Aus der Klinik für Gynäkologie
der Medizinischen Fakultät Charité – Universitätsmedizin Berlin

DISSERTATION

New insights into the pathogenesis of adenomyosis

"Is the dislocation of the endometrial fragments into the myometrium evident in adenomyosis uteri?"

zur Erlangung des akademischen Grades
Doctor medicinae (Dr. med.)

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von

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Preface

The data presented in this dissertation was published as a first author in the following international peer-reviewed journals:

- Ultramicro-trauma in the endometrial-myometrial junctional zone and pale cell migration in adenomyosis. Ibrahim MG, Chiantera V, Frangini S, Younes S, Köhler C, Taube ET, Plendl J, Mechsner S. Journal of Fertility and Sterility, December 2015.

- Myofibroblasts are evidence of chronic tissue microtrauma at the endometrial-myometrial junctional zone in uteri with adenomyosis. Ibrahim MG, Sillem M, Plendl J, Chiantera V, Sehouli J, Mechsner S. Journal of Reproductive Sciences, January 2017

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List of abbreviations

AM	Adenomyosis
ASMA	Alpha Smooth Muscle Actin
BM	Basement Membrane
CIN	Cervical Intraepithelial Neoplasia
ECM	Extra-Cellular Matrix
EM	Endometriosis
EMI	Endometrial-Myometrial Interface
EMJZ	Endometrial Myometrial Junctional Zone
EMT	Epithelial-Mesenchymal Transformation
ES	Endosalpingosis
G	Gravidity
HMB	Heavy Menstrual Bleeding
IM	Inner Myometrium
MRI	Magnetic Resonance Imaging
NSAID	Non-steroidal Anti-Inflammatory Drug
OM	Outer Myometrium
P	Parity
SMCs	Smooth Muscle Cells
TGFβ1	Transforming Growth Factor Beta 1
TGFβR1	Transforming Growth Factor Beta Receptor 1
TGFβR2	Transforming Growth Factor Beta Receptor 2
TGFβR3	Transforming Growth Factor Beta Receptor 3
TIAR	Tissue Injury And Repair
TVUS	Trans-Vaginal Ultra-Sonography

Zusammenfassung

Einleitung

Die Adenomyoseis Erkrankung betrifft häufig Frauen im gebärfähigem Alter. Mikroskopisch zeichnet lässt sich die Erkrankung durch das Vorkommen von ektopen endometrialen Drüsen und Stromazellen tief im Myometrium aus. Obwohl die Pathogenese der Adenomyose bis heute nicht geklärt wurde, ist wohl von einer Translokation von Fragmenten des basalen Endometriums durch Mikro-Dehiszenz im inneren Myometrium auszugehen. Als ursächlich wird eine mögliche uterine Hyperperistaltik diskutiert. Die Mikro-Dehiszenzen werden mikroskopisch im Bereich der Übergangzone zwischen dem basalen Endometrium und dem inneren Myometrium verortet. In der vorliegenden Studie wurden betroffene und nicht betroffenen uterine Gewebe, mikroskopisch und ultramikroskopisch, auf Mikro-Traumata und Translokationen des basalen Endometriums hin untersucht und immunhistochemisch charakterisiert.

Methodik

Zweiunddreißig Patientinnen wurden in die Studie eingeschlossen, davon waren 18 klinisch und histopathologisch von AM betroffen, 14 wiesen klinisch und histopathologisch keine AM auf. Die uterinen Proben wurden mittels Transmissions-Elektronenmikroskopie charakterisiert. Weiterhin wurden folgende Nachweise Immunhistochemisch angefertigt: van Gieson-Färbung (extrazelluläre Kollagenfaserfärbung), Myofibroblasten (ASMA, Kollagen I), glatte Muskulatur (Desmin), Transforming Growth Factor Beta Rezeptor 1, 2 und 3 (TGFβR1, R2 und R3), Zellkontakte (ECadherin) und Immunzellen (CD45, CD68).

Ergebnisse

Sowohl mikroskopisch als auch ultra-mikroskopisch zeigten sich mehrere Veränderungen in den AM-Uteri: 1. Es zeigte sich eine unkoordinierte Anordnung der glatten Muskelfasern im inneren Myometrium der AM-Kohorte. Die Anordnung der myometrialen glatten Muskelzellen der Kontrollgruppe war hingegen in paralleler Orientierung zu den basalen endometrialen Drüsen. 2. Der Übergang Endometrium/Myometrium in AM-Uteri war äußert unregelmäßig, hingegen glatt in den Kontrollproben. Es ließ sich allerdings keine Dislokation einzelner Zellen darstellen, diese blieben immer im Verband, auch waren die Zell-Zellkontakte nicht

gestört. 3. Die sogenannten Pale-Zellen in den basalen endometrialen Drüsen konnte sowohl in AM-Uteri als auch in der Kontrollgruppe nachgewiesen werden. In AM-Uteri stellte sich eine Migration der Pale Zellen ins Stroma dar, die an mehreren Stellen eine Ultra-Mikroruptur der basalen Membran in endometrialen Drüsen verursachten. 4. Als Mikrotraumatisierungsfolge, zeigten sich sowohl die ASMA-Expression im basalen Endometrium als auch die Kollagen-Expression im inneren Myometrium in der AM-Gruppe signifikant gegenüber den Kontrollproben erhöht.

Schlussfolgerung

Mehrere morphologisch-histologische Veränderungen bestärken die Theorie, dass es in der Endo-Myometrialen Übergangszone zu einer Mikrotraumatisierung kommt, die möglicherweise ursächlich an der Pathogenese der Adenomyosis uteri zu sein scheint. Dabei bleibt aber der Zellverbund intakt. Hingegen scheint der Nachweis von migrierenden Pale Zellen insbesondere in Patientinnen mit Adenomyosis uteri die These zu stärken, dass Gründerzellen mit an der Pathogenese der Adenomyosis beteiligt sein könnten. Um diese Hypothese zu bestätigen bedarf es jedoch weiterer in vivo und vitro Charakterisierung der Pale Zellen.

Abstract

Introduction

Adenomyosis (AM) is a prevalent disease among women in the reproductive-age. It is histopathologically defined by the ectopic presence of endometrial tissue deep in the underlying myometrium. Although the disease pathogenesis is so far unclear, a translocation of fragments of the basal endometrium into the myometrium, through micro-dehiscences in the inner myometrium, is the most widely accepted theory. These micro-dehiscences are caused by a uterine hyperperistalsis. The latter induces a tissue micro-trauma at the endometrial myometrial junctional zone (EMJZ) in AM-uteri. In our study, possible microscopic and ultra-microscopic evidence of micro- trauma and corresponding tissue-translocation in the EMJZ was investigated.

Materials and methods

Uterine wall biopsies were collected from clinically and histopathologically diagnosed AM (n=18) and non-AM (n=14) patients, to study any structural difference in favour of a micro-trauma at EMJZ. The biopsies were examined with Transmission Electron Microscopy (TEM), Van Gieson stain (for extra cellular collagen fibres) and immune-labelled for markers of: myofibroblasts (ASMA, collagen I), mature smooth muscle (desmin), Transforming Growth Factor beta receptor 1 (TGFβR1), TGFβR2, TGFβR3, cell-cell contact (E-cadherin) and hematopoietic cells (CD45, CD68).

Results

The EMJZ in AM-uteri showed both microscopic as well as ultra-microscopic changes as following: (1) A disarray of the smooth muscle fibres in the inner myometrium of AM-uteri was evident, compared to the parallel arrangement in non-AM uteri. (2) A disruption of the smooth interface between the endometrium and myometrium in AM was clearly seen, but lacked in non-AM. Nevertheless, neither cell disruption nor translocation of fragments of the basal endometrial glands into the stroma in AM-uteri was seen. (3) Interestingly, uterine pale cells were described in the basal endometrial glands in both AM and non-AM-uteri. However, only in the AM group were these cells migrating into the stroma, through ultra-microruptures of the glandular basement membrane at different locations. (4) As a consequence of tissue trauma, both ASMA-immunolabeled stromal cells in the endometrium as well as collagen I

immunolabeling in the inner myometrium were significantly higher in AM uteri than in non-AM uteri.

Conclusion

The different morphological changes at the EMJZ support the theory of occurrence of a micro-trauma in AM-uteri being part of the pathogenesis of the disease. However, there is no evidence of a translocation of the basal endometrium in AM uteri. Moreover, the migrating uterine pale cells in AM-uteri demand an in-depth in-vitro characterization to elucidate if they are involved in the disease pathogenesis.

1. Introduction

1.1 The different layers of the uterus

The uterus is anatomically divided into the inner layer (the endometrium) and the outer layer (the myometrium). The endometrium is composed mainly of endometrial epithelial glands and stroma. It is further subdivided into the functional (inner most) layer and the basal (outer) endometrium. The former is hormone-sensitive, so that it sheds with the falling of estrogen and progesterone blood levels, shortly before menstruation, and forms the main constituent of the menstrual blood (1). The basal endometrium is not subjected to cyclic changes, and is from which the functional endometrium regenerates (2). It is thought that stem cells reside in the basal endometrium and help regeneration of the functional layer (3, 4).

The myometrium is the muscular layer of the uterus. It is further subdivided into two layers: inner myometrium (IM) and outer myometrium (OM). The smooth muscle cells (SMCs) are arranged in bundles in the IM, and are arranged parallel to the basal endometrium (5, 6). However, SMCs in the OM are oriented in different directions and sometimes intersecting with each other. This unique SMC orientation ensures cycle phase-dependent rhythmic contractions (uterine peristalsis) in the IM. In the proliferative phase, and while the functional endometrium regenerates, the myometrium exhibits retrograde rhythmic waves of contractions. They help the upward transport of the sperms into the fallopian tube to meet the oocyte just after the occurrence of ovulation (7). Afterwards, and if fertilisation occurred in the fallopian tube, the fertilised oocyte (blastocyst) will be transported downwards into the uterine cavity, to implant in the uterine wall. This type of peristalsis predominates in the secretory phase. If pregnancy didn’t occur, the fertilised oocytes together with the shed functional endometrium will be expulsed by anterograde waves of contraction into the menstrual blood (8). The SMCs in the OM take over during menstruation and during labor and exert an expulsive peristalsis to expel the shed endometrium and the oocyte, or the full-term baby during labor, respectively (7) (9).

Interestingly, the area of both basal endometrium and inner myometrium is called the “endometrial-myometrial junctional zone (EMJZ)” (10); others also call it “junctional zone” (11) as well. Both have the same embryological origin. It is not to be confused

with “endometrial-myometrial interface (EMI)”. The latter is just the tangential line between the basal endometrium and inner myometrium (10).

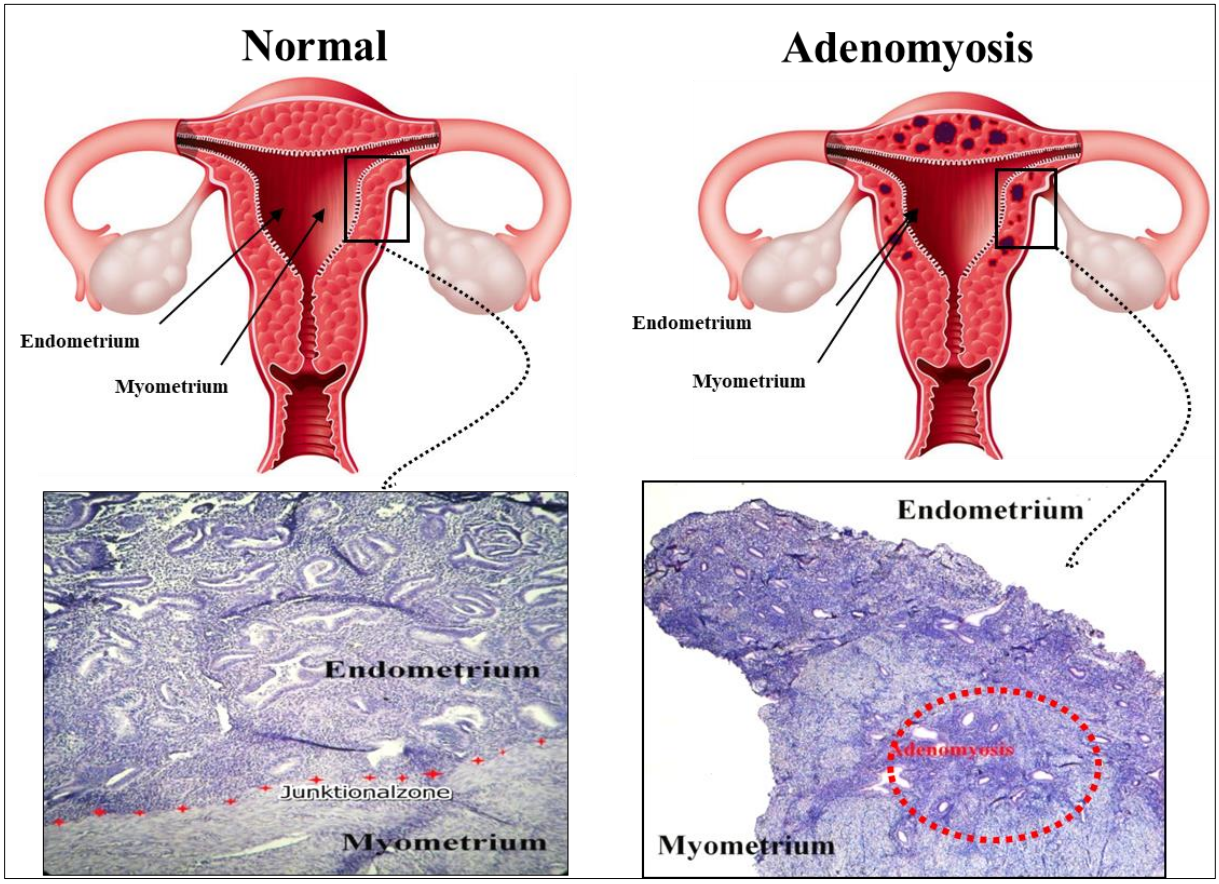

Figure 1: *Histology of a normal and adenomyotic uterus.*

Left panel: *The normal uterus has an inner layer of endometrial glands and stroma (endometrium) as well as an outer layer of smooth muscle cells (myometrium). Furthermore, the endometrial-myometrial interface (EMI) is uninterrupted (red dots).*
Right panel: *The presence of an island of endometrial glands and stroma (red punctuated circle) deep in the myometrium hallmarks the diagnosis of adenomyosis.*

1.2 What is adenomyosis?

Adenomyosis (AM) is defined by the ectopic presence of endometrial glands and stoma deep in the myometrium, and they are usually seen surrounded by hypertrophic and hyperplastic smooth muscle cells (Figure 1) (12, 13). The lesion is commonly diffuse in the myometrium (diffuse type), but sometimes can be localised and forms a focal type (14). The disease is usually associated with endometriosis (EM), which is also an oestrogen-dependent disease too (15). EM is characterised by the presence of ectopic endometrial tissue (endometrial glands and stroma) outside

the uterus, usually in the abdominal cavity (16). Three anatomical variants of EM are described: superficial peritoneal endometriosis; deep infiltrating endometriosis (affecting the urinary bladder, intestine, vagina, recto-vaginal wall, sacro-uterine ligament, or located deeper than 5 mm in the peritoneum); and ovarian endometriosis (17).

The diagnosis of AM is suspected on clinical symptoms of dysmenorrhea (painful menstruation) and menorrhagia (heavy menstrual bleeding) (18). Its prevalence is higher among patients with infertility (19), and among those who have endometriosis (20). Moreover, high resolution trans-vaginal ultrasonography (TVUS) (21) and magnetic resonance Imaging (MRI) (22) have a high specificity and sensitivity to diagnose AM (23, 24). The final diagnosis is usually made based on histopathological examination. The detection of endometrial glands and stroma at least 2.5 mm under the endometrial myometrial interface is diagnostic of AM (25).

Symptomatic AM patients usually undergo a conservative treatment, starting with non-steroidal anti-inflammatory drugs (NSAID), and extending to hormonal treatment in NASID-resistant cases. The latter is based on depriving the adenomyotic lesions from estrogen (e.g. Gonadotropin-releasing hormone-agonist “GnRH-agonist” in depot form), or continuous intake of oral contraceptive pills to inhibit endogenous production of estrogen (15). In severely resistant cases, and with completion of family planning, a hysterectomy is the effective treatment to ameliorate the patient complaints. Nevertheless, and as the disease prevails in young patients in the reproductive-age group, hysterectomy is not an option for this group of patients (26).

1.3 Adenomyosis pathogenesis

1.3.1 Tissue Injury And Repair theory (TIAR)

As the pathogenesis of AM is still unknown, many theories have been formulated to explain the development of the disease. Uterine peristalsis in AM patients becomes more intense (hyperperistalsis) and loses its synchronisation (dysperistalsis) (7, 27). Hyperperistalsis exerts shearing stress at the EMI, which in turn leads to tissue micro-traumatisation. As the EMI lacks any supporting submucosal layer of connective tissue as found normally in other tissues (e.g intestine and bronchi) (28), the effects of this shearing stress mostly involve the inner myometrium as well.

As the midline region at the intersection with the level of fallopian tubes entrance into the uterus is the embryological fusion site of the two paramesonephric ducts (Müllerian tubes) (9), the shearing stress is believed to be maximum at this site. Shearing stress is presumed to induce micro-dehiscence in the inner myometrium (2), which facilitates the translocation of dislocated basal endometrial glands into the myometrium. Moreover, micro-trauma induces a cascade of enzymatic reactions, ending up with increased local oestrogen production (paracrine effect). The latter binds to oxytocin receptors, normally expressed on SMCs in the myometrium, and causes more uterine peristalsis, hence more micro-trauma follows. This presumed theory is called “tissue injury and repair” (TIAR) (9, 20, 29) (Figure 2).

