METHOD ARTICLE

Tissue Factor expression in circulating human platelets is a property of large size platelets

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Abstract

Tissue Factor (TF), the key activator of the blood coagulation cascade and of thrombus formation, is also expressed by circulating human platelets. Despite the documented in-depth characterization of platelet TF carried out in the past fifteen years, the platelet-associated TF still remains a rather controversial issue. Some authors still fail to identify TF in platelets, especially when assessment in platelet rich plasma (PRP) or washed platelets is carried out. This study aims to extend the characterization of the subset of TF-positive platelets in PRP from healthy subjects and to verify how different centrifugation forces, used to prepare the PRP, could affect the analysis of TF-positive platelets. Data indicate that the expression of TF in circulating human platelets is a property of large size platelets both in terms of TF protein as well as in terms of TF mRNA. Therefore, particular attention must be paid to the preparation and collection of the PRP since this subset of cells can easily be lost if centrifugation protocols are not optimized thus affecting subsequent analysis, whether it is flow cytometry, functional activity tests, proteome or transcriptome analysis.

Introduction

In the past fifteen years evidence has been accumulated that Tissue Factor (TF), the transmembrane glycoprotein key activator of the blood coagulation cascade and of thrombus formation, is also expressed by circulating human platelets, suggesting that these cells can control the generation of thrombin contributing not only to the propagation of the clotting cascade but also to the initiation phase [1].

Upon activation with the classical platelet agonists such as thrombin, ADP, etc., platelets express on their surface a functionally active TF protein able to bind Factor VIIa thereby triggering thrombin generation [2-6]. Of interest, in pathological conditions such as essential thrombocythemia, cancer, diabetes, and coronary artery disease the number of TF-positive platelets significantly increases thus providing an additional explanation of the higher thrombogenicity documented in those clinical settings [1]. In addition to the protein, and despite the fact that platelets do not have a nucleus, they also contain megakaryocyte-derived TF pre-mRNA and mRNA that can be used for *de novo* protein synthesis [3,5-7].

Until recently, the transfer of TF-positive microparticles released from TF-expressing cells (activated monocytes, granulocytes or endothelial cells) has been considered the main mechanism responsible for the presence of TF in platelets, based on the findings reported at the beginning of this century by Rauch et al., who first documented by immunoelectron microscopy the microparticle transfer mechanism [8]. In the same period of time, the failure to detect TF in megakaryocytes further corroborated the concept that platelets could not derive TF from their parental cells but they have to acquire the protein from circulating microvesicles [2].

Although the microparticle-transfer mechanism may contribute to the presence of TF in platelets, later on we and others have shown that not only human platelets, but also human megakaryocytes contain TF mRNA [2,5,9]. Moreover, we have recently also provided compelling evidence that TF is an endogenously synthetized protein that characterizes human megakaryocyte maturation. In the absence of any crosstalk with other cells or microparticles, a finely-tuned mechanism is responsible for the direct transfer of TF from megakaryocytes to a subset of platelets (~25-35%), where it contributes to their thrombin generation capacity [7].

Despite the documented in-depth characterization of platelet TF carried out in the past fifteen years, some authors still fail to identify TF in platelets, especially when assessment in platelet rich plasma (PRP) or washed platelets is carried out [10,11].

The current study was undertaken to further extend the characterization of the subset of TF-positive platelets in PRP from healthy subjects by using flow cytometry, thrombin generation assay and Real Time PCR. We also verified how different centrifugation forces, used to prepare the PRP, could affect the analysis of TF-positive platelets.

