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Circulating monocyte-platelet aggregates are a robust marker of platelet activity in cardiovascular disease

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HIGHLIGHTS

- Monocyte-platelet aggregates (MPA) are a robust marker of platelet activity and inflammatory monocytes.
- MPA are higher in patients with cardiovascular disease.
- MPA are highest in patients with peripheral artery disease (PAD).

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ABSTRACT

Background and aims: Platelets are a major culprit in the pathogenesis of cardiovascular disease (CVD). Circulating monocyte-platelet aggregates (MPA) represent the crossroads between atherothrombosis and inflammation. However, there is little understanding of the platelets and monocytes that comprise MPA and the prevalence of MPA in different CVD phenotypes. We aimed to establish (1) the reproducibility of MPA over time in circulating blood samples from healthy controls, (2) the effect of aspirin, (3) the relationship between MPA and platelet activity and monocyte subtype, and (4) the association between MPA and CVD phenotype (coronary artery disease, peripheral artery disease [PAD], abdominal aortic aneurysm, and carotid artery stenosis).
Methods and results: MPA were identified by $CD14^+$ monocytes positive for $CD61^+$ platelets in healthy subjects and in patients with CVD. We found that MPA did not significantly differ over time in healthy controls, nor altered by aspirin use. Compared with healthy controls, MPA were significantly higher in CVD (9.4% [8.2, 11.1] *vs.* 21.8% [11.5, 44.1], $p < 0.001$) which remained significant after multivariable adjustment ($\beta = 9.1$ [SER = 3.9], $p = 0.02$). We found PAD to be associated with a higher MPA in circulation ($\beta = 19.3$ [SER = 6.0], $p = 0.001$), and among PAD subjects, MPA was higher in subjects with critical limb ischemia (34.9% [21.9, 51.15] *vs.* 21.6% [15.1, 40.6], $p = 0.0015$), and significance remained following multivariable adjustment ($\beta = 14.77$ (SE = 4.35), $p = 0.001$).
Conclusions: Circulating MPA are a robust marker of platelet activity and monocyte inflammation, unaffected by low-dose aspirin, and are significantly elevated in subjects with CVD, particularly those with PAD.

1. Introduction

Platelets play a key role in the pathogenesis of atherosclerosis and thrombosis [1]. Upon activation, platelets degranulate and P-selectin translocates to the platelet surface [2]. Surface bound P-selectin binds to P-selectin glycoprotein ligand-1 (PGSL-1) on monocytes, allowing a monocyte-platelet aggregate (MPA) to form [3,4]. *Ex vivo* measurement of MPA in the circulation has been proposed to represent a robust biomarker of platelet activation *in vivo* [5,6]. Cross-talk between platelets and monocytes is considered a crucial pathophysiological

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mechanism linking thrombosis and inflammation and contributes to the pro-inflammatory action induced by activated platelets [7]. Several studies have found increased MPA in patients with coronary artery disease (CAD), aortic stenosis, unstable angina, and acute myocardial infarction [8–11]. However, there is a paucity of data comparing MPA across CVD phenotypes or their association with disease severity.

Circulating monocytes are a heterogeneous cell population with three dominant subtypes distinguished by their differential surface expression of the LPS co-receptor CD14 and FCγIII receptor CD16: classical $CD14^{++}CD16^{-}$, intermediate $CD14^{++}CD16^{+}$, and nonclassical $CD14^{+}CD16^{++}$ [12]. These distinct subtypes differ phenotypically in their infiltrative [13], migratory [14], inflammatory [15], and gene expression profiles [16]. Classical monocytes are reported to be phagocytic with no inflammatory attributes [17]. Intermediate monocytes have been shown to contribute to atherosclerosis [18] and display both phagocytic and inflammatory functions while the non-classical subtype performs *in vivo* patrolling and have a pro-inflammatory phenotype [17]. Platelets can act as effector cells to promote monocyte inflammation [19], however it remains unclear if platelets preferentially bind to a specific monocyte subtype.

In the present study, we sought to assess [1] MPA measurement over time in a group of healthy volunteers [2], *ex vivo* and *in vitro* effect of aspirin on MPA within healthy subjects, [3] activity of platelets and inflammatory status of monocytes in MPA, and [4] circulating MPA in subjects with various phenotypes of CVD, including CAD, peripheral artery disease (PAD), abdominal aortic aneurysm (AAA), and carotid artery stenosis (CAS).

2. Patients and methods

2.1. Study population

The study was performed in accordance with policies of the New York University School of Medicine Institutional Review Board, and informed consent was obtained from each subject. As previously described [20], healthy volunteers > 21 years of age were recruited for a reproducibility study of platelet activity. Exclusion criteria included history of cardiovascular disease, use of medications known to affect platelet function (e.g. non-steroidal anti-inflammatory drugs [NSAIDs], anti-histamines, and selective serotonin reuptake inhibitors) 5 days prior to baseline phlebotomy, platelet count $< 100 \times 10^9$/L or $> 450 \times 10^9$/L, chronic kidney disease, anemia, or any known hemorrhagic diathesis.

Patients with CVD were recruited into ongoing studies (thrombosis [THoR] registry, platelet activity and cardiovascular events [PACE] and platelet activity and vascular thrombosis and bleeding [PIVOTAL]) investigating the role platelet activity and platelet transcriptomics in CVD [19–22]. For this cohort, subjects > 21 years of age with diagnosed CAD, CAS, PAD or AAA were recruited from the New York University Langone Medical Center Tisch Hospital, Bellevue Hospital, or the Veterans Affairs NY Harbor Healthcare System. Subjects were excluded if they used NSAIDs (other than aspirin) in the past week, antithrombotic therapy, platelet count $< 100 \times 10^9$/L or $> 450 \times 10^9$/L, renal failure (Cr Cl < 30 ml/min or on dialysis), presence of co-existing inflammatory disease (e.g. rheumatoid arthritis, lupus, etc.), cancer, active infection, anemia, or any known hemorrhagic diathesis.

2.2. Study design

Demographics and medical history were obtained via direct interview and medical record, if available. Healthy volunteers had a blood collection weekly for four consecutive weeks and took 81 mg aspirin daily for 7 consecutive days between weeks 3 and 4. Subjects fasted overnight and refrained from intensive exercise and tobacco use prior to an early-morning phlebotomy to avoid circadian changes in platelet activity. A cohort of patients with established CVD was included for comparison.

