Membrane-type matrix metalloproteinases: membrane-type 1 matrix metalloproteinase, membrane-type 2 matrix metalloproteinase and membrane-type 3 matrix metalloproteinase in endometriosis and adenomyosis

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1. INTRODUCTION

1.1 Endometriosis

Endometriosis is a gynecological condition in which endometrial glands and stroma occur outside the uterine cavity mostly in the peritoneum, ovaries and rectovaginal-septum, however, rarely it is found in extra-pelvic regions such as the brain, liver and the lungs (Giudice and Kao, 2004). Its prevalence varies among different populations; however, recent reports have shown that it affects about 0.7-8.6% of women of the reproductive age and 15.4-71.4% of women experiencing chronic pelvic pain (Vercellini et al., 2014; Ghiasi et al., 2020). In Germany, the prevalence of endometriosis in 2019 increased to 0.66% from 0.53% reported in 2010 (Göhring et al., 2022).

Reduced parity, early age at menarche, short menstrual cycle length and low body mass index (BMI) among women are potential risk factors while smoking and increased parity have been associated with a lesser risk (Vercellini et al., 2014; Wang et al., 2020a). Endometriosis is typically associated with dysmenorrhea, dyspareunia, dyschezia, dysuria and infertility (Bulletti et al., 2010; Agarwal et al., 2019).

1.1.1 Types of endometriosis

Three main types of endometriosis have been identified depending on the site of localization of the endometrial implants: ovarian, peritoneal and deep infiltrating endometriosis (Nisolle and Donnez, 1997). Even though chronic pelvic pain is common among the three types, severity of pain correlates poorly with the location of the lesions (Hsu et al., 2010). Nodules/implants in the ovary form ovarian endometriomas or ovarian cysts which are usually filled with menstrual blood while those that occur in sites more than 5 mm below the tissue borders of the peritoneum such as in the bladder, rectovaginal septum, ureter and bowel lead to deep infiltrating endometriosis (Koninckx et al., 1992; Hsu et al., 2010; Kho et al., 2018). Ovarian endometriomas/cysts are often associated with infertility whereas deep dyspareunia, dyschezia and bowel movement discomfort during menses are common among patients with deep infiltrating endometriosis (Hsu et al., 2010; Wang et al., 2020a). Superficial/peritoneal endometriosis typically occur on the pelvis or in the soft tissues of the pelvis less than 5 mm deep (Hsu et al., 2010; Kho et al., 2018; Wang et al., 2020a).

1.1.2 Pathogenesis of endometriosis

The pathogenesis of endometriosis is still debated and different theories including retrograde menstruation, coelomic metaplasia, oxidative stress, inflammation, stem and progenitor cell, lymphatic spread and endometriosis disease theory have been proposed (Sourial et al., 2014; Laganà et al., 2019; Wang et al., 2020a; Signorile et al., 2022).

1.1.2.1 Implantation hypothesis

Implantation of endometrial tissue by retrograde menstruation is the most accepted theory and according to it, during menstruation some menstrual blood containing endometrial tissues flows retrograde through the fallopian tubes into the pelvic cavity where they implant and develop into endometriotic lesions (Sampson, 1927a). Although, retrograde menstruation occurs in 70-96% of women of reproductive age, it does not explain why only a small percentage of these women get endometriosis (Halme et al., 1984; Liu and Hitchcock, 1986). This suggests possible involvement of other factors including disruptions in the pelvic peritoneum (Young et al., 2013), inflammation, oxidative stress (Samimi et al., 2019) and genetic/epigenetic modifications (Koninckx et al., 2019), in the successful implantation of endometrial tissue in ectopic sites. Different studies have reported presence of endometrial cells in the peritoneal fluid of patients with and without endometriosis (Bartosik et al., 1986; Kruitwagen et al., 1991), with high cellular similarities in patients with and without endometriosis (Kruitwagen et al., 1991), providing further evidence on the role of other factors in the pathogenesis of endometriosis. In addition, high levels of white and red blood cells have been reported in peritoneal fluid of women during the menstrual phase compared to non-menstrual phases, showing that menstruation is associated with inflammation (Bokor et al., 2009). On the other hand, the numbers of natural killer cells in the menstrual effluent of patients with endometriosis are lower and they exhibit reduced cellular cytotoxicity capacity compared to patients without endometriosis, suggesting that they might play a role in the pathogenesis of endometriosis (Warren et al., 2018; Schmitz et al., 2021).

Sampson's theory is supported by past reports that women with endometriosis show abnormal myometrial contractions and higher amounts of menstrual blood compared to those without endometriosis (Halme et al., 1984; Salamanca and Beltrán, 1995; Bulletti et al., 2002). In addition, inoculation of menstrual endometrium into different sites of the pelvic cavity induced endometriosis in non-human primates (Hastings and Fazleabas, 2006; Harirchian et al., 2012). Furthermore, the anatomical localization of endometriotic

lesions as well as their high prevalence in the posterior part of the pelvic compartment and the left side of the pelvis provides further evidence for menstrual regurgitation (Jenkins et al., 1986; Chapron et al., 2006; Scioscia et al., 2011).

However, Sampson's theory has been criticized due to the fact that it does not adequately explain the occurrence of endometriosis in extra-pelvic regions (Signorile and Baldi, 2010), but recently, Laganà et al. (2019) and Yovich et al. (2020) indicated that Sampson already suggested/observed that besides retrograde menstruation the dissemination of endometrial tissue might also happen through the lympathic/vascular system (Sampson, 1927b).

1.1.2.2 Coelomic metaplasia theory

According to this theory, endometriosis arises from abnormal differentiation of cells lining the mesothelium of the visceral and abdominal peritoneum (Gruenwald, 1942), however, different factors such as hormonal, environmental and immunological are also thought to trigger the transformation of these cells into endometrial-like cells (Burney and Giudice, 2012; Sourial et al., 2014). This theory is thought to be responsible for ovarian endometriosis and endometriosis in patients with Mayer-Rokitansky-Küster-Hauser syndrome (MRKH) (Zheng et al., 2005; Mok-Lin et al., 2010; Troncon et al., 2014). However, there is insufficient data supporting the occurrence of endometriosis without the endometrium (Konrad et al., 2019a). In addition, past studies have demonstrated the role of eutopic endometrium in recurrence of endometriosis (Bulletti et al., 2001; Shakiba et al., 2008). It is however not well understood how the coelomic epithelium abnormally differentiates into two different cell types namely endometrial epithelial and stromal cells in almost a similar way in different locations in the body (Konrad et al., 2019a; Mecha et al., 2021).

1.1.2.3 Oxidative stress and inflammation

Oxidants or reactive oxygen species (ROS) are highly unstable molecules produced during normal body metabolic processes and can react with different cellular components due to their instability (Scutiero et al., 2017). The body has defense mechanisms especially the antioxidants that react with ROS to inactive them, however, excessive production of ROS leads to an imbalance in the processes of ROS production and neutralization by antioxidants (Gupta et al., 2006; Scutiero et al., 2017). For instance, high amounts of iron, ferritin and hemoglobin have been reported in peritoneal fluid of

patients with endometriosis versus controls and it is known that high amounts of iron enhance different reactions that lead to production of ROS (Defrère et al., 2008). ROS release oxidative stress signals that trigger release of pro-inflammatory molecules, which cause inflammation by recruiting different cells of the immune system such as macrophages (Sourial et al., 2014; Morris et al., 2021). This inflammatory environment promotes production of cytokines that mediate different physiological processes such as angiogenesis necessary for development and establishment of endometriosis (Sourial et al., 2014; Morris et al., 2021). Recently, Burns et al. (2018) suggested the role of neutrophils and macrophages in initiating the formation of endometrotic lesions. In addition, the peritoneal fluid of women with endometriosis contains high numbers of activated macrophages (Morris et al., 2021).

Furthermore, women with endometriosis show increased levels of oxidative stress markers (Carvalho et al., 2012) and decreased antioxidant enzymes (Turgut et al., 2013; Turkyilmaz et al., 2016) as opposed to those without endometriosis. Consequently, the build-up of ROS may play a role in the development and progression of endometriosis (Sourial et al., 2014).

1.1.2.4 Stem and progenitor cell theory

Stem cells are undifferentiated cells that have the ability to differentiate into other cell types (Sourial et al., 2014; Signorile et al., 2022). Based on this theory endometriotic lesions arise from endometrial stem/progenitor cells of the endometrium or bone marrow which may subsequently spread to different ectopic sites through the oviducts during retrograde menstruation or through vascular circulation (Taylor, 2004; Gargett et al., 2014; Maruyama, 2022). However, as with the coelomic metaplasia theory, stem cells need to differentiate into endometrial epithelial and stroma cells in an equivalent manner in different areas in the body (Konrad et al., 2019a; Mecha et al., 2021).

1.1.2.5 Lymphatic spread theory

The lymphatic spread theory proposes that ectopic endometrial implants could be due to dissemination of endometrial cells through the lymphatic or hematogenous vessels (Burney and Giudice, 2012; Jerman and Hey-Cunningham, 2015; Signorile et al., 2022). It has thus been thought to explain the presence of endometrial tissue in the lymphatic system as observed in patients with endometriosis suggesting that the lymphatic system could be responsible for endometrial tissue dissemination (Mechsner et al., 2008; Noël et

al., 2008; Beavis et al., 2011). On the other hand, lymph nodes from animal models induced with endometriosis showed significantly higher levels of endometrial stromal and immune cells as compared to controls (Hey-Cunningham et al., 2011). The lymphatic theory thus provides a possible explanation for occurrence of endometriosis in distant regions such as the lungs and brain (Jubanyik and Comite 1997; Mecha et al., 2021).

1.1.2.6 Endometriosis disease theory

Based on this theory, development of typical, ovarian or deep infiltrating endometriosis occurs due to genetic factors (Koninckx et al., 2019). Mild/minimal endometriosis is regarded as early lesions of the disease that occur periodically in all women and most of which can be cleared by the body's immune system. Consequently, lesions with 'normal' cells degenerate naturally whereas those with genetic abnormalities and clinical manifestations advance into a more serious disease (Koninckx et al., 1999, 2019) possibly due to cellular modifications such as genetic mutations frequently observed in predisposed persons (Koninckx et al., 1999). The epigenetic/genetic theory is an update of the endometriotic disease theory and it provides some insights into the different genetic factors that contribute to cellular modifications (Koninckx et al., 2019).

However, up to date the pathogenesis of endometriosis is still debated because none of the suggested theories fully explains the different facts about the disease. Maybe a combination of the different theories would possibly work, but there is need for more research to improve our understanding of the pathogenesis, progression and pathophysiology of endometriosis as well as its management and treatment.

1.1.3 Diagnosis and treatment of endometriosis

The gold standard for diagnosis of endometriosis is laparoscopy together with histological confirmation **(**Kennedy et al., 2005; Dunselman et al., 2014; Kho et al., 2018). Ovarian endometriomas can be diagnosed using transvaginal ultrasound sonography (TVUS) and magnetic resonance imaging (MRI), however, use of MRI in diagnosis of peritoneal endometriosis has not been fully established (Dunselman et al., 2014; Amro et al., 2022). It is also difficult to differentiate endometriosis from ovarian cancer using MRI (Van Holsbeke et al., 2009). Because, MRI provides information on the localization of deep endometriosis at the time of imaging, it is mainly used as an indication for laparoscopy (Ulrich et al., 2014; Amro et al., 2022).

Endometriosis can be treated through surgical or medical means. Surgery involves the complete removal of the endometrial implants during laparoscopy. Pain after surgery can be managed by hormonal medications such as levonorgestrel-releasing intrauterine devices (LNG-IUD) (Ulrich et al., 2014). Analgesics and hormonal medications, progestogen, anti-progestogens, aromatase inhibitors and gonadotropin-releasing hormone (GnRH) agonists and antagonists are mainly used to manage endometriosis-related pelvic pain, however, these medications have been associated with adverse side effects (Dunselman et al., 2014; Ulrich et al., 2014). It is therefore necessary to consider and rule out other factors that could also contribute to pelvic pain before the commencement of the hormonal therapy (Dunselman et al., 2014).

1.2 Adenomyosis

Adenomyosis, also known as endometriosis interna, is characterized by the presence of endometrial tissue in the myometrium and is mainly associated with enlarged uterus with hypertrophic myometrium, abnormal uterine bleeding, pelvic pain and reduced fertility (Gordts et al., 2018; García-Solares et al., 2018; Guo, 2020). However, these symptoms are not always specific to adenomyosis and also in some patients adenomyosis is asymptomatic thus leading to delayed diagnosis (Gordts et al., 2018; García-Solares et al., 2018).

Approximately 2% of women in the general population and 24.4% of women of the reproductive age suffer from adenomyosis (Puente et al., 2016; Morassutto et al., 2016). This however, varies among distinct populations due to variations in diagnostic techniques used for diagnosis of adenomyosis (Guo, 2020). Using transvaginal ultrasound sonography Puente et al. (2016) and Hashim et al. (2020) reported an 80.6% and 7.5% frequency of adenomyosis in women with infertility, respectively.

Women with a history of depression, early menarche, short menstrual cycles and increased BMI are more likely to develop adenomyosis (García-Solares et al., 2018). Utero-surgical procedures such as ceaserean section have also been associated with increased risk for adenomyosis, because they may lead to disruption of the endometrial-myometrial interface and thus allow migration and implantation of endometrial tissue in the myometrium (García-Solares et al., 2018; Guo, 2020).

Adenomyosis and endometriosis are highly related and they share different molecular characteristics. The two conditions are also associated with similar challenges and the clinical and medical strategies used in management of both conditions are highly similar

(Guo, 2020). Besides, adenomyosis has been reported in 21.8-79% of women with endometriosis (Kunz et al., 2005; Di Donato et al., 2014).

1.2.1 Pathogenesis of adenomyosis

Just like endometriosis, pathogenesis of adenomyosis is not well understood (Vannuccini et al., 2017; Guo, 2020), however, two theories; invagination of endometrial glands and metaplasia have been suggested (Vannuccini et al., 2017; García-Solares et al., 2018; Guo, 2020).

1.2.1.1 Invagination theory of adenomyosis

This theory is mainly based on tissue injury and repair mechanism recently suggested (Leyendecker et al., 2009; Leyendecker and Wildt, 2011; Leyendecker et al., 2015). In the absence of pregnancy, the uterus is subject to peristatic activities due to myometrial contractions aimed at different functions such as removal of menstrual debris and sperm transportation (Shaked et al, 2015). Similarly, uterine peristalsis can also be activated by oxytocin through its receptor (OTR) whose expression as well as oxytocin production in the myometrium is mainly regulated by estrogen (Kunz et al., 1998; Guo, 2020). Moreover, overexpression of OTR and high uterine contractile amplitude have been reported in adenomyotic patients compared to controls (Guo et al., 2013).

The invagination theory proposes that repeated overstretching of the endometrium due to peristatic activities in the junctional zone causes tissue auto-traumatization which disrupts the muscle fibres of the myometrium consequently leading to invagination of the basal layer into the myometrium hence the establishment of adenomyosis (Vannuccini et al., 2017; García-Solares et al., 2018; Guo, 2020). Using 3D reconstructions of the human endometrium, Yamaguchi et al. (2021) demonstrated that endometrial glands extend into the myometrium most likely via invagination leading to adenomyosis.

1.2.1.2 Metaplasia theory of adenomyosis

According to this theory, adenomyotic lesions, like endometriosis, could also arise from abnormal differentiation of embryonically dislocated pluripotent müllerian remnants or from differentiation of potent stem cells (Vannuccini et al., 2017; García-Solares et al., 2018; Guo, 2020). Although Wang et al. (2020a) recently proposed a model illustrating the role of stem cells in the establishment of deep infiltrating endometriosis, there is no data demonstrating abnormal differentiation of stem cells into endometrial cells;

epithelial and stromal cells at different ectopic sites (Mecha et al., 2021) and thus more research is needed to demonstrate the role of stem cells as well as pluripotent müllerian remnants in the establishment of adenomyosis (Vannuccini et al., 2017).

1.2.3 Diagnosis and treatment of adenomyosis

Histology is the main method used for diagnosis of adenomyosis. However, the main drawback of histology is the variability in evaluation of the depth of myometrial invasion and classification as adenomyosis (Abbott, 2017). Recently, Andres et al. (2018) demonstrated that two-dimensional (2D) and three-dimensional (3D) TVUS are highly effective in diagnosis of adenomyosis with a specificity of 64% and 56% as well as a sensitivity of 84% and 89%, respectively. Similarly, Bazot and Daraï (2018) also indicated the role of MRI and TVUS in diagnosis and classification of adenomyosis. Van den Bosch et al. (2015) lately published a consensus report explaining the terms, definitions and measurements to be used in sonographic evaluation of different uterine masses including adenomyosis. However, no agreement has been reached on the use of non-histological methods for diagnosis of adenomyosis; hence more studies are needed (Andres et al., 2018).

Currently, there are no standard guidelines for treatment and management of adenomyosis and no drug has been approved for treatment (Chen et al., 2019). However, treatment depends on the presenting symptoms and can involve either medical or surgical interventions (Abbott, 2017). Nonsteroidal anti-inflammatory drugs (NSAIDs) are used for management of pain related to adenomyosis but they have been associated with adverse side effects such as headache, drowsiness and indigestion (Marjoribanks et al., 2015; Abbott, 2017; Chen et al., 2019). Hormonal treatments including oral progestins, aromatase inhibitors, danazol, GnRH (Badawy et al., 2012; Fawzy and Mesbah, 2015) and LNG-IUD (Sheng et al., 2009) have also been shown to be effective in treatment of adenomyosis.

Surgical treatment mainly depends on the need to maintain/restore fertility or not. Hysterectomy is mainly associated with strongly reduced recurrence and it is the main preferred method for patients with no need for future fertility (Abbott, 2017). On the other hand, conservative surgery which mainly depends on the extent and localization of the disease can be done where patients wish to maintain the uterus (Farquhar and Brosens, 2006).

High intensity focused ultrasound (HIFU) is a non-invasive technique previously introduced for treatment of malignant tumors involving the liver, pancreas, bone and breast, however, it has emerged as an alternative for treatment of different gynecological conditions such as leiyomas, fibroids and adenomyosis (Huang et al., 2020; Marques et al., 2020; Jeng et al., 2020; Li et al., 2021; Peng et al., 2021). HIFU has been reported to be effective in relieving adenomyotic symptoms in patients with adenomyosis (Huang et al., 2020; Marques et al., 2020; Li et al., 2020, 2021; Peng et al., 2021) especially when combined with mirena or with GnRH and mirena as well as with laparascopic excision as opposed to HIFU alone (Huang et al., 2020; Li et al., 2021; Peng et al., 2021). However, there is limited data on ovarian reservation and pregnancy outcomes after HIFU (Marques et al., 2020). Recently, Jeng et al. (2020) reported pregnancies in cases of HIFU-ablated uterine fibroids and adenomyosis.

1.3 Matrix metalloproteinases (MMPs)

Matrix metalloproteinases are zinc-dependent endopeptidases involved in extracellular matrix remodeling and in different physiological processes such as cell proliferation, migration, apoptosis, angiogenesis and wound healing (Chevronnay et al., 2012; Cui et al., 2017). MMPs are classified based on their domain structure and substrate specificity into; collagenases (MMP1, 8, and 13), gelatinases (MMP2 and MMP9), matrilysins (MMP7 and 26), stromelysins (MMP3, 10, and 11), and MT1-6-MMPs (MMP14, 15, 16, 17, 24, and 25) (Bałkowiec et al., 2018).

Structurally all MMPs comprise of the signal peptide, predomain, catalytic domain, hinge region (linker peptide) and hemopexin domain (Fig. 1). Matrilysins lack the hinge region and hemopexin domain. Some secreted MMPs; MMP11, 21 and 28, transmembrane MT-MMPs: MT1-MMP, MT2-MMP and MT3-MMP, and GPI anchored MT-MMPs; MT4-MMP and MT6-MMP have a furin-like motif in the C-terminus after the prodomain. In addition, transmembrane MT-MMPs contain a transmembrane and cytosolic domain (Nagase et al., 2006; Jaoude and Koh, 2016; Cui et al., 2017; Laronha and Caldeira, 2020). Figure 1 shows a representative of the MMPs structure.

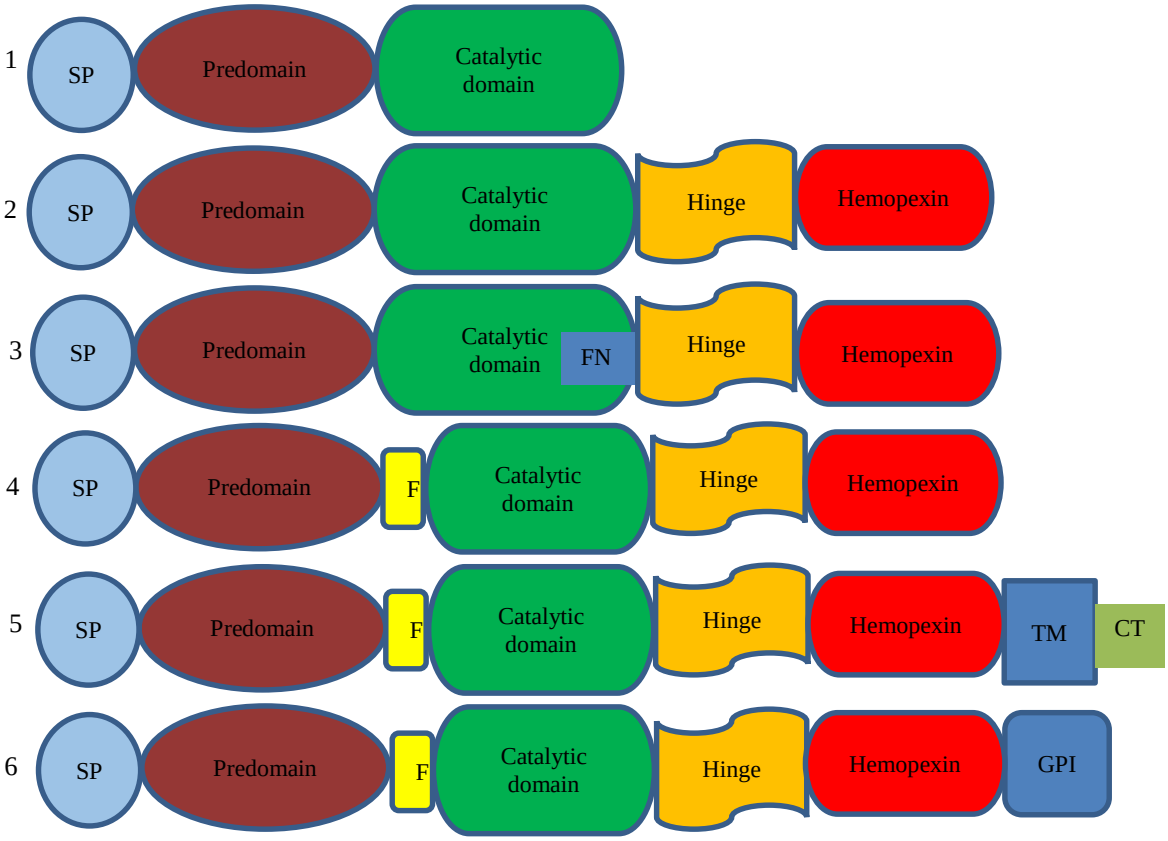

Figure 1. Representative figure showing the structure of MMPs. 1, matrilysins (MMP7 and 26); 2, collagenases (MMP1, 8,13); stromelysins (MMP3 and 10) and other MMPs (MMP12, 19, 20, 22 and 27); 3, gelatinases (MMP2 and 9); 4, secreted MMP11, 21 and 28; 5, transmembrane MT-MMPs (MT1-MMP, MT2-MMP, MT3-MMP and MT5-MMP); 6, GPI-anchored MT-MMPs (MT4-MMP and MT6-MMP). SP, signal peptide; FN, fibronectin; F, furin motif; TM, transmembrane; CT, cytoplasmic domain; GPI, glycosylphosphatidylinositol. Adapted from (Jaoude and Koh, 2016) with some modifications.

Since MMPs are produced as inactive enzymes, they undergo activation through different mechanisms such as pro-MMP cleavage and activation by oxidative stress molecules like reactive oxygen species (Jaoude and Koh, 2016). The first step in activation of MMPs is the cleavage of the pro-MMP, also referred to as the cysteine switch, in which cysteine is cleaved from a cysteine and zinc intermolecular complex, thus exposing the active site (Van Wart and Birkedal-Hansen, 1990). Both in vitro and in vivo studies have indicated the role of oxidative stress molecules in regulating the expression patterns of different MMPs in different cell types (Ovechkin et al., 2006; Moshal et al., 2006; Shin et al., 2007).

On the other hand, MMPs' activities can be inhibited through endogenous or exogenous inhibitors (Jaoude and Koh, 2016). Tissue inhibitors of matrix metalloproteinases (TIMPs); TIMP-1, TIMP-2, TIMP-3 and TIMP-4, inhibit MMP activity endogenously by

binding to MMPs in a 1:1 ratio (Itoh, 2015; Cui et al., 2017). All the TIMPs have the ability to inhibit soluble MMPs, however, TIMP-1 is a poor inhibitor of the membrane-type matrix metalloproteinases; MT1-MMP, MT2-MMP, MT3-MMP and MT5-MMP. Notably, GPI-anchored MT-MMPs can be inhibited by all the four TIMPs (Itoh, 2015) while, tissue inhibitor of matrix metalloproteinase 3 (TIMP3) can inhibit all MMPs and is the best MMP inhibitor in vivo (Baker et al., 2002). Exogenously, MMPs can be inhibited by use of different synthetic MMPs inhibitors such as batimastat and marimastat to regulate their activity (Goffin et al., 2005; Skarja et al., 2009).

