

High *In vivo* Platelet Activity in Female Fibromyalgia Patients

Milovanovic M¹, Nilsson S², Haakara PI³, Post C⁴ and Gerdle B⁵

¹Department of Social and Welfare Studies, Linköping University, Linköping, Department of Internal Medicine, Vrinnevi Hospital, Norrköping, Sweden

²Primary Health Care and Department of Medical and Health Sciences, Linköping University, Norrköping, Sweden

³Department of Neurobiology, Society and Caring Sciences, Karolinska Institutet, Stockholm, Sweden

⁴Department of Clinical and Experimental Medicine, Linköping University, Linköping, Sweden

⁵Division of Community Medicine, Department of Medical and Health Sciences, Faculty of Medicine and Health Sciences, Linköping University, Pain and Rehabilitation Center, Anaesthetics, Operations and Specialty Surgery Center, Region Östergötland, Linköping, Sweden

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Corresponding author: Micha Milovanovic, PhD, Department of Welfare and Care, Faculty of Medicine and Health Sciences, Linköping University SE-601 74 Norrköping, Sweden, Tel: +46700896320; E-mail: micha.milovanovic@liu.se

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Abstract

Introduction: Fibromyalgia (FMS) is a pain syndrome characterized by chronic widespread pain and hyperalgesia/allodynia. Many affected are women and risk factors are unidentified. Today, a certain number of set criteria of disease signs and symptoms must be met for the diagnosis to be made. These criteria are used because of the lack of reliable biomarkers or other medical examination. The current study examines if *in vivo* platelet activity varies between FMS and controls without FMS.

Material and Methods: The study involves 24 females (age 38 + 9 (SD) years) with diagnosed FMS. 25 healthy females (age 50 + 12 (SD) years) without FMS served as controls. After sampling the whole platelet population was separated according to density with a linear Percoll™, into 17 density fractions. Platelet counts was carried out in all fractions using a routine cell counter. In addition, a flow cytometer was used to measure platelet bound fibrinogen without platelet agonist, reflecting *in vivo* platelet activity.

Results: The study groups did not differ with respect to the distribution of platelets in the gradient. FMS sufferers demonstrated a significant higher platelet bound fibrinogen in most of the platelet density fractions. In particular, significant differences ($p < 0.05$) were obtained in fractions numbers 2-14 and 16. In difference, fractions numbers 1, 15 and 17 did not show any significant variance.

Discussion: This is the first study to examine *in vivo* platelet activity in FMS. The results indicate that FMS is associated with elevated *in vivo* platelet activity compared to individuals without FMS. The clinical significance and the biochemical mechanisms regarding platelet heterogeneity are still uncertain. The results stimulate

further research to elucidate the importance of platelet diversity in FMS.

Keywords: Fibromyalgia; Fibrinogen; Platelets; Platelet activity; Platelet heterogeneity

Introduction

Fibromyalgia syndrome (FMS) is a chronic pain syndrome [1] associated with several symptoms including; pain, fatigue, sleep problems, depression, digestive problems, intestinal disorders and anxiety [2]. In 1990, the American College of Rheumatology (ACR) established the now generally used criteria for FMS. ACR agreed that patients must have widespread pain in combination with widespread pain hypersensitivity for mechanical pressure, which is captured using manual palpation in at least 11 of 18 standardized anatomical locations (tender points) [1,3]. FMS is an independent diagnosis accepted by the World Health Organization (WHO). The population prevalence of FMS in the western world is 2-4% and it affects women more often than men. It generally starts as a local pain condition which spreads through the body over time; the risk factors for this spreading are mainly unknown [4].

Pain medicine lacks objective biomarkers to guide the diagnosis and choice of treatment. Hence, there is a lack of reliable and objective blood tests (biomarkers) that can be used as a part of clinical assessment of patients that fulfill the ACR criteria of FMS. As pain by definition is a subjective experience it has been pointed out that biomarkers for pain are an impossibility [5]. Bäckryd has proposed that "nocimarker" would be a better term than pain biomarker for denoting attempts to find objective, measurable correlates to the neurobiological processes involved in different pain conditions [6]. FMS is associated with central alterations (e.g. central hyperexcitability with disinhibition and possibly

facilitation of nociceptive afferent activity) [7]. However, controversies exist regarding the role of peripheral factors e.g. peripheral nociceptive inputs, muscle and nociceptive C-fibre alterations and systematic alterations for maintaining central mechanisms [8].

