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Full Length Article

# Individual variations in platelet reactivity towards ADP, epinephrine, collagen and nitric oxide, and the association to arterial function in young, healthy adults

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ABSTRACT

*Introduction:* Platelet aggregation and secretion can be induced by a large number of endogenous activators, such as collagen, adenosine diphosphate (ADP) and epinephrine. Conversely, the blood vessel endothelium constitutively release platelet inhibitors including nitric oxide (NO) and prostacyclin. NO and prostacyclin are also well-known vasodilators and contribute to alterations in local blood flow and systemic blood pressure.

*Materials and methods:* In this study we investigated individual variations in platelet reactivity and arterial functions including blood pressure and flow-mediated vasodilation (FMD) in 43 young, healthy individuals participating in the Lifestyle, Biomarkers and Atherosclerosis (LBA) study. Platelet aggregation and dense granule secretion were measured simultaneously by light transmission and luminescence. FMD was measured with ultrasound.

*Results:* The platelet function assay showed inter-individual differences in platelet reactivity. Specifically, a sub-group of individuals had platelets with an increased response to low concentrations of ADP and epinephrine, but not collagen. When the NO-donor S-nitroso-*N*-acetyl-DL-penicillamine (SNAP) was combined with high doses of these platelet activators, the results indicated for sub-groups of NO-sensitive and NO-insensitive platelets. The individuals with NO-sensitive platelets in response to SNAP in combination with collagen had a higher capacity of FMD of the arteria brachialis.

*Conclusions:* Platelet reactivity towards ADP, epinephrine and NO differs between young, healthy individuals. Some individuals have a more effective response towards NO, both in the aspect of platelet inhibition *ex vivo*, as well as vasodilation *in vivo*.

## 1. Introduction

Platelet aggregation and granule secretion can be induced by a large number of endogenous activators, like collagen, adenosine diphosphate (ADP) and epinephrine. It has previously been shown that platelet responses to different agonists are independent of age, sex or platelet number (within normal range) in healthy humans [1]. Collagen is a powerful platelet activator and plays a crucial role to stop bleeding during vascular injury when collagen is exposed in the subendothelial matrix. Collagen-induced platelet activation is mediated *via* the major platelet collagen receptor glycoprotein (GP) VI [2]. ADP can induce primary aggregation and dense granule secretion even if it is considered a weaker platelet activator than collagen. ADP is released from dense granule of activated platelets and thereby exert an autocrine and paracrine effect that contributes to a more potent secondary platelet aggregation. ADP induces different cellular effects on platelets through

*Abbreviations:* ADP, adenosine diphosphate; ApoA1, apolipoprotein A1; ApoB, apolipoprotein B; ATP, adenosine triphosphate; BMI, body mass index; BP, blood pressure; hs-CRP, high sensitive C-reactive protein; CVD, cardiovascular disease; FMD, flow-mediated vasodilation; GP, glycoprotein; HDL-C, high density lipoprotein cholesterol; LBA-study, lifestyle biomarkers and atherosclerosis study; LDL-C, low density lipoprotein cholesterol; MAP, mean arterial pressure; 2-MeS-ADP, 2-(Methylthio)adenosine 5′-diphosphate trisodium salt hydrate; NO, nitric oxide; PPP, platelet poor plasma; PRP, platelet rich plasma; SNAP, S-nitroso-*N*-acetyl-DL-penicillamine; STEMI, ST segment elevation myocardial infarction; $VO_2$, maximal oxygen uptake

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two different receptors, P2Y1 and P2Y12 [3–6]. Epinephrine is also considered a weak platelet activator that gives rise to platelet aggregation, secretion and a potentiating effect of other agonists by stimulating platelet adrenergic receptors [7].

In physiological conditions, platelets circulate in the bloodstream close to the vessel wall without becoming activated. This is mainly through the endothelial release of a number of potent platelet inhibitors, including nitric oxide (NO), adenosine and prostacyclin by the vascular endothelium. NO and prostacyclin are also well-known vasodilators that contribute to alterations in local blood flow, as well as systemic blood pressure. During vascular disease, the endothelium becomes dysfunctional and loses its ability to produce vasodilators and platelet inhibitors [8]. A dysfunctional endothelium also displays a more adhesive phenotype which is associated with exposed sub-endothelial proteins that attracts platelets and promotes thrombus formation [9]. Platelets play important roles in the development of cardiovascular disease (CVD) by thrombus formation and subsequent ischemic events [10,11].

The aim of the present study was to investigate individual variations in platelet reactivity in young healthy individuals and evaluate whether this correlate to blood pressure, vasodilation or cardiorespiratory fitness.

## 2. Method

### 2.1. Study population

In this project, a sub-population of participants in the Lifestyle, Biomarkers and Atherosclerosis (LBA) study [12] was studied. The LBA-study consists of 834 young, healthy, non-smoking volunteers enrolled from October 2014 to June 2016. The study was conducted at Örebro University in Sweden and the study design was approved by the regional ethical review board in Uppsala, Dnr: 2014/224. All subjects gave their written informed consent to participate. Out of the total 834 subjects in the LBA-study, 43 individuals were randomly selected for comprehensive platelet assessments (some measurements were performed on less subjects since it was not practically possible to run all activators on all subjects). Inclusion criteria for the LBA-study were: age of 18–25 years, non-smoker and no diagnosis of chronic diseases. Only subjects who declared to not have used any anti-platelet therapy (such as aspirin) within 10 days before blood collection were included for platelet analysis. Subjects with C-reactive protein (CRP) ≥ 10 mg/L were excluded from this sub-population.

