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Increased platelet function during frailty

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Frailty is highly associated with cardiovascular diseases (CVD) and diabetes mellitus (DM). Aging, CVD, and DM are all associated with an increase in platelet function. Therefore, the aim of this study was to evaluate platelet function during frailty. We selected a total of 37 older adults who were divided into two groups, frail ($n = 16$) and robust ($n = 21$), with a mean age of 72.4 ± 4.4 years (range: 65–84 years) in robust adults and 72.6 ± 6.6 years (range: 65–88 years) in frail adults; 20 young healthy volunteers, with a mean age of 22.9 ± 2.7 years (range: 20–30 years), were included as a control platelet function was determined using the lumi-aggregometer (aggregation) and flow cytometry (platelet activation). We also performed Western blot to evaluate the intraplatelet activation pathways involved in activation. Platelet count decreased and mean platelet volume, aggregation, and P-selectin expression increased during aging compared with young adults was found. We observed an increase in P-selectin expression in frail adults compared with robust adults. We also evaluated the characteristics of the study population to explain this difference and found a higher prevalence of DM and a tendency toward hyperglycemia in frail adults compared with robust adults. In agreement with this, high doses of glucose were able to increase platelet aggregation and P-selectin expression through thrombin receptors and p38 phosphorylation.

Increase in life expectancy worldwide has generated growth in the populations of older adults in almost every country [1,2]. A greater state of physical and mental vulnerability, known as frailty, can occur during aging. This is defined as an increase in susceptibility to adverse outcomes, including disability, dependency, falls, need for long-term care, and mortality [3]. This is also characterized by a multisystem deregulation that can be clinically identified by weight loss, increase in weakness, poor endurance and energy, slowness, and low physical activity [4].

Among the most prevalent diseases in frailty are cardiovascular disease (CVD), hypertension, osteoarthritis, diabetes mellitus (DM), chronic obstructive pulmonary disease, dementia, and depression [2,5,6]. The highest association is seen in CVD and DM as they share common biological pathways with frailty such as inflammation [7,8]. Markers of inflammation such as C-recative protein (CRP), tumor necrosis factor α (TNFα), and interleukin (IL)-6 are increased during frailty [9], CVD [10,11], and DM [8].

There is also a high prevalence of frailty in patients with CVD (18%–54%) [12], and this is a powerful predictor of mortality in cardiovascular patients regardless of age,

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underlying severity of the disease, presence of other diseases, and disability [7,12]. As well, during frailty there is a higher occurrence of four to five risk factors for CVD; the most associated is DM [13]. Atherosclerosis is the most prevalent CVD [14], and platelets play a significant role in both its initiation and spread. The adhesion of platelets to endothelial cells at the sites of atherosclerotic lesions increases the recruitment and activation of monocytes through the release of cytokines, chemokines, pro-inflammatory molecules, and other molecules (IL-1β, PF4, RANTES, PAF, and CD40L) [15]. The release of these inflammatory mediators and stimulators of cell proliferation in the local microenvironment alters the chemotactic and adhesive properties of endothelial cells, contributing to the development of the lesions [16]. Platelets also play an important role during arterial thrombosis, which occurs more frequently after rupture or erosion of an unstable atherosclerotic plaque, exposing potent thrombogenic elements to the bloodstream [15]. In addition, the importance of platelets in CVD is demonstrated by the alteration in platelet function that occurs in DM patients who present with atherosclerosis and cardiovascular disorders [17]; consistent with this, platelets from diabetic patients have shown an increase in adhesiveness, aggregation, cytoplasmic calcium, thromboxane production, and specific platelet protein levels [18–20], and some of these changes have been directly explained by hyperglycemia [21–24].

Furthermore, during aging several changes in platelet count [25] and function have been reported (increase in: aggregation [26–29], cytoplasmic calcium [30], β-thrombloglobulin levels, phosphoinositide turnover [31], release of ATP in response to stimulation [29], and excretion of urinary thromboxane [32]).

Despite all this evidence supporting an association between frailty and diseases with platelet dysfunction, the state of platelet function during frailty has not been thoroughly assessed. In this study, we found higher platelet activity during frailty and a possible mechanism involving high doses of glucose.

Methods

Study participants

A total of 37 adults aged 65 and older were randomly selected from the PIEI-ES study [33]. They were divided into two groups, frail ($n = 16$) and robust ($n = 21$). The mean age was 72.4 ± 4.4 years (range: 65–84 years) in the robust group and 72.6 ± 6.6 years (range: 65–88 years) in the frail group, with no significant difference between the groups. In addition, 20 young healthy volunteers were included as a control, with a mean age of 22.9 ± 2.7 years (range: 20–30 years) (Table 1). Participants taking any medication affecting platelet function for 10 days prior to venipuncture and those with primary and secondary hemostasis or acute illness at the time of venipuncture were excluded. Also, participants whose samples showed hemolysis and lipemia were discharged.

Table 1. Characteristics of the patients

Variable	Robust	Frail	Total
Sex, *n* (%)			
Female	14 (66.7%)	15 (93.8%)	29 (78.4%)
Male	7 (33.3%)	1 (6.3%)	8 (21.6%)
Age[a], y	72.4 ± 4.4	72.6 ± 6.6	72.5 ± 5.4
Glucose[a], mmol/L	6.2 ± 0.3	7.9 ± 1.3	
Glycosylated hemoglobin[a], mmol/L	49.0 ± 1.5	54.0 ± 3.0	

[a]Mean ± SEM.

Every participant gave informed consent to participate in this study. The protocol was authorized by the ethics committee of the Universidad de Talca in accordance with the Declaration of Helsinki (approved by the 18th World Medical Assembly in Helsinki, Finland, 1964). Because of the sensitive nature of the questions asked in this study, survey respondents were assured that raw data would remain confidential and would not be shared.

Frailty status

Frailty status was determined as described by Fried et al. [4] with some modifications. A participant was considered frail if they exhibited three or more of the following symptoms/criteria: slowness, weakness, weight loss, exhaustion, and low physical activity. Participants were classified as robust if they had none of these and pre-frail if they had one or two of them. The criteria have been defined elsewhere [33]. Briefly, slowness was defined according to a cutoff (<0.8 m/s) on 3-m walking at a usual pace, adjusted for sex and height. Weakness was assessed by measuring strength with an electronic handgrip dynamometer, according to a sex-specific cutoff (males: <27 kg, females <15 kg). Unintentional weight loss was defined as self-reported loss of at least 5 kg in the previous 6 months [2]. Participants were determined to have exhaustion if they answered positively to either of two questions from the Center for Epidemiological Studies Depression Scale: "I felt that anything I did was a big effort" and "I felt that I could not keep on doing things" [2]. Finally, low physical activity was defined by difficulty in walking as assessed with two questions: "Do you have difficulty walking a block?" or "Do you have difficulty climbing several flights of stairs without resting?" [34].

Preparation of human platelet suspensions

To obtain platelet-rich plasma (PRP), we took venous blood samples (10 mL) in citrate tubes (3.2%; 9:1 v/v; Becton Dickinson Vacutainer Systems, Franklin Lakes, NJ). The samples were centrifuged (DCS-16 Centrifugal Presvac RV, Presvac, Buenos Aires, Argentina) at 240*g* for 10 min; then two-thirds of the PRP was removed and the samples were centrifuged one more time at 650*g* for 10 min to obtain the platelet-poor plasma (PPP). Finally, the PRP was resuspended with the PPP and adjusted to a concentration between 200 and 300×10^3 platelets/μL (Bayer Advia 60 Hematology System, Tarrytown, NY) for the aggregation and surface expression of P-selectin assays.