Blood-including the subcomponents serum and plasma - reflects systemic aspects. Serum serotonin levels were significantly lower in FMS as compared to control individuals [9]. Behm and Associates investigated the immune role, specifically mononuclear cell cytokine production in FMS and reported lower levels of IL-5, IL-6, IL-8, IL-10 and IFN- γ [10]. Other studies have also reported alterations in the signature of cytokines in FMS [11,12]. Another study examined the peripheral benzodiazepine receptors on the leukocyte surface. There was an increased level of the receptors in monocytes [13]. Recently our group reported significantly increased plasma levels of lactate and glutamate in a cohort of chronic widespread pain (mainly FMS; 15 out of 17 subjects) [8]. There are very few studies regarding platelets features in FMS. Nevertheless, platelets collected from FMS patients had increased levels of magnesium and lower levels of adenosine triphosphate, in comparison with controls [14].

In whole blood, platelets are the smallest corpuscular body. They differ in density within the span of 1.04–1.08 kg/l [15,16]. Platelet organelles are key determinants of density; high-density cells have more α and dense granules [15]. In some studies it has been reported that platelet density increases as they get older [17,18], whereas other studies have come to an opposite view [19,20]. Furthermore, other scientists take the view that platelet density does not change in the circulation [21-23]. The clinical impact of platelet heterogeneity has been investigated for a long period of time. Platelet density is increased in conjunction with acute myocardial infarctions (AMI) [24]. ST-elevation AMI is further characterized by an inverse relationship between density and the inflammatory response [25]. The activity of inflammatory bowel disease is linked to small high-density platelets [26]. Low peak platelet density characterizes essential thrombocythemia [27] and pre-eclampsia severity is associated with large platelets having low peak density [28].

The study aims to investigate if platelet *in vivo* activity differ in FMS compared to a control group (CON) without FMS.

Material and Methods

Subjects

The study was approved by the local ethics committee of Linköping University, Sweden (reg. number: 2012/269-32). All participants gave informed consent. 24 female patients affected by FMS, aged 38 \pm 9 years (mean \pm SD) participated in the study. All FMS subjects were enrolled for this study from patients seeking care at the Pain and Rehabilitation Centre of the University Hospital, Linköping, Sweden. Patients fulfilling the criteria of the American College of Rheumatology criteria for FMs were included in the study [1]. The clinical diagnoses of FMS were retrospectively confirmed from the patient's case

histories and clinical examinations. 25 healthy females aged 50 \pm 12 years (mean \pm SD) were used as a CON. All of the CON group were enrolled in the study when visiting a nearby medical center for a normal routine check. **Table 1** summarizes clinical data at the time of entry to the study.

Table 1 Demographic data and pharmacological treatments at study inclusion for fibromyalgia syndrome patients (FMS) and controls (CON).

	FMS	CON	p-value
Subjects (n)	24	25	
Gender (male/female)	0/24	0/25	
Age (years)	38 \pm 9	50 \pm 12	NS
Body mass index (kg/m ²)	28 \pm 7	23 \pm 3	p < 0.05
Diabetes (n)	0	0	NS
A2-blockers (n)	0	2	NS
ACE-inhibitors (n)	2	3	NS
Acetaminophen (n)	18	0	p < 0.05
Antidepressant (n)	22	0	p < 0.05
β -blockers (n)	1	5	NS
Ca ²⁺ -blockers (n)	0	6	NS
Diuretics (n)	0	5	NS
NSAID (n)	11	0	p < 0.05
Statins (n)	0	3	NS
Vitamin B12 (n)	1	0	NS

Laboratory investigations

All blood samples were collected in Vacutainer™ tubes (Becton and Dickinson, New Jersey, USA.). Venous blood (7.5 mL) was anticoagulated with 2.5 mL 0.129 M disodium citrate. In order to separate platelets according to density a linear Percoll™ (GE Healthcare Bio-Sciences AB, Sweden) gradient was used [29,30]. The following substances were mixed in order to provide the two Percoll™ solutions (1.09 and 1.04 kg/L) for the gradient (**Table 2**).

