ORIGINAL RESEARCH PAPER
Volume-8 | Issue-9 | September - 2019 | PRINT ISSN No. 2277 - 8179 | DOI : 10.36106/ijsr
INTERNATIONAL JOURNAL OF SCIENTIFIC RESEARCH

IDENTIFICATION OF DISEASE SPECIFIC PROTEINS ASSOCIATED WITH ENDOMETRIOSIS IN INDIAN WOMEN

Gynecology

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ABSTRACT

Endometriosis is one of the major gynecological disorder affecting women in their reproductive age. The major cause of endometriosis is still unknown and there no proper noninvasive marker to diagnosis the disease at early stage. The aim of this study was to investigate the role of Human neutrophil peptides 1, 2 and 3, cytokines and matrix metalloproteinase's in pathogenesis of endometriosis. The present study characterizes the detection of HNP1-3, IL-6, IL-8, MMP-7 and MMP-9 in serum may provide novel non-invasive markers and diagnostic test to identify different stage of endometriosis

KEYWORDS

Endometriosis, Human Neutrophil Peptides 1, 2 And 3, Cytokines And Matrix Metalloproteinase's

INTRODUCTION

Endometriosis is a burdensome disease, being one of the most common benign diseases affecting women of reproductive age about 5-10% and is a common cause of infertility[1,2]. It is characterized by the presence and growth of ectopic endometrial tissue outside the endometrial cavity[3]. Women with endometriosis frequently suffer from symptoms including non menstrual pelvic pain, painful menstrual cramps, and pain during intercourse, fatigue and infertility [4,5,6,7] which can lead to a substantial reduction in quality of life. This complex disease is associated with infertility and pelvic pain [8]. A definitive diagnosis of endometriosis relies on laparoscopic visualization9 of endometriotic lesions requiring a surgical procedure, and a scoring system has been developed to assess before extent of the disease.

The vaginal ultra sound and the existing biomarker such as CA-125 are not diagnostically useful in early stages of the disease. According to previous reports, the levels of certain proteins and growth factors are involved in inflammation, angiogenesis, proliferation, differentiation and tissue remodeling, but none of these are sufficiently sensitive and specific to be translated in to a non- invasive test for clinical diagnosis[10]. HNP1-3 belongs to family of α- defens, plays an important role as immune-regulatory effects and involve in innate immunity response against any infections[11]. HNP1-3 may play a role in various inflammatory reactions. An increase in peritoneal inflammation as evidenced by elevated peritoneal fluid cytokine levels is well established in women with endometriosis[12]. While it is uncertain if the elevated cytokines levels/inflammation is a cause for or a result of the disease, it is clear that these cytokines may have profound effects which can lead to the establishment and further progression of the disease[13].

There is a possible use of specific markers for the early diagnosis of endometriosis, but while peritoneal markers are very much variable under the hormonal influence and related to the peritoneal fluid amount, the investigation of serum markers revealed many interesting molecules. The serum markers are used for its specificity, sensitivity and its ability to relate to the disease activity allowing disease follow-up.

Endometriosis is considered to be an inflammatory process in which activated immune-related cells secreted large amounts of cytokines[14] which attract more immune cells and promote growth of ectopic endometrial cells, contributing to the occurrence and development of endometriosis [15,16]. The quality of endometrial cells in peritoneal fluid of women with endometriosis is different from women with normal pelvis. Viable endometrial cells from human endometriotic biopsies but not from human endometrial biopsies are invasive in an in vitro collagen invasion assay, because they have a higher proportion of potentially invasive E-cadherin-negative epithelial cells[17] .Previous studies have indicated a link between raised serum levels of IL-6 or IL-8 and endometriosis. Human neutrophil peptides 1, 2, and 3 (HNP 1–3) belonging to -defensin family play a crucial role in innate immunity against infections , may exert immunoregulatory effects and have various inflammatory reactions

Inflammatory cytokines (IL- 6 and IL-8) produced by endometrial cells probably contribute to this adhesion process[18,19] of endometrial cells to fibronectin