Materials and Methods

Antibodies and reagents. Antibodies were as follows: mouse anti-human GpIIb (CD41a-PerCP-Cy5.5 monoclonal antibody, mAb, BD Biosciences, cat. 333148); mouse IgG_1 PerCP-Cy5.5 (BD Biosciences, cat. 347202); mouse IgG_1 FITC (BD Biosciences, cat. 345815); mouse anti-human TF mAb (Sekisui Diagnostics, cat. 4507CJ and cat. 4508CJ), goat anti-human TF polyclonal Ab (Sekisui Diagnostics, cat. 4501). Reagents were as follows: CTI (cat. CTI-01) were from Haematologic Technologies Inc; BD CompBeads (cat. 552843) from BD Biosciences; ADP, TRAP-6, U46619 and EDTA from Sigma-Aldrich; paraformaldehyde and Triton X-100 from Carlo Erba. Actichrome TF and Imubind® Tissue Factor were from Sekisui Diagnostics; all the reagents for thrombin generation assays were from Stago; iQ™ SYBR® Green supermix was from Bio-Rad Laboratories; QIAzol Lysis Reagent, miRNeasy Mini kit for RNA extraction from Qiagen; all other reagents for RT and RealTime PCR experiments were from Life Technologies.

Blood collection and PRP preparation. Blood was collected by venipuncture of the antecubital vein of healthy volunteers (n=10, 5 males and 5 females, mean age 37±5 years) who did not take antiplatelet drugs within 10 days before blood donation and who gave their informed consent to participate in the study. Whole blood (WB) was drawn with a 19-gauge needle without venous stasis into citrate (1/10 volume of 0.129M sodium citrate)- and corn trypsin inhibitor (CTI, 50µg/ml)-containing tubes (Vacutainer, Becton Dickinson) discarding the first 4 ml. For platelet rich plasma (PRP) preparation, WB was centrifuged (room temperature, centrifuge brake off) at different g as indicated in Figure 1. The top ¾ and the bottom ¼ of the PRP, and the remaining blood were analyzed with the Sysmex XE-2100 Automated Hematology Analyzer to determine the platelet count, the platelet recovery (defined as the percentage of platelet count in PRP where the number of platelets in WB was set to 100%), the Mean Platelet Volume (MPV), the Immature Platelet Fraction (IPF), and the Platelet Distribution Width (PDW). In order to obtain samples enriched in small and large platelets, WB was centrifuged at 200g for 10 minutes and sequential aliquots of 200µl of PRP were taken and analyzed with the Sysmex XE-2100 Automated Hematology Analyzer. TF expression and activity were evaluated in the PRP fractions with the lowest (small platelets) and highest (large platelets) MPV. Only platelet preparations devoid of leukocyte contamination assessed as previously described [12] were used for TF analysis.

Inter-operator variability was 14%, while intra- and inter-individual variability were 5.6% and 19%, respectively.

Platelet TF analysis by flow cytometry. Conventional flow cytometry analysis of the physical parameters, side (SSC) and forward (FSC) scatter of the platelets recovered in the top ¾ of the PRP prepared as reported in Figure 1 was performed on a BD FACSCalibur instrument (Becton Dickinson) equipped with a 15-mW, air-cooled, 488-nm argon-ion laser. Cytometer performances were checked daily by running BD CompBeads.

Surface TF expression was assessed in platelets ($1x10^6$) with low (~7 fL) and high (~11 fL) MPV, under resting conditions or upon stimulation for 15 minutes at room temperature with ADP (10µM), TRAP (25µM) or tromboxane A2 analogue (U46619, 1µM). Platelets were labeled with a saturating concentration of centrifuged (17,000g, 5 minutes, 4°C) antibodies against human TF (cat. 4507CJ) and CD41a in PBS containing 10% FBS and 2mM EDTA. For TF intracellular staining, cells were fixed for 2 hours with 1% paraformaldehyde, permeabilized for 10 minutes with a 0,1% Triton X-100 PBS solution and labeled for 15 minutes at room temperature in the dark with saturating concentration of antibodies against TF (cat. 4508CJ) and CD41a. Fluorochrome-conjugated isotype controls were used in all the experiments to quantify the background labeling. A total of 10,000 events per sample were acquired. All the data were analyzed with DIVA Software (Becton Dickinson) and reported as percentage±SD of TF positive cells.

