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Platelet density distribution in essential thrombocytemia

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Running head: Platelet density in essential thrombocytemia

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Abstract

Background: Essential thrombocythaemia (ET) is characterised by high platelet counts and slightly increased bleeding risk. Why severe haemorrhages do not occur more frequently is not known. Variations of platelet density (kg/L) depend mainly on cell organelle content, in that high-density platelets contain more α- and dense granules. This study compares ET patients and healthy volunteers with respect to platelet density subpopulations.

Methods: Platelet density distribution was examined in ET patients (n=2) and healthy subjects (n=2) served as controls. A linear Percoll™ -gradient containing prostaglandin E_1 was employed to separate platelets according to density. The platelet population was subsequently divided by density into 16 or 17 subpopulations. Determination of platelet counts was carried out. Platelet *in vivo* activity i.e. platelet bound fibrinogen was measured in each density fractions using a flow cytometer. To characterize platelet subpopulations we determined intracellular concentrations of CD40Ligand and P-Selectin in all fractions.

Results: Patients and controls demonstrated similar platelet density distribution. High-density platelets had more surface bound fibrinogen in conjunction with signs of platelet release reactions i.e. with few exceptions they contained less CD40L and P-Selectin. Peak density platelets showed less surface bound fibrinogen. These platelets contained less CD40L and P-Selectin than nearby more dense populations. The light platelets had more surface bound fibrinogen than peak platelets together with elevated concentrations of CD40L.

Conclusion: In ET, the malignant platelet production could exist together with platelets originating from normal megakaryocytes. It is also possible that clonal megakaryocytes produce platelets covering the entire density span. The “normal” density distribution offers a tenable explanation as to why serious bleedings do not occur more frequently.

Introduction

Essential thrombocytosis (ET) is a myeloproliferative disorder [1, 2]. The disease is characterized by excessive platelet production and a slightly elevated risk for bleedings [3]. ET platelets are dysfunctional [1] and they have low density (kg/L) [4]. Bleedings are related to platelet abnormalities but the relationship is weak [5].

In health, platelet density differs within the span 1.04 to 1.08 kg/L [6-8]. The variation depends mainly on platelet organelle content in that high-density platelets have more α- and dense granules. They further contain more glycogen [8]. Platelets density is related to size and metabolic activity [6]. Some authors take the view that density decreases as platelets circulate [9, 10]. Other studies suggest that platelets recently created are less dense and that density increases with age [11-13]. Finally, evidence exists indicating that platelet density does not change *in vivo* [14-16].

Platelet CD40L is located either in platelet α or in dense granula [17]. CD40L is rapidly translocated to the platelet surface upon activation [18]. In resting platelets P-Selectin is located in α-granula. The molecule is moved to the cell surface upon activation [19, 20].

Previous work from our laboratory shows that healthy individuals have high density platelets with more surface bound fibrinogen *in vivo*. Consequently, they circulate more activated [21]. We also described that low-density populations display more membrane bound fibrinogen indicating platelet activity. In contrast, the study also demonstrates that peak density platelets were less activated as estimated from *in vivo* platelet-bound fibrinogen [21].

The current study evaluates if the clonal platelet production in ET affects platelet density variations. In particular, it investigates and characterizes platelet density subpopulations with respect to platelet *in vivo* activity. To further characterize these different populations, we also determined intracellular concentrations of CD40L and P-selectin.

Material and methods

The study was approved by the research ethical committee at the Faculty of Health Sciences, Linköping, Sweden, and informed consent was obtained from all patients. We included two ET patients and two healthy blood donors served as controls. The diagnosis was established according to 2001 WHO diagnostic criteria. Peripheral blood samples (7.5 ml) were collected and anticoagulated with 2.5 ml 0.129 M disodium citrate. The separation procedure has been