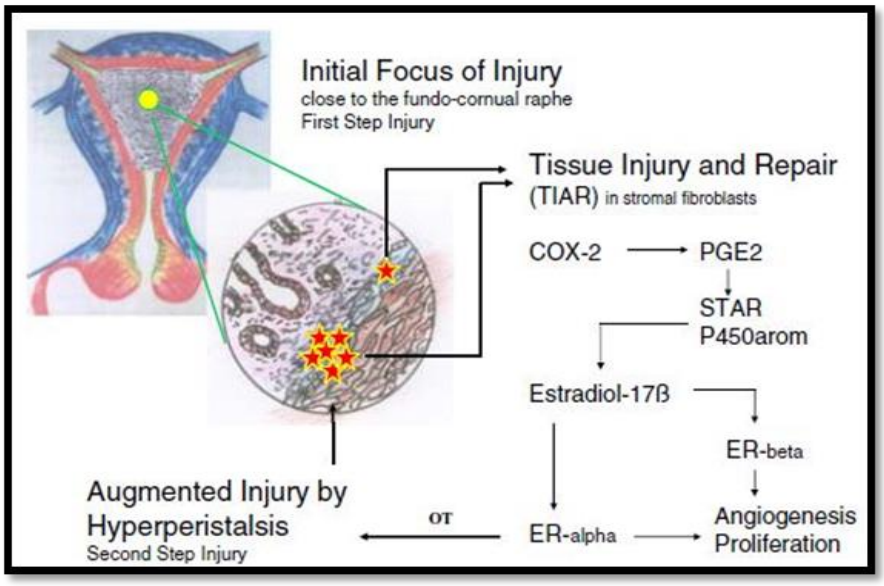

Figure 2 : *Tissue Injury and Repair theory (TIAR).*

The fundocornual raphe (the intersection area between the midline of the uterus with a line traversing the entrance of fallopian tube) is the site of maximum tissue injury (shearing stress) caused by uterine hyperperistalsis. Accordingly, a cascade of tissue injury occurs, resulting in the production of prostaglandins which stimulates the paracrine production of estrogen in the local tissue. The later activates the oxytocin receptors on the smooth muscle cells in the myometrium, which ensures a state of hyperperistalsis. Then, this continues in a vicious circle (9).

1.3.2 Myofibroblasts and tissue trauma

Upon tissue trauma, Transforming Growth Factor beta 1 (TGFβ1) is released and recruits the fibroblasts to the site of tissue injury. The latter undergo a myofibroblastic transformation into myofibroblasts, which acquire a contractile and a secretory

phenotype. The transformed fibroblasts acquire a contractile apparatus so that they can move to the site of tissue injury. Moreover, they produce collagen, to help repair the tissue injury and replace the lost extra-cellular matrix. Again, the myofibroblasts contract and exert a remodelling effect on the granulation tissue to minimize the scarred area (30, 31). Acquisition of alpha smooth muscle actin (ASMA) in the stress fibres in the cytoplasm of the myofibroblasts hallmarks the de-novo development of myofibroblasts. However, SMCs express not only ASMA but also desmin (being a marker of differentiated smooth muscle cells). Interestingly, smooth muscle-like cells are frequently seen in all different forms of EM lesions (32-38). They express uterine markers: oxytocin receptors, vasopressin receptors, oestrogen and progesterone receptors (39), which may point to a uterine origin. Furthermore, the immature cells are usually concentrically arranged in direct contact to the endometrial glands, while the mature SMCs are predominantly detected at the periphery of the endometriosis lesions.

TGFβ1 is secreted from endometrial epithelial glands, stroma and immune cells infiltrating the endometrium (e.g macrophages). Its level is cycle phase-dependent, with its highest peak around the time of menstruation, where tissue trauma and shedding of endometrium occur. It helps tissue repair, the regulation of cell proliferation, differentiation, extracellular matrix (ECM) synthesis, fibrosis and angiogenesis (40).

In our study we reappraised the pathogenesis of AM. The EMJZ, being the area of interest regarding the AM pathogenesis, was studied using transmission electron microscopy to search for any ultrastructural evidence of micro-trauma in AM patients. Furthermore, the immunolabeling of different markers of myofibroblastic metaplasia (ASMA, Desmin, Collagen I) in addition to TGFβ1 receptors (TGFβ1 receptor 1 “R1”, R2 and R3) in endometrial cells and SMCs in the EMJZ was studied. E-cadherin as a marker of cell-cell contact was also studied. Van Gieson stain was used to describe the arrangement of the SMCs fibres as well as the extra-cellular collagen fibres in the IM.

2. Research questions

In our study, we focused on answering the following research questions:

1. Is there any microscopic or ultra-microscopic evidence of tissue trauma at EMJZ in AM patients?
2. Is there any microscopic or ultra-microscopic evidence of translocation of the basal endometrial glands into the stroma of AM uteri?
3. Does the immunolabeling of the TGFβR1, R2 and R3 differ between AM and non-AM uteri?

3. Methods

3.1 Patients

In a case–control study, 2 groups were assigned: one as the AM group (n =18) and the other as non-AM group (n = 14). All patients included in the study were premenopausal and clinically diagnosed either with ultrasonography (tiny intramyometrial cysts, symmetrical or asymmetrical thickening of the myometrial walls, hypoechoic linear striation in heterogenic myometrium, ill-defined endometrial-myometrial interface) and/or MRI (thickened junctional zone ≥ 12 mm, tiny myometrial cysts, globular enlagerd uterus) to have AM or not (41, 42). However, the histological diagnosis to confirm or to exclude adenomyosis was the only deciding factor to include the patients in one of both groups. The whole uteri were obtained intact during laparoscopy-assisted vaginal hysterectomy, performed at the Charité University of Medicine in Berlin between 2012 and 2014. The uterine manipulators used during surgery were one-size smaller than the measured uterine length to avoid a possible squeezing effect on the inner endometrium that may be exerted by an over-sized manipulator. Macroscopically, and during specimen dissection no injuries were seen in the endometrium. Wedge-shape biopsies were obtained from the anterior and posterior walls and the fundus of the uterus at the crossing point of two lines: the longitudinal midline of the uterus with the line at the level of entrance of the fallopian tubes into the uterus. This corresponds to the embryological fusion site of both Müllerian tubes and the site of the presumed maximal shearing stress during uterine peristalsis (9) (Figure 3). Histopathological confirmation or exclusion of AM lesions followed in all patients. The indications for all hysterectomies were benign gynecological disorders. All relevant clinical data of the patients included were documented (Table 1).

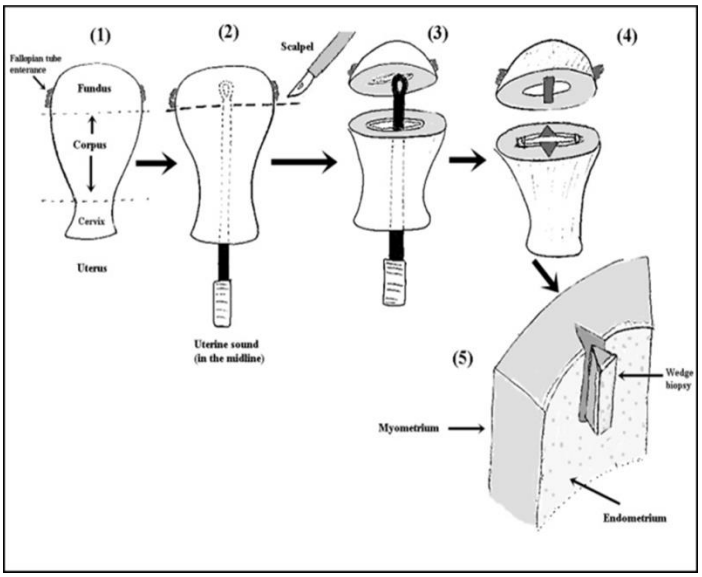

Figure 3: *A diagrammatic illustration of specimen collection from the uterus (intra-operative).*

The uterus is anatomically composed of three parts; fundus, corpus and cervix (1). A uterine sound was passed into the uterine cavity till the fundus (2). A scalpel was used to incise the uterus transversely at the level of the fallopian tubes' entrance into the uterus (3). A wedge-shaped biopsy was taken from the anterior wall (4). A magnified view of the wedge biopsy shows the basal endometrium and the inner and the outer myometrium included in the obtained biopsy (5).

Table 1: *The relevant clinical data of non-adenomyosis and adenomyosis groups.*

AM: Adenomyosis, CIN: Cervical Intraepithelial Neoplasia, G: gravidity, P: parity, EM: Endometriosis, ES: Endosalpingosis= ectopic cystic glands lined with fallopian tube-epithelium, Free: No Endometriosis, Myoma or Endosalpingosis, a: Patients having adenomyosis, HMB: heavy menstrual bleeding, metrorrhagia: irregular uterine bleeding, Atrophic: atrophic endometrium, #: unkown.

Patient	Age	G	P	Cycle phase	Indication of hysterectomy	Histopathology	Symptoms
1	47	3	3	Proliferative	HMB	Myoma	HMB
2	46	0	0	Proliferative	EM, bilateral tubo-ovarian abscess	EM+Myoma	Chronic lower abdominal pain
3	35	2	2	Proliferative	CIN III	Free	Abnormal cytology
4	45	2	1	Proliferative	HMB, myoma	Myoma	HMB
5	36	0	0	Proliferative	EM	EM+Myoma	HMB
6	44	0	0	Proliferative	Ovarian borderline tumor	Myoma+ES	Ovarian cyst
7	42	1	1	Proliferative	Uterine prolapse and stress urinary incontinence	Free	Stress incontinence
8	43	#	#	Proliferative	EM	EM only	Ovarian cyst
9	45	0	0	Proliferative	Recurrent endometriotic cysts	Endometriotic cyst	Dysmenorrhoea, Dyspareunia
10	46	10	9	Secretory	Chronic lower	No myoma, no	Chronic lower

					abdominal pain	AM	abdominal pain
11	44	2	1	Secretory	Multiple myomas	myomas	Dysmenorrhoe, HMB , metrorrhagia
12	42	1	1	Atrophic	Recurrent EM	Free	Dyspareunia, dysuria, recurrent lower abdominal pain
13	49	2	2	Atrophic	Stress incontinence	Free	Stress incontinence
14	34	2	2	Atrophic	Chronic lower abdominal pain	myomas, peritoneal EM, no AM	Chronic lower abdominal pain
15a	44	1	1	Proliferative	HMB	AM only	HMB
16a	41	0	0	Proliferative	Pelvic adhesions and EM	AM+myoma	Chronic lower abdominal pain
17a	47	0	0	Proliferative	Recurrent EM with right ovarian cyst	AM+EM+myoma	Recurrent ovarian cyst
18a	30	2	2	Proliferative	Rectovaginal EM	AM only	Dyspareuna, Dyschezia
19a	33	#	#	Proliferative	Rectovaginal EM	AM+myoma	Dyspareunia
20a	45	2	2	Proliferative	Rectovaginal EM	AM+EM	Dyspareuna, dyschezia
21a	42	0	0	Proliferative	EM	AM+EM	HMB, Dyspareunia, Dysuria, Dyschezia, chronic.lower abdominal pain
22a	37	0	0	Proliferative	AM	AM	HMB, dysmenorrhoea
23a	39	0	0	Proliferative	Chronic lower abdominal pain	EM+AM	Dysmenorrhoea
24a	51	3	3	Secretory	Myoma	AM+myoma	chronic perimenopausal bleeding
25a	47	1	1	Secretory	Endometriosis	AM+Myoma+ES	Dysmenorrhea – premenstrual lower abd pain – HMB, Dyspareunia
26a	52	3	1	Atrophic	Adenomyosis	AM+Myoma	Dysmenorrhea
27a	42	2	2	Atrophic	Myoma	AM+Myoma+ES	HMB
28a	43	0	0	Atrophic	Recurrent EM, endometriotic cysts	AM+EM	Dysmenorrhea
29a	35	0	0	Atrophic	AM	AM+EM	HMB, Dysmenorrhoe
30a	38	1	1	Atrophic	Dysmenorrhoea, endometrial polyp	EM+AM	Dysmenorrhoea
31a	45	0	0	unknown	Endometrioma	EM+AM	Dysmenorrhea, Dyspareunia
32a	43	0	0	unknown	HMB	EM+AM	HMB, Dyspareunia

The specimens were further subdivided into two groups according to the type of the investigation applied:

3.2 Group I for immunohistochemistry, immunofluorescence and van Gieson stain

Biopsies (AM=18, non-AM=14) were immediately fixed in 4% paraformaldehyde for paraffin embedded sections, as described previously, for: subsequent van Gieson staining (to evaluate the collagen fibers as a part of the extra-cellular matrix, they were stained red while the cytoplasm of all other cells were stained brown); and for immunohistochemistry staining for markers of myofibroblastic metaplasia (ASMA, collagen I), smooth muscle metaplasia (desmin), cell-cell-contact (E-cadherin), TGFβR1, TGFβR2 and TGFβR3, and immunofluorescence studies for CD45 (a marker for haematopoietic cells) and CD68 (a marker for macrophages).

3.2.1 Immunohistochemistry

The paraffin blocks of AM and non-AM uteri were cut into 4-mm-thick sections. Deparaffinization in xylene followed by rehydration in a series of decreasing ethanol concentrations was done as described previously. Antigen retrieval was done by cooking in the steamer (Multi Gourmet, type 3216; Braun, Germany) in the appropriate buffer solution for 20 minutes, followed by cooling down for 20 minutes. To minimize background staining, there was blocking with 10% fetal calf serum for 30 minutes and avidin–biotin blocking agents (Avidin/Biotin blocking kit, SP-2001; Vector Laboratories, Canada) for 10 minutes each followed. Incubation with the primary antibody (Table 2) for 60 minutes, followed by the suitable secondary antibody (Table 3) for 40 minutes at room temperature (RT) was done to mark the target protein. Streptavidin-AP was added for 40 minutes at RT, followed by 20 minute incubation with a 2-solution DAB kit (Invitrogen, Darmstadt, German) to achieve the final color. Finally, there was counterstaining with Mayer's hemalum solution (Merck K G a.A., Darmstadt, Germany) for 35 seconds, followed by covering with a drop of Eukitt quick hardening mounting medium (Sigma-Aldrich, Darmstadt, Germany), and cover slips were the last steps. As a negative control, all the above-mentioned steps were done, but the primary antibody was omitted.

Table 2: *Primary antibodies used for immunohistochemistry.*

Primary antibody	Species	Dilution	Buffer	Company
TGFβRI (v-22), sc-398	Rabbit polyclonal IgG	1:500	Citrate (pH=6)	Santa Cruz
TGFβRII (E-6), sc-17792	Mouse monoclonal IgG2a	1:25	Citrate (pH=6)	Santa Cruz
TGFβRIII (A-4), sc-74511	Mouse monoclonal IgG1	1:2000	Citrate (pH=6)	Santa Cruz
Smooth muscle actin, clone 1A4	Mouse monoclonal IgG	1:50	Target 9 (pH=9)	Dako
Collagen I (ab34710)	Rabbit polyclonal IgG	1:100	Citrate (pH=6)	abcam
Desmin	Mouse monoclonal IgG	1:100	Target 9 (pH=9)	Dako

Table 3: *Secondary antibodies used for immunohistochemistry and immunofluorescence.*

Secondary antibody	Species	Dilution	Company
Biotin-SP conjugated AffiniPure (H+L)	Rabbit anti-mouse IgG	1:400	Jackson ImmunoResearch
Biotin-SP conjugated AffiniPure (H+L)	Mouse anti-rabbit IgG	1:400	Jackson ImmunoResearch

3.2.2 Immunoreactive score

All slides were examined by light microscopy (Carl Zeiss Axiophot Microscope, Göttingen, Germany) under different magnification powers (25, 100, 200, and 400). The whole field of view was examined under a magnification power of 200 and evaluated for both extent and intensity of staining. The extent of staining was calculated according to the percentage of the positively stained tissue (endometrial glands, stroma, or myometrium) over the total area. It ranges from 0% to 100%. The intensity of staining was subjectively divided into grades: 0: no staining, 1: mild, 2: moderate, and 3: intense staining. The immunoreactive score was calculated as the spatial extent (as a percentage), multiplied by the intensity of the staining, with a maximum score of 300. Photographs were taken with a Powershot 65 camera (PC1049; Canon) at different magnifications (10, 20, 40, or 100 lenses) and edited with Photoscape software (Mooii Tech, Canon, Japan).

3.2.3 Immunofluorescence

The 4-mm-thick paraffin slides were deparaffinized and rehydrated as usual. Antigen retrieval in a citrate buffer (pH = 6) for 20 minutes was followed by cooling for 20 minutes. Blocking with fetal calf serum (FCS) for 20 minutes was followed by incubation with the primary antibody of CD45 (ab10559; Rabbit polyclonal IgG; Abcam) and CD68 (Ab-3[clone:KP1]; Mouse monoclonal IgG1; Dianova) for 60 minutes at room temperature, which was followed by 1-hour incubationwith the suitable secondary antibody, away from light. The slides were covered with DAPI Fluoromount-G (SouthernBiotech) and examined under the microscope (Axiophot).