The matrix metalloproteinase's (MMPs) is a tranquil of the enzymatic constituent, the enzyme inhibitory component, tissue inhibitors of metalloproteinases[20] which plays a crucial role during the normal development, growth of the endometrium and many other physiological processes[21,22.] Matrix metalloproteinase's (MMPs) and tissue inhibitors of metalloproteinase's (TIMPs) are expressed in the human endometrium during the menstrual cycle, in particular to induce substantial extracellular matrix breakdown underlying menstruation The aberrant expression of MMPs is associated with the pathophysiology of many diseases as it balances between MMP and Tissue inhibitors of metalloproteinase's (TIMP) [23.] Most MMPs are expressed in human endometrium[24, 25] Levels of MMP-7 cells levels strongly increase at menstruation and subsequently remain elevated during the proliferative phase[26,27] and MMP-9 increases in stromal cells during the menstrual phase, but the protein is found throughout the cycle in stromal, epithelial, and inflammatory cells [28,29]

Accordingly, we aim to investigate the role of serum Human neutrophil peptides 1, 2 and 3, cytokines and matrix metalloproteinase's as early markers of endometriosis.

MATERIALS AND METHODS

Patient enrollment

This prospective control case study was carried out from August 2016

to February 2019 at MHRT Hospital & Research Centre, Deccan College of medical Sciences & Princess Esra Hospital Hyderabad. All the patients who were undergoing laparoscopy for infertility treatment were enrolled for this study

One hundred and Eighty Six Patients with mean age 29.2 2 ± 3.2 years were recruited in, MHRT Hospital & Research Centre, Hyderabad. All patients in this study received laparoscopy on account of gynecological indications such as suspected endometriosis, pelvic masses, pelvic pain, infertility, and uterine leiomyoma. Of 186 patients, 107patients with pathologically confirmed endometriosis were assigned to endometriosis group whereas the remaining 79 patients were in the control group.

The Exclusion criteria were the patients with Genital tuberculosis, pelvic pain, Tio mass, PID.The inclusion Criteria were all the infertile patients with primary and secondary infertility between age group 22-36 were enrolled. Control cases were enrolled for the patients who were admitted for laparoscopic sterilization for the study.

The extent of endometriosis was classified in accordance with the American Society of Reproductive Medicine (ASRM) revised system[30] stage I & II (n=72), stage III & IV (n=35). Total of 79 healthy women (mean age 30.1 ± 4.2 years) who had normal hormonal profile, no pelvic pain and came for the sterilization were enrolled in the present study(Fig:1) This study was approved by the ethics committee of MHRT Hospital & Research Centre, Hyderabad, and written informed consents were obtained from all participants.

Samples collection

Collection and preparation of peritoneal fluid

Laparoscopy was performed for all the enrolled patients. Peritoneal fluid of 10ml was collected at the beginning of the laparoscopy. Peritoneal fluid samples were centrifuged at 3000rpm for 10 min. Supernatants were collected and stored frozen at −80 ◦C until further used.

Collection of Blood Samples and isolation of Peripheral blood mononuclear cells (PBMCs)

Peripheral blood sample of 5ml was collected in heparinized tube and in plane tube from all the enrolled subjects in the present study. Blood samples were diluted with normal saline in ration 1:1. Diluted sample processed with density gradient medium in ratio 3:1. Then tube was centrifuged at 3000rpm for 45 minutes. After centrifugation, the Buffy coat was transferred into fresh eppendroff tube and washed twice with PBS. Finally cells were resuspended with PBS. Plane tubes were centrifuged and serum was stored in another tube.

HNP1-3 and cytokine evaluations

The HNP1-3 were evaluated for all the samples (peritoneal fluid and serum) by enzyme-linked immunosorbent assay (ELISA)(HNP 1–3; Hycult Biotechnology B.V., Uden, Netherlands). IL-6 and IL-8 were evaluated (peritoneal fluid and serum) by specific ELISA kits from R&D (Minneapolis, MN, USA) for all the collected samples.

Molecular analysis using RT-qPCR (MMP7 and MMP9)

For molecular analysis total RNA was isolated from PBMCs by Guanidinium thiocyanate (GITC) method. cDNA was constructed from RNA which was isolated from all the samples. Construction of cDNA was confirmed by GAPDH PCR for all samples. Real time PCR was kept for all the samples. All the samples were kept in duplicate. All the samples were kept for MMP-7, MMP-9 and GAPDH primer to find out the fold difference between all the samples.

