DIFFERENTIAL FEATURES IN COMPOSITION OF CORONARY THROMBUS OF WOMEN WITH ST-SEGMENT ELEVATION MYOCARDIAL INFARCTION

Running title: Composition of coronary thrombus of women

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ABSTRACT

Objectives: To characterize sex differences in the composition of coronary thrombus in patients with ST-segment elevation myocardial infarction (STEMI), especially in the young (age ≤55 years).

Background: Women have smaller coronary vessels than men and their vascular lesions can be influenced by different exposure to circulating estrogens throughout life. These factors could determine a different composition of the coronary thrombus in women with STEMI.

Methods: A prospective, multicenter study was conducted on patients with STEMI and coronary thrombus was aspirated immediately before percutaneous coronary intervention (PCI) using a suction catheter (ProntoV3® or Export®). Histopathology, immunohistochemistry and ELISA techniques were used for the quantitative determination of fibrin, p-selectin and von Willebrand factor (vWF) within thrombi.

Results: Thrombi were collected from 100 patients (50 men and 50 women; 13 women and 13 men of <55 years). Women presented similar baseline characteristics and pain-to-balloon elapsed time than men. Thrombi from women showed a trend to a lower concentration of fibrin than those from men [median=1.2 ng/mg (IQR 3.5) vs median=2.2 ng/mg (IQR 5.9), p=–0.102]. No differences were found between sexes in p-selectin and vWF concentration in thrombi. However, thrombi from young women showed lower levels of p-selectin [median=2.2 ng/mg (IQR 4.5) vs 6.5 ng/mg (IQR 4.8), p=0.004], fibrin [median=1.1ng/mg; (IQR: 3.4) vs 4.1 ng/mg (IQR 15.6), p=0.014] and vWF [median=3.2 ng/mg (IQR 10.6) vs 25.8 ng/mg (IQR 15.0), p=0.003] than those from young men.

Conclusions: Thrombi from young women with STEMI showed a lower content of fibrin, p-selectin and vWF than those from men.

Word count: 250

Key words: thrombus, ST-elevation myocardial infarction, women.

INTRODUCTION

Intracoronary occluding thrombosis is the cause of the majority of ST-segment elevation myocardial infarctions (STEMI) (1). Primary percutaneous coronary intervention (PCI) and antithrombotic drugs have largely improved the treatment of STEMI patients in recent years and have shown to reduce mortality (2–3). However, the overall mortality remains high and it is believed that women with STEMI are at a higher risk of adverse outcomes compared with men (4-6). Clinical studies exploring sex contribution in

STEMI are limited by the confounding effect of age (7-8), due to the fact that younger women presenting STEMI have higher risk-adjusted in-hospital mortality than men (9-11). Nevertheless, the composition of the occluding thrombus, its changes with time of evolution and the potential differences between sexes and age remain unknown. Therefore, profiling intracoronary thrombus composition in women and men may be a strategy to identify potential distinguished features in coronary thrombosis between sexes in order to clarify differential pathophysiological mechanisms of coronary thrombosis (12,13).

We have previously investigated the molecular composition of aspirated coronary thrombi from STEMI patients in several circumstances (14,15). Nonetheless, the effect of sex on the thrombogenic response in STEMI had never been systematically investigated *in vivo*. This study was specifically designed to characterize sex-related differences in thrombi

composition after accounting for the confounding effect of age. To this aim, a comprehensive immunohistochemical investigation of the aspirated thrombi was undertaken in order to: 1) characterize the potential sex-dependent differences in the composition of the coronary thrombus in patients with STEMI; and 2) elucidate the contribution of age (i.e., estrogenic status) as a modulator of these sex-dependent differences in the thrombi composition of STEMI patients.

METHODS

Population Study

This is a prospective, multicenter study in which three university hospitals participated. Intracoronary thrombi were obtained from 100 patients (50 women and 50 men) with STEMI undergoing primary PCI. STEMI was defined following the criteria of the ESC/ACCF/AHA/WHF (16). In all cases PCI was performed within 12 h from the onset of pain (except in 1 woman and 2 men) and the visualization of the occlusive clot on angiography (TIMI flow grade 0–1). No patients with MI of embolic mechanism and/or using cocaine were included in our cohort. We neither included any patient on oral anticoagulant treatment. Demographic and clinical data referring to previous medical history and index event were collected from all patients. We did a subgroup analysis to assess if there were differences between sexes in patients aged 55 years old or younger and those who were older. Informed consent was obtained from each patient and the study protocol conforms to the ethical guidelines of the 1975 Declaration of Helsinki. The study received a priori approval by the human research committee of University Hospital Vall d'Hebron and the other participating centers.