3.2.4 Van Gieson stain

This stain was used to evaluate the collagen fibers in the EMJZ. Slides with paraffin-embedded sections were deparaffinised and rehydrated, as previously described, followed by transfer to an autostainer (Leica XL). Slides were stained with resorcinol for 12 minutes, and immersed in Weigert's hematoxylin (Sigma-Aldrich) for 13 minutes, rinsed with distilled water, and stained with Van Gieson solution for 8 minutes.

3.3 Group II for transmission electron microscopy

Due to drop-off of some collected uterine specimens following an exhaustive slicing during the preparation of the samples for transmission electron microscopy with consequently insufficient stock of the samples and/or loss of the area of interest, only biopsies of AM (n=12) and of non-AM (n=9) were fixed in Karnovsky fixative (7.5% glutaraldehyde& 3% paraformaldehyde in phosphate-buffered saline) for transmission electron microscopy (TEM). They were then washed in 0.1 mol/L cacodylate buffer (cacodylic acid sodium salt trihydrate; Roth, Karlsruhe, Germany), incubated in 1% osmium tetroxide (Chempur; Karlsruhe, Germany) for 120 minutes, dehydrated in an ascending series of ethanol, and washed in the intermedium propylene oxide (1,2epoxypropan; VWR, Germany). Specimens were embedded subsequently in a mixture of agar 100 (epoxy resin), DDSA (softener), MNA (hardener), and DMP 30 (catalyst; all obtained from Agar Scientific; Stansted, United Kingdom). Polymerization was done at 45°C and 55°C each for 24 hours. Semithin and ultrathin sections were cut using an ultramicrotome Reichert Ultracut S (Leica,

Wetzlar, Germany). Semithin sections (0.5 mm) were stained with modified Richardson solution19 for 45 seconds on an electric hot plate adjusted to 80 °C. Sections were checked under a light microscope (Olympus CX 21; Olympus, Stuttgart, Germany) to ensure that EMJZ is clearly identified. Ultrathin (80 nm) sections were mounted on Nickel grids (Agar Scientific) and examined with a transmission electron microscope (Zeiss EM 900; Oberkochen, Germany). Photos were taken and edited by an Adobe Photoshop Program (Adobe Systems; Unterschleissheim, Germany).

3.4 Ethical approval

The study was approved by the local research and ethics committee at the Charité University of Medicine, Berlin, Germany (EA4/071/07), and all participants gave written consent.

3.5 Statistical analysis

All statistical analyses were performed using Graph Pad prism version 4.20 Student t test and one-way analysis of variance were used. All statistical tests were 2-sided and with a 95% confidence interval ($P < 0.05$).

4. Results

4.1 Smooth muscle cells orientation in the inner and outer myometrium

Van Gieson stain allowed the visualisation of the orientation of the inner myometrial smooth muscle fibers in relation to the basal endometrial glands. Collagen fibres were stained red and the cytoplasm of the epithelial, stromal and smooth muscle cells stained brown.

In the non-AM group (n=14), the interface between the basal endometrium and the inner myometrium was regular and uninterrupted in 42.8% (6/14) of the patients. While the smooth muscle fibers in the inner myometrium appeared to follow a parallel arrangement to the basal endometrial glands, they were arranged in bundles in diverse orientation in the outer myometrium (Figure 4A). No focal globular elastosis could be detected.

In the AM group (n=18), disorientation of the EMI at different locations was obvious in 88.8 (16/18) of the patients. The basal endometrial glands were seen dipping down below the EMI into the inner myometrium, being surrounded by collagen fibers (Figure 4B).

Furthermore, smooth muscle fibers located in the inner myometrium lost their parallel arrangement to the basal endometrial glands compared to the non-AM group. Most of these smooth muscle bundles were obliquely arranged, but otherwise perpendicular to the basal endometrium (Figure 4C), mimicking the orientation-pattern in the outer myometrium. Again, no focal globular elastosis was detected.

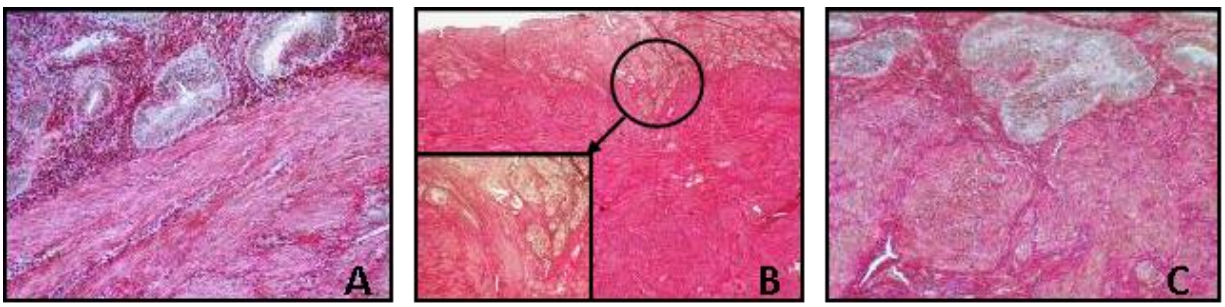

Figure 4 : *Characterisation of the inner myometrium using Van Gieson stain.*

Collagen fibers stain red while the cytoplasm stains brown. (A) The inner myometrium in a non-AM patient. The smooth muscle fibers are arranged parallel to the basal endometrial

glands. x200. (B) The Endometrial-Myometrial Interface (EMI) in adenomyosis. The basal endometrium is seen dipping down (circle) into the inner myometrium disrupting the regular EMI. x100 (inset x200).(C) The inner myometrium in adenomyosis. The smooth muscle fibers are arranged in diverse directions. x200.

4.2 Ultrastructural changes at EMJZ in AM and non-AM uteri

The ultrastructure of the EMJZ was analysed and changes in the basal endometrial epithelial and stromal cells, in addition to the myocytes in EMJZ, were studied in AM (n=12) and non-AM-uteri (n=9).

4.2.1 Changes in endometrial-myometrial interface

In the non-AM group, the interface between the stromal cells of the basal endometrium and the myocytes in the inner myometrium was regular and uninterrupted (Figure 5). However, the EMI in the AM group was interrupted. Moreover, smooth muscle cells were seen surrounding some basal endometrial glands, intermingling with endometrial stromal cells.

4.2.2 Changes in basal endometrium and inner myometrium

In the non-AM group, the glandular epithelial cells had euchromatic nuclei with smooth and regular nuclear membranes and an even distribution of the peripheral chromatin (Figure 6). Their cytoplasm was rich in ribosomes and mitochondria. Multiple desmosomes were identified between their adjoining lateral cell borders (Figure 7). The basement membrane of the basal endometrial glands was intact and the glands were completely surrounded by endometrial stromal cells. The endometrial stromal cells in the non-AM group had regular and relatively large nuclei with peripheral evenly distributed chromatin. Small blood vessels were located near the basal glands. The myocytes in the inner myometrium were parallel to each other (Figure 8).

In the AM uteri, the epithelial cells of the basal endometrial glands had euchromatic nuclei with peripherally arranged chromatin. However, the nuclear shapes were highly irregular and the nuclear membranes had multiple infolding and grooving. The desmosomes at the cell borders were clearly seen in all patients (Figure 9 and 10).

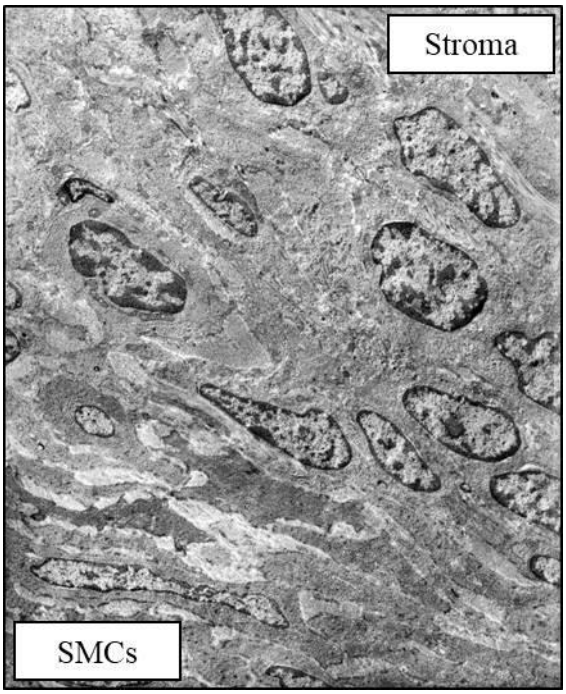

Figure 5: *The interface between the stromal cells in the basal endometrium and the smooth muscle cells (SMCs) in the inner myometrium is regular and uninterrupted in non-AM. x2500*

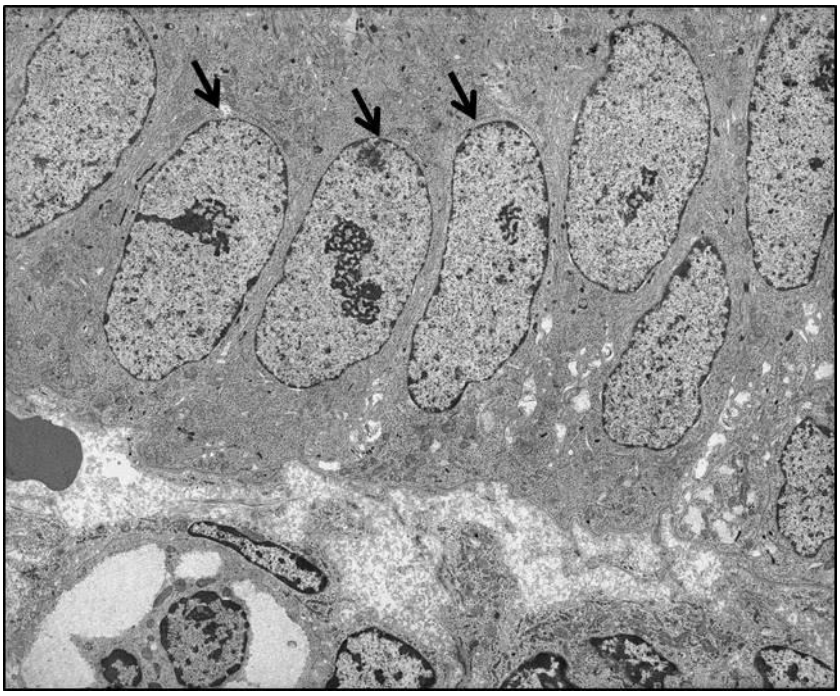

Figure 6: *Smooth nuclear membranes (black arrows) of the epithelial cells in the basal endometrium and an even distribution of the peripheral chromatin in non-AM uteri. x2000*

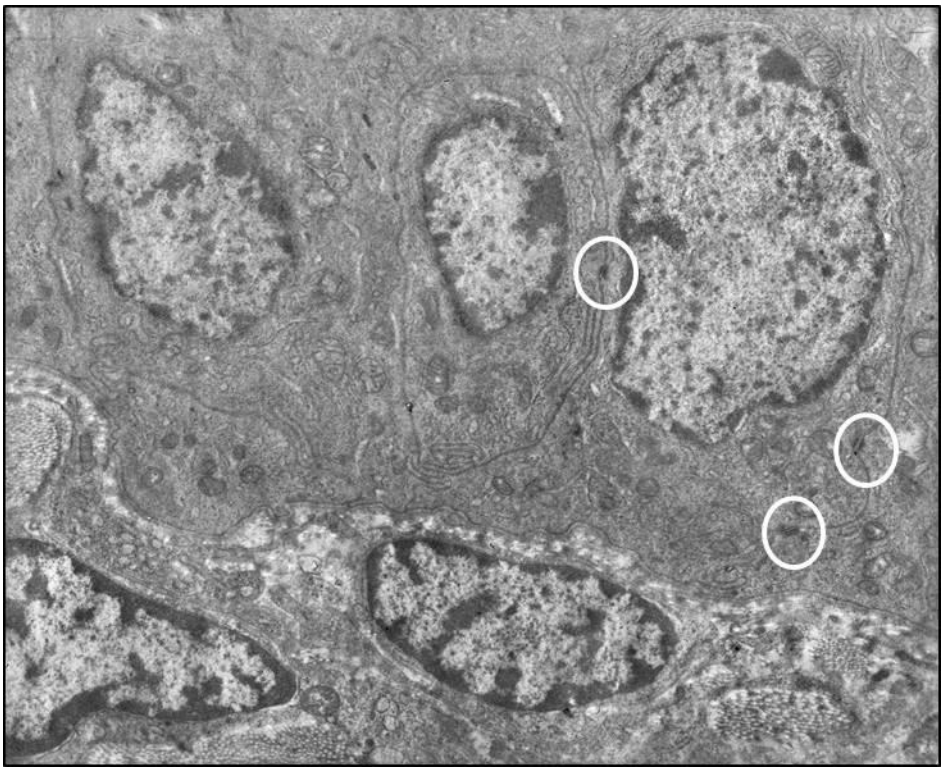

Figure 7: *Desmosomes (white circles) are seen at the lateral borders of the epithelial cells in the basal endometrial glands in non-AM. x5000*

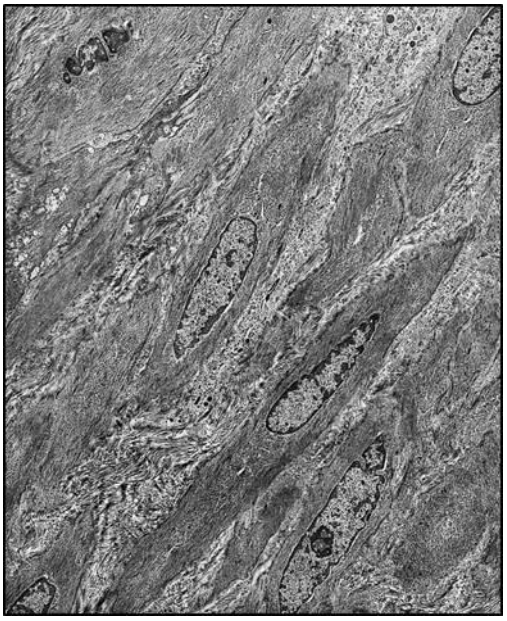

Figure 8: *The smooth muscle cells in the inner myometrium in non-AM uteri are arranged parallel to each other. x2500*

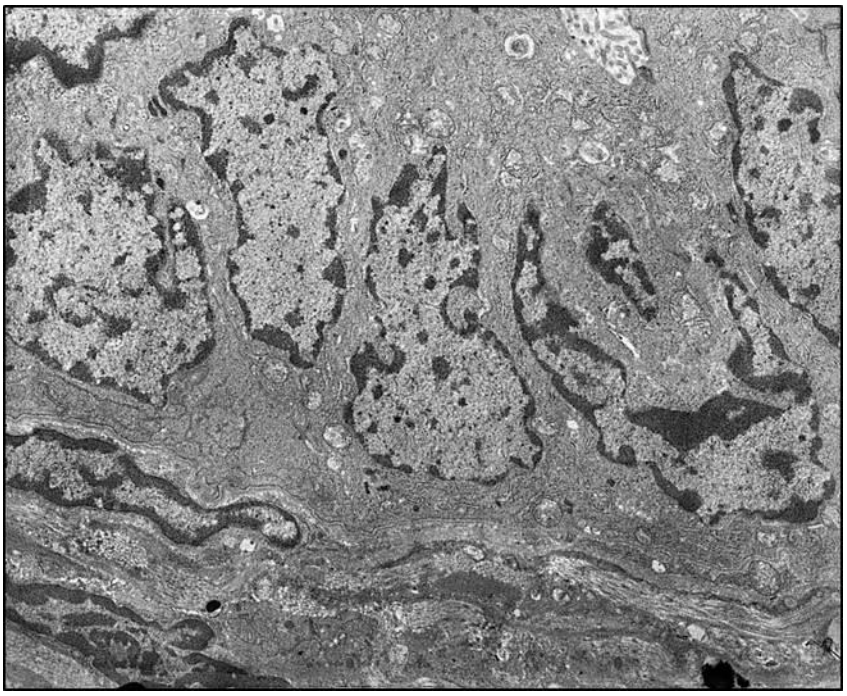

Figure 9: *The epithelial cells in the basal endometrial glands of AM have infolding of their nuclear membranes.. x4000*

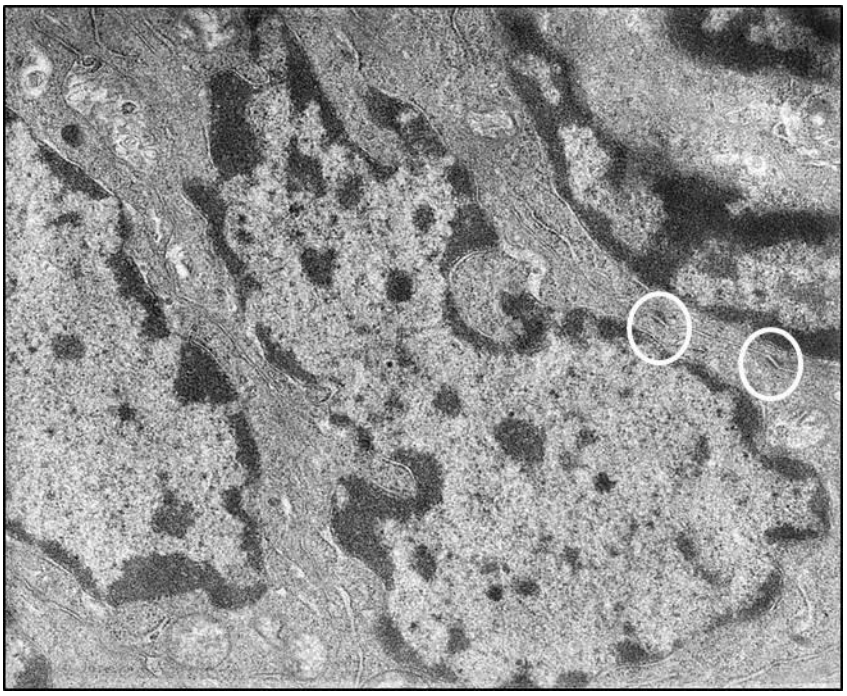

Figure 10: *A magnification of figure 9 showing two desmosomes (white circles) between the cell membranes of two neighbouring glandular epithelial cells (white circles). x10000*

Moreover, the smooth muscle fibers in the inner myometrium were arranged in different directions (Figure 11).

