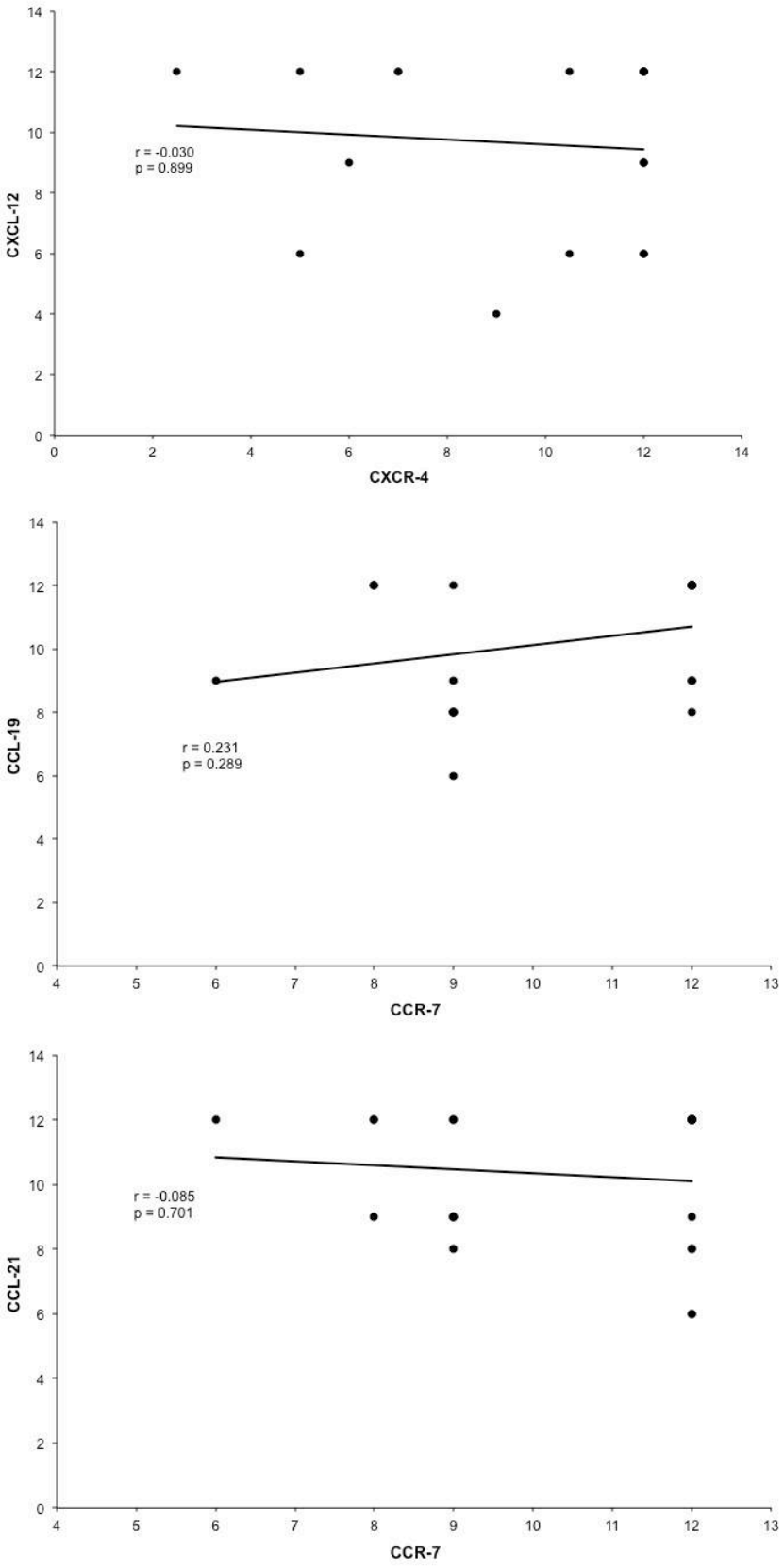

Figure 10. Scattergrams between the scores of the chemokine receptors in the DIE lesions and their specific ligands in the corresponding PSLN

However, the median IRS for all ligands was high in the lymph nodes, as expected for these chemokines, and although we could not find a statistically significant difference, the lymphatic cells in the lymph nodes compromised by endometriotic lesions appeared to present higher expression of these ligands as the cells in the lymph nodes without involvement with the disease, as represented in Figure 11.

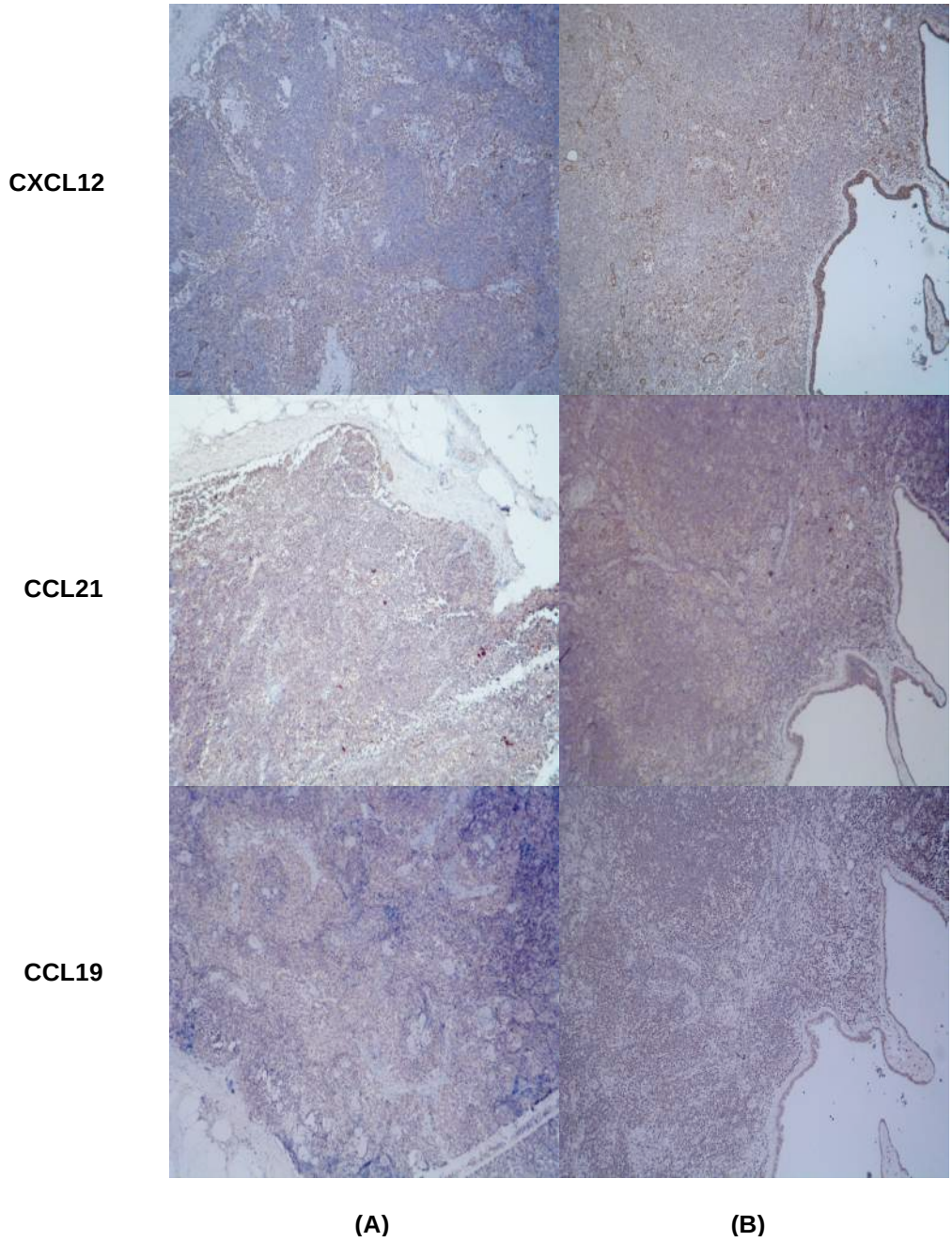

Figure 11. Expression pattern of the chemokine ligands in the pelvic sentinel lymph nodes (PSLN) not affected with endometriosis (A) and compromised with the disease (B).

3.2 Peritoneal fluid concentration level of chemokines among patients with and without endometriosis

The six chemokines CXCL12 (SDF-1), CCL19 (MIP-3beta), CCL21 (6Ckine), CCL2 (MCP-1), CCL5 (RANTES) and CXCL8 (IL-8) were assessed in the PF of a subgroup of patients with (n=36) and without (n=27) endometriosis all together for the first time with the multiplexing Luminex® x-MAP® technology described.

The mean age of the women with endometriosis in this subgroup was statistically lower than that of the women in the control group (p=0.05), and the use of hormones and the cycle phase did not show any statistically significant association with that group of women (p>0.05). Table 7 shows the main characteristics of patients included in this study's subgroup.

