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Aspirin resistance may be identified by miR-92a in plasma combined with platelet distribution width

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ABSTRACT

Objective: Aspirin is a widely used drug for prevention of thrombotic events in cardiovascular patients, but approximately 25% of patients experience insufficient platelet inhibition due to aspirin, and remain in risk of cardiovascular events. This study aimed to investigate the value of circulating miR-92a and platelet size as biomarkers of the individual response to aspirin therapy.

Methods: Blood samples were collected from 50 healthy blood donors without antithrombotic medication and 50 patients with intermittent claudication on daily aspirin therapy. Based on results from the arachidonic acid stimulated aggregation test on Multiplate®analyzer (ASPItest), patients were defined as aspirin resistant ($n = 10$) or aspirin responders ($n = 40$). Plasma levels of miR-92a were evaluated by RT-qPCR analysis and platelet distribution width (PDW) was used to assess platelet size variability. Receiver operating characteristic curves for miR-92a levels and PDW were used to set cut-off values for discrimination between aspirin responding and aspirin resistant patients.

Results: When defining aspirin resistance as an ASPItest ≥30 U, the optimal cut-off values for discrimination of aspirin responders and aspirin resistant patients were found to be PDW > 11.8 fL and a relative expression level of miR-92a > 4.5. Using these cut-off values we could define a PDW/miR-92a-score with a specificity of 97.5% and a sensitivity of 80.0% in relation to detect aspirin resistance. The corresponding positive and negative predictive values were found to be 88.9% and 95.1%, respectively.

Conclusion: Aspirin resistance can potentially be identified by miR-92a levels in plasma combined with PDW.
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1. Introduction

Atherosclerosis is one of the main underlying processes that lead to coronary heart disease and cerebrovascular disease, which together are responsible for a large proportion of cardiovascular diseases (CVDs). CVDs are the most frequent cause of deaths worldwide, but in developed countries prevention and treatment interventions have caused the mortality rate to decline over the last two decades [1].

Antiplatelet therapy is a cornerstone in relation to prevent future thrombotic complications in patients with CVD, and the indication for use of antiplatelet agents covers a large spectrum of vascular diseases, from primary prevention in patients at high risk of thrombotic events to secondary prevention in patients suffering a range of CVD including peripheral arterial disease (PAD) [2] .Therefore, an important question is whether or not patients who suffer events do so because of insufficient effect of the antiplatelet therapy.

Platelets play a central role in the maintenance of hemostasis as well as in the generation of thrombosis. Circulating platelets may be activated due to reduction in the endothelial antithrombotic properties, or due to atherosclerotic risk factors, including hypertension, hypercholesterolemia, cigarette smoking and diabetes. Inflammatory events can lead to endothelial activation, which in turn stimulates activated platelets to attach [3], and in this way platelet-derived chemokines are deposited on the surface of the inflamed vessel wall, which leads to monocyte recruitment and progression of atherosclerosis [4]. Upon rupture of an atherosclerotic plaque, large amounts of thrombogenic substances are exposed to the circulating platelets, resulting in platelet recruitment, activation and aggregation [3].

Aspirin is a widely used drug for prevention of thrombotic events in cardiovascular patients. The antithrombotic effect of aspirin is achieved

Abbreviations: ASPItest, arachidonic acid stimulated aggregation test; BMI, body mass index; COX, cyclooxygenase; CVD, cardiovascular disease; eNOS, endothelial nitric oxide synthase; HCT, hematocrit; IPF, immature platelet fraction; KLF4, Krüppel-like factor 4; MPV, mean platelet volume; NO, nitric oxide; NPV, negative predictive value; PDW, platelet distribution width; PLT, platelet count; PPP, platelet-poor plasma; PPV, positive predictive value; ROC, receiver operating characteristic; TXA_2, thromboxane A_2; WBC, white blood cell count.

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by acetylation of a key serine residue, which irreversibly inhibits the platelet cyclooxygenase 1 (COX1) enzyme, preventing the synthesis of thromboxane A_2 (TXA_2) and its enhancement of platelet activation [5]. A recent meta-analysis has revealed that approximately 25% of patients experience insufficient platelet inhibition due to aspirin, and remain in risk of cardiovascular events despite their antiplatelet therapy [5]. The laboratory tests routinely used to test platelet function show high inter- and intra-individual variability, and there is poor correlation between results obtained with the different tests [5–7]. Multiple electrode aggregometry is a well-validated method for measuring platelet function in vitro [8]. It accesses single pathways of platelet activation, and does not necessarily accurately reflect the multifaceted pathways of platelet activation in vivo [6]. Furthermore, the tests have to be performed within 30–120 min from blood sampling, which makes the test impractical in a clinical setting. Therefore, it has not gained widespread use.

MicroRNAs are short, single-stranded non-coding RNAs that act as posttranscriptional regulators of gene expression [9]. Platelets contain numerous microRNAs, some of which are released into circulation, when platelets are activated [10]. Extracellular microRNAs are remarkably stable, they are protected from degradation as they are packed in microvesicles or released as part of protein complexes or lipoproteins [9]. Circulating levels of the microRNA miR-92a have been associated with cardiovascular disease [11,12], and we hypothesized that plasma levels of this microRNA may reflect in vivo platelet reactivity. Platelet size as determined by mean platelet volume (MPV) and platelet distribution width (PDW) has been associated with platelet reactivity after antiplatelet therapy [13]. The hypothesis of the present study is that circulating miR-92a and/or platelet size variability may be biomarkers for the individual response to aspirin therapy.