Imaging flow cytometry, performed by ImageStream^X Mk II (Amnis), was also used to analyze platelet intracellular TF expression as well as to calculate the average diameter of platelets within the low and the high MPV fractions. Small and large platelets, stained as described above, were acquired with 60X magnification at low flow rate/high sensitivity and with 488nm and 785nm lasers for FITC-fluorescence and side-scatter (i.e. granularity inside a single cell) detection, respectively. Image analysis was performed using IDEAS^{TM} software. TF positive platelets were analyzed by gating on focused events. Doublets and aggregates of platelets were excluded from further analysis by selecting only the single cell population defined as events with an Aspect Ratio (minor axis/major axis of a selected event) higher than 0.6 and an Area lower than 50µm^2. In order to get an accurate size estimation of platelets, a mask was created tight around the platelet images and the Erode function was used to decrease the default brightfield mask M01 by 3 pixel rows (Erode3).

TF antigen determination. TF antigen levels were assessed in platelet lysates by ELISA (Imubind® Tissue Factor) according to the manufacturer's instructions. For this assay, platelets were solubilized with 15mM octyl-β-D-glycopyranoside for 15 minutes at 37°C, sonicated for 1 minute and diluted with 25mM HEPES–saline buffer. Values are reported as picograms of TF antigen per microgram of proteins.

Thrombin generation assay. Freshly isolated platelets were tested for their capacity to promote thrombin generation using the Calibrated Automated Thrombogram (CAT) assay. Briefly, triplicate samples of $4x10^6$ platelets/well, unless otherwise indicated, were incubated for 10 minutes with platelet-free normal pooled plasma (Pool Norm) in round-bottom 96-well microtiter plates (Immulon 2HB). Thrombin generation was started by the addition of a $CaCl_2$/fluorogenic substrate mixture (FluCa Kit) and fluorescence was read for 60 minutes in a Fluoroskan Ascent® reader (Thermo Labsystems). When platelet-associated TF was tested as rate limiting contributor of thrombin generation, CAT assay was performed in the presence of phospholipids (4µM, MP reagent) as the only trigger.
In order to assess the contribution of TF to thrombin generation, the CAT assay was performed in the presence of a neutralizing anti-TF antibody (cat. 4501, 100µg/ml). Phospholipid contribution to thrombin generation was evaluated by incubation of platelet samples with lactadherin (10-100nM), in the absence of exogenously added phospholipids. In order to correct for inner filter effects and substrate consumption, all thrombin generation measurements were calibrated against the fluorescence curve obtained after the addition of fixed amount of thrombin-α_2-macroglobulin complex. Thrombin generation curves were analyzed by dedicated software (Thrombinoscope BV). Lag-Time (min), ETP (Endogenous Thrombin Potential, nM x min) and Velocity Index (nM thrombin/min) were used as main parameters describing thrombin generation.

FXa generation. The TF activity assay (Actichrome® TF) was performed according to the manufacturer's protocol. Briefly, platelet lysates (20µg) were mixed with factor VIIa and factor X and incubated at 37°C for 15 minutes in the presence of a high concentration of phospholipids (4µM, MP reagent). Sample absorbance was read at 405nm in a microplate reader (Mithras LB 940, Berthold Technologies) after the addition of a specific chromogenic substrate (Spectrozyme-FXa). The amount of active TF was calculated from a standard curve generated using known amounts of human TF.

To quantify the contribution of TF to the FXa generation, the assay was performed after incubation of samples (30 minutes at 37°C) with a specific neutralizing antibody against human TF (cat. 4501, 100µg/ml).

