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Spontaneous and induced platelet aggregation in apparently healthy subjects in relation to age.

A. Kuhnla¹, M. Reinthaler^{1,2}, S. Braune¹, A. Maier¹, Gerhard Pindur³, A. Lendlein^{1,4}, F. Jung^{1,3,*}

¹ Institute of Biomaterial Science and Berlin-Brandenburg Centre for Regenerative Therapies, Helmholtz-Zentrum Geesthacht, 14513 Teltow, Germany

² Department of Cardiology, Charité - Universitätsmedizin Berlin, University Hospital, Campus Benjamin Franklin, 12203 Berlin, Germany

³ Institute of Clinical Haemostaseology and Transfusion Medicine, Saarland University Hospital, 66421 Homburg, Germany

⁴ Institute of Chemistry, University of Potsdam, 14476 Potsdam, Germany

Corresponding author:

Prof. Dr. Friedrich Jung

Email: friedrich.jung@hzg.de

Abstract

Thrombotic disorders remain the leading cause of mortality and morbidity, despite the fact that anti-platelet therapies and vascular implants are successfully used today. As life expectancy is increasing in western societies, the specific knowledge about processes leading to thrombosis in elderly is essential for an adequate therapeutic management of platelet dysfunction and for tailoring blood contacting implants. This study addresses the limited available data on platelet function in apparently healthy subjects in relation to age, particularly in view of subjects of old age (80 – 98 years).

Apparently healthy subjects between 20 and 98 years were included in this study. Platelet function was assessed by light transmission aggregometry and comprised experiments on spontaneous as well as ristocetin-, ADP- and collagen-induced platelet aggregation. The data of this study revealed a non-linear increase in the maximum spontaneous platelet aggregation (from $3.3 \% \pm 3.3 \%$ to $10.9 \% \pm 5.9 \%$). The maximum induced aggregation decreased with age for ristocetin (from $85.8 \% \pm 7.2 \%$ to $75.0 \% \pm 7.8 \%$), ADP (from $88.5 \% \pm 4.6 \%$ to $64.8 \% \pm 7.3 \%$) and collagen (from $89.5 \% \pm 3.0 \%$ to $64.0 \% \pm 4.0 \%$) in a non-linear manner (linear regression analysis). These observations indicate that during aging, circulating platelets become increasingly activated but lose their full aggregatory potential, a phenomenon that was earlier termed “platelet exhaustion”. In this study we extended the limited existing data for spontaneous and induced platelet aggregation of apparently healthy donors above the age of 75 years. The presented data indicate that the extrapolation of data from a middle age group does not necessarily predict platelet function in apparently healthy subjects of old age. It emphasizes the need for respective studies to improve our understanding of thrombotic processes in elderly humans.

Introduction

Aging is a multi-factorial process, which is characterized by progressive structural and functional alterations of proteins, cells, tissues and organs, leading to chronic conditions and multimorbidity [1–4]. Especially atherosclerosis is a disease that develops over decades resulting in cardiovascular disease (CVD), particularly in coronary artery disease, stroke or peripheral artery disease. Symptoms, if they occur, generally do not begin until middle age. Beyond the age of 60 years, however, coronary heart disease (CHD) becomes the leading cause of death [5–8]. Key elements of thrombus formation are platelets, anucleated cell fragments from megakaryocytes, required for the maintenance of the vasculature. Under physiological conditions they circulate in a quiescent state for approximately 10 days before they are cleared by macrophages in spleen and liver. However, in the case of vascular injury (e.g. by trauma, plaque rupture, detachment of endothelial cells, etc.), they respond immediately by adhering to exposed extracellular matrix proteins undergoing activation. Platelet activation is, next to changes of cytoskeletal proteins, activation of integrins and binding of fibrinogen or von Willebrand factor, characterized by increased adhesiveness to other platelets or cells, exocytosis of granule contents, exposure of phosphatidylserine and thrombin generation [9]. This cascade of events results in fibrin clot formation and cessation of blood loss after vascular injury, which is the main role of platelets.

Despite the successful application of current anti-platelet therapies and vascular implants, thrombotic diseases remain the leading cause of mortality and morbidity. Thus, substantial stratification of patients (patient groups), a better understanding of the platelet activation and their interaction with implant surfaces, as well as the thrombotic processes are required to improve the therapeutic management of platelet function in the patient [10].

