行政院國家科學委員會專題研究計畫　成果報告

探討細胞外質分子之表現與子宮內膜異位症病程發展的關聯性之研究

計畫類別：個別型計畫
計畫編號：NSC91-2314-B-038-051-
執行期間：91 年 08 月 01 日至 92 年 07 月 31 日
執行單位：臺北醫學大學生物醫學材料研究所

計畫主持人：楊維中

計畫參與人員：李惟宸

報告類型：精簡報告

處理方式：本計畫可公開查詢

中　華　民　國 92 年 10 月 29 日

附件一

行政院國家科學委員會補助專題研究計畫 ■ 成 果 報 告 □期中進度報告

Studies of the Expression Patterns of the Extracellular Matrix, the Regulatory Mechanism, and Their Association with the Progression of Endometriosis

計畫類別：■ 個別型計畫　□ 整合型計畫
計畫編號：NSC 91 － 2314 － B － 038 － 051 －
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計畫主持人：楊維中
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成果報告類型(依經費核定清單規定繳交)：■精簡報告 □完整報告

本成果報告包括以下應繳交之附件：
□赴國外出差或研習心得報告一份
□赴大陸地區出差或研習心得報告一份
□出席國際學術會議心得報告及發表之論文各一份
□國際合作研究計畫國外研究報告書一份

處理方式：除產學合作研究計畫、提升產業技術及人才培育研究計畫、列管計畫及下列情形者外，得立即公開查詢
□涉及專利或其他智慧財產權，□一年□二年後可公開查詢

執行單位：台北醫學大學生物醫學材料研究所
中 華 民 國 92 年 10 月 29 日

中文摘要

胚胎著床牽涉胚胎與子宮內膜細胞的黏合作用, 其上皮細胞及內皮細胞及其所分泌細胞外質(extracellular matrix, ECM)的成份及功能, 扮演進行胚胎著床時重要的決定因素. 而子宮內膜異位症(endometriosis)是由於子宮內膜組織因不明原因附著於不適當的位置, 導致胚胎著床不易, 增加流產的機率,造成不孕. 全世界有 3%-5%的婦女患有子宮內膜異位, 在台灣, 平均每四位上不孕症專科求診的婦女病患即有一位患有子宮內膜異位症. 病變的子宮內膜組織具類似惡性腫瘤的性質, 可轉移, 滲透, 侵犯及附著於周邊的器官及組織. 這些作用必須透過破壞包覆於細胞周圍的細胞外質(ECM)及重組(remodeling). 僅管細胞外質的破壞及重組已被認為是胚胎著床及癌腫瘤進行轉移的必要過程, 然而, 對於調節細胞外質重組的酵素-金屬蛋白質酵素(Matrix metalloprotease, MMP)和其抑制劑(TIMPs)於子宮內膜異位症患者體內異常之表現, 及其調節機轉及訊息傳遞之機制並不清楚. 因此, 本研究著重在觀察細胞外質金屬蛋白酶之表現與所扮演之調節角色作系統性及全面性的分析, 並評估其用於疾病診斷標記之可行性. 本研究選用初期子宮內膜異位症患者及正常生育年齡之婦女自願捐贈之血液及腹腔液為研究對象,發現其中游離型 TIMP-1 之表現在未經治療之子宮內膜異位症患者血液及腹腔液以西方墨點法被偵測到(72%), 但經 GnRH analog 治療後之患者(21%)及正常受試者則無法被偵測到. ELISA 分析顯示 TIMP-1 之總濃度在各組間並無明顯差異推測此現象可能與 TIMP-1 無法與 MMP 做結合以抑制 MMP 的活性或 MMP 的表現量減少有關.ELISA 結果顯示 MMP9 濃度在未經治療之子宮內膜異位症患者較已經治療之患者為低($p < 0.05$)低. 本研究發現血清中 TIMP-1 可作為早期子宮內膜異位的診斷標計(markers)之一.

關鍵詞：細胞外質(extracellular matrix, ECM), 細胞外質金屬蛋白酶 (matrix metalloproteinase, MMP),子宮內膜異位症(endometriosis)

ABSTRACT

Objectives: To investigate the expression of matrix metalloproteinases (MMPs) and their inhibitors, tissue inhibitors of matrix metalloproteinases (TIMPs) in serum in women with endometriosis.

Design: Molecular studies in serum.

Setting: Sera were collected at the department of obstetrics and gynecology in Taipei Medical University Hospital. The experiments were carried out in the Graduate Institute of Biomedical Materials in Taipei Medical University.

Patients: Sera were collected from women with endometriosis (stage 1 and 2) without receiving GnRHa treatment, with GnRHa treatment, pregnent and non-pregnant women.