Table 2 List of Solutions.

Percoll™ solutions	1.09 kg/L	1.04 kg/L
H ₂ O	11.42 g	19.14 g
Percoll™	32.84 g	8.88 g

Platelet-inhibitory solution	4.50 g	3.00 g
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To avoid *in vitro* platelet activity, the Percoll™ solutions contained a platelet inhibitory solution (Na₃EDTA and prostaglandin E1).

A two-chamber gradient maker was used to produce linear gradients. The gradients were manufactured in 50 mL test tubes covering the density span of 1.09 kg/L to 1.04 kg/L. 7.63 g of the 1.09 kg/L Percoll™ mixture was layered in the bottom of the test tube. Then, 13.08 g of the 1.09 kg/L and 12.48 g of the 1.04 kg/L Percoll™ solutions were employed into two-chamber gradient makers to make the gradient. Subsequently, 10 mL citrate anticoagulated whole blood was carefully layered on top of a 50 mL test tube with the completely produced gradient. The tube was thereafter centrifuged at 2767 g for 1½ hours. After centrifugation, the underside of the test tube was punctured and the contents was separated by gravity into 17 different density fractions [30]. By this setting every fraction holds about 2 mL of the test tube content. Platelet counts were determined in all fractions using a CELL-DYN 4000 (Abbott Diagnostics, Illinois, USA). Platelet bound fibrinogen (%) were also measured in each fractions with a Beckman Coulter EPICS XL-MCLTM Flow Cytometer (Beckman Coulter, Inc., California, USA). Platelets were identified with a PE-conjugated antibody against GPIb (Dako AS, Denmark). A FITC-conjugated chicken antihuman fibrinogen polyclonal antibody (Biopool AB, Sweden) discriminated membrane bound fibrinogen [31]. As no agonist was added platelet bound fibrinogen in this setting mirrors *in vivo* platelet activity.

Statistics

Microsoft Excel® was used for the statistical evaluations. In text and tables are reported mean values ± one standard deviation (SD). The unpaired Student's t test were employed for evaluating quantitative data. p-values ≤ 0.05 were regarded to indicate significance.

Results

Back-ground data

No significant difference in age was found (Table 1). The Body Mass Index (BMI) was higher in FMS than in CON (p < 0.05) (Table 1). Except demographic data, Table 1 also shows as expected differences in medication where the most notable was the differences in consumption regarding Acetaminophen, Antidepressant and NSAID medicines (p < 0.05).

Laboratory data

The distribution of platelets in the 17 density fractions is demonstrated in Figure 1. There was no difference between the groups. Figure 2 shows *in vivo* platelet activity i.e. % fibrinogen bound platelets in 17 different density fractions. FMS compared to the CON, showed significantly more fibrinogen bound platelets in most of the platelet density fractions. Especially, numbers 2-14 and 16 displayed

significantly higher platelet *in vivo* activity (p < 0.05). In contrast, the platelet from fractions (numbers 1, 15 and 17) did not circulate activated in FMS (Figure 2).

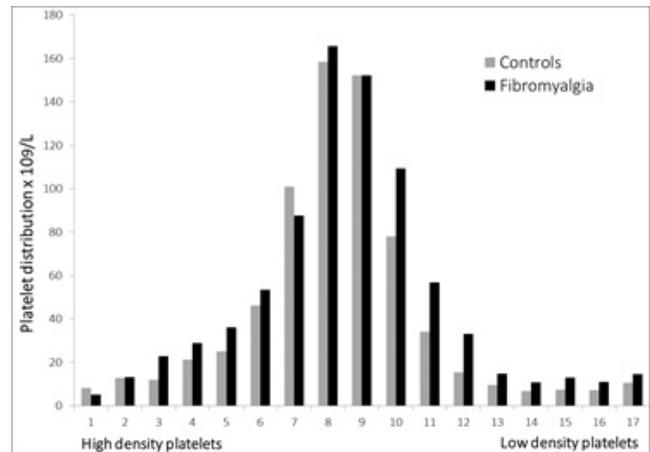


Figure 1 The distribution of platelets (×10⁹/L) in the gradient for FMS patients and controls. Fraction no. 1 contains platelets having the highest density and fraction no. 17 contains platelets having the lowest density.