Pharmacological treatment before PCI

All patients undergoing PCI received loading doses of acetylsalicylic acid (ASA 250–300 mg p.o.), P2Y12 antagonists (clopidogrel [600 mg p.o], ticagrelor [180 mg p.o] or prasugrel [60 mg p.o]) and unfractionated heparin (50-70 UI/kg/i.v.). No patients received glycoprotein IIb/IIIa inhibitors or thrombolytic therapy.

Thrombus collection during PCI

PCI of the culprit lesion was performed according to the current European guidelines (3) and thrombus aspiration was performed prior to angioplasty using a suction catheter (ProntoV3® or Export®, Medtronic®). All the material obtained through aspiration (10–200 mg each) of the thrombus was collected in a Cell-Strainer with a mesh size of 70µm (1-14 mg of weight), immediately washed in saline buffer to eliminate attached red blood cells, and stored at -80ºC until analytical determinations (14,15).

Immunofluorescence analysis of thrombus composition

Thrombi were immunolabeled as previously described (14,15). Briefly, the defrosted sample was rinsed with cold phosphate buffered saline solution (PBS) and cut in small pieces of 4µm-thick using a cryostat (Leica CM 3050 S). Clot sections were fixed with 1% paraformaldehyde during 10 min and blocked in PBS supplemented with 1% BSA and 2% FCS for 30 min at room temperature. Samples were incubated with the following primary antibodies: mouse monoclonal anti-vWF (1/50 dilution, ab68545 AbcamUK), mouse monoclonal anti-p-selectin/CD 62P (25µg/mL, R&D Systems, UK), rabbit polyclonal fibrinogen/FITC (1/50 dilution, DAKO, Spain) and further incubated in blocking solution for 30-45 min at 4ºC. Secondary antibody (1µg/mL, goat anti-mouse AlexaFluor488,

InvitrogenUK) diluted in blocking solution was added to the primary labeled samples (or the corresponding negative control) for 30-45 min at 4ºC.

Immunofluorescence was detected at 488/560nm (X20) using a confocal microscopy system (Olympus Spectral Confocal Microscopy FV1000) and analysis of fluorescence was quantified with Image J software (National Institutes of Health, Bethesda, MD, USA) as a mean gray value intensity after background subtraction.

Quantitative analysis of thrombus composition by ELISA

A piece of defrosted thrombi (3-10 mg) were washed in cold PBS to remove excess of blood, immersed in 0.5mL of cold PBS and homogenized at 21500min^{-1} (Heidolph DIAX 600) to obtain tissue homogenates. Homogenates were stored overnight at -20ºC until breakage of the fibrin fibers following kit recommendations. Fibrin (Biomatix EKC33680), p-selectin (ab100631, abcam) and vWF (ab189571, abcam) determinations in thrombi were done by Enzyme-Linked ImmunoSorbent Assay (ELISA). Colorimetric detection was carried out at 450nm using a microplate reader (Multiskan RC, Labsystems). Concentrations of fibrin, p-selectin and vWF were calculated from standard curves: 0-200ng/mL of fibrin, 0-50ng/mL of p-selectin and 0-30ng/mL of vWF. Data were expressed as ng of fibrin/mg, ng of p-selectin/mg and ng of vWF/mg.

Statistical analysis

Continuous variables are described as mean ± SD or as median (interquartile range, IQR) for time delay; categorical variables as absolute and relative frequencies of patients in each category. Continuous variables were checked for normal distribution by the Kolmogorov-Smirnov statistic and potential associations between clinical and biological parameters were tested by Student's t-test –or by the Mann-Whitney U test for non-normally distributed variables for independent samples, as appropriate. Comparison for categorical variables

was made by Chi^2 or Fisher´s exact test. Bivariate or multivariate correlations between variables were determined by Spearman or Pearson correlation coefficients. A $p \leq 0.05$ two side was considered significant. All statistical analyses were performed with SPSS v.24.

Results

Baseline characteristics of STEMI patients

Table 1 summarizes demographic characteristics, cardiovascular risk factors, previous medical history and characteristics of index event in men and women.

Women and men presented similar baseline characteristics. Regarding the characteristics of the acute episode of STEMI, women showed a similar pain-to-balloon elapsed time [median=3.4 hours (IQR 17.8) vs 3.5 hours (IQR 22.3), p=0.99; mean 6.70±5.75 vs 5.49± 5.74 hours, p=0.59] but higher creatin-kinase MB (CK-MB) peak levels [median=367ng/mL (IQR 2976) vs 219ng/mL (IQR 853), p=0.002] than men. However, we repeated the analysis excluding 3 patients with a pain-to-balloon elapsed time above 12 hours and we observed differences between both sexes with a shorter pain to balloon time in men [[median=3 hours (IQR 1.7) vs 3.7 hours (IQR 3.5), p=0.029; Therefore, higher CK-MB peak levels were maintained in women [median=367 ng/mL (IQR 351) vs 219 ng/mL (IQR 179), p=0.003]. Left anterior descending coronary artery (LAD) occlusion as culprit artery was more frequent in women than in men (57% vs 30%, p=0.009). No differences in the pharmacological treatment received before PCI were observed between both sexes. The sub-analysis of baseline characteristics stratifying by age showed that in patients >55 years, women had more frequently hyperlipidemia [24 (65%) vs 14 (38%), p=0.03] (Supplemental material, Table 1). Nonetheless, in the subgroup of patients ≤55 years there were no differences between sexes regarding baseline characteristics (neither in