### 2.2. Body composition

Body composition measurements were performed with the subject in a fasting state. Height and waist circumference were measured to the nearest 0.5 cm, height with a fixed stadiometer and waist circumference with a measuring tape [13]. Weight was measured, body mass index (BMI) calculated, and body fat (%) assessed, using an impedance body composition analyzer (Tanita Europe B.V. Tanita BC-418 MA, Amsterdam, Netherlands).

### 2.3. Blood pressure

Brachial blood pressure was measured after 15 min rest using a digital automated device (GE Healthcare, Dinamap V100, Buckinghamshire, UK) with Dura-Cuf (GE Medical Systems, GE Criticon Dura-cuf, Milwaukee, WI, US). Blood pressure (BP) was measured in the left arm with the test subject in the supine position. At least three measurements were performed with two-minute intervals. The measurement was ended when the difference between the two latest systolic pressures was < 5 mmHg. An average of the two last measurements was reported for systolic BP, diastolic BP, mean arterial blood pressure (MAP) and heart rate.

### 2.4. Flow-mediated dilation

Endothelial-dependent flow-mediated vasodilation (FMD) was assessed in the brachial artery by high-resolution ultrasound with a 12 MHz linear array transducer (GE Healthcare, Vivid E9, Chicago, Illinois, US), with simultaneous ECG registration (Cardiolex, EC Sense, Solna, Sweden), according to guidelines [14]. The test subjects were instructed not to exercise the day of examination and they were examined after over-night fasting. FMD measurements were performed in a temperature-controlled room with the test subject in the supine position. The right brachial artery was examined with ultrasound proximal to the antecubital fossa and visualized in a longitudinal view. A blood pressure cuff was placed on the forearm, distal of the examination area, and inflated to at least 50 mmHg above the systolic pressure, in order to stop the blood flow. After 5 min of occlusion, the cuff was deflated, and blood flow was restored. The diameter of the brachial artery was measured in end diastole from longitudinal images, and the media-adventitia interface on the near- and far wall was identified manually with electronic calipers. The baseline diameter was measured before occlusion, and the diameter increase, as a response to the increased blood flow after cuff deflation, was measured 30, 45, 60, and 90 s after cuff deflation. Multiple diameter measurements (at least three) were made on each image. The absolute diameter increase was calculated as (maximal diameter-baseline diameter) and the relative diameter increase, expressed as percentage of baseline diameter, was also calculated ((maximal diameter-baseline diameter)/baseline diameter). To assure that an increase in blood flow velocity had occurred, the blood flow velocity was measured with pulsed Doppler directly after the cuff deflation (within 15 s) and compared to baseline (measured before occlusion). All FMD examinations were performed by two experienced ultra-sonographers with experience from approximately two hundred FMD examinations per year.

### 2.5. Cardiorespiratory fitness

A submaximal exercise test was performed on a Monark bike (Monark Sports and Medical, Monark 939E, Vansbro, Sweden) with simultaneous ECG registration to monitor heart rate (Cardiolex, EC Sense, Solna, Sweden). The exercise test was performed to assess cardiorespiratory fitness by calculating maximal oxygen uptake ($VO_2$) [15] as previously described [12].

### 2.6. Blood sample preparation

Venous blood was collected from over-night fasting subjects, using a 21-gauge butterfly needle (Vacuette®, Greiner Bio-One International GmbH, Kremsmünster, Austria) and immediately when blood flow was established the tourniquet was released. After blood collection all tubes were gently inverted several times. Blood, plasma and serum for analysis of glucose, cholesterol, high density lipoprotein cholesterol (HDL-C), low density lipoprotein cholesterol (LDL-C), triglycerides, apolipoprotein A1 (ApoA1), apolipoprotein B (ApoB), high sensitive C-reactive protein (hs-CRP) and insulin was analyzed at the accredited clinical chemistry laboratory at Örebro University Hospital. All handling of blood samples was performed according to Örebro University Hospital guidelines. For further details see previous publication [12].

### 2.7. Platelet aggregation and ATP-secretion

For analysis of platelet function, venous blood was drawn into syringes (S-Monovette®, Sarstedt, Nümbrecht, Germany) containing trisodium citrate (3,2%). Since platelets are highly reactive cells, and blood drawing procedure as well as sample handling *ex vivo* can induce activation or desensitization of platelet receptors [16], this protocol included non-vacuum syringes for blood collection.