2. Materials and methods

2.1. Study population

A total of 50 healthy blood donors and 50 patients with intermittent claudication were enrolled in the study during June and July 2014 at Lillebaelt Hospital, Kolding, Denmark. Blood donors were excluded if they were using any medication or if they had donated blood within the last two months before blood sampling. The patients were all prescribed aspirin (75 mg, 100 mg or 150 mg per day) as monotherapy, which they confirmed to have been taking for at least 10 days before blood sampling. Patients were excluded if they were using any anti-platelet therapy other than aspirin (e.g. clopidogrel), if they had active cancer or if they had undergone surgery or received blood transfusion within the last month before blood sampling. All participants gave written informed consent and the study was conducted in agreement with the Helsinki-II-declaration and approved by Regional Ethical Committee for the region of Southern Denmark (S-20140016) and the Danish Data Protection Agency.

2.2. Sample collection

Non-fasting venous blood samples were obtained using a 21 gauge needle (Becton-Dickinson, Franklin Lakes, NJ, USA) after a minimum of venous stasis. From each person the first 3 mL of blood was collected into a K_2-EDTA containing tube (Becton-Dickinson, Franklin Lakes, NJ, USA) and used for hematological analysis. The next 2 × 3 mL of blood were collected into Hirudin containing tubes (Roche Diagnostics International Ltd., Rotkreuz, Switzerland) and then 10 mL of blood into a K_2-EDTA containing tube (Becton-Dickinson, Franklin Lakes, NJ, USA), these samples were used for aggregation analysis and miRNA analysis, respectively.

2.3. Hematological parameters

EDTA anticoagulated blood samples were analyzed using the fully automated Sysmex XE 5000 analyzer (Sysmex, Kobe, Japan). All tests were performed within 30 min of blood sampling to minimize changes in the cells size. The parameters obtained were platelet count (PLT), mean platelet volume (MPV), platelet distribution width (PDW), immature platelet fraction (IPF), hematocrit (HCT) and white blood cell count (WBC).

2.4. Platelet aggregation tests

Platelet aggregation was measured by multiple electrode aggregometry (MEA) using Multiplate® Analyzer (Roche Diagnostics, Rotkreuz, Switzerland). In the single use test cells hirudin anticoagulated whole blood was diluted 50% with saline and incubated for 3 min at 37 °C. Platelet aggregation was then stimulated by addition of different agonists 1) arachidonic acid (AA) 0.5 mM and 1.0 mM, 2) collagen 3.2 μg/mL, 3)TRAP-6-peptide 32 μM, 4) Ristocetin 0.2 mg/mL and 0.77 mg/mL and 5) adenosine diphosphate (ADP) 6.5 μM as agonists, using the specifications supplied by the manufacturer [14]. All tests were performed in duplets within 30–120 min after blood sampling. Aspirin resistance was defined according to the result of the arachidonic acid stimulated aggregation test (ASPItest) using 30 U as cut-off value, as recommended by the manufacturer and commonly used by others [15,16].

2.5. MicroRNA analysis

In order to obtain platelet-poor-plasma (PPP) 10 mL of EDTA anticoagulated whole blood was centrifuged at 3000 g for 15 min (acceleration 5, brake 6, temperature 18 °C). After centrifugation the plasma phase was carefully transferred to a clean tube, leaving approximately 1 mL of plasma on top of the buffy coat. The centrifugation step was repeated and again approximately 1 mL was left in the bottom of the tube when the PPP was transferred into two cryo-tubes for storage at −80 °C.

MicroRNA was isolated from 300 μL of PPP using Nucleospin®miRNA Plasma (Macherey-nagel, Germany) and according to manufactures protocol. As a mean of normalization, all samples were spiked with 5 μL Cel-miR-39 (2.75×10^{-12} M) (RiboTask, Odense, Denmark). The spike was added after removal of the plasma proteins in the same step as isopropanol. MicroRNA was eluted using 30 μL of RNAse free water, and the samples were frozen at −20 °C.

cDNA synthesis was performed using TaqMan®MicroRNA Reverse Transcription Kit and Custom TaqMan MIR RT Pool (both from Applied Biosystems, Foster City, CA). The RT-primer pool contained microRNA-specific stem-loop primers for miR-92a (ThermoFisher assay-ID: 000431) and cel-miR-39 (ThermoFisher assay-ID: 000200). The reaction was performed using 3 μL of RNA-sample in a total volume of 10 μL.

As the microRNA level in PPP is relatively low, a preamplification step was performed using 5 μL of the RT-product in a total reaction volume of 25 μL containing TaqMan®PreAmp Master Mix and Custom TaqMan MIR PreAmp Pool (Applied Biosystems, Foster City, CA).

The final quantification was performed using Custom TaqMan®Array MicroRNA Cards (TLDA) from Applied Biosystems. A total of 1.2 μL of the preamplification products were diluted with TaqMan®Universal Master Mix II, no UNG and loaded on the array. The microRNA was analyzed in triplets using the ABI Prism 7900HT, and outliers were removed before calculating the mean Ct-value.

Ct-values were normalized using the exogenous cel-miR-39, and the expression level calculated as $2^{-\Delta Ct}$ ($\Delta Ct = Ct_{miR\text{-}92a} - Ct_{cel\text{-}miR\text{-}39}$).

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