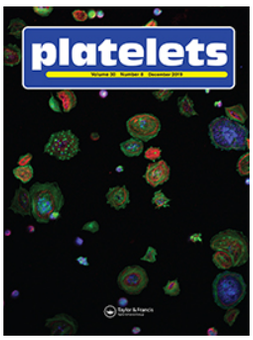

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Arachidonic acid causes lysis of blood cells and ADP-dependent platelet activation responses in platelet function tests

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Abstract

The use of arachidonic acid (AA) to stimulate platelets is considered as a specific approach to study aspirin treatment efficacy. However, very high concentrations of AA are used, and it has been previously reported that AA can induce cell lysis in other settings. Several clinical studies have reported decreased responses to AA in whole blood tests in the presence of clopidogrel. Our aim was to investigate whether unspecific effects contribute to AA-induced aggregation and platelet activation in light transmission aggregometry (LTA) in platelet-rich plasma (PRP), and in assays using whole blood, multiple electrode aggregometry (MEA, Multiplate®), and flow cytometry.

We report that cell lysis, especially of red blood cells, does occur at concentrations of AA used in the clinical tests and that ADP is very important for the AA-induced platelet activation responses. In flow cytometry, very limited platelet activation was detected before reaching AA concentrations in the millimolar range, where cell lysis also occurred, making it problematic to develop a reliable flow cytometry assay using AA as reagent.

We conclude that cell lysis and ADP release contribute to AA-induced platelet responses, most markedly in whole blood assays. This finding could potentially explain some differences between studies comparing methods using whole blood and PRP and also how clopidogrel treatment could influence AA-induced aggregation results in previously published studies. Our findings highlight some issues with AA as reagent for platelet activation, which also have an impact on how platelet activation assays using AA should be interpreted.

Keywords

Arachidonic acid, aspirin, flow cytometry, platelet aggregation, platelet function tests

History

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Introduction

Platelet inhibition by aspirin is the cornerstone treatment for prevention of arterial thrombosis in patients with acute cardiovascular disease [1,2]. It has long been recognized that some patients still suffer from recurrent events, and different platelet function tests have been developed to identify individuals with "aspirin resistance" (reviewed by [3,4]).

The use of arachidonic acid (AA) to stimulate platelets is often considered as a specific approach to study aspirin treatment efficacy [5]. AA is supposed to enter the cell and to be transformed by the COX-1 enzyme to thromboxane A_2 (TXA_2), which activates the platelet via the thromboxane TP receptors. However, COX-independent aggregation in response to AA has been previously described [6]. A recent review also questions if platelet function tests using AA really can tell whether a patient is responding to their aspirin treatment [7]. In addition, aggregation responses to the TXA_2 mimetic U46619 have been shown to be dependent on autocrine ADP release [8].

Traditionally, light transmission aggregometry (LTA) in platelet-rich plasma (PRP) has been initiated by addition of AA in high (milllimolar) doses [4]. Since 2006, whole blood multiple electrode aggregometry (MEA) with the instrument Multiplate® [9] has made platelet aggregometry more accessible to the clinic, and a number of studies have been performed using this instrument to study aspirin resistance in patients. Data available on the internet either do not mention ADP receptor inhibition as a potential confounder [10] or even claim that Multiplate testing with AA (the "ASPItest") is not affected by clopidogrel treatment [11].

Studies including comparisons between LTA and whole blood aggregometry generally show poor agreement in identification of individuals with high residual platelet activity [12–17]. As an alternative approach, there have been attempts to develop flow cytometry protocols with AA as a platelet agonist [18–20]. However, several studies report a very narrow span of concentrations where platelets become activated before they experience loss of platelet-sized particles, which suggests lysis of the cells. Hübl et al. [18] also mention that red blood cell lysis was observed at much lower AA concentrations than the ones where platelet lysis was observed. Platelet lysis by AA *in vitro* has also been mentioned as a potential confounding factor in LTA [3], but this review did not show any results or provide references to where this data can be

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found. To the best of our knowledge, it hasn't yet been investigated if lysis of cells in response to AA also occurs in whole blood aggregometry, and if this could have an impact on the results. We therefore set out to perform such a study, and can here report that ADP activation is indeed very important for AA-induced platelet aggregation. In addition, AA caused cell lysis, especially among red blood cells. This in turn potentiates ADP-dependent aggregation responses, which could potentially explain some of the differences between studies comparing methods using whole blood and PRP, and which also has an impact on how platelet activation assays using AA should be interpreted. In flow cytometry, very limited platelet activation was detected before reaching AA concentrations in the millimolar range, where cell lysis also occurred, making it problematic to develop a reliable flow cytometry assay using AA as reagent.