Relative fold calculation

Relative fold values were calculated for the transcripts to identify their expression levels in related samples. The difference in expression of each transcripts was calculated by using 2-Δ Δ Ct methods. It was further verified by pfaffl methods. Melt curve was analyzed to exclude the primer dimer. PCR efficiency was determined which was found to be 1 and accepted for further calculation

Statistical analysis

The differences were considered statistically significant at $P < 0.05$. All data are shown as means ± SD. Gene expression was expressed as relative quantification ($RQ = 2^{-\Delta\Delta CT}$) as per Livak method[31] and further validated by Pfaffl method[32]. All statistical analysis was performed using GraphPad Prism version 5.0 (GraphPad Software, Inc., San Diego, CA, USA).

RESULT

The demographic and clinical details of the study groups listed in (Table I). There was no significant difference in age, BMI, age at menarche was observed in comparison with case and control groups**.**

HNP1-3 concentration Evaluations

Concentrations of HNP 1–3 in the peritoneal fluid and Serum of patients with endometriosis found that concentrations of these α-defensins were statistically significantly increased compared with control women (P<0.001,P<0.002 respectively Table II, Fig IIA , Fig IIB). This difference was significant both in the case of grade I/II and grade III/IV of endometriosis (Table II). Furthermore, increased concentrations of HNP 1–3 significantly correlated with more advanced stages of the disease .But there was no much difference in level of HNP 1-3 in peritoneal fluid and serum sample in patients with different stage of endometriosis (**Figure IIC)**

Cytokine Evaluation

IL6 Evaluation

Endometriosis was also associated with significantly increased concentrations of IL-6

Concentrations of IL6 in the peritoneal fluid and Serum of patients with endometriosis found that concentrations of these cytokines were statistically significantly increased compared with control women (P<0.002, P<0.004 respectively Table II, Fig IIIA , Fig IIIB).Increased concentration of IL-6 was observed in peritoneal fluid in patients with different stage of endometriosis compare with controls.

But there was no much difference in level of IL-6 in peritoneal fluid and serum sample in patients with different stage of endometriosis (**Figure IIIC**).

IL8 Evaluation

Endometriosis was also associated with significantly increased concentrations of IL-8 .Concentrations of IL8 in the peritoneal fluid and Serum of patients with endometriosis found that concentrations of these cytokines were statistically significantly increased compared with control women (P 0.01,P<0.02 respectively Table II, Fig IVA , Fig IVB).

Increased concentration of IL-8 was observed in peritoneal fluid in patients with different stage of endometriosis compare with controls. Difference was not observed in level of IL-8 in peritoneal fluid and serum sample in patients with different stage of endometriosis (**Figure IVC**).

MMPs Evaluation

MMP-7 Concentration Evaluation

Endometriosis was also associated with significantly increased concentrations of Matrix metalloproteinase's. Concentrations of MMP-7in the peritoneal fluid and Serum of patients with endometriosis found that concentrations of these cytokines were statistically significantly increased compared with control women **(P 0.03,P<0.04 respectively Table II, Fig VA , Fig VB)**.Increased concentration of MMP-7 was observed in peritoneal fluid in patients with different stage of endometriosis compare with controls. Concentration of MMP-7 was found to be significantly high in grade III/IV (421.13 ± 87.15 and 441.92 ± 93.25 in peritoneal fluid and serum respectively) when compare to grade I/II (**Figure VC**).

MMP-9 Concentration Evaluation

Endometriosis was also associated with significantly increased concentrations of Matrix metalloproteinase's. Concentrations of MMP-9 in the peritoneal fluid and Serum of patients with endometriosis found that concentrations of these cytokines were statistically significantly increased compared with control women **(P 0.01, P 0.008 respectively Table II, Fig VIA , Fig VIB)**.Increased concentration of MMP-9 was observed in peritoneal fluid in patients with different stage of endometriosis compare with controls. Concentration of MMP-9 was found to be significantly high in grade III/IV in peritoneal fluid (339.23 ± 78.23) and serum (330.24 ± 71.12) when compare to grade I/II and controls.