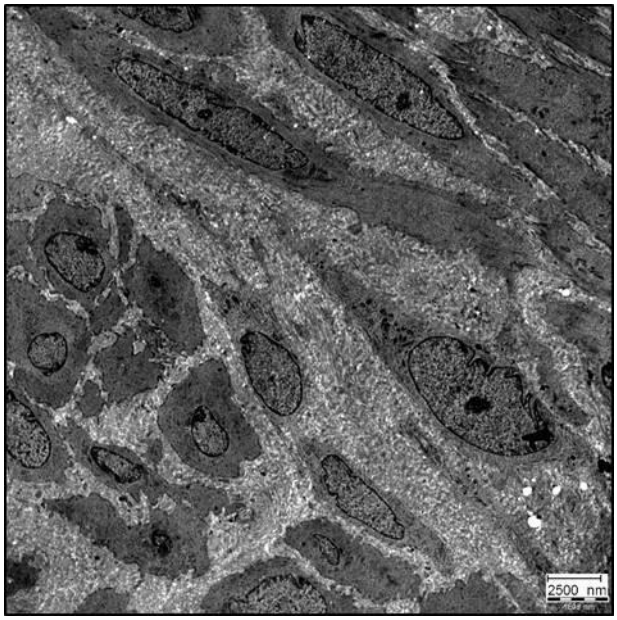

Figure 11: *The inner myometrial smooth muscles are seen arranged in diverse directions in AM uteri. x2500*

Furthermore, a special type of cell was seen in one AM patient, situated between the glandular epithelial cells in the basal endometrium. They had multiple heterogeneous electron-dense vesicles of different sizes and lacked nuclei. Hence, we propose the name “vesiculated cells” (Figure 12). Tight junction were evident on the upper lateral borders of neighbouring endometrial glandular epithelial cells (Figure 13)

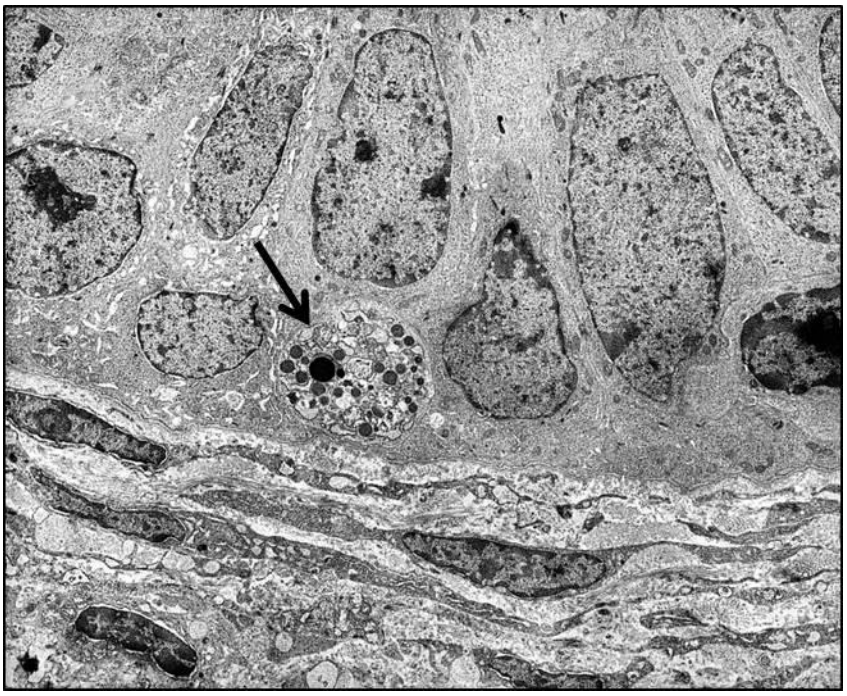

Figure 12: *A vesiculated cell (black arrow) situated eccentrically in the basal endometrial gland in AM. The cytoplasm is full of multiple heterogeneously electron-dense vesicles of different sizes. x2500*

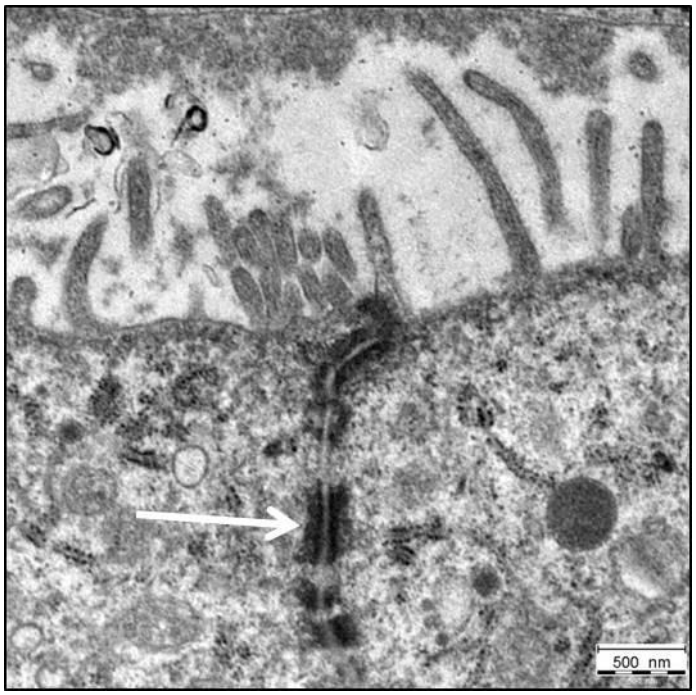

Figure 13: *A tight junction (white arrow) can be seen on the upper lateral cell borders of the basal glandular epithelial cells. x10000*

4.2.3 Uterine pale cells in basal endometrium

Another cell population, found in both groups (20 out of 21 patients - 95%), was characterized by heterochromatic nuclei and mitochondrial and ribosomal abundance in their cytoplasm. Their cytoplasm appeared more electrolucent than those of the surrounding glandular epithelial cells; hence the name “pale cells” (Figure 14). And being seen in the almost all collected uterine samples, we propose their description as “uterine pale cells”.

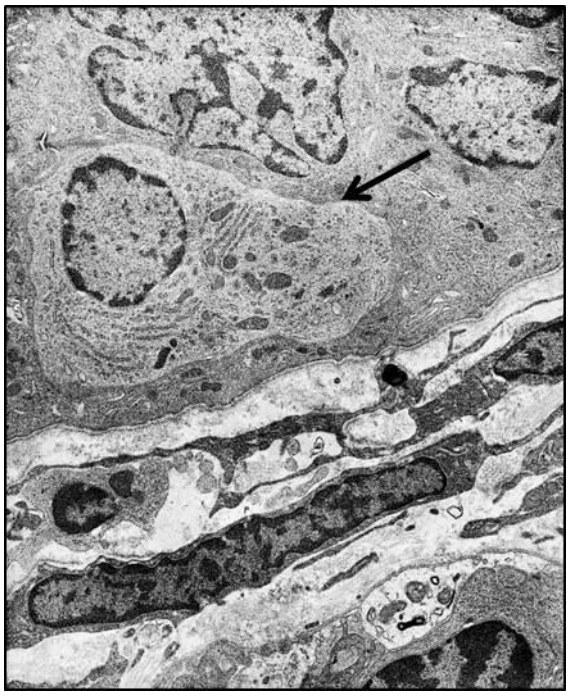

Figure 14: *A pale cell in AM (black arrow). Its cytoplasm is rich in mitochondria and ribosomes and appears more electrolucent than the surrounding glandular epithelial cells. x5000*

- *Location*

Uterine pale cells were seen in the basal endometrial glands in between the glandular epithelial cells in both groups. They were typically eccentrically located in the endometrial glands in most included patients (20/21); away from the gland lumen and in close contact to the basement membrane (BM) of the glands (Figure 15). In only three patients, they appeared concentrically located and in close relation to the gland lumen (Figure 16).

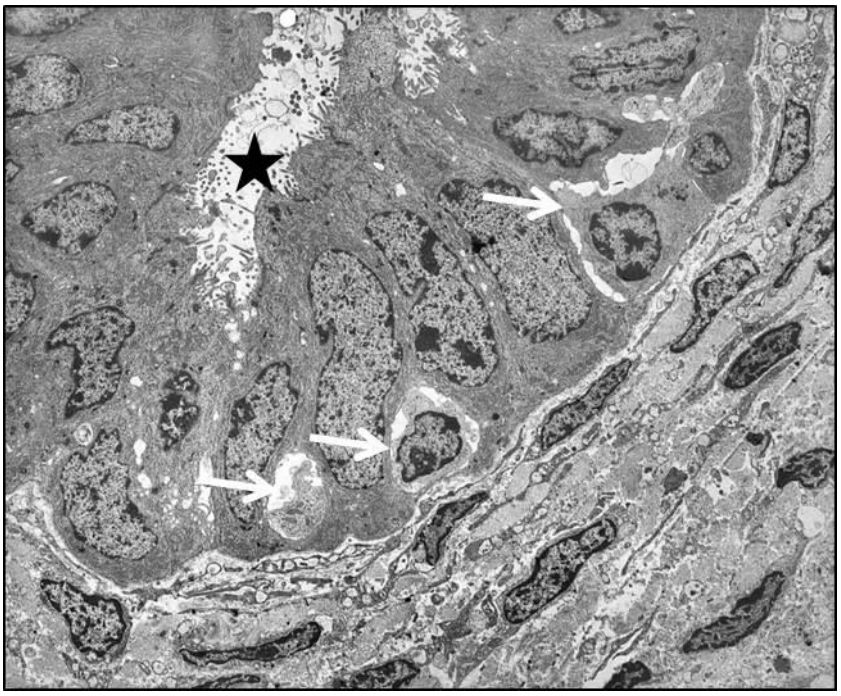

Figure 15: *The eccentric position of the uterine pale cells (white arrows) in the basal endometrial gland, away from the gland lumen (black star). x1600*

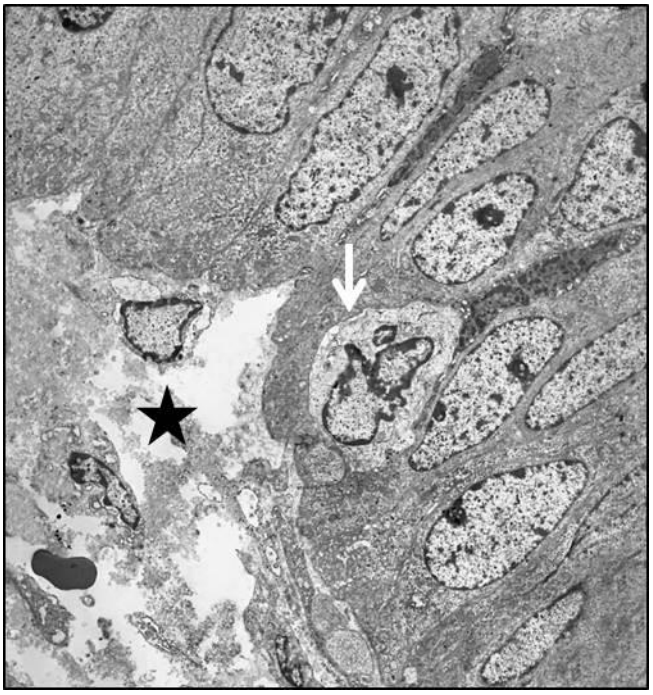

Figure 16: *The pale cell (white arrow) is seen close to the endometrial gland lumen (black star).x1600.*

- *Desmosomes*

Although desmosomes were clearly seen on the membranes of the glandular epithelial cells in the basal glands in both groups, none of the uterine pale cells exhibited a desmosome in all patients (Figure 17).

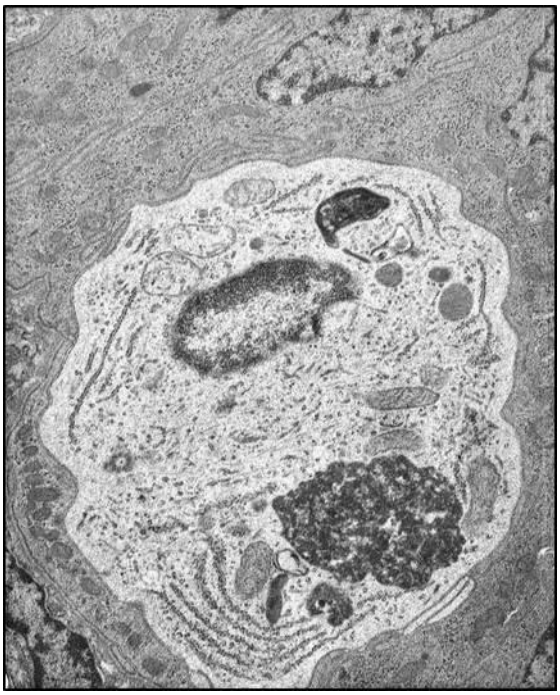

Figure 17: *Desmosomes are lacking on the cell border of the uterine pale cell. x8000*

- *Migration steps*

Furthermore, each pale cell was located in a compartment enclosed by the neighbouring glandular epithelial cells. Well-defined cellular interdigitations were extending from the basal parts of the neighbouring endometrial glandular epithelial cells and interposing between the pale cell above and the basement membrane below. A thin channel was seen between those cellular interdigitations of the glandular cells, running from the compartment occupied by the pale cell to the endometrial stromal compartment (Figure 18).

In AM (9/12, 75%) and in non-AM (6/9, 66.7%) these cellular interdigitations became less complex, with fewer interdigitations in some locations. Furthermore, only a narrow cellular protrusion could be seen interposed between the pale cell above and the basement membrane of the endometrial gland below (Figure 19).

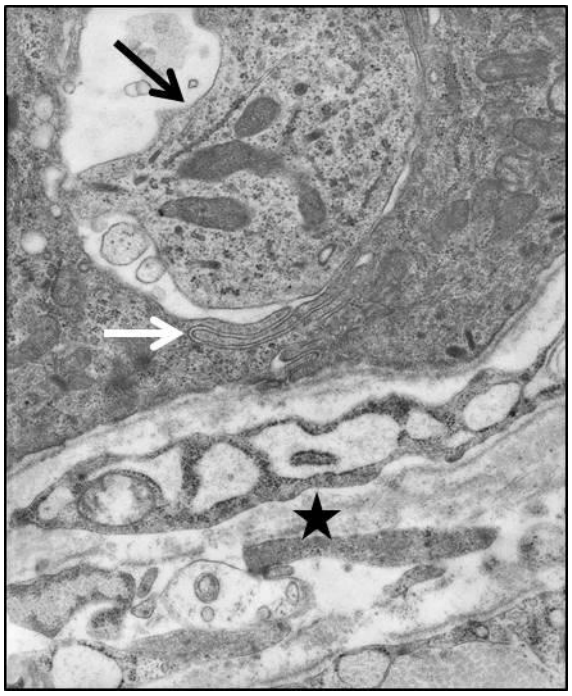

Figure 18: *The uterine pale cell (black arrow) is enclosed by two glandular epithelial cells and thick cellular interdigitations are seen separating the basal part of the pale cell from the endometrial stroma (star). Note the narrow extra-cellular channel (white arrow) running from the pale cell compartment to the stromal compartment. The pale cell border is partially detached from the surrounding epithelial cells. x12500*

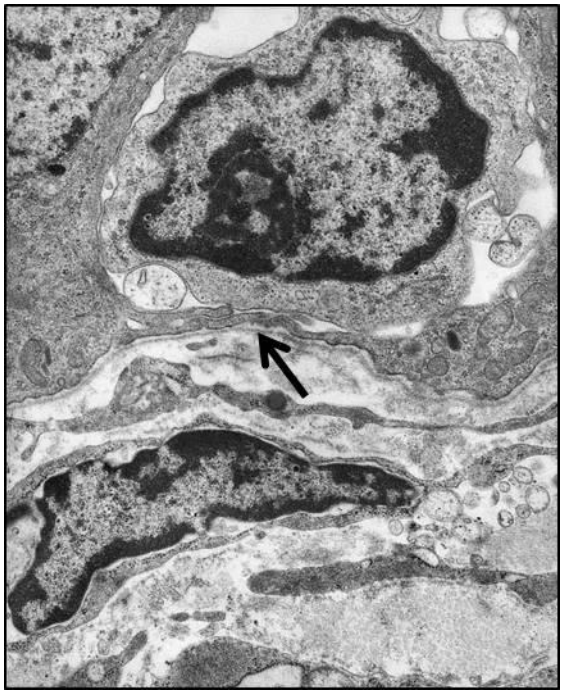

Figure 19: *Marked thinning out of the cellular interdigitations (black arrow) at the basal border of the pale cell. The pale cell border is almost totally detached from the neighbouring glandular epithelial cells.x10000*

The pale cell borders were partially detached from the surrounding epithelial cell borders in 58.3% (7/12) and 55.6% (5/9) of the AM and non-AM groups respectively (Figure 20). They were completely detached from the surrounding epithelial cell borders in 2 out of 12 patients (16.7%) with AM uteri but not in the non-AM group (Table 4 and 5).