The syringes were gently inverted several times and centrifuged

**Table 1**
Baseline characteristics of study population.

| | All (n = 43) | | Females (n = 27) | | Males (n = 16) | | |
|---|---|---|---|---|---|---|---|
| | Mean | SD | Mean | SD | Mean | SD | p values |
| Age (Year) | 22 | 2 | 22 | 2 | 22 | 2 | 0.382 |
| Height (cm) | 175 | 10 | 170 | 6 | 185 | 9 | < 0.001 |
| Weight (kg) | 71 | 13 | 65 | 9 | 80 | 12 | < 0.001 |
| BMI (kg/m$^2$) | 23 | 3 | 23 | 3 | 23 | 2 | 0.499 |
| Body fat (%) | 23 | 9 | 29 | 6 | 13 | 4 | < 0.001 |
| Waist circumference (cm) | 77 | 8 | 74 | 7 | 82 | 5 | < 0.001 |
| ApoA1 (g/L) | 1.58 | 0.32 | 1.68 | 0.33 | 1.42 | 0.23 | < 0.01 |
| ApoB (g/L) | 0.77 | 0.19 | 0.80 | 0.21 | 0.71 | 0.14 | 0.124 |
| ApoB/A1 ratio | 0.50 | 0.14 | 0.49 | 0.14 | 0.52 | 0.16 | 0.619 |
| Glucose (mmol/L) | 5.17 | 0.29 | 5.09 | 0.26 | 5.31 | 0.30 | < 0.05 |
| HDL-C (mmol/L) | 1.33 | 0.36 | 1.41 | 0.39 | 1.19 | 0.27 | 0.052 |
| Cholesterol (mmol/L) | 4.06 | 0.79 | 4.27 | 0.85 | 3.70 | 0.53 | < 0.01 |
| LDL-C (mmol/L) | 2.21 | 0.70 | 2.29 | 0.77 | 2.09 | 0.54 | 0.321 |
| Triglycerides (mmol/L) | 0.87 | 0.47 | 0.94 | 0.54 | 0.73 | 0.28 | 0.095 |
| Orosomucoid (g/L) | 0.69 | 0.16 | 0.69 | 0.18 | 0.67 | 0.13 | 0.722 |
| hs-CRP (mg/L)* | 0.50 | 0.82 | 0.61 | 1.55 | 0.38 | 0.43 | 0.187 |
| Insulin (mU/L) | 8.13 | 3.81 | 9.17 | 4.04 | 6.37 | 2.66 | 0.018 |
| Systolic BP (mmHg) | 117 | 14 | 109 | 7 | 132 | 9 | < 0.001 |
| Diastolic BP (mmHg) | 64 | 5 | 64 | 5 | 64 | 6 | 0.841 |
| MAP (mmHg) | 83 | 6 | 80 | 5 | 87 | 6 | < 0.01 |
| Heart rate (beats/min) | 63 | 12 | 65 | 13 | 58 | 7 | < 0.05 |
| VO2 (ml/kg/min) | 40.1 | 8.2 | 37.2 | 6.2 | 45.1 | 9.0 | < 0.01 |
| FMD (%) | 7.6 | 2.8 | 8.3 | 3.1 | 6.4[#] | 1.8 | < 0.05 |

BMI = body mass index, ApoA1 = apolipoprotein A1, ApoB = apolipoprotein B, HDL-C = high density lipoprotein cholesterol, LDL-C = low density lipoprotein cholesterol, hs-CRP = high sensitive C-reactive protein *shown as median and interquartile range since not normally distributed, BP = blood pressure, FMD = flow-mediated vasodilation (measured as vessel diameter increase) [#]$n = 15$, MAP = mean arterial pressure, VO2 = oxygen uptake, SD = standard deviation. Mean differences between females and males were analyzed by Student's *t*-test.

without delay. Platelet rich plasma (PRP) was prepared by centrifugation at 190 × *g* for 22 min in room temperature. The upper ¾ of the PRP was transferred to a new tube and the remaining PRP was centrifuged again at 2000 × *g* for 20 min in room temperature to obtain platelet poor plasma (PPP). Both PRP and PPP were kept in room temperature until measurement was conducted. Platelet count in PRP was not adjusted since it do not affect the aggregatory response (within normal ranges) [1] and might even induce more pre-analytical errors [17–19]. Platelet aggregation and dense granule secretion were recorded simultaneously during stirring at 1200 rpm in a CHRONO-LOG® 700 aggregometer (Chrono-Log Corporation, Havertown, USA) by measuring changes in light transmission and luminescence. PRP was incubated in 37 °C for 120 s before addition of luciferin-luciferase used according to manufacturer's instructions (Chrono-Lume®, Chrono-Log Corporation). Donor-specific PPP was used as blank. The different activators were added in the PRP 120 s after luciferin-luciferase and thereafter aggregation and adenosine triphosphate (ATP)-secretion were recorded for at least 10 min. The activators used were: the stable ADP-analog 2-(Methylthio)adenosine 5′-diphosphate trisodium salt hydrate (2-MeS-ADP, Sigma-Aldrich®, Saint Louis, USA) used in 10, 30 and 100 nM. epinephrine (Chrono-Log Corporation) used in 0.1, 0.3 and 1 μM or collagen used in 0.3, 1 and 3 μg/mL (Chrono-Log Corporation). The NO-donor S-nitroso-*N*-acetyl-DL-penicillamine (SNAP, Sigma-Aldrich®) 1 μM, was added 60 s prior to agonists. At the end of each measurement an ATP-standard (Chrono-Par®, Chrono-Log Corporation) was added to facilitate calculation of ATP-secretion. Data is shown as maximal aggregation and maximal ATP-secretion. The ATP-secretion reflects platelet dense granule secretion.

Since our platelet aggregation data indicated inter-individual variations in response to 2-MeS-ADP and epinephrine, we defined a subgroup of high responders to low dose activator as light transmission > 70%. The aggregation data also indicated inter-individual variations in response to NO, therefore we used a cut-off value of light transmission < 45% in response to 1 μM SNAP in combination with high dose of activator to define NO-sensitive platelets. Light transmission ≥ 45% in response to 1 μM SNAP in combination with high dose of activator is referred to as NO-insensitive platelets.

Control experiments were conducted to test the reproducibility of our experimental conditions. Platelet responsiveness over time was tested at 3, 6 and 8 h from blood collection to analysis. Platelet responsiveness was also tested during different stages of the menstrual cycle (early, intermediate and late phase) (n = 3).

### 2.8. Statistical analyses

All statistical calculations were performed using IBM SPSS Statistics version 24 for Windows (IBM Corp, Armonk, New York, USA). Normal distribution was checked for all variables with Kolmogorov Smirnov test and Shapiro-Wilk test. Descriptive data are presented as mean and standard deviation, except for hs-CRP, which is presented as median and interquartile range (Q1–Q3), because of a skewed distribution. Unpaired Student's *t*-test were used to analyze differences between genders. As the platelet response variables were not normally distributed, bivariate correlations (Spearman's rank correlation coefficient) were used to study associations between platelet response and body composition, blood pressure measurements, and cardiorespiratory fitness. Correction for multiple comparisons was made according to Bonferroni. The Bonferroni correction resulted in the following: Significant level < 0.05 requires $p < 0.0016$, significant level < 0.01 requires $p < 0.0003$, and significant level < 0.001 requires $p < 0.00003$. Mann-Whitney *U* test was used to analyze differences between the different sub-groups. Associations between FMD and NO-sensitivity was further analyzed by a simple linear regression with aggregation in response to SNAP and a high dose collagen as dependent variable and diameter increase (%) as independent variable.