There was no difference in value when compare with peritoneal fluid and serum samples (**Figure VIC**)

MMP-7 expression levels by Real-time PCR

The expression level of MMP-7 was found to be down regulation in

control samples compare to grade I/II and grade III/IV endometriosis patients. Our data has revealed highly significant up regulation of MMP-7 in grade III/IV endometriosis patients when compare to controls and grade I/II (p=0.008) **(Figure VII)**.

MMP-9 expression levels by Real-time PCR

Expression level of MMP-9 was revealed up regulation in grade I/II and grade III/IV compare to control subjects which was statistically significant (p=0.002). The expression level of MMP-9 was found to be high in grade III/IV compare to grade I/II **(Figure VIII)**.

The results of our study are promising and the clinical application of large samples is necessary to determine the effectiveness of the potential biomarkers

DISCUSSION

Endometriosis is a benign, chronic, estrogen-dependent inflammatory disease. Endometriosis is affecting approximately 10% of reproductive age women and 35–50% of women with infertility and pelvic pain[33]. There is evidence which supports the patho-physiological significance of HNP, Cytokines and MMPS

In the present study we demonstrated that in women with endometriosis there is a significantly higher serum and peritoneal level o HNP1-3, IL-6, IL-8, MMP-7 & MMP-9, compared to healthy controls. Concentrations of HNP1-3 in peritoneal fluid were both significantly higher in those women with advanced stages of endometriosis (stage III and IV) compared to those in the early stages (stage 1 and II) of endometriosis. The HNP1-3 concentrations are positively correlated with the severity of disease

There was no difference were observed in peritoneal fluid and serum samples. Melweski et al [11] demonstrated that the level of HNP1-3 is associated with endometriosis and also demonstrated that increased percentage of peritoneal neutrophils is also related to endometriosis
This suggests that HNP1–3 in peritoneal fluid of patients with endometriosis originate from infiltrating neutrophils, cells known to be the principal source of these α-defensins in human organism[34,35].

Cytokines play an important role in regulating the immune cells and peritoneal microenvironment is considered as substantial immunological factor. Function and development of NK cells influence by cytokine present in the peritoneal fluid. Elevated serum CA-125 , IL-6 and IL-8 are considered to be biological markers for differential diagnosis in endometriosis[36].Previous studies found conflicting results regarding serum levels of IL-6 , IL-8 in endometriosis; however, most of them were in favour of a significant increase in IL-6 , IL-8 serum or peritoneal fluid (PF) levels in the case of endometriosis .In the present study, we demonstrated that women with endometriosis had significantly increase level of IL6 in peritoneal fluid compared with healthy controls. Concentrations of IL6 in peritoneal fluid were both significantly higher in those women with advanced stages of endometriosis (stage III and IV) compared to those in the early stages (stage 1 and II) of endometriosis. TheIL6 concentrations are positively correlated with the severity of disease.

There was no difference were observed in peritoneal fluid and serum samples. Some investigator also has shown that the level of IL-6 were significantly high in the peritoneal fluid of endometriosis patients compared with the peritoneal fluid of controls without endometriosis[37]. Our result is consistent with previous reports showing increased levels of inflammatory cytokines in the serum of endometriosis patients[38, 39]. Our study revealed that the level of IL8 significantly high in women with endometriosis compared with women without endometriosis in peritoneal fluid serum. Levels of IL8 in peritoneal fluid and serum were significantly higher in those women with stage III and IV of endometriosis (advanced stages) compared to those in the stage 1 and II (early stages) of endometriosis. The IL8 concentrations are positively correlated with the severity of disease. There was no difference were observed in peritoneal fluid and serum samples. Previous report[40] suggested that the peritoneal fluid from patients with endometriosis contained significantly increased level of IL-8 than those in women without endometriosis which is consistent with present study.

Previous study was demonstrated that increased levels of inflammatory cytokines were found in the serum and peritoneal fluid in patients with endometriosis compare with control groups[41] TNF-α, IL-6, IL-8, IL-10, VEGF and MCP-1 were found to be more predominant in the in women with endometriosis.