Methods

Venous blood was collected from healthy volunteers not taking any drugs that interfere with platelet function for the last 10 days into 3.2% sodium citrate tubes (Vacuette®, Greiner Bio One, Kremsmünster, Austria) for LTA, and hirudin tubes (Roche Diagnostics GmbH, Mannheim, Germany) for MEA and flow cytometry. The procedure for blood collection was approved by the regional ethics review board in Linköping, Sweden (approval No. 2012/382–31).

To obtain PRP, whole blood was centrifuged at 150 g for 15 min at room temperature.

LTA was performed using a Chronolog instrument (Model 560, Chrono-Log, Haverston, PA, US) and MEA using the Multiplate® instrument (Roche Diagnostics GmbH). For consistency, the Multiplate® ASPItest (AA) and ADPtest (ADP) reagents were used for all experiments. These were dissolved and stored according to the manufacturer's instructions. Some blood samples were pretreated with acetylsalicylic acid (ASA, Sigma-Aldrich, St. Louis, MO, USA, final concentration 100 μM) or the direct acting ADP receptor inhibitor cangrelor (The Medicines Company, Parsippany, NJ, USA, final concentration 10 μM) for 10 min before the start of experiments. Some control experiments were also performed using AA from other manufacturers (Hart Biologicals, Hartlepool, UK, and DiaSys/Rolf Greiner Biochemica, Flacht, Germany).

In parallel, whole blood or PRP samples were treated with abciximab (Eli Lilly, Indianapolis, IN, USA, final concentration 20 μg/mL) and incubated in the aggregometers with the same stirring and temperature conditions as for aggregating samples. Subsampling for flow cytometry was performed from these cuvettes when aggregation had occurred in the aggregating sample.

For flow cytometry, 3 μL of blood or PRP from the aggregometer cuvettes was added to 33 μL HEPES buffer (137 mM NaCl, 2.7 mM KCl, 1 mM $MgCl_2$, 5.6 mM glucose, 1 g L^{-1} bovine serum albumin, 20 mM HEPES, pH 7.4, chemicals from Sigma-Aldrich) containing antibodies (0.03 μg mL^{-1} PE-anti-human-CD41 (GPIIb), 0.52 μg mL^{-1} PE-Cy5-anti-humanCD62P (P-selectin) or PE-Cy5-isotype control, and 0.56 μg mL^{-1} FITC-PAC-1 (final concentrations, all from Becton Dickinson, Franklin Lakes, NJ, USA) or 6.9 μg mL^{-1} FITC-anti-human- CD235a (glycophorin A, from Invitrogen, Frederick, MO, USA)). For dose-response and temperature experiments, this mixture also contained AA (ASPItest reagent). The samples were incubated for 10 min at room temperature before dilution in 700 μL of HEPES buffer.

Analysis on a Gallios™ flow cytometer (Beckman Coulter, Brea, CA, USA) was carried out immediately after dilution and finished within 1 h. The flow cytometer was equipped with three lasers (405, 488, and 638 nm). Acquisition was performed for 60 s or until 5000 platelets had been collected in a temporary platelet gate. To allow for sensitive detection of small particles, we used the ultra-wide angle of detection (submicron particle setting) for forward scatter (FSC) and the lowest possible FSC threshold. Gating for particles of different size was done according to Figure 3. The gate for normal-sized platelets in resting samples ("Platelet-sized particles") was used to set the limit for the gate for "Smaller particles." Events included in this gate and positive for CD41 were classified as platelet microparticles, while particles positive for CD235a were classified as erythrocyte microparticles. To enable comparisons between samples, the number of detected microparticles was divided by the acquisition time in the same sample. For analysis of platelet activation, all CD41-positive particles in the gate "Platelet-sized particles" were evaluated for positivity for P-selectin and PAC-1. Gating for positivity was performed as previously described [21], using a PE-Cy5-isotype control antibody for P-selectin and with addition of 10 mM EDTA to the buffer for PAC-1 to prevent its binding to platelets.

Statistics

Statistical analysis was performed using GraphPad Prism v.5.04 (GraphPad Software, La Jolla, CA, USA). Student's paired *t*-test, analysis of variance (ANOVA), or repeated measures ANOVA, followed by Bonferroni's post-hoc test were performed to compare single-paired data sets, multiple data sets, and multiple paired data sets, respectively.