## 3. Results

### 3.1. Study population

The study population consisted of 27 females and 16 males with a mean age of 22 years, see Table 1. Measurements of body composition

showed no difference in BMI between genders. Blood samples provided a similar lipid profile between genders, however with higher total cholesterol ($p < 0.01$) and ApoA1 ($p < 0.01$) in females compared to males. Females had lower levels of blood glucose ($p < 0.05$), while males had lower level of insulin ($p < 0.05$). The inflammatory markers CRP and orosomucoid did not differ between genders. BP measurements showed a higher MAP ($p < 0.01$) and systolic BP ($p < 0.001$) in males than females, while diastolic BP had no gender differences. Heart rate was lower in males than females ($p < 0.05$) and maximal oxygen consumption ($VO_2$) was higher in males than females ($p < 0.01$). These gender differences in BP, heart rate and maximal $VO_2$ is expected [12]. The baseline characteristics of the study population are presented in Table 1.

### 3.2. Platelet aggregation and dense granule secretion

Platelet aggregation and dense granule secretion in response to three known platelet activators was studied in PRP from a total of 43 young, healthy individuals. The response to low, intermediate and high doses of the stable ADP analog 2-MeS-ADP, epinephrine and collagen was tested. In addition, the high dose of each activator was combined with pre-treatment by the NO-donor SNAP to mimic the endothelial contribution of platelet inhibition.

Platelets exposed to the low dose of collagen, 0.3 μg/mL, showed none, or very little, aggregation and no dense granule secretion. With 1 μg/mL collagen, both aggregation and dense granule secretion ranged from a low to a high response. At the high dose collagen, 3 μg/mL, aggregation response was high and dense granule secretion detected in all individuals. Representative original traces of platelet aggregation and dense granule secretion in response to 0.3–3 μg/mL collagen are shown in Fig. 1A.

Platelet aggregation and dense granule secretion in response to the low dose of 2-MeS-ADP, 10 nM, ranged from a low to a high response. With 30 nM 2-MeS-ADP aggregation and secretion also ranged from a low to a high response, however, with a higher mean compared to the low dose. The high dose of 2-MeS-ADP, 100 nM, resulted in a high aggregation response in all subjects, while secretion ranged from a low to a high response (Fig. 2A and D).

Platelets exposed to the low dose of epinephrine, 0.1 μM, showed a range of aggregation responses from low to high, while secretion only ranged from a low to an intermediate response. With 0.3 μM epinephrine, both aggregation and secretion ranged from a low to a high response, however, with higher mean values compared to the low dose. The high dose of epinephrine, 1 μM, resulted in a high aggregation response in almost all subjects, while secretion ranged from a low to a high response, however, with a higher mean value compared to the intermediate dose (Fig. 2B and E).

After pre-treatment by the NO-donor SNAP, the mean platelet aggregation and secretion in response to the high dose of each activator were decreased. Platelet responses to SNAP in combination with epinephrine or collagen ranged from none to full inhibition of aggregation and secretion (Fig. 2B–C and E–F). SNAP in combination with 2-MeS-ADP showed no full inhibition of platelet aggregation (Fig. 2A and D).

Platelet aggregation in response to 2-MeS-ADP, epinephrine and NO showed large inter-individual variations, see Fig. 2A–C. The results indicate specific sub-groups of individuals with platelets that responded strongly to low concentrations of 2-MeS-ADP and epinephrine but not collagen. To further evaluate the individual response to the low doses of 2-MeS-ADP and epinephrine the population was divided into two sub-groups of high and low responders using a cut-off at light transmission > 70%. Almost all high responders in response to the low dose epinephrine was also defined as high responders to low dose 2-MeS-ADP. However, the high responders in response to 2-MeS-ADP showed a range from low to high response towards the low dose of epinephrine, see Fig. 3A.

For collagen, the only inter-individual variation in platelet aggregation and dense granule secretion was seen for the intermediate dose (Fig. 2C and F). This range is expected of an intermediate dose close to the half maximal effective concentration (EC50) of an agonist and the same pattern is seen in the intermediate doses of 2-MeS-ADP and epinephrine (Fig. 2A–B).

When SNAP was combined with high doses of epinephrine and collagen, the results indicated that there are two sub-groups of NO-sensitive and NO-insensitive platelets. To further evaluate this phenomenon, subjects were classified as NO-sensitive if the light transmission was < 45% in response to 1 μM SNAP in combination with high dose of activator (Fig. 2). Representative original traces of platelet aggregation from two independent donors, tested the same day, show a distinct reduction in platelet aggregation in the NO-sensitive platelets in comparison to the NO-insensitive platelets in response to 1 μM SNAP in combination with 3 μg/mL collagen (Fig. 1B). Further analyses of the sub-groups of NO-sensitive platelets show that almost all individuals with NO-sensitive platelets in response to SNAP in combination with collagen were also NO-sensitive in response to SNAP in combination with epinephrine, see Fig. 3C. Epinephrine was the only of the activators used in this study to show both a sub-group of high responders to low dose activator and a defined sub-group of NO-sensitive platelets. However, none of the individuals with a strong platelet response towards a low dose of epinephrine was defined as NO-sensitive, see Fig. 3B.

Platelet responses of the whole cohort (n = 43) to all the activators used and NO is summarized in Fig. 2. Control experiments revealed that the difference in reactivity towards activators and NO was not dependent on preparation time, menstruation cycle or gender (data not shown).