To prepare washed platelets for Western blotting analysis, a 10-mL sample of venous blood was obtained in a syringe

with extraction buffer (9:1 v/v; 12 mmol/L theophylline, 0.11 μmol/L prostaglandin E_1 [PGE1; Sigma-Aldrich, St. Louis, MO], ACD, pH 7.4) and centrifuged at 240*g* for 10 min. After this, PRP was extracted and centrifuged again at 650*g* for 10 min to obtain a platelet pellet. The pellet was mixed with 500 μL of washing buffer (137 mmol/L NaCl, 2.7 mmol/L KCl, 2 mmol/L $MgCl_2$, 9.5 mmol/L $NaHCO_3$, 0.36 mmol/L $Na_2HPO_4 \times 2\ H_2O$, 5.5 mmol/L glucose, 0.2 μmol/L PGE1, pH 7.4) and centrifuged at 175*g* for 5 min, and the supernatant were centrifuged again to obtain a pellet at 650*g* for 10 min. Finally, the pellet was resuspended with dilution buffer (137 mmol/L NaCl, 2.7 mmol/L KCl, 1 mmol/L $MgCl_2$, 9.5 mmol/L $NaHCO_3$, 0.36 mmol/L $Na_2HPO_4 \times 2\ H_2O$, 2.6 mmol/L $CaCl_2$, 5.5 mmol/L glucose, pH 7.4) to a concentration of 500×10^3 platelets/μL.

Platelet aggregation assay

This assay was realized by light transmission according to Born and Cross [35], using a lumi-aggregometer (Chrono-Log, Havertown, PA). Briefly, we incubated 410.5 μL of PRP with 5.5 μL of TRAP-6 (2.5 or 10 μM) as agonist and then registered platelet aggregation for 6 min. Platelet aggregation (maximal amplitude [%], area under the curve, and lag time [sec]) was determined with AGGRO/LINK software (Chrono-Log). In addition, the inhibition of platelet aggregation was calculated according to the following formula: percentage of inhibition of platelet aggregation = 100 − [(%AgX × 100) / %AgC], where AgX = average aggregation of inhibitor, and AgC = average aggregation of positive control.

Measurement of P-selectin surface expression

To assess platelet activation, P-selectin surface expression was measured by modifying methods previously described by Fuentes et al. [36]. Briefly, we pre-incubated 50 μL of PRP with 5.5 μL of TRAP-6 (2.5 or 10 μmol). All conditions were incubated for 30 min in the dark and at room temperature with saturated concentrations of anti-CD62P-PE and anti-CD61-FITC. After this, an Accuri C6 flow cytometer (BD Biosciences, San Diego, CA) was used to analyze P-selectin expression on the platelet surface; we acquired 10,000 events per conditions. To work specifically with the platelets and distinguish from different alterations (electronic noise or with possible contamination), we selected the platelet by CD61 positivity (specific platelet marker), cell size, and complexity using forward scatter (FSC) versus side scatter (SSC). Fluorescence intensities of differentially stained populations were expressed as the mean channel value using the BD Accuri C6 Software (BD Biosciences).

Western blotting analysis

The analyses were performed by Rasheed et al. as previously described with modifications [37]. Briefly, washed platelets were lysed with 200 μL of lysis buffer (50 mmol/L Tris, 1% Triton X-100, 10% glycerol, 150 mmol/L NaCl, pH 7.5) containing protease inhibitor cocktail. Equal quantities of total proteins (80 μg/mL for basal expression assays or 50 μg/mL for glucose stimulation assays) were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions and transferred to a polyvinyl difluoride (PVDF) membrane. Subsequently, the membrane was blocked with 5% milk diluted in a saline buffer with 0.1% Tween-20 solution. The proteins were detected with an antibody against anti-p-PKCα (Cell Signalign, Danvers, MA), anti-p-p38 (Santa Cruz Biotechnology, Santa Cruz, CA), anti-p38α/β (Santa Cruz Biotechnology), or anti-γ-tubulin (Biolegend, San Diego, CA) as a control. Specific reactivity was detected with an enhanced chemiluminescence method, employing an antibody linked to horseradish peroxidase. The area and intensity were quantified using ImageJ software (National Institutes of Health and the Laboratory for Optical and Computational Instrumentation [LOCI], University of Wisconsin, Madison, WI).

Glucose stimulation assay

For the determination of platelet function in the presence of glucose, we used a fasting blood sample from healthy young adults ($n = 3$) employing the method described by Sudic et al. with modifications [22]. Briefly, 390 μL of PRP was incubated with 20 μL of D-glucose (Sigma Aldrich) at concentrations of 5.5 to 27.7 mmol/L at room temperature (20°C–25°C) for 20 min. Subsequently, a subthreshold concentration of TRAP-6 or ADP (<30% aggregation [38]) was added and incubated for 6 min. Then, platelet aggregation, P-selectin expression, and total or phosphorylated p38 were recorded according to the methods described above (aggregometry, flow cytometry, and Western blot, respectively). A control was included; for this, the platelets were incubated with 20 μL of vehicle (0.01 mmol/L phosphate-buffered saline [PBS], pH 7.4) and a subthreshold stimulation of ADP or TRAP-6. Also, during these assays, the inhibition of p38 protein phosphorylation was carried out with platelets previously incubated with the p38 inhibitor (SB203580, Santa Cruz Biotechnology) at 2 μmol/L concentration for 10 min at room temperature.

Blood cell count and metabolite analysis

The blood cell count was performed in a BC 3600 analyzer (Mindray-Valtek, Santiago, Chile). Plasma glucose (glucose oxidase) and HbA1c (IA) were measured in a BS 300 analyzer (Mindray-Valtek, Santiago, Chile).

Statistical analysis

Data were analyzed using GraphPad Software version 5.0 (La Jolla, CA) and expressed as the mean ± SEM. Two or more measurements were made for each test. Differences among groups were analyzed with Student's *t* test or analysis of variance (ANOVA). For detection of differences in the prevalence of DM, Pearson's χ^2 test was used. p values < 0.05 were considered to indicate significance. Single triplicates and independent assays were performed for each donor.

Results

Decrease in platelet count and increase in median platelet volume during aging

To characterize the platelet population in the different groups, we performed platelet counts. We obtained normal platelet counts in the three study groups, with means of $350.6 \pm 17.3 \times 10^3/\mu L$ for the young group, $219.3 \pm 12.2 \times 10^3/\mu L$ for the robust group, and 214.6

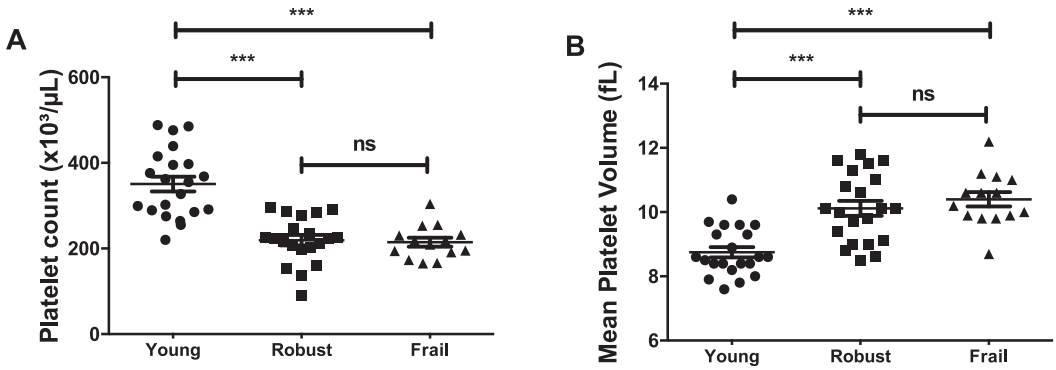

Figure 1. Characteristics of platelets during aging. Whole blood with EDTA was obtained for the determination of **(A)** platelet count and **(B)** mean platelet volume in a group of young adults ($n = 20$), robust elderly ($n = 21$) and frail elderly ($n = 16$). The results were expressed as the mean ± SEM. ***$p < 0.0001$. ns=not significant.

± $10.4 \times 10^3/\mu L$ for the frail group. Nevertheless, the number of platelets for the young population was significantly increased in comparison with those for the robust ($p < 0.0001$) and frail ($p < 0.0001$) populations (Figure 1A). Similarly, the mean platelet volume (MPV) was below normal range in the three groups: 8.8 ± 0.16 fL young, 10.1 ± 0.23 fL robust, and 10.4 ± 0.22 fL frail; however, MPV was significantly decreased in the young in comparison with the robust ($p < 0.0001$) and frail ($p < 0.0001$) populations (Figure 1B). These results show that the number of platelets decreases while the MPV increases with aging.