Intervention(s): None.

Main outcome measure(s): Western blot analysis and enzyme linked immunoadsorbent assay.

Results: Total 102 serum samples were investigated for the expression of TIMP-1 by western blot analysis. Up to 72% of women (23/32) with endometriosis (stage 1 and 2) were detected positively for TIMP-1 in serum, whereas 28% (8/29) in endometriosis patients after GnRHa treatment, and none of pregnant and infertile women were detected the presence of TIMP-1. ELISA showed that no significant difference in total concentrations of TIMP-1 between the patients with and without receiving GnRHa treatment. However, the level of MMP9 was significantly decreased in patients without GnRHa treatment.

Conclusions: TIMP-1 could be a useful serum marker for early diagnosis of endometriosis.

Key words:

ECM, extracellular matrix; TIMP, tissue inhibitors for metalloproteinase; MMP, matrix metalloproteinase; endometriosis.

Introduction

The etiology of endometriosis is not well understood. It is observed that the diseased endometrium behaves like the tumorous tissues that are capable of migration, invasion, adhesion, and growth out of the uterine. Around the world, about 5% of women with reproductive age are suffered from endometriosis. Large numbers infertile women diagnosed endometriosis, suggesting that endometriosis is the major reason causes infertility [1]. The aberrant adhered endometrium may interfere the embryonic implantation that may cause infertility. Administration of gonadotropin-releasing hormone analog (GnRHa) to regulate the hormone secretion during the cycles has been widely used in the treatment of endometriosis for many years. Laparoscopic surgery by removing the adhered endometrium is also a common treatment for endometriosis. However, the recurrence, the side effect of hormone therapy, and increased difficulty during operation in severe adhesion between the organs are the problems. Therefore, early diagnosis and treatment are critical to avoid the severe endometriosis caused infertility. Yet, no specific markers are identified for diagnosis of endometriosis.

This study aimed to monitor the levels of MMPs and TIMPs in serum and compare their differences between the patients with endometriosis and normal controls. Additionally, to investigate the effect of GnRH analog treatment on MMP/TIMP expression in serum in endometriosis patients. Identification of serum marker with high specificity may facilitate the development of convenient and non-invasive method for diagnosis and prognosis of endometriosis.

Background

The extracellular matrix (ECM) remodeling is relevant to the progression of endometriosis [2]. ECM including collagens, structural glycoproteins such as fibronectin, fibrinogen, and laminin, and proteoglycans, which often associate to each other and constitute a well-organized network structure surrounding the cells, protects organs and maintains the body shape. The adhesion of endometrial cells to the ECM was expected to play a central role in the pathogenesis of endometriosis [3]. ECM turnover is involved in the regulation of cell behavior. Matrix metalloproteinases (MMPs) and their inhibitors, tissue inhibitors for MMPs (TIMPs) play crucial roles in regulation of ECM turnover. TIMPs inhibit the activity of MMPs by binding to the active MMPs at the Zn chelating active site. TIMP-1 and TIMP-2 can inhibit most of MMPs except membrane type 1-MMP (MT1-MMP), which can not be inhibited by TIMP-1. Additionally, TIMP-1 and TIMP-2 can complex to proMMP9 and proMMP2, respectively [4]. The inappropriate expression of MMPs and TIMPs is associated with numerous disease states that involve invasive processes such as tumor growth and regression. The MMPs have recently been implicated involved in the development of endometriosis [5, 6]. The regulatory mechanism of balancing between MMPs and TIMPs is complex. Hormones and cytokines may regulate the MMP's activities [7-9]. The reported levels of MMPs and TIMPs seemed somewhat controversial depended on the measurement method and the collection of specimen from different tissues and different stages of endometriosis. MMPs appear to be over-expressed in endometriotic lesions, but expression levels are decreased following GnRH agonist therapy by analyses of peritoneal fluid in a rat model [10]. Greater expression of MMP-2 and MT1-MMP and lower expression of TIMP-2 were found in eutopic endometrium with endometriosis [11]; Both uterine endometrium

and ectopic endometriotic tissue from women with endometriosis expressed significantly lower levels of TIMP-3 than endometrium from normal women [12], suggesting that increased proteolytic activity may be one of the reasons for the invasive properties of the endometrium during the development of endometriosis. Another report showed that only MMP-3 mRNA levels were elevated in endometrial tissues collected from the intact uterine and from under the skin, whereas MMP-2 and TIMP-1 were ubiquitously expressed at all times in both euterine and ectopic tissues in a rat endometriosis model [13]. Gilabert-Estelles, J. *et al.* reported that the increased MMP-3 in endometrium of women with endometriosis might contribute to the invasive potential of endometrial cells. Once the ectopic ovarian endometriotic cyst was developed, an increase in PAI-1 and TIMP-1 was detected and significant proteolytic activity was no longer observed [14]. In an *in vitro* cell culture system, high MMP3, moderated MMP1 and 2, low MMP9, and more TIMP-1 than TIMP-2 were detected in the cultured medium in uterine endometrial cells from endometriosis patients [15].