Table 7. Clinical features of patients included in subgroup #2

Variable	**Group**		**Total (N = 63)**	**p**
	Control (N = 27)	Endometriosis (N = 36)		
Age (years)				**0.005**
Mean (SD)	37.4 (9)	31.3 (7.4)	33.9 (8.6)	
Median (min.; max.)	37 (20; 50)	29 (18; 47)	33 (18; 50)	
Hormone, n (%)				0.372*
No	18 (66.7)	20 (55.6)	38 (60.3)	
Yes	9 (33.3)	16 (44.4)	25 (39.7)	
Cycle Phase, n (%)				0.184**
No cycle	1 (3.7)	7 (19.4)	8 (12.7)	
Follicular	8 (29.6)	6 (16.7)	14 (22.2)	
Luteal	3 (11.1)	5 (13.9)	8 (12.7)	
Unknown	15 (55.6)	18 (50)	33 (52.4)	
ASRM stage, n (%)				
I		8 (22.2)	8 (22.2)	
II		13 (36.1)	13 (36.1)	
III		7 (19.4)	7 (19.4)	
IV		8 (22.2)	8 (22.2)	
Diagnosis/Localisation, n (%)				
Peritoneal		7 (19.4)	7 (19.4)	
Retrocervical		13 (36.1)	13 (36.1)	
Rectovaginal		14 (38.9)	14 (38.9)	
Endometrioma		2 (5.6)	2 (5.6)	

Student's t-test; * Chi-square test; ** Likelihood ratio test

Among the six markers assessed, the concentration levels of IL-8 (p<0.001), MCP-1 (p=0.014) and MIP-3beta (p=0.022) were statistically higher in the PF of women with endometriosis compared with controls, as represented in Table 8. The use of hormone medications did not statistically influence the PF level of any protein evaluated in this study (p>0.05), as shown in Supplementary Table 6; in the same way, no proteins evaluated showed any statistically significant difference between the

phases of the menstrual cycle among all women enrolled in this analysis (p>0.05), as presented in Supplementary Table 7.

Table 8. Description of the six chemokines according to group and the results of the comparative tests; the unit of measurement is pg/ml.

Variable	Group	Mean	SD	Median	P25	P75	Min.	Max.	N	p
CXCL-8	Control	16.91	14.80	10.72	6.84	21.86	3.11	57.00	27	**<0,001**
	Endometriosis	66.72	107.19	22.64	14.83	76.55	4.95	500.00	36	
CCL-2	Control	255.22	194.69	192.65	91.03	396.39	17.61	748.98	27	**0.014**
	Endometriosis	789.77	1342.75	366.39	179.55	741.25	47.83	7657.04	36	
CCL-5	Control	314.01	938.46	31.84	20.02	84.63	9.87	4787.33	27	0.107
	Endometriosis	790.31	2512.29	73.58	23.17	374.35	7.05	14101.64	36	
CCL-21	Control	485501	756865	174036	64026	442512	17170	2500000	27	0.123
	Endometriosis	252993	488830	93131	60199	175808	37262	2500000	36	
CXCL-12	Control	11339	4797	11942	7942	14588	2121	19461	27	0.445
	Endometriosis	12552	5741	12487	8917	16191	1613	23968	36	
CCL-19	Control	240.48	237.00	194.42	96.79	285.84	0.00	1149.97	27	**0.022**
	Endometriosis	335.93	241.84	280.74	158.36	496.17	0.00	1233.78	36	

Mann-Whitney's test

Analysing the comparisons between the stages of the disease we found that two of the studied chemokines, i.e. RANTES and 6-Ckine, were statistically higher in women with endometriosis in advanced stages (III-IV) compared with women in the initial stages (I-II) of the disease, as shown in Supplementary Table 8 (p=0.046 and p=0.010, respectively), although these two chemokines did not show any statistically significant difference between patients with and without endometriosis in our analysis.

When we compared the different sites of endometriosis presentation, only IL-8 showed PF levels with a statistically significant difference between the three localisations (p=0.033). Using Dunn's multiple comparisons test we found that the PF concentrations of IL-8 were statistically higher in women with rectovaginal DIE compared with women with retrocervical DIE (p=0.012), as presented in Supplementary Tables 9 and 10.

The ROC curves for the three chemokines that showed differences between the groups represented in Figure 12 demonstrated that IL-8 was the chemokine that could better discriminate women with endometriosis, once its area under the curve (AUC) is larger than the other two evaluated curves obtained for MCP-1 and MIP-3beta and thus it assumes larger values of sensitivity and specificity. However, the confidence intervals of the diagnostic measures between the three chemokines overlap, meaning that in our present analysis these three chemokines did not show statistically significant differences to detect endometriosis. Table 9 shows the established cut-off points and the diagnostic measures.

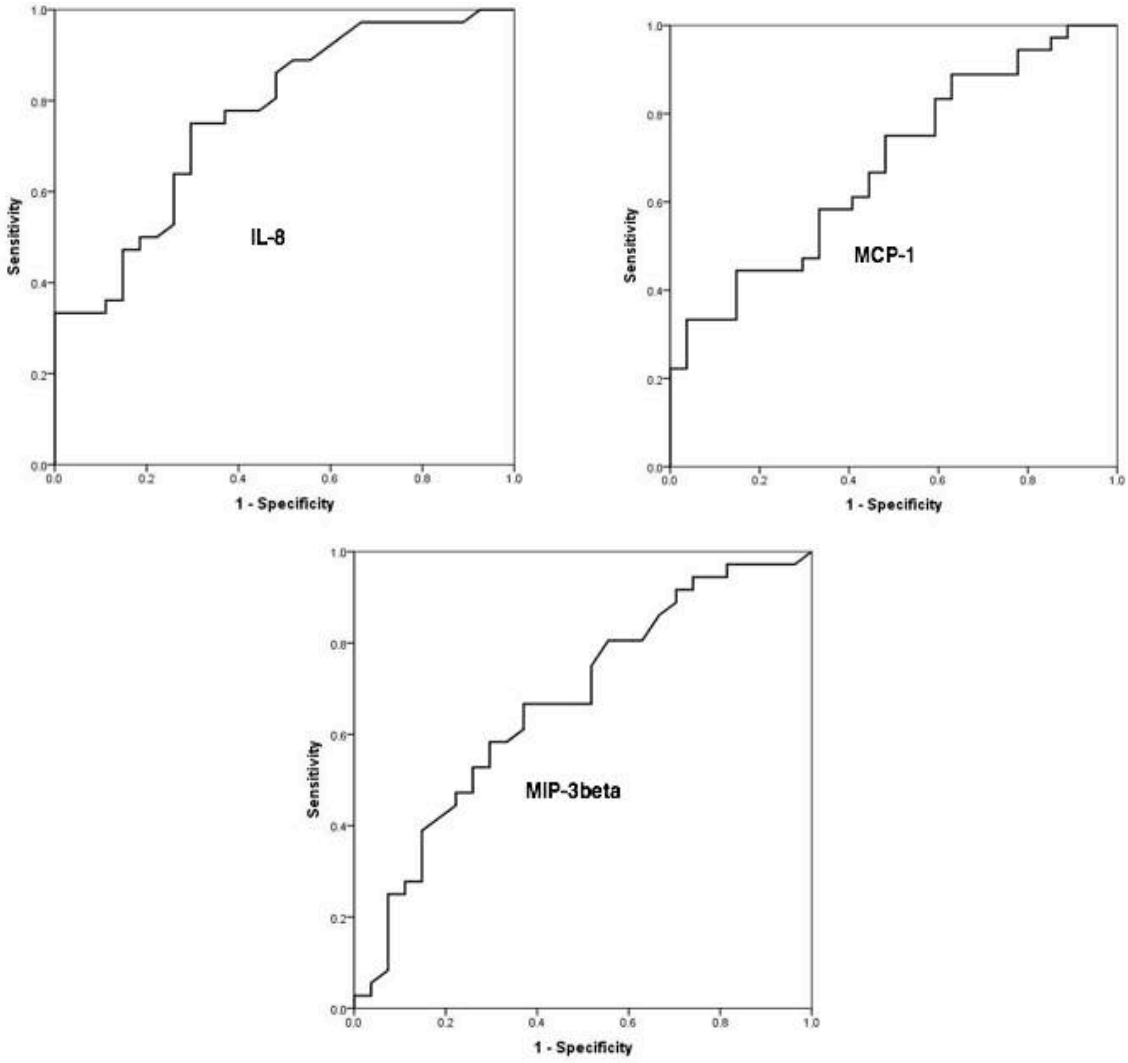

Figure 12. ROC curves of IL-8 (CXCL-8), MCP-1 (CCL-2) and MIP-3β (CCL-19) for discrimination of patients with endometriosis

Table 9. Established cut-off points and diagnostic measures

Variable	Cut-off	AUC (95% CI)	Sensitivity (95% CI)	Specificity (95% CI)
CXCL-8	16.22	0.767 (0.650 - 0.884)	75.0 (57.8 - 87.9)	70.4 (49.8 - 86.2)
CCL-2	295.46	0.682 (0.551 - 0.813)	58.3 (40.8 - 74.5)	59.3 (38.8 - 77.6)
CCL-19	214.49	0.670 (0.534 - 0.806)	66.7 (49.0 - 81.4)	63.0 (42.4 - 80.6)

Evaluation of each individual cytokine for the power of prediction of endometriosis is demonstrated in Table 10. Here we show that MCP-1 together with the other two chemokines did not statistically influence the prediction of endometriosis (p=0.263), whereas patients with IL-8 values above the established cut-off point have a chance of endometriosis 5.38 times that of women with values under the cut-off; similarly, women with MIP-3beta values above the established cut-

off point present a chance of endometriosis 3.73 times that of women with values under the cut-off.

Table 10. Results of the prediction model of probability of endometriosis with the established cut-offs for the chemokines IL-8 (CXCL-8), MCP-1 (CCL-2) and MIP-3β (CCL-19)

Variable	Coefficient	Std. Error	Wald Statistics	df	OR	95% CI for OR		p
						Lower	Upper	
CXCL-8	1.68	0.61	7.71	1	5.38	1.64	17.64	**0.005**
CCL-2	0.74	0.66	1.26	1	2.10	0.57	7.66	0.263
CCL-19	1.32	0.65	4.15	1	3.73	1.05	13.23	**0.042**
Constant	-1.63	0.64	6.59	1	0.20			**0.010**

Finally, we evaluated the power of prediction for all combination of the three investigated marker cytokines (Table 11). Women having in their PF concentrations of the three chemokines below the respective cut-off points have an estimated probability of endometriosis of only 16.3%. Importantly, when the three chemokines are present in concentrations above the established cut-off points, the likelihood of endometriosis is 89.1%.