Results

Figure 1A shows that platelet aggregation consistently appeared in 15 out of 15 donors when using hirudinized whole blood in MEA with 0.5 mM AA (standard anticoagulant and procedure for the ASPItest, two donors were also tested at two separate occasions with consistent results). Interestingly though, the same AA reagent only induced aggregation in citrated PRP from six out of eight tested donors in parallel experiments performed with LTA (where citrate is the standard anticoagulant). Increasing the AA concentration to 1 mM only changed the LTA responses for one of these non-responsive donors. To evaluate whether the lack of response could be due to stability problems with the reagent, one of the responders was re-tested both with newly dissolved ASPItest reagent and with reagent stored for 18 days at −20°C and responded equally well to both reagents (data not shown).

To test whether AA could induce cell lysis in aggregometer samples, hirudinized whole blood was incubated in MEA cuvettes with 0.5 mM AA in the presence of abciximab in order to allow subsampling for flow cytometry. These samples were incubated with the same stirring and temperature conditions as for parallel aggregating samples, and samples were collected when maximal aggregation had been detected in the parallel sample without abciximab. Figure 1B shows that microparticles of platelet and erythrocyte origin were detected in MEA cuvettes incubated with 0.5 mM AA. Over 90% of the single platelets were positive for P-selectin, indicating platelet activation and alpha granule release. However, in citrated PRP samples incubated in LTA cuvettes following the same procedure as described above, only 10–30% P-selectin positive platelets were detected, and the percentages were similar in samples with and without AA (17.5 ± 6.2 vs. 17.8 ± 8.7, $n = 4$). The same was true for the numbers of platelet microparticles, which were higher than for MEA samples, but did not differ between samples with and without AA (18.9 ± 4.1 vs. 17.2 ± 5.3, $n = 4$).

To test whether ADP released from platelets or other blood cells could play a role for AA-induced platelet aggregation, we treated blood from normal donors with aspirin (ASA) or cangrelor