Material and Methods

Blood collections

Bloods were collected at the obstetrics and gynecology clinic in Taipei Medical University Hospital. The collected samples were classified into four groups, women with endometriosis untreated and treated with GnRHa, infertile women with the reasons other than endometriosis, and women with normal pregnancy. The procedure of sample collection was approved by the institutional committee on the use of human subjects in research at Taipei Medical University. Each blood donator who volunteered to join the research was given a short interview and understood well that this donation was used only for the purpose of research.

Serum preparation

The serum is prepared from 5 ml blood collected from the patient. The blood samples were stand for coagulation at room temperature. The blood cells were then removed by centrifugation at 3000 rpm for 20 min. Serum albumin was depleted by running through a cibarcon blue column (Amersham Bioscience Ltd., Sweden). The total protein concentration is measured based on the Bradford method [16]. The sera were then kept at -20°C for long-term storage.

Antibodies

MMP antibody panel starter kit including polyclonol antibodies to MMP-1, MMP-2, MMP-3 and another MMP-9/TIMP starter kit including polyclonol antibodies to MMP-9, TIMP-1, TIMP-2, and in additional, rabbit polyclonol antibodies raised against human MMP9 and TIMP-1 respectively were all purchased from Chemicon International, Inc. (Temecula, CA, USA) for western blot analyses.

Western blot

Fifteen microgram of serum samples collected from the patients and prestained molecular weight markers (Amersham Bioscience Ltd., UK) were prepared in non-reducing condition without boiling the samples before loading and separated on a 12% sodium

dodecylsulfate-polyacrylamide gel using the mini-gel electrophoresis system (Hoffer, USA). Subsequently, the gel was transferred to a PVDF membrane (Millipore, USA). The blot was then blocked with 5% skim milk in TBS (20 mMTris, 500mM NaCl, pH8.0) and probed with the desired primary antibody. After extensively washes with TBST (TBS with 0.1% tween-20), Secondary antibody conjugated with alkaline phosphatase was added. Followed by extensively washes with TBST, the blot was soaked in the BCIP/NBT liquid substrate system (Sigma, USA) for colorimetric development resulting the purple bands of the protein. The developed membrane was then rinsed with deionized water, air dried, and stored the image.

Measurement of total TIMP-1 and MMP1, 2 3, 8, and 9 concentration in serum by enzyme linked immunoadsorbent assay (ELISA)

Total concentration of TIMP-1 as well as the activity of various MMPs were measured by using the Biotrak ELISA system (Amersham Pharmacia Biotech, UK). The experiment protocols were followed based on the description provided by the manufacture. Briefly, to measure the total concentration of TIMP-1, adding the serum samples in the 96-microtiter wells which have been pre-coated with antibodies specifically recognize TIMP-1. After proper incubation, washed off the unbound materials and subsequently added the labeled TIMP-1 antibody with alkaline phosphatase. After extensively washes, the addition of substrate containing chromogen that reacts with the enzyme can be monitored at the absorbance 450 nm. The concentration of TIMP-1 can then be calculated by interpolation from a standard curve. To determine the concentration of MMPs, both endogenous levels of free active MMP and pro MMP that has been activated were measured by interaction with the added modified pro detection enzyme and the chromogenic peptide substrate. The concentration of active MMP was determined by measuring the resultant colour read at 405 nm and interpolation from a standard curve.

Statistical analysis

The data was plotted using Kaleidagraph (Synergy Software, Reading, PA, USA). Statistical analysis was performed by Student's two-tailed *t*-test. A p value of less than 0.05 was considered significant.

Results

Screening the expressions of MMPs/TIMPs in serum from patients with Endometriosis before and after GnRHa therapy.

Firstly, western blot analysis was carried out to monitor the expression of various MMP/TIMPs. The antibodies raised against to human MMP1, MMP2, MMP3, MMP8, MMP9 and their inhibitors TIMP-1 and TIMP-2 were used for screening in the analyses. The results indicated that TIMP-1 was detected positively in sera from patients with endometriosis before GnRHa treatment (Figure 1A, lane5). In contrast, the signal of TIMP-1 was disappeared in serum from patients with endometriosis after GnRHa treatment (Figure 1B, lane 5) and in the controls. Additionally, there were no significant difference or at non-detectable level in the blots probing with other MMPs and TIMPs between the groups (Figure 1A & B). Subsequently, the expression of TIMP-1 in serum was further investigated in larger case numbers.