### 3.3. NO-sensitivity

To further analyze the relationship between platelet reactivity and vasculature, the difference in platelet inhibition by SNAP and the endothelial-dependent FMD was analyzed. The subjects with NO-sensitive platelets, in response to SNAP and collagen, had a significantly larger FMD than the subjects with the NO-insensitive platelets, see Fig. 4. The response was larger both in relative diameter increase (%), $p = 0.011$, and in absolute diameter increase (mm), $p = 0.040$. There was no significant difference in baseline diameter between the groups, however, there was a higher increase in blood flow velocity after the arterial occlusion in the NO-sensitive sub-group compared to the NO-insensitive sub-group (data not shown). Furthermore, the linear regression analysis showed that the aggregation in response to SNAP and collagen can be explained to 16.7% ($r^2$) by the diameter increase (%) of the brachial artery ($p = 0.028$). For the subjects with NO-sensitive platelets in response to SNAP and epinephrine, there was no difference in FMD, either in relative diameter increase (%), $p = 0.658$, or absolute diameter increase (mm), $p = 0.437$.

### 3.4. Platelet reactivity and blood pressure

It is well known that platelets release vasoactive mediators and that vasoactive mediators released by the endothelium also affect platelets. Therefore, we analyzed the correlation between platelet reactivity and the BP variables systolic BP, diastolic BP and MAP. None of the platelet reactivity variables correlated to the BP variables tested (data not shown). Furthermore, there were no differences in terms of blood pressure parameters (diastolic BP, systolic BP and MAP) or FMD, between the sub-groups of high and low responders to low dose 2-MeS-ADP or epinephrine (data not shown).

### 3.5. Biomarkers and fitness

The platelet reactivity towards 2-MeS-ADP, epinephrine, collagen and NO, did not correlate to inflammatory markers like hs-CRP, blood

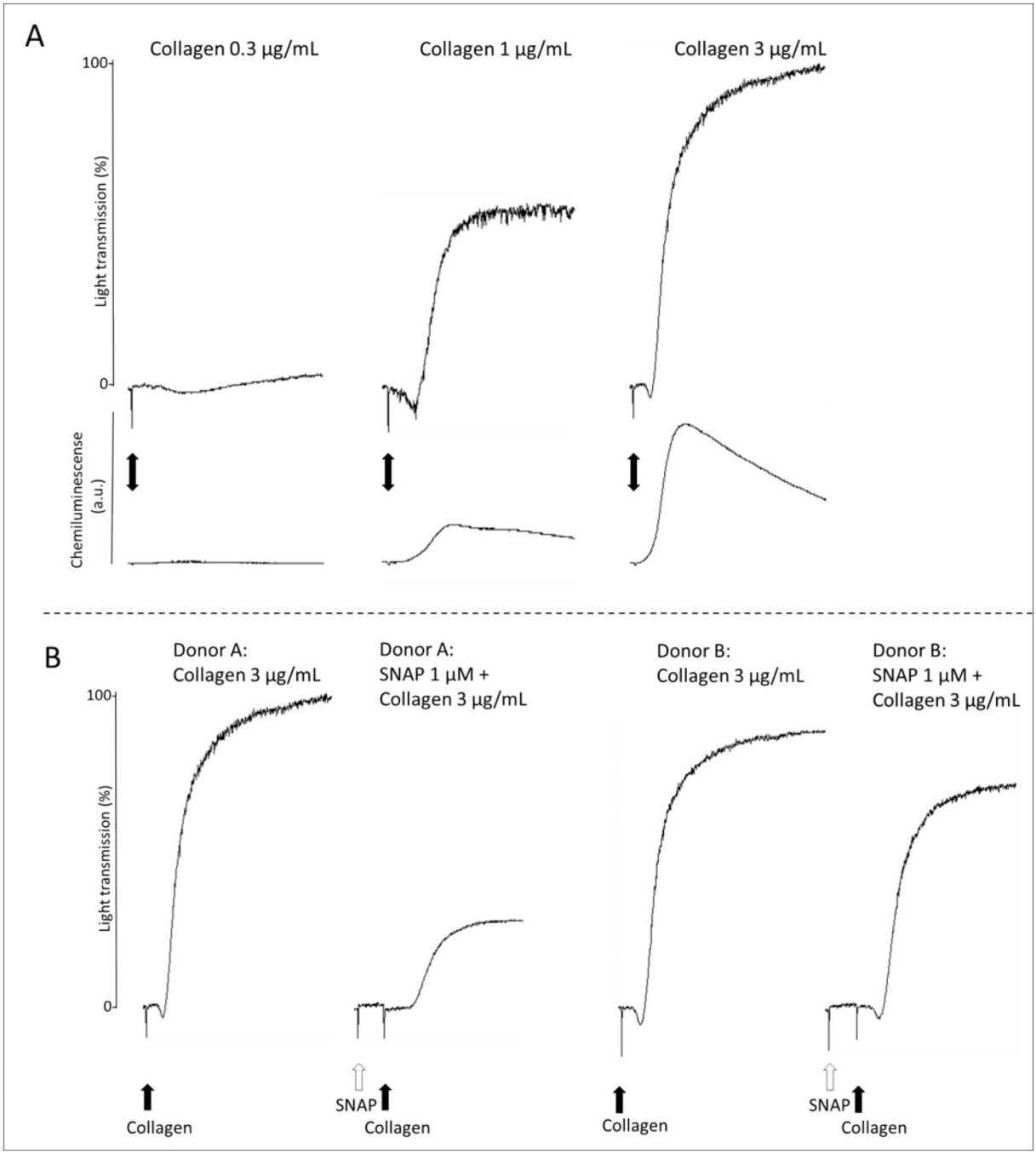


**Fig. 1.** Platelet aggregation and ATP-secretion and example of inter-individual differences in NO-sensitivity. Representative original traces of platelet aggregation and ATP-secretion measured as light transmission and chemiluminescence respectively, in platelet rich plasma from young healthy volunteers. A. Aggregation and ATP-secretion in response to different doses of collagen. B. Aggregation in response to 3 µg/mL collagen in presence or absence of 1 µM of the NO-releasing drug S-nitroso-*N*-acetyl-DL-penicillamine (SNAP), for two independent donors, measured the same day. SNAP was added 60 s prior to collagen, addition of SNAP is indicated by unfilled arrows and addition of collagen is indicated by filled arrows.