1.3.1 Membrane type-matrix metalloproteinases (MT-MMPs)

MT-MMPs form a subgroup within the MMP family and is also subdivided into two types; the transmembrane MMPs, MT1-MMP, MT2-MMP, MT3-MMP, and MT5-MMP and the glycosylphosphatidylinositol (GPI) anchored MT-MMPs, MT4-MMP and MT6-MMP (Itoh, 2015). MT-MMPs have a furin motif at the C-terminus through which they are activated intracellularly by furin. The activated MT-MMPs act on the cell surface where they perform different roles such as cleavage and activation of other proMMPs to their active forms (Cui et al., 2017). Although all 6 MT-MMPs have the ability to cleave different components of the extracellular matrix, MT1-MMP has a wider range of substrates than all the other MT-MMPs (Itoh, 2015). Furthermore, MT-MMPs can also cleave some cytokines and receptors such as endoglin, CD44 and betaglycan (Kajita et al., 2001; Velasco-Loyden et al., 2004; Hawinkels et al., 2010; Tu'uhevaha et al., 2012).

1.3.1.1 Membrane type 1-matrix metalloproteinase (MT1-MMP, MMP14)

Among the 6 MT-MMPs identified in humans, MT1-MMP is the most widely studied enzyme (Itoh, 2015). MT1-MMP is expressed in different cells including the trophoblasts and feto-placental endothelial cells (Hiden et al., 2013), epithelial cells (Han et al., 2016; Placido et al., 2021), stromal cells (Tobar et al., 2014) and mesenchymal cells (Lu et al., 2010). Together with TIMP2, MT1-MMP activates proMMP2 on the cell surface. It also activates proMMP13, however, TIMP2 is not required in this activation (Itoh, 2015).

Studies on MT1-MMP in different pathological and physiological processes have demonstrated the role of MT1-MMP in cell migration and invasion (Yan et al., 2015; Hui et al., 2015; Chen et al., 2020; Vos et al., 2021), proliferation (Chen et al., 2020; Vos et al., 2021) and angiogenesis (Hui et al., 2015; Chang et al., 2016; Vos et al., 2021). MT1-MMP null mice exhibit dwarfism, osteopenia, poor alveolization, postnatal angiogenesis

defects and alterations in cytoskeleton and lamina structure suggesting a role of MT1-MMP in development and maintenance of cell integrity (Holmbeck et al., 1999; Zhou et al., 2000; Irie et al., 2005; Gutiérrez-Fernández et al., 2015).

MT1-MMP has also been associated with poor prognosis in colorectal cancer (Cui et al., 2019), lung cancer (Wang et al., 2014) gastric cancer (Dong et al., 2015; Wang et al., 2021) and muscle invasive bladder cancer (Wang et al., 2020b). It has also been reported to regulate different genes that are involved in epithelial-mesenchymal transition (EMT) in different cell types (Yan et al., 2015; Garmon et al., 2018). In addition, MT1-MMP is a novel prognostic marker for diffuse large B-cell Lymphoma (DLBCL) (Yin et al., 2020), glioma (Zhai et al., 2022) and lung cancer (Zheng et al., 2021).

Furthermore, impaired expression of MT1-MMP has been demonstrated in serum and plasma samples of patients with different pathological conditions including gastric cancer, breast cancer, preeclampsia and vestibular schwannomas (Laudański et al., 2010; Kasurinen et al., 2018; Ren et al., 2020; Sumawan et al., 2020). Similarly, concentrations of MT1-MMP and MMP13 are low in peritoneal fluid of patients with endometriosis compared to patients without endometriosis (Laudanski et al., 2005). However, MT1-MMP levels in serum and endocervical mucus of patients with and without endometriosis have not been explored.

1.3.1.2 Membrane type 2-matrix metalloproteinase (MT2-MMP, MMP15)

MT2-MMP is the second MT-MMP to be identified with a 73.9% similarity to MT1-MMP but is less well studied compared to MT1-MMP (Ito et al., 2010). It is expressed in different tissues such as the kidney, liver, heart, parts of the gastrointestinal tract and placenta (Abdelrahman et al., 2022). Like MT1-MMP it also activates proMMP2, but without the involvement of TIMP2 (Itoh, 2015). The role of MT1-MMP and MT2-MMP in embryogenesis and placental formation has been demonstrated in mice embryos lacking MT1-MMP and MT2-MMP which showed retarded growth, dilated vasculature, enlarged pericardia and defects in labyrinth formation (Szabova et al., 2010).

MT2-MMP has been implicated in angiogenesis (Lafleur et al., 2002; Plaisier et al., 2006; Chen et al., 2010), invasion (Ito et al., 2010; Liu et al., 2016; Majali-Martinez et al., 2020; Fakhari et al., 2022), migration (Ito et al., 2010; Fakhari et al., 2022), proliferation (Ito et al., 2010; Fakhari et al., 2022), and epithelial-mesenchymal transition (Liu et al., 2016). Also, MT2-MMP involvement in cancer progression has also been widely investigated (Ito et al., 2010; Bodnar et al., 2013; Chen et al., 2014; Liu et al., 2016).

1.3.1.3 Membrane type 3-matrix metalloproteinase (MT3-MMP, MMP16)

MT3-MMP is the third member of MT-MMPs and is expressed in different tissues including the placenta (Majali-Martinez et al., 2016), brain (Yoshiyama et al., 1998) and ovary (Puttabyatappa et al., 2014). It also activates proMMP2, a process which is enhanced in the presence of TIMP2 and TIMP3 (Itoh, 2015; Cui et al., 2017). Unlike MT1-MMP and MT2-MMP, MT3-MMP does not degrade collagen type I matrices, hence it does not support cell invasion through collagen matrices. However, it promotes cell invasion into fibrin matrices (Itoh, 2015).

Previous studies have indicated the role of MT3-MMP in angiogenesis (Lafleur et al., 2002; Plaisier et al., 2004, 2006) as well as in cell proliferation, migration and invasion in different types of cancer (Cao et al., 2016; Wu et al., 2017; Shen et al., 2017). Using mouse models Shi et al. (2008) demonstrated that MT3-MMP partially complements MT1-MMP in peri-cellular collagen degradation. Furthermore, loss of MT3-MMP in mice leads to retarded growth of the skeletons while loss of both MT1-MMP and MT3-MMP results in perinatal death and abnormalities in skeletal development and cranial bone formation suggesting that both enzymes could be involved in bone development (Shi et al., 2008; Itoh, 2015).

1.3.2 MT-MMPs in the endometrium and endometriosis

In the human endometrium, mRNA and/or protein expression of MT1-MMP (Zhang et al., 2000; Määttä et al., 2000; Chung et al., 2002; Goffin et al., 2003; Plaisier et al., 2006; Londero et al., 2012), MT2-MMP (Zhang et al., 2000; Goffin et al., 2003; Plaisier et al., 2006), MT3-MMP (Goffin et al., 2003; Plaisier et al., 2004, 2006), MT4-MMP (Plaisier et al., 2004) and MT5-MMP have been described (Plaisier et al., 2004; Gaetje et al., 2007). Both, Chung et al. (2002) and Londero et al. (2012) have reported increased MT1-MMP expression in ectopic endometrium versus eutopic endometrium as well as in patients with endometriosis versus patients without endometriosis, respectively. Similarly, MT1-MMP mRNA expression was shown to be higher in pigmented endometriotic lesions compared to normal eutopic endometrium (Ueda et al., 2002). In addition, MT5-MMP mRNA expression was higher in eutopic endometrium of patients with endometriosis compared to normal endometrium (Gaetje et al., 2007). Although MT1-MMP protein and mRNA expression has been described in endometriosis, its expression in adenomyosis has not been explored. Similarly, expression of MT2-MMP and MT3-MMP in endometriosis and adenomyosis has not been analysed.

1.4 Betaglycan

Betaglycan also known as transforming growth factor β (TGF-β) type III receptor is abundantly expressed in many cell types (Gatza et al., 2010; Bilandzic and Stenvers, 2011). It binds different ligands of the TGF-β family and as a TGF-β co-receptor enhances their interaction with their respective type II receptors on the target cells (Bilandzic and Stenvers, 2011; Villarreal et al., 2016). Structurally, it consists of a large extracellular domain, a single-pass transmembrane and a short intracellular domain without kinase activity (López-Casillas et al., 1994; Bilandzic and Stenvers, 2011).

In humans, betaglycan mRNA/protein expression was found in the placenta, maternal decidua and fetal membranes (Ciarmela et al., 2003), ovary (Liu et al., 2003) and uterus (Florio et al., 2005). Its role in endometriosis, reproduction and as a tumor suppressor in different types of cancers (Gatza et al., 2010; Lee et al., 2010; Omwandho et al., 2010; Nishida et al., 2018; Grgurevic et al., 2020; Mwaura et al., 2022) has been described. Moreover, the testis of betaglycan null mice exhibit reduced fetal Leydig cell function, distorted cord formation and altered cell organization suggesting the role of TGF-β superfamily in early testis development (Sarraj et al., 2010).

Betaglycan undergoes proteolytic cleavage/shedding to generate a soluble form (sBG) which can be detected in different body fluids such as milk, serum, plasma, endocervical mucus as well as in the extracellular matrix (Andres et al., 1989; Zhang et al., 2001; Velasco-Loyden et al., 2004; Grgurevic et al., 2020; Mwaura et al., 2022). Unlike the membrane bound form, sBG sequesters ligands from their receptors and thus antagonizes TGF-β signaling (Bilandzic and Stenvers, 2011). The regulatory mechanisms involved in betaglycan shedding are not well understood, however, past studies have demonstrated the role of pervanadate and MMPs especially MT1-MMP and MT3-MMP in betaglycan shedding in different cell types (Velasco-Loyden et al., 2004; Kudipudi et al., 2019). Using a pan-MMP inhibitor, GM6001 and TIMP3, we recently revealed the role of MMPs in betaglycan shedding in endometriotic epithelial 12Z cells (Mwaura et al., 2022). TGF-β1, TGF-β2 and TNF Protease Inhibitor 2 (TAPI2) reduce betaglycan shedding (Blair et al., 2011; Kudipudi et al., 2019; Mwaura et al., 2022). Although MT1-MMP and MT3-MMP have been implicated in betaglycan shedding, they have not been analysed in endometrial cells.

1.5 Objectives

MMPs play important roles in the menstrual cycle as well as in development and establishment of endometriosis. Despite the fact that MT1-MMP, MT2-MMP and MT3-MMP have been reported in the endometrium, their exact role in endometriosis and adenomyosis need to be explored in more detail. Of note, MT1-MMP expression in patients with and without endometriosis has been reported, but its expression in adenomyosis has not been investigated. Thus it will be interesting to explore and compare the expression patterns of MT1-MMP, MT2-MMP and MT3-MMP in eutopic endometrium of patients with and without endometriosis as well as in ectopic endometrium: adenomyosis, ovarian, peritoneal and deep infiltrating endometriosis.

MT1-MMP expression is impaired in serum and plasma samples of patients with different disease conditions and in peritoneal fluid of women with endometriosis, thus, it will be of importance to investigate MT1-MMP levels in serum and endocervical mucus samples of patients with and without endometriosis and whether it can be suitable as a non-invasive biomarker for diagnosis of endometriosis.

MT1-MMP and MT3-MMP have been shown to play a role in betaglycan shedding in different cell types, thus, it will be interesting to elucidate the role of MT1-MMP and MT3-MMP in betaglycan shedding in endometriotic cells.

The main aim of this study was to explore the localization pattern of MT1-MMP, MT2-MMP and MT3-MMP in eutopic and ectopic endometrium. In addition, we also studied the levels of MT1-MMP in serum and endocervical mucus of patients with and without endometriosis. Furthermore, we investigated the expression of MT1-MMP, MT2-MMP and MT3-MMP proteins in endometrial and endometriotic cells, and the role of MT1-MMP and MT3-MMP in betaglycan shedding in endometriotic cells.

2. MATERIALS AND METHODS

2.1 Materials

In this study, tissue, serum and endocervical mucus samples collected and stored in a database since 2009 were used. The study was approved by the Ethics Committee of the Medical Faculty of Justus-Liebig-University, Giessen, Germany (registry number 95/09). All participants gave a written informed consent.

2.1.1 Preparation of tissue samples

All tissue samples used in this study were obtained from patients undergoing laparoscopy for endometriosis or hysterectomy mostly due to pelvic pain at the center for Gynecology and Obstetrics at Justus-Liebig University, Giessen, Germany. Similarly, tissues were obtained from patients who did not show endometriosis after laparoscopy and were used as controls. Pathological evaluation, which involved microscopic examination of the tissue preparations and histological evaluation for any malignancy was carried out by the Institute of Pathology at the University Hospital, Giessen. Where necessary, CD10 immunohistochemical staining was used to confirm diagnosis of endometriosis.

We analysed tissue samples from four groups: healthy endometrium (ctrl), endometrium with endometriosis (EM-En), endometrium with adenomyosis (EM-Ad), and all tissues with adenomyosis (Ad). Patients who had both endometriosis and adenomyosis were grouped together. We also analysed samples from ovarian, peritoneal and deep infiltrating endometriosis (Tables 1, 2 and 3) and used three healthy fallopian tubes and placentas, as positive controls. Due to scarcity of tissue samples, we were not able to use the same patient samples for all proteins (MT1-MMP, MT2-MMP and MT3-MMP), nevertheless, 99 of 152 samples used for MT2-MMP were also used for MT3-MMP and 100 of 113 samples used for MT3-MMP were the same with those used for MT2-MMP.

Dating of endometrial tissue was followed using a questionnaire based on the dates of the last menstrual cycle and histological evaluation by the pathologist. Even though the classification of deep infiltrating endometriosis (DIE) is still unclear (Gordts et al., 2017), MRI and Enzian score (Tuttlies et al., 2005; Haas et al., 2011) were used in determination of DIE. Tables 1, 2 and 3 show a summary of the tissue samples used for each protein.

Table 1: Overview of the tissue samples used for MT1-MMP

Tissues	**EM EN$^-$**	**EM EN$^+$**	**OV**	**PE**	**DIE**
All samples	n=3	n=13	n=6	n=2	n=4
Median age±SD	45±4.4	45±14.5	38±8.4	36±8.5	37±5.7
Adenomyosis	n=20				
Adenomyosis only	n=11				
Proliferative	n=2	n=7			
Secretory	n=1	n=5			
Unknown		1			
Rectum					3
Rectosigmoid				1	
Sigmoid colon					1
Bladder				1	

EM EN$^-$, endometrium without endometriosis; EM EN$^+$, endometrium with endometriosis; OV, ovarian endometriosis; PE, peritoneal endometriosis; DIE, deep infiltrating endometriosis; SD, standard deviation; lig, ligamentum; adenomyosis means patients with both endometriosis and adenomyosis; adenomyosis only means patients with adenomyosis only.

Table 2: Overview of the tissue samples used for MT2-MMP

Tissues	**EM EN$^-$**	**EM EN$^+$**	**OV**	**PE**	**DIE**
All samples	n=14	n=42	n=23	n=26 (27)	n=28 (29)
Median age±SD	40±9.1	42.5±7.1	36±5.4	33±4.6	32±5.0
Leiomyoma	n=36				
Adenomyosis	n=41				
Adenomyosis only	n=17				
Proliferative	n=6	n=19			
Secretory	n=8	n=23			
Bladder				3	2
Uterosacral lig				3	3
Ovarian fossa				3	
Pouch of Douglas				2	
Round lig of uterus				2	
Pelvic wall				2	
Rectum				2	11
Rectosigmoid					3
Rectovaginal septum				3	5
Paraurethral/rectal				1	1
Sigmoid colon				2	2
Vagina					1
Intestine					1
Scar/Symphysis				2	
Lig latum uteri				1	
Mesovarium				1	

EM EN$^-$, endometrium without endometriosis; EM EN$^+$, endometrium with endometriosis; OV, ovarian endometriosis; PE, peritoneal endometriosis; DIE, deep infiltrating endometriosis; SD, standard deviation; lig, ligamentum; n=26 (27), means 27 samples from 26 patients; adenomyosis means patients with both endometriosis and adenomyosis; adenomyosis only means patients with adenomyosis only.

Table 3: Overview of the tissue samples used for MT3-MMP

Tissues	**EM EN$^-$**	**EM EN$^+$**	**OV**	**PE**	**DIE**
All samples	n=12	n=35	n=18	n=17 (18)	n= 15 (17)
Median age±SD	43±7.4	43±7.4	35.3±4.0	31±3.7	33±4.6
Leiomyoma	n=30				
Adenomyosis	n=31				
Adenomyosis only	n=13				
Proliferative	n=6	n=15			
Secretory	n=6	n=20			
Bladder				1	1
Uterosacral lig					3
Ovarian fossa				2	
Pouch of Douglas				3	
Round lig of uterus				2	
Pelvic wall				2	
Rectum					4
Rectosigmoid					1
Rectovaginal septum				1	5
Paraurethral/rectal				2	1
Sigmoid colon				1	2
Scar/Symphysis				2	
Lig latum uteri				1	
Mesovarium				1	

EM EN$^-$, endometrium without endometriosis; EM EN$^+$, endometrium with endometriosis; OV, ovarian endometriosis; PE, peritoneal endometriosis; DIE, deep infiltrating endometriosis; SD, standard deviation; lig, ligamentum; n=17 (18), means 18 samples from 17 patients; adenomyosis means patients who with both endometriosis and adenomyosis; adenomyosis only means patients with adenomyosis only.

2.1.2 Serum and endocervical mucus from patients with and without endometriosis

In this study, we also analysed serum and endocervical mucus samples from patients with and without endometriosis. Contraceptive use and cycle phases of the patients invoved in the study were monitored using questionnaires and anamnesis as previously described (Hackethal et al., 2011). Similarly, pain during menstruation from a scale of 0 (no pain) to 10 (strongest pain) was monitored using questionnaires. Patients on contraception included those using dienogest only or plus ethinylestradiol and progesterone-based contraceptions. Approximately three milliliters of anticoagulant-free venous blood was obtained from each patient and centrifuged at 3000 × g for 15 minutes at 4 °C. Aliquots were made and stored at -80 °C until use. Similarly, endocervical mucus samples were obtained from patients in the afternoon using a cotton swab and immediately placed in a 10 ml tube containing 500 µl of ice-cold 1 × PBS (Gibco, without Ca^{2+} and Mg^{2+}, pH 7.2) and 5 µl of protease inhibitor cocktail (Sigma, Germany). After centrifugation (3000 × g) for 15 minutes at 4 °C, supernatants were collected, weighed and stored at -80 °C as aliquots awaiting further analysis. Table 4 shows an overview of the serum and endocervical mucus samples used.

Table 4: Overview of the serum and endocervical mucus samples used

	Serum samples		**Mucus samples**	
	EN^-	**EN^+**	**EN^-**	**EN^+**
N	61	71	87	106
Median age ±SD	27±7.9	34±7.1	28±8.2	33±7.1
BMI (kg/m^2)	21.8±6.3	23.7±5.2	21.8±4.6	22.9±4.4
Smoking n (%)	n= 12 (19.7)	n=17 (23.9)	n=23 (26.4)	n=24 (22.6)
Allergy n (%)	n=31 (50.8)	n=34 (47.9)	n=44 (50.6)	n=58 (54.7)
Menstrual phase (n)				
Proliferative	19	15	34	37
Secretory	28	16	47	48
Menstruation	8	9	-	-
Unknown	6	31	6	21
Contraception use (n)				
Yes	28	35	22	36
No	33	36	65	70
Fertlity				
Yes	12	26	24	39
No	8	19	14	30
Unknown	41	26	49	37
Pain (n)				
Dysmenorrhea				
Yes	48	44	70	83
No	13	25	17	22
Unknown	-	2	-	1
Dyspareunia				
Yes	26	39	40	62
No	32	30	41	44
Unknown	3	2	6	-
Dyschezia				
Yes	18	26	27	44
No	43	45	56	62
Unknown	-	-	4	-
Dysuria				
Yes	11	15	19	29
No	50	56	68	77
Unknown	-	-	-	-

BMI is given as median ±SD (standard deviation). EN^-, without endometriosis; EN^+, with endometriosis; BMI, body mass index. Unknown; data not available and Pain; yes denotes mild to strong pain on a nominal rating scale (NRS) of 2-10.

2.1.3 Chemicals and reagents

All chemicals and other reagents used are listed below.

Table 5: Chemicals and reagents

Description	Article No.	Company
Acrylamide (30%)	3029.2	Roth, Germany
Ammonium persulphate (APS)	9592.2	Roth, Germany
N,N,N′,N′Tetramethylethylenediamine (TEMED)	2367.3	Roth, Germany
Bovine serum albumin (BSA)	820451	Millipore, Germany
10 x Cell lysis buffer	9803S	Cell Signaling Technology, USA
Ethanol	32205-M	Sigma-Aldrich, Germany
Methanol	32213-M	Sigma-Aldrich, Germany
Glycin	3908.2	Roth, Germany
Hydrochloric acid (HCl)	K025.1	Roth, Germany
Phenylmethylsulfonyl fluoride (PMSF)	P7626	Sigma-Aldrich, Germany
Sodium dodecyl sulfate (20% SDS)	1057.1	Roth, Germany
Xylol (Xylene)	4436.2	Roth, Germany
Neoclear	109843	Merck Millipore, Germany
Tris BASE	4855.2	Roth, Germany
Trypan blue dye (0.4%)	1450013	Biorad, Germany
Tris buffered saline Tween-20 (TBST) tablets	09-7510-100	Medicago AB, Sweden
Halt ™ protease Inhibitor Cocktail	78430	Thermo Scientific,USA
Signalfire™ ECL reagent	6883S	Cell Signaling Technology, USA
Phosphate buffered saline (PBS) tablets	18912-014	Fisher Scientific, Germany
Citric acid	818707	Merck Millipore, Germany
Sodium citrate	106448	Merck Millipore, Germany
30% Hydrogen peroxide	9681.1	Roth, Germany

Triton®X 100	3051.2	Roth, Germany
Bovin serum albumin	T844.4	Roth, Germany
DAB solution (diaminobenzidine)	K3468	Dako, Germany
Meyer's hematoxylin	11932	Morphisto GmbH, Germany
Real antibody diluent	S2022	Dako, Germany
Eukitt	03989	Sigma-Aldrich, Germany
BOUIN-HOLLANDE solution	10153.02500	Morphisto, Germany
Spectra™ Multicolor High Range Protein Ladder	26625	Thermo Scientific, USA
Wash buffer	WA126	R & D, Germany
Reagent diluent	DY995	R & D, Germany
Stop solution	DY994	R & D, Germany
LDS sample loading buffer	84788	Thermo Scientific, USA
4′,6-Diamidino-2-phenylindoldihydrochlorid (DAPI)	D9542	Sigma Aldrich, Germany

Table 6: Solutions and reagents for immunohistochemistry (IHC)

Description	**Preparation**
Phosphate buffered saline (0.01 M PBS)	2 tablets of phosphate buffered saline in 1000 ml of distilled water
Citrate buffer, pH 6.0	Solution A - 21.01 grams of citric acid in 1000 ml of distilled water (0.1 M) Solution B - 29.41 grams of sodium citrate in 1000 ml of distilled water (0.1 M) Then mix 150 ml of solution B with 1850 ml of distilled water, pH to 6.0 with solution A
3% Hydrogen peroxide in methanol	5 ml of 30% hydrogen peroxide plus 45 ml of ice cold methanol
1.5% Bovine serum albumin (BSA)	1 gram of BSA + 70 ml of 0.01M PBS + 21 µl of Triton-x100
DAB solution (hydrogen peroxide	1 ml of substrate buffer + 1 drop chromogen was mixed to form a working solution

Chromogen-3,3' diaminobenzidine from the EnVision+™ system	substrate for Horseradish Peroxidase (HRP). Freshly prepared
Hematoxylin	A given volume of hematoxylin was filtered using a filter paper, the solution was refreshed after every 2 weeks

2.1.4 Antibodies

2.1.4.1 Antibodies used for western blot

Table 7: Primary and secondary antibodies used for western blots

Antibody	Company	Product	Host	Dilution
MT1-MMP	NSJ Bioreagents	R31266	Rabbit	1:1000
MT3-MMP	Invitrogen	PA5-79680	Rabbit	1:1000
GAPDH	Cell signaling	2118S	Rabbit	1:1000
Anti-rabbit IgG-HRP	Cell Signalling Technology, USA	7074S	Goat	1:1000

2.1.4.2 Antibodies used for immunohistochemistry and immunofluorescence (IF)

Table 8: Antibodies used for IHC and IF

Antibody	Company	Product	Host	Use/Dilution
MT2-MMP	Invitrogen	PA5-13184	Rabbit	IHC (1:100), IF (1:200)
MT3-MMP	Invitrogen	PA5-79680	Rabbit	IHC (1:100), IF (1:200)
MT1-MMP	Abnova	PAB18771	Rabbit	IHC (1:50)
MT1-MMP	NSJ Bioreagents	R31266	Rabbit	IF (1:200)
Anti-rabbit AF 555	ThermoFisher Scientific	A31572	Donkey	IF (1:500)

Dako envision + Dako system-HRP labelled polymer	K4003	Rabbit	IHC, Ready to use

2.1.5 ELISA kits

Table 9: ELISA Kits

Name	Species	Company	Product
Total MT1-MMP (MMP14) Duoset	Human	R & D, Germany	DY918-05
TGF-beta RIII/betaglycan Duoset	Human	R & D, Germany	DY242
Pierce™ BCA protein assay kit		Thermo Scientific, USA	23225
Ancillary Reagent Duoset Kit		R & D, Germany	DY-008
Cell Counting Kit - 8		Sigma-Aldrich, Germany	96992

2.1.6 Protein gel sample loading buffer

Table 10: Western blot buffers

Buffer/solution	Ingredients
Stacking gel buffer (200 ml)	Tris Base, 12.12 grams SDS, 4 ml Filled up to 200 ml with distilled water, pH to 6.8 with HCl
Separating gel buffer (500 ml)	Tris base, 90.825 grams SDS, 10 ml Filled up to 500 ml with distilled water, pH to 8.8 with HCl
10 × SDS running buffer (1L)	Tris base 30.2 grams Glycin 144.0 grams SDS 50 ml (or 10 grams) Dissolved in 1L of distilled water

Stripping buffer	Glycin 7,5 grams 450 ml of distilled water, pH to 1.9 with HCl
10 × blotting (transfer) buffer (1L)	Tris base, 6,06 grams Glycin, 28,83 grams Dissolved in 1L of distilled water
1 × blotting buffer (1L)	100 ml of 10 × blotting buffer 700 ml of distilled water 200 ml of methanol
Washing buffer (TBST Tween-20 tablets)	One tablet of TBST tween diluted in 500 ml of distilled water (1 × TBST)
Blocking buffer (100 ml)	100 ml of 1 × TBST 5 grams BSA
1 × Cell Lysis buffer	For 2500 ml; 250 µl lysis buffer of 10× lysis buffer 2200 µl distilled water 25 µl protease inhibitor 25 µl of 1 mM PMSF

2.1.7 Cell culture and transfection reagents

Table 11: Cell culture and transfection reagents

Description	Product	Company
1 × PBS without Ca^{2+} and Mg^{2+} pH 7.2	20012068	Thermo Fisher Scientific, Germany
DMEM high glucose	31053028	Thermo Fisher Scientific, Germany
DMEM (F12)	21041033	Thermo Fisher Scientific, Germany
Penicillin/Streptomycin (100 ×) (Pen-Strep)	15140-122	Thermo Fisher Scientific, Germany
IST (Insulin Transferrin Selenium)	51500056	Gibco, Germany
Recovery cell culture freezing medium	12648010	Gibco, Germany
Accutase	A11105-01	Gibco, Germany

Fetal calf serum (FCS)	FCS.ADD.0500	Biosell, Germany
Optimem media	31985062	Thermo Fisher Scientific, USA
Lipofectamine™ RNAiMAX reagent	13778100	Thermo Fisher Scientific, USA
MT1-MMP-siRNA	CRH2935	Cohension Biosciences, Germany
MT3-MMP-siRNA	AM16708	Thermo Fisher Scientific, USA

2.1.8. Equipment

Table 12: Equipment

Description	**Company**
TC10™ Automated cell counter	Bio-Rad, Germany
Cell culture CO_2 incubator	Thermo Fisher Scientific, Germany
SDS gel electrophoresis chambers	Bio-Rad, Germany
Vertical electrophoresis system	Bio-Rad, Germany
Trans-Blot Turbo transfer system	Bio rad, Germany
M200 microplate reader	Tecan, Switzerland
Centrifuge Rotina 380R	Hettich, Germany
Micro centrifuge 200R	Hettich, Germany
Gourmet cooker "MultiGourmet plus FS20R"	Braun, Germany
Photomicroscope DM2000 LED	Leica, Germany
pH meter CG 841	Schott, Germany
Light microscope Leitz Laborlux S	Leica, Germany
Ultrasonic homogenizer Bandelin Sonopuls	Bandelin, Germany
Heraeus Megafuge 1.0R	Themo Fisher Scientific, USA
Light microscope Dialux 20	Leica, Germany
Rotary microtome	Leica, Germany
Incubator Heratherm	Thermo Fisher Scientific, USA
Fusion pulse TS	Vilber Lourmat, Germany
Paraffin embedding machine TP1050	Leica, Germany

2.1.9 Miscellaneous materials

Other materials not listed in the tables above are listed on the table below.