cardiovascular risk factors nor in pain-to balloon elapsed time) (Supplemental material, Table 2). However, in the whole cohort and in the subgroup of >55 years, STEMI was more frequently located in LAD in women compared with men ($p<0.009$ and $p<0.001$, respectively). (Table 1 and supplemental Table 1).

Differences between sexes in thrombus composition in STEMI patients

Thrombi from women with STEMI tended to have lower concentration of fibrin than men, but without reaching statistical significance [median=1.2ng/mg (IQR 3.5) vs 2.2 ng/mg (IQR 5.9) fresh tissue, p=0.102], despite similar pain-to-balloon elapsed time between sexes. No differences in p-selectin and vWF concentration within thrombi were found between women and men (Figure 1).

There was a negative correlation between age and the content of p-selectin and vWF (R=-0.396, $p<0.0001$) within the thrombi (R=-0.226, p=0.024). Nonetheless, there was no correlation between age and the content of fibrin (R=0.029, p=0.776), (Figure 2).

Thrombi composition in young STEMI patients (age ≤55 years)

Sex-dependent differences in thrombi composition were detected in younger patients (age ≤55 years) (Figure 3A). On the other hand, thrombi from patients of both sexes >55 years had a similar amount of fibrin, p-selectin and vWF (Figure 3B). Thrombi from young women showed lower levels of p-selectin [median=2.2 ng/mg (IQR 4.5) vs 6.5 ng/mg (IQR 4.8), p=0.004], fibrin [median=1.1 ng/mg (IQR 3.4) vs 4.1 ng/mg (IQR 15.6), p=0.014] and vWF [median=3.2ng/mg (IQR 10.6) vs 25.8ng/mg (IQR 15.0), p=0.003] than the thrombi from men of the same age (Figure 3A). Despite the fact that in this subgroup of age, women received new antiplatelets more frequently than men, although without statistical

signficance (supplemental Table 1), the concentration of p-selectin between both groups of patients was similar (p = 0.096).

Representative confocal images of thrombi from patients ≤55 years and >55 years immunolabeled with p-selectin, fibrin and vWF are shown in Figure 4.1 and 4.2, respectively.

Discussion

We present the first prospective study specifically designed to evaluate sex- and age-dependent differences in the composition of thrombi in patients with STEMI *in vivo*. The results obtained in our study indicate that men and women with STEMI showed a similar composition in the thrombus regarding the content of the thrombogenic substrates p-selectin, fibrin and vWF. However, sex had a significant impact in thrombus composition at younger ages. Specifically, when considering the group of patients of ≤55 years of both sexes, young women had significantly lower levels of fibrin, p-selectin and vWF content in their thrombi than young men.

Autopsy studies have suggested differences in the underlying plaque morphology associated with acute MI in women versus men (17-19). Although the rupture of a thin-cap fibroatheroma plaque has been recognized as the most prevalent pathological substrate of STEMI, coronary thrombosis arising from eroded (intact fibrous cap) plaque was frequently reported, especially in women and young people (20). However, there is currently no systematic *in vivo* investigation of sex differences in the composition of the aspirated thrombus in the context of STEMI. According to previous *"post mortem"* research, we found a similar composition of thrombus in patients >55 years of age regardless of sex, as detected by immunobased techniques (17). However, there are no data on the potential sex-

dependent differences in thrombus composition in younger patients (≤55 years) with STEMI, in which the effect of sex hormones (i.e., estrogen profile) can have a differential pathophysiological role.

The relevance of this problem has been highlighted by the European Society of Cardiology, which issued a statement recommending the analysis of the sex-dependent pathophysiological mechanisms in MI that could influence the outcome of the diseases between both sexes. In this respect, our findings go in opposite direction to the clinical evolution of young women with STEMI, as young women have been consistently shown to have lower thrombogenic burden than young men, despite receiving a worse prognosis. Some authors have attributed this poorer prognosis to a worse cardiovascular risk profile in women compared to younger men (5). In this sense, previous studies have reported that cardiovascular risk factors are able to trigger an increase of circulating tissue factor levels contributing to the thrombus formation (21). In contrast to other authors (22), we could not find differences between sexes in this regard, probably due to the low number of patients ≤ 55 years.