Increase in platelet aggregation during aging

Given the results of the platelet count and MPV in aging, we proceeded to assess platelet function with the platelet aggregation assay. PRP ($200-300 \times 10^3$ platelets/μL) was pre-incubated for 3 min, and the percentage of platelet aggregation, area under the curve, and lag time were recorded by light transmittance for 6 min after a stimulation with 2.5 or 10 μmol/L TRAP-6. Figure 2A and B illustrates representative results for platelet aggregation in all groups. As shown in Figure 2C, stimulation with 10 μmol/L TRAP-6 activated platelet aggregation ≥65% in all study group study (81.7 ± 0.5% young, 82.2 ± 1.5% robust, and 82.8 ± 2.2% frail), demonstrating platelet functionality in all subjects. Stimulation with 2.5 μmol/L TRAP-6 caused an increase in platelet aggregation in the robust ($p = 0.0003$) and frail ($p < 0.0001$) elderly populations in comparison with the young, with mean aggregation values of 19.8 ± 3.7%, 22.2 ± 4.4%, and 5.0 ± 1.1%, respectively (Figure 2C). These differences were maintained even when the results were adjusted for the platelet count of the PRP (Figure 2D) and by analyzing the area under the curve (Figure 2E). Additionally, calculation of lag times revealed that the young population had a longer lag time than the robust ($p < 0.0001$) and frail ($p < 0.0001$) elderly populations (Figure 2F), with mean times of 211.6 ± 35.0, 45.1 ± 15.9, and 39.6 ± 12.9 s, respectively. This indicated that platelet aggregation under 2.5 μmol/L TRAP-6 in the young, in such cases, was not accomplished. Overall, these results show that platelet aggregation is increased during aging.

Increase in platelet activation during frailty

Another way to investigate platelet function is by determining the surface expression of P-selectin, which was assessed by flow cytometry according to the description under Methods. Initially, basal expression of P-selectin in platelets from all participants was determined (Figure 3A, D) and there was no significant statistical difference between the different groups studied. Furthermore, stimulation with 10 μmol/L TRAP-6 caused ≥66% platelet activation in all participants, proving functionality in platelets from all samples (Figure 3B, E); the group means were (young) 78.8 ± 2.6%, (robust) 84.4 ± 1.7%, and (frail) 87.9 ± 2.1%. However, after stimulation with 2.5 μmol/L TRAP-6 (Figure 3C), the platelets from the robust (32.7 ± 4.7%; $p = 0.0488$) and frail (52.8 ± 4.1%) populations were more activated than the platelets from the young population (22.2 ± 2.8%; $p < 0.0001$) (Figure 3E).

Furthermore, platelet activation was more significantly increased in the frail group than in the robust group ($p = 0.0018$). In summary, these results indicated higher platelet activation during aging and particularly during frailty.

No change in basal expression of p-PKCα and p-p38 during aging or frailty

Because of the changes observed in platelet function during aging and frailty, we proceeded to determine the basal expression of p-PKCα in the three study groups (Supplementary Figure E1A, online only, available at www.exphem.org), PKCα is a central protein in the full activation of platelets [39]. For this, a total lysate of washed platelets was evaluated by Western blotting. We

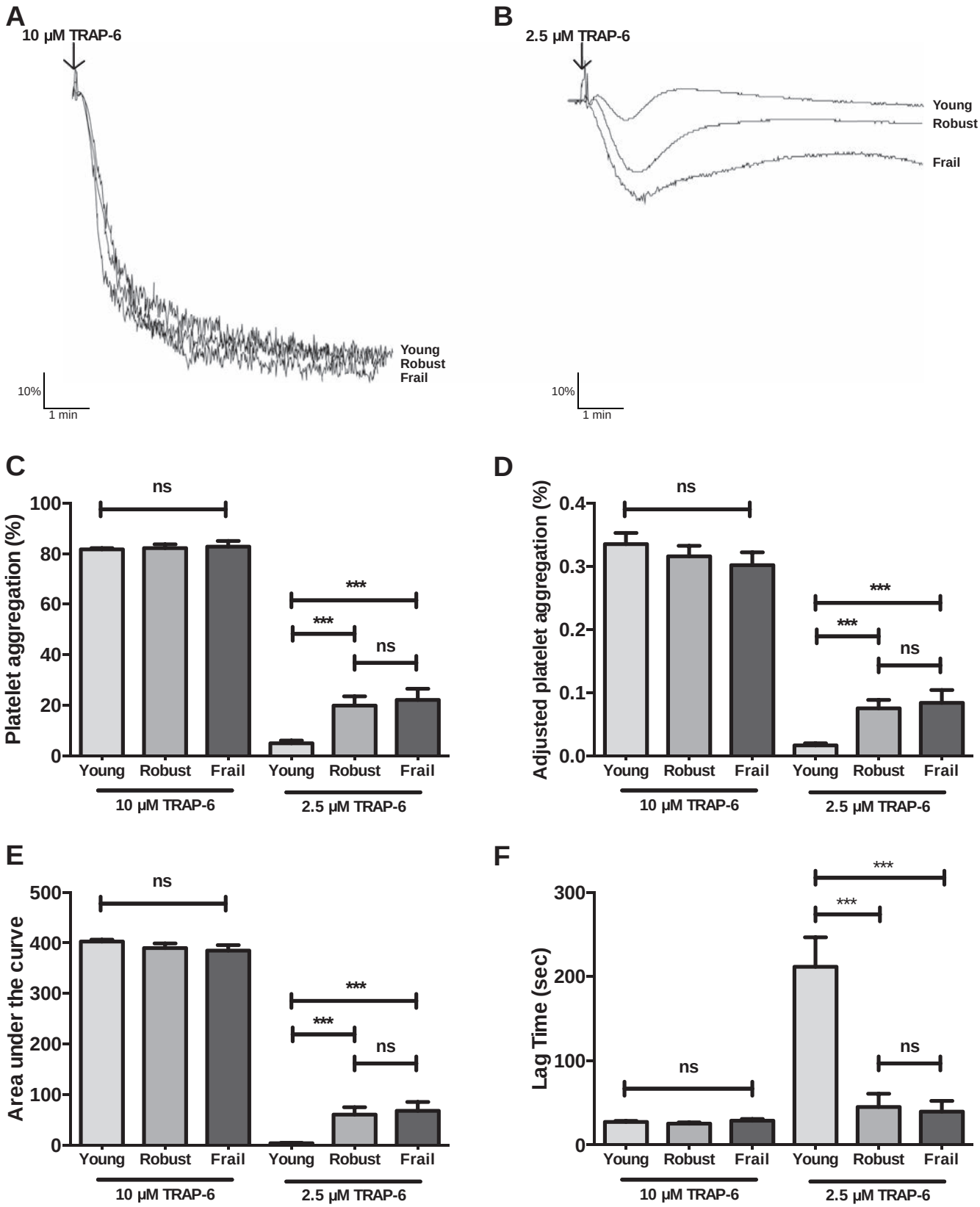

Figure 2. Platelet aggregation during aging. Platelet aggregation was obtained by incubation of PRP with **(A)** 10 or **(B)** 2.5 μmol/L TRAP-6. **(C)** The percentage of aggregation, **(D)** percentage adjusted for the number of platelets in the PRP, **(E)** area under the curve, and **(F)** lag time were calculated in young ($n = 20$), robust elderly ($n = 21$), and frail elderly ($n = 16$) adults. The results are expressed as the mean ± SEM. ***$p < 0.001$. ns=not significant.