2.3. Blood collection

For healthy controls and patients with CVD, peripheral blood samples were drawn using a 19 gauge needle without the use of a tourniquet. A subset of patients with CVD scheduled to undergo arterial revascularization had blood samples collected from the radial artery prior to incision. After an initial 2 cc discard, blood was collected into tubes containing 3.2% (0.105 mol/L) sodium citrate for platelet activity measures. Samples were processed immediately. Prior data from our group demonstrated excellent intraclass correlation coefficient (0.71, $p < 0.0001$) for MPA between arterial and venous collection [23].

2.4. Measurement of monocyte-platelet aggregates

MPA were identified in citrate anticoagulated blood as previously described [24]. Briefly, whole blood was fixed with 1% formaldehyde (Sigma) 15 min after blood collection. Fixed whole blood was stained with 5 μL CD61-FITC (Dako) to identify platelets and 5 μL CD14-APC (BD Biosciences) to identify monocytes. After lysis of red blood cells, monocytes were collected based on side-scatter properties and positive staining for CD14 using an Accuri C6 flow cytometer (BD Biosciences, San Jose, CA). MPA were identified as having a positive stain for CD14 and CD61, and were recorded as a percent of 2000 total monocytes collected.

2.5. Platelet activity measurements

Platelet activation was determined by platelet surface expression of GPIIb/IIIa (PAC-1), P-selectin, and CD40 with whole blood flow cytometry, as previously described [20]. Briefly, activated GPIIb/IIIa was assessed with a fluorescein isothiocyanate (FITC)-conjugated PAC-1 antibody (BD Biosciences), P-selectin expression was determined with a FITC-conjugated anti-CD62P antibody (BD Biosciences), and CD40 expression was determined with a FITC-conjugated anti-CD40 antibody (BD Biosciences). Gates were established to include platelets with and without aggregation to monocytes. Monocytes were identified by staining with CD14-APC (BD Biosciences), and platelets were identified by staining with CD42-PE (BD Biosciences). The expression of platelet activation markers were assessed individually.

2.6. Monocyte type measurements

Monocyte characterization was performed by flow cytometry analysis on whole blood collected in sodium citrate. Ten thousand monocytes were collected based on their forward-side-scatter properties. Monocyte subsets were identified by double immunostaining for CD14 and CD16. Monocytes were grouped into classical ($CD14^{++}CD16^{-}$), intermediate ($CD14^{++}CD16^{+}$), and non-classical ($CD14^{+}CD16^{++}$). In each monocyte category, the percent of CD42b-positive events were recorded. To further assess the monocyte type composition within MPA, CD42b positive or CD42b negative monocytes were divided into classical ($CD14^{++}CD16^{-}$), intermediate ($CD14^{++}CD16^{+}$), and non-classical ($CD14^{+}CD16^{++}$) phenotypes and reported as percent of total monocytes.

2.7. Aspirin in vitro experiments

Aspirin (Sigma) was diluted to a stock concentration of 300 μM in 0.01% DMSO. Whole blood was incubated with or without 30 μM aspirin, corresponding to a dose of 81 mg aspirin [25], 15 min after blood collection for 30 min at 37 °C. Following the incubation period, blood was then fixed with 1% formaldehyde for 15 min. Assessment of MPA was performed as described.

2.8. Statistical analysis

Data were analyzed using standard descriptive and multivariable methods. Skewed continuous variables (Shapiro–Wilk) are presented as median [interquartile range] and examined using non-parametric tests. Measurements of MPA were compared between weeks 1, 2, and 3 using the Friedman test. Difference between MPA pre- and post-aspirin was measured with the Wilcoxon Signed Rank test. Correlations were analyzed using Spearman's test. Circulating MPA was compared between groups using non-parametric independent sample tests (Kruskal–Wallis for multiple group comparisons and Mann–Whitney for comparisons between 2 groups). Multivariable linear regression analysis was used to estimate the association between CVD status and circulating MPA, controlling for age, sex, race/ethnicity, body mass index (BMI), smoking status, and family history of CVD. For analysis of phenotypes of CVD, the presence of CAS, CAD, PAD and AAA as well as diabetes, hyperlipidemia and hypertension were additional covariates into the model. A two-sided p-value < 0.05 was considered statistically significant. Statistical analysis was conducted with SAS 9.4 and the R program for scientific computing (available at www.r-project.org).

3. Results

Baseline characteristics of healthy controls that presented for four consecutive visits are shown in Supplemental Table 1. Median age was 29.0 [24.5, 36.5], 50% were female and 54% were white.

3.1. MPA over time

Circulating MPA was evaluated in whole blood of healthy controls (n = 48) for three consecutive weeks by flow cytometry (Fig. 1A). Values did not significantly differ between weeks 1, 2, and 3 (8.7% [8.0, 11.2] in week 1, 9.7% [8.4, 10.4] in week 2, and 8.9% [7.2, 11.4] in week 3, $p = 0.93$; Fig. 1B and C).

3.2. Demographics and MPA

Next, we assessed if MPA was associated with demographics and clinical variables within our healthy cohort. Healthy controls aged 21–49 with a median age of 28 [25.4, 33] were compared to healthy controls aged 51–72 with a median age of 59.5 [54, 64]. MPA values