Table 13: Miscellaneous materials

Description	Company
Eppendorf tubes and pipette tips	Eppendorf, Germany
Cell Counting slides, Dual chamber	Bio-Rad, Germany
Dako Pen	Dako, Germany
0.45 nm PVDF membrane	Merck Millipore, Germany
Sterile plastic ware for cell culture	Greiner Bio-One, Germany
SuperFrost Plus slide	Langenbrinck GmbH, Germany

2.2 Methods

2.2.1 Preparation of paraffin sections

To prepare paraffin sections, tissues samples were transferred to Bouin's solution in the operating room and then shifted to a rolling mixer in the laboratory to ensure uniform fixation. After 2 days, Bouin's solution was discarded and the tissue rinsed using 70% ethanol. This was repeated for three consecutive days, changing the ethanol solution daily. Thereafter, the tissue was stored in 70% ethanol at room temperature awaiting further processing. Tissue embedding was done using an embedding machine as described in Table 14 below.

Table 14: Paraffin embedding protocol using an embedding machine (TP1050, Leica Germany)

Tissue cuts	Number	Time	Temperature	Solution
Dehydration	1×	2 hr	RT	Ethanol (80%)
	1×	2 hr	RT	Ethanol (96%)
	3×	3 hr	RT	Ethanol (100%)
Clarification	1×	1 hr	RT	Xylol (100%)
	2×	45 min	RT	Xylol (100%)
Embedding	3×	40 min	RT	Paraffin

RT indicates room temperature

After embedding, the paraffin-embedded tissue biopsies were cooled overnight at 4 °C and serially sectioned to 5 µm using a rotary microtome. The serial sections were then transferred to a warm water bath (distilled water, 45 °C) to stretch them before applying them to SuperFrost Plus slide. The tissue samples were then stained with HE (hematoxylin-eosin stain) and mucin 1 (MUC1), an antibody used to detect endometrial/endometriotic glands/cells (Konrad et al., 2019a), to ensure that suitable samples were selected for evaluation of eutopic and ectopic endometrial epithelial cells.

2.2.2 Immunohistochemistry

2.2.2.1 Immunohistochemistry protocol on Bouin-fixed tissue sections

Immunohistochemistry of Bouin-fixed specimens was performed according to the previously described protocol (Konrad et al., 2018). The EnVision Plus system (DAKO, Germany) was used according to the manufacturer's instructions. Briefly, deparaffinization and rehydration was done by continuous incubation of the slides in neoclear and ethanol as follows:

- One time for 20 min (minutes) in neoclear in an incubator set at 40 °C
- Two times for 20 min in neoclear at room temperature
- Two times for 5 min in ethanol (100%)
- Two times for 5 min in ethanol (96%)
- Two times for 5 min in ethanol (70%)
- Washing for 5 min in distilled water on a shaker.

The slides were heated in a gourmet cooker (Braun, Germany) with citrate buffer, pH 6.0 at 100 °C for 20 minutes to unmask antigens (epitope exposure) on the sections. Then, the samples were left in the citrate buffer for another 20 minutes to cool down. This was followed by rinsing three times in 1× PBS buffer for 5 minutes each. The slides were then incubated with 3% hydrogen peroxide in methanol to inhibit endogenous peroxidase activity, followed by rinsing 3 times in PBS buffer for five minutes each. To inhibit unspecific binding, the tissue samples were incubated in 1.5% BSA (Roth, Germany) for 20 minutes. The primary antibodies (Table 9) were prepared in Real Antibody diluent (Dako, Germany) using a dilution resulting in maximum signal and low background (signal to noise ratio). The area outside the tissue was then wiped dry, leaving approximately 0.5×0.5 cm area around the tissue moist. A square was drawn around the tissue with a Dako PAP-Pen, to prevent the primary antibody from running off the slide. The primary antibody was pipetted (95 µl) and evenly spread on the tissue. The slides were incubated at 4 °C overnight in a moist chamber to protect them from dehydration.
Thereafter, the slides were rinsed three times in 1 × PBS 5 minutes each to remove excess unbound primary antibody. Then a few drops of the corresponding secondary antibody (ready to use) (Table 8) was added dropwise on the tissues and incubated at room temperature for 30 minutes in a moist staining chamber. This was followed by rinsing of the slides in 1 × PBS 3 times, 5 minutes each.

To visualize the antibody binding in colour, three drops of DAB solution (Table 6) were added under a hood. The intensity of coloration was observed under a microscope, to determine the optimal staining time for each antibody. The remaining DAB solution on the tissues was discarded and the slides rinsed 3 times with distilled water 5 minutes each. Counterstaining was done using hematoxylin according to Mayer for 15 minutes, followed by a 10-minute blueing in running tap water. The slides were then dehydrated with consecutive incubation in ethanol and xylol in the following order:

- Two times for 2 min in ethanol (70%)
- Two times for 2 min in ethanol (96%)
- Two times for 2 min in ethanol (100%)
- Once for 2 min in xylol (100%)
- Once for 5 min in xylol (100%)

Finally, the slides were mounted with Eukitt quick-hardening mounting medium.

2.2.2.2 Negative and positive control for IHC

To test for the specificity of the detection system and ensure that the results obtained are due to the interaction of the epitope of the target molecule with the antibody, we included a positive and negative control. From the protein database we identified the placenta and fallopian tubes from the female reproductive tract that express MT1-3-MMPs, and used them as positive controls. The negative control was prepared by omission of the primary antibody (incubation with antibody diluent only). The other steps were the same as for other samples.

2.2.2.3 Evaluation and quantification of the staining intensity

Analysis of the specific localization of the proteins was done using a light microscope Dialux 20 (Leica, Germany). Digital images were obtained using Leica DM 2000/Leica MC170/Leica application suite LAS 4.9.0 (Leica, Germany) and processed with Adobe photoshop CS6. Following staining, the staining intensity was quantified using the HSCORE (negative = 0, weak = 1, moderate = 2, and strong = 3) and the percentage of stained glands. The HSCORE was calculated using the formula: 3 × % of strongly stained glands + 2 × % of moderately stained glands + 1 × % weakly stained glands, giving a range of 0 to 300. All glands or cysts were used for evaluation of the HSCORE and percentage of stained glands/cysts.

2.2.3 MT1-MMP ELISA

The levels of MT1-MMP in serum and endocervical mucus samples of patients with and without endometriosis were quantified using the human total MMP14/MT1-MMP Duoset ELISA kit (DY918-05, 0.625-20 ng/ml) according to the manufacturer' instructions. Briefly, a 96-well plate was coated by addition of 100 µl of the working concentration of the capture antibody to each well and incubated overnight. The wells were then washed 3 times using 1 × wash buffer and incubated with blocking solution (300 µl of 1 × reagent diluent) for one hour at room temperature. After washing the wells, 100 µl of prepared standards, serum and endocervical mucus samples were added to corresponding wells and incubated for 2 hours at room temperature. Then, the wells were again washed 3 times and incubated with 100 µl of working concentration of detection antibody for 2 hours at room temperature. This was followed by rinsing the wells 3 times and incubation with 100 µl of streptavidin- HRP working solution for 20 minutes at room temperature. After repeating the washing step, 100 µl of substrate solution was added and the plate was incubated for 20 minutes protected from light at room temperature. Thereafter the reaction was stopped by addition of 50 µl of the stop solution to each well and absorbance was determined using the M200 microplate reader (Tecan, Switzerland) set at 450 nm/540 nm.

2.2.4 Cell culture experiments

Immortalized endometrial and endometriotic (T-HESC and 12Z) and primary stromal cells were used for the cell culture experiments. The immortalized 12Z cell line was kindly provided by Prof. Anna Starzinski-Powitz (Department of Biology, University of Frankfurt, Germany) whereas immortalized T-HESC cell line was purchased from American Type Culture Collection (ATCC) (Cat. No. CRL-4003). Primary endometrial stromal cells were obtained from endometrial tissues of women (aged 40-50 years) who went through laparascopic hysterectomy because of endometriosis and leiomyoma. The patients were not under any hormonal therapy before surgery. All experiments were performed consistent with the required guidelines and the study was approved by the Ethics committee as described (section 2.1). Primary endometrial stromal cells were isolated from endometrial tissues following the protocol previously described (Swangchan-Uthai et al., 2012) with few changes. After surgery, tissues were washed with 1 × PBS without Ca^{2+} and Mg^{2+} and cut into small pieces of about 1-3 mm. Thereafter, tissues were digested in DMEM/F12 media with 1 mg/ml of *Clostridium*

histolyticum collagenase at 37 °C for 2 hrs in a shaking water bath. Then the suspension was passed through cell strainers (70 µM) and the filtrate consisting of both endometrial epithelial and stromal cells was centrifuged at 500 × g for 5 minutes. The cell-pellet was re-suspended in DMEM F12 supplemented with 10% fetal calf serum (FCS), 1% 100 × pen/strep and 1% insulin transferrin selenium X (ITS) and maintained in a humidified incubator 37 °C, 5% CO_2. Stromal cells attach strongly to the cell culture plates after 18 hrs (Fortier et al., 1988), hence after pre-plating for 18 hrs, the media containing floating epithelial cells and other cell types was discarded and the remaining stromal cells were mantained in fresh media. Stromal cell markers; CD10 and CD248, and the epithelial cell marker mucin 1 (MUC1) were used to assess the purity of the stromal cells.

2.2.4.1 Endometriotic epithelial (12Z) cells

Endometriotic epithelial cells were cultured in DMEM high glucose (4.5 g/L) without phenol supplemented with 10% FCS, 1% 100 × pen/strep and 1% L-glutamine in a humidified incubator (37 °C, 5% CO_2). The media was replaced every two to three days.

2.2.4.2 Endometrial stromal (T-HESC) and primary stromal cells

Primary stromal and THESC cells were cultured in DMEM F12 with L-glutamine without phenol red supplemented with 10% FCS, 1% 100 × pen/strep and 1% ITS. The cells were incubated in a humidified incubator at 37 °C, 5% CO_2. Medium was changed every 2-3 days.

2.2.4.3 Passaging and splitting of cells

At about 80% confluency, cells were passaged or split by dissociation using accutase. For this purpose, medium was removed, cells rinsed with 1 × PBS without Ca^{2+} and Mg^{2+} and harvested with accutase (0.25%) for 5-10 minutes at 37 °C. An equal amount of medium was added to inactivate accutase and the cells were centrifuged at 1000 × g. The supernatant was discarded, the cell pellet re-suspended in fresh cell culture medium and seeded in new cell culture flasks for maintenance or counted and prepared for further experiments.

2.2.4.4 Freezing and thawing of cells

For cell freezing, cell pellets (after dissociation as described in 2.2.4.3) were resuspended in freezing media, aliquoted and incubated at -20 °C for 1 hour followed by overnight incubation at -80 °C. The frozen cells were later transferred to liquid nitrogen for long-

term storage. To thaw cells, a vial was quickly transferred to a 37 °C water bath for 5 minutes, cells were re-suspended in cell culture media and centrifuged at 1000 × g for 5 minutes. The supernatant was discarded and the cell pellet was re-suspended in medium and plated in cell culture flasks containing fresh cell culture medium.

2.2.4.5 Cell counting

For cell counting, 10 µl of cell suspension was transferred to a CASY tube containing 10 µl of 0.4 % of trypan blue dye and mixed by pipetting up and down without forming bubbles. Measurement of the cells was done using an automated cell counter (Bio-Rad, Germany).

2.2.5 Immunofluorescence

2.2.5.1 Immunofluorescence staining of cultured cells

After cell counting, 8×10^4 cells were seeded in a 6-well plate in 2 ml culture medium containing 10% FCS and incubated at 37 °C, 5% CO_2. At about 60% confluency, medium was discarded and cells were rinsed twice with ice-cold 1 × PBS on ice. Then cells were fixed using 95% ice-cold methanol for 20 minutes at -20 °C. The methanol was discarded and cells were washed 3 times 5 minutes each using 1 × PBS with gentle shaking. This was followed with blocking in 3% BSA in 1 × PBS for one hour at room temperature without shaking. After repeating the washing step, cells were incubated overnight with the respective primary antibody (Table 8) diluted in 3% blocking solution at 4 °C. Then cells were rinsed and incubated in the dark with their respective secondary antibody (Table 8) at room temperature for one hour. Following rinsing, cells were incubated with 1 ml nuclei staining buffer (0.5 µg/ml DAPI) for 15 minutes at room temperature protected from light followed by subsequent washing using 1 × PBS. One ml of 1 × PBS was added to each well and the plate was kept at 4 °C protected from light. Images were taken using an inverse Olympus microscope (Olympus, Germany).

2.2.6 Cell lysate preparation and western blot

2.2.6.1 Cell lysate preparation

Cell lysates were prepared on ice using cell lysis buffer (Cell Signaling Technology, USA) according to the supplier's protocol. Approximately $1.5 \times 10^5 – 2.0 \times 10^5$ cells were sub-cultured in a 6-well plate for 48 hours, washed with 1 × PBS and lysed in 250 µl of

1 × lysis buffer containing phenylmethylsulfonyl fluoride (PMSF) and protease inhibitor (Table 10). After 10 minutes, the cells were detached from the culture plate using cell scrapers and transferred to 1 ml Eppendorf tubes. The lysed cells were sonicated (10 seconds, 1 pulse, 50% power) and centrifuged at 14,000 × g for 15 minutes at 4 °C. Thereafter, supernatants were collected and stored at -80 °C awaiting further analysis.

2.2.6.2 Protein quantification

To ensure that equal amounts of proteins are loaded on each gel, protein quantification was determined using Pierce^{TM} BCA protein assay kit (Table 9). This method is based on the reduction of Cu^{2+} in copper (II) sulfate to Cu^{1+} by proteins in the sample resulting in a purple color formation that is proportional to the amount of protein in the test sample. The kit contents were prepared as described by the manufacturer, 10 µl of standards or samples were added to the bottom of a 96-well plate. Then 200 µl of working reagent mixture (reagent A and B mixed in a ratio of 50:1, respectively) was added to each well. The plate contents were mixed on a shaker for 1 minute before being incubated at 37 °C for 30 minutes. Absorbance values were measured at or near 562 nm on a microplate reader (Tecan, Switzerland).

2.2.6.3 SDS-polyacrylamide gel electrophoresis

The SDS polyacrylamide gel electrophoresis separates proteins based on their size and the gel percentage depends on the molecular weight of the proteins involved (20% gel, 4–40 kDa; 15% gel, 12–45 kDa; 12.5% gel, 10–70 kDa; 10% gel, 15–100 kDa; 8% gel, 25–100 kDa and 4-6% gel >200 kDa. Based on the size of the proteins investigated, the separating (12%) and stacking gels were prepared as shown in Table 15 and 16, respectively.

Table 15: SDS-PAGE separating gel preparation

Separating gel	**8%**	**10%**	**12%**	**14%**
Distilled water	3.9 ml	3.3 ml	2.8 ml	2.27 ml
1.5 mM Tris-HCL P.H 8.8	2 ml	2 ml	2 ml	2 ml
Acrylamide	2.13 ml	2.67 ml	3.2 ml	3.73 ml
10% (w/v) APS	25 µl	25 µl	25 µl	25 µl

Temed	7.5 µl	7.5 µl	7.5 µl	7.5 µl

Table 16: SDS-PAGE stacking gel preparation

Stacking gel	
Distilled water	2.29 ml
0.5 mM Tris-HCL PH 6.8	1 ml
Acrylamide	0.67 ml
10 % (w/v) APS	42 µl
Temed	7.5 µl

A gel cast with 1.5 mm spacers was assembled and 1-2 ml of distilled water was added to check leakage. Then, the separating gel was added to the gel cast and overlaid with distilled water to ensure a straight level surface of the polyacrylamide gel after polymerization. It was left to polymerize for about 25-30 minutes. After gently removing the distilled water and drying using a blotting paper, stacking gel was prepared and added to the separating gel. Immediately a gel comb was carefully inserted to avoid introduction of air bubbles.

After 30 minutes the gel assembly was taken out of the gel holder cassettes and transferred to the tank transfer system. The inside and outside chambers were filled with 1 × running buffer and the comb was carefully removed without destroying the wells which were rinsed with running buffer to remove any unpolymerised acrylamide. Appropriate volumes of cell lysates (2.2.6.2) were mixed with loading dye in a ratio of 3:1 (sample to dye), heated for 5 minutes at 95 °C and then set on ice. Briefly, 30 µg of each protein sample and 5 µl of protein marker were loaded to their respective wells and electrophoresed at 70 V until the samples had migrated through the stacking gel into the separating gel. Then the voltage was changed to 120 V and left running until the blue dye started to run off the gel at the bottom end.

Shortly before the end of the SDS-PAGE, a 0.45 µm pore size PVDF membrane (Merk Millipore, Germany) was cut to the size of the gel, soaked in methanol for 1 minute, double distilled water for 2 minutes and finally in 1 × blotting buffer plus 20% methanol.

The gel was carefully removed from the tank and glass plates, and placed in 1 × transfer buffer for 10 minutes. A transfer sandwich containing: blotting papers, PVDF membrane, gel and blotting papers from bottom to top was carefully prepared in a transfer cassette and proteins transferred to a PVDF membrane using the Trans-Blot Turbo transfer system (Bio-Rad, Germany) at a voltage of 25 V and a current of 1.0 A for 30 minutes.
To block non-specific binding, the membrane was incubated with blocking buffer (3% BSA in 1 × TBST) with gentle shaking for one hour at room temperature and washed 3 × each 5 minutes in 1 × TBST before overnight incubation with the respective primary antibodies (Table 7) at 4 °C. The membrane was washed 3 times 5 minutes each in 1 × TBST and then incubated with secondary antibody (Table 7) for one hour at room temperature. After repeating the washing step, the membrane was detected using a chemiluminescent HRP substrate (Cell Signaling Technology, USA). Images were taken using Fusion pulse TS (Vilber Lourmat)**.**

2.2.6.4 Stripping of the membranes

In order to reprobe the western blot membranes with other antibodies, stripping was done to remove the first primary and secondary antibodies used. The membranes were washed 3 times in 1 × TBST 5 minutes each and then incubated in 50 ml stripping buffer solution (Table 10) with gentle shaking for 15 minutes and changing the solution 3 times. The membranes were again washed three times before blocking with 3% BSA in 1 × TBST for one hour at room temperature. The western blot procedure was repeated as before with other antibodies.

2.2.7 siRNA transfection experiments

Endometriotic epithelial 12Z cells (2 x 10^5) were sub-cultured in 6-well plates. At 70 % confluency, they were transfected with either negative siRNA or MT1-MMP-siRNA and MT3-MMP-siRNA (Table 11) using lipofectamine ^{TM} RNAiMAX reagent in medium without antibiotics according to the manufacturer´s instructions. Briefly, lipofectamine transfection reagent and siRNAs were diluted in 150 µl of optimum media each, in a ratio of 1:3 (siRNA: lipofectamine transfection reagent). Equal (150 µl) amounts of diluted transfection agent and siRNA were mixed and left to settle for 5 minutes at room temperature. Then 250 µl of the mixture was added to the cells containing 750 µl of medium without antibiotics to make a final concentration of 100 nM (MT1-MMP-siRNA) and 10 nM, 20nM and 50 nM MT3-MMP-siRNA. After 24 hours, culture medium was

changed to complete culture medium and cells incubated for another 24 hours. A western blot was used to check the efficiency of the knockdown and supernatants were collected for betaglycan ELISAs.

2.2.8 Betaglycan ELISA

The amount of betaglycan in the cell culture supernatants of 12Z cells after MT1-MMP and MT3-MMP siRNA transfections were quantified using the Human TGF-βR3/betaglycan Duoset ELISA kit (Table 9) according to the manufacturer' instructions. Briefly, a 96-well plate was coated by addition of 100 µl of the working concentration of the capture antibody to each well and incubated overnight. The wells were emptied, washed 3 times using 1 × wash buffer and blocked by incubating with 300 µl of 1 × reagent diluent for one hour at room temperature. Wells were again emptied and washed 3 times using 1 × wash buffer; thereafter, prepared standards and cell culture supernatants (100 µl) were added to each corresponding well and incubated for 2 hours at room temperature. Then, wells were washed 3 times and incubated with 100 µl of working concentration of detection antibody for 2 hours at room temperature. Following washing, the plate was incubated with 100 µl of streptavidin-HRP working solution for 20 minutes at room temperature. After repeating the washing step, 100 µl of substrate solution was added and the plate was incubated for 20 minutes protected from light at room temperature. The reaction was stopped by addition of 50 µl of stop solution to each well and the absorbance determined immediately using the M200 microplate reader (Tecan, Switzerland) set at 450 nm/540 nm. Values were normalized against the total protein concentrations of each cell lysate.

2.2.9 Cell viability assay

After siRNA transfection, endometriotic epithelial 12Z cells (1×10^4) were seeded into a 96-well culture plate and incubated for 48 hours at 37 °C. Then, 10 µl of cell counting kit (CCK8) solution was added to each well and cells were incubated for 4 hours at 37 ^{0}C. Absorbance was determined at 450 nm using the M200 microplate reader (Tecan, Switzerland).

2.2.10 Statistical analysis

In this study, the mean and standard error of the mean (SEM) or SDs were used for analysis. Mann-Whitney's test was used for comparisons of 2 groups and Kruskal-Wallis

test for more than 2 groups. For betaglycan ELISAs Dunnet's test was used. Spearman's test was used to analyse the correlation of MT1-MMP serum and endocervical mucus samples with cycle days. GraphPad Prism software (version 5.01 Inc. La Jolla, CV, USA) was used for all the statistical analysis. P-values ≤ 0.05 were considered significant.

3. RESULTS

3. MT1-MMP, MT2-MMP and MT3-MMP in eutopic and ectopic endometrium

Matrix metalloproteinases play important roles in the female reproductive system especially in the endometrium (Salamonsen et al., 2000; Grzechocińska et al., 2017). Because of the membrane associated localization, MT-MMPs have been associated with different activities such as pericellular proteolysis, cell migration, invasion and angiogenesis (Hotary et al., 2000; Plaisier et al., 2006). Thus in this study, the expression pattern of MT1-MMP, MT2-MMP and MT3-MMP in eutopic and ectopic endometrium was determined by immunohistochemistry.