Another aspect challenging societies and health care systems is the increased life expectancy, which surpassed the age of 75 years in western countries and is predicted to break the 90 years

barrier by 2030 with a probability of more than 50 % in some countries [11]. This increased life expectancy, however, is associated with an increase in the emergence of chronic health conditions and multimorbidity. As shown by a range of health surveys in Europe, prevalences for musculoskeletal disease, cardio-metabolic conditions and CVD were significantly elevated for persons older than 75 years compared to younger people [12]. The American Heart Association reported an increase in the prevalence of CVD between 12.1 % for males and 13.6 % for females for people between 60 years to 79 years and people beyond the age of 80 years [13]. In view of this, there is a need to understand age-related alterations of platelet function until old age. Until today it is uncertain whether the reported platelet activation, increased coagulation state and reduced fibrinolytic capacity observed in the elderly are caused by aging or coexisting atherosclerosis [14]. Most of the studies dealing with aging and platelets were collected from the general patient population so that it cannot be excluded that those changes reflect the increasing incidence of diseases with age, such as arterial occlusive diseases and those that are associated with platelet function disorders [15–20]. To exclude these confounding processes from age-related alterations, some studies in apparently healthy animals or humans were performed, however, with partly contradictory results. Moreover, the age range of tested healthy individuals is limited to middle-aged donors (< 75 years) for a majority of the studies [20–26]. Assumptions about platelet function of healthy individuals of old age (> 75 years) are often achieved through extrapolation of the findings in the middle-aged group. For parameters like platelet count, mean platelet volume and plasminogen level, linear extrapolation from middle to old age was found to describe age-related changes inappropriately [27–31]. This raised the question whether the same applies for the function of platelets, emphasizing the need for studies including donors of old age [32]. In previous studies addressing age-related changes in platelet function in old age, test subjects were rather patients than healthy donors, which makes the separation of age or disease-related influences on platelet function very difficult [15,33].

In the present work, spontaneous and induced platelet aggregation of apparently healthy donors up to the age of 98 years was studied, aiming to extend the limited existing data on platelet function in old age (> 75 years).

Materials and Methods

Study population and blood preparation

The study was designed in accordance with the ethical guidelines of the journal [34] and received an approval of the ethics committee of the Charité University Medicine Berlin (EA2-018-16). At the participating study centers, apparently healthy donors in an age range from 20 years to 98 years (females) and 20 years to 96 years (males) were selected according to the criteria of the Nordkem-workshop and as described earlier [35,36]. Briefly, the participating blood donors did not receive any pharmaceuticals affecting platelet function (e.g. platelet surface receptor blockers, prostaglandin metabolism inhibitors, substances reducing calcium availability) for at least 10 days. Donors with diagnosed lipid metabolism disorder, hypertension or diabetes mellitus were excluded from the study. Following a standardized atraumatic protocol, blood was withdrawn from the cubital vein and collected in S-Monovettes® (Sarstedt, Nümbrecht, Germany), containing sodium citrate as an anticoagulant in a final concentration of $0.106 \text{ mol} \cdot \text{L}^{-1}$. For homogenization of anticoagulant and blood, the tubes were slowly agitated immediately after blood collection and samples were discarded if there was any evidence of clotting. In accordance with the British Society of Haematology [37], donors were only included in the study if the platelet count was within the range of $150 - 600 \cdot 10^9 \cdot \text{L}^{-1}$. Platelet rich plasma (PRP) and platelet poor plasma (PPP) were obtained by centrifugation of citrated whole blood at 140 g for 20 minutes, or at 1500 g for 20 minutes, respectively.

Light transmission aggregometry (LTA)

Platelet aggregation was assessed by light transmission aggregometry (LTA) according to Born, which is regarded as the gold standard for the evaluation of platelet function [37]. Following the recommendations of the International Society on Thrombosis and Haemostasis [38], the platelet count of PRP was not adjusted to avoid possible artifactual inhibition of platelet aggregation [39,40]. Using an APACT 4004 (Haemochrom Diagnostica, Essen, Germany), aggregation index curves were recorded for 10 minutes, describing the changes in light transmission intensity. The maximal extent of aggregation expressed as percentage (% TA) was obtained in a single measurement for each donor and parameter. To calibrate the light transmission intensity, donor-specific PPP (0 % Turbidity) and PRP (100 % Turbidity) were used. Including a range of standard agonists at standard single doses [37,41,42], induced platelet aggregation was evaluated for ristocetin at $1 \text{ mg} \cdot \text{mL}^{-1}$, ADP at $10 \text{ } \mu\text{M}$ and collagen at $5 \text{ } \mu\text{g} \cdot \text{mL}^{-1}$ final concentrations in PRP. All agonists were purchased from Haemochrom Diagnostica (Essen, Germany). Additional, spontaneous aggregation of PRP was determined.