Present study revealed that women with endometriosis had significantly high level of MMP-7 in peritoneal fluid and in serum compared with healthy controls without endometriosis. Expression levels of MMP-7 in peritoneal fluid were both significantly higher in those women with advanced stages of endometriosis (stage III and IV) compared to those in the early stages (stage 1 and II) of endometriosis. There was no difference were observed in peritoneal fluid and serum samples. The expression levels of MMP7 are positively correlated with the severity of disease. Matsuzaki et al. [42] also demonstrated that MMP-7 expression levels were significantly increased in the endometrial epithelial cells from patients with deep infiltrating endometriosis. We have demonstrated that women with endometriosis had significantly increase expression of MMP-9 in peritoneal fluid compared with healthy controls.

Concentration of MMP-9 in peritoneal fluid were significantly high in women with stage III and IV of endometriosis (advanced stages) compared to stage I and II of endometriosis. The MMP-9 expression levels are positively correlated with the severity of disease. There was no difference were observed in peritoneal fluid and serum samples. Previous report suggested that [43] the presence of MMP-9 in both peritoneal fluid and serum of endometriosis patients. MMP-9 is mainly synthesized and secreted from macrophages and neutrophil leukocytes[44].The MMP-9 synthesis is positively correlated with different stages of endometriosis. Past studies have revealed the correlation between pelvic focal inflammation and endometriosis, as the altered immune cell functions in peritoneal cavity[45]. Within peritoneal fluids, there were large amounts of macrophage-derived substances, especially in endometriosis patients whose macrophages are activated in the peritoneal cavity[46, 47]. Several investigator s suggested that level of MMP-9 were significantly high in patients with endometriosis compare with controls.

Present study demonstrated that HNP1-3, MMP7, MMP9, IL6 and IL8 can be used as panel of biomarker for the diagnosis and prognosis of endometriosis. Serum is an interesting potential source of biomarkers because it allows repeated measurements, is easily obtained, and is highly suitable for high-throughput measurements. The level of different biomarkers did not reveal any significant difference in peritoneal fluid and serum samples. Peripheral blood can be used to detect the level or concentration of all above mentioned markers instead of peritoneal fluid which is invasive procedure.

Serum biomarkers could avoid the repetitive laparoscopic procedure which is invasive and many patients will not opt for that. This serum biomarker can also be used to know the recurrence of endometriosis and pre ART assessment. This above mention serum markers can be used panel of biomarkers for the diagnosis and prognosis of endometriosis which can avoid the 2nd and 3rd laparoscopic procedure in stage III and IV. Serum biomarkers will not only help shorten the time to diagnosis, but also pave the way to new therapies.

Blood is regarded as potential source of biomarkers because it is conveniently obtained with good repeatability [48] .Many factors in plasma have been regarded as potential biomarkers for the early diagnosis of endometriosis, including glycoprotein's, growth factors, hormones, or proteins related to immunology or angiogenesis[49]. Inflammatory factors have been implicated in the progression of endometriosis. Clinical studies have confirmed close relationship between cytokines and endometriosis, suggesting that cytokines could be used as predictors of this disease.[50] For Example, Drosdzol-Cop et al [51] found adolescent patients with endometriosis displayed significantly higher serum IL-4, Othman Eel et al [52] observed serum interleukin-6 measurements discriminate between women with endometriosis and without endometriosis. Consistent with these findings, the results showed the remarkable elevated concentration of IL-4 and IL-6 in endometriosis.

CONCLUSION

The present study characterizes the role of Human neutrophil peptides 1-3, cytokines and matrix metalloproteinase's for diagnosis of endometriosis, suggesting the feasibility of using HNP 1-3, Selected Cytokines and MMPS as a noninvasive diagnostic test for the detection of endometriosis.