TF mRNAs detection by Real Time PCR. In order to detect mature TF mRNA and unspliced TF pre-mRNA transcripts in small and large platelets, cells, devoid of leukocyte contamination assessed as previously described [12], were lysed in QIAzol Lysis Reagent. 200ng of extracted RNA were then reverse transcribed by using 200 U SuperScript® Reverse Transcriptase [7]. Real-time quantitative PCR was carried out on iQ5 iCycler Optical System (Bio-Rad Laboratories).

cDNA (40ng) was incubated in 20µL iQ™ SYBR® Green supermix containing specific primers (TF for: 5'-TGATGTGGATAAAGGAGAAAACTACTGT-3'; TF rev: 5'-TCTACCGGGCTGTCTGTACTCTT-3'; pre-TF for: 5'-CAGCCCGGTAGAGTGTATGG-3'; pre-TF rev: 5'-CCCTGAGGGTGGAGCTACT -3').

GAPDH amplification was used to correct for differences in input RNA levels. Specificity of amplified products was verified by melting curves analysis obtained by stepwise increase of the temperature from 55°C to 95°C at the end of amplification. Relative expression of TF mRNA and of TF pre-mRNA was calculated by setting the expression of TF pre-mRNA transcript in small platelets to 1.

Statistics. Results are expressed as mean±SD and were analyzed by Student's paired t-test or U-Mann Whitney, as appropriate. A p-value lower than 0.05 was considered to be statistically significant. Analyses were performed using SPSS statistical package.

Results and Discussion

The PRP to be used for different kind of analysis on isolated platelets (flow cytometry, functional activity tests, proteome or transcriptome analysis etc.) must be prepared taking into account that 1) the PRP has to be representative of the entire population of platelets present in whole blood and 2) the PRP has to be devoid of leukocyte contamination. In order to fulfill the second point, it is common practice to collect the top ¾ of the PRP, keeping a safe distance from the buffy coat. However, there is not a standardized method to prepare PRP, with the exception for the methodological guidelines for standardization of light transmission aggregometry, prepared by the Platelet Physiology Subcommittee of the Scientific and Standardization Committee of the International Society on Thrombosis and Haemostasis [13]. The centrifugation force and time applied in PRP preparation are important variables that can have a critical impact on PRP quality, as illustrated in Figure 1.

When citrated blood is centrifuged at three different centrifugation forces, platelet sedimentation is, as expected, highest with the highest g used. This is easily tangible looking at the PRP turbidity, which decreases as the centrifugation force increases (Figure 1A). Collection of the top ¾ of the resulting PRP leads to a partial recovery of platelets, which ranges from ~50%, with the lowest g, to ~20%, with the highest g, of the number of platelets in whole blood (Table 1).

Beyond the fact that the recovery of platelets is only partial, far more important consequence of the habit to collect only part of the PRP is the loss of the platelet fraction with the largest size as evidenced by the values of MPV, PDW and IPF that are lower than those of whole blood in all the three centrifugation settings. At 200g, which is the centrifugation force most used for PRP preparation, a 70% decrease in IPF compared to whole blood (1.3±0.9 *vs* 4.7±3.5, $p<0.05$) was observed. Since the IPF is enriched in large platelets, the drop in the IPF influences the MPV and PDW values, which significantly decrease accordingly (MPV: 8.4±0.5 *vs* 11.2±1.2, $p<0.001$; PDW: 9.4±1.6 *vs* 13.7±2.4, $p<0.01$) (Table 1 and Figure 1C). Flow cytometry analysis of the platelet population recovered in the top ¾ of the PRP with the three different centrifugation forces further supports this finding and provides a clear view of the physical characteristics of the cells. Indeed, while the platelet population obtained at 100g is very similar to the one identified in whole blood, the FSC being almost the same in the two samples (FSC mean fluorescence intensity: 6.50±0.49 and 6.81±1.15, respectively, $p=0.788$), the loss of the large size cells observed with the higher speed centrifugations results in

platelet populations with a lower FSC (200g: 4.84±0.37; 350g: 3.16±0.61, $p<0.01$ *vs* whole blood) (Figure 1B).
These data indicate that particular attention must be paid to the preparation and collection of the PRP since such pre-analytical variables can significantly affect subsequent analysis whether it is flow cytometry, functional activity tests, proteome or transcriptome analysis.