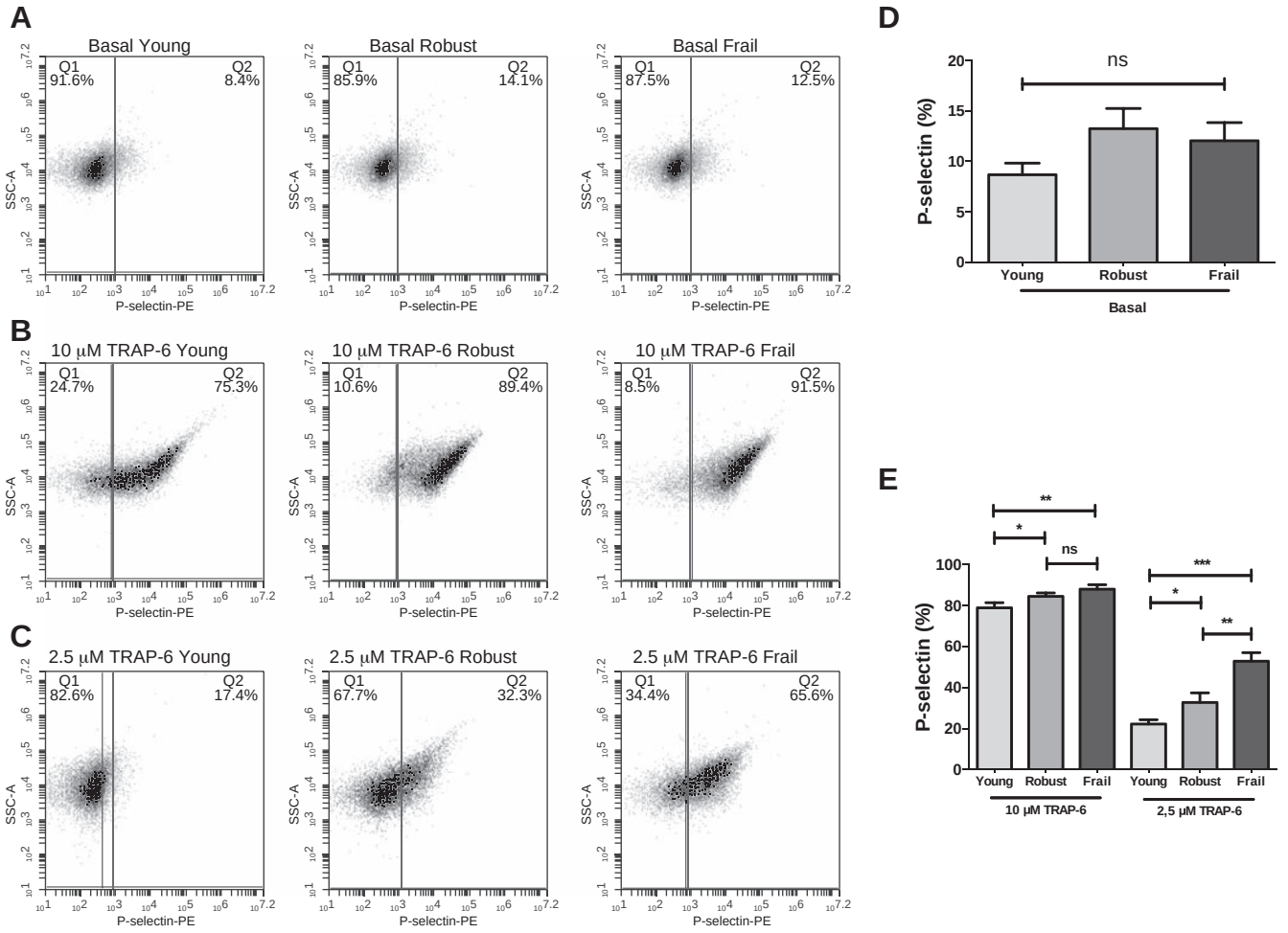

Figure 3. Platelet P-selectin surface expression during aging and frailty. The expression of P-selectin on the platelet surface was determined by flow cytometry in PRP. **(A–C)** Representative graphs showing the positivity for P-selectin and its complexity using forward scatter (FSC) versus side scatter (SSC). The expression of P-selectin was determined in the PRP **(D)** under basal conditions and after incubation with **(E)** 10 or 2.5 μmol/L TRAP-6. The results are expressed as the mean ± SEM, in young ($n = 13$), robust elderly ($n = 21$) and frail ($n = 16$) adults. ***$p <$ 0.001, **$p < 0.01$. *$p < 0.05$. ns=not significant.

did not find any significant difference in basal expression of p-PKCα between the three groups. As p38 is known to be important in signaling mediated by glucose in many cells [40–44], and also has a significant role in platelet activation, thromboxane production, and granule secretion [45–47], p-p38 basal expression was also investigated (Supplementary Figure E1B). However, there were no significant differences in expression of p-p38 in a basal condition in any of the groups.

DM increase and a tendency toward hyperglycemia during frailty

Given the increase in platelet activation during frailty and as this difference was not reflected in the basal conditions of P-selectin, p-PKC, or p-p38, it was reasonable to think that these changes were caused by a stimulus. Thus, we evaluated the characteristics of the population under study to determine what conditions could be affecting platelet function during frailty. The analysis of the population in the PIEI-ES study revealed that frail people had significantly($p = 0.0016$) more DM (42%) than robust (30%) and pre-frail (36%) people (Supplementary Figure E2A, online only, available at www.exphem.org). In addition, according to a regression analysis, a person with DM had a higher probability of being frail (odds ratio [OR] = 1.74), and this difference persisted after a multivariable analysis regardless of age, female gender, body mass index, education level, monthly income, current smoker status, drug consumption, physical activity, and fruit and vegetable intake (data not shown). Furthermore, in the sample evaluated in this study, we found that frail people tended to be hyperglycemic in comparison to robust people, with a p value of 0.0738 (Supplementary Figure E2B), with mean glucose levels of 7.9 ± 1.3 mmol/L (range: 4.6–19.8 mmol/L) in the frail and 6.2 ± 0.3 mmol/L

(range: 4.7–10.4 mmol/L) in the robust groups. In agreement with this, frail people also tended to have higher HbA1c values in comparison to robust people ($p = 0.0980$), with means of 54 ± 3.0 mmol/mol (range: 37–89 mmol/mol) in the frail and 49 ± 1.5 mmol/mol (range: 36–66 mmol/mol) in robust (Supplementary Figure E2C). These data indicate a higher prevalence of DM in the frail population with a tendency toward hyperglycemia; in addition, HbA1c levels indicate a risk for diabetes' complications.

Increase in platelet aggregation with glucose and TRAP-6 stimulus

Based on the previous results, we proceeded to evaluate the role of glucose in platelet aggregation (Figure 4). For this, the platelets from young healthy volunteers were incubated with increasing concentrations of glucose and a subthreshold concentration of ADP or TRAP-6. Incubation of the platelets with different glucose concentrations did not cause any change in platelet aggregation (Figure 4A, B), and after adding a subthreshold stimulus of ADP no change was observed in comparison to the vehicle (Figure 4C, D). However, after stimulation with 22.2 mmol/L glucose and a subthreshold concentration of TRAP-6, a significant increase in platelet aggregation in comparison to the vehicle was observed ($p = 0.0010$) (Figure 4E, F), increasing the aggregation 9.7% from 5.0 ± 0.6% (vehicle) to 14.7 ± 1.2% (22.2 mmol/L glucose and TRAP-6). When comparing aggregation levels obtained with different glucose concentrations, it was determined that there was no statistically significant difference between them.

Because of the role of p38 in the signaling mediated by glucose inside different cell types, we wanted to know if the increase in platelet aggregation caused by glucose and TRAP-6 that we had seen was mediated by p38 signaling. To assess this, we pre-incubated the given condition with 2 μmol/L SB203580 (p38 inhibitor). Platelet aggregation was inhibited by 77.6% from 14.7 ± 1.2% to 3.3 ± 0.9% (4.5-fold), with a p value of 0.0006, diminishing to levels close to that of the vehicle. Overall, these results indicate that high doses of glucose are capable of increasing platelet aggregation, and this effect is dependent on stimulation with TRAP-6. Inhibition with SB203580 suggests that this effect was mediated by p38 signaling.

Increase in P-selectin surface expression with glucose and TRAP-6 stimulus

To further investigate the role of glucose in platelet function, we determined the surface expression of P-selectin by flow cytometry. We selected two concentrations of glucose —11.1 mmol/L (a value close to the levels observed in our frail population) and 22.2 mmol/L (the value with a significant increase in aggregation). Only 22.2 mmol/L glucose values stimulated with a subthreshold concentration of TRAP-6 were able to significantly increase P-selectin expression compared with the vehicle ($p = 0.0034$) (Figure 5); P-selectin expression increased 11.7%, from 30.0 ± 2.0% to 41.7 ± 2.5%. Inhibition with SB203580 caused a 1.9-fold reduction, from 41.7 ± 2.5% to 21.8 ± 3.8% ($p = 0.0019$). These results show an increase in platelet activation mediated through glucose and TRAP-6 by p38 signaling.

