UNDERSTANDING THE ROLE OF MATRIX METALLOPROTEASE-7 IN PATHOGENESIS OF HUMAN OVARIAN ENDOMETRIOSIS

2.1. INTRODUCTION

Matrix metalloproteinases (MMPs) are a group of zinc requiring proteolytic enzymes that are involved in remodelling and degradation of extracellular matrix (ECM). MMPs are essential in orchestrating proper physiological functioning of the endometrium and hence, derangement of MMP regulation is considered to be a critical factor in the pathogenesis of endometriosis [Page-McCaw *et al.*, 2007]. In our previous studies, we have reported the roles and regulation of MMPs, especially MMP-9 (gelatinase B) and MMP-3 (stromelysin-1) in clinical and mouse model of endometriosis [Paul *et al.*, 2008, 2010]. Our studies have also demonstrated elevated expression of MMP-2 that is involved in promoting angiogenesis during pathogenesis of endometriosis [Jana *et al.*, 2016a].

Matrix metalloprotease-7 (MMP-7) or matrilysin-1 is the smallest secreted protease of the MMP family. Unlike other MMPs, MMP-7 does not possess the hemopexin domain at the c-terminal; the protease lacks substrate specificity and can cleave a vast array of ECM proteins as well as cellular ligands [Klein *et al.*, 2011]. It has strong stromelysin–like activity and degrades elastin, collagen IV, laminin, fibronectin, proteoglycans and gelatins. MMP-7 is secreted as inactive proform and the zymogen is activated by proteases like trypsin, plasmin and stromelysin-1 (MMP-3), but not by tissue collagenase (MMP-1), gelatinase A (MMP-2), gelatinase B (MMP-9). MMP-7 can intern activate other MMPs, including proMMP-1 and proMMP-9, but not proMMP-2 or proMMP-3 [Klein *et al.*, 2011]. Thus, in normal physiological responses, MMP-7 is involved with many cellular signaling pathways that trigger from proteolytic actions. In disease like cancer, the magnitude of MMP-7 expression can be a determining factor for metastasis. Several studies reported over expression of MMP-7 in different cancers, including breast cancer, gastric cancer, and colorectal cancer [Kesh *et al.* 2015; Sizemore *et al. 2012*; Zeng *et al.*2002]. MMP-7 induces tumor progression and metastasis by ECM degradation, promoting chemo-resistance and inhibiting apoptosis in cancer cells [Liu *et al.*, 2008; Zeng *et al.*,2002]. Apart from ECM components, MMP-7 acts as a sheddase for several bio-ligands including TNF-α, Fas ligand, heparin-binding epidermal growth factor (HB-EGF), E-cadherin and b4-integrin etc [Klein *et al.*, 2011; McGuire *et al.*, 2003; Page-McCaw *et al.*, 2007]. MMP-7 null mice manifest impaired wound healing due to disrupted reepithelialization [Dunsmore *et al.*, 1998]. MMP-7 is synthesized mainly by epithelial cells that require extensive/periodic cellular remodeling, including

cancer cells, and normal ovarian epithelial, glandular endometrial cells [Klein *et al.*, 2011; Sizemore *et al., 2012*; Zeng *et al.,* 2002]. Because endometriosis developed from the ectopic implantation of endometrial cells, the disease is highly associated with MMP responses, and ECM remodeling. Therefore, the role of MMP-7 in the pathogenesis can be an intriguing factor for disease pathogenesis. The present chapter aims to understand the association of MMP-7 in human ovarian endometriosis.

2.2. OBJECTIVES

To understand the involvement of MMP-7 activity during progression of human ovarian endometriosis in relation to control women.

2.3. MATERIAL AND METHODS

Chemicals: Casein, Triton X-100, protease inhibitors mixture, 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (BCIP/NBT), and 3,3',5,5'-tetramethylbenzidine (TMB), and 3, 3 diaminobenzidine (DAB) were obtained from Sigma Aldrich Inc, St. Louis, MO, USA. Pre-stained protein molecular weight markers were purchased from Fermentas Inc, Washington, DC, USA. ELISA kits were purchased from R&D system, USA. Antibodies were obtained from Santa Cruz Biotechnology Inc, California, USA; Abcam and Cell Signaling Technologies, USA. All other chemicals were purchased from Sisco Research Laboratories, Mumbai, India.