Statistical Analysis

The correlation between maximal aggregation and age was tested by calculating Spearman's r coefficient. To visualize the results, simple linear regression was performed and the 95 % confidence interval was calculated. P-values less than 0.05 were considered as statistically significant. The statistical analysis was compiled in GraphPad Prism version 6.00 for Windows (GraphPad Software, La Jolla California, USA).

Results

Maximum spontaneous platelet aggregation was tested for $n = 104$ apparently healthy donors in an age range of 20 - 98 years (mean age 42 ± 24 years, females $n = 44$). The overall mean of the spontaneous aggregation was $4.5 \% \pm 4.2 \%$. The lowest mean values were observed for the people in their twenties (20-group: $3.3 \% \pm 3.3 \%$) and the highest for people in their nineties

(90-group: $10.9 \% \pm 5.9 \%$). Values increased and were positively correlated with donor age (Spearman's correlation coefficient $r = 0.31$, P-value 0.0013) (Table 1 and Figure 1, upper row). Linear regression and 95 % confidence intervals are visualized in Figure 1. The coefficient of determination (R^2) of the linear regression analysis was 0.16 and represents a poor model fit (Table 1). The multiple comparison analysis between all five age groups (20, 30, mid-age (40-79), 80 and 90) revealed that the means of all groups differed from the mean of the test subjects in their nineties (Figure 1, lower row).

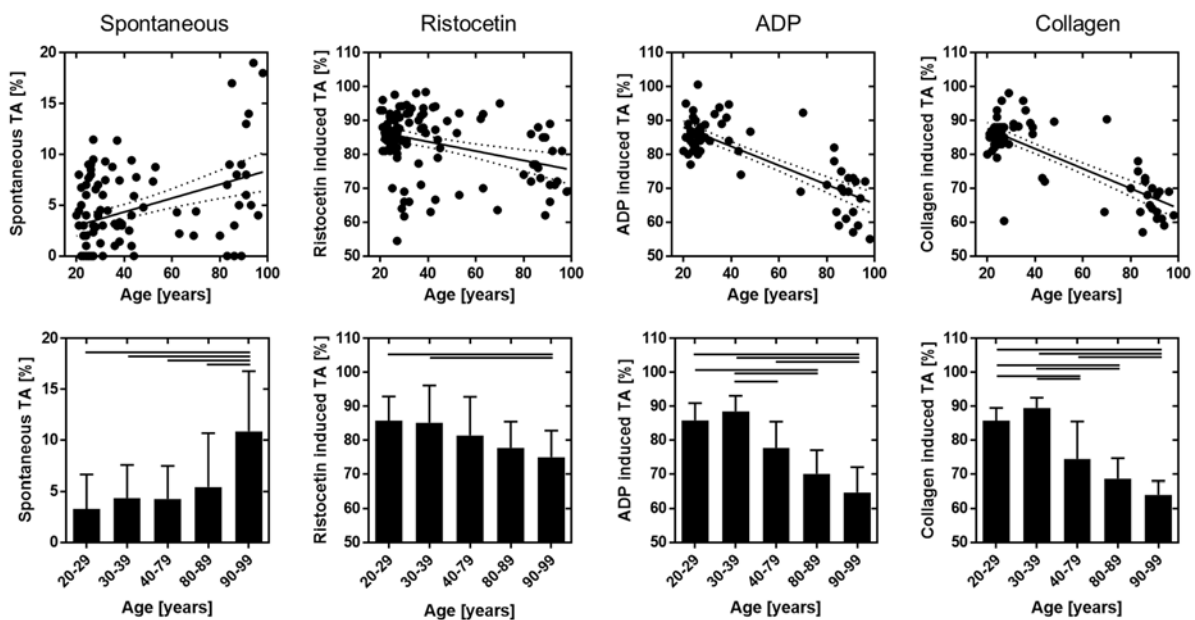


Figure 1. Age-related spontaneous and induced platelet aggregation. Linear regression fit and 95 % confidence interval (upper row) as well as arithmetic mean and standard deviation of the age groups (lower row) are displayed for the maximum spontaneous, ristocetin-, ADP- and collagen-induced platelet aggregation (%).

