行政院國家科學委員會專題研究計畫 成果報告

纖維蛋白溶解系統在骨盆腔炎症的表現及其機制探討(第 3 年)
研究成果報告(完整版)

計 畫 類 別 ：個別型
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計 畫 主 持 人：蔡秀婷
共 同 主 持 人：楊順發、王博輝

報 告 附 件 ：出席國際會議研究心得報告及發表論文

公 開 資 訊 ：本計畫涉及專利或其他智慧財產權，2 年後可公開查詢

中 華 民 國 101 年 10 月 25 日

中文摘要：骨盆腔炎症包括了子宮內膜炎、骨盆腔腹膜炎、輸卵管卵巢囊腫、輸卵管炎、輸卵管卵巢炎等，在定義上泛指女性的急性骨盆腔逆行性感染。在現今社會中不孕症夫婦的比例有逐年增加的趨勢，而骨盆腔炎症就是造成女性不孕症的最主要原因。骨盆腔炎症的症狀與許多骨盆相關疾病相似，所以很難經由一般的實驗室檢查來診斷，因此針對骨盆腔炎症較具專一性、特異性的檢查在臨床診斷上就顯得很重要。纖維蛋白溶解系統(plasminogen activator/ plasmin system)是哺乳動物血液中用來限制過度纖維蛋白(fibrin)形成及分解血塊的酵素系統，另和基底膜的降解、組織纖維化及組織重組非常有關係。而此系統亦參與組織修補、血管新生、巨噬細胞活化及腫瘤轉移等其它生理功能，並在這些過程中擔任蛋白水解的工作。然而，近年來亦有越來越多的研究發現纖維蛋白溶解系統與不同的發炎反應有密切的相關性。因此，本計畫擬探討纖維蛋白溶解系統在骨盆腔炎症的相關性表現。我們收集 70 位正常人及 64 位骨盆腔炎症患者的血液，萃取其 genomic DNA，利用 PCR-RFLP 及直接定序的方式，探討正常人及骨盆腔炎症患者的 u-PA、u-PAR、及 PAI-1 不同位點的基因多型性。並進一步以探討正常人及骨盆腔炎症患者的差異性，並進一步以病患同意授權之資料，對骨盆腔炎症的發生原因、細菌感染與否及一些臨床診斷如 WBC 數目、Neutrophils 數目及 CRP 數值等做進一步的分析。並進一步探討這些酵素的表現量與其預後的相關性。研究結果顯示，u-PA 及 u-PAR 的蛋白表現(Mean±SE)有顯著性差異(u-PA 值：健康的對照組: 0.55±0.06 ng/mL，骨盆腔炎症患者: 0.57±0.03 ng/mL，p=0.002；u-PAR 值：健康的對照組: 1192.46±51.98 pg/mL，骨盆腔炎症患者: 1372.04±68.20 pg/mL，p=0.04)，骨盆腔炎症患者，治療前、治療後血漿中 u-PAR 的蛋白表現(Mean±SE)亦有顯著差異(治療前: 1372.04±68.20 pg/mL，治療後:1220.06±58.14 pg/mL，p=0.03)。而血漿中 PAI-1 的蛋白表現在正常人與骨盆腔炎症患者，以及骨盆腔炎症患者治療前與治療後之間則無顯著的差異。u-PA、u-PAR、及 PAI-1 的基因多型性不會增加婦女罹患骨盆腔炎症的易感性，亦不會影響正常人或骨盆腔炎症患者血漿中 u-PA、u-PAR、及 PAI-1 蛋白的表現量。結論: 血漿中 u-PA 及 u-PAR 的蛋白表現量可作為預測婦女是否罹患骨盆腔炎症的指標，以及骨盆腔炎症標靶治療的臨床應用。

中文關鍵詞： 纖維蛋白溶解系統、骨盆腔炎症、基因多型性

英文摘要： Objective: To determine expression levels of urokinase-type plasminogen activator (uPA), soluble urokinase-type plasminogen activator receptor (suPAR), plasminogen activator inhibitor-1 (PAI-1) in plasma and to identify gene polymorphisms specific to patients with pelvic inflammatory disease (PID) and healthy controls.
Methods: Enzyme-linked immunosorbent assay and polymerase chain reaction-restriction fragment length polymorphism were used to measure plasma levels and polymorphisms in uPA, suPAR, and PAI-1 among seventy healthy controls and sixty-four PID patients before and after they received routine treatment protocols.
Results: The levels of plasma uPA (ng/ml) and soluble suPAR (pg/ml) were significantly elevated in PID patients (uPA: 0.57±0.03； suPAR: 1372.04±68.20) when compared to that in normal controls (uPA: 0.55±0.06, p=0.002； suPAR: 1192.46±51.98, p=0.04)； moreover, suPAR decreased significantly after treatment when compared to levels noted in same patients (1220.06±58.14； p=0.003) after they received treatment. The increased expression of suPAR was significantly correlated with WBC counts (r=0.382, p=0.002, n=64) in blood as well as the plasma levels of CRP (r=0.441, p<0.0001, n=64) and uPA (r=0.426, p<0.0001, n=64) of PID patients prior to receiving

treatment.
Conclusions: Elevated plasma suPAR could be a biological marker for the diagnosis of PID and may reflect a new focus in targeted therapy for pelvic inflammatory disease.

英文關鍵詞： urokinase-type plasminogen activator (uPA), soluble urokinase-type plasminogen activator receptor (suPAR), plasminogen activator inhibitor-1 (PAI-1), single nucleotide polymorphism (SNP), and pelvic inflammatory disease (PID)

行政院國家科學委員會補助專題研究計畫期中報告

（計畫名稱）

中文：纖維蛋白溶解系統在骨盆腔炎症的表現及其機制探討

英文： The mechanism and expression of Plasminogen activator/ plasmin system in patients with pelvic inflammatory disease

計畫類別：■ 個別型計畫　□ 整合型計畫

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共同主持人：王博輝
計畫參與人員：楊順發

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

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：中山醫學大學 護理系

中　華　民　國　101　年　10　月　25 日

目　　錄

Significantly increased concentration of soluble urokinase-type plasminogen activator receptor in the blood of patients with pelvic inflammatory disease

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No conflicts of interest.

Keywords: urokinase-type plasminogen activator (uPA), soluble urokinase-type plasminogen activator receptor (suPAR), plasminogen activator inhibitor-1 (PAI-1), single nucleotide polymorphism (SNP), and pelvic inflammatory disease (PID)

中文摘要:

骨盆腔炎症包括了子宮內膜炎、骨盆腔腹膜炎、輸卵管卵巢囊腫、輸卵管炎、輸卵管卵巢炎等，在定義上泛指女性的急性骨盆腔逆行性感染。在現今社會中不孕症夫婦的比例有逐年增加的趨勢，而骨盆腔炎症就是造成女性不孕症的最主要原因。骨盆腔炎症的症狀與許多骨盆相關疾病相似，所以很難經由一般的實驗室檢查來診斷，因此針對骨盆腔炎症較具專一性、特異性的檢查在臨床診斷上就顯得很重要。纖維蛋白溶解系統(plasminogen activator/ plasmin system)是哺乳動物血液中用來限制過度纖維蛋白(fibrin)形成及分解血塊的酵素系統，另和基底膜的降解、組織纖維化及組織重組非常有關係。而此系統亦參與組織修補、血管新生、巨噬細胞活化及腫瘤轉移等其它生理功能，並在這些過程中擔任蛋白水解的工作。然而，近年來亦有越來越多的研究發現纖維蛋白溶解系統與不同的發炎反應有密切的相關性。因此，本計畫擬探討纖維蛋白溶解系統在骨盆腔炎症的相關性表現。我們收集70位正常人及64位骨盆腔炎症患者的血液，萃取其genomic DNA，利用PCR-RFLP及直接定序的方式，探討正常人及骨盆腔炎症患者的u-PA、u-PAR、及PAI-1不同位點的基因多型性。並進一步以探討正常人及骨盆腔炎症患者的差異性，並進一步以病患同意授權之資料，對骨盆腔炎症的發生原因、細菌感染與否及一些臨床診斷如WBC數目、Neutrophils數目及CRP數值等做進一步的分析。並進一步探討這些酵素的表現量與其預後的相關性。研究結果顯示，u-PA及u-PAR的蛋白表現(Mean±SE)有顯著性差異(u-PA值：健康的對照組: 0.55±0.06 ng/mL，骨盆腔炎症患者: 0.57±0.03 ng/mL，p=0.002；u-PAR值：健康的對照組: 1192.46±51.98 pg/mL，骨盆腔炎症患者: 1372.04±68.20 pg/mL，p=0.04)，骨盆腔炎症患者，治療前、治療後血漿中u-PAR的蛋白表現(Mean±SE)亦有顯著差異(治療前: 1372.04±68.20 pg/mL，治療後:1220.06±58.14 pg/mL，p=0.03)。而血漿中PAI-1的蛋白表現在正常人與骨盆腔炎症患者，以及骨盆腔炎症患者治療前與治療後之間則無顯著的差異。u-PA、u-PAR、及PAI-1的基因多型性不會增加婦女罹患骨盆腔炎症的易感性，亦不會影響正常人或骨盆腔炎症患者血漿中u-PA、u-PAR、及PAI-1蛋白的表現量。結論: 血漿中u-PA及u-PAR的蛋白

表現量可作為預測婦女是否罹患骨盆腔炎症的指標，以及骨盆腔炎症標靶治療的臨床應用。

Abstract

Objective: To determine expression levels of urokinase-type plasminogen activator (uPA), soluble urokinase-type plasminogen activator receptor (suPAR), plasminogen activator inhibitor-1 (PAI-1) in plasma and to identify gene polymorphisms specific to patients with pelvic inflammatory disease (PID) and healthy controls.