Human Study: Serum and ectopic samples were collected from 40 women with ovarian endometriosis-associated complications, attending the gynecology unit of Spectrum Clinic & Endoscopy Research Institute, Kolkata, India. The study protocol was approved by the Human Ethics Committee of IICB and SCERI. All participants gave written informed consent for participation. The clinical diagnosis of ovarian endometriosis was confirmed by laparoscopy and all biopsy samples and serum were collected during proliferative phase of the menstrual cycle. Control samples (n=15) were collected from women who were undergoing laproscopic surgery, exhibited no visible evidence of endometriosis upon laparoscopy. Briefly, we collected early and late stages of ovarian endometriotic samples for early stages (I+II, n=20) and late stages (III+IV, n=20). Serum and eutopic endometrium from normal women were used as control. The stages of

endometriosis were indexed according to the score of revised ASRM. After collection, all serum and biopsies were stored at -80ºC for future experiments.

Cell and tissue extraction: Tissues were suspended in PBS containing protease inhibitors and minced at 4 °C. The suspension was centrifuged at 12,000 g for 15 min, and supernatant was collected as PBS extracts. The pellet was further, extracted in lysis buffer (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1% Triton X-100, and protease inhibitors) and centrifuged at 12,000 g for 15 min to obtain Triton X-100 (Tx) extracts. In vitro cells were directly homogenized in lysis buffer containing protease inhibitor cocktail and centrifuged at 12,000 g for 15 min to obtain for whole cell extract. Protein estimation was done by Lowry and Bradford method.

Casein zymography: For assay of MMP-7 activity, extracts (30 µg protein/lane) or cell supernatant (10µl/lane) were electrophoresed in 12% SDS-polyacrylamide gel containing 1 mg/ml gelatin under non-reducing conditions. The gels were washed twice in 2.5% Triton X-100 and then incubated in stromelysin assay buffer (100 mM Tris-HCl, pH 7.4, 5 mM $CaCl_2$, 0.001% (w/v) sodium azide and 0.005% (v/v) Brij 35) for 48 h at 37°C. Gels were stained with 0.1% Coomassie blue followed by destaining. The zones of casinolytic activities appeared as negative staining. Quantification of zymographic bands was done using densitometry linked to proper software (Lab Image, Kapelan Gmbh, Leipzig, Germany).

Hematoxylin & Eosin staining: Tissues were sectioned into 2-3 mm^2 pieces. The tissue samples were fixed in 4% paraformaldehyde, dehydrated and embedded in paraffin wax. After deparaffinization, three consequitive wash in xylene was done followed by rehydration in graded alcohols (2 times in 100% EtOH, 3 min; 2 times in 95% EtOH, 3 min and in 70% EtOH for 3min). After washing in distilled water for 5 min the slides were immersed in filtered hematoxylin solution for 6 min, followed by washing in tap water for 5 min. Then the slides were counterstained in Eosin for 15 seconds followed by dehydration in graded alcohols (2 times in 95% EtOH, 3 min and 2 times in 100% EtOH). After 2 consecutive washing in xylene for 5 min, slides were mounted with DPX and cover slip. The images were assessed under an Olympus microscope. Images were captured using Camedia software (E-20P 5.0 Megapixel) and processed under Adobe Photoshop version 7.0

Western blotting: Tissue (100 µg) or cell (40µg) extracts were resolved by 10% reducing SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes. The membranes were blocked for 2 h at room temperature in 3% BSA solution in 20 mM Tris-HCl, pH 7.4 containing 150 mM NaCl and 0.02% Tween 20 (TBST) followed by overnight incubation at 4ºC in 1:500 dilution of the respective primary antibodies in TBST containing 0.3% BSA. The membranes were washed five times with TBST and then incubated at 4ºC in 1:10000 dilution of the respective secondary antibodies in TBST containing 0.3% BSA. The blots were again washed 4 times with TBST followed by a last washing of TBS for 15 min under shaking condition at room temperature. After washing of secondary antibody, the bands were visualized using 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium substrate solution. The secondary antibody used for the study are goat anti-rabbit IgG-ALP conjugated (SC-105206), rabbit anti-goat IgG-ALP conjugated (SC-105218). Once the band appears the reaction should immediately be stopped by addition of distilled water.

Immunofluorescence: Deparaffinised and rehydrated sections were subjected to antigen retrieval by trypsin (0.05% trypsin, 0.1% $CaCl_2$) and blocking was performed using 5% BSA in TBS (20 mM TrisHCl, pH 7.4 containing 150 mM NaCl) for 2 hr at room temperature followed by the incubation over night at 4 °C in anti-MMP-7 (SC-8068) primary antibody solutions (1:100 dilutions in TBS with 1% BSA) in a humid chamber. The tissue sections were washed four times with TBST (20mM TrisHCl, pH 7.4 containing 150mM NaCl and 0.025% Triton X-100) followed by incubation with texus red secondary antibody (SC-3919, Santa Cruz Biotechnology, USA) solution (1:200 dilutions in TBS containing 1% BSA) for 2 h at room temperature. Tissue sections were washed four times with TBST followed by counter staining with DAPI.