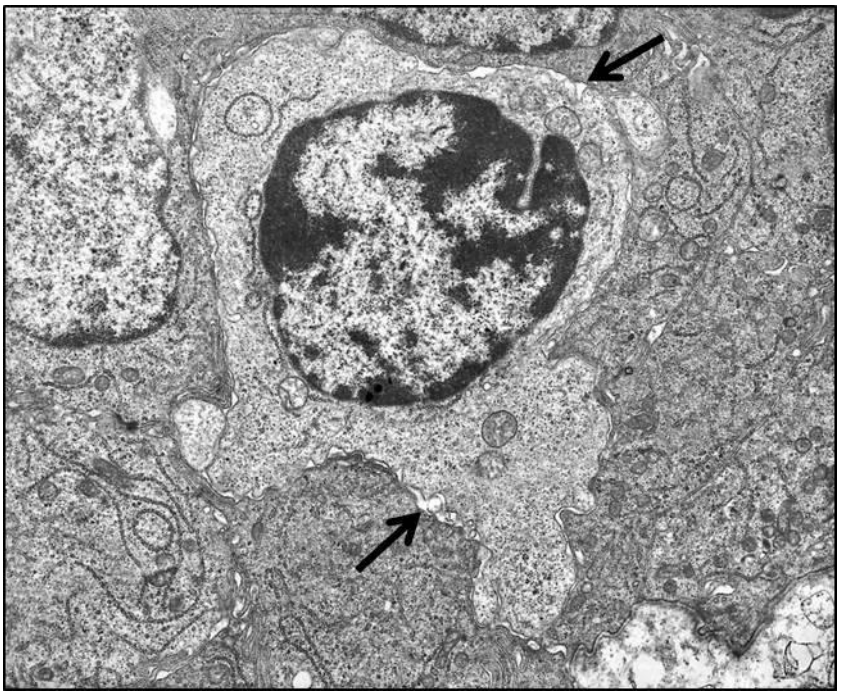

Figure 20: *The pale cell border is partially detached (black arrows) from the surrounding glandular epithelial cells.x5000*

In the basement membrane of the basal endometrial glands, multiple ultra-microruptures extending through the lamina rara and lamina densa were evident in 25% (3/12) of the AM group. Cellular buds (pseudopods) of the uterine pale cells protruded through these ultra-microruptures in 16.7% (2/12) of the AM group (Figure 21), while they were lacking in non-AM. Additionally, the pale cell cytoplasm was almost translocated through an ultra-microrupture of the basement membrane into the surrounding stroma in 16.7% (2/12) of AM, but not in non-AM. Cellular organelles were seen not only in the translocated part of the pale cell but also at the point of basal membrane ultra-microrupture (Figure 22 and 23). In non-AM uteri, neither total detachment, ultra-microrupture of the basement membrane, pseudopods nor cytoplasmic translocation were detected.

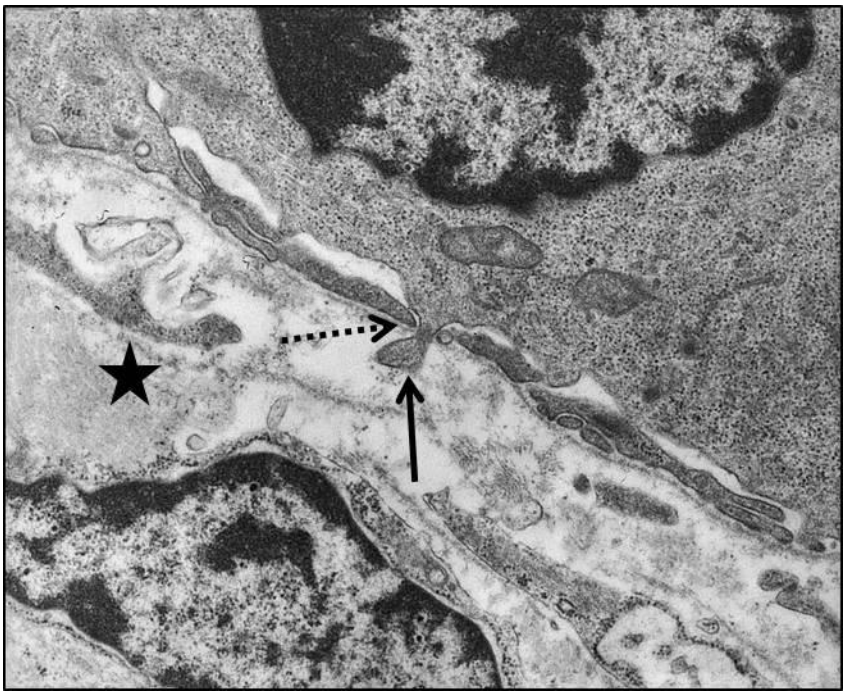

Figure 21: *The cellular pseudopod (black arrow) of a pale cell is projecting into the stroma (star) through an ultra-microrupture in the basal gland basement membrane (dotted black arrow). x12500*

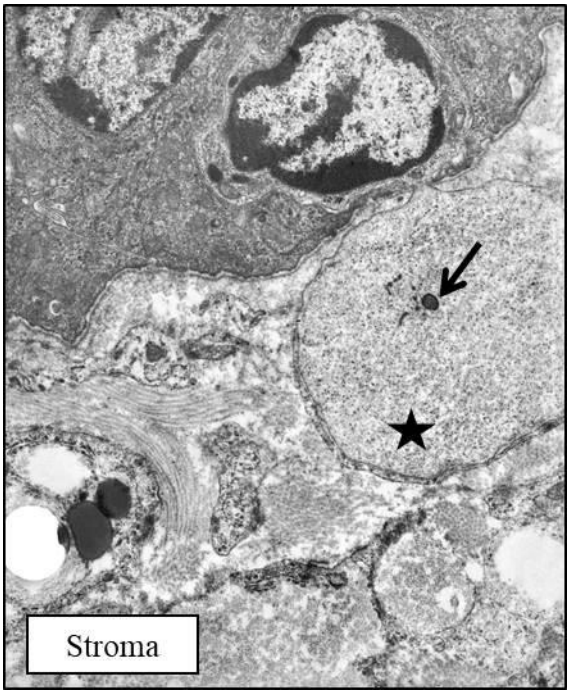

Figure 22: *The pale cell cytoplasm (black star) is almost extra-glandular (inside the stromal compartment) with marked shrinkage of the intra-glandular part. A cellular organelle (black arrow) is seen in the extra-glandular part of the cytoplasm x4000.*

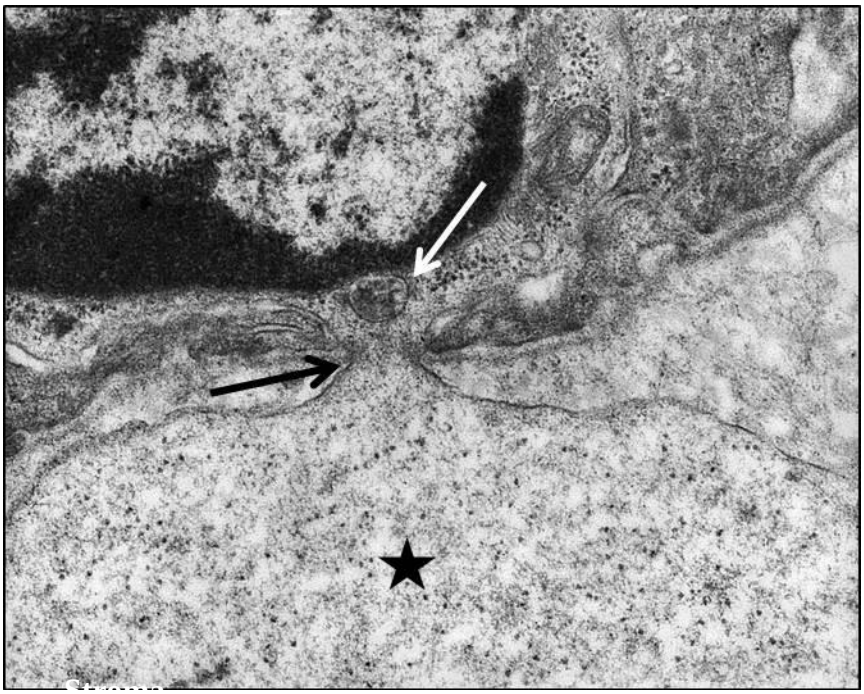

Figure 23: *A magnification of figure 22. A cellular organelle (white arrow) is located intracytoplasmic in the pale cells (black star), at the site of ultra-microrupture of the basement membrane (black arrow). x20000.*

Table 4: *The frequency of occurrence of the different migration steps of the uterine pale cells in non-AM group.*

Pat.	Lack of desmosomes	Thinned cellular interdigitations	Partial detachment	Total detachment	Pseudopods	Basement membrane ultra-microrupture	Cytoplasm translocation
1	yes	yes	yes	_	_	_	_
2	yes	yes	yes	_	_	_	_
3	yes	yes	_	_	_	_	_
4	yes	yes	yes	_	_	_	_
5	yes	yes	yes	_	_	_	_
6	yes	yes	_	_	_	_	_
7	yes	_	_	_	_	_	_
8	yes	_	_	_	_	_	_
9	yes	_	yes	_	_	_	_
Total	**100% (9/9)**	**66.7% (6/9)**	**55.6% (5/9)**	**0% (0/9)**	**0% (0/9)**	**0% (0/9)**	**0% (0/9)**

Table 5 : *Frequency of occurrence of the different migration steps of the uterine pale cells in AM group.*

Pat.	Lack of desmosomes	Thinned cellular interdigitations	Partial detachment	Total detachment	Pseudopods	Basement membrane ultra-microrupture	Cytoplasm translocation
10a	yes	yes	yes	_	_	_	_
11a	yes	yes	_	_	_	_	_
12a	yes	_	_	_	_	_	_
13a	yes	yes	yes	yes	yes	yes	yes
14a	yes	yes	yes	_	_	_	_
15a	yes	yes	yes	_	_	_	_
16a	yes	yes	yes	_	_	yes	_
17a	yes	yes	yes	_	_	_	_
18a	yes	yes	yes	yes	yes	yes	yes
19a	yes	yes	_	_	_	_	_
20a	yes	_	_	_	_	_	_
21a	yes	_	_	_	_	_	_
Total	**100% (12/12)**	**75% (9/12)**	**58.3% (7/12)**	**16.7% (2/12)**	**16.7% (2/12)**	**25% (3/12)**	**16.7% (2/12)**

- *CD45 and CD68 immunolabeling of the uterine pale cells*

To exclude a hemopioetic origin of the uterine pale cells, CD45 staining (a marker for haematopoietic cells) and CD68 (a marker for macrophages) were used. The glandular epithelial and stromal cells in the basal endometrium in both groups were immunolabeled for neither CD45 nor CD68 (Figure 24).

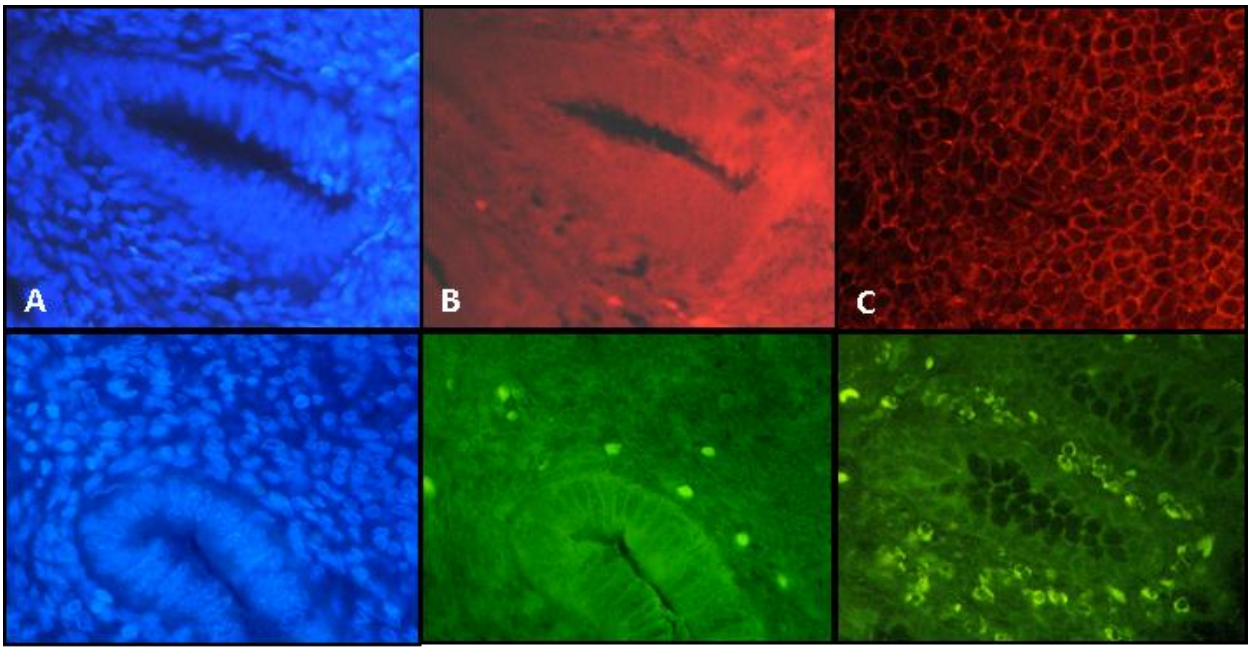

Figure 24 : *CD45 (A - B) and (D - E) CD68 immunolabeling in the basal endometrium of adenomyosis uteri.*

The glandular epithelial cells lack any immunolabeling of either CD45 or CD68. Dapi nuclear stain (A-D). However, positive controls are clearly immunolabeled (C: CD45 immunlabeled immune cells in human spleen and F: CD68 immunolabeled macrophages around the human intestinal crypts).

4.3 E-cadherin immunolabeling in basal endmetrium in AM and non-AM uteri

In order to investigate the cell-cell contact between the epithelial cells in the basal endometrial glands, we studied the immunolabeling of E-cadherin on the membrane of the basal glandular epithelial cells. No statistically significant difference between the two groups ($p>0.05$) was found (Figure 25).

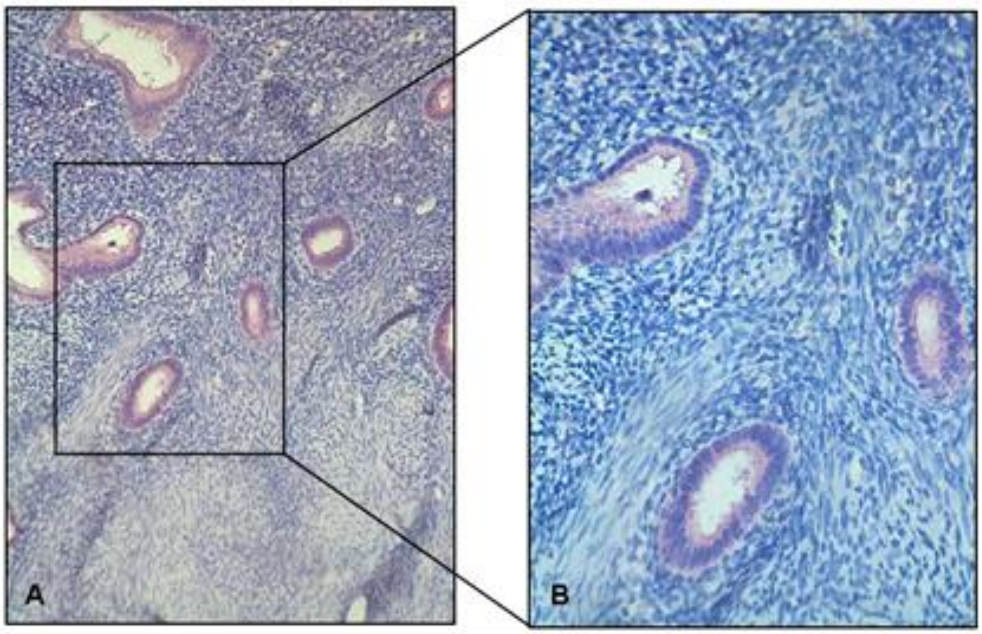

Figure 25 : *E-cadherin immunolabeling on the membrane of the basal endometrial glands at the EMJZ in AM patient.*

The glandular epithelial cells in the basal endometrium exhibit a strong immunolabeling of E-cadherin. This excludes a disruption in the cell-cell contact between the epithelial cells in the basal endometrium in AM-uteriA: x 200, B: x400.

There was no difference between the anterior, posterior or the fundus region of the uterus regarding findings from TEM, Van Gieson staining or E-cadherin immunolabeling.

4.4 ASMA, collagen and desmin immunolabeling in EMJZ

ASMA-immunolabeling was almost absent in the stromal cells of non-AM uteri, except for vascular smooth muscle cells. The latter were considered as internal positive control. However, the stromal cells in the basal endometrium in AM uteri express clearly abundant ASMA. In both groups, the endometrial glandular epithelial cells didn't express any ASMA. The immunoreactive score of ASMA staining was significantly higher in the AM compared to the non-AM group (p=0.0027) (Figure 26).