Methods: Enzyme-linked immunosorbent assay and polymerase chain reaction-restriction fragment length polymorphism were used to measure plasma levels and polymorphisms in uPA, suPAR, and PAI-1 among seventy healthy controls and sixty-four PID patients before and after they received routine treatment protocols.

Results: The levels of plasma uPA (ng/ml) and soluble suPAR (pg/ml) were significantly elevated in PID patients (uPA: 0.57±0.03; suPAR: 1372.04±68.20) when compared to that in normal controls (uPA: 0.55±0.06, p=0.002; suPAR: 1192.46±51.98, p=0.04); moreover, suPAR decreased significantly after treatment when compared to levels noted in same patients (1220.06±58.14; p=0.003) after they received treatment. The increased expression of suPAR was significantly correlated with WBC counts (r=0.382, p=0.002, n=64) in blood as well as the plasma levels of CRP (r=0.441, p<0.0001, n=64) and uPA (r=0.426, p<0.0001, n=64) of PID patients prior to receiving treatment.

Conclusions: Elevated plasma suPAR could be a biological marker for the diagnosis of PID and may reflect a new focus in targeted therapy for pelvic inflammatory disease.

Introduction

Pelvic inflammatory disease (PID) is currently the most common inflammatory disease worldwide [1-3] and is associated with serious long term consequences, such as infertility, chronic pelvic pain, and ectopic pregnancy [4-6]. The plasminogen activation system has been reported to play an important role in mediating fibrin degradation in the inflammatory process [7-9]). Plasminogen activator (PA) is a specific serine protease that binds to a cellular receptor and then converts the inactive protein plasminogen to the active enzyme plasmin [10, 11]. Two such plasminogen activators that are noted to be important in the inflammatory process include urokinase (uPA) and tissue type (tPA). Urokinase plasminogen activator and its receptor (uPAR) are thought to regulate pericellular matrix degradation, cell migration and cell adhesion during the inflammatory process [12, 13, 14]. Plasminogen activator activity in bovine endometrial tissues is significantly increased among cows with severe endometritis [15]. Urokinase-type plasminogen activator receptor (uPAR) has been reported to play an important role in the defense against pathogens [13, 14, 16]. It has been demonstrated that uPAR may become cleaved at its cell surface anchor to form a free soluble receptor (suPAR) in blood, and plasma suPAR concentrations have been noted to be elevated among patients with rheumatoid arthritis [12] and women with preeclampsia [17]. Plasminogen activator inhibitors (PAIs) contribute to the

down regulation of plasminogen activators and are present in body fluids and tissues [9, 18]. Up-regulation of uPA and PAI-1 have been found in the peritoneal fluid of women suffering from PID when compared with normal controls [18, 19]. The increased concentration of uPA was thought to stimulate fibrinolysis and prevent adhesion formation; however, an increase in PAI-1 likely reflects an underlying response that reduces fibrinolysis during the inflammatory process [18]. However, using laparoscopy to obtain peritoneal fluid is risky and inappropriate in most cases. Therefore, it is highly recommended that the significance of non-invasive strategies on the diagnosis and therapy of pelvic inflammatory disease be explored.

Single nucleotide polymorphisms (SNPs) in uPA system genes, such as uPA (rs4065) C/T SNP, uPAR (rs344781) T/C SNP, and PAI-1 (rs1799889) 4G/5G SNP, which are located within the promoter or other regulatory regions, has been suggested to affect gene expression [20-23]. Recently epidemiological studies have found that these gene polymorphisms may increase susceptibility to inflammation-related disease processes, including sepsis [22], atherosclerosis [23], rheumatoid arthritis [12] and acute pancreatitis [24]; however, their potential association with pelvic inflammatory disease has not been investigated. We hypothesized that gene polymorphisms of the *uPA* system could be associated with the development of pelvic inflammatory disease. Therefore, we determined the relationship between plasma protein concentrations, of

uPA, suPAR, and PAI-1 and pelvic inflammatory disease. Furthermore, we evaluated the distributions of uPA, uPAR, and PAI-1 gene polymorphisms among PID patients and normal controls and assessed whether protein expression levels of these genes were altered among different genetic polymorphisms to estimate the impact of these polymorphisms on expression levels.

Materials and Methods

Subjects and specimen collection

This was a hospital-based case-control study. We worked with gynecologists at Chung Shan Medical University Hospital in Taichung, Taiwan. Between April 2006 and August 2011, sixty-four women who were diagnosed with PID by gynecologists (Wang PH, Tee YT, and Lin LY) based on the criteria of the national guidelines for pelvic inflammatory disease [25, 26] were recruited as a case group. Seventy healthy women who visited the Department of Obstetrics and Gynecology or the Department of Family Medicine for a health examination, e.g., cervical Papanicolaou smear and breast examination, were randomly selected as healthy controls. Healthy controls were matched with respect to demographic and clinical data, i.e., age (matched to within five years), race, ethnicity, socioeconomic status, resident area, cigarette

smoking status, and alcohol drinking status. Healthy women with risk factors associated with PID were excluded from the control group. The diagnosis of PID conformed to the minimal criteria determined by the Centers for Disease Control and Prevention (CDC) including lower abdominal pain or pelvic pain of no other origin with one of the following criteria: uterine tenderness or adnexal tenderness or cervical motion tenderness. To maximize specificity and reduce the chance of a delayed or missed diagnosis, in addition to the criteria mentioned above, the patients were required to have at least one of the following minor criteria: oral temperature > 38.3 degrees Celsius, abnormal vaginal or cervical mucopurulent discharge, an abundance of white blood cells (WBCs) on microscopic inspection of vaginal secretions, elevated C-reactive protein (CRP), elevated erythrocyte sedimentations, or laboratory documentation of Neisseria gonorrhea or Chlamydia trachomatis. Women who were pregnant, breast feeding, taking oral contraceptives or antibiotics to treat other forms of inflammatory diseases, who had systemic diseases or cancers that originated from the pelvic organs (e.g., the cervix or ovaries) or who had undergone a gynecologic operation within 2 months prior to admission were excluded from the study. Whole blood was collected from the seventy healthy controls and sixty-four PID patients before and after they received treatment based on the routine protocols suggested by the CDC. The recommend parenteral regimens were cefotetan or cefoxitin plus

doxycycline or clindamycin plus gentamicin. All PID patients were admitted to the ward unit of the Department of Obstetrics and Gynecology, Chung Shan Medical University Hospital. They were given antibiotics intravenously for at least 3 days and for an additional 24 hours after they were afebrile. Thereafter, oral antibiotics were given until day 14 of treatment. Pre-treatment blood samples were obtained before PID patients received treatment protocols, and post-treatment blood samples were obtained one week after treatment was initiated. All blood samples were analyzed for nonspecific inflammatory markers, such as WBC and C-reactive protein (CRP) [27, 28]. In addition, plasma samples were analyzed for the expression of uPA, suPAR, and PAI-1. Both the technician who measured levels of uPA, suPAR, and PAI-1 as well as the clinical laboratory staff who measured WBC counts, neutrophil counts, lymphocytes, and CRP were blinded to this study. The blood samples obtained for measurement of uPA, suPAR, and PAI-1 were placed in tubes containing EDTA and were immediately centrifuged and stored at -80 °C. The study was performed with the approval of the Chung Shan University Hospital Institutional Review Board, and written informed consent was obtained from each patient.

Measurements of uPA, suPAR, and PAI-1 levels by ELISA

The uPA, suPAR, and PAI-1 levels in the plasma samples were analyzed using human uPA, suPAR, and PAI-1 ELISA kits, respectively (R&D Systems, Abingdon, UK). From each plasma sample, 100 μL was directly transferred to the microtest strip wells of the ELISA plate and was then assayed according to the manufacturer's instructions. Absorbance was measured at 495 nm in a microtest plate spectrophotometer, and plasma levels of uPA, suPAR, and PAI-1 were quantified by a calibration curve that used human uPA, suPAR, and PAI-1 as a standard, respectively [29].

Genomic DNA extraction

Venous blood from each subject was drawn into Vacutainer tubes containing EDTA and stored at 4 °C. Genomic DNA was extracted using QIAamp DNA blood mini kits (Qiagen, Valencia, USA) according to the manufacturer's instructions. DNA was dissolved in TE buffer [10 mM Tris (PH 7.8), 1 mM EDTA] and then quantitated by measuring OD260. The final preparation was stored at −20 °C and used as a template for polymerase chain reaction (PCR) [30].

Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP)

The primer sequences and the restriction enzyme for analysis of uPA, uPAR, and PAI-1 gene polymorphisms have been described elsewhere [30]. PCR was performed in a 10 μl volume containing 100 ng DNA template, 1.0 μl of 10× PCR buffer (Invitrogen, Carslbad, CA, USA), 0.25 U of Taq DNA polymerase (Invitrogen), 0.2 mM dNTPs (Promega, Madison, WI, USA) and 200 nM of each primer (MDBio Inc. Taipei, Taiwan). A 10 μl aliquot of PCR product was subjected to digestion by each restriction enzyme (New England Biolabs, Beverly, MA) at 37 or 55 ^{O}C for 4 h. After digestion, the products were separated on a 3% agarose gel stained with ethidium bromide. As a result, for the C/T SNP of uPA, the T allele yielded 187- and 104-bp products, while the C alleles yielded a 291-bp product; for the T/C SNP of uPAR, the C allele yielded 200- and 108-bp products, while T alleles yielded a 308-bp product; for the 4G/5G SNP of PAI-1, the 5G allele yielded 74-, 56-, and 33-bp products, while the 4G alleles yielded 107-and 56-bp products.