Enzyme-Linked Immunosorbent Assay (ELISA): ELISA for total MMP-7 was performed using ELISA kit (DY907, R&D system) for human serum and tissue extracts as per the user manual instructions. In brief, 96-well microplate was coated with prescribed dilution of the capture antibody for overnight in room temperature. Each well was aspirated with Wash Buffer for a total of three times and blocked with plates by adding 300µl reagent diluent for 1 hr followed by 3 times washing. Then sample (serum/cell sup 100µl, tissue 300µg) or standards in reagent diluents, or an appropriate diluent, was added and incubated for 2 hr at RT followed by 3 times washing. 100 µl of

the detection antibody, diluted in reagent diluents was added to each well and incubated for 2 hours at RT followed by 3 times washing. Next 100 µl of the working dilution of streptavidin-HRP was added to each well for 20min in dark followed by washing. 100 µl of TMB (3,3',5,5'-Tetramethylbenzidine) substrate Solution was added for colour development followed by addition of 50 µl of stop solution (2N sulfuric acid) to each well. OD was measured at 450 nm using a ELISA reader with reference corrections of 570 nm.

RNA isolation: For isolation of total cellular RNA, cell/ tissues were kept in 500 µl of Trizol reagent in sterilized centrifuge tubes and stored at -70°C. During RNA extraction the samples were homogenized properly at 4°C with electrical homogenizer. 100 µl of chloroform (Trizol: Chloroform = 5:1) was next added and shaken vigorously by hand for 30 sec and incubated at room temperature for 5 min. The samples were then centrifuged at 10,000x g for 15 min at 4°C. This separates the mixture into a lower red phenol-chloroform phase containing protein, a white interphase containing DNA and a colorless upper aqueous phase containing RNA. The upper aqueous phase was then transferred to a sterile centrifuge tube and equal volume of isopropyl alcohol (1:1) was added followed by proper mixing followed by centrifugation at 10,000x g for 15 min at 4°C. The precipitated RNA was washed properly with 500 µl of ice cold 75% ethanol and was centrifuged at 10,000x g for 5 min at 4°C. The pellet was air-dried partially for 15 min at room temperature and carefully dissolved in RNase free sterile 0.1% DEPC treated water by incubating for 10 min at 55°C. The purity of extracted RNA was judged by 260/280 ratio and was quantified by measuring the absorbance at 260 nm through Nanodrop 200c (Thermo Scientific, USA). The dissolved RNA was then stored at -80°C.

2.3.9. Reverse transcriptase-PCR: Complementary (c)DNA (1µl) was prepared using iscript cDNA synthesis kit (Bio-Rad, USA) and amplified in 20 µl of total reaction volumes using 10 pmoles of each primer (primers were designed with Primer 3 software), 0.2 mM of each dNTPs, 1.5 mM of $MgCl_2$, 1U of Taq polymerase in a standard PCR buffer (Invitrogen, USA). PCR was carried out for 35 cycles of: (i) denaturation for 1 min at 94°C, (ii) primer-template DNA annealing for 1 min at 55-59° C, and (iii) DNA synthesis at 72°C for a time based on expected fragment size (1 min/kb) using the following primers indicated in the Table 1. The PCR products were

analyzed by electrophoresis in 2% agarose gels and visualized by ethidium bromide staining. PCR product sizes were estimated by 100 bp marker (Invitrogen) in each case.

Table 1. Primer used in the RTPCR study

Name	Forward (5->3)	Reverse (5'->3')	Tm(°c)
MMP7	TGGGGAACTGCTGACATCAT	CCCTAGACTGCTACCATCCG	64.5
B-ACTIN	ATCATGTTTGAGACCTTCAACA	CATCTCTTGCTCGAAGTCCA	62.1

Statistical analysis: The statistical analysis of the data was done using GraphPad Instat-3 software using one-way analysis of variance (ANOVA) followed by Student–Newman–Keuls test. Protein band intensities were quantified by densitometric analysis using Lab image software. Data were fitted using Sigma plot represented as means ± SEM. $p < 0.05$ was accepted as level of significance; *** highly significant $p < 0.001$; ** significant $p < 0.01$; * less significant $p < 0.05$; NS not significant for $p > 0.05$.

2. 4. RESULTS

Study population characterization. The study was conducted with a total of 40 ovarian endometriosis patients in proliferative phase of their menstrual cycles, and 15 control women without any endometriosis with the same phase. Same menstrual phase was selected in order to exclude any hormone-mediated discrepancies in the study. Among 40 ovarian endometriosis patients, 20 belong to early stages (I+II) and 20 belong to late stages (III+IV) of ovarian endometriosis. Details of the demography of the studied population are mentioned in Table 2. No significant age differences were observed in control or endometriosis patient populations. The studied control population had no infertility related problems. However, for early and late stages of endometriosis respectively ~60 (12/20) and 70% (14/20) population were suffering from infertility. Dysmenorrheal pain was present in ~13.3, 45 and 75% of the population for control, early and late ovarian endometriosis respectively. Chronic pelvic pain was present in ~20, 60 and 80% of the population for control, early and late ovarian endometriosis respectively.