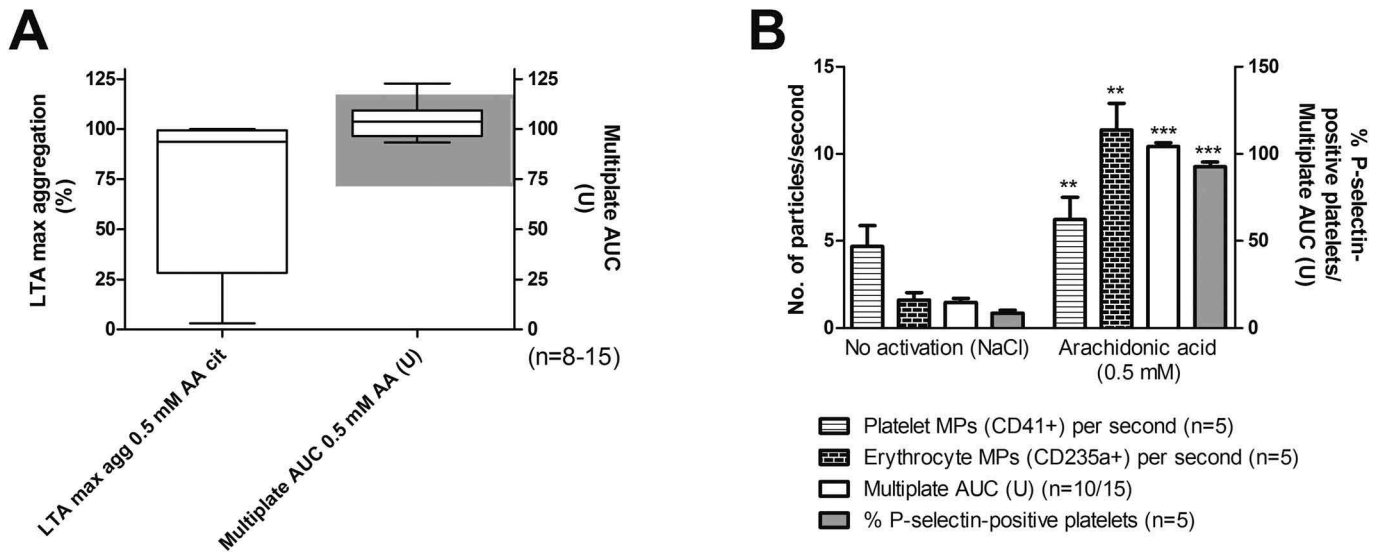

Figure 1. Arachidonic acid induces consistent platelet aggregation in whole blood but also formation of microparticles of platelet and erythrocyte origin. (A) Platelet aggregation in response to AA (0.5 mM final concentration) in blood from healthy donors, comparing light transmission aggregometry (LTA) in citrated PRP ($n = 8$) with multiple electrode aggregometry (MEA, Multiplate®) in hirudinized whole blood ($n = 15$). The box whiskers indicate the maximum and minimum values. The gray square marks the normal reference range for the Multiplate® ASPItest. (B) P-selectin exposure and microparticles (MPs) of platelet and erythrocyte origin detected by flow cytometry in blood sub-sampled from a MEA cuvette after 6 min incubation. The bars show mean and standard error of the mean (SEM). Stars denote significant differences as compared to samples without AA activation (NaCl) */**/*** = $p < 0.05/0.01/0.001$.

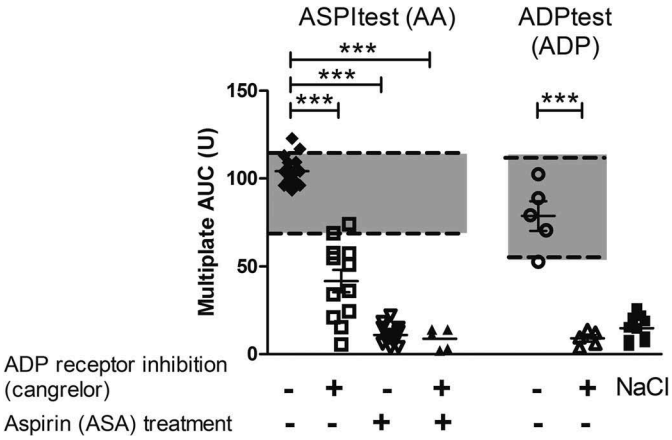

Figure 2. Whole blood aggregation in response to arachidonic acid is partly dependent on ADP. The Multiplate® ASPItest (AA) and ADPtest was performed on blood from normal donors with or without pretreatment with the ADP P2Y12 receptor inhibitor cangrelor (10 μM) or aspirin (ASA, 100 μM) (n = 5–15). Samples with saline solution (NaCl) added instead of agonist are included as reference. Symbols show results for each individual mean, and standard error of the mean (SEM) is also indicated. The gray boxes marks the normal reference ranges for the ASPItest and ADPtest. Stars denote significant differences between groups, *** = $p < 0.001$.

(a direct acting inhibitor of the ADP receptor P2Y12, used as substitute for non-direct acting drugs such as clopidogrel and prasugrel) before analysis using the MEA ASPI or ADPtests. Figure 2 shows that platelets treated with cangrelor also showed significantly decreased aggregation to AA in the ASPItest, confirming that the aggregation was partly ADP dependent. Cangrelor treatment of citrated PRP also reduced AA-induced aggregation in LTA (see Supplement Figure S1). Interestingly, hirudinized PRP from the same donors showed very low aggregation responses to AA in LTA, only one out of eight tested samples showed aggregation above 25% (Supplement Figure S1). Hirudinized PRP from two donors was also tested with MEA but very low aggregation responses were observed. The low aggregation response to AA in hirudin PRP with both techniques suggests that the red blood cells present in MEA are very important for the consistent aggregation response to AA observed with this technique.

Dose-response studies with AA were then performed in whole blood and PRP using flow cytometry. Figure 3 illustrates that most platelets were lysed at 0.75 mM AA, and that both platelets and other blood cells were lysed in the presence of 1 mM AA. In whole blood, the lysis was clearly detectable as a color change in the tubes at AA concentrations of 0.75 and 1 mM (see Supplement Figure S2). The more pronounced lysis in flow cytometry as compared to the aggregometry studies could potentially have been explained by the temperature, as flow cytometry samples were incubated at room temperature. However, when this hypothesis was tested, only minor differences in cell lysis (microparticle formation) were observed when comparing parallel samples incubated at room temperature and 37°C (n = 3–5, data not shown).

Figure 4 shows the increase in microparticles and activated platelets in response to increasing concentrations of AA. Figure 4C–D show that platelet activation in whole blood was initiated at lower concentrations than in PRP, and started at the same point as the erythrocyte microparticles started to rise (Figure 4B). For PRP samples, a marked platelet activation was only observed at concentrations where most platelets were already lysed (as shown in Figure 3A).