3.1 Localization of MT1-MMP, MT2-MMP and MT3-MMP in the placenta and fallopian tube

We searched in the protein database (www.proteinatlas.org) tissues from the female reproductive system that express the MT-MMPs; MT1-MMP, MT2-MMP and MT3-MMP and used them as positive controls: the placenta and fallopian tubes. MT1-MMP, MT2-MMP and MT3-MMP are localized in the trophoblast cells of the placenta (Fig. 2A, C and E) and the glandular cells of the fallopian tube (Fig. 2B, D and F) respectively, comparable to the localizations shown in the protein database. No staining was observed in the negative control (Fig. 2G).

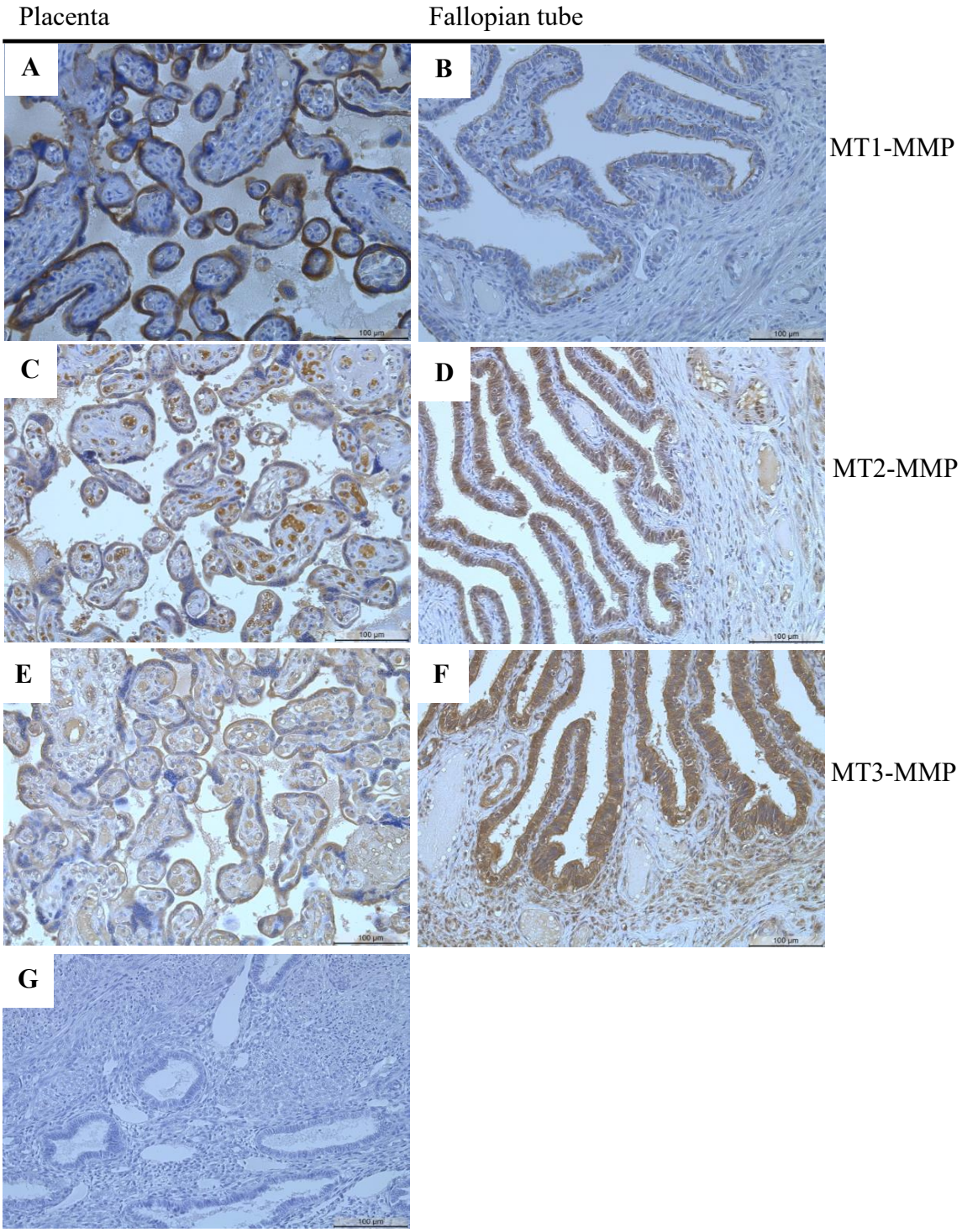

Figure 2. Immunohistochemical detection of MT1-MMP in the placenta (A) and fallopian tube (B), MT2-MMP in the placenta (C) and fallopian tube (D) and MT3-MMP in the placenta (E) and fallopian tube (F), G; negative control for IHC. Counterstaining was performed with hematoxylin. Magnification 200x. Scale bars 100 µm.

3.2 Localization of MT1-MMP in eutopic and ectopic endometrium

3.2.1 Localization of MT1-MMP in eutopic endometrium of patients with and without endometriosis

In order to investigate the localization pattern of MT1-MMP in eutopic endometrium, we analysed tissue samples from patients with and without endometriosis. Analysis of MT1-MMP showed weak or no staining in the glands (14%) of the proliferative and secretory phase of patients without endometriosis (Fig. 3A and B). However, analysis of MT1-MMP localization in patients with endometriosis showed strong to moderate MT1-MMP staining in the glandular epithelial cells of endometrial glands (76%) of the proliferative and secretory phase (Fig. 3C and D). Even though MT1-MMP staining was weak in cases without endometriosis, in both cases, MT1-MMP was preferentially localized in the basal and apical regions of the surface epithelium (Fig. 3A-D). Strong to moderate MT1-MMP staining was also observed in some stromal cells (Fig. 3C and D) and luminal epithelial cells (Fig. 3E, black arrow). Few epithelial and stromal cells also showed MT1-MMP staining in the nuclei (Fig. 3D and E, red arrows). However, no or only weak MT1-MMP expression was detected in endometrial endothelial cells of the blood vessels (Fig. 3E and F, blue arrows).

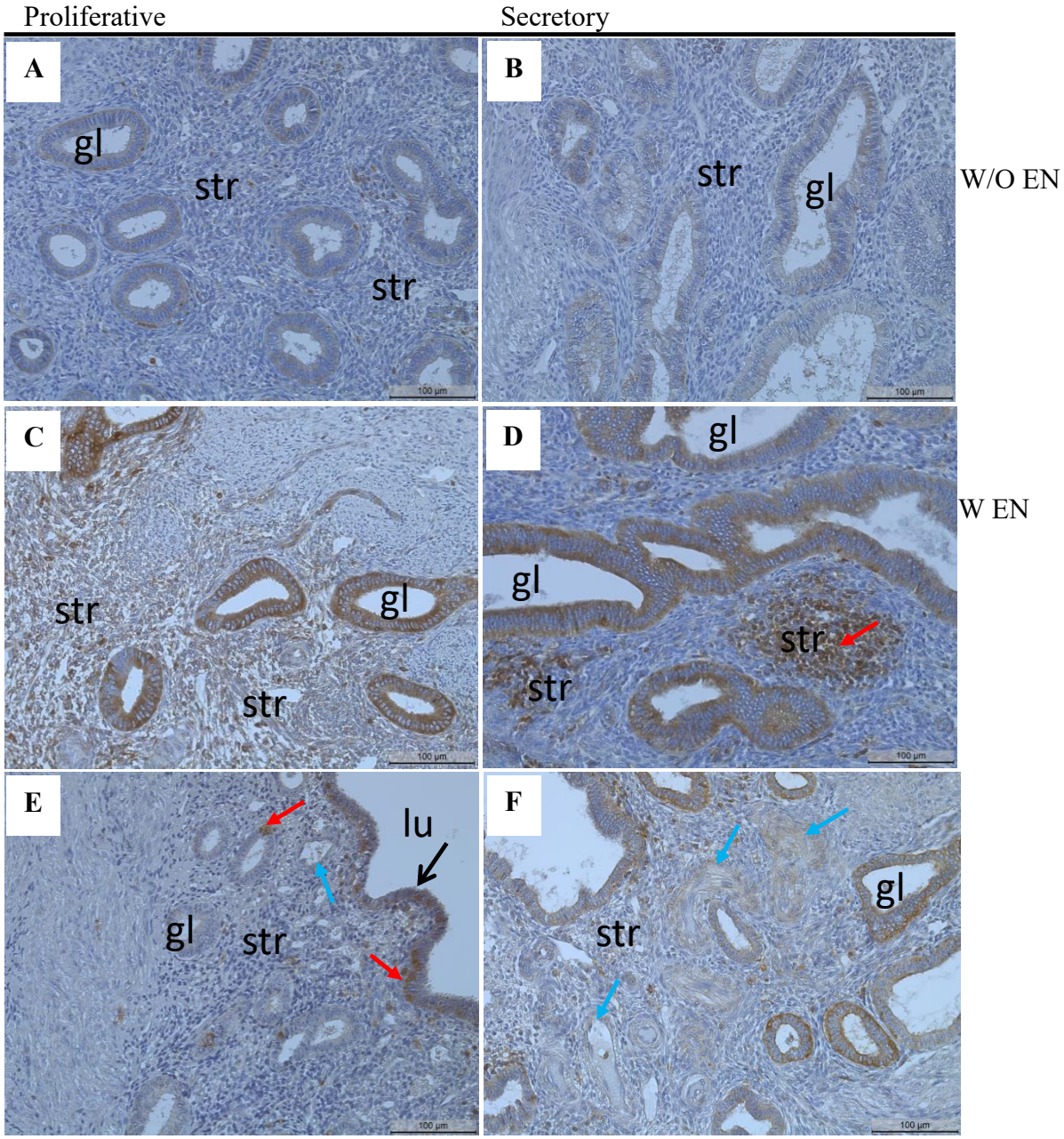

Figure 3. Faint expression of MT1-MMP in the proliferative (A) and secretory (B) endometrium without endometriosis. Strong to moderate detection of MT1-MMP in the proliferative (C) and secretory (D) endometrium with endometriosis. Sometimes strong to moderate staining was observed in some stromal cells (C and D). Some luminal epithelial were also stained (E, arrow, sample tissue from a patient without endometriosis). Few cells also showed positive staining in the nuclei (D and E, red arrows). Faint/no staining was observed in endothelial cells of the blood vessels (E and F, blue arrows). W/O EN, without endometriosis; W EN, with endometriosis; gl, gland; str, stroma; lu, lumen. Counterstaining was performed with hematoxylin. Magnification 200x. Scale bars 100 µm.

3.2.2 Localization of MT1-MMP in adenomyosis

Investigation of MT1-MMP localization in adenomyotic lesions showed a preferential MT1-MMP localization in the glands (92%) of the proliferative and secretory phase with a strong positivity both in the basal and apical regions (Fig. 4A and B). Faint or no staining was observed in the smooth muscle cells of the myometrium (Fig. 4A and B) and the pericytes of the vessel structures (Fig. 4C).

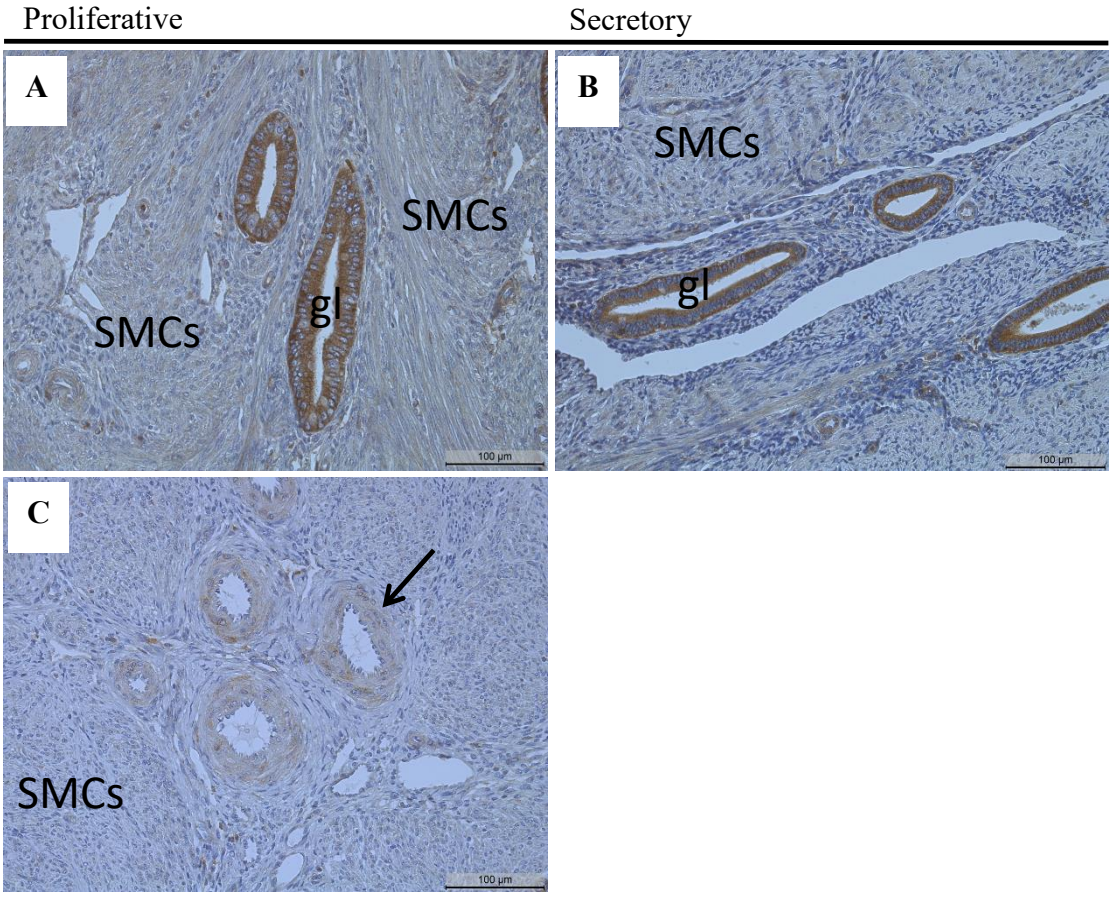

Figure 4. Representative immunostaining showing MT1-MMP localization in adenomyotic lesions of the proliferative (A) and secretory phases (B). Faint or no staining was observed in the smooth muscle cells (A and B) and pericytes of the vessel structures (C). gl, gland; SMCs, smooth muscle cells. Magnification 200x. Scale bars 100 µm.

Table 17: Quantification of MT1-MMP staining in eutopic endometrium and adenomyosis using HSCORE and percentage of stained glands

	EM	AD
	HSCORE	
Mean	91	149
SEM	15.9	15.5
P		0.024
N	16	20
Age	45±6.6	44.5±5.0
	Percentage of stained glands	
Mean	65	92
SEM	9.2	3.0
P		0.037
N	16	20
Age	45±6.6	44.5±5.0

Age is given as median±SD (standard deviation). EM, eutopic endometrium; AD, adenomyosis; SEM, standard error of the mean; n.s., not significant; N, number of samples. Analysis was performed using the Mann–Whitney test. $P \leq 0.05$ was considered significant.

Since, we did not have enough samples in the control group we pooled eutopic endometrium samples with and without endometriosis together and compared them with adenomyosis. Table 17 shows the comparison of MT1-MMP staining in eutopic endometrium and adenomyosis using the HSCORE and the percentage of stained glands. MT1-MMP localization showed a significantly lower HSCORE in eutopic endometrium compared to adenomyosis (Table 17). Similarly, the percentage of MT1-MMP positive glands was significantly lower in eutopic endometrium compared to adenomyosis (Table 17).

3.2.3 Localization of MT1-MMP in peritoneal, ovarian and deep infiltrating endometriosis

To further explore the expression pattern of MT1-MMP in endometriosis, we analysed tissue samples with peritoneal, ovarian and deep infiltrating endometriosis. Fig. 5 shows the localization of MT1-MMP in peritoneal, deep infiltrating and ovarian endometriosis.

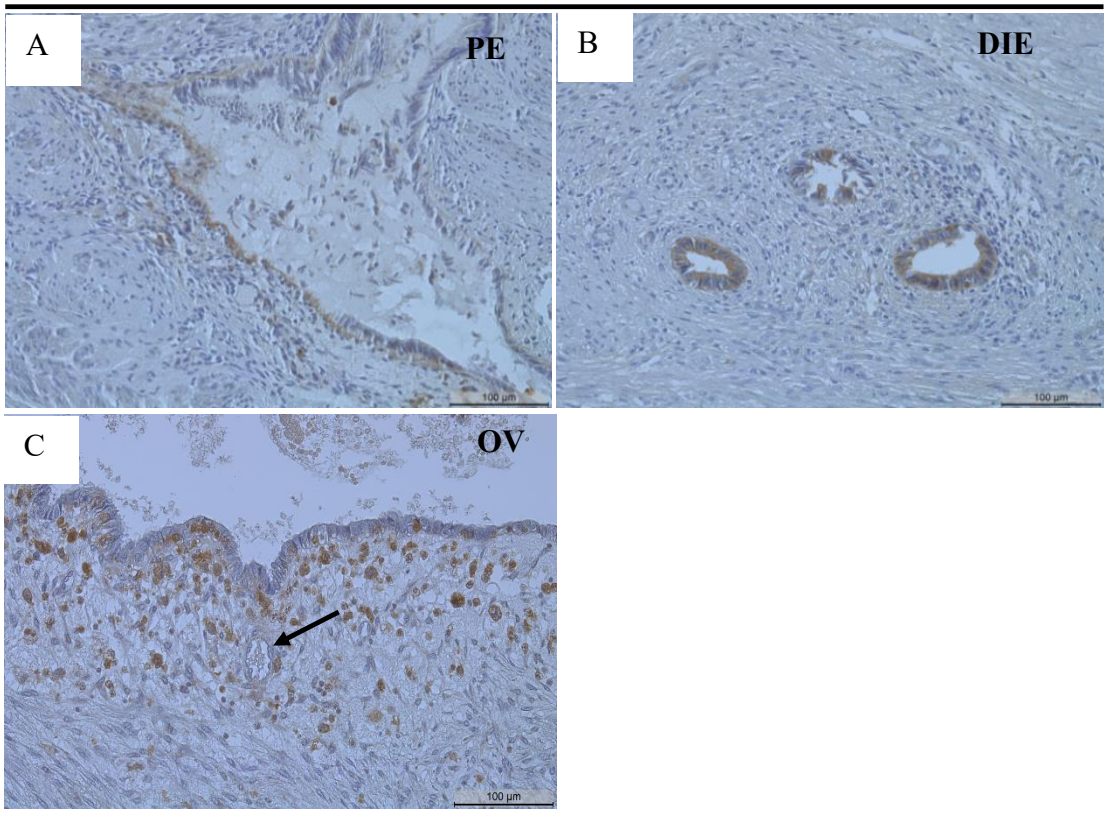

Figure 5. Localization of MT1-MMP in peritoneal (A, cyst), deep infiltrating (B, rectum) and ovarian endometriosis (C, cyst). PE, peritoneal; DIE, deep infiltrating and OV, ovarian endometriosis. Counterstaining was performed with hematoxylin. Magnification 200x. Scale bars 100 µm.

Fig. 5A shows a cyst in peritoneal endometriosis with faintly stained epithelial cells. No staining was observed in the surrounding stromal cells (Fig. 5A). In the representative image of deep infiltrating endometriosis, a tissue sample from the rectum is faintly stained in the cylindrical epithelial cells. Similar to peritoneal endometriosis, the surrounding stromal cells are also not stained (Fig. 5B). Fig. 5C shows a typical cyst in the ovary with a monolayer of few positive cuboidal epithelial cells surrounded by strongly and faintly stained stromal and endothelial (arrow) cells, respectively. Interestingly, in all the three endometriotic entities, most epithelial cells showed faint MT1-MMP staining as opposed

to adenomyotic lesions and eutopic endometrium of patients with endometriosis (Fig. 5A-C).

We did not have enough samples of peritoneal and deep infiltrating endometriosis, so we quantified MT1-MMP staining in eutopic endometrium (with and without endometriosis), adenomyosis and ovarian endometriosis as shown in Table 18. Quantification of MT1-MMP staining using the HSCORE showed that MT1-MMP staining intensity was significantly lower in ovarian endometriosis compared to eutopic endometrium and adenomyosis (Table 18). Similarly, the percentage of MT1-MMP positive glands was also significantly lower in ovarian endometriosis compared to adenomyosis (Table 18).

Table 18: Quantification of MT1-MMP staining in eutopic endometrium, adenomyosis and ovarian using HSCORE and percentage of stained glands

	EM (a)	OV (b)	Adenomyosis (c)
		HSCORE	
Mean	91	31	149
SEM	15.9	10.2	15.5
P		[a,b] 0.0325	[b,c] 0.0003
N	16	6	20
Age	45±6.6	38±8.4	44.5±5.0
		Percentage of stained glands	
Mean	65	32	92
SEM	9.2	3.0	10.5
P		[a,b] n.s.	[b,c] 0.0002
N	16	6	20
Age	45±6.6	38±8.4	44.5±5.0

Age is given as median±SD (standard deviation). SEM, standard error of the mean; EM, endometrium; AD, adenomyosis; OV; ovarian endometriosis; n.s., not significant; N, number of samples. P≤ 0.05 was considered significant. For example [a,b] n.s. means that group a is not significantly different from group b. Analysis was performed using the Mann–Whitney test.

3.3 MT1-MMP in serum and endocervical mucus samples of patients with and without endometriosis

Different studies have reported dysregulation of MT1-MMP in serum and plasma samples of patients with different health conditions such as cancer, preeclampsia, vestibular schwannomas (Laudański et al., 2010; Ren et al., 2020; Sumawan et al., 2020) as well as in peritoneal fluid of patients with and without endometriosis (Laudanski et al., 2005). Based on this, we sought to quantify the levels of MT1-MMP in serum and endocervical mucus samples of patients with and without endometriosis.

3.3.1 Levels of MT1-MMP in serum samples of patients with and without endometriosis

MT1-MMP levels in serum samples of patients with (n=71) and without (n=61) endometriosis were analysed using ELISAs (Table 9).

Table 19: MT1-MMP levels in serum samples of patients with and without endometriosis during the cycle phases

A	Menstrual (a)	Proliferative (b)	Secretory (c)			
Samples (n)	17	33	45			
Median age	30±8.3	31±8.6	29±7.4			
Mean (ng/mL)	0.73	2.17	0.68			
SEM	0.2	1.7	0.3			
Range (ng/mL)	0-2.9	0-57.2	0-7.7			
P-value		^{a,b} n.s.	^{b,c} n.s. $^{a,c}\leq 0.05$			
B	**Without endometriosis**			**With endometriosis**		
Phases	Menstrual (a)	Proliferative (b)	Secretory (c)	Menstrual (d)	Proliferative (e)	Secretory (f)
Samples (n)	8	18	29	9	15	16
Median age	26.5±10.4	27±8.7	28±7.6	31±6.3	37±6.3	32±6.6
Mean (ng/mL)	0.7	0.6	0.8	0.7	4.1	0.6
SEM	0.3	0.2	0.3	0.3	3.7	0.4
Range (ng/mL)	0.1-2.9	0-3.3	0-7.7	0-2.2	0-57.2	0-5.7
P-value		^{a,b} n.s.	^{a,c} n.s. ^{b,c} n.s.		$^{d,e}\leq 0.05$	$^{d,f}\leq 0.05$ ^{e,f} n.s.
				^{a,d} n.s.	^{b,e} 0.042	^{c,f} n.s.

Age is given as median±SD (standard deviation). SEM, standard error of the mean; n.s., not significant; n, number of samples. P≤ 0.05 was considered significant. For example ^{d,e} 0.05 means that group d is significantly different from group e. Analysis was performed using the Kruskal–Wallis test and the Mann–Whitney test.

Table 19 shows the results of the analysis of MT1-MMP levels in serum samples of patients with and without endometriosis during the phases of the menstrual cycle. We grouped the serum samples with and without endometriosis into the three phases of the

cycle and compared the levels of MT1-MMP between the groups. MT1-MMP levels in the phases of the cycle showed significantly higher (7%) levels in the menstrual phase compared to the secretory phase of patients with and without endometriosis (Table 19A). However, no differences were observed in MT1-MMP serum levels between the menstrual and proliferative phase of patients with and without endometriosis (Table 19A). Further grouping based on patients with and without endometriosis revealed that MT1-MMP serum levels in patients with endometriosis were significantly reduced in the menstrual phase (83%) and secretory phase (85%) compared to the proliferative phase (Table 19B). Similarly, MT1-MMP serum levels were about 85% lower in the proliferative phase of patients without endometriosis compared to the proliferative phase of those with endometriosis (Table 19B). No significant differences were observed in MT1-MMP serum levels in the different cycle phases of patients without endometriosis (Table 19B).

Table 20 shows the results of MT1-MMP levels in serum samples of patients with and without endometriosis and using or not using contraceptives. Comparison of MT1-MMP serum levels between patients with and without endometriosis showed significantly higher (85%) levels in patients with endometriosis compared to those without endometriosis (Table 20A).

We further sought to find out if use of contraceptives affected the levels of MT1-MMP in serum and observed significantly higher (14%) levels in patients without endometriosis and not using contraception compared to patients with endometriosis and not receiving contraception (Table 20B). Additionally, MT1-MMP serum levels in patients without endometriosis and receiving contraceptives were significantly reduced (75%) compared to patients with endometriosis and receiving contraception (Table 20B).