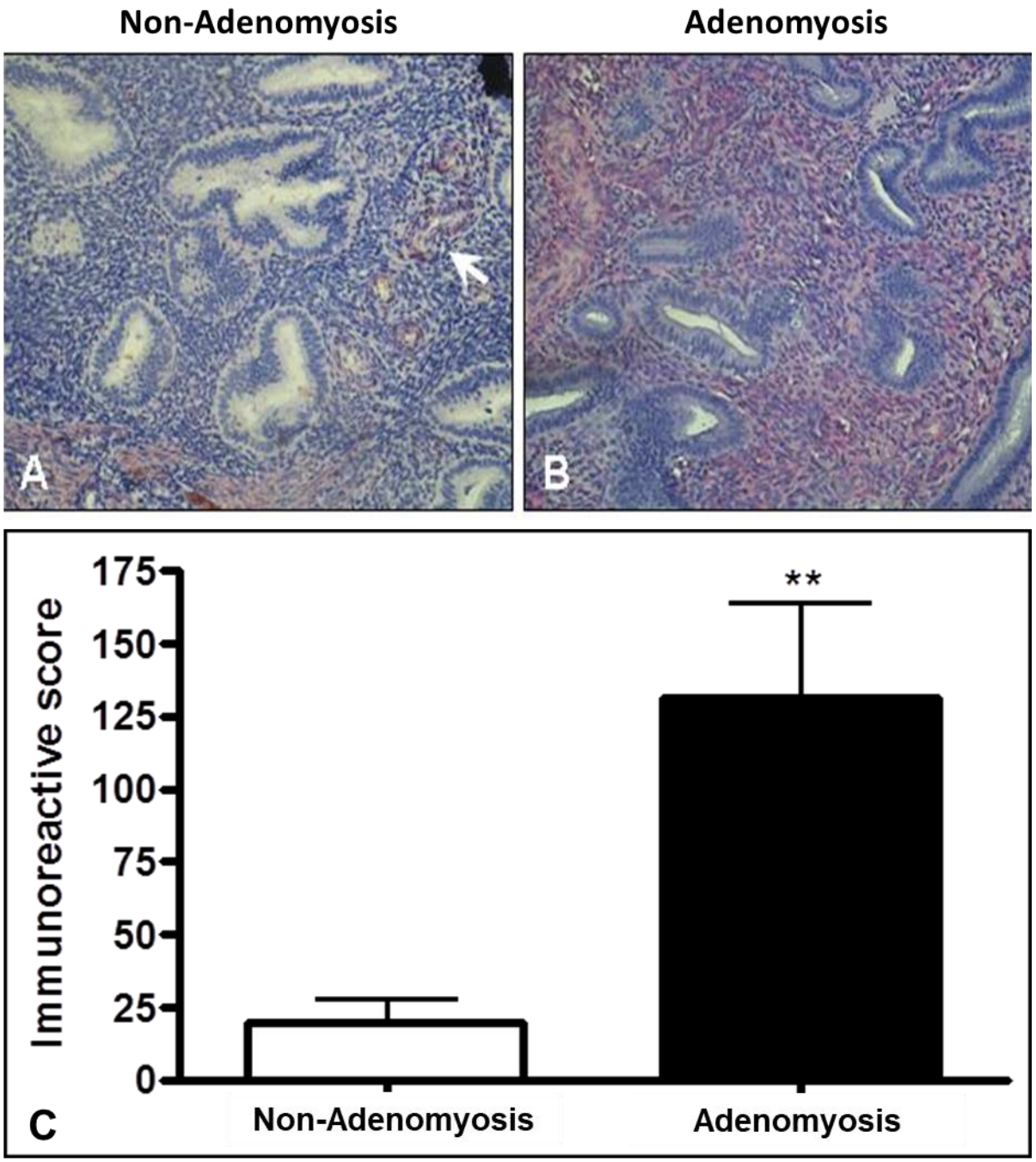

Figure 26: *ASMA cytoplasmic immunolabeling in the basal endometrium (representative sample).*

ASMA-immunolabeling (red) is almost absent in the stromal cells of non-adenoymosis uteri, except for vascular smooth muscle cells (arrow) (A). However, the stromal cells in the basal endometrium in AM uteri express clearly abundant ASMA (B). In both groups, the endometrial glandular epithelial cells didn't express any ASMA. x200. The immunoreactive score of ASMA staining is significantly higher in adenomyosis compared to non-adenomyosis group (C) (p=0.0027).

Collagen I was expressed in the cytoplasm of the basal endometrial epithelial and stromal cells and in the smooth muscle cells in the inner (IM) and outer myometrium (OM). Its immunolabeling in the inner myometrium was significantly higher in AM than in control (p=0.0185) (Figure 27).

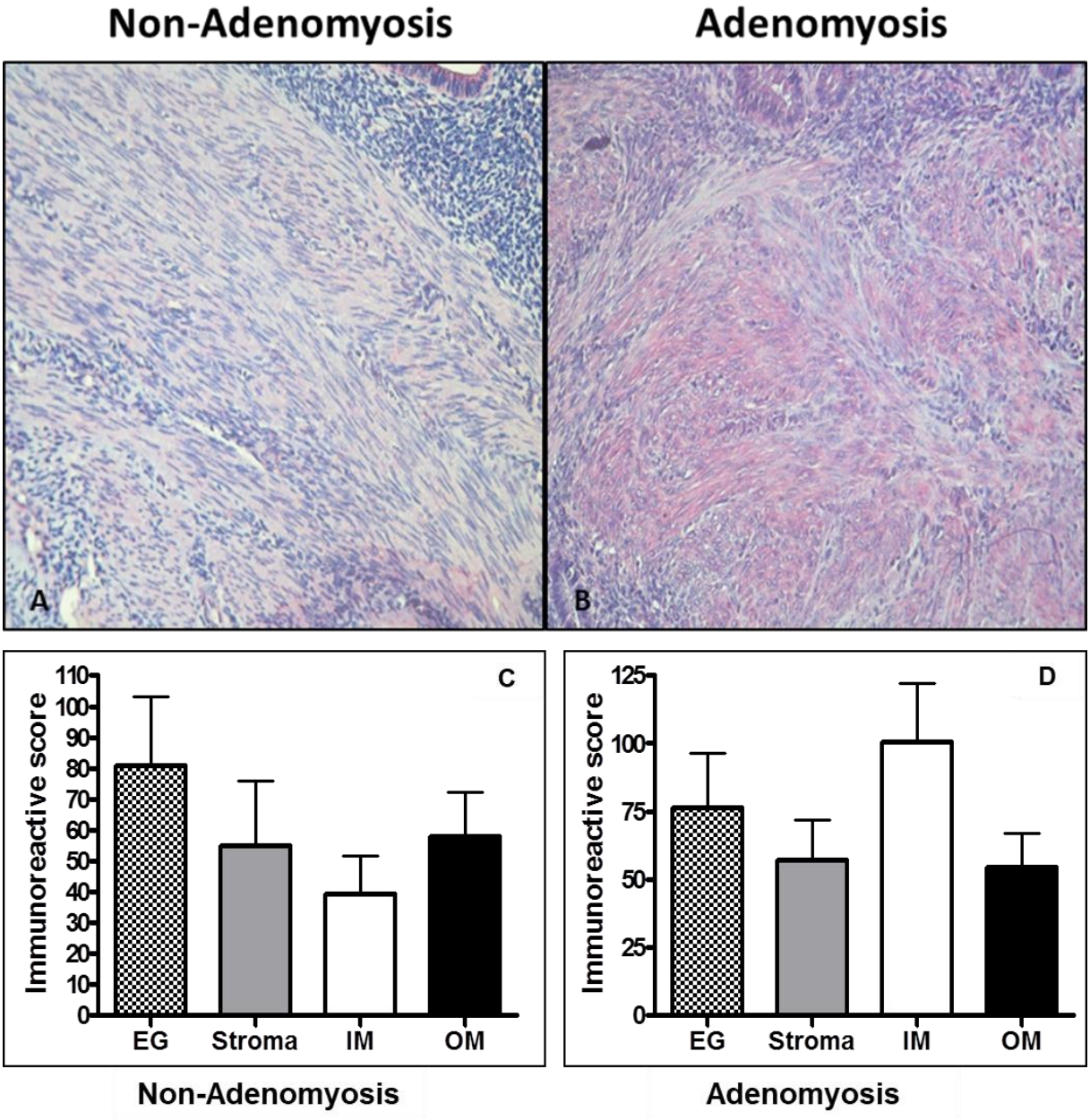

Figure 27: *Collagen I cytoplasmic immunolabeling (red) is higher in the inner myometrium (IM) in (B) adenomyosis than in (A) non-adenomyosis uteri (representative sample).*

Note the basal endometrial glands and stroma in the upper right (A) and left corners (B), marking the boundary of the endometrial-myometrial junctional zone. x200. Collagen I immunolabeling in the different layers of (C) non-adenomyotic uteri (p=0.4380) and (D) adenomyotic uteri (p=0.2435) was not significantly different. However, the immunoreactive score of the collagen I immunolabeling is significantly higher in the IM of adenomyosis uteri (D), compared to non-adenomyosis (C) (p=0.0185).EG: endometrial glands, IM: inner myometrium, OM: outer myometrium.

In the EMJZ, desmin immunolabeling was restricted to the smooth muscle cells in the inner and outer myometrium. Both glandular epithelial cells as well as stromal cells weren't immunolabeled for desmin (Figures 28 and 29)

4.5 TGFβR1, 2 and 3 receptor immunolabeling in EMJZ

TGFβR1, 2 and 3 immunolabeling was localised to the cytoplasm of the glandular epithelial and stromal cells in both the AM and non-AM groups. TGFβR1, 2 and 3 immunolabeling in the basal glandular epithelial cells didn't show any significant difference between both groups ($p=0.4508$, 0.5726 and 0.1692 respectively). The same was observed in the stromal cells in the basal endometrium in both groups ($p=0.0884$, 0.7525 and 0.4554 respectively).

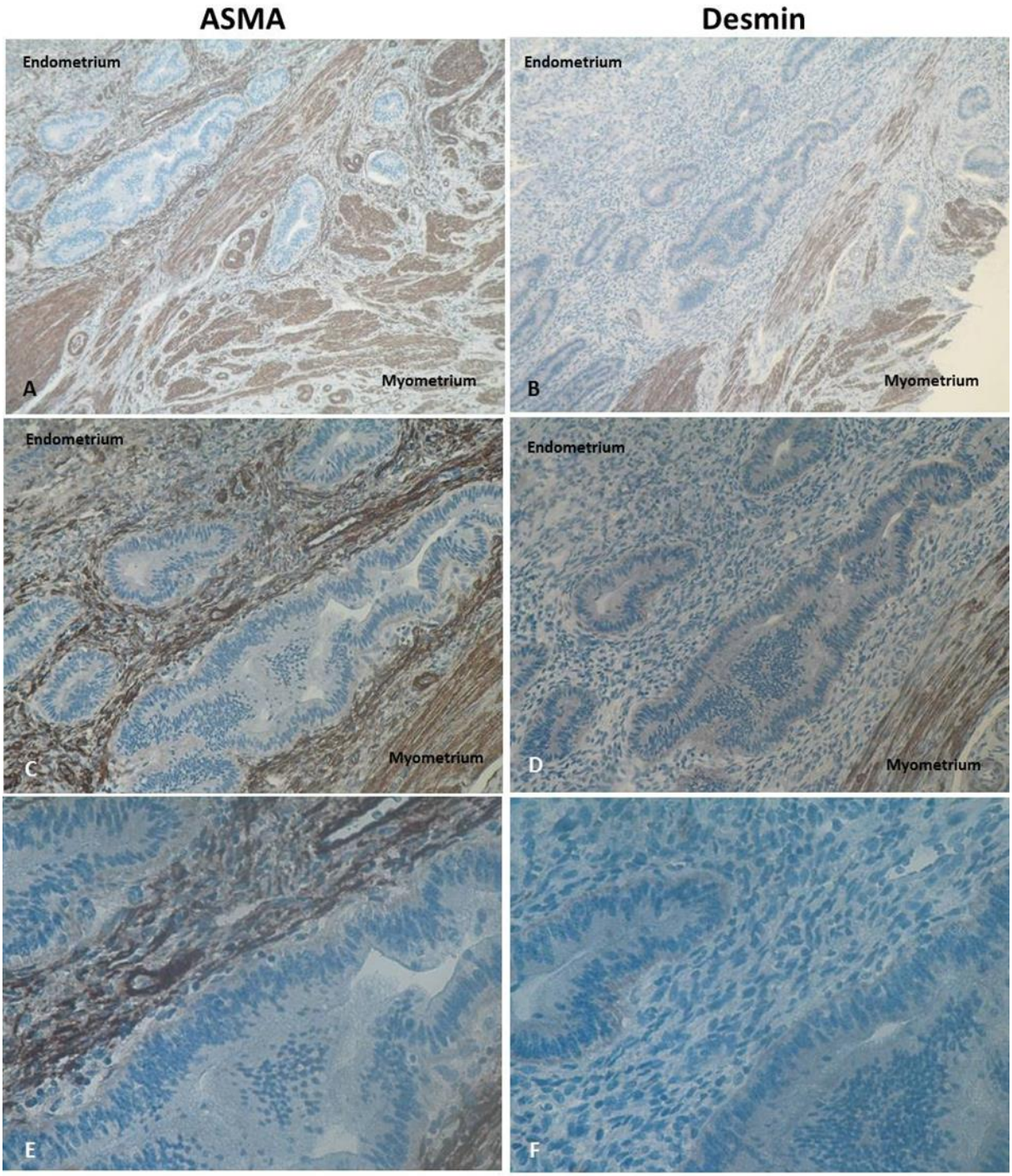

Figure 28: *ASMA and desmin immunolabeling in the basal endometrium in AM (serial sections, representative sample).*

ASMA cytoplasmic immunolabeling is evident in the stromal cells and in the inner myometrial cells (A,C and E). However, desmin cytoplasmic immunolabeling was only evident in the inner myometrial cells (B,D and F) and the stromal cells don't express Desmin at all. (A-B) x100, (C-D) x200, (E-F) x400.

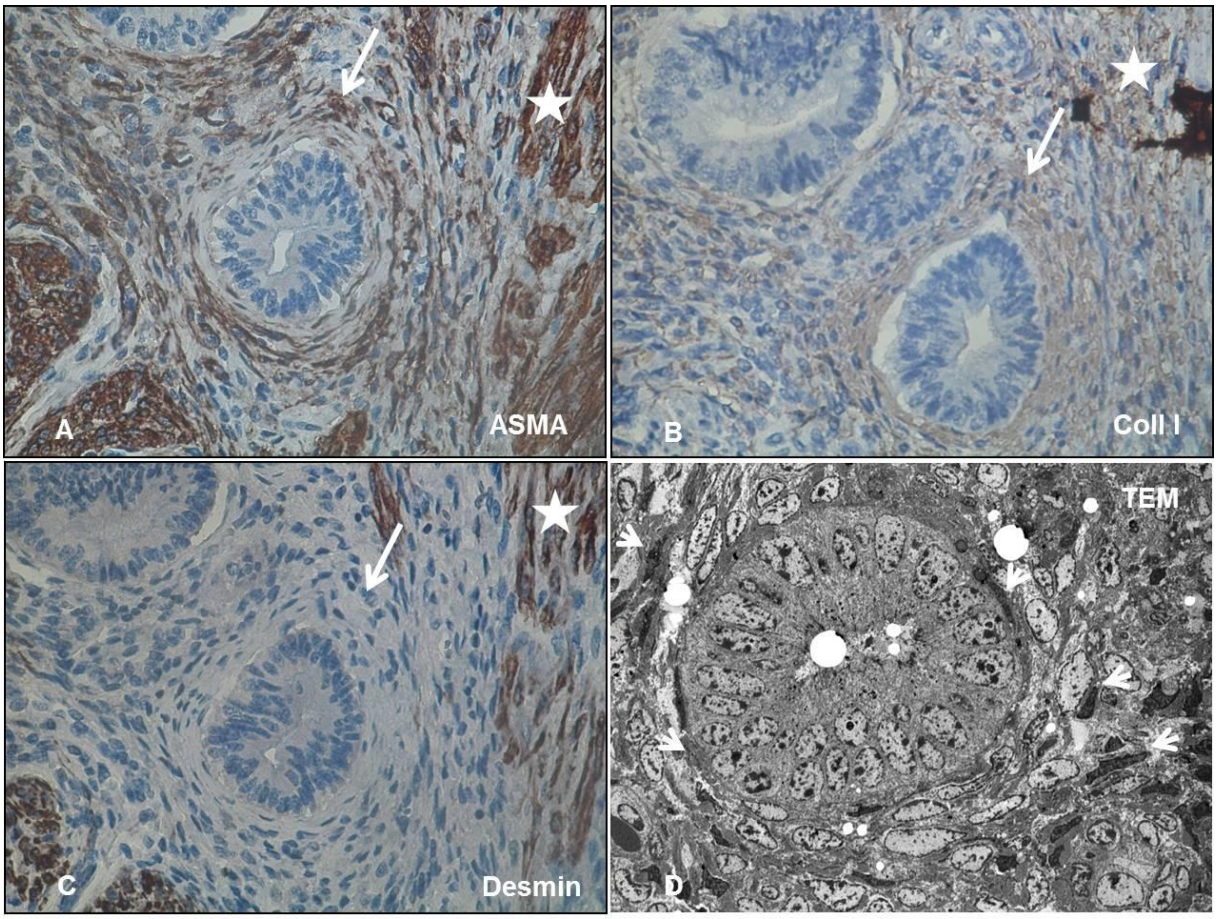

Figure 29: *Serial sections of the Endometrial-Myometrial Junctional Zone of adenomyotic uteri (representative sample).*

ASMA-expressing stromal cells (white arrows) are concentrically arranged around the basal endometrial glands (A), while they express collagen I (B) but do not express desmin (C). Smooth muscle cells in the inner myometrium (stars in A, B and C) express ASMA, collagen I and desmin.x400. Myofibroblasts are seen spindle-shaped with heterochromatic nuclei and electro-dense cytoplasm (short white arrows) (D). TEM: Transmission Electron Microscopy.