described in detail [22]. In short, two isotonic Percoll™ (GE Healthcare Bio-Sciences AB, Sweden) solutions (1.04 and 1.09 kg/L) were fabricated by mixing appropriate volumes of Percoll™, H_2O and the following platelet inhibitory solution: A: 2.7 mmol/L theophylline dissolved in 0.15 mol TRIS chloride buffer (pH 7.4 at 25°C), B: 1 mg/L prostaglandin E_1 and 1 mL 95% ethanol in H_2O, C: 0.15 mol/L Na_2 citrate and 0.13 mol/L Na_3EDTA (pH 7.4 at 25°C). A two-chamber gradient maker was used to manufacture linear Percoll™ 50 mL gradients covering the density span; 1.04 to 1.09 kg/L. Subsequently, citrate anticoagulated whole blood was carefully layered on top of the gradient. It was then centrifuged at 2767 x g for 1.5 hours thus separating platelets by density [22]. Thereafter, the bottom of the test tube was perforated and the gradient divided into 16 or 17 aliquots (density fractions) each containing approximately 2 mL of the test tube content. In all fractions platelet counts and platelet fibrinogen binding without antagonist challenge were determined. Furthermore, to ensure the efficacy of the inhibitory solution platelet bound fibrinogen was analyzed after ADP challenge (8.5 µmol/L). Subsequently, cells were lysed using Triton X-100 (final concentration 1 %). After removing cell debris, in all platelet density fractions intracellular concentrations of CD40L and P-Selectin were determined.

Platelet counting was done electronically. Platelet bound fibrinogen was determined with, a Beckman Coulter EPICS XL-MCL™ Flow Cytometer (Coulter Corp., USA) as previously described [23]. In brief, a fluorescein isothiocyanate-conjugated chicken antihuman fibrinogen polyclonal antibody (Biopool AB, Sweden) was applied to detect surface bound fibrinogen. Platelets were identified with a phycoerythrin-conjugated antibody against GPIb (Dako AS, Denmark). The number of platelets (%) having more surface bound fibrinogen than a predetermined control was used as an experimental parameter. Commercial ELISA-kits were employed to determine CD40L and P-Selectin (R&D, UK).

Results

ET patient no. 1 (Figure no. 1) showed one density peak (diagram no. 1). High-density platelets displayed more cell bound fibrinogen than peak platelets, both spontaneous (diagram no. 2) and after ADP challenge (diagram no. 3). The two densest fractions contained platelets with less intracellular CD40L and P-Selectin (diagrams nos. 4+5) than the neighbouring less dense platelets. Peak density platelets, were less activated than the densest populations as estimated from platelet bound fibrinogen (diagram no. 2). The lightest platelets contained more intracellular CD40L and P-Selectin than the neighbouring populations (diagrams nos. 4+5).

Finally, diagram no. 5 shows that the lightest platelets had more intracellular P-Selectin than peak platelets.

The second ET patient, (Figure no. 2) confirmed previous results and showed one platelet density peak. Again high-density platelet populations displayed more platelet bound fibrinogen, both spontaneous and after ADP challenge (diagrams nos. 2+3). In addition, these populations contained less CD40L (diagram no. 4) and P-Selectin (diagram no. 5) than the adjoining less dense platelets. Compared with the neighbouring more dense populations peak density platelets again demonstrated less platelet bound fibrinogen in conjunction with lower intracellular CD40L and P-Selectin. Low-density populations displayed more surface bound fibrinogen than the nearby platelets indicating platelet activity (diagrams nos. 2+3). The amount of intracellular CD40L was higher than the surrounding more dense platelet (diagram no. 4). Finally, these platelets also contained more intracellular P-Selectin (diagram no. 5) than peak platelets.

Figure no. 3 displays results for the first healthy subject. The platelet distribution in the gradient revealed one single peak (diagram no. 1). High-density platelets again displayed more spontaneous platelet bound fibrinogen and ADP challenge did not affect this finding (diagrams nos. 2+3). Similar to ET patients these platelets had lower intracellular levels of CD40L and P-Selectin (diagrams nos. 4+5). The densest platelets, however, constitute an exception as they contained more intracellular P-Selectin. The subject further had peak density platelets with less surface bound fibrinogen (diagrams nos. 2+3). These populations further demonstrated lower intracellular CD40L and P-Selectin content (diagrams nos. 4+5) than the surrounding platelets. Finally, again the lightest platelets had more surface bound fibrinogen and more intracellular CD40L and P-Selectin (diagrams nos. 4+5) than peak platelets.

The investigation of platelets from the second healthy participant demonstrated similar results (Figure no. 4). Again one density peak was found (diagram no. 1). Without agonist challenge, high-density platelets showed enhanced platelet fibrinogen binding (diagram no. 2). In agreement with previous subjects these platelets displayed lower amounts of intracellular CD40L and P-Selectin (diagrams nos. 4+5). Peak density platelets again showed less platelet bound fibrinogen than the most dense platelets, both unstimulated (diagram no. 2) and after ADP challenge (diagram no. 3). Once more these platelet populations displayed low amounts of intracellular CD40L and P-Selectin (diagrams nos. 4+5). Finally, the lightest platelets showed more surface bound fibrinogen (diagram no. 3) than peak platelets. The

lightest platelets also demonstrated more intracellular CD40L and P-Selectin (diagrams nos. 4+5) than peak density platelets.