	Control	**Early Stages**	**Late Stages**
	N=15	**N=20**	**N=20**
Age (Mean±SD)	32.2±5.9	33.4±4.5	32.8±7.6
Infertile	-	12/20	14/20
Dysmenorrhea	2/15	9/20	15/20
Chronic pelvic pain	3/15	12/20	16/20

Table 2: Demographic profile of the study population. Control subjects (n=15) and endometriosis patient samples (n = 40) on the basis of severity (as standardized by rASRM) were collected and grouped as early stages (I and II), and late stages (III and IV). All individuals of the study population were in the proliferative phase of the menstrual cycle.

Histological study of the normal eutopic endometrium versus diseased ectopic endometrium. To understand the structural differences in the eutopic and ectopic endometrium, control uterus and diseased ectopic endometrioma from both in early and late stages of ovarian endometriosis were subjected to heamatoxylin and eosin staining (Figure 1). Histological study of the control endometrium showed organised single

layered glandular epithelium, whereas in ectopic endometium the glandular cells are diffused and irregularly developed. In early ovarian endometrioma the glands are scarce and limited to development of small endometrium lining and glands within the stroma. In late stages of ovarian endometriosis, the glandular cells are vastly present and distributed in a distorted manner order to develop functional endometrial glands and lining. We also found multi layered and diffused glandular cells, which were structurally completely different from the eutopic glands. The irregular distribution of endometrial cells all over the lesion suggested increased invasiveness of the diseased cells (Fig. 1). We also found increased iron load in the endometriotic lesions which were elevated with disease severity. Also increased blood vessel formations were observed with progression of ovarian endometriosis.

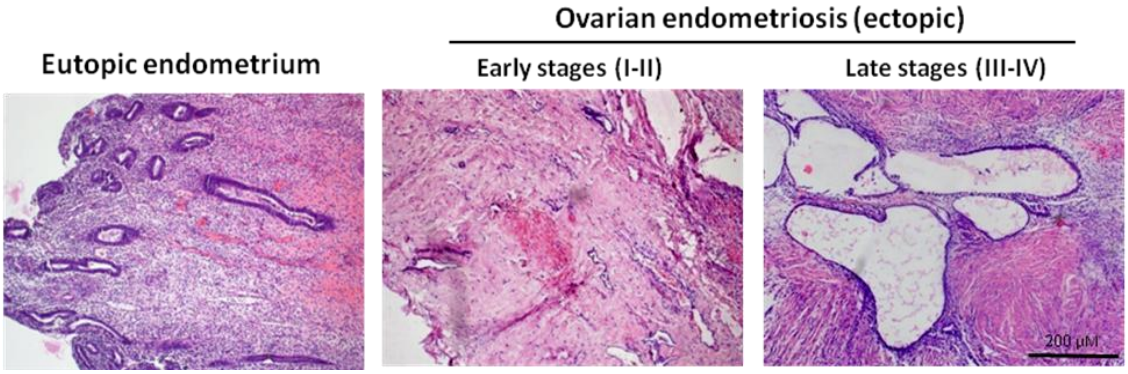

Figure 1: Histology for the control eutopic and ovarian ectopic endometrium. Hematoxylin and eosin staing for formalin fixed human endometrium from control eutopic (left panel) and ectopic ovarian endometrioma of early (middle panel) and late (right panel) stages of patients.

Status of MMP-7 levels in serum of women with or without endometriosis. MMP-7 expressions in serum of 15 control women and 40 endometriosis patients were determined through ELISA. The expression for serum MMP-7 was ~2 fold increased in endometriosis patients than the control group, and statistically significant (p=0.035) changes were observed (Fig. 2A). However, when the serum MMP-7 expressions were evaluated based on severity in only patient groups, we found that the late endometriosis patients contain very significantly increased (p=0.009) expressions of serum MMP-7 than early stages of ovarian endometriosis patients. The MMP-7 levels were ~2.5 fold elevated in late stages than early stages of ovarian endometriosis patients (Fig. 2B).

However, since the expression of MMP-7 does not necessarily depict the potency for MMP-7 functionality, the activity assay was performed with casein zymography. As depicted in Figure 2C and D, MMP-7 activity was found to be significantly elevated in the serum of late endometriosis patients in comparison to the control and early endometriosis patients.