Table 11. Probability of endometriosis according to the positivity of the chemokines

CXCL-8	CCL-2	CCL-19	Probability of endometriosis (%)
			16.3
X			51.2
	X		29.0
		X	42.1
X	**X**		68.8
X		**X**	79.7
	X	**X**	60.4
X	**X**	**X**	89.1

3.3 Potential of malignant transformation of rectovaginal deep-infiltrating endometriosis (DIE)

In this third subgroup of assessed patients the women's age in the endometriosis group was statistically lower than that of the women in the control group ($p<0.001$); however, all patients were at the premenopausal stage, except for one patient with ovarian endometriosis. The other evaluated characteristics did not present associations with the group of women ($p>0.05$), as shown in Table 12.

The immunolocalisation of *ARID1A* (BAF250a protein) was interpreted in both epithelial and stromal cells of DIE lesions, endometriotic lesions compromising the

PSLN, ovarian endometriosis or endometriomas and the eutopic endometrium from control subjects. Endometriotic lesions affecting the PSLN were identified in 7/30 (23.3%) cases of rectovaginal/bowel DIE patients.

Table 12. Clinical features of patients included in subgroup #3

Variable	Group			Total (N = 70)	p
	Control (N = 20)	Rectovaginal DIE (N = 30)	Ovarian endometriosis (N = 20)		
Hormone, n (%)					0.957
No	14 (70)	20 (66.7)	14 (70)	48 (68.6)	
Yes	6 (30)	10 (33.3)	6 (30)	22 (31.4)	
Age (years)					**<0.001****
Mean (SD)	42.5 (4.0)	32.4 (7.0)	35.5 (9.1)	36.3 (8.4)	
Median (min.; max.)	42.5 (34; 50)	31 (21; 46)	34.5 (24; 65)	36.5 (21; 65)	
Cycle Phase, n (%)					0.140#
No cycle	0 (0)	3 (10)	3 (15)	6 (8.6)	
Folicular	3 (15)	8 (26.7)	6 (30)	17 (24.3)	
Luteal	10 (50)	6 (20)	5 (25)	21 (30)	
Unknown	7 (35)	13 (43.3)	6 (30)	26 (37.1)	
ASRM stage, n (%)					0.279*
I or II		8 (26.7)	2 (10)	10 (20)	
III or IV		22 (73.3)	18 (90)	40 (80)	
Clonal loss of BAF250a, n (%)					0.676
No	15 (75)	19 (63.3)	14 (70)	48 (68.6)	
Yes	5 (25)	11 (36.7)	6 (30)	22 (31.4)	

Chi-square test; * Fisher's exact test; # Likelihood ratio test; ** Oneway ANOVA

Control group older than rectovaginal DIE and Ovarian endometriosis patients ($p < 0.001$ and $p = 0.005$ respectively)

The immunohistochemical staining of BAF250a in the epithelium and in the stroma was not always assessable for all samples because o the lack of adequate tissue in the slides after IHC was performed and therefore the IRS was only considered for those with both expressions assessed. The epithelial and stromal expression was assessable in 25/30 (83.3%) of DIE cases, 5/7 (71.4%) cases of endometriotic lesions affecting the PSLN, all cases (20/20) of ovarian endometriosis and the EE from all controls (20/20). Nuclear staining should be clearly present if the expression of BAF250a is to seen as positive, as represented in Figure 13.

Complete loss of BAF250a expression was not present in any of the 77 benign tissue samples from the 70 subjects included as well as the additional three samples from the two cases of EESS evaluated. Nevertheless, we did identify a partial loss also referred to as ‘clonal loss’ of BAF250a among all samples assessed. This phenomenon was present in 36% (9/25) of DIE, 40% (2/5) of endometriosis lesions compromising the PSLN, 30% (6/20) of ovarian endometriosis, and also in 25% (5/20) of eutopic endometrium from controls, as shown in Figure 14. Additionally, we also identified it in one of the two cases of EESS affecting the bowel/rectum represented in Figure 15.

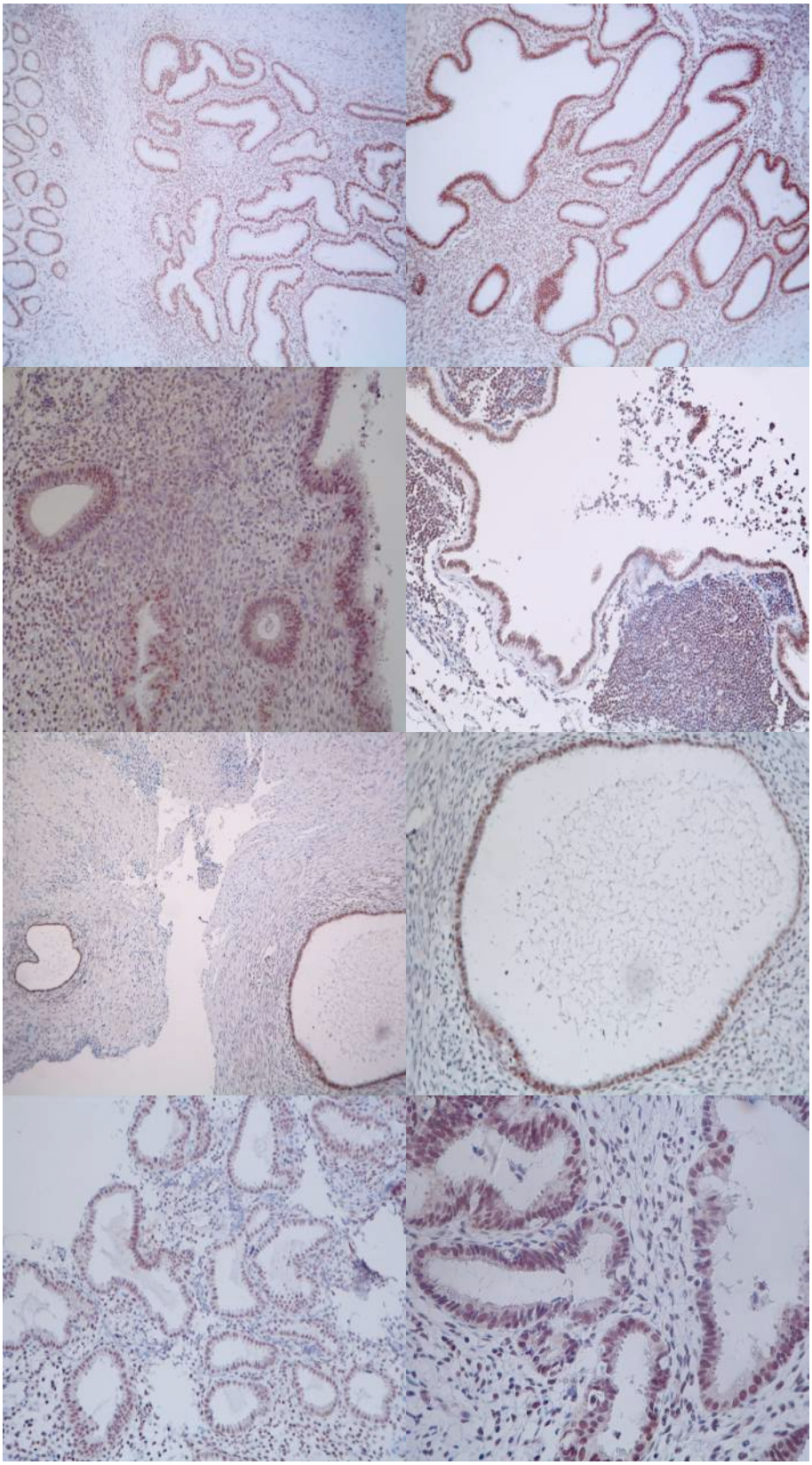

Figure 13. Positive BAF250a expression in endometriosis and normal endometrium represented by nuclear staining in (A1/A2) rectovaginal DIE (x100/x200); (B1/B2) endometriosis lesions in PSLN (x200); (C1/C2) ovarian endometriosis (x100/x200); (D1/D2) normal endometrium (x200/x400)