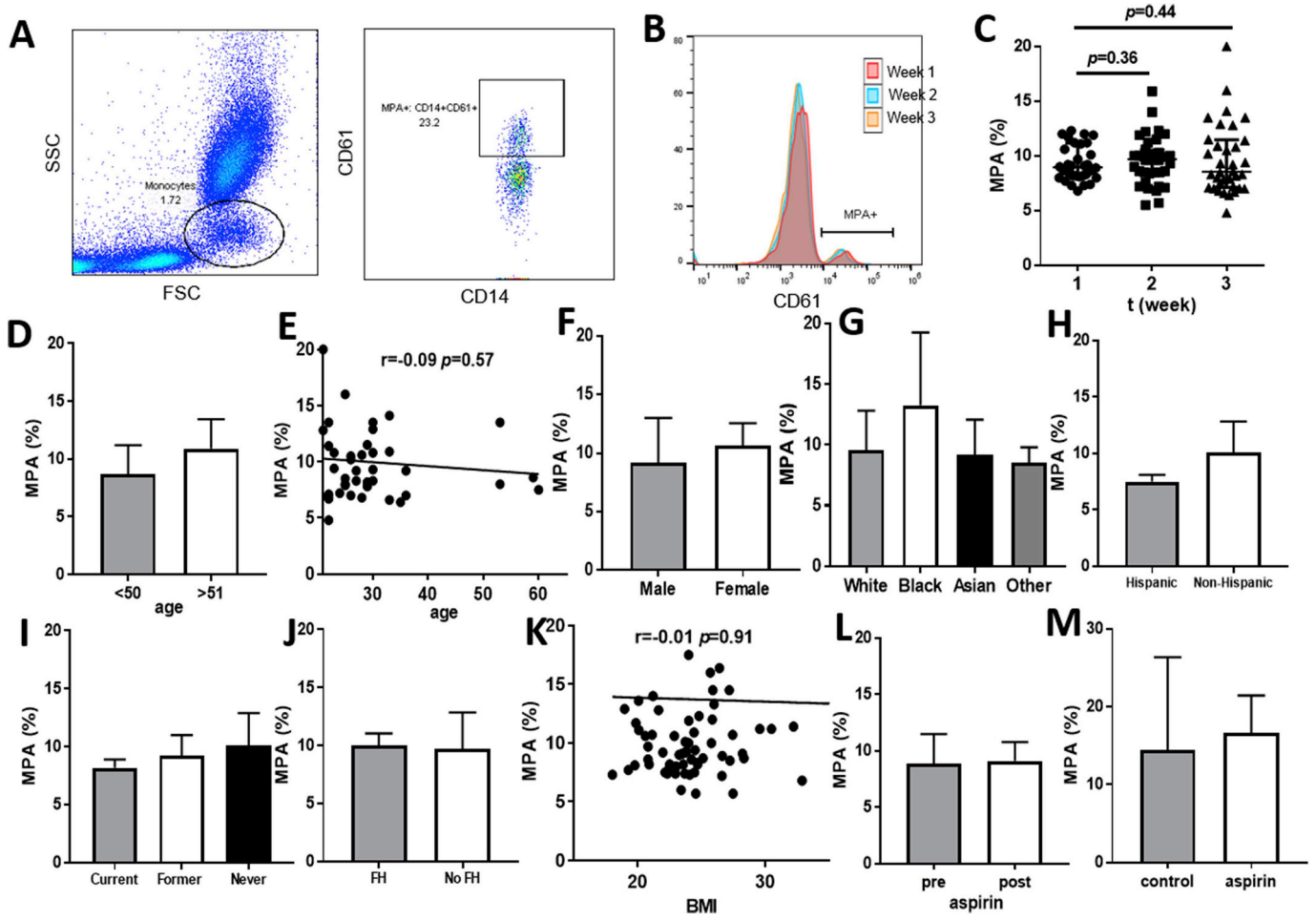

Fig. 1. Monocyte-platelet aggregates are independent of baseline characteristics and unaffected by low-dose aspirin in healthy controls.
(A) Monocyte-platelet aggregates (MPA) characterization by flow cytometry. Monocytes were first gated by characteristic forward scatter (FSC)-side scatter (SSC), shown in the circle, and expression of CD14, shown in the middle panel. The percentage of $CD14^+$ events that were also $CD61^+$ were considered MPA, shown in the right panel. To assess MPA measurements over time, healthy controls (n = 48) were analyzed over three consecutive weeks. (B) An example of a single subjects MPA over three weeks. (C) MPA percent over time in the healthy control cohort. Monocyte-platelet aggregates (MPA) did not significantly differ with age (D and E), sex (F), race (G), ethnicity (H), smoking status (I), family history (FH) of cardiovascular disease (CVD) (J), or body mass index (BMI) (K) in the healthy control cohort. (L) Monocyte-platelet aggregates (MPA) were assessed in healthy controls (n = 48) before and after seven days of *in vivo* aspirin monotherapy (81 mg daily, $p = 0.40$). (M) Healthy donor whole blood (n = 5) was incubated with a physiological dose of 30 μM aspirin *in vitro* (aspirin) or buffer (control) before assessment of MPA ($p = 0.63$).

did not significantly differ between groups, (8.7% [8.0, 11.1] in younger controls *vs.* 10.9% [10.0, 13.3] in older controls, $p = 0.44$; Fig. 1D), and there was no significant correlation between age and MPA ($r = 0.09$, $p = 0.51$; Fig. 1E). Furthermore, MPA was not significantly different when stratified by sex (Fig. 1F), race (Fig. 1G), ethnicity (Fig. 1H), smoking status (Fig. 1I), family history of CVD (Fig. 1J), or body mass index (Fig. 1K).

3.3. Aspirin does not affect MPA

Healthy volunteers had their platelet function measured before and after 1-week of low-dose aspirin (81 mg daily). Aspirin compliance was evaluated by measuring platelet aggregation in response to arachidonic acid. Platelet aggregation decreased from 90.6% [89.3, 92.5] to 13.1% [8.2, 21.1] ($p < 0.0001$) demonstrating excellent COX-1 inhibition (data not shown). MPA did not significantly differ with low-dose aspirin (pre-aspirin 8.9% [7.3, 11.5] *vs.* post-aspirin 9.1% [7.3, 10.8], $p = 0.40$; Fig. 1L). To confirm these findings, we investigated the effect of *in vitro* aspirin on MPA. MPA did not differ when whole blood was incubated with aspirin at physiological concentrations (30 μM [25]) (no aspirin 14.4% [11.5, 26.4] *vs.* aspirin 16.6% [10.5, 21.5], $p = 0.63$; Fig. 1M).

3.4. Platelet activity and monocyte subtype in MPA

Platelet activity of platelets aggregated versus not aggregated to monocytes was assessed via surface expression of PAC-1, P-selectin, and CD40. All activation markers investigated were significantly increased on platelets aggregated with monocytes versus platelets not aggregated to monocytes ($p < 0.05$ for each comparison; Fig. 2A and B). To assess the robustness of MPA as a marker of platelet activity, we investigated the correlation between these measures. MPA was significantly correlated with P-selectin and CD40 (Fig. 2C and D). No significant correlation was detected between MPA and platelet PAC-1 expression (Supplemental Fig. 1).

We next assessed if platelets preferentially aggregate to specific monocyte subtypes. Monocytes from healthy controls were stratified into monocyte subtype: classical ($CD14^{++}CD16^{-}$), intermediate ($CD14^{++}CD16^{+}$), or nonclassical ($CD14^{+}CD16^{++}$), then each subtype was individually assessed for percent of platelets attached. We found that proportionally more intermediate and non-classical monocytes were aggregated to platelets than classical monocytes ($p < 0.05$, Fig. 3A and B). We then investigated if platelet aggregation to monocytes within whole blood was monocyte subtype specific. Classical monocytes are less likely to be associated with platelets ($p = 0.024$) while a significantly greater proportion of nonclassical monocytes are found in MPA within the circulation ($p = 0.027$). No significant difference was noted in the intermediate monocyte population (Fig. 3C). These data demonstrate that platelets preferentially bind to the nonclassical pro-inflammatory monocyte subtype.