Surprisingly, women did not present a longer time from the onset of pain to PCI than men, unlike observations from most epidemiological studies (4-6), but in line with the results shown by Guagliumi et al. (17), This suggests different physiopathological mechanisms in thrombi formation between sexes depending on age, in agreement with other studies showing a different atherosclerotic substrate between women and men (23). Ramananthan et al. showed a higher fibrin level in the microstructure of the coronary clot in men than in women with coronary artery disease, which may be due to the fact that men in general have an underlying propensity to form denser and more compact clots than women (23).

On the other hand, we found a greater peak of CK-MB in women than in men, despite a similar time elapsed since the onset of pain. This fact could be related to an extensive MI due to a more frequent location in LAD in women than in men in our cohort, regardless of age.

Thus, in the present study we report on the changing nature of the composition of coronary thrombi between sexes depending on age. Given that age was the most important factor to distinguish the composition of thrombi between men and women, 55 years was established as the cut age to perform a sub-analysis of the levels of fibrin, p-selectin and vWF in the thrombi of patients (around menopause *versus* when the protective effect of estrogens on the development of coronary artery disease is still present in women) (24,25). Numerous studies have pointed out that estrogen may modulate the risks and outcomes of atherosclerotic disease. For instance, premenopausal women are less likely to experience coronary atherosclerosis than men of the same age, but after menopause, women are equally or more likely than men of the same age to develop coronary atherosclerosis (26). Platelets are one potential target for estrogen modulation within the cascade of factors involved in ischemic atherosclerotic events (26). Furthermore, plasma fibrinogen concentrations are decreased by continuous estrogen therapy (26). In addition, lower plasma levels of p-selectin have been observed in women during the reproductive period than in men (27) but no significant sex difference was observed when the average age was 55 years (27). On the other side, a recent study by Chaudhary R et al studied the thrombogenicity of patients with angina but with non-obstructive disease. These authors found a higher thrombogenicity in women than in men in plasma. However, the clinical

scenario was different than in our study and the authors performed *in vitro* techniques (28,29).

In contrast to previous studies, in which thrombosis has been generally studied in *in vitro* and *ex vivo* models, both in peripheral or post-mortem vessels (30,31), the method used in the present study, based on the aspiration of a coronary thrombus during PCI, offers the advantage of providing human biological samples *in vivo* from ongoing thrombosis (14,15,32).

The different outcomes between sexes after STEMI, especially in young women, have been described for many years (4,10). In our study, the time between pain and treatment strategies were comparable between sexes, further supporting the concept that age, and not other variables, plays an important role in the composition of the thrombus. In addition, the existence of cardiovascular risk factors could potentially influence the thrombogenic burden within the thrombi. Nonetheless, some differences in biological mechanisms could be relevant in the differences found in thrombus composition between sexes in young patients with STEMI. However, our study lacked statistical power to detect differences in risk factors and, therefore, these results should be interpreted with caution. The differences observed in the composition of thrombi between sexes in young patients with STEMI may support the notion that antithrombotic drugs should be administered according to the risk bleeding assessment, which includes: sex, age, weight, previous history and renal function, in order to reduce a higher bleeding risk observed in women with STEMI (33).

Study Limitations

Our research has several limitations. Firstly, due to the *in vivo* nature of our study, the analysis was performed on the main aspirated piece of the thrombus and did not include the total volume of retrieved fragments or the distal embolic debris. Secondly, potential distortion of the samples might have occurred during thrombus-aspiration. However, the contribution of distortion is expected to be negligible because thrombi are stiffer than *in vitro* clots and fibrin fibers bear outstanding elastic properties. The preserved appearance of cells and fibrin fibers in the microscope images confirmed that distortion was minimal. Finally, there was a low number of patients aged ≤55 years, nevertheless, consistent differences in the composition of thrombus between sexes were still detectable.

Further directions

Further studies are necessary on the knowledge of potential differences between sexes in biological mechanisms involved in coronary thrombi composition in young patients with acute coronary syndromes.

Conclusions

In conclusion, our study suggests that among patients with STEMI, young women have a lower content in p-selectin, fibrin and vWF within the thrombi than young men.

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Figure Legends:

Table 1. Baseline characteristics of STEMI patients

Foot notes: Data correspond to mean value (SD), median (IQR) and frequencies of main characteristics related with demographic, cardiovascular risk factors and percutaneous intervention. P values ≤ 0.05 were considered statistically significant.

Figure 1. Thrombi composition in STEMI patients

Not differences were shown between sexes in p-selectin, fibrin and vWF concentrations obtained in thrombi from STEMI patients (men n=50 and women n=50), and analyzed by ELISA technique. Data correspond to median value (IQR) of p-selectin, fibrin and vWF and expressed as ng of compound per mg of tissue.

Figure 2. Effect of age in thrombi composition

Dispersion graphs show the correlation between thrombi composition and age in 100 STEMI patients. P-selectin and vWF concentration are decreasing as patients are older: p-selectin (Pearson coefficient R=0.226, p=0.024) and vWF (R=0.396, p<0.0001). In contrast, age shows no effect on the fibrin content of thrombi (R=0.029, p=0.776).