Table 20: MT1-MMP levels in serum samples of patients with and without endometriosis and with or without contraception

A	**Without endometriosis (a)**	**With endometriosis (b)**
Samples (n)	61	71
Median age	27±7.9	34±7.1
Mean (ng/mL)	0.7	1.3
SEM	0.2	0.8
Range (ng/mL)	0-7.7	0-57.2
P-value		[a,b] 0.0016

B	**Contraception**			
	w/o EN, w/o c (a)	w/o EN, w c (b)	w EN, w/o c (c)	w EN, w c (d)
Samples (n)	33	28	36	35
Median age	28±9.2	25±4.9	34±7.3	34±6.9
Mean (ng/mL)	0.8	0.5	0.7	2.0
SEM	0.3	0.2	0.2	1.6
Range (ng/mL)	0-7.7	0-3.3	0-5.7	0-57.2
P-value		[a,b] n.s.		[c,d] n.s.
			[a,c] 0.043	[b,d] 0.030

Age is given as median±SD (standard deviation). SEM, standard error of the mean; w/o EN, w/o c, without endometriosis and contraception; w/o EN, w c, without endometriosis and with contraception; w EN, w/o c, with endometriosis and without contraception; w EN, w c, with endometriosis and contraception; n.s., not significant. For example [a,b] n.s. means that group a is not significantly different from group b. Analysis was performed using the Mann–Whitney test.

3.3.2 Levels of MT1-MMP in endocervical mucus samples of patients with and without endometriosis

MT1-MMP levels in endocervical mucus samples of patients with (n=106) and without (n=87) endometriosis were analysed using ELISAs (Table 9) and the results are shown in Table 21.

Table 21: MT1-MMP levels in endocervical mucus samples of patients with and without endometriosis during the cycle phases

A	**Proliferative (a)**	**Secretory (b)**
Samples (n)	66	100
Median age	31±7.8	31±7.3
Mean (ng/mL)	4.4	3.2
SEM	0.2	0.2
Range (ng/mL)	0-14.0	0-9.3
P-value		[a,b] 0.0001

B	**Without endometriosis**		**With endometriosis**	
	Proliferative (a)	Secretory (b)	Proliferative (c)	Secretory (d)
Samples (n)	32	49	34	51
Median age	31±9.1	27±7.4	30.5±6.4	34±6.5
Mean (ng/mL)	4.1	2.8	4.6	3.5
SEM	0.3	0.2	0.4	0.3
Range (ng/mL)	0-7.6	0-6.9	0-14	0-9.3
P-value		[a, b] 0.0006	[a, c] n.s.	[c,d] 0.010 [b,d] n.s.

Age is given as median±SD (standard deviation). SEM, standard error of the mean; n.s., not significant; n, number of samples. P≤ 0.05 was considered significant. For example a,b<0.0001 means that group a is significantly different from group b. Analysis was performed using the Mann–Whitney test.

We grouped both samples with and without endometriosis into the proliferative and secretory phase and compared them. Interestingly, MT1-MMP concentrations in the proliferative phase of both patients with and without endometriosis were significantly higher (38%) compared to the secretory phase of both patients with and without endometriosis (Table 21A).

Further grouping based on patients with and without endometriosis showed remarkably higher (46%) levels in the proliferative phase compared to the secretory phase of patients without endometriosis (Table 21B). Similarly, MT1-MMP levels were significantly higher (31%) in the proliferative phase compared to the secretory phase of patients with endometriosis (Table 21B).

Table 22: MT1-MMP levels in endocervical mucus samples of patients with and without endometriosis and with or without contraception

A	**Without endometriosis (a)**	**With endometriosis (b)**
Samples (n)	87	106
Median age	28±8.2	33±7.1
Mean (ng/mL)	3.2	3.6
SEM	0.2	0.2
Range (ng/mL)	0-7.6	0-14
P-value		[a,b] n.s.

B	**Contraception**			
	w/o EN, w/o c (a)	w/o EN, w c (b)	w EN, w/o c (c)	w EN, w c (d)
Samples (n)	65	22	70	36
Median age	30±8.5	27±7.2	32.5±7.2	33±6.8
Mean (ng/mL)	3.4	2.7	3.8	3.2
Range (ng/mL)	0-7.6	0-5.4	0-9.3	0-14
SEM	0.2	0.4	0.2	0.4
P-value		[a,b] n.s.		[c,d] 0.003 [b,d] n.s.

Age is given as median±SD (standard deviation). SEM, standard error of the mean; w/o EN, w/o c, without endometriosis and contraception; w/o EN, w c, without endometriosis and with contraception; w EN, w/o c, with endometriosis and without contraception; w EN, w c, with endometriosis and contraception; ns, not significant. P≤ 0.05 was considered significant. For example [a,b] n.s. means that group a is not significantly different from group b. Analysis was performed using the Mann–Whitney test.

Table 22 shows the results of MT1-MMP levels in endocervical mucus samples of patients with and without endometriosis, with or without contraception. MT1-MMP levels in endocervical mucus of patients with and without endometriosis showed no significant difference between the groups (Table 22A). However, MT1-MMP levels in endocervical mucus of patients with endometriosis and with contraception were significantly reduced (16%) compared to patients with endometriosis and without contraception (Table 22B).

3.3.3 Relationship of MT1-MMP levels in serum and endocervical mucus with different clinical characteristics

We also investigated if MT1-MMP levels in serum and endocervical mucus samples are correlated with different clinical characteristics such as cycle days and pain. Besides, we explored the relationship between MT1-MMP levels in serum and endocervical mucus of

patients with and without endometriosis with BMI, age and pain: dysmenorrhea, dysuria, dyschezia and dyspareunia, during menstruation.

There were no significant associations between MT1-MMP levels in serum and endocervical mucus of patients with and without endometriosis and cycle days in all phases of the menstrual cycle (Fig. 6 and 7, Table 23A). We grouped the serum and mucus samples, both with and without endometriosis based on the pain level on a scale of 1-10; pain scale of 0-3 (no pain) and 4-10 (with pain) and compared the two groups. We did not detect any significant differences in MT1-MMP levels in serum and endocervical mucus of patients with pain compared to those without pain (Table 23B and C). However, the age of serum samples of patients with endometriosis was significantly higher as compared to patients without endometriosis (Table 23B).

Fig. 6 shows the correlation of MT1-MMP levels in serum samples of patients with and without endometriosis and cycle days: menstrual, proliferative and secretory phases.

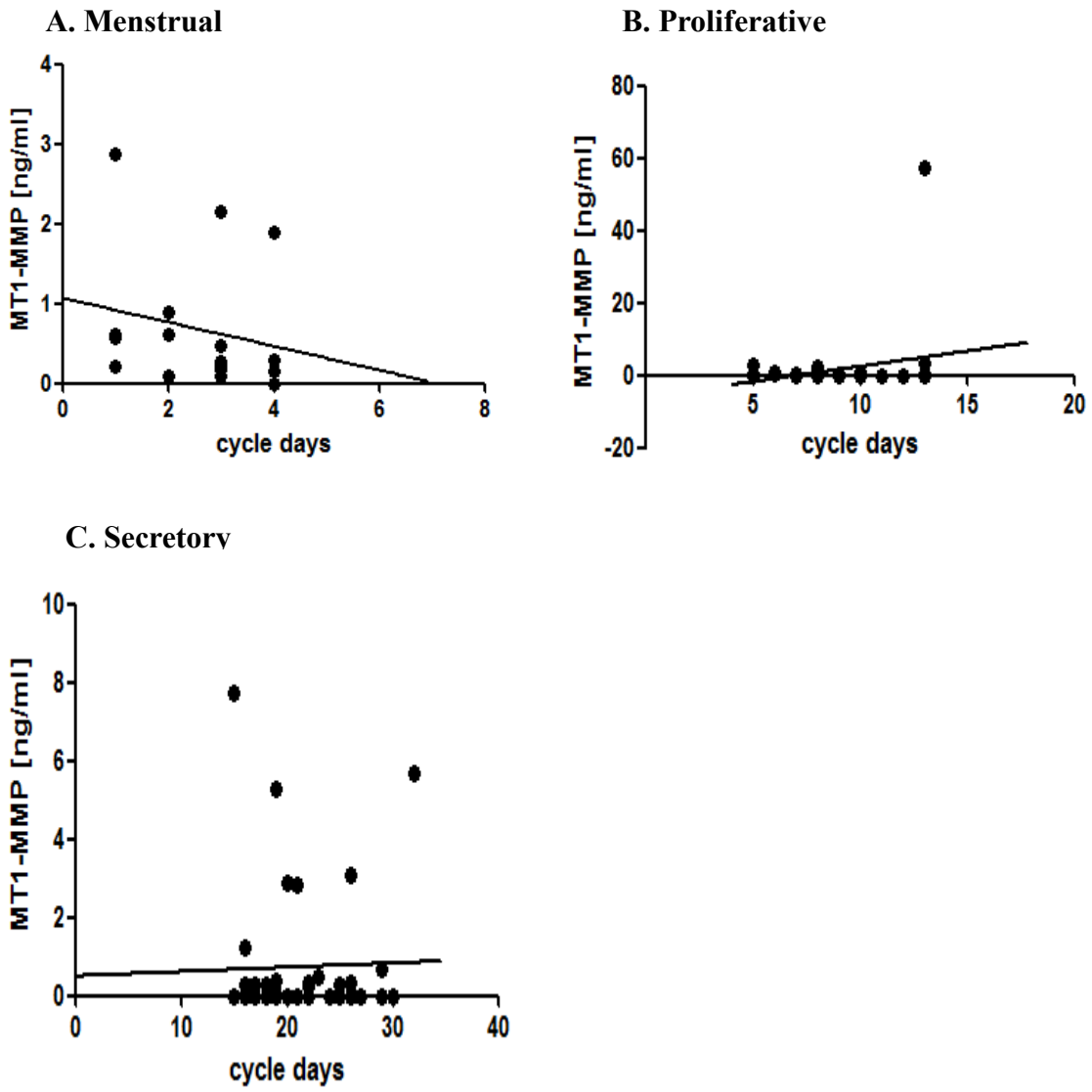

Figure 6. Correlation of MT1-MMP levels in serum samples of patients with and without endometriosis with cycle days. A, menstrual (day 1-4, n=17); B, proliferative (day 5-14, n=33) and C, secretory (day 15-32, n=44). For example, day 1-4 means the first four days of the menstrual cycle.

Fig. 7 shows the correlation of MT1-MMP levels in endocervical mucus samples of patients with and without endometriosis and cycle days: proliferative and secretory phases.

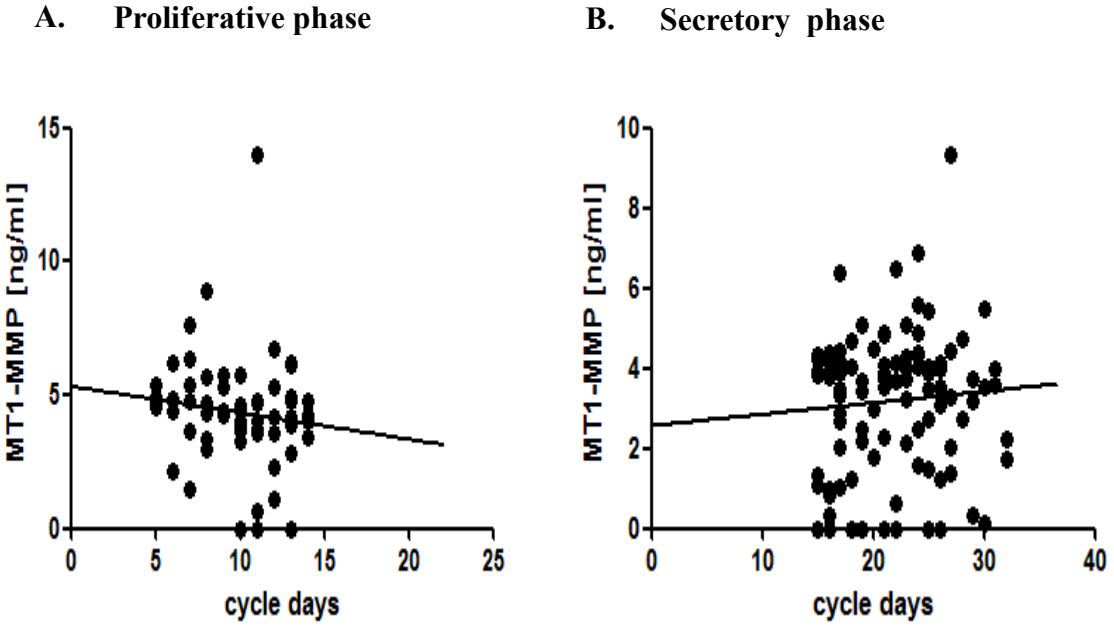

Figure 7. Correlation of MT1-MMP levels in endocervical mucus samples of patients with and without endometriosis with cycle days. A, proliferative phase (day 5-14, n=66) and B, secretory phase (day 15-32, n=100). Analysis was performed using the Spearman's test.

Table 23 shows the correlation of MT1-MMP levels in serum and endocervical mucus samples of patients with and without endometriosis with different clinical characteristics.

Table 23: Relationship of MT1-MMP levels in serum and endocervical mucus samples with different clinical characteristics

A	**Correlation of MT1-MMP levels**				
Serum MT1-MMP levels			**Endocervical mucus MT1-MMP levels**		
Cycle phase	**Spearmann**	**P**	**Cycle phase**	**Spearmann r**	**P**
Menstrual (n=17)	-0.32	n.s.	Proliferative (n=66)	-0.23	n.s.
Proliferative (n=33)	-0.18	n.s.	Secretory (n=100)	0.04	n.s.
Secretory (n=44)	0.03	n.s.			
B	**Serum MT1-MMP levels**				
				Mean (ng/mL)±SEM	
	W/o EN	**W EN**	**P**	**Without pain**	**With pain**	**P**
BMI (kg/m^2)	23.9±0.8	24.4±0.6	n.s.			
Age	28.9±1.0	34.8±0.8	0.0001			
Dysmenorrhea	130			0.97±0.3	1.10±0.7	n.s.
Dysuria	131			1.18±0.5	0.22±0.1	n.s.
Dyschezia	132			1.24±0.6	0.52±0.2	n.s.
Dyspareunia	127			0.77±0.2	1.46±1.1	n.s.
C	**Endocervical mucus MT1-MMP levels**				
				Mean (ng/mL)±SEM	
	W/o EN	**W EN**	**P**	**Without pain**	**With pain**	**P**
BMI (kg/m^2)	22.9±0.5	23.4±0.4	n.s.			
Age	30.8±0.9	33.9±0.7	n.s.			
Dysmenorrhea	n=192			3.18±0.3	3.49±0.2	n.s.
Dysuria	n=193			3.39±0.2	3.52±0.3	n.s.
Dyschezia	n=189			3.46±0.2	3.50±0.2	n.s.
Dyspareunia	n=187			3.56±0.2	3.39±0.2	n.s.

n, number of samples; n.s., not significant; SEM, standard error of the mean; BMI, body mass index W/o EN, without endometriosis; W EN, with endometriosis. Cycle days (1–32); without pain (pain scale = 0–3); with pain (pain scale 4–10). BMI and age are given as mean±SEM Analysis was performed using the Spearman's correlation test and the Mann–Whitney test.

3.4 Localization of MT2-MMP in eutopic and ectopic endometrium

3.4.1 Localization of MT2-MMP in eutopic endometrium of patients with and without endometriosis

Similar to MT1-MMP, MT2-MMP localization in eutopic endometrium was also analysed with tissue samples from patients with and without endometriosis. Study of

MT2-MMP showed low to moderate staining in the glands (57%-70%) of the proliferative and secretory phase of patients with and without endometriosis. MT2-MMP localization in the glandular component was observed in the basal and apical regions (Fig. 8A-E) and sometimes also in the nuclei (Fig. 8B). Additionally, stromal cells (Fig. 8A-C and E) and luminal epithelial cells (Fig.8E, red arrow) were also moderately stained. Endothelial cells of the blood vessels also demonstrated moderate MT2-MMP staining (Fig. 8E, black arrows).

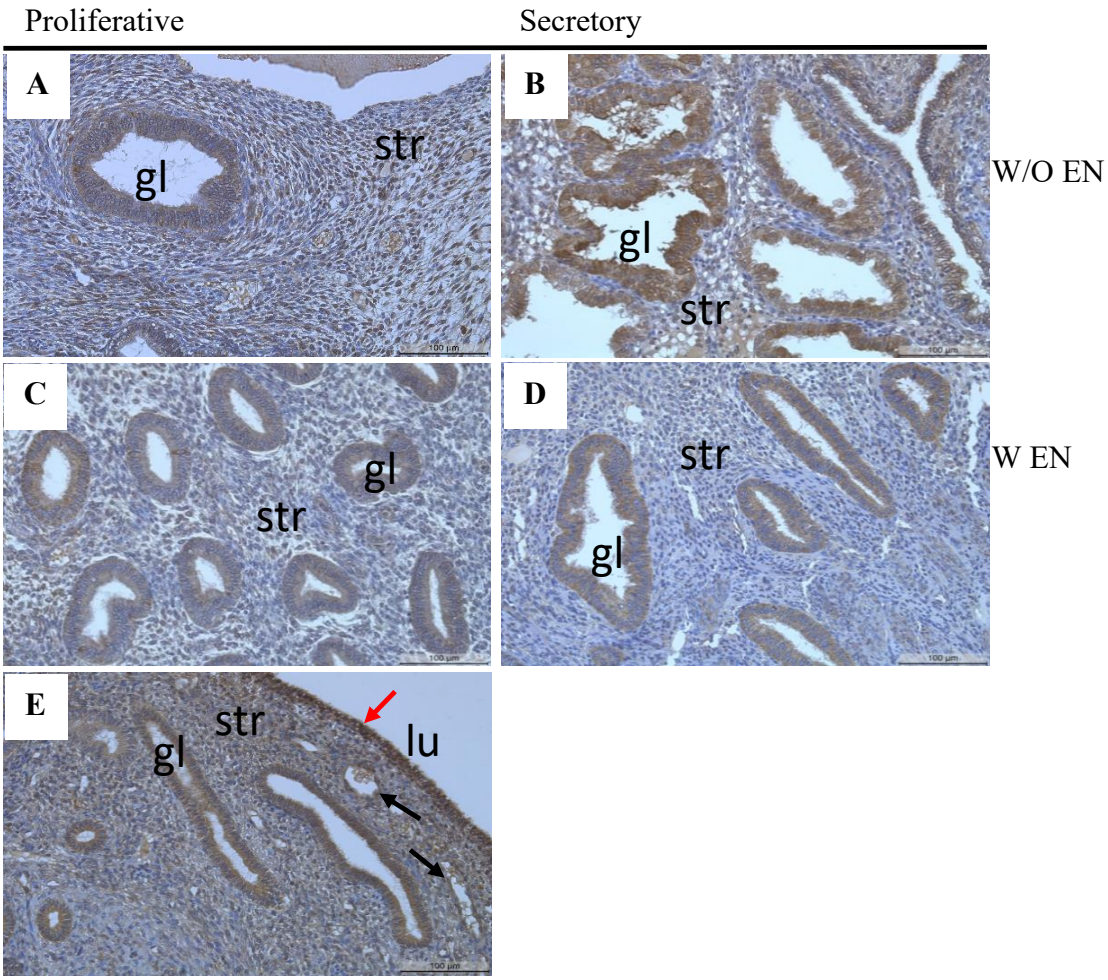

Figure 8. Immunohistochemical detection of MT2-MMP in the proliferative (A) and secretory (B) endometrium without endometriosis and proliferative (C) and secretory (D) endometrium with endometriosis. Sometimes, moderate staining of the surrounding stromal (A-C and E) and endothelial cells (E) could be observed. Some luminal epithelial cells also stained positive for MT2-MMP (E, red arrow). W/O EN, without endometriosis; W EN, with endometriosis; str, stroma; gl, gland; lu, lumen. Counterstaining was performed with hematoxylin. Magnification 200x. Scale bars 100 µm.

3.4.2 Localization of MT2-MMP in adenomyosis

Positivity of MT2-MMP was also identified in the glandular epithelial cells (81%-84% of stained glands) of patients with adenomyosis in both the proliferative and secretory phase (Fig.9A and B). In some tissues MT2-MMP staining in the glandular cytoplasm was apicolateral (Fig.9A) and others showed staining in the apical and basal regions (Fig. 9B). Also, the nucleus in some cells stained positive for MT2-MMP (Fig. 9A). The smooth muscle cells of the myometrium demonstrated strong MT2-MMP staining (Fig. 9A-B). Additionally, the pericytes and endothelial cells of the vessel structures in the myometrium showed strong MT2-MMP staining (Fig. 9C, arrow).

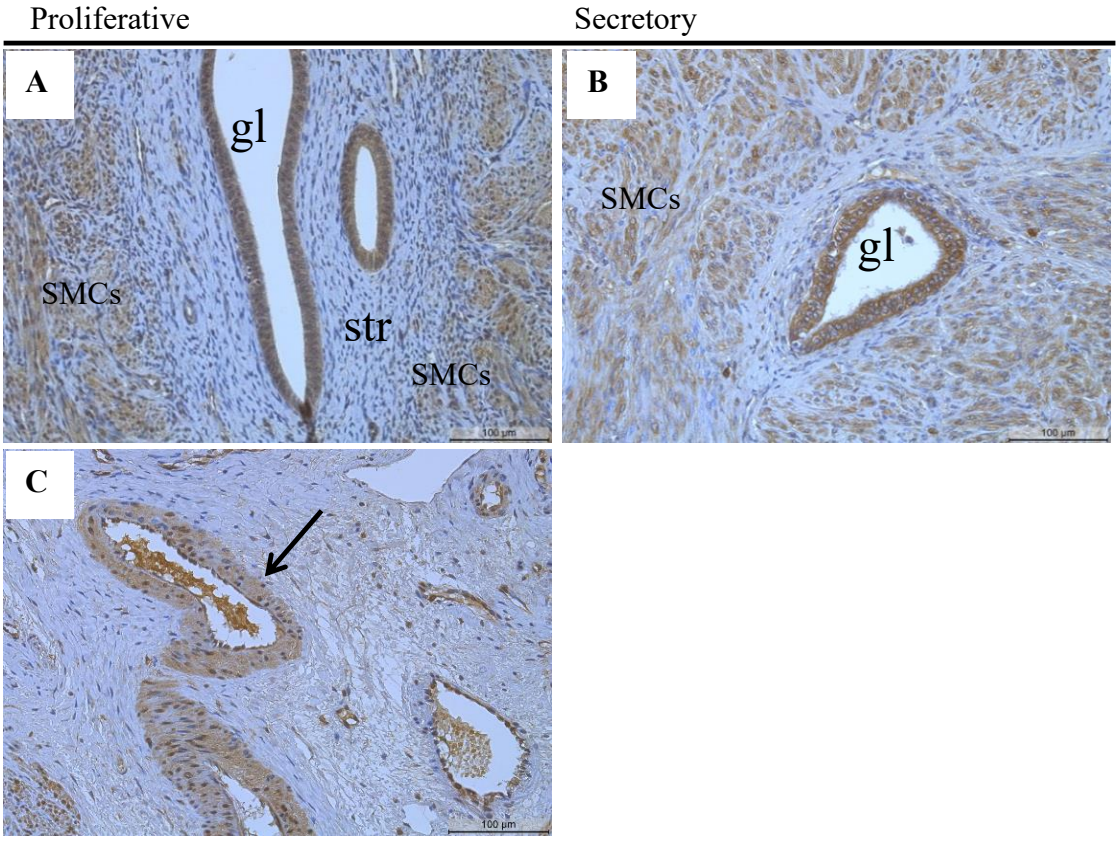

Figure 9. Immunohistochemical detection of MT2-MMP in the proliferative (A) and secretory phases (B) of patients with adenomyotic lesions and in blood vessels (C). gl, gland; SMCs, smooth muscle cells; str, stroma. Counterstaining was performed with hematoxylin. Magnification 200x. Scale bars 100 µm.

Comparison of MT2-MMP HSCORE and percentage of stained glands in eutopic endometrium and adenomyosis is shown in Table 24 below.

Table 24: Comparison of MT2-MMP in the proliferative and secretory phases of endometrium of patients with and without endometriosis and adenomyosis using the HSCORE and percentage of stained glands

	ctrl (healthy)		Em-En		Em-Ad		Ad	
				HSCORE				
	P	**S**	**P**	**S**	**P**	**S**	**P**	**S**
Mean	67	59	61	56	67	70	90	83
SEM	4.6	8.1	8.2	9.3	11.7	6.8	6.8	5.2
P-value	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
N	6	8	8	10	10	14	19	22
Age	35±13	41±5.3	33±6.7	41±6.3	43.5±5.5	45±5.9	44±5.4	45±5.8
				Percentage of stained glands				
	P	**S**	**P**	**S**	**P**	**S**	**P**	**S**
Mean	57	65	62	59	70	69	84	81
SEM	7.4	7.9	7.2	9.1	9.1	5.7	5.0	4.6
P-value	n.s	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
N	6	8	8	10	10	14	19	22
Age	35±13	41±5.3	33±6.7	41±6.3	43.5±5.5	45±5.9	44±5.4	45±5.8

Age is given as the median ± SD (standard deviation). N, number of samples; SEM, standard error of the mean; ctrl, control group (normal endometrium), Em-En, Endometrium with endometriosis; Em-Ad, Endometrium with adenomyosis; Ad, Adenomyosis; P, proliferative; S, secretory; n.s., not significant. $P \leq 0.05$ was considered significant. Analysis was performed using the Kruskal–Wallis test.

Quantification of MT2-MMP expression using the HSCORE and the percentage of stained glands showed no significant differences between the four groups in both the proliferative and secretory phase (Table 24). Similarly, no significant differences were

observed between the eutopic endometrium of patients with compared to patients without endometriosis (Table 24). Thus we decided to merge the datasets for further comparison.