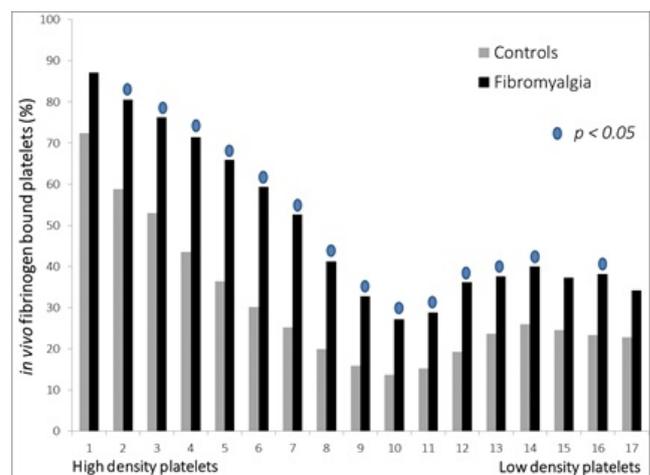


Figure 2 Platelet bound fibrinogen (%) i.e. *in vivo* platelet activity, is shown for FMS patients and healthy controls. The densest platelets are found in fraction no. 1 and the lightest in fraction no. 17.

Discussion

The current study shows that FMS is characterized by substantial alterations in heterogeneity of *in vivo* circulating platelets. Indeed, compared with controls FMS patients showed a higher *in vivo* platelet activity i.e. platelet bound fibrinogen. To the best of our knowledge, this is the first report investigating FMS with respect to platelet bound fibrinogen in different platelet density subpopulations. One can assume that the current method has several weaknesses. One of these are the preanalytical effect on platelet activation. It can probably

be considered that if platelets are treated as described it is impossible to avoid an activation of the platelets, which will affect the results obtained. However our opinion is that the effect is comparable in the two groups, i.e. we have dealt with and performed all analyzes in exactly the same way on every individual in both groups. If the activation of the platelets had occurred in the preanalytical process, it had happened in both groups. In any case, the result shows a significant difference whether a preanalytical activation has appeared or not. A cautious conclusion that we can draw from our findings is that the disease is connected to *in vivo* activated platelets.

It is also well known that platelet function is affected by anti-platelet drugs in the body. However, some individuals exhibit strong reactions while others only have weak platelet inhibition. Earlier studies have shown that individual variation exists when this drug is given [32]. In the current study none of the controls received any anti-platelet drugs, still, it is proposed that NSAID drugs affect platelet aggregation to varying degrees [33]. In the current study 11 of the FMs did receive NSAID. It is possible that the drug influenced the results of platelet fibrinogen binding (**Figure 2**). One can postulate that without NSAID FMS would have an even higher percentage of platelet fibrinogen binding.

Also, it is well-known that platelet activity measurements can depend on the platelet number, nevertheless, platelet distribution (**Figure 1**) was similar in both groups, i.e., no differences were found between the groups. For this reason we do not see the platelet count as an important factor that could have affected our obtained results.

The main criticism of this study is probably that we have not selected an age-matched control group. Obviously there is a difference in age between both groups, but not significant. The control group consists of some older individuals. It is however very difficult to comment on whether the age of patients alter platelet activity. There are no studies that support the latter. We have assumed that the age difference between the groups should not affect platelets. Moreover, for obvious reasons we only selected women in the control group because FMS group consisted of women.

The present method used in this work is relatively simple to perform, for that reason, it should be possible to use the method as a supportive diagnostic tool in the clinical examination of patients with widespread pain. However, it is important to verify the present results in other cohorts of FMS. It is also important to investigate if the finding of activated platelets is specific for FMS or could be connected to chronic pain of different etiologies.

The clinical significance of the observed platelet heterogeneity i.e. activated *in vivo* platelets remains unclear; including what may be the origin and consequence of disparities. One cannot judge the clinical relevance of our findings based on the present study. For this reason, it is important to make further attempts to investigate why the platelets are activated in FMS and in such studies include assessments of co-morbidities.

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Conflict of Interest Statement

We declare that no economic relationships exists that can be construed as a conflict of interest.

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