Increase in phosphorylation of p38 with glucose and TRAP-6 stimulus

To confirm the role of p38 in glucose signaling inside platelets, the phosphorylation of p38 with glucose and TRAP-6 stimuli was assessed in a total lysate of PRP under the conditions described before (Figure 6A). The incubation of 11.1 or 22.2 mmol/L glucose with the PRP did not increase the phosphorylation of p38. Also, incubation of 11.1 mmol/L glucose with a subthreshold concentration of TRAP-6 did not increase phosphorylation in comparison to the vehicle. However, after incubation with 22.2 mmol/L glucose and a subthreshold concentration of TRAP-6, phosphorylation of p38 increased significantly compared with that of the vehicle ($p = 0.0099$). This increased from 0.8 ± 0.1 RU up to 1.3 ± 0.1 RU (1.6-fold), a level of phosphorylation similar to that observed with 10 μmol/L TRAP-6 (1.5 ± 0.2 RU) and thus a full aggregate concentration. In addition, these results were confirmed by inhibition with SB203580 (2 μmol/L), which caused a significant decrease in the effect seen with glucose 22.2 mmol/L and TRAP-6 ($p = 0.0034$) and decreasing phosphorylation from 1.30 ± 0.1 RU to 0.8 ± 0.1 RU (1.6-fold), and thus the same levels as the vehicle (0.8 ± 0.1 RU). Whether the changes observed in p-p38 were a product of a change in the total protein of p38 (Total p38) was evaluated under all the conditions described above (Figure 6B). This indicated that there was no significant difference between any of them. Together these results confirm that glucose platelet activation is mediated through p38 phosphorylation, indicating that the glucose effect specifically modifies its phosphorylation and not the total protein levels.

Discussion

In the present study, we found an increase in platelet function during aging, and among the aged population, the frail group showed increased platelet activation compared with the robust group; this was expressed through an augmentation in the surface expression of P-selectin. In addition, we evaluated a possible mechanism for this altered function with high doses of glucose. We found that high doses of glucose could increase platelet function (aggregation and activation) through thrombin receptors and p38 phosphorylation.

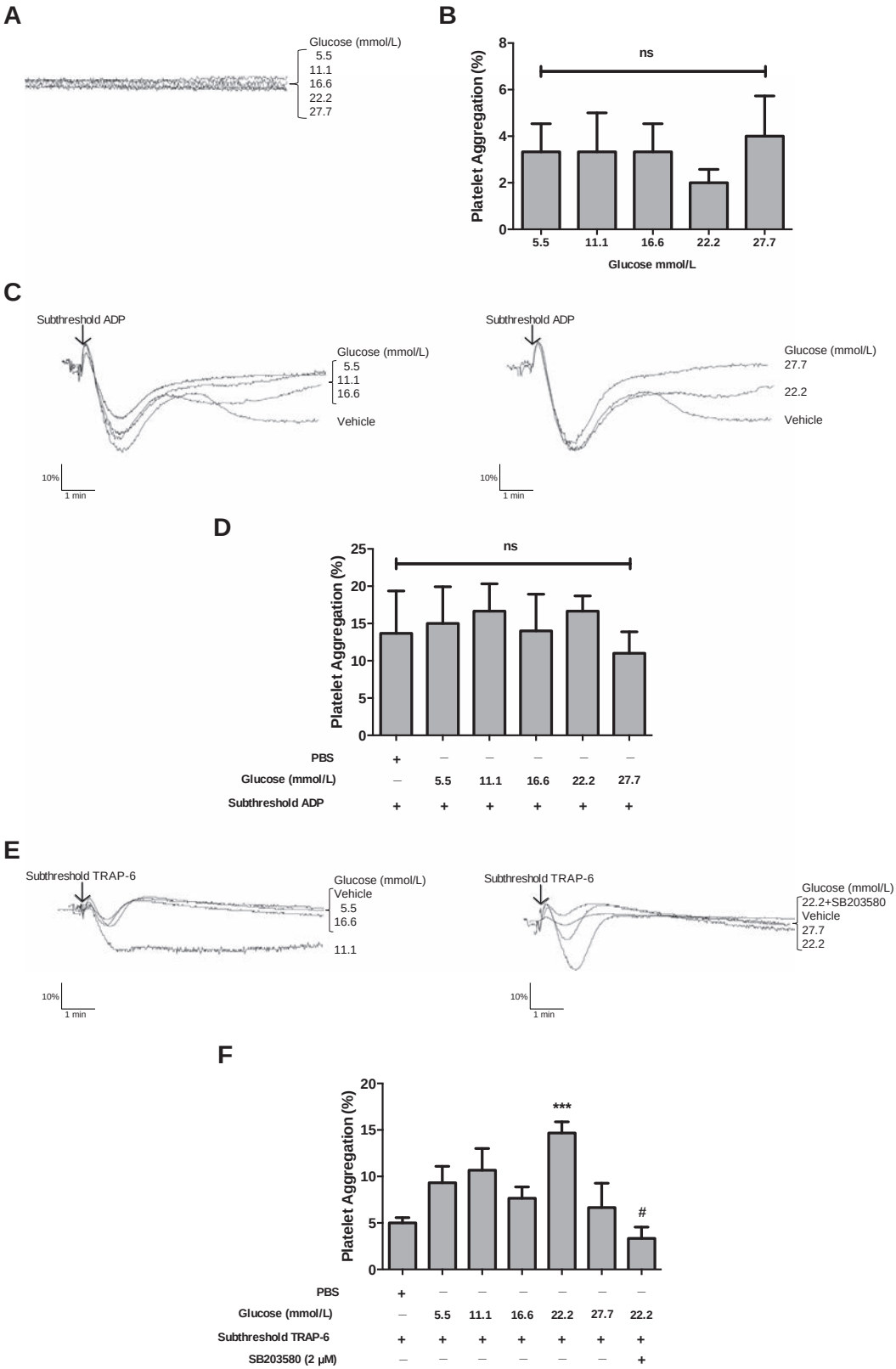

Figure 4. Platelet aggregation induced by glucose and TRAP-6 stimuli and its inhibition by SB203580. The PRP was incubated with 5.5 −27.7 mmol/L glucose **(A, B)**. Subsequently, it was stimulated with subthreshold concentrations of ADP **(C, D)** or TRAP-6 **(E, F)**. For the inhibition of p38, the PRP was pre-incubated with 2 μmol/L SB203580 prior to stimulation with glucose and TRAP-6. Bars represent the mean ± SEM (n=3), ***$p < 0.001$ compared with the vehicle (PBS). $^{\#}p < 0.001$ compared with glucose 22.2 mmol/L. ns=not significant.