Figure 3. Thrombi composition in young (age ≤ 55 years) and (age >55 years) STEMI patients

Thrombi from young women patients (Figure 3A, n=13) shown lower concentrations of p-selectin [2.2 ng/mg (IQR 8.8)], fibrin (1.1ng/mg (IQR 16.4)) and vWF [3.2 ng/mg (IQR 40.3)] than the ones from men of the same age range (n=13): p-selectin [5.6ng/mg (IQR 9.7), p=0.004], fibrin (4.1ng/mg (IQR 64.9), p=0.014) and vWF [25.8ng/mg (IQR 54.3) fresh tissue, p=0.003]. On the contrary, there were no differences between sexes when the patients are aging >55 years old (Figure 3B). Data correspond to median value (IQR) of p-

selectin, fibrin and vWF concentrations obtained in thrombi analyzed by ELISA and expressed as ng of compound per mg of tissue.

Figure 4A. Immunofluorescence analysis of thrombus composition by confocal microscopy in patients with ≥55 years. Representative confocal images of thrombus from patients after immunolabelling thrombi sections using fibrin, p-selectin and vWF specific antibodies. (A= woman; B= man)

Figure 4B. Immunofluorescence analysis of thrombus composition by confocal microscopy in patients with <55 years. Representative confocal images of thrombus from patients after immunolabelling thrombi sections using fibrin, p-selectin and vWF specific antibodies. (A= woman; B= man)

Table 1. Differences in baseline characteristics of whole cohort with ST-elevation myocardial infarction

	Men **N = 50**	**Women** **N = 50**	**P value**
Age (years), Mean (SD)	65 (13)	67 (14)	0.534
Past Medical History, n (%)			
Active smoking	25 (50)	18 (36)	0.157
Alcohol intake	7 (14)	2 (1)	0.001
Hypertension	27 (54)	25 (50)	0.692
Systolic blood pressure, mm Hg	136 (122-146)	130 (128–143)	0.087
Diastolic blood pressure, mm Hg	72 (76–85)	74 (73–85]	0.094
Hyperlipidemia	21 (42)	27 (54)	0.228
LDL-cholesterol, mg/dl	120 (90-150)	117 (92-152)	0.073
Diabetes mellitus	13 (26)	12 (23)	0.723
Fasting glucose, mg/dl	90 (80-120)	98 (82-122)	0.08
Previous PCI	0	0	1.000
Atrial fibrillation	0	0	1.000
Peripheral arterial disease	4 (8.0)	4 (8.0)	0.952
Chronic kidney disease	2 (4)	3 (6)	0.020
COPD	4 (8)	1 (2)	0.498
Treatment before PCI, n (%)			
ASA	50 (100)	50 (100)	1.000
Clopidogrel	42 (84)	37 (74)	
Ticagrelor	5 (10)	8 (16)	

Pasugrel	3 (4)	5 (10)	0.317
Heparin	50 (100)	50 (100)	
Antihipertensive treatment	26 (53)	25 (50)	0.89
Lipid-lowering treatment	19 (38)	19 (38)	1.000
Clinical presentation			
Pain-ballon time (hours), median (IQR)	3.5 (22.3)	3.4 (17.8)	0.991
CK-MB peak (ng/mL), median (IQR)	219 (853)	367 (2976)	0.002
Infarct related artery, n (%)			
LAD artery	15 (30)	29 (52)	0.009
Right coronary artery	30 (60)	19 (38)	0.098
Circumflexe artery	5 (8)	2 (4)	0.056
Multivessel disease	13 (25)	19 (38)	0.203
Post-PCI TIMI flow grade, n (%)			
Flow grade 2	2 (4)	5 (9)	
Flow grade 3	48 (96)	45 (91)	0.422
Blush < 3 (%)	18 (35)	22 (44)	0.158

Footnotes: PCI.percutaneous coronary intervention; LAD: left anterior descending coronary artery.