Discussion

The current study reveals similarities between platelets of ET patients and healthy individuals with respect to platelet density (Figures nos. 1-4). All participants showed peak density platelets with less surface bound fibrinogen. Most of these platelets had lower intracellular P-Selectin and CD40L levels than nearby more dense populations. In addition, high-density platelets were more activated *in vivo* i.e. they displayed more membrane bound fibrinogen than peak platelets. High density cells contained less intracellular CD40L and P-Selectin than neighbouring less dense platelets. The finding indicates release reactions occurring *in vivo*. Finally, the study confirms previous work [21] in showing that the lightest platelets were more activated *in vivo*. They further contained more P-Selectin than peak platelets. ET platelets could be a mixture of cells produced by clonal and healthy megakaryocytes. As a consequence, the latter cells could be responsible for platelet density diversity. It is also tenable that malignant megakaryocytes produce platelets having different density. Thus, similarities to healthy individuals with respect to platelet density distribution could explain why bleeding do not occur more frequently in ET.

In recent years interest for platelet subpopulations has increased most notable is the description of coat platelets [24]. These cells express high levels of procoagulant proteins after maximal stimulation with collagen and thrombin. Coat platelets constitute approximately 30% of the total platelet population [24]. For instance Alzheimer´s disease is characterized by an increased number of coat platelets and these cells are associated with disease progression [25]. Others have described a subpopulation of circulating activated platelets in acute coronary syndromes. These platelets are impossible to stimulate further [26]. These examples indicate that it is possible that platelet subpopulations could be important in human pathophysiology.

The Percoll™ gradients included a "blocking solution" containing prostaglandin E_1 to avoid artifacts due to platelet activation during separation. The control experiment i.e. ADP challenge did not raise platelet bound fibrinogen significantly (Figures 1-4). Thus, the inhibition solution blocks platelet activity effective. As a consequence, with a high degree of certainty it is possible to conclude that high and low density platelet subpopulations circulate more activated.

High density platelets demonstrate more surface bound fibrinogen indicating *in vivo* activation. These cells also contained less CD40L and P-Selectin than neighbouring less dense platelets. The latter findings suggest *in vivo* release reactions. The lightest platelets could be contaminated with extracellular P-Selectin and CD40L from plasma located on the top of the gradient. It may explain why these platelets contain more CD40L and P-Selectin than neighbouring more dense platelets. The location of CD40L in unstimulated platelets is not known. However, α-granule release is associated with CD40L translocation [20]. The densest platelets with signs of platelet *in vivo* activity contained less CD40L and P-Selectin. The finding indicates [20] that CD40L liberation is closely related with α-granule degranulation.

Low density platelets contained more P-Selectin indicating enhanced α-granule content. Earlier work [21] shows that these low density populations have less dense bodies. Thus, low levels of other intracellular constituents determining platelet density such as dense granule and glycogen could explain why these cells have low densities despite enhanced α-granule content. A previous study from our laboratory concluded that platelets utilize intracellular fibrinogen when activated in surroundings depleted of Ca^{2+} and fibrinogen [21]. The conclusion was premature. In contrast, to our previous hypothesis this study shows that platelet bound fibrinogen with a high degree of certainty reflects *in vivo* platelet activity.

The current study demonstrates that ET patients and healthy volunteers display similar platelet density patterns. Some caveats exist most notable is the low sample size. However, each subject has been investigated extensively. The work makes it tenable to hypothesize that platelet density variations in ET could at least partly explain why bleedings do not occur more frequently. The present study stimulates to further research to evaluate the role of platelet subpopulations in human physiology and in disease state.

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Figure legends

Figure 1: Results for first ET patient. Diagram no. 1 shows the platelet distribution in the gradient. Diagram no. 2 displays the spontaneous platelet surface fibrinogen binding. Figure no. 3 shows the platelet fibrinogen binding after stimulation with ADP. Graphic no. 4 demonstrates for all the fractions the total amount of P-Selectin per fraction divided by the platelet counts. Last diagram illustrates the total quantity of CD40L per aliquots adjusted for the number of platelets.