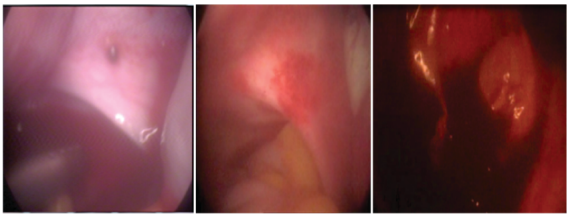
In conclusion, our present findings indicate that Women with

endometriosis have elevated levels of peritoneal and serum fluid levels of HNP 1–3, IL-6, IL-8, MMP-7 AND MMP-9 which can be used as markers for diagnostic test for Endometriosis. Further studies are necessary to clarify and confirm the role of Human Neutrophil Peptides, Pro-inflammatory Cytokines and Matrix Metalloproteinase's in the pathogenesis of endometriosis, and moreover to find a suitable predictive model for early diagnosis.

Acknowledgements
We would like to thank ICMR Government of India to support the project. All the authors equally contributed to the research work.

DISCLOSURE
All authors have no conflict of interest.

Fig 1 Depicts Different stages of Endometriosis identified by laparoscopy during the study



Stage I Stage II Stage III

Table I Illustrates the Comparison of socio- demographic and clinical details of case and control groups

Characteristics	Endometriosis group (n=107)	Control group (n=79)
Age (yr)	**30.5**± 5.5	**30.1**±4.2
Body mass index (kg/m^2)	**25.2**±2.0	**25.1**±1.2
Age at menarche (yr)	**12.7**±1.0	**12.6**±1.1
Symptoms :Pelvic Pain	**95 (96.7%)**	**39(38%)**
Dyspareunia [n (%)]	**35(36%)**	**13(12.5%)**
Dysmenorrhoea		
Mild	**32(32.8%)**	**20 (19.5%)**
Moderate	**5 (5.1)**	**4 (3.9%)**
Severe	**5 (5.1)**	**1(0.9%)**
No dyspareunia & dysmenorrhoea	**30 (30.8%)**	**33(31.9%)**
Primary infertility [n (%)]	**84(86.2%)**	
Secondary infertility [n (%)]	**23(23.7%)**	

Data are presented as mean ± SD
NR, not reported

Table II Illustrates the Concentrations of HNP 1–3, IL-6, IL-8, MMP7, MMP9 in the peritoneal fluid and serum of patients with endometriosis and control women.

	Control	Endometriosis		P value
		Stage I/II	Stage III/IV	
HNP 1–3 (Peritoneal Fluid)	207.3 ± 93.2	342.62 ± 145.32	490.32 ± 287.96	<0.001
IL-6 (Peritoneal Fluid)	16.85 ± 5.26	40.44 ± 15.62	108.56 ± 50.24	<0.002
IL-8 (Peritoneal Fluid)	10.89 ± 2.82	24.29 ± 3.98	40.78 ± 12.25	0.01
PF MMP-7 (Peritoneal Fluid)	110.25 ± 21.24	316.12 ± 81.12	421.13 ± 87.15	0. 03
PF MMP-9 (Peritoneal Fluid)	107.23 ± 22.12	209.23 ± 56.89	339.23 ± 78.23	0.01
HNP 1–3 (Serum)	187.68 ± 87.56	329.25 ± 111.32	478.81 ± 267.28	<0.002
IL-6 (Serum)	14.54 ± 3.63	35.76 ± 13.42	101.42 ± 49.71	<0.04
IL-8 (Serum)	09.36 ± 03.92	22.39 ± 08.56	40.62 ± 13.32	<0.02
PF MMP-7 (Serum)	101.34 ± 29.41	317.26 ± 89.12	441.92 ± 93.25	0. 04
PF MMP-9 (Serum)	99.22 ± 19.12	201.73 ± 55.27	330.24 ± 71.12	0.008

a) Data are presented as means ± SD.
b) P value as computed by one-way ANOVA

Fig II Depicts the Concentrations and Comparison of HNP 1–3, in the peritoneal fluid and serum of patients with and without endometriosis

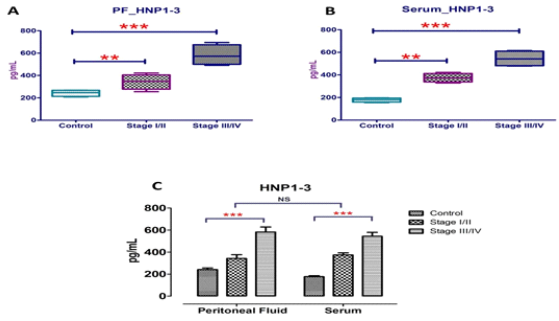


Fig III Depicts the Concentrations and comparison of IL6 in the peritoneal fluid and serum of patients with and without endometriosis