3.4.3 Localization of MT2-MMP in peritoneal, ovarian and deep infiltrating endometriosis

We also investigated the localization of MT2-MMP in peritoneal, deep infiltrating and ovarian endometriosis to find out how it compares with eutopic endometrium.

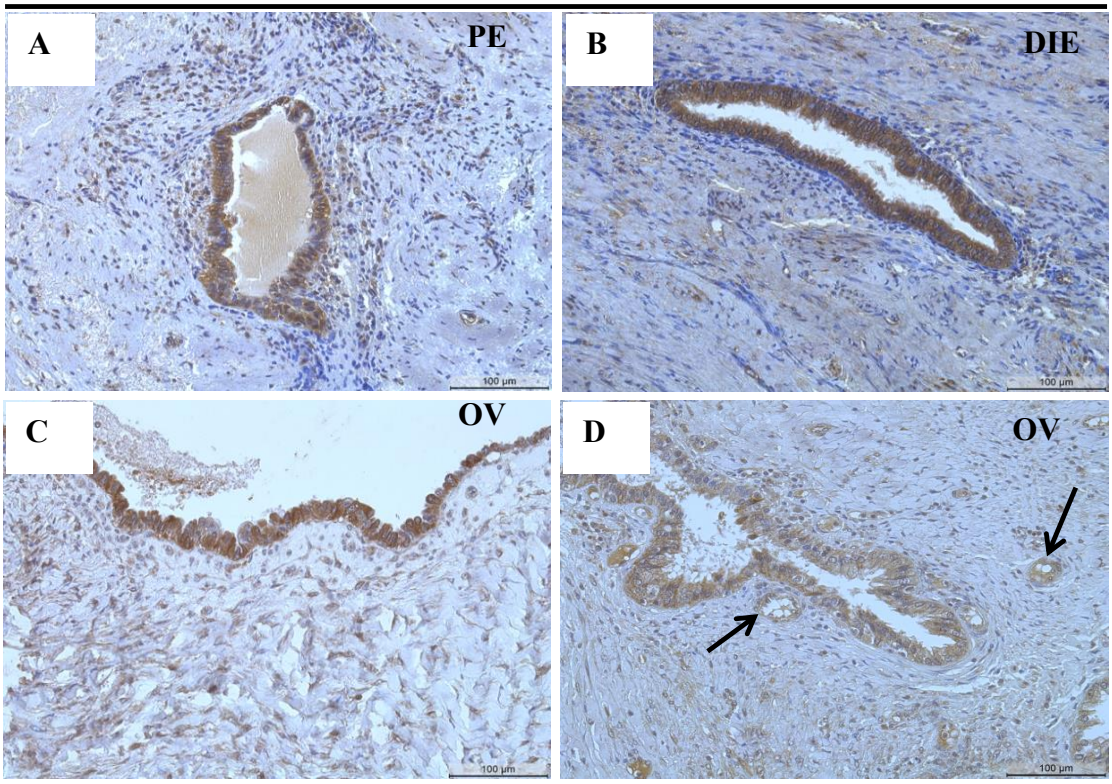

Figure 10. Localization of MT2-MMP was detected in peritoneal (A, scar) deep infiltrating (B, rectosigmoid) and ovarian (C and D, cyst) endometriosis. PE, peritoneal; DIE, deep infiltrating and OV, ovarian endometriosis. Counterstaining was performed with hematoxylin. Magnification 200x. Scale bars 100 µm.

Fig. 10 shows that MT2-MMP localization in the three endometriotic entities which was greatly reduced in the glands/cysts (33%-42%) compared to eutopic endometrium (65%). The photograph representing peritoneal endometriosis shows a scar with moderately stained cylindrical epithelial cells and faintly stained adjacent stromal cells (Fig. 10A). Also, representing deep infiltrating endometriosis is a tissue from the rectosigmoid with moderately stained cylindrical epithelial cells. The adjacent stroma cells did not stain for MT2-MMP (Fig. 10B). Fig. 10C shows a cyst in ovarian endometriosis with positively stained cuboidal epithelial cells and adjacent negative stroma cells. Nuclei staining were

also observed in some epithelial cells (Fig. 10C). Endothelial cells in ovarian lesions also stained positive for MT2-MMP (Fig. 10D, arrows).

Table 25 shows the comparison of MT2-MMP HSCORE and percentage of stained glands in eutopic and ectopic endometrium. We pooled the eutopic endometrium samples of both patients with and without endometriosis together and made comparisons with the three different endometriotic entities. Quantification of MT2-MMP staining using the HSCORE revealed that MT2-MMP staining intensity was significantly lower in ovarian, peritoneal and deep infiltrating endometriosis compared to eutopic endometrium (Table 25). Similarly, the percentage of ectopic positive MT2-MMP glands was also significantly lower in the three endometriotic entities compared to the eutopic endometrium (Table 25).

Table 25: Comparison of MT2-MMP in eutopic and ectopic endometrium using the HSCORE and percentage of stained glands

	EM (a)	OV (b)	DIE (c)	PE (d)
		HSCORE		
Mean	64	33	45	38
SEM	3.5	5.9	5.8	5.9
P	n.s.	a,b<0.001	a,c≤0.05	a,d<0.01
N	56	23	29	27
Age	42±7.6	36±5.4	32±5.0	33±4.6
		Percentage of stained glands		
Mean	65	33	42	36
SEM	3.2	5.8	5.1	5.7
P	n.s	a,b<0.001	a,c ≤0.01	a,d<0.001
N	56	23	29	27
Age	42±7.6	36±5.4	32±5.0	33±4.6

The age is given as the median ± standard deviation (SD). N, number of lesions; (a-d; groups); EM, eutopic endometrium; OV, ovarian endometriosis; DIE, deep infiltrating endometriosis; PE, peritoneal endometriosis; SEM, standard error of the mean; n.s, non-significant. P≤ 0.05 was considered significant. For example a,b<0.001 means that group a is significantly different from group b. Analysis was performed using the Kruskal–Wallis test.

3.5 Localization of MT3-MMP in eutopic and ectopic endometrium

3.5.1 Localization of MT3-MMP in eutopic endometrium of patients with and without endometriosis

Investigation of MT3-MMP localization in eutopic endometrium showed strong MT3-MMP staining in most of the glands of the proliferative and secretory phase of patients with and without endometriosis (Fig. 11A-D). In both cases the surface epithelium in the basal and apical regions was stained (Fig. 11A-D). Additionally, luminal epithelial cells demonstrated moderate MT3-MMP staining (Fig. 11C, arrow). Some stromal cells were also stained (Fig. 11A and E). Strong MT3-MMP staining was also observed in endometrial endothelial cells of the blood vessels (Fig. 11E).

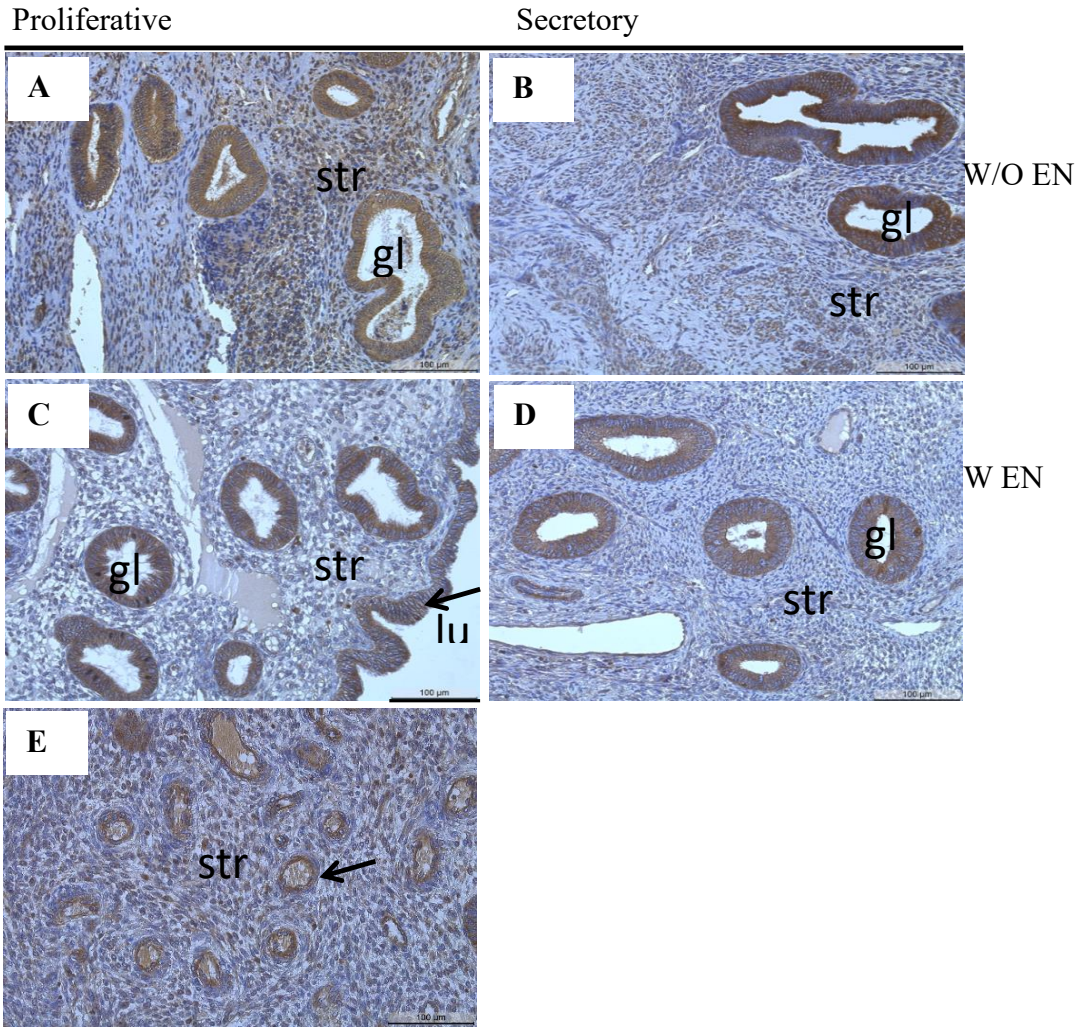

Figure 11. Detection of MT3-MMP showed strong positive staining in the glandular epithelial cells of the proliferative (A) and secretory (B) endometrium without endometriosis and proliferative (C) and secretory (D) endometrium with endometriosis. Luminal epithelial cells (C, arrow), stromal cells (A and E) and endothelial cells of the blood vessels (E) were also stained. gl; gland; lu, lumen; str, stroma; W/O EN, without endometriosis; W EN, with endometriosis. Counterstaining was performed with hematoxylin. Magnification 200x. Scale bars 100 µm.

3.5.2 Localization of MT3-MMP in adenomyosis

Localization of MT3-MMP in adenomyosis was found mainly in the proliferative and secretory glands (95%) of patients with adenomyotic lesions with pronounced staining in both the apical and basal regions (Fig.12A and B). Moreover, strong to moderate staining was also observed in the smooth muscle cells of the myometrium (Fig. 12A and B). The pericytes and endothelial cells in the myometrium demonstrated strong MT3-MMP staining (Fig. 12C).

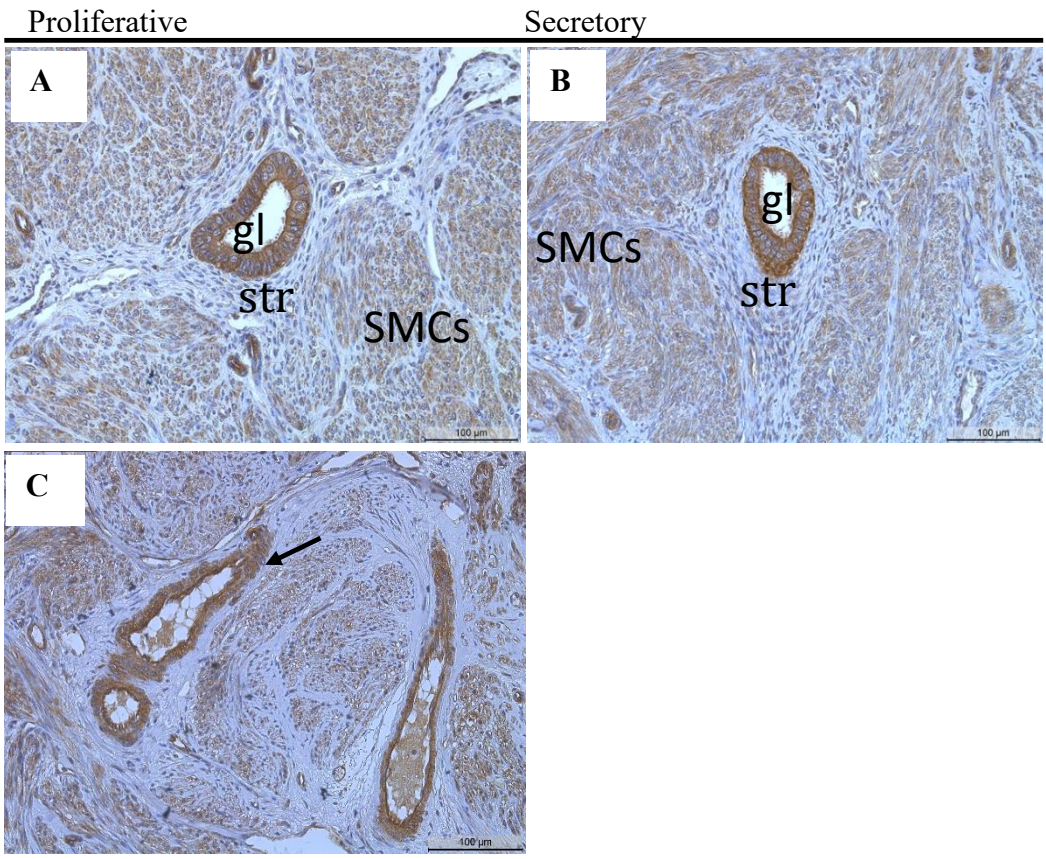

Figure 12. Localization of MT3-MMP was found in the glandular epithelial cells in the proliferative (E) and secretory (F) phase of patients with adenomyotic lesions. The smooth muscle cells (A and B), pericytes and endothelial cells (C) also showed strong MT3-MMP staining. gl; gland, SMCs; smooth muscle cells; str; stroma. Counterstaining was performed with hematoxylin. Magnification 200x. Scale bars 100 µm.

Comparison of MT3-MMP staining using the HSCORE and percentage of stained glands in eutopic endometrium with and without endometriosis is shown in Table 26.

Table 26: Comparison of MT3-MMP in the proliferative and secretory phases of endometrium of patients with and without endometriosis and adenomyosis using the HSCORE and percentage of stained glands

	ctrl (healthy)		Em-En		Em-Ad		Ad	
				HSCORE				
	P	**S**	**P**	**S**	**P**	**S**	**P**	**S**
Mean	191	136	168	128	182	150	161	146
SEM	21.6	19.5	21.8	9.5	14	14.6	13.9	16.3
P-value	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
N	6	6	6	11	9	9	15	16
Age	41±13	43±6.0	32.5±5.8	42±6.0	45±5.0	45±6.8	45±5.1	43.5±6.7
				Percentage of stained glands				
	P	**S**	**P**	**S**	**P**	**S**	**P**	**S**
Mean	99	89	98	99	100	98	95	95
SEM	0.7	7.4	1.9	0.4	0.0	1.2	3.6	3.2
P-value	n.s.	n.s	n.s.	n.s.	n.s.	n.s.	n.s	n.s.
N	6	6	6	11	9	9	15	16
Age	41±13	43±6.0	32.5±5.8	42±6.0	45±5.0	45±6.8	45±5.1	43.5±6.7

Age is given as the median ±SD (standard deviation). N, number of samples; SEM, standard error of the mean; ctrl, control group (normal endometrium); Em-En, Endometrium with endometriosis; Em-Ad, Endometrium with adenomyosis; Ad, Adenomyosis; P, proliferative; S, secretory; n.s., not significant. $P \leq 0.05$ was considered significant. Analysis was performed using the Kruskal–Wallis test.

MT3-MMP HSCORE across the menstrual cycle was highly similar between the four groups (ctrl, EM-En, EM-Ad, and Ad, Table 26). Furthermore, quantification using the percentage of MT3-MMP positive glands demonstrated a high percentage (89-100%) with no differences between all groups (Table 26). Because we did not observe any differences in eutopic endometrium with and without endometriosis, we merged the datasets for further comparison.

3.5.3 Localization of MT3-MMP in peritoneal, ovarian and deep infiltrating endometriosis

We further analysed MT3-MMP staining in peritoneal, deep infiltrating and ovarian endometriosis.

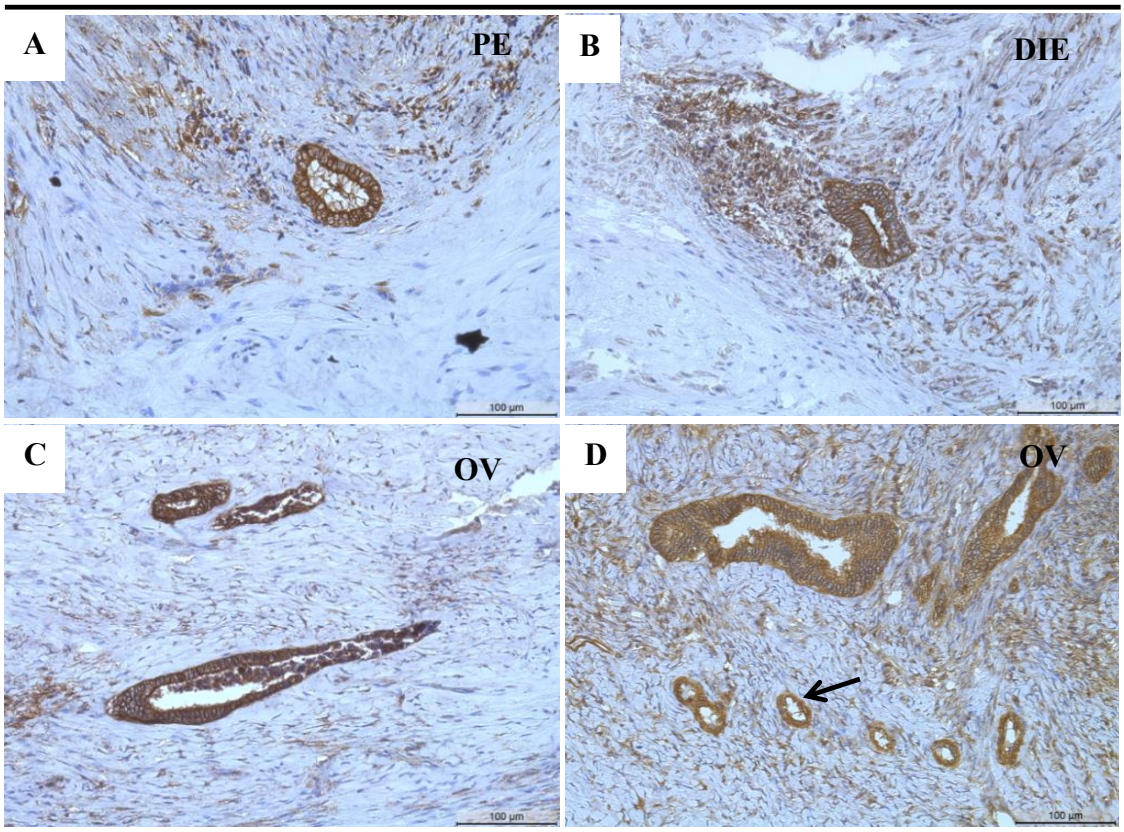

Figure 13. Localization of MT3-MMP in peritoneal (A, scar) deep infiltrating (B, bladder) and ovarian endometriosis (C, ovary). PE, peritoneal endometriosis; DIE, deep infiltrating endometriosis; OV, ovarian endometriosis. Counterstaining was performed with hematoxylin. Magnification 200x. Scale bars 100 µm.

Fig. 13 shows MT3-MMP localization in endometriotic lesions. In Fig. 13A representing peritoneal endometriosis is a scar with strongly stained cylindrical epithelial cells and some few positive stromal cells. A similar staining pattern was also observed in tissue from the bladder representing deep infiltrating endometriosis but with more positive adjacent stromal cells (Fig. 13B). Comparable to peritoneal and deep infiltrating endometriosis, in ovarian endometriosis the cylindrical epithelial cells from tissue samples of the ovary showed strong MT3-MMP staining (Fig.13C and D). Similarly, endothelial cells in ovarian lesions showed strong MT3-MMP staining (Fig. 13D, arrow).

Table 27 shows the comparison of MT3-MMP using the HSCORE and the percentage of positively stained glands in eutopic and ectopic endometrium.

Table 27: Comparison of MT3-MMP in eutopic and ectopic endometrium using the HSCORE and percentage of stained glands

	EM (a)	**OV (b)**	**DIE (c)**	**PE (d)**
			HSCORE	
Mean	157	120	101	96
SEM	7.0	5.5	10.1	6.4
P	n.s.	n.s.	a,c<0.001	a,d <0.001
N	47	18	17	18
Age	43±8.0	35.5±4.4	32±5.0	31±3.7
			Percentage of stained glands	
Mean	98	96	83	85
SEM	1.0	2.6	4.6	4.2
P	n.s.	n.s.	a,c≤0.05	n.s.
			b,c≤0.05	n.s.
N	47	18	17	18
Age	43±8.0	35.5±4.4	32±5.2	31±3.7

Age is given as the median ± standard deviation (SD). N=number of lesions; (a-d; groups); EM, eutopic endometrium; OV, ovarian endometriosis; DIE, deep infiltrating endometriosis; PE, peritoneal endometriosis; SEM, standard error of the mean; n.s., non-significant. P≤ 0.05 was considered significant. For example a,b<0.001 means that group a is significantly different from group b. Analysis was performed using the Kruskal–Wallis test.

Comparison of MT3-MMP HSCORE between eutopic endometrium and the three endometriotic entities showed a significantly higher MT3-MMP HSCORE in eutopic endometrium compared to peritoneal and deep infiltrating endometriosis (Table 27). Contratingly, the percentage of MT3-MMP positive glands was significantly reduced only in deep infiltrating endometriosis compared to eutopic endometrium (Table 27).

3.6 Expression of MT2-MMP and MT3-MMP in eutopic endometrium of patients with and without endometriosis using the HSCORE and the percentage of stained glands

Since the samples used for analysis of MT2-MMP and MT3-MMP in eutopic endometrium were almost similar, we compared the expression of the two proteins in eutopic endometrium of patients with and without endometriosis. Using both the HSCORE and the percentage of stained glands, MT3-MMP expression in eutopic endometrium of patients with and without endometriosis was significantly higher compared to MT2-MMP (Table 28). Table 28 presents the comparison of MT2-MMP and MT3-MMP expression in eutopic endometrium of patients with and without endometriosis using the HSCORE and percentage of stained glands.

Table 28: Comparison of MT2-MMP and MT3-MMP expression in eutopic endometrium of patients with and without endometriosis using the HSCORE and percentage of stained glands

All endometrium samples	MT2-MMP	MT3-MMP
	HSCORE	
Mean	64	157
SEM	3.5	7.0
P		0.0001
N	56	47
Age	42 ±7.6	43±8.0
	Percentage of stained glands	
Mean	65	98
SEM	3.2	1.0
P		0.0001
N	56	47
Age	42 ±7.6	43±8.0

Age is given as the median ± standard deviation (SD). N=number of samples; SEM, standard error of the mean; n.s., non-significant. P≤ 0.05 was considered significant. Analysis was performed using the Mann–Whitney test.

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3.7 Expression of MT1-MMP, MT2-MMP and MT3-MMP in endometrial and endometriotic cells

Since immortalized endometrial and endometriotic cells as well as primary endometrial cells are used as models of endometriosis in vitro, we used immunofluorescence to find out if the cells express MT1-MMP, MT2-MMP and MT3-MMP. MT1-MMP was localized in the cytosol and intermediate filaments in endometriotic epithelial (12Z), endometrial stromal (T-HESC) and primary stromal cells (Fig. 14). Similarly, MT2-

MMP was also localized in the nucleoplasm, cytosol and vesicles in 12Z, T-HESC and primary stromal cells (Fig. 15). Besides, we further observed MT3-MMP localization in the cytosol and vesicles in 12Z, T-HESC and primary stromal cells (Fig 16).