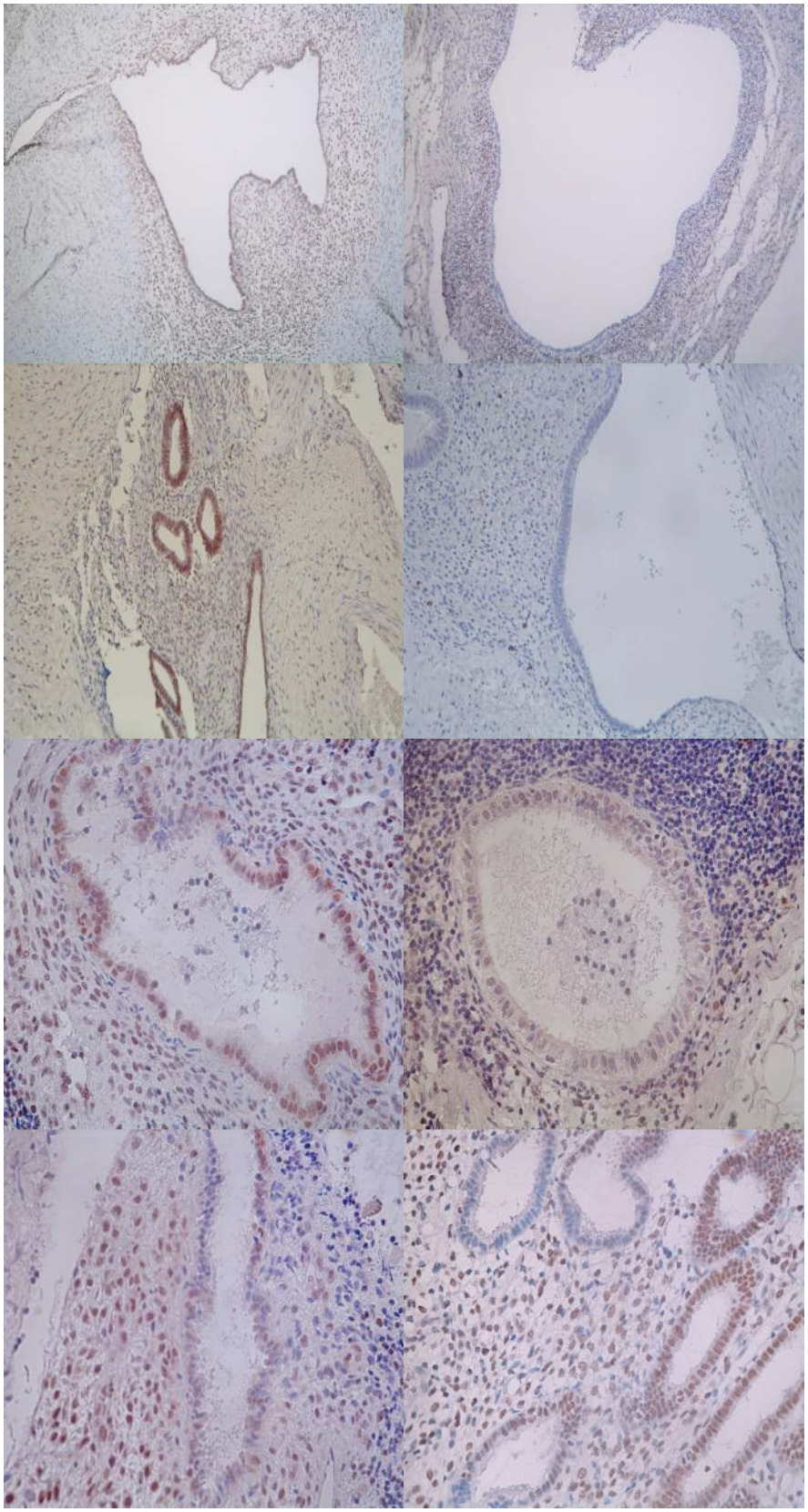

Figure 14. Clonal loss of BAF250a represented in the same sample for each endometriosis presentation or normal endometrium showing patchy staining; (A1/A2) ovarian endometriosis (x100); (B1/B2) rectovaginal DIE (x100); (C1/C2/D1) endometriosis foci in PSLN (x400); (D2) normal endometrium (x400)

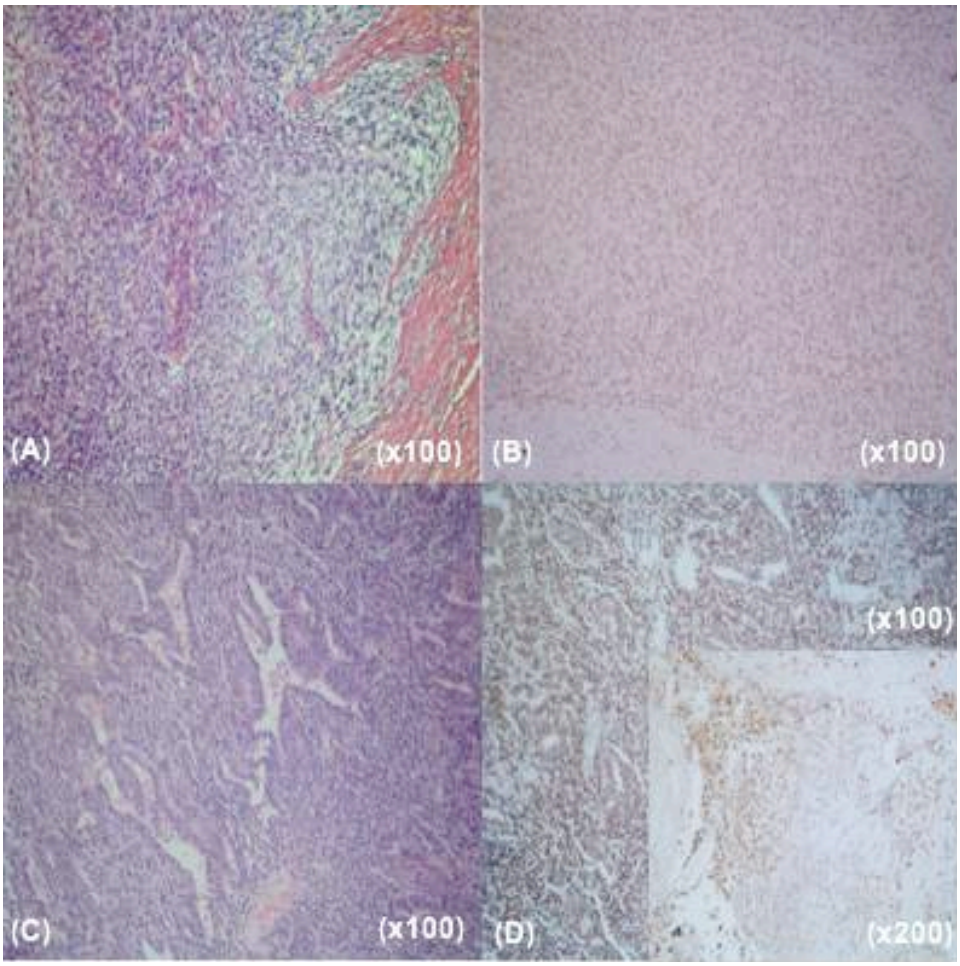

Figure 15. Primary extragenital endometrial stromal sarcoma (EESS) of the bowel. (A) and (C) representing hemaetoxilin-eosin staining and corresponding BAF250a positive staining (B) and clonal loss of BAF250a expression (D).

Interestingly, in our analysis, the phenomenon of clonal loss of BAF250a seemed more frequent among women who were not using hormone medications. However, although less than half of women taking hormone medications presented clonal loss of BAF250a expression compared with women not receiving hormones, a statistically significant association between the use of hormones and the occurrence of clonal loss of BAF250a was not possible (p=0.106), as presented in Table 13.

Table 13. Description of clonal loss of BAF250a occurrence according to the use of hormones and results of association test

Clonal loss of BAF250a	**Hormone**		**Total (N = 70)** n (%)	**p**
	No (N = 48) n (%)	Yes (N = 22) n (%)		
No	30 (62.5)	18 (81.8)	48 (68.6)	0.106
Yes	18 (37.5)	4 (18.2)	22 (31.4)	
Total	**48 (100)**	**22 (100)**	**70 (100)**	

Chi-square test

The IRS for BAF250a was calculated for all samples including endometriosis groups and controls without the disease and there was no statistically significant difference (p=0.885) between the scores from all endometriosis presentations and the EE from controls, as represented in Figure 16.

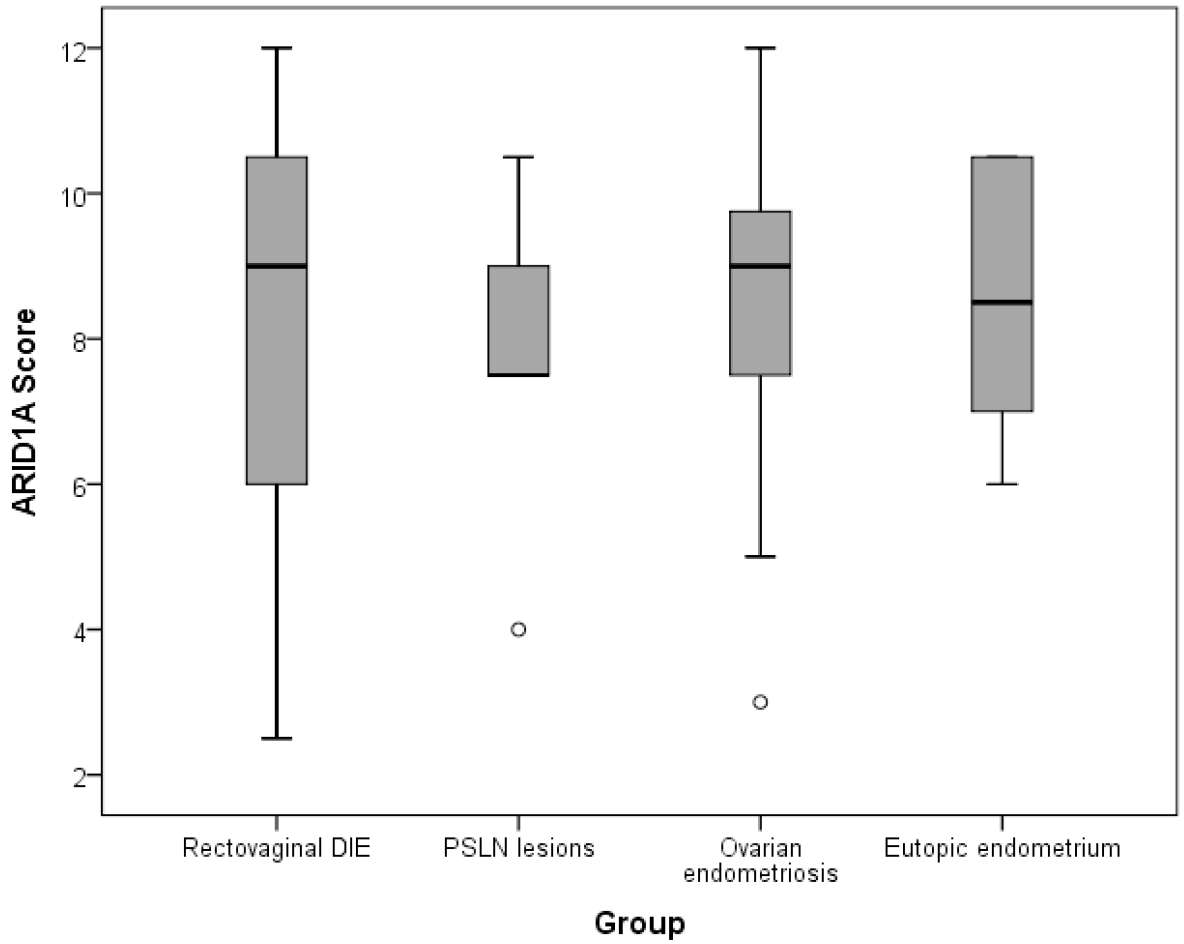

Figure 16. ARID1A immunoreactivity score (IRS) for all groups and results of comparative tests showing no statistically significant difference (p=0.885) between the groups (calculated with Kruskal-Wallis test)

4 DISCUSSION

Endometriosis may be related to cancer in three different ways: 1) occurring with any kind of cancer simultaneously; 2) mimicking cancer; and 3) evolving into cancer. This study focused on two aspects of this association, which were endometriosis mimicking and evolving into cancer. Endometriosis may mimic cancer at least in two ways: in the clinical presentation, presenting itself in similar localisations and causing anatomic distortion; and in the mechanism of the disease's spread, showing a rate between 11% to 21% of regional lymph node involvement within endometriosis, disclosing the capacity of the lymphatic spread of the disease [42,77].