Our results are in contrast to the ones reported by Hübl et al. [18], who developed a protocol using citrate PRP diluted in phosphate-buffered saline (PBS) and a relatively low concentration of AA, 0.08 mM. This protocol was used by the same group in a subsequent study [22]. At this concentration of AA, they claim to see a peak in platelet activation (P-selectin exposure) after incubation for 10 min at 37° followed by 20 min staining at room temperature (with only 25% further dilution at this step, meaning that activation by AA could potentially proceed here as well). As the dilution factor for the PRP was relatively similar between our protocols, we tested whether the difference in buffer and incubation time could be the factors explaining why we did not find the same results. However, we did not observe this peak

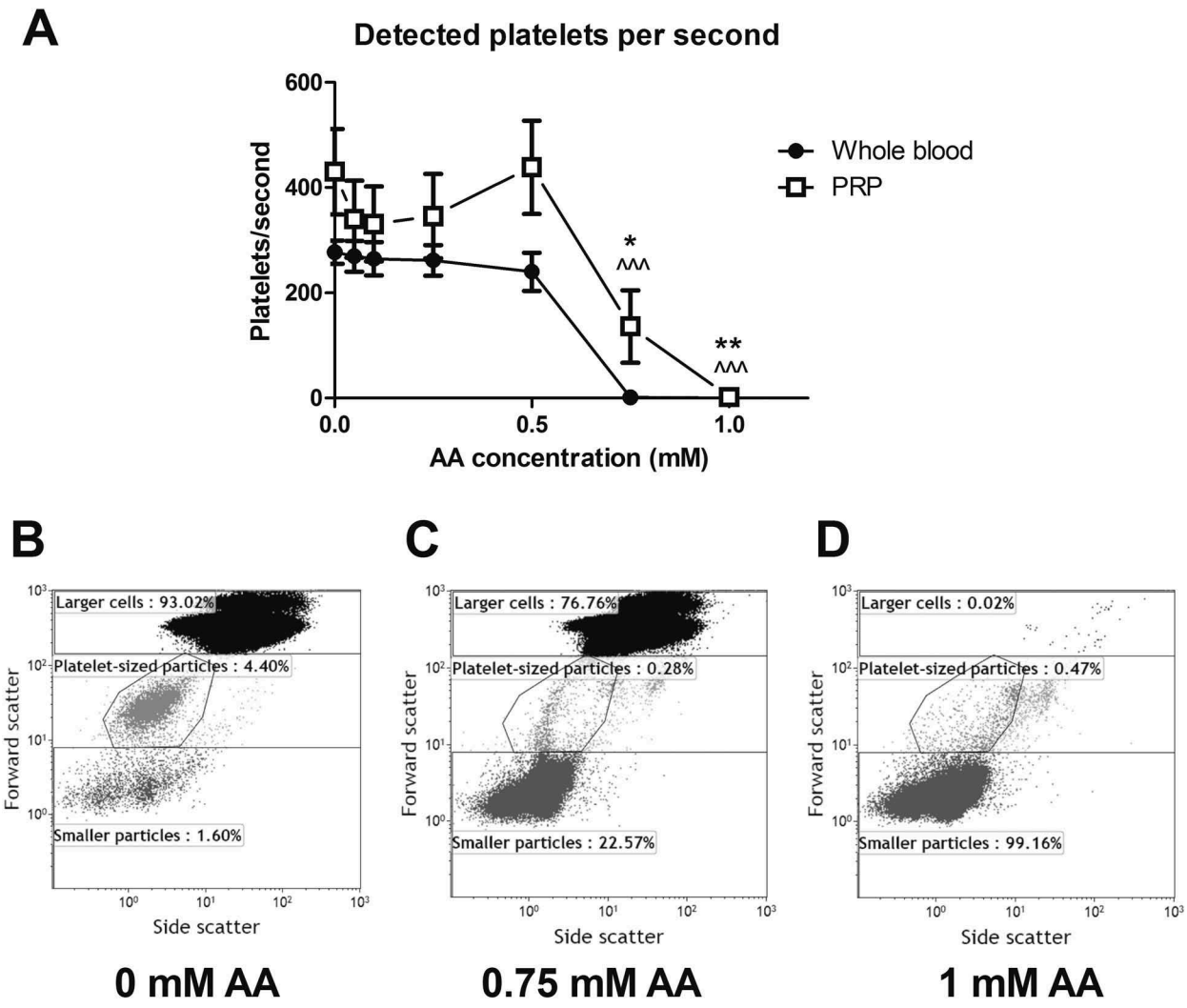

Figure 3. Arachidonic acid causes lysis of blood cells detected by flow cytometry. (A) Change in number of platelets detected by flow cytometry in PRP (white squares) and whole blood (black circles) from normal donors treated with increasing concentrations of AA ($n = 6$ for 0, 0.5, and 1, $n = 5$ for 0.75, and $n = 4$ for 0.05, 0.01, and 0.25 mM AA). The graph shows mean and standard error of the mean (SEM). Symbols (PRP: *, Whole blood: ^) denote significant differences as compared to samples without AA activation, */**/*** = $p < 0.05/0.01/0.001$. (B) Dot plots from the whole blood samples from one of the donors, showing how the disappearance of platelet-sized particles and the larger (red and white) blood cells is accompanied by an increase in smaller particles ("microparticles") in the presence of increasing concentrations of AA.

in P-selectin exposure, even though we changed the buffer to PBS and incubated for 10 + 20 min, neither in citrate nor hirudinized PRP (Supplement Figure S3). All five donors tested here did show aggregation to AA in both LTA and MEA (results included in Figures 1 and 2), and their platelets showed high activation in response to PAR1-activating peptide (TRAP) in flow cytometry tubes run together with the AA dose response (data not shown).