Statistical analysis

The experimental results are presented as the mean ± SE. The estimated parameters (uPA, suPAR, and PAI-1 levels) were skewed quantitative variables even we attempted transform them to achieve normality, so non-parametric statistics methods were used. A Mann-Whitney U test was used to compare differences in

plasma levels of uPA, suPAR, and PAI-1, as well as WBC counts and C-reactive protein (CRP) levels between healthy women and PID patients prior to receiving the treatment protocols. A Wilcoxon signed-rank test was used to test the difference in these parameters between pre-treatment and post-treatment plasma. Spearman's rank correlation analysis was used to estimate the correlations between each plasma level of uPA, suPAR, and PAI-1 and inflammatory markers, such as WBC count and C-reactive protein levels.

We tested the difference in the genotype frequencies in uPA, uPAR, and PAI-1 gene polymorphisms between PID patients and normal controls using Fisher's exact test. A Kruskal-Wallis test was used to detect differences in the concentrations of plasma protein among the three genotypic frequencies of uPA, uPAR, and PAI-1, then a Bonferroni correction was performed for post Hoc multiple comparisons.

The adjusted odds ratio (AOR) and the 95% confidence intervals (CIs) for the uPA, uPAR, and PAI-1 gene polymorphisms for PID were estimated using multiple logistic regression models after adjusting for age, WBC count and plasma CRP levels. In response to multiple analyses, we adjusted the *P* values using Hommel method by WinPepi software, version 10.0.

P values <0.05 were considered to be statistically significant. The data were analyzed using SAS statistical software (Version 9.1, 2005; SAS Institute Inc., Cary,

NC) and SPSS version 13.0 (SPSS Inc., Chicago, IL, USA) statistical software.

Results

With the exception of age (p=0.008), there was no significant differences in the distribution of demographic characteristics between PID patients and healthy controls (data not show). WBC counts were significantly increased in patients with PID (11391.7±650.2) prior to receiving treatment compared to those in healthy controls (7103.1±302.9, p<0.0001) and those in PID patients after receiving treatment (6462.7±278.4, p<0.0001) (Figure 1A). In addition, plasma CRP levels were significantly increased in patients with PID (6.85±0.80) prior to receiving treatment compared with those in healthy controls (0.38±0.03, p<0.0001) and those in PID patients after receiving treatment (2.06±0.32, p<0.0001) (Figure 1B).

Plasma expression (mean±SE) of uPA, suPAR, and PAI-1 are shown in Figure 2. Plasma suPAR levels were significantly increased (p=0.04) in patients with pelvic inflammatory disease prior to receiving treatment (1372.04±68.20) when compared with healthy controls (1192.46±51.98) and significantly decreased (p=0.003) after the women received treatment (1220.06±58.14) (Figure 2B). There was also a significantly increased plasma level of uPA in PID patients (0.57±0.03) compared to

that in normal controls (0.55±0.06; p=0.002) (Figure 2A), however, there was no significant difference in the concentration of PAI-1 between healthy controls and patients with pelvic inflammatory disease before and after they received treatment (Figure 2C). In addition, the magnitude of the increase in plasma suPAR was positively correlated with WBC counts (r = 0.382, p=0.002, n=64), blood CRP levels (r = 0.441, p<0.0001, n=64), and plasma levels of uPA (r = 0.426, p<0.0001, n=64). In addition, plasma levels of uPA were significantly correlated with plasma levels of PAI-1 (r = 0.26, p=0.03, n=64) from PID patients prior to receiving treatment (Figure 3 A-D).

The genotype distributions of uPA, uPAR, and PAI-1 are shown in Table 1. There was no association between gene polymorphisms of uPA, uPAR, and PAI-1 and pelvic inflammatory disease. There was also no relationship between plasma concentrations of uPA, suPAR, and PAI-1 and their genetic polymorphisms (Table 2).

Discussion

The activation and migration of leukocytes have been reported to play a crucial role in pelvic inflammatory disease [31, 32]. The plasminogen activation system has been shown to be associated with the activation and migration of leukocytes [13, 33,

34]. In a study by Dorr et al., the authors recruited 10 patients with PID and 9 controls to estimate the concentrations of u-PA-Ag and PAI-1 Ag in peritoneal fluid and plasma among PID patients and controls. They found that u-PA-Ag and PAI-1-Ag levels in peritoneal fluid were significantly elevated in PID patients compared to controls; however, PAI-1-Ag levels in plasma showed no differences between these two groups [19]. The authors' use of laparoscopy was inconvenient, and the small sample size of their study limited the veracity of the author's findings of an association between plasminogen activation system and PID. To develop non-invasive predictors for the development of PID, we estimated plasma concentrations of uPA, suPAR, and PAI-1 in sixty-four patients with PID and seventy healthy controls. We found that there was significantly increased plasma suPAR concentration among patients with PID compared to that in healthy controls and that these levels decreased significantly when compared with that in PID patients after receiving treatment. Also, a significantly increased plasma concentration of uPA in PID patients compared to that in normal controls was found. However, we found no association between PID susceptibility and plasma concentrations of PAI-1. The plasminogen activation system was thought to contribute to the development of fibrinolysis, immunity, and pathology. The degradation of extracellular matrix (ECM) components is mediated by PA system and is considered to be a crucial factor in inflammatory cell migration [13]. uPAR is a

glycosylphosphatydilinositol (GPI)-anchored protein, which consists of three domains including D1, D2, and D3 [13]. The release of active soluble uPAR fragments was reported to play an important role in the recruitment and activation of inflammatory cells, including monocytes and neutrophils, to infected sites to defend against invading microorganisms as well as to amplify tissue-remodeling events [13]. It has been demonstrated that uPAR contributes to neutrophil migration into the peritoneal cavity during lipopolysaccharide-induced peritonitis [16], and increased suPAR could be an indicator of tissue remodeling for tissue repair in inflammation [12]. We suggest that the significantly increased plasma soluble uPAR concentration in our recruited sixty-four PID patients could facilitate the interaction of uPAR and its ligands, which could result in the attraction and recruitment of immune cells to sites of infection and also contributes to the clearance of invading pathogens and the remodeling of damaged tissues [12].

Furthermore, we found that increased concentrations of plasma suPAR were significantly correlated with WBC counts and plasma levels of CRP among PID patients prior to treatment. Our results are consistent with that of Slot et al., [12]. Slot et al., recruited fifty-one subjects with rheumatoid arthritis (RA) and fifty-three healthy controls to measure suPAR concentrations in the plasma of RA patients and healthy controls. They found that suPAR concentrations were significantly increased

among RA patients compared to those of healthy controls ($p<0.001$). In addition, plasma suPAR levels were positively correlated ($p<0.01$) with CRP levels in RA patients. We suggest that plasma suPAR levels are significantly correlated with white blood cells and plasma CRP levels in PID patients, indicating the potentially important role of uPAR for amplifying the concentrations of WBC to defend against pathogens, thereby inducing an inflammatory response. We believe that determining plasma uPAR levels could be a useful biological marker for predicting PID. Moreover, interrupting the interaction between uPAR and its ligands could be a strategy for preventing the development of disease or decreasing the severity of pelvic inflammatory disease.

It has been reported that circulating concentrations of uPA and its inhibitor PAI-1 are detected following bacterial infection [13]. In the present study, we found a significantly elevated plasma concentration of uPA in PID patients but a lack of significantly decreased uPA level when compared with that in PID patients after receiving treatment as well as non-relationship between PID and plasma concentration of PAI-1. uPA has been suggested to be one of the earliest mediators of fibrinolysis, however, uPA-deficient mice did not have fibrinolytic disorders. In contrast to uPA, in uPAR-knockout mice revealed a failure in support a major role of uPAR in fibrinolysis [35]. It has been reported that the ability of uPAR to modulate cell

migration is based on two types of molecular interactions, one is its ability to interact with uPA, and the other is its ability to bind $\mathcal{B}2$-integrins [13, 34]. The binding of uPA facilitated the generation of plasmin; however, its ability to activate and mobilize leukocytes occurred through its interaction with $\mathcal{B}2$-integrins [13, 34]. Moreover, Wiersinga et al., found that uPAR plays a more important role in facilitating phagocytosis in defense against pneumonia-derived septic melioidosis than in inducing pulmonary fibrinolytic activity [14]. We presumed that uPA played a minor role and that other proteins played more important roles in the binding of uPAR, which, in turn, modulates leukocyte migration and immune response in pelvic inflammatory disease. The increased plasma concentration of uPA in PID patients but a lack of similar to suPAR, which significantly decreased after receiving treatment, indicating that uPA could be work as a mediater of fibrinolysis for degradation of extracellular matrix components, which is beneficial to cell migration in inflammatory response, but the regulation of concentration of suPAR plays a major role than that of uPA for the development of PID [7-9, 13]. Also, we found a significant correlation between plasma concentrations of suPAR and uPA, as well as a significant correlation between uPA and PAI-1. PAI-1 is an acute-phase protein, and its levels are regulated in response to inflammation or injury [36, 37]. Plasma uPA has a significant, positive relationship with suPAR and PAI-1 among PID patients, indicating that the

fibrinolytic system was both activated by the interaction of uPA and uPAR and inhibited by the effect of PAI-1, acting as part of a negative feedback loop that regulates the expression of uPA [36, 37]. Future studies that investigate the molecular basis of the interaction between uPAR and its ligands during pelvic inflammatory disease are needed.