TIMP-1 was detected positively in serum from the patients with Endometriosis before GnRHa therapy.

Figure 2 showed that by western blot analyses, most of the serum samples from women with endometriosis at early stages were TIMP-1 positive, whereas sera from normal and women with endometriosis after receiving GnRHa treatment were TIMP-1 negative. Additionally, a higher molecular weight band at 120-140 kDa was found especially in patients with endometriosis after receiving GnRHa treatment and the normal controls (Figure 2, lane 10-15) implying that a complex form of TIMP-1 with proMMP9 may exist. It was confirmed by probing the blot with antibody raised against MMP9 (data not shown). In this study, total 102 serum samples including 32 women with endometriosis (stage 1 and 2) without receiving GnRHa treatment, 29 women diagnosed endometriosis with GnRHa treatment, 19 pregnant women, 22 non-pregnant including 7 infertile women without endometriosis were investigated for TIMP-1 expression by western blot analyses and the results were summarized in Table 1. A 30 kDa band corresponding to the free form TIMP-1 appeared in 23 serum samples from women with endometriosis not receiving GnRHa treatment (23/32, 71.9%), whereas only 4 samples (4/19, 21%) from women with endometriosis receiving GnRHa treatment was detected positively for TIMP-1. The free form of TIMP-1 in the other serum samples from pregnant and infertile women was at non-detectable level. The results suggest that free form of TIMP-1 was extensively found in serum from patients with endometriosis at early stages (stage I & II). Further analyses found that, the nine TIMP-1 negative sera from patients with endometriosis without receiving GnRHa treatment were all the recurrent cases who have diagnosed endometriosis at lease one year ago and ever received treatment in the other hospital.

To further understand the evidence of increased free form TIMP-1 in serum of endometriosis patient, ELISA analysis was employed to measure the total concentrations of TIMP-1 as well as varies MMPs in serum. The results indicated that no significant difference in the total concentration of TIMP-1 in serum between the examed groups (Figure 3). Additionally, the concentrations of MMP1, 2, 3, 8, and 9 were measured respectively as well. The results showed that the concentration of MMP9 in serum from patient with endometriosis before GnRHa treatment was significantly lower compared to the patients have received GnRHa treatment (Figure 4), while no significant differences in concentration between the tested groups for the other MMPs (data not shown). It implicated that MMPs may be uneffectively inhibited as shown that most of TIMP-1 remains to present as free form as we observed in serum in endometriosis patients at early stages of endometriosis.

Discussions

In this study, we reported that the expression of free form TIMP-1 was distinct in serum in women with endometriosis at early stages. Similar results were reported by measuring the concentrations of MMPs and TIMPs in uterine endometrial cells of endometriosis patients by ELISA indicating that more TIMP-1 was secreted by the uterine endometriotic cells [5]. Another study observed that once the ovarian endometriotic cyst was developed, increased TIMP-1 was detected, which resulted in decreased proteolytic activity [14]. However, controversial finding also reported decreased TIMP-1 secretion and increased MMP activity were measured at localized endometrium tissues or in peritoneal fluid and their detection method was different [17-19].

In this study, we reported that increased free form TIMP-1 was found in patients with endometriosis and total concentration of TIMP-1 was not different in between the patients and normal controls, suggesting that less complex of TIMP-1 with MMP was formed, which may result in impaired inhibitory activity for proteolysis at early stages during endometriosis development. Additionally, this study found that cases detected positively for free form TIMP-1 in serum from patients with endometriosis did not include the recurrent cases who have endometriosis and had ever received treatment before. The regulatory mechanism of balancing MMPs and TIMPs is complex. Further studies are required to dissect the mechanism of resulting in the presence of free form TIMP-1 in patients with endometriosis at early stage.

Serum detection is a convenient and non-invasive approach for disease diagnosis. This study provides important information that presence of free form TIMP-1 in serum could be a useful marker for early diagnosis of endometriosis.