The maximum ristocetin induced aggregation was tested for $n = 113$ in an age range of 20 - 98 years (mean age = 42 ± 24 years, females $n = 45$). ADP-, as well as collagen-induced aggregation was tested for $n = 76$ donors in an age range of 20 – 98 years (mean age = 44 ± 28 years, females $n = 30$). The overall means were $83.6 \% \pm 9.4 \%$ for ristocetin, $80.3 \% \pm 13.0 \%$

for ADP and $80.6 \% \pm 10.2 \%$ for collagen. For ristocetin, the lowest mean values were observed for the 90-group ($75.0 \% \pm 7.8 \%$) and the highest for the 20-group ($85.8 \% \pm 7.2 \%$). This was not the case for the maximal ADP and collagen induced aggregation. Here, the highest mean values were observed for the 30-group (ADP: $88.5 \% \pm 4.6 \%$, collagen: $89.5 \% \pm 3.0 \%$) and the lowest for the 90-group (ADP: $64.8 \% \pm 7.3 \%$, collagen: $64.0 \% \pm 4.0 \%$).

Agonist induced platelet aggregation tests were negatively correlated with donor age (Table 1 and Figure 1, upper row). The strongest negative correlation was observed for ADP-induced platelet aggregation ($r = -0.55$, P-value < 0.0001), compared to ristocetin ($r = -0.26$, P-value 0,006) and collagen ($r = -0.28$, P-value < 0.0001).

Table 1. Results of correlation and linear regression analysis of age-related changes of platelet aggregation. Sample size n, Spearman's coefficient r, equation and R² of linear regression analysis, as well as respective P-values, are given for age-related changes in spontaneous and induced aggregation.

Platelet aggregation	n	Spearman correlation		Linear regression		
		r	P-Value	Equation	R ²	P-Value
Spontaneous	104	0.31	0.0013	$y = 0.06792 \cdot x + 1.624$	0.16	< 0.001
Ristocetin-induced	113	-0.26	0.006	$y = -0.1385 \cdot x + 89.31$	0.12	< 0.001
ADP-induced	76	-0.54	< 0.0001	$y = -0.2604 \cdot x + 91.72$	0.31	< 0.0001
Collagen-induced	76	-0.28	< 0.0001	$y = -0.2935 \cdot x + 93.43$	0.63	< 0.0001

Except for ristocetin, linear regression analysis revealed higher R² values for agonist induced compared to spontaneous platelet aggregation (ristocetin: R² = 0.12, ADP R² = 0.31, collagen R² = 0.63). However, the linear regression model did not reach an accurate goodness of fit (near 1) in any of the cases.

For ristocetin-induced aggregation, the multiple comparison tests showed that the mean of the 90-group differed significantly from the means of people in their twenties (20-group) and thirties (30-group) (Figure 1, lower row). For ADP-induced platelet aggregation, means of the 80- and 90-groups differed significantly from the 20- and 30-groups, but not from the mid-age group (40-79-group). The latter differed significantly from the 30-group, which showed the highest mean of all age groups. The means of both old-age groups (80- and 90-group) did not differ significantly from each other. The statistical analysis revealed the same differences for the collagen induced aggregation. Here, the mean of the mid-age group differed from the means of the 20- and 30-groups. Also, the 30-group had the highest mean value, even though, it was not significantly different to the mean of the 20-group.

For the maximal spontaneous platelet aggregation, correlations were similar for females and males (Figure S1, Table S1). For induced aggregation the effects were more profound in females than in males. However, those observed differences were not significant and not further considered in view of the relatively small n for each sex (Figure S2 – S4, Table S1).

Discussion

The majority of studies addressing age-related changes of platelet function in apparently healthy humans include donors of a maximum age of 75 years [20–26]. Assumptions about platelet function in older individuals have been made by extrapolation of these findings, but epidemiological studies assessing further platelet parameters reveal the limitations of this approach. While e.g. platelet counts are stable during middle age (< 60 years) they decrease in older people [27–29]. Also, platelet indices - such as the mean platelet volume - were shown to increase during middle age and to decrease in men beyond 80 years [30]. In the present work apparently healthy humans between 20 and 98 years were included to address the limited data for platelet function - particularly aggregation - of apparently healthy subjects of old age.