Figure 2: Data for second ET patient. Diagram no. 1 reveals the distribution of platelets in the gradient. For each fraction the spontaneous platelet fibrinogen binding is given in diagram no 2. Figure 3 illustrates the platelet fibrinogen binding when stimulating with ADP. In graphic no. 4 displays for all fractions, the total amount of P-Selectin per divided by the platelet counts is shown. The last diagram shows the total quantity of CD40L per aliquots corrected for platelet counts.

Figure 3: The figure demonstrates data for blood donor no. 1. Diagram no. 1 displays the distribution of platelets in the gradient. For each platelet subpopulation the spontaneous platelet fibrinogen binding is revealed in diagram no. 2. Figure no. 3 presents the platelet fibrinogen binding after ADP stimulation. Graphic no. 4 shows for each fraction the total amount of P-Selectin divided by the platelet counts for all the fractions. Last diagram demonstrates the total quantity of CD40L per aliquots adjusted for platelet counts.

Figure 4: Results for healthy donor no. 2. Diagram no. 1 demonstrates the distribution of platelets in the gradient. Diagram no. 2 confirms the spontaneous platelet fibrinogen binding. Figure no. 3 shows reveals the ADP induced platelet fibrinogen binding. Diagram no. 4 illustrates the total amount of P-Selectin per fraction divided by the platelet counts for all the fractions. The very last graphic shows the total quantity of CD40L per aliquots corrected by the platelet counts.