References

1. SURREY ES, HALME J. Endometriosis as a cause of infertility. Obstet Gynecol Clin North Am 1989;16:79-91.
2. SILLEM M, PRIFTI S, NEHER M, RUNNEBAUM B. Extracellular matrix remodelling in the endometrium and its possible relevance to the pathogenesis of endometriosis. Hum Reprod Update 1998;4:730-5.
3. SPUIJBROEK MD, DUNSELMAN GA, MENHEERE PP, EVERS JL. Early endometriosis invades the extracellular matrix. Fertil Steril 1992;58:929-33.
4. GOMEZ DE, ALONSO DF, YOSHIJI H, THORGEIRSSON UP. Tissue inhibitors of metalloproteinases: structure, regulation and biological functions. Eur J Cell Biol 1997;74:111-22.
5. SILLEM M, PRIFTI S, KOCH A, NEHER M, JAUCKUS J, RUNNEBAUM B. Regulation of matrix metalloproteinases and their inhibitors in uterine endometrial cells of patients with and without endometriosis. Eur J Obstet Gynecol Reprod Biol 2001;95:167-74.
6. CHUNG HW, WEN Y, CHUN SH, NEZHAT C, WOO BH, LAKE POLAN M. Matrix metalloproteinase-9 and tissue inhibitor of metalloproteinase-3 mRNA expression in ectopic and eutopic endometrium in women with endometriosis: a rationale for endometriotic invasiveness. Fertil Steril 2001;75:152-9.
7. OSTEEN KG, BRUNER KL, SHARPE-TIMMS KL. Steroid and growth factor regulation of matrix metalloproteinase expression and endometriosis. Semin Reprod Endocrinol 1996;14:247-55.
8. BRUNER KL, EISENBERG E, GORSTEIN F, OSTEEN KG. Progesterone and transforming growth factor-beta coordinately regulate suppression of endometrial matrix metalloproteinases in a model of experimental endometriosis. Steroids 1999;64:648-53.
9. BRUNER-TRAN KL, EISENBERG E, YEAMAN GR, ANDERSON TA, MCBEAN J, OSTEEN KG. Steroid and cytokine regulation of matrix metalloproteinase expression in endometriosis and the establishment of experimental endometriosis in nude mice. J Clin Endocrinol Metab 2002;87:4782-91.
10. SHARPE-TIMMS KL, ZIMMER RL, JOLLIFF WJ, WRIGHT JA, NOTHNICK WB, CURRY TE. Gonadotropin-releasing hormone agonist (GnRH-a) therapy alters activity of plasminogen

activators, matrix metalloproteinases, and their inhibitors in rat models for adhesion formation and endometriosis: potential GnRH-a-regulated mechanisms reducing adhesion formation. Fertil Steril 1998;69:916-23.

11. CHUNG HW, LEE JY, MOON HS, et al. Matrix metalloproteinase-2, membranous type 1 matrix metalloproteinase, and tissue inhibitor of metalloproteinase-2 expression in ectopic and eutopic endometrium. Fertil Steril 2002;78:787-95.
12. CHUNG HW, WEN Y, CHUN SH, NEZHAT C, WOO BH, LAKE POLAN M. Matrix metalloproteinase-9 and tissue inhibitor of metalloproteinase-3 mRNA expression in ectopic and eutopic endometrium in women with endometriosis: a rationale for endometriotic invasiveness. Fertil Steril 2001;75:152-9.
13. COX KE, PIVA M, SHARPE-TIMMS KL. Differential regulation of matrix metalloproteinase-3 gene expression in endometriotic lesions compared with endometrium. Biol Reprod 2001;65:1297-303.
14. GILABERT-ESTELLES J, ESTELLES A, GILABERT J, et al. Expression of several components of the plasminogen activator and matrix metalloproteinase systems in endometriosis. Hum Reprod 2003;18:1516-22.
15. SILLEM M, PRIFTI S, KOCH A, NEHER M, JAUCKUS J, RUNNEBAUM B. Regulation of matrix metalloproteinases and their inhibitors in uterine endometrial cells of patients with and without endometriosis. Eur J Obstet Gynecol Reprod Biol 2001;95:167-74.
16. BRADFORD MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 1976;72:248-54.
17. GOTTSCHALK C, MALBERG K, ARNDT M, et al. Matrix metalloproteinases and TACE play a role in the pathogenesis of endometriosis. Adv Exp Med Biol 2000;477:483-6.
18. SZAMATOWICZ J, LAUDANSKI P, TOMASZEWSKA I. Matrix metalloproteinase-9 and tissue inhibitor of matrix metalloproteinase-1: a possible role in the pathogenesis of endometriosis. Hum Reprod 2002;17:284-8.
19. SHARPE-TIMMS KL, KEISLER LW, MCINTUSH EW, KEISLER DH. Tissue inhibitor of metalloproteinase-1 concentrations are attenuated in peritoneal fluid and sera of women with endometriosis and restored in sera by gonadotropin-releasing hormone agonist therapy. Fertil Steril 1998;69:1128-34.