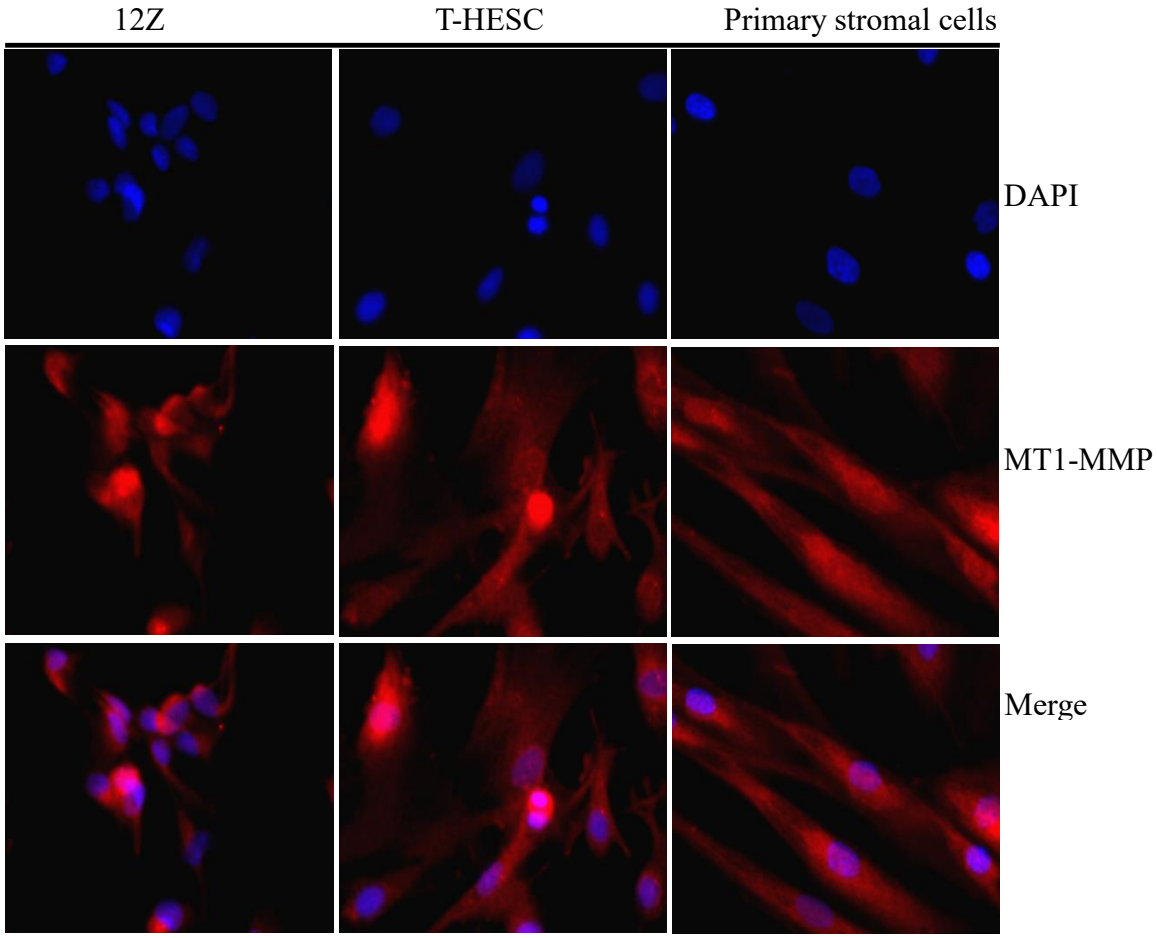

Figure 14. Immunofluorescence staining of MT1-MMP in the cytosol and intermediate filaments in endometriotic epithelial (12Z), endometrial stromal (T-HESC) and primary stromal cells.

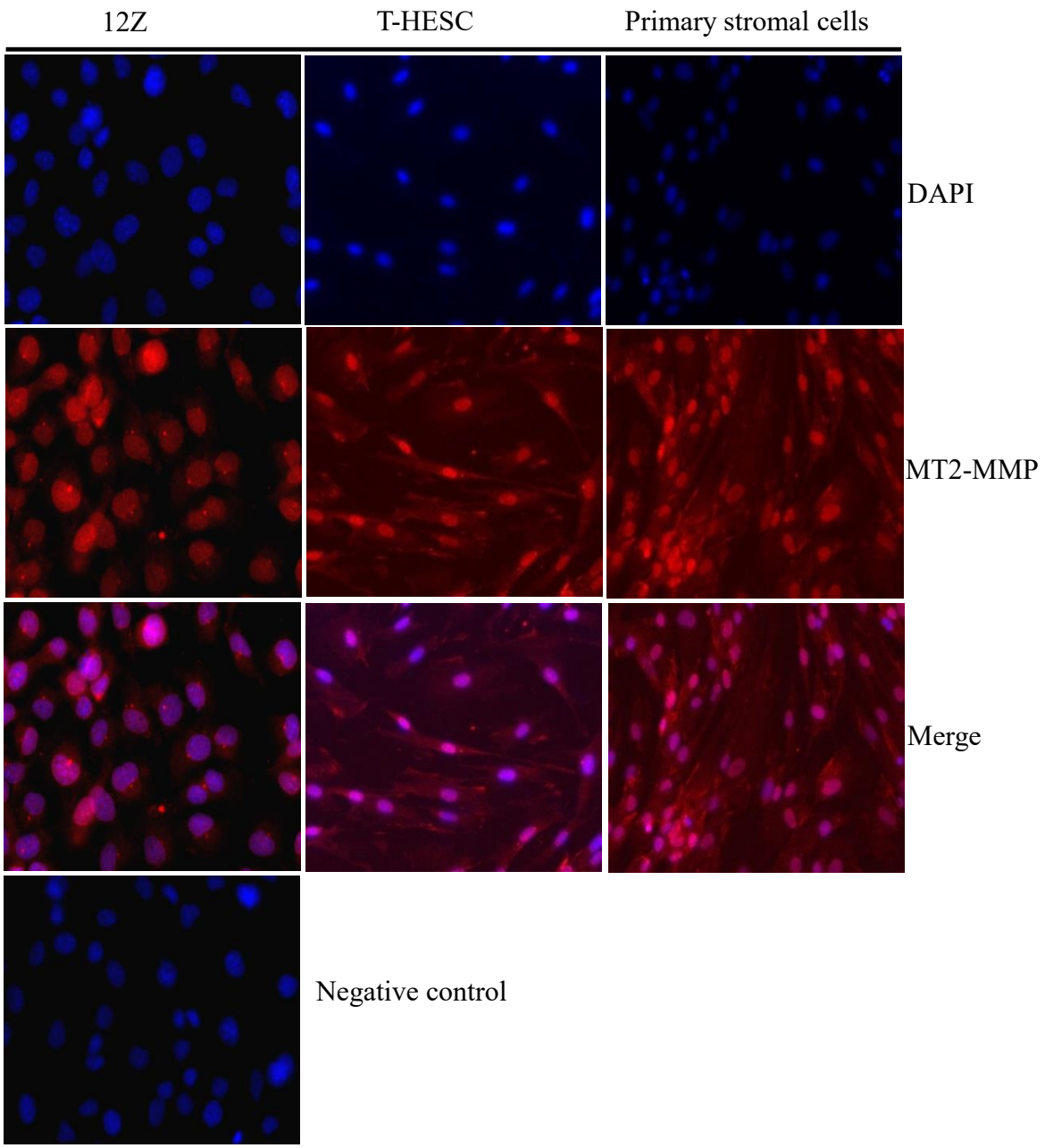

Figure 15. Immunofluorescence staining of MT2-MMP in the nucleoplasm, cytosol and vesicles in endometriotic epithelial (12Z), endometrial stromal (T-HESC) and primary stromal cells. Negative control for IF was prepared by omission of the primary antibody.

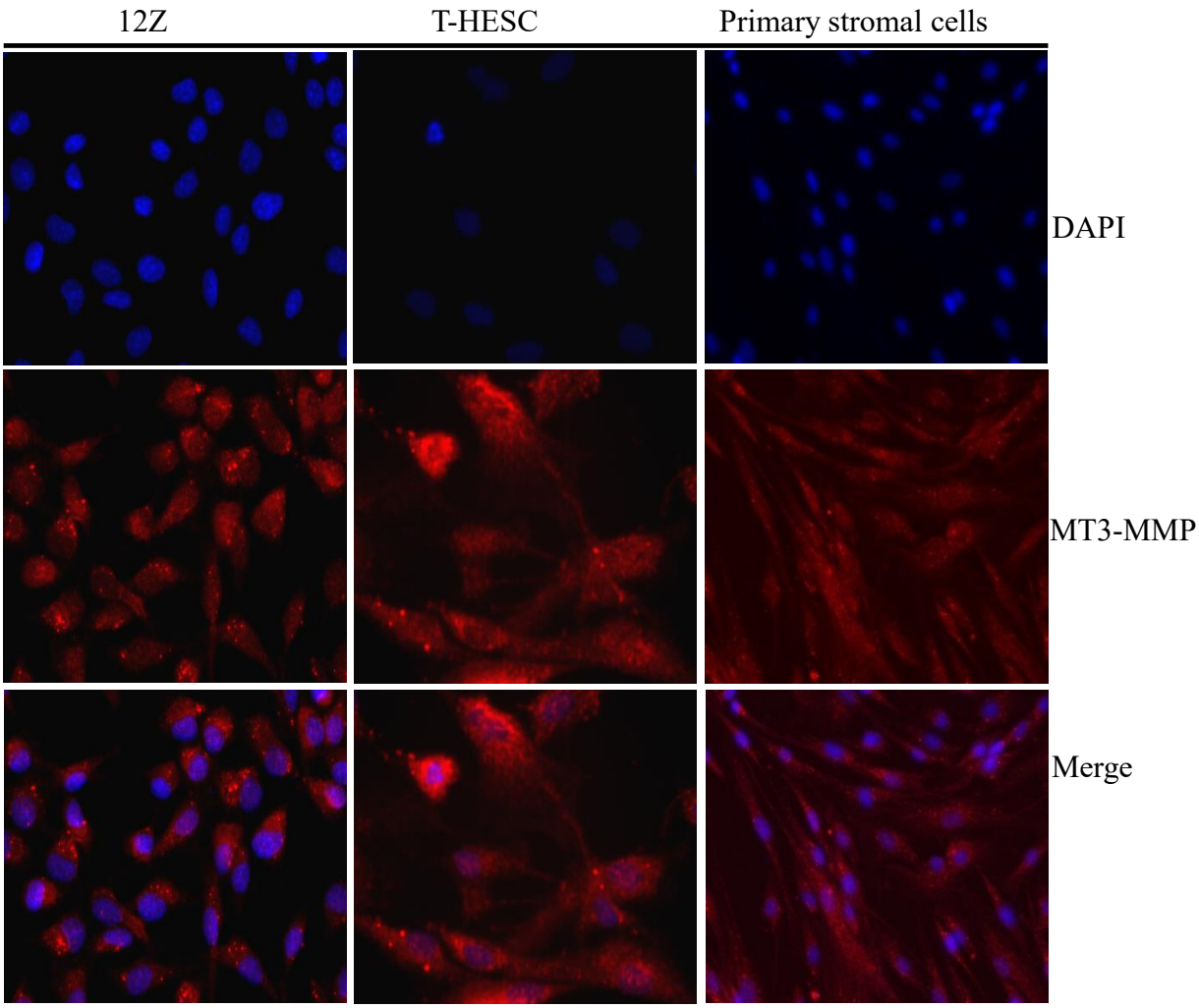

Figure 16. Immunofluorescence staining of MT3-MMP in the cytosol and vesicles in endometriotic epithelial (12Z), endometrial stromal (T-HESC) and primary stromal cells.

3.8 Role of MT1-MMP and MT3-MMP in betaglycan shedding

Studies in different cell types have shown that MT-MMPs especially MT1-MMP and MT3-MMP are involved in betaglycan shedding (Velasco-Loyden et al., 2004; Bilandzic & Stenvers, 2011). Moreover, our recent study demonstrated that GM6001 and TIMP3 blocked the effects of MMPs on betaglycan shedding (Mwaura et al., 2022). Based on this we analysed the role of MT1-MMP and MT3-MMP in betaglycan shedding in human endometriotic epithelial 12Z cells by siRNA gene silencing. The efficiency of each siRNA gene knockdown was confirmed using western blots (Fig. 17A and B). MT1-MMP and MT3-MMP gene silencing had no effect on betaglycan shedding in 12Z cells (Fig. 18A and B).

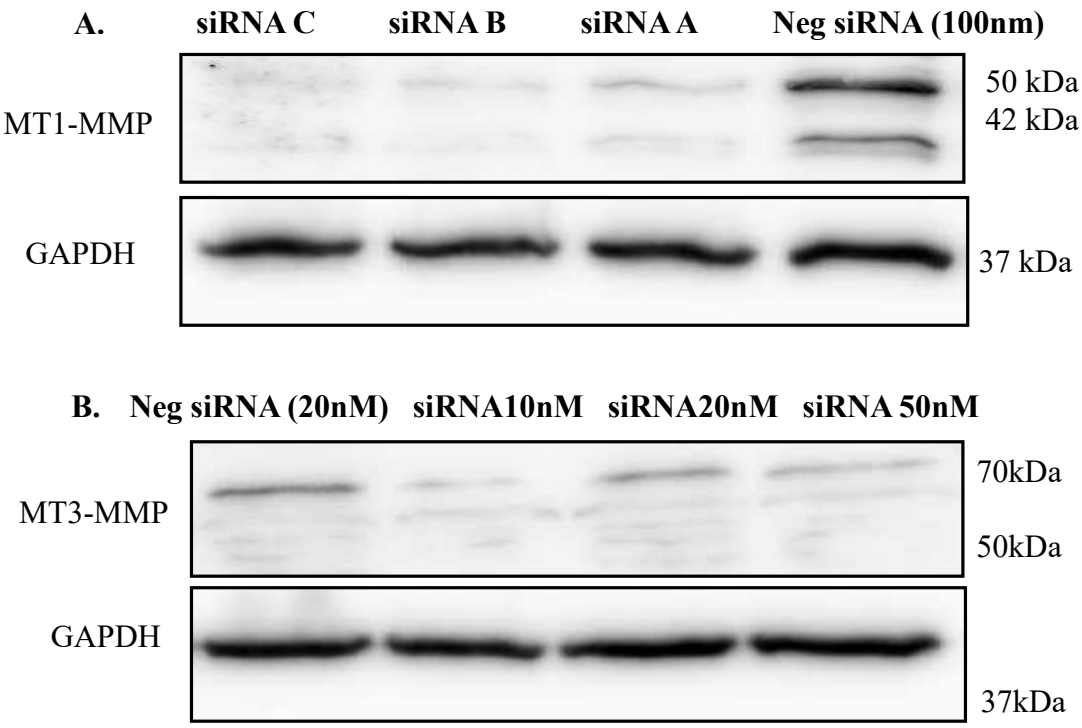

Figure 17. Endometriotic epithelial 12Z cells were transfected with MT1-MMP-siRNA, MT3-MMP-siRNA or negative control siRNA. A western blot confirmed silencing of MT1-MMP and MT3-MMP after 48 hour transfection. siRNA transfection reduced MT1-MMP (A) and MT3-MMP (B) protein expression.

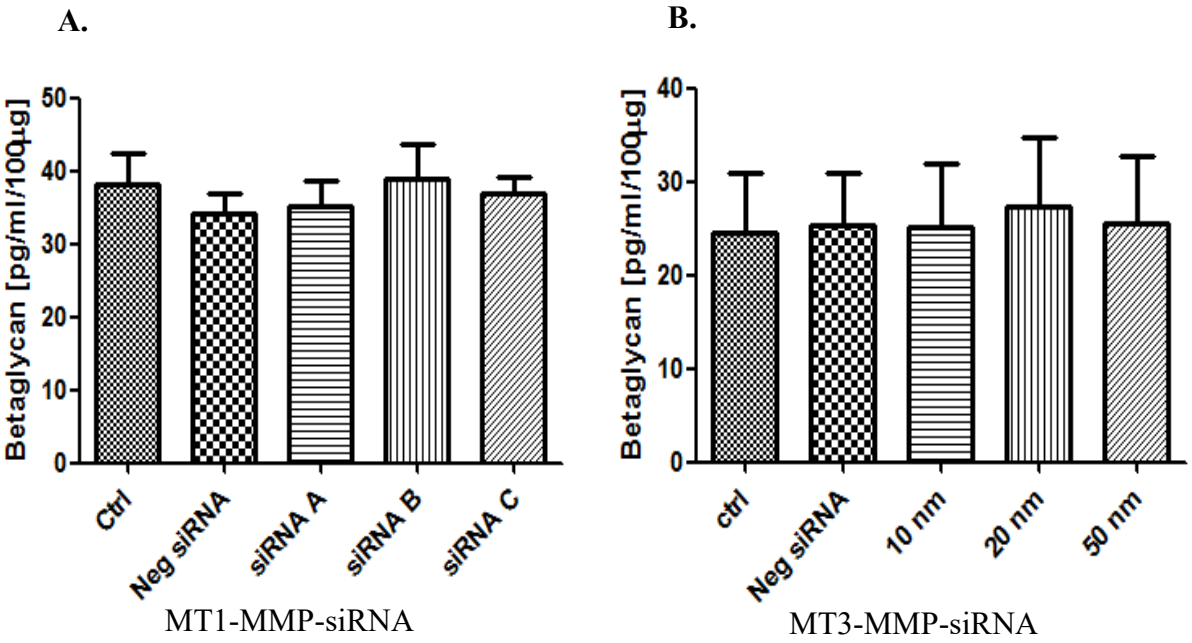

Figure 18. Endometriotic epithelial 12Z cells were transfected with MT1-MMP-siRNA, MT3-MMP-siRNA or negative control siRNA. After 48 hour transfection, supernatants were collected and analysed for soluble betaglycan. Analysis of individual knockdowns showed no effect on betaglycan shedding (A and B). Each bar graph represents data obtained from three independent experiments (mean ± SEM). Dunnett's test was used for statistical analysis.

3.9 Effect of MT1-MMP and MT3-MMP-siRNA knockdown on viability of 12Z cells

To measure cell survival of 12Z cells after MT1-MMP and MT3-MMP-siRNA transfection, we analysed cell viability using the CCK8 reagent 48 hours after transfection. MT1-MMP and MT3-MMP siRNA transfection had no effect on the viability of 12Z cells (Fig. 19).

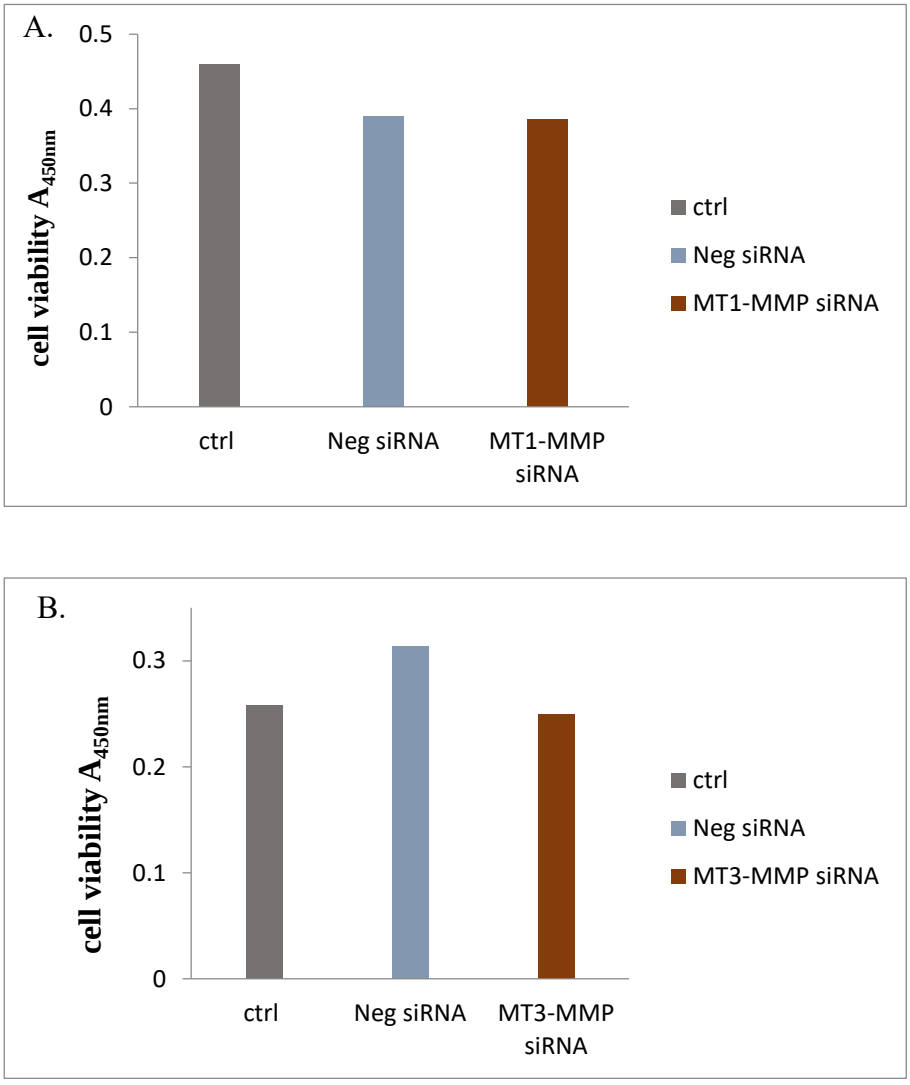

Figure 19. Endometriotic epithelial 12Z cells (1×10^4) transfected with either MT1-MMP siRNA or MT3-MMP-siRNA were seeded in a 96-well plate. After 48 hours 10 µl of CCK8 reagent was added and the cells incubated for additional 4 hours. Absorbance was determined at 450 nm using a microplate reader. Neither MT1-MMP nor MT3-MMP siRNA had an effect on 12Z cell viability. Each bar graph represents data obtained from two independent experiments (mean ± SD).

4. DISCUSSION

Matrix metalloproteinases are key enzymes involved in extracellular matrix remodeling, proliferation, cell migration, invasion and tissue repair in different tissues (Stamenkovic, 2003; Nawaz et al., 2018). Different MMPs are expressed in the endometrium and are associated with menstruation and establishment and development of endometriosis (Pitsos and Kanakas, 2009; Bałkowiec et al., 2018; Ke et al., 2021). In the present study, we explored the expression and localization patterns of MT1-MMP, MT2-MMP and MT3-MMP in eutopic and ectopic endometrium using immunohistochemistry. We also studied the levels of MT1-MMP in serum and endocervical mucus samples of patients with and without endometriosis. Furthermore, the protein expression of MT1-, MT2- and MT3-MMP in endometrial and endometriotic cells and the role of MT1-MMP and MT3-MMP in betaglycan shedding was investigated.

4.1 Expression and localization pattern of MT1-MMP in eutopic and ectopic endometrium

Given that different studies have implicated MT1-MMP in different physiological and pathological conditions (Itoh, 2006; Kondratiev et al., 2008; Bartolomé et al., 2009), we investigated the role of MT1-MMP in endometriosis and adenomyosis. Furthermore, previous studies on MT1-MMP and endometriosis have reported increased MT1-MMP mRNA/protein expression in eutopic endometrium of patients with endometriosis and pigmented endometriotic tissues compared to normal endometrium (Chung et al., 2002; Ueda et al., 2002; Londero et al., 2012). However, MT1-MMP expression in other endometriotic entities like adenomyosis has not been analysed up to date. Thus we explored the expression pattern of MT1-MMP in eutopic endometrium of patients with and without endometriosis, adenomyosis and the three endometriotic entities: peritoneal, ovarian and deep infiltrating endometriosis.

The results confirmed localization and expression of MT1-MMP in epithelial and stromal cells of eutopic endometrium and ovarian endometriosis in agreement with earlier reports (Zhang et al., 2000; Määttä et al., 2000; Chung et al., 2002; Ueda et al., 2002; Goffin et al., 2003; Plaisier et al., 2006; Londero et al., 2012). In addition, we demonstrate here for the first time MT1-MMP protein expression in epithelial cells of adenomyotic lesions. Although we did not compare MT1-MMP expression in the phases of the menstrual cycle, we observed preferential MT1-MMP expression in endometrial epithelial cells compared

to stromal cells in the proliferative and secretory phases of the cycle as demonstrated in previous reports (Zhang et al., 2000). On the other hand, Goffin et al. (2003) demonstrated that MT1-MMP mRNA expression is increasingly present during the menstruation phase associating it with menstruation.

Furthermore, MT1-MMP was virtually absent in the smooth muscle cells and endothelial cells of the blood vessels (Zhang et al., 2000; Plaisier et al., 2004, 2006) in accordance with our results. However, Plaisier et al. (2006) detected moderate MT1-MMP expression in the perivascular smooth muscle cells of the myometrium. The absence of MT1-MMP staining in the endothelial cells of the blood vessels suggests that it may not be involved in angiogenesis in the human endometrium. Contrastingly, studies in other tissues have indicated the involvement of MT1-MMP in angiogenesis (Zhou et al., 2000; Lafleur et al., 2002; Stratman et al., 2009) suggesting that different factors could be responsible for vascular development in different tissues.

Of note in this study is the reduced MT1-MMP staining in cases without endometriosis compared to patients with endometriosis, indicative of increased expression of MT1-MMP in patients with endometriosis. In the past, different reports have shown increased MT1-MMP mRNA and protein expression in patients with endometriosis compared to healthy controls (Chung et al., 2002; Londero et al., 2012). However, in contrast to the findings of Londero et al. (2012) we observed reduced MT1-MMP expression in ovarian endometriosis compared to eutopic endometrium. In another study, Jana et al. (2016) reported increased MT1-MMP protein expression in ovarian endometriosis that increased with the disease stage. However, they did not compare MT1-MMP expression in ovarian endometriosis and eutopic endometrium. Furthermore, we observed increased MT1-MMP expression in adenomyosis compared to eutopic endometrium and ovarian endometriosis. Matsuda et al. (2001) found an increased MT1-MMP mRNA expression in uterine tissues of mice with experimentally induced adenomyosis compared to control uteri but did not analyse MT1-MMP expression in ovarian endometriosis.

Elevated MT1-MMP expression has been linked to enhanced activation and expression of MMP2 in patients with endometriosis versus healthy controls (Chung et al., 2002; Ueda et al., 2002). We suggest that increased MT1-MMP expression in adenomyosis observed in our study could be responsible for activation and increased MMP2 expression in adenomyosis (Inagaki et al., 2003; Tokyol et al., 2009) and endometriosis (Wenzl and Heinzl, 1998).

4.2 MT1-MMP in serum and mucus

Diagnosis of endometriosis is a key challenge among endometriosis patients. This is due to different factors such as lack of specific symptoms that are associated with endometriosis (Agarwal et al., 2019). For instance, the main symptoms associated with endometriosis, pelvic pain and infertility, could also be due to other gynecological problems such as irritable bowel syndrome, adenomyosis and fibroids, thus making it difficult and time-consuming to diagnose the correct cause (Agarwal et al., 2019). In addition, normalization of symptoms among patients and care-givers and stigmatization associated with menstrual cramps also leads to delay in diagnosis of endometriosis (Ballard et al., 2006).