Figure 1: Patient no. 1

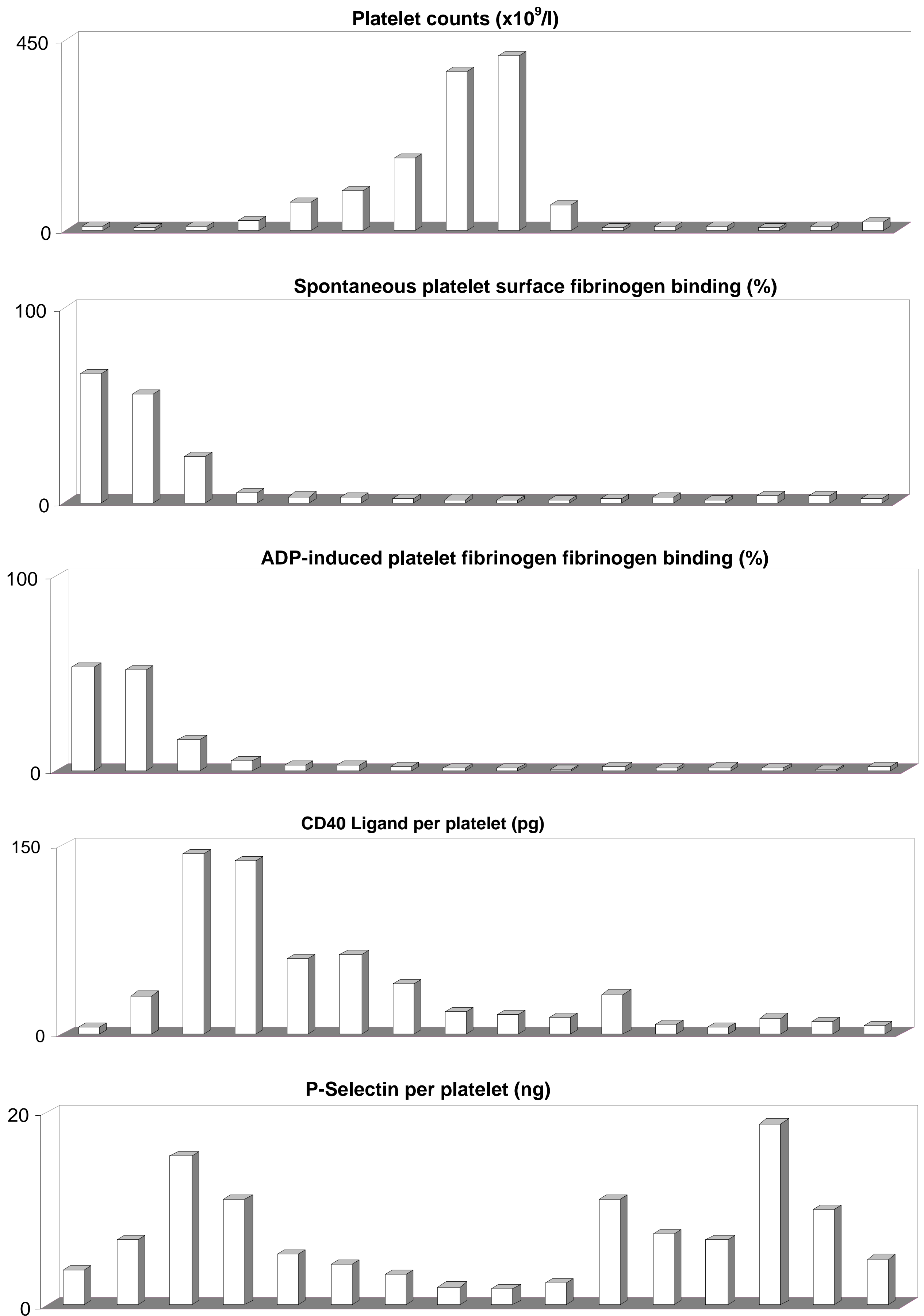

Figure 2: Patient no. 2

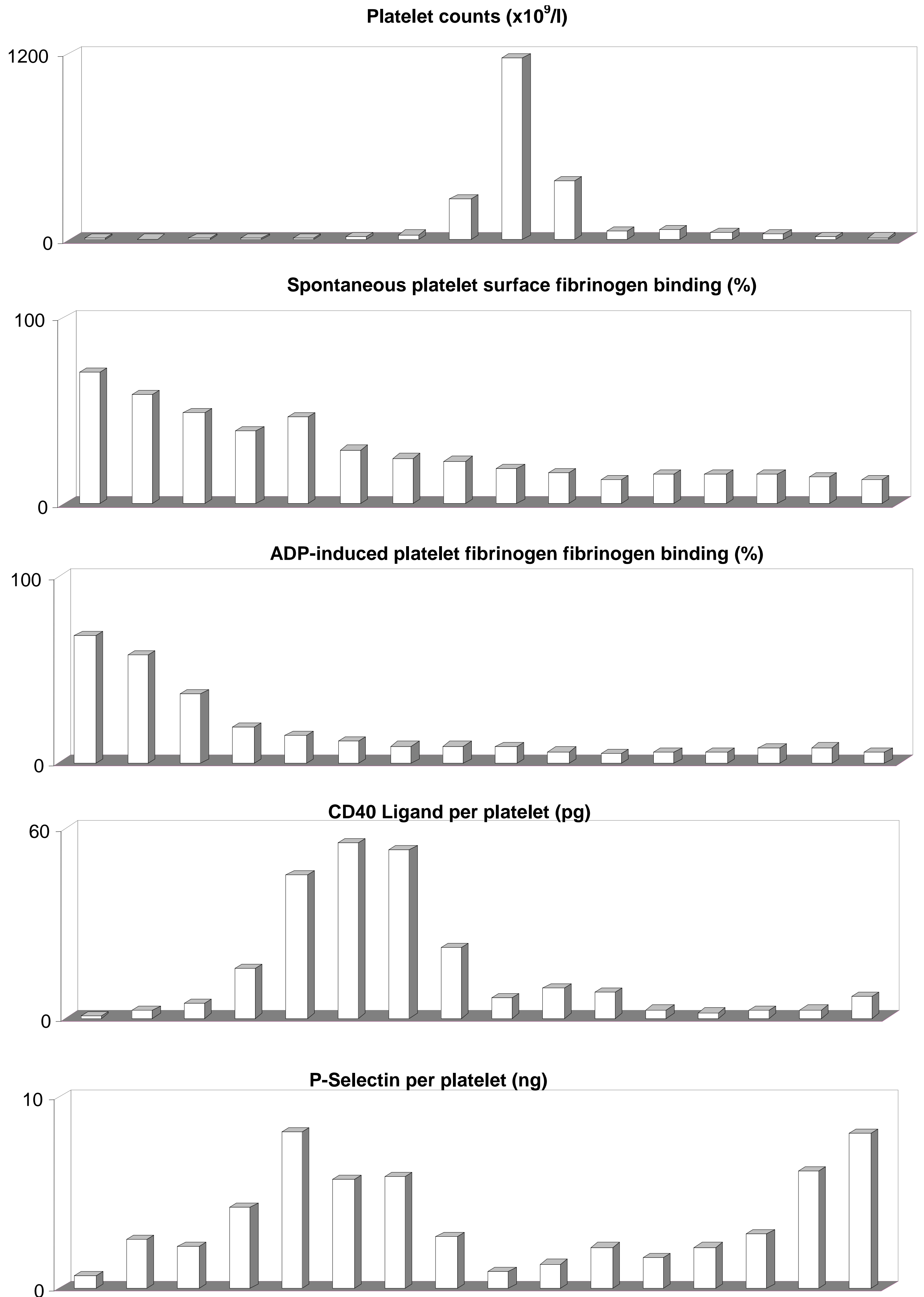