Table 1. Summary of western blot analyses for the rate of tested positively for TIMP-1 in serum from the patients with endometriosis with and without receiving GnRHa treatment.

Patient	TIMP-1 (numbers of positive /total numbers of sample)	Percentage of positive detection (%)
Non-Pregnant	0/15	0
Pregnant	0/19	0
Endo-GnRHa	23/32	71.9
Endo+GnRHa	8/29	27.6

Figure legends

Figure 1 Western blot analysis of MMPs/TIMPs in serum from patients with endometriosis (A) without GnRHa treatment (B) with GnRHa treatment. Serum sample from the patient, 15 ug for each lane was loaded and separated on a 12% acrylamide gel and transferred to a PVDF membrane. After blocking, the membrane was probed respectively with a panel of antibodies against to MMP or TIMP, lane M, molecular weight marker, lane 1, anti-MMP1 antibody, lane2, anti-MMP2 antibody, lane 3, anti-MMP3 antibody, lane 4, anti-MMP9 antibody, lane 5, anti-TIMP-1 antibody, lane 6, anti-TIMP-2 antibody, lane 7, purified human TIMP-1 probed with anti TIMP-1 antibody as positive control. The figure represents one of three independent experiments with similar results.

Figure 2. Detection of the presence of TIMP-1 in serum in the patients with endometriosis by western blot analysis. Lane |M, protein molecular weight marker, lane 1, purified human TIMP-1 as positive control, lane2-9, eight serum samples from the patient with endometriosis without GnRHa treatment, lane 10-12, three serum samples from the patient with endometriosis and have received GnRHa treatment, lane 13-15 normal serum controls. All eight patients' serum samples were detected positively for TIMP-1 expression.

Figure 3. Measurement of total concentration of TIMP-1 in serum by ELISA.

Figure 4. Measurement of total concentration of MMP9 in serum by ELISA.