5. Discussion

5.1 Findings of tissue injury in EMJZ in AM uteri

In our study, we provided new findings of tissue injury at the EMJZ in AM uteri. The latter exhibits a fissured EMI together with diversely-arranged smooth muscle cells in the inner myometrium. Furthermore, the high collagen I immunolabeling in the IM as well as the high ASMA immunolabeling in the basal stroma of AM uteri support the presumed increased mechanical tension at the EMJZ. Moreover, infolding of the nuclear membrane of the basal endometrial glands in AM may add a further indication of a tissue injury. In what follows, these different microscopic and ultra-microscopic findings in AM uteri will be discussed in detail.

5.1.1 Finding 1: Fissuring of EMI and diversely-arranged SMCs in IM in AM uteri

In non-AM uteri the basal endometrium meets the inner myometrium at a smooth regular interface. Contrarily, the EMI in AM uteri is disrupted at multiple points, where the basal endometrium dips into the underlying myometrium, resulting in fissuring of the smooth regular EMI in AM. The same was seen in AM (2) as well as in endometriosis patients (27) in previous studies.

This fissuring is also seen with TEM. The myocytes are arranged around the basal endometrial glands in AM in a way that the smooth regular interface to the basal endometrial glands and stroma is completely lost. Again, non-AM uteri exhibit a regular and smooth EMI. A previous study showed also ultrastructural differences between the myocytes in IM and OM in AM. The earlier exhibited smaller nuclei (43).

Fissuring of EMI in AM uteri may explain the previously published finding of communication between the uterine cavity and adenomyotic lesions during sonohysterography in a case series of four patients (44). This may represent microscopic tracks, created between the myometrial muscle bundles secondary to chronic tissue trauma, and thorough which the contrast media during sonography can extravaste into the uterine wall. Furthermore, this fissuring may be a reasonable

explanation for accidental extravasation of methylene blue staining into the uterine wall during a chromopertubation (45).

The loss of the parallel arrangement of SMC bundles in the IM in AM mimics the disarray of the muscle fibers at surgical uterine injuries (e.g. Caesarean section scar, myomectomy scar or endomyometrial ablation) in the course of tissue healing (46). Furthermore, increased intrauterine pressure like in dysperistalsis induces an upregulation of stress facos at endometrial myometrial interface (47). Experimentally-induced AM in mice led to loss of the parallel arrangement of the myometrial muscle cells (48, 49).This may be of a valuable for the understanding of the pathogenesis of AM, as abnormal muscular orientation is linked to a suboptimal smooth muscle contraction leading to muscular hypertrophy (50). The altered contractility of the inner myometrium is associated with obvious ultrastructural hypertrophy of the inner myometrial myocytes in AM (6). Furthermore, the activation of β-catenin (essential for maintenance of normal tissue architecture) in the uteri of mutant mice led to an abnormal myometrial structure with an interwoven appearance associated with increased myometrial thickness and an accelerated proliferation rate of the myocytes (51). In AM, the cellular density seems to decrease together with the increased nuclear hypertrophy in both the IM and OM and the muscle fascicles rearrange again which can partially explain the abnormal peristalsis in AM (6) (5).

This supports the concept of tissue injury at the EMJZ in AM with an abnormal repair pattern. The regenerating smooth muscle fibers in the inner myometrium tend to be re-arranged in diverse directions, giving the interwoven appearance of the inner myometrium instead of the linear parallel pattern.

5.1.2 Finding 2: High collagen I immunolabeling in IM of AM uteri

Collagen I is a major component of the extra-cellular matrix (ECM) in the uterus representing 80% of the whole collagen (52), and mainly secreted by fibroblasts(53). In our study, collagen I immunolabeling in the IM in AM uteri was significantly higher than in non-AM uteri. This may support the presumption of increased shearing stress at the EMJZ in AM than found in non-AM. The same was previously seen in other studies (54, 55). The shearing stress consequently induces a cascade of events in the attempt to repair the injured tissue with subsequent collagen deposition.

Furthermore, and in an in-vivo study, collagen I expression was upregulated upon induction of AM by tamoxifen (56). Collagen I is thought to interrupt any communication between the superficial endometrial glands and the adenomyotic lesions (57).

However, the non-significant difference in collagen I immunolabeling in the basal stroma in both AM and non-AM can support the concept of the scarless endometrial repair seen normally following menstruation.

5.1.3 Finding 3: High ASMA immunolabeling in the basal stroma in AM uteri

Mechanical tension at the site of the wound injury is the stimulus for the transformation of the fibroblasts into myofibroblasts which in turn contract to minimise the size of the granulation tissue and hence minimize the scar size (58). ASMA is a good marker for identifying those myofibroblasts in the different tissues, being an integral component of the cellular contractile apparatus (59).

The stromal cells in the basal endometrium in the non-AM group expressed ASMA in our study. This may support the fact that normal uterine peristalsis induces a (physiological) shearing stress at the EMJZ during the normal menstrual cycle. Nevertheless, ASMA immunolabeling was absent in four patients. The latter points to a transient existence of the myofibroblasts during the normal tissue healing which subsequently disappear as soon as tissue healing is completed (31).

In AM uteri, ASMA immunolabeling in the stromal cells was significantly higher than that in non-AM group. Again, this points to an increased shearing stress at the EMJZ in AM which in turn induces a myofibroblastic metaplasia hallmarked by the high ASMA immunolabeling. In accordance to our results, myofibroblasts in adenomyosis overexpressed ASMA and collagen I (54, 55). Functional inhibition of ASMA in the myofibroblasts in-vitro reduced the force of contraction of these cells, together with inhibiton of collagen I production. This may propose a close correlation between ASMA and collagen I production in functionally active myofibroblasts (60).
We speculate then that the basal stromal cells (59) and the inner myometrial cells could intermingle with each other and therefore giving the microscopic fissuring seen

at the EMI (2).. Supporting the presumption of cellular intermingling at the EMJZ in AM, the stromal cells were previously seen in AM interposed between the muscle fascicles in the IM. The muscle fascicles themselves became thinner and lost their architecture (57). Furthermore, collagen I can stimulate the endometrial epithelial cell proliferation (61) which might favour the dipping of the endometrial glands into the myometrium, building up new lesions.

5.1.4 Finding 4: Infolding of the nuclear membrane of the basal endometrial glands in AM uteri

The nuclear membranes of the glandular epithelial cells in the basal endometrium of AM uteri are irregular and exhibit multiple nuclear membrane infolding and grooving. Contrarily, the nuclear membranes of the basal endometrial epithelium in the non-AM group are smooth and regular without any infolding. Similar ultrastructural changes were seen by another group, where the epithelial cells exhibited larger nuclei in AM than non-AM (43).These nuclear irregularities may be caused by (pathological) mechanical stress secondary to hyperperistalsis (62). On the other hand, it may reflect an abnormal cellular behaviour in favour of invasion, as seen in the well-differentiated endometrial adenocarcinoma (63). Supporting the last assumption, the nuclear membrane of the endometriotic epithelial cells in 19 patients with ovarian EM was also irregular (64).

5.2 No evidence of a translocation of the basal endometrial glands into the myomterium in AM uteri

To date, it was supposed that stromal invasion precedes the glandular invasion into the myometrium during the course of AM development (49). Involution of the IM and widening of the extracellular spaces were considered crucial steps in easing the endometrial invasion (48, 49). Mehasseb showed the increased invasiveness of the stromal cells isolated from uteri of adenomyotic patients when co-cultured with myometrium from AM patients, reflecting the necessity of stromal-muscular interaction in the invasion process (6). Moreover, Matrix Metalloproteinase (MMP) exhibit higher concentration (especially MMP-2 and MMP-9) in the uterine fluid (lavage) from AM uteri (65), and the endometrial stromal cells isolated from AM express higher levels of MMP-2 and 9 than non-AM(66). Moreover, and based on

MRI imaging of AM uteri, smooth muscle proliferation and hyperplasia precede the endometrial invasion (67). Leyendecker found that menstrual blood of adenomyotic patients contains basal endometrial fragments with a stem cell-like character. Through a process of retrograde menstruation of these fragments into the abdominal cavity or their translocation into the myometrium, EM or AM is developed respectively (68). Nevertheless, it was observed that apoptosis and bcl-2 expression (anti-apoptosis) in the adenomyotic lesions differ completely from that occurring in both epithelial and stromal cells of the basal endometrium, supporting that AM is not just developed from the translocation of the basal endometrial glands (69), (70).

In our study, no evidence of a translocation of the basal endometrial tissue into the myometrium was found, as described in the following:

5.2.1 Intact desmosomes and adherens junctions between the basal glandular epithelial cells

The cell polarity and arrangement in the different organs are usually maintained through a big group of different cell-cell contact types. The desmosomes and the adherens junctions are important members in this group.

Desmosomes are clearly seen - with TEM - between the endometrial epithelial cells in the basal endometrial glands in AM and non-AM patients with no significant difference between both groups. Moreover, the glandular epithelial cells in AM are completely intact without any evidence of any cell disruption. Furthermore, the immunolabeling of E-cadherin – a component of the adherens junction – shows no significant difference between both groups. This goes well with the previously published data showing the non-significant difference in the E-cadherin immunolabeling either in the basal endometrial glands or in the adenomyotic glands (71).

Accordingly, and as the basal endometrium lacks any evidence of cell-cell disruption in the glandular epithelium, the theory of translocation of fragments of the basal endometrium into the myometrium as a step in the AM pathogenesis should be reappraised again.

5.2.2 Migrating non-hematopoietic uterine pale cells in the basal endometrial glands

Based on TEM, a unique cell population is described which was located among the epithelial cells of the basal glands at the EMJZ in both groups. In the basal endometrial glands, they are mostly eccentrically-located with direct opposition to the basement membrane of the basal glands, while fewer are concentrically-located near to the glandular lumen. An abundance of the cytoplasmic organelles (mitochondria and ribosomes) was characteristic in those cells, which may point to a high cellular activity.

Furthermore, the cytoplasm appears paler than the surrounding glandular epithelial cells with the light microscope (Van Gieson stained) and more electro-lucent with TEM; hence the name “pale cells”. In this regard, they resemble the chromophobe cells in the anterior pituitary gland which function as reserve cells. The cytoplasm of the chromophobes is rich in ribosomes and Golgi apparatuses reflecting high metabolic activity, but with sparse secretory granules. The same was previously seen by another group (43). Their cytoplasm appeared paler than the surrounding chromophil cells and refused any histological stain, the same as the uterine pale cells at the EMJZ did (72) .

Uterine pale cells have a unique feature, being motile. They lack any desmosome with the neighbouring glandular epithelial cells. The same was described previously, as the pale cells were seen in the peritoneal endometriotic lesions (pEM) (73). Moreover, another research group described the abundance of single E-cadherin negative epithelial cells among E-cadherin positive glandular epithelium in both endometriotic lesions as well as the eutopic endometrium (74, 75). The loss of E-cadherin guarantees not only a free mobility of the cells in the surrounding tissue but also is a characteristic of acquisition of cell invasiveness. The latter was approved in an in-vitro study, where the E-cadherin negative epithelial cells (isolated from peritoneal endometriotic lesions) were more abundant and solely invasive into the collagen gel than those isolated from normal endometrium.

Additionally, these cells detach their intact cell borders partially or completely from the neighboring glandular epithelial cells. Surprisingly, while the basement membrane

of the basal endometrial glands is intact in the non-AM group, it shows multiple ultramicroruptures at different sites - and only in opposition to the uterine pale cells - in the AM group. The basement membrane is found everywhere when the epithelial or the endothelial cells come in contact with the extra-cellular matrix. It is composed of two layers: lamina rara (less electro-dense) and lamina densa (more electro-dense). Collagen IV, laminin and heparan sulphate are the unique constituents of the basement membranes (76). The basement membrane is cycle phase independent and is even seen in the endometrium of postmenopausal patients (77).

It is known that the basement membrane is considered a barrier to invasion by malignant epithelial cells. It is continuous and shows no breaks with light microscopy in endometriotic lesions (78).

In our study (for the first time) multiple ultramicroruptures were seen only in the basement membrane of the basal endometrial glands in AM but not in non-AM. Through these ultra-microruptures, the uterine pale cells were extending cytoplasmic processes (pseudo-pods) mimicking an amoeboid movement to migrate into the stroma.

Ultrastructural abnormality of the basement membrane has been previously described in the ectopic endometrium of AM uteri and in ovarian endometriomas, being tortuous (64). The normal endometrium, even during the time of menstruation, doesn’t show any basement membrane discontinuity, although in atypical endometrial hyperplasia and endometrial carcinoma the basement membrane is largely disrupted (79). As these ultramicroruptures of the basement membrane in AM uteri are seen only with TEM, we would prefer to use the term “ultramicrorupture” for more precise description.

The uterine pale cells exhibit ultra-microscopic processes (pseudopods), sometimes partially extending into the underlying endometrial stroma, suggesting an active migration process into the stroma. In this context, this resembles the migration process of the leucocytes from the blood stream to the extracellular tissue in response to trauma or inflammatory stimuli (especially IL-1β). Leucocytes migrate

through the basement membrane and resulting defects in the basement membrane are usually repaired by the vascular endothelium.

Furthermore, the cell organelles are seen translocated from the uterine pale cell's body into the pseudopod too. Similar ultrastructural changes were previously seen in the polymorph nuclear leucocyte (PMNL) migrating between the endothelial cell and the pericyte during inflammation (80). Both suggest an active migration process.

To exclude a haematopoietic phenotype of the uterine pale cells, immunolabeling with CD 45 and CD 68 was done. The uterine pale cells were proven to be neither haematopoietic cells nor macrophages invading the endometrial glands. It is known that the endometrial stromal cells normally lack CD45 expression (81) and that the endometrial stem cells do not express the haematopoietic markers (CD34 and CD45) as well (82). Nevertheless, CD45+ Leucocytes are increased in endometritis, correlate with the degree of inflammation, and are usually located beneath the surface epithelium and around the superficial glands and the blood vessels. These changes lack in a healthy endometrium (83) which is the same case in our study as none of the included patients had endometritis.

In previous studies, and in the context of the eccentric and concentric localisation of the uterine pale cells, a unique cell population was described expressing Musashi-1, co-localised with Notch and Telomerase (84, 85). These cells were also eccentrically and concentrically located in the glandular epithelium compartment in the endometrium and endometriotic lesions. Musashi-1 is a known neural stem-cell marker (86), (87).

Upon characterization of the endometrium with different stem cell markers (Musashi-1 (86), SSEA/ SOX-9 (88), LGR5 (89) and N-cadherin (4)), it is strongly believed that endometrial stem cells reside in the basalis endometrium. Their role in the pathogenesis of endometriosis is widely studied (90). Nevertheless, their role in adenomyosis still demands further research.

Whether uterine pale cells could be the previously described Musahi-1 expressing endometrial epithelial cells, demands an in-vitro isolation for further characterisation.

Based on the previous findings, we presume that the AM develops not through the translocation of the basal endometrial glands into the stroma but might be through the active migration of a unique non-haematopoietic cell population (uterine pale cells). Ultra-microperforations seen in the basement membrane of the basal glands in AM together with the pseudopods formation support the active migration of these cells.

5.3 Does the immune-expression of the TGFβR1, R2, R3 at the EMJZ show any difference between AM and non-AM patients?

Upon mechanical tissue injury (e.g. skin), TGFβ1 is secreted and mediates the transformation of the fibroblasts into myofibroblasts. The latter contract and minimise the size of the granulation tissue in the course of the healing process (58).

Although in our study no significant difference between AM and non-AM regarding the immunolabeling of the three different TGFβ receptors is evident (55), there is a preferential immunolabeling of TGFβR2 over the other two receptors in non-AM and AM (though non-significant in AM). The insignificant difference in TGFβ receptors between both groups can be explained as follows: It seems that TGFβ1 action is controlled not through regulating the expression of its receptors, but rather through its local activation. The normal glandular endometrial cells, as well as the eutopic endometrium of the EM express the urokinase-type Plasminogen Activator (uPA) which activates the plasminogen into plasmin. The latent TGFβ1 is cleaved by plasmin into active TGFβ1. Plasmin, plasminogen and also uPA are higher in the chocolate cyst fluid than in the peritoneal fluid of the EM. This supports the presumption of a local activation of TGFβ1 (91). Moreover, the TGFβ1 level in the uterine lavage was found to be higher in AM than in non-AM (65). TGFβ1 helps the normal endometrial tissue repair following menstruation by inhibiting the unnecessary endometrial stromal cell (ESC) proliferation and stopping their further migration. This could prevent the excessive repopulation of the endometrium by ESC. Furthermore, they stimulate the contractility of ESC to minimise unwanted expansion of the regenerating tissue (58) and even stimulate ASMA expression in the decidualised stromal cells (92).