lipids such as LDL-, HDL- and total-cholesterol or fitness parameters such as $VO_2$ or body composition in this sub-study of the LBA-study cohort (data not shown).

## 4. Discussion

In the present study, 43 participants in the LBA cohort were enrolled in a sub-study for in-depth analysis of platelet responses. Specifically, we aimed to clarify the possible presence of individual differences in platelet reactivity towards physiological important platelet activators and inhibitors, and to elucidate correlations with blood pressure, vasodilation or cardiorespiratory fitness. The key findings in this study were the existence of inter-individual variations in platelet reactivity towards an ADP analogue (*i.e.* 2-MeS-ADP) and epinephrine as well as the presence of NO-sensitive and insensitive individuals. We also found a more potent endothelial function *in vivo* in the NO-sensitive group which had a significantly higher FMD compared to the NO-insensitive group. This indicates a connection between platelet function *ex vivo* and vascular function *in vivo* in regard to endothelial function measured as FMD. The relationship between NO action on platelets and NO production from endothelium may have an impact in early cardiovascular changes that may contribute to the development of atherosclerosis

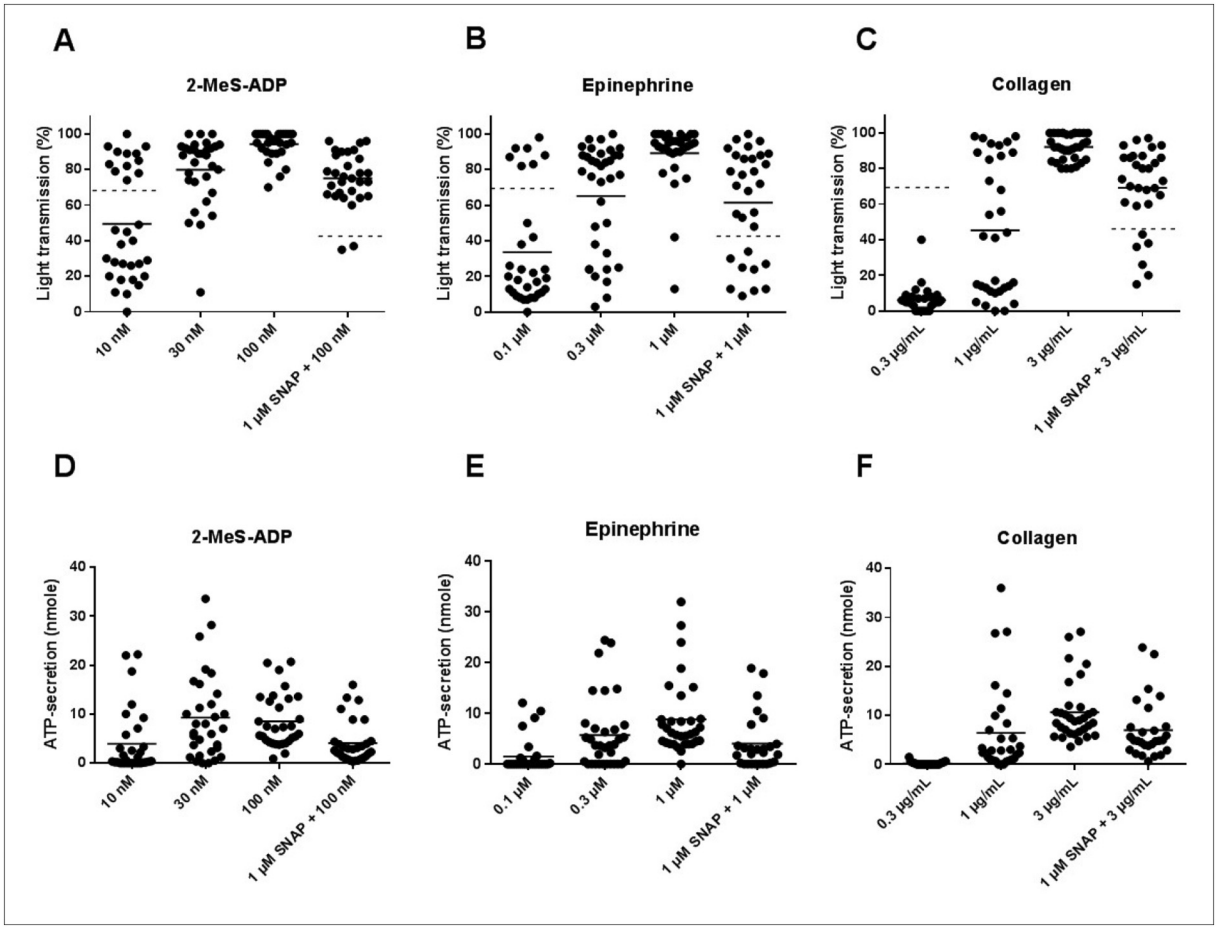


**Fig. 2.** Individual variations in platelet reactivity. Inter-individual differences in platelet aggregation (A–C) and ATP-secretion (D–F) in response to different doses of the activators 2-MeS-ADP (A and D), epinephrine (B and E) and collagen (**C** and **F**). High dose of the activators was also combined with the inhibitory, NO-releasing drug, S-nitroso-*N*-acetyl-DL-penicillamine (SNAP), added 60 s prior to the activators. Data is shown as maximal aggregation, measured by light transmission and the amount of released ATP measured with chemiluminescence (n = 28–36). Mean values are indicated as black lines in the graphs. Cut-off values for sub-groups of high responders to low dose activator and NO-sensitive platelets are indicated as dashed lines at light transmission > 70% and < 45%, respectively.