In our present study, we found no association between genetic polymorphisms and their gene expression levels in plasma for the sixty-four PID patients and seventy healthy controls. We were also unable to find an association between gene polymorphisms of the uPA system and pelvic inflammatory disease. We hypothesize that SNPs of the uPA system do not have a strong impact on the risk for PID and may partially explain our results.

Conclusion

The expression of soluble urokinase-type plasminogen activator receptor was significantly increased in the plasma of patients with pelvic inflammatory disease. The presence of soluble urokinase-type plasminogen activator receptor may be a non-invasive marker predicting the development of pelvic inflammatory disease. The lack of association between PID susceptibility and gene polymorphisms of uPA, uPAR, and PAI-1 as well as their gene expression may be evidence that these gene

variants do not have a strong impact on susceptibility to PID among Taiwanese women.

Acknowledgements

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Table 1. Adjusted odds ratio (AOR) and 95% confidence intervals (CIs) of pelvic inflammatory disease associated with genotypic frequencies of uPA, uPAR, and PAI-1.

Variable	Controls (n=70) (%)	Patients (n=64) (%)	OR (95% CI); *P* value	AOR (95% CI) ; *P* value	Hommel Adjusted *P* value
u-PA					
CC	60 (85.7%)	54 (84.4%)	1.00	1.00	
CT	10 (14.3%)	10 (15.6%)	1.11 (0.43-2.87); p=0.82	1.21 (0.30-4.83); p=0.78	p=0.97
TT	0 (0%)	0 (0%)	—	—	
u-PAR					
TT	15 (21.4%)	20 (31.3%)	1.00	1.00	
TC	46 (65.7%)	31 (48.4%)	0.50 (0.22-1.13); p=0.09	0.90 (0.27-2.99); p=0.87	p=0.97
CC	9 (12.9%)	13 (20.3%)	1.08 (0.36-3.19); p=0.88	0.97 (0.19-4.85); p=0.97	p=0.97
TT	15 (21.4%)	20 (31.2%)	1.00	1.00	
TC or CC	55 (78.6%)	44 (68.8%)	0.60 (0.27-1.30); p=0.19	0.91 (0.28-2.93); p=0.88	p=0.97
PAI-1					
4G/4G	24 (34.3%)	29 (45.3%)	1.00	1.00	
4G/5G	34 (48.6%)	31 (48.4%)	0.75 (0.36-1.56); p=0.44	0.46 (0.15-1.37); p=0.16	p=0.80
5G/5G	12 (17.1%)	4 (6.3%)	0.27 (0.07-0.96); p=0.04	0.17 (0.02-1.18); p=0.07	p=0.42
4G/4G	24 (34.3%)	29 (45.3%)	1.00	1.00	
4G/5G or 5G/5G	46 (65.7%)	35 (54.7%)	0.62 (0.31-1.26); p=0.19	0.39 (0.13-1.11); p=0.07	p=0.42

The odds ratios (ORs) with their 95% confidence intervals (CIs) were estimated using logistic regression models.

The adjusted odds ratios (AORs) with their 95% confidence intervals (CIs) were estimated using multiple logistic regression models after controlling for age, WBC, and CRP levels.

Hommel adjusted *P* value was in response to multiple analyses.

Table 2. The plasma concentrations of uPA (ng/ml), suPAR (pg/ml), and PAI-1 (ng/ml) for different genotypes.

Variables	Plasma concentrations in different genotypes (mean±SE)			p value	Hommel Adjusted *P* value
	uPA genotypes				
	PID patients and Controls (n=134)				
uPA	CC (n=114) 0.5721±0.043	CT (n=20) 0.5236±0.091		p=0.66 ♣	p=0.86
	Controls (n=70)				
uPA	CC (n=60) 0.5639±0.077	CT (n=10) 0.53±0.166		p=0.86 ♣	p=0.86
	PID patients (n=64)				
uPA	CC (n=54) 0.5811±0.032	CT (n=10) 0.5171±0.089		p=0.45♣	p=0.86
	uPAR genotypes				
	PID patients and Controls (n=134)				
suPAR	TT (n=35) 1269.93±82.86	TC (n=77) 1246.72±55.73	CC (n=22) 1401.75±137.78	p=0.46♣♣ p=1.0♣♣♣	p=0.91
suPAR	TT (n=35) 1269.93±82.86	TC or CC (n=99) 1281.17±53.10		p=0.91♣	p=0.91
suPAR	CC (n=22) 1401.75±137.78	TC or TT (n=112) 1253.97±46.05		p=0.22♣	p=0.91
	Controls (n=70)				
suPAR	TT (n=15) 1168.48±116.99	TC (n=46) 1185.11±55.13	CC (n=9) 1270.04±230.41	p=0.84♣♣ p=1.0♣♣♣	p=0.91
suPAR	TT (n=15) 1168.48±116.99	TC or CC (n=55) 1199.0±58.51		p=0.81♣	p=0.91
suPAR	CC (n=9) 1270.04±230.41	TC or TT (n=61) 1181.02±50.05		p=0.57♣	p=0.91
	PID patients (n=64)				
suPAR	TT (n=20) 1346.01±115.0	TC (n=31) 1338.14±110.97	CC (n=13) 1492.93±173.27	p=0.71♣♣ p=1.0♣♣♣	p=0.91
suPAR	TT (n=20) 1346.01±115.0	TC or CC (n=44) 1383.88±92.98		p=0.81♣	p=0.91
suPAR	CC (n=13) 1492.93±173.27	TC or TT (n=51) 1341.23±80.39		p=0.40♣	p=0.91

	PAI-1 genotypes				
	PID patients and Controls (n=134)				
PAI-1	4G/4G (n=53) 5.1533±0.334	4G/5G (n=65) 5.3121±0.294	5G/5G (n=16) 5.5367±0.506	p=0.83♣♣ p=1.0♣♣♣	p=0.83
PAI-1	4G/4G (n=53) 5.1533±0.334	4G/5G or 5G/5G (n=81) 5.3564±0.255		p=0.62♣	p=0.83
PAI-1	5G/5G (n=16) 5.5367±0.506	4G/4G or 4G/5G (n=118) 5.2408±0.22		p=0.63♣	p=0.83
	Controls (n=70)				
PAI-1	4G/4G (n=24) 4.9193±0.39	4G/5G (n=34) 5.6983±0.40	5G/5G (n=12) 5.1805±0.489	p=0.37♣♣ p=0.52♣♣♣	p=0.83
PAI-1	4G/4G (n=24) 4.9193±0.39	4G/5G or 5G/5G (n=46) 5.5632±0.324		p=0.23♣	p=0.83
PAI-1	5G/5G (n=12) 5.1805±0.489	4G/4G or 4G/5G (n=58) 5.3760±0.29		p=0.77♣	p=0.83
	PID patients (n=64)				
PAI-1	4G/4G (n=29) 5.347±0.52	4G/5G (n=31) 4.8885±0.420	5G/5G (n=4) 6.6054±1.419	p=0.42♣♣ p=0.65♣♣♣	p=0.83
PAI-1	4G/4G (n=29) 5.347±0.52	4G/5G or 5G/5G (n=35) 5.0847±0.40		p=0.68♣	p=0.83
PAI-1	5G/5G (n=4) 6.6054±1.419	4G/4G or 4G/5G (n=60) 5.1101±0.33095		p=0.26♣	p=0.83

♣ The significant difference was analyzed by Mann-Whitney U test.

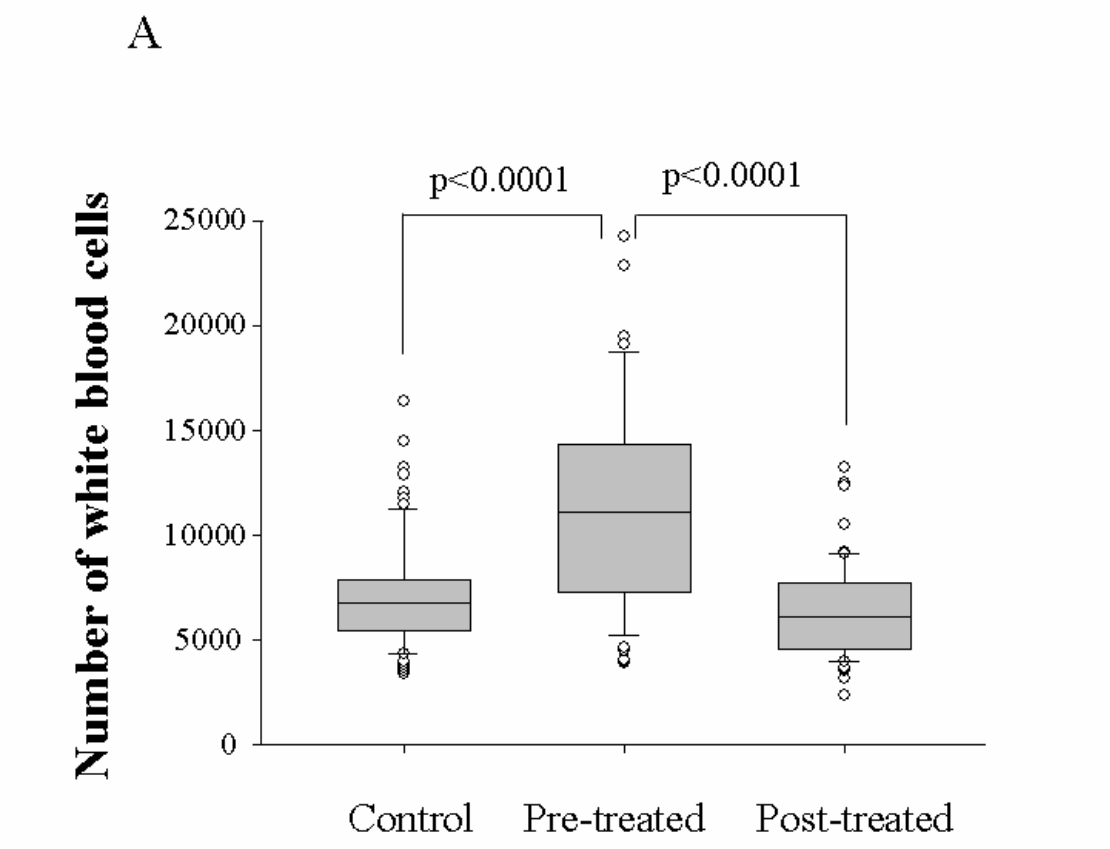
♣♣The significant difference was analyzed by Kruskal-Wallis test.