6. Conclusion

Based on our results, we could reappraise the pathogenesis of adenomyosis as following and provide a new insight into the pathogenesis of the disease:

First, the shearing stress induced by physiological and pathological peristalsis is confined to the EMJZ and is evident in the significantly high immunolabeling of ASMA and collagen I in the basal endometrium and in the IM in AM uteri respectively. Moreover, both fissuring of the EMI in AM as well as the intermingling appearance of the smooth muscle bundles in the IM of AM uteri support a shearing stress at EMJZ..

Second, it might be the uterine pale cells – but not the whole basal endometrial gland – which (may be under certain conditions) may be directly involved in the pathogenesis of AM. The uterine pale cells migrate into the stroma of the basal endometrium and subsequently into the myometrium in AM patients. They probably migrate actively and are not just being passively translocated through the basement membrane of the basal endometrial glands.

The migration of uterine pale cells may be staged in the following steps: loss of desmosomes, partial detachment of the cell border from the neighbouring cells, thinning-out of the basal cellular interdigitations, complete detachment of the cell border from the neighbouring cells, pseudopod formation, ultra-microrupture of the basement membrane and finally cytoplasmic translocation (Figure 30).

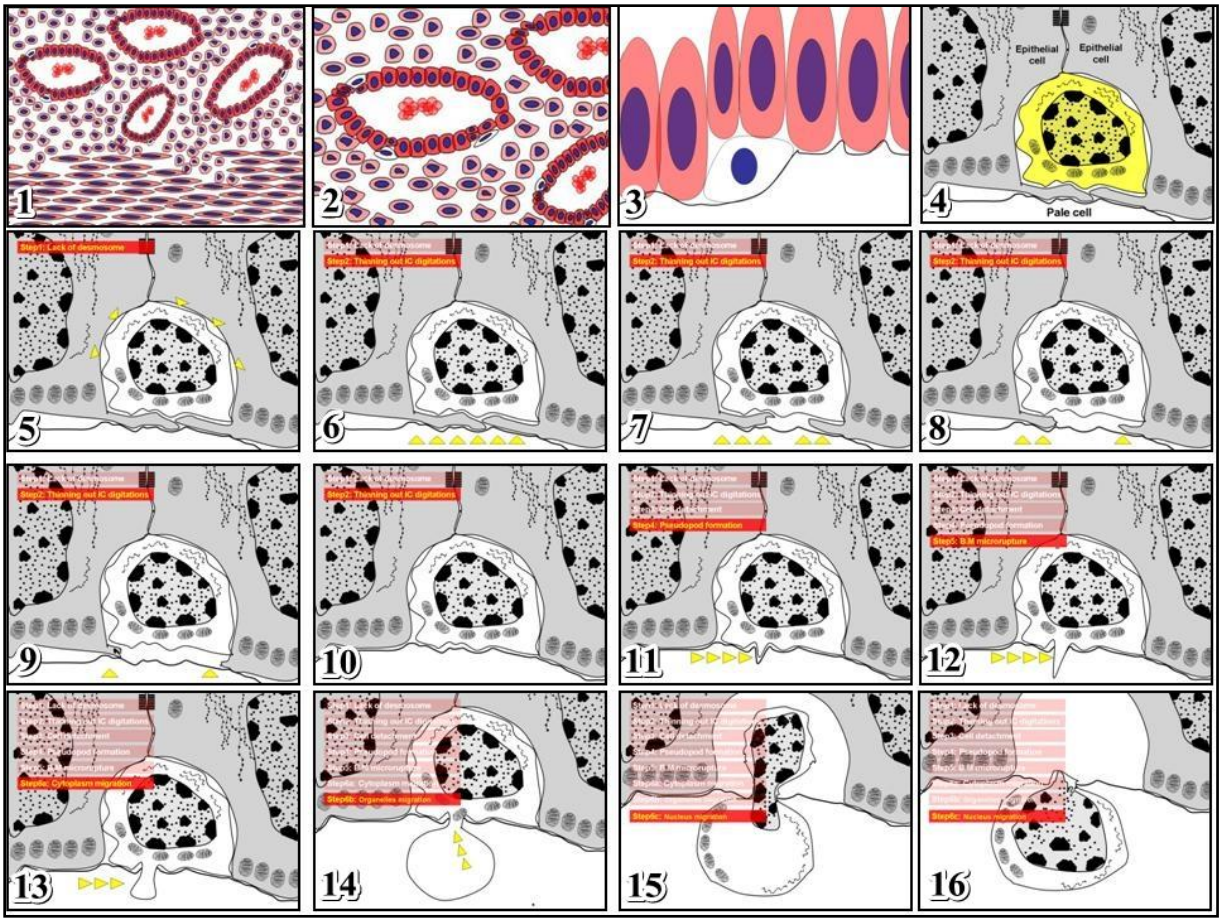

Figure 30: *Collective diagrammatic illustrations of the different migration steps of the uterine pale cells into the stroma.*

The uterine pale cells are usually eccentric between the glandular epithelial cells (1-3). The pale cell (yellow colored) doesn't have a desmosomal junction with the neighboring epithelial cells (4 & 5). The intercellular digitations (IC) of the glandular epithelial cells (vertical yellow arrow heads) recede so that the pale cell becomes directly exposed to the basement membrane (6-10). A pseudo-pod of a pale cell (horizontal yellow arrow head) appears pushing the basement membrane (11) which in turn becomes ruptured (12). The cytoplasm of the pale cell is seen migrating into the stroma with the intra-cytoplasmic organelles (13 & 14). And finally the nucleus follows into the stroma and the pale cell becomes completely translocated in the stroma (15 & 16).

Although most of the uterine pale cells are eccentrically located, few uterine pale cells are located on the luminal side of the basal glands, supporting our speculation that these cells have the ability to migrate bidirectionally (93): either through the uterine cavity into the abdominal cavity to develop into EM (concentric uterine pale cells), or deep into the myometrium to develop into AM (eccentric uterine pale cells) (Figure 31).

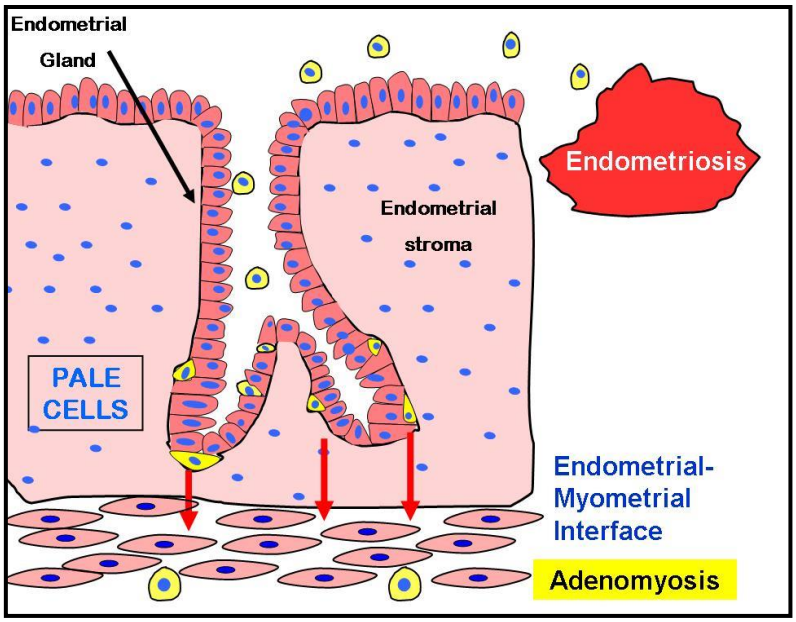

Figure 31: *Diagrammatic illustration of the uterine pale cells' role in the common pathogenesis of EM and AM.*

The uterine pale cells, being located eccentrically in the basal endometrial glands can migrate into the myometrium (below), where they develop into adenomyotic lesions. On the other hand those which are in close contact with the glandular lumen (concentric position) can migrate through the uterine cavity into the peritoneal cavity, where they develop into peritoneal EM. Yellow cells: uterine pale cells.

Loss of cell-cell contact of the uterine pale cells to the neighbouring glandular epithelial cells ensures a free cell motility and might be the first step in an epithelial-mesenchymal transformation (EMT) process (94-97). The latter is a described event occurring in adenomyosis (98)

Lastly, we have not only answered the scientific questions set out at the start, but can also invite more questions for further research studies to better understand the disease pathogenesis. Further phenotypic as well as in-vitro characterisation of these uterine pale cells using specific stem cell markers should be done. Under what conditions these uterine pale cells could migrate into the stroma remain of great concern.

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8. Affidavit

I, [Mohamed Gamaleldin Saleh Ali, Ibrahim] certify under penalty of perjury by my own signature that I have submitted the thesis on the topic [New insight into the pathogenesis of adenomyosis. Is the dislocation of the endometrial fragments into the myometrium evident in adenomyosis uteri?]. 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 or in spirit on publications or presentations of other authors are, as such, in proper citations indicated. The section on methodology (in particular practical work, laboratory requirements, statistical processing) and results (in particular images, graphics and tables) corresponds to the URM (s.o) and are answered by me. My contribution in the selected publication for this dissertation corresponds to those that are specified in the following joint declaration with the responsible person and supervisor.

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.

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Date Signature

Detailed Declaration of Contribution (1)

[Mohamed Gamaleldin Saleh Ali Ibrahim] had the following share in the following publication:

- Ultramicro-trauma in the endometrial-myometrial junctional zone and pale cell migration in adenomyosis: [Ibrahim MG, Chiantera V, Frangini S, Younes S, Köhler C, Taube ET, Plendl J, Mechsner S.], [titles], [Journal of Fertility and Sterility], [2015]

Contribution in detail:
Before he started with the electron microscopy study, he did a review of literature about the ultrastructure of endometrium in all menstrual cycle phases and presented it in the journal club in the endometriosis lab.

He shared - together with the supervisor - in the selection of the patients to be included in the study, collected all uterine biopsies intra-operatively by himself, fixed the samples in two different ways for both the transmission electron microscopy as well as the immunohistochemistry/immunofluorescence staining, shared in the paraffinisation process, prepared the paraffin slides of the uterine samples using the microtome, did all steps of the immunohistochemistry/immunofluorescence by himself, calculated the immunoreactive scores using the light microscopy himself and finally discussed the results with the supervisor.

Furthermore, he provided the paraffin slides to Dr. ELTaube in the pathology department to be further stained with the van Gieson stain. He took photos of areas of interest to be further discussed with the results of the other methods used.

He delivered the uterine samples himself to the department of anatomy at the free university of Berlin to be further prepared for transmission electron microscopy study. He examined himself all semi-thin and ultrathin slices using the transmission electron microscopy there, took photos of areas of interest after being trained by the technicians there, and finally discussed the results together with both the supervisor as well as Prof. Plendl (the head of the anatomy department).

Mr. Ibrahim did the statistical analysis (supplemental table 1 and 2 in the published paper), collected the clinical data of all included patients from the available medical records in the hospital, and critically discussed the results with all other co-authors, drafted the manuscripts. After final agreement of all authors he submitted the papers in the corresponding journals. He responded together with the supervisor to the reviewers' comments and finally both paper were accepted and published on-line

Signature, date and stamp of the supervising University teacher

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Signature of the doctoral candidate

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Detailed Declaration of Contribution (2)

[Mohamed Gamaleldin Saleh Ali Ibrahim] had the following share in the following publication:

- Myofibroblasts Are Evidence of Chronic Tissue Microtrauma at the Endometrial-Myometrial Junctional Zone in Uteri With Adenomyosis: [Ibrahim MG, Sillem M, Plendl J, Chiantera V, Sehouli J, Mechsner S], [titles], [Journal of Reproductive Sciences], [2017]

Contribution in detail:

He shared - together with the supervisor - in the selection of the patients to be included in the study, collected all uterine biopsies intra-operatively by himself, fixed the samples in two different ways for both the transmission electron microscopy as well as the immunohistochemistry staining, shared in the paraffinisation process, prepared the paraffin slides of the uterine samples using the microtome, did all steps of the immunohistochemistry by himself, calculated the immunoreactive scores using the light microscopy himself and finally discussed the results with the supervisor.

Furthermore, he carried out the in-vitro experiment himself, preparing the necessary culture media, following the standard protocols used in the endometriosis lab. He did all steps for the cytohistochemistry using immunofluorescence antibodies himself, examined the slides using the microscopy and discussed the results with the supervisor.

He delivered the uterine samples himself to the department of anatomy at the free university of Berlin to be further prepared for transmission electron microscopy study. He examined himself all semi-thin and ultrathin slices using the transmission electron microscopy there, took photos of areas of interest after being trained by the technicians there, and finally discussed the results together with both the supervisor as well as Prof. Plendl (the head of the anatomy department).

Mr. Ibrahim did the statistical analysis (figure 1 and 2 in the published manuscript), collected the clinical data of all included patients from the available medical records in the hospital and critically discussed the results with all other co-authors, drafted the manuscripts. After final agreement of all authors, he submitted the papers in the corresponding journal. He responded together with the supervisor to the reviewers' comments and finally the paper was accepted and published on-line.

Signature, date and stamp of the supervising University teacher

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Signature of the doctoral candidate

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9. Curriculum Vitae

Mein Lebenslauf wird aus datenschutzrechtlichen Gründen in der elektronischen Version meiner Arbeit nicht veröffentlicht

10. List of publications

M.G.Ibrahim, V. Chiantera, S. Frangini, S. Younes, C. Köhler, E. Taube, J. Plendl and S. Mechsner. Ultramicro-trauma in the Endometrial-Myometrial Junctional Zone and Pale Cell Migration in Patients with Adenomyosis. New Insight on Adenomyosis Pathogenesis. Journal of Fertility and Sterility (IF: 4,4). 2015

M.G.Ibrahim, E. Delarue, E. Abesadze, M. Haas, J. Sehouli, J. Plendl, V. Chiantera and S. Mechsner. Abdominal Wall Endometriosis.Myofibroblasts as a Possible Evidence of Metaplasia. A case report. Journal of Gynecologic and Obstetric Investigation (IF: 1,6). 2016

V. Chiantera, E. Abesadze**, M.G.Ibrahim**, A. M. Dückelmann and S. Mechsner. An atypical manifestation of inguinal endometriosis in the extra pelvic part of the round ligament. International Journal of Reproduction, Contraception, Obstetrics and Gynecology (IF: 0,7). 2016

M.G.Ibrahim, M. Sillem, J. Plendl, V. Chiantera, and S. Mechsner. Myofibroblasts are Evidence of Chronic Tissue Microtrauma at the Endometrial-Myometrial Junctional Zone in Uteri with Adenomyosis. Journal of Reproductive Sciences (IF: 2,4). 2017

M.G.Ibrahim, M. Sillem, J. Sehouli, J. Plendl, V. Chiantera and S. Mechsner. Arrangement of myofibroblastic and smooth muscle-like cells in superficial peritoneal endometriosis and a possible role of transforming growth factor beta 1 (TGFβ1) in myofibroblastic metaplasia. Journal of Archives of Gynecology and Obstetrics (IF: 2,0). 2018

M.G.Ibrahim, EA Elghonaimy , S. Schäfer, M. Vennemann, S. Kliesch, L. Kiesel, M. Götte, and A.N. Schüring. Seminal plasma (SP) induces an instant TGFβ1–independent upregulation of EMT- and myofibroblastic metaplasia markers in endometriotic and endometrial cells. Journal of Archives of Gynecology and Obstetrics (IF: 2,0). 2018

Coauthroship in the World Endometriosis Research Forndation (WERF) EPHect Working Group

Becker CM, Laufer MR, Stratton P, Hummelshoj L, Missmer SA, Zondervan KT, Adamson GD; WERF EPHect Working Group. World Endometriosis Research Foundation Endometriosis Phenome and BiobankingHarmonisation Project: I. Surgical phenotype data collection in endometriosis research. Fertility Sterilty (IF: 4,7). 2014.

Allison F. Vitonis, Katy Vincent, Nilufer Rahmioglu, Amelie Fassbender, Germaine M. Buck Louis, Lone Hummelshoj, Linda C. Giudice, Pamela Stratton, G. David Adamson, Christian M. Becker, Krina T. Zondervan, Stacey A. Missmer and WERF EPHect Working Group. World Endometriosis Research Foundation Endometriosis Phenome and biobanking harmonization project: II. Clinical and covariate phenotype data collection in endometriosis research. Fertility Sterilty (IF: 4,7). 2014.

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Amelie Fassbender, Nilufer Rahmioglu, Allison F. Vitonis, Paola Vigano, Linda C. Giudice, Thomas M. D'Hooghe, Lone Hummelshoj, David Adamson, Christian M. Becker, Stacey A. Missmer, Krina T. Zondervan and the WERF EPHect Working Group. World Endometriosis Research Foundation Endometriosis Phenome and BiobankingHarmonisation Project: IV. Tissue collection, processing, and storage in endometriosis research. Fertility Sterilty (IF: 4,7), 2014.

11. Acknowledgment

Thanks…

To my late father … *who inspired me in my life, and to him I am forever grateful*

To my mother … *under her feet lies my heaven and who holds my hand for a moment but my heart for a lifetime*

To my wife … *the arms that hold me at my weakest times, the eyes that see my beauty at my ugliest time and the heart that loves me at my worst*

To my mentor … *it would be impossible to count all the ways that you've helped me in my career. Thank you so much for all that you've done. I only hope I can return the favour sometime in the future.*

To all my patients … *whose life was not about to wait for the storm to pass, but about learning how to dance in the rain*.