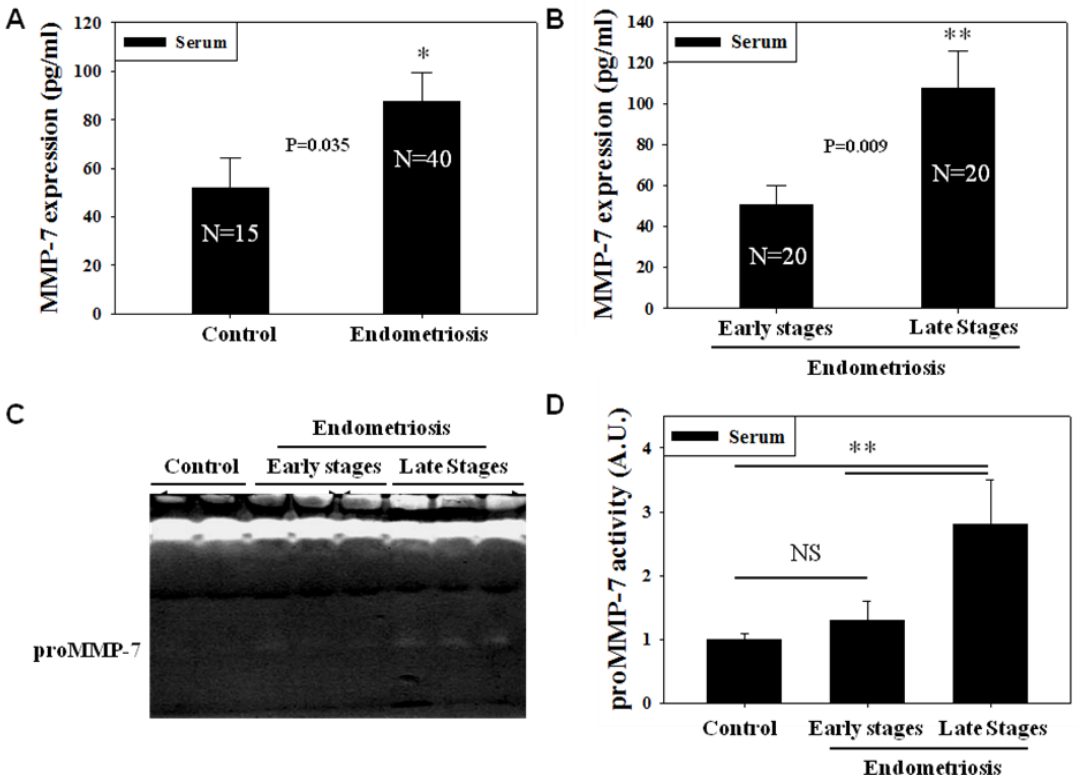

Figure 2: Status of serum MMP-7 in ovarian endometriosis. (A) Expression of MMP-7 in serum of control and ovarian endometriosis patients. (B) Serum MMP-7 expression in early and late stages of ovarian endometriosis. (C) Comparison of serum MMP-7 activity in representative casein zymography for control and early, late stages of endometriosis. and (D) quantification of the caseinolytic activity of MMP-7. Number of patients for early stages (n=20), late stages (n=20), control (n=15).

Involvement of MMP-7 with disease severity of ectopic ovarian endometriosis. As increased MMP-7 was observed in serum of late stages of endometriosis patients, we investigated whether ectopic endometriotic lesions are the source for the elevated MMP-7 responses. ELISA was performed with whole tissue lysate of ovarian endometriotic lesions between early and late stages of ovarian endometriosis patients. The MMP-7 expressions for late endometriosis lesions were observed ~4 fold higher than the early

ovarian endometriosis and were significantly (p=0.0074) elevated (Fig. 3A). Moreover, the ectopic tissue levels for MMP-7 expressions were ~10 folds elevated than the serum, suggesting endometriotic lesions as the principal source for MMP-7 in systemic fluid. The mRNA levels for MMP-7 further confirmed with RT-PCR studies, where the expressions for MMP-7 were found to elevate with severity of the disease (Fig. 3B). Casein zymography were performed for endometriotic tissues in order to understand the pro and active MMP-7 status in endometriosis patients. Zymography analysis found that both pro and active MMP-7 activities were increased with severity of ovarian endometriosis. The (active) MMP-7 activity were significantly elevated (p=0.004) in the ovarian endometrioma of late stage patients in comparison to the early stage patients (Fig. 3C, D).