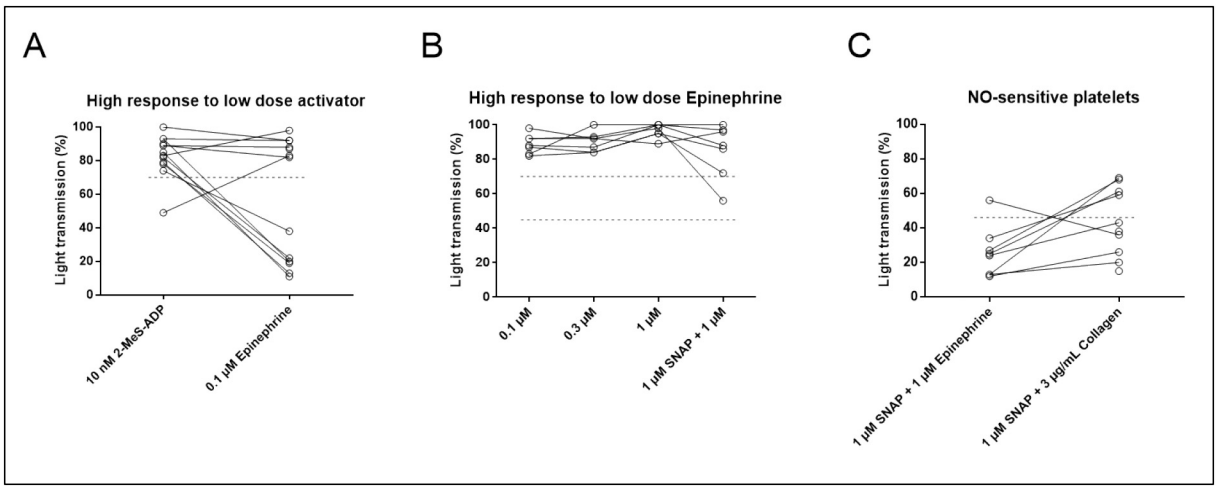


**Fig. 3.** Sub-groups of individuals presenting altered platelet reactivity. The sub-groups of high responders to low dose activator and subjects with NO-sensitive platelets were defined by cut-off values at light transmission > 70% and < 45%, respectively. The cut-off lines are indicated as dashed lines in the figure. Data is shown as maximal aggregation, measured by light transmission. A. All individuals with a strong platelet response towards a low dose of 2-MeS-ADP (n = 12) and/or epinephrine (n = 7). B. Response to different doses of epinephrine alone or in combination with the inhibitory, NO-releasing drug, S-nitroso-*N*-acetyl-DL-penicillamine (SNAP), added 60 s prior to the activators, shown for the individuals with high response to low dose epinephrine (n = 7). C. All individuals with NO-sensitive platelets in response to SNAP in combination with epinephrine (n = 7) or collagen (n = 6).

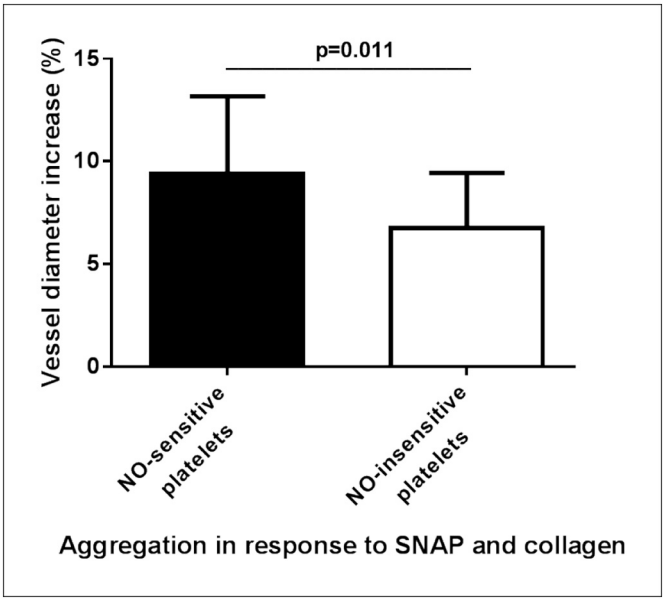


**Fig. 4.** Flow-mediated vasodilation and NO-sensitive platelets. Difference in flow-mediated vasodilation (FMD), measured as diameter increase (%), and platelet aggregation in response to 1 μM of the NO-releasing drug S-nitroso-*N*-acetyl-DL-penicillamine (SNAP), added 60 s prior to 3 μg/mL collagen. Endothelial-dependent FMD was analyzed in the brachial artery by high-resolution ultrasound and platelet aggregation was measured with light transmission aggregometry. Cut-off value to identify NO-sensitive platelets was set at light transmission < 45% in response to SNAP in combination with collagen. Data are shown as mean values and standard deviations, Mann-Whitney *U* test was used to analyze differences between the NO-sensitive (n = 7) and the NO-insensitive (n = 23) sub-group.

[8,10].