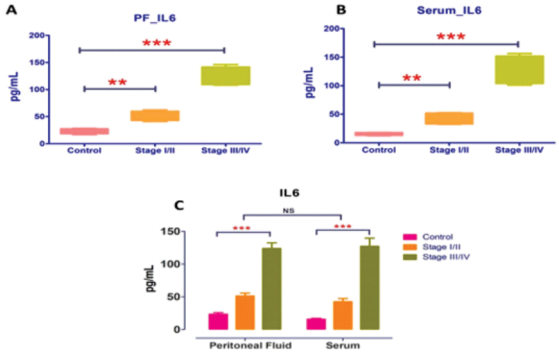


Fig IVDepicts the Concentrations and comparison of IL8 in the peritoneal fluid and serum of patients with and without endometriosis

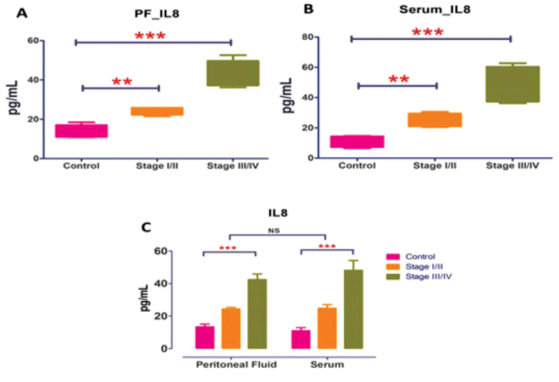


Fig V Depicts the Concentrations and comparison of MMP 7 in the peritoneal fluid and serum of patients with and without endometriosis

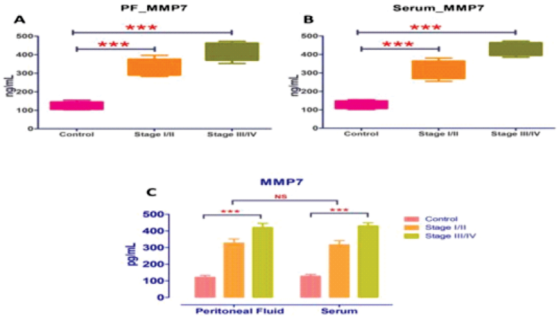


Fig VI Depicts the Concentrations and comparison of MMP 9 in

the peritoneal fluid and serum of patients with and without endometriosis

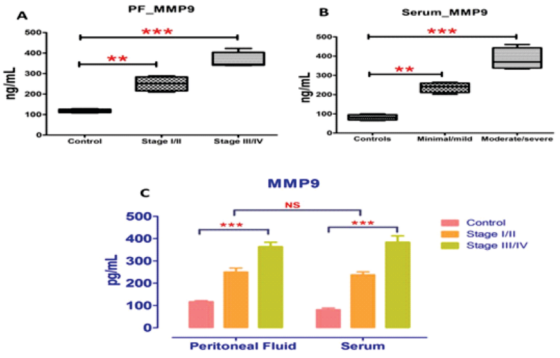


Fig VII Depicts the MMP-7 expression levels by Real-time PCR

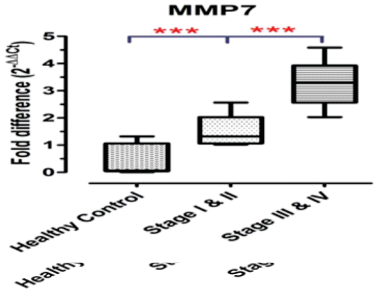
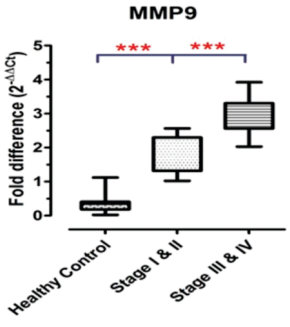


Fig VIII Depicts the MMP-9 expression levels by Real-time PCR



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