3.5. MPA are elevated in patients with CVD

We next sought to examine if circulating MPA were higher in patients with CVD. Baseline characteristics of healthy controls (n = 73) and CVD subjects (n = 345) are shown in Supplemental Table 2.

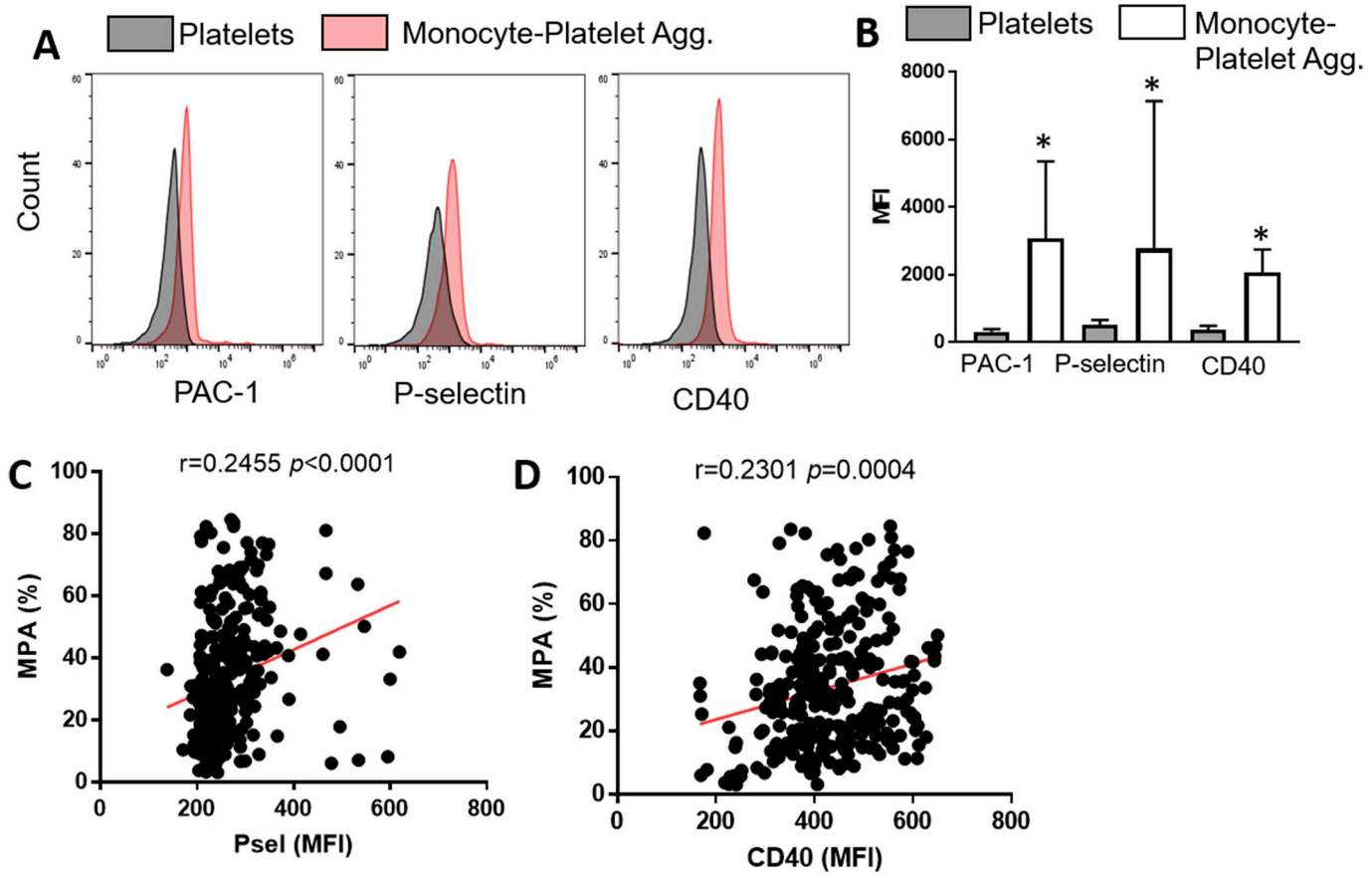

Fig. 2. Platelets are more hyperactive in monocyte-platelet aggregates. Surface expression of PAC-1, P-selectin, and CD40 was assessed on platelets alone versus platelets aggregated to monocytes within the same healthy donor blood sample.
(A) Representative flow cytometry plot of analysis, and (B) quantification of platelet activation markers (*$p < 0.05$ between platelets alone versus platelets aggregated to monocytes). (C) Monocyte-platelet aggregates (MPA) are significantly correlated with platelet P-selectin (Psel) ($r = 0.2455$, $p < 0.0001$) and (D) CD40 surface expression ($r = 0.2301$, $p < 0.0004$) (n = 302). MFI = Mean Fluorescence Intensity.