Clinical laboratory testing of platelet function is often performed with high concentrations of a battery of platelet activators. Although, protocols for platelet function testing reveals the effect of anti-platelet drug therapies or inherited platelet disorders, such tests are less effective for predicting risk assessment for acute atherosclerosis-related cardiovascular disease [20]. For example, we recently showed that standard protocol for platelet testing did not discriminate between healthy volunteers and patients suffering of acute ST segment elevation myocardial infarction (STEMI) [21]. Dysfunctional/hyper-reactive platelet responses are most likely easier to detect when using sub-maximal concentrations of platelet activators [22]. This was taken in consideration in our study design where we selected concentrations to achieve a range of low, intermediate and high doses of the different platelet activators. The high doses of platelet activators used in the present study is still substantially lower compared to many clinical laboratory testing protocols [20].

It should be mentioned that the total number of participants in this sub-study was small, but the results indicate that there were no differences in platelet response between females and males in the LBA cohort. According to our control experiments on platelet aggregation and dense granule secretion performed at early, intermediate and late phase of the menstrual cycle, there were no differences in platelet reactivity at various stages of the menstrual cycle.

Our platelet functional assay showed inter-individual differences in aggregation in response to both 2-MeS-ADP and epinephrine. Previous studies have associated a gain of function genetic polymorphism affecting the alpha2 adrenergic receptor (α2AR) to hypertension and enhanced epinephrine induced platelet aggregation [23,24]. However, our data showed that there was no correlation between BP/MAP and agonist-induced platelet activation. A significant difference between our and the majority of published papers is that the study population was young and normotensive. This may be the principal reason for observed lack of correlation to BP. However, the genetic defect described in the α2AR could still be a possible contributing factor to the subpopulation of high responders to a low dose epinephrine. Another alteration of the subsequent intracellular signaling following epinephrine stimulation of the adrenergic receptors is a polymorphism affecting the β3-subunit of the activated G-protein. This polymorphism is both associated to increased epinephrine-induced platelet aggregation [25]. Furthermore, sub-populations of high responders to both 2-MeS-ADP and epinephrine may be explained by similarities in receptor-induced intra-cellular signaling. Regarding intracellular signal transduction pathways, it is well-established that α2AR and P2Y12 share a similar Gi/z signaling mechanism. In fact, it has previously been shown that individuals with low response to epinephrine also can have a reduced response to ADP [26].

Inter-individual differences were not observed when using collagen as platelet activator in our platelet aggregation and dense granule secretion assay, except in the intermediate dose (*i.e.* at a dose close to an EC50 value) were it is expected. Interesting, collagen can induce secretion of dense- and alpha-granule without causing full aggregation, which could lead to a hypersecretory phenotype of platelets [27]. Increased platelet reactivity has previously been recorded in hypertension [28], angina pectoris and myocardial infarction [29,30] where administration of NO-donating drugs can restore the platelet reactivity to normal levels [31].

Prostacyclin and NO released from the healthy endothelium reduce the platelet aggregatory ability. However, in pathophysiological conditions with endothelial dysfunction like hypertension and coronary artery disease, platelets may develop resistance towards NO-induced inhibition [32,33]. Our data indicate two different sub-groups of platelet populations, referred to as NO-sensitive and NO-insensitive. The NO-sensitivity detected in platelets *ex vivo* may also have a physiological impact on other vascular cells such as endothelial cells and smooth muscle cells *in vivo*. This could explain the higher FMD response in the NO-sensitive sub-group compared to the NO-insensitive sub-group. The increase in blood flow velocity after the arterial occlusion in the FMD assay was also higher in the NO-sensitive sub-group compared to the NO-insensitive sub-group. This could either be a consequence of, or contribute to, the significantly higher increase in vasodilation response reported in the NO-sensitive sub-group. It should be noted that the measurement of blood flow velocity was not as standardized as the other vessel examinations in the LBA-study, such as the baseline diameter, which did not differ between the NO-sensitive and NO-insensitive sub-group.

The approach to analyze possible relationships between FMD and platelet activation has previously been studied by Haynes et al. in a substantially older population than the LBA-study. Like our data Haynes et al. did not detect any relationships between FMD and platelet activation [34]. However, our data show a relationship between platelet inhibition by NO and FMD.

One limitation in our study is the lack of continuous measurement of diameter and flow velocity during the FMD examination. In the guidelines from Thijssen et al. [35] the recommendation is a continuous measurement of diameter and flow from one minute before cuff inflation until three minutes after cuff deflation. Unfortunately, we did not have the technical possibilities to follow that protocol. The diameter was measured with multiple calipers in each image, but we did not have the technical equipment to make a continuous edge detection or wall tracking to capture the true peak diameter. Instead we measured the diameter at repeated times, 30, 45, 60 and 90 s after cuff deflation, and we cannot be sure that we captured the true peak diameter without a continuous recording.

Earlier findings in the LBA-study showed an association between high aerobic fitness and low risk of future CVD events [12]. It has also recently been shown that an increased platelet reactivity can correlate to low cardiorespiratory fitness in young, healthy women. Heber et al.

showed that an intervention with exercise for at least two months in subjects with a low cardiorespiratory fitness decreases platelet reactivity towards the levels of the subjects with a higher cardiorespiratory fitness [36]. However, our data do not show any correlations between platelet reactivity and fitness parameters such as $VO_2$ or body composition. This could be due to a more complex association between platelet function and physical activity since hard exercise does not result in a decreased platelet reactivity [36].

In conclusion, platelet reactivity to 2-MeS-ADP, epinephrine and NO differs between young, healthy individuals. Furthermore, a sub-group in this study has a more effective response towards NO both in the aspect of platelet inhibition *ex vivo* as well as vasodilation *in vivo*. This indicates that variations in platelet response to externally applied NO *ex vivo* may be physiologically relevant in the aspect of endothelial function *in vivo*.

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## Declarations of interest

None.

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