Figure 1

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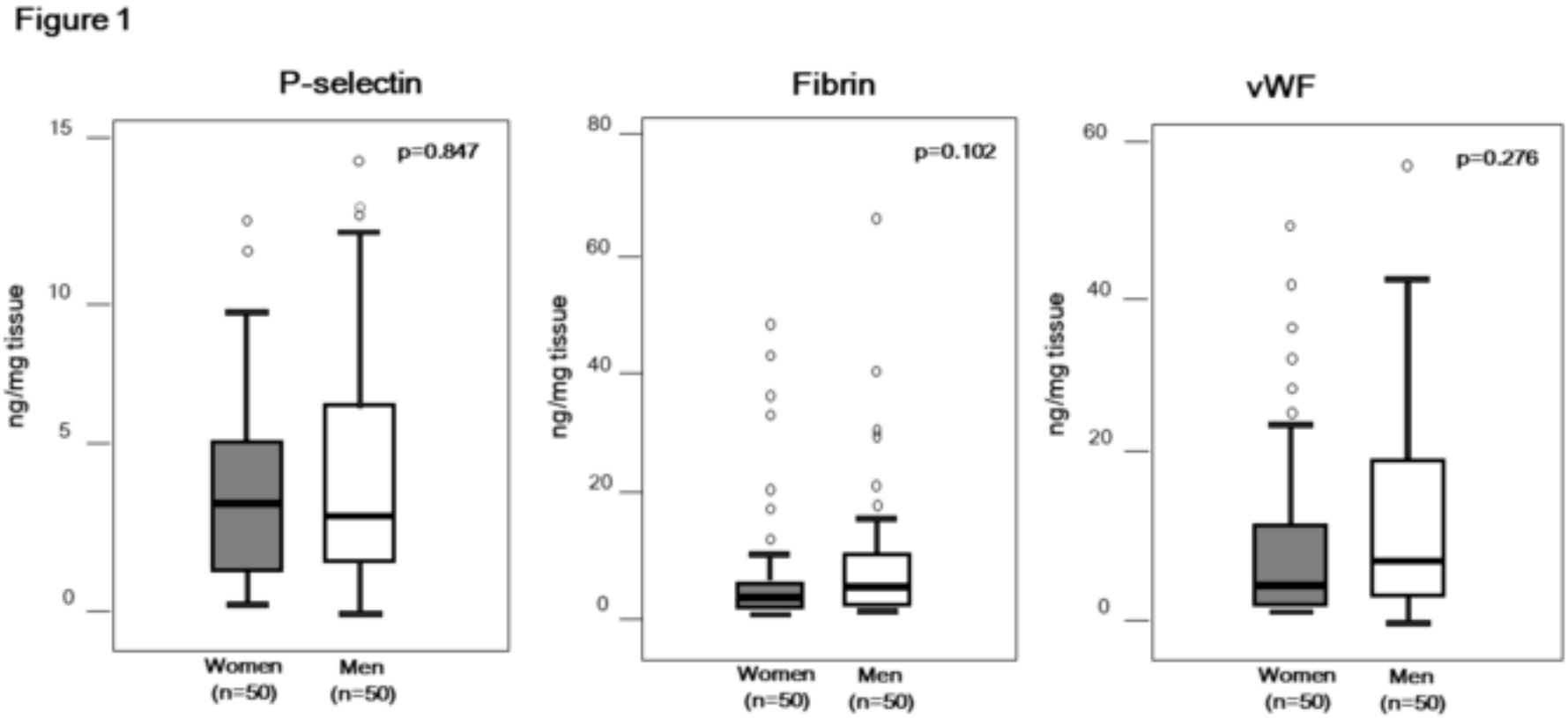