Figure 1

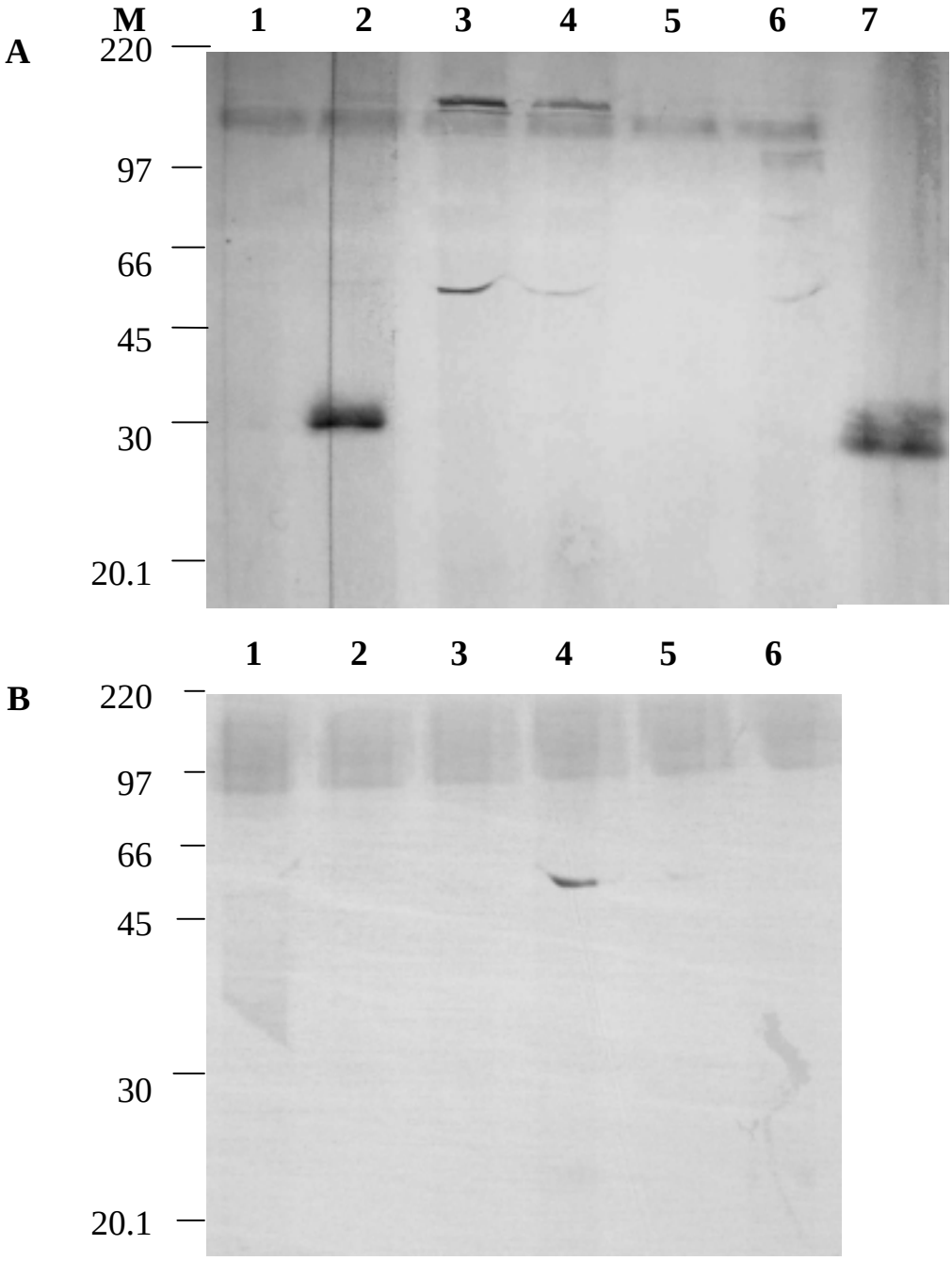


Figure 2

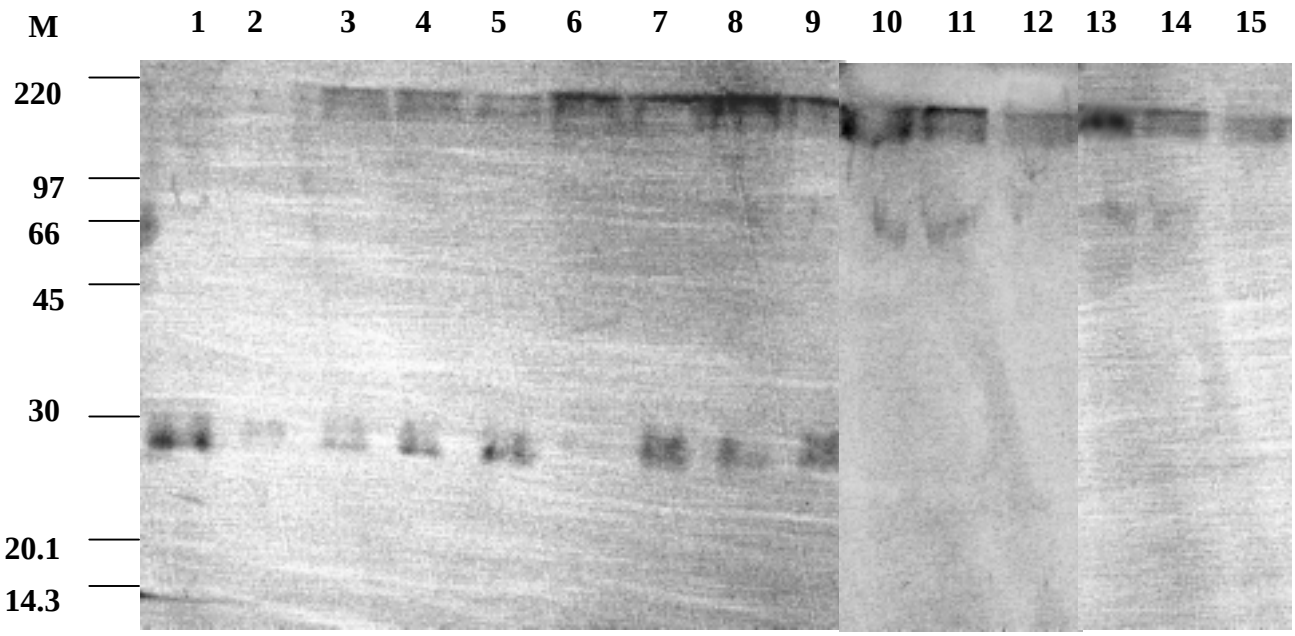
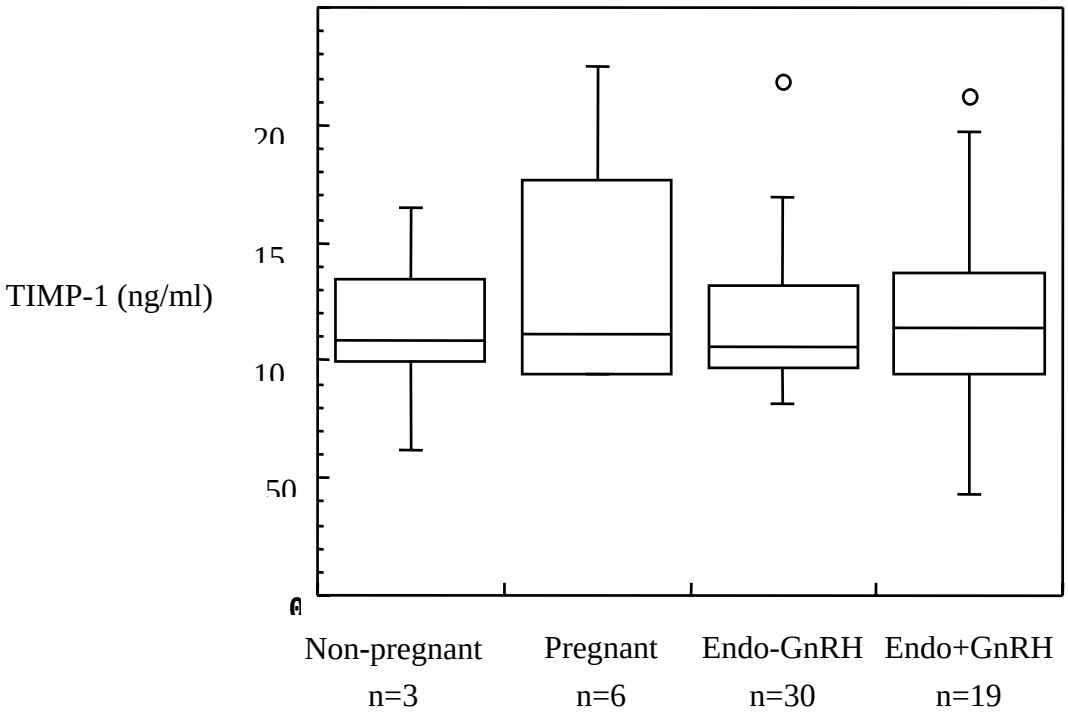