Discussion

It has been previously reported that AA may fail to induce aggregation in LTA with PRP in some healthy volunteers [13,23]. However, in accordance with our results, these studies found no such results with MEA in the same populations. We found that microparticles of both platelet and erythrocyte origin increased in MEA cuvettes incubated with 0.5 mM AA. In addition, over 90% of the platelets were activated, as shown by exposure of P-selectin. In our dose-response study by flow cytometry, we found that platelet activation by AA in whole blood was initiated at lower concentrations than in PRP, and started at the same point as the first erythrocyte microparticles became detectable. For PRP samples, a marked platelet activation was only observed at AA concentrations where most platelets were already lysed. This makes it hard to develop a reliable flow cytometry test of platelet activation in response to AA. Our activation profiles are actually very similar to the ones reported by Rubak et al. using diluted whole blood [19], with very little platelet activation below 0.4 mM AA. In accordance to our findings, both Rubak and Hübl [18] mention drastic cell fragmentation with higher concentrations of AA (1 mM was the highest concentration tested in both papers). Another whole blood flow cytometry protocol was used by Frelinger et al. in two papers [20,24] with 0.4–0.8 mM AA. No dot plots are shown in these papers, thus it is not possible to confirm whether cell fragmentation was occurring, but interestingly, an ADP-dependent component was reported by these authors as well. Red blood cell lysis is a potential source of ADP, and also Hübl et al. mention observations of red cell lysis and suggest that ADP will contribute to AA-induced platelet activation in whole blood flow cytometry. Therefore, they used PRP in their subsequent study [22]. However, we could not confirm their findings of a peak in platelet activation at lower AA concentrations, even though we changed our buffer to PBS and extended our incubation time in order to adapt to their settings.

