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# Platelet aggregation is dependent on platelet count in patients with coronary artery disease☆

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ABSTRACT

*Introduction:* Platelet function testing in whole blood is widely used to evaluate the effect of antiplatelet agents, but it is not known whether results are affected by whole blood parameters. This study investigated the importance of platelet count, haematocrit, red blood cells (RBC), and white blood cells in whole blood platelet aggregometry.

*Materials and methods:* We included 417 patients with coronary artery disease on aspirin mono-therapy and 21 aspirin-naïve healthy individuals. Blood sampling was performed one hour after aspirin ingestion. The antiplatelet effect of aspirin was evaluated using the VerifyNow® Aspirin assay and multiple electrode aggregometry (MEA, Multiplate®) induced by collagen (1.0 μg/mL) and arachidonic acid (1.0 or 0.75 mmol/L). Measurements of whole blood parameters were performed to evaluate the three major cell lines in circulating blood.

*Results:* In patients, platelet count correlated significantly with platelet aggregation ($MEA_{collagen}$, $p<0.0001$; $MEA_{arachidonic\ acid}$, $p<0.0001$; VerifyNow®, $p=0.03$). Haematocrit and RBC correlated inversely with MEA induced by collagen ($p_{haematocrit}<0.001$; $p_{RBC}=0.07$) and with VerifyNow® ($p_{haematocrit}<0.0001$; $p_{RBC}<0.0001$), but not with MEA induced by arachidonic acid ($p_{haematocrit}=1$; $p_{RBC}=0.87$). White blood cells correlated significantly with platelet aggregation ($MEA_{collagen}$, $p<0.001$; $MEA_{arachidonic\ acid}$, $p<0.0001$; VerifyNow®, $p=0.05$). Similar associations were observed in aspirin-naïve healthy individuals.

*Conclusions:* Whole blood aggregometry is dependent on all major cell lines in whole blood. Importantly, platelet aggregation is significantly associated with platelet count even within the normal range.



## Introduction

In recent years, residual platelet aggregation during antiplatelet treatment has been recognised as a novel risk factor for acute coronary syndromes [1]. Platelet function testing enables the detection of increased residual platelet aggregation and is particularly interesting with respect to individualising and optimising antiplatelet treatment [2]. The pharmacological efficacy of aspirin and other antiplatelet drugs displays considerable inter-individual variability, possibly resulting from a variety of complex mechanisms [2,3]. We have previously shown that the extent of residual platelet aggregation depends on the method employed [4]. As modern whole blood platelet aggregometry is increasingly used as a research tool and for clinical purposes [5,6], evaluating its dependence on whole blood parameters is important.

Whole blood aggregometry is encumbered by the fact that whole blood contains a variety of compounds affecting platelet aggregation, including the three major cell lines: platelets, red blood cells (RBC), and white blood cells (WBC). This may potentially interfere with the interpretation of test results. It has been suggested that different platelet function tests may not be equally dependent on whole blood parameters [7,8]. Recently, it was reported that whole blood aggregometry is particularly dependent on platelet count [9–11]. Previous studies have reported that RBC and WBC also influence whole blood aggregometry [12,13].

We recently evaluated whole blood platelet aggregometry in a large population of patients with stable coronary artery disease (CAD) treated with low-dose aspirin [14]. In the present study, we evaluated this study population and a smaller group of aspirin-naïve healthy individuals to investigate whether whole blood aggregometry is influenced by the three major cell lines in circulating blood.

*Abbreviations:* ADP, adenosine diphosphate; ATP, adenosine triphosphate; CAD, coronary artery disease; MEA, multiple electrode aggregometry; RBC, red blood cells; WBC, white blood cells.

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## Materials and methods

### Design

We included 417 patients angiographically diagnosed with CAD. Among 1,500 patients invited by mail, a total of 830 did either not respond or declined to participate; of the remaining 670 patients accepting the study invitation, 253 were either not eligible or were excluded according to the criteria described below. All patients were above the age of 18 years and were treated with non-enteric coated aspirin 75 mg once daily prior to and during study participation.

Furthermore, we investigated 21 healthy individuals not treated with aspirin. This group was originally enrolled in a study evaluating the comparability between different platelet function tests [4]. We decided to include this population of aspirin-naïve healthy individuals to test whether the associations between platelet aggregation and whole blood parameters observed in our main study population were also present in the absence of CAD and aspirin-induced platelet inhibition.

Exclusion criteria for patients were: aspirin intolerance, any acute disease, use of anticoagulants or any drugs other than aspirin known to affect platelet function (i.e. thienopyridines, ticagrelor, dipyridamol, and nonsteroidal anti-inflammatory drugs), platelet count $< 120 \times 10^9$/L, pregnancy, any ischaemic event or revascularisation procedure (percutaneous coronary intervention or coronary artery bypass grafting) within the previous 12 months, and inability to give informed consent. Exclusion criteria for healthy individuals were identical, except that no chronic disease was accepted within this study population.