♣♣♣ The significant difference was analyzed by Bonferroni correction for post Hoc multiple comparisons.

Hommel adjusted *P* value was in response to multiple analyses.

p value <0.05 was considered significant.

Figure 1


A
p<0.0001
p<0.0001
Number of white blood cells
25000
20000
15000
10000
5000
0
Control
Pre-treated
Post-treated

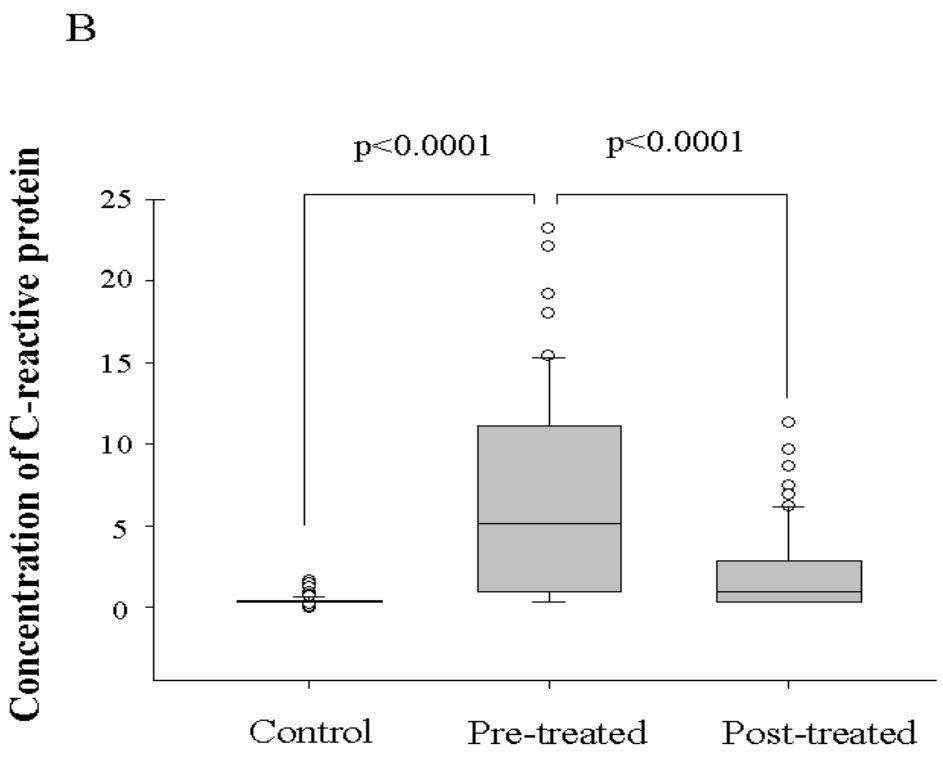
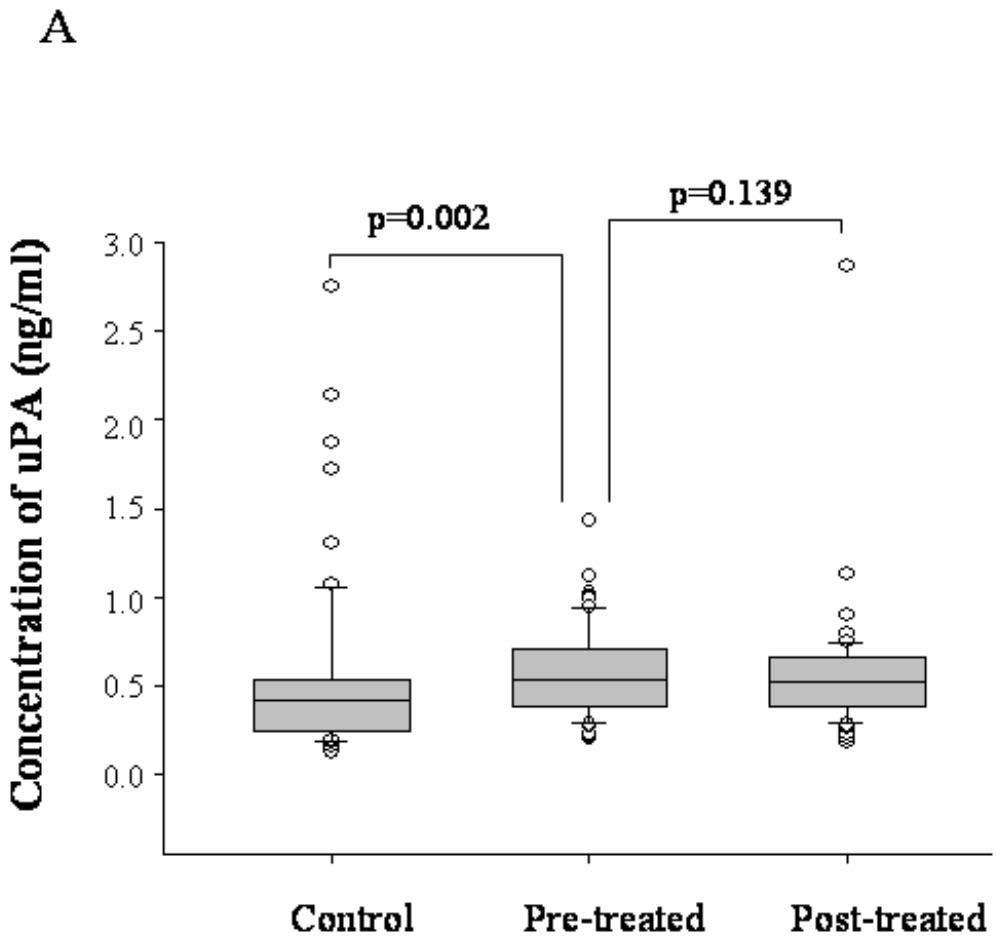


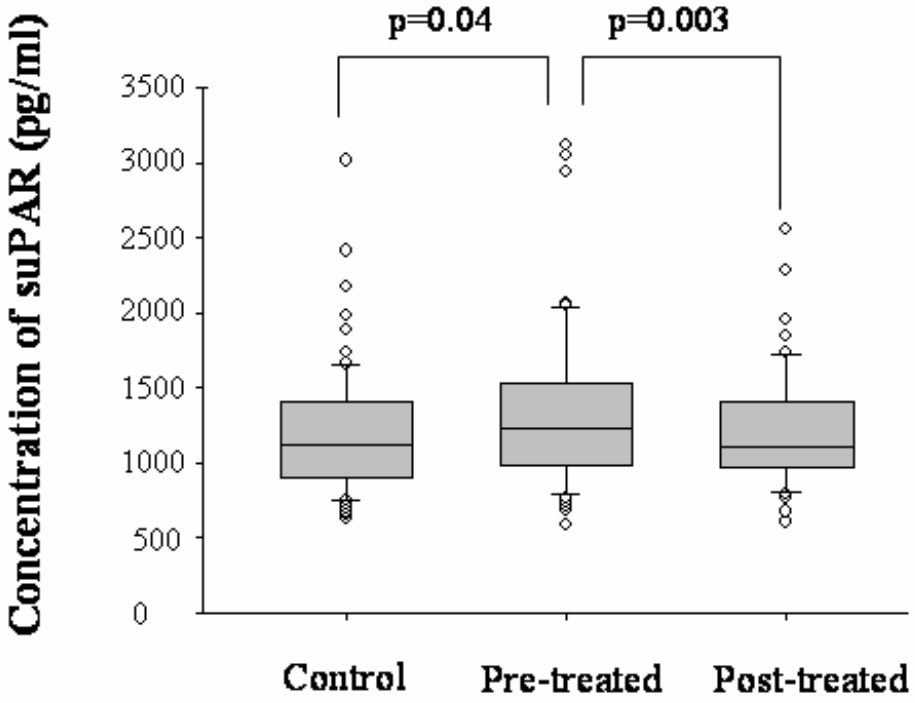
Figure 1. (A) WBC counts and **(B)** the CRP level in blood were significantly increased in sixty-four patients with PID prior to receiving treatment compared with those in seventy healthy controls ($p<0.0001$) and those in PID patients after receiving treatment ($p<0.0001$).

p value <0.05 was considered significant.

Figure 2.