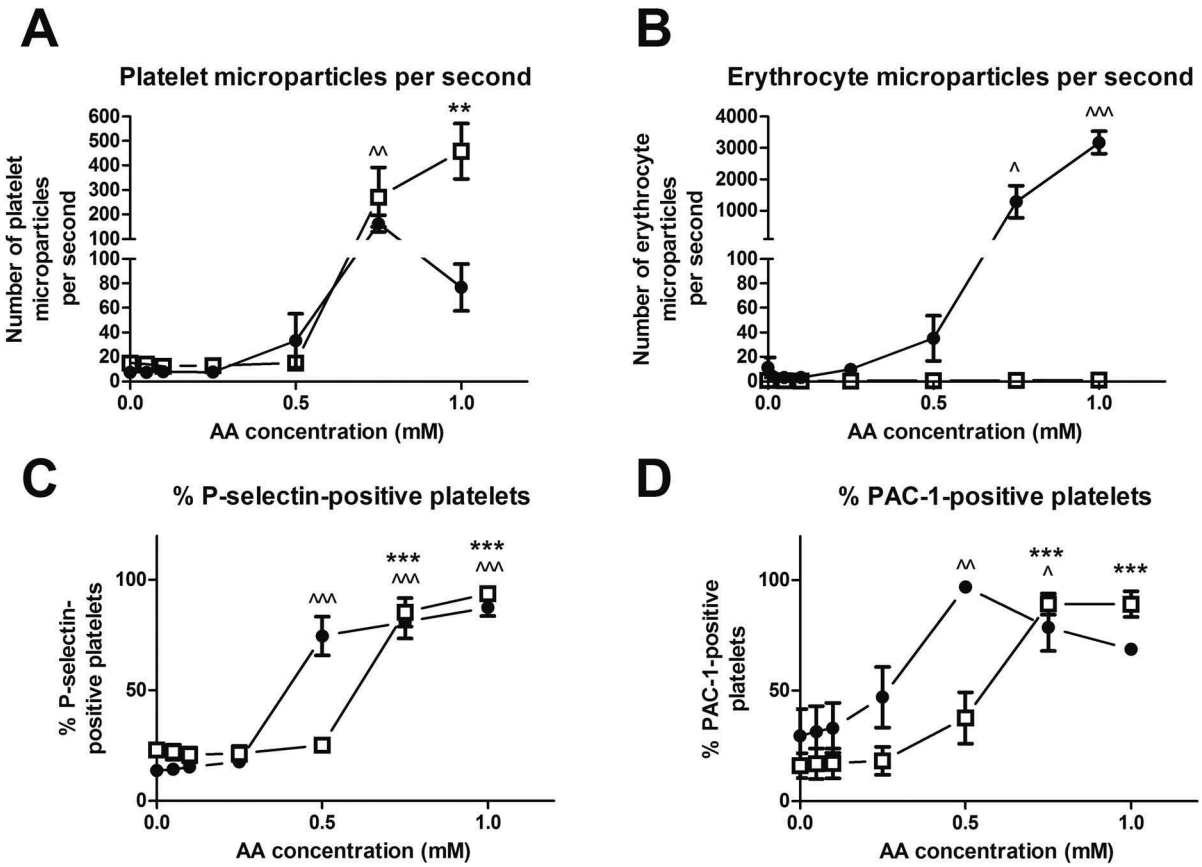

Figure 4. Arachidonic acid induces a dose-dependent cell lysis and platelet activation detectable by flow cytometry. Formation of (A) Platelet (CD41+) microparticles, (B) Erythrocyte (CD235a+) microparticles, (C) platelets positive for P-selectin (marker of alpha granule exocytosis), and (D) platelets positive for PAC-1 (marker for the active conformation of fibrinogen receptor GPIIb/IIIa) in response to increasing concentrations of arachidonic acid (AA) in PRP (white squares) and whole blood (black circles) from normal donors ($n = 6$ for 0, 0.5, and 1, $n = 5$ for 0.75, and $n = 4$ for 0.05, 0.01, and 0.25 mM AA). The graphs show mean and standard error of the mean (SEM). Symbols (PRP: *, Whole blood: ^) denote significant differences as compared to samples without AA activation, */**/*** = $p < 0.05/0.01/0.001$. Note the break in the Y-axis in Figure 4A and 4B in order to visualize the changes at low concentrations. Also note that the very low numbers of intact platelets at 1.0 mM AA (PRP) and 0.75 and 1.0 mM AA (whole blood) (see Figure 3A) make the percentages of activated platelets less reliable.

The finding that platelets treated with the ADP receptor inhibitor cangrelor showed decreased aggregation to AA in the ASPItest confirmed that whole blood aggregation tested by MEA was also partly ADP dependent. Low responses were seen in MEA samples treated with physiological saline instead of platelet agonist. This suggest that ADP release from intact red blood cells due to stirring, etc., which has been described as an important contributor in some individuals [25], did not contribute much to the observed aggregation in our donors. The contribution to the aggregation response from ADP released from activated platelets and lysed cells could be one explanation to previous publications that reported a decreased response to AA in whole blood tests in individuals treated with clopidogrel, but never proposed any possible mechanism to explain this phenomenon [26–28].

The more pronounced lysis in flow cytometry as compared to the aggregometry studies could potentially be explained by the dilution of the blood or PRP in buffer. The presence of proteins has been reported to protect cells from AA-induced lysis [29]. However, our buffer contained bovine serum albumin which should have protected the cells. In fact, the results with PBS were relatively similar, showing that the buffer had little influence on the lysis. Another difference was the temperature, but only minor differences in cell lysis were observed when comparing parallel samples incubated at room temperature and 37°C. A more likely explanation is the lower concentration of cell membranes available for interactions with AA. Early studies report that AA also interacts directly with cell membranes to increase their ion permeability [6,30].