In the present analysis, cancer metastasis-related chemokines were evaluated for the first time in rectovaginal DIE and in the endometriotic lesions affecting the PSLN, in order to better understand this complex process of lymphatic spread of cells with regard to endometriosis. The findings showed that the two chemokine receptors involved with the process of metastasis in cancer, CCR7 and CXCR4, presented high expression in the rectovaginal DIE lesions as well as in the endometriotic lesions compromising the PSLN; however, there were no statistically significant differences ($p>0.05$) between their expressions in the EE from patients without endometriosis. Furthermore, we had the impression that the expression of these receptors in the EE from controls was more expressive in the glands than in the stroma, although we did not find statistically significant difference in the final IRS, which included the evaluation of the stroma. These results seem to be in contrast with previous findings regarding the profile of these two receptors in breast cancer. Studies have shown that both CCR7 and CXCR4 appear to be statistically significant higher expressed in breast cancer cells than in adjacent normal breast tissue [61,78]. However, this was identified in adjacent normal stroma and not among normal mammary glands [78]. Hence, the staining pattern of those receptors in healthy tissue still needs to be clarified.

The receptor CXCR4 has already been identified in endometriosis and in the EE from controls by IHC previously [66]. The findings revealed a significantly higher expression of this receptor among endometriosis lesions than EE from controls, although the authors used a smaller sample size (11 patients and 8 controls) and their score system for IHC included the intensity only (0-3). Furthermore, they did not specify what subtype(s) of endometriosis they assessed. Previously, Furuya et al

(2007) also identified the expression of CXCR4 in ovarian endometriosis and endometriosis-associated ovarian cancer (EAOC) [67].

To the best of our knowledge, no one has assessed the receptor CCR7 by IHC in endometriosis yet. Interestingly, in our analysis, the CCR7 immunolocalisation in the majority of cases was in the cell membrane but in some cases nuclear staining was also present; coincidentally or not, patients who had positive PSLN (compromised with endometriotic lesion) presented the nuclear expression of CCR7 in the respective DIE, although some patients with this same expression's pattern did not present lymph node involvement. On the other hand, the expression of CCR7 in the EE from controls was always in the cell membrane. The nuclear staining of CCR7 in the DIE lesions might be related to the chance of lymph node involvement with the disease.

The ligands CXCL12, CCL19 and CCL21 were also expressed in the rectovaginal DIE lesions, in the endometriotic lesions compromising the PSLN and in the EE from controls in our analysis. CXCL12 and CCL21 presented higher IRS in DIE than in the EE from controls whereas CCL19 showed lower IRS in DIE than in the EE; however, no results showed any statistically significant difference ($p>0.05$). CXCL12 was assessed by IHC once before in ovarian endometriosis and endometriosis-associated ovarian cancer (EAOC), but its expression's pattern was not well described in those lesions. The staining pattern of the other two ligands – CCL19 and CCL21 – has not been described in endometriosis yet.

Thus, our study presents for the first time the immunohistochemical expression pattern of the most important cancer-related chemokine receptors – CXCR4 and CCR7 – and ligands – CXCL12, CCL19 and CCL21 – in rectovaginal DIE lesions as well as in the endometriotic lesions compromising the PSLN, in comparison to their expression pattern in the EE from control patients without the disease. It would be interesting to compare the expression pattern of those chemokines in the eutopic endometrium from patients with endometriosis as well, and preferably from the same patients with rectovaginal DIE who were assessed. Unfortunately, this was a retrospective study and we did not have samples from our cohort of patients to perform this analysis but this should be considered in further prospective studies in the future.

We also tried to find a correlation between the expressions of the chemokine receptors in the DIE lesions with the expressions of their specific ligands in the PSLN

cells. Even though no statistical correlation was identified within the scattergrams, looking at the IRS for the three analysed axes (CXCR4-CXCL12, CCR7-CCL19 and CCR7-CCL21), we saw that all ligands showed a high expression (IRS) in the lymphatic cells of the PSLN assessed, as expected for these proteins. Furthermore, as recently found by Leconte et al (2014) for the CXCR4-CXCL12 axis in rectovaginal DIE, the endometriotic stromal cells were attracted by CXCL12 [79] and perhaps we should focus on them in future analysis to understand better this complex process of cell migration within endometriosis. Hence, our findings should encourage future research using different methods on these three chemokine axes in endometriosis, such as primary cell culture and transwell migration assay, not only considering the peritoneal implantation of endometriotic stromal cells as they did but also taking into count the endometriotic lesions affecting the PSLN.

The lymphatic dissemination of endometriotic cells as well as the lymph node involvement in endometriosis may not have the same relevance as the lymphatic dissemination of tumour cells and lymph node involvement in malignancies, at least yet, as endometriosis presents itself as a benign disease. However, this knowledge could bring new insights not only into the pathogenesis and the better understanding of this enigmatic disease, but might also contribute to new treatment strategies as well as new therapies in the near future.

The search for a marker capable to differentiate patients with and without endometriosis is challenging [80]. The difficulty is not only finding the most relevant candidates but also validating them as a reliable diagnostic test for use in clinical practice. Chemokines comprise a class of many proteins and some of them have shown satisfactory results as putative markers for endometriosis [64,65]. However, different studies on chemokines and endometriosis have shown controversial results [65]. In our analysis we included α-chemokines and β-chemokines and for the first time, using a multiplexing assay technology, we assessed together the three chemokines systematically selected as the main candidate markers among all chemokines for endometriosis. We also investigated three cancer-related chemokines of our interest.

We found statistically significant differences between the PF levels of IL-8 (CXCL8), MCP-1 (CCL2) and MIP-3β (CCL19) in patients with endometriosis compared with controls without the disease, as already presented. The results of IL-8 and MCP-1 are in agreement with those reported in our systematic review [65],

where most of the included studies found statistically significant higher levels of IL-8 (94% of studies) and MCP-1 (54.5% of studies) in the PF of patients with endometriosis compared with controls. MIP-3β was assessed in the PF in only one previous publication [81] and their results are in accordance with our present findings, revealing statistically higher levels of this chemokine in the PF of patients with endometriosis compared with controls. Regarding our results for RANTES (CCL5), which showed no statistically significant difference in the PF levels between the group with the disease and controls, we are also in agreement with the majority of previous publications as 57.2% of the authors included in the systematic review [65] found no statistically significant difference for this chemokine in the PF. The PF concentration of SDF-1α and β (CXCL12) did not show statistically significant difference between endometriosis patients and controls in our findings, in contrast to the one previous publication which found higher concentrations of this chemokine in the PF of DIE patients compared with controls [79]; however, the authors used only the SDF-1α antibody whereas our antibody included the α and β fractions from this heterodimer chemokine, which could explain this controversial result. Finally, the PF concentration of 6Ckine (CCL21) is reported for the first time as far as we are aware and did not show any statistically significant difference between endometriosis patients and controls in the present analysis.

Interestingly, all the six chemokines in this analysis, including the cancer-related ones, did not reveal any statistically significant difference in their concentration levels between the patients with PE and DIE. As regards the biology of the disease, these findings lead us to believe that the chemokine concentrations in the PF do not depend on the mass or the size of the lesion, as PE lesions have much less mass than DIE lesions.

As regards the three chemokines that did disclose PF levels statistically different between the groups, IL-8 alone had a sensitivity of 75% and specificity of 70.4% in the diagnosis of endometriosis, and MCP-1 alone showed 58.3% and 59.3% respectively and MIP-3β presented sensitivity of 66.7% and specificity of 63%. Most interestingly, when all three were considered together as a panel of markers and were above the cut-off points, the multiple logistic regression analysis revealed a probability of endometriosis of 89.1%.

An ideal test for endometriosis should be non-invasive and therefore the PF is not the best sample to be tested and used as a diagnostic tool. However, the

research of biomarkers in endometriosis usually includes the PF evaluation, once the disease occurs mainly in the pelvic/abdominal cavity, and the PF could express specific substances (proteins) released by the endometriotic lesions. Unfortunately, we did not have serum samples from the same cohort of patients, which we could include in the present analysis. Hence, our findings should encourage future studies to investigate this possible and putative panel of markers in the serum of patients with and without endometriosis. Agic et al (2008) performed a similar study using the chemokine receptor CCR1 mRNA, MCP-1, and CA125 measurements in peripheral blood and found that the association of those markers improved the diagnostic accuracy of the test compared with each of them alone, including CA125, the unique marker used in the current clinical practice for this purpose [82]. Vodolazkaia et al (2012) also investigated several markers, and in their analysis two models with four markers each – anexin V, VEGF, CA125 and glycodelin or anexin V, VEGF, CA125 and sICAM-I – were selected and highlighted as potential blood markers for endometriosis [83].