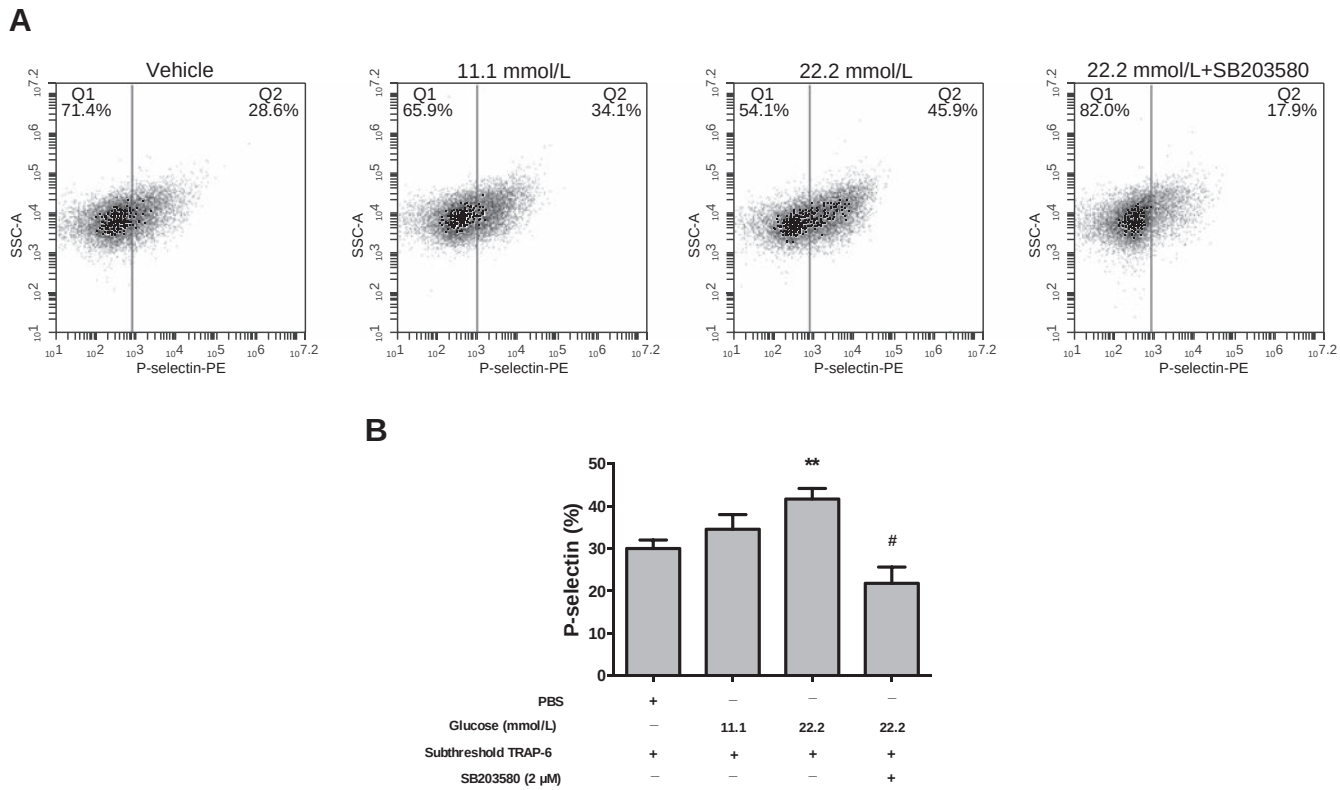

Figure 5. Surface P-selectin expression induced by glucose and TRAP-6 stimuli and its inhibition by SB203580. PRP was pre-incubated with SB203580 (2 μmol/L) and then stimulated with 11.1 or 22.2 mmol/L glucose and a subthreshold concentration of TRAP-6. **(A)** Representative graphs show positivity for P-selectin and its complexity by forward scatter (FSC) versus side scatter (SSC). **(B)** The results are expressed as the mean ± SEM ($n = 3$). $^{**}p < 0.01$ compared with the vehicle (PBS). $\#p < 0.01$ compared with glucose 22.2 mmol/L.

This mechanism could explain, in part, the high rate of acute myocardial infarctions associated with hyperglycemia [48] and, in turn, the high incidence of DM and CVD seen in frailty.

A possible weakness of our study is that a single platelet agonist (TRAP-6) was used, but this may be justified as Heijnen et al. [49] demonstrated that only thrombin was able to promote glucose mobilization; even when using high concentrations of ADP (10 μM) no significant differences were obtained with respect to the control. Also, thrombin caused a marked activation in platelets, concomitant with a substantial redistribution of GLUT-3 to the platelet cell surface membranes, not to the ADP (10 μM).

Also, Dörmann et al. [50] showed that thrombin induced the procoagulant activity of platelets, finding that 80% of platelets had procoagulant activity when stimulated with thrombin. This effect was specific as blocking the PAR-1 receptor (thrombin receptor) with bradykinin (antagonist of PAR-1) completely inhibited the effect. Finally, Kramer et al. demonstrated that only strong agonists were able to activate p38 with the consequent platelet activation [51].

These results are supported by platelet characteristics observed in the aging group: a decrease in platelet count and increase in MPV. Santimone et al. also found these changes—a normal platelet count (234 ± 1.0×10^3 platelets/μL) and MPV (8.63 ± 0.01fL)—but significantly different in comparison to younger people [25]. Even though there is no clear explanation for this change in platelet characteristics during aging, it could reflect a diminishing effect in hematopoietic stem cells in old age [52] or the reduction in thrombopoietin from birth to adulthood [53]. On the other hand, the increase in MPV could be explained as a compensatory mechanism; because of the decrease in platelet count, the bone marrow produces a greater number of reticulated (younger) platelets and therefore has a larger volume [25]. Also, an elevated MPV is associated with greater platelet functionality [54] and has been related to the presence of DM, acute myocardial infarction, coronary atherosclerosis, and risk of cardiovascular accident [54–57].

With respect to platelet function, we found an increase in aggregation and activation during aging. Even though an increase in platelet aggregation has been reported by several investigators [26–28], Jones [58] has highlighted that most of these studies were conducted in middle-aged populations and not in the elderly, not truly concluding what occurs with platelet function during aging. In this study we specifically

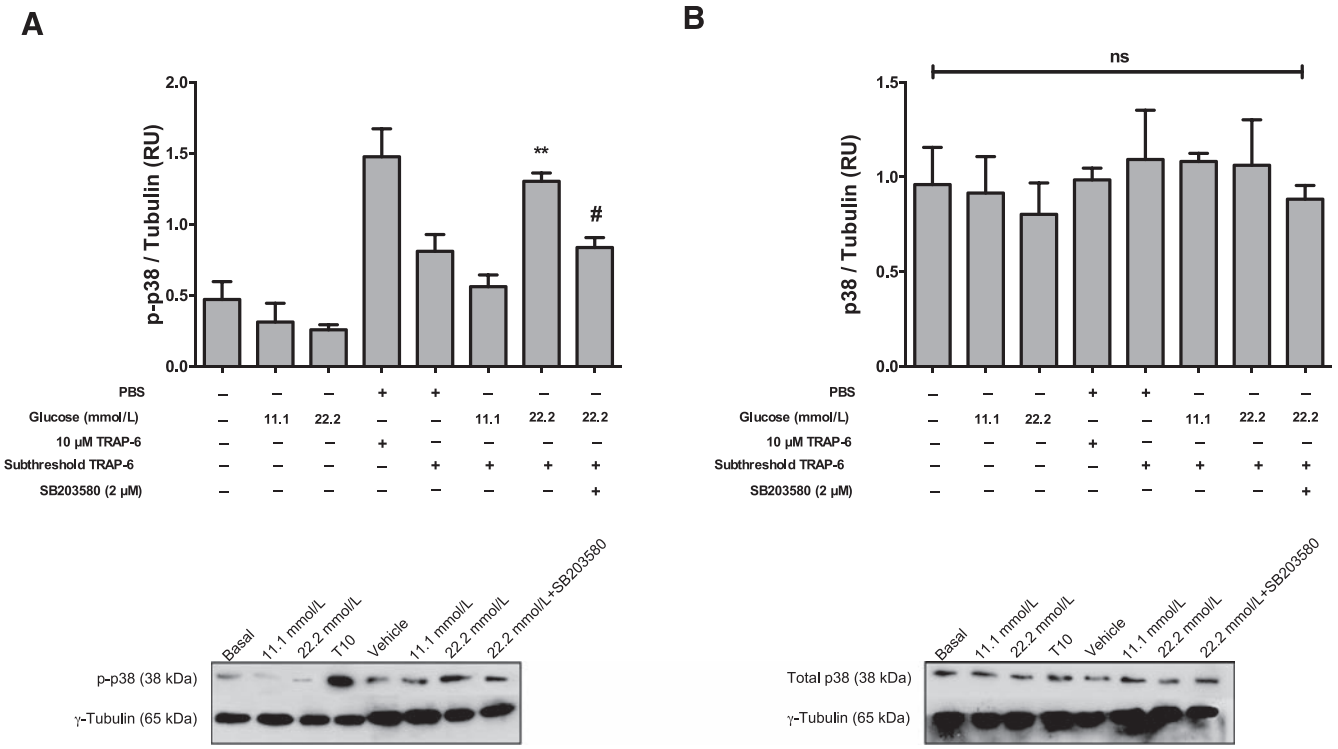

Figure 6. p38 phosphorylation induced by glucose and TRAP-6 stimuli. PRP was pre-incubated with or without SB203580 (2 μmol/L) and stimulated with 11, 1, or 22.2 mmol/L glucose and a subthreshold concentration of TRAP-6. Subsequently, the total lysate of the PRP was analyzed to determine the expression of p-p38 and p38. The expression of both **(A)** p-p38 and **(B)** total p38, was normalized against the expression of γ-tubulin and expressed in relative units (RU). The results are expressed as the mean ± SEM ($n = 3$), $^{**}p < 0.01$ compared with the vehicle (PBS). $\#p < 0.01$ compared with glucose 22.2 mmol/L. ns=not significant.

evaluated a population of older adults (65–88 years old), confirming that platelets from older adults had a higher sensitivity to a subthreshold concentration of an agonist. In addition, we observed higher expression of P-selecting during aging, a change that to our knowledge has only been reported in rodent models [59,60]. The increase in platelet function, particularly in aggregation, is associated with the presence of risk factors for CVD (DM, hypertension, hypercholesterolemia, smoking, and hyperhomocysteinemia) and with the presence of CVD (angina and acute myocardial infarction) [61].