The lack of aggregation in response to AA in LTA for some donors is interesting, although it was not in focus of this study. From other publications, it is hard to find data regarding the frequency of normal donors with low response to AA, but it seems clear from published figures that data do exist [13,23]. All donors were asked and denied taking any aspirin or NSAID for the nearest 10 days, but of course we cannot exclude the possibility that some of them gave an incorrect answer. We found no indications supporting that AA reagent stability would be the explanation to the lack of response, as one of the responders showed platelet aggregation also with AA reagents stored dissolved for 18 days at −20°C. Surely this question should be more thoroughly investigated, but as it was an incidental finding in this study, we just wanted to report it for others to further investigate.

The cangrelor treatment of citrated PRP showed that AA-induced aggregation in LTA was also dependent on ADP. However, AA showed very low potency to cause aggregation in hirudinized PRP. It is known since long that ADP only is able to cause secondary aggregation, secretion, and thromboxane production in situations with close platelet-to-platelet contact and in low calcium such as in citrated PRP [31], which might explain the higher aggregation responses in citrated PRP. However, the fact that hirudin blood always showed aggregation in MEA suggests

that the red blood cells present here are indeed very important for the consistent aggregation response to AA observed with this technique. The prevention of platelet-to-platelet contacts by abciximab in the subsampling experiments from the aggregometers could also be one potential explanation to why no significant P-selectin exposure was observed in the PRP from the LTA cuvettes.

In conclusion, we report that AA in concentrations commonly used for platelet function testing is able to cause lysis of blood cells and that ADP is clearly contributing to the AA-induced activation response, especially in whole blood. This could explain the previously reported ADP dependence of the residual response to AA in whole blood flow cytometry [20] and the decreased responses to AA in whole blood tests that have been reported in the presence of clopidogrel [26–28]. It could also contribute to the higher residual activity and bigger variation reported for whole blood-based tests in aspirin-treated patients [12,14]. The higher residual activity observed in aspirin-treated donors when MEA was performed with higher doses of AA [14] could also potentially be due to a larger influence of cell lysis. For this study, we did not have an ethical approval for collection of blood from patients, which means that our results still need to be confirmed in a patient population. However, we have no reason to believe that blood cells from patients should be less sensitive to lysis than the ones from our donors. Hopefully our results can raise the awareness and lead to studies by others with access to suitable patients to confirm our findings. An increased awareness among clinicians that ADP receptor inhibitor treatment potentially affects the MEA ASPItest is important, especially as documents from the inventors are available that claim the opposite [10].

Our results suggest that further investigation of platelet function tests using AA-induced platelet activation and the correct interpretation of these test results could be warranted. In flow cytometry, very limited platelet activation was detected before reaching concentrations where cell lysis also occurred. This makes it problematic to develop a reliable flow cytometry test using AA as reagent. The unreliable responses among normal donors in LTA, which did not seem to be due to reagent stability, are another issue for further investigation. We also repeated testing using AA from other manufacturers (Hart Biologicals and DiaSys/Rolf Greiner Biochemica) with the same results (data not shown), which indicate that the cell lysis was not specific to the ASPItest reagent.

The implications of these findings in the clinical setting would be that patients on ADP-receptor antagonists, especially if they use the more efficient drugs available today, could show decreased responses to AA, even though they do not take their aspirin. Another finding, although not in the focus of this study, was that a low response to AA in LTA does not necessarily mean that the donor is taking aspirin. However, the limited amount of data makes further studies necessary before any conclusions can be drawn regarding that issue. In the light of this, we support the statement that measurements of thromboxane B_2 in serum or 11-dehydro-TXB_2 in urine are probably the only specific way to evaluate the aspirin effect [3,5].

On the other hand, it is possible that an approach that measures a more general platelet activation response, in order to capture high residual platelet reactivity instead of efficacy of specific anti-platelet drugs, could be more powerful to identify patients with higher risk for adverse events. Previous studies report both positive [24,32] and negative findings [33], probably depending on study population and platelet function test employed. Thus, the optimal platelet function test for prediction of outcome in patients with cardiovascular diseases or other conditions where platelet function is part of the problem has yet to be identified. Interesting work lies ahead in order to evaluate whether new approaches for platelet function testing could be developed, and whether these could help to improve the clinical outcome for patients with platelet-related disorders.

Declaration of interest statement

The author has no conflicts of interest in relation to this study.

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Supplementary Material

Supplemental data for this article can be accessed on the publisher's website.

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