In order to identify the subsets of TF positive platelets, we performed TF intracellular staining on low (~7 fL) and high (~11 fL) MPV enriched platelet fractions, which were then analyzed by conventional flow cytometry (Figure 2 A and B) as well as by Imaging Flow Cytometry (Figure 2 C and D). The results indicate that in the high MPV fraction ~40% of platelets are TF positive (Figure 2 B and D) compared to ~15% found in the low MPV fraction (Figure 2 A and C). The use of Imaging Flow Cytometry, which besides the study of the antigenic properties also allows the visualization of the morphology (brightfield, BF, Figure 2 C and D) of each single cell analyzed, showed that events with a high MPV are indeed platelets with an average diameter of 4.1±0.3µm and not aggregates of small platelets (Figure 2 C and D).

TF antigen levels, quantified in platelet lysates by Elisa, confirmed that the fractions containing mostly large platelets contained 3-fold more TF compared to those containing predominantly small platelets (0.29±0.1 *vs* 0.08±0.03 pg TF/µg proteins, in large and small platelets, respectively; $p<0.01$).
Large and small platelets were then evaluated for their capacity to promote thrombin generation. In our experimental setting, where the contribution of the intrinsic pathway of coagulation has been virtually removed by the presence of CTI during the blood withdrawal, thrombin generation is dependent on the presence of TF, the trigger of the reaction, and on phospholipids, which are required for the assembly of the coagulation factor complexes. It should be considered that large platelets contain more phospholipids than small platelets, when comparing the same number of cells. Indeed, when the effect of lactadherin (100nM), which specifically binds to phosphatidylserine, was tested in the CAT assay, a full inhibition of thrombin generation was observed in small platelet samples whereas it was only partial in large platelets (Figure 3A). Therefore, in order to make the platelet-associated TF the rate limiting step in the thrombin generation assay, experiments were performed in the presence of exogenously added phospholipids. In these experimental conditions, the kinetic rate of

thrombin generation increased linearly with the platelet counts only when large cells were analyzed (Lag Time: 27.5±1.7 *vs* 22±2.1 min in $2x10^6$ and $4x10^6$ platelets, respectively); by contrast, thrombin generation in small platelets did not change, even when a double number of small platelets was used, being similar to that measured in cell-free control plasma (Figure 3B). Moreover, comparison of the two platelet fractions showed that large size platelets were able to generate thrombin with a faster kinetic rate compared to the small size platelets, as evidenced by the significantly lower lag time observed in large compared to small platelets (22.4±2.2 *vs* 26.3±2.8 min, respectively; $p<0.01$; Figure 3C).

Preincubation of samples with a neutralizing anti-TF antibody highlighted a significant increase in lag time ($p<0.01$) and a trend toward a reduction of the endogenous thrombin potential (ETP) and velocity of thrombin formation (Velocity Index) only in large size platelets.

These data indicate that TF-dependent thrombin generation is a feature of platelets with high MPV as confirmed also in FXa generation experiments since only platelets with high MPV were able to generate FXa in a TF-dependent manner (Figure 3D). By contrast, TF antigen present in small size platelets does not support the hemostatic function. Indeed, only 4.4 % of the Xa generation was TF-specific (i.e. inhibited by the specific neutralizing anti -TF antibody) in the small platelets compared to 33.6 % of the total Xa-generation in the large platelets (Figure 3D).

Whether this lack of functional activity in small platelets is due to a different conformation of the protein [13] or to other mechanisms will be a matter of future investigations. It is worth mentioning on this regard that beyond its role in coagulation, TF is also recognized as a signaling receptor and, in small platelets, it may play one of the several other functions ascribed to this protein [14].