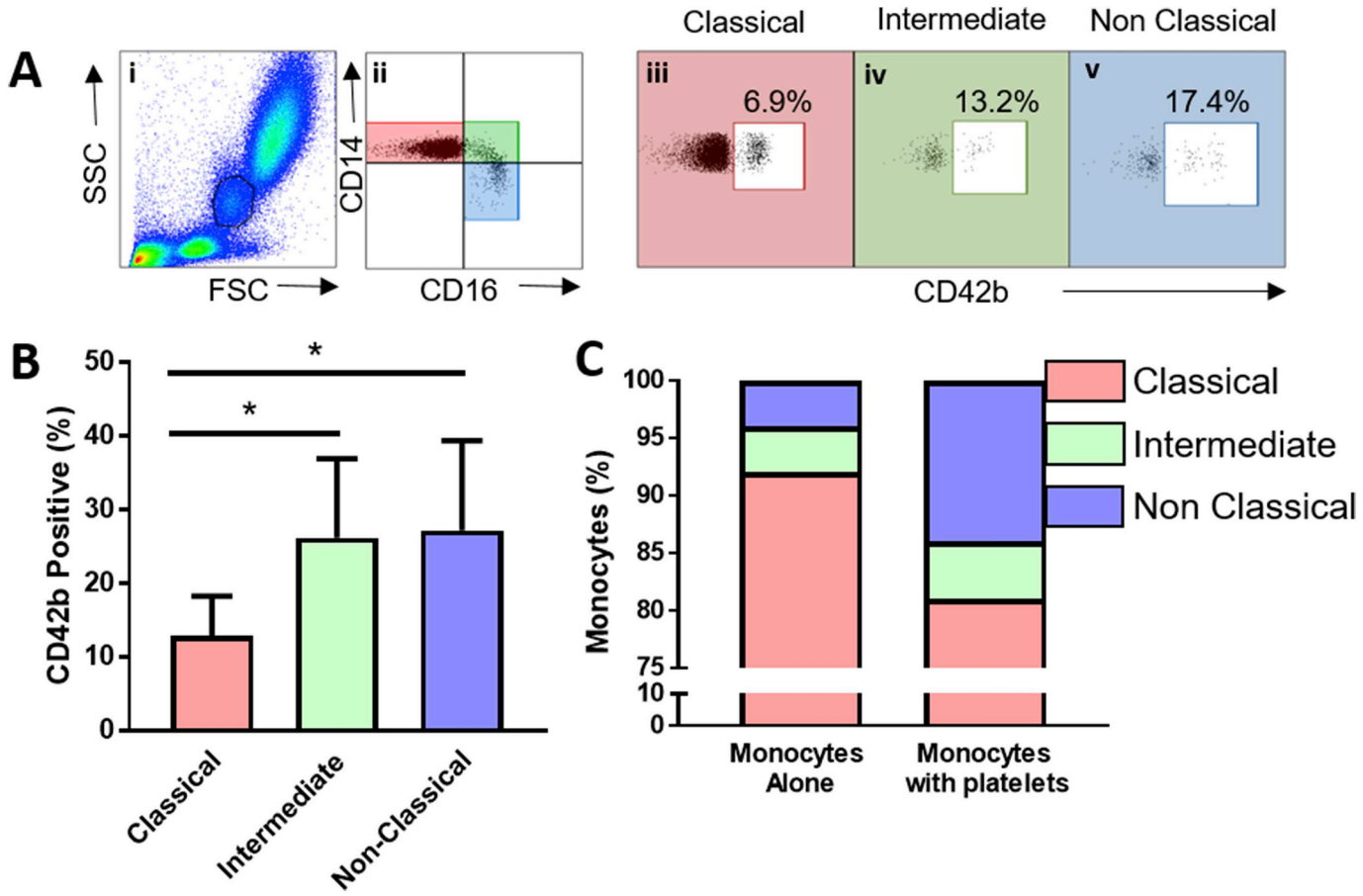

Fig. 3. Intermediate ($CD14^{++}CD16^{+}$) and non-classical ($CD14^{+}CD16^{++}$) monocytes are associated with monocyte-platelet aggregates.
(A) Monocytes were first isolated by their characteristic forward scatter (FSC) side scatter (SSC) (i) then split into monocyte phenotypes based on CD14 and CD16 expression. Classical monocytes ($CD14^{++}CD16^{-}$) are shown in red, intermediate monocytes ($CD14^{++}CD16^{+}$) are shown in green, and nonclassical monocytes ($CD14^{+}CD16^{++}$) are shown in blue (ii). Each monocyte subtype was then divided and MPA assessed (panel iii-v). MPA was reported as the percent of each monocyte subtype positive for the platelet marker CD42b. (B) Healthy donor monocytes (n = 6) were divided into subtypes then assessed for percent MPA, $*p < 0.05$ as determined via one-way ANOVA with Tukey's multiple comparison test. (C) Assessment of platelet aggregation to monocytes in whole blood as determined by monocyte subtype. Classical monocytes are less likely to be associated with platelets ($p = 0.024$), while non classical monocytes are more likely to be associated with platelets ($p = 0.027$), as determined by a paired students t-test. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

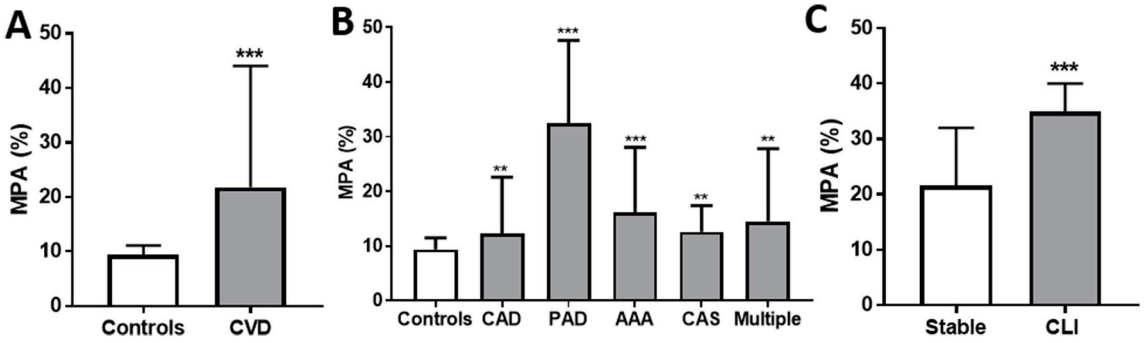

Fig. 4. Monocyte-platelet aggregates are increased in cardiovascular disease.
(A) Healthy subjects on aspirin monotherapy (n = 64) were brought in for assessment of monocyte platelet aggregates (MPA). Subjects with established cardiovascular disease (CVD) on aspirin were analyzed for comparison (n = 351). After adjustment for differences in smoking status, sex, age, race, and BMI, the CVD group is associated an increased number of MPA ($*p = 0.02$). MPA was determined by quantification of CD14 and CD61 positive events. (B) Subjects with established CVD on aspirin (n = 345) were broken up by specific phenotypes, including carotid artery disease (CAD), peripheral artery disease (PAD), abdominal aortic aneurysm (AAA), carotid artery stenosis (CAS), and multiple phenotypes and had MPA assessed. (C) MPA levels are significantly elevated in patients with PAD presenting with critical limb ischemia (CLI) $***p < 0.0001$; $**p < 0.001$.

Table 1
Linear regression analysis of CVD cohort by disease phenotype.