Spontaneous platelet aggregation

The results of this study revealed a positive correlation (Spearman) between age and spontaneous platelet aggregation, independent of the donor gender (see Figure 1 and Table 1). These findings are in line with previous data, e.g. of the HAPARG study (“Haemostatic Parameters as Risk Factors in Healthy Volunteers”), which reported an increase in the spontaneously enhanced platelet aggregation for healthy donors between 30 and 65 years [43]. The present data confirm this trend including donors of young (20-group) and old age (80- and 90 group). The available literature reveals that spontaneous platelet aggregation and hyperaggregability are in principle positively correlated with the risk of vascular diseases such as atherosclerotic heart disease [43,44]. The observed slight increase of spontaneous platelet aggregation in donors without a history of CAD might be indicative of low-grade subclinical thrombosis evolving during aging [45,46]. Thus, our results support previous findings suggesting spontaneous platelet aggregation as a marker for predicting future coronary events and mortality, even in low risk groups [44]. For the donors of old age, it appears plausible that further comorbidities and cardiovascular risk factors might coevolve and contribute to the sudden increase in platelet aggregation, such as age-related immune-senescence, the associated rising prevalence of acute systemic inflammatory and infectious diseases or prodromal atherosclerosis [47–49]. For instance, in subjects without evident CVD, levels of C-reactive protein (CRP) have been reported to increase with age [50]. The prothrombotic effects of CRP might support platelet reactivity and suggest CRP to be an important risk factor associated with age-related diseases like atherosclerosis, beyond its function as inflammatory biomarker [51,52]. Also, several parameters of the hemostatic system are reported to be altered and to very likely affect platelet function. One factor that is discussed to influence age-related alterations of platelet function is nitric oxide (NO). NO influences several signalling pathways e.g. the activation of sGC and intracellular cGMP and can inhibit platelet activation, adhesion and aggregation [53–55]. Not only endothelial production, but platelet synthesis of NO decreases

with age too. Also, diminished bioavailability of NO due to an elevated clearance by O_2^- is discussed. Generation of the latter is augmented in oxidative stress, which is associated with aging - e.g. of the heart tissue - and cardiovascular disease in principle [56–59]. Thus, an age-related decrease of NO is associated with a reduction of its inhibitory function and can lead to an increase in platelet hyperaggregability. Findings on age-related decrease in the fibrinolytic activity are in line with these observations [60]. Other factors, which are involved in regulating platelet function, are the von Willebrand factor (vWF), fibrinogen, β -thromboglobulin and platelet factor 4. The respective plasma levels are described to elevate during aging (and cardiovascular disease) and can have an enhancing effect on platelet reactivity [61–64].

Induced platelet aggregation

In contrast to the increase in spontaneous platelet aggregation, we found that platelet aggregation in response to ristocetin, ADP and collagen decreased with increasing age for females and males. Previous data on the relation of age on stimulated platelet function are not conclusive. While some studies concluded that there were no differences between young and old donors [65–67], others – e.g. the Northwick Park Heart Study - described an increase in the induced platelet aggregation (e.g. ADP-induced) in donors of higher age [20,21,23,24,60,68,69]. Interestingly, in a range of studies, epinephrine as well as serotonin levels were shown to be elevated in plasma of elderly humans, while thresholds to induce platelet aggregation with these agonists were lower (higher sensitivity) in this age-group compared to younger study participants [70–73]. Particularly these results have led to the longstanding opinion that the responsiveness of platelets increases with age almost linearly. However, the majority of these studies assessed platelet function in a restricted age range and only little data are available how this changes in people of old age and on a molecular level. One study on induced platelet aggregation (collagen, ADP and epinephrine), which was conducted in the frame of the Framingham Heart Study (1363 females, 1058 males, 26 - 82