A
p=0.002
p=0.139
Concentration of uPA (ng/ml)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
Control
Pre-treated
Post-treated

B


p=0.04
p=0.003
Concentration of suPAR (pg/ml)
3500
3000
2500
2000
1500
1000
500
0
Control
Pre-treated
Post-treated

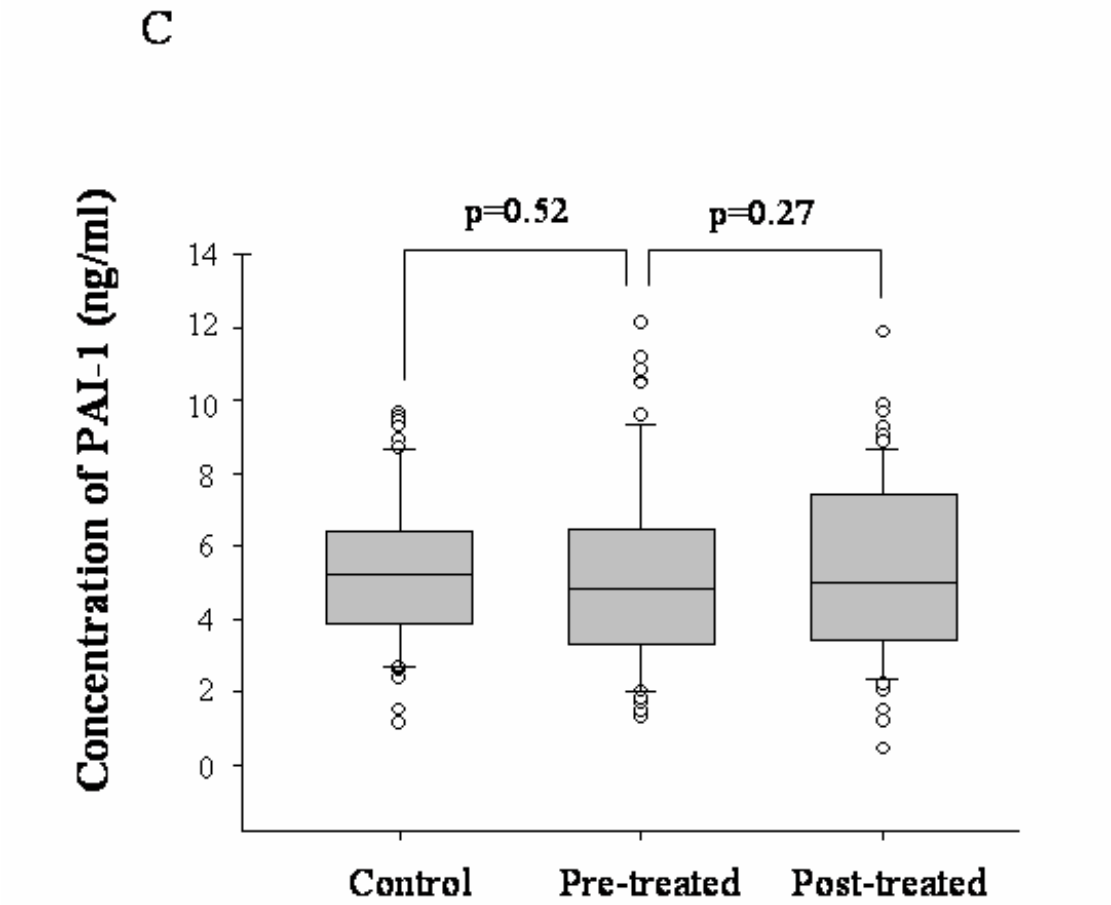


Figure 2.

A. A significantly increased expression of plasma uPA was found in sixty-four patients with PID prior to receiving treatment compared with the seventy healthy controls (p=0.002).

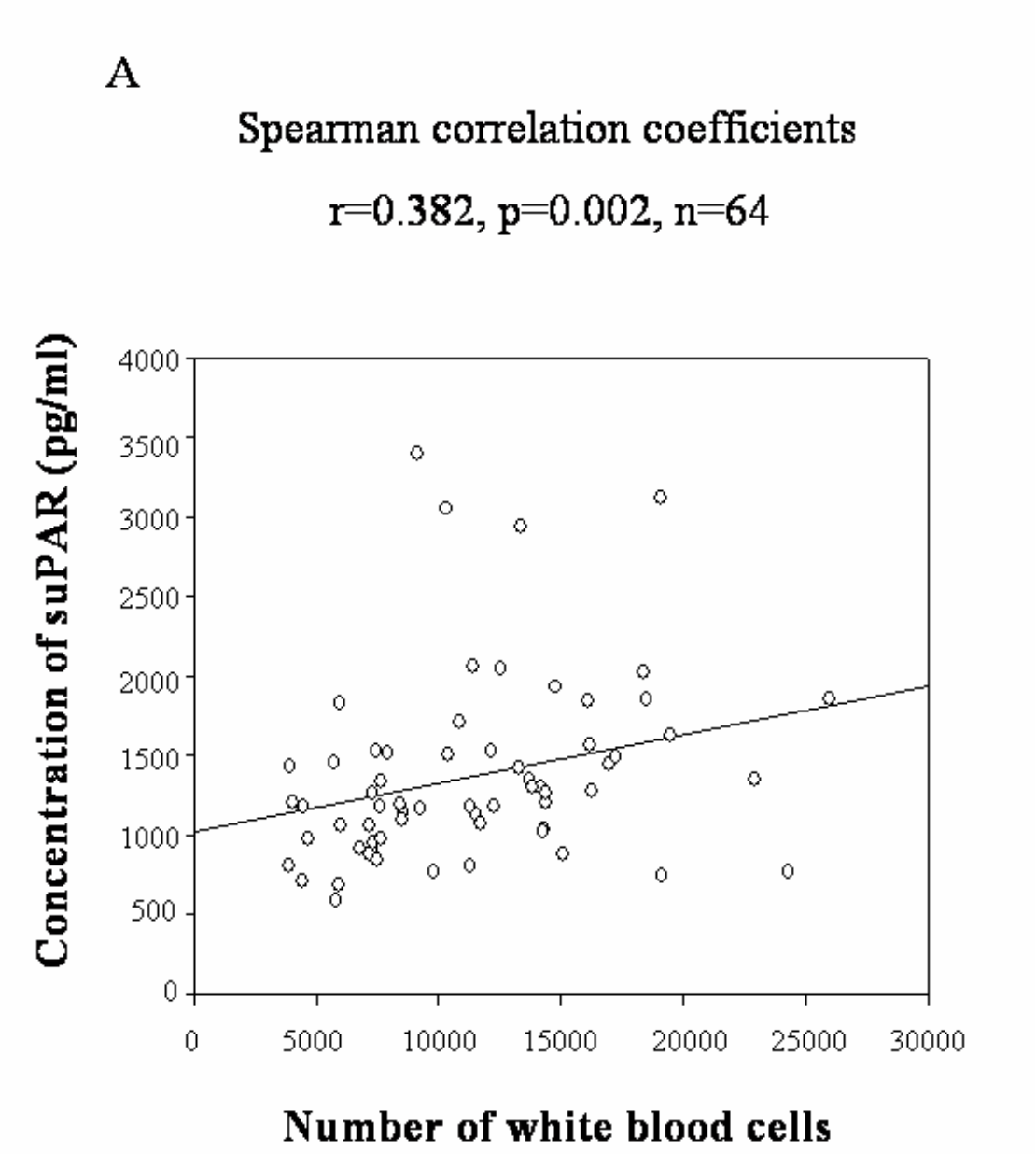
B. A significantly increased expression of plasma suPAR was found in sixty-four

patients with PID prior to receiving treatment compared with the seventy healthy controls (p=0.04) and PID patients after receiving treatment (p=0.003).

C. There was not a significant difference in the concentration of PAI-1 between seventy healthy controls and sixty-four patients with PID before and after receiving treatment.

p value <0.05 was considered significant.

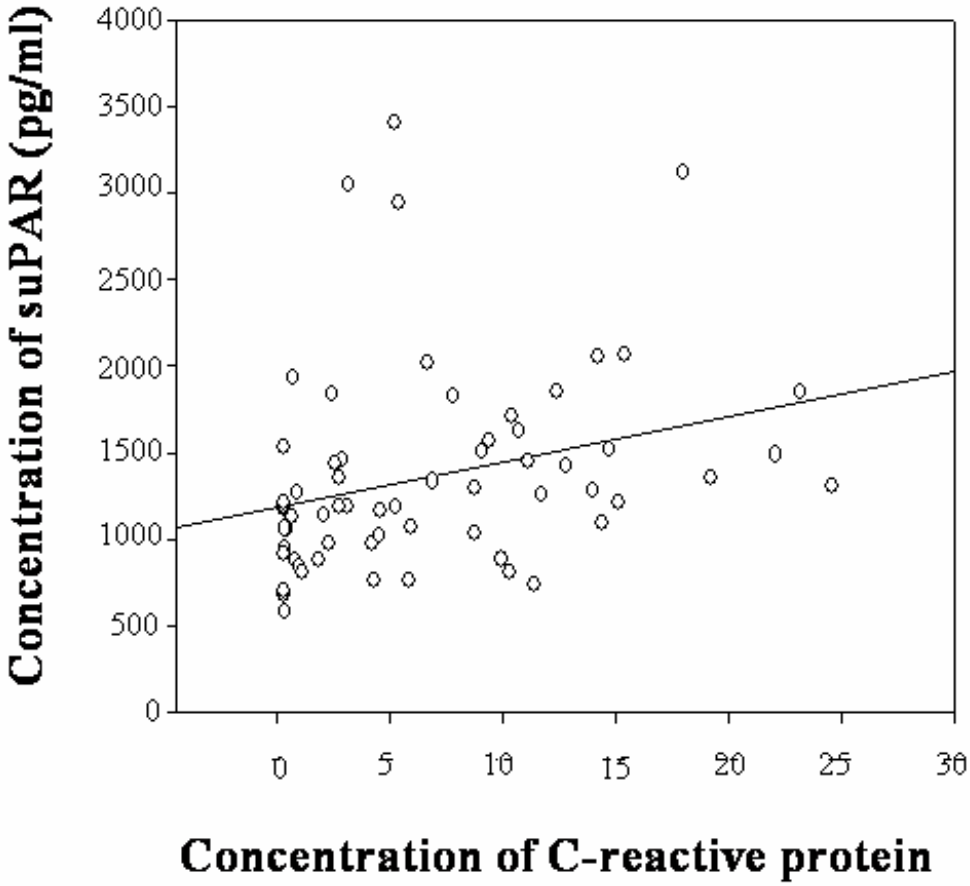
Figure 3


A
Spearman correlation coefficients
r=0.382, p=0.002, n=64
Concentration of suPAR (pg/ml)
4000
3500
3000
2500
2000
1500
1000
500
0
0
5000
10000
15000
20000
25000
30000
Number of white blood cells