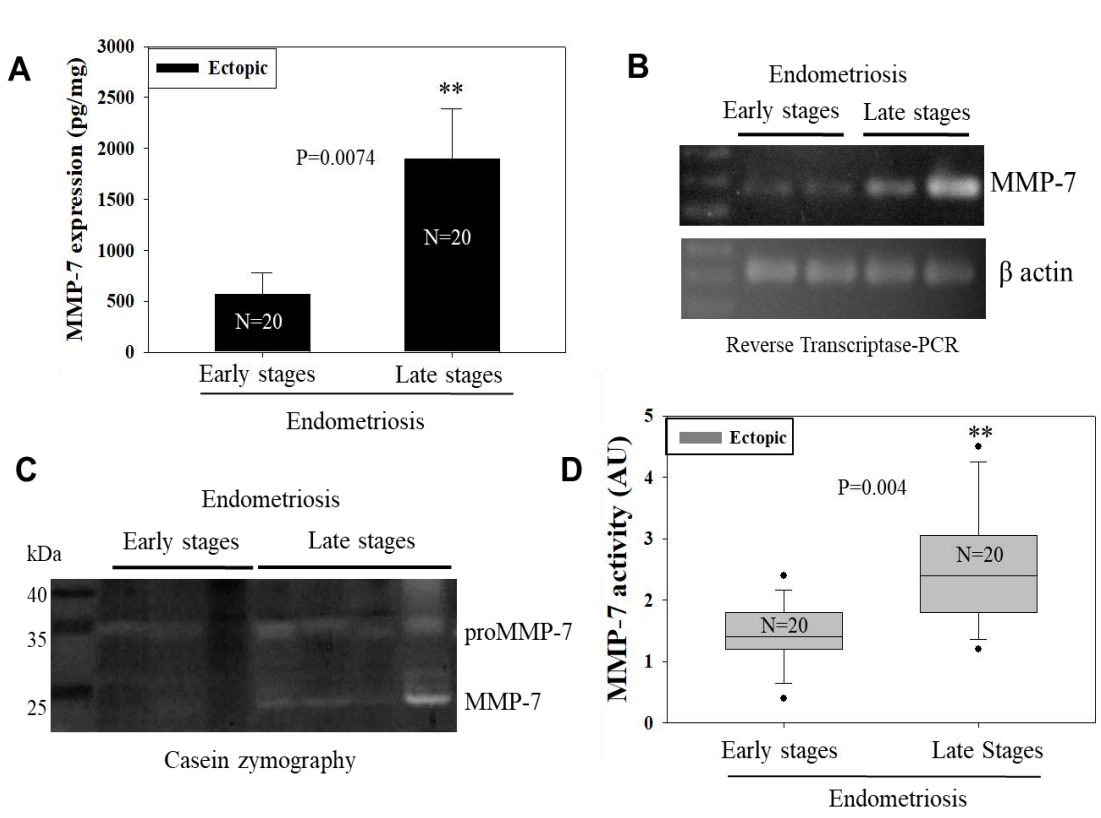

Figure 3: Involvement of MMP-7 in ectopic ovarian endometriosis. (A) MMP-7 expression of the same population by ELISA (B) mRNA status of MMP-7 in ectopic endometrium in early and late stages of endometriosis. (C) Casein zymography for MMP-7 activity and (D) quantification.

MMP-7 is expressed by endometriotic glandular epithelial cells. To indentify MMP-7 positive cell population in endometriosis, immunoflourescence study was performed with anti-MMP-7 antibody. The glandular epithelial cells were found to be expressing MMP-7 in ovarian endometrioma and were found to be increased with the disease severity (Fig. 4A). When quantified the population of MMP-7 positive cells between early and late ovarian endometriosis, we found that the MMP-7 positive cells were very significantly elevated (~10 folds) in higher stages of ovarian endometrioma compared to the early stages of the same (Fig. 4A, B).

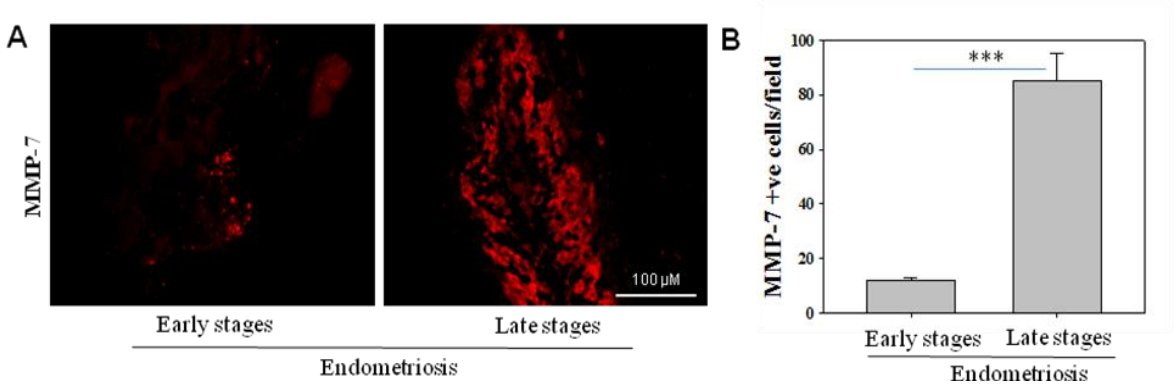

Figure 4: MMP-7 localization in ectopic ovarian endometriosis. (A) Immunofluorescence study for MMP-7 localization in early and late stages of ovarian endometriosis patients. (B) MMP-7 positive cells were quantified from 6 different fields of early and late stages ovarian endometriosis (n=3/group) and presented in histogram.