Furthermore, our results indicate an increase in platelet activation during frailty. We did not find a significant difference in platelet aggregation in the frail population in comparison to the robust; this could be explained by the lack of sensitivity of the method in comparison to flow cytometry [62]. It has been reported by others that compared with determination with light transmission aggregometry, platelet function determined by flow cytometry has an added value in diagnosis of mild platelet function disorders [63] and that results from aggregometry were poorly correlated with the results of expression of P-selectin determined by flow cytometry in patients who received antiplatelet treatment [64]. In agreement with this, Nguyen et al. found no difference in the platelet aggregation in frail adults in comparison to robust with full concentration of different agonists [65].

Also, no significant difference in the basal expression of P-selectin, p-PKCα, and p-p38 was found in any of the three study groups. This suggests that the superactivation of platelets during frailty could be caused by a stimulus, new studies should be conducted to determine p-PKCα and p-p38 levels in the presence of levels of subacute TRAP-6 in frail adults. As the alterations of aging are multifactorial, there are many prevalent conditions in frail older adults that could generate an increase in platelet function: oxidative stress [38], inflammation [66], endothelial dysfunction [15], alterations in calcium homeostasis [67]. These, in turn, are associated with the presence of comorbidities such as hypertension [68], DM [69], dyslipidemia [70], and cancer [71]. To find this stimulus, the characteristics of the whole population of the PIEI-ES study and of the subjects sampled in this study were assessed. The analysis highlighted that there was a significant difference in the presentation of DM between the frail and robust groups and that when DM was present there was a greater probability of frailty. In addition, among

the sampled population, frail adults tended to have higher glycemia and HbA1c values than robust adults, with an average value of 54 ± 3.0 mmol/mol (range: 37–89 mmol/mol). These results are in accordance with what was observed in the Women's Health and Aging Studies, where HbA1c levels as low as 48 mmol/mol had a significantly higher association with frailty than in the reference group; in addition, this association increased as the HbA1c value increased [72]. In addition, some studies have shown impaired platelet function in diabetic patients with an average HbA1c of 61 mmol/mol, which represents a fasting glucose of 9.7 mmol/L [73], values close to those presented in our study.

On the basis of these results, the ability of glucose to alter platelet function was evaluated, it is also important to note that in our experiments, high doses of glucose, compared with the levels found in patients, were used to obtain the same glucose effect on platelets from patients (chronic effect); over a shorter time, high concentrations of glucose must be used (acute effect), which has been reported by several authors [21,22,74].

We found that high doses of glucose (22.2 mmol/L) and a subthreshold concentration of TRAP-6 were able to increase platelet aggregation and activation. Sudic and colleagues also found an increase in platelet P-selectin expression with high doses of glucose (30 mmol/L) and ADP or TRAP [22].

Tang and colleagues also found an increase with the stimulation of collagen receptors and high doses of glucose (25 mmol/L) [21]. They also reported no difference in aggregation with the stimulation of TRAP and glucose, this could be explained by the differences in methodology; while Tang and colleagues incubated platelets with glucose for an hour at 37°C, we incubated platelets for only 20 min at room temperature. Some studies have reported that incubation of platelets at 37°C could accelerate the aging and activation of platelets [75,76]; hence this could have clouded the activation with TRAP.

According to our results, glucose has the *in vitro* capacity to aggregate and degranulate the platelets, which may favor thrombotic complications. This is consistent with what has been seen in diabetic patients, in whom plasma glucose levels positively correlate with the ability to form platelet aggregates [77]. Furthermore, the effects observed with the stimulation with TRAP-6 are not irrelevant, as it has been shown that activation with thrombin is capable of generating greater procoagulant activity in platelets (between 60% and 80%) [50] and also increases the transport of glucose to the cell, through the release of GLUT-3 to the platelet membrane [49].

Likewise, it has been proposed that the pathological changes induced by hyperglycemia are mediated in various cells by activation of the p38 protein [40–44]. While in the platelet, p38 has been found to be capable of increasing thromboxane production, granule secretion, and activation of $\alpha IIb\beta_3$ [45–47]. In this study, we found that platelet superactivity produced by glucose and TRAP-6 was mediated through the phosphorylation of p38. Similarly, the phosphorylation of p38 through glucose and collagen in platelets has also been described [21]. The inhibition seen with SB203580 in the platelet aggregation and expression of P-selectin, restoring it to levels observed in the vehicle, suggests that the inhibition of p38 was able to eliminate the effect of glucose, but not the platelet activation of TRAP-6 by other paths. In other studies, the inhibition of p38 has been shown to eliminate the change in shape, adhesion, and platelet aggregation [47,78].

Although the results of this study do not directly prove that the increase in platelet activation in frail older adults is due to hyperglycemia, we consider that one of the mechanisms that could be involved in platelet alterations has been proposed. Even when the effects seen with glucose were only seen with high doses, it is likely that the effects produced by lower concentrations are visible in platelets with longer incubation times; however, the viability of platelet samples for their study is short. One of the limitations of this study is the small number of patients, which prevents us from finding a significant association between glucose levels or HBA1c and platelet hyperactivity. New studies should be carried out with larger numbers of patients.

Therefore, there is a high prevalence of DM during aging, and its effects are associated with an increased risk of manifesting the frailty phenotype as DM and frailty share similar biological bases (such as inflammation) [8]. Currently, it is well defined that there is a platelet superactivity in patients with DM [18]. In this study we found that in addition to this, frail older adults also had greater platelet activation, which could probably be associated with the high prevalence of DM observed in this group. Also, this superactivation can be induced by uncontrolled levels of blood glucose, which can enter the platelet through the GLUT-1 and GLUT-3 receptors [79], favored by the stimulation with thrombin, which inside the cell is able to generate activation of the p38 protein, producing an increase in the secretion of platelet granules, with the consequent expression of P-selectin and the increase in the exposure of GPIIb/IIIa, which allows greater platelet aggregation. Thus, a state of superactivation that favors atherosclerosis and thrombosis in patients with frailty is established (Figure 7).

The results of this study are important because they allow us to understand one of the probable causes of the high incidence of CVD in the frail population.