There is therefore an urgent need for reliable, sensitive and specific biomarkers for non-invasive diagnosis of endometriosis which would potentially reduce diagnostic delays especially in women with mild or moderate endometriosis and those experiencing pain/infertility but with normal ultrasound findings (Fassbender et al., 2015). Despite the fact that different studies centered on the relevance of different molecules such as glycoproteins, growth factors, hormones, angiogenic factors and inflammatory molecules in the pathogenesis of endometriosis have been done, no biomarkers are currently validated for non-invasive diagnosis of endometriosis (Vodolazkaia et al., 2012; Fassbender et al., 2015; Nisenblat et al., 2016).

Thus, in this study we tried to establish a non-invasive biomarker for diagnosis of endometriosis. A previous report on MT1-MMP levels in peritoneal fluid of women with and without endometriosis revealed that the levels are impaired and this could contribute to the establishment of endometriosis (Laudanski et al., 2005). In this study we analysed the levels of MT1-MMP in serum and endocervical mucus of patients with and without endometriosis. We found significantly higher MT1-MMP levels in serum samples of patients with endometriosis compared to those without endometriosis consistent with previous studies on cancer and preeclampsia patients (Laudański et al., 2010; Kasurinen et al., 2018; Dong et al., 2019; Ovayolu et al., 2021). Interestingly, in our study one of the patients with endometriosis had significantly higher MT1-MMP levels (57.2 ng/mL) in serum as opposed to others and we could not find any specific or special clinical characteristic of the patient in our database that could explain such high levels.

In recent studies, we and others have shown that different proteins in endocervical mucus are dysregulated in endometriosis and that some of these proteins contribute to or enhance

the establishment and maintenance of endometriosis (Konrad et al., 2016; Grande et al., 2017; Mwaura et al., 2022). We analysed MT1-MMP levels in endocervical mucus samples of patients with and without endometriosis. Our results showed significantly higher MT1-MMP levels in the proliferative phase compared to the secretory phase of both patients with and without endometriosis. A similar trend was also observed when we separated patients with and without endometriosis as opposed to earlier reports in tissue samples showing enhanced MT1-MMP mRNA during the secretory and menstruation phases (Chung et al., 2002; Goffin et al., 2003).

Although we did not find differences in MT1-MMP levels in endocervical mucus samples of patients with versus those without endometriosis, we observed a significant reduction in MT1-MMP levels in patients with endometriosis using contraception compared to patients with endometriosis but without contraception. We have in the past observed that contraceptives especially dienogest alone or in combination with ethinylestradiol reduced clusterin levels in endocervical mucus of patients with endometriosis (Konrad et al., 2016). Similarly, Endrikat et al. (2013) indicated that administration of different concentrations of estradiol valerate/dienogest oral contraceptive reduced cervical mucus production. Thus, we suggest that the reduced MT1-MMP levels in cases with endometriosis using contraception could be due to the contraceptives by possibly reducing the thickness of the endometrium similar to reduced clusterin levels (Endrikat et al., 2013; Konrad et al., 2016). However, the specific contraceptives that decrease MT1-MMP levels need to be determined in future experiments. Previously, expression of MMPs and TIMPs in the cervical mucus plug of pregnant and non-pregnant women has been associated with cervical remodeling and proteolytic processing in pregnancy and pre-term labor (Becher et al., 2010).

4.3 Relationship of MT1-MMP levels in serum and endocervical mucus with different clinical characteristics

Different clinical characteristics in women have been associated with endometriosis (Hemmings et al., 2004; Ashrafi et al., 2016). For instance, studies on laparoscopy and chronic pelvic pain have indicated that 33% of women who undergo surgery because of chronic pelvic pain have endometriosis as opposed to 35% with no visible pathology (Howard, 2003). In our study we investigated the correlation of MT1-MMP levels in serum and endocervical mucus samples of patients with and without endometriosis with cycle days, age, BMI and pain; dysuria, dysmenorrhea, dyschezia but could not detect

any associations between serum and endocervical mucus MT1-MMP levels in patients with and without endometriosis in agreement with earlier findings in gastric cancer patients (Kasurinen et al., 2018), however in serum samples, patients with endometriosis showed higher age as opposed to patients without endometriosis. In uterine leiyomyoma, MT1-MMP mRNA expression is increased and positively correlated with expression of myostatin and activin A and intense dysmenorrhea (Tsigkou et al., 2015).

4.4 Expression and localization pattern of MT2-MMP in eutopic and ectopic endometrium

Despite identification of MT2-MMP protein expression in the human endometrium (Zhang et al., 2000; Goffin et al., 2003; Plaisier et al., 2006), its expression pattern in endometriosis and adenomyosis has not been explored, therefore, we investigated the expression pattern of MT2-MMP in eutopic and ectopic endometrium. We identified expression and localization of MT2-MMP in the human endometrium throughout the menstrual cycle without cyclic variations in agreement with previous studies (Zhang et al., 2000; Goffin et al., 2003). We also observed a preferential localization of MT2-MMP in human endometrial epithelial cells compared to stromal cells similar to other reports (Zhang et al., 2000; Plaisier et al., 2006). Similarly, in adenomyotic lesions MT2-MMP was preferentially localized in epithelial cells. To our knowledge, this is the first study to show MT2-MMP localization in adenomyotic lesions.

Moreover, MT2-MMP was also localized in vessel strucures of the blood vessels (pericytes and endothelial cells) and smooth muscle cells of the myometrium as previously reported (Zhang et al., 2000; Plaisier et al., 2006). Contrastingly, Plaisier et al. (2004) hardly detected MT2-MMP mRNA in human endometrial microvascular endothelial cells (hEMVECs). Studies on MT-MMPs and vascularization in the human endometrium have revealed that MT2-MMP, MT3-MMP and MT4-MMP vascular expression corresponds with angiogenic periods of the menstrual cycle, suggesting a possible role of these proteins in angiogenesis (Plaisier et al., 2004, 2006). MT1-MMP, MT2-MMP, MT3-MMP and MMP2 mRNA expressions and the process of tubulogenesis are enhanced in endothelial cells grown within fibrin (Lafleur et al., 2002).

We revealed for the first time expression and localization of MT2-MMP mainly in the epithelial cells of the three endometriotic entities; peritoneal, ovarian and deep infiltrating endometriosis. We further demonstrated that MT2-MMP HSCORE and percentage of stained glands in eutopic endometrium of patients with and without endometriosis were

highly identical. Notably, in this study MT2-MMP protein expression was greatly reduced in the three endometriotic entities compared to eutopic endometrium. Using whole genome DNA microarray analysis, Eyster et al. (2007) revealed reduced MT2-MMP gene in ectopic endometrium compared to eutopic endometrium.

4.5 Expression and localization pattern of MT3-MMP in eutopic and ectopic endometrium

MT3-MMP is the third transmembrane metalloproteinase and different studies have shown that it is overexpressed in different human metastatic melanoma cells (Tatti et al., 2011), hepatocellular carcinoma (Arai et al., 2007), Dupuytren's disease (Johnston et al., 2008), gastric cancer (Cao et al., 2016), prostate cancer (Jiang et al., 2017) and colorectal carcinoma (Wu et al., 2017). It has also been shown to promote cell migration and epithelial to mesenchymal transition in neural crest cells (Roth et al., 2017). Although expression and localization of MT3-MMP in the endometrium has been described (Goffin et al., 2003; Plaisier et al., 2004, 2006), its expression in the ectopic endometrium and the relationship to endometriosis has not been explored.

In our experiments, we investigated the expression and localization pattern of MT3-MMP in eutopic endometrium of patients with and without endometriosis and ectopic endometrium: adenomyosis, peritoneal, ovarian and deep infiltrating endometriosis. Our results showed expression and localization of MT3-MMP in the human endometrium, with a preferential localization in the epithelial cells as opposed to stromal cells in agreement with previous reports (Plaisier et al., 2006). Interestingly, for the first time we identified MT3-MMP protein localization in the epithelial cells of adenomyotic lesions. Although in our study MT3-MMP was slightly increased in the proliferative phase, no associations were observed in MT3-MMP expression and the phases of the menstrual cycle. However, Goffin et al. (2003) reported that MT3-MMP mRNA expression was increased during the proliferative phase of the menstrual cycle.

Besides, MT3-MMP was also localized in the vessel structures (endothelial cells and the pericytes) as well as the smooth muscle cells of the myometrium. Different studies have demonstrated expression of MT3-MMP in endothelial cells of the endometrium associating it with endometrial tube formation by endothelial cells (Plaisier et al., 2004, 2006). Of note, in this study is the high similarity in MT3-MMP protein expression between patients with endometriosis versus those without endometriosis.

We also observed MT3-MMP expression in epithelial cells and to a lesser extent in stromal cells of the three endometrotic entities. Comparable to MT2-MMP, we noted reduced MT3-MMP expression in endometriosis but not in adenomyosis. In addition, MT3-MMP expression in peritoneal and deep infiltrating endometriosis was reduced compared to eutopic endometrium.

Furthermore, we noted that MT3-MMP expression using the HSCORE and percentage of stained glands in eutopic endometrium of patients with and without endometriosis was about 2.5 and 1.5-fold higher compared to MT2-MMP, respectively, which is probably due to the fact that MT3-MMP cleaves more substrates compared to MT2-MMP (Itoh, 2015).

4.6 Relevance of MT1-MMP, MT2-MMP and MT3-MMP expression in endometriosis and adenomyosis

As discussed above, MT2-MMP and MT3-MMP expression in eutopic endometrium was highly similar to adenomyosis. Furthermore, we also observed increased MT1-MMP expression in adenomyosis compared to eutopic endometrium. Recently, based on protein abundance of calycyphosine (CAPS) and msh homeobox 1 (MSX1) we suggested that adenomyotic glands are highly identical to eutopic endometrial glands (Maier et al., 2020). Our present findings provide further evidence on our earlier suggestion that adenomyotic glands arise from endometrial glands (Maier et al., 2020). This would also suggest that adenomyosis and endometriosis are different entities based on pathogenesis. The most accepted theory on pathogenesis of endometriosis is retrograde menstruation which involves tissue breakdown during menstruation while adenomyosis is mostly due to invagination but without endometrial tissue breakdown. In a recent study, Yamaguchi et al. (2021) using 3D reconstructions, demonstrated that eutopic endometrial glands branched and extended into the myometrium especially along areas adjacent to blood vessels in consistent with previous studies in support of the invagination theory of adenomyosis (García-Solares et al., 2018; Maier et al., 2020).

From our present and previous results (Maier et al., 2020), we suggest that the use of LNG-IUD might reduce cell migration after laparoscopy and hence reduce recurrence of endometriosis. However, inhibitors of cell invagination in adenomyosis need to be identified.

Furthermore, we observed different expression patterns of MT1-MMP in ovarian endometriosis, MT2-MMP and MT3-MMP in ovarian, peritoneal and deep infiltrating

endometriosis versus eutopic endometrium. This is in agreement with our recent hypothesis (Konrad et al., 2020) and that of Chung et al. (2002) that most alterations in expression patterns occur after but not before implantation. The differences could also be due to different interactions of the endometrial implants with discrete microenvironments (Koninckx et al., 1999; Konrad et al., 2020).

4.7 Expression of MT1-MMP, MT2-MMP and MT3-MMP in endometrial and endometriotic cells

To find out whether endometrial and endometriotic cells are good models for MT-MMPs studies, we investigated MT1-MMP, MT2-MMP and MT3-MMP protein expression in endometrial stromal (T-HESC), endometriotic epithelial (12Z) and primary endometrial stromal cells using immunofluorescence. All MT-MMPs were expressed in endometrial stromal, endometriotic epithelial and primary endometrial stromal cells; however, they were localized inside the cell instead of on the cell membrane. Similar findings were also reported by Plaisier et al. (2006) who observed MT1-, MT2, MT3-, MT4-, MT5- and MT6-MMP localization inside the cell but not on the cell membrane in hEMVEC cells.

A key feature of membrane proteins including MT-MMPs is their potential to go through endocytosis and internalization (Conner and Schmid, 2003; Osenkowski et al., 2004). Endocytosis is a complex process involving different molecules aimed at delivering the target proteins to endosomes for either recycling to the plasma membrane or degradation by lysosomes (Osenkowski et al., 2004). MT1-MMP undergoes endocytosis from the cell membrane via clathrin- and caveolin-dependent mechanisms, similar to MT3-MMP (Osenkowski et al., 2004; Itoh et al., 2015). However, not much information is available on MT2-MMP recycling and endocytosis. We suspect that MT1-, MT2-, and MT3-MMP protein expression inside the cell rather on the cell membrane could be a mechanism of regulating their activities on the cell membrane.

4.8 Role of MT1-MMP and MT3-MMP in betaglycan shedding

Different studies have elucidated the role of MMPs, especially MT1-MMP and MT3-MMP in betaglycan shedding in different cell types (Velasco-Loyden et al., 2004; Kudipudi et al., 2019). Similarly, in our recent study using human endometriotic epithelial cells we revealed involvement of MMPs in betaglycan shedding (Mwaura et al., 2022). In the present study we investigated the role of MT1-MMP, MT3-MMP in betaglycan shedding in human endometriotic epithelial cells (12Z) using gene silencing/knockdown.

We downregulated MT1-MMP and MT3-MMP expression using siRNAs, collected cell culture supernatants and analysed them for betaglycan levels using ELISAs. Downregulation of MT1-MMP and MT3-MMP had no effect on betaglycan shedding. Even though our knockdown experiments showed that MT1-MMP and MT3-MMP are not involved in betaglycan shedding, they may participate in activation of other proteins such as proMMP2 that could probably play a role in betaglycan shedding. It is also unclear whether or not a complete knockdown of MT1-MMP and MT3-MMP could decrease the levels of soluble betaglycan. Based on our present and previous (Mwaura et al., 2022) findings we suggest that other MMPs apart from MT1-MMP and MT3-MMP could be responsible for betaglycan shedding in human endometriotic epithelial (12Z) cells.

4.9 Limitations

Even though we did some in vitro tests, most of our experiments were on in vivo samples and hence the different functions of the studied proteins in endometriosis and adenomyosis need to be investigated further using isolated primary endometrial and endometriotic epithelial and stromal cells. In addition, further studies in animal models need to be done.

5. Conclusions

Our findings on the localization of MT1-, MT2-, and MT3-MMP in eutopic and ectopic endometrium in endometriosis and adenomyosis demonstrated that the proteins investigated are mainly localized in the glandular epithelial and luminal cells of eutopic endometrium with no cycle variations. Also, the results showed that MT2-MMP and MT3-MMP protein expressions between patients with and without endometriosis were highly similar. In addition, MT2-MMP and MT3-MMP protein abundance in adenomyosis were nearly unchanged, but increased for MT1-MMP. Furthermore, we revealed the reduced but heterogenous MT1-MMP protein expression in ovarian endometriosis, MT2-MMP in ovarian, peritoneal and deep infiltrating endometriosis as well as MT3-MMP in peritoneal and deep infiltrating endometriosis. These findings further support our recent observations that adenomyotic glands originate from endometrial glands and that most variations occur after but not before implantation and could be due to varied interactions of the endometrial implants with the distinct microenvironments.

Our results further revealed for the first time that MT1-MMP levels in endocervical mucus of patients with and without endometriosis are highly similar. Interestingly, we were also able to show that MT1-MMP levels in endocervical mucus samples of patients with endometriosis are decreased by hormonal contraception. Moreover, we also demonstrated that MT1-, MT2- and MT3-MMP proteins are expressed in endometrial and endometriotic cells and gene knockdown of MT1-MMP and MT3-MMP has no effect on betaglycan shedding in endometriotic epithelial cells. Further investigations are needed to better understand the functions of these proteins in endometriosis and adenomyosis.

6. ZUSAMMENFASSUNG

Endometriose ist eine gutartige gynäkologische Erkrankung, die durch das Vorhandensein von Endometriumgewebe; Endometriumdrüsen und Stroma außerhalb der Gebärmutter gekennzeichnet ist. Obwohl Endometriose etwa 0,7–8,6 % Frauen im gebärfähigen Alter betrifft, ist ihre Pathogenese nicht gut verstanden und es gibt keinen zuverlässigen nicht-invasiven Biomarker für die Diagnose. Matrix-Metalloproteinasen werden signifikant im Endometrium und auch bei Endometriose exprimiert. Das Ziel dieser Studie war es, die Rolle von membranartigen-lokalisiarten MMPs in der Pathogenese der Endometriose zu untersuchen.

Dazu analysierten wir Gewebeproben von Patientinnen mit und ohne Endometriose, adenomyotische Läsionen und die drei Endometriose-Entitäten; ovarielle, peritoneale und tief infiltrierende Endometriose und bewerteten ihren HSCORE und den Prozentsatz gefärbter Drüsen. Außerdem untersuchten wir die MT1-MMP-Spiegel in Serum- und Endozervixschleimproben von Patientinnen mit und ohne Endometriose. Darüber hinaus untersuchten wir auch die MT1-, MT2- und MT3-MMP-Proteinexpression in endometrialen und endometriotischen Zellen und die Rolle von MT1-MMP und MT3-MMP bei der Betaglycan-Ablösung von endometriotischen Epithelzellen.

Unsere Ergebnisse zeigten, dass alle untersuchten MT-MMPs im menschlichen Endometrium mit hohen Ähnlichkeiten zwischen Frauen mit und ohne Endometriose exprimiert werden, mit Ausnahme von MT1-MMP, das wir nur in wenigen Proben bewerteten. Variationen in der Proteinexpression wurden mit einer reduzierten MT1-MMP-Expression bei ovarieller Endometriose, MT2-MMP bei ovarieller, peritonealer und tief infiltrierender Endometriose sowie MT3-MMP bei peritonealer und tief infiltrierender Endometriose gefunden. In ähnlicher Weise war bei der Adenomyose MT1-MMP erhöht, während die Häufigkeit von MT2-MMP und MT3-MMP hochgradig identisch mit eutopischem Endometrium war. Folglich schlagen wir vor, dass Variationen höchstwahrscheinlich nach der Implantation des endometriotischen Gewebes an den ektopischen Stellen auftreten und Endometriumdrüsen die einzige Quelle für adenomyotische Drüsen sein könnten. Dies könnte auch darauf hindeuten, dass Endometriose und Adenomyose in der Pathogenese im Berug auf Invasion versus Invagination unterschiedlich sind.

Darüber hinaus haben wir gezeigt, dass die MT1-MMP-Spiegel im Endozervixschleim von Patientinnen mit und ohne Endometriose sehr ähnlich waren. Darüber hinaus fanden

wir, dass die MT1-MMP-Konzentration in endozervikalen Schleimproben von Patientinnen mit Endometriose durch hormonelle Kontrazeptiva verringert wurde, aber weitere Untersuchungen sind erforderlich, um die spezifischen beteiligten Kontrazeptiva zu ermitteln.

Unsere Ergebnisse zeigten ferner, dass MT1-MMP-, MT2-MMP- und MT3-MMP-Proteine in endometrialen und endometriotischen Zellen exprimiert werden und die Herunterregulierung von MT1-MMP und MT3-MMP keinen Einfluss auf die Ablösung von Betaglycan hatte. Zusammengenommen liefern diese Ergebnisse neue Ideen zur Funktion von MT1-, MT2- und MT3-MMP in der Pathophysiologie der Endometriose und Adenomyose. Es sind jedoch weitere Experimente erforderlich, um die Rolle dieser Proteine bei der Adenomyose und Endometriose zu verstehen, wobei primäre Endometrium- und Endometriosezellen sowie Tiermodelle verwendet werden sollten.

7. SUMMARY

Endometriosis is a benign gynecological disease characterized by the presence of endometrial tissue; endometrial glands and stroma outside the uterus. Even though endometriosis affects about 0.7-8.6% women of the reproductive age, the pathogenesis is not well understood and there is no reliable non-invasive biomarker for diagnosis. Matrix metalloproteinases are significantly expressed in the endometrium and also in endometriosis. The aim of this study was to investigate the role of membrane-type MMPs in the pathogenesis of endometriosis.

To achieve this, we analysed tissue samples from patients with and without endometriosis, adenomyotic lesions and the three endometriotic entities; ovarian, peritoneal and deep infiltrating endometriosis and evaluated their HSCORE and percentage of stained glands. Also, we studied the levels of MT1-MMP in serum and endocervical mucus samples of patients with and without endometriosis. In addition, we also investigated MT1-, MT2- and MT3-MMP protein expression in endometrial and endometriotic cells and the role of MT1-MMP and MT3-MMP in betaglycan shedding in endometriotic epithelial cells.

Our results showed that all studied MT-MMPs are expressed in the human endometrium with high similarities between women with and without endometriosis except for MT1-MMP which we only evaluated in few samples. Variations in protein expression were found with a reduced MT1-MMP expression in ovarian endometriosis, MT2-MMP in ovarian, peritoneal and deep infiltrating endometriosis as well as MT3-MMP in peritoneal and deep infiltrating endometriosis. Similarly, in adenomyosis, MT1-MMP was increased while MT2-MMP and MT3-MMP abundance was highly identical to eutopic endometrium. Consequently, we suggest that variations most likely occur after implantation of the endometriotic tissue in the ectopic sites and endometrial glands could be the sole source of adenomyotic glands. This could also suggest that endometriosis and adenomyosis do not share a common pathogenesis but are distinguishable by, invasion versus invagination, respectively.

In addition, we demonstrated that MT1-MMP levels in endocervical mucus of patients with and without endometriosis were highly similar. Furthermore, we revealed that MT1-MMP concentration in endocervical mucus samples of patients with endometriosis was decreased by hormonal contraceptives, but further investigations are needed to ascertain the specific contraceptions involved.

Our results further revealed that MT1-MMP, MT2-MMP and MT3-MMP proteins are expressed in endometrial and endometriotic cells and downregulation of MT1-MMP and MT3-MMP had no effect on betaglycan shedding. Taken together, these findings provide new evidence on the function of MT1-, MT2- and MT3-MMP in the pathophysiology of endometriosis and adenomyosis. However, more experiments are needed to understand the role of these proteins in adenomyosis and endometriosis using primary endometrial and endometriotic cells as well as animal models.

8. ABBREVIATIONS

°C	Degree Celsius
µg/ml	Microgram per milliliter
µl	Microliter
2D	Two dimensional
3D	Three dimensional
A	Ampere
APS	Ammonium persulphate
BCA	Bicinchoninic acid
BMI	Body mass index
BSA	Bovine serum albumin
CAPS	Calcyphosine
CD10	Cluster of differentiation 10
cm	Centimetre
CO_2	Carbon dioxide
DAB	3,3′-Diaminobenzidine
DAPI	4′,6-Diamidino-2-phenylindoldihydrochlorid
DIE	Deep infiltrating endometriosis
DLBCL	Diffuse large B-cell lymphoma
DMEM	Dulbecco’s modified eagle’s medium
DNA	Deoxyribonucleic acid
ECL	Enhanced chemiluminescence
ELISA	Enzyme-linked Immunosorbent Assay
EMT	Epithelial-mesenchymal transition
FCS	Fetal calf serum
Fig	Figure
g	Gravitational force
g/L	Grams per liter
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase

Abbreviations

GnRH	Gonadotropin-releasing hormone
GnRH	Gonadotropin-releasing hormone agonists
GPI	Glycosylphosphatidylinositol
HCl	Hydrochloric acid
HE	Hematoxylin-eosin stain
HIFU	High intensity focused ultrasound
hr	hour
HRP	Horseradish peroxidase
HSCORE	Histological SCORE
IF	Immunofluorescence
IHC	Immunohistochemistry
ITS	Insulin-transferrin-selenium
kDa	Kilodaltons
L	Liter
LNG-IUD	Levonorgestrel-releasing intrauterine device
M	Molar
min	Minute
ml	Milliliter
mm	Millimetre
mM	Millimolar
MMPs	Matrix metalloproteinases
MRI	Magnetic resonance imaging
MRKH	Mayer-Rokitansky-Küster-Hauser syndrome
mRNA	Messenger ribonucleic acid
MSX1	Msh homeobox 1
MT-MMPs	Membrane type matrix metalloproteinases
MUC1	Mucin 1
ng/ml	Nanogram per milliliter
nm	Nanometer
nM	Nanomolar

Abbreviations

NSAIDs	Nonsteroidal anti-inflammatory drugs
OTR	Oxytocin receptor
OV	Ovarian endometriosis
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate-buffered saline
PE	Peritoneal endometriosis
Pen-Strep	Penicillin/streptomycin
pg/ml	Picogram/milliliter
PMSF	Phenylmethylsulfonyl fluoride
PVDF	Polyvinylidene fluoride
ROS	Reactive oxygen species
RT	Room temperature
sBG	Soluble betaglycan
SDS	Sodium dodecyl sulfate
siRNA	Small interfering RNA
TAPI 2	TNF Protease Inhibitor 2
TBST	Tris buffered saline Tween-20
TEMED	N,N,N′,N′-Tetramethylethylenediamine
TGF-β	Transforming growth factor β
TGF-βR3	Transforming growth factor beta receptor III
TIMPs	Tissue inhibitor of matrix metalloproteinases
TVUS	Transvaginal ultrasound sonography
V	Voltage
w/v	weight per volume

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10. REFERENCES

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12. DECLARATION

I declare that I have completed this dissertation single-handedly without the unauthorized help of a second party and only with the assistance acknowledged threin. I have appropriately acknowledged and rerefenced all text passages that are derived literally from or are based on the content of published or unpublished work of others, and all information that relates to verbal communicatons. I have abided by the principles of good scientific conduct laid down in the charter of the Justus Liebig University of Giessen in carrying out the investigations described in the dissertation.

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Place, Date Jane Bosibori Maoga

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