Parameter	β	Standard error	*p*-value
CVD			
CAD only	0.663	5.209	0.899
PAD only	19.346	5.995	**0.001**
AAA only	4.736	7.747	0.541
CAS only	0.206	5.823	0.972
Multiple	5.057	5.589	0.366
Smoker: Current	2.385	3.495	0.495
Smoker: Former	−0.157	2.398	0.948
Race: Black	7.838	3.365	**0.020**
Race: Asian	−4.135	4.093	0.312
Race: Other	3.597	3.613	0.320
Age	−0.004	0.089	0.962
BMI	−0.279	0.185	0.131
Female	−2.272	2.158	0.293
Family History of CVD	−3.494	2.269	0.124
Diabetes	1.890	2.385	0.428
Hyperlipidemia	5.286	3.914	0.177
Hypertension	2.363	2.916	0.418

CVD = cardiovascular disease; CAD = coronary artery disease; PAD = peripheral artery disease; AAA = abdominal aortic aneurysm; CAS = carotid artery stenosis; BMI = body mass index.

Circulating MPA were higher in CVD than controls (21.8 [11.5, 44.1] *vs.* 9.4 [8.2, 11.1], $p < 0.0001$; Fig. 4A). After adjustment for age, sex, race/ethnicity, smoking status, BMI, and family history of CVD, circulating MPA remained significantly associated with CVD (β = 9.1 (SER = 3.9), $p = 0.02$).

Each phenotype of CVD within our patient cohort (CAD, PAD, AAA, CAS, or polyvascular disease) had significantly higher MPA than healthy controls (Fig. 4B). We next performed a multivariable linear regression analysis to investigate the association of different phenotypes of CVD with MPA. After adjustment for age, sex, race/ethnicity, smoking status, BMI, hyperlipidemia, hypertension, diabetes, and each phenotype of disease, PAD was significantly associated with an increase in MPA (β = 19.3 (SE = 6.0), $p = 0.001$; Table 1). The only other variable associated with increasing MPA was black race (versus white; β = 5.1 (SE = 3.4), $p = 0.02$). Next, we categorized PAD by severity of disease (Demographics in Supplementary Table 3). We found that PAD subjects with versus without critical limb ischemia (CLI) had an increased MPA (34.9% [21.9, 51.15] *vs.* 21.6% [15.1, 40.6], $p = 0.0015$). This difference remained statistically significant after multivariable adjustment (β = 14.77 (SE = 4.35), $p = 0.001$).

4. Discussion

The binding of platelets and monocytes in CVD highlights the integral overlap between inflammation and thrombosis in cardiovascular disease [7]. The role of platelets in the recruitment of leukocytes to sites of vascular injury is a well-established immune response [26]. Activated platelets bind to leukocytes in circulating blood, where the binding of platelet P-selectin to PSGL-1 on leukocytes is the dominant molecular event in these interactions. The interaction of platelets with monocytes has physiological inflammatory consequences, and increasing evidence indicates they contribute to the propagation of inflammatory damage in disease [19,27]. Given the non-standardized nature of MPA measurements [28], we first investigated the reliability of this biomarker over time. We found that our measurement of circulating MPA in whole blood did not differ over time, and was independent of baseline demographics. Additionally, we found MPA measurements to be unaffected by aspirin. However, we found that MPA significantly correlated with platelet activity markers associated with increased adhesion, e.g. surface expression of P-selectin and CD40. Altogether, these data strongly suggest that circulating MPA is a robust marker of platelet activity *in vivo*.

Human monocytes display a range of heterogeneity, with at least three subtypes routinely reported, defined by expression of the surface markers CD14 and CD16. Each subtype is hypothesized to have a unique function (i.e. chemokine release, migration) and the association between monocyte subtype and MPA is relatively unexplored. In our cohort of healthy individuals, we found intermediate ($CD14^{++}CD16^{+}$) and non-classical ($CD14^{+}CD16^{++}$) monocytes had increased aggregability to platelets than classical monocytes ($CD14^{++}CD16^{-}$, Fig. 3B–C). The significance of this observation is currently unknown, however given the key role of non-classical monocytes in the monitoring of the endothelium [29] we hypothesize platelets play an essential role in this process. A recent study suggests that the interaction of platelets with monocytes induces a phenotypic switch in monocytes from healthy donors, by inducing CD16 expression, however whether this occurs *in vivo* is unknown [30]. In addition to these findings, it is thought that $CD16^{+}$ monocytes aggregated to platelets (i.e. intermediate and nonclassical monocytes) exhibit increased adhesiveness to activated endothelium, further highlighting the effector function of platelets to these circulating cells [30]. Thus, identification of $CD16^{+}$ monocyte-platelet aggregates may represent a superior biomarker for platelet activity *in vivo*.

Circulating MPA have been shown to be elevated in subjects with PAD [31], CAD [8], unstable angina [10], and acute MI [11], and are heightened in acute versus stable coronary subjects [32]. However, this is the first study to investigate circulating MPA levels across the CVD spectrum. We found a significant increase in MPA in patients with each phenotype of CVD, and in particular patients with PAD. Notably, differences remained significant following adjustment for age, sex, race/ethnicity, smoking status, BMI, hyperlipidemia, hypertension, diabetes, and CVD phenotype (Fig. 4B, Table 1).

Following additional adjustment for clinical phenotype, circulating MPA was most linked with PAD. Among patients with PAD, MPA was higher in those with the most severe subtype of PAD, CLI, and remained so after adjustment for demographics and clinical risk factors. Prior data from our group found increased platelet activity in patients with PAD and the potent effect of PAD platelets on monocyte inflammation and migration [19]. Our new data suggest that the platelet-monocyte interaction is significantly pronounced in PAD and requires understanding of this observation. Circulating inflammatory MPA may represent a biomarker and therapeutic target in PAD. Patients with PAD are at heightened risk of a platelet mediated events, including myocardial infarction, stroke, death and acute limb ischemia [33,34]. Data from clinical trials suggest that aspirin may not be particularly effective in decreasing cardiovascular events in patients with PAD [34,35], in contrast to the well-described effect of aspirin in patients with CAD and prior stroke [36]. These data demonstrate that in the setting of PAD, antiplatelet therapies with different therapeutic targets may be required. Our data demonstrating that aspirin did not change circulating MPA is consistent with prior data in patients with CAD showing no association between MPA and aspirin or its downstream targets [37–39]. However in our current study we did not directly assess MPA and aspirin use in patients with CVD. Of note, clopidogrel was able to decrease circulating MPA [39] and was particularly effective in reducing cardiovascular events compared to aspirin [36,40].