years), revealed that aggregability decreased with age [25]. In a later study on 109 patients (38 - 92 years) suffering from angina pectoris with and without acute coronary syndromes, multivariate analyses revealed age as the strongest predictor of decreased ADP-induced platelet aggregation [33]. Women of younger age (< 45 years) had significantly higher agonist-induced aggregation response than both men and post-menopausal women (60 - 65 years) [74]. As reported by Knight and coworkers, ADP and thrombin-induced fibrinogen binding and P-selectin expression on platelets were lower in old (mean age: 52 years) compared to young subjects (mean age: 25 years) [15]. Further, the ability to polymerize actin, as part of the thrombin-induced platelet activation process, was shown to decrease with age [75]. In this context, the work of Meade and coworkers may shed some light on the contrary findings in the present literature [20]. They concluded an age-related increase in the ADP-induced platelet aggregability based on the dose of ADP, at which aggregation proceeds at half its maximum velocity (ADP ED50). However, the estimated maximum response on the agonist (ADP EMR) was shown to decrease with aging, which is in good agreement with the data of our study. These results indicate that the age-related increase in the spontaneous platelet aggregation may be associated with an exhaustion of the platelets. Interestingly, a similar phenomenon was described for women exhibiting pre-eclampsia during their pregnancy [76]. Here, activation of the coagulation system and endothelial cell dysfunction (e.g. reduced production of antiaggregatory prostacyclin and nitric oxide) are associated with an increase of circulating platelet aggregates and platelet aggregation, which is followed by metabolic exhaustion of the platelets due to chronic overstimulation [77–80]. For patients with hemolytic uremic syndrome, thrombotic thrombocytopenic purpura, obstructive pulmonary disease or malignant cancer, severe cases of platelet exhaustion were reported, e.g. as an impaired ability to respond to epinephrine, ADP and collagen [81–85].

Linearity of the data

The linear regression and the group wise analysis revealed that the observed increase in the spontaneous platelet aggregation only poorly fits a linear model ($R^2 = 0.16$). Particularly, for the people in their nineties ($10.9 \% \pm 5.9 \%$), maximum aggregation values increased suddenly (doubling compared to the previous age group), after a continuous but considerably smaller increase between the donors in their twenties (20-group: $3.3 \% \pm 3.3 \%$) and donors in their eighties (80-group: 5.5 ± 5.2). Similarly, R^2 values for agonist induced aggregation revealed an only poor model fit for the linear regression (ristocetin: $R^2 = 0.12$, ADP: $R^2 = 0.31$, collagen: 0.63). The maximal response on ADP and collagen occurred in the group of donors in their thirties (30 group, see Figure 1). However, for both agonists, the increase was below 4 % and not significantly different to the mean values of the younger donors (20-group). Despite the biological relevance of the level of differences might be debatable, taken together, our data support previous findings of O'Donnell et al. that the age-dependent changes in platelet aggregation might not be linear and that trends e.g. from middle age groups cannot be extrapolated into older age groups [25]. As discussed earlier by Jones, it appears plausible that platelet function might change at different stages of aging, similarly to and possibly associated with the alterations in platelet counts [32]. Overall, the present literature reveals that the mechanisms underlying age-related changes in platelet function – particularly in people of old age – are by far not understood. This may also stem from the difficulties in unraveling the influence of age on platelet function from those of (subclinical) chronic disease and the increasing use of prescription medication.

Conclusion

The results of this study support earlier findings that age-related changes in spontaneous and induced platelet aggregation are not linear but may change at different stages of aging, as reported for other parameters such as the platelet count. These changes were particularly

prominent in very old donors (90+) and, thus, support earlier assumptions that extrapolation of platelet function from data of young or middle age humans into old age may not be adequate [32]. The increase in spontaneous and the decrease in agonist induced platelet aggregation (ristocetin, ADP, collagen) indicated a chronic increase in the activation of circulating platelets, which might be associated with platelet exhaustion, as observed in other clinical disease patterns such as pre-eclampsia or cancer. With this study, there is now increasing evidence that in the elderly, platelets develop a reduction in their ability to react to physiological agonists *in vitro*. These results seem to indicate the need for more profound analysis of platelet function – such as platelet platelet adhesion – in humans particularly of old age (> 75 years) to expand our knowledge about the processes leading to thrombosis in the elderly. A deeper understanding of these age-related processes would be essential for improving the therapeutic management of platelet dysfunction and might support approaches for tailoring cardiovascular implants hemocompatible. However, a major remaining challenge will be to distinguish between alterations related to age and those related to low-grade subclinical changes in the hemostatic system.

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