Comparison of MMP-7 in eutopic endometrium of women with or without endometriosis. Since development of endometriosis is reported to affect uterine functionality (Paul et al, J Pineal Res 2008), MMP-7 expressions were measured with ELISA in eutopic endometrium of control and ovarian endometriosis patients. Significant decreases in MMP-7 expressions were observed (p=0.0392) in eutopic endometium of endometriosis affected patients, compared to unaffected polpulation **(**Fig. 5A). The mRNA levels for MMP-7 further confirmed with RT-PCR studies, where the expressions for MMP-7 were found to decreased in endometriosis affected endometrium compatedto the control endomterium (Fig. 5A inset). However, when casein zymography were performed with eutopic tissue extracts, an interesting notion was observed. We found that endometriosis affected eutopic endometrium express majorly active form of MMP-7 (~25 kDa), where as the control uterus retain only inactive proform of MMP-7 protein (~35 kDa) (Fig. 5B). Therefore, when MMP-7 activity were quantified in eutopic

endometrium of control versus endometriosis patients, significantly elevated MMP-7 activity (p=0.026) were observed in the eutopic endometrium of ovarian endometriosis patients than women without endometriosis (Fig. 5C).

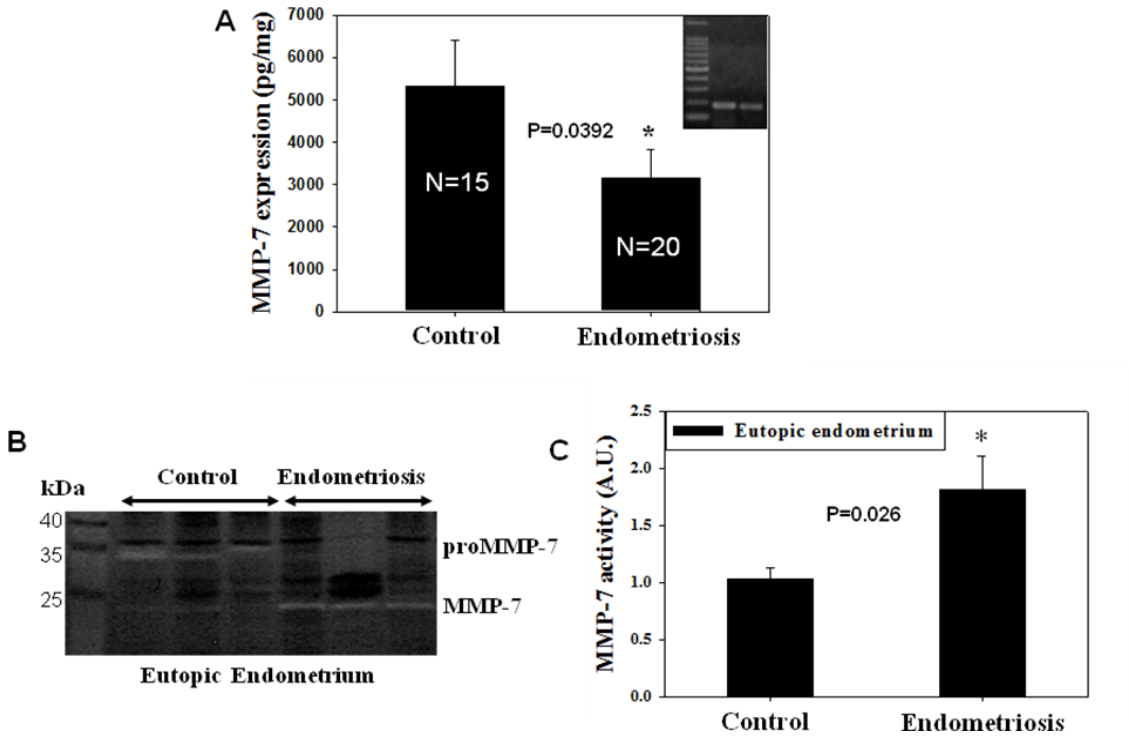

Figure 5: Involvement of MMP-7 in eutopic endometrium. (A) Status of MMP-7 expression (protein) in eutopic endometrium between women with and without endometriosis. Inset shows the mRNA status of the same through RT-PCR. (B) Representative casein zymography for pro and active MMP-7 activity in eutopic endometrium of women with and without endometriosis and quantification of the caseinolytic activity of active MMP-7 in histogram (C).