Our findings indicate a heightened cross talk between platelets and monocytes in the setting of PAD, and provide novel insight to the heightened risk of CVD in patients with PAD. MPA are likely to be of significant relevance to patients with PAD as the vasculature of the lower limbs generally has larger vessel lengths making them more susceptible to inflammatory monocyte entry to the sub endothelium, a process elevated by the presence of platelet-bound monocytes [30]. Multivariate adjustment of CVD patients also revealed that black subjects have elevated MPA compared to white subjects. Blacks are known to have increased platelet activity [41], increased MPA [42], and are at higher risk for PAD and its complications [43].

Platelet activity tracks with a range of inflammatory vascular

disorders, and recent studies have demonstrated the importance of the effector cell properties of platelets in inflammatory and immune response [27,44]. Our study highlights the interplay between active platelets and inflammatory monocytes in CVD and demonstrates the importance of circulating MPA as a predictive marker of CVD and disease severity. Additionally, we find that the platelet-monocyte interaction is particularly elevated in PAD. Future therapeutic studies should investigate targeting of MPA in this high-risk population.

Conflicts of interest

The authors declared they do not have anything to disclose regarding conflict of interest with respect to this manuscript.

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Author contributions

N. Allen, T. Barrett, M. Nardi and J. Berger designed the research. N. Allen performed the research. N. Allen, T. Barrett, Y. Guo. and J. Berger analyzed the data. N. Allen, T. Barrett, Y. Guo, M. Nardi, B. Ramkhelawon, C. Rockman, J. Hochman and J. Berger wrote the manuscript. All authors critically revised the manuscript.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.atherosclerosis.2018.12.029.

References

[1] J.J. Badimon, A. Zaman, G. Helft, Z. Fayad, V. Fuster, Acute coronary syndromes: pathophysiology and preventive priorities, Thromb. Haemostasis 82 (1999) 997–1004.
[2] A.D. Michelson, M.R. Barnard, H.B. Hechtman, H. MacGregor, R.J. Connolly, J. Loscalzo, et al., In vivo tracking of platelets: circulating degranulated platelets rapidly lose surface P-selectin but continue to circulate and function, Proc. Natl. Acad. Sci. U. S. A. 93 (1996) 11877–11882.
[3] A. Celi, R. Lorenzet, B. Furie, B.C. Furie, Platelet-leukocyte-endothelial cell interaction on the blood vessel wall, Semin. Hematol. 34 (1997) 327–335.
[4] P.S. Frenette, C.V. Denis, L. Weiss, K. Jurk, S. Subbarao, B. Kehrel, et al., P-Selectin glycoprotein ligand 1 (PSGL-1) is expressed on platelets and can mediate platelet-endothelial interactions in vivo, J. Exp. Med. 191 (2000) 1413–1422.
[5] E. Shantsila, G.Y. Lip, The role of monocytes in thrombotic disorders. Insights from tissue factor, monocyte-platelet aggregates and novel mechanisms, Thromb. Haemostasis 102 (2009) 916–924.
[6] A.D. Michelson, M.R. Barnard, L.A. Krueger, C.R. Valeri, M.I. Furman, Circulating monocyte-platelet aggregates are a more sensitive marker of in vivo platelet activation than platelet surface P-selectin: studies in baboons, human coronary intervention, and human acute myocardial infarction, Circulation 104 (2001) 1533–1537.
[7] J.E. Freedman, J. Loscalzo, Platelet-monocyte aggregates: bridging thrombosis and inflammation, Circulation 105 (2002) 2130–2132.
[8] M.I. Furman, S.E. Benoit, M.R. Barnard, C.R. Valeri, M.L. Borbone, R.C. Becker, et al., Increased platelet reactivity and circulating monocyte-platelet aggregates in patients with stable coronary artery disease, J. Am. Coll. Cardiol. 31 (1998) 352–358.
[9] R. Badr Eslam, T. Gremmel, A. Schneller, M. Stegfellner, A. Kaider, C. Mannhalter, et al., High levels of platelet-monocyte aggregates after valve replacement for aortic stenosis: relation to soluble P-selectin and P-selectin glycoprotein ligand-1 genes, Thromb. Res. 129 (2012) 453–458.
[10] S. Zeng, X. Zhou, L. Ge, W.J. Ji, R. Shi, R.Y. Lu, et al., Monocyte subsets and monocyte-platelet aggregates in patients with unstable angina, J. Thromb. Thrombolysis 38 (2014) 439–446.
[11] M.I. Furman, M.R. Barnard, L.A. Krueger, M.L. Fox, E.A. Shilale, D.M. Lessard, et al., Circulating monocyte-platelet aggregates are an early marker of acute myocardial infarction, J. Am. Coll. Cardiol. 38 (2001) 1002–1006.
[12] L. Ziegler-Heitbrock, P. Ancuta, S. Crowe, M. Dalod, V. Grau, D.N. Hart, et al., Nomenclature of monocytes and dendritic cells in blood, Blood 116 (2010) e74–80.
[13] C. Weber, K.U. Belge, P. von Hundelshausen, G. Draude, B. Steppich, M. Mack, et al., Differential chemokine receptor expression and function in human monocyte subpopulations, J. Leukoc. Biol. 67 (2000) 699–704.
[14] F. Geissmann, S. Jung, D.R. Littman, Blood monocytes consist of two principal subsets with distinct migratory properties, Immunity 19 (2003) 71–82.
[15] E. Shantsila, B. Wrigley, L. Tapp, S. Apostolakis, S. Montoro-Garcia, M.T. Drayson, et al., Immunophenotypic characterization of human monocyte subsets: possible implications for cardiovascular disease pathophysiology, J. Thromb. Haemostasis: JTH 9 (2011) 1056–1066.
[16] K.L. Wong, J.J. Tai, W.C. Wong, H. Han, X. Sem, W.H. Yeap, et al., Gene expression profiling reveals the defining features of the classical, intermediate, and non-classical human monocyte subsets, Blood 118 (2011) e16–31.
[17] R. Mukherjee, P. Kanti Barman, P. Kumar Thatoi, R. Tripathy, B. Kumar Das, B. Ravindran, Non-classical monocytes display inflammatory features: validation in sepsis and systemic lupus erythematous, Sci. Rep. 5 (2015) 13886.
[18] M. Wildgruber, T. Aschenbrenner, H. Wendorff, M. Czubba, A. Glinzer, B. Haller, et al., The "Intermediate" CD14++CD16+ monocyte subset increases in severe peripheral artery disease in humans, Sci. Rep. 6 (2016) 39483.
[19] R. Dann, T. Hadi, E. Montenont, L. Boytard, D. Alebrahim, J. Feinstein, et al., Platelet-Derived MRP-14 induces monocyte activation in patients with symptomatic peripheral artery disease, J. Am. Coll. Cardiol. 71 (2018) 53–65.
[20] B. Shah, V. Valdes, M.A. Nardi, L. Hu, E. Schrem, J.S. Berger, Mean platelet volume reproducibility and association with platelet activity and anti-platelet therapy, Platelets 25 (2014) 188–192.
[21] M.J. Kraakman, M.K. Lee, A. Al-Sharea, D. Dragoljevic, T.J. Barrett, E. Montenont, et al., Neutrophil-derived S100 calcium-binding proteins A8/A9 promote reticulated thrombocytosis and atherogenesis in diabetes, J. Clin. Invest. 127 (2017) 2133–2147.
[22] E. Montenont, C. Echagarruga, N. Allen, E. Araldi, Y. Suarez, J.S. Berger, Platelet WDR1 suppresses platelet activity and is associated with cardiovascular disease, Blood 128 (2016) 2033–2042.
[23] B. Shah, S.P. Sedlis, X. Mai, N.S. Amoroso, Y. Guo, J.D. Lorin, et al., Comparison of platelet activity measurements by use of arterial and venous blood sampling, J. Thromb. Haemostasis: JTH 11 (2013) 1922–1924.
[24] B. Shah, N. Allen, B. Harchandani, M. Pillinger, S. Katz, S.P. Sedlis, et al., Effect of colchicine on platelet-platelet and platelet-leukocyte interactions: a pilot study in healthy subjects, Inflammation 39 (2016) 182–189.
[25] A.K. Pedersen, G.A. FitzGerald, Dose-related kinetics of aspirin. Presystemic acetylation of platelet cyclooxygenase, N. Engl. J. Med. 311 (1984) 1206–1211.
[26] M. Ghasemzadeh, E. Hosseini, Intravascular leukocyte migration through platelet thrombi: directing leukocytes to sites of vascular injury, Thromb. Haemostasis 113 (2015) 1224–1235.
[27] A.S. Weyrich, G.A. Zimmerman, Platelets: signaling cells in the immune continuum, Trends Immunol. 25 (2004) 489–495.
[28] A. Burdess, A.E. Michelsen, F. Brosstad, K.A. Fox, D.E. Newby, A.F. Nimmo, Platelet activation in patients with peripheral vascular disease: reproducibility and comparability of platelet markers, Thromb. Res. 129 (2012) 50–55.
[29] G. Thomas, R. Tacke, C.C. Hedrick, R.N. Hanna, Nonclassical patrolling monocyte function in the vasculature, Arterioscler. Thromb. Vasc. Biol. 35 (2015) 1306–1316.
[30] G. Passacquale, P. Vamadevan, L. Pereira, C. Hamid, V. Corrigall, A. Ferro, Monocyte-platelet interaction induces a pro-inflammatory phenotype in circulating monocytes, PLoS One 6 (2011) e25595.
[31] J.F. Dopheide, J. Rubrech, A. Trumpp, P. Geissler, G.C. Zeller, K. Bock, et al., Leukocyte-platelet aggregates-a phenotypic characterization of different stages of peripheral arterial disease, Platelets 27 (2016) 658–667.
[32] J. Sarma, C.A. Laan, S. Alam, A. Jha, K.A. Fox, I. Dransfield, Increased platelet binding to circulating monocytes in acute coronary syndromes, Circulation 105 (2002) 2166–2171.
[33] B.A. Golomb, T.T. Dang, M.H. Criqui, Peripheral arterial disease: morbidity and mortality implications, Circulation 114 (2006) 688–699.
[34] R.L. Pande, T.S. Perlstein, J.A. Beckman, M.A. Creager, Secondary prevention and mortality in peripheral artery disease: national Health and nutrition examination study, 1999 to 2004, Circulation 124 (2011) 17–23.
[35] J.S. Berger, M.J. Krantz, J.M. Kittelson, W.R. Hiatt, Aspirin for the prevention of cardiovascular events in patients with peripheral artery disease: a meta-analysis of randomized trials, Jama 301 (2009) 1909–1919.
[36] C. Antithrombotic Trialists, C. Baigent, L. Blackwell, R. Collins, J. Emberson, J. Godwin, et al., Aspirin in the primary and secondary prevention of vascular disease: collaborative meta-analysis of individual participant data from randomised trials, Lancet 373 (2009) 1849–1860.
[37] T. Gremmel, C.W. Kopp, D. Seidinger, G.A. Giurgea, R. Koppensteiner, S. Steiner, et al., The formation of monocyte-platelet aggregates is independent of on-treatment residual agonists'-inducible platelet reactivity, Atherosclerosis 207 (2009)