Moreover, as endometriosis has a complex pathophysiology that leads to chronic inflammation, ectopic growth and invasion of distant organs, mimicking malignancies, the biochemical diagnosis becomes very challenging once the same proteins could also be altered in other diseases, as happens with the antigen CA125. Accordingly, a mathematical model, as recently suggested by Galazis et al (2014) and supported by our group, which gathers biochemical markers, clinical parameters and radiological findings could improve the accuracy of a non-invasive diagnostic tool for endometriosis [84,85].

The risk of malignant transformation in endometriosis has been suggested in the literature lately, in most cases in relation to ovarian cysts of endometriosis (endometriomas), as already stated. We focused this investigation on rectovaginal DIE presentation and endometriotic lesions compromising the PSLN, since no one else has done it yet. As previously presented, in this analysis we did not find the complete loss of BAF250a, as described by others, among any endometriosis cases. However, we did identify the partial or clonal loss of BAF250a among all lesions evaluated, including rectovaginal DIE and endometriotic lesions compromising the PSLN. Although Samartzis et al. (2012) have already described the presence of this phenomenon among endometriosis lesions [71], we described for the first time the occurrence rate of clonal loss of BAF250a expression among these important

endometriosis presentations – rectovaginal DIE and endometriotic lesions compromising the PSLN – as well as in ovarian endometriosis and in eutopic endometrium from controls. Unfortunately, we did not have enough tissue samples from this cohort of patients to provide a sufficient amount of DNA to screen *ARID1A* mutations. As previously described and validated [70,72,86], immunohistochemical staining showing the complete and the clonal loss of BAF250a expression can be a surrogate marker for *ARID1A* mutations for EAOC and uterine endometrioid carcinomas. Thus, it would be timely to investigate in future studies whether this is also true for endometriosis.

The occurrence of this phenomenon in the 'normal' eutopic endometrium from controls may not be expected, however it was already reported among low-grade and high-grade uterine endometrioid carcinomas and complex atypical endometrial hyperplasia [72,87]. Hence, we might hypothesise that our findings could be related to patients with higher risk for developing endometrial hyperplasia. The mean age of this group was 42.5 years, supporting this hypothesis, but as most of the samples came from patients who were subjected to hysterectomy because of myomatosis or uncontrolled uterine bleeding, the follow-up of these cases is not possible.

Interestingly, among uterine endometrioid carcinomas *ARID1A* mutations often co-occurred with mutations of the tumour suppressor gene *PTEN* [88]. As recently published in an animal model, steroid hormones intervene in the endometrial tumorigenesis of *PTEN* ablation [89]. Our results showed more cases of clonal loss of BAF250a among women not receiving hormone medications than women undergoing hormone treatment, although this association was not statistically significant, as already presented. However, it would be important to investigate in future studies the role and possible influence of steroid hormones on *ARID1A* mutated cases.

The present study reports the occurrence rate of clonal loss of BAF250a among benign endometriosis (not related to EAOC or other malignancies) including for the first time a specific group with rectovaginal/bowel DIE and endometriosis foci affecting the PSLN, which were the main focus of this investigation, as the previous study by Samartzis et al. (2012) evaluated DIE lesions but they did not specify the site of those lesions [71], besides ovarian endometriosis and EE from controls without the disease. Our findings show that the frequency of clonal loss of BAF250a expression found among rectovaginal/bowel DIE and endometriosis compromising

the PSLN was similar to the one found in ovarian endometriosis. As the majority of publications regarding this phenomenon are related to ovarian presentation of endometriosis and the higher risk for EAOC, we highlight our findings with regard to rectovaginal/bowel DIE as well as endometriotic lesions affecting the PSLN.

Moreover, considering the previous finding that primary endometrioid carcinomas and extragenital endometrial stromal sarcomas affecting the bowel are related to benign endometriosis, we also intended to investigate the expression of BAF250a among those lesions. As primary presentations of these two neoplasms are very rare we could assess only two cases of EESS. One patient was 45 years old at the time of diagnosis and surgery. Hysterectomy was also performed and endometrium was negative, confirming the primary presentation of EESS in the bowel. Interestingly, this patient was confirmed to have adenomyosis after pathological examination of the uterus. However, the immunohistochemical staining showed retention of BAF250a expression, as already presented. The second patient was 59 years old by the time of diagnosis and had no history of endometriosis. Hysterectomy also revealed a normal endometrium. Unlike the other case, here the immunohistochemical staining disclosed the clonal loss of BAF250a, as previously shown.

Hence, we believe that more efforts should be made to investigate these rare cases of EESS and endometrioid carcinoma affecting the bowel to clarify the possible link between them and rectovaginal/bowel DIE. Ideally, multicentre studies would improve the quality of the findings as a greater number of cases could be enrolled. Finally, patients should not be alarmed at the risk of malignant transformation of rectovaginal/bowel DIE at this point as the value of our findings as a predictor of malignant transformation in endometriosis still needs to be clarified.

Taking into account the objectives proposed we might conclude:

1. The cancer metastasis-related chemokine receptors as well as their ligands are highly expressed in rectovaginal DIE and endometriotic lesions in PSLN.

1.1. There was no correlation between the IRS of chemokine receptors in rectovaginal DIE and the respective chemokine ligand in the corresponding PSLN.

1.2. The use of hormones, the phase of the menstrual cycle and the stage of disease did not influence the IRS of cancer-related chemokines overall, except for CCL19 whose IRS was higher in the group undergoing

hormones and in the luteal phase of the cycle, and lower in the advanced stages of disease. The receptor CXCR4 IRS was directly related to the size of the lesion, and the other chemokines' IRS did not show any association with the size of the lesion.

2. IL-8 ($p<0.001$), MCP-1 ($p=0.014$) and MIP-3β ($p=0.022$) had statistically significant higher concentrations in PF of women with endometriosis compared with controls. When IL-8 (CXCL8) is increased in the PF and when it is assessed in combination with MCP-1 (CCL2) and MIP-3β (CCL19) as a panel of markers, the likelihood of identifying women with endometriosis is enhanced.

3. Complete loss of BAF250a was never found in the present analysis. However, all forms of endometriosis assessed including rectovaginal/bowel DIE and endometriotic lesions compromising the PSLN presented clonal loss of BAF250a protein expression. This phenomenon was also present in one case of EESS of the bowel.

5 SUPPLEMENTARY DATA

S. Figure 1. Approval from Ethic Committee

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Datum: 17.08.11

Untersuchungen zum Vorkommen von Endometriose-assoziierten Lymphgefäßen und Persistenz von Endometriosezellen in Lymphknoten
Hier: Antrag an die DFG: „Invasionsfaktoren und tief infiltrierende Endometriose"
Antragsnummer: EA4/038/07
Vorgang vom 14.07.11, Eingang am 05.08.11

Sehr geehrte Frau Dr. Mechsner,

hiermit bestätigen wir Ihnen den Eingang Ihres Schreibens vom 14.07.11 mit der Bitte um Erteilung eines Ethik-Votums.

Die Ethikkommission bestätigt, dass die im Rahmen Ihres Antrages an die DFG geplanten Untersuchungen durch das vorliegende Ethik-Votum EA4/038/07 abgedeckt sind.

Mit freundlichen Grüßen

Prof. Dr. Ralf Stahlmann
-Vorsitzender-

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S. Table 1. Revised ASRM classification of endometriosis, 1996.

THE AMERICAN FERTILITY SOCIETY
REVISED CLASSIFICATION OF ENDOMETRIOSIS

Patient's Name _____ Date _____

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

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

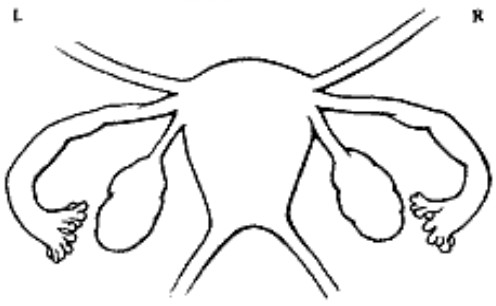
PERITONEUM	**ENDOMETRIOSIS**	<1cm	1-3cm	>3cm
	Superficial	1	2	4
	Deep	2	4	6
OVARY	R Superficial	1	2	4
	Deep	4	16	20
	L Superficial	1	2	4
	Deep	4	16	20
	POSTERIOR CULDESAC OBLITERATION	Partial	Complete	
		4	40	
OVARY	**ADHESIONS**	<1/3 Enclosure	1/3-2/3 Enclosure	>2/3 Enclosure
	R Filmy	1	2	4
	Dense	4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