Figure 3: blood donor no. 1

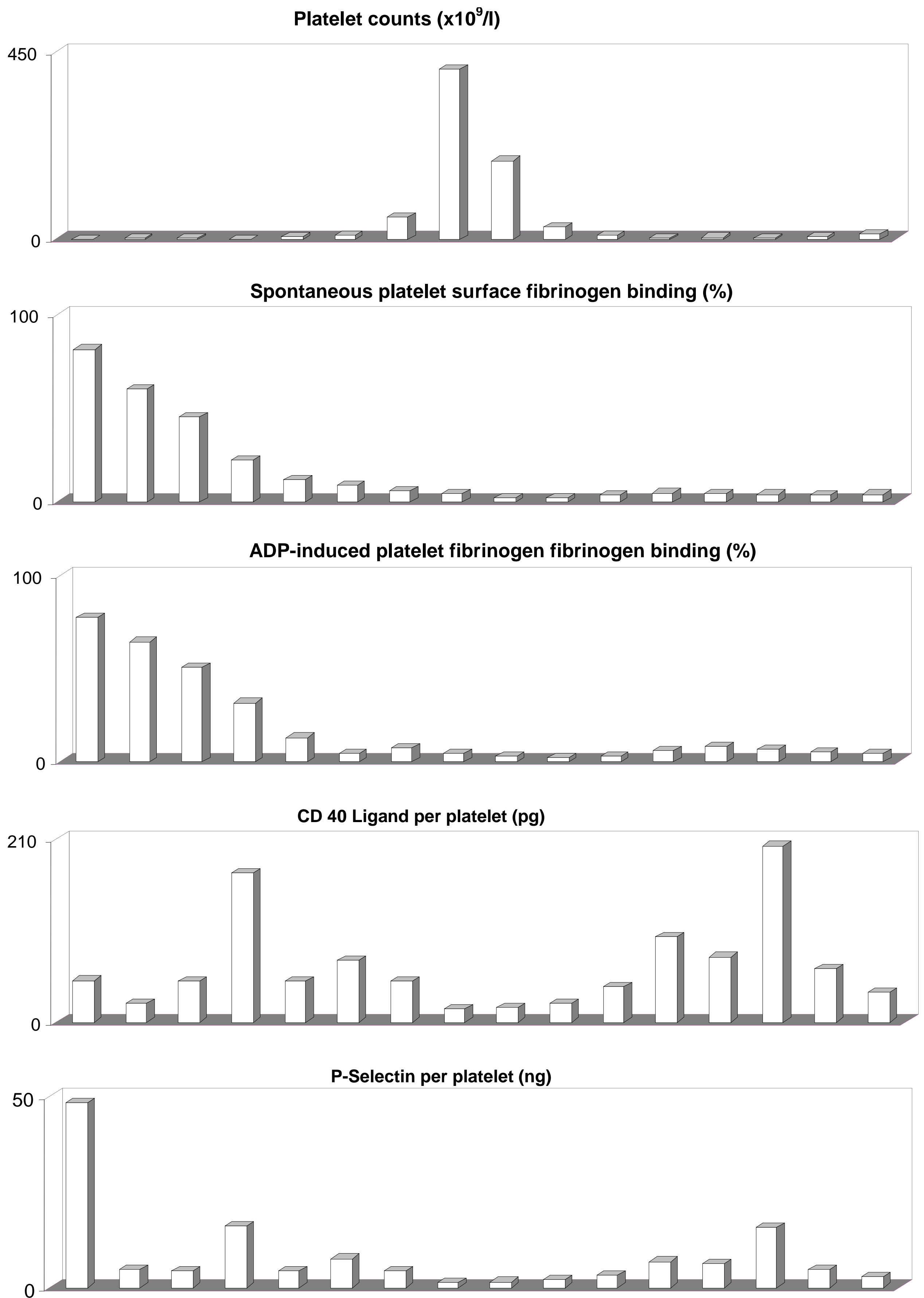

Figure 4: blood donor no. 2