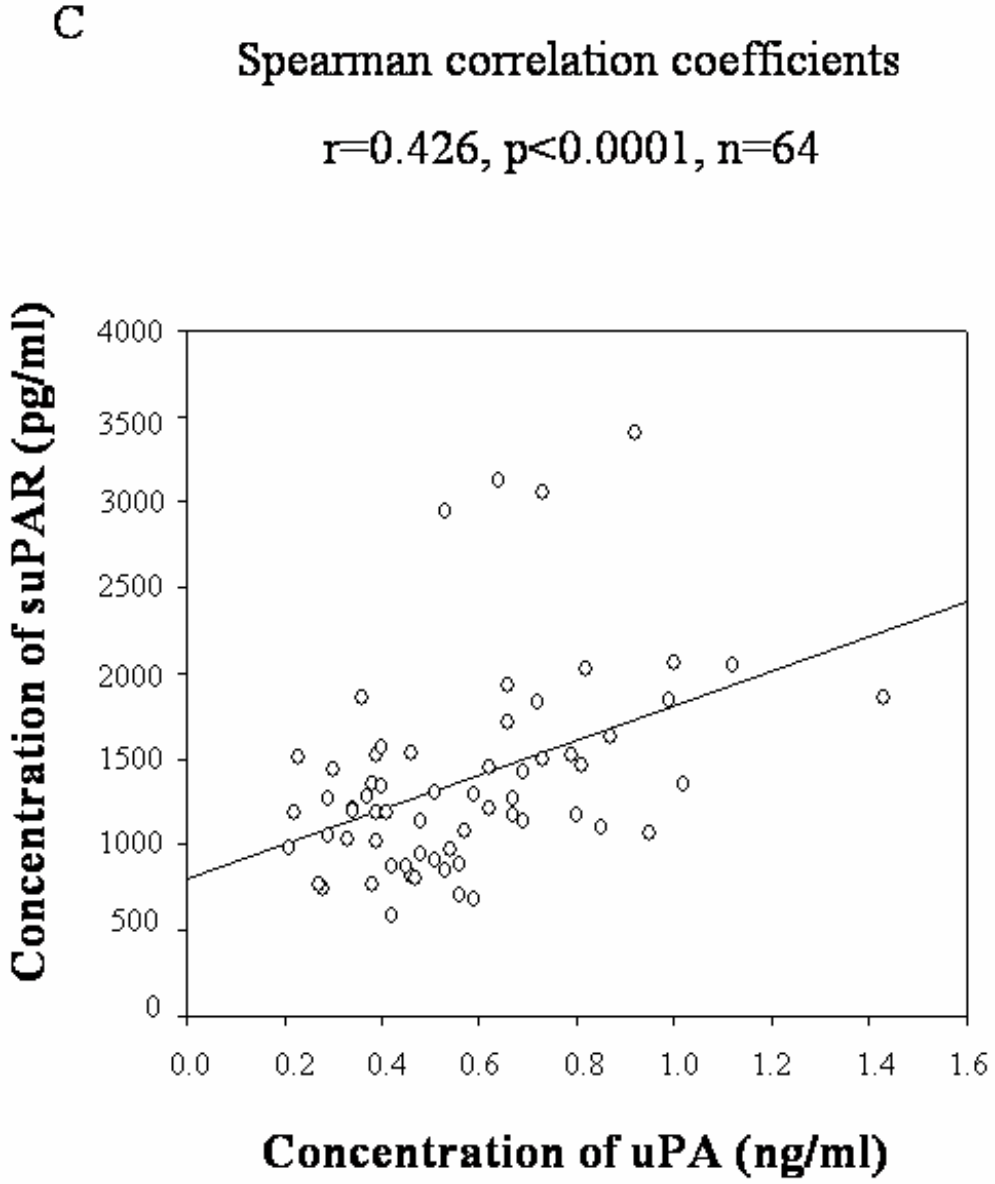
B

Spearman correlation coefficients

r=0.441, p<0.0001, n=64



C


Spearman correlation coefficients
r=0.426, p<0.0001, n=64
Concentration of suPAR (pg/ml)
4000
3500
3000
2500
2000
1500
1000
500
0
0.0
0.2
0.4
0.6
0.8
1.0
1.2
1.4
1.6
Concentration of uPA (ng/ml)

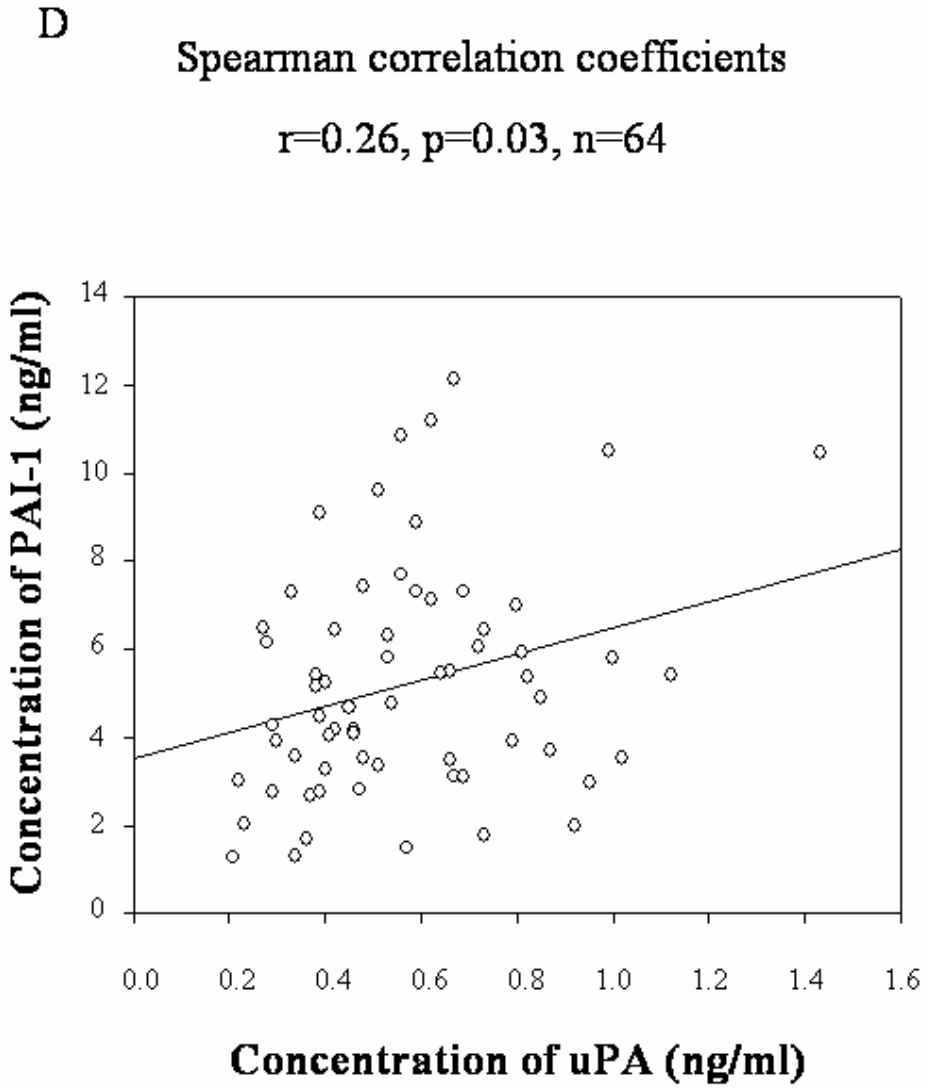


Figure 3. There was a significant correlation between suPAR and **(A)** WBC counts (Spearman correlation coefficients r = 0.382, p=0.002, n=64), **(B)** CRP (r = 0.441, p<0.0001, n=64), and **(C)** u-PA (r = 0.426, p<0.0001, n=64) as well as a significant correlation between **(D)** u-PA and PAI-1 (r = 0.26, p=0.03, n=64) in the blood of sixty-four patients with PID prior to receiving treatment.

p value <0.05 was considered significant.