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

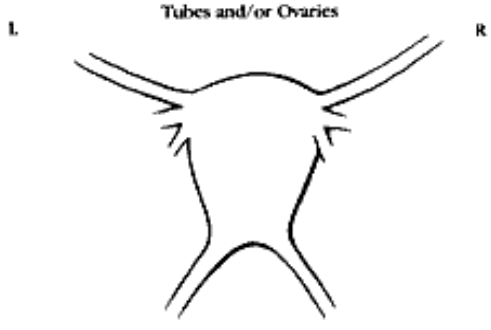
Additional Endometriosis: _____

Associated Pathology: _____

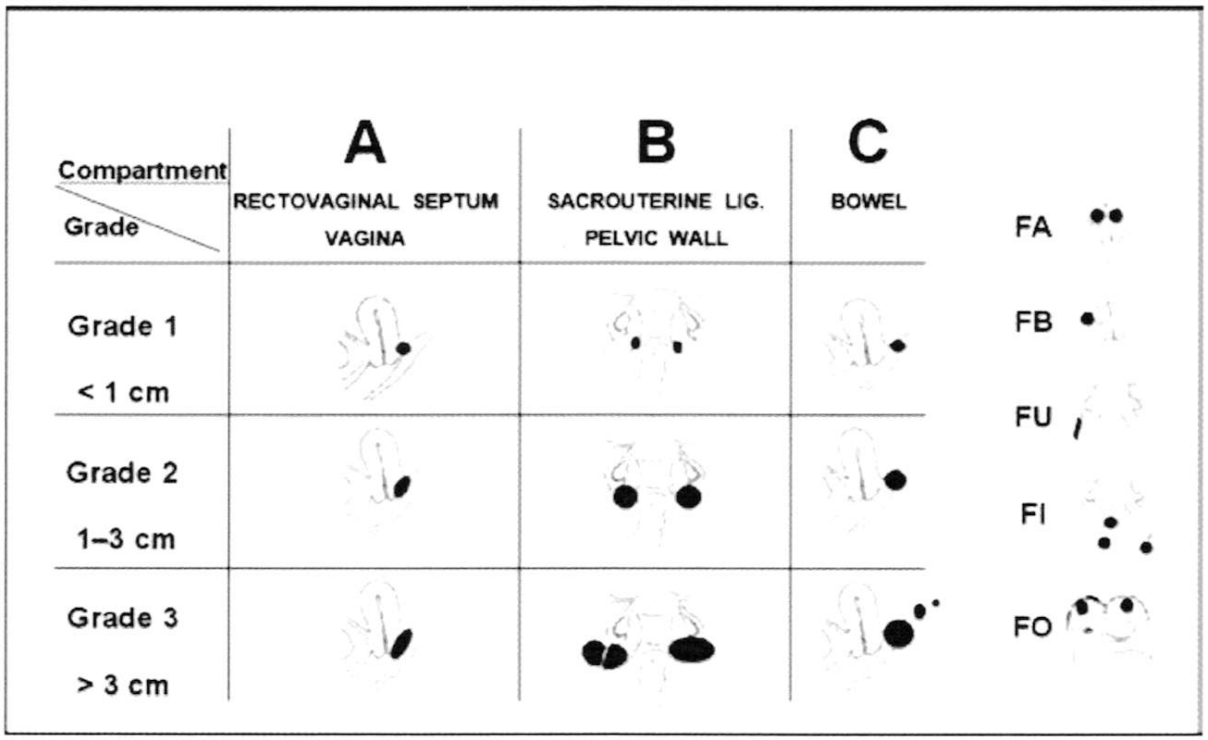
To Be Used with Normal Tubes and Ovaries



To Be Used with Abnormal Tubes and/or Ovaries



S. Table 2. Revised ENZIAN score, 2011



The revised Enzian classification [32]

S. Table 3. Chemokine immunoreactivity score according to the use of hormonal medications

Variable	Hormone	Mean	SD	Median	P25	P75	Min.	Max.	N	p
CXCL-12	No	8.20	2.40	8.0	7.3	10.0	1	12	30	0.705
	Yes	8.65	1.59	9.0	7.5	9.5	6	12	13	
CXCR-4	No	9.24	3.26	10.5	6.5	12.0	1	12	29	0.318
	Yes	10.62	1.75	12.0	9.0	12.0	7	12	13	
CCL-19	No	7.22	2.75	7.8	6.0	9.0	0	12	30	**0.045**
	Yes	9.08	2.74	10.5	7.0	11.3	3	12	13	
CCL-21	No	9.52	2.47	10.3	7.5	12.0	5	12	30	0.969
	Yes	9.81	1.81	9.0	9.0	12.0	7	12	13	
CCR-7	No	9.32	1.95	9.0	8.0	12.0	6	12	30	0.232
	Yes	8.50	2.10	8.0	7.0	9.8	5	12	13	

Mann-Whitney's test

S. Table 4. Chemokine immunoreactivity score according to the phase of the menstrual cycle

Variable	Cycle Phase	Mean	SD	Median	P25	P75	Min.	Max.	N	p
CXCL-12	Follicular	8.50	2.55	9.0	7.5	10.3	3	12	9	0.519
	Luteal	8.30	1.63	8.0	7.4	9.3	6	12	15	
CXCR-4	Follicular	9.61	3.32	10.5	7.5	12.0	3	12	9	0.829
	Luteal	10.11	2.61	11.3	8.8	12.0	5	12	14	
CCL-19	Follicular	5.61	3.34	6.0	3.0	8.3	0	10.5	9	**0.041**
	Luteal	8.47	1.98	8.5	6.9	10.5	6	12	15	
CCL-21	Follicular	8.94	2.57	9.0	6.8	11.3	5	12	9	0.446
	Luteal	9.67	2.45	10.5	8.3	12.0	5	12	15	
CCR-7	Follicular	9.56	2.07	9.0	8.5	12.0	6	12	9	0.640
	Luteal	9.27	2.01	9.0	7.8	12.0	6	12	15	

Mann-Whitney's test

S. Table 5. Chemokine immunoreactivity score according to the stage of the disease (ASRM)

Variable	ASRM stage	Mean	SD	Median	P25	P75	Min.	Max.	N	p
CXCL-12	I or II	8.42	3.88	9.5	6.3	10.9	1	12	6	0.708
	III or IV	8.85	2.07	9.0	8.0	10.3	3	12	17	
CXCR-4	I or II	10.67	2.16	12.0	8.5	12.0	7	12	6	0.516
	III or IV	9.59	3.23	12.0	6.5	12.0	3	12	17	
CCL-19	I or II	9.75	2.82	10.5	7.6	12.0	5	12	6	**0.036**
	III or IV	6.68	3.11	7.0	4.5	8.3	0	12	17	
CCL-21	I or II	10.50	1.64	10.5	9.8	12.0	8	12	6	0.431
	III or IV	10.15	1.42	10.0	9.0	12.0	8	12	17	
CCR-7	I or II	10.17	1.92	10.5	8.5	12.0	7	12	6	0.431
	III or IV	9.44	1.82	9.0	8.0	12.0	7	12	17	

Mann-Whitney's test

S. Table 6. Description of chemokines according to the use of hormone and the results of comparative tests

Variable	Hormone	Mean	SD	Median	P25	P75	Min.	Max.	N	p
CXCL-8	No	29.61	40.38	16.73	9.62	28.42	3.11	227.63	38	0.308
	Yes	69.33	122.82	21.34	10.23	81.28	4.02	500.00	25	
CCL-2	No	363.93	366.77	261.79	152.45	414.33	29.57	1823.60	38	0.407
	Yes	859.72	1578.70	361.56	119.69	745.63	17.61	7657.04	25	
CCL-5	No	345.19	820.65	54.95	21.48	366.80	8.53	4787.33	38	0.725
	Yes	952.49	3005.73	47.62	18.25	223.70	7.05	14101.64	25	
CCL-21	No	393502	670450	130524	58155	383167	17170	2500000	38	0.725
	Yes	290527	551510	125876	66321	228187	29241	2500000	25	
CXCL-12	No	11970	5601	12045	8659	16167	2121	23968	38	0.811
	Yes	12127	5055	12269	8377	15291	1613	20797	25	
CCL-19	No	308.96	278.05	255.07	106.73	411.04	0.00	1233.78	38	0.817
	Yes	273.83	178.98	211.34	153.43	316.32	0.00	810.03	25	

Mann-Whitney's test

S. Table 7. Description of chemokines according to the cycle phase and the results of comparative tests

Variable	Cycle Phase	Mean	SD	Median	P25	P75	Min.	Max.	N	p
CXCL-8	Follicular	30.55	31.10	15.47	10.35	47.94	6.84	111.90	14	0.525
	Luteal	34.46	26.04	24.56	11.49	56.31	9.74	79.12	8	
CCL-2	Follicular	321.54	271.06	241.27	140.34	436.89	47.83	1081.68	14	0.973
	Luteal	402.56	436.25	282.00	106.90	471.10	82.16	1412.97	8	
CCL-5	Follicular	208.79	351.34	58.56	20.76	232.28	11.70	1292.70	14	0.330
	Luteal	343.70	404.04	144.99	31.48	649.49	11.70	1115.87	8	
CCL-21	Follicular	544333	864224	121803	62237	618920	27804	2500000	14	0.482
	Luteal	221897	367832	101769	39921	183629	29241	1121000	8	
CXCL-12	Follicular	12815	5433	12993	9173	17030	2690	23024	14	0.868
	Luteal	12112	5218	12474	8426	16023	2883	19461	8	
CCL-19	Follicular	313.34	304.02	237.39	111.94	366.10	96.79	1233.78	14	0.525
	Luteal	324.96	204.20	301.11	166.96	488.39	77.52	696.17	8	

Mann-Whitney's test

S. Table 8. Description of chemokines according to the stage of the disease in women with endometriosis and the results of comparative tests

Variable	ASRM stage	Mean	SD	Median	P25	P75	Min.	Max.	N	p
CXCL-8	I or II	38.11	33.58	21.34	13.45	67.42	4.95	111.90	21	0.374
	III or IV	106.77	155.46	32.39	16.73	108.93	9.25	500.00	15	
CCL-2	I or II	532.95	596.69	361.56	195.11	685.74	47.83	2580.31	21	0.409
	III or IV	1149.33	1939.43	422.11	163.91	1081.68	82.16	7657.04	15	
CCL-5	I or II	769.29	3057.29	70.04	18.47	184.23	7.05	14101.64	21	**0.046**
	III or IV	819.74	1557.12	263.03	47.62	1115.87	8.53	6179.70	15	
CCL-21	I or II	273258	606055	71283	51895	127066	37262	2500000	21	**0.010**
	III or IV	224621	266816	134635	92777	202701	58583	1121000	15	
CXCL-12	I or II	12154	5179	12344	8170	15776	2690	23968	21	0.657
	III or IV	13108	6596	12629	9619	19905	1613	23024	15	
CCL-19	I or II	331.40	285.90	240.59	122.23	516.41	84.84	1233.78	21	0.294
	III or IV	342.27	171.39	304.16	263.31	450.24	0.00	696.17	15	

Mann-Whitney's test

S. Table 9. Description of chemokines according to the site of the disease in women with endometriosis and the results of comparative tests