Comparison of MMP-7 between eutopic and ectopic endometrium from women with endometriosis. We compared MMP-7 levels between eutopic and ectopic endometrium from individual endometriosis patients in order to understand ectopic functionality in comparison to eutopic. Casein zymography revealed that active MMP-7 is prevalent in both ectopic and eutopic endometrium of endometriosis patients, and active MMP-7 activity is significantly elevated in eutopic endometrium (p=0.023) than ectopic irrespective of the disease severity (Fig. 6A, B). When MMP-7 expressions were assessed in ectopic and eutopic endometrium of the same endometriosis patients (n=20),

eutopic endometrium showed significantly elevated MMP-7 expression (p=0.023) compared to the ectopic endometrium (Fig. 6C). Inset shows the MMP-7 mRNA status for the same.

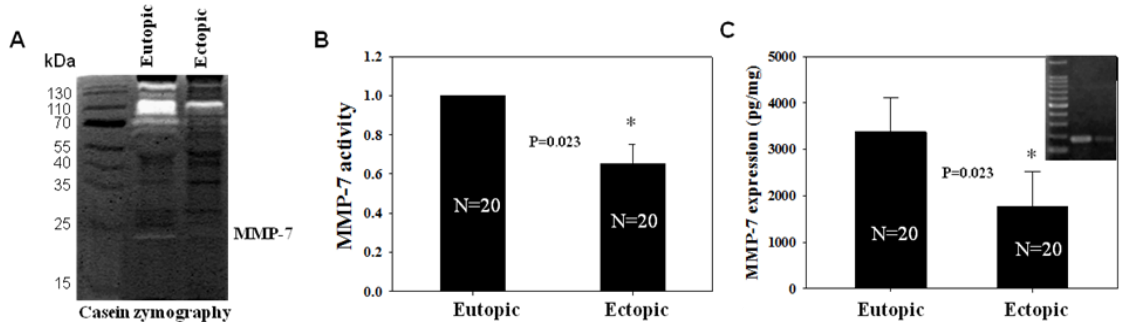

Figure 6: Eutopic versus ectopic MMP-7 levels in ovarian endometriosis patients. (A) Casein zymography for MMP-7 activity in endometrium of eutopic and ectopic origin of endometriosis patients. (B), Quantification of the MMP-7 activity. (C) Total MMP-7 expression of MMP-7 measured by ELISA. Inset shows the MMP-7 RTPCR status for the same.

2.5. DISCUSSIONS

MMP-7 is the smallest of MMP family of proteins due to lack of hemopexin domain. This also confers the protease lesser substrate specificity and allows to cleave a vast array of ECM components and nonECM bio-ligands. Although earlier studies have identified MMP-7 in endometriosis [Bruner-Tran *et al.,* 2006], no study still date reports detailed involvement of MMP-7 with ovarian endometriosis. The present study shows significant upregulation of MMP-7 expressions and activities, especially in higher stages of ovarian endometriosis both in serum and ectopic tissues. Presence of MMP-7 in a benign disease like endometriosis indicates its increased invasive properties. Moreover, endometriosis is reported to be recurrent and has a tendency to spread over multiple organs within the peritoneum; although whether the process is lymphatic or vascular dependent is still unknown [Samani *et al.,* 2017]. This also indicates that the disease has a metastatic nature and may be involved with the EMT process. MMP-7 is reported mainly to be expressed in cancer cells and involved with cancer metastasis. Recent study with MMP-7 knockout mice reported to have reduced prostate tumorigenicity, by means of suppression of invasiveness [Zhang *et al.,* 2016]. The major source for MMP-7 in ovarian endometriosis is glandular epithelium, although stromal-derived paracrine

signals are required for progesterone to inhibit endometrial epithelial proMMP-7 expression [Bruner-Tran *et al.,* 2006]. Herein, we report an upregulation of MMP-7 activity in the eutopic tissues of endometriosis effected patients compared with the control eutopic endometrium. However, the total expression for MMP-7 is found significantly higher in control endometrium. The discrepancies can be explained with substrate zymography, where women without endometriosis are found to have only proform of MMP-7. On the other hand, the endometriosis affected eutopic endometrium has active MMP-7, enabling the disease affected endometrium to have higher MMP-7 functionality. In summary, the present study found ovarian endometriosis progression is associated with elevated MMP-7 responses both in serum and ectopic lesions of affected women.