Since it has been previously reported that large platelets are more reactive compared to small platelets [15-17], we tested how platelet stimulation affected cell surface expression of TF in small and large platelets. Upon stimulation with ADP, TF is readily exposed on the cell surface of the large platelets while the percentage of low MPV platelets that expresses the protein on the cell surface is almost undetectable compared to the high MPV platelets (31.0 %±11.2 *vs* 3.0%±1.4, respectively; $p<0.01$; Figure 4, black bars).

Similar results were obtained after platelet stimulation with other agonists such as TRAP-6 and the tromboxane A2 analogue U46619 (51.7%±13.1 *vs* 1.3%±0.4 and 43.1%±10.6 *vs* 1.1%±0.3, respectively, in large and small platelets; $p<0.001$; Figure 4, grey bars).

Thus, the expression of TF on the surface of activated platelets is a feature mainly of the high MPV cells, it is not agonist-related and confirm previously published data regarding the increased reactivity of large size platelets [17]. Taken together, these data further indicate a different functional role of these two subsets of platelets, being the high MPV fraction haemostatically more reactive.

Since platelets contain not only the TF protein but also megakaryocyte-derived TF pre-mRNA and mRNA, we analyzed the presence of these two transcripts within small and large platelet fractions.

TF pre-mRNA is the most prevalent species present in platelets [4,7] and it was indeed found both in small and large platelet fractions (Figure 5). In particular, levels of pre-mRNA were 4-fold higher in large platelets compared to small ones.

By contrast TF mRNA, which was detected in all large platelet preparations, was amplified only in 4 out of 9 samples of small platelets. In those subjects who have detectable TF mRNA levels in small platelets, TF mRNA expression was 5-fold lower compared to large platelets (Figure 5). Since platelet activation controls the RNA processing and *de novo* protein synthesis [4,5], the detection of TF mRNA mainly in large platelets may reflect the higher reactivity of these cells compared to small platelets.

The presence of TF mRNA within platelets is still a matter of debate. Our data suggest that TF mRNA is mainly stored in large platelets which are those frequently lost if PRP is not carefully prepared and therefore enriched in low MPV platelets. In this case, the failure to detect TF mRNA is very likely, as documented also in this study. Of note, interindividual variability may also account for the lack of consistency in TF mRNA detection in small platelets.

All together these data provide further evidence that the platelet population is heterogeneous and that not all platelets are equally committed to fulfill the haemostatic role. It is conceivable that the different functions ascribed nowadays to platelets are carried out by

several subsets of platelets, produced by megakaryocytes and released into the bloodstream with different transcriptome and proteome contents.

In conclusion, our study further characterizes the expression of TF in circulating human platelets providing evidence that it is a property of large size platelets. It is worth mentioning that when analyses are carried out on isolated platelets, this subset of cells can easily be lost if centrifugation protocols are not optimized.

Since TF is involved in several important functions besides haemostasis, such as inflammation, cell differentiation and migration, angiogenesis [18,19]; the finding that platelets express TF is of particular relevance since it paves the way for the evaluation, in future studies, of new mechanisms potentially involved in the etiopathogenesis of human disorders such as coronary artery disease but also cancer, inflammation and immune mediated disease.

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Declaration of Interest

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Legends to Figures

Figure 1. Effect of different centrifugation forces used in PRP preparation on platelet recovery. For PRP preparation, whole blood (WB) was centrifuged at different g as indicated. **(A)** Images highlighting the differences in platelet sedimenattion according to the different centrifugation of the WB. **(B)** Flow cytometry analysis of the physical parameters, side (SSC) and forward scatter (FSC), of the platelet recovered in the top ¾ of the PRP. **(C)** Platelet Distribution Width (PDW) data graph generated by Sysmex XE-2100 Automated Hematology Analyzer. *p<0.05 vs WB.