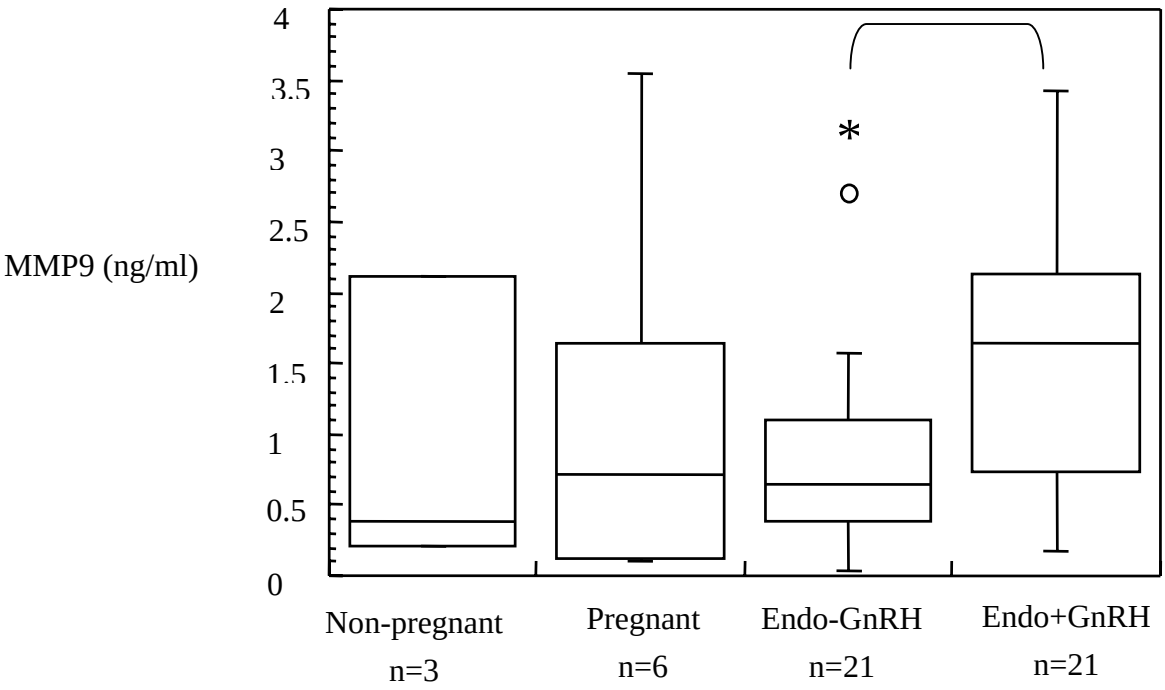


Figure 3



n, number of samples

Figure 4



* P<0.005, n, number of samples