Figure 2

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Figure 2

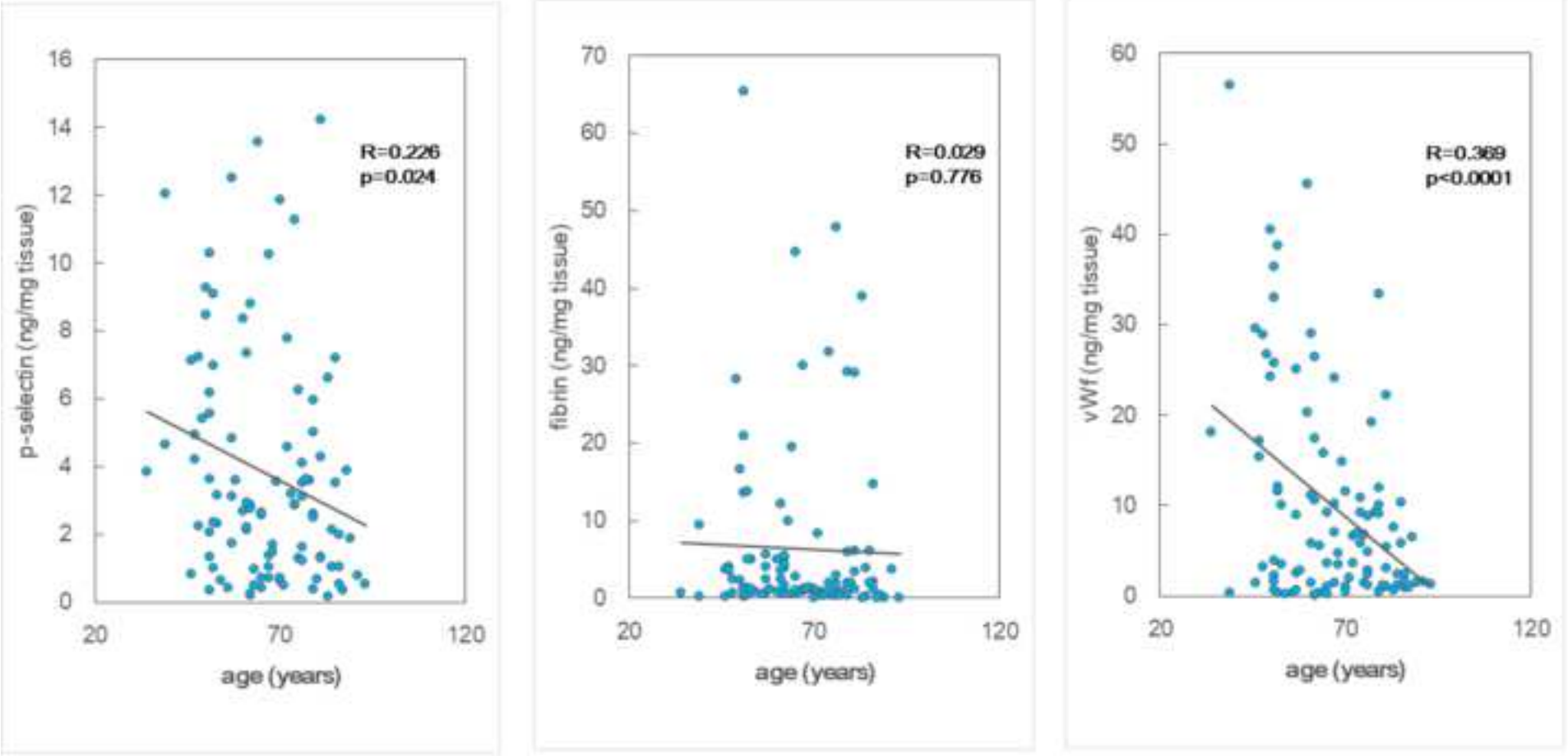

Figure 3

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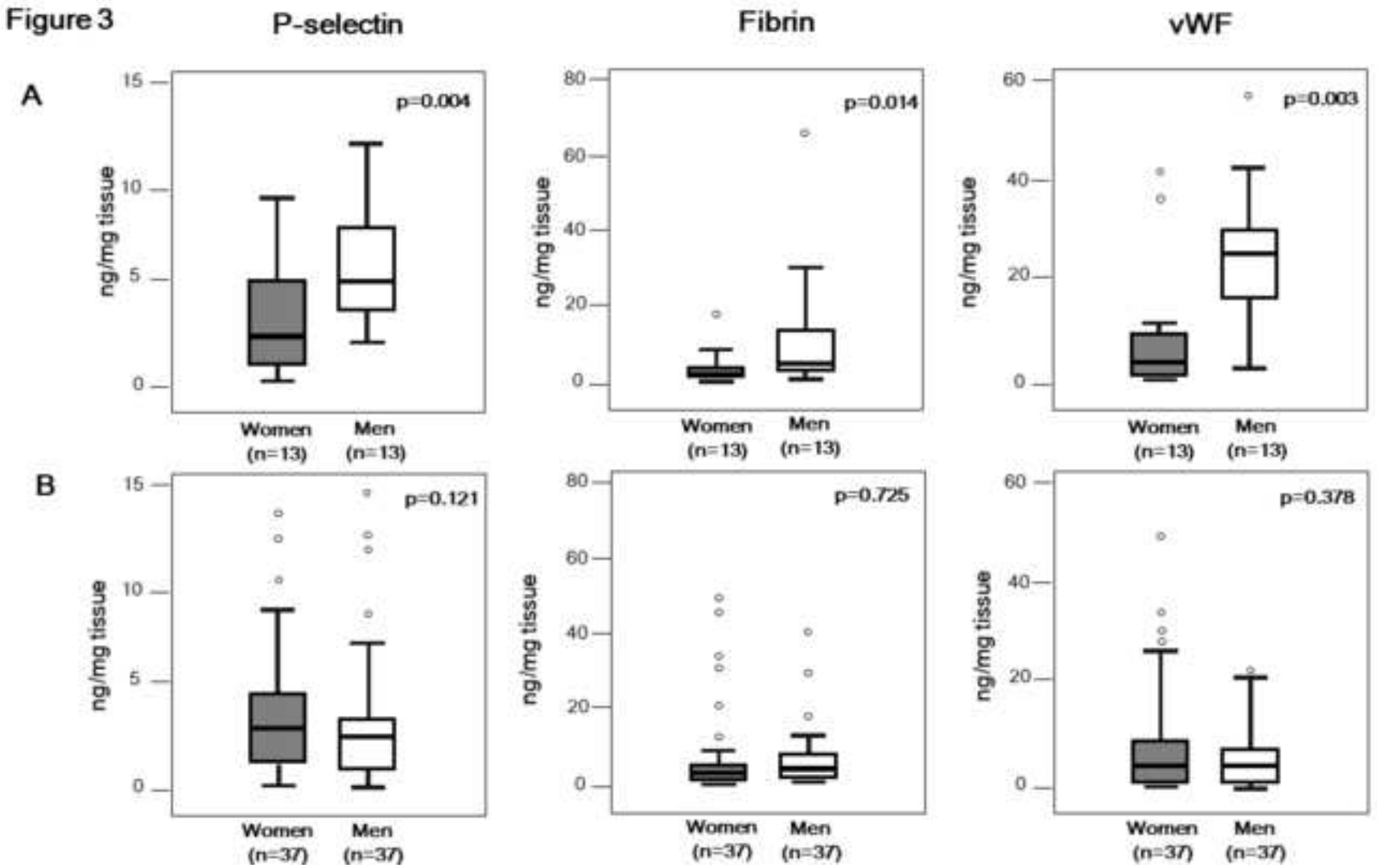