Figure 2. Intracellular TF expression in platelets according to their Mean Platelet Volume. (**A-B)** Representative dot plot analysis of intracellular TF expression in low (Small) and high (Large) MPV platelets, performed by FACSCalibur. Numbers in the upper right quadrant are mean±SD, (n=10). **(C-D)** Evaluation of intracellular Tissue Factor expression and platelet size in Small and Large platelet fractions by Imaging Flow Cytometry. Representative channel images of brightfield (BF, gray), Tissue Factor (green) and side scatter (SSC, magenta) and composite images of SSC and BF, acquired by ImageStream^X Mk II, at 60X magnification. Platelet diameter (Ø, mean±SD) as calculated by Amnis IDEAS data analysis software is reported.

Figure 3. Platelet TF functional activity measured by thrombin generation (A-C) and by FXa (D) assays. (A). Small (left panel) and large (right panel) platelet samples were incubated with lactadherin in order to assess the contribution of phospholipids to thrombin generation. Curves obtained in a representative experiment are shown (n=3). (**B)** Representative curves of thrombin generated by different amount ($2x10^6$ or $4x10^6$ cells) of small (left panel) and large (right panel) platelets (n=3). (**C)** The contribution of TF to thrombin generation was evaluated by preincubation of small and large platelets with a neutralizing anti-TF antibody. Representative curves are shown. Histograms report the effect of the neutralizing anti-TF antibody on the Lag Time, ETP and Velocity Index (setting to 100 the mean values of the untreated sample). Data are mean±SD (n=5).*p<0.01. (**D)** FXa generation evaluated in small and large platelet samples in the presence or absence of a neutralizing anti-TF antibody. Histograms report mean±SD of FXa, calculated setting 100 the mean value of FXa generated by untreated samples (n=9). *p<0.05 vs untreated sample.

Figure 4. Evaluation of surface TF expression on small and large platelets in resting and stimulated conditions. Small (**A**) and large (**B**) platelets were analyzed under resting conditions or upon stimulation with ADP (10µM), TRAP-6 (25µM) and the tromboxane A2 analogue, U46619 (1µM). The percentage of TF positive platelets was reported in histograms as the mean±SD (n=10). *$p<0.05$, $^{\#}p<0.01$ vs resting conditions.

Figure 5. Evaluation of TF mRNA and TF pre-mRNA by Real Time PCR. Histograms reported the relative expression of TF mRNA and of TF pre-mRNA in small and large platelets, calculated setting to 1 the expression of TF pre-mRNA transcript in small platelets (n=9).

TABLE

Table 1. Modifications of platelet parameters according to different centrifugation forces used in PRP preparation.

	Fraction	PLT recovery (%)	IPF (%)	MPV (fL)
Whole Blood		100	4.7 ± 3.5	11.2 ± 1.2
100g, 10' PRP	***top ¾***	48 ± 15.8 *	4.6 ± 2.7	10.2 ± 0.8
	bottom ¼	26.1 ± 9.5	3.4 ± 1.9	11.1 ± 1.5
	remaining blood	20.6 ± 6	2.9 ± 2	11.4 ± 0.2
200g, 10' PRP	***top ¾***	35.6 ± 14.9 *	1.3 ± 0.9 *	8.4 ± 0.5 *
	bottom ¼	33.5 ± 20.7	7.6 ± 4.6	11.1 ± 1
	remaining blood	21.2 ± 11.6	3.5 ± 2.3	8.0 ± 0.6
350g, 15' PRP	***top ¾***	22.0 ± 10.2 *	0.3 ± 0.02 *	6.9 ± 0.6 *
	bottom ¼	25.9 ± 12.4	0.3 ± 0.1	8.6 ± 1.3
	remaining blood	41.3 ± 11.7	3.7 ± 2.3	12.1 ± 0.3

PRP= platelet rich plasma; PLT=platelet; IPF=Immature Platelet Fraction; MPV=Mean Platelet Volume.
*p<0.05 *vs* Whole Blood