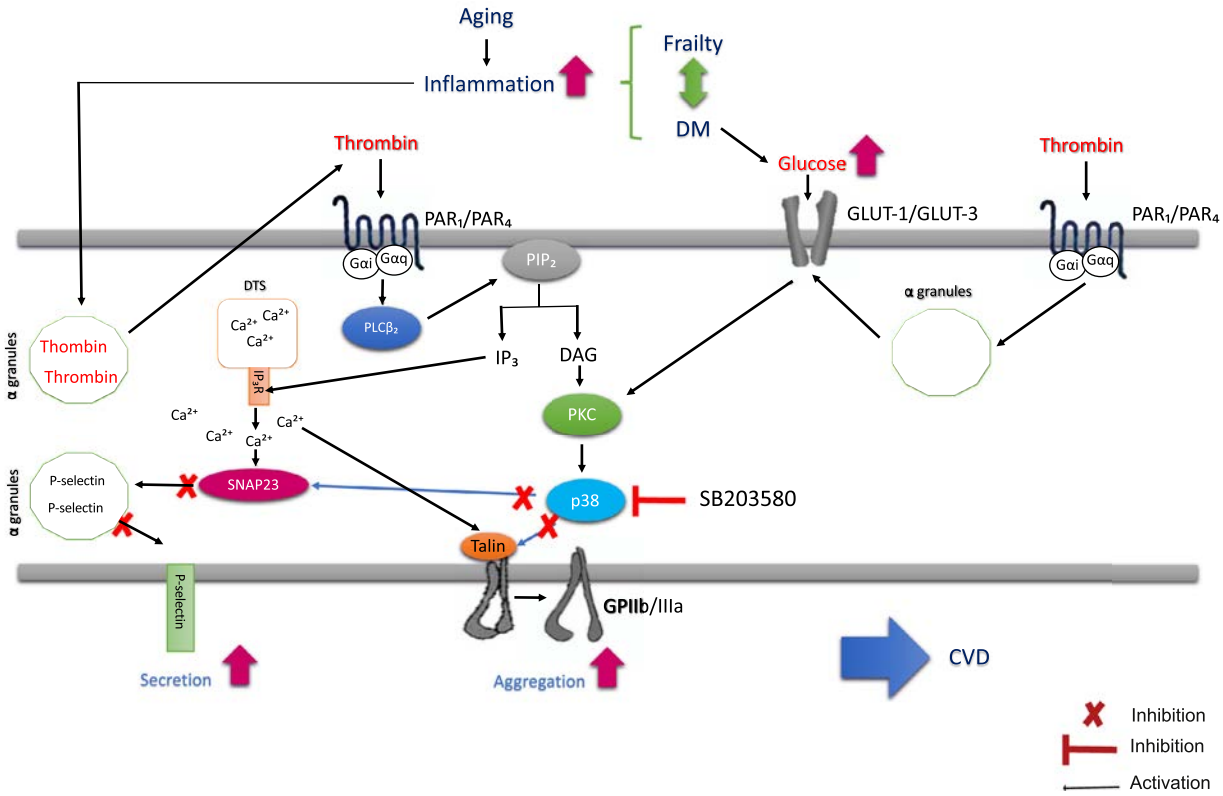

Figure 7. Platelet superactivation during diabetes mellitus and its association with frailty. DM=diabetes mellitus; PAR=protease-activating receptors; PLC=phospholipase C; PIP_2=phosphatidylinositol 4,5-bisphosphate; DAG=diacylglycerol; IP_3=inositol triphosphate; IP_3R=inositol triphosphate receptor; DTS=dense tubular system; SNAP23=protein associated with synaptosome-23; PKC=protein kinase C; GLUT=glucose transporter; CVD=cardiovascular disease.

Given that CVD produces high mortality rates in this population, the identification of individuals at higher risk is of vital importance. Expression of P-selectin and the MPV markers that have been identified is promising in the detection of cardiovascular risk [80]. We consider that a prospective study is needed for this study population, to establish the individuals at greater risk and to define the strategies necessary for the prevention of cardiovascular events in them. In addition, for a better understanding of the role of DM in platelet function during frailty, it is necessary to develop studies on frail patients with and without DM and with or without hyperglycemia.

Conclusions

We found higher platelet function during aging and particularly in a frail population. Although there are many possible sources for this increase during frailty, we propose that higher doses of glucose could be one of these mechanisms. Therefore, alterations in platelet function may be a contributor to the development of CVD in frailty.

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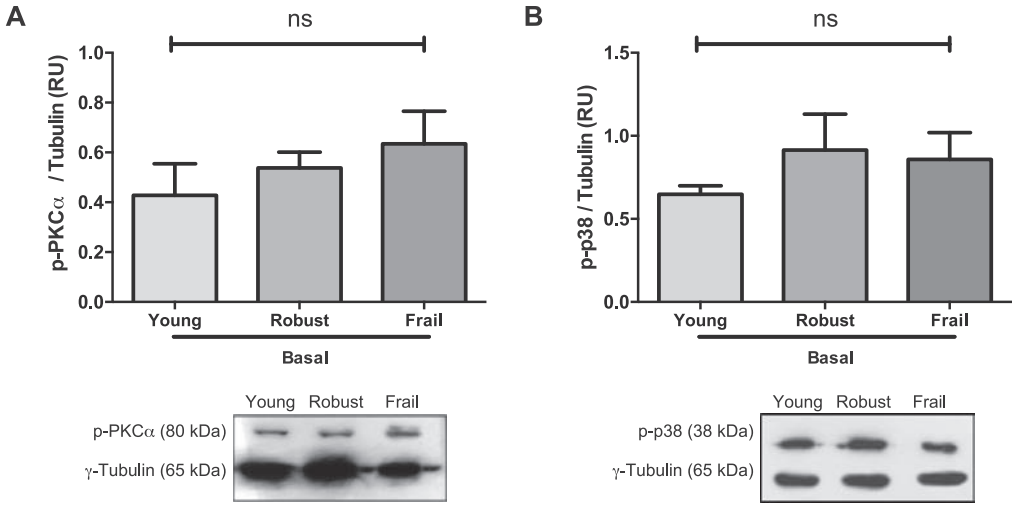
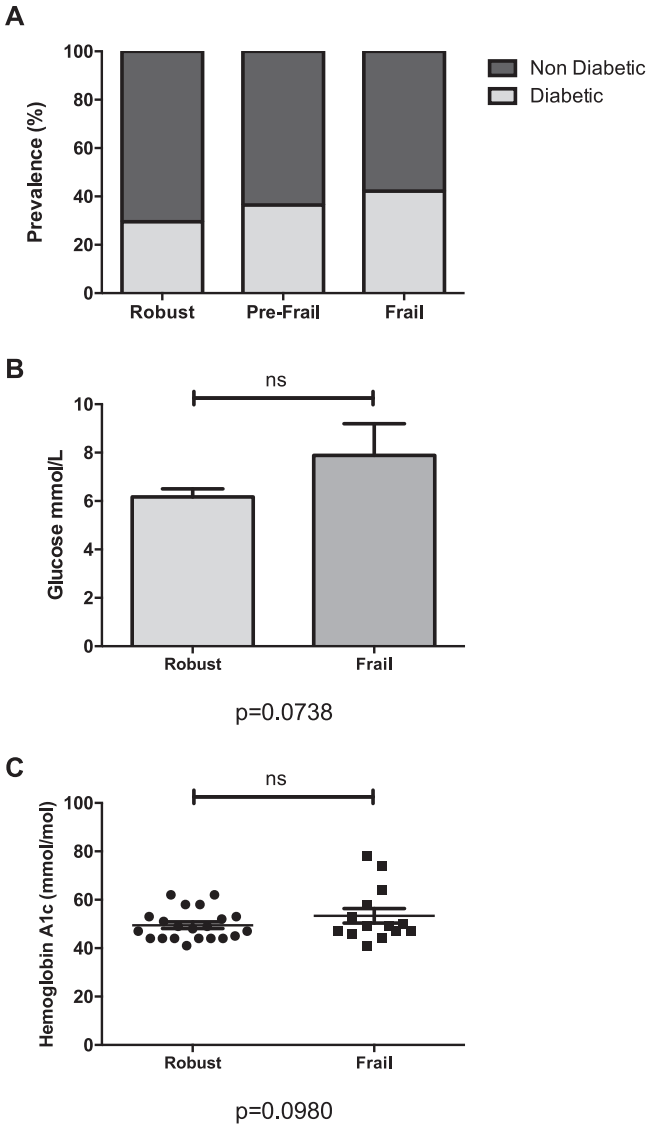


Supplementary Figure E1. Basal expression of p-PKCα and p-p38 in frailty. Basal expression of **(A)** p-PKCα and **(B)** p-p38 was determined in a total lysate of washed platelets. The results were normalized against the expression of γ-tubulin and expressed in relative units (RU). Results are expressed as the mean $\pm$ SEM of $n = 5$. ns=not significant.



Supplementary Figure E2. Diabetes mellitus, glycemia, and glycosylated hemoglobin during frailty. **(A)** Prevalence of diabetes mellitus was determined in robust ($n = 440$), pre-frail ($n = 469$), and frail ($n = 296$) adults. Pearson's χ^2 test was used to determine differences between groups, where $p = 0.0016$. Levels of **(B)** glycemia and **(C)** glycosylated hemoglobin (HbA1c) were determined in robust ($n = 21$) and frail ($n = 14$) adults. Results are expressed as the mean ± SEM. ns=not significant.