Variable	Diagnosis/ Localisation	Mean	SD	Median	P25	P75	Min.	Max.	N	p
CXCL-8	Peritoneal	35.76	36.47	21.86	11.71	79.12	4.95	96.55	7	**0.033**
	Retrocervical	29.23	25.65	18.77	12.46	36.70	8.76	96.55	13	
	Rectovaginal	124.50	154.19	67.14	21.21	140.83	9.74	500.00	14	
CCL-2	Peritoneal	898.31	922.54	411.74	123.85	1412.97	55.80	2580.31	7	0.571
	Retrocervical	361.95	179.61	361.01	240.77	441.65	122.50	742.28	13	
	Rectovaginal	1222.84	1994.77	560.91	191.65	1267.16	47.83	7657.04	14	
CCL-5	Peritoneal	181.27	180.98	184.37	17.35	366.71	17.35	471.10	7	0.531
	Retrocervical	1170.66	3888.07	61.48	18.00	106.63	7.05	14101.64	13	
	Rectovaginal	694.68	1628.67	107.58	24.80	535.98	15.44	6179.70	14	
CCL-21	Peritoneal	469874	900371	77662	65049	273613	37262	2500000	7	0.841
	Retrocervical	101862	49608	92777	61060	130204	38222	202701	13	
	Rectovaginal	230499	399211	101467	55585	184815	37827	1559600	14	
CXCL-12	Peritoneal	11141	3924	10056	8812	14476	5084	16155	7	0.170
	Retrocervical	15335	5431	15396	12245	20504	6478	23968	13	
	Rectovaginal	11573	6057	10543	7382	15771	1613	23024	14	
CCL-19	Peritoneal	322.07	192.94	240.59	157.81	521.34	122.23	541.03	7	0.271
	Retrocervical	258.32	187.34	217.64	140.02	299.04	84.84	810.03	13	
	Rectovaginal	412.59	308.11	339.53	219.58	570.53	0.00	1233.78	14	

Kruskal-Wallis' test

S. Table 10. Results of multiple comparisons of IL-8 (CXCL-8) between the sites of the disease

Comparisons	Z value	p
Peritoneal VS Retrocervical	0.20	0.843
Peritoneal VS Rectovaginal	-1.89	0.059
Retrocervical VS Rectovaginal	-2.51	**0.012**

Dunn multiple comparisons

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Affidavit

I, Giuliano Moysés Borrelli certify under penalty of perjury by my own signature that I have submitted the thesis on the topic *"Investigation of invasion factors in deep-infiltrating endometriosis"* and I wrote this thesis independently and without assistance from third parties; I used no other aids than the listed sources and resources.

All points based literally on publications of other authors, which are in proper citations – URM (uniform requirements for manuscripts) indicated. The section on methodology (in particular practical work, laboratory requirements, statistical processing) and results (in particular images, graphics and tables) correspond to the URM and are answered by me. My interest in any publications to this dissertation corresponds to those that are specified in the following joint declaration with the responsible person and supervisor. All publications resulting from this thesis and which I am author correspond to the URM and I am solely responsible.

The importance of this affidavit and the criminal consequences of a false affidavit (section 156,161 of the Criminal Code) are known to me and I understand the rights and responsibilities stated therein.

Giuliano M. Borrelli, MD

August 20th, 2015

Curriculum Vitae

Oral presentations

- ESGE (European Society for Gynaecological Endoscopy) 23rd Annual Congress – Brussels, Belgium – September 2014; Title: *Partial Loss of BAF250a (ARID1A) in deep endometriosis and compromised pelvic sentinel lymph nodes: a normal phenomenon or an early event of malignant transformation in endometriosis?* Borrelli GM, Abrão MS, Taube ET, Darb-Esfahani S, Chiantera V, Mechsner S.

- 12th World Congress on Endometriosis – Sao Paulo, Brazil May 2014; Title: *Endometriosis and Cancer: How should we look to this association? BAF250a and cancer-related chemokines expression in endometriosis lesions and pelvic lymph nodes*. Borrelli GM, Abrão MS, Mechsner S.

Publications

Borrelli GM, Carvalho KI, Kallas EG, Mechsner S, Baracat EC, Abrão MS. Chemokines in the pathogenesis of endometriosis and infertility. *J Rep Immunol* 2013; 98(1-2): 1-9.

Borrelli GM, Abrão MS, Mechsner S. Can chemokines be used as biomarkers for endometriosis? A systematic review. *Hum Reprod* 2014; 29(2):253-66.

Borrelli GM, Abrão MS, Mechsner S. Reply: Biochemical markers for endometriosis: a long way to go. *Hum Reprod* 2014; 29(10):2353.

Borrelli GM, Kaufmann AM, Abrão MS, Mechsner S. Addition of MCP-1 and MIP-3β to the IL-8 appraisal in the peritoneal fluid enhances the probability of identifying women with endometriosis. *J Reprod Immunol* 2015; 109:66-73.

Borrelli GM, Abrão MS, Taube ET, Darb-Esfahani S, Köhler C, Kaufmann AM, Chiantera V, Mechsner S. Immunohistochemical expression of cancer-related chemokines in rectovaginal deep-infiltrating endometriosis and pelvic sentinel lymph nodes: a possible role in the lymphatic spread of endometriosis. *Reprod Sci* 2015 Jul 12. pii: 1933719115592711. [Epub ahead of print]

Awards

YEP Award (T. Schollmeyer) for the best YEP Abstract – ESGE 23rd Annual Congress 2014 – Brussels, Belgium.

My CV will not be published in the electronic version of my work for privacy reasons.

Giuliano M. Borrelli, MD

August 20th, 2015

Declaration of any eventual publications

Giuliano Moysés Borrelli had the following share in the following publications:

- Borrelli GM, Carvalho KI, Kallas EG, Mechsner S, Baracat EC, Abrão MS. Chemokines in the pathogenesis of endometriosis and infertility. *J Reprod Immunol* 2013; 98(1-2):1-9.

Contribution: first author. **G.M.B.** selected all the studies included in the review and wrote the first version of the manuscript. **K.I.C.**, **E.G.K.**, **S.M.**, **E.C.B.** read the first version and helped to improve the work giving new ideas. **G.M.B.** wrote the final version after discussion with all colleagues. **M.S.A.** critically reviewed and approved the final version before publication.

- Borrelli GM, Abrao MS, Mechsner S. Can chemokines be used as biomarkers for endometriosis? A systematic review. *Hum Reprod* 2014; 29(2):253-266.

Contribution: first author. **G.M.B**. and **M.S.A**. assessed and selected the studies for eligibility. **G.M.B.** extracted data from the studies and prepared the manuscript. **M.S.A**. and **S.M.** critically reviewed and corrected the manuscript. **S.M.** supervised the project and, together with **G.M.B.**, edited the final version of the manuscript.

- Borrelli GM, Abrao MS, Mechsner S. Reply: Biochemical markers for endometriosis: a long way to go. *Hum Reprod* 2014; 29(10): 2353.

Contribution: first author. **G.M.B.** wrote the manuscript (letter). **M.S.A.** and **S.M.** gave suggestions and corrected the final version for publication.

- Borrelli GM, Kaufmann AM, Abrão MS, Mechsner S. The addition of MCP-1 and MIP-3β to the IL-8 appraisal in the peritoneal fluid enhances the probability of identifying women with endometriosis. *J Reprod Immunol* 2015; 109:66-73.

Contribution: first author. **S.M.** performed the surgical procedures. **G.M.B.** helped to collect the samples and prepared them (centrifugation) for storage. **G.M.B.** and **S.M.** designed the study. **G.M.B.** selected the samples included in the study and performed all experimental tests. **G.M.B.** assessed all data and prepared the first version of the manuscript. **A.M.K.**, **M.S.A.** and **S.M.** critically reviewed and corrected the first version. **G.M.B.** prepared the final version for publication.

- Borrelli GM, Abrão MS, Taube ET, Darb-Esfahani S, Köhler C, Kaufmann AM, Chiantera V, Mechsner S. Immunohistochemical expression of cancer-related chemokines in rectovaginal deep-infiltrating endometriosis and pelvic sentinel lymph nodes: a possible role in the lymphatic spread of endometriosis. *Reprod Sci* 2015 Jul 12. pii: 1933719115592711. [Epub ahead of print]

Contribution: first author. **S.M.**, **C.K.** and **V.C.** performed the surgical procedures and collected the material. **G.M.B.** and **S.M.** designed the study. **G.M.B.** selected and sliced all paraffin blocks, and prepared the slides for immunohistochemistry. **S.D.E.** and **E.T.T.** helped **G.M.B.** with the immunohistochemical staining and analysis. **G.M.B.** assessed all data and prepared the first version of the manuscript. **M.S.A.**, **A.M.K.**, and **S.M.** critically reviewed and corrected the manuscript. **A.M.K.** discussed and gave important suggestions regarding this project. **S.M.** supervised the project and, together with **G.M.B.**, edited the final version of the manuscript.

- Borrelli GM, Abrão MS, Taube ET, Darb-Esfahani S, Chiantera V, Schneider A, Mechsner S. Clonal loss of BAF250a (ARID1A) in rectovaginal deep-infiltrating endometriosis and compromised pelvic sentinel lymph nodes: a normal phenomenon or an early event of malignant transformation in endometriosis? *Histopathol* 2015; submitted.

Contribution: first author. **S.M.**, **A.S.** and **V.C.** performed the surgical procedures. **G.M.B.** and **S.M.** designed the study. **G.M.B.** selected and sliced all paraffin blocks, and prepared the slides for immunohistochemistry. **S.D.E.** and **E.T.T.** helped **G.M.B.** with the immunohistochemical staining and analysis. **G.M.B.** assessed all data and prepared the first version of the manuscript. **M.S.A.** and **S.M.** critically reviewed and corrected the manuscript. **A.S.** discussed and gave important suggestions regarding this project. **S.M.** supervised the project and, together with **G.M.B.**, edited this final version of the manuscript.

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