Written informed consent was obtained from all participants. The study was conducted in accordance with the Helsinki II declaration, and the study protocol was approved by the Central Denmark Region Committees on Biomedical Research Ethics.

### Study population

We investigated a population of stable CAD patients treated with low-dose aspirin [14]. Patients were recruited from the Western Denmark Heart Registry and included from November 2007 to May 2009. The Western Denmark Heart Registry collects data on all interventional procedures performed in interventional centers in the western part of Denmark [15]. Current medication was registered on the day of blood sampling. Healthy individuals were recruited by advertising at Aarhus University Hospital, Skejby, Denmark.

### Compliance

Compliance was evaluated by face-to-face interviews and pill counting and subsequently confirmed by measurements of serum thromboxane $B_2$. In order to optimise compliance and uniform pharmacokinetics, patients received a tablet dosage box containing seven tablets of non-enteric coated aspirin (Hjerdyl®; Sandoz, Copenhagen, Denmark) in separate compartments. Patients were instructed to take these tablets the last seven days before blood sampling.

### Blood sampling

Standardised blood sampling on patients was performed one hour after aspirin ingestion. Both patients and healthy individuals were resting for 30 minutes before sampling. Samples were drawn from an antecubital vein into vacuum tubes through a 19-G butterfly needle using a minimum of stasis.

### Platelet aggregation tests

Platelet aggregation was evaluated in whole blood using two different techniques: multiple electrode aggregometry (MEA, Multiplate®; Dynabyte, Munich, Germany) and the VerifyNow® Aspirin assay (Accumetrics Inc., San Diego, CA, USA). All analyses were performed within 2 hours of sampling. Multiplate® is a whole blood impedance aggregometer [16,17]. Collagen (1.0 μg/mL, Collagen Reagent Horm; Nycomed, Linz, Austria) and arachidonic acid (1.0 mmol/L, Medinova Scientific, Glostrup, Denmark) served as agonists in patients, whereas only arachidonic acid (0.75 mmol/L, Medinova Scientific, Glostrup, Denmark) was used in the original study on healthy individuals [4]. Aggregation was recorded for 6 minutes and reported as the area under the curve (aggregation units x min) [6]. The VerifyNow® Aspirin assay is based on turbidimetric optical detection of platelet aggregation. This test inherently employs arachidonic acid as the agonist, and results are reported as Aspirin Reaction Units [18]. Blood for platelet aggregometry was collected in 3.6 mL (MEA) and 2.7 mL (VerifyNow®) tubes containing 3.2% sodium citrate (Terumo, Leuven, Belgium).

### Platelet count, red blood cells, and white blood cells

Platelet count, haematocrit, RBC, and WBC were measured with the Sysmex XE-2100 haematology analyser (Sysmex, Kobe, Japan). We measured both haematocrit and RBC to optimise the comparability with previous studies. Blood was collected in 3.0 mL tubes containing EDTA (Terumo, Leuven, Belgium) and was analysed within 90 minutes of sampling.

### Serum thromboxane $B_2$

Serum thromboxane $B_2$ is regarded as a reliable estimate of the platelet inhibiting effect of aspirin [19]. Furthermore, it confirms whether or not patients are compliant with aspirin therapy. Serum thromboxane $B_2$ was determined according to Patrono *et al.* [20], with the modification that serum was collected after 1 hour of clotting, and that an ELISA technique was used (Cayman Chemical, Ann Arbor, MI, USA). Blood was collected in 5.5 mL non-anticoagulated glass tubes (Terumo, Leuven, Belgium) and allowed to clot at 37 °C. Subsequently, it was centrifuged for 10 minutes at 2,600 *g* and the supernatant serum was stored at −80 °C until analysis.

### Statistics

Continuous data are presented as mean ± SD if data were normally distributed and as medians (25th, 75th percentile) if not. Distributions of categorical variables were compared with the $\chi^2$-test and presented as absolute counts and percentages. All variables were tested for normality using the D'Agostino-Pearson normality test. Correlations were calculated using Spearman's rank correlation coefficient. Multiple linear regression analysis was used to identify determinants of whole blood platelet aggregation by adjusting for whole blood parameters and baseline characteristics. Statistical analyses were performed using GraphPad Prism® version 5.0 (GraphPad Software, La Jolla, CA, USA) and STATA® version 10.0 (StataCorp, College Station, TX, USA).

## Results

Baseline characteristics of patients and healthy individuals are shown in Table 1, and levels of platelet aggregation, platelet count, haematocrit, RBC, and WBC are shown in Table 2. A relatively high prevalence of previous myocardial infarction (67%), percutaneous coronary intervention (90%), and coronary artery bypass grafting (21%) was observed within the patient population. All patients returned empty pill boxes and claimed to be adherent to aspirin