608–613.
[38] N. Kuzniatsova, E. Shantsila, G.Y. Lip, A.D. Blann, The membrane expression of P-selectin, but not monocyte-platelet aggregates, is influenced by variability in response to aspirin in patients with coronary artery disease, Platelets 25 (2014) 142–143.
[39] U. Klinkhardt, R. Bauersachs, J. Adams, J. Graff, E. Lindhoff-Last, S. Harder, Clopidogrel but not aspirin reduces P-selectin expression and formation of platelet-leukocyte aggregates in patients with atherosclerotic vascular disease, Clin. Pharmacol. Therapeut. 73 (2003) 232–241.
[40] J.S. Berger, W.R. Hiatt, Medical therapy in peripheral artery disease, Circulation 126 (2012) 491–500.
[41] R. Qayyum, L.C. Becker, D.M. Becker, N. Faraday, L.R. Yanek, S.M. Leal, et al., Genome-wide association study of platelet aggregation in African Americans, BMC Genet. 16 (2015) 58.
[42] A.R. Folsom, N. Aleksic, A. Sanhueza, E. Boerwinkle, Risk factor correlates of platelet and leukocyte markers assessed by flow cytometry in a population-based sample, Atherosclerosis 205 (2009) 272–278.
[43] M.H. Criqui, V. Vargas, J.O. Denenberg, E. Ho, M. Allison, R.D. Langer, et al., Ethnicity and peripheral arterial disease: the san Diego population study, Circulation 112 (2005) 2703–2707.
[44] M.T. Rondina, A.S. Weyrich, G.A. Zimmerman, Platelets as cellular effectors of inflammation in vascular diseases, Circ. Res. 112 (2013) 1506–1519.