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評值

本研究內容與原計畫相符程度約達 100%。
本研究對婦科醫學而言具有顯著的貢獻，此研究結果可得知與骨盆腔炎症有關的基因型態以及協助診斷骨盆腔炎症的生物指標，以作為臨床應用之參考。

論文接受函

Ms. Ref. No.: CCA-D-12-00894R1
Title: Significantly increased concentration of soluble urokinase-type plasminogen activator receptor in the blood of patients with pelvic inflammatory disease
Clinica Chimica Acta

Dear Dr. Hsiu-Ting Tsai,

A final disposition of "Accept" has been registered for the above-mentioned manuscript.

For further assistance, please visit our customer support site at http://help.elsevier.com/app/answers/list/p/7923. Here you can search for solutions on a range of topics, find answers to frequently asked questions and learn more about EES via interactive tutorials. You will also find our 24/7 support contact details should you need any further assistance from one of our customer support representatives.

Kind regards,

Clinica Chimica Acta

Comments from the Editors and Reviewers:

本研究計畫之研究成果

(A) 本研究計畫之期刊論文著作目錄（*通訊 作者）

1. **Hsiu-Ting Tsai***, Tsung-Hsien Lee, Shun-Fa Yang, Long-Yau Lin, Yi-Torng Tee, Po-Hui Wang. Markedly elevated soluble E-cadherin in plasma of patient with pelvic inflammatory disease. Fertil Steril. (in press) (IF: 3.958, Rank: 4/77=0.051); (SCI 期刊)

2. Yuan-Hung Yeh, Po-Hui Wang, Long-Yau Lin,Yi-Torng Tee, Ming-Chih Chou, Shun-Fa Yang , **Hsiu-Ting Tsai *.** Significantly increased concentration of soluble urokinase-type plasminogen activator receptor in the blood of patients with pelvic inflammatory disease. (Accepted) Clin Chim Acta. (Impact Factor: 2.389, Rank: 6/31=0.19); (SCI 期刊)

3.Hsiao PC, Wang PH, Tee YT, Yang SF, Su PH, Chen YC, Lin LY, **Tsai HT***. Significantly elevated concentration of plasma monocyte chemotactic protein 1 of patients with pelvic inflammatory disease. Reprod Sci. 2010 Jun;17(6):549-55. (IF: 2.586, Rank: 12/77=0.15); (SCI 期刊)

4. **Tsai HT**, Su PH, Lee TH, Tee YT, Lin LY, Yang SF, Wang PH. Significant elevation and correlation of plasma neutrophil gelatinase associated lipocalin and its complex with matrix metalloproteinase-9 in patients with pelvic inflammatory disease. Clin Chim Acta. 2011 Jun 11;412(13-14):1252-6. (Impact Factor: 2.389, Rank: 6/31=0.19); (SCI 期刊)

5. Wang PH, Liu YF, **Tsai HT**, Tee YT, Lin LY, Hsieh YH, Yang SF. Elevated Plasma Osteopontin Level is Associated With Pelvic Inflammatory Disease. Reprod Sci. 2010 Nov;17(11):1052-8 . (IF: 2.586, Rank: 12/77=0.15); (SCI 期刊)

6.Lee SA, Wang PH, Chiou HL, Chou MC, **Tsai HT***, Yang SF. Markedly elevated plasma myeloperoxidase protein in patients with pelvic inflammatory disease who have A allele myeloperoxidase gene polymorphism. Fertil Steril. 2010 Mar 1;93(4):1260-6. Epub 2009 Jan 14. Erratum in: Fertil Steril. 2010 Aug;94(3):1172. (IF: 3.958, Rank: 4/77=0.051); (SCI 期刊)

(B) 本研究計畫之研討會論文:

1. 會議名稱：4th International Conference in Quantitative Genetics
   時間：101年06月17日至101年06月22日
   地點：英國 愛丁堡
   發表題目：Elevated concentration of soluble urokinase-type plasminogen activator receptor in the blood of patients with pelvic inflammatory disease

國科會補助大學教師出席國際會議心得報告書

<table>
<tr><td>報 告 人</td><td>蔡秀婷</td><td>學校</td><td>中山醫學大學</td></tr>
<tr><td rowspan="2">會 議 名 稱</td><td>中文：第四屆國際性基因醫學研討會</td><td rowspan="2">會議地點</td><td>國家： 英國</td></tr>
<tr><td>英文：4th International Conference in Quantitative genetics</td><td>城市：愛丁堡</td></tr>
<tr><td rowspan="2">發表論文題目</td><td colspan="3">中文：纖維蛋白溶解酶原活化因子接受器在骨盆腔患者血液中之表現</td></tr>
<tr><td colspan="3">英文：Elevated concentration of soluble urokinase-type plasminogen activator receptor in the blood of patients with PID</td></tr>
<tr><td colspan="4">心 得 報 告</td></tr>
<tr><td colspan="4">參加會議經過：此會議屬於國際性基因醫學研討會，主要探討各種基因表現與蛋白質表現及疾病的相關性，會中有來自世界各國的學者，發表及分享最新研究發現、及基因研究成果在臨床之應用。次會議中，我們發表有關纖維蛋白溶解系統在骨盆腔患者血液中之表現，探討活化纖維蛋白溶解系統導致骨盆腔發炎的可能機轉，以及纖維蛋白溶解系統相關蛋白質基因多型性與其蛋白質表現量及罹患骨盆腔炎症的相關性。會議中也與各國學者經驗分享及交流，獲益良多。經由此次會議，吸收他人的經驗及成果。給了我很多的啟示及激勵，讓我感受到教學與研究是一體的，從研究的發現及實務經驗，與國際性的研究交流下，更能促進研究與教學的成長。</td></tr>
</table>

國科會補助計畫衍生研發成果推廣資料表

日期:2012/10/25

國科會補助計畫	計畫名稱: 纖維蛋白溶解系統在骨盆腔炎症的表現及其機制探討
	計畫主持人: 蔡秀婷
	計畫編號: 98-2314-B-040-014-MY3　　學門領域: 婦產科

無研發成果推廣資料

98 年度專題研究計畫研究成果彙整表

計畫主持人：蔡秀婷	計畫編號：98-2314-B-040-014-MY3
計畫名稱：纖維蛋白溶解系統在骨盆腔炎症的表現及其機制探討	

成果項目			量化			單位	備註（質化說明：如數個計畫共同成果、成果列為該期刊之封面故事...等）
			實際已達成數（被接受或已發表）	預期總達成數(含實際已達成數)	本計畫實際貢獻百分比		
國內	論文著作	期刊論文	0	0	100%	篇	
		研究報告/技術報告	0	0	100%		
		研討會論文	1	1	100%		
		專書	0	0	100%		
	專利	申請中件數	0	0	100%	件	
		已獲得件數	0	0	100%		
	技術移轉	件數	0	0	100%	件	
		權利金	0	0	100%	千元	
	參與計畫人力（本國籍）	碩士生	2	2	100%	人次	
		博士生	1	1	100%		
		博士後研究員	0	0	100%		
		專任助理	0	0	100%		
國外	論文著作	期刊論文	6	6	100%	篇	
		研究報告/技術報告	0	0	100%		
		研討會論文	1	1	100%		
		專書	0	0	100%	章/本	
	專利	申請中件數	0	0	100%	件	
		已獲得件數	0	0	100%		
	技術移轉	件數	0	0	100%	件	
		權利金	0	0	100%	千元	
	參與計畫人力（外國籍）	碩士生	0	0	100%	人次	
		博士生	0	0	100%		
		博士後研究員	0	0	100%		
		專任助理	0	0	100%		

其他成果 (無法以量化表達之成果如辦理學術活動、獲得獎項、重要國際合作、研究成果國際影響力及其他協助產業技術發展之具體效益事項等，請以文字敘述填列。)	本研究結果可供臨床醫師協助診斷骨盆腔發炎之參考，並有助於瞭解骨盆腔發炎之病理生理機轉。未來可望藉由研究結果，研發相關之標跋治療。此外，計畫執行期間也培訓學生實驗操作、資料收集及分析之技能，並鼓勵學生參加學術研討會論文發表，以期培育台灣未來之研究人才。

	成果項目	量化	名稱或內容性質簡述
科教處計畫加填項目	測驗工具(含質性與量性)	0	
	課程/模組	0	
	電腦及網路系統或工具	0	
	教材	0	
	舉辦之活動/競賽	0	
	研討會/工作坊	0	
	電子報、網站	0	
	計畫成果推廣之參與（閱聽）人數	0	

國科會補助專題研究計畫成果報告自評表

請就研究內容與原計畫相符程度、達成預期目標情況、研究成果之學術或應用價值（簡要敘述成果所代表之意義、價值、影響或進一步發展之可能性）、是否適合在學術期刊發表或申請專利、主要發現或其他有關價值等，作一綜合評估。

1. 請就研究內容與原計畫相符程度、達成預期目標情況作一綜合評估

■達成目標

□未達成目標（請說明，以 100 字為限）

□實驗失敗

□因故實驗中斷

□其他原因

說明：

2. 研究成果在學術期刊發表或申請專利等情形：

論文：■已發表 □未發表之文稿 □撰寫中 □無

專利：□已獲得 □申請中 ■無

技轉：□已技轉 ■洽談中 □無

其他：(以 100 字為限)

本研究計畫研究成果共有 6 篇已刊登或已被接受於 SCI 期刊之論文發表，以及 1 篇之國際會議論文發表。

3. 請依學術成就、技術創新、社會影響等方面，評估研究成果之學術或應用價值（簡要敘述成果所代表之意義、價值、影響或進一步發展之可能性）(以 500 字為限)

本研究結果可供臨床醫師協助診斷骨盆腔發炎之參考，並有助於瞭解骨盆腔發炎之病理生理機轉。未來可望藉由研究結果，研發相關之標跋治療。