Figure 4A

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Figure 4A

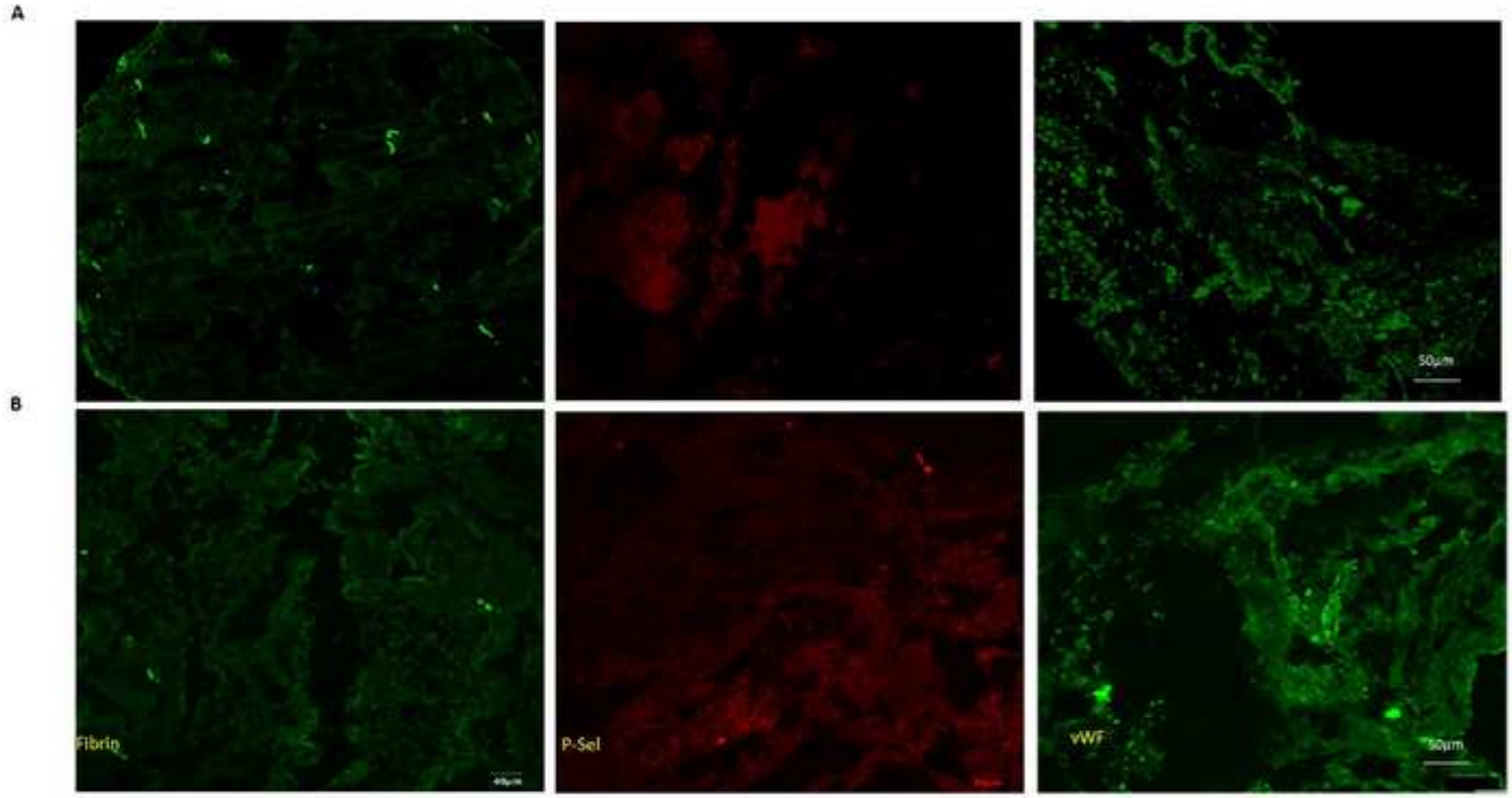

Figure 4B

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Figure 4B